LECTIN NATURE OF ~~*VICIA FABA*~~ α-GALACTOSIDASE I

FROM "VICIA FABA"

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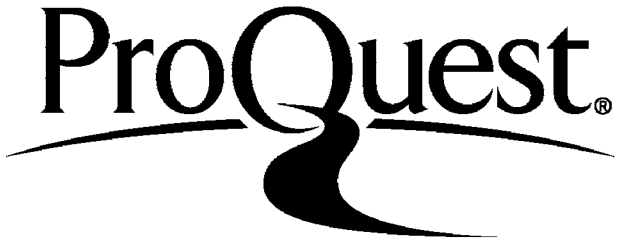

To Bhagawan

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ABSTRACT

α−Galactosidase I (EI) preparations from *Vicia faba* seeds have been reported to possess glucose/mannose-specific lectin activity. Attempts have been made to obtain more information concerning the structural component responsible for this activity. In particular, the enzyme has been examined to see whether it is contaminated with the 'classical' lectin, favin, which also occurs in *V. faba* seeds.

EI and favin were purified and new methods tried in an endeavour to improve the purification procedures. These included the use of an EI-antibody column for EI and a Sephadex G-100 affinity column for favin.

The EI preparation was examined by a number of analytical techniques including SDS-PAGE, isoelectric focusing and chromatofocusing. No evidence of contamination by favin was revealed. Microheterogeneous subunits of EI were detected with both α−galactosidase activity and haemagglutinin activity.

Immunoprecipitation experiments with antibodies for favin and EI have shown that the two proteins are structurally related. Western blotting experiments with EI using favin antibodies revealed the M_r 40,000 monomeric subunit of the enzyme and a minor component with M_r 20,000 ie. a peptide similar to the β−subunit of favin, which is known to contain the glucose/mannose-combining sites of this lectin. Western blotting experiments with EI using EI antibodies showed the presence of a M_r 28,000 fragment together with EI monomer. However, the former band was not seen on the Western blot using favin antibodies.

When Western blotting experiments were carried out using favin β−subunit antibodies, as with favin antibodies, the enzyme monomer was stained and the peptide with M_r 20,000 was again detected in the EI lane. However, when this experiment was repeated and an empty well was placed between the wells that contained EI and favin, no band corresponding to M_r 20,000 (or M_r 28,000) was detected in the EI lane. This observation indicated that the M_r 20,000 (or M_r 28,000) component observed in EI in the previous Western blots was the result of excess favin flowing across to the adjoining well containing EI.

As EI and favin cross-reacted with antibodies to EI, favin and β−subunit and as no contaminant appeared to be present it is concluded that the lectin properties displayed by EI is probably due to an amino acid sequence in the enzyme which is homologous to favin.

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LIST OF ABBREVIATIONS

M_r	Molecular weight
TCA	Trichloroacetic acid
V. faba (Fava bean)	*Vicia faba*
PBS	Phosphate Buffer Saline
I(EI), II, II^1 and II^2	α−Galactosidases from *V. faba*
Favin	Classical Lectin from *V. faba*
HRP	Horse-Radish Peroxidase
SDS	Sodium Dodecyl Sulphate
Temed	N,N,N′,N′ -Tetramethylene diamine
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
PMSF	Phenylmethane Sulphonyl Fluoride
PCMB	P-Chloromercuribenzoate
EPNP	1,2-Epoxy-3(p-nitrophenoxy) propane
TLCK	N-Tosyl-L-Lysine Chloromethyl Ketone
TPCK	N-Tosyl-L-Phenylalanine Chloromethyl Ketone
Con A	Concanavalin A
3-O-methyl...column	3-O-methyl-N-hexanoyl-glucosamine-Sepharose column
DBA	Dibromoacetone
IA	Iodoacetamide
Gal	Galactose
Man	Mannose
PNPG	P-nitrophenyl-α−D-galactopyranoside

INTRODUCTION

1.1 Occurrence

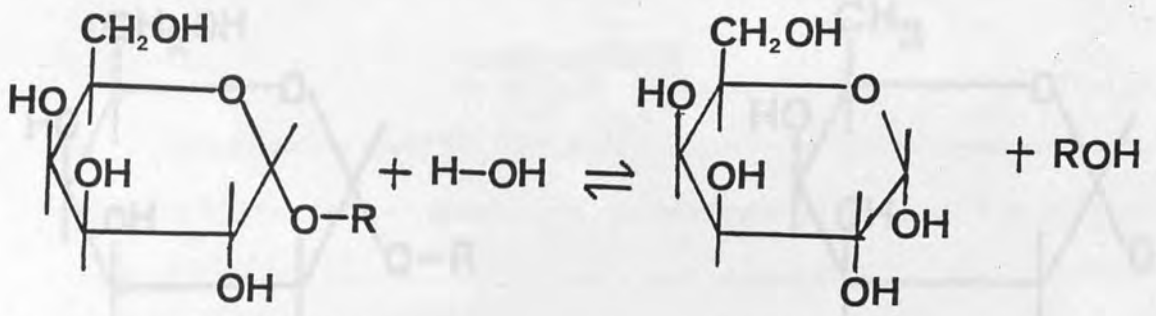
α−Galactosidases are found widely distributed in nature in plants [1-6], animals [7-11], and micro-organisms [12-16]. In plants the enzyme is usually distributed throughout the tissues including leaves, roots, stems and bark [17]. Particularly high levels of activity have been detected in the seeds [18-19] of many leguminous plants; in most cases (but not with exalbuminous seeds) activity is higher in the germinating seeds than in the resting forms [20].

Aqueous buffers are normally used to extract the enzyme from plant tissues and a high percentage of the total activity is usually obtained in the soluble fraction. However, in some instances the activity is bound to water-insoluble material. In spinach leaves, for example, bound activity is present in the mitochondrial, chloroplast and microsomal fractions [21] whilst in mature *Cucurbita pepo* leaves the enzyme is present in cell wall fractions [22]. Recent studies have also shown the presence of α−galactosidase in plant cell vacuoles [23] and in protein bodies [24].

In most instances ammonium sulphate and acid precipitations, gel filtration, ion exchange and affinity chromatography have been used for the purification of the soluble enzymes [25]. However, they have only been obtained on a few occasions as homogenous preparations; eg. from the seeds of *Vicia faba* [25], *Saccharomyces carlsbergensis* [26], *Medicago sativa* [27], and *Bacteroides ovatus* [28].

1.2 General Properties

α−Galactosidase hydrolyses the glycosidic bonds of compounds which contain non-reducing terminal α−D-galactopyranosyl residues. Although it is a reversible reaction the equilibrium strongly favours hydrolysis:



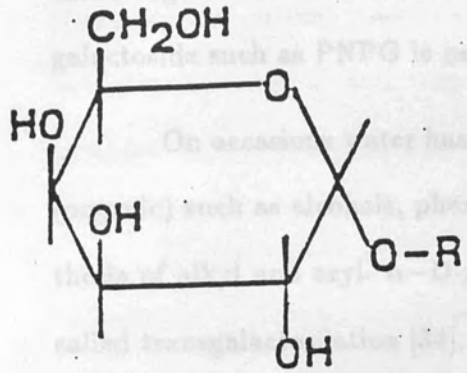
α-D-galactopyranoside D-galactose

In the above reaction R may represent alkyl, aryl, monoglycosyl or polyglycosyl groups. In general, the specificity of action of glycosidases is directed towards the glycon part of the substrate [29]. Thus, changes in the configuration of the -OH groups of the glycone moiety can either slow down or completely arrest the reaction. The configurations at C-1, C-2, C-3 and C-4 are critical for the hydrolysis. Hence, this enzyme will not hydrolyse β−D-galactosides or, for example, substrates that contain terminal D-glucose or D-mannose residues with changed configurations at C-4 and at C-2 and C-4, respectively. However, α−D-fucopyranosides, β−L-arabinopyranosides and D-glycero- α−D-galactoheptosides are hydrolysed by the enzyme as these compounds possess hydroxyl configurations at C-1, C-2, C-3 and C-4 which are the same as those in α−D-galactopyranose (Fig. 1) [30-31]. Substituents at C-5 of the glycon have relatively little effect on the rate of hydrolysis by α−galactosidase.

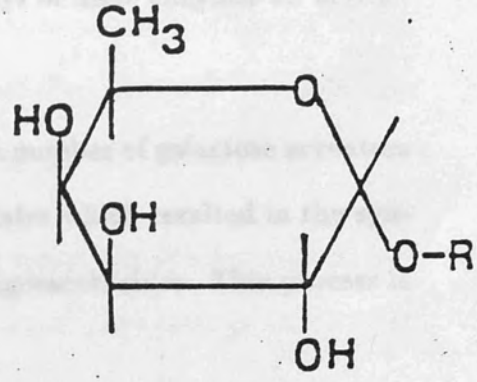
Changes to the aglycon moiety of the substrate have only a limited effect

Figure 1

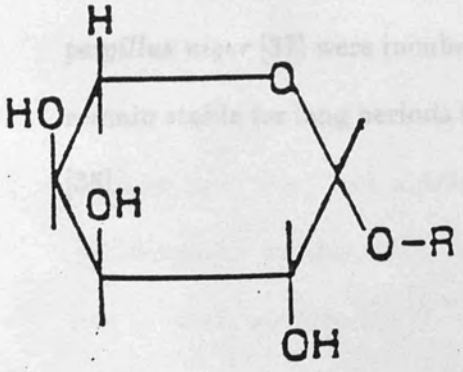
α-D-Galactopyranoside and related glycosides



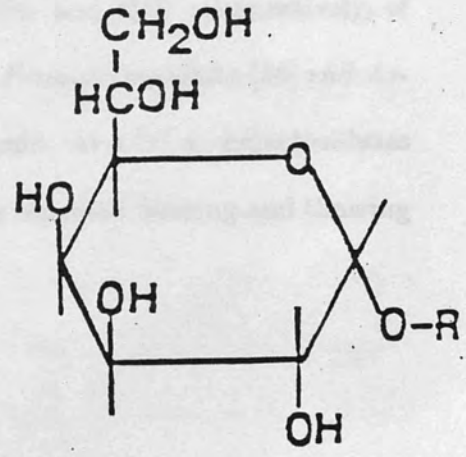
α-D-Galactopyranoside



α-D-Fucopyranoside



β-L-Arabinopyranoside



D-Glycerol-α-D-galactopheptoside

on the rate of hydrolysis. Thus, α−galactosidases from *Calvatia cyanthiformis* [1] and *Prunus amygdalus* [32] are able to hydrolyse methyl and phenyl α−D-galactosides and naturally occurring oligosaccharides such as melibiose, raffinose and stachyose. However, aryl-D-galactosides are better substrates than the latter three oligosaccharides and hence for standard assays of these enzymes an aryl-D-galactoside such as PNPG is usually used [33].

On occasions water has been replaced by a number of galactose acceptors (organic) such as alcohols, phenols and carbohydrates which resulted in the synthesis of alkyl and aryl- α−D-galactosides and oligosaccharides. This process is called transgalactosilation [34].

The pH optima for most α−galactosidases are acidic, usually within the pH range 4 to 6. However, in the case of α−galactosidase IV from *Cucurbita pepo*, an alkali pH optimum was recorded [35].

α−Galactosidases in general show stability over a wide range of pH values and temperatures. The latter has enabled the purification of the enzyme to be achieved at room temperatures, conditions under which many other enzymes lose substantial amounts of activity. Approximately 70% and 75% , respectively, of the activity remained when α−galactosidases from *Prunus amygdalus* [36] and *Aspergillus niger* [37] were incubated at 55 ^{0}C for 20 min. At 4 ^{0}C α−galactosidases remain stable for long periods but activity is lost on repeated freezing and thawing [38].

1.3 Multimolecular Forms

As methods for the fractionation of enzyme proteins improved, the existence of multimolecular forms of many became evident and the term "isoenzyme" was eventually introduced to describe any form of enzyme heterogeneity [39]. However, the current recommendations are that those proteins or glycoproteins with similar catalytic activities which are composed of polypeptide subunits, the products of separate genes, should alone be classified as "isoenzymes".

In addition to genetic factors, enzyme heterogeneity can be caused by : (a) artefact formation due to deamidization, binding of substrates or proteolytic action and (b) aggregate formation produced by the association of monomer units [40].

Multimolecular forms of many enzymes from plant sources have been observed. Separations of these have been achieved on the basis of differences in M_r (eg. using gel-filtration) or ionic charge (eg. using ion-exchange chromatography or gel-electophoresis) [41].

The first glycosidase, and one of the first enzymes, to be reported to occur in multiple forms was $\alpha-$galactosidase from coffee beans which was resolved into two fractions by Courtois and Petek in 1961 using an alumina column [42]. Apart from studing pH optima and substrate specificity, the French workers did little to show how the forms differed. However, in 1968 Dey and Pridham [43] used gel- filtration to demonstrate that extracts of resting *V. faba* seeds contained two $\alpha-$galactosidase fractions (I and II) with a large difference in M_r. A full kinetic study carried out on these two forms indicated that $\alpha-$galactosidase I (M_r 209,000) possessed a specific activity which was 10-15 greater than form

II (M_r 40,000) [44-45]. A later investigation showed that enzyme II could be further separated on a cation exchange column into two fractions, II^1 and II^2 [46]. Sedimentation equilibrium studies indicated that I, II^1 and II^2 had M_r values of 160,000 ± 2850, 45,730 ± 3073 and 43,390 ± 1409 respectively. Polyclonal antibodies raised against the three forms all showed cross reactivity although I and II^2 appeared to exhibit a greater degree of homology than I and II^1 when immunotitration was carried out [47].

