THE LECTIN NATURE AND OTHER CHARACTERISTICS OF α-GALACTOSIDASES FROM VICIA FABA SEEDS

by

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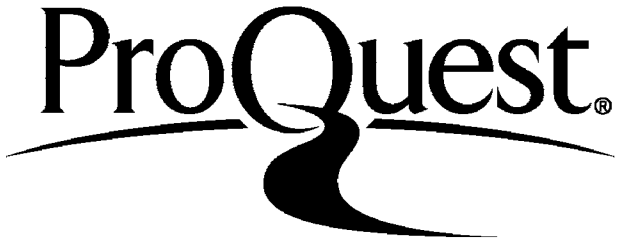

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ABSTRACT

Three forms of α-galactosidases (I, II^1 and II^2 with apparent molecular weights of 160,000, 45,000 and 43,400 respectively), which had been reported earlier were purified from mature Vicia faba seeds. The purification procedure was improved by the use of a substrate-affinity step. All three forms displayed catalytic and hemagglutinin activities. Various experimental results suggested that the enzyme preparations were free from favin (apparent molecular weight 50,000), a lectin which also occurs in V.faba seeds.

The sites for catalytic and lectin activities appeared to reside at separate loci in all forms of the enzyme, and the lectin activity was glucose/mannose specific.

The use of equilibrium dialysis and spectrophotometric technique indicated the presence of 8, 4 and 2 lectin binding sites in α-galactosidases I, II^1 and II^2 respectively, and 4 catalytic sites in α-galactosidase I.

All three forms of the enzyme were glycoproteins, and in the case of α-galactosidase I a constituent glycopeptide was isolated and investigation of its structure by fast atom bombardment-mass spectrometry suggested that an asparagine-glucosamine linkage was present and that α-galactosidase I was a 'mannose-rich' type glycoprotein.

α-Galactosidase I was found to be altered in structure upon incubation with endo-β-N-acetylglucosaminidase H (Endo H), which was possibly due to the removal of glycan chains.

The effects of a range of concentrations of urea and methyl-α-D-mannoside on α-galactosidase I were examined. Conversion of this high molecular weight tetramer into enzymically active low molecular weight forms, possibly trimers, dimers and monomers, was achieved. The three forms of the enzyme were separated into further active forms on isoelectric focusing and chromatofocusing.

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LIST OF ABBREVIATIONS

Asn	Asparagine
Con A	Concanavalin A
Endo H, CI, CII and D	Endo-β-N-acetylglycosaminidase H, CI, CII and D
EDTA	Ethylenediaminetetra-acetic acid
FAB-MS	Fast atom bombardment-mass spectrometry
Fuc	Fucose
GlcNAc	N-acetylglucosamine
Man	Mannose
MW	Molecular weight
PBS	Phosphate buffered saline
SDS	Sodium dodecyl sulphate
SDS-PAGE	Polyacrylamide gel electrophoresis in the presence of Sodium dodecyl sulphate
Temed	N,N,N',N'-tetramethylene diamine
EI, II, II^1 and II^2	α-Galactosidases I, II, II^1 and II^2
Forms I, II, II^1 and II^2	α- Galactosidases I, II, II^1 and II^2
Hex	Hexose unit

CHAPTER 1

INTRODUCTION

1.1. Occurrence

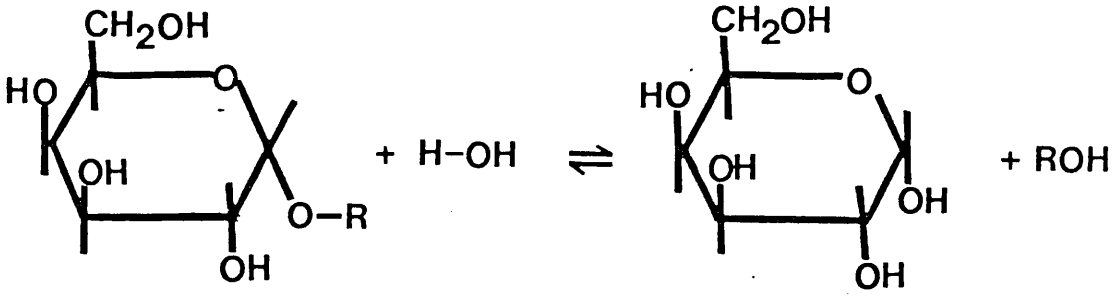
α-Galactosidases [α-D-galactosyl-galactohydrolases (EC 3.2.1.22)] occur widely in nature [1] and have been found in many species of plants, microorganisms and animals: seeds are a particularly rich source of the enzyme. However, highly purified and homogeneous preparations have been reported in relatively few cases.

Preparations with high specific activities have been obtained from the seeds of Prunus amygdalus (sweet almond) [2], Phaseolus vulgaris (kidney bean) [3], Vicia faba (broad bean) [4], Vicia cracca (vetch) [5] and Coffea sp. (coffee) [6]. Homogeneous preparations have been obtained by Harpaz et al. [7] from Glycine max (soybean) and from Vigna radiata (mung bean) by Hankins and Shannon [8]. Only one crystalline preparation of the enzyme has been reported and this was obtained from the fungus Mortierella vinacea [9].

α-Galactosidases from most organisms can be generally obtained by simple extraction with aqueous buffers. However, bound forms of the enzyme occur in chloroplast, mitochondrial and microsomal fractions from Spinacia oleracea (spinach) leaves [10], and there is evidence to suggest that α-galactosidase is bound to the cell walls in mature Cucurbita pepo (squash) leaves [11], Pisum sativum (pea) [12] and Vigna radiata hypocotyls [13,14]. There have also been reports that α-galactosidases are present in protein bodies [15] and leaf vacuoles [16].

1.2. Reactions of α-Galactosidases

α-Galactosidases hydrolyse the glycosidic bonds of compounds possessing non-reducing terminal α-D-galactopyranosyl residues. The reaction is reversible, although the equilibrium usually strongly favours hydrolysis:



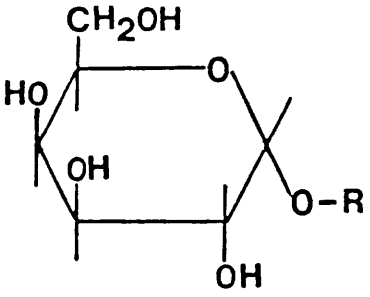
α-D-galactopyranoside D-galactose

With most α-galactosidases, R may represent alkyl, aryl, monoglycosyl or polyglycosyl residues.

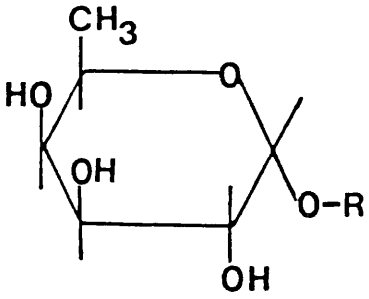
Glycosidases usually show a specificity that is relatively high with respect to the glycon moieties of their substrates, whilst large structural variations are permitted in the aglycon [17]. Changes in the configurations of hydrogen or hydroxyl groups on any single carbon atom of the glycon may reduce the rate of reaction or completely inhibit it. In the case of α-galactosidase, the glycon residue of the substrate must be in the pyranoid ring form and possess the α-D-galactosyl configuration on carbon atoms 1, 2, 3 and 4 in order that significant rates of hydrolysis may occur. Hence, most α-galactosidases hydrolyse α-D-fucopyranosides, β-L-arabinopyranosides and D-glycero-α-D-galactoheptoside (Fig.1), as well as α-D-galactopyranosides. Such

Figure 1

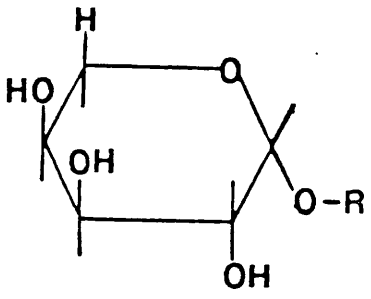
α-D-Galactopyranoside and related glycosides



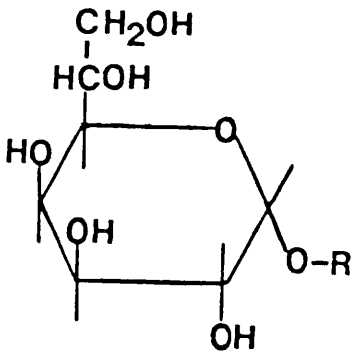
α-D-Galactopyranoside



α-D-Fucopyranoside



ß-L-Arabinopyranoside



D-Glycerol-α-D-galactoheptoside

is the case with the V.faba and P.amygdalus enzymes where the relative rates of hydrolysis are in the order: α-D-galactoside > α-D-fucoside > β-L-arabinoside: the substituent group at C-5 is, therefore, not of primary importance [1].

Wide limits of specificity exist for the aglycon moiety. α-Galactosidases from Calvatia cyanthiformis, P.amygdalus and V.faba, for example, are able to hydrolyse methyl, phenyl and naturally-occurring α-D-galactosides such as melibiose, raffinose and stachyose [1,18]. In general, aryl-α-D-galactosides are better substrates than alkyl derivatives or galactose-containing disaccharides [1,18-20]. The ease of hydrolysis of non-reducing terminal α-D-galactosyl residues of oligosaccharides normally decreases with increasing chain length [5,18, 21-24]. Several α-galactosidases hydrolyse galactomannans where single galactose residues are attached by α-1,6-linkages to a backbone of β-1,4-linked mannosyl residues [1].

Water can be replaced by a variety of organic galactose acceptors (alcohols, phenols and carbohydrates) and in this way alkyl and aryl-α-D-galactosides and oligosaccharides have been synthesized by transgalactosylation [1].

α-Galactosidases with both acidic and alkaline pH optima occur in plant tissues, however, most enzymes exhibit maximum activity between pH 4.0 and 6.0. In general, they exhibit a single broad pH optimum when assayed with, supposedly, natural substrates such as galactosyl-sucroses [1]. There are, however, examples of α-galactosidases which display two acidic pH optima using synthetic substrates (e.g. p-nitrophenyl-α-D-galactoside). These include the enzymes from Trigonella foenum-graecum (fenugreek) [25] and V.faba [21] seeds.

Only one example of an α-galactosidase with an alkaline (pH 7.5) pH optimum is recorded in the literature and this was detected in the mature leaves of Cucurbita pepo by Gaudrealt and Webb [26] (see also p. 30).

1.3. Multimolecular Forms

Since the first International Conference on Molecular Forms of Enzymes in 1961 [27] and as separation methods have improved, an ever-increasing number of enzymes have been reported to exist in more than one form. The term 'isoenzyme' was first introduced by Markert and Moller [28] to describe any category of enzyme heterogeneity without reference to the origin of diverse forms or the nature of the molecular differences between them. Current recommendations are, however, that only those proteins with very similar catalytic activities and with separate genetic origins should be classed as isoenzymes.

In some instances it is not clear whether all multiple forms detected in vitro are also present in vivo, or whether some are artefacts of purification procedures, but the readiness of some isolated enzymic forms to aggregate and dissociate or be modified by covalent changes suggest that, in the natural state, heterogeneity may not be as great as indicated by many in vitro studies [29].

Several glycosidases occur in multiple forms in higher plants, including N-acetylglucosaminidase from Canavalia ensiformis (jack bean) [30] and α-glucosidase from Beta maritima (sugar beet) [31], α-galactosidase and α-mannosidase from Dolichos biflorus (horsegram)

[32] and β-mannosidase from Ceratonia siliqua (carob), Gleditsia triacanthos (honey locust) and Medicago sativa L. (lucerne) [33].

The occurrence of multimolecular forms of α-galactosidase was first reported by Petek and Dong [34]. Two forms were obtained from the seeds of Coffea sp. by fractionation on alumina columns. The enzymes from this source could not be resolved by Sephadex gel filtration [35], which relies on molecular weight differences, but three active forms were separable by polyacrylamide gel electrophoresis (PAGE) at pH 8.3, indicating different charge properties [36].

Resting seeds, in general, appear to possess two forms of α-galactosidase which are separable by gel filtration. An examination of seeds from 20 different legume species for α-galactosidase activity showed that one higher molecular weight form representing most of the total activity (commonly referred to as I: MW range 120,000-190,000) was always present and the majority of species also contained a second low molecular weight enzyme (II; MW 30,000-40,000) [37]. The reported preponderence of high molecular weight α-galactosidases must, however, be assessed with the knowledge that association of low molecular weight forms could occur during isolation procedures (see Section 1.4).

Dey and co-workers [1,21,38] have examined the multiple forms of α-galactosidases in resting V.faba seeds and isolated three components, α-galactosidases I, II^1 and II^2. Enzyme I was separable from EII^1 and EII^2 by gel filtration and EII^1 and EII^2 were separable from each other by CM-cellulose chromatography. The specific activity of I was approximately ten times greater than II^1 or II^2 [39]. The molecular weight of EI, estimated by gel filtration, was 209,000 and

EII^1 and II^2 were both 38,000. Molecular weights determined by sedimentation equilibrium, however, gave values of 160,000 ± 2850; 45,730 ± 3073 and 43,390 ± 1409 for I, II^1 and II^2 respectively [40].

Dey and co-workers [39] reported that SDS-PAGE of EI and II^2 both yielded a major protein band (MW 41,300 ± 1200) together with two minor components with higher electrophoretic mobilities. The molecular weight data obtained by the two procedures suggested that EI was a tetramer of EII^2. Examination of EII^1 by SDS-PAGE confirmed the molecular weight value obtained by the sedimentation equilibrium method and showed that II^1 was a larger (MW 42,650 ± 950) protein than EII^2.

The relationships between the three α-galactosidases were also investigated by immunochemical methods using polyclonal antibodies prepared against all forms. These indicated a close structural similarity between EI and II^2 [39]. Other seed α-galactosidases from several legume species, e.g. Lens culinaris (lentil) appear to be composed of identical subunits with molecular weights of approximately 40,000 [41]. There have been no reports of the existence of two monomeric forms of α-galactosidases in any single higher plant species other than in the seeds of Vicia faba, however future examination of the low molecular weight α-galactosidase fractions from other plants may well reveal mixtures of components similar to EII^1 and II^2.

1.4. Interconversion

The patterns of multiple forms of plant glycosidases *in vivo* appear to vary with the physiological changes occurring in the tissues. For example, tomato (*Lycopersicon esculentum*) fruits in the early stages of development contain a high molecular weight form of polygalactouronase (PGI) and on ripening a second form of the enzyme (PGII) appears [42]. In the same fruit the levels of the three forms of ß-galactosidase also change with ripening [43]. During germination seeds of carob, honey locust and lucerne changes in the two forms of α-mannosidase have also been noted [33].

The factors which induce these changes and the resulting effects on tissue components are of great interest and considerable commercial importance in relation to fruit ripening, seed germination and seedling vigour. For example, in the tomato fruit changing patterns of polygalacturonases [42] and β-galactosidases [43] may control the ripening process and a full understanding of these relationships may lead to improved horticultural and storage practices.

Changes in the distribution of multiple forms of enzymes could arise via protein synthesis, complete or partial degradation of one or more forms or the association and dissociation of subunits [27,40,44].

Glycosidases which occur in monomeric and polymeric forms can often be made to associate and dissociate, respectively, *in vitro*, and these changes may mirror those occurring *in vivo*. For example, the formation of oligomeric enzymes in tissues could simply occur by monomer association in response to the synthesis of increased levels of monomers or a change in pH (see also, p. 29).

In the case of the α-galactosidases, those from V.radiata seeds can be reversibly interconverted by changing the pH. The tetrameric form is favoured at pH 4.0 and the monomer at pH 7.0 [45]. Similar changes occur with G.max α-galactosidases [46]. Monomeric (MW 40,000) α-galactosidase from Lens culinaris readily aggregates to an oligomer, I, (MW160,000) when the enzyme is concentrated. Incubation with 6 M urea and 2-mercaptoethanol is required, however, to dissociate this tetramer to monomeric (inactive) forms [41].

In the case of V.faba seed α-galactosidase, storage of partially purified form II (now known to be a mixture of monomers II^1 and II^2) at 4°C and pH 5.5 resulted in an increase in the specific activity of the preparation due to the aggregation of the monomers and the formation of the more active tetramer, I. It was claimed that this conversion did not occur if II had previously been passed through a gel filtration column [40,47].

Dey and co-workers [48] also reported that procedures used for the purification of V.faba α-galactosidase, e.g. citric acid and ammonium sulphate precipitations, enhanced aggregation of the monomers.

Interconversion of the multiple forms A (MW 123,000) and B (MW 21,400) of α-galactosidase from coconut (Cocos nucifera) kernel has been shown to occur in vitro and in this instance a high molecular weight enzymically inactive protein, C, appeared to facilitate the conversion of B to A [49,50].

1.5. Physiological Significance of α-Galactosidases

1.5.1. Hydrolysis of reserves

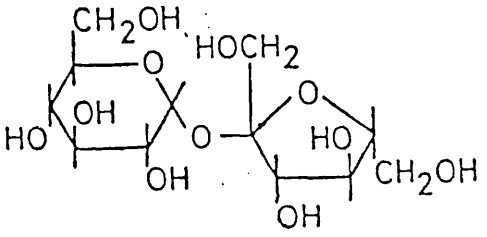
In the plant kingdom galactose-containing oligo- and polysaccharides and glycolipids are common tissue components [1,51-54]. The majority of plant oligosaccharides are derivatives of sucrose and appear to be synthesized by the transfer of galactopyranosyl, glucopyranosyl or fructofuranosyl residues to the disaccharide.

Raffinose (6-O-α-D-galactosylsucrose) is the first member of a series of homologous oligosaccharides, known as the 'raffinose family' (Fig.2a) where additional 1,6-linked α-D-galactopyranosyl residues are bonded to the galactosyl moiety of the trisaccharide [55]. Members of the series possess up to 7 galactose residues and the trivial names stachyose, verbascose and ajugose are used for the tetra-, penta- and hexa-saccharides, respectively. Isomers of the raffinose series, e.g. planteose (Fig.2b) and sesamose, have also been isolated from seeds, but these carbohydrates are less common than those of the 'raffinose family'. All of these oligosaccharides appear to be stored as 'energy reserves' and raffinose and stachyose are also found as mobile 'reserves' in the sieve-tube exudates of some plants (e.g. white ash, elm and linden) [56,57] and are believed to protect plants from frost damage in some instances (e.g. spinach) [58,59,60].

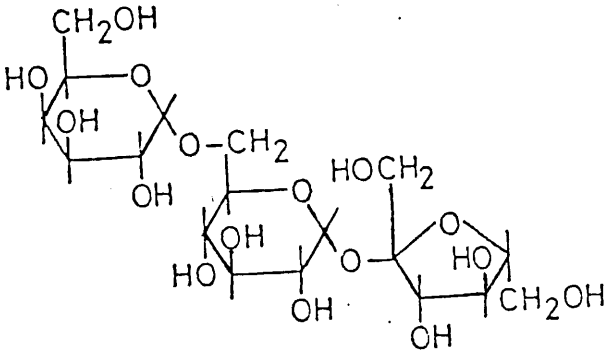
In addition to oligosaccharides, galactose-containing polysaccharides are also found as reserve carbohydrates in many leguminous plants [61,62]. Galactomannans, for example, occur in the endosperms of albuminous seeds of the Leguminosae family [63]. These polysaccharides

Fig. 2a The Raffinose family of oligosaccharides

Sucrose (0-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside).



Raffinose (0-α-D-galactopyranosyl-(1→6)-0-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside)



Stachyose (0-α-D-galactopyranosyl-(1→6)-0-α-D-galactopyranosyl-(1→6)-0-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside)

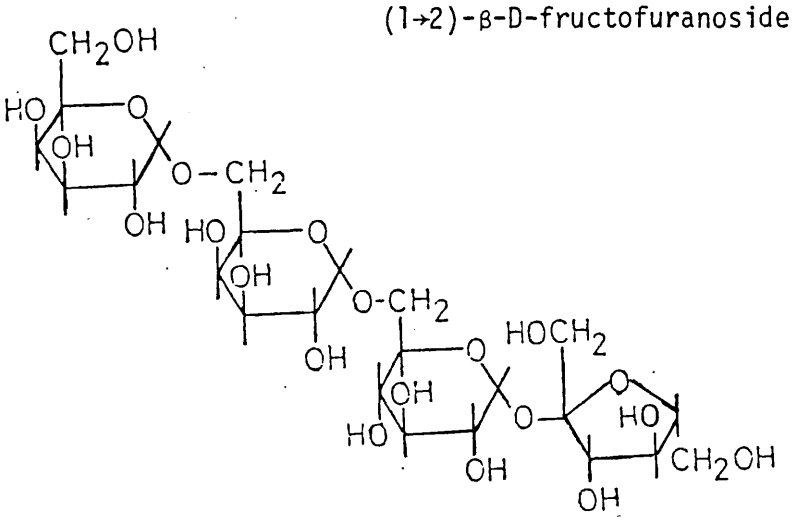


Fig. 2a (continued)...

Verbascose (O-α-D-galactopyranosyl-(1→6)-O-α-D-galactopyranosyl-(1→6)-O-α-D-galactopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→2)-β-D fructofuranoside).

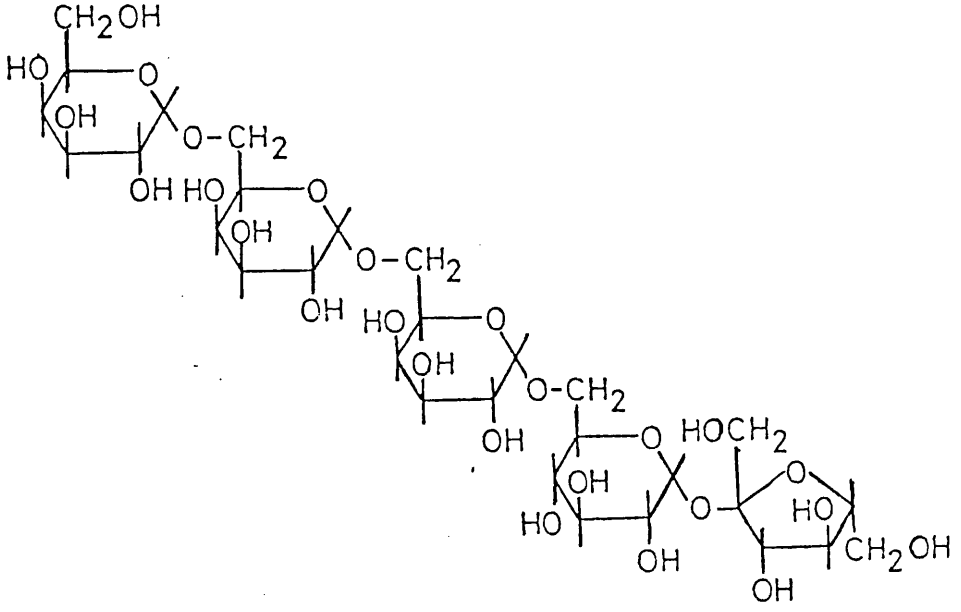
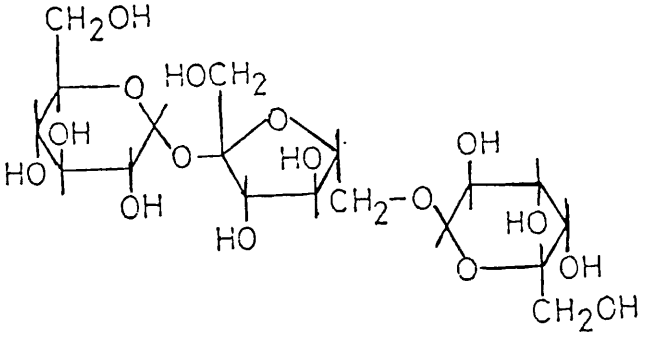


Fig. 2b

Planteose (O-α-D-galactopyranosyl-(1→6)-O-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside



are composed of a α-1,4-mannan backbone to which is attached varying numbers of α-D-galactopyranosyl residues of β-1,6-linkages: the mannose/galactose ratios are species-specific [64].

Seed galactomannans appear to have a double physiological function. Firstly, they retain water by solvation and thereby prevent complete dehydration which would cause protein denaturation, and in particular the denaturation of those enzymes essential for seed germination. In this connection the D-galactosyl side-branches of the polymer may be regarded as hydrophilic parts of the molecule. Secondly, galactomannans serve as energy reserves for germinating seeds [64].

The main function of the α-galactosidase in plants appears to be the release of galactose from the storage oligo- and poly-saccharides [55, 59, 60, 64, 65], which is the first step in their utilization as a source of energy and a supply of components for plant growth [64]. Some α-galactosidase activity appears to be present in all mature seeds [1,66-72].

McCleary and Matheson [68] have examined α-galactosidase activity and galactomannan and galactosyl sucrose depletion in germinating albuminous legume seeds. With lucerne, guar (Cyamopsis tetragonolobus) and carob, raffinose and its homologues are mobilized at an early stage of germination and the degradation of the galactomannans follows. The α-galactosidases from these seeds are separable into multiple forms, A, B and C, by ion-exchange chromatography. One of the forms (C) can be further resolved, into C_1 and C_2 by molecular sieving. The multiple forms exhibit a degree of tissue specificity.

α-Galactosidases A and B are located in the cotyledon-embryo region of the seeds, whereas form C is restricted to the endosperms of lucerne and guar seeds: in the case of carob this enzyme occurs in both the endosperm and cotyledon-embryo regions.

On germination, the changes in the levels of the activities of the multiple forms vary with the plant species, but overall the total enzyme activity increases [1,66-70]. α-Galactosidase A in lucerne, carob and soybean, which is present before germination, shows a slight increase in activity followed by a decrease, whereas this activity in guar seeds decreases gradually during germination. The activity of the endosperm α-galactosidase (C) increases during the germination of all four legume seeds with the least change occurring in soybean.

These changes in activities have been related to mobilization of oligo- and poly-saccharide reserves. Lucerne, carob and guar seeds contain significant amounts of galactomannan (8,19,20 mg/20 g seeds respectively) and the major contribution to the total increase of α-galactosidase activity on germination is due to enzyme C. As C is located in the endosperm where all the galactomannan is found, this suggests that this molecular form is primarily involved in galactomannan hydrolysis. The limited increase in the activity of C in soybean can be equated to the low galactomannan content of this seed. Galactosyl-sucrose oligosaccharides are also found in endosperms, hence C must also be involved in their degradation.

The presence of α-galactosidases A and B in the cotyledon-embryo region of soybean suggests that these enzymes are responsible for the hydrolysis of raffinose and homologous oligosaccharides which are

present in this tissue: the changes in the levels of activities of A and B parallel the depletion of these oligosaccharides.

Reid [63] studied the metabolism of galactomannan and galactose-containing oligosaccharides in fenugreek seeds. Working with seeds in first phase of germination (24 hr after imbibition), he showed that the level of galactomannan remained constant in the endosperm and that the level of stachyose and verbascose decreased. The depletion of stored galactomannan occurred in the second phase (48 hr after imbibition): both the oligo- and poly-saccharides completely disappeared from the endosperm at the end of this phase. α-Galactosidase activity was measured separately in the embryo and the endosperm tissues at different stages of germination [66]. In the early stages very low levels of α-galactosidase were detectable in the endosperm. The activity increased significantly in later stages and Reid associated this with galactomannan breakdown. Extracts of both resting and germinating embryos, on the other hand, contained high α-galactosidase activity which showed little change throughout germination. This activity is believed to be associated with the mobilization of oligosaccharide reserves in the embryo which contains no galactomannan.

Analysis of extracts of Cucurbita pepo seeds revealed changes in the levels of three forms, LI, LII and LIII, of the enzyme [73]. In dry seeds LIII was predominant, but during germination the levels of LI and LII increased. This change was accompanied by an increase in the total enzyme activity and a rapid depletion of ajugose, verbascose, stachyose and raffinose.

Studies have also been made with exalbuminous seeds which show different patterns of α-galactosidase activity from those with endosperms. Reserves of oligosaccharides such as raffinose and stachyose, have been observed to accumulate during maturation of such seeds from several species of legumes. This accumulation is paralleled by an increase in total α-galactosidase activity [1]. On germination the oligosaccharides are utilized in the very early stages and would appear to constitute an important soluble reserve for the developing seedling.

Vicia faba seeds synthesize monomeric form(s) of α-galactosidase during maturation and, when fully mature, possess the tetrameric form of the enzyme with high specific activity [39,40,44]. At maturity the seeds contain the highest levels of α-galactosidase I and the highest total α-galactosidase activity found in any stage of seed/seedling development. Hence, the mature seed is in suitable condition to effect the rapid hydrolysis of oligosaccharide reserves which occurs during early germination. Following germination, the total α-galactosidase activity declines, form I of the enzyme disappears and the monomer(s) activity increases. This situation is, therefore, different from that of albuminous seeds where an increase in total α-galactosidase activity occurs during seed germination.

These studies with Vicia faba were made with intact seeds and the changes observed are likely to be more complex and to differ in embryo and cotyledon tissues, as shown in the case of Pisum seeds [74].

The cotyledons of wild Pisum elatius and cultured P.sativum (peas) in the resting (mature) state possess relatively high levels of α-galactosidase activity which do not change during the first 24 hr of

germination, after which activity decreases. However, in the embryos where the activity is again initially high the activity rises between 24 and 48 h germination. This rise may relate to the high metabolic activity of the embryo region of the seed and the special requirements of the seedling.

α-Galactosidases and their substrates in plant leaves have received limited attention. Thomas and Webb [73] reported that the immature, galactosyl-sucrose importing leaves of Cucurbita pepo can rapidly hydrolyse stachyose and verbascose, which enter the tissues from the phloem. These tissues cannot synthesize or store oligosaccharides. Mature, exporting leaves are responsible for their synthesis, but here no hydrolysis is detectable even if the leaf petioles are freeze-blocked [75]. In this case the oligosaccharides continue to accumulate despite the fact that the mature leaf tissues contain comparatively high levels of acid α-galactosidase activity [75-77].

Three forms of acid α-galactosidases (LI, LII and LIII) with distinct properties were detected in the leaves of Cucurbita pepo [78]. They varied in their susceptibility towards metal inhibition and in their K_m values for the hydrolysis of PNPG and raffinose and they exhibited different pH optima.

The more recent discovery of alkaline α-galactosidaseLIV in Cucurbita pepo [26] raises the possibility that the distribution of this enzyme might be responsible for the different patterns of utilization of galactosyl-sucrose by mature and immature leaves. The high specificity of LIV for stachyose, the major sugar translocated in C.pepo, and the fact that LIV was observed to undergo a 6-fold decrease

(cf. the constant total acid α-galactosidase activity) in activity over the developmental period during which immature importing leaves become nett exporting organs, supports this hypothesis.

It is clear that α-galactosidases are responsible for the hydrolysis of galactose-containing reserves in plant tissues, but little is known about the regulation of activity of these enzymes. From the previous discussion it is probable that protein synthesis, and possibly degradation, are controlling factors. With maturing seeds, for example, the synthesis of α-galactosidase is presumably a primary mechanism which prepares the seed for germination [40].

In the case of lettuce (Lactuca sativa L.) seeds, protein synthesis has been shown to increase levels of α-galactosidase during imbibition and in this special case, light also appears to be a controlling factor [79]. Activity levels remain approximately constant when the seeds imbibe in the dark, but following a short stimulus with red light (5 sec pulses cumulative to 2 min with intervening periods of darkness), α-galactosidase activity increases rapidly and this can be prevented with cycloheximide or by removing the seed axis. Gibberellic acid also serves as a stimulus in the dark [80,81].

These observations have been interpreted to mean that red light causes the release of a diffusable factor (gibberellic acid?) from the axis, which in turn stimulates the synthesis of α-galactosidase in the cotyledons and endosperm. In this case α-galactosidase synthesis may be an important factor in the breaking of the dormancy of the seed [81].

In maturing exalbuminous seeds, for example Vicia faba [40] where α-galactosidase activity and galactosyl-sucrose reserves increase

in parallel, there is a need to postulate that enzyme and substrate do not interact, otherwise the oligosaccharides would not accumulate. Similarly, acid α-galactosidase in mature stachyose exporting leaves of Cucurbita pepo must be prevented in some way from hydrolysing the export product [11]. In both situations α-galactosidase could be inhibited or compartmentalized [1]. No information regarding inhibition in vivo is available but there is evidence that α-galactosidase may be physically prevented from binding with potential substrates.

It is believed that the acid α-galactosidases in mature Cucurbita pepo leaves are sited in the apoplast, perhaps attached to the cell wall or the outer plasmalemma [11]. Cell wall-bound α-galactosidase has also been reported to occur in P.sativum epicotyls [12] and V.radiata hypocotyls [13]. As previously mentioned (p.14), α-galactosidases have also been located in leaf vacuoles and seed cotyledon protein bodies. This latter location is not unexpected, as protein bodies are thought to be derived from vacuoles [82,83].

If bound forms of α-galactosidases exist, what is the detailed nature of the binding? In the case of the cell wall, ionic and/or hydrogen bonds may link the enzyme protein with the wall polysaccharides or glycoproteins. Covalent bonding would not appear to be important, as much of the bound activity can be extracted under relatively mild conditions, for example using high salt concentrations. In the case of enzyme in vacuoles and protein bodies, it may form part of the lumen contents or be associated with the membranes of these organelles. Hydrophobic forces may hold α-galactosidases in an internal membrane location or the enzyme could be more loosely bound to the membrane surface via ionic and/or hydrogen bonds.

A further and novel possibility for α-galactosidase binding is that lectin interactions are involved. This suggestion is supported by the fact that lectins (see p.34) have been shown to occur in locations similar to those where α-galactosidase is reported to be bound [8,84-90]. In addition, plant α-galactosidases appear to be mannose-containing glycoproteins [39,41] and some α-galactosidases also possess haemagglutinin (lectin) activity [8,39,46] (see pp.39 and 43).

Several models for α-galactosidase binding, based on lectin interactions can, therefore, be proposed:

1. α-Galactosidase may be bound via its glycoprotein glycan chains to lectins present in cell membranes or associated with the cell wall;
2. The lectin sites of α-galactosidase may bind to the glycan chains of glycoproteins embedded in membranes or cell walls or to the wall polysaccharides themselves.

1.5.2. Lectins

These possible modes of binding of α-galactosidase, therefore, warrant a brief consideration of 'classical' (non-enzymic) plant lectins, and those enzymes which have lectin-like properties.

(i) General

Lectins were defined by Goldstein *et al.* [91] as "sugar-binding proteins or glycoproteins of non-immune origin which agglutinate cells and precipitate glycoconjugates". More recently Kocourek and Horejsi

[92] modified this definition by making the additional comment that they are "devoid of enzymic activity towards sugars to which they bind and do not require free glycosidic hydroxyl groups on these sugars for their binding" [see also ref.91].

The presence in plant tissues of proteins with the ability to cause agglutination of red blood cells has been known since 1888 [93]. Because of this property, they were originally called 'haemagglutinins' and, as they were first extracted from plants they became known as 'phytohaemagglutinins'. The term 'lectin' has generally replaced 'haemagglutinin' and 'phytohaemagglutinin', however, not all lectins can agglutinate cells (e.g. abrin and ricin [94],.see p.35). There are several reviews of the early history of these proteins.[95,96]. Goldstein and Hayes [97], Lis and Sharon [98], Kauss [99] and Barondes [100] have also recently published general reviews of the subject.

To date over 50 plant lectins have been isolated and highly purified forms have been obtained. Amongst the best characterized lectins are concanavalin A (from jack bean (Canavalia ensiformis)) [101], soybean agglutinin (from Glycine max) [102], wheat germ lectin (from Triticum vulgaris) [103] and potato (Solanum tuberosum) lectin [104].

