# STUDIES OF BARLEY LIMIT DEXTRINASE

by

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## SUMMARY

Limit dextrinase (LD) is potentially an important starch degrading enzyme in barley because α-and β-amylase cannot hydrolyse the α-1,6-bonds in amylopectin and limit dextrins. The methods used to date to measure the activity of this enzyme in crude extracts of germinated seed or malt are subject to interference from other starch degrading enzymes present in the extract. This problem has limited the study of this enzyme in contrast to research on α-and β-amylase. An immunochemical approach utilizing a monospecific antibody has the potential to overcome this problem. Immunochemical methods have been used increasingly in investigations of plant proteins and enzymes to study their biochemistry and physiology.

Limit dextrinase was purified from barley malt to electrophoretic homogeneity and partially characterised. The enzyme is a large, protein ($M_r$ 104,000), LD has an optimum activity at pH 5.5 and 55°C but is rapidly inactivated at 70°C. Isoelectric focusing revealed six isoenzymes (pI 4.2-4.6 ).

The purified protein was used to immunise a rabbit. The IgG fraction, which contained the anti-LD antibodies, was purified from the antiserum. These anti-LD antibodies are functionally monospecific for LD as determined by immunoblotting and crossed immunoelectrophoresis. Two immunoassays were developed; an ELISA to measure LD in crude extracts, and immunoblotting following IEF to detect isoforms of LD.

An excellent correlation between the reduction in enzyme activity (Red Pullulan assay) and immunological activity (ELISA) was observed when extracts of malt flour were heated to inactivate enzymes, (e.g. mashing). This indicates the ELISA is measuring active enzyme.

There was a significant variation in enzyme and immunological activity between genetically diverse cultivars of barley. In contrast, there was minor variation in the isoenzyme patterns detected by IEF-immunoblotting but greater variation in *Hordeum spontaneum*. The latter could be useful as genetic markers.

Wheat -barley addition lines were used to locate the gene encoding LD. A specific banding pattern was associated with chromosome 1. However, evidence from wheat nullisomic-tetrasomic lines, indicates this difference in banding pattern is complex and is not necessarily due to a single structural gene on homeologous chromosomes.

Small quantities of LD were detected with the ELISA in kernels at various stages of grain development. Limit dextrinase was present in small quantities at the onset of anthesis, but increased rapidly five days later, reaching a maximum at 21-23 days, then decreasing slightly, but remaining elevated through to maturity. The increase up to day 21 was solely due to a 'free' form of the enzyme. The 'free' LD decreased rapidly after 21 days and at the same time a 'bound' form increased remaining elevated through to maturity.

There were minor variations in the banding intensity of IEF-immunoblotting patterns of LD observed during development. The same isoforms found in developing barley grain

were also found in malt or germinated barley. Dissections of kernels at 21 days post-anthesis demonstrated that LD was located in the embryo and endosperm, with a trace in pericarp tissue. The IEF-immunoblotting patterns were identical in these tissues.

Several barley cultivars were shown to differ in the rate of appearance of LD during germination studies with maximum activities at 4, 6 and 10 days. In micromalting, enzyme and immunological activity increased at similar rates but did not reach a plateau by day four (start of kilning). This slow rate of appearance may limit the potential fermentability of the wort, for example; a low activity could result in large quantities of non-fermentable dextrins in a brewers wort.

During kilning, enzyme activity was lost more rapidly (up to a 58% reduction) than immunological activity (up to a 24 % reduction) and there was a difference in the sensitivity of Clipper and Skiff.

The reduction in immunological activity found during a standard EBC mashing was similar to previous reports using enzyme assays. Activity began to decrease after 60°C, decreasing by 23 % over 5 min between 65-70°C fifty percent of the initial activity remained after 5 min at 70°C but no activity after 1 h at 70°C. Enzyme activity (RP assay), was only detected when cysteine was added to the mash water. Cysteine brought about a more rapid decline in both enzyme and immunological LD activity.

A 'bound' form of LD detected with the anti-LD antibodies exists in mature and germinated seed and is released using thiol reducing agents or papain. These agents also activate the enzyme depending upon the concentration used. In germinated seed, the 'bound' form represents about 11 % of the total extractable activity; the majority is probably synthesized *de novo*.

Limit dextrinase may be important to malting quality through its effect on the apparent attenuation limit (AAL) by controlling the amount of non-fermentable dextrins in wort. In a study using 39 cultivars of barley which were malted under standard conditions, there was no significant relationship between AAL and malt LD activity when cultivar differences in AAL were taken into account. In another study, malting for longer periods (up to 10 days), increased LD activity and wort AAL. An analysis of variance to explain the variation in AAL in this study showed enzyme (but not immunological) activity of the malt, cultivar differences, % extract and the length of malting, all contributed significantly to the variation in AAL but cultivar differences was the main factor.

## STATEMENT OF ORIGINALITY AND CONSENT TO PHOTOCOPY OR LOAN

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text.

I give consent to the librarian of the Barr-Smith Library, the University of Adelaide, or his/her appointed agent to make this thesis, or any part thereof, available for photocopying or loan.

Mike Sissons

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## LIST OF PUBLICATIONS

Some of the material in this thesis has been presented at Scientific meetings and submitted for publication.

Sissons, M. J., Lance, R. C. M. and Sparrow, D. H. B. (1989). The Development of Immunochemical Approaches. In' Proceedings of the 39th Australian Cereal Chemistry Conference' (T. Westcott and Y. Williams eds.). Perth, Cereal Chemistry Division, Royal Australian Chemical Institute, pp 202-205.

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Sissons, M. J., Lance, R. C. M. and Sparrow, D. H. B. (1991). Studies on Limit Dextrinase in Barley: 2. Application of an ELISA and Immunoblotting to Studies of Genetic Variability and Malting Effects. *J. Cereal Science* (submitted).

## Addendum: Corrections to thesis

Page 1, middle of page:
The work of Kneen and Spoerl in 1948 indicated a starch degrading enzyme activity which was not an amylase. This work predates that of Hobson *et al.* 1954.

Page 20, line 128 and page 187 line 8 from foot:
The term "wavy' and "wavy distribution" is a term used by Enevoldsen and Schmidt (1973) to describe the shape of the distribution of the various groups of dextrins found in beer.

Page 28, line 8 from foot:
RO water refers to water purified by reverse osmosis using membranes.

Page 51, Fig. 4a:
A schematic diagram of the ELISA chequerboard assay design. This grid pattern shows the layout of a 96 well microtitre plate used for an ELISA. Each grid represents a well where solutions are added. Serial dilutions of antibody occur across the plate and antigen down the plate.

Page 89-90 missing information:
the word missing is 'separate'.

Page 97, Fig. 4.18:
CE refers to a crude extract and CCE to a concentrated preparation of crude extract.

Page 110, last line of text:
It should be noted that pI values quoted are only approximate values.

Page 120, line 3:
"cultivars within day" refers to no replication of micromalt cans for each of the three days of malting.

# CHAPTER 1

# LITERATURE REVIEW

*"Limit Dextrinase is a name of ancient currency in the brewing industry and has been used with respect to barley malt to describe an enzymatic activity that would liberate fermentable sugars from non-fermentable dextrins" (Enevoldsen, 1975).*

## 1.1 Introduction

This chapter presents a review of the literature relevant to limit dextrinase up to early 1988. Literature since 1988 is discussed in the individual chapters.

The hydrolytic enzymes that degrade starch are classified as those that hydrolyse :

(1) only α-1,4 bonds (α-amylase, E. C. 3.2.1.1 and β-amylase, E. C. 3.2.1.2)

(2) both α-1,4 and α-1,6 bonds (amyloglucosidase E. C. 3.2.1.3, (not found in barley seed) and α-glucosidase, E. C. 3.2.1.20)

(3) only α-1,6 bonds (limit dextrinase E. C. 3.2.1.41 and isoamylase E. C. 3.2.1.68).

The last group of enzymes are collectively known as debranching enzymes. Limit dextrinase, also known in the literature as R-enzyme, pullulanase and pullulan 6-glucanohydrolase is an α-dextrin 6-glucanohydrolase and differs from isoamylase in that the latter does not hydrolyse pullulan.

The first debranching enzymes were discovered in 1951 in broad beans and potatoes and termed R-enzyme (Hobson *et al.* 1954). MacWilliam and Harris (1959) reported a biochemical separation into two forms of debranching activity in studies with malted barley and broad beans. One of these forms, able to hydrolyse α-1,6 bonds in amylopectin but apparently with no effect on α-dextrins, was termed R-enzyme. The second enzyme (called limit dextrinase) was shown to hydrolyse α-1,6 bonds in α-dextrins, but did not affect amylopectin. Later work, with potato found only one form of debranching enzyme that could hydrolyze both α-dextrins and amylopectin (Drummond *et al.*, 1970). These investigators showed debranching enzyme action on amylopectin was dependent on the concentration of enzyme. Thus, limit dextrinase and the R-enzyme were considered to be one and the same enzyme.

One of the problems with the earlier studies are the methods used to assay limit dextrinase are not specific and are subject to interference from other enzymes in crude extracts. The discovery of the substrate pullulan, a linear polymer of α-1,6 linked α-maltotriose units produced by the bacterium *Pullularia pullulans* (Bender and Wallenfels, 1961) is not significantly hydrolysed by the amylases (Lee and Pyler, 1982). Pullulan improved the specificity of assay towards limit dextrinase. By the mid 1970's reports in a range of plant species; broad bean, (Gordon *et al.*, 1975), oat, (Dunn and Manners, 1975), sorghum,

(Hardie *et al.*, 1976), rice (Iwaki and Fuwa, 1981) and barley, (Manners and Yellowlees, 1971) had shown that only one debranching enzyme exists.

Isoamylase (from bacteria, yeast and some plants) also known in the literature as debranching enzyme or glycogen 6-glucanohydrolase, differs from limit dextrinase (from higher plants) and pullulanases (a bacterial limit dextrinase). Isoamylase does not hydrolyse pullulan but readily hydrolyses glycogen (Manners, 1985a). Limit dextrinase and pullulanase hydrolyse glycogen only slowly. To avoid confusion throughout this review the term limit dextrinase (LD) will be used in preference to other names.

## 1.2 Properties of Limit Dextrinase

Limit dextrinase is a hydrolase enzyme catalysing the hydrolysis of α-1,6 glucosidic bonds found in amylopectin, α- and β-limit dextrins and pullulan.

### 1.2.1 Purification

Limit dextrinase has been isolated to varying degrees of purity from a range of plant species (Table 1.1). The techniques used to isolate the enzyme usually involve ammonium sulphate fractionation of the crude extract followed by anion exchange chromatography then either preparative isoelectric focusing, chromatofocusing, gel filtration or cyclodextrin affinity chromatography. Recoveries of purified protein vary from 1.5 to 15 % with most studies obtaining 3%. The degree of purification varies from 15 (Dunn and Manners, 1975) to 1000 fold (Maeda *et al.*, 1978). The latter was obtained in barley using cyclodextrin affinity chromatography as the final step.

Data on the degree of purification are subject to error because the method used to measure enzyme activity during the purification (see 1.3) can overestimate activities. Contaminating carbohydrases may hydrolyse maltotriose, the main product of LD action on pullulan. Hence calculations of the degree of purity and yield using specific activity of the crude extract as a basis are misleading.

The classical test of protein homogeneity (Suelter, 1985) is a single band on sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). In only four reports was this achieved, two in barley (Maeda *et al.*, 1978; Lecommandeur *et al.*, 1988) and two in rice (Iwaki and Fuwa, 1981; Yamada and Izawa, 1979a).

## 1.2.2 Enzyme Properties

Knowledge of the properties of LD so far obtained includes; molecular weight, isoelectric point, optimum pH and temperature for enzyme activity, thermal and pH stability, substrate specificity, enzyme kinetic data for various substrates (km and Vmax, see Table 1.3), and the inhibitors of activity. Some of these data are summarised in tables 1.1, 1.2, 1.3 and figure 1.1 from a range of plant species. Information on amino acid composition, N-terminal sequence, location of the active site and primary structure has not been published.

Limit dextrinase is a large protein with estimates of molecular weight varying depending upon the plant source and the method used for measurement. In barley, estimates range from about 80 kd using gel filtration to 103 kd using SDS-PAGE (Table 1.1). The protein is thought to be a monomer and is larger than α-amylases I (52 kD kd, MacGregor, 1978b) and the β-amylase 'free' (54, 56, 58 and 59.7 kD, Lundgard and Svensson, 1987) and 'bound' form (64 kd, Sopanen and Laurière, 1989). The pH optimum for LD from many plant species studied is similar (pH 5.0-5.6); the exceptions being broad bean, maize and peas. The purified enzyme from various sources is inactivated within 15 min when heated to 65-70°C (Iwaki and Fuwa, 1981; Hardie *et al.*, 1976) and slightly longer (15-60 min) when crude extract is used (Kruger and Marchylo, 1978; Lee and Pyler, 1984) presumably due to the protective effect of the extract on the unpurified protein.

## 1.2.3 Effect of Inhibitors

In general, heavy metals at concentrations around 0.1 mM inhibit LD activity (Maeda *et al.*, 1979; Iwaki and Fuwa, 1981). Chelating agents such as ethylenediaminetetraacetic acid (EDTA) at 1 mM have variable effects, for example in rice and wheat EDTA had no effect on activity (Iwaki and Fuwa, 1981; Kruger and Marchylo, 1978) but about 50 % inhibition occurs in sorghum (Hardie *et al.*, 1976) and barley (Maeda *et al.*, 1979). Para-hydroxymercuribenzoate (at 0.1 mM ), which reduces sulphydryl bonds, did not inhibit activity in sorghum (Hardie *et al.*, 1976) and wheat (Kruger and Marchylo, 1978) but inhibited activity by 50 % in barley (Maeda *et al.*, 1979). This information suggests that metal ions or sulphydryl groups are not necessary for enzyme activity for some plant LDs but are for others. The inhibition of barley LD activity by EDTA indicates that a cofactor, probably a divalent cation, is required for activity but the identity of this cation is not known. Other substances found to be inhibitors of barley LD were potassium permanganate, and β-cyclodextrin (Maeda *et al.*, 1979).

| Enzyme source | Molecular weight (kD) | pH Optima | Isoelectric point | Temp. Optima (°C) | Specific activity (U/g) | References |
|---|---|---|---|---|---|---|
| **Oat**-ungerminated | 80 (GF)[a] | 5.0 | ND | ND | 0.8 | Dunn & Manners, (1975) |
| -ungerminated | 85 (GF) | 5.6 | ND | ND | 16.8 | Yamada, (1981c) |
| **Wheat**-immature | ND | ND | 4.32-4.4 | ND | ND | Kruger & Marchylo, (1978) |
| **Maize**-ungerminated | 110 (GF) | 6.0 | ND | ND | 6.5 and 5.5[b] | Manners *et al.*, (1969) |
| **Barley**-germinated | 102 (SDS)[b] | ND | ND | ND | ND | Lecommandeur *et al.*, (1988) |
| -germinated | ND | 5-5.3 | ND | ND | 0.068 | Manners *et al.*, (1971) |
| -malted | 103 (SDS) 80 (GF) | 5-5.5 | | 50 | 10 | Maeda *et al.*, (1978, 1979) |
| -malted | ND | ND | 4.8-5.0 | ND | ND | Lenoir *et al.*, (1984) |
| **Rice**-immature | 70 (GF) | 5.6 | ND | ND | 16.5 | Yamada & Izawa, (1979a) |
| -ungerminated | 70 (GF) | 5.6 | ND | ND | 30 | Yamada, (1981d) |
| -germinated | 70 (GF) | 5.6 | 5.0-7.0 | ND | 10 | Yamada *et al.*, (1980) |
| -germinated | 100 (SDS) | 5.5 | 4.9 | 60 | 43 | Iwaki & Fuwa, (1981) |
| **Sorghum**-malt | 90 (GF) | 5-5.4 | ND | ND | 1.1 | Hardie *et al.*, (1976) |
| **Broad bean** | 80 (GF) | 6.6 | ND | ND | 1.0 | Gordon *et al.*, (1975) |
| **Pea** | 180 (GF) | 6.0 | ND | ND | 1.1 | Yellowlees, (1980) |

**Table 1.1**

Characteristics of cereal limit dextrinases.

a. Gel filtration. b. SDS-PAGE. ND not determined.

| Substrate | Oat | Rice | Barley (germinated) | Sorghum (malt) | Broad bean | Pea |
|---|---|---|---|---|---|---|
| Panose | 0 | 0 | 0 | 0 | 0 | 0 |
| 6-α-glucosylmaltotriose | 0 | 0 | 0 | N.D. | 0 | 0 |
| 6-α-maltosylmaltotriose | 120 | 50 | N.D[a]. | 220 | 75 | 90 |
| 6-α-maltotriosylmaltotriose | 220 | 170 | N.D. | 250 | 210 | 170 |
| 6-α-maltosylmaltotetraose | 290 | 210 | N.D. | 300 | 195 | 230 |
| 6-α-maltotriosylmaltotetraose | 380 | 260 | 250 | 400 | 320 | 280 |

**Table 1.2**

Action of limit dextrinase on singly-branched oligosaccharides. The initial rate of hydrolysis of each substrate is given relative to the rate of hydrolysis of pullulan (100). a. not determined. Table was reproduced from Manners, (1985a).

| Substrate | Kinetic Data | Oat | Rice | Barley | Sorghum | Broad bean |
|---|---|---|---|---|---|---|
| Amylopectin | Km[a] | 1.4 | 6.0 | 4.2 | N.D. | 1.2 |
| | Vm[b] | 2.6 | 3.5 | 13.0 | 14.0 | 10.0 |
| Amylopectin β-limit dextrin[h] | Km | 1.5 | 4.0 | 6.7 | 2.5 | 1.0 |
| | Vm | 16.0 | 15.0 | 460.0 | 260.0 | 65.0 |
| Glycogen β-limit dextrin[h] | Km | N.D.[c] | 7.0 | >20.0 | 30.0 | 17.0 |
| | Vm | N.D. | 5.5 | >330.0 | 300.0 | 50.0 |
| Pullulan | Km | 0.23[d] | 0.06[e] | 0.08[f] | 0.20[g] | N.D. |

**Table 1.3**

Action of limit dextrinase on various polysaccharides.

a. Km is expressed as mg/ml.

b. Vm initial rate of hydrolysis relative to pullulan (100).

c. not determined.

d. Yamada, (1981c). e. Iwaki and Fuwa, (1981).

f. Maeda *et al.*, (1979). g. Hardie *et al.*, (1976). h. Manners, (1985a).

### 1.2.4 Polymorphism

The technique of isoelectric focusing (IEF) with high resolving power, sensitivity, relative ease of operation and sensitive protein detection (silver staining) has been used to determine the isoelectric points (pI's) of LD and to examine its polymorphism. The number of detectable isoenzymes is much fewer than found in α-amylase (MacGregor, 1978a) and β-amylase (LaBerge and Marchylo, 1983). Kruger and Marchylo (1978) found two isozymes of LD in immature wheat with pI of 4.32 and 4.40. Two or three closely associated isomers (pI 4.70 to 5.0 ) have been found in extracts of barley and malted barley depending upon the cultivar tested (Manners and Yellowlees, 1973). In sweet corn two multiple forms of the enzyme have been separated using a hydroxylapatite column (Hardie *et al.*, 1976). In rice Yamada *et al.* (1980) found eight isozymes with pI's in the range 5.0-7.0.

### 1.2.5 Substrate Specificity

#### 1.2.5.1 Action of Limit Dextrinase on Oligosaccharides

The ability of plant LDs to hydrolyse various oligosaccharide substrates (two to seven glucose residues) has been studied extensively (Dunn and Manners, 1975; Yamada and Izawa, 1971; Yamada *et al.*, 1980). In general, the following is known about the specificity of the enzyme: (1) at least one α-1,4 glucosidic bond on either side of the α-1,6 bond is required before hydrolysis can occur (Fig. 1.1). Thus, isomaltose, panose and 6-α-glucosylmaltotriose are not hydrolysed. (2) the rate of hydrolysis increases with the length of the side and main chains (Table 1.2). Thus, 6-α-maltotriosylmaltotetraose is hydrolysed more rapidly than 6-α-maltosylmaltotriose which is hydrolysed more rapidly than 6-α-maltosylmaltose.

#### 1.2.5.2 Polysaccharides

Evidence that LD can cause the partial hydrolysis of amylopectin is an increase in iodine staining power, β-amylolysis limit (10 - 15%) and reducing sugar production (Kruger and Lineback, 1987). The enzyme hydrolyses amylopectin much more slowly than pullulan (Table 1.3). The plant LDs do not hydrolyse glycogen and phytoglycogen at concentrations which show action upon amylopectin (Manners and Yellowlees, 1971) except for LD from germinated rice endosperm which can hydrolyse 20% of the α-1,6 linkages in oyster

| Code | Substrate | Product | Hydrolysis |
|---|---|---|---|
| A | Isomaltose | None | No |
| B | Panose | None | No |
| C | 6-α-glucosylmaltotriose | None | No |
| D | 6-α-maltosylmaltose | Maltose | Yes |
| E | 6-α-maltosylmaltotriose | Maltose & Maltotriose | Yes |
| F | 6-α-maltotriosylmaltotetraose | Maltotriose & Maltotetraose | Rapid |

**Figure 1.1**

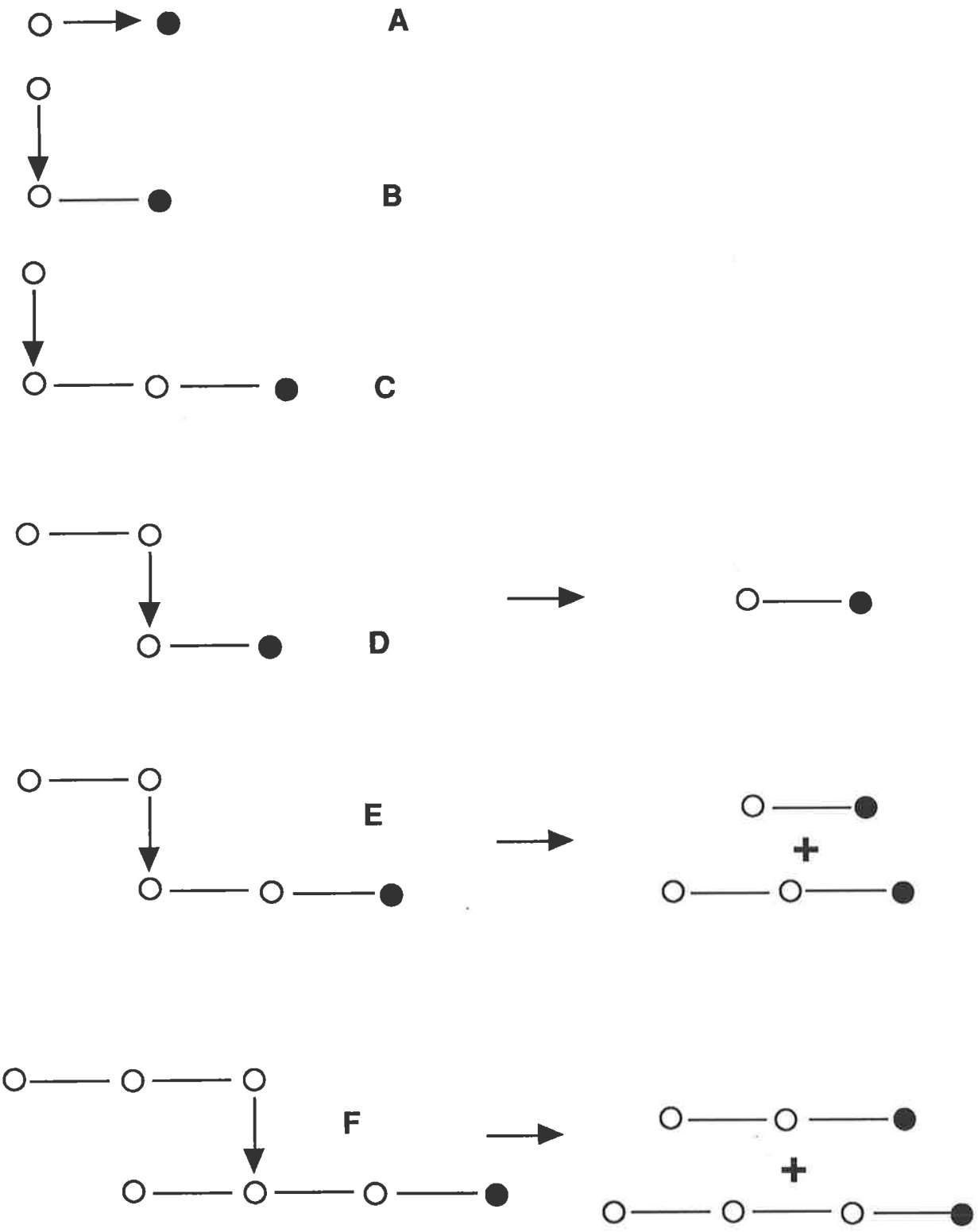
Action of limit dextrinase upon various oligosaccharides.

○ : Glucose ——— : α–1,4 bond

● : Reducing end ⟶ : α–1,6 bond

Information in this table was cited from Serre and Laurière (1989).

**Substrate** **Product**



glycogen (Iwaki and Fuwa, 1981). Both these polysaccharides appear to possess long branched external chains which hinder sterically the formation of the enzyme-substrate complex.

There is no evidence that LD alone will hydrolyse starch granules but the presence of LD does increase the rate of starch granule hydrolysis together with a mixture of α- and β-amylase (Maeda *et al.*, 1979).

Complex polysaccharides than pullulan are hydrolysed more slowly presumably due to their more complex branched structures. The unbranched chain can be of varying length, but the length of the branched chain is more critical in determining the rate of hydrolysis. Reaction rate is also affected by the degree of polymerization of the substrate (Hardie *et al.*, 1976).

Pullulan is thought to be hydrolysed by an "endo" type mechanism i.e. an enzymic reaction started from α-1,6 bonds at any place in the substrate molecule resulting in products of varying molecular weight (Manners and Yellowlees, 1971; Drummond *et al.*, 1970; Hardie and Manners, 1974; Yamada and Izawa, 1979b; Iwaki and Fuwa, 1982). Using H-NMR spectroscopy, the products released during hydrolysis of pullulan were found to be in the α-configuration (Shiomi and Yamada, 1982).

Polysaccharides, such as amylopectin and glycogen show a different pattern of degradation. Gel-filtration analyses of the hydrolysis products produced after LD action upon amylopectin and glycogen do not produce any middle molecular weight glucans, suggesting that the enzyme degraded preferentially the outer α-1,6 bonds (Iwaki and Fuwa, 1982), probably due to reasons of steric hindrance.

## 1.3 Methods of Assay

The assay for LD has been a problem for many years. The ideal assay should be specific, sensitive and easy to perform. Kruger and Lineback (1987) have reviewed assays for LD and only a brief overview will be given here. The substrates used in the early methods were α- or β-limit dextrins. These are prepared by the partial hydrolysis of amylopectin by a bacterial α- or β-amylase, respectively. The activity of LD is measured by incubating plant extracts with the limit dextrin substrate and the reducing sugars released are measured by the method of Nelson (1944). The problem with this assay is that the extract contains other enzymes such as α-amylase, β-amylase and α-glucosidase, which are present in variable amounts in crude plant extracts, and these can also degrade limit dextrins. As a consequence, LD activity is overestimated.

Another non-specific technique is the measurement of the absorbance of the amylose-iodine complex. When LD attacks amylopectin or β-limit dextrin, linear dextrin chains of varying length are produced which can bind iodine and increase the staining intensity.

However, other amylolytic enzymes present in extracts can degrade the products of LD action and reduce binding of the iodine (Yamada *et al.*, 1980).

The discovery of the substrate pullulan by Manners and Yellowlees (1973) improved the assay of this enzyme. Pullulan is not hydrolysed by α-amylase, β-amylase and α-glucosidase to a significant extent (Lee and Pyler, 1982) and offered promise as a specific assay. Pullulan is produced by *Pullularia pullulans* and *Aureobacterium pullulans* and is a polysaccharide polymer composed of repeating maltotriose units linked by α-1,6 glucosyl bonds (Fig. 1.2). Pullulan does not exist as a natural substrate in plants. Limit dextrinase cleaves the α-1,6-glucosyl bond releasing maltotriose, a substrate which is only slowly hydrolysed by amylases. However, maltotriose is degraded by enzymes from extracts of malt and endogenous α-glucosidase and the additional reducing groups produced may lead to erroneous results. Significant amounts of α-glucosidase have been found in embryos dissected from germinated barley kernels (MacGregor, 1987). The enzyme can hydrolyse α-1,4 and α-1,6 bonds in small dextrins (Jorgensen, 1964). Lee and Pyler found that adding an excess of yeast α-glucosidase to the reaction mixture converted all reducing groups to glucose, which was then assayed. Although this overcomes the problem of interference from other hydrol ases in the extract the method is time-consuming because of the long period of dialysis required for the removal of endogenous reducing sugars and has low sensitivity (Lenoir, 1985).

Since the hydrolysis of pullulan causes a slight reduction in the viscosity of the medium a viscosity assay was developed for measuring LD in plant extracts (Hardie and Manners, 1974). This assay does not suffer from interference by other enzymes in the crude extracts but is also time consuming and has a low sensitivity because of the very small changes in viscosity involved (Lee and Pyler, 1984).

An alternative method for measuring LD is the rocket immunoelectrophoresis assay which has been applied in barley and malt (Daussant *et al.*, 1987). This assay is specific and does not suffer from the problems described above but is slow to perform. Serre and Laurière (1989) cite "assay sensitivity is, however, inadequate for some extracts containing low levels of limit dextrinase (Daussant, personal communication)".

### 1.3.1 Detection of Limit Dextrinase Activity in Electrophoretic Gels

There are two methods for detecting enzyme activity in gels: (1) The proteins present in the flour extract are first separated by either isoelectric focusing or electrophoresis (non-reducing) and a second overlay gel (containing amylopectin β-limit dextrin) is placed on the separating gel. After incubation the overlayed gel is stained with iodine. LD hydrolyses the α-1,6 linkages in the amylopectin β-limit dextrin to release unbranched chains of α-1,4 linked glucose units that are sufficiently long to give a blue colour with iodine (Kruger and Marchylo, 1978). The presence of α-amylase in extracts hinders characterisation of LD by

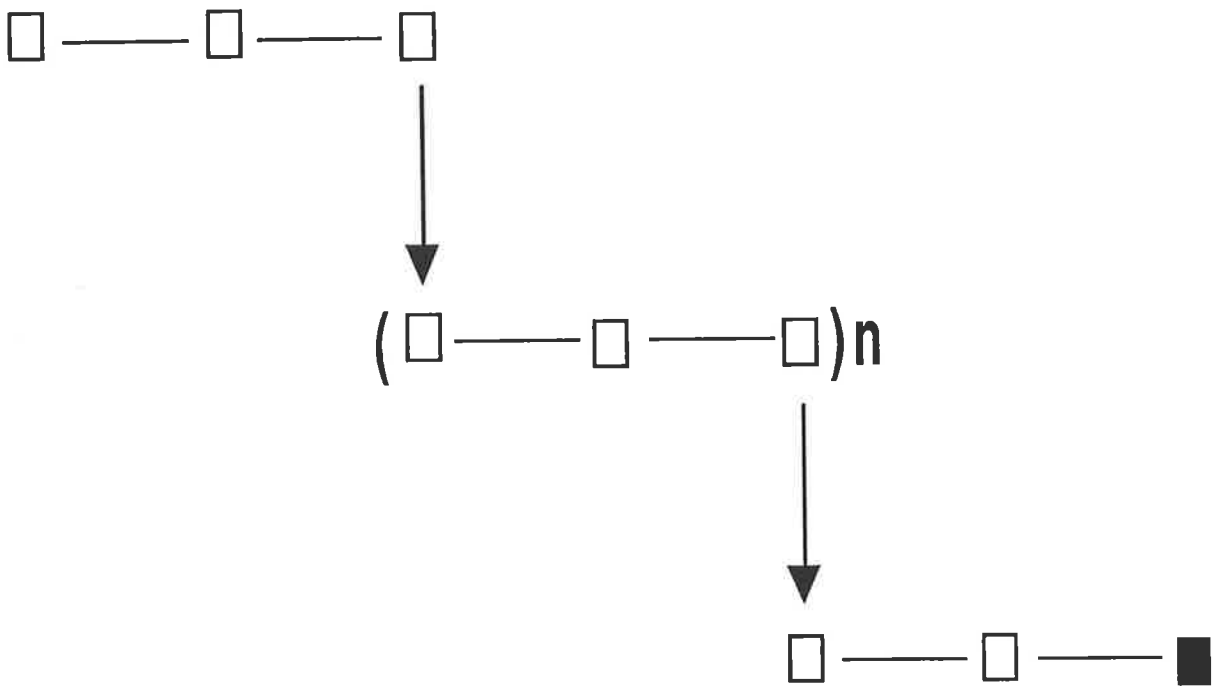



**Figure 1.2**

Schematic diagram of the pullulan polymer.

□ : Glucose ——— : α–1,4 bond

■ : Reducing end ⟶ : α–1,6 bond

this method unless α-amylase is removed from the extract for example by immunosorption with an α-amylase immune serum prior to isoelectric focusing (Lenoir *et al.*, 1984). (2) The overlay gel contains a pullulan-reactive red substrate which after incubation shows the presence of LD activity by clear bands on a red background (Yang and Coleman, 1987). However with both of these methods the resolution and sensitivity of the banding patterns obtained is poor. An alternative to zymogram detection of enzymes in gels is immunoblotting. This requires a monospecific antibody to an enzyme. Immunoblotting has been used to examine the isoenzymes of the α-amylase inhibitor from wheat and rye (Sadowski *et al.*, 1986).

## 1.4 Synthesis of Limit Dextrinase

Studies on the changes in LD during seed life (ripening and germination) have used pullulan based assays to measure LD in crude extracts. These assays are not completely reliable (see 1.3) and so results should be interpreted with caution. Mature cereals show varied amounts of LD activity decreasing in the order; oats, barley, wheat and rye (Yamada, 1981a).

The presence of limit dextrinase in ungerminated cereals might be related to incipient sprouting of a small amount of the cereal sample or synthesis of the enzyme during kernel development. The molecular forms of LD were examined in developing kernels of wheat (Kruger and Marchylo, 1978) using β-limit dextrin zymograms. Two bands were found, believed to be LDs. Extracted protein from the bands caused an increase in reducing sugars after incubation with pullulan suggesting these bands were LD. Limit dextrinase was present during ripening and the pullulan hydrolysing activity remained until full maturity.

Manners and Yellowlees (1973) reported a 10 fold increase in LD activity of barley and rye and a 4-5 fold increase in wheat after 4 days germination. Lee and Pyler (1984) found activity did not appear until after three days of germination whereas Manners and Yellowlees (1973) detected activity at day 1. Reports also vary in the timing of maximum activity during germination (petri dish experiments) of four days (Manners and Yellowlees, 1973), seven days (Grieg, 1963) 10 days (Lee and Pyler, 1984) and with malting of 11 days (Pratt *et al.*, 1981). These differences reflect variation in the cultivars tested and the methods used to assay LD and therefore it is difficult to be certain that LD appearance during germination is slow. The use of a specific and sensitive assay for LD and the assessment of several cultivars is required to be certain whether the synthesis of this enzyme is initiated at a later period during germination than say α-amylase. The implication is that in a conventional 4-5 day malt, LD does not have time to develop fully. There may therefore be potential for improving the breakdown of the starch if the enzyme can be synthesized more rapidly. Cultivars may differ in their rates of LD synthesis and in the amount of enzyme synthesized.

Hardie (1975) using de-embryonated and dehusked barley seed (half seeds) showed that gibberellic acid (GA) induced the formation of limit dextrinase in a dose dependent way.

At concentrations below $10^{-8}$ M no stimulation occured in barley half seeds. Enzyme activity increases more rapidly during germination and malting with the addition of GA to the steep water but did not produce earlier appearance of the enzyme (Lee and Pyler, 1984). Maximum stimulation was achieved with 2 mg/kg in the steep water.

### 1.4.1 Mechanism of Increase in Limit Dextrinase Activity during Germination

The increase in LD activity during germination could be due to *de novo* synthesis or activation of a pre-existing enzyme or a combination of both. There are four ways of demonstrating *de novo* synthesis of an enzyme. (1) The tissue can be exposed to a radioactive amino acid, and after a suitable time the enzyme is purified and the specific activity determined. (2) The tissue is exposed to deuterium oxide so that the heavy atoms enter amino acids and hence proteins. Enzymes that are synthesized *de novo* show a shift in the profile of enzyme activity towards higher density, measured after centrifugation. This technique was used to demonstrate the *de novo* synthesis of α-amylase (Filner and Varner, 1967). Hardie (1975) showed, using dehusked barley germinated with deuterium oxide, a shift in the buoyant density of LD determined by equilibrium sedimentation in a CsCl gradient. This provides good evidence for *de novo* synthesis of LD. In the same study, LD synthesis was shown to be inhibited by both cycloheximide, a protein synthesis inhibitor and actinomycin, an RNA synthesis inhibitor which add additional support to this mechanism. (3) A quantitative increase in enzymatic proteins (immunological activity) which parallels an increase in enzymatic activity supports the view for *de novo* synthesis of the enzyme. Direct proof of *de novo* synthesis may be provided by combining the *in vivo* incorporation of labelled amino acids into proteins with the antigenic identification of proteins. Neither of these approaches have been used for the study of the mechanism of increase in LD activity during germination. This approach was used to show *in vivo* synthesis of α-amylase I and II isoenzymes during wheat germination. Seeds were germinated in water containing $^{14}C$-labeled amino acids . By immunoelectrophoresis of the extracts of germinated seeds with an immune serum specific for α-amylases of germinated seeds, the two precipitin bands corresponding to the enzyme became labelled (Daussant *et al.*, 1977). (4) A cDNA probe to the mRNA of a protein would give direct indication of synthesis. For example, Higgins *et al.* (1976), using *in vitro* translation techniques, demonstrated that gibberellic acid increased the level of translatable mRNA for α-amylase and the rate of α-amylase synthesis *in vivo* was correlated with the level of mRNA for α-amylase.

In some plant species, the germination process also involves the activation by reductants capable of breaking disulphide linkages. In non-glutinous rice seeds, Yamada (1981b) considers the conversion of a 'bound' form into a 'free' form by reductants and proteolytic enzymes is enough to account for the increase in LD activity during germination without invoking *de novo* synthesis. Yamada hypothesized that during maturation in rice,

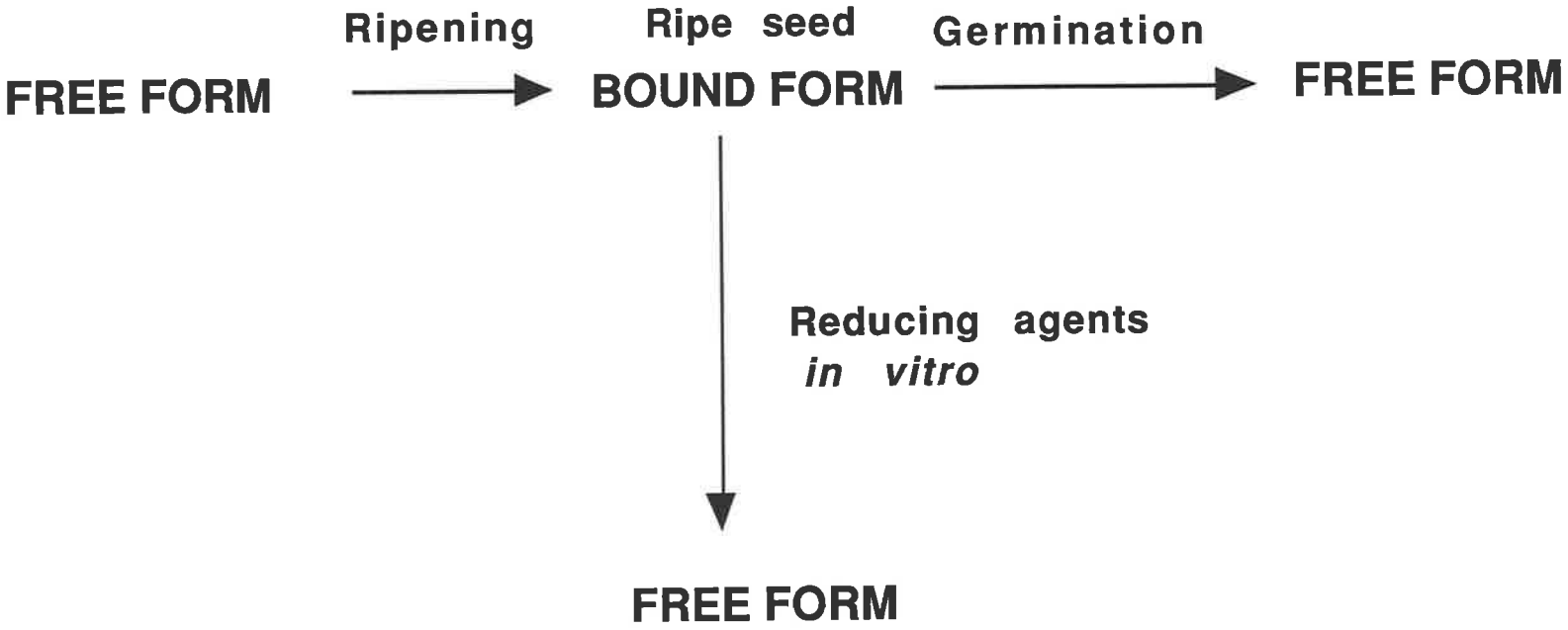



**Figure 1.3**

Mechanism of conversion of bound to free limit dextrinase in rice (Yamada, 1981b).

the active form ('free') of the enzyme appears after anthesis reaching a maximum amount until 20 days post-anthesis, then decreases, whereas the inactive form ('bound') appears after 20 days post-anthesis increasing rapidly until maturity. It has been shown by incorporation of radioactive amino acids that the enzyme is synthesized in an active form and is then gradually converted into an inactive form which accumulates in the mature seed (Yamada, 1981b). During germination this pool of inactive enzyme is converted to the active or 'free' form (Fig. 1.3). Aisien *et al.* (1983) measured the changes in LD activity in the embryo and endosperm during germination of sorghum. They showed an early rise (activity increased rapidly after 24 h) in LD activity and suggested this was due to the enzyme existing in the endosperm of the ungerminated seed as a 'bound' form.

Incubation of rice, oat, barley, wheat and rye flour with a range of thiol reductants and papain causes increased enzyme activity possibly due to reduction of disulphide bonds (Yamada, 1981c). In peas, the LD is present in a pre-cursor form and is released by protease action during germination (Yellowlees, 1980). Treatment of barley kernels with metabisulphite prior to extraction increases the total LD activity recovered (Lenoir *et al.*, 1984). Whether this is due to stimulation of enzyme activity or the conversion of an inactive ('bound') to active ('free') form of the enzyme is uncertain. This raises the possibility that some of the barley LD could be present in the 'bound' form, which is released during germination. From the evidence available to date it is not possible to say how much of the enzyme present during germination is synthesized *de novo*. The situation for LD in barley is uncertain, for example; is LD present in developing and mature seed?, how much enzyme is present in the 'bound' form compared to the quantity synthesized during germination?,and what is the mechanism of release of 'bound' enzyme? Further studies are required to answer these questions and to determine the importance of the 'bound' form of LD in barley.

There has been no work published on the location of the enzyme during synthesis and secretion. It was suggested by Hardie (1975) that "enzyme induction within the endosperm involving protein synthesis implies that it must occur in the aleurone layer, which is the only living part of the endosperm in mature seed". The localisation of LD in seeds is important for understanding its function during grain development, maturation and germination. Immunohistochemistry techniques have been used to determine the location of α-amylase (Gibbons, 1979, 1981) and β-amylase (Laurière *et al.*, 1986). Utilizing these techniques to determine the exact cellular distribution of LD would be invaluable.

## 1.5 Effect of Barley Genotype and Environment on Limit Dextrinase Activity

Very little is known about the genetic variability for LD activity. Kneen and Spoerl (1948) measured limit dextrinase in eight barley cultivars grown at seven locations in North America and found differences in activity. Lee and Pyler (1984), measured LD activity in

extracts of 10 day germinated barley from seven cultivars grown at four locations over three growing seasons. They found that cultivar, growing location, and season all had a significant effect upon activity. There was no interaction between cultivar and growing location and season, but the interaction between location and year was significant. The range in activity between the small number of cultivars tested was 2.5 fold. These results suggest that differences exist in the ability of these cultivars to produce LD and therefore there is potential to manipulate the development of LD in a breeding program to produce barley with a high LD activity. However, many more cultivars need to be tested from a broad genetically diverse population (including 'wild' populations) to better define the extent of the variability.

## 1.6 Limit Dextrinase - Role in Malting and Brewing

One of the earliest reports of the presence of LD in malt was hinted at from the work of Kneen and Spoerl (1948) who wrote " it appears that limit dextrins resulting from the action of malt enzymes on starch contain anomalous α-1,6 glucosidic linkages. Apparently these linkages are resistant to carbohydrases such as α- and β-amylase. It might be hypothesized then that the action of malt limit dextrinase could be primarily one of breaking the α-1,6 glucosidic linkage". Support for this hypothesis came later from the work of Hopkins and Wiener (1955). They showed, using a semi-pure preparation of α-amylase, 12-16% of the endosperm starch was not degraded. When malt extract was added to starch, all the starch was degraded (by α- and β-amylase) and presumably, another enzyme in malt was capable of degrading the α-1,6 bonds which are not attacked by α-amylase and β-amylase. Possible candidates were α-glucosidase and the R-enzyme described earlier (Hobson *et al.*, 1954). Hopkins described the R enzyme as limit dextrinase because the substrate, limit dextrin, is degraded by malt and not by the amylases. Later work showed that the limit dextrinase described by Hopkins was the R-enzyme (see section 1.1) which was responsible for breaking the α–1,6 bonds (Hopkins and Wiener, 1955). Amyloglucosidase (from *Aspergillus niger*) hydrolyses both α-1,4 and α-1,6 bonds but is not present in barley.

The starch granules (ungerminated) are first attacked by α-amylase since β-amylase and LD cannot hydrolyse starch granules unless they have been first gelatinized and liquefied (Maeda *et al.*, 1979). Gelatinization and cooling disrupt the starch granules and amylose and amylopectin are solubilized. Both α-amylase and β-amylase attack the α-1,4 bonds which form the straight chains of the amylose and amylopectin molecules. The α-1,6 bonds which form the branch points in amylopectin are attacked by LD (Fig. 1.4).

During malting, the synthesis of hydrolytic enzymes and the breakdown of cell walls and protein occur. Starch hydrolysis is limited during malting, but if excessive enzyme attack on large starch granules occurs this can lead to high malting losses. The hydrolysis of

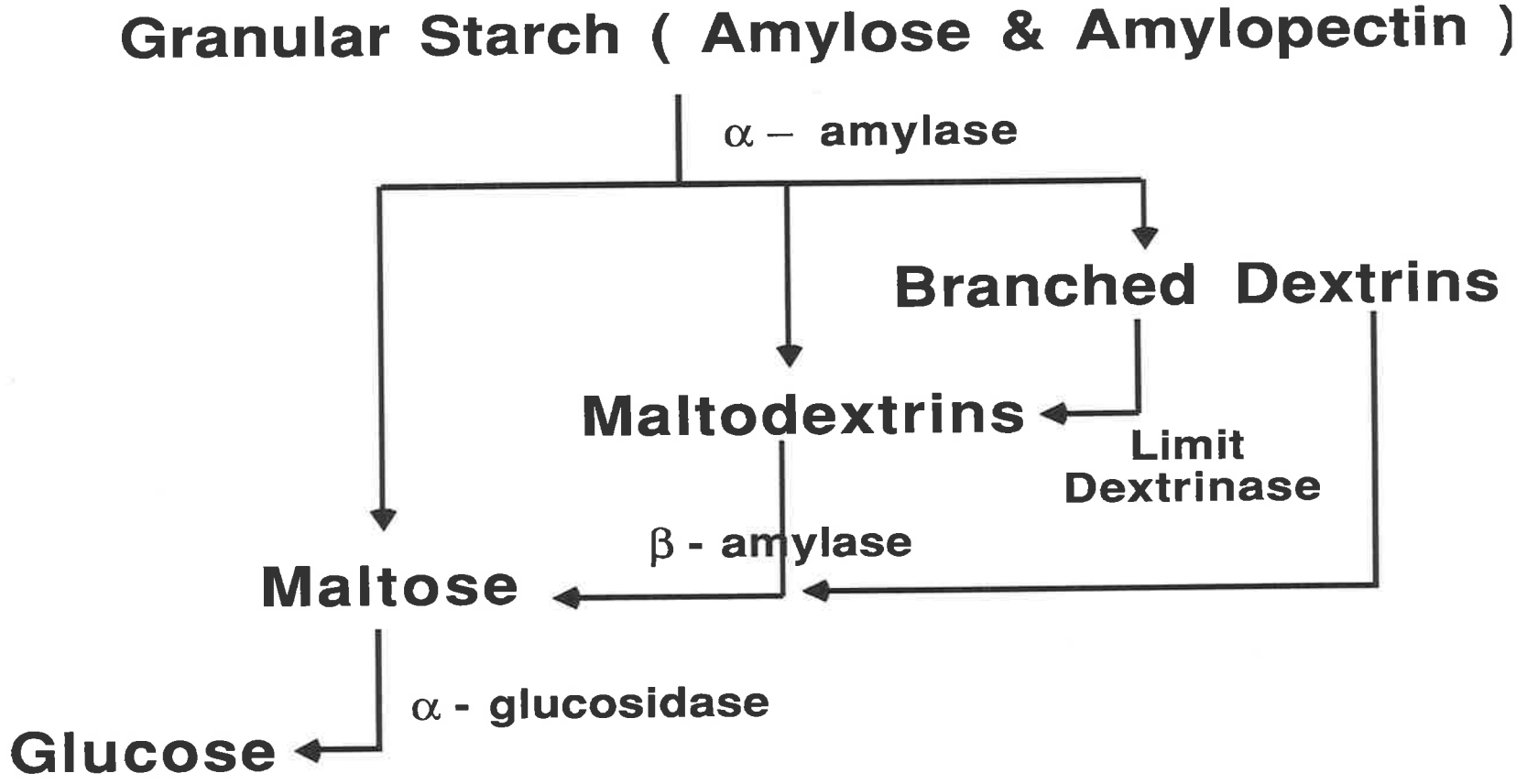



**Figure 1.4**

Pathways for the degradation of starch by starch degrading enzymes in germinating barley (modified from Palmer and Bathgate, 1976).

amylopectin by the amylases is incomplete and requires LD to convert the residual α- and β-limit dextrins into linear maltosaccharides.

Examination of a range of LD activities in different commercial malts reveals a large variation in enzyme activity (Manners, 1985a; Grieg, 1963). For example in Golden Promise LD activity was 15 units /500 seeds compared to Ymer of 257 units/500 seeds. High levels of amylases, LD, β-glucanases and peptidases may be required to improve barley malting quality. The role that LD plays in malting quality has yet to be addressed.

### 1.6.1 Effect of Kilning on Limit Dextrinase Activity

Kilning stabilises green malt by drying the seed to prevent further germination. During this process of controlled dehydration, the required colour and flavour of malt develops. Heat labile enzymes like β-amylase (Narziss *et al.*, 1973), endo β-1,4;1,3 glucanases (Loi *et al.*, 1987), and limit dextrinase (Lee and Pyler, 1984) are lowered during the kilning of malt. It is difficult to compare the stability of LD between different studies because the temperature/moisture relations and the cultivar used differ. Kilning loss is a function of temperature, moisture and cultivar. No loss in LD activity of malt heated at 50°C for 24 h occurs but over 50 % of the initial activity is lost after 3 h at 75°C (Kneen and Spoerl, 1948). Similarly, Lee and Pyler (1984) reported a small loss in activity at 65°C after 3 h but when the kiln temperature was increased to 82°C activity declined by more than 80 % within 3 h. In contrast, Manners and Yellowlees (1973) kilned barley malt for an unspecified period at 88°C and found no loss in LD activity. Their assay for LD suffers from the effects of α-glucosidase activity which could explain the apparent LD activity at this temperature. Pratt *et al.* (1981) reported a procedure for producing a malt with a high LD activity. A slow removal of moisture during the first 10-24 h of kilning at 35°C followed by an increase in temperature (but not exceeding 70°C) to dry the malt was advocated as part of a patented scheme designed to produce high LD malt. Kilning by this procedure reduced the activity of the green malt by only 18 %. Given the lability of LD to high temperatures, monitoring LD activity during kilning may be a good and sensitive indicator of the "status" of the kiln.

### 1.6.2 Effect of Mashing on Limit Dextrinase Activity

The process of mashing refers to the conversion of barley malt into a fermentable extract suitable for the production of beer. During this process the enzymic degradation of starch occurs (see Palmer and Bathgate, 1976 and Palmer, 1989; for a more detailed discussion of mashing and mashing systems). At the starch gelatinization temperature, α-amylase reduces starch viscosity by attacking both amylose and amylopectin randomly at α-

1,4-linked regions, releasing some fermentable sugars and exposing many non-reducing ends which β-amylase attacks. Starch granules do not gelatinize at one particular temperature but rather over a range (large granules, 61-63°C and small granules, 75-80°C). Published values for the gelatinization temperature range of barley starch granules are quite variable (Bathgate and Palmer, 1973; Banks *et al.*, 1973; Kang *et al.*, 1985) probably because several different techniques have been used to determine the values. The action of the starch degrading enzymes produces an extract containing a mixture of fermentable sugars and non-fermentable dextrins commonly referred to as brewers extract or the wort. The action of the LD in mashing is to hydrolyse the α-1,6 glycosidic bonds in starch and in combination with other starch degrading enzymes to degrade starch to fermentable sugars. A typical wort contains 91% carbohydrate, 6% protein and other minor material. This carbohydrate fraction consists of 22% α-dextrins and 75% fermentable sugars (Palmer, 1989).

The temperature of the mash is an important parameter affecting extract and the sugar composition of the wort. Mashing below 62°C may limit gelatinization of starch and result in a loss of extract. Mashing above 65°C will decrease the maltose and increase the dextrin materials in the wort because of the heat lability of β-amylase and LD and therefore reduce fermentability. These enzymes may be rate limiting enzymes in the generation of fermentable sugars from starch. During the course of a typical mash at 65°C the majority of the β-amylase will be destroyed (Narziss *et al.*, 1973). Although much less is known about limit dextrinase, the enzyme may have a lower heat stability than β-amylase. Harris *et al.* (1957) found that 96% of the starch is converted to fermentable sugars when malt is mashed at 40°C. This loss in the ability to degrade starch was thought to be caused by a decrease in LD activity and these investigators concluded that LD can work at 40°C but not at 65°C. This conclusion is based upon the finding that when a semi-pure preparation, thought to be a LD, was added together with salivary α-amylase, the resultant combination brought about an almost complete conversion of starch into fermentable sugars. To show that LD can degrade dextrins in mashing it may be necessary to measure the activity of LD during a typical mash and relate the activity to its action upon the starch with respect to carbohydrate profile and fermentable sugars.

Limit dextrinase activity during an ASBC scheduled mash was stable up to 55°C but declined rapidly as the temperature increased to 70°C (Lee and Pyler, 1984). Hopkins and Wiener (1955) found LD survived 65°C for 2 h but was inactivated at 70°C. A similar heat lability is seen with β-amylase, this suggests LD will only be effective in degrading soluble starch to a limited extent during mashing. In each of these studies different methods of assay were used and this may explain the different results. Even if LD activity is lost rapidly, the maintenance of activity may not lead to greater starch breakdown. It would be necessary to measure the action or the ability of LD to degrade α-1,6 linkages during a mashing cycle and relate this to the enzyme activity once improved methods of assaying the enzyme are available.

The analysis of the dextrins present in wort and beer may give an indication of the action of LD. Many of the dextrins (50 %) in wort and beer have a DP above 9 glucose units and are branched (Enevoldsen and Schmidt, 1974). This shows that the majority of the interchain linkages in amylopectin survives mashing, and suggests LD plays a minor role in starch degradation. These studies were conducted using malt with low LD activity. In malts with high activity the situation may be quite different. In fact Enevoldsen (1975) cited the following "the older brewing literature contains several examples which show that lightly kilned malt, green malt and prolonged conversion time leads to increased fermentability and reduced levels of dextrins". Thus LD could be important to manipulating the dextrin profile of the final beer.

The carbohydrate distributions of 22 different beers were examined and shown to have a characteristic 'wavy' distribution in their dextrin profiles (Enevoldsen and Schmidt, 1973). About half of the dextrins were oligosaccharides with a DP<10 and half had a DP>10. This pattern was similar to that found in wort and samples analysed during the mashing process. The typical 'wavy' distribution found in beers was significantly modified when pullulanase was added to the mash. The proportion of maltose and maltotriose increased and most of the DP>10 dextrins were converted to linear dextrins (DP 4-6) and fermentable sugars. In addition, increasing the quantity of pullulanase used led to greater conversion of the higher DP dextrins to fermentable sugars. However, the pullulanase used in this study was not pure and probably contained peptidases, since the total soluble nitrogen of the wort increased. It is therefore not clear whether the increase in fermentable sugars was solely due to LD. Thus, there is potential to manipulate the beer dextrin profile. Whether this can also be achieved by choosing to malt cultivars with high LD activity is not known. MacWilliam and Harris (1959) found that worts produced from green malt had a lower percentage of dextrins and more maltose and maltotriose. Such worts when fermented had higher fermentability compared to conventional brews using kilned malt.

The changes in the dextrin profile may alter the aftertaste (Ragot *et al.*, 1989) and mouthfeel (Brefort *et al.*, 1989) of the beer. This may be a desirable feature so that the manipulation of LD may serve the purpose of modifying characteristics of a beer, perhaps to suit a particular market.

The results of studies by Enevoldsen indicate that LD has limited action during mashing, since many of the higher DP dextrins are not degraded. The enzyme may have more action if:

(1) optimum conditions of malting and kilning are chosen (germinate barley for longer to increase synthesize of LD; reduce kilning temperature)

(2) the conditions of mashing are optimized to enhance the survival of the enzyme

(3) a malt high in LD is added to worts to convert starch or dextrins to yield fermentable sugars

(4) commercial pullulanase is added to the wort

(5) cultivars with high LD activity are chosen

(6) LD with improved heat stability will permit use at temperatures above 63°C

By manipulation of malting and mashing procedures, the activity of LD could be controlled leading to a wort composition with a desired content of non-fermentable dextrins. Changing the mashing program to lower temperatures or using malts that have a high LD activity may improve fermentability. To date there is no information about the relationship between malt LD activity and the action of the LD during mashing. The addition of exogenous enzymes has a number of constraints which will not be discussed here.

### 1.6.3 Limit Dextrinase - Role in Speciality Brewing, Distilling and Related Industries

The activity of LD may be important for the production of low calorie beers. Low calorie beers are usually produced by adding amyloglucosidase to the fermented wort which converts the dextrins to glucose. This has several problems (1) the amyloglucosidase is heat stable. Therefore if beer containing even minor amounts of amyloglucosidase is accidently mixed with other types of beer, the enzyme will degrade residual β-limit dextrins in the beer to free glucose producing unwanted sweetening. (2) it is thought in the brewing industry that amyloglucosidase can alter yeast metabolism which causes undesirable changes in flavour. The yeast prefers maltose instead of the large amounts of glucose produced. (3) The enzyme acts on the branched α-1,6 bonds in the residual dextrins relatively slowly at fermentation temperatures, and consequently fermentation time may have to be extended to achieve the desired fermentation. Pratt *et al.* (1981) suggested as an alternative, the addition of a malt containing a high LD activity to the mash could be used to degrade the dextrins to fermentable sugars, thereby reducing the calorie content of the beer.

Malts containing high LD or exogenous enzymes (pure malt LD or bacterial pullulanase) might find uses in brewing from barley. The addition of pullulanase to a mash of barley flour together with 10-20% malt added (to supply α- and β-amylases) is sufficient to yield adequate amounts of fermentable sugars providing mashing temperatures are kept below 63°C to prevent denaturation of the pullulanase (Enevoldsen, 1975). Recently, thermostable pullulanase activity has been described in a number of microorganisms. A pullulanase from *Clostridium thermohydrosulfuricum* has been purified which has optimum activity at 90°C (Saha *et al.*, 1988). This enzyme may be especially useful to the starch processing industry (Saha and Zeikus, 1989).

The production of dry beer with low sugar content represented about 30% of the Japanese beer market in 1988-89 and is also becoming popular in the U.S.A. (G. Allen, Joe White Maltings, personal communication). The role of LD in controlling the level of non-fermentable dextrins may be particularly important in the production of dry beer. Either

adding pure malt LD (or partially pure enzyme) or using a malt high in LD activity to wort prior to fermentation should reduce the dextrin content of the beer. This may be a preferred option to extending the fermentation time which is common practice for producing dry beers.

Despite the use of bacterial and fungal sources of β-amylase, pullulanase, α-1,6 glucosidase, amyloglucosidase and α-1,4 glucosidase for conversion of starch to maltose, these sources introduce increased costs and sometimes unwanted flavours. The production of malts containing a high LD activity may also have value to the starch processing industry (Pratt *et al.*, 1981).

### 1.6.4 Relationship between Limit Dextrinase Activity, Wort Fermentability and Alcohol Production

There are three main factors determining the apparent attenuation limit of the wort (Enari, 1975):

(1) carbohydrate composition of the malt
(2) nitrogen content of the wort
(3) the supply of minerals for the yeast .

The carbohydrates of wort consist of fermentable sugars (glucose, fructose, maltose, maltotriose) and non-fermentable carbohydrates or dextrins. The apparent attenuation limit (AAL) is a measure of the total amount of fermentable sugars in the wort. A common method to measure AAL is to compare wort specific gravity before and after fermentation to the limit (see chapter 2, section 2.6.3). These changes are very small and the method assumes that the metabolism of the carbohydrate fraction of the wort by the yeast is solely responsible for the change in specific gravity. It is possible however, that changes in the amino acid composition of the wort during fermentation could contribute to specific gravity changes.

Fermentable sugars are formed from the starch of malt and adjuncts during mashing. The major factors determining the amount of fermentable sugars formed are the mashing conditions (temperature changes and pH, Hobson *et al.*, 1954) and the activities of the starch degrading enzymes (amylases, α-glucosidase and limit dextrinase) produced during malting. Given a particular mashing schedule, fermentable sugar production will be dependent upon the amount of the starch degrading enzymes produced. Malt enzyme potential varies with barley cultivar (intrinsic potential), the procedure for malting i.e. length and temperature of germination, use of additives such as gibberellic acid, the number and frequency of steeps and steeping, abrasion techniques, and the kilning regimen used. It remains to be shown whether the variation in LD activity produced by these variables can directly change the apparent attenuation limit.

Enari (1975) reports that the total nitrogen content of the barley and the free amino nitrogen content of the wort affects the AAL. With increasing barley protein content, both wort amino nitrogen and AAL increase. Amino acids are important for yeast metabolism and fermentation will be slow if these are not sufficient.

The relationship between malt LD activity and AAL also called fermentability, is not clear. Bathgate *et al.* (1978) showed a negative correlation between malt LD activity and fermentability. Limit dextrinase was found not to be a limiting factor in determining AAL. These results were based on one cultivar (Golden Promise), and the malt was overmodified and therefore there may have been a shortage of substrate (AAL ranged from 85.8% to 88.4%). In contrast, Lee and Pyler (1984) obtained a correlation between fermentability (ranging from 68.3% to 75.5%), alcohol production and the LD activity of malt flour when the flours containing varying amounts of LD were added to fermenting wort. In an earlier report, Greig (1963) found no correlation between LD activity and alcohol production. These differences might be explained by the choice of the assay since the substrate used, α-dextrin, is not specific for LD (see 1.3).

The addition of pullulanase or malt extract containing a high LD activity to the wort before fermentation can increase AAL. For example, the addition of pullulanase to wort produced marked improvements in AAL from 81.1% to 85.2 % (Willox *et al.*, 1977) and from 71.2% to 76.4% (Enevoldsen, 1975). Pratt *et al.* (1981) increased AAL from 66.2 % to 80.8 % by addition of a high LD malt to the wort. There are no reports where a pure malt LD has been added to wort and shown to increase fermentability and alcohol production directly.

## 1.7 Role of Limit Dextrinase in Starch Degradation

In 1970 Erlander and Griffin (1970) suggested that debranching enzymes were involved in the synthesis of starch. The mechanism of action was thought to require hydrolysis of phytoglycogen. Later work by Yamada and Izawa (1971) showed no action on phytoglycogen.

A more likely role of the enzyme is involvement in the degradation of starch. There is no evidence that LD hydrolyses starch granules. However, partially purified LD from malted barley accelerated the rate of digestion of barley starch granules by the action of the purified α-and β-amylases to the same extent as a dialysed crude extract from malted barley (Maeda *et al.*, 1978). Perhaps the function of LD is to degrade branched α-dextrins released from amylopectin by limited amylase breakdown to unbranched linear dextrins. The resultant linear dextrins can then be hydrolysed by the α-and β-amylases and α-glucosidase to glucose.

Evidence for the in vivo role of LD points to synergistic action with β-amylase hydrolysing the initial products of granule degradation by α-amylase to small dextrins. A possible role for LD in starch degradation is shown in figure 1.4.

## 1.8 Conclusions

Limit dextrinase is present in small quantities in malt and is difficult to quantify accurately. The existing assays for measuring LD are either not specific or have low sensitivity. The availability of a monospecific antibody which recognises LD offers the ability to overcome difficulties with measuring LD and extend the possibilities for studying the enzyme. Immunochemical techniques could be developed once an antibody is available and be used for example, to quantify activity, detect isoenzymes and follow the changes in activity during the malting and brewing process.

In rice, the LD present in germinated seed is thought to arise by activation of the enzyme synthesized during kernel development. The presence of this type of enzyme in barley is not well understood.

The high sensitivity of immunoassay methods would be useful for investigating the presence and changes in LD during kernel development in barley because of the small quantities present.

Studies on LD are important for malt quality because LD is the only endogenous enzyme that can debranch the starch-derived compounds present in brewing. As a consequence, the ratio of fermentable to non-fermentable sugars in wort may be manipulated through control of the action of LD. This would also allow manipulation of another malt quality parameter, the AAL, which is an important criterion used by brewers in assessing malt quality. The precise relationship between this parameter and malt LD activity requires investigation.

A study of the genetic variability for LD in barley is a necessary prerequisite to making improvements in the activity of this enzyme through breeding. This may improve malt extract and fermentability because LD is an important hydrolytic enzyme involved in starch degradation. The first step would be to determine the extent of variation in enzyme activity between genotypes. The identification of cultivars with high activity could then be used in breeding studies to incorporate this useful characteristic into malting cultivars.

This thesis describes the development of immunoassays to measure LD activity and its isoenzymes in mature, germinated barley seed and in malt. These assays were then used to study genetic variability, changes in immunological and enzyme activity and the nature of the isoenzymes, during kernel development and changes during malting, kilning and mashing.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Protein Assay

The protein concentration of samples was measured using the 'Bradford' dye-binding method (Bradford, 1976) supplied in kit form (BioRad, Australia). Bovine IgG was used as a standard.

## 2.2 Enzyme Assays

### 2.2.1 Column Fractions and Purified Enzyme

Limit dextrinase activity of chromatography fractions and the purified enzyme were determined by incubating 0.1 ml of the test sample with 0.4 ml of a pullulan solution (0.5% w/v in 50 mM acetate buffer, pH 5.5) for 30 min at 50°C. Enzyme activity was stopped after 30 min by heating in a boiling water bath for 5 min and an aliquot assayed for reducing sugars (Dygert, 1965). Maltotriose was used as the standard and as a blank, extract incubated without added pullulan was used. A unit of activity is defined as the amount of enzyme catalysing the conversion of pullulan to 1 µmol of maltotriose per min at 50°C, pH 5.0. Specific activity is defined as units of enzyme per mg protein.

Calculation of units of activity:
Y (units) = (A x 1000) / 30
where A = mg maltotriose equivalents / molecular weight of maltotriose (504.4)
and maltotriose equivalents =

[mg/ml for test sample (reading from the standard curve) – mg/ml for blank] x dilution factor.

