Analysis of defense responses in the barley-*Rhynchosporium secalis* pathosystem

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Summary

The imperfect fungus *Rhynchosporium secalis* (Oudem) J.J. Davis causes scald of barley (*Hordeum vulgare* L.) and several other grasses. In susceptible plants the fungus penetrates the cuticle and grows subcuticularly for at least 9-14 days. It then attacks the underlying epidermis and causes the typical scald lesions. In resistant cultivars, the fungus may germinate and penetrate the cuticle but forms sparse subcuticular hyphae that cause little damage to the plant cells. No hypersensitive response (HR) has been reported in this system and the resistance is suggested to be mediated through accumulation of pathogenesis related (PR) proteins (PRPs). During the subcuticular phase, the fungus is believed to interact with the plant mainly through the apoplast. In this investigation the resistance response of barley towards *R. secalis* was studied by examining the *in vitro* interaction between various components of the apoplastic fluid and the fungus.

Isolates of *R. secalis*, collected across Australia, were tested for their differential virulence on the near-isogenic barley cultivars, Atlas (*Rrs2*), and Atlas 46 (*Rrs1*, *Rrs2*). Isolate H2.5, with the largest differential response toward Atlas (susceptible) and Atlas 46 (resistant), was identified and used along with the cultivars for further studies on the defense responses.

Barley intercellular washing fluid (IWF), which represents the apoplastic fluid, was found to possess antifungal activity when incubated with *R. secalis* conidia. A bioassay, developed based on this, was used to measure the levels of antifungal activity in IWF collected from Atlas and Atlas 46 before and after inoculation with *R. secalis*. IWF from uninoculated Atlas showed the lowest antifungal activity whereas inoculation of this cultivar provided IWF with the highest activity. IWF from uninoculated Atlas 46 displayed an intermediate activity that also increased, to a lesser extent, after the plants were inoculated. Analysis of the compounds that had antifungal activity and/or increased in response to inoculation led to the purification in IWF of seven proteins.

The first protein isolated was a small (<3kDa) peptide containing hydroxyproline. This peptide had no detectable physiological activity and its purification was largely based on reverse phase HPLC, a denaturing technique. No protein sequence could be obtained for this protein but the percentage of each amino acid was determined. A

blocked amino-terminal end and resistance to protease and to deglycosylation impeded sequencing and further characterization of this protein. Another protein with no detectable antifungal activity was also isolated. This 14-kDa protein was sequenced and found to be a PR-4 protein previously reported in barley.

The remaining five PR proteins all had strong antifungal activity against *R. secalis*. The proteins ranged in molecular weight from 22 to 32 kDa. These were isolated using several non-denaturing HPLC techniques in order to preserve their biological activities. Purified proteins were sequenced by N-terminal or internal micro-sequencing. A β(1-3) glucanase, a chitinase and three thaumatin-like (TL) proteins were identified. Four of these proteins have been reported previously but one of the TL proteins is a novel protein. Enzymatic and physiological activity of these proteins, when used individually and in various combinations, was studied. The purified chitinase, β(1-3) glucanase and all the three TL proteins degraded the cell walls of *R. secalis* conidia. At least one of the TL proteins was also shown to have β-glucanase activity. This is in agreement with the recent report that TL proteins exert their antifungal activity by affecting the cell wall, not the membrane as was thought previously.

The amount of each antifungal protein in inoculated and uninoculated Atlas and Atlas 46 IWF was quantified using HPLC. The proteins under investigation appeared to be coordinately regulated, *i.e.* if one increased so did the others. Atlas had the lowest levels of each protein. Whereas inoculated Atlas had the highest levels. Some of the proteins increased up to 16 times following inoculation of this susceptible cultivar. The resistant cultivar Atlas 46 was found to have an intermediate level for all the proteins prior to inoculation. Only a modest increase in the protein levels was observed following inoculation of Atlas 46.

The more pronounced increase of protein levels following inoculation of the susceptible plant Atlas, is consistent with the notion that heightened PRP level is a general stress response. However, the detrimental effect of the proteins on *R. secalis* conidia and the relatively high protein levels in the uninoculated resistant plant, Atlas 46, suggest that protein levels at the onset of infection define the fate of the interaction. The possible function of comparatively high PRP levels in non-inoculated Atlas 46 is discussed in relation to the rapid induction of similar proteins in other plant-pathogen systems.

Declaration

This work contain no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contain no material previously published or written by another person, except where due reference has been made in the text.

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Seyed-Reza Zareie-Abarghoui

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Dedication:

I would like to dedicate this thesis to my wife, Mitra, for her love and support and to my daughter, Parnian, for her patience and understanding during all these years. I also dedicate this thesis to my parent.

Abbreviations

ηm	nanometer
2-D	two dimensional
aa	amino acid
AGP	arabinogalactan protein
BSA	bovine serum albumin
CHS	chalcone synthase
d.f	degree of freedom
DB	doublet
DD	double deionised
DTT	dithiothreitol
F pr.	"F" probability
FDA	fluorescin diacetate
FW	fractured cell wall
G%	germination percentage
GP	gel permeation
GPC	gel permeation chromatography
GRP	glycine-rich protein
GT	germ tube
HFBA	heptafluorobutanoic acid
HI	hydrophobic interaction
HIC	hydrophobic interaction chromatography
HMW	high molecular weight
HPLC	high pressure liquid chromatography
HR	hypersensitive response
HRGP	hydroxyproline-rich glycoprotein
HVPE	high voltage paper electrophoresis
IG	internal granulation
IWF	intercellular washing fluid
LiBA	Lima Bean Agar
LMW	low molecular weight
LRR	leucine-rich repeat
LSD	least significant differences
LTP	lipid transfer protein
m.s	mean of squares
mAU	milli absorbance units
MWCO	molecular weight cut off
NIP	necrosis-inducing peptide
PAGE	polyacrylamide gel electrophoresis

PAL	phenylalanine ammonia-lyase
PBS	phosphate buffer saline
PDA	potato dextrose agar
PEG	polyethylene glycol
pI	isoelectric points
PI	protein inhibitor
pM	pico mol
PM	plasma membrane
PR	pathogenesis related
PRGP	proline rich glycoproteins
PRP	pathogenesis related proteins
RLK	receptor-like protein kinase
ROI	reactive oxygen intermediate
RP	reverse phase
RPC	reverse phase chromatography
s.e.m	standard errors of means
s.s	sum of squares
SAX	strong anion exchange (chromatography)
SDS	sodium dodecylsulfate
SGF	spore germination fluid
TBS	tris buffer saline
TFA	trifluoroacetic acid
TL	thaumatin-like
TLC	thin layer chromatography
TMV	tobacco mosaic virus
Tris	trishydroxymethylaminomethanol
UC	University of California
v.r	variance ratio
WCX	weak cation exchange (chromatography)

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Chapter 1:

Literature review

1.1 Scald of barley

1.1.1 Disease features

Scald of barley is one of the major leaf diseases of barley (*Hordeum vulgare*) worldwide. The disease has been reported to cause grain yield losses of up to 65% in experimental conditions (Ender *et al.*, 1983). In South Australia it has been identified as the most damaging of the leaf and stem diseases of barley with an averaged 15% inflicted yield loss (Abbott *et al.*, 1991). The damage to the crop is essentially due to a decrease in both grain quality and yield. Current control measures appear to be unable to successfully control the disease, a phenomenon mainly attributed to the restricted number of resistance genes as well as high genetic variability of the pathogen in nature. Although barley resistance to scald has been studied extensively and many resistance genes have been used in breeding programs, none have resulted in durable control of the disease (Abbott *et al.*, 1991).

Scald, alternatively called leaf blotch, is caused by the imperfect fungus *Rhynchosporium secalis* (Oud.) J.J. Davies. In addition to barley, the pathogen may attack rye plants (*Secalis cereale*) and a number of other grasses (Mathie, 1982). The pathogen infects aerial tissues (mostly leaves), and in susceptible plants causes small

water-soaked lesions around 12-14 days post-inoculation. These lesions, characteristic to scald, develop to 1-2 cm long, bluish gray oval-shaped areas that turn to pale grayish brown at the center. In barley, dark-brown margins around the dried lesions usually give them a scalded appearance (Auriol *et al.*, 1978). Total collapse of the leaves has also been reported in the case of young and susceptible barley plants. Despite some controversial reports, barley, rye grass and barley grasses all appear to be susceptible to isolates of *R. secalis*. That is, there is no specificity within the above mentioned host range (Mathie, 1982). Scald of barley is especially prevalent in cold semi-humid climates as the optimal temperature range for both plant infection and spore production is 15-20°C. Survival of *R. secalis* has been shown to be on seeds, plant residues and volunteer plants. Splashing is the main resort by which the fungal spores spread in short distances (Mathie, 1982). Although airborne dispersion has rarely been noted, movement of the fungus along with plant debris by strong winds is conceivable. This may be particularly important in windy regions such as southern regions of Australia.

1.1.2 Pathogenicity and virulence of *R. secalis*

After spores of *R. secalis* land on their host plants many of them germinate. However, some fail to do so and disintegrate on the plant surface (Ali, 1974). This has been associated with resistance of some barley cultivars. Spores that germinate require the presence of free water or >95% humidity (Brown, 1991; Jones and Ayres, 1974). Although surface hyphae can occasionally grow directly into stomatal pores, studies on the infection pattern using electron microscopy indicated that direct penetration through the plant cuticle is the main ingress technique by the pathogen (Jones and Ayres, 1974). In both resistant and susceptible cultivars penetration is usually accompanied by localised cell collapse (Wevelsiep *et al.*, 1991). It is notable, however, that the fungus does not penetrate the host living cells. After perforation of the host cuticle, the pathogen appears to be confined between the plant cuticle and the underlying epidermis for at least 10-14 days during which only a subcuticular growth occurs. This stage is known as the biotrophic or subcuticular phase.

During the subcuticular phase in susceptible plants, thick hyphae are formed which later develop into a rather massive stroma (Jones and Ayres, 1974; Jorgensen *et al.*,

1993). Following formation of the stroma, the host's underlying cells start to collapse. Later, the pathogen gradually grows into the mesophyll while host cells collapse and disintegrate in advance of the penetrating hyphae. This stage is known as the necrotrophic phase which leads to development of lesions 10-14 days post-inoculation. In contrast, in resistant plants the subcuticular hyphae are sparse and commonly confined to leaf margins. The fungus appears to be unable to terminate the subcuticular phase and invade the underlying cells (Ali, 1974; Jorgensen *et al.*, 1993).

Commencement of the necrotrophic phase in susceptible plants, which is characterised by destruction of host underlying cells, appears to be coincident with a number of other phenomena around 10-14 days post-inoculation. These are:

- Sporulation. This phenomena is also seen in the resistant plants but only to a limited extent (Ali, 1974; Jorgensen *et al.*, 1993).
- Stabilisation of induced electrolyte loss which increases upon infection by the fungus (Jones and Ayres, 1972).
- Drop in CO_2 fixation as a result of epidermis destruction (Jones and Ayres, 1974).

Detection in diseased leaves of fungal phytotoxic including necrosis-inducing peptides (Wevelsiep *et al.*, 1991; Rohe *et al.*, 1995), a cellobioside called rhynchosporoside (Auriol *et al.*, 1978) and a toxic glycoprotein (Mazars *et al.*, 1989b).

1.1.3 Pathogenicity factors

Phytotoxins are the most extensively investigated pathogenicity factors of *R. secalis*. Preliminary evidence on the involvement of toxins in the barley-*R. secalis* interaction comes from histological studies conducted during the necrotrophic phase of the scald. Necrotic lesions start with the collapse of epidermal cells, followed by mesophyll cells and accompanied by swelling and degeneration of chloroplasts in advance of the invading hyphae (Jorgensen *et al.*, 1993; Jones and Ayres, 1974).

Ayesu-Offei and Clare (1971) demonstrated for the first time that *R. secalis*, isolate

SA-1, produced an autoclave-resistant, fast-effective (1-2 h), non-specific toxin(s) in culture media. However, the toxin(s) was not characterised any further (Ayesu-Offei and Clare, 1971).

A later report indicated that some isolates of *R. secalis* produced a low molecular weight, non-specific, heat resistant toxin in media. The toxin, a cellobioside of 1,2-propanediol called rhynchosporoside, was also found in infected susceptible plants. However, the authors did not explain why resistance and susceptibility to the toxin did not correspond to resistance and susceptibility to the fungus (Auriol *et al.*, 1978).

Isolates of *R. secalis* were also shown to produce a high molecular weight (HMW) glycoprotein toxin(s) in media and in barley (Mazars *et al.*, 1989a). The glycoprotein(s) was characterised as a heat and mild acid-resistant, non-specific toxin with a molecular size of ~400 kDa (Mazars *et al.*, 1989b). The toxin(s) appear to generate the scald symptoms at concentrations as low as 5 μg/l. At this concentration, disintegration of host cells leads to scald lesions on barley leaves after approximately 48 hours. Histological studies indicated that in response to the toxin, plants blocked their xylem vessels, a phenomenon also observed during infection by the pathogen (Mazars *et al.*, 1989a).

More recent studies by Knogge and co-workers demonstrated that a new class of necrosis-inducing peptides (NIPs) with molecular weights of <10 kDa are produced by at least some *R. secalis* isolates. The peptides appear to be non-specific and cause necrosis within 48 hours when injected into the plants in quantities less than 10 μg per plant (Wevelsiep *et al.*, 1991). As discussed in the next section, one of these toxins (NIP1) also appears to function as the product of the avirulent gene *AvrRrs1*.

1.1.4 Barley defense responses against *R. secalis*

The first line of barley defense against *R. secalis* has been found on the plant surfaces where germination of the fungal spores may be reduced or blocked in resistant plants (Ali, 1974; Hahn *et al.*, 1993). This was suggested to be caused by the presence of preformed inhibitors on the plant surfaces (Ali, 1974).

Penetration of *R. secalis* is known to be associated with the formation of haloes and papillae in the cell walls of the host's underlying epidermal cells. These structural barriers appear to be more prominent and longer lasting in resistant cultivars (Jorgensen *et al.*, 1993). However, since *R. secalis* does not penetrate the plant cells in any stage of the disease cycle (Jorgensen *et al.*, 1993; Lehnackers and Knogge, 1989) the cell wall barriers are less likely to play any role in resistance against this fungus. Alternatively, papillae may contain toxic compounds that diffuse out and affect the penetrating pathogen. Accumulation of peroxidases in barley papillae, as a response to infection by powdery mildew, has been previously shown (Scott-Craig *et al.*, 1995).

Once penetration occurs, avirulent strains of *R. secalis* differ from the virulent ones by their confined growth and inability to invade and kill the host cells in the later stages of disease development. Unlike many other plant/pathogen systems (pathosystems), the hypersensitive response (HR) is not observed in the barley-*R. secalis* interaction. However, a pronounced induction of pathogenesis related proteins (PR proteins, PRPs) in barley challenged with *R. secalis* has been shown. In 1993, Hahn and coworkers showed that genes for a peroxidase and the thaumatin-like protein Hv-1 were strongly induced in barley attacked by *R. secalis*. The induction, detectable as early as 9 hours postinoculation, was faster and more pronounced in the resistant cultivars compared to the susceptible ones (Hahn *et al.*, 1993). Later, Roulin and coworkers (1997) found that various isoforms of another PRP, β(1-3) glucanase, were also induced when barley was inoculated with the pathogen. Both the gene and encoded protein were studied by these authors and showed to be induced as early as one day post inoculation in a resistant cultivar. The induction did not occur until day 2 postinoculation in a susceptible cultivar of barley (Roulin *et al.*, 1997). The antimicrobial peptide thionin has also been reported to be induced in barley infected by *R. secalis* (Broekaert *et al.*, 1997). The increase was found to be specifically induced by an avirulent, but not by a virulent strain of the pathogen.

1.1.5 Induction of resistance in the *R. secalis* / barley interaction

Recently it was shown that some isolates of *R. secalis* produced small toxic peptides, NIPs, which non-specifically induced scald-like necrotic lesions in barley cultivars. One of these peptides, NIP1, appeared to be produced only in fungal isolates that were avirulent on *Rrs1*-bearing barley cultivars such as Atlas 46. The isolates were virulent on the near-isogenic cultivar Atlas, which did not contain the *Rrs1* gene. When purified NIP1 was applied to resistant cultivars containing *Rrs1*, PRPs were induced with similar kinetics as in the response to the avirulent fungal strains (Hahn *et al.*, 1993). It was concluded that NIP1 was the product of an avirulent gene in *R. secalis* and that NIP1 specifically activated defense responses in barley cultivars carrying the *Rrs1* gene (Hahn *et al.*, 1993).

In 1995, Rohe and coworkers reported that they successfully introduced the *nip1* gene into a virulent strain of *R. secalis* and that the new transgenic pathogen was avirulent to barley cultivars that express *Rrs1*. Hence, it was concluded that barley lines that express the resistance gene *Rrs1* have developed a natural recognition mechanism that is able to identify a pathogen by detecting one of its pathogenicity factors, a phytotoxin (Rohe *et al.*, 1995).

1.2 Resistance and induction of resistance in plants

1.2.1 Compatible and incompatible plant/pathogen interactions

Compatibility in a plant/pathogen interaction is characterised by susceptibility in the plant and virulence in the pathogen. Under this condition, the pathogen infects and colonises the plant which results in tissue damages known as disease symptoms. An incompatible interaction, on the other hand, involves a resistant plant and an avirulent pathogen. This interaction, associated with an effective host defense response, is characterised by reduced pathogen ingress and colonistion.

Scald resistance in barley generally follows the "gene-for-gene" concept (Hahn *et al.*, 1993). According to this hypothesis, for each resistance (*R*) gene in the plant there is a complementary avirulence (*Avr*) gene in the pathogen. Resistance is manifested by the resistance gene only if the corresponding avirulence gene is expressed in the interacting pathogen. This implies a molecular recognition mechanism. The process is believed to be initiated by recognition of the avirulence gene products, namely elicitors, by the plant resistance gene product, *i.e.* the hypothetical receptor (Lindsay *et al.*, 1993; Yashikawa *et al.*, 1993).

The matching gene pairs, *R-Avr*, determines the type and magnitude of the expressed incompatibility. Resistance to different pathotypes in plants could be controlled by *R* genes at different loci. A *R-Avr* gene pair generally dominates over gene pairs that otherwise condition for compatibility. In addition, the *R-Avr* gene pairs that confer stronger incompatibility generally show a non-allelic dominance (epistasis) over other gene pairs conferring a lower degree of incompatibility. However, additive effect (gene dosage) has also been observed in both plants and diploid/dikaryotic fungal pathogens (Crute and Pink, 1996).

It is known that, in addition to *R* genes, there are other genes whose functions are important in expression of resistance. It is believed that these genes encode components of the signal transduction pathway, the pathway that leads to expression of defense genes (Crute and Pink, 1996).

Activation of plant defense responses, *i.e.* induction of PRPs, phytoalexins, etc., is believed to be the result of a cytoplasmic signal triggered by formation of the hypothetical elicitor/receptor complex. While the receptor molecules involved in this process are hypothesised to be membrane proteins, elicitors are likely to be either cell wall components or secreted microbial metabolites (Dixon *et al.*, 1994). These are further explained in the next sections.

1.2.2 Elicitors

Like the recognition itself, elicitors can be either non-specific or specific. The non-specific elicitors, generally cell-wall derivatives (Yashikawa *et al.*, 1993), are recognised by a number of different plants spread across unrelated species. Examples of non-specific elicitors, comprising the majority of the known elicitors, are branched β-glucans from *Phytophthora megasperma* f.sp. *glycinea* and chitinous substances found in a variety of fungi including *P. megasperma* f.sp. *glycinea* (Yashikawa *et al.*, 1993). Non-specific elicitors are believed to be responsible for general or non-specific resistance in plants.

Besides the ability to detect non-specific elicitors found in many pathogens, plants appear to be able to recognise specific elicitors. These compounds are expressed by certain races or pathovars of a pathogen only. The race/cultivar specific elicitors are believed to be direct or indirect products of avirulent genes, and are capable of inducing a resistance response. This resistance, mediated by specific recognition of the elicitors, is explained according to the "gene-for-gene" concept (Flor, 1971). Certain cell-wall glycoproteins from *P. megasperma* f.sp. *glycinea* and from *Colletotrichum lindemuthianum* and cellular envelopes from *Pseudomonas syringae* pv. *glycinea* are amongst the first elicitors reported to function in a specific manner (Yashikawa *et al.*, 1993). More recently, it was shown that the *Pto* resistance gene in tomato is responsible for mediation of resistance against some races of *P. syringae* pv. *tomato*, the causal agent of tomato bacterial speck. The resistance is mediated only if the bacteria express the avirulence gene, *AvrD*. In other words, both *Pto* and *AvrD* gene products are necessary to give rise to an incompatible reaction in this pathosystem. The *AvrD* gene product, void of any elicitor activity by itself, is believed to transform a constitutively

present bacterial compound into syringolide elicitors (Yashikawa *et al.*, 1993).

Another example of specific elicitors is reported in the interaction of tomato with *Cladosporium fulvum*, the causal agent of tomato leaf mould. In 1982, DeWit and Spikman reported the presence of a specific elicitor in the intercellular fluids of tomato leaves infected with *C. fulvum*. The elicitor, highly expressed *in planta*, was found to be a peptide of 27 amino acids that induces a hypersensitive reaction in tomato plants that express the *Cf9* resistance gene. The successful transformation of a virulent isolate into an avirulent one by introduction of the *Avr9* gene was solid evidence for the role of avirulence gene and elicitor in this system (DeWit *et al.*, 1992).

In plant/virus interactions, it has been speculated that the viral coat protein serves as the signal molecule or elicitor (Yashikawa *et al.*, 1993). In tobacco plants that express the *N* resistance gene, the avirulent tobacco mosaic virus (TMV) strain induces a hypersensitive response and hence resistance. It has been shown that the virulent line differs from the avirulent one only in one nucleotide in the coat protein coding region of the viral gene. This results in the expression of a phenylalanine residue in the avirulent strain instead of a serine in the coat protein sequence of the virulent strain.

As for the role of specific avirulence gene products in pathogens, virulence and/or cell maintenance have been suggested (Staskawicz *et al.*, 1995; Lindsay *et al.*, 1993). Recently, an association between avirulence and pathogenicity has been shown in the Arabidopsis pathogen *P. syringae* pv. *maculicola*. Isolates of *P. syringae* that lack the *AvrRpm1* gene do not induce any HR in resistant Arabidopsis cultivars. However, they also appear to be unable to infect either the resistant or previously susceptible cultivars. This lack of pathogenicity on both resistant and susceptible cultivars is taken to mean that the *AvrRpm1* gene is required by *P. syringae* pv. *maculicola* to infect Arabidopsis (Ritter and Dangel, 1995).

The dual role of elicitors is also exemplified by toxicity of cryptogein, a proteinaceous elicitor from *Phytophthora cryptogea*. While 0.1 μM cryptogein is deadly to tobacco cells, at lower concentrations it elicits typical defense responses such as medium alkalinization, oxidative burst, hypersensitive-like lesions and induced resistance (Blein *et al.*, 1991; Viard *et al.*, 1994). NIP1, produced by some isolates of *R. secalis*, also seems to fit into this suggested dual role (see section 1.1.5). The *AvrRrs1* gene product, NIP1, is both a non-specific toxin and also a specific elicitor to

barley plants with *Rrs1* (Hahn *et al.*, 1993; Rohe *et al.*, 1995).

It, therefore, appears that although during evolution pathogens acquired the ability to produce phytotoxins as their pathogenicity factors, some plants used this opportunity to develop a matching mechanism to identify the toxic products before they reach a toxic concentration. This rendered the phytotoxins an elicitor role in the resistant plants.

1.2.3 Receptors

The existence of cell surface receptors, with a role in the initiation of cellular/molecular signaling, has now been widely reported in plants. However, the study of the structure of receptors and transmembrane signaling in plants is still in its infancy.

Recognition of non-specific elicitors by receptor-like binding sites on plant cell membranes has been speculated in a number of systems. These studies have been mostly with elicitors from *Phytophthora* spp. (Yashikawa *et al.*, 1993; Dixon *et al.*, 1994). A recent study indicated that there were about 2900 binding sites per parsley protoplast for a glycoprotein elicitor from *P. megasperma* f.sp. *glycinea* (Hahlbrock *et al.*, 1995). The high affinity binding, detected using radioiodinated elicitor in binding assays, appears to be competitive, reversible, saturable and highly specific in terms of both elicitor structure and plant species.

The presence of a specific receptor on tomato cells for the *Avr-9* gene product from *C. fulvum* has also been shown. However, the AVR-9 peptide appears to bind equally to membrane proteins from both *Cf-9* and *Cf-0* expressing plants. Since the AVR-9 peptide does not induce a defense response in *Cf-0* expressing plants, it was concluded that binding alone may not be consequential for recognition (Staskawicz *et al.*, 1995).

Predicted protein structures based on cDNA sequences and similar models from animal cells have been extensively used in the study of putative plant receptors. Receptor-like protein kinases (RLKs) on the plant cell surfaces basically consist of three structural domains (Walker, 1994): a large extracytoplasmic domain, a single trans-membrane spanning segment, and a cytoplasmic domain with serine and/or threonine kinase activity. RLKs are divided into three categories based on their extracytoplasmic

region: the S-domain class, the leucine-rich repeat (LRR) class and the epidermal growth factor-like repeats. Apart from a S-receptor kinase, postulated to mediate self-recognition during pollination, the biological function of the other RLKs is unclear (Walker, 1994; Song *et al.*, 1995). LRRs are known, however, to function as extracellular receptors and to be involved in protein-protein interactions in many organisms (Staskawicz *et al.*, 1995; Walker, 1994). They are also the most common gene products from cloned resistance genes in plants (Staskawicz *et al.*, 1995).

1.2.4 Resistance determined by factors other than elicitor/receptor interaction

Other factors besides elicitors and receptors are occasionally involved in the "gene-for-gene" interaction. For instance, suppressors are known to interfere with the background elicitor/receptor interaction to suppress the resistance responses by the plant. Germination fluid of *Mycosphaerella pinodes* (telemorph *Ascochyta pinodes*), a pea pathogen, is known to contain low-molecular weight peptides capable of furnishing seemingly non-virulent pathogens with virulence. The peptides, when administered with non-pathogenic fungi such as *Stemphylium sarcinaefurume* and *Alternaria alternata*, appear to render them pathogenic on their non-host plants. This suppression of resistance, observed in pea and other legumes, leads to an absence of phytoalexin accumulation. The fungal peptides, which are known as non-specific suppressors, and the suppression phenomena are now explained with a model called the suppressor model. The interaction of *Phytophthora infestans* with its host, potato, also appears to fit this model but this suppressor functions in a specific manner, acting only on certain cultivars. In addition, while high molecular weight non-specific elicitors from *P. infestans* appear to play a dominant role in the induction of disease, some isolates of the pathogen still manage to overcome the plant resistance. These virulent isolates are known to release low molecular weight glucans that compete with the elicitors for binding sites on the plant's putative receptors. Hence, lack of recognition in some potato plants renders them susceptible to attack by these pathogens. This susceptibility is known to be associated with lack of phytoalexin accumulation (reviewed in Yashikawa *et al.*, 1993).

1.2.5 Signal transduction pathway in plants responding to pathogens

It appears that a number of biochemical events link elicitor perception on the cell surface to the activation of defense genes in the plant's nucleus. Most of the information on the mechanism of plant/pathogen interactions comes from the more convenient system of suspension cell cultures which have been treated with elicitors. However, some phenomena such as HR, callose accumulation and spatial relationships may not be observed using this method (Hahlbrock *et al.*, 1995; Tenhaken *et al.*, 1995).

Major biochemical events, following contact of elicitor on plant cell surface and leading to induction of defense genes, are reviewed below.

1.2.5.1 Membrane depolarisation

It has been shown that depolarisation of the plant's plasma membrane (PM) is one of the first detectable responses after the plant is challenged with an elicitor. Transient influx of Ca^{2+} and H^+ and efflux of K^+ and Cl^- are observed 2-5 min post-elicitation (Dixon *et al.*, 1994; Hahlbrock *et al.*, 1995). Addition of elicitors to plant cells appears to produce a concentration-dependent depolarisation in the plant's PM in less than 2 minutes. A subsequent repolarisation, less than 10 min later, gives the appearance of an action potential to this phenomenon (Mayer and Ziegler, 1988; Pelissier *et al.*, 1986). Ion channel inhibitors, that stop flux of one or more ions, and kinase inhibitors also generally block other plant responses to the elicitors. In particular, the role of Ca^{2+} appears to be crucial. Not only does a Ca^{2+} ionophore appear to induce plant defense responses but in general defense responses are abolished in the absence of Ca^{2+} in the media. Increased cytosolic Ca^{2+} following elicitation has also been demonstrated (Dixon *et al.*, 1994; Lindsay *et al.*, 1993; Hahlbrock *et al.*, 1995). Cellular acidification, which is not well-understood, and alkalinisation of the media immediately after elicitation are some other events linked to the ion flux (Blein *et al.*, 1991; Dixon *et al.*, 1994). Transient activation of the plasma membrane ion channels is, therefore, the first important event in the cascade of events toward expression of plant defense responses.

1.2.5.2 The oxidative burst

In many systems, almost immediately after activation of the PM ion channels, a rapid burst of the reactive oxygen intermediates (ROIs) is observed. This oxidative burst, mediated by O_2^-, H_2O_2 and $OH^\bullet$, is a potential antimicrobial event with a central role in the expression of HR (Dixon *et al.*, 1994; Tenhaken *et al.*, 1995). In a suspension cell culture, such as soybean culture, ROI appears 2-3 min post elicitation and results in oxidative cross-linking of the structural proline- and hydroxyproline-rich glycoproteins (PRGPs and HRGPs) rendering them insoluble. This phenomenon, which makes cell walls more resistant to microbial invasion, has also been observed in wound sites and in the interaction of soybean with *P. syringae* pv. *glycinea* carrying the *AvrD* gene. No such structural changes are observed in the corresponding compatible interaction (Dixon *et al.*, 1994). It appears that ROI plays a pivotal role in the orchestration of the HR in plant/pathogen systems (Tenhaken *et al.*, 1995). Recent observations indicate that the oxidative burst may include two successive phases, a rapid initial burst followed by a longer and slower second burst (Chandra *et al.*, 1996). While non-specific elicitors and compatible interactions lead to either no burst or only the first phase, incompatible interactions appear to be associated with the second burst only.

1.2.5.3 Protein phosphorylation

Protein phosphorylation appears to play an extensive role in signal transduction (Dixon *et al.*, 1994). While detectable levels of protein phosphorylation generally occur 5 to 30 min post-elicitation and after the oxidative burst, changed patterns of protein phosphorylation in the plant cell's cytoplasm, nucleus and membrane may be observed as soon as 1-5 min post-elicitation. Kinase inhibitors, K252a and staurosporin, appear to stop alkalinization of the media, ROI, production of phenylalanine ammonia-lyase (PAL), production of ethylene and defense responses. The inhibition is reported to occur even part way through the elicitation, indicating a need for on-going phosphorylation to maintain the activated status (Chandra and Low, 1995; Felix *et al.*, 1991). The reversal of phosphorylation upon removal of elicitors and its dependency on Ca^{2+} have been shown in parsley and soybean. In some cells, treatment with different elicitors (chitin oligomers and xylanase) results in phosphorylation of the same protein

but different responses (Dixon *et al.*, 1994).

1.2.5.4 Gene translation

Translation of defense related genes starts as soon as 5 to 10 min post-elicitation. Hence, it is believed that only a few biochemical reactions separate the perception of pathogens to activation of the genes (Lindsay *et al.*, 1993). Although details of this causal relationship are not clear, there is evidence to conclude that a system similar to that of vertebrates and insects exists in plants. In both vertebrate innate immunity and insect immunity responses, activation of cell surface receptors and subsequent protein phosphorylation lead to the release and translocation of transcription factor(s) into the nucleus. The last stage of the signal transduction pathway occurs in the nucleus where transcription factors function with *cis*-elements of appropriate gene promoters to mediate transcription and hence expression of the genes. Insect and mammalian cytoplasmic domains of receptors and transcription factors show significant homology (Dixon *et al.*, 1994; Hultmark, 1994). In plants, it has been shown that two DNA binding proteins in bean cells, KAP1 and KAP2, migrate to the nucleus after elicitation. The proteins, capable of specifically recognising elements of a chalcone synthetase gene (*chs 15*), are believed to function as the means to achieve rapid induction of transcriptional activation of the gene (Dixon *et al.*, 1994; Lindsay *et al.*, 1993). Protein phosphorylation is the mechanism suggested to mediate the release of putative transcription factors from their cytoplasmic inhibitors in this system (Dixon *et al.*, 1994).

In addition to the biochemical and enzymatic aspects, cytoplasmic changes are also reported to be associated with the onset of plant resistance induction in response to pathogen invasion. Changes in the cytoskeleton arrangements have been observed after fungal pathogens attempt to penetrate plant cells. The responses include local depolarisation of the microtubular network and consequent migration of the cell cytoplasm and the nucleus to penetration sites. These are immediately followed by cell wall apposition and the hypersensitive response. The latter phenomena happen almost at the same time that defense genes are activated (Gross *et al.*, 1993). Following is a summary of the events in the signal transduction pathway and an estimate of the timing (Dixon *et al.*, 1994):

- Ion flux (H^+,Ca^{2+},Cl^-,K^+) → starts at 2-5 min
- Oxidative burst → immediately thereafter
- Protein phosphorylation → within 5-30 min
- Gene activation/deactivation → immediately thereafter

1.3 Plant defense responses

Agrios (1988) suggested that plant defense responses may be classified into two groups, structural barriers and biochemical defense (Agrios, 1988). In reality, structural barriers, exemplified by callose and PRGP precipitation, are also types of biochemical responses but with a different mode of action. Nevertheless, biochemical defense responses, but not the structural barriers, are believed to play an important role in the barley-*R. secalis* interaction (Lehnackers and Knogge, 1989; Hahn *et al.*, 1993). A review of plant defense responses, with special reference to inducible biochemical responses, is presented in this section.

1.3.1 Classes of induced resistance

Inducible defense responses in plants may be divided into three classes based on the timing, mechanism and scope of the expressed resistance: (1) immediate, (2) local and (3) systemic responses.

The immediate responses, are exemplified by the hypersensitive response, oxidative burst (Dixon *et al.*, 1994) and start of callose and phenolic deposition that later form haloes and papillae (Hahlbrock *et al.*, 1995). Although the cascade of events leading to HR is rather unclear at present, the other immediate responses appear to be independent of gene transcription (Dixon *et al.*, 1994; Lindsay *et al.*, 1993). This independency leads to a rapid expression of defense, compared to transcription-dependent local and systemic responses. The immediate plant responses generally constitute the first line of induced defense against pathogens. It is evident that in pathosystems where HR occurs, it plays a major role in expression of resistance and restriction of the pathogen (Hahlbrock *et al.*, 1995). While HR is characteristic of many incompatible reactions in plants, local and systemic responses are not necessarily confined to incompatible reactions. That is, they may also be observed, generally to a lesser extent, in susceptible plants (Staskawicz *et al.*, 1995; VanLoon, 1999).

Local and systemic responses are expressed by activation of pathogenesis-related genes and deactivation of non- pathogenesis-related genes (Hahlbrock *et al.*, 1995). Local gene expression generally leads to accumulation of numerous PR and structural

proteins as well as metabolites such as phytoalexins. While local responses are limited to the vicinity of the infection site, systemic gene activation occurs in distant tissues, possibly including the whole plant. Similar to the local gene activation, systemic responses also result in a gradual accumulation of PR proteins and enzyme activity (Ye *et al.*, 1989; Hahlbrock *et al.*, 1995). Local and systemic defense genes have been shown to be activated independent of HR induction (Jakobek and Lindgren, 1993; Keller *et al.*, 1996). It is, however, unclear if local and systemic responses may also be induced independent of each other. Compared to the rapid HR, local and systemic responses are rapid to intermediate and slow to intermediate respectively.

Almost all the PR proteins and phytoalexins have been shown to have one or another type of antimicrobial activity (see below). In pathosystems where immediate responses do not play an important role in resistance against pathogen ingress, such as that of barley-*R. secalis*, the role of local defense responses is probably crucial. In barley a large number of PR proteins have been shown to exist and be induced in response to different pathogens (see below). However, no phytoalexin has been isolated from this plant (Oku *et al.*, 1994).

PR proteins belong to the larger class of defense-related proteins. They accumulate in response to pathogen attack, elicitors, sensitisers (such as salicylic acid and synthetic sensitisers) and sometimes physical injury. They are also found in seed and storage organs as part of the plant's natural developmental pattern (Bowles, 1990). Study of PR proteins constitutes a significant part of this thesis. These are reviewed in more details in the reminder of this section.

1.3.2 Pathogenesis-related proteins

The term pathogenesis related proteins (PRP) was first coined in 1980 (Antoniw *et al.*) for what was formerly known as *b*-proteins. The concept, itself, has been understood since 1970. A PR protein is defined as a plant protein that is induced in response to pathological or related environmental challenge (Cutt and Klessig, 1992; Van Loon, 1999). However, PR genes are shown to be under developmental and tissue specific control as well as stress regulation. While developmental and tissue specific

controls usually regulate these genes individually, a coordinated expression of many PR proteins occurs following stress (Cutt and Klessig, 1992).

When qualifying a protein as "pathogenesis-related", the characteristic of induction in response to pathological challenge is the sole requirement (Van Loon, 1999). Hence, this characteristic takes priority over other identifying characteristics such as sequence similarity or enzymatic activity. Nevertheless, antimicrobial activity, on top of induciblity, has been frequently used as an additional argument to qualify known or novel proteins as PRPs (Mayer *et al.*, 1996). Other commonly occurring characteristics of PR proteins include a high stability at low pH and a remarkable resistance to proteinase (Heitz *et al.*, 1999). These probably help the proteins to resist the unfavorable extracellular conditions and to resist microbial degradation.

PR proteins commonly accumulate in what Matile (1975) defined as the "lytic compartment" of plants, being the vacuoles and the apoplast (quoted in Boller, 1987). A characteristic apparently shared by all the PR proteins is the presence of basic and acidic isoforms. Following pioneer studies in tobacco, it was believed that subcellular localization of PRPs was correlated with their isoelectric points (pI). Negatively charged proteins were suggested to be extracellular (intercellular) while the positively charged isoforms were thought to accumulate intracellularly essentially in the vacuole (Bowles 1990; Ward *et al.*, 1991). However, more recent studies showed that localisation of many PRPs is independent of their pI (Linthorst, 1991; Collinge *et al.*, 1993; Roulin *et al.*, 1997). In addition, the cytosol is suggested as a possible compartment for certain PRPs (Xu *et al.*, 1994).

PR proteins are traditionally grouped based on similarities in molecular weight, serological and nucleotide or amino acid similarities and enzymatic activity (Cutt and Klessig, 1992; Van Loon, 1999). The classification presented here is based on the grouping proposed in 1994 by Van Loon and coworkers (quoted in Van Loon, 1999) as well as that by Bowles (1990) and others (see below). Pathogenesis-related proteins 1 to 9, small antimicrobial peptides and the cell wall structural proteins are reviewed here.

1.3.2.1 Pathogenesis-related protein 1

Pathogenesis-related protein 1 (PRP-1, PR-1 protein) was one of the first PR proteins

reported. PR-1 proteins are generally referred to as the most abundantly induced PR proteins in plants (Ward *et al.*, 1991; Muradov *et al.*, 1993; Buchel and Linthorst, 1999). PR-1 proteins are generally around 14 kDa. No glycosylation is reported in these proteins (Cutt and Klessig, 1992). No sequence homology between PR-1 proteins and any other proteins has been identified and until recently no activity was associated with these proteins. In 1995, Niderman and coworkers reported that three PR-1 proteins isolated from tomato inhibited the germination of *Phytophthora infestans* zoospores (Niderman *et al.*, 1995). More recently, a basic PR-1 from broad bean was shown to strongly inhibit the differentiation of rust infection hyphae and hence stop the disease (Rauscher *et al.*, 1999).

The levels of two basic isoforms of PR-1 were found to increase strongly in response to pathological stimuli in barley (Bryngelsson *et al.*, 1994). Other forms, including some acidic isoforms may also exist (Bryngelsson *et al.*, 1994; Moradov *et al.*, 1994). The characterized PR-1 proteins have been shown to be extracellular and have 140 amino acids (aa) in the mature peptide. The PR-1 proteins have molecular weights of ~15.0 and ~15.2 kDa. The isoelectric points for these were estimated to be 10.5 and 11.0 respectively (Bryngelsson *et al.*, 1994). Characterised barley PR-1 proteins are blocked at the N-terminus as judged by Edman degradation.

Barley PR-1 proteins have been shown to increase markedly in response to powdery mildew attack from 6-10 hours postinoculation at the mRNA level and from 3 days postinoculation at the protein level. A marked increase in the gene translation was also recorded in response to treatment with ethylene, salicylic acid, methyl jasmonate, 2,6-dichloro-isonicotinic acid and irradiation with UV but not in response to wounding (Muradov et *al.*, 1993; Muradov et *al.*, 1994; Bryngelsson *et al.*, 1994).

1.3.2.2 Pathogenesis-related protein 2

Plant PR-2 proteins endohydrolyse β(1-3) glucans (Leubner-Metzger and Meins; 1999). β(1-3) glucanases and chitinases (see below) were among the first PR proteins reported to have *in vitro* enzymatic activity (Kauffman *et al.*, 1987). They are also probably the best characterised PR proteins mainly because of their strong and synergistic *in vitro* antifungal activity (Cutt and Klessig, 1992). Substrates of these

enzymes constitute major parts of many fungal cell walls. Accordingly, the antifungal activity of both proteins is believed to be caused by hydrolysis of cell wall components of fungi (Bowles, 1990; Malehorn *et al.*, 1993; Sela-Buurlage *et al.*, 1993). Plant β(1-3) glucanases vary in molecular weight from 30 to 40 kDa (Bol *et al.*, 1990, 1990; Cutt and Klessig 1992).

Characterised β(1-3) glucanases in barley include isoforms GI to GVI and ABG2. In response to pathogen attack or elicitors, many studies point to induction of chiefly one basic β(1-3) glucanase isoform (Jutidamrongphan *et al.*, 1992; Xu *et al.*, 1992; Stevens *et al.*, 1996; Reiss and Bryngelsson 1996; Kaku *et al.*, 1997; Roulin *et al.*, 1997). This has been shown to be the extracellular 32.3-kDa GII in most cases. In addition, an intermediate induction for the intracellular isoform GI, a weak induction for the extracellular GIII and no induction for GIV, GV or GVI were recorded (Roulin *et al.*, 1997). In one instance, strong induction of the 32.6-kDa acidic isoform ABG2 has also been shown (Malehorn *et al.*, 1993).

Increase in the β(1-3) glucanase activity in barley leaf extracts has been reported to begin at 1 (Jutidamrongphan *et al.*, 1992) or 2 (Roulin *et al.*, 1997) days postinoculation depending on the experimental system.

1.3.2.3 Pathogenesis-related protein 3 and 8

Plant PR-3 and PR-8 (and also PR-11) proteins are all chitinases but classified in different groups because of sequence homology, gene/protein structure and serological characteristics (Linthorst, 1991; Kragh *et al.*, 1993; Colling *et al.*, 1993; Neuhaus, 1999). PR-3 proteins include chitinases class I, II and some other minor groups. PR-8 proteins include chitinases class III.

Class I chitinases are characterised by two distinct domains in their primary structure: a cysteine-rich chitin-binding domain at the N-terminus and a conserved chitinase domain. Class II chitinases are very similar to those in class I but lack the chitin-binding domain. Hence, class II chitinases are smaller molecules with significantly lower affinity for chitin. The chitin-binding domain, also known as the hevein domain, does not seem to be necessary for chitinase activity. However, in tobacco and barley that have both types of chitinases, class I enzymes have markedly

more specific activity than those of class II (Linthorst, 1991; Kragh *et al.*, 1993). Class III chitinases lack the hevein domain and their hydrolysing domains are also very different from that of either class I or II.

There is a close relationship between chitinase (EC 3.2.1.14) lysozyme (EC 3.2.1.17) and chitisonase (EC 3.2.1.99) (Boller, 1987; Dixon and Harrison, 1990; Colling *et al.*, 1993; Pozo *et al.*, 1998). Many plant chitinases are shown to possess lysozyme activities and hence hydrolyse peptidoglycans, a significant component of bacterial cell walls. Some plant chitinases were also shown to hydrolyse chitosan. This is a partially or fully deacetylated derivative of chitin found in the cell wall of many fungi (Grenier and Asselin 1990; Pozo *et al.*, 1998). The multifunctional nature of chitinases, although widespread (Mayer *et al.*, 1996), may not be universal. Some plant proteins have been shown to be either chitinases or chitosanases (Grenier and Asselin, 1990; El Ouakfaoui and Asselin, 1992). Regardless of this, none of the potential chitinase substrates are detected in plants and hence the enzyme is believed to be essentially targeted to non-self organisms (Boller, 1987; Oswald *et al.*, 1992). Plant chitinases vary in molecular weight from 25 to 36 kDa (Boller, 1987; Bol *et al.*, 1990, 1990; Colling *et al.*, 1993).

Amongst 4 detected isoforms of barley leaf chitinase, only two were found to increase in response to pathogen attack. These are an acidic 26-kDa chitinase (Reiss and Bryngelsson 1996) and the 25-kDa basic chitinase 2 (Kragh *et al.*, 1993). The acidic chitinase has not been further characterised but its induction has been recorded in response to *Puccinia hordei*, *Ery*siphe *graminis* (both 10 days PI) and to *Drechslera teres* (4-5 days PI) (Reiss and Bryngelsson 1996). Chitinase 2 is an extracellular class II chitinase that has been found to increase from day 4 after inoculation with *Erysiphe graminis* f.sp. *hordei* (Kragh *et al.*, 1993). No induction of other barley chitinases have been reported.

Other studies have also verified the induction of chitinases in response to pathogen attack in barley (Boyd *et al.*, 1994a, and 1994b; Vallelian-Bindschedler *et al.*, 1998) but the isoforms and/or subcellular localisation of the enzyme were not investigated in these studies.

1.3.2.4 Pathogenesis-related protein 4

PR4 proteins are a group of small proteins with molecular weights ranging from 13 to 15 kDa (Cutt and Klessig 1992; Linthorst, 1991). PR-4 proteins are homologous to the potato wound-induced proteins, Win1 and Win2, and also to the rubber tree antifungal protein hevein (Hejgaard *et al.*, 1992; Svensson *et al.*, 1992; Neuhaus, 1999). PR4 proteins display a compact structure with 6 cysteine residues in their structure (Svensson *et al.*, 1992; Neuhaus, 1999). The proteins display *in vitro* antifungal activity but the mechanism is yet to be determined. No enzymatic activity has been detected for almost any of isolated PR-4 proteins (Hejgaard *et al.*, 1992; Cutt and Klessig 1992). The only exception is a tobacco PR-4 protein, reported to have *in vitro* chitinase activity (Neuhaus, 1999).

Up to three isoforms of PR-4 protein have been identified in barley leaves (Kragh *et al.*, 1990; Hejgaard *et al.*, 1992). All these were found to be ~14 kDa, basic, extracellular and with strong affinity for chitin binding (Hejgaard *et al.*, 1992). However, the chitin-binding affinity appeared to be unrelated to protein domains rendering a similar affinity in PR-3 proteins (Hejgaard *et al.*, 1992). No chitinase activity was detected in these proteins. The proteins were shown to increase from day 3 after inoculation with powdery mildew or following treatment with an elicitor, nickel chloride. Two of the isoforms were further characterised and shown to be 13.6 and 13.7 kDa (Hejgaard *et al.*, 1992).

1.3.2.5 Pathogenesis-related protein 5

PR-5-type proteins share one universal characteristic, a large (~65%) sequence homology to the sweet-tasting 22-kDa protein thaumatin (Cutt and Klessig 1992). There is also 57% homology at the amino acid level with the maize bifunctional amylase/proteinase inhibitor (Richardson *et al.*, 1987). Furthermore, these proteins have a limited amount of sequence similarity to the 5' upstream region of PR-1 genes (Cutt and Klessig 1992). PR-5-type proteins are 15-30 kDa (Grenier *et al.*, 1999) and while most of them are simply referred to as thaumatin-like (TL) proteins some are called osmotin, permatin or similar names because of particular phenomena associated with their expression or function (Linthorst, 1991; Vigers *et al.*, 1991). Most TL

proteins display significant *in vitro* antifungal activity (Grenier *et al.*, 1999). The proteins are also known to have strong synergism with nikkomycin, a nucleoside-peptide antibiotic and a chitin synthase inhibitor (Roberts and Selitrennikoff, 1990; Hejgaard *et al.*, 1991; Vigers *et al.*, 1991; Cheong *et al.*, 1996). Salts at concentrations about 100 millimolar suppress the antifungal activity of TL proteins. Until recently no enzymatic activity has been associated with TL proteins (Roberts and Selitrennikoff, 1990; Hejgaard *et al.*, 1991; Cheong *et al.*, 1996). A model proposed by Roberts and Selitrennikoff (1990) suggested that TL proteins exert their antifungal activity by affecting the cell membrane and that salts suppress the antifungal activity by relieving the osmotic pressure that otherwise burst the fungal cells. However, TL proteins were recently demonstrated to lyse fungal cell walls and bind to β(1,3) glucans (Trudel *et al.*, 1998a; Trudel *et al.*, 1998b). Furthermore, Grenier and coworkers, in 1999, showed that some TL proteins endohydrolyse certain types of β(1,3) glucans *in vitro*.

Up to 6 different TL proteins have been identified in barley. Amongst these, only induction of three proteins, Hv-1, IFW19 and Barperm1, has been reported (Bryngelsson and Green, 1989; Hahn *et al.*, 1993; Grenier *et al.*, 1999; Skadsen *et al.*, 2000). Increased levels of barley TL proteins have also been reported by other researchers including Boyd *et al.* (1994a and 1994b), Vale *et al.* (1994) and Vallelian-Bindschedler *et al.* (1998). However, from these reports it could not be concluded if the induced proteins refer to any of the known barley TL-proteins.

1.3.2.6 Pathogenesis-related protein 6

PR-6 proteins are proteinase inhibitors (PIs) (Heitz *et al.*, 1999). These are a diverse group of proteins with molecular weights ranging from 8 to 22 kDa. There are four classes of PIs, including serine-, cysteine-, asparatic- and metallo- proteinase inhibitors. PIs, with their one or more active sites, specifically bind to protease and hence stop the enzymes from further proteolytic activity.

Barley contains a cysteine-rich peptide related to the Bowman-Birk subgroup of serine-PI (Stevens *et al.*, 1996). The gene associated with this peptide, *bsi1*, has been found to be induced in barley seedlings challenged with *Stagonospora* (*Septoria*) *nodorum*.

1.3.2.7 Pathogenesis-related protein 7

PR-7 proteins are endoproteinases (Van Loon, 1999). However, with the exception of a tomato proteinase, P69, little information is available about these proteins. P69 is a subtilisin-like protein of serine-proteinase type with two isoforms, P69E and P69F. The gene for P69E is found to be expressed only in roots whereas the one for P69F is expressed only in hydathodes (Vidhyasekaran, 1997; Jorda L. *et al.*, 2000). Recently, a very similar proteinase in potato (Avarova *et al.*, 1999) and a putative cysteine protease in barley root (Schlichter *et al.*, 1998) have also been reported.

1.3.2.8 Pathogenesis-related protein 9

Plant PR-9 proteins are peroxidases, a group of heme- and Ca^{2+}-containing proteins that catalyse the oxidation by H_2O_2 of a wide range of substrates (Chittoor *et al.*, 1999). Peroxidases are one of the most studied plant enzymes and a large number of defense responses are associated with their activity. These include hydroxycinnamyl oxidation/polymerisation, phenol oxidation, polysaccharide cross-linking, lignification, suberization, cross-linking of extensin monomers as well as direct antimicrobial activity (Kerby and Somerville, 1989; Chittoor *et al.*, 1999; Kristensen *et al.*, 1999). Consistent with these functions, most plant peroxidases appear to be targeted at the plant cell wall (Kristensen *et al.*, 1999). Another characteristic of some PR-9 proteins is that they have two co-existing, glycosylated and non-glycosylated, forms. Both forms have been found to display similar enzymatic activity but the glycosylated form has been suggested to be more resistant to degradation by environmental factors (Kerby and Somerville, 1992).

Increased levels of extracellular peroxidase have been shown to be associated with local (Holden and Rohringer, 1985; Scott-Craig *et al.*, 1995; Tamas and Fric, 1995; Young *et al.*, 1995) and systemic resistance (Ward *et al.*, 1991). Simultaneous accumulation of peroxidase and cell wall structural proteins, such as extensins, is suggested to mediate the enhanced defense responses in acquired resistance (Bowles, 1990). This mixture of accumulated PRPs is activated, upon infection, by an oxidative burst which leads to cross-linkage of the structural PRPs and hence increased resistance of plant cell walls against pathogen ingress (see section 1.3.2.10 for more details).

A total of 12 peroxidases were found in barley tissues (Kristensen *et al.*, 1999). Of these, 6 to 7 isoforms, with isoelectric points ranging from 3.8 to 9.6, have been detected in the extracellular space (Kerby and Somerville, 1989; 1992; Kristensen *et al.*, 1999). Most of these proteins were shown to increase in response to infection although detailed studies have been conducted with only a few of them (Kerby and Somerville, 1989; Kristensen *et al.*, 1999).

Studies concerned with the overall levels of PR proteins have also confirmed the induction of barley peroxidases in response to different pathogens including *R. secalis* (Hahn *et al.*, 1993; Vale *et al.*, 1994; Boyd *et al.*, 1994a and 1994b; Vallelian-Bindschedler *et al.*, 1998). However, the isoforms and/or subcellular localisation of the proteins can not be deduced from these studies.