Lectins are widely distributed in nature. The seeds of plants, especially legumes, are a particularly rich source, but they are also present in other plant tissues such as roots, leaves, tubers, fruits and phloem exudates [97,98,105-108]. Lectins occur not only as soluble cytoplasmic constituents, but also as components of plant cell wall [109],

membranes [110], vacuoles and protein bodies [8,84-89]. They are not exclusively found in plants, but have been found in animal tissues [111,112] and in microorganisms [111].

(ii) Detection and carbohydrate specificities of lectins

Lectins with multiple carbohydrate-binding sites (multivalent) are normally detected by their ability to agglutinate erythrocytes and further by demonstrating that the agglutination can be inhibited by simple or complex carbohydrates and their derivatives [113,114]. Lectins such as ricin, with single carbohydrate-binding sites, are unable to agglutinate cells [94,115].

The activity of multivalent lectins is normally measured in haemagglutinin (HA) units: one unit is the amount that produces 50% agglutination of red blood cells under standard conditions. The agglutination is normally estimated by visual means or, sometimes, by the use of a spectrophotometer. Errors of ± 20% are possible with the visual determination: the spectrophotometric technique is more accurate but it is laborious and requires relatively large amounts of lectin. An alternative procedure for the assay of multivalent lectins involves complex formation with specific polysaccharides or substituted proteins bearing several specific carbohydrate residues/molecule followed by turbidity measurements [97]. This type of interaction is concentration and temperature dependent. When all of the lectin sites are occupied by the binding component maximum turbidity occurs. If there is a further increase in the concentration of the binding material, then the precipitate dissolves. This behaviour can be compared to antibody-antigen systems [116].

The specificity of a lectin is usually defined in terms of monosaccharide(s) or simple oligosaccharides that inhibit lectin-induced haemagglutination (or glycan precipitation) reactions. This is referred to as the hapten-inhibition technique which was originally developed by Landsteiner to determine antibody specificity [117].

Using this procedure all legume lectins, for example, have been found to fall into two main categories:

(i) Those specific for D-galactose (e.g. Ricinus communis) and its derivatives, and

(ii) Those specific for D-glucose and D-mannose (e.g. P.sativum) and their derivatives [118].

The configuration of the hydroxyl group on C-4 is thus an important factor in specificity.

In general, many lectins have been found to tolerate variation of configuration at C-2 of the glycosyl residues for which they exhibit primary specificity. Concanavalin A (Con A) and the lectins from L.culinaris and V.faba (favin) exhibit primary specificity for D-mannose but they will also bind D-glucose and to a lesser extent N-acetylglucosamine [119-121]. Little variation at C-3 is usually tolerated by lectins [121]. Configurations at the anomeric carbons of the binding glycosyl residues are also often important in lectin interactions. Some lectins such as Con A [101] and Bandeiraea simplicifolia [122] and Lotus tetragonolobus (asparagus pea) [102] lectins exhibit pronounced anomeric specificity prefering α-D-configurations to the corresponding β-D-structures; whereas other lectins, such as those from soybean [123]

and castor bean (Ricinus communis) [124], appear to be indifferent to configurational changes at C-1.

Many lectins have been found to interact mainly with the non-reducing, terminal residues of glycan chains. Con A, however, binds with internal 2-O-α-D-mannopyranosyl residues in glycoproteins, as well as the terminal units. D-Mannose-specific lectins such as favin and those from P.sativum and L.culinaris seeds, have been reported to interact with the reducing D-mannose residue of 2-O-β-N-acetylglucosaminyl-D-mannose [97] (cf. Kocourek and Horejsi's definition, p. 33).

For some lectins no efficient monosaccharide inhibitors of haemagglutination have been found; only oligosaccharides are effective. Potato lectin, for example, is inhibited by β-1,4-oligomers of N-acetyl-glucosamine and here, an extended carbohydrate-binding site is proposed which preferentially binds to the pentasaccharide of the series. With decreasing chain length binding becomes weaker [104]. The lectin from Datura stramonium is also inhibited by oligomers of N-acetylglucosamine but not by the monosaccharide itself [125,126]. A considerable number of lectins have been found which only interact with glycosyl residues in complex structures such as glycoproteins [127-129].

Seeds from some legume species commonly contain two lectins with specificities directed towards the same sugar(s). However, Vicia cracca seeds contain two lectins, one specific for galactosamine and the other for glucose [130].

An examination of amino acid composition, molecular size, and other molecular properties of many lectins, has shown that they have

very little in common except that they are all proteinaceous [97,98]. Molecular weights vary from 36,000 for wheat germ lectin [131] to 265,000 for the lima bean lectin [132], and lectins with 2, 4 and 6 subunits have been reported [133]. Most lectins are oligomeric, but not all have identical subunits [97].

The overall amino acid composition of lectins reflects that of many other types of plant protein. They are usually rich in aspartic acid, serine and threonine and low in sulphur-containing amino acids. Some lectins, such as wheat germ lectin and potato lectin however, contain high levels of cystine. The lectin from potato tuber is rich in hydroxyproline which is characteristic of only a limited number of plant proteins [104,115].

Information regarding the primary amino acid sequences of lectins is accumulating. The most interesting feature appears to be that lectins isolated from leguminous plants generally possess extensive homology proximal to their N-terminal residues. This probably explains why lectins exhibiting different binding specificities often exhibit immunological cross-reactivity [118,134].

Most lectins are haemagglutinins and, therefore, have more than one carbohydrate-binding site per molecule. It is possible to determine the number of binding sites by fluorescence quenching or equilibrium dialysis procedures [135].

Many plant lectins are also glycoproteins with carbohydrate contents as high as 50% (e.g. potato lectin) [104], but some, such as Con A and peanut (Arachis hypogea) agglutinin, are devoid of any covalently bound sugar [136].

Many lectins have been shown to contain divalent metal ions, mainly Ca^{2+} and Mn^{2+}, which are required for activity. Detailed information on the role of metal ions and their spatial orientation is available for Con A [97], which is a tetramer of identical protomers (MW 25,500), and binds one Ca^{2+} and one Mn^{2+} ion per molecule [136].

(iv) Lectins with catalytic activity

According to the definition of Koucorek and Horejsi [92], lectins should be devoid of catalytic activity towards the carbohydrate residues they bind: however a number of glycosidases have been reported to agglutinate red blood cells. A tetrameric (MW 160,000) phytohaemagglutinin from V.radiata, for example, has been purified and has been found to possess high α-galactosidase activity [8,137]. Evidence that the haemagglutinin and catalytic activities both reside on a single protein species was derived from purification studies where both activities were observed to co-purify at all stages. A monomeric (MW 40,000) form of α-galactosidase isolated from the same seed possessed no haemagglutinin activity [8].

The V.radiata 'α-galactosidase-haemagglutinin' was reported to display two unique characteristics. Firstly, the lectin activity was inhibited by xylose and inositol, in addition to galactose, thus exhibiting a specificity distinct from all known 'classical' lectins. (The same carbohydrates also inhibited the α-galactosidase activity). Secondly, the enzyme displayed post-agglutination 'clot-dissolving' activity. The resulting disaggregated erythrocytes were thought to be permanently altered since they were no longer agglutinable by the

V.radiata bean extract, although re-precipitation could be effected by some other legume lectins of unreported specificity [8,138]. Both the 'clot-dissolving' and the catalytic activities of the V.radiata α-galactosidase-haemagglutinin exhibited the same pH optimum between 6 and 7 [8].

Hankins and co-workers [137] have shown that four other legume seeds (Pueraria thunbergiana, Thermopsis caroliniana, Lupinus arboreus and Phaseolus limensis) possess α-galactosidase-haemagglutinins which are immunologically similar, both to one another and to the enzyme isolated from V.radiata, and possess similar specificities and kinetic properties.

In addition to the legumes discussed above, an α-galactosidase with haemagglutinin activity was also isolated from Glycine max by Del Campillo and Shannon [46]. This enzyme was shown to be comparable to the V.radiata α-galactosidase-haemagglutinin [8]. Only the tetrameric form of the enzyme exhibited lectin activity, the catalytic and lectin activities were reported to co-elute from a Sephacryl S-200 gel filtration column, and both activities were inhibited by the same spectrum of sugars.

In the case of Glycine max and P.limensis seeds, 'classical', N-acetylgalactosamine-specific lectins accompany the α-galactosidase-haemagglutinins: the two activities in both seeds are readily separable by normal purification procedures [7,46,137].

Many legume seeds contain α-galactosidases which are apparently devoid of haemagglutinin activity but appear to be structurally similar (as shown by immunological techniques) to the α-galactosidase-haemagglutinins [37]. α-Galactosidase-haemagglutins are discussed further in the Results and Discussion section (p.134).

Two other enzymes appear to possess haemagglutinin activities. Hill and Hanke [139] have shown that the Cl^--stimulated ATPase from Limonium vulgare binds to galactosamine-Sepharose and can be eluted with galactose, and α-mannosidase from P.vulgaris seeds exhibits mitogenic activity, a property commonly associated with lectins [140].

(v) Biological activities of lectins

Lectins exhibit a number of interesting biological activities. These have been reviewed by Lis and Sharon [98]. In addition to agglutinating animals cells, lectins have also been observed to clump plant cells [141].

Some lectins are mitogenic and, for example, stimulate the transformation of small resting lymphocytes to large blast-like cells which may undergo mitotic division [94,142]. The apparent restriction of certain mitogenic lectins to seeds has led to the suggestion that they might be involved in differentiation and developmental processes in the seed embryo by controlling the rates of cell division [143]. There is, however, no evidence to indicate that lectins which are mitogenic against animal cells also exhibit this activity against plant cells [115].

The mitogenic action of lectins is a result of binding to cell surface receptors, presumably glycoproteins, which in turn stimulates cell metabolism [144]. Bowles has discussed the possibility that lectins as membrane components may be involved in the regulation of various cellular events including the modulation of enzyme activity [145]. She refers, for example, to Limonium vulgare ATPase, an apparent

enzyme-lectin, the catalytic activity of which can be increased by N-acetylgalactosamine and galactose [see also ref.139].

In animal cells, lectins have been observed to stimulate various specific functions including the secretion of insulin by pancreatic islet cells and the oxidation of glucose by adipocytes [146]. Studies with protein reagents using resealed erythrocytes ghosts indicate that Ricinus lectin binds to the outer membrane surface and renders the proteins on the inner surface chemically more reactive [147]. This is, perhaps, a demonstration of mechanism whereby lectins could modify intracellular reactions by binding to the outside of the plasmalemma. It remains to be seen whether endogenous plant lectins are associated with regulation of cellular metabolism.

Lectins appear to play a role in the relationships between plants and other organisms. For example, an antiparasitic function has been demonstrated in the case of wheat germ lectin. Mirelman and co-workers [148] and Barkai-Golan and co-workers [149] have observed that this lectin, which is N-acetylgalactosamine-specific, inhibits the growth of some chitinous fungi: chitin is a polymer of N-acetyl-galactosamine. It has, therefore, been proposed that wheat germ lectin protects wheat seed against fungi during imbibition and germination and early seedling growth.

In the case of plant/animal interactions, a protective function of an endogenous seed lectin against beetle infestation of P.vulgaris has been claimed. Bruchid beetles are killed when feeding on black beans (P.vulgaris), but not by cowpeas (V.unguiculata) which are lectin-free [150].

Lectins are involved in recognition interactions. Specific complementary molecules appear to play an important role in the recognition between pollen grains and stigma surfaces in higher plants. The lectin Con A binds to wall-bound carbohydrate-containing materials, probably glycoproteins, on the pollen grain surface and it is believed that the stigma surface may contain specific lectin-like components which are responsible for initiating acceptance or rejection of pollen grains and, hence, controlling fertilization [115].

Recent evidence also suggests that similar mechanisms may play an important part in determining host-symbiant specificity. For instance, lectins have been directly implicated in the legume-Rhizobium interaction relating to the nitrogen-fixation process [151-153]. This symbiosis is specific in that certain species of Rhizobium can only associate with a particular legume. Evidence for lectin involvement in the clover bacterial symbiosis system came from the finding that 2-deoxyglucose but not 2-deoxygalactose or glucose, inhibited the attachment of R.trifolli to clover roots [154]. Dazzo and Hubbell had previously shown that the capsular polysaccharides of R.trifolli contained combined 2-deoxyglucose and residues of this sugar are presumed to serve as receptors for lectin derived from the clover [100].

1.5.3. Plant glycoproteins

The possible involvement of glycoprotein glycan chains in binding α-galactosidases to cell constituents was discussed on p. 33. For this reason, and because α-galactosidases [39,41,155] and perhaps all glycosidases [62] and some lectins [156] contain covalently linked

carbohydrate, it is pertinent to briefly discuss the nature of plant glycoproteins and the basis of the enzymic methods we have used to help establish the partial structures of the glycan chains in V.faba α-galactosidase I.

Glycoproteins are defined as macromolecules with polypeptide backbones to which are attached one or more glycan chains usually consisting of 2-15 monosaccharide residues: two or more different monosaccharides are covalently bound in these chains [156,157].

The early studies on glycoproteins were limited to those of animal origin. However, it was later found that similar glycoprotein structures were also present in plants and microorganisms [158].

Carbohydrates in glycoproteins

The monosaccharides present in glycoprotein glycan chains include D-galactose, D-glucose (rarely), D-xylose, D-fucose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine and sialic acids (animal glycoproteins only). L-Arabinose, which is a common sugar in plant glycoproteins, has not unambiguously been demonstrated as a component of animal glycoproteins. D-Xylose, which in animals is confined to proteoglycans, is also found in plant glycoproteins [159].

A glycoprotein structure is usually verified by identifying the covalent linkage between the carbohydrate and the polypeptide: i.e. by isolating glycopeptides obtained, for example, by proteolytic (pronase) digestion. Only six different amino acids have been seen to be involved in these linkages. These are asparagine, serine, threonine, hydroxyproline, hydroxylysine and cysteine [121].

Three groups of glycopeptide linkages are known to occur in glycoproteins:

(a) N-Glycosidic linkages: N-acetylglucosamine linked to the amide group of asparagine;

(b) O-Glycosidic linkages: N-acetylglucosamine, mannose or galactose linked to the hydroxyl groups of serine or threonine, galactose linked to the hydroxyl groups of hydroxylysine and hydroxyproline or arabinose linked to hydroxyproline [160];

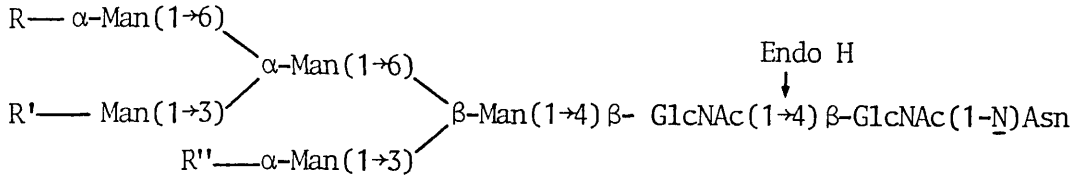
(c) S-Glycosidic linkages: between glucose and cysteine [161] and galactose and cysteine (so far detected only in two animal glycoproteins) [162].

Until recently, threonine was only considered to be a glycopeptide component of animal glycoproteins, however a 'threonine linkage' has now been described in glycoproteins isolated from maize (Zea mais) root slime cap [163] and from the red algae (Rhodophyta) [164].

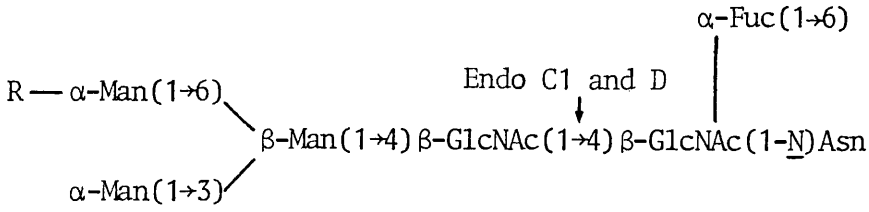
Structure of asparagine-linked glycan chains

The carbohydrate moieties of the various mannose-containing glycoproteins are of two main types; namely those posessing 'high-mannose' chains and those with 'complex-chains' [165]. 'High-mannose' chains contain glycosidically-bound mannose and N-acetylglucosamine residues only (Fig.3a). The 'complex chains' contain, in addition, galactose, fucose (Fig.3b) and sialic acids. In both types, mannosyl residues are usually present as 'clusters' of various sizes, referred to as oligomannosyl cores. The cores are larger in the 'high-mannose' chains than in the 'complex-chains'.

(a) 'High-mannose' (e.g. from ovalbumin)



(b) 'Complex-chain' (e.g. from fetuin)



R,R',R'' = monosaccharides or oligosaccharides

Figure 3

Structures of mannose-containing glycopeptides and the linkages hydrolysed by endoglycosidases H, CI and D

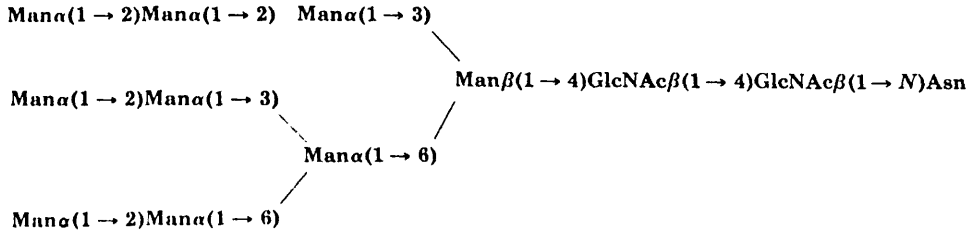
(see also Results and Discussion, p.142).

Enzymes hydrolysing complex carbohydrates are valuable tools for structural studies since they often display high specificities for their substrates [160]. Such is the case with the endo-β-*N*-acetylglucosaminidases which hydrolyse internal glycosidic linkages in specific glycoproteins or glycopeptides and release oligosaccharides. This family of enzymes can effectively be used to distinguish between the two major groups of mannose-containing glycoproteins. Endo H, from *Streptomyces plicatus*, is specific for the hydrolysis of 'high-mannose' glycoproteins [166] and endo CI, CII (both from *Clostridium perfringens*) [167] and endo D (from *Diplococcus pneumoniae*) [168] for the hydrolysis of 'complex-type' glycoproteins.

Failure of an endo-β-*N*-acetylglucosaminidase to cleave glycan chains cannot be unambiguously interpreted; it does not necessarily mean in the case of endo H, for example, that the material is not of 'mannose-rich' type: microfeatures of the structure can inhibit the enzyme activity.

Amongst the 'high-mannose' type glycoproteins soybean agglutinin has been reported to contain 4.5% mannose and 1.5% glucosamine (Man_9-$GlcNAc_2$) linked to asparagine. Treatment of the glycopeptide (Man_9-$GlcNAc_2$)-Asn, isolated from soybean agglutinin with endo H, liberated two oligosaccharide fractions, which on methylation were shown to be $Man_9GlcNAc$ and GlcNAc-Asn, hence, indicating that a chitobiose unit was present in the core region, which is typical of the *N*-glycosidically linked glycoproteins [169]. Lis and Sharon also demonstrated the presence of a branched structure in the core [α-Man(1→3)[α-Man(1→6)]-β-Man(1→4) β-GlcNAc(1→4)GlcNAc] of soybean agglutinin. On the basis

of the results obtained from methylation studies of released oligosaccharide it was proposed that the agglutinin contained a mixture of Asn-linked oligosaccharide chains. However, Dorland and co-workers [170] have recently presented data based on high resolution [^{1}H]-NMR spectroscopy of the oligopeptide isolated from soybean agglutinin showing that the carbohydrate moiety is homogeneous and possesses the following structure:



Favin, a lectin isolated from V.faba seeds, is also believed to possess 'mannose-rich' glycan moieties. These chains were shown to contain mannose and glucosamine residues and to be attached to the protein via an asparagine residue [171].

Earlier preliminary studies in this laboratory on the three forms of α-galactosidases from V.faba seeds indicated that they all contained covalently-bound carbohydrate (mannose, glucosamine, xylose and glucose) that could be released by acid hydrolysis [39]. The glycoprotein nature of these enzymes has been further examined in the study described in this thesis (p.139).

CHAPTER 2

MATERIALS AND METHODS

General laboratory chemicals were obtained from BDH Ltd, Poole, Dorset and were of 'Analar' grade where possible. P-Nitrophenyl-α-D-galactopyranoside (PNPG), 4-methylumbelliferyl-α-D-galactopyranoside and Triton X-100 were purchased from Koch-Light, Colnbrook, Bucks. Methyl-α-D-mannoside-Agarose, proteinase from Streptococcus griseus, sheep erythrocytes, carbohydrates used for inhibition assays, bovine serum albumin and standard proteins for molecular weight estimations by gel filtration were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey. Chromatofocusing kit, standard proteins of known values for isoelectric focusing, Sephadex G-100, Sephacryl S-200 and Concanavalin A-Sepharose were purchased from Pharmacia (G.B.) Ltd, London, U.K. CM-Cellulose (CM-52) was obtained from Whatman Biochemicals, Maidstone, Kent. Melibiose-Sepharose was obtained from Pierce Chemical Co.; Rockford, Il; USA. Isoelectric focusing kit was obtained from LKB, Bromma, Sweden. Endo-β-N-acetylglucosaminidase H was obtained from Seikagaku Fine Biochemicals, Tokyo, Japan. The 3-O-methyl-N-hexanoylglucosamine-Sepharose and favin were gifts from Dr A.K. Allen of Charing Cross Hospital Medical School, London, U.K. All the buffers used were prepared by the procedures described in Methods in Enzymology, Vol.1 [172]. All operations were carried out at 4°C unless otherwise stated.

2.1. Enzyme Assays

2.1.1. Quantitative assay

α-Galactosidase activity was assayed by following the initial rate of substrate hydrolysis. An appropriately diluted enzyme solution was added to a mixture of McIlvaine buffer [173] pH 5.5 and 10 mM PNPG (0.1 ml) preheated to 30°C; the final volume was 1.0 ml. The temperature was maintained for 15 min after which the reaction was stopped by addition of 0.1 M sodium carbonate (5 ml). The absorbance was measured at 405 nm. Enzyme activity is expressed as Katal (Kat), where one Katal is the amount of enzyme activity effecting the conversion of 1 mol of substrate per second.

2.1.2. Detection of α-galactosidase after isoelectric focusing

Following isoelectric focusing, enzyme activity was detected by the use of fluorescent substrate, 4-methylumbelliferyl-α-D-galactoside. The gels were covered with a solution of substrate (5 mg/10 ml), McIlvaine buffer, pH 5.5, and incubated for 5-10 min at room temperature. The enzyme activity was located using U.V. light and the gels were photographed using a Polaroid camera.

2.1.3. Qualitative observations using incident fluorescent light microscopy

(a) Using potato starch (acetone-washed) - four tubes were prepared containing 500 µl starch suspensions (10 mg/ml) in phosphate buffered saline (PBS; 7.2 g NaCl, 1.42 g Na_2HPO_4, 0.43 g KH_2PO_4) pH 7.2; tube (iii) contained, in addition, 0.1 M D-mannose. α-Galactosidase I

(10 µl; 80 nKat/ml) was added to tubes (i), (ii) and (iii) and incubated for 2 h. No enzyme was added to tube (iv). The suspensions were then mounted on microscope slides for observing fluorescence, using a Leitz Epifluorescent system (Ploenopak incident fluorescent illumination with H_2 filter block, which provides violet and blue light, 390-490 nm, for excitation). 4-Methylumbelliferyl-α-D-galactoside solution (1 mg/ml; 10 µl) in PBS was added to the slides prepared from tubes (i), (iii) and (iv) and observed under the microscope immediately and at definite intervals. Samples of tube (ii) and (iv) were controls.

(b) Using sheep erythrocytes - two tubes (i) and (ii) were prepared with 500 µl of 2% (v/v) red blood cell suspension in PBS, pH 7.2. α-Galactosidase I (10 µl; 80 nKat/ml) was added to tube (i) and the sample was incubated at room temperature for 2 h. Slides were prepared from both tubes for observations under the fluorescence microscope. The substrate was then added to the slide prepared from tube (i) and the reaction was followed as in (a).

2.1.4(a) Assay of the yeast α-mannan precipitation reactions

The α-galactosidases (EI 0.2 mg/ml; EII^1 0.10 mg/ml; and EII^2 0.05 mg/ml) were dialysed against 0.1 M sodium acetate-HCl buffer, pH 6.1. Serial dilutions of yeast α-mannan (10 mg/ml; in sodium acetate buffer) were made using 200 µl volumes. The enzyme and α-mannan solutions (200 µl of each) were pre-incubated separately at 25°C for 15 min then mixed and the incubation continued for a further 10 min. The turbidities were then measured at 420 nm in a Unicam SP 1800 spectrophotometer. Following the turbidity

measurements, the suspensions were centrifuged in a MSE Superspeed 65 centrifuge at 30,000 rev/min for 30 min and the supernatant obtained in each case was assayed for enzyme activity as described in Section 2.1.1.

2.1.4(b) Quantitative measurement for α-galactosidase I binding to starch

Potato starch suspensions in PBS, pH 7.2 (500 µl; 10% w/v) were placed in microcentrifuge tubes containing 0.1 M D-mannose, D-glucose, melibiose, maltose, sucrose or raffinose: one control reaction mixture contained only starch and PBS. α-Galactosidase I (10 µl, 80 nKat/ml) was added to each tube and the suspensions incubated for 2 h at 20°C. The tubes were shaken at intervals and finally centrifuged; each supernatant (50 µl) was assayed for α-galactosidase activity as described in Section 2.1.1. The concentration of the haptens in the assay was uninhibitory to the enzyme.

In a separate experiment, α-galactosidase I-starch incubation mixture in PBS was treated with Aspergillus niger α-amylase (final conc., 25 units/ml) for 1 h at 37°C followed by centrifugation and assayed for α-galactosidase I activity in the supernatant as described in Section 2.1.1.

2.1. Protein Assays

2.2.1. Measurement of protein

Protein was determined by the method of Lowry et al. [174] using crystalline bovine serum albumin as the standard.

2.2.2. Detection of protein on gels

After electrophoresis, the gels were stained for protein with 0.2% (w/v) Coomassie brilliant blue (R250) in 50% (w/v) TCA for 15-20 min at 60°C and then destained at room temperature with a solution containing 7.5% (v/v) acetic acid and 12.5% (v/v) isopropanol. (The time for destaining could be decreased by placing the gels at 30°C).

2.3. Column Chromatography

2.3.1. Sephadex G-100 and Sephacryl S-200 gel filtration

Sephadex G-100 (2.5 cm x 90 cm and 5.0 x 90 cm) and Sephacryl S-200 (2.5 cm x 90 cm) columns were packed by the method described by Andrews [175]. The columns (2.5 cm x 90 cm) were eluted with appropriate buffers at a flow rate of 30 ml/h and fractions (3.ml) were collected. The flow rate for the columns (5.0 cm x 90 cm) was 100 ml/h and fractions (10 ml) were collected. The columns were water-jacketed, with tap water flowing through the jackets, and therefore run at room temperature.

2.3.2. Affinity chromatography

(i) Melibiose-Sepharose affinity chromatography

A 5.0 ml plastic syringe fitted with a porous polyethylene gel support disc was used as a column. Melibiose-Sepharose was equilibrated with 0.1 M McIlvaine buffer, pH 5.5, and packed with this gel to a height of 5 cm at a flow rate of 30 ml/h using a peristaltic pump. The enzyme sample which was in the same buffer, was applied on the column at 15-20 ml/h. The flow was then stopped for 30 min to allow the enzyme to bind the column. The column was washed with the buffer and 2 ml fractions were collected. The eluent was monitored for protein at 280 nm using a Beckman 25 spectrophotometer. When the E_{280} was nil, the sample bound to the column was eluted with 50 mM PNPG in the equilibrating buffer and fractions (2.0 ml) collected until 100 ml eluting buffer had passed through the column. The post-PNPG fractions were dialysed and assayed for α-galactosidase activity (Section 2.1.1).

(ii) 3-O-Methyl-N-hexanoylglucosamine-Sepharose

The three forms of α-galactosidases were applied directly to a column (5.0 x 1.0 cm) of 3-O-methyl-N-hexanoylglucosamine-Sepharose equilibrated with 1 M NaCl and eluted with 1 M NaCl. Fractions (10 ml) were collected and examined for α-galactosidase (as described in Section 2.1.1) and lectin (as described in Section 2.8.1).activities. The column was washed until the E_{280} of eluate was below 0.015. Smaller fractions were then collected and any protein bound to the column was eluted with 1 M-NaCl containing 0.1 M methyl-α-D-glucoside. The fractions collected were dialysed against 3 changes of 1 M-NaCl (2 litres) for 24 h and assayed for α-galactosidase and lectin activities. The method used was essentially similar to that described by Allen et al. [121].

(iii) 'Methyl-α-D-mannoside- Agarose' affinity chromatography

A 5.0 ml plastic syringe fitted with a porous polyethylene gel support disc was used as a column. 'Methyl-α-D-mannoside-Agarose' was equilibrated with 0.1 M potassium phosphate buffer, pH 7.0, containing 0.1 M sodium chloride and the column was packed with gel to a height of 5 cm at a flow rate of 30 ml/h using a peristaltic pump. The enzyme sample which had been dialysed against 0.1 M potassium phosphate buffer, pH 7.0, was applied on the column at a flow rate of 15-20 ml/h. The flow was then stopped for 30 min to allow the enzyme to bind to the column. The column was washed with the buffer and fractions (2 ml) were collected. The eluent was monitored for protein at 280 nm using a Beckman 25 spectrophotometer. When E_{280} was nil, the sample bound to the column was eluted with 0.5 methyl α-D-mannoside in the equilibrating buffer and fractions (2 ml) collected until E_{280} was nil again. The fractions were assayed for α-galactosidase activity as described in Section 2.1.1. The enzyme active fractions were pooled, dialysed against potassium phosphate buffer, concentrated and assayed for lectin activity, as described in Section 2.8.1.

(iv) Concanavalin A-Sepharose affinity chromatography

A 20 ml plastic syringe fitted with a porous polyethylene gel support disc was used as a column. Con A-Sepharose was equilibrated with 0.1 M potassium phosphate buffer, pH 7.0, containing 0.1 M sodium chloride and the column packed with this gel to a height of 20 cm at a flow rate of 100 ml/h using a peristaltic pump. The enzyme sample which had been dialysed against 0.1 M potassium phosphate buffer, pH 7.0, was applied on the column at 60 ml/h. The column was washed with buffer and fractions (2 ml) were collected. The eluent was monitored

for protein at 280 nm using a Beckman 25 spectrophotometer. When the E_{280} was nil, the sample bound to the column was eluted with a linear methyl-α-D-mannoside gradient prepared using a mixing chamber containing 0.1 M potassium phosphate buffer, pH 7.0 (100 ml), and a reservoir containing 0.01 M methyl-α-D-mannoside (100 ml) in the same buffer. Fractions (1.5 ml) were collected at a flow rate of 60 ml/h. The fractions were examined for α-galactosidase activity, as described in Section 2.1.1.

2.3.3. Ion-exchange chromatography (cation)

Whatman ion-exchange cellulose, CM-52, was prepared as described by the manufacturer's instruction sheet and equilibrated with McIlvaine buffer, pH 3.5, and a column (1 cm x 14 cm) packed at a rate of 20 ml/h. The enzyme preparation was dialysed against McIlvaine buffer (pH 3.5) for 2-3 h and subsequently applied to the column. Elution was carried out with the same buffer until the absorbance at 280 nm was negligible. The bound enzyme was then eluted with a linear sodium chloride gradient prepared using a mixing chamber containing 0.05 M sodium chloride (100 ml) and a reservoir containing 0.5 M sodium chloride (100 ml) in McIlvaine buffer, pH 3.5. Fractions (2.0 ml) were collected at a flow rate of 15 ml/h.

2.4.1. Purification of α-galactosidases I, II^1 and II^2

Purification of the enzymes was carried out according to Figure 4. One thousand grams of seed powder from dormant broad beans was used for each batch of purification. The testas were removed and

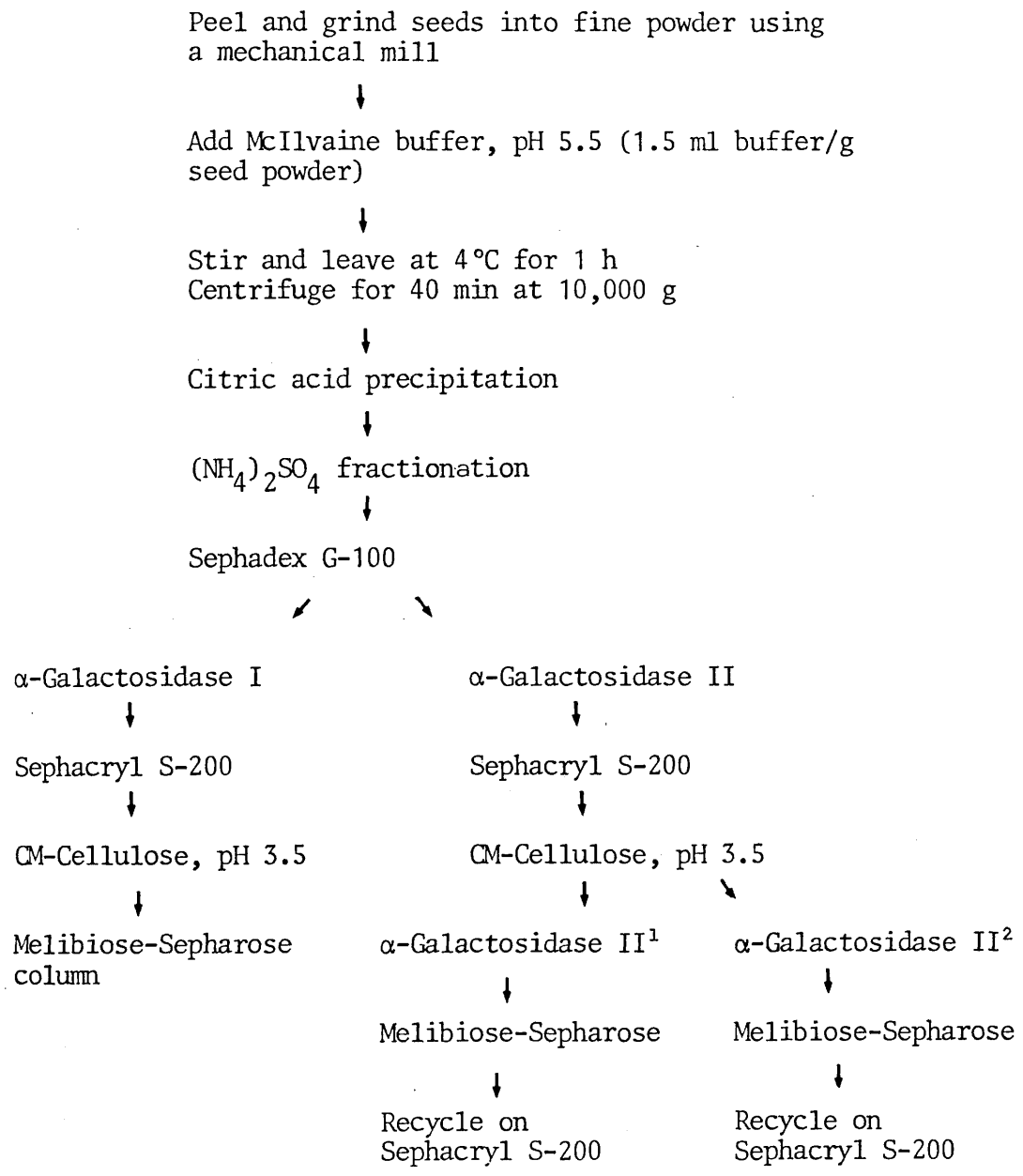


Figure 4

Purification of α-galactosidases I, II^1 and II^2 from Vicia faba seeds

the beans ground in a mechanical grinder. The resultant bean powder (1,000 g) was suspended in McIlvaine buffer, pH 5.5 (1,500 ml), stirred and left for 1 h at 4°C. The slurry was then centrifuged at 10,000 g for 40 min using a 6 x 250 ml MSE 18 rotor and the cell debris discarded. The supernatants (1,010 ml) were pooled and the pH of this crude extract was lowered to pH 3.2 by gradual addition of 1 M citric acid, with continuous stirring, which was maintained for 1 h after the last drop of citric acid had been added. The precipitated material was centrifuged off at 10,000 g and the supernatants were again pooled and the pH adjusted to pH 5.5 by the addition of a saturated solution of disodium hydrogen orthophosphate.. This fraction (1440 ml) was then made 25% saturated with ammonium sulphate by the addition of the solid salt and stirred for 3 h at 4°C. This sample was then centrifuged and the precipitate obtained, discarded. The ammonium sulphate concentration of the resultant supernatant was raised to 65% and stirring continued for a further 3 h at 4°C. The precipitated material was collected by centrifugation and the precipitate suspended in McIlvaine buffer, pH 5.5, and dialysed overnight against the same buffer. This 25-65% ammonium sulphate fraction (ca 100 ml) was applied to a 5.0 cm x 90 cm Sephadex G-100 column and eluted with McIlvaine buffer, pH 5.5, containing 0.1 M potassium chloride. Fractions (10 ml) were collected, assayed for α-galactosidase activity and the absorbance at 280 nm was measured. Enzymically active fractions corresponding to forms I and II were pooled separately. From this stage the two forms I and II were purified separately.

