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THE NATURE OF VICIA FAB

$\alpha$ -GALACTOSIDASES

by

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## ABSTRACT

Two molecular forms I and II (separable by gel filtration) from mature Vicia faba seeds have been studied. The extractability of the enzymes and the in vitro conversion of the low MW form, II, to the larger oligomer, I has been examined over a range of salt concentrations. Specific and total activities of the preparations were high when strong salt solutions were used for extraction. It would appear that  $\alpha$ -galactosidase I, in comparison with II, is best extracted from the seeds if solutions of high ionic strength are used.

The effects of these salt solutions on the relative levels of the two forms have been investigated by gel filtration. Interpretation of the gel elution profiles obtained is, however, complicated by the in vitro conversion of form II to form I, which is favoured by high salt concentrations and some routine procedures in the purification of  $\alpha$ -galactosidases, such as ammonium sulphate fractionation.

The relationships between the multiple forms have been studied.  $\alpha$ -galactosidase II can be resolved into enzymes II<sup>1</sup> and II<sup>2</sup> by CM-cellulose chromatography.  $\alpha$ -Galactosidases I, II<sup>1</sup> and II<sup>2</sup> have been highly purified. Form I (MW 160,000) is a tetramer of enzyme II<sup>2</sup> as shown by SDS-PAGE. Immunological studies on the three forms have been carried out and the evidence suggests that they are structurally related, although forms I and II<sup>2</sup> are more closely related than forms I and II<sup>1</sup>.

On hydrolysis, monosaccharides are released from the three purified enzymes, suggesting they are glycoproteins. The three  $\alpha$ -galactosidases also agglutinate red blood cells indicating that they possess lectin activity.

To Salim

## ACKNOWLEDGEMENTS

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Lastly, I wish to extend my gratitude to Sally Montagnon for her patience and skill in typing this thesis.

## ABBREVIATIONS

MW	molecular weight
SDS	sodium dodecyl sulphate
PAGE	polyacrylamide gel electrophoresis
SDS-PAGE	polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate
Mann/Gal	mannose/galactose
Con A	concanavalin A
TCA	trichloroacetic acid
Anti-I	anti-( $\alpha$ -galactosidase I)-antiserum
Anti-II <sup>1</sup>	anti-( $\alpha$ -galactosidase II <sup>1</sup> )-antiserum
Anti-II <sup>2</sup>	anti-( $\alpha$ -galactosidase II <sup>2</sup> )-antiserum
Form I, II, II <sup>1</sup> and II <sup>2</sup>	$\alpha$ -galactosidase I, II, II <sup>1</sup> and II <sup>2</sup>
Enzyme I, II, II <sup>1</sup> and II <sup>2</sup>	$\alpha$ -galactosidase I, II, II <sup>1</sup> and II <sup>2</sup>

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## **INTRODUCTION**

## 1 $\alpha$ -D-Galactosidases : Nature and Properties

### 1.1 General

Glycosidases, the enzymes that catalyse the hydrolysis of the glycosidic bonds of simple glycosides, oligo- and polysaccharides, have attracted the continuous attention of biologists for about 150 years. The occurrence of these enzymes has been reported in animals (1-6), micro-organisms (7-12) and plants (13-23).

Recently, there has been a considerable increase in the interest in these enzymes because of their usefulness as tools for studying the structures of oligo- and polysaccharides and glycoproteins (24).

The study described in this thesis is concerned with the preparation of three highly purified forms of  $\alpha$ -galactosidase (EC 3.2.1.22) from Vicia faba seeds and an investigation of their structural relationships, glycoprotein compositions and lectin activities.



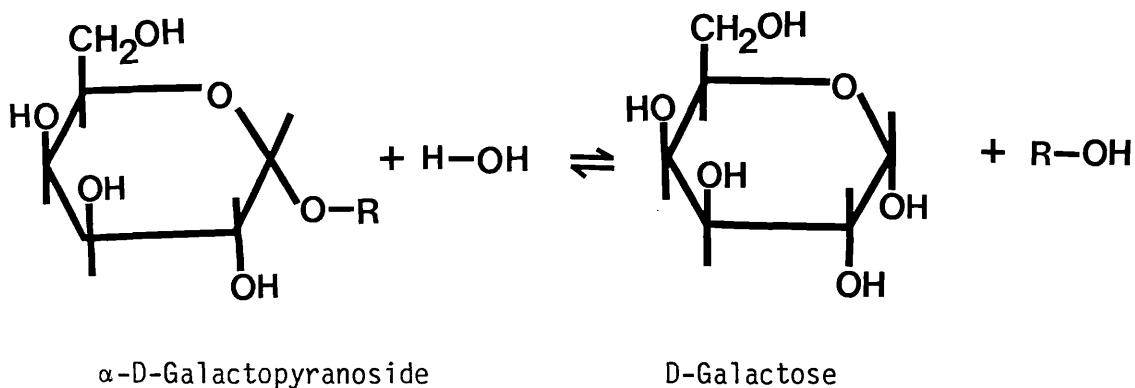
## 1.2 Occurrence

The wide distribution of  $\alpha$ -galactosidase activity is illustrated in the review by Dey and Pridham (25), only in a few cases have attempts been made to purify the enzyme. Harpaz et al (26) have obtained a homogeneous preparation from soybeans and multistage purifications of the enzyme from sweet almonds (27), germinating kidney beans (15), dormant broad beans (28), common vetch seeds (29) and coffee beans (30) have been carried out. Of a number of micro-organisms which produce  $\alpha$ -galactosidase, Mortierrella vinacea has been found to be an excellent source for the large scale production of the enzyme (31) and Suzuki et al (32) were able to obtain the first crystalline preparation of  $\alpha$ -galactosidase from this fungus.

Galactosidases can generally be obtained in soluble form by tissue homogenisation with aqueous buffers. However, in spinach leaves, the enzyme has been detected in chloroplast, mitochondrial and microsomal fractions (33).

## 1.3 Reaction

$\alpha$ -Galactosidases are generally known to hydrolyse the  $\alpha$ -D-galactosidic linkages of substrates possessing non-reducing terminal galactose residues. They catalyse the following reversible reaction:-



With most enzymes, R may represent alkyl, aryl, monoglycosyl or polyglycosyl residues.

#### 1.4 Multimolecular forms of $\alpha$ -galactosidase

The occurrence of isoenzymes in plant and animal cells is widespread. Since the first international conference on multiple molecular forms of enzymes in 1961 <sup>(34)</sup>, an ever increasing number of enzymes have been reported to exist in more than one molecular form. The term 'isoenzyme', first introduced by Markert and Moller <sup>(35)</sup> has been used in several senses by various workers. The word has been and to some extent is still being used to describe any category of enzyme heterogeneity, without any implication as to the origin of the diverse forms or the nature of the molecular differences between them. Current recommendations however, are that only those different molecules which exhibit a particular type of catalytic activity but have distinct genetic origins should be classed as isoenzymes.

Multiple forms of a given enzyme are still frequently referred to as isoenzymes, even though the information to justify their inclusion within the recommended restricted usage of this term may be lacking (36). At present, the molecular basis for enzyme multiplicity is known to be of several different kinds. In addition to genetic reasons, multiple forms may arise from other causes which are categorized below (34):-

- i) Artefacts - caused by deamidization, binding of substrates, proteolytic action; for example, yeast hexokinase and chicken aldolase.
- ii) Aggregates - multiple forms with different electrophoretic mobilities produced by association of monomer units; for example, serum choline esterase and  $\beta$ -galactosidase.
- iii) Conformers - enzymes with the same amino acid sequences and molecular weights but differing conformations which result in different electrophoretic mobilities; examples are mitochondrial malate dehydrogenase and brain-type creatine kinase.

Although there has been much discussion about the possibility that multiple forms of some enzymes are artefacts, very few of these have been clearly identified (34). As separation methods have improved, increasing numbers of multiple forms of enzymes

have been discovered. Whether many of these forms are present in vivo or arise during the purification processes is not known, but the readiness of some isolated enzymic-forms to aggregate and/or dissociate suggests that in the natural state less heterogeneity prevails (37).

The occurrence of multimolecular forms of  $\alpha$ -galactosidase was first reported by Petek and Dong (38). Two forms were obtained from the seeds of coffee by fractionation on alumina columns. The enzyme from coffee could not be resolved by Sephadex-gel filtration (39) which relies on molecular weight (MW) differences but three active forms were separable by polyacrylamide gel electrophoresis (PAGE) at pH 8.3 (40) indicating different charge properties.

An  $\alpha$ -galactosidase preparation obtained from Aspergillus niger could not be resolved into multiple forms by Sephadex G-200, Biogel 200, DEAE-Sephadex or DEAE-cellulose chromatography. However, on CM-cellulose columns, three active forms were obtained (41).  $\alpha$ -Galactosidase from M. vinacea (32) gave a single band when examined by PAGE. However, elution of the enzyme from a DEAE-Sephadex column yielded three peaks of activity. This latter study illustrates that it is not always possible to separate multiple forms of an enzyme by the use of different techniques which rely on similar protein properties.

Wakabayashi (42) found that the  $\alpha$ -galactosidase from bottom yeast

could be resolved into at least four active components by zone electrophoresis. A single major form was reported to be present in mouse liver as judged by the failure to detect multiple components on ion-exchange chromatography and electrophoresis (43). However, Lusi and Paigen (43), have questioned whether minor forms of the enzyme exist. Human tissues in contrast to mouse liver (43), contain two major forms of  $\alpha$ -galactosidase, A and B (44-46), which appear to be quite distinct as judged by enzymological (47), immunochemical (48-50) and genetic studies (51, 52).

Following the first reports of multiple forms of  $\alpha$ -galactosidase in coffee (38), the majority of work on these higher plant enzymes has been focused on seed tissues particularly in relation to their oligosaccharide contents and the utilization of these (mainly the raffinose family of carbohydrates) during germination.

The plant  $\alpha$ -galactosidases that have been studied in detail include those from coffee (30, 38, 40), plantain (53), broad bean (28, 54, 55), and water melon (56). Many seeds have been found to contain two forms which are separable by gel filtration. The larger forms have MWs which are reported to range from 125,000 to 209,000 and the smaller forms from 23,000 to 57,000 (25, 39, 57). In the case of the legumes, seeds from twenty different species (20 genera) have been examined for  $\alpha$ -galactosidase activity (57). All species contained one high MW form (commonly referred to as

I) and most species also possessed the second lower MW enzyme, II. Additionally,  $\alpha$ -galactosidase I always represented a significant fraction of the total enzyme activity. In the above study with leguminous seeds, the MWs of enzyme I ranged from 120 - 190,000 and of II from 30 - 42,000 as determined by Sephacryl S-200 gel filtration.

There have been fewer studies with other plant genera but two forms of the enzyme which differ in MW have been separated from the extracts of Cucumis sativus leaves (58). The two forms I and II were partially purified using ammonium sulphate fractionation, Sephadex-gel filtration and DEAE-Sephadex chromatography and their MWs as determined by gel filtration, were 50,000 and 25,000 respectively. The higher MW form comprised ca 84% of the total  $\alpha$ -galactosidase activity in crude extracts from the mature C. sativus leaves.  $\alpha$ -Galactosidase activity has also been studied in leaves of Cucurbita pepo (59) and by the use of DEAE-cellulose chromatography, three forms of the enzyme were detected and their distribution in the plant studied (60). Gatt and Baker (33) who looked at the enzyme in spinach leaves have found only one form of  $\alpha$ -galactosidase in the leaves. Two forms of the enzyme were found in the case of Cocos nucifera kernal (61, 62), Cajanus indicus seeds (63, 64) and Lens esculenta seeds (65).

Dey and Pridham (14, 28, 55) have studied the multiple forms of  $\alpha$ -galactosidase in Vicia faba seeds. They have shown that

extracts of dormant V. faba seeds contain two  $\alpha$ -galactosidases differing in MW. The two forms, I and II, were purified 3,660 and 337 fold, respectively and both behaved as homogeneous protein preparations when examined by PAGE. The MWs estimated by Sephadex-gel filtration studies were 209,000 and 38,000 for I and II, respectively. The kinetic properties of the two forms were examined and it was shown that the specific activity of I was higher (ten times) than that of II (66). Enzymes I and II were later re-examined by ion-exchange chromatography (25, 67, 68). No further resolution of either enzyme was affected with DEAE cellulose columns. However, when II was applied to a CM-cellulose column which was then eluted stepwise with buffer of increasing pH, two active fractions, II<sup>1</sup> and II<sup>2</sup> were obtained. The total activity of the peak for II<sup>1</sup> was three times higher than that of the II<sup>2</sup> fraction. Enzyme I could not be resolved further by passage through a CM-cellulose column. The MW of both II<sup>1</sup> and II<sup>2</sup>, estimated by gel filtration was 38,000. MW determinations by sedimentation equilibrium techniques gave values for enzymes I, II<sup>1</sup> and II<sup>2</sup> of  $160,400 \pm 2850$ ,  $45,730 \pm 3073$   $43,390 \pm 1409$  respectively (68, 69).

### 1.5 Interconversion of multiple forms of enzymes

The interconversion of multiple forms of enzymes in vitro is not uncommon. For example  $\beta$ -galactosidase from mouse liver has a subunit MW of 63,000 but the enzyme is apparently

a dimer at neutral pH and aggregates further under acidic conditions to form a tetramer with an apparent MW of 240,000 (70). The  $\beta$ -galactosidase from Escherichia coli dissociates into an inactive monomer in the presence of  $\text{Ag}^+$  but when the monomer is treated with excess of thiol, an enzymically active dimer is formed in addition to an active tetramer (71). Umezurike (72), working with  $\beta$ -glucosidase from Botryodiplodia theobromae demonstrated that storage of the purified high MW enzyme (350,000 - 380,000) led to its dissociation into low MW 'species' with MWs 170,000 - 180,000, 83,000 - 87,000 and 45,000 - 47,000. The rate of the association-dissociation processes must be slow, making it possible for the different components in the system to be readily separable by gel filtration and gel electrophoresis.

When an  $\alpha$ -galactosidase preparation from human tissues (eg. liver, kidney) containing A and B forms was incubated with neuraminidase at pH 5.0, form A disappeared gradually while B increased (45, 73): contrary to these suggestions, the two forms cannot be converted by neuraminidase (47, 74). Schram et al (50) working on  $\alpha$ -galactosidase from human liver have shown that during storage,  $\alpha$ -galactosidase B is converted to a form with some kinetic and physical-chemical properties similar to those of  $\alpha$ -galactosidase A (see also ref. 75). This form remains immunologically identical to  $\alpha$ -galactosidase B (contrast ref. 75).



Regarding  $\alpha$ -galactosidase in plants, interconversion of multiple forms A and B from coconut kernal has been reported (61, 62). Sephadex-gel filtration of a partially purified preparation (P) of  $\alpha$ -galactosidase in the presence of 0.1 M potassium chloride resolved the enzyme activity into two fractions, A (the major component) and B. On repeating the gel filtration of P in the absence of potassium chloride in the column buffer, only a single peak of activity corresponding to the lower MW enzyme, B, appeared. It, therefore, appears that the conversion of A to the lower MW form B is inhibited by the presence of potassium chloride. Conversion of B to an  $\alpha$ -galactosidase with the same apparent MW as A was also observed. This occurred when enzyme B was mixed with an inactive protein fraction C and then eluted with McIlvaine buffer containing 0.1 M potassium chloride on a Sephadex column. In the absence of C no conversion took place.

Mung bean possesses an  $\alpha$ -galactosidase which can be reversibly converted by pH changes from a tetrameric form to a monomeric form (76). At pH 7.0 or above, the tetramer is dissociated into the low MW monomer (40,000) and as the pH is lowered from 7 to 4, the monomers re-associate into a form with MW 160,000. Since dissociation occurs at pH values near the isoelectric pH, the major forces involved in aggregate formation may be electrostatic. Therefore, the mung bean  $\alpha$ -galactosidase appears to undergo a pH-dependent noncovalent association-dissociation reaction similar to that reported for soybean  $\alpha$ -galactosidase (26).

Interconversion of forms I and II of V. faba  $\alpha$ -galactosidases has been observed both in vivo (66) and in vitro (68, 69). Soaking the resting bean seeds in water resulted in a 65% decrease in total  $\alpha$ -galactosidase activity which could be roughly equated with a fall in the amount of I. After 60 h, no further marked alteration in activity was observed (66). Examination of the total  $\alpha$ -galactosidase activity and enzyme patterns obtained by Sephadex-gel filtration of extracts of immature V. faba seeds showed that at early stages of development only a low level of enzyme II exists and the level increases with maturation. At later stages, there is a marked increase in specific activity of the extract which can be related to the appearance of I and decrease of II. It was not claimed, however, that I is derived from II in vivo (68).

Storage at 4<sup>0</sup>C for several days, of a partially purified preparation of fraction II (obtained from a batch of seeds which appeared to be mature but only possessed enzyme II) resulted in an increase in specific activity with a concomitant formation of the high MW enzyme I (68, 69). It was claimed that the conversion did not occur if II had previously been passed through a molecular sieve (67). Most of the work cited above was carried out using partially purified preparations of the enzyme, however, more recent work by Hustler (77) with crude bean extracts shows that differences in Sephadex-gel profiles can arise from in vitro reactions occurring during the purification process.

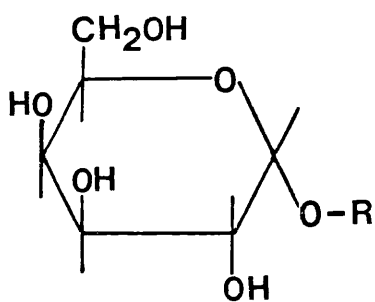
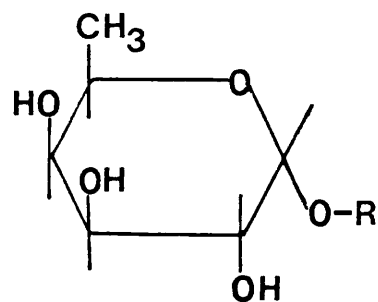
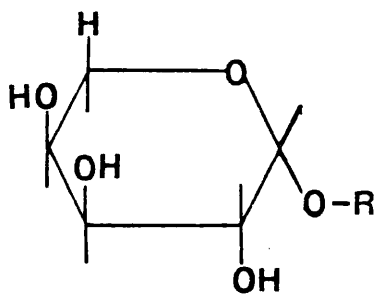
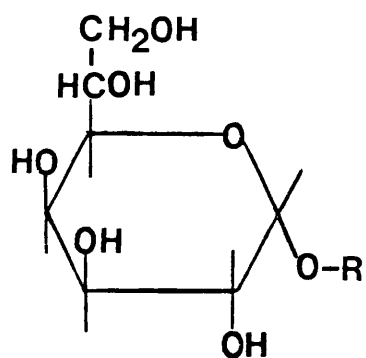
## 1.6 Specificity

### 1.6.1 Simple glycosides and oligosaccharides

Glycosidases usually show a specificity that is high with respect to the glycone moieties of the substrate and its anomeric configurations, whilst large structural variations are permitted in the aglycone (78). In general, changes in the configuration of hydrogen and hydroxyl groups on any single carbon atom of a glycoside substrate is sufficient to reduce the rate or completely inhibit the hydrolytic action of the corresponding glycosidase.

In the case of  $\alpha$ -galactosidase, the substrate must resemble the structure of an  $\alpha$ -D-galactopyranoside, particularly with respect to ring size and hydroxyl configuration on carbon atoms 1, 2, 3 and 4, in order that significant rates of hydrolysis may occur. Hence, the enzyme should hydrolyse  $\alpha$ -D-fucopyranosides,  $\beta$ -L-arabinopyranosides and D-glycero- $\alpha$ -D-galactoheptopyranosides (Fig. 1) as well as  $\alpha$ -D-galactopyranosides (79, 80). This does not always appear to be the case (81-85).

Wide limits of specificity exist for the aglycone moiety. Thus the  $\alpha$ -galactosidase from Aerobacter aerogenes is capable of hydrolysing melibiose and methyl, ethyl, phenyl and o-nitrophenyl  $\alpha$ -D-galactopyranosides (86). Similarly,  $\alpha$ -galactosidases from Calvatia cyanthiformis, Prunus amygdalus and Vicia faba are able to hydrolyse methyl, phenyl and naturally occurring  $\alpha$ -

Fig. 1.  $\alpha$ -D-Galactopyranoside and related glycosides $\alpha$ -D-Galactopyranoside $\alpha$ -D-Fucopyranoside $\beta$ -L-ArabinopyranosideD-Glycero- $\alpha$ -D-galacto-heptoside

galactosides including melibiose, raffinose and stachyose. (85, 25). In general, aryl  $\alpha$ -D-galactosides are better substrates than alkyl derivatives or disaccharides (25, 80, 85, 87).

The naturally occurring and synthetic  $\alpha$ -D-galactosides hydrolysed by various  $\alpha$ -galactosidases are reviewed by Dey and Pridham (25). The ease of hydrolysis of non-reducing terminal  $\alpha$ -galactosyl residues of oligosaccharides normally decreases with increasing chain length (29, 55, 82, 84, 85, 88). A free reducing group in an oligosaccharide is reported to reduce the rate of hydrolysis. For example, melibiose is hydrolysed less rapidly than raffinose by  $\alpha$ -galactosidases from Cajanus indicus (64).

### 1.6.2 Polysaccharides

Several  $\alpha$ -galactosidases hydrolyse  $\alpha$ -D-galactosyl linkages in polysaccharides such as galactomannans (15, 32, 89). Galactomannans of leguminous seeds are composed of  $\beta$ -(1  $\rightarrow$  4) linked D-mannopyranosyl residues. D-Galactopyranosyl groups are attached to these backbones by  $\alpha$ -(1  $\rightarrow$  6)-linkages. The percentage of mannose residues thus substituted ranges from 20-100 depending on the source of the galactomannan. When the galactose to mannose ratio approaches 1.0 (as in the polysaccharide from alfalfa or clover), it is not possible to remove all the galactose in vitro even after several successive additions of coffee bean  $\alpha$ -galactosidase (90).  $\alpha$ -Galactosidase from germinated fenugreek seeds (91) is capable of partially

hydrolysing galactomannan (Mann/Gal 1.2:1) obtained from the same source.

Agrawal and Bahl (15) using an enzyme preparation from Phaseolus vulgaris obtained a 27 - 30% removal of total galactose from locust bean (Mann/Gal 3.75:1) and guar (Mann/Gal 1.5:1) galactomannans. The  $\alpha$ -galactosidases, I and II from Cajanus indicus (64) hydrolysed guar and locust bean gums and the hydrolysis rates appeared to be higher than those for  $\alpha$ -galactosidases from P. vulgaris and Vicia faba (68) as shown in Table 1.  $\alpha$ -Galactosidase II from Vicia faba did not hydrolyse locust bean gum whereas enzyme I showed a 6% release of galactose after 45 min. Variations in hydrolysability according to the source of the galactomannan substrate have been found. For example, the galactomannan from Gleditsia ferox (Mann / Gal 3.7:1) was reduced by coffee bean  $\alpha$ -galactosidase to a water soluble material, whereas galactomannans from white clover (Mann / Gal 1.6:1), genista (Mann / Gal 4.1:1) were only slightly hydrolysed (89).

An  $\alpha$ -galactosidase from Lens esculenta (65) however failed to liberate galactose from guar (Mann / Gal 1.5:1), locust (Mann / Gal 3.7:1) and tara (Mann / Gal 2.9:1) gums irrespective of the varying Mann / Gal ratios. Similar results have been obtained with a purified  $\alpha$ -galactosidase preparation from germinating Vicia sativa seeds (29).

Table 1. Hydrolysis of galactomannan by  $\alpha$ -galactosidases.

Source of enzyme	Substrate	Amount of enzyme milliunits	Period of incubation h	Galactose released %	
<u>P. vulgaris</u> seeds (15)	guar gum	1160	24	27	
	locust bean gum	1160	24	30	
<u>C. indicus</u> seeds (64)	guar gum	20	2	10	11.3
	locust bean gum	20	2	9.1	12
<u>V. faba</u> seeds (68)	locust bean gum		3/4	6	0

\*  $\alpha$ -galactosidases I and II.

In conclusion it would appear that, the degree of hydrolysis of galactomannan is controlled not only by the Mann / Gal ratio but also by the distribution pattern of the  $\alpha$ -galactosyl residues on the galactomannan backbone.

### 1.7 Transgalactosylation

In addition to the hydrolysis of  $\alpha$ -D-galactosides,  $\alpha$ -galactosidases from various plant and microbial sources catalyse trans  $\alpha$ -D-galactosylation reactions from aryl  $\alpha$ -D-galactopyranosides and galactose-containing oligosaccharides to alcohols and mono- and oligo- saccharide acceptor molecules (92 - 94).

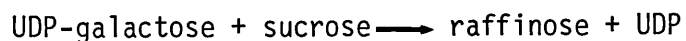
D-Galactosyl transferase activity has been extensively studied especially with respect to parameters such as galactosyl donor and acceptor specificity, acceptor concentration, pH, temperature and source of enzyme (80, 87, 90, 91, 95). The subject is also reviewed by Dey and Pridham (25).

In general, galactose is transferred preferentially to the primary alcohol group of an oligosaccharide acceptor. When yeast  $\alpha$ -galactosidase is incubated with melibiose (0- $\alpha$ -D-galactopyranosyl-(1  $\rightarrow$  6)-D-glucopyranose) for example, galactose is transferred from one molecule to another with the formation of mannotriose (0- $\alpha$ -D-galacopyranosyl-(1  $\rightarrow$  6)-0- $\alpha$ -

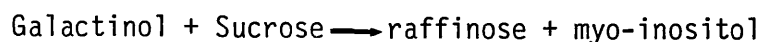
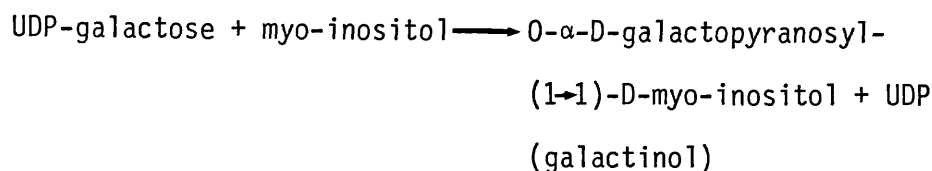


D-galactopyranosyl-(1 → 6)-D- glucopyranose (96, 97). The transfer of a D-galactose residue from melibiose to sucrose producing raffinose and its isomer planteose has been reported in the case of V. faba  $\alpha$ -galactosidase (13). Most  $\alpha$ -galactosidases have a low transfer / hydrolysis ratio, but preparations from kidney beans (98) and wheat (99) have high ratios. In the case of the enzyme isolated from kidney beans (98) transgalactosylation from galactinol to raffinose with the formation of stachyose occurs more readily than hydrolysis of galactinol.

Before the discovery of sugar nucleotides, such transfer reactions were thought to be a major route for oligosaccharide synthesis (25). It is doubtful whether  $\alpha$ -galactosidases have any synthetic function in vivo. The possible biosynthetic pathway for the formation of raffinose in several plant species has been examined by a number of workers, some (100 - 102) of whom have concluded that the galactose is derived directly from UDP-galactose with sucrose acting as an acceptor.



Kandler and Hopf (103, see also 104 - 106) however consider that the  $\alpha$ -galactoside, galactinol is an intermediate in this reaction



## 1.8 Physiological significance of $\alpha$ -galactosidases

### 1.8.1 Hydrolysis of reserves.

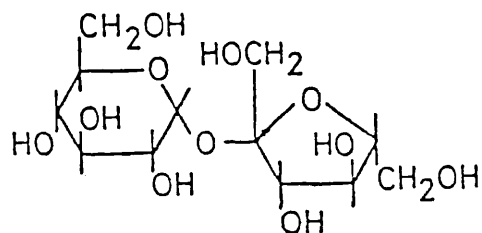
In addition to sucrose, a variety of other oligosaccharides occur widely in the plant kingdom (107 - 110). The vast majority of these oligosaccharides may be considered as derivatives of sucrose. In all cases, they appear to be synthesized by the transfer of galactopyranosyl, glucopyranosyl or fructofuranosyl residues (usually from nucleotide derivatives) to sucrose.

Raffinose is the basis of a series of homologous oligosaccharides which was named the 'raffinose family' by French (97). As shown in Fig.2, raffinose is 6-O- $\alpha$ -D-galactosylsucrose and the tetrasaccharide stachyose, contains a second galactosyl residue attached via an  $\alpha$ -(1  $\rightarrow$  6)-linkage to the galactosyl moiety of raffinose. Further addition of galactose gives rise to verbascose, ajugose and longer chain oligosaccharides to DP 9.

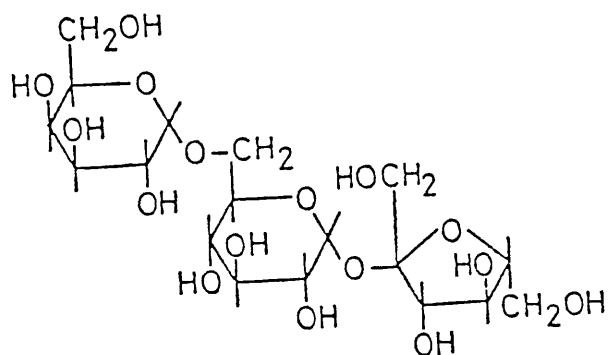
In storage tissues, these oligosaccharides are commonly present (109, 111 - 115), in amounts equal to or greater than that of

Fig. 2. The Raffinose family of oligosaccharides

Sucrose (0- $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -D-fructofuranoside).



Raffinose (0- $\alpha$ -D-galactopyranosyl-(1 $\rightarrow$ 6)-0- $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -D-fructofuranoside)



Stachyose (0- $\alpha$ -D-galactopyranosyl-(1 $\rightarrow$ 6)-0- $\alpha$ -D-galactopyranosyl-(1 $\rightarrow$ 6)-0- $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -D-fructofuranoside)

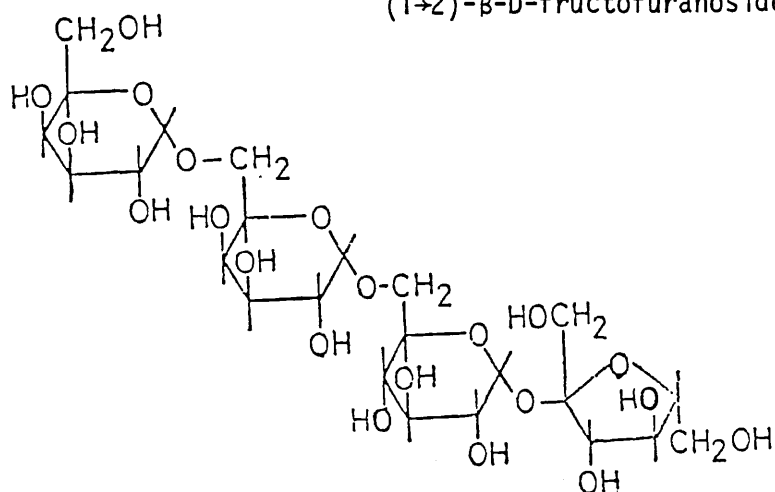


Fig. 2 cont.

Verbascose (0- $\alpha$ -D-galactopyranosyl-(1 $\rightarrow$ 6)-0-D-galactopyranosyl-(1 $\rightarrow$ 6)-0- $\alpha$ -D-galactopyranosyl-(1 $\rightarrow$ 6)-0- $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -D fructofuranoside).

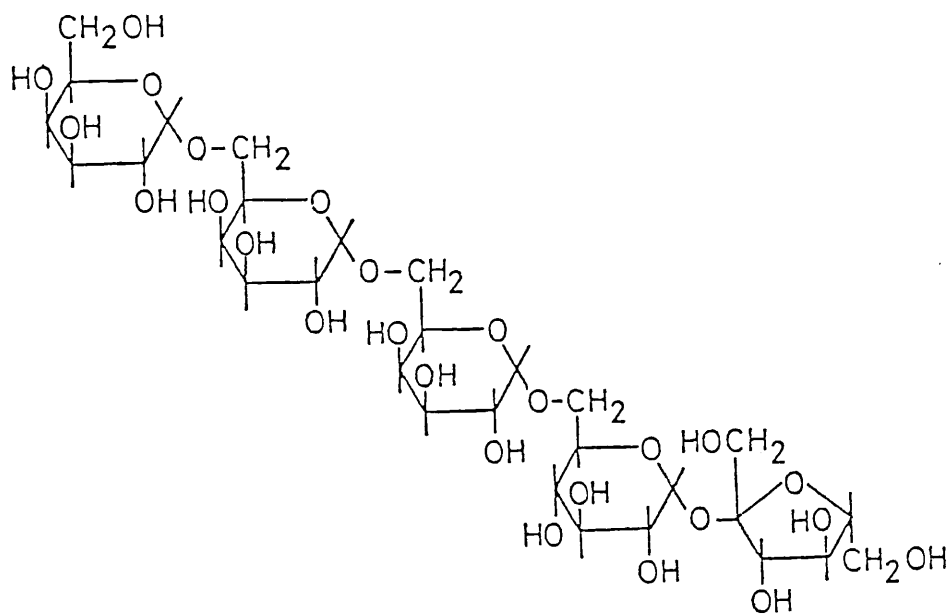
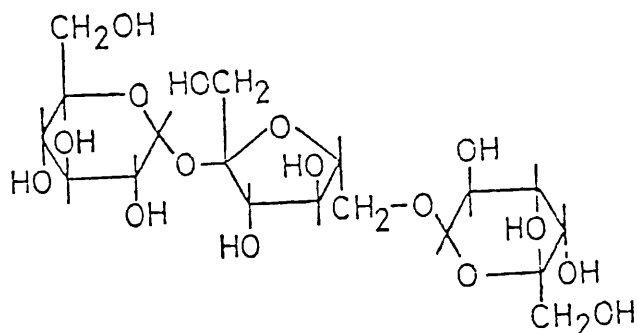


Fig. 3.

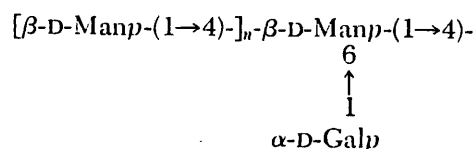
Planteose (0- $\alpha$ -D-galactopyranosyl-(1 $\rightarrow$ 6)-0- $\beta$ -D-fructofuranosyl-(2 $\rightarrow$ 1)- $\alpha$ -D-glucopyranoside



sucrose. More than one member of this family of oligosaccharides normally occurs together with sucrose in storage tissues and the mixture of galactosylsucroses appears to form a major reserve of soluble low MW carbohydrates (97).

An isomer of raffinose with the trivial name planteose (Fig. 3) which was discovered in seeds of Plantago species (116) is found in several plant species (117) commonly in association with stachyose and raffinose. Sesamose, a tetrasaccharide present together with planteose in large amounts in the seeds of Sesamum indicum has been tentatively identified as a higher homologue of planteose (118) containing two galactosyl residues attached to the fructose moiety.

In addition to oligosaccharides, galactomannans also occur as reserve carbohydrates in many leguminous seeds (119, 120). The seed galactomannans occur in the endosperms of seeds of a wide range of species of Leguminosae (121, 122). With the exception of two cases (119, 123) the sole source of the galactomannan has been found to be the endosperm. Plant galactomannans are composed of linear chains of (1 → 4) - linked β -D-mannopyranosyl residues having single stubs of α -D-galactopyranosyl groups joined by (1 → 6)-linkages along the chain. The fundamental structure of the plant galactomannan is the following (122).



$\alpha$ -Galactosidases are responsible for cleaving the galactose residues from all of these galactose-containing reserve materials.

There have been many studies with the raffinose family of oligosaccharides, which support this statement. For example, Gould and Greenshields (124) studied the distribution and changes of these carbohydrates in ripening and germinating seeds of Phaseolus vulgaris. They found that on maturity, the bean seed contained raffinose oligosaccharides and sucrose in the embryo and cotyledons, a situation which persisted throughout the dormancy period. On germination, the galactosylsucrose derivatives disappeared leaving sucrose, glucose and fructose. There have been similar observations with other seeds for example, Japanese red pine (125), black pine (125), sunflower (125) and cotton (126) where raffinose was found to be metabolized during germination.  $\alpha$ -Galactosidase activity has also been shown to increase during the germination of seeds of various species, notably, fenugreek (127, 128), guar (129, 130), lucerne (130), carob (130), soybean (130), runner bean (131, 132), coffee (133), cotton (126) and various others (25, 134). In most of these, a concomitant depletion of  $\alpha$ -D-galactosidic

reserve carbohydrates takes place. A large amount of stachyose has been found in peas and the work of Lee and Shallenberger (135) indicates that  $\alpha$ -galactosidase is responsible for the breakdown of the tetrasaccharide during the early stages of germination.

It has been noted by Shiroya (126) that raffinose is metabolized in germinating cotton seeds in the same way as in the seeds of pines, and other plants no free galactose being detected throughout all stages of germination. Similarly, when planteose, is metabolized during germination of sesame seeds, no free galactose is detected (125).

When germination of cotton seeds (126) was carried out under reduced pressure, raffinose and stachyose remained unchanged, although  $\alpha$ -galactosidase activity was detected. On introducing air, the seeds began to germinate and at the same time, raffinose and stachyose disappeared. This suggests that oxygen is necessary not only for germination but also for the disappearance of these sugars. As aerobic conditions are required for galactose utilization, raffinose is doubtless readily hydrolysed under the same conditions.

The failure to detect free galactose in tissues where active hydrolysis of galactosylsucrose derivatives is occurring, suggests that galactose utilization is very efficient and that the rate of utilization of the monosaccharide is at least as high

as the rate of removal of galactose from the oligosaccharides by  $\alpha$ -galactosidase. Pridham et al (136) examined the fate of raffinose in germinating V. faba seeds and suggested that the concerted action of  $\alpha$ -galactosidase and sucrose-UDP-glucosyltransferase may form an important mechanism for the rapid utilization of raffinose and other galactosylsucrose derivatives. Pazur (137) has suggested that the hydrolytic cleavage of galactosyl residues is followed by phosphorylation of the galactose by galactokinase. This enzyme is very active in the early stages of germination of pea seeds (135) and is also present in broad bean seeds (136, 138), hence the free galactose is converted to galactose-1-phosphate. The latter reaction presumably is at least partly responsible for the failure of the seeds to accumulate free galactose during germination. Galactose-1-phosphate, can no doubt be converted to UDP-galactose by UTP-galactose-1-phosphate uridyl transferase, a ubiquitous enzyme which has been detected in germinating V. faba seeds (Pridham, J.B. and Walter, M.W. unpublished results).

In contrast, galactose and manninotriose are detectable in germinating Stachys, Verbascus, and Ajuga seeds (see ref. 126) which contain raffinose and its higher homologues. Invertase is, therefore, presumably responsible for the appearance of manninotriose.

McCleary and Matheson (130) have looked at  $\alpha$ -galactosidase activity and galactomannan and galactosylsucrose oligosaccharide

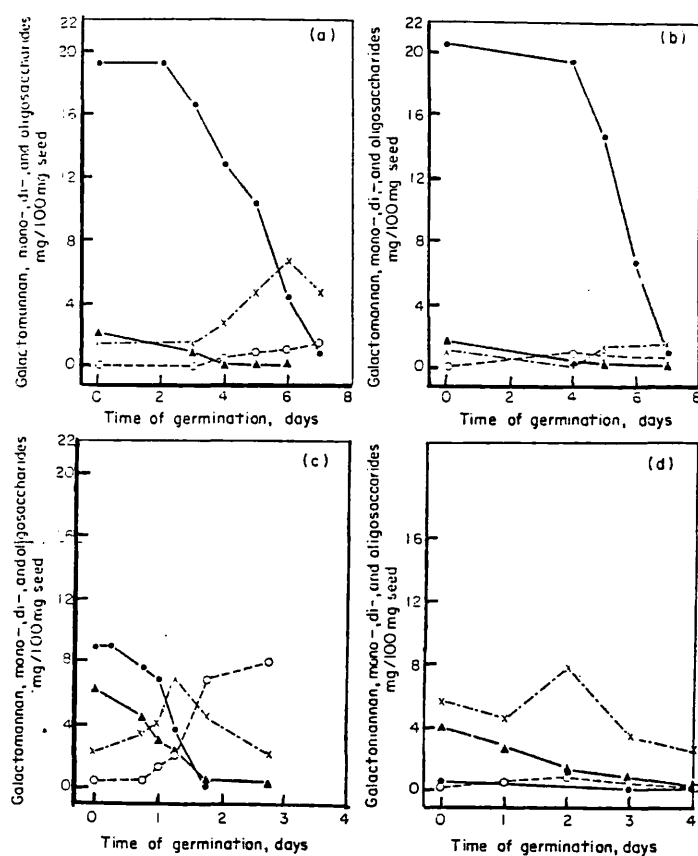


depletion in germinating legume seeds. On germination, seeds of lucerne, guar and carob are initially depleted of the raffinose series of oligosaccharides and then the galactomannans disappear (Fig. 4)<sup>(130)</sup>. This breakdown of reserves was accompanied by a rapid increase and then a decrease in total  $\alpha$ -galactosidase activity (Fig. 5). The  $\alpha$ -galactosidase activity in lucerne, guar, carob and soybean was separated into multiple forms, A, B and C by DEAE - cellulose chromatography. Lucerne and guar seeds contain two forms (A and C), carob has three forms (A, B and C) and soybean four forms (A, B, C<sub>1</sub> and C<sub>2</sub>); enzyme C was resolved into C<sub>1</sub> and C<sub>2</sub> by Sephadex G-200 column chromatography.

When the seeds were separated into endosperm and cotyledon plus embryo,  $\alpha$ -galactosidases A and B were always found in the cotyledon-embryo fraction whereas  $\alpha$ -galactosidase C was found in the endosperm. However in the case of carob seeds, enzyme C was present in both, the endosperm and the cotyledon-embryo region (Table 2).

On germination, the changes in the levels of activities varied.  $\alpha$ -Galactosidase A of lucerne, carob and soybean were present before germination (Fig. 6) and showed a slight increase and decrease on germination whereas the enzyme from guar decreased gradually. Changes in the levels of  $\alpha$ -galactosidase C were much larger except for soybean and the cotyledon-embryo enzyme C of carob (Fig. 7).

Fig. 4. Depletion of galactomannan, oligosaccharides and monosaccharides on the germination of  
 a) Carob; b) Guar; c) Lucerne; and d) Soybean.



● Galactomannan; ▲ Oligosaccharides;  
 × Disaccharides; ○ Monosaccharides.

Reproduced from McCleary and Matheson (ref.130)

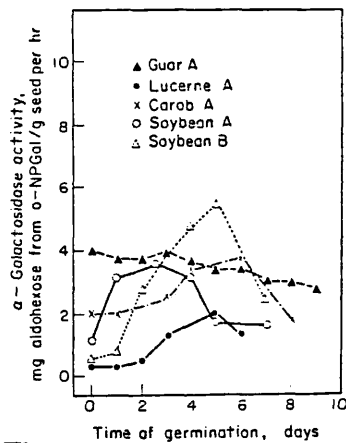
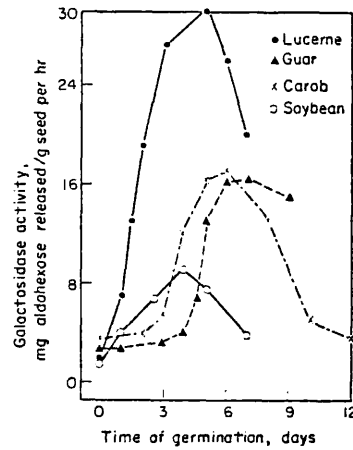
Fig. 5. Changes in total  $\alpha$ -galactosidase activity on germination

Fig.6

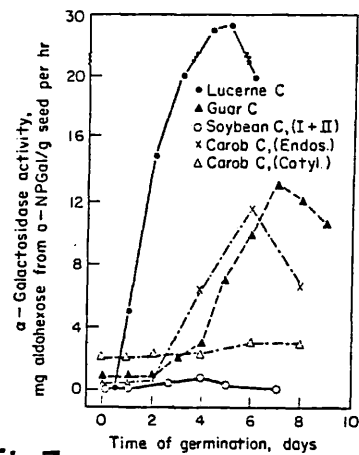


Fig.7

Fig. 6. Changes in  $\alpha$ -galactosidases A and B activities on germination.Fig. 7. Changes in  $\alpha$ -galactosidase C activities on germination.

Table 2. Location of multiple forms (A, B, C,) of  $\alpha$ -galactosidase in lucerne, guar, soybean and carob seeds (130).

