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STRUCTURAL AND DEGRADATIVE STUDIES OF STARCH GRANULES FROM CEREALS AND TUBERS. PRELIMINARY INVESTIGATIONS INTO THE CLONING OF GLUCOAMYLASE AND XYLANASE STRUCTURAL GENES FROM ASPERGILLUS niger (ATCC. 1084).

BY

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Dedication.

The author would like to dedicate this manuscript to Beethoven.

DECLARATION.

I hereby declare that this thesis contains no material which has been submitted for the award of any other degree or diploma in any University.

To the best of my knowledge, no material described herein has been previously published by another person, except when due reference is made in the text.

If accepted for the award of Ph.D., this thesis will be available for loan and photocopying.

Ronald H. McHale.

NOMENCLATURE AND ABBREVIATIONS.

ATP	Adenosine 5'-triphosphate
ADP	Adenosine 5'-diphosphate
DTT	Dithiothreitol
NAD	Nicotine-adenine-dinucleotide
EDTA	Ethylenediaminetetraacetate
PVP	Polyvinyl-pyrrolidone
TRIS	2-Amino-2-hydroxymethyl-1,3-propanediol
PEG	Polyethyleneglycol
PAGE	Polyacrylamide gel electrophoresis
PGK	Phospho-glycerate kinase

SYMBOLS AND UNITS.

Abs	Absorbance
°C	Degrees Centigrade (Celcius)
cm	Centimetre(s)
g	Gram(s)
hr	Hour(s)
l	Litre(s)
M	Molar
mg	Milligram(s)
min	Minute(s)
ml	Millilitre(s)
mM	Millimolar

μg	Microgram(s)
μl	Microlitre(s)
Mr	Relative molecular weight
N	Normality
nM	Nanomole(s)
pg	Picogram(s)
rpm	Revolutions per minute
eg.	For example
et al	and others
Fig(s)	Figure(s)
SD	Standard deviation for given set of results
v/v	volume:volume
w/v	weight:volume
>	Greater than

≃	Aproximately equal to
%	Percentage
I.D.U.	International Diastatic Units

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Summary.

The work presented within this thesis is in two parts. The first section investigates structural aspects of starch granules and changes which occur after treatment with carbohydrases.
The second part concentrates on the degradation of granular starch from an industrial viewpoint, in particular utilizing the methods of molecular biology to identify polysaccharide degrading enzymes and their introduction into industrially useful microrganisms.

1. Susceptibility of starch granules to alpha-amylases.

Starch granules from barley prepared by different procedures were shown to vary in their susceptibility to salivary alpha-amylase. Further, the different visual degradation patterns produced on the exterior of potato, maize, mannioc and barley starch granules after incubation with alpha-amylases were shown to be granule type specific.
The storage protein fraction of barley, the hordeins,were shown to have an affinity for the starch granule surface during granule isolation. The presence of these proteins on the granule surface were shown to hinder the enzymic hydrolysis of these granules and their dispersion during gelatinization. The hordein fraction appeared to be glycosylated with mannose and fucose type residues.
Tuber starch granules were more resistant to degradation by alpha-amylases and differences in enzyme susceptibility were detected between barley cultivars and within large and small granule populations within a cultivar. The adsorption of barley and salivary alpha-amylase to large and small granules of barley at 25°C differed with granule size, cultivar and source of enzyme.
Calcium ions although known to be important for the maximum activity of cereal alpha-amylases do not appear to be involved in the initial binding of the enzyme to the granule surfaces.

These observations suggest there are structural differences between granule types and may be economically important for starch related industries where the efficient degradation of granular starch is paramount for good yield of soluble saccharides.

2.0 Binding of Concanavilin-A to Starch granules.

The lectin Concanavilin-A was found to have different binding affinities for starch granules types.

In the presence of Concanavilin-A, adsorption and enzymic activity of salivary alpha-amylase for the large and small starch granules of barley were shown to be related to lectin concentration. Theoretically less lectin was required to completetly inhibit the degradation of small granules from barley than large granules.

These results indicate structural differences exist in the surface architecture of starch granules and this is discussed.

Based on Concanavilin-A/starch granule interactions a fluorometric method was developed for assessing damage to wheat starch granules after milling. Since existing tests for starch damage are indirect eg. release of reducing equivalents after treatment with alpha-amylase, infra-red reflectance analysis, this new method may provide a direct assessment of granule damage caused by the milling process.

3.0 Substituted Starch and Amylose.

The susceptibility to enzymic degradation of hydroxpropylated starch granules and amylose was shown to be dependant on the type of amylase and degree of substitution. A new test for measuring endo-amylase activity in the presence of exo-amylase activity suitable for automated analysis was developed and assessed.

By substituting intact and enzymically degraded starch granules by hydroxpropylation followed by debranching and elution by column chromatography, an insight into the structural organization of granules could be obtained. This method, together with the inclusion of HPLC for increased resolution and rapidity of substituted products, may provide a future technique for studying and comparing the structural organization of intact starch granules before and after enzymic modifications.

4.0 Identification and cloning of potential Xylanase and Glucoamylase gene sequences form Aspergillus.

Starch and xylan induced c-DNA libraries were produced and potential glucoamylase and xylanase gene sequences identified.

A potential xylanase c-DNA primer extension product was ligated to a protein-A fusion vector and the protein product vizualized immunologically. This construct was introduced into a yeast expression vector and recombinants were screened for their ability to produce xylan degrading enzymes and utilization of xylan as the sole carbon source.

The genomic sequence complimentary to a potential xylan degrading enzyme was isloated and mapped.

Potential c-DNA fragments and primer extension products corresponding to approximately 90% of a glucoamylase gene were identified.

The introduction, secretion and controlled expression of amylolytic and cellulolytic degrading enzymes into industrially important microrganisms eg. industrial yeast, is gaining momentum as a greater understanding of the genes involved in key metabolic processes are identified.

A number of useful genes have already been cloned and are under commercial scale trials, this is discussed later in this manuscript.

1.0 INTRODUCTION.

1.1 STARCH GRANULE MORPHOLOGY.

The number, size and morphology of starch granules present in the seeds and tubers of plants are dependent on the source. A morphological account of starch granules isolated from potato, maize, mannioc, barley and other sources has recently been documented by *Greenwood*, [1,2,3]. Within a plant species the size, shape and proportional representation of each class of granule type is virtually constant and can be used as a criterion of identification of plant species from which the starch granule was obtained. In this thesis starch granules from barley, maize, mannioc and potato were utilized for experimental procedures, however reference is made to other starch types where considered appropriate.

1.2 STARCH GRANULE STUCTURE

1.2.1. Chemical Structure.

Starch granules are composed of two distinct homopolymers of alpha-D- glucose, namely amylose and amylopectin. The manner in which these two macromolecular components are synthesized and combined within the plastid accounts for the unique chemical and physical characteristics displayed by individual granule types.

The molecular structure of starch granules varies with its source and method of isolation. The structure of amylopectin from gelatinized starch granules isolated from barley [4,5], wheat [6], rice [7],and pea [8], have been investigated utilizing the microbial debranching enzymes pullulanase and isoamylase. The hydrolyzed products from enzymic debranching were fractionated by column chromatography, and some of the fine structural features have been determined.

Temperature has been shown to induce structural changes within the amylose and amylopectin components of developing rice [9]. The amylose content was observed to decrease and the number of B-chains in the amylopectin component were shown to increase [10].

A proportion of starch in some cereal, tuber species [11] and maize mutants [12] is composed of an intermediate material between amylose and amylopectin.

The amylose present in amylomaize starch granules has a small number of alpha 1,6 branch points at various positions along its backbone. This intermediate material was shown to have a chain length of 50-100 glucose units and only reaches significant levels in starches high in amylose [13]. Amylose is essentially a linear molecule composed of alpha -1,4 linked D-glucopyranosyl residues, the chain length of normal amylose is approximately 3,000 glucosyl residues.

In contrast, amylopectin is a branched homopolymer composed of linear alpha -1,4 -D-glucopyranosyl residues with alpha -1, 6-D-glucopyranosyl branch points on average every 20-25 glucosyl residues and is therefore approximately 4-5% branched. It has a molecular weight of approximately 10^6-10^7 daltons.

Intact starch granules are associated with surface and integral lipids. The surface lipids occur as droplets, spheresomes, components of membranes or organelles which may be associated with storage proteins at least in the case of cereal starches. These lipids are not considered to be true components of the granule structure but adhere to the exterior of the granule during purification and may form inclusion complexes with amylose at the granule surface [14]. They can be removed by treatment with various polar solvent mixtures eg. n-propanol-water at ambient temperatures without any obvious granule disruption.

True starch lipids in contrast, are integral components of the granule. In normal and high amylose cereal starches there are larger quantities of internal starch lipids than surface lipids [15], while waxy cereal, potato, and bean starches have only small amounts of surface lipids and probably contain no internal lipids [14].

Integral lipids of cereal starch granules, appear to consist exclusively of monoacyl lipids which may occur within amylose molecules or be trapped between the amylose and amylopectin. Extraction of internal starch lipids requires the use of solvents e.g.water saturated n-butanol at reflux temperatures of 90-100°C for efficient removal and results in considerable granule disruption. Conditions for the efficient extraction and characterization of starch lipids have been reviewed recently by *Morrison et al* [16].

The formation of a stable complex between amylose and monoacyl lipids has attracted attention recently from industry, since these complexes reduce amylose solubility thereby increasing gelatinization temperature [17], reduce stickiness and freeze-thaw stability [18], retard retrogation and therefore increase storage time [19]. Amylose/lipid complexes have greater resistance to alpha-amylolysis [20]. This may have important consequences if the starch is utilized for human, animal consumption or industrial processes which require the use of enzymes.

A very comprehensive study of the relationship between amylose and lipid content has been carried out by *Morrison et al* [21], on maize, barley, rice, oat, millet and sorghum. They concluded that there was a fundamental relationship between amylose and lipid content but that the relationship was affected by a number of varietal differences and environmental factors.

1.2.2. Physical Features.

Intact starch granules from different sources have been shown to possess three X-ray diffraction patterns the A, B and C-forms [22,23,24], indicating that there is a certain degree of crystalline order inherent in their structure.

Cereal starches are associated mainly with the A-form [25], potato and amylomaize with the B-form and smooth pea and various bean starches with the C-form [26].

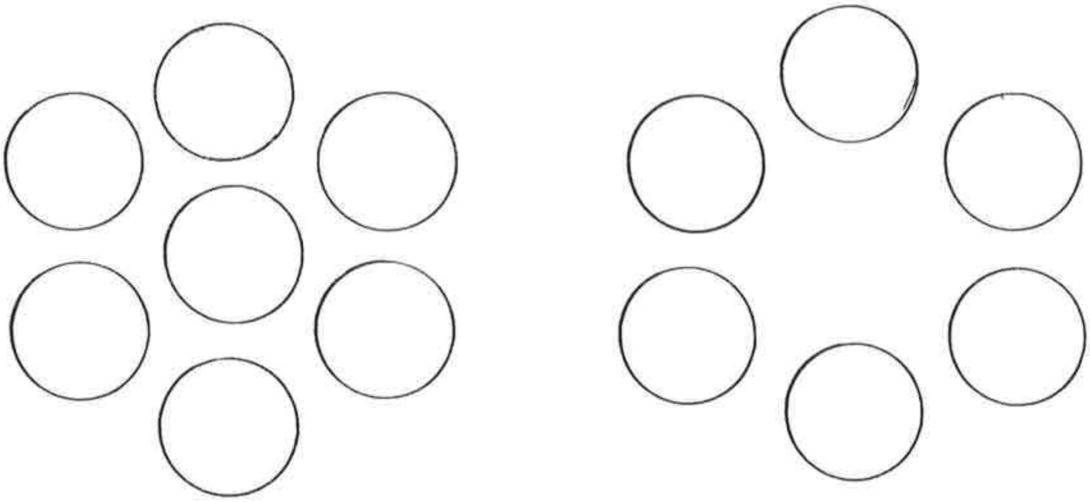
Changes in temperature have been shown to affect the X-ray diffraction pattern of soya bean starch granules during their development, from an A to a B- form [27,28].

Wu and Sarko [29], using computer techniques have proposed structural models to account for the X-ray diffraction patterns obtained from intact granules and those of amylose crystallites formed in aqueous solutions. These models are illustrated in Plate.1. Each circle represents an amylose double helix, water molecules are present between duplexes in the A-structure and in the central cavity of the B-structure. Amylose, essentially a linear structure, capable of retrograding readily in aqueous solutions, might be expected to form the crystalline component of the intact starch granule. In contrast the highly-branched component, amylopectin, is less likely to form crystalline structures.

The ability of amylose to complex with iodine is due to the entrapment of iodide ions in the central cavity of a helical amylose molecule [30], and is dependant on the amylose molecule having a structural conformation corresponding to an ordered V-form, depicted in Plate.2

Although the B-form of amylose does not complex readily with iodine [31], potato starch granules (which produce a B-form X-ray diffraction pattern), on exposure to iodine vapour readily stain blue-black, suggesting that some of the amylose present within the granule is present in a V-form. There is little evidence from X-ray crystallography to suggest that this structural conformation exists in the intact granule. Cereal starch lipids have been shown to form inclusion complexes with amylose consistent with amylose being present in a V-form. No evidence from X-ray diffraction studies has shown amylose to be present in any conformation other than the A, B or C-forms in intact unmodified granules. Therefore any amylose present in a V-conformation within the granule is not detected by X-ray diffraction and therefore exists in a conformation which may prevent it from forming a crystalline structure.

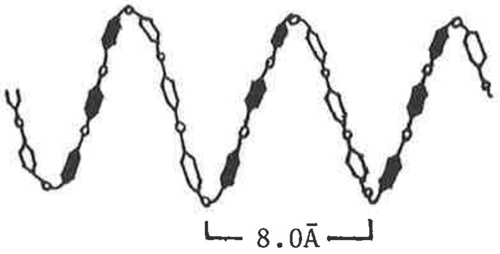
Plate. 1



A-Amylose

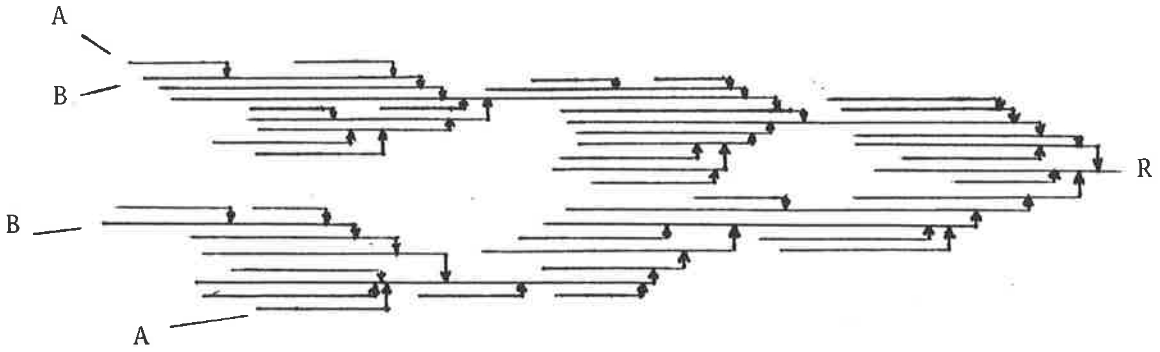
B-Amylose

Plate. 2.



Helical Amylose Molecule

Plate. 3



Cluster Model Of Amylopectin.

It has been suggested that amylopectin may be the crystalline component within intact starch granule. Thus amylose can be leached preferentially from potato starch granules leaving an intact crystalline granule which maintains the same X-ray diffraction pattern as intact potato granules [32]. Waxy maize starch has the same crystallinity pattern as normal maize starch, and amylomaize has a less ordered pattern than normal maize starch. Recently, *Gidley et al* [33] have shown that malto-oligosaccharides possessing a degree of polymerization of $\simeq 11$ can form crystalline structures having sharp X-ray diffraction patterns characteristic of A,B,and C-type starch polymorphs. They have also shown that smaller oligosaccharides of D.P.$\simeq$6 can form double helices in the presence of larger chains.The polymorphic form they obtained was dependant on chain length, concentration and temperature. The retrogration of amylose and amylopectin unit chains is promoted by small molecular weight saccharides, dissacharides were more effective than glucose [34]

Recently *Manners et al.* [35] proposed the "Cluster model" in an attempt to explain the physical, chemical and enzymic properties of the amylopectin molecule dispersed in aqueous solutions, this is depicted in Plate 3.

Bertroft [36] has proposed that a unit cluster has a molecular weight of 3 x10^4 daltons. If models based on a Cluster type structure are correct then the work carried out by *Gidley et al.*[33] and [34] may relate to the in-vivo nucleation and crystallization of starch in the developing amyloplast, so that there may be significant potential for crystallization in amylopectins between chains within unit clusters and chains between unit clusters.

Gelatinization of starch granules is promoted by increased temperatures in aqueous systems and has been well documented [37]. An increase in temperature results in granule swelling until a critical point is reached where granule integrity is lost completely and irreversibly. This phenomenon, gelatinization, is granule-type-specific, each species possessing unique gelatinization characteristics, namely transition temperature, onset of gelatinization and gelatinization temperature. The process of gelatinization usually occurs over a 5-10°C temperature range. Morphology displayed during gelatinization is also related to the origin of the granule [38], and different granule classes within a plant species have different gelatinization points e.g. in barley the large granules gelatinize at temperatures of 55-60°C, whereas the small granules are not affected until temperatures above 70°C [39]. This is significant industrially and will be referred to later.

Physical differences between starch granule types, for example gelatinization temperature, morphological changes during gelatinization, differential enzyme susceptibilities, fine structure and X-ray diffraction patterns suggest that there are structural differences between starch granule types. Most normal starch granules consist of two homopolymers amylose and amylopectin in an approximate ratio of 20-30% and 70-75% respectively. Differences in mutant starch granule properties may in some cases be related to the structural changes caused by the mutation. Differences in gelatinization temperature and enzyme susceptibility between amylomaize and waxy maize starch granules are known, however the rationale for these differences is not understood. Similarly differences between normal potato and barley granules exist in enzyme susceptibility and gelatinization properties, but the amylose/amylopectin ratios are similar. In some cases there appears to be a strong link between structural, physical, chemical and biological properties, whereas in other examples the relationship is not as close.

The quantities of amylose and amylopectin present cannot satisfactorily explain the differences in the major physical properties of granule types. Therefore the way in which these two components are orientated within the intact granule may be responsible for the unique properties displayed by individual granule types. Any experimental procedure capable of elucidating this orientation is likely to provide insight regarding the chemical, physical and biological structure and functional relationships of starch granules.

1.3 BIOSYNTHESIS OF STARCH GRANULES.

1.3.1. Granule Synthesis.

The biosynthesis of developing starch granules within the amyloplast is not well understood. The synthesis of starch granules within plastids occurs by the successive transfer of glucosyl residues to the reducing terminus of an already existing glucan chain primer [39], by granule bound and cytoplasmic starch synthases (E.C.2.4.1.21, E.C.2.4.1.11) respectively, ADP-glucose is thought to be the main glucosyl donor [40]. K^+ ions appear to be required for maximum activity [41] however this response to K^+ ions appears to be dependant on the enzyme source [42].

In waxy maize, the granule bound starch synthase is absent, suggesting that it may be involved in the synthesis of the amylose fraction [43]. At some crucial length a branching enzyme (E.C.2.4.1.18) possibly associated with one of the starch synthases, cleaves a section of the growing chain and links it to an already existing polysaccharide chain, either producing an intrachain linkage to the same chain or interchain linkage to a separately synthesized amylose molecule.

It is postulated that a prerequisite for branching enzyme activity is a substrate molecule in a stable alpha-helical configuration. This limits the substrate to an oligosaccharide with a degree of polymerization greater than 30 glucosyl residues [44]. Studies on the debranching enzymes of normal and mutant maize endosperms have shown that they consist of a series of isoenzymes, some of which appear to be associated with starch synthases [45]. This is well reviewed by Priess [46].

The synthesis of starch granules appears to be a highly ordered and genetically defined process considering the constancy of granule size, structure and granule class representation within a selected plant species. However, the substrate specificities of branching enzymes, their regulation, the extent of their association with soluble and granule-bound starch synthases, the effects of these specificities and associations on the development, structure and morphology of starch granules remain to be elucidated.

The use of mutants with lesions affecting starch synthesis coupled to progress in molecular biology should elucidate the mechanisms involved and aid in the identification of regulatory systems which appear to impose such stringent structural constraints on this storage polysaccharide.

1.3.2. Granule Development.

In cases where there are different granular sizes, eg.barley and wheat, it is generally accepted that starch granules which increase in size and become the large granules at maturity, are synthesized first. The smaller granules are synthesized at a later stage in development and remain small at maturity. The gelatinization properties and size distribution during the development of barley, rye and wheat have been investigated recently [47]. The results suggest that the gelatinization temperature is also affected during granule development, being lower in starch at maturity than at earlier stages of development. This change in gelatinization temperature was greater for large granules than for small ones, suggesting that granule structure changes during development.

Small starch granules of wheat generally increase in amylose content throughout development, the larger size class of granules also increase in amylose content but at a later stage in development [48,49]. Similar studies carried out on normal and high amylose barley starch granules [50] have shown that in normal barley cultivars, there was no apparent bimodal distribution of granule sizes until 27 days after anthesis, although all granule types increased in size. However from 27 days onwards a discontinuity in size was apparent. A cultivar high in amylose displayed no granule size distinction at any stage of development, furthermore the granules were smaller than those of normal barley starch. As observed in developing wheat, potato [51], barley [52] and pea starches [53] amylose content increased during development. This suggests that in the same plant species the synthesis of large and small granules may be under separate genetic controls and that the structural composition of the granule appears to change throughout development.

1.4. STARCH DEGRADATION *IN-VIVO* AND *IN-VITRO*.

1.4.1. *In-vivo* Degradation.

The physical state of the granule is important for an accurate assessment of starch degradation. Gelatinized starch is always more susceptible to enzymic degradation than intact granules. The only enzymes known to attack starch granules are the alpha amylases (E.C. 3.2.1.1) glucoamylases (E.C. 3.2.1.3) and a beta-amylase purified from Bacillus polymyxa [54]. Cereal alpha-amylases are Ca^+ requiring enzymes with a pH optimum between between pH 4.75 and pH 6.0 [55] In the presence of a full complement of Ca^+, alpha-amylases are more resistant to extremes of pH, temperature, urea, or to proteolytic degradation [56]. In the case of Aspergillus oryzae a single sulphydryl group is assumed to be involved in Ca^+ binding [57]. Heavy metal ions eg. Cu^+,Hg^+, Ag^+ and Pb^+ inhibit alpha-amylases [58,59]. Alpha-amylase iso-enzymes have been characterized extensively in barley, because of their importance in starch degradation industrially.

During germination alpha-amylase synthesis occurs de-novo in the aleurone layer and Ca^+ ions have been implicated in the secretion of iso-enzyme-2 [60]. However, in rice and maize seeds the scutellum is a major source of alpha-amylase during germination [61,5]. Denaturing PAGE, electrophoresis of purified alpha-amylase from barley indicates one protein band of molecular weight 45,000 daltons [62]. On native gel electrophoresis or isoelectric focusing a number of differentially charged protein bands were separated [63,64,65]. These have been classified into two groups, one having a pI of 5.8 and the other of 4.5.

Individual c-DNA clones corresponding to the two isoenzymes have been isolated[66] and the genes have been mapped. The low pI enzyme group are located on chromosome 1 and the high pI group on chromosome 6 [67,68]. Alpha-amylase can be induced in barley aleurone layers by gibberellic acid, but the exact derivative of giberellic acid involved is still not known [69]. Enzyme regulation is thought to be primarily transcriptional as indicated by the increase in m-RNA coding for alpha-amylase after gibberellic acid treatment [70,71]. Gibberellic acid appears to differentially regulate the two alpha-amylase groups [72] and to specifically stimulate the translation of particular proteins, eg. alpha-amylase and to repress the translation of others [73].

1.4.2. *In-Vitro* -Degradation.

MacGregor and co-workers [74] have separated alpha-amylase iso- enzymes from germinating barley utilizing ion-exchange chromatography. They obtained enough purified iso-enzymes to investigate the binding and hydrolysis patterns of starch granules from barley [75] and concluded that iso-enzyme 2 was the most prominent iso-enzyme present in malting barley. Iso-enzyme 1, the minor component, was more efficient in degrading barley starch granules than iso-enzyme 2. In a later study a number of iso-enzymes present within isoenzyme group 1 and group 2 were further separated by chromatofocusing [76]. Differential binding to intact starch granules has been observed for isoenzymes of group 1 and group 2 [77]. Group 1 isoenzymes the minor component, binds strongly to intact barley starch granules whereas those of group 2, the major component, bind only weakly. *MacGregor et al* [74] have studied the hydrolysis of intact large and small starch granules isolated from barley kernels using purified group 2 iso-enzymes and proposed that in order to account for the hydrolysis products, the iso-enzyme possessed between nine and eleven subsites at its active centre. It was suggested that the catalytic site of the enzyme was situated between subsite six and seven and each subsite has a characteristic affinity for a glucosyl residue of the oligosaccharide substrate. A similar subsite hypothesis has been proposed for bacterial [78] and fungal [79] amylases and the binding of various lectins to their respective carbohydrate moieties [80].

Whether these distinct isoenzymes within the major groups are the result of post translational modifications, e.g. glycosylation, limited proteolysis, or are derived through differential initiation of transcription is not known. The possibility of artifacts arising from the extraction procedures cannot be excluded. A very large increase in both the quantity and the number of iso-enzyme species can be greatly enhanced by the addition of gibberellic acid during germination of barley [81,82]. Maltsters have exploited this observation to enhance and synchronize germination and more recently the introduction of absicic acid in combination with gibberlic acid has been shown to decrease malting losses, increase fermentability and prevent wort decolourization caused by excessive proteolysis which occurs when gibberellic acid is used alone [83].

There are significant differences in the susceptibility of various starches to hydrolysis by alpha-amylases. A number of studies have been initiated on the hydrolysis of intact starch granules [84,13,50] and the pattern of degradation visualized using a scanning electron microscope [85,86,87,88]. These studies have shown that the degradation of intact starch granules is dependant both on the source of the enzyme and granules. The large starch granules of barley are more resistant to degradation than small ones, similarly there are differences between starch granule types eg. potato starch granules are extremely difficult to degrade at 37°C and high enzyme concentrations or temperatures above 50°C are required to disrupt granules [84] before hydrolysis occurs readily.

The pattern of granular attack is markedly dependant on granule type. In waxy and normal genotypes of sorghum and maize, all granules were extensively eroded in contrast to selective granule by granule degradation observed for rice, potato, sago, amylomaize and arrowroot starch granules. The extent and pattern of erosion can be altered by the enzyme used during granule degradation and this makes it difficult to compare interlaboratory results where different enzyme sources, activities and purification methods have been adopted. In all cases certain areas on the granule surface appear to be more susceptible to initial attack. Only recently have data become available [74] on the hydrolysis products and the action pattern of cereal alpha-amylase on intact starch granules. These findings suggest that cereal alpha-amylases have a random action pattern on high molecular weight substrates whereas a non-random attack was observed on small molecular weight substrates.

1.5 STARCH DEGRADATION: A MOLECULAR BIOLOGICAL APPROACH.

In order to render the intact starch granule more susceptible to enzymic degradation on an industrial scale, high temperatures are employed for granule dispersion. This energy demand adds considerably to the cost of production. Often the enzymes necessary for starch hydrolysis and product profile are closely associated with the raw material, eg. distillation and brewing industries. A fine balance must be maintained between maximum production and denaturation of the enzymes necessary for granule hydrolysis.

In traditional fermentation processes there is often strict legislation preventing the use of unnatural additives which include the use of unpurified starch and cellulose degrading enzymes. Purified or partially purified enzyme preparations are utilized eg. in brewery mashes, but their use adds to production costs in what is already a highly competitive industry. During industrial syrup production the enzymes required for the degradation of starchy materials are not as intimately associated with the substrate and the starch granules can be initially dispersed without the risk of enzyme denaturation, by pressure cooking or steam injection. Thermostable starch degrading enzymes are then added to induce liquification and saccharification. In both industries, considerable time and energy is required to disrupt an insoluble starch granule in order that it be effectiviely degraded by amylolytic enzymes. There could be commercial advantage in degrading intact starch granules to fermentable sugars in good yield.

There is increasing industrial awareness in utilizing molecular biological techniques to increase the efficiency of industrial processes eg. by the manipulation of industrial strains of yeast, bacteria, or fungi. In the case of yeast, difficulties in utilizing this new technology on an industrial scale have been considerable, mainly because strains of industrial microorganisms are often poorly classified genetically. This is still true in the distillation and wine industries and to a lesser extent the brewing industry.

Classical genetic breeding has been used to incorporate improved characters into industrial strains of yeast. However, a necessary prerequisite for classical breeding is the production of haploid spores. There was little problem encountered with laboratory strains of yeast which produce an abundance of viable spores, however industrial strains sporulate rarely and their spores are usually non viable. Most industrial strains of yeast are polyploid, therefore the formation of auxotrophs for use as selection markers has restricted progress. Protoplast fusions have produced homo and heterologous fusants, however their polyploid genotype often possess several nuclei associated within a cell and this led to unstable hybrids. (One of the main reasons industrial strains had been chosen initially apart from their practical industrial attributes, was for their exceptional stability under environmental stress, this was a direct result of their polyploid genotype and their reluctance to sporulate).

Loss of chromosomes at each mitotic division of colony outgrowth during protoplast fusion experiments also resulted in the recovered variants differing markedly in their chromosome number. The selection of heterologous fusants also posed a formidable problem but this was overcome by the use of petite mutants. Two industrial strains with selected characteristics, could be treated with Mn^+ ions creating point mutations [89] in the mitochondrial genome resulting in respiratory deficiencies resistant to certain mitochondrial specific antibiotics eg. chloramphenicol, oligomycin, or erythromycin. The heterologous fusants were selected by complementation of petite mutations, which displayed restored growth on non-fermentable substrates, or on the basis of their double antibiotic resistance.

These two techniques required the complementation of cytoplasmic markers, and neither procedure guarantees karyogamy. Dominant selection markers are also required in each of the two strains. Such markers are rare in industrial strains of Saccharomyces cerevisae, but do include Cu^+ resistant genes known to be present in one wine yeast [90], and in a number of brewing strains [91,92], ALG1: lactic acid growth in the presence of antimycin A [93], ROC resistance to the disinfectant Roccal.[91] and ANT., antibiotic resistance[91,94].

The technique of protoplast fusion has been used successfully to construct brewing [95], and baking yeast strains [96]. A serious drawback with this technique, is the introduction of an unfavourable background with the new character, which can lead to undesirable properties e.g. off flavours in the final product[97]. Construction of a glucoamylase (E.C. 3.2.1.3) secreting strain of Saccharomyces cerevisae and Saccharomyces uvarum utilizing protoplast fusion and transformation is well described in a number of recent publications [97,98,99,100]. The glucoamylase gene from Saccharomyces diastaticus was transferred but recipient strains had poor ability to degrade intact starch granules since it possesses low debranching activity and therefore incompletely debranches the amylopectin component of starch.

However, the presence of yeast recognition sequences allowed the expression of the glucoamylase its correct transcription, translation, processing and secretion in its new yeast host. The introduction and expression of this gene provided evidence regarding the practicalitites of manipulating industrial microrganisms and initiated debate for legislation in the use of genetically engineered strains in industry.

The use of recombinant DNA methodology in contrast to protoplast fusion has the advantage of inserting an autonomously-replicating plasmid or integrating into the host genome, single or multiple copies of a required gene without changing the genetic background. This is therefore the method of choice for genetic manipulations of industrial yeast strains.

However, there are limitations since little information is available regarding the genes which are involved in regulating the industrial performance of a given strain. In cases where a character is controlled by a single gene e.g. the introduction of cellulytic, starch or xylan degradation capacities to yeasts there is the formidable problem of selecting the appropriate gene coding for an enzyme, its isolation, integration and adequate expression and secretion in its new host.

Where the genetic characters have not been identified and are important targets for genetic manipulation eg. ethanol tolerance and production, pH and temperature tolerance, carbohydrate assimilation, flavour aspects, these may be controlled by a large number of genes. Current recombinant DNA techniques are suitable for single copy traits or those controlled by only a small number of genes.

A further problem which is not often considered is that of screening. This may be relatively easy for a new enzyme providing it is secreted in an active form, although an inactive gene product can be detected immunologically. However, it may be difficult to screen at the laboratory level for the potential industrial performance of new strains and obtain meaningful results. The use of recombinant DNA for industrial improvement of microrganisms, although limited at present, is still an extremely powerful method when target genes can be identified.

Site-specific mutagenisis enables not only deletion and modification of genes involved in expression but also allows for the insertion of modified genes for specific tasks. The gene product can also be targeted to a particular cell compartment if desired.

Thus an immense potential exists for progress in molecular biology which is hindered by our present basic biochemical understanding.

2.0 MATERIALS AND METHODS.

All chemicals used during the course of this work were of analytical grade as specified by their respective manufacturers unless otherwise stated.

2.1 STARCH GRANULE ISOLATION AND PURIFICATION.

2.1.1 ISOLATION OF STARCH GRANULES.

Starch granules were isolated from barley essentially by the method of *MacGregor et al.* [101] with the substitution of sodium azide for mercuric chloride since mercuric chloride has been shown to inhibit subsequent granule hydrolysis by alpha-amylase [102]. Starch granules, free from the plant debris, were washed with sodium acetate (0.05 M, pH 6.0) five times and then a further five times with distilled water.

After individual washings the granules were collected by centrifugation using a Sorvall RC-5B superspeed centrifuge at 2000 xg. for 30 min. The granules were vacuum dried at 30°C over anhydrous calcium chloride for 48 hr. This low speed centrifugation allowed the separation of broken or fragmented granules from intact granules, without loss of intact small granules. The purification procedure was monitored by electron microscopy from washings prepared by centrifugation at 15000 r.p.m. (14,400 x g.) for 15 min..

2.1.2. SEPARATION OF LARGE AND SMALL STARCH GRANULES.

Large and small starch granules of barley were separated using a modification of the discontinuous sucrose density gradient technique initially carried out by *Duffus et al* for wheat starch granules [48]. Sucrose (0.5ml. of a 95% w/v aqueous solution) was overlaid with 5 ml. of a 60% w/v aqueous sucrose solution. Large and small starch granules were layered onto the surface and the gradient centrifuged at 40 x g for 6 min. in a swing out rotor. Small granules remained within the 60% sucrose layer, which were carefully removed. The large and small granule aggregates were resuspended from the pellet and reloaded onto a similar gradient until a satisfactory separation was achieved as judged by light microscopy.

2.1.3. PROTEIN REMOVAL.

Surface albumins and globulins were removed from the large and small starch granules (4.0g.) of barley by two 2hr. extractions with sodium chloride (20 ml, 0.5 N) at 25°C. Hordein was removed by two 3hr. extractions using 20 ml. of the aqueous solvent mixture propan-1-ol (50% v/v), acetic acid (1% v/v) and 2-mercaptoethanol (1% v/v) at 25°C in an orbital shaker (100 r.p.m.). The extraction supernatants were dialysed overnight and the precipitated material was collected by centrifugation and lyophylised.

2.1.4. REMOVAL OF STARCH GRANULE SURFACE PROTEIN USING PROTEINASES.

Surface proteins were removed from starch granules using Pronase E (Type XXV, Sigma Chemical Company). The proteinase was shown to be free from exo or endo-amylase activity. Intact starch granules (2 mg/ml) were incubated with pronase E (1 mg/ml) in Tris/HCl buffer,(pH 7.3, calcium chloride, 8 mM) for twelve hr. at 37°C. The granules were collected by centrifugation (2,300 x g) for 10 min., resuspended in sodium acetate buffer (0.2M, pH 5.5, calcium chloride 1mM) agitated briefly and recovered by centrifugation, the washing procedure was repeated five times.

2.1.5. REMOVAL OF LIPIDS AND AMYLOSE DETERMINATION.

This was carried out as previously described by *Morrison et al.* [103].

2.2. BINDING OF ALPHA-AMYLASES TO BARLEY STARCH GRANULES.

Alpha-amylase (5.7 I.D.U.), isolated from germinated barley was incubated with purified starch granules (2.75 mg/ml).in sodium acetate buffer, (0.02M, pH 5.5) with calcium chloride, (1 mM) in a total volume of 10 ml at 25°C in a shaking water bath. For incubations with salivary alpha- amylase, phosphate buffer (0.02 M, di-hydrogen ortho-phosphate, di-sodium hydrogen otho-phosphate pH7.0, sodium chloride 1mM) was used. Aliquots (1ml) were removed at appropriate time intervals and centrifuged at 15,000 x g for 2 min. to pellet the granules. The supernatant solution was divided equally. To one aliquot Somogyi-Nelson reagent (0.5ml) was added to inactivate the enzyme. This sample was subsequently used to determine the extent of granule degradation. Alpha-amylase activity was determined in the second aliquot (10 µl.) using cross-linked amylose (x15, 20 mg.) as substrate. Incubation was carried out for 3 min. and activity was assessed as described by *Mateescu et al* [104]. The free enzyme activity present in solution was expressed as a percentage of the initial activity

2.3. LECTIN BINDING.

Fluorescein isothiocyanate-(F.I.T.C.) labelled Concanavilin-A was obtained from Sigma Chemical Co. and was dialysed in the dark for twelve hr. at 4°C against incubation buffer, (Tris-HCl, 0.01M, pH 7.8) containing calcium chloride (1mM) and magnesium chloride (1mM) before use. No decrease in fluorescence was observed after dialysis.

2.4. INCUBATION OF STARCH GRANULES WITH F.I.T.C.- LABELLED CONCANAVILIN-A.

Aliquots of Concanavilin-A in incubation buffer were added to acid-rinsed glass flasks containing starch granules (1-30 mg), dispersed in incubation buffer (section 2.3, total volume 3 ml). The suspensions were incubated at 25°C on a shaking water bath for 60 min. The incubation mixtures were then centrifuged in acid-rinsed glass tubes for 5 min. at 3,000 x g. The fluorescence of the supernatant was measured in a spectrofluorometer (Amico-S.P.F.-125), exitation 490nm and emission 515nm.

2.5. INITIATING DAMAGE ON WHEAT STARCH GRANULES.

Wheat starch granules (1g) were milled, (Macrone Micronising Mill) for fixed periods of time. After milling, the damaged granules were dispersed in an aqueous solution of sodium chloride (0.1M, 50 ml). The mixture was centrifuged at 5000 x g for 2 min. and the granular pellet was resuspended in Tris-HCl buffer (pH 8.0, 0.1M, 1% w/v SDS). This mixture was vortexed for 3 min. and centrifuged as above for 2 min. The resulting pellet was washed (x 3) with glass distilled water and the granules were dried under vacuum . Dried granules (1-30mg) were taken for incubation with F.I.T.C.-labelled Concanavilin-A (100µg) as described previously Measurement of free and bound lectin was carried out as indicated above.

2.6. FLUORESCENT MICROSCOPY.

The distribution of F.I.T.C.-labeled Concanavilin-A bound to the starch granule surface was visualized using a Zeiss microscope with Epi-fluorescent condenser 111 R.S. and B.G.12 filter.

2.7. SCANNING ELECTRON MICROSCOPY.

Purified and modified starch granules were gently layered onto the surface of carbon adhesive paste bound to pin-type stubs and coated under vacuum with a thin layer of gold-palladium. Granules were examined in an electron microscope (JEOL JEM 100c.x. A.S.I.D.).

2.8. PURIFICATION AND ACTIVITY OF ALPHA-AMYLASE.

Total barley alpha-amylase was extracted according to *MacGregor et al.* [105] from the whole kernels of cultivars Clipper, Betzes or C.I. 3576 and germinated for 5 days at 25°C in the dark. Separation of alpha-amylase-1 and alpha-amylase-2 from barley cultivar Clipper was carried out as above but Polyvinylpyrilodone (1mg/ml) was added to the malt extract before the heat inactivating stage.

Alpha-amylase activity was determined at 37°C using a 1% w/v solution of "Merk" soluble potato starch (0.5 ml) in sodium acetate buffer (0.2M, pH 5.5) with calcium chloride (1mM) as substrate. Two µl of purified total alpha-amylase and purified alpha-amylase-2 liberated 66 µg and 42 µg of apparent glucose respectively in 3 min.

2.9. ENZYMIC DIGESTION OF LARGE AND SMALL STARCH GRANULES.

Purified large and small starch granules (2.75 mg/ml) were incubated with alpha-amylase (175 µl) as described above (section 2.8). Portions of the digest (0.15 ml) were removed at intervals over a 60 min. period and the enzyme inactivated with mercuric chloride (1% w/v). The samples were centrifuged at 15,000 x g for 3 min. and the supernatants were assayed for total soluble carbohydrate[105] using a L.K.B. Biochrom Ultraspec 5040.

2.10. PAPER CHROMATOGRAPHY OF CARBOHYDRATES.

Descending paper chromatograph was performed at room temperature on Whatman 1 Chr chromatography paper using the solvent butan-1-ol- pyridine-water (6:4:3) The chromatograms were developed using the alkaline silver nitrate procedure outlined by *Trevelyan et al* [107].

2.11. PAPER ELECTROPHORESIS AND ANALYSIS OF HORDEIN FOR THE PRESENCE OF CARBOHYDRATE.

Hordein (1 mg) isolated from kernels of barley cultivar Clipper was suspended in 5 ml. of water and added to di-Ethyl-Ether (20ml). The mixture was agitated for 5 min. and the insoluble hordein fraction was allowed to sediment. The ether was decanted and residual ether was removed under a stream of nitrogen. The hordein was hydrolyzed with 1 M HCl (1.5 ml) for 3 hr. at 100°C under nitrogen. The hydrolysate was neutralized with 1 M sodium hydroxide and the solution was added to a 5 ml. column of Dowex ion exchange resin (CO_3 form, 200-400 mesh). The column was washed with 5 bed volumes and the eluate was collected and lyophilized. The sample was dissolved in 50µl of water and an aliquot (10µl) was spotted onto Whatmans filter paper (1 Chr.) Paper electrophoresis was carried out as described by *Tate* [108].

Reducing sugars were detected as outlined by *Trevelyan et al* [107].

2.12. PREPARATION OF SALIVARY ALPHA AMYLASE.

Salivary alpha amylase was prepared from saliva collected from the author in the morning before food or beverage. Saliva, (10 ml) was added to phosphate buffer (0.2M, pH6.8, 1mM sodium chloride) and centrifuged for 30 min. at 20,000 r.p.m. (16,000 x g). The supernatant was carefully decanted and the resulting enzyme solution utilized in further experiments. The enzyme solution was assayed for the presence of maltase activity by paper chromatography after incubation with maltose and by incubation with alpha-PNPG, the activity was 40 I.D.U./ml.

2.13. DIGESTION OF PURIFIED BARLEY STARCH GRANULES WITH PHOSPHOLIPASE-C.

Barley starch granules (2.5 mg/ml) were incubated for 4 hr at 37°C with Phospholipase-C Type X11, (2.0 U/ml) obtained from Sigma Chemical Co., in Tris/HCl buffer (0.02 M, pH 7.3). After incubation the starch granules were centrifuged at 10,000 x g for 5 min., washed twice with water and three times with sodium acetate buffer (0.2M, pH5.5, calcium chloride 1Mm). Enzyme digestions and binding experiments were carried out as described previously (section 2.2 and 2.9).

2.14. DIGESTION OF STARCH WITH BETA-AMYLASE.

Gelatinized starch and derivatized starch granules or soluble starch were incubated with of Beta-amylase (30 U), Type 1-B, Sigma Chemical Company, in sodium acetate buffer (0.05M, pH 4.8, and or pH 5.5) at 30°C. Aliquots were removed at appropriate periods and analysed for the presence of reducing sugars [109] or total carbohydrate [106].

2.15. DIGESTION OF GELATINIZED STARCH WITH ISO-AMYLASE AND PULLULANASE.

Isoamylase [E.C. 3.2.1.68] was obtained from "Sigma" Chemical Company.

Gelatinized starch granules (200 mg) were dispersed in dimethylsulphoxide (1.0 ml) overnight and precipitated with five volumes of ethanol. The precipitated carbohydrate was vacuum dried and dispersed in boiling water prior to enzyme incubation. Incubation was carried out in sodium acetate buffer (0.05M, pH 3.5, Isoamylase 300 I.U./ml) for 12 hr. at 37°C.

2.16. DIGESTION OF GELATINIZED STARCH GRANULES WITH PULLULANASE.

Pullulanase [E.C. 3.2.1.41] was obtained from "Sigma" Chemical Company.

Modified and native starch granules were boiled in double distilled water until no clear gel was visible, approximately 5 min. The suspensions were allowed to return to room temperature prior to incubation with pullulanase. Incubation with pullulanase (2 I.U.was carried in sodium acetate buffer (0.05M, pH 5.5) at 30°C for 12 hr. Where a double incubation with isoamylase and pullulanase was required, the incubation buffer was adjusted to the pullulanase incubation requirements after isoamylase incubation.

After pullulanase and isoamylase treatment, the solution was adjusted to pH 7.0, heated to boiling point to inactivate the enzymes and centrifuged at 15,000 xg for 5 min. The supernatant was adjusted to 0.05 M sodium hydroxide and applied to the appropriate chromatography column for fractionation.

2.17. GEL PERMEATION CHROMATOGRAPHY OF DERIVATIZED AND NON DERIVATIZED POLY/OLIGO-SACCHARIDES.

Derivatized and non derivatized carbohydrates in a total volume of 4 ml were applied to glass columns (1.6cm x 78cm) containing Sepharose CL-2B, Sephadex G-150 or Sephadex G-50 (all gel filtration media were obtained from Pharmacia Fine Chemicals). The columns were equilibrated and eluted with sodium hydroxide (0.05 M) and sodium azide (0.02%) at 25°C. A carbohydrate recovery of 92-96% was observed. All samples were analysed in triplicate

2.18. HYDROXPROPYL SUBSTITUTION OF STARCH GRANULES.

Samples of purified starch granules (2 gm) were mixed with sodium hydroxide (0.25 M) and di-sodium sulphate (0.85 M) in a total volume of 2.4 ml at 25°C in air tight glass containers. The samples were placed in a water bath at 35°C and propylene oxide (0-400 µl) were added. Incubation was carried out for 24 hr. with constant agitation. The alkali conditions were neutralized with sulphuric acid (0.01 M) and the substituted granules centrifuged at 15000 x g for 5 min. The supernatant was decanted and the substituted granules were washed (x5) with double distiled water and dried.

When required for enzymic degradation the granules were solubilized by boiling for 5 min in double distilled water and buffered to the required pH.

2.19. DETERMINATION OF MOLAR SUBSTITUTION.

Hydroxypropyl substitution of derivatized starch granules was determined by the spectrophotometric method developed by *Johnson* [110].

2.20. PREPARATION AND INCUBATION OF CEREAL ALPHA-AMYLASE USING CROSS LINKED AMYLOSE.

Amylose obtained from Sigma Chemical Co., was cross linked with 1-chloro-2,3-epoxypropane as described by *Mateescu et al* [104]. Incubations were performed using cross-linked amylose (30mg) and starch granule free incubation supernatant (50 µl) for 3 min. at 37°C in sodium acetate buffer (0.02 M, pH5.5, calcium chloride 1mM) unless otherwise indicated. Colorimetric determination of enzymic activity was as outlined in reference [104].

2.21. GROWTH CONDITIONS FOR ASPERGILLUS SPECIES.

Conidia were germinated in a liquid medium containing potassium di-hydrogen phosphate (1.5%), ammonium sulphate (0.5 %), magnesium chloride (0.06%), calcium chloride (0.06%), proteose peptone (0.15%), urea (0.03%),and either sucrose (2%), soluble starch (1 %), obtained from B.D.H. Chemical Company, Sydney or oat spelt xylan (0.5 %) obtained from Sigma Chemical Company. For the isolation of m-RNA, cultures were incubated at 30°C under continuous agitation for 48 hr. in the case of sucrose and 96hr. for starch and xylan.

2.22. DETERMINATION OF XYLANASE AND AMYLASE ACTIVITIES.

Xylanase and amylase activities were monitored in the culture supernatant for 2 to 6 days growth in xylan or soluble starch. Hyphae were recovered by filtration.The medium (100 ml), was centrifuged at 10,000 x g for 30 min. and the supernatant concentrated 20 fold with an Amicon Ultra-filtration cell equipped with a P.M. 10 membrane. Aliquots were assayed for enzymic activity using pullulan as substrate for glucoamylase activity, soluble potato starch for total amylase activity, alpha-phenyl-D-glucopyranoside for alpha-glucosidase and oat spelt xylan for total xylanase activity.

2.23. ISOLATION OF GENOMIC DNA FROM ASPERGILLUS SPECIES.

Hyphae were collected as described above and washed several times with double distilled water. The hyphae (~15 gm wet weight) were frozen immediately in liquid nitrogen and ground finely in a precooled mortar and pestle. The fragmented hyphae were transferred to a glass flask containing phenol/chloroform (20 ml), Sarcosyl N30 (1.5 ml of a 30% aqueous solution) and 18 ml. of DNA extraction buffer (Tris-HCl 0.02M, pH8.5, Magnesium chloride 0.05M, EDTA 0.005M). The mixture was agitated gently with a magnetic stirrer for 1.5 hr. at 4°C. then centrifuged for 10min. at 10,000 x g. The aqueous phase was removed and re-extracted with fresh phenol/chloroform solution (20 ml) for 15 min. Phase separation was achieved by centrifugation as before and the upper aqueous phase removed. Sodium acetate (3 ml of a 3 M aqueous solution, pH 4.8) were added and mixed gently. Ethanol (60 ml) was added and the mixture agitated gently by inversion. The mixture was placed at -20°C for 1 hr. and the genomic DNA was collected as a precipitate by centrifugation at 15000 x g for 30 min. at 4°C. The precipitated DNA was washed several times with ethanol (80%) and dried under a gentle stream of nitrogen The dried DNA pellet was dissolved in TE buffer (Tris-HCl 0.01M, EDTA, 0.001M, pH7.5).

2.24 RESTRICTION DIGESTION OF GENOMIC DNA.

Genomic DNA (10 µg) was digested overnight in Eppendorf tubes with the appropriate restriction endonucleases (obtained from Pharmacia or Boehringer Molecular Biological Products) the amounts and conditions were as specified by the manufacturers.

2.25. ISOLATION OF m-RNA FROM STARCH AND XYLAN INDUCED CULTURES OF ASPERGILLUS SPECIES.

Hyphae (30g) were frozen in liquid nitrogen and ground in a mortar and pestle as indicated above (2.23). Homogenization buffer (30 ml of an aqueous solution of guanidine-HCl 6 M, sodium acetate 0.2 M and mercapto-ethanol 1 mM, pH 5.2) were added, and the suspension passed through a French Pressure Cell at 12,000 p.s.i.

The suspension was centrifuged at 10,000 x g for 20 min. at 4°C. and the supernatant decanted. Total RNA was precipitated by the addition of 1/2 a volume of Ethanol (95%) and refrigeration at -20°C for 1 hr. The precipitated RNA was collected by centrifugation at 15,000 x g for 20 min. at 4°C. The supernatant was discarded and the RNA pellet was dissolved in homogenization buffer (15 ml with the addition of EDTA 0.01 M). RNA was re-precipitated dissolved in 10 ml. of Tris-HCl (0.1 mM, pH 8.5), urea (7 M), EDTA (0.1 M), and SDS (0.1%w/v). An equal volume of phenol/chloroform was added and the mixture agitated till an emulsion was formed. Phases were separated by centrifugation at 10,000 x g for 10 min at 4°C. The upper aqueous phase was carefully removed and the phenol/chloroform phase re-extracted with 10 ml. of Tris-HCl buffer (0.1 M, pH 8.5), EDTA (0.1 M), SDS (0.1%w/v). The suspension was mixed and the phases separated as above. The upper aqueous phases were pooled and re-extracted with phenol/chloroform solution.

The RNA was precipitated from solution by the addition of potassium acetate to a concentration of 0.1 M (pH 5.0), 2 volumes of ethanol (95%) and refrigeration at -20°C overnight. The RNA was collected by centrifugation at 15,000 x g for 30 min. at 4°C and washed twice with ethanol (70%v/v). The RNA pellet was dried under a stream of nitrogen, dissolved in diethyl pyrocarbonate treated double distilled water and stored until required at -80°C.

2.26. PURIFICATION OF POLY ADENYLATED m-RNA

Poly-Adenylated m-RNA was purified by affinity chromatography with oligo-d- Thymidine linked cellulose, (Boehringer Mannheim) as outlined by Perbal [111]. The m-RNA was stored as a precipitate under ethanol at -80°C until required.

2.27. PREPARATION OF c-DNA FROM POLY-ADENYLATED m-RNA

Approximately 1 µg. of m-RNA was dissolved in sterile water. Oligo-d-T, (12-18) primer (100 ng) was annealed to the m-RNA after heating at 95°C for 1min and quickly cooling in ice. First strand synthesis was obtained using Tris-HCl (0.025 M pH8.3), Sodium chloride (0.004 M), Magnesium chloride (0.003 M) and DTT (0.0025 M), human placental RNAase inhibitor (5 U), AMV Reverse Transcriptase (Pharmacia, 5 U) , d-GTP, d-ATP, and d-TTP (1.25 mM), 1 µl. of ^{32}P-d-CTP (10 µCi/ml.) in a total volume of 30 µl. Each incubation was carried out at 50°C for 15 min. An aliquot of d-CTP (1 µl of a 10 mM solution) was added to the incubation mixture and incubation was continued for a further 60 min. The reaction was terminated by the addition of EDTA to a final concentration of 0.02 M.

An equal volume of phenol/chloroform was added to the mixture and mixed, the upper aqueous phase was recovered and the c-DNA/m-RNA hybrid precipitated with ethanol, sodium acetate . After washing with sodium acetate and drying, the sample was dissolved in sterile water (10 µl) and an aliquot was removed and run on an 8% w/v polyacrylamide gel in order to assess the approximate size range of the hybrid. To the remaining sample, second strand synthesis buffer was added. This consisted of Tris-HCl (0.2 M, pH7.5), magnesium chloride (8 mM), ammonium sulphate (10 mM), potassium chloride (40 mM), beta-NADH

(0.15 mM), d-NTP's (50 µM), E.coli RNAase H (2 U) obtained from Pharmacia, E.coli DNA ligase (5 U) obtained from Pharmacia and Klenow Fragment of DNA Polymerase1 (10 U) obtained from BRESA, to a final volume of 50 µl.

Incubation was carried out at 15°C for 1 hr. then at 30°C for a further 1 hr. The reaction was terminated by the addition of EDTA to 0.02 M and the mixture was extracted with phenol/chloroform, precipitated and dried. The c-DNA was incubated with S1- nuclease obtained from B.R.L. Research Laboratories (10 U in sodium acetate 0.05 M, pH 4.5, sodium chloride 0.2M, zinc sulphate 0.01M, glycerol 0.05%) total volume 30 µl for 45min. The reaction was stopped by the addition of 3 µl of Tris -HCl buffer (1 M, pH8.0). The S-1 nuclease treated c-DNA was then precipitated and dried as described previously.

The c-DNA was dissolved in Tris-HCl (0.05 M, pH 7.4), magnesium chloride (0.07 M), DTT (0.01 M) and Klenow Fragment of DNA Polymerase1 (1 U). Incubation was at 30°C for 30 min. and the c-DNAwas then treated with phenol/chloroform, precipitated and dried. Eco-R1 or Hind-111 linkers were attached to the c-DNA in an overnight ligation with T-4 DNA ligase. Restriction endonuclease digestion was carried out with Hind 111 or Eco-R1 and the digested c-DNA was separated from unligated linkers on a 1% w/v agarose gel. DNA >150 base pairs was isolated and purified using DEAE membranes as outlined by *Smith et al* (section 2.35). The c-DNA was now ready for cloning into plasmid vector pUC19.

2.28. TRANSFORMATION OF E. coli CELLS.

E.coli strains HB-101, JM-101 or N4380-1were prepared for transformation by the SOB/SOC method of Hanahan [112].

Transformation efficiencies were between 10^6-10^7/µg of plasmid DNA.

Transformants were detected by plating onto LM agar and ampicillin. Recombinant plasmid identification was aided by the use of X-gal in the plates for JM-101 transformants. The

N4830-1 strain was used in combination with the protein-A gene fusion plasmid pRIT-2T (Pharmacia). Transformants were selected for ampicillin resistance or in some instances an attempt was made to assess expression by the utilization of specific substrates.

2.29 ISOLATION OF PROTEIN-A GENE FUSION PRODUCTS.

Protein-A gene fusion products were visualized on 8 or 12.5% w/v SDS-PAGE after purification of the expressed product by affinity chromatography as outlined by the suppliers.

2.30. PREPARATION AND ^{32}P-LABELLING OF OLIGO-NUCLEOTIDE PROBES.

Two 30-mer single stranded oligo-nucleotide probes complementary to the C-terminal and middle sequences of the Aspergillus awamori glucoamylase structural gene were synthezised by Dr. W. Bottomley (C.S.I.R.O. Division of Plant, Industry, Black Mountain, Canberra) using an Applied Biosystems oligo-nucleotide synthesizer. The crude fragments were partially purified and end-labeled with gamma ^{32}P-ATP using T4- polynucleotide kinase (Pharmacia) as outlined by *Maniatas et al* [113].

2.31. CONDITIONS USED FOR PRIMER EXTENSION

Total m-RNA (10 µg) dissolved in Tris/acetate buffer (0.05 M, pH 8.3), Sodium chloride (0.05 M), EDTA, (0.5 mM), DTT (0.005 M), Magnesium acetate (0.008 M), d-ATP, d-TTP, d-GTP (0.5 mM), ^{32}P-d-CTP, (2 µl, 10 mCi/ml), RNAase inhibitor (Pharmacia, 5 U), AMV Reverse Transcriptase (Pharmacia, 10 U) and 2 µl of oligo-nucleotide primers (50 ng) total volume of 50 µl. Fragments of c-DNA (~50 ng) were also utilized for primer extension. Single strand DNA was prepared using a strand separating gel [114].

Incubation was carried out at 60°C for 15 min, 50°C for 30 min. and 40°C overnight. Cold d-CTP (1 µl of a 10 mM solution) was added 15 min. after the start of the reaction. At the end of the reaction an aliquot of the reaction mixture was run on a 5% w/v polyacrylamide gel and the size of the DNA products assessed by autoradiography. Primer extension products from the glucoamylase probes were dissolved in restriction endonuclease buffer. For the C-terminal probe a double digest using Sal 1, and Pst 1 was carried out and for the probe complimentary to the middle segment of the glucoamylase structural gene, a double digest using Rsa 1 and Pst 1 was utilized.

The material was incubated for 4 hr. and the reaction mixture extracted with phenol/chloroform, ethanol precipitated and dried as before. Samples were electrophoresed on a 1.5% w/v agarose gel and fragments corresponding to >400 bp were removed. The eluted fragments were ligated to a Blue-scribe vector and used in transformations of E.coli (J.M101). White ampicillin resistant colonies were picked and the inserts excised and analysed on 8% w/v polyacrylamide and 1.5% w/v agarose gels after digestion with the appropriate restriction endonucleases. The potential xylanase primer extension products were treated with S-1 nuclease and DNA polymerase-1 (Klenow Fragment) and ligated to a Sma 1 digested Blue Scribe vector.

Selection was carried out as outlined above.

2.32. PREPARATION OF ^{32}P-LABELED PROBES FOR SOUTHERN HYBRIDIZATION.

Probes for Southern hybridization were prepared by Nick Translation [115] or using the random primer method [116].

2.33. CONDITIONS USED FOR SOUTHERN HYBRIDIZATIONS.

Southern hybridization was carried out by the method of *Reed,* [117] on Nytran N.Y. membranes (Schleicher and Schuell W. Germany), in some cases P.E.G. 6000 (10%) was substituted [118] for dextran sulphate.

2.34. AGAROSE AND POLYACRYLAMIDE GEL ELECTROPHORESIS.

Agarose and polyacrylamide gel electrophoresis was carried out as described by *Maniatas et al* 119], [120] respectively.

2.35 RECOVERY OF DNA FROM AGAROSE GELS.

Recovery of DNA fragments from agarose gels was carried out by the procedure of *Smith et al* [121] using D.E.A.E. membranes NA 45, 0.45 μm, (Schleicher and Schuell, W. Germany).

2.36. "DOT-BLOTS".

DNA fragments were dissolved in sterile water and denatured by boiling for 3 min. at 98°C. Aliquots (1 μl containing ≃50-100 pg) were spotted directly onto Nytran NY membranes. The membranes were baked for 2 hr. at 80°C under vacuum prehybridized and hybridized as above (2.35).

2.37. SMALL SCALE ISOLATION OF PLASMIDS.

Small scale plasmid isolation and purification was carried out as described by *Dale, et al* 122].

2.38. IN-SITU HYBRIDIZATION.

In- situ colony hybridization was carried out as outlined by *Dale,et al* [123] from a method originally proposed by *Grunstein et al* [124].

3.0. RESULTS.

3.1. STARCH GRANULE PREPARATION.

Starch granules were prepared as described in the Materials and Methods, (section 2.1), however starch granules have also been prepared by lyophilization [125,126].

To ascertain if any quantifiable damage could be detected both granule preparations were compared by subjecting them to enzymic degradation. Freeze dried starch granule preparations were significantly more susceptible to degradation by salivary alpha-amylase, Fig. 1. (SD: 1.52).

3.2. PROTEIN ADHERING TO THE STARCH GRANULE SURFACE OF BARLEY.

The results depicted in Plate.4, lane 6-9 show SDS-PAGE of salt soluble and hordein proteins remaining on the granule surface after isolation. Clipper granules appeared to possess little affinity for salt soluble proteins (lane 9, Plate. 4) when compared to Betzes (lanes 7, Plate. 4). The salt soluble proteins isolated from kernels of barley cultivars Betzes, C.I.3576 and Clipper are shown in lane 2-4 respectively. The starch granule surface of Betzes and Clipper appear to have differing affinities for hordein fractions, lane 6and 8. The incomplete reduction of disulphide bonds in the Clipper hordein sample (lane 8) cannot be excluded.

Hordein adhering to the starch granule surface, particularly small starch granules, and their detrimental effects on the yield of hot water extract during the brewing process has been

Fig.1 Susceptibility Of Freeze Dried v Vacuum Dried Starch Granules To Salivary Alpha-Amylase.