Pooled enzyme I was concentrated to about 10 ml by ultrafiltration using a PM10 membrane. This enzyme was applied to a 2.5 x 90 cm Sephadex G-100 column as described above, with the exception that

3 ml fractions were collected. The latter column was replaced with a Sephacryl S-200 column, of the same size, which was found to give a better resolution. Three ml fractions were collected for this column as well. The sample was concentrated and dialysed against McIlvaine buffer, pH 3.5, and the sample (ca 10 ml) was applied to a CM-cellulose column equilibrated with the same buffer. The column was washed with this buffer (100ml) after which the enzyme was eluted with a linear gradient from 0.05 M sodium chloride in McIlvaine buffer, pH 3.5 (100 ml) to 0.5 M sodium chloride in McIlvaine buffer (100 ml). The active fractions were pooled, concentrated and finally recycled through a CM-cellulose column.

The enzyme was pooled, concentrated and dialysed against McIlvaine buffer, pH 5.5 (5 L) and applied to a Melibiose-Sepharose column (1.0 cm x 5.0 cm), which had been equilibrated with the same buffer. The dialysed sample (ca 5 ml) was applied in 1-2 ml batches. The bound proteins were washed with the same buffer and when the E_{280} was negligible, the bound enzyme was eluted with 50 mM PNPG in the eluting buffer; fractions (2 ml) were collected, dialysed, assayed for α-galactosidase activity (see Section 2.1.1), pooled and concentrated.

The same purification procedure was followed for enzyme II fraction obtained from the second gel filtration column, except that a 0.05 M sodium chloride to 0.4 M sodium chloride gradient was used to elute the enzyme from the CM-cellulose column. Two peaks of activity, II^1 and II^2, were obtained when fraction II was applied to the ion-exchange column. The two enzymes were pooled, concentrated and separately passed through the Melibiose-Sepharose affinity column.

The purified enzymes were stored at 4°C with azide as an added preservative. Since purified α-galactosidases II^1 and II^2 tended to aggregate upon storage, the enzymes were recycled on a Sephacryl S-200 gel filtration column (2.5 cm x 90 cm) to remove polymeric material.

2.4.2. Purification of α-galactosidases I and II from Vigna radiata and the 'clot-dissolving' properties of form I

α-Galactosidases I and II from V.radiata obtained by the method used by Hankins and Shannon [8]. The enzyme obtained from the 30-60% ammonium sulphate precipitation followed by Sephacryl S-200 chromatography. The enzyme activity was resolved into two molecular weight forms, EI (MW 160,000) and EII (MW 40,000). The two forms were pooled separately, and concentrated using an Amicon ultrafiltration cell. α-Galactosidase activity was determined by the method described in Section 2.1.1.

α-Galactosidases I and II from V.radiata were added to erythrocytes (in PBS; pH 7.0) in the presence and absence of D-galactose and incubated at room temperature for 2h. The four samples of erythrocytes were washed three times with PBS and incubated separately with EI from V.radiata and with purified forms of α-galactosidases I, II^1 and II^2 from V.faba seeds.

2.5(a) Polyacrylamide Gel Electrophoresis in the Presence of Sodium Dodecyl Sulphate (SDS-PAGE)

SDS-PAGE was performed as described by Laemmli [176] with a slight modification. Slab gel electrophoresis was carried out in a water-jacketed BioRad apparatus. Stacking and separating gels were 5% and 12.5% respectively, and were prepared from a stock solution of 30% (w/v) acrylamide and 0.8% (w/v) N,N'-bismethylene acrylamide. The final concentrations in the 12% separating gel (15 mm x 120 mm) were as follows: 12.5% acrylamide, 0.1% N,N'-bismethylene acrylamide, 0.1% (w/v) sodium dodecyl sulphate (SDS), 0.03% (v/v) Temed (N,N,N',N'-tetramethylene diamine and 0.03% (w/v) ammonium persulphate, and 0.38 M Tris-HCl buffer, pH 8.8. The stacking gel (1.5 mm x 30 mm) contained: 0.1% (w/v) SDS, 0.05% (v/v) Temed, 0.05% (w/v) ammonium persulphate and 0.13 M Tris-HCl buffer, pH 6.8. Ten ml stacking gel and 30 ml separating gel were adequate for one slab gel. The electrode buffer, pH 8.3, consisted of 0.38 M glycine, 0.01% (w/v) SDS and 0.05 M Tris-HCl buffer, pH 6.8. Protein samples were mixed with the sample buffer which contained 0.13 Tris-HCl buffer, pH 6.8, 10% (v/v) glycerol, 1.25% SDS and 0.015% bromophenol blue. The samples were heated for 3 min at 100°C and after cooling they were applied onto the gel; maximum volume of the sample not exceeding 50 µl/track. Electrophoresis was performed at 12.5 mA/slab gel until the samples entered the stacking gel (ca 45 min), after which the current was increased to 40 mA/gel. The gels were removed when the dye front was within 1.0 cm from the bottom of the gel. The total time taken for the run was 3 h. The gels were stained for protein, as described in Section 2.2.2. Protein standards used were obtained as a kit from Bio-Rad

and consisted of Myosin (MW 200,000), β- galactosidase (MW 130,000), phosphorylase (MW 90,000), bovine serum albumin (MW 65,000) and ovalbumin (MW 45,000).

(b) Gradient gels

When SDS-PAGE was carried out using gradient gels (by Dr D.J. Bowles, Department of Biochemistry, University of Leeds), the procedure followed was essentially similar to that described for homogeneous gels (Section 2.5a). The only difference was that the separating gel was a 10-15% gradient gel.

The 10-15% gradient gel was prepared by using two solutions containing 10 and 15% (final concentration) acrylamide. The final concentration of the other reagents in the two solutions were the same and were as follows: 0.1% N,N'-bismethylene acrylamide, 0.1% (w/v) SDS, 0.03% (v/v) Temed, 0.3% (w/v) ammonium persulphate and 0.38 M Tris-HCl buffer, pH 8.8.

The casting apparatus was connected via a peristaltic pump to a gradient mixer. 20-25 ml ethanol solution (20%, v/v) in buffer was placed into the mixing chamber of the gradient mixer and pumped into the casting apparatus, leaving the connecting tube filled with liquid.

The polymerization mixtures (ammonium persulphate and Temed) were mixed with the two solutions containing 10 and 15% acrylamide. The low concentration mixture was then immediately poured into the mixing chamber of the gradient mixer and the high concentration mixture into the other chamber. The stirrer was started and the valve between

the two chambers was opened. A flow rate of approximately 10 ml/min was used to pump the gradient into the casting apparatus.

When all of the gradient had been delivered, a saturated sucrose solution was added to both chambers of the gradient mixer and pumping was continued until the gradient was displaced into the cassettes by a cushion of dense underlay solution. Pumping was stopped when the interface between the gradient solution and the dense underlay was about to reach the bottom of the cassettes. The tubing to the inlet of the casting apparatus was closed using a screw clamp. The gradient gel slab was allowed to polymerise completely before taking it out of the casting apparatus (at least 1-2 hours).

2.6. Dissociation of α-Galactosidase I Using Urea

To α-galactosidase I (395 nKat/ml; 2 ml) in McIlvaine buffer, pH 5.5, solid urea was added to final concentrations of 1.5, 2.5, 3.0 and 5.0 M. The samples were incubated in a waterbath at 30°C for 2 h and then applied separately to calibrated Sephacryl S-200 columns. Following elution with McIlvaine buffer, pH 5.5 (see Section 2.3.1), fractions (3.0 ml) were collected and assayed for enzyme activity as described in Section 2.1.1.

The enzyme fraction isolated from the Sephacryl S-200 column (elution volume 260-290 ml) after treatment with 5.0 M urea was subjected to CM-cellulose chromatography. A mixture of the enzyme isolated from the Sephacryl S-200 column and purified α-galactosidase II^2 (~60 nKat of each) was subjected to CM-cellulose chromatography (see Section 2.3.3) and the column fractions assayed for enzyme activity (see Section 2.1.1).

2.7.1. Chromatofocusing

The manufacturer's instructions for packing and running the column were followed. The gel and the buffers used were degassed immediately before use. The column (1.0 cm x 15.0 cm) was packed initially at the maximum rate allowed by the column outlet.. After all the suspension had run into the column it was continued to be packed at a linear flow rate of 100 cm h^{-1} until the gel had settled and the column was equilibrated with 0.02 M ethanolamine-acetic acid buffer, pH 9.4. The sample was dialysed against the starting buffer, and was applied to the column after running on 5 ml of eluent (Polybuffer 96, pH 6.0), to prevent the sample proteins from being exposed to extremes of pH. The column was then eluted with 250 ml of Polybuffer 96 at a flow rate of 30-40 ml/h. Fractions (2.0 ml) were collected. The pH of each fraction was monitored to ensure that there were no anomolies in the pH gradient. Each was also monitored for protein content at 280 nm and assayed for α-galactosidase activity as described in Section 2.1.1.

2.7.2. Isoelectricfocusing

Ready-prepared LKB Ampholine PAG-plates (thin-layer polyacrylamide gels containing Ampholine carrier ampholytes for analytical electrofocusing, pH range 3.5-9.5) were used. The method described by the manufacturer's instruction sheet was followed. The instruments used were LKB 2117 Multiphor and LKB 2103 Power Supply. Initially, the gel was placed on a Multiphor cooling plate with some insulating fluid (light paraffin oil) spread in between. Two electrode strips were saturated evenly with 1 M NaOH and 1 M H_3PO_4, respectively. The wet

strips were placed on either side of the gel, i.e. the former on the cathode side, and the latter on the anode side. The samples were applied in 2-5 µl droplets (protein conc. 2 mg/ml) directly onto the gel surface. After the apparatus was set up as per instructions, the power supply, also from LKB was connected, with the current set at 50 mA. The voltage was increased at 10 min intervals, until it reached 1500 V. The experiment was run for 1.5 h. The pH gradient was estimated by using standards of known pI values. The plates were cut so that the part containing the standard proteins could be stained for protein, and in the part containing the enzymes, α-galactosidase activity could be located. The former part of the gel was placed in a fixing solution (500 ml distilled water, containing 57.5 g trichloroacetic acid and 17.25 g sulphonic acid) for approximately an hour. The gel was placed in destaining solution (500 ml ethanol and 160 ml acetic acid, made up to 2 L with distilled water) for 5 min, in staining solution (0.46 g Coomassie Brilliant Blue R-250 in 400 ml destain) for 10 min at 60°C. The gel was then replaced in destaining solution with several changes of destain, until the background was clear.

The part of the gel containing the enzymes was stained for activity, as described in Section 2.1.2.

2.8.1. Haemagglutinin Assays

The method used is described by Allen *et al.* [103]. Blood cells from rabbits were harvested by centrifugation, resuspended in Alsever's solution, pH 6.1, the volume of Alsever's being equal to the original volume of blood. The cells were washed twice with phosphate

buffered saline (PBS), pH 7.2, and a 1.5% suspension of the blood cells prepared in PBS. To assay for agglutination, a serial dilution of the lectin was made in PBS (100 µl) and 200 µl of the 1.5% suspension of erythrocytes added. The tubes were shaken at 15 min intervals and the extent of agglutination was assessed after 2 h on a scale of 0-++++. One unit is the amount required to cause half-maximal agglutination of the cells. The assay is semi-quantitative with a range of error of ±20% [121].

2.8.2. Inhibitory Effect of Various Carbohydrates on the Agglutinating Activity of Lectins

To determine the 50% inhibition values for various sugars, the method of Lis et al. [177] was used. The degree of agglutination was determined on the visual serological scale, rather than spectrophotometrically. A serial dilution of the carbohydrate was made in 100 µl phosphate-buffered saline, a constant amount of lectin and 200 µl of 1.5% suspension of blood was added. The tubes were shaken at intervals and after 2h the degree of agglutination was assessed. The half maximal agglutination (++) was taken as 50% inhibition. The concentration of carbohydrates required to cause 50% inhibition was calculated.

2.9. Sephacryl S-200 Gel Filtration of D-Glucose-Treated α-Galactosidase I

α-Galactosidase I (395 nKat; 2 ml) was dialysed against potassium phosphate buffer (pH 7.2) containing 0.5 M D-glucose for 2 h.

The enzyme was stored at 4°C for 22 h and applied to a calibrated Sephacryl S-200 gel filtration column equilibrated with the same buffer containing 0.1 M D-glucose. The fractions (3.0 ml) obtained were dialysed against three changes of potassium phosphate buffer (total volume 15 L) for 22 h and assayed for catalytic and lectin activities (as described in Sections 2.1.1 and 2.8.1 respectively).

2.10. Protection of Catalytic and Haemagglutinin Activities of α-Galactosidases by Specific Carbohydrates During Heat Treatment

α-Galactosidases I, II^1 and II^2 (2 samples of each) were dialysed separately against 0.1 M potassium phosphate buffer, pH 7.0, and 0.1 M McIlvainebuffer, pH 5.5. The samples were incubated for 1 h at 75°C in the presence and absence of D-mannose and D-galactose respectively and then cooled immediately by placing in an ice bath and dialysed against the respective buffers (2 changes, total volume 10 L) for at least 8 hours.

The catalytic (see Section 2.1.1) and lectin (see Section 2.8.1) activities of each sample were then measured.

2.11. Metal-Ion Content and its Effect on the Catalytic and Haemagglutin Properties of α-Galactosidase I

The metal-ion content of native and demetallized enzymes was determined by atomic absorption spectrometry, using a Unicam SP90A Series 2 Atomic Absorption Spectrometer. The method described by the manufacturer's instruction sheet for metal-ion detection was followed.

The procedure for demetallization was essentially the same as that described by Galbraith and Goldstein [178].

The native enzyme samples (1.0 ml; 1.5 mg/ml) were prepared for metal analysis by dialysis against four changes of deionized water (1 L) for 48 hours.

Demetallization of the samples (1.0 ml; 1.5 mg/ml) was effected by sequential dialysis against deionized water (2 changes; total volume 2 L) for 24 h, 0.1 M EDTA (4 changes; total volume 1.2 L), deionized water (4 changes; total volume 1.2 L) for 48 hours, 1 M acetic acid (4 changes; total volume 1.2 L) for 48 hours and then finally against deionized water (5 changes; 1.5 L) for 48 hours. The samples were then examined for catalytic and lectin activity (as described in Sections 2.1.1 and 2.8.1 respectively) and analysed for metal content.

2.12.1. Equilibrium Dialysis

This experiment was performed in an Equilibrium Dialon dialyser in 250 µl dialysis cells in potassium phosphate buffer, pH 7.0. The dialysis membranes were boiled in 1% Na_2CO_3 and washed exhaustively with distilled water before use.

A constant amount of [^{14}C]-D-mannose (ca 1·3 µCi) and varying of cold D-mannose (0-0.12 mg) and protein (0.13, 0.31 and 0.17 mg for EI, EII^1 and EII^2 respectively) were placed in opposite compartments of the dialysis cells. The total volume was made up to 200 µl in each

compartment. The cells were rotated for 24 h at 25°C to establish equilibrium. One hundred µl of the samples were then withdrawn from each compartment and placed in scintillation vials. NCS tissue solubilizer (500 µl) was added to each of the vials, which were then incubated for 2 h at 60°C. Glacial acetic acid (20 µl) and toluene-PPO (10 ml; 5 g PPO/l toluene) was added to each vial upon cooling. The radioactivity in each vial was then determined in a Beckman LS7500 liquid scintillation counter.

In the study of the number of catalytic sites, the same procedure was followed, except that D-galactose was used instead of D-mannose.

2.12.2. Spectrophotometric Analysis

The method used was essentially the same as that described by Neurohr et al. [179]. The difference spectra (lectin- carbohydrate versus lectin) were measured with a Perkin-Elmer 550S spectrophotometer attached to a Perkin-Elmer chart recorder (Model No.561).

Aliquots (2.0 ml) of enzyme solutions (approximately 0.4 mg/ml EI and 0.2 mg/ml of EII^1 and EII^2) were dialysed against potassium phosphate buffer, containing 0.1 M NaCl (pH 7.0) and then added to both sample and reference cells (semi-microquartz cuvettes of 1 cm path length). The base-line was recorded into the instrument's memory unit to be subtracted automatically from subsequent spectra. The spectra were recorded between 300 and 250 nm. 1 M D-Mannose solution (5 µl aliquots) were then added to the sample cuvette while the corresponding reference cuvette received the same amount of phosphate buffer. The difference spectrum was again recorded between 300 and 250 nm. The amount of D-mannose solution used did not absorb between these wavelengths.

2.13.1. Isolation of a Glycopeptide

Carboxymethylation of α-galactosidase I

The method used was essentially similar to that of Konigsberg [180]. Pure α-galactosidase I (0.2 μmole) was dialysed against distilled water and freeze-dried. The sample was dissolved in 500 μl of 3 M Tris-guanidine-HCl buffer, pH 8.5, through which N_2 had been bubbled for 15 min. On dissolving the enzyme preparation in the buffer, N_2 was allowed to blow gently over the surface for a further 30 min. 3.2 μmole of dithiothreitol (DTT; the equivalent of 4 moles of DTT per mol of the disulphide bridges, assuming 4 disulphide bridges per molecule) was added. The sample was incubated at 37°C for 30 min. 16 μmoles (5 moles per mole of DTT in the sample) of iodoacetate dissolved in 500 ul of 0.3 M guanidine-HCl buffer, pH 8.3, was added and the sample was incubated for a further 30 min at room temperature for alkylation of cysteine residues to S-carboxymethyl-cysteine residues. The pH was controlled between pH 8.0 and 8.5 by the addition of solid Tris. The reduced carboxymethylated protein was separated from the reactants by dialysing versus 5% acetic acid. The sample was then dried down by rotary evaporation.

Proteolytic digestion of α-galactosidase I

The conditions used were the same as those used by Allen et al. [121] for the digestion of the V.faba lectin. The enzyme (2.5 mg/ml) was digested at 37°C with proteinase from S.griseus in Tris-acetate buffer at pH 7.8, containing 1 mM $CaCl_2$ and in the presence of toluene for a total of 144 h. Initially proteinase equal to 2% of the weight of α-galactosidase was added, followed by a further 1% of the weight of α-galactosidase, after 48 h. The sample

was applied to a Biogel P-4 column (5.0 cm x 80 cm) equilibrated with water. The sample was eluted with water and fractions (5 ml) were collected at a rate of 25 ml/h. 0.2 ml portions of the fractions were assayed for neutral sugar by the anthrone method [181].

Amino acid analysis

The samples were dissolved in 1 ml H_2O and 50 µl aliquots were taken for amino acid analysis. The aliquots were dried *in vacuo* in hydrolysis tubes. 6 M HCl (25 µl) containing norleucine (2 nmol) as an internal standard was added. The tubes were sealed under vacuum and heated on a heating block at 110°C for 16 h. The tubes were opened and dried in vacuum. The samples were analysed on a Beckman 121MB amino acid analyser using the method described by Mayes *et al.* [182].

2.13.2. Acetolosis of glycopeptides

The sample from peak 1 (Fig.28) of the Biogel P4 column was dissolved in a mixture of glacial acetic acid, acetic anhydride and sulphuric acid (in a ratio of 10:10:1, v:v:v; 1.0 ml). The sample was incubated at 60°C and aliquots (0.2 ml) were taken after 30 min, 2 h, 5 h and 7 h. The samples were quenched with approximately 1 ml H_2O and extracted into $CHCL_3$. The $CHCl_3$ was washed 3 times with H_2O and evaporated by blowing with N_2. The samples were freeze-dried to remove any traces of H_2O before analysis by fast atom bombardment-mass spectroscopy (F.A.B-M.S.) (Dell; A. and Oates, J: personal communication).

2.13.3. Endo H treatment of native α-galactosidase I

Endo H (10 milliunits) was added to α-galactosidase I (4500 OD units) in citrate-phosphate buffer (1 ml; pH 5.5). The solution was incubated at 37°C for 15 h and analysed for α-galactosidase activity as described in Section 2.1.1. The samples were then applied to a Sephacryl S-200 column and eluted as described in Section 2.3.1. The fractions (3.0 ml) were assayed for α-galactosidase activity. The fractions collected just beyond the estimated included volume of the column (440 ml) were tested for the presence of carbohydrate by the anthrone method [181]. The peaks containing α-galactosidase activity were pooled separately, concentrated using an Amicon PM10 filter, and dialysed against phosphate buffer, pH 7.0. The samples were applied to Con A-Sepharose columns and eluted as described in Section 2.3.2(iv) using a methyl-α-D-mannoside gradient. (Any non-binding components in the α-galactosidase I preparation had been removed from the sample by application to the Con A-Sepharose column prior to Endo H treatment [39]).

CHAPTER 3

RESULTS AND DISCUSSION

The main aim of this study was to obtain highly purified samples of α-galactosidases I, II^1 and II^2 from V.faba seeds and to investigate further the haemagglutinating (lectin) properties of the three forms. Preliminary studies by Dey et al. [39] had shown that they all agglutinated rabbit erythrocytes. In particular, it was necessary to clearly establish that these enzymes were true lectins and to eliminate the possibility that their carbohydrate-binding properties were due to contamination by other lectins or that the binding occurred via their catalytic sites. V.faba seeds contain at least one other classical lectin, favin (also known as the V.faba lectin), with glucose/mannose specificity [121]. The lectin properties of α-galactosidase I from V.radiata and the α-galactosidases from V.faba were also compared.

In addition to this main theme, the glycoprotein nature of α-galactosidase I has been examined (in collaboration with Professor H. Morris and Dr A. Dell at The Imperial College of Science and Technology). More information concerning the subunit structure has been obtained.

Finally, the binding of the α- galactosidase to cell constituents has been investigated.

3. Isolation of V.faba α-Galactosidases and an Investigation of Their Lectin Properties

3.1. Lectin Activities of the Multiple Forms of α-Galactosidases from V.faba seeds

3.1.1. Purification of α-galactosidase-haemagglutinins from V.faba seeds

α-Galactosidases I, II^1 and II^2 (EI, EII^1 and EII^2) were isolated from testa-free, powdered V.faba seeds. For each preparation approximately 1 kg of seed powder was used and the process required 2 weeks to complete. The purification procedure followed was similar to that described by Dey et al. [39], the main difference being that the Con A-Sepharose column was omitted in order to avoid contamination of the α-galactosidases with this lectin. At each stage both the enzyme and lectin specific activities were determined. Unlike the observations made by Hankins and Shannon [8], who purified α-galactosidase from V.radiata, the enzyme/lectin specific activity ratio did not remain constant during the various stages.

A typical purification procedure is summarized in Table 1. The stage involving the addition of citric acid was necessary to remove the bulk of the storage proteins and a number of glycosidases other than α-galactosidases [183]. This step resulted in a significant increase in the volume of the preparation. To overcome the latter effect, the activity was concentrated by ammonium sulphate precipitation. After the first three stages there was a seven-fold increase in the specific activity of the enzyme and a decrease in the lectin specific activity.

The preparation obtained at Stage 3 was then applied to a large (5 x 90 cm) Sephadex G-100 column (Stage 4) in order to separate fractions EI and EII (MW 160,000 and 40,000 respectively ; Fig.5a). Previously it

TABLE 1 Purification of α-galactosidases from V.faba seeds *

Stage	Volume (ml)	Activity (nkat/ml)	Protein (mg/ml)	α-Galactosidase specific activity (nkat/mg) [A]	Lectin activity (HA/mg) [B]	Ratio A/B	Recovery
1. Crude extract	900	16	130.00	0.12	17,600	7.0×10^{-6}	-
2. Citric acid precipitation (pH 3.0)	1000	15	30.00	0.50	20,440	2.4×10^{-5}	104
3. $(NH_4)_2SO_4$ - (25-65% saturation)	95	117	130.00	0.90	12,300	7.3×10^{-5}	77
4. Sephadex-G100 gel filtration							
. α-galactosidase I	90	57	4.0	14.2	1,560	9.1×10^{-3}	35
. α-galactosidase II	75	48	1.4	34.3	2,200	1.5×10^{-2}	25
5. Sephacryl-S200 gel filtration							
. α-galactosidase I	8	306	10.1	30.2	2,700	0.01	17
. α-galactosidase II	6.5	244	2.63	93			11
6. CM-cellulose ion-exchange chromatography							
. α-galactosidase I	10	118	0.01	11,800	650	18.1	8.2
. α-galactosidase II[1]	2	50	0.41	122	7,710	1.6×10^{-2}	0.7
. α-galactosidase II[2]	4	210	0.18	1,167	1,800	0.65	5.6
7. Melibiose-Sepharose affinity chromatography							
. α-galactosidase I	3	250	0.02	12,500	770	16.2	5.2
. α-galactosidase II[1]	2	38	0.23	165	4,400	3.7×10^{-2}	0.5
. α-galactosidase II[2]	2.5	170	0.12	1,417	1,560	0.91	2.9

* Recovery has been calculated relative to Stage 1. Weight of seed powder taken = 1 kg.

had been reported that this stage minimized the aggregation of the low molecular weight fraction, EII, to EI [184], however, in the present study there was considerable conversion of EII to EI after 10 days, when EII was stored at 4°C (see p.84).

After Stage 4 there was a marked reduction in the total lectin activity which was probably due to the removal of favin. Although this lectin is reported to have a low affinity for Sephadex G-100, it is bound by large columns of the gel [121]. In a parallel study, the $(NH_4)_2SO_4$ fraction (Stage 3) was applied to a smaller (2.5 x 90 cm) Sephadex G-100 gel filtration column and here the enzyme and lectins were observed to co-elute in the two fractions EI and EII (Fig.6a).

EI and EII derived from Stage 4 were applied separately (Stage 5) to a Sephacryl S-200 column (2.5 x 90 cm) (Fig.5b&c) which gave a better resolution of mixture of EI and EII and a faster flow rate than Sephadex G-100: the resulting enzyme fractions were concentrated by ultrafiltration. This step resulted in an increase in the specific activities of the enzymes (EI, 2-fold, and EII, 3-fold).

The two forms (EI and EII) were dialysed against McIlvaine buffer (pH 3.5) for 2-3 h and applied separately to CM-cellulose columns (Stage 6; Fig.7). The columns were washed with buffer until the absorbance at 280 nm was nil and then eluted with a sodium chloride gradient. EI eluted as a single, enzymically-active peak with coincident lectin activity (Fig.6b) and the enzyme specific activity rose 390-fold in comparison with Stage 5; however, the lectin specific activity decreased by 75% (Table 1). This decrease suggests that either further loss of contaminating lectins and/or inactivation of some of the lectin sites on EI due to prolonged exposure to low pH, had occurred. The α-galactosidase II fraction was resolved into two forms, EII^1 and EII^2 on the CM-cellulose column, again

Figure 5

Gel filtration of α-galactosidases from V.faba seeds

(a) Sephadex G-100 gel filtration elution profile of α-galactosidases (from Stage 3, Table 1)

(b) Sephacryl S-200 gel filtration elution profile of α-galactosidase I (from Stage 4)

(c) Sephacryl S-200 gel filtration elution profile of α-galactosidase II (from Stage 4)

See Section 2.3.1. for further details.

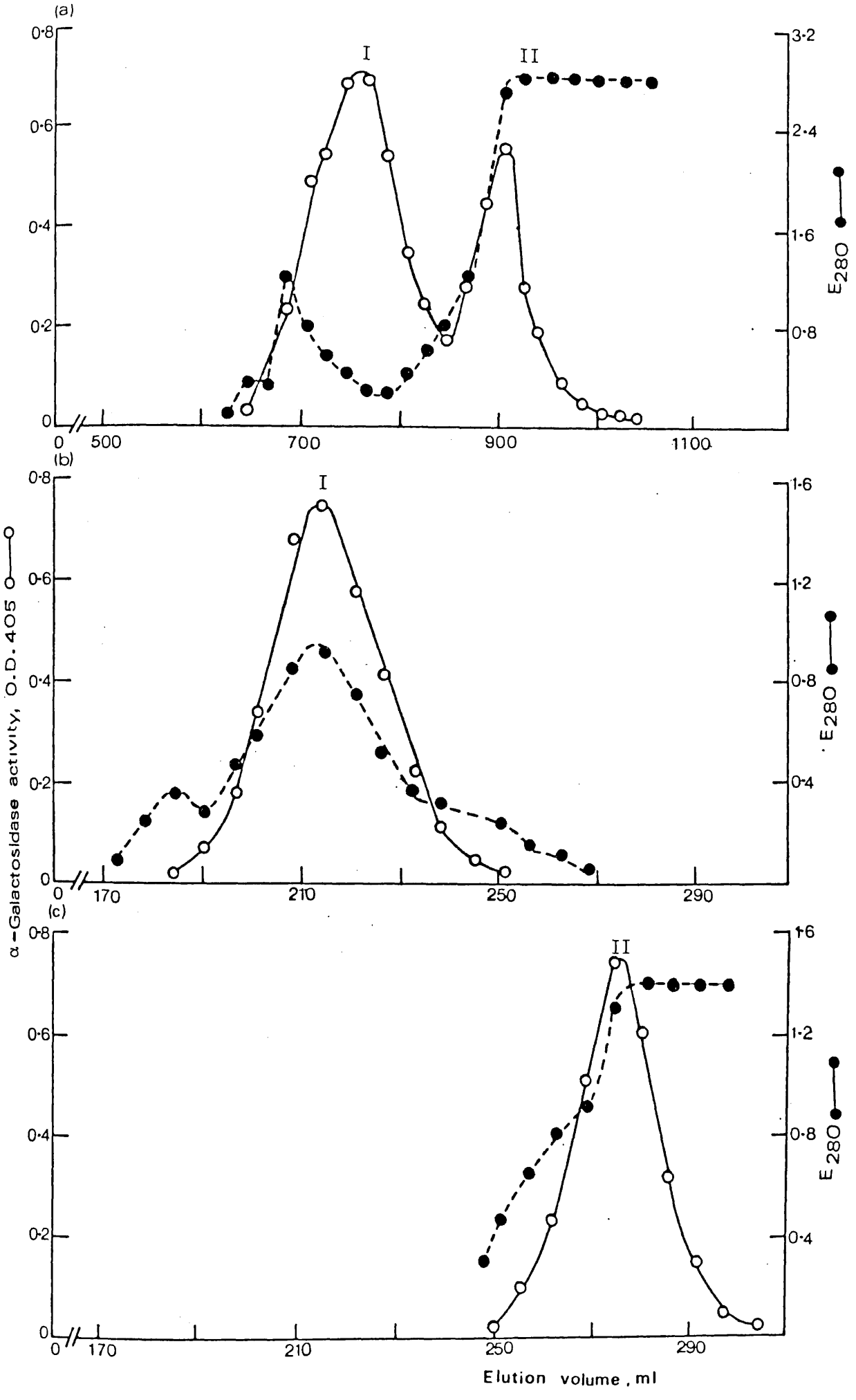

(a)
I
II
0·8
0·6
0·4
0·2
0
3·2
2·4
1·6
0·8
E280
0
500
700
900
1100
(b)
I
0·8
0·6
0·4
0·2
0
1·6
1·2
0·8
0·4
E280
0
170
210
250
290
α-Galactosidase activity, O.D. 405
(c)
II
0·8
0·6
0·4
0·2
0
1·6
1·2
0·8
0·4
E280
0
170
210
250
290
Elution volume, ml

Figure 6

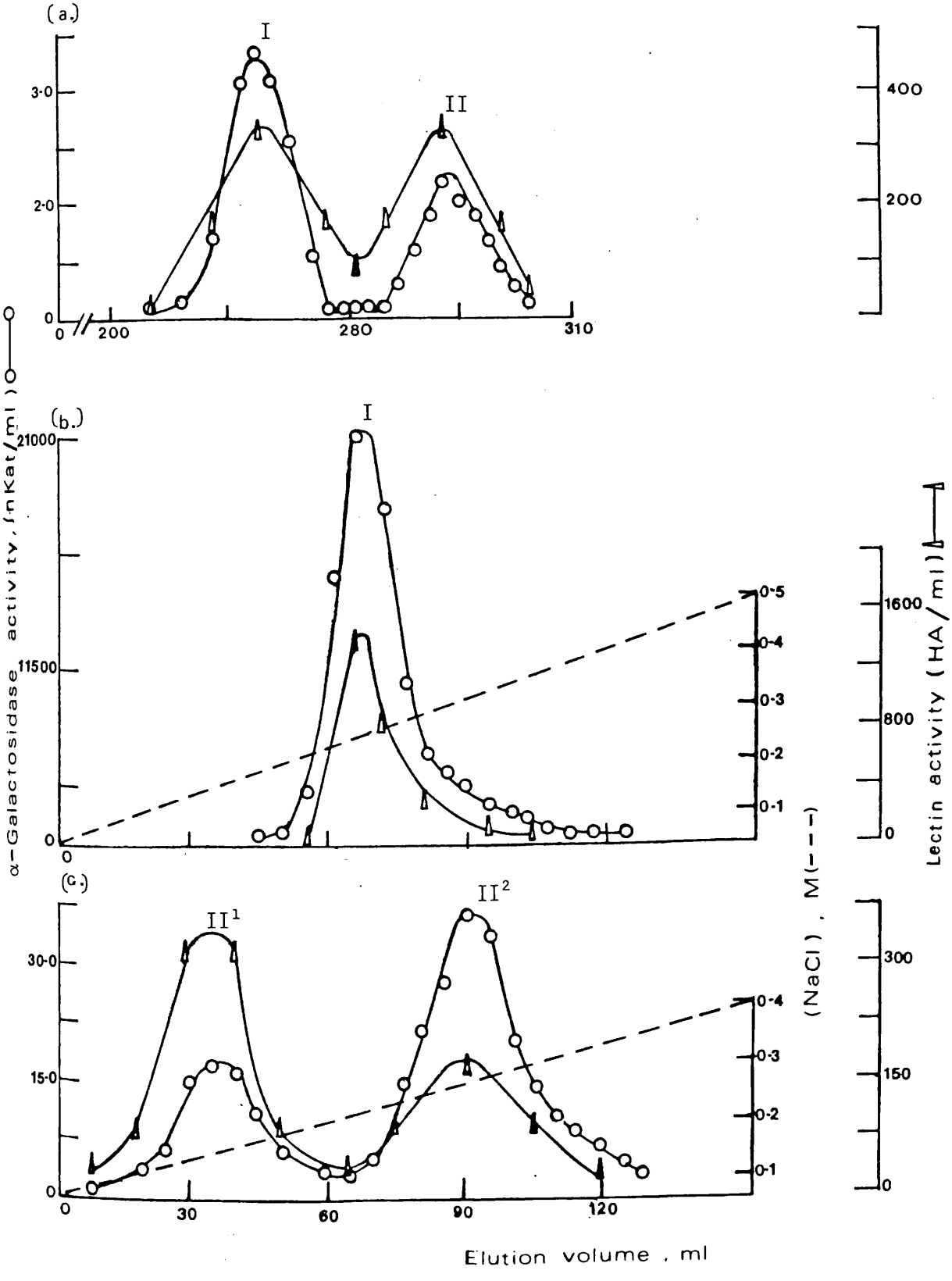
Co-elution of catalytic and lectin activities of V.faba α-galactosidases

(a) Sephadex G-100 gel filtration of α-galactosidases I and II (Stage 4; Table 1)

(b) CM-Cellulose chromatography of α-galactosidase I (Stage 5; Table 1)

(c) CM-Cellulose chromatography of α-galactosidase II (Stage 5; Table 1)

Experimental details are similar to those described for Figures 5 and 7.