Organ	Seed			
	Lucerne	Guar	Carob	Soybean
Cotyledon-embryo	A,B	A,B	A,B,C	A,B
Endosperm	C	C	C	C

In lucerne, carob and guar, seeds that contain significant amounts of galactomannan (8, 19, 20 mg/100 g seed respectively), the major part of the total  $\alpha$ -galactosidase increase on germination was due to enzyme C. From its location in the endosperm where all the galactomannan is found, enzyme C appears to be primarily involved in galactomannan hydrolysis. However, since galactosylsucrose oligosaccharides are also found in the endosperm of these three seeds (lucerne, carob and guar), this enzyme is also likely to be involved in their breakdown. In soybean, however, there was only a slight increase in the  $\alpha$ -galactosidase C activity on germination which is consistent with the low galactomannan content of these seeds.

The presence of  $\alpha$ -galactosidases A and B in the cotyledon-embryo region of soybean, suggests that these enzymes are responsible for the hydrolysis of the raffinose family of oligosaccharides present in this seed tissue. The changes in levels of activities of A and B paralleled the depletion of the oligosaccharides.

The pattern of disappearance of galactomannan has been studied in fenugreek seeds by Reid (139). Working with fenugreek seeds soaked in water for 24 h, Reid has shown that after a further 24 h (first phase of germination), the level of galactomannan remained constant in the endosperm and that the level of stachyose and verbascose decreased. The dissolution of galactomannan occurred in the second phase (lasting 24 h after the first phase). The

galactomannan reserve and the raffinose family of sugars completely disappeared from the endosperm at the end of this phase. During the third and final phase, galactomannan is absent and the endospermic carbohydrates of low MW also disappear completely and rapidly.

$\alpha$ -galactosidase activity was measured in the embryo and endosperm homogenates from fenugreek seeds at different stages of germination (128). Embryo and endosperm tissues were examined separately because galactomannan is present only in the former. In the endosperm, the early stages of germination (up to 15 h), were marked by the virtual absence of  $\alpha$ -galactosidase followed by a significant increase in activity (the activity reaching 75 mu per seed), suggesting its association with galactomannan breakdown. Extracts of resting and germinating embryos, on the other hand, contained high  $\alpha$ -galactosidase activity (ca 62 mu per seed) which changed only slightly (ca 70 mu per seed) throughout germination. No in vitro work has so far been carried out to establish whether the  $\alpha$ -galactosidases of the endosperm and embryo are distinct enzymes with different substrate specificities.

The correlation between the changes in endosperm  $\alpha$ -galactosidase activity and the different stages of galactomannan mobilisation is so close as to leave little doubt about its direct involvement in the breakdown process. It is interesting to note that the embryos of fenugreek seeds possess high  $\alpha$ -galactosidase

activities for which galactomannan cannot be the natural substrate since the galactomannan is present in the endosperm. It has been suggested (139) that the embryo enzyme may be responsible for the hydrolysis of the raffinose family of oligosaccharides.

Raffinose and its higher homologues have been found in the sieve tube exudates of trees. In white ash, most of the carbohydrate in the exudate appears to be raffinose and stachyose and in the case of elm and linden, raffinose and stachyose accompany sucrose (113, 114). It is therefore probable that raffinose and sucrose take part in the translocation of photosynthetic products from the leaves to other parts of the plant (126). Studies on the metabolism of galactosylsucroses have been mostly confined to seed tissues where it is acknowledged that hydrolysis is affected by  $\alpha$ -galactosidase. It would seem reasonable to expect that those plants that transport these oligosaccharides in their vascular tissues possess  $\alpha$ -galactosidases which are involved in their breakdown.

Thomas and Webb (60) have discovered that immature, galactosylsucrose-importing leaves of Cucurbita pepo possess the ability to rapidly hydrolyse raffinose, stachyose and verbascose which enter the tissues from the phloem. These tissues cannot synthesize or store the oligosaccharides. In contrast, mature, exporting leaves are able to synthesize these oligosaccharides but no in vivo hydrolysis is detectable (140 - 142). However,

the presence of comparatively high levels of  $\alpha$ -galactosidase in the mature leaves suggests that the enzyme may have an alternative role in these tissues other than the degradation of transport sugars.

The results presented by Thomas and Webb (60) show that  $\alpha$ -galactosidase activity is distributed throughout Cucurbita pepo. In all tissues examined, a similar set of three forms (LI, LII and LIII) of the enzyme are present in at least trace amounts. Each form shows distinct properties (59). LIII is more stable than LI or LII and is less susceptible to metal ion inhibition and the  $K_m$  values obtained for the hydrolysis of p-nitrophenyl- $\alpha$ -D-galactopyranoside and raffinose respectively were 0.37 and 11.1 mM for LI, 0.57 and 12.5 mM for LII and 0.042 and 5.6 mM for LIII. However, the most striking difference between the three forms was their response to changes in pH. The pH optimum for LI was found to be 5.7 with ca 50% reduction in activity at pH 4.2 and 6.8. For LII, the pH optimum was 4.6 with ca 50% reduction in activity at pH 3.5 and 5.8. Both forms showed little activity either below pH 3 or above pH 7.0. LIII showed optimum activity at 5.6, but it had appreciable activity over a wide range of pH with ca 50% activity still present at pH 2.5 and 7.0. A combination of the appropriate forms would thus enable the enzymes to operate in the range of compartments with 'local' pH values. In addition, the presence of the LIII form would enable the enzyme to function in regions where the pH is poorly controlled and hence variable.



Three forms of  $\alpha$ -galactosidase, LI, LII and LIII, similar to those identified in C. pepo leaves were found in the seeds of C. pepo (60). Analysis of the seed extracts revealed comparable changes in the enzymic forms during imbibition. In dry seeds, LIII was the predominant form while upon imbibition and germination the levels of LI and LII increased. The shift from LIII to LI and LII was accompanied by an increase in total activity with a rapid depletion of ajugose, verbascose, stachyose and raffinose.

Changes in enzymic forms have also been observed in Vicia faba (68). Green, immature seeds of V. faba contain a low specific activity, monomeric form of  $\alpha$ -galactosidase (form II which is probably a mixture of forms II<sup>1</sup> and II<sup>2</sup>) and as the seeds mature, the total activity rises and a tetrameric enzyme, I, is formed. In resting seeds, the high specific activity enzyme I, appears to reach a maximum level. This has been related to the need for the seed to effect a maximum rate of breakdown of reserves, in the early stages of germination (25). After germination, the enzyme pattern in V. faba seeds is reversed i.e. there is a fall in total and specific activity and enzyme I rapidly disappears leaving only form II (II<sup>1</sup> and II<sup>2</sup>?).

Generally speaking, the presence of  $\alpha$ -galactosidases and their substrates in dry seeds suggests that they are in some way spatially separated in maturing seeds.

A further physiological role for  $\alpha$ -galactosidase occurs in the case of Poterioochromonas malhamensis (143), a wall-less alga which grows in brackish waters where the salinity is constantly changing. In this case, osmotic regulation is mediated by the formation and degradation of isofloridoside ( $\alpha$ -galactosyl-(1 $\rightarrow$ 1)-glycerol).  $\alpha$ -Galactosidase activity has been demonstrated in cell homogenates of P. malhemensis. An increase in external osmotic pressure causes an increase in the activity of this enzyme: when the synthesis of  $\alpha$ -galactosidase is suppressed using actinomycin D, the in vivo degradation of isofloridoside is slowed down, indicating a function of the enzyme in osmotic regulation.

### 1.8.2 Lectins

The function of glycosidases, and in particular  $\alpha$ -galactosidases, in plant cells may be closely related to the lectin properties of these enzymes. The presence in plant tissues of proteins with the ability to cause agglutination of erythrocytes has been known since 1888 (144 see also ref. 145). Their early history has been reviewed (146, 147).

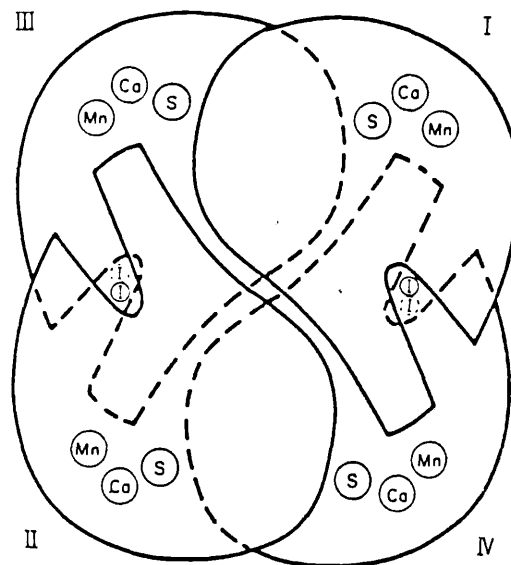
Plant agglutinins are commonly referred to as phytohaemagglutinins (or phytoagglutinins). However, as cell-agglutinating proteins also occur in organisms other than plants, the more general term 'lectin' was proposed by Boyd (147). In

the broadest sense, lectins may simply be defined as those proteins (or glycoproteins) of plant, animal or microbial origin which bind to cell surfaces through specific carbohydrate-containing receptor sites (145). Binding is reversible and all lectins have more than one specific carbohydrate-combining site (148). Kocourek and Horejsi (149) have recently suggested the following definition: lectins are sugar-binding proteins or glycoproteins of non-immune origin which 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. 150).

Lectins have, so far, been found predominantly in the seeds of plants, particularly those of legumes. In this case virtually all of the lectins are metal ion-requiring tetrameric glycoproteins, possessing identical or nearly identical subunits (151, 152). The best studied lectin structure is that of Concanavalin A which has been shown to be a tetramer of identical protomers, each of which has a MW 25,500 (148). The schematic representation of the Con A tetramer is shown in Fig. 8. In addition to seeds, lectins are widely distributed in other plant tissues, for example, roots, leaves and bark (146, 147, 153). Lists of lectins that have been purified from plant and animal sources along with their chemical and biological properties are given in reviews by Sharon and Lis (154) and Brown and Hunt (148).

Apart from agglutination of red blood cells, lectins exhibit

Fig. 8. Schematic representation of Concanavalin A tetramer.



The manganese and calcium sites are indicated by Mn and Ca respectively.

The saccharide-binding site is indicated by S and the major cleft by I.

Reproduced from Brown and Hunt (ref. 148).

a number of other properties including the ability to agglutinate plant cells and to precipitate polysaccharides and glycoproteins. They exhibit widely differing specificities regarding cell-agglutination and the susceptibility of these reactions to inhibition by specific carbohydrates also varies. Some of the lectins specifically bind to blood group glycoproteins and have, therefore, been used for blood typing and in investigations of the chemical basis of blood group specificity. Some lectins are 'mitogenic' and, for example, stimulate the transformation of lymphocytes from small 'resting' cells into large blast-like cells which may ultimately undergo mitotic division (154).

The biological effects of most lectins which are discussed above can be inhibited in a fairly specific manner by low MW carbohydrates. This has led to the conclusion that lectins act by binding to specific carbohydrate residues in polysaccharides or glycoproteins on the cell surface (145).

Although lectins are better known for their capacity to agglutinate erythrocytes, most can, in addition, 'clump' other animal cell types. Striking changes can be observed in lectin agglutinability accompanying neoplastic transformation, the infection of cells with some non-oncogenic viruses and in the case of some normal developmental processes. Transformed cells can usually be agglutinated at a much lower concentration of lectin than is required for the corresponding normal cell type (148) although this is not always the case (155, 156, see also

148). Such selective lectin agglutination of transformed cells was first observed by Aub et al (157). It has been suggested that the degree of agglutination by lectins depends on the relative contribution of carbohydrate receptors on the surface of the cell; in normal cells, the receptors are dispersed whereas after malignant transformation, a redistribution of sites occurs which results in 'clustering', an arrangement favourable to cell agglutination (154).

Whilst lectins have been widely used as reagents in studies on cell agglutination, mitosis etc. (148, 152, 158) surprisingly little attention has been given to the potential effects they may exert in vivo in animals which consume diets rich in lectins (151, 159 - 161). It is especially interesting to note that the foods in which lectins are found are becoming increasingly important in the diet of a health conscious society (151, 158, 160) and include such staples as potatoes, peas, beans, soybeans, nuts and wheat germ (162).

Some lectins are extremely toxic to animals. Castor beans, for example, contain the highly poisonous lectin known as ricin (148). The fact that lectins are found in those legumes which constitute an important source of human dietary protein raises the question as to their possible nutritional significance (151). The growth-inhibiting effect produced in animals by feeding them uncooked beans, particularly from P. vulgaris, would appear to be at least in part, caused by lectins (160). In this connection,

a comparative study on the growth of rats has been carried out using five varieties of kidney beans (163). Two varieties of seed containing haemagglutinin were found to be significantly more toxic than the three other varieties devoid of significant lectin activity. At least one outbreak of food poisoning has been traced to the lectin of the raw red kidney bean (164). Jaffe (165) proposed that the toxic effect of lectins when ingested orally may be due to their ability to bind specific receptor sites on the surface of the intestinal epithelial cells and hence interfere with intestinal absorption. Brady *et al.* (166) on the other hand, have shown that wheat germ agglutinin can traverse the wall of the human ileum without degradation. There is no conclusive evidence, however, that dietary wheat germ agglutinin is toxic, but *in vitro*, it can cause moderate agglutination of both foetal and adult illeal cells (167).

The isolation and characterization of a lectin from tomato fruit has been reported (162). The presence of a lectin in the tomato is dietetically significant as it is primarily found in the fluid and gel phase of the fruit. It is therefore, likely that mastication releases most of the lectin hence allowing it to interact with the cells, secreted glycoproteins and microflora of the mouth. It has been demonstrated that the lectin binds to the mucosal cells from all areas of the mouth (168). Tomato lectin is resistant to denaturation by acid and by proteolytic enzymes and is, therefore, likely to reach the intestinal tract intact. The tomato is one of the primary vegetable sources of

vitamins and minerals in the U.S. diet (169) and it is estimated that the average person ingests at least 100 - 200 mg of the lectin per year. The biological consequences to the host of the consumption of this common dietary lectin are currently being investigated (162).

The jack bean lectin, concanavalin A, produces hypersecretion of nasal mucus in humans and jejunal mucus in the rat. This effect has been termed 'mucotractive'. It has been suggested that the mucotractive effect of a suitable inhaled/ingested lectin might be useful for the treatment of patients with cystic fibrosis (161).

#### 1.8.2.i Lectins with $\alpha$ -galactosidase activity

The phytohaemagglutinin from mung bean (Vigna radiata) has been purified and found to possess a high  $\alpha$ -galactosidase activity. It is a tetrameric glycoprotein (MW 160,000) composed of identical subunits with MW 45,000 (170). Hankins and Shannon (170) have shown that both the lectin and enzymic activities reside on a single protein species. The most recent work (76) on this enzymic phytohaemagglutinin has shown that the tetramer can be reversibly dissociated into a low MW form, possibly a monomer, which is devoid of haemagglutinin activity although still enzymically active. Hypocotyls from V. radiata contain an  $\alpha$ -galactosidase-haemagglutinin (MW 170,000) which in the absence



of D-galactose and mercaptoethanol, dissociates into smaller units exhibiting preferentially either  $\alpha$ -galactosidase or lectin properties (171).

Hankins et al (172) have shown that four other legume species (Pueraria thunbergiana, Thermopsis caroliniana, Lupinus arboreus and Phaseolus limensis) possess  $\alpha$ -galactosidase-haemagglutinins which are similar immunologically as well as with respect to specificity and kinetic behaviour, both, to one another and to that isolated from Vigna radiata. In addition to the legumes discussed above, an  $\alpha$ -galactosidase with haemagglutinin properties has been found in soybean (Glycine max) (172, 173). This soybean  $\alpha$ -galactosidase-haemagglutinin (173) is comparable to that described for mung bean (170).

The  $\alpha$ -galactosidase-haemagglutinins from soya and lima beans can be readily separated from the N-acetyl-galactosamine specific lectin which is present in these plants (26, 174). The conclusion drawn from these observations is that at least two distinct classes of proteins (non-homologous, but perhaps evolutionarily related) with lectin activity exist in legume seeds (172).

Many legume seeds contain  $\alpha$ -galactosidases which are apparently devoid of lectin activity (57) and which are probably tetrameric with MWs ranging from 150,000 to 190,000. The  $\alpha$ -galactosidase-haemagglutinins and the enzymes without lectin activity appear to be structurally similar, as shown by immunological techniques,

but there is no information available to account for the different carbohydrate binding properties.

#### 1.8.2.ii Proposed role of lectins

Although lectins have been successfully employed to probe a wide variety of cell surfaces and activities, not much is known about their normal function in vivo (148). Most of the theories advanced to explain the role of lectins in plants are based on their characteristic ability to bind carbohydrates. This specific interaction with carbohydrates or carbohydrate-containing molecules is unlikely to be fortuitous, and it seems reasonable to suppose that this property has some relevance in vivo. It has been proposed that lectins may have the following functions in plants (145, 148, 152) :

(i) they serve as plant antibodies (175). Lectins have a superficial similarity to antibodies, however their comparatively narrow range of specificities makes a precise correlation unlikely. Nevertheless, it is possible to see how the presence of a toxin or a precipitating agent could protect a plant against attack from a variety of organisms. A protective function of phytohaemagglutinin against beetle infestation of Phaseolus vulgaris has been demonstrated. Bruchid beetles are killed by a diet of black beans, but not by cowpeas which are agglutinin free (176). Some lectins may protect plants against the spread of

viral infections. It has been observed that certain plant viruses contain surface glycoproteins and that their presence correlates with the manner in which the virus is transmitted from plant to plant. For example, barley stripe mosaic virus (BSMV) and cowpea mosaic virus are both transmitted via the seeds of the host plant, and both have surface-associated glycoproteins (177). Thus lectins (usually concentrated in the seed) may confer on the plant a selective advantage by protecting it against seed-transmitted viruses. Barley lectin binds to and precipitates BSMV in vitro and renders the virus non-infectious (see ref. 148).

ii) they might be involved in carbohydrate transport (178, see also ref 145). No experiments appear to have been designed to test this hypothesis, but the fact that the developing seed, a major nutrient 'sink' in the plant, is the main source of lectins might provide circumstantial evidence in favour of this proposal. Intracellular transport of sugars or polysaccharides from the cytoplasm to the cell wall, for example, could be facilitated by lectins but again, this has not been tested.

iii) cell division in embryogenesis may be controlled by lectins (179). It has been suggested that lectins might be involved in differentiation and developmental processes in the seed embryo by controlling the rates of cell division. There is however no evidence to indicate that lectins which are mitogenic against animal cells also exhibit this activity against plant cells.

iv) they are involved in 'recognition' interactions. Recently, the existence of specific and complementary molecular mechanisms located at the cell surface has been demonstrated for plant and fungal cells in relation to mating behaviour (147, 180) and morphogenesis (181). Specific complementary molecules appear to play an important role in the recognition between pollen grains and stigma surfaces in higher plants. The lectin, Concanavalin A, binds to certain wall-bound carbohydrate-containing materials on the pollen grain surface, in a species-specific manner, and it has therefore been suggested that the protein pellicle on the stigma surface may contain lectin-like receptors responsible for initiating acceptance or rejection of the pollen grain (182). It would appear then, that surface-localised, complementary macromolecular mechanisms may be widely involved in recognition processes. In several instances, at least one of the complementary pairs of molecules possesses lectin-like activity. Recent evidence also suggests that similar mechanisms may play an important part in determining host-parasite and host-symbiont specificity. For instance, lectins have been directly implicated in the legume-Rhizobium interaction (183 - 185). The major sites of Rhizobium entry into the legume root, the root hairs, have been shown to bind erythrocytes heavily to their surfaces (183). Legume lectins from kidney bean (183) and soybean (184) will also bind to Rhizobium cells and it has been suggested, therefore, that surface-localised legume lectins provide receptor sites for the specific binding of Rhizobium, prior to infection.

The identification of  $\alpha$ -galactosidase-haemagglutinins may indicate that these enzymes are bound to cell components such as membranes and cell walls, by lectin-glycan bonds (Dey, P.M. and Pridham, J.B., unpublished).

### 1.8.3 Glycoprotein nature of hydrolytic enzymes

Glycoproteins are usually defined (186, 187) as macromolecules that are composed of a polypeptide backbone to which are attached one or more carbohydrate moieties. The latter usually consist of monosaccharide residues that are composed of two or more of the sugars: D-mannose, D-galactose, D-glucose, L-fucose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine and sialic acid (188).

Early studies on glycoproteins were limited to those of animal origin. The first proof of the presence of glycoproteins in higher plants was obtained about twenty years ago with the isolation of an asparaginyoligosaccharide from soybean agglutinin (189) and of the glycopeptide from bromelain, the proteolytic enzyme from pineapple (190).

Plant glycoproteins include enzymes, lectins and structural proteins (188). There is experimental evidence to suggest that some plant glycosidases (and perhaps all) are glycoproteins (191). Thus, there is the interesting possibility that proteins exist with catalytic and lectin sites and glycosyl residues.

Evidence of the glycoprotein nature of glycosidases has been obtained by gel-staining techniques, carbohydrate analysis and Concanavalin A Sepharose binding studies. Thus in the case of almond emulsion  $\beta$ -glucosidase, the periodate-metabisulphite procedure has been used to locate the enzyme on polyacrylamide gels (21). Two forms, I and II, of sugar beet  $\alpha$ -glucosidase have been purified and shown to contain 2.7 and 8.8% neutral carbohydrate respectively (192). The existence of glycan chains in  $\alpha$ -rhamnosidase from saracen corn seeds is indicated by the fact that the enzyme binds to Con A columns (193). Partially purified Vicia faba  $\alpha$ -galactosidases, the subject of this thesis, were also reported to contain bound carbohydrate; 4% in the case of forms II<sup>1</sup> and II<sup>2</sup> and 8% in enzyme I (68).

## MATERIALS AND METHODS

General laboratory chemicals were obtained from BDH Ltd., Poole, Dorset and were of 'Analar' grade where possible. *p*-Nitrophenyl- $\alpha$ -D-galactopyranoside (PNPG), 4-methylumbelliferyl  $\alpha$ -D-galactopyranoside and Triton X-100 were purchased from Koch-Light, Colnbrook, Bucks. 6-Bromo-2-naphthyl- $\alpha$ -D-galactoside, dimethylformamide, fast blue RR salt, methyl  $\alpha$ -D-mannoside, agarose type II, bovine serum albumin and standard proteins for molecular weight estimations by gel filtration were obtained from Sigma (London) Chemical Co., Kingston-Upon-Thames, Surrey. Molecular weight markers for SDS-PAGE were bought from Bio-Rad Laboratories, Watford. Sephadex G-100 and Concanavalin A-Sepharose were purchased from Pharmacia (G.B.) Ltd., London. CM-cellulose (CM-52) was obtained from Whatman Biochemicals, Maidstone, Kent. Vicia faba seeds (Bunyard Exhibition, long pod variety) were acquired from Suttons Seeds, Reading. All the buffers used were prepared by the procedures described in Methods in Enzymology, Vol.1 (194). All operations were carried out at 4°C, unless otherwise stated.



## 2.1 Enzyme assays

### 2.1.1 Quantitative assay

$\alpha$ -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 (195), pH 5.5 and 10 mM PNPG (0.1 ml) preheated to 30°C; the final assay 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 sec.

### 2.1.2 Detection of $\alpha$ -galactosidase after analytical electrophoresis

Following polyacrylamide gel electrophoresis (PAGE), immunoelectrophoresis and immunodiffusion enzyme activity was detected using the following methods.

i) By the use of fluorescent substrate, 4-methyl umbelliferryl- $\alpha$ -D-galactoside. Polyacrylamide gels or agarose coated slides were covered with strips of Whatman (no.2) paper previously soaked in a solution of substrate (5 mg/10 ml McIlvaine buffer, pH 5.5) and incubated for 5-10 min at 37°C. The

paper was then removed gently and the enzyme activity located using a uv light.

ii) By the use of the histochemical substrate, 6-bromo-2-naphthyl-  $\alpha$ -D-galactoside (BNG). The stock substrate solution contained 0.3% (w/v) BNG in McIlvaine buffer, pH 5.5 (20 ml). The following solutions were prepared using the stock substrate solution.

Soln. A. McIlvaine buffer, pH 5.5, (20 ml) containing substrate solution (1 ml) and diazonium salt (20 mg).

Soln. B. McIlvaine buffer, pH 5.5 (20 ml) containing substrate solution (1 ml).

Soln. C. Diazonium salt (20 mg) in McIlvaine buffer (20 ml).

All the solutions were freshly prepared. After electrophoresis, the gels were immersed in Soln A for 4 h; then transferred to Soln. B for 3 h after which they were put into Soln. C for 3 h. The whole process was carried out at room temperature. Following detection of enzyme activity (reddish-brown bands), the gels were stored in 7% acetic acid.

## 2.2 Protein assays

### 2.2.1 Measurement of Protein

Protein was determined by the method of Lowry et al (196), using crystalline bovine 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 (R 250) in 50% (w/v) TCA for 15-30 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 the gels could be decreased by placing the gels at 30°C).

## 2.3 Column Chromatography

### 2.3.1 Sephadex-gel filtration

Sephadex G-100 columns (2.5 cm x 90 cm and 5.0 cm x 90 cm) were packed by the method described by Andrews (197). The columns (2.5 cm x 90 cm) were eluted with the 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) collected. The columns were water jacketed and therefore run at room temperature, with tap water flowing through the jackets.

### **2.3.2 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 0.5 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 a flow rate of 60 ml/h. The column was washed with the buffer and fractions (2 ml) were collected. The eluent was monitored for protein at 280 nm using an LKB uvicord. When the  $E_{280}$  was nil, the sample bound to the column was eluted with 0.5 M methyl- $\alpha$ -D mannoside in the equilibrating buffer and fractions (2 ml) collected until the  $E_{280}$  was nil again.

### **2.3.3 Ion-exchange chromatography (cation)**

Whatman ion-exchange cellulose, CM-52, was prepared as described in the manufacturer's instruction sheet and equilibrated with McIlvaine buffer, pH 3.5 and a column (1 cm x 14 cm) packed at

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 of the eluate 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 5.5. Fractions (2.0 ml) were collected at a flow rate of 15 ml/h.

## 2.4 Electrophoresis

### 2.4.1 Polyacrylamide gel electrophoresis (PAGE)

PAGE was carried out under cationic conditions according to the method of Reisfeld et al. (198) with a slight modification. Electrophoresis was performed in gel tubes (0.5 cm x 7.5 cm); the separating gel (6.5 cm) consisted of 7.5%(w/v) N, N, N', N'-tetramethylethylene diamine (Temed), 0.14%(w/v) ammonium persulphate, potassium acetate buffer, pH 3.5. The stacking gel (0.5 cm) was made with 2.5% (w/v) acrylamide, 0.6% (w/v) N', N'-methylene bisacrylamide, 0.06% (v/v) Temed, riboflavin (5 $\mu$ g/ml), 0.1 M potassium acetate buffer, pH 6.8. The stacking gel was polymerized (15 - 30 min) by placing a fluorescent lamp behind the gel tubes about 3" away. Prepared gel tubes were inserted in a Shandon PAGE apparatus and the electrode compartments (anode

uppermost) filled with  $\beta$ -alanine acetate buffer, pH 3.5 diluted 1:1 with water. The samples (up to 70  $\mu$ l) containing 10  $\mu$ l of 10% (v/v) glycerol and 5  $\mu$ l of 1%(w/v) methyl green were applied to the gels. Electrophoresis was carried out at 4 mA/gel for 2.5 h at room temperature, with tap water flowing through the water jacketed apparatus. After electrophoresis, the gels were immediately removed from the glass tubes by gently rimming them with a 22-gauge syringe needle through which a thin stream of water was passed. The gels were stained for enzyme activity and protein as described in sections 2.1.2 and 2.2.2.

#### **2.4.2 Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE)**

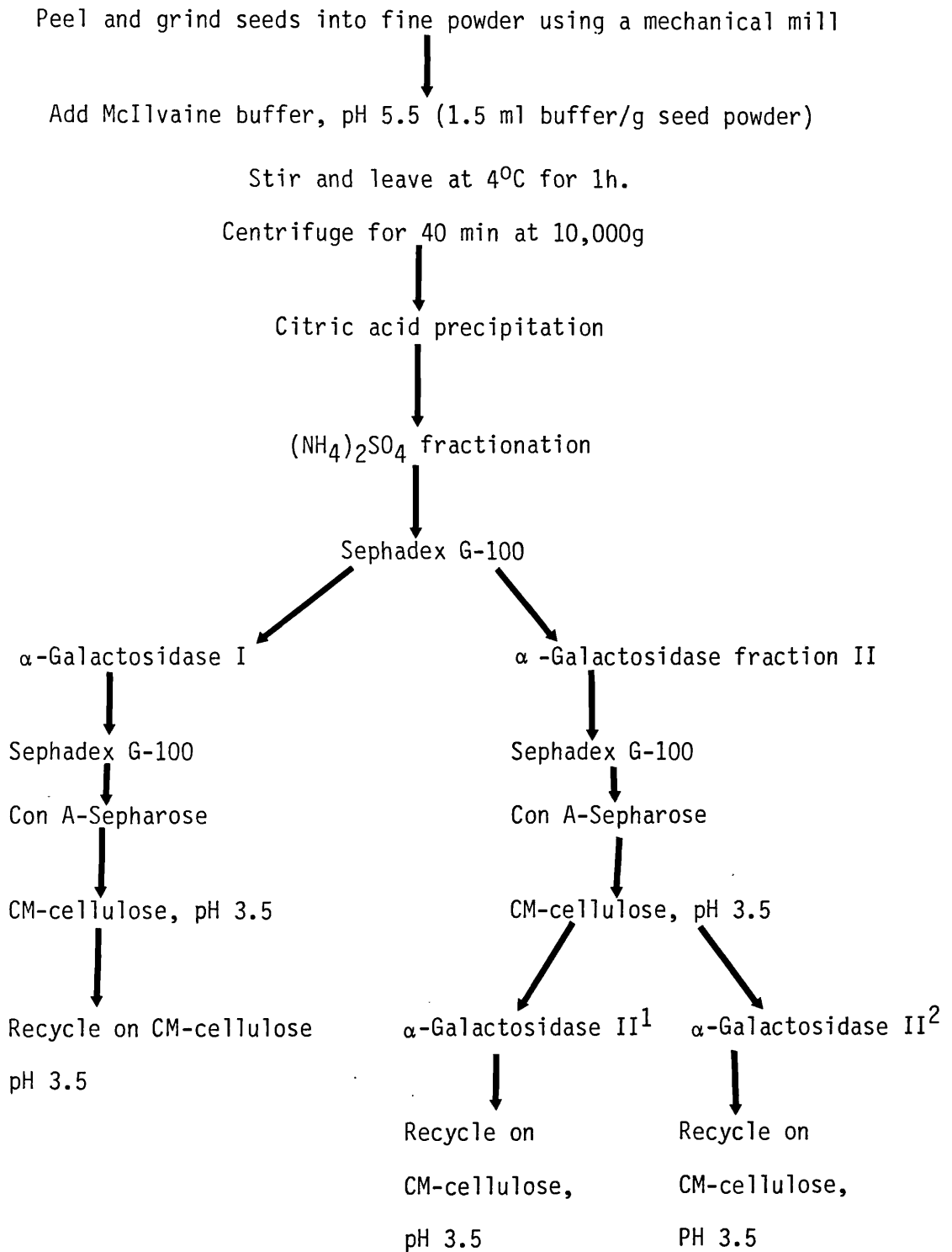
SDS-PAGE was performed as described by Laemmli (199) with a slight modification. Slab gel electrophoresis was carried out in a Bio Rad apparatus while the Shandon system was used for cylindrical gels; both apparatus were water jacketed. Stacking and separating gels containing 5% and 8% acrylamide respectively 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 separating gel (1.5 mm x 120 mm) were as follows : 8% acrylamide, 0.1% (w/v) sodium dodecyl sulphate (SDS), 2 mM ethylenediaminetetra-acetic acid (EDTA), 0.03% (v/v) Temed and 0.03% (w/v) ammonium per sulphate, 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, 2 mM EDTA, 0.05% (v/v) Temed, 0.1% (w/v) ammonium persulphate and 0.13 M Tris-HCl buffer, pH 6.8. 10 ml stacking gel and 30 ml separating gel solutions were adequate for one slab gel. The electrode buffer, pH 8.3 consisted of 0.38 M glycine, 2 mM EDTA, 0.1% (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 M Tris-HCl buffer, pH 6.8, 10% (v/v) glycerol, 1.25% SDS, 2 mM EDTA and 0.015% bromophenol blue. The samples were heated for 3 min at 100°C and after cooling, they were applied to the gel; maximum volume of sample not exceeding 60 µl/track. Two slab gels could be run at the same time. 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 2 h and 45 min. 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).

## 2.5 Purification of α-galactosidases I, II<sup>1</sup> and II<sup>2</sup>

Purification of the enzymes was carried out according to Fig. 9. 1,000 g of seed powder from dormant broad beans was used for each

Fig. 9. Purification of  $\alpha$ -galactosidases I, II<sup>1</sup> and II<sup>2</sup> from Vicia faba seeds





batch of purification. The testas were removed and 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 10,000g for 40 min using a 6 x 250 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 10,000g 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 (105 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  $\alpha$ -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 PM 10 membrane. This enzyme was re-applied to a 2.5 cm x 90 cm Sephadex G-100 column as described above with the exception that 3 ml fractions were collected. The fractions containing  $\alpha$ -galactosidase were pooled, concentrated and dialysed against 0.1 M potassium phosphate buffer, pH 7.0 (5 lit). The dialysed sample (8 ml) was applied in 2 ml 'batches' to a Con A-Sepharose column (2 cm x 20 cm) equilibrated with 0.1 M potassium phosphate buffer, pH 7.0 containing 0.5 M sodium chloride. The unbound proteins were washed with the same buffer and when the  $E_{280}$  was negligible, the bound enzyme was eluted with 0.5 M methyl  $\alpha$ -D-mannoside in the eluting buffer; fractions (2 ml) were collected. The bound and unbound fractions were assayed for enzyme activity and the active fractions were pooled and concentrated separately. The concentrated bound enzyme fraction was dialysed against McIlvaine buffer, pH 3.5 and the sample (6 ml) applied to a CM-cellulose column equilibrated with the same buffer. The column was washed with this buffer (40ml) after which the enzyme was eluted with a linear sodium chloride gradient from 0.05 M sodium chloride in McIlvaine buffer, pH 5.5 (100 ml) to 0.5 M sodium chloride in McIlvaine buffer (100 ml). The active fractions were pooled, concentrated and finally recycled through the CM-cellulose column. The unbound  $\alpha$ -galactosidase I fraction from the Con A-Sepharose stage was treated similarly.

The same purification procedure was followed for enzyme II fraction obtained from the Sephadex G-100 stage except that a 0.05 M sodium chloride - 0.4 M sodium chloride gradient was used to elute the enzyme from the CM-cellulose column. Two peaks of activity II<sup>1</sup> and II<sup>2</sup> were obtained when fraction II was applied on the ion-exchange column. These two enzymes were pooled and concentrated separately and recycled through the CM-cellulose column. The purified enzymes were stored at 4°C with thymol added as a preservative.

## 2.6 Immunological methods

### 2.6.1 Production of anti-( $\alpha$ -galactosidase) antisera

Rabbits of the lop variety were used to raise the antisera. The following procedure was used for each antigen I, II<sup>1</sup> and II<sup>2</sup>. 125  $\mu$ g of purified antigen in McIlvaine buffer (0.5 ml) was mixed with Freund's complete adjuvant (0.5 ml) and emulsified using a 23 gauge, 4 cm long metal tube with leur fittings at each end to which 1 ml syringes were attached. On day 1, the emulsion was injected subcutaneously into the rabbit at two sites on the shoulder (each side of the vertebral column). The rabbits were given successive injections on days 110, 125 and 162, each injection consisting of 125  $\mu$ g of emulsified antigen (on day 125, Freund's incomplete adjuvant was used). Blood was collected, on day 192, from the marginal ear vein, allowed to stand for 1 h at

room temperature and overnight at 4°C. It was then centrifuged at 3,000g for 30 min at 4°C. The supernatant was removed and tested for the presence of  $\alpha$ -galactosidase antibodies by immunoprecipitation, immunodiffusion and immunoelectrophoresis (sections 2.6.2, 2.6.3 ad 2.6.4). 1 ml aliquots of antisera were stored at -20°C in 1.2 ml capacity plastic microcentrifuge tubes without any preservative.

### 2.6.2 Quantitative Immunoprecipitation

Immunoprecipitation was carried out in 0.4 ml plastic microcentrifuge tubes by adding variable amounts of antiserum (0 - 50  $\mu$ l for anti I and anti II<sup>2</sup>, 0 - 100  $\mu$ l for anti II<sup>1</sup>) to constant amounts of enzyme. The total amount of antiserum was maintained at 50  $\mu$ l (100  $\mu$ l in the case of II<sup>1</sup>) by addition of control antiserum. Where dilutions of antiserum had to be made, control antiserum was used as the diluting medium. After adding the components and mixing, the tubes were incubated for 15 min at room temperature. Finally, the tubes were centrifuged for 6 min in a microcentrifuge (Quickfit Instruments). The supernatants were assayed for  $\alpha$ -galactosidase activity as described in section 2.1.1.

### 2.6.3 Immunodiffusion : The Ouchtelony method (200)

Glass plates (8.3 x 9.2 cm) were overlaid with 11.5 ml of molten 1% (w/v) agarose in 25 mM sodium citrate buffer, pH 6.0. Using a template, a hexagonal arrangement of wells (4 mm) was punched out with the holes spaced 5 mm apart. Four identical patterns of wells were cut, one at each corner of the plate. The appropriate antiserum (12  $\mu$ l) was placed in the central well of each pattern and the corresponding antigen added in increasing amounts (2-12  $\mu$ l) to the surrounding wells. On a second plate the appropriate antiserum was placed in the central well as before, except that I, II<sup>1</sup> and II<sup>2</sup> antigens were added to the surrounding wells. After the addition of antiserum and antigens, the plates were stored for 24 h in plastic containers lined with moist tissue paper to allow diffusion to take place. The plates were examined occasionally for the appearance of white precipitin lines. After the diffusion was complete, the plates were pressed and washed in 0.15 M sodium chloride for 4 h at room temperature and 20 h at 4°C with one change of the sodium chloride solution. The plates were then washed with water for 2 h. Following this, the plates were pressed and stained for enzyme activity by the fluorescent method (section 2.1.2.). After enzyme staining, the plates were washed, pressed, dried under a stream of hot air and stained for protein as described in section 2.2.2.

#### 2.6.4 Immuno-electrophoresis

The agarose plates were prepared as for immunodiffusion. Three antigen wells were cut in the agarose layer using a 4 mm well cutter. Antiserum troughs (0.2 cm x 6 cm) were cut with a scalpel but not removed. The plates were then placed on the cooling plate of the electrophoresis apparatus (Shandon) and the enzyme solution (12  $\mu$ l) added to each antigen well. Three plates were run each time. The electrode compartments were filled with 25 mM sodium citrate buffer, pH 6.0. Electrophoresis was performed at 10V/cm for 2.5 h at room temperature. The actual voltage across the wicks was measured using a voltmeter. At the end of electrophoresis, the plates were taken out from the tank and the pre-cut agarose strip removed. The appropriate antiserum (100  $\mu$ l) was added to each trough and the plate incubated for 24 h at room temperature in a humid atmosphere. After this stage the procedure was the same as for immunodiffusion described in section 2.6.3.

#### 2.7 Haemagglutinin assay

The method used is described in ref. (201). 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 the 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  $\mu$ l) and 200  $\mu$ l of the 1.5% suspension of the 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  $\pm$  20% (cf. ref. 202).

## 2.8 Amino acid and amino sugar analyses

The methods of Allen et. al. (202) and Allen and Neuberger (203) were used. Purified  $\alpha$ -galactosidases I, II<sup>1</sup> and II<sup>2</sup> (200  $\mu$ g each) were dried in vacuo in hydrolysis tubes with p-fluorophenyl-alanine (100 nmol) as an internal standard. The hydrolysing acid, 3 M p-toluenesulphonic acid (0.5 ml) was added, and the solution was thoroughly de-aerated by repeated freezing and thawing under vacuum. The tubes were then sealed and heated in an oven at 110°C for 24 h. After cooling the tubes, p-toluenesulphonic acid was partially neutralised by the addition of water (0.5 ml) and 1M sodium hydroxide (1 ml) as recommended by Liu (204, 205) for direct application to the analyser. Cysteine values were obtained from the analysis of cysteic acid residues resulting from performic acid oxidation (206) and subsequent hydrolysis of the protein in constant-boiling 6 M HCl at 110°C for 24 h. For the determination of glucosamine and

galactosamine, protein samples were hydrolysed in 3 M *p*-toluene sulphonic acid at 100°C for 24 h.

Analyses were done on a Locarte Mini analyser fitted with an automatic-loading attachment. The standard system for analysis was that of Mayes *et al*, (207) which involved successive elution of the column (0.9 cm x 25 cm) with 0.2 M sodium citrate buffers, pH 3.2 for 33 min, pH 4.35 for 110 min and 1.0 M sodium citrate buffer, pH 6.65 for 160 min, at a flow rate of 30 ml/h. The temperature was set at 50°C for the first buffer, and at 60°C thereafter. The amino sugars were separated by elution on the column with 0.2 M sodium citrate buffer, pH 4.25 for 200 min at 50°C(203). The absence of hydroxyproline from the hydrolysates was demonstrated by elution with 0.2 M sodium citrate buffer, pH 2.8 as described by Allen and Neuberger (208).

## 2.9 Neutral-sugar analysis

The method of Chambers and Clamp (209) was used. This involved methanolysis of dry samples (100 µg) of the purified  $\alpha$ -galactosidases I, II<sup>1</sup> and II<sup>2</sup> with mannitol (25 nmol) as an internal standard in 1 M methanolic-HCl at 90°C for 4 h followed by re-acetylation and trimethylsilylation. The sugars were measured by gas-liquid chromatography (g.l.c.) using a Perkin-Elmer F.11 gas chromatograph fitted with dual columns and a flame ionisation detector.



## RESULTS AND DISCUSSION

### PART 1 Factors Affecting the Extraction and the Relative Proportions of V. faba $\alpha$ -Galactosidases

Although multiple forms of  $\alpha$ -galactosidases occur in extracts of Vicia faba seeds in characteristic proportions depending on the physiological state of the seed, it is not known whether these proportions reflect the state of affairs in vivo. Hence the effect of different extraction and purification procedures on the enzyme patterns was investigated.

Obviously the enzyme forms present in a tissue extract may not represent the in vivo situation as all forms may not be quantitatively extracted by a particular procedure. Furthermore, after enzyme extraction the levels of the various forms may change through secondary modifications (210) such as association of subunits. The latter has been observed in the case of hepatic  $\beta$ -galactosidases which interconvert in vitro in response to changes in the ionic strength of the medium (211, 212).

In the case of  $\alpha$ -galactosidases from V. faba seeds, conversion of form II to form I (see introduction p. 11) in vitro has been reported (68, 69). This conversion was first observed when an ammonium sulphate fraction of a preparation of enzyme II was stored at 4°C over a period of several days. When this was examined by Sephadex G-100 gel filtration the higher molecular weight form I was also found to be present. Furthermore, conversion in vitro has been reported by Hustler (77) who has shown that some processes in the purification procedure facilitate the conversion of  $\alpha$ -galactosidase II to  $\alpha$ -galactosidase I. Hustler (77) observed that in crude V. faba

extracts, enzyme II was present at a higher level than I but partially purified extracts (eg. after ammonium sulphate fractionation) showed the reverse pattern (Fig. 10).

Hence, enzyme extraction and subsequent treatments are likely to seriously interfere with any attempt to describe the pattern of multiple forms of  $\alpha$ -galactosidases in vivo. It was therefore decided to study the extractability of the different forms of V. faba  $\alpha$ -galactosidases and to see what factors might affect changes in their relative levels.

### 3.1 Preparation of $\alpha$ -galactosidase extracts

Resting, testa-free Vicia faba seeds (20 g) were finely powdered in a mechanical mill and extracted at 4°C for 1 h in the appropriate medium (30 ml). The extract was then centrifuged at 10,000g for 40 min and the supernatant used as the source of  $\alpha$ -galactosidase.