### 2.2.2 Extracts of Barley and Malt Flour

A dyed Red-Pullulan assay (McCleary, 1991) was used to measure enzyme activity (see for details 2.5) and an ELISA to measure LD immunological activity (see 4.3.3.1).

## 2.3 Electrophoretic Methods

### 2.3.1 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

The method for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was based upon that of Laemmli (1970). Samples were appropriately diluted in 'sample buffer' (0.065 M Tris-HCL, SDS 2 % w/v, glycerol 10 % v/v, bromophenol blue 0.002 % w/v, 2-mercaptoethanol - 5 µl/100 µl) and loaded into the wells of the stacking gel using a microsyringe. Electrophoresis was performed for 2.5-3 h at 40 mA constant current. The gel compositions used for the stacking and resolving gels are given in table 2.1.

**Table 2.1**

Gel composition of separating and stacking gels used for SDS-PAGE.

**Separating Gel**

| Composition of gel | Volume (ml) | |
|---|---|---|
| | Final polyacrylamide concentration | |
| | 8% | 10% |
| Acrylamide stock [1] | 5.0 | 6.25 |
| Double strength buffer (0.75 M Tris-HCL, pH 8.8, 0.2% SDS) | 12.5 | 12.5 |
| Deionised water | 7.50 | 6.25 |
| TEMED[2] | 0.035 | 0.035 |
| Ammonium persulfate (10% w/v) | 0.087 | 0.087 |

Stacking Gel

| Composition of gel | Volume (ml) (Final polyacrylamide concentration 3 %) |
|---|---|
| Acrylamide stock [1] | 1.0 |
| Double strength buffer (0.25 M Tris-HCL, pH 6.8, 0.2% SDS) | 5.0 |
| Deionised water | 3.9 |
| TEMED[2] | 0.011 |
| Ammonium persulfate (10%) | 0.044 |

1. Acrylamide stock: Separating gel; 39 % (w/v) acrylamide and 1.04 % bisacrylamide (w/v). Stacking gel; 30 % acrylamide and 0.80 % bisacrylamide.
2. TEMED: N N N', N'-tetramethylethylenediamine.

### 2.3.2 Isoelectric Focusing (IEF)

Polyacrylamide gels 0.3 mm thick (see table 2.2 for gel composition) were cast onto the hydrophilic side of gel bond PAG film (FMC BioProducts, USA) using the LKB gel casting unit (Pharmacia, Sweden) according to the instructions supplied by the manufacturer.

The catholyte used was 0.1 M NaOH and the anolyte 0.1 M $H_2SO_4$. The gel was prefocused for 30-45 min at 2,200 V, 50 mA, 5 W constant power or until the current was stable. The distance between electrodes was 9.5 cm. Samples (20 µl) were applied to 1.5 x 0.5 cm paper applicators (Whatman 3 MM) placed about 1 cm from the cathode. The applicators were removed from the gel after 30 min electrofocusing. When the purified LD was subject to IEF, the sample of enzyme was concentrated by ultrafiltration using a centricon microconcentrator PM 30 membrane (Amicon, Australia) centrifuged at 10,000 g for 5 min. Electrofocusing was complete after 5000 Vh (2,200 V, 50 mA, 15 W, constant voltage, about 2-2.5 h).

The pH gradient of the gel was measured by cutting 1.0 x 0.5 cm strips, soaking them in 2 ml of deionised water containing KCL (1 M), mixing occasionally for 1-2 h and measuring pH. The pH of the solution was measured with a Corning meter (Ciba Corning Diagnostics, England) coupled to an Orion electrode (Orion Research, Switzerland). Estimates of pI were obtained from a reference line relating migration

distance with pH. Alternatively, commercial pI markers were used (Sigma, low pI markers).

**Table 2.2**

Gel composition used for isoelectric focusing

| Composition of gel | Volume (ml) |
|---|---|
| Deionised water | 11.8 |
| Glycerol | 1.8 |
| Acrylamide monomer (30 % acrylamide; 0.8 % bis-acrylamide w/v) | 3.3 |
| Ampholytes pH 3.5-5.0 (LKB, Sweden) | 1.0 |
| TEMED | 0.025 |
| Ammonium persulphate (10 % w/v) | 0.050 |

### 2.3.3 Silver Staining

The procedure for silver staining of SDS-PAGE and IEF gels (Table 2.3) was modified from that described by the supplier of the developer solution used (Bio-Rad, Australia) to reduce cost.

## 2.4 Preparation of Cyclohexaamylose Sepharose 6B

Cyclohexaamylose Sepharose 6B was prepared by coupling cyclohexaamylose (Sigma) to epoxy-activated Sepharose 6B (Pharmacia, Sweden) by a modification to the procedure described by Vretblad (1974). Epoxy-activated Sepharose 6B (6 g) was suspended in RO water (18 ml) for 15 min, washed on a sintered glass funnel for 1 h and resuspended in 0.1 M sodium hydroxide. The gel was dried by suction and resuspended in coupling solution (800 mg cyclohexaamylose in 0.1 M sodium carbonate buffer containing dimethylsulphoxide, 50 % v/v with the pH adjusted to 10-10.5 using 0.1 M sodium hydroxide) and incubated at 40°C for 16 h with gentle shaking. After coupling, the gel was washed with about 500 ml of water, then 500 ml of an aqueous D-glucose solution (25 mg/ml) followed by a further wash with water, then equilibrated with 50 mM acetate buffer, pH 5 for 2 h.

**Table 2.3**

Silver Staining Procedure for Polyacrylamide Gels.

| Reagent | Reagent contact time (min) for different gel types | | Volume (ml) |
|---|---|---|---|
| | IEF (<0.5mm) | SDS-PAGE (0.75mm) | |
| 30% methanol/10% TCA/3.5% sulphosalicylic acid | 60 | – | 200 |
| 30% methanol/12% TCA | 120 | – | Few changes |
| 40% methanol/10% acetic acid | – | 60 | 400 |
| 10% ethanol/5% acetic acid | 30 | 30 | 400 |
| 10% ethanol/5% acetic acid | 30 | 30 | 400 |
| reducer (dithiothreitol 0.05% w/v) | 30 | 30 | 200 |
| Deionised water | 5 | 5 | 200 |
| Deionised water | – | 10 | 400 |
| Silver nitrate (0.1% w/v) | 30 | 30 | 200 |
| Deionised water | 1 | 2 | 400 |
| Developer (Bio-Rad, Australia) | 1 | 1 | 200 |
| Developer | 5 | 5 | 200 |
| Developer | 5 | 5 | 200 |
| Stopping solution (5% acetic acid) | 5 | 5-10 | 400 |

## 2.5 Red Pullulan Assay

The Red Pullulan (RP) assay (obtained from Megazyme Australia) was used to measure the LD activity in crude extracts of malt and germinated barley seed. The assay was performed according to the instructions supplied by the manufacturer (Megazyme, Australia) but with modifications to improve the assay. A description of the method and the modifications follows:

(1) The substrate was prepared by dissolving the Red Pullulan in a vigorously stirred solution of 0.5 M potassium chloride (2 % w/v). This solution was stored on ice before use because the substrate is unstable at room temperature.

(2) Barley grain or malt was ground in a Udy mill to pass a 0.5 mm sieve. Flour (1 gm) was suspended in extraction buffer (4 ml; 200 mM sodium acetate buffer containing cysteine (20 mM). The pH was adjusted to pH 5.0 using NaOH and incubated for either 5 h or 17 h at 30°C with occasional mixing. The slurries were centrifuged at 1,500 g for 10 min and the supernatant used in the assay immediately or stored at -20°C for later analysis.

(3) The assay was performed as follows:

The substrate (0.5 ml) and the enzyme extract (1 ml) were pre-equilibrated at 40°C then mixed and incubated for exactly 20 min at this temperature. The reaction was stopped by adding 2.5 ml of ethanol (80 % v/v modified from the 98 % stated by the manufacturer), mixed vigorously and allowed to stand at room temperature for 20 min then centrifuged at 50,000 g for 10 min (modified from 1000 g). Blanks were prepared by adding ethanol immediately after mixing the substrate with the extract. The absorbance of the supernatant was measured at 510 nm. Samples with an absorbance exceeding 1.0 were repeated in dilution (acetate buffer containing 1 mg/ml BSA). The standard curve relating limit dextrinase activity to absorbance is linear over the absorbance range of 0.1 to 1.0 (after subtraction of the blank absorbance value). This relationship varies with the batch of substrate used and each batch of RP should be standardised against known units of activity of purified barley limit dextrinase determined using the assay described in 2.2.1. All analyses were performed with batch MRP90201. The standard reference line applicable to this batch is :

$$\mathbf{RP = (A + 0.056) / 0.008992}$$

where: RP is malt limit dextrinase activity (U/ml); 0.008992 is the slope of the calibration line; A is the absorbance of the reaction minus absorbance of blank; 0.056 is the intercept.

## 2.6 Micromalting and Methods of Malt Analysis

### 2.6.1 Micromalting Procedure

Micromalting was performed by the procedure described by Sparrow *et al.* (1987) except that some modifications to the schedule were made (Table 2.4).

### 2.6.2 Mashing and Determination of Extract

Extracts were measured using modifications to the procedure described in the EBC methods of analysis. These modifications were the use of small (10 g) flour samples and the amount of water added is treated as a variable compared with the constant value (800) used in the EBC methods (MacLeod *et al.* 1991). A brief description of the procedure follows:

(1) Malt is ground to fine grind specifications and 10 g of flour is weighed (±0.010 gm) into a 100 ml erlenmeyer flask.

(2) The mashing program is shown in table 2.5

The formulae to calculate extract on a dry base are derived from ASBC tables:

$$P=((-466.947+(675.656 \times SG)) - (208.71 \times SG^2))$$

where SG is the average specific gravity of the wort from duplicate readings and P is the plato or gms of extract/100 g solution.

The percent extract on a dry base (E) is calculated as

$$E=(E1 \times 100)/(100-M)$$

where

$$E1=P(D+M)/(100-P)$$

M is malt moisture and D is the final mash dilution factor

$$D=(((MS-(FK+FL))/FL)) \times 100$$

**Table 2.4**

Micromalting Schedule.

| Step | Cycle time (h) | Temp. (°C) | Time rollers on (%) |
|---|---|---|---|
| **Wash** | 0.17 (10 min) | 15 | 100 |
| **Rinse** | 0.25 (15 min) | 15 | 100 |
| **Pre-condition** | 0.25 (15 min) | 15 | 100 |
| **Steep 1** | 8 | 15 | 25 |
| **Air rest 1** | 8 | 15 | 20 |
| **Steep 2** | 8 | 15 | 25 |
| **Air rest 2** | 94 | 15 | 22 |
| **Kiln 1** | 6 | 50 | 100 |
| **Kiln 2** | 8 | 55 | 100 |
| **Kiln 3** | 10 | 63 | 100 |
| **Kiln 4** | 4 | 70 | 100 |
| **Tank drain** | 0.25 (15 min) | 20 | 100 |

**Table 2.5**

Mashing program.

| Step | Temp (°C) | Time (min) | Add water (ml) | Comment |
|---|---|---|---|---|
| 1 | 46 | 0-30 | 40 | Start at 46°C |
| 2 | 70 | 30-57 | - | Ramp at 0.89°C/min |
| 3 | 70 | 60-120 | 15 | Hold at 70°C |
| 4 | 20 | 120-125 | 5 | Cool mash to room Temperature |
| 5 | | 125-140 | 15 | Add water and record flask weight |
| 6 | | | | Centrifuge 1,500 g, 10 min. |
| 7 | | Measure two replicate samples for specific gravity: record to ± 0.0001° after 2 min. | | |

where MS is the weight after mashing, FK is the flask weight and FL is flour weight. This procedure allows the rapid determination of HWE on small amounts of malt. The method is repeatable and closely correlated with the EBC procedure (MacLeod *et al.* 1991b)

### 2.6.3 Measurement of Apparent Attenuation Limit

The apparent attenuation limit (AAL) was measured by the EBC method with modifications to suit the smaller wort volumes. A brief description of the method follows:

(1) 40 ml of wort prepared from mashing the malt sample (see 2.6.2) was transferred to 100 ml erlenmeyer flasks and weighed (W1).

(2) The wort was heated quickly in a microwave to boiling point and boiled briefly to arrest any remaining enzyme activity, cooled and sterile water added to bring back to the original weight (W1).

(3) To 40 ml of wort sample, 1 g of dried yeast culture (Lager yeast, NCYC 1324, Intek, South Australia) was added and reconstituted in wort at 40°C for 5-10 min.

(4) The contents of the flask were sealed with a U-tube water trap attached to the flask via a one hole rubber stopper. Sterile water was added to the U-tube to maintain anaerobic conditions and prevent evaporation of ethanol. The rubber stopper was also fitted with a 20 G needle to which a sterile 3-way stopcock and 1 ml syringe were attached to allow sampling of the flask contents without breaking the air tight seal.

(5) The flask contents were equilibrated to 25°C and incubated for 60 h with gentle shaking in a reciprocating water bath.

(6) Samples of about 1 ml were collected at intervals before the yeast was added, about 36 h into the fermentation and at the completion of fermentation, 60 h.

(7) Fermentation was stopped by centrifuging a 20 ml sample of the yeast suspension at 10,000 g for 10 min to sediment the yeast cells.

(8) The specific gravity of the supernatant was measured (in duplicate) at 20°C.

(9) Calculation of the AAL :

$$AAL\ (\%) = ((OG-FG) \times 100) / OG$$

where OG is the original gravity in degrees of gravity and FG is the final gravity (attenuation limit) in degrees of gravity.

degrees of gravity = (specific gravity x 1000)-1000.

### 2.6.4 Moisture Determination

The moisture content of flour samples used for extract determination was measured using an Infralyser 400 (Technicon, USA). The infralyser was calibrated against oven moistures (IOB method) from several hundred different malt samples obtained from the Waite Institute Barley Improvement Program.

The moisture content of green or partially kilned malt was measured by oven drying described in the recommended methods of the Institute of Brewing (section 1.2).

### 2.6.5 Preparation of Malt Flour for Extraction of Limit Dextrinase

The standard procedure chosen for the extraction of LD from malt was gentle mixing of one part flour with four parts acetate buffer (200 mM) containing 20 mM cysteine , the pH of the buffer adjusted to 5.0 with NaOH and the extract incubated at 30°C for 5 or 16 h.

# CHAPTER 3

# PURIFICATION AND CHARACTERIZATION OF BARLEY LIMIT DEXTRINASE

## 3.1 INTRODUCTION

The use of an immunochemical assay to measure LD in crude plant extracts may overcome the inherent problems associated with assays of this enzyme (see section 1.3 for a full discussion). Detailed discussions on the application of immunochemical methods in cereal science and technology are available in reviews by Daussant and Bureau (1988) and Vaag and Munck (1986).

An immunoassay has the potential to be very specific for the identification of an antigen be it a protein, peptide, or carbohydrate in a complex mixture such as a plant crude extract. This is made possible by the high specificity of the antibody-antigen reaction where the antibody recognises a binding site unique to the antigen of interest. However, the antibody may recognise a similar site on an unrelated molecule thus producing a cross reaction. In this study it was therefore important to ensure the antibody was monospecific recognising only LD in crude plant extracts. This is more likely if immunization is performed using highly purified enzyme. The production of an antibody to LD involves the immunisation of an animal with highly purified limit dextrinase. The present chapter describes the methods used to purify this enzyme from barley malt.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Plant Material

Lightly kilned barley malt (cv. Clipper) was a gift from Joe White Maltings Pty., Ltd., Adelaide. To improve the yield of LD from malt the whole malted grain was treated with a strong reducing agent prior to extraction as described by Lenoir *et al.* (1984). Malt (1 kg) was incubated with potassium metabisulphite (0.2% w/v; 9 mM) at 45°C for 24 h, washed extensively in water, blotted dry on paper towelling and homogenized (1 kg/1.5 L) in 100 mM phosphate-citrate buffer, pH 6.8 at 4°C using an Omnimixer (Sorval, Du Pont, U.S.A.). The slurry was incubated for 2-3 h at 30°C and insoluble material removed by centrifugation at 10,000 g for 30 min at 4°C.

### 3.2.2
### Purification of Limit Dextrinase from Malt Extract

There are many procedures for purifying LD described in the literature (see 1.2.1). Only a few of these studies show evidence of obtaining a pure enzyme. Since the production of a monospecific antibody requires the immunisation with purified enzyme a modification of the purification method of Maeda *et al.* (1978) was used (see 3.4.1).

#### 3.2.2.1
#### Ammonium sulphate fractionation

The supernatant extract was first fractionated with 0-30 % w/v ammonium sulphate, after centrifugation the second supernatant was fractionated again with 30-60 % w/v ammonium sulphate. The majority of LD activity was found in the supernatant of this fraction. The LD was recovered by centrifugation at 10,000 g for 30 min at 4°C. All subsequent purification steps described were performed at 4°C.

#### 3.2.2.2
#### Desalting

The ammonium sulphate precipitate was redissolved in a minimum volume of 10 mM Tris-HCL, pH 7.5 (500 ml) and desalted on a Sephadex G 25 M (Pharmacia, Sweden) column (30 cm long x 11 cm diameter). The void volume (900 ml) was collected then immediately applied to the ion-exchange column.

#### 3.2.2.3
#### Anion-exchange chromatography

A suspension of DEAE Trisacryl (Pharmacia LKB, Sweden) was packed in a column (12.5 x 5 cm) and equilibrated with a 10 mM Tris-HCL, 100 mM NaCl (pH 7.5) buffer. The desalted enzyme preparation was loaded on the column at a flow rate of 300 ml/h. The column was washed with the equilibration buffer and fractions (20 ml) collected. The bound limit dextrinase was eluted by a linear gradient of NaCl from 100-500 mM at a flow rate of 75 ml/h (gradient volume 1200 ml). The eluent was collected in 10 ml fractions. All fractions were assayed for limit dextrinase by the method described in 2.2.1. The absorbance (280 nm) of the column eluent was monitored continuously with a Uvicord SII single wavelength detector (Pharmacia LKB, Sweden). The eluted fractions containing pullulan-hydrolysing activity were pooled and reduced in volume to approximately 15 ml by ultrafiltration using a YM 30 or PM 30 membrane (Amicon Scientific, Australia).

### 3.2.2.4
### Gel filtration chromatography

A suspension of Sephacryl S200 (Pharmacia, Sweden) was packed in a column (72 x 2.6 cm) and equilibrated with 50 mM sodium acetate buffer, pH 5 containing 100 mM NaCl. The enzyme solution was loaded on the column and eluted with this buffer at a flow rate of 11 ml/h. The eluent was collected in 5 ml fractions. Fractions containing LD activity were pooled.

### 3.2.2.5
### Affinity chromatography

A suspension of cyclohexaamylose Sepharose 6B (prepared as described in 2.4) was packed in a column (9.6 x 1.5 cm) and equilibrated with 50 mM sodium acetate buffer, pH 5. The enzyme solution was loaded onto the column at 10-20 ml/h. The column was washed with acetate buffer at 10 ml/h and the bound LD eluted with acetate buffer containing cycloheptaamylose (18 μg/ml) at a flow rate of 10 ml/h. The eluent was collected in 2 ml fractions. Fractions containing LD activity were pooled and dialysed (molecular weight cut-off 12,000-14,000 daltons) against acetate buffer to remove bound cycloheptaamylose. The dialysed protein was reduced in volume by ultrafiltration using a PM 30 membrane (Amicon Scientific, Australia). This preparation was checked for purity by SDS-PAGE.

### 3.2.3
### Characterization of Barley Limit Dextrinase

### 3.2.3.1
### Molecular weight determination

The molecular weight of the affinity purified enzyme (2 and 6 μg) was estimated using SDS-PAGE. Both 8 % and 10 % polyacrylamide gels were used for the estimation. After electrophoresis the gel was fixed and protein visualized with silver stain reagent (see 2.3.3). The molecular weight of the limit dextrinase was determined from a calibration line obtained from a plot of the molecular weight (plotted on a $\log_{10}$ scale) against distance migrated from the interface of the stacking and separating gels. A range of commercial low and high molecular weight standards were used.

### 3.2.3.2 Isoelectric point determination

Analytical isoelectric focusing of LD was performed in ultrathin (0.3 mm) polyacrylamide gels. The gels were cast in a LKB ultramould gel casting unit (Pharmacia, Sweden) onto gel bond. The gel contained the following final concentrations: 5.3 % (w/v) acrylamide; 0.045 % (w/v) N,N'-methylene-bis-acrylamide (Bio-Rad, Australia); 2.2 % (w/v) ampholyte (Ampholines, LKB), pH range 3.5-5; 10 % (v/v) glycerol; 0.14 % (v/v) TEMED; 0.027 % (w/v) ammonium persulfate (see 2.3.2 for details).

### 3.2.3.3 Effect of temperature on activity and stability

The effect of temperature on enzyme activity was determined by incubating a reaction mixture consisting of 400 µl pullulan (0.5 % w/v in acetate buffer), 10 µl purified enzyme, and 90 µl 50 mM acetate buffer, pH 5 at selected temperatures (30, 35, 40, 45, 50, 55, 60, 65, and 70°C) for 30 min The reducing sugars were measured as described in 2.2.1.

For studies on the effect of exposure time to high temperature on enzyme stability, the enzyme solution (12 µl purified enzyme diluted to 100 µl with 50 mM acetate buffer, pH 5) was incubated at 30°C and 55°C for 0.16, 0.5, 1, 2, 5, 23, and 48 h and the residual activity measured as described in 2.2.1.

### 3.2.3.4 Effect of pH on activity

The effect of pH on enzyme activity was studied by measuring LD activity as described in 2.2.1, except that the pH of the assay buffer was varied from 3 to 8. For pH values between 3 and 5.5, 100 mM sodium acetate buffer was used; between pH 4 and 7, 100 mM citrate-phosphate buffer was used and between pH 6-8, 100 mM phosphate buffer was used.

## 3.3 RESULTS

### 3.3.1 Enzyme Purification

The average results of four purifications are shown in Table 3.1. Limit dextrinase was purified 135 fold on average with a range of 90-600 fold. The degree of purification is usually expressed relative to the specific activity of the crude extract. The determination of

| Step | Total volume (ml) | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Fold purification |
|---|---|---|---|---|---|
| **Crude extract** | 1918 | 9990 | 1561 | 0.16 | - |
| **Ammonium sulphate** | 500 | 5551 | 276 | 0.05 | 1 |
| **Ion exchange** (DEAE Trisacryl) | 183 | 290 | 57 | 0.20 | 4 |
| **Gel filtration** (Sephacryl S200) | 60 | 37 | 32 | 0.86 | 17 |
| **Affinity** (Cyclohexaamylose Sepharose 6B) | 2.21 | 0.37 | 1.81 | 4.89 | 98 |

**Table 3.1.** Purification of limit dextrinase. Data in the table are the mean of four different purifications.

LD activity in the crude extract by the method described in 2.2.1 may be an overestimate because contaminating carbohydrases may hydrolyse maltotriose which is one of the products of LD action upon pullulan. The degree of purification is therefore expressed relative to the specific activity of the ammonium sulphate fraction.

Two discrete peaks of LD activity were found after ion exchange chromatography (Fig. 3.1). The majority of the LD activity was bound to the column and was eluted at a salt concentration of 250 mM. The LD in both peaks was later analysed by IEF-immunoblotting using the anti-LD immune serum. Identical banding patterns were obtained. This suggests the conditions used for binding were not optimal in terms of maximum retention of LD on the column. The nature of the LD activity in the unbound fraction was not investigated further.

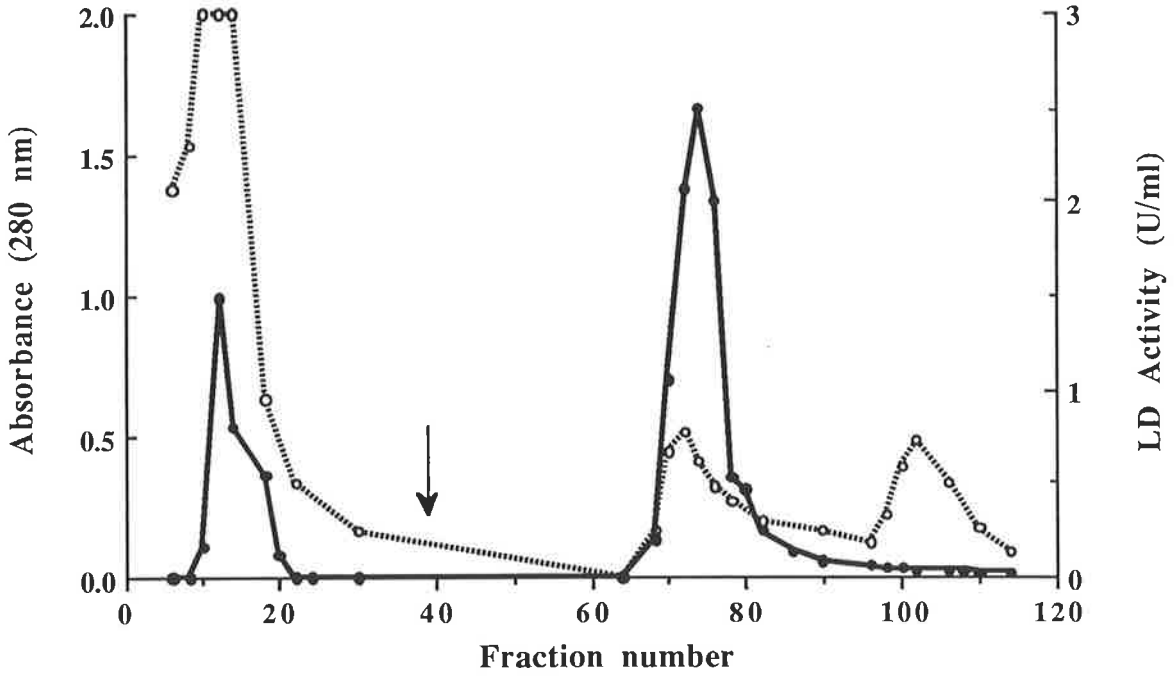



**Figure 3.1**

Ion exchange chromatography on DEAE Trisacryl of the desalted $(NH_4)_2SO_4$ fraction. Limit dextrinase activity (●); absorbance at 280nm (○). The arrow indicates the application of the NaCl gradient.

The gel filtration procedure separated LD from the majority of unwanted protein in the sample (Fig. 3.2) producing a good purification (Table 3.1). Despite the improvement in the purification, SDS-PAGE shows there are many contaminating proteins still present (data not shown).

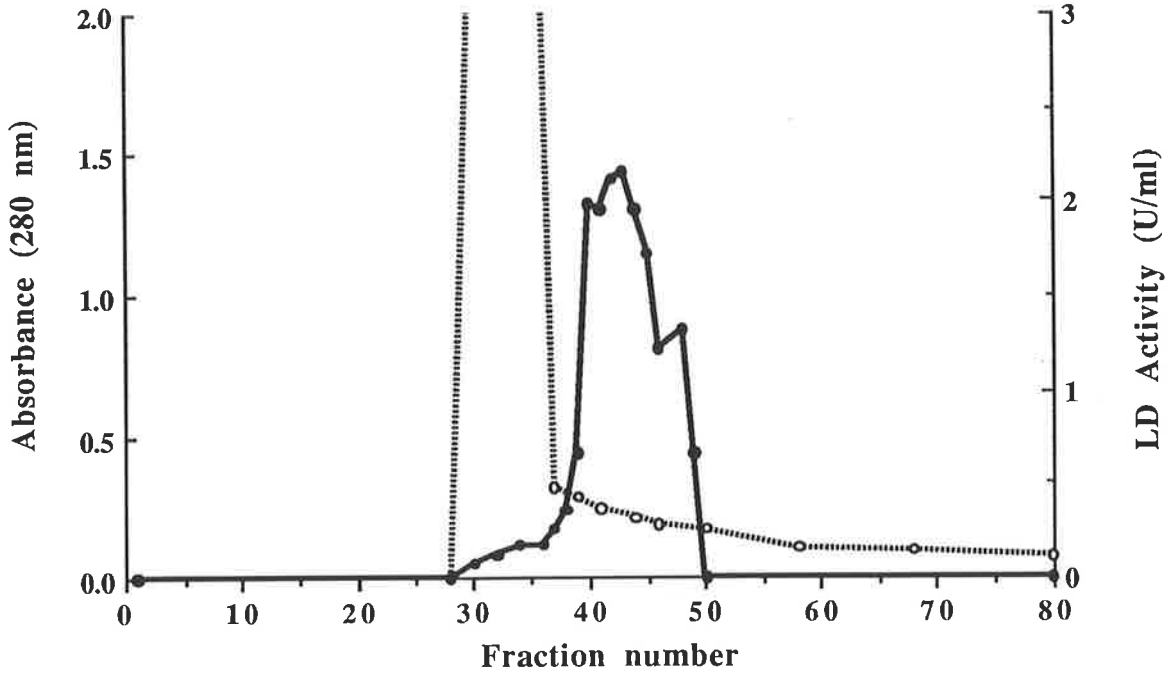



**Figure 3.2**

Gel filtration chromatography on Sephacryl S200 of the pooled fractions (68-80) from the ion exchange separation. The sample was loaded onto the column and eluted with 50 mM acetate buffer containing 100 mM NaCl, pH 5. Limit dextrinase activity (●); absorbance at 280nm (○).

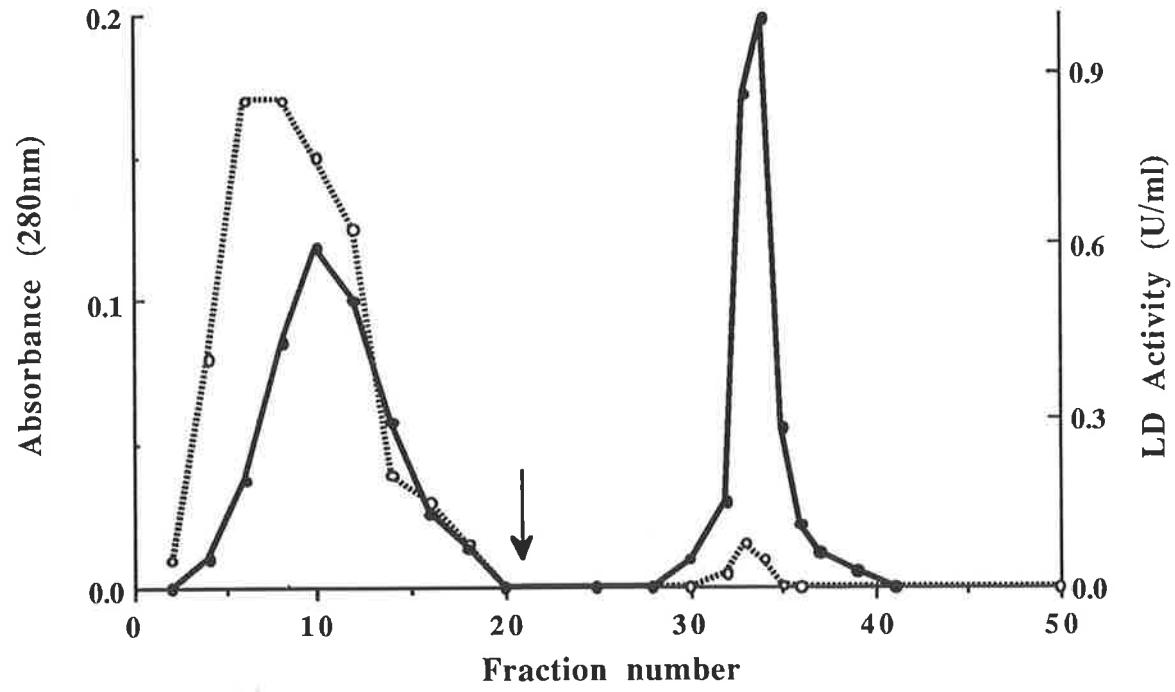
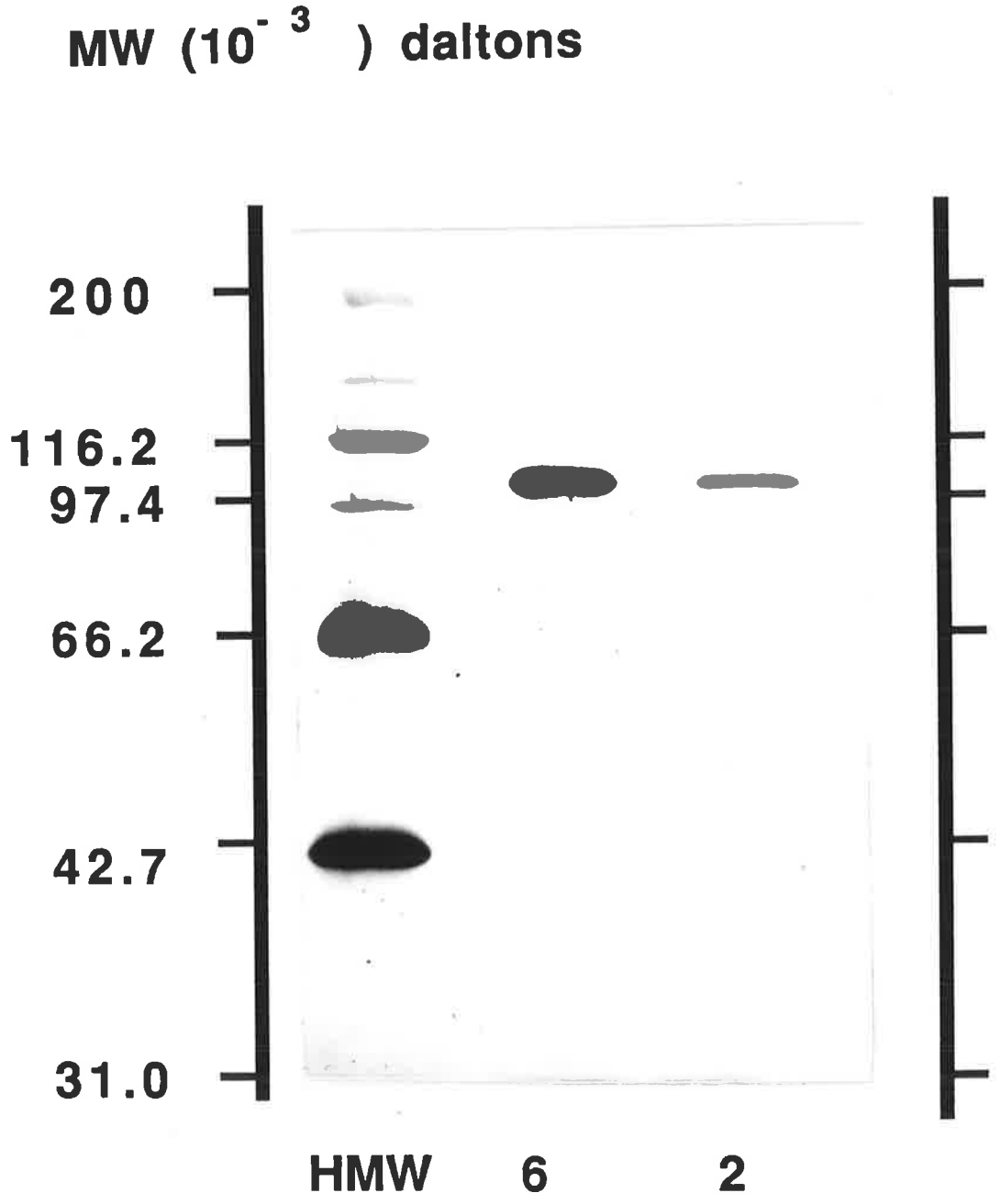



**Figure 3.3**

Affinity chromatography on cyclohexaamylose-Sepharose 6B. Limit dextrinase activity (●); absorbance at 280nm (○). The arrow indicates the application of cycloheptaamylose (18 µg/ml).

**Figure 3.4**

Silver stained SDS-PAGE of purified barley malt limit dextrinase. Molecular weight markers (HMW) used were (from bottom to top), ovalbumin (43kD), bovine serum albumin (66kD), phosphorylase b (97kD), β–galactosidase (116kD) and Myosin (200kD). Purified enzyme applied; 6 and 2 μg.


MW (10- 3 ) daltons
200
116.2
97.4
66.2
42.7
31.0
HMW
6
2

The final purification step using an affinity matrix incorporating an inhibitor of LD was required to purify the LD. About 35% of the LD activity loaded onto the affinity column did not bind and appeared in the column wash (Fig. 3.3).

The purity of the final enzyme preparation was assessed by SDS-PAGE (Fig. 3.4). Two bands were obtained; a major staining band ($M_r$ 104,000 ) and a very faint staining band of lower molecular weight ($M_r$ ca. 100,000) which was only visible with silver staining.

### 3.3.2 Characterization

#### 3.3.2.1 Molecular weight determination

The relative molecular weight ($M_r$) of the purified enzyme was 104,700 (Fig. 3.5).

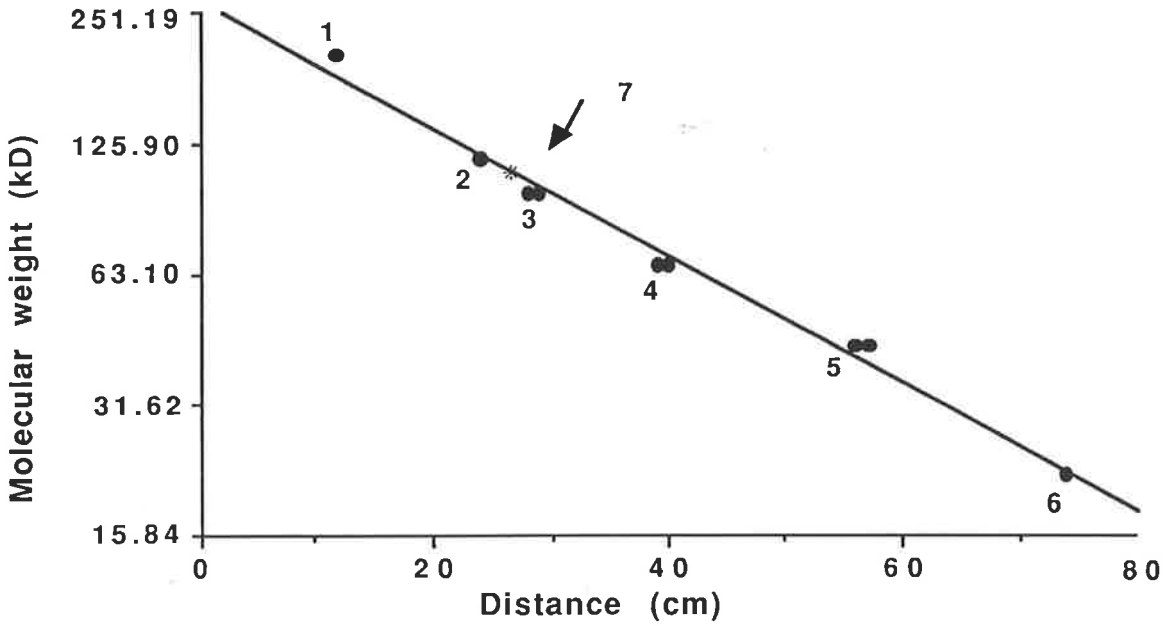
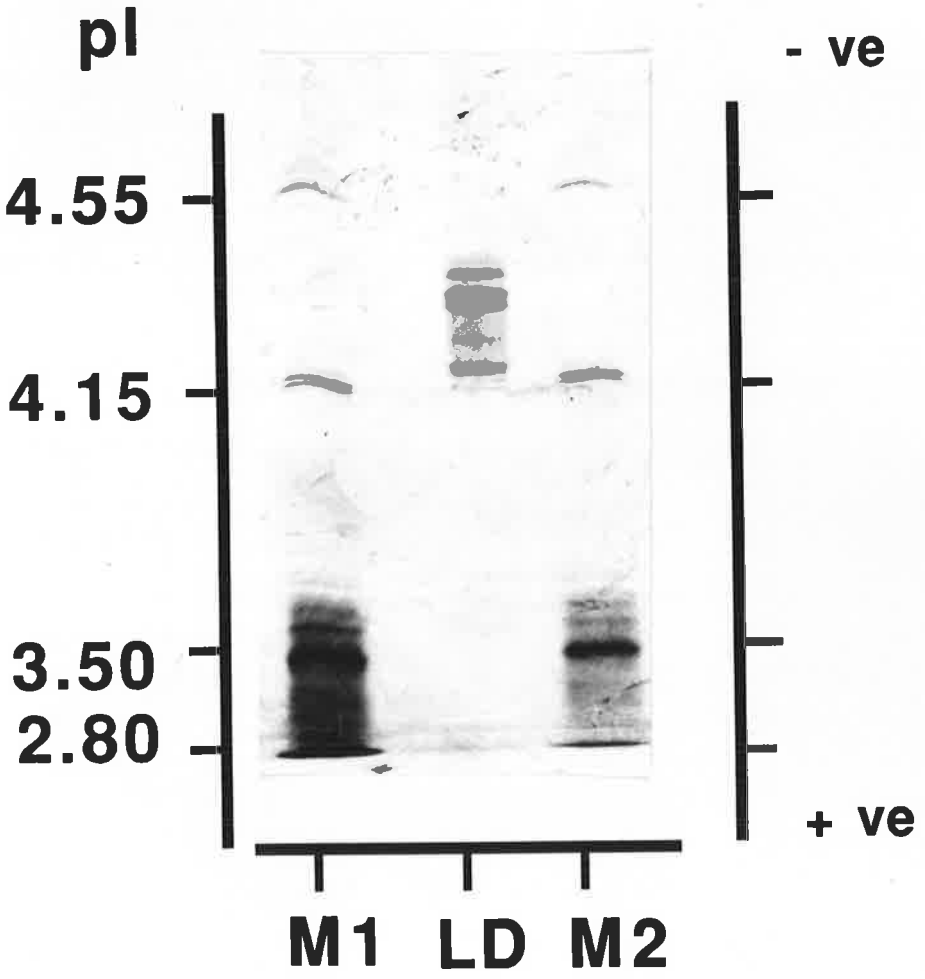



**Figure 3.5**

Calibration line for determination of molecular weight of limit dextrinase. The distance travelled from the interface of the stacking and separating gels for the enzyme was measured from the gel shown in figure 3.4. The molecular weights of the marker proteins are: 1, myosin (200kD); 2, β–galactosidase (116kD); 3, phosphorylase b (97kD); 4, bovine serum albumin (66kD); 5, ovalbumin (43kD) and 6, trypsin inhibitor (21.5kD). The $M_r$ of the purified barley malt LD (7, indicated by an asterisk) was found to be 104,700 daltons.

**Figure 3.6**

Isoelectric focusing of LD. Purified enzyme (5 μg) was subject to isoelectric focusing using a 0.4 mm polyacrylamide gel with a pH 3.5-5 gradient. The separated proteins were fixed and visualized with silver stain. Apparent pI was calculated from the position of reference proteins of known pI (Pharmacia 2.5-6.5 calibration kit) applied diluted 1:8 (M1) and 1:16 (M2).


pI
- ve
4.55
4.15
3.50
2.80
+ ve
M1
LD
M2

### 3.3.2.2
### Isoelectric point

Examination of the purified enzyme in a narrow pH gradient (pH 3.5-5.0) resolved at least six bands with apparent pI's ranging from 4.2 to 4.5 (Fig. 3.6). These same six bands were also detected in IEF-immunoblots of the purified enzyme using the anti-LD immune serum (data not shown).

### 3.3.2.3
### Effects of temperature on activity and stability

The effect of temperature on the activity and stability of limit dextrinase is illustrated in figures 3.7 and 3.8, respectively. The optimum temperature for activity was 55°C. Activity declined rapidly above 55°C and was not detected after 30 min at 70°C.

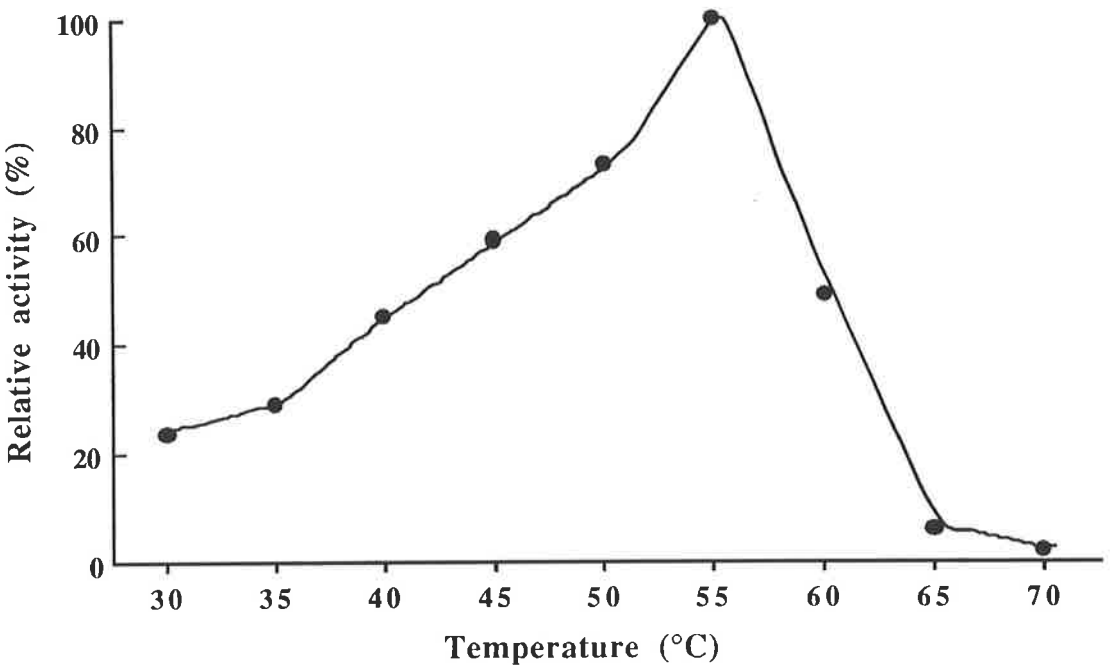



**Figure 3.7**

Effects of temperature on barley limit dextrinase activity. The purified enzyme was assayed as described in Materials and Methods (2.2.1) after incubation for 30 min at temperatures between 30°C and 70°C.

The stability of LD was assessed by measuring the changes in activity over time at 30°C and 50°C. The relative activity declined to 70% of maximum within 20 h at 30°C but remained stable for the period of measurement (49h). At the higher temperature the relative activity declined to 50% of maximal within 5 h, and also remained stable to 49 h.

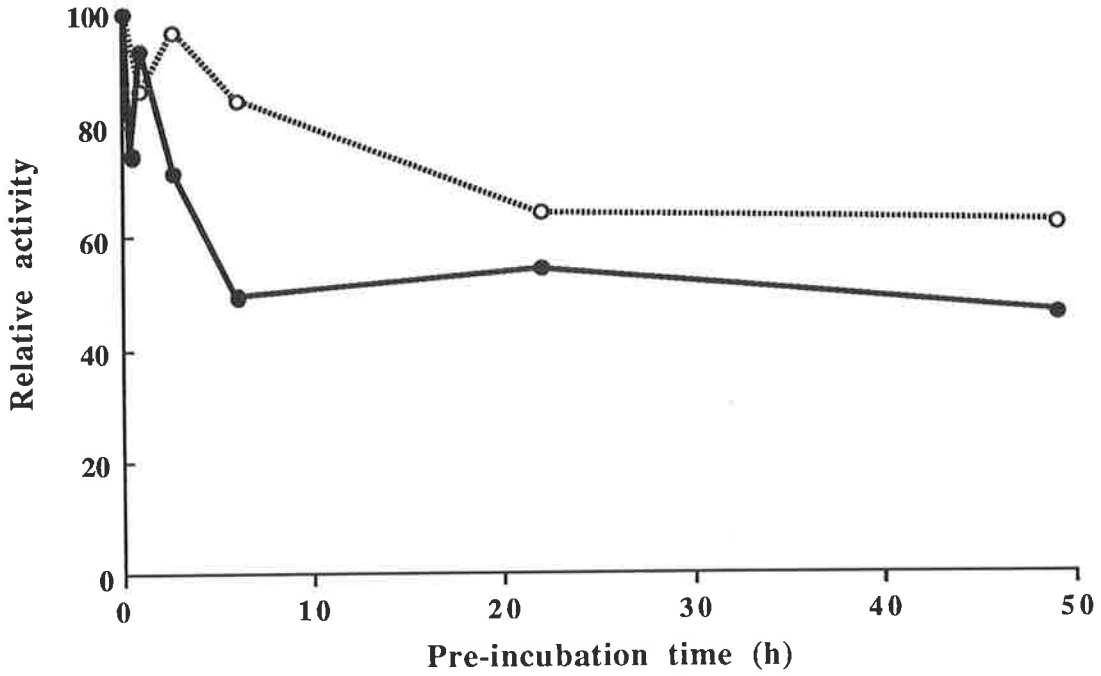



**Figure 3.8**

Effect of temperature on the stability of the purified barley limit dextrinase. The enzyme was assayed as described in Materials and Methods (2.2.1) after incubation at either 50°C (●) or 30°C (○) for various times.

### 3.3.2.4
### Effect of pH on activity

The effect of pH on LD activity is shown in figure 3.9. There were differences in LD activity in acetate and phosphate compared with the citrate-phosphate buffer for the same pH with higher activity obtained in the citrate-phosphate buffer. However, the optimum pH for activity was 5.5 regardless of the buffer used.

## 3.4
## DISCUSSION

### 3.4.1
### Purification of Barley Limit Dextrinase

Limit dextrinase has been purified to varying degrees in a range of plant species. The highest specific activity reported for barley is 10 U/mg (Maeda *et al.*, 1978). In order to obtain LD in high purity and good yield, which is essential for antibody production, the method of Maeda was used with the following modifications:

(1) The ammonium sulphate fraction was desalted by gel filtration rather than the time consuming dialysis.

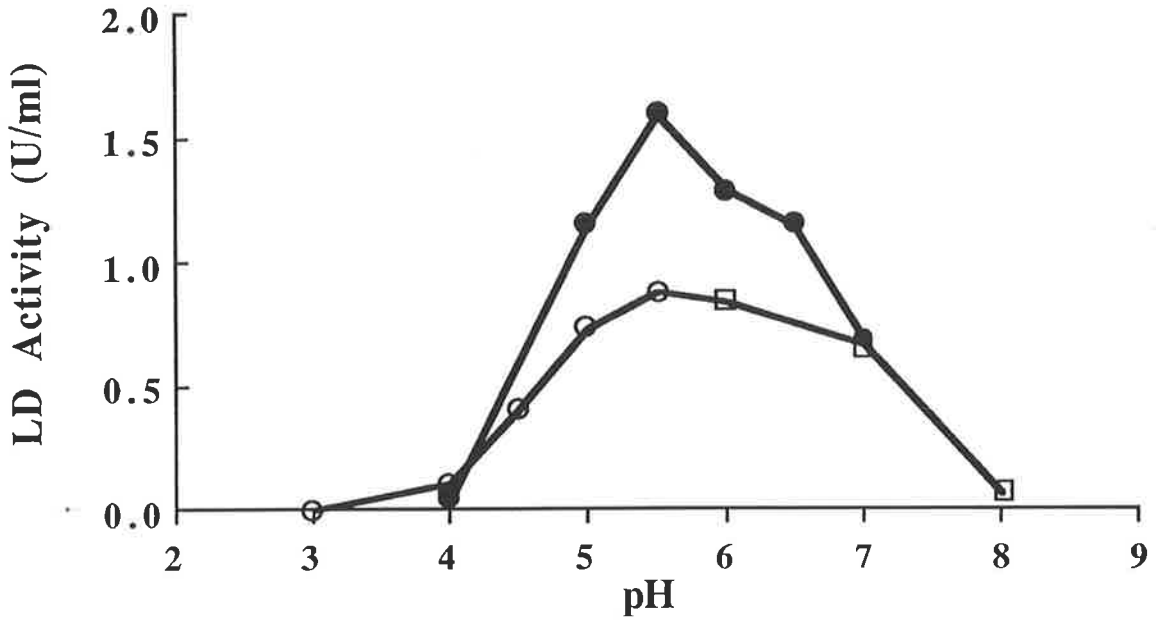



**Figure 3.9**

The effect of pH on limit dextrinase activity. The activity of purified limit dextrinase was assayed at pHs between 3 and 8 using three buffers: acetate, (○) and phosphate (□) to cover the range 3-5.5 and 6-8, respectively, and citrate-phosphate buffer (●) to cover the range 4-7.

(2) DEAE Trisacryl was preferred to DEAE cellulose, the latter readily compresses thus reducing flow rates. Also, the DEAE cellulose bound some components of the crude extract irreversibly.

(3) For gel filtration Sephacryl S200 was used which allows higher flow rates and thus faster throughput than Sephadex G100.

(4) A different procedure for preparing the affinity column was employed (see below).

Maeda used the manufacturers procedure for coupling the cyclohexaamylose to the Sepharose 6B. This was tried but the resulting gel was found to bind the LD too strongly so that even a 10 mg/ml solution of cycloheptamylose (1000 times higher than necessary) could not elute the enzyme. This is a problem since these concentrations are used to elute any bound α–amylase on the column. Instead a procedure modified from that of Vretblad, (1974) was used which allowed the successful purification of LD (see 2.4).

In the elution pattern of the affinity chromatogram (Fig. 3.3) two LD activity peaks were obtained. When the unbound fraction was again loaded onto the regenerated affinity column about 10% more enzyme bound and this could be eluted with cycloheptaamylose (results not shown). It was found that the re-use of the affinity column (regenerated between uses) led to a gradual decline in the capacity of the column to bind LD. Later results with the immunoassay show that the bound and unbound LDs have identical immunogenicity (results not shown). A similar problem was obtained by Maeda *et al.* (1978) using this type

of affinity column. Lecommandeur *et al.* (1988) used β-cyclodextrin as the ligand and only detected LD (using an anti-LD antibody) in the cycloheptaamylose eluted fraction. A β-cyclohexaamylose-Sepharose 6B column prepared by the procedure described in 2.4, did not however, produce different results (data not shown). These findings suggest that failure of the column to bind LD is related to the conditions chosen for chromatography and the capacity of the matrix. The effects of the buffer ionic strength and pH, sample volume, flow rate and the concentration of cycloheptaamylose were studied and at best only 67% of the LD loaded could be bound to the column (Fig. 3.3).

The limit dextrinase was not absolutely pure. A very faint protein staining band of lower molecular weight (ca. 100kD) was detected but only with silver staining (Fig. 3.4). Maeda *et al.* (1978) and Lecommandeur *et al.* (1988) report a single band on Coomassie stained SDS-PAGE of their enzyme preparations. No results with silver staining were reported. Silver staining is known to be about 100 times more sensitive than Coomassie blue so it is possible that these preparations contained impurities. Whether this minor band is LD or a degradation product caused by the purification procedure or an impurity is uncertain. Nevertheless, its presence represents only a very small percentage of the total protein visible in the gel.

### 3.4.2 Characterization of Limit Dextrinase

The molecular weight of the barley malt LD was found to be 104,700 daltons which is similar to other reports in barley of 102 kD (Lecommandeur *et al.*, 1988) and 103 kD (Maeda *et al.*, 1978). The molecular weight of other plant LDs varies from 70 kD in rice (Yamada *et al.*, 1980) to 180 kD for peas (Yellowlees, 1980).

The number of detectable isoenzymes are much fewer than found in α– and β–amylase (MacGregor, 1978 and LaBerge and Meredith, 1969). Two or three closely associated isomers (pI 4.70-5.0) have been reported in barley malt (Lenoir *et al.*, 1984). In the present study up to six closely spaced isoforms were found (Fig. 3.6). One or more of these bands may be due to the minor band in the LD preparation (Fig. 3.4). To determine if all of these bands are true isoenzymes a physical separation of each band or the identification of enzyme activity of each band *in situ* is required (see chapter 4). There are currently no zymogram procedures sufficiently sensitive enough to identify or resolve discrete bands of activity.

The pH optimum of most plant LDs is similar (pH 5.4-6.0). Amongst the plant LDs the enzyme is readily inactivated at 70°C with a temperature optimum for activity of 50-55°C (Kruger and Marchylo, 1978; Lee and Pyler, 1984; Iwaki and Fuwa, 1981, Hardie *et al.*, 1976). The enzyme is stable at 50°C for many hours but is rapidly inactivated at temperatures above 65°C. Similar results (Lee and Pyler, 1984) were obtained when malt extracts were held at 65°C for up to 1 h and the activity was lost within 30 min. This loss in activity is presumably due to denaturation and consequent loss of the enzyme function.

# CHAPTER 4

# DEVELOPMENT OF IMMUNOCHEMICAL METHODS

## 4.1 INTRODUCTION

Limit dextrinase is present in small quantities in malt and is difficult to quantify accurately. The availability of a monospecific antibody which recognises LD should overcome difficulties of sensitivity and with the accuracy of measurement, and also extend the possibilities for studying the enzyme. Immunochemical techniques could be developed once an antibody is available and be used for example, to quantify activity (using an ELISA), detect isoenzymes (using immunoblotting) and study the biochemistry of the enzyme. In the case of LD, there is a need for a specific, sensitive and rapid assay for measuring enzyme activity in plant extracts.

Much of the knowledge concerning LD obtained to date has relied on enzyme assays for the quantification of LD activity (Lee and Pyler, 1984; Manners and Yellowlees, 1973; Serre and Laurière, 1989). The most commonly used substrate for the assay of LD is pullulan which is not hydrolysed by the amylases (Lee and Pyler, 1984; Serre and Laurière, 1989). The enzyme α–glucosidase, present in germinated seed, appears to be able to hydrolyze α-1,4 and α-1,6 linkages in small dextrins and therefore may partially degrade pullulan (Jorgensen, 1964). Pullulan is degraded by LD mainly to maltotriose and linear oligosaccharides which are then assayed. Enzyme activity is proportional to the total amount of reducing sugars produced. However, maltotriose is also degraded by extractions of malt and perhaps endogenous α-glucosidase and the additional reducing groups produced may lead to erroneous results (Lee and Pyler, 1982). These authors found that adding an excess of yeast α-glucosidase to the reaction mixture converted all G3 molecules to glucose, which was then assayed. Although this overcame the problem of interference from other hydrolases in the extract the method has been reported to have low sensitivity and be time-consuming to perform (Serre and Laurière, 1989). Also, the products of initial hydrolysis of pullulan by LD, are linear oligosaccharides, not just maltotriose. Yeast α-glucosidase rapidly cleaves maltotriose, but has limited action on the next higher oligosaccharide i.e. $6^3$-α-D-maltotriosyl-D-maltotriose (Chiba *et al.*, 1962).

More recently, two assays (Serre and Laurière, 1990; McCleary, 1991) specific for both plant and bacterial LD, use a dyed-pullulan substrate which is not hydrolysed by α-glucosidase. These assays are easy and quick to perform. Their sensitivity is however no better than a rocket immunoelectrophoresis assay used to measure LD in barley and malt (Daussant *et al.*, 1987) but has inadequate sensitivity for some extracts (see 1.3).

The detection of LD and isoenzymes in electrophoretic gels is commonly carried out using zymogram techniques (Serre and Laurière, 1989; Lenoir *et al.*, 1984) with either β–limit dextrin and/or

pullulan substrates. These methods have poor resolution and are not suitable for studying isoenzyme variation.

Utilising a specific anti-enzyme antibody, it would be possible to study isoenzyme variation. The techniques of IEF and immunoblotting offer high resolution and sensitivity and are increasingly being applied to the study of cereal proteins (Daussant and Bureau, 1988).

This chapter describes the production of monospecific antibodies, the development of an enzyme linked immunosorbant assay (ELISA) for measuring LD in extracts of malt flour and immunoblotting procedures for detecting LD isoenzymes.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Immunisation Schedule

Polyclonal antibodies were prepared from a rabbit employing the following immunisation schedule based on the method of Harboe and Ingild (1973). Purified LD (200µg in 50 mM acetate buffer, pH 5) mixed with an equal volume of Freund's complete adjuvant (Commonwealth Serum Laboratories, Australia), and held on ice for about 10 min was injected (0.2-0.3 ml) subcutaneously at multiple sites in the lower back and hind leg region. A booster injection was given 28 days after the first injection (100-150 µg of LD in Freund's incomplete adjuvant) and a second 3 weeks later. Blood was collected 8 days after the final booster and the serum tested for the presence of antibodies to LD using a chequerboard ELISA technique (Johnstone, and Thorpe 1987)

### 4.2.2 Assessment of Antibody Titre

Chequerboard ELISA technique:

(1) Standards containing purified LD in a dilution series (final concentrations 25, 8.3, 2.77, 0.925, 0.309 and 0.103 µg/ml) were prepared in coating buffer (50 mM carbonate buffer, pH 9.6). Aliquots (100µl) were added to appropriately marked wells of a 96-well microtitre plate (Nunc-Immuno Plate MaxiSorp, Denmark) and incubated overnight in a 10°C cold cabinet.

(2) The solutions were removed from the wells by inverting the plate and tapping sharply onto a workbench. Each well was then washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (BioRad, Australia).

(3) Excess sites were blocked with skim milk powder (5 % w/v) in PBS/Tween) and incubated for 2-3 h at 37°C.

(4) Plates were washed as in (2)

(5) Dilutions of immune serum (1:50, 1:500, 1:4,500, 1:13,500, 1:40,500, 1:121,000) were prepared in PBS/Tween and 100µl added to appropriately labeled wells and the plate incubated for 3 h at 37°C.

(6) Plates were washed as in (2)

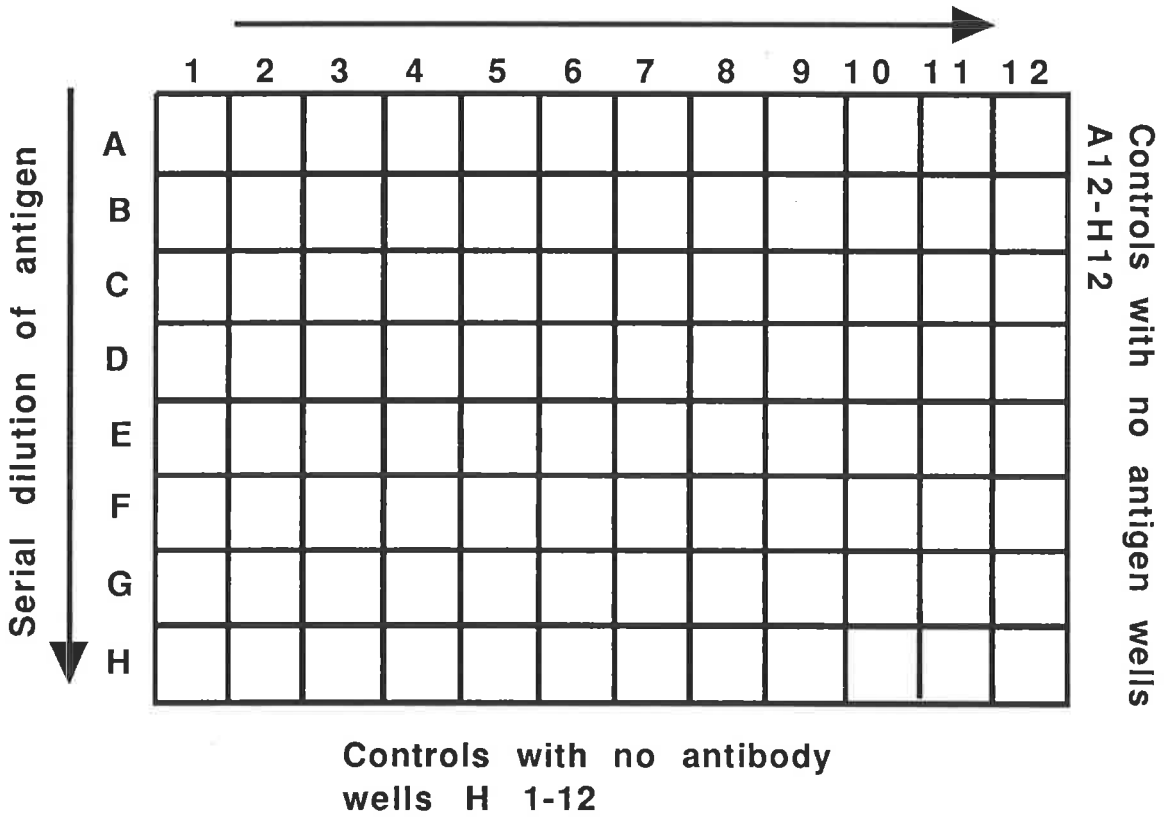
(7) 100µl of a 1:1000 dilution of goat anti-rabbit horseradish peroxidase antibody conjugate (BioRad, Australia) was added, and the plate incubated for 1h at 37°C.

(8) Plates were washed as in (2)

(9) 50µl of substrate solution was added to each well (prepared by adding 100 ml of ABTS buffer to 10µl of hydrogen peroxide). After 5-10 min, the reaction was stopped by adding 50 µl of oxalic acid (3% w/v). The absorbance of each well was measured at 414 nm using an ELISA plate reader (Titertek Multiscan, Flow Laboratories, Australia).

**Figure 4a**

A schematic diagram of the ELISA chequerboard assay design.



### 4.2.3 Separation of IgG from other Serum Proteins

The immunoglobulin fraction of the serum was purified using a Protein G Sepharose 4B column (Pharmacia, Sweden). The Protein G Sepharose supplied as a gel was packed into a plastic PD10 column according to the instructions supplied by the manufacturer. The column was equilibrated in 20mM sodium phosphate buffer, pH 7 containing 0.1 mM phenylmethylsulfonylfluoride (PMSF). The immune serum was centrifuged at 10,000 g for 5 min and the supernatant (1.25 ml) mixed with an equal volume of the above buffer and loaded onto the column at a flow rate of about 10 ml/h. The column was washed with 20mM sodium phosphate buffer, pH 7 and the immunoglobulin bound to the column was eluted with 100 mM glycine-HCL, pH 3. Fractions (ca. 1 ml) were collected and the absorbance of the column eluent monitored continuously at 280 nm. To preserve the activity of the acid labile IgG, eluted fractions were neutralised by adding a sufficient volume of 1M Tris-HCL, pH 9. The pH was checked with indicator paper. The protein concentration of each fraction was measured using the Bradford assay (see 2.1) and the purity of the separated IgG assessed by SDS-PAGE (see 2.3.1). Fractions containing IgG were pooled, concentrated by ultrafiltration on a PM 10 membrane (Amicon Scientific, Australia) and dialysed against PBS. Sodium azide (0.02% w/v) was used as an antimicrobial agent and aliquots were stored at -20°C.

### 4.2.4 Conjugation of Polyclonal IgG Antibodies to Horseradish Peroxidase

A solution containing 5 mg (250,000 U) of HRP (Boehringer Mannheim, Australia) dissolved in 1 ml of water was gently mixed with 0.2 ml of 100 mM sodium periodate for 20 min. The free periodate remaining after the reaction was removed by fractionation on a gel filtration column (PD10 column, Pharmacia, Sweden ) equilibrated in 1 mM citrate buffer, pH 4.5. The HRP-aldehyde was eluted from the column with 1 mM citrate buffer, pH 4.5 and the coloured fractions were collected (these contain the conjugate). The HRP-aldehyde conjugate (5 mg) was mixed gently for 3 h with the dialysed IgG (10 mg) prepared as described in 4.2.3, and the pH adjusted to 9.5 with 1 M sodium carbonate buffer, pH 9.5. Excess sites were blocked by incubation with 2 M ethanolamine, pH 9.6 overnight at 4°C. The conjugate was precipitated with a saturated ammonium sulphate, pH 7 solution. The pellet was resuspended in the original coupled volume with PBS and then dialysed against PBS. Aliquots were stored at 4°C in the presence of 0.1% (w/v) merthiolate (an antimicrobial agent). Immunological activity was tested as described in 4.3.3.1 and 50µl aliquots were stored at 4°C.