(a.)
I
II
3·0
2·0
0
0
200
280
310
400
200
(b.)
I
21000
11500
0
0
0·5
0·4
0·3
0·2
0·1
1600
800
0
(c.)
II¹
II²
30·0
15·0
0
0
30
60
90
120
0·4
0·3
0·2
0·1
300
150
0
α-Galactosidase activity, (nKat/ml)
(NaCl), M(- - -)
Lectin activity (HA/ml)
Elution volume, ml

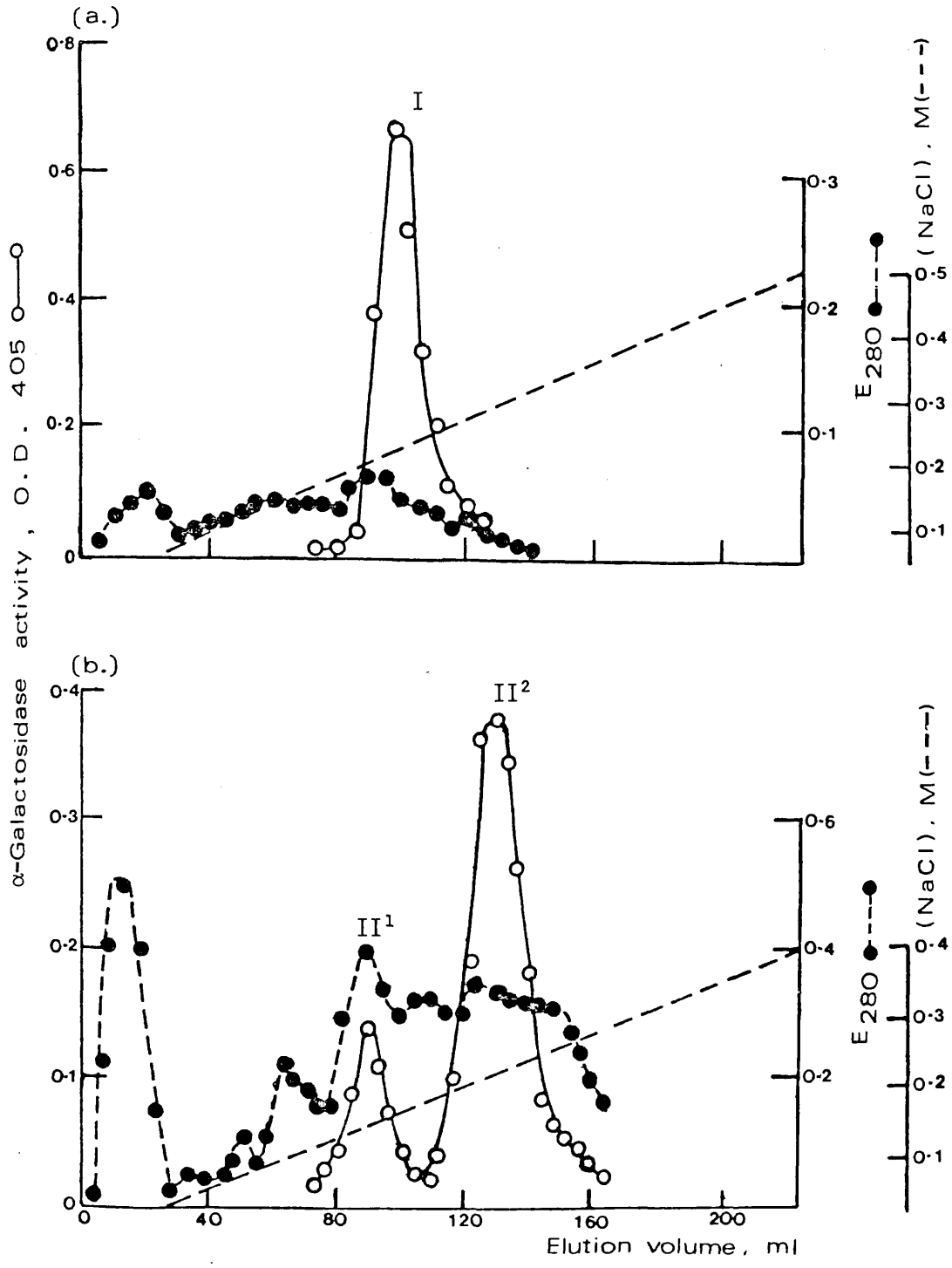


Figure 7

CM-Cellulose chromatography of α-galactosidases from V.faba seeds
(a) α-Galactosidase I (from Stage 5, Table 1);
(b) α-Galactosidase II (from Stage 5, Table 1).
The enzyme was resolved into two peaks, II^1 and II^2.
See Section 2.3.3. for further details.

with coincident lectin and enzyme activity (Fig.6c). The total enzyme activity of II^2 was found to exceed that of II^1 by a factor of 3 (Fig.7) if EII was applied to the CM-cellulose column within 10 days of the initial extraction of the enzyme from the seeds. The activity ratios changed in favour of II^1 if the enzyme preparation was stored for longer periods prior to applying to the column (see p.78).

The enzyme preparations at Stage 6 in this study were found to have higher specific catalytic activities (EI and EII^2 approximately 10-fold and EII^1 2-fold, respectively) than those obtained by Dey et al. [39] from CM-cellulose columns. The lower activities in the earlier work can probably be partly explained by the additional Con A-Sepharose step used by Dey et al. where not all of the enzyme activity was bound by the Con A-Sepharose column.

The final purification step (Stage 7) involved the use of a Melibiose-Sepharose affinity column which is highly specific for binding α-galactosidases [46].

The α-galactosidases (from Stage 6) were individually applied to the column in batches of one ml (Fig.8) and with all enzyme forms (using flow rates of less than 10 ml/h) the activities were completely bound to the columns. The enzymes were eluted with PNPG in McIlvaine buffer, pH 5.5. Following dialysis, the three α-galactosidases were subjected to analysis for catalytic and lectin activities. The enzyme/-lectin ratios in the case of EI were found to be essentially similar to the fraction that was originally applied to the column. Hence contamination of EI with favin was not apparent. With EII^1 and EII^2 the enzyme specific activities increased after affinity chromatography, but the lectin-specific activities decreased, suggesting further loss of contaminating lectin.

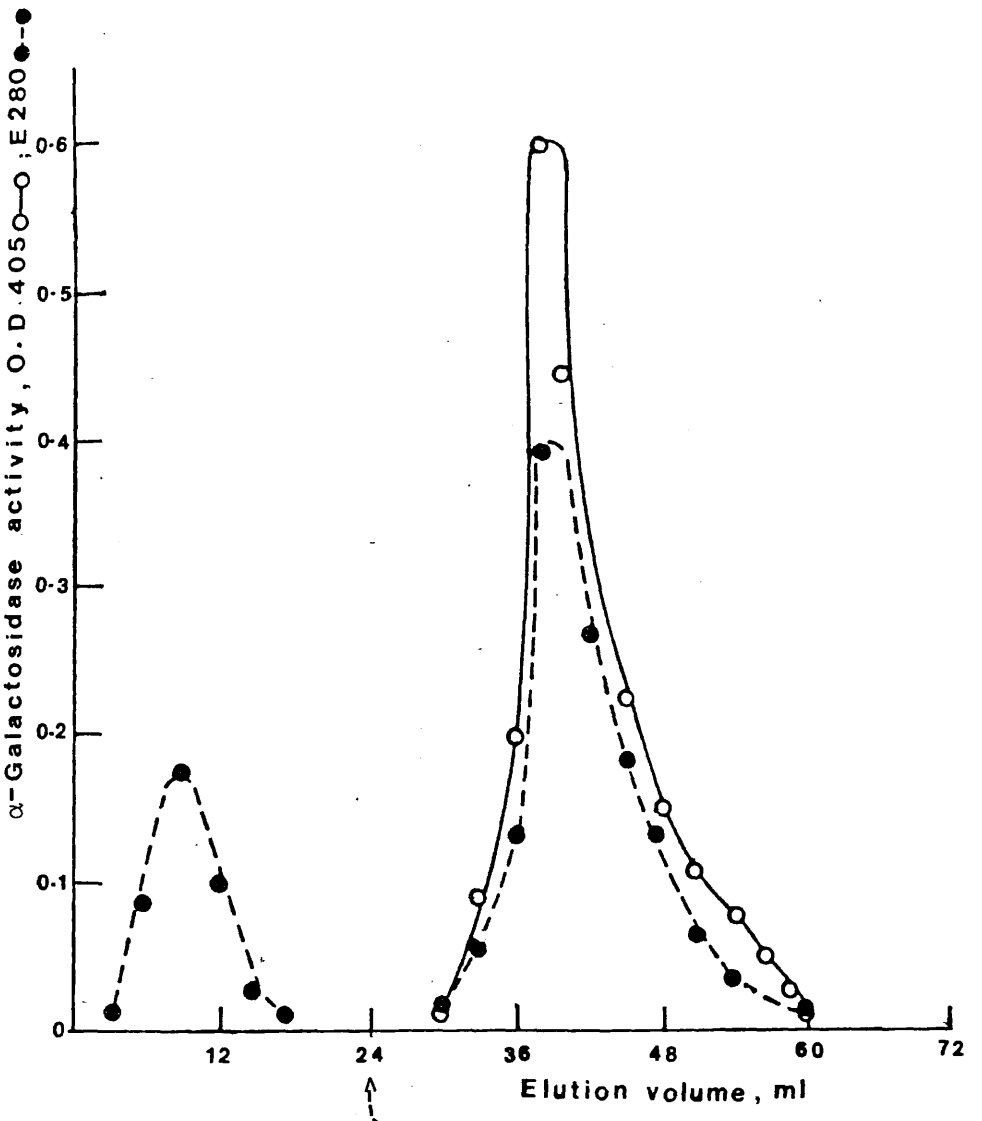


Figure 8

Affinity chromatography of α-galactosidase I using a Melibiose-Sepharose column.
See Section 2.3.2(i) for experimental details.

It is of interest to compare the haemagglutinating activities of EI, EII^1 and EII^2 obtained in the final stage of purification. Assuming EI is a tetramer of EII^2 (MW 41,000) and a MW of 43,000 for EII^1 [39], the activities/molecule subunit for EI and per molecule EII^1 and EII^2 are in a ratio 1:5:2, i.e. there is a relatively high haemagglutinating activity associated with the monomer EII^1. The situation is, therefore, different from that described for the monomeric α-galactosidases from the seeds of V.radiata, P. thunbergiana, T.caroliniana, L.arboreus, P.limensis and G.max, which are reported to possess no blood-clotting activity [8,46,137]. Hypocotyls from V.radiata contain an α-galactosidase-haemagglutinin (MW 170,000) which in the absence of D-galactose and mercaptoethanol dissociates into smaller units, probably monomers, these exhibit either catalytic or haemagglutinating properties, but not both [13].

3.1.2. Further attempts to detect favin contamination of α-galactosidase I

A further attempt was made to establish that the lectin activity of EI from V.faba was not due to a complex formed between EI and favin. α-Galactosidase I, obtained from Stage 7 of the purification procedure, was dialysed against 0.1 M potassium phosphate buffer (pH 7.2) containing 0.5 M glucose (a hapten to dissociate possible EI-favin complexes) for 2 h and the enzyme then stored at 4°C for 22 h. It was then applied to a Sephacryl S-200 gel filtration column which had previously been equilibrated with 0.1 M glucose in potassium phosphate buffer. The elution volume of EI was found to be identical to that obtained with untreated enzyme (elution volume 214 ml) on an unequilibrated column. Hence, there was no apparent change in the molecular weight of EI (Fig.9) on treatment with glucose, which suggests that no significant amounts of favin were bound to EI. Furthermore, the haemagglutinin activity associated with the EI fraction from the glucose-treated column was 96% of that applied to the column

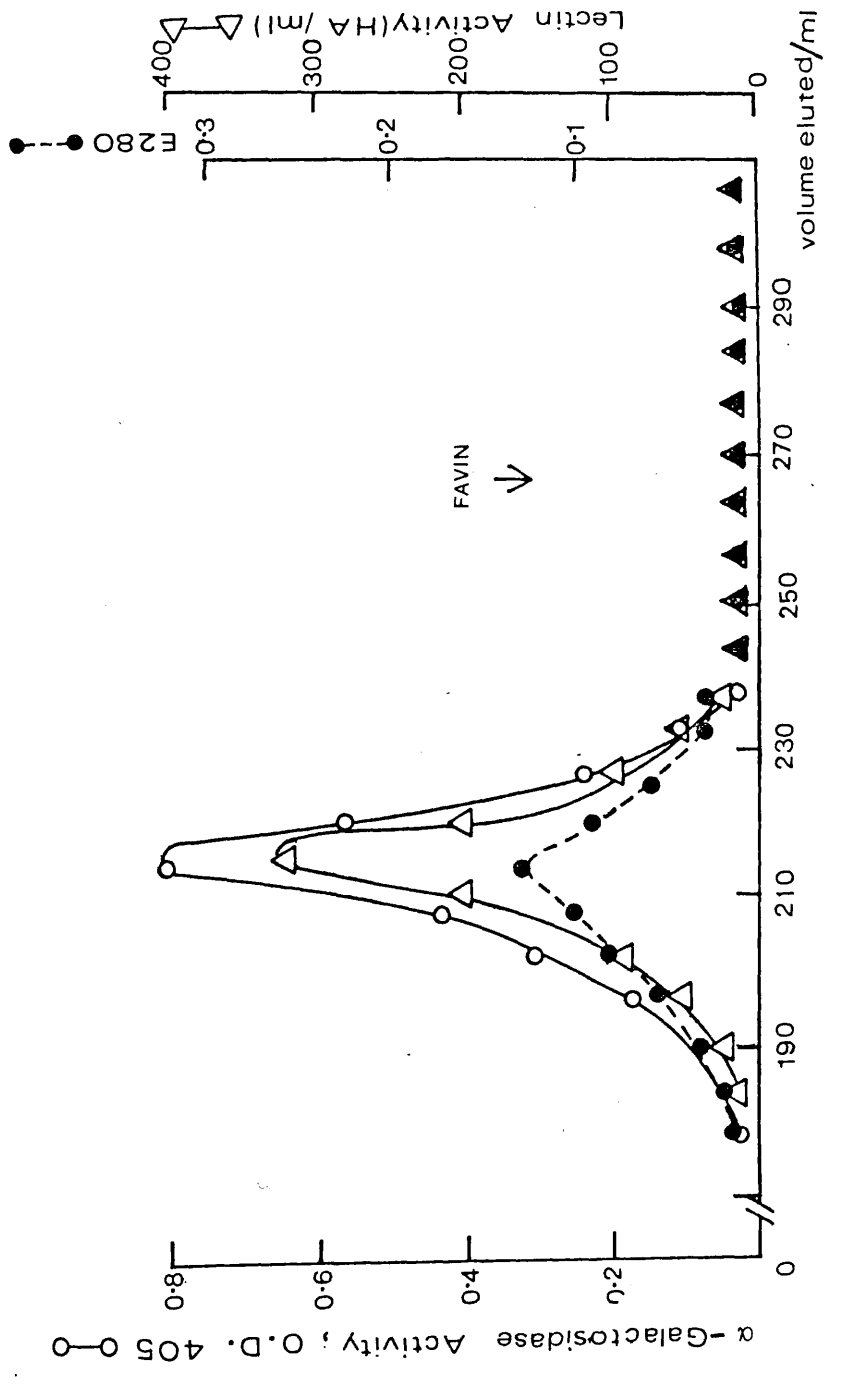


Figure 9

Sephacryl S-200 gel filtration profile of α-galactosidase I (from Stage 7, Table 1) in the presence of D-glucose. Elution was with D-glucose containing buffer. Arrow shows hypothetical elution volume of favin.
See Section 2.9 for experimental details.

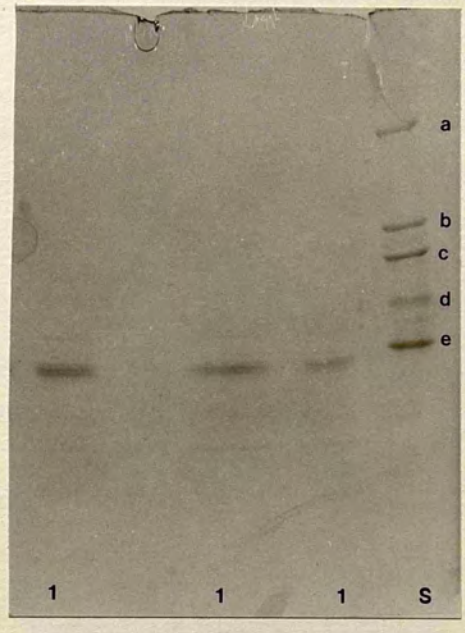
and no fraction with haemagglutinin activity corresponding to the elution volume of favin (or its subunits) was detectable in the column fractions upon dialysis.

3-O-Methyl-N-hexanoyl-α-glucosamine-Sepharose strongly absorbs favin [121]. When all three forms of V.faba α-galactosidase (from Stage 7; Table 1) were separately examined on this affinity gel, no catalytic or haemagglutinating activity appeared to be absorbed, again giving no indication of favin contamination.

3.1.3. The homogeneity and monomer composition of V.faba α-galactosidases

The homogeneity of the three α-galactosidase forms (from Stage 7; Table 1) was investigated by SDS-polyacrylamide slab gel electrophoresis (Fig.10). α-Galactosidase I migrated as a single protein band (MW 44,500) with evidence of microheterogeneity, EII^2 as three bands (MW 44,600 - major; and 52,500 and 90,000 - minor) and EII^1 as one major band with MW 49,545. The molecular weights of the favin subunits are 22,500 and 18,000 and there was no indication of the presence of either of these in any of the enzyme forms under the conditions that were used (Fig.11, see also Fig.10).

The results of SDS-PAGE described above and those described by Dey et al. [39] are similar. There are clear indications that EI is a tetrameric form composed of monomers with molecular weight approximating to 44,500. Although the present study indicated that EI was only microheterogeneous, the work of Dey et al. [39] showed that the dissociation of EI by SDS produced mainly EII^2 and also small, but significant, amounts of two other proteins with molecular weights greater than EII^2. However, the specific activity of EI in the present study was 10 times greater than that observed by Dey et al. In view of the observed microheterogeneity of EI it is likely that this enzyme is not a single molecular



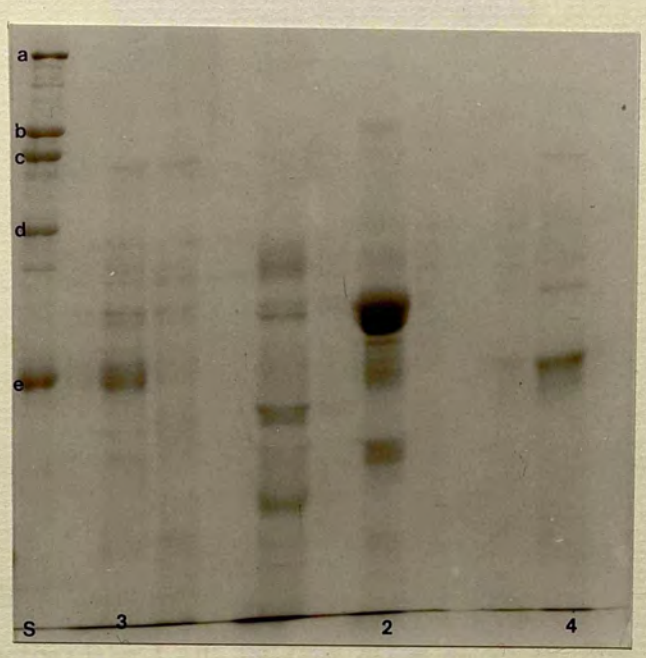


Figure 10

SDS-PAGE of V.faba α-galactosidase I, II[1] and II[2]

S represents the track for marker proteins:
(a) myosin, MW 200,000; (b) ß-galactosidase, MW 130,000; (c) phosphorylase b, MW 90,000; (d) bovine serum albumin, MW 65,000; (e) ovalbumin, MW 45,000.

1. α-Galactosidase I (ca 5 µg) (from Stage 7; Table 1)
2. α-Galactosidase II[1] (25 µg) " " "
3. α-Galactosidase II[2] (10 µg) " " "
4. α-Galactosidase II[2] (5 µg) " " "

Experimental details are given in Section 2.5(a).

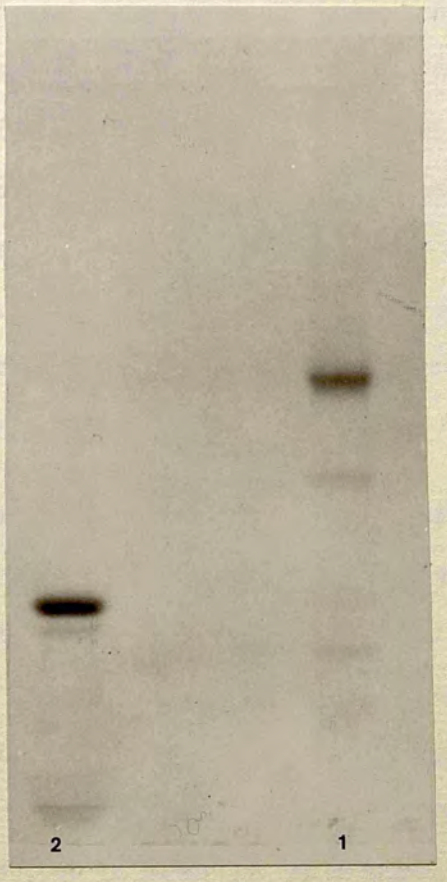


Figure 11

SDS-PAGE pattern of α-galactosidase I and favin from V.faba seeds

(1) α-Galactosidase I (10 µg;from Stage 6, Table 1)
(2) Favin (15 µg).

For experimental details see Section 2.5(b).

species, but a family of closely related tetramers composed of near identical monomers with molecular weight approximating to 44,500. This would be expected, for example, if EI was synthesized from monomers with different degrees of glycosylation [185]. Mixtures of monomers might also result from minor post-translational degradations of polypeptide chains. If such suggestions are correct, then the relative amounts of different monomers (both free and associated) may well vary with the age and treatment received by the seeds prior to isolation of the enzymes.

In addition to SDS dissociation, the effect of urea on α-galactosidase I was investigated. Treatment of the enzyme with varying concentrations (2.5-5.0 M) of urea for 2 h at pH 5.5 followed by examination on the Sephacryl S-200 columns, appeared to yield a range of enzymically active dissociation products (Fig.12). With 2.5 M urea (Fig.12b) a small proportion of enzyme I was converted to a form with molecular weight 80,000 (a dimer?). At a higher concentration (3.5 M; Fig.12c) most of enzyme I was dissociated to lower molecular weight forms, mostly dimer and possibly some trimer (MW 120,000). Dissociation of enzyme I largely to the monomer was achieved with 5.0 M urea (Fig. 12d). The fractions containing this monomer were pooled and subjected to CM-cellulose ion-exchange chromatography. A single peak of enzyme activity which corresponded to enzyme II^2 (which is separable from EII^1: Fig.13b; see also Fig.7b) was eluted with a continuous NaCl gradient. Purified EII^2 was also mixed with the monomer produced by 5 M urea and the two proteins were observed to elute as a single peak (Fig.13c) providing further proof of the identity of the monomer.

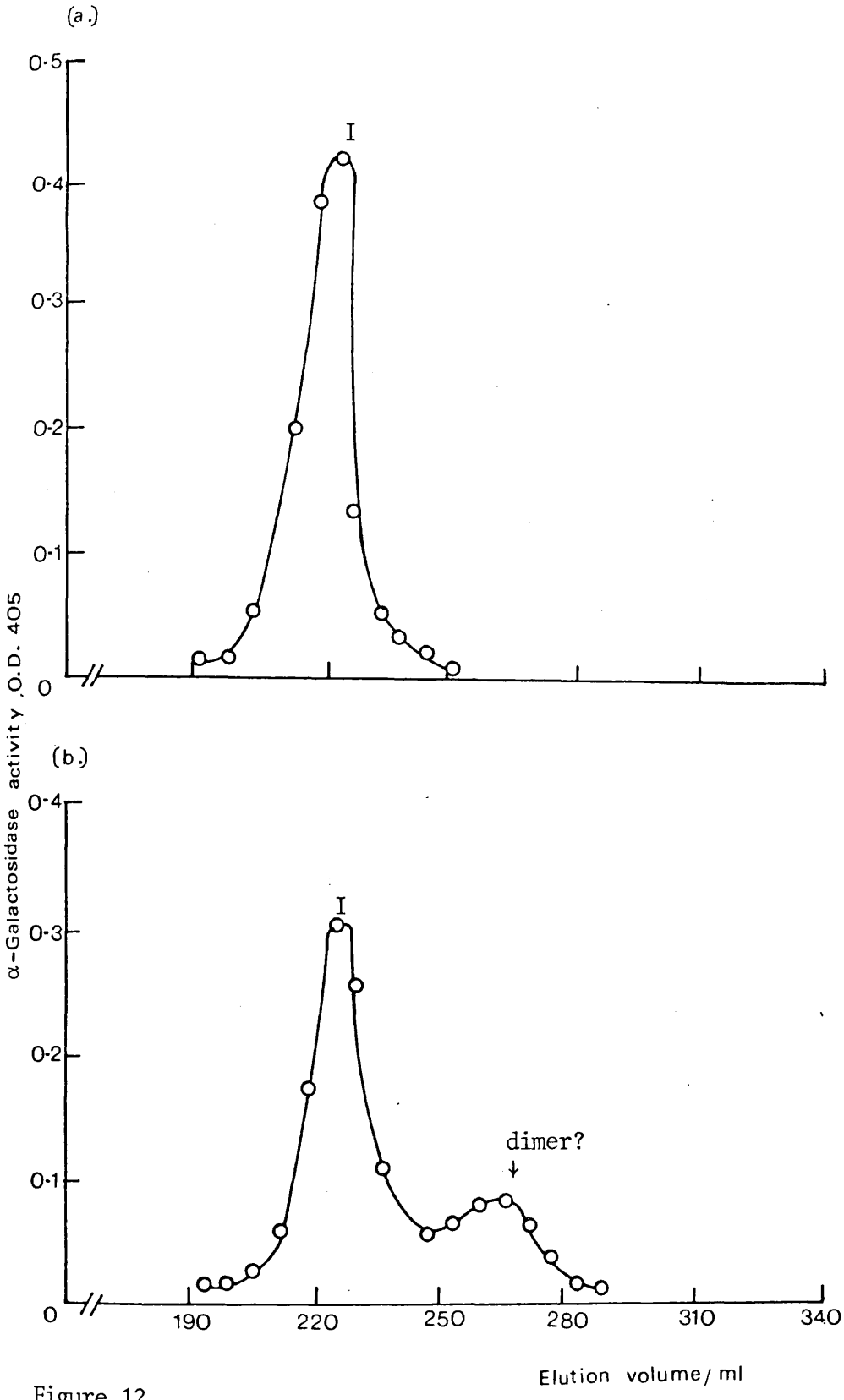


Figure 12

Treatment of α-galactosidase I (from Step 7, Table 1) with urea and its effect on Sephacryl S-200 gel filtration profile:
(a) untreated; (b) 2.5 M urea treated.
See Section 2.6 for experimental details.

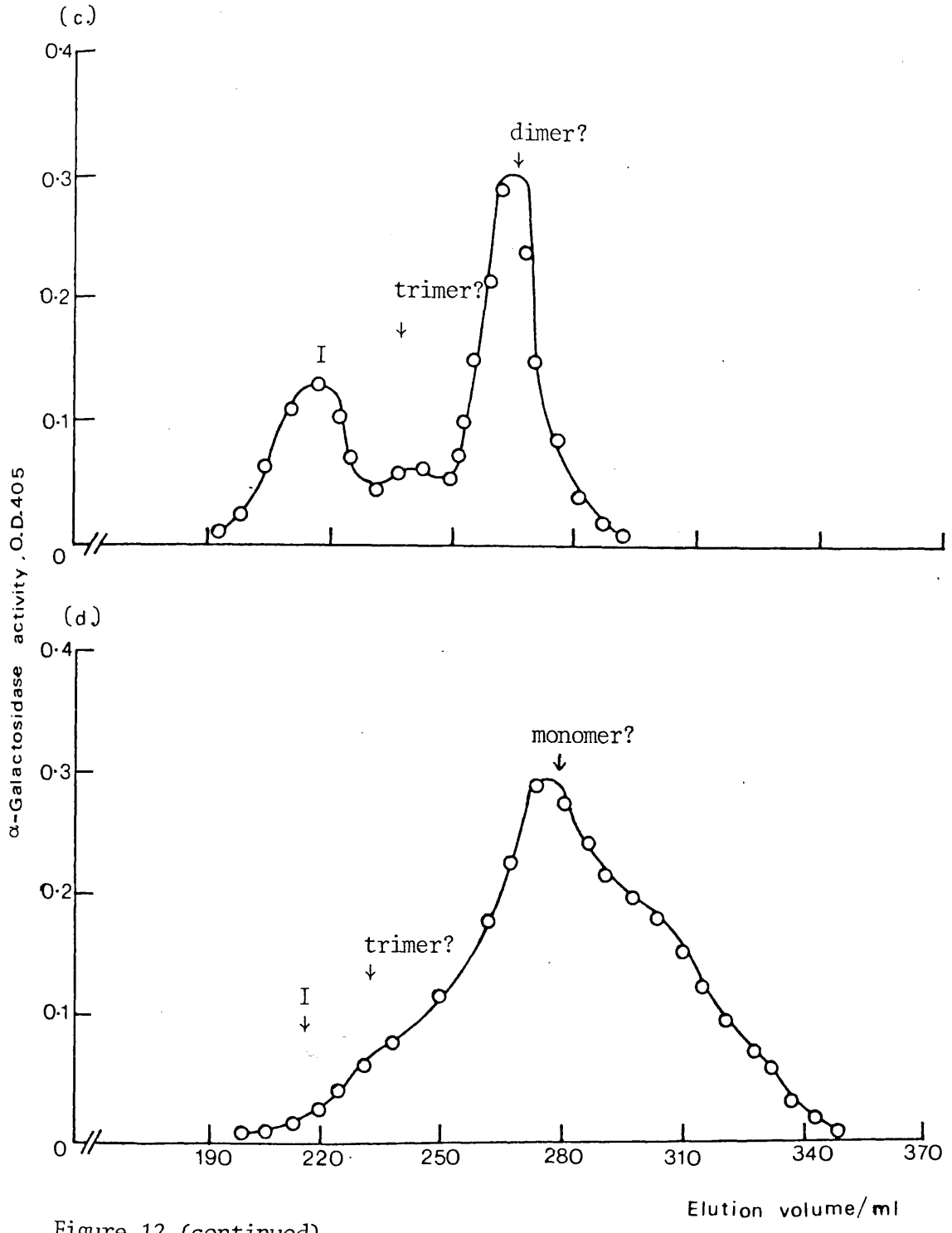


Figure 12 (continued)

Treatment of α-galactosidase I (from Step 7, Table 1) with urea and its effect on Sephacryl S-200 gel filtration profile.
(c) 3.5 M urea; and (d) 5.0 M urea treated.
See Section 2.6 for experimental details.

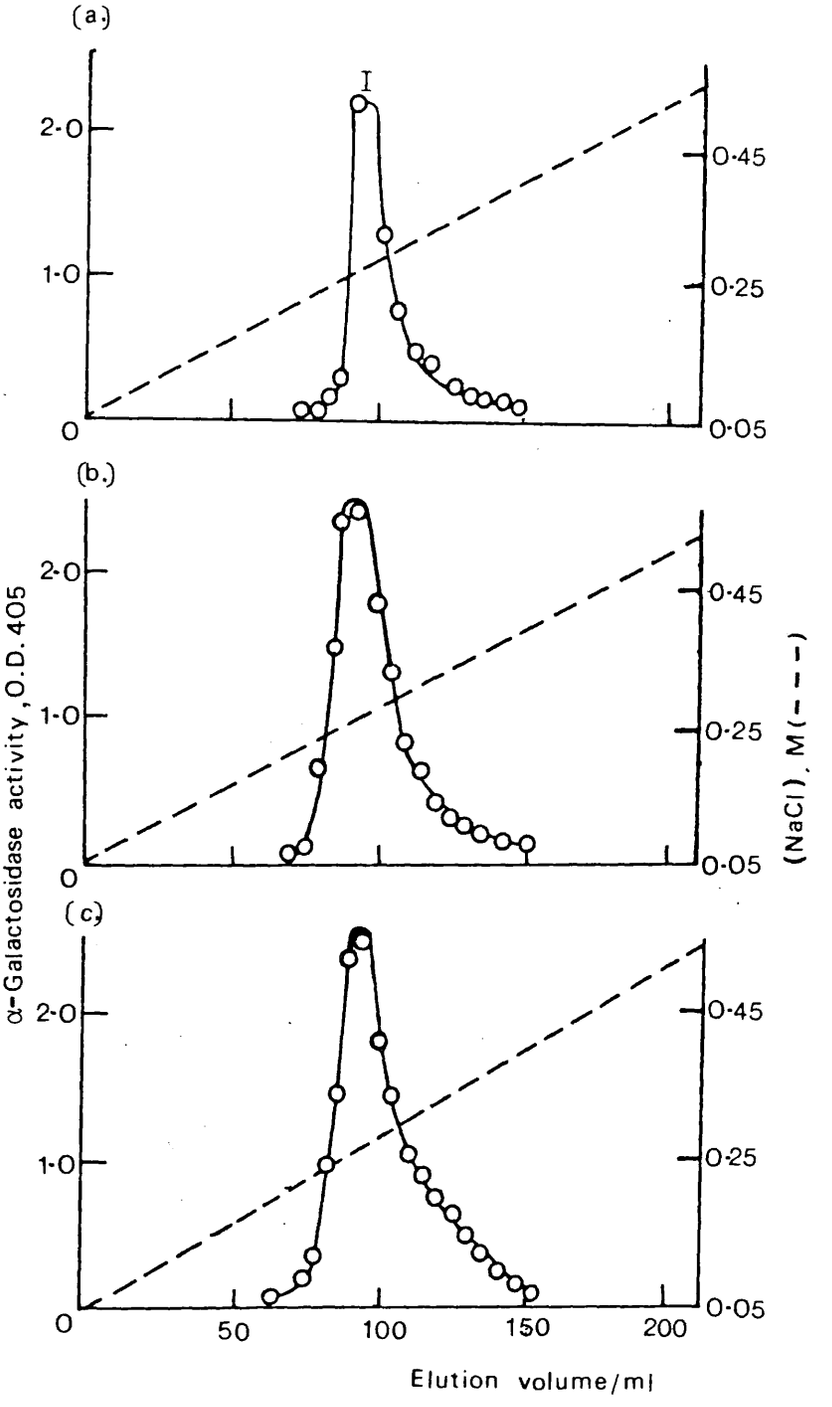


Figure 13

CM-cellulose chromatography of (a) native α-galactosidase I (Stage 7, Table 1); (b) enzyme isolated after treatment with 5.0 M urea (elution volume 250-290 ml, Fig. 12 d) and (c) a mixture (1.1 with respect to activity) of isolated enzyme as used in (b) and α-galactosidase II[2] (Step 7, Table 1). See Section 2.6 for experimental details.