### 3.2. Variation of extraction procedures

Cotyledon powder obtained from resting V. faba seeds was extracted separately with water and a series of salt, buffer and detergent solutions and after centrifugation, the  $\alpha$ -galactosidase activities and protein concentrations of the extracts were

Fig. 10. Sephadex G-100 Eluent patterns of  $\alpha$ -galactosidases from dormant *V. faba* seeds.

Fig. 10.1.  $\alpha$ -galactosidase applied after multi-stage purification<sup>(39)</sup>.

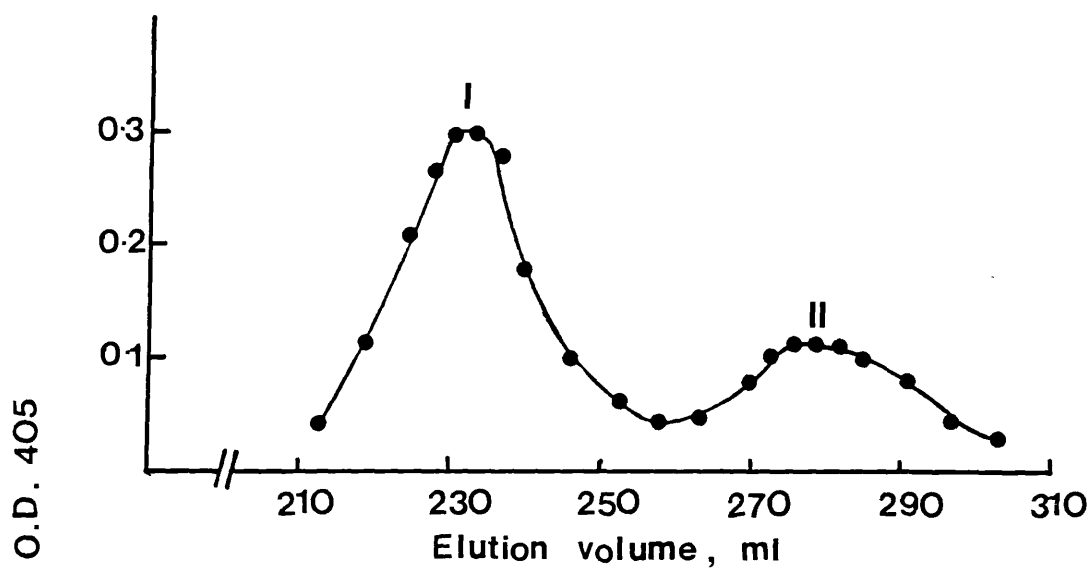
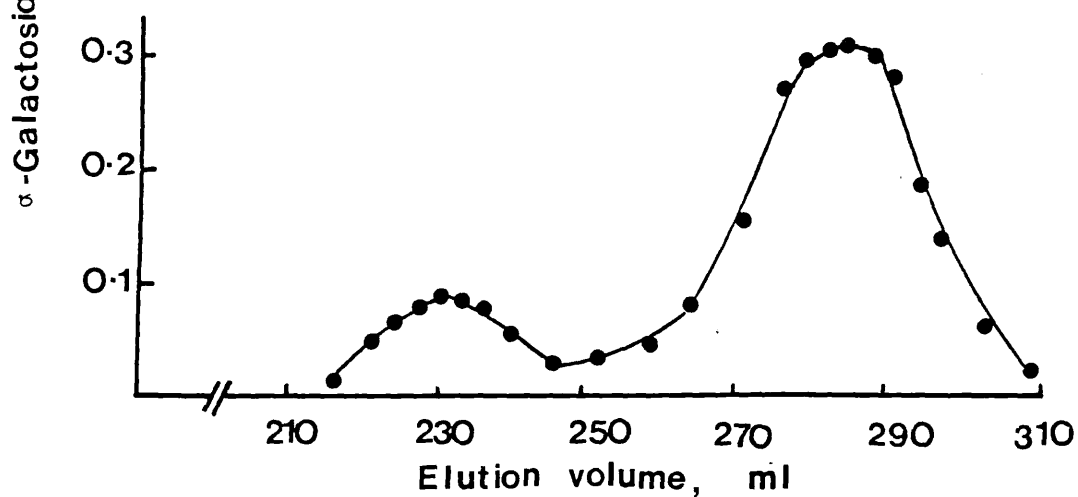


Fig. 10.2. Crude extract of  $\alpha$ -galactosidase applied direct to the column (77).



measured. Table 3 shows the effect of increasing the ionic strengths of the media on the  $\alpha$ -galactosidase activity extracted. The lowest total activity was obtained with the water extraction and the influence of salt concentration was marked. In the case of sodium chloride solution and sodium acetate buffer, maximum release of activity required a minimum salt concentration of 0.5 M. Similarly, with McIlvaine buffer, concentrations between 5 and 10 times that normally used were needed for maximum extraction of enzyme activity. The specific activities were significantly greater with the higher concentrations of sodium chloride and McIlvaine buffer, although this was not apparent with acetate buffer. For comparative purposes, the extraction of the enzyme with increasing concentrations of Triton X-100 was also examined. High total activities comparable to those found with the more concentrated salt solutions were observed with 0.1% detergent but activity was lost as the Triton concentration was increased. It is therefore, clear, as expected, that the composition of the extraction medium is important in terms of total activity extracted and the specific activity (cf. 213). In these studies it would appear that  $\alpha$ -galactosidase activity is at least in part bound to cellular components. However, a further possibility is that different salt concentrations initially extract similar levels of activity and subsequently there is enzyme modification in vitro which results, in the case of high salt concentration, in greater activity. In this connection, it should be noted that 0.12 M potassium chloride has been reported

**Table 3. Extraction of  $\alpha$ -galactosidase from mature Vicia faba seeds using various conditions**

Extraction medium	Volume of extract (ml)	Total enzyme activity (nkat)	Total protein (mg)	Sp. act. (nkat/mg protein)
Water	12.0	156.1	1356	0.11
Sodium Chloride				
0.05M	13.0	213.2	2574	0.08
0.1 M	13.0	199.0	2555	0.08
0.5 M	13.0	270.4	2145	0.13
1.0 M	13.0	270.4	1417	0.19
Triton (w/v)				
0.1%	13.0	251.0	1742	0.14
0.5%	12.5	211.2	1513	0.14
1.0%	12.0	198.0	1200	0.16
5.0%	12.5	111.3	1950	0.06
Acetate buffer (pH 5.5)				
0.05M	12.5	191.3	1162	0.16
0.1 M	12.5	218.8	1250	0.17
0.5 M	13.0	245.7	1586	0.15
1.0 M	12.0	244.8	1440	0.17
McIlvaine buffer (pH 5.5)				
x0.5 concn	13.0	210.6	1547	0.14
x1 concn	13.0	227.5	1560	0.15
x5 concn	12.0	298.8	1440	0.21
x10 concn	12.0	314.4	1500	0.21

20 g seed powder was extracted with the appropriate extracting medium and left to stand at 4°C for 1 h. It was then centrifuged at 10,000 g for 40 min and the supernatant assayed for enzyme activity and protein as described in sections 2.1.1. and 2.2.1.

to activate V. faba  $\alpha$ -galactosidases (69). Table 4 shows the effect of adding various levels of salts and Triton X-100 to an  $\alpha$ -galactosidase preparation obtained by extracting seed powder with deionized water. Over the range of concentrations examined, none of the salt or buffer solutions had a marked effect on the enzyme activity over a 2 h period, suggesting that in vitro modification had not occurred. Triton was observed to inhibit the enzyme activity at the higher concentrations.

### 3.3 Sephadex-gel filtration of V. faba $\alpha$ -galactosidase preparations obtained by extraction of seed powder with different media

Having completed the preliminary work on different extraction procedures, it was decided to examine the levels of the multiple molecular forms of  $\alpha$ -galactosidase in these extracts. In all cases examined, the extracts were applied to Sephadex G-100 columns which were subsequently eluted with the corresponding salt or buffer solutions. Fractions were collected and  $\alpha$ -galactosidase activity assayed. The extraction time and the time between applying the extract to the column and assaying the activity was kept constant in each case.

The pattern obtained with 0.1 M sodium chloride extract is shown in Fig. 11.1. Only one peak of enzyme activity was detected corresponding to the lower MW (40,000) fraction, II. The enzyme preparation obtained with 0.25 M sodium chloride showed three

**Table 4. The effect of increasing ionic strength and detergent concentration on  $\alpha$ -galactosidase activity extracted with water**

Final concentration in assay mixture	Enzyme activity (nkat)
Water	0.13
Sodium Chloride	
0.05M	0.14
0.1 M	0.14
0.5 M	0.14
1.0 M	0.14
Triton (w/v)	
0.1%	0.14
0.5%	0.11
1.0%	0.11
5.0%	0.10
Acetate buffer (pH 5.5)	
0.05M	0.13
0.1 M	0.13
0.5 M	0.14
1.0 M	0.15
McIlvaine buffer (pH 5.5)	
x 0.5 concn	0.14
x 1 concn	0.14
x 5 concn	0.13
x10 concn	0.13

20 g bean powder was mixed and stirred with water (30 ml) and left to stand at 4°C for 1 h. The mixture was then centrifuged at 10,000g for 40 min and the supernatant was assayed for  $\alpha$ -galactosidase activity. This crude aqueous extract (0.5 ml) was mixed with the appropriate medium (0.5 ml) and allowed to stand at 4°C for 2 h before assaying for  $\alpha$ -galactosidase activity; the activity of the aqueous extract was re-measured at this stage.



Fig. 11. Examination of NaCl extracts of *V. faba* seed tissue by Sephadex G-100 chromatography.

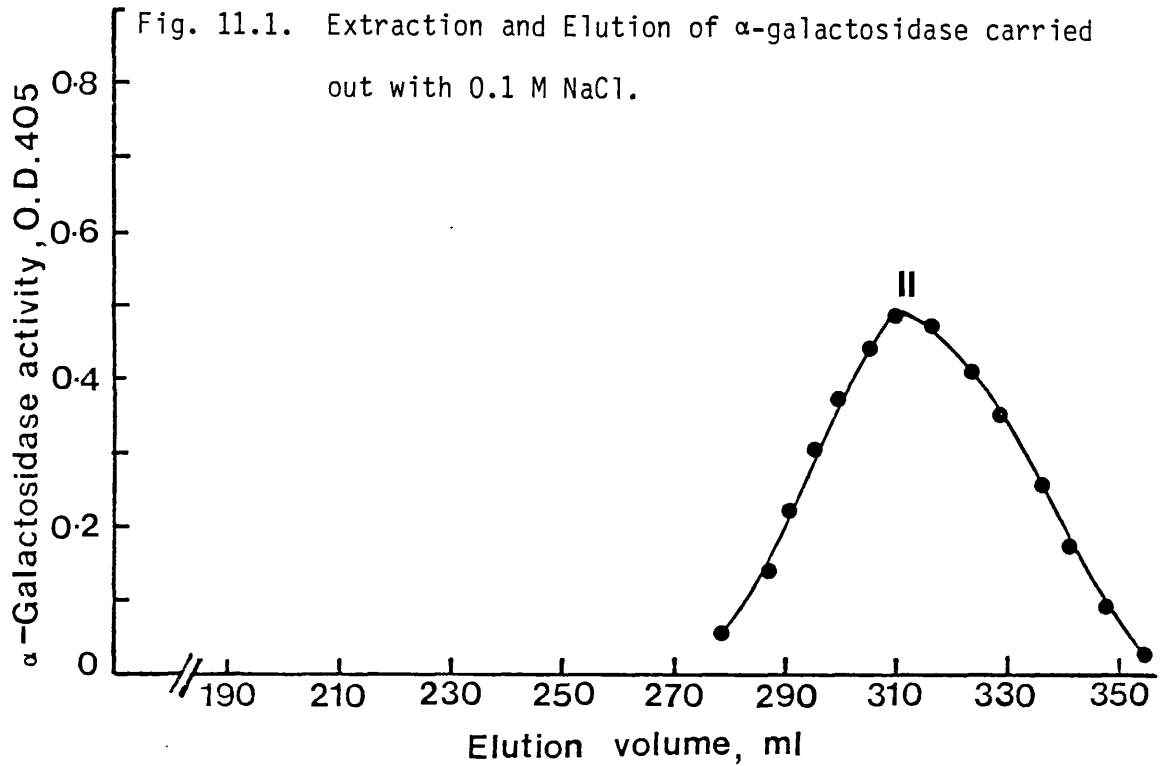
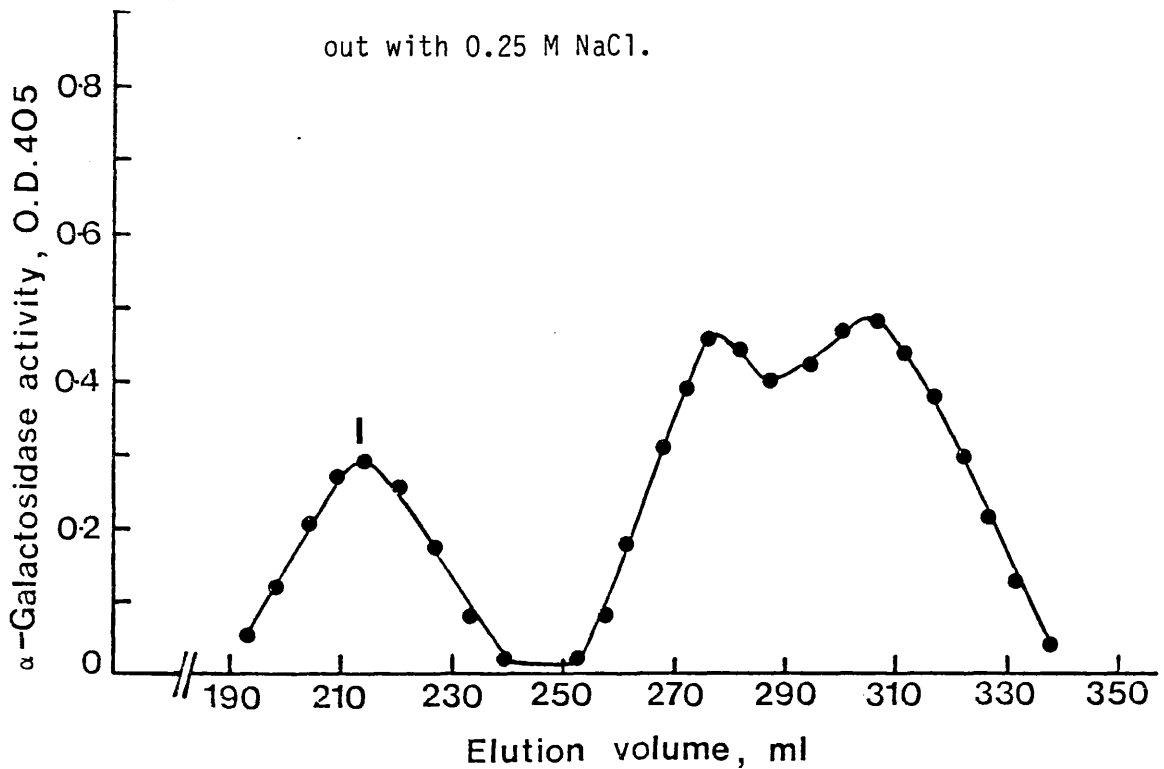


Fig. 11.2. Extraction and Elution of  $\alpha$ -galactosidase carried out with 0.25 M NaCl.



peaks of activity (Fig. 11.2), corresponding to I (MW 209,000) and II, together with a third peak positioned between I and II with an apparent molecular weight of 70,000 (a dimer?). This is the first report of the presence of an intermediate form of  $\alpha$ -galactosidase. Figures 11.3 and 11.4, show the elution profiles of enzyme preparations obtained by extraction with 0.5 M and 1.0 M sodium chloride, respectively. Here again, as with 0.25 M sodium chloride, three peaks of  $\alpha$ -galactosidase activity are seen, although the proportions of the enzyme forms vary at each concentration.

It is clear, therefore, that the highest levels of  $\alpha$ -galactosidase I are found in the extracts obtained with high salt concentrations. This could result because high ionic strengths are needed to remove form I from the tissues and/or the high salt causes a conversion of II to I perhaps via the intermediate form.

In order to investigate the latter possibility, an enzyme extract in 0.1 M sodium chloride was applied to a Sephadex G-100 column which had been equilibrated with 0.5 M sodium chloride and was eluted with the same medium (Fig. 11.6). A control experiment was carried out where the original extract was applied to a column which was equilibrated and eluted with 0.1 M sodium chloride (Fig. 11.5). The elution times were the same for both experiments. A comparison of Figs. 11.5 and 11.6 clearly shows that during elution of the column with 0.5 M sodium chloride,  $\alpha$ -galactosidase I and the intermediate form are produced.

Fig. 11.3. Extraction and Elution of  $\alpha$ -galactosidase carried out with 0.5 M NaCl.

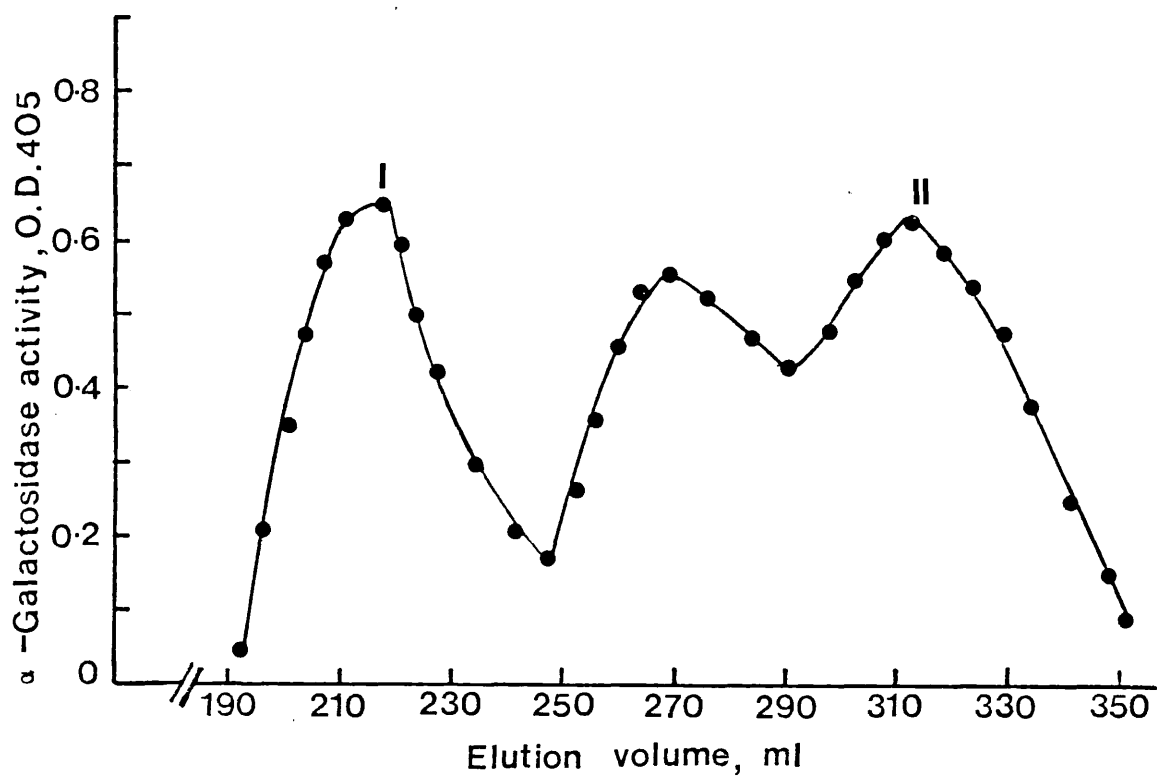


Fig. 11.4. Extraction and Elution of  $\alpha$ -galactosidase carried out with 1.0 M NaCl.

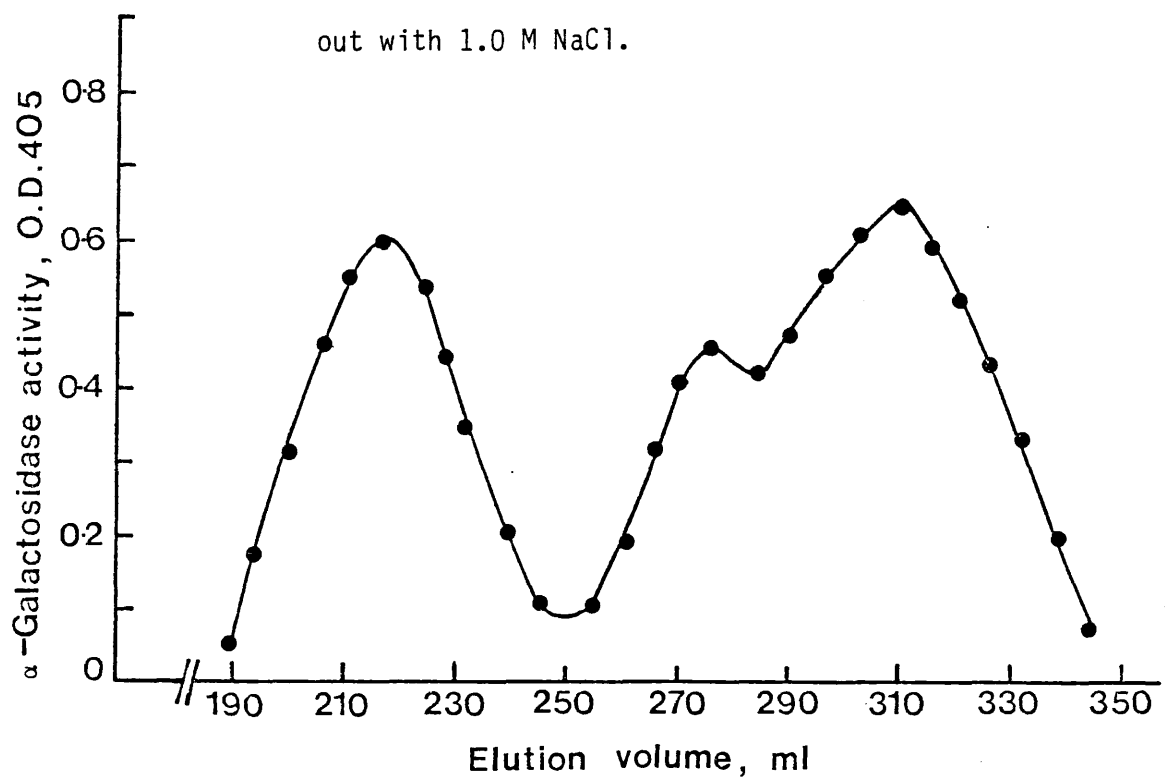


Fig. 11.5. Extraction and Elution of  $\alpha$ -galactosidase with 0.1 M NaCl.

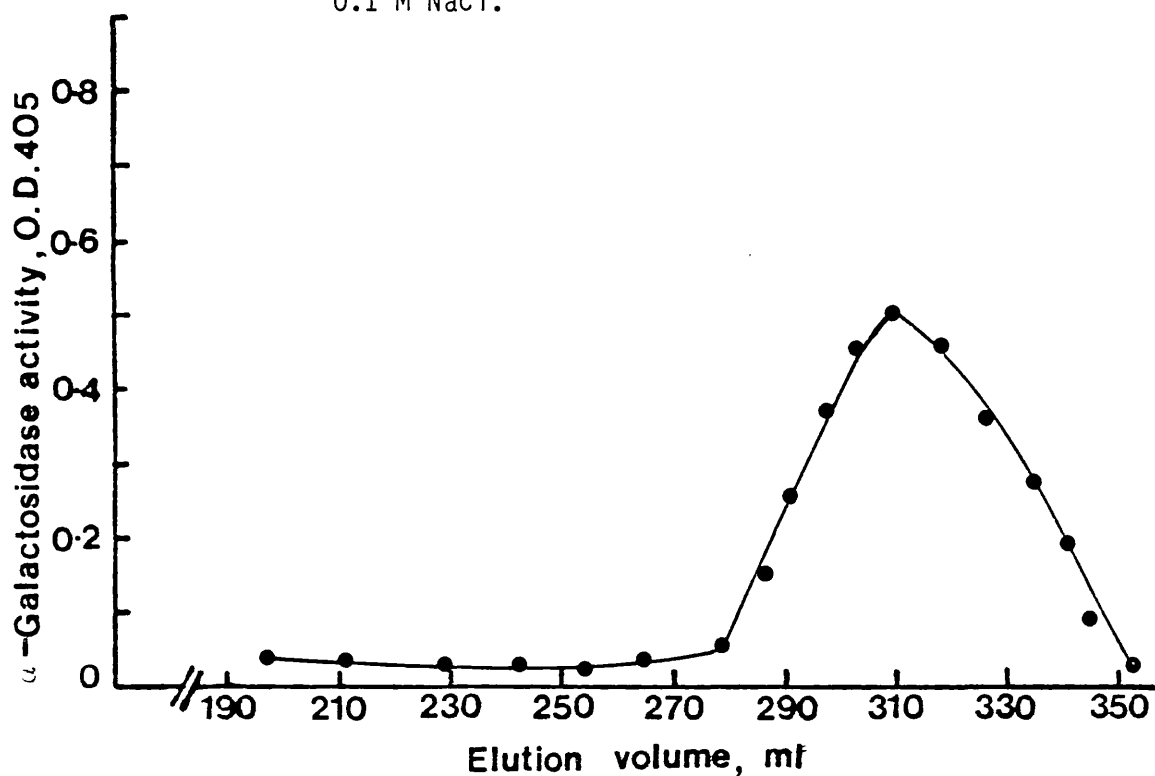
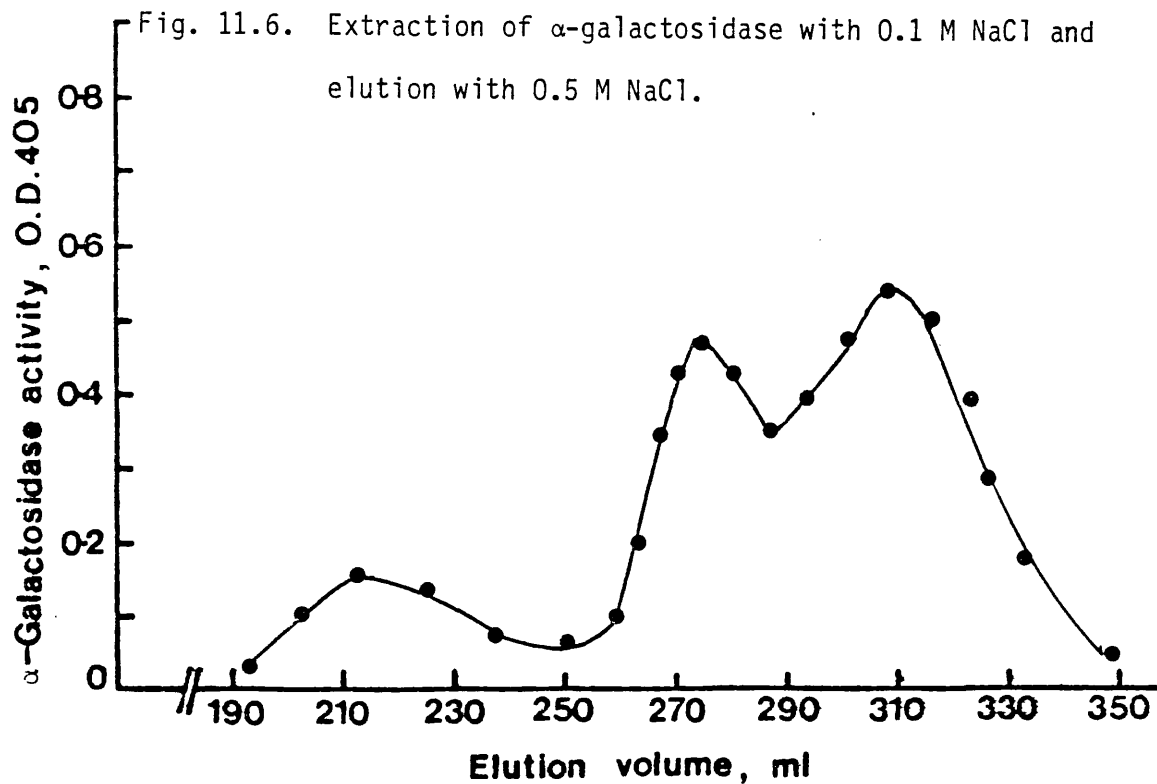


Fig. 11.6. Extraction of  $\alpha$ -galactosidase with 0.1 M NaCl and elution with 0.5 M NaCl.



Inspection of Fig. 11.3 where activity was extracted with 0.5 M sodium chloride and the column eluted with the same salt solution shows higher levels (ca. 3.5 fold) of  $\alpha$ -galactosidase I than in Fig. 11.6 where extraction was effected with 0.1 M sodium chloride and elution with 0.5 M sodium chloride. At first sight, the explanation for this difference would appear to be that, in the case of Fig. 11.3, more conversion of II to I had occurred because of the longer exposure time (extraction and elution) to the high (0.5 M) salt concentration. However, it should be noted that this time difference was only 20%, which would be unlikely to account for a 3.5 fold difference in the level of enzyme I. Therefore, it would seem that enzyme I is present in vivo and is extracted more efficiently or perhaps only by the high salt concentrations. The apparent absence of enzyme I in preparations where extraction and column elution were both achieved with 0.1 M sodium chloride (see Fig. 11.1), may be due to the fact that the high molecular weight form was not extracted or it was extracted and then dissociated.

To test the latter theory, enzyme extracted with 0.5 M sodium chloride was applied to a Sephadex G-100 column, using 0.1 M sodium chloride as eluent and in this case, the major activity detected was  $\alpha$ -galactosidase II (Fig. 11.7). In the control experiment with 0.5 M sodium chloride as extractant and eluent, both forms I and II were present (Fig. 11.3). This gives some support for the possibility that form I can be extracted with 0.1 M sodium chloride but it then dissociates.

Fig. 11.7. Extraction of  $\alpha$ -galactosidase with 0.5 M NaCl and elution with 0.1 M NaCl.

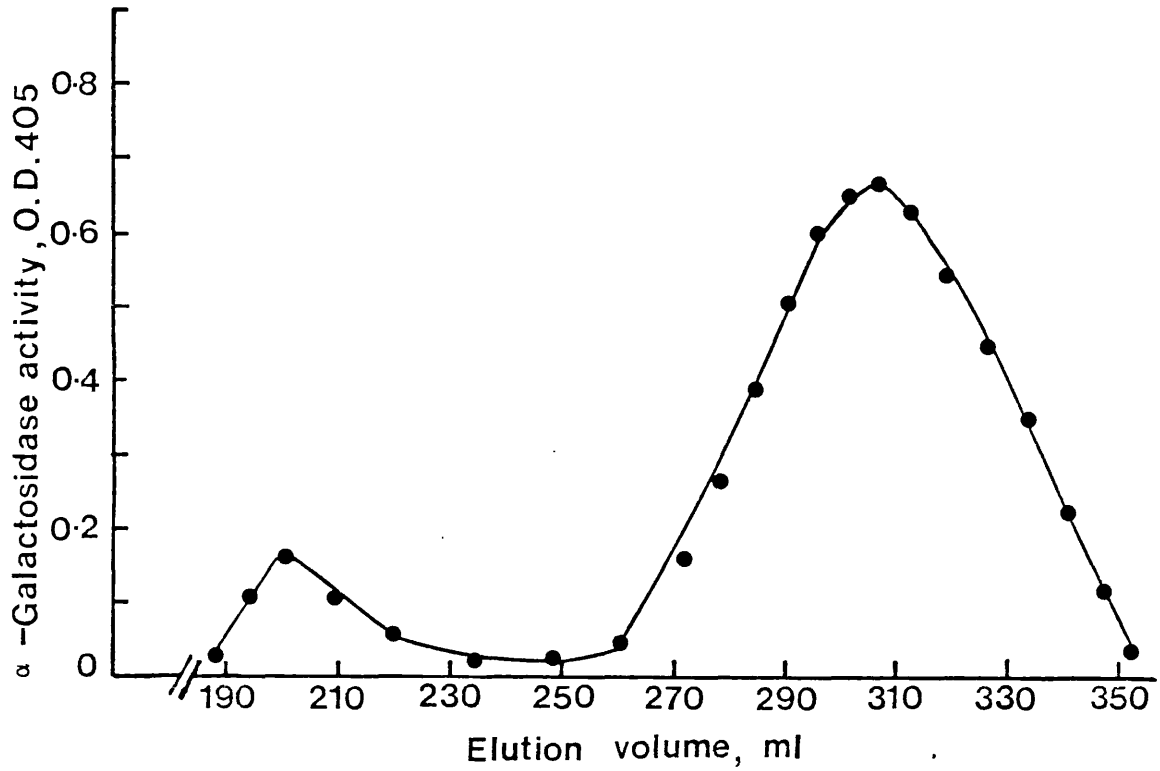
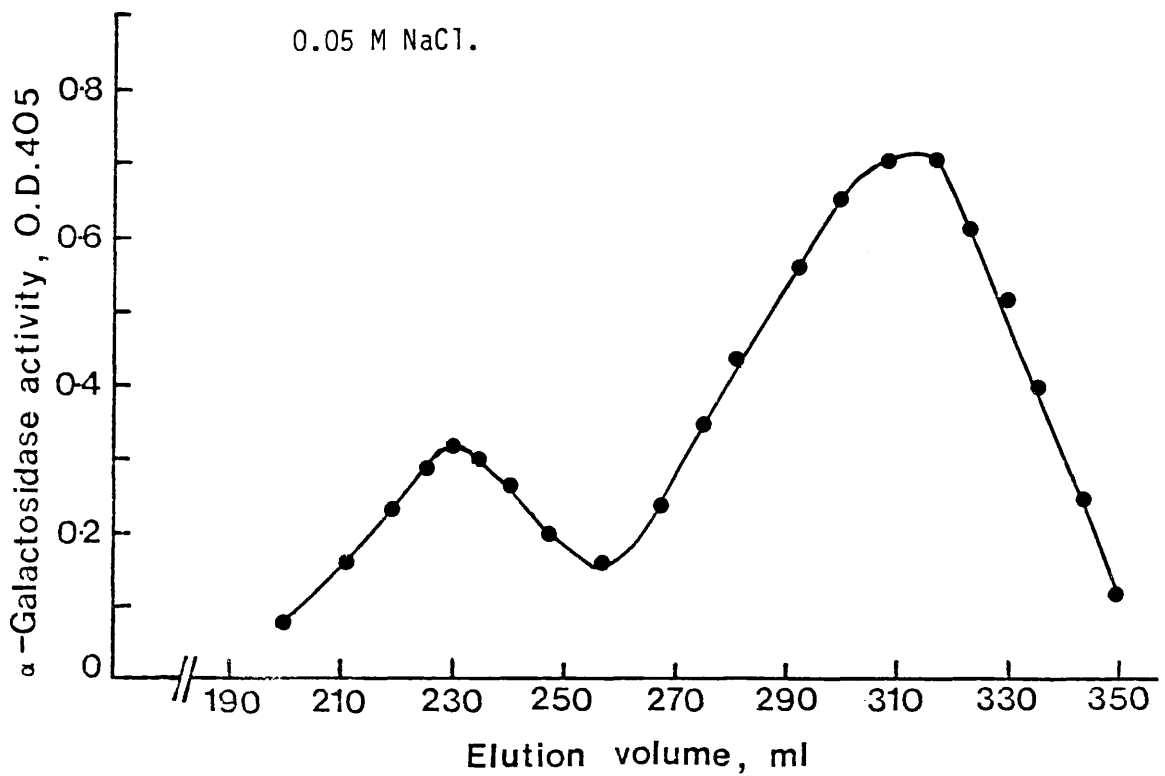


Fig. 11.8. Extraction and elution of  $\alpha$ -galactosidase with 0.05 M NaCl.



An unexpected result was obtained in two separate experiments when an enzyme extract prepared in 0.05 M sodium chloride was applied to a Sephadex column and eluted with the same molarity salt solution. Here (Fig. 11.8), significant levels of  $\alpha$ -galactosidase I were detected when a pattern similar to that shown in Fig. 11.1 had been expected. No clear explanation for this observation can be given (but see p.89).

Following the detailed work on the influence of various concentrations of sodium chloride on extraction and interconversion of multiple forms of  $\alpha$ -galactosidase, the effects of solutions of other salts and buffers which had been used and were to be used in conjunction with  $\alpha$ -galactosidase extraction and purification were investigated. The conditions were identical to those used for the 'sodium chloride experiments'.

Potassium chloride plays a role in the association/dissociation of coconut  $\alpha$ -galactosidase (61, 62), and it activates V. faba  $\alpha$ -galactosidases (69). It was also routinely used as a buffer component for elution of Sephadex-gel columns. Hence, its effect on the pattern of multiple forms was examined. At low concentration (0.1 M) for both tissue extraction and column elution, only the low MW fraction, II, of  $\alpha$ -galactosidase could be detected (Fig. 12.1). At higher concentrations (0.5 M), enzyme I and the intermediate form were apparent in addition to II (Fig. 12.2). The effects were, therefore, rather similar to those of sodium chloride.

Fig. 12. Examination of KCl extracts of *V. faba* seed tissue by Sephadex G-100 chromatography.

Fig. 12.1. Extraction and Elution of  $\alpha$ -galactosidase with 0.1 M KCl.

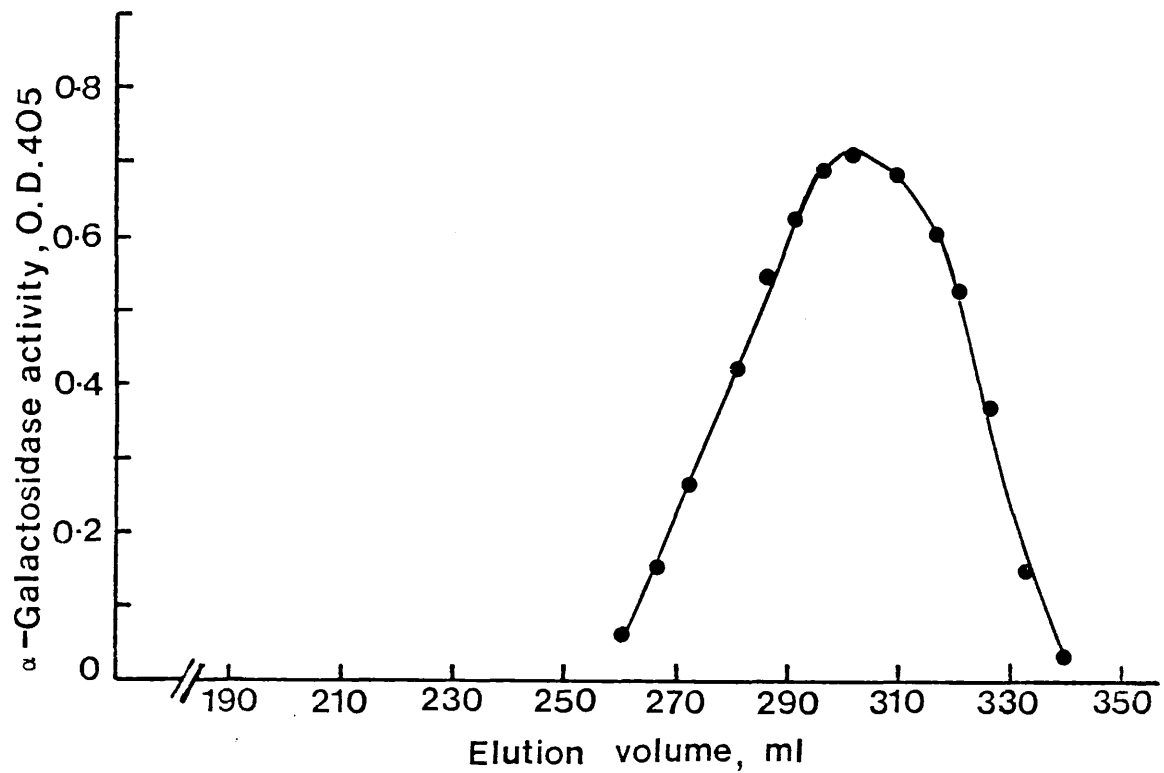
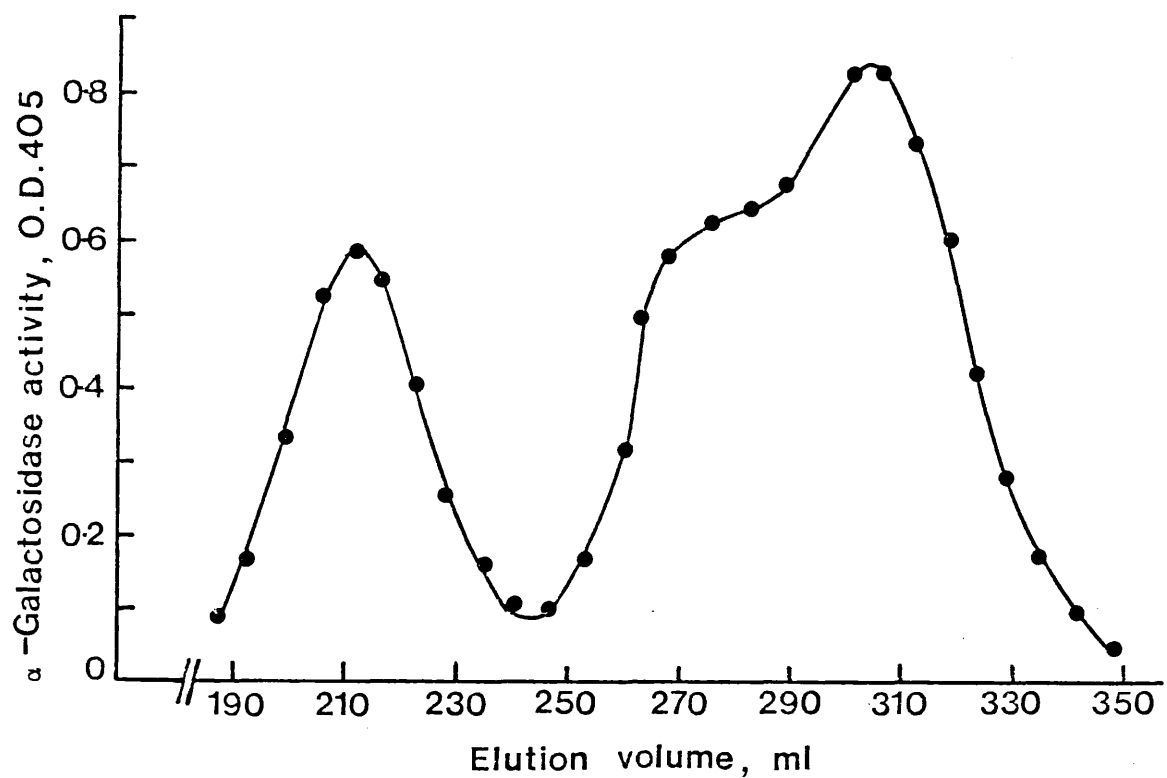


Fig. 12.2. Extraction and Elution of  $\alpha$ -galactosidase with 0.5 M KCl.





Much of the previous work on multimolecular forms of V. faba  $\alpha$ -galactosidase had utilized McIlvaine (citrate/phosphate) buffer, pH 5.5, in purification procedures. When this buffer was used for enzyme extraction and elution, the pattern shown in Fig. 13.1 was observed; I and II were present with II predominating. The addition of either 0.1 M sodium chloride or 0.1 M potassium chloride to the McIlvaine buffer produced no significant change (Figs. 13.2 and 13.3). However, a 5 fold concentrated McIlvaine buffer, pH 5.5, containing 0.1 M potassium chloride exhibited a marked effect on the enzymic forms present. Figure 13.4, in comparison with Fig. 13.3, shows that there was a significant increase in enzyme I, a decrease in II and the appearance of the intermediate form which was at higher levels than II. Thus the concentrated buffer seems to favour the intermediate form and enzyme I at the expense of fraction II. This led to the examination of the effect of citrate, a McIlvaine buffer component, on the levels of these enzymes. Citrate ions have been implicated in the conversion of II to I (77). The elution profile obtained with an enzyme extract in 0.1 M sodium citrate buffer, pH 5.5, shows (Fig. 14) some II, a predominance of the intermediate form and a negligible amount of form I. The pattern difference was further investigated using 0.1 M pH 8.6, and 0.5 M, pH 9.1, solutions of unbuffered sodium citrate. In both cases (Figs. 15.1 and 15.2),  $\alpha$ -galactosidase I was present at high levels and the difference between the pattern for 0.1 M sodium citrate solution and that for 0.1 M citrate buffer can, presumably be accounted for by the difference in pH. The relatively high pH

Fig. 13. Examination of McIlvaine buffer extracts of *V. faba* seed tissue by Sephadex G-100 chromatography.