An examination of I by SDS-PAGE considered in conjunction with the M_r values and the immunological data suggested that I was a tetramer of II^2 [25].

1.4 Interconversion

Protein molecules can often aggregate and disaggregate in solution in response to changes in pH, protein concentration, salt concentration and long term storage [40,48].

For example, extraction of β−galactosidase from human liver with phosphate buffers containing higher salt concentrations resulted in the aggregation of the low M_r form A (M_r 80,000) to produce a structure with M_r 160,000-170,000.

In the case of *V. faba* α−galactosidases, a tetrameric α−galactosidase similar to native form I was produced on storage of form II at 4 ^{0}C in McIlvaine buffer (pH 5.5) for several days [50].

Aggregation has been also observed in the case of the monomer α−galactosidase II from *Lens culinaris* which was converted to the tetrameric form upon concentrating the enzyme solution [51].

Interconversion has also been observed in the case of multiple forms of

α–galactosidase from coconut (*Cocos nucifera*) where form B (M_r 21,400) was converted into form A (M_r 123,000) in the presence of a high M_r enzymically inactive form C [52-53].

Dey and co-workers examined in detail the factors affecting the interconversion of the multiple forms of *V. faba* α–galactosidase. It was discovered that some routine procedures used in enzyme purification, such as acid precipitation and gel-filtration at high salt concentrations, could significantly affect the relative proportions of the various forms [54]. There is, therefore, a need for caution when attempting to relate enzyme forms occurring *in vitro* with those found *in vivo*.

1.5 Galactose Reserves and the Role of α–Galactosidase in Plants

In addition to sucrose, an ubiquitous mobile reserve substance in plants, and storage polysaccharides such as starch and fructans, there are also galactose-containing oligosaccharides and polysaccharides which function as reserves [55-57], particularly in seeds. Amongst the numerous oligosaccharides that have been isolated from plant tissues is the 'raffinose family' of sugars which have structures based on sucrose where the glucosyl moiety is substituted with α–D-galactopyranosyl residues (n = 1 to 7) at C-6 (Fig. 2) [58].

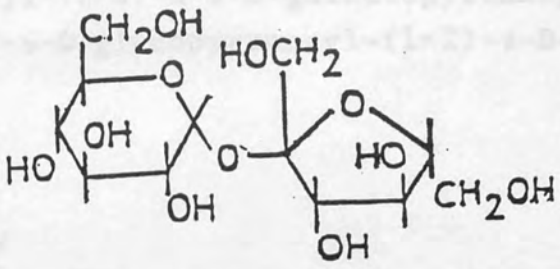
The tri-, tetra-, penta- and hexa-saccharides of this family have been given the trivial names raffinose, stachyose, verbascose and ajugose, respectively. The lower homologues in this series are translocated in the phloem [59] as well as stored in leaves [60], roots [61] and seeds, and the higher members only appear to be present in storage organs. Other galactosylated sucroses with substitution on the fructose residue or on both the fructose and glucose moieties are known but have received little attention from biochemists [62-63].

The polysaccharide galactomannan, is stored in large quantities in the seeds of many albuminous species where it is an important reserve substance which is required on germination [64-65]. The detailed structure of this material is species specific, but all galactomannan possess a $\beta 1 \rightarrow 4$-linked 'backbone' with varying numbers of α–D-galactosyl side chains linked to C-6 of the mannose residues [56].

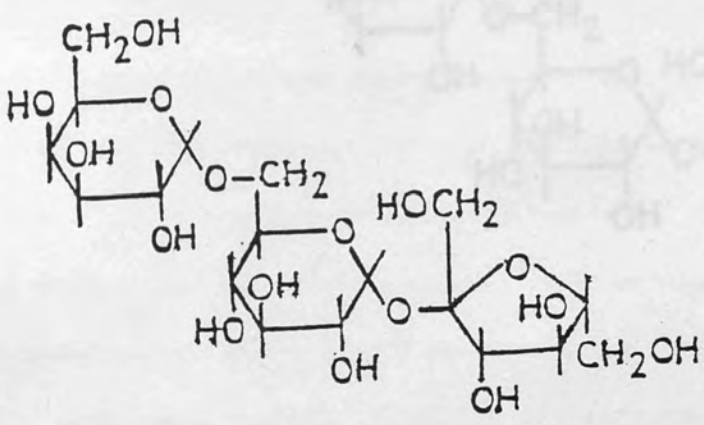
Figure 2a

The Raffinose family of oligosaccharides

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



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



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

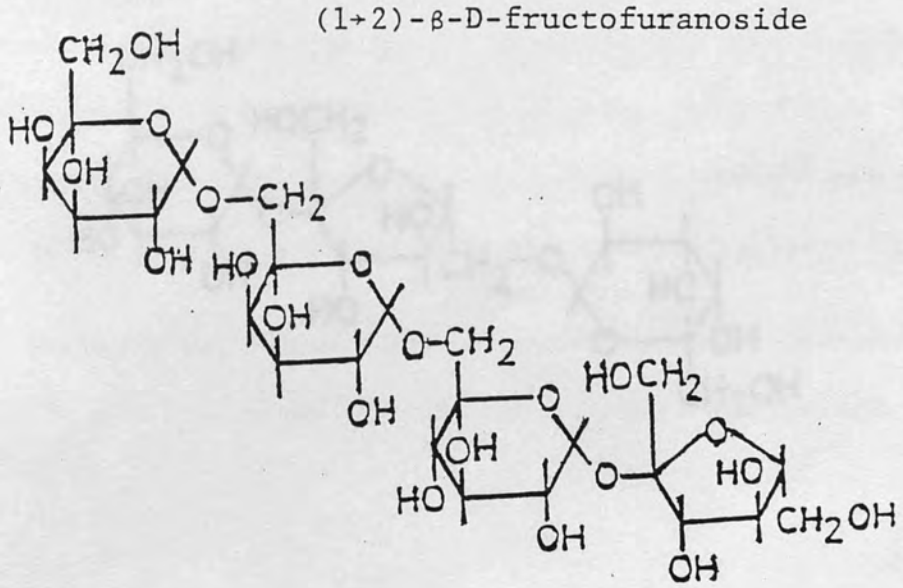


Figure 2 a (continued)

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

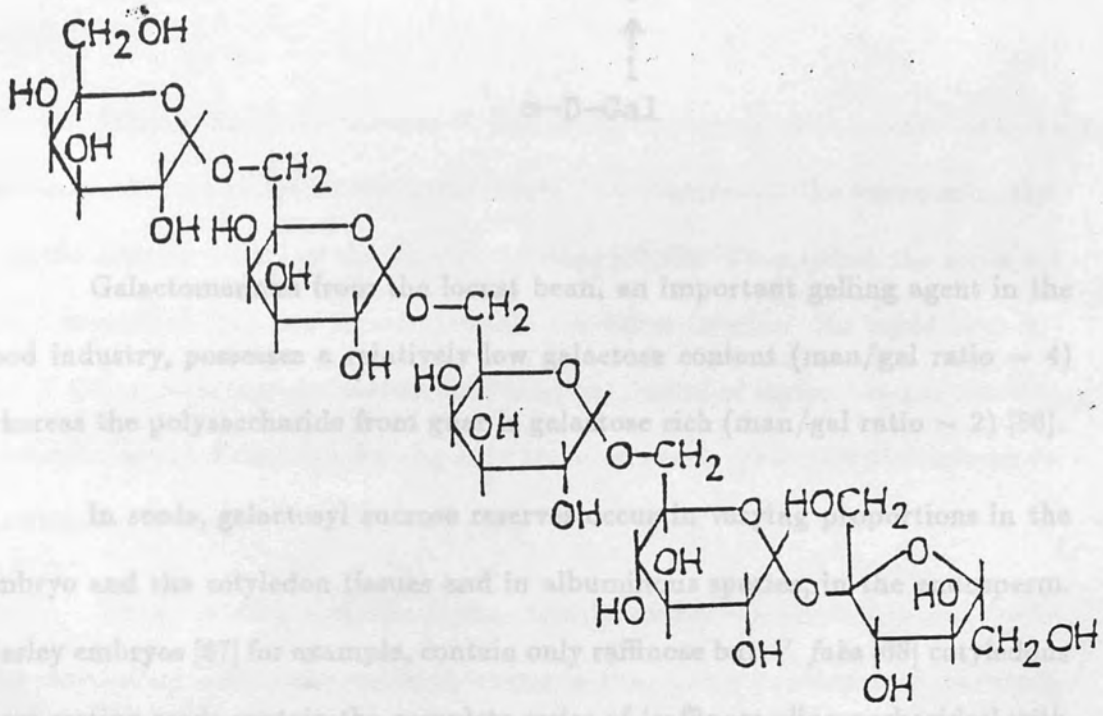
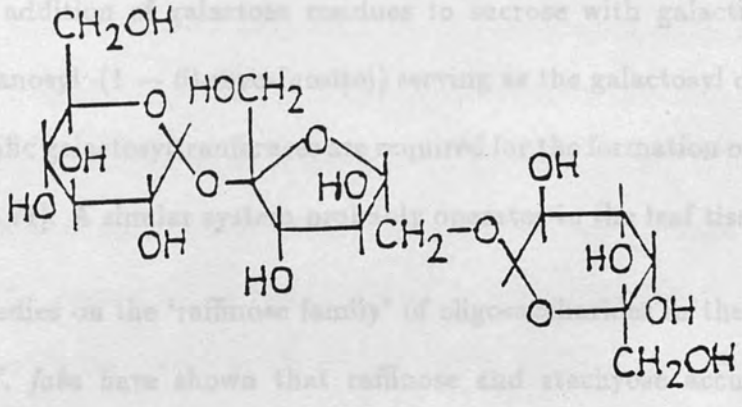
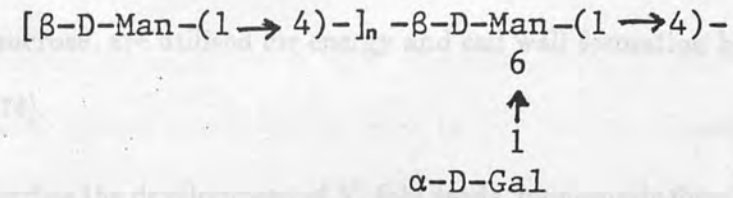


Figure 2b

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





Galactomannan from the locust bean, an important gelling agent in the food industry, possesses a relatively low galactose content (man/gal ratio $\sim$ 4) whereas the polysaccharide from guar is galactose rich (man/gal ratio $\sim$ 2) [66].

In seeds, galactosyl sucrose reserves occur in varying proportions in the embryo and the cotyledon tissues and in albuminous species, in the endosperm. Barley embryos [67] for example, contain only raffinose but *V. faba* [68] cotyledons from resting seeds contain the complete series of 'raffinose oligosaccharides' with stachyose predominating. Raffinose and stachyose are probably present in the leaves of most dicotyledonous plants [66] and can be translocated within the plant [66,69].

The synthesis of raffinose and its homologues in seeds is achieved by sequential addition of galactose residues to sucrose with galactinol (O- α-D-galactopyranosyl -(1 $\rightarrow$ 6)-myo-inositol) serving as the galactosyl donor [70]. Different specific galactosyltranferases are required for the formation of each oligosaccharide [66,71]. A similar system probably operates in the leaf tissues [72-73].

Studies on the 'raffinose family' of oligosaccharides in the exalbuminous seeds of *V. faba* have shown that raffinose and stachyose accumulate as the

seeds mature [55,56]. Parallel to this is an increase in the total activity of α–galactosidase [55]. During the early stages of germination these oligosaccharides are rapidly hydrolysed by the action of this enzyme and the products, galactose and sucrose, are utilised for energy and cell wall formation by the developing seedling [74].

During the development of *V. faba* seeds, monomeric form(s) of α–galactosidase are first synthesised and in the latter stages of maturation the tetrameric, high specific activity form I of the enzyme appears [46,48]. Thus, when the seeds are fully developed they are in a favourable condition to effect the rapid hydrolysis of the oligosaccharide reserves following imbibition of water. As germination proceeds form I disappears leaving only the monomeric form(s) with low specific activities.

The co-existence of the oligosaccharides and α–galactosidase activity in the developing exalbuminous seeds suggests that there are separate compartments for the enzyme and its potential substrates, otherwise the oligosaccharides would be hydrolysed [41]. This suggestion is supported by the observation that α–galactosidase is localised in seed protein bodies [75].

Most studies on 'galactosyl reserve' α–galactosidase systems have been carried out with seeds: other plant organs mainly contain reserve oligosaccharides. *Cucurbita pepo* leaves have been studied: this plant synthesizes stachyose from sucrose in its mature leaves and then transports the tetrasaccharide via the phloem to the young developing leaves where it is metabolised [76,35]. It has been shown that the leaves contain several forms of α–galactosidase. Three of the forms (LI, LII and LIII) possess pH optima in the acidic range [76] whilst the fourth form (LIV) has optimum activity at pH 7.5 [35]. LIV is highly specific for stachyose and

appears to decrease in activity *in vivo* as the leaf progresses from the immature to the mature 'exporting' state. It is believed that this form may be responsible for the hydrolysis of the stachyose in the immature 'importing' leaves [35].

Despite the high level of 'acid' $\alpha-$galactosidase activity that can be extracted from mature leaves, no activity *in vivo* can be demonstrated. Hence 'freeze-blocking' the petiole causes a large accumulation of raffinose and stachyose in the lamina but no free galactose is produced. This is probably because the 'acid' $\alpha-$galactosidases are cell wall bound. In the translocation process it must be assumed that stachyose is loaded in to the phloem symplastically, hence avoiding the apoplastic enzyme. In the immature leaves, unloading the phloem presumably brings stachyose into contact with the symplastic, alkaline $\alpha-$galactosidase which causes hydrolysis.