In the absence of urea, dialysis of α-galactosidase I for 2 h against buffers at pH 3.5 and at pH 7.0 yielded no dissociation products (when applied to Sephacryl S-200 columns equilibrated with buffers of appropriate pH). The tetrameric α-galactosidases from V.radiata [45] and G.max [46] seeds both dissociate to monomers at pH 7.0 and this can be reversed by lowering the pH to 4.0.

The structural complexities of α-galactosidases I, II^1 and II^2 purified by the 7-stage procedure were investigated further by the use of chromatofocusing, which can be regarded as isoelectric focusing on an ion-exchange column and it is one of the most powerful tools for resolving protein mixtures. For example, it has been used for the purification of β-N-acetylhexosaminidase isoenzymes, where separation cannot be achieved by any other physicochemical method [186], and for the separation of human α-foetoprotein from albumin and other serum proteins [187].

Following application of the α-galactosidase sample, the column was eluted with Polybuffer 96 (pH range 9.0 to 6.0), the resulting fractions were assayed for α-galactosidase activity and the pH was also measured. The elution profiles are shown in Figure 14. This method separated EI into multiple enzymically active fractions with different pI values ranging from pH 8.75 to 7.35. In the case of EII^1 and EII^2 both were again resolved into several components, but it was not clear whether any of these were common to both enzymes or to EI. The activity pattern for EII^1 shows one major component (pI 8.4) which is apparently absent from the EII^2 profile. The latter shows the presence of 5 distinct fractions with pI values of 8.75, 8.0, 7.35, 6.9 and 6.35.

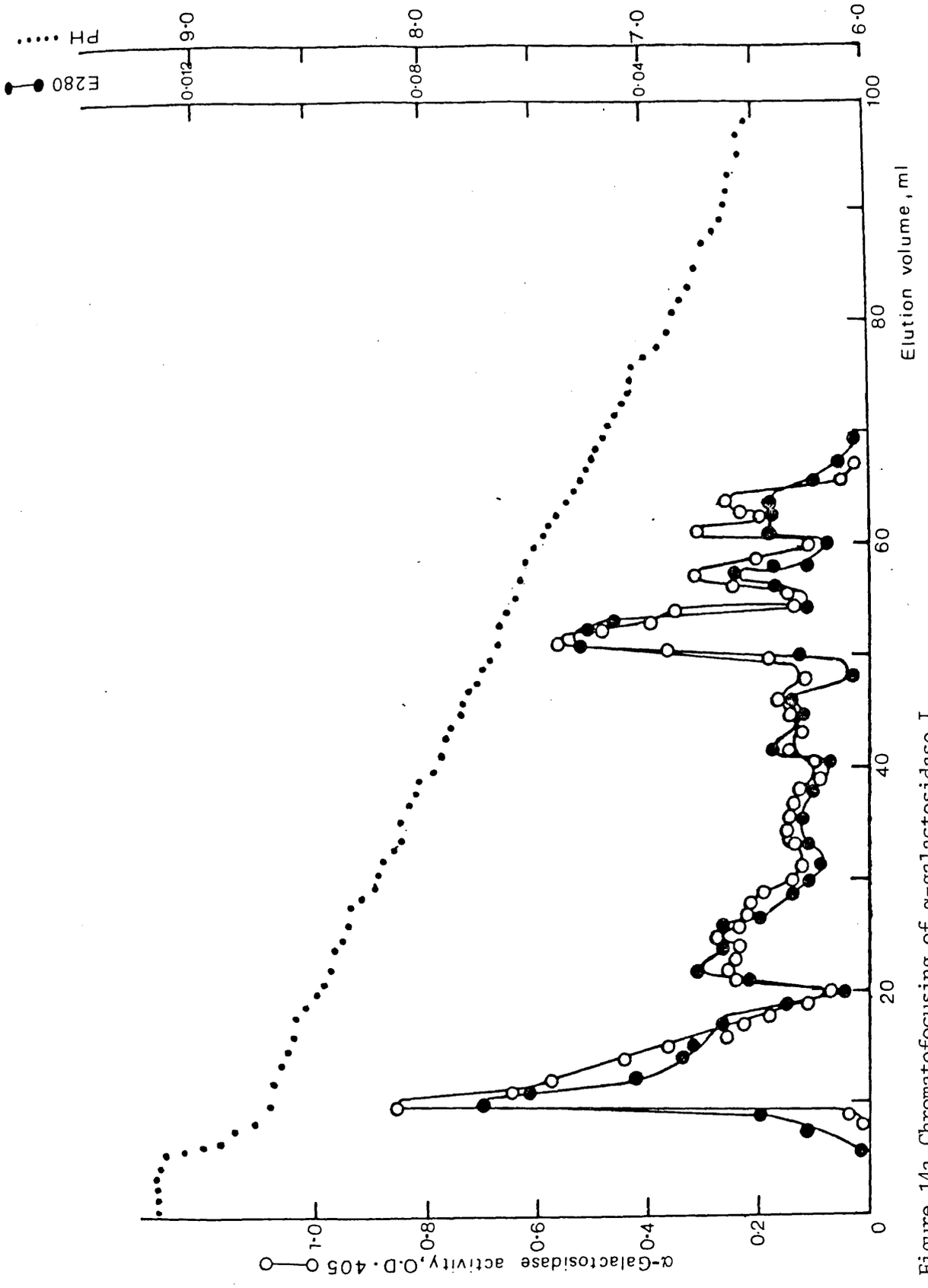


Figure 14a Chromatofocusing of α-galactosidase I (from Stage 7, Table 1), from V.faba seeds
See section 2.7.1 for experimental details

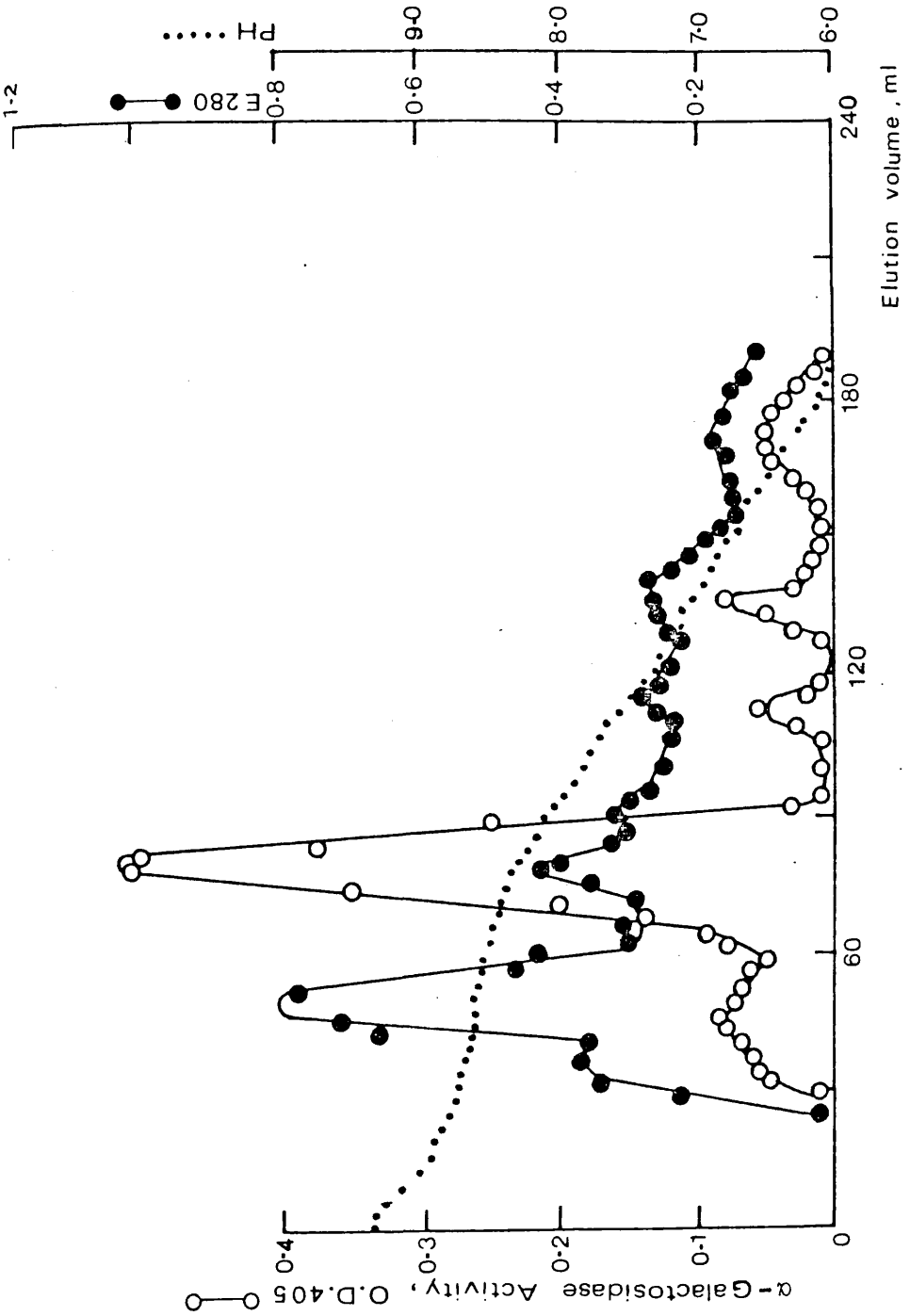


Figure 14b Chromatofocusing of α-galactosidase II[1] (from Stage 7, Table 1) from V.faba seeds

See Section 2.7.1. for experimental details

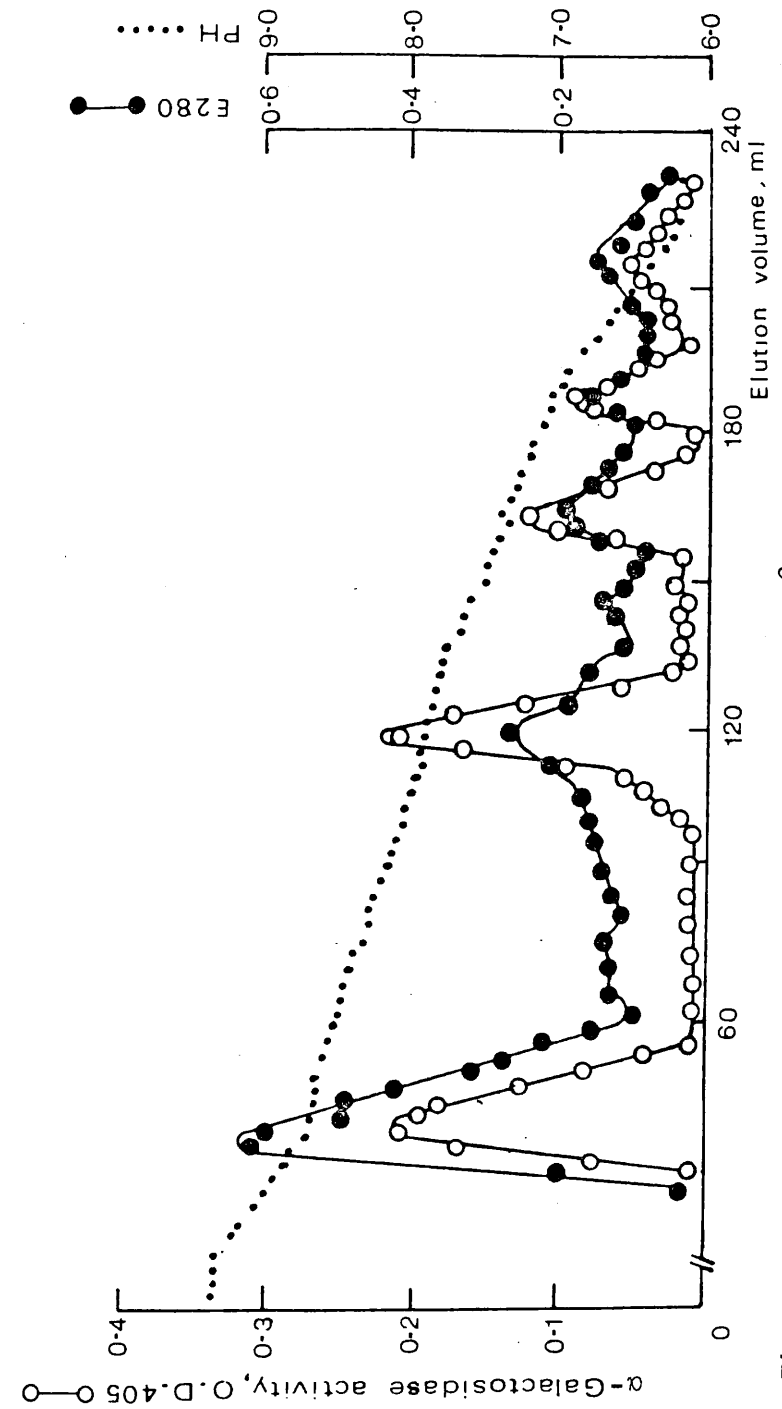


Figure 14c Chromatofocusing of α-galactosidase II[2] (from Stage 7, Table 1) from V.faba seeds

See Section 2.7.1. for experimental details.

The results of the chromatofocusing study were in the main confirmed by isoelectric focusing (Fig.15) which again revealed the complex nature of all three α-galactosidase fractions: enzyme I produced a rather diffuse pattern which equates with the apparent large number of peaks obtained by chromatofocusing (Fig.14a).

Isoelectric focusing has previously been used to study α-galactosidases. For example, examination of the monomeric (II; MW 40,000) and tetrameric (I; MW 160,000) forms of the enzymes from imbibed V.radiata seeds produced a single identical peak in both cases with a pI value of 7.8 [45]. Similarly, α-galactosidases I and II from L.culinaris (with approximately the same molecular weights as the corresponding V.radiata enzymes) both exhibited an apparent isoelectric point of pH 8.5 which was presented as evidence that EI consisted of 4 identical subunits of EII [41]. The dimeric and tetrameric forms of α-galactosidase-haemagglutinins (MW 71,000 and 160,000 respectively) from V.radiata hypocotyls were, however, reported to possess different isoelectric points with pH 4.5 for the tetrameric form and 8.5 for the dimeric form [13].

In the case of Con A, more complex isoelectric focusing patterns have been obtained. A homogeneous crystalline sample of this lectin was reported to separate into at least eight protein bands. Upon pre-incubation with D-mannose the main protein bands with isoelectric points between pH 5.9 and 7.3 became insignificant and two new protein bands appeared in the region between pH 7.6 and 8.0. This shift of isoelectric points towards the pH range 7.6 to 8.0 was enhanced by increasing the D-mannose concentration, hence implying changes in the conformation of Con A resulting from binding of carbohydrates [188].

Figure 15

Isoelectric focusing of α-galactosidases (a) I; (b) II^1 and (c) II^2 (from Stage 7, Table 1) from V.faba seeds

Experimental details are given in Section 2.7.2.
The gels were stained for enzymic activity as described in Section 2.1.2.

Returning to the preliminary results obtained from the chromatofocusing and isoelectric focusing studies with the V.faba α-galactosidases, the complex profiles may be further indications of the micro-complexities of those enzymes which were discussed on p.91 resulting from in vivo processing reactions. On the other hand, in vitro reactions producing heterogeneity during enzyme purification cannot be ruled out, although efforts were made to avoid this by working and storing the enzymes at low temperatures.

It is clear that much work needs to be done to establish relationships between pI values and the physical and chemical structures of proteins in general, and in particular to discover the source of the α-galactosidase components with different pI values. Are they artefacts or genuine cell components?

3.2. The Carbohydrate Specificity of the Lectin Sites of α-Galactosidases I, II^1 and II^2 from V.faba Seeds

3.2.1. Hapten inhibition of haemagglutination reactions

Having demonstrated that the three forms of α-galactosidases from V.faba seeds agglutinated red blood cells and, hence that they were lectins, a study of the carbohydrate specificities was made. This was investigated firstly by the hapten inhibition technique [189]. Table 2 shows the effects of various carbohydrates on the agglutination of rabbit erythrocytes by α-galactosidases I, II^1 and II^2. The data clearly indicate that D-mannose and, to a lesser extent, D-glucose together with low molecular weight derivatives of these monosaccharides

TABLE 2

Carbohydrate inhibition of the lectin activities of α-galactosidases I,II[1] and II[2] from V.faba seeds [121]

Sugar	Minimum concentration (mM) causing 50% inhibition of 6-haemagglutinin units *		
	I	II[1]	II[2]
D-Glucose	31.3	8.36	19.60
3-O-Methyl-α-D-glucopyranose	31.3	8.36	19.60
Methyl-α-D-glucopyranoside	62.5	16.72	39.20
Methyl-β-D-glucopyranose	250	100	125
N-Acetyl-glucosamine	31.3	8.36	19.60
D-Mannose	15.6	4.68	9.40
Methyl-α-D-mannopyranoside	3.1	2.00	1.00
D-Galactose	1000	1000	1000
Methyl-α-D-galactopyranoside	1000	1000	1000
D-Xylose	250	100	125
L-Arabinose	250	125	250
Melibiose	100	62.5	125
Stachyose	100	62.5	125
Raffinose	100	62.5	125
Sucrose	125	-	-
Galactomannan (clover)	0.12 mg/ml	0.06 mg/ml	0.10 mg/ml
Galactomannan (guar gum)	0.09 mg/ml	0.05 mg/ml	0.10 mg/ml
Glycogen	0.03 mg/ml	0.02 mg/ml	0.03 mg/ml
Starch (soluble)	0.03 mg/ml	0.02 mg/ml	0.03 mg/ml
α-Mannan (Baker's yeast)	-	0.03 mg/ml	0.03 mg/ml

* Experimental details are given in Section 2.8.2.

were significantly effective in producing inhibition of agglutination than D-galactose and its derivatives.

A further indication of the mannose specificity of the lectin site of α-galactosidase I was obtained by passing the enzyme through a 'methyl-α-D-mannoside-Agarose' column, an affinity column for glucose/-mannose-binding proteins. The enzyme was fully absorbed, but could be eluted from the column with 0.5 M methyl-α-D-mannoside. The enzyme/-lectin ratio of the preparation applied to the column was similar to that for the enzyme fraction eluted from the column by methyl-α-D-mannoside (Table 3; Fig.16).

Methyl-α-D-glucoside (Table 2) was four times more effective as a haemagglutinin inhibitor than the β-D-anomer, thus suggesting α-D-specificity as has been observed with glucose/mannose specific lectins [121].

It should be noted that 3-O-methyl-D-glucose has some inhibitory effect on the haemagglutination activity of all three α-galactosidases, however, favin is much more sensitive to this glucose derivative [121].

Some oligosaccharides and polysaccharides also acted as rather weak agglutination inhibitors of the α-galactosidases. The oligosaccharides included sucrose, melibiose, raffinose and stachyose, and here the inhibitory effect may have been a function of the glucosyl residues all of these compounds possess. Hapten inhibition by galactomannans is presumably due to the mannosyl residues in the 'backbones' of these polymers.

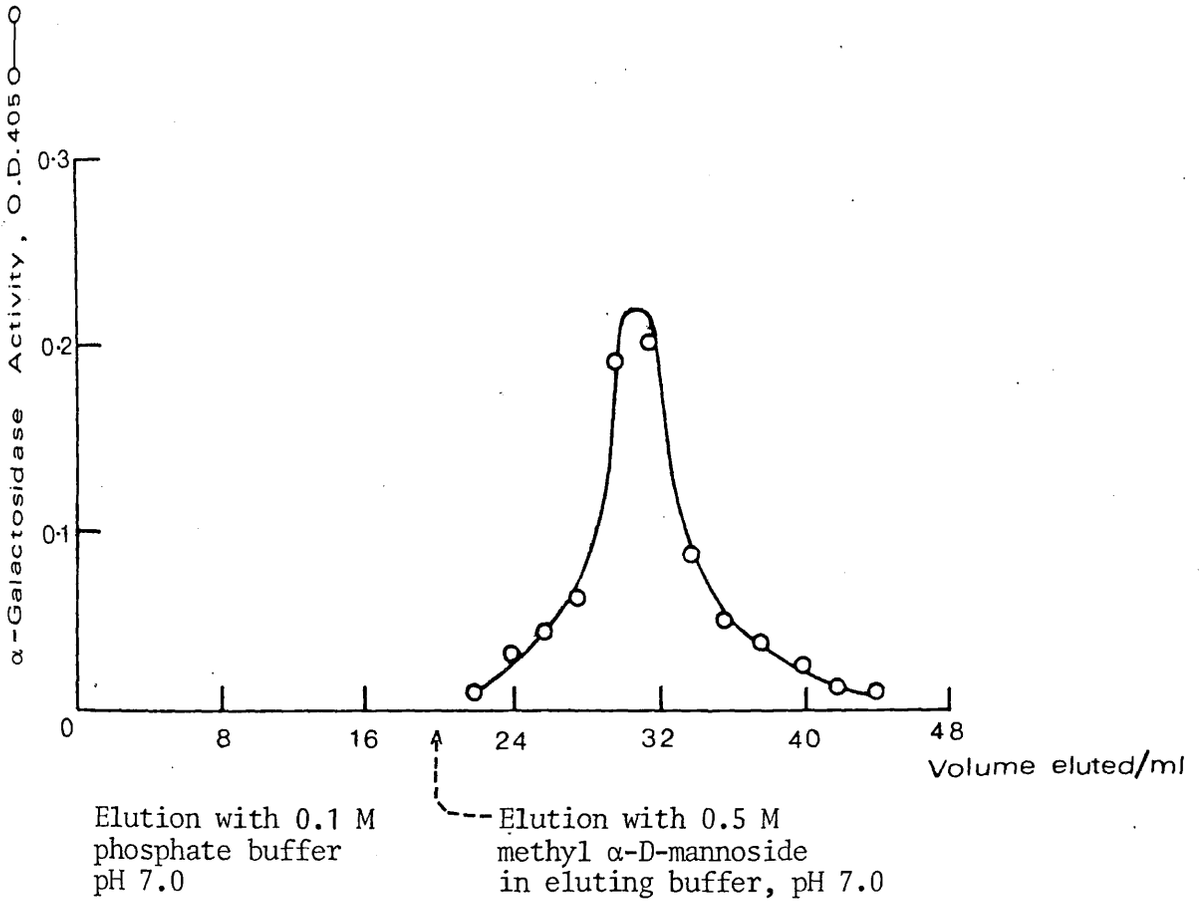


Figure 16

Methyl-α-D-mannoside-Agarose affinity chromatography of α-galactosidase I (from Stage 7, Table 1) from V.faba seeds

See Section 2.3.2(ii) for experimental details.

TABLE 3 The behaviour of α-galactosidase I on a methyl-α-D-mannoside-Agarose column

	Volume (ml)	Protein (mg)	Specific activity (nkat/mg) [A]	Lectin activity (HA/mg) [B]	Ratio A/B
Enzyme applied to column*	3.0	0.02	12,500	770	16.2
Enzyme fraction eluted with methyl α-D-mannoside	1.2	0.04	11,400	790	14.4

* purified by 7-stage procedure (see Table 1); see Section 2.3.2(iii) for experimental details.

The lectin specificities of the V.faba α-galactosidases I, II[1] and II[2] are, therefore, similar to 'classical' lectins found in many plant tissues, but not to the 'so-called' α-galactosidase-haemagglutinins from V.radiata and G.max, which are reported to be galactose-specific (see p. 39). In legume seeds the 'classical' glucose/mannose specific lectins include favin (ex. V.faba), Con A (ex. C.ensiformis) and lectins from L.culinaris and P.sativum. Inhibition of haemagglutinins by 3-O-methyl-D-glucose could be used to divide these lectins into two groups: favin and the lectins from L.culinaris and P.sativum are strongly inhibited by this sugar derivative, whereas Con A and the V.faba α-galactosidases are not. In the case of 3-O-methyl-D-glucose-sensitive lectins, it has been suggested that inhibition may be caused by hydrophobic binding of methyl groups to these proteins and that non-sensitive lectins do not possess hydrophobic binding sites [121].

3.2.2. Precipitation reactions of α-galactosidases with polysaccharides

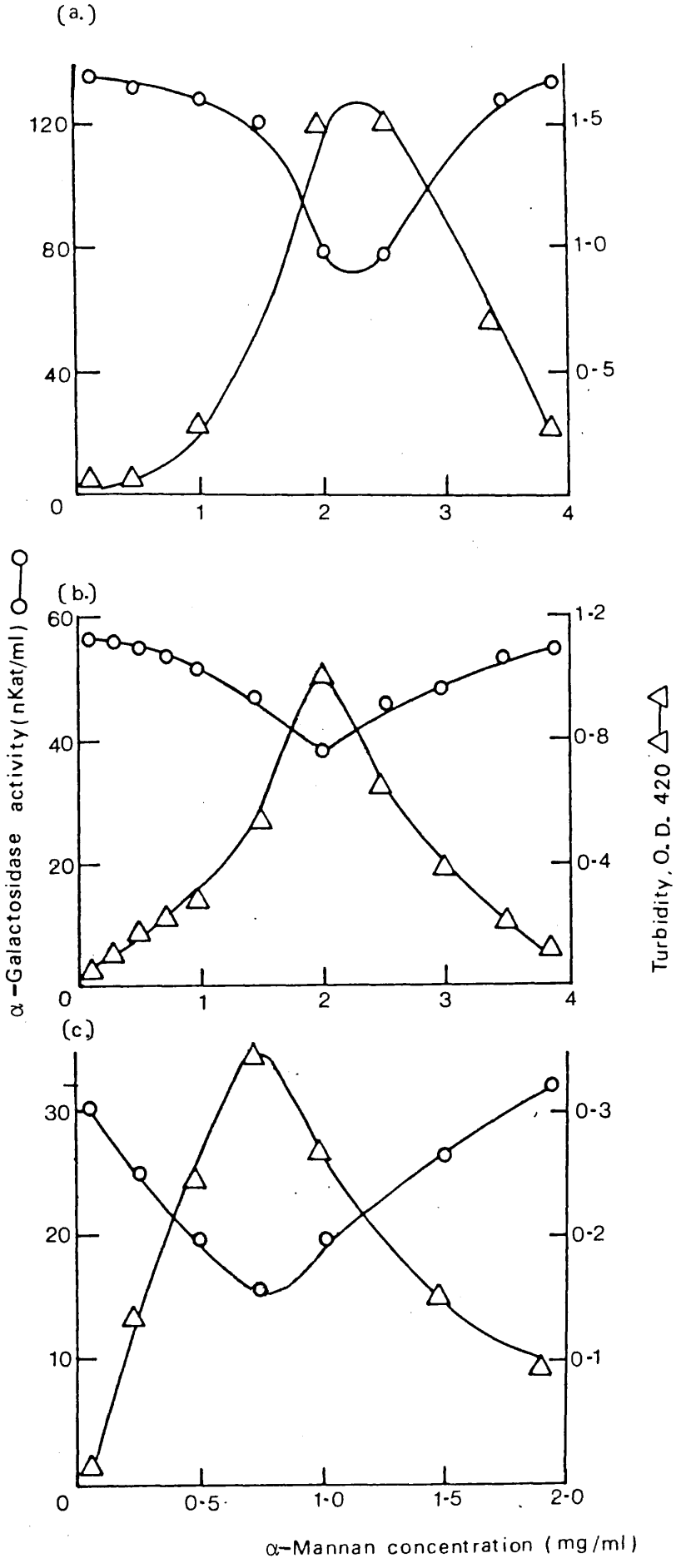
In addition to haemagglutinin reactions, all forms of the V.faba α-galactosidases were observed to precipitate yeast α-mannan, glycogen and soluble starch. With yeast α-mannan normal bell-shaped curves were obtained with increasing concentrations of polysaccharides (Fig.17) and a reciprocal relationship was observed between turbidity and enzyme activity in the supernatant with all forms of the enzyme. This could be fully accounted for by changes in the amounts of the enzymes bound to the α-mannan.

Interactions between lectins and polysaccharides are dependent on the concentrations of both components. In the case of the α-galactosidases and α-mannan it was found that optimum turbidity, under

Figure 17

Interaction of α-galactosidases (a) I; (b) II^1 and (c) II^2 (from Stage 7, Table 1) from V.faba seeds with yeast α-mannan

Experimental details are given in Section 2.1.4(a).


(a.)
120
80
40
0
1
2
3
4
1·5
1·0
0·5
(b.)
60
40
20
0
1·2
0·8
0·4
(c.)
30
20
10
0
0·5
1·0
1·5
2·0
0·3
0·2
0·1
α-Galactosidase activity (nKat/ml)
Turbidity, O.D. 420
α-Mannan concentration (mg/ml)

standard conditions, was produced when the molar concentrations of EII^2 was four times greater than that of EI: in the case of EII^1 only half that of EII^2 was required.

There appears, therefore, to be a relationship between the number of binding sites per molecule of each of the enzymes and the concentration required for optimum turbidity at constant α-mannan concentration, which relates well to the actual number of binding sites determined by equilibrium dialysis (see p.127).

As expected from the hapten inhibition studies, the turbidity produced with all three α-galactosidases and α-mannan could be prevented by including D-mannose (0.1 M) in the incubation mixtures.

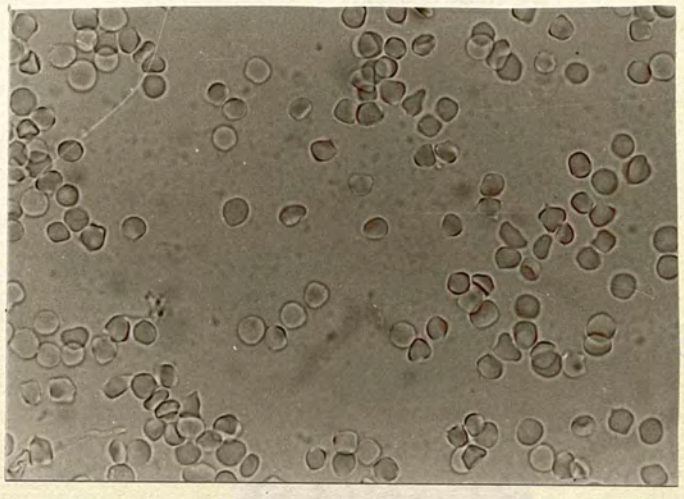
3.2.3. Binding of α-galactosidase I to starch granules and sheep erythrocytes

A further demonstration of lectin binding using α-galactosidase I was achieved by fluorescence microscopy relying on the presence of free catalytic sites in the bound complex. These were detectable after a short incubation of the complex with an α-galactosidase substrate, 4-methylumbelliferyl-α-D-galactoside, which on hydrolysis released the fluorescent 4-methylumbelliferone aglycon.

The haemagglutinin activity of α-galactosidase I is clearly illustrated in Figure 18 which shows the agglutination of sheep erythrocytes by the enzyme.

Figure 19(a) shows that after 2 min incubation with the enzyme substrate there is an intense fluorescence surrounding the agglutinated

(a)



(b)

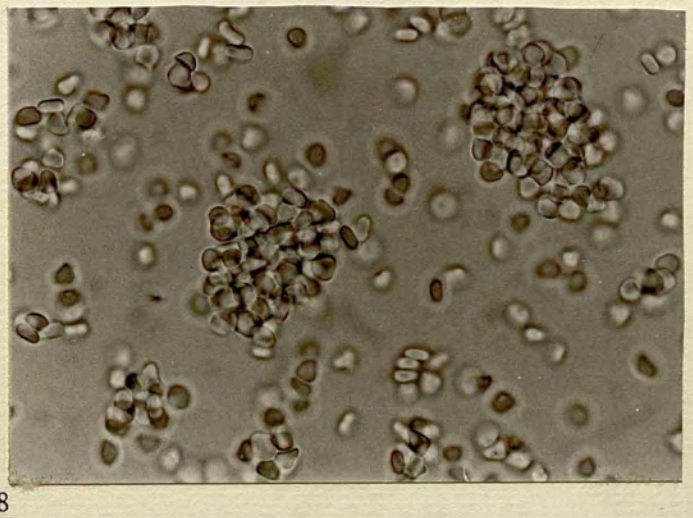


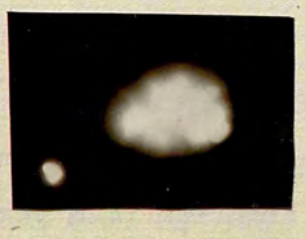
Figure 18

Agglutination of sheep erythrocytes:

(a) sheep erythrocytes in PBS (x500)
(b) sheep erythrocytes agglutinated by incubating with α-galactosidase I (from Stage 7, Table 1) from V.faba seeds.

Experimental details are described in Section 2.1.3(b).

(a)



(b)

Figure 19

Display of fluorescence by α-galactosidase I (from Stage 7, Table 1) from V.faba seeds bound to agglutinated erythrocytes, 2 min (a) and 15 min (b) after addition of 4-methylumbelliferyl-α-D-galactoside

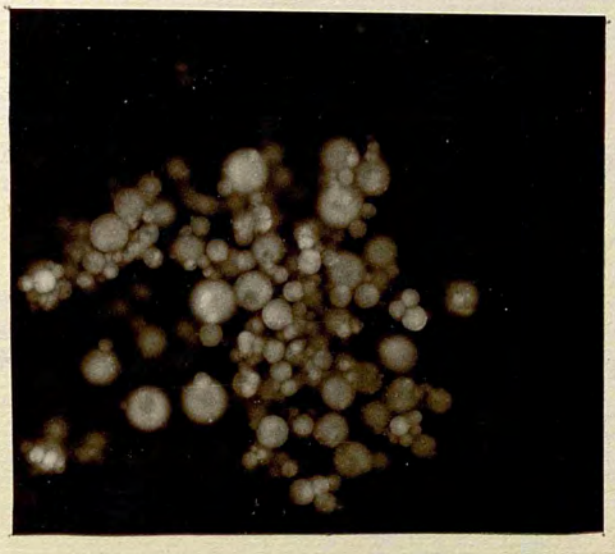
Experimental details are given in Section 2.1.3.

cells which is 600-fold (estimated from the film exposure time) greater than the natural fluorescence displayed by untreated cells in a control experiment. The dark background in Figure 19(a) indicates that no significant diffusion of newly liberated 4-methylumbelliferone from the surface of agglutinated cells into the surrounding medium has taken place. This should be contrasted with the light background, resulting after 15 min incubation in Figure 19(b).

When α-galactosidase I was incubated with potato starch granules the bound enzyme could again be demonstrated by fluorescence microscopy. Unlike the erythrocytes, starch granules do not display a natural fluorescence. Figure 20(a) represents an early stage (2 min) during the incubation with 4-methylumbelliferyl-α-D-galactoside and α-galactosidase I with a dark background surrounding the fluorescing starch particles. Furthermore, it was shown that by incubating the granules with 4-methylumbelliferone no granule fluorescence was produced. Again, after 15 min incubation (Fig.20b) the fluorescence has spread into the background as a result of product diffusion.

The production of granule fluorescence was inhibited by 0.1 M D-mannose in the incubation mixture. In addition, studies with various hapten inhibitors on the distribution of α-galactosidase I between the starch granules and the suspension medium (Table 4), supports this assumption. D-Glucose and D-mannose and oligosaccharides with terminal non- reducing α-D-glucopyranosyl residues (sucrose and maltose) all inhibited the binding, whereas melibiose and raffinose with α-D-galactopyranosyl residues, did not.