Fig. 13.1. Extraction and Elution of  $\alpha$ -galactosidase with McIlvaine buffer, pH 5.5.

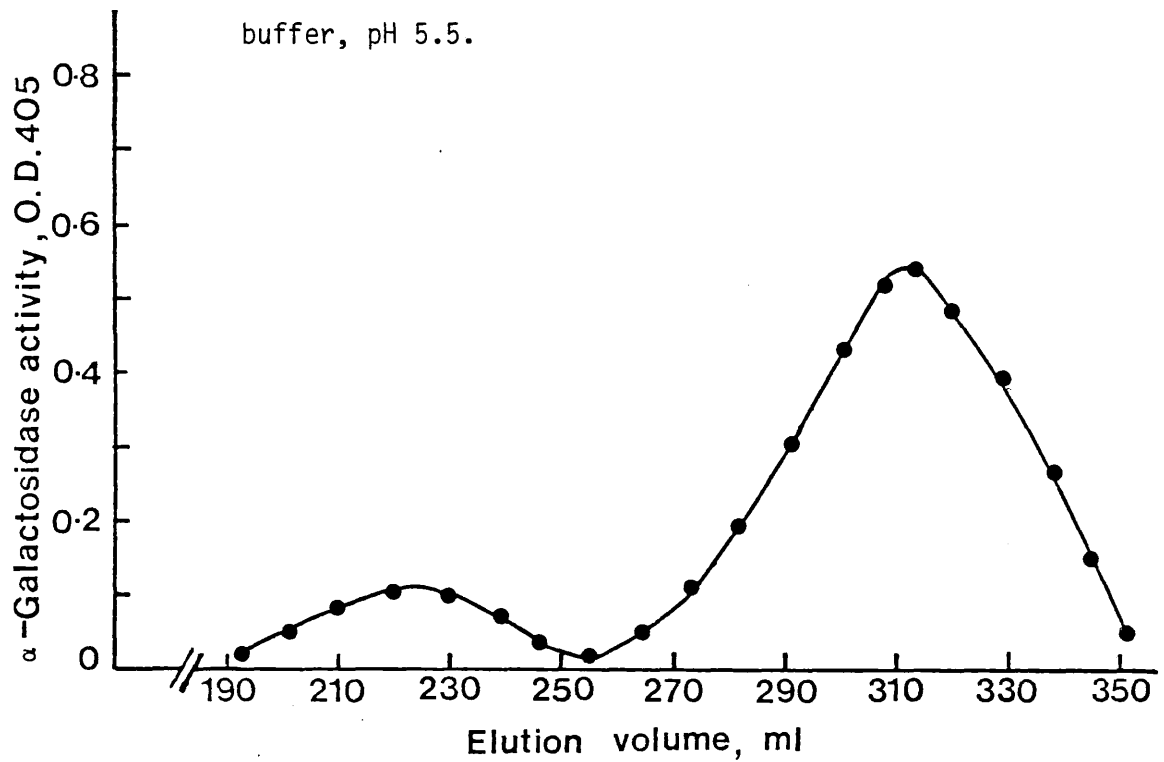


Fig. 13.2. Extraction and Elution of  $\alpha$ -galactosidase with McIlvaine buffer containing 0.1 M NaCl, pH 5.5.

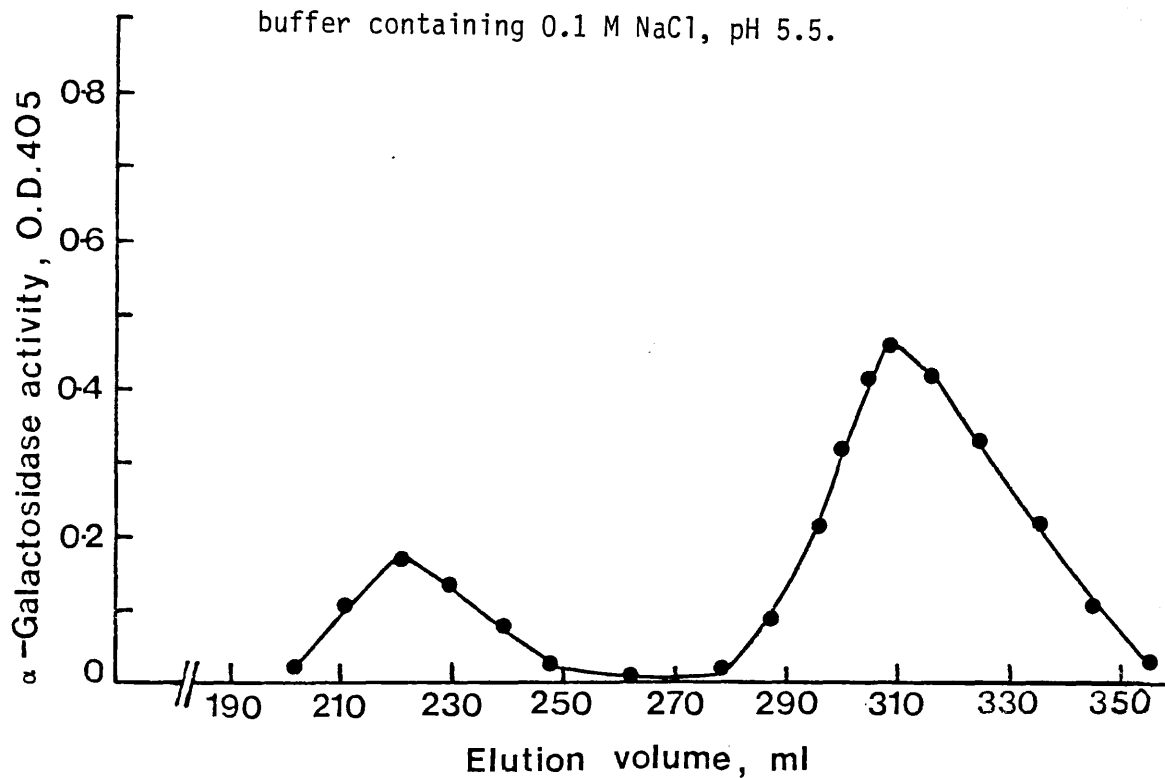


Fig. 13.3. Extraction and Elution of  $\alpha$ -galactosidase with McIlvaine buffer containing 0.1 M KCl, pH 5.5.

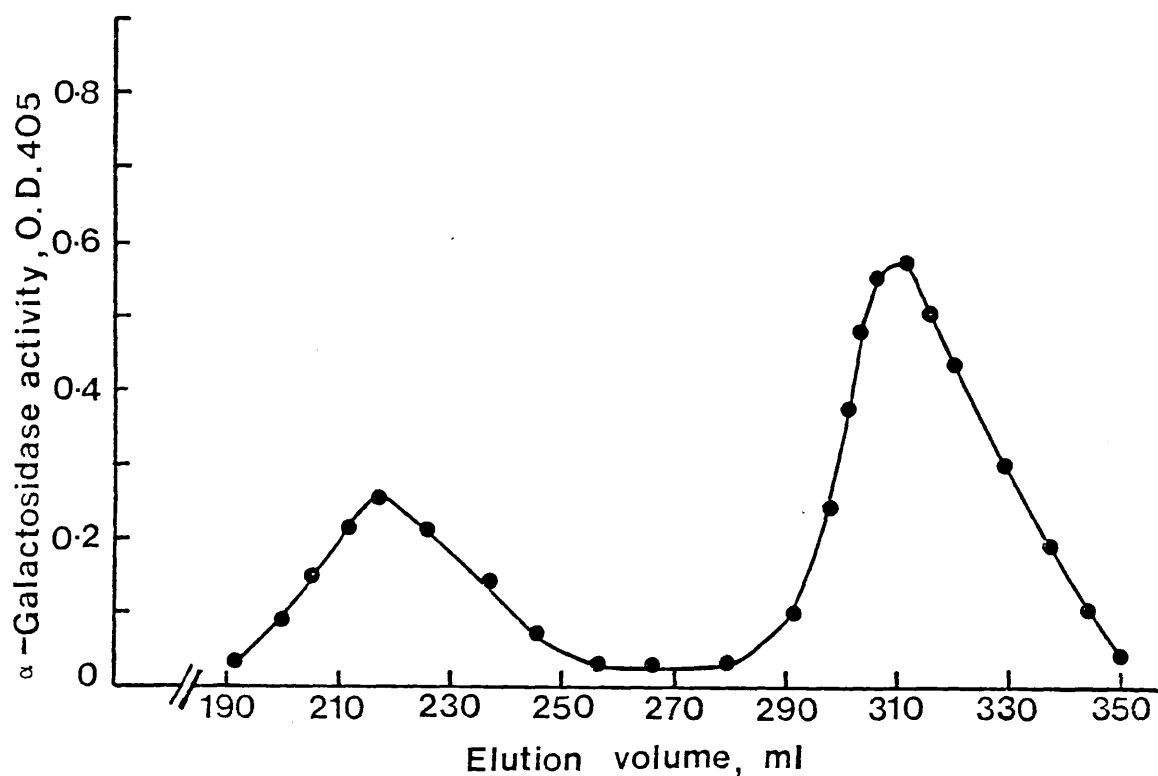


Fig. 13.4. Extraction and Elution of  $\alpha$ -galactosidase with a 5 fold concentrated McIlvaine buffer containing 0.1 M KCl, pH 5.5.

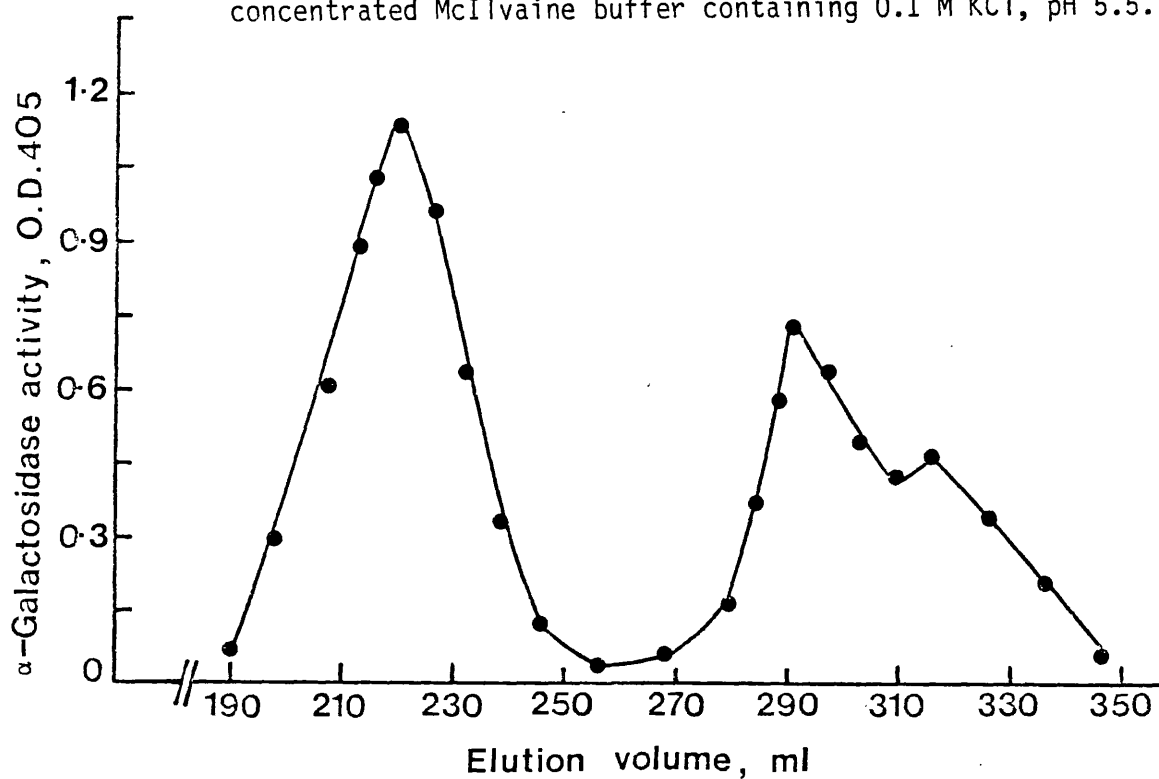


Fig. 14. Examination of a 0.1 M Citrate buffer extract of V. faba seed tissue by Sephadex G-100 chromatography, using the same buffer, pH 5.5 as eluent.

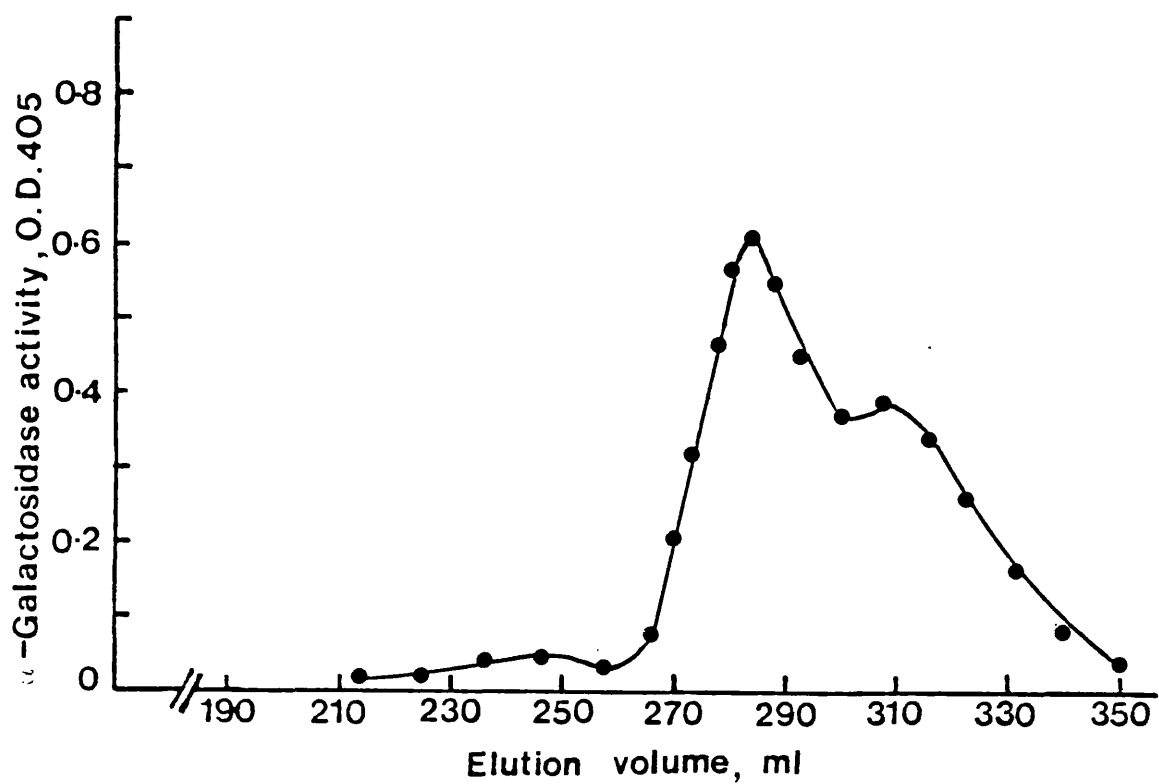


Fig. 15. Examination of Sodium citrate extracts of *V. faba* seed tissue by Sephadex G-100 Chromatography.

Fig. 15.1. Extraction and Elution of  $\alpha$ -galactosidase with 0.1 M Sodium citrate, pH 8.6.

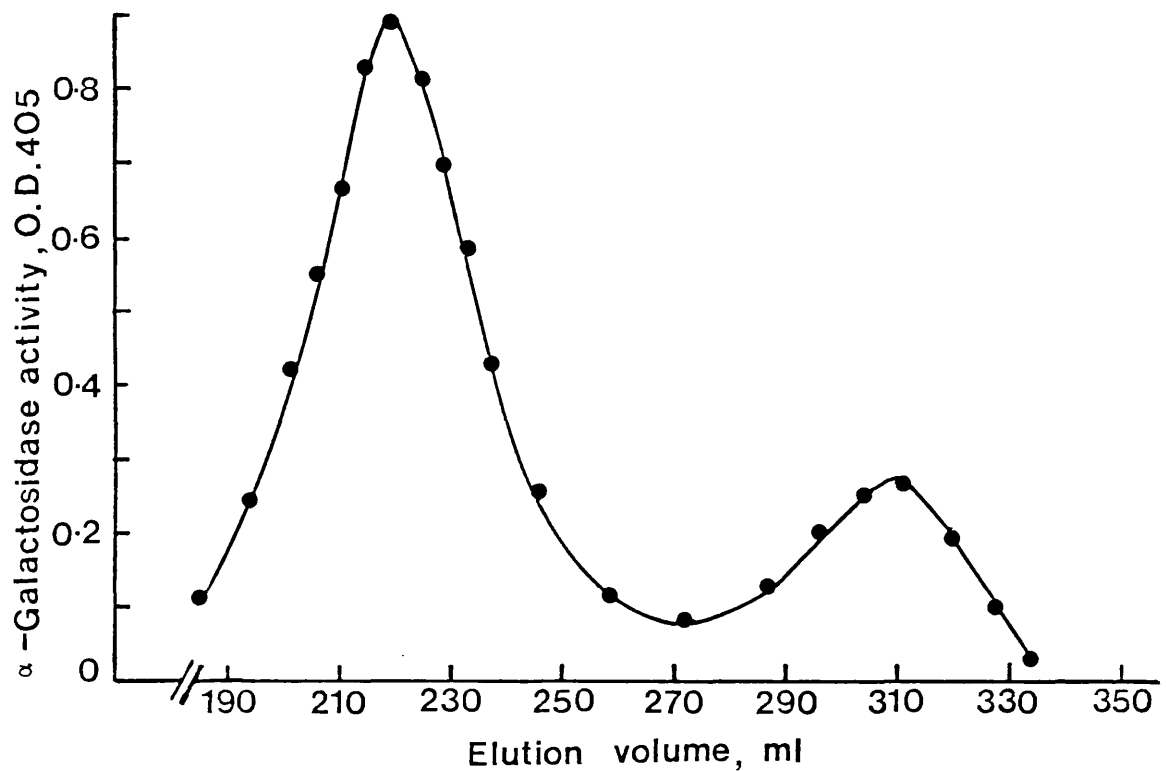
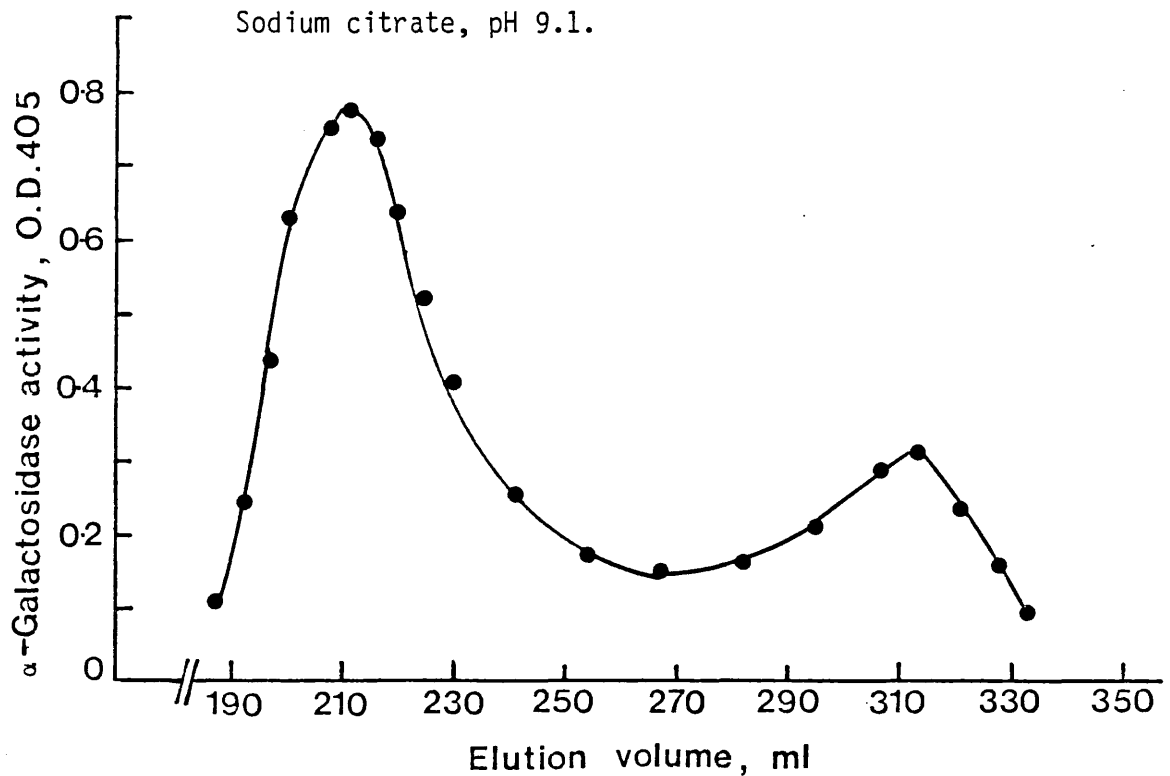


Fig. 15.2. Extraction and Elution of  $\alpha$ -galactosidase with 0.5 M Sodium citrate, pH 9.1.



of the unbuffered citrate solution may also explain the lower recoveries of activity from the columns; 41% and 59% for 0.1 M and 0.5 M sodium citrate, respectively.

Sodium phosphate, is the other McIlvaine buffer component, so the effect of sodium phosphate buffer, pH 5.7, as extractant and eluent was examined. At a concentration of 0.1 M, the pattern shown in Fig. 16.1 was observed which was identical to those obtained with sodium chloride and potassium chloride at the same concentrations. With 0.5 M sodium phosphate buffer, however, the pattern (Fig. 16.2) was different. Only activities corresponding to I and II could be detected; there was no trace of the intermediate form which was found in the case of 0.5 M sodium chloride (Fig. 11.3). Similar results (Figs. 17.1 and 17.2) were obtained with 0.1 M and 0.5 M sodium acetate buffers, pH 5.5 and with unbuffered sodium acetate solutions at the same concentrations (Figs. 18.2 and 18.3). However, in the case of 0.05 M sodium acetate solution, pH 7.3, only one enzymically active peak corresponding to enzyme I (Fig. 18.1) was found. This was the only case, including studies with 0.05 M sodium chloride (Fig. 11.8), where II was not detected in an enzyme extract. It is important to mention here, however, that the total recovery of enzyme on gel filtration was only 17 and 21% in duplicate experiments (cf. 0.05 M sodium chloride; 63%). With extracts using other salt solutions, even when the recovery was low (ca. 40%), for example, with 0.1 M sodium citrate (Fig. 15.1), both forms were present. The explanation for this unexpected

Fig. 16. Examination of Phosphate buffer extracts of *V. faba* seed tissue by Sephadex G-100 chromatography.

Fig. 16.1. Extraction and Elution of  $\alpha$ -galactosidase 0.1 M with Sodium phosphate buffer, pH 5.7.

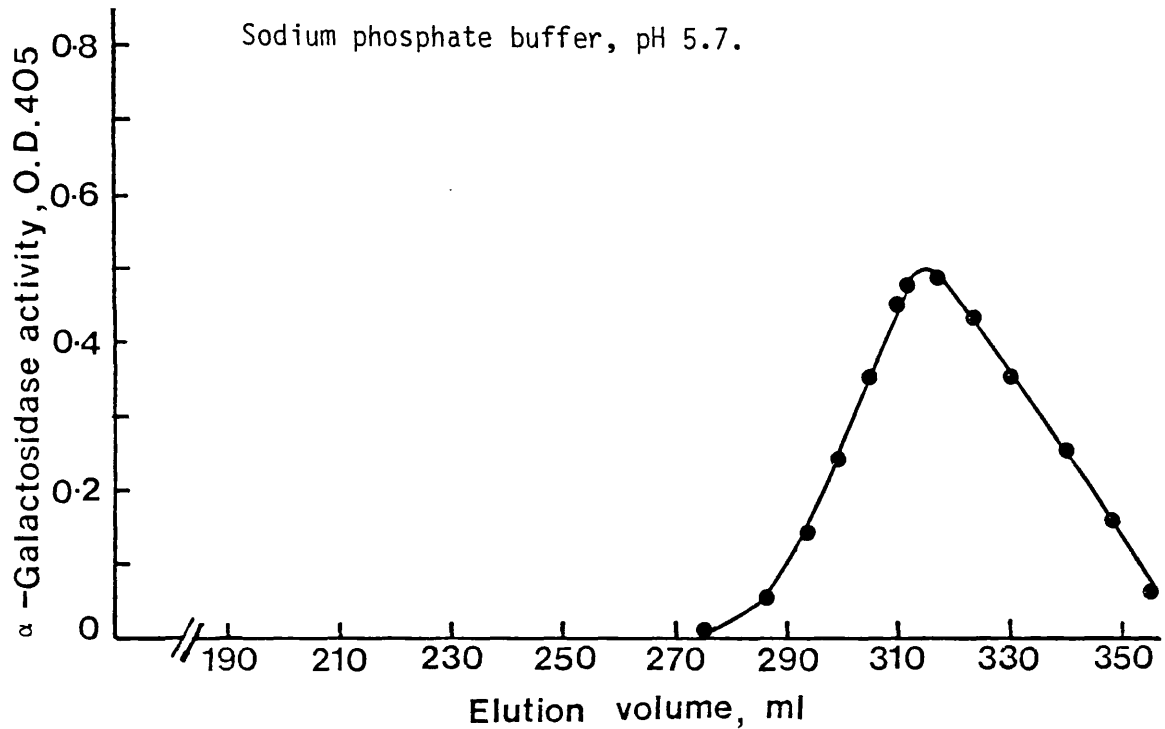


Fig. 16.2. Extraction and Elution of  $\alpha$ -galactosidase with 0.5 M Sodium phosphate buffer, pH 5.7.

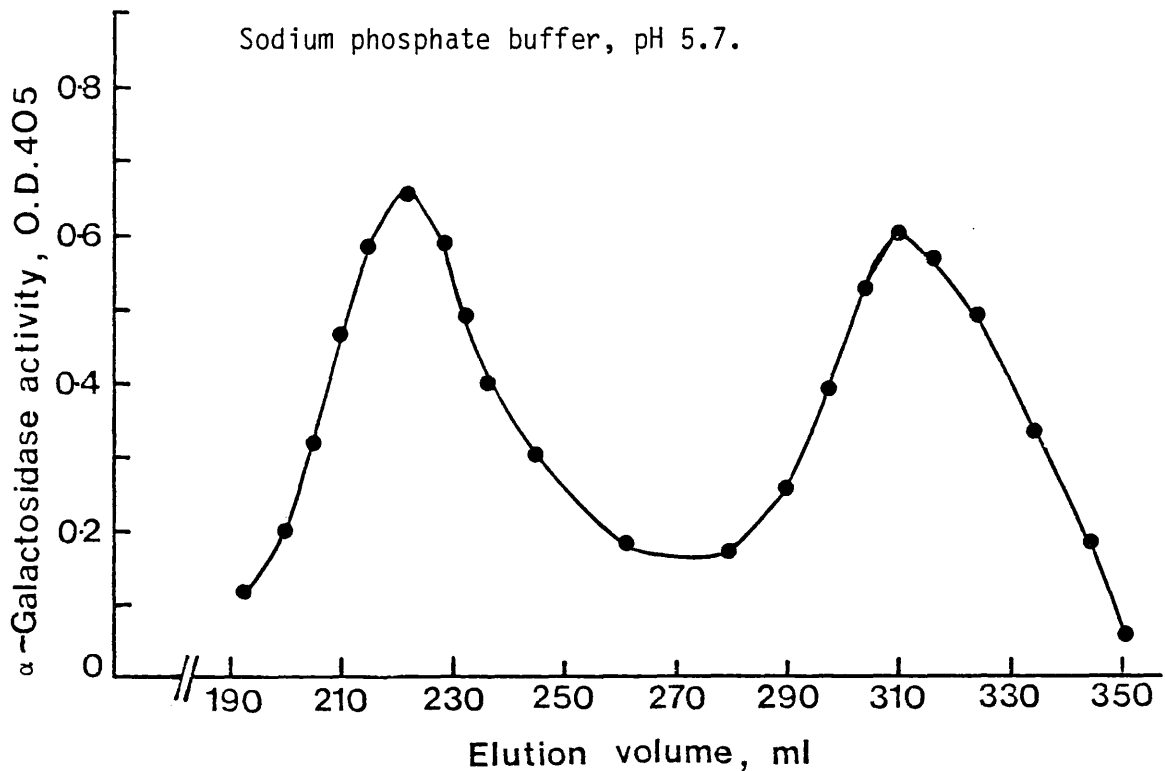


Fig. 17. Examination of Acetate buffer extracts of *V. faba* tissue by Sephadex G-100 chromatography.

Fig. 17.1. Extraction and Elution of  $\alpha$ -galactosidase with 0.1 M Acetate buffer, pH 5.5.

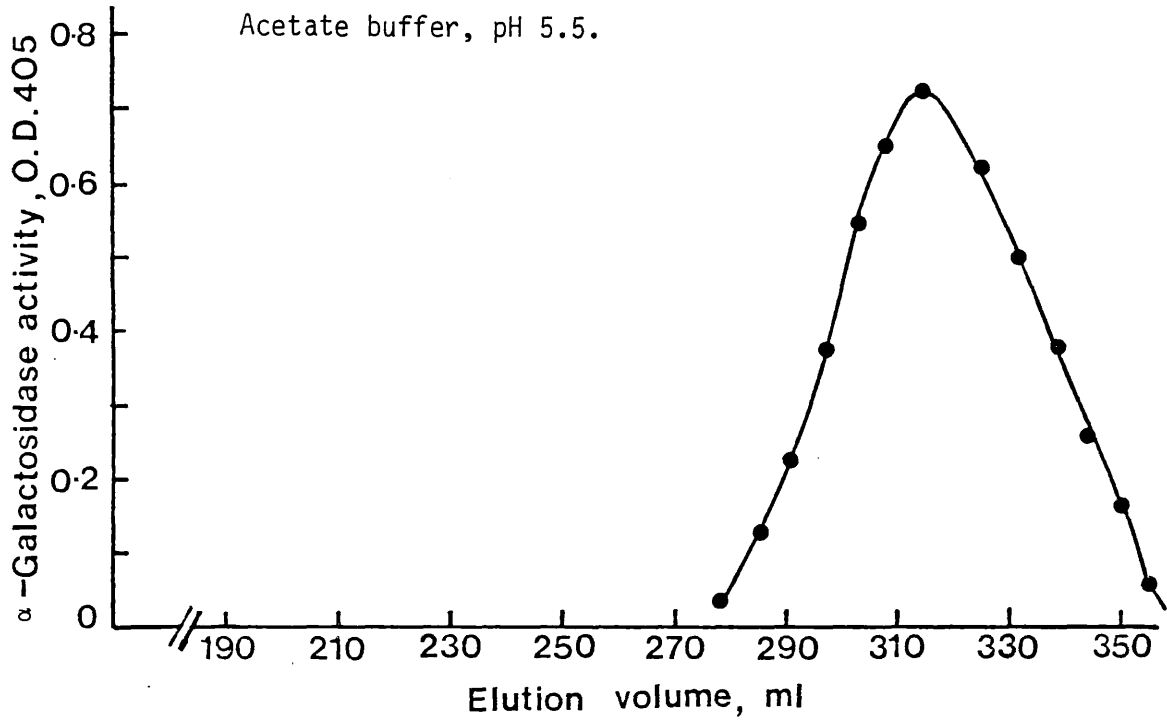


Fig. 17.2. Extraction and Elution of  $\alpha$ -galactosidase with 0.5 M Acetate buffer, pH 5.5.

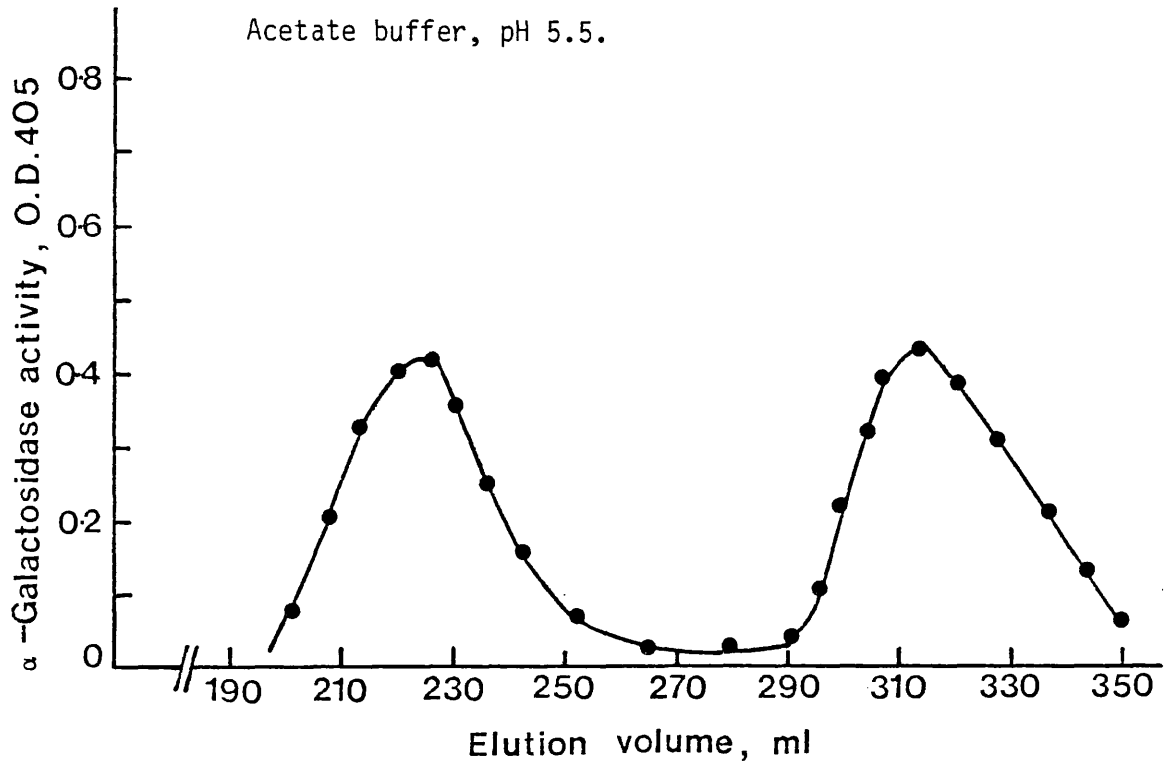




Fig. 18. Examination of Sodium acetate extracts of V. faba seed tissue by Sephadex G-100 chromatography.

Fig. 18.1. Extraction and Elution of  $\alpha$ -galactosidase with 0.05 M Sodium acetate, pH 7.3.

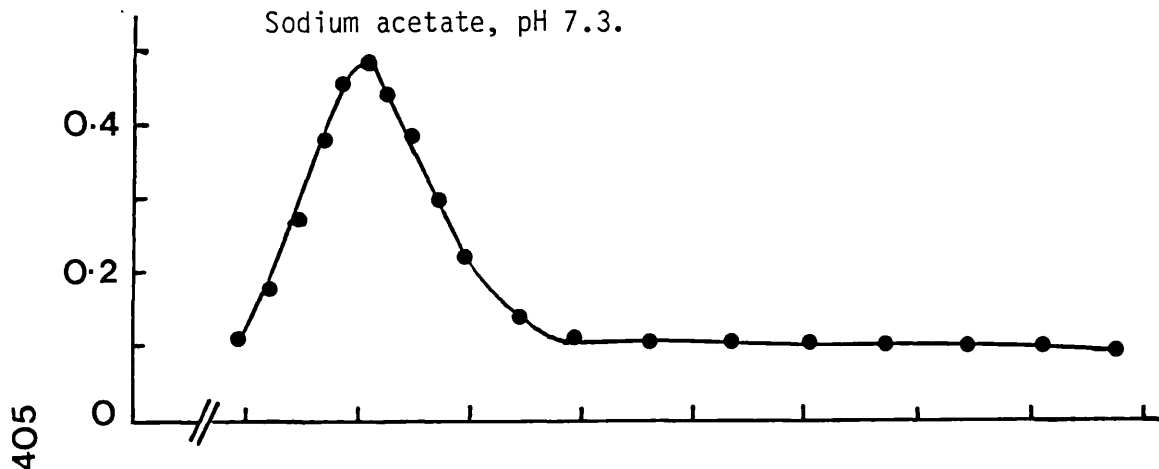


Fig. 18.2. Extraction and Elution of  $\alpha$ -galactosidase with 0.1 M Sodium acetate, pH 7.5.

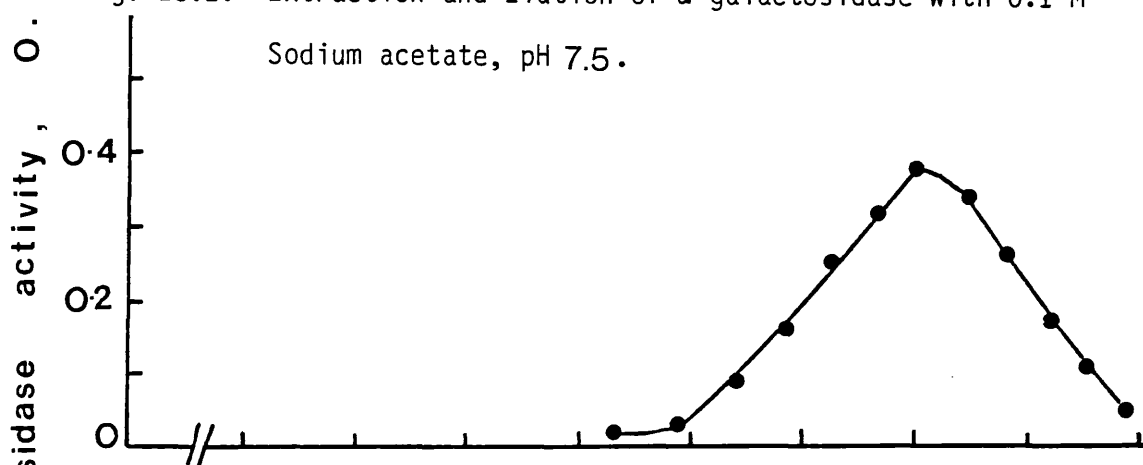
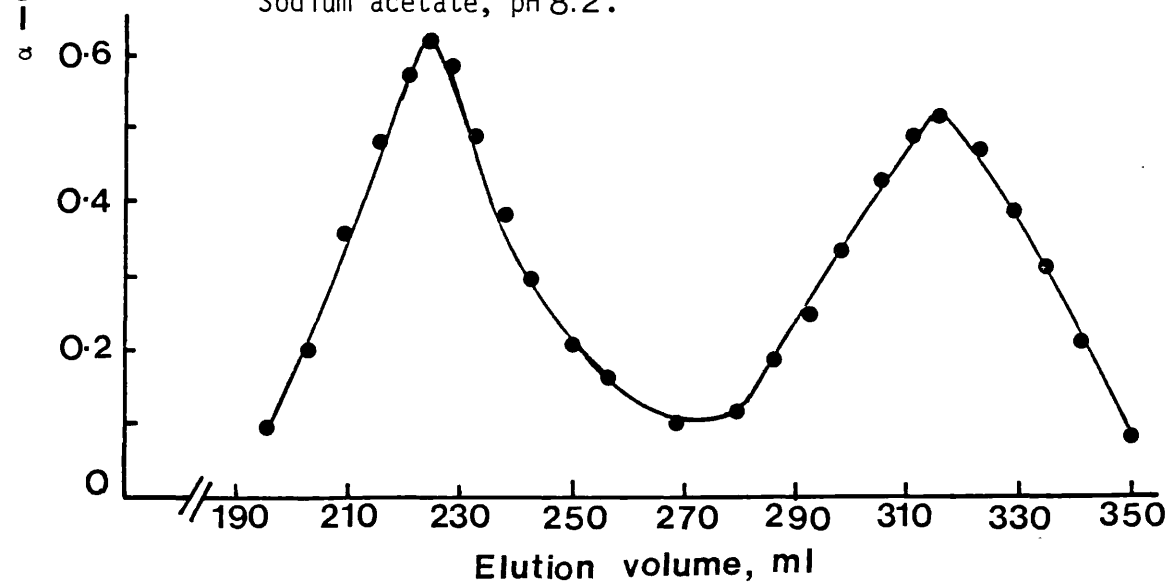


Fig. 18.3. Extraction and Elution of  $\alpha$ -galactosidase with 0.5 M Sodium acetate, pH 8.2.



result with low levels of unbuffered sodium acetate (0.05 M) is not clear especially when compared with the results obtained with sodium chloride at the same concentration. However, the pH of the former solution, pH 7.3, was higher than that of the sodium chloride solution, pH 6.8 and perhaps at low ionic strength and high pH,  $\alpha$ -galactosidase II is unstable. At high ionic strength (see Figs. 18.2 and 18.3), form II would appear to be able to tolerate this pH.

This whole investigation clearly indicates the difficulties in trying to extrapolate from the pattern of multiple forms as observed in vitro to the forms as they exist in vivo. In summary it would seem that  $\alpha$ -galactosidase I is present in vivo and that high concentrations of sodium chloride, and probably other salts, favour its extraction from seed powders and almost certainly enhance conversion of the low MW enzyme to the larger aggregates. There is an apparent anomaly in the case of 0.05 M sodium acetate and an attempt has been made to explain this on this page.

The effect of citrate on the multiple forms is of interest. Inspection of the patterns obtained with salt and buffer solutions at the 0.1 M level shows that the high MW forms of the enzymes are only present in the cases where citrate is present (sodium citrate solution and sodium citrate buffer). It is possible that citrate is acting as a chelating agent (214, 215) which aids extraction of the high MW enzymes and/or favours

aggregation of form II. In vitro studies by Hustler (77) have shown that citrate does enhance the conversion of II to I. In this case and in the present work, this could conceivably be achieved by the removal of divalent cations which interfere with aggregation of subunits. The difference between the effects of 0.1 M sodium citrate and 0.1 M sodium citrate buffer may be a function of pH where in the former case the higher pH facilitates chelation by citrate and hence the formation of relatively 'high' level of  $\alpha$ -galactosidase I. At the lower pH of sodium citrate buffer, the aggregation would appear to go no further than the dimer. The effect of divalent ions, such as  $Mg^{2+}$  and  $Ca^{2+}$ , on the polymerization of  $\alpha$ -galactosidase monomers is an area worthy of further investigation.

Complex changes in multiple forms in response to ionic strength and pH in the case of animal  $\beta$ -galactosidases have been observed. For example, the concentration of sodium chloride in the eluting medium has been shown to have a marked effect on the gelfiltration patterns of human liver acid  $\beta$ -galactosidase (212). In the absence of sodium chloride, three peaks of activity were resolved with MWs of 36,000, 67,000 and 130,000. In the presence of 1 M sodium chloride, a major peak of activity of MW greater than 400,000 was observed with shoulders corresponding to lower MW forms. At an intermediate ionic strength, a progressive decrease in the amounts of the three MW forms was observed with an increase in the high MW form. Similarly Sephadex G-200 profiles of marmoset kidney  $\beta$ -galactosidase

indicated aggregation of the enzyme in response to treatment with 1 M sodium chloride (216).

Hoeksema et al (217) have detected multimeric and dimeric forms of human fibroblast  $\beta$ -galactosidase by gel chromatography. Two buffer systems were used; in the first, 10 mM sodium phosphate/10 mM sodium chloride, pH 7.0, the major peak observed was a low MW (65,000) form but a minor peak of MW 600,000 was also present. In the second buffer, 0.1 M sodium acetate/0.1 M sodium chloride, pH 4.5, the dimer with MW 130,000 - 150,000 predominated and the multimer was absent.

## RESULTS AND DISCUSSION

### PART 2 Isolation and Characterization of V. faba $\alpha$ -Galactosidases

The main aim of this project was to obtain highly purified samples of the previously reported  $\alpha$ -galactosidases I, II<sup>1</sup> and II<sup>2</sup> from Vicia faba seeds (25) and to examine their structural relationships by immunochemical techniques. Minor goals were to discover more about the glycoprotein nature of the enzymes and to establish whether they possessed lectin activity as had been claimed earlier in the case of Vigna radiata seed  $\alpha$ -galactosidase (170).

#### 4.1 Purification of $\alpha$ -galactosidases

The three forms of the enzyme were isolated from powdered resting V. faba beans from which the testas had been removed. Fourteen preparations of the enzymes were extracted and purified over a 4-month period. In each case 1 kg (each week) of seed powder was used and the process required approximately 2 weeks to complete.