Although exalbuminous seeds in general contain high levels of the 'raffinose family' of oligosaccharides as food reserves they are devoid of any detectable amounts of galactomannan. Albuminous seeds on the other hand commonly contain relatively high levels of galactomannan and low levels of 'raffinose oligosaccharides' [77]. Studies on the deposition of galactomannan in the endosperm of developing seeds of fenugreek (*Trigonella foenum-graecum*) have revealed that this process begins near to the embryo and in cells adjacent to the cotyledons and ends in the outer periphery of the endosperm [78]. The single layer of cells surrounding the endosperm, the aleurone layer, remains unfilled. This layer plays a key role in the post-germinative catabolism of galactomannan [79].

Little knowledge has so far been accumulated regarding the mechanism of biosynthesis of galactomannan in seeds. Working on fenugreek seeds Reid and Meier [80] showed that the 'raffinose family' of oligosaccharides (mainly

stachyose) is deposited in the endosperm concurrently with galactomannan. Recent studies by Campbell and Reid [81] on galactomannan formation have shown that homogenates prepared from developing endosperms of fenugreek seeds can catalyse the transfer of D-[^{14}C]galactosyl units from UDP-D-[U $-^{14}$ C]galactose to galactomannan. GDP-D-mannose appears to be the mannose donor for the synthesis of the galactomannan 'backbone'.

Studies on the depletion of the 'raffinose family' of oligosaccharides and galactomannan in albuminous seeds have shown that this occurs in two phases. In the case of fenugreek seed endosperms which contain galactomannan (the main polygalactosyl reserve) stachyose is also present together with smaller amounts of raffinose, verbascose and sucrose. In the first 24 hr of germination there is a rapid depletion of the 'raffinose oligosaccharides' along with a build-up of sucrose and galactose: no free mannose is detectable. During the next 24 hr there is a marked decrease in the galactomannan content and finally all oligo- and polysaccharides in the endosperm are removed [77]. $\alpha-$Galactosidase activity was measured separately in the embryo and the endosperm over the same time intervals [82]. Very low levels of activity were detected in the endosperm in the early stages of germination but the activity increased significantly in the latter stages and this was considered to be associated with the galactomannan breakdown. In contrast $\alpha-$galactosidase activity in the embryos was at a high level in resting seeds and remained so throughout germination: this activity is presumably required for the early mobilisation of oligosaccharide reserves in the embryo which contains no galactomannan [82].

A detailed study of the hydrolysis of galactosyl reserves and the role of $\alpha-$galactosidase was carried out by Mcleary and Matheson working on the

seeds from lucerne (*Medicago sativa*), guar (*Cyamopsis tetragonolobus*) and carob (*Ceratonia siliqua*) which are all albuminous legumes [83]. Three different forms of α−galactosidase were present in all species which were separable by a combination of ion-exchange chromatography and gel-filtration techniques. The different forms displayed tissue specificity; forms A and B were confined to the embryos of all three species whilst form C could only be detected in the endosperms of lucerne and guar seeds, but was present in both the endosperm and embryo in the case of carob seeds [83].

During germination the total α−galactosidase activity in all of the three species increased. However, when the activities of the individual forms were analysed, A and B showed a slight increase during the initial stages of germination followed by a gradual decrease in later stages [83]. Regarding form C, there was a gradual increase in activity during the early stages of germination followed later by a rapid increase and finally a decrease in activity. In time course experiments it was noticed that the increase in the activity of form C coincided with the galactomannan depletion thereby suggesting that the primary role of this enzyme was to hydrolyse the polysaccharide reserves. The small amount of α−galactosidase C activity present in the early stages of germination may be concerned with the removal of the low levels of oligosaccharides which accompany galactomannan in the endosperms. Forms A and B of the enzyme presumably degrade the oligosaccharides in the embryos [83].

All three of the above forms of α−galactosidase were also reported by Mcleary and Matheson to be present in soya beans (*Glycine max*) and the tissue distribution was the same as in the three other species. However enzyme C was at a very low level in the endosperm of soya which may relate to the small

galactomannan reserves accumulating in the seeds from this species [83].

The mannan 'back-bones' of galactomannans are hydrolysed by the combined action of β−mannanase and β−mannosidase [84].

Galactosyl residues that are released on germination from galactomannan and galactosyl sucroses by α−galactosidase are rapidly metabolised. Very little galactose accumulates in intact seeds. The galactose moves from the endosperm to the cotyledon/embryo tissues by simple diffusion. It is proposed that the liberated galactose is here converted to galactose-1-phosphate by the action of galactokinase. This enzyme has been found, for example, to be very active in the germinating pea seeds [85]. Galactose-1-phosphate can then be converted to UDP-D-galactose by the action of a pyrophosphorylase and this in turn to UDP-D-glucose by the action of UDP-D-galactose-4′-epimerase [66]. The above reactions and the utilization of UDP-D-glucose and UDP-D-galactose by the growing seedling are outlined in Fig. 3.

Although α−galactosidase is undoubtedly involved in the mobilisation of galactose-containing oligo- and poly-saccharide reserves there has been little work on the regulation of these processes apart from the possible involvment of cell 'compartments' and the existence of different α−galactosidases for hydrolysing low and high M_r reserves. This is discussed above.

Spyropoulos and Reid [86] have tentatively proposed a regulatory mechanism for galactomannan breakdown in germinating fenugreek seeds. They showed that in endosperms isolated from seeds that had been incubated for 5 hr in a small volume of water (as opposed to a large volume) α−galactosidase activity was inhibited and galactomannan breakdown decreased. The α−galactosidase

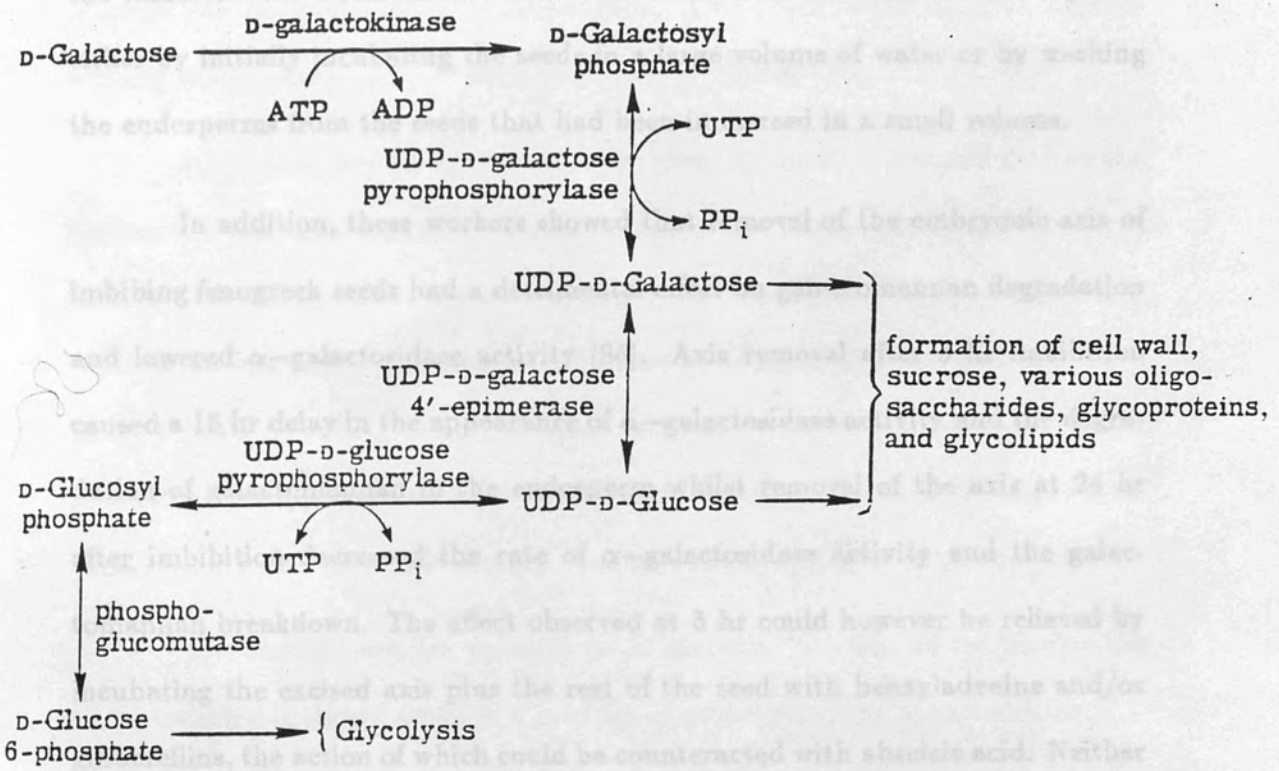


Figure 3

Metabolism of D-galactose in plants

inhibition observed could be prevented by pre-washing the endosperms and reimposed by adding back the washings. The above observations were explained on the basis that the newly imbibed endosperms contained a compound (or compounds) which inhibited α−galactosidase synthesis in the aleurone layer. Furthermore, the inhibitor was water soluble and could be leached out from the endosperms either by initially incubating the seeds in a large volume of water or by washing the endosperms from the seeds that had been immersed in a small volume.

In addition, these workers showed that removal of the embryonic axis of imbibing fenugreek seeds had a detrimental effect on galactomannan degradation and lowered α−galactosidase activity [86]. Axis removal after 5 hr imbibition caused a 15 hr delay in the appearance of α−galactosidase activity and the degradation of galactomannan in the endosperm whilst removal of the axis at 24 hr after imbibition decreased the rate of α−galactosidase activity and the galactomannan breakdown. The effect observed at 5 hr could however be relieved by incubating the excised axis plus the rest of the seed with benzyladenine and/or gibberellins, the action of which could be counteracted with abscicic acid. Neither of these treatments (gibberellin and/or benzyladenine) however could relieve the inhibition brought about by the axis excision at 24 hr.

Based on these observations Spyropoulos and Reid proposed a dual role for the embryonic axis in the regulation of the degradation of galactomannan in fenugreek seeds. In the imbibition stage it is assumed that the embryonic axis controls the synthesis of α−galactosidase and that this role is mediated by diffusible factors possibly gibberellin and/or benzyladenine which presumably counteract the diffusible inhibitor in the endosperm. Following germination, it appears that the axis acts as a sink for the galactomannan breakdown products released in the

endosperm and taken up by the cotyledons.

Working on lettuce seeds (*Lactuca sativa L.*), Leung and Bewley [87-88] have shown that exposure to red light or treatment with gibberellic acid causes an increase in the level of $\alpha-$galactosidase activity in germinating seeds and that this increase can be inhibited by cyclohexamide. This effect of red light on enzyme activity is not observed if the embryonic axis is removed from the seed.

Further studies on lettuce seeds by these workers have shown that in the presence of red light, axis plays a crucial role in the increase in $\alpha-$galactosidase activity [88]. Removal of the axis at any stage during imbibition, while being exposed to red light, inhibited the increase in $\alpha-$galactosidase activity. The activity could however be relieved if the detipped seeds were incubated with the irradiated tips. On the other hand there was no recovery in activity if the detipped (irradiated or non- irradiated) seeds were incubated with non-irradiated axis.

The requirement for the axis could however be replaced by placing the non-irradiated detipped seeds in a mixture of gibberelins and benzyladenine.

On the basis of these observations it was concluded that on exposure to red light the axes produced and/or released a diffusible factor to the cotyledons (and possibly to endosperm) which caused *de novo* synthesis of $\alpha-$galactosidase protein.

1.6 Lectins

1.6.1 Nature and Properties

Proteinaceous materials that agglutinate red blood cells have been known since 1888 when such a haemagglutinin (or phytohaemagglutinin) was first detected in crude extracts of castor bean [89]. In recent times the name "haemagglutinin" has been replaced by "lectin", a term used to describe all proteins and glycoproteins of non-immune origin which combine with carbohydrate derivatives but do not covalently modify the combining ligands [90-91]. This, therefore, distinguishes lectins from enzymes which have carbohydrates as substrates. Lectins may or may not agglutinate red blood cells which requires multiple cross-linking of the cells via their surface glycoprotein glycosyl residues with the formation of a complex structure which eventually 'clots'. Lectins with one carbohydrate binding site per molecule [92-93], eg. ricin and abrin, obviously cannot achieve the necessary cross-linking. The activities of multivalent lectins (ie. those with more than one carbohydrate binding site) are measured in haemagglutinin units (HA) [90,94]. One unit is defined as the amount of lectin which produces 50% agglutination of red blood cells under standard conditions. Agglutination is usually measured by visual means and, hence, subject to about 20% error. An adaptation of the method using a spectrophotometer is not popular as relatively large amounts of lectins are required and the procedure is tedious [95].

An alternative method of investigating the carbohydrate binding capacity of lectins is to replace red blood cells with specific polysaccharides or glycopeptides and then measure the resulting turbidity spectrophotometrically [90].

Many lectins from plant [96], microbial [97] and animal [98] sources have

now been purified. The first to be purified to homogeneity was Concanavalin A (Con A) from jack bean seeds [99] and approximately 100 others, since, have been fully or partially purified.

Seeds are a particularly rich sources of lectins, although their presence has also been noted in other plant organs including leaves [100], fruits [101] and roots [102]. In addition to being present as soluble cytoplasmic constituents they have also been located bound to the cell walls [103], vacuoles [104] and protein bodies [105]. Separation of lectins from other proteins is normally based on the use of affinity columns containing immobilised glycosyl residues to which the lectins combine [106-108]. Elution is achieved with concentrated carbohydrate solutions which compete with the column-bound residues for the binding sites on the lectin.