### 4.2.5 Immunoblotting of SDS-PAGE Gels for Determining the Specificity of the Antiserum

The electrophoresis of purified enzyme or grain extracts was performed in 8 % polyacrylamide gels containing SDS as described in 2.3.1. The separated proteins were transferred electrophoretically from the gel to a nitrocellulose membrane (Trans-Blot, 0.45µm, BioRad, Australia) using a mini-transblot cell (BioRad, Australia) according to the instructions supplied by the manufacturer. The buffer used was 25 mM Tris, 192 mM glycine, pH 8.8 containing 20 % (v/v) methanol. Transblotting was carried out for 3 h at 50 V, and 0.3 A. After transfer, the membrane was washed briefly in a rinse buffer (10 mM Tris-HCL, pH 7.4, 150 mM NaCl, 1mM EDTA and 0.1% Triton X100) and processed for immunoblotting as described below.

Immunoblotting

(1) Excess sites on the nitrocellulose membrane were blocked with rinse buffer containing skim milk powder (5 % w/v) or BSA (1 % w/v) and shaken gently overnight at 20-25°C.

(2) The blocking agent was discarded and the membrane washed for 5 min in rinse buffer and incubated with immune serum (1:1000 dilution in rinse buffer containing 1% w/v BSA) for 2 h with gentle shaking.

(3) Excess antibody was washed off the membrane by rinsing for 5 min with 5 changes of rinse buffer

(4) The membrane was incubated with the second antibody, goat anti-rabbit alkaline phosphatase conjugate (BioRad, Australia) diluted 1:2000 in rinse buffer for 2 h with gentle shaking.

(5) The membrane was washed as in step 3

(6) Bound alkaline phosphatase was detected by incubating for 1-4 min in substrate solution (1 mg/ml Fast Red TR (Sigma, USA) in naphtol AS-BI solution 50 mg naphtol phosphate (Sigma, USA), 20 ml N, N-dimethyl formamide, 20 ml water). The reaction was stopped by discarding the substrate solution and washing the membrane in several changes of deionised water.

### 4.2.6 Immunoblotting of IEF gels

The development of the method used is discussed in 4.3.7. Isoelectric focusing was performed on samples as described in 2.3.2. Immediately after focusing, proteins were transferred from the gel to nitrocellulose by passive diffusion for 45 min at 25°C. A sandwich arrangement consisting of (from bottom to top); gel, wet nitrocellulose membrane (moistened in PBS and carefully layered onto the gel to avoid trapping air bubbles) and three layers of 3 MM paper (moistened in PBS). After transfer, the membrane was blocked in BSA (1 % w/v in PBS or Tris buffered saline containing tween 20) for 2 h or overnight at room temperature and incubated with the primary antibody (anti-LD antiserum) for 1 h at room temperature. Bound antibodies were detected in initial studies with goat anti-rabbit horseradish peroxidase (BioRad, Australia) followed by the substrate, diaminobenzidine. Later, an alternative substrate was tested which showed improved sensitivity compared with other antigen/antibody systems (Blake *et al.*, 1984). In this case bound antibodies were detected with anti-rabbit alkaline phosphatase conjugate followed by the substrate nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Promega, Australia). Details of both procedures are given below:

#### 4.2.6.1 Method using horseradish peroxidase

This method was used initially but proved less sensitive than that described below. The only variations were the use of a goat anti-rabbit horseradish peroxidase as the second antibody and a different substrate (0.1 g imidazole, 0.01 g diaminobenzidine in 100 ml of TBST to which 50 µl of hydrogen peroxide is added).

#### 4.2.6.2 Method using alkaline phosphatase conjugate

(1) After proteins were transferred to nitrocellulose, the membrane was rinsed briefly in TBST (10 mM Tris-HCL, pH 8.0, 150 mM NaCl, 0.05 % Tween 20). Nonspecific protein binding sites were blocked by incubating the membrane in TBST containing skim milk powder (5 % w/v) or BSA (1 % w/v) for either 1 h or overnight at 20-24°C with gentle shaking.

(2) The blocking solution was replaced with TBST containing a 1:1000 dilution of the primary antibody and incubated for 1h with gentle shaking.

(3) The membrane was washed with TBST three times for 10 min each to remove unbound antibody.

(4) The second antibody, goat anti-rabbit alkaline phosphatase conjugate (BioRad, Australia) diluted 1:7500 in TBST was incubated for 1h with gentle shaking.

(5) Washed as for step 3.

(6) The membrane was blotted dry on filter paper and the colour development solution added (66 µl of NBT and 33 µl of BCIP in 10 ml of 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$ buffer). Reactive areas turned purple, usually within 1-15 min.

(7) When the colour had developed sufficiently the reaction was stopped by rinsing the membrane in deionised water several times and allowing the membrane to dry.

### 4.2.7 Crossed Immunoelectrophoresis

The method described for crossed immunoelectrophoresis below was based on the method of Weeke and Ingild (Harboe, 1973). Preliminary studies using rocket immunoelectrophoresis were carried out to obtain an antigen/antibody ratio that gives a suitable rocket from crossed immunoelectrophoresis.

First Dimension

(1) A gel was prepared by dissolving agarose, type A (Sigma, USA) in 50 mM Tris-barbital buffer, pH 8.2 (1 % w/v) and poured onto a glass plate (18 x 10.5 x 0.15 cm) placed on a horizontal table.

(2) After congelation of the agarose three wells (4 mm in diameter) were punched in the gel by means of a gel puncher.

(3) Samples of an extract of malt (1:2 dilution), purified enzyme and albumin containing bromophenol-blue were applied to each of the wells.

(4) Electrophoresis was performed at 200 V (constant) for 2 h or until the albumin marker had migrated about 5 cm.

Second Dimension

(1) The tracks containing the LD and extract were cut out of the first dimension gel and placed onto a glass plate adjacent to a second agarose gel containing anti-LD immune

serum (1:1000 dilution) which had been prepared 1 h earlier to allow the agarose to set.

(2) Electrophoresis was performed at 70 V constant voltage at 15°C for about 12 h. The buffer used was 50 mM Tris-barbital buffer, pH 8.2.

(3) After electrophoresis, the gel was blotted dry for 15 min, washed three times with PBS containing Tween 20 (0.05 %), blotted dry again then dried in an oven (55°C). The dried gel was stained with Coomassie blue R250 to visualize protein and destained.

### 4.2.8 Crossed Immuno-Isoelectric Focusing

Crossed immuno-isoelectric focusing is a powerful technique for analysing complex mixtures of macromolecules. The method combines the high resolution of isoelectric focusing in one dimension with the detection selectivity of immunoelectrophoresis in a second dimension. The following procedure was used :

Polyacrylamide gels 0.75 mm thick, were prepared (see below) and poured between two glass plates held in a sandwich which was clamped together and supported in a gel casting unit (Hoeffer Scientific, USA).

**Table 4.1**

Gel composition used for first dimension (isoelectric focusing)

| Composition of gel | Volume (ml) |
|---|---|
| Deionised water | 12.79 |
| Glycerol | 2.0 |
| Acrylamide monomer (30 % acrylamide; 0.8 % bis-acrylamide w/v) | 4.0 |
| Ampholytes pH 3.5-5.0 | 0.55 |
| Ampholytes pH 4-6 | 0.36 |
| Ampholytes pH 3-10 | 0.19 |
| TEMED | 0.02 |
| Ammonium persulphate (10 % w/v) | 0.09 |

Polymerization was complete after about 30 min. The solution used for the catholyte was 1M NaOH (degassed for 1 h) and for the anolyte, 0.02 M acetic acid. Prefocusing was for 60 min at; 800 V, 20 mA, 20 W constant voltage or until the current was stable. Samples (up to 80 µl) were applied to wells at the cathode end. The sample used was a 1:2 dilution (in water, 295 µl, glycerol, 75 µl and ampholyte, 30 µl) of a concentrated preparation of an extract of malt prepared as described below. Electrofocusing was carried out at; 800 V, 20 mA, 20 W and constant voltage mode for 15 h at 12°C then at; 2000 V 20 mA, 20 W and constant voltage mode for 1 h to enhance band sharpening. The pH gradient of the gel was measured using a surface pH electrode. After focusing, tracks were cut out from the gel and processed for either protein detection, immunoblotting or crossed immunoelectrophoresis.

Protein staining

Proteins were fixed in the gel by immersion in 12 % trichloroacetic acid for 10 min. The gel was washed in destain (40 % ethanol, 10 % acetic acid) for 5 min then stained for protein (40 % ethanol, 10 % acetic acid, 0.125 % Coomassie blue, R250) until bands became visible. The background was removed by destaining.

Preparation of concentrated extract of malt

In order to obtain an immunoprecipitate it was necessary to concentrate the proteins. A concentrated extract was prepared from 100 g of an extract of Clipper malt. After extraction the protein fraction was treated with 60 % ammonium sulphate. The precipitate was recovered by centrifugation, redissolved in 250 mM Tris-HCL, pH 6.8 and dialysed extensively against this buffer. The dialysed solution was concentrated by ultrafiltration on a PM 10 membrane at 10°C. The concentration of total protein increased from 9.2 to 74 mg/ml (8 fold increase).

Second dimension

At the pH of the electrophoresis buffer (8.2) most of the proteins separated in the first dimension have a net negative charge and migrate towards the anode when an electric field is applied. As the proteins migrate, those that are recognised by the antibodies in the antiserum incorporated into the gel mixture form antigen-antibody complexes (see Harboe and Inglid, 1973, for details on the preparation of antiserum containing gels). If there is a sufficient concentration of these complexes, an immunoprecipitate will form which takes on a rocket shape. Each rocket is unique for a specific protein.

After IEF, a strip of the polyacrylamide gel containing the separated proteins was cut and placed adjacent to the agarose gel containing the anti-LD immune serum (diluted 1:1500). Details of this procedure are outlined in 4.2.7 and in Harboe and Ingild (1973).

## 4.3 RESULTS AND DISCUSSION

### 4.3.1 Separation of IgG from other Serum Proteins

The Protein G column resolved two protein containing peaks (Fig.4.1). Fractions 1-5 (diluted 1/80), 11-13 (diluted 1/5), 14 (diluted 1/2), 15 and the column load (undiluted) were electrophoresed in an SDS polyacrylamide gel as described in 2.3. Albumin (66 kd) was the major protein in fractions 1-5 and immunoglobulin in fractions 11-15 (Fig. 4.2). The immunoglobulin separated into the heavy (51 kd) and light (23 kd) chain because samples were reduced with 2-mercaptoethanol which breaks the disulphide linkages joining these chains.

The fractions 3, 11-13 and the column load were examined for presence of the anti-LD antibody using the ELISA described in 4.2.2. There was no evidence of any specific binding to LD in the contents of fraction 3 which is consistent with the electrophoretic findings showing an absence of immunoglobulins (Fig. 4.3). Fractions 11-13 contained specific anti-LD antibodies and the greatest binding was in fraction 12, consistent with a more intensely staining band (Fig. 4.2). An unexpected result was that the unfractionated serum had as good or better binding than fraction 12. Nevertheless, the removal of unwanted proteins in the serum helps to reduce non-specific binding in the ELISA used to quantitate LD in extracts (see 4.3.3) and in immunoblotting. Based on these results, fractions 11-15 were pooled, concentrated by ultrafiltration, dialysed against PBS and a bacterial inhibitor, merthiolate, added (0.1 % w/v). The protein concentration of the immunoglobulin preparation was 5.2 mg/ml. Aliquots were stored at -20°C.

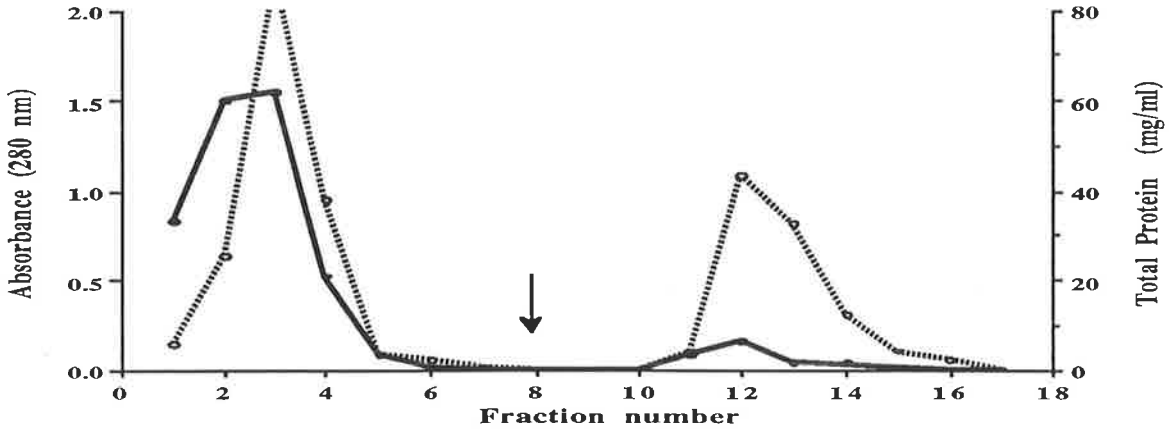
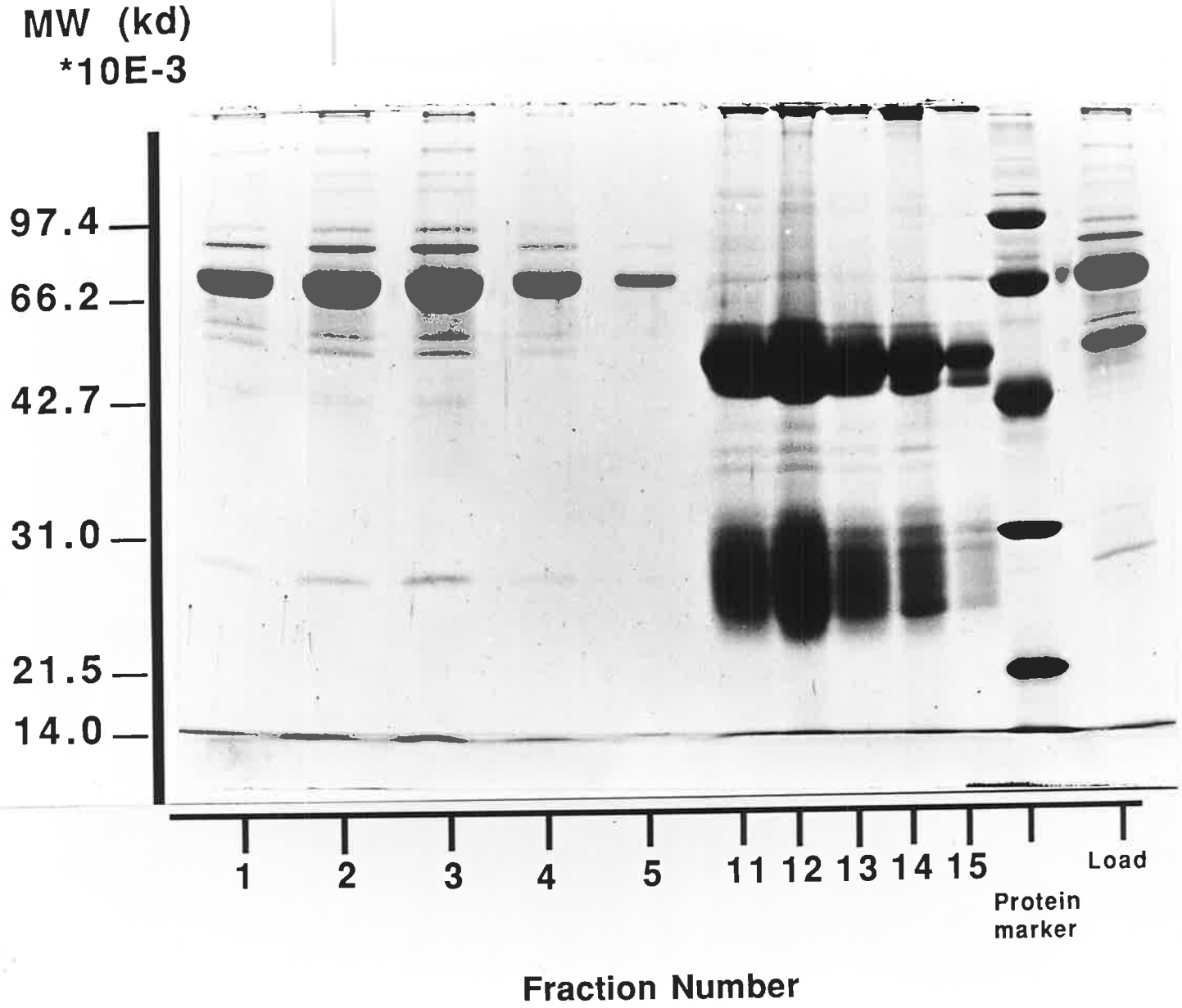



**Figure 4.1**

Fractionation of immune serum on Protein G Sepharose. Serum (1.25 ml) diluted 1:1 with equilibration buffer (20 mM sodium phosphate buffer, pH 7 containing 0.1 mM PMSF) was loaded onto a Protein G Sepharose column. Fractions (1 ml) were collected. Bound IgG was eluted from the column with 0.1 M glycine-HCL, pH 3 (Arrow). Absorbance at 280 nm, dashed line (○); Total protein, (●).

**Figure 4.2**

Electrophoresis of fractions from the Protein G Sepharose chromatography.


MW (kd)
*10E-3
97.4
66.2
42.7
31.0
21.5
14.0
1
2
3
4
5
11
12
13
14
15
Protein marker
Load
Fraction Number

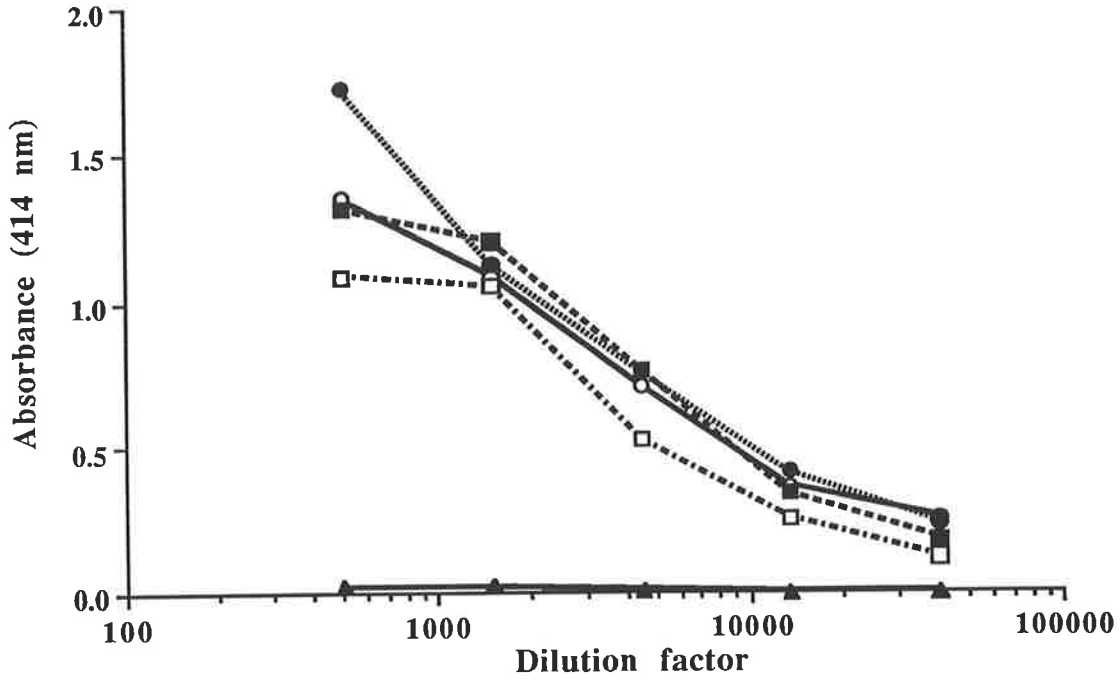



**Figure 4.3**

Titration curves produced from using the indirect ELISA (see 4.2.2) on fractions from the Protein G Sepharose fractionation. Fraction 3, (▲); Fraction 11, (○); Fraction 12, (●); Fraction 13, (□); Column load, (■).

### 4.3.2 Antibody Titre and Specificity

Titre

The titre of the immune serum is defined as the highest dilution of antiserum at a given antigen concentration which produces an absorbance in the ELISA above that produced by the pre-immune serum. The pre-immune serum is used to measure the amount of non-specific binding. This can for example be due to antibodies raised in the immunised animal against malt or cereal proteins present in the animal diet. If these protein escaped proteolytic degradation in the gut of the animal and enter the lymphatic system they would stimulate the immune system to produce antibodies. The titre was estimated as 1:121,500 (Fig. 4.4).

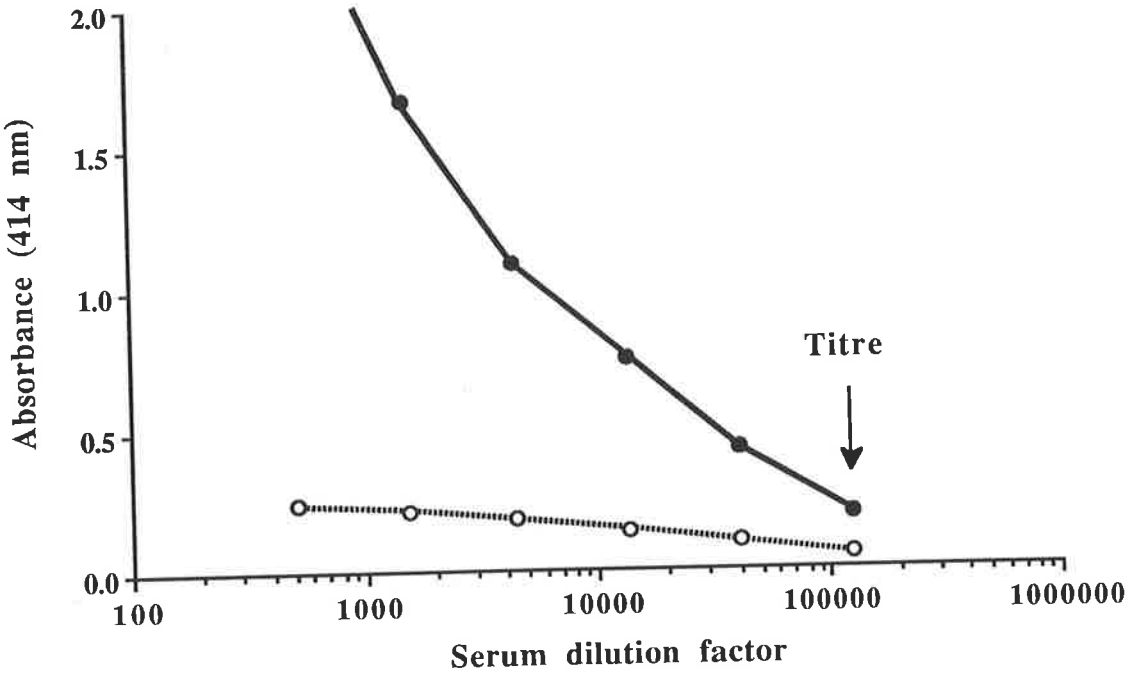



**Figure 4.4**

Determination of antiserum titre. Immune and pre-immune serum were diluted in PBS/BSA/Tween and tested for specific binding to purified LD (925 ng/ml) in the ELISA (see 4.2.2). Immune serum, (●); pre-immune serum, (○).

Specificity-tested by SDS-PAGE and immunoblotting

For the immunoassay to have a high sensitivity and specificity a monospecific antisera is required. Monospecific polyclonal antisera contain specific antibodies that are reactive to one macromolecular entity. Every specific antibody produced by the immune system in response to the injected LD will come from a lymphocyte line derived from an original single lymphocyte. Because of the multiplicity of antigenic sites on any single immunogen, a large number of different and distinct lymphocytes may be stimulated following immunization. As a consequence antisera usually contain a mixture of different antibodies of varying specificity and affinity.

At a high dilution, only the antibodies with the highest affinities will, for practical purposes, be reacting. Under these conditions the apparently heterogeneous antiserum may behave as a homogeneous or monospecific antiserum. The specificity of antisera should be verified by sensitive techniques such as crossed immunoelectrophoresis and immunoblotting. However, the antibody should be tested in a system of similar sensitivity to which it will be used, e.g. immunoblotting, when the ELISA is the intended assay of choice. Antibodies functionally monospecific in precipitation-in-gel techniques might contain weak non-specific antibodies that become evident in blotting due to the much higher sensitivity of immunoblotting.

Precipitation-in-gel techniques do have a place. The "graphical" relationship of the precipitation peaks can be used and interpreted in a way that is not possible with blotting methods. The specificity of the anti-LD antibodies was determined by immunoblotting of SDS polyacrylamide gels and by crossed immunoelectrophoresis.

Non-specific reactions are more difficult to interpret. A truly monospecific antibody may react with several bands, but there may be several explanations for this. (1) The antigen in question might be present in many forms, (precursors, intermediates more or less fully modified by post-translation; fragments either degraded *in vivo* or *in vitro* during extraction and fractionation or induced by sample treatment). 2) Inadequate reduction can lead to multimeric proteins migrating as a series of bands and even proteins with only internal thiol bridges will migrate differently as the partially reduced proteins have a smaller Stoke's radius. (3) The specificity of the antibody can be relative, and increasing the sensitivity of a method may lead to detection of bands not previously evident e.g. Fast Red staining is much less sensitive than the NBT/BCIP substrate.

The method used for the electrotransfer of proteins from the SDS gel was very efficient. After transfer, the gel was silver stained and no proteins were detected. When the pre-immune serum was tested (see 4.2.5) at a dilution of 1:100, no bands were visible until after 15 min contact with the Fast Red substrate when three to four faint bands were seen (Fig. 4.5a). At a 1:1000 dilution, no bands were seen (data not shown). These results show that the serum of the rabbit prior to immunization had antibodies recognising some malt proteins. These low affinity interactions represent non-specific binding since the bands disappear with dilution. When immune serum (diluted 1:1000) was tested, a band of molecular weight coincident with the purified enzyme was obtained. There is a minor band just detectable below this band. These bands appeared within 1 min exposure to the substrate (Fig. 4.5b). Immune serum diluted 1:100 did show several bands in addition to that of LD (results not shown) but these non-specific bands disappeared using a 1:1000 dilution.

When the SDS-PAGE and immunoblotting was repeated using the BCIP/NBT substrate, the antibody was found to recognise two distinct bands against the purified enzyme and extracts of malt flour, a major and a minor band (Fig. 4.5d, e). These bands were the same as those seen in the silver stained gel of the purified LD (Fig. 3.5). The minor band may be a degradation product produced during the purification of LD or a contaminating protein in the enzyme preparation to which antibodies have been raised. In order to clarify this, it would be necessary to purify the 100 kD band which is present in very small quantities because it is only detected in silver stained gels. This was considered to be a major project by itself and with time limiting was not attempted. However, a minor contaminant in the enzyme preparation can produce a strong immunological reaction and thus make the antiserum polyspecific. The minor staining band seen in figure 3.5 also showed as a lightly staining band on the immunoblot (Fig. 4.5b) indicating this protein (ca. 100 kD) has low antigenicity. Based on the relative concentration of this band compared to the strong

antibody binding of the 104 kD band, its possible interference was considered not to be a significant problem. Although the nature of this protein remains to be resolved the antiserum is functionally monospecific for LD.

The problem of unwanted specificities is often related to the high sensitivity of immunoblotting which visualizes antigen-antibody reactions not detected by other immunochemical methods. Thus, for polyclonal antibodies, monospecificity is an operational term defined by the method in question. The presence of unexpected bands in immunoblots may reflect either nonspecific or specific staining. The former might include genuine nonspecific staining of non-immunological origin such as binding of antibodies to proteins through binding of Fc-receptors. The latter may result from the antiserum containing antibodies of different specificities because of pre-immune antibody populations or induced by impurities in the immunogen preparation. The following controls were considered suitable for immunoblotting experiments (see table below).

| | |
|---|---|
| Pre-immune serum instead of primary antibody | Control for presence of pre-immune antibodies |
| Omission of antigen | Control for impurities in antisera, buffers and second antibody which bind non-specifically to the membrane |
| Omission of second antibody | Control for cross-reactions between antigen on blots and second antibodies or nonimmunological interaction between detection system and primary antibodies |
| Omission of all antibodies | Control for endogenous enzyme activity |
| Omission of primary antibody | Control for the presence of immunoglobulin in antigen preparation and/or binding of second antibody to the antigen |

The results of the control immunoblots shows the absence of the problems described in the above table (Fig. 4.5c) but the antiserum does contain pre-immune antibodies (Fig. 4.5a). Routinely, an antiserum should be diluted and be as high avidity as possible. Appropriate dilution of the sample load eliminates unwanted binding (Fig. 4.5d). Only the high affinity binding of the anti-LD antibody interaction remains (a single band corresponding to LD is just visible at a 1:81 dilution of the extract, Fig. 4.5d). Dilution of the antiserum will enable the very much lower titre of unwanted antibodies to disappear from the blot (Fig. 4.5e). As the antiserum dilution is increased, only the higher titre antibody

interaction is visible (corresponding to LD) on the blot and unwanted antibodies are sufficiently diluted so as not to be detected.

Malt extract examined by SDS-PAGE and immunoblotting reveals one band with a minor band just below under the following conditions; antigen diluted 1:27, antiserum diluted 1:3000 or antigen diluted >1:100, antiserum diluted 1:1000. Note that with the ELISA (see 4.3.3), an antigen dilution of 1:300 to 1:1000 and an antiserum dilution of 1:1000 has been routinely used in this study. At these dilutions, only one band corressponding to LD is just visible by immunoblotting.

Specificity tested by crossed immunoelectrophoresis

A false impression of the specificity and complexity of the antiserum may be obtained if attention is not paid to the multiple antigen/antibody equivalence concentrations operating in a potentially complex antiserum. Every polyclonal antiserum must first be treated as if it were a complex antiserum before it is proven to be monospecific to the antigen of interest. Crossed immunoelectrophoresis is better able to accommodate differences in antibody titre than immunoelectrophoresis or Ouchterlony (Dunbar, 1987). The sensitivity of crossed immunoelectrophoresis depends upon the smallest area of a precipitate to be recognised which depends upon the ratio of antigen to antiserum. Increasing the volume of sample and diluting the antiserum further can increase the sensitivity of the method. For most proteins the sensitivity will be 0.1-1 mg/ml. It is possible that cross reacting antibodies present in the antiserum do not result in visible rockets because equivalence is not achieved and no precipitation occurs.

Crossed immunoelectrophoresis was used to assess the monospecificity of the immune serum. A single rocket was obtained showing no trailing and was similar in shape and position in the gel to the rocket produced by the purified LD (Fig. 4.6). The absence of

**Figure 4.5a**

Test for antibodies to malt proteins in the pre-immune serum. Purified enzyme (3 μg; LD) and crude extract of malt (70 μg protein; CE) were subject to SDS-PAGE and the separated proteins electroblotted onto nitrocellulose. Immunoblotting was performed as described in 4.2.5 except that the primary antibody used was pre-immune serum (diluted 1:100). The blot was left in contact with the substrate overnight.

LD CE

**Figure 4.5b**

Monospecificity of polyclonal antisera assessed by immunoblotting. Purified enzyme (3 μg) and crude extract of malt (70 μg protein) were subject to SDS-PAGE. One half of the gel was fixed and silver stained and the other half processed for immunoblotting as described in 4.2.5. The antisera was diluted 1:1000 and the substrate solution allowed to react for 2 min.

# WESTERN BLOT

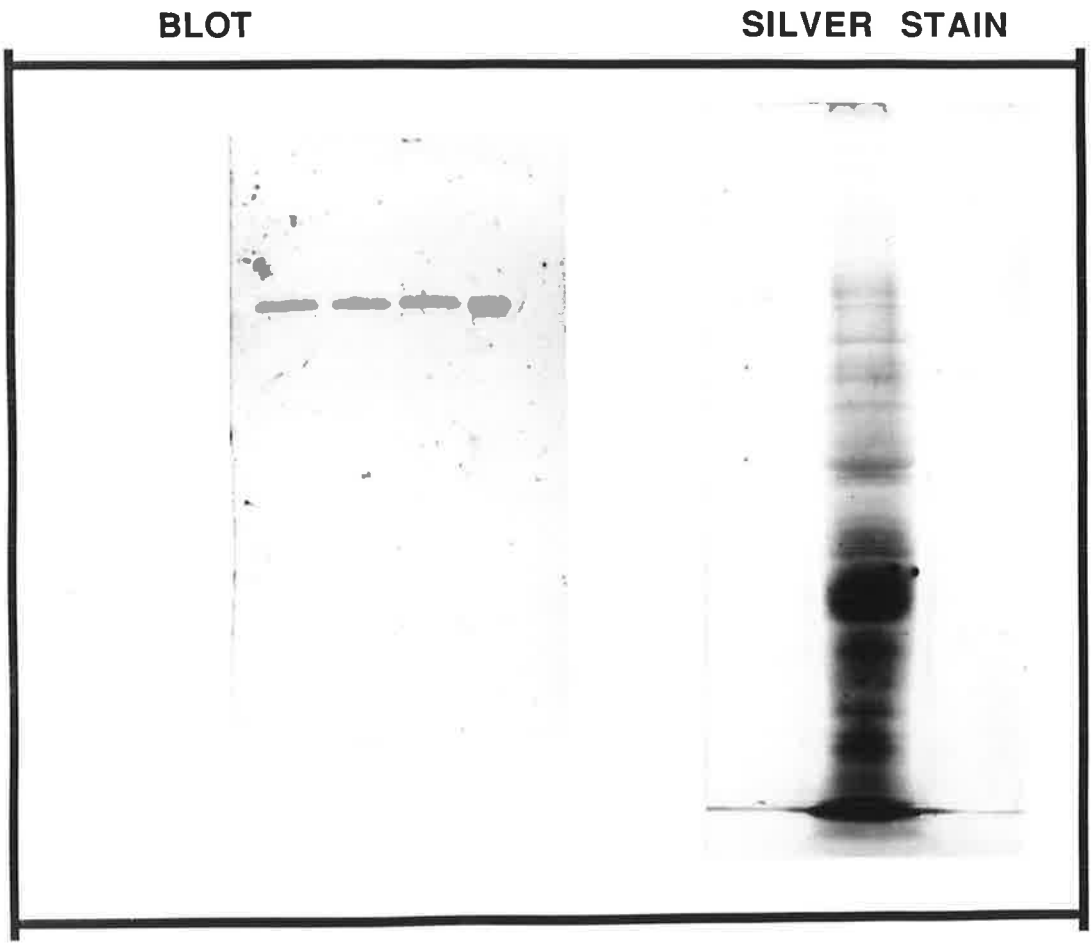


**Figure 4.5c**

Monospecificity of polyclonal antisera assessed by immunoblotting-control blot. Flour from Clipper malt was extracted as detailed in section 2.5, analysed by SDS-PAGE, (extract diluted 1:3 in sample buffer) and immunoblotted as described in 4.2.5 except that the NBT/BCIP substrate was used.

**A**, positive control-addition of both antibodies

**B**, omission of the immune serum

**C**, omission of the 2nd antibody

**D**, omission of both antibodies.

Blots B-D were in contact with the substrate for 60 min and blot A for 4 min.

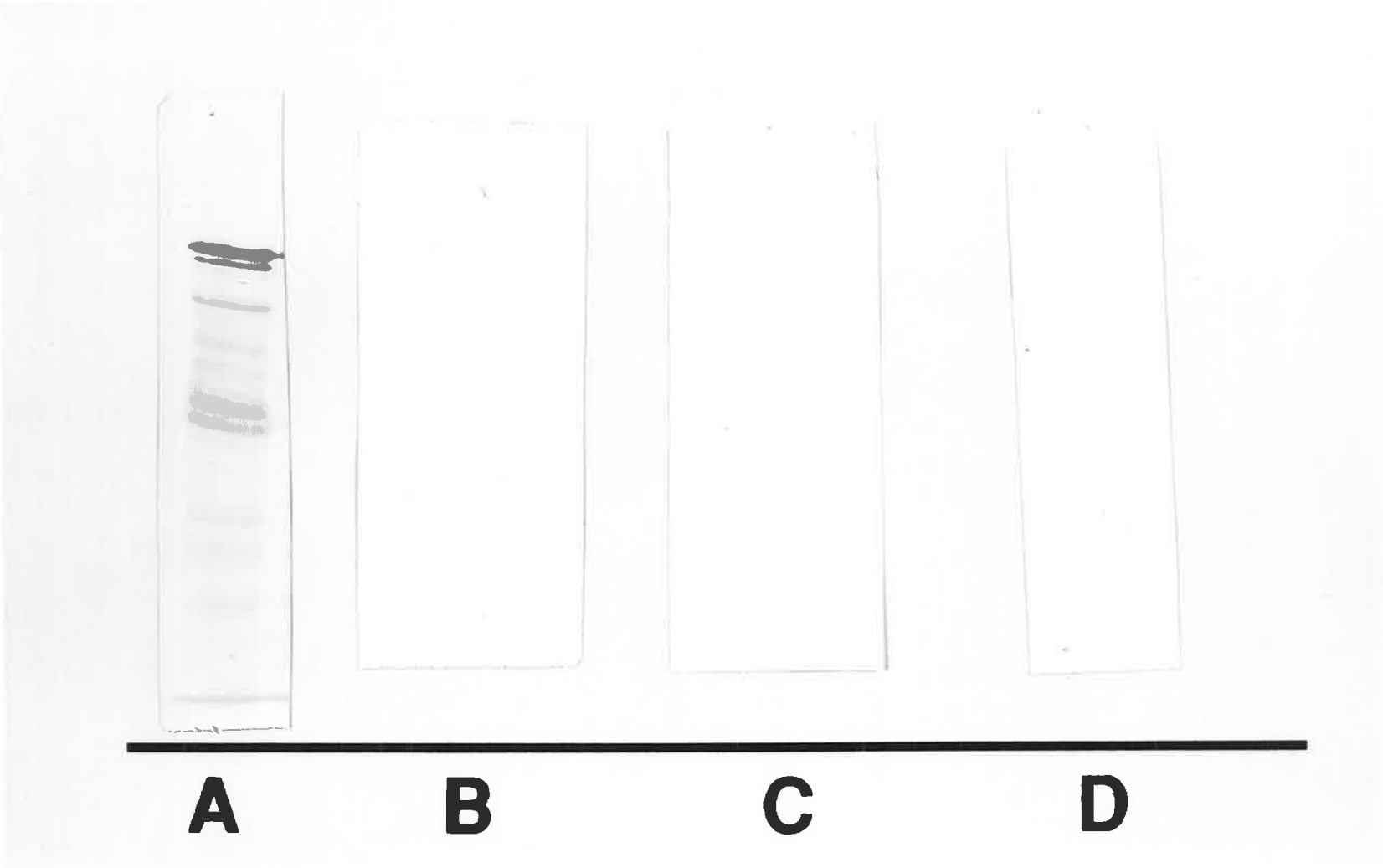

A
B
C
D

**Figure 4.5d**

Monospecificity of polyclonal antisera assessed by immunoblotting-effect of antigen dilution. Conditions as for figure 4.5c. The extract of malt was diluted in SDS sample buffer (1:3, 1:9, 1:27, 1:81, 1:243) and applied in duplicate to the gel Substrate contact time was 4 min.

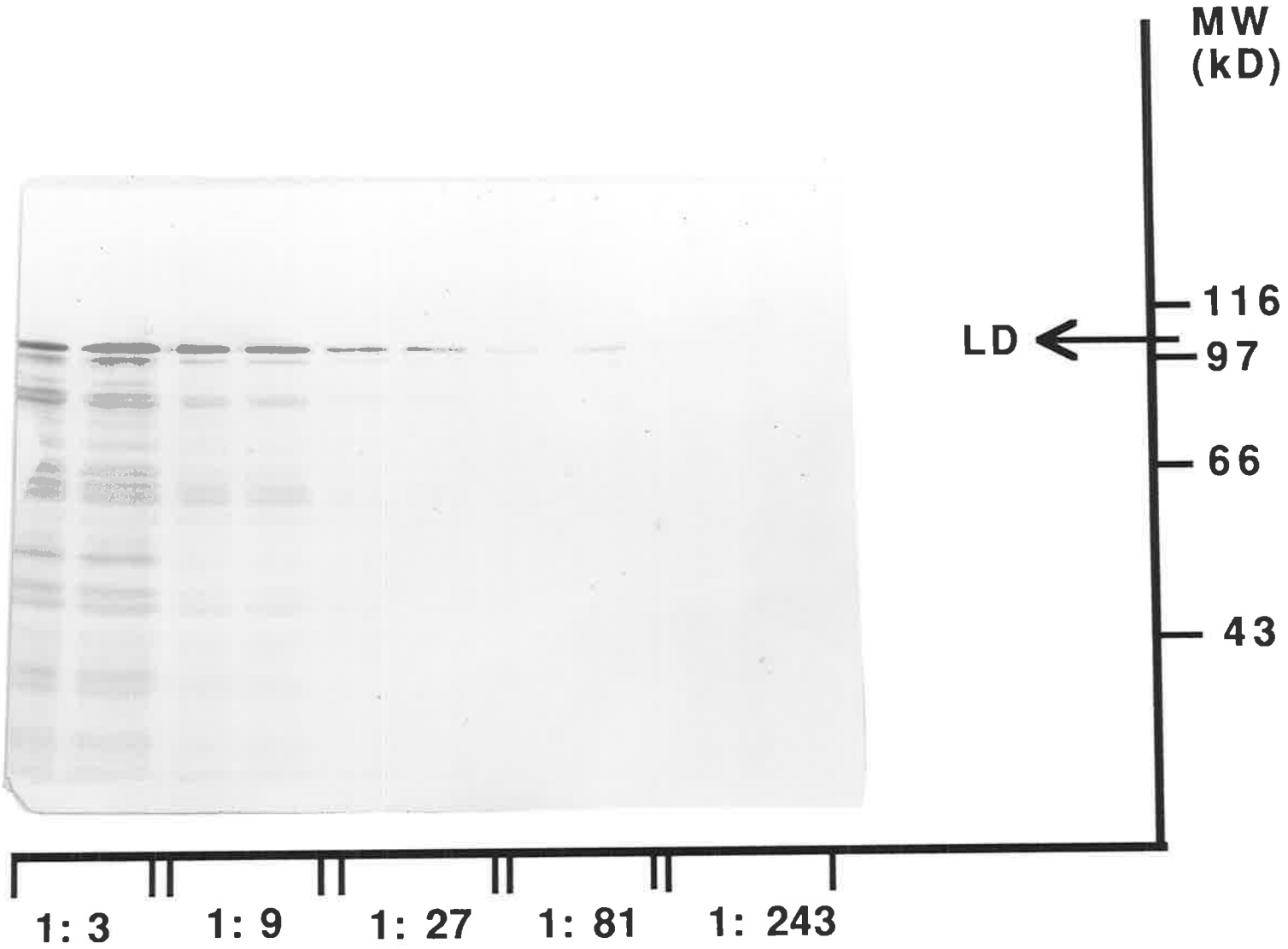

MW
(kD)
116
97
66
43
LD
1: 3
1: 9
1: 27
1: 81
1: 243

**Figure 4.5e**

Monospecificity of polyclonal antisera assessed by immunoblotting-effect of 1st. antibody dilution at two dilutions of extract. Conditions as for figure 4.5c.

| Extract dilution | Antiserum dilution |
|---|---|
| 1:9 | **A**, 1:1000 |
| | **F**, 1:3000 |
| | **G**, 1:9000 |
| 1:27 | **E**, 1:1000 |
| | **B**, 1:3000 |
| | **C**, 1:9000 |
| | **D**, 1:27000 |

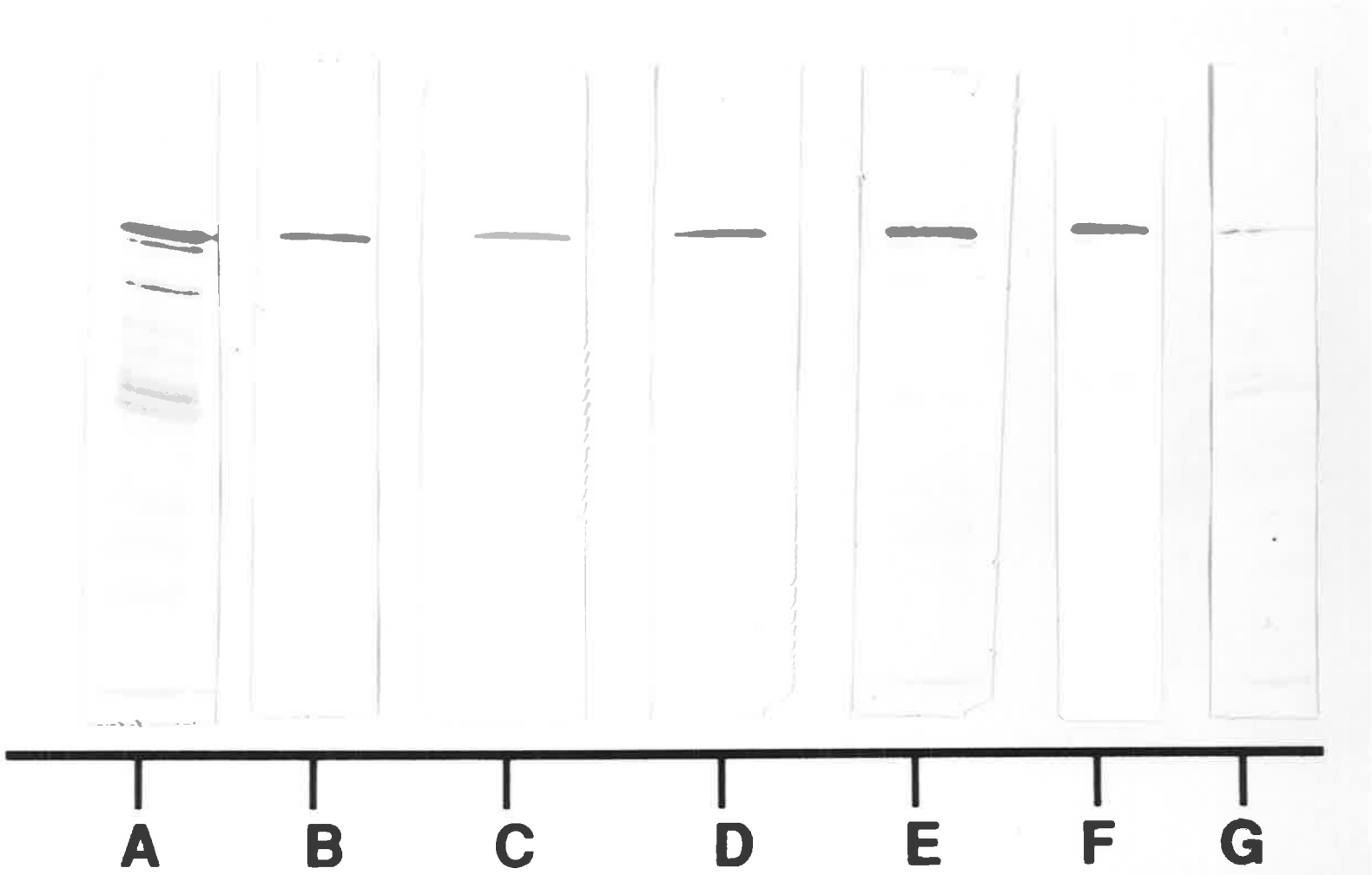
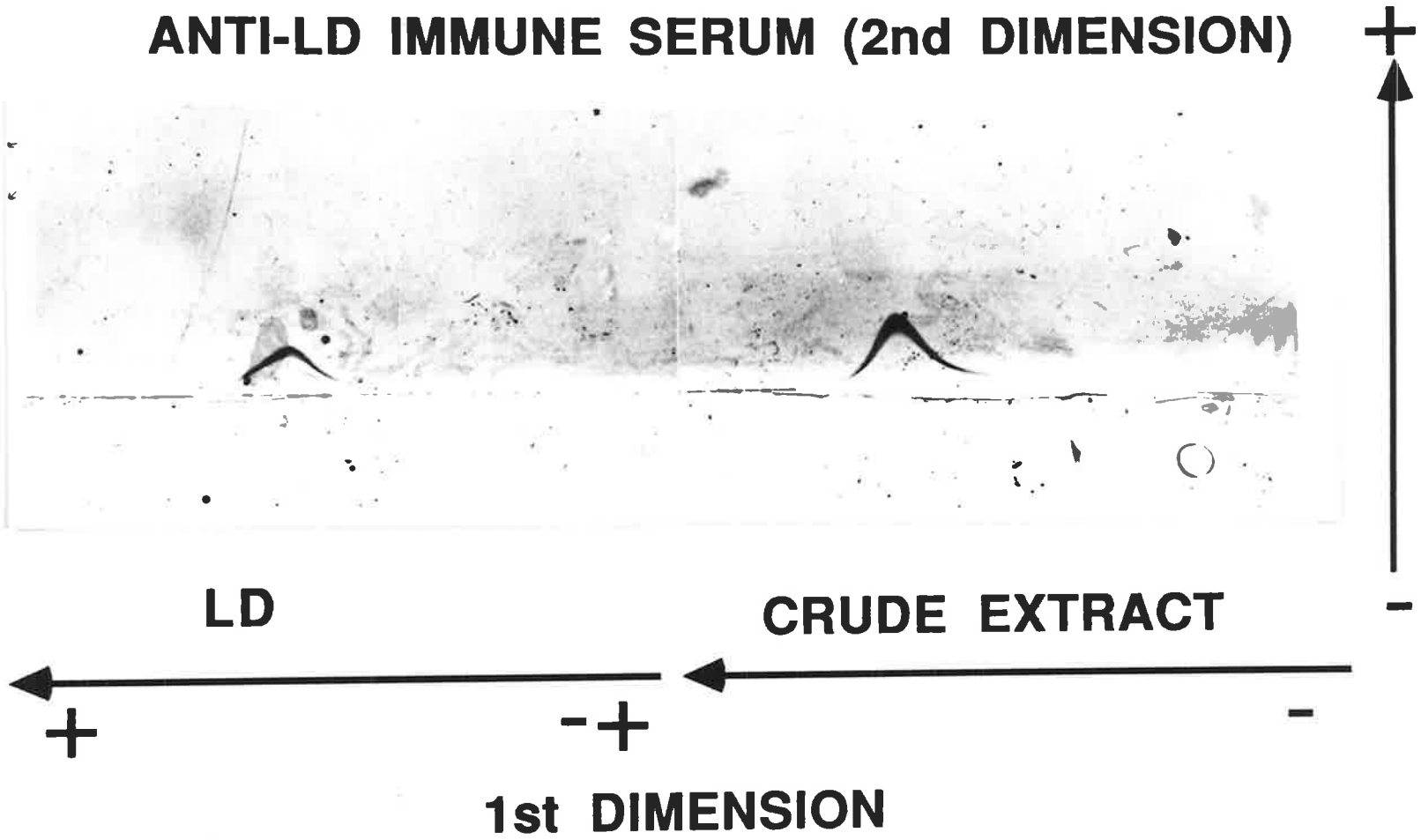

A
B
C
D
E
F
G

**Figure 4.6**

Crossed immunoelectrophoresis of polyclonal antisera. An extract of malt flour and purified LD were separated by electrophoresis on a 1 % agarose gel. The selected sample lane from the first dimension was cut and laid alongside the antisera-containing gel (diluted 1:1000) with its gel surface facing upwards. At the pH of the buffer (8.6), the majority of the proteins migrate towards the anode in an electric field. Immunoprecipitates were detected with Coomassie R-250 staining.


ANTI-LD IMMUNE SERUM (2nd DIMENSION)
+
-
LD
CRUDE EXTRACT
+
-+
-
1st DIMENSION

multiple rockets when tested against a crude extract of malt flour indicates the antiserum contains antibodies specific for only LD. Daussant *et al.* (1987) prepared an immune serum against purified LD from germinated barley and tested the specificity of the serum using a less sensitive method, immunoelectrophoresis. Their results indicated their serum was monospecific.

### 4.3.3 Development of an ELISA for Measuring Limit Dextrinase in Crude Extracts

The ELISA is potentially a very sensitive assay procedure and if applied to the measurement of a specific enzyme (including all the molecular forms) a monospecific antiserum is necessary. There are three types of ELISA (Engvall, 1980). These have been classified as competitive (C) and non-competitive (A and B) (Fig. 4.7). The former technique involves a reaction step in which unlabelled and labeled antigen compete for a limited number of antibody sites. With the latter, the antigen (B) or antibody (A) to be measured is first allowed to react with the antibody (antigen) attached to a solid phase followed by measurement of the binding of enzyme-labeled immune reactant.

Although competitive ELISA techniques are specific and easy to execute, they have some problems :

(1) Purified antigen in relatively large amounts is required. This is not feasible with LD since a typical yield from a purification is only 200-500 μg which was insufficient to cover the range of investigations of this enzyme in this study. A similar drawback also applied to method B.

(2) It is necessary to incubate the enzyme-labelled antibody with a crude plant extract, which may contain proteases and noncompetitive enzyme inhibitors. This is a more serious problem since these substances, when present, may alter the activity of the enzyme used as a label. This difficulty is avoided with the noncompetitive ELISA.

The noncompetitive ELISA allows the possibility of binding several enzyme-labeled antibody molecules to a single antigen molecule which has multiple epitopes, thus providing an element of amplification. The direct, noncompetitive ELISA method (Fig. 4.7 method A) was chosen for the assay of LD.

The development of an ELISA for quantifying antigen (LD activity) levels in crude samples of mature, germinated or malted barley seed was adapted from the method described in 4.2.2. The following variables were studied :

(1) Quantity of anti-LD IgG (1st. antibody) to coat the microtitre plate
(2) Dilution of conjugate (2nd antibody)
(3) Incubation times and temperatures
(4) Dilution of sample

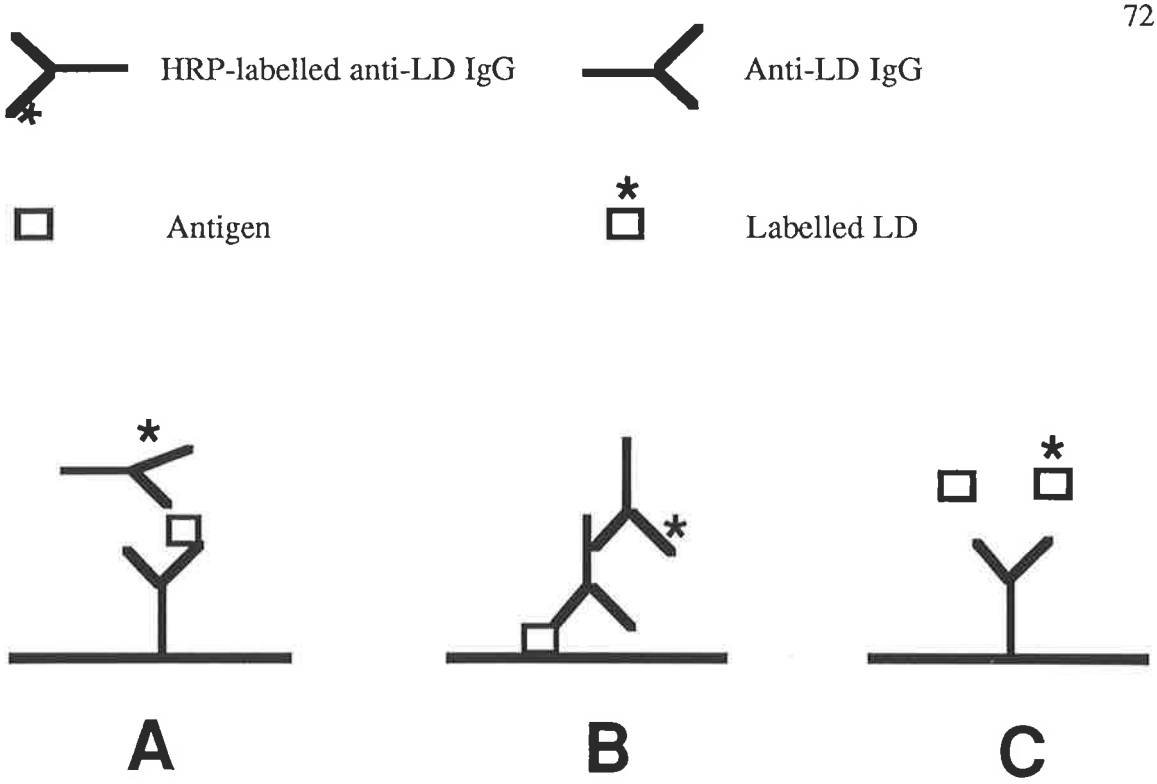



**Figure 4.7**

Three ELISA techniques used in antigen determination. **A**: coat with anti-LD IgG; incubate with serial dilutions of purified LD or unknown sample; incubate with HRP-labelled anti-LD IgG; substrate addition. The activity is related to the amount of LD in the sample or standard (direct, non-competitive). **B**: coat with serial amounts of the purified LD or with unknown sample; incubate with anti-LD IgG; incubate with HRP-labelled anti-LD IgG; substrate addition. The activity is related to the amount of LD coated (indirect, non-competitive). **C**: Coat with anti-LD IgG; incubate with standard amounts of labelled LD mixed with serial dilutions of the purified LD or with the unknown sample; substrate addition. The activity is inversely related to the amount of the unlabeled LD (direct, competitive).

(1) Determination of the optimum quantity of coating antibody (anti-LD IgG or 1st. antibody)

The sensitivity of the assay can be adjusted by varying the coating concentration of first antibody (Fig. 4.8). At concentrations of IgG to 1 µg/ml, LD is detectable with accuracy over a very narrow range (100-250 ng/ml). At 3 µg/ml IgG, a more useful linear range of 20-1000 ng/ml is obtained. Increasing the coating quantity of IgG increases total binding (higher absorbance) and improves sensitivity at the expense of range. At 10 µg/ml IgG, the sensitivity is better than 20 ng LD/ml and the linear range is up to 150 ng/ml.

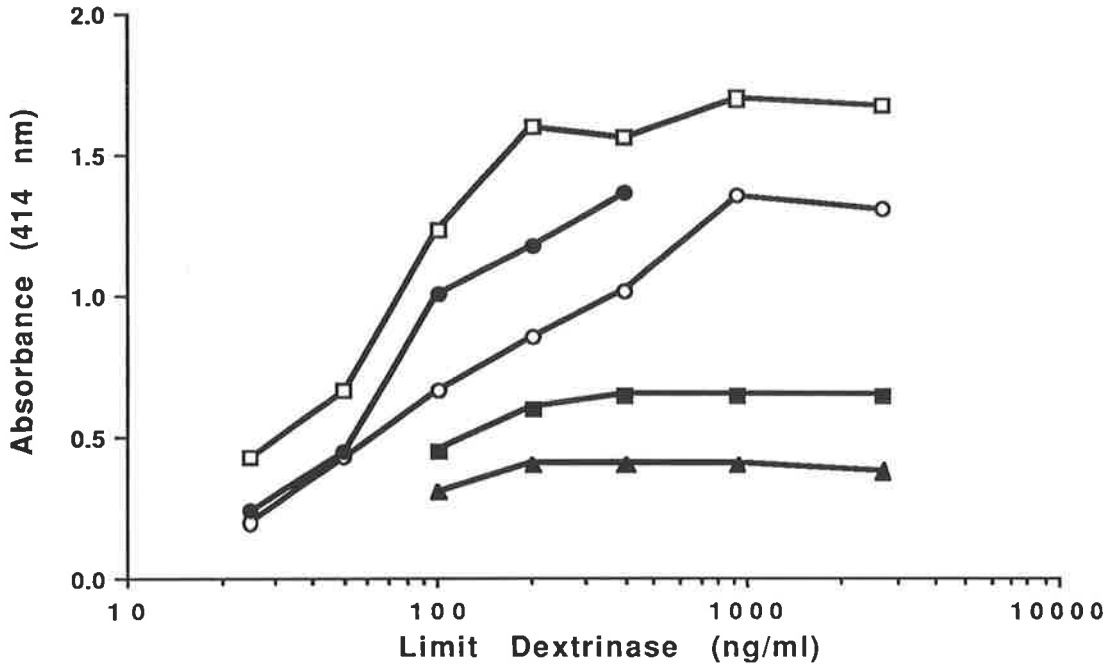



**Figure 4.8**

Titration curves for ELISA with varying concentrations of anti-limit dextrinase IgG. A chequerboard ELISA was set up varying the anti-limit dextrinase IgG and antigen concentrations. Anti-limit dextrinase IgG (μg/ml); 0.3, (▲); 1.0, (■); 3.0, (○); 5.0, (●); 10.0, (□). Anti-LD HRP conjugate (first antibody) diluted 1:1000.

(2) Optimum dilution of anti-LD horseradish peroxidase conjugate (2nd. antibody)

Increasing the concentration of the second antibody at a fixed first antibody concentration increases the total binding and shifts the titration curve to the left (Fig. 4.9). At lower dilutions of the second antibody, the standard curve is more sensitive i.e. the curves are steeper in the range from 10-30 ng/ml.

Optimum dilution of sample

Variation in the quantity of LD in test samples required a preliminary test to determine the dilution of sample that would produce an absorbance in the ELISA falling on the linear part of the reference line. Typically, a dilution of an extract of malt from 1:1000-1:2000 is suitable (Fig. 4.10).

| Dilution of extract | Absorbance |
|---|---|
| 1:500 | 1.8 |
| 1:1000 | 0.9 |
| 1:2000 | 0.42 |

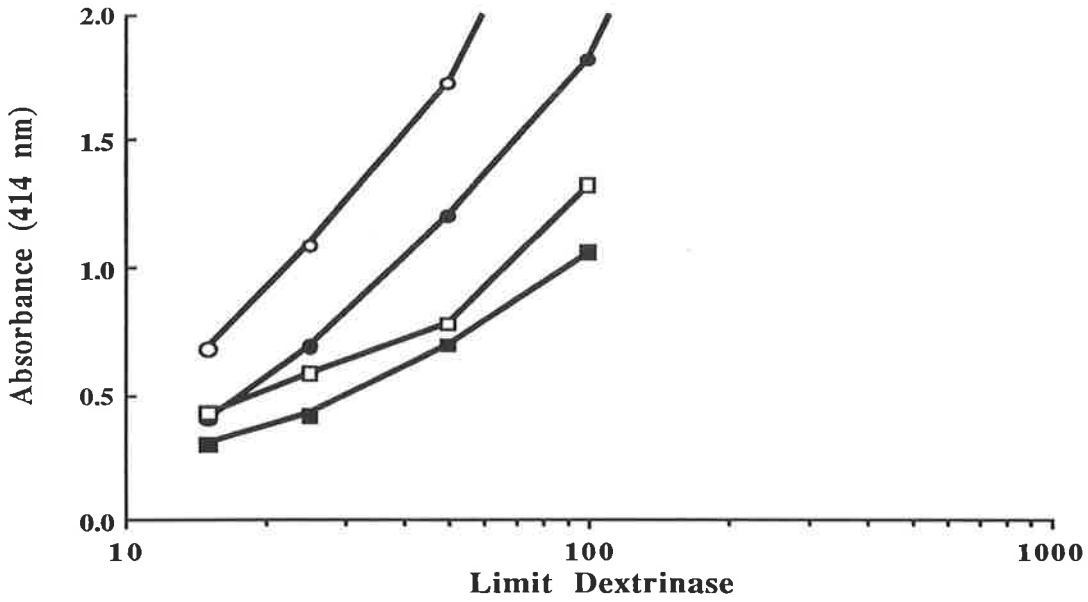



**Figure 4.9**

Titration curves for ELISA with varying concentrations of anti-LD horseradish peroxidase conjugate (second antibody). Anti-LD IgG (first. antibody) held at 3 µg/ml. A chequerboard ELISA was set up varying the second antibody and antigen concentrations. Dilution factor of second antibody: 1:500, (○); 1:1000, (●); 1:1500, (□); 1:2000, (■).

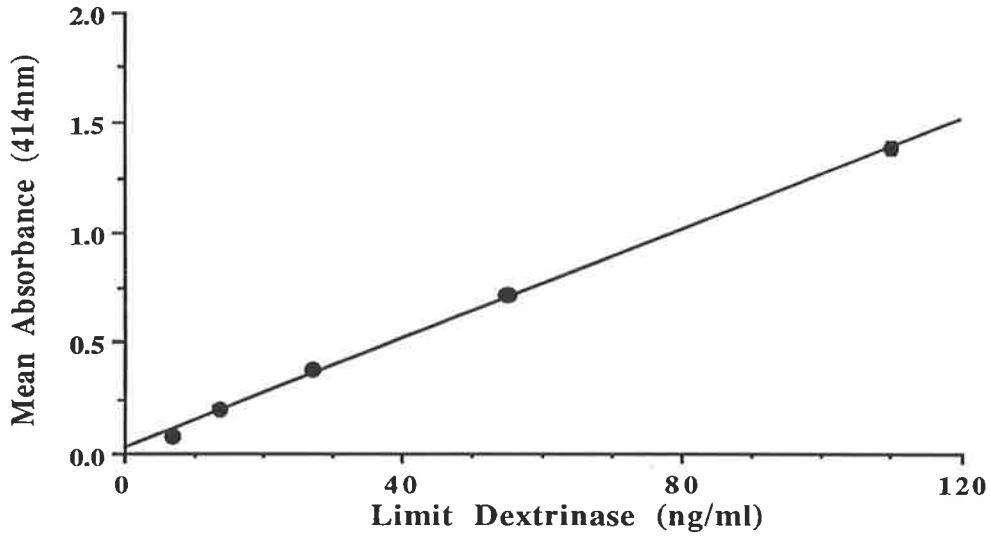



**Figure 4.10**

Standard curve for the limit dextrinase ELISA. Purified LD diluted in assay buffer was measured in the ELISA by the procedure outlined in 4.3.3. Each point is a mean absorbance of four determinations. The standard errors are too small to show on the graph (0.001-0.027). Linear regression analysis of the data produced a significant line: Absorbance = 0.124 + 0.0134 x LD ; $R^2$ 0.997.

Based upon these trial experiments the final procedure chosen for the ELISA for measuring LD in extracts is listed below:

#### 4.3.3.1 ELISA protocol for measuring limit dextrinase activity in malt extracts.

1. Microtiter plates (Nunc-Immuno Plate MaxiSorp, Denmark) are coated with 100 µl of the IgG anti-LD antibody (2.5 µg/well or 1:1000 dilution) diluted in coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6) by incubating plates at 37°C for 90 min or overnight at 4° C.

2. Plates are washed three times with PBS containing Tween 20 (0.05% v/v) and tapped dry onto paper towelling

3. Excess binding on the plates is blocked by incubation with 200 µl of PBS/Tween containing BSA (0.1% w/v) at 37°C for 60 min.

4. Plates are washed as for step 2

5. Dilutions (100 µl) of purified limit dextrinase (7-220 ng/ml), an extract of malt (1:500-1:2000) or blanks (PBS/Tween/BSA) are incubated at 37°C for 60 min.

6. Plates are washed as for step 2

7. Anti-LD horseradish peroxidase conjugate (1:1000 dilution) is added to wells (100 µl) and incubated at 37°C for 60 min.

8. Plates are washed as for step 2.

9. The plates were developed by addition of 150 µl of substrate solution (540 mg 2,2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid and 50 µl of 30 % (v/v) hydrogen peroxide in 0.5 L of citrate buffer, 26 mM) for 7 min. The reaction was stopped by addition of 50 µl of oxalic acid (3% w/v).

10. The colour developed was measured as absorbance at 414 nm using a microplate reader. The test was performed in three replicates including standards on each plate. The absorbance of the blank (omission of antigen) was subtracted from the absorbance of each well.

11. Results are calculated using the regression equation (y=a+bx) applicable to each run for the 'standard curve' relating absorbance of dilutions of purified LD to the enzyme concentration.

$$\text{Concentration} = \frac{\text{(absorbance corrected for blank-intercept)}}{\text{slope}}$$

### 4.3.4 Evaluation of the ELISA

The introduction of a new assay for measuring LD requires the following evaluation analysis:

(1) Specificity testing
(2) Assay linearity with volume of sample assayed
(3) Interference from components in the extract upon the ELISA tested
(4) Evidence that the ELISA measures the native enzyme which has enzyme activity
(5) Assay reproducibility
(6) Comparison with other assays for measuring LD activity

#### 4.3.4.1 Specificity

The anti-LD antibody used in the ELISA is specific for LD as shown by immunoblotting and crossed immunoelectrophoresis (Figures 4.5a and 4.6). As a further test commercial preparations of starch hydrolytic enzymes from barley and other sources were assayed in the ELISA at total protein concentrations of 1 and 10 µg/ml (Table 4.2). These commercial enzymes are not homogeneous and contain varying amounts of other enzymes. The concentrations refer to total protein based on the information supplied with the product and therefore do not necessarily relate to actual amounts of the enzyme. At the higher concentration tested, there was some interference from α-amylase and β-amylase (Table 4.2) equivalent to 3 ng/ml LD (detection limit of the ELISA). However, since these enzyme preparations are not highly purified, they may contain LD. Only by testing purified starch hydrolytic enzymes from barley could it be concluded that the ELISA is free from interference. In the α-amylase ELISA specific for this enzyme an absorbance above 1.5 was obtained with purified α–amylase at 1.5 µg/ml (MacLeod *et al.*, 1989, personal communication). When purified α–amylase was tested at this concentration against the LD specific ELISA, the absorbance was not higher than the buffer blank (data not shown). The immunoblotting results (see 4.3.2) indicate that interference from these enzymes is absent.