1.3.2.9 Antimicrobial peptides

These are a group of plant peptides that because of their molecular size (2 to 10.5 kDa) and other common features are classified together. Generally, these molecules have 4 to 8 disulfide bonds and hence a compact structure that helps them to resist the harsh condition of the extracellular environment (Broekaert *et al.*, 1997). Although many of these peptides have been shown to exert their antimicrobial activity by affecting the plasma membrane, a few were found to inactivate hydrolytic enzymes. Cations, at concentrations less than 10 millimolar, have been found to suppress the antifungal activity of many of these peptides. While many of the antimicrobial peptides are readily extractable from plant seeds, they have also been isolated from other tissues such as leaf and stem. Antimicrobial peptides may be divided into 5 major classes based on amino acid sequence homology and molecular structure. These are thionins, plant defensins, lipid transfer proteins, hevein- and knottin-type peptides, and Ib-AMP-like peptides (Broekaert *et al.*, 1997).

Thionins, originally called viscotoxins, are a large group of basic peptides with molecular weights of ~5 kDa. These peptides have toxic effect on a range of organisms including fungi and bacteria (Bohlmann, 1994). Thionins have 3 to 4 disulfide bonds and are found mainly in cereals. Thionins accumulate in the apoplast and to a lesser extent in vacuoles. While different thionin isoforms are specifically found in leaves and

seeds, no difference has been detected between the apoplastic and vacuolar thionins (Bohlmann, 1994; Broekaert *et al.*, 1997). Amongst plant antimicrobial peptides, leaf-specific thionins have been frequently reported to be induced in barley in response to infection and other related elicitors (Andersen *et al.*, 1992; Bohlmann, 1994; Boyed *et al.*, 1994a and 1994b). Induction in response to an avirulent, but not a virulent strain, of *R. secalis* has also been reported (Broekaert *et al.*, 1997).

Plant defensins, originally called γ thionins, are very similar in toxicity, localisation, isoelectric point, molecular weight and sequence to thionins with 4 disulfide bonds. However, their peptide structure differs from that of thionins (Broekaert *et al.*, 1997; Broekaert *et al.*, 1995). Lipid transfer proteins (LTPs) also have 4 disulfide bonds but their molecular weight is different (~8-10 kDa) (Molina *et al.*, 1993; Broekaert *et al.*, 1997). Moreover, LTPs are located in the cell wall and also in the outer wax layer close to the leaf surface (Broekaert *et al.*, 1997). This suggests that LTPs may be one of the agents responsible for suppressing germination of *R. secalis* conidia on barley leaves first reported by Ali (1974) and later by Hahn and coworkers (1993). Nevertheless, the level of LTP gene translation was found not to change following infection by this pathogen (Broekaert *et al.*, 1997).

Hevein- and knottin-type peptides (~3 – 4 kDa), and Ib-AMP-like peptides (~2 kDa) are amongst the smallest antimicrobial peptides reported in plants. Amongst these, hevein is interesting in its ability to bind chitin although it displays weak antifungal activity (Broekaert *et al.*, 1997).

1.3.2.10 Cell wall structural proteins

These proteins are directly involved in strengthening, repairing or altering the plant cell walls and thus decreasing wall vulnerability to microbial enzymes and mechanical pressure. Major proteins classified in this class include proline- and hydroxyproline-rich glycoproteins (PRGPs and HRGPs), Glycine-rich proteins (GRPs) and enzymes involved in production and/or modification of suberin, lignin, cell wall-bound phenolics and callose (Showalter and Varner, 1989; Bowls, 1990).

Amongst the structural proteins, PRGPs and HPGPs have been studied extensively and detected in the extracellular matrix of many plants (Showalter and Varner, 1989;

Bowles, 1990; Jose and Puigdomenech, 1993). Both PRGPs and HRGPs, like other PR proteins, are under developmental and/or stress regulation. Both are also shown to be able to cross-link and incorporate into the cell wall, a phenomenon triggered by an oxidative burst in conjunction with peroxidase enzymes. The stimulus-dependent cross-linking is mainly through oxidatively coupling of tyrosine residues from two adjacent protein chains. This link forms a novel phenolic amino acid, isodityrosine (Fry, 1982), and lead to insolubilization of the generated polymer. Both PRGPs and HRGPs may be insolubilized *in vitro* by low concentrations of H_2O_2 (Jose and Puigdomenech, 1993; Kjellbom *et al.*, 1997). Other functions suggested for these proteins include formation of nucleation sites for deposition of lignin and agglutination of bacteria by positively charged HRGPs (Showalter and Varner, 1989).

HRGPs are generally heavily glycosylated. This is mainly through a hydroxyproline-O-glycosidic linkage that attaches up to four arabinose molecules to each hydroxyproline residue (Lamport, 1967). Three major groups of HRGP are recognized in the cell wall of plant species: lectins, arabinogalactan proteins (AGPs) and extensins (Jose and Puigdomenech, 1993). Although lectins are widespread proteins in the plant kingdom, lectins that contain hydroxyproline have only been found in the Solanaceae family (Jose and Puigdomenech, 1993; Van Damme *et al.*, 1998). The other two HRGPs, extensins and AGPs, have been detected in both mono- and di-cotyledonous species. Extensins contain 40 to 60% proteinacous moiety whereas protein content of AGPs is only 2 to 10% (w/w). Both AGP and extensin could be present in soluble or cross-linked insolubilized forms (Showalter and Varner, 1989; Jose and Puigdomenech, 1993; Kjellbom *et al.*, 1997). However, extensins have isoelectric points in the range of 10-12 and are generally tightly absorbed to the negatively charged cell wall. AGPs are negatively charged and freely move in the apoplastic fluid (Showalter and Varner, 1989; Jose and Puigdomenech, 1993).

PRGPs, compared to HRGPs, constitute a smaller group of cell wall structural proteins. They represent a vastly diverse group of proteins with some being glycosylated and others not (Jose and Puigdomenech, 1993). Some may even have up to 50% of their proline hydroxylated. Serine that is typically high in HRGP is either absent or at a lower level in PRGPs (Jose and Puigdomenech, 1993).

1.3.2.11 Other PR proteins

There are many more PR proteins that are not reviewed here mainly because they have less relevance to this project. Some of these, like celery mannitol dehydrogenase (Williamson *et al.*, 1995) or barley oxalate oxidase (Gregersen *et al.*, 1997), have been less studied. While others, such as phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) that are involved in the biosynthesis of some phytoalexins and other secondry metabolites such as lignin, have been frequently used as markers of defense response in different plants (Bowles, 1990; Dixon and Harrison, 1990; Strange, 1993;).

1.4 Scope

Many research groups have investigated the phytotoxins, elicitors and other factors related to *R. secalis*, although less attention has been paid to plant defense responses against this pathogen. Only three reports were found in which barley reactions in response to infecting *R. secalis* were analysed using molecular tools (see Section 1.1.4). These studies identified the enhanced levels of PR proteins as the main response of barley plants against the pathogen ingress. On the other hand, none of these studies, or any others, investigated the potential physiological effects of induced PR proteins on the pathogen. Most studies of this type in barley have traditionally focused on the interaction of barley and the globally more damaging pathogen *Blumeria graminis* f.sp. *hordei*.

The focus of this thesis is on defense responses of barley against *R. secalis*. Particular emphasis is on pathogen-induced compounds of barley with *in vitro* antifungal activity against the pathogen.

Results in this thesis are divided into 5 chapters. In Chapter 3 some of the basic observations pivotal to the subsequent experiments are presented. The plant/pathogen system and the bioassay that were used throughout this thesis are introduced in this chapter. A technique to fractionate plant intracellular washing fluid (containing apoplastic fluid) into low- and high-molecular weight compounds was also described in this chapter. The low-molecular weight compounds were analysed in Chapter 4. The high-molecular weight compounds received more attention and were analysed in Chapters 5, 6 and 7.

Chapter 2:

General materials and methods

2.1 Materials

Acetonitrile, ammonium sulfate and silver nitrate were obtained from BDH. Glycine was from APS Finechem, Australia. Acrylamide monomer, bisacrylamide was from BioRad. Trifluoroacetic acid was from Hewlett Packard. Ammonium formate and sodium acetate were obtained from Ajax Chemicals, Sydney, Australia. Iodoacetamide, dithiothreitol (DTT), ovalbumin, polyethylene glycol (MW 6000), trishydroxymethylaminomethanol (Tris), ammonium bicarbonate and trypsin were purchased from Sigma-Aldrich. Lima Bean Agar (LiBA) was acquired from Difco.

An Ultropac TSK 6 3000 SW™ chromatography column was purchased from LKB. Macrosphere GPC 60A™ and Macrosphere WCX™ columns were bought from Altech Associates. A PL-SAX™ column was bought from Polymer Labs. A Brownlee HIC-300 Aquapore™ column was purchased from Applied Biosystems, USA. Spectra/Por™ dialysis membranes were acquired from Spectrum™, USA. Microcon™3, Centricon™3, Ultrafree™-MC and Ultrafree™-CL filters were bought from Millipore.

Seeds and fungal isolates were provided by Dr. Dara Melanson from the Cereal Pathology Unit at the South Australia Research and Development Institute (SARDI). All the isolates, collected in Asutralia, had been subjected to single cell isolation and stored on porcelain beads (Lange and Boyd, 1968).

2.2 Methods

2.2.1 Statistical design

A minimum of fifteen plants were harvested for each treatment. Pooled IWF from each treatment was used only once, forming one experimental unit. All the treatments associated with each experiment were carried out at the same time. Experiments intended for statistical analysis were repeated three times. Repeats were carried out sequentially and not simultaneously, unless otherwise noted. Results were analyzed according to a Randomized Complete Block Design. For each analysis replica mean of square (REP m.s.) was compared to residual mean of square to justify the choice of the Randomized Complete Block Design compared to a Completely Randomized Design. Validity of each analysis was also checked for homogeneity and constant variance by plots of Residual versus Fitted Values. When required, logarithmic transformation was used. Statistical significance was at the 5% level. Pairwise comparisons, carried out based on least significant differences (LSD), were also at the 5% level. Statistical analyses were conducted in Genstat-5 and charts were produced using Microsoft Excel.

2.2.2 The pathogen

Rhynchosporium secalis was grown on Lima Bean Agar (LiBA) plates at 15°C in the dark. The fungus was subcultured weekly. Conidia were only harvested from 1- to 3-week old cultures. Older cultures were discarded. To harvest conidia, a bacteriological loop was gently scraped against the colonies until enough spore mass was collected inside the loop.

For long term storage, each isolate was also kept on porcelain beads according to Lange and Boyd (1968). In order to maintain a consistent pathogenicity throughout the project, every 3 months all the culture plates were discarded and new cultures were prepared from the porcelain beads.

2.2.3 The plant

Near isogenic cultivars Atlas (*Rrs1*) and Atlas 46 (*Rrs1*, *Rrs2*) as well as cultivar Clipper (nil resistance gene) were used. Seeds were surface sterilization following the method of Hoj and coworkers (1989) and planted in pasteurised University of California (UC) soil mix. Plants (8/pot) were grown in a glasshouse under containment cylinders made of clear PVC with the top covered with greaseproof papers (see Figure 2-1). It was shown that application of containment cylinders stopped contamination of the plants with undesirable microorganisms including powdery mildew detected in the environment. Not only were no disease symptoms observed on plants grown inside the cylinders, but also no microbial growth was detected on leaf segments of these plants incubated on PDA for 10 days.

Plants were inoculated at 2-leaf stage (2-3 weeks after planting). To prepare the inoculum, conidia of *R. secalis* were suspended in sterilized distilled water and adjusted to 1 x 10^6 spores/ml with the aid of a haemocytometer. A small amount of Tween-20 (final concentration of 0.05%) was added to the suspension to assist with adherence of spores to leaves. For inoculation, plants were transferred into a growth room (12 hours photoperiod, 20/15°C) and greaseproof paper was temporarily removed. Plants were

Figure 2-1: Barley plants grown inside containment cylinders
Cylinders were made of clear PVC but the top was covered with greaseproof paper to control the excessive humidity that otherwise accumulated inside the cylinders. Cylinders were 80 cm high and 50 cm in diameter. Each cylinder contained up to 8 pots of 8 plants.

sprayed with 2.5 ml/pot of the *R. secalis* conidia suspension. Greaseproof paper was replaced and cylinders were covered with a piece of black cloth. After 48 hours incubation, the cloth was removed and plants were allowed to grow in the growth room. For extraction of IWF, plants were harvested on day three postinoculation, unless otherwise indicated. Alternatively, for disease assessment, plants were kept in the growth room until needed.

2.2.4 IWF extraction

Intercellular washing fluid (IWF) was extracted following the method of Jutidamrongphan and coworkers (1991), except that after extraction IWF was not dried. Instead, extracted IWFs were filter-sterilized, using a 0.4 µm Ultrafree®-MC or -CL filter (Millipore). Sterile IWF was stored at 4°C for short term (1-2 days) or frozen at -20°C for long term storage.

When required, IWF was fractionated into low- and high-molecular weight components, using Microcon or Centricon ultrafilters (Millipore) with exclusion limits of 3 kDa or 10 kDa. Filters were first washed with double deionized water and samples (typically 500 µl for Microcon or 2 ml for Centricon) were ultrafiltered until the minimum volume was reached. Retentate was recovered according to the procedure supplied by Millipore.

2.2.5 High pressure liquid chromatography

High pressure liquid chromatography (HPLC) of samples was carried out in a Hewlett Packard 1090 Liquid Chromatograph using a 1 ml injection loop. Proteins were detected by UV absorbance. The absorbance of all the chromatograms, except for the reverse phase, was recorded at 280 ηm. The reverse phase chromatogram was recorded at 214ηm absorbancy for higher sensitivity. Peak area, which is directly and linearly correlated with the amount of protein detected, was calculated using the reverse phase chromatogram and a standard integration procedure available as part of the HP

1090 control software. Quantification by peak area was used to estimate the amount of pure proteins.

When a marker was required, 20 µl of a universal marker mixture made of 2 mg/ml blue dextran, 0.85 mg/ml ovalbumin and 1.5 mg/ml RNase-A was used. The pH and salt levels were adjusted to that of the appropriate eluent.

The chromatography methods are outlined in the following sections.

2.2.5.1 Gel permeation chromatography with a LKB Ultropac TSK 3000 SW™ column

This is also known as GP chromatography or GPC. The following conditions were applied. Column dimension: 7.5 x 300 mm. Chromatography temperature: room temperature (24°C). Flow rate: 0.5 ml/min. Eluent: 50 mM ammonium formate and 1 mM DTT in water, pH 7.5. Samples were 100 µl or smaller. Chromatography was run for a total of 45 min and fractions were collected every minute.

2.2.5.2 GP chromatography with a Altech Macrosphere GPC 60A™ column

The following conditions were applied. Column dimension: 4.6 x 250 mm. Chromatography temperature: room temperature (24°C). Flow rate: 0.2 ml/min. Eluent: 50 mM ammonium formate and 1 mM DTT in water, pH 7.5. Samples were 30 µl or smaller. Chromatography was run for a total of 40 min and fractions were collected every minute.

2.2.5.3 Anion exchange chromatography with a Polymer Labs PL-SAX™ column

This strong anion exchange chromatography technique is also known as SAX chromatography or simply as SAX. The following conditions were applied. Column dimension: 4.6 x 100 mm. Chromatography temperature: room temperature (24°C).

Flow rate: 0.4 ml/min. Eluent A: 10 mM ammonium formate in water, pH 9.0. Eluent B: 1 M ammonium formate, pH 9.0. Samples, typically 500 µl, were adjusted to the pH and salt levels of eluent A and injected. Chromatography was run for a total of 24 min and fractions were collected every minute. The gradient was according to Table 2-1.

programmed time (min)	eluent B
0	0%
1.5	0%
11.5	20%
16.6	100%

Table 2-1: Eluent gradient for SAX HPLC
The delay between formation of gradient and detection was approximately 6.5 min.

programmed time (min)	eluent B
0	0%
2	0%
22	50%
25	100%

Table 2-2: Eluent gradient for WCX HPLC
The delay between formation of gradient and detection was approximately 14.7 min.

2.2.5.4 Cation exchange chromatography with an Altech Macrosphere WCX™ column

This is a weak cation exchange chromatography technique, also known as WCX chromatography or simply as WCX. The following conditions were applied. Column dimension: 4.6 x 150 mm. Chromatography temperature: room temperature (24°C). Flow rate: 0.4 ml/min. Eluent A: 10 mM ammonium formate and 1 mM DTT in water, pH 7.5. Eluent B: 400 mM ammonium formate and 1 mM DTT in water, pH 7.5. Samples, typically 500 µl, were adjusted to the pH and salt levels of eluent A and injected. Chromatography was run for a total of 40 min and fractions were collected every minute. The gradient was according to Table 2-2.

2.2.5.5 Hydrophobic interaction chromatography with a Brownlee HIC-300 Aquapore™ column

This is also known as HI chromatography or HIC. The following conditions were applied. Column dimension: 2.1 x 30 mm. Chromatography temperature: room

temperature (24°C). Flow rate: 0.2 ml/min. Eluent A: 1.2 M ammonium sulfate, 150 mM sodium acetate and 1 mM DTT in water, pH 6.8. Eluent B: 0.5 M ammonium sulfate, 62.5 mM sodium acetate and 1 mM DTT in water, pH 6.8. Samples were typically 500 µl and adjusted to salt levels of eluent A before injection. Chromatography was run for a total of 37 min and fractions were collected every minute. A linear gradient of 0 to 100% eluent B (over 0 to 25 min) was used. The delay between formation of gradient and detection was estimated to be 10 min.

2.2.5.6 Reverse phase chromatography with Vydac Peptide and Protein C18™ columns

This is also known as RP chromatography or RPC. Two columns with different dimensions were used:

2.2.5.6.1 Vydac 4.6 x 300 mm Peptide and Protein C18™ column

Chromatography with this column was performed at 40°C with a flow rate of 0.6 ml/min. Eluent A: 0.05% trifluoroacetic acid (TFA) in water. Eluent B: 70% acetonitrile, 0.04% TFA in water. Samples, typically 500 µl, were adjusted to 0.05% TFA before injection. Only the protein peaks were collected when the Vydac column was used.

Three different gradient systems (methods) were used with this column:

1- Method RPC-LMW: This chromatographic method was used to isolate and detect peptides in the low molecular fraction of IWF. Chromatography was run for a total of 42 min. The gradient was according to Table 2-3.

Time (min)	Buffer B concentration
0	5%
5	5%
20	50%
35	100%

Table 2-3: Eluent gradient for RP HPLC method RPC-LMW
There is a delay between formation of gradient (just before the eluent mixture enters the column) and detection (after the eluent leaves the column). This was approximated to be 6.2 min.

2- Method RPC-2: This was a general purpose method to (1) verify the purity of the isolated proteins, (2) eliminate salts and

buffers remaining from previous chromatographic steps and (3) quantify the proteins. This chromatography was run for a total of 36 min with the gradient according to Table 2-4

programmed time (min)	eluent B
0	5%
5	40%
30	100%

Table 2-4: Eluent gradient for RPC HPLC method RPC-2
The delay between formation of gradient and detection was approximately 6.2 min.

programmed time (min)	eluent B
0	5%
5	45%
25	60%
30	100%

Table 2-5: Eluent gradient for RPC HPLC method RPC-TL
The delay between formation of gradient and detection was approximately 6.2 min.

3- Method RPC-TL: This method was developed, with the above three aims in mind, to specifically resolve TL proteins. Chromatography was run for a total of 36 min. The gradient is shown in Table 2-5.

2.2.5.6.2 Vydac 2.4 x 300 mm Peptide and Protein C18™ column

This column was used for peptide mapping. The chromatography was performed at 40°C with a flow rate of 0.2 ml/min. Eluent A: 0.05% trifluoroacetic acid (TFA) in water. Eluent B: 70% acetonitrile, 0.04% TFA in water. Samples, typically 400 µl, were adjusted to pH 3-4 with 0.1% TFA before injection. Two gradients (methods) were used:

1- Method 22PEPM2: This was used to isolate peptides following tryptic digestion of proteins except for TL proteins. The chromatography was performed for 80 min with a gradient according to Table 2-6.

2- Method 22PEPM3: This was used to isolate peptides and compare TL proteins following tryptic digestion of these proteins. The chromatography was performed for 110 min with a gradient according to Table 2-7.

programmed time (min)	eluent B
0	5%
1	5%
61	60%
70	100%

Table 2-6: Eluent gradient for RPC HPLC method 22PEPM2
This gradient was used in peptide mapping of non-TL proteins. The delay between formation of gradient and detection was approximately 10 min.

programmed time (min)	eluent B
0	5%
1	5%
91	60%
100	100%

Table 2-7: Eluent gradient for RPC HPLC method 22PEPM3
This gradient was used in peptide mapping of TL proteins. The delay between formation of gradient and detection was approximately 10 min.

2.2.6 Protein digestion and peptide mapping

Reductive alkylation and tryptic digestion were carried out according to Rosenberg (1996). Purified samples were dried in a Speed Vac™ centrifuge and reconstituted in 50 µl of resuspension buffer (8 M urea and 0.1 M ammonium bicarbonate pH 8.0 in water). The proteins were reduced by the addition of 5 µl 45 mM DTT and incubation at 50°C for 15 min. Samples were cooled and alkylated by adding 5 µl of 0.1 M iodoacetamide aquatic solution and incubation in the dark at room temperature for 15 min.

Tryptic digestion was carried out with an enzyme:substrate ratio of 1:25. For this, trypsin was dissolved in 145 µl of 33 mM ammonium bicarbonate pH 8.0 and added to the samples which were incubated in the dark at 37°C for 24 hours. Digestion was stopped by freezing the samples at -20°C. Peptide fragments were resolved using reverse phase HPLC. Commonly an 80-min gradient (method 22PERM2) was used. However, for digested TL proteins a longer 110-min gradient (method 22PERM3) was used (for gradient details see section 2.1.5.6.2).

2.2.7 SDS-PAGE

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted in a BioRad™ Mini-protean II cell following a procedure recommended by the manufacturer. Generally, an amount equivalent to 10 µl IWF was dried in a Speed Vac centrifuge and reconstituted in 10 µl of a denaturing loading buffer containing 50mM DTT. Samples were boiled for 2 min and loaded onto 12.5% polyacrylamide gels.

To visualise proteins, gels were stained following the procedure of Dunn and Crisp (1994).

2.2.8 Protein sequencing

Protein sequencing was carried out by the Nucleaic Acid and Protein Chemistry Unit at the Dartment of Plant Science of The University of Adelaide, using a Hewlet Packard G1000A Protein Sequencer. Protein sequences were matched against the non-redundant databases using BLAST at the NCBI internet site (http://www.ncbi.nlm.nih.gov/BLAST/).

Chapter 3:

Basic physiological and biochemical observations

3.1 Introduction

In Chapter 1 it was explained that barley induced defense responses against *R. secalis* were the focus of this thesis. Similar studies of barley responses were conducted from 16 hours (Kerby and Somerville, 1989) to 10 days (Reiss and Bryngelsson 1996) postinoculation. However, the majority of the studies appear to be conducted on day 2 to 3 postinoculation (Hejgaard *et al.*, 1992; Bryngelsson *et al.*, 1994; Tamas and Fric, 1995; Roulin *et al.*, 1997). The focus of this chapter and most of the subsequent chapters is on barley defense responses three day postinoculation. Another common feature amongst many of the studies outlined above is the use of near-isogenic resistant and susceptible cultivars (Kerby and Somerville, 1989; Kragh *et al.*, 1990; Wubben *et al.*, 1996; Gregersen *et al.*, 1997; Roulin *et al.*, 1997). This allows a comparison between the defense response of resistant and susceptible cultivars. In the current study, two near isogenic cultivars of barley were used: Atlas, with the *Rrs2* resistance gene, and Atlas 46, with the *Rrs1* and *Rrs2* resistance genes (Dyck and Schaller, 1961). By using these cultivars, the first aim of the present chapter was to study the differential virulence of a number of *R. secalis* isolates. Differential virulence in this case was simply defined as the level of disease symptoms on the susceptible cultivar, Atlas, minus that on the resistant cultivar, Atlas 46. Isolates that had the largest differential virulence were, therefore, assumed to be responding to the presence of the *Rrs1* gene in

Atlas 46. These isolates were hypothesised to be expressing the avirulence gene *Avr1*, according to the gene for gene concept.

After identifying isolates that had maximum virulence differences on Atlas and Atlas 46, attention was drawn to the mechanism of resistance in the plants. It was previously explained that during the subcuticular phase, the pathogen interacts with the plant essentially through the apoplast. Accordingly, the barley apoplastic fluid was considered for further studies. However, since the apoplastic fluid was not directly extractable, water was infiltrated into the leaves and then gently centrifuged out. The new fluid, containing the apoplastic fluid, was known as intracellular washing fluid (IWF).

A preliminary observation indicated that IWF had strong antifungal activity against spores of *R. secalis*. The activity was stronger in the IWF from Atlas 46 compared to that from Atlas. In addition, the activity was more pronounced in the IWF from the inoculated plants compared to that from the un-inoculated ones. This served as the first evidence on the role of IWF compounds in defense mechanism of barley against *R. secalis* and on the inducible nature of antifungal compounds in the IWF.

Plants have been shown to produce a large number of different compounds in response to pathogen attack or related stimuli (see Chapter 1). In barley, a number of PRPs were shown to be induced in response to elicitation. However, in response to *R. secalis* attack, only two barley PRPs were studied and shown to increase (Hahn *et al.*, 1993; Roulin *et al.*, 1997). Antifungal affect of barley extracts on spores of *R. secalis* spores were reported as early as 1972 (Ali, 1972) but no compound with demonstrated activity against this pathogen has been previously isolated.

Fungal spores have been frequently used in bioassays of plant antifungal compounds. Spores were shown to germinate at a lower percentage and/or germinate more slowly when they were exposed to total extracts (Ali, 1970), to phenolic compounds (Smith, 1982) and to proteins (Niderman *et al.*, 1995; Xu and Reddy, 1997) from plants. However, a factor that sometimes interferes with this type of bioassay is the simultaneous presence, in extracts, of stimulatory and inhibitory compounds. Plant extracts have been shown to contain a variety of compounds that stimulate germination of fungal spores (Ayres and Owen, 1970; Strange, 1993; Vidhyasekaran, 1997). This may complicate the bioassay and mask the stimulatory and/or the inhibitory activity. In

such occasions successful detection of either activity may be dependent on early separation of the antagonistic compounds, which once achieved leaves a fraction highly stimulatory to the fungal germination and another one inhibitory to it.

3.2 Materials and methods

3.2.1 Materials

Bradford protein assay kit was purchased from BioRad™. BSA was bought from Sigma-Aldrich. Sodium dihydrogen phosphate, disodium hydrogen phosphate and EcoLum™ scintillation liquid were purchased from ICN. C^{14}-mannitol was a gift from Dr. Robert Reed from the Department of Plant Sciences, The University of Adelaide.

3.2.2 Plants and disease

Near isogenic cultivars Atlas (*Rrs1*) and Atlas 46 (*Rrs1*, *Rrs2*) as well as cultivar Clipper (nil known resistance gene) were used. Plants (8/pot) were grown under the conditions outlined in Chapter 2 and inoculated at the 2-leaf stage (2-3 weeks after planting). Inoculated plants were incubated for 48 hours in the dark before returning to the normal growth conditions.

Disease symptoms were scored following the method of Jackson and Webster (1976) (Table 3-1). All leaves on each plant were scored, averaged and recorded as the disease score for that plant. Disease scores for 8 plants in each pot were averaged and used as one replica for data analysis.

score	symptoms
0	no visible symptoms
1	very small lesions confined to leaf margins
2	small lesions not only confined to leaf margins
3	large coalescing lesions involving a majority of the leaf area
4	total collapse of the leaf

Table 3-1: The disease scoring system

3.2.3 Spore germination fluid

A volume of 1 ml IWF from Atlas 46 was placed in a 1.5 ml polypropylene (Eppendorf) centrifuge tube and *R. secalis* conidia to a final concentration of 1 x 10^7 spores/ml was added. This was incubated at 20°C while gently shaking. After 18 hours, the mixture was centrifuged at 1000g for 5 min and the supernatant was collected. The spore germination fluid (SGF) produced by this method was checked under microscope to be free of *R. secalis* conidia.

3.2.4 Antifungal bioassay of IWF

A volume of 60 µl of IWF was placed at the bottom of 1.5 ml polypropylene (Eppendorf) centrifuge tubes and dried in a Speed Vac™ vacuum-centrifuge for 1 to 2 hours. Before the experiment started, conidia of *R. secalis* isolate H2.5 were harvested from a Lima Bean Agar (LiBA) plate using a bacteriological loop. These were suspended in sterilized distilled water and adjusted to 1 x 10^7 spores/ml with the aid of a haemocytometer. Six µl of the spore suspension was added to the bottom of each tube and briefly mixed with a pipette tip to assist with reconstituting the dried IWF. Tubes were sealed and incubated at 20 °C in the dark. After 12 hours, the assay was stopped by placing the tubes on ice. The bioassay mix was placed on a heomocytometer, covered with the cover slip and examined under the microscope at 400X magnification. Spores detectable in 20 randomly chosen small square of the heamocytometer were counted and assessed for the phenotype under study. The examined volume (5 x 10^{-3} µl) normally contained 50 spores.

As part of the bioassay preparation, dried IWF was reconstituted in one tenth of its original volume. IWF concentration in the bioassay was, therefore, calculated to be 10X.

3.2.5 Heat and chemical treatment of IWF

For heat treatment, 60 µl of IWF was placed in a centrifuge tube that was recapped

and placed in boiling water for 5 min. This was dried in a Speed Vac™ and bioassayed at the final concentration of 1 x 10^7 spore/ml as explained above. For the control treatment, 60 µl of IWF was dried without heating and subjected to the bioassay.

For proteinase K treatement, 6-µl of either IWF or water was placed in 1.5 ml centrifuge tubes and dried in a Speed Vac™. A volume of 3 µl of 0.1 mg/ml proteinase K was added to these, briefly mixed and incubated at 37 °C for 30 minutes. For bioassay, 3 µl of *R. secalis* conidia previously adjusted to 2 x 10^7 spore/ml was added in these to gain a final concentration of 1 x 10^7 spore/ml.

Similarly, for dithiothreitol (DTT) treatment, 6-µl of either IWF or water was placed in 1.5 ml centrifuge tubes and dried in a Speed Vac™. A volume of 3 µl of DTT solution adjusted to 1 mM, 10 mM or 100 mM was added to the samples, briefly mixed and incubated at the room temperature (25°C) for 30 minutes. For bioassay, 3 µl of *R. secalis* conidia previously adjusted to 2 x 10^7 spore/ml was added to these to obtain a final concentration of 1 x 10^7 spore/ml.

3.2.6 Radiation measurement

A volume of 4 ml of scintillation liquid was added to a 10-µl sample and mixed well. This was read in a Beckman LS 3801 liquid scintillation reader. For each experiment every measurement was repeated three times and averaged. Data were recorded as CPM/ml.

3.2.7 Total protein

Total protein was measured using a BioRad™ protein assay kit based on the Bradford protocol. Absorbance was measured using a Beckman™ DU-68 spectrophotometer. Standards were prepared using aqueous solution of BSA.

3.3 Results

3.3.1 Interaction of *R. secalis* with barley

3.3.1.1 Time-course study

A preliminary time-course study was conducted on nine isolates of *R. secalis* to determine the best time for disease assessment under the experimental conditions. Isolates F1, F2, F3, F5, H1.1, H2.5, WA3076, D7.2 and K8 were separately inoculated onto cultivars Atlas, Atlas 46 and Clipper that were then incubated in the dark for 48 hours before returning to the normal growth conditions. Symptoms were scored and recorded on days 9, 11, 13, 15 and 17 postinoculation. Results, summarized in Table 3-2, indicated that scald lesions started to appear at around day 9. However, typical symptoms were observed after day 13 for most treatments. On average, symptoms were most easily assessed on day 15. After that, plants started to produce dense foliage that interfered with the visual assessment of the disease. In most cases disease symptoms increased rapidly until day 15 and then a plateau phase started, probably because plant growth started to overcome the pathogen damage at this stage.

Following this trial, all further disease assessments were conducted only on day 15 postinoculation.

In Figure 3-1 averaged disease scores produced by all isolates on individual cultivars are shown. It appears that, in general, disease progress in Atlas and Atlas 46 was at a similar rate but Atlas 46 had much reduced symptoms because the symptoms started later on this resistant cultivar. On the other hand, disease progress on the universal susceptible cultivar, Clipper, appeared to occur at a much higher rate than that on either Atlas or Atlas 46.

3.3.1.2 Differential virulence of pathogen isolates

For further experiments in this thesis it was required that at least one pathogen isolate

fungal isolate	barley cultivar	days postinoculation				
		9	11	13	15	17
F1	Clipper	0	0.55	2.2	4	4
	Atlas	1	1.15	1.25	1.45	1.25
	Atlas 46	0	0	0.1	0.35	0.3
F2	Clipper	0.25	1.5	2.55	3.4	4
	Atlas	0	0.25	0.45	0.75	0.5
	Atlas 46	0	0	0.25	0.25	0.15
F3	Clipper	0.25	1.4	2	3.75	4
	Atlas	0.25	0.5	0.5	0.85	0.95
	Atlas 46	0	0.1	0.25	0.25	0.3
F5	Clipper	0	1.75	2.15	3.5	4
	Atlas	0.35	0.35	0.4	0.4	0.35
	Atlas 46	0	0	0.1	0.15	0.25
H1.1	Clipper	1.35	2.5	3	3.6	4
	Atlas	0.5	1.35	2.1	2.15	2.1
	Atlas 46	0.1	0.25	0.85	0.9	0.85
H2.5	Clipper	1	1.4	2	2.75	4
	Atlas	0.45	0.6	1.75	2	2.9
	Atlas 46	0	0.2	0.25	0.25	0.6
WA3076	Clipper	1	1.55	2.35	4	4
	Atlas	0.75	0.9	1.85	2.5	3
	Atlas 46	0.35	0.85	1.75	2.6	3.5
D7.2	Clipper	0	0	0.35	1.5	2.85
	Atlas	0	0	0.1	0.4	0.75
	Atlas 46	0	0	0.1	0.1	0.25
K8	Clipper	0.25	0.55	0.75	1.2	2.55
	Atlas	0	0	0	0	0.3
	Atlas 46	0	0	0	0	0

Table 3-2: Average disease rating of barley cultivars in time
Symptoms were scored based on the method of Jackson and Webster (1976). Data are average of two measurements in two independent experiments.

with a large difference in virulence on Atlas and Atlas 46 be available. An experiment was conducted to study the virulence of 20 isolates of *R. secalis* on cultivars Atlas and Atlas 46. Isolates included F1, F2, F3, F4, F5, F6, H1.1, H2.5, WA3076, D7.2, K8, A12, D5.1, R5, H1A, H2A, D7A, A12A, D5A and R5A.

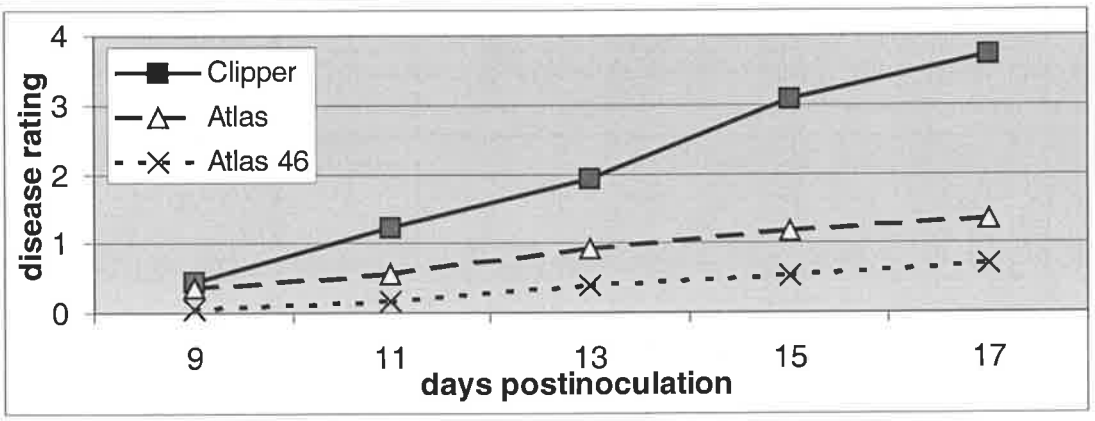


Figure 3-1: Averaged disease rating for cultivars Clipper, Atlas and Atlas 46

Following inoculation, plants were incubated for 48 hours in the dark before returning to the normal growth conditions. Symptoms were recorded on day 15 postinoculation. Results, presented in Figure 3-2, are consistent with findings of the previous experiment. In general, Atlas had less symptoms than Atlas 46 although the difference varied amongst the isolates. Based on this difference in virulence on Atlas and Atlas 46, isolates were arranged in the following series:

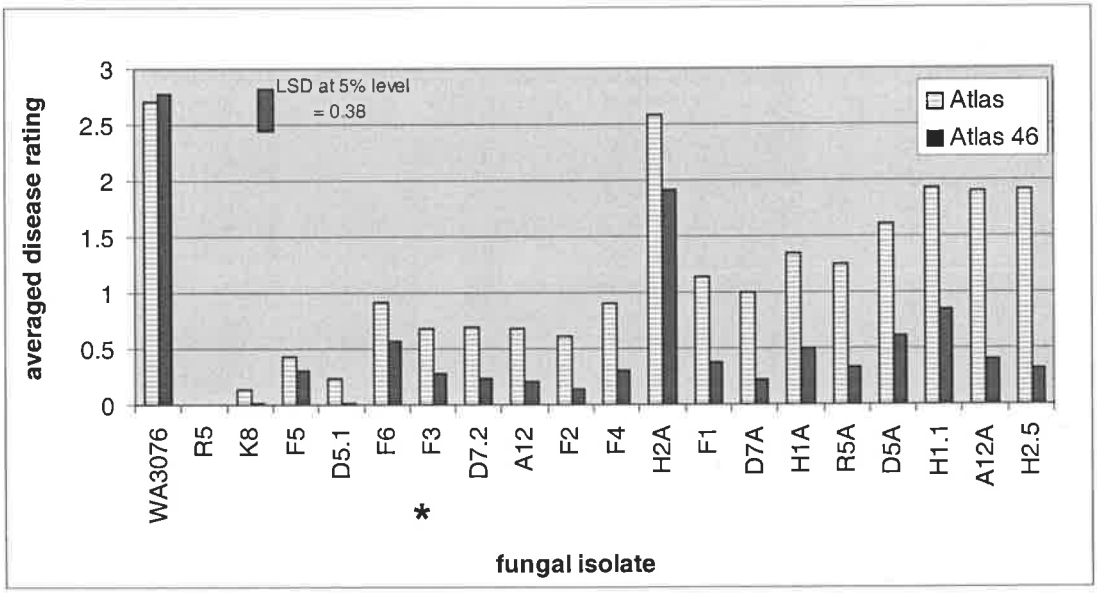


Figure 3-2: Differential virulence of 20 pathogen isolates on barley cultivars
Disease score of 20 isolates on Atlas and Atlas 46 are shown. Data are average of three observations. Isolates are sorted, ascending from left to right, according to their differential responses, defined as disease score on Atlas minus that on Atlas 46. The least significant difference (LSD) between the cultivars was calculated to be 0.38 at the 5% level. Accordingly, isolate F3 and all the isolates on its right had significant differential virulence whereas isolates on its left did not. Isolate H2.5, on the far right, had the highest differential virulence and used in further studies.

WA3076<R5<K8<F5<D5.1<F6<F3<D7.2<A12<F2<F4<H2A<F1<D7A<H1A<
R5A<D5A<H1.1<A12A<H2.5

Following this experiment, isolate H2.5 with maximum differential virulence was selected for further studies. All the subsequent experiments were conducted with this isolate and cultivars Atlas and Atlas 46.

3.3.2 IWF antifungal activity

3.3.2.1 Development of a bioassay to detect IWF activity

As outlined in Chapter 2, *R. secalis* was routinely grown on plates of Lima Bean Agar (LiBA). Preliminary assessment of barley IWF antifungal activity against the fungus was conducted in this medium. In order to do this, surface of a LiBA plates was evenly inoculated by a loopful of spores from a 1- to 3-week old *R. secalis* culture, 10 days prior to the experiment. This was incubated at 15°C in the dark to allow the fungus to grow. Holes, approximately 5 mm in diameter, were made into the agar gel using a cork borer and a volume of 10 µl of IWF from cultivar Atlas 46 was added to these holes. Plates were again incubated for another 7 days with daily inspection under a dissecting microscope with 40X magnification. However, no distinguishable inhibition zone or other antifungal effects were detected (data not shown).

Agar gel used in growth media is known to be capable of inhibiting the antimicrobial activity of some plant compounds (Stoessl and Unwin, 1969). It was hypothesised that the antifungal activity of the small amounts of IWF used in these experiments might be diminished in the agar gel. Accordingly, further studies were conducted by incubating *R. secalis* spores with IWF without any extraneous additives such as LiBA. Spores but not hyphae were used for this study because of practical implications. The fungus did not produce any aerial hyphae that could be harvested and used for experiments involving IWF. Instead, it produced hyphae that grew inside the agar gel. It also produced a large number of spores that were on the media surface and could be readily harvested.

IWF incubated with spores of *R. secalis* displayed marked antifungal activities (see next section). Following preliminary tests, an IWF concentration of 10X was chosen as the standard for all the subsequent experiments. Other concentrations tested were 1X and 5X, with too little effects, and 20X that almost completely lysed all the spores (data not shown). To achieve 10X concentration, 60µl of IWF was dried in a Speed Vac™ vacuum-centrifuge and reconstituted in 6 µl of a conidia suspension previously adjusted to 1 x 10^7 conidia per milliliter of deionised water. Other spore concentrations were also considered. However, at lower spore concentrations too few spores were readily seen under the microscope (at 400X) whereas at higher concentrations too many overlapping or aggregating spores were present (data not shown).

The bioassay carried out according to the above conditions was routinely used in the rest of this thesis. There were modifications to this method in each chapter that, when applicable, are explained at the start of each chapter. More details of the above method, used in rest of this chapter, are explained in Section 3.2.2.3.

3.3.2.2 Effects of IWF on *R. secalis* conidia

Volumes of 60 µl of water (as the control) or IWF from cultivar Atlas 46 were dried in a Speed Vac™ vacuum-centrifuge, reconstituted in 6 µl conidial suspension (10^7/ml) and bioassayed. Results are shown in Figure 3-3. Conidia incubated in water appeared to undergo little changes (Figure 3-3-B) whereas those incubated in IWF underwent significant changes (Figure 3-3-A). At least three different types of deformities, some of them fatal, were identified amongst the IWF-incubated spores. IWF was also found to affect percentage germination and germ tube length of conidia. The deformities, including "fractured cell wall", "doublets", "internal granulation", and the IWF-induced germination are explained below. Percentages of spores affected by each phenomenon, reported below, were calculated based on three separate experiments.

3.3.2.2.1 Internal granulation (IG)

Cytoplasm of *R. secalis* conidia was normally uniform and transparent (see Figure 3-3-F). However, following incubation in IWF many of the conidia were found to contain several distinct particulate or imperfect spherical structures (granules). This condition was called internal granulation (see Figure 3-3-E). The internal granules varied in

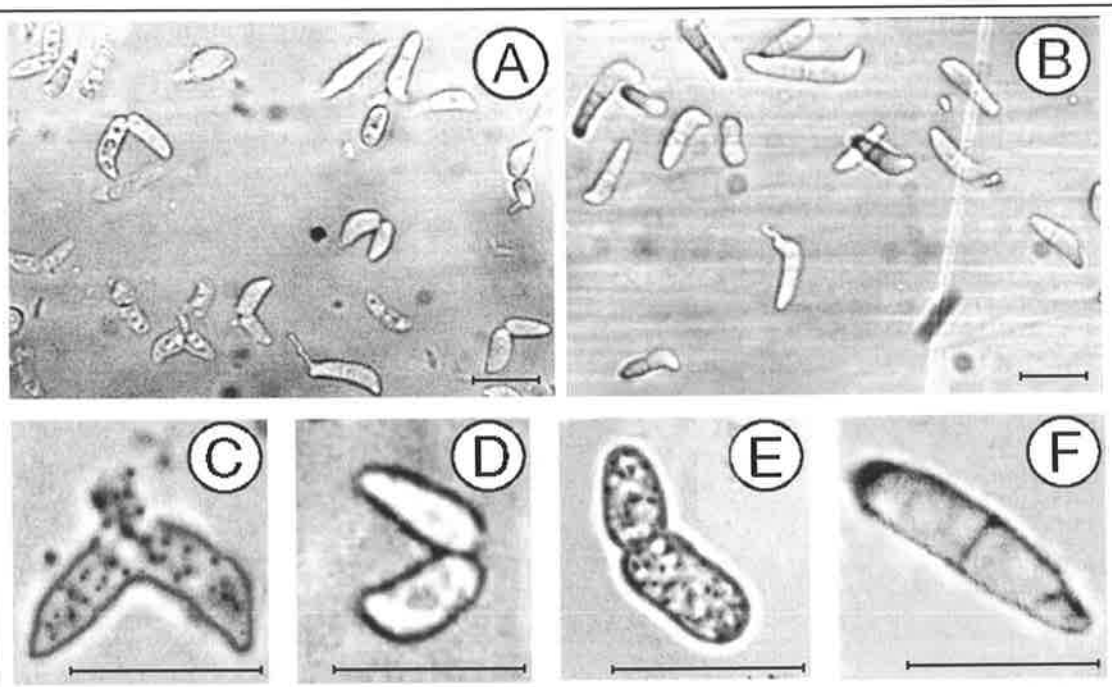


Figure 3-3: Effects of IWF on *R. secalis* conidia
A and **B**, at the top, show conidia of *R. secalis* after 12 hours incubation in 10X IWF and in water respectively. At the bottom different forms of conidia following incubation in IWF or water are shown. **C:** A spore with fractured cell wall (FW). **D:** A doublet (DB) spore. **E:** A spore with internal granulation (IG). **F:** A spore with normal appearance.
Bars in the photographs represent 10 μm.

appearance from relatively transparent to completely dark. The size of the granules ranged from 0.25 to 2 μm. Larger granules were usually light whereas smaller ones were dark in appearance. The variation in intensity and appearance of the internal granulation may represent various stages of this phenomenon but more study is required to properly characterise this phenomena. Percentage of IG was calculated based on the following formula: number of IG spores ÷ total number of intact conidia.

In this formula, percentage of IG is calculated based on intact conidia, thus excluding broken cell parts and conidia with fractured cell wall (explained below). Many of conidia with fractured cell wall were also internally granulated. Fractured conidia were excluded from the present calculation because they will be counted in the fractured cell wall assay (see next). This prevented spores from being counted in both assays.

In average, 37% (Std.Dev.: 12.9%) of spores had IG following incubation in IWF. An average of 8.4% (Std.Dev.: 9.1%) of spores incubated in water also had IG.

3.3.2.2.2 Fractured cell wall (FW)

This phenomenon was the result of rupture of the fungal cell walls. The rupture generally occurred just next to the septum of bicellular spores of *R. secalis* (see Figure 3-3-C). Cells affected by the FW phenomena were found to be either void of cell content or heavily granulated and presumably non-viable. In Figure 3-3-C it is shown how the granulated content of a fractured spore is released into the medium. Released cell contents were believed to be the source of debris also found in the media after incubating spores with IWF. Percentage of FW was calculated based on the following formula: number of FW spores ÷ total number of spores.

In average, 45.2% (Std.Dev.: 6.1%) of spores incubated in Atlas 46 IWF were affected by the FW phenomenon. Only an averaged 2% (Std.Dev.: 2.8%) of spores incubated in water showed FW formation.

3.3.2.2.3 Doublets (DB)

Following incubation in IWF a relatively large number of spores were found to form doublets. A doublet spore was defined as a pair of oblong fungal cells that appear to be loosely adhering to each other at one end (see Figure 3-3-D). It will be shown in Section 3.3.2.4 that a doublet spore was in fact formed from a bicellular *R. secalis* conidia after partial lysis of the septum. Percentage of DB was calculated based on the following formula: number of DB spores ÷ total number of intact spores.

An averaged 31% (Std.Dev.: 7.8%) of spores were found to form doublets following incubation in IWF. No doublet was found in water-incubated spores. Doublets were interesting in the view that they were specifically occurring in IWF with a relatively high percentage.

Since both FW and DB formation degraded the septum of the bicellular conidia, it was suggested that these effects were related. This hypothesis will be further addressed in this chapter (Section 3.3.2.4) and also in Chapters 5 and 7.

3.3.2.2.4 Germination

Both germination percentage (G%) and germ tube (GT) length of conidia incubated in water and in IWF were measured. Percentage of germination was calculated based

on the following formula:

number of germinating spores ÷ total number of intact spores.

Conidia incubated in water had a low 10.2% (Std.Dev.: 14.2) germination with an average germ tube length of 7.5 µm (Std.Dev.: 2.9). Conidia incubated in IWF had an average 27% (Std.Dev.: 17) germination and 8.1 µm (Std.Dev.: 0.5) germ tube length. It was noted that the germination in water was not only lower but also much more variable than that in IWF or nutrient solutions (data not shown).

3.3.2.3 Comparative study of IWF antifungal activity

IWF from Atlas, inoculated Atlas, Atlas 46 and inoculated Atlas 46 were studied for their effects on conidia of *R. secalis*. Following 12 hours incubation in 10X IWF, conidia were examined for germ tube (GT) length, percentage of germination (G%), internal granulation (IG), fractured cell wall (FW) and doublet (DB) formation. Results are summarised in Figure 3-4.

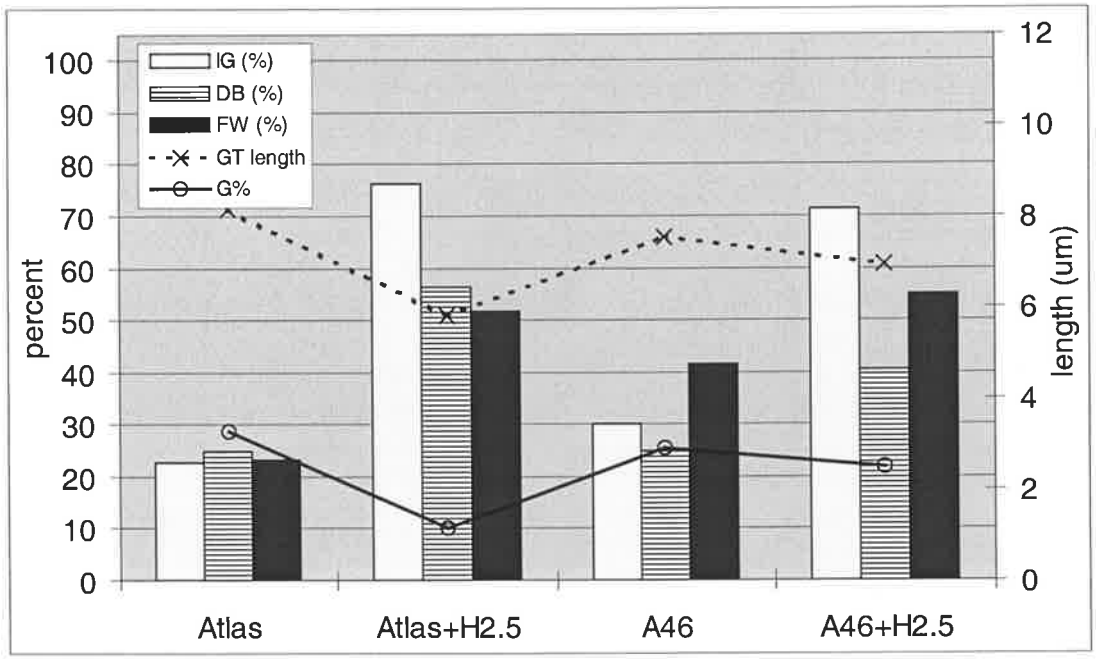


Figure 3-4: Comparative antifungal activity of IWFs from inoculated and uninoculated Atlas and Atlas 46
Data are average of three measurements. Except for germ tube length, all the other factors were significantly different amongst the treatments.

Bioassay data were compared in a Randomized Complete Block Design (see Appendex 1). It was found that GT length was not significantly different amongst the treatments. All the other factors were significantly different. Pairwise comparisons indicated that both Atlas and Atlas 46 had significantly higher DB, FW, IG and significantly lower G% after inoculation. FW was significantly higher in un-inoculated Atlas 46 compared to that in un-inoculated Atlas.

In summary, it was concluded that infection of barley with the scald agent, *R. secalis*, caused measurable increases in IWF antifungal activity on day 3 postinoculation. Although there were no significant differences between the inoculated cultivars, inoculated Atlas 46 seemed to have less antifungal activity than inoculated Atlas. However, between uninoculated plants, Atlas 46 had a significantly stronger FW formation. Other antifungal factors also seemed to be more pronounced in Atlas 46 but the differences were not statistically significant.

3.3.2.4 Time-course study

A time-course study was conducted to understand how normal *R. secalis* conidia produce IG-, DB- and FW-deformed spores. A 60-µl volume of HMW IWF from Atlas was dried and reconstituted in 6 µl of conidial suspension (10^7/ml). This was transferred onto a haemocytometer and covered with a cover slide. The slide edges were sealed with Vaseline to prevent drying of the assay mixture throughout the experiment. The haemocytometer was mounted on a microscope and without moving examined at 0, 0.5, 1, 2, 3, 4, 6 and 12 hours. Results, displayed in Appendix 2, showed that:

- Spores with IG, DB and FW were first seen as early as 0.5 hour after incubation in IWF and then their numbers increased with time.
- DB was formed by incomplete lysis of the septum in bicellular conidia of *R. secalis* not by adherence of two single cell spores, as it first seemed.
- DB and FW were mostly formed independently. In other words, most conidia directly formed either DB or FW spores. Hence, DB was not a transient stage between normal and FW spores as previously hypothesised.

- Fractured spores released cell contents into the media that were the source of debris in the IWF.

3.3.2.5 Inhibition of IWF antifungal activity by *R. secalis*

It was shown earlier in this chapter that barley IWF had a certain level of antifungal activity against conidia of *R.* secalis. However, when spores were harvested from old, non-viable cultures and incubated with 10X IWF, an unusually high level of FW and DB formation was detected (data not shown). To further investigate this, viable conidia of *R. secalis* were killed by heating to 100°C for 5 min. The heat-killed conidia were bioassayed with 10X IWF and again a very high level of FW and DB formation was detected (data not shown). It was hypothesised that perhaps viable spores produced compounds that protected them against the IWF antifungal chemicals. A study was conducted to test this hypothesis.