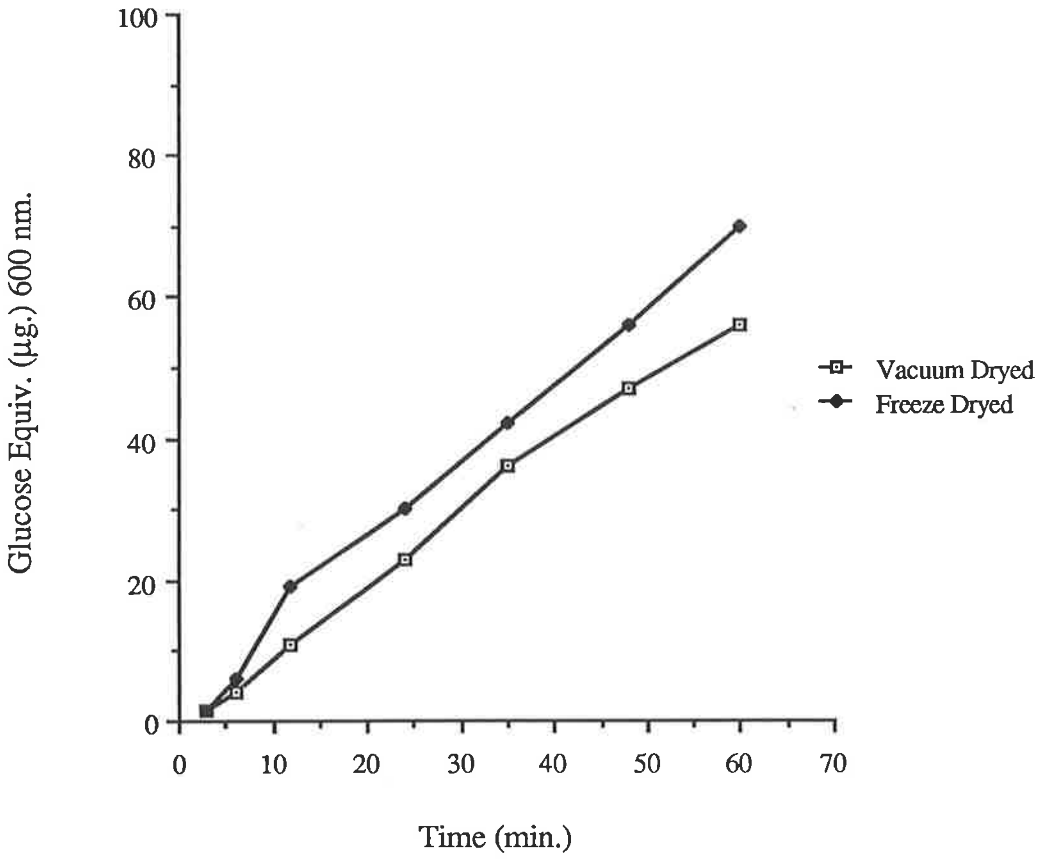


Fig. 2 Barley Granule Susceptibility To Alpha-amylase After Protein And Lipid Removal.

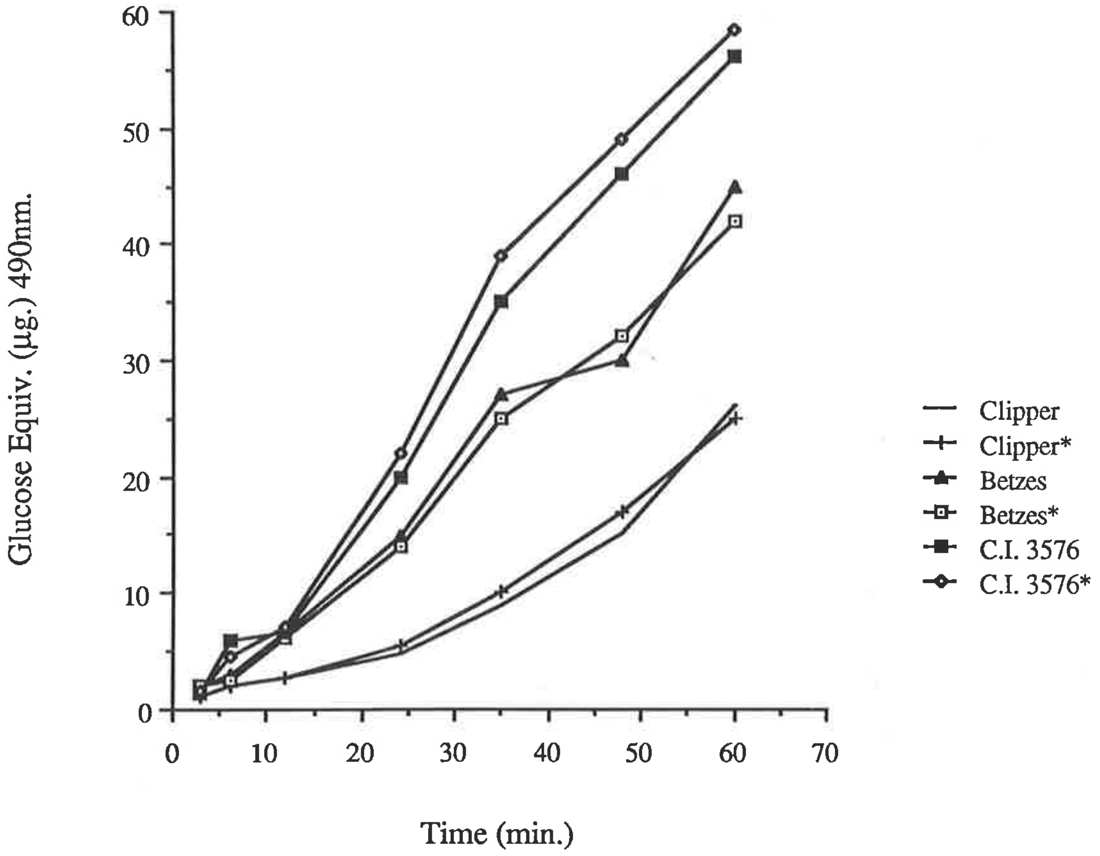


PLATE. 4

SDS-PAGE:

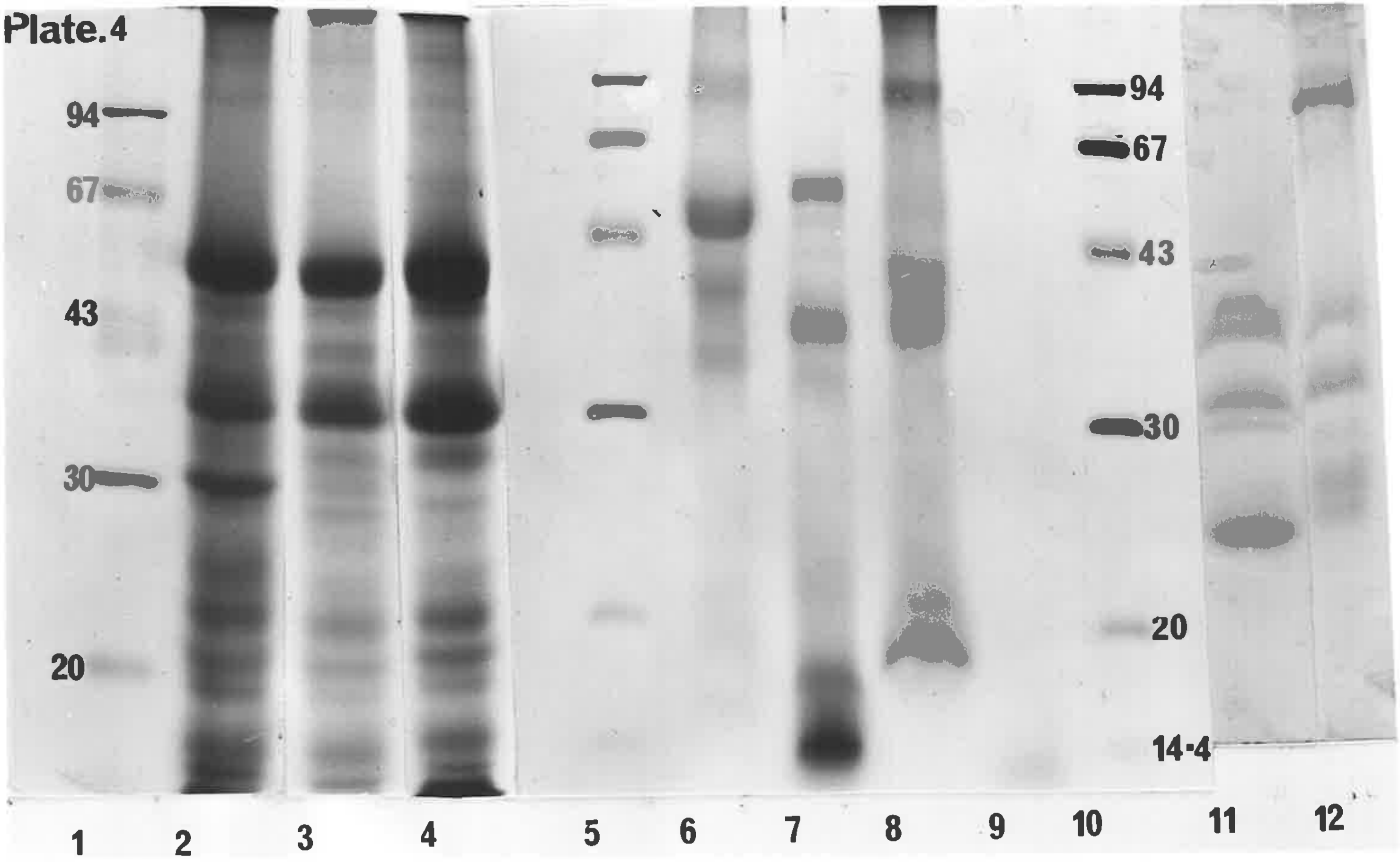
Lanes 2-4: Total Na Cl soluble proteins extracted from whole kernels of Betzes, C.I. 3576 and Clipper. Lanes 2-4 respectively.

Lanes 6 and 8: Propan-1-ol (50%, v/v), mercaptoethanol (1%, v/v) and acetic acid (1%, v/v) soluble surface proteins extracted after starch granule isolation from Betzes and Clipper respectively.

Lanes 7 and 9: Na Cl (0.5M) soluble proteins present on starch granule surface after isolation from Cultivars Betzes and Clipper respectively.

Lanes 11 and 12: "Schieff's" staining of propanol (50% w/v), mercaptoethanol (1% w/v), acetic acid (1%) soluble starch granule surface proteins from Clipper and Betzes respectively.

Plate.4



reported [127]. To study the effect of adhering lipids and proteins on starch granule degradation, purified starch granules isolated from barley cultivars Clipper, C.I. 3576, and Betzes were incubated at 37°C with and without* their presence. The results presented in Fig. 2 show that removal of surface lipids and remaining salt soluble proteins have little effect on granule degradation rate (SD: 1.74). In contrast the removal of the hordein proteins* renders the starch granules more susceptible to degradation, Fig. 3 (SD: 0.96).

During traditional brewing and distillery processes, starch granule degradation occurs over a temperature gradient during mashing, where temperatures reach 65°C and 68°C respectively. To establish whether the removal of hordein had any effect on this industrial process, granules with* and without protein were incubated at 65°C. The results are presented in Fig. 4 and infer considerable inhibition of granule degradation when hordeins are present even at these elevated temperatures (SD: 1.63). Hordein (1-3 mg.) extracted from barley cultivar Clipper was solubilized in ethanol (70% v/v) and added to a purified starch granule preparation from cultivar Clipper. Granules were dried in vacuum and incubated as described previously (section 4.9). Results in Fig. 5 (SD: 0.51). demonstrate that considerable inhibition of granule degradation again occurs in the presence of hordeins.

Starch granules and soluble starch were incubated with Pronase-E to assess the presence of exo and endo-amylase activities in these commercial proteinase preparations. Only Pronase-E displayed the absence of amylase contamination under the conditions utilized in Materials and Methods section (4.1.4.). Pronase-E was used in a 4 hr. incubation with starch granules from cultivar Clipper where surface hordeins were not removed prior to incubation with alpha-amylase. Results depicted in Fig. 6 (SD: 0.97) indicate similar experimental findings to the results obtained above.

Hordein isolated from barley cultivar Clipper, Betzes and C.I. 3576 were stained with Sheiff's reagent [128] after separation on SDS-PAGE to determine if any carbohydrate was associated with this fraction, Plate. 4 (lane 11-12). These results suggest carbohydrate is present and further analysis after acid hydrolysis and paper electrophoresis showed

Fig. 3 Barley Granule Susceptibility To Alpha-amylase After Removal Of Hordeins.

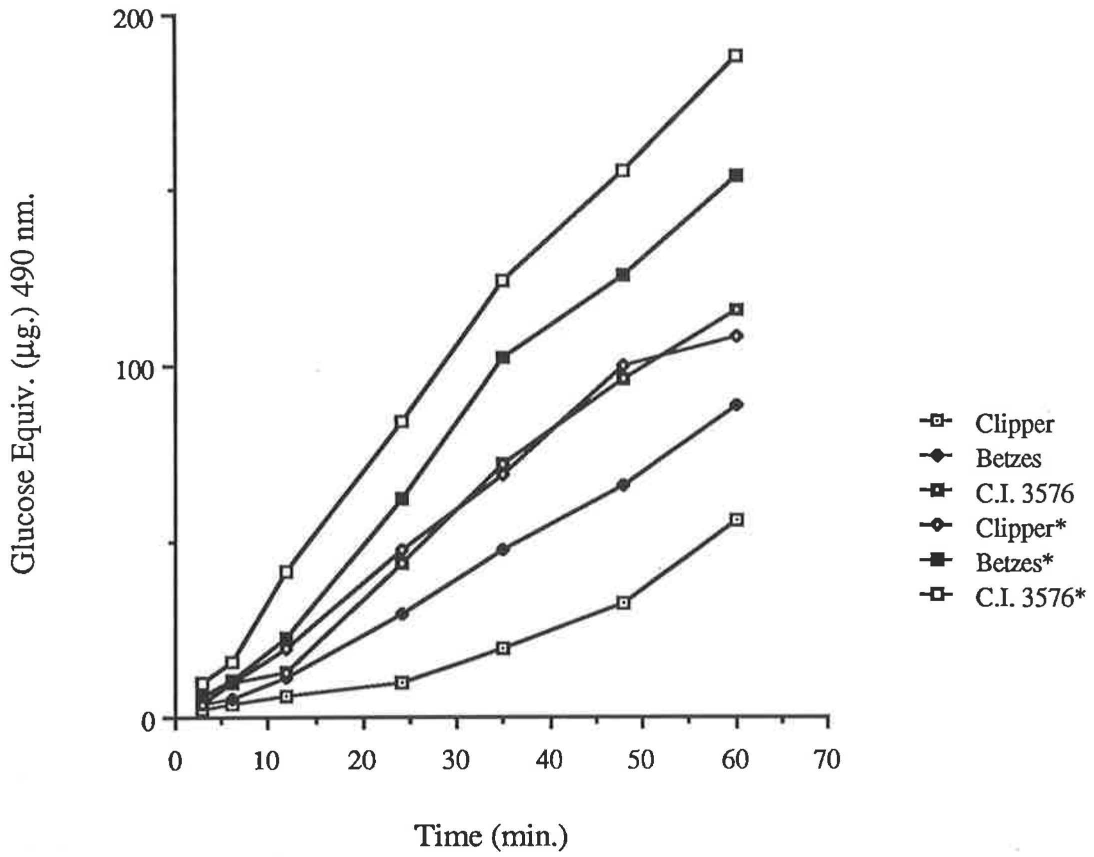


Fig.4 Clipper barley granule susceptibility to amylase action at 65ºC,inthe presence* or absence of the hordein proteins.

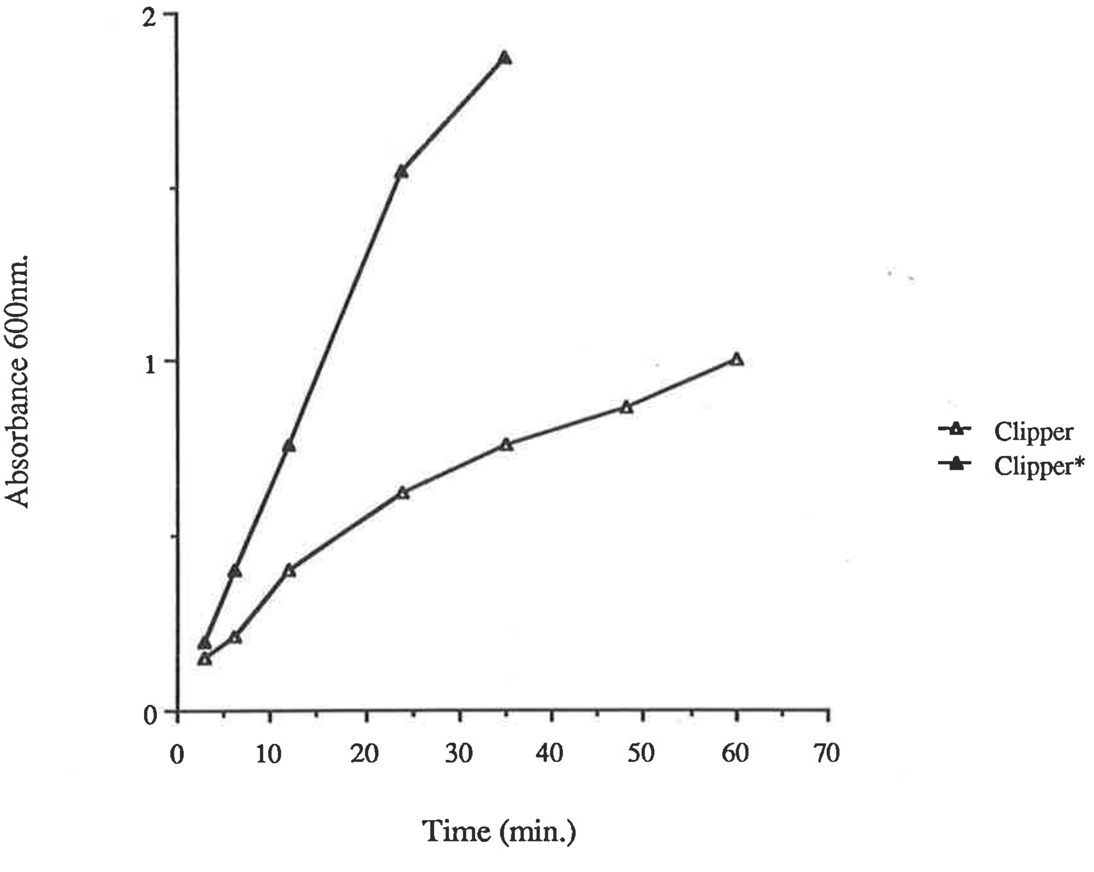


Fig. 5 Effect Of [Hordein] On Barley Granule Suseptibility To Alpha-amylase.

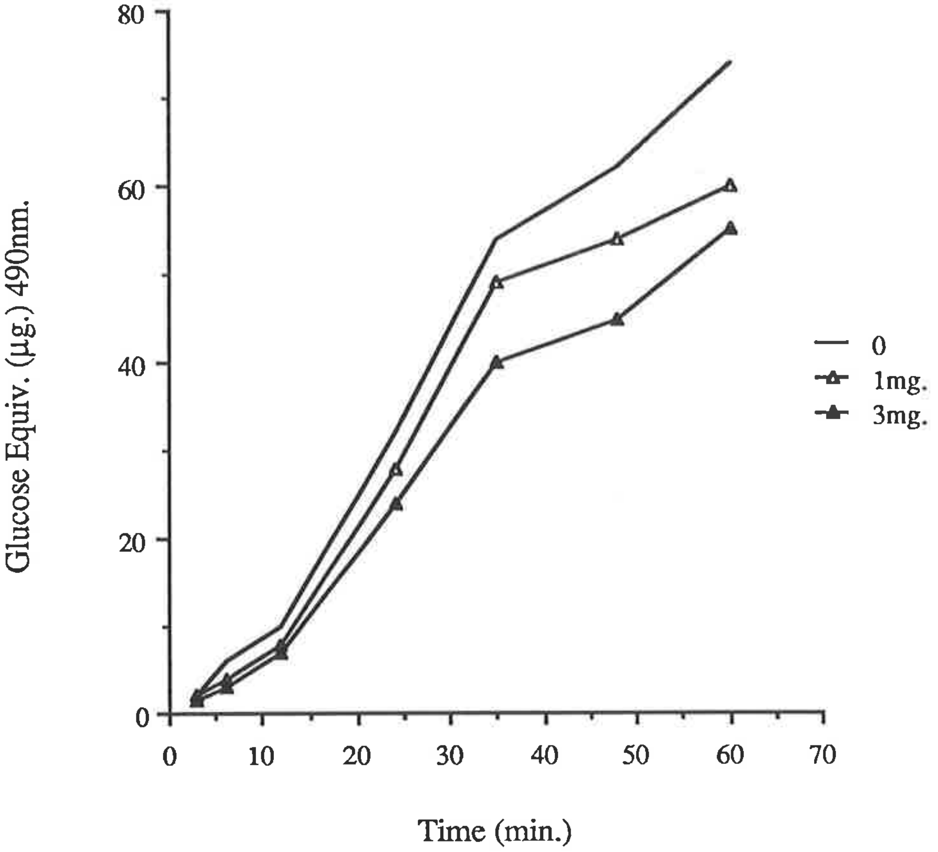
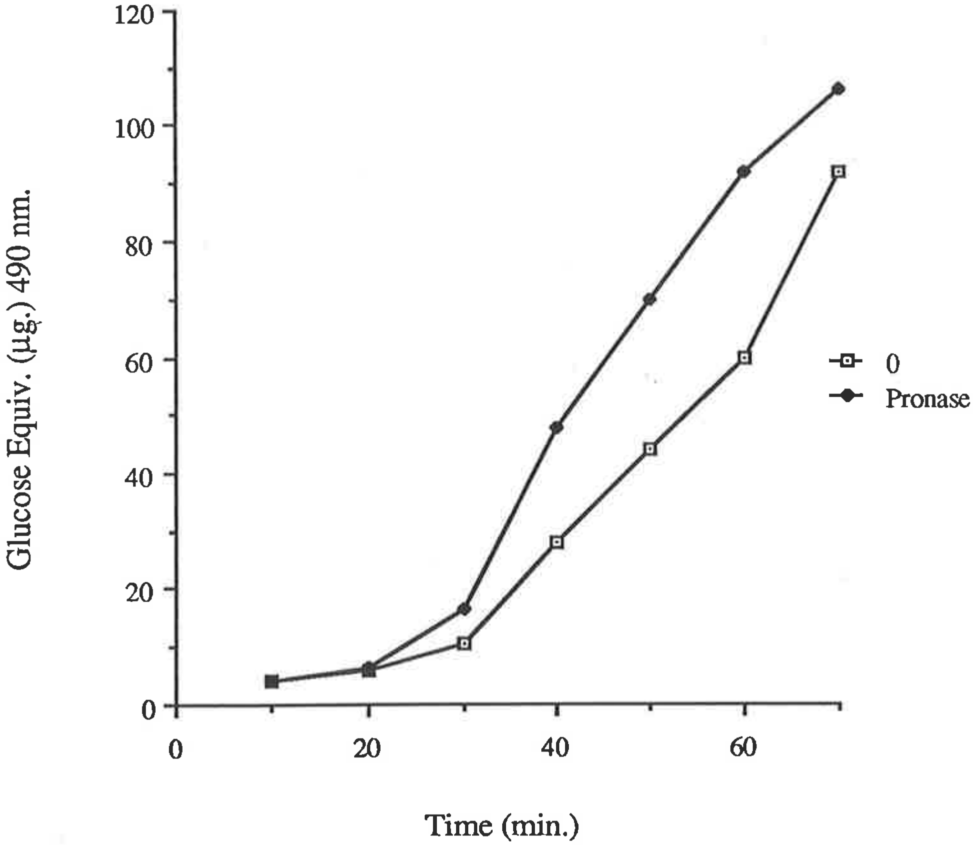


Fig.6 Amylase Degradation Of Barley Granules After Preincubation With Pronase-E.



predominant spots migrating with the same R.F. value as alpha-D-mannosepyranoside and L-fucose.

3.3. Effect Of Calcium Ions On Alpha-amylase Binding To Intact Barley Starch Granules.

Alpha-amylase purified from cultivar Clipper was incubated with barley starch granules isolated from cultivar Betzes with and without calcium chloride. Results presented in Table.1 (SD: 2.04) indicate that there are only small differences in the amount of enzyme bound in incubations with or without calcium ions. Although free calcium ions are required for the structural integrity of the enzyme with regard to its activity [55,56], they do not appear to be involved in binding of enzyme to the granule surface.

3.4. Phospholipid/Calcium Ion Interactions Between Starch Granules And Barley Alpha-amylase.

Monoacyl phospholipids at the surface of intact cereal starch granules and their possible interactions with free enzyme or enzyme complexed with calcium ions was investigated. A Phospholipase-C preparation free of contaminating amylase activity was used to cleave the phosphate moiety from monoacylphospholipids which may protrude from the starch granule. The results of starch granule degradation and the amount of Clipper alpha-amylase bound (%) to starch granules isolated from cultivar Betzes were determined after treatment with Phospholipase-C. The results are presented in Fig. 7 (SD: 0.52) and show degradation rate was not significantly affected. Further the amount of alpha-amylase bound (%), was not altered.

Table 1

Effect Of Calcium Ions On The Binding (%) Of Cereal Alpha-Amylase To Barley Starch Granules.

Time (min.)	Calcium Chloride (2mM.)	Calcium Chloride (none)
3	18.0	20.0
6	20.0	23.0
12	18.1	21.0
24	16.6	23.0
35	26.6	22.5
48	30.4	23.2
60	21.3	24.8

Fig.7 Barley Granule Suseptibility To Amylase After Phospholipase-C Preincubation.

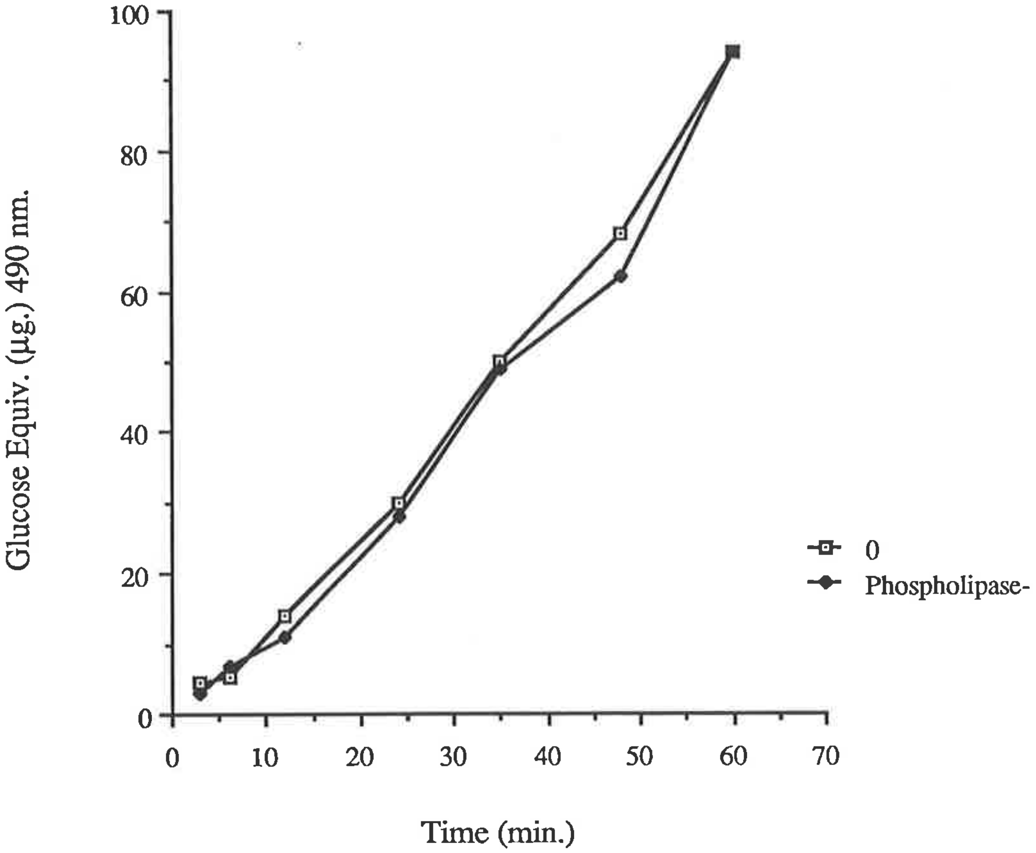
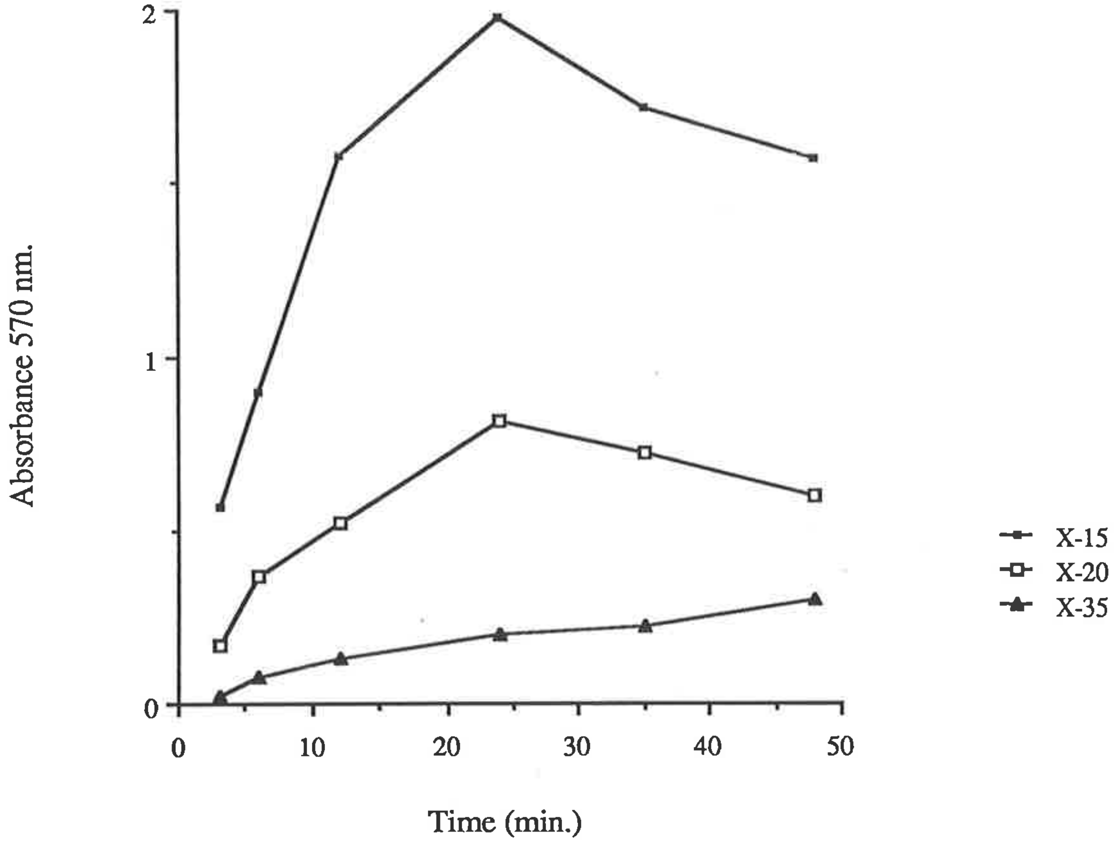


Fig.8 Effects Of Increasing Cross linking On Alpha-Amylase Activity.



3.5. Assessment Of Cross Linked Amylose For The Determination Of Alpha-Amylase Activity.

Cross linked amylose preparations (X-10, X-15, X-20, X-35) [16], were assessed for their suitability as substrates for the determination of cereal alpha-amylase activity during binding experiments. Incubations resulting from alpha-amylase (75 µl) purified from cultivar Betzes with differently crosslinked substrates are shown in Fig.8 (SD: 0.31) and indicate the sensitivity of the method is significantly reduced as crosslinking is increased. Amylose X-15 was selected as an appropriate substrate, with an incubation period of 3 min. and an aliquot (50 µl) of incubation mixture free of starch granules. Longer periods of incubation with higher enzyme activities lead to decreases in iodine binding.

3.6. Percentage Amylose Of Integral Lipid Free and Lipid Containing Starch Granules Isolated From Large And Small Starch Granules Of Barley and Unfractionated Potato, Mannioc, Waxy and Normal Maize.

Integral lipid was removed from purified starch granules using the method of *Morrison et al* [16]. Results presented in Table. 2 show the amount of amylose before and after lipid removal from starch granule preparations.

Increases in the amylose content of the small granules isolated from barley indicated that they may have a much greater association with integral lipids than the larger granules.

Table 2

Amylose Content As A Percentage Of Dry Matter of Barley,Potato And Maize Starch Granules Before[Apparent Amylose %]And After[Amylose %]Removal Of Integral Lipids.

Starch Type.	Amylose (%)	ApparentAmylose (%).
Clipper (large granules)	27.9	19.6
Betzes (" ")	28.8	20.0
C.I. 3576(" ")	28.9	24.0
Clipper (small granules)	20.0	15.2
Betzes (" ")	22.2	12.2
C.I. 3576(" ")	23.5	20.0
Waxy Maize	2.5	N.D.
Maize	28.1	N.D.
Potato (unfractionated)	28.4	N.D.
Mannioc (unfractionated)	24.1	N.D.

3.7. Digestion Of Starch Granules With Barley Alpha-Amylase From Potato, Normal And Waxy Maize, Mannioc, Fractionated And Unfractionated Barley.

3.7.1. Barley Alpha-Amylase Digestion Of Starch Granules and Electron Micrographs Of Digested And Undigested Granules.

Electron micrographs of intact potato, maize, large and small granules from barley are shown in Plates. 5 a-e. Electron micrographs illustrated in Plates.5 f-m show the well documented attack pattern of alpha-amylase incubated with intact normal and waxy maize, large and small granules purified from barley and potato granules. The susceptibility of these and mannioc starch granules to barley alpha-amylase isolated from cultivar Betzes is shown in Figs. 9-11 (SD: 1.03).

Root starches (B-form) are significantly more resistant to enzymic attack than the cereal starches (A-form). However the degree of susceptibility to initial enzyme attack differs significantly within cereal cultivars eg. in fractionated and unfractionated Clipper, C.I.3576 and Betzes (Fig. 3, 9 and 10). Large and small granules of these cultivars also differ in their susceptibility to alpha-amylase. Attack appears to be dependent on the source of the enzyme, ie. glucoamylase appears to degrade intact starch granules via surface erosion and does not cause the formation of pits [129] and the granule type eg. potato starch granules appear to be degraded from the distal ends whereas cereal granules are degraded in well defined areas often concentrated at the midrib (Plates. 5 i and 2 j respectively).

Granule degradation is also dependent on enzyme concentration, low enzyme concentrations do not result in the well defined pitted regions clearly observed when higher enzyme concentrations are used. Small starch granules from barley appear to be degraded by surface erosion and show no apparent detectable pitting even at high enzyme concentrations [130]

Plates 5 a-b.

Electron Micrographs.

Plate. 5 a:	Intact potato starch granules, (magnification x 3,600)
Plate 5 b:	Intact corn starch granules, (magnification x 2,100)

Plates.5a-b

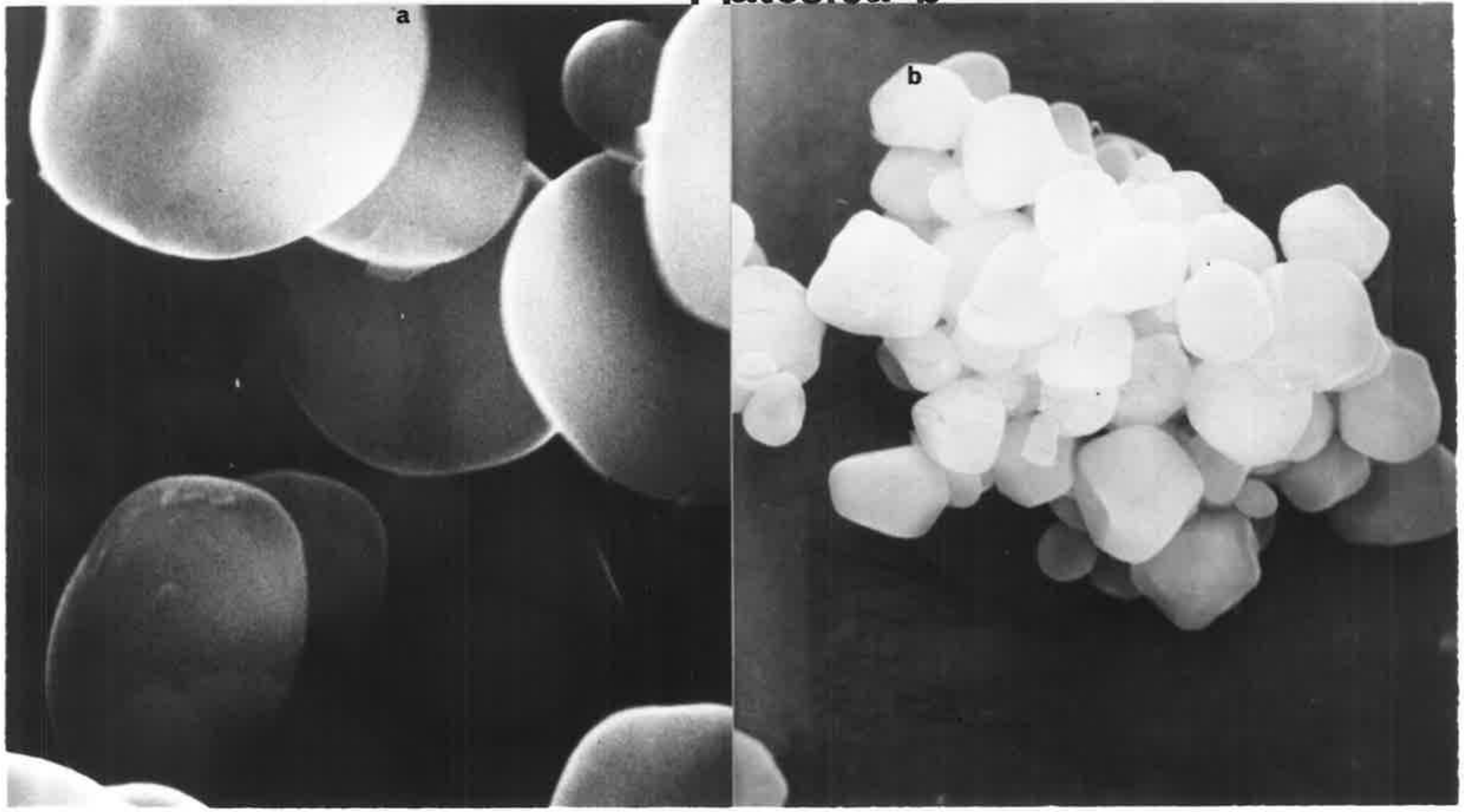


Plate. 5 c.

Electron Micrographs

Plate 5 c: Intact large starch granules of barley from cultivar Clipper.

Plate.5c

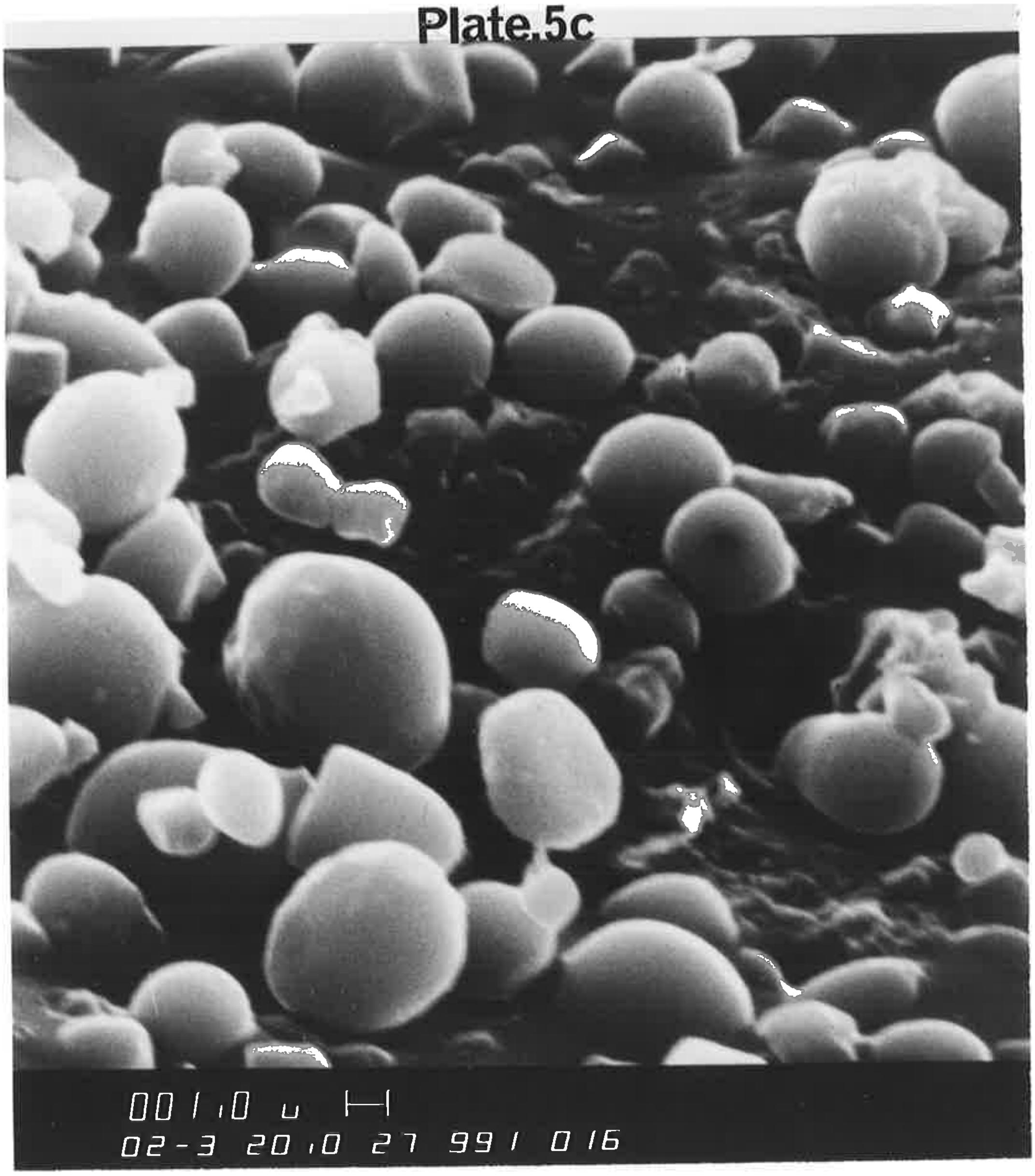
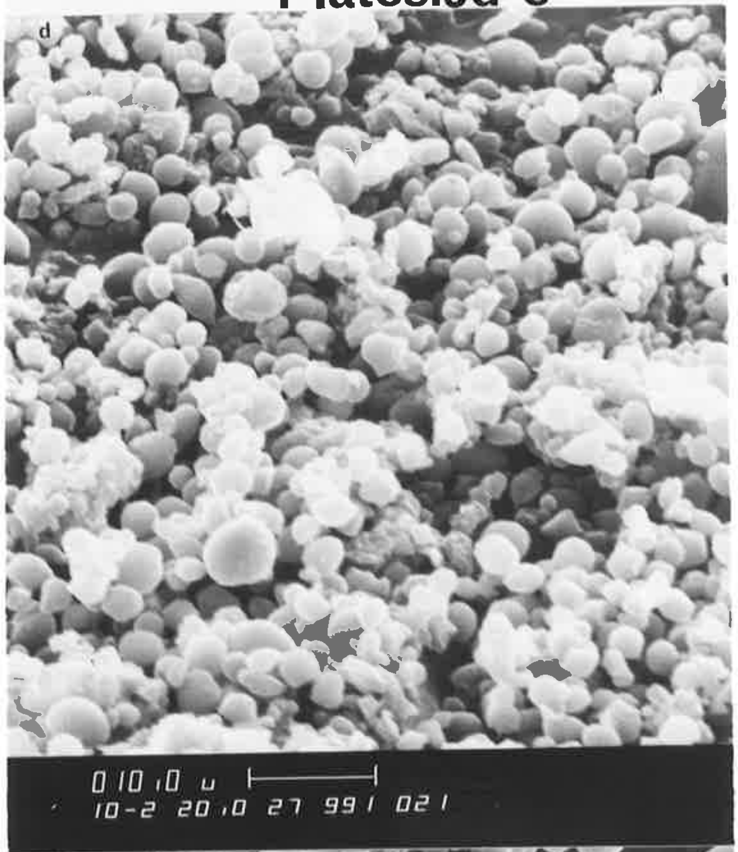


Plate. 5 d-e.

Electron micrographs.

Plate 5 d and e : Intact small granues of barley from cultivar Clipper.

Plates.5d-e



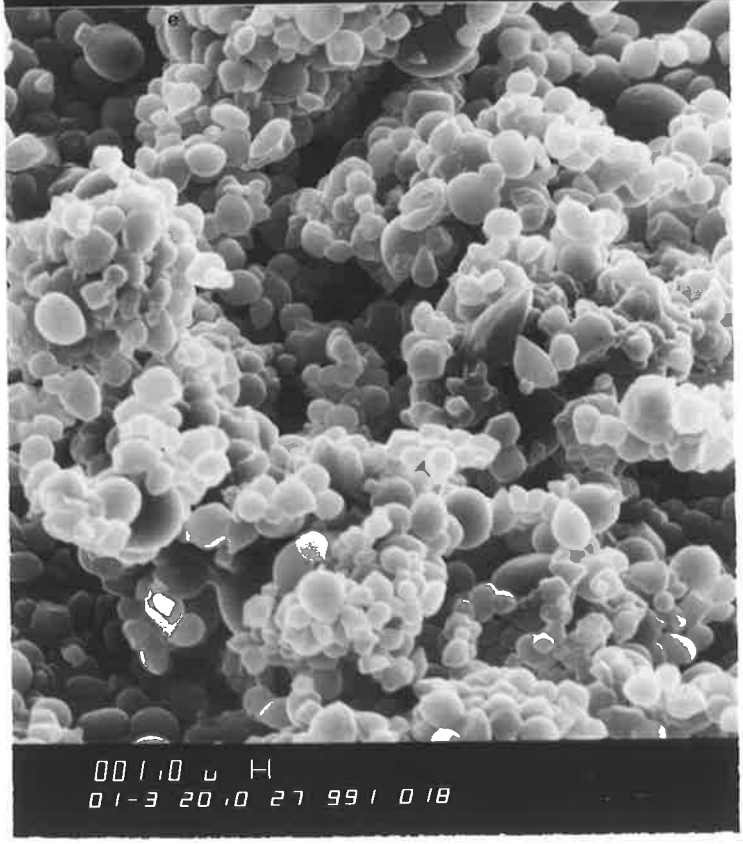


Plate. 5 f-i.

Electron Micrographs.

Plate 5 f: Alpha-amylase (5.7 I.D.U.) treated normal maize starch granules (1hr.).

Plate. 5 g: Alpha-amylase (5.7 I.D.U.) treated waxy maize granules (1 hr.).

Plate. 5 h: " " " " large granules from barley cultivar Clipper.

Plate. 5 i " " " " potato granules.

Plates.5f-i

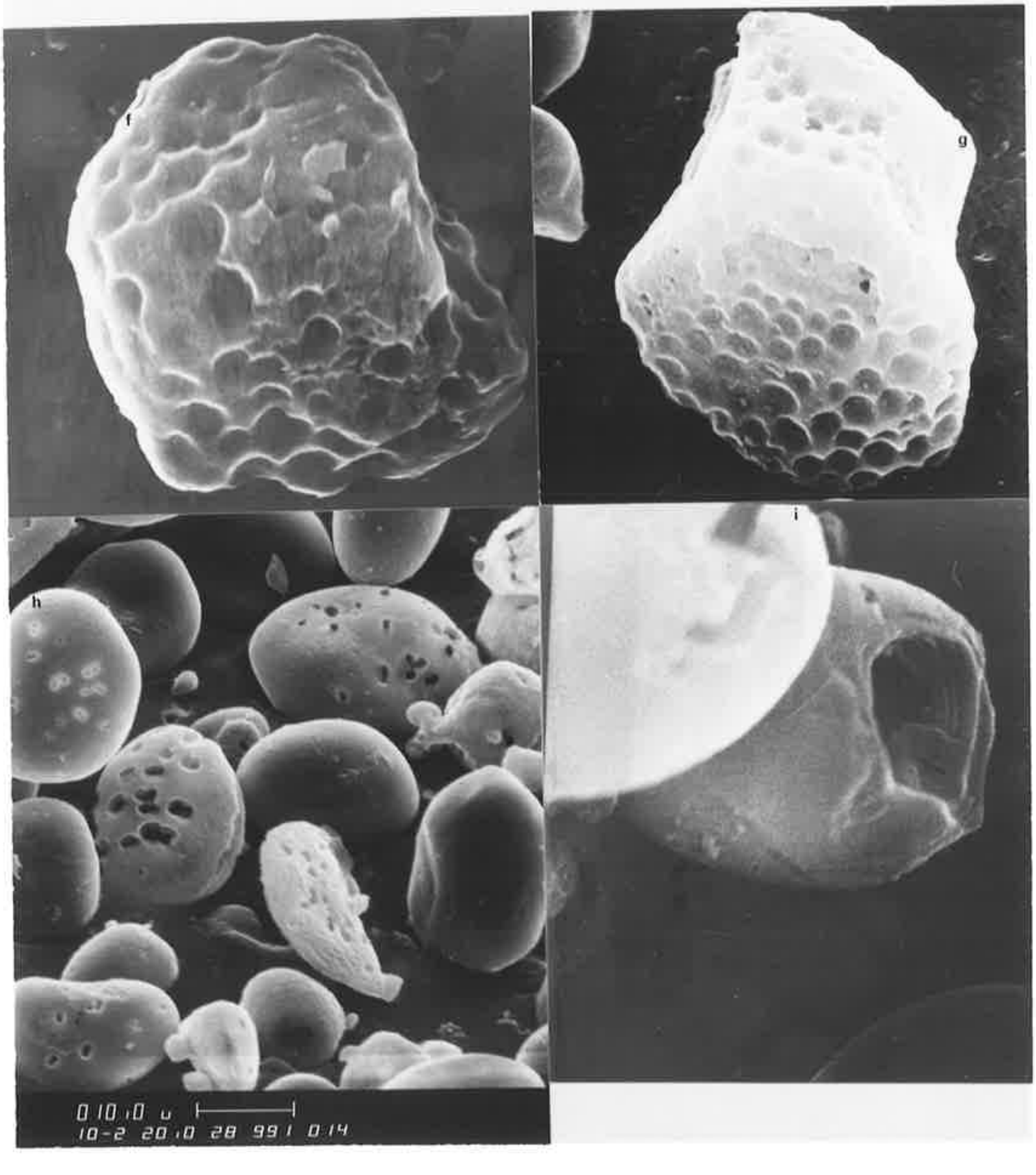


Fig. 9 Susceptibility of Small Granules To Barley Alpha-amylase.

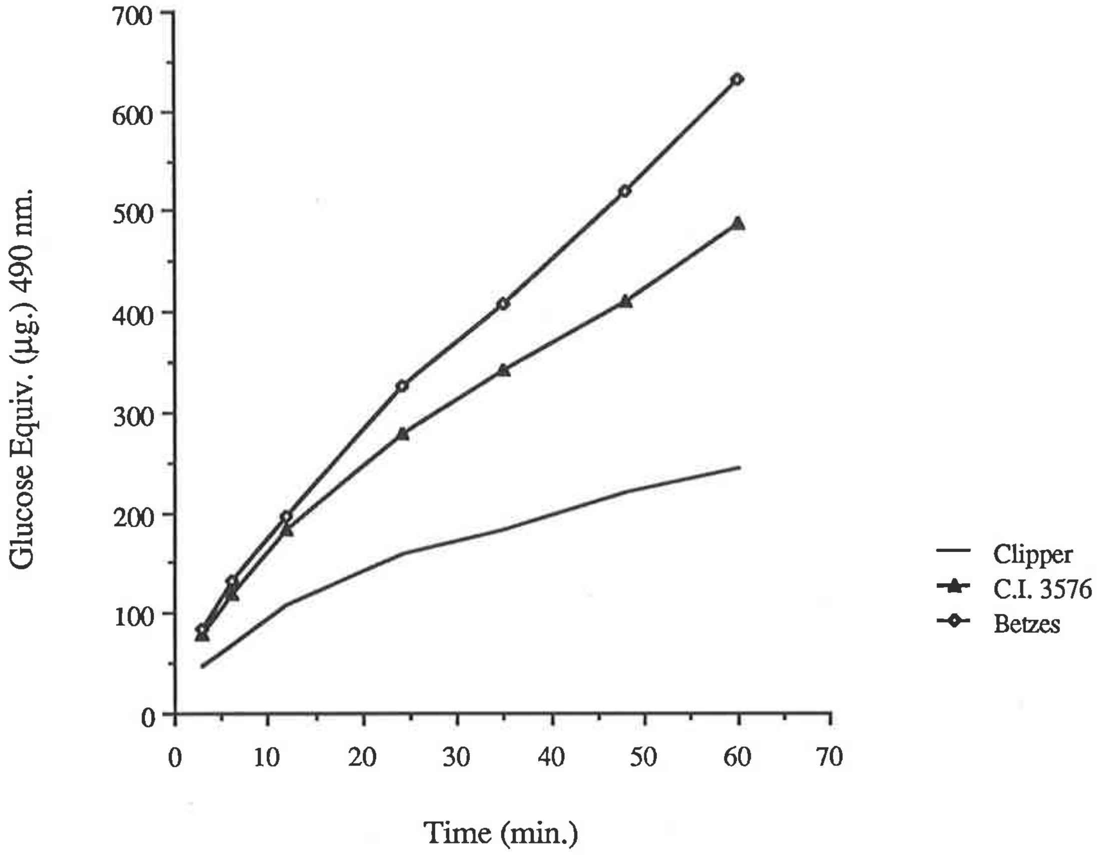


Fig.10 Susceptibility of Large Granules To Barley Alpha-amylase.

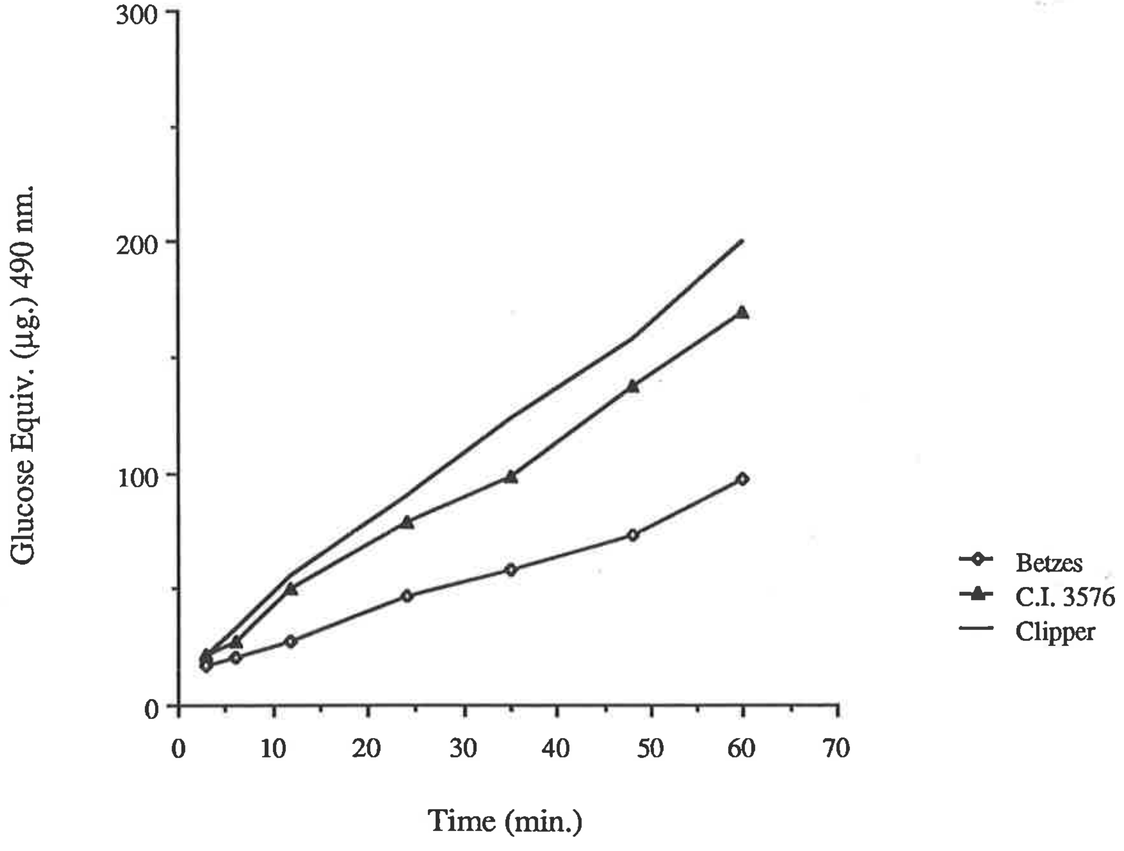


Fig. 11 Susceptibility of Tuber and Maize Granules To Barley Alpha-amylase.

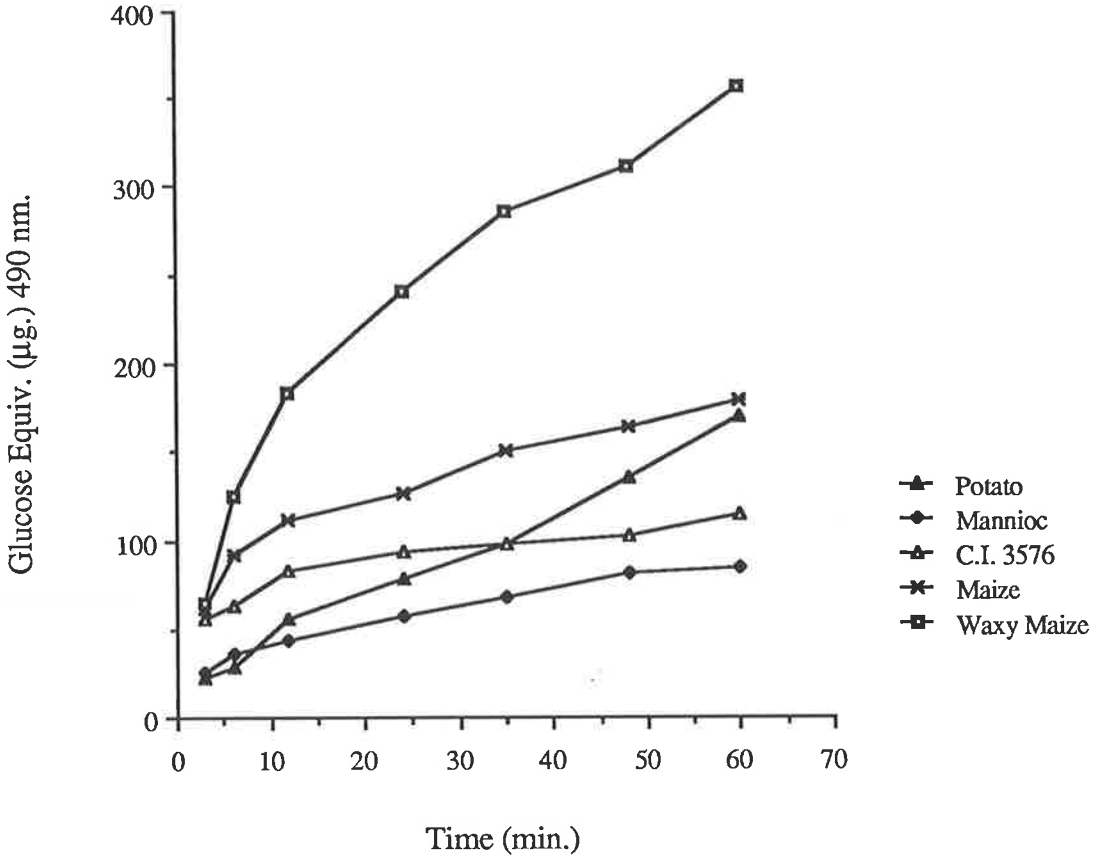
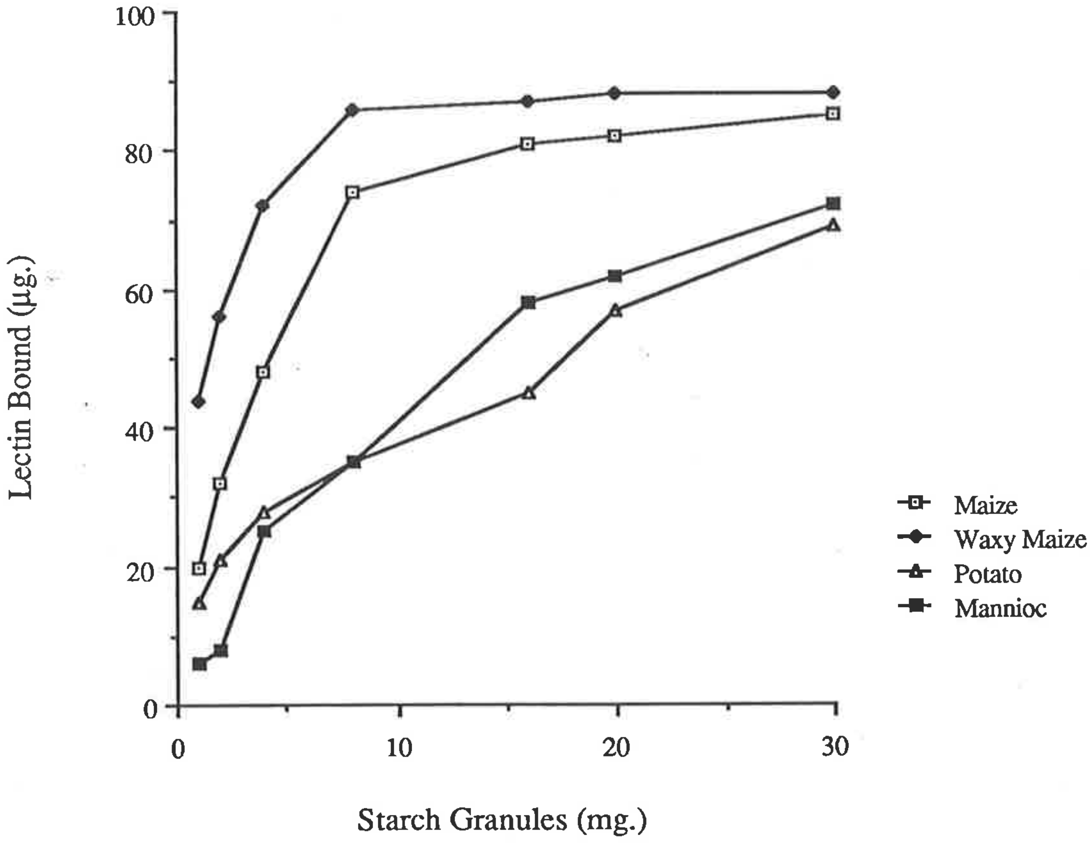


Fig.12 Lectin Affinity For Tuber and Maize Granules



and Plate. 5 k-m (a degradation characteristic of glucoamylase).

The results presented in Plate. 5 0, show a freeze fractured large starch granule from barley, cultivar Clipper, after digestion with barley alpha-amylase (5.7 I.D.U.) for 60 min. Degradation was observed to occur at susceptible areas on the granule surface and internally.

3.8. Binding Of Concanavilin-A to Intact Starch Granules.

Conanavilin-A possessing affinity for branched a-1,4-glucopyranoside residues was used to investigate whether structural differences on the starch granule surface could be differentiated between starch granules types and within large and small granules derived from the same cultivar eg. large and small barley granules.