(a)



(b)

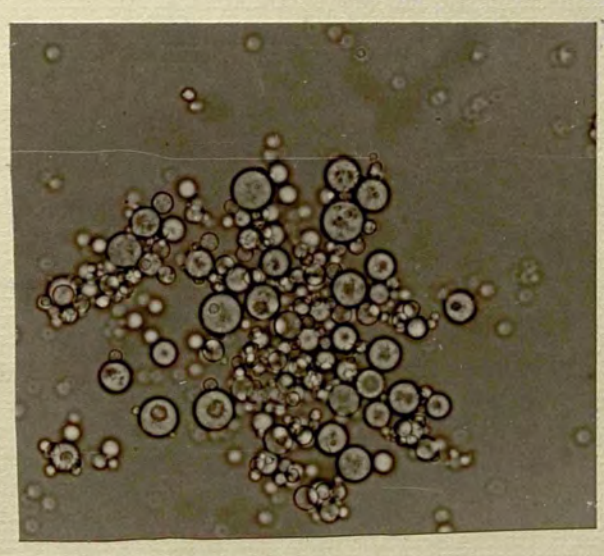


Figure 20

Display of fluorescence by starch granules after incubating (2 h) with α-galactosidase I (from Stage 7, Table 1) from V.faba seeds followed by the addition of 4-methylumbelliferyl-α-D-galactoside and leaving for (a) 2 min, and (b) 15 min.

Experimental details are given in Section 2.1.3(a).

TABLE 4

Binding of α-galactosidase I to starch granules and the effect of various carbohydrates*

Sugars (final concentration 100 mM)	α-Galactosidase activity (%)	
	Supernatant	Starch granules
Control	48.6	51.4
D-Mannose	81.4	18.6
D-Glucose	80.3	19.7
Maltose	64.6	35.4
Sucrose	62.3	37.7
D-Melibiose	45.4	54.5
Raffinose	43.6	56.6

* For experimental details see Section 2.1.4(b).

Treatment of the starch granule-α-galactosidase complex with α-amylase (25 units incubated with the complex for 1 h at 37°C) released 80% of the bound enzyme into the supernatant. Extensive lectin binding to glycan components of amyloplast membrane fragments on the granules appeared to be unlikely as washing the granules with acetone had no apparent effect on the degree of binding.

In addition to demonstrating the lectin binding properties of α-galactosidase I, this work also suggests the possibility of using enzyme-lectins as cytochemical reagents which can be readily detected by virtue of their free catalytic sites, after binding to cell receptors.

3.3. Evidence that V.faba α-Galactosidases Possess Separate Catalytic and Lectin Sites

Having clearly demonstrated that the three forms of α-galactosidases from V.faba seeds were glucose/mannose specific lectins, further attempts were then made to show that the lectin sites and enzyme sites on the protein molecules were situated at different loci. The effects of heat, pH, monosaccharides and demetallization on the two types of activity were, therefore, investigated.

3.3.1. Heat stability of catalytic and haemagglutinin activities

Examination of Figure 21 indicates that the haemagglutinin activities of all three forms of α-galactosidases are more stable than the catalytic activities when incubated at 65°C for periods up to approximately 30 min. Furthermore, α-galactosidase I is more stable with

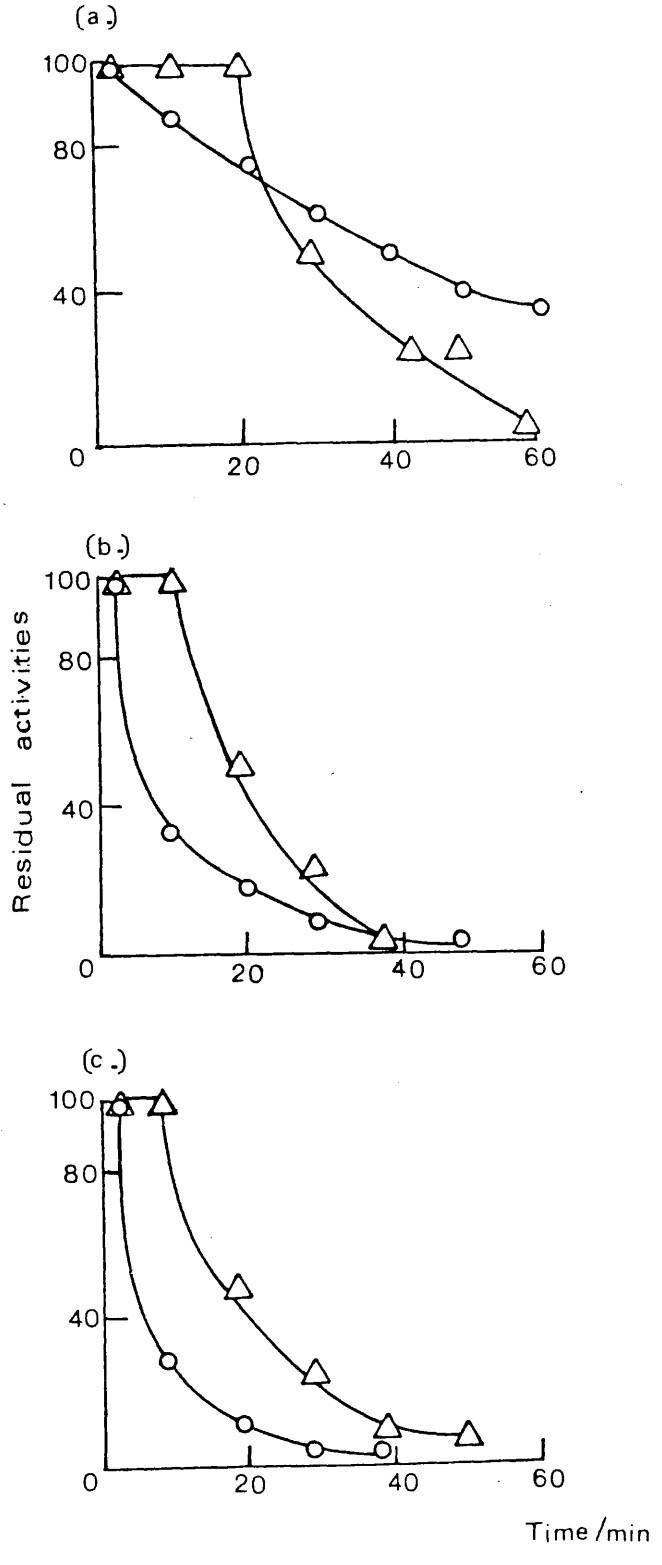


Figure 21

Stability of catalytic and lectin activities of α-galactosidases (a) I; (b) II^1 and (c) II^2 (from Stage 7;Table 1) from V.faba seeds

Experimental details are similar to those described in Section 2.10, except that the incubating temperature was 65°C.

O—O catalytic activity Δ—Δ lectin activity

respect to both activities than the monomers II^1 and II^2. The two types of activities also respond differently to heat in the presence of different monosaccharides (Fig.22): D-galactose in the incubation mixture appears to protect the catalytic functions of the V.faba enzymes but not their haemagglutinin activities, whereas D-mannose has the opposite effect.

The protection of lectin sites against heat inactivation by the use of specific monosaccharides has been reported in the case of "classical" glucose/mannose specific lectin from L.culinaris. In the presence of D-glucose or D-galactose and in the absence of these sugars it retained its lectin activity at 65°C for 30 min, but at 75°C activity was lost in both cases. At 72°C D-glucose at a concentration of 0.1 M afforded strong protection, but the same concentration of D-galactose had little effect [190].

3.3.2. The effect of pH on haemagglutinin and catalytic activities of α-galactosidases from V.faba seeds

Figure 23 shows that EI, II^1 and II^2 all exhibited dual pH optima for the hydrolysis of PNPG. All three forms were found to exhibit an optimum at pH 3.5. The second optimum for EI and II^2 was at pH 5.65, whereas the maximum optimum for EII^1 occurred at 5.4. These results indicate, as expected, that the catalytic sites of EI and EII^2 are similar, but they differ from those of EII^1.

On comparing the pH optima (in the pH range 5.0-8.5) for the haemagglutinin and catalytic activities, a difference of 1-2 pH was observed (Fig.24), with all three α-galactosidases, indicating different sites for these activities.

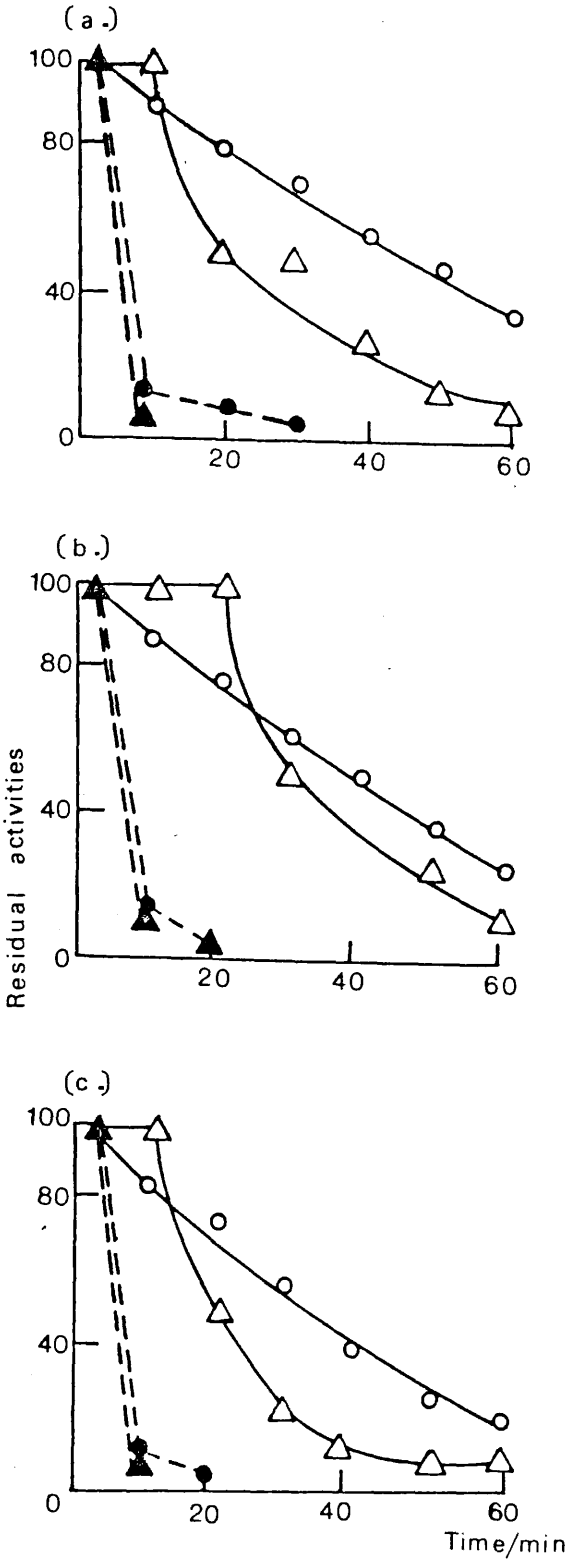


Figure 22

Protection of catalytic and lectin activities (at 75°C) of α-galactosidases (a) I; (b) II^1 and (c) II^2 (from Stage 7, Table 1) from V.faba seeds in the presence of D-galactose and D-mannose.

o—o catalytic activity remaining in presence of D-galactose

●—● catalytic activity remaining in absence of D-galactose or, in presence of D-mannose

△—△ lectin activity remaining in presence of D-mannose

▲—▲ lectin activity remaining in absence of D-mannose or, in presence of D-galactose

Experimental details are given in Section 2.10.

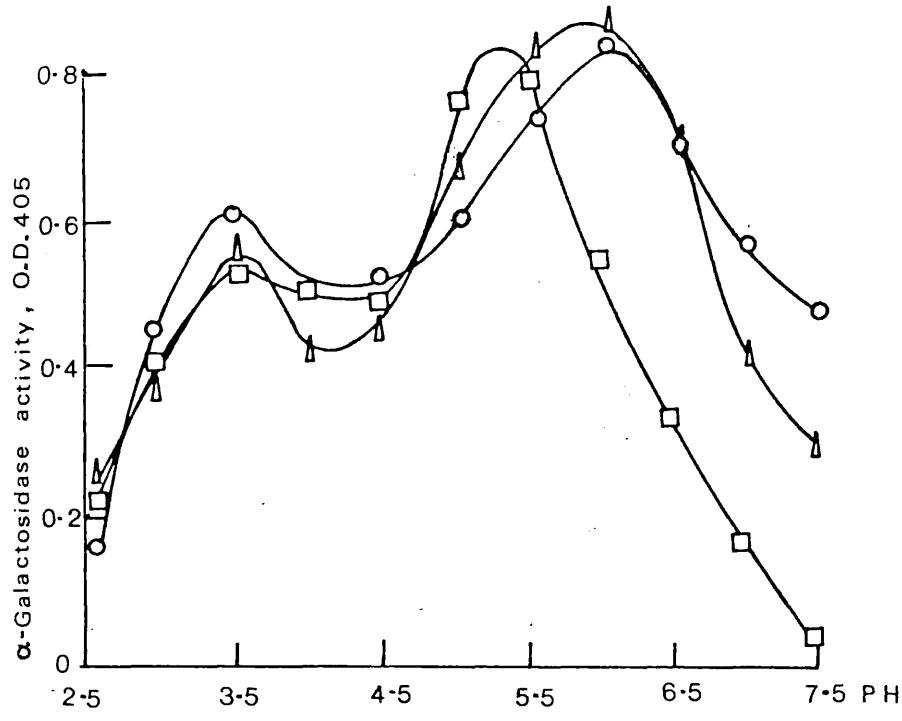


Figure 23

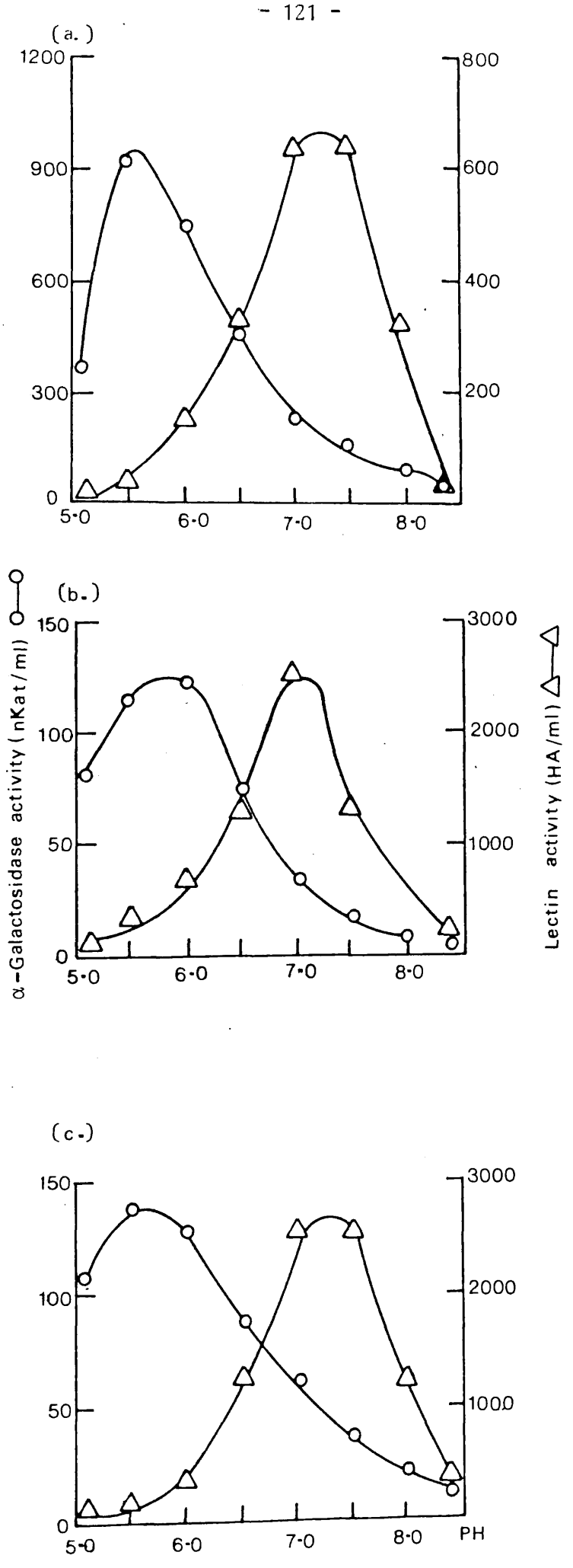
pH optima of α-galactosidases I (O—O); II^1 (□—□) and II^2 (∣—∣) (from Stage 7, Table 1) from V.faba seeds

Reactions were carried out at various pH values and assayed for α-galactosidase activity as described in Section 2.1.1.

Figure 24

pH optima of catalytic and lectin activities of α-galactosidases (a) I, (b) II^1 and (c) II^2 (from Stage 7, Table 1) from V.faba seeds

Reactions were carried out in potassium phosphate buffer at various pH values and assayed for α-galactosidase activity and lectin activities as described in Sections 2.1.1. and 2.8.1 respectively.


(a.)
1200
900
600
300
0
800
600
400
200
5·0
6·0
7·0
8·0
(b.)
α-Galactosidase activity (nKat/ml)
150
100
50
0
3000
2000
1000
Lectin activity (HA/ml)
5·0
6·0
7·0
8·0
(c.)
150
100
50
0
3000
2000
1000
5·0
6·0
7·0
8·0
PH

3.3.3. Comparative effects of monosaccharides on the haemagglutinin and catalytic activities of V.faba α-galactosidases

Enzyme kinetic studies using D-glucose, D-mannose and D-galactose showed that only D-galactose significantly inhibited the hydrolysis of PNPG by the three α-galactosidases from V.faba seeds and the inhibition was competitive (Table 5; see also [4]). As reported earlier, however, (see Table 2) D-glucose and D-mannose, but not D-galactose inhibit the haemagglutinin activities of these enzymes. These two studies, therefore, again suggest that separate catalytic and lectin sites exist.

3.3.4. Metal ion content and the effect of demetallization on the catalytic and haemagglutinin activities of α-galactosidases from V.faba seeds

The presence of Ca^{2+} and Mn^{2+} in lectins and the effect of such ions on their carbohydrate-binding properties has been discussed in several papers [178,191,192]. The published data suggest that the metal content, as well as the general mode of metal-ion binding, may be very similar for many lectins and that divalent ions, particularly Ca^{2+} and Mn^{2+} are essential for lectin activity [193].

The metal ion content of purified α-galactosidases I, II^1 and II^2 was determined after dialysis against several changes of deionized water and compared with demetallized samples of these same enzymes obtained by sequential dialysis against 0.1 M EDTA, deionized water, 1 M acetic acid and deionized water. The samples were analysed by atomic absorption spectrometry. The results (Table 6) showed that calcium was the major metal component of all the enzyme forms. The

TABLE 5

Inhibition of the catalytic activity of α-galactosidases I, II[1] and II[2] by galactose, mannose and glucose

	Monosaccharide concentration (mM)*	% Inhibition		
		I	II[1]	II[2]
D-Galactose	5	70	60	65
	25	90	95	90
	100	100	100	100
D-Mannose	25	10	5	10
	100	38	35	40
	500	60	55	60
D-Glucose	25	10	8	10
	100	40	35	38
	500	58	55	55

* included in the α-galactosidase assay mixture; see Section 2.1.1. for experimental details.

TABLE 6 Metal ion content of α-galactosidases I, II[1] and II[2] from V.faba (estimated by atomic absorption Spectrometry)

	Calcium ion content g.ion Ca^{2+}/mol enzyme	Magnesium ion content g.ion Mg^{2+}/mol enzyme	Manganese ion content g.ion Mn^{2+}/mol enzyme	Zinc ion content g.ion Zn^{2+}/mol enzyme
I	5.60 (1.2 mol Ca^{2+}/ 40,000 mw subunit)	0.40	0.15	0.16
Demetallized I	3.20 (0.8 mol Ca^{2+}/ 40,000 mw subunit)	0.30	ND	ND
II[1]	0.60	0.30	0.13	0.25
Demetallized II[1]	0.40	0.15	ND	ND
II[2]	0.40	0.20	0.10	0.23
Demetallized II[2]	0.30	0.10	ND	ND

ND = not detectable. See Section 2.11. for experimental details.

Ca^{2+}/Mg^{2+} ratios in EII^1 and EII^2 were significantly lower than in the case of EI and generally speaking, the zinc and manganese levels were low in all enzymes compared with calcium and magnesium levels.

On demetallization with EDTA all of the Mn^{2+} and Zn^{2+} ions and varying proportions (25-50%) of Ca^{2+} and Mg^{2+} appeared to be removed from the three enzyme forms. After EDTA/acetic acid treatment, EI contained 3.5 g ion/mol of enzyme (0.9 g ion/mol of subunit) of firmly bound metal ions, and EII^1 and EII^2 contain 0.55 and 0.4 g ion/mol of enzyme, respectively.

As observed in the present study and studies of Galbraith and Goldstein [178], Mn^{2+} ions can normally be readily removed from lectins by EDTA/acetic acid treatment, but this is not always the case with alkaline earths. Arachis hypogea, for example, contains 0.98 mol of Ca^{2+} and 0.78 Mg^{2+} per subunit, none of which is lost on dialysis against EDTA and acetic acid [179].

Demetallization of V.faba α-galactosidase I increased catalytic activity by 60% but decreased haemagglutinin activity by 87% (Table 7). Studies with alkaline α-galactosidase from C.pepo have shown that Ca^{2+}, Mg^{2+} and Mn^{2+} inhibit the catalytic activity of this enzyme and chelating agents such as EDTA appear to stimulate it [26].

The one important observation from the present studies is that the level of metal ions in α-galactosidase I reciprocally affects the haemagglutinin and catalytic activities. This supports the notion that separate sites on the protein are involved in these two functions, as was the case with heat treatment, pH and enzyme and lectin carbohydrate inhibition studies.

TABLE 7

Effect of demetallization on catalytic and lectin activities of α-galactosidase I from V.faba seeds

	Native	Demetallized
α-Galactosidase activity (nkat/ml)	50,000	80,000
Lectin activity (HA/ml)	320	40

3.4. Determination of the Number of Carbohydrate-Binding Sites and Association Constants of α-Galactosidases from V.faba Seeds

With strong evidence for different loci for the lectin and catalytic sites of all three forms of V.faba α-galactosidases, a determination of the number of lectin sites on each enzyme and the number of catalytic sites in the case of α-galactosidase I alone was attempted.

The number of lectin and catalytic sites were estimated by equilibrium dialysis and the values for the association constants for the carbohydrate-binding by equilibrium dialysis and a spectrophotometric method.

(i) Equilibrium dialysis:

In this case samples of the enzymes were dialysed against solutions containing a fixed [^{14}C]-monosaccharide (D-galactose or D-mannose) and varying amounts of the unlabelled monosaccharide. The data were evaluated according to the method of Scatchard [194] where the number of molecules of monosaccharide bound per molecule of protein (B) is plotted against B/U where U is the free sugar concentration.

α-Galactosidase I combined with D-galactose with an association constant of 1.67×10^4 M^{-1}. The intercept on the abscissa in Fig.25(i) represents the maximum number of sugar-binding sites, which is four for the tetrameric α-galactosidase I, thus suggesting a single galactose-binding (catalytic) site per subunit.

In the case of D-mannose, the association constants were 1.37×10^4 M^{-1}, 2.18×10^3 M^{-1} and 3.14×10^3 M^{-1} and the numbers of lectin sites is eight, four and two for EI, II^1 and II^2 respectively

Figure 25

Determination of the number of binding sites and association constants of :

(i) D-galactose for the catalytic sites of α-galactosidase I

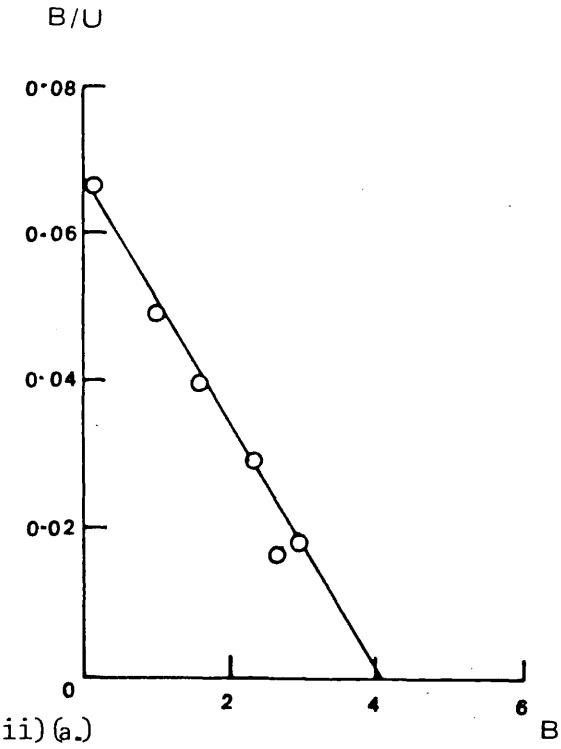
and of:

(ii) D-mannose for the lectin binding sites of α-galactosidases (a) I; (b) II^1 and (c) II^2

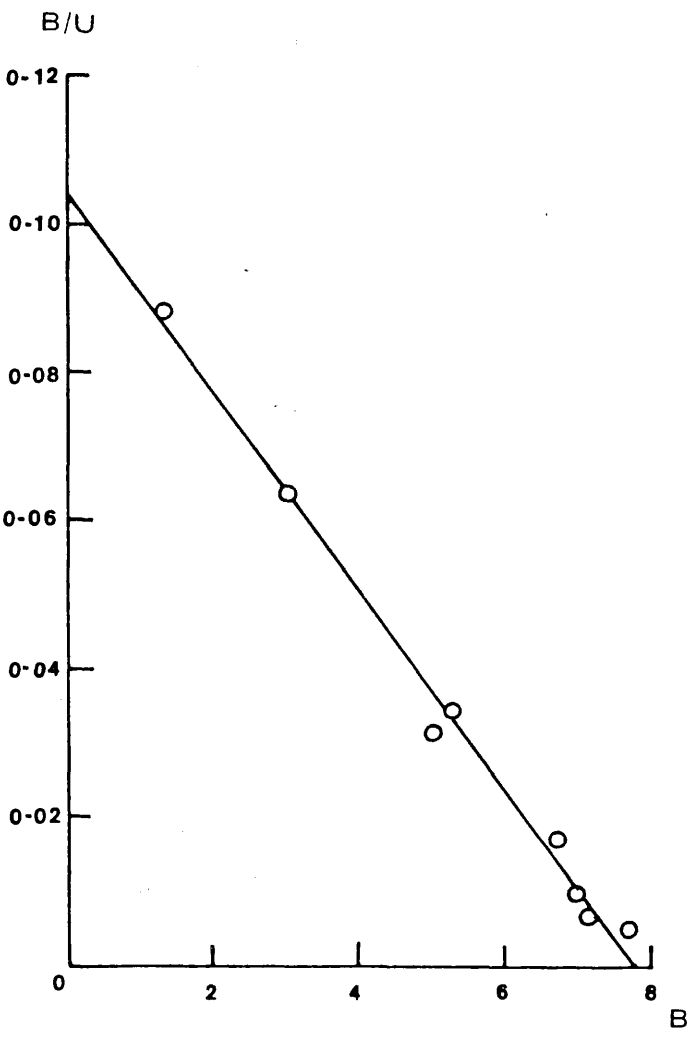
(from Stage 7, Table 1) of V.faba seeds.

Experimental details are given in Section 2.12.1.

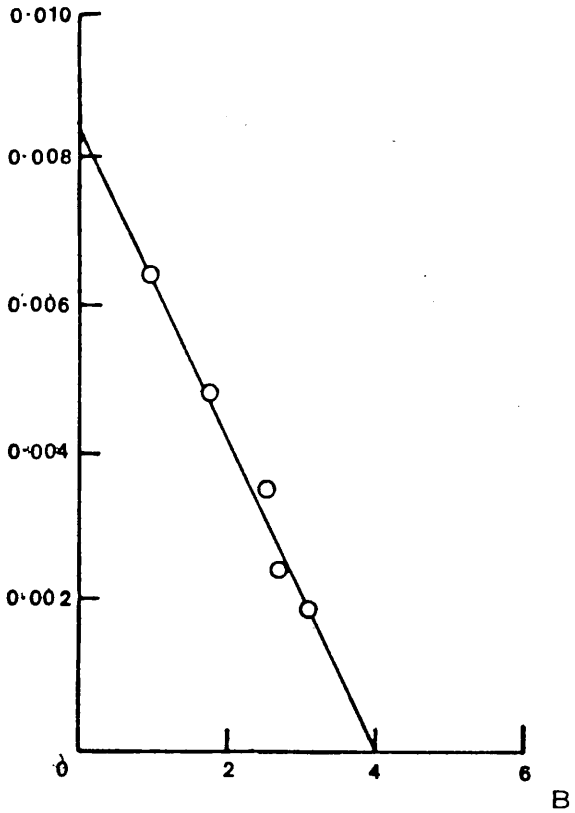
(i)



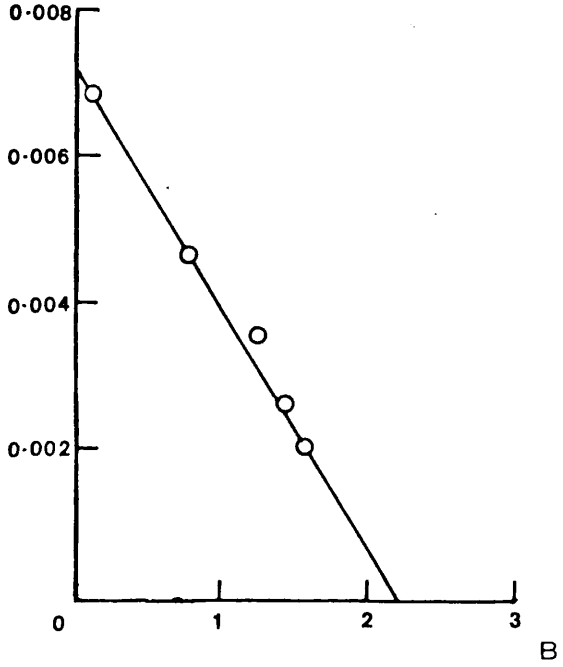
(ii) (a.)



(b)


B/U
0·010
0·008
0·006
0·004
0·002
0
2
4
6
B

(c)


B/U
0·008
0·006
0·004
0·002
0
1
2
3
B

(Fig.25ii). For EI this represents two mannose-binding sites per monomer unit. These studies were repeated using a fixed concentration of [^{14}C]-D-mannose and varying amounts of unlabelled D-galactose. The D-galactose did not significantly alter the affinity of the enzymes for D-mannose as might be expected if separate sites for the two monosaccharides exist.

(ii) Spectrophotometric method:

Changes in the ultraviolet difference spectra occur when specific sugars are added to solutions of lectins. These changes allow binding constants to be determined [195].

The spectra of the three forms of α-galactosidase from V.faba were obtained in the presence of D-mannose in varying concentrations. Figure 26 shows titration curves obtained from the difference spectra between 300 and 250 nm for the binding of D-mannose to the α-galactosidases. The association constants obtained by this method were $1.48 \times 10^4\ M^{-1}$, $2.51 \times 10^3\ M^{-1}$ and $3.05 \times 10^3\ M^{-1}$ for α-galactosidases I, II^1 and II^2, respectively. These constants were determined from the free monosaccharide concentration at 50% saturation using the relationship:

$$K = 1/[S_{free}] \quad \text{with } [S_{free}] = [S_{total}] - 0.5\ nP$$

where nP represents the protein subunit concentration. The results are in good agreement with the equilibrium dialysis values.

The difference spectra induced by D-mannose in the case of all three α-galactosidases showed single broad characteristic peaks between

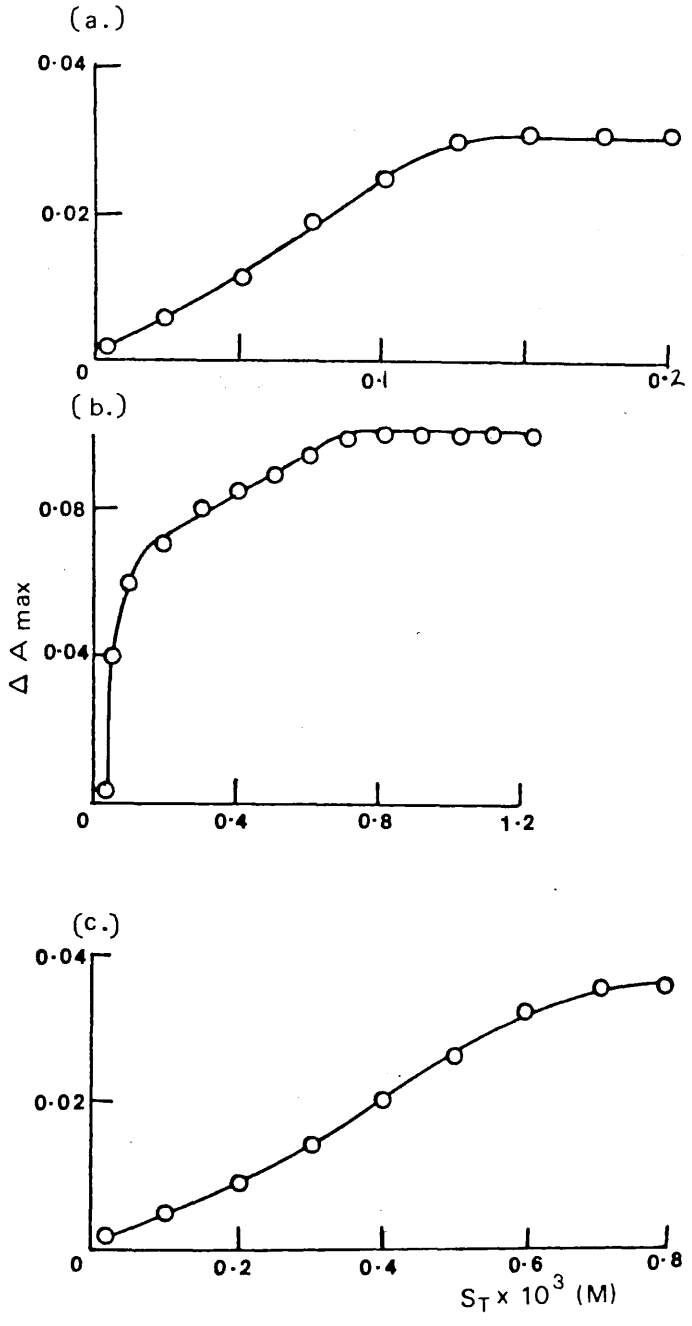


Figure 26

Effect of D-mannose concentration (S_T) on the difference spectra of α-galactosidases (a) I; (b) II^1 and (c) II^2

Experimental details are given in Section 2.12.2.

282.5 and 279 nm. In contrast, Arachis hypogea agglutinin with D-mannose exhibits two tyrosine peaks at 285 and 279 nm. L.culinaris, Sophora japonica (Japanese pagoda) and Solanum tuberosum lectins and wheat germ lectin gave two peaks each at 292 nm and 284-287 nm (which are characteristics of tryptophanyl residues) when they were treated with methyl-α-D-mannoside, lactose, di-N-acetylchitobiose and di-N-acetylchitobiose respectively [195]. The spectrophotometric method, therefore, helps to identify aromatic amino acid residues at, or near to, the sugar-binding site, which in the case of the V.faba enzymes appear to be one or more tyrosine residues.

It is presumed that a lectin must have at least two binding sites per molecule in order to agglutinate cells [196]. Abrus precatorius (jequirity bean) lectin, for example, exists as a tetramer of two pairs of non-identical subunits and has two binding sites/molecule [135], suggesting that one type of subunit does not possess binding sites. The glucose/mannose specific tetrameric lectins (composed of two pairs of non-identical subunits) from V.cracca [197] and P.sativum [198] also have two binding sites/molecule, as does the dimeric lectin, with identical subunits, from L.culinaris [199]. In the case of wheat germ lectin also a dimer with two identical subunits, there are two non-interacting binding sites per subunit for β-1,4-linked oligomers of N-acetylglucosamine [200,201].