To prepare the hypothetical fungal detoxifying compound(s), conidia were germinated in 1 ml of 1X IWF from Atlas 46. Conidia incubated in the un-concentrated IWF were found to germinate ~50% which was much higher than the germination percentage in water (~10%). The generated spore germination fluid (SGF) was collected after 18 hours incubation. A volume of 60 µl of SGF was mixed with 60 µl of Atlas 46 IWF, incubated for 10 minutes at room temperature and dried in a Speed Vac™ vacuum-centrifuge. For the control, 60 µl of Atlas 46 IWF was dried. Both IWF and "IWF+SGF" were reconstituted in 6 µl of a conidia suspension (1 x 10^7 conidia/ml) and bioassayed for percentages of germination, FW activity and IG formation. Results are summarised in Table 3-3. Bioassay data were compared in a Randomized Complete Block Design (see Appendex 1). It was found that both IG and FW activity were

bioassay	IWF	IWF + SGF
FW (%)	40.3 (±8.1)	22.3 (±2.5)
IG (%)	58.3 (±8.5)	33 (±4)
germination (%)	16.7 (±3)	30.3 (±7.2)

Table 3-3: Inactivation of IWF by SGF
Conidia of *R. secalis* were bioassayed following incubation in IWF or IWF+SGF. For each treatment, FW and IG formation and percentage of germination are shown. Data are average of three measurements. Standard deviations are shown in parentheses.

significantly different between the treatments. The difference in IG activity was approaching significance with the F probability being 5.5%. The result of this experiment, therefore, indicated that IWF antifungal activity was deactivated by the fungal products present in SGF.

3.3.3 Basic characteristics of IWF

3.3.3.1 Inactivation of IWF

Inactivation of IWF by heat, proteinase K and the reducing compound dithiothreitol (DTT) was investigated.

IWF was extracted from Atlas 46. To establish a control for the other treatments, this was directly bioassayed for IG, DB, FW formation and G%. For the boiling treatment, 60 µl IWF was heated in a boiling water bath for 5 min, dried in a Speed Vac™ vacuum-centrifuge and bioassayed in a similar manner to the control. For DTT and proteinase K treatments, water or 20 times concentrated IWF was incubated with 1 mM DTT, 10 mM DTT, 100 mM DTT or proteinase K and bioassayed. Results, shown in Figure 3-5, indicated that boiling destroyed the IG, DB and FW activities. Percentage of germination was not very different between the boiled and the control IWF treatment. Proteinase K slightly decreased the IG activity but was mostly ineffective on DB and FW activities. Proteinase K, by itself, decreased the germination and because of this interference it was not clear if the enzyme affected the effect of IWF on germination of *R. secalis*. DTT was also found to suppress the germination of spores at any concentration and to induce internal granulation at 10 mM or more. This interfered with the bioassay and hence with evaluation of the IWF antifungal activities. Nevertheless, decreases in DB and FW formation at respectively 10 and 100 mM DTT were observed.

Absence of IG, DB and FW formation in heat-treated IWF is consistent with the hypothesis that the IWF compounds(s) responsible for these activities are proteinaceous in nature. However, ineffectiveness of proteinase K suggests that either the active compound(s) are not protein or are proteins unusually resistant to protein digestion.

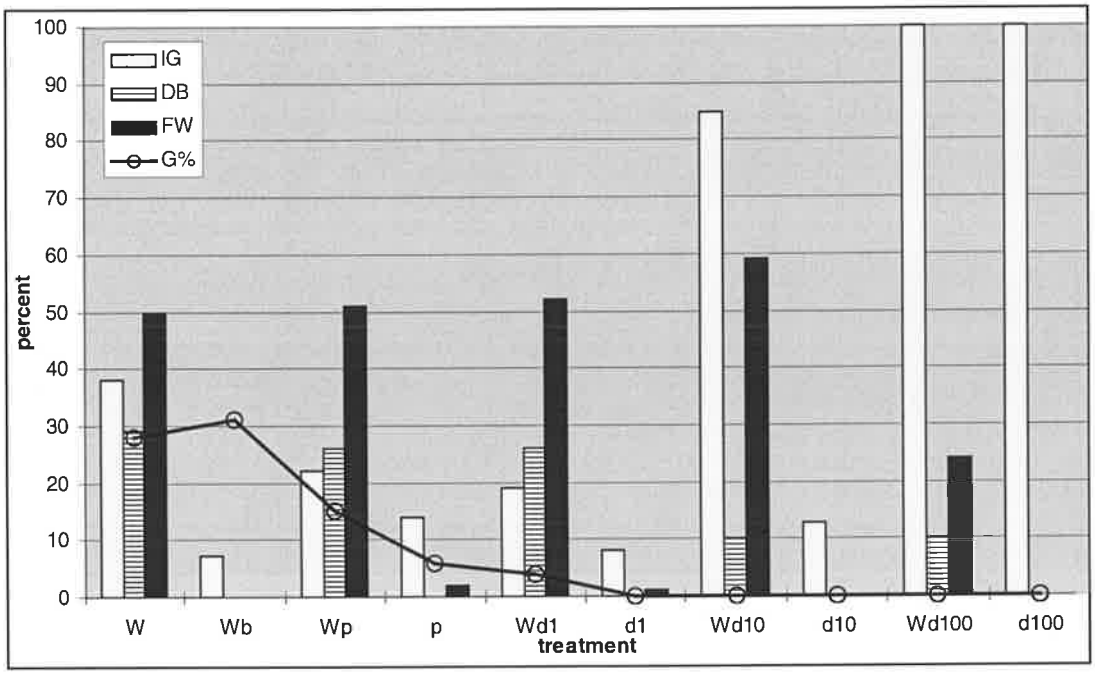


Figure 3-5: Treatment of IWF by heat, proteinase K and DTT
Treatments included control IWF (W), boiled IWF (Wb), proteinase K + IWF (Wp), proteinase K (p), 1mM DTT + IWF (Wd1), 1mM DTT (d1), 10mM DTT + IWF (Wd10), 10mM DTT (d10), 100mM DTT + IWF (Wd100) and 100mM DTT (d100). These were bioassayed for IG, DB and FW activities. GT len. was not assessed in this experiment.

3.3.3.2 High- and low-molecular weigh fractionation of IWF

Determination of the molecular weight of the IWF active compound or compounds could provide important information about them. Ultrafiltration with an exclusion limit of 3 kDa was used to divide IWF from Atlas 46 into high- and low-molecular weight fractions. These fractions were then bioassayed for the presence of IWF active compound(s).

Following the ultrafiltration of typically 500 µl of IWF, the high-molecular weight (HMW) compounds were retained on the membrane in a volume that generally amounted to ~25 µl. This was ~20 times smaller than the original IWF volume. The filtrate that constituted the low-molecular weight (LMW) fraction was ~475 µl. Typically, only 95% of proteins are recoverable following ultrafiltration according to the manufacturer, Millipore™. The concentration of the original IWF was assumed to be the unit (1X) and hence concentrations of the HMW and LMW fractions of IWF

were calculated to be:

$C_{HMW} = 500 \div 25 \times 95\% = 19$ times IWF (= 19X)

$C_{LMW} = 500 \div 475 \times 95\% = 1$ times IWF (= 1X)

For bioassay of the HMW fraction, 3.2 µl of HMW IWF from Atlas 46 was dried in a Speed Vac™ centrifuge, reconstituted in 6 µl spore suspension and bioassayed. The final concentration in the bioassay mixture was calculated to be ($3.2 \times 19 \div 6 =$) ~10X. For bioassay of the LMW fraction, 60 µl of LMW IWF from Atlas 46 was dried in a Speed Vac™ centrifuge, reconstituted in 6 µl spore suspension and bioassayed. The final concentration in the bioassay mixture was calculated to be ($60 \times 1 \div 6 =$) 10X. For control purpose, assays of spores incubated in 10X un-fractionated IWF and in water were also included in this experiment.

Results, shown in Figure 3-6, indicated that all the IG, DB and FW forming compounds were essentially retained in the HMW fraction. These strongly suggested that IG, DB and FW forming compounds had molecular weights markedly larger than 3 kDa. This was consistent with the idea that the IWF compound(s) were protein.

On the other hand, spore germination was found to be markedly lower in the LMW

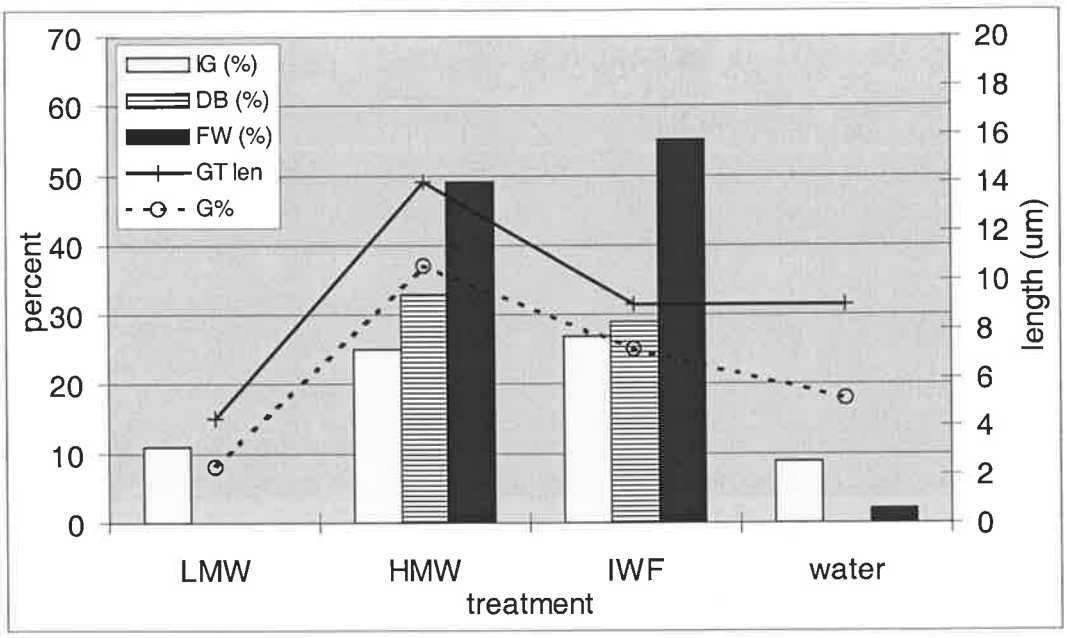


Figure 3-6: Bioassay of LMW and HMW IWF
Data are average of two measurements. Results for spores incubated in LMW IWF, HMW IWF, un-fractionated IWF and water are shown.

fraction compared to that in IWF or even that in water. Moreover, IWF was also found to have lower germination compared to HMW IWF. These data strongly suggest IWF contained germination suppressor(s) with molecular weight smaller than 3 kDa and germination stimulator(s) with molecular weight larger than 3 kDa.

3.3.3.3 Comparative total protein concentration in IWF

Total protein concentration in IWF from Atlas, inoculated Atlas, Atlas 46 and inoculated Atlas 46 were measured following the Bradford protocol. Results, shown in Table 3-4, indicated that IWF protein content was higher in inoculated plants especially in inoculated Atlas. Total protein concentration in LMW and HMW IWF were also measured and shown to follow the same pattern as un-fractionated IWF.

In general, the pattern of total protein concentrations in IWF, HMW IWF and LMW IWF appeared to be very similar to that of IWF antifungal activity (see Figure 3-4). However, no statistically valid regression was found between the IWF, HMW IWF or LMW IWF total protein contents and the IWF antifungal activities (data not shown). Accordingly, although these data suggest that the IWF antifungal compounds are proteinaceous, this is not backed by statistical evidence.

treatment	IWF	LMW IWF	HMW IWF
Atlas	34.0	1.6	29.7
Atlas + H2.5	61.2	2.1	56.3
Atlas 46	39.5	3.6	30.6
Atlas 46 + H2.5	42.3	5.4	34.2

Table 3-4: Comparative total protein concentrations in IWF, LMW IWF and HMW IWF
Protein concentrations (μg/ml) are average of two measurements.

3.3.3.4 Relationship between IWF and apoplast

Extraction of IWF involved infiltration of water into leaves. Accordingly, the concentration of compounds in IWF was always smaller than that in the original apoplastic fluid. A study was conducted to determine the concentration of IWF

compared to that of the apoplastic fluid. A solution of (radioactive) C^{14}-mannitol was used in this study to enable the calculation of the dilution factor at any stage of IWF extraction.

IWF was extracted from leaves of barley cultivar Atlas as outlined in Chapter 2 section 2.2.2 except for the following modifications. Leaves (0.2 g) were cut into 4 cm pieces and vacuum-infiltrated for 5 min using 10 ml solution of 10 mM phosphate buffer pH 7.5 containing 10 mM C^{14}-mannitol. Infiltrated leaves were blot-dried, weighed and centrifuged at 1000g for 5 min. The whole procedure was carried out in a way that from the start of the infiltration to the end of centrifugation it only took 15 min. This was to help decrease the adsorption of mannitol into the leaf cells. The experiment was repeated three times and the results are summarized in Table 3-5.

The final concentration of C^{14}-mannitol in IWF (C_{IWF}) was dependent on concentration of C^{14}-mannitol in the infiltration solution (C_{inf}), the volume of the infiltration solution (V_{inf}) and the volume of apoplast in leaves prior to infiltration (V_{apo}). In mathematical terms it may be expressed as:

$$C_{IWF} = C_{inf} \times V_{inf} \div (V_{inf} + V_{apo}) \qquad \leftarrow \textbf{Formula 3-1}$$

$$\Rightarrow V_{apo} = V_{inf} \times (C_{inf} - C_{IWF}) \, C_{IWF} \qquad \leftarrow \textbf{Formula 3-2}$$

With the values from Table 3-5:

$$\Rightarrow V_{apo} = 0.06 \times (657000 - 391000) \div 391000 = 0.041$$

radiation in infiltration solution (C_{inf})	657000 CPM / ml (±16600)
radiation in IWF (C_{IWF})	391000 CPM / ml (±72000)
original leaf weight	0.20 g (±0)
leaf weight after vacuum-infiltration	0.26 g (±0.02)
IWF volume (V_{IWF})	0.03 ml (±0.01)
weight of infiltrated solution (V_{inf})*	0.06 ml (±0.02)

Table 3-5: IWF, apoplast and leaf weight ratios
Averaged data and (± standard deviation) are presented. The white rows contain values that were directly measured whereas the gray row contains a calculated value.
The infiltration solution contained C^{14}-mannitol and radiation was measured as count per minute (CPM).
*: V_{inf} is a calculated value and equals leaf weight after vacuum-infiltration minus original leaf weight. One milliliter of solutions was assumed to weigh one gram.

In Formula 3-1 and 3-2 the compound of interest (C^{14}-mannitol) was originated from the infiltrating liquid. When the compound of interest is in the apoplast, as it normally happens during extraction of IWF, Formula 3-1 will be transformed into:

$C_{IWF} = C_{apo} \times V_{apo} \div (V_{apo} + V_{inf})$ ←**Formula 3-3**

V_{apo} and V_{inf} were already known:

$\Rightarrow C_{IWF} = C_{apo} \times 0.041 \div (0.041 + 0.06)$

$\Rightarrow C_{IWF} = C_{apo} \times 0.4$ ←**Formula 3-4**

$\Rightarrow C_{apo} = C_{IWF} \div 0.4 = C_{IWF} \times 2.5$ ←**Formula 3-5**

This implies that, assuming the ratio V_{apo} : $(V_{apo} + V_{inf})$ is constant, average concentration of any chemical in apoplast is 2.5 times that in IWF.

Other information concluded from this experiment is:

- Percentage of apoplast in leaf (V:W) = V_{apo} ÷ leaf weight = 0.041 ÷ 0.20 = 20%
- Percentage of infiltrated solution to leaf weight (V:W) = V_{inf} ÷ leaf weight = 0.06 ÷ 0.20 = 30%
- Efficiency of IWF extraction = $V_{IWF} \div V_{inf}$ = 0,03 ÷ 0.06 = 50%

It is noted that in all the above calculations, fluids in xylem and phloem were assumed to be part of the apoplastic fluid.

3.3.3.5 Relationship between IWF and leaf

Data presented here were not collected in one set of specific experiments. Rather, they were collected whenever IWF was extracted. Fresh leaf weights were measured immediately after leaves were cut from the pots. Leaf weights after infiltration were measured after leaves were infiltrated in vacuum and blotted dry. The pH and volume of IWF were measured immediately after the IWF extraction. Following extraction of IWF, leaves were dried in a 50°C-oven and weighed. Results are shown in Table 3-6. Data were analysed in Randomized Complete Block Design but no significant

difference between Atlas, inoculated Atlas, Atlas 46 and inoculated Atlas 46 were detected (data not shown).

It was concluded that inoculation and cultivars have no detectable effect on the factors studied in Table 3-6. It was also concluded that previously observed differences in protein concentrations and antifungal levels between Atlas and Atlas 46, with and without inoculation, were not introduced during extraction of IWF. Instead, these differences were direct result of differences in apoplastic makeup.

	number of plants	IWF pH	dry leaf weight (g)	leaf weight after infiltration	IWF volume
Atlas	39 (±7)	6.3 (±0.6)	0.94 (±0.08)	13.1 (±1)	1.5 (±0.4)
inoculated Atlas	38 (±9)	6.5 (±0.5)	0.91 (±0.08)	13.0 (±0.5)	1.5 (±0.6)
Atlas 46	37 (±9)	6.5 (±0.7)	0.88 (±0.06)	12.9 (±0.5)	1.3 (±0.4)
inoculated Atlas 46	39 (±9)	6.5 (±0.7)	0.89 (±0.08)	13.1 (±0.5)	1.5 (±0.3)

Table 3-6: Statistics of IWF and plant factors
Averaged number of plants, pH of IWF, dry leaf weight, leaf weight after infiltration and IWF volume from Atlas, inoculated Atlas, Atlas 46 and inoculated Atlas 46 are shown. All data are based on 10 grams fresh leaf weight. Values in parentheses are standard deviations.

3.4 Discussion

3.4.1 IWF

Many research groups have used water to infiltrate and extract IWF from plant leaves (Kerby and Somerville, 1989; Ye *et al.*, 1989; Jutidamrongphan *et al.*, 1992; Roulin *et al.*, 1997). Others used buffers, with salt concentrations ranging from 32 to 600 mM (Christ and Mosinger, 1989; Roulin and Buchala, 1995; Hogue and Asselin, 1986; Kragh *et al.*, 1990). While some comparative studies have not revealed any quantitative difference between water- and buffer-extracted IWFs, there are indications that pure water may lead to lower yield for some proteins in some plants (Hogue and Asselin, 1986; Hammond-Kosack, 1992). On the other hand, high ionic strength could led to cytoplasmic contamination (Hogue and Asselin, 1986). In the current study only water was used to infiltrate leaves for extraction of IWF. Although this could have led to lower yield of some compounds, it ensured that cytoplasmic contamination was not likely.

Extraction of IWF unavoidably destroys any uneven distribution of compounds throughout the apoplast in its natural state. In other word, IWF, at its best, is only an extract of "unstructured" apoplastic fluid. As mentioned above, differences in solubility and absorption to the stationary components of the apoplast could also define how much of each component of apoplastic fluid enters into IWF. In particular, negatively charged cellulose in the plant cell wall may interfere with extraction of positively charged compounds such as some proteins. Accordingly, neutral or negatively charged compounds may enter IWF more freely than the positively charged compounds.

Providing that IWF has no cytoplasmic contamination, it may be used to determine subcellular localisation of proteins and other compounds. Compounds that are detected in IWF would have an extracellular localisation whereas those present in total leaf extract but absent in IWF must be intracellular. However, it should be noted that the classic IWF preparation does not extract all the plant apoplastic fluid (Hogue and Asselin, 1986). In this Chapter it was shown that approximately half of the apoplast was held inside the leaves after IWF extraction (see Section 3.3.3.5). If this is not

considered then interpretation of results may be misleading. Roulin and coworkers (1997) who after extraction of IWF used the remaining leaves as the source of intracellular proteins gave one example of this. These authors found the extracellular PRP-2 isoforms GII and GIII in both IWF and the leaf extract and concluded that these proteins must be present in both intra- and inter-cellular spaces. One way of gradually eliminating apoplastic compounds from leaves would be repeated infiltration-centrifugation but it could also encourage cytoplasmic contamination because of accumulative cell wall damage.

3.4.2 The bioassay

The widely used agar gel diffusion assay was found to not suit the bioassay of IWF under the experimental conditions in this study. Agar was previously found to interfere with the fungitoxic activity of an extract from barley (Stoessl and Unwin, 1969). The small volume of IWF used in the present study may also contribute to this problem.

The use of fungal spores to bioassay plant extracts is the approach routinely used in this thesis. However, the concept of this bioassay is not new. As early as the 1970's barley extracts were shown to contain compounds that affect *R. secalis* conidia (Ayres and Owen, 1970; Ali, 1972). On the other hand, results obtained with conidia of *R. secalis* may not be readily extended to hyphae because spores and hyphae of fungi do not necessarily display similar levels of sensitivity to antifungal compounds (Smith, 1982).

In studies with scald, the subcuticular hyphae and, to a lesser extent, fungal spores on the leaf surface were identified as targets of the barley defense responses (Ali 1974; Wevelsiep *et al.* 1991; Jorgensen *et al.* 1993). It would, therefore, be desirable to understand the effect of barley defense mechanisms or compounds on both spore and hyphae of *R. secalis*. However, in the present project only the effects of plant compounds on spores of *R. secalis* were studied.

The reasons for using the spore bioassay in the format outlined in this chapter are as follow:

- Volumes as small as 6 µl could be bioassayed.
- Exogenous factors that might complicate the interaction of the spores and IWF are not required.
- Unlike hyphae, spores are readily harvested from *R secalis* cultures on Lima Bean Agar.
- Because spores are individual units observations on spores can be easily quantified whereas observations on hyphae are more difficult to quantify. An example of this is percentage of FW, which is simply the number of affected spores divided by the total number of spores.
- Bioassay sensitivity was increased by measuring more than one factor, *i.e.*, IG, DB and FW formation as well as percentage of germination and germ tube length.

Ten times-concentrated IWF was used in bioassays in this Chapter. This was concentrated enough to demonstrate the antifungal activity of IWF but not so strong as to lyse all the spores and thus mask the differences between the cultivars or the treatments. This concentration, *i.e.* 10X, was also found not to be widely different from the calculated concentration of the apoplast, *i.e.* 2.5X (see section 3.3.3.4). In fact, although the average concentration of apoplastic fluid is ~2.5, its local concentration in certain tissues may significantly exceed this. Previous studies indicated that accumulation of many antifungal and pathogenesis-related compounds follow a tissue-specific pattern in the apoplast. Examples of this are accumulation around the infection sites (Bailey, 1982; Scott-Craig *et al.*, 1995) and accumulation in certain tissues of leaf or stem (LaRosa *et al.*, 1992; Young *et al.*, 1995; Wubben *et al.*, 1996; Gregersen *et al.*, 1997; Kristensen *et al.*, 1999).

Since only the average of the apoplastic concentration was calculated in Section 3.3.3.4, further studies are required to understand the actual range of the concentration across the apoplast particularly in the points of interaction between the plant and the pathogen.

3.4.3 Plant resistance and IWF activity

From twenty isolates of *R. secalis* assayed on the near-isogenic cultivars, Atlas (*Rrs1*) and Atlas 46 (*Rrs1*, *Rrs2*), 14 isolates had significantly more symptoms on Atlas compared to that on Atlas 46. Amongst these, isolate H2.5 had the largest differential virulence and was used for further studies. Since the apoplast is a major point of interaction between barley and *R. secalis* for at least a few days after inoculation, studies were concentrated on the apoplastic fluid or, as it is called after extraction, IWF.

Infection of barley with the scald agent, *R. secalis*, was found to have a measurable effect on the IWF. These effects, including an increase in total protein concentration and an increase in antifungal activity, were observed in whole IWF as well as in low- and high-molecular weight fractions of IWF from both Atlas and Atlas 46. Comparison between the two cultivars indicated that IWF from Atlas 46 had smaller increases of protein concentration and antifungal activities on day 3 postinoculation. However, antifungal activity and the protein concentration of IWF from uninoculated Atlas 46 were somewhat higher than those of IWF from uninoculated Atlas.

A similar pattern of protein concentrations and antifungal activity suggests that the IWF antifungal compounds may be proteins. The finding that the compound(s) responsible for IG, DB and FW activities were specifically present in the HMW IWF indicates that they are larger than 3 kDa in size. This is consistent with the compound(s) being protein(s). On the other hand, the LMW IWF contained one or more germination suppressors with molecular weights smaller than 3 kDa. Non-proteinaceous low molecular weight compounds as well as small peptide could be suspected in this case.

Boiling of IWF for 5 min destroyed the IG, DB and FW activities of IWF. This was probably because the compounds responsible for these activities were large molecules with tertiary structure and thus sensitive to heat. That is, they are proteins. Spores treated with boiled IWF had only slightly higher germination that those treated with un-boiled IWF. This increase is not large enough to indicate that the proposed germination suppressor(s) were destroyed. This is, again, consistent with these compound(s) being small peptide(s) or small non-proteinaceous compound(s).

Proteinase K was found not to destroy DB and FW activities although a slight

decrease in IG formation was evident. No conclusion is made based on the result of this experiment. Many plant proteins, including some of the so-called pathogenesis-related proteins (PRPs) with antifungal activity, are known to be resistant to proteolysis (Pierpoint, 1983; Linthorst, 1991). However, should proteinase K treatment have destroyed the antifungal activities of IWF, it would have been concluded that the compound behind the destroyed activity was a protein. The effect of the enzyme on the proposed germination suppressor(s) could not be analysed because of proteinase K interference with the assay of spore germination.

DTT partially decreased the FW and DB activities. DTT is known for its ability to reduce disulfide bonds found in many proteins (Edelstein and Bollag, 1992). Accordingly, high concentrations, *i.e.* 10 to 100 mM, of DTT can destroy the activity of proteins with vulnerable disulfide bounds. Partial decrease in the activity of the antifungal compounds may be due to the short time of incubation (30 minutes). It is, therefore, conceivable that the compound(s) responsible for FW and DB activities have disulfide bonds in their structure. The effect of DTT on germination suppression and FW activity could not be analysed because of the reducing agent interfering with the assay for these activities.

Further analysis of the low molecular weight and the high molecular weight fractions of IWF are the focus of the remaining chapters of this thesis. Isolate H2.5, cultivars Atlas and Atlas 46 as well as the spore bioassay introduced in the present chapter form the basis of the studies in this thesis.

3.4.4 Fungal defense

A finding in this chapter was that germinating conidia of *R. secalis* were able to inactivate IWF antifungal compounds. Pathogens are known to be able to overcome or avoid plant defense responses by a variety of compounds or strategies (Boller, 1987). *Colletotrichum lindemuthianum* is known to produce an extracellular inhibitor of bean β(1,3) glucanase (Albersheim and Valent 1974). The antifungal peptides thionins, found in the cell wall of barley, were reported to be degraded by the fungal pathogen *Fusarium culmorum* (Ebrahim-Nesbat *et al.*, 1990). Many fungi are also known to

metabolise antifungal phytoalexins (VanEtten *et al.*, 1982).

In the present study, spore germination fluid (SGF) was prepared by growing *R. secalis* conidia in 1X IWF. When mixed with IWF, the SGF was found to deactivate the antifungal compounds of IWF. Considering that the base media for preparation of SGF was IWF, it is clear that the germinating spores not only deactivated the antifungal compounds in the media but also released an excess of deactivating compounds into the SGF. This implied that the interaction of IWF and *R. secalis* spores was not as simple as it might seem first. Instead it was a two-way interaction with the outcome of the interaction dependent on the balance of the fungal and plant compounds. Although this was considered during subsequent experiments in this thesis, no attempt was made to purify the fungal compounds.

Chapter 4:

Low molecular weight pathogenesis-related compounds implicated in the barley-*R. secalis* interaction

4.1 Introduction

4.1.1 Objective

In chapter 3 some of the basic biological and biochemical features of barley IWF were discussed. For further studies, IWF was divided into low- and high- molecular weight fractions. This chapter explores the finding in the IWF low-molecular weight (LMW) fraction.

The LMW IWF comprised that fraction of IWF that passed through an ultrafilter with a 3 kDa exclusion limit. The study on LMW IWF was aimed at detection of possible differences between inoculated and uninoculated plants and between compatible and non-compatible interactions. Two approaches were used to analyze the LMW IWF: one based on biological activity (bio-activity) and the other based on biochemical characteristics. With the first approach a compound or group of compounds with more relative abundance in inoculated plants and an ability to suppress *R. secalis* spore germination was detected. The second approach led to detection of a

low molecular weight hydroxyproline-containing peptide with no detectable biological activity against *R. secalis*. Both compounds are more abundant in the inoculated plants and could therefore be defined as "pathogenesis related".

4.1.2 Low molecular weight defense compounds in plant apoplast

The low molecular weight defense compounds phytoalexins and antimicrobial peptides are well characterised components of defense responses in plants. Phytoalexins are defined as low molecular weight antimicrobial compounds produced by plants in response to microbial attack or related elicitation (Deverall, 1982). First phytoalexins were isolated from the intercellular spaces of pea and bean challenged with fungal pathogens. These phytoalexins, called pisatin and phaseollin respectively, were shown to be closely related pterocarpans with a molecular weight of 314 and 322 Da (cited in Deverall, 1982). Suppression of fungal germination is a characteristic of many phytoalexins and has been frequently used to detect and purify the compounds (Deverall, 1982; Smith, 1982). Almost all the phytoalexins reported so far are hydrophobic compounds, mostly with aromatic structures. To date, no phytoalexin has been isolated from barley, although the presence of such a compound was speculated (Oku *et al.*, 1975; Oku *et al.*, 1994).

Amongst the major plant antimicrobial peptides, defensins, lipid transfer proteins (LTPs) and to a lesser extent thionins are secreted into the intercellular space (Broekaert *et al.*, 1997). Barley LTPs are relatively large at ~9 kDa, while defensins and thionins have a molecular weight of ~5 kDa. Barley antimicrobial peptides have been found to have an inhibitory effect on fungal growth *in vitro*. *In planta*, they appear to be either induced as a result of pathogen attack or have an apparent protective role (Broekaert *et al.*, 1997; Bohlmann, 1994; Molina *et al.*, 1993).

Another group of compounds known to accumulate in the plant apoplast includes proline- and hydroxyproline-rich PR glycoproteins (PRGP and HRGP). These compounds generally have molecular weights larger than 20 kDa but because of their importance in relation to the finding in this chapter are briefly reviewed here.

PRGP and HRGP are best known for their structural role in the plant cell wall (Jose and Puigdomenech, 1993). Moreover, many of these have been shown to increase following pathogen attack or similar stress conditions. Although insolubilization of these proteins in penetration sites may actually decrease their availability in extracts such as IWF, this is less likely to occur in the *R. secalis*/barley interaction. This is because the phenomenon of insolubilization is principally dependent on oxidative burst and hence induction of hypersensitive response (HR) (Dixon *et al.*, 1994; Tenhaken *et al.*, 1995) that is largely absent in the *R. secalis*/barley interaction (Lyngs Jorgensen *et al.*, 1993; Lehnackers and Knogge, 1989). To date, no cell wall-associated proline- or hydroxyproline-rich proteins have been reported in barley leaves. However, arabinogalactan-proteins (AGPs), a group of highly glycosylated hydroxyproline-rich proteins, are found in wheat and may also exist in barley (Fincher and Stone, 1974). The wheat AGP is estimated to have a molecular weight of ~22 kDa with only 8% protein moiety most likely positioned at the core of the molecule (Fincher *et al.*, 1974).

4.2 Materials and methods

4.2.1 Material

Anisole, BSA, heptafluorobutanoic, perchloroethylene, trifluoromethanesulfonic acid and tricine were from Sigma-Aldrich. Acetone, ammonium sulfate, ethanol, ammonia, chloroform, ethyl acetate, heptafluorobutanoic acid (HFBA) and formic acid were from BDH. Acetic acid and sodium hydroxide were from APS Finechem, Australia. Pre-stained Ultra-low marker was from BioRad. Pyridine was from AJAX Chemicals, Australia.

4.2.2 Plant and pathogen

Near isogenic barley cultivars Atlas and Atlas 46 were used. Plants were inoculated at the 2-leaf stage as explained in Chapter 2. *R. secalis* isolate H2.5 was used for both inoculation and the bioassay.

IWF samples were extracted following the procedure outlined in Chapter 2 on day three postinoculation unless stated otherwise. Fractionation of the IWF into low- and high-molecular weight (LMW and HMW) components was achieved using a Microcon™3 or Centricon™3 ultrafilter (Millipore) with a 3 kDa exclusion limit. Filtrates, containing sub-3kDa compounds of IWF, were collected. Concentration of LMW IWF was equal to that of the original IWF, *i.e.* 1X (see section 1.3.3.2).

4.2.3 Bioassay

The bioassays used in this chapter were dependent on spore germination. It was shown in Chapter 3 that conidia of *R. secalis* H2.5 germinated poorly in water. Addition of a small amount of unfractionated Atlas IWF (*i.e.* containing both HMW and LMW components) to all the treatments appeared to remedy this problem by

stimulating a steady level of germination. The stimulatory effect of IWF appeared to be equally present in IWFs extracted from either cultivars, Atlas or Atlas 46. However, IWFs also contained antifungal compounds (see Chapter 3). IWF from uninoculated Atlas, with the least antifungal activity, was used to maintain the germination while minimizing the antifungal activity introduced by the unfractionated IWF.

A volume of 60 µl of sample (LMW IWF or water) was mixed with 6 µl of Atlas IWF in a 1.5ml polypropylene (Eppendorf) test tube and dried in a Speed Vac™ vacuum-centrifuge for 1 to 2 hours. Before the experiment started, conidia of *R. secalis* isolate H2.5 were harvested from a 1-2 weeks old plate, suspended in sterilized distilled water and adjusted to 1 x 10^7 spores/ml with the aid of a haemocytometer. Six µl of the spore suspension was added to the bottom of each tube and briefly mixed with a pipette tip to aid reconstituting the dried sample. Tubes were sealed and incubation at 20 °C in the dark. After 12 hrs, the percentage germination, length of germ tubes (µm), appearance of the spores and presence of cellular debris were recorded using a microscope with 120 times magnification.

In this standard bioassay, concentrations of the sample and the added Atlas IWF were 10X and 1X, respectively. In certain conditions when sample strength might have been depleted during purification, more than 60 µl sample was used. These variations are noted in the text.

4.2.4 Reverse phase chromatography (RPC)

Reverse phase chromatography of LMW samples was carried out by high-pressure liquid chromatography (HPLC) using a Hewlett Packard 1090 Liquid Chromatograph™ and a Vydac 4.6 x 300 mm Peptide and Protein C18™ column. Solvent system and gradient conditions were according to the method RPC-LMW outlined in Chapter 2 (section 2.1.5.6.1).

Performance of the column used in this chapter was estimated by a recurring chromatographic technique. A volume of 500 µl of an aqueous solution of bovine serum albumin (BSA, 40 µg/ml) was chromatographed. The peak area was calculated and the eluted protein was collected and again injected for chromatography by the same

column. The ratio between peak area the second time to that the first time was used as the column performance factor. The performance factor of the Vydac® 4.6 x 300 mm C18 Peptide and Protein column was calculated to be 80%.

4.2.5 Protein precipitation

Five hundred microliters of LMW IWF was dried in a Speed Vac® centrifuge and reconstituted in 45 µl water. Five microliters of 50 mg/ml BSA aqueous solution was added to make a final concentration of 5 mg/ml BSA in a volume of 50 µl. This was precipitated with either acetone or ammonium sulfate (Edelstein and Bollag 1992).

For acetone precipitation, protein sample was mixed with 250 µl cold (-20°C) acetone and incubated at -20°C for 3 hrs. The mixture was centrifuged at 15000g in an Eppendorf benchtop centrifuge for 15 min. The supernatant was removed with a pipette and both the supernatant and precipitate were dried and reconstituted in 100 µl double deionised (DD) water.

For ammonium sulfate precipitation, the salt was added to the protein sample until saturation at the room temperature was reached. The mixture was centrifuged at 15000g in an Eppendorf benchtop centrifuge for 15 min. The supernatant was collected as above but not dried. and the precipitate was solubilised in 500 µl water.

4.2.6 Liquid two-phase fractionation

A liquid two-phase system, comprising cold (4°C) ethyl acetate and water, was used to fractionate LMW IWF into lipophilic, hydrophilic and amphiphilic phases. Equal volumes of cold ethyl acetate were added to LMW IWF in 2-ml polypropylene Eppendorf tubes on ice. The tubes were vigorously mixed for 5 minutes and then centrifuged in an Eppendorf benchtop centrifuge for 2 minutes at 15000g. The upper fraction, comprising the lipophilic phase, and the lower fraction, comprising the hydrophilic phase, were collected using a pipette. The remaining of both water and ethyl acetate, or interface, was left in the tube and treated as the amphiphilic phase.

Fractions intended for the bioassay were dried in a Speed Vac® vacuum-centrifuge.

4.2.7 High voltage paper electrophoresis

High voltage paper electrophoresis (HVPE) was carried out according to Murphy *et al.* (1987). A volume of 100 µl of hydrophilic LMW IWF was concentrated in a Speed Vac™ centrifuge until it had reduced to approximately 5 µl. It was then spotted in the middle of a 80 × 20 cm Whatman™ 20chr chromatography paper and air-dried. The paper was saturated with formic acid/acetic acid buffer (28.4 ml 98% formic acid, 59.2 ml glacial acetic acid in 1 L water, pH 1.75) and electrophoresed at 3000 V for 20 min in formic/acetic acid buffer with distilled perchloroethylene as the inert buffer. The paper was air-dried and extracted in a process explained in the following paragraph.

Fifteen cm from each end of the paper electrophorogram were discarded. The middle 50-cm was cut into 2 cm strips parallel to the direction of electrophoresis in a way such that each strip contained one sample. Each strip was then cut into 25 2-cm pieces. Each piece was extracted three times with 50µl DD water followed by a 5-min centrifugation in an Eppendorf centrifuge at 15000g. The extracts from each piece were pooled and filtered through a 0.4 µm Ultrafree™-MC filter before drying in a Speed Vac™ vacuum-centrifuge. Dried extracts were subjected to the routine bioassay explained previously.

4.2.8 Tricine SDS PAGE

Protein samples were resolved using the tricine-SDS polyacrylamide gel system of Schagger and Van Jagow (1987). The running gel was composed of 16% total acrylamide monomer (T) and 3% cross-linking bisacrylamide (C). This was overlaid with a 10%T 3%C spacer gel and a 4%T 3%C stacking gel. Samples were dried in a Speed Vac™, reconstituted in 20µl loading buffer and loaded into the wells. Gels were run in a Hoofer Electrophoresis Unit model SE600 at 15°C for 10 hours. To visualise proteins, gels were stained following the procedure of Dunn and Crisp (1994). BioRad Pre-stained Ultra-low marker was used to monitor the gel running condition and

estimate molecular weight.

4.2.9 Protein sequencing and amino acid analysis

Amino-terminal protein sequencing was carried out as outlined in Chapter 2.

Amino acid analysis was performed following acid-hydrolysis of protein samples according to Fincher and Stone (1974). Hydrolysed samples were analysed by the Nucleic Acid and Protein Chemistry Unit, Department of Plant Sciences, The University of Adelaide.

4.2.10 Chemical deglycosylation

Chemical deglycosylation of proteins was carried out by two different procedures, (1) hydrochloric acid method and (2) trifluoromethanesulfonic acid method.

(1): For the hydrochloric acid method, protein extracted from 2.5 ml LMW IWF was lyophilized in a 1.5 ml test tube using a Speed Vac™ centrifuge. A 500 µl volume of hydrochloric acid solution pH 1.0, was added and the mixture was incubated for 1 hr at 105°C (Derek *et al.*, 1973). The solution was cooled to room temperature and dried in a Speed Vac™. The deglycosylated protein was reconstituted in 100 µl water.

(2): For the trifluoromethanesulfonic acid method, protein extracted from 2.5 ml LMW IWF was lyophilized and deglycosylated using trifluoromethanesulfonic acid according to the Florman and Wasserman method described by Rosenberg (1996). The O-deglycosylated protein was dried overnight and reconstituted in 1 ml DD water.

4.3 Results

Results are divided into two sections based on the two major approaches used: the bio-activity approach and the biochemical approach. There is, however, some overlap between the two approaches.

4.3.1 Analysis of LMW IWF: bio-activity approach

In Chapter 3 it was shown that spores of *R. secalis* germinate poorly in water but an even smaller percentage was found to germinate in LMW IWFs. Spores also appeared to produce shorter germ tubes in LMW IWF. The effect of LMW IWF isolated from different treatments upon spore germination is investigated below. As explained in section 4.2.4, a small amount of unfractionated Atlas IWF was added to all the bioassays.

4.3.1.1 Suppression of germination by LMW IWF

Comparison of LMW IWFs and the control was conducted in triplicate. For each repeat, 60 µl LMW IWF extracted from Atlas, inoculated Atlas, Atlas 46 or inoculated Atlas 46 was mixed with 6 µl Atlas IWF and bioassayed. Controls included 6 µl Atlas IWF and 60 µl water. Both germination percentage (G%) and germ tube (GT) length were measured. The results are summarised in Figure 4-1. Data were also statistically analysed (see Appendix 3).

Analysis of variance of G% indicated a very significant difference between treatments. Pairwise comparisons showed that germination in all LMW IWFs was significantly lower than the controls (water). While LMW IWFs from Atlas and inoculated Atlas were not significantly different from each other, LMW IWF from inoculated Atlas 46 caused a significantly lower G% than that from Atlas 46. A comparison between Atlas and Atlas 46 and between inoculated Atlas and inoculated Atlas 46 did not reveal any significant difference in G%. In summary, it appeared that uninoculated plants had similar levels of germination inhibitor. The inhibitor increased upon inoculation in both plants but significantly more in the resistant cultivar Atlas 46.

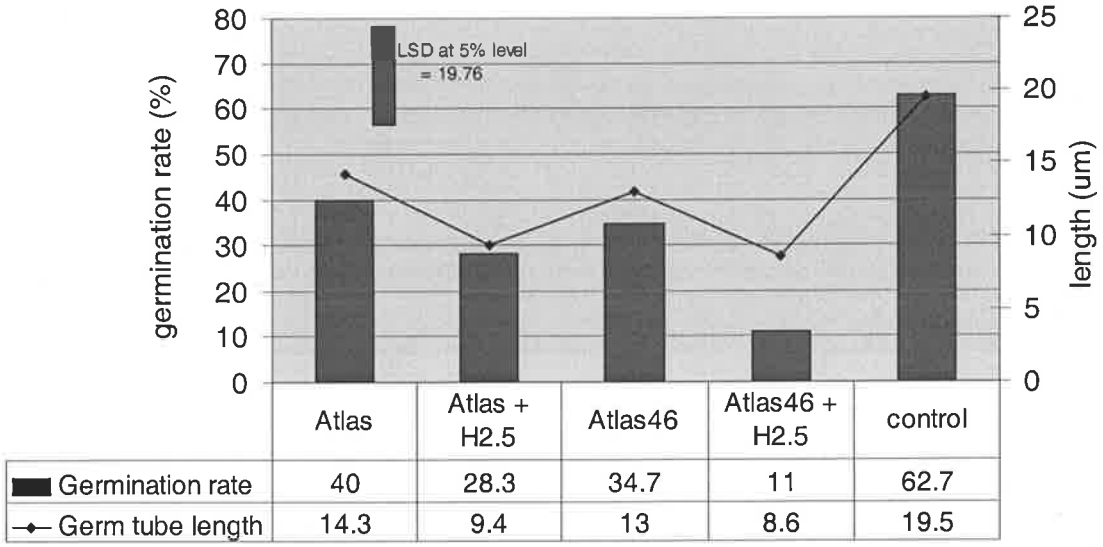


Figure 4-1: Germ tube length and germination percentage in *R. secalis* conidia incubated in LMW IWF

GT length and G% were measured after 12 hrs incubation of spores in LMW IWF from different sources. "+ H2.5" indicates that the source plants were inoculated with *R. secalis* isolate H2.5. The bar of least significant differences (LSD), on top left side of the graph, is for comparison between the G% only. GT lengths failed to show any significant differences and hence LSD is not applicable to them.

Analysis of variance for GT length showed that, with an F value of 5.5%, differences between treatments were just not significant at the 5% level (see Appendix 3).

Considerable time was spent to compare the antifungal effect of LMW IWF with that of LMW IWF mixed with Atlas IWF but no conclusive result was achieved. This was mainly due to poor and inconsistent germination of H2.5 in water and in LMW IWF alone. It was, however, noted that Atlas IWF may have a synergistic harmful effect on the spores when mixed with LMW IWF. It was frequently observed that LMW IWF mixed with Atlas IWF led to higher germination suppression and more spore damage than what was expected from each alone.

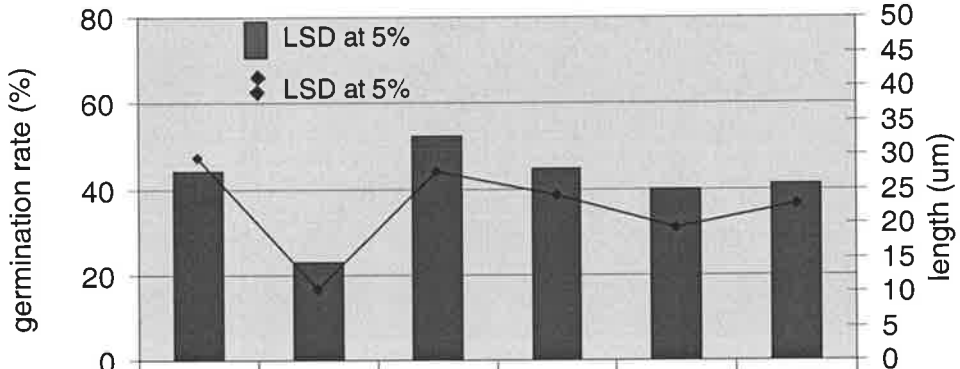
4.3.1.2 Two-phase fractionation of LMW IWF

To purify the germination inhibitor detected in LMW IWF, a fractionation technique based on liquid two-phase systems was used. Although both chloroform and ethyl acetate are immiscible with water and could potentially be used in a liquid two-phase system, preliminary tests showed that conidia germinated in chloroform-treated samples

produced malformed germ tubes. Ethyl acetate is not as immiscible with water as chloroform (Merck Index, 1983) but ethyl acetate-treated samples did not have any unexpected effect similar to that of chloroform-treated samples.

Equal volumes of cold ethyl acetate and LMW IWF from inoculated Atlas 46 were thoroughly mixed and after centrifugation separated into hydrophilic, amphiphilic and lipophilic fractions. For controls, sterilized distilled water was mixed with ethyl acetate and fractionated likewise. To bioassay these, fractions containing equivalent to 100 µl LMW IWF were mixed with 6 µl Atlas IWF and dried in a Speed Vac® vacuum-centrifuge. The bioassay results are summarized in Figure 4-2. It was found that (1) controls were not different from each other and (2) amongst the LMW IWF fractions, almost all the LMW IWF antifungal activity was contained in the hydrophilic (aqueous) fraction.

Analysis of variance confirmed that neither G% nor GT lengths were significantly different after incubation in the control lipophilic (L), hydrophilic (H) or amphiphilic (A) fractions. Comparisons of LMW IWF fractions were carried out in a Randomized Complete Block Design with controls fitted as covariants (see Appendix 3). It was



	LMW-L	LMW-H	LMW-A	CNTRL-L	CNTRL-H	CNTRL-A
germination rate	44	22.7	52.3	44.7	40	41
germ tube length	29.5	10.4	27.6	24	19.4	22.8

Figure 4-2: Germ tube length and germination percentage in *R. secalis* conidia incubated in LMW IWF fractions

GT length and G% were measured after 12 hrs incubation of conidia in lypophilic (L), hydrophilic (H) and amphiphilic (A) fractions of LMW IWF (LMW) and control (CNTRL). The solid LSD bar is for comparison between G% values and the lined bar is for comparison between GT lengths. The bars are not intended for comparisons involving controls as they were compared only to each other and failed to show any significant differences.

found that both G% and GT length in L, H and A fractions were significantly different. Covariance effects also appeared to be significant, which justified its use in the analysis. In both analyses, pairwise comparisons indicated that only fraction H significantly reduced the germination of spores.

The following points summarise the two-phase fractionation experiment:

- Comparison of the controls indicated that treatment with ethyl acetate did not adversely affect the spores.
- Ethyl acetate did not seem to have any detrimental effect on the LMW IWF active agent(s).
- The active agent(s) is a hydrophilic compound.

4.3.1.3 HVPE fractionation of hydrophilic LMW IWF

In an attempt to determine the charge characteristics of the proposed germination inhibitor compound(s), the hydrophilic fraction of LMW IWF was subjected to high voltage paper electrophoresis (HVPE). A 150 µl volume of the hydrophilic fraction of LMW IWF was concentrated, applied to HVPE and the fractions generated were bioassayed. However, no marked antifungal activity was detected in any of the fractions (data not shown). Pooled fractions also appeared to contain no significant activity.

To analyse the reason for this, an amount equivalent to 100 µl of hydrophilic fraction of LMW IWF was applied to a 2-cm x 2-cm piece of Whatman™ 20chr chromatography paper which was dried without addition of HVPE buffers or conducting electrophoresis. The paper was then extracted as explained in section 4.2.7. The extract was bioassayed but no activity was detected. It was concluded that the active compound might not be recovered from the chromatography paper regardless of the HVPE occurring or not. Further analysis of the active compound(s) in LMW IWF was stopped at this stage due to time constraints.

4.3.2 Analysis of LMW IWF: biochemical approach

4.3.2.1 Preliminary observations

It was found in Chapter 3 that LMW IWF contained low levels of protein as judged by the Bradford protein assay (see Table 3-4). However, this could not be confirmed by tricine SDS PAGE that can resolve proteins as small as ~1 kDa (Schagger and Van Jagow, 1987). Up to 150µl LMW IWF was applied to a tricine SDS PAGE but no protein band was detected (Figure 4-3).

LMW IWF was analyzed using reverse phase chromatography (RP chromatography or RPC). A preliminary observation on samples obtained 3 days post-inoculation suggested that at least one compound, detected at 17.6 min, increased after inoculation. Figures 4-4 and 4-5 represent typical chromatograms produced from Atlas and Atlas 46 LMW IWF, respectively. Infected (broken line) and non-infected (solid line) samples are compared in each chart. Following this finding a more thorough time-course study of changes in barley LMW IWF was carried out using RP chromatography. This is presented in the following section.

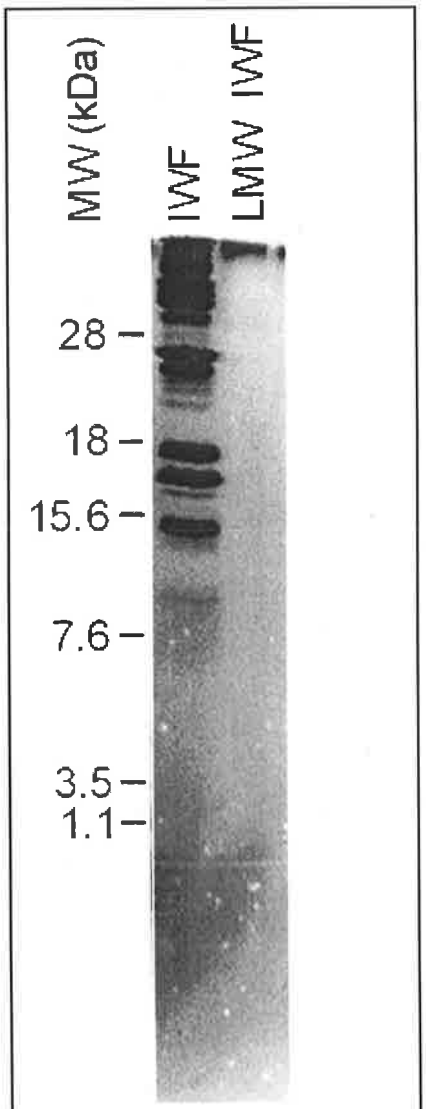


Figure 4-3: Protein profile of IWF and LMW IWF

4.3.2.2 A putative pathogenesis-related compound in the LMW IWF

Barley LMW IWF was collected on days 1, 2, 3 and 9 post-inoculation and applied to RP chromatography. Analysis of the results from 3 independent replicates indicated that all the chromatograms had 3 major peaks: a large shapeless peak at approximately 5.5 min and two

symmetrical ones at 17.6 and 18.7 min. The first peak was the void, containing a mixture of unbound compounds that are most likely non-proteins. The other two peaks were likely to be hydrophilic proteins.

The two major symmetrical peaks were named after their detection times (Pk17 and Pk18) and their areas were calculated as being indicative of the detected amounts.

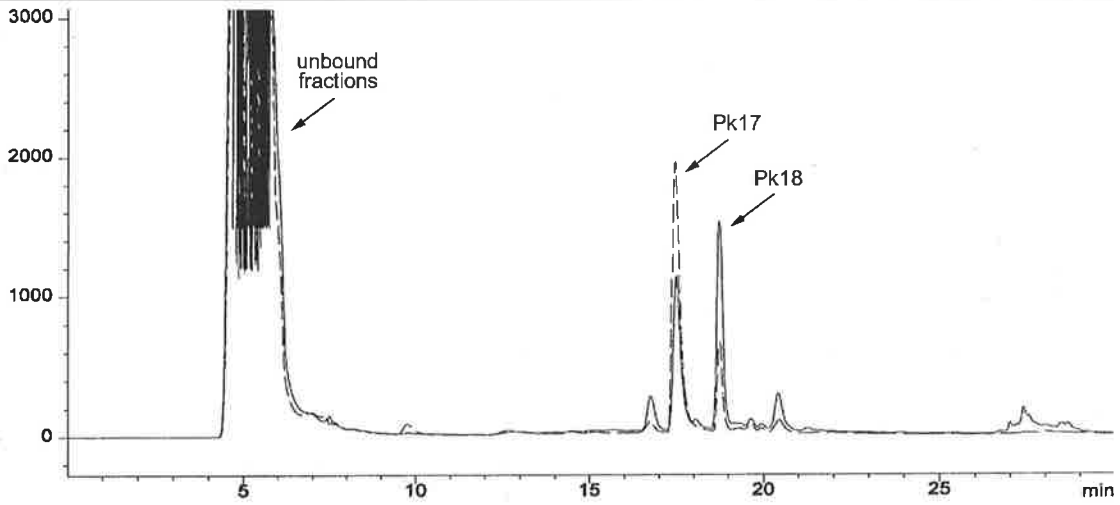


Figure 4-4: Comparative chromatogram of LMW IWF from control and inoculated Atlas on day 3 postinoculation
Solid line shows the control LMW IWF and the broken line shows the LMW IWF from the inoculated plants. Detection is based on absorbance at 214 nm. The vertical axis measures the absorbance at mili Absorbance Unit (mAU). Pk17 and Pk18 are shown on the chromatogram.