The effects of FITC- labeled Concanavilin-A incubated with starch granules isolated from normal and waxy maize (SD: 2.14), potato and mannioc (SD: 1.63) respectively are presented in Fig. 12. The results for large and small starch granules from barley are presented in Fig. 13 (SD: 1.54) and 14 (SD: 2.17) respectively. (The approximate size of the starch granules studied are shown in Table. 3)

Results demonstrate the different affinities of lectin for the starch granule types. Small starch granules from barley have a much higher affinity for lectin than the larger granules. Initial binding affinity of lectin for starch granules were in the order, small starch granules from barley > waxy maize > normal maize > large starch granules from barley > mannioc > potato.

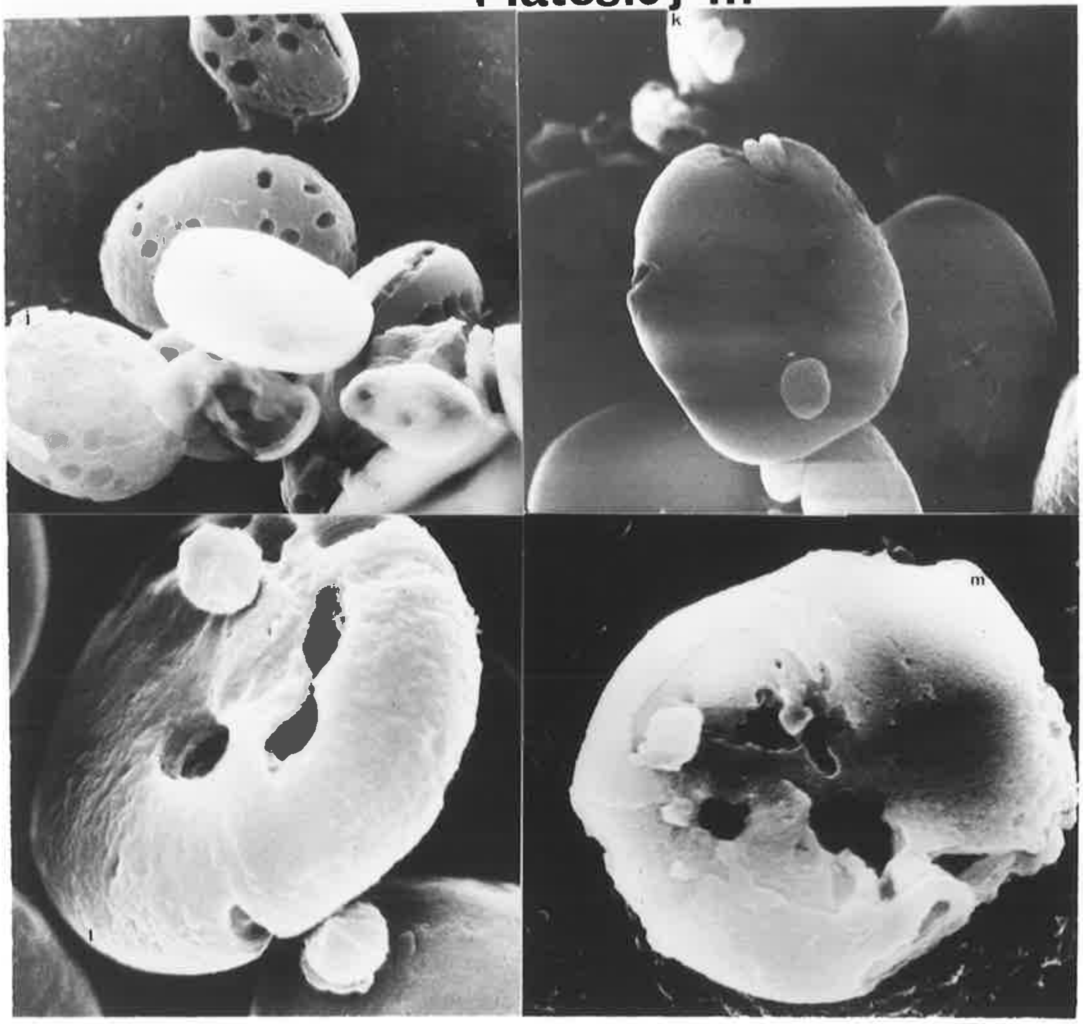
Tuber starches showed significantly less affinity for lectin than their cereal counterparts. The distribution of lectin bound to the exterior of granules isolated from barley cultivars Clipper, Betzes and C.I. 3576 visualized by fluorescent and phase contrast microscopy are shown in Plates. 6, 7 and 8 respectively. Results of F.I.T.C.-labeled lectin bound to starch granules from normal and waxy maize and potato are illustrated in Plates. 9 a-b, 10 a-c, and 11 a-c . From the photographs there appears to be some selectivity in terms of the areas on which the lectin binds. The binding of lectin to starch granules was cooperative Fig. 15, (SD: 0.78) and binding was reversed (95%), when alpha-methyl-D-mannose was introduced into the incubation mixture.

Plates. 5 j-m.

Electron Micrographs.

Plates. 5 j-m: Large and small starch granules from barley cultivars Clipper, Betzes and C.I. 3576 (l and m), after incubation with alpha-amylase (5.7 I.D.U.) for 1 hr.

Plates.5 j-m



Plates. 5 n-o.

Electron Micrographs.

Plate. 5 n: _Alpha-amylase treated large granules from barley cultivar C.I. 3576 in the presence of Concanavilin-A.

Plate. 5 o: Freeze fractured large granule from barley cultivar Clipper after incubation with alpha-amylase (5.7 I.D.U.) for 1 hr.

Plate.5n

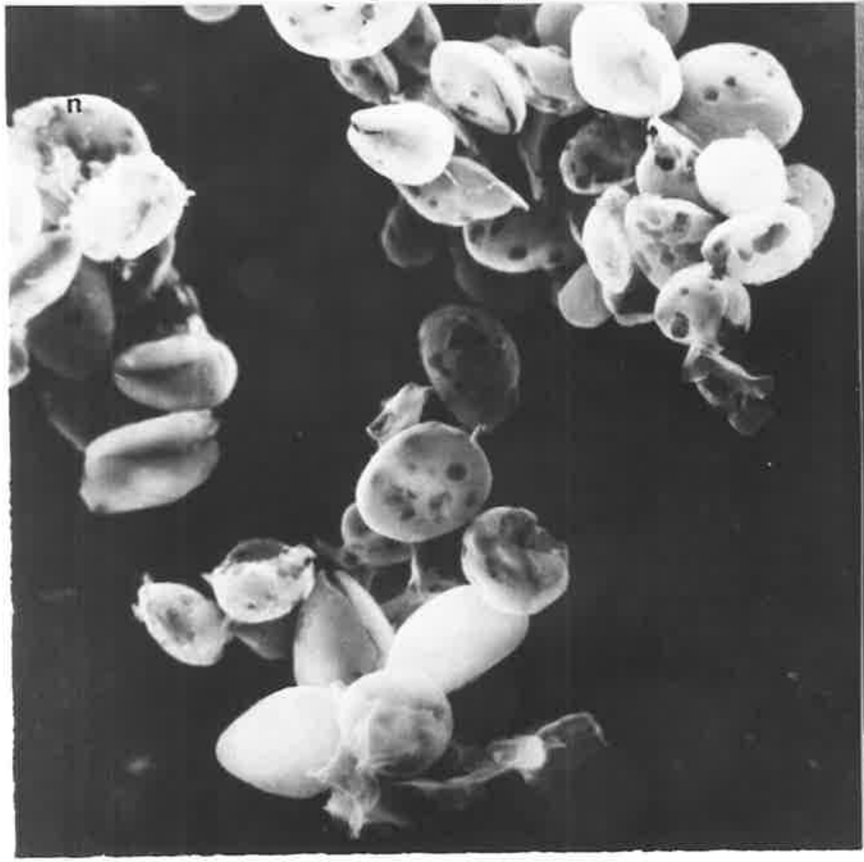


Plate.5o

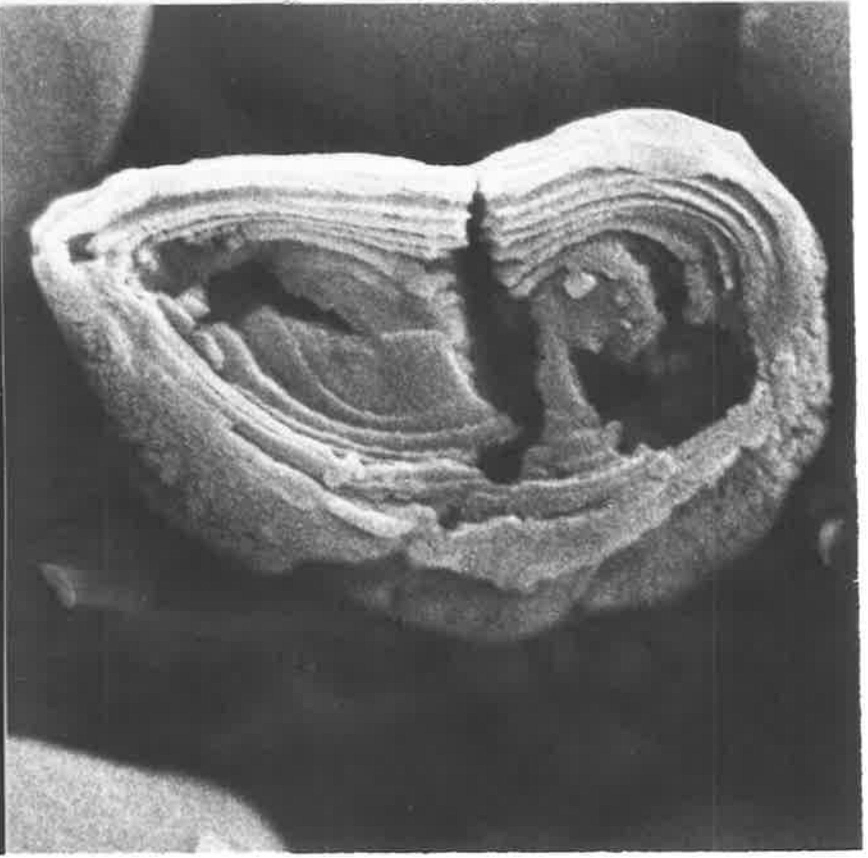


Table 3

Starch Granule Size (μm).

Granule Type.	Size
Potato	7-40
Barley	1-35
Wheat	2-40
Mannioc	5-35
Maize	10-15

Fig.13 Lectin Affinity For Barley (large granules).

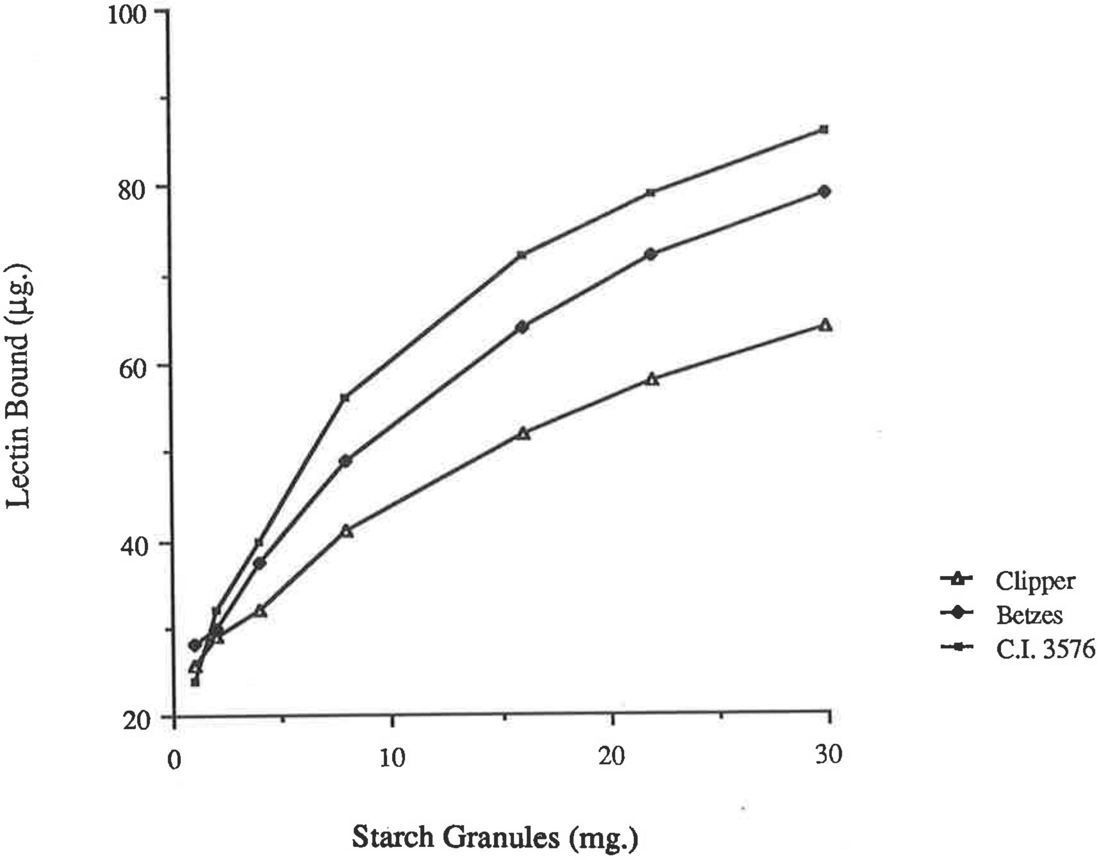


Fig.14 Lectin Affinity for Barley (Small granules)

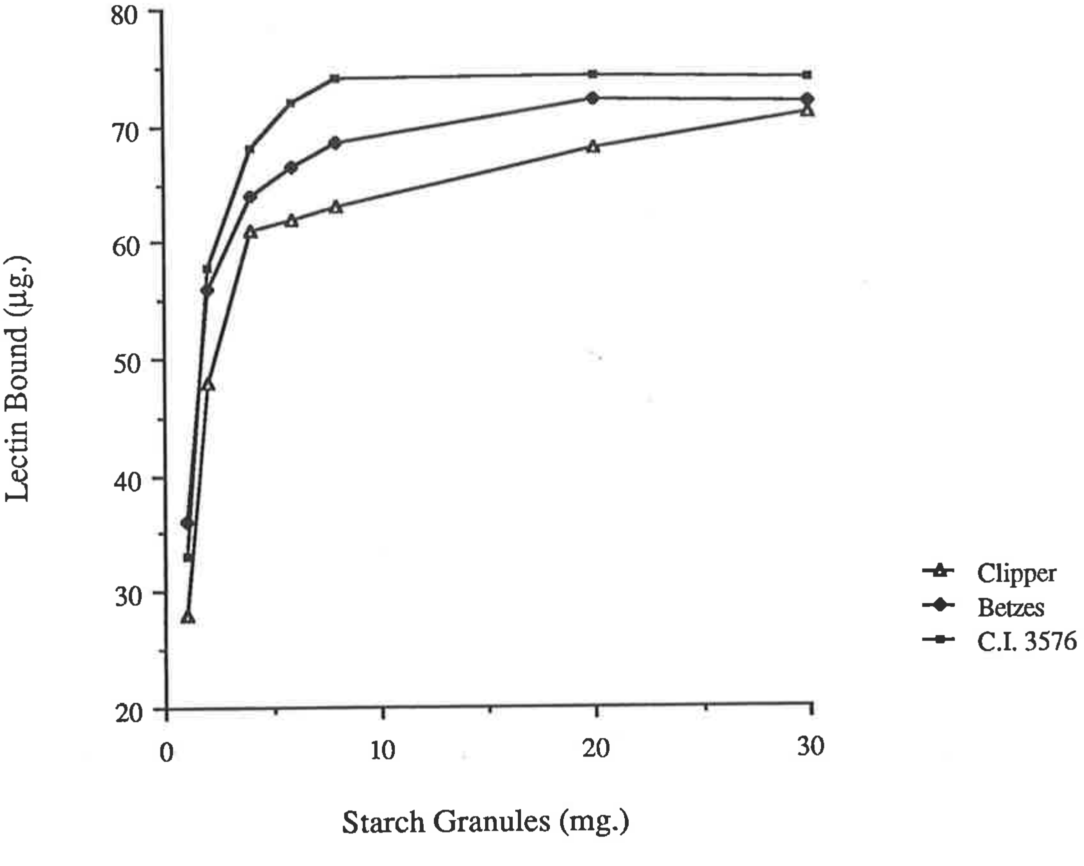


Fig.15 Illustration Of Lectin Cooperativity Using Barley Starch Granules.

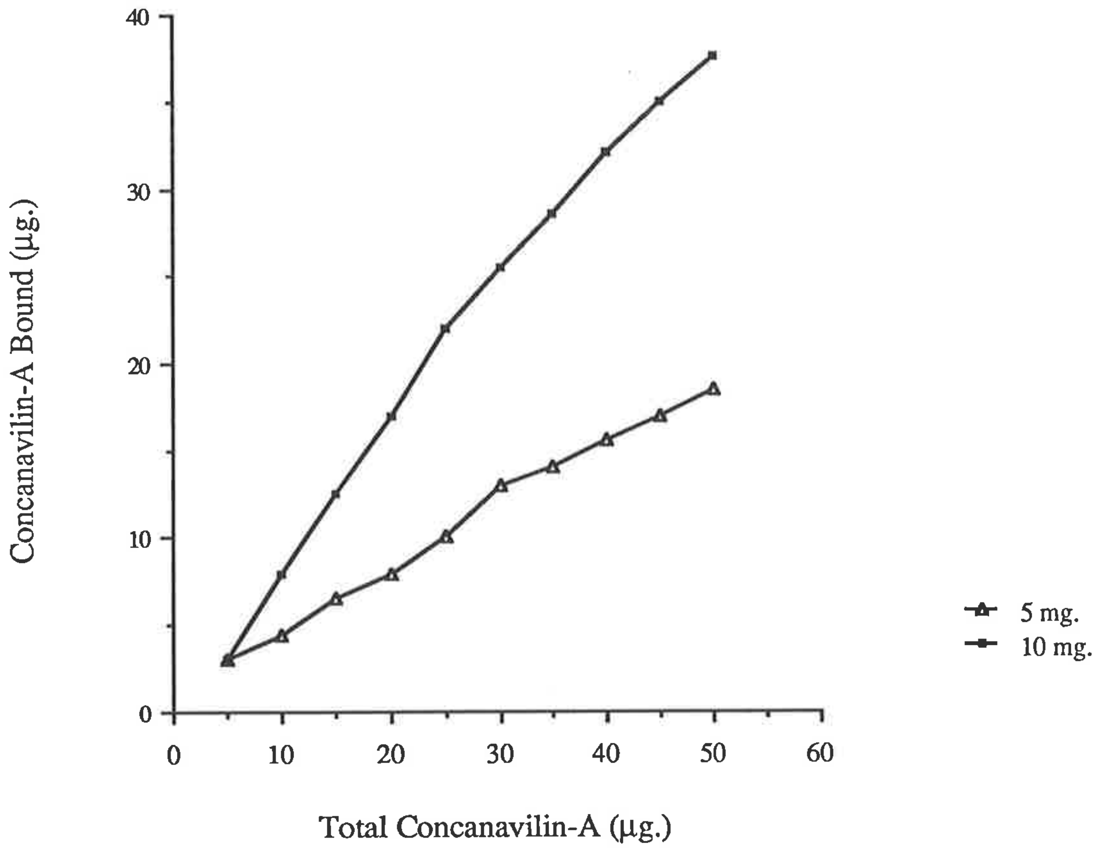
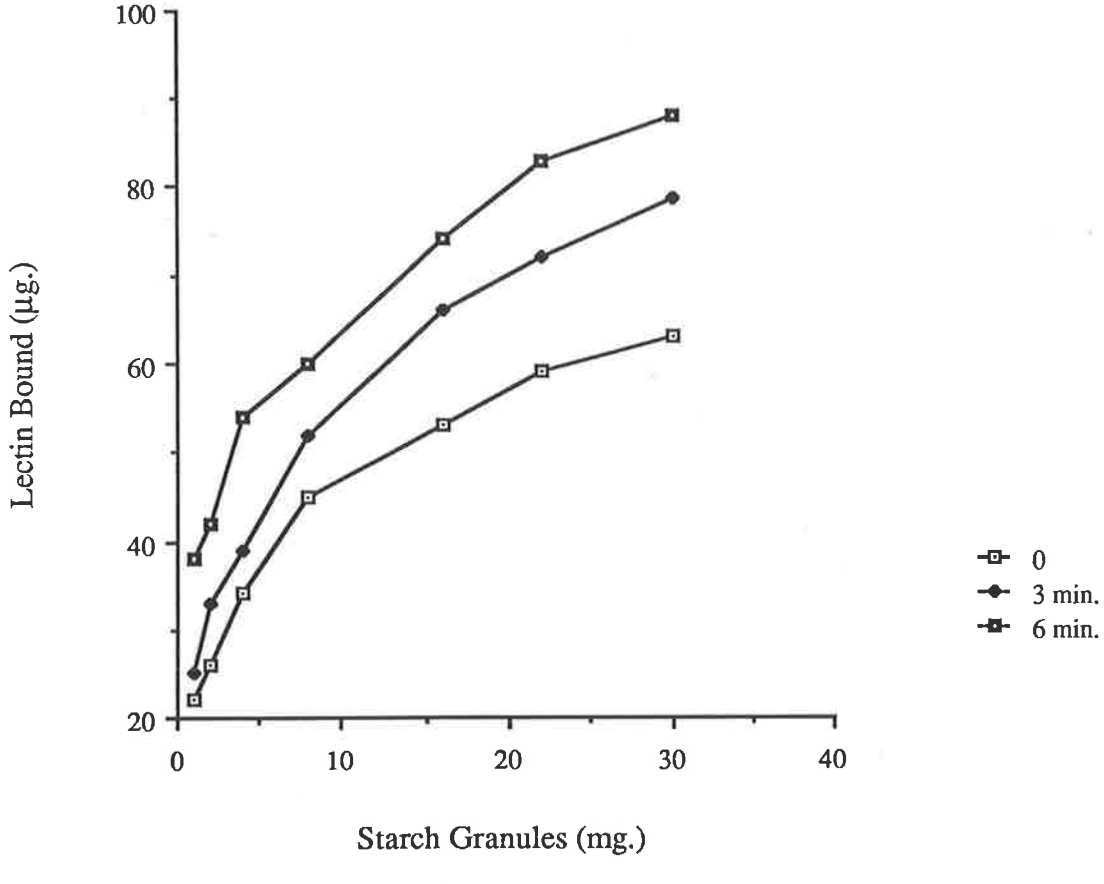


Fig.16 Lectin Affinity For Damaged Wheat Starch Granules.



Plates. 6-8.

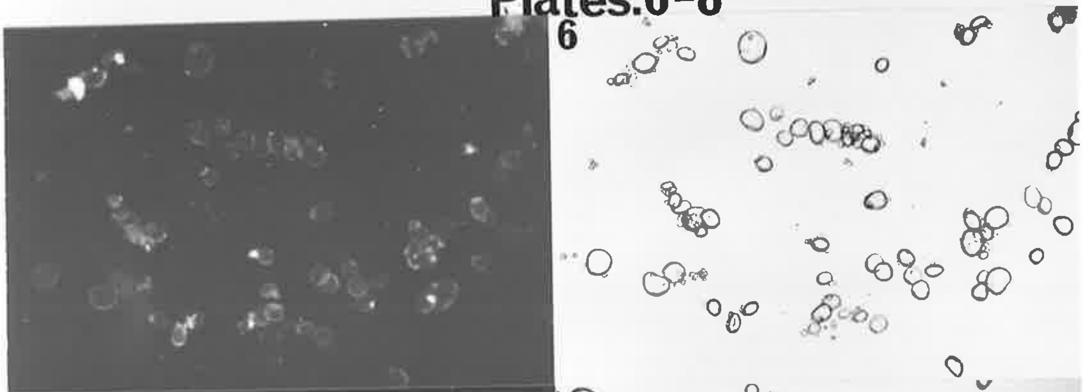
Fluorescent Microscopy.

Plate. 6: Large and small starch granules from barley cultivar Clipper, incubated with F.I.T.C. labeled Concanavilin-A.

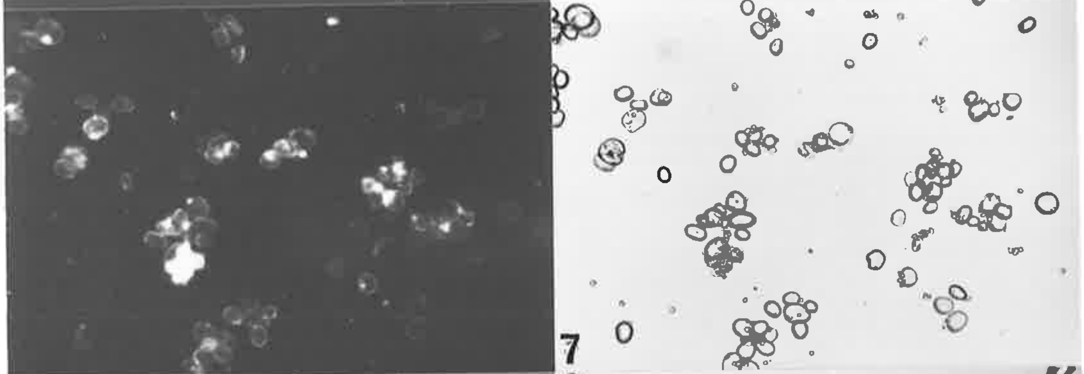
Plate. 7: Large and small granules from barley cultivar Betzes, incubated with F.I.T.C. labeled Concanavilin-A.

Plate. 8: Large and small granules from barley cultivar CI 3576, incubated with F.I.T.C. labeled Concanavilin-A.

Plates.6-8



6



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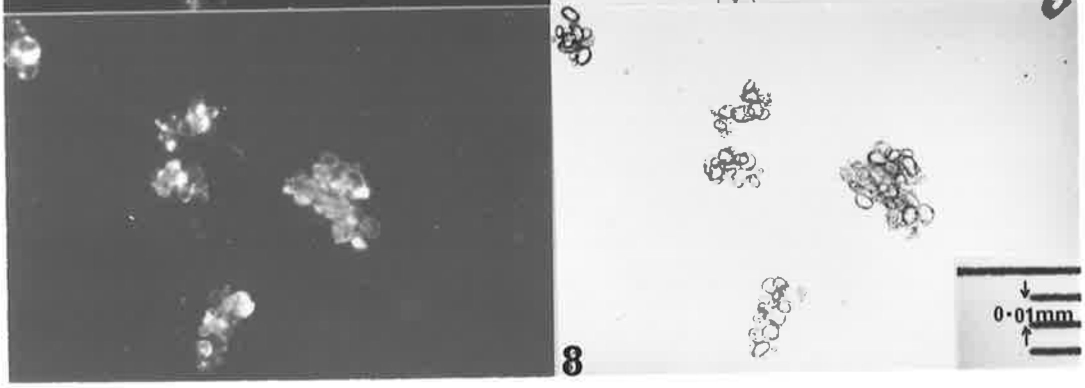


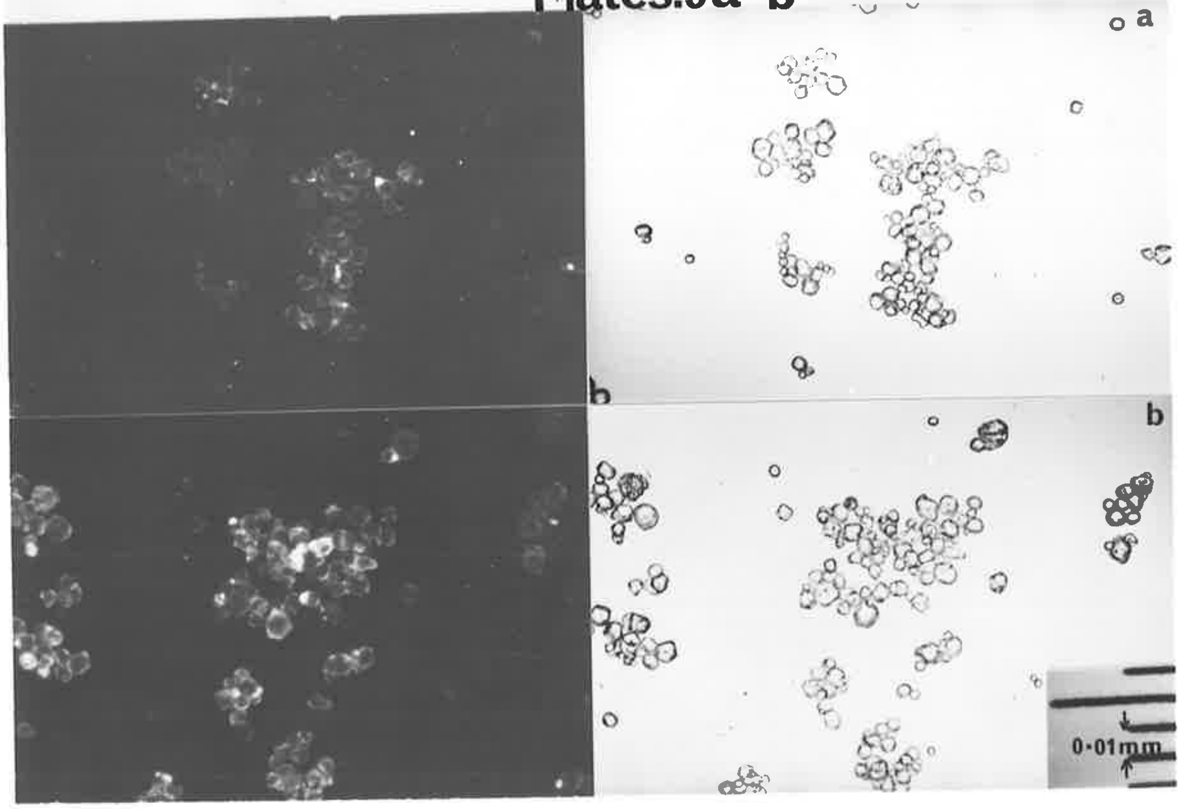
8

Plates. 9 a-b.

Fluorescent Microscopy.

Plate. 9 a-b: Normal maize starch granules incubated with F.I.T.C. labeled Conanavilin-A.

Plates.9a-b

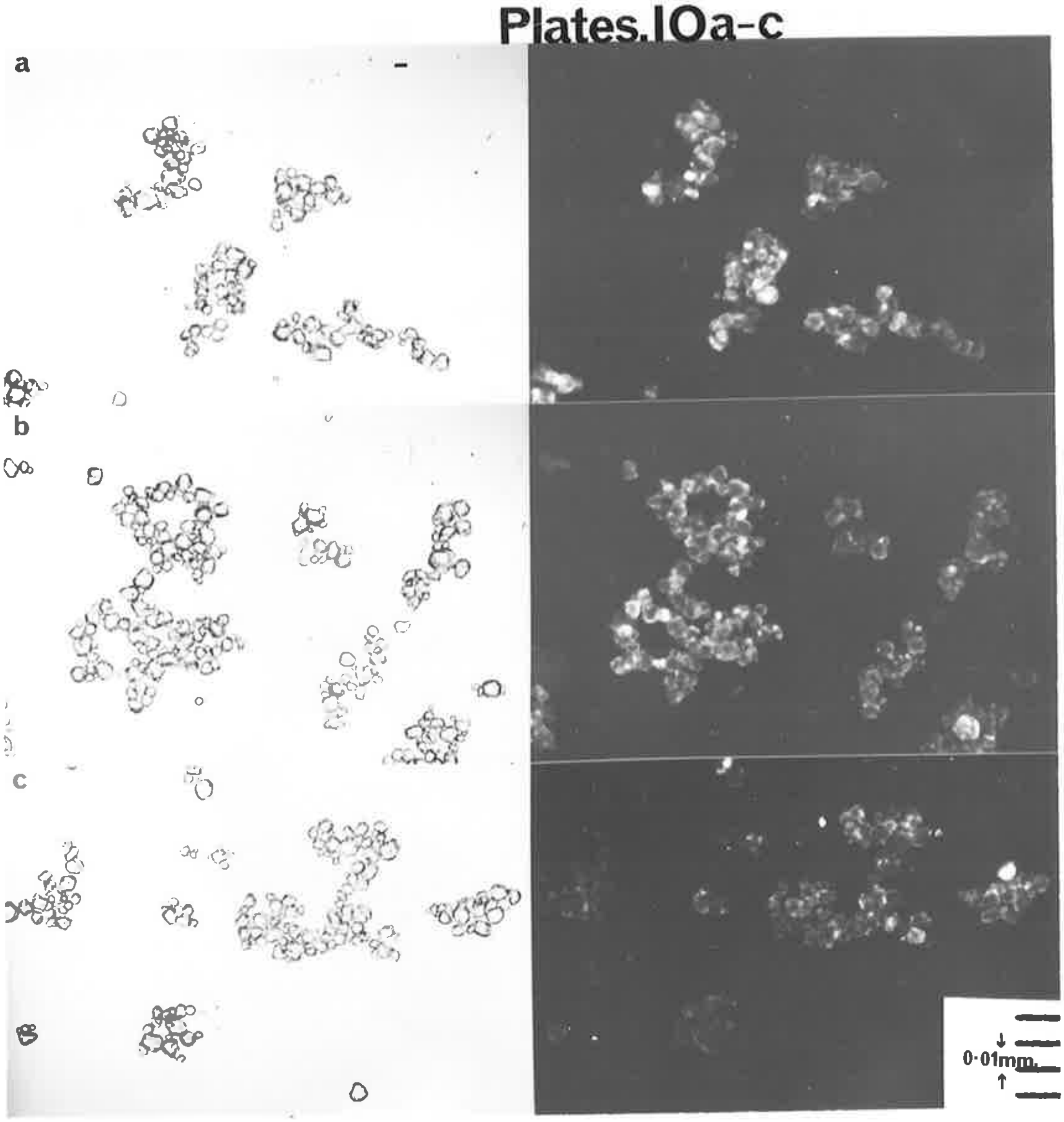


Plates. 10 a-c.

Fluorescent Microscopy.

Plate. 10 a-c: Waxy maize starch granules incubated in the presence of F.I.T.C. labeled Concanavilin-A.

Plates.10a-c

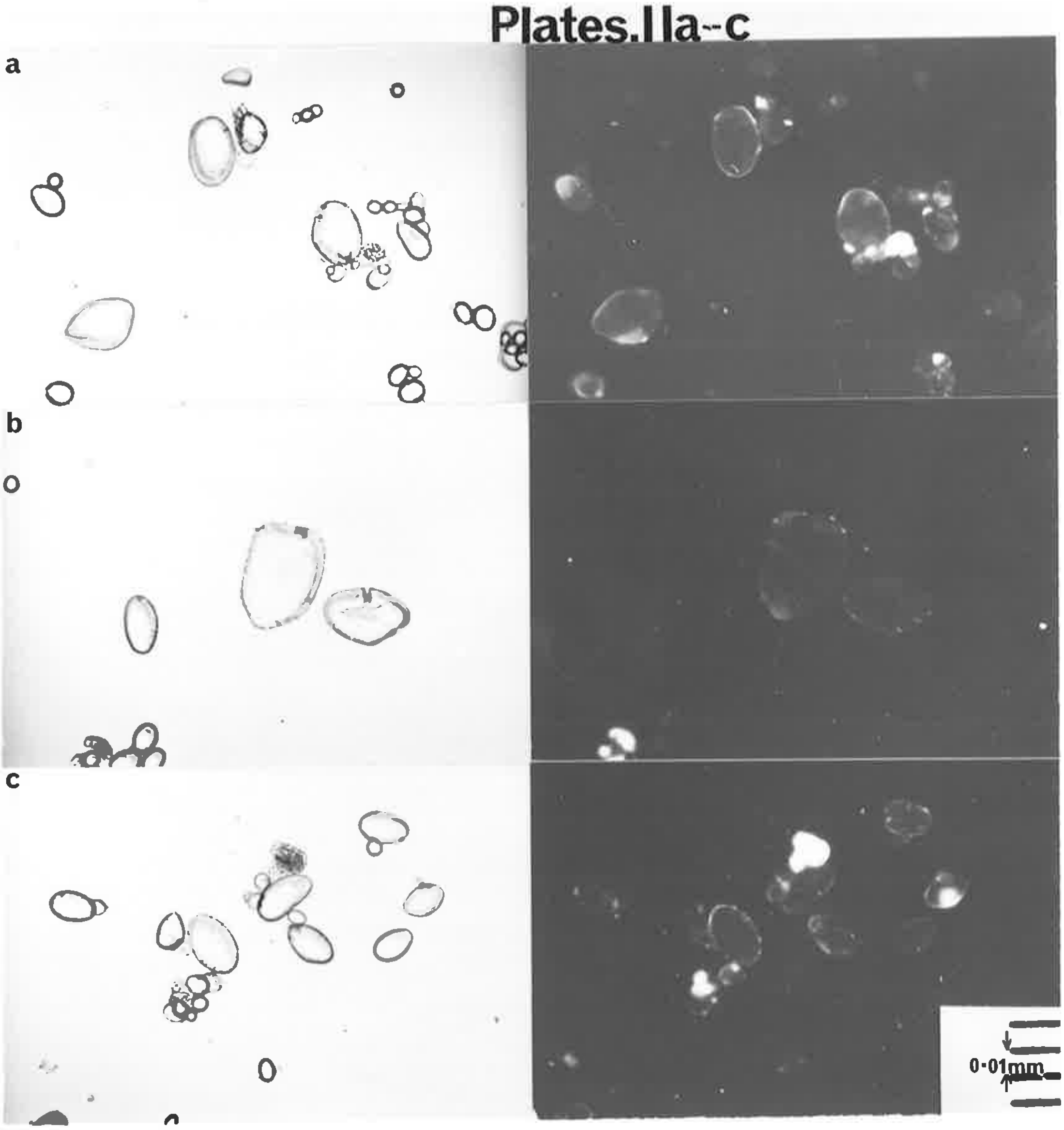


Plates. 11 a-c.

Fluorescent Microscopy.

Plate. 11 a-c: Potato granules incubated in the presence of F.I.T.C. labeled Concanavilin-A.

Plates.IIa-c



Large starch granules from barley, incubated in the presence of Concanavilin-A and barley alpha-amylase, are depicted in Plate. 5 n. The characteristic pitting shown to occur in the absence of lectin, remains unchanged.

3.9 Binding of F.I.T.C.-Labeled Concanavilin-A to Unfractionated Intact And Damaged Wheat Starch Granules.

The degree of granular damage is a parameter used to assess the quality of a flour samples potential during the breadmaking. Since newly acessible areas will become available for lectin binding during granule damage a new test was evaluated, based on the amount of lectin bound to damaged wheat starch granules.

Figure.16 (SD: 0.51) shows the binding affinity of F.I.T.C.-labeled Concanavilin-A for intact and damaged starch granules of wheat cultivar Warigal. Damaged wheat starch granules possessed a greater affinity for lectin than undamaged ones. Further, the milling time of the starch granules ie. the degree of damage, is positively related to the amount of lectin bound to the granules.

3.10. Adsorption Of Clipper Alpha Amylase-2, To Large and Small Barley Starch Granules.

Results presented in Figs.17 (SD: 2.64) and 18 (SD: 2.76) indicate the affinity of alpha-amylase-2 purified from cultivar Clipper, for large and small starch granules of barley (cultivars Clipper, Betzes and C.I. 3576). Incubations were carried out at 25°C. Similar adsorption of enzyme occurred with both types of granules under the conditions in these experiments. There were only small differences in the degree of binding between cultivars.

Fig.17 Adsorption of Barley Alpha-amylase to Large Granules From barley.

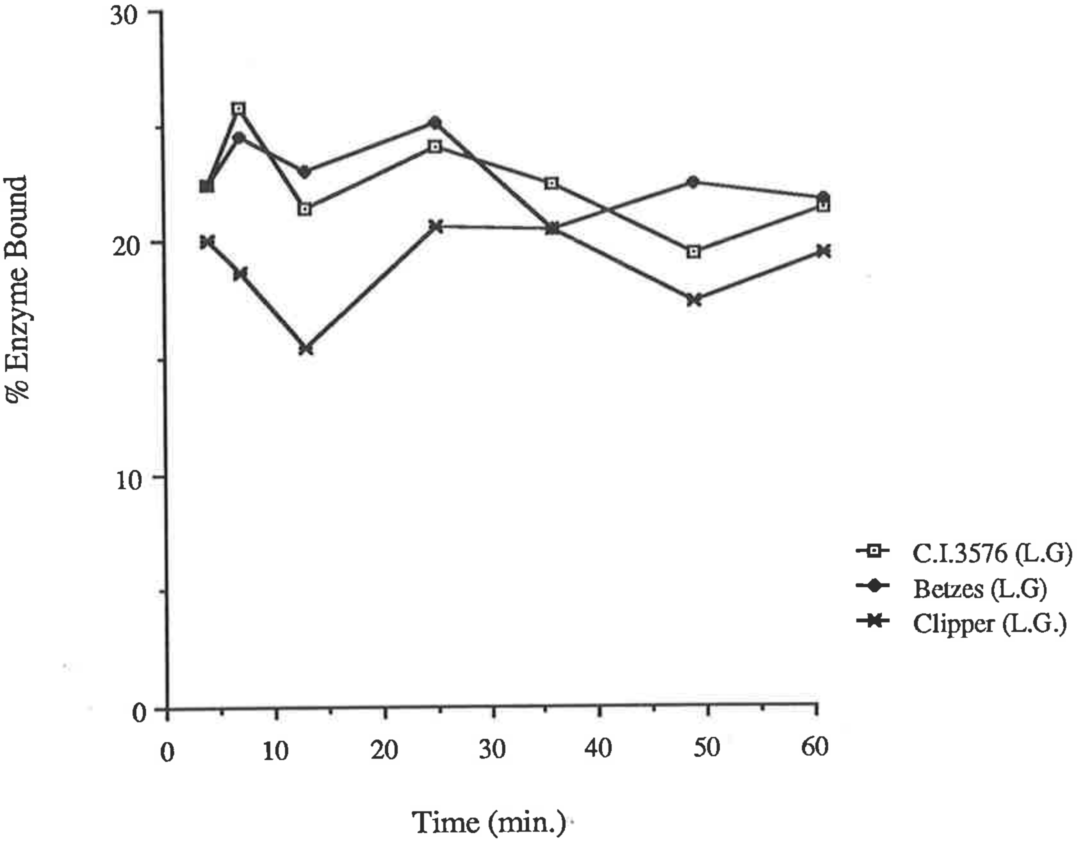
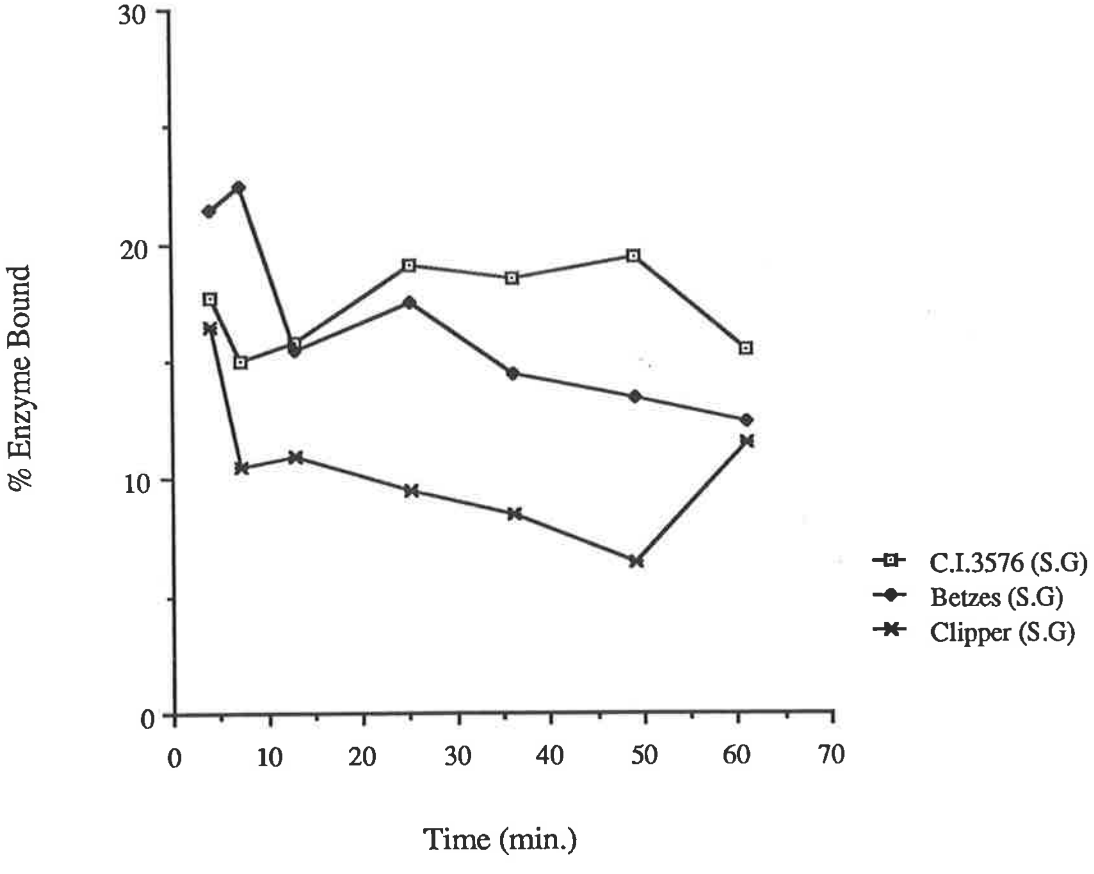


Fig. 18 Adsorption of Barley Alpha-amylase to Small Granules From Barley.



3.11. Adsorption Of Salivary Alpha-Amylase To Large And Small Starch Granules Of Barley.

Since cereal a-amylases have maximum activity at pH 5.5 they are clearly of little value in binding studies involving Concanavilin-A which binds optimally at pH>7.0. Salivary a-amylase was therefore substituted for the cereal enzyme.

Figure 19 (SD: 2.81) and 20 (SD: 2.32) show the affinity of salivary alpha-amylase for the exterior of the large and small granules of barley. The enzyme has a greater affinity for barley starch granules than that of the cereal amylase. Differences in the affinity of the salivary enzyme for both starch granule sizes was also evident, with the large granules showing a greater enzyme affinity.

3.12. Adsorption Of Salivary Alpha-Amylase To Large And Small Barley Starch Granules In The Presence Of Concanavilin-A.

Results presented in Fig. 21 (SD: 2.05) and 22 (SD: 1.77) show a decrease in adsorption of salivary alpha-amylase for purified large and small starch granules isolated from barley cultivar C.I. 3576 with and without increasing amounts of Concanavilin-A. Affinity of salivary alpha-amylase decreased as lectin concentration increased in both granule types.

3.13. Inhibition Of Salivary Alpha-Amylase Degradation Of Barley Starch Granules By Concanavilin-A.

Fig.19 Adsorption Of Salivary Alpha-amylase To Large Granules From Barley.

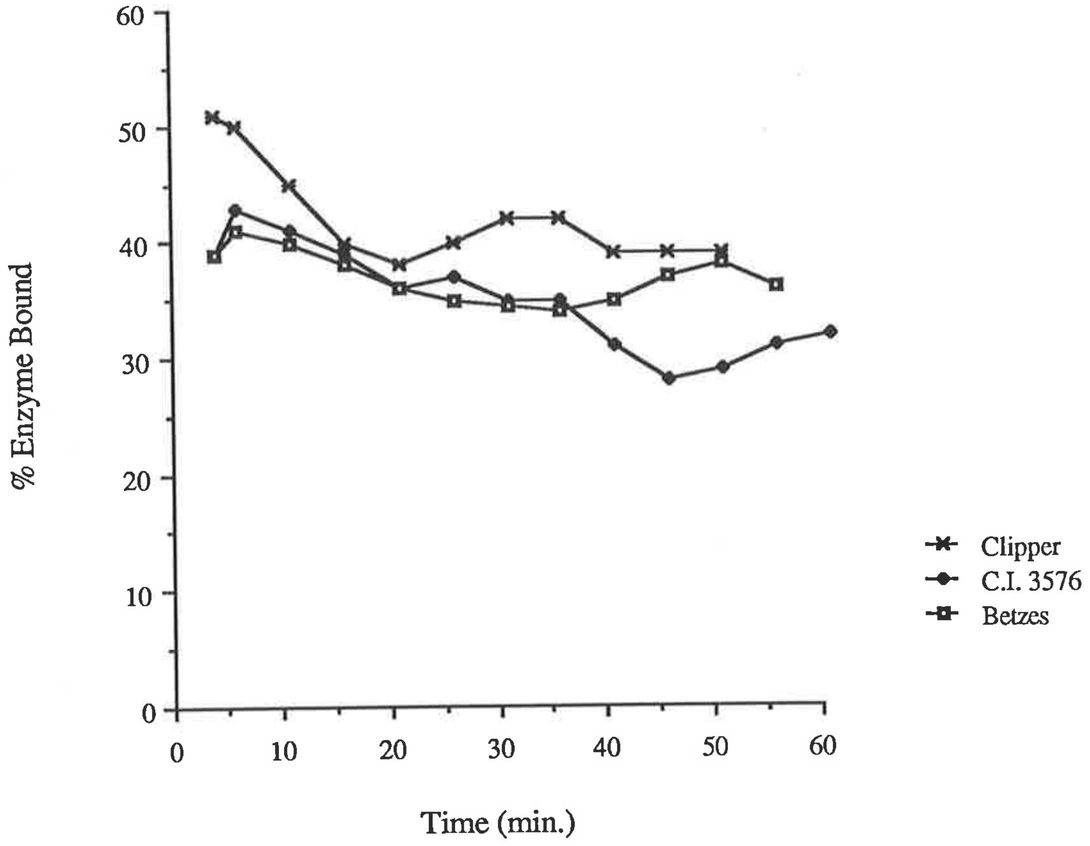


Fig. 20 Adsorption Of Salivary Alpha-amylase To Small Granules From barley

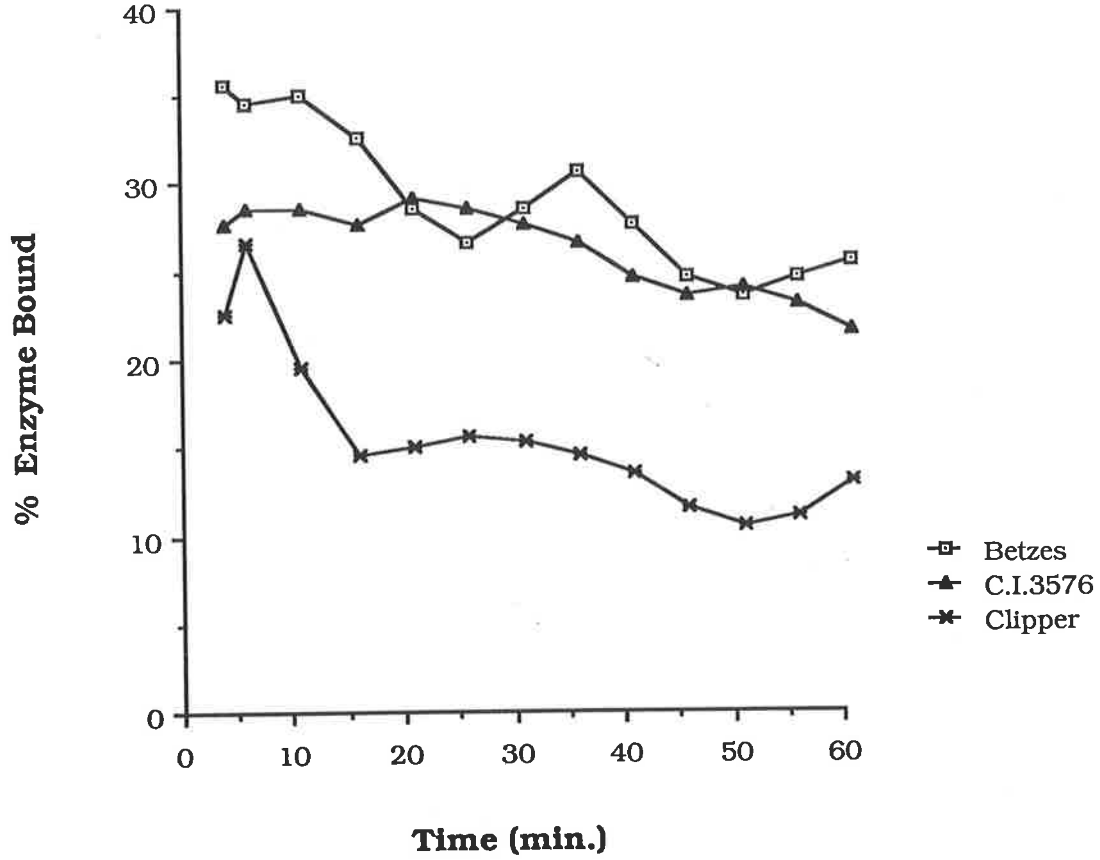


Fig.21 Amylase Bound(%) To ~~Small~~ LARGE Granules In The Presence Of Increasing [Lectin].

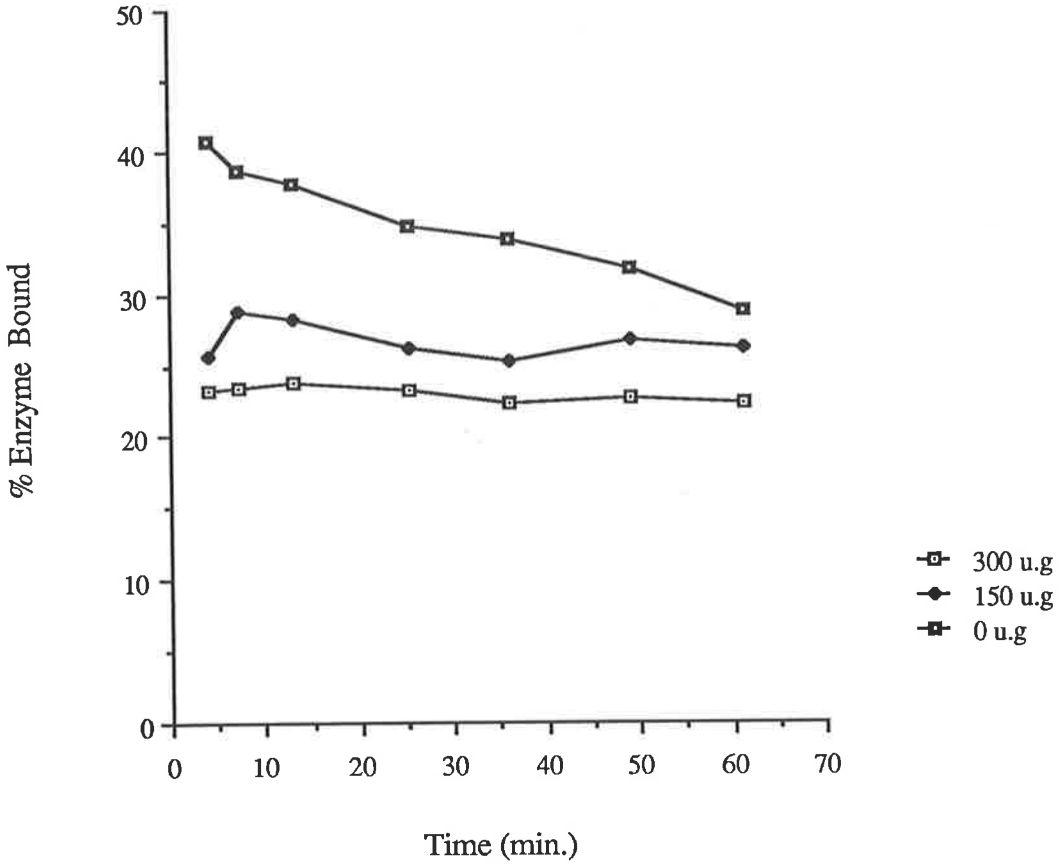


Fig.22 Enzyme Bound(%) To Small Granules In The Presence Of Increasing [Lectin].

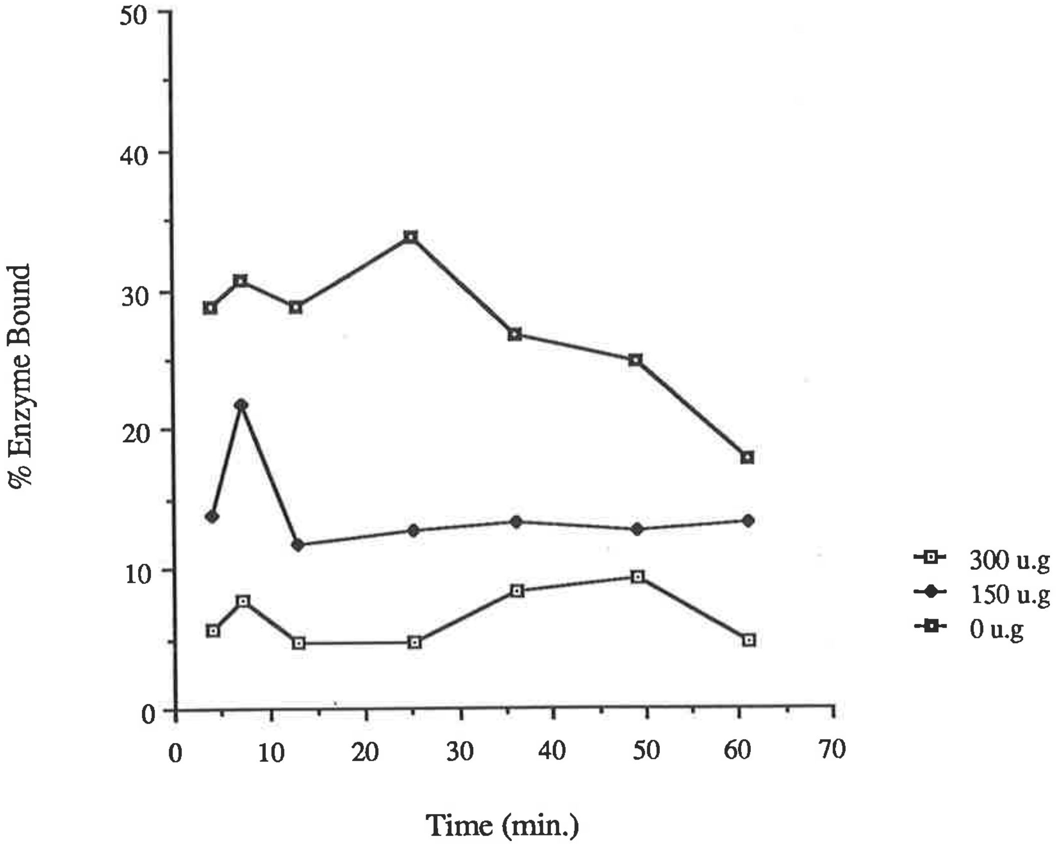


Fig.23 Inhibition Of Salivary Amylase By Increasing [Lectin] (large granules)

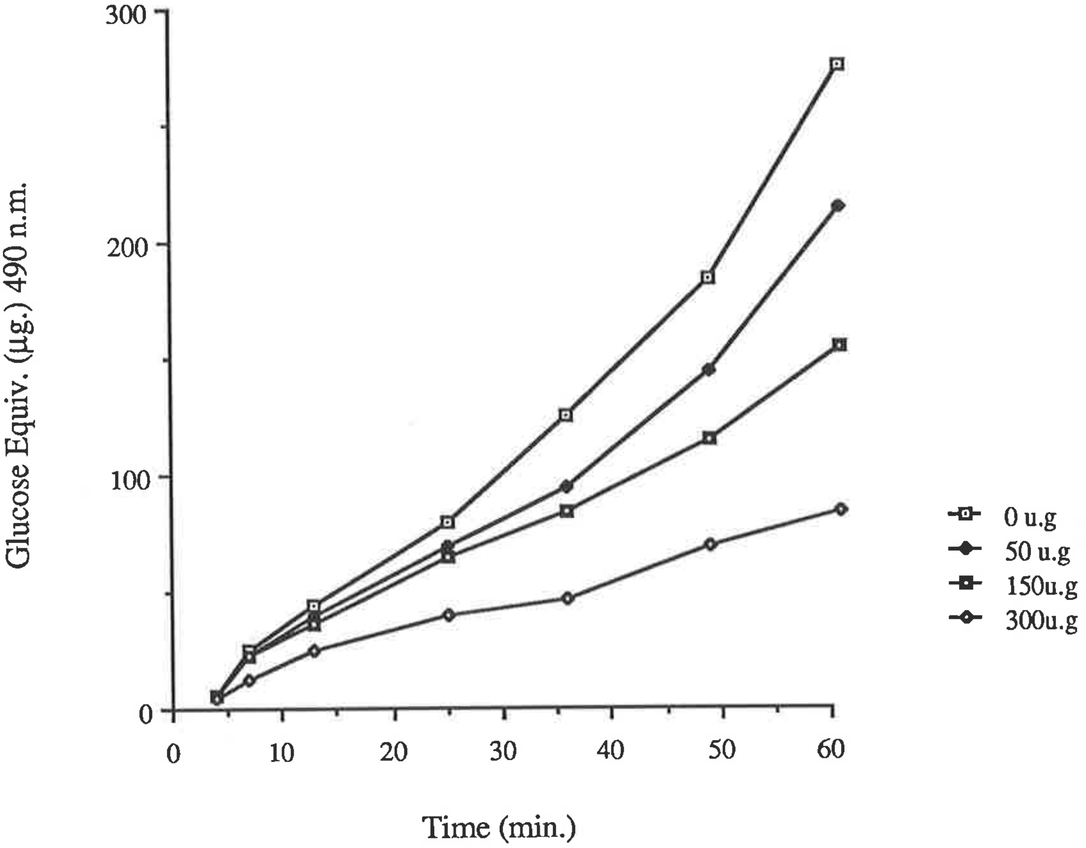
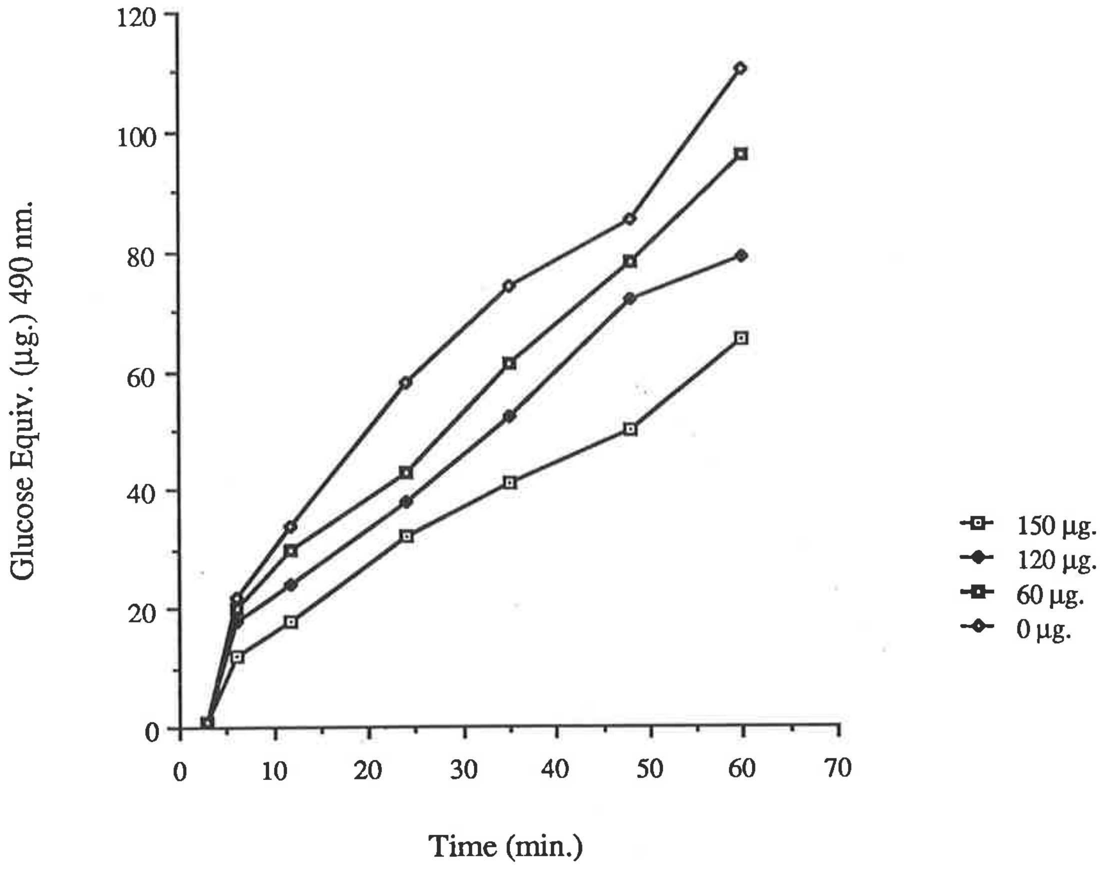


Fig.24 Inhibition Of Salivary Amylase By Increasing [Lectin] (Small Granules).