Lectins exhibit specificity towards the glycosyl residues to which they bind. These may be single, terminal, non-reducing residues or occasionally one or more glycan intra-chain units [109-110].

The carbohydrate specificity of a lectin is determined by the hapten inhibition test, developed by Landsteiner [111] to study antibody specificity. Basically solutions of different concentrations of a range of carbohydrates are tested to see which is most effective in inhibiting haemagglutination. Most lectins exhibit specificity towards one of the following sugars [90]: D-mannose (D-glucose), 2-Acetamido-2-deoxy-D-glucose, D-galactose, 2-Acetamido-2-deoxy-D-galactose and Fucose.

The configuration of the hydroxyl group at C-4 is an important factor in defining specificity. However, many lectins are able to tolerate variation of the configuration at C-2. For example, Con A and favin (from *V. faba*) interact with

both mannose and glucose residues [112-113]. Some lectins also appear to tolerate variation at C-3 of the combining sugar. Lectins may also display specificity towards the anomeric configuration of glycosyl residues as is seen with Con A and the lectin from *Bandeiraea simplicifolia* [114-115]. Both lectins display specificity towards the α anomers of mannose (glucose) and galactose respectively. However, *Glycine max* [116] and *Ricinus communis* [117] lectins show no preference for a particular anomeric configuration.

The majority of plant lectins have been shown to be glycoprotein in nature. However, there are exceptions as in the case of Con A [118]. Results of *in vitro* translation experiments have shown that Con A is initially synthesised as a glycoprotein [119], but that there is post-translational modification which removes the glycan chains to release the protein prior to its final deposition in protein bodies [119,120].

Plant lectins are generally dimers or tetramers and their M_r values range between 35,000 for wheat germ agglutinin [121] and 265,000 for lima bean lectin [122].

The examination of the primary structure of lectins from 10 species of leguminosae has shown that they possess extensive homology [123]. When the polypeptide chains of these lectins were aligned in a circular permutation, amino acids at specific points were shown to be invariant or highly conserved. These amino acids included the two in Con A responsible for binding Ca^{2+} and Mn^{2+}, and the amino acids which contributed to the three dimensional structure of the hydrophobic binding cavity of Con A.

Usually each subunit of a lectin which possesses a binding site is spe-

cific for the same sugar although exceptions to this have been observed in few instances. For example with anti-B lectin of *Bandeiraea simplicifolia* a tetrameric stucture made of two different subunits, one shows specificity towards α–N-acetylgalactosamine and the other to α–galactose [124-125]. Most plant lectins require metal ions such as Ca^{2+} , Mn^{2+} and Mg^{2+} for their activity.

1.6.2 Role of Lectins

Although many lectins have been isolated from numerous natural sources their roles in nature are not well understood.

In view of the mitogenic properties of plant lectins it has been suggested that they may be involved in cell division. This concept is supported, for example, by the observation that Con A and *Phaseolus vulgaris* lectin (PHA) stimulate pollen germination [126]. However, in contrast no increased cell division was observed when soya bean root or tobacco pith segments were tested *in vitro* in the presence of PHA or soya bean agglutinin [127].

Attention has also been focused on the role of lectins in plant defence against pathogenic organisms [128]. Hence, lectins may control the response of tobacco cells to avirulent and virulent strains of *Pseudomonas solanacearum* [129]. When the avirulent strain is incubated with the tobacco tissue the bacterial cells became enveloped by plant cell wall material. The virulent bacteria on the other hand remain between the tobacco cells where they multiply. The response to the avirulent strain is thought to be mediated by a tobacco cell wall lectin which combines with the surface glycan receptors of the bacteria thus immobilizing the cells. The surface glycans of the virulent strains are structurally different and are not recognised by the plant lectin. Similar observations have been made with

other phytopathogenic bacteria and fungi [130-131].

Lectins may also control insect pests. The Brucid beetle for example, can use cowpeas (*Vigna sinensis*) as a nutritional source but not bean (*Phaseolus vulgaris*) seeds [132]. Preparations of the latter when fed to beetles are lethal but not cowpea meal. A major difference between the two species is that *Phaseolus vulgaris*, but not cowpea, possesses a toxic lectin which probably affects the digestive system of the beatle.

There are strong indications that lectins are involved in the symbiotic relationship between legumes and nitrogen- fixing bacteria. It has been shown that legumes are nodulated by specific *Rhizobium* isolates and it was postulated that this phenomenon was the result of bacterial recognition by the legume root-tip lectin [133-135]. This theory is supported by the fact that lectin isolated from soya bean when tested against different strains of *Rhizobium* only agglutinates those which infect the soya bean roots. In conclusion, it would appear that soya bean lectin is present in the root tips where it mediates the specific attachment of the appropriate symbiont bacteria to the host.

1.6.3. Favin

In addition to Con A, one of the best studied lectins is favin which accompanies $\alpha-$galactosidase in the broad bean (*V. faba*) seed.

The first observation of agglutination of erythrocytes by fava bean extract was made in 1949 by Boyd and Reguera [136] who showed that it produced 'clots' with human erythrocytes of all blood groups. Cregor and Grifford [137] later showed that fava extracts could agglutinate erythrocytes from three unrelated species (guinea-pig, rabbit, albino-rat).

The carbohydrate binding properties of crude favin extracts were investigated by hapten inhibition which showed that the lectin was glucose/mannose specific [138].

Purification of favin was first achieved by Tomita and co-workers [139] using a Sephadex G-50 column. This was followed by a more detailed characterisation by Wang and colleagues [140] who isolated the lectin using Sephadex G-75 column. Mr estimation by the sedimentation equilibrium method gave a value of 50,000 whilst SDS-PAGE of the purified favin showed three bands of M_r 18,000, 16,000 and 9,000. The bands at 16,000 and 9,000 also stained with periodate-Schiffs reagent thus suggesting they were glycoproteins. On the basis of the above results and crystallographic data Wang's group proposed a tetrameric structure for favin of two M_r 18,000 and two 9,000 subunits. They confirmed that it was a glucose/mannose specific lectin.

Following the above work, Johnson and colleagues [141] also purified favin using a Sephadex G-150 column. They again identified a tetrameric structure composed of two non-identical pairs of subunits, however, the Mr values of the subunits obtained in Johnsons's group were somewhat different to the results obtained by Wang and others. Johnson and colleagues proposed a structure made of two pairs of subunits, one pair of M_r 17,400, whilst the other is M_r 14,300. As mentioned earlier, Wang's group suggested a tetrameric structure made of two pairs of subunits with M_r's 18,000 and 9,000. The presence of carbohydrate in favin was confirmed and estimated to be 3% in total.

A detailed study of the specificity of carbohydrate binding by favin was carried out by Allen and co-workers [113] who showed that 3-O-methyl substituted monosaccharides were more tightly bound than their parent sugars: this suggested

the presence of a hydrophobic region at the carbohydrate binding site of favin. Favin displays anomeric specificity being more specific for the $\alpha-$ than the $\beta-$ forms of D-glucose and D-mannose and their derivatives. Allen and colleagues [113] confirmed observations made in earlier work of the presence of a carbohydrate moiety attached to favin and they showed that it contained mannose and N-acetylglucosamine residues.

The primary and the subunit structure of favin was finally elucidated by Cunningham and co-workers [142-143] who showed it to be composed of two pairs of subunits α (M_r 5,571) and β (M_r 20,700). The $\alpha-$subunit consisted of 51 amino acid residues [142] whilst the $\beta-$subunit contained 182 residues [143]. The carbohydrate moiety was found attached to asparagine at position 168 in the $\beta-$chain [143]. In addition to the above subunits a number of minor components were found to be present in the purified favin no matter what purification procedure was followed. Whilst the predominant minor component was the β'-subunit, others included B1, B2, B3 and B4 (M_r 26,500-30,000) which were assumed to be various precursor forms of the $\beta-$subunit. Two fragments, C1 and C2 were identified to be the hydrolysis products of the $\beta-$subunit, and finally the high M_r form was shown to be the polymerised aggregate (HMV) of the $\beta-$subunit. Analysis of the β'-subunit showed that it was essentially the $\beta-$subunit minus the glycan chain and a few amino acid residues from the -COOH terminal end: the M_r of this chain was estimated to be 18,900.

1.6.4. Enzyme-Lectins

In addition to the presence of 'classical' lectins in plants, a few glycosidases, mainly from the Leguminosae, have been reported to agglutinate red blood

cells. These have been called "enzyme-lectins" [144].

The first such claim was made by Paus and Steen [145] using an α−mannosidase preparation from *Phaseolus vulgaris*. Hankins and co-workers [146], later showed that *Vigna radiata* α−galactosidase (a tetrameric protein; M_r 160,000) was able to agglutinate rabbit erythrocytes. A multistage purification procedure co-purified both the lectin and enzyme activities at each stage, thereby suggesting that both activities resided on the same protein. At neutral pH the tetrameric enzyme dissociated to a monomer form which retained enzyme activity but lost all haemagglutinin activity. Unlike 'classical' lectins, haemagglutination by the tetrameric enzyme was rapidly followed by dispersal of the 'clot' [144].

Studies on the carbohydrate binding specificity of this, so called, 'enzyme-lectin' showed that it was galactose specific; xylose and myo-inositol were also able to inhibit the lectin activity [144,146]. The latter two compounds were also competitive inhibitors of the catalytic activity of this protein. A consideration of these properties led Dey and Pridham [147] to conclude that the catalytic and lectin functions of the tetrameric *Vigna radiata* α−galactosidase resided at the same site on the protein and that there was only one such site per monomer unit, hence, the monomeric enzyme could not agglutinate erythrocytes. Agglutination by the tetramer resulted from cross- linking of the cells via membrane galactosyl residues binding to the enzyme/lectin sites. Disposal of the 'clots' resulted from the subsequent hydrolysis of the galactosyl residues. By definition such a protein cannot be defined as a lectin.

This galactose-specific 'lectin-like' activity is possessed by α−galactosidases from four other types of legume seeds [148]: the enzymes were immunologically related to *Vigna radiata* α−galactosidase and to one another. The above observa-

tions prompted Hankins and colleagues to suggest that each of these plants may contain a homologue from a specific class of enzyme-haemagglutinins.

Del Campillo and Shannon [149-150] have also shown that a highly purified form of α–galactosidase from *Glycine max* seeds displays haemagglutinin properties in addition to enzymic activity.

This thesis describes further studies on the *V. faba* seed tetrameric glycoprotein, α–galactosidase I, which possess both α–galactosidase and glucose/mannose specific lectin activities at separate loci [25]. If such a molecule exists it is an interesting new discovery and it is, therefore, essential to obtain unequivocal evidence that it is a true enzyme-lectin and, in particular, to ensure that the lectin activity is not a function a of contaminating 'classical lectin'. In this connection it should be noted that α–galactosidase and favin co-occur in *V. faba* seeds.

The work described has been partly to this end and partly to examine alternative methods for purifying *V. faba* α–galactosidase and favin.

METHODS AND MATERIALS

CHEMICALS

General laboratory chemicals were obtained from BDH Ltd., Poole, Dorset and were 'Analar' grade. P-Nitrophenyl α−D-galactopyranoside (PNPG), was purchased from Koch-Light, Colnbrook, Bucks. Protease from *Staphylococcus aureus* strain V8 (referred to below as V8 protease), agarose Type II medium EEO, 4-chloro-1-naphthol, DBA, IA, PMSF, EPNP and one batch of M_r markers for SDS-PAGE were obtained from Sigma (London) Chemical Co. Kingston-upon-Thames, Surrey. Sephadex G-100, Epoxy-Sepharose 6B, Pharmalite, Protein A-Sepharose, CNBr activated-Sepharose 4B, Concanavalin A-Sepharose, Chromatofocusing Kit and the second batch of M_r markers for SDS-PAGE were purchased from Pharmacia (G.B.) Ltd., London, U.K. CM-Cellulose (CM-52) was obtained from Whatman Biochemicals, Maidstone, Kent. Immobilised Melibiose was obtained from Pierce Chemical Co., Rockford, Il, USA. Anti-rabbit goat serum coupled to horse-radish peroxidase was obtained from Miles Laboratories, Il, USA. Amberlite-XAD and nitrocellulose sheets were obtained from Bio-Rad Laboratories Ltd., Watford, Herts. All the buffers were prepared by the procedures described in Methods in Enzymology [151]. All operations were carried out at 4 ^{0}C unless otherwise stated.

2.1 Enzyme Assay

2.1.1 Quantitative Assay

α−Galactosidase activity was assayed by following the initial rate of substrate hydrolysis. An appropriately diluted enzyme solution was added to a mixture of McIlvaine buffer [152] (pH 5.5) and 10 mM PNPG (0.1 ml) preheated to 30 ^{0}C; the final volume was 1.0 ml. The temperature was maintained for 15 min after which the reaction was stopped by addition of 0.1 M Na_2CO_3 (5 ml). The absorbance was measured at 405 nm. Enzyme activity is expressed as Katal (Kat), where 1 Katal is the amount of enzyme activity effecting the conversion of 1 mol of substrate per second.

2.1.2 Detection of Bound EI Activity on 'Mannan' and 'Mannose' Columns

Following attempts to elute enzyme activity from the two columns (see Section 2.4.3 (iii) and 2.4.3 (iv)) they were individually treated with 10 mM PNPG (5 ml in McIlvaine buffer; pH 5.5).The PNPG was left in contact with the column materials for 20 min after which they were eluted with PBS (pH 5.5; 25 ml) and the fractions (3 ml) collected were examined at 405 nm for the product of the enzyme reaction.