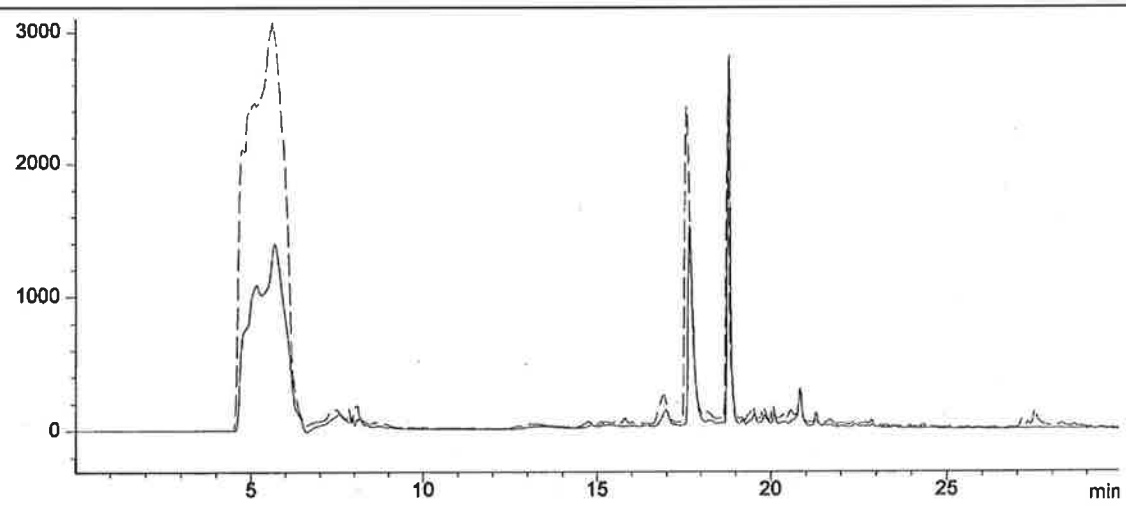
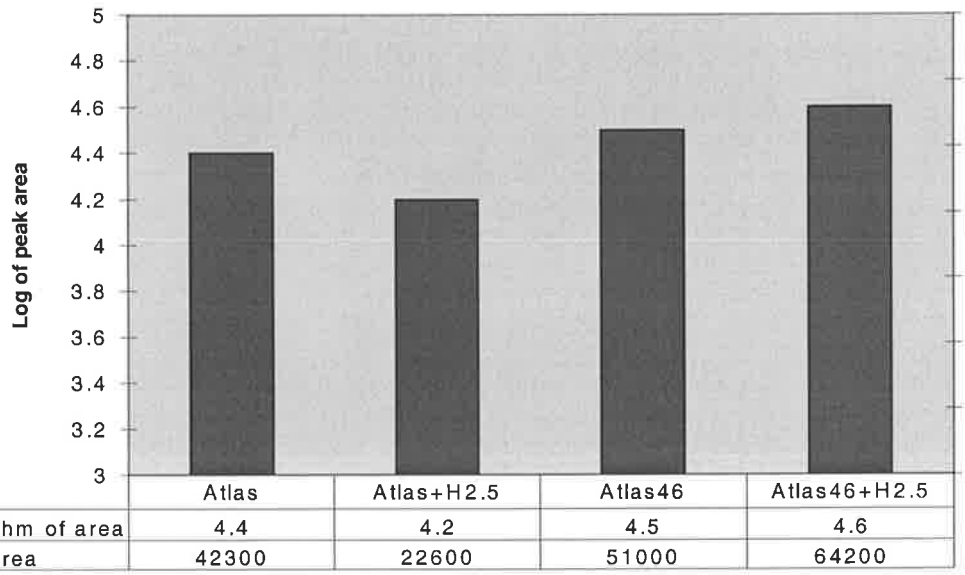


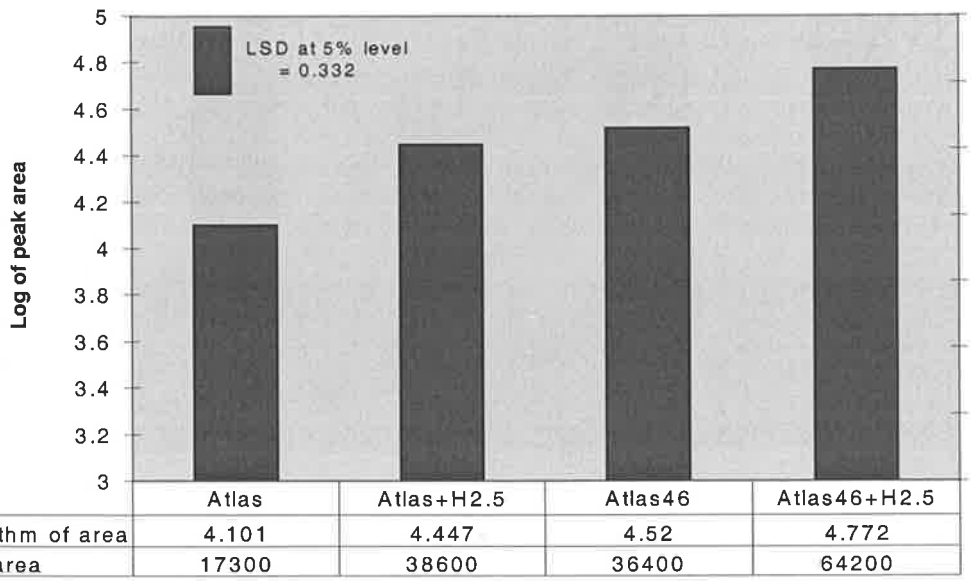
Figure 4-5: Comparative chromatogram of LMW IWF from control and inoculated Atlas 46 on day 3 postinoculation
Solid line shows the control LMW IWF and the broken line shows the LMW IWF from the inoculated plants. Detection is based on absorbance at 214 nm. The vertical axis measures the absorbance at mili Absorbance Unit (mAU).

Estimated quantities of both peaks in each day were applied separately to analysis of variance after logarithmic transformation. Results indicated that levels of Pk17, on day 2 to 9, were significantly different between the treatments (see Appendix 3). Figures 4-6, 4-7, 4-8 and 4-9 summarize the values for Pk17 level on days 1, 2, 3 and 9 respectively.



	Atlas	Atlas+H2.5	Atlas46	Atlas46+H2.5
Logarithm of area	4.4	4.2	4.5	4.6
Peak area	42300	22600	51000	64200

Figure 4-6: Comparison of Pk17 area for day 1 post-inoculation
Both log-transformed and original values are shown. "+ H2.5" indicates that the source plants were inoculated with isolate H2.5. No significant differences between the treatments were found.

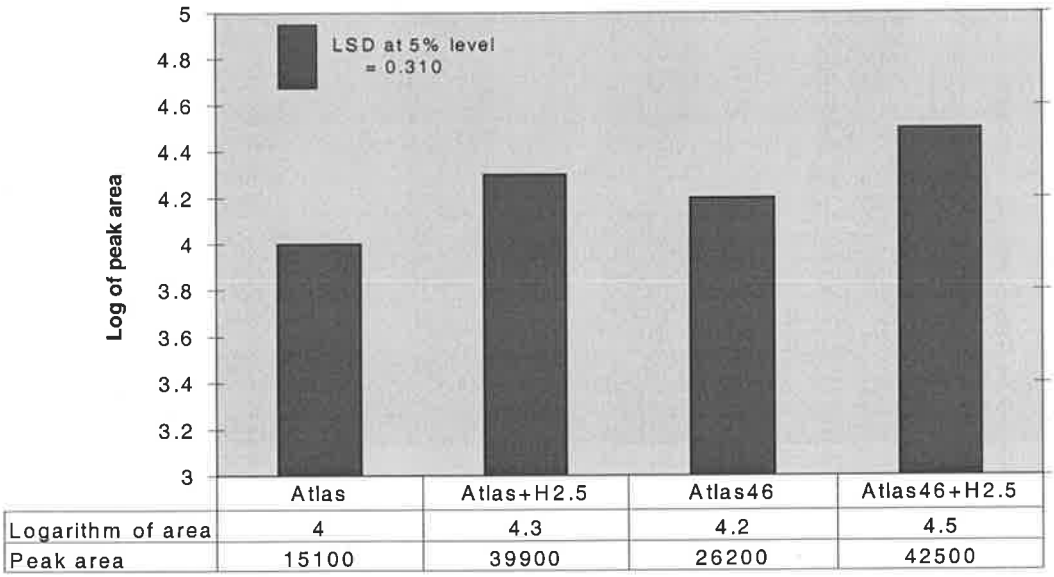


	Atlas	Atlas+H2.5	Atlas46	Atlas46+H2.5
Logarithm of area	4.101	4.447	4.52	4.772
Peak area	17300	38600	36400	64200

Figure 4-7: Comparison of Pk17 area for day 2 post-inoculation
Both log-transformed and original values are shown. "+ H2.5" indicates that the source plants were inoculated with isolate H2.5.

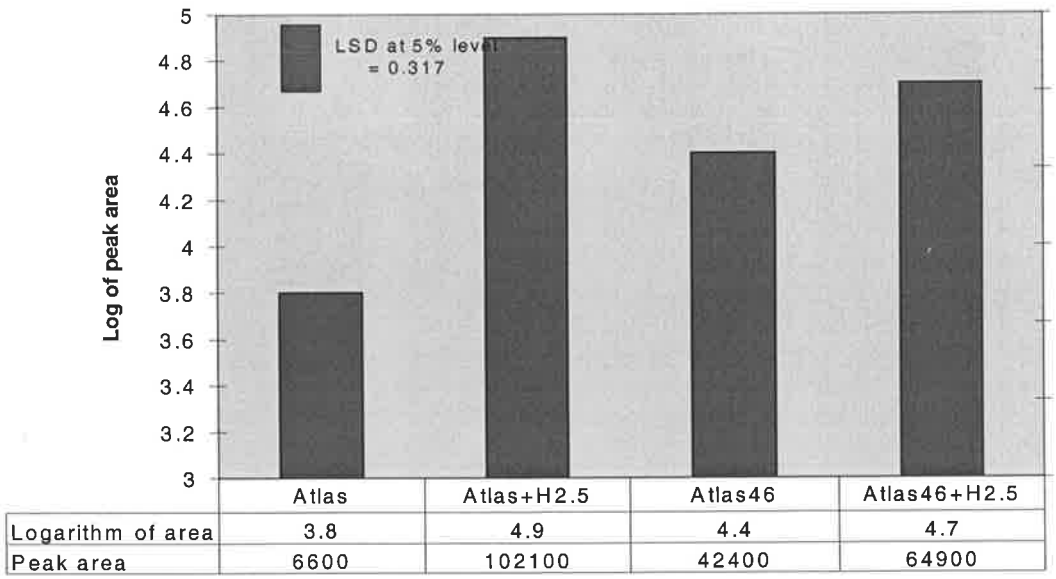
The following conclusions apply for Pk17:

- In uninoculated plants, Pk17 is of significantly higher abundance in Atlas 46 than that in Atlas on days 2 and 9.



	Atlas	Atlas+H2.5	Atlas46	Atlas46+H2.5
Logarithm of area	4	4.3	4.2	4.5
Peak area	15100	39900	26200	42500

Figure 4-8: Comparison of Pk17 area for day 3 post-inoculation
Both log-transformed and original values are shown. "+ H2.5" indicates that the source plants were inoculated with isolate H2.5.



	Atlas	Atlas+H2.5	Atlas46	Atlas46+H2.5
Logarithm of area	3.8	4.9	4.4	4.7
Peak area	6600	102100	42400	64900

Figure 4-9: Comparison of Pk17 area for day 9 post-inoculation
Both log-transformed and original values are shown. "+ H2.5" indicates that the source plants were inoculated with isolate H2.5.

- In Atlas 46, Pk17 shows no significant increases upon inoculation.
- In Atlas, Pk17 shows a significant increase in inoculated plants on days 2 to 9

No significant variation in the levels of Pk18 was detected (see Appendix 3). Following this finding, the study was more focused on Pk17 although, for comparison purposes, Pk18 was also included in some of the subsequent experiments.

Homogeneity of Pk17, and also of Pk18, was examined by elution from a RPC column under conditions different from that originally used to isolate the compounds. For every experiment, 500 µl IWF-equivalent of Pk17 was dried in a Speed Vac™ centrifuge, reconstituted in 500 µl buffer A and applied to RP chromatography. In total, 5 different chromatographic methods were used for this purpose. These are shown in rows 3 to 7 of Table 4-1. The original method, RPC-LMW that was used to purify Pk17 and Pk18, is shown in row 2 of this table.

In all the chromatography experiments, Pk17 consistently eluted in one single peak, indicating that it was homogenous. On the other hand, Pk18 was found to be comprised of three similar compounds. (data not shown). However, Pk18 was not purified any further because of its relatively less importance in this study.

method	temperature	eluent A	eluent B
RPC-LMW	40°C	0.05% TFA[1]	70% acetonitrile, 0.04% TFA
RPC-LMW1	20°C	0.05% TFA	70% acetonitrile, 0.04% TFA
RPC-LMW2	60°C	0.05% TFA	70% acetonitrile, 0.04% TFA
RPC-LMW3	40°C	0.05% HFBA[2]	70% acetonitrile, 0.04% HFBA
RPC-LMW4	40°C	0.05% TFA	70% ethanol, 0.04% TFA
RPC-LMW5	40°C	0.05% HFBA	70% ethanol, 0.04% HFBA

Table 4-1: RPC elution systems used to purify and analyse the purity of the low-molecular weight peptides
The first method, RPC-LMW, was used to purify Pk17 and Pk18 (for more details see Chapter 2). The other methods, RPC-LMW1 to RPC-LMW5, were only used to analyse the homogeneity of the compounds.
[1]: TFA is abbreviation for trifluoroacetic acid.
[2]: HFBA is abbreviation for heptafluorobutanoic acid.

4.3.2.3 Characterization of the LMW IWF compounds.

The ability of acetone and ammonium sulfate in precipitation of Pk17 and Pk18, under the conditions that precipitate most proteins, was analysed. Since precipitation may not occur if total protein concentration is less than 1 mg/ml (Edelstein and Bollag 1992) and the concentration of Pk17 and Pk18 was unknown, a small amount of BSA was added to both solutions of Pk17 and Pk18 before the analysis.

A volume of 500 µl of LMW IWF from Atlas 46 was concentrated 10 times, adjusted to 5 mg/ml BSA and precipitated with 83% acetone or saturated ammonium sulfate. To detect Pk17 and Pk17, both the precipitate and supernatant were collected and analysed with RP chromatography. Neither Pk17 nor Pk18 precipitated with acetone. However, Pk18 was almost completely precipitated in saturated solution of ammonium sulfate while Pk17 solubility was only partially lost. Fifty five percent of Pk17 was recovered from the ammonium sulfate supernatant and 45% was in the precipitate as assessed by Pk17 peak size following RP chromatography. As expected, almost all the BSA was recovered from the precipitates of the acetone or ammonium sulfate solutions.

Bradford protein concentration assay was used to detect Pk17 and Pk18 in solutions. But even at 20X concentration no protein was detected. In section 4.3.2.1, no protein was detected in LMW IWF as judged by tricine-SDS PAGE. To directly analyse Pk17 and Pk18 by this technique, both compounds were purified from 250 µl Atlas 46 LMW IWF and subjected to tricine-SDS PAGE. However, no protein band was detected following visualisation by silver staining (data not shown).

Pk17 and Pk18, purified from 250 µl Atlas 46 LMW IWF, were subjected to amino acid analysis. It was found that both compounds did have peptide moieties in their structure. The analysis also indicated the presence of yet unknown moieties in the structure of both compounds. Table 4-2 presents a comparison of peptide composition in Pk17, Pk18 and also an arabinogalactan-peptide (AGP) from wheat endosperm (Fincher *et al.*, 1974).

Attempts to sequence the Pk17 peptide through Edman N-terminal sequencing failed as it appeared that the peptide is blocked at the N-terminal. The first cycle of the degradation produced amino acids glycine, alanine and valine but no more amino acids

were detectable in subsequent cycles.

Pk17 was subjected to reductive alkylation and a subsequent tryptic digestion. However, neither the alkylation nor the enzyme affected the peptide as judged by its unchanged position and appearance on chromatograms produced following these steps (data not shown). Attempts to cut the alkylated peptide with the non-specific stronger protease, proteinase K, also failed. Ovalbumin was used as the positive control in both alkylation and digestion steps.

amino acid	Pk17	Pk18	wheat AGP[1]
ASX	10.9	6.3	6.3
GLX	10.5	7.3	12.2
SER	4.7	7.2	9.6
HIS	1	0	0.6
GLY	14.4	15.3	4.8
THR	6.1	3.3	6.1
CYS	ND	1	0
ALA	7.7	6.7	24.3
ARG	1.8	0	0.9
TYR	1.6	4.6	3.4
VAL	10.7	6.4	6.0
MET	ND	1.3	1.0
PHE	3.2	5	0.5
ILE	5.5	6.6	1.4
LEU	7.5	11.2	1.4
LYS	3.4	0	2.1
PRO	8.7	15.8[2]	1.3
HYP	2.5	2.0[2]	16.7
total	100	100	100

Table 4-2: Amino acid analysis of the peptides in LMW IWF
Amounts are in picomol. ASX denotes aspartic acid or asparagine. GLX denotes glutamic acid or glutamine. HYP is hydroxyproline. Tryptophan could not be measured by the method used.
ND: Not detrmined.
[1]: reproduced from Fincher *et al.*, 1974.
[2]: Due to interference of unknown moiety, these values are only approximates.

Resistance to enzymatic digestion and results of the amino acid analysis suggested that Pk17 is probably highly glycosylated. Pk17 extracted from 25 ml Atlas 46 IWF was incubated in HCl pH 1.0 for 1 hr at 105°C (Derek *et al.*, 1973) or treated with trifluoromethanesulfonic acid and anisole scavenger (Rosenberg, 1996). The products were then applied to RP chromatography but no changes in the elution time was detected, indicating that Pk17 did not undergo any changes. Treatment with HCl followed by trifluoromethanesulfonic acid did not affect Pk17 either.

Although RP chromatography is generally considered a denaturing procedure, some proteins especially LMW species may retain their structure or re-nature following the

chromatography. A volume of 100 µl of LMW IWF was applied to the RP chromatography. One fraction per minute was collected, dried in a Speed Vac centrifuge and applied to the bioassay. However, no germination inhibition or other biological activities were detected (data not shown).

4.4 Discussion

4.4.1 Statistical design

Throughout the experiments sampling units were made of pooled IWF from 15 plants or more to compensate for variation between individual plants. While this should have helped to smooth out individual data points, there was still relatively large variation between replicates. This was attributed to carrying out the repetitions at different times and thus introducing a new source of variation. To account for this effect, replicates were recognized as a block factor in statistical analysis. This choice of design was also confirmed in analysis of variance by showing that residual mean square in Randomized Complete Block Design was notably smaller than that in a comparable Completely Randomized Design.

4.4.2 The bioassay

Biological activity and bioassays were of prime importance in the study of IWF. In the case of LMW IWF, bioassays were dependent on spore germination, an infrequent phenomenon in either LMW IWF or water. An overall low germination level meant that differences in germination were, if not impossible, difficult to detect. This was rectified by adding a small amount of Atlas IWF to all treatments. It was explained in Chapter 3 that isolate H2.5 germinated poorly in the absence of host plants or appropriate plant stimulants. IWF, being a plant extract, was highly stimulatory to spore germination. Since spore damage associated with IWFs is minimal in 1X Atlas IWF, this was added to all treatments including the control.

It is believed that addition of Atlas IWF assisted with the bioassay in two major ways. Firstly, it increased and stabilized the overall germination level to approximately 50%. This in turn magnified the potential differences. Secondly, it might restore some of the activity that was lost because of ultrafiltration. It is conceivable that any fractionations, including ultrafiltration, could potentially separate co-factors or diminish

effects that were otherwise stronger because of synergistic effects. Addition of HMW IWF or simply IWF to all the treatments may restore these effects. Since the same composition was added equally to all the treatments, the only source of variation was the LMW IWFs. Whether the effects were the result of an interaction between component of the LMW- and HMW-IWF or caused by LMW IWF alone is another matter that could be investigated separately.

4.4.3 Analysis of LMW IWF through the bio-activity approach

R. secalis spore germination was the only factor that appeared to be affected in the presence of LMW IWF. Two germination parameters, G% and GT length, were studied in every experiment.

Comparison between LMW IWFs from inoculated and uninoculated Atlas and Atlas 46 indicated a highly significant difference (F=0.4%) for G% and an approaching significance (F=5.5%) for GT length in LMW IWFs from different treatments. The highest level of germination constantly occurred in the control treatment that did not have any LMW IWF. LMW IWFs from both resistant and susceptible cultivars reduced germination of *R. secalis* conidia to a similar level. LMW IWF isolated from both cultivar after inoculation reduced the germination levels even further, although this was markedly more for the LMW IWF collected from the resistant cultivar Atlas 46. The data presented here indicate that LMW IWF contains a compound that inhibits the germination of *R. secalis* spores. The fact that this compound increased in inoculated resistant plants is suggestive of the specific nature of this factor. Accordingly, non-specific compounds such as salts, that may inhibit spore germination in certain concentrations, are probably not involved.

Barley is known to contain the enzymes PAL (Boyed *et al.*, 1994a and 1994b; Kervinen *et al.*, 1998) and CHS (Gregersen *et al.*, 1997). Although these enzymes have been shown to be involved in the production of flavonoid phytoalexins in plants, no barley phytoalexins have been identified so far (Oku *et al.*, 1994). Phytoalexins from many plants have been shown to inhibit spore germination or germ tube elongation (Smith, 1982; Coxon, 1982; Oku *et al.*, 1994). For purification purposes, two-phase

systems have been frequently used to separate the hydrophobic phytoalexins from hydrophilic compounds abundantly present in the plant inter-cellular space (Deverall, 1982; Ingham, 1982). Although phytoalexins are not necessarily hydrophobic, detection in barley of PAL, CHS and a recently reported flavonoid 7-O-methyltransferase (Christensen *et al.*, 1998) suggest that a pathway involved in production of an aromatic phytoalexin in barley may exist. Barley LMW IWF suppressed germination of *R. secalis* spores but when it was applied to an ethyl acetate-water two-phase system the active agent was almost entirely sequestered in the aqueous phase. There was no indication of a hydrophobic active compound in the solvent phase.

The two-phase fractionation experiment indicated that the LMW IWF active agent(s) is a polar compound and could therefore be purified by procedures based on molecular charge such as HVPE. However, this could not be shown due to a problem in recovering the active compound from the chromatography paper. It is possible that the active compound was absorbed to the paper. Future purification could be focused on chromatography techniques such as thin layer chromatography (TLC). In the case of TLC a mobile phase composed of 70% propanole (or propane-1-ole) and 30% water would be a good starting point.

The expression pattern of the antifungal activity in LMW IWF was similar to that of Pk17, that is, more in the resistant and inoculated plant. Yet no antifungal activity was detected in bioassay of LMW IWF HPLC fractions. It is not known if Pk17 and Pk18 have any effect on *R. secalis* conidia or whether their activity was lost because of the RP chromatography. Nevertheless, detection of hydroxyproline in Pk17 and Pk18 suggest that these peptides belong to plant structural proteins that do not normally have any direct antimicrobial activity.

4.4.4 Analysis of LMW IWF through the biochemical approach

Since Pk17 and Pk18 are present in uninoculated as well as in inoculated plants it is most likely that both compounds were of plant origin. Pk17 also appeared to increase early enough in the course of infection to suggest that it may play a role in plant resistance.

Amino acid analysis provided the only evidence on the proteinaceous nature of Pk17 and Pk18. The analysis also indicated that glycine accounts for approximately 15% of the protein moiety in both Pk17 and Pk18. The hydrophobic amino acids valine, leucine and proline as well as the polar glutamine and asparagine (or their acid forms) are at fairly high levels in both Pk17 and Pk18. The higher amount of proline in Pk18 may explain its higher hydrophobicity and hence, later elution from the reverse phase column. The basic amino acids lysine, arginine and histidine were either absent or at low levels in both peptides. The amino acid cysteine, involved in protein cross-linking, was also at very low levels. Both Pk17 and Pk18 appeared to contain hydroxyproline, an amino acid specific to structural proteins in plant cell walls (Jose and Puigdomenech, 1993). Tyrosine, an amino acid involved in insolubilization of structural proteins, was present in low amounts.

Cell wall structural proteins have been classified into three major groups: proline-rich glycoproteins (PRGPs), hydroxyproline-rich glycoproteins (HRGPs) and glycine-rich proteins (GRPs) (Bowls, 1990; Jose and Puigdomenech, 1993). The proteins are under both developmental and stress regulation. In particular, many of them were shown to increase in abundance response to pathogen attack (Bowles, 1990; Jose and Puigdomenech, 1993). PRGPs and HRGPs induce a rapid hardening of the cell walls through insolubilization cross-linking that particularly relies on tyrosine residues. The cross-linking is triggered by oxidative-burst, a phenomenon correlated with the hypersensitive response (Dixon *et al.*, 1994; Tenhaken *et al.*, 1995). The insolubilization may lead to an apparent decrease in levels of HRGPs and PRGPs in extracts such as IWF. However, this apparent decrease probably does not occur in the *R. secalis*/barley interaction that lacks the hypersensitive response and the oxidative-burst (Lyngs Jorgensen *et al.*, 1993; Lehnackers and Knogge, 1989).

In plants, hydroxyproline is generally specific to a group of proteins collectively known as HRGPs. These include lectins, extensins and arabinogalactan glycoproteins (Jose and Puigdomenech, 1993). Hydroxyproline-containing lectins are essentially limited to the Solanaceae family. Extensins are basic (positively charged) and tightly absorbed to the cell wall. They are usually extractable only by high-salt solutions. However, acidic arabinogalactan proteins (AGPs) are extremely soluble and are extractable by water alone (Jose and Puigdomenech, 1993; Showalter and Varner, 1989). Similarly, both Pk17 and Pk18 were extracted from barley apoplast with water.

Other similarities include the ability of many AGPs to stay soluble in a saturated solution of ammonium sulfate that normally precipitates most proteins (Fincher *et al.*, 1983; Showalter and Varner 1989). AGPs may also be soluble in presence of organic solvents. For example, wheat AGPs were extracted with 80% ethanol (Fincher and Stone, 1974). AGPs, and generally most HRGPs, are unable to be resolved by SDS-PAGE. They are also resistant to enzymatic digestion and to protein staining procedures. A high degree of glycosylation is one of the reasons suggested to be responsible for these unusual characteristics (Lamport, 1969; Stuart and Varner, 1980).

Typical AGPs are rich in hydroxyproline, serine, alanine, glycine and probably threonine (Fincher *et al.*, 1983; Showalter and Varner, 1989). Nevertheless, almost all AGPs are highly glycosylated and 90-98% of their molecular weight is due to a carbohydrate moiety (Fincher *et al.*, 1983). A 22-kDa AGP isolated from wheat endosperm is comprised of 92% polysaccharide and a peptide core estimated to contain only 20 amino acids (Fincher and Stone, 1974). Like many other glycoproteins, AGPs have their carbohydrate moiety attached to serine, threonine or hydroxyproline residues (Fincher *et al.*, 1983). However, Pk17 and Pk18 are relatively rich in glycine and contain only 2 to 2.5% hydroxyproline. The other two amino acids capable of establishing glycosidic links, serine and threonine, are in intermediate levels in both Pk17 and Pk18. Nevertheless, neither trifluoromethanesulfonic acid (Rosenberg, 1996) nor HCl (Derek *et al.*, 1973) deglycosylation procedures appeared to affect the chromatographic characteristics of Pk17. Accordingly, glycosylation of Pk17 and Pk18 was not proved although not all glycosylated proteins are deglycosylated by these procedures (Rosenberg, 1996). Future deglycosylation attempts could be focused on treatment with anhydrous hydrogen fluoride (Mort and Lamport, 1977). Alternatively, oxalic acid or alkaline degradation (Fincher and Stone, 1974) may provide useful information regarding the carbohydrate composition of the peptides.

Pk18 and especially Pk17 appeared to be highly soluble even in acetone or ammonium sulfate solutions. Many proteins are soluble in water but precipitate in the presence of salts or organic solvents, a phenomenon explained by a reduced level of interaction between water molecules and proteins (Knuth and Burgess, 1987; Rosenberg, 1996). Water molecules normally surround protein molecules and prevent them from directly interacting with each other. Some salts and organic solvents can disturb this condition. This promotes inter-protein interactions leading to absorption of

protein molecules to each other at the polar or especially hydrophobic patches. The result is protein aggregations that are large enough to precipitate (Roe, 1989; Rosenberg, 1996). Since acetone is less effective in precipitating proteins smaller than 15 kDa (Edelstein and Bollag 1992), the results of salt precipitation are more significant in this case. The solubility of Pk17 and Pk18 in precipitating conditions suggests that no significant polar or hydrophobic inter-molecular interaction was formed between these molecules. Since proteins are inevitably made of polar or hydrophobic structural units, one explanation is that these are masked with sugars that are neither polar nor hydrophobic. Glycosylation may also explain the resistance of Pk17 and Pk18 to enzymatic digestion, colorimetric quantification and to protein staining following SDS-PAGE.

Amino acid analysis suggested that each Pk17 molecule contains approximately 125 amino acids with a molecular weight of ~13 kDa. This does not agree with the fact that Pk17 passed through an ultrafilter membrane with a 3-kDa molecular weight cut off (MWCO). For a protein to be 3 kDa or smaller, it must have less than 30 amino acids. A similar inconsistency was also reported by Fincher and Stone (1974) who analysed the wheat AGP, a glycoprotein that was demonstrated to have ~20 amino acids.

It is possible that some of the low-abundant amino acids detected for Pk17 and Pk18 are from contaminating proteins. However, this was not confirmed by any of various RP chromatographies that were conducted to check the purity of Pk17. Another more likely explanation is the existence of isoforms with small differences in amino acid compositions. These isoforms might not be separated by the purifying chromatographies. Moreover, since the nominal MWCO of an ultrafilter is essentially for globular proteins, it is possible for linear proteins with a much larger size to pass through a membrane with a 3-kDa MWCO. A linear structure is also in agreement with the peptide structure suggested for AGPs (Fincher *et al.*, 1983), although the actual size is dependent on the amount of carbohydrates attached to the protein core.

Chapter 5:

Cell-wall-degrading proteins implicated in the barley-*R. secalis* interaction

5.1 Introduction

5.1.1 Objective

The study of intercellular washing fluid (IWF) throughout this thesis has been based on the partitioning of IWF into low- and high-molecular weight fractions. In Chapter 4 the low-molecular weight (LMW) fraction was discussed. This and the next two chapters explore the characteristics of the high-molecular weight (HMW) fraction.

Preliminary experiments indicated that the interaction of barley cultivar Atlas with isolate H2.5 was compatible while that of cultivar Atlas 46 was incompatible (see Chapter 3). It was also shown that IWF from both Atlas and Atlas 46 possessed antifungal activity and that this activity was markedly higher in both cultivars after inoculation with the pathogen. Although the IWF isolated from inoculated Atlas had slightly higher antifungal activity, the IWF from inoculated Atlas 46 was selected for further study because it represented the incompatible interaction. Accordingly, the objective of this chapter was to identify the HMW antifungal compound(s) present in

the IWF of the resistant plant after inoculation. The study was focused on compounds with cell wall lytic activity. Preliminary analysis suggested that these compounds were proteinaceous in nature. Hence, subsequent purification strategies were focused on methods developed to purify proteins.

5.1.2 The choice of purification techniques

Two major techniques have been used to isolate proteins in general, and plant bioactive proteins in particular: chromatography and polyacrylamide gel electrophoresis (PAGE). Chromatography techniques, being rather versatile in terms of pH and buffer conditions, have been applied by many researchers especially when biological activity is of prime importance (Roberts and Selitrennikoff, 1990; Hrmova and Fincher, 1993; Kragh *et al.*, 1993; Cheong *et al.*, 1996). PAGE methods, on the other hand, are less complicated and usually result in higher resolution of the proteins. However, they are generally restricted by their specific pH, ionic strength and buffers that may not be compatible with the requirements of a labile protein. Nevertheless, many non-denaturing PAGE systems have been used to characterise or purify bioactive plant proteins (Pozo *et al.*, 1998; Rothe *et al.*, 1998; Grenier and Asselin, 1990). To overcome the poor resolution of non-denaturing gels, some researchers combined the high resolution of a denaturing SDS-PAGE separation with the ability of many proteins to re-nature following electrophoresis (Trudel and Asselin, 1989; Trudel *et al.*, 1998). This usually means that no reducing agent, such as β-mercaptoethanol or DTT, is added to the protein samples as the proteins may unfold irreversibly (Trudel and Asselin, 1989; El Ouakfaoui and Asselin, 1992; Trudel *et al.*, 1998).

In this project both chromatography and PAGE techniques were examined for purification of the antifungal proteins. However, high performance liquid chromatography (HPLC) was eventually used to successfully purify the proteins of interest. This technique was relatively fast, suitable for small IWF samples and had better resolution than other types of chromatography. Reducing SDS-PAGE was routinely used to monitor the protein profiles during the course of HPLC purification.

5.2 Materials and methods

5.2.1 Materials

Ammonium acetate was obtained from Ajax Chemicals, Sydney, Australia. Hydrogen orthophosphate was obtained from BDH.

5.2.2 Intercellular washing fluid

IWF was extracted from barley cultivar Atlas 46 three days post-inoculation as outlined in Chapter 2. This was fractionated into low- and high-molecular weight components, using Centricon™ ultrafilters with an exclusion limit of 3 kDa or 10 kDa. Retentate was recovered according to the procedure supplied by Millipore™. Ultrafilters were used.

The concentration of HMW IWF was calculated in the form of "X times IWF". To do this, the volume of recovered HMW IWF was divided by the volume of the original IWF, multiplied by the performance of the ultrafilters. The recovery of ultrafilters is estimated by the manufacturer to be 95%, *i.e.* 5% of the proteins are not recoverable following ultrafiltration.

5.2.3 Dialysis

Samples, typically 100-500 µl, were dialyzed according to the method of Overall (1987). Dialysis was carried out at 4°C for a total of 24 hours against two changes of 500 ml of water or the bioassay buffer (10 mM ammonium acetate pH 6.0 and 1 mM DTT). For fractions generated by HI chromatography (HIC), 4% polyethylene glycol (PEG, MW 6000) was added to the first change of buffer. This was to compensate for the high osmosis pressure caused by the high salt concentration in the HIC buffer. In the absence of PEG, dialysis membranes occasionally burst. Overall (1987) estimated

the protein recovery of this method to be 90%.

5.2.4 Estimation and adjustment of concentration for the bioassay

IWF was fractionated by ultrafiltration or chromatographic steps. However, every purification step changed the concentration of proteins in the fractions generated. Performance of a method was used to estimate the concentration of proteins in these fractions that, in turn, was used to re-calibrate the protein concentration for subsequent bioassays. This standardized the bioassay and ensured that the concentration of a given protein was relatively constant in bioassays carried out after each purification step. Nevertheless, this method is only suitable for detecting major bioactive compounds.

Adjusting the concentrations required the calculation of three values: column performance, method performance and IWF-equivalent concentration. These are explained below.

- **Column performance (Pc)** = second peak area ÷ first peak area

In order to calculate the performance of a particular column, an ovalbumin solution containing 20 μg was applied to the column and chromatographed. The eluted protein was collected and if required, concentrated in a Speed Vac™ before re-chromatographing by the same method. The peak areas in the first and the second chromatograms were calculated and used as above. Column performance for non-denaturing purification steps used in this chapter are shown in Table 5-1. Performance of an ultrafiltration device was estimated by the manufacturer to be 95%. Performance of dialysis was estimated to be 90% (Overall, 1987)

- **Method performance (Pm)** = Pc ÷ peak width

Peak width, measured in minutes, was the width of the ovalbumin peak in the first chromatogram explained above. Method performance for non-denaturing purification steps used in this chapter are shown in Table 5-1.

- **IWF-equivalent concentration** = Pm × starting concentration × volume of sample

purification step	Pc	Pm	volume of sample (ml)	volume of generated fractions (ml)	concentration of generated fractions
Ultrafiltration	NA	95%[1]	20	1	19 X
SAX chromatography	80%	40%	1	0.4	19 X
WCX chromatography	85%	39%	0.8[2]	0.4	14.8 X
HI chromatography	78%	25%	1.2[3]	0.2	22.2 X
GP chromatography	70%	10%	0.6[3]	0.2	6.7 X
Dialysis	NA	90%[1]	0.2	0.2	6.03 X

Table 5-1: Estimated chromatography performance and typical concentration factors

Purification steps, column performance (Pc), method performances (Pm) and concentration factors used for purification of a TL protein from barley IWF are shown. Columns and methods are explained in section 5.2.3 (GP chromatography was with a Macrosphere column).
[1]: Performance estimated by others.
[2]: Two active fractions from the previous step were pooled.
[3]: Three active fractions from the previous step were pooled.
NA: not applicable.

÷ volume of generated fraction

Where:

- "Starting concentration" is IWF-equivalent concentration of the sample before applying to the method. This is 1 for IWF.
- "Volume of sample" is the original volume of the sample prior to the purification step.
- "Volume of generated fraction" is the volume of the sample after the purification step.

As the purification proceeded, sample concentrations were calculated for each purification step. A typical concentration account is shown in Table 5-1. The concentrations were adjusted only if the samples were intended for bioassay. Samples with too high a concentration were diluted before the bioassay. When the concentration was too low, sample volumes were reduced in a Speed Vac™ before dialysis, which was a prerequisite of the bioassay.

5.2.5 Bioassay

Protein samples intended for bioassay were dialysed against the bioassay buffer (10 mM ammonium acetate pH 6.0 and 1 mM DTT) and adjusted to 10X IWF-equivalent concentration (for adjustment of concentration and related topics see section 5.2.4). Three µl of each sample was placed at the bottom of a 1.5 ml polypropylene (Eppendorf) centrifuge tube. To this was added 3 µl *R. secalis* conidia suspension already adjusted to 2 x 10^7 spores per milliliter in the bioassay buffer. The 6-µl assay preparation was briefly mixed with a pipette tip and sealed in the test tube. The final concentrations were 10 mM ammonium acetate pH 6.0, 1 mM DTT, 5X protein and 1 x 10^7 spores/ml. Variations to this bioassay, when applicable, are explained in the text.

Samples were incubated overnight (12 hours) at 20°C in the dark. To terminate the bioassay, samples were placed on ice and examined under the microscope the same day. Depending on experimental requirements, one or all of the characteristics listed below were recorded. Note that "intact spores" refers to the spores with a superficially intact cell wall. This exclude the (non-viable) spores with fractured cell walls.

- Percentage of doublet (DB) spores, calculated as:

  number of DB spores ÷ total number of intact spores.

- Percentage of internally granulated (IG) spores, calculated as:

  number of IG spores ÷ total number of intact spores.

- Percentage of germinated spores, calculated as:

  number of germinated spores ÷ total number of intact spores.

- Percentage of spores with a fractured cell wall (FW), calculated as:

  number of FW spores ÷ total number of spores.

- Percentage of viable spores, calculated as:

  number of viable spores ÷ total number of spores.

  A spore viability test based on the procedures of Widholm (1972) and Heslop-

Harrison and Heslop-Harrison (1970) was used. This involved preparing a stock of 5 mg/ml fluorescin diacetate (FDA) in acetone that was stored at -20°C. On the day of the experiment a working solution was prepared by dissolving 10 µl of the stock solution in 500 µl water. Three µl of the FDA working solution was added to the sample and incubated for five minutes at room temperature. Spores were observed under an Olympus™ microscope equipped with a UV light source and a barrier filter 47. Spores that fluoresced were considered to be viable.

For an expanded description of the FW, DB and IG spore phenotypes see Chapter 3.

5.3 Results

The results are presented in 3 sections. Development of the bioassay procedure and how this affected the purification strategies are discussed in the first section. Section 2 explores the different antifungal activities detected in HMW IWF. In Section 3, a bioassay of these antifungal activities is used to purify a number of proteins.

5.3.1 Optimization of the bioassay and chromatography

The final purification strategy used in this thesis is presented in section 5-3-3. But before the final strategy was known many different issues had to be resolved and many methods were tried. The optimization process of both bioassay and chromatography that occurred throughout the purification procedures is explained in this section.

Development of a purification protocol for the bioactive components was reliant on two major elements: (1) the IWF fractionation and (2) the bioassay. IWF fractionation was mainly achieved through chromatography techniques based on anion exchange, hydrophobic-interaction and gel permeation. These were routinely followed by the bioassay to identify the active fraction(s) for possible further purification or biochemical analysis. However, a number of problems were encountered during purification of the bioactive proteins. These included (1) intolerance of the bioassay to the presence of chromatographic buffers and (2) inactivation of proteins in the course of purification. Modification and optimization of the procedures that rectified these problems are discussed in the following.

5.3.1.1 Interference of salts with the bioassay

Salts and buffers are unavoidable components of most purification techniques including chromatography. They also cause serious interference with the bioassay and thus, identification of the IWF active fractions following chromatography.

Almost all forms of chromatography require specific ionic strength and pH conditions for proper separation (Roe, 1989; Edelstein and Bollag, 1992). Often, the

ionic strength and pH are adjusted independently using different salts. However, as it will be shown later, it is possible to use one salt to maintain both factors simultaneously. Commonly fractions generated by chromatography had a salt concentration ranging from 10 to over 1200 mM. This posed two obvious problems. Firstly, the levels of salts in the chromatography fractions were frequently unequal. This had to be brought to the same level before fractions could be compared for their biological activity. Secondly, it was desirable to reduce the salts to a common low level that allows a comparison between results of the *in vitro* studies and the situation occurring in the plant. A third, more serious, problem with salts was not obvious until the later purification steps. The chromatographic fractions eluted with high salt concentrations, including all the HIC fractions, had no detectable activity. After the salts were removed, however, many regained the strong antifungal effect also displayed by the source IWF (data not shown). This was interpreted as interference by salts with the original/normal antifungal activity of IWF or its components. However, whether the salts actually deactivated the proteins or rendered the environment unsuitable for the antifungal effect is not known.

Salts were also found to upset the normal characteristics of *R. secalis* spores. For instance, ammonium acetate (NH_4CH_3COO) at concentrations higher than 10 mM prevented the spores from germinating. At 50 mM, not only germination was completely suppressed, but spores also started to show signs of internal granulation (IG). Nonetheless, a viability assay showed that neither of these salt concentrations was actually lethal to the spores after 3 hours incubation (data not shown). Analysis of *R. secalis* membrane potentials indicated that while spores retain their normal transmembrane charges at 10mM ammonium acetate, they are under-polarized at 50mM (Reed *et al.,* unpublished data).

In order to desalt chromatographic fractions, two methods were considered: (1) dialysis and (2) removal of volatile salts under vacuum. Since the number of samples was relatively large and could easily reach 30 per chromatography run, the second option seemed more practical and was explored further.

Volatile salts are a small group of chemicals, usually ammonium salts, that may be sublimed at room temperature under low pressure. When used in chromatography, volatile salts can be conveniently removed from the fractions by vacuum. A

preliminary study was conducted to find out how successfully ammonium acetate (NH_4CH_3COO) and ammonium formate (NH_4HCOO) might be removed from small samples. A 6-µl volume of either 500 mM ammonium acetate, 500 mM ammonium formate or water was placed in 1.5 ml centrifuge tubes and placed under vacuum in a Speed Vac™ centrifuge overnight. Six microliter of conidial suspension, adjusted to 10^7 spores/ml, was added to the tubes and examined after 12 hours incubation. No differences in germination or spore appearance were detected, indicating that both volatile salts were removed under vacuum.

Following on from this, the effect of volatile salts on HMW IWF was investigated. HMW IWF (equivalent to 60 µl IWF) was dried and reconstituted in 6 µl 500 mM ammonium acetate, 500 mM ammonium formate or water. These were incubated at room temperature for 3 hours, placed in a Speed Vac™ centrifuge overnight and examined for levels of germination, doublet (DB), fractured cell wall (FW) and internal granulation (IG) formation. No differences between the activity of IWF were detected, indicating that the ammonium salt treatment had no marked effect on HMW IWF antifungal activity (data not shown). Curiously, when instead of HMW IWF, unfractionated IWF was used, the number of DB was markedly lower in the samples treated with the ammonium salts.

When accompanied with their corresponding hydroxyl acids, both ammonium acetate and ammonium formate may act as buffers at a pH range of 7 to 9 (Perrin and Dempsey, 1974). Preliminary chromatography showed that ammonium formate alone may be used for adjusting pH as well as maintaining the required ionic strength in ion exchange as well as gel permeation chromatography. Visual inspection of the chromatograms produced from either gel permeation or ion exchange chromatography of HMW IWF with the formate buffer indicated that protein separation was achieved (for examples see Figure 5-5-A and 5-7-A). To investigate if fractions produced using the above mentioned chromatography/buffer conditions had biological activity, 2 ml HMW IWF was fractionated by either gel permeation or ion exchange chromatography. Generated fractions were placed under vacuum overnight, reconstituted in 6 µl conidial suspension (10^7/ml) and bioassayed. Some of the fractions from each chromatography appeared to be biologically active (for examples see Figure 5-5-B and 5-7-B). It was concluded that ammonium formate is suitable for maintaining pH and ionic strength

during chromatography and has the added advantage that it is easily removed without any undesirable effect on the IWF antifungal activity. Although not used further, ammonium acetate also seemed to be as applicable as ammonium formate for this study.

Despite the original successful use of volatile salts with certain types of columns, these salts did not prove useful with the Brownlee HIC-300 column used for hydrophobic interaction chromatography. Neither ammonium acetate nor ammonium formate, applied at concentrations up to 2 M, led to retention of HMW IWF proteins or marker proteins by the column. Other less volatile salts such as ammonium bicarbonate (NH_4HCO_3) and ammonium iodide (NH_4I) were also tested unsuccessfully. However, two non-volatile mixtures were successful: (1) 2 M ammonium sulfate mixed with 125 mM sodium acetate pH 6.8 (recommended by the column manufacturer) and (2) 1 M *di*-potassium hydrogen orthophosphate ($(NH_4)_2HPO_4$) pH 6.8. Since phosphate might interact with some enzymes (Harris, 1989a) it was decided that a buffer system containing ammonium sulfate and sodium acetate would be used. The concentration of the salts was later optimized from that shown above (see section 5.2.3.5). For the bioassay, fractions were dialyzed against distilled water, dried briefly and reconstituted with a suspension of 10^7 conidia/ml. For consistency proposes, other chromatographic samples intended for bioassay were also desalted only by dialysis even if volatile salts were used. The *in vacua* removal of volatile salts was abandoned, although ammonium formate continued to be used in chromatography when possible.

5.3.1.2 Inactivation of proteins

5.3.1.2.1 Inactivation by drying

Degradation of conidia cell walls, manifested as DB formation and FW activity, was the focus of this study (see section 5-3-2). However, this activity greatly diminished as the purification reached its second chromatographic step (WCX). This was unexpected, as the concentrations were kept relatively constant between the purification steps (see section 5-2-5). It was observed that if the protein fractions were not dried prior to the bioassay, the activity would stay at a much higher level. To further investigate this, IWF, HMW IWF and the active fractions obtained by SAX and by WCX (first and second chromatography steps, respectively) were used in a comparative study of

bioactivity of dried and non-dried proteins. The percentage of DB formation was used as an indication of the cell wall lytic activity of the protein samples.

Firstly, all the samples were dialyzed against distilled water. For the dried treatment, an amount, equivalent to 30 µl IWF was completely dried under vacuum in a Speed Vac™. These samples were reconstituted in 6 µl of *R. secalis* conidia suspension previously adjusted to 10^7 spores/ml. For the non-dried treatments samples were concentrated in a Speed Vac™ centrifuge until, without drying, they reached a concentration equivalent to 10X IWF. Three µl of the 10X IWF-equivalent sample was placed in a 1.5 ml centrifuge tube and mixed with 3 µl of conidial suspension previously adjusted to 2 x 10^7 spore/ml. All the samples were incubated for 12 hrs at 20°C in the dark. DB percentage of the samples was determined with the aid of a microscope. Results, summarized in Table 5-2, confirmed that drying diminished DB activity. It also suggested that the higher the purity of the sample the more sensitive it was to drying.

Following this finding, samples intended for bioassay were not taken to dryness.

5.3.1.2.2 Inactivation by oxidation

Bioactive fractions from SAX chromatography had markedly lower activity after one week storage at 4°C. Proteins which had been further purified by WCX did not have any detectable activity after storage at 4°C for a week. Further, HIC- and GPC-purified proteins did not have any activity when bioassayed immediately after chromatography. However, when a small amount (1-3 mM final concentration) of the reducing agent DTT was added to these fractions, the activity was restored (data not shown).

sample source	%DB by dried samples	%DB by non-dried samples
IWF	53	70
HMW IWF	47	68
SAX active fraction	30	51
WCX active fraction	8	18

Table 5-2: Comparison of drying and non-drying bioassay strategies
Data are average of two measurements. IWF and its derivatives were all from inoculated Atlas extracted on day 3 post-inoculation. SAX fraction 4 and WCX fraction 28 were used. Equivalent to 30 µl IWF of protein samples were adjusted to 10^7 spore/ml and results were recorded as percentage of DB (DB%) after 12 hours incubation.

Moreover, the presence of 1 or 2 mM DTT in the chromatography buffers, not only led to reproducible detection of activity after each chromatography run, but also improved protein storage markedly. Purified samples containing 1 to 3 mM DTT retained their activities for a minimum of one week at 4°C. Samples that were deactivated after this period could again be recovered by further addition of the same amount of DTT. However, not all the activity was recoverable and this process was not repeatable beyond 2-3 times.

Following this finding, 1 mM DTT was routinely included in all the chromatography buffers except for the first chromatography, SAX. DTT was not added to the SAX buffers prior to the chromatography but instead added to all the generated fractions after this chromatography. This was to avoid the possible binding of DTT to the positively charged SAX column at pH 9.0.

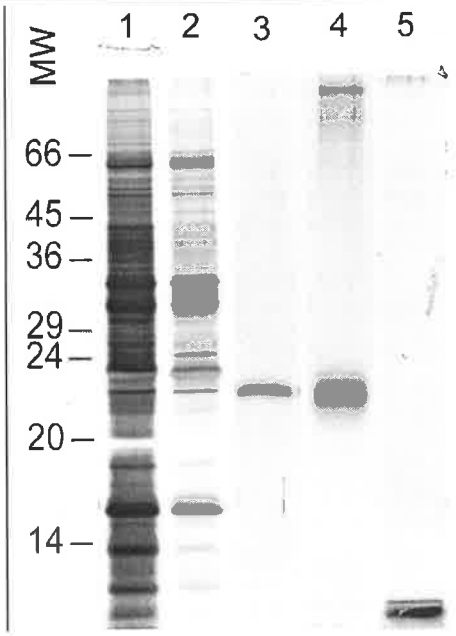
R. secalis conidia were viable in the presence of 1-3 mM DTT, as judged by the viability assay, but they did not germinate (data not shown). This did not affect the bioassay and the purification procedure because at this stage only DB activity was used as a bioassay to purify proteins. Hence, bioassays of the purified fractions were conducted with 1 mM final concentration of DTT and also a buffer of 10 mM ammonium acetate pH 6.0 to increase the reproducibility of the assay.

5.3.1.3 IWF storage problems

Originally IWF and the proteins fractions produced from IWF were stored at 4°C. However, comparative SDS-PAGE profile of stored proteins frequently showed that some of the IWF proteins were not detected after a certain period of storage at 4°C. Two instances of this are discussed here.

IWF was extracted from inoculated Atlas 46 and immediately stored at –20°C or 4°C. After two weeks, 10 µl of each were analysed with SDS-PAGE. Results are shown in lanes 1 and 2 in Figure 5-1. It was found that although some proteins were similarly preserved at –20°C and 4°C, many others were only detectable in the frozen sample.

Figure 5-1: Effect of storage on IWF proteins
Lanes 1 and 2 are profiles of Atlas 46 IWF (10 µl) stored for 2 weeks at -20°C and 4°C respectively.
Lanes 3, 4 and 5 are profiles of a purified IWF protein (Pr22-1) after 0, 7 and 14 days storage at 4°C. In lane 3 note the protein band at ~22 kDa. This becomes diffused in lane 4 and finally disappears in lane 5. A band at the end of gel in lane 5 was often detected.



Also in Figure 5-1, lane 3 contains the profile of an IWF protein immediately prepared after it was purified. Lane 4 and 5 show the same fraction after 1 and 2 weeks storage at 4°C respectively. The diffused appearance of the protein in lane 4 and its absence in lane 5 suggest that the protein had degraded (likely digested). This was further evident by simultaneous appearance at the bottom in lane 5 of a new band probably produced by fragments of the degraded protein. Protein digestion, precipitation and surface absorption have been frequently blamed for inability to detect proteins after storage (Edelstein and Bollag, 1992).

Following these findings, IWF and its fractions were stored with the minimum time at temperatures other than -20°C. In addition, the purification time was reduced to a minimum by conducting the chromatographic steps immediately after one another.