3.14. Theoretical Quantity Of Concanavilin-A Required to Inhibit Salivary Alpha-Amylase Degradation Of Large And Small Barley Starch Granules.

Figures. 25 (SD: 2.03) and 26 (SD: 2.31) show the theoretical quantity of lectin required to completely inhibit the degradation of large and small starch granules of barley, cultivar C.I.3576. Extrapolation of the terminal area of the line suggests the approximate amount of lectin required to obtain complete inhibition of starch granule degradation. For large starch granules, lectin concentrations between 650-700 µg/13.5 mg. are required. For small starch granules the concentration is dependant on the which part of the line is utilized for extrapolation, if the terminal area of the line is used, a concentration of 250-300 µg/13.5 mg. is required, earlier points would require a concentration of 300-400 µg/13.5

3.15. Hydroxpropyl Substitution Of Gelatinized Starch And Amylose.

Increasing hydroxypropyl substitution and its effects on the degradation of "Merk" soluble starch by alpha and beta-amylases is shown in Table.4 and 5 respectively. Increasing the degree of substitution decreased the degradation rate. Inhibition of exo-acting enzymes, eg. beta-amylase, is also affected and reducing groups released by beta-amylase were readily detected in substitutions less than 0.018M. The combination of alpha and beta-amylase on the degradation of substituted starch (0.018M) was investigated. Initially, the substituted starch was incubated with alpha-amylase (5.7 I.D.U.) for 10 min., the remaining polysaccharide precipitated with ethanol (97% v/v), lyophilized and a sample incubated with beta-amylase (30 I.D.U). The results are presented in Fig. 27 (SD: 1.21)

Fig.25 Theoretical [Lectin] Required For Complete Inhibition (Large Granules).

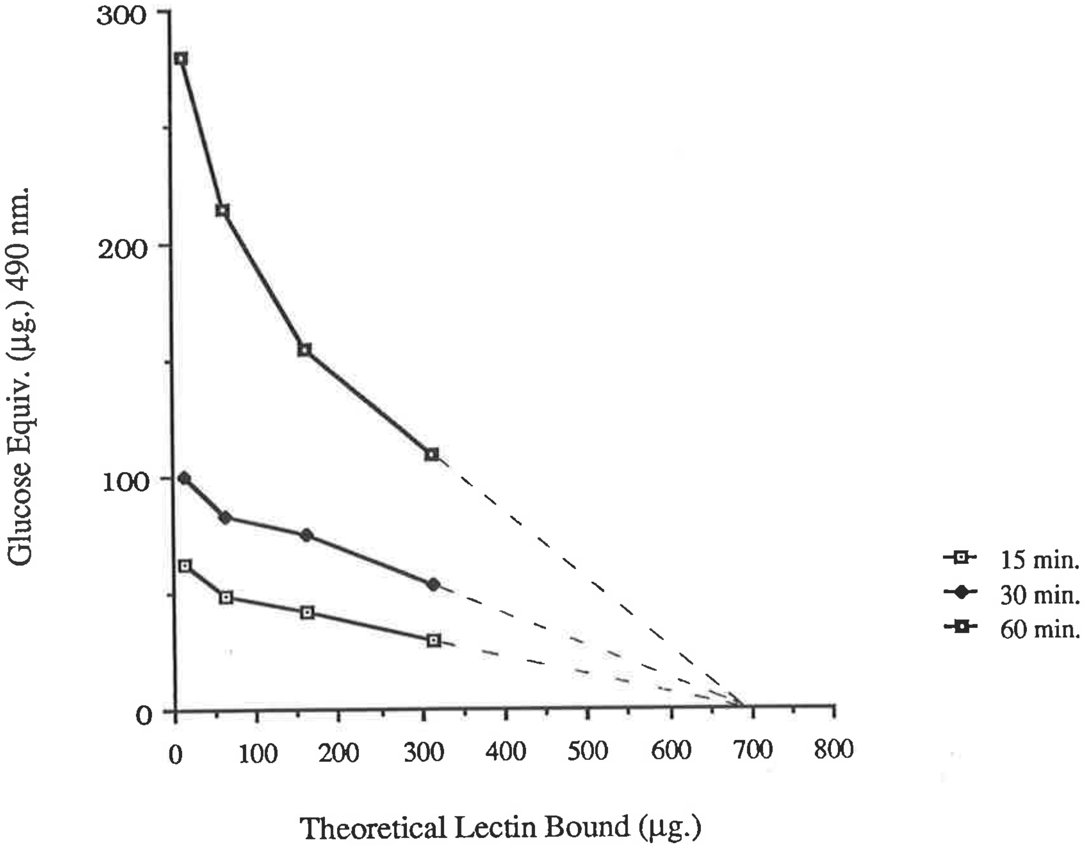


Fig.26 Theoretical [Lectin] Required For Complete Inhibition (Small Granules).

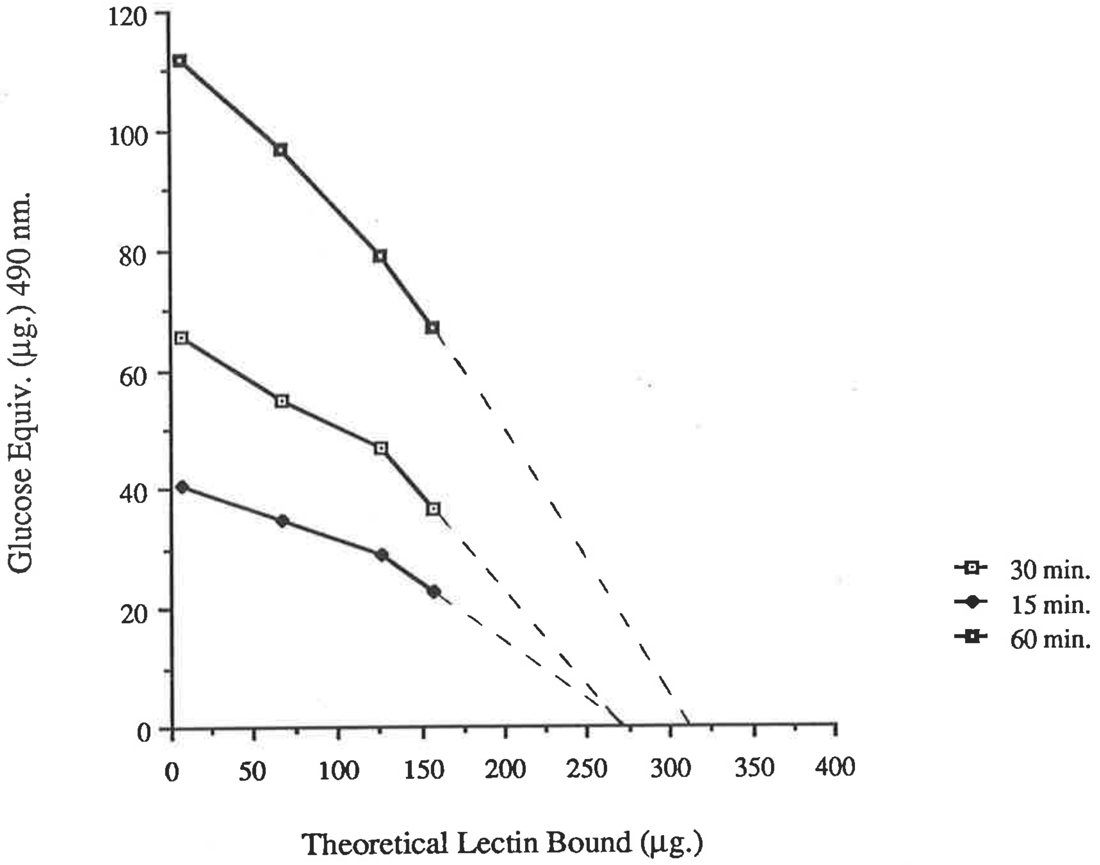


Table 4

Susceptibility Of Substituted Soluble Starch* To Salivary Alpha-Amylase

Degree Of Substitution	Time (min.)	Activity (Abs. 600 nm.)
0.03	6	0.650
"	15	0.924
0.07	6	0.515
"	15	0.769
0.12	6	0.366
"	15	0.594
0.27	6	0.230
"	15	0.205
0.32	6	0.012
"	15	0.064

*Merk Soluble Starch.

Table 5

Susceptibility Of Substituted Soluble Starch To Beta-Amylase.

Degree Of Substitution (M)	Time	Enzyme Activity (Abs. 600 nm.). pH.4.8	pH.5.5
0.03	6	0.570	0.446
"	15	0.595	0.491
0.07	6	0.400	0.285
"	15	0.495	0.342
0.12	6	0.305	0.185
"	15	0.215	0.079
0.27	6	0.004	-
"	15	-	-
0.32	6	-	-
"	15	-	

Fig.27 Beta-amylase incubation of Alpha-amylase treated, substituted starch.

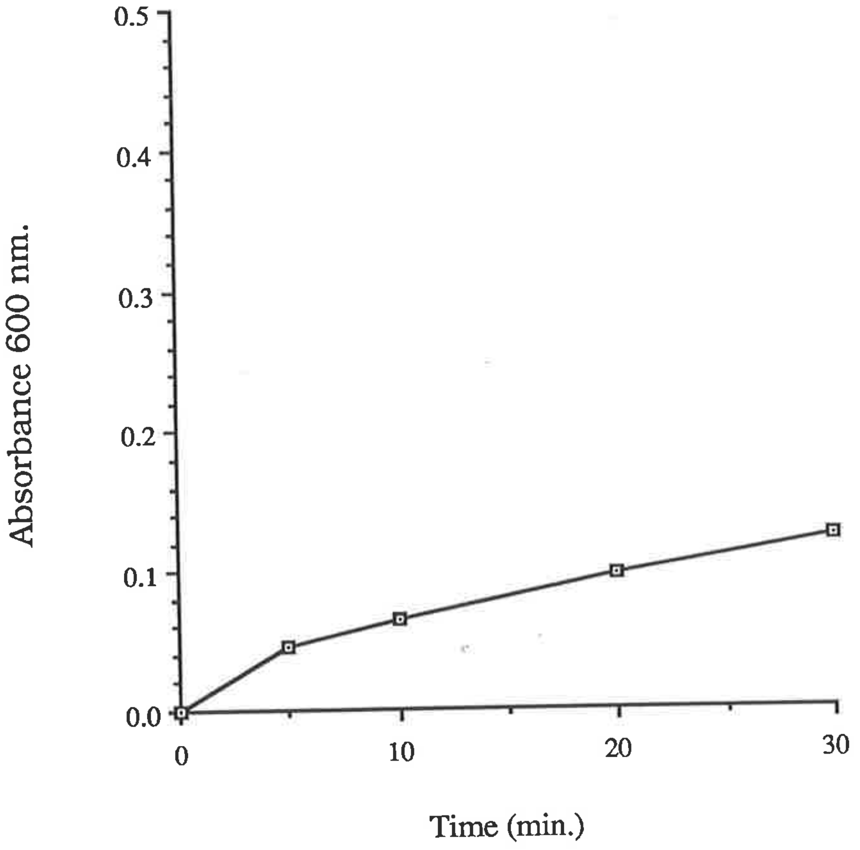
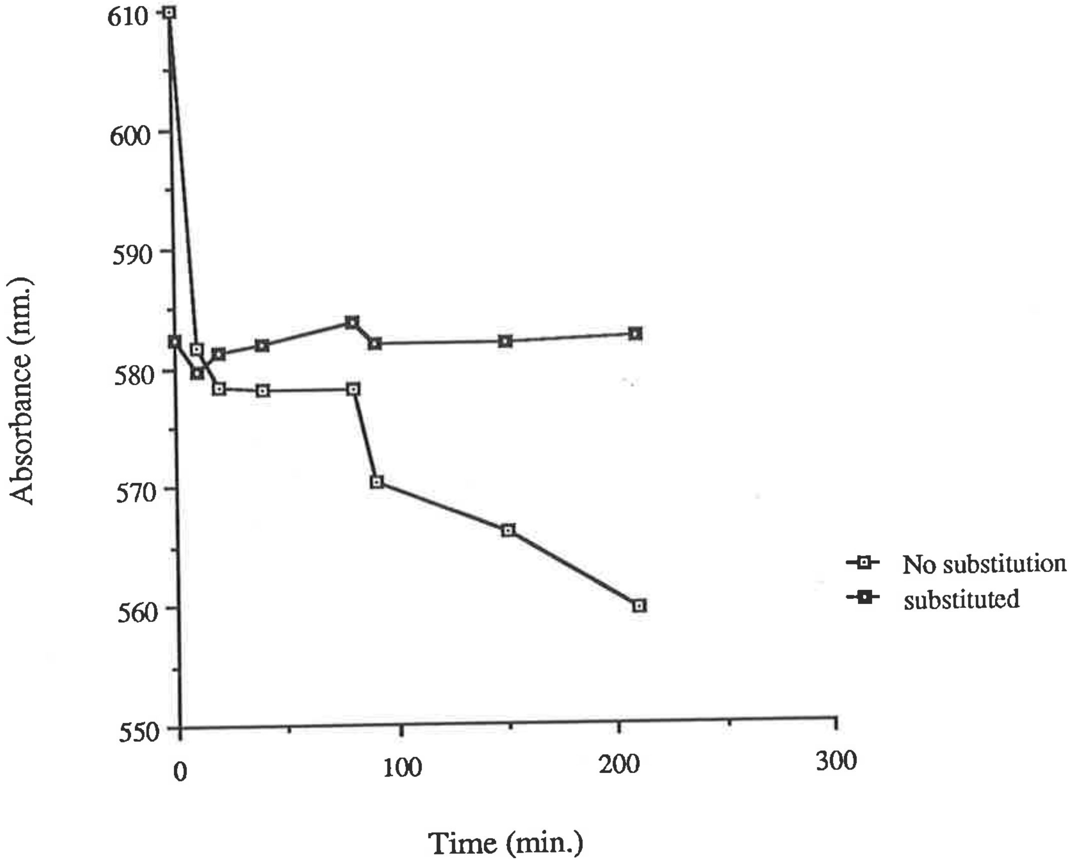


Fig.28 Substituted And Unsubstituted Starch incubated with Beta-amylase..



and show little release of reducing groups. A change in the wavelength of maximum absorbance of beta-amylase incubations with substituted (0.018) and unsubstituted soluble starch over a two and a half hours are presented in Fig. 28 (SD: 1.76). Little decrease in absorption occurred in the substituted sample in marked contrast to the decrease observed for the unsubstituted sample. To assess the effect of substitution and amylase degradation on a more homogeneous substrate than soluble starch, a preparation of amylose (Molar substitution 0.018) was prepared. An alternative detection method of assessing polysaccharide degradation, based on a decrease in iodine staining, with modified amylose and starch as substrates is illustrated in Fig. 29 (SD: 2.14). The results show a decreases in iodine affinity for both substrates. Changes in wavelength and maximum absorbance of modified and unmodified amylose after incubation for twenty min. with beta-amylase are shown in Fig. 30 (SD: 1.78). One fifth of the quantity of beta-amylase utilized in the modified amylose incubation reduced the absorbance of unsubstituted amylose from 1.3 at 615 nm. to 0.32 at 576 nm. The wavelength change was accompanied by a decrease in absorbance at that wavelength and was most noticeable in the unmodified substrates where the initial absorbance and wavelength changes were greater initially and less finally than that of the unmodified substrates, demonstrating greater degradation in the unmodified substrates. The slight decrease in absorption observed for modified substrates maybe due to degradation of those amylose chains which are not substituted or were substituted and remained susceptible in the presence of beta-amylase, resulting in a product of shorter chain length and lower iodine affinity.

Results for modified starch incubated with increasing concentrations of alpha-amylase are given in Fig. 31 (SD: 1.45). Increasing the amount of enzyme enhanced the degradation reflected in an increase in free reducing groups. Incubation with alpha and beta-amylase on modified and unmodified amylose is shown in Fig. 32 (SD: 1.64).

A negligible amount of reducing groups were released from the modified substrate in contrast to the unmodified substrate. The potential of this modified substrate as a method of assaying

Fig.29 Alpha-amylase degradation Of Substituted Starch And Amylose.

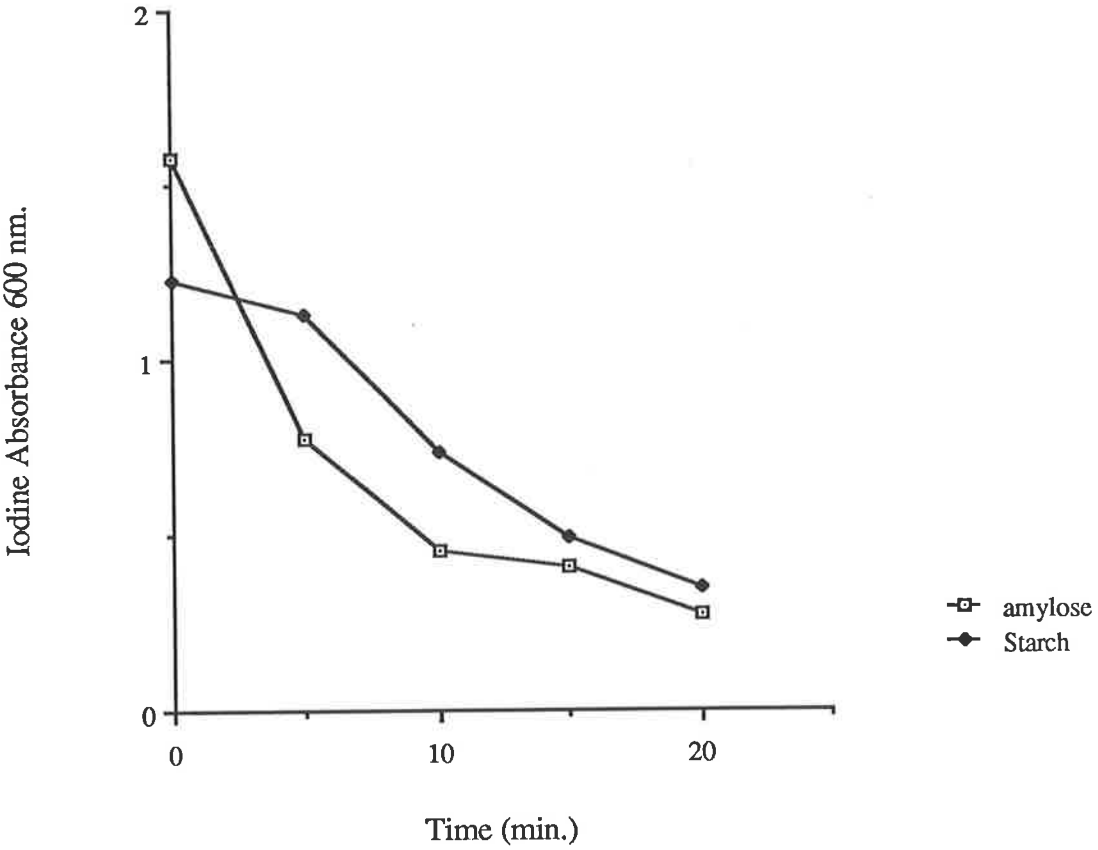


Fig.30 Beta-amylase Degradation Of Substituted And Unsubstituted Amylose.

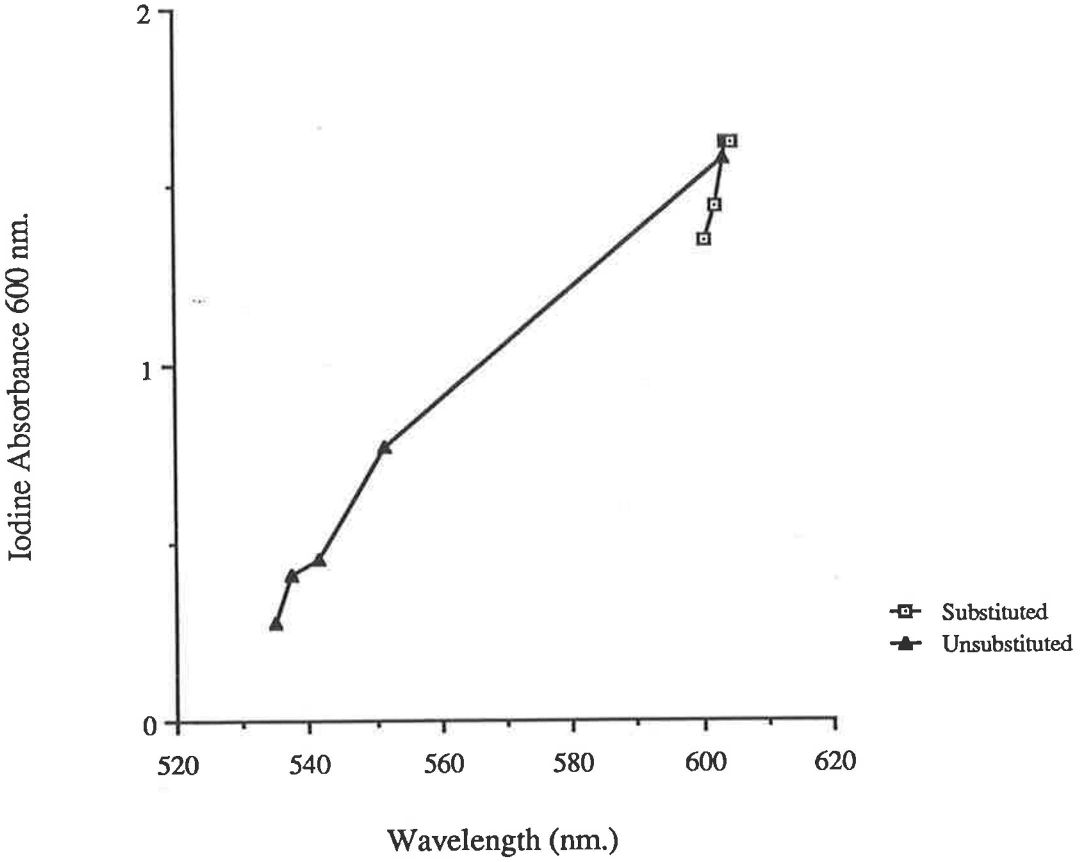


Fig.31 Substituted Starch Incubated With Increasing Quantities Of Alpha-Amylase.

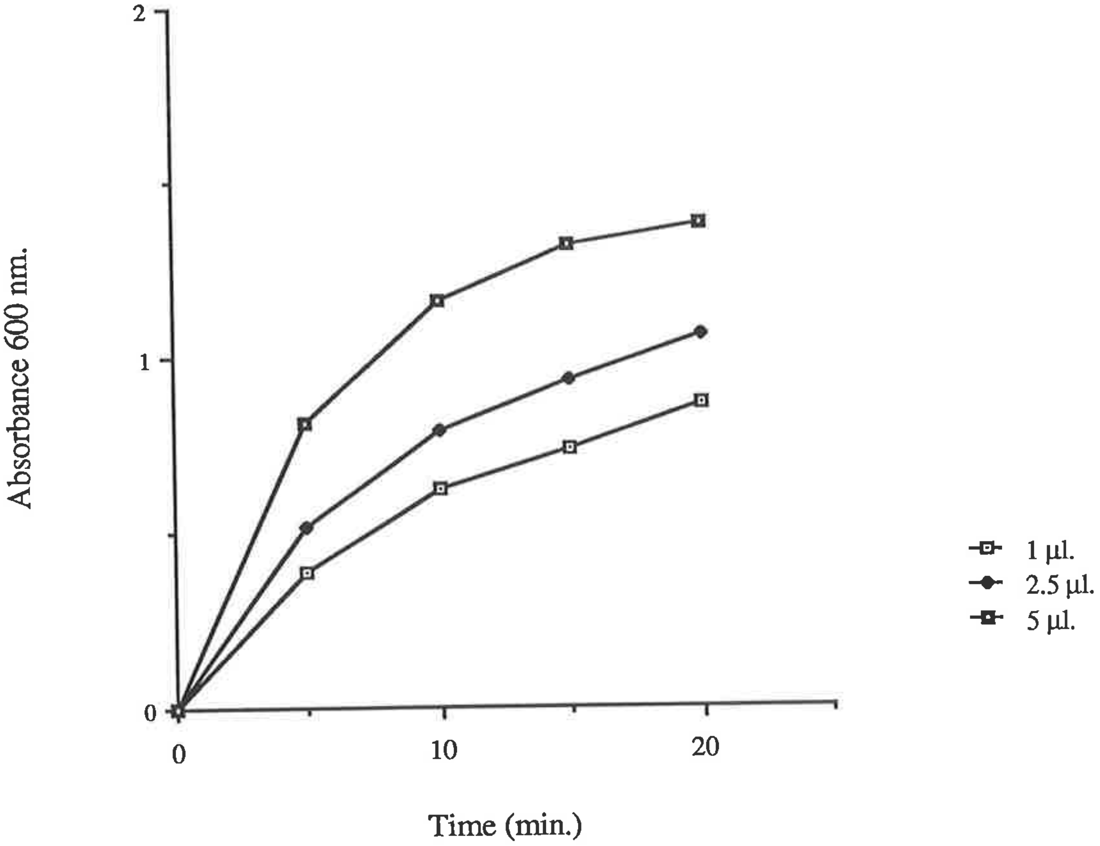
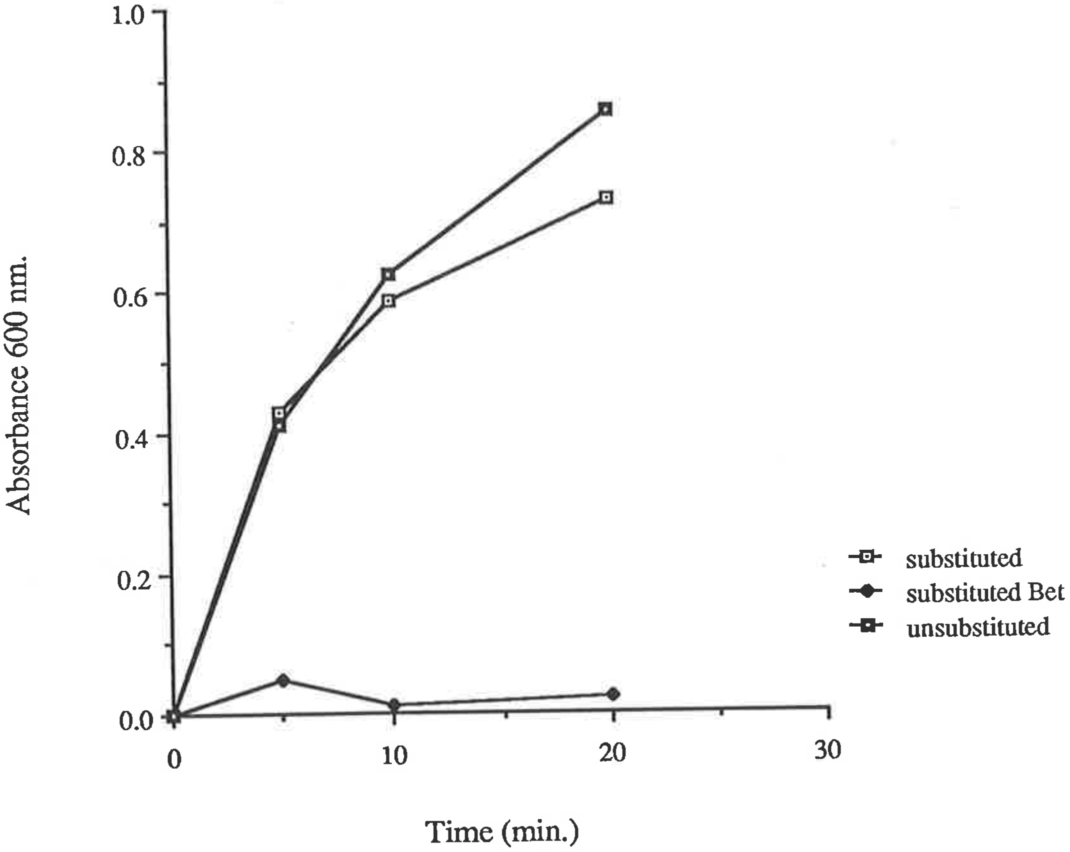


Fig.32 Amylose, Substituted And Unsubstituted Incubated with Alpha And Beta-amylase.



endo-amylases in the presence of exo-amylases was assessed. Substituted starch was incubated with malted barley extracts possessing a range of amyloytic activities expected to occur within samples of a plant breeding programme. The samples were equally divided, one set were subjected to heat treatment to denature beta-amylase.

Results are presented in Table. 6 (SD: 1.71). and clearly demonstrate the effectiveness of this substrate for assessing endo-amylase activity. The effects of heat treatment on the stability and activity of alpha-amylase is also highlighted, the inactivation of alpha-amylase is not of the same order of magnitude in all cases.

3.16. Substitution Of Intact And Enzymically Modified Starch Granules.

Intact and enymically modified starch granules from maize, barley and tapioca respectively were substituted as previously described. Any detrimental effect to the structural integrity of the granule by this procedure was assessed by observing samples of substituted granules under the electron microscope.

Plates. 12 a and b illustrate substituted granules from barley cultivar C.I. 3576 (Molar substitution 0.12) and mannioc (Molar substitution 0.27) Little change in granular shape appears to have occurred, suggesting that granular integrity is maintained.

An assessment on the effect substitution has on the wavelength and absorbance of iodide complexed starch was investigated using isolated substituted fractions of amylose and amylopectin. Results are presented in Table. 7 for potato starch granules and in Table. 8 for starch granules of normal maize. An increase in substitution resulted in decreases in maximum wavelength and absorbance values. The extent of substitution in the fractionated components of substituted potato and normal maize starch granules were assessed. Results are presented in Table. 9 (SD: 0.011). Wavelength of maximum absorbance for the substituted amylose component in Tables. 7 and 8 was significantly lower than that of

Table. 6

Activity Of Malted Barley Extracts On Substituted Soluble Starch

Sample	Time.(min.)	Activity (Abs. 600nm)	Activity, Heated (Abs.600 nm.)
1	10	0.361	0.260
"	20	0.648	0.406
2	10	0.072	0.074
"	20	0.258	0.153
3	10	0.212	0.080
"	20	0.433	0.206
4	10	0.282	0.043
"	20	0.433	0.209
5	10	0.406	0.191
"	20	0.712	0.438
6	10	0.291	0.184
"	20	0.624	0.432

Plates.12 a-b.

Electron Microscopy.

Plate. 12 a: Intact mannioc starch granules.

Plate. 12 b: Hydroxypropylated (0.3M) mannioc starch granules, crystals are Na_2SO_4.

Plates.12a-b

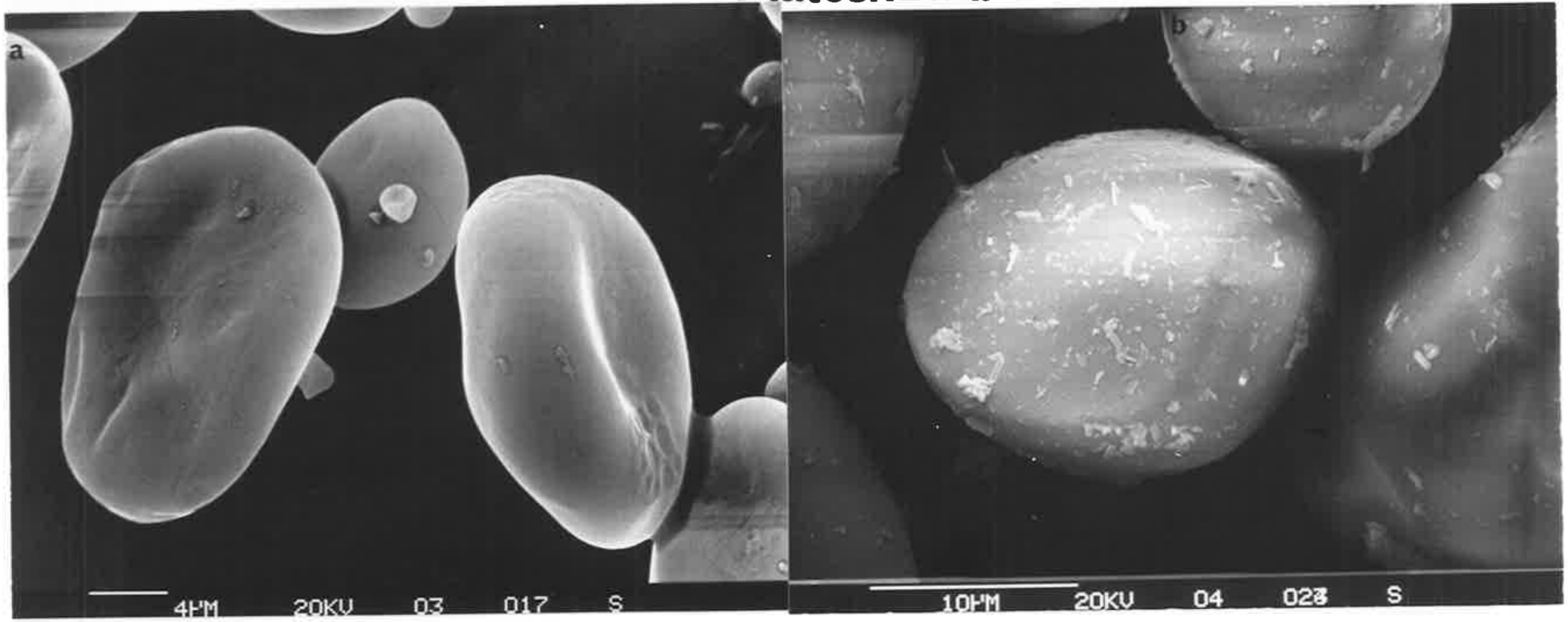


Table 7

Fractionation Of Potato Starch Granules.

Mol.Substitution	Fraction	Wavelength Max.	Absorbance (600 nm).
0	Complete	595.5	1.160
0	Amylose	630.2	1.079
0	Amylopectin	579.9	1.111
0.01	Amylose	609.0	0.900
0.01	Amylopectin	560.0	0.982
0.03	Amylose	605.0	0.880
0.03	Amylopectin	565.0	1.000
0.18	Amylose	576.2	0.812
0.18	Amylopectin	578.8	0.862

Table 8

Fractionation Of Normal Maize Starch Granules

Molar Substitution	Fraction	Wavelength Max.	Absorbance(600nm)
0	Unfractionated	597.7	1.255
0	Amylose	625.0	1.380
0	Amylopectin	565.0	1.100
0.03	Amylose	600.0	0.930
0.03	Amylopectin	570.0	0.500
0.18	Amylose	598.6	0.722
0.18	Amylopectin	563.6	0.675

Table 9

Distribution Of Hydroxypropyl Substituents Of Fractionated Starch Granules

Starch Type	Fraction	Degree Of Substitution (M)	Substitution (Abs.590 n.m.)
Potato	Amylose	0.010	0.092
Potato	Amylopectin	0.010	0.079
Potato	Amylose	0.030	0.278
Potato	Amylopectin	0.030	0.213
Potato	Amylose	0.180	0.930
Potato	Amylopectin	0.180	1.089
Maize	Amylose	0.010	0.106
Maize	Amylopectin	0.010	0.071
Maize	Amylose	0.030	0.386
Maize	Amylopectin	0.030	0.106
Maize	Amylose	0.180	n.d.
Maize	Amylopectin	0.180	n.d.

unsubstituted amylose. This caused difficulties in assessing the degree purification achieved in fractionated amylose and amylopectin samples.

Substitution changes the solubility and structural properties of the amylose component and may prevent it complexing efficiently with thymol during the fractionation procedure, some may remain in solution possibly co-purifying with the amylopectin component. The "salting out" method proposed by *Muetgeert et al* [131] was used as an alternative purification procedure, however this method did not result in a satisfactory fractionation as determined by wavelength of maximal absorbance.

3.16.1 Fine Structural Distribution of Hydroxypropyl Groups Of Intact Starch Granules.

The fine structure and distribution of hydroxpropyl groups present on starch granules was investigated by subjecting modified starch to column chromatography before and after debranching with iso-amylase and pullulanase.

3.16.1.2 Elution Profiles Of Substituted And Unsubstituted Normal Maize Starch Granules.

Figures 33 a and b show the chromatographic profile wavelengths and absorbance values of amylose and amylopectin from normal maize starch fractionated through a column of Sepharose CL2B. Total carbohydrate was measured at 490 nm., wavelength and absorption values were obtained from scanning between 500-650 nm. and amylose detected by iodine/potassium iodide. Reducing groups were measured at 600 nm. and hydroxypropyl substitution at 590 nm.

Elution profiles obtained from normal intact maize starch granules after degradation to 35% by salivary alpha-amylase are shown in Figs. 34 a-c. Comparison of Figs. 33 and 34

Fig.33 a. Fractionated Maize Granules.

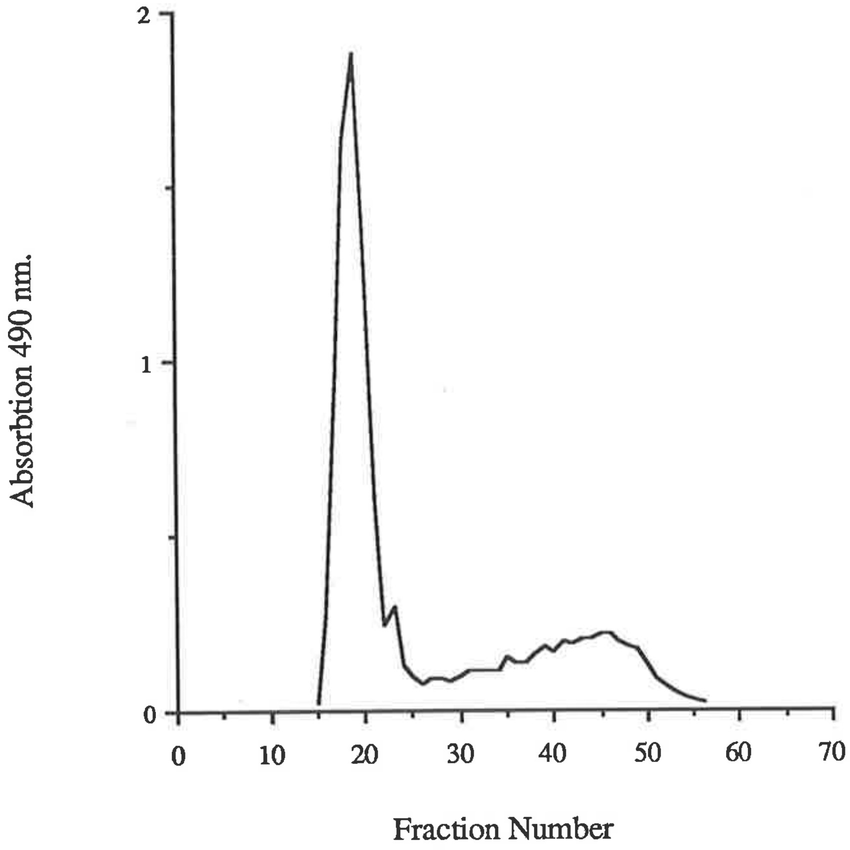


Fig. 33 b. Fractionated Maize Granules.

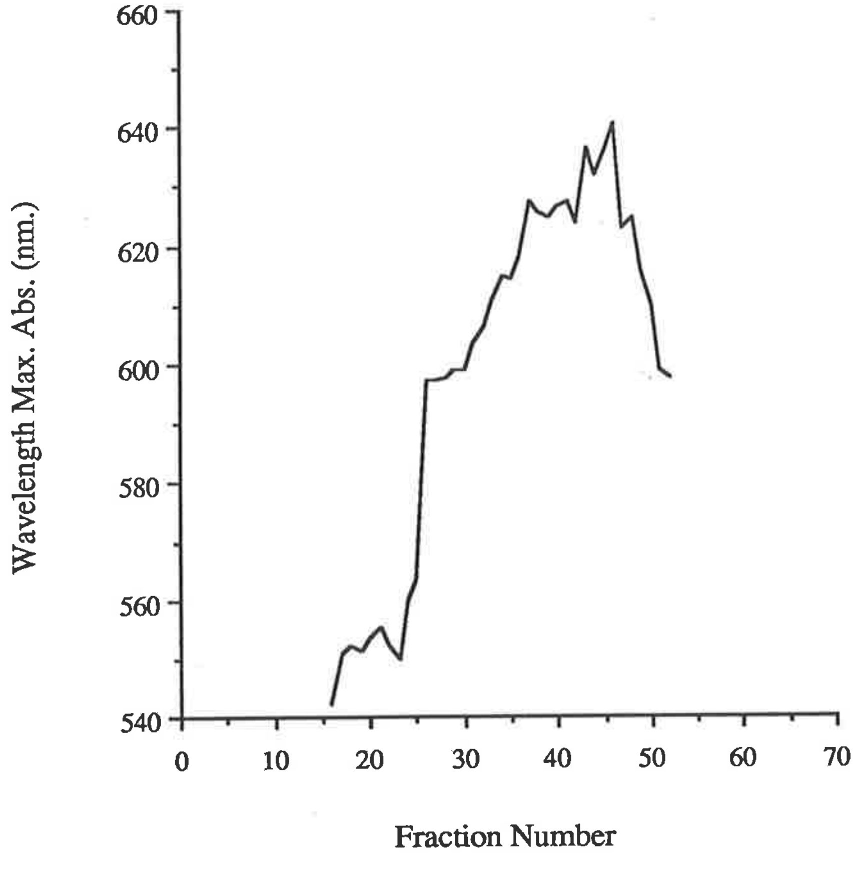


Fig. 34 a. Alpha-amylase Degraded (35%) Maize granules. Fractionated.

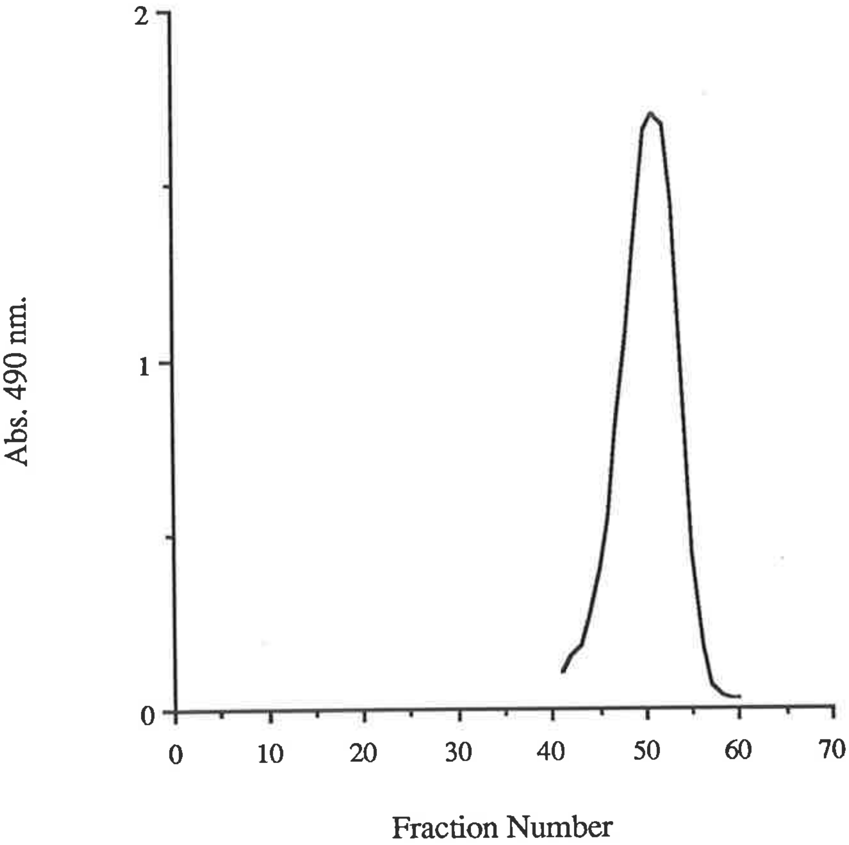


Fig. 34 b. Maize Granules. Alpha-amylase Degraded(35%). Fractionated.

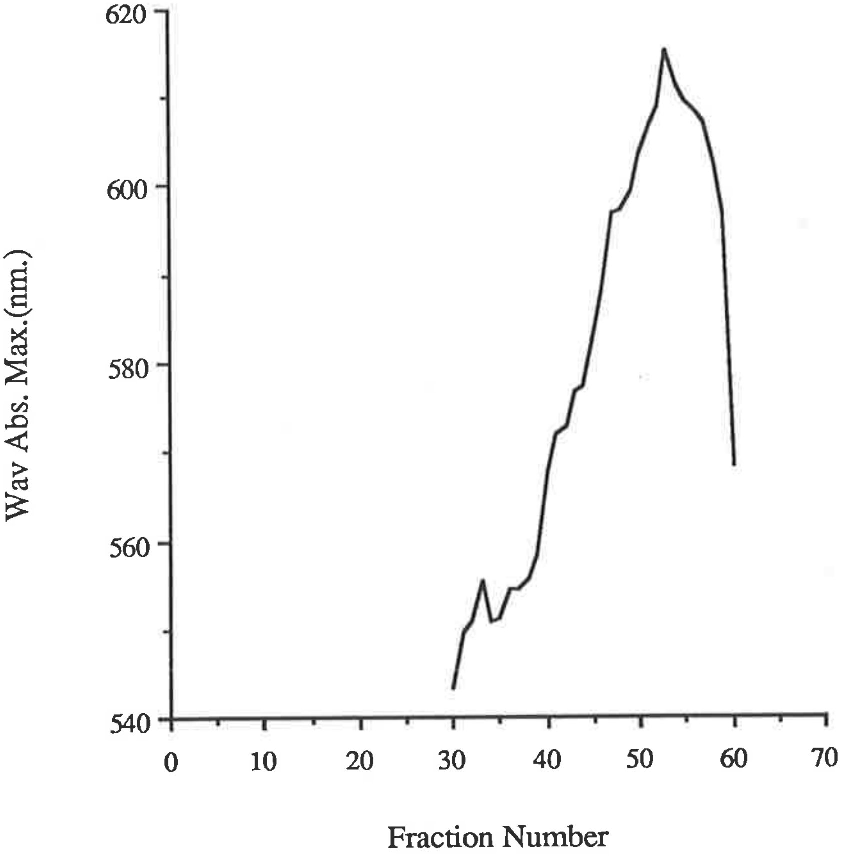


Fig. 34 c. Alpha-amylase Degraded(35%). Maize Granules. Fractionated.

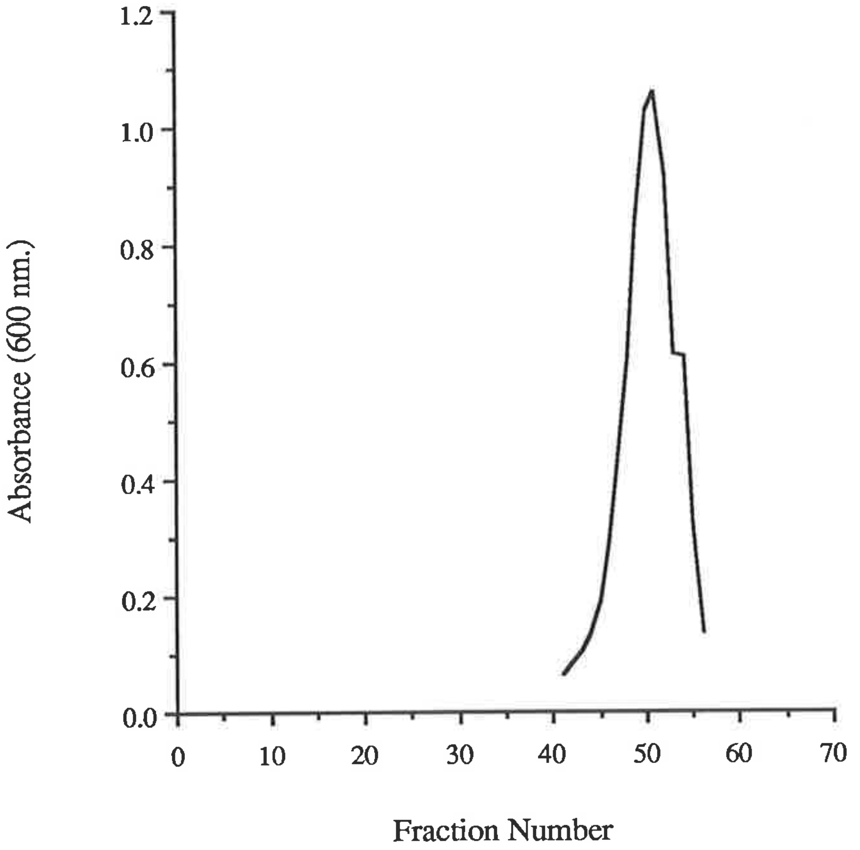
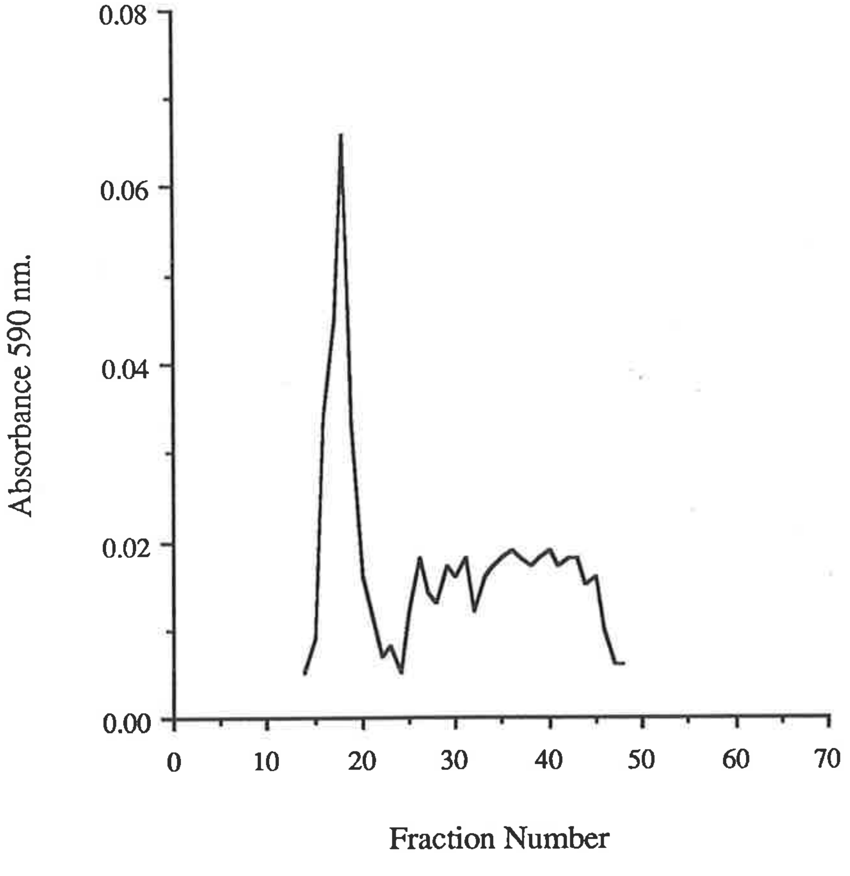


Fig. 35. Fractionated. Substituted Maize Granules



indicates that alpha-amylase degradation promotes losses in the amylose and amylopectin fractions producing lower molecular weight sugars. Figure 35 shows that hydroxypropyl groups are distributed over the amylose and amylopectin components of substituted granules. The elution profile indicated in Figs. 36 a-c for maize starch after substitution, debranching and passage through a chromatographic matrix of Sepharose CL2B. A distribution profile of amylose and unit chains derived from the debranched amylopectin fraction occurs between fractions 35-60. A major hydroxpropylated peak occurs between fraction 45-60, this contains debranched substituted and unsubstituted chains of amylopectin and amylose.

Increased resolution of substituted and unsubstituted granules after debranching was obtained by passage through a column containing Sephadex G-50.

An elution profile and absorbance values for debranched maize is shown in Figs. 37 a-c. Comparison of substituted (0.03 M) maize starch, Fig. 38 a-c, with Figs. 37 a-c demonstrates both amylose and amylopectin components were substituted. Debranched maize starch granules degraded by 35% is shown in Fig. 39 a and b. A decrease in the amylose fraction and chains derived from the debranched amylopectin component is apparent when compared with the elution profile of maize starch without amylase treatment.

Comparison of Figs. 37, 38 and 39 show little of the amylose component still remains after degradation. Figures 40 a-d illustrates the elution profile of substituted maize starch granules (0.03M) degraded 15-20% with alpha-amylase.

Figures 41 a and b depict the elution profile of substituted maize starch granules treated with beta-amylase after debranching and alpha-amylase degradation. A portion of the amylose fraction is resistant to exo-amylase attack in both samples, suggesting it is resistant and substituted.

In contrast chains derived from the debranched amylopectin fraction are degraded extensively by beta-amylase suggesting they are not substituted to the same extent as the amylose fraction, further the smaller unit chains appear to be more extensively degraded than the larger unit chains.

Fig. 36 a. Substituted, Debranched And Fractionated Maize Granules.

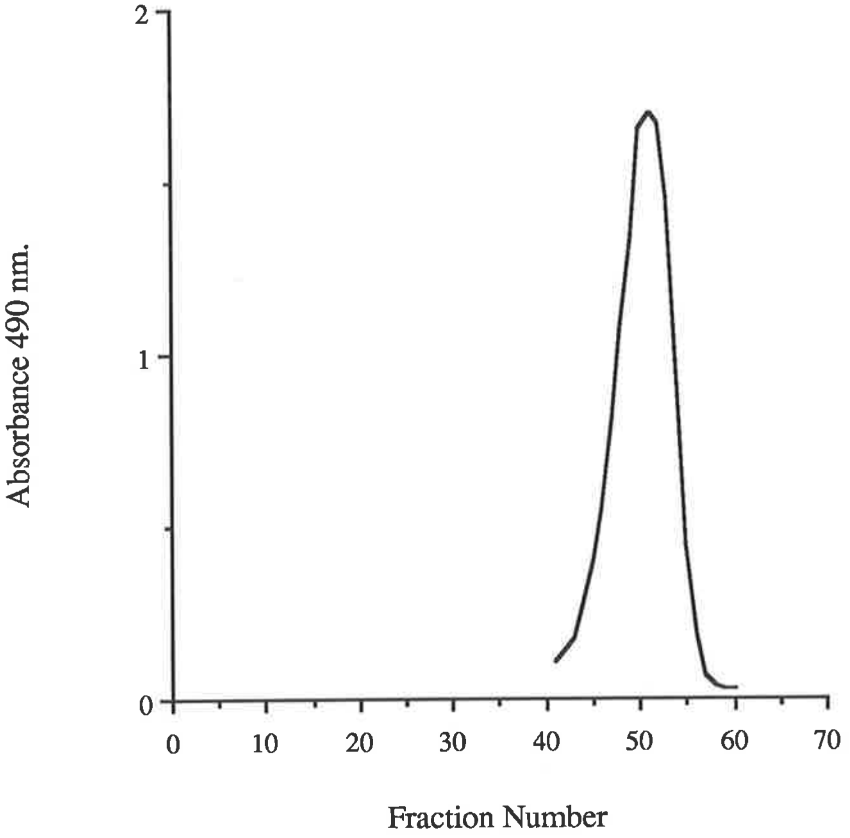


Fig. 36 b. Substituted, Debranched And Fractionated Maize Granules.

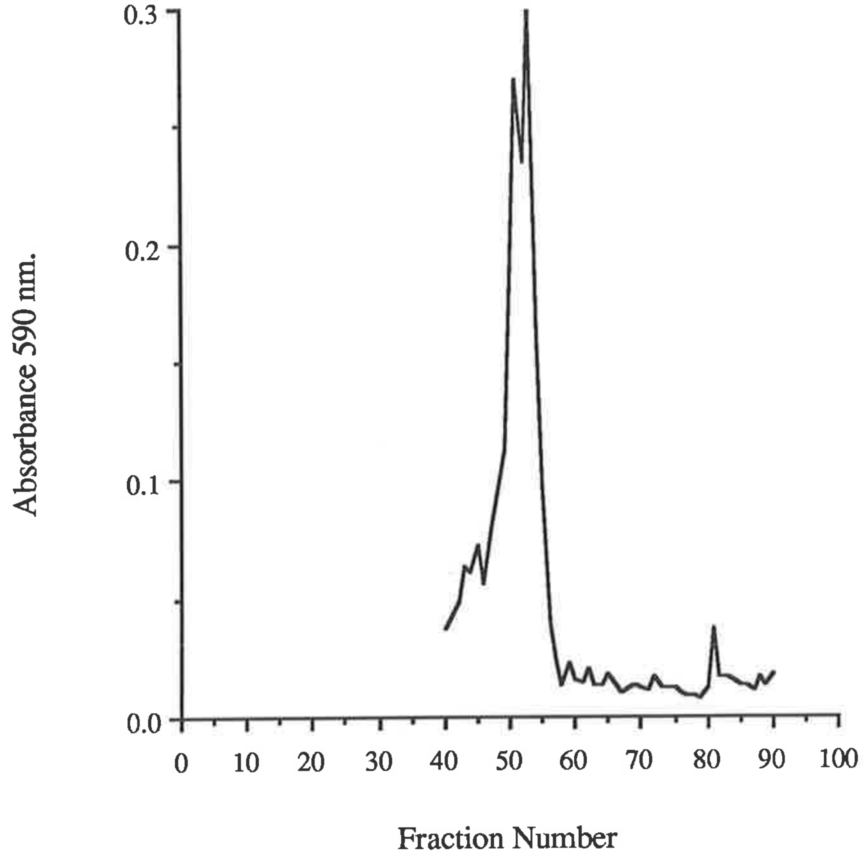


Fig. 36 c. Substituted, Debranched And Fractionated Maize Granules.

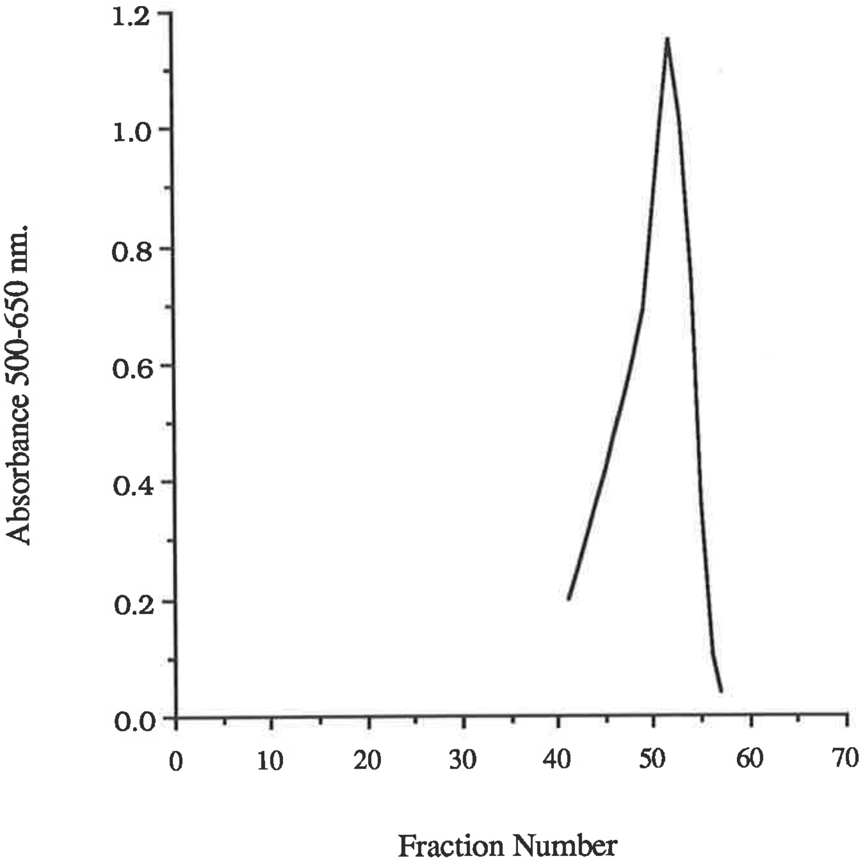


Fig. 37 a. Fractionated Maize Starch. Debranched.

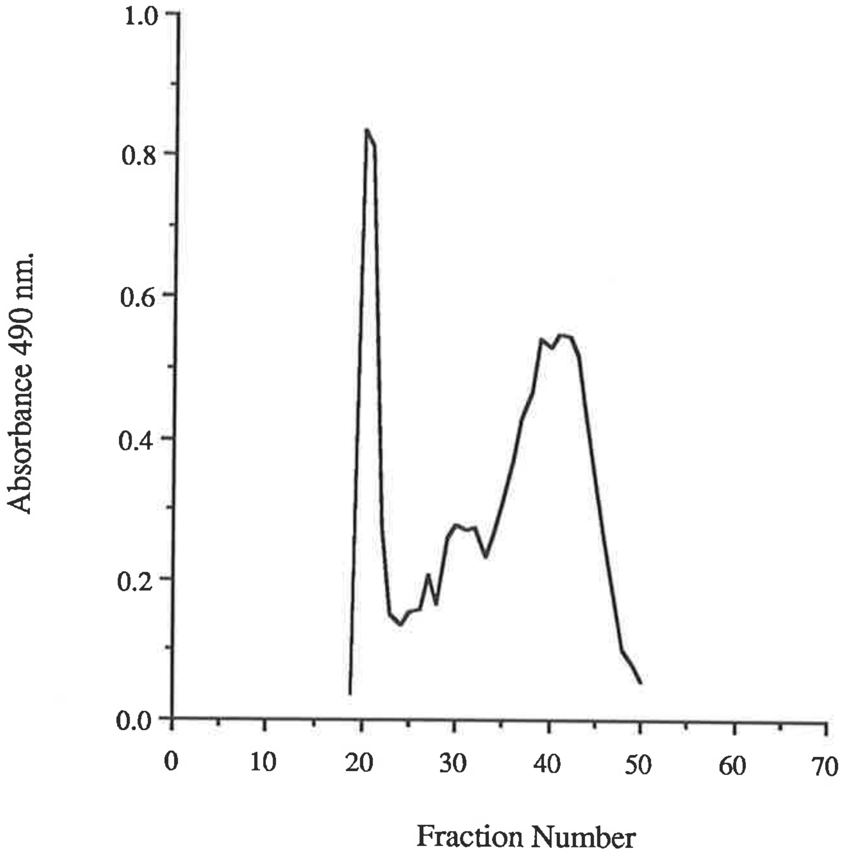


Fig. 37 b. Fractionated Debranched Maize Granules.