**Table 4.2**

Effect of various starch degrading enzymes upon the LD ELISA. The following commercially (Sigma, USA) available enzymes (α–amylase, type VIII-A from barley malt; β–amylase, type IIB from barley malt; amyloglucosidase from *Aspergillus* and α–glucosidase, type IV from brewers yeast) were assayed by the ELISA at final concentrations of 1 and 10 μg/ml. Results are expressed as the mean absorbance of three determinations.

| Enzyme | Protein concentration (μg/ml) | Mean absorbance (414 nm) | SD r=3 |
|---|---|---|---|
| α–amylase | 1 | 0.085 | 0.0040 |
| | 10 | 0.110 | 0.0068 |
| α–glucosidase | 1 | 0.087 | 0.0037 |
| | 10 | 0.081 | 0.0146 |
| β–amylase | 1 | 0.107 | 0.016 |
| | 10 | 0.139 | 0.0356 |
| amyloglucosidase | 1 | 0.084 | 0.0046 |
| | 10 | 0.076 | 0.0095 |
| buffer blank | - | 0.093 | 0.0012 |
| LD | 3 ng/ml | 0.109 | 0.0058 |
| LD | 7 ng/ml | 0.195 | 0.0165 |
| LD | 14 ng/ml | 0.341 | 0.0187 |
| LD | 100 ng/ml | 1.544 | 0.0232 |

### 4.3.4.2 Assay linearity

Samples of extract are diluted so the absorbance falls within the linear range of the "standard curve" (Fig. 4.10). The final LD activity expressed as µg/ml is obtained by multiplying the reading obtained from the "standard curve" (expressed in units of ng/ml) by the extract dilution factor and converting to µg/ml. Some samples require different dilutions depending upon the concentration of LD in the sample. It is important that a linear relationship exists between the quantity of extract assayed and the absorbance recorded otherwise erroneous results will be obtained after correcting for dilution in the calculation. By multiplying the volume of diluted extract assayed by the dilution factor for the sample, the actual volume of undiluted extract assayed is calculated. This is a more convenient way to express the amount of sample assayed rather than expressing as a dilution. Thus, over the range of 0-200 nL of extract assayed, there is an excellent linear relation with the absorbance (Fig.4.11).

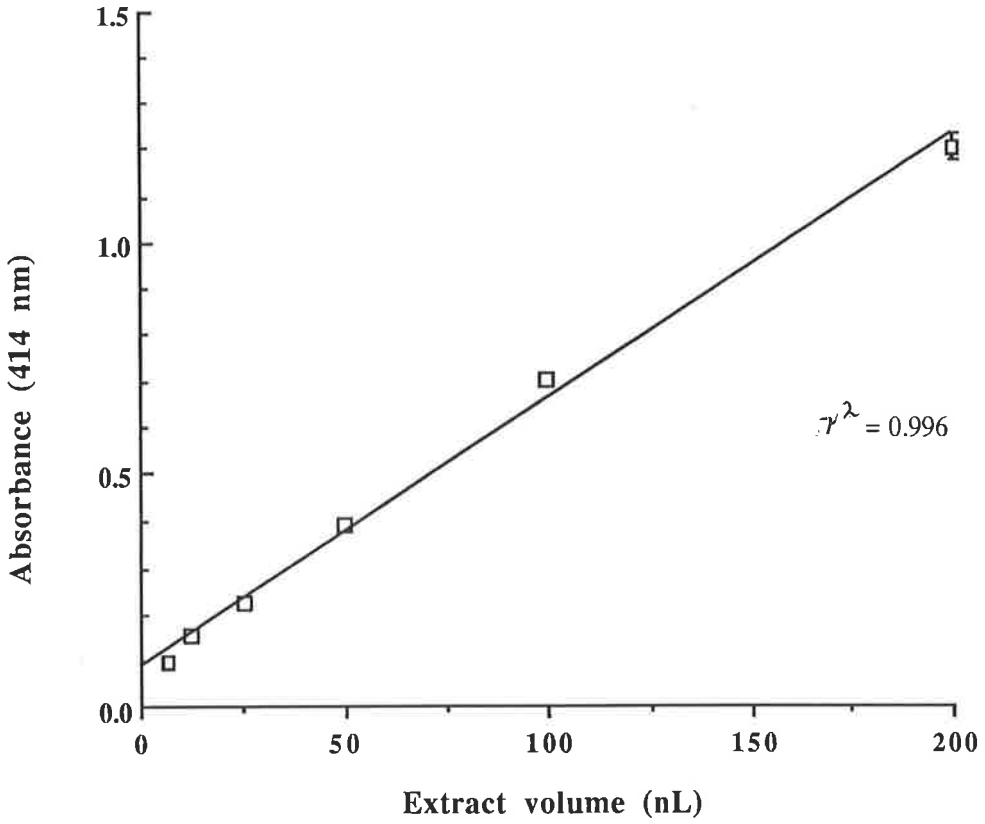



**Figure 4.11**

Linearity of the ELISA with volume of malted barley seed extract. Data points (❑) are the mean ± sd of three replicates. The continuous line is the linear regression line of 'best fit'.

### 4.3.4.3 Potential interference from an extract of malt

In the crude extract there could be substances which could interfere with the accuracy of the ELISA. Different quantities of crude extract were added to known quantities of purified LD and the total activity resulting from the pure enzyme and that in the extract measured by the ELISA. If the extract does not interfere with the measurement of LD then adding a fixed amount to different, but known quantities of purified LD will cause a parallel shift in the "standard curve" (Fig. 4.12). Interference from proteases in the extract degrading the purified LD could for example, cause a non-parallel shift.

The same result is obtained when purified LD standards are 'spiked' with a semi-pure (Sephacryl S200 fraction from the purification, see 3.2.2.4) LD preparation (results not shown).

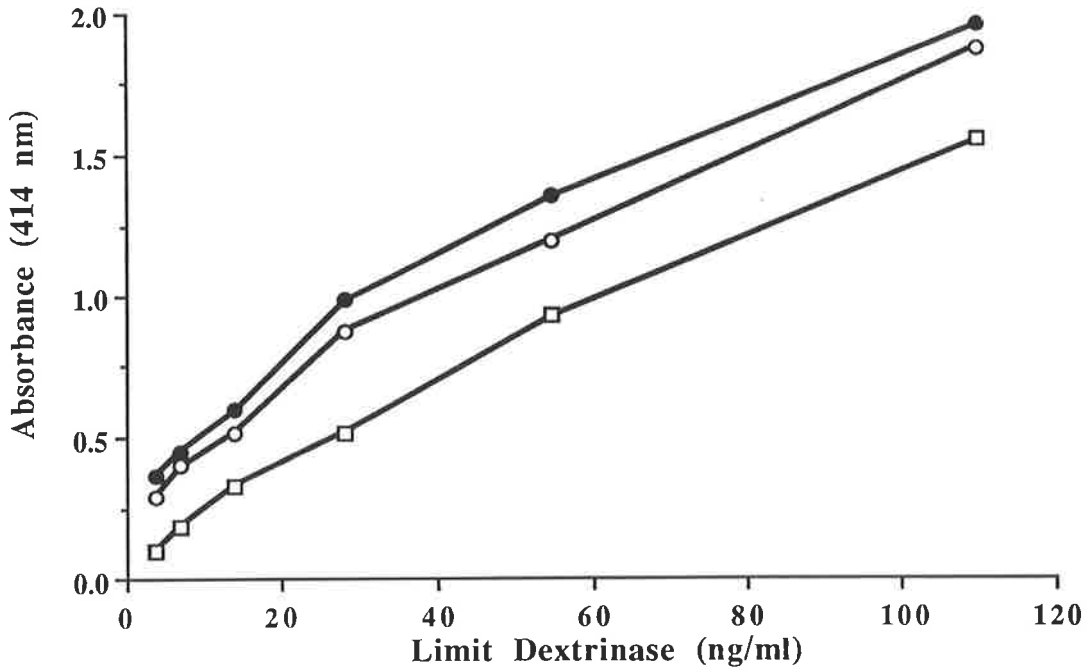



**Figure 4.12**

Accuracy of the ELISA. Purified LD standards were spiked with either 0, 13 or 50 nL of crude extract (in a volume of 1ml) to give final concentrations of 110, 55, 28, 14, 7, 3.5 ng/ml and assayed by ELISA (see 4.3.3.1). LD plus 13 nL extract, (○); LD plus 50 nL extract, (●); LD alone, (□).

#### 4.3.4.4 Evidence that the specific anti-limit dextrinase antibody recognises active enzyme.

The location of antibody binding sites can be quite independent of the enzymes active site. Immunological activity may therefore have no relation to enzyme activity, i.e. the antibody may recognise protein irrespective of enzyme activity. The ELISA developed here is intended to be used to measure the enzyme activity of LD in order to study the changes during physiological and biochemical processes. This problem was addressed by Vaag and Munck (1986) who developed an ELISA for measuring residual amyloglucosidase activity in low-calorie beers. They demonstrated that immunological activity of amyloglucosidase was destroyed at a rate that almost equals the destruction of the enzymatic activity.

The relationship between immunological and enzyme activity can be defined by manipulating enzyme activity and measuring the changes in the immunological activity with the ELISA. High temperature reduces enzyme activity (Lee and Pyler, 1984) but the choice of temperature is important. When extracts of malt flour are heated at 65°C, activity decreases too quickly to be measured accurately, and at 55°C activity did not decline sufficiently (see 3.3.2.3). A temperature of 60°C produced the desired reduction in enzyme activity (80 % decline over a 2 h period). The only suitable assay for measuring LD activity in extracts of malt flour are those methods using a dyed-pullulan substrate. The red pullulan assay was used to measure the enzyme activity of the malt extracts (Megazyme, Australia).

Heating the extract of malt reduced both the enzyme and immunological activity at an almost identical rate (Fig. 4.13). After 2 h only the ELISA can still detect a further decline in activity probably because it is the more sensitive assay. These results suggest the antibody binding sites are destroyed at a similar rate to the enzyme active site, presumably due to thermal denaturation, and supports the hypothesis that the ELISA recognises protein with enzyme activity. Daussant *et al.* (1987) evaluated a rocket immunoelectrophoresis assay for LD in a similar manner but used purified LD to compare the immunoassay with an enzyme assay because they experienced difficulties in measuring enzyme activity in extracts of malt flour. They concluded their immunological assay was measuring enzyme activity.

The close agreement between the two assays suggests that some of the antigenic sites may be located at the enzymes active site. The dependence of immunological activity on the stability of the active site can be tested by using site specific inhibitors.

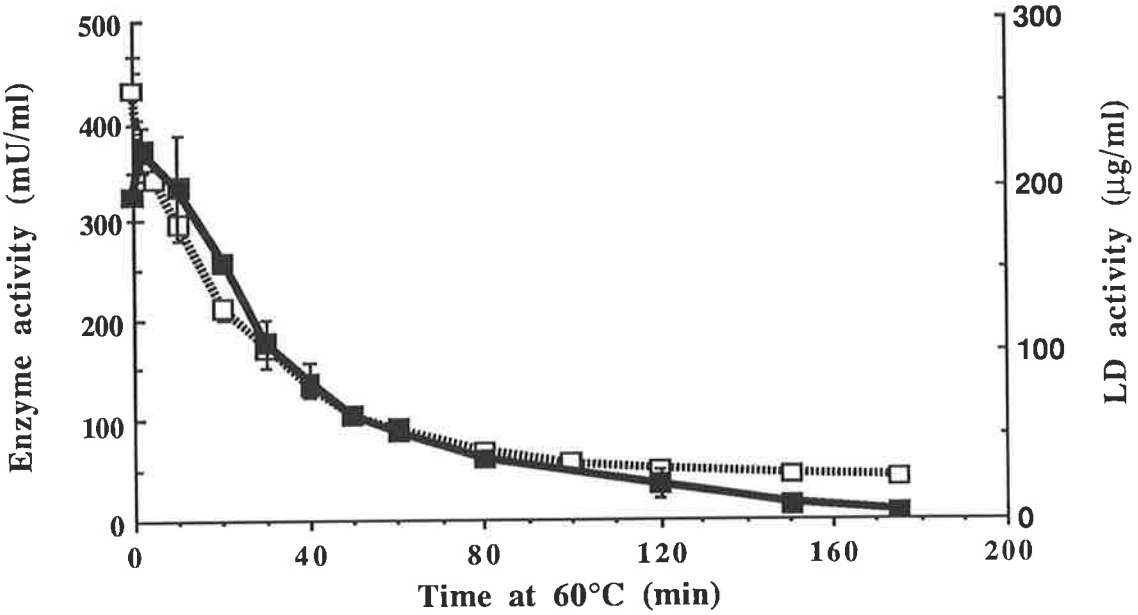



**Figure 4.13**

Effect of heat on enzyme and immunological (LD) activity of limit dextrinase. Barley malt (cv. Clipper) was ground in a Udy mill to pass through a 0.5 mm sieve. An extract was prepared by mixing 20 g flour with 80 ml of 0.2 M acetate buffer, pH 5 containing 20 mM cysteine for 5 h at 30°C. Aliquots (200µl) of this extract were incubated at 60°C in a water bath for 0, 2, 5, 10, 30, 40, 50, 60, 80, 100, 120, 150, 180, 210 and 240 min then immediately transferred to an ice water bath until all the samples were collected. After centrifugation, the supernatant was assayed for LD activity by the RP assay and ELISA. Enzyme activity, (❑); LD activity by ELISA, (■).

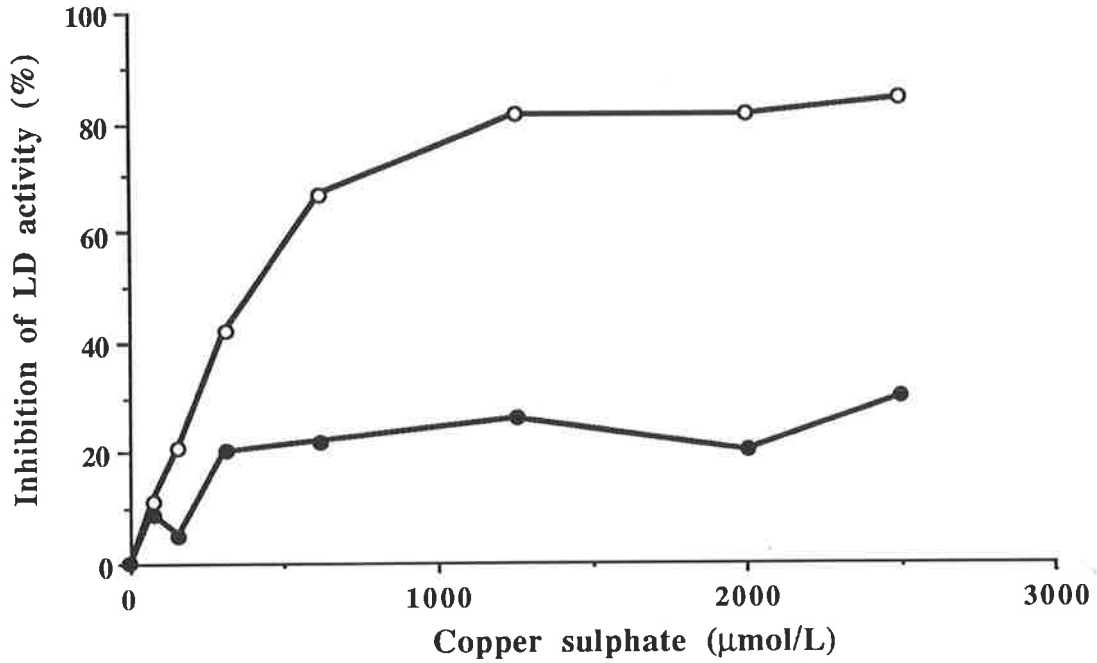



**Figure 4.14**

Inhibition of enzyme and immunological activity by copper. Purified LD (100 µl) was incubated with copper sulphate (final concentrations: 0, 78, 156, 312, 625, 1,250, 2,000, and 2,500 µmol/L in a final volume of 200 µl) at 37°C for 30 min. For the ELISA, 10 µl of a 1:1000 dilution of each sample was assayed. Enzyme activity was measured by the method described in Materials and Methods (2.1). Enzyme activity, (○); LD activity by ELISA, (●). On the y-axis activity is expressed as a percent inhibition defined as:

$$\% \text{ Inhibition} = 100 \times \frac{[\text{activity in absence of Cu minus activity in presence of Cu}]}{[\text{activity in absence of Cu}]}$$

Copper is an inhibitor of LD activity but the mechansim of inhibition is not known (Maeda *et al.*, 1979). Purified LD was incubated with copper sulphate at a range of concentrations to reduce enzyme activity (Fig. 4.14). Copper reduced enzyme activity by more than 85 % at 1.25 mM. In contrast, immunological activity was only inhibited by 30 % at the highest concentration of $CuSO_4$ tested (2.5 mM). It is possible that $Cu^{++}$ replaces another divalent cation at the active site (EDTA is known to inhibit barley LD activity suggesting that a divalent cation is involved at the active site, see 1.2.3) or at a substrate binding site thereby causing inhibition of enzyme activity. Some of the antigenic sites may be located at the active site and if these are blocked when copper binds, antibody binding would be decreased. However, other antigenic sites are likely to be present at other locations on the molecule not affected by copper, which would explain why immunological activity was less affected. The anti-LD antibodies appear to be measuring enzyme protein which has significantly reduced activity, i.e. inactive enzyme, but probably not substantially denatured. However, this kind of inhibition is very specific and may not be a significant problem for the immunoassay. This is because in the malting and brewing process, high temperatures cause the denaturation of enzyme activity and under these conditions, a good relationship between the enzyme and immunological assays exist.

#### 4.3.4.5
#### Assay reproducibility

All samples assayed using the ELISA were measured in triplicate. The precision based upon comparing results of replicate tests of the same sample within a single assay or between different assays (on the same day) using different plates has a CV of 3-6 %. To minimise variation between assays performed on different days, for each study presented in this thesis, all of the samples relevant to the particular study were assayed on the same day. This procedure was also applied to the assay of enzyme activity using the RP assay.

#### 4.3.4.6
#### Comparison of the ELISA with other enzyme assays

Below is a list of the main assays available to measure LD activity (Fig. 4.15).

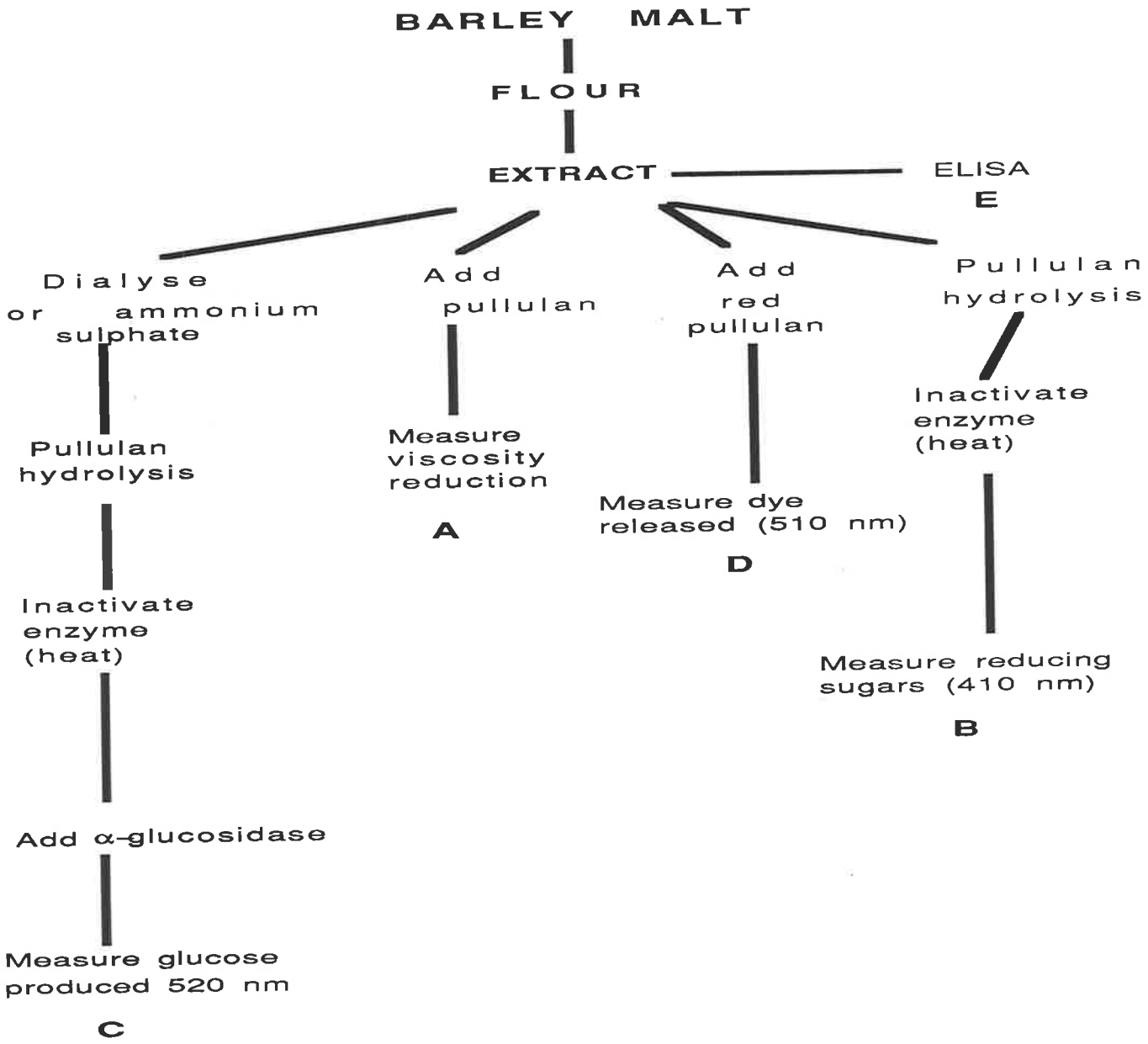
**A** The reduction in the viscosity of pullulan caused by LD has been used to assay this enzyme in extracts of barley seed. This method is unsuitable for the analysis of large numbers of samples (see 1.3).

**B** The measurement of the reducing sugars produced by LD hydrolysis of pullulan (in excess). This method is not suitable for measuring LD in crude extracts (see 1.3) but is acceptable when dealing with purified preparations of LD.

**Figure 4.15**

Assays for measuring limit dextrinase activity in barley malt.

| Method | Reference |
|---|---|
| A | Hardie and Manners (1974) |
| B | Manners and Yellowles (1973) |
| C | Lee and Pyler (1984) |
| D | McCleary (1991) and Serre and Laurière (1990) |
| E | Immunoassays |


BARLEY MALT
FLOUR
EXTRACT
ELISA
E
Dialyse
or ammonium
sulphate
Pullulan
hydrolysis
Inactivate
enzyme
(heat)
Add α-glucosidase
Measure glucose
produced 520 nm
C
Add
pullulan
Measure
viscosity
reduction
A
Add
red
pullulan
Measure dye
released (510 nm)
D
Pullulan
hydrolysis
Inactivate
enzyme
(heat)
Measure reducing
sugars (410 nm)
B

**C** To overcome the difficulties with the previous assay (B), LD activity is measured by adding an excess of yeast α–glucosidase after pullulan hydrolysis to convert all the residual sugars to glucose. A blank containing no pullulan is run to estimate starch-derived glucose. The increase in glucose production is due to the LD activity in the sample.

**D** The measurement of the quantity of dye released from a dyed-pullulan substrate is used to measure LD activity. These assays are specific and rapid to perform making them suitable for the assay of large numbers of samples.

**E** Immunochemical assays such as the ELISA developed here and the electroimmunodiffusion assay of Daussant *et al.* (1987).

An attempt was made to compare assays B, C, D and E. A major problem with assays B and C are the low sensitivities and high background absorbance caused by endogenous reducing sugars. Although extensive dialysis reduced some blank absorbance (blank is defined as the absorbance resulting from the extract incubated without pullulan), the increase in glucose formed due to LD action was either very small or nonexistent. Also, many samples tested in these assays gave a lower absorbance than the blank i.e. the glucose produced when extract was incubated with pullulan was lower than the blank. Thus, useful comparisons between assays B and C with the ELISA were not possible using crude extracts. This illustrates a major problem with these types of assays in that they do not accurately measure LD in a crude extract. Reliable results could not be obtained with method C. This assay has low sensitivity and is very time consuming to perform due to the extensive dialysis required for the removal of endogenous reducing sugars. Similar problems with this assay were described by Lenoir (1985).

Another approach for comparing the different assays is to add an extract of malt to known quantities of purified LD and assay the resultant mixture for total LD activity. If the assay is only measuring LD, and this was established for the ELISA (see Fig. 4.12), the effect of adding an extract of malt to the purified LD will be to cause a shift in the "standard curve" by the amount of LD in the extract. The accuracy of the assay could be affected by components in the extract which interfere with the assay mechanism for measuring LD. The likely effect of this is to produce a non-parallel shift in the spiked 'standard curve'. The effect of spiking the pure enzyme with extract upon the shift in the standard curve is shown for three assays (Fig. 4.16a-c). The relationship between known amounts of the purified enzyme and the measurement of activity in terms of reducing equivalents is shown in figure 4.16a. The LD activity of the extract is about 7 mg RE/ml and as increasing amounts of the purified enzyme are added, the "standard curve" should be shifted to the left by the amount of activity in the extract alone. The enzyme plus extract line becomes non-linear after about

300 mU/ml of purified LD plus extract has been added (Fig. 4.16a). This suggests that the quantity of substrate (pullulan) used to measure enzyme activity has become limiting, i.e. the assay is outside the linear range.

For the RP assay (Fig. 4.16b) spiking the pure enzyme with extract produces a parallel shift to the left by the amount of LD activity in the extract. This indicates the RP assay can accurately measure LD in the presence of the crude extract i.e. there is no interference caused by the extract in this assay.

Similarly for the ELISA (Fig. 4.16c) a parallel shift in the standard curves with increasing amounts of extract added was obtained. This suggests the ELISA is capable of accurately measuring LD from a crude extract of malt (see also Fig. 4.12).

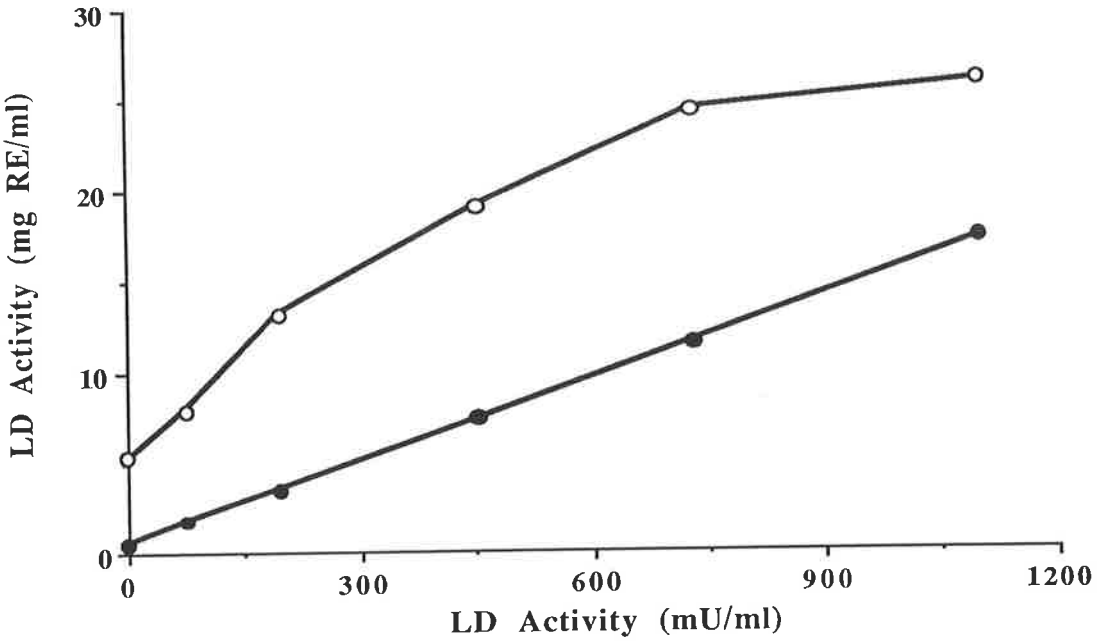



**Figure 4.16a**

Effect of germinated barley seed extract upon the accuracy of the pullulan/reducing sugar assay (B). Purified LD standards of known activity (measured using the assay in 2.1) were spiked with or without crude extract and then assayed using method 'B'. Purified enzyme alone, (●); purified enzyme plus extract, (○).

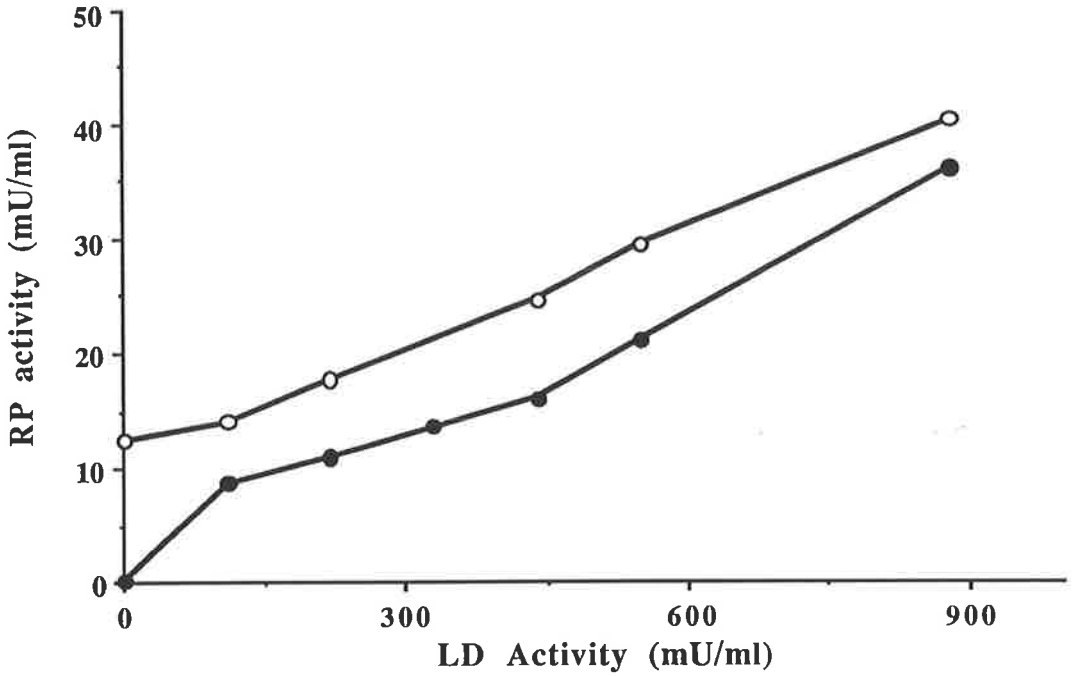



**Figure 4.16b**

Effect of germinated barley seed extract upon the accuracy of the RP assay (D). Purified LD standards of known activity (measured using the assay in 2.1) were spiked with or without crude extract and then assayed as described in 2.5. Purified enzyme alone, (●); purified enzyme plus extract, (○).

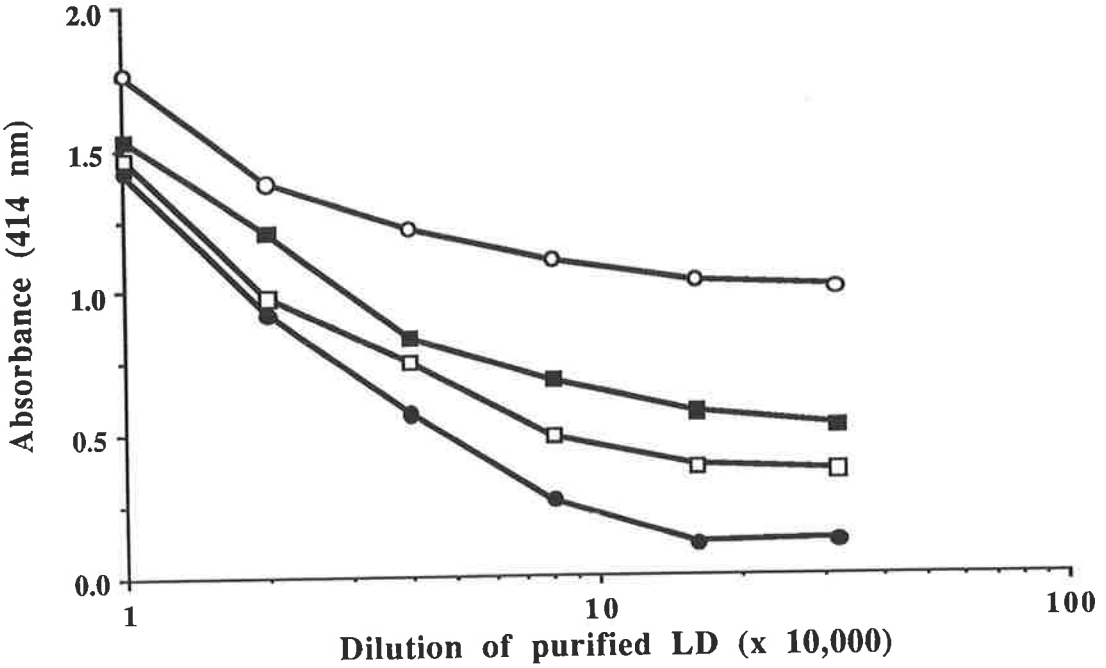



**Figure 4.16c**

Effect of germinated barley seed extract upon the accuracy of the ELISA (E). Purified LD standards of known activity (measured using the assay in 2.1) were spiked with or without crude extract and then assayed by the ELISA as described in 4.3.3. Purified enzyme alone, (●); purified enzyme plus 50 nL extract, (○); purified enzyme plus 20 nL extract, (■); purified enzyme plus 10 nL extract, (□).

### 4.3.5 Extraction of Limit Dextrinase

In any extraction process the aim is to obtain all the component of interest in a stable form so that accurate and reproducible measurement is possible. Lee and Pyler (1984) report optimal extraction was achieved after 2.5 h at 20°C using a phosphate-citrate buffer, pH 6.8. Other reports indicate that more LD is extracted using reducing agents (Yamada, 1981b and 1981c). Lenoir *et al.* (1984) noted that although the use of reducing agents allows a better extraction of LD, optimum extraction is obtained only after steeping the kernels in potassium metabisulphite solution prior to extraction.

The effect of various buffers on the extraction of LD was studied. In early studies performed before the dyed-pullulan substrates became available, the pullulan/reducing sugar method was used. Due to problems with quantifying LD enzyme activity in crude extracts using this method an attempt was made to quantitate activity by precipitating proteins with ammonium sulphate and assaying the redissolved proteins. This was necessary to

LD from the high concentration of reducing sugars present in the extract, but the results from this procedure were not reproducible. Later, the Red-Pullulan substrate became available which allowed a better estimate of LD activity (Table 4.3).

The procedure recommended by the manufacturer of the RP assay is to extract for 17 h in 200 mM acetate buffer containing 20 mM cysteine, apparently to obtain maximum extraction. This procedure is time consuming so a comparison was made between a 5 and a 17 h extraction. There was no significant difference ($p < 0.05$) in enzyme and immunological activity of LD extracted after 5 h compared to 17 h (Table 4.3). Acetate buffer containing a reducing agent extracted between 60 % more enzyme and immunological activity relative to acetate buffer alone. These results suggest that thiol reducing agents were required to extract maximal levels of LD from the extracts of malt flour. This is consistent with other reports of the need for reducing agents. However, Lee and Pyler found extraction with citrate-phosphate buffer (pH 6.8) was comparable to using cysteine (7 mM). Further studies presented in this thesis indicate that maximum extraction of LD with cysteine is achieved with concentrations above 15 mM (see Table 4.4).

The possibility that cysteine enhances LD activity per se was tested on an extract of malt and activity was measured using the RP assay (Table 4.4). There was no change in the LD activity of flour extracts prepared with cysteine in the extraction buffer after incubation with cysteine at the concentrations and incubation periods tested. This suggests that all the LD has been extracted. However, when flour extracts were prepared without cysteine in the extraction buffer and then cysteine was added to the supernatant, the LD activity increased at cysteine concentrations between 10-60 mM and incubations for up to 30 min. There was no significant difference in activity after 30 min incubation at the concentrations tested. If LD is present in a 'bound form' which has no activity and this is released to a form which has activity ('free from'), then the data presented here suggests that cysteine activates enzyme activity. Immunological activity was not measured on these samples but a similar study in chapter 7 (Fig. 7.11) indicates there is no change in immunological activity. To resolve this question it is necessary to test the effect of cysteine upon the activity of the purified enzyme. This experiment was not undertaken because of the relatively large quantities of purified enzyme required using the RP assay. These data and the results in 7.4 strongly suggests that cysteine acts in two ways, (1) to increase the extraction of LD from flour extracts of germinated or malted seed, possibly by releasing LD from a bound form, and (2) to activate (in terms of an increase in the catalytic rate) the enzyme (see chapter 7 for a more detailed discussion).

**Table 4.3**

Effect of various chemicals upon enzyme extraction. Barley grain (cv. Clipper) was micromalted, kiln dried and the malt ground in a Udy mill to pass a 0.5 mm screen. Malt flour (2 g) samples were extracted with 8 ml of extraction solution for either 5 or 17 h at 30°C. Insoluble material was removed by centrifugation at 1,000 g for 10 min. The supernatant was assayed for LD activity by the ELISA (dilution 1:1000) and the RP assay. Data are the mean of six determinations; SD refers to the standard deviation.

| Extraction Solution | Mean ELISA Activity ± SD (µg/ml) | Mean RP Activity ± SD (mU/ml) | Mean ELISA Activity ± SD (µg/ml) | Mean RP Activity ± SD (mU/ml) |
|---|---|---|---|---|
| | 5 hour extraction | | 17 hour extraction | |
| Citrate-phosphate buffer 100 mM, pH 6.8 | 25 ± 2.3 | <15 | 32 ± 2.0 | <15 |
| Sodium acetate buffer 200 mM, pH 5 | 31 ± 6.4 | 140 ± 8.1 | 33 ± 6.9 | 164 ± 20.5 |
| Acetate buffer plus 20 mM Glutathione | 41 ± 7.2 | 159 ± 20.3 | 46 ± 8.5 | 179 ± 33.8 |
| Acetate buffer plus 20 mM Cysteine | 47 ± 2.4 | 170 ± 22.1 | 53 ± 2.7 | 218 ± 11.3 |
| Acetate buffer plus 10 mM Dithiothreitol | 50 ± 7.5 | 197 ± 32.4 | 50 ± 4.9 | 224 ± 21.9 |

**Table 4.4**

Effect of cysteine on release or activation of LD from malt flour extracts. Barley malt (cv. Clipper) was extracted with either acetate buffer alone or acetate buffer containing 20 mM cysteine for 5 h at 30°C. The extracts were centrifuged and cysteine added (at the final concentrations shown in the table) to 3 ml aliquots of the supernatant and incubated at 30°C for either 10, 30 or 60 min. After incubation a 1 ml subsample was assayed for activity using the RP assay (see 2.5). Data on activity are the mean of 2 replicate estimations ± standard deviation.

| Cysteine concentration (mM) | Incubation Time (min) | Extract with cysteine | Extract without cysteine |
|---|---|---|---|
| | | LD activity (mU/ml) | |
| **0** | **10** | 173 ±13 | 43 ± 2 |
| 10 | | 173 ± 8 | 63 ± 3 |
| 20 | | 184 ± 3 | 81 ± 4 |
| 60 | | 165 ± 1 | 95 ± 4 |
| 120 | | 182 | 99 ± 2 |
| **0** | **30** | 164 ± 16 | 44 ± 3 |
| 10 | | 177 ± 15 | 71 ± 2 |
| 20 | | 184 ± 12 | 88 ± 4 |
| 60 | | 165 ± 11 | 102 ± 5 |
| 120 | | 167 | 108 ± 7 |
| **0** | **60** | 175 ± 26 | 43 ± 4 |
| 10 | | 175 ± 12 | 81 ± 3 |
| 20 | | 179 ± 11 | 96 ± 3 |
| 60 | | 162 ± 14 | 97 ± 5 |
| 120 | | 174 | 107 ± 6 |

### 4.3.6 Crossed Immuno-Isoelectric Focusing

Ideally, to investigate the various isoforms of LD seen after IEF, the technique of crossed immuno-Isoelectric focusing should be applied to purified enzyme. However, such experiments require a relatively large quantity of pure enzyme which was not available. Instead, this technique was developed using an extract of malt. The polymorphism detected by immunoblotting after IEF of an extract of malt (see 4.3.7) could be due to isoforms of the enzyme or multiple antibodies in the serum recognising different proteins. If the purified enzyme contained an impurity, this could have induced, during immunization, the formation of corresponding antibodies. The following results support the first hypothesis.

One single heavy precipitin band covers the whole area of the three constituents separated by IEF (Fig. 4.17). This strongly suggests that the proteins detected by immunoblotting are recognised by a single population of antibodies (shown to be specific for LD earlier) and these proteins are probably isoenzymes of LD. The origin of these isoforms, whether due to physiochemical reasons, post-translational modifications of the main constituent or genetic expression of isoenzyme, calls for further studies. To answer these questions it would be necessary to isolate and characterise the individual proteins. Their amino acid sequence for example could provide useful information about their structure and help to understand their physical properties. There are several techniques which might enable the successful separation of these isoenzymes. In IEF, immobiline pH gradients (which have resolution better than 0.001 pH units), if used on a preparative scale may enable isolation. Chromatofocusing, another high resolution method with peak widths ranging down to 0.04-0.05 pH units, could prove useful and is suitable for preparative work.

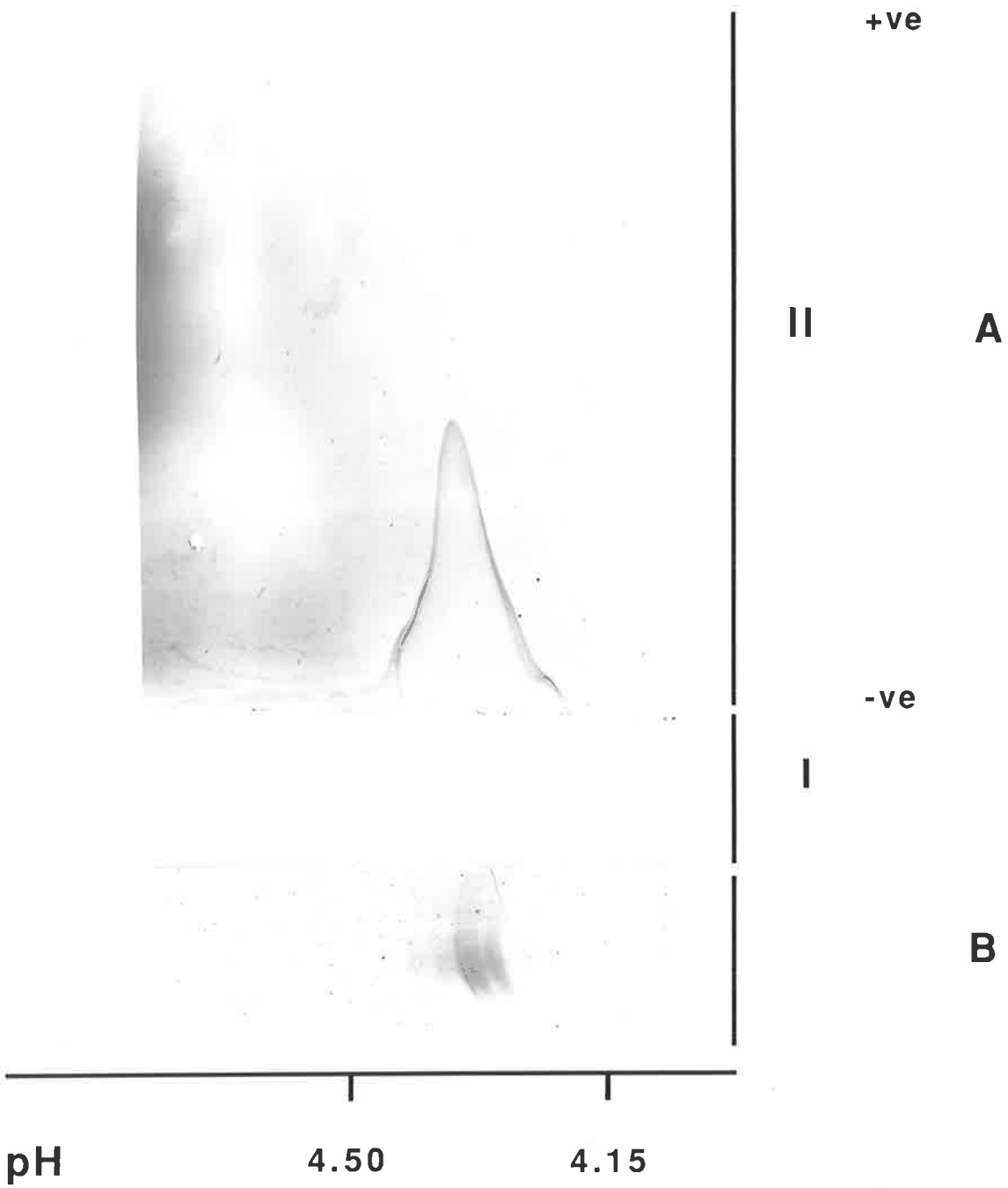
### 4.3.7 Development of an Immunoblotting Procedure for Isoelectric Focusing Gels

To be certain that the isoforms seen after IEF or immunoblotting of IEF gels (pH range 4.1-4.5) are true isoenzymes they must be separated and tested for enzyme activity. The separation of the isoenzymes is difficult due to the close spacing of the bands (Fig. 3.7) and attempts at using chromatofocusing and immobiline described above proved unsuccessful.

Another approach for assessing the enzyme activity of the bands, is to be able to stain each band *in situ* for enzyme activity. A commonly used method for detection of LD activity in gels uses amylopectin β-limit dextrin as a substrate incorporated into an overlay gel. The overlay gel is stained with iodine and where the substrate has been degraded by LD a blue

**Figure 4.17**

Crossed Immuno-isoelectric focusing. A: Crossed immunoelectrophoresis; (I) location of polyacrylamide gel strip containing the separated proteins; (II) agarose gel containing a 1:1500 dilution of anti-LD serum. B: Immunoblotting pattern. See section 4.2.8 for experimental details.


+ve
II
A
-ve
I
B
pH
4.50
4.15

colour is produced (Kruger and Marchylo, 1978). However, the presence of α–amylase in malt extracts hinders characterisation by this method because β–limit dextrin is a substrate for these enzymes. Prior immunoabsorption of extracts with anti-α–amylase immune serum to reduce this interference removes most of the unwanted staining (Lenoir *et al.*, 1984). However, the zymograms specific for LD published to date show broad, diffuse bands with poor resolution. There are no reports using a narrow pH range (4-5) for the zymogram and better resolution may be possible using this range. The zymograms do not match the resolution possible with immunoblotting, and therefore are not suitable for separating the isoenzymes of LD. The characterisation of reducing sugars (Gabriel and Wang, 1969) formed after immersion of the IEF gel in a solution of pullulan has also been used (Lenoir *et al* , 1984; Lenoir, 1985) but again poor resolution was obtained. More recently, Yang and Coleman (1987) describe a method for the detection of pullulanase activity in polyacrylamide gels after electrophoresis. A pullulan-reactive red agar gel was overlayed onto the separating gel and the site of enzyme activity was indicated by clear bands developed on a red background. This method may have promise for the characterization of putative isoenzymes of LD.

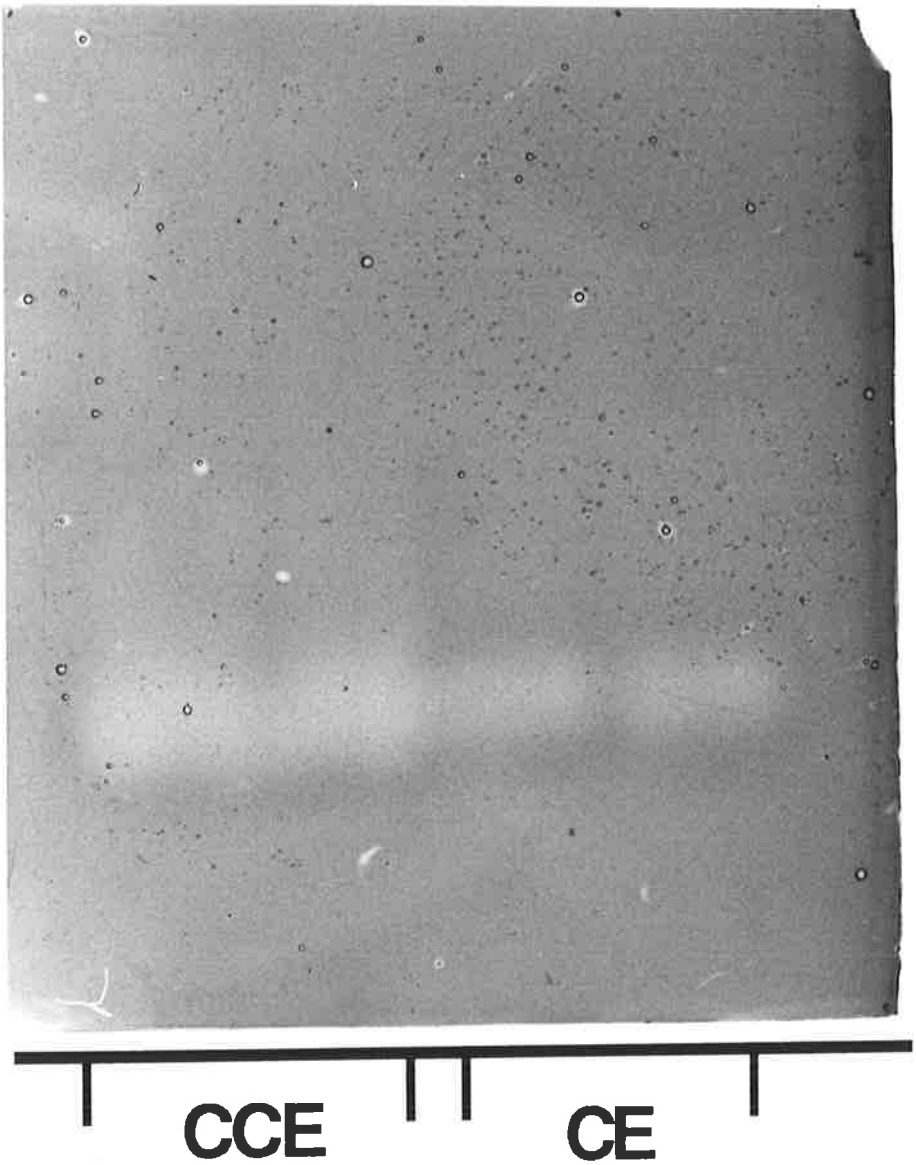
Attempts at using either red-pullulan overlay gels or incubating the IEF gel in a solution of Red-Pullulan have proved unsuccessful. In both methods broad, diffuse bands were obtained (Fig. 4.18 shows the results with the former method) and again they do not approach the resolution of the bands obtained when purified LD is subjected to IEF (Fig. 3.1c). An alternative approach is to detect the isoenzymes using immunoblotting.

There are no reports in the literature where isoenzymes of LD have been detected using immunoblotting. Thus a method based on separation of the isoforms using IEF and transfer of the proteins to a membrane which could be probed with the anti-LD antisera was required. The development of such a procedure is described below. The following variables were considered important to the success of the method :

(1) Choice of enzyme labeled second antibody and substrate
(2) First and second antibody dilutions
(3) Optimum conditions for the transfer of the proteins from the gel to the membrane
(4) Optimum blocking solution
(5) Sample preparation requirements
(6) Avoidance of artifacts
(7) pI determination

**Figure 4.18**

Detection of LD activity using Red-Pullulan overlay gel. Extracts of malt flour (pre-concentrated by ultrafiltration 3-4 fold) were electrophoresed on an 8 % polyacrylamide gel by the method described in 2.3.1 except that SDS and reducing agents were not used. After electrophoresis, the gel was soaked twice in fresh 25 % isopropanol and 50 mM acetate buffer (pH 5) for 10 min, immersed in acetate buffer for 10 min, blotted dry and overlaid on top of the pullulan-reactive red agar plate (prepared by mixing 12 ml of 4.2 % (w/v) agarose in 100 mM acetate buffer, pH 5 with 12 ml of a heated 2.5 % (w/v) Red Pullulan solution (see 2.5), into a mould 14 x 8 x 0.2 cm ). The plate was covered with a lid, tightly sealed with parafilm and incubated at 37°C for 15 h to detect LD activity and the overlay gel immediately photographed.


CCE
CE

Crude extract proteins were separated in a pH 3.5-9.5 gradient and prepared for immunoblotting as described in 4.2.5 revealed a series of closely spaced bands between pH 4-5 (Fig. 4.19). Loading the sample at various positions on the gel between the cathode and anode did not change the final focused position of the protein. If a narrower pH gradient (3.5-5) is used, there is an enhanced resolution (Fig. 4.20). The optimum position for loading the sample was 1-2 cm from the cathode (data not shown but the optimum location for a pH 3.5-5 gradient gel with a longer interelectrode distance is shown in figure 4.24). All preliminary experiments were conducted using a crude extract of malt prepared by the method described in 4.3.5 and separated by IEF using a pH 3.5-5 gradient unless otherwise stated.

(1) Choice of enzyme labeled second antibody and substrate

Horseradish peroxidase (HRP) was the first enzyme conjugate used for immunological detection on blotted membranes. The HRP conjugate and substrate are the least expensive and provide rapid detection results compared to gold and alkaline phosphatase (AP) labeled antibodies. However, the blots are subject to fading with exposure to light, HRP activity can be inhibited by azide in the buffers and non-specific colour precipitation due to endogenous peroxidase enzymes and metal bearing proteins can occur. The AP enzyme offers a number of advantages and was compared with HRP. The clarity of bands detected by the two procedures is similar (Fig. 4.21) but it took about 6 hours for the bands to become visible using AP compared to only 2 min with HRP. Also, the background staining was greater with AP, therefore detection of anti-LD using the HRP system was preferred.

Much of the early development work on the IEF/blotting technique used the HRP system but another substrate for the alkaline phosphatase was tried which is claimed by the manufacturer to be 10-20 fold more sensitive than HRP (Blake *et al.*, 1984). The substrate is a combination of 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitro blue tetrazolium (NBT) (Promega Corporation, Australia). This AP/BCIP/NBT system produces a stable reaction product that will not fade when exposed to light. Immunoblots using the HRP or AP/BCIP/NBT were compared. The AP/BCIP/NBT system gave sharper and clearer bands with improved resolution (see Fig. 4.20).

(2) Effect of varying the first and second antibody dilutions

First antibody dilution

IEF and immunoblotting were performed in a pH 3.5-5 gradient using the HRP second antibody and the 3, 3'-diaminobenzidine (DAB) substrate. The pattern obtained using a 1:500 dilution of the first antibody (anti-LD immune serum) was identical with

**Figure 4.19**

Isoelectric focusing and immunoblotting of extracts of malt flour over a pH 3.5-9.5 range. Extracts of barley malt (cv. Clipper) were prepared as described in 2.6.5 and the proteins separated by IEF as detailed in 2.3.2 and 3.2.3.2. Immunoblotting was performed by the method detailed in 4.2.6.2. Samples were applied at different locations on the gel (indicated by an arrow), see also figure 4.24.

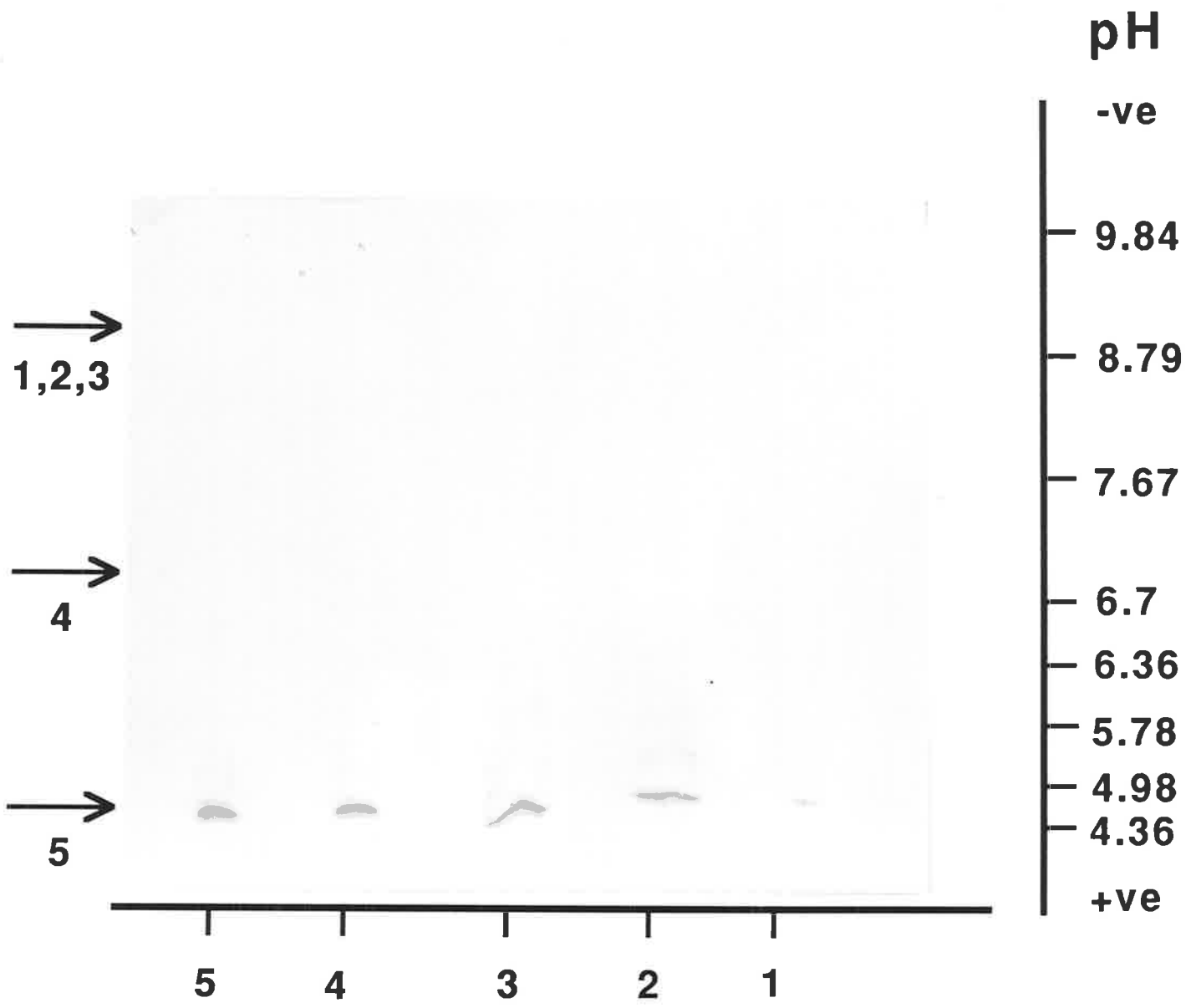

pH
-ve
9.84
8.79
7.67
6.7
6.36
5.78
4.98
4.36
+ve
1,2,3
4
5
5
4
3
2
1

**Figure 4.20**

Isoelectric focusing and immunoblotting of extracts of malt flour over a narrow pH range. Extracts of germinated barley or malt and germinated wheat were prepared as described in 2.6.5 and the proteins separated by IEF as detailed in 2.3.2 and 3.2.3.2. Immunoblotting was performed by the method detailed in 4.2.6.2 (alkaline phosphatase conjugate and the BCIP/NBT substrate, left side of diagram) and by the method detailed in 4.2.6.1 (goat anti-rabbit horseradish peroxidase, on the right side of the diagram).

Tracks: 1-3, Clipper malt
4, 5, Chinese Spring (wheat); germinated
6, 7, Betzes (germinated)
8, 40 µl of Clipper malt

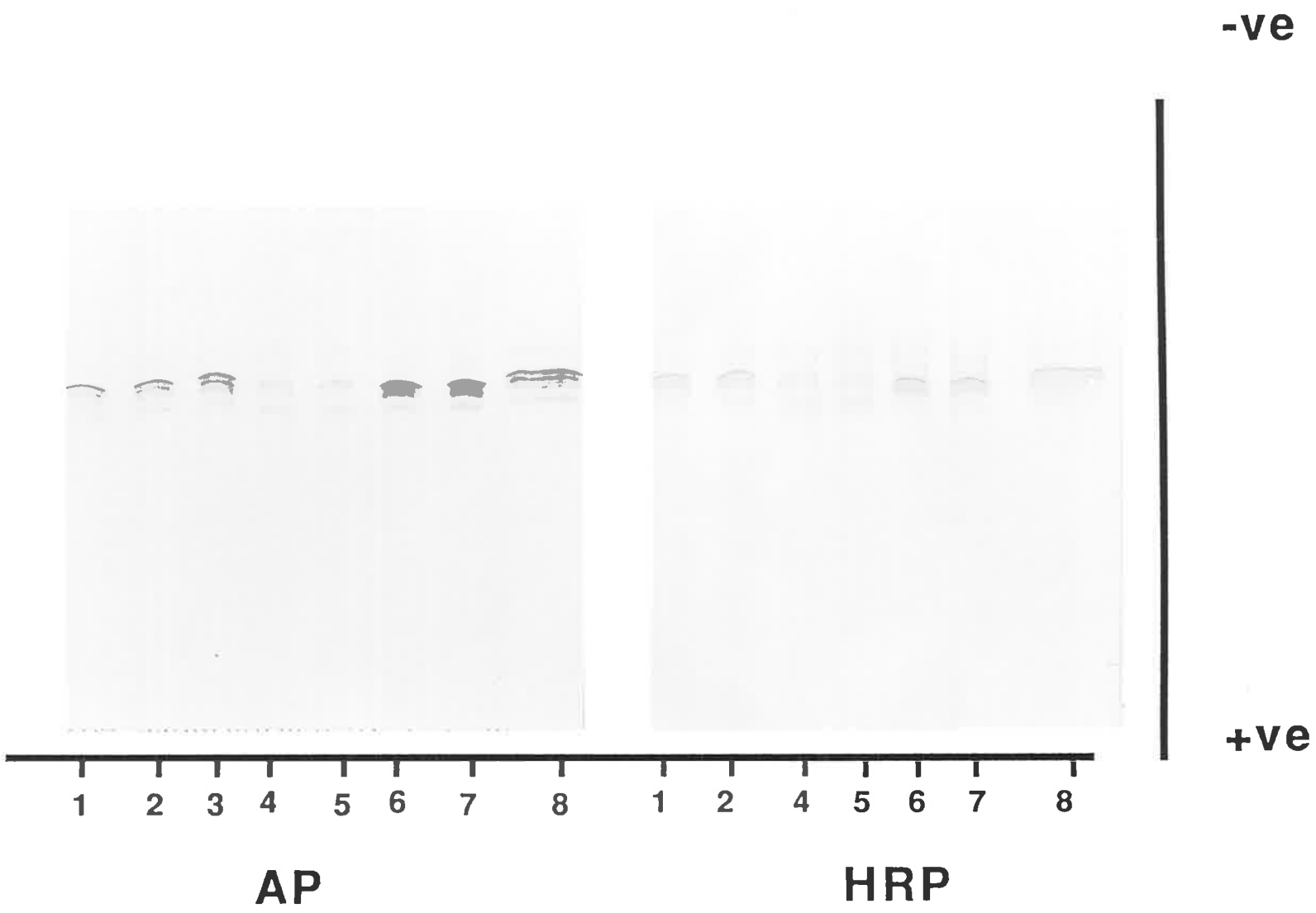
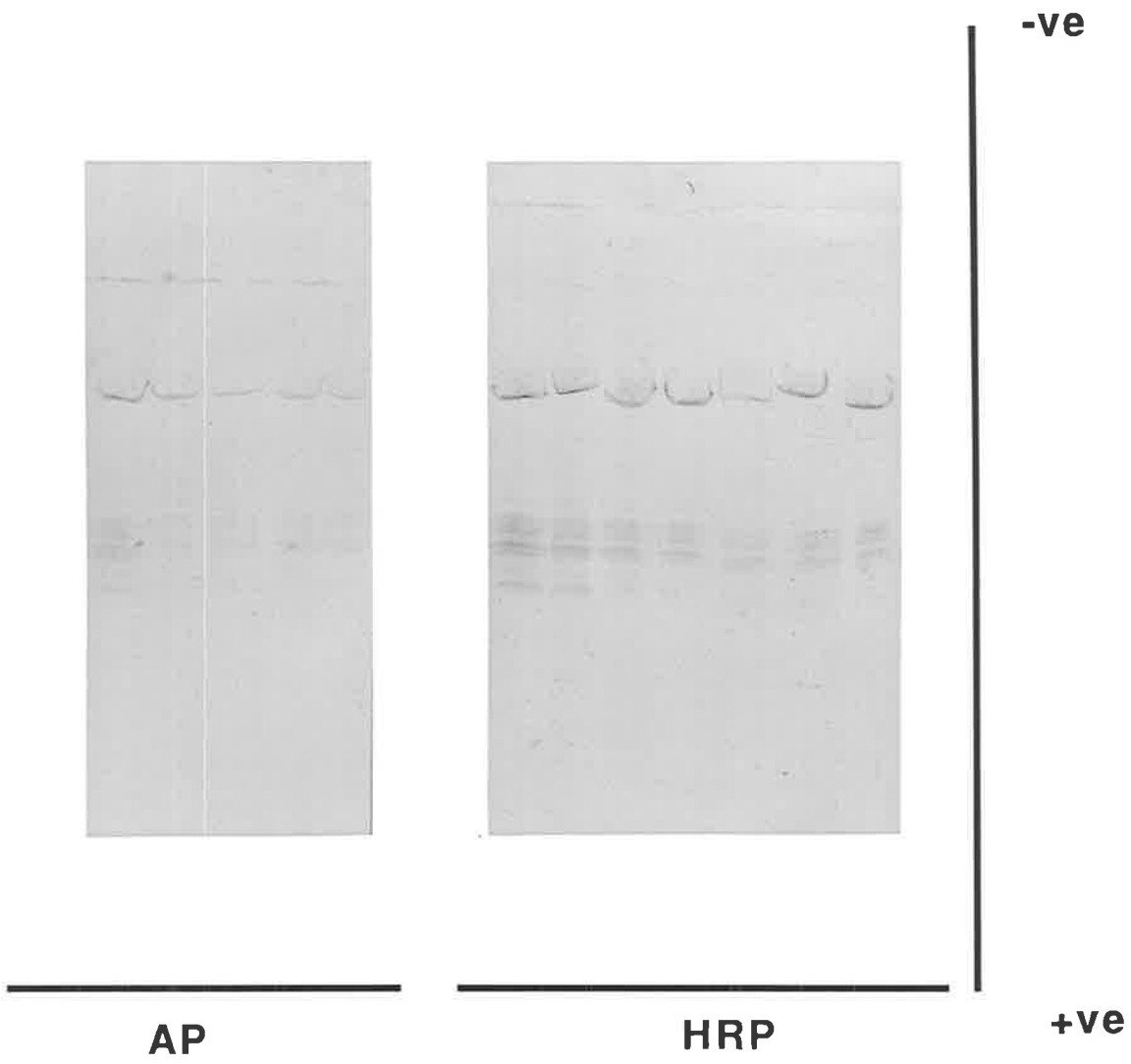

-ve
+ve
1
2
3
4
5
6
7
8
1
2
4
5
6
7
8
AP
HRP

**Figure 4.21**

Comparison of different enzyme second antibody conjugates for immunoblotting. An extract of Clipper malt was prepared and the proteins separated by IEF as detailed in 2.3.2 and 3.2.3.2. Immunoblotting was performed by the method detailed in 4.2.6 2 except that for the second antibody, one half of the membrane was incubated with goat anti-rabbit alkaline phosphatase conjugate (AP) and the other half with goat anti-rabbit horseradish peroxidase conjugate (HRP). The substrate used for the alkaline phosphatase was Fast Red (see 4.2.5) and for HRP, DAB. The staining obtained at the location where the sample applicators were placed is an artifact.