5.3.2 Forms of bioactivity in HMW IWF: what to purify

The following experiments were carried out before developments outlined above took place. Accordingly, volatile salts were removed *in vacua* and no DTT was added to the samples.

5.3.2.1 Preliminary resolving of bioactivity in HMW IWF

In previous chapters, it was explained that IWF exhibits a complex range of activities on spores of *R. secalis*. These included effects on spore germination, internal granulation (IG), fractured cell walls (FW), formation of doublet spores (DB) and generation of cellular debris (see section 3.3.2.2). It was likely that a number of different compounds were responsible for these effects. To clarify this, IWF was initially separated into LMW and HMW fractions. However, most of the antifungal activity was found to be present in the HMW fraction. As the next step, gel permeation chromatography (GPC) was used to separate the various compounds associated with antifungal activity.

A volume of 4 ml of IWF was ultrafiltered, resulting in 100 µl HMW IWF. This was applied to a LKB® Ultropac TSK 3000 SW column at room temperature (24°C). The manufacturer recommendation of 40°C column temperature was originally used but no activity was detected in the fractions generated under this condition. Starting from 10 min, 35 fractions were collected at a rate of 1 fraction/min. Sixty microliter of each fraction was subjected to vacuum overnight, reconstituted in 6 µl of conidial suspension (10^7/ml) and bioassayed. The results, presented in Figure 5-2, can be summarized as follow:

- DB formation and FW activity were co-purified in a single peak (Figure 5-2-B).
- Doublet (DB) formation and fractured cell wall (FW) activity were mostly detected in the same fractions.
- Internal granulation (IG) was observed in distinctly different fractions than those inducing FW activity or DB formation (Figure 5-2-B).
- IG was detected in 3 major and 2 minor peaks. In the major peaks, not only more spores were affected but the granulation effect inside the cells appeared to be more pronounced. The major IG peaks also coincide with Lower germination percentages.
- FW activity and pronounced IG in the cells led to loss of viability.

Figure 5-2 (continued):

HMW IWF was separated by gel permeation chromatography and one fraction per minute was collected from 10 to 45 min. Fractions were numbered according to their collection time (fraction #11 at 10-11 min, fraction #12 at 11-12 min and so on) and bioassayed. The chromatogram and the bioassay results are shown. Micrographs showing the spores following incubation in fractions 20, 27 and 29 are displayed at the bottom. Each picture presents a normal microscopic view of the spores (top) and a view taken at under UV after staining with FDA (bottom). Viable spores fluoresce in the FDA stained view. Bars in the photographs represent 10 μm.

A: Gel permeation chromatogram measured by milli absorbance units (mAU) at 280 ηm. No protein was detected before 10 min (not shown). **B**: Doublet (DB), fractured cell wall (FW) and internal granulation (IG) in each fraction. **C**: Viability and germination rates for each fraction. **D**: Spores incubated in fraction 20, as the control. **E**: Spores incubated in fraction 27 showing that most of the spores were affected by either DB or FW. **E1**: Arrow points to a (non-viable) FW spore. **E2**: Arrow points to a (viable) DB spore. **E3**: Arrow points to a germ tube of a germinating DB spore. **F**: Spores incubated in fraction 29 which show high IG and low viability.

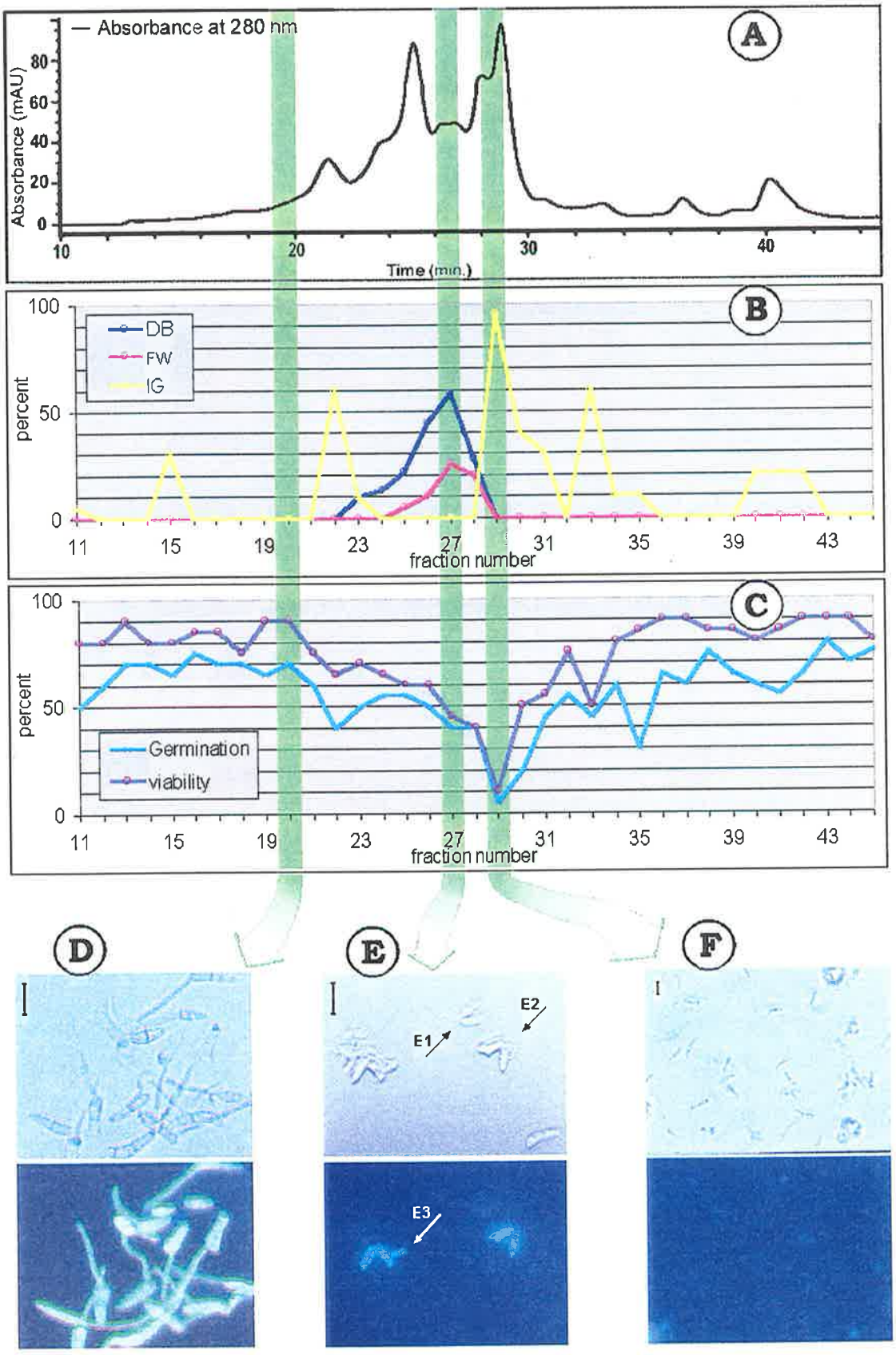


Figure 5-2: Preliminary resolving of HMW IWF bio-activities using GP chromatography
(For legends see the opposite page)

Unlike DB formation and FW activity, IG did not co-migrate with any other bioactivity. Accordingly, it was concluded that IG is a phenomenon independent of either DB formation or FW activity. Occurrence of IG in 5 non-continuous peaks implied that at least 5 compounds may be responsible for this phenotype (Figure 5-2-B). A close examination indicated that spores with pronounced IG neither germinated nor were viable (Figure 5-2-F). Spores affected by IG, unlike FW, appeared to lose viability without any visible damage to the cell wall (data not shown).

The biological activity peak exemplified by fraction 27 includes both FW activity and DB formation (Figure 5-2-E). Both FW activity and DB formation appeared to degrade the septum of the bicellular conidia. The likely reason that spores with FW were non-viable is because of cell wall damage and a consequent rupture of the protoplast (see E1 in Figure 5-2-E). On the other hand, DB spores were viable and occasionally germinated (see E2 and E3 in Figure 5-2-E). Therefore, the two activities appeared to be different despite their co-migration on the GPC column.

In Chapter 3 (section 3.3.2.4), a time-course study indicated that *R. secalis* conidia incubated in HMW IWF directly formed DB or FW spores. It was found that DB formation was not a transient stage between normal and FW spores. The effect of various concentrations of purified proteins upon DB formation and FW activity is investigated later in this chapter.

5.3.2.2 The choice of bioassay for protein purification

In view of the presence of numerous IG-causing compounds in the IWF and the complexity that this would pose to a purification procedure it was decided not to investigate this phenomenon further. Loss of viability was also not followed because of similar complications. DB- and FW-causing agents were considered to be simpler to purify. However, for a given concentration the percentage of DB formation was markedly greater than that of FW activity (see Figure 5-2-B). Hence less protein was required for a bioassay to detect DB formation. This and the fact that, compared to FW activity, DB formation was a more reproducible phenomenon led to the conclusion that DB formation presents a better bioaasay for use in subsequent purification experiments.

The preliminary fractionation of HMW IWF conducted in this section was carried

out by GPC because this technique was the least likely to (1) denature or (2) irreversibly bind proteins to the column. Accordingly, the different activities existing in HMW IWF should be retained after fractionation. However, this method generally lacks high resolution and is best suited as the last step in purification protocols (Harris, 1989b). The purification of the DB-causing agents explained in the following section was conducted independently of this preliminary separation.

5.3.3 Purification of the cell wall degrading enzymes from IWF

In order to purify the putative DB-causing agent(s), HMW IWF was fractionated by anion exchange, cation exchange, hydrophobic interaction and gel permeation chromatography in succession. Each time active fractions were collected and applied to the next chromatographic step. The protein profiles of fractions were monitored by SDS-PAGE. When it appeared that an active fraction contained only one protein band, its homogeneity was confirmed with RP chromatography before further characterisation. Figure 5-3 presents a diagrammatic summary of the non-denaturing

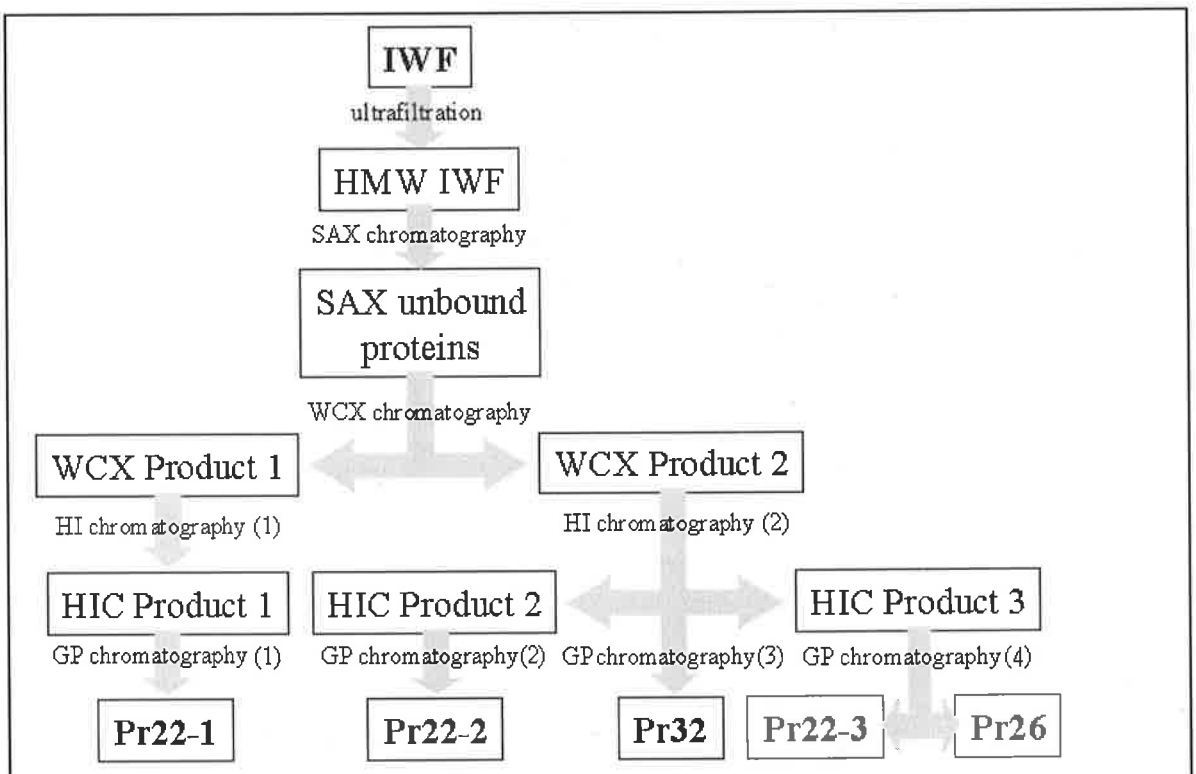


Figure 5-3: Non-denaturing purification steps of the cell wall degrading proteins

purification procedures. Details are explained below.

5.3.3.1 Extraction and preparation of HMW IWF

Barley plants (cultivar Atlas 46) were grown for 2 to 3 weeks and inoculated with *R. secalis* isolate H2.5 as outlined in Chapter 2. Thirty four grams of leaves (~ 130 plants) were harvested 3 days post-inoculation and IWF was extracted as outlined in Chapter 2. This typically yielded ~5 ml IWF which was concentrated to a final volume of 500 µl using ultrafilters. Early experiments were carried out using Centricon-3 ultrafilters with an exclusion limit of 3 kDa. However, no difference between bioactivity of retentates produced by this and the 10-kDa filter was detected (data not shown). Accordingly, later experiments were conducted using Centricon-10 ultrafilters with an exclusion limit of 10 kDa at a much faster flow rate. No DB-formation activity was detectable in any of the filtrates.

5.3.3.2 Anion exchange chromatography

HMW IWF was adjusted to 10 mM ammonium formate pH 9.0 and loaded onto a PL-SAX anion exchange chromatography column. A gradient of 10–1000 mM ammonium formate pH 9.0 at 400 µl/min was applied. One fraction per minute for 24 minutes was collected and bioassayed following overnight dialysis. Results indicated that almost all the DB-forming activity was detected in fractions 3 to 5. These fractions contained the SAX-unbound proteins that were subsequently pooled and carried through to the next purification step. Profiles of elution, cell wall lytic activities and protein analysis by SDS-PAGE are shown in Figure 5-4. Figure 5-5 shows a comparative SDS-PAGE profile of IWF and pooled SAX-unbound proteins.

The SAX chromatography began with a 1.5 min isocratic zone (Figure 5-4-A). This was to improve the separation of the unbound from the bound proteins. As shown in Figure 5-4-C, at pH 9.0 a large number of inactive (bound) proteins were removed from the active (unbound) proteins. Under more basic conditions more proteins bound to the column and the bioactivity began to divide between the unbound and bound fractions. This was not used because it did not improve the final purity of the proteins when

combined with the other chromatographic steps (data not shown). At pH a lower than 9.0 more inactive proteins were in the unbound fraction.

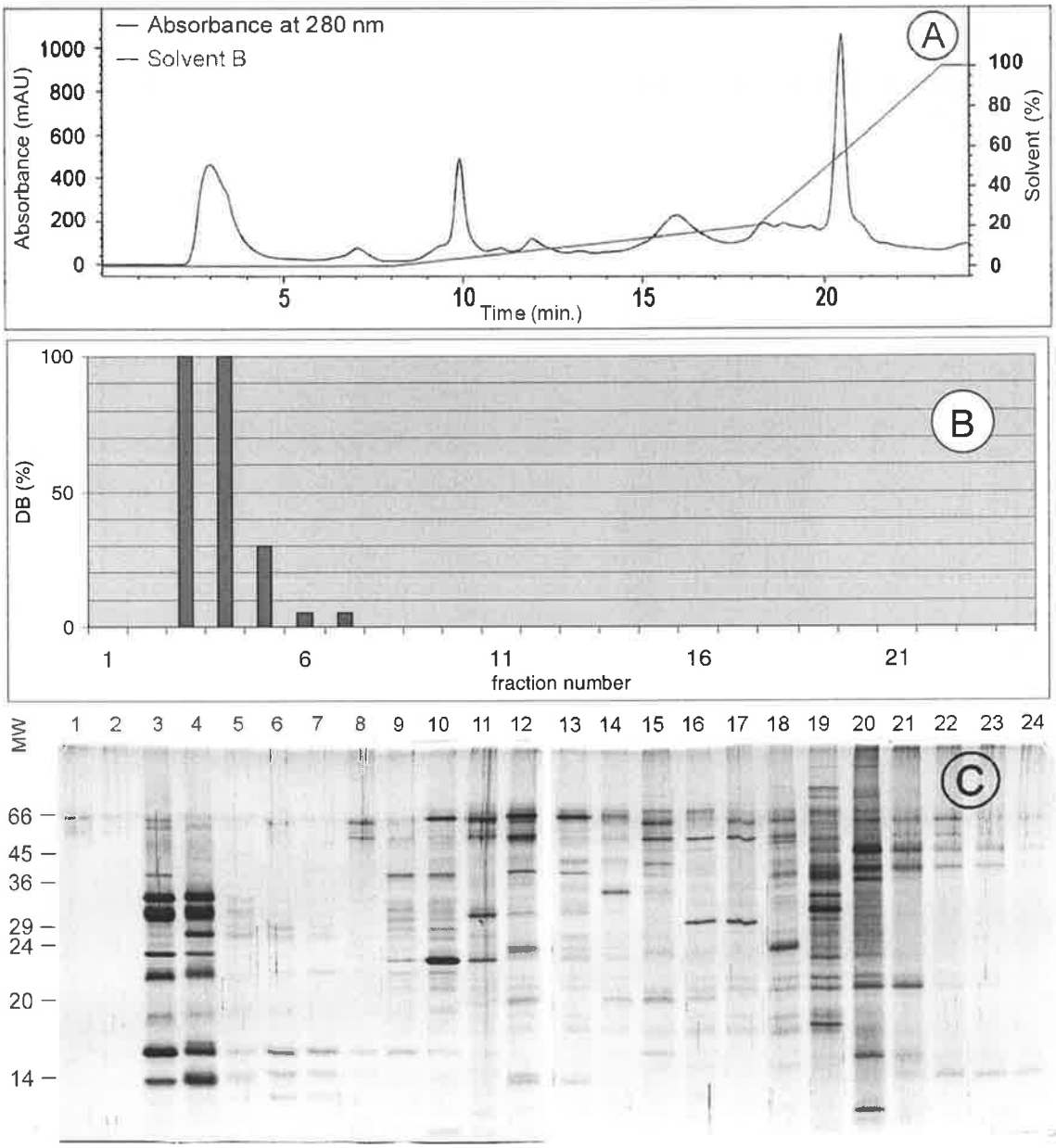
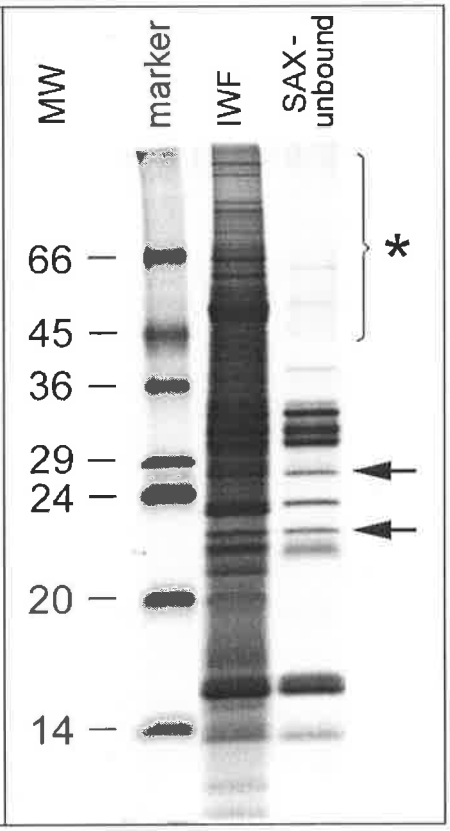


Figure 5-4: Anion exchange chromatography of HMW IWF

HMW IWF was subjected to SAX chromatography and one fraction per minute was collected from 0 to 24 min (fraction #1 at 0-1 min, fraction #2 at 1-2 min and so on). Fractions were bioassayed for the DB formation. Almost all the biological activity was detected in the unbound proteins eluted from 2.5 minute. Bound proteins started to elute from 8.5 minute.

A: The SAX chromatogram detected at 280 ηm. Numbers on the left refer to absorbance measured in milli absorbance unit (mAU). Salt gradient is shown on the right. **B**: Results for the DB bioassay. Percentage of the doublet spores (DB) is shown on the left. **C**: SDS PAGE profiles of the fractions. Numbers on top of the gels refer to fraction numbers. Numbers on the left side refers to molecular weights (MW) measured in kDa.

Figure 5-5: SDS PAGE profile of SAX-unbound proteins
IWF and SAX-unbound proteins are shown. Arrows point to SAX-unbound protein bands that were not always present. Star indicates a zone of the unbound fraction where normally no protein band was detected. Artifactual bands were sometimes detected at 45 to 60 kDa. These have been reported to form when DTT-containing samples were visualized with silver staining (Edelstein and Bollag, 1992). Molecular weights (MW) measured in kDa are shown on the left.



5.3.3.3 Cation exchange chromatography

Unbound fractions from SAX at pH 9.0 that contained DB activity were pooled and adjusted to pH 7.5, 1 mM DTT. If necessary, this was ultrafiltered to reduce the volume to 500-1000 µl and loaded onto a Macrosphere WCX cation exchange chromatography column. Proteins were eluted at 400 µl/min using a gradient of 10–400 mM ammonium formate pH 7.5. One fraction per minute was collected from 5 to 35 minutes and bioassayed after dialysis. Results presented in Figure 5-6, show that the bioactivity was spread into 2 broad peaks. The first included fractions 22-24. These were pooled and defined as WCX Product 1. The second peak, included fractions 26 to 30, were pooled and defined as WCX Product 2. Both products were purified further.

5.3.3.4 Hydrophobic interaction chromatography

WCX Product 1 and 2 were ultrafiltered when necessary to reduce the volumes to approximately 500 µl. These were adjusted to 1 mM DTT, 1.2 M ammonium sulfate

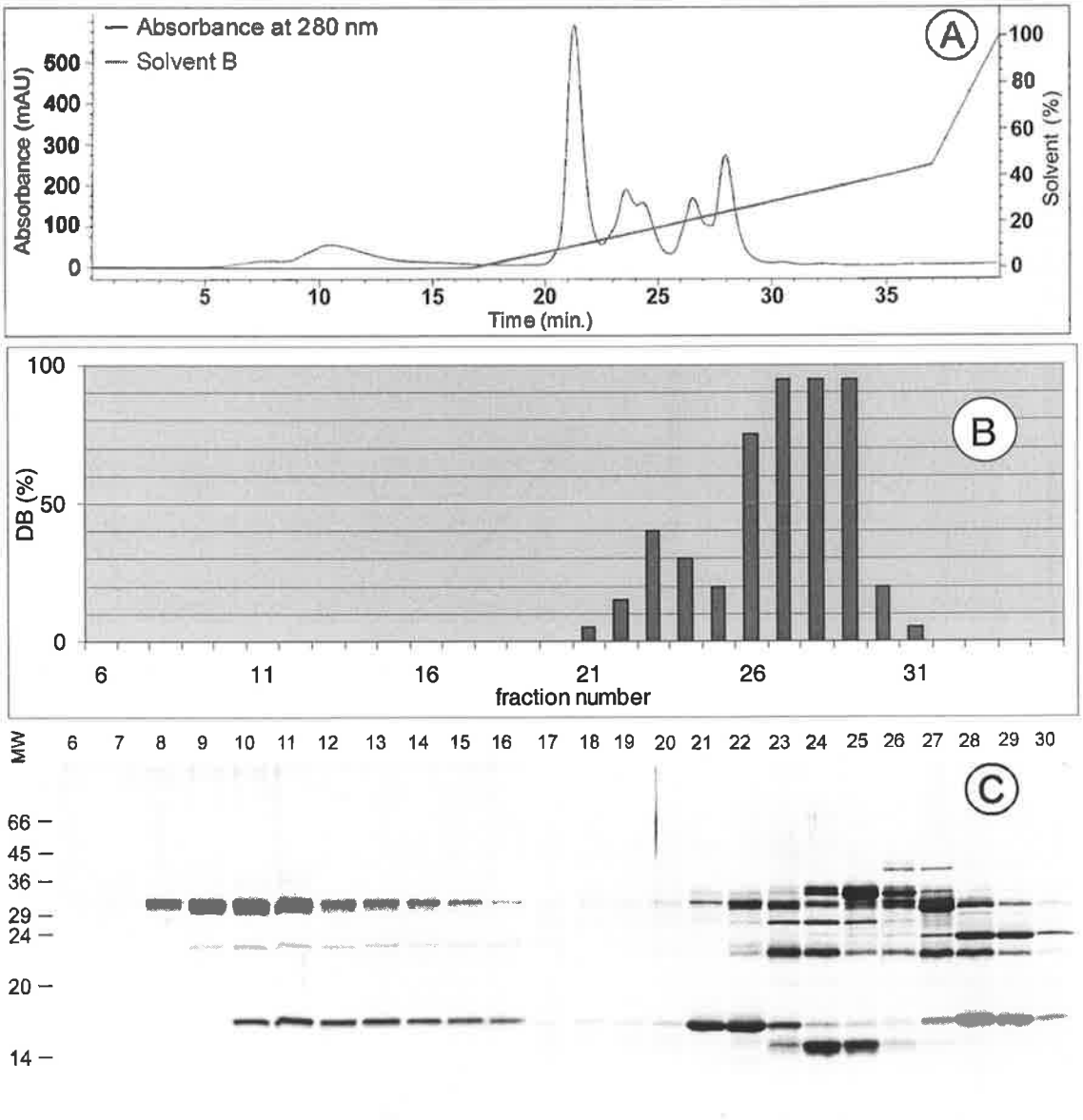


Figure 5-6: Cation exchange chromatography of SAX-unbound proteins

SAX unbound proteins were chromatographed using a Macrosphere WCX column. One fraction per minute was collected from 5 to 35 min (fractions #6 to 35). The activity was detected in fractions 21 to 31. Two zones were identified based on distribution of the lytic activity and the protein bands. First zone, WCX Product 1, included fractions 22 and 24. The second zone, WCX Product 2, included fractions 26 to 29.

A: The WCX chromatogram detected at 280 ηm. Numbers on the left refer to absorbance measured in milli absorbance unit (mAU). Salt gradient is shown on the right. **B**: Results for the DB bioassay. Percentage of the doublet spores (DB) is shown on the left. **C**: SDS PAGE profiles of fractions 6-30. Numbers on top of the gels refer to fraction numbers. Numbers on the left side refers to molecular weights (MW) measured in kDa.

and 150 mM sodium acetate pH 6.8 and applied to HI chromatography (HIC). A gradient of (1.2 M ammonium sulfate and 150 mM sodium acetate) to (0.5 M ammonium sulfate and 62.5 mM sodium acetate) at 200 µl/min was used for both products. One fraction per minute was collected from 5 to 37 minutes and bioassayed after dialysis. Results for each of the two WCX Products were:

- **WCX Product 1:** Activity was detected in one continuous zone chiefly including fractions 15 to 17 (Figure 5-7). These fractions were pooled, identified as HIC Product 1 and applied to the next purification step.

- **WCX Product 2:** Activity was detected in 3 distinct peaks (Figure 5-8). The first peak, including fractions 14-16, contained up to 4 protein bands. These fractions were combined (HIC Product 2) and advanced to the next purification step. The second peak included fractions 19-22 that again contained more-than-one protein band. These were also pooled (HIC Product 3) and further purified. The third peak comprised fractions 27-29. These fractions appeared to contain only a single 32-kDa protein band (Pr32). Fractions containing Pr32 were combined and stored at -20°C. For consistency purpose, the sample of Pr32 intended for bioactivity studies (section 5-3-5) was first chromatographed by a GPC Ultropac TSK 3000 SW column. This was for uniform treatment of all the proteins and did not improve the purity of Pr32.

5.3.3.5 Gel permeation chromatography

An Ultropac TSK 3000 SW column used in the preliminary fractionation (see section 1-3-2) is best suited for proteins in the range of 100-3000 kDa (manufacturer specifications). A Macrosphere GPC 60A column that is more suitable for smaller proteins became available at a later stage in this study and was used in the following experiments.

HIC products were ultrafiltered to reduce the volumes to approximately 30 µl and loaded onto a GPC 60A column. An isocratic gradient comprising of 50 mM ammonium formate pH 7.5 and 1 mM DTT was applied. Starting from 15 minutes, 20 fractions at the rate of one per minute were collected. These were bioassayed following dialysis. Results for each of the three HIC products were:

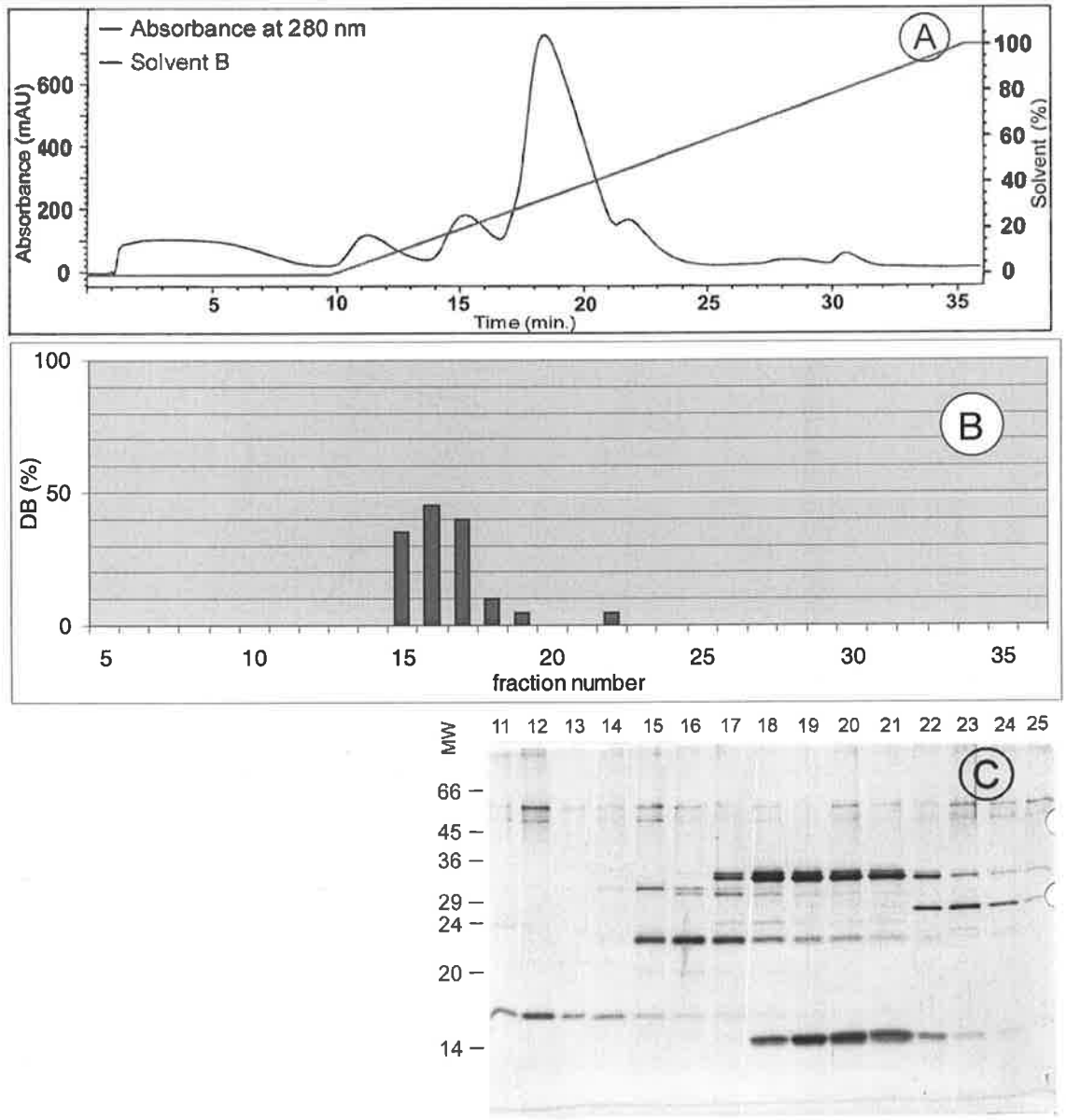


Figure 5-7: Hydrophobic interaction chromatography for WCX Product 1
WCX Product 1 was subjected to HI chromatography. One fraction per minute was collected from 4 to 36 min (fractions #5 to 36). The activity was mainly detected in fractions 15 to 17 (HIC product 1).
A: The HI chromatogram detected at 280 ηm. Numbers on the left refer to absorbance measured in milli absorbance unit (mAU). Salt gradient is shown on the right. **B**: Results for the DB bioassay. Percentage of the doublet spores (DB) is shown on the left. **C**: SDS PAGE profiles of fractions 11-25. Numbers on top of the gels refer to fraction numbers. Numbers on the left side refers to molecular weights (MW) measured in kDa.

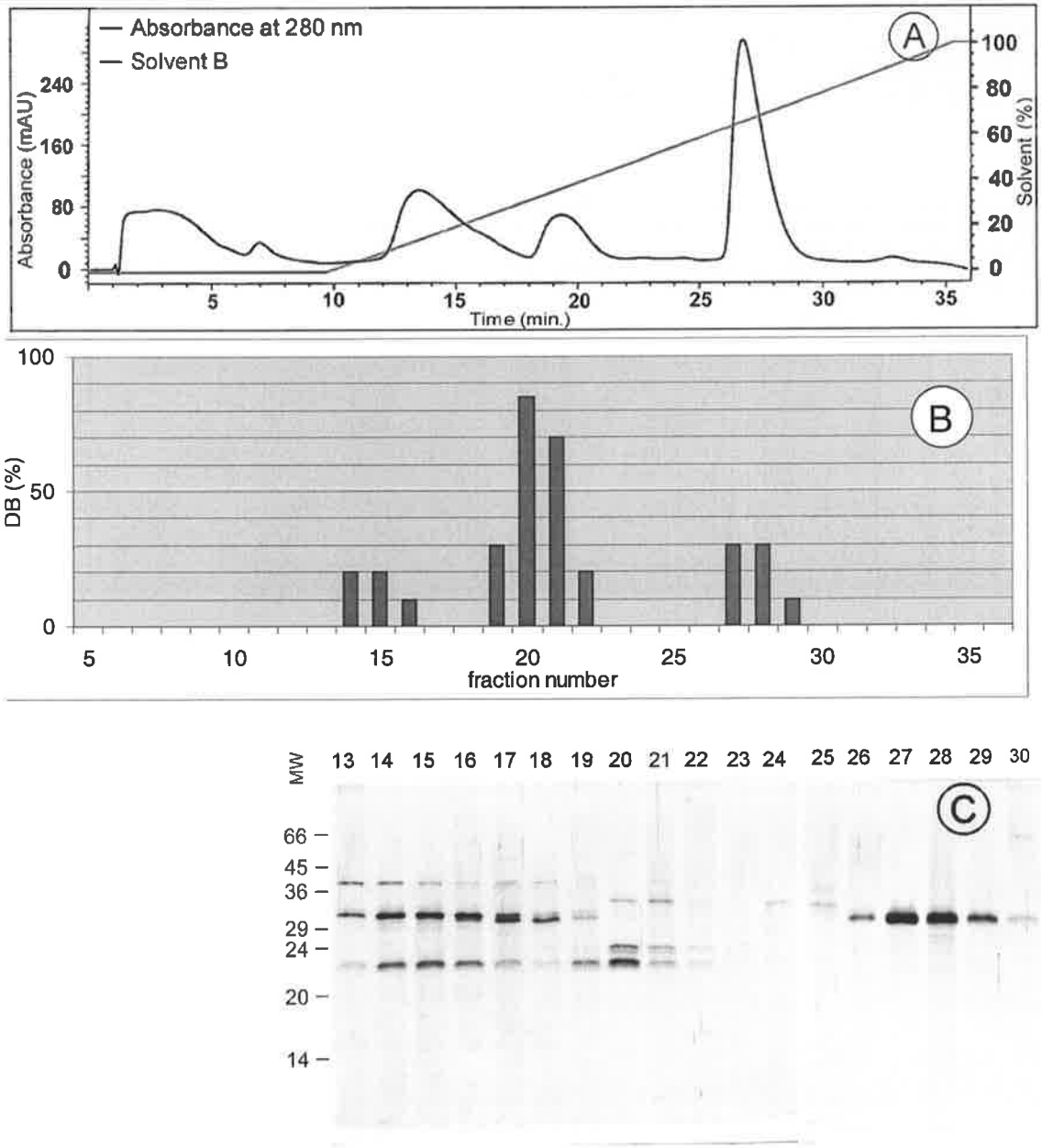


Figure 5-8: Hydrophobic interaction chromatography for WCX Product 2
WCX Product 2 was subjected to HI chromatography. One fraction per minute was collected from 4 to 36 min (fractions #5 to 36). The activity was detected in 3 zones including fractions 14 to 16 (HIC product 2), fractions 19 to 22 (HIC product 3) and fractions 27 to 29 (HIC product 4). Only the latter fractions appear to have a single protein band.
A: The HI chromatogram detected at 280 ηm. Numbers on the left refer to absorbance measured in milli absorbance unit (mAU). Salt gradient is shown on the right. **B**: Results for the DB bioassay. Percentage of the doublet spores (DB) is shown on the left. **C**: SDS PAGE profiles of fractions 13-30. Numbers on top of the gels refer to fraction numbers. Numbers on the left side refers to molecular weights (MW) measured in kDa.

- **HIC Product 1:** Activity was detected in one zone comprising fractions 25-31. This corresponded with one protein band in the SDS-PAGE profile (Figure 5-9). Fractions containing this protein (Pr22-1) were pooled and stored at –20°C until further characterisation.

- **HIC Product 2:** Activity was again detected in one zone comprising fractions 27-35. This contained one protein band at ~22 kDa as judged by the SDS-PAGE profiles (Figure 5-10). Fractions containing this protein (Pr22-2) were pooled and stored at –20°C until further characterisation.

- **HIC product 3**: Activity was detected in a broad zone comprising fractions 25 to 34. This appeared to implicate 2 proteins that were partly overlapping as judged by SDS-PAGE profiles (Figure 5-11). The first protein with the maximum abundance in fraction 26 was spread between fractions 24 to 34. This protein was estimated to be slightly less than 25 kDa but called Pr25 for compatibility with further data. The second protein was detected in fractions 27 to 35 with the peak in fraction 29. This protein was approximately 22 kDa in size (Pr22-3). Examination of the SDS-PAGE profiles (Figure 5-11-C) showed that Pr25 was reasonably pure in fractions 24 to 26. These fractions were combined and stored at –20°C until further characterisation. The other protein, Pr22-3, had traces of Pr25 and required further purification.

Considerable time was spent to determine the conditions required for separation of Pr25 from Pr22-3 in any of the SAX, WCX, HI or GP chromatographic steps. However, complete separation was not achieved with the available columns. Nevertheless, a combination of partial separation delivered by WCX and GPC chromatography was used to produce sufficiently pure protein to characterize Pr22-3. To achieve this, all the steps involving purification of Pr22-3 were repeated, except that following WCX chromatography, only fraction 26 was collected and applied to HIC. Following GPC, fractions 29-34 were collected (data not shown). Although this did not completely remove Pr25, its level was reduced to less than 5% (w/w) as judged by the RPC purity check (section 5-3-3-6). This sample of Pr25 was stored at –20°C until further characterisation.

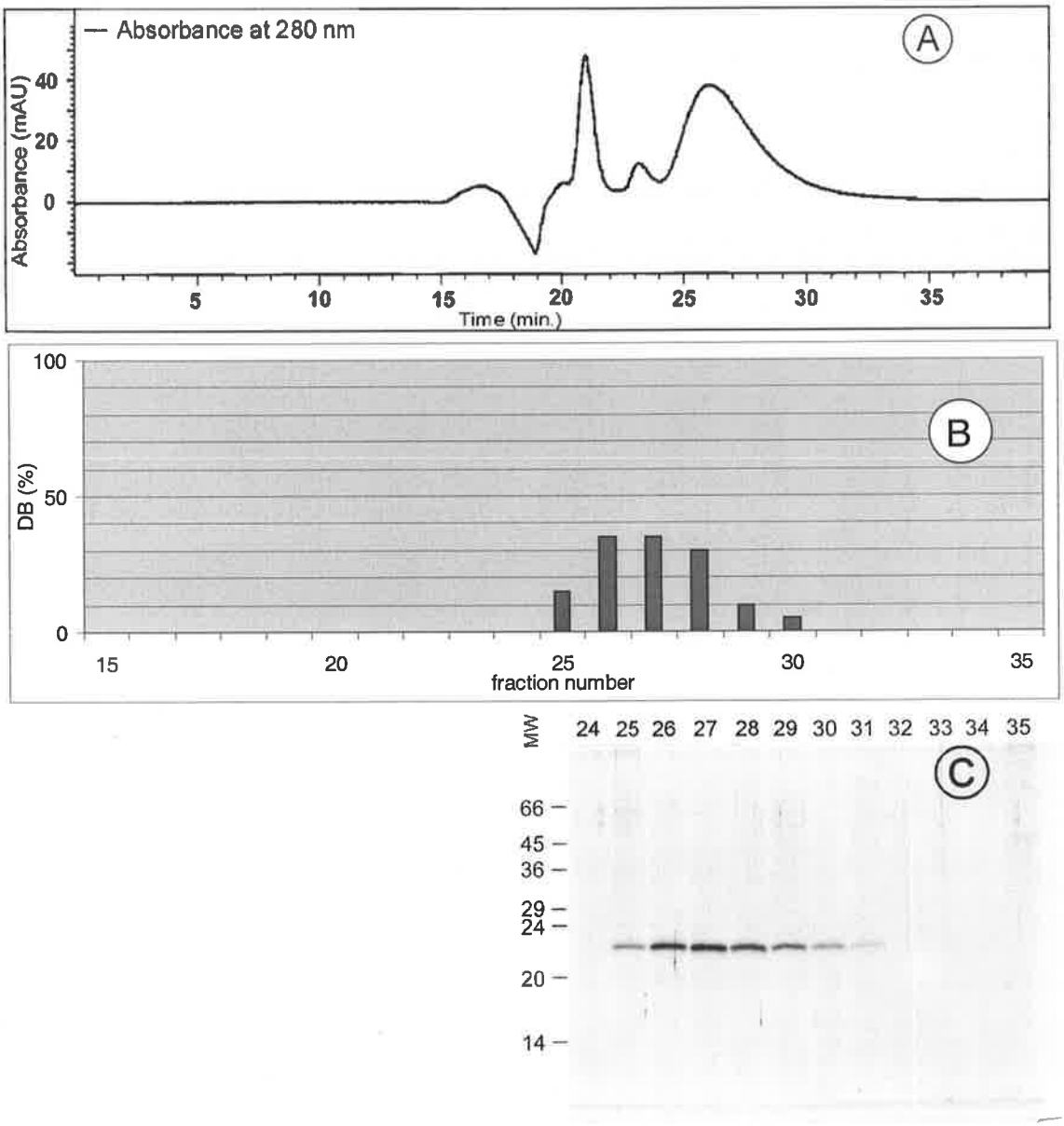


Figure 5-9: Gel permeation chromatography for HIC Product 1

HIC Product 1 was subjected to a Macrosphere GPC 60A column for gel GP chromatography. One fraction per minute was collected from 14 to 35 min (fractions #15 to 35). The activity was detected in fractions 25 to 30. These appeared to contain one protein band at ~22kDa (Pr22-1).

A: The GP chromatogram detected at 280 ηm. Numbers on the left refer to absorbance measured in milli absorbance unit (mAU). Salt gradient is shown on the right. **B**: Results for the DB bioassay. Percentage of the doublet spores (DB) is shown on the left. **C**: SDS PAGE profiles of fractions 24-35. Numbers on top of the gels refer to fraction numbers. Numbers on the left side refers to molecular weights (MW) measured in kDa.

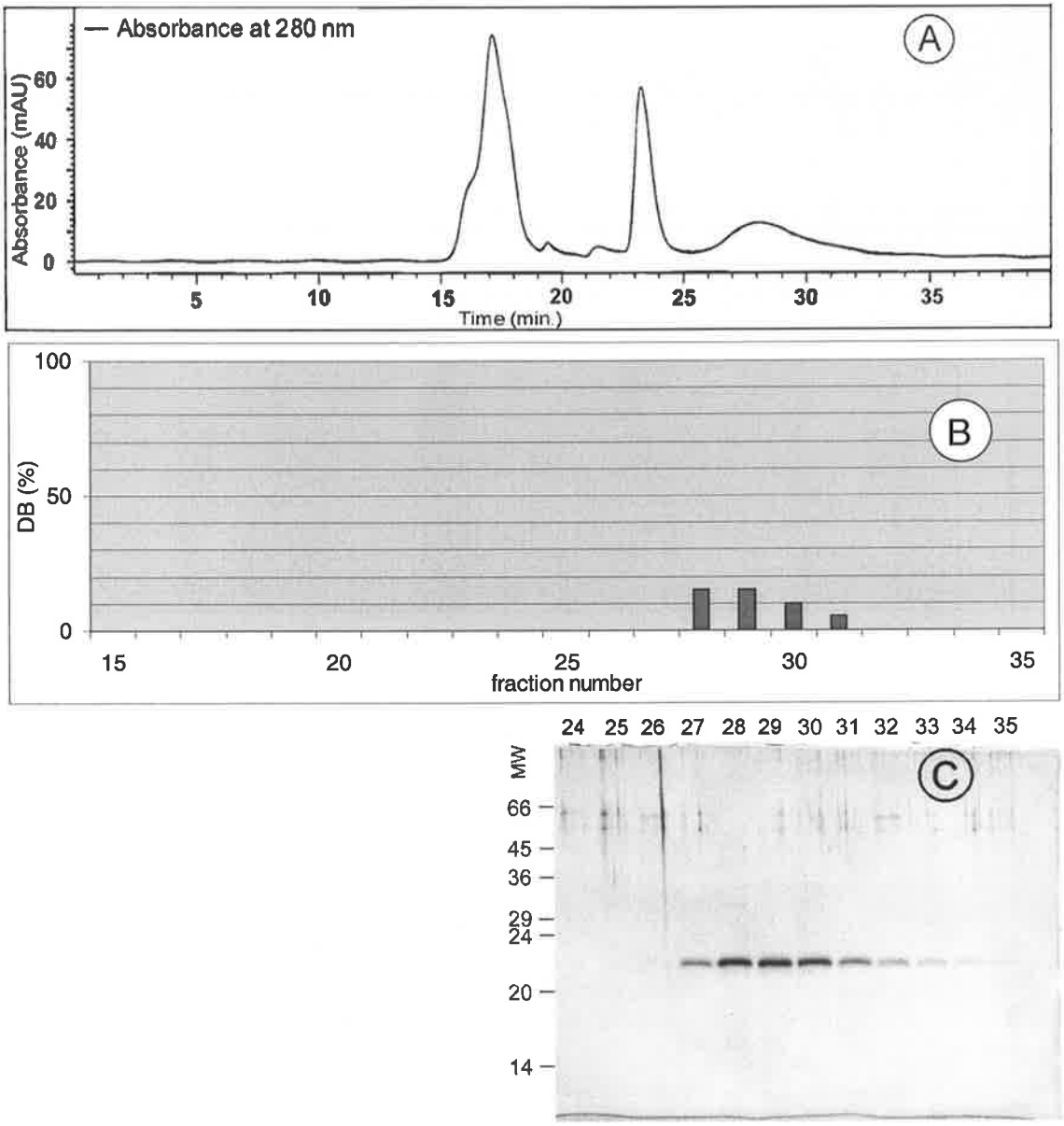


Figure 5-10: Gel permeation chromatography for HIC Product 2

HIC Product 2 was subjected to a Macrosphere GPC 60A column for gel GP chromatography. One fraction per minute was collected from 14 to 35 min (fractions #15 to 35). The activity was detected in fractions 28 to 31. These, again, correspond to a 22 kDa protein band (Pr22-2) in the SDS PAGE profiles.

A: The GP chromatogram detected at 280 ηm. Numbers on the left refer to absorbance measured in milli absorbance unit (mAU). Salt gradient is shown on the right. **B**: Results for the DB bioassay. Percentage of the doublet spores (DB) is shown on the left. **C**: SDS PAGE profiles of fractions 24-35. Numbers on top of the gels refer to fraction numbers. Numbers on the left side refers to molecular weights (MW) measured in kDa.

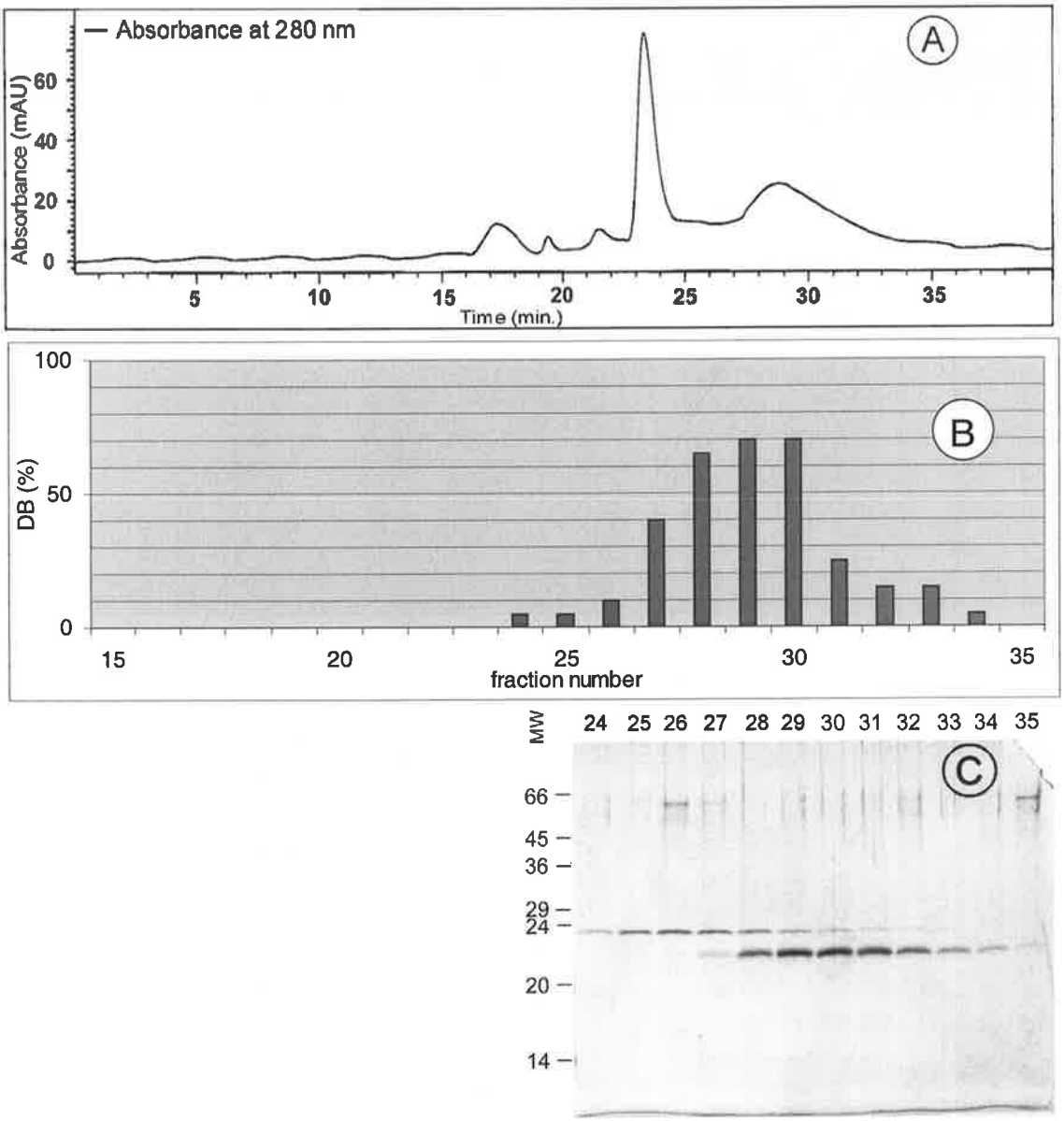


Figure 5-11: Gel permeation chromatography for HIC Product 3

HIC Product 3 was subjected to a Macrosphere GPC 60A column for gel GP chromatography. One fraction per minute was collected from 14 to 35 min (fractions #15 to 35). The activity was detected in fractions 24 to 34. SDS PAGE analysis indicated that 2 protein bands were present in these fractions: a ~22 kDa protein (Pr22-3) and a protein just under the 24kDa mark (Pr25).

A: The GP chromatogram detected at 280 ηm. Numbers on the left refer to absorbance measured in milli absorbance unit (mAU). Salt gradient is shown on the right. **B**: Results for the DB bioassay. Percentage of the doublet spores (DB) is shown on the left. **C**: SDS PAGE profiles of fractions 24-35. Numbers on top of the gels refer to fraction numbers. Numbers on the left side refers to molecular weights (MW) measured in kDa.

5.3.3.6 Reverse phase chromatography

Pr22-1, Pr22-2, Pr22-3, Pr32 and Pr25 were applied to a reverse phase chromatography (RPC) column. RPC was used to confirm the homogeneity of the protein samples before further characterisation. Since RPC is basically a denaturing method the products of this chromatography were not used in experiments concerned with biological activity. They were, instead, used for protein sequencing and SDS-PAGE-related studies (see Chapter 6). Two different gradients were routinely used with RP chromatography. The first gradient, RPC-2, was a general-propose method used for all the proteins. The second gradient, RPC-TL, was a shallower gradient developed to better resolve the three Pr22 proteins (for details see Chapter 2).

Data from RPCs was also used to quantify the amount of protein. This was achieved by comparing the peak area (at 210 ηm) of a protein sample with that generated from 5 μg ovalbumin eluted under the same conditions.