The eight lectin binding sites for D-mannose detected in the case of α-galactosidase I from V.faba seeds represents the largest number of sites so far reported for a tetrameric lectin and suggests that the monomer II^2, with two sites gives rise to a tetramer in which all sites are exposed. Anomolous results, produced by dissociation of

α-galactosidase I during the equilibrium dialysis are unlikely to have occurred. Except in the presence of SDS or urea the quaternary structure of EI appears to be stable within a pH range of 3.5 to 7.0 (see p. 95).

The association constants reported for lectins have also been found to vary considerably, although they are usually in the range of 10^3-10^4 M^{-1}: an exception is the L.culinaris lectin which binds D-mannose with a Ka of 2.3 x 10^2 M^{-1} [133]. The glucose/mannose specific lectin from V.cracca has an association constant for glucose-binding of approximately 2.0 x 10^3 M^{-1} [197] which approximates the value for D-mannose binding by α-galactosidases II^1 and II^2. Mormodica charantia and Ricinus communis lectins exhibit association constants of 1.8 x10^4 and 1.65 x 10^4 respectively, when binding p-nitrophenyl-β-D-galactopyranoside [135,196]: the latter is similar to the high value obtained in the present studies for D-mannose binding by α-galactosidase I. Association constants of the order 10^6-10^7 M^{-1} have been reported for the lectin binding to natural receptors (see p. 157).

3.5. Comparison of the Lectin Activities Displayed by α-Galactosidases from V.radiata and V.faba seeds

Unlike the V.faba α-galactosidases, the haemagglutinin activities of some other legume seed α-galactosidases, such as those from V.radiata [8] and G.max [46] are reported to be galactose-specific (see Introduction, p.39). The question arises, therefore, whether these latter enzymes possess one type of site which is responsible for both lectin and catalytic activities.

One of the first observations made with the tetrameric V.radiata α-galactosidase I was that at pH 7.0, it agglutinated erythrocytes and subsequently the clot dissolved. The monomeric form (II) of the enzyme did not cause agglutination. It was suggested that the agglutination by α-galactosidase I was followed by some change in the red blood cell surface which caused clot dissolution; dissolved cells could not be reagglutinated with the crude extract. Galactose in the medium prevented agglutination by V.radiata α-galactosidase I [8].

The above properties of the V.radiata enzymes were re-investigated (see Fig.27) and compared with those of the α-galactosidases from V.faba which all possessed haemagglutinin activity, but not the 'clot-dissolving' property.

Red blood cells that received a separate preliminary treatment (2 h) with the tetrameric (EI) or monomeric (EII) forms of V.radiata α-galactosidases (prepared as described by Hankins and Shannon [8], see Materials and Methods section, p. 61) were washed with PBS buffer and again incubated separately with EI, EII^1 and EII^2 from V.faba and EI from V.radiata. It was confirmed that the erythrocytes which had been treated with V.radiata α-galactosidases I or II and re-isolated could not be agglutinated by further addition of V.radiata enzyme I. Agglutination did occur in both cases, however, in the presence of all three forms of the glucose/mannose specific V.faba α-galactosidases.

When the preliminary incubation of red blood cells with V.radiata EI and EII was repeated in the presence of 3 mM galactose (an inhibitor of α-galactosidase activity), the treated cells were agglutinable by the V.radiata EI as well as the V.faba enzymes I, II^1 and II^2. These results

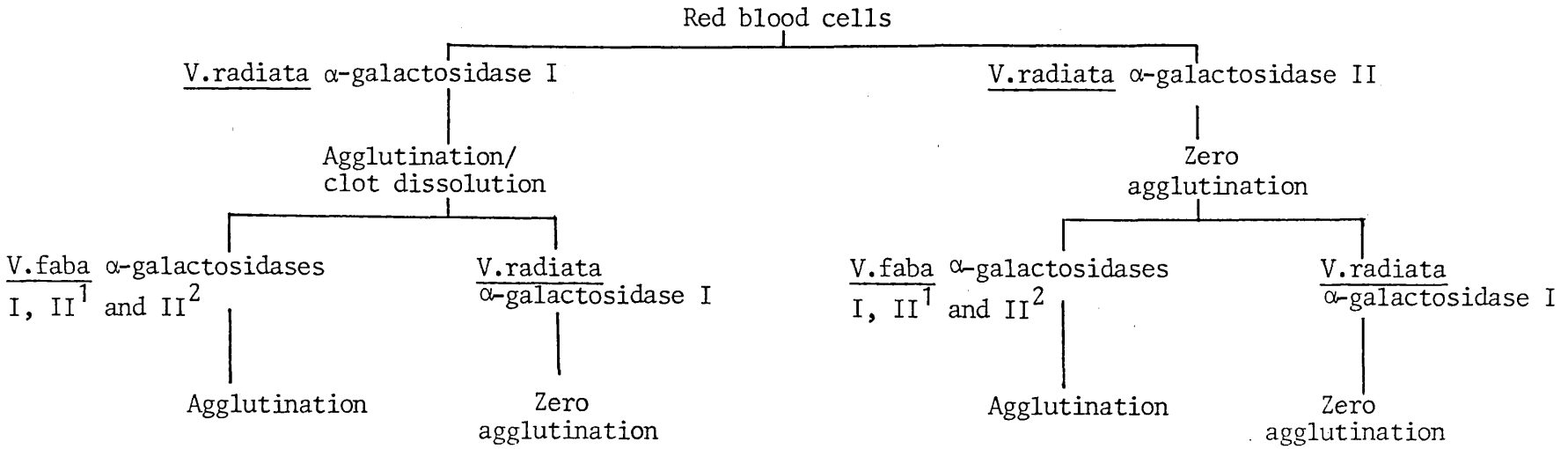


Figure 27

Treatment of rabbit red blood cells with V.radiata and V.faba α-galactosidases

suggest that V.radiata α-galactosidases I and II removed 'receptors' for V.radiata EI from the red blood cell surface (hence inhibiting subsequent agglutination by V.radiata I), but not those for glucose/-mannose specific V.faba enzyme-lectins: presumably the former receptors were galactosyl residues.

In the absence of further evidence, therefore, the agglutination and 'clot-dissolving activities' of V.radiata α-galactosidase I can be explained in terms of multiple galactose-binding catalytic sites which first combine with galactose 'receptors' on the erythrocyte surface, producing agglutination, then slowly hydrolyse the galactosidic bonds causing loss of the clot.

Agglutination of the red blood cells followed by clot-dissolution has also been described in the case of galactose oxidase. Here, as with V.radiata EI, the enzyme presumably combines via its catalytic sites to cell surface galactose residues, causing clot formation, then after oxidation of the hydroxyl groups at C-6 of the monosaccharide residue(s), 'product' and enzyme separate and the clot dissolves [202].

It is not known to what extent the V.faba enzyme-lectins remove galactose from red blood cells. It may be minimal because of a rapid aggregation of red blood cells via glucose/mannose-specific lectin cross-links resulting in steric hindrance of hydrolytic activity. There are, however, several reports in the literature of the enzymic removal of galactose residues from cell surfaces; for example in the conversion of B-type to O-type erythrocytes by coffee α-galactosidase [203].

The assumption that agglutination and clot-dissolving activities of V.radiata α-galactosidase I involves a single type of galactose-binding catalytic site is supported by a number of other observations. As mentioned in the Introduction (p. 39) the catalytic and haemagglutinin activities of this enzyme are equally inhibited by galactose, xylose and inositol. The two properties also respond similarly to pH changes and to heat treatment and are equally protected from heat inactivation by galactose [8] (cf. the V.faba enzymes, p.117).

These comparisons of V.radiata α-galactosidase I with the V.faba enzymes lead to the conclusion that the former is not a lectin as defined by Kocourek and Horejsi (p.33). The V.faba α-galactosidases on the other hand, are true lectins.

The tetrameric α-galactosidase isolated from the seeds of G.max would appear to be similar to V.radiata EI. It also agglutinates erythrocytes, clot dissolution follows and galactose inhibits both the catalytic and haemagglutinin activities [46].

4. The Glycoprotein Nature of α-Galactosidase I from V.faba Seeds

In the second part of this study on α-galactosidases, the nature of the carbohydrate associated with form I of the enzyme was investigated. Previously, Dey et al. [39] reported the presence of combined mannose and glucosamine in the protein, together with glucose and xylose. Mannose and glucosamine are well established components of glycoproteins (see Introduction, p. 45) but the significance of combined glucose and xylose in the enzymes is not entirely clear. Xylose has been reported to occur in a limited number of glycoproteins, e.g. S.japonica lectin [204] and Ananas sativa (pineapple) bromelain [156] and may be present in α-galactosidases from V.faba seeds. Dey et al. [39] suggested that glucose might have been derived from a contaminant of the enzymes (cf. also [121]) perhaps bound in the form of starch to the lectin sites. A further possibility is that combined glucose is present in nascent forms of the enzymes, as in the case of animal 'high-mannose' glycoproteins which contain glucose as intermediary stages of biosynthesis and it is then removed by specific α-glucosidases during final processing [160].

It was considered that further details of the glycan structures present in α-galactosidase I were required as a basis for future studies on the possible role of these moieties in the binding of the enzyme to cell components and the regulation of α-galactosidase activity. Hence, an analysis of the glycan components of α-galactosidase I was undertaken.

4.1. Isolation of a Glycopeptide from α-Galactosidase I from V.faba seeds

Following pronase digestion of α-galactosidase I from V.faba seeds the products were separated on a Biogel P-4 gel filtration column. Analysis of the column fractions with anthrone/H_2SO_4 reagent revealed a large number of carbohydrate-containing materials (Fig.28) which was unexpected in view of the long (6 days) digestion period. These results suggest structural heterogeneity in the carbohydrate moieties of the glycopeptides. Ovalbumin is an example of a glycoprotein exhibiting such heterogeneity [160].

When the gel filtration fractions were pooled, freeze-dried and analysed for amino acids, the first nine fractions were found to contain only four detectable amino acids, namely, glycine, asparagine/-aspartic acid, glutamine/glutamic acid and serine in approximately equal molar ratios. The amino acids known to occur in glycopeptide linkages are asparagine, serine, threonine, cysteine, hydroxyproline and hydroxy-lysine [121] and of these only asparagine and serine were present in the glycopeptides from α-galactosidase I and it is, therefore, probable that the glycan chains are linked via one, or both, of these amino acids.

It is possible that the four amino acids present in the glyco-peptides are present in the sequence Gly/Glx-Asn-Gly/Glx-Ser. This would conform to the Asn X Ser/Thr sequence which was proposed as a requirement for the attachment of the carbohydrate moiety to the amide of asparagine (in an N-type linkage) in plant [205] and animal [206] glycoprotein synthesis.

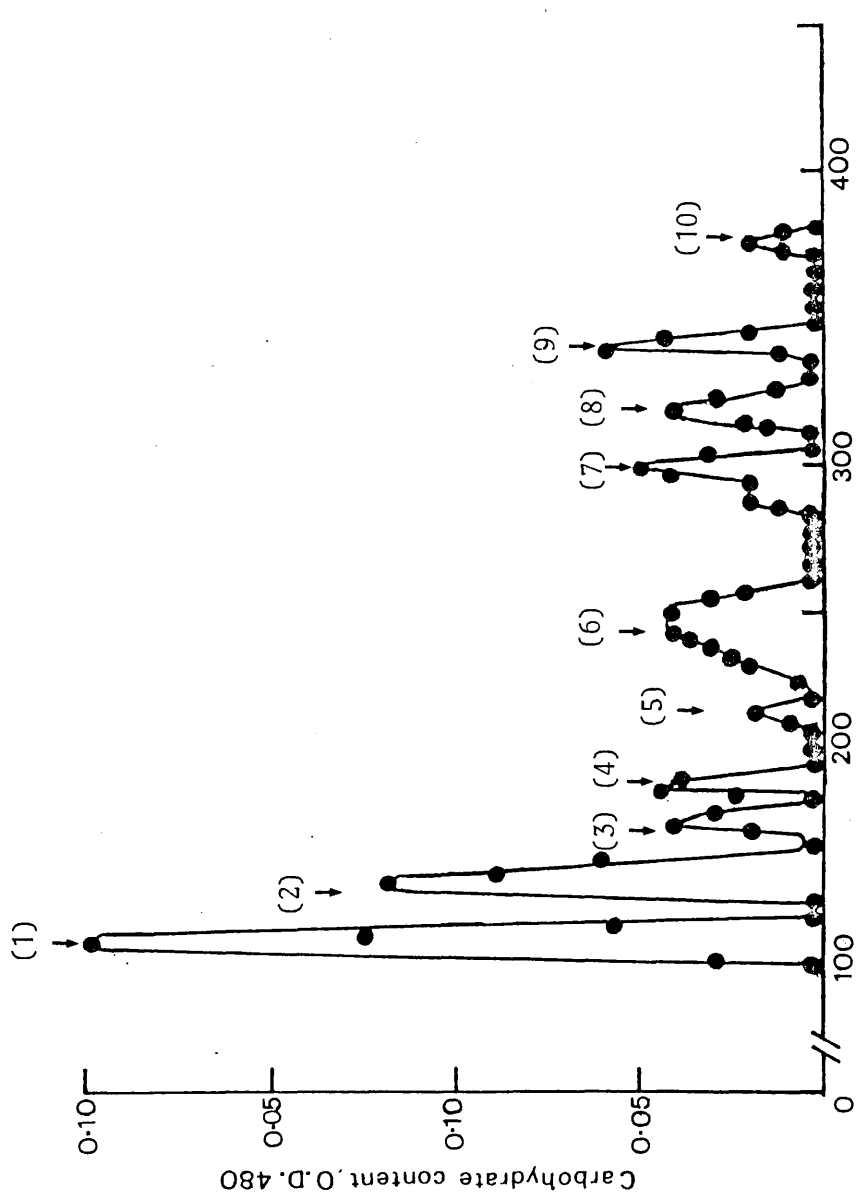


Figure 28

Biogel P-4 gel filtration of pronase digested α-galactosidase I (from Stage 7, Table 1) from *V.faba* seeds

Experimental details are given in Section 2.13.1.

The last peak (No.10, Fig.28) which was eluted from the Biogel column appeared to contain all of the amino acids and little carbohydrate, probably represents in the main, a mixed, low molecular weight peptide fraction.

The Asn-linked oligosaccharides of glycoproteins are of two major types, namely 'high-mannose' glycan and 'complex' glycans [165]. The 'high-mannose' types are composed of mannose and N-acetylglucosamine (Fig.3a; Introduction, p. 46) and the 'complex' chains in addition contain galactose, fucose (Fig.3b) and (in animal glycoproteins) sialic acids [207].

Two lines of investigation were pursued in order to confirm the presence of Asn-linkage in α-galactosidase I and to differentiate between the two classes of Asn-linked carbohydrate chains.

4.2. Endo-H Digestion of α-Galactosidase I from V.faba seeds

A preliminary study was made with endo-H (see p. 47) to determine whether it would remove glycan chains from α-galactosidase I, and hence indicate that the glycoprotein was "mannose-rich".

After incubation of α-galactosidase I with endo H the products were applied to a Sephacryl S-200 column (Fig.29). α-Galactosidase I activity was recovered in two fractions, one of which co-eluted with the native enzyme (MW 160,000) and the other at a higher elution volume corresponding to a molecular weight of 80,000. This suggested that the tetrameric enzyme had been split into active dimeric forms as a result of the action of endo H. Additional evidence that the cleavage of the

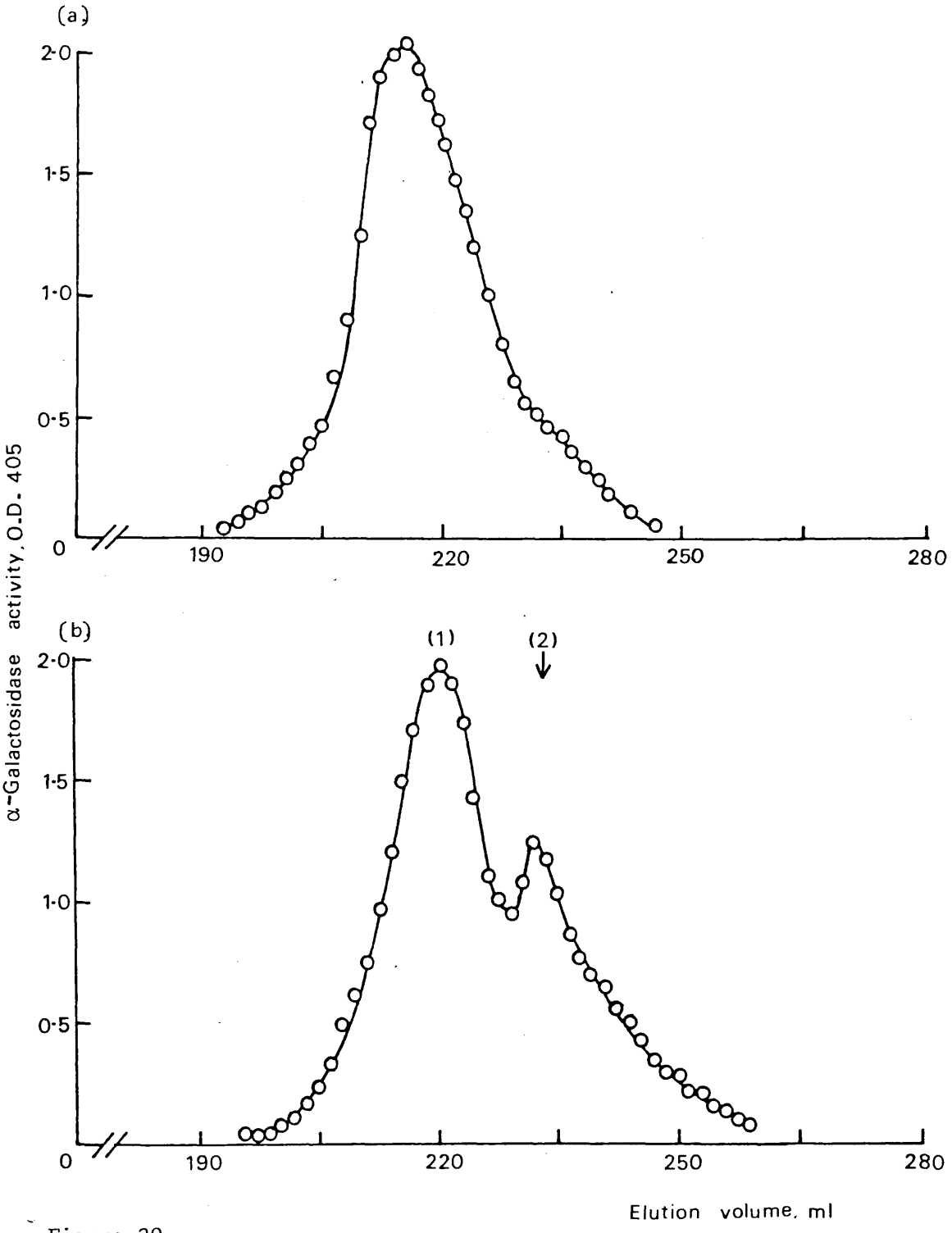


Figure 29

Sephacryl S-200 gel filtration of (a) native, and (b) endo H-treated α-galactosidase I (from Stage 7, Table 1) from V.faba seeds

Experimental details are given in Section 2.13.3.

carbohydrate chains from the glycoprotein had occurred was obtained by testing the column eluent for carbohydrate with anthrone/H_2SO_4 reagent. Carbohydrate-containing fractions eluting at approximately 440 ml (i.e. beyond the inclusion range of the column) were observed, thus indicating that at least some of the glycan chains had been removed from α-galactosidase I.

The two enzymically active peaks (Fig.29; 1 and 2) from the Sephacryl S-200 column were separated and applied to Con A-Sepharose columns to check whether the samples retained their affinity for the lectin (Fig.30).

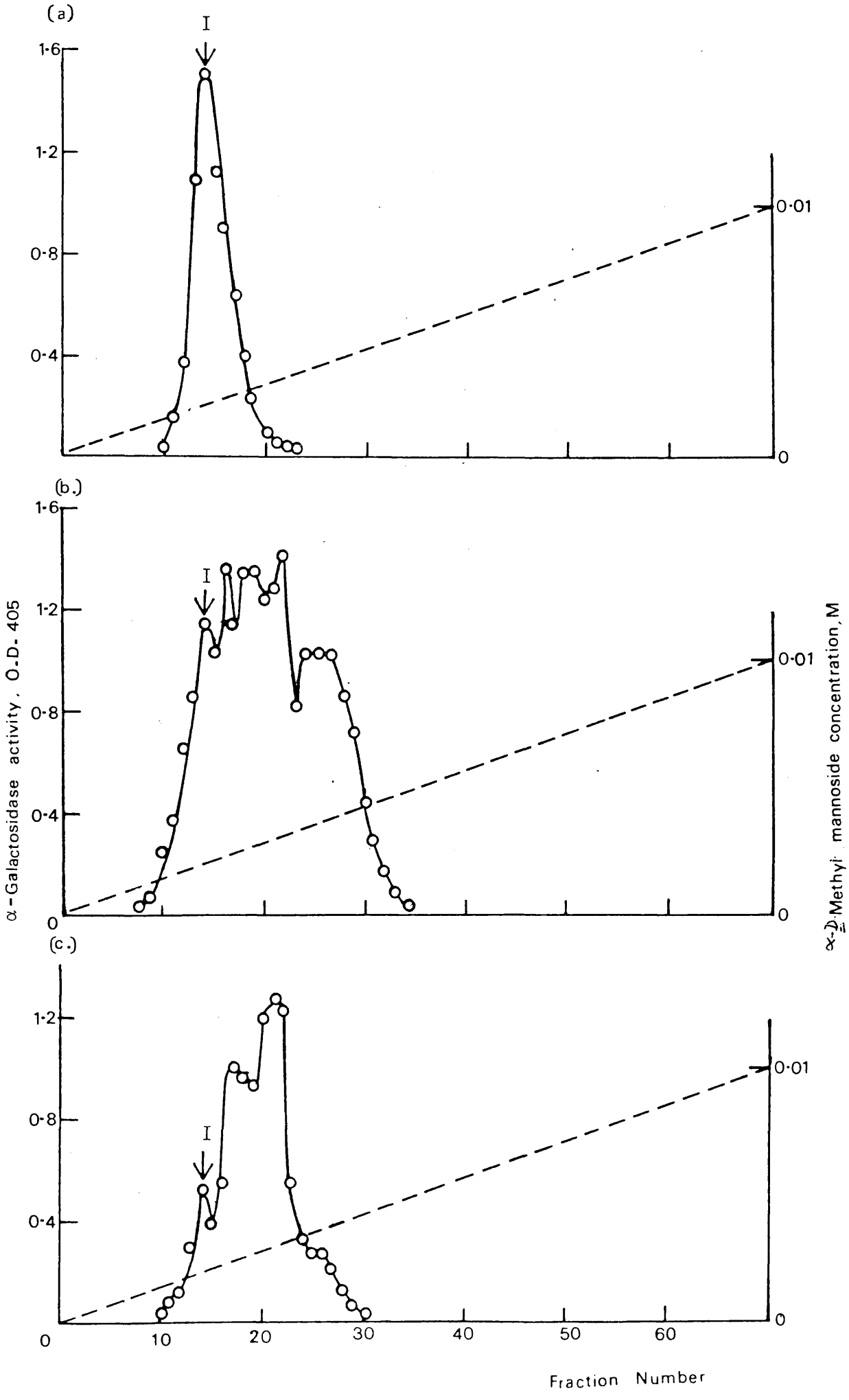
In both cases all of the α-galactosidase activity was bound to the column, suggesting that at least some glycan chains remained attached to α-galactosidase I (fraction 1) as well as the putative dimer (fraction 2). The enzyme activities were eluted from the columns with a methyl-α-D-mannoside gradient (Fig.30) and broad heterogeneous peaks were observed with both the fractions (cf. native α-galactosidase I), particularly in the case of fraction 1 from the Sephacryl S-200 column.

These differences in the elution patterns of the treated and untreated enzymes confirmed that endo H had altered the structure of α-galactosidase I and produced fractions with higher affinity for Con A, i.e. requiring higher concentrations of methyl-α-D-mannoside for elution compared with untreated α-galactosidase I. Furthermore, in view of the known specificity of endo H it can be concluded that α-galactosidase I contains 'high-mannose' oligosaccharides linked to Asn.

Figure 30

Concanavalin A-Sepharose affinity chromatography of: (a) native α-galactosidase I, and (b) fraction 1, and (c) fraction 2, from Sephacryl gel filtration column (Fig.29b) of Endo H-treated α-galactosidase I (from Stage 7; Table 1) from V.faba seeds.

Experimental details are given in Section 2.13.3.


(a)
I
1·6
1·2
0·8
0·4
0·01
0
(b.)
I
1·6
1·2
0·8
0·4
0
0·01
0
α-Galactosidase activity, O.D. 405
α-D-Methyl mannoside concentration, M
(c.)
I
1·2
0·8
0·4
0·01
0
0
10
20
30
40
50
60
Fraction Number

4.3. Acetolysis/FAB-MS of the Glycopeptide Isolated from α-Galactosidase I from V.faba Seeds

Recently a rapid procedure to determine the nature of the glycan chains in glycoproteins/glycopeptides has been devised. In essence, this consists of acetolysis followed by identification of the released oligosaccharide by Fast Atom Bombardment-Mass Spectrometry (FAB-MS) [Dell, A and Oates, J; Personal communication].

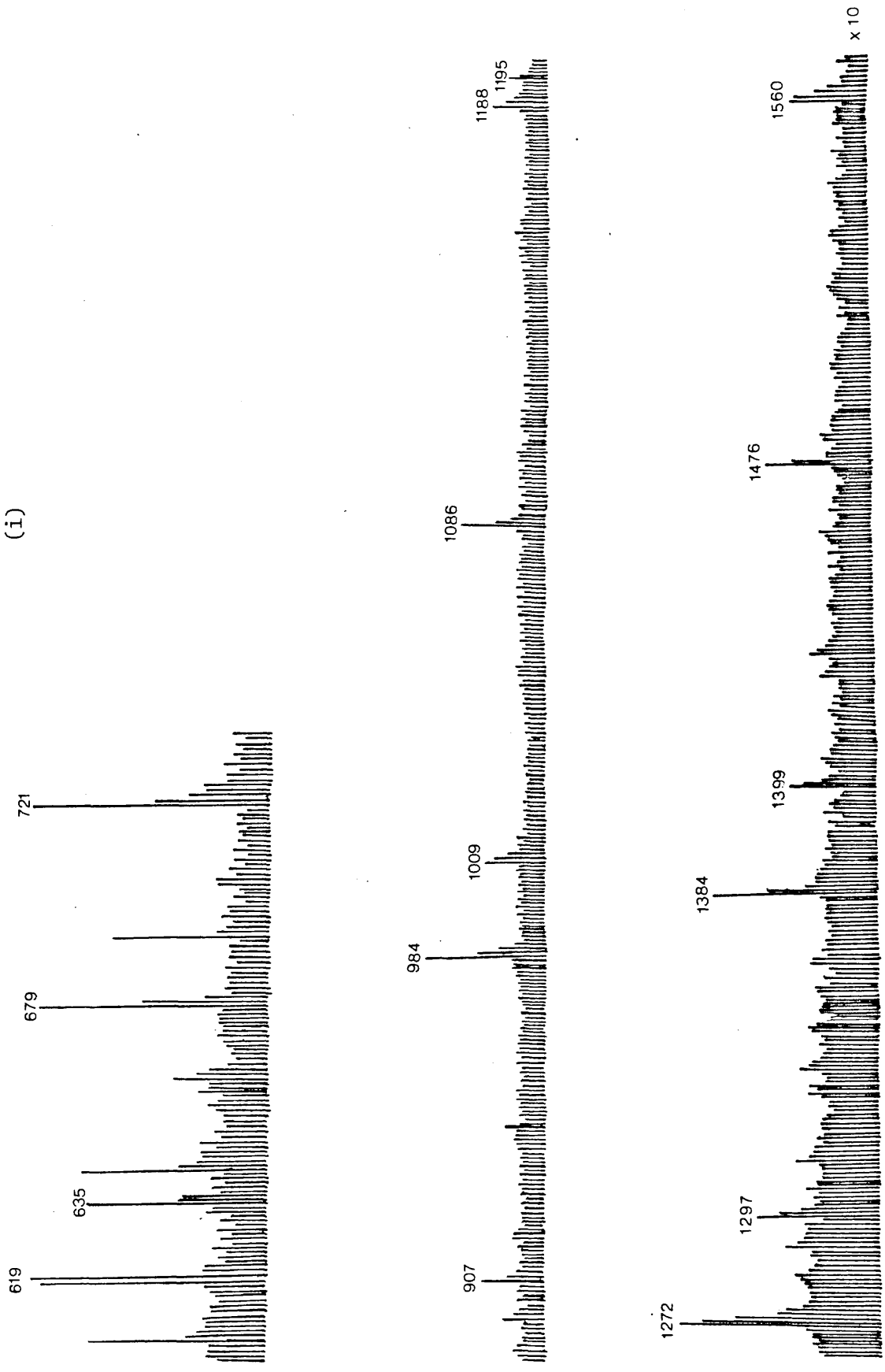
A fraction (Fig.28: Peak 1) obtained from α-galactosidase I by pronase digestion was acetolysed for various times and the products analysed by FAB-MS. The 30 min acetolysed sample did not produce any detectable ions. However, after 2 h a signal was obtained at m/z 907 which corresponds to the acetylated A-type [208] ion of composition Hex_3^+. The 5 and 7 h acetolysis mixtures yielded signals 288 m.u. apart at m/z 619, 907, 1195, 1483 and 1771 corresponding to Hex_2^+, Hex_3^+, Hex_4^+, Hex_5^+ and Hex_6^+, respectively.

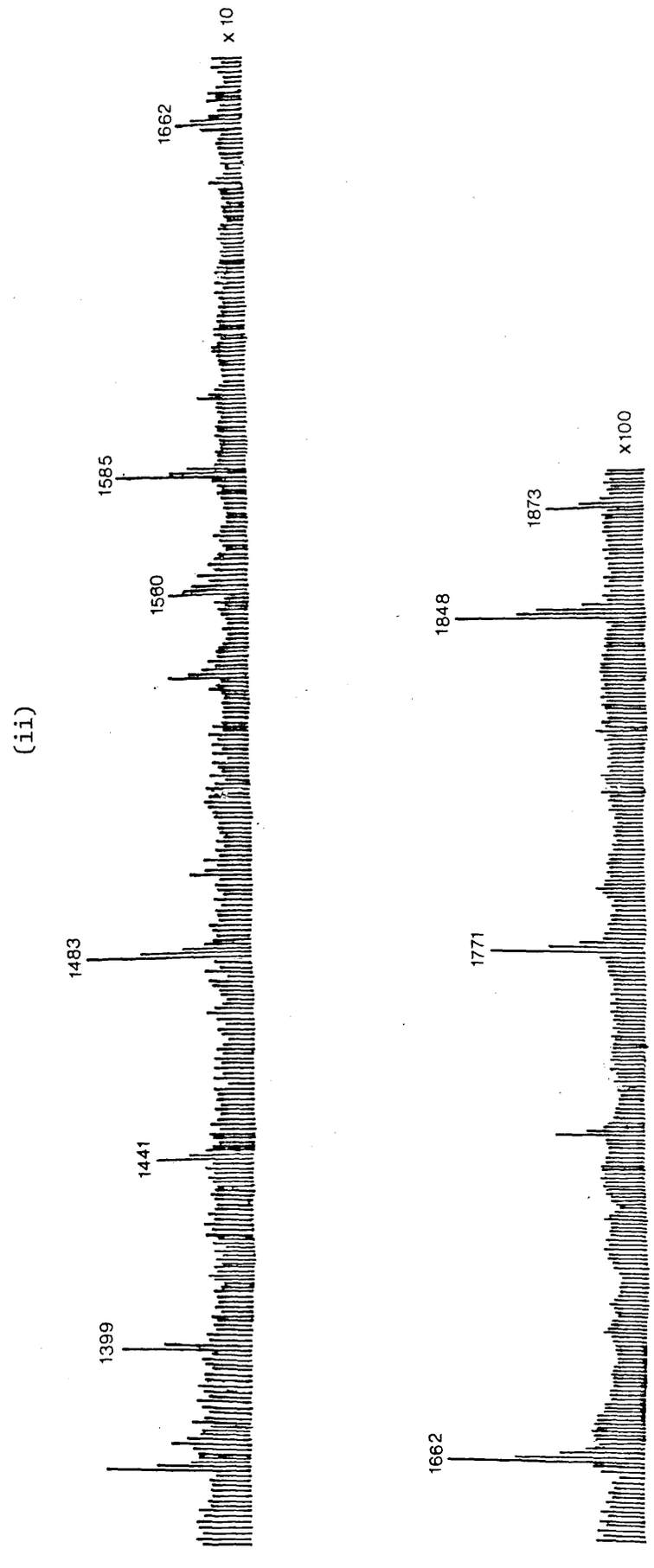
A-type ions at m/z 907, 1195, 1483 and 1771 are accompanied by signals at m/z 984, 1272, 1560 and 1848, which are the ammonium cationized molecular ions for the fully acetylated species corresponding to three, four, five and six hexose units. The FAB-MS spectrum obtained from the 5 h acetolysed sample is shown in Figure 31. The FAB-MS spectrum obtained for the acetolysed glycopeptide isolated from α-galactosidase I was similar to that obtained with acetolysed ribonuclease, an established 'high-mannose' type glycoprotein [Dell, A and Oates, J; unpublished results]. The production of acetylated fragments containing up to six hexose residues is strong evidence that the glycan chains in α-galactosidase I are of a 'high-mannose' type [Dr A. Dell; personal communication].

Figure 31

Acetolysis FAB-MS of glycopeptide isolated from α-galactosidase I (from Stage 7; Table 1) from V.faba seeds.

Experimental details are given in Section 2.13.2.


(i)
619
635
679
721
907
984
1009
1086
1188
1195
1272
1297
1384
1399
1476
1560
x10


(ii)
1399
1441
1483
1580
1585
1662
x 10
1662
1771
1848
1873
x100

The presence of xylose in α-galactosidase I which was reported by Dey *et al*[39] was not confirmed by acetolysis/FAB-MS study. No pentose residues were observed in any of the mass spectra. Furthermore, it is perhaps unlikely that a xylose residue would be present in an endo-H susceptible 'high-mannose' glycopeptide unless it was remote from the site of action of the enzyme, or possibly present attached to an amino acid other than Asn.

Hence, the glycopeptide isolated from α-galactosidase I appears to resemble that isolated from soybean agglutinin (see p.48).

5. Bound α-Galactosidases

The existence of cell bound forms of α-galactosidase was discussed in the Introduction (p. 32): the detailed nature of this binding to cell constituents is not known, although it may involve cell wall constituents and/or membranes.

The present study with V.faba α-galactosidases has shown that these enzymes are both lectins and 'mannose-rich' glycoproteins. The question arises therefore whether binding of the enzymes to cell 'constituents' can occur via lectin interactions, i.e. the lectin sites of α-galactosidase-binding to carbohydrate receptors (e.g. glycoprotein glycans or polysaccharides) in the cell or the glycan chains of α-galactosidase interacting with bound 'classical' lectins, also present in the cell.

Attempts were therefore made to identify such linkages by extracting resting V.faba seed tissues with methyl-α-D-mannoside and comparing the enzyme activity released with that obtained by extraction with other agents.