2.1.3 Preparation of the Substrate Solution for Horse-radish Peroxidase

4-Chloro-1-naphthol (18 mg) was dissolved in absolute methanol (6 ml) and gently poured into Tris-HCl-saline (pH 7.4 ;30 ml) and mixed well. H_2O_2 (12 μl) was added and after mixing, the entire solution was carefully poured onto the nitrocellulose sheet that had been incubated with the two (primary and secondary) antibodies.

2.2 Protein Assay

2.2.1 Measurement of Protein

Protein was determined by the method of Bradford [153] using crystalline bovine serum albumin as the standard.

2.2.2 Detection of Protein on Polyacrylamide Gels

After electrophoresis (or isoelectric focusing), the gels were stained for protein with 0.2% (w/v) Coomassie brilliant blue (R250) for 20 min and then destained at room temperature with a solution containing 30% (v/v) methanol and 5% (v/v) acetic acid. (The time for destaining could be decreased by placing the gels at 37 ^{0}C.)

2.2.3 Detection of Proteins on Agarose Gels

After 48 hr of diffusion, the agarose gels were stained for proteins with 0.5% (w/v) Coomassie brilliant blue G for 20 min and then destained (with the same solution that was used for destaining SDS-PAG ; see Section 2.2.2) until the background became clear.

2.2.4 Detection of Proteins on Nitrocellulose Sheets

Following the transfer of proteins from the SDS-PAG to a nitrocellulose sheet (through Western blotting) one half of the paper (that included the M_r markers) was stained for proteins using 0.1% (w/v) amido black solution for 2 min and destained (with the same solution that was used for destaining SDS-PAG; see Section 2.2.2) until the background was clear.

2.3.1 Haemagglutinin Assays

The method used is described by Roitt in "Essential Immunology" [154]. Blood cells from rabbits were harvested by centrifugation, resuspended in Alsever's solution, (pH 6.1), using a volume equal to the original volume of blood. The cells were washed twice with 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 (or EI) was made in PBS (0.1 ml) and the 1.5% suspension of erythrocytes (0.2 ml) then added. The tubes were shaken at 15 minute intervals and the extent of agglutination was assessed after 2 hr on a seriological scale. One haemagglutinin (HA) unit is the amount required to cause half-maximal agglutination of the cells. The assay is semi-quantitative with an error range of $\pm$ 20% .

2.4 Column Chromatography

2.4.1 Sephadex G-100 Gel Filtration

Sephadex G-100 (2.5 cm x 90 cm and 5.0 cm x 90 cm) columns were packed by the method described by Andrews [155]. Following the application of the enzyme preparation in small volumes (2% of the bed volume), the column (5 cm x 90 cm) was eluted with appropriate buffers at a flow rate of 50 ml/hr and 10

ml fractions were collected whilst the column (2.5 cm x 90 cm) was eluted with appropriate buffers at a flow rate of 60 ml/hr and 3 ml fractions were collected.

2.4.2 Cation-exchange Chromatography

Whatman ion-exchange cellulose, CM-52, was pre-washed as described in the manufacturer's (Whatman) instruction sheet, equilibrated with McIlvaine buffer (pH 3.5) and a column (0.5 cm x 17 cm) prepared. The EI preparation was dialysed against McIlvaine buffer (pH 3.5) for 8 hr (with two changes) and subsequently applied to the column. Elution was carried out with the same buffer until the absorbance of the fractions (3 ml) collected at 280 nm was nil. The bound enzyme was then eluted with a linear NaCl gradient prepared by using a mixing chamber containing 0.05 M NaCl (100 ml) and a reservoir containing 0.5 M NaCl (100 ml) in McIlvaine buffer (pH 3.5). Fractions (3 ml) were collected at a flow rate of 30 ml/hr and tested for EI activity at 405 nm (see Section 2.1.1) and the absorbance at 280 nm. Fractions showing EI activity were also tested for haemagglutination (see Section 2.3.1).

2.4.3 Affinity Chromatography

2.4.3 (i) Immobilised Melibiose Affinity Chromatography

A plastic syringe (0.5 ml x 10 ml) fitted with a porous polyethylene gel-support disc was used as a column. Immobilised melibiose affinity gel was packed into this column and equilibrated with McIlvaine buffer (pH 5.5). EI in McIlvaine buffer (Stage 7, Fig. 4; 5 units, 1 ml of EI; maximum capacity of the column) was applied and the flow was then stopped for 15 min to allow the enzyme to bind. The column was washed at a flow rate of 50 ml/hr with the same buffer and fractions

(3 ml) were collected. The eluent was monitored for protein at 280 nm, and for EI at 405 nm (see Section 2.1.1). When the absorbance at 280 nm was zero, the bound enzyme was eluted with the equilibrating buffer (50 ml) containing 10 mM PNPG and 0.5 M NaCl and fractions (3.0 ml) collected, dialysed and assayed for α−galactosidase activity (see Section 2.1.1) and haemagglutination (see Section 2.3.1).

2.4.3 (ii) Concanavalin A-Sepharose Affinity Chromatography of EI

EI (from Stage 6, Fig. 4 ; 50 units, 10 ml) following dialysis against sodium acetate buffer (pH 5.5) was applied to a Con A-Sepharose affinity column (0.5 ml x 5 ml) that had been equilibrated with sodium borate buffer (pH 6.5) followed by sodium acetate buffer (pH 5.5) containing 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 1 mM $CaCl_2$. The column was washed with the latter buffer at a flow rate of 50 ml/hr (containing the metal ions) and fractions (3ml) collected until the absorbance at 280 nm was nil. The column was next eluted with this buffer (50 ml) containing 0.2 M methyl α−D-mannoside and fractions (3 ml) collected were tested for α−galactosidase activity (see Section 2.1.1) and absorbance at 280 nm.

2.4.3 (iii) Synthesis of Mannan-Sepharose 6B for use as an Affinity Column

Epoxy Sepharose 6B (1.5 g) was allowed to swell for 15 min in distilled water and then washed extensively with the same. The washed gel was filtered through a sintered glass funnel and suspended in PBS (pH 11.0; 50 ml) and then mixed with PBS (pH 11.0; 100 ml) that contained dissolved yeast α−mannan (100 mg). The coupling reaction was carried out according to the manufacturer's instructions (Pharmacia Biotechnology) [156] and the product formed was packed

in a syringe (0.5 ml x 5 ml) that contained a 'porous polyethylene disc' which functioned as a support for the gel.

The column material was washed with PBS (pH 7.2; 50 ml) and partially purified EI (from Stage 6, Fig. 4; 25 units, 5 ml) was applied to the column. The flow was stopped for 15 min for the enzyme to bind to the column and then the column was washed with PBS (pH 7.2) at a flow rate of 50 ml/hr until the absorbance of the fractions (3 ml) at 280 nm was nil. The column was eluted with PBS (pH 7.2; 50 ml) containing 0.5 M methyl α−D-mannoside. When the EI did not appear in the fractions, the column was eluted with PBS (pH 5.5; 50 ml) containing 0.5 M methyl α−D-mannoside, which did not remove the enzyme activity.

In further attempts to elute the enzyme, the column was washed with PBS (pH 5.5; 50 ml) that contained 0.5 M methyl α−D-mannoside, 15 mM EDTA and 15 mM EGTA.

2.4.3 (iv) Synthesis of Mannose-Sepharose 6B and use as an Affinity Column

The preparation and the use of this column was essentially the same as that described above (see Section 2.4.3 (iii)) except that mannose (and not mannan) was used for coupling to Epoxy Sepharose 6B.

To serve as a control for the two columns described above, an ethanolamine derivative of the epoxy-Sepharose was prepared. Epoxy Sepharose 6B (1.5 g) was swelled, washed and then treated in a flask with 1 M ethanolamine (100 ml). The resulting gel was poured into a syringe (0.5 ml x 5 ml) that contained a 'porous polyethylene disc' and washed with PBS (pH 7.2; 100 ml) prior to use.

2.4.3 (v) Purification of EI Antibodies using a Protein A-Sepharose Affinity Column

A Protein A-Sepharose column (1 ml) was used for the purification of EI antibodies. The antiserum (2 ml) containing the EI antibodies was diluted to 5 ml with PBS (pH 7.2) and applied to the column. The column was then washed with the same buffer at a flow rate of 25 ml/hr, and fractions (1 ml) collected until the absorbance at 280 nm was nil. The column was next eluted with glycine-HCl (pH 2.2) and fractions (1 ml) which showed a high absorbance at 280 nm were pooled, dialysed against PBS (pH 7.2) and concentrated using polyethylene glycol 6000. The protein content in the above solution was estimated using Bradford's reagent (2.2.1) and the molecular properties of the proteins were studied using SDS-PAGE (see Section 2.5.1).

Coupling of Purified EI Antibodies to CNBr-activated Sepharose 4B

Coupling was carried out as described in "Affinity Chromatography" (Pharmacia Biotechnology)[156]. CNBr activated Sepharose 4B (0.35 g) was swollen in 1 mM HCl (100 ml) for 15 min and washed with 0.1 M bicarbonate buffer (pH 8.75, 25 ml). The swollen and washed gel was immediately poured into the same buffer (4 ml) which contained dissolved EI antibodies (purified; 1.2 mg). This mixture was placed in a plastic vial and incubated for 2 hr at room temperature in an inverting mixer. Following the completion of the reaction, the contents were taken out of the vial and washed extensively with PBS (pH 7.2).

2.4.3 (vi) Purification of EI using the Immobilised EI Antibody-Sepharose Affinity Column

EI (from Stage 6, Fig. 4; 25 units, 5 ml) was gently applied to the immobilised EI antibody-Sepharose affinity column (1 ml) pre-conditioned with PBS (pH 7.2). The column was washed with the same buffer at a flow rate of 25 ml/hr and the fractions (1 ml) collected were tested for protein content at 280 nm and EI activity at 405 nm (see Section 2.1.1). When the absorbance of the fractions at 280 nm was nil, the column was eluted with glycine-HCl (pH 2.2) (chosen from 5 different solvent systems on the basis of the conditions under which EI was most stable, as seen in Table 2) and fractions (1 ml) collected were monitored at 405 nm (see Section 2.1.1) and 280 nm. Those fractions showing a high absorbance at 405 nm were pooled, dialysed against PBS (pH 7.2) and concentrated to 3 ml using polyethylene glycol 6000.

2.4.3 (vii) Use of a 3-0-Methyl-N-Hexanoyl-D-Glucosamine-Sepharose Column for the Purification of Favin

V. faba seeds (100 g) were pulverised in a mechanical grinder and the powder extracted with 0.15 M NaCl (200 ml) at 4 ^{0}C overnight. The insoluble material was then removed by centrifuging at 10,000 rpm for 1 hr. The supernatant was decanted and treated with 80% $(NH_4)_2SO_4$ over a period of 1 hr and stirred for a further 1 hr. This solution was centrifuged at 10,000 rpm for 40 min and the supernatant discarded. The precipitate was extracted with PBS (pH 7.2; 15 ml) and extensively dialysed against several changes of the same buffer for 24 hr at 4 ^{0}C . The contents of the dialysis bag were centrifuged for 30 min at 7500 rpm and the supernatant was applied in 1 ml aliquots to a 3-O-methyl-N-hexanoyl

glucosamine-Sepharose column (0.5 ml x 5 ml) pre-equilibrated with the above buffer. The column was washed with PBS (pH 7.2) at a flow rate of 50 ml/hr until the absorbance of the fractions (1 ml) at 280 nm was nil. The column was next eluted with PBS (25 ml; pH 7.2) containing 0.2 M methyl α−D-mannoside and the fractions (1 ml) collected were tested for α−galactosidase activity (see Section 2.1.1) and the protein content at 280 nm. Those that showed a high reading at 280 nm were pooled, dialysed and tested for haemagglutinin activity (see Section 2.3.1). Finally the above fractions were concentrated and stored at -20 ^{0}C for future use.

2.4.3 (viii) Use of Sephadex G-100 Affinity Chromatography for the Purification of Favin

The initial steps involved in the purification of favin were similar to those in Section 2.4.3 (vii) except that PBS (pH 7.2) (and not 0.15 M NaCl) was used in the extraction of soluble matter from the (*V. faba*) seeds powder.

Following dialysis of the $(NH_4)_2SO_4$ concentrate, it was applied to a Sephadex G-100 column (1.75 cm x 27 cm) pre-equilibrated with the same buffer and washed with PBS (pH 7.2) until the absorbance at 280 nm was nil. The column was next eluted with PBS (pH 7.2; 200 ml) containing 0.5 M glucose at 50 ml/hr and the fractions (3 ml) collected were tested as in Section 2.4.3 (vii) prior to concentrating and storing at -20 ^{0}C for future use.

2.4.4 Simultaneous Extraction and Separation of EI, EII and Favin

Seed powder (1 kg) was extracted with McIlvaine buffer (pH 5.5; 2000 ml), and the initial stages of separation was carried out up to Stage 4 as in Fig. 4. The column was further washed with the eluting buffer until the absorbance of the washings at 280 nm was nil. The Sephadex G-100 column was next eluted with McIlvaine buffer (pH 5.5) containing 0.5 M glucose and the fractions collected (each of 10 ml) were tested for protein content at 280 nm and α−galactosidase activity (see Section 2.1.1). Those fractions showing a high absorbance at 280 nm were dialysed and tested for haemagglutinin activity (see Section 2.3.1). Finally they were pooled and concentrated to a final volume of 3 ml using an Amicon apparatus with a PM-10 membrane.