All the isolated proteins were individually concentrated to 100 μl using Centricon-10 ultrafilters and 20 μl of each were chromatographed on a RPC column. Protein peaks were collected and again run on SDS-PAGE to positively identify the peak associated with each protein. As discussed previously, except for Pr22-3 all the proteins were pure. Pr22-3 contained a small amount of Pr25, estimated to be 4.8 % of the total protein weight. Figure 5-12 shows the RP chromatogram of Pr22-3. Table 5-3 presents a comparison of elution times for all the proteins resolved by RP as well as other

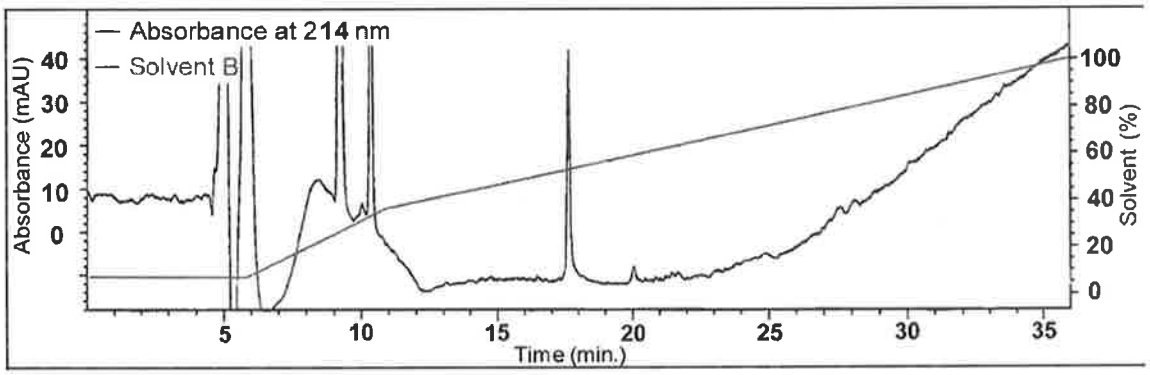


Figure 5-12: Reverse phase chromatogram of Pr22-3
The large peak detected at 17.8 min belongs to Pr22-3 (method: RPC-2). The small peak at 20 min is the contaminant Pr25. Comparison of peak areas of the two proteins indicated that Pr22 constituted ~4.8% of this sample. The peak detected at 10.4 min is DTT. Peaks before that are also other non-proteinaceous compounds as judged by SDS PAGE.

chromatographic methods. Concentrations calculated for the ultrafiltered proteins were 43.7 µg/ml (Pr22-1), 21.1 µg/ml (Pr22-2), 33.3 µg/ml (Pr22-3), 66.0 µg/ml (Pr25) and 42.8 µg/ml (Pr32). Note that these values reflect both the amounts of the proteins available in IWF and the efficiencies of the purification strategies. Since each protein was purified with a different strategy and efficiency, it is inappropriate to compare these values in order to assess the original proportions in the IWF. A modified method with uniform purification strategy for individual proteins is used in Chapter 7 to estimate the proportions of each protein in IWF.

protein	chromatographic methods				
	WCX[1]	HIC[1]	GPC	RPC (RPC-2)	RPC (RPC-TL)
Pr22-1	23	15.2	26.5	18.1	18.5
Pr22-2	26	14	28	18.1	18.2
Pr22-3	27	19.3	29	17.8	16.3
Pr25	28	28.5	26	20	25
Pr32	26.5	19.7	25.5	25	32.8

Table 5-3: Peak elution times
Elution times are measured in minutes. For details of the methods see Chapter 2.
[1]: values for WCX and HIC are approximate.

5.4 Discussion

5.4.1 Non-denaturing protein purification

In order to study the activity of proteins or to purify proteins based on bioactivity it is very useful to maintain their native structure. Although reversible oxidation of some proteins in this study (section 5.3.1.2.2) indicated that these proteins renatured after losing activity, the goal was to avoid denaturation of the proteins.

While polyacrylamide gel electrophoresis (PAGE) is primarily an analytical tool, it has been used to purify semi-quantitative amounts of proteins (Patterson, 1994; Fischer *et al.*, 1989; Trudel *et al.*, 1998b). However, many components of PAGE may promote the modification of proteins. SDS and the reducing agent β-mercaptoethanol, two components of denaturant gels, unfold and denature almost all proteins (Edelstein and Bollag, 1992; Smith, 1994). Yet many, including some antifungal proteins, were reported to renature after SDS and the reducing agent were removed (Michaud and Asselin, 1995). On the other hand, some proteins were only found to be active if reduced (Van der Wel and Bel, 1980; Trudel *et al.* 1998a).

When denaturation in PAGE appears to be a problem, non-denaturing PAGE systems that do not contain SDS and/or a reducing agent may be used (Walker, 1994). However, even conditions of a non-denaturing PAGE have the potential to inactivate some proteins (Edelstein and Bollag, 1992). If caused by optional additives the problem may be avoided. Examples of these additives include β-mercaptoethanol that attaches to cysteine residues, persulfate that oxidizes methionine (Patterson, 1994) and finally primary amines (such as Tris), phosphate and borate buffers that inactivate some enzymes (Harris, 1989a). Other deleterious effects are caused by more mandatory elements such as acrylamide that may react with cysteine residues and covalently bind with proteins in a native or denatured condition (Patterson, 1994).

Unlike PAGE, chromatography is mostly a preparative technique. In general, it has lower resolution but has more flexibility in terms of pH, buffer type and separation mechanisms. It is probably because of this flexibility that chromatography is the method of choice when biological activity is of prime importance. However, proteins

may unfold under some chromatography techniques. For instance, RPC is generally considered to be denaturing because of the solvents, extreme pH and surface effects implicated in this technique (Roe, 1989; Knuth and Burgess, 1987). Other chromatographic methods are commonly much more gentle, although it is possible that some sensitive proteins unfold via surface effects present under any chromatographic technique (Roe, 1989; Knuth and Burgess, 1987). In summary, chromatography is more reliable to purify proteins in their native state, an important criterion in the present study where the proteins were repeatedly assayed for biological activity.

5.4.2 The purification strategy

On average it takes three successive and different chromatography techniques to purify a protein. The most commonly used procedures include precipitation with ammonium sulfate, then ion exchange, followed by affinity and gel permeation chromatography (Harris 1989b). In the purification strategy outlined in this chapter no ammonium sulfate precipitation was used. Instead, the proteins were concentrated by ultrafiltration and then separated on the basis of charge by two successive ion exchange chromatographies, SAX and WCX. These were followed by HIC that, like RPC, resolved the proteins based on their hydrophobicity. The last chromatographic step, GPC, separated the proteins based on size. The commonly used technique of affinity chromatography was inappropriate because it is only suitable for proteins with well-known biological characteristics (Angal and Dean, 1989). Such information was not available for the cell wall degrading agents of IWF. In other words, no particular enzymatic activity, such as β(1-3) glucanase or chitinase, or related chemical affinity, such as absorption to potential substrates, was exploited to purify the proteins. This broadened the detection limit and allowed the purification of different types of proteins based on antifungal activity alone.

Whatever the strategy, a goal in protein purification is to use the minimum number of steps to purify a protein (Harris, 1989b; Roe, 1989). An important factor in deciding the number of steps is the sequence, or ordering, of the chromatographic steps. It is helpful if the product of a certain chromatography could be applied directly to the next chromatography. If this is not possible extra processing of samples, such as desalting, is

required. If repeated, these could markedly impact on the efficiency of a method (Harris, 1989b). The salt level in samples usually increases as the purification proceeds through more chromatography steps. One exception is GPC, where it is possible to readjust the salt to any, usually lower, level. GPC is also exceptional in its flexibility with pH or salt content of samples prior to chromatography. HIC requires high levels of salts and ion exchange chromatographies require low level of salts (Roe, 1989).

In the strategy used here concentrated IWF samples were applied to SAX, WCX, HIC and GPC columns. This ordering required minimum manipulation of the samples. The product of each chromatography was applied to the next step with only pH adjustment or addition of salts. Although no desalting was required, sample volumes were reduced before GPC and occasionally before other chromatography steps. The present purification protocol is interesting for its ability to purify a diverse range of proteins with minimum sample manipulation. Nevertheless, one of the purified proteins (Pr22-3) was found to contain ~5% contaminant Pr25. Low contamination is not always avoidable and has been reported by others (Hrmova and Fincher, 1993).

One aspect of the present strategy was to use SAX to absorb the bulk of inactive proteins at the start of purification. Other purification strategies also benefited from this approach (Kragh *et al.*, 1990; Hrmova and Fincher, 1993; Cheong *et al.*, 1996). This method appears to be very useful in separating strongly basic proteins from other neutral or acidic ones.

5.4.3 Purification of other similar proteins

Different strategies have been previously used to isolate antifungal proteins from plants in general and barley in particular. Some of these strategies, with more relevance to the present project, are compared with the strategy used in this chapter.

At least two different methods have been described previously for the purification of biologically active TL proteins from barley. Hejgaard and coworkers (1991) purified proteins R and S from barley grains by $(NH_4)_2SO_4$-precipitation, WCX (CM-Sephadex), GPC (Sephadex G-50) and WCX again. This strategy was relatively inefficient and lengthy because samples required desalting or concentration prior to each

chromatography step. The second strategy used to purify TL proteins in barley is based on pachyman-affinity and the ability of TL protein to renature after SDS-PAGE (Trudel *et al.*, 1998a; Trudel *et al.*, 1998b). Acid or SDS and heat treatment were used to desorb the TL proteins from pachyman. Proteins that do not bind to pachyman or irreversibly lose activity because of desorption treatment or SDS-PAGE are not suited to this method.

Barley β(1-3) glucanases have been isolated from grain and other tissues by different groups (Hoj *et al.*, 1989; Leah *et al.*, 1991; Hrmova and Fincher, 1993). To extract grain proteins, Hrmova and Fincher (1993) used $(NH_4)_2SO_4$-precipitation, anion exchange (DEAE-cellulose), WCX (CM-Sepharose), chromatofocusing and finally GP (Bio-Gel P-60) chromatography. Samples had to be desalted prior to DEAE and chromatofocusing but no further processing was required. Three β(1-3) glucanase isozymes (GI, GII, GIII) were purified using this method. Three out of four chromatographies in this strategy exploited protein charge while hydrophobicity was almost not used. This method is comparatively complicated but appears to be necessary for separating closely related isoforms of β(1-3) glucanase.

Chitinases of barley have been purified repeatedly (Jacobsen *et al.*, 1990; Kragh *et al.*, 1990; Leah *et al.*, 1991; Swegle *et al.*, 1992). The procedure of Kragh and coworkers (1990) for the extraction of chitinases from IWF is explained here. In this case IWF was applied to anion exchange (DEAE), chitin affinity and cation exchange (Mono S) chromatography. The method is dependent on absorption to chitin and does not suit proteins with no chitin affinity, which includes class II and III chitinases (Kragh *et al.*, 1993). Nevertheless, barley PR-4 proteins, with no chitinase activity, were found to have chitin affinity and were purified by this method.

The method outlined in this chapter, with four chromatography steps, was not optimized for any single protein. As mentioned above, affinity for potential substrates, such as β(1,3) glucan or chitin, was not used either, as this could limit the type of proteins purified or detected. In accordance with the experimental requirements, the method was, instead, applicable to all the proteins with antifungal activity. It will be shown in Chapter 6 that the purified proteins belong to a broad range of plant proteins.

5.4.4 Lack of activity by proteins

In this study high salt concentration, drying and oxidation led to loss of protein activity during the course of purification. The loss of biological activity under high salt concentration was particularly evident in the case of HIC products which required dialysis in order to detect DB activity. Without dialysis, these fractions contained 250 to 600 mM ammonium sulfate and 31 to 75 mM sodium acetate in the bioassay mixture. Routine bioassays were conducted with 10 mM ammonium acetate, but it is not known if this level of salt also suppressed the activity to some extent.

The salt content of media has been reported to strongly influence antifungal activity of plant antimicrobial peptides (Broekaert *et al.*, 1997) and TL proteins (Roberts and Selitrennikoff, 1990; Cheong *et al.*, 1996). Plant antimicrobial peptides, with molecular weights of 10 kDa or less, lose their antifungal activity at salt concentrations well below 10 mM whereas TL proteins require about 10 times more salt concentration to lose activity. The maximum antifungal effects of maize and pumpkin TL proteins have been detected at the minimal salt concentrations. Increasing the salt concentration from 0 mM to 30 mM NaCl decreased the activities by a factor of 4 to 6. The effects were greatly diminished at 100 mM and completely disappeared at 200 mM NaCl (Roberts and Selitrennikoff, 1990; Cheong *et al.*, 1996). It will be shown in Chapter 6 that some of the purified proteins were TL proteins.

Drying also suppressed the activity of cell wall lytic proteins and was found to be irreversible under the experimental conditions (see section 5.3.1.2.1). Proteins are known to readily unfold as they lose water (Allison *et al.*, 1996; Carpenter and Crowe, 1988). While some proteins are able to refold to their original structure after reconstitution, others may not be able to do this and hence lose their native structure and activity. It has been reported that the composition of the solution prior to drying, as well as that of the reconstituting solution, have a definite role in the ability of proteins to refold to their native condition (Allison *et al.*, 1996; Carpenter and Crowe, 1988).

Oxidation is yet another phenomenon that affected the activity of the IWF proteins. *In vitro* loss of activity as a result of exposure to atmospheric oxygen is known for many proteins (Edelstein and Bollag, 1992). This is commonly caused by oxidation of thiol-containing residues, especially cysteine. The phenomenon could be reversed and proteins may recover their activity after the oxidized thiol groups are reduced (Edelstein

and Bollag, 1992). In this chapter, partially purified IWF proteins completely lost their biological activity while stored at 4°C. The addition of a final concentration of 1-3 mM DTT restored the lost activity. This strongly suggested oxidation as the likely cause for the loss of activity. Purified proteins, being more prone to oxidation, did not have any activity in the absence of DTT, even when they were bioassayed immediately after purification. All the purified proteins were found to lose activity but the rate of this phenomenon for each protein was not studied here. Proteins remain active in IWF probably because of its intrinsic reducing condition and the protective environment generated by high concentration of other proteins. Both of these factors are removed by purification. It is noted that although oxidation of the proteins is the likely cause of this phenomenon, it is difficult to completely reject the possibility that DTT, or the IWF intrinsic reducing agent, exerts its effect by affecting the fungus cell wall.

Oxidation and reduction of the IWF proteins will be further addressed in Chapters 6 and 8.

Chapter 6:

Biochemical and biological characterisation of the purified cell-wall-degrading proteins

6.1 Introduction

6.1.1 Objective

Five cell wall lytic proteins, Pr22-1, Pr22-2, Pr22-3, Pr25 and Pr32, were purified in Chapter 5. However, except for their molecular weight and activity, almost no more was known about them. This chapter explores the analytical experiments that led to the identification of these proteins. Some of biochemical and biological characteristics of the purified proteins were also analysed. Not surprisingly, all the purified proteins were found to belong to previously characterised families of cell wall lytic proteins. Some of these proteins relevant to the findings in this chapter are reviewed here.

6.1.2 β(1-3) glucanases

β(1-3) glucanases (EC 3.2.1.39) cleave the β1→3 link in glucan structures. The enzyme tolerates some degree of substitution or branching and can endohydrolyse both

β(1-3) glucans and β(1-3, 1-6) glucans (Xu *et al.*, 1992; Hrmova and Fincher, 1993). Both these polysaccharides contribute to the structure of cell walls in many fungal pathogens (Wessels and Sietsma 1981; Xu *et al.*, 1992). Hydrolysis of these cell wall components is believed to be the reason behind *in vitro* antifungal activity of plant β(1-3) glucanases (Bowles, 1990; Malehorn *et al.*, 1993; Sela-Buurlage *et al.*, 1993). The polysaccharide β(1-3, 1-6) glucan is also present in plants in the form of callose precipitation in sieve tubes and in wounded tissues (Boller, 1987). Another closely related polysaccharide, β(1-3, 1-4) glucan, is a major component of plant cell walls but it can not be hydrolyzed by β(1-3) glucanases (Hrmova and Fincher, 1993). Figure 6-1-A shows the structure of β(1-3) glucan and its 1→3 link.

Table 6-1 presents characterised β(1-3) glucanases in barley cultivars based on different gene and protein studies. Between 44 to 81% amino acid identity is detected between these isoforms (Xu *et al.*, 1992; Malehorn *et al.*, 1993). There are indications that more isoforms may also exist (Jutidamrongphan *et al.*, 1992; Trudel *et al.*, 1998a). A β(1-3) glucanase reported by Leah and coworkers (1991) is extremely similar to isoform GII. All characterised barley β(1-3) glucanases except GVI and ABG2 are basic. GII and GIII are extracellular, GV and GI are believed to be cytosolic and GIV is probably vacuolar (Xu *et al.*, 1994; Roulin *et al.*, 1997). Optimum pH for GI, GII and GIII activity is found to be 4.8 (Hrmova and Fincher, 1993).

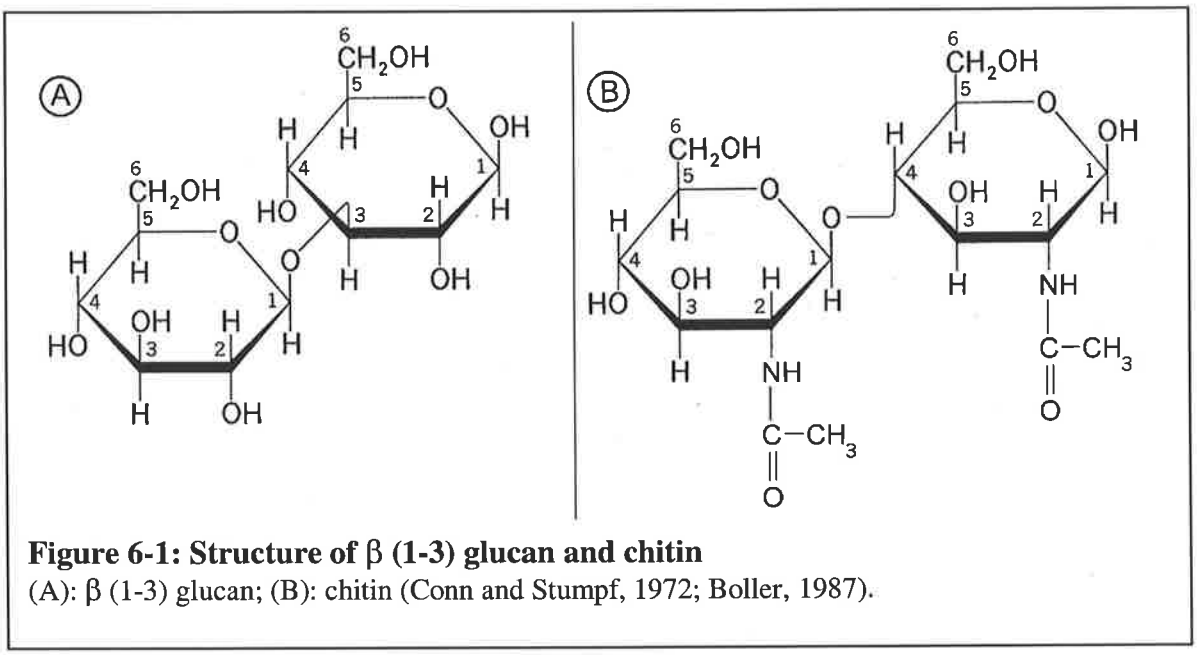


Figure 6-1: Structure of β (1-3) glucan and chitin
(A): β (1-3) glucan; (B): chitin (Conn and Stumpf, 1972; Boller, 1987).

name	MW (kDa)	pI[1]	source	reference
GI	33	8.6	leaf, grain	Xu *et al.*, 1992; Roulin *et al.*, 1997
GII	32.3	9.5	leaf, grain	Xu *et al.*, 1992; Roulin *et al.*, 1997
GIII	32.4	9.8	leaf	Xu et al., 1992; Roulin et al., 1997
GIV	35	10.7	leaf	Xu *et al.*, 1992; Roulin *et al.*, 1997
GV	34	7.5	-	Xu *et al.*, 1992; Roulin *et al.*, 1997
GVI	32.9	4.6	-	Xu *et al.*, 1992; Roulin *et al.*, 1997
ABG2	32.6[1]	4.9	leaf, grain	Malehorn *et al.*, 1993

Table 6-1: β(1-3) glucanses in barley
[1]: Isoelectric points are theoretical values calculated based on deduced amino acids.
ND: not determined.
- : may not be expressed.

6.1.3 Chitinases

Plant chitinases endohydrolyse chitin (Legrand *et al.*, 1987; Linthorst, 1991; Pozo *et al.*, 1998), a significant component of cell walls in most fungi and arthropods (Wessels and Sietsma 1981; Boller, 1987; Mayer *et al.*, 1996). Fungal cell walls may have up to 60% chitin in their structure (Colling *et al.*, 1993), a characteristic that could explain the *in vitro* antifungal activity of plant chitinases (Bowles, 1990; Sela-Buurlage *et al.*, 1993). Chitin, shown in Figure 6-1-B, is built of *N*-acetyl-β-D-glucosamine (GlcNAc) units polymerised by β(1-4) links.

It was explained in Chapter 1 that plant chitinases have been classified into class I, II, III and a few less significant groups (Neuhaus, 1999). Class I chitinases are characterised by two distinct domains in their structure: a chitin-binding domain and a hydrolysing domain. Class II chitinases are similar to those in class I but lack the chitin-binding domain. Accordingly, class II chitinases are smaller molecules and lack any affinity for chitin. Class III chitinases lack the chitin-binding domain and their hydrolysing domains are also very different from that of either class I or II. One implication of this diversity is antibodies raised against one chitinase type may not detect other chitinases (Neuhaus, 1999). It was also reported that purification by chitin-binding chromatography, successfully applicable to class I chitinases, is not effective in purification of chitinases of class II and III (Kragh *et al.*, 1993)

At least eight chitinases have been identified in barley cultivars. These are listed in Table 6-2. It is likely that more chitinase isoforms exist in barley (Grenier and Asselin, 1990; Leah *et al.*, 1991; Rothe *et al.*, 1998). CHI26, with only 6-amino acid differences, is very close to chitinase C (Leah *et al.*, 1991). Barley chitinases have been found to be grain specific (K, T and C) or leaf specific (1, 2 and 6) (Kragh *et al.*, 1993). Chitinases K, T and 1 are class I, chitinases C and 2 are class II and chitinase 6 is class III. Chitinase 1 is intracellular and likely to be vacuolar while chitinases 2 and 6 are extracellular. This localisation is consistent with characteristics of class I, II and III chitinases in other plants (Kragh *et al.*, 1993). Optimum pH for chitinase 1 activity was studied and found to be 4.1 (Kragh *et al.*, 1990). Amongst the leaf-specific chitinases of barley, chitinase 1 and 2 are blocked at the N-terminal, as judged by Edman degradation, whereas chitinase 6 is not (Kragh *et al.*, 1993).

Grenier and Asselin (1990) reported that barley also contains specific chitosanases with no or minimal effect on chitin. The proteins were extracted from the extracellular space and increased in response to elicitation. Using a non-reducing SDS-PAGE system, authors estimated the proteins to be 19 and 22 kDa. Both proteins were found to have basic and acidic isoforms. One of the basic isoforms of the 19-kDa protein lysed spores of a yeast. A later report by the same group suggested that some of these proteins may be thaumatin-like proteins (Grenier *et al.*, 1999) but this needs further clarification.

name	MW (kDa)	pI	source	reference
(acidic chitinase)	26	acidic	leaf	Reiss and Bryngelsson 1996
chitinase K	33	8.7	grain	Jacobsen *et al.*, 1990; Swegle *et al.*, 1992
chitinase T	33	9.8	grain	Jacobsen *et al.*, 1990; Swegle *et al.*, 1992
chitinase C	28	9.7	grain	Swegle *et al.*, 1989; Swegle *et al.*, 1992
CHI26	25.9	ND	grain	Leah *et al.*, 1991
chitinase 1	30	9.1	leaf	Kragh *et al.*, 1990
chitinase 2	24.8[1]	≥9.8	leaf	Kragh *et al.*, 1993
chitinase 6	30	≥9.8	leaf	Kragh *et al.*, 1993

Table 6-2: Chitinases in barley
[1]: reported to migrate as a 27 kDa protein (Kragh *et al.*, 1990; 1993).
ND: not determined.

6.1.4 Thaumatin-like proteins

These are a group of proteins with extensive (~65%) sequence homology to the sweet-tasting 22-kDa protein thaumatin (Cutt and Klessig 1992). In addition, because of the extensive homology and unusually highly conserved cysteine residues it is expected that all these proteins have an almost identical folding pattern (Cutt and Klessig 1992; Svendsen, 1996; Skolnick and Fetrow, 2000). Many TL proteins are known to be antifungal at 5-10 μg/ml *in vitro* (Hejgaard *et al.*, 1991; Vigers *et al.*, 1991), some so potent that they can lyse fungal hyphae in minutes (Roberts and Selitrennikoff 1990; Cheong *et al.*, 1996). Thaumatin itself is only slightly antimicrobial (Vigers *et al.*, 1991). *In vitro* antifungal activity of TL proteins rapidly diminishes by increased concentration of mineral salts in the media (Roberts and Selitrennikoff, 1990; Cheong *et al.*, 1996).

Much of the pioneer research on TL proteins found that they have no known enzymatic activity such as chitinase, glucanase or protease (Roberts and Selitrennikoff, 1990; Hejgaard *et al.*, 1991; Cheong *et al.*, 1996). Consequently, it was suggested that these proteins exert their antifungal effects by permeabilizing the cell membranes, an effect that in turn increases the turgor pressure and causes the cells to burst (Roberts and Selitrennikoff, 1990). Further studies indicated that TL proteins react with the cell wall rather than with the membrane (reviewed by Grenier *et al.*, 1999). Recently many, but not all, TL proteins were shown to bind and/or endohydrolyse some β(1,3) glucans, especially pachyman (Trudel *et al.*, 1998a; Trudel *et al.*, 1998b; Grenier *et al.*, 1999). No digestion of laminarin, widely used as substrate for β(1-3) glucanases, was detected. The ultrastructural difference between laminarin and pachyman was suggested as a possible reason behind this. Laminarin is a random-coil oligomeric β(1,3) glucan with few β(1,6) links while pachyman is a β(1,3) glucan with a more complicated helical structure (Trudel *et al.*, 1998b). Baker's yeast cell wall, a rich source of complex β(1,3) glucans with smaller amounts of β(1,6) glucans and mannan was also strongly digested (Grenier *et al.*, 1993; Trudel *et al.*, 1998a; Grenier *et al.*, 1999).

Known TL proteins in barley are listed in Table 6-3. Protein R and IFW19 (both only partially sequenced) and Barperm1 have only one known amino acid difference between them (Trudel *et al.*, 1998b; Skadsen *et al.*, 2000). They probably represent different forms of one protein. IWF15 (only partially sequenced) has no known

sequence difference with Hv-1 but its molecular weight may be different (Trudel *et al.*, 1998b). Despite a similar MW and pI, protein R and protein S have only ~60% amino acid homology and are, therefore, considered rather distant within the TL family (Hejgaard *et al.*, 1991). The activity of barley TL proteins toward pachyman varies. IFW19 hydrolyses pachyman whereas IFW15 and IFW16 do not (Grenier *et al.*, 1999). IFW15, IFW16, IFW19 and Protein R bind strongly to pachyman (Grenier *et al.*, 1999; Osmond *et al.*, 1999) while only weak binding was detected for Protein S (Osmond *et al.*, 1999). IFW15, IFW19, protein R and protein S have *in vitro* antifungal activities (Bryngelsson and Green, 1989; Grenier *et al.*, 1999). Protein S was found to suppress fungal growth 2-3 times more than protein R (Hejgaard *et al.*, 1991).

IFW(15,16,19) proteins have been detected in the extracellular fluid of barley leaves (Trudel *et al.*, 1998b) whereas protein R, protein S, barperm1 and barperm2 were extracted from barley seed (Hejgaard *et al.*, 1991; Skadsen *et al.*, 2000). Recently, Skadsen and coworkers (2000) suggested that, like chitinases, barley TL proteins could be classified into seed- and leaf-specific types. However, this is not entirely consistent with previous findings.

name	MW (kDa)	pI	source	reference
protein R[2]	23	9-10	grain	Hejgaard *et al.*, 1991
protein S	23	9-10	grain	Hejgaard *et al.*, 1991
Hv-1	19	3.4	leaf	Bryngelsson and Green, 1989; Hahn *et al.*, 1993
IFW15	23	acidic	leaf	Trudel *et al.*, 1998b; Grenier *et al.*, 1999
IFW16	16	acidic	leaf	Trudel *et al.*, 1998b; Grenier *et al.*, 1999
IFW19[2]	24	basic	leaf	Trudel *et al.*, 1998b; Grenier *et al.*, 1999
barperm1[2]	≥21	7.46[1]	grain	Skadsen *et al.*, 2000
barperm2	≥21	ND	grain	Skadsen *et al.*, 2000

Table 6-3: PR-5-type proteins in barley
[1]: Isoelectric point is calculated based on deduced amino acid sequence of the protein.
[2]: possibly different forms of one protein based on amino acid homology.
ND: not determined.

6.2 Materials and methods

6.2.1 Materials

laminarin (from *Laminaria digitata*) and laminarinase (from Trichoderma species, activity: 100-400 units/g, lot: 60H0344) were purchased from Sigma-Aldrich™.

Pr22-1, Pr22-2, Pr22-3, Pr25 and Pr32 proteins purified in Chapter 5 were used.

6.2.2 Assay for enzymatic release of reducing sugars

Substrates included 2.5% (w/v) laminarin in 10 mM ammonium acetate pH 6.0 and 1 mM DTT aquatic solution. Samples were assayed for the release of reducing sugars according to the Somogyi-Nelson method explained elsewhere (Paleg, 1959). Absorbance was measured at 660 ηm using a Beckman™ DU-68 spectrophotometer.

6.3 Results

Proteins purified in Chapter 5 were analysed in this chapter. Two types of analysis were conducted, biochemical and biological analysis. Accordingly, results are presented in two sections.

6.3.1 Biochemical analysis of the cell wall lytic proteins

6.3.1.1 N-terminal protein sequencing

Pr22-1, Pr22-2, Pr22-3, Pr25 and Pr32 were applied to a RPC column to remove the salts and quantify the amount of protein. One hundred pico mol (pM) of each protein was collected and the N-terminus sequenced by the Edman method. Results are as follow:

- Pr22-1: Sixty pM of one protein sequence was detected. Seventeen amino acid residues were sequenced and found to be completely identical to a number of barley TL proteins including protein R (Hejgaard *et al.*, 1991), Barperm1 (Skadsen *et al.*, 2000), IFW19 (Grenier *et al.*, 1999) and the deduced sequence for a permatin with the NCBI accession number of T05973. There was also 100% identity to the deduced sequence of a wheat trimatin. These are shown in Figure 6-2-A. Protein R, also known as THHR_HORVU, is a 23-kDa TL protein with an isoelectric point of 9-10 that was previously isolated from barley grain (Hejgaard *et al.*, 1991).

- Pr22-2: Only 15 pM of one protein sequence was detected. Seventeen residues were sequenced and these were identical to Pr22-1 (Figure 6-2-A).

- Pr22-3: One hundred pM in one sequence was detected. Fifteen residues were sequenced and found to be completely homologous to the amino-terminal sequence of protein S, also known as THHS_HORVU. This is another 23-kDa TL protein, with an isoelectric point of 9-10, that was also isolated from barley grains (Hejgaard *et al.*, 1991). A comparison of the sequences is shown in Figure

6-2-B.

- Pr25: No protein was detected. The protein was blocked at the N-terminal end.
- Pr32: One hundred pM protein of one sequence was detected. Fifteen residues were sequenced and found to be completely homologous to the N-terminal sequence of the barley β(1-3) glucanase isoform GII (Hoj *et al.*, 1989). This is a 32.4 kDa protein with an isoelectric point of 9.8 (Xu *et al.*, 1992). A comparison of the sequences is shown in Figure 6-3

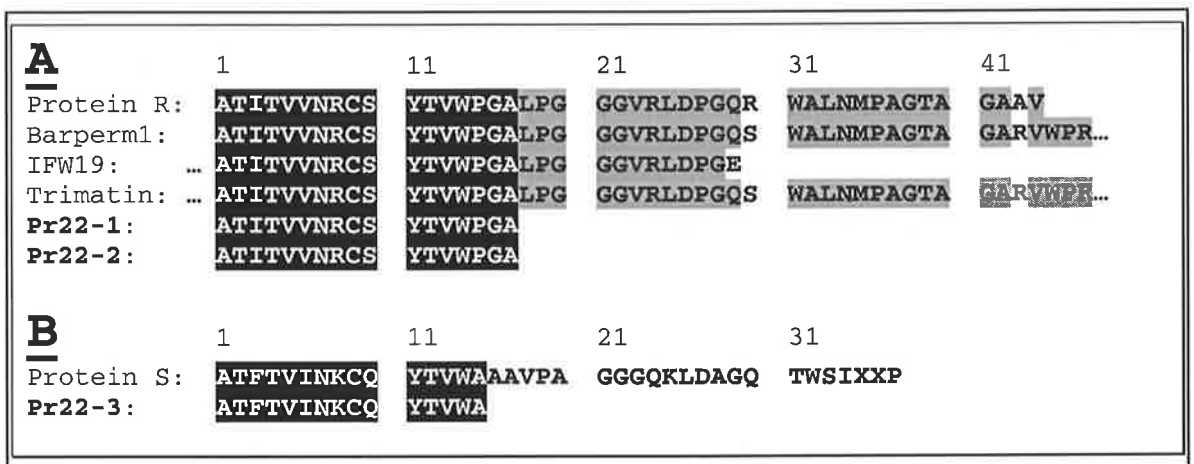


Figure 6-2-terminal sequence of Pr22-1, Pr22-2 and Pr22-3
A: The N-terminal 17 amino acids of Pr22-1 and Pr22-2 are completely homologous to each other and to the N-terminal sequence of protein R (NCBI accession numbers P33044), Barperm1 (T04370), IFW19 (not found at NCBI) a barley permatin (T05973) and a wheat trimatin (T05973).
B: The N-terminal 15 amino acids of Pr22-3 are completely homologous to the N-terminal sequence of protein S (P33045). There is ~60% homology between protein R and protein S (Hejgaard *et al.*, 1991).
In each group black boxes show 100% identity between the proteins whereas gray boxes denote dominant but less than 100% identity. Numbers on the top refer to mature protein sequences.

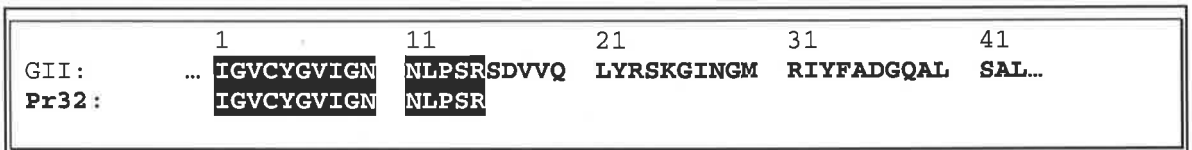


Figure 6-3: N-terminal sequence of Pr32
The N-terminal 15 amino acids of Pr32 show 100% identity to the N-terminal sequence of β-1→3 glucanase isoform GII (P15737). Black boxes denote 100% homology between the amino acids. Numbers on the top refers to mature protein sequences.

6.3.1.2 Peptide mapping of the TL proteins

Pr22-1 and Pr22-2 were eluted at different points in WCX, HI and RP chromatography (See Table 5-3 in Chapter 5) yet, the 17 N-terminal amino acids of the proteins were identical. The powerful technique of peptide mapping was used to further characterise these proteins as well as Pr22-3 which is clearly a different but related protein.

Fifty micrograms of purified Pr22-1, Pr22-2 and Pr22-3 were dried in a Speed Vac™ centrifuge and along with the positive control (15 µg ovalbumin) and the negative control (no protein) were treated by reductive alkylation and tryptic digestion as outlined in section 2.2.1. Following 24 hours incubation, samples were subjected to RP chromatography (110-min run). Results indicated that despite close resemblance, Pr22-1 and Pr22-2 were not the same protein (Figure 6-4). As expected, Pr22-3 was clearly different from the other two proteins (Figure 6-5).

In order to further characterise the differences between Pr22-1 and Pr22-2, a peptide peak specific to each protein was selected for sequencing. These were peak "A", specific to Pr22-1, and peak B, specific to Pr22-2 (see Figure 6-4). Peak A was sequenced and 20 amino acids were identified (see Figure 6-6). A comparison of this Pr22-1 internal sequence to existing sequences indicated that the first 17 amino acids perfectly matched a segment (amino acids 25-41) from Barperm1 and T05973. However, the last 3 residues of the 20-amino acid fragment were not present in these sequences.

Peak B, specific to Pr22-2, appeared to contain more than one peptide. Unfortunately no sequence could be produced for this peak.

6.3.1.3 Mobility of the TL proteins in polyacrylamide gel

Comparative mobility of the three TL proteins was briefly analysed by SDS-PAGE under reduced and non-reduced conditions. Fifty nanograms of purified Pr22-1, Pr22-2 and Pr22-3 was dried in a SpeedVac™ and reconstituted in either a reducing loading buffer containing 50 mM DTT or a non-reducing loading buffer with no DTT. These were then subjected to SDS-PAGE following the procedure outlined in section 3.4.

Results, presented in Figure 6-7, indicate that:

Under reducing conditions the TL proteins migrated at approximately 22 kDa whereas when non-reduced they migrated faster at 19-20 kDa.

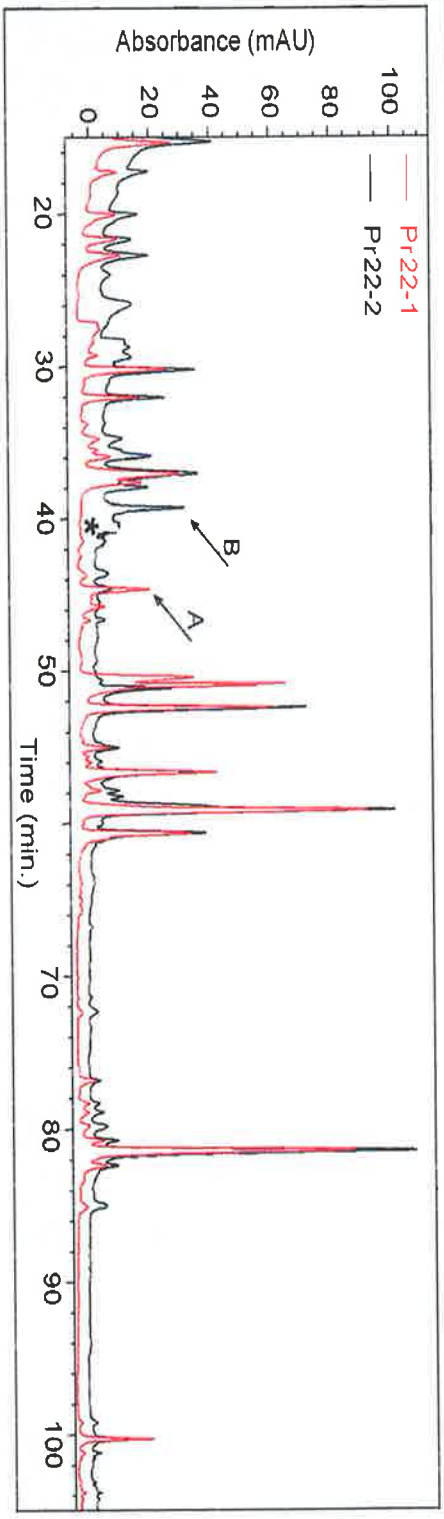


Figure 6-4: Comparative peptide mapping of Pr22-1 and Pr22-2
Similarity of the two chromatograms, detected by 214 ηm absorbance, is indicative of the close similarity of the two proteins. A peak specifically present in Pr22-1 (A) and one from Pr22-2 (B) were collected for sequencing.
*: A bubble trapped in the flow cell interfered with the detection of Pr22-2 at 40.3 to 41.2 min.

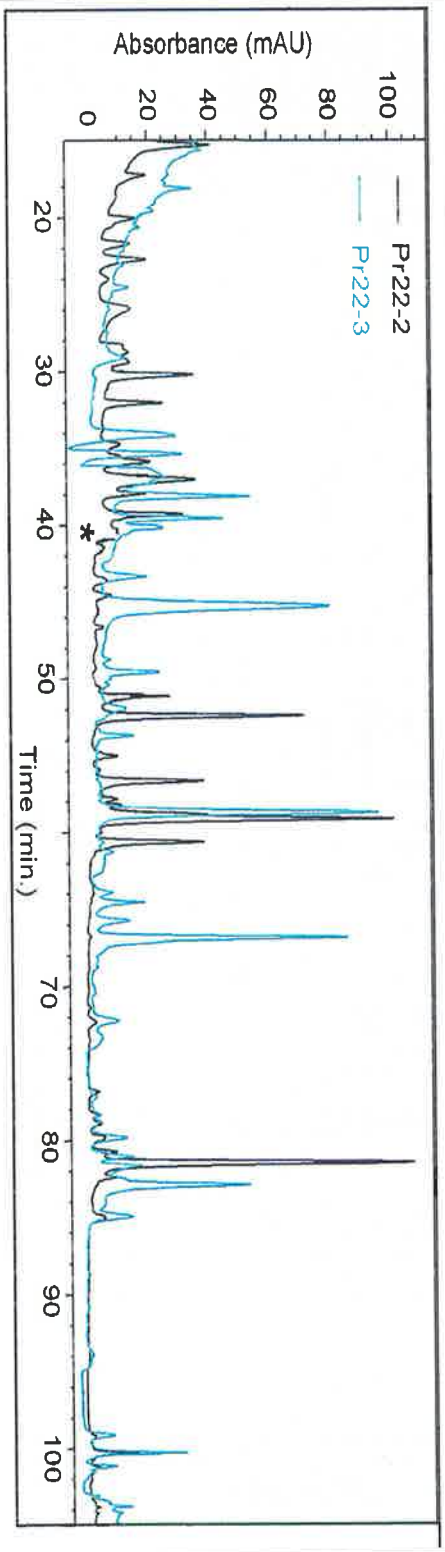


Figure 6-5: Comparative peptide mapping of Pr22-2 and Pr22-3
The peptide mapping chromatograms as detected by 214 ηm absorbance are shown. Despite 80% amino acid identity between the two proteins, very few similarities are identified by peptide mapping.
*: Trapped bubble, as above.

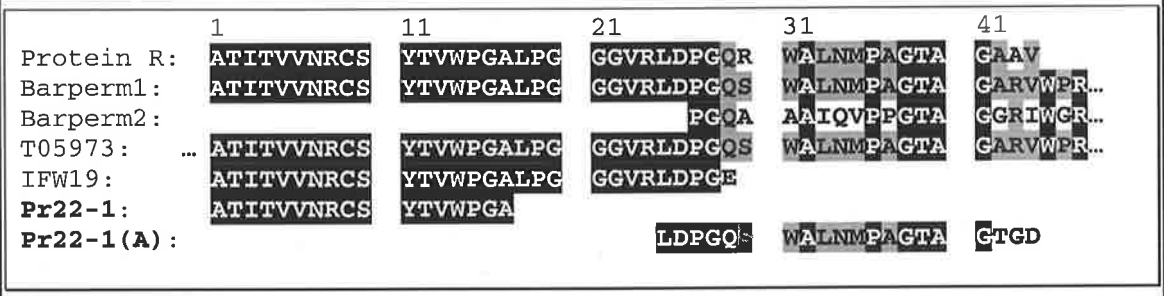
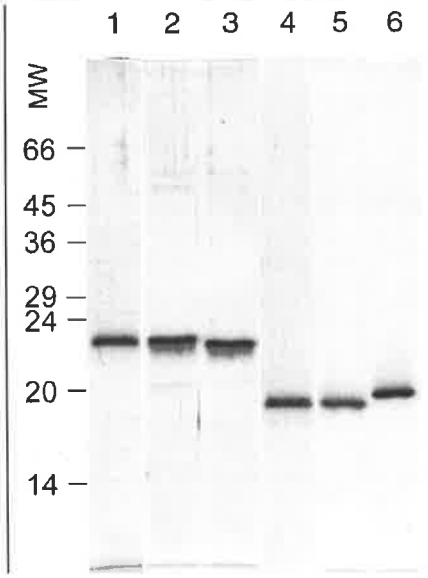


Figure 6-6: Internal sequence of Pr22-1
Pr22-1 sequences for the 17-residue amino-terminal and the 20-residue peak A internal segment (A) are shown. Since trypsin is specific for cleavage at arginine (R) or lysine (K) (Rosenberg, 1996), position 24 of Pr22-1 should be one of these residues. Black boxes show 100% identity between the proteins whereas gray boxes denote dominant but less than 100% identity. Numbers on the top refer to mature protein sequences.

- Under reducing conditions Pr22-1 and Pr22-2 migrated at the same level while Pr22-3 migrated slightly faster.
- Under non-reducing conditions Pr22-1 and Pr22-2 migrated at ~19 kDa while Pr22-3 migrated at ~20 kDa.

Since all the TL proteins migrated very close to each other under reducing conditions it was concluded that they have very close molecular mass (~22 kDa). The migration difference between the reduced and non-reduced proteins is an indication that disulfide bonds play a significant role in the tertiary structure of these proteins. These results are consistent with the notion that Pr22-1 and Pr22-2 are similar to each other while Pr22-3 is different from both.

Figure 6-7: In-gel mobility of the TL proteins under reduced and non-reduced conditions
SDS PAGE under reducing conditions: **1**: Pr22-1, **2**: Pr22-2, **3**: Pr22-3.
SDS PAGE under non-reducing conditions: **4**: Pr22-1, **5**: Pr22-2, **6**: Pr22-3.



Non-reduced Pr25 migrated slightly faster (~0.5 kDa) than the reduced form whereas no difference in the mobility of reduced and non-reduced Pr32 was detected (data not shown).

6.3.1.4 Internal microsequencing of Pr25

Pr25 could not be sequenced directly with the Edman method that is reliant on an unblocked amino terminal end of proteins. Subsequently, the method of internal micro sequencing was explored. This involves enzymatic or chemical generation of internal peptide fragments that are then purified and sequenced. Produced fragments are not generally blocked.

Fifty micrograms of Pr25 was collected following RP chromatography. This was dried and treated by the reductive alkylation and tryptic digestion procedures described in section 2.2.1. The digested protein was subjected to an 80-min RP chromatography and peptide peaks were collected. A symmetrical peak that eluted at 48.7 min was selected and sequenced. Twenty three residues were identified (Figure 6-8). These were found to have a 100% identity to the deduced amino acid sequence of barley chitinase 2b, a homologue (~98%) of chitinase 2 reported by Kragh *et al.* (1993). Chitinase 2 is a leaf-specific extracellular protein with a molecular weight of 24.8 and a pI of ≥9.8.

Figure 6-8: Internal micro sequencing of Pr25
Twenty three residues of Pr25 were sequenced and found to be 100% identical to the deduced amino acid sequence of chitinase 2b (NCBI accession number Q43765). Being cut by trypsin, residue 183 of Pr25 should be arginine (R) or lysine (K). An undigested R residue at position 195 is unexpected. Black boxes show 100% identity between the proteins. Numbers on the top refer to mature protein sequences.

6.3.2 Biological characteristics of the cell wall lytic proteins

Although DB formation was used as the bioassay throughout the purification procedure, the biological activity of the purified proteins was re-investigated.

Pr22-1, Pr22-2, Pr22-3, Pr25 and Pr32 purified by the non-denaturing procedures were dialyzed against the bioassay buffer (10 mM NH_4HCOO, pH 6.0 containing 1 mM DTT) and assayed for a number of biological activities. All the assays were conducted at the final concentrations of the bioassay buffer. Samples were incubated for 12 hours.

6.3.2.1 Cell wall degradation

In Chapter 5 a preliminary purification of HMW IWF suggested a possible association between DB formation and FW activity (see section 5.3.2). Throughout the purification only the assay of DB formation was used as the assay, while FW activity was largely ignored. To further study the relationship between FW activity and DB formation the effect of purified proteins on these activities was investigated. The DB/FW association and the biological activity of the purified proteins at a concentration of 1 μM were addressed in a comparative bioassay.

Protein concentrations were calculated based on RPC peak area (section 5.3.3.6). Protein concentrations that were either too high or too low were adjusted by addition of the bioassay buffer or by ultrafiltration, respectively. Purified proteins were bioassayed at a final concentration of 1 μM protein and 10^7 conidia per ml. Percentages for both DB formation and FW activity were recorded. Results, presented in Figure 6-9, indicated that Pr25 and Pr32 had comparable activities, with approximately 50% of the spores forming doublets and no FW activity. Pr22-1, Pr22-2 and Pr22-3 had more pronounced effects with 60 to 100% DB formation and 13 – 33% FW activity. Note that in accordance with the definitions, DB formation is measured amongst intact spores, that is, excluding FW activity. Accordingly, for example, in Pr22-3 one third of the spores were affected by FW and the other two third were all doublets.

The association of DB formation and FW activity was further investigated in a concentration study involving Pr22-3. It was shown that FW activity was not necessarily detected every time DB formation was observed. No FW activity was

detectable at low protein concentrations when DB formation was also low. Figure 6-10 shows that Pr22-3 at 0.125 μM caused only 21% DB formation and no detectable FW activity. Similarly, increased concentration of Pr32 induced not only more DB but also some FW spores (data not shown).

Phenotypes of FW and DB spores following incubation with different proteins were

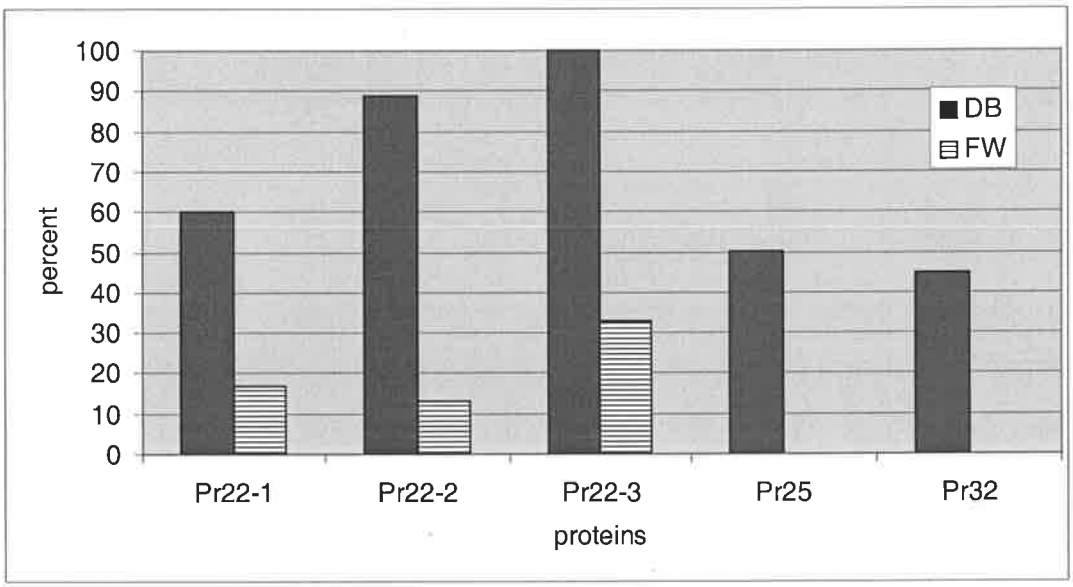


Figure 6-9: Comparative effect of the cell wall lytic proteins
Data are average of two measurements. Pr22-2 and Pr22-3 appeared to have the highest activities. All the proteins were at the final concentrations of 1 μM (That is, 22 μg/ml for Pr22-(1,2,3), 26 μg/ml for Pr25 and 32 μg/ml for Pr32).

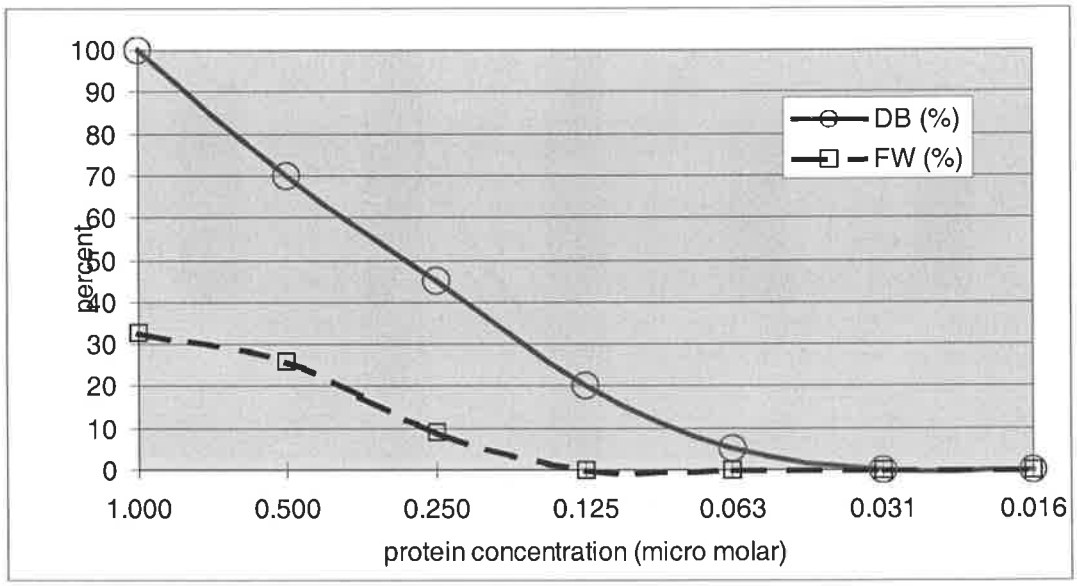


Figure 6-10: Effect of different Pr22-3 concentrations on spores of *R. secalis*
Both DB formation and FW activity are shown.

examined by light microscopy but no difference between the treatments was noticed. Figure 6-11 shows both DB and FW spores as viewed under 1000X magnification. For both phenomena, cell wall degradation was the likely cause. The septum area of the bicellular spores appears to be particularly vulnerable to both FW and DB effects. Nevertheless, in Figure 6-11-C cell walls fractured in places other than the septum are evident. This was especially the case for multicellular spores.