Resting, testa-free V.faba seeds were finely powdered, then equal weights of powder were extracted by grinding in a mortar with phosphate buffered solutions (pH 7.0) of varying concentrations of NaCl, methyl-α-D-mannoside or Triton X-100 or buffer alone. As far as possible the degree of grinding was kept the same in all cases. The extracts were centrifuged and the supernatants retained. Each pellet was then extracted three times with the appropriate medium. The total α-galactosidase activity solubilized by each extraction medium was then determined. Table 8 shows that with increasing concentrations of NaCl

TABLE 8

Extraction of α-galactosidase from mature Vicia faba seed powder using various media

Extractant (0.1 M potassium phosphate buffer, pH 7.0)	Activity per extraction O.D.405		Total enzyme activity extracted O.D.405
0.1 M potassium phosphate buffer	(i) 144 (ii) 252	(iii) 55 (iv) 26	477
Sodium chloride (M)			
0.05	(i) 408 (ii) 400	(iii) 100 (iv) 37	945
0.10	(i) 416 (ii) 325	(iii) 106 (iv) 77	924
0.25	(i) 420 (ii) 416	(iii) 104 (iv) 40	980
0.5	(i) 352 (ii) 446	(iii) 174 (iv) 85	1057
1.0	(i) 452 (ii) 334	(iii) 179 (iv) 88	1053
Triton (% w/v)			
0.1	(i) 289 (ii) 417	(iii) 100 (iv) 34	838
0.25	(i) 119 (ii) 489	(iii) 261 (iv) 20	889
0.50	(i) 299 (ii) 461	(iii) 62 (iv) 21	843
1.0	(i) 104 (ii) 387	(iii) 76 (iv) 27	594
5.0	(i) 144 (ii) 206	(iii) 43 (iv) 14	407

continued .....

TABLE 8 (continued)....

Extractant (0.1 M potassium phosphate buffer, pH 7.0)	Activity per extraction O.D.405		Total enzyme activity extracted O.D.405
0.1 M potassium phosphate buffer	(i) 144 (ii) 252	(iii) 55 (iv) 26	477
Methyl-α-D-mannoside			
0.1	(i) 308 (ii) 282	(iii) 42 (iv) 18	650
0.25	(i) 340 (ii) 282	(iii) 107 (iv) 74	803
0.5	(i) 535 (ii) 325	(iii) 61 (iv) 18	939
0.8	(i) 536 (ii) 648	(iii) 174 (iv) 104	1462
1.0	(i) 651 (ii) 683	(iii) 322 (iv) 45	1701
D-Glucitol			
0.1	(i) 259 (ii) 230	(iii) 37 (iv) 29	555
0.25	(i) 311 (ii) 272	(iii) 66 (iv) 19	668
0.5	(i) 361 (ii) 283	(iii) 78 (iv) 30	752
0.8	(i) 379 (ii) 296	(iii) 87 (iv) 39	801
1.0	(i) 484 (ii) 301	(iii) 79 (iv) 35	899

Seed powder (5g) was extracted four times (i-iv) with the appropriate medium, by grinding in a pestle and mortar at 10 min intervals for 1 min over a period of 30 min. Extracts were centrifuged at 15,000 rpm for 40 min and the supernatant assayed for enzyme activity as described in the Experimental section (Section 2.1.1).

or methyl-α-D-mannoside there was increased solubilization of α-galactosidase activity.

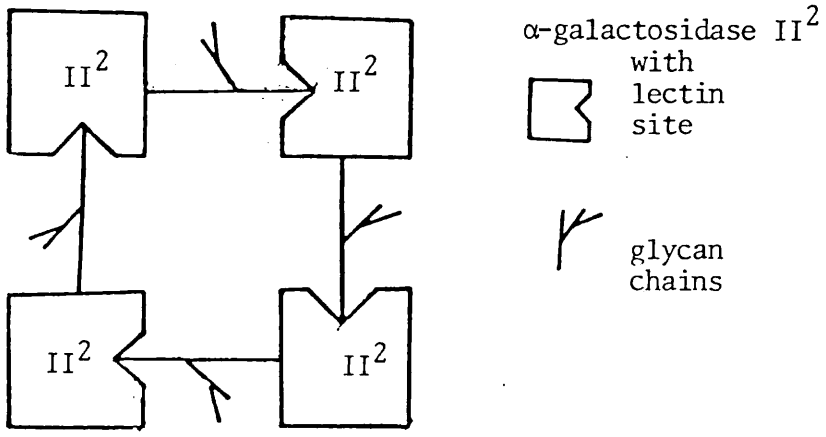
In the case of 1 M NaCl, the total activity extracted was approximately twice that obtained with 0.1 M potassium phosphate buffer alone. With 1 M methyl-α-D-mannoside this was raised to approximately three-fold. In the case of the detergent, Triton X-100, there appeared to be increased extraction of the enzyme, compared to buffer alone, at 0.1% concentrations, but at higher levels there was in general, decreasing activity, presumably as a result of enzyme inhibition. NaCl and methyl-α-D-mannoside at 1 M concentrations did not inhibit activity when added to a soluble α-galactosidase preparation in phosphate buffer.

The most interesting observation (Table 8) is that buffered solutions of methyl-α-D-mannoside solubilized more α-galactosidase activity than the corresponding concentrations of NaCl. The latter is normally considered to release ionically bound protein: dehydration effects may also facilitate this release. Solubilization by methyl-α-D-mannoside could indicate lectin binding, with the monosaccharide derivative specifically competing, for example, with cell receptors (glycoprotein glycans?) for the α-galactosidase lectin sites. A further possibility is that a non-specific dehydration reaction by methyl-α-D-mannoside might be responsible for the release of bound α-galactosidase activity. However, with D-glucitol (a straight chain monosaccharide alcohol which is unlikely to compete strongly with cell lectin receptors) was used as an extractant, significantly less (approximately 52% at 1 M sugar concentration) α-galactosidase was extracted than with methyl-α-D-mannoside.

In conclusion, the binding of α-galactosidase to cell components via lectin-glycan bonds in seed powder is a possibility which requires further study and, in particular, a detailed investigation of the effect of different carbohydrates on the solubilization of activity.

In this connection, it is of interest to note that Bowles et al [209] claimed that the galactose-specific lectin in Ricinus communis seed mitochondria was membrane-bound and could be solubilized with a lactose solution, hence suggesting the existence of bonds between lectin sites and galactosyl receptors in the membrane.

In addition to binding to cell components, other interactions are possible between α-galactosidase glycan chains on one enzyme molecule and lectin sites on another. This could result in a novel type of secondary intermolecular binding which would allow protein units to associate as polymeric quaternary structures, as shown below for α-galactosidase I:



It was argued that if such a structure existed, it would possibly dissociate to monomer units in the presence of a competing hapten. However, when 1.2 M methyl-α-D-mannoside was incubated with α-galactosidase I for 24 h at 4°C and the mixture then examined on a methyl-α-D-mannoside equilibrated column, no α-galactosidase monomer could be detected. This does not necessarily mean that inter-monomer lectin links do not exist, as the affinity of the enzyme-lectin sites for low molecular weight haptens may be much smaller than the complex chains of mannose residues present on the enzyme. Wheat germ lectin, for instance, has been shown to possess two classes of binding sites for cell surface components of human erythrocytes: high affinity sites (4.9×10^6 per cell; apparent $K_A = 1.27 \times 10^7\ M^{-1}$) and low affinity sites (3.3×10^6 per cell; apparent $K_A = 1.x\ 10^6\ M^{-1}$) [210].

Treatment of α-galactosidase I with endo H, which specifically removes 'mannose-rich' glycan chains from the enzyme (see p.47), yielded an enzymically active fraction with an apparent molecular weight of 80,000 corresponding to a dimer (Fig.29) but no monomer could be detected in this preliminary study. It would appear, therefore, that the glycan chains are important in some way for the stabilization of the quaternary structure of α-galactosidase I, possibly via lectin bonds.

However, if α-galactosidase II[2] possesses two lectin sites and the tetrameric enzyme, I, has eight (see p.127), then this suggests that all the latter are unoccupied (and available for binding with mannose on the equilibrium dialysis study) and, hence cannot be involved in inter-monomer binding. There could, however, conceivably be other sites with low affinity for mannose and a high affinity for complex

glycans which are involved in holding the monomers together. Information relating to the possible intermolecular lectin bonds is, therefore, conflicting and before firm conclusions can be reached, a detailed study of the association and dissociation of the α-galactosidases under various conditions is required.

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The lectin nature of α-galactosidases from *Vicia faba* seeds

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α-Galactosidase from *Vicia faba* seeds has been resolved into three molecular forms, I, II^1 and II^2, respectively. Enzyme I is a tetramer (M_r 160000) consisting of identical sub-units (M_r 44000 ± 2000). All three forms display lectin activity with glucose/mannose specificity. Enzyme I has been further studied with respect to its lectin specificity and various factors affecting this property. The results indicate that the catalytic and the lectin sites reside in the same protein molecule. The results presented are unique in that the enzyme activity is specific for galactose and its lectin activity is specific for glucose/mannose.

Vicia faba *α-Galactosidase* *Lectin* *Multimolecular form*

1. INTRODUCTION

Carbohydrate-binding proteins which agglutinate erythrocytes and precipitate glycoconjugates are very common in plant tissues. These phytohaemagglutinins or lectins may be simple proteins or glycoproteins and they exhibit considerable binding specificity towards carbohydrates. The nature and properties of plant lectins have been reviewed in [1,2]. Leguminous seeds are a rich source of lectins many of which possess homologous segments of amino acids and, hence, may have an evolutionary relationship [3–5].

Few examples of enzymes possessing lectin activity have been reported but α-mannosidase from *Phaseolus vulgaris* seeds [6] and α-galactosidases obtained from several species of leguminous seeds [7] have been shown to be lectins.

α-Galactosidases from *Vigna radiata* (mung bean) seeds possess monomeric and tetrameric forms of the enzyme (M_r 40000–45000 and 160000, respectively) [8,9]. The tetramer is a lectin possessing D-galactose specificity.

2. METHODS

α-Galactosidase activity was assayed using *p*-nitrophenyl α-D-galactoside (PNPG) as substrate according to [11].

Lectin activity was assayed using a 1.5% suspension of rabbit blood erythrocytes, as in [12,13]. The sugar specificity of the lectin was analysed by observing 50% inhibition of agglutination on a visual serological scale [13].

Protein was determined as in [14] using bovine serum albumin as a standard.

This paper is mainly concerned with a study of α-galactosidase I from *Vicia faba* seeds, a tetrameric glycoprotein with glucose/mannose lectin specificity. This enzyme occurs together with its monomer, II^2, and a second low M_r enzyme, II^1, in resting seeds [10].

3. RESULTS AND DISCUSSION

The procedure used for the purification of the three forms of α-galactosidase from resting *Vicia faba* seeds was essentially similar to that in [10], although the con A–Sepharose stage was omitted to avoid possible contamination with concanavalin A. In addition, enzymes I and II were recycled twice through a Sephadex G-100 column and not recycled through CM-cellulose.

At each stage in the purification (see table 1) both the specific α-galactosidase and lectin activities were measured. Unlike the observations in [8], the lection/enzyme activity ratio did not remain the same during the purification. In stages I–III

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Table 1

Purification of α-galactosidase and the accompanying lectin activity from *Vicia faba* seeds[a]

Purification stage		Vol. (ml)	Enzyme act. (nkat/ml)	Protein (mg/ml)	(A) Spec. act. (nkat/mg)	(B) Lectin act. (HA/mg)	Ratio A/B
I. Crude extract		900	16	130.0	0.12	17600	7.0×10^{-6}
II. Citric acid precipitation		1000	15	30.0	0.50	20440	2.4×10^{-5}
III. $(NH_4)_2SO_4$ fractionation (25–65%)		95	117	130.0	0.90	12300	7.3×10^{-5}
IV. Sephadex G100 gel filtration	EI	90	57	4.0	14.2	1560	9.1×10^{-3}
	EII	75	48	1.4	34.3	2200	1.5×10^{-2}
V. CM-cellulose chromatography	EI	10	118	0.01	11800	650	18.1
	EII^1	2	50	0.41	122	7710	1.6×10^{-2}
	EII^2	4	210	0.18	1167	1800	0.65
VI. Melibiose–Sepharose	EI	3	250	0.02	12500	770	16.2
VII. α-Methyl mannoside–agarose	EI	1.2	456	0.04	11400	770	14.8

[a] 1 kg of seed powder was used

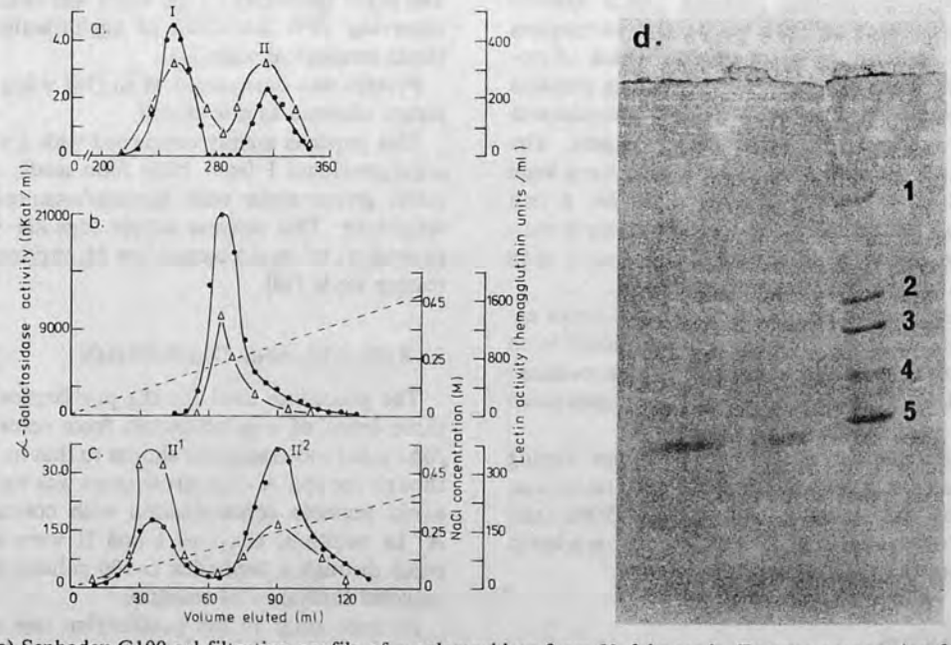

Fig. 1. (a) Sephadex G100 gel filtration profile of α-galactosidase from *V. faba* seeds. Enzyme preparation from stage III of table 1 was used; (b) CM-cellulose chromatography of α-galactosidase I; (c) CM-cellulose chromatography of α-galactosidase II; (●—●) α-galactosidase activity; (△—△) hemagglutinin activity; (– – –) NaCl gradient; (d) SDS–polyacrylamide slab gel electrophoresis of *V. faba* α-galactosidase I. The two wells to the left show enzyme samples (from stage V, table 1; 20 μg and 10 μg, respectively) stained with Coomassie blue. The following marker proteins (M_r $\times 10^{-3}$) were applied to the right well: 1, myosin (200000); 2, β-galactosidase (116000); 3, phosphorylase *b* (94000); 4, bovine serum albumin (68000); 5, ovalbumin (43000).

there was a 7-fold increase in enzyme specific activity whereas the lectin activity decreased. In the following stage (IV), lectin and enzyme (M_r 160000) activities co-eluted from a Sephadex G-100 column (fig. 1a) and there was a marked reduction in total lectin activity, presumably due to the removal of *Vicia faba* lectin (M_r 47500; [13]) and a further significant increase in enzyme purity. On passing enzyme I through CM-cellulose (fig. 1b; stage V) more lectin activity was lost, although the enzyme specific activity rose 800-fold in comparison with stage IV. (This enzyme preparation had a 10-fold higher specific activity than reported for enzyme I [10] although the amount of protein recovered was comparable. The loss of activity can presumably be explained by the greater number of purification stages used in [10].) Enzyme II from stage IV was resolved into two fractions, II^1 and II^2 on CM-cellulose (fig. 1c). Both of these fractions possessed higher specific agglutination activities than enzyme I. α-Galactosidase and lectin activities co-eluted in all cases in stage V.

When α-galactosidase I was examined by flat-bed SDS gel electrophoresis [15] it migrated as a single protein band (M_r 44000 ± 2000) which exhibited microheterogeneity. In comparison, the largest subunit of *V. faba* lectin has an M_r of 22500 [13].

To establish that none of the α-galactosidase fractions were contaminated with the *V. faba* lectin isolated in [13], which is devoid of α-galactosidase activity (N. Sumar, unpublished), all 3 purified forms were passed separately through a 3-*O*-methyl-D-glucosamine–CH-Sepharose affinity column [13] but no detectable α-galactosidase or lectin activity was absorbed. Furthermore, α-galactosidase I was retained entirely by an immobilized melibiose column (1.3 × 2.5 cm, 0.05 M acetate buffer, pH 4.0 at 4°C; Pierce and Warriner, UK) which is known to bind lectins and carbohydrate-binding proteins with α-D-galactose specificity [16]. On elution of this column with 10 mM PNPG and dialysis of the desorbed material the enzyme/haemagglutinin ratio was measured and shown to be essentially similar (stage VI, table 1) to that of the fraction applied to the column. Hence no contamination of enzyme I with the *V. faba* lectin was apparent. The binding of enzyme I to the melibiose affinity column could have occurred via catalytic and/or galactose-specific lectin sites on the enzyme. However, the presence of galactose-specific lectin sites appears unlikely as enzyme I was fully absorbed when passed through a methyl α-D-mannoside–agarose column (1.3 × 5 cm, 0.01 M phosphate buffer, pH 7.2 at 20°C; Sigma, London): this material is known to bind glucose/mannose-specific lectins. When the column was eluted with 0.5 M methyl α-D-mannoside and the resulting fraction dialysed, the enzyme/haemagglutinin ratio (stage VII, table 1) was similar to that observed at stage VI; the small difference is due to the loss of enzyme activity at stage VII brought about by the relatively high pH (7.2) used in this affinity step.

The lectin specificity of α-galactosidase I was further investigated by the hapten inhibition technique [17]. Table 2 shows the effects of various carbohydrates on rabbit erythrocyte agglutination. The data clearly show that D-mannose and to a lesser extent D-glucose and their low-M_r derivatives were significantly more effective in producing in-

Table 2

Sugar inhibition of hemagglutinin activity of α-galactosidase I from *Vicia faba* seeds

Sugar	Minimum concentration (mM) causing 50% inhibition of 6 hemagglutinin units [13]
D-Glucose	31.3
3-*O*-Methyl-D-glucopyranoside	31.3
Methyl-α-D-glucopyranoside	62.5
Methyl-β-D-glucopyranoside	250
N-Acetyl-glucosamine	31.3
D-Mannose	15.6
Methyl-α-D-mannopyranoside	3.1
D-Galactose	1000
Methyl-α-D-galactopyranoside	1000
D-Xylose	250
D-Arabinose	250
Melibiose	100
Stachyose	100
Raffinose	100
Sucrose	125
Galactomannan (clover)	0.125 mg/ml
Galactomannan (guar)	0.094 mg/ml
Glycogen	0.032 mg/ml
Starch (soluble)	0.003 mg/ml

hibition than D-galactose and its derivatives. Polysaccharides possessing glucosyl or mannosyl residues also acted as agglutination inhibitors.

α-Galactosidase I precipitated soluble starch and glycogen from solutions, and plots of turbidity against polysaccharide concentrations gave the normal characteristic bell-shaped curves [18,19]. Fig. 2 shows the interaction of yeast α-mannan with α-galactosidase I and the reciprocal relationship between the turbidity and the enzyme activity in the supernatant. Changes in the latter could be accounted for by changes in the activity in the precipitate. Methyl α-D-mannopyranoside at final conc. 50 mM prevented precipitation of the mannan in these experiments.

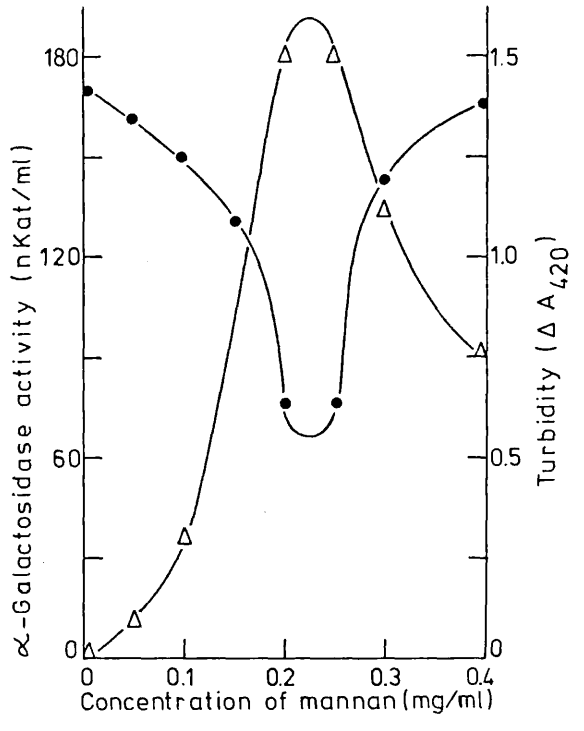

Fig. 2. Interaction of α-galactosidase I with yeast mannan. α-Galactosidase I (0.2 mg/ml) from stage V of table 1 was added to various concentrations of mannan solution in 0.1 M sodium acetate–HCl buffer (pH 6.1) and incubated at 25°C for 10 min. The turbidity formed was measured at 420 nm. The turbid solutions were centrifuged and the total activity in the supernatant and the precipitate was measured: (●—●) α-Galactosidase activity; (Δ—Δ) hemagglutinin activity.

In view of the close similarity between lectin and enzyme activities in *V. radiata* α-galactosidase reported in [8,20], a comparison of these activities was made with α-galactosidase I. Fig. 3 shows a

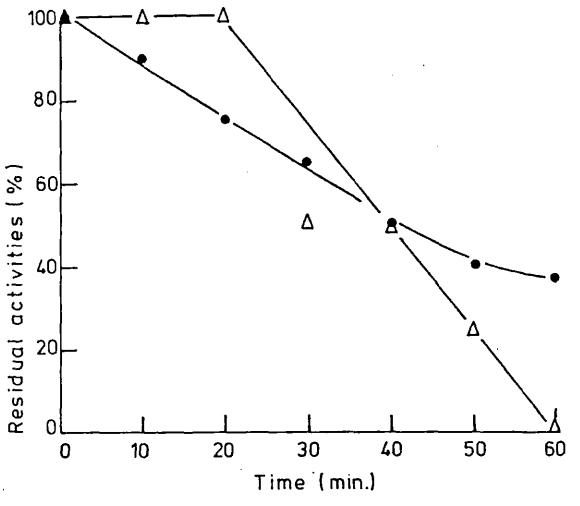

Fig. 3. Heat stability of α-galactosidase I. The enzyme (stage V of table 1) was incubated at 65°C in McIlvaine buffer (pH 5.5) for various time intervals and then the α-galactosidase (●—●) and hemagglutinin (Δ—Δ) activities were measured.

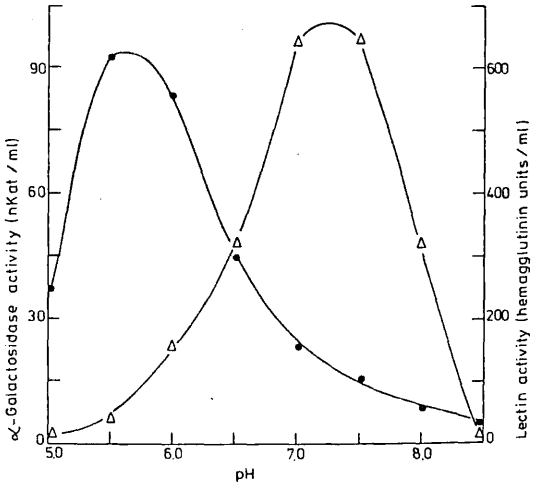

Fig. 4. pH optimum of hemagglutinin and enzyme activities of α-galactosidase I. Reactions were carried out in 0.1 M potassium phosphate buffer at various pH values using the enzyme from stage V of table 1; (●—●) α-galactosidase activity; (Δ—Δ) hemagglutinin activity.

difference in the heat stabilities of the lectin and catalytic activities of the *V. faba* enzyme. Fig. 4 shows that the optimum pH for agglutination is almost two units removed from that for the hydrolysis of PNPG.

A clear difference between the lectin and catalytic activities of α-galactosidase I can also be seen when the data in table 2 are compared with enzyme inhibition studies [21]. D-Galactose and galactose-containing oligosaccharides inhibited the enzyme activity but significantly higher concentrations of these compounds were needed to inhibit haemagglutination.

4. CONCLUSIONS

α-Galactosidase I possesses both enzymic and lectin activities and the latter is glucose/mannose specific. It seems very unlikely, in view of the purification procedure used, the affinity column studies and the molecular mass differences, that the enzyme preparation is composed of two proteins, an α-galactosidase and the *V. faba* lectin. All results suggest that the sites for both catalytic and lectin functions reside at separate loci on the same protein, and, hence, both in terms of lectin specificity and site separation, *V. faba* α-galactosidase I differs from the haemagglutinating α-galactosidase in [8]. In the latter case enzyme and lectin activities would appear to reside at identical or nearly identical sites. It is possible to explain the observations [8] in terms of a tetrameric α-galactosidase. Thus in the case of their agglutination studies the enzyme may first bind via its catalytic sites to terminal α-D-galactosyl residues on the surfaces of the red blood cells and then slowly hydrolyse, thereby causing the blood-clot to dissolve. It is also worth noting that the monomeric form of the enzyme from *Vigna radiata* [9] possessed no lectin activity, unlike the monomer II^2 of α-galactosidase I from *V. faba*. (Preliminary studies have shown that both II^1 and II^2 are also glucose/mannose-specific lectins.) In the case of the *Vigna* enzyme, this could result if monomer units possessed only single binding sites.

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BINDING OF α-GALACTOSIDASE I FROM VICIA FABA TO POTATO STARCH GRANULES AND SHEEP ERYTHROCYTES.

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Key Word Index - Vicia faba; Leguminosae; broad bean; α-galactosidase; lectin seed.

Abstract - α-Galactosidase I from Vicia faba seeds has been shown to bind to potato starch and sheep erythrocytes. With the aid of fluorescence microscopy and using 4-methylumbelliferyl α-D-galactoside as the substrate it has been demonstrated that the binding is via the lectin sites of the enzyme leaving catalytic sites free and detectable. The lectin site is specific for D-glucose/D-mannose residues.

INTRODUCTION

Three forms of α-galactosidases, I, II^1 and II^2 have been isolated from Vicia faba seeds and purified [1]. All forms hydrolyse α-D-galactosides but they also behave as lectins with D-glucose/D-mannose specificies [2]. There is evidence in the case of α-galactosidase I that these two activities reside in the same protein molecule but on separate loci [2]. The 'classical' lectin, favin [3] which, again, is D-glucose/D-mannose specific, also occurs in V.faba seeds, however, there are no indications that favin is a contaminant of purified α-galactosidase I. The latter enzyme appears to be a unique example of a protein possessing separate catalytic and lectin sites. In the case of other, so-called, enzyme-lectins, for example, α-galactosidase from Vigna radiata [4], both activities would appear to reside on the same or same type of site [5].

In this communication we report the binding, via lectin sites, of α-galactosidase I to starch granules and sheep erythrocytes. The mode of binding of this enzyme and perhaps others, to cell constituents is of considerable importance as it introduces a new concept of in vivo localisation of enzymes. It has been postulated, for example, that α-galactosidases are compartmentalised in maturing seeds in order to separate enzyme from substrate (galactosyl-sucrose derivatives) [2,6,7] and also in the case of mature stachyose-exporting leaves of Cucurbita sp. [8]. In both cases it is conceivable that lectin binding of

the enzyme to cellular constituents is involved [9].

RESULTS AND DISCUSSION

The haemagglutinin (lectin) activity of α-galactosidase I is clearly illustrated in Fig. 1 which shows the agglutination of sheep erythrocytes by the enzyme. The bound enzyme with free and functional catalytic sites can be observed by fluorescent light microscopy after the addition of 4-methylumbelliferyl α-D-galactoside to the agglutinated cells (Fig. 2). This substrate is not itself fluorescent, but after enzymic hydrolysis the 4-methylumbelliferone released is strongly fluorescent in blue light. Fig. 2a shows that after a short incubation with substrate there is an intense fluorescence surrounding the agglutinated cells which is about 600-fold (estimated from the film exposure time) greater than the natural fluorescence displayed by untreated cells in a control experiment. The dark background in figure 2(a) indicates that no significant diffusion of newly liberated 4-methylumbelliferone from the surface of the agglutinated cells into the surrounding medium has taken place. This should be contrasted with the light background, resulting after 15 min incubation, in Fig. 2(b) in which 4-methylumbelliferone has diffused into the surroundings.

When α-galactosidase I was incubated with potato starch granules the bound enzyme could again be demonstrated by fluorescence microscopy. Unlike the erythrocytes, starch granules do not display a natural fluorescence. Fig. 3(a) represents an early stage during the incubation with 4-methylumbelliferyl α-D-galactoside and α-galactosidase I, with a dark background surrounding the fluorescing starch particles. Furthermore, it was shown that the fluorescence of the granules was not produced by incubating them with 4-methylumbelliferone. Again, at a later stage (Fig. 3b) the fluorescence has spread into the background as a result of product diffusion. It is assumed that the binding of α-galactosidase I to the granules occurred via the interaction between the lectin sites of the enzyme and the starch glucosyl residues while the substrate binding/catalytic sites remained free. The production of granule fluorescence was inhibited by 0.1M D-mannose in the incubation mixture. In addition, studies with various hapten inhibitors on the distribution of α-galactosidase activity between starch granules and the suspension medium (Table I) support this assumption. D-Glucose and D-mannose and oligosaccharides with terminal non-reducing α-D-glucopyranosyl residues all inhibited the binding wherease melibiose and raffinose, with terminal α-D-galactopyranosyl residues, did not. Furthermore, treatment of the starch granule - α-galactosidase complex with α-amylase released 80% of the bound

enzyme into the supernatant. Extensive lectin binding to glycan components of amyloplast membrane fragments on the granules was unlikely as washing the granules with acetone had no apparent effect on the degree of binding.

These results indicate that α-galactosidase binds to the glycosyl residues of sheep erythrocytes/starch granules via its D-glucose/D-mannose specific lectin sites [2] leaving the catalytic site free and detectable. This work also suggests the possibility of using enzyme-lectins as cytochemical reagents which can be very readily detected after binding to cell receptors.

EXPERIMENTAL

Enzyme preparation: The procedure used for the purification of α-galactosidase I from resting seeds of Vicia faba was the same as that described earlier [1]. The enzyme activity was assayed using p-nitrophenyl-α-D-galactoside (PNPG) as the substrate [10,11].

Qualitative observations using incident fluorescence light microscopy: (a) Using potato starch (acetone-washed) - Four tubes were prepared containing 500 µl starch suspensions (10 mg/ml) in phosphate buffered saline, (PBS; 7.2g NaCl, 1.42g Na_2HPO_4, 0.43g KH_2PO_4) pH 7.2; tube (iii)contained, in addition, 0.1M mannose. α-Galactosidase I (10 µl; 80 nKat/ml) was added to tubes (i), (ii) and (iii) and incubated for 2h. No enzyme was added to tube (iv). The suspensions were then mounted on microscope slides for observing fluorescence using a Leitz Epifluorescent system [Ploenopak incident fluorescent illumination with H_2 filter block, which provides violet and blue light (390-490 nm) for excitation]. 4-Methylumbelliferyl α-D-galactoside solution (1 mg/ml; 10 µl) in PBS was added to the slides prepared from tubes (i), (iii) and (iv) and observed under the microscope immediately and at definite intervals. Samples of tube (ii) and (iv) were taken as controls.

(b) Using sheep erythrocytes - Two tubes, (i) and (ii), were prepared with 500 µl of 2% (v/v) red blood cell suspension in PBS, pH 7.2. α-Galactosidase I (10 µl; 80 nKat/ml) was added to tube (i) and the sample was incubated at room temperature for 2h. Slides were prepared from both tubes for observation under the fluorescence microscope.

Quantitative measurement of α-galactosidase I binding to starch

Potato starch suspensions in PBS, pH 7.2 (500 µl, 1% W/V) were placed

in microcentrifuge tubes containing 0.1M D-mannose, D-glucose, melibiose, maltose, sucrose or raffinose: one control reaction mixture contained only starch and PBS. α-Galactosidase I (10 µl, 80 nKat/ml) was added to each tube and the suspensions incubated for 2h at 20°C. The tubes were shaken at intervals and finally centrifuged; each supernatant (50 µl) was assayed for α-galactosidase activity. The concentration of the haptens in the assay was uninhibitory to the enzyme.

In a separate experiment, α-galactosidase I - starch incubation mixture in PBS was treated with Aspergillus niger α-amylase (final concn., 25 units/ml) for 1h at 37° followed by centrifugation and assay for α-galactosidase activity in the supernatant.

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LEGENDS

Fig. 1. Agglutination of sheep erythrocytes; (a) sheep erythrocytes in PBS (X 500), (b) sheep erythrocytes agglutinated by incubating with α-galactosidase I from V. faba (X 500).

Fig. 2. Display of fluorescence by α-galactosidase I agglutinated erythrocytes after 2 min (a) and 15 min (b) after addition of 4-methylumbelliferyl-α-D-galactoside solution.

Fig. 3. Display of fluorescence by potato starch granules after incubating (2 h) with α-galactosidase I followed by the addition of 4-methylumbelliferyl-α-D-galactoside solution and leaving for (a) 2 min and (b) 15 min.

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TABLE I

Effect of carbohydrates on the binding of α-Galactosidase I to potato starch granules*

Carbohydrate (final conc. 100mM)	α-Galactosidase activity (%)	
	Starch Granules	Supernatant
None	51.4	48.6
D-Glucose	19.7	80.3
D-Mannose	18.6	81.4
Maltose	35.4	64.6
Sucrose	37.7	62.3
Melibiose	54.5	45.6
Raffinose	56.6	43.6

*See Experimental section for details.

Fig. 1

See Thesis p. 110

Fig. 1

Li...

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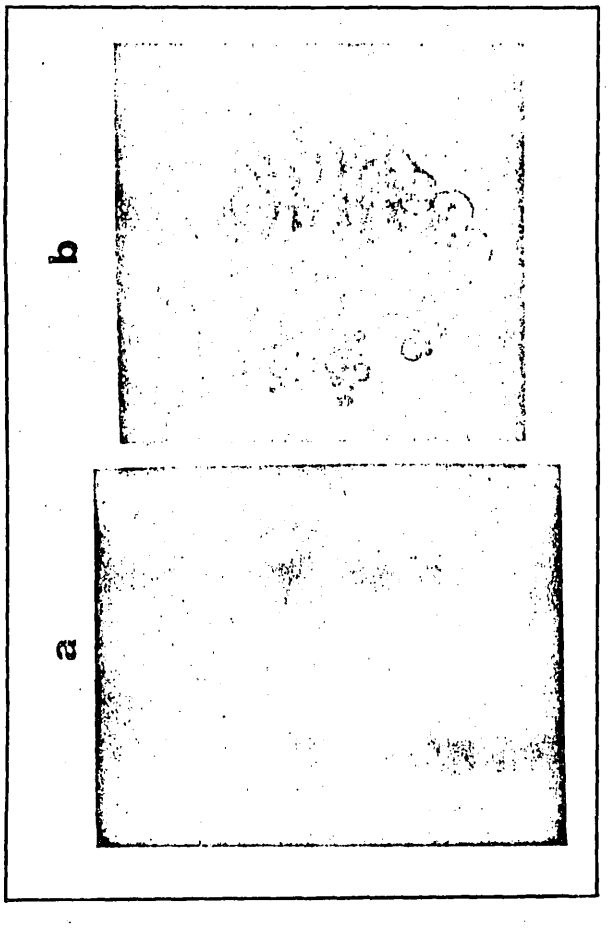
Fig. 3



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