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

SDS-PAGE was performed as described by Laemmli [157] with a slight modification. Slab gel electrophoresis was carried out in a Shandon vertical slab unit 200 apparatus. Unless specified, the stacking and separating gels were 5% and 13.5% acrylamide, respectively, and they were prepared from a stock solution of 29.2% (w/v) acrylamide and 0.8 (w/v) N,N′-bismethylene acrylamide. The final concentrations in the 13.5% separating gel (15 mm x 120 mm) were as follows: 13.5% acrylamide, 0.1% N,N′-bismethylene acrylamide, 0.1% (w/v) SDS, 0.03% (v/v) Temed, 0.05% (w/v) ammonium persulphate, and 0.38 M Tris-HCl buffer (pH 8.8). The 5% stacking gel (1.5 mm x 30 mm) contained: 5% acrylamide, 0.1% N,N′-bisacrylamide 0.1% (w/v) SDS, 0.05% (v/v) Temed, 0.05% (w/v) ammonium persulphate and 0.13 M Tris-HCl buffer (pH 6.8). The

stacking gel (10 ml) and separating gel (30 ml) were adequate for one slab gel. The electrode buffer, pH 8.3, consisted of 0.38 M glycine, 0.01% (w/v) SDS and 0.05 M Tris.

Protein samples were mixed with the sample buffer which contained 0.13 M Tris-HCl buffer (pH 6.8), 10% (v/v) glycerol, 10% SDS,1% 2-mercaptoethanol and 0.015% bromophenol blue. The samples were heated for 3 min at 100 ^{0}C and after cooling they were applied onto the gel with the volume of the sample not exceeding 0.15 ml/track. Electrophoresis was performed at 25 mA until the samples entered the stacking gel (ca 30 min), after which the current was increased to 40 mA/gel. The gels were removed when the dye front was 1.0 cm from the bottom of the gel. The total time taken for the run was 4 hr. The gels were stained for protein, as described in Section 2.2.2. Two different Protein standards were used, obtained as kits from Pharmacia and Sigma. The Pharmacia kit consisted of: phosphorylase b (M_r 94,000), albumin, bovine serum (M_r 67,000), albumin, egg (M_r 43,000), carbonic anhydrase (M_r 30,000), soybean trypsin inhibitor (M_r 20,100) and α–lactalbumin (M_r 14,400) while the Sigma kit consisted of: albumin, bovine serum (M_r 66,000), albumin, egg (M_r 45,000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36,000), carbonic anhydrase (M_r 29,000), trypsinogen (M_r 24,000), trypsin inhibitor (Mr 20,100), and α–lactalbumin (M_r 14,400).

2.5.2 Isolation of the M_r 20,000 Favin β–Subunit

An SDS-PAG with 14% separating and 5% stacking gel (see Section 2.5.1) was used for the separation of the individual subunits of favin. Purified favin (see Section 2.4.3 (viii); 1 mg) was applied to a large well that had been cut in one half of the gel (A) and M_r markers and favin (5 μg) were applied

to two standard wells in the other half of the gel (B). When the electrophoretic run was complete the two halves of the gel were separated and (B) stained for proteins to locate the M_r 20,000 band of favin. (B) was used to locate the main 20,000 M_r band in (A) which was removed with a scalpel blade and inserted in a dialysis 'tube' containing electrode buffer (the same as that used in SDS-PAGE; 4 ml). All air bubbles were removed prior to closing the open end of the dialysis 'tube' and it was placed in a DNA sub-cell apparatus such that the length of the gel slice was perpendicular to the direction of the current. Finally, the electrode buffer was poured into the apparatus until the dialysis bag was just covered. A potential of 55 V was applied for 3 hr and then reversed for 30 sec prior to removing the dialysis 'tube'. The solution in the 'tube' was carefully removed with a pasteur pipette and the gel slice was removed with forceps. The interior of the bag was washed once with the electrode buffer (1 ml) and pooled with the original solution. This was dialysed against PBS (pH 7.2) overnight with two changes. Finally this solution was carefully applied to a capillary column containing Amberlite-XAD pre-equilibrated with PBS (pH 7.2). The column was eluted with PBS (pH 7.2) and fractions (1 ml) were collected and tested for proteins using Bradford's reagent (see Section 2.2.1) The fractions that contained proteins were pooled, dialysed against PBS (pH 7.2) and concentrated to 3 ml using an Amicon apparatus (PM-10 membrane).

2.6.1 Purification of α–galactosidase I (EI) from *V. faba* Seeds

Purification of E1 was carried out according to the Fig 4. Seed powder (1 kg) from resting broad-beans (*V. faba*) was used for each purification batch. The beans were ground using a mechanical grinder and the resultant powder suspended in McIlvaine buffer (pH 5.5) (2000 ml), stirred and left for 1 hr at 4 ^{0}C. The slurry was then centrifuged at 10,000 rpm for 40 min and the debris discarded. The supernatants (950 ml) were pooled and the pH of this crude extract lowered to pH 3.2 by gradual addition of 1 M citric acid, with stirring which was continued for 1 hr after addition of the acid. The precipitated material was centrifuged down at 10,000 rpm for 40 min and the supernatants again pooled and the pH adjusted to pH 5.5 by the addition of a saturated solution of Na_2HPO_4. Solid $(NH_4)_2SO_4$ was then added to 25% saturation with continuous stirring for 3 hr at 4 ^{0}C and the solution obtained was centrifuged. The precipitate obtained was discarded. The $(NH_4)_2SO_4$ concentration of the resultant supernatant was raised to 65% and the stirring continued for a further 5 hr at 4 ^{0}C. The resultant solution was centrifuged for 40 min at 10,000 rpm and the precipitated material suspended in McIlvaine buffer, (pH 5.5) and dialysed overnight with several changes against the same buffer. This 25-65% $(NH_4)_2SO_4$ fraction (100 ml) was applied to a Sephadex G-100 column (5 cm x 90 cm; see Section 2.4.1) and eluted with McIlvaine buffer (pH 5.5) containing 0.1 M KCl. Fractions (10 ml) were collected, assayed for α–galactosidase activity (see Section 2.1.1) and the absorbance at 280 nm was measured. The fractions were also tested for haemagglutinin activity (see Section 2.3.1). Enzymically active fractions corresponding to forms EI and EII were pooled separately.

Pooled EI was concentrated to 10 ml by ultrafiltration using an

Grind the seeds in to a fine powder using a mechanical mill

↓

Add McIlvaine buffer (pH 5.5; 2 ml buffer 1 g of seed powder)

↓

Stir and leave at 4 ^{0}C for 1 hr and centrifuge for 40 min at 10,000 rpm. (Stage 1)

↓

Citric acid precipitation (pH 3.2) (Stage 2)

↓

$(NH_4)_2SO_4$ fractionation (pH 5.5) (Stage 3)

↓

Sephadex G-100 gel-filtration (pH 5.5; 5 cm x 90 cm) (Stage 4)

↙ ↘

$\alpha-$Galactosidase I $\alpha-$Galactosidase II (Stored at 4 ^{0}C)

↓

Sephadex G-100 gel-filtration (pH 5.5; 2.5 cm x 90 cm) (Stage 5)

↓

CM-cellulose cation exchange chromatography (pH 3.5) (Stage 6)

↓

Immobilised melibiose affinity chromatography (pH 5.5) (Stage 7)

Fig. 4 Purification of $\alpha-$galactosidase I from *V. faba* seeds.

Amicon apparatus with a PM-10 membrane. This enzyme was re-cycled through a Sephadex G-100 column (2.5 cm x 90 cm; see Section 2.4.1) as described above, with the exception that 3 ml fractions were collected. The fractions that showed enzyme activity were pooled, concentrated and dialysed against McIlvaine buffer (pH 3.5) and the resulting solution (10 ml) applied to a CM-cellulose column (0.5 cm x 17 cm) (see Section 2.4.2) equilibrated with the same buffer (see Section 2.4.2). The column was washed with this buffer until the absorbance of the fractions (3 ml) at 280 nm was nil and then the enzyme was eluted with a linear gradient of NaCl in McIlvaine buffer (pH 3.5) from 0.05 M (100 ml), to 0.5 M (100 ml).

The enzyme fractions were again pooled, concentrated and dialysed against McIlvaine buffer (pH 5.5) and applied to an immobilised melibiose affinity column (0.5 cm x 10 cm; see Section 2.4.3 (i)), which had been equilibrated with the same buffer: the sample (10 ml) was applied in 1 ml aliquots at a time. The column was washed with McIlvaine buffer and when the absorbance at 280 nm was negligible, the bound enzyme was eluted with 10 mM PNPG and 0.5 M NaCl in the eluting buffer; fractions (3 ml) were collected, dialysed, assayed for $\alpha-$galactosidase activity (see Section 2.1.1) and those which showed a high value were pooled and concentrated.

2.7.1 Chromatofocusing of EI

The manufacturer's instructions ("Chromatofocusing", Pharmacia Biotechnology) [158] for packing and running the column were followed. The gel and the buffer used were degassed prior to use. The column (0.5 cm x 15 cm) was packed initially at the maximum flow rate allowed by the column outlet. After all the col-

umn material had been poured into the column, more buffer was passed through the column to complete the packing of the gel. At this stage the column was equilibrated with 0.02 M ethanolamine-acetic acid buffer (pH 9.4). The concentrated enzyme solution (from Stage 7, Fig. 4; 10 ml, 100 units) was dialysed against the starting buffer and applied to the column that had been pre-treated with eluent (Poly buffer 96, pH 6; 5 ml). This prevented the protein from being exposed to extreme conditions of pH. The column was next eluted with Poly buffer 96 (250 ml) at a flow rate of 40 ml/hr. Each of the fractions (2 ml) collected was tested for pH, absorbance at 280 nm and enzyme activity at 405 nm (see Section 2.1.1).

The individual peaks of EI activity were pooled separately and concentrated by the centrifugation technique using Amicon tubes (0.5 cm x 5 cm) with PM-10 membranes attached to one end of the tube with a 'cut-off' value of M_r 30,000 to remove the Poly buffer. The tubes were washed three times with PBS (pH 7.2; 0.5 ml) and concentrated as above. The concentrated EI was washed off with PBS (pH 7.2; 0.3 ml) and tested for agglutination with rabbit erythrocytes (see Section 2.3.1).

2.8.1 Isoelectric Focusing of Native EI and Favin

The glass plates were set up as described in the LKB [159]. The gel solution was prepared by the method described in "Isoelectric Focusing" by Pharmacia Biotechnology [160]. The final composition of the gel solution was as follows: Stock Acrylamide (5 ml), glycerol (8 ml), Pharmalite (3.8 ml; pH 3-10). The volume was made up to 60 ml with distilled water. The acrylamide used was from a freshly prepared 30% stock solution and the final concentration in the gel solution was 5% . The solution was degassed under vacuum for 30-45 min and

freshly prepared 4.6% (w/v) ammonium persulphate (0.4 ml) added. Using a glass pipette the final solution was carefully applied through the side opening into the mould. When the polymerisation was complete (as shown by the difference in refractive index in and around the gasket) the thin glass plate containing the gel was carefully taken out of the mould and placed on a LKB Multiphor cooling plate which had been treated with light paraffin oil to act as an insulator. The isoelectric focusing procedure described by the manufacturer's (LKB) instruction sheet was then followed. The instruments used were a LKB 2117 Multiphor apparatus with a LKB 2103 power supply. The cathode strip was saturated with 1 M NaOH and the anode with 1 M H_3PO_4. The strips were then placed on either side of the gel and the protein samples, on pieces (2 mm x 3 mm) of Whatman No. 3 paper, were placed on the gel surface. Electrophoresis for 1.5 hr was carried out with a final current of 50 mA at 1500 V. The pH gradient was estimated by extracting the pharmalyte from various parts of the gel and measuring the pH value. The remaining gel sheet was placed in a fixing solution (11.5% trichloroacetic acid and 3.5% sulphosalicylic acid) for approximately 1 hr and then destained with a solution containing ethanol (500 ml), acetic acid (160 ml) and distilled water (1440 ml) for 5 min, and then in staining solution, Coomassie Brilliant Blue R250 0.2% (w/v) for 10 min. The gel was then replaced in destaining solution (see Section 2.2.2) with several changes of destain, until the background was clear.

2.9.1 Proteolytic Hydrolysis of EI and Favin using V8 Protease from *Staphylococcus aureus*

Proteolytic hydrolysis was carried out according to the method of Cleveland [161]. Samples of purified EI (from Stage 7, Fig. 4; 5 μg and 10 μg) and of

favin (see Section 2.4.3 (viii); 10 μg and 20 μg) together with M_r marker proteins were run separately on a 14% SDS-PAGE. The gel (see Section 2.5.1) then was stained with Coomasie Blue (R250) for 20 min and destained (see Section 2.2.2) until the bands corresponding to the subunit of EI and β−subunit of favin could be clearly seen. These bands were carefully cut out of the gel using a scalpel and washed several times with 0.13 M Tris-HCl buffer (pH 6.8).

The four gel slices were carefully placed in four separate wells of a second SDS-PAGE (15% separating and 5% stacking gel) (see Section 2.5.1) and using a thin spatula each gel slice was pushed to the bottom of each well. Any air bubbles trapped between the gel slices and the stacking gel were squeezed out by carefully aligning the slices inside the wells. A mixture of M_r markers was placed in a 5th well.