-ve
AP
HRP
+ve

respect to banding intensity, resolution and clarity to a 1:1000 dilution but at a 1:2000 dilution the bands were very faint. The most economical use of the antiserum consistent with good results was a 1:1000 dilution.

Second antibody dilution

The dilution of the AP using the BCIP/NBT substrate requires a higher dilution than commonly used with enzyme labelled second antibodies to avoid high backgrounds (Fig. 4.22). A dilution of 1:7500 was found to be satisfactory with a 1 h incubation. The manufacturers recommend a 30 min incubation but the bands take too long to develop. Blotting time can be reduced by increasing the concentration of this antibody at the expense of consumption of a relatively expensive antiserum.

(3) Optimum transfer time

After diffusion transfer to nitrocellulose was completed (45 min) the gel was fixed and stained with Coomassie blue. Only a faint smear was visible suggesting most of the protein had transferred to the membrane. However, silver staining reveals many more proteins indicating that diffusion transfer is not quantitative.

(4) Optimum blocking solution

The blocking agent recommended by the manufacturer of the BCIP/NBT substrates (Promega Corporation, Australia) is a 1% (w/v) BSA solution in TBST. Dried skim milk powder (5 % w/v) is a cheaper alternative to BSA but gave slightly higher backgrounds (Fig. 4.22).

(5) Sample preparation requirements

The presence of cysteine in the extraction buffer is essential to obtain detectable bands without prior concentration of the sample before electrophoresis (Fig. 4.23). One of the intended uses of the procedure is to screen different cultivars for banding pattern differences and it is necessary to have a simple, rapid and reproducible blotting procedure. The need to pre-concentrate the sample would add considerable time to the processing of the sample. Sample preparation for IEF is important, for example the sample should have a low conductivity to avoid distortions in the bands. A relatively high buffer concentration is used in the extraction of the LD (200 mM sodium acetate) which might cause problems. Desalted samples (by diafiltration using Amicon PM 10 membranes and conventional dialysis) produced an identical banding pattern to the non-desalted sample. The standard procedure developed was to add 20 µl of undiluted extract to 1.5 x 0.5 cm paper applicators which were left on the gel for 30 min during electrofocusing. Extending the contact time tended to

**Figure 4.22**

Effect of blocking agent and second antibody dilution. Extracts of barley malt (cv. Clipper) were prepared as described in 2.6.5 and the proteins separated by IEF as detailed in 2.3.2 and 3.2.3.2. Immunoblotting was performed by the method detailed in 4.2.6.2 with modification: **A**, blocking with 1 % BSA, second antibody diluted 1:2000
**B**, blocking with skim milk powder (5 % w/v), second antibody diluted 1:7500
**C**, blocking with skim milk powder (5 % w/v), second antibody diluted 1:2000.

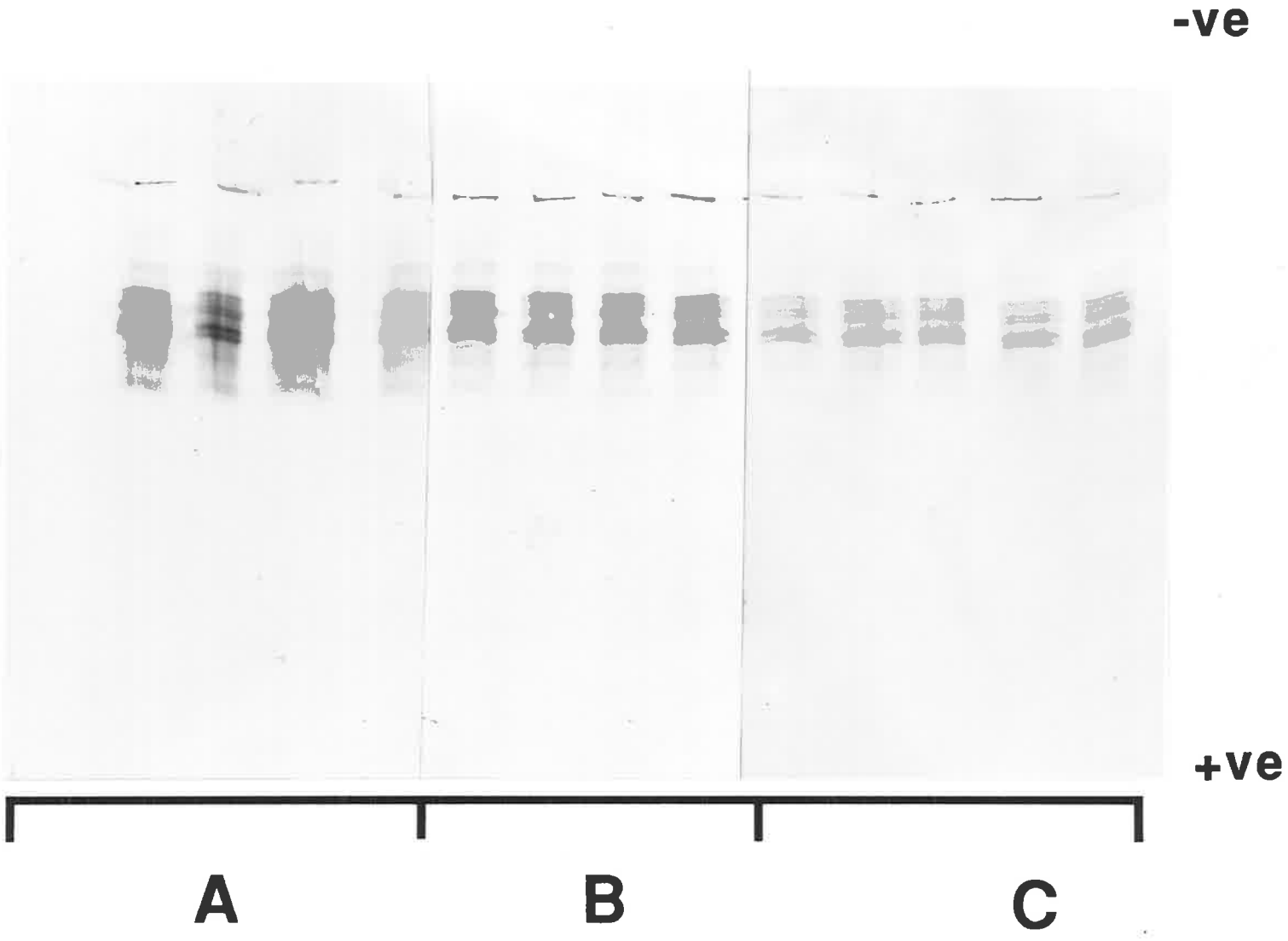
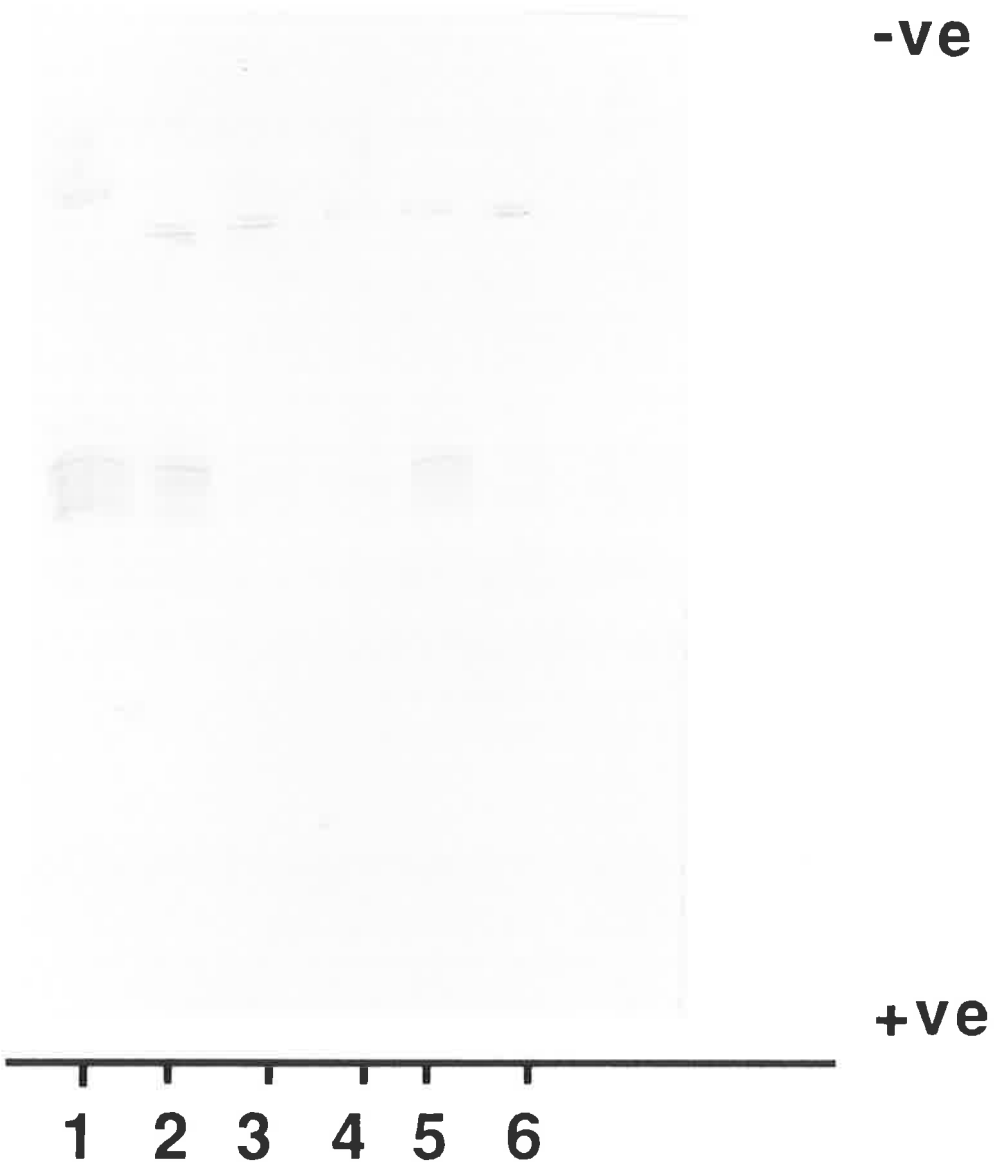

-ve
+ve
A
B
C

**Figure 4.23**

Importance of sample preparation on immunoblotting. Extracts of Clipper malt were prepared as described in 2.6.5 except that cysteine was either omitted or included in the buffer. Proteins were separated by IEF as detailed in 2.3.2 and 3.2.3.2. Immunoblotting was performed by the method detailed in 4.2.6.2. Tracks 1, 2 and 5, extract buffer containing cysteine; 3, 4 and 6 extracted without cysteine.


-ve
+ve
1
2
3
4
5
6

produce streaking and a longer focusing time was required. However, extending the electrofocusing time may produce pH gradient drift i.e. the pH gradient in the gel becomes unstable and decays faster than the proteins migrate, since proteins move more slowly as they approach their isoelectric point. Leaving the applicators in contact with the gel for 30 min avoided these problems.

The Whatman 3 MM papers were found to be better than silicon rubber application strips (Pharmacia, Sweden).

The optimum position on the gel to load the sample was determined by applying the sample at various points diagonally across the gel between the two electrodes. Samples applied near the anode gave little or no pattern. Possibly the low pH near the anode solution (sulphuric acid, 0.05 M) denatures the enzyme causing precipitation at the application site. Sample is best applied near the middle of the gel since there is always a slight temperature rise near the anode and cathode during gradient formation. When LD is separated using a narrow pH gradient, focusing is near the middle of the gel, so this is not a desirable position. Application near the cathode produced a good result. Focusing was complete after 5000 volthours (Fig. 4.24).

(6) Avoidance of artifacts

The band heterogeneity seen after IEF of the purified LD (Fig. 3.6) could be an artifact but many proteins reveal microheterogeneity after high resolution IEF (Gianazza and Righetti, 1980a) Some of the possible explanations for microheterogeneity seen in the purified enzyme and in immunoblots of crude extracts are:

(i) Physiochemical reasons i.e. the different bands may represent alternative stable configurations. This could be caused by

(a) Glycosylation; the isolated IEF zones could correspond to a non-uniform distribution of saccharide units between different molecules. There is no evidence in the literature on whether LD from barley is a glycoprotein. Detection using the periodic acid-Schiff stain could be used to stain LD in polyacrylamide gels. However, each of the bands may have different carbohydrate content which would alter their pI. This has been suggested for carboxypeptidase 1 from malted barley which consists of two species of pI 5.65 and 5.73 (Breddam, 1983; cited in Gianazza and Righetti, 1980).

(b) Peptidase action during extraction resulting in forms with different pI's; Adding various peptidase inhibitors (Table 4.5) to the extraction buffer did not alter the IEF banding pattern of LD (Fig. 4.25). This suggests the microheterogeneity is not likely to be due to peptidase degradation of the enzyme during extraction.

**Table 4.5**

Endopeptidase inhibitors used to investigate whether the IEF banding patterns are caused by peptidase action.

| Inhibitor | Concentration | Type of endopeptidase |
|---|---|---|
| **Leupeptin** | 10 μM | thiol endopeptidase |
| **pCMPS**[1] | 10 μM | thiol endopeptidase |
| **PMSF**[2] | 1 mM | serine endopeptidase carboxypeptidase |
| **EDTA**[3] | 1-10 mM | metalo endopeptidase |
| **Pepstatin** | 10 μM | aspartic acid endopeptidase |

1. p-Chloromercuriphenol sulphonic acid
2. Phenylmethylsulfonylfluoride
3. Ethylenediaminetetraacetic acid

(c) Microheterogeneity may arise by proteolytic modification of the enzyme during germination. To test this two barley varieties, Schooner and [illegible] were germinated for 11 days and extracts prepared from the freeze-dried grain and analysed by IEF-immunoblotting. There was no variation in the banding pattern between the days 1-11. The bands in the early germinated seed were less well stained (due to a lower concentration of LD in these samples, see chapter 7 for data) compared to 11 day samples (Fig. 4.26). This is evidence that the microheterogeneity is not due to proteolytic modification of the enzyme during germination.

(ii) Post-translational modification of the main constituent. Modifications *in vivo* occur to many proteins during translation and after release from the polysome. The amino acids can be covalently modified which affects their pI.

(iii) Genetic expression of isoenzymes. Each band represents a specific protein (isoenzyme) which is coded by a gene. Differences in amino acid composition could also account for the charge difference and hence pI.

The enzyme, α–amylase is a complex mixture of components which are revealed by IEF. Some of the heterogeneity is caused by proteolytic modification of the enzyme during

**Figure 4.24**

Optimum location for sample application to IEF gels. Extracts of Clipper malt were prepared as described in 2.6.5. Proteins were separated by IEF as detailed in 2.3.2 and 3.2.3.2 except the interelectrode distance for this experiment was 20 cm to see if separation could be improved. Immunoblotting was performed by the method detailed in 4.2.6.2. The position on the gel where the sample (indicated by a number) was applied is shown by an arrow on the vertical axis of the diagram.

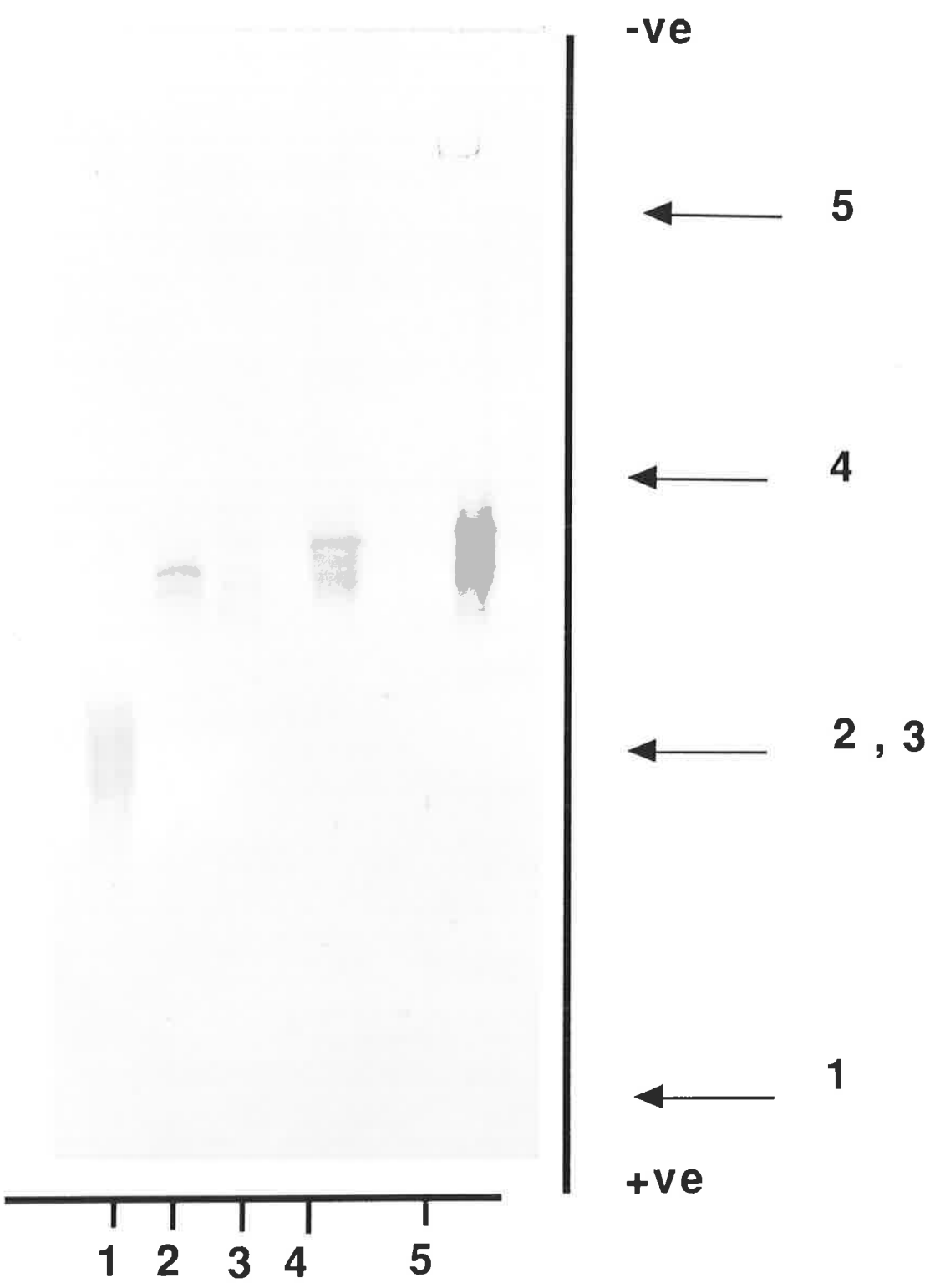

-ve
5
4
2 , 3
1
+ve
1 2 3 4 5

**Figure 4.25**

Effect of peptidase inhibitors on immunoblotting pattern. Extracts of Weir malt were prepared as described in 2.6.5 except the extraction buffers used had a peptidase inhibitor added. Proteins were separated by IEF as detailed in 2.3.2 and 3.2.3.2 and immunoblotting was performed by the method detailed in 4.2.6.2.

Tracks: 1, control extract
2, EDTA (10 mM)
3, pepstatin (10 μM)
4 and 5, PMSF (1 mM)
6 and 7, PCMPS (10 μM)
8 and 9, leupeptin (10 μM)

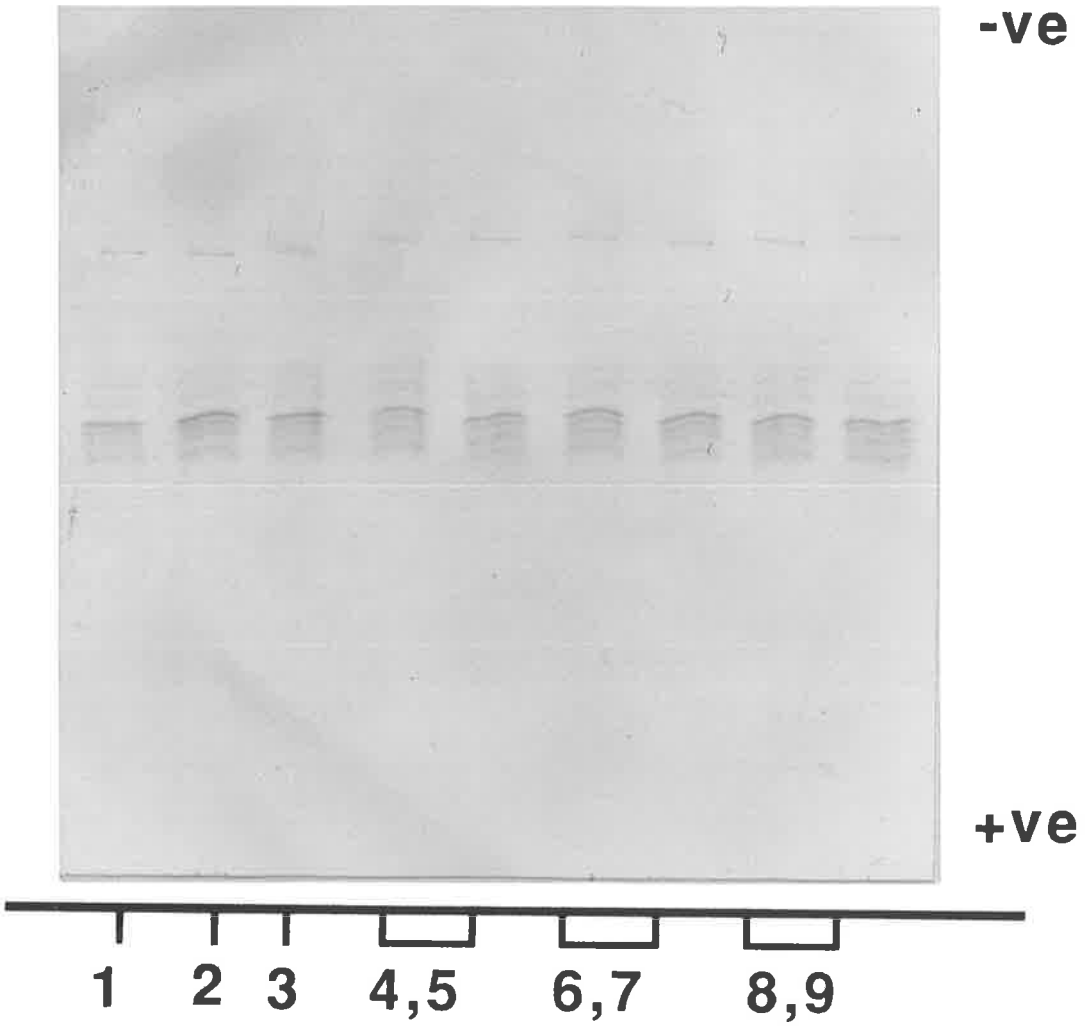
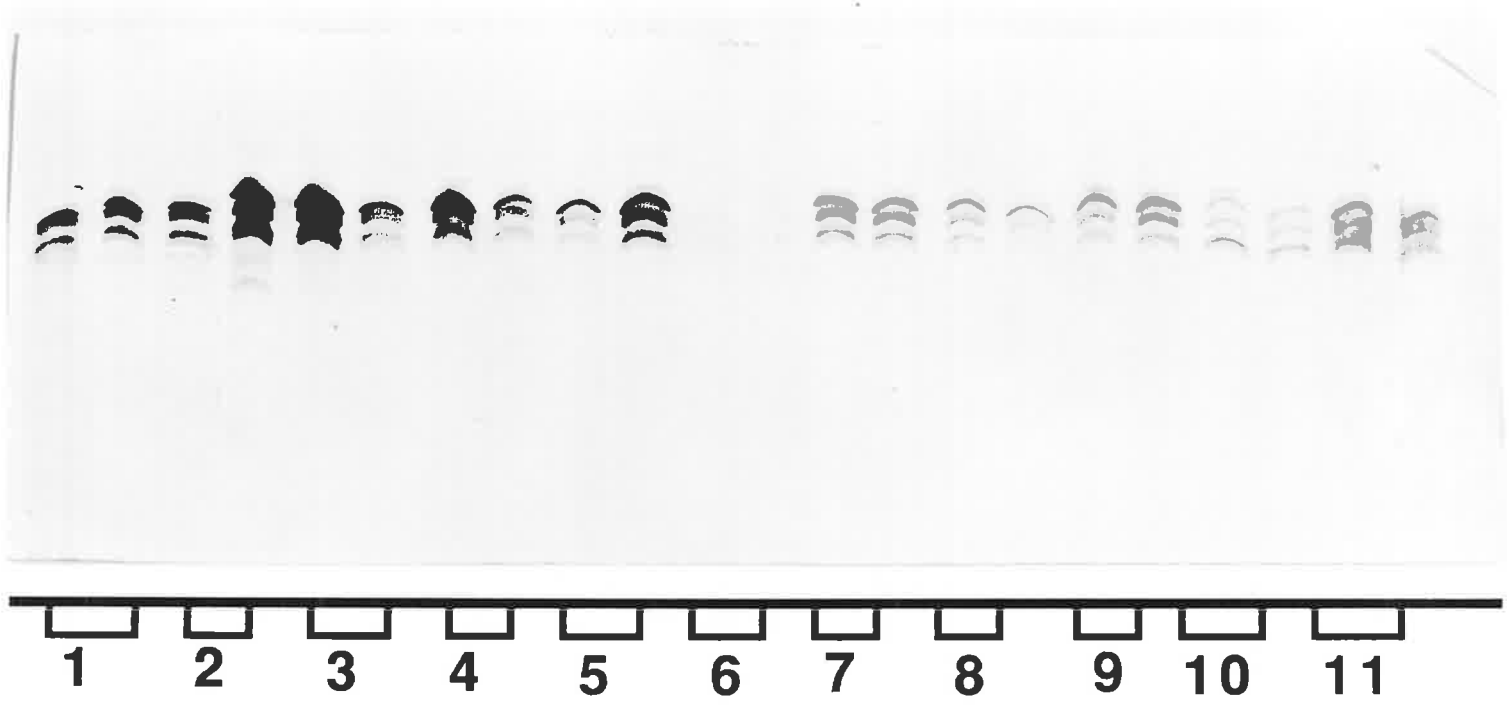

-ve
+ve
1
2
3
4,5
6,7
8,9

**Figure 4.26**

Effect of germination on banding pattern microheterogeneity. Extracts of germinated barley (cv. Fuji Nijio) were subject to IEF over the pH 3.5-5.0 range. Grains were germinated for 1-11 days and prepared as described in 5.2.2.1, except that the frozen grain was freeze-dried. Extracts were prepared as described in 4.3.5 and IEF as detailed in 2.3.2 and 3.2.3.2. Immunoblotting was performed by the method in 4.2.6.2. Samples are in duplicate. Numbers refer to the day of germination.

-ve




+ve

extraction (Hayes *et al.*, 1988). However, α–amylase isoenzymes are the product of two gene families.

(7) pI determination

Three methods for measuring the pH profile of the gel after isoelectric focusing were tested. (i) Use of a surface pH electrode suitable for gels. A surface electrode easily tore the ultrathin (0.4 mm thick) gels and was not suitable. (ii) Small pieces of gel (1 x 1 cm square) were added to tubes containing 1 M KCL in deionised water and left for 1-2 h to allow the amphyloytes to diffuse. The pH of the solution was measured. A typical calibration line is shown in figure 4.27. (iii) Standard commercial protein markers of known pI were tested (Pharmacia, Sweden). A comparison of the pI of the reference proteins given by the manufacturer and their pI measured by method (ii) shows discrepancies (see the table below). The conditions used for the IEF are not identical to those suggested by Pharmacia and the true pI may differ from those stated by the manufacturer. It was found however, that method (ii) when applied to different experiments produced slightly different results. The need for the advantages of using a mixture of purified pI markers was first suggested by Righetti (1975). Reference proteins (method iii) were used in IEF experiments mentioned in later chapters.

**Table 4.6**

Comparison of pI determination using commercial markers (method iii) and method (ii).

| **Reference protein** (Pharmacia) | **Stated pI value** method (iii) | **Measured pI value** (method (ii) |
|---|---|---|
| soybean trypsin inhibitor | 4.55 | 4.93 |
| glucose oxidase | 4.15 | 4.01 |
| amyloglucosidase | 3.50 | 3.68 |

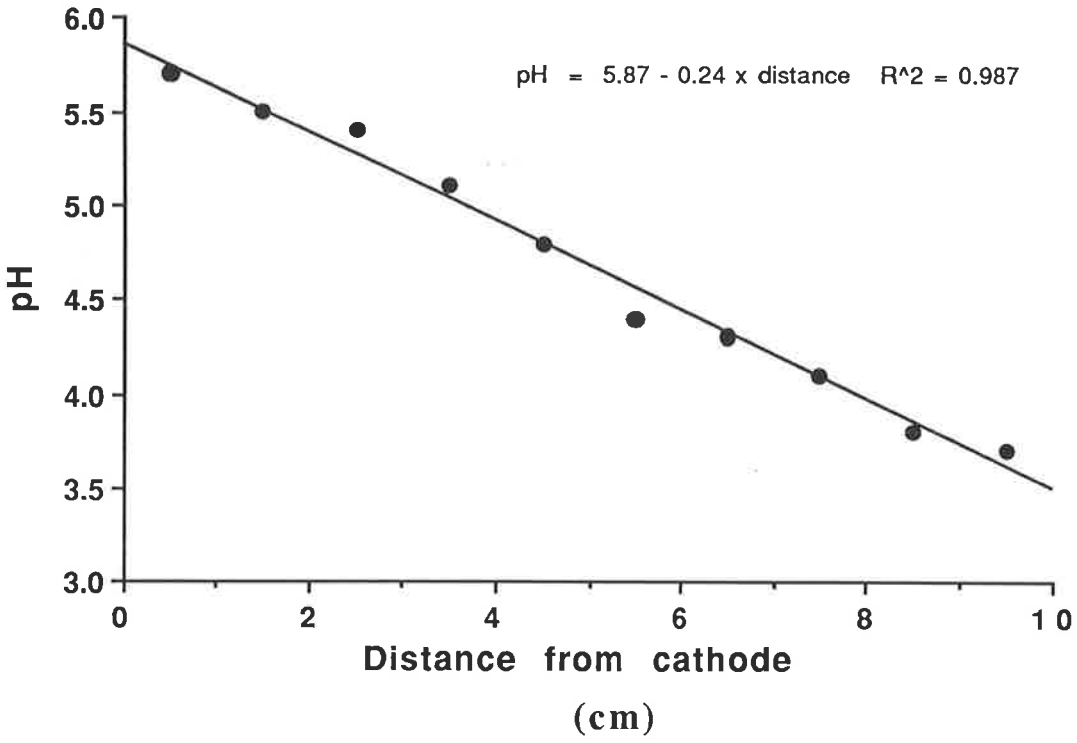



**Figure 4.27**

Calibration line to determine pH of the gel. Immediately after focusing sections of 1 x 1 cm square strips of gel were cut from the cathode end of the IEF gel (pH 3.5-5.0) and placed into 2 ml of deionised water containing 1 M KCL, mixed for 2 h and the pH measured.

# CHAPTER 5

# STUDIES OF THE GENETIC VARIABILITY OF LIMIT DEXTRINASE

## 5.1 INTRODUCTION

If a relationship between the level of LD activity and the malting quality exists there may be good reason to introduce cultivars with improved LD levels. If for example, a high activity of LD improves starch degradation and fermentability (Enevoldsen, 1975), breeding for cultivars with this desired character would be useful. A study of the genetic variability for LD in barley is a necessary prerequisite to making improvements in the activity of this enzyme through breeding. The first step would be to determine the extent of variation in enzyme activity between genotypes. The identification of cultivars with high activity could then be used in breeding studies to incorporate this useful characteristic into malting cultivars. Earlier reports indicated some genetic variation in activity (Lee and Pyler, 1984; Kneen and Spoerl, 1948) but only a few cultivars were tested in these studies. The extent of variability in activity in a wide genetic range of barley has not been studied.

Specific isoenzymes of LD may be important to malt quality. The α-amylase I isoenzyme is known to hydrolyse more starch than the more predominant α-amylase II isoenzyme (Bertoft and Kulp, 1986) and may therefore be more important to malt quality. It is possible that the qualitative and/or quantitative balance of the LD isoenzymes (which may have different structural genes) could be important in starch degradation and screening barleys for a desired isoenzyme may be required. The detection of LD isoenzymes in electrophoretic gels has utilized zymogram techniques (Serre and Laurière, 1989 and Lenoir *et al.*, 1984) with either β-limit dextrin or pullulan substrates. These methods have poor resolution and are therefore not suitable for studying isoenzyme variation. By employing the IEF and immunoblotting procedures described in chapter 4, a superior resolution and sensitivity would be possible. The combination of these techniques is being applied increasingly to the study of cereal protein and isoenzyme variation (Daussant and Bureau, 1988).

The purpose of this chapter is to obtain information on the variability of LD activity in cultivated barley and *Hordeum spontaneum.* C. Koch. Isoenzyme variability will be examined in a genetically diverse sample of barleys and, in addition, an attempt to locate the structural gene coding for the enzyme by use of the wheat-barley addition lines will be described. Furthermore, wheat nullisomic-tetrasomic lines will be used in an attempt to elucidate the chromosomal location of LD in the homoeologous chromosomes in hexaploid wheat.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Cultivar Differences in Limit Dextrinase Activity and Isoenzymes Detected by IEF-Immunoblotting

#### 5.2.1.1 Plant material

Thirty nine cultivars were selected (Table 5.1) from a trial of 135 cultivars of wide genetic diversity grown at the same site (Charlick Experimental Station; Strathalbyn, South Australia) in the 1989 season. Samples of mature grain (30 g) from each of the thirty nine cultivars and one control sample (Clipper) were micromalted as described in 2.6.1. Each sample was micromalted on three separate occasions (three different micromalting batches) so as to replicate the experiment. Malt was stored in air-tight screw capped containers in a controlled temperature room (15°C) until required for analysis.

In studies of the isoenzyme variation, grain samples of 131 cultivars (Appendix 5.1) from a similar trial (Charlick Experimental Station) in the 1988 season were germinated in petri dishes as follows: Ten seeds from each cultivar were surface sterilised in 3 % (w/v) Miltons solution for 4 min (Milton Pharmaceutical Company, N.S.W.), washed in sterile water, and blotted dry on paper towelling. The seeds were placed on two circles of Whatman 1 filter paper in the lid of a 9 cm diameter petri dish. Sterile water (4 ml) was added, and the petri dish lid sealed with Parafilm®. All manipulations were performed in a laminar flow hood. Germination was at 15°C for 11 days. After germination, the roots and coleoptile were removed and the remaining seed frozen in liquid nitrogen and ground in a pre-cooled pestle and mortar. During grinding, liquid nitrogen was added to keep the grain frozen. The finely ground material was extracted in 100 mM acetate buffer pH 5, containing 20 mM cysteine, for 4 h at 30°C. The extracts were centrifuged at 10,000 g for 10 min and the supernatants stored at -20°C until required for analysis. Seeds that failed to germinate were discarded.

#### 5.2.1.2 Analyses

Malt samples: About 5 g of kiln dried malt was ground in a Udy mill to pass through a 0.5 mm sieve. Flour moisture was measured as described in 2.6.4 and enzyme extracts were prepared from 1 g of flour (see 2.6.5). Limit dextrinase activity was measured by the ELISA (see 4.3.3.1) and the RP assay (see 2.5). Results, expressed per gram of dry weight

are means of three independent measurements (each cultivar was micromalted three times on different occasions).

The stored frozen extracts of the germinated samples were thawed in lots of twenty and analysed by IEF and immunoblotting as described in 2.3.2 and 4.2.6, respectively. The LD activity was measured by the ELISA and protein concentration by the Bradford assay (see 2.1).

#### 5.2.1.3 Statistical methods

Analysis of variance was used to determine if the variation in activity observed between cultivars was statistically significant. This analysis was performed using the program JMP™ (SAS Institute Inc., Cary, NC, USA).

## 5.3 RESULTS AND DISCUSSION

### 5.3.1 Cultivar Differences in Limit Dextrinase Activity

The LD activity of the thirty nine cultivars is shown in tabulated form in Table 5.1 and as histograms in figures 5.1 and 5.2. The ELISA activity values are distributed normally with an equal number of observations above and below the mean (Fig. 5.1). The distribution of activity measured by the RP assay is skewed toward the lower values in the range with most cultivars having activity below 0.4 U/g. In comparison, the distribution of the ELISA activities were 'normally' distributed. The median for the RP data is about half the mean whereas with the ELISA data, both these statistics are similar.

| | **ELISA** (μg/g) | **RP** (mU/g) |
|---|---|---|
| Maximum | 51.94 | 1664 |
| Minimum | 16.12 | 51 |
| Median | 33.42 | 230 |
| Mean | 32.66 | 462 |
| Standard deviation | 7.15 | 445 |

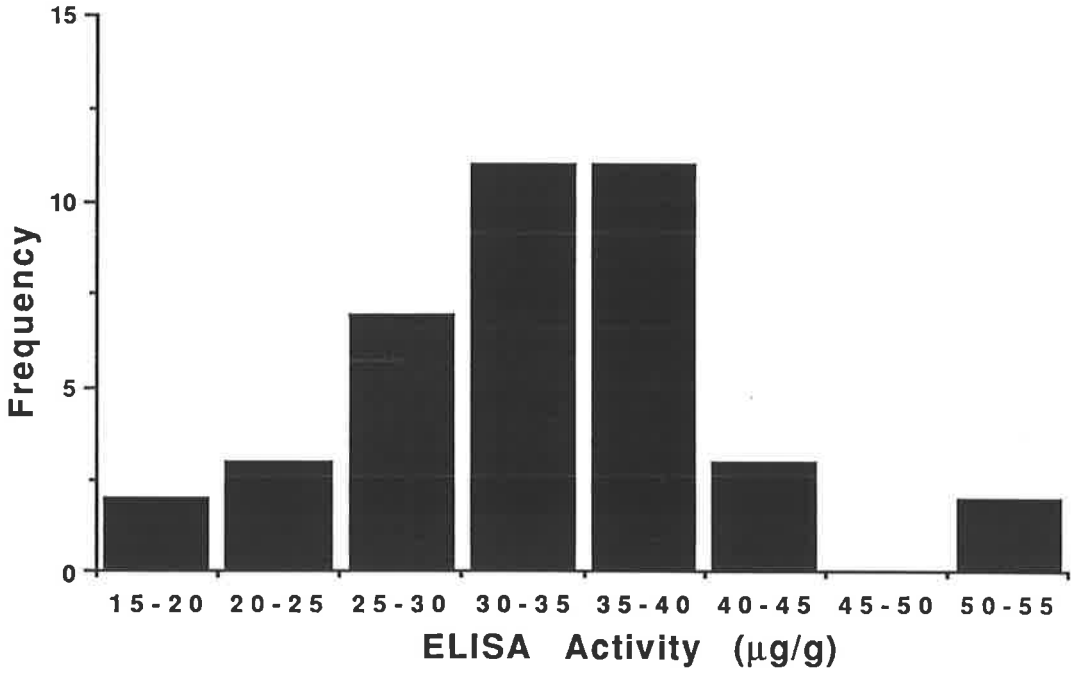



**Figure 5.1**

Frequency distribution of LD activity. ELISA activity (mean of three replicates) of the malts of thirty nine different cultivars.

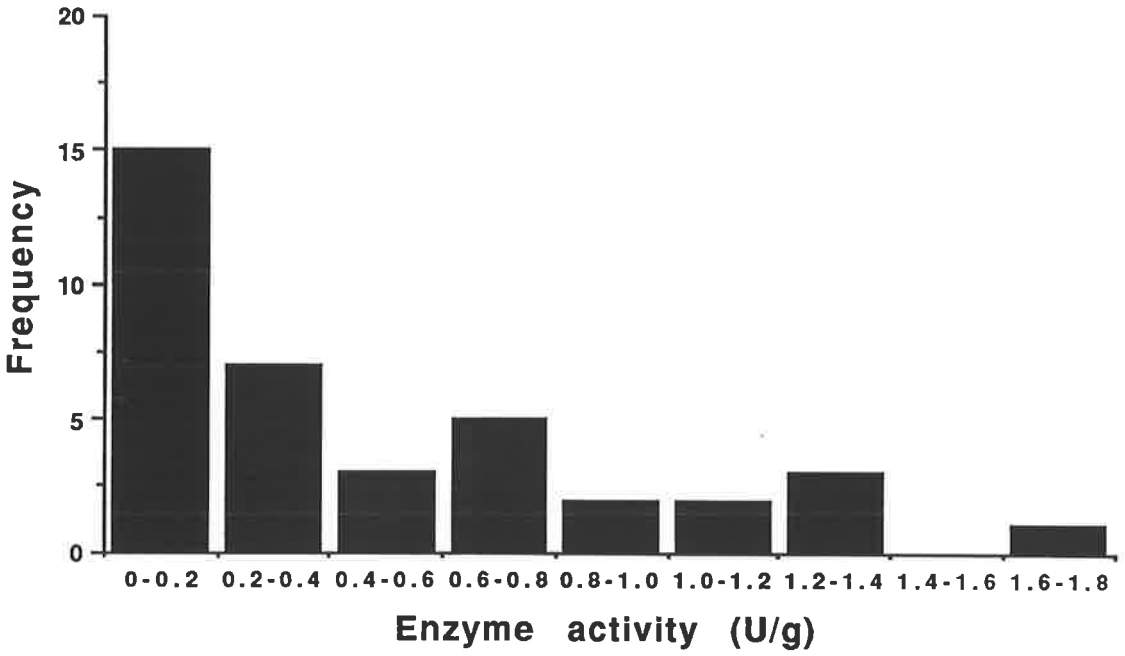



**Figure 5.2**

Frequency distribution of LD activity. Enzyme activity (mean of three replicates) of thirty nine different malts.

**Table 5.1**

Barley cultivars used to study variation in LD activity. WI is a Waite Agricultural Research Institute accession number. Data are the mean of three replicates of LD activity measured by the ELISA and RP assay. The ELISA data are sorted from low to high activity.

**a**, Cross between WI-2231 and (A. Deba x Clipper)

**b**, Cross between *Foma Ant* 13 and Kristina

**c**, Cross between WI-2335 and (Zephyr x Ketch)

| Cultivar | Mean ELISA activity (μg/g ± SD) | Mean Enzyme activity (mU/g ± SD) |
|---|---|---|
| Galleon | 16.12 | 61.74 |
| Forrest | 19.16 | 72.33 |
| Xia Gang Da Hao | 20.20 | 68.65 |
| Shannon | 20.89 | 230.02 |
| O'Connor | 22.85 | 446.71 |
| Prior A | 25.63 | 649.55 |
| Malebo | 27.42 | 650.65 |
| Triumph | 27.69 | 880.83 |
| Ketch | 27.74 | 266.59 |
| Piroline | 28.60 | 63.48 |
| G/Promise | 29.20 | 229.95 |
| 2 EBYT 16 | 30.40 | 1110.43 |
| Dampier | 31.15 | 93.98 |
| Stirling | 31.38 | N.D. |
| WI-2585[c] | 31.76 | 219.57 |
| Chevron | 31.79 | 675.67 |
| Mosane | 32.16 | 1191.80 |
| M 737 | 32.89 | 77.39 |
| G/Archer | 33.42 | 51.09 |
| Excelsior | 33.42 | 68.56 |
| Parwan | 34.30 | 489.77 |
| Grimmett | 34.45 | 695.40 |
| Princess | 34.50 | 107.59 |
| WI-2477[a] | 35.18 | 1273.80 |
| Schooner | 35.46 | 1234.67 |
| Clipper | 36.14 | 176.07 |
| Proctor | 36.48 | 134.16 |
| Clermont | 36.55 | 219.53 |
| Br. 1239 | 38.03 | 58.60 |
| WI-2575[b] | 38.22 | 194.40 |
| Weeah | 38.40 | 222.52 |
| Kirin Choku | 38.44 | 114.14 |
| Skiff | 38.74 | 488.98 |
| Lara | 38.81 | 727.50 |
| Varde | 41.28 | 1279.03 |
| Minn | 41.29 | 949.82 |
| Hu Mai Si Hao | 41.55 | 1664.13 |
| Hiproly | 51.06 | 230.13 |
| Qi Wu Qi | 51.94 | 198.81 |
| | LSD = 2.80 | LSD = 29.24 |

The LD immunological activity varied 3-3.5 fold compared to a 32 fold range in activity with the RP assay.

The variation in activity observed could be due to differences in LD between cultivars, the effects of micromalting the same sample on different occasions (block effect) or an unexplained variation (error). Analysis of variance (ANOVA) was applied to test the variation in LD activity and the null hypothesis of no difference in activity between cultivars. This showed the observed differences in LD activity were not due to a block effect, i.e. the three micromaltings were uniform. Therefore, it is reasonable to combine the activity values for each cultivar from the different batches of malt as a mean value. The 1-way ANOVA shows there were significant differences in the LD activity between cultivars, i.e. the null hypothesis of no difference between cultivars is rejected (Table 5.2).

**Table 5.2**

ANOVA of the ELISA activity data; ($r^2$ 0.69 for model, number of observations 117)

| Source | Df | Sum of Squares | Mean Square | F Ratio |
|---|---|---|---|---|
| Cultivar | 39 | 6678.5329 | 175.751 | 4.5426*** |
| Error | 78 | 3017.7915 | 38.690 | |
| Total | 116 | 9696.3245 | | |

*** refers to a 0.01 % significance level.

The least significant difference (LSD) statistic was calculated to determine which cultivars had significantly different ELISA and RP activities (Table 5.1).
For example the calculation of the LSD for the ELISA:

$$LSD = 1.96 \times \sqrt{(2 \times EMS)/Df}$$
$$= 1.96 \times \sqrt{(2 \times 38.69)/39}$$
$$= 2.803$$

i.e. if the difference between the cultivar means exceeds 2.803 then a significant difference exists at the 5 % level.

**Table 5.3**

ANOVA of the RP activity data; ($r^2$ 0.78, number of observations 120)

| Source | Df | Sum of Squares | Mean Square | F Ratio |
|---|---|---|---|---|
| Day of malting | 2 | 41524.0 | 20762.0 | 4.9317** |
| Cultivar | 39 | 1156294.6 | 29648.6 | 7.0426*** |
| Error | 78 | 328371.3 | 4209.0 | |
| Total | 119 | 1526190.0 | | |

** refers to a 0.1 % and *** to 0.01 % significance level.

The situation for the RP results (Table 5.3) is different. Both cultivar and the time of malting contribute significantly to the variation in activity. There was no replication of cultivars within day of malting and no interaction between cultivar and day of malting. The model explains 78 % of the observed variation in RP activity.

Relationship between ELISA and RP assays

The relationship between the ELISA and RP data on matching samples shows a wide scatter of points (Fig. 5.3) and a statistical analysis indicates only a very low correlation between these assays ($r^2$ 0.06). Several of the samples read high in the ELISA but low by the RP assay which accounts for the poor correlation and vice versa. This was further illustrated by preparing serial dilutions of extracts and measuring the activity of the same dilution by both assays. The assays respond equally to increasing quantities of LD protein (Fig. 5.4) but there is a difference in the slopes of the relationship between cultivars. For example, a small increase in RP activity is associated with a larger increase in ELISA activity for Hiproly and Qi Wu Qi but a large increase in RP for Mosane and Hu Mai Si Hao is associated with only a small increase in ELISA activity. This suggests that when comparing the LD activity measured by either the ELISA or the RP assay on many different cultivars it is not surprising that a poor correlation showing much scatter is obtained (Fig. 5.3). The relationship is worse at low RP activities where there is more scatter. The reproducibility of the RP assay between assays on different days and between different users has only recently been shown to be poor in samples with low LD activities (McCleary, 1991; Henry, 1992). It is therefore possible the reason for the poor correlation between the ELISA and the RP assay, especially at low RP activities, is due to the poor reproducibility of the RP assay.

The reason for the poor correlation between the ELISA and RP assay could be due to (1) different cultivars may vary in the nature and number of their antigenic sites and (2) the

Km for the Red-Pullulan substrate may differ markedly when tested with extracts prepared from different cultivars; in some cultivars the dye attached to the substrate may interfere with binding of the enzyme (3) the ELISA measures total antigenic protein whereas the enzyme assay measures the degradation of a substrate and there are possibly cultivar differences in the specific activities of LD. Additionally, there may be differences in the amount of individual isoenzymes and their relative specific activities.

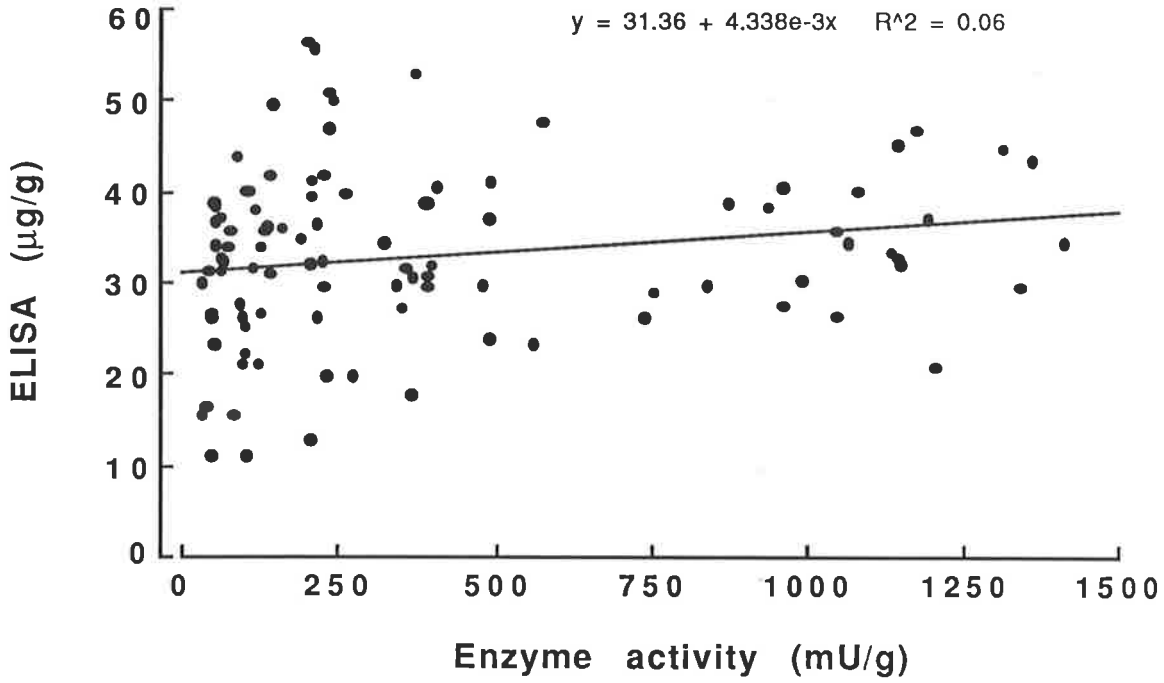



**Figure 5.3**

Relationship between the ELISA and RP assay. Data are the LD activity measured by the ELISA and RP assay of 117 malts (39 cultivars replicated three times) described in 5.2.1.1. A linear regression line (and its function) are included.

The results of the study presented here shows there are significant differences in the LD activity of cultivars (Table 5.1).

### 5.3.2.1 Cultivar differences in limit dextrinase detected by IEF-immunoblotting

The plant material used for these analyses differed from the previous experiment where variability in LD activity was studied because that material was not available for the IEF-Immunoblotting analysis. Instead the cultivars examined were the same (plus many more) but were obtained from a crop harvested in the 1988 season. The objective of the study was to see if there was any variability in the IEF banding patterns of 131 genetically diverse barley cultivars or breeding lines.

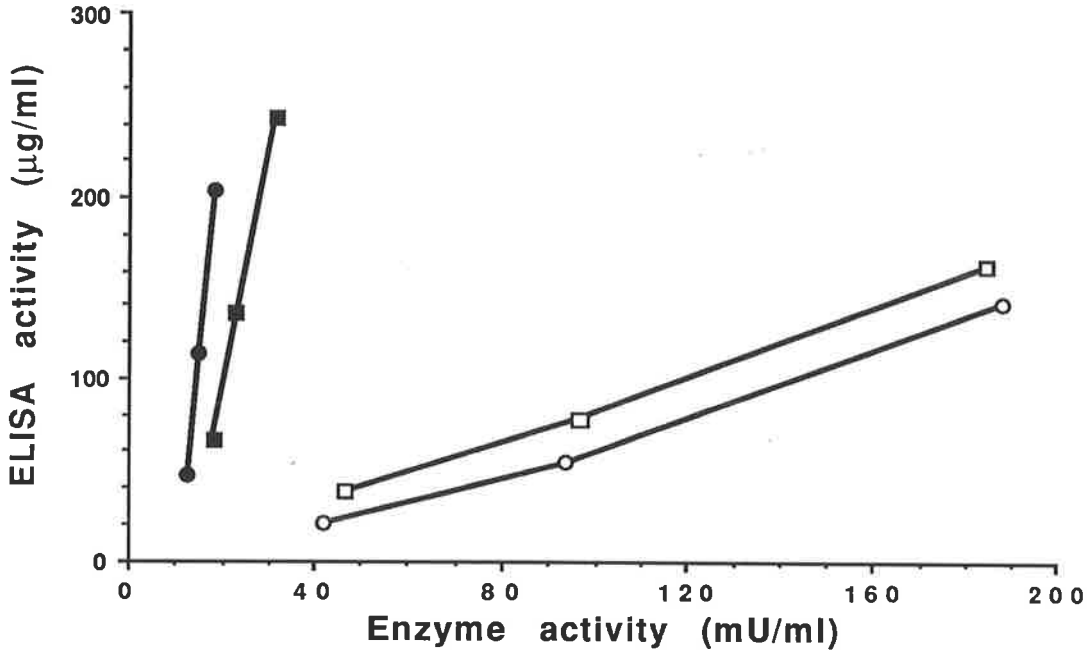



**Figure 5.4**

Relationship between ELISA and RP assay. Extracts of malt flour prepared from four different cultivars were diluted in assay buffer and LD activity measured by the ELISA and RP assay. Results are not corrected for dilution. The cultivars tested were: Hu Mai Si Hao, (○); Mosane, (□); Hiproly, (■) Qi Wu Qi, (●).

The same banding pattern was found in 128 of the 131 cultivars, and a sample of these patterns is shown in figure 5.5. Three of the cultivars, all from China (Jia Ding Hong Jin Zhu Tou, Al Jia Erling and Xia Gang Da Mai), showed an extra higher pI band with a similar intensity of staining as the main band; two of these patterns are shown in figure 5.5. Lenoir *et al.* (1984) analysed five different malts and found no differences in the zymogram pattern, therefore the analysis of many cultivars may be required to show further differences in banding patterns. Some patterns showed poor resolution but the basic pattern was still discernible after careful study, and a photograph lacks the required clarity to show these patterns.

The LD activity and protein content of the 131 samples of barley extracts were measured by the ELISA and Bradford assay (Appendix 5.2). The purpose was to see if there was any relationship between the specific activity and the banding pattern, i.e. a high specific activity may be associated with an extra band or fewer bands or a completely different pattern. The specific activity of the three Chinese cultivars was not different from the other 128 cultivars examined (Appendix 5.2) and therefore is not related to the different banding pattern. To estimate the concentration of each band in the blot, densitometry could be used to scan the profiles. At the present time this technique is not reliable. Therefore it is not possible to show a relationship between banding pattern and quantitative levels of activity.

In all the patterns, there was one band which stained with greatest intensity suggesting this particular protein was present in greater abundance or has a higher avidity to the antiserum. A repeat analysis of samples producing unclear banding patterns did not change the result so that for some samples a good blot was not possible. Given the limitations of the method for studying banding pattern variation it appears that there is minor variability in banding patterns for LD.

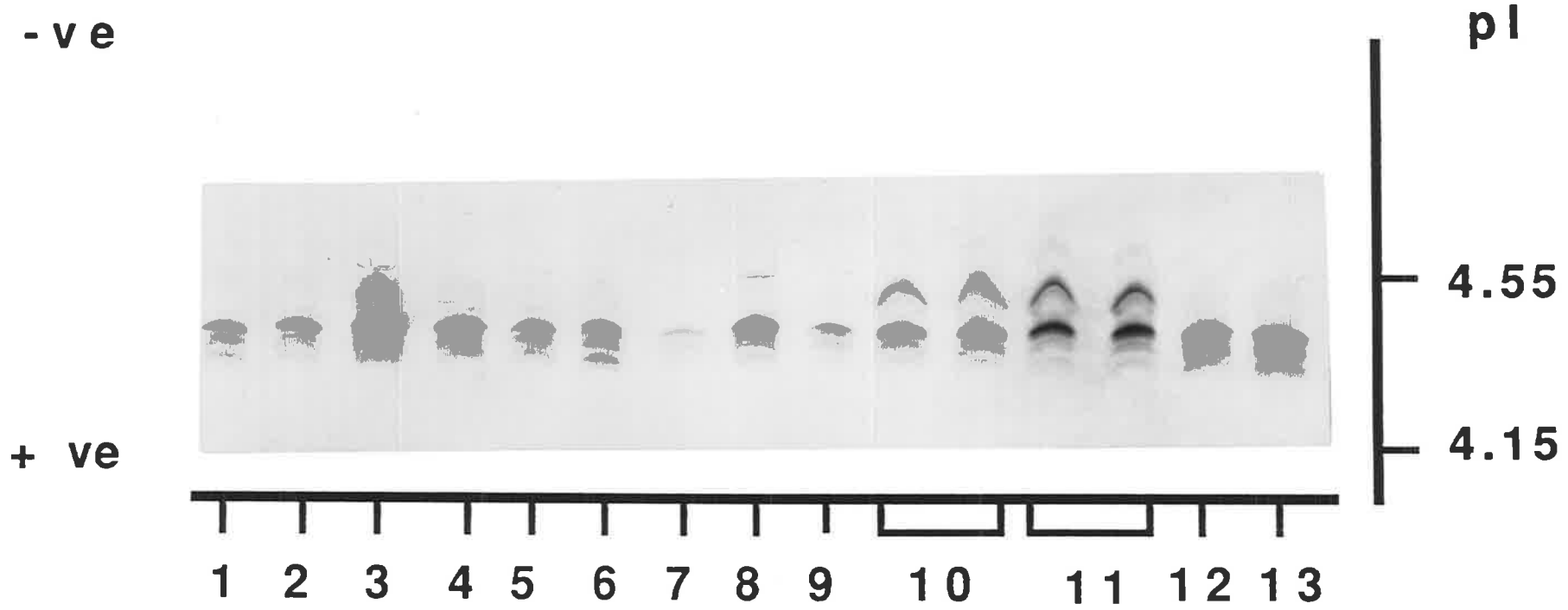
Specific isoenzymes of LD may be important to meet quality, for example MacLeod *et al.* (1990) noted that in addition to the importance of total potential amounts of endo (1-3;1-4)-β-glucanases, the qualitative and/or quantitative balance of the isoenzymes of endo (1-3;1-4)-β-glucanase may also be of significance to malting quality. It is then necessary to relate those cultivars with a different banding pattern to a functional difference. For example, cultivars with an additional or different band may have a different overall specific activity, or may be able to hydrolyse its substrates at a faster rate.

Improved levels of enzyme activity are major objectives in barley breeding programmes in Australia. To select for higher LD activity many samples may need to be screened. Screening samples using the IEF-immunoblotting procedure may be useful if a variant banding pattern is associated with a higher specific activity. Screening for the desired character then becomes possible by analysing samples for the unique LD banding pattern which is associated with high LD activity.

**Figure 5.5**

A sample of the variation in isoenzymes of LD. Thirteen of the 131 samples analysed as described in 5.2.1.2 are presented here.

1-Azuma Golden
2-Menuet
3-Puebla
4-Gold Marker
5-Kirin Choku
6-Kirstina
7-Conquest
8-Piroline
9-Mosane
10-Jia Ding Hong Jin Zhu Tou
11-Al Jia Erling
12-WI-2607
13-WI-2606


-ve
+ ve
pI
4.55
4.15
1 2 3 4 5 6 7 8 9 10 11 12 13

### 5.3.2.2 Isoenzyme variation in *Hordeum spontaneum*. C. Koch

Genetic variation present in the wild relatives of crops represents a major resource for plant improvement. Barley (*Hordeum vulgare* L) and its wild progenitor (*Hordeum spontaneum* C. Koch) provide an ideal system for introgressing wild or "exotic" germplasm into adapted breeding populations (Frey, 1976). Wild barley is found in the Mediterranean basin in the Fertile Cresent, and weedy forms reach from Morocco to Eastern Asia. It occurs in a wide range of habitats and is highly polymorphic in terms of morphological variation and isoenzyme diversity (Brown *et al.*, 1978). There are significant differences in the α-amylase, β-amylase, β-glucanase and LD activities between wild and domestic barley (Ahokas and Naskali, 1990). Wild barley thus represents a pool of biochemical and genetic variation in the grain. This variation could be used by barley breeders to improve malting quality.

The variation in banding pattern for LD isoenzymes was investigated in a subset (Table 5.5) of wild populations (obtained by Dr. A. H. D. Brown CSIRO Canberra) chosen from those used by him for his comprehensive study of allozyme diversity among and within Israel populations of *H. spontaneum* (Brown *et al.*, 1978). Seven of the eighteen samples tested differed in their banding pattern showing an additional higher pI band (six of them are shown in Fig. 5.6). The additional band, which was not seen in any of the 131 samples of *H. vulgare*, showed the same intensity of staining as the main band seen in *H. vulgare*. The variability in the *H. spontaneum* could be useful for studying the segregation of the banding pattern and determine the genetics of inheritance of the LD genes controlling the patterns. Importantly, the variant bands may differ in their thermal stability.

There was no relation between the banding pattern and specific activity in the 19 *H. spontaneum* tested (Table 5.5).

**Figure 5.6**

Limit dextrinase isoenzyme variation in *H. spontaneum.* Numbers are sample numbers described in table 5.5. Samples showing an additional higher pI band are numbers: 2, 5, 6, 10, 12 and 15.

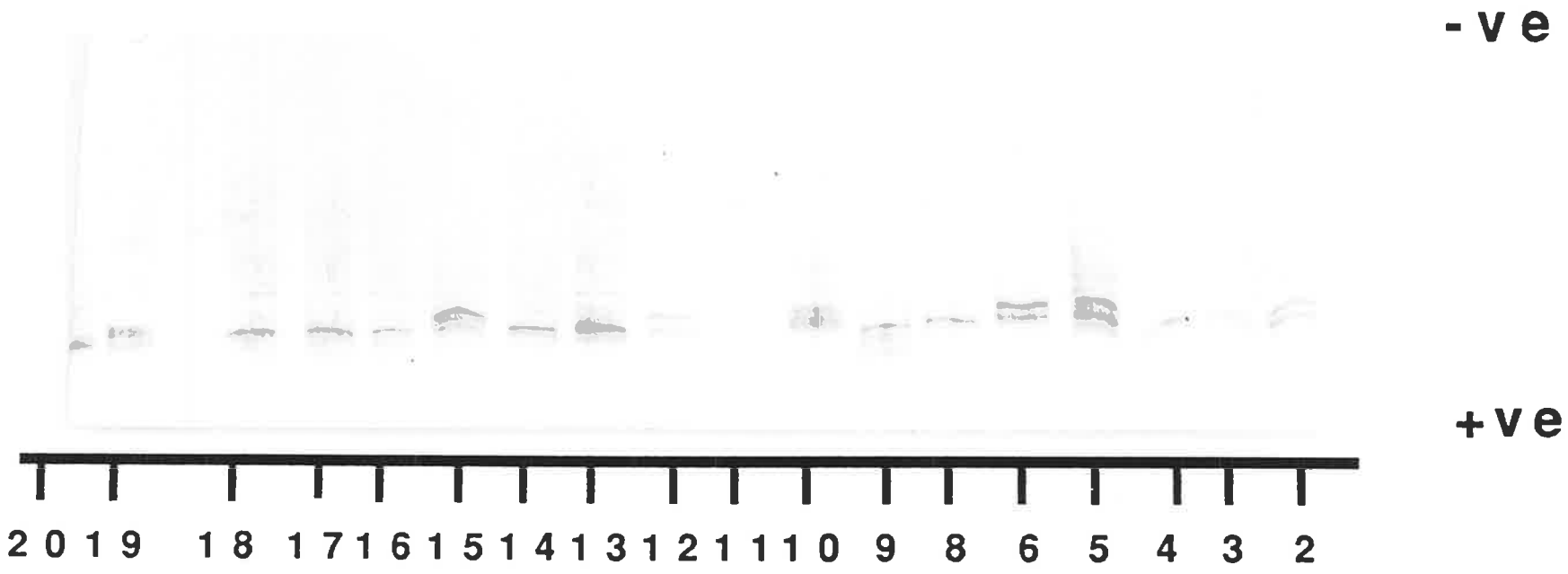

-ve
+ve
20 19 18 17 16 15 14 13 12 11 10 9 8 6 5 4 3 2

**Table 5.5**

Populations of *H. spontaneum* used in the IEF-blotting studies. Seeds of these cultivars were germinated as described in 5.2.2.1 and analysed for LD activity as described in 5.2.1.2.

1. and 2. Although not clear in figure 5.6, the higher pI band was confirmed in a duplicate IEF/blot for both samples (not shown). 3. Not shown in figure 5.6.

| Site in Israel | Accession Number | Sample Number | LD Activity (μg/g grain) | LD specific activity (μg/mg protein) | Banding pattern type (see Fig. 5.6) |
|---|---|---|---|---|---|
| **Coastal** | | | | | |
| Akhziv | 77127 | 11 | 5.6 | 0.4 | no bands visible |
| Atlit | 77129 | 16 | 48.0 | 4.2 | |
| Herzliyija | 77134 | 13 | 126.0 | 15.6 | |
| **Central Range** | | | | | |
| Bar Giyyora | 71283 | 5, 19 | 57.2 | 4.9 | additional band |
| Rash Pinna | 77140 | 6 | 81.2 | 5.3 | additional band |
| Rash Pinna | 77140A | 3 | 30.4 | 3.1 | |
| Afiq | 77128 | 9 | 72.0 | 4.2 | |
| Talpiyyot | 77144A | 15 | 64.4 | 5.2 | additional band[1] |
| Talpiyyot | 77144B | 12 | 39.2 | 2.1 | additional band |
| Tel Hay | 77145 | 20 | 67.6 | 6.3 | |
| Tel Hay | 77145A | 14 | 41.2 | 8.6 | |
| **Rift Valley** | | | | | |
| Beit Shean | 77131 | 17 | 84.4 | 8.2 | |
| Mechola | 77137 | 18 | 62.8 | 6.1 | |
| Sede Baker | 77141 | 8 | 78.4 | 5.9 | |
| Bor Mashah | 71285 | 4 | 40.8 | 4.3 | |
| Wadi Qily | 77135 | 10 | 46.0 | 3.3 | additional band[2] |
| Rivivim | 77139 | | 25.2 | 3.0 | additional band[3] |
| Eyzariya | 77130 | 2 | 80.4 | 8.1 | additional band |

| **Barley (number of samples)** | **Mean ELISA activity ± sd (Range)** | |
|---|---|---|
| | For grain mass (μg/g) | For protein (μg/mg) |
| Wild (18) | 58.38 ± 27.54 (6-126) | 5.49 ± 3.31 (0.4-15.6) |
| Cultivated (39) | 33.20 ± 7.62 (16-52) | Not determined |

It is not statistically valid to make comparisons between the mean and variance of *H. vulgare* with *H. spontaneum* because the variances are significantly different (F=13.06, with 17 and 38 degrees of freedom). There is certainly a wider range for the wild barley (range is 101 units for the wild compared to 36 for cultivated). The maximum immunological activities in the wild barleys were 2-3 times higher than the top activity in the cultivated barleys. Ahokas and Naskali (1990) found a similar variation in LD activity. They measured the LD activity in 5 day germinated extracts of 175 different wild barley strains (*H. spontaneum* ), and showed an eight fold difference in activity.