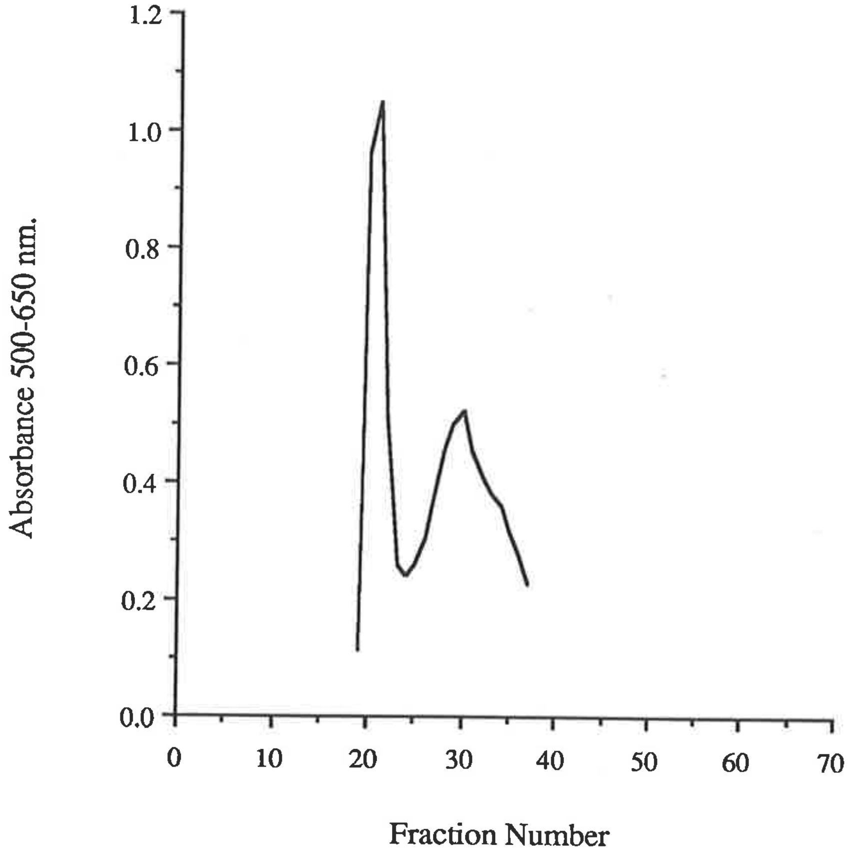


Fig. 37 c. Fractionated, Debranched Maize Granules.

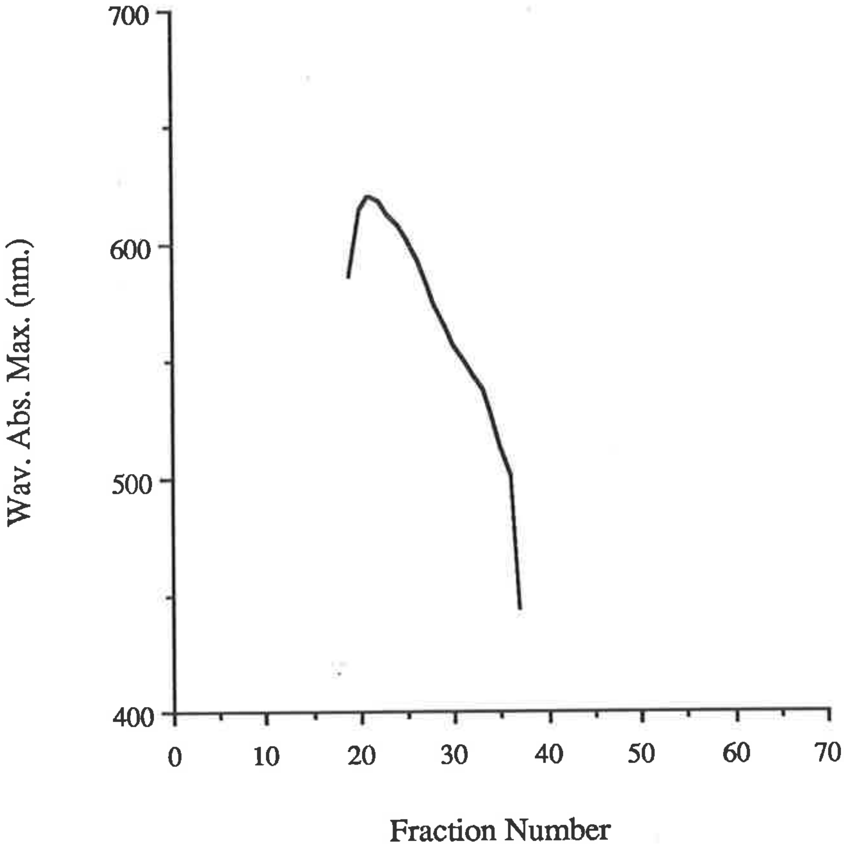


Fig. 38 a. Fractionated, Substituted, Debranched Maize Granules.

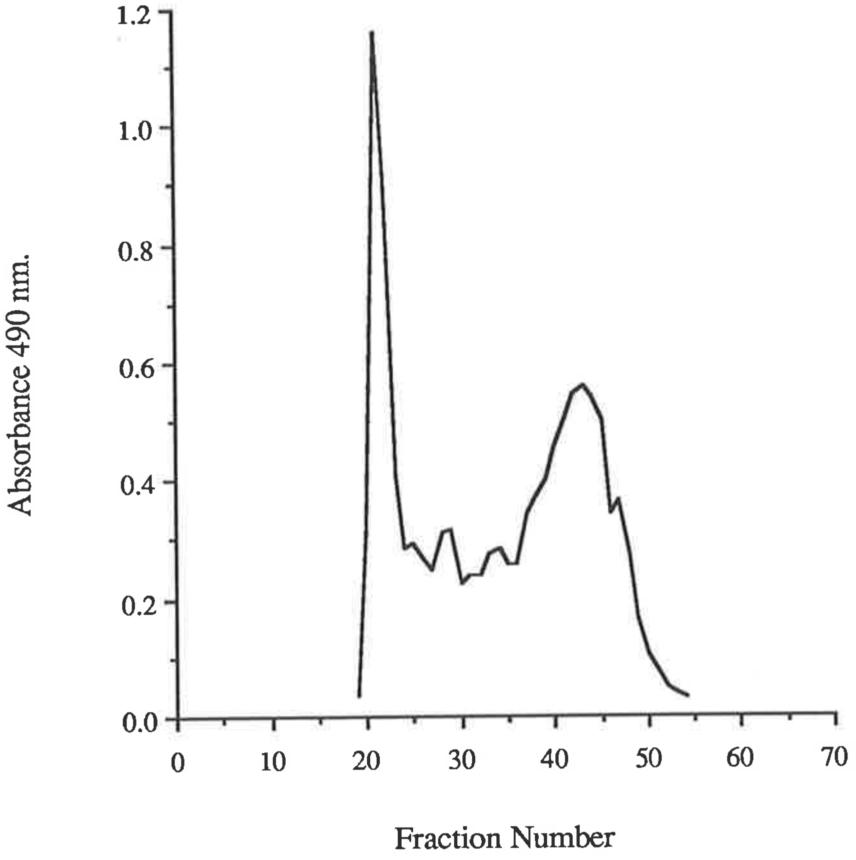


Fig. 38 b. Fractionated, substituted, Debranched Maize Granules.

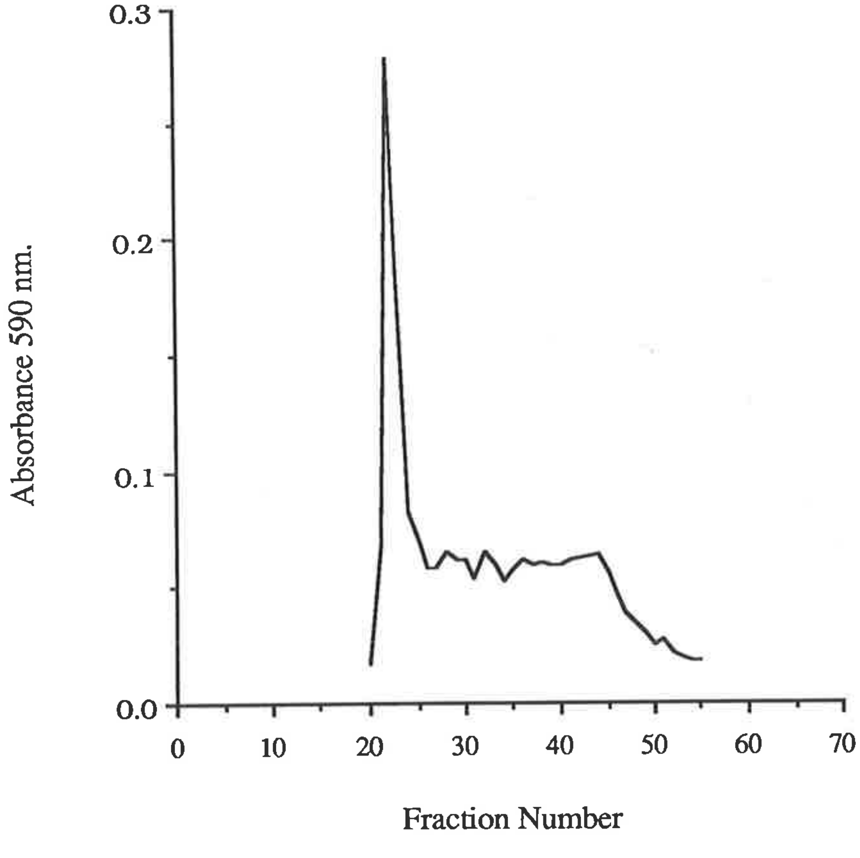


Fig. 38 c. Fractionated, Substituted, Debranched Maize Granules.

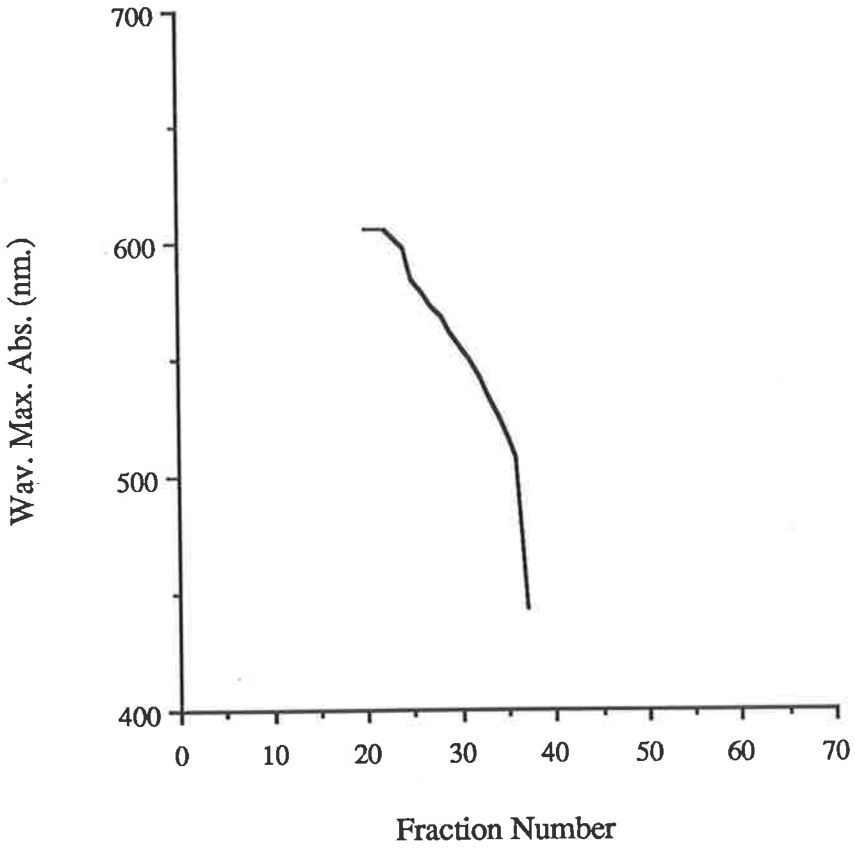


Fig. 39 a. Alpha-amylase Degraded(35%). Debranched. Fractionated Maize Granules.

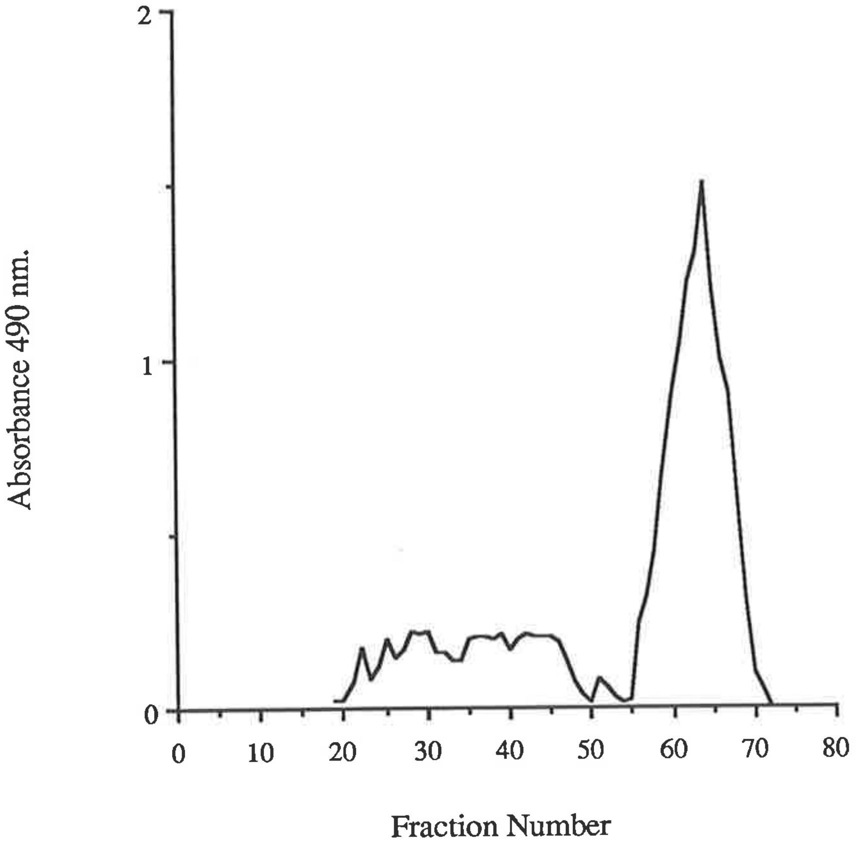


Fig. 39 b.Alpha-amylase Degraded(35%). Debranched. Fractionated Maize Granules.

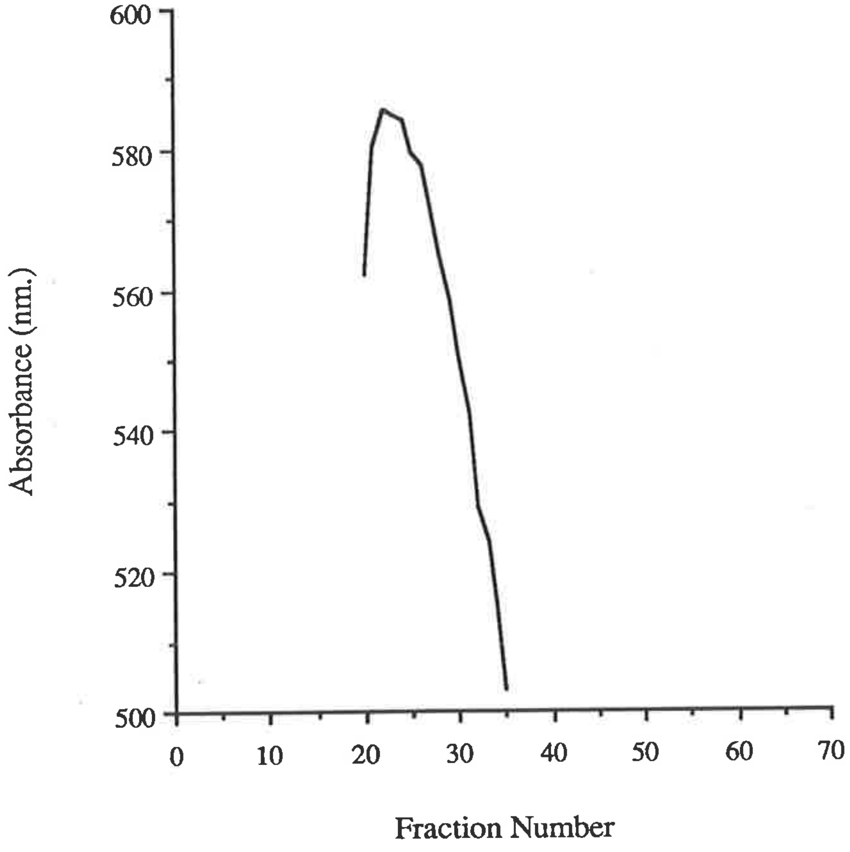


Fig.40 a. "Alpha"(15-20%). Debranched. Substituted. Fractionated.

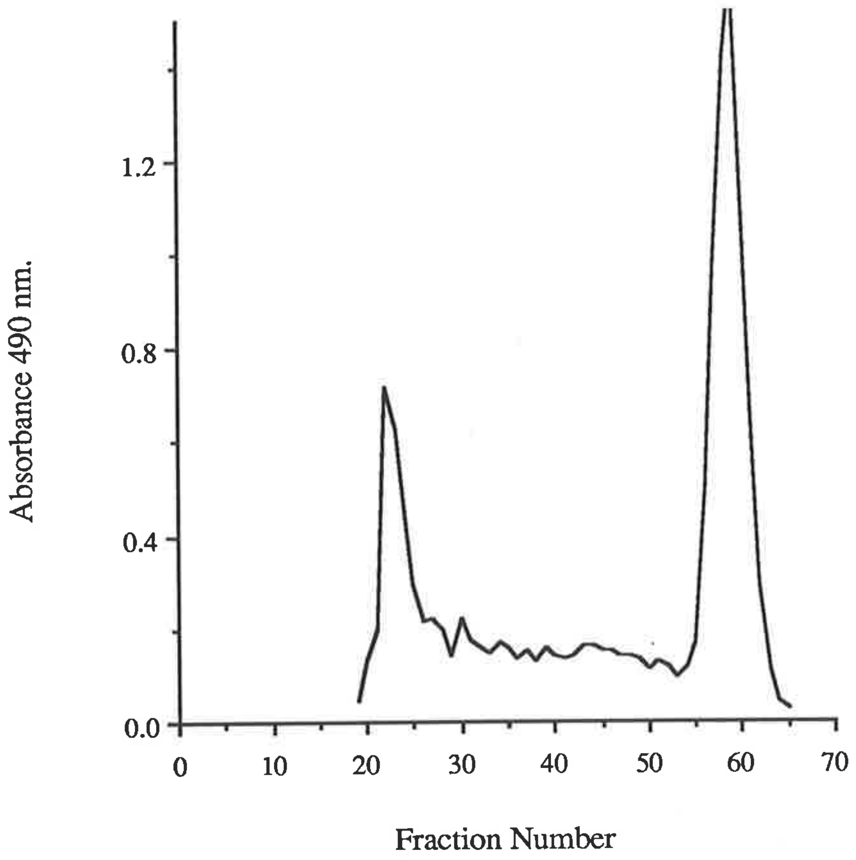


Fig.40 b. "Alpha"(15-20%). Substituted. Debranched. Fractionated.

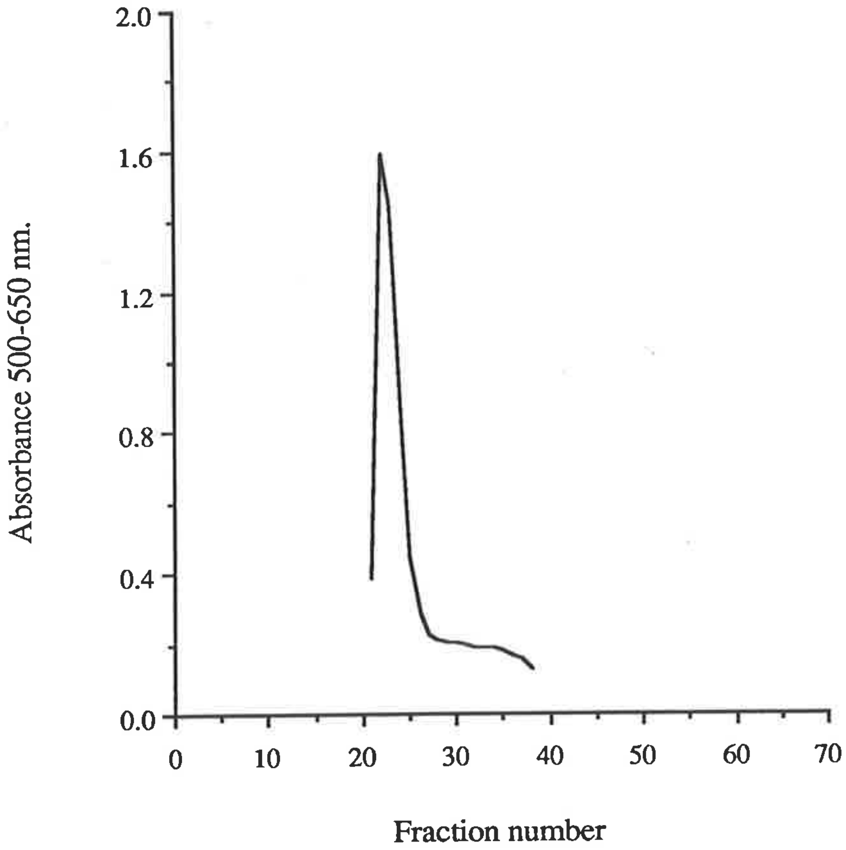
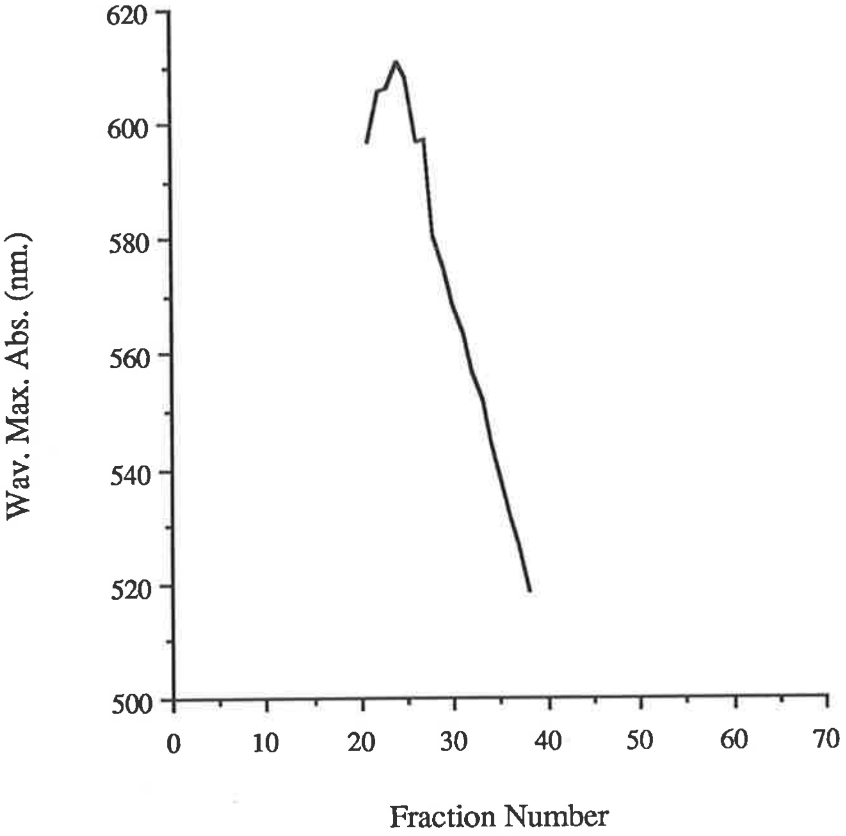


Fig.40 c. "Alpha"(15-20%). Substituted. Debranched. Fractionated.



Alpha-amylase attack was directed towards the amylopectin portion of the granules initially with emphasis on the smaller unit chains (comparison of Fig. 37a with Fig. 41a). Both unit chains are degraded by alpha-amylase but the smaller unit chains are degraded to a greater extent by beta-amylase, suggesting the small chains are not as accessible to substitution as the large unit chains. The small unit chains within the intact granule maybe in a physical state or location preventing substitution to the same extent as the larger unit chains. Both amylose and amylopectin fractions are accessible to alpha-amylase, at least initially, since some the amylose is not degraded but remains susceptible to substitution infers that a proportion of the amylose is present on the granule exterior but not in an accessible form to the enzyme. The elution profile of substituted (0.03 M) maize starch granules is presented in Figs. 42 a-c after overnight degradation by alpha-amylase and demonstrates a portion of the substituted amylose is resistant. Substituted and unsubstituted low molecular weight products are also present.

3.16.3 Elution Profile Of Substituted And Unsubstituted Mannioc Starch Granules.

Figures. 43 a-c show the elution profiles of unsubstituted, debranched mannioc starch granules passed through columns of Sephadex G-50. The amylose component was eluted at the void volume, the two peaks correspond to the A- and B- unit chains present in the original amylopectin.

The data for substituted (0.03 M), debranched mannioc starch illustrated in Figs. 44 a-d have a similar profile to the unsubstituted sample. Figure. 44 d shows decreased absorbtion due to the effects of substitution. This effect can be compared with the unsubstituted sample in Fig. 43 c Hydroxypropyl groups are associated with both amylose and amylopectin fractions, Fig. 44 b. Degradation of starch granules to10-15% before substitution followed by debranching with salivary alpha-amylase indicates that the amylose component was degraded

Fig. 41 a. Substituted, Debranched, Beta-amylase Treated, Maize Granules.

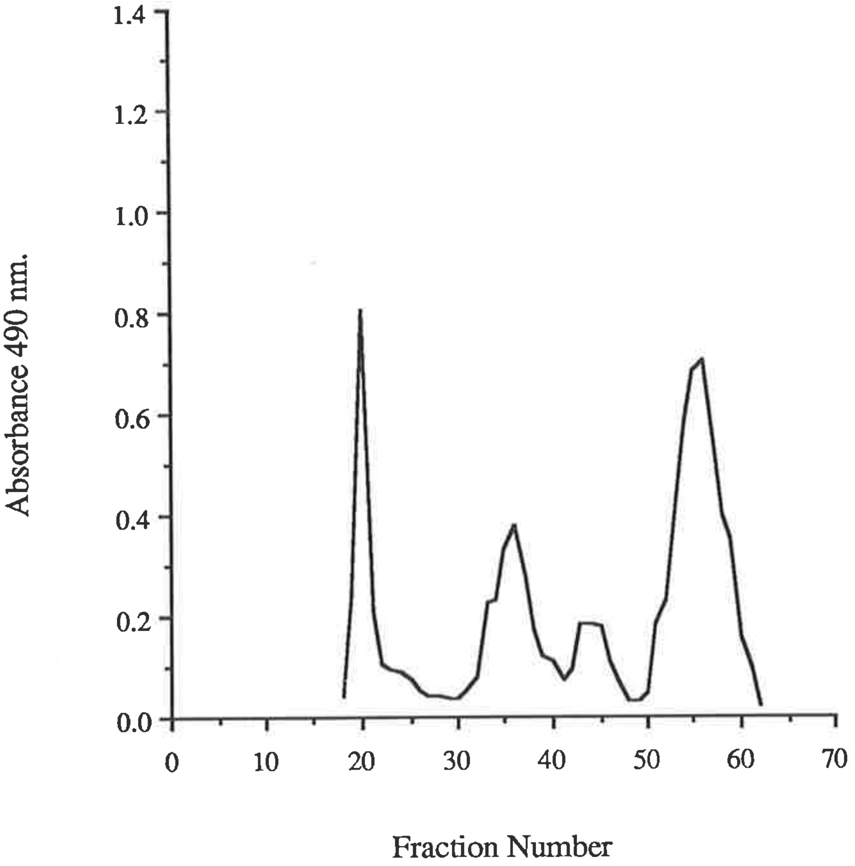


Fig.41b. "Alpha"(15-20%).Substituted, Debranched, Fractionated, Beta-amylase.

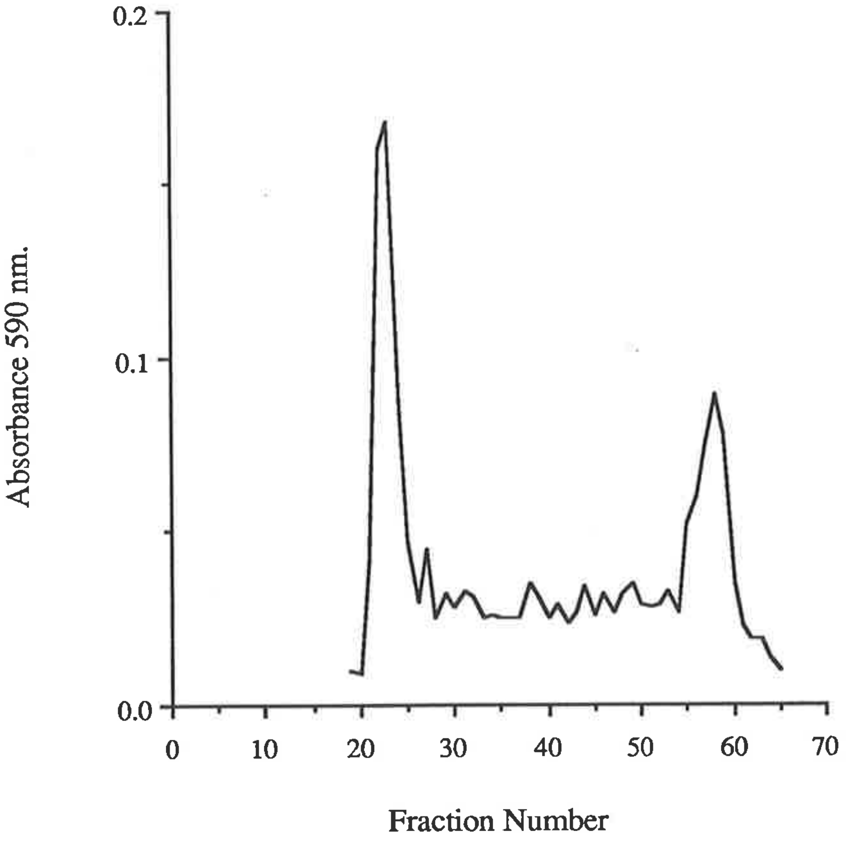


Fig. 42 a. Substituted, Alpha-amylase Degraded(24 hr.). Maize Granules.

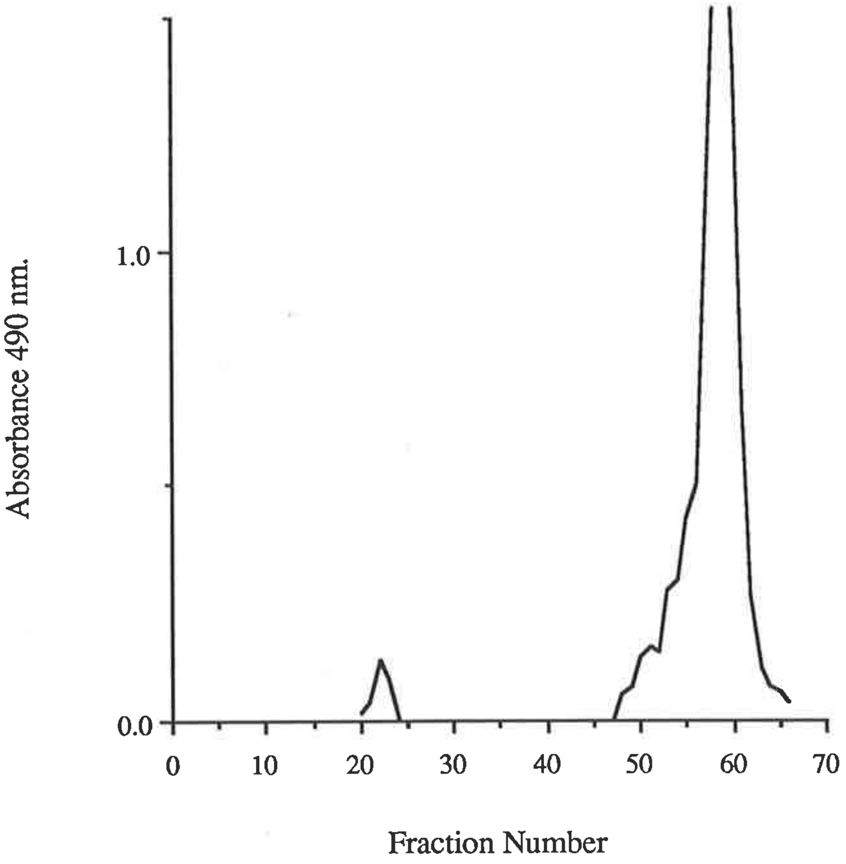


Fig. 42 b. Substituted, Alpha-amylase degraded(24 hr.). Maize Granules.

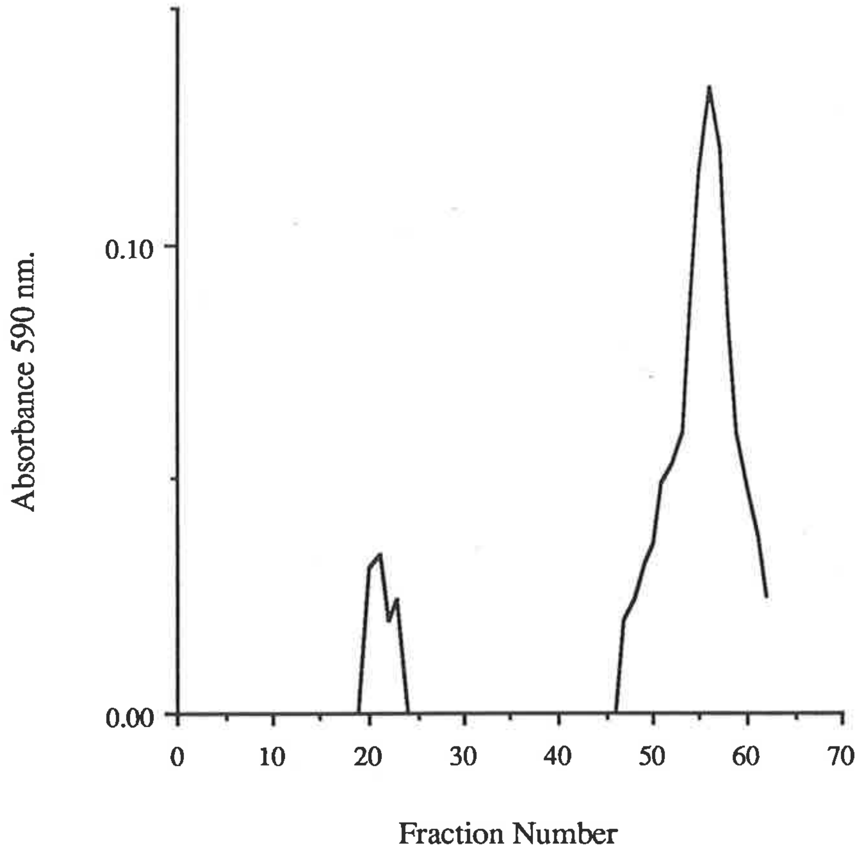


Fig.42c. Substituted. Alpha-amylase Degraded(24 hr.). Debranched. Maize Granules.

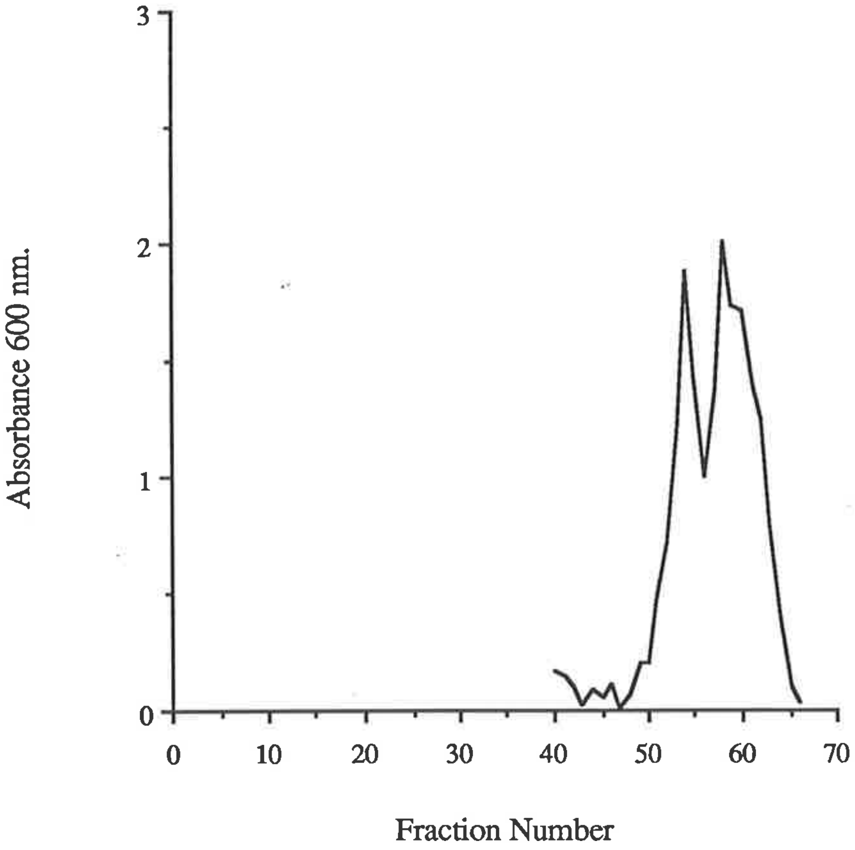


Fig. 43 a. Debranched, Fractionated, Mannioc Granules.

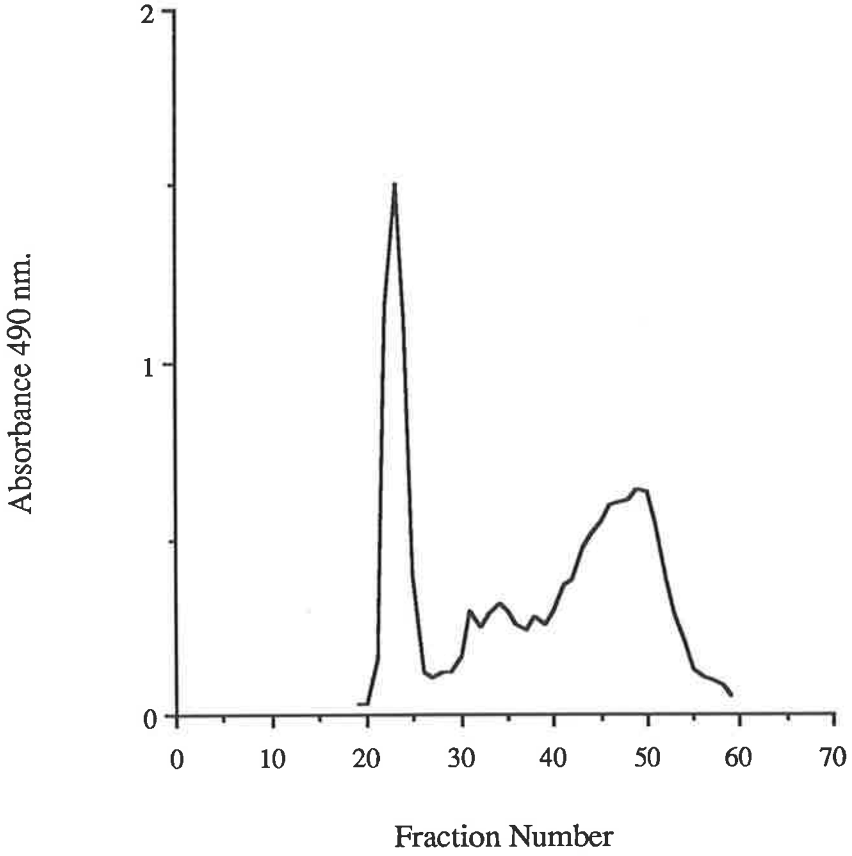


Fig. 43 b. Debranched, Fractionated Mannioc Granules.

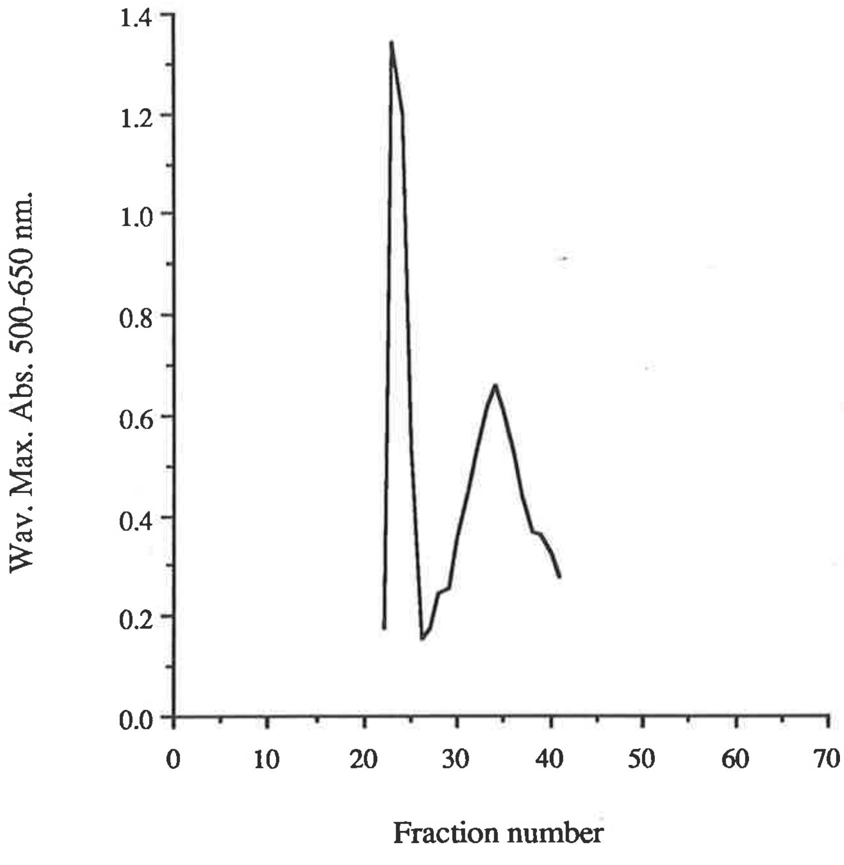


Fig. 43 c. Debranched, Fractionated Mannioc Granules.

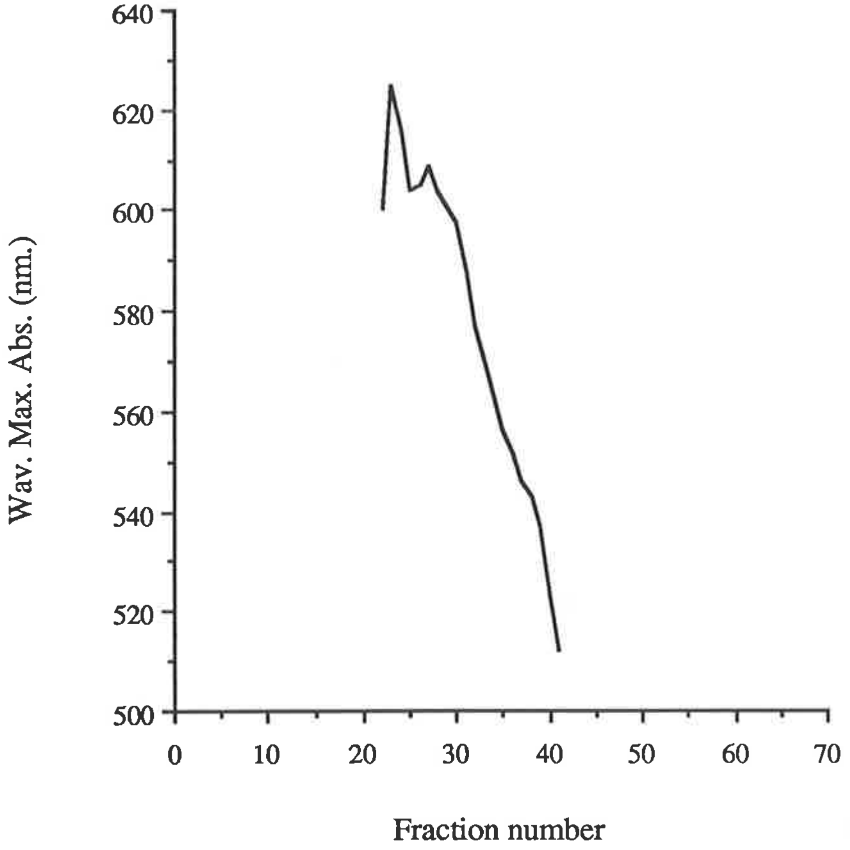


Fig. 44 a. Substituted. Debranched. Fractionated Mannioc Granules.

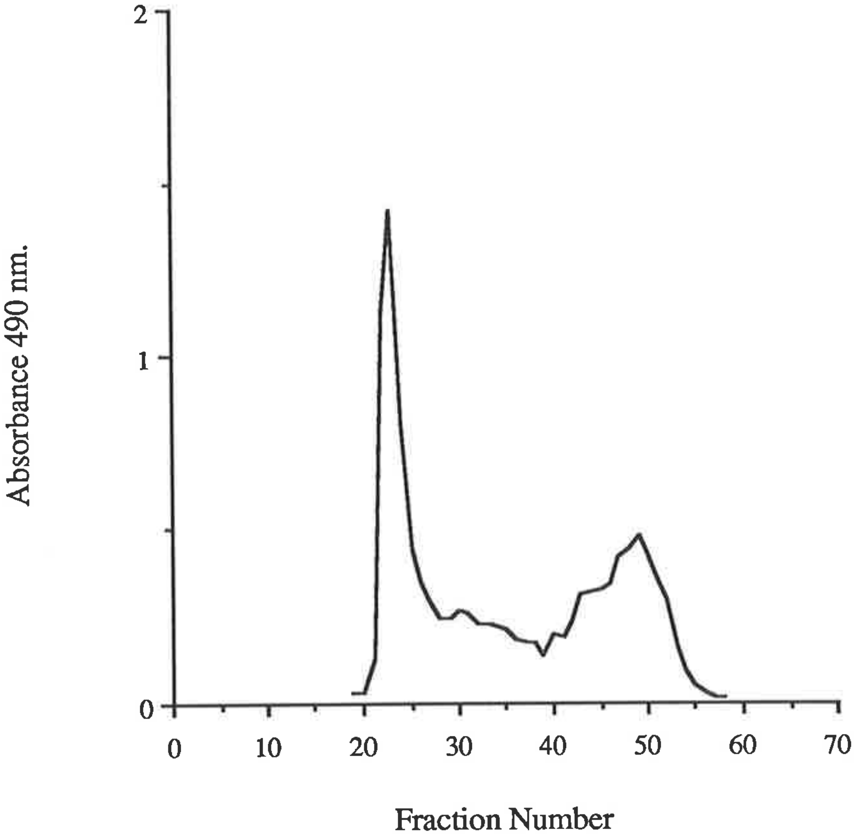


Fig. 44 b. Substituted. Debranched. Fractionated Mannioc Granules.

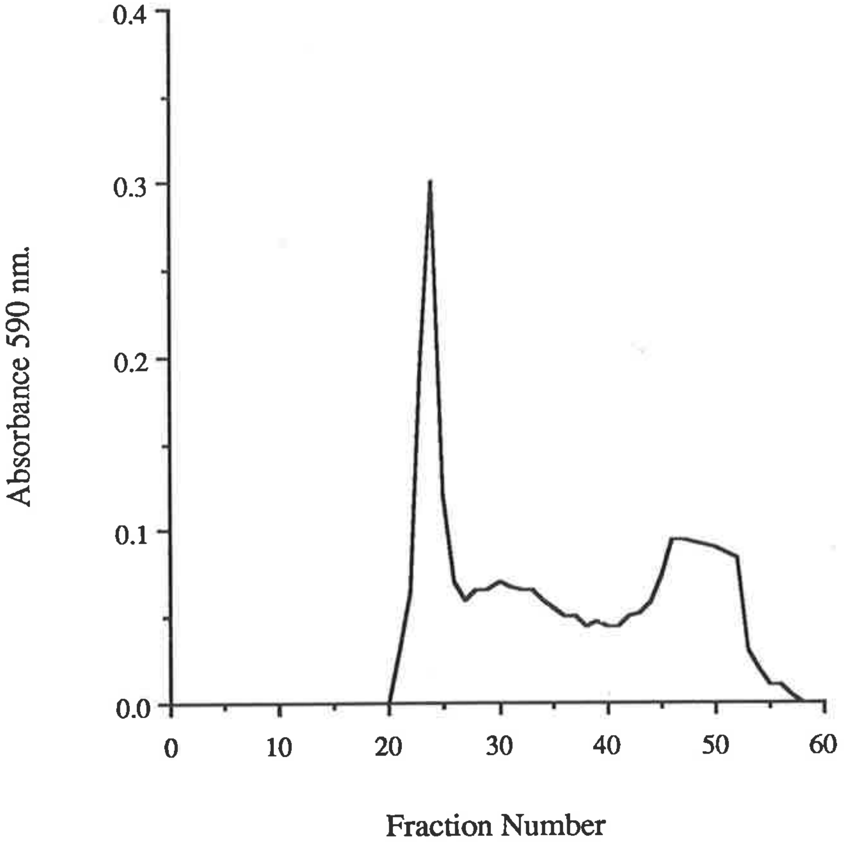


Fig. 44 c, Substituted, Debranched, Fractionated mnnioc Granules.

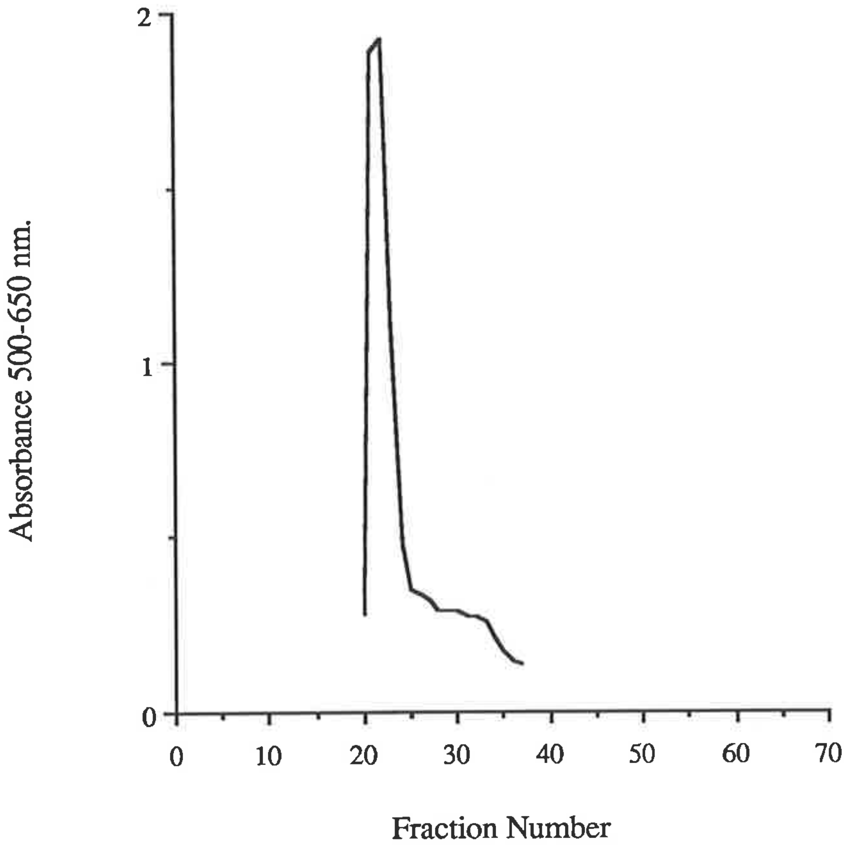
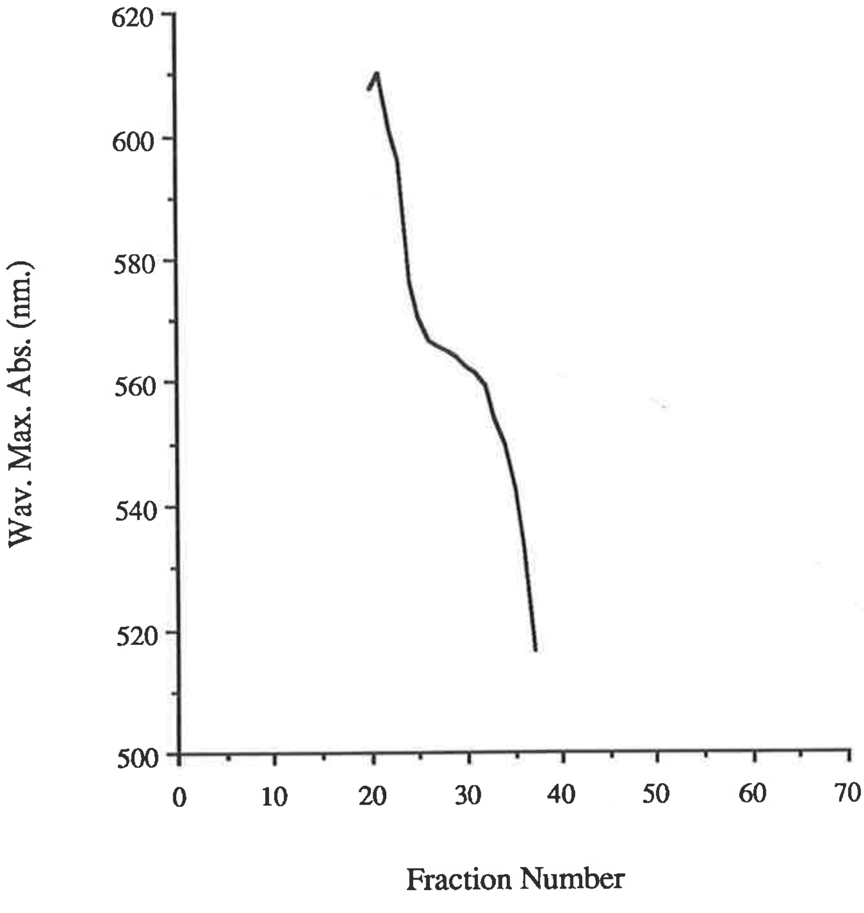


Fig. 44 d, Substituted, debranched, Fractionated Mannioc Granules.



to a greater extent than the amylopectin component, Fig. 45 a, as shown by the loss of the amylose and the substituted peak in Fig. 45 b. Amylose degradation was similar in the unsubstituted, debranched starch granules degraded by 10-15% Fig. 46 a and b.

Initial degradation appears to be directed preferentially towards the amylose component, then the amylopectin fraction. Small unit chains were extensively degraded in comparison with the large unit chains. Small unit chains appear to be substituted to a lesser extent than larger chains.

3.16.4 Elution Profile Of Substituted And Unsubstituted Large Starch Granules Of Barley.

Unsubstituted large starch granules of barley cultivar C.I. 3576 were debranched and eluted from a column of Sephadex G-150. The results are presented in Figs. 47 a Two peaks were obtained, one corresponding to amylose and the other to the small chains derived from amylopectin. Complete separation of the small chains was not obtained using Sephadex G-150. Figures 48 a-d show substituted (0.12 M), debranched large granules from cultivar C.I. 3576 eluted from a column of Sephadex G-50. The increase in material between fraction 30-40 in Fig. 48 a, is possibly incomplete debranching caused by stereochemical hindrance of pullulanase or isoamylase at substitution levels in excess of (0.03 M).

The distribution of hydroxypropyl groups on the amylose fraction and unit chains is presented in Fig. 48 b. Both amylose and unit chains were substituted. The results depicted in Figs. 49 a-b show decreases in amylose and unit chains and increases in smaller molecular weight material after substitution, debranching and beta-amylase degradation. Increased resolution provided by H.P.L.C. techniques is necessary to resolve the precise origins of these degradation products.

Fig. 45 a. Substituted, "Alpha"Degraded(15%), Debranched, Mannioc Granules.

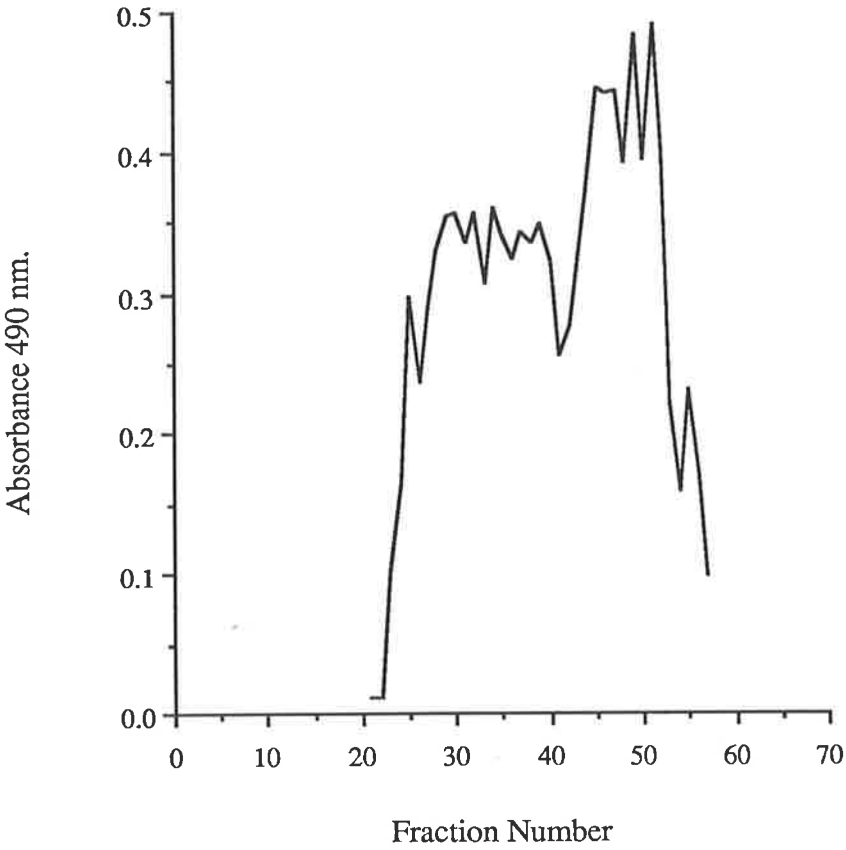


Fig. 45 b. Substituted, "Alpha"Degraded(15%), Debranched, Mannioc Granules.

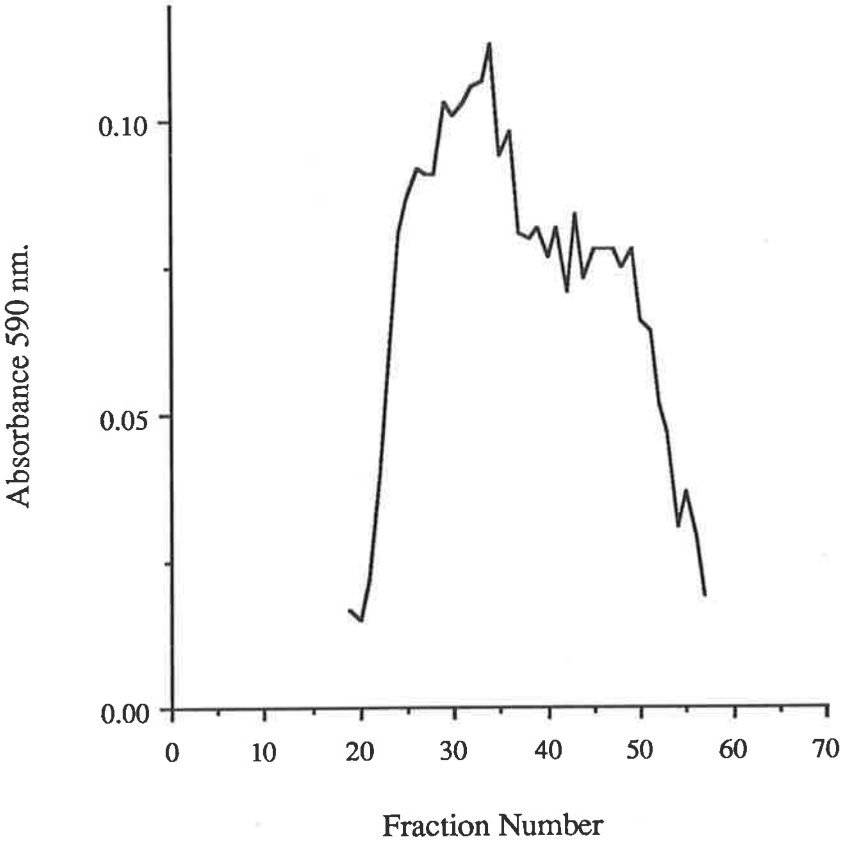


Fig. 45 c. Substituted, Alpha-Amylase(15%), Debranched, Mannioc Starch.

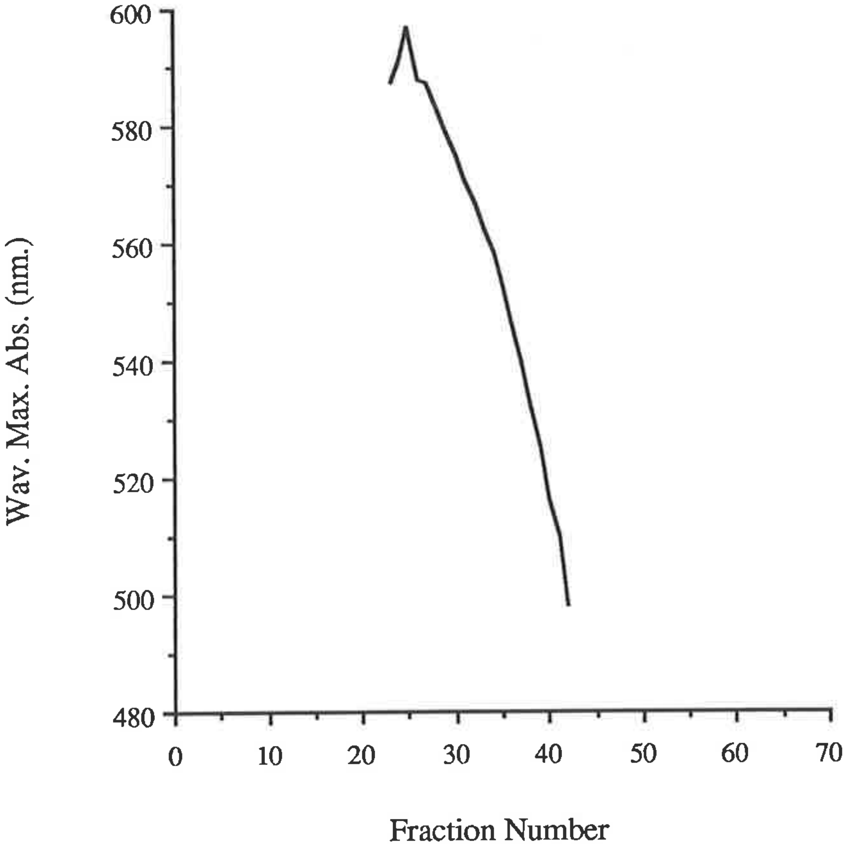


Fig. 45 d. Substituted, Alpha-amylase(15%), Debranched, Mannioc Granules.