A typical purification procedure is summarized in Table 5. The stage involving addition of citric acid (Stage 2) was necessary to remove the bulk of the seed storage proteins and a number of glycosidases other than  $\alpha$ -galactosidase (218). This resulted in a 5-fold increase in specific activity and also a significant increase in the volume of the preparation. To overcome the latter, the protein was next precipitated with ammonium sulphate (Stage 3) and the fraction dialysed against McIlvaine buffer (pH 5.5). No improvement in the specific activity occurred at this stage. The preparation was then rapidly applied to a Sephadex

Table 5. Purification of *V. faba*  $\alpha$ -galactosidases

Stage	Volume (ml)	Activity (nkat/ml)	Protein (mg/ml)	Specific activity (nkat/mg)	Recovery* (%)
1. Crude extract	1010	16.0	88.0	0.18	-
2. Citric acid precipitation	1438	9.1	9.2	1.0	81
3. (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 25-60%	68	124.4	98.0	1.3	52
4. Sephadex G-100					
$\alpha$ -Galactosidase I	9.0	253.7	6.1	41.6	14
$\alpha$ -Galactosidase II	11.0	165.3	62.9	2.6	11
5. Sephadex G-100, recycle					
I	3.0	426.6	5.4	79	8
II	4.0	229.5	37.0	6.2	6
6. Con A-Sepharose					
I	5.0	184.0	0.8	230.0	4.5
II	5.0	114.4	3.1	36.9	3.5
7. CM-cellulose chromatography					
I	1.0	307.0	0.24	1279.0	2.0
II <sup>1</sup>	2.5	43.6	1.08	40.4	0.7
II <sup>2</sup>	2.5	48.4	0.56	86.4	0.56
8. CM-cellulose chromatography					
I	1.0	180.0	0.14	1286.0	1.1
II <sup>1</sup>	1.0	60.0	0.78	76.9	0.37
II <sup>2</sup>	1.0	72.0	0.63	114.3	0.44

\* % Recovery has been calculated relative to the first stage, ie. crude extract. Weight of seed powder taken = 1 kg.

G-100 column (Stage 4): resolution of the high and low MW activities (Fig. 19.1) was relatively poor but the main purpose of this stage was to minimise the aggregation of the low MW form II to I; gel filtration had been reported to achieve this (67).  $\alpha$ -galactosidases I and II thus obtained were separately pooled, concentrated by ultrafiltration and then recycled through Sephadex G-100 columns (Stage 5, Figs. 19.2 and 19.3). The specific activities of the two forms were increased 2-fold by this last step in the procedure and the two forms were then dialysed against potassium phosphate buffer (pH 7.0) and applied to Con A-Sepharose columns (Stage 6) in 2 ml 'batches' (Fig. 20). In both cases, most of the activity was bound to the column, however 17% of enzyme I ( $I_{ub}$ ) and 20% of II ( $II_{ub}$ ) were not absorbed and were eluted by the phosphate buffer. The bound enzymes were then desorbed from the columns with buffered 0.5 M methyl- $\alpha$ -D mannoside. This step resulted in 2- and 5-fold purification of  $\alpha$ -galactosidase I and II, respectively. Enzymically active fractions I, II,  $I_{ub}$  and  $II_{ub}$  from Stage 6 were then separately pooled, concentrated and dialysed against McIlvaine buffer (pH 3.5) for 2-3 h. The enzymes were subsequently applied to CM-cellulose columns (Stage 7) which were washed until the absorbance of the washings at 280 nm was nil. The columns were then eluted with sodium chloride gradients (0.05 - 0.4 M). Considering first the enzymes which bound to Con A-Sepharose, enzyme I eluted as a single peak (Fig. 21.1) and there was a 7-fold increase in its specific activity. The  $\alpha$ -galactosidase II fraction was resolved into two forms ( $II^1$  and



Fig. 19. Purification of  $\alpha$ -galactosidases from *V. faba* seeds.

Fig. 19.1. Sephadex-gel Elution profile of I and II; Stage 4

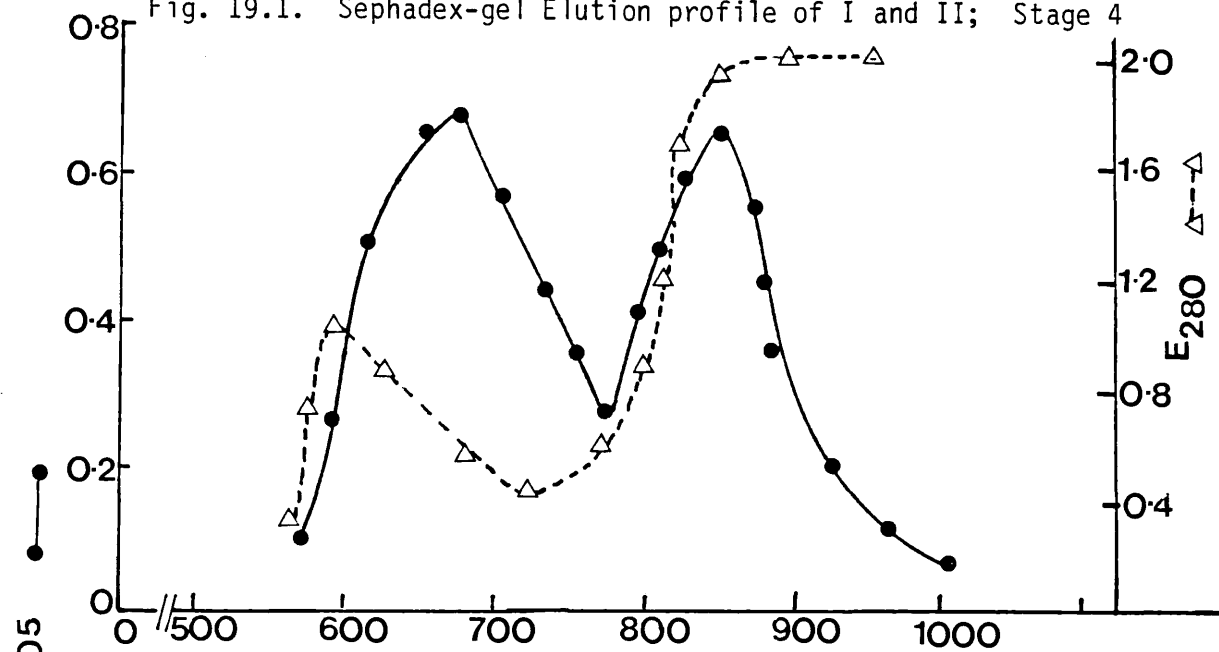


Fig. 19.2. Sephadex-gel Elution profile of I. Stage 5.

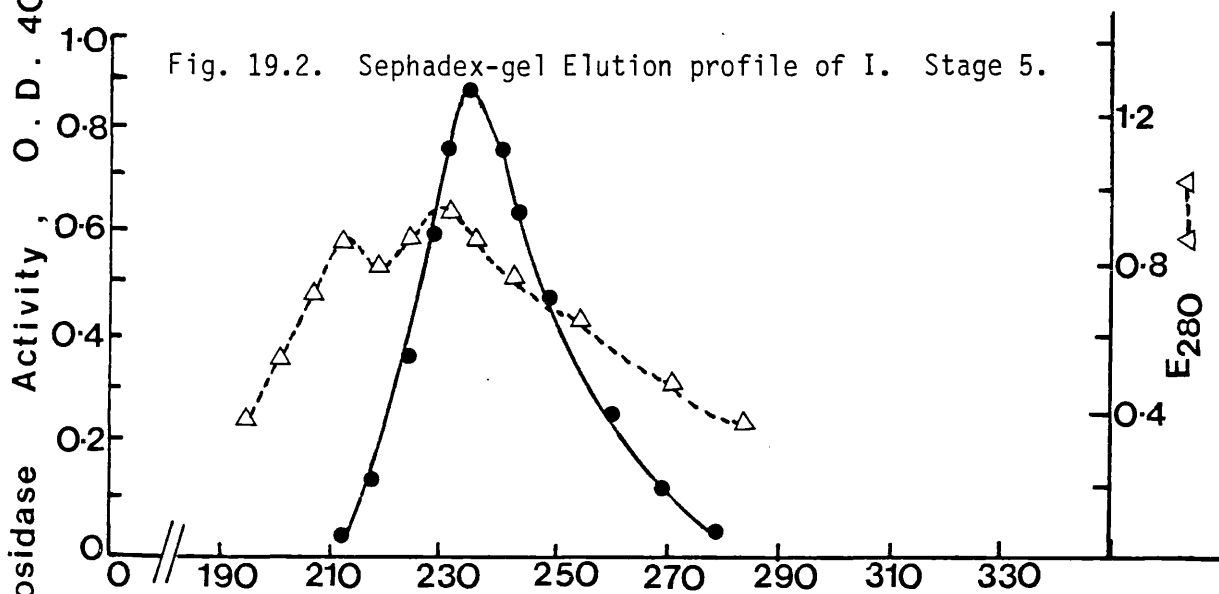
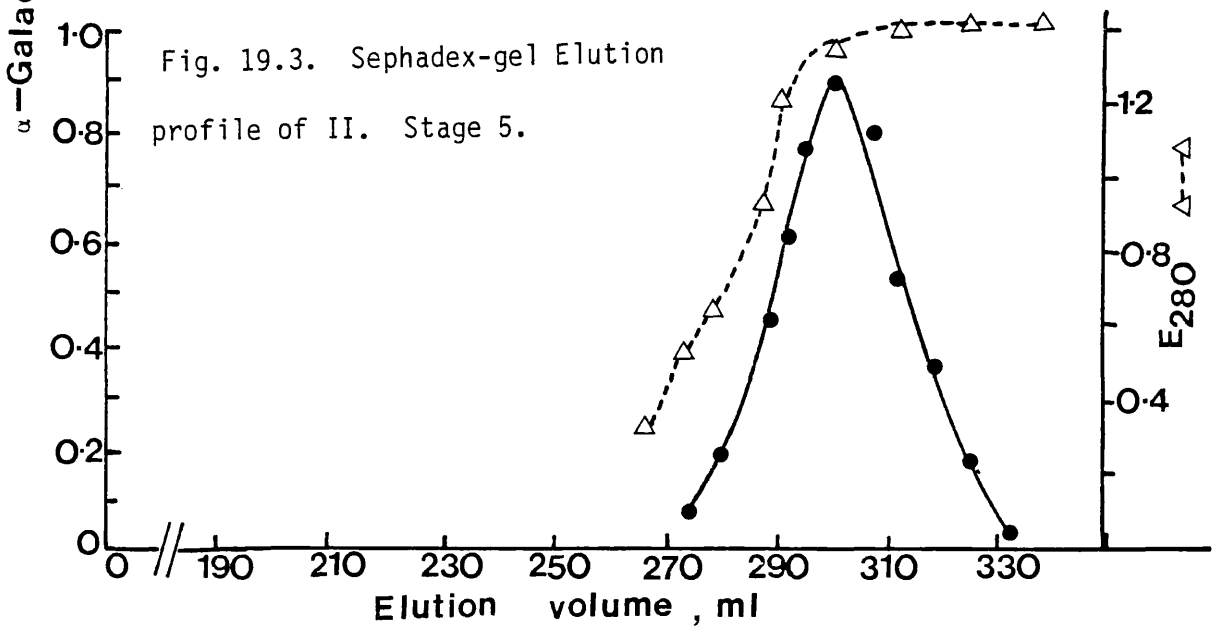


Fig. 19.3. Sephadex-gel Elution profile of II. Stage 5.



Purification of  $\alpha$ -galactosidases

Fig. 20. Con A-Sepharose Elution profile of  $\alpha$ -galactosidase.  
Stage 6.

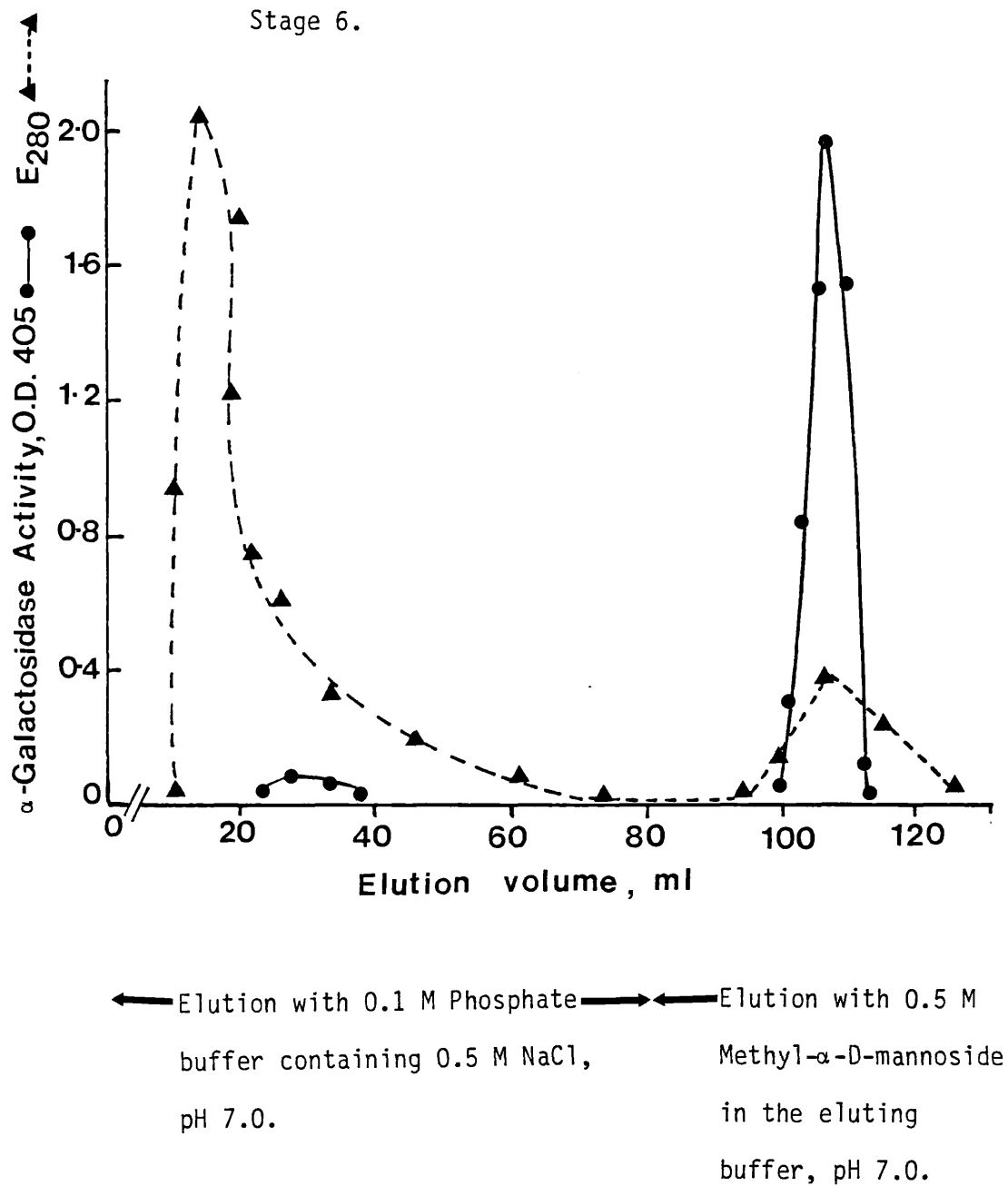


Fig. 21. Purification of  $\alpha$ -galactosidases on ion-exchange columns

Fig. 21.1. CM-cellulose chromatography of  $\alpha$ -galactosidase I

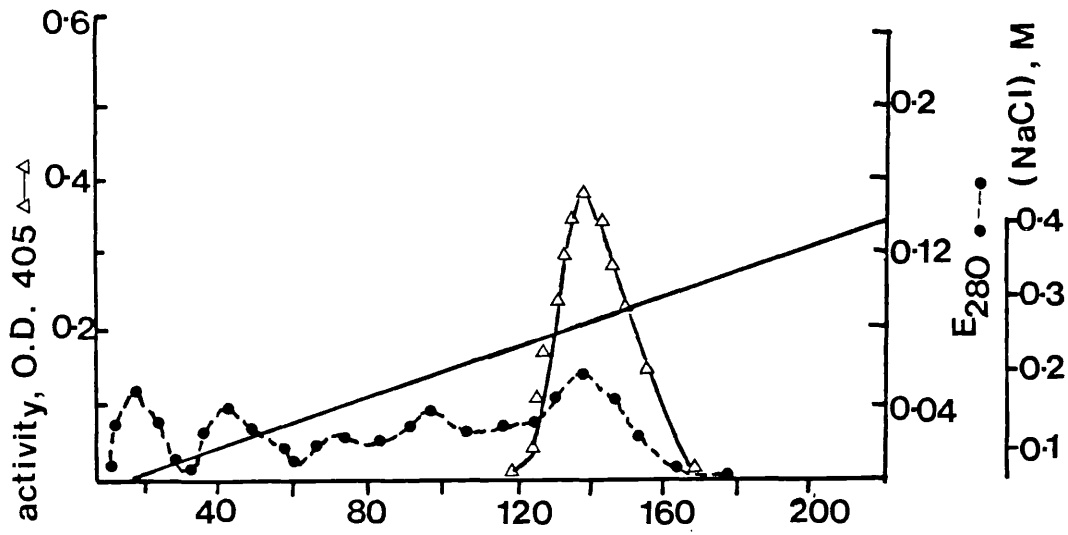
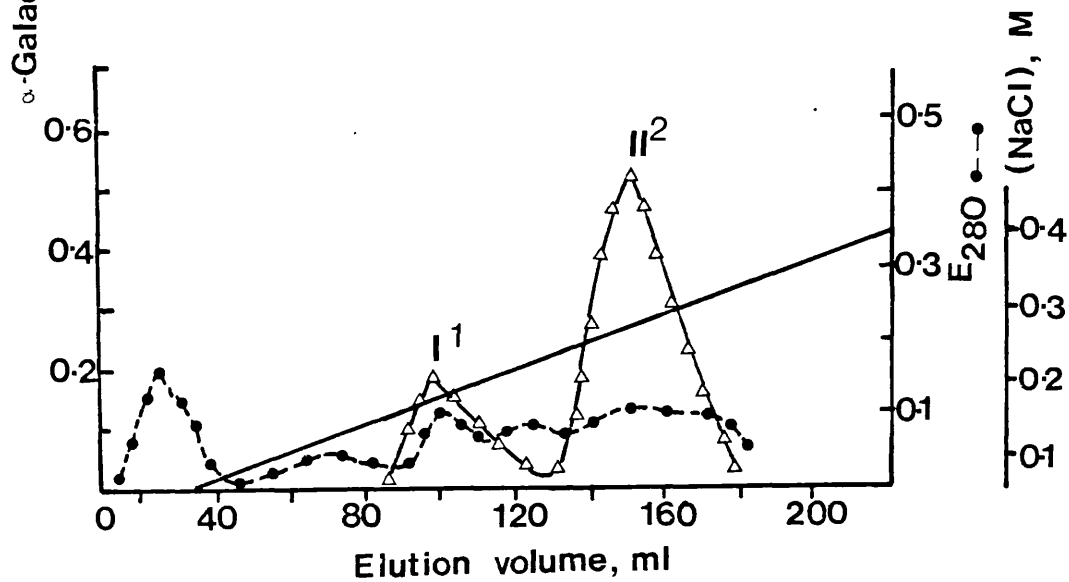


Fig. 21.2. CM-cellulose chromatography of  $\alpha$ -galactosidase II



II<sup>2</sup>, Fig. 21.2) and the specific activity of II<sup>2</sup> was twice that of II<sup>1</sup>. In terms of total activity, II<sup>2</sup> exceeds II<sup>1</sup> by a factor of 3 (Fig. 21.2), however this difference was found to be variable and was probably dependent on the batch of seeds being used.

It is interesting to note that  $\alpha$ -galactosidases I and II<sup>2</sup> elute at similar sodium chloride concentrations.

When II<sup>1</sup> and II<sup>2</sup> were recycled, separately through CM-cellulose columns to improve homogeneity, further purification was achieved and the specific activity ratio (II<sup>2</sup>/II<sup>1</sup>) was 1.5. The loss of total enzyme activity at the CM-cellulose stages was rather high; somewhat similar losses on CM-cellulose have also been reported for  $\alpha$ -galactosidase B from human liver (see Table V ref. 50).

#### 4.2 Electrophoretic examination of $\alpha$ -galactosidases

PAGE of the final preparations of  $\alpha$ -galactosidases I, II<sup>1</sup> and II<sup>2</sup> resulting from Stage 8 were carried out under anionic and cationic conditions. Under anionic conditions (219) with Tris buffer, pH 8.3, the electrophoretic mobilities of the three forms were zero. This result contrasts with earlier reports that purified  $\alpha$ -galactosidase I and fraction II from V. faba gave single bands on polyacrylamide gels using the same Tris-glycine buffer system (28). However, Hadocava and Benes (220) have

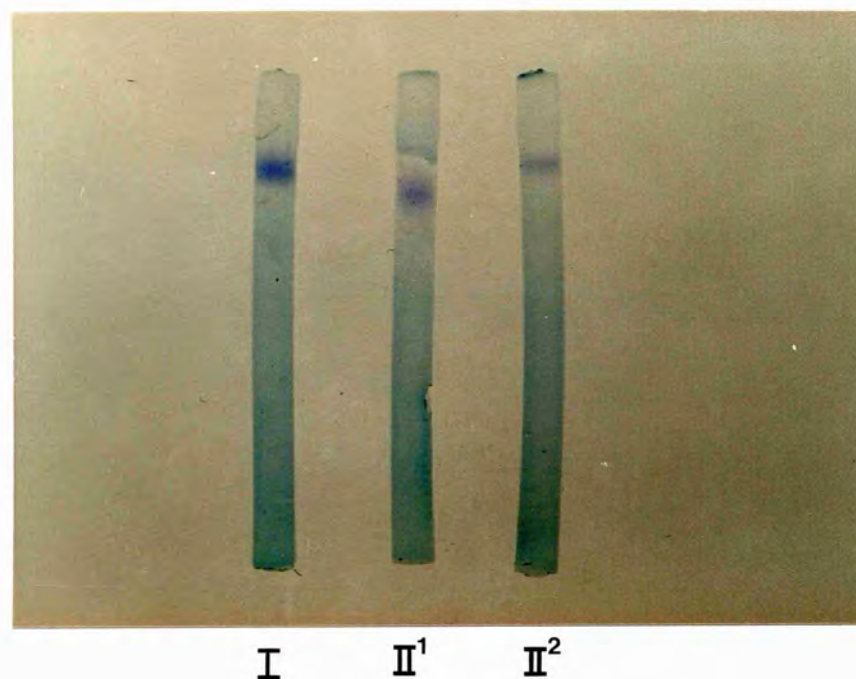
reported that this system was unsuitable for the resolution of multiple forms of  $\alpha$ -galactosidases from Zea mays and Vicia faba root tips.

PAGE of the V. faba enzymes with  $\beta$ -alanine-acetate buffer at pH 3.5, using the method recommended by Hadocava and Benes (220) resulted in all forms migrating towards the cathode as single bands (Figs. 22.1 and 22.2) which were stained for both protein and  $\alpha$ -galactosidase activity. The relative migrations of  $II^2$  and I on PAGE are similar, however  $II^1$  migrates faster. When  $\alpha$ -galactosidase II eluted from Con A-Sepharose (Stage 6, Table 5) was subjected to PAGE, it was resolved into two active bands corresponding to  $II^1$  and  $II^2$  (Fig. 22.2).

On PAGE, at pH 3.5, enzymes I and  $II^2$  appear less basic than  $II^1$ , yet they both elute at a higher salt concentration from CM-cellulose columns in comparison to  $II^1$ . This can be attributed to the fact that only a part of the charged regions of the enzyme interact with the ion-exchanger and that elution is not a function of the total charges on the proteins.

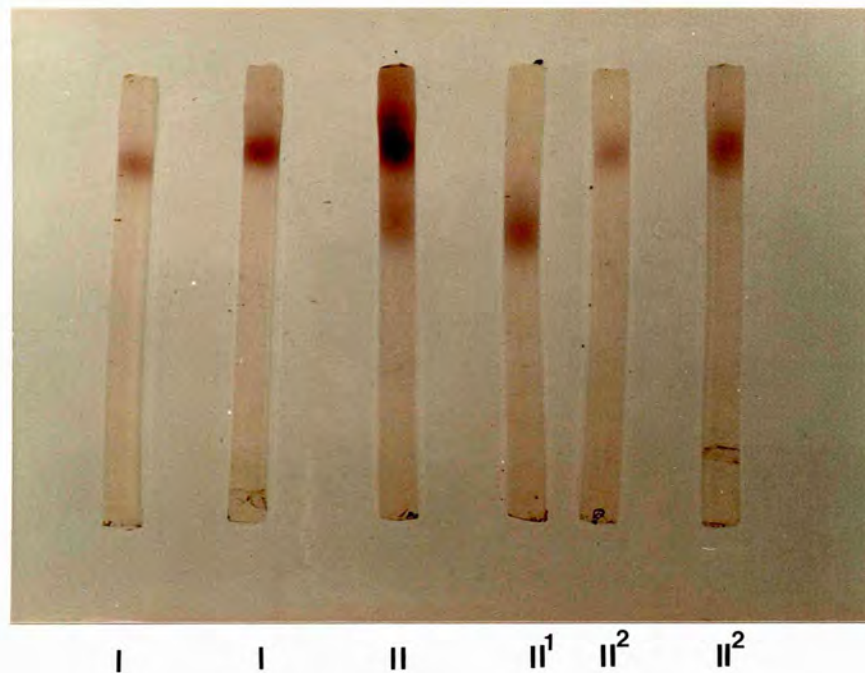
Some heterogeneity was observed when the purified enzyme samples were examined by SDS-PAGE: this was more evident on slab gels (Fig. 23.2) than on cylindrical gels (Fig. 23.1). Enzyme I dissociated into a major subunit with MW  $41,300 \pm 1200$  and two minor bands which had slightly higher mobilities. There was

Fig. 22.1 Polyacrylamide gel electrophoresis of  $\alpha$ -galactosidases I, II<sup>1</sup> and II<sup>2</sup> (stained with Coomassie blue)



Experimental details are given in section 2.4.1. 10, 21 and 26  $\mu$ g of purified (after Stage 8 of the purification procedure)  $\alpha$ -galactosidases I, II<sup>1</sup> and II<sup>2</sup>, respectively were applied on the gels and stained for protein as described in section 2.2.2.

Fig. 22.2 Polyacrylamide gel electrophoresis of  $\alpha$ -galactosidases (stained for enzyme activity)

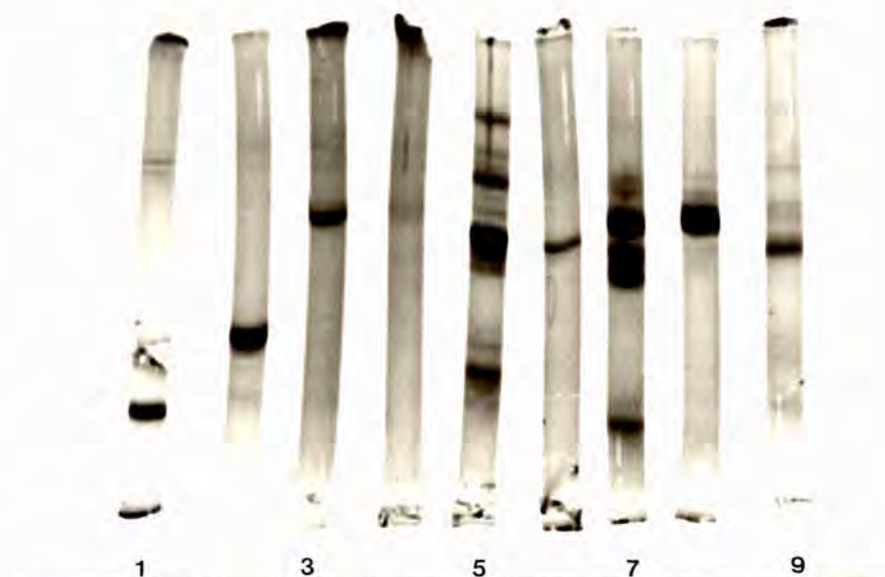


Experimental details are given in section 2.4.1.

5 and 10  $\mu$ l of I, 40  $\mu$ l of II<sup>1</sup> and 20 and 30  $\mu$ l of II<sup>2</sup> (after Stage 8 of the purification procedure) and 25  $\mu$ l of  $\alpha$ -galactosidase II (after Stage 6 of the purification procedure) were applied on the gels and stained for  $\alpha$ -galactosidase as described in section 2.1.2 ii.

Fig. 23 SDS-PAGE of  $\alpha$ -galactosidases I, II<sup>1</sup> and II<sup>2</sup>

Fig. 23.1 Disc gel electrophoresis



Experimental details are given in section 2.4.2. Gels 1 - 4 represent marker proteins;  $\beta$ -lactoglobulin (MW 18,400), trypsinogen (MW 24,000), pepsin (MW 34,700) and ovalbumin (MW 43,500). Gel 5. 20  $\mu$ g of  $\alpha$ -galactosidase I obtained from Stage 6 of the purification procedure. Gel 6. 10  $\mu$ g of  $\alpha$ -galactosidase I after Stage 7 of the purification procedure.

Gel 7. 20  $\mu$ g of  $\alpha$ -galactosidase II applied after Stage 6 of the purification procedure.

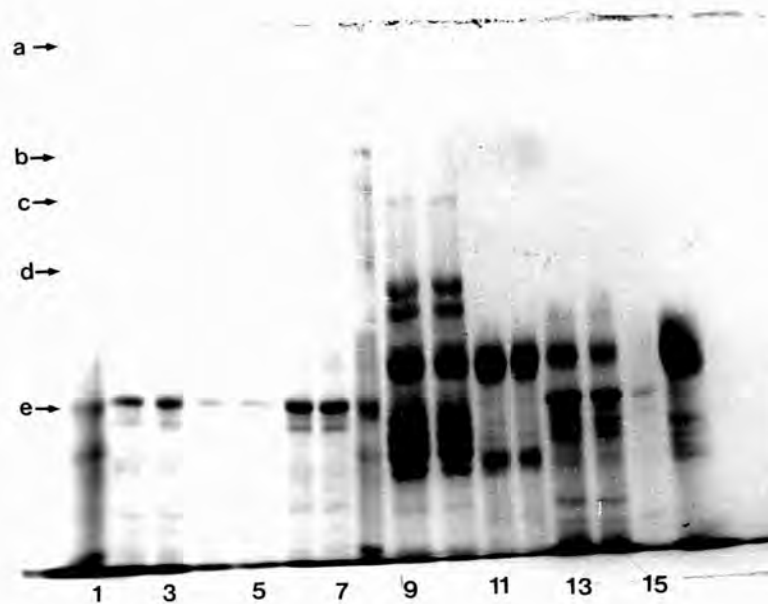
Gel 8. 20  $\mu$ g of  $\alpha$ -galactosidase II<sup>1</sup> applied after Stage 7 of the purification procedure.

Gel 9. 14  $\mu$ g of  $\alpha$ -galactosidase II<sup>2</sup> applied after Stage 7 of the purification procedure.

The gels were stained for protein as described in section 2.2.2.



Fig. 23.2. SDS-polyacrylamide slab gel electrophoresis of  $\alpha$ -galactosidases I, II<sup>1</sup> and II<sup>2</sup>



Experimental details are given in section 2.4.2.

Tracks 1 and 8 represent MW markers a) myosin, MW 200,000

b)  $\beta$ -galactosidase, MW 130,000 c) phosphorylase b, MW 90,000

d) bovine serum albumin, MW 65,000 e) ovalbumin, MW 45,000

Tracks 2 - 7 show varying amounts (10, 8 and 15  $\mu$ g) of

enzyme I, applied after Stage 7 of the purification procedure.

Tracks 9-10 show enzyme II applied after Stage 6.

Tracks 11-12 show enzyme II<sup>1</sup> (20  $\mu$ g) applied after Stage 7.

Tracks 13-14 show enzyme II<sup>2</sup> (15  $\mu$ g) applied after Stage 7.

Tracks 15-16 represent  $\alpha$ -galactosidases II<sup>2</sup> and II<sup>1</sup> after Stage 8; shown more clearly in Fig. 23.3.

Fig. 23.3 SDS-PAGE of  $\alpha$ -galactosidases I, II<sup>1</sup> and II<sup>2</sup> obtained from Stage 8 of the purification procedure



1.  $\alpha$ -Galactosidase II<sup>2</sup>
2.  $\alpha$ -Galactosidase II<sup>1</sup> and II<sup>2</sup>
3.  $\alpha$ -Galactosidase II
4.  $\alpha$ -Galactosidase I

clear evidence of microheterogeneity in the major subunit. The pattern for  $\alpha$ -galactosidase II<sup>2</sup> was identical to that of enzyme I (Fig. 23.3). The two minor protein bands are thought to be proteolytic degradation products (cf. ref. 221). Enzyme II<sup>1</sup> gave a major band exhibiting a MW of  $42,650 \pm 950$  with some faster migrating minor bands. The low carbohydrate content (2-5%) of these enzymes is unlikely to seriously interfere with estimations of MW by the SDS-PAGE method (222).

The purification of  $\alpha$ -galactosidases from various plant and animal sources is compared in Table 6. In the two cases cited in Table 6, the purified animal  $\alpha$ -galactosidases have very low specific activities. Inspection of the data for the higher plant enzyme preparations shows clearly that Coffea is an excellent source of high specific activity  $\alpha$ -galactosidases with acceptable recovery of total activities. A satisfactory comparison can be made of the present purification of  $\alpha$ -galactosidase I with the previous attempt by Dey and Pridham (28). In the former, the specific activity is almost doubled at the expense, however, of a significant loss of total activity (ca 90%). No comment can be made concerning II<sup>1</sup> and II<sup>2</sup> as fraction II was not resolved in the earlier investigation (28).

#### 4.3 Quaternary structure of the $\alpha$ -galactosidases

Comparison of the MWs of enzymes II<sup>2</sup> and I obtained by SDS-PAGE ( $41,300 \pm 1200$ ) and sedimentation equilibrium methods ( $43,390 \pm$

Table 6. Comparison of the specific activities and yields of some purified  $\alpha$ -galactosidases.

Source	Total enzyme activity (nkat)*	Total protein (mg)*	Specific activity (nkat/mg)	Purification (fold) <sup>+</sup>	Recovery (%)	Ref.
<i>Mortierella vinacea</i>	222,285	6.34	3515	17.0	11.8	32
Human plasma						
$\alpha$ -Galactosidase A	3.66	15.90	0.23	694	11.0	223
Mouse liver	836.9	98.0	8.54	464	40.0	43
<i>Prunus amygdalus</i>	329.6	1.34	246	2000	55.7	27
<i>Coffea canephora</i>						
I	2955.3	0.22	13,433	848	9.0	30
II	5866.6	0.16	36,666	2311	19.0	
<i>Lens esculenta</i>						
I	3150	4.2	750	4090	27	65
<i>Vicia faba</i>						
I	982.2	1.34	733	3660	8.7	28
II	821.4	12.08	68	337	7.2	
<i>Vicia faba</i>						
I	180	0.14	1286	6430	1.1	Present
II <sup>1</sup>	59.3	0.78	76	380	0.37	
II <sup>2</sup>	71.2	0.63	113	565	0.44	Study

+ Purification relative to the crude extract.

\* Total protein and enzyme activity per Kg tissue.

1409 and  $160,400 \pm 2850$ , ref. 69), strongly suggests that  $\alpha$ -galactosidase I is a tetramer of  $II^2$ . The tetrameric nature of V. faba enzyme I would appear to be similar to  $\alpha$ -galactosidases extracted from a number of other leguminous plants (26, 57, 170, 172). Mung bean  $\alpha$ -galactosidase, for example, is reported to be a tetramer with MW 160,000 which on SDS-PAGE gives a single protein band with  $MW 45,000 \pm 2500$  (170). In the case of  $\alpha$ -galactosidases from broad bean, although two low MW forms  $II^1$  and  $II^2$  are present, only  $II^2$  seems to aggregate to form I. In mung bean (170), only one low MW form has been reported. It should be noted, however, that imbibed mung beans were used as the source of the  $\alpha$ -galactosidases whereas in the present study, resting broad beans were used and the pattern of multiple enzyme forms may be different in the two seeds because of the different physiological states of the tissues.

Del-Campillo (224), has shown that soybean seed  $\alpha$ -galactosidase (MW 160,000) dissociates in the presence of SDS into two sub units with MWs 38,000 and 40,000.

Purified S-carboxymethylated  $\alpha$ -galactosidase A from human placenta (221) showed one component with MW 65,000 when examined by SDS-PAGE but electrophoresis of the non-S-carboxymethylated enzyme showed two components:- a major band of MW 67,500 and a diffuse band with MW 47,000. It was suggested that the smaller component was a degradation product and that the enzyme is a dimer with a MW of approximately 150,000.

## 5 Immunochemical Studies

Further information on the structural relationships between the three forms of  $\alpha$ -galactosidase from broad bean was obtained by immunological studies.

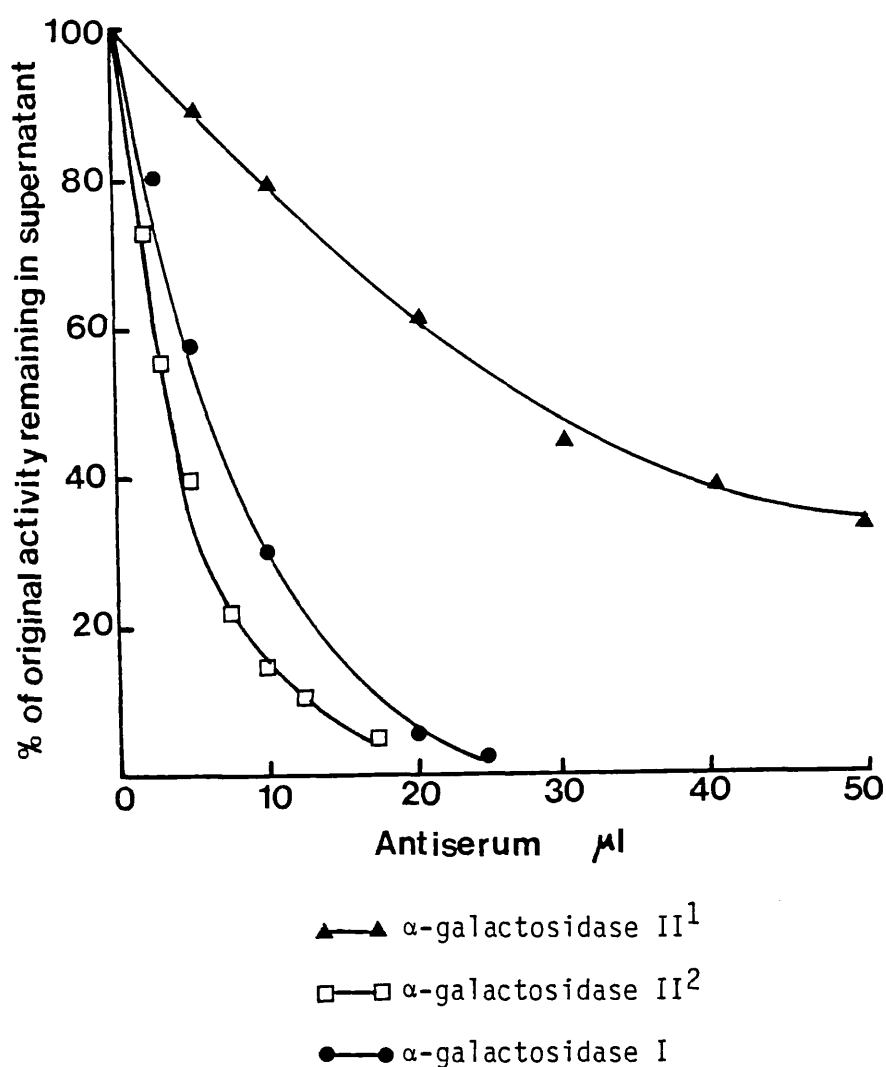
Antibodies to purified I, II<sup>1</sup> and II<sup>2</sup> were raised by separate, multiple subcutaneous injections of the enzymes mixed with Freund's adjuvant into rabbits. The blood serum in each case was used as the source of antibodies without further purification and each anti-( $\alpha$ -galactosidase) serum was subjected to immunoprecipitation, immunoelectrophoresis and immunodiffusion using all three antigens.

### 5.1 Quantitative Immunoprecipitation

Figure 24 shows the results of immunoprecipitation analyses where varying quantities of antisera were added to constant amounts of enzyme activity and after incubation and centrifugation, the activities of the supernatants were assayed. Anti- $\alpha$ -galactosidase I reacted with all forms (I, II<sup>1</sup> and II<sup>2</sup>) of the enzyme producing precipitation of activity, the maximum reaction occurring between anti-I and enzyme I, as expected (Fig. 24.1). The reaction between anti-I antiserum and enzyme II<sup>2</sup> was found to be stronger than that between anti-I and enzyme II<sup>1</sup>. Precipitation reactions between anti-II<sup>2</sup> and anti-II<sup>1</sup> sera

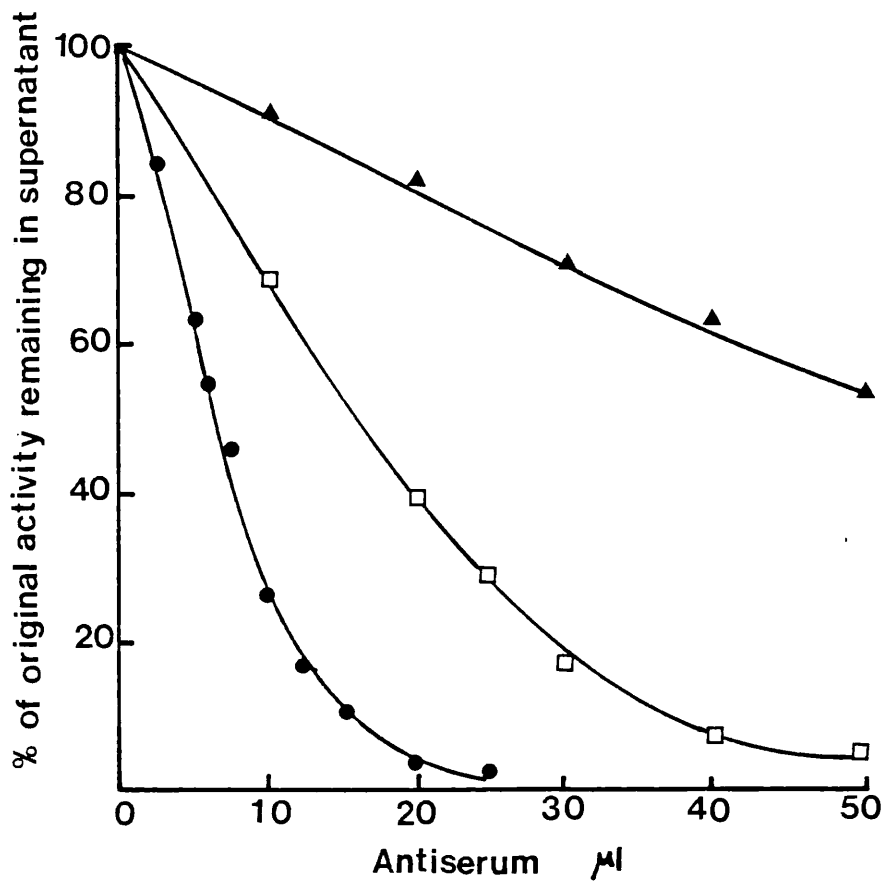
Fig. 24. Immunoprecipitation of  $\alpha$ -galactosidases

Fig. 24.1. Immunoprecipitation of  $\alpha$ -galactosidases I, II<sup>1</sup> and II<sup>2</sup> with anti-( $\alpha$ -galactosidase II<sup>2</sup>) serum.



Increasing volumes of antiserum were added to tubes containing purified preparations of  $\alpha$ -galactosidases I, II<sup>1</sup> and II<sup>2</sup> from Stage 8 of the purification procedure. After incubation and centrifugation as described in section 2.6.2.,  $\alpha$ -galactosidase activity was determined for each of the supernatants. The points shown represent means of duplicate incubations.