The wild barley parents (Table 5.5) were backcrossed to Clipper four to five times. For each wild parent a marker enzyme of known chromosome location was present (Brown *et al.*, 1978). If the variant banding pattern for LD present in the wild parent was also found in any backcross line this would mean the gene coding for the banding pattern was linked to the marker enzyme and therefore it would be possible to locate the chromosome that carries the structural gene for LD. Using a similar procedure, the linkage relationships between the gene locus encoding a variant banding pattern for 1,3;1,4-β-D-glucan-4-glucanohydrolase EI and malate dehydrogenase *Mdh 1* locus, which occurs on the long arm of chromosome 5 in barley, was used to locate the *Glb* locus (MacLeod *et al.*, 1991a) The backcross lines used and the enzyme marker, loci and chromosome are shown in Table 5.6. There was no variation in banding pattern between any of these lines and Clipper indicating the variant isoenzyme pattern seen in the selected wild parents was not linked to the marker enzymes (Fig. 5.7).

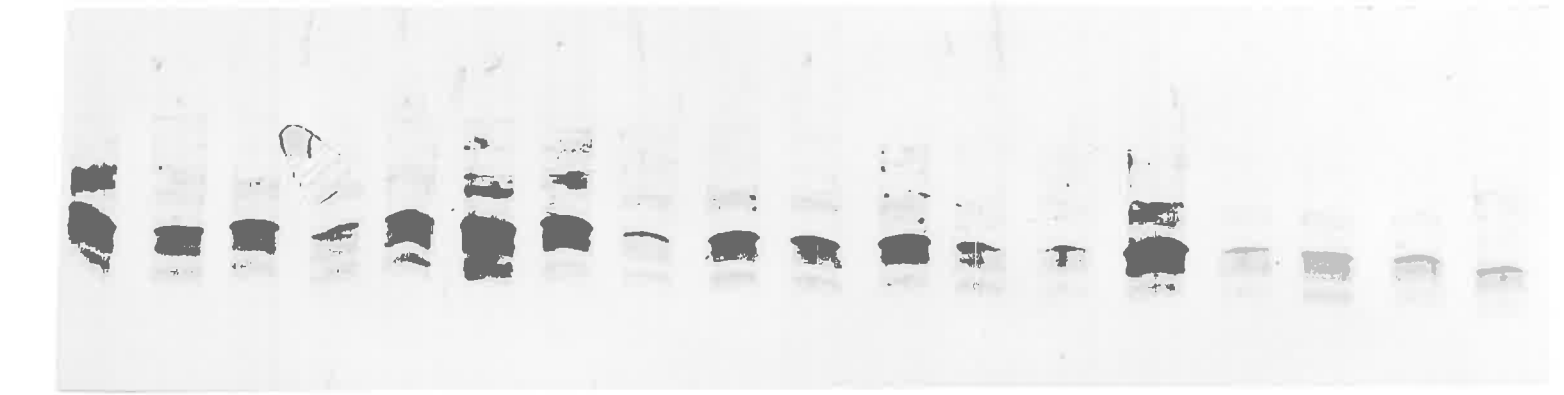
| Sample number | Accession number | Locus | Chromosome |
|---|---|---|---|
| 6 | 77144 | *Adh* 1 | 4 |
| 1 | 77130 | *Acp* 2 | 4 |
| 2 | 77128 | *Est* 2 | 3 |
| 3 | 77128 | *Est* 2 | 3 |
| 7 | 77145 | *Est* 2 | 3 |
| 4 | 71283 | *Est* 2 | 3 |
| 8 | 77127 | *Acp* 2 | 4 |
| 9 | 77141 | *Aco* 2 | 5 ? |
| 10 | 77127 | *Aco* 1 | 6 |
| 11 | 77127 | *Gpi* 1 | 5 |
| 12 | 77137 | *Pgd* 1 | ? |
| 15 | 71284 | *Mdh* 1 | 5 |
| 14 | 77131 | *Est* 2 | 3 |
| 5 | 77135 | *Aco* 1 | 6 |
| 13 | 71284 | *Aco* 1 | 6 |
| 16 | 77131 | *Aco* 1 | 6 |
| 17 | 77127 | *Acp* 2 | 4 |

**Table 5.6**

Marker enzymes and their chromosome location in backcross lines of *H. spontaneum* x Clipper.

**Figure 5.7**

Limit dextrinase isoenzyme variation in backcross lines of *H. spontaneum.* x Clipper. Numbers refer to the backcross line and their identification is given in Table 5.6.


-ve
+ve
17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1
Clipper

## 5.3.3
## Attempts to Locate the Chromosome carrying the Structural Gene for Limit Dextrinase

### 5.3.3.1
### Studies with wheat-barley addition lines

#### 5.3.3.1.1
#### Introduction

The chromosome location of the structural gene(s) coding for LD is presently unknown. The wheat cultivar Chinese Spring for which compensating nullisomic-tetrasomic lines (Sears, 1966) and six euplasmic wheat-barley addition lines are available (Islam *et al.*, 1981) has added a valuable tool for genetic analysis of isoenzymes.

The antiserum against barley LD also recognises LD isoenzymes in wheat (Chinese Spring, Fig. 4.20) indicating cross reactivity between these species. This is useful because the analysis of wheat-barley chromosome addition lines, in which single chromosomes from barley have been added to Chinese Spring (Wheat), may allow the chromosomal location in barley of the structural gene for LD to be identified. The presence in Betzes of a band(s) which is not present in Chinese Spring and then present in a specific chromosome combination, would provide direct evidence for chromosome location. A complete set of additions, except for barley chromosome 5 was kindly supplied by Dr. A.K.M.R. Islam, (Waite Agricultural Research Institute).

#### 5.3.3.1.2
#### Methods

Ten seeds from Chinese Spring (CS), Betzes (B) barley, and the six euplasmic addition lines 1, 2, 3, 4, 6 and 7 were germinated for 8 days in the dark at 15°C. After germination, seeds were freeze-dried, ground in a ball mill and the flour extracted with 50 mM acetate buffer containing 20 mM cysteine, pH 5 for 3 h at 30°C. The supernatant (undiluted) obtained after centrifugation at 10,000 g was analysed by IEF-immunoblotting as described in chapter 4 section 4.2.6.

In another study designed to improve the resolution of the technique used above, CS B and 1α, 1 β (short and long arm, respectively) ditelocentric chromosome addition lines were examined. Seeds were germinated only this time more seeds were used and the extraction volume altered from 1 g/4 ml to 1 g/2 ml. Also, fresh reagents were prepared to give the best possible chance of obtaining good electrofocusing results.

**5.3.3.1.3**

**Results and Discussion**

The LD isoenzyme patterns are complex and to assist in their interpretation the following features were considered. The number of bands, their grouping patterns and the relative intensity of each band. Some of the common features found in repeated analyses of the same samples were variations in the intensity of the entire pattern, which reflects the concentration of LD in the volume of sample loaded (20µl) and wavy bands, probably due to an uneven pH gradient in a localised region of the gel or a high salt concentration in the sample.

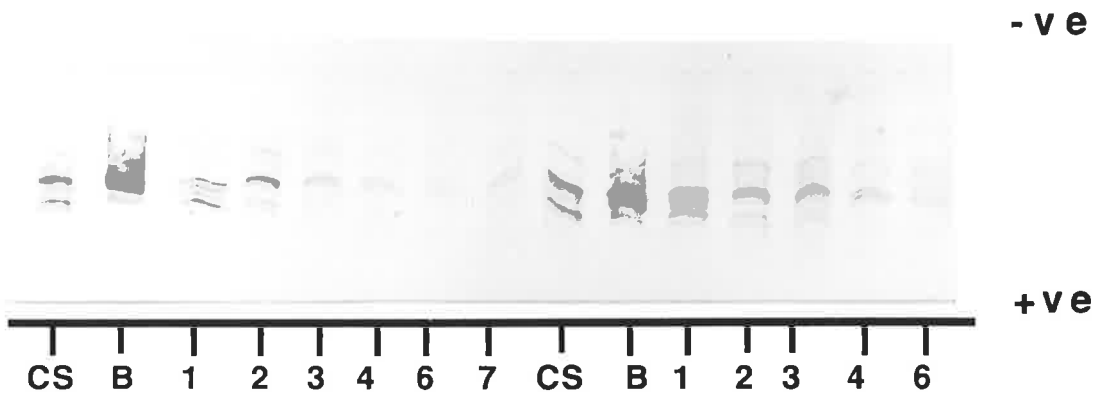
The isoenzyme patterns for additions 2, 3, 4, 6 and 7 are the same as CS but addition 1 has an extra band (Fig. 5.8A). This band may be from the barley chromosome addition 1 but the patterns for B and CS are not well resolved. When this experiment was repeated to see if these results were reproducible, i.e. a separate group of seeds were germinated and extracted, a blot showing more bands in every sample was obtained. For example, a group of higher pI bands was clearly seen this time but these bands were just detectable or not at all in the first experiment (cf. A and B in Fig. 5.8). This difference probably reflects the higher concentration of enzyme in the second experiment since more seeds were germinated and also the electrofocusing was improved. Once again, the banding pattern for chromosome 1 appears to be different to the other addition lines but the difference is small (Fig. 5.8B). These results indicate that the extra band probably comes from the barley parent. However, there is still the problem of poor resolution in Betzes (B) which may be reflected in the lower specific immunoreactivity compared to CS i.e. the concentration of LD in Betzes extracts may be too low.

In another study using the 1α, 1 β ditelocentric chromosome addition lines, a much better resolution of B was obtained (Fig. 5.9). The B and CS are different in terms of the positioning of the groups of bands and their relative intensity, for example, in B the predominantly staining band is not as dominant in CS (see diagram in Fig. 5.9). The wheat-barley (WB) addition 1 is identical to the samples in figure 5.8A and B and very similar to Betzes (it appears to be lacking in one band which is difficult to see in a photograph) but is different to CS (Fig. 5.9). The long arm of barley chromosome 1 has the same pattern as chromosome 1 whereas the short arm pattern is the same as CS. Overall, the results suggest there is a difference in barley chromosome addition 1 which is more like barley than wheat and this difference is localised on the long arm of the chromosome 1.

**Figure 5.8**

Use of wheat-barley addition lines to investigate chromosome location of LD. CS, Chinese Spring (wheat); B, Betzes barley and euplasmic addition lines 1, 2, 3, 4, 6 and 7.

A




B

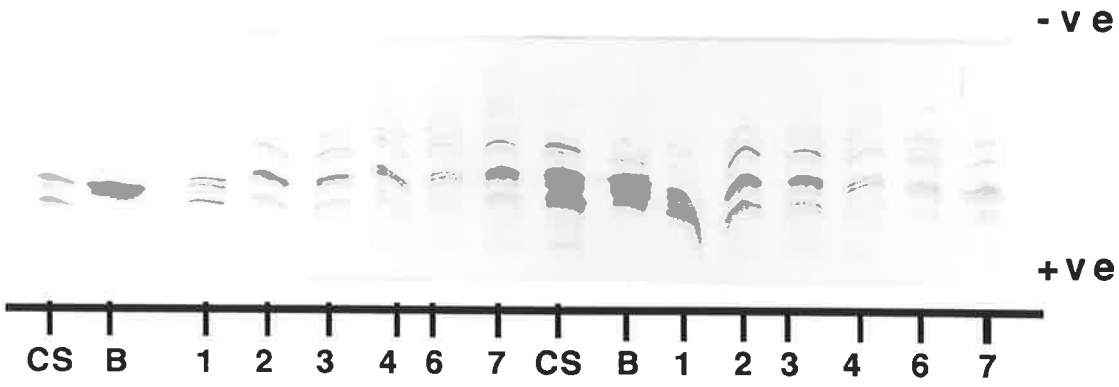
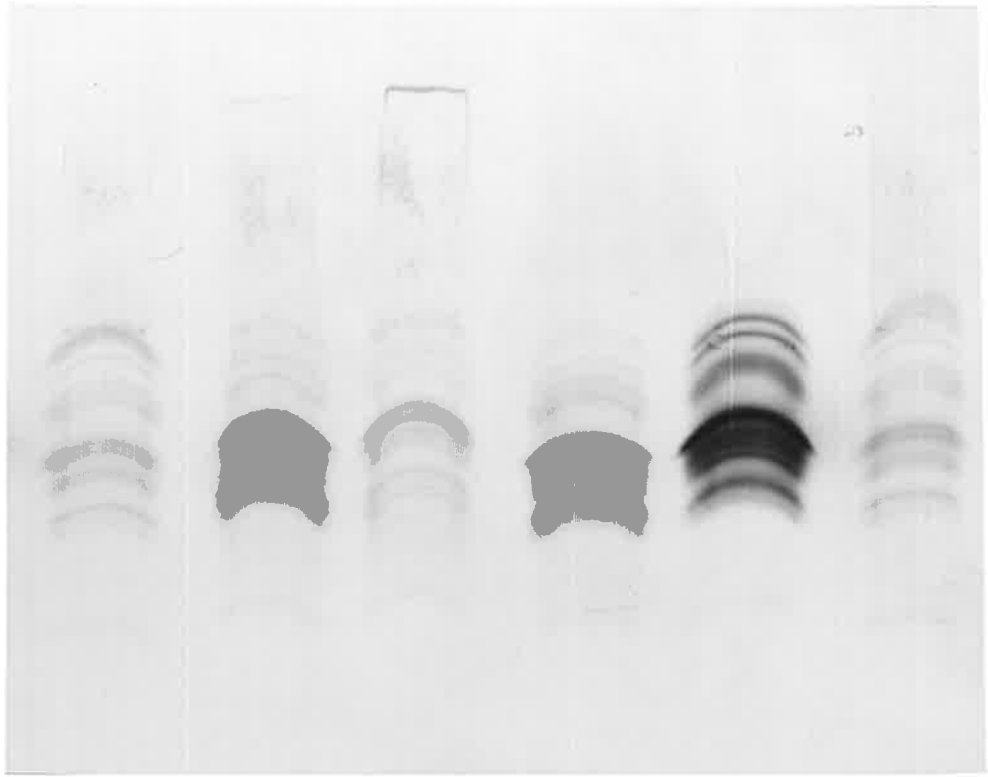


**Figure 5.9**

Use of wheat-barley addition line chromosome 1 to investigate location of LD structural gene. CS, Chinese Spring (wheat); B, Betzes barley and euplasmic addition lines 1, 1α (short arm) and 1β (long arm).


- v e
+ v e
CS
1β
1α
1
B
CS

### 5.3.3.2 Studies with wheat nullisomic-tetrasomic lines

#### 5.3.3.2.1 Introduction

A typical analysis of protein structural genes in wheat usually involves the use of one or more nullisomic-tetrasomic (NT) lines. In such genotypes, the loss of one complete pair of chromosomes (nullisomic) is compensated by a double (tetrasomic) dose of another pair of chromosomes within the same homoeologous chromosome group. The homologous proteins produced by homoeologous genes often differ enough in electrophoretic properties to identify individual genes (Hart, 1973). There are two important criteria to be used as evidence for the existence of a locus (1) a repeatable, resolvable band or set of bands appears on the gel system (2) zones of activity can be located to specific chromosomes because the presence or absence of the zone should be controlled by the presence or absence of the gene.

Most of the 42 possible NT combinations are available in Chinese Spring (4A and 2A are not available) and the usual approach followed in identifying the chromosomal location of an enzyme structural gene is to first screen NT lines, in which each chromosome is nullisomic in turn (to identify the critical homoeologous group) to confirm the gene locations and to observe dosage effects of genes carried on the tetrasome. Instead of investigating all of the NT lines, only homoeologous chromosomes involving 7A, 7B and 7D were studied (N.B. Barley chromosome 1 is 7H) because this is the chromosome where the gene for LD is suspected. This should show whether the bands seen in the IEF-immunoblots are controlled by chromosomes of homoeologous to barleys 7H. Invoking the simplest model, a specific group of bands should disappear in the nullisomic and be increased in the tetrasomic relative to the control wheat with unaltered homoeologous group. This result should be reproducible in repeat analyses. If the banding pattern does not show this consistency, then it is highly likely the patterns are due to environmental variation for example differences between seeds of the same plant or between seeds of different plants or caused by post-translational modifications occuring in the plant or during extraction or germination.

#### 5.3.3.2.2 Methods

Seed of the three pairs of NT lines of chromosome 7A, 7B, 7D and Chinese Spring (kindly supplied by Dr. Shepherd, Waite Agricultural Research Institute) were germinated for 7-8 days and prepared as described for the addition lines. Each of the flour extracts of Chinese Spring was prepared from different seeds and not a pool of flour so that variation

between groups of CS seeds could be tested (Fig. 5.11 only). Two independent germination studies using seed from different plants were performed to ensure the results obtained were repeatable.

**5.3.3.2.3**

**Results and discussion**

Study 1: Hypotheses (Fig. 5.10)

(1) The upper pI group of 4 bands (see diagram in Fig. 5.10) are controlled by the 7A genome. Nullisomics 7A-7D and 7A-7B showed a decrease in the relative intensity of these bands compared to the CS control. However these bands were not increased in the tetrasomics, 7D-7A and 7B-7A.

(2) The lowest pI bands (three; see diagram in Fig. 5.10) are controlled by the 7D genome. Nullisomics 7D-7B and 7D-7A showed absence of the lowest two pI bands and a decrease in the relative intensity of the remaining band compared to the CS control. Lines with tetrasomic 7D; 7B-7D in particular and 7A-7D were increased in relative intensity in this region especially the lowest pI band, supporting this hypothesis.

Study 2: Hypotheses (Fig. 5.11)

(1) The upper pI group of 4 bands are controlled by the 7A genome. In this experiment the result is very clear showing an absence in the nullisomics and a greater predominance in the tetrasomics.

(2) The lowest pI bands (three) are controlled by the 7D genome. The lowest pI band is consistently lacking in the 7D nullisomics and there appears to be only one of the three in this lower group visible in the nullisomics. The lines with tetrasomic 7D show these lower two bands with slightly greater intensity than the controls but this is less clear.

(3) The middle group of bands (see diagram Fig. 5.10) are controlled by the 7B genome. There is a reduction in this pattern in the nullisomics which is enhanced in the 7A-7B but less so in the 7D-7B. This was not seen in study 1.

The evidence presented here for the wheat LD isoenzyme system does not conclusively confirm the involvement of the loci on homoeologous group 7H in barley i.e. the banding pattern variation is controlled by a structural gene on these chromosomes. The gene for LD should be on each of the three genomes and the NT lines should show a dosage effect. Control by the 7A genome was clear in study 2 but not as convincing in study 1. Also, the evidence for control of bands by the 7B genome is poor. The results presented are not consistent and until one can be confident of getting reproducible patterns, it is not possible to name the chromosome location for the LD structural gene. Not all the bands decreased or disappeared by removal of any homoeologous group 7 chromosome, which suggests that some bands are controlled by more than one chromosome. A loss of one chromosome may not therefore lead to a loss of the bands. Other NT lines should be tested

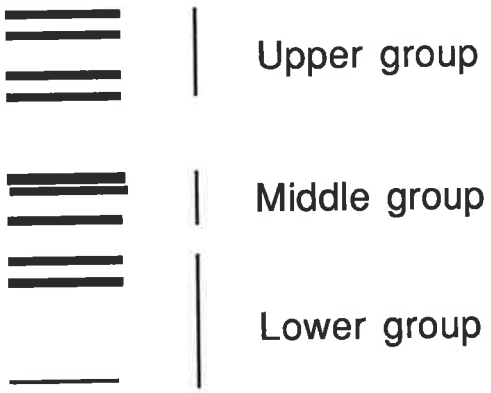
to see if there is any control on the banding pattern which would provide additional evidence for genetic control of the banding pattern. It is also possible that within a homoeologous group more than one gene within a complex locus could control a band. Thus removal of say 7A, may not result in a loss of a band if say 7B also controls expression of the same band.

Wheat (CS)-barley (*H. Chilensi*) addition lines could also be tested. A different genome may produce a different set of bands between wheat and barley which could allow the chromosome location to be identified.

**Figure 5.10**

Study 1: Use of nullisomic-tetrasomic lines from wheat chromosome 7A, 7B and 7D to investigate gene control of LD isozymes. Duplicate samples were loaded onto the gel at different locations.

| | |
|---|---|
| CS; | Chinese Spring |
| 1; | 7A-7B (nullisomic-tetrasomic) |
| 2; | 7A-7D |
| 3; | 7B-7A |
| 4; | 7B-7D |
| 5; | 7D-7A |
| 6; | 7D-7B |



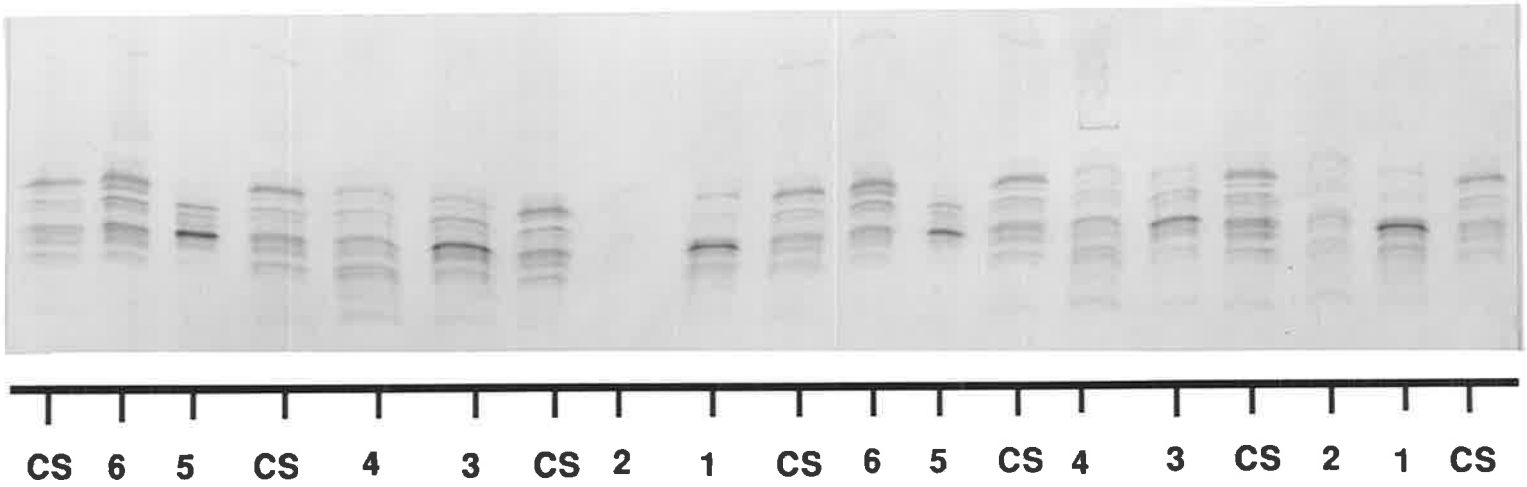
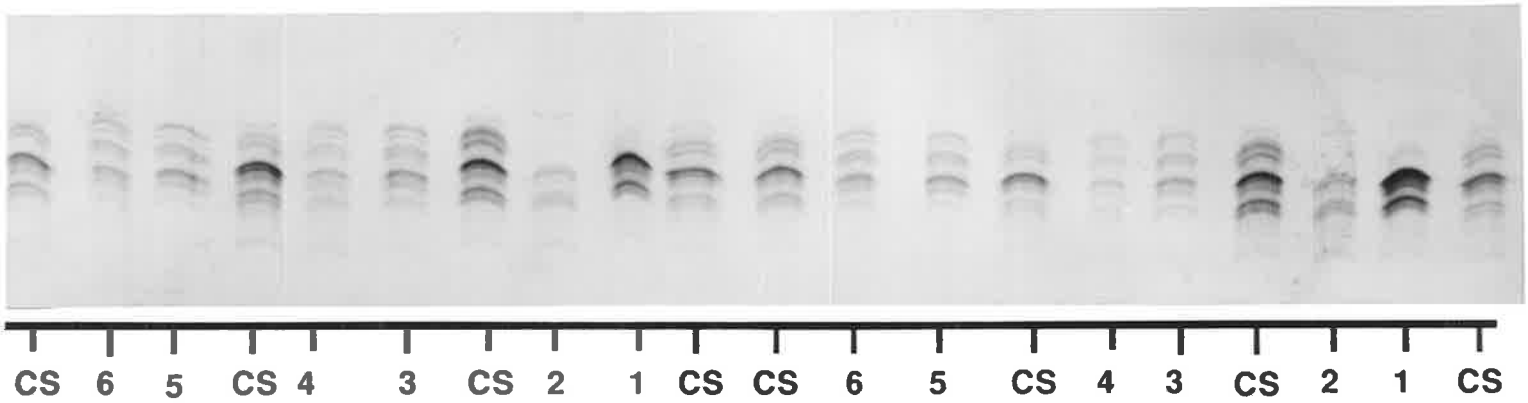

CS 6 5 CS 4 3 CS 2 1 CS 6 5 CS 4 3 CS 2 1 CS

**Figure 5.11**

Study 2: Use of nullisomic-tetrasomic lines from wheat chromosome 7A, 7B and 7D to investigate gene control of LD isozymes. Duplicate samples were loaded onto the gel at different locations. The CS samples were extracts prepared from different seed.

| | |
|---|---|
| CS; | Chinese Spring |
| 1; | 7A-7B (nullisomic/tetrasomic) |
| 2; | 7A-7D |
| 3; | 7B-7A |
| 4; | 7B-7D |
| 5; | 7D-7A |
| 6; | 7D-7B |


CS 6 5 CS 4 3 CS 2 1 CS CS 6 5 CS 4 3 CS 2 1 CS

# CHAPTER 6

# CHANGES IN LIMIT DEXTRINASE DURING KERNEL DEVELOPMENT

## 6.1 INTRODUCTION

Chrzaszcz and Janicki (1936) reported finding starch liquefying and dextrinizing enzymes in immature kernels of wheat, oat, barley and rye. Sandstedt and Beckord (1946) showed that α-amylase activity in wheat pericarps increased during the first week after pollination and then decreased. Many reports on the presence of α-amylase in immature kernels of wheat, barley, rye and triticale are available which indicate a transitory α-amylase exists shortly after anthesis and that the enzyme activity is very low compared to that found in germinated kernels (Hill and MacGregor, 1988).

Information on the presence of LD during seed development is less well documented. The enzyme is present in mature kernels of most cereals but with lower activity than produced during germination (Manners, 1985; Serre and Laurière, 1990). The reason the enzyme occurs in ungerminated cereals is not clear but could be due to synthesis of the enzyme during kernel development or the presence of a bound form of the enzyme (see chapter 7 for a detailed discussion). Why there should be LD in the mature grain is not known but the enzyme may assist α-amylase in the degradation of starch in the endosperm adjacent to the crushed cell layer and in the outer pericarp (MacGregor and Dushnicky, 1989a,b).

There are three published reports where the activity of LD was monitored during kernel development in wheat and barley (Kruger and Marchylo, 1978; Laurière *et al.*, 1985; MacGregor and Dushnicky, 1989b). Kruger and Marchylo studied the appearance of α-amylase isoenzymes during development in ten wheat cultivars and found in their IEF-zymograms, two bands with pI values of 4.32 and 4.40. Sections of the gel containing these bands were excised, macerated in water and extracted and the eluted proteins degraded pullulan, suggesting these proteins were LD. These bands, which were only found in the endosperm, appeared just after anthesis and remained until full maturity. MacGregor and Dushnicky (1989b) performed isoelectric focusing on extracts of endosperm and embryo tissues from barley at 19 days after anthesis. In their zymograms they used the amylopectin β–limit dextrin substrate and found a relatively low pI (ca. pI 4) enzyme thought to be LD in the crushed cell layer, embryo, proximal and distal endosperm. If the enzyme responsible for this band was LD, then it would produce a staining band because the enzyme hydrolyses α-1,6-linkages in the amylopectin β–limit dextrin substrate to release unbranched chains of α-1,4-linked glucose units.

Based upon these studies information on the quantitative changes in LD activity during development of the barley kernels and in dissected tissues is lacking. The purpose of this study was to measure LD activity in the developing kernels of barley using the sensitive ELISA and to follow changes in the isoenzymes with immunoblotting.

## 6.2 MATERIALS AND METHODS

### 6.2.1 Barley Material

Barley (cv. Skiff) was grown at the Waite Agricultural Research Institute in the 1990 season. A large number of ears were tagged at the onset of anthesis but to ensure a sufficient number of plants for later analyses, three groups of plants, each group reaching anthesis on a different day, were marked with colour coded tags to assist identification. The tagging was as follows: white tags, plants reached anthesis on 15/10/90 (day 9); black tags, plants reached anthesis on 18/10/90 (day 12); green tags, plants reached anthesis on 22/10/90 (day 16). At three day intervals after anthesis until maturity, a number of tagged heads was collected at random throughout the plots. The heads were stored at -20°C until analysed.

### 6.2.2 Preparation of Samples for Analysis

The frozen heads were removed from the freezer and the kernels separated from the rachis. Some of the kernels (of known weight and number) were freeze dried and the remainder stored frozen. Immediately after drying, the samples were weighed and transferred to a desiccator to minimise moisture uptake. The change in weight after drying was used to calculate the percentage moisture in the samples. The freeze-dried grains were ground in a ball mill (Spex Industries, U.S.A.) and the flour carefully transferred (a fine paint brush was used to ensure all the flour was collected) to pre-weighed tubes. Extracts were prepared from the flour as described in chapter 2 section 2.6.5. The volume of extraction buffer used was adjusted to the weight of the flour (4 ml/g).

### 6.2.3 Analyses

The LD activity of the extracts was measured with the ELISA. It was not possible to detect any enzyme activity with the RP assay, despite adding cysteine to the extraction buffer, presumably because this assay has insufficient sensitivity to detect the relatively low

enzyme activity in these samples compared to malt. It is possible the anti-LD antibody detects some inactive LD, perhaps a 'bound' form (see chapter 7). Results are expressed as activity per 10 kernels.

### 6.2.4 Immunoblotting Analysis

The presence of LD isoenzymes in the samples from days 15, 18, 21, 24 and 27 post-anthesis (PA) of the white tagged samples and the mature Skiff (the final harvested barley at the completion of the experiment, see Table 6.1) and malted Skiff were analysed by IEF/immunoblotting as described in 4.2.6.

### 6.2.5 Dissection Studies

Frozen kernels were thawed and dissected at room temperature. Kernels harvested at 21 and 24 days PA were used because immunological activity was maximum. Kernels were pooled to obtain a sufficient number of tissues for analyses and were dissected as follows. Outer pericarp (clear layer), inner pericarp (green layer), embryo including the scutellum, and endosperm with associated aleurone. The endosperm was cut into two to give proximal and distal endosperm halves. A total of 80 kernels were dissected. During the dissections, the tissues were kept on ice until sufficient material was obtained, then the tissue frozen in liquid nitrogen and freeze-dried. The dried samples were ground in a ball mill and the flour weighed and extraction buffer ( 50 mM acetate buffer, pH 5 containing 20 mM cysteine) added in the ratio 1 g/4 ml. The samples were extracted at 30°C for 3 h then centrifuged at 10,000 g for 10 min. There was sufficient endosperm flour to carry out extractions in the presence and absence of cysteine in order to measure both bound and free LD.

## 6.3 RESULTS AND DISCUSSION

### 6.3.1 Changes in Limit Dextrinase During Kernel Development

The expression of activity per dry weight is not appropriate because the dry weight changes during development (Fig. 6.1). Activity was expressed per number of tissues to allow for an increase in weight during development. After 15-20 days PA, the dry kernel weight of 10 tissues was constant.

**Table 6.1**

Schedule for the tagging of barley plants from anthesis to maturation (harvest). * No samples were taken from green tagged plants on day 0.

| **Days after anthesis at which heads were collected** | | |
|---|---|---|
| Colour code used for tagged plants | | |
| **White** | **Black** | **Green** |
| 0 (15/10/90) | - | - |
| 3 | 0 (18/10/90) | - |
| 7 | 4 | * (22/10/90) |
| 9 | 6 | 3 |
| 12 | 9 | 6 |
| 15 | 12 | 9 |
| 18 | 15 | 12 |
| 21 | 18 | 15 |
| 24 | 21 | 18 |
| 27 | 24 | 21 |
| 30 | 27 | 24 |
| 33 | 30 | 27 |
| 36 | 33 | 30 |
| 39 | 36 | 33 |
| 42 | 39 | 36 |
| 45 | 42 | 39 |
| 51 (harvested) | 48 (harvested) | 45 (harvested) |

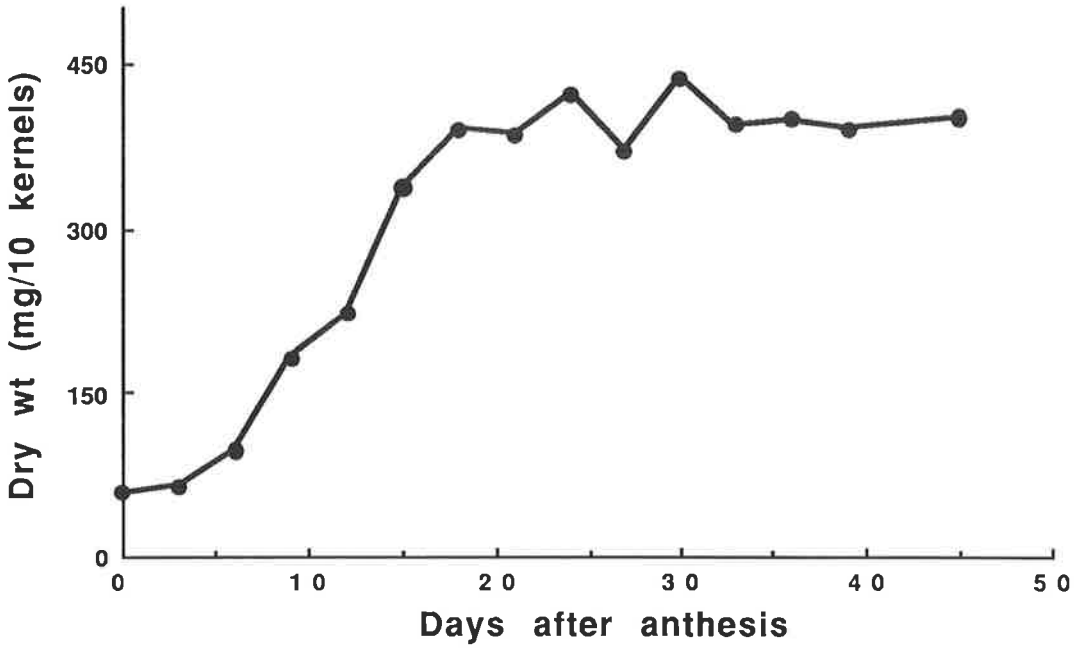



**Figure 6.1**

Changes in the ratio of dry weight per 10 kernels between anthesis and maturity (white tag samples). Data are the mean (n=3) ± sd (too small to be seen).

When extracts were prepared with cysteine added to the extraction buffer, LD activity was detected just after anthesis, remaining at low levels until day 5 then increasing rapidly to a level 8-9 fold above that before day 5 (Fig. 6.2). Activity fluctuated between days 25-42, decreasing slightly but remaining elevated. When the extractions were performed without cysteine, a similar increase in activity occurs but it reaches a peak at day 20 and then the activity decreases rapidly to a level slightly above that before anthesis (Fig. 6.2). There is a possibility that these changes were environmentally induced but this cannot be deduced with the available data. While there were no extremes of rainfall during the period of kernel development (Fig. 6.3), there were several days where the maximum temperatures exceeded 30°C, a high temperature during October (Fig. 6.4). The first couple of hot days coincide with a fall in 'free' enzyme which may be significant or just coincidental.

Yamada (1981b) suggested that LD extracted without a reducing agent is free enzyme, and when extracted with a reducing agent a total of free plus bound is obtained. The arithmetic difference between total and free LD activity gives a measure of the quantity of bound enzyme by definition. There was virtually no bound enzyme until 21 days PA and then the quantity of bound enzyme increased rapidly reaching a plateau which was sustained through to maturity. Thus, the increase in the total activity to 21 days PA is solely due to the free enzyme but after day 21 the quantity of free enzyme decreases and at the same time

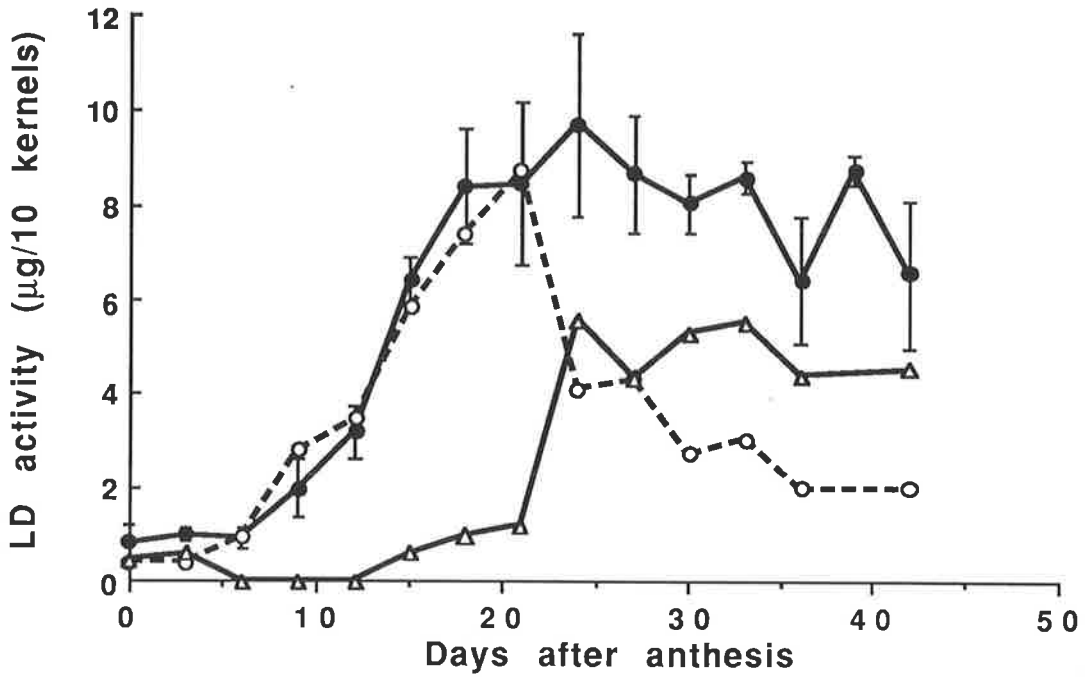



**Figure 6.2**

Limit dextrinase immunological activity in whole kernels of developing barley. Data are the mean activity of three samples. The error bars are standard deviations. Total (●); 'Bound' (Δ); 'Free' (○), dashed line.

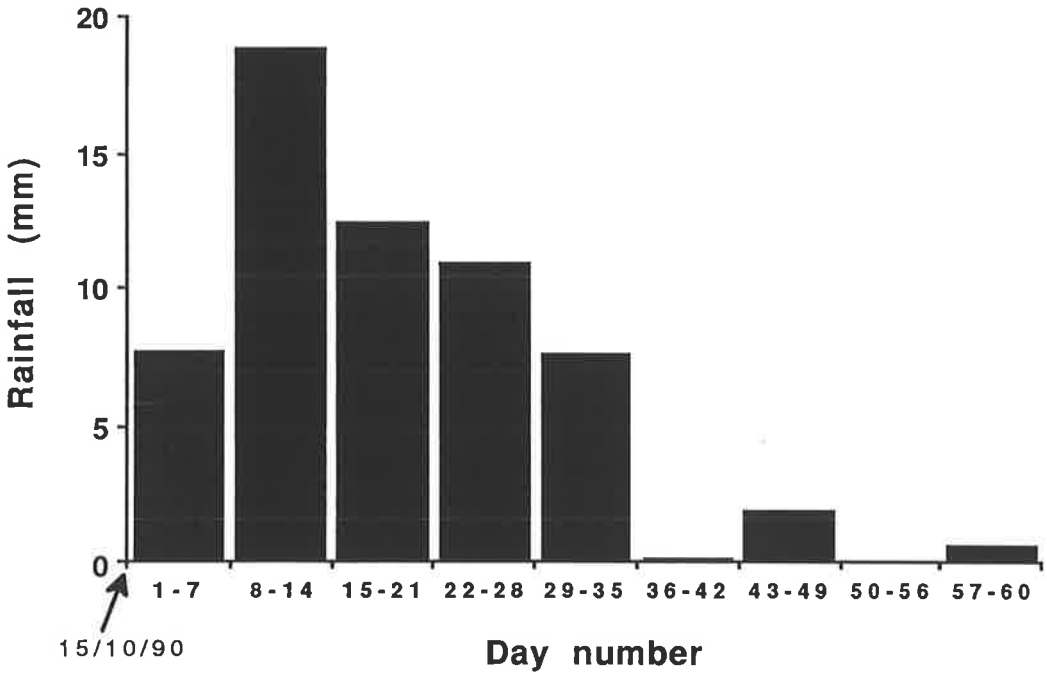



**Figure 6.3**

Rainfall distribution at the Waite Agricultural Research Institute where experimental plots of Skiff barley were grown. Barley plants selected with a white tag reached anthesis on day 9, black tags on day 12, and green tags on day 16. Day 0 was 15th October, 1990.

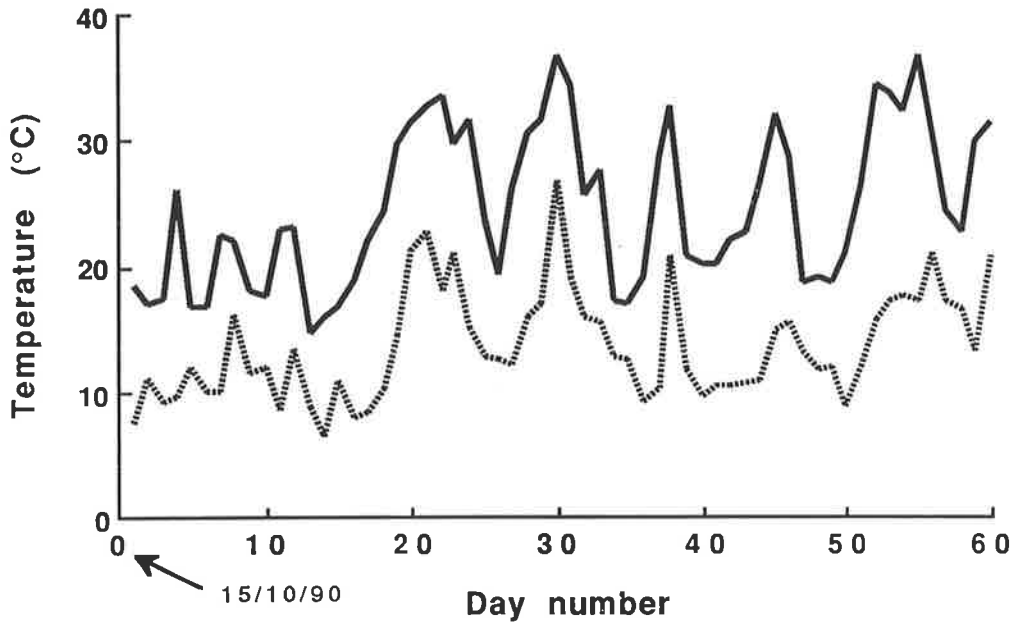



**Figure 6.4**

Variation in maximum (▬▬▬) and minimum (▪▪▪▪▪▪) temperatures at the Waite Agricultural Research Institute where experimental plots of Skiff barley were grown. The identification of anthesis is as described in figure 6.3.

bound enzyme is formed. Similar results were obtained by Laurière *et al.* (1985) using zymogram staining. They found a free form of the enzyme appeared soon after anthesis, and then a bound form gradually appeared at the expense of the free form after 20 days PA. However, they were unable to quantify these changes. The zymogram method they used is not completely specific for LD and therefore the changes observed may not reflect changes solely in LD.

Yamada (1981b) measured the changes in LD activity during development and germination in rice using an assay based on reducing sugars. The free form increased in activity up to day 20 PA and then decreased, whereas the bound form was absent until day 20 then increased continuously until maturity. In germination Yamada (1981b) showed the opposite occurs, there was a decrease in the bound and an increase in the free enzyme. Yamada hypothesised that the LD produced during germination in rice results from an activation of the bound form which accumulates during development of the kernel and that LD is not synthesized during germination.

A possibility is that during the early phase of development, synthesis of LD occurs and the free form is converted to a bound form in the mature seed. Thus, in ungerminated seed there would be a pool of LD which could be released for the purpose of starch hydrolysis during germination. Good evidence for this mechanism exists in rice where Yamada (1981b) showed, by incorporation of radioactive amino acids, that the free enzyme is synthesized and is gradually converted into a bound form which accumulates in the mature seed. However, the situation in rice could be different to barley.

During endosperm development the synthesis of many proteins occurs (Bewley and Black, 1983). In maize, there is an increase in protein content in the endosperm until 20-30 days PA. In this period, the synthesis of enzyme and structural proteins for endosperm growth and metabolism occurs. After 30 days PA only storage protein synthesis continues as the endosperm cells are presumably crushed by the expanding starch (Ingle *et al.*, 1965).

The precise role of LD is not known but it could have a similar role to α-amylase which is synthesized in small quantities during kernel development to degrade starch in the pericarp and endosperm. The changes in LD are similar to the variation in activity of α-amylase I from the outer pericarp of developing Bonanza barley (MacGregor and Dushnicky, 1989a). The α-amylase activity increases rapidly immediately after anthesis and then decreases to low levels by 17 days PA with a peak of activity (8-9 fold increase) at 7 days. In other cereals like oat, rye, rice, triticale, wheat and maize, α-amylase has been reported to be present in kernels during growth and maturation. In general, α-amylase appears in cereal kernels shortly after anthesis, rapidly reaches a peak of activity, and then disappears or declines to low levels in most cereals as the kernels mature (see Hill and MacGregor, 1988 for a review).

The α-amylase inhibitor is present in small amounts 7 days PA and then accumulates rapidly between 7 and 21 days PA reaching a plateau (Robertson and Hill, 1989). Possibly

this protein functions to prevent α-amylase synthesis in the middle to late stages of maturation thereby preventing premature starch degradation before germination.

The β–amylase activity in the pericarp of wheat reaches a maximum at 23 days PA then vanishes during ripening (Daussant and Laurière, 1990).

The study by Kruger and Marchylo (1978) described earlier, found two isoenzymes of LD just after anthesis. Unfortunately they did not quantify activity and their data are only qualitative relying on zymograms. Thus, it seems to be a general phenomenon, that for some of the starch degrading enzymes activity increases soon after anthesis reaching a peak then decreases as maturation is approached.

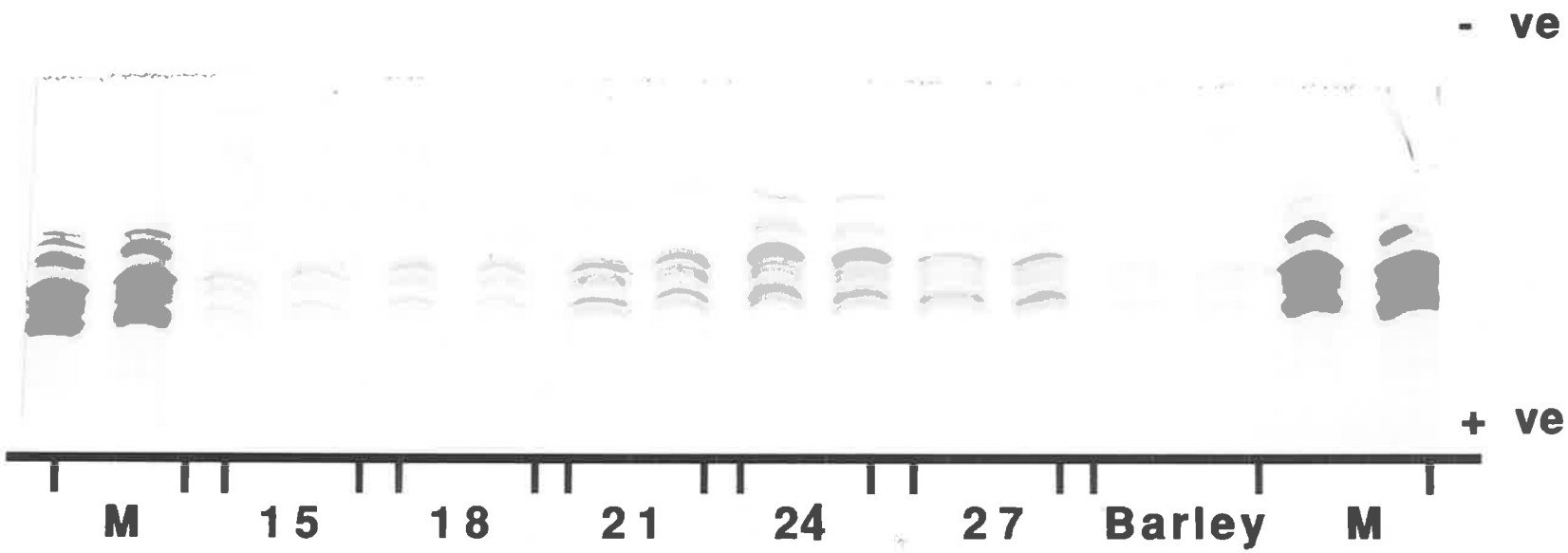
The role of LD in the developing grain is not known. Kruger and Marchylo suggested that it is involved in the production of starch from a phytoglycogen precursor. Limit dextrinase may have a similar role to pericarp α-amylase I whose fall in activity coincides with the disappearance of starch from the pericarp during maturation. It is thought that pericarp α-amylase I is responsible for starch degradation in this tissue (MacGregor and Dushnicky, 1989a) and LD may be needed to assist α-amylase I by breaking the α-1,6 bonds in amylopectin. Some starch degradation occurs in immature barley endosperms and α-amylase II is the major enzyme responsible for granule degradation (MacGregor and Dushnicky, 1989b). The free LD present in the immature kernels could also be present in the endosperm and assist α-amylase II with starch degradation. This raises the question, in what tissue is LD present? In the zymograms of endosperm and embryo tissues, MacGregor and Dushnicky (1989b) found a relatively low pI enzyme that produced a blue colour on dextrin gels. It was thought this protein was LD. The presence of LD in wheat endosperm tissue has been reported (Kruger and Marchylo, 1978).

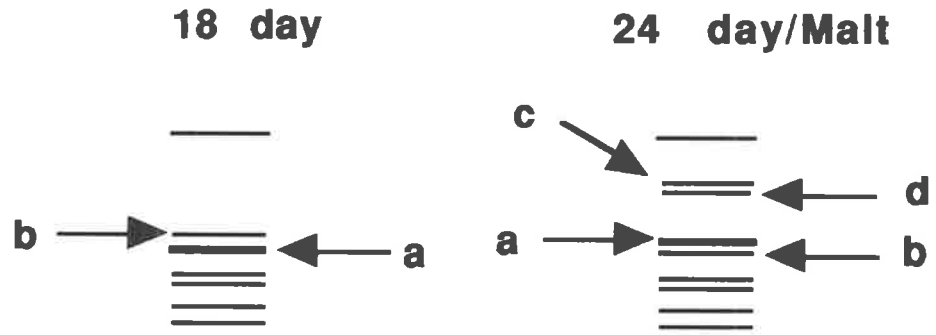
### 6.3.2 Analysis of Isoenzyme Variation during Development

The nature of the isoenzymes at different stages of development is shown in figure 6.5. The intensity of the banding pattern reflects quantitative changes as measured by the ELISA, for example a much heavier staining pattern occurs in malt compared to mature barley (ELISA: 35-45 μg/g in malt compared to 10-15 μg/g in mature barley). There are some differences in the banding patterns during development: (1) bands a and b (indicated with an arrow in Fig. 6.5) are present in malt and days 24and 27 PA material but in days 15, 18 and 21 and mature barley, there is a switch in the intensity of the bands; (2) the absence of bands, c and d (Fig. 6.5) in mature and 15, 18 and 21 PA material but present in malt and day 24-27 PA material. This could be due to the lower avidity of these proteins which have not appeared in the blot because they are below the detection sensitivity of the method. This is likely since bands c and d cannot be seen in mature barley but reappear in malt. Alternatively, the proteins representing bands c and d, may be another form of the enzyme which is not expressed before day 24. The appearance of bands c and d and the switch in

**Figure 6.5**

Isoelectric focusing on a pH 3.5-5 gradient and immunoblotting detection of LD in whole kernels of malt (M), 15, 18, 21, 24 and 27 days post-anthesis samples and mature barley (Barley).


- ve
+ ve
M
15
18
21
24
27
Barley
M


18 day
b
a
24 day/Malt
c
d
a
b

bands a and b may be related to the rapid increase in the presence of the bound enzyme (Fig. 6.2).

The pattern in days 24 and 27 has the same number and position of bands as in malt. This is good evidence that there are no changes in the isozymes during germination. Additional evidence for this are the absence of changes in banding patterns in petri dish germinations from mature to 11 day germinated grain (Chapter 4, Fig. 4.26).

### 6.3.3 Location of Limit Dextrinase in Different Tissues of the Developing Kernel

To determine the tissue location of LD in immature kernels, samples harvested at 21 and 24 days PA were used because total LD activity was then at its highest level (Fig. 6.2). Limit dextrinase was largely confined to the embryo and endosperm, with a trace of activity in the pericarp (Table 6.2). This is quite a different situation to the distribution of α-amylase activity in developing kernels of barley where the majority of activity is located in the outer pericarp (MacGregor and Dushnicky, 1989b). The highest activity was found in the distal endosperm with a slightly higher activity in the embryo than proximal endosperm. Extraction of endosperm tissue in buffer without cysteine gives a measure of the quantity of free LD. Based on this data the proportion of bound enzyme in the proximal endosperm was about 40 % and in the distal endosperm, 60 %. These proportions approximate the activity of bound and free LD in whole kernels (Fig. 6.2) between days 21-24 PA.

**Table 6.2**

Limit dextrinase immunological activity in immature barley tissues. Data are the mean immunological activity ± sd (4 replicate estimations).

| Tissue | LD activity (μg/10 tissues) | |
|---|---|---|
| | With cysteine | Without cysteine |
| Outer pericarp | 0.27 ± 0.022 | N.D. |
| Inner pericarp | 0.19 ± 0.052 | N.D. |
| Embryo | 3.38 ± 0.25 | N.D. |
| Proximal endosperm | 4.20 ± 0.93 | – |
| Proximal endosperm | – | 2.35 ± 0.22 |
| Distal endosperm | 7.41 ± 1.74 | – |
| Distal endosperm | – | 2.99 ± 0.54 |

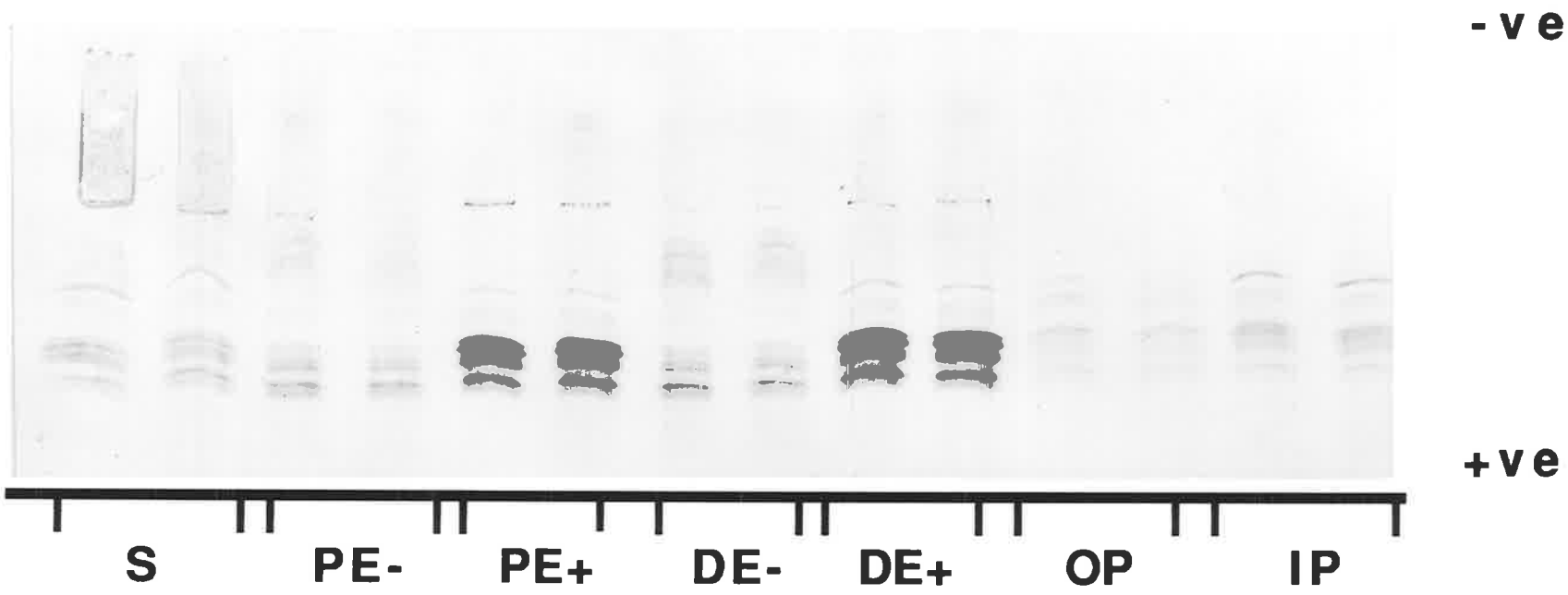
N.D. Not determined because there was insufficient sample.

Isoelectric focusing and immunoblotting revealed the type of LD present in the different tissues (Fig. 6.6). As LD levels in the pericarp tissue are low, a long incubation time with the BCIP/NBT substrate (see chapter 4, section 4.3.7) was required to permit visualization of the bands. Under these conditions some non-specific staining near the cathode resulted. In all the tissues an identical banding pattern was obtained. Bands a and b (refer to diagram in figure 6.5) were decreased significantly in staining intensity when extracted in buffer without cysteine. These two bands predominate in cysteine extracted material which suggests that this isoform could be released by cysteine and might be the 'bound' form. Alternatively, cysteine may bring about a conformational change in the structure of one or more isoenzymes which alters the pI. At day 24 PA, there is a rapid rise in the quantity of the bound form (Fig. 6.2) which is only detected in extracts treated with cysteine. This would explain the reduction in antibody binding of bands a and b (Fig. 6.6). Interestingly, in whole kernels extracted with cysteine there is a slight change in the intensity of staining of bands a and b between days 21 and 24 (Fig. 6.5). The action of cysteine involves breaking disulphide bridges between the enzyme and another protein(s), as occurs with β–amylase (Sopanen and Laurière, 1989; Guerin *et al.*, 1991) possibly allowing exposure of antigenic sites for antibody binding. The bound β–amylase is not inactive but has a reduced enzyme activity possibly due to steric hindrances which prevent substrates from reaching the active site. The RP assay was not able to detect any activity in the bound LD treated with cysteine. Either the bound enzyme has no activity, or the RP assay is not sensitive enough to detect the small quantities of LD present in this fraction. The latter possibility is likely since the ELISA can detect bound LD, though it is possible the antibody is detecting a form of LD which has no catalytic activity. Alternatively, cysteine may invoke conformational changes in the protein structure which allows more binding of the antibody resulting in a more intensely staining pattern.

Two antigenically distinct β–amylases have been found in developing wheat kernels. One form in the pericarp and the well known β–amylase synthesized during seed development in the starchy endosperm (Daussant and Laurière, 1990). The isoelectric focusing patterns of the two forms are very similar, but they have different molecular weights and show different developmental patterns. The data in figure 6.6 suggest there is no variability in the LD isoenzymes between the different tissues examined except for proportion changes and very low amounts of LD in the pericarp however, LD may have been high in pericarp earlier on.

**Figure 6.6**

Isoelectric focusing on a pH 3.5-5 gradient and immunoblotting detection of LD in scutellum/embryo (S), proximal endosperm extracted without cysteine (PE-), proximal endosperm extracted with cysteine (PE+), distal endosperm extracted without cysteine (DE), distal endosperm extracted with cysteine (DE+), outer pericarp (OP) and inner pericarp (IP).


-ve
+ve
S
PE-
PE+
DE-
DE+
OP
IP

# CHAPTER 7

# STUDIES OF THE CHANGES IN LIMIT DEXTRINASE DURING GERMINATION, MALTING, KILNING AND MASHING AND 'BOUND' AND 'FREE' FORMS

## 7.1 INTRODUCTION

During germination a range of hydrolytic enzymes is synthesized, these include; α-amylase, endo-1,3-β-glucanase, endo-1,3;1,4-β-glucanase, endopeptidases xylan degrading enzymes (MacGregor, 1990) and α-glucosidase (MacGregor, 1987). Barley limit dextrinase is also thought to be synthesized during germination (Hardie, 1975) whereas in peas (Shain and Mayer, 1968) the enzyme is released from an inactive form present in the ungerminated seed. In ungerminated barley, LD activity is not detectable (Lenoir *et al.*, 1984; Manners and Yellowlees, 1973) but with the addition of reducing agents or papain to the extraction buffer an increase in activity occurs (Yamada, 1981), suggesting barley LD is also present in a 'bound form' .

Unlike α-amylase II which reaches maximum synthesis after 4-5 days germination (Hill and MacGregor, 1988), maximum activities of LD are reported only after 10 days (Lee and Pyler, 1984). This slow rate of appearance may limit the potential fermentability of the wort, an important malt quality parameter (see chapter 8).

Limit dextrinase has been thought to be temperature labile and a significant reduction in activity can occur during kilning (Kneen and Spoerl, 1948; Lee and Pyler, 1984) and mashing before the starch gelatinization temperature (63°C) is reached (Lee and Pyler, 1984). This lability combined with the slow appearance of the enzyme would explain the very low activity of LD in malt.

The purpose of this chapter was to measure the changes in LD activity during the germination, and phases of malting, kilning and during mashing. The LD activities associated with different cultivars of barley are investigated. In addition, some studies of the 'bound and 'free' forms' of LD are presented.

## 7.2 MATERIALS AND METHODS

### 7.2.1 Germination Studies

Seed of Schooner, Skiff, Lara (malting quality barley s) and Galleon (feed barley) were surface sterilized for 4 min in 3% Miltons solution (Milton Pharmaceutical Company,

N.S.W.), washed in sterile water and blotted dry. Fifty seeds were placed onto two filter papers inside each petri dish (two dishes per sampling time). Sterile water (4 ml) was added, and the dishes sealed with parafilm. All manipulations were performed in a laminar flow hood. Germination was for 0, 2, 3, 4, 6, 8, 10 and 12 days at 15°C. After germination rootlets and coleoptiles were removed and the seed frozen in liquid nitrogen and stored at -20°C until all samples were collected. Seeds were selected for uniform germination. After all the seeds were collected, they were freeze-dried, and ground in a Udy mill to pass through a 0.5 mm screen. Extracts were prepared from 1 g of flour extracted in 4 ml of 200 mM acetate buffer, pH 5, containing cysteine (20 mM) and incubated for 5 h at 30°C. Samples were centrifuged and the supernatant assayed for LD activity by the ELISA and the RP assay as described in 4.3.3.1 and 2.5, respectively. The moisture content of the seeds was not measured and results are not corrected for moisture.

In a preliminary study, Proctor and Fuji Nijio were germinated for 1, 2, 3, 4, 5, 7, 8, 9, 10 and 11 days as described above except that fewer seeds were germinated and as a consequence there was insufficient flour to assay for LD activity using the RP assay.

### 7.2.2 Malting, Kilning and Mashing Studies

#### 7.2.2.1 Malting

Mature grain of Clipper and Skiff (barley protein 11.8 %) was micromalted as described in 2.6.1 (see also Table 2.4) and replicated as three 30 g samples per sampling time. At the beginning of air rest 3 (after completion of steeping), samples (three 30 g samples of each cultivar) were removed from the micromalter at 24, 48, 72, and 95 h (beginning of kilning) and at the completion of kilning. The grain was weighed, then immediately frozen in liquid nitrogen and stored at -20°C until analysis. To maintain the air flow characteristics in the micromalter chamber, as each can was removed it was replaced by another can containing barley grain (ca. 30 g). A 5 g subsample of frozen malt was freeze-dried, and after drying the roots and shoots were removed and the grain ground in a Udy mill. Extracts were prepared from the flour (in acetate buffer containing cysteine) and the supernatant assayed for LD activity by the ELISA and RP assay. Grain moisture was measured on thawed grain as described in 2.6.4. Enzyme activities determined on freeze-dried grain were corrected for varying moisture contents between green and kiln-dried malt.

### 7.2.2.2 Kilning

In a separate study to that described in 7.2.2.1, Clipper and Skiff were micromalted as described in 2.6.1 (see also Table 2.4) and during kilning, samples were removed from the micromalter at 0 (green malt), 6, 12, 16, 20, 24, 26 h during kilning and at 28 h (end of kilning). At each sampling time four cans for each cultivar were removed from the micromalter and weighed, then immediately frozen in liquid nitrogen and stored at -20°C until analysis. The preparation of extracts, assays for LD and moisture measurements are as described in 7.2.2.1.

### 7.2.2.3 Mashing

Clipper malt was ground to EBC fine-grind specifications and mashed according to the method described in 2.6.2. During mashing, a flask was removed from the mash bath at 10, 20, 30, 35, 40, 45, 50, 55, 60, 70, 80, 90, 95, 100, 110, 120 min (total of 16 flasks) and portions of the mixture transferred into four tubes which were immediately cooled in an ice-water bath (1°C). Once all the samples were collected they were centrifuged at 10,000 g at 4°C for 15 min and the supernatant assayed for LD activity by the ELISA.

To measure enzyme activity with the RP assay, the above procedure was modified by adding cysteine (20 mM) to the mash water (see 7.3.4). Sampling times used were 15, 30, 35, 40, 45, 50, 55, 60, 65, 77, 90 and 120 min.

## 7.2.3 Studies of 'Bound' and 'Free' Forms of Limit Dextrinase

### 7.2.3.1 Effect of reducing agents and papain upon 'bound' and 'free' limit dextrinase

Ungerminated barley (cv. Schooner) seed (20 g) or freeze dried 7 day germinated seed (see 5.2.1.1 for details) was ground in a Udy mill to pass a 0.5 mm screen and the flour extracted with 100 mM acetate buffer, pH 5.0 (extraction buffer) for 5 h at 20-24°C with constant stirring. After centrifugation at 1,500 g for 15 min at 4°C, the supernatant was stored on ice. These fractions of the ungerminated and germinated seed extracts are called UGS and GS, respectively. The pellets were resuspended in extraction buffer and extracted for 15 min, centrifuged and the pellet washed again. These pellet fractions of the ungerminated and germinated seed extracts are called UGP and GP, respectively. To release

'bound' LD the washed pellet was resuspended in 'incubation buffers'. The 'incubation buffers' were 100 mM acetate buffer, pH 5 containing either 2-mercaptoethanol (1 or 10 mM) or papain (1 mg/ml, Sigma P4762 from papaya latex) in the presence of either 1 or 10 mM 2-mercaptoethanol or cysteine (20 mM). The suspension was incubated at 30°C for 17 h, centrifuged and the LD in the supernatant assayed using the ELISA. As a control extraction, a pellet fraction was treated with 100 mM acetate buffer, pH 5 alone.

#### 7.2.3.2 Determination of the proportion of 'bound' and 'free' limit dextrinase

The procedure described above was followed, except that cysteine (20 mM) was used to extract the 'bound' enzyme. The LD activity of each of the fractions extracted with or without cysteine (UGP, UGS, GP and GS, ) was measured (replicated 10 times) using the ELISA. The volume of each fraction was recorded in order to calculate the proportion of enzyme in each fraction.