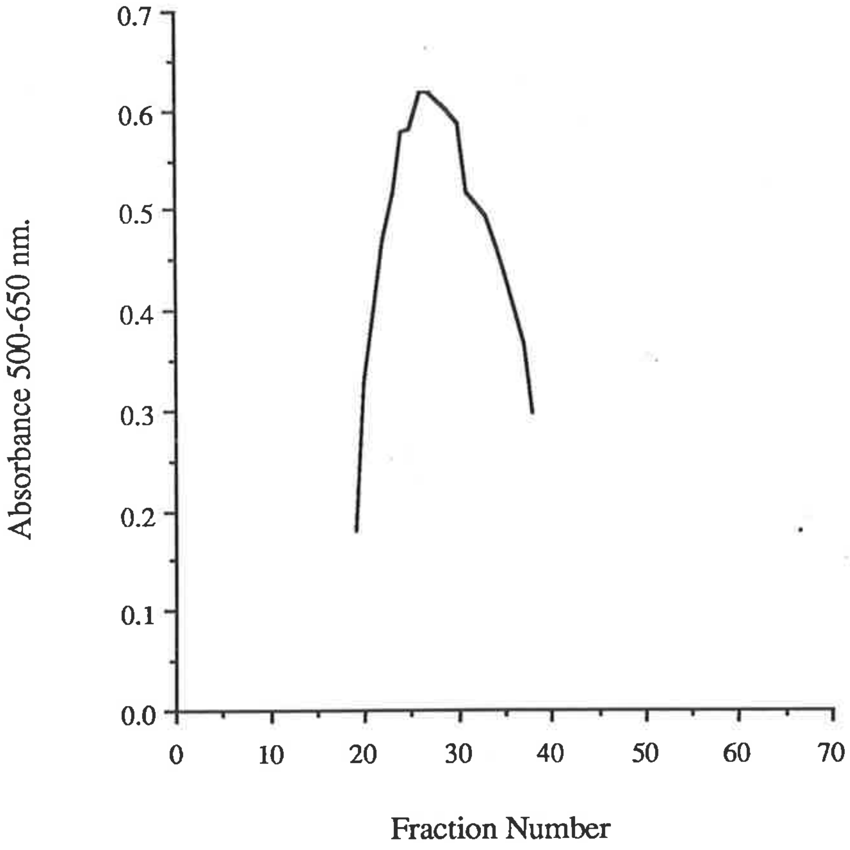


Fig. 46 a. Debranched "Alpha"(15%). Mannioc Granules.

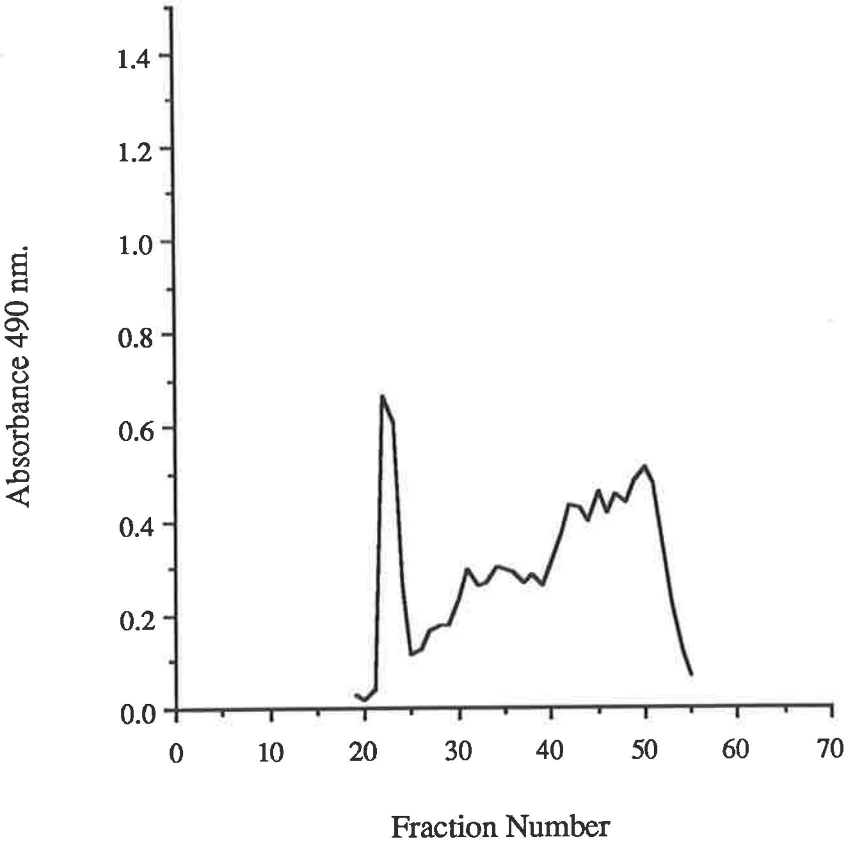


Fig. 46 b. Alpha-amylase(10-15%). Debranched. Mannioc Granules.

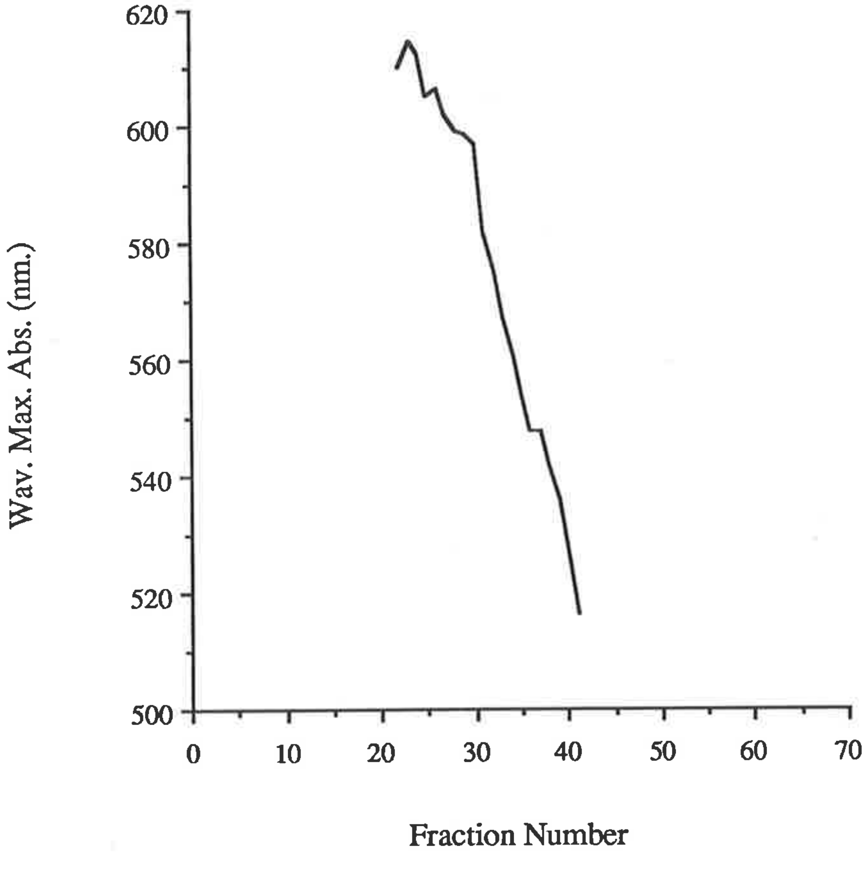


Fig. 47 a. Barley. Large granules. Debranched.

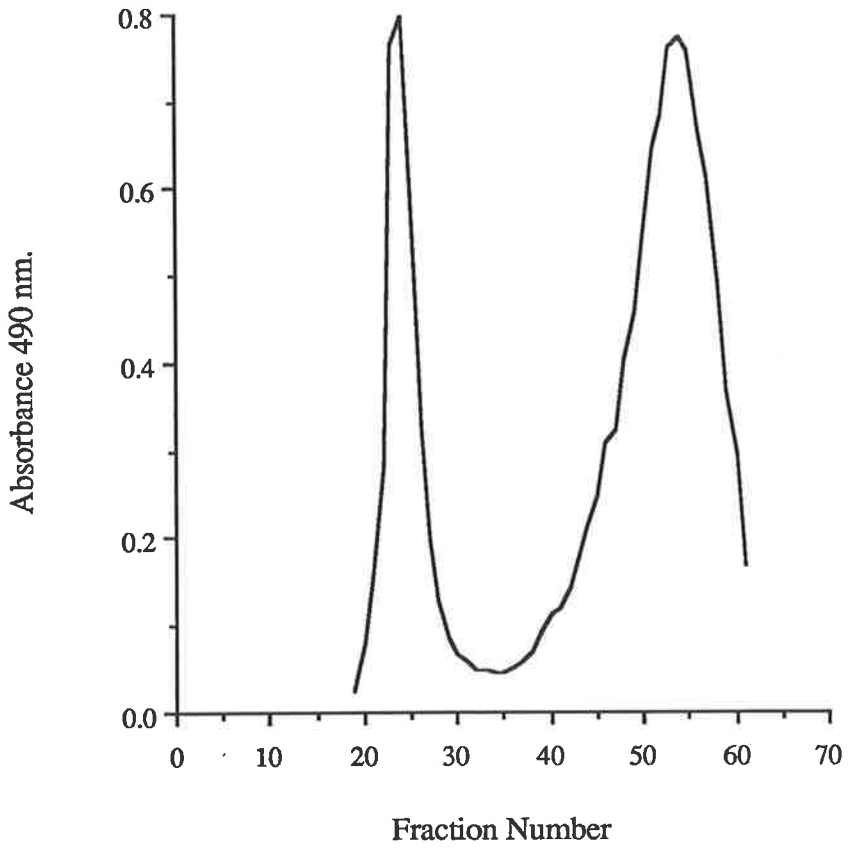


Fig. 48a. Barley. Large granules. Substituted. Debranched.

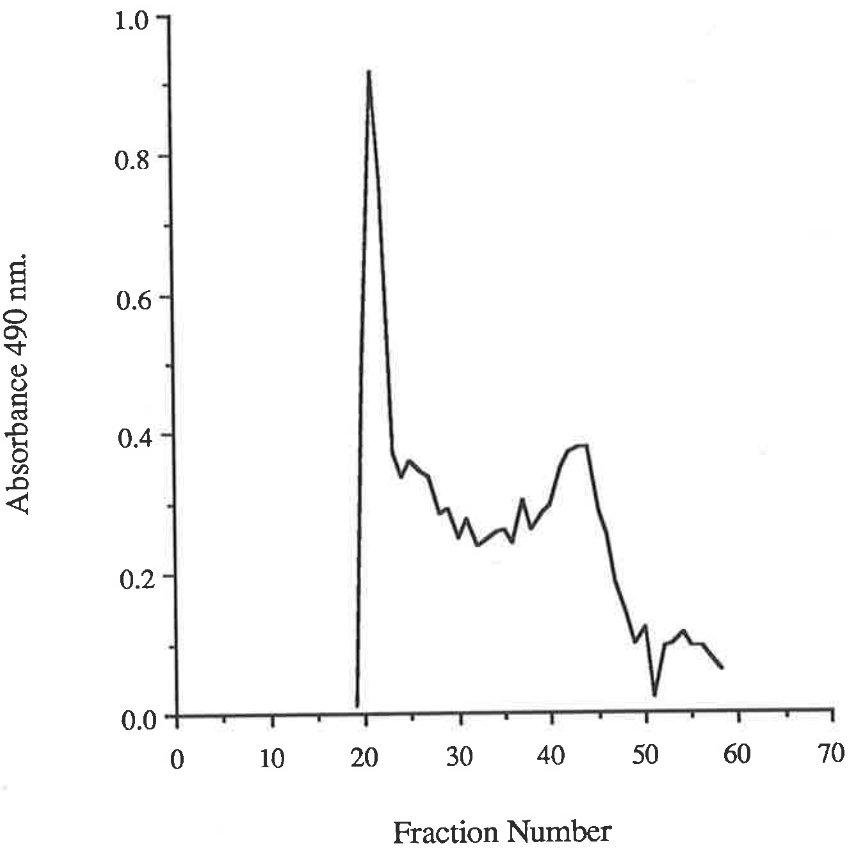


Fig. 48 b. Barley. Large granules. Substituted. Debranched.

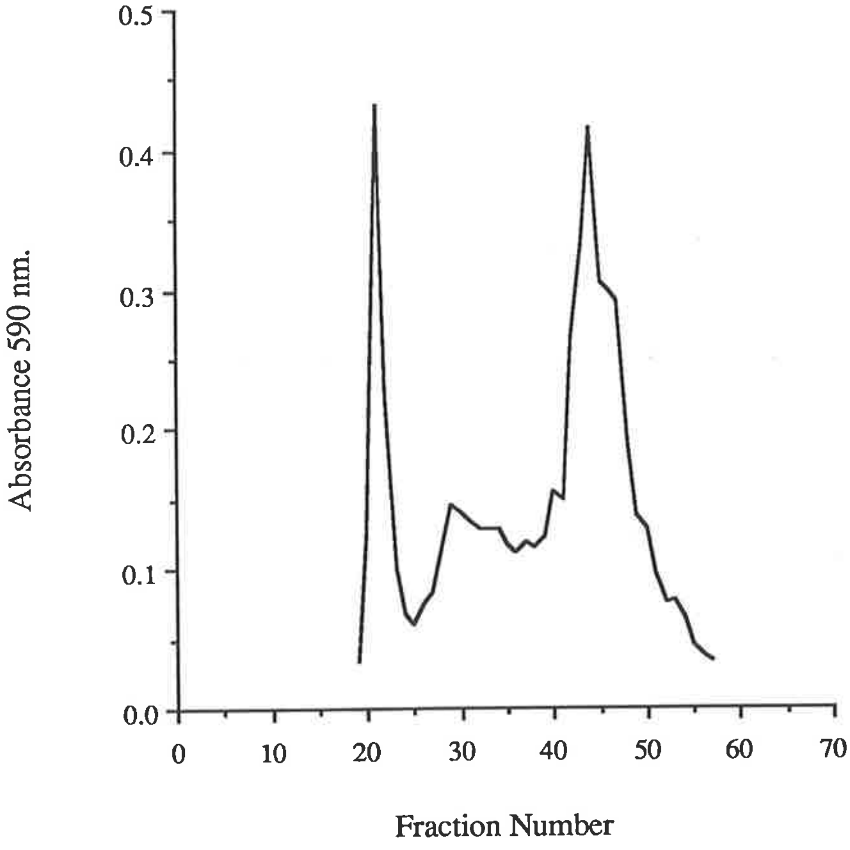


Fig. 48c. Barley. Large Granules. Substituted. Debranched.

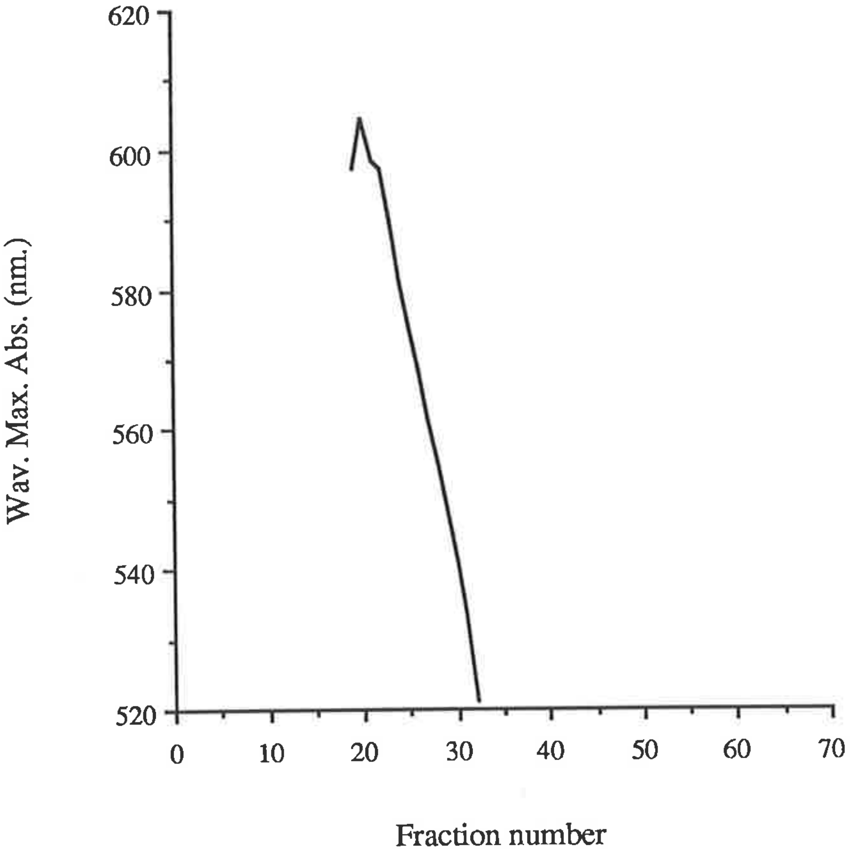


Fig. 48 d. Barley. Large granules. Substituted. Debranched.

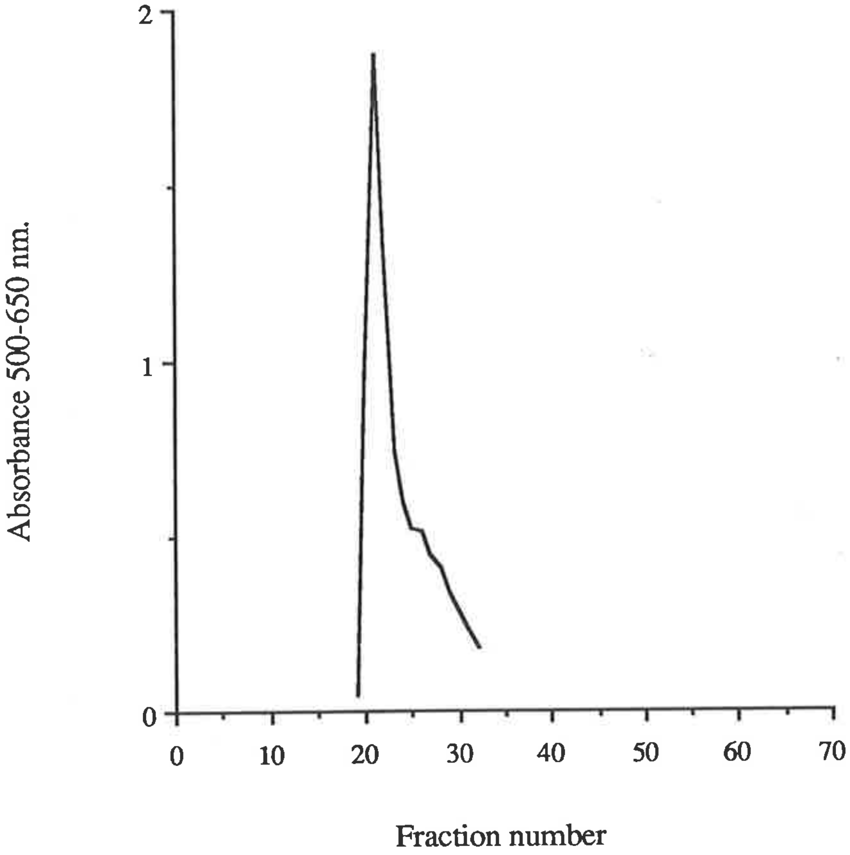


Fig.49 a. Barley. Large granules. Substituted. Debranched.Beta-amylase Degraded.

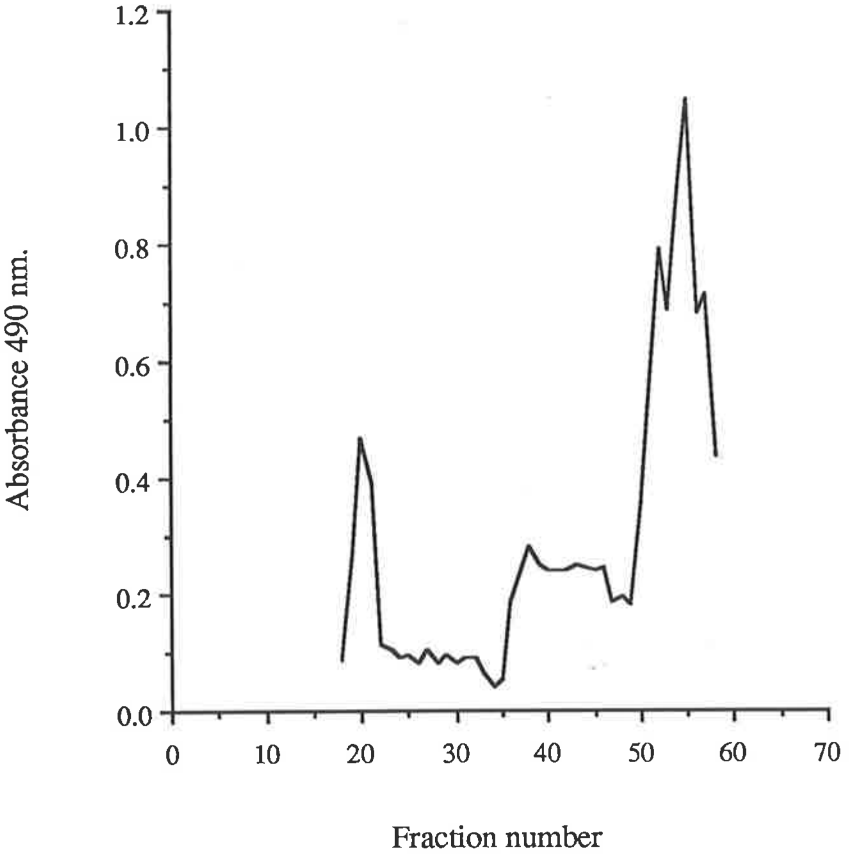
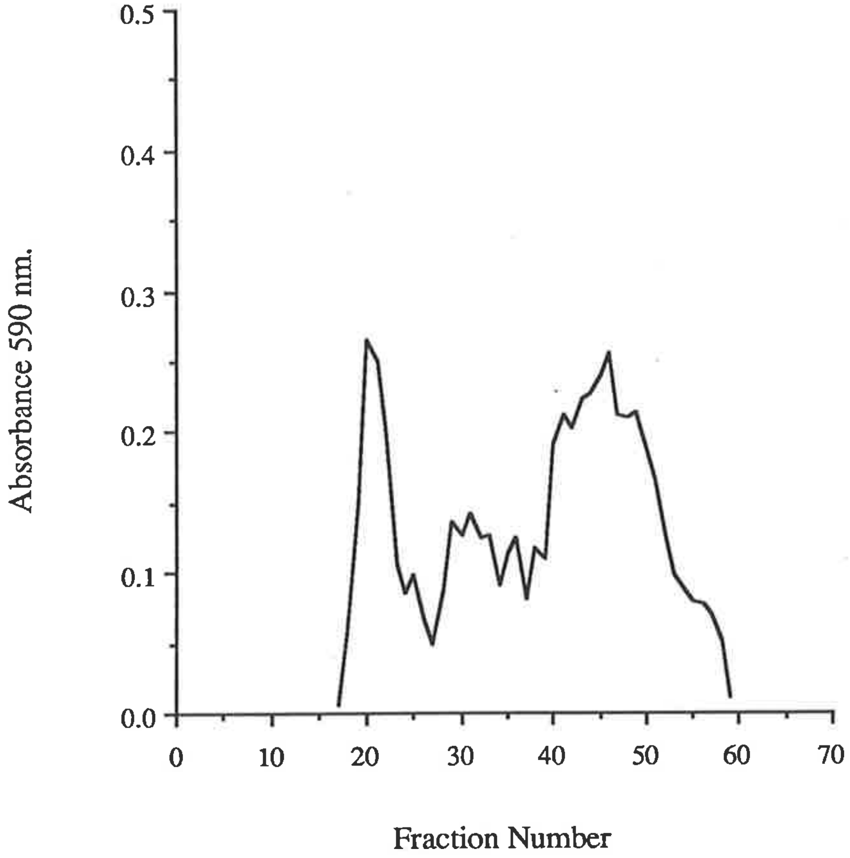


Fig. 49 b. Barley. Large granules. Substituted. Debranched. Beta-amylase Degraded.



3.16.5 Elution Profile Of Substituted And Unsubstituted Small Starch Granules Of Barley.

Figures 50 a-d show the absorbance values of substituted debranched small starch granules from barley eluted from a column of Sephadex G-50. The elution profile is similar to that obtained for large starch granules of barley. The data in Figure. 50 b suggest that the larger unit chains were not as heavily substituted as the smaller ones.

3.17 Extracellular Production Of Starch And Xylan Degrading Enzymes From Aspergillus niger (ATC. 1084) and Asp. awamori (NRRL. 3112).

3.17.1 Growth and Measurement of amylases and xylanases.

Conidia were inoculated into liquid culture media containing soluble starch or oat spelt xylan for 1-4 days. Enzymes were measured as outlined in section 2.22.

Results showed that starch and xylan degrading enzymes are most active by day 4 and day 5 respectively. Aspergillus niger (ATC. 1084) produced greater total xylanase activity than A. awamori (NRRL 3112), however, both produced similar quantities of glucoamylases. Since A. niger possessed higher xylanase activity it was chosen for further experimentation.

3.18 0 Synthesis Of c-DNA.

Synthesis was carried out as described in section 2.27 from starch and xylan induced Aspergillus niger (A.T.C. 1084) m-RNA and cloned into the EcoR 1 site of pUC19.

Two hundred separate recombinant clones were chosen from starch induced material and 120

Fig. 50 a. Barley. Small Granules. Debranched.

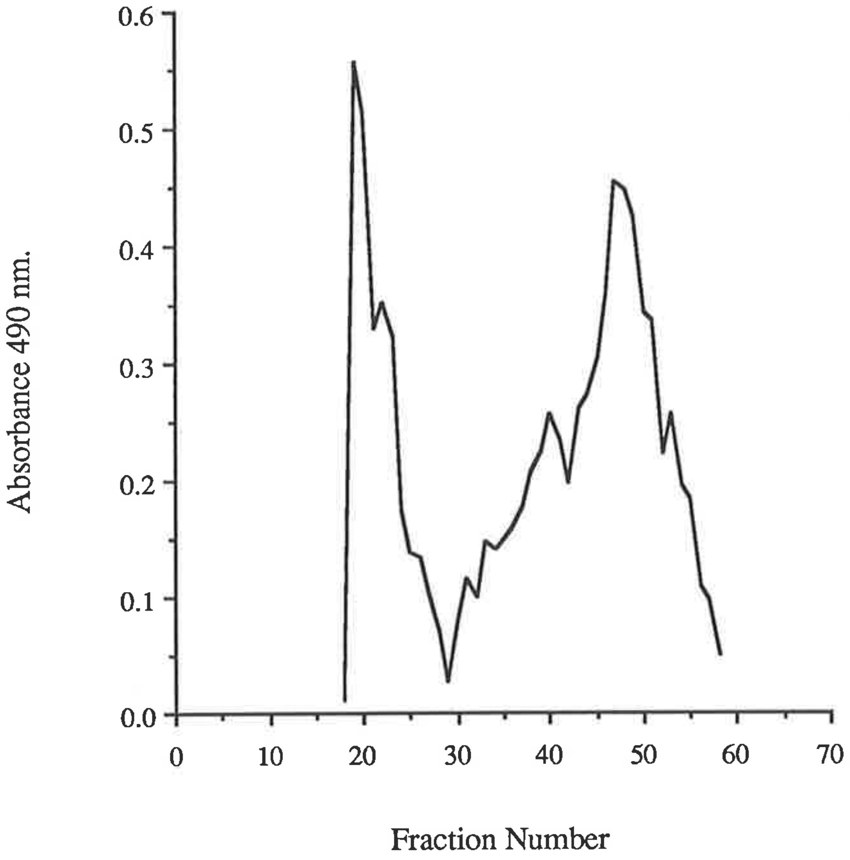


Fig. 50 b. Barley. Small granules. Substituted. Debranched.

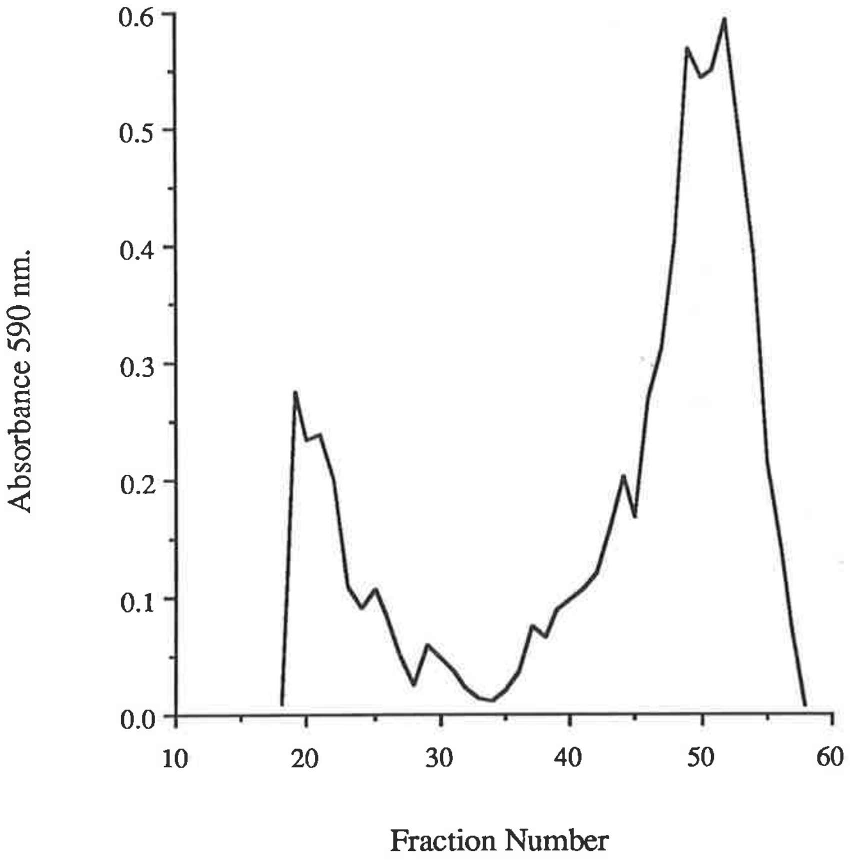
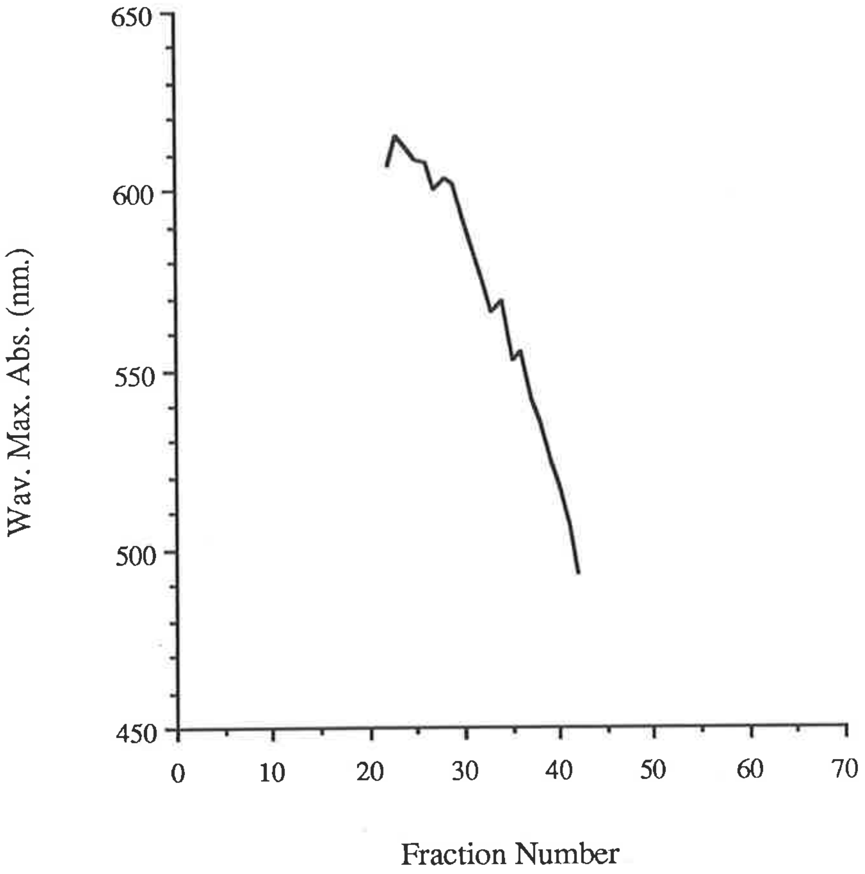


Fig. 50 c. Barley Small Granules, Debranched.



clones from xylan induced material.

3.19. Xylan Induced Library.

Two individual c-DNA clones from the xylan induced library were isolated from the EcoR 1 site of plasmid pUC19. Fragments, ~400 bp and 650 bp referred to as c-16 and c-52 respectively are illustrated in Plates. 13 a, column 1 and 2 respectively.

3.19.1 Hybridization To c-DNA

The isolated fragments, c-52 and c-16 were nick translated, labeled with ^{32}P-CTP then hybridized to a subsample of the xylan induced c-DNA previously digested with EcoR 1. The results are illustrated in Plate 13 b, c-52 represented 23% and c-16 represented 17% of the total xylan induced c-DNA library.

3.19.2 Isolation Of Genomic DNA Fragments Corresponding To c-52 Sequence.

Genomic DNA was separated on a 1% agarose gel after digestion with Pst 1, EcoR 1, Hind 111, Cla 1 and Dra 1. Gels were prepared for Southern hybridization with nick translated c-16 and c-52. The results are presented in Plate. 14 a and 14 b respectively.

Hind 111 digested genomic DNA was separated on a 1% agarose gel. The portion of the gel Between 1.5 and 3.0 Kbp was isolated and this material was ligated to a phosphatased Hind 111 digested pUC19 and used to transform E. coli (JM101).

Recombinant colonies were replica-plated, prepared for In-situ hybridization as described in

Plates. 13 a-b.

"Dot-Blots"

Plate. 13 a: PAGE (5%), of xylan induced c-DNA fragments c-16 (lane 1) and c-52 (lane 2).

Plate. 13 b: "Dot-Blot" analysis of DNA fragment c-52 with a representative sample from the total xylan induced c-DNA library.

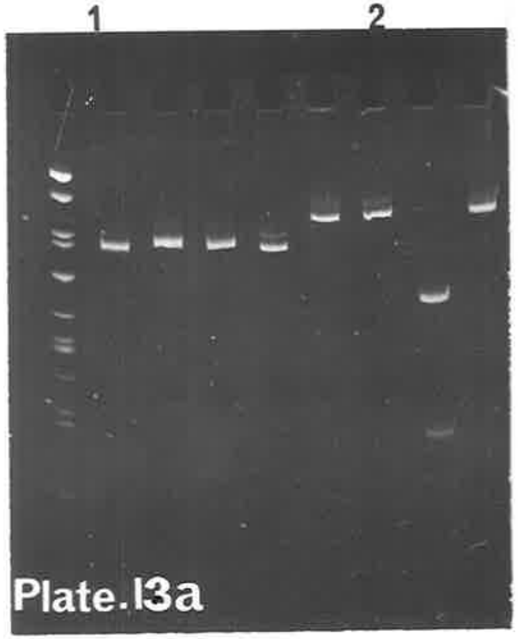
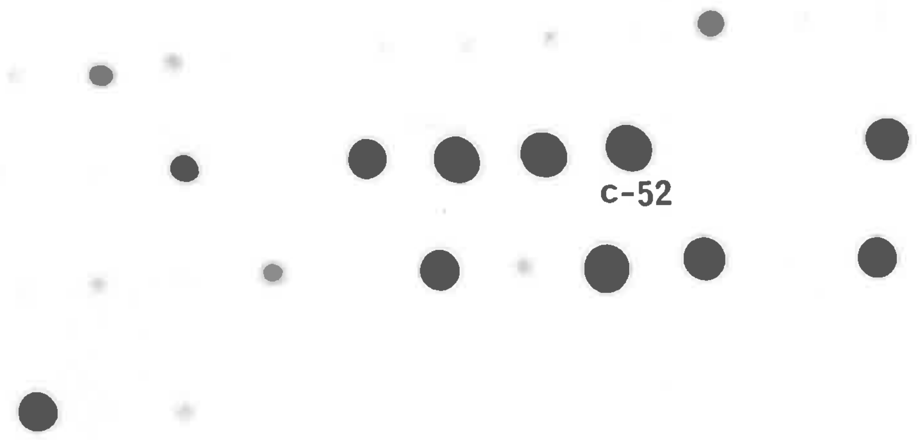


Plate.13a

Plate.13b



Plates.14 a-b.

Southern Hybridization.

Plate. 14 a: Southern hybridization of c-16 with restriction digested genomic DNA from Aspergillus niger (ATC.1084).

Plate. 14 b: as above using c-52 as DNA probe.

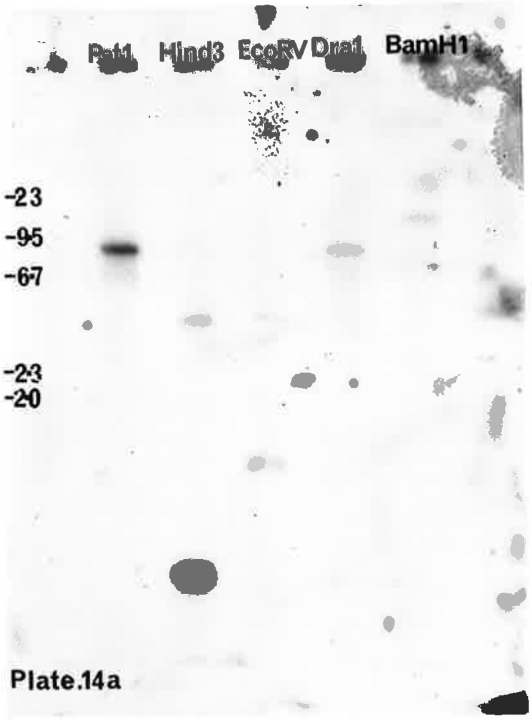


Plate.14a

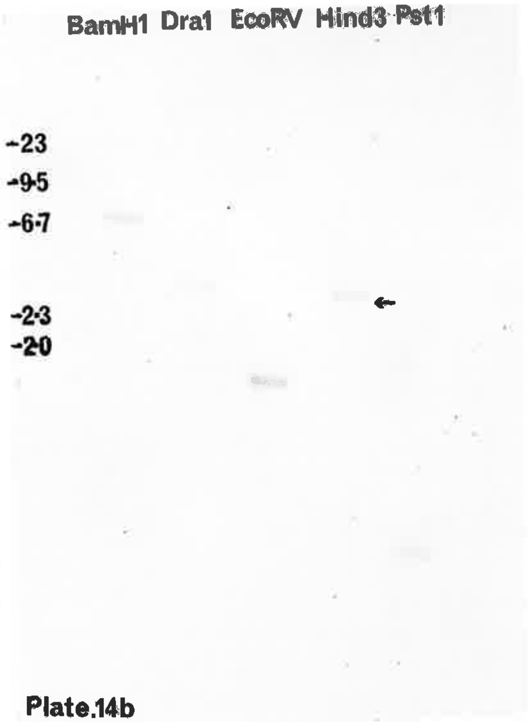


Plate.14b

Section.2.38 and hybridized to nick translated c-52, (Plate. 15 a).

After overnight autoradiography, positive colonies were isolated and rescreened by "dot blots", Plate 15 b. A genomic fragment complementary to c-52, 52/58G was isolated from a recombinant plasmid and mapped with restriction enzymes. These results are depicted in Plate. 16 b. Lane 1 and 2 represent an Eco R1/Pst 1 double digest of the primer extension product of c-52 and the genomic fragment 52/58G. Similar sized fragments are released from both inserts further suggesting they have a degree of homology.

3.19.3 c-52 Primer Extension.

Primer extension of c-52 was carried out as described in section 2.31. Extension products were ligated to the Sma 1 site of Blue scribe and used to transform E.coli (J.M.101). Fragments ~1,100 bp were isolated from recombinant plasmids "dot blotted" and hybridized with P^{32}-c-52. A fragment with homology to c-52 was isolated and mapped with restriction enzymes. These results are presented in Plate. 16 a (lane 6, depicts a Pvu 11 digest of a primer extension product).

3.19.4 Identification Of Primer Extension/Protein-A Fusion.

A primer extension fragment c-52/20 complimentary to c-52 was excised and isolated from Blue Scribe with Pvu 11. The fragment was digested with Bal 1 (1U) for 4 min., ligated into the Sma 1 site of pRIT-2T and used to transformed E.coli (N4380-1). Six recombinant clones with inserts >1000bp were isolated and the protein-A fusion products synthesized as outlined by Pharmacia. The fusion products were isolated by IgG-Sepharose affinity chromatography and visualized on 12% PAGE, Plate. 17 a. Fusion proteins (recombinant

Plates. 15a-b.

Plate. 15 a: Identification of genomic fragments homologous to c-52 by *In-situ* hybridization of c-52.

Plate. 15 b: "Dot-Blot" analysis of c-52 with itself, primer extension c-52/20 and potential genomic sequences homologous to c-52 from the *In-situ* hybridization indicated above.

Plate.15a

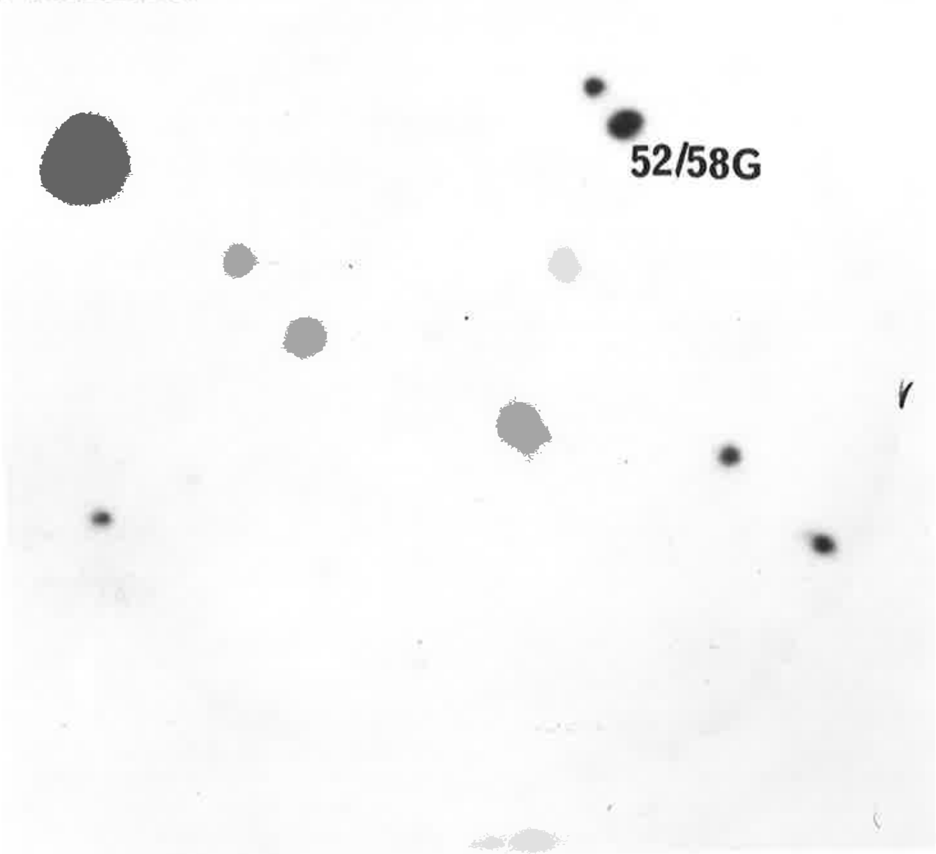
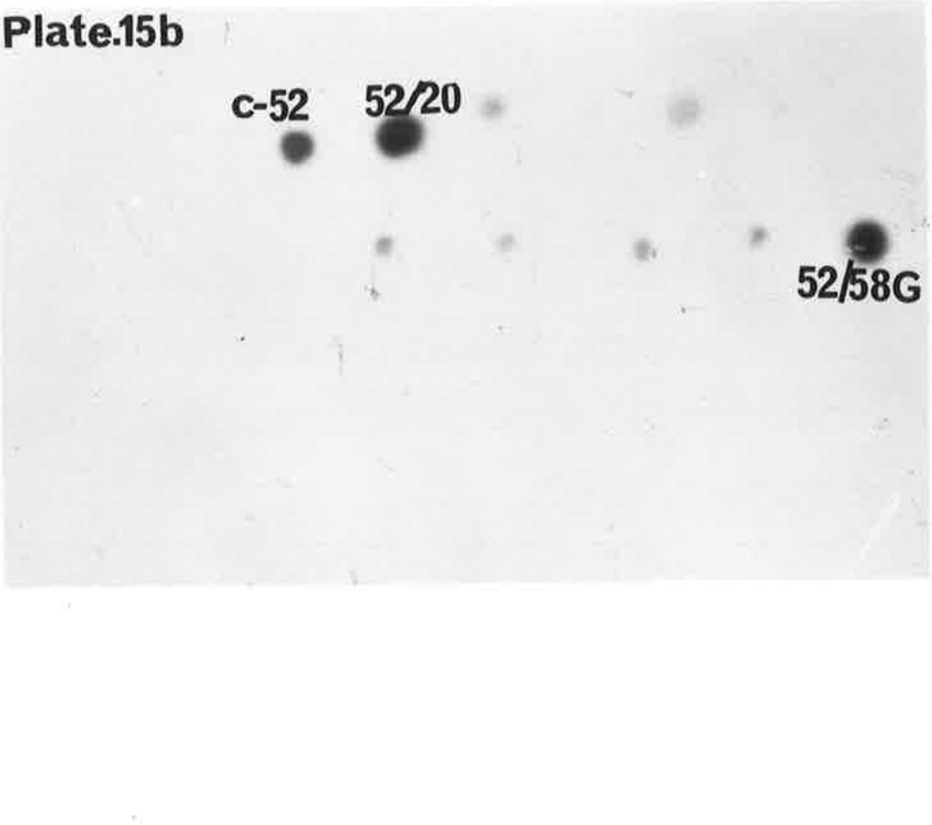


Plate.15b



Plates 16 a-b.

Plate. 16 a: Digestion of primer extension product, c-52/20 with restriction enzyme Pvu 11 (lane 6).

Plate. 16 b: Restriction enzyme digestion of primer extension product c-52/20 and genomic sequence homologous to c-52, 52/58G.

Lane. : Hae 111 digested lambda dv markers.

Lane. m: Hind 111 digested lambda phage DNA markers.

Lane.1: _52/20 primer extension product digested with restriction enzymes Eco R1 and Pst 1.

Lane.2: 52/58G digested with Eco R1 and Pst 1.

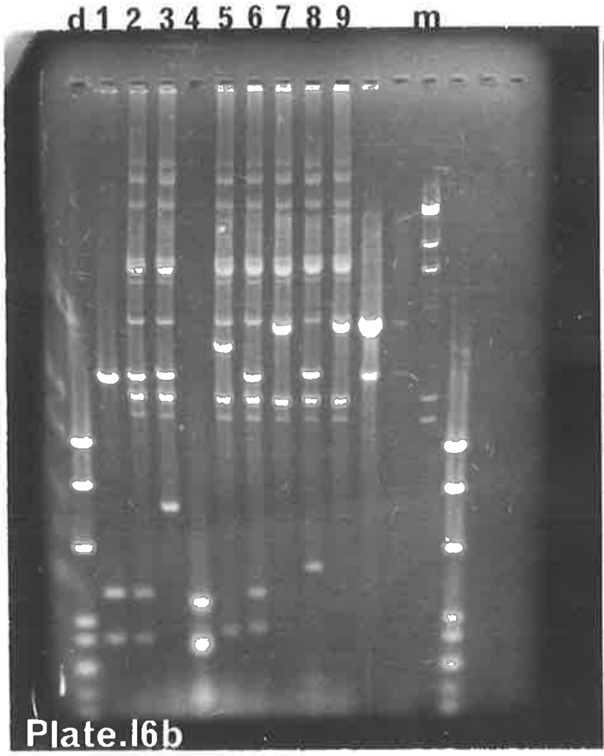


Plate.16b

Plate.16a

Plates. 17 a-b.

SDS-PAGE of protein fusion products from a ligation of primer extension c-52/20 and plasmid pRIT-2T.

Plate. 17 a: SDS-PAGE (10%), of protein-A fusion products from Bal 1 deletions of c-52/20 after purification through IgG-Sepharose affinity chromatography, a and b represents the fusion products, c is protein-A.

Plate. 17 b: Identification of protein-A fusion products present in SDS-PAGE analysed by the affinity of Horseradish peroxidase-IgG for protein-A, c' and to protein-A/c-52/20 fusion protein, a' and b'.

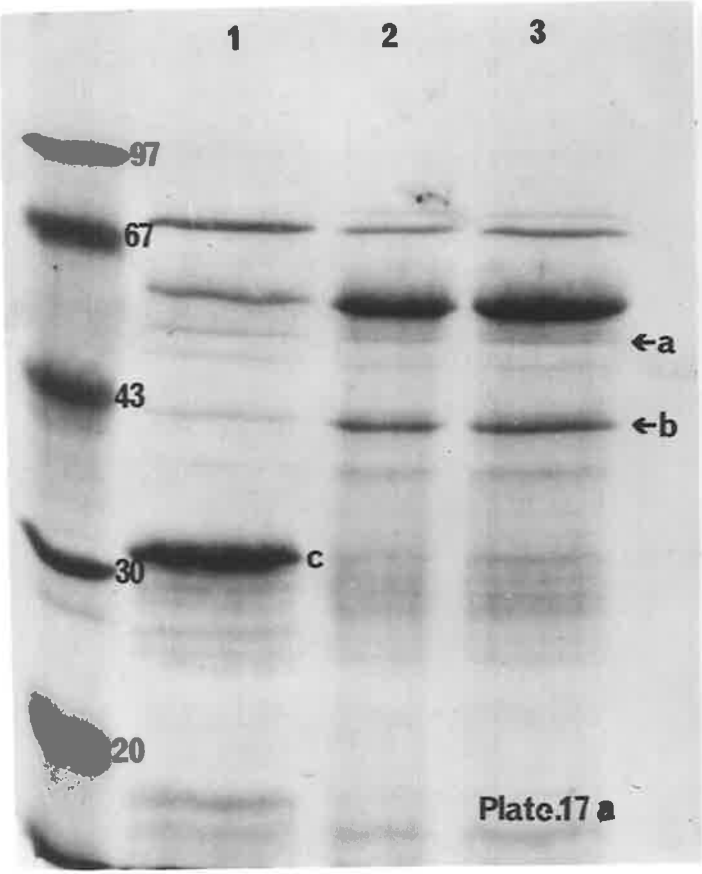


Plate.17 a

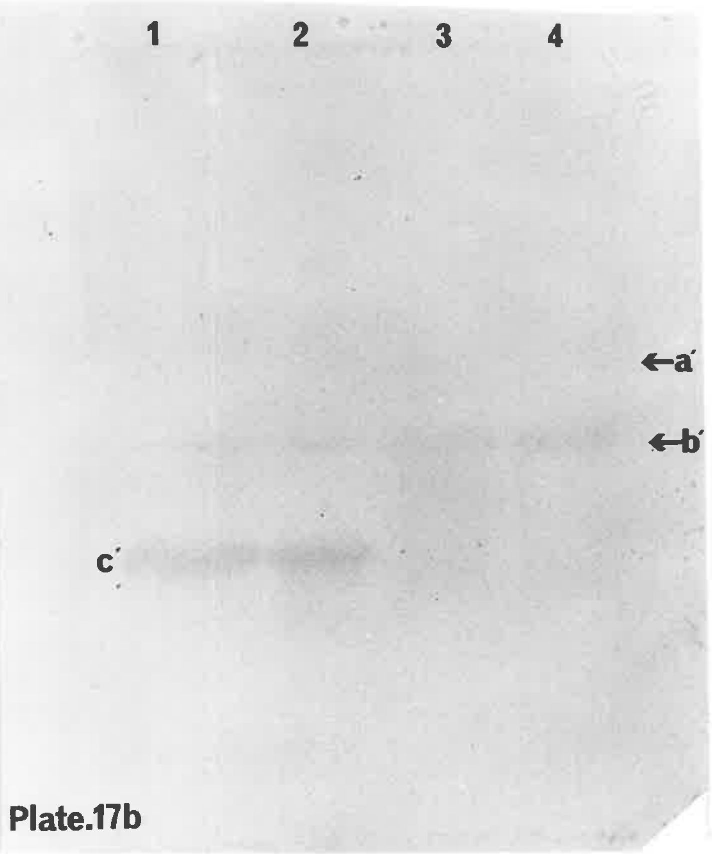


Plate.17b

clone 2) corresponding to ~43,000 and 58,000 Daltons was transferred to nitrocellulose membranes and hybridized with IgG. Alkaline phosphatase-labelled goat anti-rabbit Ig-G was used to detect the presence of protein-A domains. These results are presented in Plate 17 b.

3.19.5 Expression Of c-52/20 Gene Fragment In Yeast.

Fragments of c-52/20 were isolated from Blue Scribe with Pvu 11. The fragments were treated with Bal-1 exo-nuclease and ligated to yeast expression vector AH. 21, previously cut with Hind 111 and blunt-ended with T-4 DNA polymerase. Recombinant plasmids were used to transform E.coli (J.M. 101) and. transformants were selected by their resistance to ampicillin. Isolated plasmids were subjected to restriction digestion to confirm the presence of the c-52/20 DNA sequence. A selection of 20 recombinant clones were chosen, amplified in E.coli and their plasmids isolated. Purified plasmids were used to transform competent yeast cells (VB-20-2A) with selection of transformants on leucine utilization. Transformed yeast cells were assessed for their ability to utilize xylan as a carbon source.

3.20 Starch Induced Library.

3.20.1. Identification Of Potential Glucoamylase c-DNA Sequences.

Two synthetic oligonucleotides (30-mers), complimentary to the carboxyl terminus and middle sequences (PL 1 and PL 2 respectively) of the mature glucoamylase gene of Aspergillus niger A.T.C. 1084, were hybridized to c-DNA "dot blots", Plate. 18 a.

Fifteen percent of the c-DNA sequences, 14 individual c-DNA sequences out of 95 selected,

Plates. 18 a-b.

"Dot-Blots".

Plate. 18 a: "Dot-Blot" analysis of starch induced c-DNA library hybridized to oligonucleotide 30-mers PL-1 and PL-2.

Plate. 18 b: Selected starch induced c-DNA inserts identified by PL-1 and PL-2 "Dot-Blots" hybridized to sample of starch induced c-DNA library.

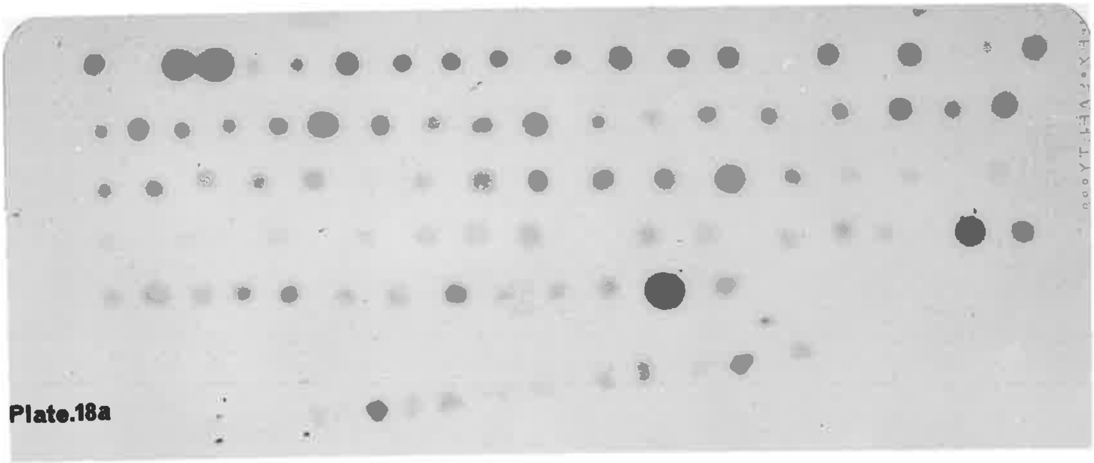


Plate.18a

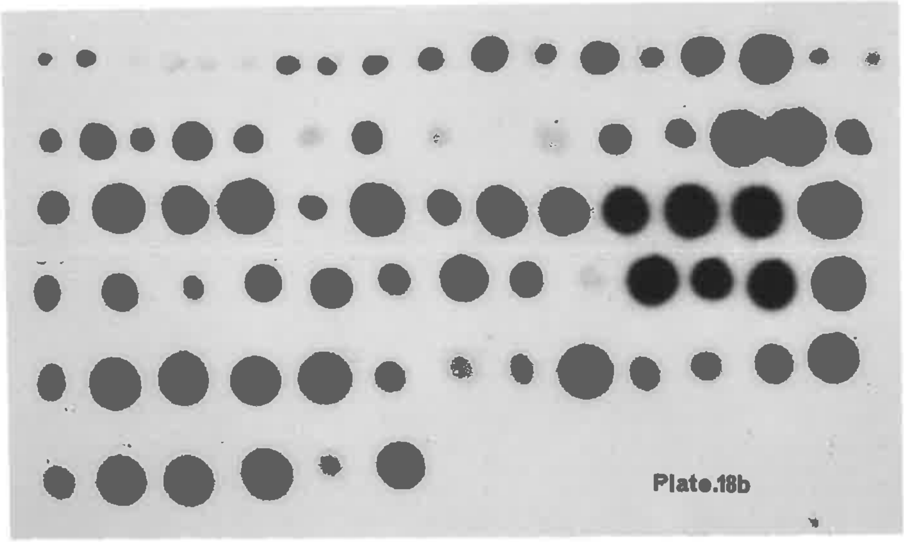


Plate.18b

showed homology to the single stranded oligonucleotide probes, Plate.18 a. Positive recombinants were digested by enzymes known to cut the mature glucoamylase gene sequence and the fragments isolated.

These sequences were labeled with ^{32}-P-CTP and hybridized to other starch induced c-DNA sequences, Plate. 18 b. A number of c-DNA fragments spanning approximately 70% of the structural gene were tentatively identified, Fig. 51.

3.20.2. Primer Extension Of N-Terminal Sequences.

Representation of the glucoamylase structural gene isolated, sequenced and mapped by *Nunberg et al* [180] together with potential c-DNA fragments, position of synthetic oligonucleotide primers and primer extension products are presented in Fig.51

Primer extensions using starch induced total RNA and the oligonucleotide 30-mer, PL2, was carried out in an attempt to obtain the complete N-terminal sequence.

Primer extension products were double digested with restriction enzymes Pst 1 and Rsa 1, ligated into the Rsa 1, Pst 1 site of Blue Scribe and used to transform E.coli J.M.101. Recombinant colonies were selected, plasmids isolated and cut with restriction enzymes Rsa 1 and Pst 1. Similar primer extensions were carried out using the C-terminal oligonucleotide primer PL-1. Primer extension products were double digested with restriction enzymes Sal 1 and Pst 1. The extension products were ligated to the Pst 1 and Sal 1 site of Blue Scribe and transformed into competent E.coli (J.M. 101). Restriction digested recombinant plasmids were separated and visualized on a 1% agarose gel, results are presented in Plates. 19 a and 19 b respectively.

Plates. 19 a-b.

Plate. 19 a: PL-1 primer extension products cut with Pst 1 and Rsa 1 after synthesis and ligated into the Rsa 1/Pst 1 sites in Blue-scribe. Extension products were excised from the vector with a Rsa 1/Pst 1 double digest separated and visualized on a 1% agarose gel, Lanes.A,B,C,D,E.

Plate. 19 b: PL-2 primer extension products cut with Sal 1 and Pst 1 after synthesis and ligated into the Sal 1/Pst 1 sites in Blue-scribe. Extension products were excised from the vector with a Pst 1/Sal 1 double digest and analysed as above, Lanes. A,B,C,D,E.

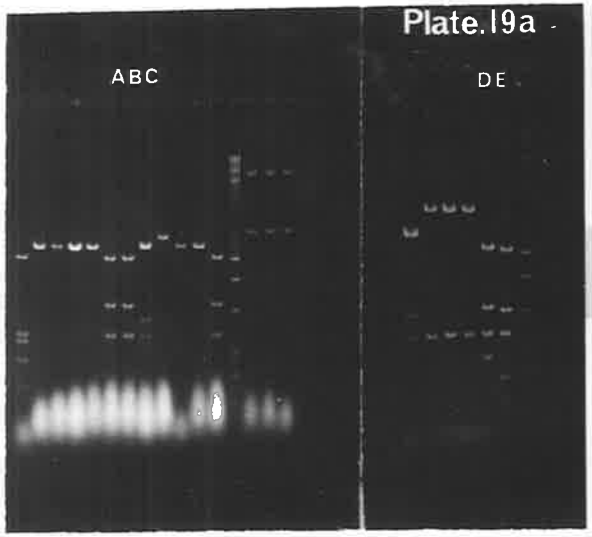


Plate.19a

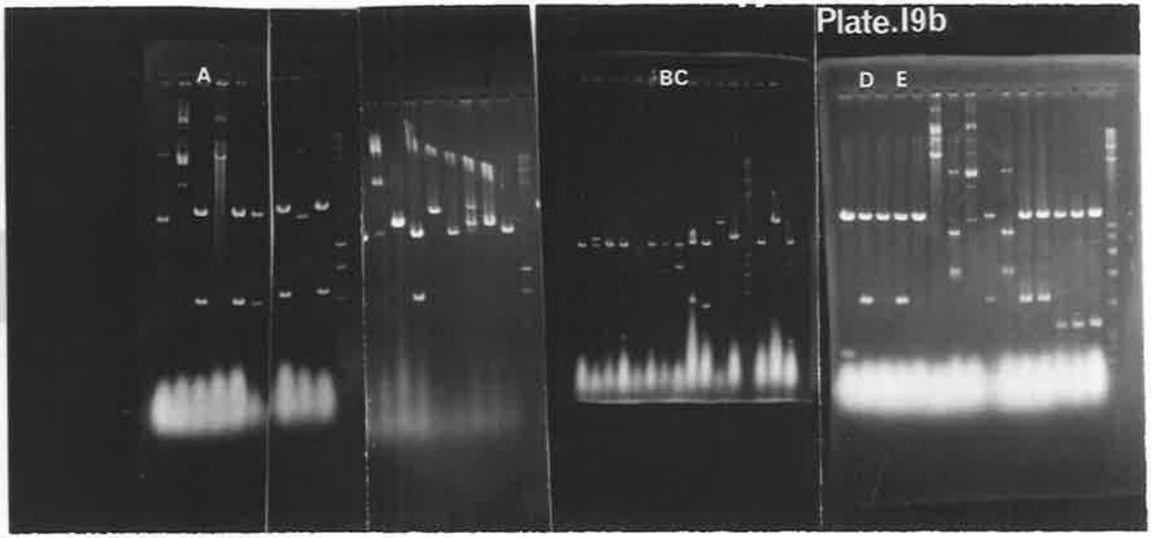
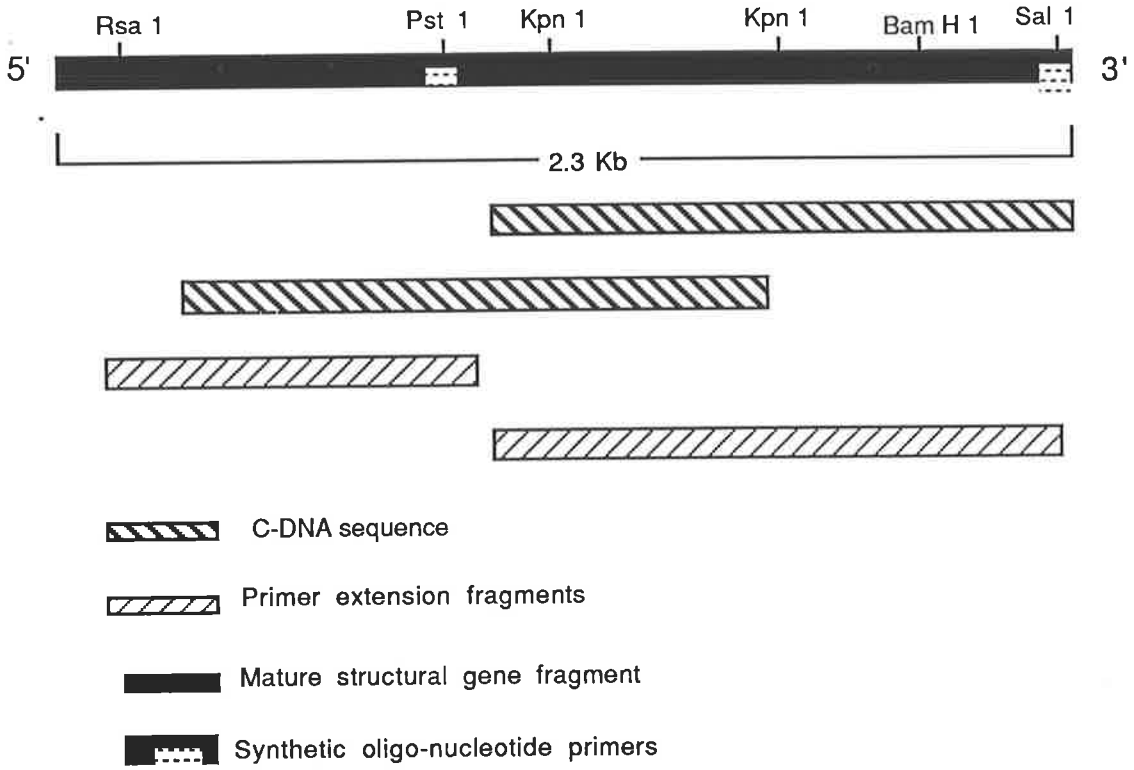


Plate.19b

Fig.51a A. niger Glucoamylase Structural Gene Fragment.