Finally, V8 protease (0.1 μg and 0.4 μg) from *Staphylococcus aureus* dissolved in 0.13 M Tris-HCl buffer (pH 6.8; 10 μl) containing 10% glycerol and 0.015% bromophenol blue was placed in the wells which contained the EI subunit obtained from the 10 μg EI sample and the β−subunit of favin obtained from the 20 μg favin sample. In a 6th well of this slab gel, was placed denatured V8 protease (0.4 μg) in the same buffer as above. Electrophoresis was carried out as described in Section 2.5.1 except that the flow of current was stopped for 30 min when the bromophenol blue dye reached the bottom of the stacking gel.

2.10 Immunological Methods

2.10.1 (i) Production of Antibodies against Purified EI

A male rabbit of the New Zealand white variety was used to raise an-

tibodies. Purified EI (from Stage 7, Fig. 4; 0.15 mg) in PBS (pH 7.2; 0.5 ml) was mixed with Fruend's complete adjuvent (0.5 ml) and emulsified in a plastic syringe (1 ml) (without the needle).

On day 1, the emulsion was injected subcutaneously into the rabbit at three different sites around the neck (near the vertebral column). The rabbit was given successive injections on days 5l, 65 and 92. Each injection volume contained 0.1 mg of emulsified antigen (in Freund's incomplete adjuvent). Blood (15 ml) was collected on day 106 from the marginal ear vein, allowed to stand for 1 hr at room temperature and then centrifuged at 3000 rpm for 30 min. The supernatant (8 ml) was collected and tested for the presence of antibodies using immunodiffusion.

2.10.1 (ii) Immunodiffusion of EI using EI antibodies

Immunodiffusion was carried out according to Ouchterlony [162]. Three clean dry glass plates (8.3 cm x 9.3 cm) were overlaid with molten 1% (w/v) agarose (11.5 ml) in 0.1 M barbital buffer (pH 8.6). Using a template, a hexagonal arrangement of wells (4 mm diameter; 7 mm apart) was punched out with a hole puncher.

(1) On the plate [a], the antiserum (20 μl) was placed in the central well and the surrounding wells contained 2-12 μl of the antigen (EI).

(2) On the plate [b], the antiserum (20 μl) was placed in the central well and 5 surrounding wells contained 2-10 μl of the antigen (EI).

(3) On the plate [c], blood serum (20 μl; control) was placed in the central well and the surrounding wells contained 2-12 μl of the antigen (EI).

EI used in the above instances was of a highly purified variety (from

Stage 7; Fig 4).

The plates were stored for 48 hr in a plastic container lined with moist tissue paper to allow the diffusion to take place. The plates were checked from time to time for the appearance of white precipitin lines. After the diffusion was complete the plates were pressed and washed with 0.15 M NaCl for 2 hr followed by 2 hr of washing with distilled water. Finally the plates were stained and destained for proteins as in Section 2.2.3.

2.10.2 (i) Production of Antibodies against Favin

The procedure was essentially the same as in Section 2.10.1 (i), except that EI was replaced by favin (see Section 2.4.3 (viii)) and the quantity used for each injection was 0.2 mg of the protein. The injections were given on days 1, 48, 62 and 79.

Blood (25 ml) collected on day 96 and the serum isolated (11 ml) was tested for antibodies using immunodiffusion (see Section 2.10.1 (ii)).

2.10.2 (ii) Immunodiffusion of Favin using Favin Antibodies

The preparation of the three glass plates were the same as in Section 2.10.1 (ii).

(1) On the plate [a], antiserum (20 μl) was placed in the central well and the surrounding wells contained 2-12 μl of antigen (favin).

(2) On the plate [b], antiserum (20 μl) was placed in the central well and 5 surrounding wells contained 2-10 μl of antigen (favin).

(3) On the plate [c], blood serum (20 μl; control) was placed in the the central well and the surrounding wells contained 2-12 μl of antigen (favin).

Favin used in the above instances were of a highly a purified variety (see Section 2.4.3 (viii)).

The rest of the procedure was exactly the same as in Section 2.10.1 (ii). Following immunodiffusion, the plates were stained and destained as in section 2.10.1 (ii).

2.10.3 (i) Production of Antibodies against the β−Subunit of Favin

The procedure was essentially the same as in Section 2.10.1 (i) except that EI was replaced by the β−subunit (of favin) and the quantity used for each injection was 0.1 mg of the protein. Injections were given on days 1, 38, 63 and 78. Blood (20 ml) was collected on day 98 and the serum isolated (9 ml) was tested for antibodies using Western blotting (see Section 2.10.3 (ii)).

2.10.3 (ii) Testing for the Presence of Antibodies against the β−Subunit through Western Blotting

Wells 1 and 3 in the SDS-PAG contained purified favin (20 μg) and well 2 contained the M_r markers. Following electrophoresis the rest of the experiment was performed identically to that in Section 2.10.6 except that favin antibodies were replaced by β−subunit antibodies and lanes 1 and 2 were stained with 0.1% amido black (see Section 2.2.4) for proteins while lane 3 was incubated with the β−subunit antibodies (primary; 1:500) followed by the horse-radish peroxidase (secondary; 1:500) antibody solution.

2.10.4 (i) Quantitative Immunoprecipitation of EI using EI Antibodies and Favin Antibodies.

Immunoprecipitation was carried out in plastic microcentrifuge tubes (1.5 ml) by adding variable amounts of EI antiserum and favin antiserum (0 - 50 μl) to

constant amount (activity) of enzyme. A constant volume (50 μl) was maintained in each tube by addition of PBS (pH 7.2). After adding the components and mixing, the tubes were incubated for 15 min at room temperature. Finally, the tubes were centrifuged (12,000 rpm) for 6 min in a microcentrifuge (Eppendorf). The supernatants were assayed for $\alpha-$galactosidase activity as described in Section 2.1.1.

2.10.4 (ii) Quantitative Immunoprecipitation of EI using Purified EI Antibodies

The procedure in this instance was the same as above (see Section 2.10.4 (i)) except that crude EI antibodies were replaced with purified EI antibodies (see Section 2.4.3 (v)).

2.10.5 Dot-Blot Studies on EI and Favin using Favin Antibodies

The dot-blot experiments were carried out according to McDougal [163] with slight modifications. EI (denatured and undenatured; 14 μg) were applied to one set of wells whilst favin (denatured and undenatured; 14 μg) were added to the remaining wells. The antigens were allowed to be in the wells for 2 hr before washing the wells once with Tris-HCl-saline (pH 7.4) followed by the application of 1% BSA (50 μl) into each well. The wells were washed after 1 hr with the above buffer before treating with the favin antibody (100 μl each) at dilutions from 1:500 to 1:8000 for 16 hr at room temperature. The nitrocellulose sheet was washed several times with Tris-HCl-saline (pH 7.4) containing 0.05% Tween 20 followed by several washes with Tris-HCl-saline (pH 7.4). The wells were next treated with anti-rabbit goat serum coupled to horse-radish peroxidase (50 μl; 1:500) for 2 hr at room temperature. The nitrocellulose sheet was washed once again with Tris-HCl-saline (pH 7.4) containing 0.05% Tween 20 followed by several washes with Tris-HCl-saline (pH 7.4). The nitrocellulose sheet was carefully taken out of the apparatus and treated with the horse-radish peroxidase substrate solution (see Section 2.1.3) and observed for the appearance of purple dots.

The order of application of the antigens and the antibodies were as follows (Fig. 5):

Wells in the region of 7-9 and A-D consisted of the undenatured EI in equal amounts. The antibodies were applied into the wells in duplicate. With the exception of the 1st pair of wells (7:A,B which was the control) the rest of the 5 pairs of wells were treated with antibody dilutions in the ratio of 1:500, 1:1000, 1:2000, 1:4000 and 1:8000. Also antibody dilutions contained 0.2 M methyl $\alpha-$D-

mannoside. The 12 wells in the region of 10-12 and A-D consisted of the denatured EI (denatured with SDS and 2-mercaptoethanol) in equal amounts. The order of application and the level of antibody dilution were the same as above. The 12 wells in the region of 7-9 and E-H consisted of the undenatured favin in equal amounts. The order of application and the level of antibody dilution was the same as above. The 12 wells in the region of 10-12 and E-H consisted of the denatured favin (denatured with SDS and 2-mercaptoethanol) in equal amounts. The order of application and the level of antibody dilution were the same as above.

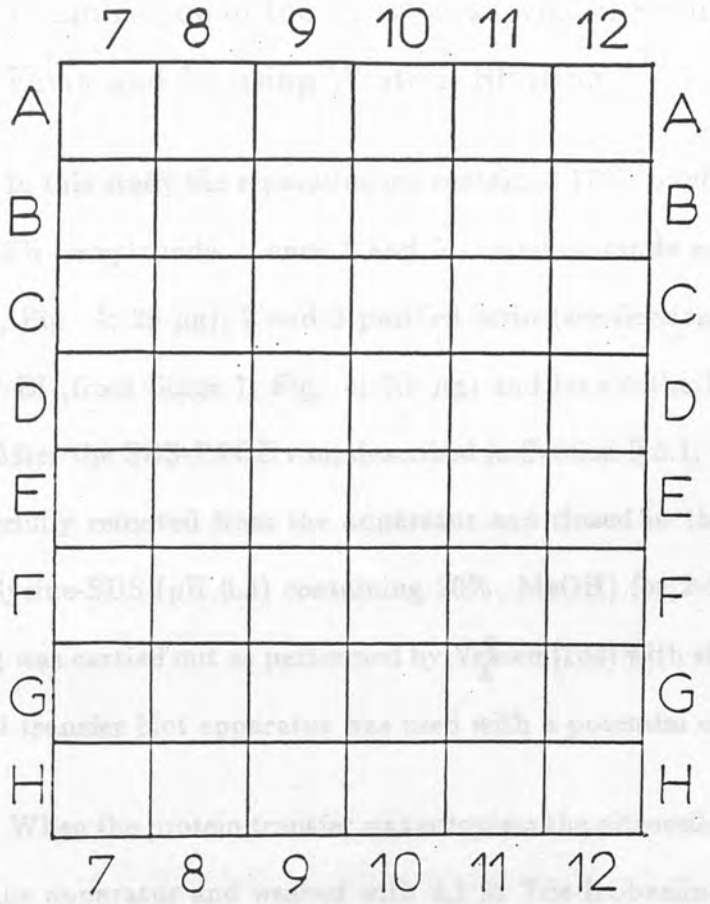


Figure 5

Mode of Antigen and Antibody Application on the dot-Blot

Wells 7-9 and A-D consisted of 14 µg undenatured EI
Wells 10-12 and A-D consisted of 14 µg denatured EI

Wells 7-9 and E-H consisted of 14 µg undenatured favin
Wells 10-12 and E-H consisted of 14 µg denatured favin

Wells 8: A,B; 9: A,B; 7: C,D; 8: C,D and 9: C,D contained 100 µl (each) of favin antibody at dilutions 1:500, 1:1000, 1:2000, 1:4000 and 1:8000 respectively.
Wells 7: A,B remained without any antibodies.

Wells 11: A,B; 12: A,B; 10: C,D; 11: C,D and 12: C,D contained 100 µl (each) of favin antibody at dilutions 1:500, 1:1000, 1:2000, 1:4000 and 1:8000 respectively.
Wells 10: A,B remained without any antibodies

Wells 8: E,F; 9: E,F; 7: G,H; 8: G,H and 9: G,H contained 100 µl (each) of favin antibody at dilutions 1:500, 1:1000, 1:2000, 1:4000 and 1:8000 respectively.
Wells 7: E,F remained without any antibodies

Wells 11: E,F; 12: E,F; 10: G,H; 11: G,H and 12: G,H contained 100 µl (each) of favin antibody at dilutions 1:500, 1:1000, 1:2000, 1:4000 and 1:8000 respectively.
Wells 10: E,F remained without any antibodies.

2.10.6 Examination of the Cross-reactivity of Favin Antibodies with Favin and EI using Western Blotting

In this study the separating gel contained 12% acrylamide and the stacking gel 5% acrylamide. Lanes 1 and 5 contained crude enzyme extract (from Stage 1, Fig. 4; 25 μg), 2 and 6 purified favin (see Section 2.4.3 (viii); 10 μg), 3 and 7 EI (from Stage 7, Fig. 4; 7.5 μg) and lane 4 the M_r markers (5 μg of each). After the SDS-PAGE run, described in Section 2.5.1, was complete the gel was carefully removed from the apparatus and rinsed in the transferring buffer (Tris-Glycine-SDS (pH 8.3) containing 20% MeOH) for 2-5 min. The Western blotting was carried out as performed by Vaessen [164] with slight modification. A Bio-Rad transfer blot apparatus was used with a potential of 55 V for 4 hr.

When the protein transfer was complete the nitrocellulose sheet was taken out of the apparatus and washed with 0.1 M Tris-HCl-saline (pH 7.4). One half of the sheet (Lanes 1-4) was stained (2-3 min) and destained for proteins as in Section 2.2.4. The other (Lanes 5-7) was saturated with 1% (w/v) BSA for 1 hr and then rinsed in 0.1 M Tris-HCl-saline (pH 7.4) and incubated in favin antibody (1:500; 25 ml) for 16 hr at room temperature. After washing three times with 0.1 M Tris-HCl-saline (pH 7.4) containing 0.05% Tween 20 followed by several washes with 0.1 M Tris-HCl saline (pH 7.4) the sheet was next incubated for 2 hr with anti-rabbit goat serum coupled to horse-radish peroxidase (secondary, 1:500; 25 ml) at room temperature. The sheet was washed several times with the detergent containing buffer (as above) followed by several washes with the same buffer without the detergent. Finally the sheet was treated with horse-radish peroxidase substrate solution (see Section 2.1.3) and observed for the appearance of purple bands.