A viability bioassay conducted on these spores indicated that only FW spores are non-viable. Viability of the DB spores, having intact cell wall, did not appear to be affected (see section 5.3.2 for original observation on this).

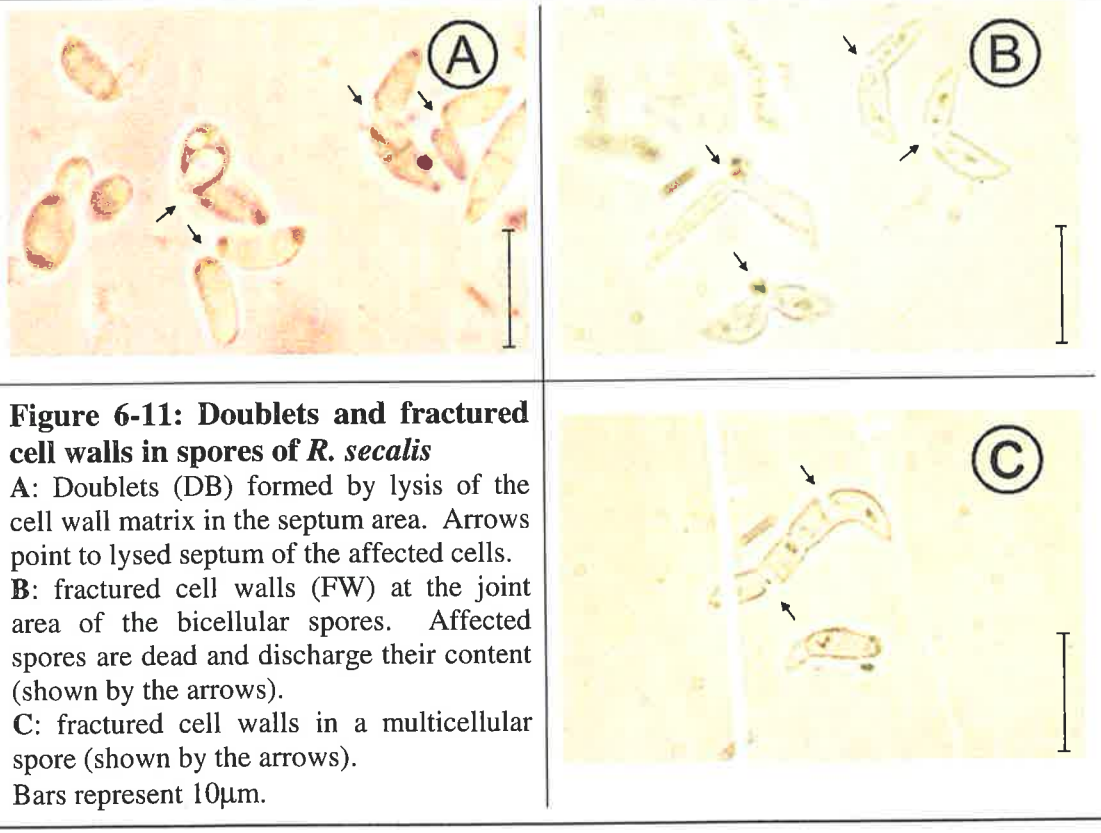


Figure 6-11: Doublets and fractured cell walls in spores of *R. secalis*
A: Doublets (DB) formed by lysis of the cell wall matrix in the septum area. Arrows point to lysed septum of the affected cells.
B: fractured cell walls (FW) at the joint area of the bicellular spores. Affected spores are dead and discharge their content (shown by the arrows).
C: fractured cell walls in a multicellular spore (shown by the arrows).
Bars represent 10µm.

6.3.2.2 Synergistic effect of the cell wall lytic proteins

Combinations of Pr22-1, Pr22-2, Pr22-3 and Pr32 were bioassayed for their effect on spores of *R. secalis*. Not enough Pr25 was available for this study. Results, summarized in Table 6-4, were indicative of a strong synergism between all the tested proteins.

Comparison of 2-protein combinations (Group 2) shows how mixtures involving Pr32 were more effective than comparable mixtures without it. Synergism between Pr22-1,2,3 proteins is demonstrated best in "Group 3" where mixtures involving 3 proteins are compared. The mixture of Pr22-1, Pr22-2 and Pr22-3 (total 90 ηM) appeared to generate more DB and FW activity than any of the proteins individually even at the higher concentration of 120 ηM (Group 1-b). The strongest effects in Group 3 occurred with mixtures containing Pr32 or Pr22-3. When both of these proteins were present not only 48-76% DB was recorded, but FW also reached ~30%. When all the four proteins are present at 30 ηM each (totaled 120 ηM , Group 4) 50% of the spores

group	available concentrations of proteins (ηM)					DB (%)	FW (%)
	Pr22-1	Pr22-2	Pr22-3	Pr32	total		
1-a	30	-	-	-	30	0	0
	-	30	-	-	30	0	0
	-	-	30	-	30	1	0
	-	-	-	30	30	0	0
1-b	120	-	-	-	120	0	0
	-	120	-	-	120	15	0
	-	-	120	-	120	20	0
	-	-	-	120	120	2	0
2	30	30	-	-	60	0	0
	30		30	-	60	2	0
	30	-	-	30	60	9	0
	-	30	30	-	60	3	0
	-	30	-	30	60	17	0
	-	-	30	30	60	23	0
3	30	30	30	-	90	28	3
	30	30	-	30	90	38	3
	30	-	30	30	90	76	33
	-	30	30	30	90	48	32
4	30	30	30	30	120	80	50

Table 6-4: Combined effect of the cell wall lytic proteins
Effects of different combinations of the cell wall lytic proteins, excluding Pr25, on spores of *R. secalis* are shown. Group 1-a includes single proteins at a concentration of 30 ηM. Group 1-b contains the same proteins at 120 ηM which is the total molarity when 30 ηM of all four proteins is present (*i.e.* in Group 4). Group 2 and 3 include combinations of 2 and 3 proteins respectively at 30 ηM each. Group 4 includes combination of all the four proteins at 30 ηM each. Bar (-) indicates nil.

formed FW and 50% of the spores with intact cell walls formed DB. However, when each protein was used individually at 120 ηM, no FW and 0-20% DB were detected (Group 1-b) had hardly generated 20% DB and no FW. In summary the combined effect of these proteins cannot be simply explained by additive effects.

6.3.2.3 Digestion of polysaccharides

Pr22-2, Pr22-3, Pr32 and laminarinase were compared for their ability to digest laminarin. One microliter of the protein samples, adjusted to 1 ηM, was mixed with 5 μl of 2.5% laminarin. After 12 hours incubation at 20°C, samples were assayed for the release of reducing sugars. Results, measured as absorbance at 660 ηm are shown in Table 6-5. Control treatments included laminarin, laminarinase, Pr22-2, Pr22-3, Pr32 and the incubation buffer only. No reducing sugar was detected in the controls, except for the laminarin treatment, which is likely caused by reducing ends of the laminarin macromolecules (Boller, 1992). Other key points are:

- Pr32 was more than twice as effective as laminarinase in digesting laminarin.
- Pr32 was approximately three times as effective as Pr22-3 in digesting laminarin.
- Unlike Pr22-3, no digestion of laminarin was detected for Pr22-2.

treatment	absorbence
laminarinase + laminarin	1.40
Pr22-2 + laminarin	0
Pr22-3 + laminarin	1.06
Pr32 + laminarin	2.98
laminarin	0.05
laminarinase	0
Pr22-2	0
Pr22-3	0
Pr32	0
bioassay buffer1 only	0

Table 6-5: Release of reducing sugars by Pr22-2, Pr22-3 and Pr32
Ability of the proteins to hydrolyse laminarin was measured by the Somogyi-Nelson method of measuring reducing sugars. Numbers in the right column present the absorbance at 660 ηm that is directly correlated to the amount of reducing sugars in samples.
[1]: Samples were incubated with a buffer of 1 mM DTT and 10 mM NH_4HCOO pH 6.0.

6.4 Discussion

6.4.1 Mobility of proteins in SDS polyacrylamide gels

Proteins with disulfide bonds in their structure often have different mobilities when analysed by SDS-PAGE under reducing and non-reducing conditions (See and Jackowski, 1990; Hames and Rickwod, 1990; Smith, 1994). Only reduced, unfolded proteins bind optimally with SDS and hence migrate proportional to their sizes. If the protein of interest is able to form polymeric structures through its disulfide bonds then it will migrate slower in non-reduced forms than in reduced form. Alternatively, a monomeric protein with a compact structure under non-reduced conditions may unfold under reduced conditions. This protein will migrate slower under reducing conditions (Hames and Rickwod, 1990). Many TL proteins such as zeamatin, protein R, protein S, IFW19, etc. that migrate at 22-23 kDa when reduced, appear at 19-20 kDa when not reduced (Roberts and Selitrennikoff, 1990; Hejgaard *et al.*, 1991; Trudel *et al.*, 1998b). Clearly, the molecular mass of these proteins is 22-23 kDa and fast migration of the unreduced forms is merely indicative of the role of S-S bonds in the tertiary structure of these proteins. This has caused some confusion in the literature lately (Trudel *et al.*, 1998b; Skadsen *et al.*, 2000). It is also noted that the molecular masses measured by SDS-PAGE are only approximates and a ±10% error in measurement is not unexpected (See and Jackowski, 1990; Hames and Rickwod, 1990).

Amongst the purified proteins, no difference in the mobility of reduced and non-reduced Pr32 was detected. This was expected because β(1-3) glucanase is a monomeric protein (Hrmova and Fincher, 1993) and has no intramolecular disulfide bonds. Examination of the amino acid sequence of GII and most other β(1-3) glucanases of barley indicates that they only have one cysteine residue. The other protein purified in this chapter, Pr25 migrated only slightly faster under non-reducing conditions. This indicates that it is a monomeric protein with intramolecular disulfide bond(s). However, because its reduced and non-reduced forms migrate close together, the S-S bonds do not appear to play a significant role in the tertiary structure of this protein. Examination of the amino acid sequence of Chitinase 2 indicates that it has

five cysteine residues. On the other hand, Pr22-1, Pr22-2 and Pr22-3 were found to migrate markedly faster when non-reduced. These are, therefore, monomeric proteins with their disulfide bonds playing a significant role in their tertiary structure. Many TL proteins, such as protein R and protein S, have 16 cysteine residues probably arranged in 8 disulfide bonds (Hejgaard *at al.*, 1991; Cutt and Klessig 1992; Grenier *at al.*, 1999). However, it is noted that none of the data produced in this chapter may be used to determine the number of disulfide bounds in the protein structures.

When analysed under reducing condition, Pr22-3 migrates slightly faster than Pr22-1 and Pr22-2. Although the difference is small and may not necessarily indicate a difference in mass it could be used to differentiate between the proteins in a gel. Under non-reducing conditions Pr22-1 and Pr22-3 migrate at ~19 kDa while Pr22-3 migrates at ~20 kDa. This may imply that Pr22-3 has a less compact tertiary structure than the other two proteins. Hejgaard and coworkers (1991) also found barley protein S (homologue to Pr22-3) to migrate slightly slower than protein R (homologue to Pr22-1 and -2). These authors estimated the molecular weight of both proteins to be ~23 kDa.

6.4.2 Cell wall degradation as the antifungal mechanism

The antifungal effects exhibited by β(1-3) glucanase and chitinase have been previously studied and attributed to the ability of these proteins to degrade fungal cell walls (Boller, 1987; Bowles, 1990). The synergistic effects of these enzymes in inhibiting fungal growth have also been studied (Cutt and Klessig 1992). *In vitro* antifungal activity of TL proteins have been reported to be almost immediate, especially on hyphal tips where cytoplasmic burst is observed (Roberts and Selitrennikoff, 1990; Cheong *et al.*, 1996). Lack of activity on some substrates led early researchers to conclude that the cell membrane, not the cell wall, is the point of action for TL proteins (Roberts and Selitrennikoff, 1990). Recently, Grenier and coworkers (1999) reported that not only do many TL proteins digest fungal cell walls but some could also hydrolyse pachyman, an insoluble β(1,3) glucan.

In the present study some of IWF antifungal proteins of barley were isolated and shown to be a 32-kDa β(1-3) glucanase (Pr32), a 25-kDa chitinase (Pr25) and three 22-kDa TL proteins (Pr22-(1,2,3)). Table 6-6 presents a summary of these proteins. At 1

μM, Pr32 and Pr25 caused a benign spore damage, termed doublet (DB), while Pr22-1, Pr22-2 and Pr22-3 caused a higher level of DB formation as well as a high number of non-viable spores with fractured cell walls (FWs) (Figures 6-9 and 6-11). A high concentration of Pr32 may also cause FW activity. The phenomenon of FW activity is probably analogous to the previously reported cytoplasmic burst of hyphal tips by other TL proteins (Roberts and Selitrennikoff, 1990; Cheong *et al.*, 1996). Microscopic studies strongly suggested that both DB formation and FW activity were essentially caused by cell wall degradation. There was no evidence to confirm the suggested impact of TL proteins on spore membrane, although the possibility is difficult to reject from the present data. Since the hydrolytic enzymes, Pr32 and Pr25, caused an effect similar to Pr22-(1,2,3) this confirms that cell wall, rather than the membrane, is the point of action for TL protein.

Three TL proteins were detected in this study: Pr22-1, Pr22-2 and Pr22-3. N-terminal sequencing, in-gel mobility and peptide mapping showed that Pr22-1 and Pr22-2 were similar to each other, although not identical. P22-3 was very different from both of these. Pr22-1 and Pr22-2 are close homologues of protein R, barperm1 and IFW19. Pr22-1 was characterised further and found to be slightly different from all the previously reported TL proteins (Figure 6-6). Pr22-2 is different from Pr22-1 as judged

protein	anti-fungal	homologue	sequenced residues	binding to pachyman	laminarin hydrolysis	reference
Pr32	+	glucanase GII	15	ND	++	this work; Xu *et al.* (1992)
Pr25	+	chitinase 2b	23[1]	ND	-	this work; Kragh *et al.* (1993)
Pr22-1	++	protein R, barperm1 and IFW19	17+20[1]	strong[2]	ND	this work; Hejgaard et al., 1991; Grenier et al., 1999
Pr22-2	++	protein R, barperm1 and IFW19	17	strong[2]	-	this work; Hejgaard *et al.*, 1991; Grenier *et al.*, 1999
Pr22-3	+++	protein S	15	weak[2]	+	this work; Osmond *et al.*, 1999; Hejgaard *et al.*, 1991

Table 6-6: Summary information on purified cell wall degrading proteins
[1]: Internal sequence
[2]: Based on homology.
ND: Not determined.

by peptide mapping, but no sequence was obtained to characterise this difference. Pr22-3 was homlogous to protein S.

Pr22-3, but not Pr22-2, was shown to digest laminarin. This is the first time a TL protein has been found to digest laminarin, although a close homologue of Pr22-3, protein S, does not seem to have been tested for laminarinase activity (Hejgaard *et al.*, 1991; Grenier *et al.*, 1999). The inability of Pr22-2 to digest laminarin was in agreement with a previous finding for IFW19 (Grenier *et al.*, 1999). Compared to the other two TL proteins, Pr22-3 had a more pronounced antifungal activity. This was also shown by Hejgaard and coworkers (1991), who compared the antifungal activity of protein R and S on *Trichoderma*, *Candida* and *Fusarium* species. Digestion of laminarin may not solely explain the higher antifungal activity of Pr22-3 because Pr32, being homologous to β(1-3) glucanase, digested this substrate more vigorously but had much less impact on *R. secalis*. However, when combined with the putative protease activity of the TL proteins, the β(1-3) glucanase activity of Pr22-3 may explain its higher antifungal activity.

Some of the reported synergistic effects of antifungal proteins of barley include that between protein S or R and chitinase C (Hejgaard *et al.*, 1991), chitinase C and GII, chitinase C or GII and a barley grain ribosome-inactivating protein (Leah *et al.*, 1990), and chitinase C or protein R and PR-4 proteins (Hejgaard *et al.*, 1992). In the present study all combinations of the proteins tested had a synergistic effect on *R. secalis* conidia (Table 6-4). The maximum effect was recorded for those interactions that involved dissimilar proteins, that is, the β(1-3) glucanase and any of the TL proteins. However, there was also a slight but noticeable synergism between different TL proteins. The detected synergism between isoforms of one protein is in agreement with the finding of Kragh and coworkers (1990) on chitinase. Reasons cited for existence of multiple protein isoforms include tissue-specific and developmental regulation, specific subcellular localisation, substrate specificity and possible synergistic effect because of broadened specificity (Cutt and Klessig, 1992; Xu *et al.*, 1992).

Previously, a TL protein (Hv-1) and β(1-3) glucanases were shown to be induced in the defense response of barley against *R. secalis* (Hahn *et al.*, 1993; Roulin *et al.*, 1997). On the other hand, it has been known that not all isoforms of a cell wall lytic enzyme may exhibit antifungal activity against a fungus (Sela-Buurlage *et al.*, 1993). It was,

therefore, desirable to demonstrate the prposed effects of the barley PR proteins on *R. secalis*. Data presented in this chapter quantitatively measured some of these effects and confirmed the suggested role of these proteins in the defense responses of barley towards *R. secalis*. Extracellular expression of Pr32, Pr25, Pr22-1 and Pr22-2 in barley leaves is in agreement with previous findings for β(1-3) glucanase isoform GII, chitinase 2 and IFW19 (Kragh *et al.*, 1993; Roulin *et al.*, 1997; Trudel *et al.*, 1998b). Expression of Pr22-3 in barley leaves and its localisation in the extracellular space had not been shown previously.

6.4.3 Cell wall of *R. secalis* conidia

A significant proportion of the cell wall of many Ascomycetes and Basidiomycetes is made of chitin and β(1,3) glucans with some β(1,6) linkages. The mature cell wall may have several layers with the outermost layer usually comprised of compounds that are not digestible by β(1,3) glucanase or chitinase (Wessels and Sietsma 1981). α(1,3) and α(1,6) linked glucans, mannoproteins, phosphomannoproteins and lipids are some of the compounds found in the outer layer of some fungal cell walls.

Prior to this study, no direct information on the structure of the cell wall of *R. secalis* was available. Partial digestion of the conidial cell wall by Pr32 (β(1,3) glucanase) or Pr25 (chitinase) indicates the presence of both β-1,3 glucan and chitin. Formation of DB, caused by uneven digestion of the fungal cell wall, indicates that the cell wall is not homogenous in structure. The cell wall around each cell appears to be more resistant than the matrix that connects the two cells together in the septum area. However, the FW activity showed that even the protective cell wall could be degraded, although this was at a lower rate and chiefly in the septum area. In the current experiments, FW activity was not observed for chitinase although higher concentrations of the enzyme may induce it.

No distinction between the DB spores formed by β(1-3) glucanase, chitinase or TL proteins was observed. This indicates that substrates of these enzymes contribute similarly to the structure of the septum. Both this study and a recent one by Grenier and coworkers (1999) show that TL proteins can hydrolyse β-1,3 glucans. The present

study also suggests that TL proteins have proteolytic activity. However, since the substrate range for TL proteins has not been completely investigated, the effect of these proteins cannot be used to analyse the cell wall composition in *R. secalis*.

Chapter 7:

Pathogenesis-related proteins implicated in the barley-*R. secalis* interaction

7.1 Introduction

7.1.1 Objective

The isolation and characterisation of five cell wall lytic proteins from barley were described in Chapters 5 and 6. The proteins, comprising Pr32, Pr25 and Pr22-(1,2,3), were shown to be a β(1-3) glucanase, chitinase and thaumatin-like (TL) proteins, respectively. In many plants, these families of proteins have been found to increase in response to pathogen attack and are, therefore, classified as pathogenesis-related proteins or PRPs (Van Loon, 1999). However, no attempt was made in the previous chapters to compare the level of the purified proteins in inoculated and uninoculated plants. In the present chapter the level of these proteins, as well as that of several other proteins with no known biological activity, was studied. Some of the latter proteins were also characterised further to determine whether they are related to known pathogenesis-related proteins (PRPs).

7.1.2 Pathogenesis-related proteins

PRPs are defined as plant proteins induced in pathological or related situations (Van Loon, 1999). The increase in the levels of PRPs is believed to be generally faster and/or more prominent in resistant compared to susceptible plants (Bowles 1990; Van Loon, 1999). However, PR proteins may be at reasonably high levels before pathogen attack. It is possible that the high levels of PRPs were induced by a previous environmental challenge. This is believed to be one of the mechanisms behind induced (or acquired) resistance. The high levels could also be regulated by tissue-specific or developmental controls, a process that builds preemptive protection against pathogen attack (Fincher 1989; Bowles 1990).

The finding in tobacco that negatively charged (acidic) PR proteins are extracellular whereas positively charged (basic) proteins are intracellular prompted the suggestion that subcellular localisation of PRPs is correlated with their isoelectric points (pI) (Bowles 1990; Ward *et al.*, 1991). However, more recent studies showed that localisation of many PRPs is independent of their pI (Linthorst, 1991). In fact, the majority of barley extracellular PR proteins are extremely basic (Hejgaard *et al.*, 1992; Collinge *et al.*, 1993; Bryngelsson *et al.*, 1994; Roulin *et al.*, 1997). A number of barley PRPs, especially those of extracellular origin, are important to the results presented in this chapter and are reviewed below.

PRP-1 is perhaps the most abundant PRPs with strong induction in response to pathogen attack in many plants (Buchel and Linthorst, 1999). The level of two basic extracellular isoforms of PRP-1 was found to increase strongly in response to pathological stimulation in barley (Bryngelsson *et al.*, 1994). The proteins had molecular weights of ~15.0 and ~15.2 kDa, with isoelectric points of 10.5 and 11.0, respectively (Bryngelsson *et al.*, 1994).

PR-2 proteins are β(1-3) glucanases (Leubner-Metzger and Meins; 1999). Barley β(1-3) glucanase, including isoforms GI to GVI and ABG2, were reviewed in Chapter 6. The extracellular isoforms GII, GIII and ABG2, as well as the intracellular isoform GI, have been shown to increase in response to inoculation (Jutidamrongphan *et al.*, 1992; Xu *et al.*, 1992; Stevens *et al.*, 1996, Reiss and Bryngelsson 1996; Kaku *et al.*, 1997; Roulin *et al.*, 1997). The cell wall lytic protein Pr32, identified in the current

research, is β(1-3) glucanase isoform GII.

PR-3 proteins are chitinases (Neuhas, 1999). At least 4 isoforms of chitinase have been detected in barley leaves (see Chapter 6). However, only an acidic 26-kDa chitinase and the 25-kDa basic chitinase 2 have been reported to increase in response to pathogen attack (Kragh *et al.*, 1993; Reiss and Bryngelsson 1996).). Pr25 isolated in this work was identified as chitinase.

Up to three isoforms of PRP-4 have been identified in stressed barley leaves and all were found to be ~14 kDa, basic and extracellular (Kragh *et al.*, 1990; Hejgaard *et al.*, 1992). A homologue of PRP-4 was also purified from barley grain (Hejgaard *et al.*, 1992; Svensson *et al.*, 1992). This protein, also known as barwin, is 13.7 kDa with a pI estimated to be between 9.3 (Hejgaard *et al.*, 1992) to more than 10 (Svensson *et al.*, 1992). Barwin has a pyroglutamate residue at the N-terminal (Svensson *et al.*, 1992) which might explain why the protein could not be sequenced by Edman's N-terminal sequencing (Hejgaard *et al.*, 1992; Svensson *et al.*, 1992). Barley PR-4 proteins were shown to increase in response to pathological stimuli (Hejgaard *et al.*, 1992).

PRP-5 shows extensive homology to thaumatin and hence are called thaumatin-like (TL) proteins (Cutt and Klessig, 1992). Up to 6 different TL proteins have been identified in barley (see Chapter 6). Amongst these, Hv-1 has been reported by different groups to increase in response to pathogen attack although its sub-cellular localisation was not elucidated (Bryngelsson and Green, 1989; Hahn *et al.*, 1993). The extracellular IFW19, a close homologue of Barperm1 and protein R, is reported to increase in barley leaves treated with $AgNO_3$ (Grenier *et al.*, 1999). Three TL proteins were identified in the research presented here, Pr22-1, -2 and -3.

PRP-9 are peroxidases (Van Loon, 1999). Up to 7 peroxidase isoforms, with pI's ranging from 3.8 to 9.6, have been detected in the extracellular space of barley leaves (Kerby and Somerville, 1989, 1992; Kristensen *et al.*, 1999). Many of these were shown to increase following inoculation with powdery mildew (Kerby and Somerville, 1989; Tamas and Fric, 1995; Kristensen *et al.*, 1999). P8.5, a peroxidase also known as Prx8 and peroxidase 1, has been characterised further and shown to be a heme-containing protein present in both glycosylated and non-glycosylated forms (Christensen *et al.*, 1992; Kristensen *et al.*, 1999; Scott-Craig *et al.*, 1995). Some other peroxidases from barley and other plants were also shown to have similar heme-

containing coexisting glycosylated and non-glycosylated forms (Kerby and Somerville, 1992).

7.2 Materials and methods

7.2.1 Materials

Pharamalyte, electrophoresis-grade urea, peroxidase-conjugated secondary anti-rabbit IGg (5000X) and 3-3'-diaminobenzidine were purchased from Sigma-Aldrich. Methanol, NaH_2PO_4 and sodium azide were from BDH. Hybond-C™ nitrocellulose and Hybond-P™ PVDF were from Amersham Pharmacia Life Science. NaCl and hydrogen peroxide (H_2O_2) were from Ajax Chemicals, Sydney, Australia. Carnation™ non-fat milk was from Nestle. Hv-1 antiserum (2000X) was a gift from Dr T. Bryngelsson at the Department of Crop Genetics and Breeding, The Swedish University of Agricultural Sciences.

7.2.2 Intercellular washing fluid

IWF was extracted from 34 g of leaves (~130 plants) of Atlas, inoculated Atlas, Atlas 46 and inoculated Atlas 46 as outlined in Chapter 2. This produced ~5 ml IWF per treatment, which was then concentrated to 100 µl using Centricon-10™ ultrafilters. The product, high-molecular weight (HMW) IWF, was purified by HPLC (see below).

7.2.3 HPLC and protein purification

High-pressure liquid chromatography (HPLC) included SAX, WCX, HI and RP chromatographic steps, outlined in Chapter 2. The purification strategy was explained in Chapter 5. Variations to the original purification strategy, when used, are explained in the text.

Data from the last purification step, RP chromatography, was used to quantify the amounts of the purified proteins. The Hewlett Packard 1090 Liquid Chromatograph used in this experiment had a diode-array detector capable of on-line spectrum

recording. When required, spectra of the purified proteins were saved for further analysis.

7.2.4 Western blot analysis

Protein samples were resolved by SDS PAGE as outlined in Chapter 2. The separated proteins were transferred to nitrocellulose or PVDF membranes using a BioRad™ Trans-Blot™ semidry transfer cell and a transfer buffer comprising of 25 mM Tris-HCl, pH 8.3, 192 mM glycine buffer plus 10% methanol, according to the manufacturer instructions. The membrane was washed in PBS (1.56 g/l NaH_2PO_4, 8.76 g/l NaCl, pH 7.2) for 5 min and incubated in 20 ml blocking buffer (PBS containing 8% non-fat milk and 0.02% sodium azide) for 1 hr at room temperature. The membrane was transferred into 20 ml fresh blocking buffer containing diluted Hv-1 antiserum (1:2000) and incubated for a minimum of 2 hour at room temperature. The membrane was washed in 3 changes of PBS, 10 min each, and then incubated in 20 ml of TBS buffer (150 mM NaCl, 50 mM Tris-Cl, pH 7.5) for 10 min. This buffer was replaced with 20 ml of fresh TBS containing 5% non-fat milk, 0.02% sodium azide and diluted (1:5000) peroxidase-conjugated secondary antirabbit Igg and incubated for 1 to 2 hrs at room temperature. The membrane was then washed in 4 changes of TBS, 10 min each. Finally, the membrane was incubated in 40 ml of the reaction mixture (25 mM Tris-HCl pH 7.5, 0.025 H_2O_2 and 0.05% 3-3'-diaminobenzidine) until (brown) bands were completely developed.

7.2.5 Two-dimensional gel electrophoresis

2-D gel electrophoresis was carried out in a BioRad™ Mini-Protean II apparatus as described by the manufacturer. For isoelectric focussing (first dimension) two pH intervals of the Pharmalyte were mixed in the following proportions, 90% pH 3-10 and 10% pH 9-11 (v:v) to obtain a pH range of 3 to 11. Proteins were visualized with the silver staining procedure outlined in Chapter 2. Isoelectric points were estimated based on the positions in the gel of horse cytochrome C (pI 10.5), human carbonic anhydrase I

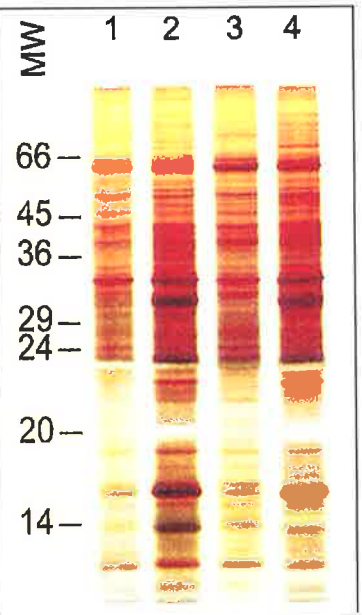
(pI 6.6), Bovine β-lactoglobulin A (pI 5.1) and *Aspergillus niger* amyloglucosidase (pI 3.6).

7.3 Results

7.3.1 Preliminary comparison of IWF extracts

Ten microliters of IWF from Atlas, inoculated Atlas, Atlas 46 and inoculated Atlas 46 were applied to SDS PAGE and visualized by silver staining. The results, presented in lanes 1 to 4 in Figure 7-1, suggest a coordinated increase of a number of proteins following inoculation of both cultivars. In attempts to resolve the proteins further, 70 µl of IWF from each treatment was analysed by 2-D gel electrophoresis (see Appendix 4). While many IWF proteins were found to increase in response to inoculation it was not possible, due to the large number of proteins, to distinguish putative PRPs that had increased substantially more than the others. It is also possible that the PRPs focused outside the gel or did not focus properly, which may be the case for extremely basic proteins including many known PRPs of barley (see section 7.1.2).

Figure 7-1: Comparison of IWF proteins from Atlas, inoculated Atlas, Atlas 46 and inoculated Atlas 46
SDS PAGE profiles of IWF from Atlas (lane **1**), inoculated Atlas (lane **2**), Atlas 46 (lane **3**) and inoculated Atlas 46 (lane **4**) are shown. Molecular weights (kDa) are displayed on the left.



7.3.2 Are the cell wall lytic enzymes PR proteins?

An experiment was conducted to determine the levels of the five cell wall lytic

proteins (Chapter 5) in resistant and susceptible plants, with and without inoculation. A purification procedure, similar to the one originally developed in Chapter 5, was used to isolate the cell wall lytic proteins from inoculated and uninoculated plants. The level of proteins was compared at three different stages throughout the purification procedure: after the first and the second chromatographic steps and at the end of the purification process.

Semi-purified samples collected from the first two steps of the purification procedure were analysed with SDS PAGE. This enabled simultaneous estimation of the level of the lytic proteins as well as that of the other proteins co purified with them. At the end of the purification procedure, quantity of the purified proteins were estimated by a chromatographic technique and compared with each other. This latter technique, chromatographic quantification, enabled a precise measurement of the protein levels but it was inapplicable to non-pure proteins. The purification step, the qualitative SDS PAGE analysis and the chromatographic quantification are discussed in the following sections.

7.3.2.1 Protein purification

Pr22-(1,2,3), Pr25 and Pr32 were purified from Atlas, inoculated Atlas, Atlas 46 and inoculated Atlas 46. The purification procedure used was similar to that outlined in Section 5.3.3 except that the final non-denaturing low-resolution GPC was replaced with a denaturing high-resolution RPC. Hence the purification sequence became SAX, WCX, HI and RP chromatography. In addition, more protein was collected in the early steps which enabled a higher recovery of proteins compared to the non-denaturing method used in Chapter 5. This was possible because the final high resolution RPC compensated for the lowered purity of the early chromatographic steps. Table 7-1 presents a summary of this method for the purification of Pr22-(1,2,3), Pr25 and Pr32. Proteins purified by this method appeared to be pure as judged by SDS PAGE analysis (data not shown).

protein	collected fractions			
	SAX	WCX	HIC	RPC
Pr22-1	3-4	22-24	15-21	19
Pr22-2	3-4	26-30	13-17	19
Pr22-3	3-4	26-30	19-23	17
Pr25	3-4	26-30	19-23	25,26
Pr32	3-4	26-30	26-30	33,34

Table 7-1: Summary denaturing purification for Pr22-(1,2,3), Pr25 and Pr32
Chromatographic methods are the same as those used in Chapter 5. RP chromatography was carried out according to the method RPC-TL (for details see Chapter 2). As before, 1 fraction/min was collected (fraction #1 at 0-1 min, fraction #2 at 1-2 min, etc.).

7.3.2.2 Comparative SDS PAGE profiles

Semipurified samples from the first two steps of the purification procedure, SAX and WCX, contained relatively few proteins compared to the IWF. Analysis with SDS PAGE provided an easy means to compare the protein composition of samples from inoculated and un-inoculated resistant and susceptible plants.

7.3.2.2.1 SAX-unbound proteins

It was shown in Chapter 5 that SAX-unbound proteins contained all the antifungal activity originally detected in the IWF. The proteins were mostly eluted in fractions 3 and 4 of the SAX chromatography (see Figure 5-4). One microliter of these fractions from Atlas, inoculated Atlas, Atlas 46 and inoculated Atlas 46 was applied to SDS PAGE and visualised by silver staining. Results are shown in Figure 7-2 in lanes 2 to 5 with fractions 3 and 4 labeled as f3 and f4, respectively. The SAX-unbound proteins have been identified by their molecular weight and include the cell wall lytic proteins plus other copurified proteins with higher and lower sizes.

Amongst the cell wall lytic proteins only the protein band associated with Pr25 is clearly separated from the rest. All the TL proteins (Pr22-1,2,3) appeared as a single band, although there was a slight difference between fractions 3 and 4 in it's migration. Pr32 could not be separated from proteins slightly higher in molecular weight. It was shown in Chapter 5 that the other SAX-unbound proteins had no detectable biological activity and were not purified at the time. These proteins were identified as Pr14, Pr16,

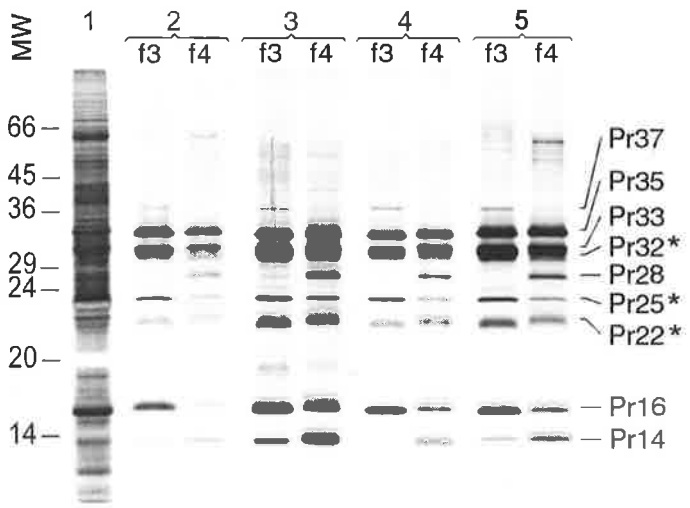


Figure 7-2: Comparison of SAX-unbound proteins from Atlas, inoculated Atlas, Atlas 46 and inoculated Atlas 46

SDS PAGE profiles of the SAX-unbound proteins from Atlas (lane **2**), inoculated Atlas (lane **3**), Atlas 46 (lane **4**), inoculated Atlas 46 (lane **5**) are displayed. Both SAX fractions 3 (**f3**) and 4 (**f4**), containing most of the unbound proteins, are shown. For comparison, a typical IWF profile from inoculated Atlas 46 is shown in lane **1**. Molecular weights (kDa) are shown on the left. Proteins, identified by molecular weight, are displayed on the right. Stars show proteins that were purified in Chapter 5.

Pr 28, Pr 33, Pr35 and Pr37 following their apparent molecular weight.

Almost all the visualised protein bands were more pronounced in the samples from inoculated plants compared to the uninoculated plants. It also appeared that the susceptible cultivar Atlas, with the lowest levels of most proteins preinoculation, had the largest protein increase following inoculation. Previously, a comparison of IWF profiles points to a number of putative PR proteins that increased in response to inoculation (see Figure 7.1). Figure 7.2 indicates that most of these proteins did not bind to the SAX column and, therefore, did not have a significant negative net charge at pH 9.0. In this context, analysis of the SAX-bound proteins indicated that some of these proteins also markedly increased in response to inoculation (data not shown). However, thses proteins were not studid any further in this project.

7.3.2.2.2 WCX antifungal fractions

WCX chromatography further resolved the SAX-unbound proteins and hence sprcad them into a number of fractions. This greatly enhanced the identification of the individual proteins when analysed by SDS PAGE.

One microliter of WCX fractions 19 to 30 from Atlas, inoculated Atlas, Atlas 46 and inoculated Atlas 46 were applied to SDS PAGE and the protein bands were visualized by silver staining. Results are shown in Figure 7-3. Again, all the bands, including proteins with or without antifungal activity, were more pronounced in the inoculated plants. Despite the higher resolution obtained with WCX chromatography, Pr32 was not separated from a protein band (Pr33) above it and Pr22-2 and Pr22-3 were not separated from each other.

The lowest levels of proteins were detected in uninoculated (Fig 6-3A), with many under the detection limit. Inoculated Atlas (Fig 6-3B), on the other hand, had the highest levels of proteins. Uninoculated Atlas 46 (Fig 6-3C) appeared to contain intermediate levels of the proteins, with a moderate increase recorded for inoculated Atlas 46 (Fig 6-3D). Except for uninoculated Atlas, all treatments appeared to contain the same proteins, although at different amounts. One exception was a ~28 kDa protein

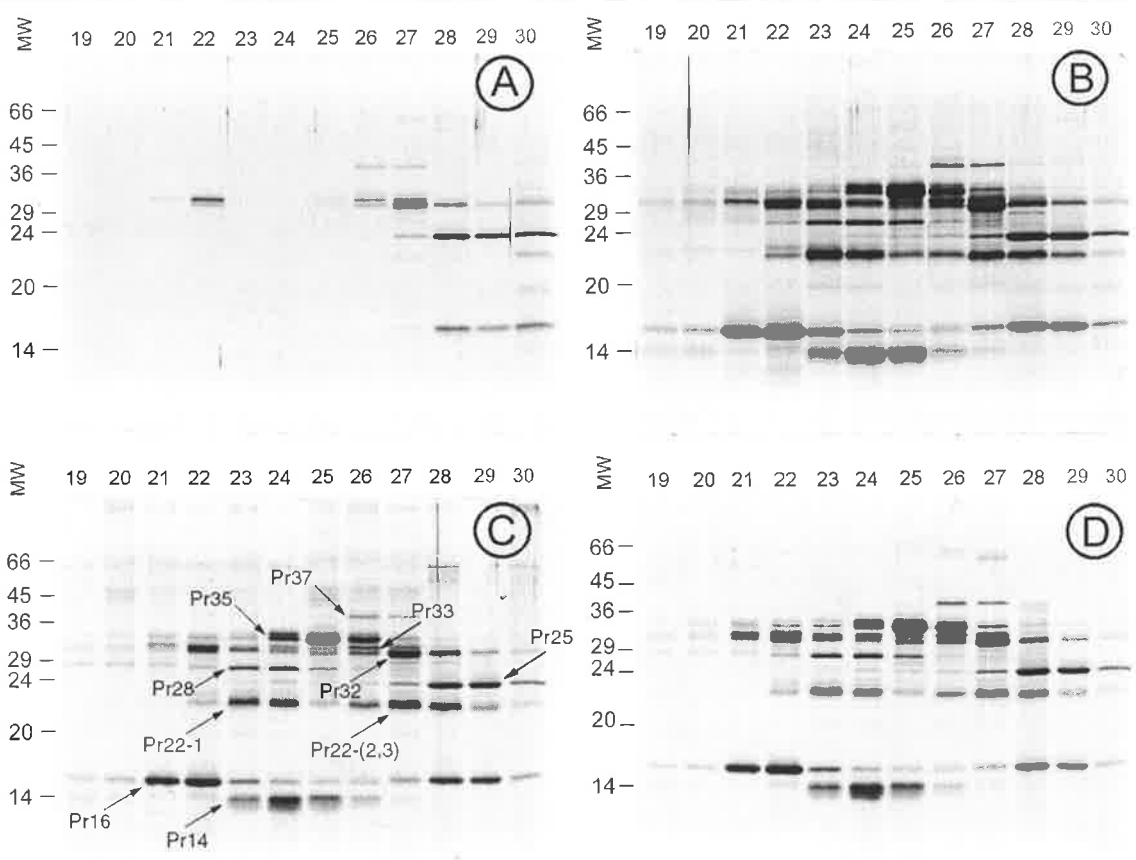


Figure 7-3: Comparison of WCX fractions containing antifungal activity
SDS PAGE comparative profiles of WCX active fractions are shown. Molecular weights (kDa) are on the left side and fraction numbers are shown on top of each gel. Proteins, identified by their molecular weight, are shown on gel C.
A: Atlas, **B**: inoculated Atlas, **C**: Atlas 46, **D**: inoculated Atlas 46.

(Pr28) that was sometimes detected in inoculated plants. In Figure 7.3, it was apparent that the intensity of bands in Pr16, Pr22 and Pr33 varied across the fractions with intensely stained bands separated by less intense bands. This suggests that the proteins were eluted in two separate peaks from the WCX column and that there may be more than one isoform. The presence of different isoforms has already been demonstrated for Pr22-1, 2, 3. In this study, the hypothesised isoforms of Pr16 and Pr33 were not differentiated. In addition, close examination shows that both Pr33 and Pr35 consist of two bands.

7.3.2.3 Quantitative measurement

Following RP chromatography, the peak area associated with each of the five purified proteins was caluclated and translated into micrograms of protein by comparison to the peak area produced from 5 μg ovalbumin. The results are presented in Table 7-2. It was concluded that:

- In the susceptible cultivar Atlas, the proteins were 6.5 times (for Pr32) to 16 times (for Pr22-3) more abundant in the inoculated plants compared to the control. Neither Pr22-1 nor Pr22-2 were detected in uninoculated Atlas.
- In the resistant cultivar Atlas 46, a higher amount of protein was observed in the inoculated plants compared the uninoculated, although the differences were not as pronounced as for Atlas.

protein	Atlas	inoculated Atlas	Atlas 46	inoculated Atlas 46
Pr22-1	ND	368 (8.1)	155 (3.4)	241 (5.3)
Pr22-2	ND	132 (2.9)	73 (1.6)	82 (1.8)
Pr22-3	35 (0.8)	564 (12.4)	177 (3.9)	255 (5.6)
total Pr22- (1,2,3)	35 (0.8)	1064 (23.4)	405 (8.9)	578 (12.7)
Pr25	94 (2.5)	1398 (37.2)	425 (11.3)	545 (14.5)
Pr32	163 (5.2)	1053 (33.7)	350 (11.2)	500 (16.0)

Table 7-2: Comparative levels of cell wall lytic enzymes found in barley IWF
Amounts are shown as pico mol and (microgram). IWF was extracted from 34 grams of leaves (~130 plants). Data are average of two measurements. ND: not detected.

- For the uninoculated plants, Atlas 46 had a markedly higher level of all the proteins. This ranged from 2.1 times (for Pr32) to 5 times (for Pr22-3) more than that detected in uninoculated Atlas. Pr22-1 and Pr22-2 were found only in Atlas 46.

- For the inoculated plants, the situation was opposite to the above, although less pronounced. The susceptible cultivar Atlas had 1.5 times (for Pr22-1) to 2.5 times (for Pr25) higher levels of the proteins compared to Atlas 46.

- In general, the amount of measured proteins in Atlas and inoculated Atlas were at the lowest and highest end of the spectrum, respectively. Atlas 46 and inoculated Atlas 46 were in the middle of this range.

- Within each plant, the detected amounts of the three TL-proteins could be arranged as Pr22-3>Pr22-1>Pr22-2.

It is also curious to note that within each plant, except for Atlas, comparable amounts (±30% pico mol) of Pr32, Pr25 and total Pr22-(1,2,3) were detected.

7.3.3 Other PR proteins in barley IWF

In addition to the five cell wall lytic proteins, a number of proteins, previously determined to have no detectable antifungal activity, appeared to increase in response to inoculation. Since this characteristic qualifies them as PRPs, some of these induced proteins were investigated further.

7.3.3.1 Pr19, an Hv-1 homologous protein

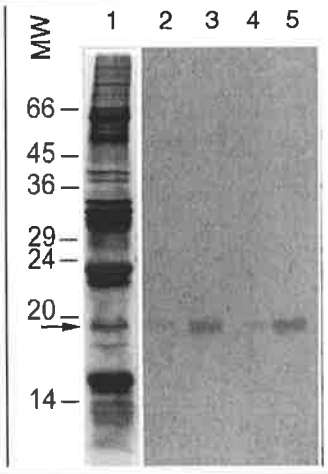
One of the putative PR proteins detected in preliminary comparison of the IWFs (Figure 7-1) was a ~19 kDa protein named Pr19. This has the same molecular weight as the barley acidic TL protein, Hv-1, that was previously reported to increase in response to *R. secalis* infection (Hahn *et al.*, 1993). The occurrence of Pr19 and its relationship to Hv-1 was studied further.

Ten microliters of IWF from Atlas, inoculated Atlas, Atlas 46 and inoculated Atlas 46 were collected 3 days postinoculation and analysed with SDS PAGE. However, after this experiment was repeated 15 times, only on 8 occasions was Pr19 detected in both cultivars following inoculation. On one occasion Pr19 was only detected in Atlas 46 and on 6 occasions Pr19 was not detectable in any treatment.

The relationship between Pr19 and Hv-1 was investigated by western blot analysis using an Hv-1-specific antibody kindly donated by Dr. Bryngelsson (Bryngelsson and Green, 1989). Twenty microliters of IWF from Atlas, inoculated Atlas, Atlas 46 and inoculated Atlas 46 were run on SDS PAGE, blotted onto nitrocellulose or PVDF membrane and probed with the Hv-1 antibody. The analysis was carried out 3 times and a typical result is displayed in Figure 7-4, lanes 2 to 5. A positive reaction for a 19 kDa protein occurred for both Atlas and Atlas 46, indicating that Pr19 is related to Hv-1. No obvious cross-reaction between the HV-1 antibody and other TL proteins, Pr22-(1,2,3), was detectable. The un-inoculated plants, Atlas and Atlas 46, consistently gave a low signal. In two experiments the inoculated plants had a markedly stronger signal and in one experiment there was no difference between inoculated and uninoculated plants. No obvious difference between Atlas and Atlas 46 was detectable in any of the experiments.

In this context, a ~19-kDa protein band previously seen in Figures 7-2-3 and 7-3-B is generated from SAX-unbound fractions and thus likely to be a basic protein. The relationship between the acidic Hv-1 and Pr19 was only investigated in un-fractionated IWF and, thus, it is not known if the 19 kDa protein in Figures 7-2-3 and 7-3-B is also related to Hv-1 and Pr19.

Figure 7-4: Western blot analysis of Pr19
A typical SDS PAGE profile of IWF containing Pr19 is shown in lane 1. The arrow points to the Pr19 band. The corresponding western blot of IWF from Atlas, inoculated Atlas, Atlas 46 and inoculated Atlas 46, respectively is shown in lanes 2, 3, 4 and 5.



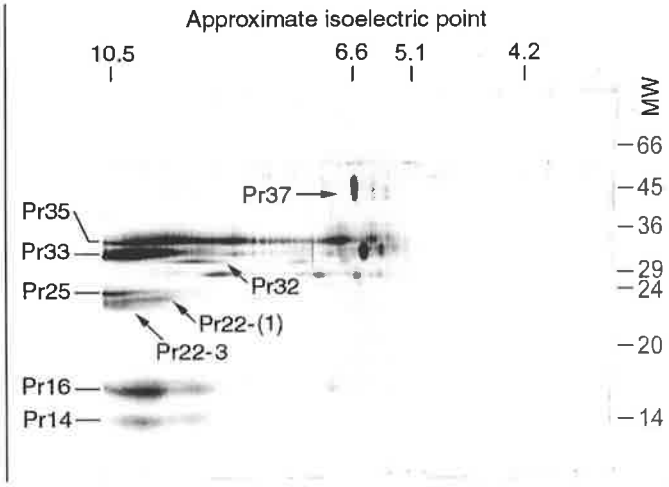
7.3.3.2 PR proteins in SAX-unbound fractions

Comparison of SAX-unbound proteins from inoculated and uninoculated plants indicated that all these proteins increased upon inoculation (see Figure 7-2 and 7-3). An amount, equivalent to 350 µl of pooled SAX fractions 3 and 4 from inoculated Atlas 46 IWF, was analysed by 2-D PAGE. Figure 7-5 shows that most of the proteins were extremely basic, which was expected since they did not bind to the negatively charged SAX column at pH 9. However, less basic proteins, positioned closer to the middle of the gel, were unexpected.

It is apparent from the 2-D gel that Pr14, Pr16, Pr33 and Pr35 contain more than one spot, although they are not individually labeled. Pr22-3 and Pr22-1 were identified based on their relative migration in SDS PAGE (see Figure 6-7). Pr22-2 migrates at the same level as Pr22-1 but because it is much less abundant than Pr22-1 and Pr22-3 it was not detectable. Pr22-(1,3) and Pr37 migrated more slowly in the second dimension than expected (see Figure 7-2 and 7-3) but this might be caused by the presence of urea in the first dimension of the 2-D gel.

Figure 7-5 also shows names of the proteins separated by 2-D PAGE. In Table 7-3 molecular weights and isoelectric points for some of these proteins are compared with those previously reported for known PR proteins. As expected, alignment was observed for the cell wall lytic proteins studies here. Moreover, Pr14 and Pr16 were found to have masses and iseolectric points close to those reported for PRP-4 and PRP-1 in barley (Hejgaard *et al.*, 1992; Svensson *et al.*, 1992; Bryngelsson *et al.*, 1994). Another

Figure 7-5: 2-D PAGE of SAX-unbound proteins
Proteins are identified according to their molecular weight and shown to the left and on the gel.



protein suspected of being related to a previously described PR protein was Pr33. This protein was found to have a high absorbance at 403 ηm (Figure 7-6), a characteristic of heme-containing peroxidases (PRP-9) (Saunders and Stark, 1964).

Pr14, Pr16 and Pr33 were purified from Atlas 46 IWF by SAX, WCX, HI and RP chromatography (see Table 7-4). As shown in Figure 7-7, Pr14 and Pr16 contained single protein bands with no detectable contaminating protein whereas Pr33 appeared as a double band. This is consistent with Pr33 being a peroxidase as many previously

barley protein	MW (kDa)	pI	reference
PRP-1	15, 15.2[1]	10.5	Bryngelsson *et al.*, 1994
Pr16	~16	~10	this work
PRP-4 (barwin)	13.7[1]	>10.0	Svensson *et al.*, 1992
Pr14	~14	~10	this work
PRP-2: GII	32.3[1]	9.5[1]	Xu *et al.*, 1992
Pr32	~32	~9	this work
PRP-3: chitinase 2	24.8[1]	≥9.8	Kragh *et al.*, 1990
Pr25	~24	~10.5	this work
PRP-5 (protein R and S)	~23	9-10	Hejgaard *et al.*, 1991
Pr22-1	~22	~10	this work
Pr22-2	~22	?	this work
Pr22-3	~22	~10.5	this work

Table 7-3: comparison of MW and pI of some detected proteins with PR proteins
Data in white rows are quoted from references cited in the right column. Data in the gray rows are based on 2-D and SDS PAGEs in this thesis. For Pr14 and Pr16 only the largest spots were considered for estimation of pI.
[1]: These values were calculated based on deduced amino acid sequences.

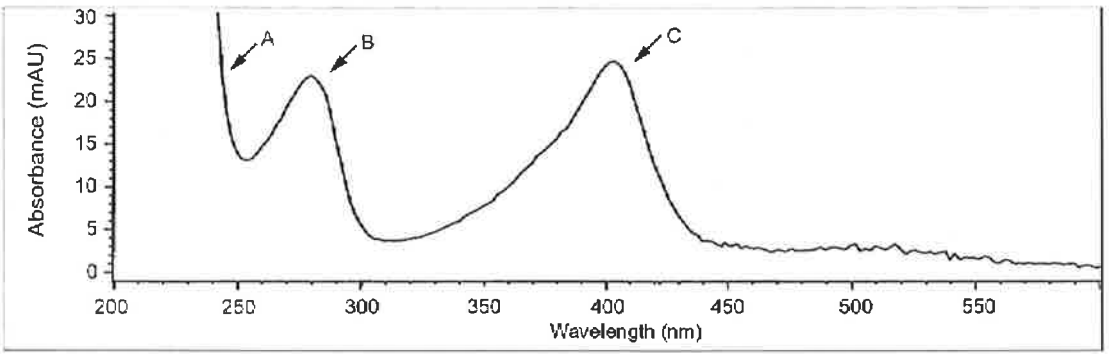


Figure 7-6: Pr33 spectrum
A: Absorbance above 250ηm was common to all the IWF compounds.
B: Absorbance peak at 280ηm, a common characteristic of proteins and other compounds with aromatic structures.
C: Absorbance peak at 403ηm. Amongst SAX-unbound proteins only Pr33 had this peak.