3.20.3. Isolation And Verification Of Potential Gene Fragments.

An attempt was made to identify and isolate the 3.4 Kbp unprocessed structural gene sequence to unequivocally verify the potential c-DNA sequences and primer extension products. Genomic DNA was digested with EcoR 1 and fractions corresponding to 3.4 Kb were removed from the gel and ligated into the EcoR 1 site of Blue Scribe. A double digest using Eco R 1 and EcoR V was also carried out, this material was ligated into the Eco R1/EcoR V site of pBR322. Both vectors were used to transform E.coli strain J.M.101. Oligonucleotide 30-mers and c-DNA sequences were used for *In-Situ* colony hybridizations.

4.0. DISCUSSION.

4.1. PREPARATION OF STARCH GRANULES.

Starch granules prepared by freeze drying were more susceptible to enzymic degradation, Fig. 1. Water is an integral component of granule structure and the freezing process may cause expansion within the granule disrupting structural integrity.

Whether all granules within plant species, populations within species or individual granules are affected in the same way is not known. However this result demonstrates that caution must be exercised when comparing granules prepared by different preparitive procedures.

4.2. STARCH GRANULE ISOLATION.

Barley starch granules are embedded in the endosperm closely associated with a matrix of storage proteins, cell wall components, lipids and other cellular constituents. During tissue disruption eg. granule isolation or industrial milling, some cellular constituents may adhere to the granule surface. It is important that adhering proteins, cell wall components and lipid material be removed if granules are used in experiments involving surface interactions eg. in enzymic studies. The presence and quantity of cell debris remaining on the surface of intact or damaged granules after isolation may effect its industrial performance.

Further, particular cell components may possess greater affinity for the surface of granule. The material that remains granule-bound after isolation may relate to associations present within the intact granule. Adhering proteins remaining after granule purification were separated by SDS-PAGE and a comparison made with proteins present in the intact kernel

(Plate 1). Inhibition, due to the presence of hordein, of granule degradation by alpha-amylase is probably due to decreased accessibility of enzyme for the granule surface. Similar results have been obtained by *Slack et al.* [132].

During granule purification, proteinaceous material adhering to the granule surface is usually removed by treatment with toluene[133]. However, as discussed by *Byers et al* [134], a poor representation of the protein fractions are obtained from barley kernels in the absence of a reducing agent eg. mercaptoethanol and extraction temperatures below 4°C. *Morrison et al.* [103], have recently reviewed the optimum conditions for removing integral and surface cereal lipids.

Increased degradation was obtained with starch granules incubated at 65°C, however, granules with hordein still demonstrated a significant decrease in degradation. Therefore, the presence of hordein may also affect the gelatinization and dispersion of granules. Pronase-E is capable of degrading granule surface proteins and able to significantly increase the ability of alpha-amylase to undertake granule hydrolysis Fig.6.

Starch granules from barley cultivar Clipper had less affinity for salt soluble proteins compared with granules isolated from barley cultivar Betzes. The isolation and purification procedures were identical in both samples. Although research to improve the malting quality of barley has considered important "Quality" aspects eg. beta-glucan [135], little progress has been made on its quality, work has actually concentrated on its quantity [136,137,138,139,140]. The molecular weight of barley beta-glucan, its fine structure, covalent or non-covalent association with cellular constituents eg. arabinoxylans and proteins during granule disruption will probably affect its ease of solubilization and enzymic degradation. It is these aspects which are likely to affect malting quality as well as its quantity.

Similarly, with protein the major concern has been determination of the total amount of protein present. This approach can be misleading since it is the hordein fraction and its capacity to adhere to the granule exterior during milling and its effects on granule dispersion during mashing which influence starch degradation, Plate. 4, Figs. 2,3,4 and 5, [132].

The use of H.P.L.C. [141] for separating and quantifying storage proteins and in determining the possible relation between starch degradation and specific hordein fractions, will be important in future work.

Paper electrophoresis showed that mannose and fucose appear to be associated with the hordein fraction. Whether a carbohydrate interaction is involved in the association of hordein proteins with starch granules or other components in the intact endosperm is presently not known. Mannose, fucose and glucose have been demonstrated to be covalently linked to certain subgroups of maize proteins [142]. A considerable amount of storage protein is not degraded by proteolysis during malting [143,144]. Endo-proteinases in malted barley kernels have been shown to be the rate limiting step in the release of amino-acids during malting and are not considered to survive kilning, although carboxypepdidases are relatively heat stable [145]. Little protein breakdown occurs during mashing [146] and the removal of wort proteins occurs by precipitation during boiling and at the cold break.

The high molecular weight hordeins (Mr. 60,000-120,000) not degraded during mashing with an affinity for the granule surface could be responsible for reducing the soluble saccharides released from starch granules during mashing and may explain the maltsters preference for the selection of prime malting quality barley with low protein content *Smith et al* [146] recently identified a gel forming fraction of barley which was negatively correlated with malting quality at high nitrogen levels, the gel was formed from hordein fraction-D and a "carbohydrate complex". The gel forming potential was related to the amount of D-hordein present.

In malting barley a number of quality components are considered to be important in assessing the potential of a cultivar for malting and its subsequent performance in the brewhouse or distillery. A barley cultivar should have the potential to modify rapidly and uniformly during germination, possess high a diastatic potential as well as optimum amounts of beta-glucan and nitrogen.

The ease with which the endosperm of barley undergoes disruption during milling and its relationship to malting quality has been assessed for a number of European varieties[147]. Although some cultivars have certain endosperm attributes which result in their ease of disruption during milling energy measurements and their modification during malting, the exact components which are responsible for these features have not yet been identified.

Recently *Allison* [148] based on studies with the parents of cultivars having good malting quality attributes and which perform well in milling energy determinations, proposed that there may exist a genetic link between cultivars which malt well and a common parental ancestry. Although attention has focused on the quantity of beta glucan and hydrolytic enzymes during the brewing process, little information is available on physical interactions between cellular components of modified kernels during milling and mashing stages of the brewing process. In particular the effects of adhering cellular constituents to the granule surface on granular susceptibility to enzymes. Introduction of heat stable endo-proteinases into barley or yeast (if yeast cell lysates are utilized as a nutrient source for the new fermentation and are added at mashing) could increase the efficiency of prime malting barley, or aid in the endosperm modification of cheaper malts or adjuncts. Adequate amounts of endo-proteinases present during malting of barley kernels have been shown to be important in determining the final hot water extract [149,150].

The amino acid requirement for yeast growth is already in excess of requirement and the increase in amino acids due to increased proteolysis would have to be monitored with regard to adverse effects in the flavour profile eg. proline, which is not assimilated by brewing yeast and is present in large amounts in the hordein fraction of barley. Possible detrimental effects to foam and head retention by increased proteolysis would also have to be assessed together with the excessive yeast growth and increased risk of contamination in the final product.

4.3. Effect Of Calcium Ions On The Binding Of Cereal Alpha-Amylase To Barley Starch Granules.

Calcium ions are required for maximum activity of cereal alpha-amylases [55,56]. It is possible that a complex of isoenzymes at the granule surface function by ionic interactions, however the results presented in Table.1 suggest that Ca^+ ions have no effect in the initial binding of enzymes to the granule. This result does not preclude the possibility that other ionic interactions are important in binding enzymes to granules, however there is no experimental evidence to support this idea.

4.4. Phospholipid And Calcium Ion Interactions Between Starch Granules And Alpha-amylase.

A calcium ion/enzyme/phosphate complex may be produced during initial adsorption of alpha-amylase at the granule surface, possibly at amylose/phosphate rich areas on the granule surface. Amylose concentration has been shown to increase during granule development this is positively related to the quantity of integral lipid in mature granules of barley [151]. Furthermore small granules isolated from barley have a higher content of amylose-lipid complexes [47], and are more susceptible to alpha-amylase.

Phosphate groups were removed from monoacylphospholipid by phospholipase-C. This should prevent ionic interactions between free or enzyme bound calcium ions and the phosphate component. Results shown in Fig.7 suggest that Ca^+/PO_4 interactions have little direct involvement in initial binding of enzyme to granules.

4.5. Percentage Amylose Of Integral Lipid Free And Integral Lipid Containing Starch Granules Isolated From The Large And Small Starch Granules Of Barley, Unfractionated Potato, Mannioc, Waxy And Normal-Maize.

Increases in amylose observed on removal of integral lipids are illustrated in Table.2, demonstrate the need for lipid removal for an accurate assessment of amylose. This is further discussed [152].

Lipids are integral components of intact starch granules. Cereal granules contain mainly mono-acyl-phospholipids [153], whereas tubers contain little integral lipid [152]. A relationship between enzyme binding or topographic distribution of amylose and amylopectin within the intact granule may be dependant on the quantity of amylose and amylopectin present within the granules.

4.6. Digestion Of Barley Starch Granules By Alpha-Amylase And Electron Micrographs Of Digested And Undigested Granules.

Large starch granules of the favourable malting quality cultivar Clipper were more susceptible to initial degradation by alpha-amylase than those of poorer malting quality cultivars. Interestingly, the small granules of Clipper were more resistant to initial degradation than those from other cultivars studied (large granules are present 10% by number but 90% by weight of endosperm starch reserves in barley). The fine structure of starch granules of this cultivar may differ from others. Small starch granules of barley are more susceptible to alpha-amylase (comparison of Figs. 9 and 10), [74]. It is the small starch granules which are thought to be digested first during germination but not in brewery mashes, since their gelatinization temperature exceeds that of their large granule counterparts.

The quantity of fermentable sugars present in the final extract produced during the brewing process is a balance of enzyme activity, together with brewer skill. The need for optimum enzyme levels and rapid endosperm modification during malting leads to inevitable losses, approximately 15%, [154] in hot water extract. The final extract recovered is the result of a balance between efficient degradation during mashing and inevitable losses of carbohydrate during malting. A barley cultivar with small granules resistant to enzyme attack may reduce malting losses without reducing enzyme activity and still maintain high hot water extracts at the end of the brewing process. Clipper possesses such attributes, some of which are related to the differential ease by which both starch granule sizes are hydrolysed by alpha-amylase Figs. 9 and 10. This may also occur during germination and mashing. Other positive factors contribute and inevitably it will be the cumulative effect of individual quality components, agronomical and biochemical, which defines a good malting variety rather than one overwhelming character.

The degradation of normal maize, potato and large granules of barley by alpha-amylases occurs at specific areas of the granule surface, (Glucoamylases do not degrade starch granules in the same way as alpha-amylases). The small starch granules of barley are degraded exclusively by surface erosion, Plate. 2 k-m, by either alpha-amylase [155,156], or glucoamylase. Beta-amylases, debranching enzymes and alpha-glucosidases do not digest intact starch granules. This indicates that the mode of alpha-amylase attack on susceptible areas may be due to the unique characteristics of the enzyme and granule type. Once attack is initiated and erosion of a particular area of the granule exterior commenced, the enzyme appears to gain access to the interior of the granule, especially in the case of the large starch granules from barley and potato, Plates. 2 f-m. The enzyme then proceeds to digest the interior of the granule in preference to the exterior. This is illustrated in the freeze fractured large starch granule from barley cultivar Clipper Plate. 3, where the enzyme has extensively digested the granule interior leaving apparently resistant material on the exterior.

Whether the enzyme digests the interior of the granule because it is more degradable or because the enzyme has become physically entrapped is not known. However, the concept of susceptible areas outside the granule allowing easy access to the interior of the granule is a novel form of enzyme entrapment and presents the enzyme with large concentrations of the substrate. It may also protect the enzyme from proteases and inhibitory molecules present during germination *In-Vivo*. The surface of the granule may be important for initial binding prior to degradation. Little information is presently available on the intact structure and changes in intact granules, during enzymic attack. There is abundant literature on gelatinized granules. In this thesis investigations were designed to probe the exterior of the granule surface in an attempt to reveal structural differences, areas of greater susceptibility which could provide initial attachment sites for alpha-amylases and to determine any topographical orientation between major granule constituents, namely amylose and amylopectin.

4.7. Binding Of Concanavilin-A to Starch Granules.

Small starch granules from barley have a greater affinity for Concanavilin-A than large ones Figs. 13 and 14. Small starch granules also have a lower amylose content Table. 2 and are more susceptible to alpha-amylase than large granules Figs. 9 and 10. Waxy maize starch granules have a greater affinity for Concanavilin-A than maize granules with a normal amylose content Fig. 12. Waxy maize is also more susceptible to alpha-amylase Fig. 11. Size does not appear to contribute to lectin affinity eg.waxy maize and normal maize granules are of similar size, (The approximate size of the starch granules utilized in these studies are presented in Table. 3) and possess distinctly different affinities for lectin Fig. 12.
This indicates that size may be less significant than topographical structural differences. Agglutination was observed for small granules of barley and both maize types in the presence of lectin, Plates. 4-8, waxy maize starch granules appear to agglutinate better than other types. Differences in granular size together with structural differences at their exterior may

affect agglutination since the large starch granules of barley and potato showed no agglutination, Plates. 4,5,6,9.

From lectin binding experiments the small granules of barley and waxy maize granules may have some structural similarities in their exterior architecture. Tuber starches which show a B-type pattern do not possess the same affinity for lectin as their cereal counterparts, thus their external structural features are probably different.

Individual potato granules displayed an affinity for lectin at their distal ends, Plate. 9 a-c. Electron micrographs of potato granules show that degradation occurred at their distal ends Plate. 2 i. *Leach et al* [157] observed a granule by granule degradation of potato starch suggesting that some granules were more resistant to degradation than others.

Lectin binding to the cereal starches showed specificity both for granule type and for areas on the granular surface. If lectin and enzyme share common binding sites then these areas would represent amylase attachment sites. The affinity of starch granule for lectin mimics the susceptibility of granule type to enzymic degradation Enzymically susceptible waxy maize, normal maize and the small starch granules from barley all display higher affinity for lectin than the less susceptible granules from potato, mannioc and large granules from barley. Concanavilin-A has been shown to have affinity for the terminal reducing residues of branched alpha- D-glucosylpyranose and alpha-D-mannosylpyranosyl molecules.

Starch is a homopolymer of branched and unbranched alpha-D-glucosyl residues, its fine structure described in terms of average mean values eg. chain lengths, degree of polymerization. Mean values from debranched gelatinized starches although useful in comparing starch types give little indication of the distribution or location of these components within intact granules, eg. whether they are evenly distributed throughout the exterior and interior of the intact granule. The way in which chains are arranged within an intact granule and the size distributions throughout the granule are not known.

It is possible that the chain lengths or non reducing termini are not uniformly distributed throughout the intact granule, that they are considerably greater at the exterior of the small starch granules of barley than the large ones. This would provide a much greater number of terminal reducing end groups, a less compact exterior possibly providing increased binding sites with affinity for lectin and enzyme.

Recent studies by *Colonna et al* [158] on amylopectins dispersed in solution and further modified by beta-amylase indicate that the external chain length is a major parameter for stable interactions between beta-limit dextrins and Concanavilin-A. They suggested that long chain length is detrimental to the formation of stable complexes since translational activation entropy increases with increasing chain length. Shorter chain lengths form more stable complexes. If the formation of lectin complexes with insoluble starch granules is similar to that for dispersion of amylopectin in solution then the preferential adsorption of lectin by waxy maize and the small granules isolated from barley may be due to a preponderance of smaller external chain lengths protruding from the exterior of these granule types thus forming more stable complexes with lectin.

Kang et al [159], suggest that small granules of barley possess a higher percentage of small unit chains than that of large granules. This infers that the large granules of barley possess greater proportions of long B-unit chains. Although chain length may offer some explanation for the differential affinity of lectin for large and small starch granules of barley, detailed structural information to explain differential binding to potato and mannioc granules is lacking. Differential lectin binding between normal and waxy maize starch granules may be directly related to the amylose content of the two granule types. Moreover amylose is known to increase in granules during development, thus less amylopectin would be present at the surface exterior of normal maize starch in comparison to the waxy cultivar. It would be of interest to determine the lectin affinity of maize mutant *ae*, which possesses amylopectin type material containing longer than average external chain lengths.

4.8. Binding Of FITC-Labeled Concanavilin-A To Intact And Damaged Wheat Granules.

Whole kernels from Australian wheat cultivar Warigal were subjected to controlled damage [160]. Flour separated from damaged kernels was used to determine whether a fluorescent method using FITC-labeled Concanavilin-A could be developed to assess the degree of damage to wheat starch granules after milling.

Results, presented in Fig. 16 suggest that the amount of granular damage is positively related to the amount of lectin bound, at least for the procedure reported in these experiments.

Damage to wheat starch granules during milling is positively related to water absorbed by them during dough mixing and is important during bread making [161]. Some damage is important for producing readily fermentable sugars for yeast and to produce dough of consistent viscosity suitable for further processing. Numerous tests for assessing the degree of damage associated with milling wheat kernels and effects on the final product have been developed [162,161]. These involve enzymic digestibility and measurement of amylose preferentially extracted from damaged granules in aqueous solutions and recently near infra-red reflectance analysis. Advantages and disadvantages of these methods have recently been examined [163,164]. The results suggest, that the procedure could be useful for the direct assessment of wheat flour damage. However, further research is required using industrially ground wheat samples with a range of quality features, before a final assessment of its value can be made.

4.9 Adsorption Of Clipper Alpha-Amylase-2, To Large And Small Barley Starch Granules.

Cross-linked amylose was used as substrate to assess residual amylase activity present in solution after adsorption experiments. Incubation for >10 min. in conjunction with high enzyme activities resulted in decreases in iodine binding. This was not reported in the original publication [104]. A number of enzyme and substrate concentrations should be assessed at different time intervals before optimal conditions are chosen. The reason for this sudden decrease in iodine binding may be due to a decrease in the availability of glycosidic bonds towards the end of the incubation period, perhaps by stereochemical hindrance or inaccessibility of enzyme to modified substrate. Soluble amylose previously released may be preferentially attacked and cleaved into smaller linear molecules below the length capable of forming stable inclusion complexes with free iodide ions, ie. cl. <20. However, under defined conditions this determination is extremely sensitive and rapid, (S.D. 2.06). Adsorption of Clipper alpha-amylase-2 by large and small starch granules of barley (Fig.17 and18) was higher at 25°C than reported previously from experiments conducted at 5°C [165]. It is possible that differences in granule purification, enzyme concentration, the influence of environment on granule structure and by differences in enzyme adsorption to starch granules isolated from different sources could explain this discrepancy. A significant amount of degradation occurs at 25°C, and an equilibrium may be established between free enzyme and that which remains bound. Further, because small starch granules are more susceptible, a greater quantity of enzyme may be present in solution at 25°C than 5°C. Small granules from barley demonstrate an initial period of rapid degradation which decreases as digestion proceeds [75]. This could explain the increase in the amount of residual enzyme present in solution at 25°C , at least for small starch granules.

Results of large and small granule digests indicate the amount of enzyme adhering to the granule fluctuates over the incubation period. This may be associated with changes in the ease of granule digestibility as enzyme gains access to new areas of the granule interior indicating differences in structure from exterior to interior.

Differing affinity of enzyme for granules isolated from different cultivars Figs.17 and 18 suggest differences in structural arrangements of starch in these cultivars.

Small granules from Clipper had the least affinity for the enzyme and are the most resistant of the small granules to enzymic degradation.

Total alpha-amylase from cereals consists of a number of isoenzyme groups [75,76] and each isoenzyme may have a distinctive mode of attack. The initial attack on starch granules may be carried out by various isoenzymes, a degree cooperation involving all, or a proportion of them. The elucidation of these mechanisms, the potential synergism occurring between groups of isoenzymes and their effect on granules and at what stage awaits the purification and isolation of isoenzymes in quantities that can be used in enzymic studies. The use of chromatofocusing as demonstrated by *MacGregor et al* [76] may also be useful in these studies.

Molecular biological techniques can be used to isolate the m-RNA transcripts coding for individual isoenzymes followed by synthesis in a eukaryiotic host in amounts capable of being used in enzymic studies. This may provide a method of producing very pure preparations with no contamination from other isoenzyme groups.

4.10. Adsorption Of Salivary Alpha-Amylase To Large And Small Starch Granules From Barley.

In comparison to barley alpha-amylase, greater amounts of salivary amylase was adsorbed onto large and small granules from barley. Larger amounts were adsorbed onto large starch granules compared to small ones Figs.19 and 20.

The binding characteristics observed previously at lower temperatures for barley alpha-amylases [165] were evident when salivary alpha-amylase was utilized at higher temperatures. Small granules of cultivar Clipper again bound least enzyme.

4.11. Adsorption Of Salivary Alpha-Amylase To The Large And Small Starch Granules From Barley, In The Presence Of Concanavilin-A.

Figures 21 and 22 demonstrates decreases in salivary alpha-amylase bound to large and small starch granules of cultivar C.I.3576 in the presence of lectin. As the lectin concentration increased the enzyme showed decreased affinity for both granule types.

The binding of Concanavilin-A to polysaccharides is pH dependent, [166] below pH 7.0 the quaternary structure of the molecule begins to disassociate, whilst at pH >7.0 multiple subunit complexes accumulate [167]. Since barley alpha-amylase activity is optimal at pH 5.5 [54], it is unsuitable for use in conjunction with Concanavilin-A. However, human salivary alpha-amylase has optimal activity at ~pH 6.9 and retains activity over a broad pH range, namely pH 3.8-9.4 [168], and is therefore more suitable for experiments with Concanavilin-A. Salivary amylase has the advantage of economy and ease of isolation, it displays higher, but similar binding profiles to its cereal counterpart and the characteristic pitting of large starch granules during degradation displayed by cereal alpha-amylase is also observed. Thus, the salivary enzyme appears to have similarities to the cereal alpha-amylases regarding its mode of attack on granular starch.

4.12. Inhibition Of Salivary Alpha-Amylase Degradation Of Barley Starch Granules By Concanavilin-A.

Increases in the amount of lectin bound to granules prior to incubation with salivary alpha-amylase caused decreases in free reducing groups and total soluble saccharides released, Figs. 23 and 24. The results imply that the lectin may compete with salivary amylase for access to common binding sites present on the granule exterior. Decreases in total soluble saccharides were not accompanied by decreases in reducing groups liberated, suggesting the lectin may not inhibit further degradation of soluble saccharides once released from the granules. Lectin bound to the granule exterior initially, may remain bound and any decrease in granule susceptibility to enzyme may be due to decreases in the number of susceptible areas present on the exterior. However, as previously indicated ≃20% of the lectin remained in an unbound form (Figs.12-14), suggesting that all sites available on the granule exterior are lectin bound. Since degradation continues and lectin appears to be in excess it may compete with the enzyme at certain sites on the granule exterior, while other sites are not available to lectin or possess lower affinity for lectin compared to enzyme.

The situation may be further complicated by the cooperative effects at increased lectin concentrations, Fig. 15. At these high lectin concentrations increased binding of lectin to the granule occurs and inhibition of enzyme activity may be amplified. By increasing lectin concentration to 300 µg/13.5 mg. starch granules (approximately three times the lectin concentration shown to be required for 80% binding to granules), enzymic degradation was was still apparent, although at a much reduced level. This may be due to reduced quantities of enzyme binding initially to the granule exterior in the presence of lectin.

Initial enzyme degradation in large granules appears concentrated in susceptible areas on the granule surface. Since the pattern of degradation for the granule exterior in the presence of lectin was not altered (Plate. 2 n), this suggests there are areas on the granule surface initially accessible to the enzyme but not to the lectin. Further, once the enzyme has gained access to the granule interior, degradation may proceed with reduced lectin inhibition. The form of the curve illustrated in Fig. 25 indicates that the inhibition of large granule degradation is high initially but decreases with time, even at increasing concentrations of lectin. In contrast, small starch granules were degraded by surface erosion [155,156] and Plates. 2 k-m. The form of the graph shown in Fig. 26 suggests that as degradation proceeded inhibition was also increased. Furthermore at higher lectin concentrations the degree of inhibition was enhanced. This is in marked contrast to the inhibition characteristics of large granules and suggests that digestion of the small starch granules proceeds exclusively from the exterior without much access to the interior. Concanavilin-A has a greater affinity for the small starch granules and it is possible that they possess a more highly branched system at their exterior than that of large granules. As degradation proceeds the enzyme may encounter increased stereochemical hindrance by a continual decrease in terminal reducing groups thereby limiting its rate of attack. The observation by *Macgregor et al* [75] concerning initial susceptibility of small barley starch granules by cereal alpha-amylase-1 for the first 1-2hr. then a decrease in granule degradation could be associated with changes in the structural organization of small granules from exterior to interior.

4.13. Theoretical Quantity Of Concanavilin-A Required to Inhibit Salivary Alpha-Amylase Degradation Of Large And Small Starch Granules Of Barley.

The lower theoretical concentrations of lectin required to inhibit enzyme degradation of small granules compared to large granules may be linked to the different mode of enzyme action on the two granule types. Initial effects on small granules are exclusively external whereas for large granules it is initially external and then via the well defined pin holes it rapidly becomes internal. Lectin, which strongly binds to the small granule exterior would prevent access of the enzyme to the surface and a subsequent decrease in degradation. However lectin affinity did not appear to be as strong for large granules and inhibition of granule degradation was not as severely inhibited as for small granules. This suggests that the enzyme gains access to the exterior of the granule and subsequently the interior via areas not occupied by lectin. The cooperativity shown to occur with lectin at high concentrations may be required to inhibit adsorption of enzyme to large granules. Therefore increased concentrations of lectin are necessary for the inhibition of large starch granules compared to small granules.

The down turn of the slope observed at the terminal area of the curve for small granules Fig. 26, notably absent in the large granules Fig. 25, is possibly due to a cumulative effect involving increased lectin affinity for the exterior of small granules accompanied by the cooperative effect demonstrated by lectin for granules at increased concentrations.

4.14.Substitution Of Soluble Starch And Amylose.

Numerous tests have been developed for assaying alpha-amylases in the presence of exo polysaccharide degrading enzymes, including amyloclastic [104], dye binding [169,170,171,172,173] and methods based on soluble substrates [168]. Enzyme activity is assessed by measuring reducing groups liberated, iodine binding, release of bound dyes from insoluble dye-substrate complexes, viscometry and polarimetry. All these methods have drawbacks. The amyloclastic and dye-binding techniques utilize insoluble substrates and are therefore not suitable for automated analytical procedures. The methods based on soluble substrates invariably utilize beta-limit dextrins prepared by time consuming methods from waxy starches or purchased and thereby often subject to economical limitations when screening large numbers of samples eg. in the early generations of a plant breeding programme. Beta-limit dextrins do not react efficiently with iodide ions and therefore are assayed by measuring the quantity of reducing groups released in the presence of enzyme. This is a disadvantage in situations were background levels of reducing groups are unfortunately high eg. in malted barley.

The substitution of soluble starch or amylose results in the hydroxypropylation of free hydroxyl groups mainly at positions 2 (80-85%), and 6 (7-15%) [174]. This produces a modified substrate which is virtually resistant to beta-amylase but is still sensitive to endo-acting alpha-amylase Tables. 4,5 and Figs. 27, 28. A further advantage in the use of substituted starch is its ease of preparation and increased solubility which makes it suitable for automated analytical procedures since higher substrate concentrations can be used. Enzyme activity can be measured by an increase in reducing groups eg. Nelson-Copper technique [109], or by a decrease in iodine staining, Figs. 29,31.

The presence of debranching enzymes resulting in an increase in reducing groups at this degree of substitution is minimal, but can be overcome by utilizing iodine rather than Nelson-Copper as the detecting agent. Alternatively since substitution increased substrate solubility and inhibits retrogration of amylose, substituted amylose can be prepared and utilized as indicated previously Figs. 30,32.

A number of laboratories have a vested interest in measuring alpha-amylases from plant extracts particularly in the case of malted barley where the amount of alpha-amylase present not only indicates the extent of endosperm modification but can also be used to indicate the starch degrading potential of a given cultivar during the brewing process. In order to assess alpha-amylase activity free from beta-amylase the crude extract from malted grain is often subjected to a heat treatment at 70°C for 15 min. Although this treatment can be effective in reducing or eliminating beta-amylase it also affects the stability of alpha-amylase as indicated in Table. 6.

Changes to more alkaline pH 8.0 would decrease but not eliminate the detrimental effects of heating. Furthermore the results clearly demonstrate that the denaturation of alpha-amylase in the extract is not constant for each cultivar, and is probably dependant on such variables as types of isoenzymes present and their individual concentrations. The protein concentration of the extract may also affect the stability of alpha-amylase during heat treatment and will not be the same in all malted samples. Heat treatment of malted barley kernel extracts for measurements of alpha-amylase is not therefore recommended.

4.15. Substitution Of Intact Starch Granules From Maize, Mannioc And Barley.

4.15.1.Substitution Of Maize Starch Granules.

Chemical substitution of the intact granule exterior was carried out in an attempt to elucidate structural differences between starch granule types, without damage to granule integrity Plates. 10 a-b. On occasion, granules were subjected to controlled enzymic degradation prior to substitution. Gelatinization of the granule followed by debranching allowed those areas which were substituted within the amylopectin and amylose fractions to be resolved. Further, since complete digestion of the substituted carbohydrate by beta-amylase does not occur, a tentative assessment of the substitution extent in particular fractions could be assessed. Those areas degraded initially by alpha-amylase and accessible to derivitization could be located on the granule exterior. The results from experiments using maize starch granules show that the amylose and amylopectin were substituted Fig. 35.

Fractionation of substituted maize starch granules on Sephadex G-50 suggest the amylose fraction was substituted to a greater extent than the smaller unit chains derived from amylopectin Fig. 38b. Degradation by alpha-amylase to 35% indicate that amylopectin and amylose fractions were degraded Figs. 39. The amylose fraction was rapidly degraded, accessible to substitution and could be present on the granule surface. Maize starch granules pretreated with alpha-amylase to 15-20%, then substituted, debranched and beta-amylase digested, suggest that a portion of the amylose fraction was resistant to alpha and beta-amylase (Figs. 40-41). Therefore a portion of the amylose was located on the granule exterior and was accessible to substitution but inaccessible to initial degradation by alpha-amylase.

Exhaustive degradation of substituted maize starch granules indicated that some of the amylose was completely resistant to alpha-amylase degradation, indicative of a high degree of substitution Fig. 42.

Maize starch granules substituted, debranched and treated with beta-amylase revealed that a portion of the amylose and A-chain fraction were resistant to degradation Fig. 41 a-c.

In conclusion, a major fraction of amylose was accessible to both initial enzymic degradation by alpha-amylase and hydroxpropylation and may be present on the granule surface. The B-chains derived from the amylopectin fraction were degraded initially by alpha-amylase and did not appear to be heavily substituted, because of their susceptibility to beta-amylase, suggesting these chains are accessible to enzyme but are not in a form suitable for substitution. The physical state of the small unit chains in association with other intact granular components may render them inaccessible to substitution.

Comparison of the fine structure in gelatinized normal and mutant maize granules has recently been carried out by *Boyer et al* [175], and during development by *Inouchi et al* [176].

4.15.2. Substitution Of Mannioc Starch Granules.

Mannioc granules substituted and debranched displayed similar substitution profiles to maize starch granules Figs. 43-44. Prior treatment of granules with alpha-amylase (10-15%) resulted in degradation of amylose Figs. 45. Large and small unit chains appeared more resistant to the enzyme when compared to maize granules. Moreover the large unit chains appear to be substituted to a greater extent than small ones. Small unit chains appear to be degraded preferentially to large chains in unsubstituted preparations Figs. 46.

In conclusion, the results suggest that in common with maize starch granules, amylose appears to be present on the granule exterior as shown by its ease of digestion with alpha-amylase and its accessibility to substitution. However a portion of larger unit chains derived from the amylopectin fraction may be orientated towards the outside as indicated by their accessibility and degree of substitution. Since they are not readily degraded by alpha-amylase they may be in a physical form or location which is stereochemically unsuitable for initial alpha-amylase attack.

Small unit chains derived from amylopectin do not appear to be degraded to the same degree as maize starch granules and they appear to be less substituted. This may be indicate a central location within the intact granule.

4.15.3. Substitution Of Large And Small Starch Granules From Barley

Preliminary investigations of substituted and debranched large granules suggest the amylose and smaller unit chains were substituted Fig.48 b. Treatment of substituted, debranched large granules with beta-amylase indicate that a proportion of the A-unit chains were degraded in preference to the B-unit chains Figs. 49.

Approximately fifty percent of the amylose was degraded. A proportion of the B-chains were substituted and resistant to beta-amylase.

Lack of alpha-amylase degraded granules prevent conclusions being formulated regarding the location of polysaccharide fractions within the granule. Substitution of small starch granules indicate the degree of substitution was greater for small unit chains than for either amylose or large unit chains, suggesting these chains have an exterior location Figs. 50.

Large and small starch granules from barley have similar proportions of amylose and amylopectin (Table. 2), but their susceptibility to degradation by alpha-amylases and gelatinization temperature [85] differed significantly. This indicates the granules differ in structure. *Macgregor et al* after studying amylopectins from large and small granules from barley, concluded they had identical structures [177]. This may indicate the way in which the macromolecular components within the intact starch granule, namely amylose and amylopectin, are orientated differently in either granule type.

MacGregor et al [177] demonstrated that debranched large and small starch granules from barley amylopectin possessed three groups of unit chains. This has been verified further by *Hizukuri et al* [178,179] utilizing laser technology. Intact starch granules from normal maize, mannioc and fractionated barley are known to possess morphological, physical and enzymic differences. The ratio of amylose and amylopectin within these starch granule types is similar. Differences in physical, morphological and enzymic parameters are probably not associated with the amount of these macromolecular components but with their orientation within the granule. This may not be true for mutant starch granules where large differences in the ratio of amylose to amylopectin eg. amylomaize, waxy maize, high amylose barley starch granules, may have a direct influence on their morphological, physical and enzymic properties. The ease of enzymic degradation of waxy maize starch compared to its high amylose counterpart has been documented [157]. Tuber starch granules eg. potato, are significantly more resistant to enzymic degradation by alpha-amylases than cereal starch granules containing similar amounts of amylose and amylopectin, indicating that differences in the susceptibility of the granules to alpha-amylases is not due soley to the ratio of these components.

Differences in X-ray diffraction patterns for tuber and cereal starches indicate their structural composition are different, however there has been little progress relating physical observations to structural and enzymic differences between and within intact granules.

Although mutant starch granules have been utilized in attempts to relate granular composition to physical and enzymic observations, the information regarding the detailed structural composition of even one normal starch granule type is very limited. Therefore the results obtained for mutants granules and their extrapolation to the physical and chemical composition of normal starch granules must be interpreted with caution.

Techniques described in this thesis may, with increased resolution and decreased analysis time provided by H.P.L.C., provide useful information regarding the structural differences between granule types perhaps aiding the association between structure, enzymic, physical and morphological observations. Although results and methods presented in this thesis may be useful in future investigations involving granule architecture, it is clear that to fully elucidate the mysteries of even one starch granule type, a multidisiplinary approach will be required.

Production of monoclonal antibodies specifically to the individual isoenzyme forms of alpha-amylase and the fluorescent labeling of enzymes directly or by methods which do not effect their activity or binding properties would be useful in identifying areas on the granule surface involved in initial enzyme attachment and subsequent degradation. Site directed mutagenisis should also play an important part in the elucidation of those structural areas on the enzyme important for granule binding. After more detailed structural studies on normal granule types the results obtained could be reinforced by detailed structural investigations using mutant granules.

4.16 Discussion Molecular Biology.

4.16.1 Extra-Cellular Production Of Starch And Xylan Degrading Enzymes From Aspergillus niger (A.T.C. 1084).

Enzymes required for starch and xylan degradation were most active at 4-5 days. *Nunberg et al* [180] have previously indicated that glucoamylase m-RNA can be induced several hundred fold if Aspergillus awamori is grown with starch as the sole carbon source. Increases in glucoamylase activity were also demonstrated. Similar increases in xylan degrading enzymes may also be expected to occur when hyphae are cultured in conditions with xylan as the sole carbon source. Isolation of m-RNA at this time period may increase the probability of obtaining m-RNA coding for starch and xylan degrading enzymes.

Recently an alpha-amylase from Aspergillus awamori has been purified and characterized [181], c-DNA transcripts, referred to later, not coding for glucoamylase may code for this alpha-amylase which can also be induced in culture conditions were starch is the sole carbon source.

4.16.2 Synthesis Of c-DNA.

The size of c-DNA fragments obtained from Aspergillus m-RNA were dependant on the Na^+ concentration utilized during first strand synthesis. High Na^+ concentrations > 40 mM produced small c-DNA fragments not greater than 800bp's. A decrease in the Na^+ concentration to 0.04.M allowed fragments up to 1,900 bp's to be synthesized.

This observation may be due to decreases in m-RNA secondary structures in the presence of lower Na^+ concentrations.

4.16.3 Potential Xylanase Transcripts.

Complimentary DNA transcripts c-16, (400bp) and c-52, (650bp) represented 40% of the xylan induced c-DNA and are therefore abundant transcripts possibly coding for enzymes required for xylan degradation.

Fragment c-16 and c-52 were nick translated with ^{32}-P-CTP and hybridized to genomic DNA cut with restriction enzymes as indicated in Plates.14 a and 14 b.

Primer extension of c-52 produced a major fragment of ≃1,100bp, c-52/20. The endo-xylanase from Aspergillus sps. is approximately 25,000 Daltons [182]. A peptide of this molecular weight would correspond to a m-RNA sequence of approximately 800bp. The presence of a signal sequence and intronic sequences may increase the size of the transcript to ≃1000bp. Thus the primer extension product is in the size range required to code for a protein with a molecular weight of approximately 25,000 Daltons.

Restriction enzyme digests were carried out to elucidate enzymes which cut twice within fragment c-52/20. Restriction enzymes EcoR I and Pst I were observed to cleave within c-52/20.

A genomic fragment with homology to c-52 was isolated as described in section 3.20 and similar enzymes were used to cleave 52/58G. Plate.16 b demonstrates that a double digest of both c-52/20 and 52/58G with restriction enzymes EcoR I and Pst I both cleaved within both sequences, producing common fragments of ~600 and ~300, lane.1 and 2. The fragment c-52/58G may correspond to the genomic sequence of c-52/20 since common fragments are released after a double digest with both restriction enzymes and after washing at the highest stringency the fragments share homology. Sequence analysis, hybridization and deletion mapping would be required to obtain further information on the genomic sequence and to establish whether it contains all or only part of the c-52/20 sequence and any intronic regions.

4.16.4 Expression Of Primer Extension/Protein-A fusion.

Bal I deletions of c-52/20 were carried out and ligated to the plasmid vector pRIT-2T as indicated in section 3.22. Ten recombinant clones were analysed for the presence of fusion products and one was demonstrated to synthesize a protein of approximately 43,000 and 58,000 Daltons linked to Protein-A, Plates. 17 a and 17 b. The protein-A domain corresponds to a molecular weight of approximately 28,000 Daltons[183,184]. The size of the fusion products was observed to depend on the time allowed for protein synthesis after temperature induction. Thus 40 min. was required to produce fusion protein products without extensive degradation which occurred at longer periods. Failure to form larger fusion products in greater amounts could be due to proteolysis within the E.coli cells possibly associated with difficulties in producing correct protein folding presumably caused by the large protein-A domain and the reducing environment.

4.16.5 Cloning Of c-52/20 Into Yeast Expression Vector AH-21.

Similar Bal 1 deletions of c-52/20 were carried out as previously described and ligated to yeast expression vector AH-21[185]. This vector carries the yeast promoter for alcohol dehydrogenase, an ATG start sequence and yeast termination signals. Yeast transformants were selected on the auxotrophic marker leucine. Transformed colonies were picked and transferred to leucine deficient agar plates with xylan as the sole carbon source. A number of yeast transformants are presently being screened for their ability to degrade xylan.

4.17 Identification of Potential Glucoamylase Sequences.

The glucoamylase gene from Aspergillus niger (BU 1), [186] A. awamori (NRRL 3112), [180] and Rhizopus oryzae (SAM0034) [187,188] have recently been cloned and sequenced. Comparison of amino acid sequences from these and that from Saccharomyces diastaticus have been discussed recently [189].

From amino acid and DNA sequence comparisons, the active site of Rhizopus glucoamylase appears to be present at the 3'-end and the starch binding domain at the 5'-end of the m-RNA sequence. Conversely the starch binding domain in Aspergillus sp. appears to be positioned at the 3' end and the catalytic site at the 5'-end of the m-RNA. The m-RNA can be further processed in the case of Aspergillus glucoamylase where splicing excises a fragment from the 3'-end, producing a protein product that degrades soluble starch but does not bind to insoluble starch granules ie. glucoamylase isoenzyme-2. This indicates the presence of a starch binding capacity at the 3'-end of the m-RNA. Also this suggests that the binding of amylases to the starch granule surface is a prerequisite to degradation.

A number of potential glucoamylase gene sequences were obtained from starch induced m-RNA as described in section 2.27. The size of c-DNA fragments obtained ranged from 450 bp to 1,650 bp and potential glucoamylase coding fragments were identified by screening a representative subsample of c-DNA fragments with two single stranded oligo-nucleotide probes complimentary to the 3'- and near the middle of the glucoamylase gene sequence, Plates.18 a and 18 b. Restriction enzyme digestion of these fragments tentatively determined their location within the mature m-RNA, Fig. 51 a.

A number of primer extensions were carried out using the synthetic oligo-nucleotide sequences as primers and with single stranded c-DNA fragments. The primer extension products,Plate 19a and 19 b were double digested with Rsa 1 and Pst 1 for N-terminal extensions and with Sal 1, and Pst 1 for C-terminal extensions. Primer extensions not digestions, suggesting secondary structures within the m-RNA may prevent the production of full length c- DNA transcripts under the conditions outlined in section 2.27. Previous workers obtained a 1.8 Kb c-DNA transcript using m-RNA previously denatured with mercuric hydroxide then isolated from agarose gels.[180], this would correspond to a c-DNA transcript lacking the last 180bp's.

Secondary structural analysis of the glucoamylase gene sequence indicates that there is a hairpin loop between residues 74-99. This observation may prevent the synthesis of full length transcripts from this m-RNA.

At the present time over 3,000 recombinant colonies harbouring genomic fragments approximately 3.4 Kb in length have been screened by oligonucleotide probes and c-DNA sequences but to date the genomic sequence for glucoamylase has not been successfully isolated. This presents difficulties in assessing the validity of the potential c-DNA sequences and obtaining the necessary N-terminal and catalytic sequences which are a prerequisite to establishing a glucoamylase gene construct for active enzyme secretion in yeast. .A private communication from John Hammond of the Microbiology Dept; at the Brewing Research Foundation, Nutfield, Surrey, U.K., indicated that they have obtained the glucoamylase sequence from *Nunberg et al* [180]. They have now cloned this full length sequence into a yeast expression vector under the control of the "strong" PFK-promoter. Selection and stability of transformed industrial brewing yeast were maintained by using copper resistance [92]. Trials are presently underway with a number of industrial brewing strains and indications are that the expression and secretion of the Aspergillus glucoamylase is still too low for commercial exploitation. However integration, plasmid modifications, alternative upstream promoters and N-terminal signal sequences are all under active investigation to increase the efficiency of secretion.

4.18. Future Trends.

Glucoamylase from Aspergillus and Rhizopus sp. have the ability to debranch amylopectin and degrade the amylose and amylopectin components. The effect of pullulanase combined with alpha-amylase on the enhanced degradation of wheat starch has been studied [190]. Alcohol production and wort fermentability have also been shown to be closely related to the amount of exogenously added pullulanase [191,192,193]. Thus the ability of a commercial yeast strain with a capacity to degrade starch directly to fermentable saccharides in good yield is commercially viable and is a real possibility in the future. Although not suitable for brewery applications, the insertion of thermostable enzymes might be useful for distillation based industries.

With presently attractive prices and the added financial incentive of vital gluten as a by product , usage of wheat for the production of starch or fermentable sugars for beverage, food processing, malt adjunct or wheat beer, is on the increase. Endo-xylanases may be useful in aiding solubilization of pentosans present in the cell walls of wheat which would otherwise present viscosity problems to brewers as well as during industrial starch processing.The introduction of an endo-xylanase into yeast may have beneficial effects during brewery or distillery processes and may allow the usage of wheat flour as an alternative carbohydrate source or increase its use as a cereal adjunct.

Hansen [194], has discussed the possibilities of aiding the food problem in many third world countries. The following method has been put forward as a possible way of providing a cheap nutritious food source for the populations of malnourished countries. He has suggested purchasing broken rice which can be obtained cheaply and the starch after gelatinization can be initially degraded by Aspergillus oryzae. This mixture is centrifuged to concentrate the proteins into an insoluble fraction, resulting in a three fold increase in protein, from 8% to 25% whereas the starch level was reduced from 89% to 32%. This protein fraction which can be dispersed in water has been shown to have a biological value akin to casein. The saccharides can then be used to culture yeast, another source of valuable vitamins.

However the introduction of the structural gene for glucoamylase to yeast directly would save considerable time and expense in production costs. Furthermore the introduction of a protein of high biological value into the yeast genome to be expressed at the end of fermentation would further enhance the value of the foodsource and indicates the potential of molecular biological techniques as an alternative strategy for supplying nutrition to malnourished countries.

5.0 Overall Conclusions.

In this thesis an attempt has been made to elucidate the fine structural details of starch granules isolated from different sources and to relate structural variations to changes in their susceptibility to degradation by α-amylases and their affinity for Concanavilin-A. Hordeins were shown to co-purify with barley starch granules surface. Introduction of a reducing agent was required to remove the proteins from the granule surfaces. Granules associated with hordein were poorly degraded by α-amylases when compared to granules free of the proteins. It is suggested that the interaction between granule and protein is of a physical nature rather than covalent.

Starch granules studied in this thesis possessed differences in the degree of degradation when subjected to treatment with salivary and cereal α-amylases.

The affinity of Concanavilin-A, a carbohydrate binding protein with defined affinity, was shown to bind differentially to starch granules isolated from different sources, independent of granule size.

This differential lectin binding may indicate distinct differences in the surface architecture of granules types.

Indications from electron microscopic studies of large granules isolated from barley showed that the interior of the granules are more easily digested than their exterior. Whether this is because the enzyme becomes physically entrapped within the granule or that the interior of the granule is more digestible than the exterior is not known. However if the latter were true this would indeed indicate structural differences do exist between interior and exterior.

Further structural studies using chemically substituted granules, debranching with pullulanase and isoamylase, partial enzymic degradation with α- and β-amylases after separation of the products by gel exclusion column chromatography, indicated that the spatial arrangement of amylose and amylopectin chains is arranged differently within granule types.

The observed structural differences would influence binding of amylases and Concanavilin-A (as discussed previously and within the text) further, since binding of the amylases is a prerequisite to digestion similarly the catalytic efficiency of the enzymes would be altered.

Accurate determination of the intitial hydrolysis products released from granule surfaces by α-amylases before the enzyme carries out further cleavages within the same chain, using starch granules from different sources and varying in fine structure may be informative in the location of those glucose residues presented to the enzyme at the granule surfaces.

Plant species carrying mutations for starch synthesis will be particularly valuable for further studies.

Methods involving the chemical modification of amylases, without changing their specificity for granular starch, which will allow irreversible binding to initial attachment sites could be an important area for further study. The identification of their location at the granule surface using monoclonal antibodies and microscopic techniques may aid in the elucidation of initial amylase binding sites at the granule surface.

Whether α-amylases produce multiple iso-enzyme complexes in the formation of "pitting" at the granule surfaces could also be studied by similiar techniques. This may aid in the resolving whether any particular isoenzyme carries out a major role in the initiation of granule degradation.

The location of the starch granule binding domain present in α-amylases, the degree of conservation at this domain between α-amylases isolated from different sources and their homology to other starch binding proteins should be important areas of future investigation.

The presence, absence or variations in this domain between the cereal iso-enzymes with relation to their ability to bind granular starch should be particularly interesting.

After over a century of intensive study the fine structure of granular starch, the spatial relationship of its macromolecular components, its synthesis, crystallization in the amyloplast or chloroplast and its final degradation are still poorly understood. With the introduction of laser and molecular biolological techniques perhaps the finer details of this important, industrial renewable biopolymer will be revealed by the end of this decade.

Finally it is clear that their are considerable industrial applications for amylases, glucoamylases and xylanases in starch and wood related industries respectively. Whether mesophilic or thermophilic enzymes are selected will depend on the industrial application and economic considerations will always remain the paramount concern.

Regarding the investigations reported in this study further work is required to gain adequate secretion and expression of glucoamylase after fragment assembly.

The xylanse requires ligation in three frameshifts to an *E. coli* or yeast expression vector and assessed for activity *In-situ* utilizing larchwood xylan and congo-red or Remazol-brilliant blue dye-linked xylan.

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Appendix

Fig. 52 b:	Yeast expression plasmid AH-21.
Fig. 52 c	Blue Scribe plasmid.
Fig. 52 d	Plasmid, pUC19.
Fig. 52 e	Protein-A fusion vector, pRIT-2T.

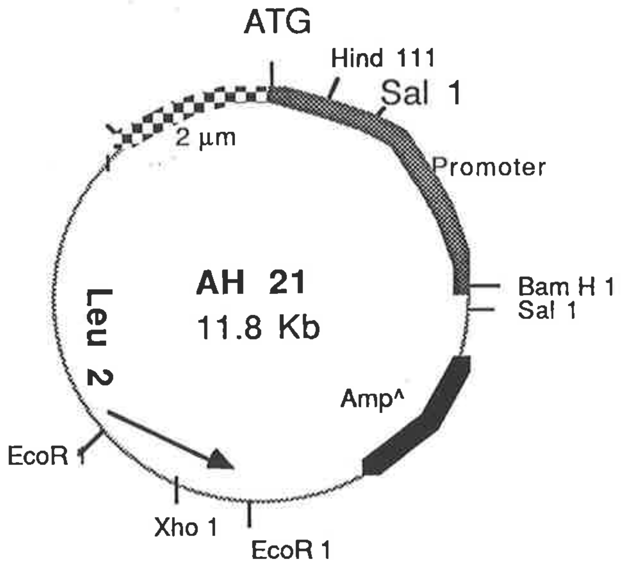
Publications from this thesis: 1. Differential Binding Of Concanavilin-A To Large And Small Starch Granules Of Barley.(In Print)

2. A New Test For Assaying Alpha-amylase In The Presence Of Beta-amylase. (Forwarded for publication, 12/87)

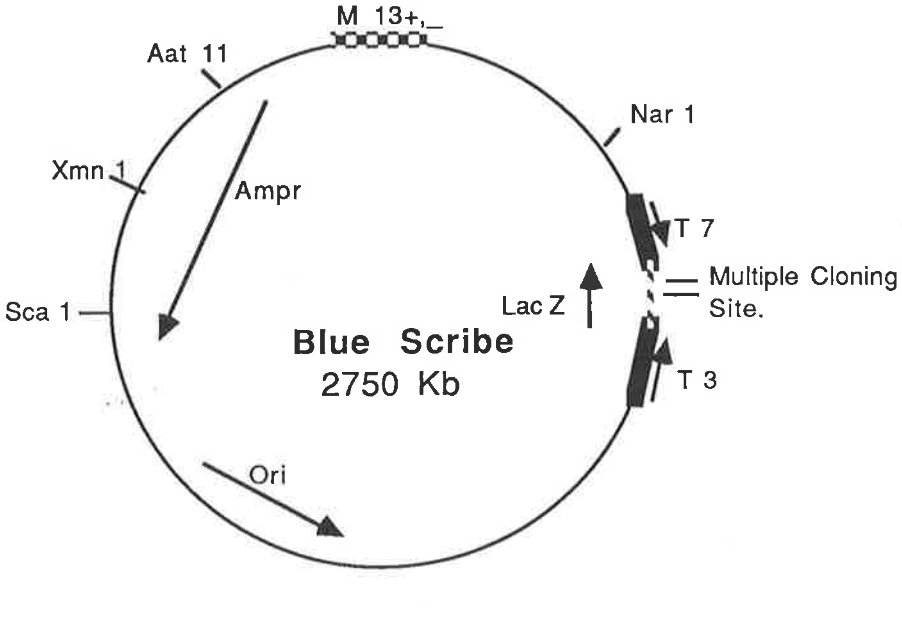
3. Inhibition Of Alpha-amylase Degradation Of Starch Granules In The Presence Of Concanavilin-A. (Forwarded for publication, 12/87).

Figure. 52 b.

Yeast Expression Plasmid, AH-21.



Blue Scribe, Fig. 52c



pUC19, Fig. 52d.

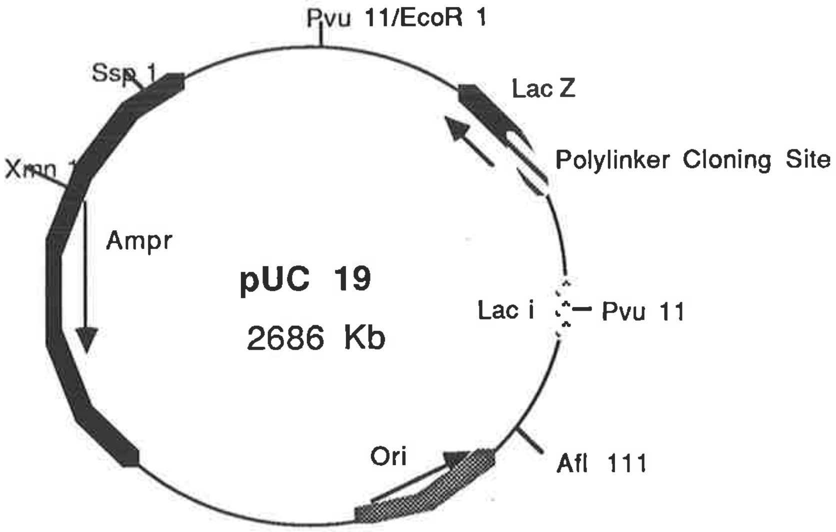
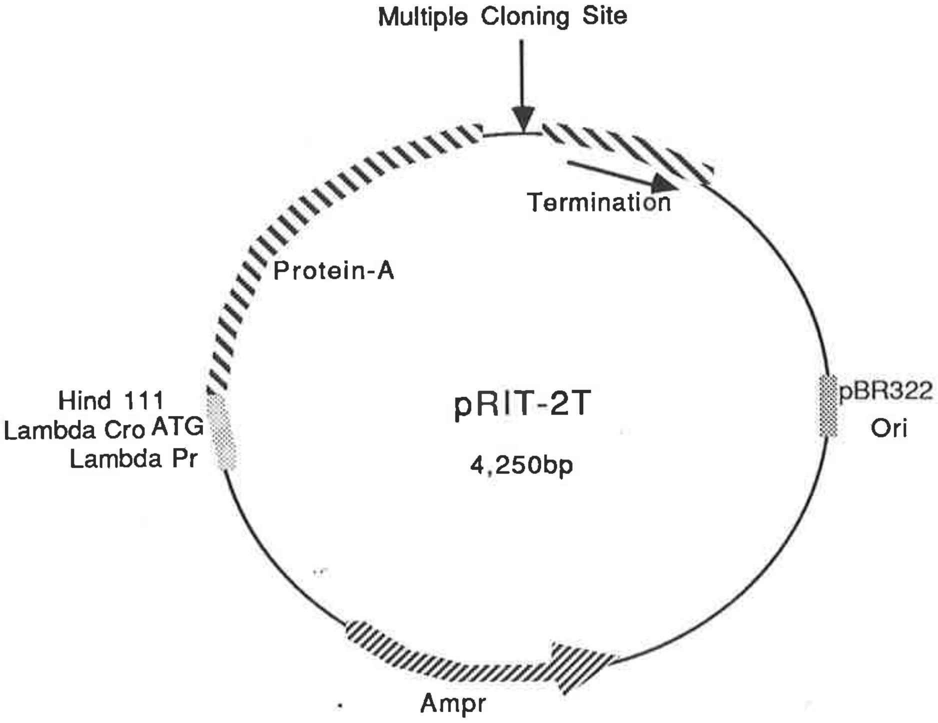


Fig. 52e pRIT-2T,



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Title: A Test For Endo-amylase Activity In The Presence Of Exo-amylases.

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Summary.

Increased hydroxpropylation of potato starch or amylose reduced the susceptibility of these substrates to attack by alpha and beta-amylases.

By limited hydroxypropyl substitution (molar substitution: 0.18) of starch or amylose, substrates were obtained which were specifically degraded by alpha-amylase.

These modified substrates enabled alpha-amylase to be determined in the presence of beta-amylase in extracts of malted barley.

1.0 Introduction.

Numerous tests have been developed for assaying alpha-amylase activity in the presence of exo-polysaccharide degrading enzymes, including amyloclastic[1], substrate dye complexes [2,3,4,5,6], and methods based on soluble substrates[7].

Enzyme activity is usually assessed by measuring the production of reducing groups, iodine binding, release of bound dyes from insoluble dye substrate complexes, viscometry and polarimetry.

All these methods have drawbacks. The amyloclastic and dye binding techniques utilize insoluble substrates and are therefore unsuitable for automated analytical procedures. The methods based on soluble substrates invariably utilize beta-limit dextrins prepared by time consuming methods from waxy starches or else purchased at high cost thus limiting their usage in screening large numbers of samples eg. in the early generations of a plant breeding programme.

Beta-limit dextrins do not react efficiently with iodide ions and therefore are assayed by measuring the quantity of reducing groups released in the presence of the enzyme. This is a problem when background levels of reducing groups are unfortunately high, eg. in malted barley.

An alternative method of assaying for alpha-amylase is proposed where limited hydroxypropylation of starch or amylose results in a soluble substrate resistant to exo-amylase attack but retains its susceptibility to endo-amylases.

2.0 Materials and Methods.

2.1 Hydroxpropylation of Starch and amylose.

Samples (2 g) of "Merk" soluble potato starch or amylose (Type 111, Sigma, Chemical Company) were mixed with sodium hydroxide (0.25 M) and di-sodium sulphate (0.85 M) in a total volume of 2.4 ml. in air tight glass containers. The samples were placed in a shaking water bath at 35°C and propylene oxide (Ajax Chemicals, LR grade, 0-400µl) was added.to produce substituted polysaccharides with a degree of molar substitution from 0.03-0.32. Incubation was carried out for 24 hr. with constant agitation (100 r.p.m.). The alkaline conditions were neutralized with sulphuric acid (0.01 M) and the treated starch or amylose centrifuged at 15,000xg for 5 min. at 5°C.

The supernatant was decanted and the substituted granules were washed (x5) with double glass distilled water and then lyophilized.

When required for enzymic degradation the substituted starches were solubilized in the required buffer by heating to 80°C for 2 min.

2.2 Incubation of substituted starch and amylase with amylases.

Solubilized substituted starch or amylose (10 mg./ml.) were incubated with alpha-amylase (5.7 International Diastatic Units), purified from barley [8], salivary alpha-amylase (obtained from the author 5.7 I.D.U.) or beta-amylase (Type 1-B,Sigma Chemical Company, 30 I.D.U.). The reactions were carried out in sodium acetate buffer (0.05 M, Ca C12 mM, pH 5.5 for cereal alpha-amylase, at pH 4.8 for beta-amylase and in Phosphate buffer 0.01 M, 1 mM Na Cl pH 7.0 for salivary alpha-amylase) all at 30°C. Aliquots were removed at appropriate periods and analysed for the presence of reducing sugars [9], total carbohydrate[10], and reaction with iodide [11].

2.3 Incubation of substituted starch with alpha and beta-amylase.

Initially, substituted starch (10 mg/ml, 0.018 M) was incubated with salivary alpha-amylase (5.7 I.D.U.) for 10 min., the remaining polysaccharide was precipitated with ethanol (90%), lyophilized and a sample incubated with beta-amylase (30 I.D.U.). Degradation products were assessed as above.

3.0 Results.

3.1 Effect of increased substitution.

The effects of increasing hydroxypropyl substitution on the degradation of "Merk" starch by alpha-amylase and beta-amylase are shown in Tables 1 and 2 respectively.

Increasing the degree of molar substitution from 0.03-0.32 showed progressive decreases in the ability of alpha-amylase and beta-amylase to degrade these substituted substrates. At molar substitutions >0.27 beta-amylase activity was completely inhibited and at pH 4.8 its activity was negligible.In contrast salivary alpha-amylase still showed activity at molar substitution 0.32.

3.2 Incubations with alpha and beta-amylases.

The results of incubations with alpha-amylase prior to treating with beta-amylase presented in Fig. 1,(SD: 0.21) indicate that little release of reducing groups occurred during the beta-amylase incubation period. There was a change in the maximum absorbance, as measured by iodine, of beta-amylase incubated with either substituted (0.018 M) or unsubstituted soluble starch over 2.5 hr. as shown in Fig. 2 (SD: 1.76). Little decrease in absorption occurred in the substituted starch sample in contrast to a marked decrease for the unsubstituted sample.

To assess the effect of hydroxypropylation and amylase degradation on a more homogeneous substrate than soluble starch, amylose (0.018 M) was prepared. An alternative detection method of assessing polysaccharide degradation, based on a decrease in iodine staining, with substituted amylose and substituted starch as substrates is illustrated in Fig. 3 (SD: 2.14). The results show a decrease in iodine affinity for both substrates.