Fig. 24.2. Immunoprecipitation of  $\alpha$ -galactosidases I, II<sup>1</sup> and II<sup>2</sup> with anti-( $\alpha$ -galactosidase I) serum.

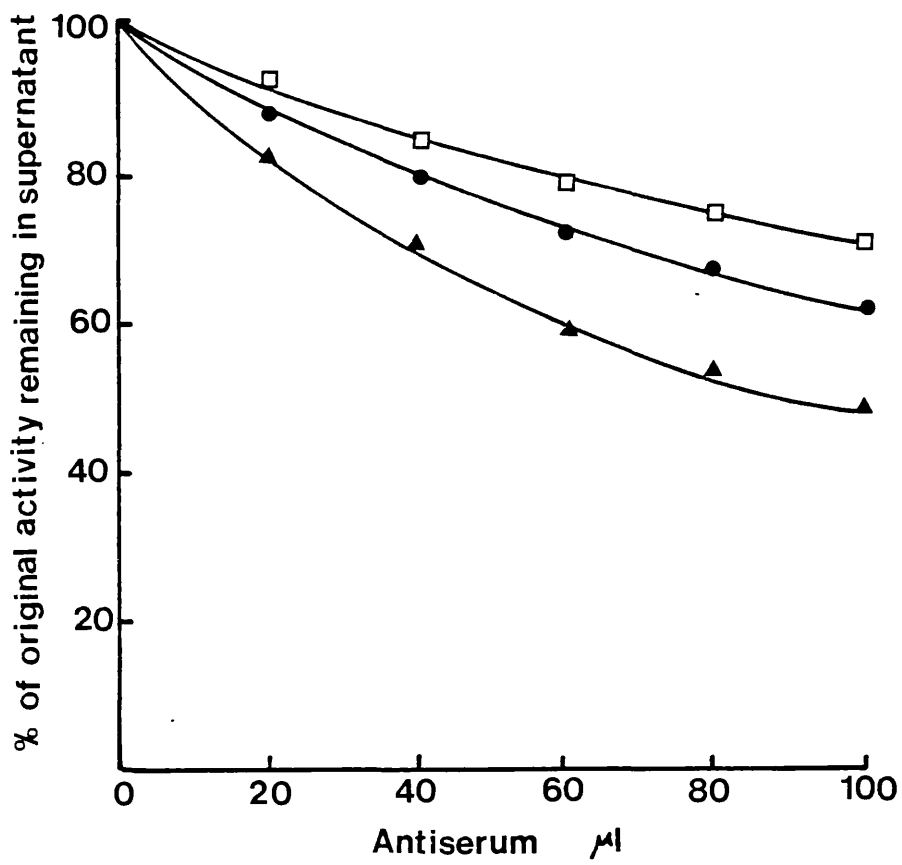


- ▲—▲  $\alpha$ -galactosidase II<sup>1</sup>
- $\alpha$ -galactosidase I
- $\alpha$ -galactosidase II<sup>2</sup>

Details as for Fig. 24.1. except that anti-( $\alpha$ -galactosidase I) serum was used.



Fig. 24.3. Immunoprecipitation of  $\alpha$ -galactosidases I, II<sup>1</sup> and II<sup>2</sup> with anti-( $\alpha$ -galactosidase II<sup>1</sup>) serum.



- $\alpha$ -galactosidase I
- $\alpha$ -galactosidase II<sup>2</sup>
- ▲—▲  $\alpha$ -galactosidase II<sup>1</sup>

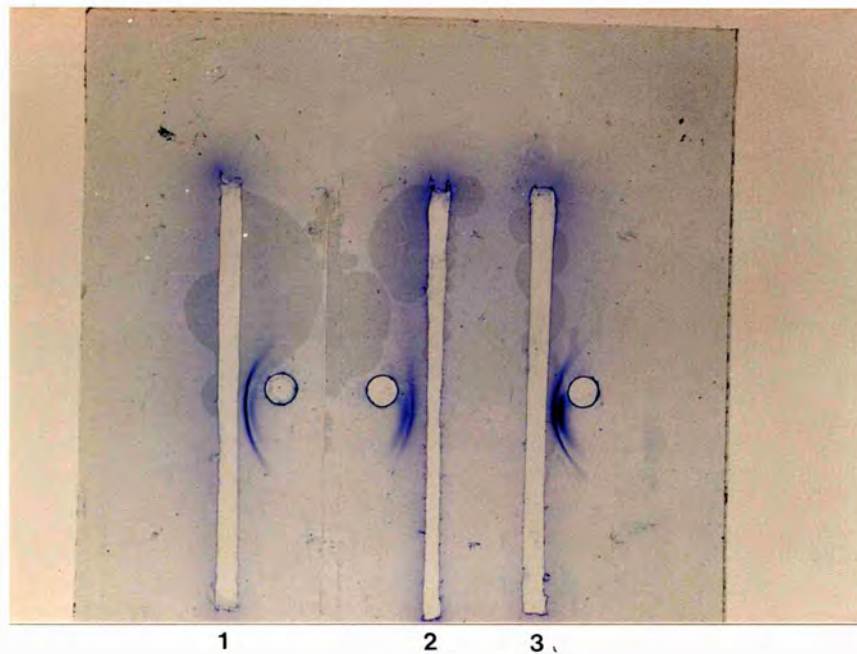
Details as for Fig. 24.1. except that anti-( $\alpha$ -galactosidase II<sup>1</sup>) serum was used.

and the three forms of  $\alpha$ -galactosidase were also studied (Figs. 24.2 and 24.3). As with anti- $\alpha$ -galactosidase I, cross reaction was observed between anti-II<sup>1</sup> and anti-II<sup>2</sup> and  $\alpha$ -galactosidases I, II<sup>1</sup> and II<sup>2</sup>. From Fig. 24.3 however, it seems that II<sup>1</sup> is a relatively poor immunogenic antigen: only 50% of the enzyme II<sup>1</sup> activity was precipitated with 100  $\mu$ l of anti-II<sup>1</sup> antiserum (cf. the titres in Figs. 24.1 and 24.2). From the gradients of the curves for each antiserum, it would appear that  $\alpha$ -galactosidases I and II<sup>2</sup> are more closely related than  $\alpha$ -galactosidases I and II<sup>1</sup>. This supports the evidence from the SDS-PAGE study that  $\alpha$ -galactosidase I is a tetramer of II<sup>2</sup>.

## 5.2 Immuno-electrophoresis

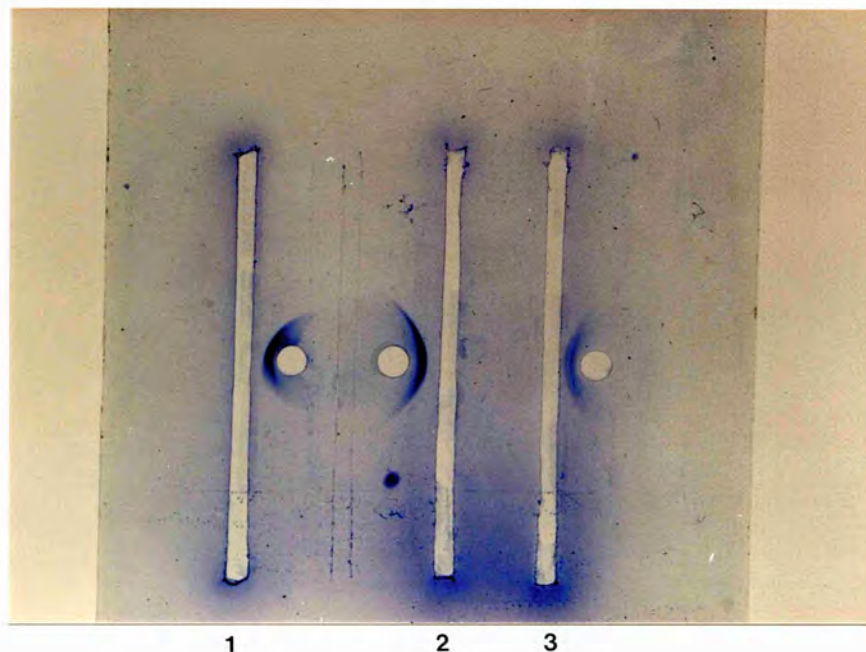
Immuno-electrophoretic studies again showed that the three antisera reacted with all the forms of the enzyme. When Coomassie blue was used to stain the gels for protein (Figs. 25.1, 25.2 and 25.3), multiple precipitation arcs were observed showing that the antisera were not monospecific. This was expected in view of the heterogeneity of the antigens. Single arcs were obtained when the gels were stained with 4-methylumbelliferyl  $\alpha$ -D-galactoside and observations made under uv light (Fig. 26).

Fig. 25.1 Immunelectrophoresis of  $\alpha$ -galactosidase I  
(stained with Coomassie blue)



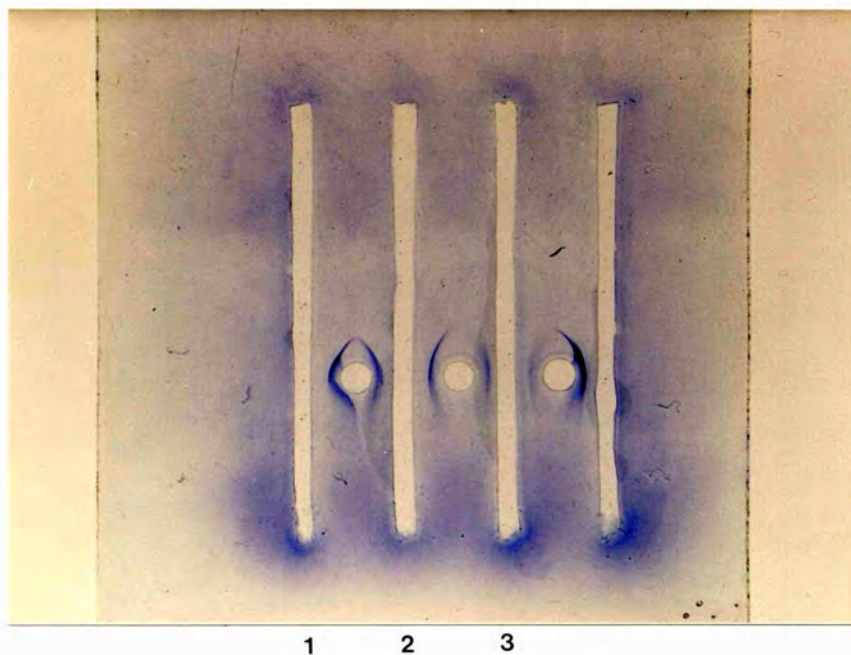
5  $\mu$ l of purified  $\alpha$ -galactosidase I (after Stage 8 of the purification procedure) were applied to the antigen wells and 100  $\mu$ l of anti-I, anti-II<sup>1</sup> and anti-II<sup>2</sup> antisera were placed in troughs 1, 2 and 3 respectively. Experimental details are given in section 2.6.4.

Fig. 25.2      Immunoelectrophoresis of  $\alpha$ -galactosidase II<sup>1</sup>  
(stained with Coomassie blue)



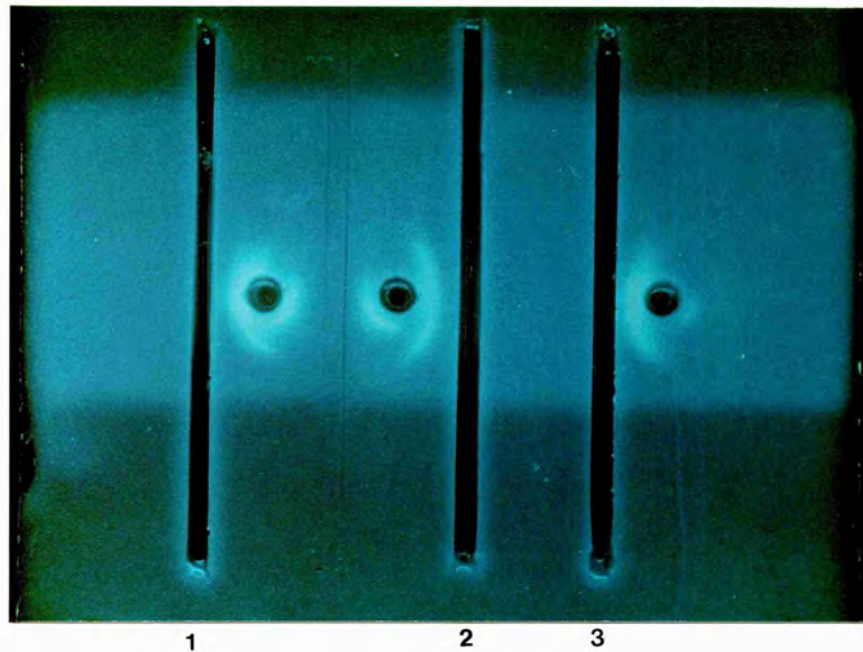
5  $\mu$ l of purified  $\alpha$ -galactosidase II<sup>1</sup> (after Stage 8 of the purification stage) were applied to the antigen wells and 100  $\mu$ l of anti-II<sup>1</sup>, anti-I and anti-II<sup>2</sup> antisera were placed in troughs 1, 2 and 3 respectively.

Fig. 25.3      Immunoelectrophoresis of  $\alpha$ -galactosidase II<sup>2</sup>  
(stained with Coomassie blue)



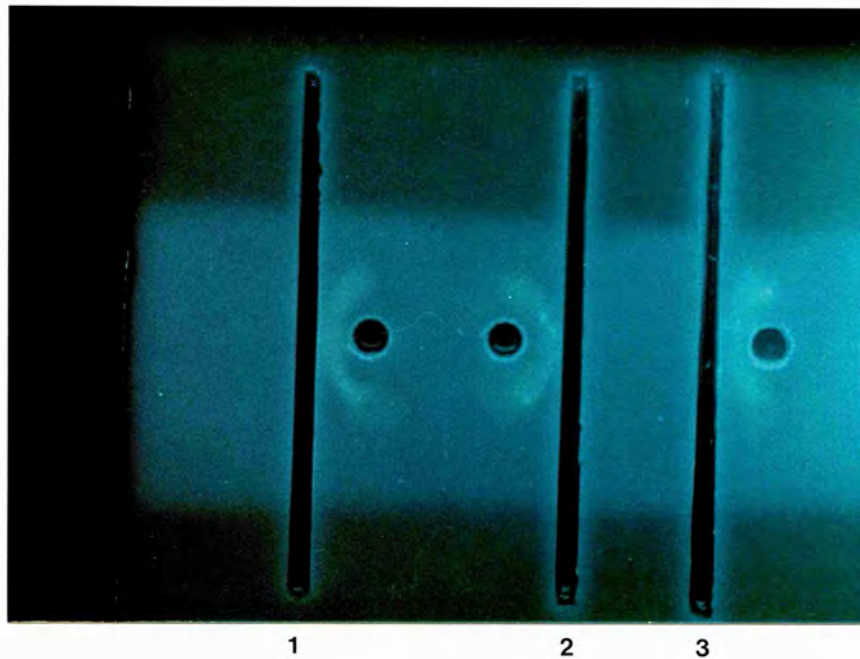
12  $\mu$ l of purified  $\alpha$ -galactosidase II<sup>2</sup> (after Stage 8 of the purification procedure) were applied to the antigen wells and 100  $\mu$ l of anti-II<sup>2</sup>, anti-I and anti-II<sup>1</sup> antisera were placed in troughs 1, 2 and 3 respectively.

Fig. 26.1 Immuno-electrophoresis of  $\alpha$ -galactosidase I  
(stained for enzyme activity)



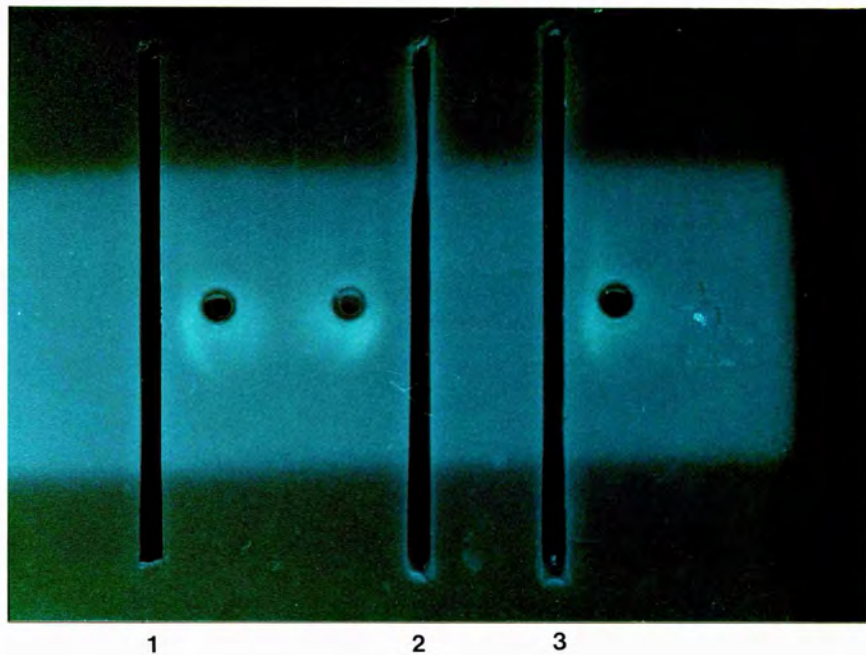
The antigen wells contained  $12_{\mu}$  l of purified  $\alpha$ -galactosidase I and troughs 1, 2 and 3 contained  $100_{\mu}$  l of anti-I, anti-II<sup>2</sup> and anti-II<sup>1</sup> antisera, respectively. The slide was stained for  $\alpha$ -galactosidase activity as described in section 2.1.2 ii.

Fig. 26.2 Immunelectrophoresis of  $\alpha$ -galactosidase II<sup>1</sup>  
(stained for enzyme activity)



The antigen wells contained 60  $\mu$ l of purified  $\alpha$ -galactosidase II<sup>1</sup> and troughs 1, 2 and 3 contained 100  $\mu$ l of anti-II<sup>1</sup>, anti-I and anti-II<sup>2</sup> antisera, respectively. The slide was stained for  $\alpha$ -galactosidase activity.

Fig. 26.3 Immunelectrophoresis of  $\alpha$ -galactosidase II<sup>2</sup>  
(stained for enzyme activity)



The antigen wells contained 20  $\mu$ l of purified  $\alpha$ -galactosidase II<sup>2</sup> and troughs 1, 2 and 3 contained 100  $\mu$ l of anti-II<sup>2</sup>, anti-I and anti-II<sup>1</sup>, respectively.



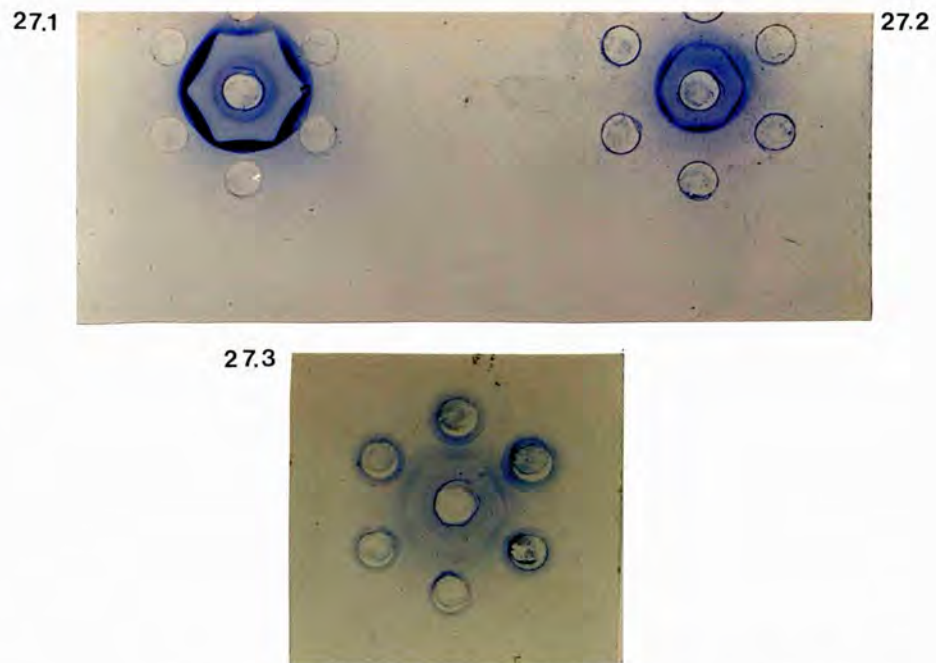
### 5.3 Immunodiffusion

Double diffusion of purified  $\alpha$ -galactosidases I, II<sup>1</sup> and II<sup>2</sup> against their respective antisera, using varying amounts of enzyme gave precipitation lines even when small quantities of enzyme (1-2  $\mu$ g protein) were used (Figs. 27 and 28). The reaction between anti-II<sup>2</sup> serum and enzymes II<sup>1</sup>, II<sup>2</sup> and I is shown in Fig. 29 where a protein stain has been used. The precipitin lines for enzymes I and II<sup>1</sup> appear to form a spur showing partial identity. However, since multiple precipitin lines were produced, it is impossible to judge which lines are due to the enzymic protein.

With anti-II<sup>2</sup> serum placed in the antiserum well and enzymes I and II<sup>2</sup> in adjacent wells, merged precipitin lines (enzyme stain) were observed indicating a reaction of complete identity between the two forms (Fig. 30). Similar results were obtained when anti-I antiserum and enzymes I and II<sup>2</sup> were allowed to diffuse (Fig. 31).

With anti-I in the antiserum well and enzymes I and II<sup>1</sup> in adjacent wells, a spur (enzyme stain) was formed suggesting partial identity between the two antigens (Fig. 32).

Fig. 27 Immunodiffusion of  $\alpha$ -galactosidases I, II<sup>1</sup> and II<sup>2</sup> (stained with Coomassie blue)



Experimental details are given in section 2.6.3.

- Fig. 27.1 The centre well contained anti-I serum. The top well contained 2  $\mu$ l of purified enzyme I. Continuing in a clockwise direction, the other wells contained 4, 5, 7, 8 and 10  $\mu$ l of the enzyme.
- Fig. 27.2 The centre well contained anti-II<sup>1</sup> serum. The surrounding wells contained 2, 4, 5, 7, 8 and 10  $\mu$ l of purified enzyme II<sup>1</sup> in the same order as described above.
- Fig. 27.3 The centre well contained anti-II<sup>2</sup> serum. The surrounding wells, starting from the top one contained 3, 5, 8, 10, 15 and 8  $\mu$ l of purified enzyme II<sup>2</sup>.

Fig. 28 Immunodiffusion of  $\alpha$ -galactosidases I and II<sup>2</sup>  
(stained for enzyme activity)

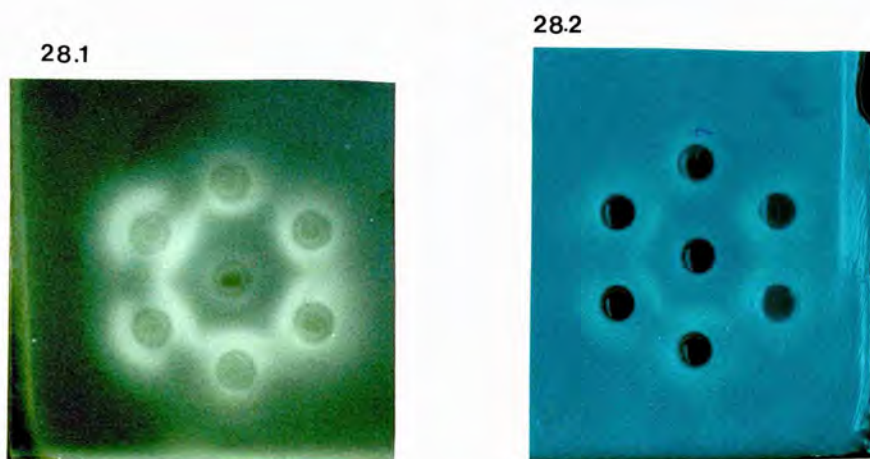
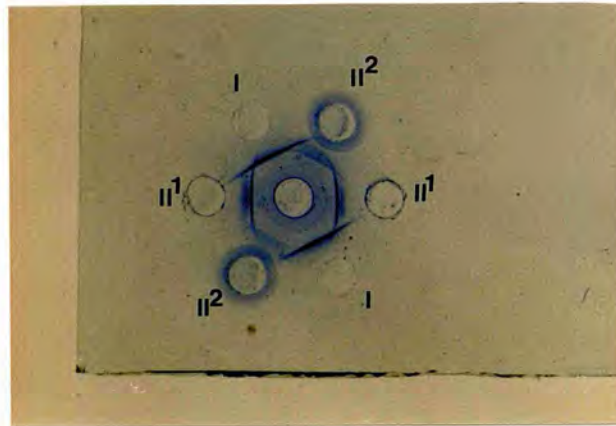


Fig. 28.1 The centre well contained anti-I serum. The top well contained 3  $\mu$ l of purified  $\alpha$ -galactosidase I. Continuing in a clockwise direction, the other wells contained 5, 7, 8, 10 and 12  $\mu$ l of the enzyme.

Fig. 28.2 The centre well contained anti-II<sup>2</sup> serum. Starting from well 1 and continuing in a clockwise direction, the wells contained 2, 4, 6, 8, 10 and 12  $\mu$ l of  $\alpha$ -galactosidase II<sup>2</sup>.

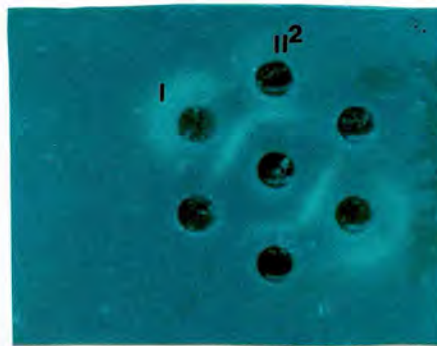
Fig. 29 Immunodiffusion of  $\alpha$ -galactosidases I, II<sup>1</sup> and II<sup>2</sup> with anti-( $\alpha$ -galactosidase II<sup>2</sup>) serum.



Anti-II<sup>2</sup> antiserum was placed in the centre well and 6, 10 and 15  $\mu$ l of purified enzymes II<sup>1</sup>, I and II<sup>2</sup> respectively were put in the surrounding wells as shown above.

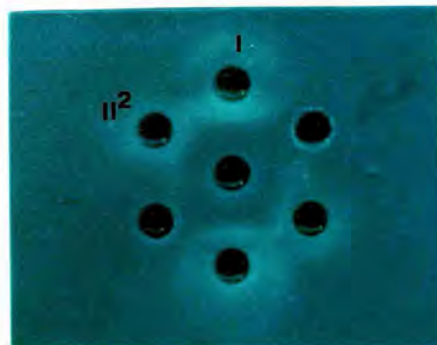
Immunodiffusion was performed as described in section 2.6.3 and the gel was stained for protein.

Fig. 30 Immunodiffusion of  $\alpha$ -galactosidases I and II<sup>2</sup> with anti-( $\alpha$ -galactosidase II<sup>2</sup>) antiserum



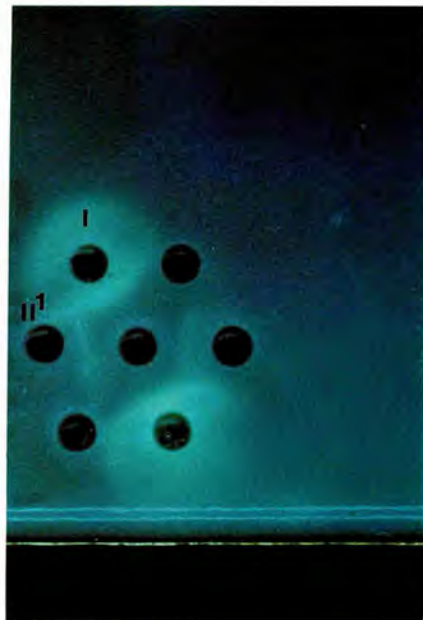
Anti-II<sup>2</sup> serum was placed in the centre well and 10 to 15  $\mu$ l of  $\alpha$ -galactosidases I and II<sup>2</sup> were put in adjacent wells as shown above.

Fig. 31 Immunodiffusion of  $\alpha$ -galactosidases I and II<sup>2</sup> with anti-( $\alpha$ -galactosidase I) antiserum



Anti-I serum was placed in the centre well and 10 and 15  $\mu$ l of  $\alpha$ -galactosidases I and II<sup>2</sup> were put in adjacent wells as shown in the figure. Immunodiffusion was carried out as described in section 2.6.3. and the gels stained for  $\alpha$ -galactosidase activity as described in section 2.1.2i.

Fig. 32 Immunodiffusion of  $\alpha$ -galactosidases I and II<sup>1</sup> with anti-( $\alpha$ -galactosidase II<sup>1</sup>) anti-serum



Anti-II<sup>1</sup> serum, was placed in the centre well. 15 and 20  $\mu$ l of  $\alpha$ -galactosidases I and II<sup>1</sup> were placed in adjacent wells as shown in the figure. Immunodiffusion was carried out as described in section 2.6.3. The gel was stained for  $\alpha$ -galactosidase activity.

## 6 Carbohydrate and amino acid compositions of $\alpha$ -galactosidases I, II<sup>1</sup> and II<sup>2</sup>

### 6.1 Carbohydrate analysis

The results of the neutral sugar analysis by methanolysis and trimethylsilylation and g.l.c. and amino sugar analysis using an amino acid analyser are given in Table 7. Enzymes I, II<sup>1</sup> and II<sup>2</sup> contain 2.7%, 2.1% and 4.4% total carbohydrate respectively. All forms contained xylose, mannose, glucose and glucosamine and in all cases, the ratio of glucosamine to mannose is the same (ca. 0.3). Mannose and glucosamine are commonly part of the core structure of plant glycoproteins. However the results presented here do not suggest any structural similarity between the oligosaccharide chains on the *V. faba* enzymes and the mannose rich glycan moieties of other plant glycoproteins such as soybean agglutinin (225, 226). Lis and Sharon (225) have demonstrated the presence in soybean agglutinin of the branched core:



This structure had previously been found in many animal, fungal and yeast glycoproteins (see ref. 225).

Xylose, which is commonly found in glycosaminoglycans from animals is not uncommon in plant glycoproteins, for example

Table 7. Carbohydrate content of  $\alpha$ -galactosidases I, II<sup>1</sup> and II<sup>2</sup>.

Enzyme	Sugars present (mol/mol protein)				Total carbohydrate (%) (w/w)
	Xylose	Mannose	Glucose	Glucosamine	
I	2.7	6.7	13.0	2.1	
	(0.25)*	(0.75)	(1.5)	(0.23)	2.7
II <sup>1</sup>	0.9	2.3	1.5	0.7	
	(0.28)	(0.9)	(0.6)	(0.27)	2.1
II <sup>2</sup>	1.9	3.2	5.1	0.94	
	(0.65)	(1.3)	(2.1)	(0.39)	4.4

\* The figures in brackets represent the % of each sugar present.

The results have been calculated using MWs 160,400, 45,730 and 43,390 for I, II<sup>1</sup> and II<sup>2</sup> respectively.



pineapple stem bromelain and various lectins (see ref. 188).

The significance of glucose which was found in the broad bean enzyme preparations is not clear; it may be a contaminant (202), perhaps starch which could be bound as a result of the lectin properties of the enzymes (see p.41). However, glucose has been reported as a glycan component of plant glycoproteins (188). If one disregards the glucose content, then, on a mol/mol basis, form I possesses twice as much total carbohydrate as II<sup>2</sup>. This could mean that the II<sup>2</sup> subunits that constitute enzyme I have, on average, a higher degree of glycosylation and that the association process 'selects' monomers with a high carbohydrate content. On a molar basis, the individual monosaccharides present are also greater in enzyme I than in enzyme II<sup>2</sup>.

It must be remembered that glycoprotein analysis is normally complicated by the fact that the carbohydrate side chains of a single glycoprotein, even when carefully isolated and purified from a genetically pure organism, are not identical in all the molecules of the glycoprotein. This microheterogeneity may arise either because of an unfinished biosynthetic sequence or a post-biosynthetic degradation of completed carbohydrate chains. Attachment of carbohydrate side chains is initiated through the action of glycosyl transferases capable of incorporating monosaccharides into the side chains of peptide-bound amino acids. Since the substrate specificities of glycosyl transferases are relative rather than absolute, the assembly of

oligosaccharide residues may be subject to error (227).

For unequivocal demonstration that a protein is indeed a glycoprotein, it is necessary to isolate from a partial hydrolysate, a low MW glycopeptide containing both amino acids and carbohydrate residues. However, where satisfactory evidence of the homogeneity of the glycoprotein is available, it is normally assumed that the carbohydrate is an integral part of the molecule and covalently linked to the polypeptide chain (227). For the preparation of the glycopeptide at least 5 mg of purified protein is needed and in the present study, this was not achieved. Hence further analysis and confirmation of the glycoprotein structures in the case of the three  $\alpha$ -galactosidases from Vicia faba seeds was not possible.

## 6.2 Amino acid analysis

The results of the amino acid analyses of  $\alpha$ -galactosidases I, II<sup>1</sup> and II<sup>2</sup> are presented in Table 8. The amino acid content has been calculated on the basis of the MWs of the native enzymes obtained by the sedimentation equilibrium method (69). The data obtained for the three forms shows that there do not appear to be any unusual features in their amino acid compositions. We have shown that form I is likely to be a tetramer of II<sup>2</sup> and, hence, on a molar basis enzymes I and II<sup>2</sup> should have similar amino acid contents. Although there are many similarities between these

Table 8. Amino acid composition of  $\alpha$ -galactosidases I, II<sup>1</sup> and II<sup>2</sup>.

	Amino acid content (mol/mol)			
	I		II <sup>1</sup>	II <sup>2</sup>
Asp	124	31 <sup>+</sup>	39	30
Thr	57	14	22	21
Ser	122	30.5	35	36
Glu	113	28	31	26
Pro	67	16.8	26	17
Gly	135	33.8	39	32
Ala	90	22.5	24	23
Cys*	Present		9	9
Val	83	20.8	29	19
Met	6	1.5	1.4	1.7
Ile	63	15.8	17	19
Leu	112	28	33	34
Tyr	36	9	11	11
Phe	51	12.8	16	22
His	22	5.5	5	5
Lys	58	14.5	16	18
Arg	57	14.21	14	19

The amino acid values are calculated as molar proportions using MWs 160,400, 45,750 and 43,390 for I, II<sup>1</sup> and II<sup>2</sup> respectively.

<sup>+</sup> represents the value for the subunit assuming that enzyme I is a tetramer.

\* Cysteine has been determined separately as cysteic acid after hydrolysis of a performic acid-oxidized sample. Cysteine was found to be present in enzyme I but the value was not calculated since the resolution of the peak from the analyser was poor.

two enzymes, anomalies do occur particularly in the case of arginine, leucine, phenylalanine, serine and threonine. These may result at least in part from the heterogeneity of the preparations which was observed by SDS-PAGE (see p.104).

A comparison of the amino acid contents of  $\alpha$ -galactosidases II<sup>1</sup> and II<sup>2</sup> shows that the former is relatively rich in the aspartic and glutamic acids and contains less arginine: this would account for the greater acidity of II<sup>1</sup> compared with II<sup>2</sup> (Fig. 21.2). Enzyme II<sup>1</sup> also appears to contain more proline, glycine and valine and less phenylalanine than II<sup>2</sup>.

**7 The nature of the  $\alpha$ -galactosidase activities, I<sub>ub</sub>, II<sup>1</sup><sub>ub</sub> and II<sup>2</sup><sub>ub</sub>, which are not bound by Con A-Sepharose**

When it was discovered (p.94) that some  $\alpha$ -galactosidase activity had little or no affinity for Con A, it was first thought that the unbound fractions were a result of overloading the columns. This was, therefore, checked by applying varying quantities of the isolated unbound enzymes to the column and measuring bound and unbound activity. Table 9 shows that irrespective of the amount of sample applied, very little enzyme was retained by the column and hence, overloading was not the cause of non-binding.

**Table 9. Effect of applying varying amounts of isolated I<sub>ub</sub> and II<sub>ub</sub> (fractions not bound by Con A-Sepharose) to Con A-Sepharose columns.**

Sample	Enzyme applied (OD units) <sup>+</sup>	Protein applied (mg)	UB* (OD units)	B# (OD units)	Activity bound %
Enzyme I <sub>ub</sub>	130	27.5	119	6	5
	780	82.5	725	20	2.5
	1300	137.5	1200	12	1
Enzyme II <sub>ub</sub>	260	74	185	7	2.6
	520	148	484	25	4.8

\* Unbound activity was obtained by eluting the column with 0.1 M potassium phosphate buffer, pH 7.0.

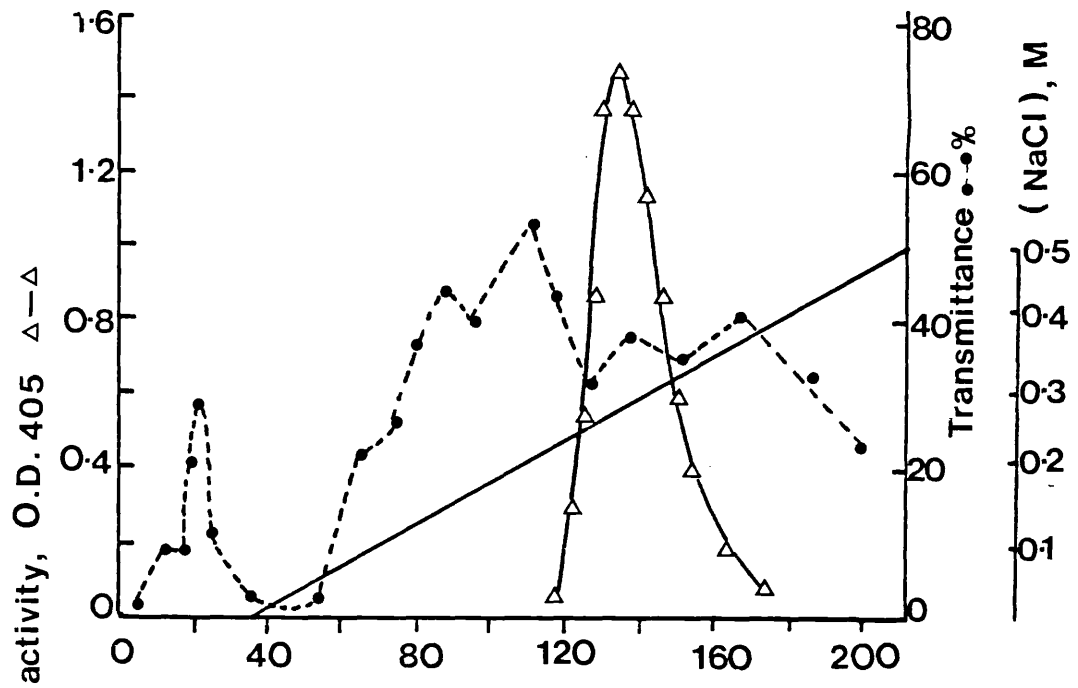
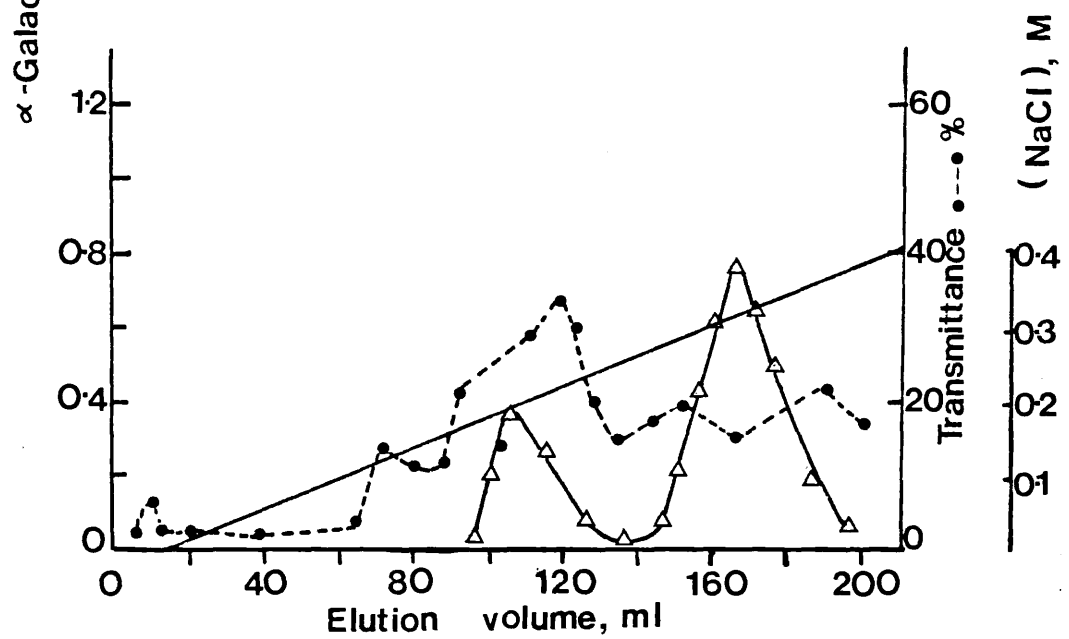
# Bound activity was eluted with 0.5 M methyl  $\alpha$ -D mannoside in 0.1 M potassium phosphate buffer, pH 7.0.

+ Absorbance at 405 nm.

Binding to Con A is dependent on the presence of glucose or mannose residues (228). Mattila (229) has used Con A-Sepharose to separate glycoproteins which exhibit heterogeneity in their glycan composition. The possibility that the bound and unbound  $\alpha$ -galactosidases had different degrees of glycosylation was therefore examined by analysing their carbohydrate contents.

Firstly, however, attempts were made to further purify the unbound fractions by CM-cellulose ion-exchange chromatography. The elution profiles for  $I_{ub}$ ,  $II^1_{ub}$  and  $II^2_{ub}$  are shown in Figs. 33.1 and 33.2. It is interesting to note that all three forms elute at a much higher concentration in comparison with their bound counterparts, suggesting they were less acidic (see Figs. 21.1 and 21.2). However, as with bound I and  $II^2$ ,  $I_{ub}$  and  $II^2_{ub}$  elute at similar sodium chloride concentrations. The three forms showed the same PAGE mobilities as the corresponding bound forms.

After recycling through CM-cellulose, carbohydrate analyses of the three Con A unbound enzymes was carried out. Table 10 shows that in comparison with the corresponding bound forms, there were quantitative differences. The total carbohydrate contents of  $I_{ub}$  and  $II^1_{ub}$  were higher than those for the bound  $\alpha$ -galactosidases I and  $II^1$  whereas bound  $II^2$  contained approximately four times more carbohydrate than  $II^2_{ub}$ . Despite recycling through the ion-exchange column, all three unbound enzyme preparations were very heterogeneous (SDS-PAGE Fig. 34) and

Fig. 33 Purification of  $\alpha$ -galactosidases on ion-exchange columnsFig. 33.1 CM-cellulose chromatography of  $\alpha$ -galactosidase I<sub>Ub</sub>Fig. 33.2 CM-cellulose chromatography of  $\alpha$ -galactosidase II<sub>Ub</sub>

**Table 10. Carbohydrate content of unbound and bound  $\alpha$ -galactosidases I, II<sup>1</sup> and II<sup>2</sup>.**

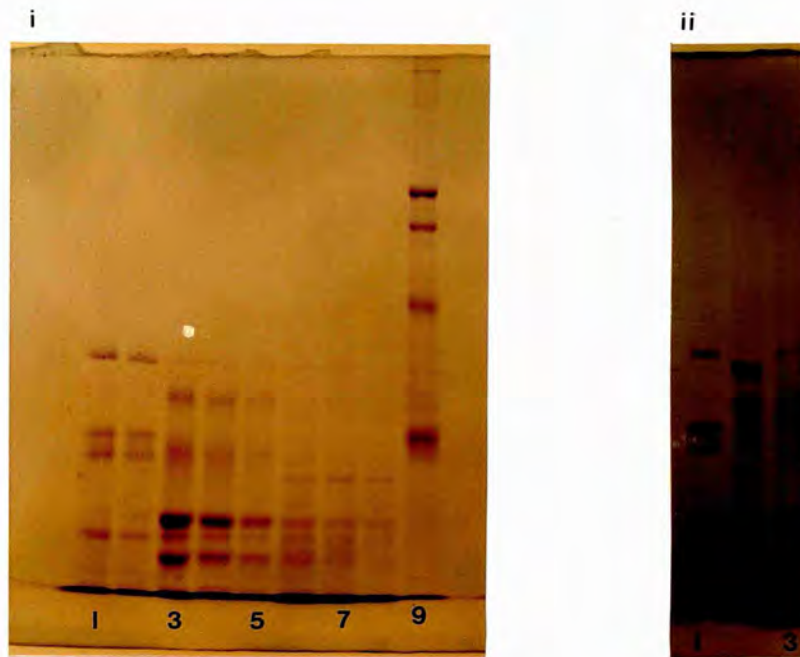
Sample	Sugars present (mol/mol protein)				Total Carbohydrate (%) (w/w)
	Xylose	Mannose	Glucose	Glucosamine	
Unbound enzyme					
I <sub>ub</sub>	5.3 (0.5)*	7.0 (0.8)	13.8 (1.6)	4.0 (0.45)	3.35
II <sup>1</sup> <sub>ub</sub>	1.4 (0.45)	3.7 (1.5)	2.2 (0.9)	1.6 (0.63)	3.48
II <sup>2</sup> <sub>ub</sub>	0.6 (0.19)	1.2 (0.49)	1.1 (0.45)	0.5 (0.21)	1.34
Bound enzyme					
I	2.7 (0.25)	6.7 (0.75)	13.0 (1.5)	2.1 (0.23)	2.7
II <sup>1</sup>	0.9 (0.28)	2.3 (0.9)	1.5 (0.6)	0.7 (0.27)	2.1
II <sup>2</sup>	1.9 (0.65)	3.2 (1.3)	5.1 (2.1)	0.94 (0.39)	4.4

\* The figures in brackets represent % of each sugar present.