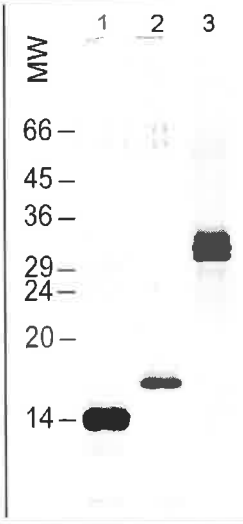
purified barley peroxidases have been shown to have glycosylated and non-glycosylated forms that migrate as double bands (Kerby and Somerville, 1992; Kristensen *et al.*, 1999). Approximately 100 pM of the proteins were individually subjected to Edman N-terminal sequencing. However, all the proteins appeared to be blocked at the N terminal and hence, no sequence was obtained. To circumvent this, 50 μg of Pr14 was dried and treated by the reductive alkylation and tryptic digestion procedures described in Chapter 2. The digested protein was subjected to an 80-min RP chromatography run (method 22PEPM2, outlined in Section 2.1.3.6.2). A symmetrical peak, eluted at 43.7 min, was collected and sequenced. Nineteen residues were identified (Figure 7-8). These were found to have a 100% identity to the deduced amino acid sequence of barley PRP-4 (Gregersen *et al.*, 1997). This, together with the pI and molecular weight data, indicates that Pr14 is the same protein previously identified as PRP-4 (barwin).

Due to time constraints of this project, it was not possible to conduct additional

protein	collected fractions			
	SAX	WCX	HIC	RPC
Pr14	3-4	24-25	18-21	17
Pr16	3-4	21-23	11-13	15
Pr33	3-4	27-28	14-15	22

Table 7-4: Summary of denaturing purification of Pr14, Pr16 and Pr33
Chromatographic methods were the same as those used in Chapter 5. RP chromatography was carried out according to the method RPC-2 (for details see Section 5.2.3.6.1). As before, 1 fraction/min was collected (fraction #1 at 0-1 min, fraction #2 at 1-2 min, etc.).

Figure 7-7: SDS PAGE profiles of Pr14, Pr16 and Pr33
Purified Pr14, Pr16 and Pr33 are shown in lanes 1, 2 and 3 respectively. Pr33, in lane 3, was consistently detected as a double band. However, this protein was believed to be a peroxidase.



experiments to further characterize Pr16 and Pr33.

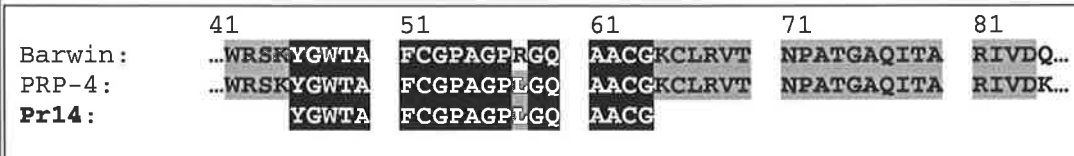


Figure 7-8: Internal micro sequencing of Pr14
Nineteen residues of Pr14 were sequenced and found to be 100% identical to the deduced amino acid sequence of PRP-4 (NCBI accession number T06169). Being cut by trypsin, residue 45 of Pr14 should be arginine (R) or lysine (K). Black boxes show 100% identity between the proteins whereas gray boxes denote dominant but less than 100% identity. Numbers on the top refers to mature protein sequences.

7.4 Discussion

7.4.1 Protein detection and identification

A range of detection and visualisation techniques were used in this chapter to identify and quantify PR proteins. These included SDS PAGE, 2-D PAGE, western blot analysis, HPLC and Spectrophotometry. SDS PAGE and western blot analysis have been used frequently by other researchers to detect and quantify PR proteins (Scott-Craig *et al.*, 1995; Cheong *et al.*, 1996; Rauscher *et al.*, 1999). However, only rarely have HPLC and spectrophotometry have been used to quantify PRP levels in plants.

The identification of Pr14, Pr22-(1,2,3), Pr25 and Pr32 as PRP-4, PRP-5 isoforms S and R, chitinase 2 and β(1-3) glucanase isoform GII, respectively, were based on HPLC and protein sequencing whereas Pr19 was shown to be related to PRP-5 isoform Hv-1 by western blot analysis. While the identification techniques used for these proteins are specific and reliable, other less specific techniques were also used in this chapter to help identify other induced proteins found in barley IWF.

Spectrophotometry was used to determine that Pr33 has an absorbance peak at 403 ηm. Absorbance at approximately 400 ηm is a characteristic of heme-containing peroxidases (Saunders and Stark, 1964). In addition, Pr33 was consistently detected as a double band. This is similar to many previously characterised barley peroxidases that have coexisting glycosylated and non-glycosylated forms (Kerby and Somerville, 1992; Kristensen *et al.*, 1999). However, neither of these serve as conclusive evidence that Pr33 is a peroxidase. Pr33 has a molecular weight of ~33 kDa and pI of ~10. A previously characterised extracellular barley pathogenesis-related peroxidase, P8.5, is 35-34 kDa in size with a pI of 8.5 (Scott-Craig *et al.*, 1995). Although the difference between the two molecular weights are within the 10% expected accuracy of SDS PAGE analysis, the difference in the pI of the proteins indicates that they are not the same. If Pr33 is a peroxidase, it is most likely similar to the less-studied peroxidase P9.6 (Kristensen *et al.*, 1999) with a pI value of 9.6. With a pI of 10 for Pr33, P9.6 has the closest pI of the reported barley extracellular peroxidases (Kerby and Somerville,

1992; Kristensen *et al.*, 1999).

Another protein, Pr16, had an apparent molecular weight of 16 kDa and a pI of ~10. Considering the expected error in estimation of these values by 2-D and SDS PAGE, it is reasonable to assume both of these values match those of PRP-1 in barley (MW~15, pI~10.5). This and the fact that Pr16 is also an extracellular PR protein, like PRP-1, strongly suggest that these two proteins are the same.

No N-terminal sequence was obtained for Pr33 and Pr16. Further studies should focus on identification of these proteins by internal protein sequencing or western blot analysis.

7.4.2 Concomitant Induction of PR proteins

Coordinated multi-component gene activation has been shown in almost all plants attacked by pathogens including barley (Boyed *et al.*, 1994a and 1994b; Tamas and Fric, 1995; Stevens *et al.*, 1996) and tobacco (Ye *et al.*, 1989; Ward *et al.*, 1990). In particular, chitinase and β(1-3) glucanase have been shown to always be coordinately induced as a consequence of pathogen attack or related stimulation (Sahai and Monocha, 1993). Comparative SDS-PAGE and 2-D PAGE of IWF samples and sub-fractions indicated that most, if not all, of the proteins increased 3 days after inoculation. The increase was consistently more pronounced in the susceptible plant Atlas.

The SAX-unbound fractions played a pivotal role in this study because they were found to contain a number of proteins that all markedly increased in response to inoculation. Many of these proteins, including Pr14, Pr19, Pr22-(1,2,3), Pr25 and Pr32, were shown to be previously identified PRPs. Others such as Pr16 and Pr33 were induced, but their relationship to known PRPs was less clear. Lack of absorbance to the SAX column by these proteins can be explained by their extremely basic pI. However, basic proteins were not the only PR proteins detected in barley IWF. Some of the SAX-bound proteins were also found to markedly increase although these proteins were not studied further. In addition, Pr19 was shown by western blot analysis to be related or equivalent to Hv-1, and therefore an extracellular acidic PR protein. Detection of a

number of basic proteins, as well as some acidic proteins, in IWF rejects the primary notion that the pI of PR proteins defines their subcellular localisation (Bowles 1990; Ward *et al.*, 1991). This report also provides the first indication on extracellular localisation of Hv-1.

The SAX-unbound fractions also appear to provide a practical means to isolate many of the more basic PR proteins from IWF in plants such as barley. For plants with a large number of acidic PR proteins, such as tobacco, this is not an efficient technique to capture the bulk of these proteins.

Most studies on barley PR proteins have focused on plant responses to powdery mildew infection by *Erysiphe graminis* f.sp. *hordei*. This pathogen has been shown to induce PRP-1 (Muradov et *al.*, 1993), PRP-2 isoform GII (Xu *et al.*, 1992), PRP-3 isoform chitinase 2 (Kragh *et al.*, 1993), PRP-4 (Hejgaard *et al.*, 1992), PRP-5 isoform Hv-1 (Bryngelsson and Green, 1989) and PRP-9 isoforms P5.2 and P8.5 (Kerby and Somerville, 1989). The causal agent for scald, *R. secalis*, has also been shown to induce PRP-2 isoforms GI, GII and GIII (Roulin *et al.*, 1997), PRP-5 isoform Hv-1 and an unidentified peroxidase isoform (Hahn *et al.*, 1993). Induction of these isoforms was also detected in the present study although induction of Hv-1 (Pr19) was not consistent. Another protein with similar inconsistent induction in inoculated plants was Pr28. This later protein was not characterised further.

Apart from Pr32 (PRP-2) and Pr19 (Hv-1), the other proteins, described in this thesis, had not been previously reported to increase in response to *R. secalis* infection of barley. These include Pr14 (PRP-4), Pr22-1 (homologue of protein R), Pr22-2 (protein R), Pr22-3 (protein S) and Pr25 (PRP-3). Other, less-studied, proteins shown to increase in this pathosystem include Pr16 (probably PRP-1), Pr33 (probably PRP-9), Pr35 and Pr37.

Previously, only the elicitor $AgNO_3$ had been shown to induce barley protein R (Grenier *et al.*, 1999) whereas induction of protein S has not been shown previously.

7.4.3 Differential induction of PR proteins

It has been suggested that plant antifungal proteins released into the extracellular

space constitute a barley defense mechanism against *R. secalis* (Hahn *et al.*, 1993; Roulin *et al.*, 1997). This probably implies that resistant plants should have higher levels or earlier induction of these proteins. Hahn and coworkers (1993) studied the induction of mRNA for two PR proteins, Hv-1 and a peroxidase, in a number of barley cultivars including Atlas and Atlas 46. The genes were found to be induced in resistant plants within 12 to 48 hours after inoculation, whereas this did not occur in the susceptible plants before 72 hours. In a similar study, Roulin and co-workers (1997) analysed the induction of PRP-2 isoforms in the susceptible barley cultivar Clipper and three near-isogenic resistant lines inoculated with *R. secalis*. Isoforms GI, GII and to some extent GIII were found to increase. In addition, the PR proteins were induced markedly faster in one of the resistant lines. The authors concluded that β(1-3) glucanase is involved in at least one of the physiologically distinct modes of barley resistance against *R. secalis*. However, the significance of the findings in this experiment is difficult to evaluate because the induction dynamics of two other resistant cultivars were comparable to the susceptible cultivar Clipper.

In the present study uninoculated Atlas was found to have the lowest levels of all the proteins studied, whereas inoculated Atlas had the highest. The resistant cultivar Atlas 46 was found to have an intermediate level prior to inoculation and only a modest increase in the amount of proteins was observed following inoculation. This may seem in sharp contrast to the findings of Hahn and coworkers (1993) who detected greater induction of mRNA in the resistant cultivar. However, induction of mRNA does not always correspond with expression of PR proteins (reviewed by Vidhyasekaran, 1997). Bryngelsson and coworkers (1994) showed that although PRP-1 genes were translated earlier in resistant barley inoculated with powdery mildew, the level of protein was actually higher in the susceptible plant at the same time. Postranslational regulation of PR proteins was also shown for PRP-5 in tobacco (LaRosa *et al.*, 1992). In fact, induction of mRNA alone is believed not to be enough to qualify a protein as pathogenesis-related (Vidhyasekaran, 1997; Van Loon, 1999). On the contrary, detection of increased levels of a protein in response to pathological induction is considered necessary and sufficient for this.

The marked increase of PR protein levels following inoculation of the susceptible plant Atlas, is consistent with the notion that heightened PRP levels is a general stress response (Sahai and Monocha, 1993). However, the resistant plant Atlas 46 had

markedly higher protein levels prior to inoculation than did Atlas. Some proteins, including Pr22-1 and Pr22-2, which displayed strong antifungal activity against the pathogen, were not detected by silver staining in uninoculated Atlas. The pre-existing presence of PR proteins is possibly more advantageous to Atlas 46 in evading pathogens than the fast induction of these proteins. Accordingly, successful infections occur more frequently in the susceptible plant Atlas compared to Atlas 46. In time, this translates to a higher colonisation level in Atlas. It is this stressful condition that most likely leads to a large induction of PR proteins by this cultivar.

Further elaboration on the induction of PR proteins in this pathosystem, especially in relation to previous findings in this thesis, is presented in Chapter 8.

Chapter 8:

General discussion

8.1 Physicochemical and biological characteristics of the thaumatin-like and other purified proteins

8.1.1 Oxidation versus reduction

Oxidation was one of the mechanisms by which the cell wall lytic proteins, purified in Chapter 5, lost activity. *In vitro* loss of activity following exposure to atmospheric oxygen is generally caused by oxidation of thiol-containing residues, especially cysteine (Edelstein and Bollag, 1992). Cysteine residues may participate in disulfide (S-S) bonds that help form the tertiary and quaternary structures of proteins (Rosenberg, 1996). This structural role of cysteine is common in extracellular proteins but is less prevalent inside the cells (Carlsson *et al.,* 1998). Proteins may also contain cysteine residues that do not participate in disulfide bridges and thereby retain their free thiol groups. This form of cysteine is sensitive to oxidation and usually does not occur in proteins found in the oxygen-rich extracellular space unless it serves a special function (Carlsson *et al.,* 1998). This is exemplified by enzymes that contain free sulphydryl groups in their active site, which is naturally very reactive. These sulphydryl groups are particularly sensitive to oxidation, that may lead to protein deactivation (Harris 1989b; Carlsson *et al.,* 1998). Depending on the conditions, two oxidized cysteine residues

may react with each other to form a disulfide bridge. This could be intramolecularly that affects the protein conformation or intermolecularly that leads to formation of protein polymers.

Reducing agents such as DTT and β-mercaptoethanol, applied at millimolar concentrations, reduce the oxidised thiol groups or protect them from oxidation (Edelstein and Bollag, 1992). Subsequently, disulfide groups will be reduced to, or kept as, free sulphydryl groups. Cleavage of a S-S bond that participates in the protein tertiary structure leads to destabilisation of the structure and likely loss of activity whereas cleavage of a S-S bonds, formed by oxidation, may reactivate a protein.

Thaumatin and many TL proteins have 16 cysteine residues believed to be arranged in 8 disulfide bonds (Van der Wel and Bel, 1980; Hejgaard *at al.*, 1991; Cutt and Klessig 1992; Grenier *at al.*, 1999). Chitinase 2 has five cysteine residues whereas β(1-3) glucanase isoform GII and most other β(1-3) glucanases of barley have only one cysteine residue. The odd number of cysteine residues in chitinase and β(1-3) glucanase implies that at least one of the residues cannot be involved in S-S bonds. Provided this residue is exposed to the environment, it may be readily oxidised resulting in loss of activity. Loss of activity reported previously for purified barley β(1-3) glucanases has been rectified by adding 160 μg/ml BSA (Hrmova and Fincher, 1993). However, it is not clear whether this was due to protection of the proteins from oxidation or from some other phenomena such as surface effects (Edelstein and Bollag, 1992).

In Chapter 5, lost activity of TL proteins, a chitinase and a β(1-3) glucanase was restored by addition of 1-3 mM DTT, an indication that these proteins may undergo oxidation of thiol groups. In a preliminary experiment in Chapter 3, addition of 1 mM DTT did not change the antifungal activity of IWF, suggesting that the active proteins were already in a non-oxidised form. This is probably achieved by intrinsic anti-oxidation factors present in the IWF (see below). Addition of 10 to 100 mM DTT to IWF caused a decrease in FW and DB formation. However, this high level of DTT is not only capable of restoring oxidised thiol but probably also cleaves some of the structural S-S bonds present in chitin and TL proteins. β(1-3) glucanase, of course, should be insensitive to excessive reduction because it only has one cysteine and thus no intermolecular S-S bonds. Later in Chapter 6, it was explained that cleavage of S-S bound, by 50 mM DTT, and unfolding of chitinase and particularly cysteine-rich TL

proteins was also involved in reduced mobility of these proteins in SDS PAGE.

8.1.2 Active and inactive TL proteins

Thaumatin, extracted from the fruit of *Thaumatococcus danielii* Benth, is normally a sweet-tasting protein with no known enzymatic activity although it may have a slight antifungal activity (Vigers *et al.*, 1991). Van der Wel and Bel (1980) found that the addition of 1.25 mM DTT renders thaumatin with strong proteolytic and autodigestion activities. The so-called native thaumatin has 8 disulfide bridges and no free sulfhydryl group, whereas in the activated form, one of the disulfide bonds is cleaved. The strong autodigestion of thaumatin, occurring optimally at pH ~8.0, may completely digest the protein in 3 hours (Van der Wel and Bel, 1980). In a process similar to activation of thaumatin, DTT restored the activity of TL proteins in the present study. This may be readily explained only if the proteins had one or more oxidation-sensitive free thiol groups. In addition, all the activated TL proteins, but not β(1-3) glucanase or chitinase, were found to rapidly degrade over time (see Figure 5-1, lanes 3 to 5). Not surprisingly, storage at -20°C stopped or markedly decreased this process.

Based on the available information it is suggested that:

- The active form of TL proteins, or one of the active forms, is the reduced form previously shown in thaumatin. Extensive homology and unusually highly conserved cysteine residues amongst the TL proteins strongly suggest an almost identical folding pattern for these proteins (Cutt and Klessig 1992; Svendsen, 1996; Skolnick and Fetrow, 2000). It is likely that cleavage of one disulfide bond is the universal mechanism for activation of the TL proteins. The resulting unbound thiol groups are probably involved in the protein active sites.

- *in vitro*, the reduced state is maintained by the agents released into the extracellular space by plant cells or, more intriguingly, by the fungus. Presence of various reducing agents in the apoplast of both fungi (Sollod *et al.*, 1992; Rebbeor *et al.*, 1998) and plants (Polle *et al.*, 1990; Flury *et al.*, 1996) including barley (Vallelian-Bindschedler *et al.*, 1998; Vanacker *et al.*, 1998) have been previously shown. One reducing agent particularly well studied and found in

many living organisms is the cysteine-containing tripeptide, glutathione, that is present at millimolar levels (Creighton, 1990; Carlsson *et al.,* 1998).

- TL-proteins are multifunctional proteins with β(1-3) glucanase and proteolytic (and autolytic) activities. Nevertheless, it is known that a single enzyme may act on different substrates in different environmental conditions such as pH and ionic strength (Mayer *et al.*, 1996; Osswald *et al.*, 1994). The multifunctional feature of TL proteins, therefore, needs to be further characterised.

Many authors have previously purified TL proteins (Hejgaard *et al.*, 1991; Vigers *et al.*, 1991; Roberts and Selitrennikoff 1990; Cheong *et al.*, 1996; Trudel and *et al.*, 1998a and 1998b). Since these proteins had been subjected to the conditions also used in this thesis it is likely that they were oxidised. This may explain why these proteins had no *in vitro* enzymatic activity (Hejgaard *et al.*, 1991; Roberts and Selitrennikoff 1990). The same proteins were found to be highly active on cell wall or membrane when applied to actively growing fungi (Hejgaard *et al.*, 1991; Vigers *et al.*, 1991; Roberts and Selitrennikoff 1990; Cheong *et al.*, 1996). One possibility is that the proteins were activated by the reducing condition prevailing in the apoplast of growing fungi where TL proteins lyse the fungi and also autolyse. As mentioned above, the reducing environment in the apoplast of fungi has been shown.

In the work of Trudel and coworkers (1998a and 1998b) where very few purification steps were taken and the oxidising condition in polyacrylamide gels was avoided by replacing persulfate with riboflavin, TL proteins may have not been oxidised at any stage. TL proteins purified by this method lysed fungal cell walls and the β(1,3) glucan pachyman in an in-gel purification-assay that this group developed. No reduction was used to activate these proteins. On the contrary, these authors reported that TL proteins reduced by 2% β-mercaptoethanol had no activity following separation by SDS PAGE. However, this amount of the reducing agent (~ 240 mM) probably unfolded the proteins in the presence of SDS.

8.1.3 Other deactivating factors

In Chapter 5, high salt concentration was a factor that led to loss of activity by the

proteins. Inactivation of TL proteins by salts has been previously reported (Roberts and Selitrennikoff, 1990; Cheong *et al.*, 1996). A model proposed by Roberts and Selitrennikoff (1990) suggested that TL proteins exert their antifungal activity by affecting the cell membrane and that salts suppress the antifungal activity by relieving the osmotic pressure that otherwise bursts the fungal cells. In the current study TL proteins were found to cause cell wall degradation, a phenomenon also caused by chitinase and β(1-3) glucanase. Salts were found to inhibit all these proteins, although the inhibition rate might not be the same for all the proteins. It is suggested that salts render the substrate or the enzymes unfit for the interaction. The influence of certain ions on the structure of proteins has been shown previously (Roe, 1989). In addition some enzymes were shown to lose, or gain, the ability to act on certain substrates at different ionic strengths or pH (Osswald *et al.*, 1994; Mayer *et al.*, 1996).

8.2 PR proteins and plant defense responses

8.2.1 The role of PR proteins

Pathogen ingress is nearly always associated with induction of PR proteins in plants (Cutt and Klessig 1992). Moreover, PRP levels are frequently higher in incompatible interactions compared to the compatible ones (Van Loon, 1999). Induced resistance is also found to be closely associated with heightened levels of PRPs in many plant/pathogen systems (Dixon and Harrison, 1990; Ward *et al.*, 1991; Strange, 1993). Several PR proteins show *in vitro* anti-microbial activity. Notably some PRPs block the penetration (Bowles, 1990), deactivate protease (Heitz *et al.*, 1999), digest cell walls (Boller, 1987; Trudel *et al.*, 1998a), digest proteins (Vidhyasekaran, 1997), interfere with reproduction (Cutt and Klessig 1992), poison the ribosome (Jensen *et al.*, 1999) or arrest pathogenic differentiation (Rauscher *et al.*, 1999) of pathogens. Furthermore,

many plants engineered to express some of these proteins had heightened resistance to pathogens (Collinge *et al.*, 1993; Datta *et al.*, 1999).

However, there are some unexplained observations that give serious doubt as to the role of PRPs in disease resistance.

- Firstly, there are a number of examples in which PR proteins were found to be induced equally or even more in the compatible interactions, compared to the incompatible interactions. Roulin and coworkers (1997) compared the levels of β(1,3) glucanase isoforms following compatible and incompatible interactions of barley with *R. secalis*. They found that although one of the resistant cultivars had a higher and faster induction of the PR proteins, another resistant cultivar had a similar induction of PR protein as the susceptible cultivar (Roulin *et al.*, 1997). Since in the study by Roulin and coworkers only PR-2 proteins were investigated, it was conceivable that some other PR proteins might have induced more or with faster kinetics in the resistant cultivar in question. Nevertheless, Gregersen and coworkers (1997) who studied the induction of 15 genes associated with different PR proteins in the barley/*Erysiphe graminis* pathosystem, also found that PR proteins may be similarly induced in both resistant and susceptible cultivars (Gregersen *et al.*, 1997). Furthermore, analysis of barley leaf proteins 4-5 days after inoculation with *Drechslera teres* indicated a more pronounced increase of many PR proteins in the compatible interaction compared to the incompatible one (Reiss and Bryngelsson 1996). Similarly, inoculation of barley cultivars with powdery mildew resulted in stronger induction of chitinase (Kragh *et al.*, 1990) and PR-1 proteins (Bryngelsson *et al.*, 1994) in susceptible cultivars compared to the resistant plants. Analysis of defense responses in other plants have also indicated that the level of PR proteins may increase at a similar rate or even more so in compatible interactions compared to incompatible ones (Cutt and Klessig 1992).

- Secondly, in a number of pathosystems despite strong induction of PR proteins there is no apparent effect on the pathogen. β(1,3) glucanase, for instance, has no detectable role in defense against viruses. However, viral infections, especially when accompanied by HR, strongly induce plant β(1,3) glucanases (Boller, 1987). Another example of this condition is found amongst transgenic plants. A

number of these plants have been found to contain a heightened levels of PR proteins but no increase in resistance when compared with control plants (Collinge *et al.*, 1993; Datta *et al.*, 1999).

All these observations have generated a lack of complete correlation between induced resistance and expression/characteristics of PR proteins. Two major explanations have been offered:

- Accumulation of PR proteins may be a result of a resistance reaction but it is also a general response to stress caused by pathogen ingress, toxins and plant- or pathogen-derived elicitors (Cutt and Klessig 1992; Reiss and Bryngelsson 1996; Gregersen *et al.*, 1997). Accordingly, induced resistance or perceived stress, through specific or non-specific pathways respectively, may determine the level of induction for PR proteins.

- Known PR proteins may not directly or solely determine disease specificity (Boller, 1987). This implies that other, yet unknown, plant factors play a more central role in disease resistance. Alternatively, the pathogen virulence factors, such as inhibitors of PR proteins (Albersheim and Valent, 1974; Ham *et al.*, 1997), may determine the fate of interactions.

To determine the conclusive role that each of these factors play in resistance needs more research. One path, that may shed some light on this, is to block induction of known PR proteins by genetic manipulation of plants and study the effect on the disease. However, this has not been yet achieved.

8.2.2 PR proteins in the barley-*R. secalis* interaction

In Chapter 3, IWF collected from uninoculated Atlas was found to have a low level of antifungal activity that increased significantly 3 days postinoculation. IWF from uninoculated Atlas 46 had an antifungal activity stronger than that in uninoculated Atlas

but, following inoculation, it did not increase to the same level of inoculated Atlas IWF (Figure 3-4). Five antifungal proteins and 3 proteins with no known activity were detected in IWF and shown to increase in the same pattern as the IWF antifungal activity (Chapter 7). A low molecular weight peptide, Pk17, also appeared to increase moderately in response to inoculation (Chapter 4). However, both its constitutive level and its induction were more pronounced in the resistant plant Atlas 46. Since the IWF proteins/peptides studied here increased upon inoculation, they all fit the definition of PR proteins/peptides. However, their role in the resistance responce of barley against *R. secalis* does not seem as straight forward.

The constitutively higher level of PRPs in the resistant plant, compared to the susceptible, is consistent with the notion that PRPs play a role in resistance. Except for the induction of Pk17, however, this study does not indicate that a faster or more pronounced induction of PRPs occurs in the resistant plants. On the contrary, a much larger induction of PR proteins was consistently detected in the susceptible plant Atlas on day 3 postinoculation. Whether this reflects the overall dynamics of the interaction is yet to be determined by detailed analysis of the PRP kinetics in a time course study involving the early hours and days of the interaction. Induction of barley PR proteins has been previously shown to be detectable as early as 16 hours postinoculation (Kerby and Somerville, 1989). Furthermore, the dynamics of Pk17, studied in Chapter 4, suggests that induction of proteins on day 1 postinoculation may not be necessarily the same as that on day 3 (see Figure 4-6).

More pronounced induction of PRPs in the susceptible plant Atlas confirms the notion that PRPs are general stress responses. Strong induction of potent antifungal PRPs in Atlas and yet the inability to overcome the pathogen progress in this plant may be explained by the ability of the fungus to not only attack but also actively defend itself following germination. The capacity of fungi to secrete compounds that inhibit plant cell wall lytic enzymes is well known (Albersheim and Valent, 1974; Ham *et al.*, 1997). In Chapter 3, *R. secalis* SGF (spore germination fluid) was shown to contain compounds that reduced the IWF antifungal effect. Although the putative inhibitor compounds of *R. secalis* were not isolated, it is conceivable that the fungus dynamically reacts to the environment by releasing offensive/inhibiting compounds. To achieve this, the fungus must first survive the early or constitutive plant defense responses, germinate and penetrate the plant. From this point a fine balance is formed between the fungus

that is increasing its mass as well as its offensive/inhibitory arsenal and the plant that increases the defense responses. The minimum mass required for the fungus, in order not to be overwhelmed by the plant defense, is probably not achieved in the resistant plant because it has more active or preformed responses. However, in the susceptible plants, the pathogen should swiftly grow to reach the critical mass/inhibition level that ensures its tolerance to the imminent yet delayed induction of plant defense.

As explained in Chapter 3, IWF, at its best, is only an extract of "unstructured" apoplastic fluid. Further investigation is required to determine the presence and the level of the detected proteins in the critical sub-cellular sites. Techniques such as *in vitro* hybridisation and immuno-histochemical microscopy could be used to determine the distribution of the proteins in sites of fungal penetration or colonisation in both resistant and susceptible plants. Further studies are also required to determine the induction levels of the PR proteins by cultivars Atlas and Atlas 46 against *R. secalis* isolates with virulence different from that of isolate H2.5. The study of Atlas 46 response to a virulent isolate (such as WA3076) could shed more light on the picture of the barley defense responses against the pathogen. Similarly, analysis of the defense response of Atlas against an avirulent isolate (such as K8) may also be of interest. None of these studies need to follow the purification process taken in this project. Probing tools, such as antibodies, are probably adequate for these studies.

In this context, some implications in the development of barley, with transgenic resistance against *R. secalis*, may also emerge from the finding in this thesis. Firstly, it was shown that TL proteins have stronger antifungal activity against this pathogen and therefore they probably present a better candidate compared to proteins such as β(1-3) glucanase or chitinase. Secondly, resistance against *R. secalis* was found to correlate with a higher level of PR protein constitutively present in the plant apoplast. A transgenic barley plant, with constitutive expression of appropriate PR proteins, would be applying a strategy that is already at work in the resistant plant Atlas 46 and thus more likely to meet success.

Appendix 1

(Literature associated with this appendix appeared in Chapter 3.)

Analysis of variance for "Comparative study of IWF antifungal activity"

******* Analysis of variance for DB *******

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
replicat stratum	2	50.2	25.1	0.16	
replicat.*Units* stratum					
cultivar	1	192.0	192.0	1.21	0.313
treatment	1	1633.3	1633.3	10.30	0.018
cultivar.treatment	1	192.0	192.0	1.21	0.313
Residual	6	951.2	158.5		
Total	11	3018.7			

***** Statistical factors *****

Table	cultivar	treatment	cultivar*treatment
rep.	6	6	3
d.f.	6	6	6
s.e.m.	7.27	7.27	10.28
LSD.	17.79	17.79	25.15

******* Tables of means *******

cultivar	Atlas	Atlas_46
	40.7	32.7

treatment	Inoculated	Un-inoculated
	48.3	25.0

cultivar	treatment	Inoculated	Un-inoculated
Atlas		56.3	25.0
Atlas_46		40.3	25.0

******* Analysis of variance for FW *******

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
replicat stratum	2	423.167	211.583	60.94	
replicat.*Units* stratum					
cultivar	1	352.083	352.083	101.40	<.001
treatment	1	1302.083	1302.083	375.00	<.001
cultivar.treatment	1	168.750	168.750	48.60	<.001
Residual	6	20.833	3.472		
Total	11	2266.917			

***** Statistical factors *****

Table	cultivar	treatment	cultivar*treatment
rep.	6	6	3
d.f.	6	6	6
s.e.m.	1.076	1.076	1.521
LSD.	2.632	2.632	3.723

******* Tables of means *******

cultivar	Atlas	Atlas_46
	37.50	48.33

treatment	Inoculated	Un-inoculated
	53.33	32.50

cultivar	treatment	Inoculated	Un-inoculated

Atlas	51.67	23.33
Atlas_46	55.00	41.67

******* Analysis of variance for IG *******

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
replicat stratum	2	135.2	67.6	0.60	
replicat.*Units* stratum					
cultivar	1	4.1	4.1	0.04	0.855
treatment	1	6768.8	6768.8	60.54	<.001
cultivar.treatment	1	114.1	114.1	1.02	0.351
Residual	6	670.8	111.8		
Total	11	7692.9			

***** Statistical factors *****

Table	cultivar	treatment	cultivar*treatment
rep.	6	6	3
d.f.	6	6	6
s.e.m.	6.10	6.10	8.63
LSD.	14.94	14.94	21.13

******* Tables of means *******

cultivar	Atlas	Atlas 46
	49.5	50.7

treatment	Inoculated	Un-inoculated
	73.8	26.3

cultivar treatment	Inoculated	Un-inoculated
Atlas	76.3	22.7
Atlas_46	71.3	30.0

******* Analysis of variance for GT length *******

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
replicat stratum	2	14.740	7.370	3.81	
replicat.*Units* stratum					
cultivar	1	0.192	0.192	0.10	0.763
treatment	1	6.409	6.409	3.31	0.119
cultivar.treatment	1	2.283	2.283	1.18	0.319
Residual	6	11.603	1.934		
Total	11	35.227			

***** Statistical factors *****

Table	cultivar	treatment	cultivar*treatment
rep.	6	6	3
d.f.	6	6	6
s.e.m.	0.803	0.803	1.135
LSD.	1.965	1.965	2.778

******* Tables of means *******

cultivar	Atlas	Atlas 46
	6.98	7.23

treatment	Inoculated	Un-inoculated
	6.37	7.83

cultivar treatment	Inoculated	Un-inoculated
Atlas	5.81	8.14
Atlas_46	6.94	7.52

******* Analysis of variance for G% *******

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
replicat stratum	2	228.50	114.25	3.16	
replicat.*Units* stratum					

cultivar	1	56.33	56.33	1.56	0.258
treatment	1	363.00	363.00	10.04	0.019
cultivar.treatment	1	176.33	176.33	4.88	0.069
Residual	6	216.83	36.14		
Total	11	1041.00			

***** Statistical factors *****

Table	cultivar	treatment	cultivar*treatment
rep.	6	6	3
d.f.	6	6	6
s.e.m.	3.47	3.47	4.91
LSD.	8.49	8.49	12.01

******* Tables of means *******

cultivar	Atlas	Atlas 46
	19.3	23.7

treatment	Inoculated	Un-inoculated
	16.0	27.0

cultivar	treatment	Inoculated	Un-inoculated
Atlas		10.0	28.7
Atlas_46		22.0	25.3

Analysis of variance for "IWF detoxification by *R. secalis*"

******* Analysis of variance for G% *******

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
repeat stratum	2	19.000	9.500	2.28	
repeat.*Units* stratum					
treatmen	1	280.167	280.167	67.24	0.015
Residual	2	8.333	4.167		
Total	5	307.500			

***** Statistical factors *****

Table	treatmen
rep.	3
d.f.	2
s.e.m.	1.667
LSD.	7.171

******* Tables of means *******

treatmen	IWF	IWF+SGF
	16.67	30.33

******* Analysis of variance for FW *******

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
repeat stratum	2	104.33	52.17	2.68	
repeat.*Units* stratum					
treatmen	1	486.00	486.00	24.92	0.038
Residual	2	39.00	19.50		
Total	5	629.33			

***** Statistical factors *****

Table	treatmen
rep.	3
d.f.	2
s.e.m.	3.61
LSD.	15.51

******* Tables of means *******

treatmen	IWF	IWF+SGF
	40.3	22.3

******* Analysis of variance for IG *******

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
repeat stratum	2	67.00	33.50	0.61	
repeat.*Units* stratum					
treatmen	1	912.67	912.67	16.54	0.055
Residual	2	110.33	55.17		
Total	5	1090.00			

***** Statistical factors *****

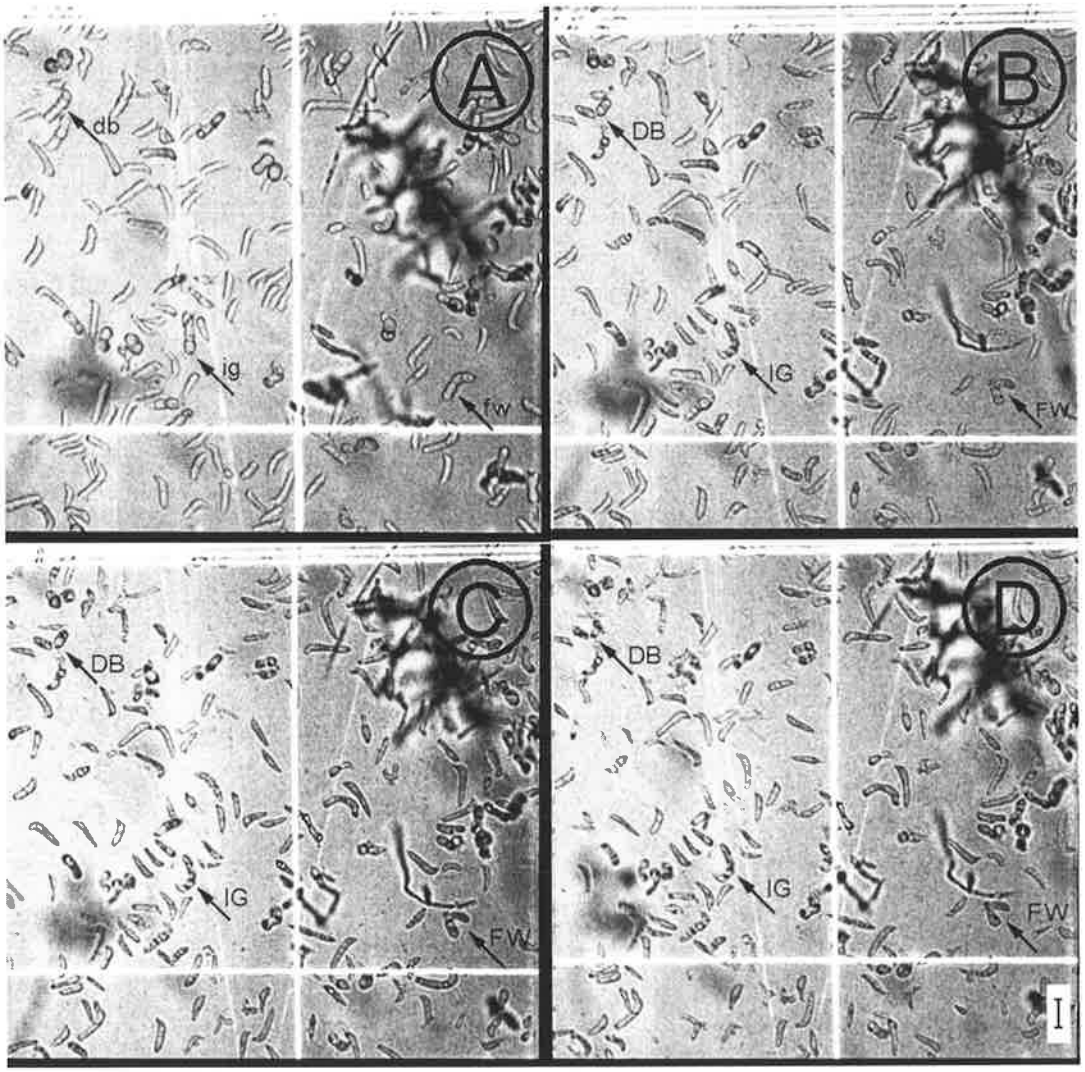
Table	treatmen
rep.	3
d.f.	2
s.e.m.	6.06
LSD.	26.09

******* Tables of means *******

treatmen	IWF	IWF+SGF
	58.3	33.7

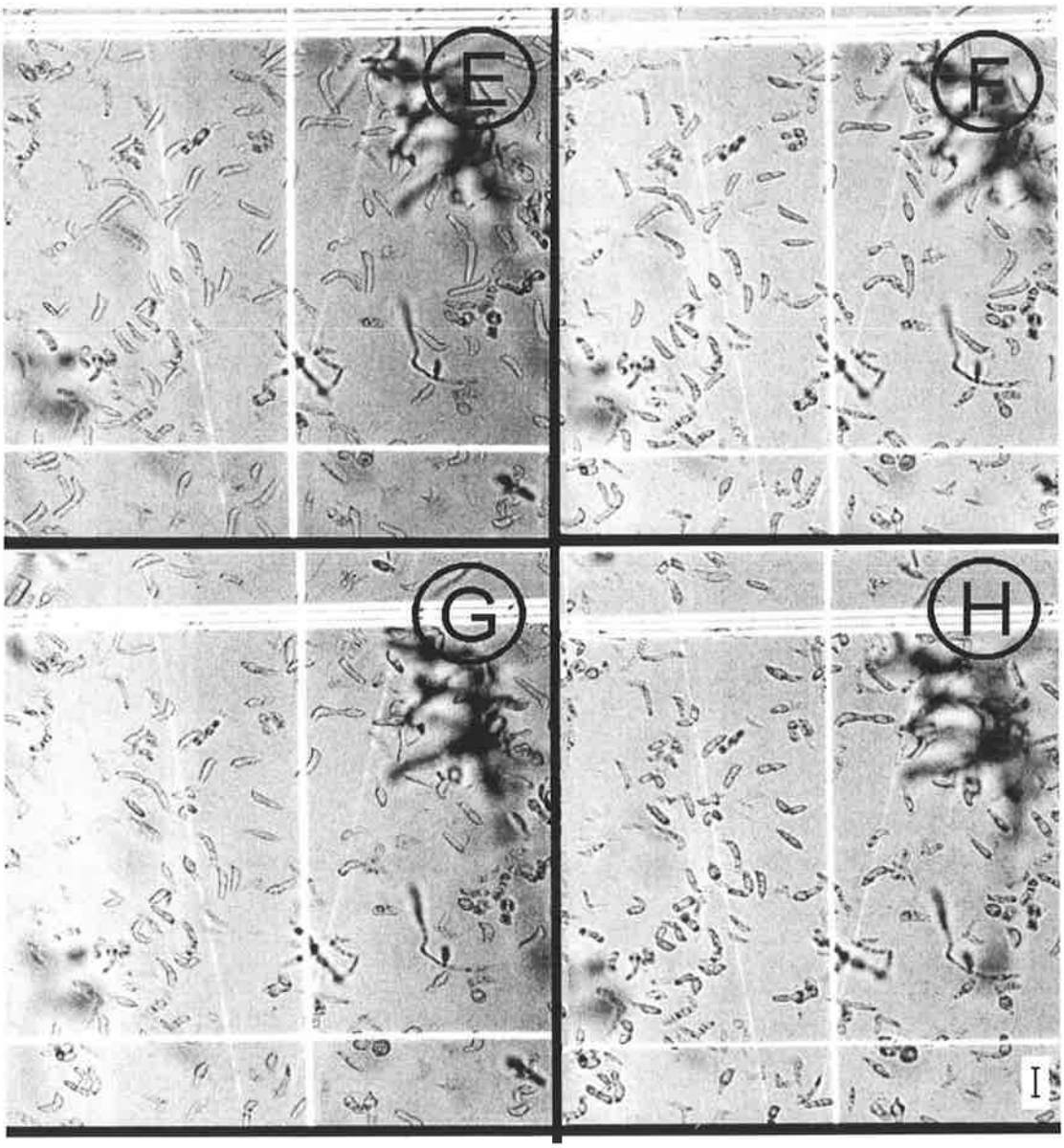
Appendix 2

(Literature associated with this appendix appeared in Chapter 3.)



Conidia of *R. secalis* incubated in IWF (10X) from Atlas 46. Photographs were taken after 1 min (A), 30 min (B), 1 hour (C) and 2 hours (D) incubation at the room temperature (~22°C). The arrows point to the first spores that formed FW, DB and IG spores. Lower case letters were used where spores had not been affected yet. Bar, on the bottom right, represents 10 μm.

(Continued next page)



(continue from previous page)

Conidia of *R. secalis* incubated in IWF (10X) from Atlas 46. Photographs were taken after 3 hours (E), 4 hours (F), 6 hours (G) and 12 hours (H) incubation at the room temperature (~22°C). Bar, on the bottom right, represents 10 μm.

Appendix 3

(Literature associated with this appendix appeared in Chapter 4.)

Analysis of variance for "Suppression of germination by LMW IWF"

******* Analysis of variance for G% *******

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP stratum	2	3944.5	1972.3	17.90	
REP.*Units* stratum					
TREAT	4	4231.3	1057.8	9.60	0.004
Residual	8	881.5	110.2		
Total	14	9057.3			

***** Statistical factors *****

Table	TREAT
rep.	3
d.f.	8
s.e.m	6.06
LSD	19.76

******* Tables of means *******

TREAT	control	Atlas	Atlas46	Atals+H2.5	Atals46+H2.5
value	62.7	40.0	34.7	28.3	11.0
group	A	B	B	BC	C

******* Analysis of variance for germ tube (GT) length*******

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP stratum	2	104.03	52.02	3.34	
REP.*Units* stratum					
TREAT	4	229.02	57.26	3.67	0.055
Residual	8	124.68	15.58		
Total	14	457.73			

***** Statistical factors *****

Table	TREAT
rep.	3
d.f.	8
s.e.m	2.28
LSD	7.43

******* Tables of means *******

TREAT	control	Atlas	Atlas46	Atals+H2.5	Atals46+H2.5
value	19.5	14.3	13.0	9.4	8.6
group	A	A	A	A	A

Analysis of variance for "Two-phase fractionation of LMW IWF"

******* Analysis of variance for G% (adjusted for covariate) *******

Variate: LMW_G%
Covariate: CNTRL_G%

Source of variation	d.f.	s.s.	m.s.	v.r.	cov.ef.	F pr.
REP stratum						
Covariate	1	71.254	71.254	25.95		0.123
Residual	1	2.746	2.746	0.39	13.47	
REP.*Units* stratum						
TREAT	2	1622.560	811.280	116.57	0.74	0.001
Covariate	1	218.454	218.454	31.39		0.011
Residual	3	20.879	6.960		8.60	
Total	8	1718.000				

***** Statistical factors *****

Table	TREAT
rep.	3
d.f.	3
s.e.m	1.773
LSD	7.978

******* Tables of means (adjusted for covariate) *******

TREAT	Amphophilic	Lypophilic	Hydrophilic
value	50.50	49.74	18.76
group	A	A	B

*******Analysis of variance for GT length (adjusted for covariate)*******

Variate: LMW_GT len.
Covariate: CNTRL_GT len.

Source of variation	d.f.	s.s.	m.s.	v.r.	cov.ef.	F pr.
REP stratum						
Covariate	1	6.2174	6.2174	9.38		0.201
Residual	1	0.6628	0.6628	2.22	5.19	
REP.*Units* stratum						
TREAT	2	34.3470	17.1735	57.49	0.82	0.004
Covariate	1	5.1684	5.1684	17.30		0.025
Residual	3	0.8961	0.2987		5.08	
Total	8	43.2364				

***** Statistical factors *****

Table	TREAT
rep.	3
d.f.	3
s.e.m	0.347
LSD	1.564

******* Tables of means (adjusted for covariate) *******

TREAT	Lypophilic	Amphophilic	Hydrophilic
value	6.81	6.07	1.55
group	A	A	B

Analysis of variance for "A putative pathogenesis-related compound in the LMW IWF"

*******Analysis of variance for Pk17 level on day 1 (log. transformation)*******

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP stratum	2	1.93582	0.96791	14.99	
REP.*Units* stratum					
TREAT	3	0.25729	0.08576	1.33	0.350
Residual	6	0.38736	0.06456		
Total	11	2.58047			

***** Statistical factors *****

Table	TREAT
rep.	3
d.f.	6
s.e.m	0.1467
LSD	0.5076

******* Tables of means *******

TREAT	Atlas46+H2.5		Atlas46	Atlas	Atlas+H2.5
value	4.614	4.479	4.399	4.210	

*******Analysis of variance for Pk17 level on day 2 (log. transformation)*******

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP stratum	2	0.92337	0.46168	16.75	
REP.*Units* stratum					
TREAT	3	0.68995	0.22998	8.34	0.015
Residual	6	0.16539	0.02757		
Total	11	1.77871			

***** Statistical factors *****

Table	TREAT
rep.	3
d.f.	6
s.e.m	0.0959
LSD	0.3317

******* Tables of means *******

TREAT	Atlas46+H2.5		Atlas46	Atlas+H2.5	Atlas
value	4.772	4.520	4.447		4.101
group	A	A	A		B

*******Analysis of variance for Pk17 level on day 3 (log. transformation)*******

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP stratum	2	1.70883	0.85442	35.38	
REP.*Units* stratum					
TREAT	3	0.39376	0.13125	5.44	0.038
Residual	6	0.14488	0.02415		
Total	11	2.24748			

***** Statistical factors *****

Table	TREAT
rep.	3
d.f.	6
s.e.m	0.0897
LSD	0.3105

******* Tables of means *******

TREAT	Atlas46+H2.5		Atlas+H2.5	Atlas46	Atlas
value	4.521	4.340	4.244	4.018	
group	A	A	AB	B	

*******Analysis of variance for Pk17 level on day 9 (log. transformation)*******

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP stratum	2	0.86543	0.43271	17.23	
REP.*Units* stratum					
TREAT	3	2.23335	0.74445	29.64	<.001
Residual	6	0.15070	0.02512		
Total	11	3.24947			

***** Statistical factors *****

Table	TREAT
rep.	3
d.f.	6
s.e.m	0.0915
LSD	0.3166

******* Tables of means *******

TREAT	Atlas+H2.5	Atlas46+H2.5		Atlas46	Atlas
value	4.941	4.722	4.429	3.793	
GROUP	A	AB	B	C	

*******Analysis of variance for Pk18 level on day 1 (log. transformation)*******

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP stratum	2	1.6760	0.8380	5.17	
REP.*Units* stratum					
TREAT	3	0.1408	0.0469	0.29	0.832
Residual	6	0.9722	0.1620		
Total	11	2.7890			

***** Statistical factors *****

Table	TREAT
rep.	3
d.f.	6
s.e.m	0.232
LSD	0.804

******* Tables of means *******

TREAT	Atlas	Atlas+H2.5	Atlas46	Atlas46+H2.5
value	3.84	3.97	4.06	4.13
group	A	A	A	A

*******Analysis of variance for Pk18 level on day 2 (log. transformation)*******

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP stratum	2	0.49379	0.24689	11.27	
REP.*Units* stratum					
TREAT	3	0.02727	0.00909	0.42	0.749
Residual	6	0.13143	0.02190		
Total	11	0.65249			

***** Statistical factors *****

Table	TREAT
rep.	3
d.f.	6
s.e.m	0.0854
LSD	0.2957

******* Tables of means *******

TREAT	Atlas	Atlas46+H2.5		Atlas46	Atlas+H2.5
value	4.095	4.064	3.996	3.979	
group	A	A	A	A	

*******Analysis of variance for Pk18 level on day 3 (log. transformation)*******

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP stratum	2	0.52378	0.26189	4.73	
REP.*Units* stratum					
TREAT	3	0.24506	0.08169	1.47	0.313
Residual	6	0.33255	0.05542		
Total	11	1.10139			

***** Statistical factors *****

Table	TREAT
rep.	3
d.f.	6
s.e.m	0.1359
LSD	0.4704

******* Tables of means *******

TREAT	Atlas+H2.5	Atlas46+H2.5		Atlas46	Atlas
Value	4.233	4.066	4.008	3.833	
group	A	A	A	A	

*******Analysis of variance for Pk18 level on day 9 (log. transformation)*******

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP stratum	2	0.06473	0.03236	0.35	
REP.*Units* stratum					
TREAT	3	1.12691	0.37564	4.09	0.067
Residual	6	0.55124	0.09187		
Total	11	1.74287			

***** Statistical factors *****

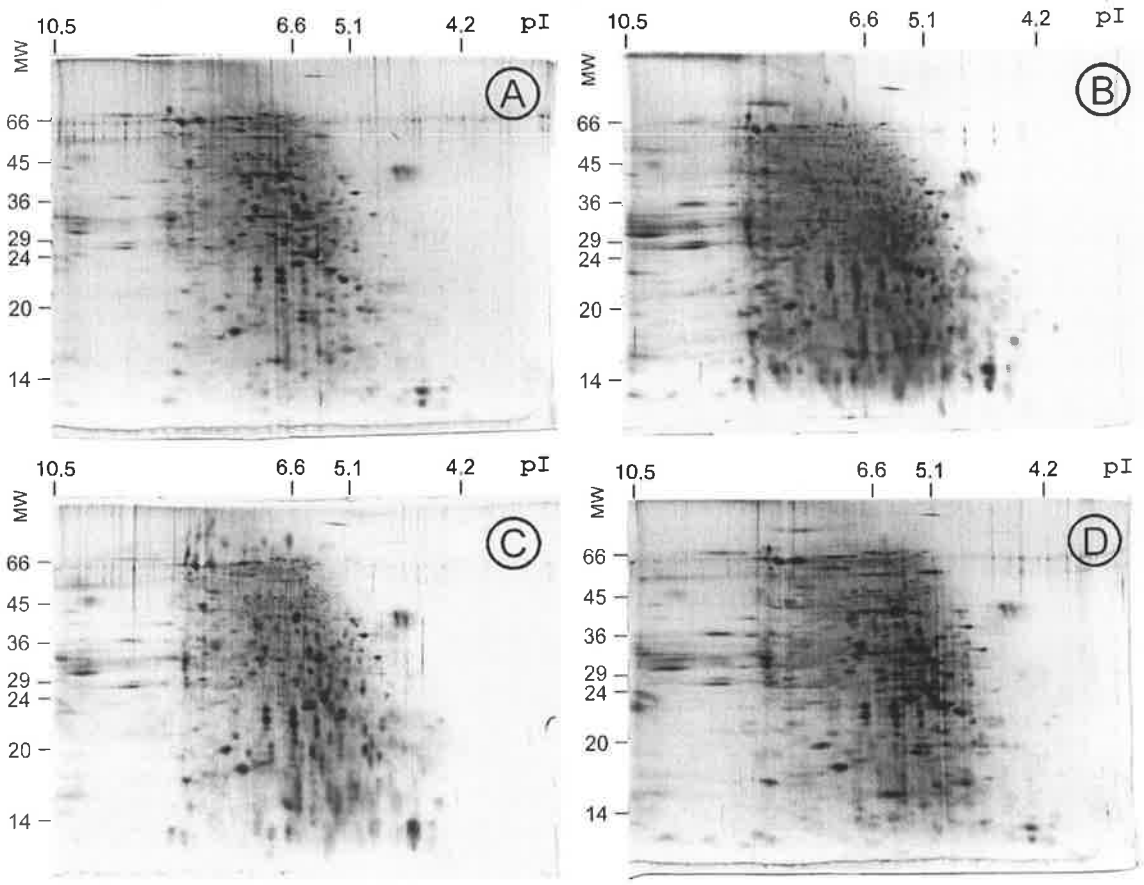
Table	TREAT
rep.	3
d.f.	6
s.e.m	0.1750
LSD	0.6056

******* Tables of means *******

TREAT	Atlas+H2.5	Atlas46+H2.5		Atlas46	Atlas
Value	4.213	4.134	4.067	3.440	
group	A	A	A	A	

Appendix 4

(Literature associated with this appendix appeared in Chapter 7.)



2-D PAGE of IWF from Atlas (A), inoculated Atlas (B), Atlas 46 (C) and inoculated Atlas 46 (D).

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