Changes in wavelength, and maximum absorbance at those wavelengths of modified and unmodified amylose after incubation for 20 min. with beta-amylase are shown in Fig.4, (SD: 1.75).

One fifth of the quantity of beta-amylase utilized in the modified amylose incubation reduced the absorbance of unsubstituted amylose from 1.3 at 615 nm. to 0.32 at 576 nm. The wavelength change was accompanied by a decrease in absorbance at these wavelengths and was most noticeable in the unmodified substrates where the initial absorbance and wavelength changes were greater initially and less finally than that of unmodified substrates, demonstrating greater degradation in the unmodified material.

3.3 Incubation of substituted starch with increasing amounts of alpha-amylase.

Results for modified starch incubated with increasing concentrations of alpha-amylase are given in Fig.5, (SD: 1.45). Increasing the amount of enzyme enhanced the degradation reflected in an increase in free reducing groups.

3.4 Incubation of substituted amylose with alpha and beta-amylase.

Results of incubations of modified and unmodified amylose with alpha-amylase and beta-amylase are shown in Fig. 6, (SD: 1.64).
A negligible amount of reducing groups were released from the modified substrate incubated with beta-amylase, in contrast to the unmodified form.

3.5 Assessment of modified substrate.

The use of this modified substrate as a method of assaying endo-amylase in the presence of exo-amylase was assessed. Substituted starch was incubated with malted barley extracts possessing a wide range of amylolytic activities normally associated with a typical plant breeding programme. The samples were equally divided, one set was subjected to heat treatment to denature beta-amylase.

Results presented in Table. 3, (SD: 1.71) clearly demonstrate the effectiveness of this substrate for assessing endo-amylase activity.

The effects of heat treatment on the activity of alpha-amylase is also shown, the inactivation of alpha-amylase by heat treatment does not cause similar decreases in alpha-amylase activity in all samples.

4.0 Discussion.

The substitution of soluble starch or amylose results in the hydroxypropylation of free hydroxyl groups mainly at positions 2, (80-85%), and 6 (7-15%) in the amylopectin component [12]. This produces a modified substrate which is virtually resistant to beta-amylase but is still sensitive to the endo-acting alpha-amylase.

Substituted starch can be prepared easily and its increased solubility makes it suitable for automated analytical procedures. Higher substrate concentrations can also be used. Enzyme activity results in an increase in reducing groups [9], or decreased affinity of polysaccharide for iodide ions.

The presence of debranching enzymes resulting in an increase in reducing groups at a molar substitution of 0.03 is minimal, however if this presented a problem the substitution of iodine for Nelson-copper reagent can be made. Alternatively since substitution increased solubility and inhibited retrogation of amylose, substituted amylose can be prepared and used as a substrate.

The slight decrease in absorption (Fig. 4) observed during beta-amylase degradation of substituted amylose when using iodine as the detection agent for modified substrates after prior treatment with alpha-amylase, maybe due to degradation of those amylose chains which are not substituted or were substituted and remained susceptible to beta-amylase. Similiarly a small amount of reducing equivalents were released from substituted amylose and starch by beta-amylase before (Fig. 6), and after (Fig. 1), alpha-amylase treatment. This is probably due to those terminal chains which remain unsubstituted.

A number of laboratories assay alpha-amylases in plant extracts particularly in the case of malted barley where the amount of alpha-amylase present not only indicates the extent of endosperm modification but also the starch degrading potential of a given cultivar. In order to asses the alpha-amylase activity in the absence of beta-amylase the crude extract from the malted grain is often heated at 70°C for 15 min. Although this treatment can be effective in reducing or eliminating beta-amylase it also affects the stability of alpha-amylase as indicated in Table. 3.

Changes to more alkaline pH 8.0 would decrease but not eliminate the detrimental effects of heating. Furthermore the results clearly demonstrate that the denaturation of alpha-amylase in the extract is not constant for each cultivar and is probably dependant on such variables as types of isoenzymes present and their individual concentrations. The protein content of the extract which may also affect the stability of alpha-amylase during the heat treatment would vary in the malted samples. The usage of limited substituted starch or amylose as indicated above presents an alternative method of directly assaying endo-amylase in the presence of exo-amylase.

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Fig.1 Beta-amylase incubation of Alpha-amylase treated, substituted starch.

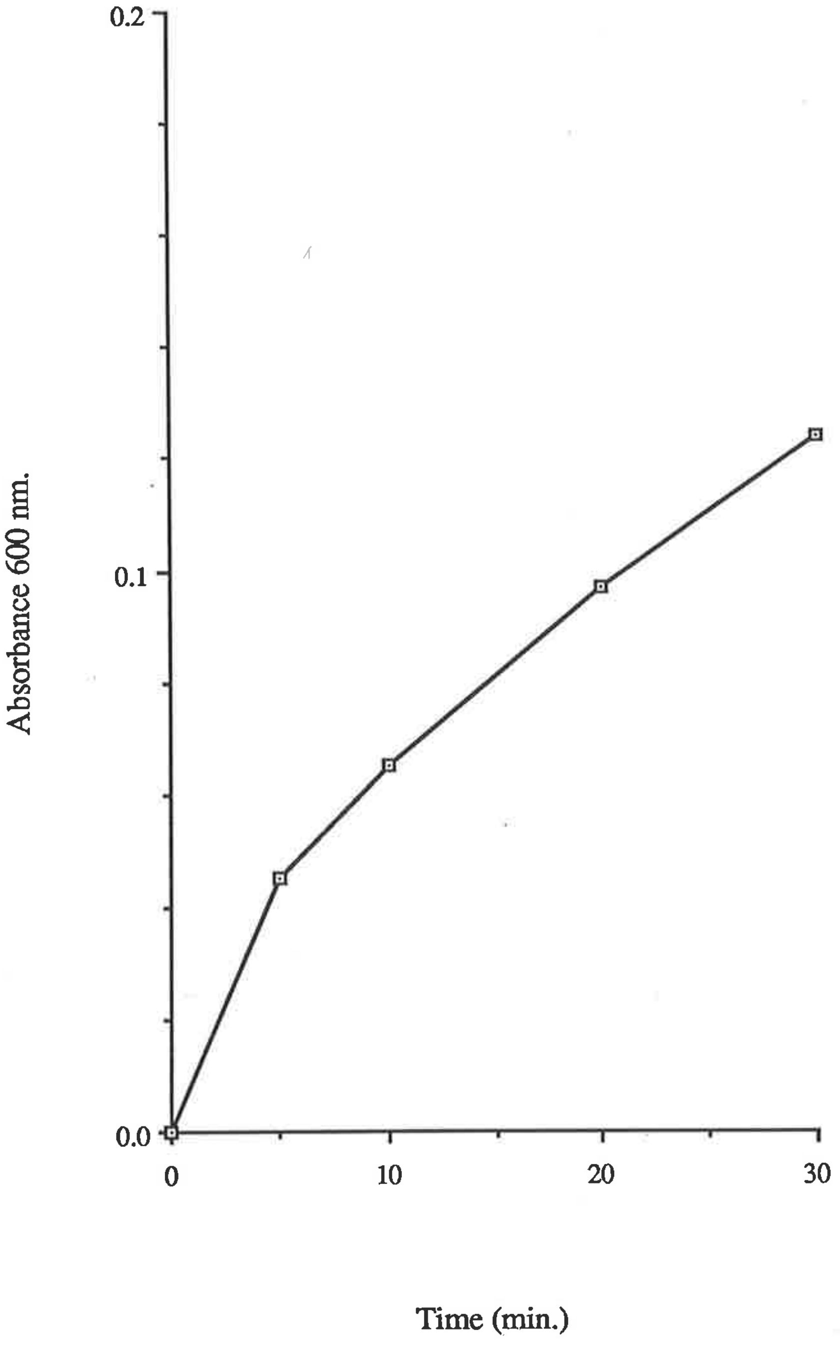


Fig.2 Substituted And Unsubstituted Starch incubated with Beta-amylase..

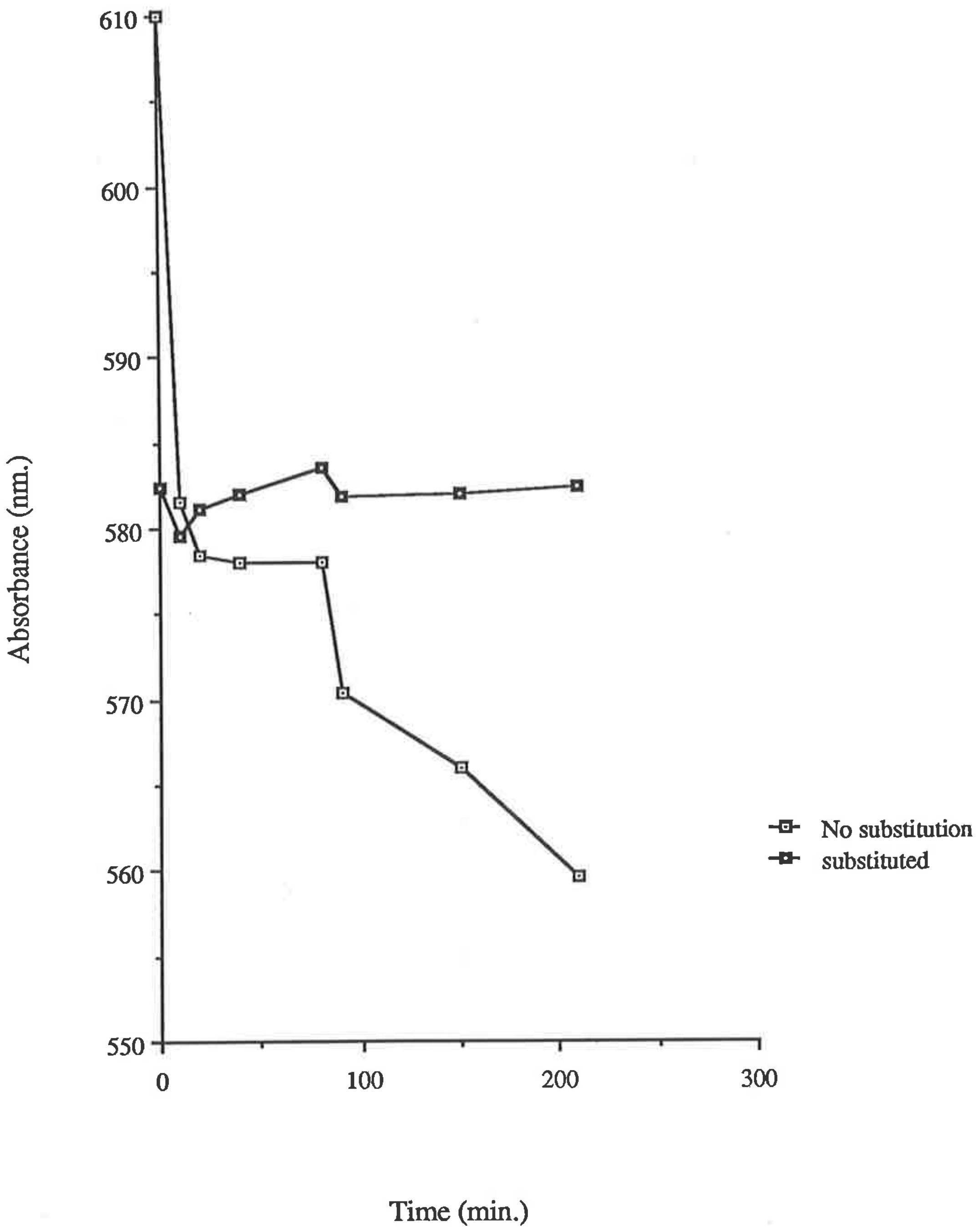
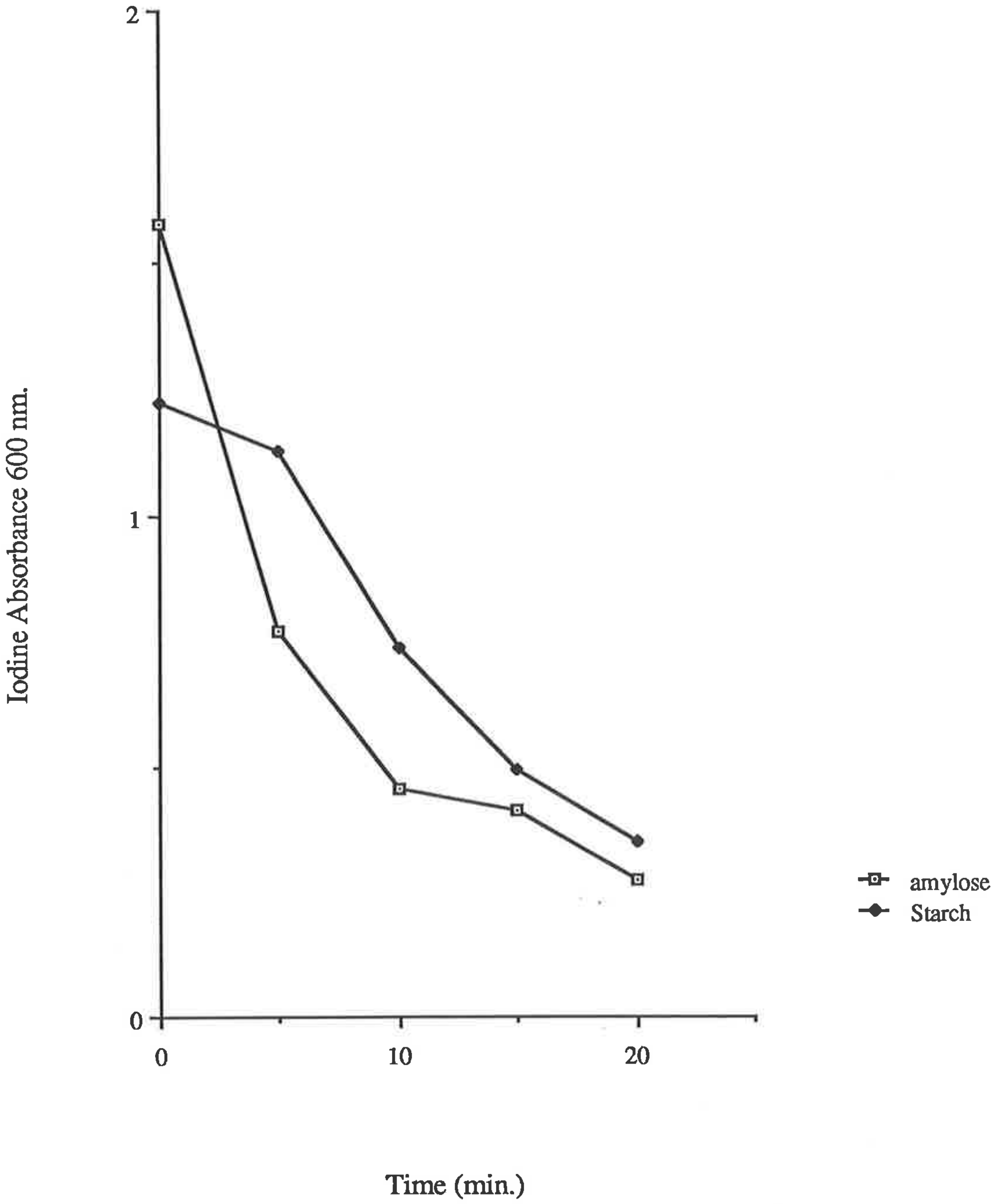


Fig.3 Alpha-amylase degradation Of Substituted Starch And Amylose.



<u>**Fig.4 Beta-amylase Degradation Of Substituted And Unsubstituted Amylose.**</u>

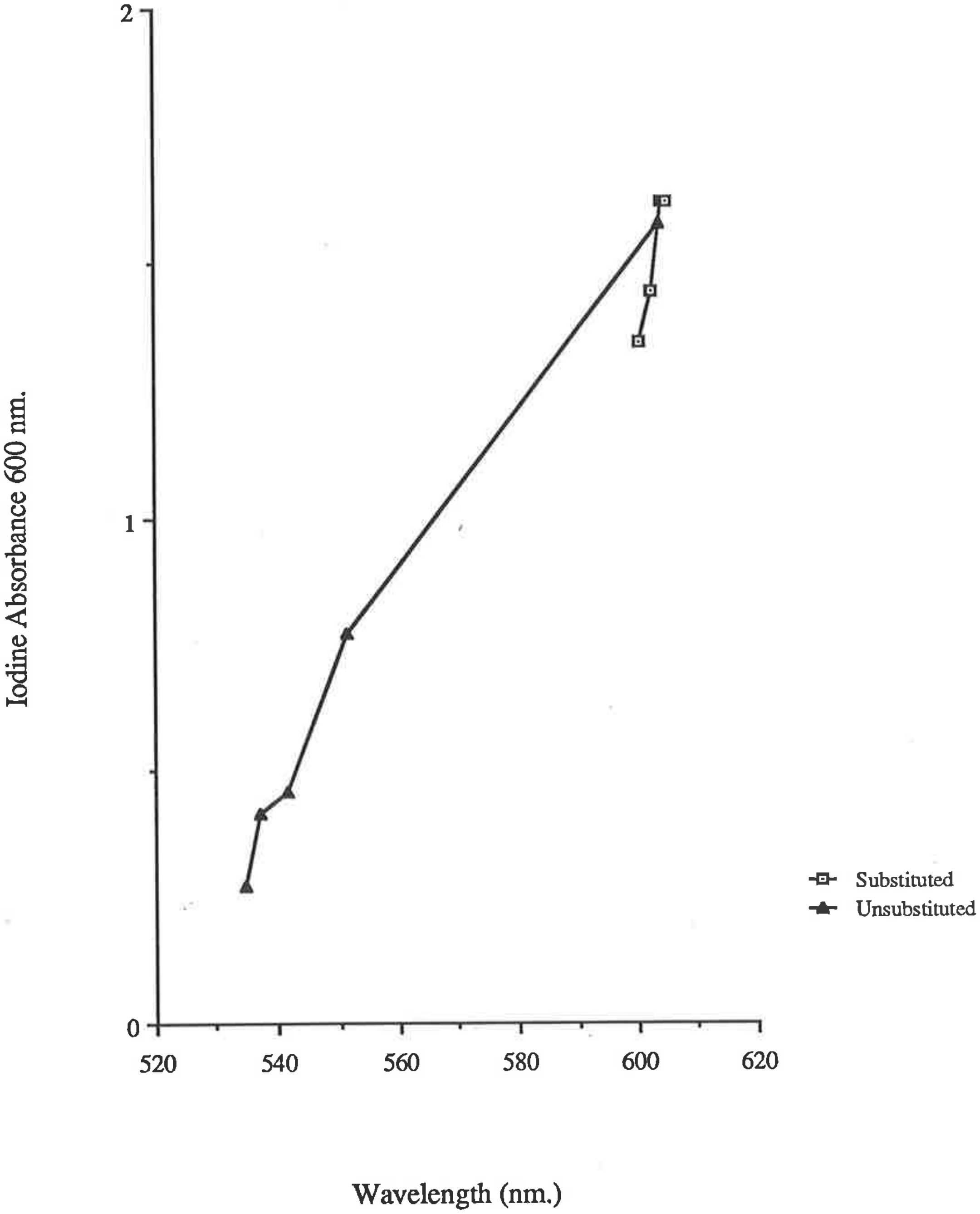


Fig.5 Substituted Starch Incubated With Increasing Quantities Of Alpha-Amylase.

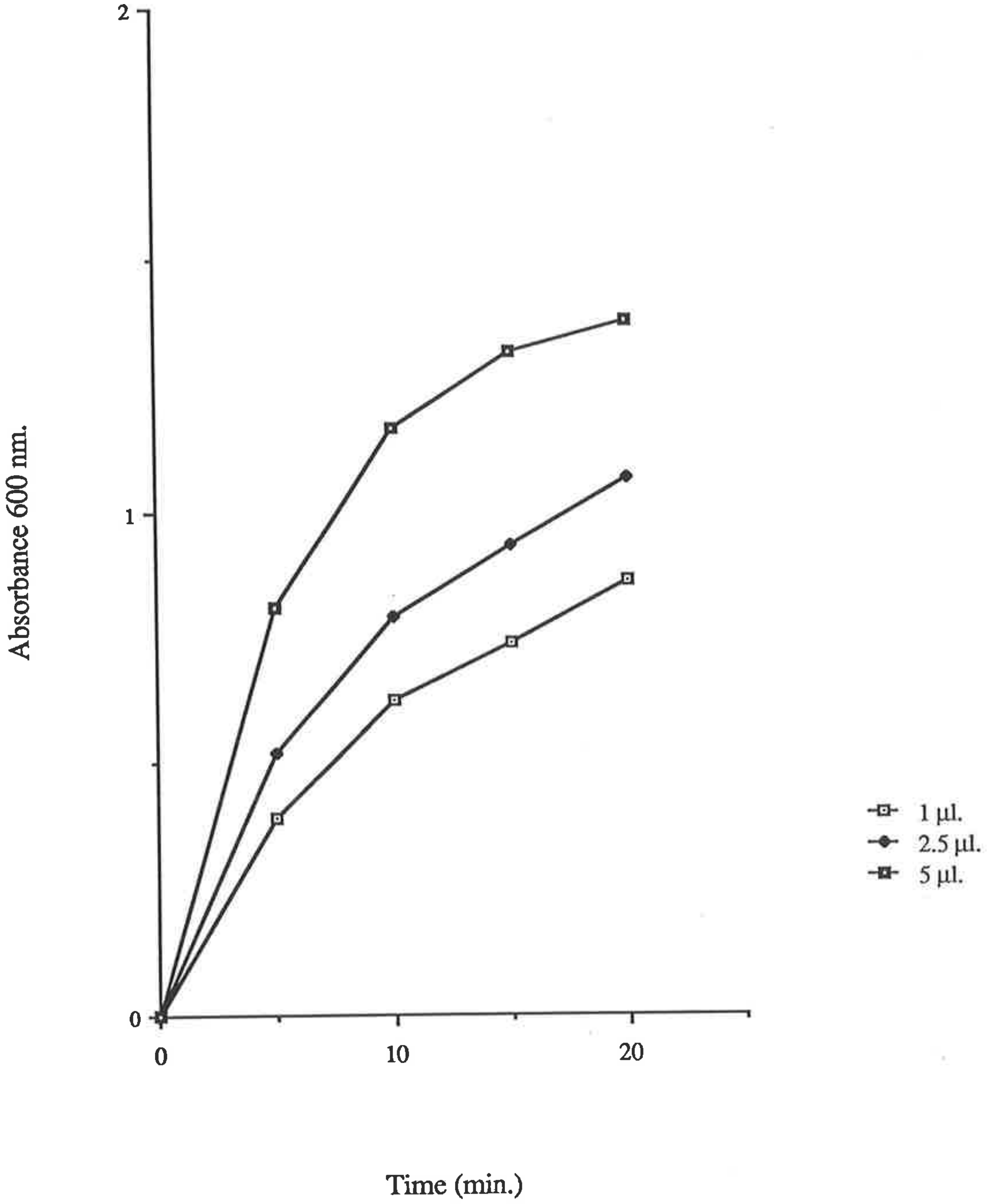
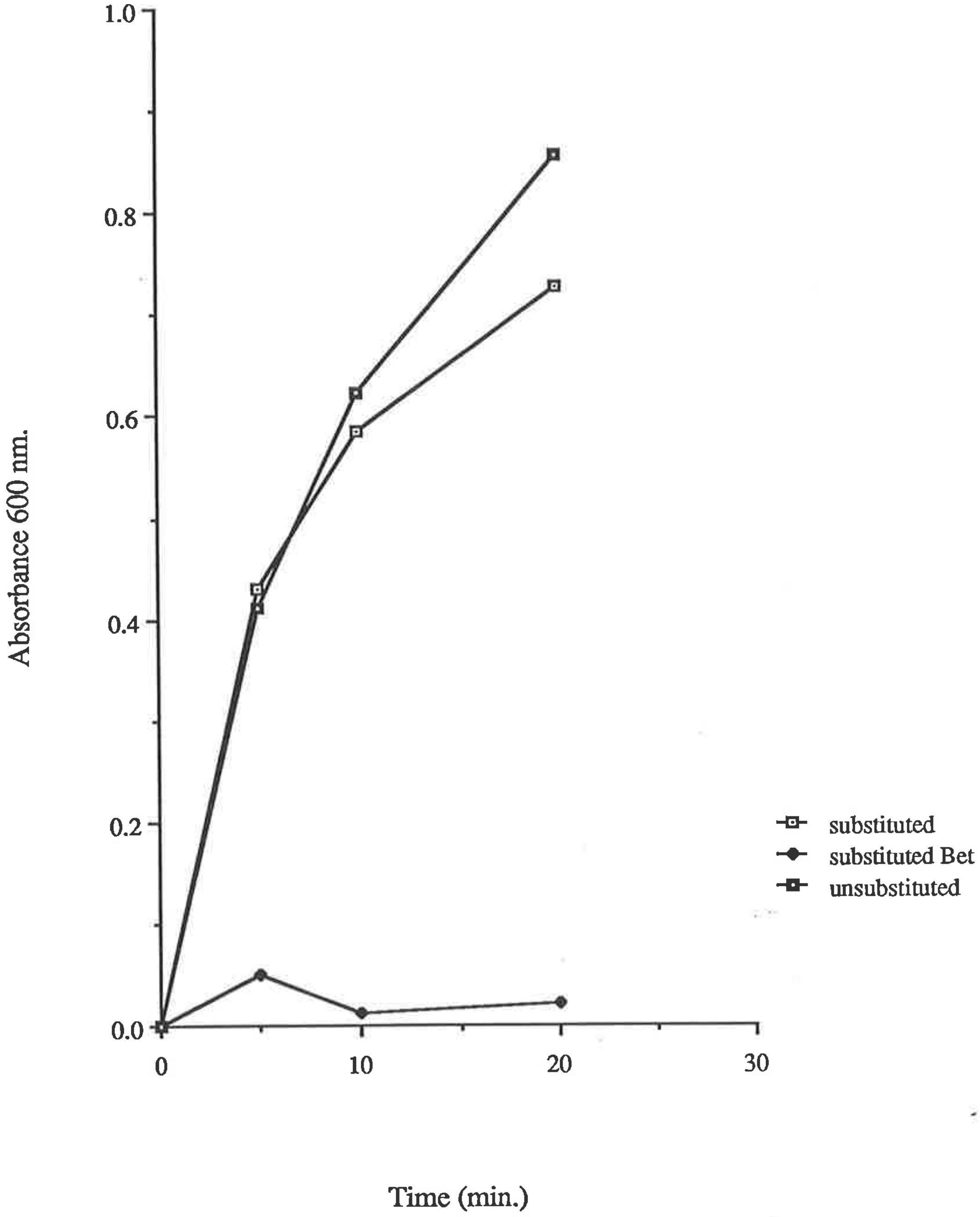


Fig.6 Amylose, Substituted And Unsubstituted Incubated with Alpha And Beta-amylase.



Title: Inhibition Of Barley Starch Granule Degradation By Salivary Alpha-amylase In The Presence Of Concanavilin-A.

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Summary.

Salivary alpha-amylase showed greater affinity for the exterior of the large starch granules of barley than small granules at 25°C. Enzymic degradation and affinity for these starch granules was decreased in the presence of lectin. Decreased degradation was greater for large granules. The extrapolated theoretical quantities of lectin required to completely inhibit the digestion of barley starch granules was found to be greater for large granules than small ones.

1.0 Introduction.

Alpha-amylases digest intact starch granules isolated from various sources in different ways.

In the case of barley starch, initial enzyme attack on the large granules appears to result in well formed pitted areas on the granule surface [1-4]; subsequently the internal material was more susceptible to degradation. Small granules from barley are degraded mainly by surface erosion [5-7].

Starch granules isolated from different sources have been shown to vary in their susceptibility to breakdown by alpha-amylases[8-11].

Previous work has indicated that the lectin, Concanavilin-A, possessed differential binding affinity for the large and small starch granules of barley as well as those of waxy and normal maize [12]. Recently tuber starch granules were shown to have less affinity for Concanavilin-A than those of cereals.

The affinity of starch granule for lectin is associated with the susceptibility of granule type to attack by alpha-amylases, thus the enzymically susceptible waxy and normal maize, as well as the small starch granules from barley all display higher affinity for the lectin than the less susceptible starch granules from mannioc, potato and the large granules from barley.

The work reported here is concerned with the binding and degradation of large and small starch granules from barley by salivary alpha-amylase in the presence of Concanavilin-A.

2.0 Materials And Methods.

2.1 Starch Granules.

Isolation and purification of large and small starch granules from barley cultivars Clipper, C.I. 3576 and Betzes have been described previously [12].

2.2 Preparation of Salivary Alpha-amylase.

A sample (10 ml.) of saliva was collected from the author in the morning before the intake of food or beverage. Saliva was added to a phosphate buffer (10 ml. of 0.2M, pH 7.0, 1 mM sodium chloride) and centrifuged for 30 min. at 32,000xg at 5°C. The supernatant was carefully decanted and the resulting enzyme solution used in further experiments, final activity 40 International Diastatic Units/ml.

2.3 Incubation of starch granules with Concanavilin-A.

Starch granules (25 mg) were incubated with lectin (Type V, Sigma Chemical company) for 60 min. as described previously [12]. Salivary alpha-amylase 5.7 I.D.U were then added to the mixture. Portions of the digest (0.25 ml.) were removed at intervals over a 60 min. period and amylase activity inhibited by the addition of 50 μl of mercuric chloride (1% w/v). The samples were centrifuged at 15,000xg for 3 min. at 25°C and the supernatants were assayed for total soluble carbohydrate [13] and reducing equivalents [14].

2.4 Binding of salivary alpha-amylase to barley starch granules.

Alpha-amylase (5.7 I.D.U), was incubated with purified starch granules (2.75 mg./ml.) in phosphate buffer (0.02 M, pH 7.0, 1mM MgCl2, 1mM CaCl2, 1mM MgCl2). Aliquots (1ml.) were removed at appropriate intervals and centrifuged at 15,000xg for 2 min. to pellet the granules. The supernatant solution was divided equally. To one aliquot Somogyi-Nelson reagent [14] (0.5ml.) was added to inactivate the enzyme. This sample was subsequently used to determine the extent of granule degradation determined by liberation reducing groups. Alpha-amylase activity was determined in the second aliquot (10 µl.) using cross-linked amylose (X-15, 20mg) as substrate. Incubation was carried out for 3 min. and enzyme activity was assessed as described by Mateescu*et al* [15]. The free-enzyme activity present in solution was expressed as a percentage of the initial activity.

3.0 Results.

The data in Figs. 1 and 2 show the affinity of salivary alpha-amylase for the exterior of the large and small starch granules of barley respectively. The enzyme had a greater affinity for the larger granules of barley.

Results presented in Figs. 3 and 4 show a decrease in salivary alpha-amylase adsorption by both the large and small starch granules of barley, cultivar C.I. 3576 in the presence of lectin. Affinity of the enzyme decreased as lectin concentration increased in both granule types.

The data shown in Figs. 5 and 6 demonstrate the reduction in granule susceptibility to enzyme in the presence of increasing concentrations Concanavilin-A.

The results in Figs. 7 and 8 show the theoretical quantity of lectin required to completely inhibit the degradation of large and small starch granules of barley, cultivar C.I. 3576. An extrapolation of the line indicates the approximate amount of lectin required for a complete inhibition of starch granule degradation. For large starch granules, lectin concentrations between 48-52µg/mg. are required. For small starch granules the concentration is dependent on which part of the line is utilized for extrapolation, if the end of the line is used then a concentration of 18.5-22µg/mg. is required, whereas when earlier points are used then the values would be between 22.2-29.6µg/mg.

4.0 Discussion.

The specificity of Concanavilin-A for the non-reducing termini of branched alpha-1,4 glucopyranosides and alpha-1,4 manno-pyranosides has been discussed [16-18].

Results of large and small granule digests with salivary alpha-amylase indicate that the amount of enzyme adhering to the granule fluctuates over the incubation period. This may be associated with the ease in which the granules are digested which is dependent on the enzyme gaining access to new internal areas of the granule thus reflecting differences in structural arrangements from exterior to interior.

Small granules from Clipper had the least affinity for the enzyme and are most resistant to enzymic degradation. The adsorption of barley alpha-amylase to barley starch at 5°C has been reported previously[19].

Figs. 3 and 4 demonstrates a decrease in salivary alpha-amylase bound to large and small starch granules of cultivar C.I. 3576 in the presence of lectin. As lectin concentration increased the enzyme had a decreased affinity for both granule types.

Increases in the amount of lectin bound to granules prior to incubation with salivary alpha-amylase resulted in a decrease in the total soluble saccharides released, (Figs. 5 and 6). The results imply that the lectin competes with salivary amylase for access to common binding sites on the granule exterior.

Decreases in total soluble saccharides were not accompanied by decreases in reducing groups liberated, suggesting that the lectin may not inhibit further degradation of soluble saccharides once released from the granules. Lectin bound to the granule surface prevents access of the enzyme to the granule and may decrease enzyme binding areas present on the granule exterior.

However, as shown previously [12] about 20% of the lectin remained in an unbound form, suggesting that all sites available on the granule surface are bound to lectin. Since degradation continues and lectin appears to be in excess it may compete with the enzyme at certain other sites on the granule exterior. Other sites are either not available to lectin or they possess lower affinity for lectin compared to the enzyme.

By increasing lectin concentration to 22.2μg/mg starch granules (approximately three times the lectin concentration required for 80% binding), enzymic degradation was still detected, although at a much reduced level. This may be due to reduced quantities of enzyme binding initially to the granule exterior in the presence of lectin.

Initial enzyme degradation in large granules appears to be associated with susceptible areas on the granule surface. Since the pattern of degradation of the granule exterior, in the presence of lectin, was not altered (Plate. 1), this suggests that the lectin and enzyme have different binding sites or differing affinities for available binding sites.

Further, once the enzyme has gained acess to the granule interior, degradation can proceed with less inhibition from the lectin. The form of the curve in Fig. 3 indicates that restricted degradation was high initially but decreases with time, even at increasing concentrations of lectin.

In contrast, small starch granules were degraded by surface erosion (Plate. 2). The form of the graph shown in Fig. 6 suggests that as degradation proceeded inhibition by lectin was also increased. Furthermore at higher lectin concentrations the extent of inhibition was also increased. This is in marked contrast to the inhibition characteristics of large granules and suggests that digestion of the small granules may proceed exclusively from the surface without much acess to the interior. Concanavilin-A has a greater affinity for the small starch granules so that it is possible that they may possess a more highly branched system at their exterior than for the large granules. As degradation proceeds the enzyme may encounter increased stereochemical hindrance by a continual decrease in terminal reducing groups, surface area and increased competion between lectin and enzyme for available binding sites, thereby limiting enzymic degradation.

The lower theoretical concentrations of lectin required to inhibit enzyme degradation of small granules compared to large granules may be linked to the different mode of enzyme action for the two granule types. Initial effects on small granules are exclusively external whereas for large granules although it is initially external it proceeds via the well defined pin holes rapidily extending internally. Lectin, which strongly binds to the exterior of small granules would prevent access of the enzyme to the surface resulting in a decreased degradation. However lectin affinity did not appear to be as strong for large granules and inhibition of granule degradation was not as severely restricted as in small granules. This suggests that the enzyme gains access to the exterior of the granule and subseqently the interior via areas not occupied by lectin. The cooperativity shown to occur with lectin at high concentrations may be required to inhibit adsorption of the enzyme by large granules. Therefore increased concentrations of lectin are necessary for the inhibition of large granules compared to small ones.

Recent studies by Colonna *et al* [20], on amylopectins dispersed in solution and further modified by beta-amylase indicate that external chain length is a major parameter for stable interactions between beta-limit dextrins and Concanavilin-A. They suggested that long chain length is detrimental to the formation of stable complexes since translational activation entropy increases with increased length. Shorter chain lengths form more stable complexes. If the formation of lectin complexes with insoluble starch granules is similiar to that for dispersions of amylopectin, then preferential adsorption of lectin by waxy maize starch and the small starch granules of barley may be due to a preponderance of smaller external chain lengths protruding from the exterior of these granule types thus forming more stable complexes with lectin.

Kang *et al* [21], suggest that small starch granules of barley possess a higher percentage of small unit chains thsan that of large granules. This infers that the large granules of barley possess greater proportions of long B-chains. Although chain length may offer some explanation for the affinity of lectin for large and small starch granules of barley, detailed structural information to explain differential binding to other starch granule types is lacking.

Differential binding of lectin to normal and waxy maize granules [12] may be directly due to the amylose content of the two granule types. Moreover amylose is known to increase in granules during development [10, 22-25], thus less amylopectin may be present at the surface of normal maize starch in comparison to the waxy cultivar.

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Fig.1 Adsorption Of Salivary Alpha-amylase To Large Granules From Barley.

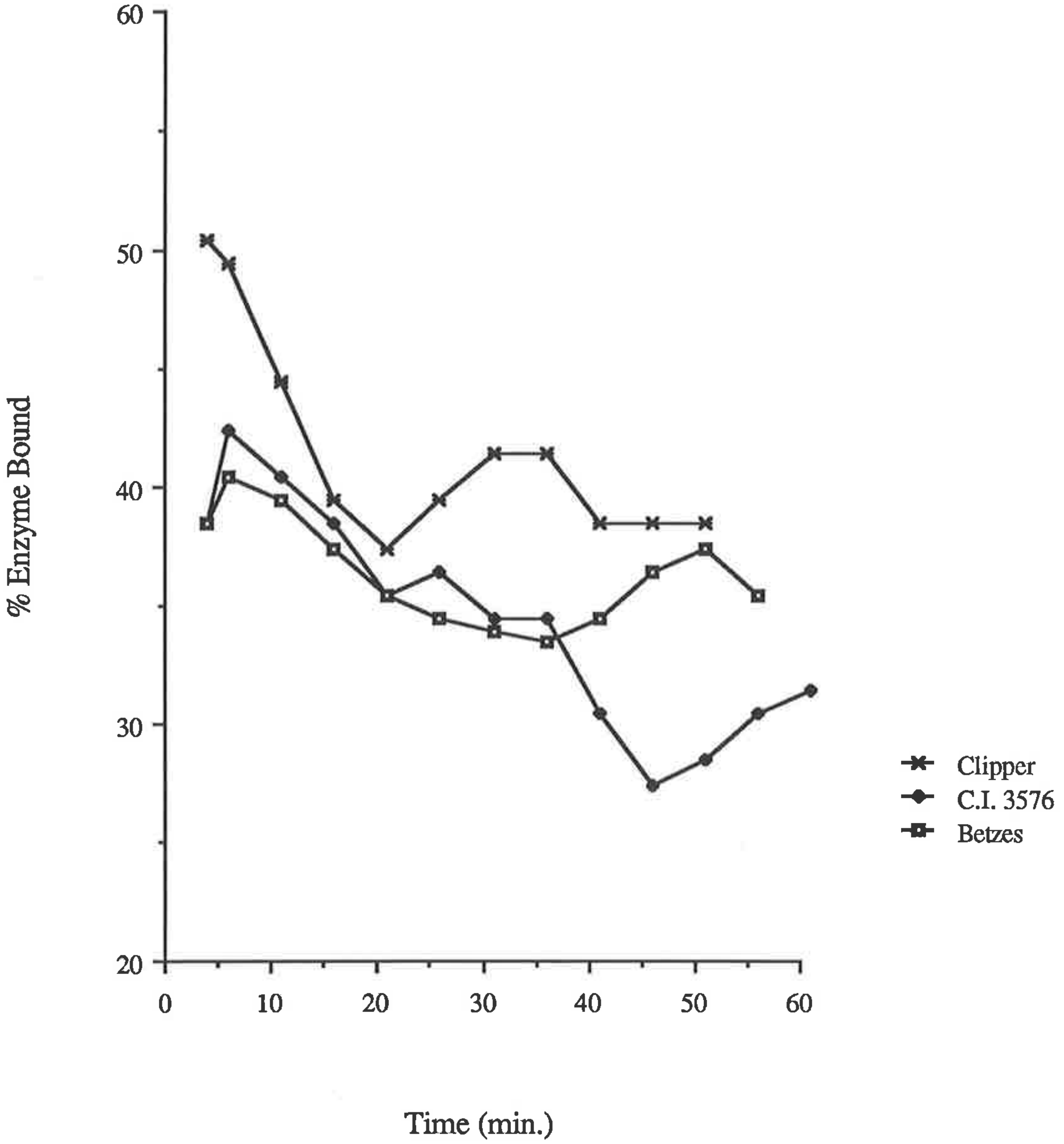

Fig. 2 Adsorption Of Salivary Alpha-amylase To Small Granules From barley

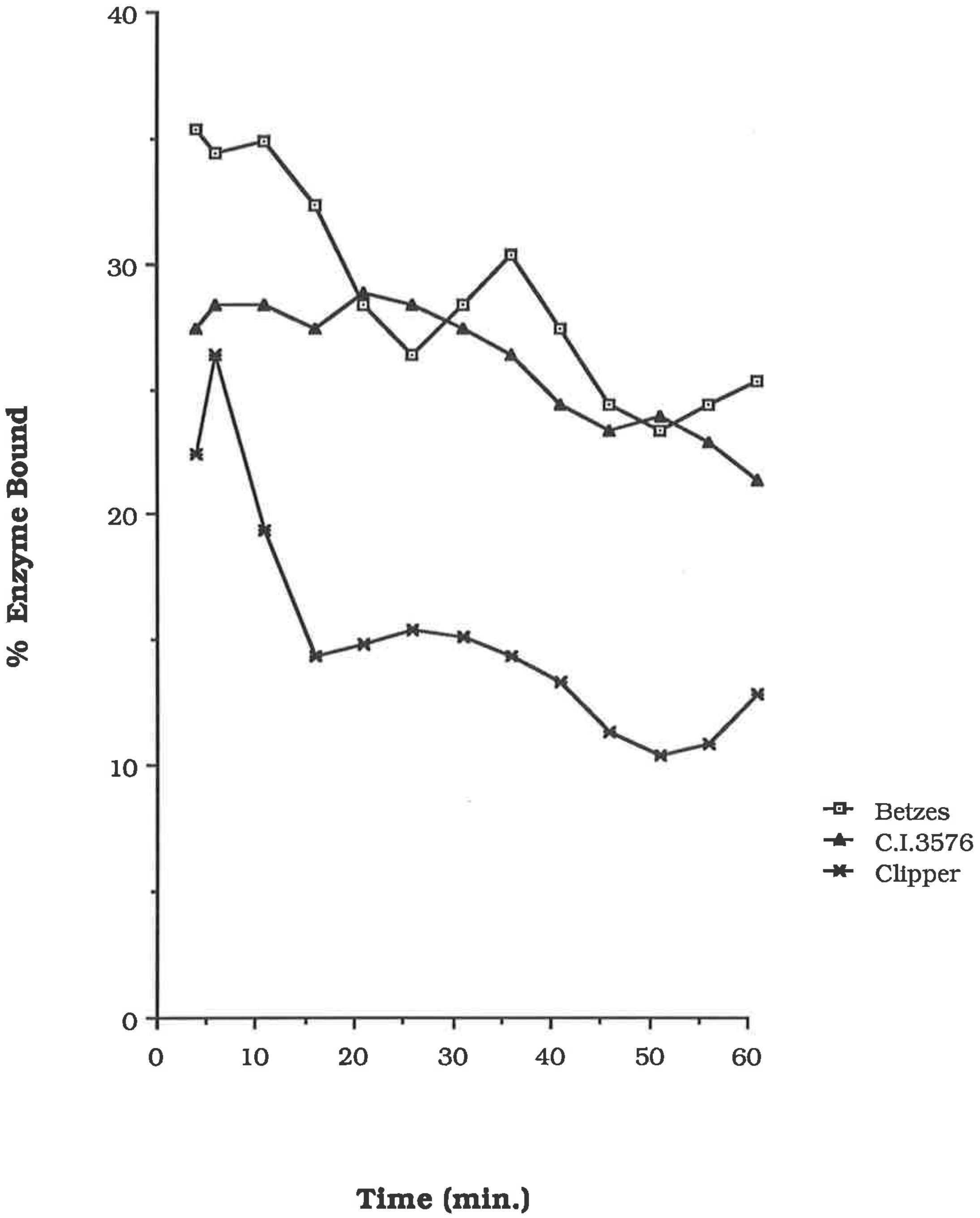

Fig.3 Amylase Bound(%) To Small Granules In The Presence Of Increasing [Lectin].

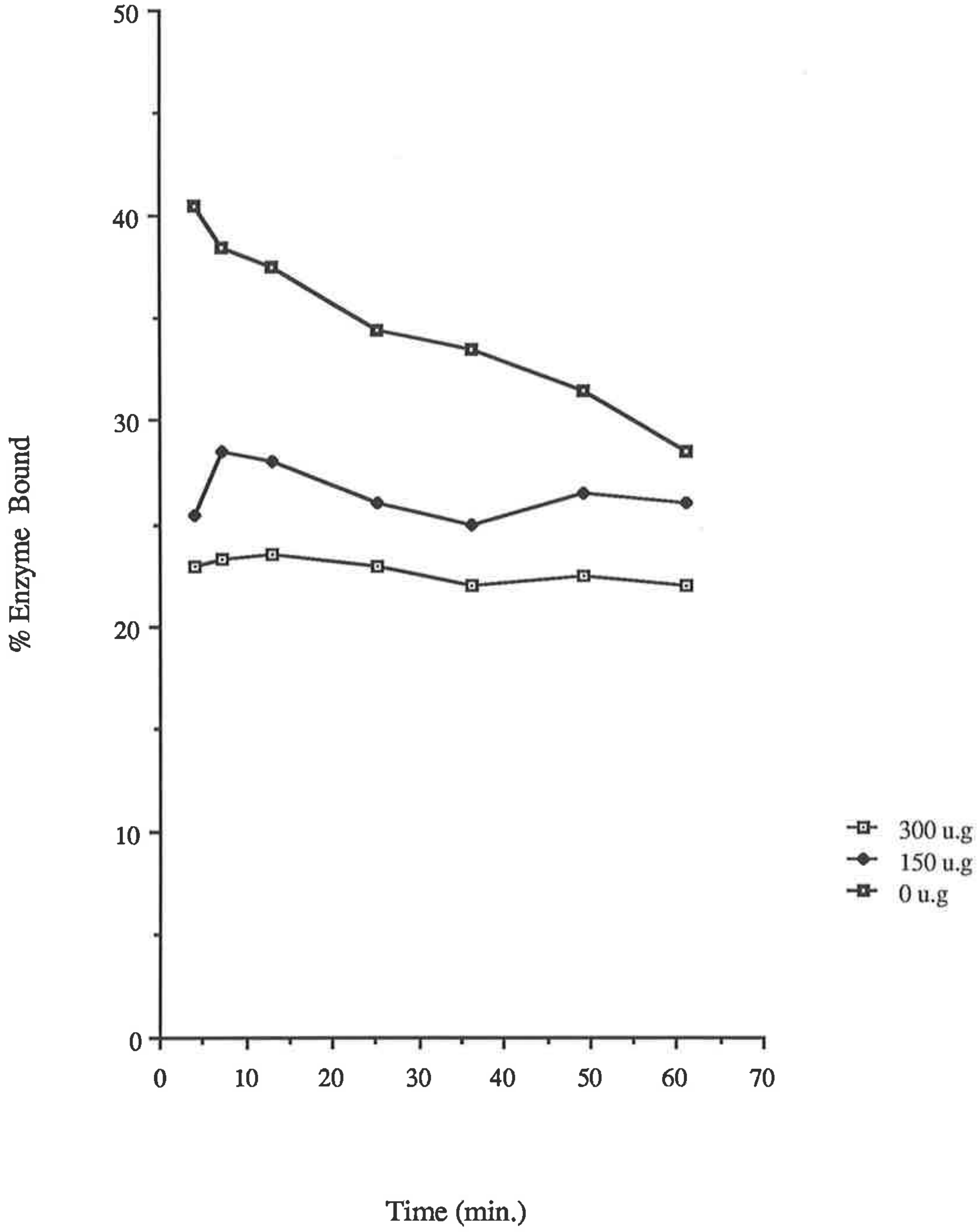

Fig.4 Enzyme Bound(%) To Small Granules In The Presence Of Increasing [Lectin].

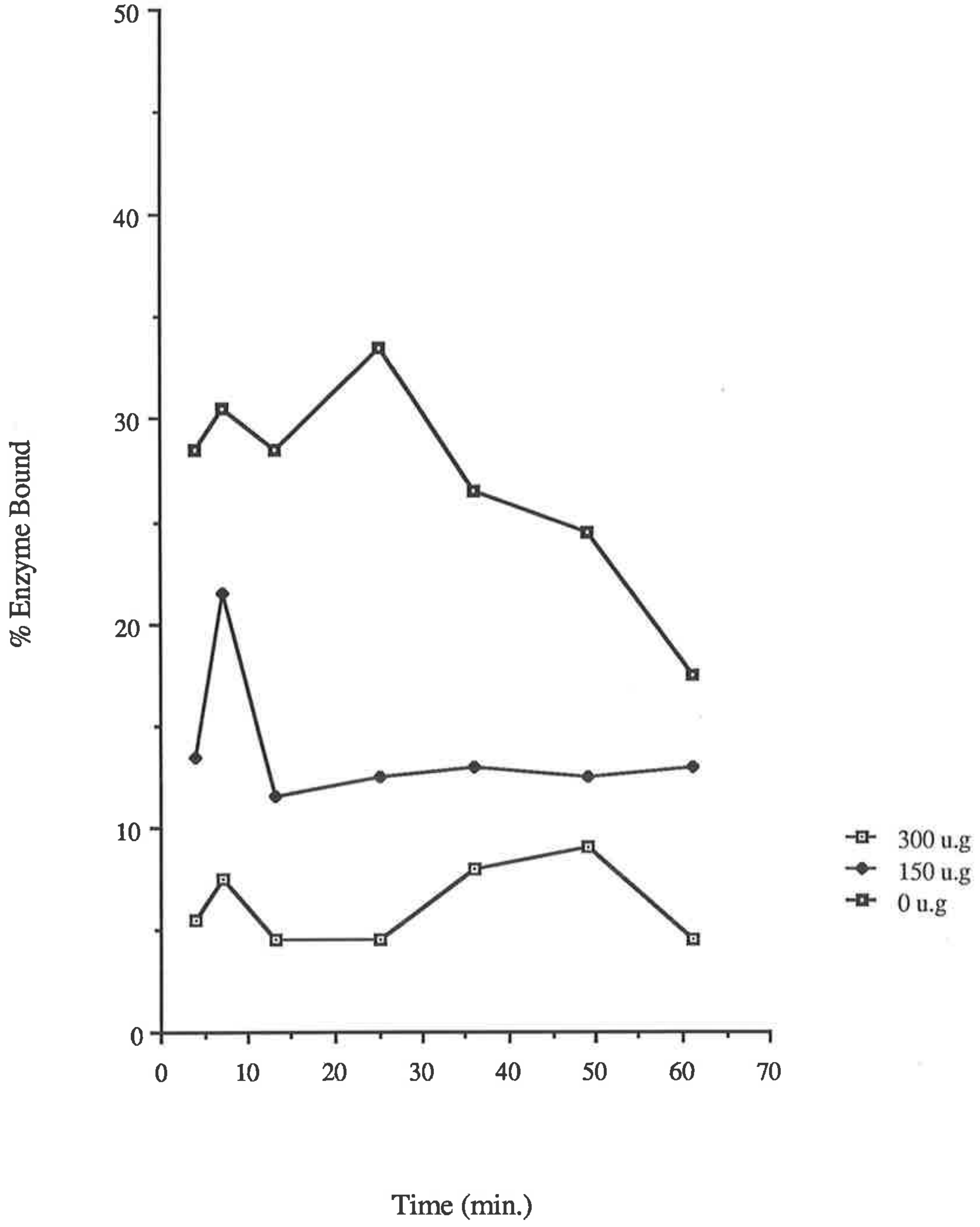

Fig.5 Inhibition Of Salivary Amylase By Increasing [Lectin] (large granules)

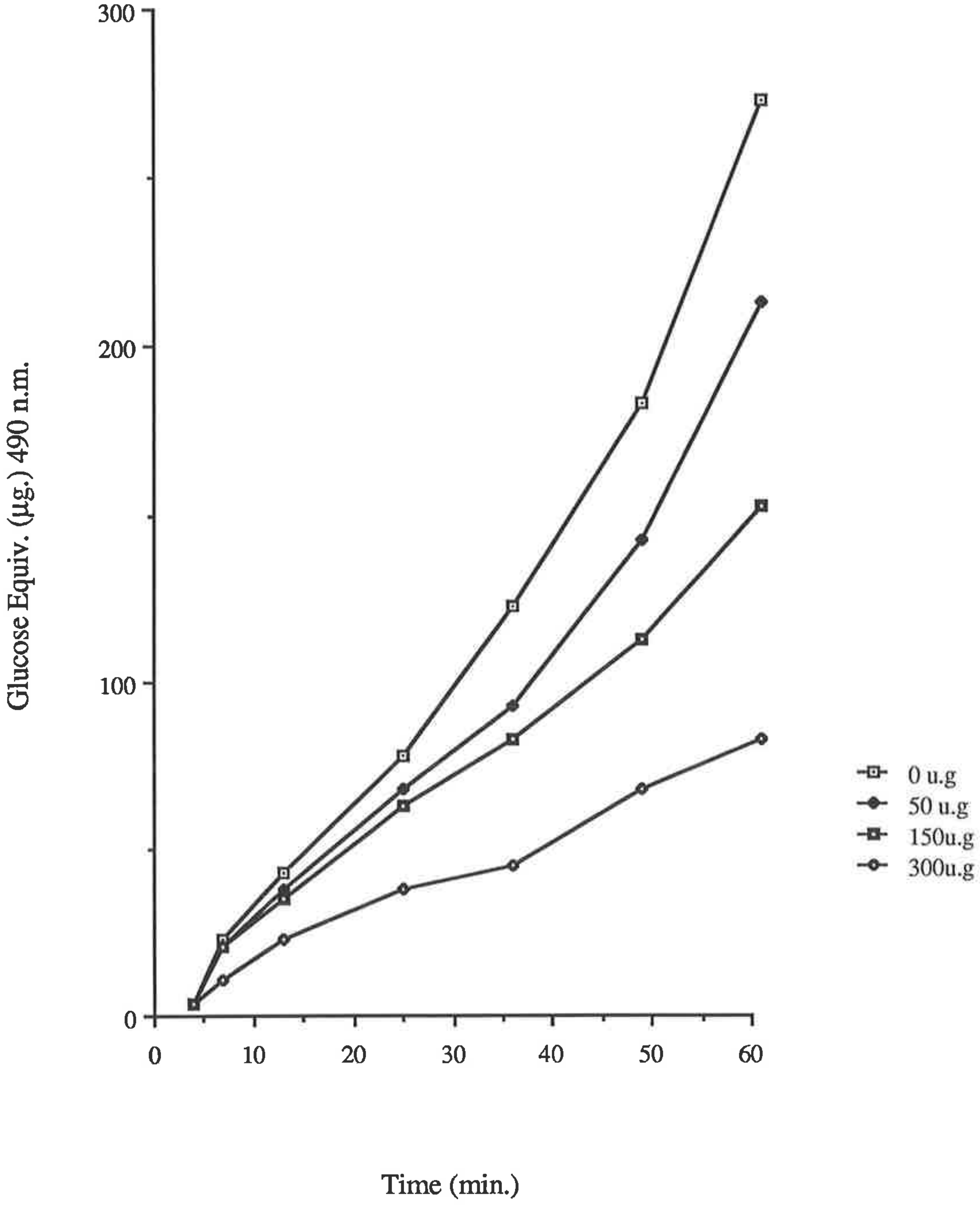

Fig.6 Inhibition Of Salivary Amylase By Increasing [Lectin] (Small Granules).

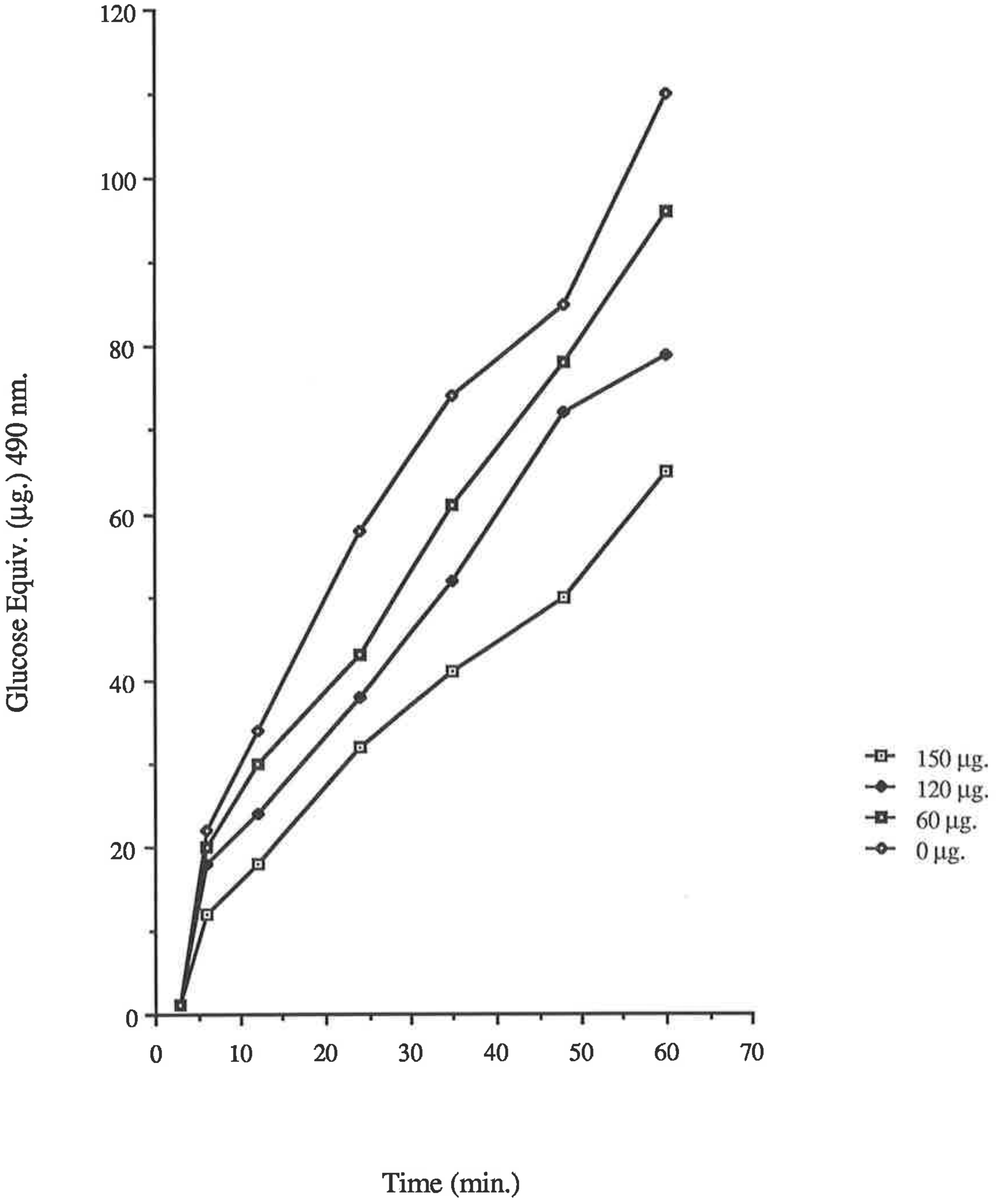

Fig.7 Theoretical [Lectin] Required For Complete Inhibition (Large Granules).

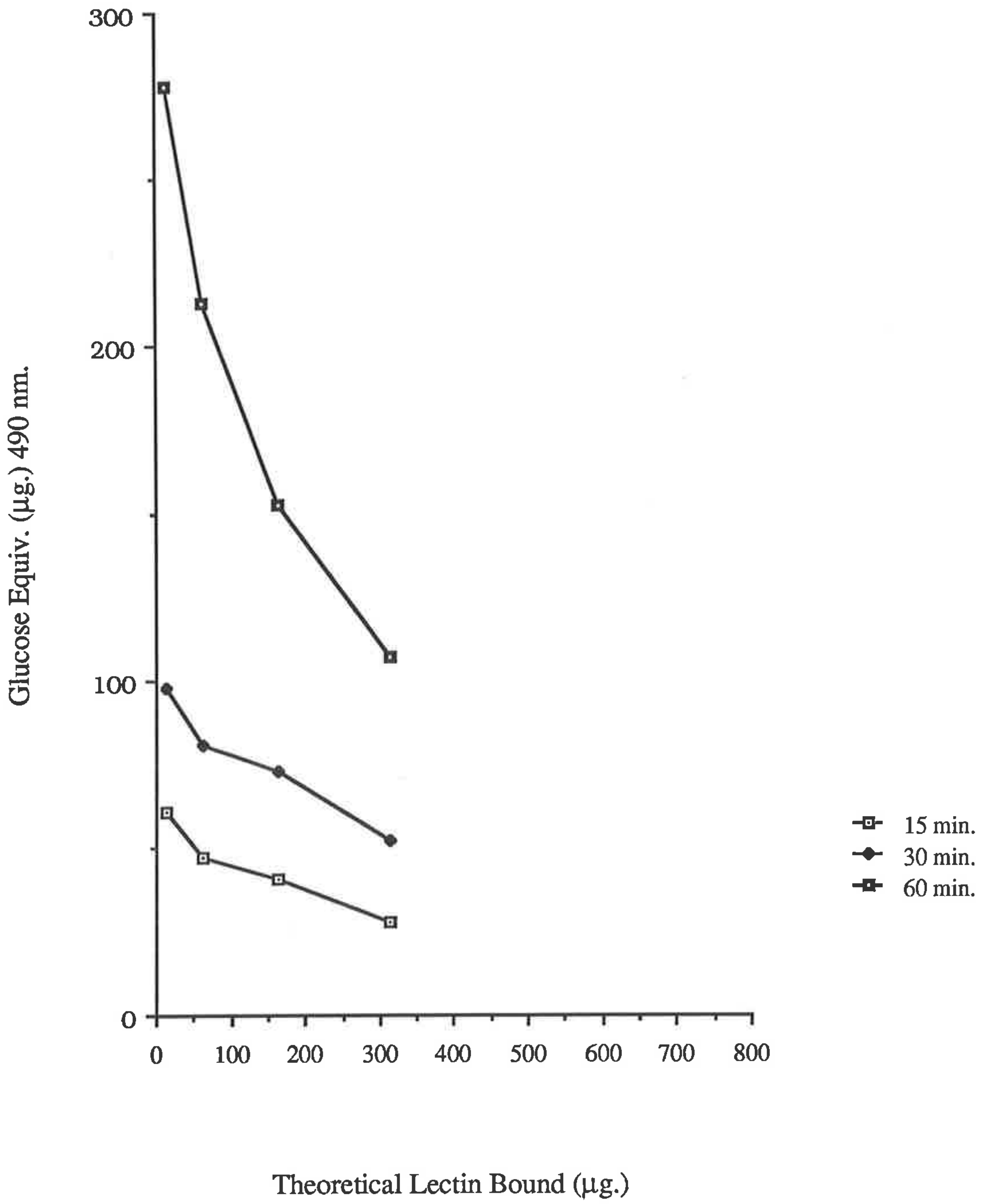

Fig.8 Theoretical [Lectin] Required For Complete Inhibition (Small Granules).