The results have been calculated using MWs 160,400, 45,730 and 43,390 for I and I<sub>ub</sub>, II<sup>1</sup> and II<sup>1</sup><sub>ub</sub> and II<sup>2</sup> and II<sup>2</sup><sub>ub</sub> respectively.



Fig. 34 SDS-PAGE of purified  $\alpha$ -galactosidases  
 $I_{ub}$ ,  $II^1_{ub}$  and  $II^2_{ub}$



- i.  $\alpha$ -Galactosidases  $I_{ub}$ ,  $II^1_{ub}$ , and  $II^2_{ub}$  were purified up to the stage 7 of the purification procedure and then electrophoresed.
- Tracks 1 and 2.  $\alpha$ -Galactosidase  $I_{ub}$  (10  $\mu$ g).
- Tracks 3 - 5.  $\alpha$ -Galactosidase  $II^1_{ub}$  (12, 12 and 8  $\mu$ g).
- Tracks 6 - 8.  $\alpha$ -Galactosidase  $II^2_{ub}$  (8, 8 and 6  $\mu$ g).
- ii.  $\alpha$ -Galactosidases I,  $II^1$  and  $II^2$  recycled through a CM-cellulose column.
- 1). 10  $\mu$ g of  $I_{ub}$ ; 2). 10  $\mu$ g of  $II^1_{ub}$ ; 3). 8  $\mu$ g of  $II^2_{ub}$ .

hence it is very difficult to comment on these results and no explanation for the low affinity of these enzymes for Con A can be advanced.

## 8 The Lectin nature of V. faba $\alpha$ -galactosidases

The occurrence, nature and properties of lectins has been briefly discussed in the Introduction (p. 35). In view of recent reports that some  $\alpha$ -galactosidases are lectins (contrast ref. 230), the possibility that V. faba  $\alpha$ -galactosidases were also lectins was next investigated by observing their effect on rabbit erythrocytes. All three purified forms possessed the ability to agglutinate red blood cells and their relative activities expressed as haemagglutinin units/mg protein were 104, 245 and 847 for I, II<sup>1</sup> and II<sup>2</sup>, respectively. The lower haemagglutinin activity of enzyme I could be explained if the lectin binding sites of the subunits are occupied in the tetramer by mannose or glucose residues present on the surface of adjacent monomer units. Further, it is of interest to speculate that such internal lectin binding might serve to hold the tetramer together, in addition to other quaternary binding forces.

V. faba seeds as well as other leguminous seeds contain non-enzymic lectins, one of which, favin from V. faba has been purified by Allen et al (202) and its structure examined in detail by Hemperly et al (231) and Hopp et al. (232). This

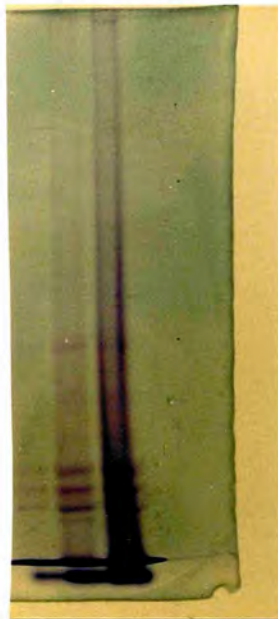
lectin is a tetramer composed of two  $\alpha$ -chains ( $M_r$  5571) and two  $\beta$ -chains ( $M_r$  20,700) which are glycosylated: it is glucose/mannose specific.

Attempts were made to exclude the possibility that the lectin activity associated with the V. faba  $\alpha$ -galactosidases was due to contamination with favin.

Favin was purified according to the method of Allen et al (202) and in the first place, it was found to possess no  $\alpha$ -galactosidase activity when *p*-nitrophenyl  $\alpha$ -galactopyranoside was used as substrate. Figure 35 shows the SDS-PAGE pattern for the purified favin. Five distinct protein bands are present. The slowest band is probably the undissociated tetramer ( $M_r$  ca 50,000) and the four bands grouped together are presumably a mixture of the  $\beta$ -chain with other polypeptides that Hopp et al (232) have been unable to remove. The  $\alpha$ -chain ( $M_r$  5571) would be obscured by the dye front in Fig. 35.

When the favin SDS-PAGE pattern was compared with those for  $\alpha$ -galactosidases I, II<sup>1</sup> and II<sup>2</sup>, no similarities were apparent and, hence, there was no evidence for a significant contamination by favin.

Fig. 35 SDS-PAGE of purified favin



Favin was purified by the method of Allen et al (202)  
5, 10 and 15  $\mu\text{g}$  of favin was applied to the gel.

## REFERENCES

1. Distler, J.J. and Jourdian, G.W. (1973) *J. Biol. Chem.* **248**, 6772-6780
2. Robinson, D., Phillips, N.C. and Winchester, B. (1975) *FEBS Lett.* **53**, 110-112
3. Alhadeff, J.A., Miller, A.L., Wenaas, H., Vedvick, T. and O'Brien, J.S. (1975) *J. Biol. Chem.* **150**, 7106-7113
4. Pierce, R.J., Price, R.G. and Fowler, J.S.L. (1977) *Biochem. J.* **167**, 765-773
5. Bishop, D.F. and Sweeley, C.C. (1978) *Biochim. Biophys. Acta* **525**, 399-409
6. Giudicelli, J., Emiliozzi, R., Vannier, C., De Burlet, G. and Sudka, P. (1980) *Biochim. Biophys. Acta* **612**, 85-96
7. Sheinin, R. and Crocker, B.F. (1961) *Can. J. Biochem. Physiol.* **39**, 55-61
8. Kaji, A. and Ichimi, T. (1969) *Appl. Microbiol.* **18**, 1036-1040
9. Bahl, O.P. and Agrawal, K.M.L. (1969) *J. Biol. Chem.* **244**, 2970-2978
10. Keegstra, K., English, P.D. and Albersheim, P. (1972) *Phytochemistry* **11**, 1873-1880
11. Iwashita, S. and Egami, F. (1973) *J. Biochem.* **73**, 1217-1222
12. Opheim, D.J. (1978) *Biochim. Biophys. Acta* **524**, 121-130
13. Pridham, J.B. and Walter, M.W. (1964) *Biochem. J.* **92**, 20p
14. Dey, P.M. and Pridham, J.B. (1968) *Phytochemistry* **7**, 1737-1739
15. Agrawal, K.M.L. and Bahl, O.P. (1968) *J. Biol. Chem.* **243**, 103-111
16. Li, Y.T. and Li, S. (1968) *J. Biol. Chem.* **243**, 3994-3996
17. Cabezas, J.A. and Vazquez-Pernas, R. (1969) *R. esp. Fisiol.* **25**, 147-152
18. Somme, R. (1970) *Acta Chem. Scand.* **24**, 72-76
19. Lee, J.W. and Ronalds, J.A. (1972) *J. Sci. Fd. Agric.* **23**, 199-205
20. Dey, P.M. (1977) *Phytochemistry* **16**, 323-325
21. Grover, A.K., Macmurchie, D.D. and Cushley, R.J. (1977) *Biochim. Biophys. Acta* **482**, 98-108
22. Killilea, S.D. and Clancy, M.J. (1978) *Phytochemistry* **17**, 1429-1431
23. Burmeister, G. and Hösel, W. (1981) *Planta* **152**, 578-586

24. Keegstra, K. and Albersheim, P. (1970) *Plant Physiol.* **45**, 675-678
25. Dey, P.M. and Pridham, J.B. (1972) *Adv. Enzymol.* **36**, 91-130
26. Harpaz, N., Flowers, H.M. and Sharon, N. (1977) *Eur. J. Biochem.* **77**, 419-426
27. Malhotra, O.P. and Dey, P.M. (1967) *Biochem. J.* **103**, 508-513
28. Dey, P.M. and Pridham, J.B. (1969) *Biochem. J.* **113**, 49-55
29. Petek, F., Villaroya, E. and Courtois, J.E. (1969) *Eur. J. Biochem.* **8**, 395-402
30. Carchon, H. and De Bruyne, C.K. (1975) *Carbohydr. Res.* **41**, 175-189
31. Suzuki, H., Ozawa, Y., Oota, H. and Yoshida, H. (1969) *Agric. Biol. Chem.* **33**, 506-513
32. Suzuki, H., Li, S.C. and Li, Y.T. (1970) *J. Biol. Chem.* **245**, 781-786
33. Gatt, S. and Baker, E.A. (1970) *Biochim. Biophys. Acta* **206**, 125-135
34. Kaplan, N.O. (1968) *Ann. N.Y. Acad. Sci.* **151**, 383-399
35. Markert, C.L. and Moller, F. (1959) *Proc. Natl. Acad. Sci. U.S.A.* **45**, 753-763
36. Moss, D.W. (1979) in *Isoenzyme Analysis, Analytical Science Monographs*, no.6, pp. 1-33, The Chemical Soc., London
37. Chester, M.A., Hultberg, B. and Ockerman, P. (1976) *Biochim. Biophys. Acta* **429**, 517-526
38. Petek, F. and Dong, T. (1961) *Enzymologia* **23**, 133-142
39. Barham, D., Dey, P.M., Griffiths, D. and Pridham, J.B. (1971) *Phytochemistry* **10**, 1759-1763
40. Harpaz, N., Flowers, H.M. and Sharon, N. (1974) *Biochim. Biophys. Acta* **341**, 213-221
41. Lee, Y.C. and Wacek, V. (1970) *Arch. Biochem. Biophys.* **138**, 264-271
42. Wakabayashi, K. (1961) *J. Faculty of Eng., Shinshu Univ.* **11**, 17
43. Lusic, A.J. and Paigen, K. (1976) *Biochim. Biophys. Acta* **437**, 487-497
44. Beutler, E. and Kuhl, W. (1971) *J. Lab. Clin. Med.* **78**, 987
45. Kint, J.A. (1971) *Arch. Int. Physiol. Biochim.* **79**, 633-634
46. Beutler, E., Guinto, E. and Kuhl, W. (1973) *Am. J. Hum. Genet.* **25**, 42-46

47. Beutler, E. and Kuhl, W. (1972) *J. Biol. Chem.* **247**, 7195-7200
48. Beutler, E. and Kuhl, W. (1972) *Nat. New Biol.* **239**, 207-208
49. Rietra, P.J.G.M., Molenaar, J.L., Hamers, M.H., Tager, J.M. and Borst, P. (1974) *Eur. J. Biochem.* **46**, 89-98
50. Schram, A.W., Hamers, M.H., Brouwer-Kelder, B., Doner-Koopman, W.E. and Tager, J.M. (1977) *Biochim. Biophys. Acta* **482**, 125-137
51. De Groot, P.G., Westerveld, A., Meera, Khan, P. and Tager, J.M. (1978) *Hum. Genet.* **44**, 305-312
52. Schram, A.W. and Tager, J.M. (December, 1981) *TIBS* 328-330
53. Courtois, J.E., Petek, F. and Dong, T. (1963) *Bull. Soc. Chim. Biol.* **45**, 95-100
54. Dey, P.M. and Pridham, J.B. (1969) *Biochem. J.* **114**, 86-87p
55. Dey, P.M. and Pridham, J.B. (1969) *Biochem. J.* **115**, 47-54
56. Ahmed, M.U. and Cook, F.S. (1964) *Can. J. Biochem.* **42**, 605-612
57. Hankins, C.N., Kindinger, J.I. and Shannon, L.M. (1980) *Plant Physiol.* **66**, 375-378
58. Smart, E.L. and Pharr, D.M. (1980) *Plant Physiol.* **66**, 731-734
59. Thomas, B. and Webb, J.A. (1977) *Phytochemistry* **16**, 203-206
60. Thomas, B. and Webb, J.A. (1978) *Plant Physiol.* **62**, 713-717
61. Balasubramaniam, K., Dey, P.M. and Pridham, J.B. (1974) *Biochem. Soc. Trans.* **2**, 1128-1130
62. Balasubramaniam, K., Dey, P.M. and Pridham, J.B. (1976) *Phytochemistry* **15**, 1445-1446
63. Dey, P.M. and Dixon, M. (1974) *Biochem. Soc. Trans.* **2**, 1130-1132
64. Dey, P.M. and Dixon, M. (1974) *Biochim. Biophys. Acta* **370**, 269-275
65. Dey, P.M. and Wallenfels, K. (1974) *Eur. J. Biochem.* **50**, 107-112
66. Dey, P.M., Khaleque, A. Palan, P.R. and Pridham, J.B. (1973) *Biochem. Soc. Trans.* **1**, 661-663
67. Dey, P.M., Khaleque, A. and Pridham, J. B. (1971) *Biochem. J.* **124**, 27p
68. Pridham, J.B. and Dey, P.M. (1974) in *Plant Carbohydrate Biochemistry* (Pridham, J.B. ed.), pp. 83-96, Academic Press, London
69. Khaleque, A. (1973) Ph.D. thesis, University of London



70. Tomino, S. and Meisler, M. (1975) *J. Biol. Chem.* **250**, 7752-7758
71. Kaneshiro, C.M., Enns, C.A., Hahn, M.G., Peterson, J.S. and Reithel, F.J. (1975) *Biochem. J.* **151**, 433-434
72. Umezurike, G.M. (1975) *Biochem. J.* **145**, 361-368
73. Ho, M.W., Beutler, S., Tennant, L. and O'Brien, J.S. (1972) *Am. J. Hum. Genet.* **24**, 256-266
74. Romeo, G., Dimatteo, G., D'Urso, M., Li, S. and Li, Y. (1975) *Biochim. Biophys. Acta* **391**, 349-360
75. Romeo, G., D'Urso, M., Pisacane, A., Blum, E., De Falco, A. and Ruffilli, A. (1975) *Biochem. Genet.* **13**, 615-618
76. Del Campillo, E., Shannon, L.M. and Hankins, C.N. (1981) *J. Biol. Chem.* **256**, 7177-7180
77. Hustler, M.J. (1977) Ph.D. thesis, University of London
78. Legler, G. (1973) *Molecular and Cellular Biochem.* **2**, 31-38
79. Pigman, W.W. (1940) *J. Am. Chem. Soc.* **62**, 1371-1374
80. Wallenfels, K. and Malhotra, O.P. (1961) *Adv. Carbohydr. Chem.* **16**, 239-298
81. Adams, M., Richtmyer, N.K. and Hudson, C.S. (1943) *J. Amer. Chem. Soc.* **65**, 1369-1380
82. Bailey, R.W. (1963) *Biochem. J.* **86**, 509-514
83. Bailey, R.W. and Howard, B.H. (1963) *Biochem. J.* **87**, 146-151
84. Li, Y.T., Li, S.C. and Shetlar, M.R. (1963) *Arch. Biochem. Biophys.* **103**, 436-442
85. Li, Y.T. and Shetlar, M.R. (1964) *Arch. Biochem. Biophys.* **108**, 523-539
86. Hogness, D.S. and Battley, E.H. (1957) *Fed. Proc.* **16**, 197-198
87. Courtois, J.E. (1959) *Proc. Intern. Congr. Biochem.*, 4th Congr., Vienna, **1**, 140-159
88. Coleman, R.L., (1968) *Biochim. Biophys. Acta* **159**, 192-193
89. Courtois, J.E. and Dizet, P.L. (1966) *Carbohydr. Res.* **3**, 141-155
90. Courtois, J.E. and Petek, F. (1966) *Methods Enzymol.* **8**, 565-571
91. Courtois, J.E. and Percheron, F. (1961) *Bull. Soc. Chim. Biol.* **43**, 167-175

92. Anagnostopoulos, C., Courtois, J.E. and Petek, F. (1954) Bull. Soc. Chim. Biol. **36**, 1115-1123
93. Courtois, J.E., Petek, F. and Dong, T. (1961) Bull. Soc. Chim. Biol. **43**, 1189-1196
94. Wohnlich, J. (1962) Bull. Soc. Chim. Biol. **44**, 567-568
95. Anagnostopoulos, C., Courtois, J.E. and Petek, F. (1955) Arch. Sci. Biol. (Bologna) **39**, 631-640
96. Blanchard, P.H. and Albon, N. (1950) Arch. Biochem. Biophys. **29**, 220-222
97. French, D. (1954) Adv. Carbohydr. Chem. **9**, 149-184
98. Tanner, W. and Kandler, O. (1968) Eur. J. Biochem. **4**, 233-239
99. Wohnlich, J. (1963) Bull. Soc. Chim. Biol. **45**, 1171-1190
100. Bourne, E.J., Pridham, J.B. and Walter, M.W. (1965) Biochem. J. **97**, 802-806
101. Pridham, J.B. and Hassid, W.Z. (1965) Plant Physiol. **40**, 984-986
102. Gomyo, T. and Nakamura, M. (1966) Agric. Biol. Chem. **30**, 425-427
103. Kandler, O. and Hopf, H. (1980) in The Biochemistry of Plants (ed. Preiss, J.), **3**, pp. 221-270, Academic Press, Inc.
104. Tanner, W. and Kandler, O. (1966) Plant. Physiol. **41**, 1540-1542
105. Tanner, W., Lehle, L. and Kandler, O. (1967) Biochem. Biophys. Res. Commun. **29**, 166-171
106. Lehle, L. and Tanner, W. (1973) Eur. J. Biochem. **38**, 103-110
107. Duperon, R. (1955) Comp. Rend. Acad. Sci. (Paris) **241**, 1817-1819
108. Courtois, J.E., Archambault, A. and Dizet, P.L. (1956) Bull. Soc. Chim. Biol. **38**, 1117-1119
109. Bourquelot, E. and Brindel, M. (1910) Comp. Rend. Acad. Sci. (Paris) **151**, 760-762
110. Courtois, J.E., Archambault, A. and Dizet, P.L. (1956) Bull. Soc. Chim. Biol. **38**, 359-363
111. Cerbulis, J. (1955) Arch. Biochem. Biophys. **58**, 406-413
112. Bradfield, A.E. and Flood, A.E. (1950) Nature **166**, 264-265
113. Zimmerman, M.R. (1957) Plant Physiol. **32**, 288-290
114. Zimmerman, M.R. (1958) Plant Physiol. **33**, 213-217

115. Dey, P.M. (1980) *Adv. Carbohydr. Chem. Biochem.* **37**, 283-372
116. Wattiez, N. and Hans, M. (1943) *Bull. Acad. R. Med. Belg.* **8**, 386-396
117. Jukes, C. and Lewis, D.H. (1974) *Phytochemistry* **13**, 1519-1521
118. Hatanaka, S. (1959) *Arch. Biochem. Biophys.* **82**, 188-194
119. Aspinall, G.O. and Whyte, J.N.C. (1964) *J. Chem. Soc.* 5058-5063
120. Dea, I.C.M. and Morrison, A. (1975) *Adv. Carbohydr. Chem. Biochem.* **31**, 241-312
121. Bailey, R.W. (1971) in *Chemotaxonomy of the Leguminosae* (Harbourne, J.B., Boulter, D. and Turner, B.L., eds.) pp.503-541, Academic Press, London, New York
122. Dey, P.M. (1978) *Adv. Carbohydr. Chem. Biochem.* **35**, 341-376
123. Larson, E.B. and Smith, F. (1955) *J. Am. Chem. Soc.* **77**, 429-432
124. Gould, M.F. and Greenshields, R.N. (1964) *Nature* **202**, 108-109
125. Hasegawa, M., Takayama, T. and Shiroya, T. (1951) *Kagaku* **21**, 593-594 and *Chem. Abstr.* (1954) **48**, 5297c
126. Shiroya, T. (1963) *Phytochemistry* **2**, 33-46
127. Sioufi, A., Percheron, F. and Courtois, J.E. (1970) *Phytochemistry* **9**, 991-999
128. Reid, J.S.G. and Meier, H. (1973) *Planta* **112**, 301-308
129. Lee, S.R. (1965) Ph.D. thesis, Univ. of Minnesota, Minneapolis and *Chem. Abstr.* (1968) **68**, 111, 694c
130. McCleary, B.V. and Matheson, N.K. (1974) *Phytochemistry* **13**, 1747-1757
131. Lechevallier, D. (1960) *Compt. Rend.* **250**, 2825-2827
132. Lechevallier, D. (1964) *Compt. Rend.* **258**, 5519-5522
133. Shadaksharaswamy, M. and Ramachandra, G. (1968) *Phytochemistry* **7**, 715-719
134. Lechevallier, D. (1962) *Compt. Rend.* **255**, 3211-3213
135. Lee, C.Y. and Shallenberger, R.S. (1969) *Experientia* **25**, 692-693
136. Pridham, J.B., Walter, M.W. and Worth, H.G.J. (1969) *J. Expt. Bot.* **20**, 317-324
137. Pazur, J.H., Shadaksharaswamy, M. and Meidell, D. (1962) *Arch. Biochem. Biophys.* **99**, 78-85

138. Kalckar, H.M., Anderson, R.P. and Isselbacher, K. (1956) *Biochim. Biophys. Acta* **20**, 262-268
139. Reid, J.S.G. (1971) *Planta* **100**, 131-142
140. Webb, J.A. and Gorham, P.R. (1964) *Plant Physiol.* **39**, 663-672
141. Webb, J.A. (1971) *Can. J. Bot.* **49**, 717-733
142. Turgeon, R. and Webb, J.A. (1975) *Planta* **123**, 53-62
143. Kreuzer, H.P. and Kauss, H. (1980) *Planta* **147**, 435-438
144. Stillmark, H. (1888) *Inaug. Dis. Dorpat.*
145. Callow, J.A. (1975) *Current Adv. Plant Sci.* **7**, 181-193
146. Boyd, W.C. (1963) *Vox. Sang.* **8**, 1-32
147. Boyd, W.C. (1970) *Ann. N.Y. Acad. Sci.* **169**, 168-190
148. Brown, J.C. and Hunt, R.C. (1978) *Int. Rev. Cytol.* **52**, 277-349
149. Kocourek, J. and Horejsi, V. (1981) *Nature* **290**, 188
150. Goldstein, I.J., Hughes, R.C., Monsigny, M., Osawa, T. and Sharon, N. (1980) *Nature* **285**, 66
151. Liener, I.E. (1976) *Annu. Rev. Plant. Physiol.* **27**, 291-319
152. Lis, H. and Sharon, N. (1973) *Annu. Rev. Biochem.* **42**, 541-574
153. Toms, G.C. and Western, A. (1971) in *Chemotaxonomy of the Leguminosae* (Harborne, J., Boulter, D. and Turner, B.L., eds.) pp. 367-462, Academic Press, London and New York
154. Sharon, N. and Lis, H. (1972) *Science* **177**, 949-958
155. Liske, R. and Franks, D. (1968) *Nature* **217**, 860-861
156. Sivak, A. and Wolman, S.R. (1972) *In Vitro* **8**, 1-6
157. Aub, J.C., Tieslau, C. and Lankester, A. (1963) *Proc. Natl. Acad. Sci. U.S.A.* **50**, 613-619
158. Goldstein, I.J. and Hayes, C.E. (1978) *Adv. Carbohydr. Chem.* **35**, 137-340
159. Jaffe, W.G. (1969) in *Toxic Constituents of Plant Foodstuffs* (Liener, I.E., ed.), pp. 69-102, Academic Press, New York
160. Liener, I.E. (1974) *J. Agr. Food Chem.* **22**, 17-22
161. Freed, D.L.J. and Buckley, H. (1978) *Lancet* 585-586

162. Nachbar, M.S., Oppenheim, J.D. and Thomas, J.O. (1980) *J. Biol. Chem.* **255**, 2056-2061
163. Werner, G., Jaffe, W.G. and Vega Lette, C.L. (1968) *J. Nutrition* **94**, 203-210
164. Public Health Lab. Service, *Br. Med. J.* (1976) *ii* 1268, see ref. 161
165. Jaffe, W.G. (1960) *Arneim. Forsch.* **12**, 1012-1016
166. Brady, P.G., Vannier, A.M. and Banwell, J.G. (1978) *Gastroenterology* **75**, 236-239
167. Weiser, M.M. (1972) *Science* **177**, 525-526
168. Aull, F., Nachbar, M.S. and Oppenheim, J.D. (1977) *J. Cell. Physiol.* **90**, 9-14
169. Rick, C.M. (1978) *Sci. Am.* **239**, 76-87
170. Hankins, C.N. and Shannon, L.N. (1978) *J. Biol. Chem.* **253**, 7791-7797
171. Haab, D. Frey, R., Thiesen, M. and Kauss, H. (1981) *Planta* **151**, 490-496
172. Hankins, C.N., Kindinger, J.I. and Shannon, L.M. (1980) *Plant Physiol.* **65**, 618-622
173. Del Campillo, E. and Shannon, L.M. (1982) *Plant Physiol.* **69**, 628-631
174. Galbraith, W. and Goldstein, I.J. (1972) *Biochemistry* **11**, 3976-3983
175. Saint-Paul, M. (1961) *Transfusion* **4**, 3-37
176. Janzen, D.H., Juster, H.B. and Liener, I.E. (1976) *Science* **192**, 795-796
177. Partridge, J.E., Shannon, L.M., Gumpf, D.J. and Colbaugh, P. (1974) *Nature* **247**, 391
178. Ensgraber, A. (1958) *Ber. dt. Bot. Ges.* **71**, 349-361
179. Howard, I.K., Sage, H.J. and Horton, C.B. (1972) *Arch. Biochem. Biophys.* **49**, 323-326
180. Wiese, L. and Hayward, P.C. (1972) *Am. J. Bot.* **59**, 530-536
181. Rosen, S.D., Kafka, J.A., Simpson, D.L. and Barondes, S.H. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2554-2557
182. Watson, L., Knox, R.B. and Creaser, E.H. (1974) *Nature* **249**, 574-576
183. Hamblin, J. and Kent, S.P. (1973) *Nature New Biol.* **245**, 28-30
184. Bohlool, B.B. and Schmidt, E.L. (1974) *Science* **185**, 269-271

185. Bhuvanewari, T.V. and Wolfgang, D.B. (1978) *Plant Physiol.* **62**, 71-74
186. Marshall, R.D. and Neuberger, A. (1968) in *Carbohydrate Metabolism and its Disorders* (Dickens, F., Randle, P.J. and Whelan, W.J., eds.), **1**, pp. 213-258, Academic Press, New York and London
187. Marshall, R.D. and Neuberger, A. (1970) *Adv. Carbohydr. Chem. Biochem.* **25**, 407-478
188. Sharon, N. (1974) in *Plant Carbohydrate Chemistry* (Pridham, J.B., ed.), pp. 235-250, Academic Press, London
189. Lis, H., Sharon, N. and Karchalski, E. (1964) *Biochim. Biophys. Acta.* **83**, 376-378
190. Murachi, T., Suzuki, A. and Takahashi, N. (1967) *Biochemistry* **6**, 3730-3736
191. Pazur, J.H. and Aronson, JR. N.N. (1972) *Adv. Carbohydr. Chem. and Biochem.* **27**, 301-340
192. Yamasaki, Y. and Suzuki, Y. (1980) *Planta* **148**, 354-361
193. Bourbouze, R., Percheron, F. and Courtois, J.E. (1976) *Eur. J. Biochem.* **63**, 331-337
194. Gomori, G.L. (1955) *Methods Enzymol.* **1**, 138-146
195. McIlvaine, T.C. (1921) *J. Biol. Chem.* **49**, 183-186
196. Lowry, O.H., Rosebrough, N.J., Lewis Farr, A. and Randall, R.J. (1951) *J. Biol. Chem.* **193**, 265-275
197. Andrews, P. (1964) *Biochem. J.* **91**, 222-233
198. Reisfeld, R.A., Lewis, U.J. and Williams, D.E. (1962) *Nature* **195**, 281-283
199. Laemmli, U.K. (1970) *Nature* **227**, 680-685
200. Ouchterlony, O. (1958) *Prog. Allergy* **5**, 1-78
201. Allen, A.K., Neuberger, A. and Sharon, N. (1973) *Biochem. J.* **131**, 155-162
202. Allen, A.K., Desai, N.N. and Neuberger, A. (1976) *Biochem. J.* **155**, 127-135
203. Allen, A.K. and Neuberger, A. (1975) *FEBS Lett.* **60**, 76-80.
204. Liu, T-Y. and Chang, Y.H. (1971) *J. Biol. Chem.* **246**, 2842-2848
205. Liu, T-Y. (1972) *Methods Enzymol.* **25**, 44-55
206. Hirs, C.H.W. (1956) *J. Biol. Chem.* **219**, 611-621

207. Mayes, R.W., Mason, R.M. and Griffin, D.C. (1973) *Biochem. J.* **131**, 541-553
208. Allen, A.K. and Neuberger, A. (1973) *Biochem. J.* **135**, 307-314
209. Chambers, R.E. and Clamp, J.R. (1971) *Biochem. J.* **125**, 1009-1018
210. Scandalios, J.G. (1974) *Annu. Rev. Plant Physiol.* **25**, 225-258
211. Cheetam, P.S.J. and Dance, N.E. (1976) *Biochem. J.* **167**, 189-195
212. Heyworth, C.M., Newmann, E.F. and Wynn, C.H. (1981) *Biochem. J.* **193**, 773-779
213. Rhodes, M.J.C. (1977) in *Regulation of Enzyme Synthesis and Activity in Higher Plants* (Smith, H., ed.), pp. 245-269, Academic Press, London and New York.
214. Martell, A.E. and Calvin, M. (1952) in *Chemistry of the Metal Chelate Compounds* (compiled by Sillen, L.G.), pp. 194-195, Academic Press, London
215. *Stability Constants, Supplements No.1, Special Pub. 25* (1971) The Chem. Soc. London
216. Pierce, R.J., Price, R.G. and Creeth, M. (1977) *Biochem. J.* **167**, 765-773
217. Hoeksema, H.L., Van Diggelen, O.P. and Galjaard, H. (1979) *Biochim. Biophys. Acta* **566**, 72-79
218. Tauber, H. (1932) *J. Biol. Chem.* **99**, 257-264
219. Davis, B.J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404-427
220. Hadocava, V. and Benes, K. (1977) *Biol. Plant.* **19**, 436-441
221. Mayes, J.S. and Beutler, E. (1977) *Biochim. Biophys. Acta* **484**, 408-416
222. Segrest, J.P. and Jackson, R.L. (1972) *Methods Enzymol.* **28**, 54-63
223. Bishop, D.F., Wampler, D.E., Sgouris, J.T., Bonefeld, R.J., Anderson, D.K., Hawley, M.C. and Sweeley, C.C. (1978) *Biochim. Biophys. Acta* **542**, 109-120
224. Del Campillo, E. (1980) Ph.D. thesis, University of California, Riverside, U.S.A.
225. Lis, H. and Sharon, N. (1978) *J. Biol. Chem.* **253**, 3468-3476
226. Dorland, L., van Halbeck, H., Viegenthart, J.F.G., Lis, H. and Sharon, N. (1981) *J. Biol. Chem.* **256**, 7708-7711
227. Sharon, N. in *Complex Carbohydrates* (1975) pp. 1-466, Addison-Wesley Publishing Company, Inc. Reading, Massachusetts, U.S.A.

228. Goldstein, I.J., Hollerman, C.E. and Smith, E.E. (1965)  
Biochemistry **4**, 876-883
229. Mattila, K. (1979) Biochim. Biophys. Acta **579**, 62-72
230. Plant, A.R. and Moore, K.G. (1982) Phytochemistry **21**, 985-989
231. Hemperly, J.J., Hopp, T.P., Becker, J.W. and Cunningham, B.A. (1979)  
J. Biol. Chem. **254**, 6803-6810
232. Hopp, T.P., Hemperly, J.J. and Cunningham, B.A. (1982) J. Biol. Chem.  
**257**, 4473-4483



## FACTORS AFFECTING THE EXTRACTION AND THE RELATIVE PROPORTIONS OF MULTIPLE FORMS OF PLANT $\alpha$ -GALACTOSIDASES

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**Key Word Index**—*Vicia faba*; Leguminosae; broad beans;  $\alpha$ -galactosidases; multiple forms.

**Abstract**—The extractability of  $\alpha$ -galactosidase activity from mature *Vicia faba* seeds and the conversion of the low molecular weight form II to the larger oligomer I, was examined over a range of salt concentrations. Specific and total activities of the preparations were high when strong salt solutions were used. Extraction of  $\alpha$ -galactosidase I, in comparison with II, requires solutions with a high ionic strength (e.g. 0.5 M NaCl). Interpretation of gel filtration patterns are, however, complicated by conversion of II to I which occurs under these conditions. This conversion is also enhanced by routine procedures used for enzyme purification, such as citric acid precipitation.

### INTRODUCTION

It has been clearly demonstrated that multiple forms of many glycosidases can be extracted from plant and animal cells and that they may have different kinetic properties [1-4]. The possible functions of these forms *in vivo* are of considerable interest. For example, high and low MW  $\alpha$ -galactosidases are detectable by gel filtration of buffered extracts of mature *Vicia faba* seeds and enzyme I (MW 160 000 by sedimentation equilibrium method) has significantly higher  $V_{max}$  values with a range of substrates than the enzyme II fraction [5, 6]: the latter is a mixture of two forms (MW 43 000 and 46 000) separable by ion-exchange chromatography [7]. It has been suggested that the presence of  $\alpha$ -galactosidase I in the resting seed favours a rapid hydrolysis of soluble reserves of galactosylsucroses which is required at the onset of germination [7]. Little of enzyme I can be detected in extracts of seed that have germinated [7].

One major problem in attempting to determine the relative proportions of the multiple forms of  $\alpha$ -galactosidase occurring *in vivo* is that of interconversion. With crude and partially purified preparations it has been shown that the enzyme II fraction readily aggregates to form enzyme I with a consequent increase in the specific activity of the preparation [6, 8]. In addition, Heyworth *et al.* [9] and Cheetam and Dance [10] have shown that the ratio of multiple forms of human liver  $\beta$ -galactosidase is markedly affected by the ionic strength of the medium.

Whilst attempting to accurately determine the proportions (high and low MW forms) of  $\alpha$ -galactosidases in resting *V. faba* seeds, a number of procedures which are commonly used for the extraction and purification of enzymes were examined to see if they affected the ratio of the multiple forms and

the enzyme activity. Detailed information of this kind is rarely reported in the literature (however, see e.g. refs. [11] and [12]).

### RESULTS AND DISCUSSION

#### *Effect of salt concentration on enzyme extraction*

Table 1 shows the effect of increasing the ionic strength of the extraction media on the recovery of  $\alpha$ -galactosidase activity from powdered resting *V. faba* seeds. The time between adding the extraction medium to the tissue and assaying the enzyme was kept constant (2 hr). Over the range of concentrations examined, none of the salt or buffer solutions had any effect on enzyme activity when tested *in vitro*. In the case of sodium chloride solution and acetate buffer, maximum release of enzyme required a minimum salt concentration of 0.5 M. Similarly, with McIlvaine (citrate-phosphate) buffer concentrations between 5 and 10 times that normally used were needed for maximum extraction. With the more concentrated solutions specific activities were significantly higher when sodium chloride solution or McIlvaine buffer was used, although this was not apparent with acetate buffer. For comparative purposes the extraction of  $\alpha$ -galactosidase with increasing concentrations of Triton X-100 was examined. High total activities, comparable with those found with the more concentrated salt solutions, were observed with 0.1% detergent but activity was lost as the Triton concentration was increased.

#### *Analysis of extracted fractions*

Enzyme preparations obtained by extracting with solutions of high and low salt concentrations were then applied to Sephadex G-100 columns which were eluted with the corresponding extraction media. Elution times were kept constant at *ca* 12 hr. (Triton

Table 1. Extraction of  $\alpha$ -galactosidase from mature *Vicia faba* seeds using various conditions

Extraction medium	Total enzyme activity (nkat)	Total protein (mg)	Sp. act. (nkat/mg protein)
H <sub>2</sub> O	1561	1356	0.11
NaCl			
0.05 M	213.2	2574	0.08
0.1 M	199.0	2555	0.08
0.5 M	270.4	2145	0.13
1.0 M	270.4	1417	0.19
Triton (w/v)			
0.1%	251.0	1742	0.14
0.5%	211.2	1513	0.14
1.0%	198.0	1200	0.16
5.0%	111.3	1950	0.06
Acetate buffer (pH 5.5)			
0.05 M	191.3	1162	0.16
0.1 M	218.8	1250	0.17
0.5 M	245.7	1586	0.15
1.0 M	244.8	1440	0.17
McIlvaine buffer (pH 5.5)			
×0.5 concn	210.6	1547	0.14
×1 concn	227.5	1560	0.15
×5 concn	298.8	1440	0.21
×10 concn	314.4	1500	0.21

extracts could not be examined in this way because of changes in the gel characteristics and the flow rate.) Only the lower MW fraction of  $\alpha$ -galactosidase (II) was detectable in the 0.1 M sodium chloride and 0.1 M acetate buffer preparations (Figs. 1a and 3a). However, at the 0.5 M levels (Figs. 1b and 3b), form I was present and with the sodium chloride a form intermediate in MW (*ca* 70 000 by gel filtration [13]) was also apparent (Fig. 1b). This enzyme, together with enzyme I, was also detected when a 5-fold concentrated McIlvaine buffer extract was passed through the column (Fig. 2b). In the case of McIlvaine buffer at normal dilution, enzyme I was also present (Fig. 2a) but at relatively low levels in comparison with the concentrated buffer extract.

To investigate the possibility that the composition of the enzyme mixture was affected after extraction by the salt concentration an  $\alpha$ -galactosidase preparation obtained with 0.1 M sodium chloride solution was applied to a column of Sephadex G-100, which was subsequently eluted with 0.5 M sodium chloride. The results (Fig. 1c) clearly show that higher MW forms are produced *in vitro* by high salt concentrations presumably from II (cf. ref. [8]). No significant changes in the column characteristics were noticed when a mixture of proteins of known MW were analysed using 0.1 or 0.5 M sodium chloride as eluant.

Returning to the problem of identifying the multiple forms of  $\alpha$ -galactosidase occurring *in vivo*, both the extractability of the different forms and the formation of oligomers *in vitro* complicate any interpretation. However, a comparison of Figs. 1(a) and 1(c) shows that little conversion of II to I occurs during the processing time and hence most of enzyme I in Fig.

1(b) must presumably have been derived from the bean tissues and high sodium chloride levels were required for its extraction (cf. Figs. 1a and 1b). Again, a concentrated acetate buffer would appear to be required for extraction of  $\alpha$ -galactosidase I (Fig. 3b). McIlvaine buffer at normal concentrations does appear to extract some  $\alpha$ -galactosidase I, but the amount extracted with buffer of five times this ionic strength removes significantly more of enzyme I from the seed tissues. The intermediary form of  $\alpha$ -galactosidase occurring with the concentrated sodium chloride solution (Figs. 1b and 1c) and McIlvaine buffer (Fig. 2b) may be a dimer.

#### *Effect of purification on the relative proportions of enzymes I and II*

A four-stage purification of  $\alpha$ -galactosidase from resting *V. faba* seeds was next carried out (Table 2). The Sephadex G-100 gel filtration patterns at each stage are shown in Fig. 4. Powdered, resting *V. faba* seeds were soaked in McIlvaine buffer (pH 5.5) for 1 hr and following centrifugation the soluble fraction was acidified to pH 3.2 with citric acid solution and the resulting precipitate discarded (Stage b). After readjusting the pH of the supernatant solution to 5.5, the enzyme activity and the multiple forms were examined. Treatment with citric acid resulted in a 2.7-fold increase in total activity and a 4-fold increase in specific activity during the operation, with a significant increase in  $\alpha$ -galactosidase I. The rise in total activity can be equated with the known higher specific activity of form I [14]. In Stage (c) of the purification, the fraction precipitating on addition of ammonium sulphate to 30–65% saturation was dissolved in McIlvaine buffer (pH 5.5) without dialysis. Little

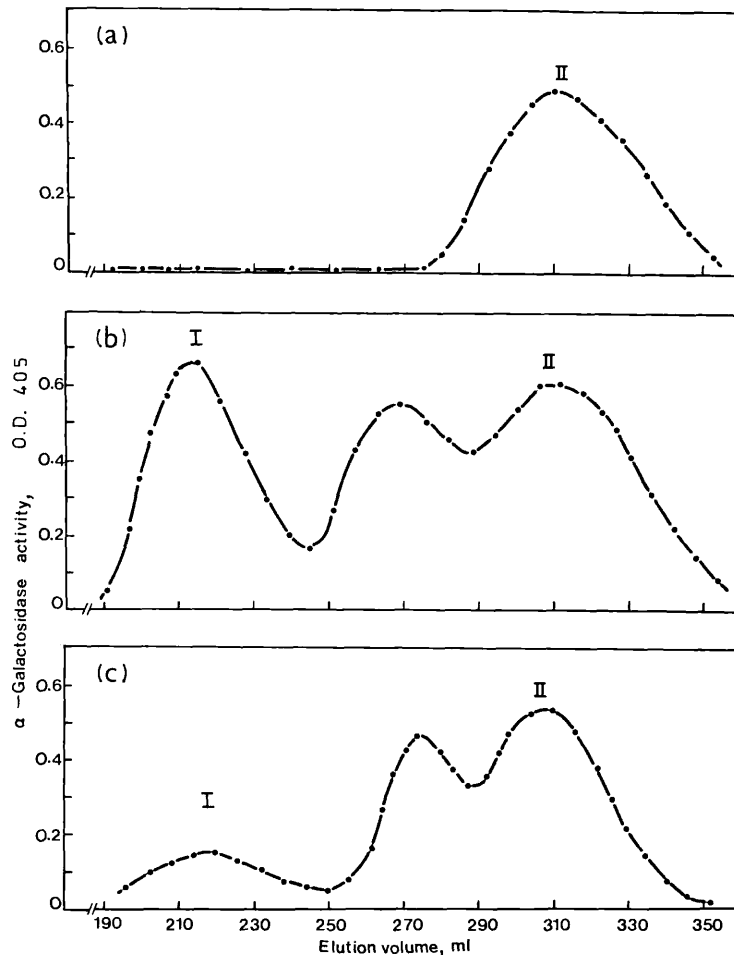


Fig. 1. Gel-filtration of crude  $\alpha$ -galactosidase preparations in varying sodium chloride concentrations on Sephadex G-100. Extraction and elution were carried out as specified in the Experimental. a, 0.1 M NaCl; b, 0.5 M NaCl; c, 0.1 M NaCl extraction followed by 0.5 M NaCl elution.

change in total activity was observed, the specific activity was raised nine-fold (Table 2) but the gel filtration pattern (Fig. 4c) remained unchanged. When the preparation from Stage (c) was dialysed against McIlvaine buffer (a stage requiring *ca* 70% of the time taken for purification) no protein was lost but the total  $\alpha$ -galactosidase activity increased 1.7-fold, which can be accounted for by a further increase in the level of form I (Fig. 4d).