## 7.3 RESULTS AND DISCUSSION

### 7.3.1 Changes in Limit Dextrinase Activity During Germination

There were significant increases in the immunological activity during germination in all six cultivars but at different rates (Fig. 7.1). The activity of the ungerminated grain was low or absent and a detectable increase occurred from the first day. Skiff, Lara, Galleon and Proctor reached maximum activity at 6 days whereas Fuji Nijio and Schooner reached their maximum at 8-10 days (Fig. 7.1). The amount of enzyme synthesized varied considerably between the cultivars tested from a low of about 40 µg/g for Galleon to a high of 160 µg/g for Schooner (4-15 fold increase).

Enzyme activity did not appear until day two or three but showed similar changes to the immunological activity except that Skiff had a lower enzyme activity than Galleon in contrast to the ELISA data (Fig. 7.2).

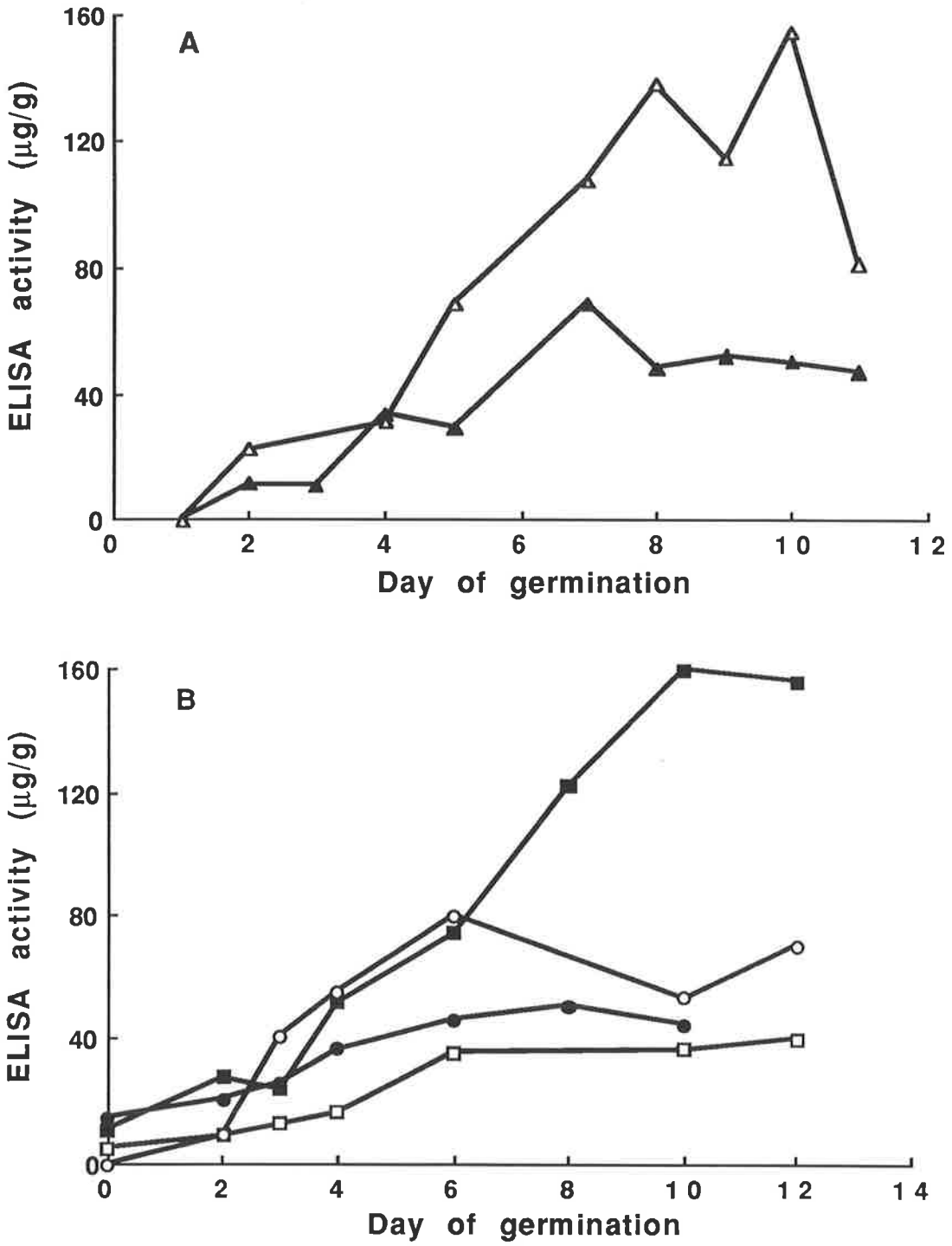



**Figure 7.1**

Limit dextrinase immunological activity in selected cultivars of barley during germination.

**A**: Proctor, ▲; Fuji Nijio, △.

**B**: Schooner, ■; Skiff, ●; Galleon, ❑; Lara, ○.

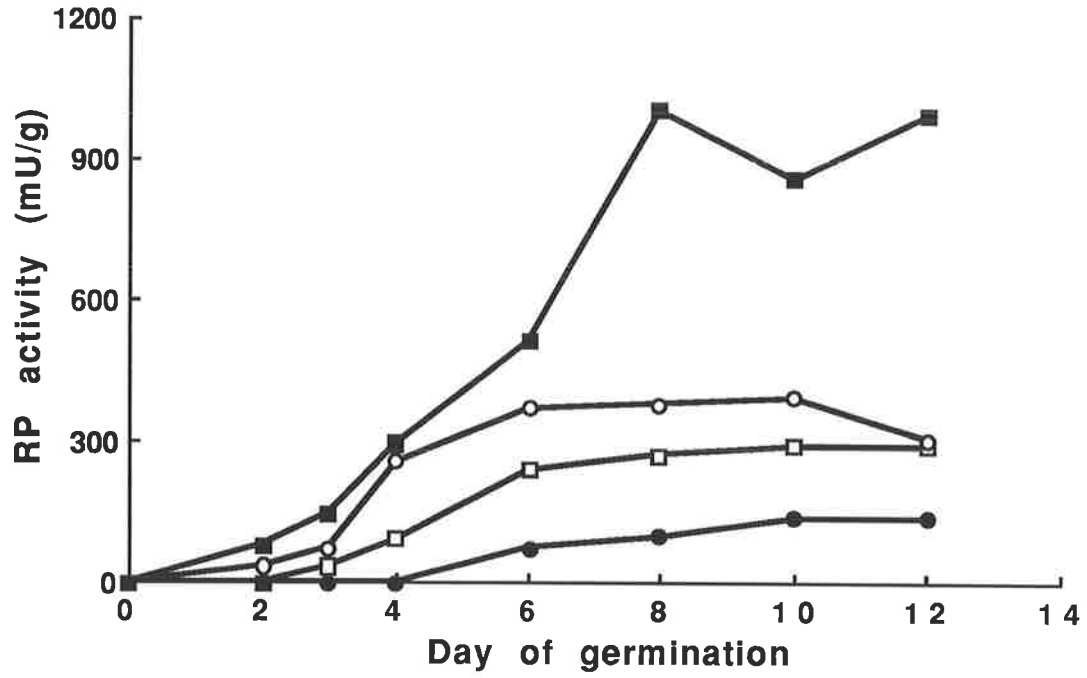



**Figure 7.2**

Limit dextrinase activity (RP) in selected cultivars of barley during germination. Schooner, ■; Skiff, ●; Galleon, ❑; Lara, ○.

The increase in activity seen during germination is probably due to *de novo synthesis* (Hardie, 1975). The results from petri dish germination shows a significant variation in the rate of synthesis of LD and in the timing of maximum synthesis. In Galleon and Skiff, germinating longer than 4 days produced only a marginal increase in both immunological and enzyme activity, whereas in Schooner and Lara, immunological and enzyme activity continued to increase rapidly beyond day four. Other studies have shown variation in the timing of maximum activity. Maximum activity was observed after four (Manners and Yellowlees, 1973), five (Grieg, 1963), or 11 days of germination (Lee and Pyler, 1984 and Pratt *et al.*, 1981), increasing 5-15 fold. In these studies different cultivars were used. The results in figures 7.1 and 7.2 suggest that the reason for the different timing of maximum activity reported by these workers is probably due to differences in the rate of synthesis of LD between cultivars. Differences in the rate of α-amylase synthesis between cultivars exists (Hill and MacGregor, 1988). These results indicate that if a high LD activity is desired in malt, then the choice of a cultivar in which LD is synthesized rapidly should be used. It is important to note that enzyme synthesis under petri dish germination conditions may be different from that in limited moisture conditions normally used in malting.

### 7.3.2 Changes in Limit Dextrinase Activity during Malting

Earlier evidence from Lee and Pyler (1984) has shown that LD is synthesized at a slow rate reaching highest activities after 7-10 days germination. Typically, malting is for 3-5 days and according to MacGregor (1987) there is insufficient time during malting for optimum levels of activity. This could have important implications for malting quality as LD is required to break the $\alpha$–1,6 bonds in amylopectin and derived dextrins. A low activity of LD could result in large quantities of non-fermentable dextrins in the beer and poor distillery yield.

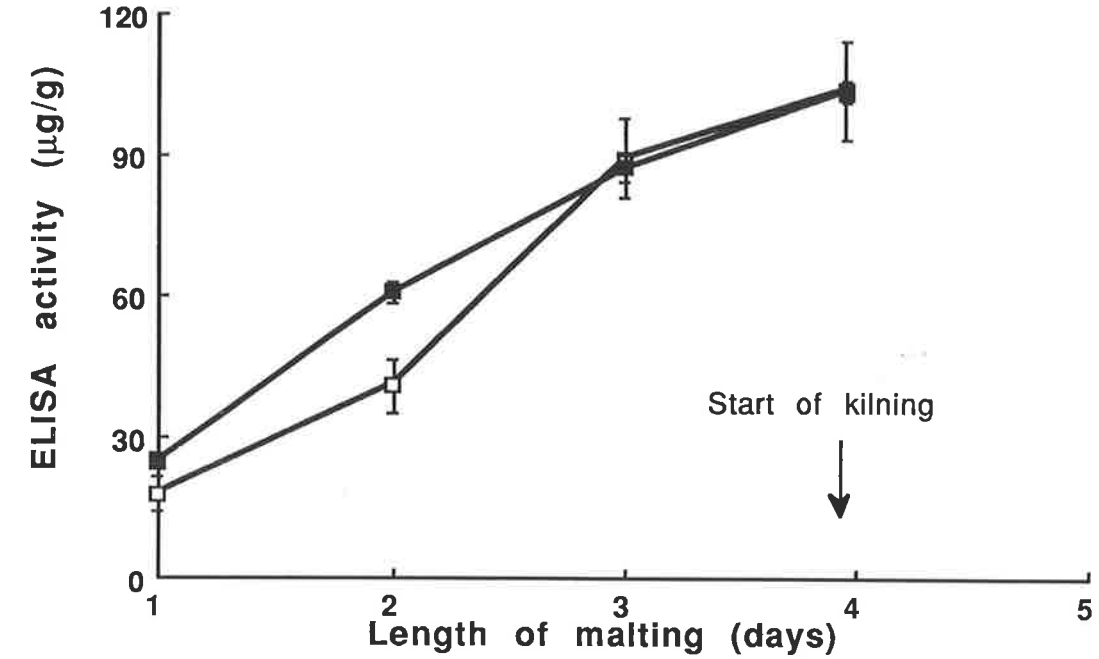
The changes in LD immunological and enzyme activity during malting are shown in figure 7.3. Whereas immunological activity was detected after steeping (day 1), enzyme activity was just detectable. This suggests the protein present at day 1 has either low activity or there is very little protein there. Since the ELISA is more sensitive than the RP assay, this would explain the virtual absence of enzyme activity in day 1 grain.

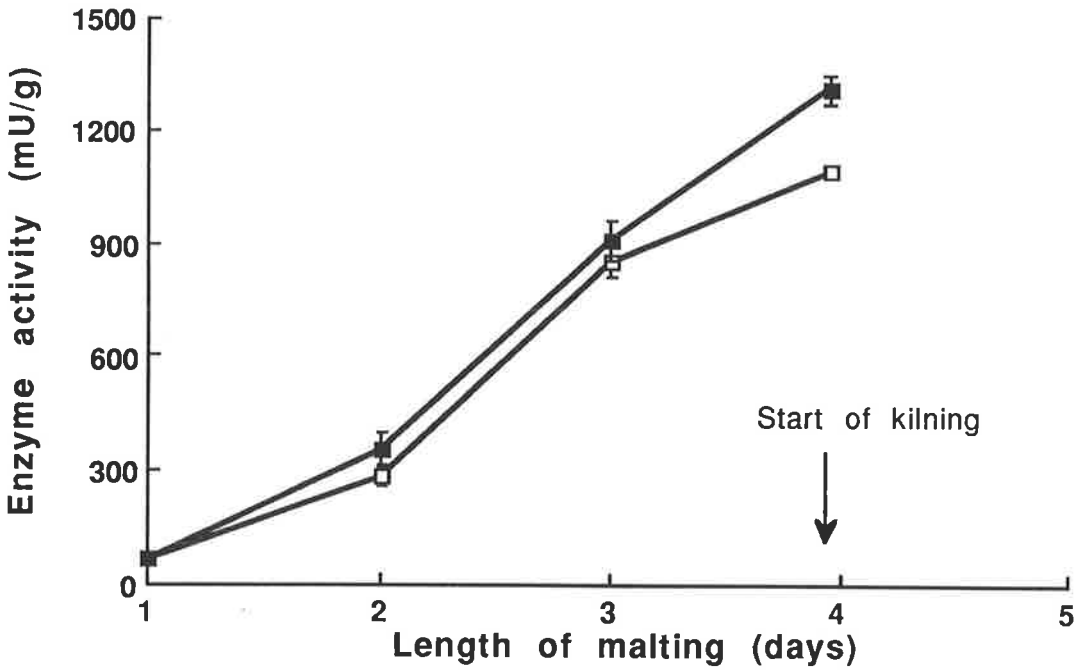
Both immunological and enzyme activity increased rapidly at a linear rate until day three and thereafter increased at a decreasing rate but showed no sign of reaching a plateau. A similar result was obtained by McCleary (1991) who measured changes in LD activity using the RP assay in Schooner malted for 3 days. One could speculate that if malting was continued for longer, a higher activity would be expected indicating there is the potential to produce malts with a higher LD activity. Results in chapter 8 show that extending the malting time from 4 to 7 days in eight cultivars produced an increase in both enzyme and immunological activity. Between day one and approaching day three, the quantity of LD produced by Skiff was less than Clipper, but after three days the quantities were the same. Enzyme activity in Skiff was significantly lower than for Clipper.

The increases in immunological activity were 4-5 fold and in enzyme activity the increase is greater. The results do indicate that the increase in enzyme activity parallels the increase in the amount of antigenic protein. This would support the idea of *de novo* synthesis. More direct evidence is from studies using deuterium oxide and measuring shifts in the buoyant density of LD (Hardie, 1975). The increases in LD of Skiff from the germination experiments are of a similar order of magnitude to the changes during malting. Actual activities cannot be compared between these studies because the germination data are not corrected for moisture.

**Figure 7.3**

Changes in LD immunological and enzyme activity during malting. Data points represent the mean (corrected for moisture on a dry weight basis) of three replicate samples of malt (each sample was assayed in the respective assay in triplicate) and the error bars are the standard error of the mean. Clipper, (■) and Skiff, (❐).


ELISA activity (µg/g)
120
90
60
30
0
1
2
3
4
5
Start of kilning
Length of malting (days)


Enzyme activity (mU/g)
1500
1200
900
600
300
0
1
2
3
4
5
Start of kilning
Length of malting (days)

### 7.3.3 Changes in Limit Dextrinase Activity during Kilning

Kilning is a process where malt is heated to (i) arrest the biological activity of the germinating grain (ii) reduce the moisture content so the finished product can be safely stored and in some cases (iii) to remove unwanted compounds, for example dimethyl-sulphide or polyphenol oxidases known to cause hazes in beer (Palmer and Bathgate, 1976). Malting is a controlled process where optimum synthesis of enzymes, maximum β-glucan degradation and minimal starch breakdown (malting loss) is desired but during kilning the high temperatures used can cause a significant loss of enzyme activity.

The moisture contents of the samples taken during kilning are shown in figure 7.4. Kilning reduced the moisture content of the green malt to 6 % with most of the moisture removed below 55°C. Palmer and Bathgate (1976) suggest that once most of the moisture is removed, the grain is heated as the cooling effect from evaporation is lost and it is during this period that protein denaturation can occur.

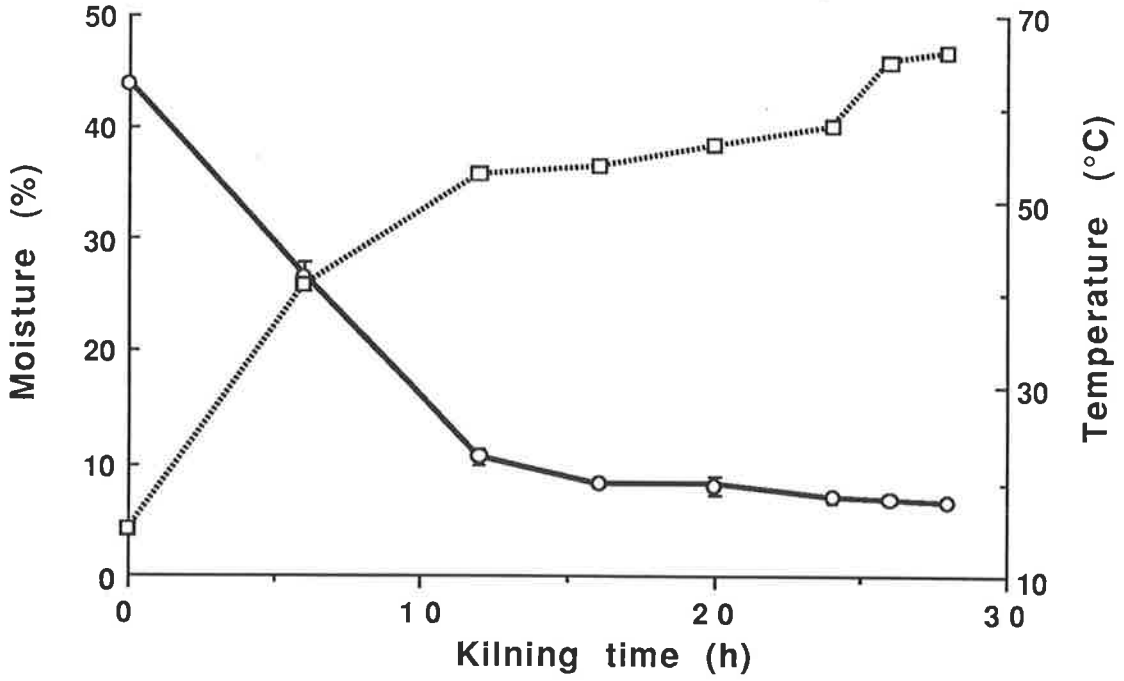



**Figure 7.4**

Changes in grain moisture during kilning. Data on moisture are the mean ± sd shown as error bars. Moisture, ○; Temperature, □.

The immunological activity in Clipper began to decrease slowly above 50°C, but for Skiff this started from 30°C (Fig. 7.5). The activity of the green malt was reduced by 18 % for Clipper and by 24 % for Skiff. These results suggest there are differences in the sensitivity of cultivars to kilning. Enzyme activity (RP assay) for both cultivars begins to

decline at kiln temperatures between 45-50°C (Fig. 7.6). The activity of the green malt by contrast (Fig. 7.7), was reduced by 30% for Clipper, and 58 % for Skiff, confirming the different thermal stabilities of LD from these cultivars. These data show however, a discrepancy between LD activity measured by the enzyme assay compared to the ELISA in samples of green malt collected during kilning. This could be explained if the ELISA detects both active and inactive protein. This result, is in contrast to the almost identical rate of decline in LD immunological and enzyme activity, in extracts of malt heated at 65°C (see 4.3.4.4).

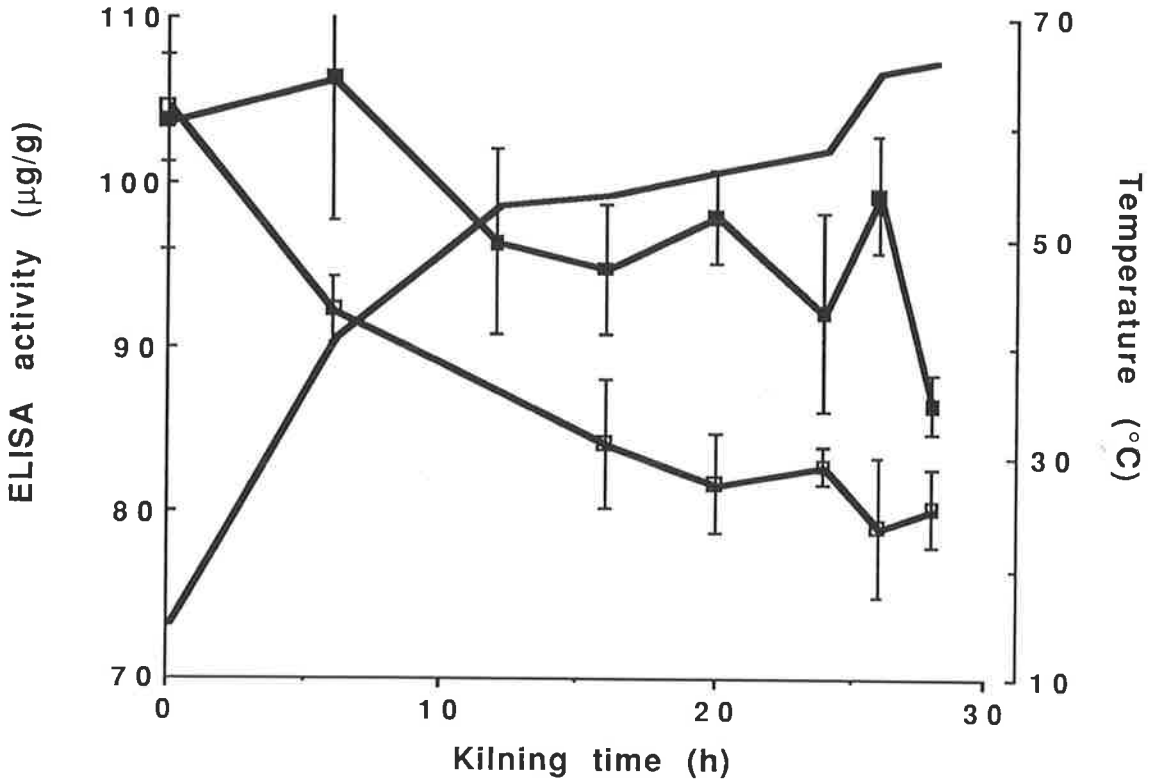



**Figure 7.5**

Changes in the immunological activity of limit dextrinase during kilning. Clipper and Skiff were micromalted and during kilning samples were removed and tested in the ELISA as described in 7.2.2.2. Temperature, (continuous line) ; Data are the mean ± standard error of the mean of four samples. Clipper, (■) and Skiff, (□).

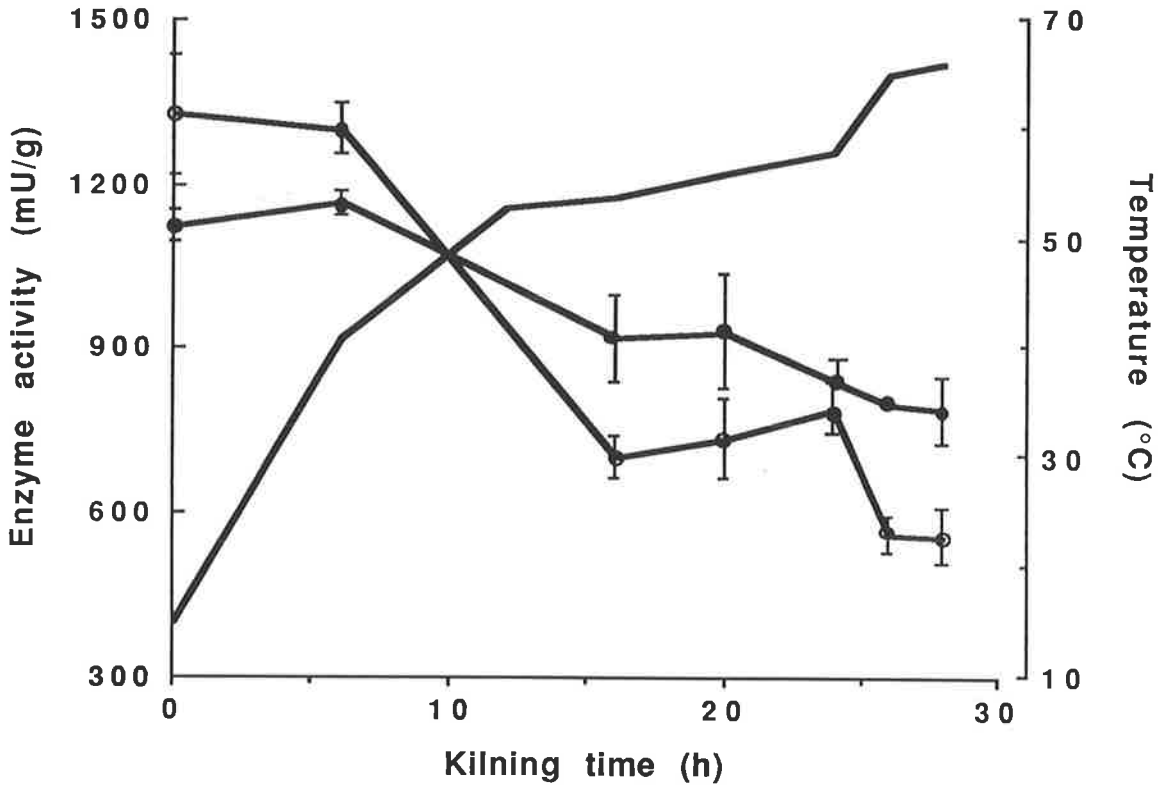



**Figure 7.6**

Changes in the enzyme activity of limit dextrinase during kilning. Clipper and Skiff were micromalted and during kilning samples were removed and tested in the RP assay as described in 7.2.2.2. Temperature, (continuous line); Data are the mean ± standard error of the mean of four samples. Clipper, (●) and Skiff, (○).

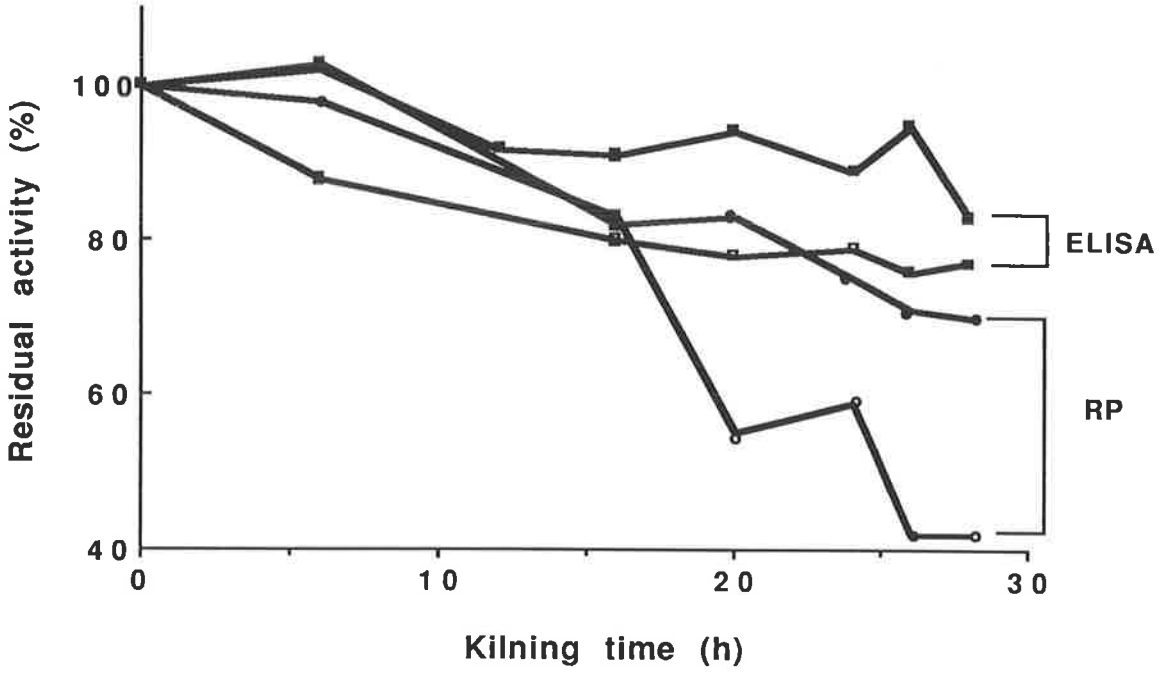



**Figure 7.7**

Comparison of the stability of LD measured by the ELISA and RP assays. ELISA data for Clipper, (■); Skiff, (□); Enzyme activity data for Clipper, (●); Skiff, (○).

In comparison with other studies, Kneen and Spoerl (1948) found no loss in LD activity when malt was heated at 50°C for 24 h, a 32 % reduction in activity after 3 h at 70°C and a 59 % loss after 3 h at 75°C. Lee and Pyler (1984) found no loss in LD activity of malt heated at 49°C for 24 h, a 9 % fall in activity after heating at 65°C for 3 h and an 80 % fall after 3 h at 82°C. These data show LD to be temperature labile since a significant reduction in activity occurs during kilning. The LD activity of the kiln dried malt is probably a function of kilning temperature, moisture and the cultivar and it is difficult to compare the stability of LD between different studies because these variables differ. Also, the temperature programs used differ with that used in the study described here.

Comparing the heat stability of other starch degrading enzymes, α-amylase I or II activity is only slightly reduced during kilning (McCleary, 1991). Kilned malt which was dried at temperatures up to 80°C, retained 38% of the endo-1,3;1,4-β-glucanase isoenzyme II activity detected in green malt (Loi *et al.*, 1987).

Pratt *et al.* (1981) reported that in contrast to conventional kilning methods where moisture is removed rapidly, a slow removal of moisture during the first 10-24 h of kilning at a lower temperature (35°C) minimises the reduction in activity caused by kilning. After the initial slow drying the temperature can be increased to dry the malt to a desired moisture content (4%) while ensuring the temperature does not exceed 70°C.

Given the lability of LD to high temperatures, monitoring LD enzyme activity during kilning may be a good and sensitive indicator of the "status" of the kiln which could be of

use to maltsters. These findings suggest that it is possible to maintain most of the activity of the green malt by choosing a suitable kilning regimen but some cultivars may be more sensitive to high temperatures. In situations where a kiln temperature exceeding 70-75°C is used it is likely that a significant reduction in LD activity will occur but this will also depend upon the moisture profile and cultivar.

### 7.3.4 Changes in Limit Dextrinase Activity during Mashing

Mashing refers to the conversion of barley malt into a fermentable extract suitable for the production of beer. Enzyme degradation of starch produces fermentable sugars that yeast can convert to alcohol (for more details see the review by Palmer and Bathgate, 1976). Some starch is degraded by α-amylase and possibly LD during malting (Bertoft, 1983) and this partially degraded starch is broken down during mashing despite not reaching the starch gelatinization temperature (Palmer and Bathgate, 1976). However, once the gelatinization temperature (ca.,63°C; see 1.6.2 for a detailed discussion) has been reached, the bulk of the starch is degraded by enzymes (Schur *et al.*, 1973). The stability of LD during mashing will affect the degree of starch degradation by this enzyme.

During mashing, LD activity was stable throughout the 30 min rest period at 45°C but declined slowly once 60°C was reached (Fig. 7.8). Between 65-70°C, LD activity declined by about 23 % over 5 min but 50 % of the initial activity remained after 5 min at 70°C. All the activity was lost during the 1 h at 70°C.

When the same mash samples were assayed using the RP assay no activity was detected. During mashing, enzymes are extracted and inactivated depending upon the temperature. The ELISA data (Fig. 7.8) indicate that inactivation occurs at temperatures exceeding 60°C. There is therefore only a limited time (ca. 45 min) for extraction of LD. Recently, McCleary (1991) using the RP assay, found that in order to detect LD activity in extracts of malt flour, a long extraction was required (10-16 h) and very little activity was detected after 1-2 h. This may explain the absence of enzyme activity during mashing.

However, enzyme activity was detectable by adding cysteine (20 mM) to the mash water. In the presence of cysteine, immunological activity declined slightly during the 30 min period at 45°C, then decreased rapidly above 50°C (Fig. 7.9). Immunological activity declined by about 76 % over 10 min between 50-65°C and was not detectable at 70°C. Enzyme activity was lost slightly more rapidly than immunological activity over the same period. These results show clearly that cysteine increased the amount of LD extracted during mashing and brought about a more rapid decline in immunological activity. These data suggest that cysteine is required before LD can be measured with the RP assay. Cysteine increases the extraction of LD from malt flour (see chapter 4 section 4.3.5) and causes activation of the enzyme (see section 7.4; McCleary, 1991). This may explain why enzyme activity was not detected and immunological activity was greater during early mashing. The

nature of this activation is questionable i.e. does cysteine enhance enzyme kinetics, or release LD from a 'bound' form? Cysteine reduces the stability of the enzyme since immunological activity is lost more rapidly and the reason for this is not known. If cysteine releases LD from a 'bound' form it may become more susceptible to thermal denaturation. Enzymes complexed to proteins are generally less susceptible to denaturation due to the protective effect when in a bound form. For example, LD is more thermal stable in crude extracts compared to the purified enzyme in solution (cf. Fig. 3.7 chapter 3 with Fig. 7.6). In the EBC and IOB mashing procedures, no chemicals are added to the water. These results do not reflect what happens to LD during conventional mashing.

The action of LD in mashing is to hydrolyse the α-1,6 glycosidic bonds in starch and in combination with other starch degrading enzymes to degrade starch to fermentable sugars. Only at mashing temperatures above 60°C did the immunological activity decline (Fig. 7.8), whereas Hopkins and Wiener (1955) report an earlier loss of activity. A comparison of the present study with that by Lee and Pyler (1984) shows the reduction in enzyme activity was very similar to the loss of immunological activity. In their study, enzyme activity increased slightly at 46°C and after 25 min at 70°C there was no activity. However, the ELISA was able to detect activity until 1 h at 70°C (but not with cysteine treatment), probably because this assay is more sensitive (Table 7.1).

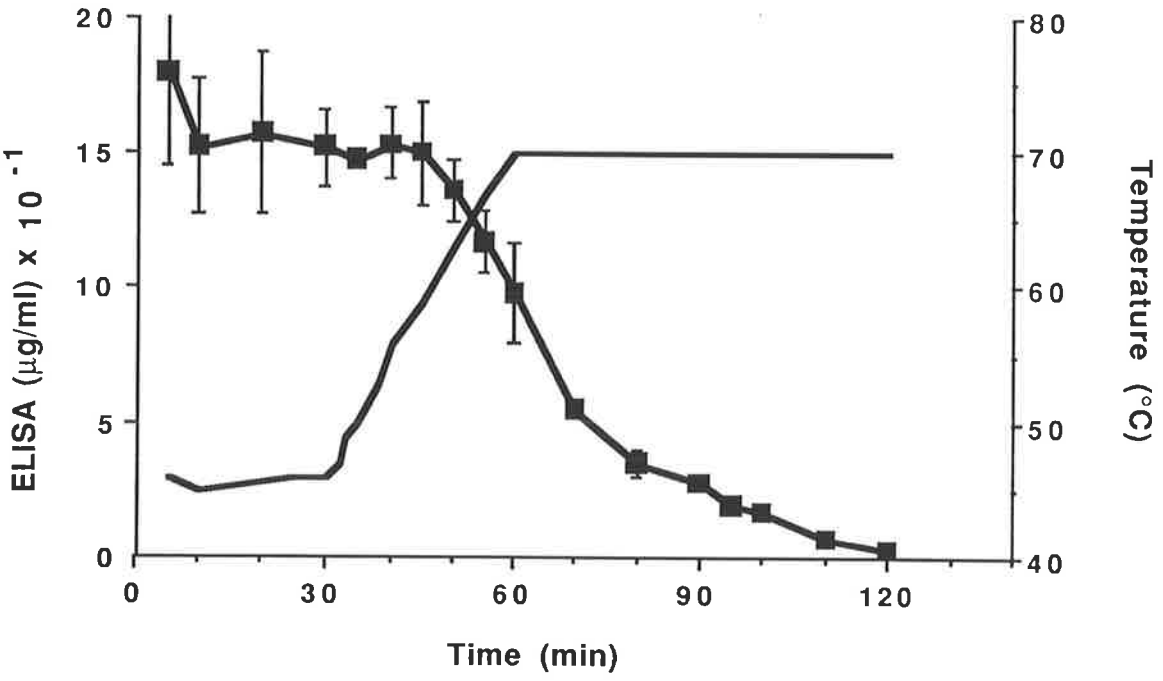



**Figure 7.8**

Changes in the immunological activity of limit dextrinase during mashing. Clipper malt was mashed by a modification of the standard EBC methods of analysis. At each sampling time (represented by a single flask) four subsamples were taken, chilled and centrifuged. Mash temperature, (continuous line); ELISA activity, mean ± se, (■).

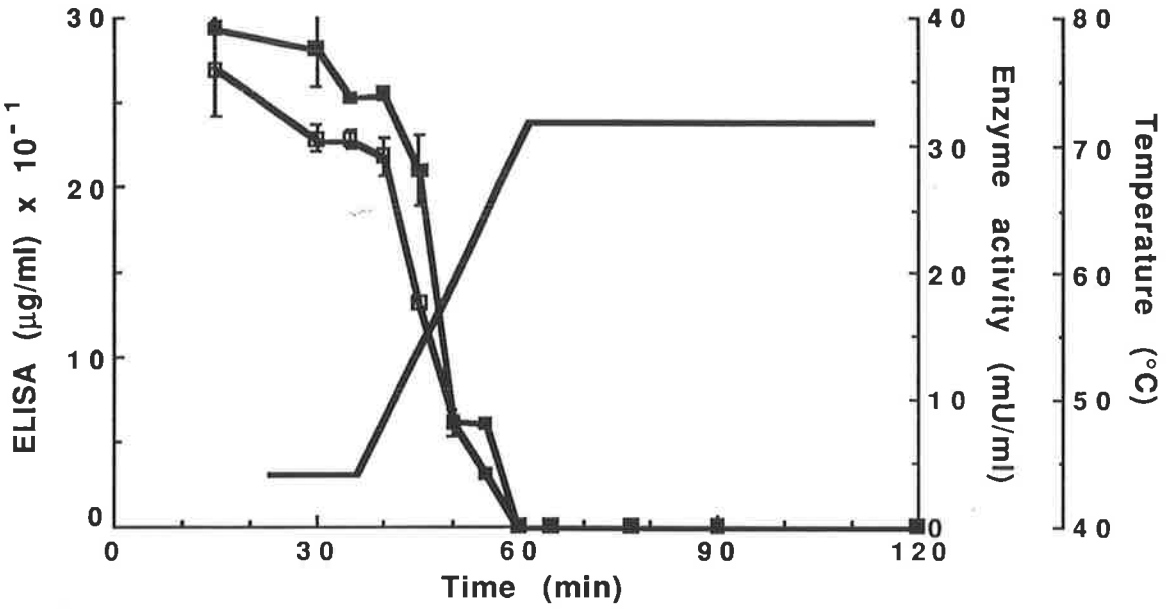



**Figure 7.9**

Changes in the immunological and RP activity of limit dextrinase during mashing. Clipper malt was mashed using cysteine in the mash water. Mash temperature, (continuous line); Mean ± SE for ELISA activity, (■); RP activity, (❑).

**Table 7.1**

Comparison of enzyme activity between different studies. Activity is expressed as a percent of initial activity.

| Immunological data from Fig. 7.8 | Enzyme activity data of Lee and Pyler (1984) | Time (min) | Temperature (°C) |
|---|---|---|---|
| 100 | 100 | 0 | 45 |
| 100 | 109 | 30 | 45 |
| 102 | 108 | 40 | 55 |
| 97 | 91 | 45 | 59 |
| 64 | 65 | 60 | 70 |
| 36 | 35 | 70 | 70 |
| 20 | 0 | 85 | 70 |
| 13 | 0 | 95 | 70 |
| 2 | 0 | 120 | 70 |

Lee and Pyler (1984) suggested that their data showed LD would be active during mashing at low temperatures (on starch solubilized by α-amylase during malting) and would

become inactivated before the starch gelatinization temperature of 63°C (Palmer, 1989) is reached. The immunological data in the present study show that at 63°C, over half of the initial activity is still present but whether this quantity of enzyme is effective in degrading starch remains to be determined. Detailed analyses of starch dextrins in wort and beer by Enevoldsen and Schmidt (1973) have shown that a very high percentage (about 25 %) of starch degradation products (a mixture of branched and unbranched dextrins) are not fermented during brewing and are present in beer. These dextrins could be hydrolyzed by LD and β-amylase. The presence of dextrins in the beer indicates that either insufficient quantities of these two enzymes are present in malt or the enzymes are heat inactivated during mashing before they have hydrolyzed the dextrins produced by α-amylase. Taylor (1974) showed that wort fermentability can be regulated by either slightly increasing or decreasing the mash temperature. Malt contains much smaller quantities of LD than ten day germinated grains and in conventional malting (4-5 days), there is insufficient time to develop maximum LD activity. If a malt with high LD activity is mashed, or the mashing procedure altered to minimise inactivation, more of the dextrins produced by α-amylase could be degraded resulting in more fermentable sugars but this remains to be tested.

The conditions of mashing are important in determining the rates of (i) solubilization and gelatinization of starch (ii) extraction of hydrolytic enzymes from malt (iii) hydrolysis of starch and (iv) thermal inactivation of enzymes. Increasing the mashing temperature from 63 to 67°C reduces the quantity of β-amylase while α-amylase is not affected (Manners, 1974). Other factors like the mash concentration and temperature profiles can affect the stability of enzymes. The immunological data show that LD is more stable in mashing than indicated by other studies, and it is likely that enzyme action occurs at temperatures up to 65°C. Variations in the mashing program may lead to improved survival of LD and as a consequence more α-1,6 bonds broken. Perhaps LD would be more valuable to the distilling industry where lightly kilned malts are mashed at lower temperatures (Manners, 1974). Studies are required to determine the optimal mashing conditions that promote LD action.

## 7.4 Studies on 'bound' and 'free' forms of limit dextrinase

### 7.4.1 Effect of Reducing Agents and Papain upon 'Bound' and 'Free' Enzyme

#### 7.4.1.1 Introduction

The purpose of the studies described in this section is to examine the effect of thiol agents and papain on the extraction and or activation of LD. It is useful to approach these

studies with reference to β-amylase. In ungerminated grain most of the β-amylase is 'bound' via S-S bridges to insoluble constituents in the starchy endosperm and these bonds can be broken *in vitro* with thiol reducing agents and papain (Laurière *et al.*, 1985). This form is partly insoluble in water and an aqueous extraction contains a mixture of a heterodimer complex between Z protein and β-amylase and monomeric Z protein and β-amylase (Hejgaard, 1976). The heterodimer can be split with thiol reducing agents and papain to release β-amylase and Z protein. Yamada (1981d) found that in ungerminated rice flour, LD can be extracted in greater quantities when the extraction buffer contains reductants or proteases. This suggests that LD may be present in a form in ungerminated cereals 'bound' via disulphide bridges to another component analogous to the situation of β-amylase.

The extraction of LD in acetate buffer may release a heterodimer complex between LD and another constituent, possibly a protein (Y). It is therefore not strictly correct to describe this activity as due solely to 'free' or monomeric LD. The studies described do not allow any distinction between monomeric LD and heterodimer forms because even if both have only partial immunochemical identity, the ELISA could not distinguish between them. For convenience, the LD extracted with acetate buffer (supernatant fraction) is referred to as 'free' LD. Acetate buffer does not extract all the LD found in ungerminated and germinated seed. Thiol reducing agents or papain are required to extract LD which is trapped in the pellet fraction remaining after centrifugation of the extract. This form of LD is tightly bound and probably exists as highly aggregated enzyme. This form is referred to as 'bound' LD.

#### 7.4.1.2
#### Methods

Refer to section 7.2.3.1 for details.

#### 7.4.1.3
#### Results

The agents used to release 'bound' β-amylase may also be effective in releasing 'bound' LD. Sopanen and Laurière (1989) found a 1 mg/ml papain solution extracted all the 'bound' β-amylase in 30 min, whereas Bureau *et al.* (1989) found 50 mM acetate buffer, pH 5.5 containing 2-mercaptoethanol (20 mM) for 16 h was effective in releasing 'bound' β-amylase. Studies to determine the optimum extraction of LD (4.3.5) show cysteine to be superior to papain or 2-mercaptoethanol. Hejgaard (1978) found major quantitative changes in β-amylase during malting so it is relevant to study the 'bound' and 'free' LD extracted from germinated and ungerminated seed.

The distributions of LD immunological activity in the four fractions are described in figures 7.10 and 7.11. Preliminary studies (data not shown) to establish the length of incubation of the various acetate extractions (UGS, UGP, GS and GP) with one of the

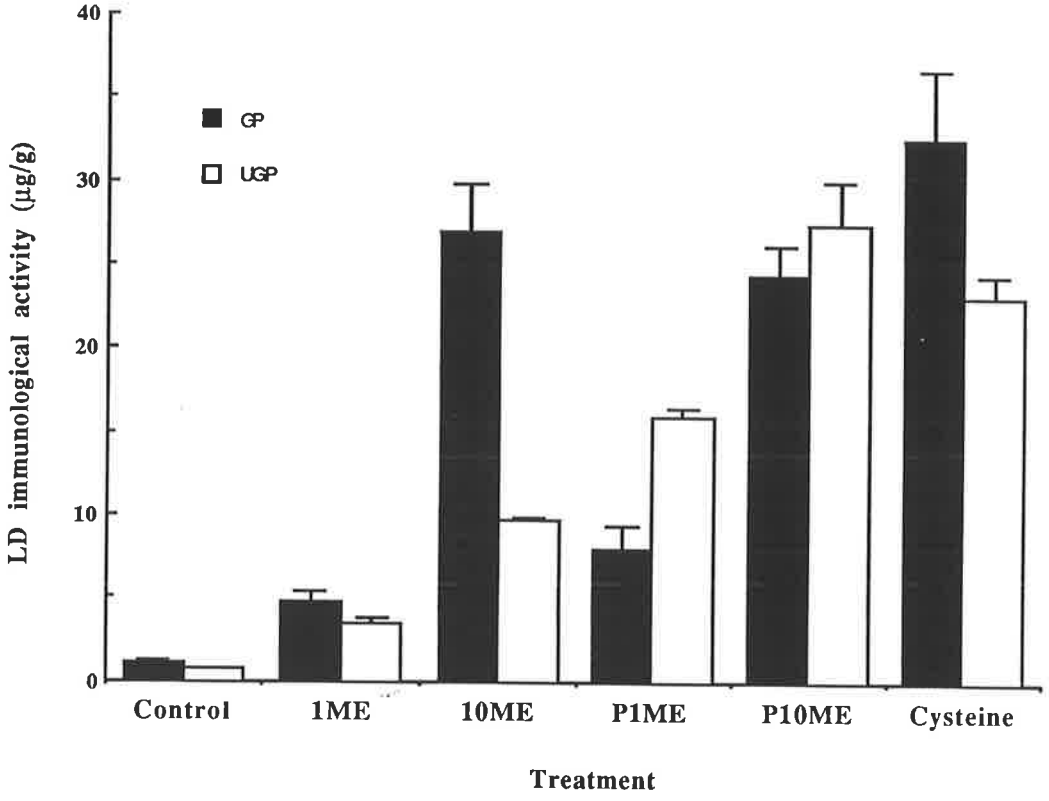



**Figure 7.10**

Release of the 'bound' fraction of LD in germinated and ungerminated barley. Germinated and ungerminated barleys were extracted with acetate buffer to remove the 'free' enzyme and, after two washes with acetate buffer, the pellet fraction was separated from the 'free' LD by centrifugation as described in 7.2.3.1. The pellet was resuspended in either acetate alone (control) or acetate buffer containing 2-mercaptoethanol at 1 and 10 mM (1ME and 10ME, respectively) or papain (1 mg/ml) in 2-mercaptoethanol (P1ME and P10ME), or cysteine (20 mM), ('incubation buffers') and incubated for 17 h at 30°C, centrifuged and the supernatant assayed for immunological activity with the ELISA.

'incubation buffers' used (cysteine), found maximum effect occurs after a 17 h incubation. The activity of extracted enzyme from cysteine treated UGP increased by 65 % and for GP by 165 % after a 17 h incubation compared to 1 h. There was no difference in the activities of the GS and UGS fractions between 1 or 17 h, but in order to treat all fractions equally, incubations were for 17 h. There was a trace of LD in the control UGP and GP fractions. Reducing agents and papain significantly increased LD immunological activity in the UGP and GP fractions. The greatest increase was produced with cysteine and P10ME except GP treated with 10ME (Fig. 7.10). The higher concentration of ME increased immunological activity in GP as much as P10ME did. In the control supernatant fractions (GS and UGS), a 7-8 fold greater quantity of immunological activity was found in GS compared with UGS (Fig. 7.11). The reducing agents and papain had no effect upon the quantity of immunological activity in these fractions.

Enzyme activity was only detected in the GS fraction using the RP assay. The reducing agents and papain increased activity 1.5-2 fold (Table 7.2). Both cysteine and P1ME and P10ME increased activity the most.

**Table 7.2**

Enzyme activity of the GS fraction measured using the RP assay. Details on this experiment are given in 7.2.3.1.

| **Treatment** | **RP activity (mU/ml) Mean ± std. deviation** |
|---|---|
| Control | 35.5 ± 0.78 |
| 2-mercaptoethanol (1ME) | 42.6 ± 1.77 |
| 2-mercaptoethanol (10ME) | 54.1 ± 4.24 |
| Papain + 2-mercaptoethanol (P1ME) | 60.0 ± 11.24 |
| Papain + 2-mercaptoethanol (P10ME) | 66.0 ± 0.14 |
| Cysteine (20 mM) | 61.4 ± 4.17 |

The sum of activities in the GS and GP cysteine treated fraction, approximates the activity in seven day germinated Schooner from petri dish germinations (see Figure 7.1B). Also, the concentration of activity in the ungerminated grain (UGS + cysteine activated UGP, about 33 μg/g) is similar to that shown in ungerminated seed (Fig. 7.1B).

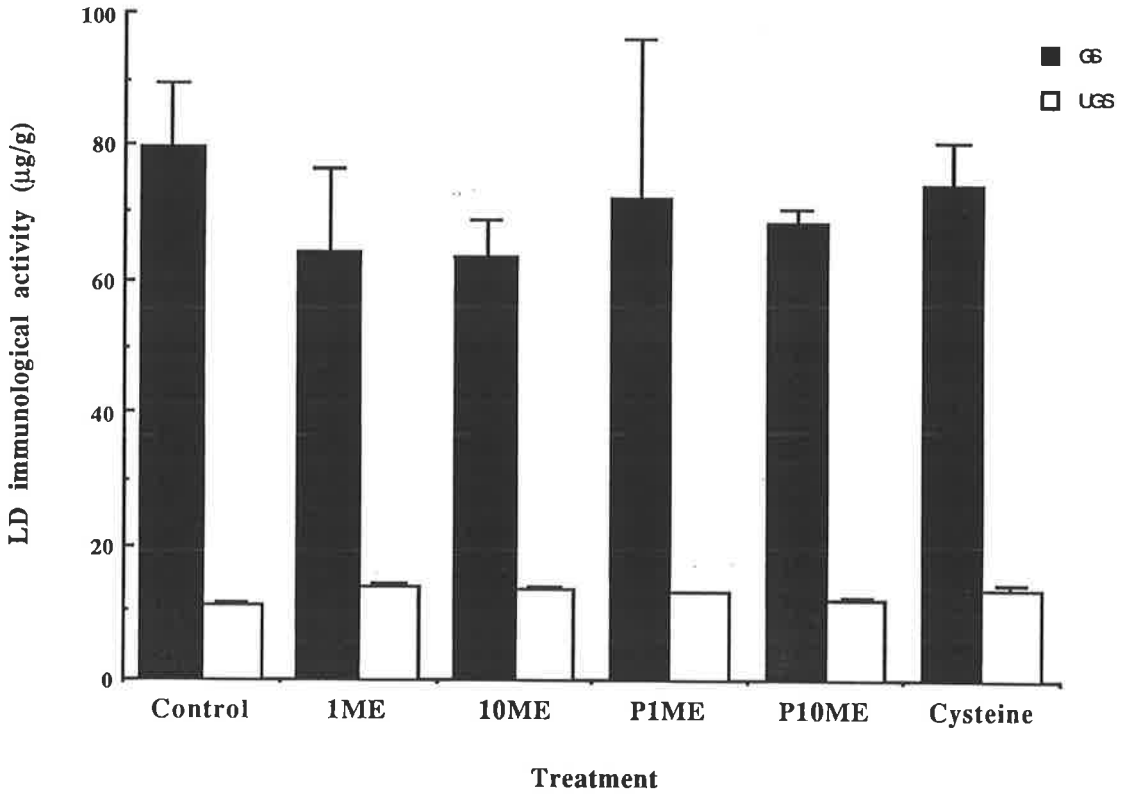



**Figure 7.11**

The effect of reducing agents and papain on extracted 'free' LD in germinated and ungerminated barley. Germinated and ungerminated barleys were extracted with acetate buffer as described in 7.2.3.1. The supernatant fractions were treated with the 'incubation buffers' and assayed for immunological activity as described in figure 7.10.

### 7.4.1.4 Discussion

Activity in ungerminated grain, described earlier (see 7.3.1), has also been reported in the ungerminated seed of other cereals using enzyme assays (Manners, 1985a ; Yamada, 1981b). However, the RP assay did not detect any enzyme activity in ungerminated seed in the present studies. This is probably because there is a much lower activity before germination (McCleary, 1991) and this assay has insufficient sensitivity compared to the ELISA. There is much more immunological activity in 7 day germinated seed (GS fraction), presumably this is due to *de novo* synthesis as suggested by Hardie (1975). The effect of reducing agents and papain on enzyme but not immunological activity of the supernatant fraction of germinated seed (GS) is a significant finding. This suggests that (1) LD could be activated; a similar study was reported in chapter 4 (section 4.3.5). Sopanen and Laurière (1989) found 'free' β-amylase activity was increased by 20 % in ungerminated barley by papain and dithiothreitol, indicating activation. (2) the 'free' fraction does not only contain a monomeric form of the enzyme. Hejgaard (1978) report the existence in water soluble extracts of barley, three electrophoretically distinct immunochemical forms; β-amylase monomer, Z protein and a complex heterodimer between the two. The effect of high concentrations of 2-mercaptoethanol (100 mM) was to break the aggregate and increase the activity of the β-amylase monomer by 17 %. The 'free' LD fraction may therefore contain a mixture of monomer LD and aggregated LD, both of which are recognised by the antibody. If reducing agents act to disrupt the aggregate they would not alter the total immunoreactivity measured by the ELISA (assuming the antibody recognizes 'bound' and 'unbound' equally) but instead would change the electrophoretic distribution of the immunoreactivity. The increase in LD monomer would lead to an increase in enzyme activity as measured. Further studies using crossed immunoelectrophoresis similar to those studies by Hejgaard (1976) should be undertaken to resolve this.

There is a trace of immunological activity in the untreated pellet fractions (GP and UGP). This might be due to (1) the presence of soluble monomer LD in traces of water trapped in the pellet (2) the antibody has immunoreactivity towards the bound form (3) the activity is a non-specific reaction. The pellets received two washes and there was no activity in the second wash so all of the LD should be in the 'bound' form and possibility (1) is unlikely. Any LD present in the pellet ('bound') is probably in a highly aggregated form. This is likely to have a low immunoreactivity due to steric problems with the antibodies attempting to bind LD. Evidence from studies with β-amylase has found the aggregate of β-amylase-Z protein to have partial immunochemical identity with β-amylase monomer (Hejgaard, 1976). Due to the low dilution required to measure LD in the UGP fraction, there is a possibility of non-specific binding to components other than LD.

Reducing agents and papain significantly increased immunological activity presumably due to release of monomeric LD from its bound state.

Release of immunological activity in the GP and UGP fractions was more effective at a higher 2-mercaptoethanol concentration (Fig. 7.10). This suggests release is dependent upon concentration of the reducing agent. Hejgaard (1976) showed the conversion of the β-amylase heterodimer to monomer was dependent upon the concentration of 2-mercaptoethanol and that 100 mM 2-mercaptoethanol was required to release all the β-amylase

### 7.4.2 Determination of the Proportion of 'Bound' and 'Free' Enzyme

#### 7.4.2.1 Methods

Refer to section 7.2.3.2 for details.

#### 7.4.2.2 Results

In another experiment, each of the four fractions was prepared in sufficient quantities to determine immunological activities on 10 replicate extractions. The volume of each fraction was measured and used to calculate total activities (Table 7.3).

**Table 7.3**

Proportion of immunological activity in 'bound' and 'free' fractions of germinated and ungerminated seed.

| Fraction | Total activity (μg LD/100 g flour) | | | |
|---|---|---|---|---|
| | Control (no reducing agent) Mean ± sd | % of total | Cysteine treated Mean ± sd | % of total |
| GS | 6857 ± 488 | 88.9 | 9082 ± 1505 | 86.2 |
| GP | 856 ± 55 | 11.1 | 1452 ± 207 | 13.8 |
| Total | 7713 | 100.0 | 10534 | 100.0 |
| UGS | 1851 ± 116 | 98.9 | 2051 ± 266 | 88.6 |
| UGP | 20 ± 1.0 | 1.1 | 263 ± 28 | 11.4 |
| Total | 1871 | 100.0 | 2314 | 100.0 |

The majority of the LD activity is present in the control extract supernatant prepared from the germinated seed (GS). There is a very small quantity of LD in UGP but a much greater amount in GP. Cysteine had no significant effect on activity in the GS and UGS fractions but released immunological activity in the GP and UGP fractions as described earlier.

### 7.4.2.3 Discussion

In the control treatment of germinated grain about 11 % of the total immunological activity is found in the pellet. The presence of LD in the pellet fraction suggests there is still some LD in a 'bound' form which has not been converted to a 'free' form during germination as is suggested for the mechanism of β-amylase release (Sopanen and Laurière, 1989). Alternatively, this activity could be an artifact of the extraction or a means of control of activity at a physiological level. The source of this 'bound' form is not known, it is unlikely to come from the 'bound' fraction in ungerminated grain because that has a much smaller quantity of 'bound' enzyme. A more likely possibility is that some of the LD synthesized *de novo* during germination becomes 'bound', possibly to regulate the supply of LD and hence enzyme activity to the developing embryo, for example to prevent breakdown of starch completely to small sugars. Alternatively, an inhibitor of LD may be synthesized during germination to bind some of the LD synthesized, thereby controlling enzyme activity. It is therefore imperative to understand the exact nature of the 'bound' form and to what it is bound.

Total extractable activity was greater in cysteine treated extracts of germinated and ungerminated seed. However, it is not known whether this gives exhaustive extraction of the enzyme since only 20 mM cysteine was tested and further studies are required.

In peas (Shain and Mayer, 1968) and sorghum (Aisien *et al.*, 1983), the LD in the germinated seed is thought to arise by activation of a 'bound' form of LD. Aisien *et al.* (1983) measured the changes in LD activity in the embryo and endosperm during germination of sorghum. They showed an early rise (activity increased rapidly after 24 h) in LD activity and suggested this was due to the enzyme existing in the endosperm of the ungerminated seed as a zymogen ('bound' form). In the case of barley the data here suggest the amounts of 'bound' enzyme do not account for the quantity of LD in germinated grain (GS fraction). These data support the view held by Hardie (1975) that LD is synthesized during germination.

The mechanism of release of 'bound' forms of LD has not been reported and the best analogy would come from the studies with β-amylase. A large portion of 'bound' β-amylase is attached, apparently via S-S-bridges, to insoluble constituents of the starchy endosperm (Sopanen and Laurière, 1989). It can be released *in vitro* either with reducing agents or with papain but the mechanism of release *in vivo* involves proteolytic action of one or

several SH-proteinases (Sopanen and Laurière, 1989 ; Guerin *et al.*, 1991). There is evidence in other plants of a 'bound' form of LD but the significance of this is unknown. Shain and Mayer (1968) showed that LD is present in a precursor form in peas and is released by protease action during germination. They also found in the pellet fraction of seed extracts treated with trypsin, an increase in LD suggesting activation, possibly due to a change in the structure of LD from an inactive to active form, by liberation from a precursor form. Yamada (1981d) found that in ungerminated rice flour, LD can be extracted in greater quantities when the extraction buffer contains reductants or proteases. This suggests that LD may be present in a form in ungerminated cereals 'bound' via disulphide bridges to another component. By extracting with a buffer containing a reducing agent, such as dithiothreitol, both the 'free' and aggregated forms were extracted. Yamada (1981d) used this to show that during germination of rice the 'bound' enzyme decreased continually, and the 'free' form increased to day six, suggesting conversion of the 'bound' to the 'free' form during germination. It was shown by incorporation of radioactive amino acids that the enzyme was converted from a 'free' to a 'bound' form during germination. This seems contradictory to the situation in barley where the majority of LD is synthesized *de novo*. There are therefore differences between species in the mechanisms by which LD appears during germination. The reasons for this are not known. Interestingly, the activity obtained in the 6 day germinated seeds was comparable to the activity obtained after a 3 h incubation with papain (Yamada, 1981d), suggesting that activation of a pre-existing form is the mechanism by which activity increases.

# CHAPTER 8

# RELATIONSHIP BETWEEN LIMIT DEXTRINASE ACTIVITY AND THE APPARENT ATTENUATION LIMIT

## 8.1 INTRODUCTION

Limit dextrinase is potentially important to malting quality by virtue of being the only enzyme produced by the barley seed during germination which hydrolyses α-1,6 glucosyl bonds in amylopectin and β-limit dextrins. During mashing, the solubilization of starch and its subsequent enzymic degradation to fermentable sugars occurs. The quantity of branched dextrins and their profile in the wort (their degree of polymerization) and beer gives an indication of the effectiveness of this enzyme during mashing. This is evident in the studies where pullulanase (a bacterial LD) was added to the wort after pitching (Enevoldsen and Schmidt, 1973). There was a dramatic decrease in the quantity of branched dextrins in the beer and an increase in maltose and maltotriose. Possibly, malts with a high LD activity may produce worts and, after fermentation, beers with a high apparent attenuation limit.

The apparent attenuation limit (AAL) gives an indication of the total amount of fermentable sugar in the wort. A wort with a higher AAL will produce more alcohol per amount of malt used than a low AAL wort. The common method for measuring the AAL is to compare wort specific gravity before and after fermentation to the limit (Analysis Committee of the EBC, Analytica-EBC). The measurement of the AAL is an important malt quality parameter used by maltsters, brewers and distillers to judge the quality of malt and the potential alcohol yield. One of the major long term markets for Australian malted barley is Japan. The Japanese have recently expressed some concern about the lower levels of AAL of Australian malts compared with malts made from Japanese cultivars. The Australian malting industry exporting malt to Japan considers AAL to be a high priority. AAL is related to diastase and improved diastase would result in improved AAL (personal discussions with South Australian Malting Companies). If LD is a limiting factor determining the maximum potential AAL, then breeding for a high LD activity should improve the quality of Australian barley.

Lee and Pyler (1984) showed that alcohol production and fermentability were correlated with LD activity. Their results were based upon the addition of malt flour from four cultivars with different LD (and α-amylase and β-amylase) activities to a Morex wort prior to fermentation. It is not possible with this type of study to be certain that the increase in fermentability was due to LD alone. Willox *et al.* (1977) and Enevoldsen and Schmidt (1973) showed that the addition of pullulanase to fermenting wort produced a marked improvement in

AAL. Perhaps of more relevance is whether there is any relationship between malt LD activity which varies between cultivar, and AAL of fermented wort prepared from the same malt.

The production of dry beer with low residual sugar content represents about 30 % of the Japanese beer market (1990 estimates) and is popular in the USA. Although amyloglucosidase can be used to reduce the carbohydrate content of the beer, the action of this enzyme is difficult to control (Pratt *et al.*, 1981). The role of LD in controlling the quantity of dextrins may be important in the production of dry beer and in low calorie beer production (Willox *et al.*, 1977; Pratt *et al.*, 1981).

This chapter presents some information on the relationship between AAL in malts with different LD activities.

## 8.2 MATERIAL AND METHODS

### 8.2.1 Relationship Between Limit Dextrinase and Apparent Attenuation Limit in Genetically Diverse Cultivars

Four day malts prepared from 38 of the genetically diverse cultivars (plus a batch control) described in chapter 5, section 5.2.1, were mashed and fermented and the percent extract (dry basis) and the AAL measured as described in chapter 2, section 2.6. Statistical analyses were performed using the JMP™ software package.

### 8.2.2 Relationship Between Limit Dextrinase and Apparent Attenuation in Selected Cultivars Malted for 4, 7 and 10 Days

Eight cultivars were selected from a trial similar to that described in 5.2.1 but grown at a different site (Waite Agricultural Research Institute). The cultivars were selected on the basis of their LD immunological activity.

Samples of mature grain from the eight cultivars and a control malt (replicated as three samples or micromalt cans per cultivar) were malted for either 4, 7 or 10 days. The procedure used for micromalting differed from that described in 2.6.1 in that the steeping was done outside the micromalter. All grain samples were scheduled to go into kiln at the same time. To do this it was necessary to hand steep the grain. This consisted of submerging micromalt cans in 15°C water for the periods described in table 2.4. At the end of steeping, cans were removed from the steep bath and placed onto the rollers of the micromalter to drain (air rest mode, see table 2.4).

The timing of steeping was staggered so that the length of air rest for day 10 grain was 240 h, day 7 grain, 168 h and day 4 grain, 96 h. During the long air rest, the lid of the micromalter was sealed with insulation tape to maintain humidity but this did not prevent carbon dioxide escape.

Enzyme and immunological activity were measured on the malt samples as described previously (chapter 5, section 5.2.1). Malt samples (10 g) were mashed and the fermentations performed. Up to 20 samples could be processed for extract and fermentability determinations each day. The selection of samples for analysis was randomized over the 8 different cultivars, three replicates and three different malting times. Included in each assay were duplicate samples of batch control malt (cv. Clipper).

## 8.3 RESULTS AND DISCUSSION

### 8.3.1 Relationship between Limit Dextrinase Activity and the Apparent Attenuation Limit in Genetically Diverse Cultivars

Extract (percent) and the AAL varied significantly between cultivars (Fig. 8.1 and 8.2). However, in this study these are quite low compared to a control malt processed in the same batch. The total protein content of the barley harvested from this field site was high due to environmental conditions at the time of growing. High grain protein has long been known to be associated with poor extract (Bishop and Day, 1933). A discussion of the variation in LD activity has been given previously (see 5.3.1). The percent extract varied between 72 % and 77 % and the AAL between 68 % and 76 %. There was no correlation between AAL and % extract and so % extract was excluded from the analysis of variance (ANOVA).

Limit dextrinase activity (measured using the ELISA or RP assay, see Appendix 8.1) varied significantly between cultivars and to determine if this contributed to the variation in AAL an ANOVA was performed. The ANOVA showed the total variation in AAL due to enzyme activity (10 %) was significant but AAL was not related to immunological activity (Appendix 8.3; model 8.3.1 and 8.3.2). The distribution of enzyme activity (RP) was skewed toward lower levels (see chapter 5, section 5.3.1). To 'normalise' the data a $\log_{10}$ transformation was applied to the data prior to statistical analysis. Most of the variation in AAL is due to cultivar differences, (this explained 80 % of the variation in AAL) and when this was included in the ANOVA, RP activity was not significant (see Appendix 8.3; model 8.3.3). Therefore malt LD activity was not related to the AAL.