The four-stage purification was completed in *ca* 24 hr and the  $\alpha$ -galactosidase pattern at Stage (b), which was reached in *ca* 2 hr, (Fig. 4b) can be compared with that obtained after storing the crude extract (see Fig. 4a) at 4° for 60 hr, over which there was a steady increase in specific activity which paralleled the accumulation of  $\alpha$ -galactosidase I (Fig. 5). It can be concluded that the normal aggregation of the protein with time is enhanced by citric acid precipitation and that it goes beyond the 60 hr storage pattern (Fig. 5) at Stage (d) mainly as a result of the long period required for dialysis. The expected continuous change in pattern during the ammonium sul-

phate fractionation (Stage c) appears to be interrupted, possibly because of 'salting-out' effects. This contrasts with the enhancement of aggregation produced by high levels of salts (see Fig. 1b) but the 'salting-out' effect can vary significantly with the nature of the ionic species [15].

It would appear, therefore, that great caution is needed when relating the pattern of multiple forms of enzymes found *in vitro* to the situation *in vivo*. Different forms may be bound to varying degrees to cell contents and hence will require different extraction conditions. On the other hand, at high salt levels false impressions may be obtained through aggregation of forms. Aggregation of  $\alpha$ -galactosidase is a slow continuous process which is enhanced by simple procedures used in enzyme preparation and undoubtedly other physical factors such as protein concentration, pH and temperature also play important roles.

Our attention has recently been directed towards the lectin nature of plant glycosidases [16, 17]. *V. faba*  $\alpha$ -galactosidases possess haemagglutinating properties

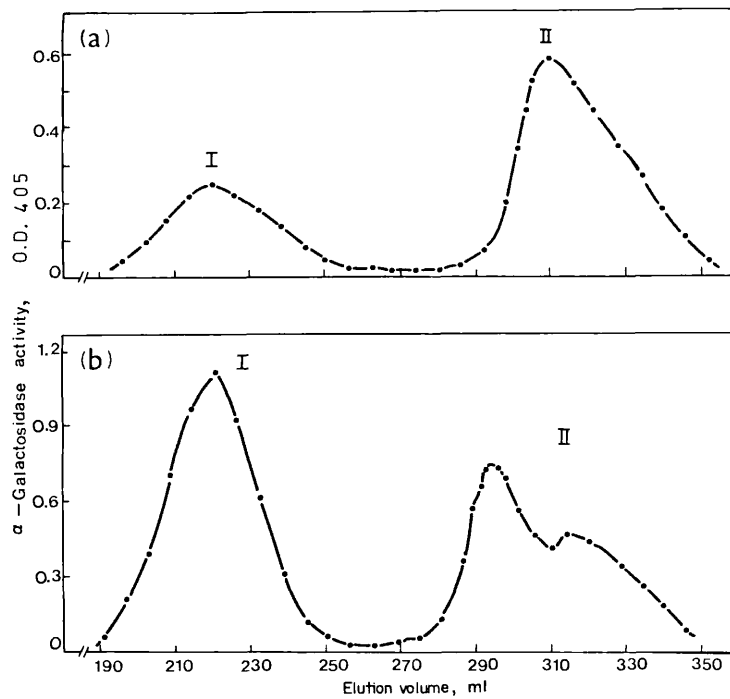


Fig. 2. Gel-filtration of crude  $\alpha$ -galactosidase preparations in different concentrations of McIlvaine buffer, pH 5.5, on Sephadex G-100. a, Extraction and elution with buffer (normal concentration); b, extraction and elution with buffer (five-fold concentration).

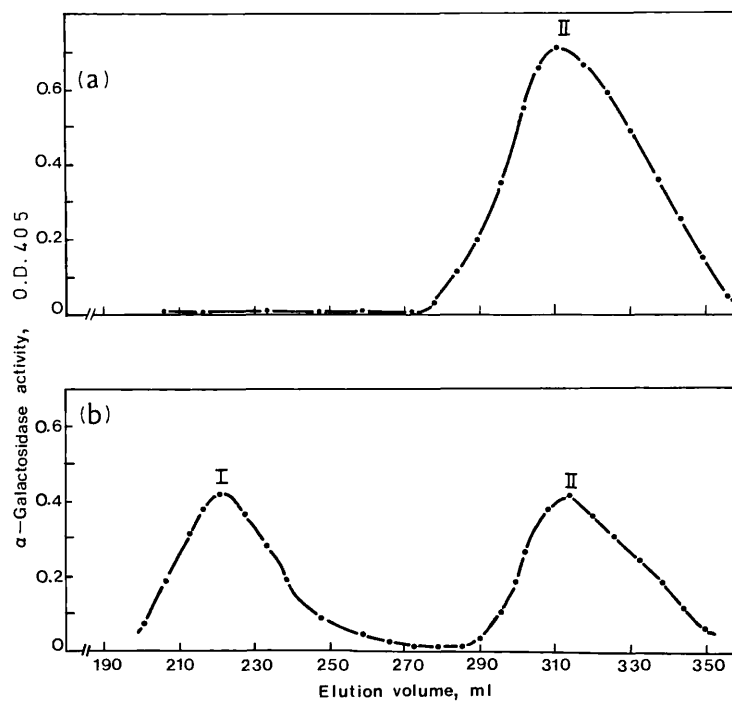


Fig. 3. Gel-filtration of crude  $\alpha$ -galactosidase preparations in different concentrations of sodium acetate buffer, pH 5.5. a, Extraction and elution with 0.1 M buffer; b, extraction and elution with 0.5 M buffer.

Table 2. Purification of  $\alpha$ -galactosidase from mature *Vicia faba* seeds

Purification stage	Total activity (nkat)	Total protein (mg)	Sp. act. (nkat/mg protein)	Purification (fold)	Recovery* (%)
(a) Crude	633.3	3575	0.18	—	—
(b) Citric acid precipitation	1700.0	2500	0.68	3.8	268.4
(c) Ammonium sulphate precipitation (30–60%)	1676.0	289	5.8	32.8	98.6
(d) Dialysis	2845.0	297	9.6	54.2	169.7

Details as described in the Experimental.

\*Expressed with respect to the preceding stage.

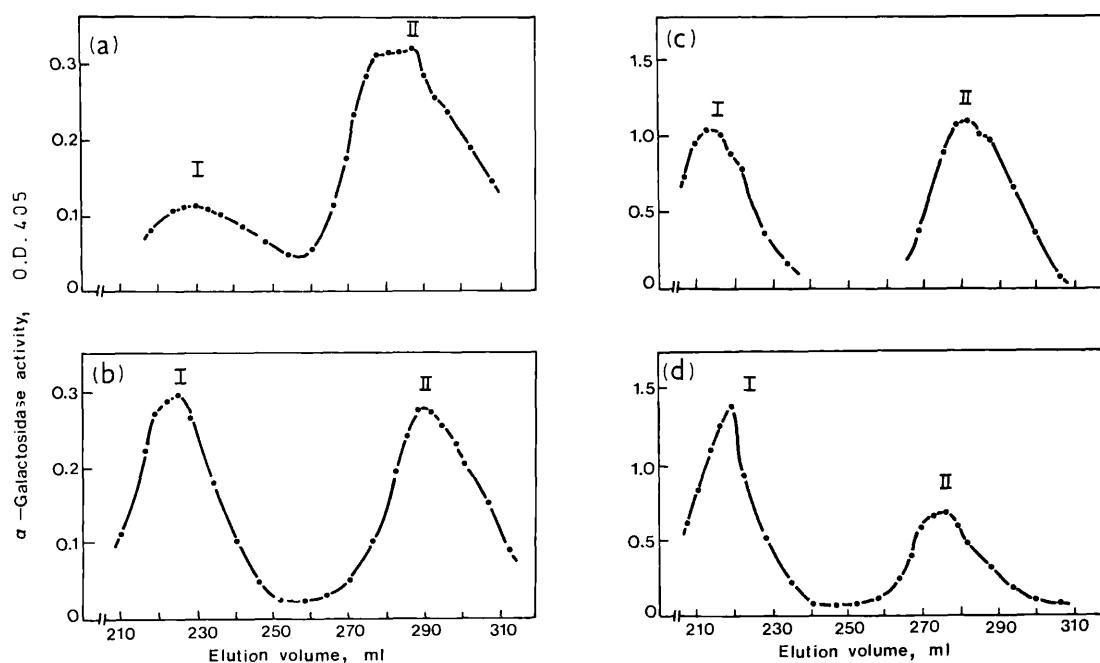


Fig. 4. Sephadex G-100 elution patterns of  $\alpha$ -galactosidase fractions at various stages of purification (Table 2). McIlvaine buffer, pH 5.5, was used for elution. a, Stage (a), crude extract; b, Stage (b), citric acid precipitation; c, Stage (c), ammonium sulphate fractionation; d, Stage (d) dialysis.

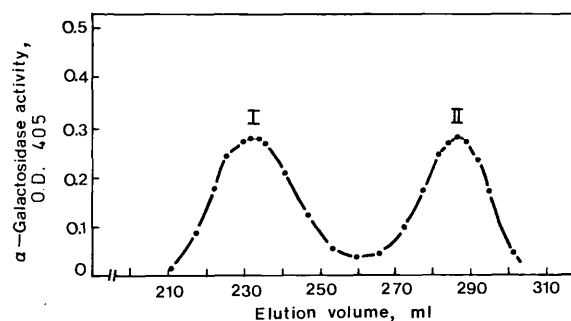


Fig. 5. Sephadex G-100 elution pattern of crude  $\alpha$ -galactosidase preparation after storage at 4° for 60 hr.

with mannose–glucose specificity and they are also glycoproteins with mannose and possibly glucose residues which leads to interesting possibilities for subunit aggregation as well as binding to cell contents. This is presently being investigated.

#### EXPERIMENTAL

Mature, testa-free *V. faba* (var. Bunyards Exhibition) seeds were finely powdered and extracted for 1 hr at 4° with the appropriate medium (ca 1 g powder per 1.5 ml). The extract was then centrifuged at 10 000 g for 40 min to obtain a soluble enzyme preparation (Stage a).

When purifying the enzyme, the pH of the supernatant was first lowered to 3.2 (Stage b) by slow addition of 1.0 M citric acid soln with constant stirring; stirring was maintained for a further 30 min after the required pH was reached. The resulting ppt was removed by centrifugation at 20000 g for 20 min and the pH of the active supernatant readjusted to 5.5 with satd Na<sub>2</sub>HPO<sub>4</sub> soln. The enzyme soln was then fractionated with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (Stage c) and the ppt obtained at 30–65% satn centrifuged down and dissolved in McIlvaine buffer (pH 5.5). This soln was then dialysed (Stage d) against McIlvaine buffer (pH 5.5, diluted 1:1 with deionized H<sub>2</sub>O) for 16 hr.

Multiple enzyme forms were examined on Sephadex G-100 (Pharmacia) columns which were prepared as described by Andrews [13]. The columns were equilibrated with the appropriate eluant. Samples (3 ml) were applied and the columns eluted at a flow rate of 30 ml/hr. Fractions (3 ml) were collected and assayed for  $\alpha$ -galactosidase activity. The elution vols. have not been corrected for the column sizes.

$\alpha$ -Galactosidase activity was measured as described by Dey and Pridham [1] using *p*-nitrophenyl- $\alpha$ -D-galactopyranoside (Koch-Light) as substrate in McIlvaine buffer pH 5.5. Protein was determined by the method of Lowry *et al.* [18]. Sp. act. is defined as nkat of enzyme activity per mg protein.

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## REFERENCES

1. Dey, P. M. and Pridham, J. B. (1969) *Biochem. J.* **113**, 49.
2. Barham, D., Dey, P. M., Griffiths, D. and Pridham, J. B. (1971) *Phytochemistry* **10**, 1759.
3. Pierce, R. J. and Price, R. G. (1977) *Biochem. J.* **167**, 765.
4. Alam, T. and Balasubramanian, A. S. (1978) *Biochim. Biophys. Acta* **524**, 373.
5. Dey, P. M. and Pridham, J. B. (1969) *Biochem. J.* **115**, 47.
6. Pridham, J. B. and Dey, P. M. (1974) in *Plant Carbohydrate Biochemistry* (Pridham, J. B., ed.) p. 83. Academic Press, London.
7. Dey, P. M. and Pridham, J. B. (1972) *Adv. Enzymol.* **36**, 91.
8. Khaleque, A. (1973) Ph.D. thesis, University of London.
9. Heyworth, C. M., Neumann, E. F. and Wynn, C. H. (1981) *Biochem. J.* **193**, 773.
10. Cheetam, P. S. J. and Dance, N. E. (1976) *Biochem. J.* **157**, 189.
11. Keegstra, K. and Albersheim, P. (1970) *Plant Physiol.* **45**, 675.
12. Thomas, B. and Webb, J. A. (1979) *Can. J. Botany* **57**, 1904.
13. Andrews, P. (1964) *Biochem. J.* **91**, 222.
14. Dey, P. M., Khaleque, A., Palan, P. R. and Pridham, J. B. (1973) *Biochem. Soc. Trans.* **1**, 661.
15. Green, A. A. (1932) *J. Biol. Chem.* **95**, 47.
16. Hankins, C. N. and Shannon, L. M. (1978) *Plant Physiol.* **253**, 7791.
17. Hankins, C. N., Kindinger, J. I. and Shannon, L. M. (1980) *Plant Physiol.* **65**, 618.
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.

## MULTIPLE FORMS OF *VICIA FABA* $\alpha$ -GALACTOSIDASES AND THEIR RELATIONSHIPS

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**Key Word Index**—*Vicia faba*; Leguminosae; broad bean;  $\alpha$ -galactosidase; lectin; multiple forms; seed.

**Abstract**—Three highly purified  $\alpha$ -galactosidases, I, II<sup>1</sup> and II<sup>2</sup> have been isolated from resting *Vicia faba* seeds. Form I (MW 160 000) is a tetramer of units of enzyme II<sup>2</sup>. Immunological evidence suggests that all the enzyme forms are closely related. The enzymes are glycoproteins and they also possess haemagglutinating activity which is glucose-mannose specific. The possible involvement of lectin interactions in the formation of the tetrameric enzyme and the binding of the enzymes to cell components is discussed.

### INTRODUCTION

$\alpha$ -Galactosidases ( $\alpha$ -D-galactoside galactohydrolases: EC 3.2.1.22) are a widely distributed group of enzymes [1] which as hydrolytic agents for structural investigations, have attracted attention over the last two decades. There has also been interest in naturally occurring multiple forms of these enzymes in plants and in their physiological function (cf. ref. [2]).

In 1968 Dey and Pridham [3] located two forms, I and II, of the enzyme in resting *V. faba* seeds and later partially purified and examined their properties [4, 5]. Sephadex gel filtration suggested that the MWs of I and II were 209 000 and 38 000, respectively, and the former enzyme possessed a significantly higher specific activity than the latter. Later work [6] showed that form II was a mixture of two enzymes, II<sup>1</sup> and II<sup>2</sup> with MWs (sedimentation equilibrium method) of  $45\,730 \pm 3073$  and  $43\,390 \pm 1409$ , respectively, which were separable on CM-cellulose columns. Examination of the MW of I by the same method gave a value of  $160\,400 \pm 2850$  [2].

This paper describes the preparation of highly purified  $\alpha$ -galactosidases I, II<sup>1</sup> and II<sup>2</sup> from *V. faba* seeds by a modified procedure involving Con A-Sepharose affinity chromatography. The enzymes so obtained have been analysed for carbohydrate content, and the subunit structure of I has been examined by SDS polyacrylamide gel electrophoresis and immunochemical methods. All forms were shown to possess lectin activity.

### RESULTS AND DISCUSSION

#### Enzyme purification

Stages 1 and 2 (see Table 1) in the purification of the  $\alpha$ -galactosidases from resting *V. faba* seeds were essentially those described by Dey and Pridham [4].

In the present work this was followed by ammonium sulphate fractionation and Sephadex G-100 chromatography. The resolved forms, I and II, were then passed separately through columns of Con A-Sepharose on which they were largely retained. In both cases, however, significant proportions of the activities applied (17% of I and 20% of II) appeared to be unbound as they were eluted from the columns with phosphate buffer (pH 7).

Enzymes bound to the columns were eluted with a buffered solution of methyl  $\alpha$ -D-mannopyranoside which resulted in two- and five-fold purifications of I and II, respectively. The resulting enzyme I fraction when applied to a CM-cellulose column and eluted with a sodium chlorate gradient, appeared as a single sharp band (Fig. 1a) and there was a seven-fold increase in specific activity with no further increase on recycling through CM-cellulose. The fraction of  $\alpha$ -galactosidase II from the Con A-Sepharose stage was resolved into two active enzymes (II<sup>1</sup> and II<sup>2</sup>: see Fig. 1b) by the ion-exchange procedure. The apparent specific activity of II<sup>2</sup> was twice that of II<sup>1</sup> and the proportions of the two total activities varied with different batches of seed. It was also noted that enzymes I and II<sup>2</sup> were eluted from CM-cellulose columns by similar sodium chloride concentrations. When II<sup>1</sup> and II<sup>2</sup> were recycled separately through CM-cellulose further purification was achieved and the specific activity ratio (II<sup>2</sup>/II<sup>1</sup>) was then 1.5.

The mobility of the three forms of the enzyme on polyacrylamide gel electrophoresis (PAGE) at pH 8.3 was zero. At pH 3.5, however, they all migrated towards the cathode as discrete bands (Fig. 2a) when examined with both protein and  $\alpha$ -galactosidase-specific reagents. The mobilities of I and II<sup>2</sup> appeared to be identical and these proteins were apparently less basic than II<sup>1</sup>. In view of these results the order of elution of the enzymes from CM-cellulose columns

Table 1. Purification of *V. faba*  $\alpha$ -galactosidases

Stage	Volume (ml)	Activity (nkat/ml)	Protein (mg/ml)	Specific activity (nkat/mg)	Recovery* (%)
1. Crude extract	1010	16.0	88	0.2	—
2. Citric acid pptn	1438	9.1	9.2	1	81
3. $(\text{NH}_4)_2\text{SO}_4$ , 25–60%	68	124.4	98	1	52
4. Sephadex G-100					
$\alpha$ -Galactosidase I	9	253.7	6.1	42	14
$\alpha$ -Galactosidase II	11	165.3	62.9	3	11
5. Sephadex G-100, recycle					
I	3	426.6	5.4	79	8
II	4	229.5	37	6	6
6. Con A-Sepharose					
I	5	184	0.8	185	4.5
II	5	114.4	3.1	36	3.5
7. CM-Cellulose chromatography					
I	1.0	307	0.24	1278	2
II <sup>1</sup>	2.5	43.6	1.08	40	0.7
II <sup>2</sup>	2.5	48.4	0.56	86	0.56
8. CM-Cellulose chromatography, recycle					
I	1.0	180.0	0.14	1286	1.1
II <sup>1</sup>	1.0	60.0	0.78	76	0.37
II <sup>2</sup>	1.0	72.0	0.63	113	0.44

\*% recovery has been calculated relative to the first stage, i.e. crude extract. Weight of seed powder taken = 1 kg.

(Fig. 1) was not expected, however in this case it can be assumed that only a proportion of the charged regions of the enzymes interact with the ion exchange material and the elution is not a function of the total charges on the proteins.

#### Structural relationships between the enzyme forms

Acid hydrolysis of the three enzymes yielded glucosamine, mannose, glucose and xylose in all

cases: the analytical data are given in Table 2. It is of interest to note that the ratio of mannose to glucosamine is comparable for all forms. These monosaccharides are commonly part of the core structure of plant glycoproteins but the results presented here do not suggest any structural relationship between the oligosaccharide chains on the *V. faba* enzymes and the mannose-rich glycan moieties of other plant glycoproteins such as soybean agglutinin [7]. Xylose is

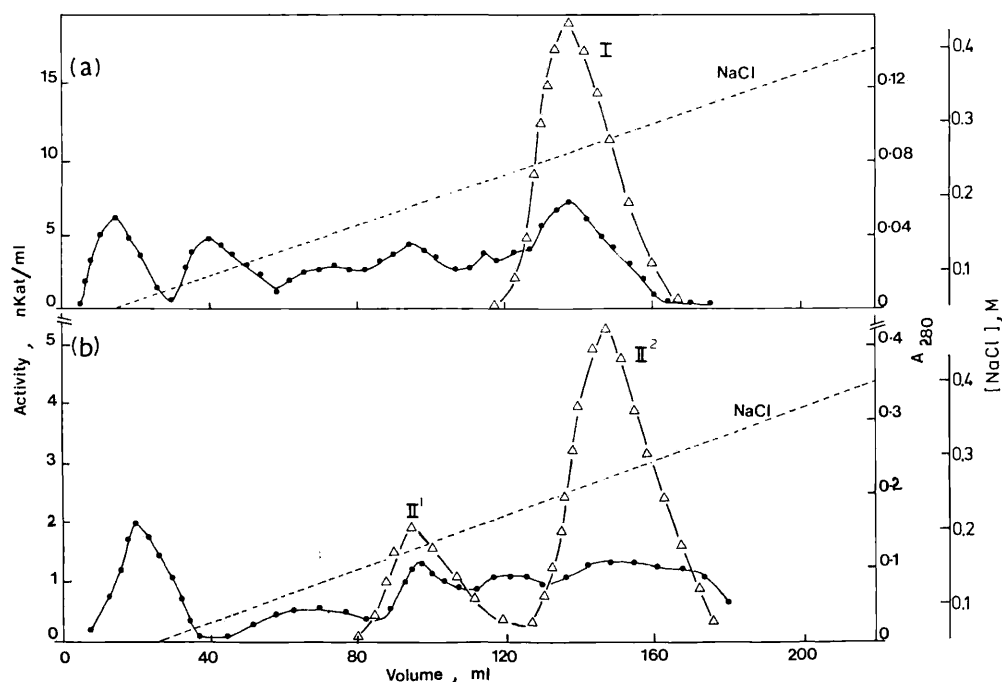


Fig. 1. Elution profiles of enzymes I, II<sup>1</sup> and II<sup>2</sup> on CM-cellulose columns. Elution procedure as described in the Experimental. (a)  $\alpha$ -Galactosidase I; (b)  $\alpha$ -galactosidases II<sup>1</sup> and II<sup>2</sup>; (●) protein ( $A_{280}$ ); ( $\Delta$ ) enzyme activity.



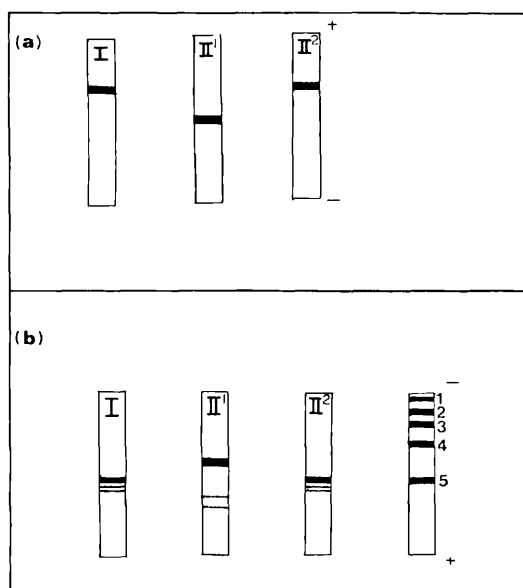


Fig. 2. (a) Polyacrylamide disc gel electrophoresis of  $\alpha$ -galactosidases I, II<sup>1</sup> and II<sup>2</sup>. (b) SDS-polyacrylamide gel electrophoresis of enzymes I, II<sup>1</sup> and II<sup>2</sup>. The standard MW markers were 1, myosin, MW 200 000; 2,  $\beta$ -galactosidase, MW 130 000; 3, phosphorylase *b* MW 94 000; 4, BSA, MW 68 000; 5, ovalbumin, MW 43 000.

not an uncommon component of plant glycoproteins [9] and glucose which is present in widely varying proportions may be a contaminant (cf. ref. [8]) of the  $\alpha$ -galactosidases; however it has also been reported as a glycan component of plant glycoproteins [9, 10].

Figure 2(b) shows the SDS-PAGE mobilities of  $\alpha$ -galactosidases I, II<sup>1</sup> and II<sup>2</sup>. In the case of forms I and II<sup>2</sup>, major identical protein bands (apparent MW 41 300  $\pm$  1200) were observed together with two minor components with higher mobilities. (In the case of enzyme I there was clear evidence of microheterogeneity in the major band.) This MW value considered together with the MWs for I and II<sup>2</sup> obtained by ultracentrifugation, suggests that I is a tetramer of II<sup>2</sup>.  $\alpha$ -Galactosidase II<sup>1</sup> exhibits an apparent MW of 42 650  $\pm$  950 on SDS gels and the sedimentation equilibrium method also indicates that the MW of II<sup>1</sup> is greater than that of II<sup>2</sup>. The electrophoretic pattern for II<sup>1</sup> (Fig. 2b) also showed the presence of minor polypeptide bands.

Rabbit antisera were raised against the three purified *V. faba*  $\alpha$ -galactosidases and each serum was titrated against the enzymes by assaying changes in the activities in the supernatants (Fig. 3). In all cases immunoprecipitation occurred although the reaction

Table 2. Carbohydrate content of *V. faba*  $\alpha$ -galactosidases

Sample	Sugars present (mol/mol)			
	Xylose	Mannose	Glucose	Glucosamine
Galactosidase I	2.7	6.7	13	2.1
Galactosidase II <sup>1</sup>	0.9	2.3	1.5	0.7
Galactosidase II <sup>2</sup>	1.9	3.2	5.1	0.94

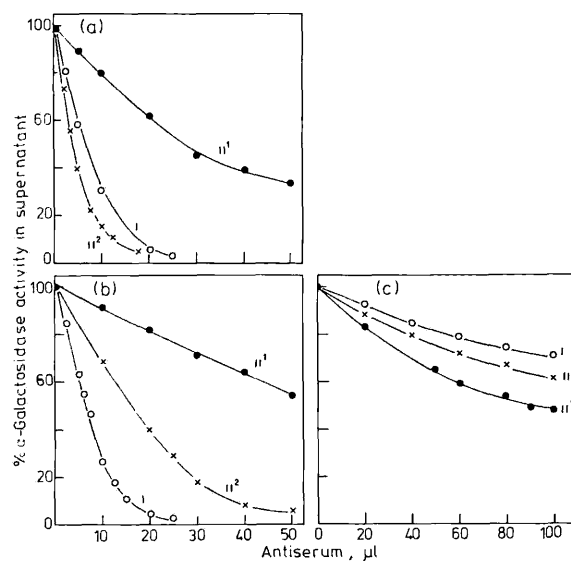


Fig. 3. Immunoprecipitation curves with  $\alpha$ -galactosidases I, II<sup>1</sup> and II<sup>2</sup> using: (a) antiserum-II<sup>2</sup>; (b) antiserum-I; (c) antiserum-II<sup>1</sup>.

was relatively poor in the case of the anti-II<sup>1</sup> serum. The immunoprecipitation titration curves for anti-I indicate homology amongst the three enzyme forms, however the slopes of the curves show that I and II<sup>2</sup> are structurally more closely related than I and II<sup>1</sup>. Similarly both the anti-II<sup>1</sup> and -II<sup>2</sup> sera reacted with all of the  $\alpha$ -galactosidases and again showed the close relationship between I and II<sup>2</sup>. Examination of these interactions by the Ouchterlony double diffusion method [11] confirmed the above findings.

*V. faba*  $\alpha$ -galactosidase I, therefore, is probably a tetramer composed of II<sup>2</sup> glycoprotein units and in this respect it would appear to be similar to  $\alpha$ -galactosidases occurring in a number of other leguminous plants [12–15]. Although *V. faba* seeds possess two forms of  $\alpha$ -galactosidase, II<sup>1</sup> and II<sup>2</sup>, of similar molecular size only the latter appears to aggregate to produce the high MW enzyme I. The situation is similar in the case of *Vigna radiata* (mung bean) seeds where examination of the high MW form (160 000) by SDS-PAGE yielded a single subunit of MW 45 000 [13]. However, there was no report of a second low MW enzyme equivalent to II<sup>1</sup> in these seeds. It should perhaps be noted that Hankins and Shannon [13] examined the  $\alpha$ -galactosidases in mung bean seeds that had imbibed water whereas in the present study resting seeds were used. The pattern of multiple forms of  $\alpha$ -galactosidase are known to change when seeds germinate [2]. Del Campillo [16], however, has reported that soybean seeds possess a high MW (160 000)  $\alpha$ -galactosidase which in the presence of SDS dissociates into two subunits with MWs of 38 000 and 40 000. Regarding enzyme II<sup>1</sup> in *V. faba* seeds, it appears from the immunological studies that it is structurally related to the smaller  $\alpha$ -galactosidase II<sup>2</sup> and hence may be the precursor of II<sup>2</sup> via post-translational modification.

The three purified *V. faba* enzymes were all shown to possess haemagglutinin activity towards rabbit erythrocytes: their relative activities expressed as

haemagglutination units per mg protein were, 104, 245 and 847 for I, II<sup>1</sup> and II<sup>2</sup>, respectively. Tetrameric forms of  $\alpha$ -galactosidase from other leguminous seeds have been reported to possess galactose-specific lectin activity [13, 14]. In contrast to *V. faba*, however, the monomeric  $\alpha$ -galactosidase from *Vigna radiata* is said to be devoid of lectin activity [17] as apparently are the low MW forms of the enzyme from other legumes [14]. Further, haemagglutination by the broad bean enzymes was specifically inhibited by  $\alpha$ -D-mannose and  $\alpha$ -D-glucose derivatives but not significantly by compounds possessing non-reducing terminal  $\alpha$ -D-galactosyl residues [Dey, P. M., Naik, S. and Pridham, J. B., unpublished results].

### Conclusions

*V. faba* seeds possess an interesting group of structurally related enzymes, two monomers and a tetramer, with one monomer presumably in equilibrium with the tetramer *in vivo* [6]. The association of subunits of enzyme II<sup>2</sup> appears to lead to a significant increase in specific activity and a reduction in glucose-mannose-specific lectin activity. The tetramer with its higher enzyme activity, would seem to be present only in resting seeds and this would be advantageous for oligosaccharide mobilization in the ensuing early stages of germination [2].

The lower haemagglutination activity of the tetramer (I) in comparison with the monomer (II<sup>2</sup>) is to be expected if the lectin binding sites of the subunits are occupied in the tetramer by mannose or glucose residues present on the surface of the monomer units. We speculate that such internal lectin bonds may function significantly in holding the tetramer together. Further work is in progress to investigate this possibility. This hypothesis helps to explain the apparent anomaly in the carbohydrate analysis data (Table 2) where  $\alpha$ -galactosidase I has a higher carbohydrate content on a molar basis than enzyme II<sup>2</sup>. We suggest that this is in part because II<sup>2</sup> is a microheterogeneous mixture of glycoproteins and that the process of association to form enzyme I preferentially selects, because of stronger binding potential, forms of II<sup>2</sup> with a relatively high carbohydrate content. In addition, internal binding of extraneous polymers such as polysaccharides and glycoproteins by the tetrameric form of  $\alpha$ -galactosidase may contribute to these differences in carbohydrate content, particularly in the case of glucose (cf. ref. [8]).

From a physiological standpoint one would also expect the enzymes to bind via their lectin sites to a variety of cell components of carbohydrate and glycoprotein nature [18]. This might include cell membranes and cell walls and thus the  $\alpha$ -galactosidases could be 'compartmentalized' or regulated perhaps by steric interactions (cf. ref. [19]), an essential requirement if their activities are to be controlled for example during the synthesis of raffinose and its homologues in developing seeds. Lectin interactions might well be modified by the level of soluble carbohydrates in the tissues.

### EXPERIMENTAL

**Enzyme assay.**  $\alpha$ -Galactosidase activity was determined using *p*-nitrophenyl- $\alpha$ -D-galactopyranoside as described ear-

lier [4]. For detection of enzyme activity on polyacrylamide gels, 6-bromo-2-naphthyl- $\alpha$ -D-galactoside was used [20]. Protein was assayed, after pptn with 10% TCA, by the method of ref. [21]. BSA was used as standard.

**Enzyme isolation and purification.** Testa free, dormant beans were ground in a mechanical grinder. The resultant bean meal (1 kg) was suspended in 1.5 l. of McIlvaine buffer pH 5.5, stirred and left for 1 hr at 4°. The slurry was subsequently centrifuged at 10 000 g for 40 min and the residue discarded. The pH of the supernatant was lowered to 3.2 by gradual addition of 1 M citric acid with constant stirring. The ppted proteins were discarded by centrifuging at 10 000 g and the pH of the supernatant raised to 5.5 with a satd soln of Na<sub>2</sub>HPO<sub>4</sub>. This fraction was then made 25% satd with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and the ppted protein centrifuged and discarded. The resultant supernatant was made 60% satd with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and stirred for 3 hr at 4°. The ppted protein was collected by centrifugation and suspended in McIlvaine buffer (pH 5.5) and dialysed against the same buffer (2 l.) overnight. This dialysed fraction was applied to a 5 × 90 cm, Sephadex G-100 column prepared according to the method of ref. [22]. McIlvaine buffer containing 0.1 M KCl was used to elute the enzyme from the column. Two peaks of  $\alpha$ -galactosidase activity I and II were obtained which were pooled and concentrated separately. From this stage onwards enzymes I and II were purified separately.  $\alpha$ -Galactosidases I and II were recycled through the Sephadex column. The active fractions were dialysed against KPi buffer (0.1 M, pH 7) and applied in 2 ml aliquots to Con A-Sepharose columns (2 × 20 cm) equilibrated with KPi buffer (pH 7) containing 0.5 M NaCl. The column was washed with buffer until all the unbound proteins had been eluted. The bound enzyme was then eluted with 0.5 M  $\alpha$ -D-methyl mannoside in the same buffer. Fractions (2 ml) were collected and the active fractions pooled and concentrated. The enzymes were then dialysed against McIlvaine buffer (pH 3.5) and the dialysed samples applied to 1.0 × 14 cm, CM-cellulose columns equilibrated with McIlvaine buffer, pH 3.5. The flow rate was 15 ml/hr. Following sample application, the column was washed with the buffer and elution of protein carried out in a linear NaCl gradient of 0.05–0.4 M in the same buffer.  $\alpha$ -Galactosidase II was further resolved into II<sup>1</sup> and II<sup>2</sup>. All three enzymes I, II<sup>1</sup> and II<sup>2</sup> were recycled through the CM-cellulose column. The purified enzymes were stored at 4° with thymol added as preservative.

**Electrophoresis.** PAGE was carried out under cationic conditions according to an earlier method [23] with a slight modification. Electrophoresis was performed in 7.5 × 0.5 cm gel tubes, the separating gel being 6.5 cm and the stacking gel 0.5 cm. The gels were run at pH 3.5 in  $\beta$ -alanine-acetate buffer diluted 1 : 1 with H<sub>2</sub>O. For SDS-PAGE, samples were heated to 100° in buffer containing 1.25% SDS and subjected to electrophoresis through 5% acrylamide stacking gels and 8% acrylamide separating gels [24]. Gels were stained with Coomassie brilliant blue-R250 [0.2% (w/v)] in 50% TCA for 15–30 min at 60° and then destained with a soln containing 7.5% (v/v) HOAc and 12.5% (v/v) *iso*-PrOH. The protein standards used were obtained as a kit from Bio-Rad.

**Amino sugar analyses.** Analyses were carried out as described in ref. [8].

**Neutral-sugar analyses.** These were carried out by GC after methanolysis and trimethylsilylation as described earlier [25].

**Assay of agglutination.** The method used is described in ref. [26]. A serial dilution of the lectin was made in 100  $\mu$ l of Pi-buffered saline (pH 7.2) and 200  $\mu$ l of a 1.5% suspension of rabbit erythrocytes was added. The tubes were shaken at

15 min intervals and the extent of agglutination was assessed after 2 hr on a scale of 0-++++. One unit is the amount of lectin required to cause half-maximal (++) agglutination of the cells. The assay is semi-quantitative with a range of error of  $\pm 20\%$  (cf. ref. [8]).

*Immunological methods.*  $\alpha$ -Galactosidases I, II<sup>1</sup> and II<sup>2</sup> were purified as described earlier. One rabbit per enzyme was used. The rabbits were injected  $\times 4$  over a period of 162 days, each time with 125  $\mu\text{g}$  of enzyme mixed with complete Freund's adjuvant; serum was collected 30 days after the last injection. Immunopptn was carried out in plastic micro-centrifuge tubes by adding variable amounts of antiserum (0–50  $\mu\text{l}$  for antiserum I and antiserum II<sup>2</sup>, 0–100  $\mu\text{l}$  for antiserum II<sup>1</sup>) to constant amounts of enzyme activity. The total vol. of antiserum was adjusted to 50  $\mu\text{l}$  (100  $\mu\text{l}$  for antiserum II<sup>1</sup>) in each case by addition of blood serum. After mixing the antiserum and enzyme, the tubes were incubated for 15 min at room temp. They were then centrifuged for 6 min and the supernatants assayed for  $\alpha$ -galactosidase activity. Each antiserum was titrated against all three enzyme forms.

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#### REFERENCES

- Dey, P. M. and Pridham, J. B. (1972) *Adv. Enzymol.* **36**, 91.
- Pridham, J. B. and Dey, P. M. (1974) *Plant Carbohydrate Biochemistry* (Pridham, J. B., ed.) p. 83. Academic Press, London.
- Dey, P. M. and Pridham, J. B. (1968) *Phytochemistry* **7**, 1937.
- Dey, P. M. and Pridham, J. B. (1969) *Biochem. J.* **113**, 49.
- Dey, P. M. and Pridham, J. B. (1969) *Biochem. J.* **115**, 47.
- Dey, P. M., Khaleque, A. and Pridham, J. B. (1971) *Biochem. J.* **124**, 27P.
- Dorland, L., van Halbeck, H., Viegenthart, J. F. G., Lis, A. and Sharon, N. (1981) *J. Biol. Chem.* **256**, 7708.
- Allen, A. K., Desai, N. N. and Neuberger, A. (1976) *Biochem. J.* **155**, 127.
- Sharon, N. (1974) *Plant Carbohydrate Biochemistry* (Pridham, J. B., ed.) p. 235. Academic Press, London.
- Lis, H. and Sharon, N. (1981) *The Biochemistry of Plants* (Marcus, A., ed.) p. 371. Academic Press, New York.
- Ouchterlony, O. (1958) *Acta Pathol. Microbiol. Scand.* **25**, 186.
- Harpaz, N., Flowers, H. M. and Sharon, N. (1977) *Eur. J. Biochem.* **77**, 419.
- Hankins, C. N. and Shannon, L. M. (1978) *J. Biol. Chem.* **253**, 7791.
- Hankins, C. N., Kindinger, J. I. and Shannon, L. M. (1980) *Plant Physiol.* **65**, 618.
- Hankins, C. N., Kindinger, J. I. and Shannon, L. M. (1980) *Plant Physiol.* **66**, 375.
- Del Campillo, E. (1980) Ph.D. thesis, University of California, Riverside, U.S.A.
- Del Campillo, E., Shannon, L. M. and Hankins, C. N. (1981) *J. Biol. Chem.* **256**, 7177.
- Basha, S. M. and Roberts, R. M. (1981) *Plant Physiol.* **67**, 936.
- Lorenc-Kubis, I., Morawiecka, B., Wieczorek, E., Wisniowska, J., Wierzba, E., Ferens, M. and Bøgg-Hansen, T. C. (1981) *Lectins: Biology, Biochemistry and Clinical Biochemistry* (Bøgg-Hansen, T. C., ed.) Vol. 1, p. 169. Walter de Gruyter Berlin.
- Hadačová, V. and Beneš, K. (1977) *Biol. Plant.* **19**, 436.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
- Andrews, P. (1964) *Biochem. J.* **91**, 222.
- Reisfeld, R. A., Lewis, U. J. and Williams, D. E. (1962) *Nature (London)* **195**, 281.
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680.
- Chambers, R. E. and Clamp, J. R. (1971) *Biochem. J.* **125**, 1009.
- Allen, A. K., Neuberger, A. and Sharon, N. (1973) *Biochem. J.* **131**, 155.

## Aims

The presence of multiple forms (high MW enzyme I and low MW enzyme II) of  $\alpha$ -galactosidase in Vicia faba seeds was first demonstrated by Dey and Pridham (14, 28, 55). Sephadex-gel elution profiles of partially purified preparations of the enzyme from mature broad beans showed that  $\alpha$ -galactosidase I was the predominant form. It was also found by the authors that enzyme I had a significantly higher specific activity than II (66). This was thought to be physiologically significant in that an enzyme with high specific activity would efficiently degrade reserve digosaccharides at the onset of germination. However in 1977, Hustler (77) showed that crude extracts of  $\alpha$ -galactosidases from dormant broad beans contained higher levels of  $\alpha$ -galactosidase II, contrary to what was shown in partially purified extracts of the enzyme. Hustler (77) maintained that  $\alpha$ -galactosidase II was being converted to  $\alpha$ -galactosidase I during the purification procedure. Although in vitro reactions were responsible for the conversion, the nature of the factor(s) involved in this process was not defined. One aim of the project was to assess, as far as possible, the in vivo levels of enzyme I and II, and also to investigate the factor(s) that might influence the interconversion of the two forms.

Another objective was to determine possible molecular relationships between the multiple forms. The working hypothesis had always been that  $\alpha$ -galactosidase I was a tetramer of II (MW $\sim$ 40,000). It was later shown that II could be resolved into two active forms II<sup>1</sup> and II<sup>2</sup> (67, 68). It was, therefore, of interest to investigate the relationship of II<sup>1</sup> and II<sup>2</sup> with enzyme I. Immunological interactions as well as amino acid and carbohydrate analyses were examined to show any correlations. During the later part of this work, reports were published showing that some legume  $\alpha$ -galactosidases displayed lectin properties (170, 172). It was therefore thought to analyse V. faba  $\alpha$ -galactosidases for haemagglutinin activity. This activity would offer interesting possibilities for subunit aggregation of the enzymes as

well as binding to cell components. However, this aspect was touched only briefly and the objective was to give lead for future work.

### Achievements

The results presented in Part I of the "Results and Discussion" section of this thesis clearly show that it is virtually impossible to extrapolate the results from *in vitro* experiments to the *in vivo* situation as far as the levels of  $\alpha$ -galactosidases I and II in the mature seeds are concerned. However, the results do indicate that  $\alpha$ -galactosidase I is present *in vivo* in spite of the observed inter conversion between the two forms during extraction. This study brings to light the problems one faces when extracting multiple forms of an enzyme and it would appear, therefore, that great caution is needed when relating the pattern of multiple forms found *in vitro* to that which might exist in the tissue.

Purification of  $\alpha$ -galactosidases I, II<sup>1</sup> and II<sup>2</sup> was achieved through the earlier reported procedure except that the glycoprotein nature of the enzyme was taken advantage of and an affinity step using Con A-Sepharose was incorporated. All three forms appeared homogeneous on polyacrylamide gel electrophoresis; some heterogeneity was observed on SDS-PAGE. Antisera to the three forms were successfully raised.

As far as the relationships between enzymes I, II<sup>1</sup> and II<sup>2</sup> are concerned firstly the SDS-PAGE pattern indicated that enzyme I was a tetramer of II<sup>2</sup>. This was further supported by immunological evidence. This technique also showed partial relationship of enzyme I with II<sup>1</sup> and II<sup>1</sup> with II<sup>2</sup>. The conclusions from the carbohydrate and amino acid analyses were, however, not as conclusive. It was to be expected that if enzyme I was a tetramer of II<sup>2</sup>, molar ratios of the carbohydrate contents should be comparable. However,  $\alpha$ -galactosidase I had a higher carbohydrate content than II<sup>2</sup>. Considering the glycoprotein nature of the enzyme, it is likely that out of a mixture of enzyme-protein species with varying degrees of glycosylation,

only those with a greater carbohydrate content are able to aggregate resulting in enzyme I.

Unlike the work reported from Professor Shannon's laboratory (170, 172), all three forms of  $\alpha$ -galactosidase from V. faba showed haemagglutinin property in addition to catalytic activity. However, the present work does not entirely clarify whether this haemagglutinin activity is due to favin (a glucose/mannose specific lectin) bound to the enzyme glycoprotein.

In conclusion, it can be said that mature V. faba seeds contain a very interesting group of structurally related  $\alpha$ -galactosidases consisting of two monomeric forms II<sup>1</sup> and II<sup>2</sup>, and a tetrameric form, I. All the evidence obtained so far points to the tetramer being composed of II<sup>2</sup> monomers. However, we have not been able to relate II<sup>1</sup> and I with respect to aggregation. It is possible that II<sup>1</sup> is first transformed to II<sup>2</sup> before aggregating to I; further work is required to verify this possibility.

The lectin nature of these  $\alpha$ -galactosidases provides a possibility of compartmentalization of the enzymes and hence separating them from their natural substrates in developing seeds.

The present work has aided towards a better understanding of the  $\alpha$ -galactosidases in Vicia faba seeds and opened new areas for future research.