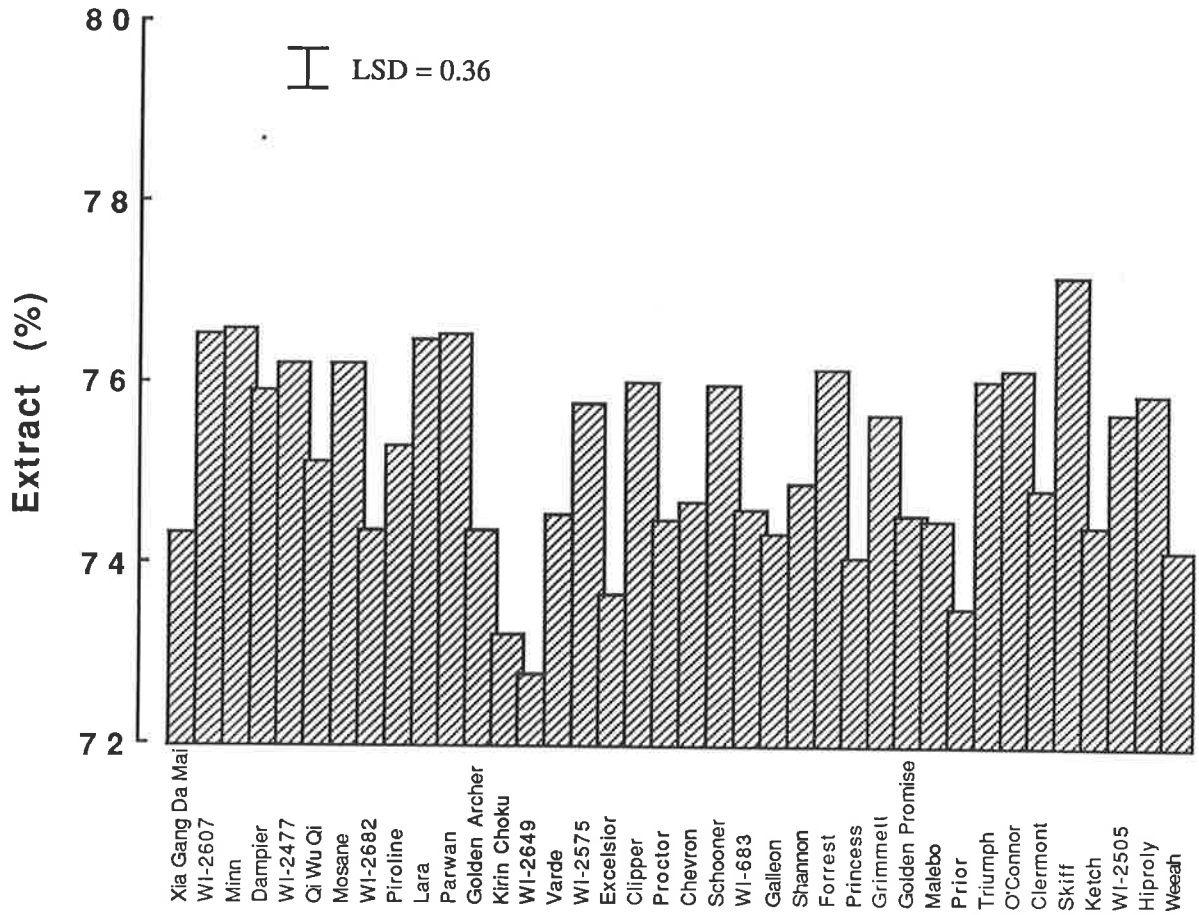



**Figure 8.1**

Variation in mean percent extract between 38 barley cultivars of genetically diverse origin. Each cultivar was micromalted on three separate occasions (three replicates) and each replicate malt sample mashed on different days.

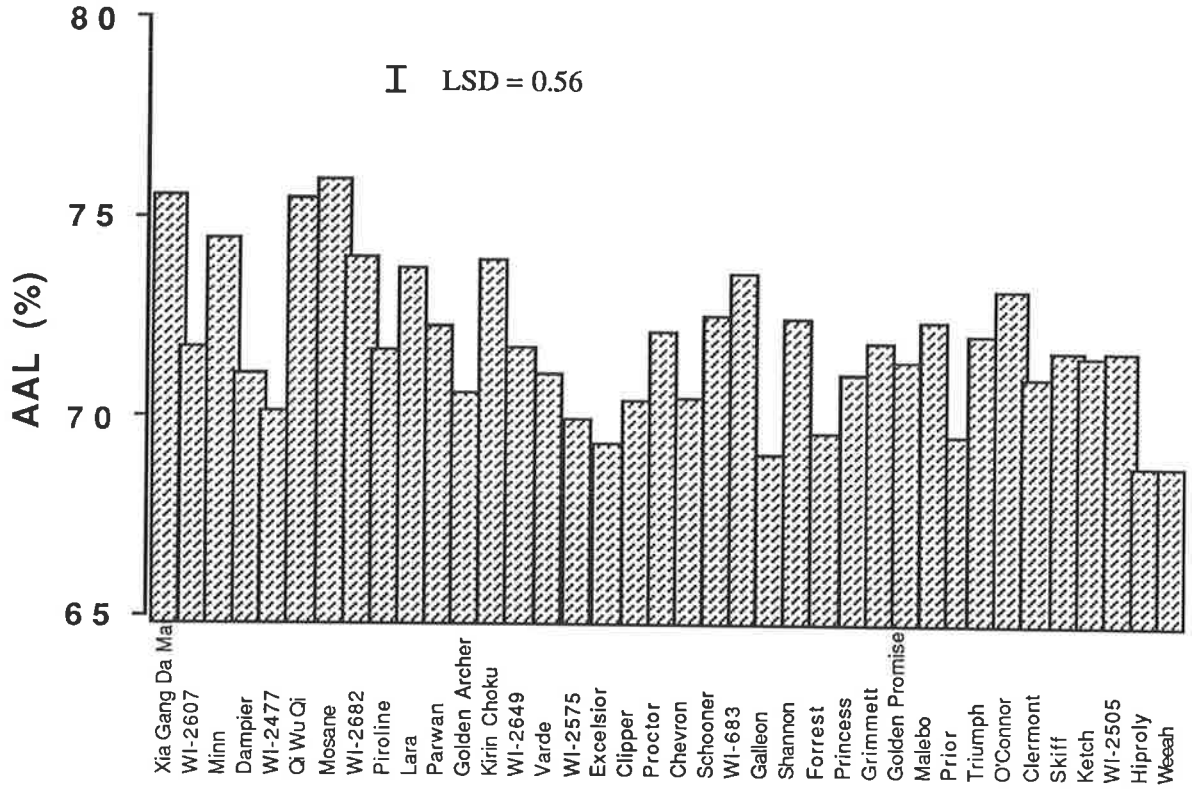



**Figure 8.2**

Variation in mean AAL between 38 barley cultivars of genetically diverse origin. Each cultivar was micromalted on three separate occasions (three replicates). Each replicate malt sample was mashed and the wort fermented on different days.

### 8.3.2 Relationship Between Limit Dextrinase and Apparent Attenuation in Selected Cultivars Malted for 4, 7 and 10 Days

The AAL of the wort depends upon many factors that were discussed in 1.6.4. Diastase levels would have a major influence upon AAL in terms of controlling the supply of fermentable sugars. The components of diastase, *viz.* α-amylase, β-amylase and LD would be the most important enzymes controlling breakdown of starch and a shortage of any of these enzymes could potentially limit AAL. In the case of LD, the presence of large quantities of dextrins in beer indicates that either LD activity is too low or the conditions of mashing prevent what enzyme there is from degrading α-1,6-bonds sufficiently. Evidence from studies where either pullulanase (Enevoldsen and Schmidt, 1973; Willox *et al.*, 1977) or malt flour with a high LD activity (Pratt *et al.*, 1981; Lee and Pyler, 1984) is added to wort, shows that AAL and alcohol production can be increased. It is possible that cultivars with elevated LD activity (for example by extending malting), may also increase AAL. The poor relationship found between AAL and malt LD activity in the previous study (Appendix 8.3; model 8.3.3) may indicate that the range of LD levels was neither too low or too high to effect AAL. Limit dextrinase may alter AAL when either LD levels are very low, in which case they would be expected to be rate limiting, or when high levels are obtained so that more dextrins can be degraded. The aim of this experiment was to investigate the relationship between malt LD activity and the AAL of worts prepared from malts germinated for more than four days.

The LD immunological and enzyme activities (Table 8.1) varied significantly between cultivars (Appendix 8.4; model 8.4.1 and 8.4.2) and were higher after 7 compared to 4 days of malting. There was no significant difference between 7 and 10 day malts. A multiple comparison table for the ELISA and RP least square means is shown in Appendix 8.5. There were very large differences in enzyme activity between cultivars e.g. Hiproly cf. Minn, but not immunological activity. This difference was found in the studies described in chapter 5, section 5.3.1). Also, the classification of significant differences in LD between cultivars (Appendix 8.5) do not match in all cases reflecting the poor relation between these two assays.

There was no statistically significant change in the percent extract between 4, 7 and 10 day malts. There was a significant relationship between AAL and enzyme (but not immunological ) activity without consideration of other factors affecting AAL (Appendix 8.4; models, 8.4.3 and 8.4.4). A full ANOVA however, indicates that cultivar, length of malting, and their interaction (Fig. 8.3), enzyme activity and % extract included as covariates were significantly related to AAL (Appendix 8.4; model, 8.4.5). The AAL increased significantly with longer malting and there were large differences between cultivars (Table 8.2).

**Table 8.1**

Changes in the LD immunological and enzyme activity in the eight cultivars malted for either 4, 7 or 10 days. Data are the mean activity of three replicate malts (see Materials and Methods).

| Cultivar | Length of malting (days) 4 | | 7 | | 10 | |
|---|---|---|---|---|---|---|
| | ELISA | $log_{10}$RP | ELISA | $log_{10}$RP | ELISA | $log_{10}$RP |
| Hiproly | 43.7 | 1.70 | 54.6 | 1.78 | 54.8 | 1.87 |
| Xia Gang Da Mai | 38.5 | 1.79 | 47.6 | 1.96 | 46.3 | 1.90 |
| Schooner | 42.9 | 2.74 | 44.9 | 2.96 | 60.3 | 3.01 |
| Minn | 39.0 | 2.79 | 44.7 | 3.09 | 46.9 | 3.01 |
| Galleon | 23.5 | 1.66 | 25.9 | 2.12 | 32.4 | 1.89 |
| Ui Wu Qi | 23.4 | 1.65 | 30.3 | 2.05 | 29.7 | 1.94 |
| Lara | 35.5 | 2.69 | 41.2 | 2.80 | 52.5 | 3.01 |
| Skiff | 31.8 | 2.68 | 37.1 | 2.77 | 41.0 | 2.90 |
| **LSD** | **5.4** | **0.14** | | | | |

**Table 8.2**

Least square means (model 8.4.5, Appendix 8.4) for AAL of 8 cultivars malted for 4, 7 and 10 days.

| Cultivar | Apparent attenuation limit (%) | |
|---|---|---|
| | Least squares mean | Standard error |
| Xia Gang Da Mai | 78.42 | 0.46 |
| Qi Wu Qi | 81.51 | 0.63 |
| Galleon | 75.47 | 0.43 |
| Hiproly | 73.91 | 0.49 |
| Lara | 78.26 | 0.42 |
| Minnesota | 79.19 | 0.50 |
| Schooner | 77.53 | 0.46 |
| Skiff | 77.78 | 0.41 |
| LSD | 0.36 | |

| Length of malting (days) | Apparent attenuation limit (%) | |
|---|---|---|
| | Least squares mean | Standard error |
| 4 | 76.59 | 0.25 |
| 7 | 78.11 | 0.14 |
| 10 | 78.58 | 0.16 |
| LSD | 0.36 | |

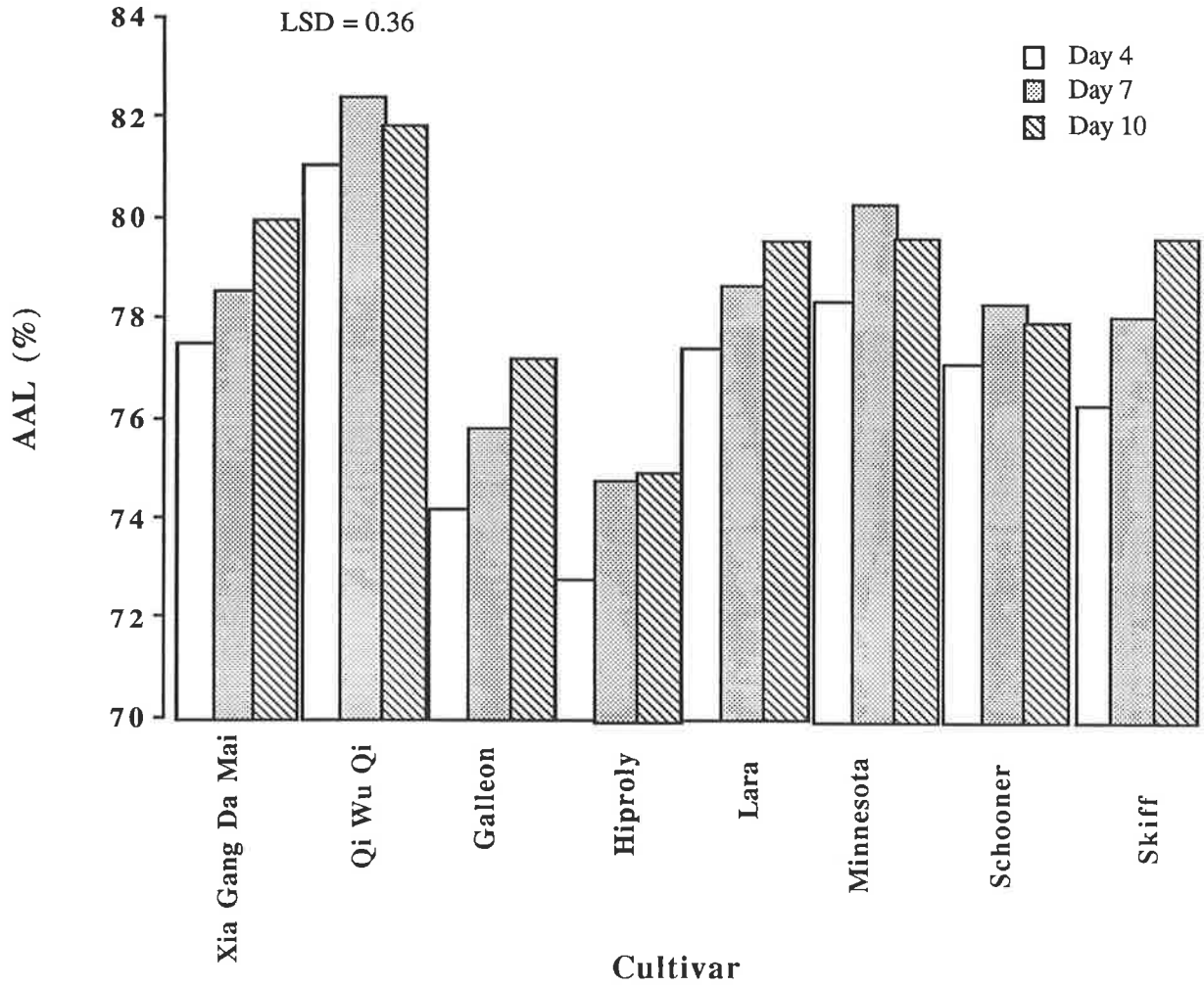



**Figure 8.3**

Effect on AAL of the interaction between cultivar and the length of malting. Data are the least square means adjusted for the contribution by extract and LD activity.

In those cultivars with a low enzyme (RP assay) activity (day 4 malts) there is a wide variation in AAL. This may be related to the poor reproducibility of this assay in samples with low activity (Henry and Butler, 1992). If all the day 4 data were removed from the analysis and a new ANOVA conducted, there is a very significant relationship between AAL and RP activity (Appendix 8.4; model 8.4.6). However, the ELISA is not significant under these circumstances.

Increasing the activity of the malt LD by extending the malting time was associated with higher AAL in worts prepared from these malts. It is not possible however, to say whether the higher LD activity in the 7 and 10 day malts was the only possible cause for the increase in AAL observed. Extending the malting time is likely to improve the degree of modification, i.e. protein and cell wall degradation, for example, Loi *et al.* (1987) suggested extended malting would allow the development of maximum 1,3;1,4 β-glucanase activity, which could degrade all the 1,3;1,4 β-glucan and increase diastase both of which could contribute to higher AAL. If higher LD activities and quantities (immunological level was increased with extended malting) were partly responsible for increased AAL, it is conceivable LD could do this by reducing the number of α-1,6 linkages during mashing, therefore increasing the quantity of fermentable sugars and hence AAL.

The measurement of AAL using the microbiological method introduces many variables that can influence the final result. These might include: (1) yeast type (Lager yeast, NCYC 1324, which can metabolise maltotriose), viability and sample size (2) the ratio of wort to yeast and the conditions of fermentation (temperature, length of fermentation and how rapidly the flask is shaken) (3) the diastase and % extract of the malts (4) the conditions of malting and mashing which influence the synthesis and degradation of the starch hydrolytic enzymes responsible for producing fermentable sugars.

Since pullulanase has been shown to alter the dextrin profile of wort and beer in a dramatic way (Enevoldsen and Schmidt, 1973), a more direct indicator of LD action than the AAL is to measure the dextrin profile in the wort and beer. Enevoldsen and Schmidt (1973) found in 22 beers that about half of the dextrins are oligosaccharides with a degree of polymerization (DP) below 10, either linear or singly-branched. The other half have a DP of 10 or more and are multiply branched. These dextrins exhibited a characteristic wavy distribution but in two of the beers this distribution was quite different, indicating somewhat different conditions for brewing these beers. The effect of LD was clearly illustrated when these workers added pullulanase to a mash sample and found the dextrin profile showed a complete absence of branched dextrins and an increase in the quantity of linear dextrins and fermentable sugars (glucose, maltose and maltotriose). The relationship between the malt LD activity and the wort dextrin profiles requires further study. With the availability of HPLC columns suited to the rapid separation of DP1 to DP12 dextrins, analysis of these profiles is much faster than the

methods used by Enevoldsen. It would first be necessary to define the relationship between AAL and the dextrin profile. In worts with widely different AAL's, how much variation in the dextrin profile exists and can this be related to the LD activity? The manipulation of the dextrin profile in the wort by controlled action of LD, may result in new types of beers with different mouthfeel and aftertaste characteristics. The dextrins present in the final beers (80 % of the total beer carbohydrates) have been shown to influence the mouthfeel (Ragot *et al.*, 1989) and the aftertaste of the beer (Brefort *et al.*, 1989).

During an EBC mash, LD activity is lost rapidly after a temperature of 65°C is exceeded (see chapter 7) and therefore LD may have little influence upon the dextrin profile. Enevoldsen and Schmidt (1973) found that during mashing there are very few changes in the dextrin pattern and virtually the same distribution is found in the wort and beer. There were small increases in the DP 5-10 dextrins and a decrease in the DP 11 and higher dextrins during mashing. These changes are thought to be due to the action of β-amylase and LD during mashing. This implies that LD action during mashing is very limited. Also, Willox *et al.* (1977) found that the concentration of maltotriose in the beer increased by 216 % when pullulanase was added to the wort before fermentation but only increased 6 % when added at the beginning of mashing. This suggests that most of the added pullulanase was unable to bring about a significant reduction in the dextrins, presumably due to thermal denaturation during mashing. The conditions of malting, kilning and mashing used in distilling, produces enough LD in malt to degrade almost all of the branched dextrins to fermentable sugars (Bathgate *et al.*, 1978). The malt used for distilling may be kilned over peat to impart the smoky flavours characteristic of whisky and during kilning the malt does not encounter temperatures above 70°C. These malts have much higher LD activity than brewery malts (Pratt *et al.*, 1981). Also, a distiller mashes at 60-65°C for 6-8 h and the wort is not boiled (Palmer, 1989), to ensure a high yield of fermentable sugar and maximum alcohol production. The results in chapter 7 shows that LD is quite stable at these temperatures. This suggests that the mashing schedule used (EBC) in the two studies presented in this chapter is unsuitable and rapid inactivation of LD may occur before the enzyme has been able to complete its action. However, in order to be certain, it would be necessary to add purified barley LD to the mash and measure the AAL, alcohol production and the dextrin profile of the fermented wort. If the quantities of purified enzyme added are adjusted to match the levels in malt and it is found there is a substantial loss in activity, then the mashing program would need to be modified to enhance the survival of the enzyme. Instead of increasing the temperature continuously from 46°C to 70°C (see chapter 2, table 2.5) a 'rest' period at 63°C where LD is stable and the saccharification temperature is reached, may improve the action of LD. Willox *et al.* (1977) showed that the AAL could be increased by modifying the mashing procedure.

The main factor explaining the variation in AAL in the studies presented here are 'cultivar' effects. Differences in endosperm structure, amylose/amylopectin ratio, the supply of

enzymes (starch, protein and cell wall degrading enzymes) are likely to have an influence upon AAL. In addition, the free amino nitrogen (FAN) content of the wort can have a major influence upon AAL. If there is insufficient FAN for yeast metabolism, fermentation rate is slower (Enari, 1975). The barley cultivar (nitrogen content, and peptidase activity) is a major determinant of the FAN level of the wort. It is likely that with conventional decoction or especially infusion mashing schedules, cultivars with varying LD activity probably do not influence the AAL of the fermented wort.

In conclusion, there was a poor relationship between AAL and LD in four day malts, but with extended malting, enzyme but not immunological activity was significantly related to AAL. The association of high malt LD activity with higher AAL does not mean that the LD was responsible for the increase of the latter since other factors (length of malting, cultivar etc.) were also related.

# CHAPTER 9

## GENERAL DISCUSSION

The successful purification of barley malt LD depended upon the affinity of the cyclohexaamylose ligand used in the final purification step. It was found that a significant amount of LD did not bind to the affinity column. If the partially purified LD applied to the affinity column consisted of a mixture of aggregated ('bound') and monomeric enzyme, the former may have a different binding constant and not bind under the conditions in which monomeric LD binds. The use of reducing agents in the preparation of the crude extract should release into the extract, monomeric and aggregated forms of LD. Maeda *et al.* (1978) using the same ligand, found the majority of the enzyme loaded onto the column bound to the matrix. In their study, purified LD was prepared from a NaCl extract which may not have released the aggregated form of LD. It would be useful to have information on the inhibitor constant of cyclohexaamylose or cycloheptaamylose or both for the bound and unbound fractions. The binding constant of the ligand when purified LD treated with or without cysteine or purified malt endopeptidase, would indicate if the latter agents alter LD binding.

In a very real sense the study of LD in many areas has been limited by the lack of a suitable sensitive and specific assay. To help overcome these problems, immunochemical assays were developed using a specific polyclonal antibody against LD. The utility of the immunochemical assays developed depends upon the specificity of the antiserum. A western blot of malt extract showed the antibody recognised two proteins; a protein corresponding to LD (104 kD), and a minor band (ca. 100 kD). The minor band was just detectable in a silver stained SDS polyacrylamide gel of the purified protein and may either be a breakdown product of LD or a contaminant. Limit dextrinase was purified from malt and it is possible that during malting, endopeptidases clipped a small portion of LD during release from the 'bound' form which might explain the minor band. Such a mechanism is responsible for the release of 'bound' β-amylase by endopeptidases. The size of the β-amylase decreases by about 5 kD (Sopanen and Laurière, 1989). The purification and characterization and sequencing of the minor (100 kD) protein band is required to determine which of these possibilities is true. Alternatively, the enzyme may have been partly degraded during the preparation of the enzyme. If the enzyme was clipped during malting, different isoenzymes would be expected, however there was no change in the isoenzyme pattern during germination. A probable explanation for this discrepancy is that the minor band is present in very small quantities and the dilution of antiserum used may be too high to detect a small change in the isoenzyme pattern. Given sufficient dilution of the sample, the minor band becomes insignificant in immunoblots and therefore the antibody should be functionally monospecific for LD.

Maltsters and brewers are generally concerned with enzyme activity in kiln-dried malt which is important in determining potential malt extract. During malting and brewing, many enzymes lose enzyme activity due to thermal denaturation, therefore it is important to establish if antibody activity is lost at a similar rate to enzyme activity. There was a good correlation between the reduction in immunological and enzyme activity of LD in extracts of malt heated at 60°C. Presumably, inactivation occurs as a result of severe conformational changes in the protein structure which destroys epitope sites and reduces enzyme activity. MacLeod *et al.* (1987) also obtained a good correlation between the reduction in α-amylase II immunological and enzyme activity by using high kilning temperatures. These studies indicate that denatured, inactive LD enzyme is not detected by the specific antiserum. However, studies on the inhibition of LD by copper, suggests the ELISA may detect inactive protein and does not bind to LD at only the active site. This type of inhibition is unlikely to affect the structural integrity of LD which would explain the persistence of antibody binding. The action of copper is not known but probably interferes with binding at the active site or a substrate binding site. This result may not be a major problem since the reduction in LD activity during malting and mashing is largely caused by thermal denaturation.

It is important that an ELISA is standardized and there is acceptable variation between assays performed on different days. A drift was detected in the LD ELISA which means that it is not appropriate to compare ELISA readings between studies performed where this drift occurred. In order to avoid this problem, the inclusion of one or more quality control flour samples in every assay would allow control over drift and 'bad' assays. A plot of the mean of the control sample would permit the pin-pointing of drifts in the assay. Drift could be caused by a fall in activity of the HRP conjugate, decreased binding of the antibodies (primary and secondary), denaturation or loss of immunological activity during storage of the purified LD standard used to coat the microtitre plate, or changes in microplate batch and manufacturer which could reduce the binding capability of the plastic. Corrections to dilutions of antibodies or preparing fresh conjugate would solve this problem. Such control measures are essential for long term use of the ELISA.

The RP assay used throughout the studies presented here has recently been shown to have poor reproducibility at low enzyme concentrations (McCleary, 1991; Henry and Butler, 1992). These findings are not inconsistent with those observed by the studies described in this thesis. An improved substrate (Azurine-CL-Pullulan) has now become available (McCleary, 1991). This substrate is claimed to be 10-15 times more sensitive and has better reproducibility than the Red Pullulan assay but the reaction curve was not linear over the absorbance range 0-2 and the values obtained were 1.5 times greater than those observed with the Red Pullulan substrate.

The availability of monospecific polyclonal antibodies and enzyme conjugates to α-amylase II, (MacLeod *et al.*, 1987) a malt endopeptidase (Phillips and Wallace, 1989), LD and monoclonal antibodies to endo β-1,3;1,4-glucanase isoenzymes I and II (Høj *et al.*,

1990) might allow determination of the activity of these enzymes from a single flour extract using one microtitre plate and coating appropriate wells with each specific antibody. These enzymes are important to malt quality, and such a test would permit a more rapid and specific estimation of these enzymes in a flour sample than with conventional enzyme assays.

The independence of the antigenicity and the activity of an enzyme is useful in fundamental studies of an enzyme (Daussant and Bureau, 1988). Some of the uses of the anti-LD antiserum exploited in this thesis were: (1) detecting the antigenic enzyme without regard to activity. (2) measurement of LD during grain filling using the ELISA where enzyme activity was not detectable either because the antigenic protein has no activity or the enzyme assay (RP) does not have the required sensitivity. (3) the detection of isoenzymes using immunoblotting where conventional zymogram methods for LD have poor resolution and sensitivity. For example immunoblotting was used for identifying the structural gene for the α-amylase inhibitor on rye chromosome 2R (Hill *et al.*, 1986) for which a zymogram technique was not possible. The IEF-immunoblotting procedure developed for LD was used to investigate genetic variability and chromosome location of the structural gene for LD.

The monospecific antiserum recognises several proteins in IEF-immunoblots of extracts of ungerminated, germinated barley and malt which are not seen in a silver stained IEF gel of the purified protein. This may be due to a difference in the sensitivity of the detection methods. It is possible that not all the IEF forms were purified but the polyclonal antibodies raised against the purified forms are likely to be cross reactive to other forms detected in crude extracts. The pullulan assay used for detecting LD hydrolysing activity during the purification may not have had the resolution and sensitivity required to isolate all forms of LD, and use of the ELISA may improve this.

The question of whether each band corresponds to a single antigen with different physiochemical properties or whether some of them correspond to the presence of parasite antibodies specific for other antigens are possibilities. The evidence from crossed immuno-isoelectricfocusing shows one single heavy precipitin band covering the whole area of the multiple bands separated by IEF indicating polymorphism of LD (Fig. 4.17). The origin of these bands whether due to physiochemical reasons, post-translational modifications of the main constituent (proteolytic conversion giving rise to charge differences, differences in glycosylation) or genetic expression or complex loci i.e. several copies of gene with minor modification, requires further study. Evidence from studies with the nullisomic-tetrasomic lines from wheat suggests some of the bands from wheat are under genetic control. The purification of the bands, their amino acid composition and substrate specificities would allow their identification as isoenzymes. Improved separation is required so the bands can be isolated, for example using immobiline IEF gels, chromatofocusing over a narrow pH range or HPLC using an ion-exchange resin may facilitate their separation. A similar approach was used to study four major isoforms of barley β-amylase. It was suggested

from knowledge of the agreement between protein and DNA sequence analysis for β-amylase that the isolated multiple forms were products of a single gene (Lundgard and Svensson, 1987).

A significant variation in both enzyme and immunological activity was found between cultivars of barley suggesting that differences exist in the ability of cultivars to produce LD. Given this variation, the screening of breeders lines for higher enzyme or immunological activity may improve quality. In contrast, there was much less variation in the IEF-immunoblotting patterns. However, only a relatively few lines (131) were tested and it is possible that if many more lines were screened (perhaps thousands), a variant with for example, superior levels of activity, thermal stability or other attribute, which are likely to be rare, may be detected. The ELISA format is well suited to early generation screening because only a small quantity of flour is required and rapid measurements of LD levels in unpurified extracts of malt is possible. However, the substitution of an immunoassay for an enzyme assay to measure enzyme activity must ensure the immunoassay detects active enzyme under the conditions the grain is exposed to during malting and brewing.

Limit dextrinase can be detected early in the endosperm and embryo/scutellum of the developing barley kernel. Extraction of flour samples taken at various stages during grain development in acetate buffer with or without cysteine reveals two forms of activity. One form (extracted without cysteine) increases in amount up to 21 days post-anthesis and then decreases, and the other form (extracted with cysteine) increases after 21 days and then decreases slightly until maturity. Using the analogy described in rice (Yamada, 1981b), the latter form could be LD bound to insoluble constituents which are released *in vitro* by thiol reducing agents or *in vivo* by endopeptidase (Guerin *et al.*, 1991; Sopanen and Laurière, 1989). The presence of LD in the developing kernel raises a number of questions: (1) Is the enzyme synthesized and for what purpose *in vivo* ? If the enzyme has activity it may, like α-amylase I, play a role in starch hydrolysis which occurs in immature barley endosperm (MacGregor and Dushnicky, 1989a, b). The purification of LD from developing grain would allow its action on starch and oligosaccharides to be tested to see if there was a potential role in starch hydrolysis in the immature kernel but this would not show the *in vivo* role. (2) What is the mechanism that controls its appearance i.e. hormonal control? (3) Is this enzyme different, in terms of physiochemical properties, from the LD found in germinated grain?

A model proposed to explain the presence of 'bound' and 'free' forms of LD as defined in this thesis (see 7.4.1.1) is presented. The following conclusions are suggested from the studies on 'bound' and 'free' forms:

(1) There is a much greater immunological and enzyme activity in the supernatant fraction of acetate extracts from germinated seed. This is most likely due to *de novo* synthesis and not release of LD from a 'bound' form. If all the LD in ungerminated seed was in the 'bound' form, and was released during germination, the amount actually detected would be less than that found in germinated seed. The parallel increase in enzyme and

immunological activity during malting and germination (see chapter 7) supports the view of *de novo* synthesis. Additional, more direct evidence was shown by Hardie (1975). However, the proportion of LD protein detected by the ELISA after one day of malting compared to four days, was much greater than the corresponding enzyme activity. Also, LD protein is present in mature seed but has no apparent enzyme activity. These suggest the presence of protein with no enzyme activity during early malting could be due to the presence of an inhibitor of LD to which it is bound. This could be the putative Y form, which may have little activity itself, and its presence may result in the slow release of the active enzyme during germination. Perhaps the selection of cultivars with reduced Y could increase LD and/or the rapidity of its appearance in a malt.

(2) The LD immunological activity in the supernatant fraction of acetate extracts ('free' form) of mature seed exceeds that in the pellet ('bound' form). Whereas in developing kernels there was more 'bound' LD. The samples used in the studies described in 7.3.5 are different to those analysed in chapter 6, for example, different cultivars were used (Schooner and Skiff) and the seed used for the 'bound'/'free' studies had been stored for over a year after harvest compared to the freshly harvested samples of Skiff assayed in chapter 6. Changes in the proportion of these two forms may have occurred which may explain some of these differences.

(3) Incubation of the supernatant fraction of acetate extracts of ungerminated and germinated seed (GS/UGS) with thiol reducing agents and papain had no effect upon immunological activity but increased enzyme activity (GS only). Possible reasons for this are (i) activation of the monomeric form. This could be tested by attempting to activate purified enzyme. (ii) release of LD from a 'bound' form (LD-Y) which exists in the supernatant. If the 'bound' form was antigenically identical to the monomeric LD this would explain why there was no change in the immunological level.

The studies on the 'bound' and 'free' forms of LD presented in this thesis raise several questions. (1) Does the mechanism of release of 'bound' LD *in vivo* involve endopeptidases as found for β-amylase (Guerin *et al.*, 1991; Sopanen and Laurière, 1989)? Evidence for this would be a clipped e.g. 100 kD LD appearing during germination and *in vitro* in response to the relevant purified endopeptidase. (2) Do the flour extracts prepared with acetate buffer contain other forms beside monomeric LD? The simplest way to test this would be to subject an acetate extract to analytical gel filtration and using the ELISA to identify the heterodimer if it exists, then to split the heterodimer with reducing agents and re-run through the column (Hejgaard and Carlsen, 1977). (3) What concentration of thiol reducing agent is required *in vitro* to completely release LD from the 'bound' form of pellet and supernatant fractions? (5) What is the nature of the putative substance Y, LD is hypothesized to be bound to? If the heterodimer complex described in (2) above is identified, using a high concentration of a thiol reducing agent to break up the complex would separate compound Y, which could then be isolated and characterized.

Maximum limit dextrinase activity is not achieved by 4-5 days malting or germination.

Malting for 7-10 days, increased LD activity and AAL. A multivariate analysis of the causes of the increased AAL shows higher LD activity (RP assay) was contributing. Extended malting also increases diastase (Pratt *et al.*, 1981) and probably improves modification. These factors may have contributed more to the increase in AAL than LD. There is good evidence that exogenous LD can alter AAL. The addition of pullulanase to wort before fermenting increases alcohol production and decreases the quantity of dextrins in the beer (Enevoldsen, 1975). This is direct evidence that LD can alter AAL. Control over the quantity of LD and its action may have potential to manipulate the oligosaccharide profile to obtain a particular type of beer without undesirable utilization of exogenous enzymes. The level of LD activity required in malt to degrade the majority of the α-1,6 linkages is not known. It is either necessary to extend the malting time to allow for maximum synthesis of LD or to alter the conditions of kilning and mashing to minimise the rate of LD inactivation so that the available enzyme is effective. Bathgate *et al* (1978) found in distillers malt (lightly kilned malt and lower temperatures used during mashing) that there was sufficient LD to degrade the majority of α-1,6 linkages to fermentable sugars which suggests that conditions of malting and mashing determine the effectiveness of LD. More studies are required to define the optimum conditions of malting and mashing producing maximum effect of LD.

## Future Research

(1) Purification and characterization LD isoenzymes in terms of substrate specificity, amino acid sequence, and their origin. Development of monoclonal antibodies specific for each isoenzyme.

(2) Structural information on LD is required to understand the action of thiol compounds and endopeptidases on enzyme activity and release of 'bound' forms. The role thiol compounds play in determining the expression of isoenzymes of LD needs further study.

(3) The purified enzyme should be sequenced and used to screen cDNA libraries to obtain a cDNA clone. The DNA sequence of the cDNA and the number of gene copies should be determined to assist in studies on the regulation of the *de novo* synthesis of LD by gibberellic acid. Other possible applications of this technology include: (i) engineer the protein to have improved heat stability once structural information is available and (ii) transformation of barley to synthesize LD more rapidly.

(4) Antibodies specific for a protein provide a unique means of localizing an enzyme at the tissue, cellular and subcellular levels where the activity of the enzyme is too weak to be detected by other methods. These studies would be useful to locate the tissue where LD synthesis occurs. The localization of LD in seeds is important for understanding its function during grain development, maturation and germination. Immunohistochemistry techniques have been used to determine the location of α-amylase (Gibbons, 1979, 1981)

and β-amylase (Laurière *et al.*, 1986). Utilizing these techniques to determine the exact cellular distribution of LD would be invaluable. In addition, the isolation of scutellum and aleurone tissue and using immunochemistry (see chapter 1, 1.4.1) to determine in which tissue *de novo* synthesis is occurring would be invaluable.

(5) Identify the chromosome location of the LD structural gene. Alternative approaches to that described in this thesis are (i) using restriction fragment length polymorphisms (RFLP), assuming polymorphism exists . Once the cDNA encoding LD is available, restriction endonuclease digestions of DNA from Chinese Spring, Betzes and the wheat-barley chromosome addition lines could be prepared and the products probed by Southern blot analysis using a labelled LD cDNA. This approach was used to identify the barley chromosome carrying the 1,3;1,4-β-glucanase genes (Loi *et al.*, 1988). (ii) A simpler and cheaper approach is the Polymerase Chain Reaction (PCR). In this system, polymorphisms are sought in the distance between two short target sequences rather than the presence or absence of restriction endonuclease sites as is the case for standard RFLPs. This technique has been used to identify polymorphism for wheat and barley α-amylase and to identify the chromosome carrying the gene for α-amylase (Weining and Langridge, 1991). A major limitation of the PCR approach is the need for extensive sequence information in order to synthesize the appropriate primers. (iii) Identification of a linkage between a variant LD IEF-immunoblotting pattern and known isoenzyme or morphological loci. Using the IEF-immunoblotting procedure three variant patterns in Chinese cultivars (see 5.3.2.1) and in several lines of *Hordeum spontaneum* have been observed. This gives a marker for allelic variation assuming the band(s) are controlled by a structural gene i.e. a genetic marker. Crossing any one of these showing the variant pattern with say Clipper, the $F_1$, $F_2$ and backcrosses with Clipper, should demonstrate simple Mendelian inheritance. By choosing marker loci known to be present on chromosome 1 (the suspected site of the LD structural gene), it may be possible to show a linkage, which would allow the gene to be mapped. Direct evidence that the locus *Glb* 1 for 1,3;1,4-β-D-glucan-4-glucanohydrolase enzyme E1 is located on barley chromosome 5 was provided by studies using the IEF-immunoblotting procedure to identifying a linkage to the malate dehydrogenase locus (*Mdh* 1) which occurs on the long arm of this chromosome (MacLeod *et al.*, 1991).

(6) A more detailed study of the conditions used for malting and brewing to maximize the effectiveness of LD to produce worts with a desired composition of non-fermentable dextrins is required. Malts germinated for different length of time should be assessed in terms of their α-amylase, β-amylase, endopeptidase, LD and glucanase activities. Dextrin profiles of the worts and beers should be examined using HPLC techniques.

The investigations described in this thesis have uncovered several future lines of research. More studies are required on the 'free' and 'bound' forms, their presence, importance and mechanism of release *in vivo* . The verification of *de novo* synthesis and the cellular location using immunochemical methods would now be possible. The availability of antibodies opens up possibilities for DNA technologies to be applied to the

study of LD. It is important to establish more precisely what role LD plays in malting quality and whether specialized beers could be produced by manipulating the activity and/or heat stability of this enzyme. Such information would be invaluable to the malting and brewing industry.

## APPENDIX 5.1

| Cultivar | Cultivar | Cultivar | Cultivar | Cultivar |
|---|---|---|---|---|
| Calif. Mariout | Cambrinus | Lami *ant* 13 | Proctor | Minerva |
| CI-5611 | WI-2472 | Manapou | Maythorpe | WI-2585 |
| Nirasaki nijo | Minn 73-75 | Miho Golden | Weeah | Hiproly |
| Candellilla | WI-2610 | Lenta | Maraini | Kaniere |
| Gerbel | WI-2680 | O/Prophete | Chevron | WI-2597 |
| WI-2664 | Velvon | La Mesita | Schooner | Izmir 9 |
| Atlas | Dampier | Kirstina | WI-683 | O'Connor |
| Drake | WI-2477 | WI-2643 | OMartin | WI-2621 |
| Maris Mink | Minn 76-14 | Harry | Galleon | |
| Tequila | WI-2611 | Orange Lemma | Arivat | |
| Minerva | WI-2681 | Cytris | Athenais | |
| WI-2665 | Bonus | Magnum | Shannon | |
| Atlas 46 | Diamant | Rupal *ant* 13 | WI-768 | |
| WI-948 | Mazurka | EBYT 23 | Betzes | |
| Akka | Mosane | Gimpel | Forrest | |
| WI-2509 | WI-2612 | Compana | Princess | |
| WI-2606 | WI-2682 | Kirin Choku | Psaknon | |
| WI-2677 | Piroline | Gold Marker | Bandulla | |
| Maja | Conquest | WI-2582 | WI-958 | |
| Noyep | Puebla | WI-2646 | I.Dwarf | |
| Nudinka | Karl | Vada | Grimmett | |
| Menuet | Beka | WI-775 | B.Korai | |
| WI-2607 | Azuma Golden | Fuji Nijo | G/Promise | |
| WI-2678 | Vaughn | Gold Spear | Malebo | |
| Rigel | Lara | WI-2583 | Prior | |
| Resibee | Manker | WI-2649 | WI-2539 | |
| Midas | Parwan | Varde | Stirling | |
| Pirouette | WI-2700 | | Ketch | |
| WI-2608 | G/Archer | Excelsior | Triumph | |
| WI-2679 | WI-2231 | Bolivia | Clermont | |
| Ymer | Estanzuela | Clipper | Skiff | |

Cultivars selected for a study of the isoenzyme variation of LD (see 5.2.1.1).

## APPENDIX 5.2

| Cultivar | LD activity (µg/ml) | Protein (mg/ml) | Specific activity (µg/mg) |
|---|---|---|---|
| Orange Lemma | 12.2 | 2.87 | 4.3 |
| Cytris | 8.8 | 3.13 | 2.8 |
| Magnum | 9.6 | 2.91 | 3.3 |
| Rupal *ant* 13 | 12.4 | 2.17 | 5.7 |
| 3 EBYT 23 | 8.6 | 2.56 | 3.4 |
| Gimpel | 9 | 1.23 | 7.3 |
| Kirin Choku | 34.8 | | |
| Gold Marker | 15.6 | 1.76 | 8.9 |
| WI-2582 | 11 | 2.23 | 4.9 |
| WI-2646 | 8.6 | 2.66 | 3.2 |
| Vada | 8.2 | 3.03 | 2.7 |
| WI-775 | 9.9 | 2.45 | 4.0 |
| Fuji Nijo | 11.8 | 1.42 | 8.3 |
| Gold Spear | 7.5 | 2.06 | 5.44 |
| WI-2583 | 11.2 | 3.24 | 3.5 |
| WI-2649 | 23.4 | 3.22 | 7.3 |
| Varde | 25.8 | 1.4 | 18.4 |
| Excelsior | 5.9 | 2.14 | 3.8 |
| Bolivia | 9.5 | 2.93 | 6.6 |
| Clipper | 11.7 | 2.81 | 4.2 |
| Proctor | 11.2 | 3.04 | 3.7 |
| Maythorpe | 11.7 | 3.51 | 3.3 |
| Weeah | 10.4 | 3.6 | 2.9 |
| Chevron | 32.7 | 3.77 | 8.7 |
| Schooner | 15.1 | 2.88 | 5.2 |
| WI-683 | 4.3 | 1.85 | 2.3 |
| O.Martin | 11 | 1.69 | 6.5 |
| Galleon | 10 | 2.57 | 3.9 |
| Athenais | 9.6 | 2.33 | 4.1 |
| Shannon | 5.6 | 2.19 | 2.6 |
| WI-768 | 9.5 | 2.35 | 4.0 |
| Betzes | 14.9 | 2.15 | 6.9 |
| Forrest | 48.6 | 2.08 | 23.4 |
| Princess | 29.8 | 1.67 | 17.8 |
| WI-958 | 14.2 | 1.96 | 7.2 |
| Grimmett | 10.3 | 2.1 | 4.9 |

| Cultivar | LD activity (μg/ml) | Protein (mg/ml) | Specific activity (μg/mg) |
|---|---|---|---|
| Minn 73-75 | 17.9 | 1.06 | 16.9 |
| WI-2610 | 10.1 | 2.29 | 4.4 |
| WI-2680 | 10.9 | 2.64 | 4.1 |
| Velvon | 9.5 | 2.46 | 3.9 |
| Dampier | 11.3 | 2.55 | 4.4 |
| WI-2477 | 9.3 | 2.72 | 3.4 |
| Minn 76-14 | 13.7 | 2.72 | 5 |
| WI-2611 | 10.6 | 2.72 | 3.9 |
| WI-2681 | 27 | 3.18 | 8.5 |
| Bonus | 9.5 | 3.04 | 3.1 |
| Diamant | 11.3 | 2.82 | 4 |
| Mazurka | 13.9 | 2.79 | 5 |
| Mosane | 20.6 | 0.92 | 22.4 |
| WI-2612 | 11.6 | 1.14 | 10.2 |
| WI-2682 | 7 | 0.87 | 8 |
| Piroline | 32.1 | 1.26 | 25.5 |
| Conquest | 7.7 | 1.02 | 7.5 |
| Puebla | 11.6 | 1.57 | 7.4 |
| Beka | 13 | 0.93 | 14 |
| Azuma Golden | 10.9 | 1.07 | 10.2 |
| Vaughn | 15.9 | 0.75 | 21.2 |
| Lara | 18.8 | 1.4 | 13.4 |
| Parwan | 7.4 | 1.3 | 5.7 |
| WI-2700 | 11.3 | 1.44 | 7.8 |
| G/Archer | 30.3 | 1.51 | 20.1 |
| WI-2231 | 8.6 | 1.97 | 4.4 |
| Estanzuela | 11.8 | 1.72 | 6.9 |
| Lami *ant* 13 | 7.6 | 1.31 | 5.8 |
| Manapou | 10.1 | 2.1 | 4.8 |
| Miho Golden | 14.6 | 3.05 | 4.8 |
| Lenta | 10.9 | 2.45 | 4.4 |
| O/Prophete | 10.2 | 1.62 | 6.3 |
| La Mesita | 9.2 | 2.57 | 3.6 |
| Kirstina | 7.1 | 2.07 | 3.4 |
| Harry | 11.1 | 1.46 | 7.6 |

| Cultivar | LD activity (μg/ml) | Protein (mg/ml) | Specific activity (μg/mg) |
|---|---|---|---|
| Orange Lemma | 12.2 | 2.87 | 4.3 |
| Cytris | 8.8 | 3.13 | 2.8 |
| Magnum | 9.6 | 2.91 | 3.3 |
| Rupal *ant* 13 | 12.4 | 2.17 | 5.7 |
| 3 EBYT 23 | 8.6 | 2.56 | 3.4 |
| Gimpel | 9 | 1.23 | 7.3 |
| Kirin Choku | 34.8 | | |
| Gold Marker | 15.6 | 1.76 | 19.8 |
| WI-2582 | 11 | 2.23 | 7 |
| WI-2646 | 8.6 | 2.66 | 4.1 |
| Vada | 8.2 | 3.03 | 2.8 |
| WI-775 | 9.9 | 2.45 | 3.3 |
| Fuji Nijo | 11.8 | 1.42 | 7 |
| Gold Spear | 7.5 | 2.06 | 5.7 |
| WI-2583 | 11.2 | 3.24 | 2.3 |
| WI-2649 | 23.4 | 3.22 | 3.5 |
| Varde | 25.8 | 1.4 | 16.7 |
| Excelsior | 5.9 | 2.14 | 12.1 |
| Bolivia | 9.5 | 2.93 | 2 |
| Clipper | 11.7 | 2.81 | 3.4 |
| Proctor | 11.2 | 3.04 | 3.8 |
| Maythorpe | 11.7 | 3.51 | 3.2 |
| Weeah | 10.4 | 3.6 | 3.3 |
| Chevron | 32.7 | 3.77 | 2.7 |
| Schooner | 15.1 | 2.88 | 11.4 |
| WI-683 | 4.3 | 1.85 | 8.2 |
| O.Martin | 11 | 1.69 | 2.5 |
| Galleon | 10 | 2.57 | 4.3 |
| Athenais | 9.6 | 2.33 | 4.3 |
| Shannon | 5.6 | 2.19 | 4.4 |
| WI-768 | 9.5 | 2.35 | 2.4 |
| Betzes | 14.9 | 2.15 | 4.4 |
| Forrest | 48.6 | 2.08 | 7.2 |
| Princess | 29.8 | 1.67 | 29.1 |
| WI-958 | 14.2 | 1.96 | 15.2 |
| Grimmett | 10.3 | 2.1 | 4.7 |

| Cultivar | LD activity (μg/ml) | Protein (mg/ml) | Specific activity (μg/mg) |
|---|---|---|---|
| I.Dwarf | 9.9 | 1.06 | 13.4 |
| G/Promise | 18.1 | 2.65 | 3.9 |
| Malebo | 21.6 | 1.52 | 6.8 |
| Prior | 4.2 | 2.47 | 8.7 |
| WI-2539 | 8.1 | 2.16 | 1.9 |
| Stirling | 14.4 | 1.45 | 5.6 |
| Ketch | 5.9 | 2.71 | 5.3 |
| Triumph | 37.2 | 2.26 | 2.6 |
| O'Connor | 19.6 | 2.83 | 13.1 |
| Clermont | 5.7 | 2.52 | 7.8 |
| Skiff | 19.2 | 1.31 | 4.4 |
| Minerva | 12.1 | 1.3 | 14.8 |
| WI-2585 | 34.9 | 1.66 | 7.3 |
| Hiproly | 26.9 | 2.59 | 13.5 |
| Kaniere | 11 | 3.75 | 7.2 |
| WI-2597 | 13.1 | 2.14 | 5.1 |
| Izmir 9 | 15.6 | 2.69 | 4.9 |
| WI-2621 | 15.8 | 2.08 | 7.5 |

Samples of barley (Appendix 5.1) were germinated and extracted as described in 5.2.1.1, and assayed for LD (ELISA) and for total protein (Bradford assay).

**APPENDIX 8.1**

| Cultivar | Extract (% dry) | ELISA Activity (µg/g) | $Log_{10}$ RP Activity (mU/g) | AAL (%) |
|---|---|---|---|---|
| WI-2677 | 74.2 | 19.63 | 1.845 | 75.7 |
| WI-2607 | 76.4 | 32.0 | 2.23 | 71.9 |
| Minn | 76.5 | 40.3 | 2.97 | 74.6 |
| Dampier | 75.8 | 30.1 | 1.98 | 71.2 |
| WI-2477 | 76.1 | 34.2 | 3.09 | 70.1 |
| WI-2681 | 75.0 | 50.3 | 2.33 | 75.6 |
| Mosane | 76.1 | 31.4 | 3.10 | 75.9 |
| WI-2682 | 74.2 | 40.14 | 3.25 | 74.1 |
| Piroline | 75.2 | 27.75 | 1.95 | 71.8 |
| Lara | 76.3 | 37.69 | 2.84 | 73.8 |
| Parwan | 76.4 | 33.18 | 2.58 | 72.4 |
| Golden Archer | 74.2 | 32.32 | 1.90 | 70.7 |
| Kirin Choku | 73.1 | 37.11 | 2.19 | 74.1 |
| WI-2649 | 72.6 | 29.54 | 3.08 | 71.9 |
| Varde | 74.4 | 40.26 | 3.14 | 71.2 |
| WI-2575 | 75.8 | 36.82 | 2.22 | 69.8 |
| Excelsior | 73.5 | 32.39 | 2.18 | 69.3 |
| Clipper | 75.9 | 35.0 | 2.20 | 70.3 |
| Proctor | 74.4 | 35.61 | 2.13 | 72.3 |
| Chevron | 74.5 | 30.83 | 2.82 | 70.4 |
| Schooner | 75.9 | 34.70 | 3.10 | 72.7 |
| WI-683 | 74.5 | 31.46 | 2.01 | 73.7 |
| Galleon | 74.2 | 15.54 | 1.79 | 69.0 |
| Shannon | 74.8 | 29.55 | 2.10 | 72.6 |
| Forrest | 76.0 | 18.46 | 1.86 | 69.5 |
| Princess | 73.9 | 31.40 | 2.04 | 71.2 |
| Grimmett | 75.5 | 29.16 | 2.60 | 72.0 |
| Golden Promise | 74.4 | 28.76 | 2.11 | 71.5 |
| Malebo | 74.4 | 26.54 | 2.74 | 72.5 |
| Prior | 73.4 | 24.86 | 1.99 | 69.5 |
| Triumph | 75.9 | 28.08 | 2.77 | 72.2 |
| O'Connor | 76.0 | 26.64 | 2.96 | 73.3 |
| Clermont | 74.7 | 22.0 | 2.63 | 71.2 |
| Skiff | 77.1 | 35.54 | 2.33 | 71.7 |
| QC | 79.3 | 37.68 | 3.12 | 72.6 |
| Ketch | 74.3 | 26.78 | 2.14 | 71.6 |
| WI-2585 | 75.6 | 30.96 | 1.98 | 71.8 |
| Hiproly | 75.7 | 49.76 | 2.14 | 68.7 |
| Weeah | 74.0 | 37.31 | 2.02 | 68.8 |
| LSD | 0.33 | 3.16 | 0.18 | 0.55 |

Data in the table are least square means for the study described in 8.2.1. The least square means were obtained from separate ANOVA for each parameter taking into account differences between replicates.

## APPENDIX 8.2

Data from a study of the relationship between Limit Dextrinase and Apparent Attenuation Limit in selected cultivars malted for 4, 7 and 10 days (see 8.2.2). The ELISA data is the mean of three determinations; enzyme activity, AAL and % extract are the mean of two determinations.

| Cultivar | Length of malting (days) | ELISA activity (μg/g) | Enzyme activity (mU/g) | AAL (%) | Extract (%) |
|---|---|---|---|---|---|
| **Hiproly** | 10 | 58.8 | 72 | 73.7 | 79.4 |
| | 10 | 49.0 | 43 | 71.9 | 73.9 |
| | 10 | 56.6 | 128 | 74.7 | 74.3 |
| | 7 | 59.4 | 67 | 72.5 | 78.9 |
| | 7 | 52.9 | 69 | 73.4 | 80.4 |
| | 7 | 51.6 | 47 | 74.5 | 74.0 |
| | 4 | 41.5 | 55 | 70.4 | 78.9 |
| | 4 | 45.9 | 50 | 72.7 | 74.0 |
| | 4 | Ot[1] | 46 | 72.5 | 74.2 |
| **Xia Gang Da Mai** | 10 | 43.0 | Ot[1] | 82.2 | 75.6 |
| | 10 | 46.5 | 67 | 79.6 | 78.3 |
| | 10 | 49.4 | 94 | 80.9 | 80.4 |
| | 7 | 53.3 | 86 | 81.2 | 76.2 |
| | 7 | 49.8 | 73 | 82.0 | 76.7 |
| | 7 | 39.8 | 123 | 80.6 | 81.5 |
| | 4[2] | 38.1 | 61 | 74.0 | 79.1 |
| | 4 | 39.0 | 63 | 79.3 | 75.3 |
| **Schooner** | 10 | 51.0 | 1133 | 78.5 | 79.3 |
| | 10 | 79.6 | 893 | 78.4 | 79.0 |
| | 10 | 50.3 | 1039 | 78.5 | 78.5 |
| | 7 | 44.2 | 640 | 78.2 | 78.8 |
| | 7 | 41.8 | 998 | 78.8 | 79.4 |
| | 7 | 48.7 | 1191 | 79.2 | 78.6 |
| | 4 | 48.4 | 481 | 77.7 | 78.5 |
| | 4 | 33.6 | 571 | 76.8 | 78.9 |
| | 4 | 46.7 | 602 | 77.2 | 78.3 |
| **Minn** | 10 | 38.3 | 644 | 78.7 | 79.1 |
| | 10 | 50.0 | 1332 | 80.6 | Ot[1] |
| | 10 | 52.3 | 1289 | 81.0 | 80.3 |
| | 7 | 51.5 | 1190 | 81.8 | 78.9 |
| | 7 | 50.0 | 1094 | 79.6 | 79.3 |
| | 7 | 47.7 | 1418 | 81.4 | 79.1 |
| | 4 | 39.5 | 688 | 79.2 | 78.6 |
| | 4 | 44.1 | 593 | 78.9 | 78.0 |
| | 4 | 33.8 | 571 | 77.7 | 79.3 |

**APPENDIX 8.2 (continued)**

| Cultivar | Length of malting (days) | ELISA activity (μg/g) | Enzyme activity (mU/g) | AAL (%) | Extract (%) |
|---|---|---|---|---|---|
| **Galleon** | 10 | 32.2 | 64 | 76.4 | 77.5 |
| | 10 | 27.7 | 43 | 75.6 | 77.8 |
| | 10 | 37.4 | 172 | 76.2 | 77.9 |
| | 7 | 25.5 | 104 | 75.1 | 78.0 |
| | 7 | 25.6 | 147 | 75.1 | 76.9 |
| | 7 | 26.5 | 148 | 75.1 | 77.6 |
| | 4 | 25.1 | 48 | 72.5 | 77.1 |
| | 4 | 22.1 | 43 | 73.5 | 76.8 |
| | 4 | 23.2 | 47 | 73.1 | 75.4 |
| **Qi Wu Qi** | 10 | 27.4 | 110 | 79.4 | 76.1 |
| | 10 | 36.9 | 86 | 79.8 | 74.2 |
| | 10 | 24.7 | 69 | 79.8 | 74.7 |
| | 7 | 26.0 | 142 | 79.3 | 73.8 |
| | 7 | 32.4 | 145 | 77.4 | 76.9 |
| | 7 | 32.5 | 69 | 78.9 | 73.9 |
| | 4 | 27.0 | 44 | 74.1 | 75.8 |
| | 4 | 19.6 | 43 | 76.9 | 75.0 |
| | 4 | 23.7 | 48 | 77.0 | 73.5 |
| **Lara** | 10 | 51.1 | 1063 | 80.6 | 77.7 |
| | 10 | 51.8 | 964 | 79.9 | 78.4 |
| | 10 | 54.7 | 1029 | 80.2 | 78.4 |
| | 7 | 42.0 | 1013 | 79.1 | 78.4 |
| | 7 | 43.0 | 591 | 80.0 | 77.7 |
| | 7 | 38.5 | 431 | 78.0 | 78.4 |
| | 4 | 37.2 | 450 | 76.9 | 77.0 |
| | 4 | 34.8 | 520 | 78.4 | 77.5 |
| | 4 | 34.5 | 510 | 78.0 | 77.6 |
| **Skiff** | 10 | 45.5 | 1478 | 80.3 | 79.7 |
| | 10 | 39.6 | 647 | 79.6 | 79.7 |
| | 10 | 37.8 | 534 | Ot[1] | Ot[1] |
| | 7 | 44.2 | 659 | 78.2 | 79.5 |
| | 7 | 30.0 | 517 | 78.1 | 78.4 |
| | 4 | 34.4 | 665 | | 78.7 |
| | 4 | 34.5 | 345 | 76.6 | 78.0 |
| | 4 | 26.4 | 462 | 76.3 | 77.6 |

1. Statistical outlier
2. For Skiff and Xia Gang Da Mai there was insufficient malt for a complete analysis.

# APPENDIX 8.3

Relationship between LD and AAL in 38 genetically diverse cultivars.

Model: 8.3.1

| Parameter | df | Sums of Squares | Mean Square | F Ratio |
|---|---|---|---|---|
| $Log_{10}$ RP activity | 1 | 53.580 | 53.580 | 12.938*** |
| Error | 110 | 455.538 | 4.141 | |

Model: 8.3.2

| Parameter | df | Sums of Squares | Mean Square | F Ratio |
|---|---|---|---|---|
| ELISA | 1 | 13.828 | 13.828 | 3.095 n.s. |
| Error | 112 | 500.322 | 4.467 | |

Model: 8.3.3

| Parameter | df | Sums of Squares | Mean Square | F Ratio |
|---|---|---|---|---|
| Cultivar | 37 | 350.398 | 9.470 | 6.329*** |
| $Log_{10}$ RP activity | 1 | 3.580 | 3.580 | 2.393 n.s. |
| Error | 70 | 104.741 | 1.496 | |

ANOVA tables for the response AAL, and the factors contributing to the total variation in AAL. Data are from 38 different malts. Significance level, 0.01 % (***) and n.s. refers to not significant.

# APPENDIX 8.4

ANOVAs to explain the variation in enzyme and immunological activity in eight cultivars malted for 4, 7 and 10 days. An ANOVA allows partitioning of the variation in LD activity attributed to cultivar differences and the length of malting. Taking into account these factors, a valid statistical comparison in enzyme activity between 4, 7 and 10 days and between different cultivars is possible.

Model: 8.4.1

Data of the LD immunological activity (ELISA) from the eight cultivars malted for varying times were analysed by ANOVA. LSD statistic = 5.43. Least squares data for length of malting derived from the ANOVA are shown at the bottom of the table.

| Source | df | Sums of Squares | Mean Square | F ratio |
|---|---|---|---|---|
| Cultivar | 7 | 5374.342 | 767.7631 | 22.229*** |
| Length of malting | 2 | 1172.511 | 586.2555 | 16.974*** |
| Error | 55 | 1899.644 | 34.539 | |

| Parameter | Least square mean | Std. error |
|---|---|---|
| 4 day | 35.201 | 1.366 |
| 7 day | 41.421 | 1.228 |
| 10 day | 45.890 | 1.229 |

## APPENDIX 8.4 (Continued)

Model: 8.4.2

Data of the enzyme activity (RP) from the eight cultivars malted for varying times were analysed by ANOVA. LSD statistic = 0.144. Least squares data for length of malting derived from the ANOVA are shown at the bottom of the table.

| Source | df | Sums of Squares | Mean Square | F ratio |
|---|---|---|---|---|
| Cultivar | 7 | 18.776 | 2.682 | 114.519*** |
| Length of malting | 2 | 1.073 | 0.537 | 22.909*** |
| Error | 59 | 1.382 | 0.023 | |

| Parameter | Least square mean | Std. error |
|---|---|---|
| 4 day | 2.178 | 0.032 |
| 7 day | 2.446 | 0.032 |
| 10 day | 2.440 | 0.032 |

ANOVA tables are presented below to explain the variation in AAL in eight cultivars malted for 4, 7 and 10 days.

Model: 8.4.3

ANOVA of AAL data using RP activity to explain variation ($r^2$ 0.27).

| Source | df | Sums of Squares | Mean Square | F Ratio |
|---|---|---|---|---|
| $Log_{10}$ RP activity | 1 | 170.675 | 170.675 | 32.042*** |
| Error | 65 | 346.230 | 5.327 | |

Model: 8.4.4

ANOVA of AAL data using ELISA activity to explain variation.

| Source | df | Sums of Squares | Mean Square | F Ratio |
|---|---|---|---|---|
| ELISA | 1 | 17.128 | 17.128 | 2.414 n.s. |
| Error | 61 | 432.787 | 7.095 | |

## APPENDIX 8.4 (Continued)

Model: 8.4.5

ANOVA table for the linear model to explain the variation in AAL. Significance level 5% (*) and 1% (**).

| Source | df | Mean Square | F ratio |
|---|---|---|---|
| Cultivar | 7 | 32.90 | 83.71** |
| Length of malting | 2 | 6.85 | 17.41** |
| Length malting x Cultivar | 14 | 0.793 | 2.02* |
| $Log_{10}$ LD activity | 1 | 1.952 | 5.0* |
| % Extract | 1 | 6.578 | 16.74** |
| Error | 37 | 0.393 | |

Revised ANOVA to explain the variation in AAL in cultivars malted for 7 and 10 days.

Model: 8.4.6

ANOVA of AAL after removing day 4 data ($r^2 = 0.94$). Length of malting was not significant for AAL.

| Source | df | Sums of Squares | Mean Square | F Ratio |
|---|---|---|---|---|
| Cultivar | 7 | 149.337 | 21.334 | 45.008*** |
| $Log_{10}$ RP (mU/g) | 1 | 8.897 | 8.897 | 18.771*** |
| % Extract | 1 | 3.926 | 3.926 | 8.282** |
| Error | 32 | 243.650 | 0.474 | |

## APPENDIX 8.4 (Continued)

Least square means for AAL of 8 cultivars (without day 4 data; model, 8.4.6).

| Cultivar | Apparent attenuation limit (%) | |
|---|---|---|
| | Least squares mean | Standard error |
| Xia Gang Da Mai | 79.36 | 0.44 |
| Qi Wu Qi | 82.01 | 0.42 |
| Galleon | 76.51 | 0.36 |
| Hiproly | 74.72 | 0.40 |
| Lara | 78.92 | 0.34 |
| Minnesota | 79.40 | 0.47 |
| Schooner | 77.75 | 0.39 |
| Skiff | 78.69 | 0.40 |
| LSD | 0.66 | |

## APPENDIX 8.5

Comparison of least square means of LD immunological activity for eight cultivars malted for 4, 7 or 10 days.

| Cultivar | | Qi Wu Qi | Galleon | Skiff | Lara | Xia Gang Da Mai | Minn | Schooner |
|---|---|---|---|---|---|---|---|---|
| | LSM | 27.20 | 27.26 | 36.62 | 43.07 | 44.22 | 45.26 | 49.37 |
| Qi Wu Qi | 27.20 | | | | | | | |
| Galleon | 27.26 | 0.06 | | | | | | |
| Skiff | 36.62 | 9.42* | 9.36* | | | | | |
| Lara | 43.07 | 15.87* | 15.81* | 6.45* | | | | |
| Xia Gang Da Mai | 44.22 | 17.02* | 16.96* | 7.60* | 1.15 | | | |
| Minn | 45.26 | 18.06* | 18.0* | 8.64* | 2.19 | 1.04 | | |
| Schooner | 49.37 | 22.17* | 22.11* | 12.75* | 6.30* | 5.15 | 4.11 | |
| Hiproly | 53.72 | 26.52* | 26.46* | 17.10* | 10.62* | 9.50 | 8.46* | 4.35 |

Significance difference at the 5 % level is indicated by *. LSMs are calculated from the ANOVA of appendix 8.4 for each of the cultivars. Data in the table are the arithmetic difference between the LSM in rows and the LSM in columns.

## APPENDIX 8.5 continued

Comparison of least square means of LD enzyme activity (RP) for eight cultivars malted for 4, 7 or 10 days.

| Cultivar | LSM | Qi Wu Qi | Galleon | Skiff | Lara | Xia Gang Da Mai | Minn | Schooner |
|---|---|---|---|---|---|---|---|---|
| | | 1.88 | 1.89 | 2.80 | 2.84 | 1.79 | 2.96 | 2.90 |
| **Qi Wu Qi** | 1.88 | | | | | | | |
| **Galleon** | 1.89 | 0.01 | | | | | | |
| **Skiff** | 2.80 | 0.92* | 0.91* | | | | | |
| **Lara** | 2.84 | 0.96* | 0.95* | 0.04 | | | | |
| **Xia Gang Da Mai** | 1.79 | -0.09 | -0.10 | -1.01* | -1.05* | | | |
| **Minn** | 2.96 | 1.08* | 1.07* | 0.16* | 0.12 | 1.17* | | |
| **Schooner** | 2.90 | 1.02* | 1.01* | 0.10 | 0.06 | 1.11* | -0.06 | |
| **Hiproly** | 1.78 | -0.01 | -0.11 | -1.02* | -1.06* | -0.01 | -1.18 | -1.12* |

Significance difference at the 5 % level is indicated by *. LSMs are calculated from the ANOVA of appendix 8.4 for each of the cultivars. Data in the table are the arithmetic difference between the LSM in rows and the LSM in columns.

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