2.10.7 Examination of the Cross-reactivity of EI antibodies with EI and Favin using Western Blotting

Lanes 1 and 5 in the SDS-PAG contained crude EI extract (from Stage 1, Fig 4; 30 μg), 2 and 6 contained purified favin (see Section 2.4.3 (viii); 10 μg), 3 and 7 contained EI (from Stage 7, Fig. 4; 7.5 μg) and lane 4 contained the M_r markers (5 μg each). Following electrophoresis the rest of the experiment was identical to Section 2.10.6 except that favin antibodies (primary antibodies) were replaced with EI antibodies and the lanes that were incubated with the EI antibodies (primary) followed by horse-radish peroxidase (secondary) antibody solution were 5-7 while lanes from 1-4 were stained and destained for proteins (see Section 2.2.4).

2.10.8 Use of Proteolytic Inhibitors in an Attempt to Study the Origin of Polypeptide Fragments in EI

Broad bean powder (100 g) was extracted with McIlvaine buffer (pH 5.5) containing 2 mM PMSF, 10 mM EPNP and 10 mM IA for 1 hr and then subjected to the initial 4 stages (EI+) of the 7 stage purification procedure (Fig. 4). A further sample of the powder (100 g) was extracted in an identical manner but excluding the protease-inhibitors and then purified to stage 4 (EI-). 10 μg of EI ((EI+) and (EI-)) was applied in duplicate in wells on either side of an SDS-PAG and following electrophoresis (see Section 2.5.1) the proteins were transferred to a nitrocellulose sheet through Western blotting (see Section 2.10.6) and one half of the sheet was stained for proteins (see Section 2.2.4) whilst the other half was incubated with favin antibodies (1:500; primary) followed by secondary antibodies (1:500). The staining procedure is described in Section 2.1.3.

2.10.9 (i) Examination of the Cross-reactivity of EI with β−Subunit (of Favin) Antibodies using Western Blotting.

Lanes 1 and 5 contained EI (10 μg each), and lanes 2 and 4 contained favin (10 μg each) respectively. Well 3 contained the M_r markers. Following electrophoresis, the rest of the experiment was identical to Section 2.10.6 except that the primary antibody used in this instance was the β−subunit of favin. Lanes 4 and 5 were incubated with the primary and the secondary antibody while lanes 1,2 and 3 were stained for protein (see Section 2.2.4).

2.10.9 (ii) Examination of Cross-reactivity of EI with β−Subunit (of Favin) Antibodies using Western Blotting

Lanes 3 and 5 contained EI (10 μg) and favin (10 μg), respectively. Lanes 2 and 4 were remained blank while lane 1 contained M_r markers. The rest of the experiment was carried out as in Section 2.10.6. Lane 1 was stained for protein (see Section 2.3.4) while lanes 2-5 were incubated with β−subunit (1:500; primary) and horse-radish peroxidase (1:500; secondary) antibody solution and stained as in Section 2.1.3.

RESULTS AND DISCUSSION

3. Studies of the Lectin Nature of α–galactosidase I from *V. faba*

3.1 Isolation and Characterisation of α–galactosidase I from *V. faba*

3.1.1 Purification of α–galactosidase I from *V. faba* Seeds

α–Galactosidase I was isolated from ground *V. faba* (broad bean) seeds. For each preparation approximately 1 kg of seed powder was used and the procedure finally adopted, after attempting a number of different techniques, was similar to that described by Dey and colleagues [25] except that the concentration of PNPG in the eluting buffer for the immobilised melibiose column (Stage 7; Fig 4) was decreased from 50 mM to 10 mM and 0.5 M NaCl added.

The various purification stages are summarised in Table 1. Stage 2, involving the addition of citric acid, was necessary to remove the bulk of the storage proteins and a number of glycosidases other than the α–galactosidases. This step significantly increased the volume of the preparation and to reduce this, the enzyme activity was concentrated by $(NH_4)_2SO_4$ precipitation. Following the first three stages of purification there was a 1.7 fold increase in the specific activity of the enzyme and a 7.2 fold increase in the lectin specific activity.

The preparation obtained at Stage 3 was then applied to a large Sephadex G-100 column (Stage 4; Fig. 4) which was eluted with McIlvaine buffer (pH 5.5) containing 0.1 M KCl in order to separate α–galactosidase fractions EI and EII (M_r 160,000 and 40,000, respectively; Fig. 6(a) and 6(b)). There was a marked reduction in the total lectin activity of the enzyme preparation due to the removal of favin (see Section 3.4). The last three Stages (5-7) were used to purify EI only.

EI fractions derived from Stage 4 were pooled, concentrated to 10 ml

TABLE 1 Purification of α−galactosidase I (EI) from *V. faba* seeds .†

Stage	Volume (ml)	Activity (nkat/ml)	Protein (mg/ml)	EI specific activity (nkat/mg)[A]	Lectin activity (HA/ml)	Lectin specific activity (HA/mg)[B]	Ratio A/B	Recovery of EI) %
1. Crude extract	950	14.5	120	0.12	640	5.3	0.02	100
2. Citric acid precipitation (pH 3.2)	1,250	7.81	40	0.19	416	10.4	0.02	70.8
3. $(NH_4)_2SO_4$- (25-65% saturation)	100	27	135	0.2	5,120	38	0.0052	19.6
4. Sephadex G-100 gel filtration								
α−galactosidase I	95	8.21	5	1.64	160	32	0.051	5.7
α−galactosidase II	80	4.4	3	1.47	96	32	0.046	5.4
5. Sephadex G-100 gel filtration								
α−galactosidase I	25	21.8	7	3.1	416	59.4	0.052	3.9
6. CM-cellulose cation exchange chromatography								
α−galactosidase I	20	16.7	0.15	111.3	211.2	1408	0.079	2.4
7. Immobilised melibiose affinity chromatography								
α−galactosidase I	10	31.3	0.09	348	320	3555.6	0.097	2.3

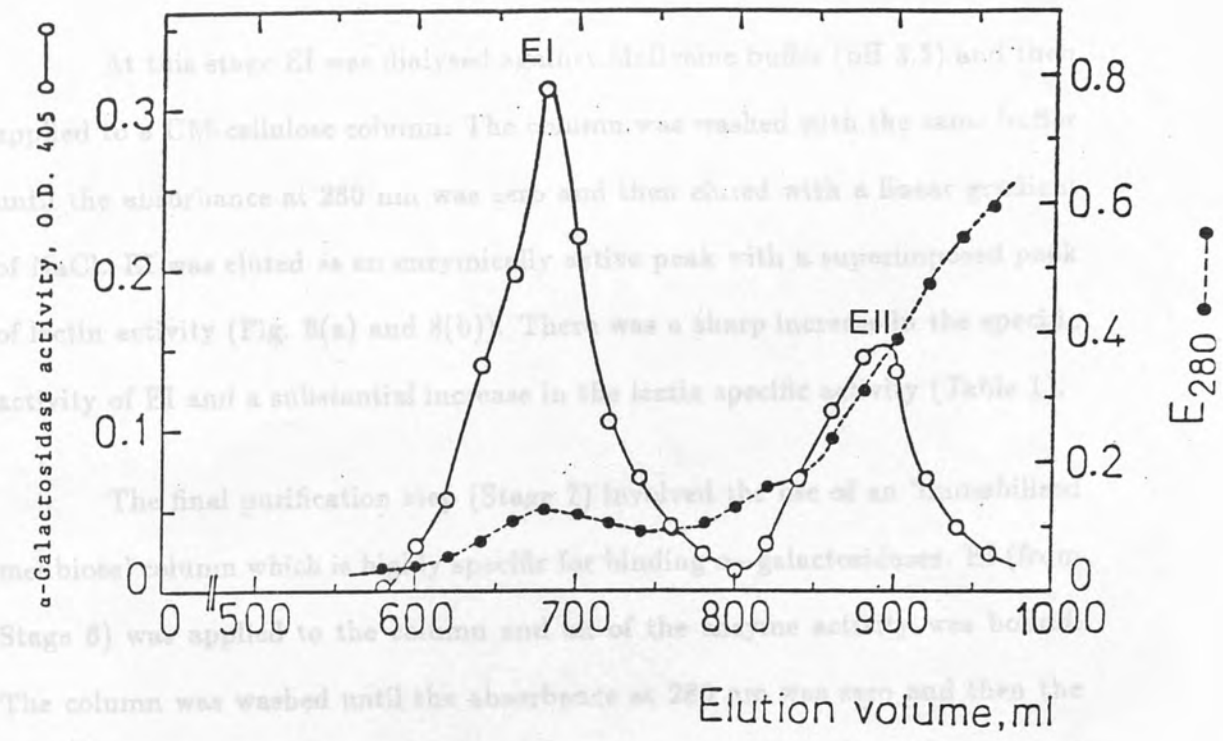
† Recovery has been calculated relative to stage 1. Weight of the seed powder=1 kg.

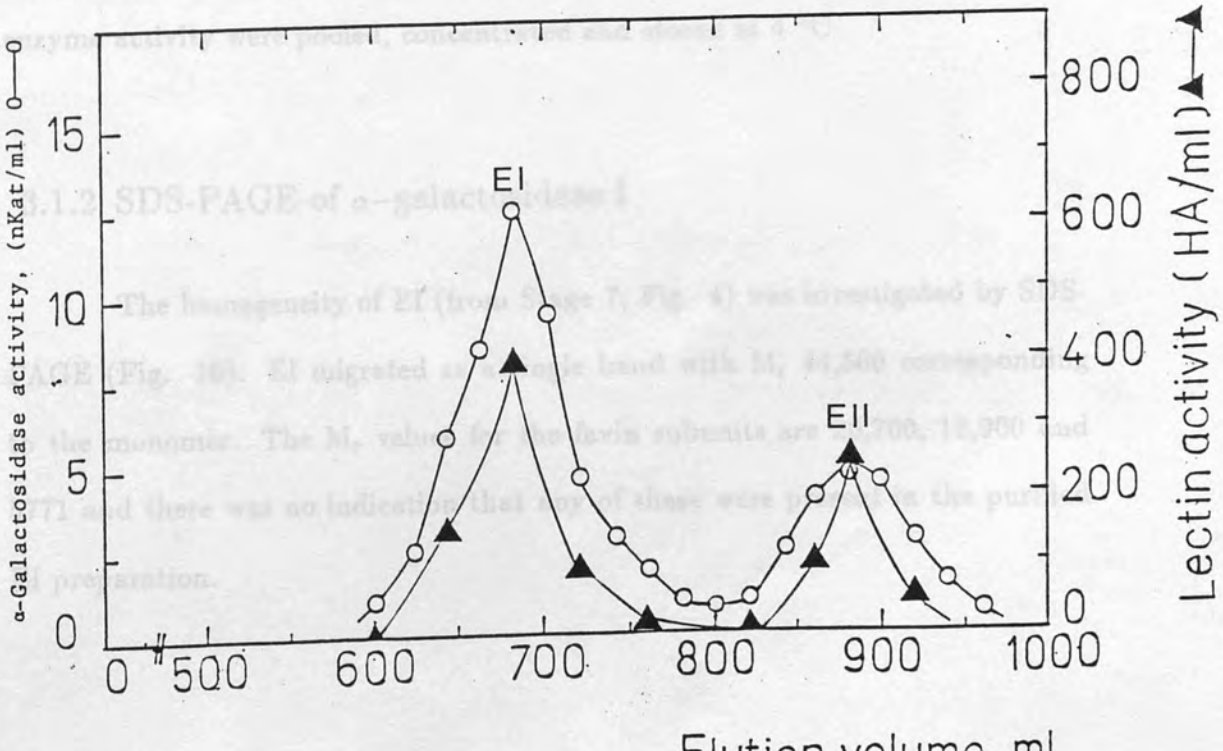
Figure 6

(a) Sephadex G-100 gel-filtration of EI and EII (Stage 4; Table 1)

(b) Co-elution of catalytic and lectin activities of EI and EII

Experimental details are given in Section 2.4.1


α-Galactosidase activity, O.D. 405
EI
EII
0.3
0.2
0.1
0
0.8
0.6
0.4
0.2
0
E_{280}
0
500
600
700
800
900
1000
Elution volume, ml


α-Galactosidase activity, (nKat/ml)
EI
EII
15
10
5
0
800
600
400
200
0
Lectin activity (HA/ml)
0
500
600
700
800
900
1000
Elution volume, ml

and applied to a second small Sephadex G-100 column and the resulting enzyme fractions again pooled and concentrated (Fig. 7(a) and 7(b)). This step resulted in a 1.9 fold increase in the specific activity of EI.

At this stage EI was dialysed against McIlvaine buffer (pH 3.5) and then applied to a CM-cellulose column. The column was washed with the same buffer until the absorbance at 280 nm was zero and then eluted with a linear gradient of NaCl. EI was eluted as an enzymically active peak with a superimposed peak of lectin activity (Fig. 8(a) and 8(b)). There was a sharp increase in the specific activity of EI and a substantial increase in the lectin specific activity (Table 1).

The final purification step (Stage 7) involved the use of an 'immobilised melibiose' column which is highly specific for binding α–galactosidases. EI (from Stage 6) was applied to the column and all of the enzyme activity was bound. The column was washed until the absorbance at 280 nm was zero and then the enzyme was eluted with McIlvaine buffer (pH 5.5) containing 10 mM PNPG and 0.5 M NaCl (Fig. 9(a) and 9(b)). The fractions containing EI were dialysed and subjected to analysis for catalytic and lectin activities and those that showed enzyme activity were pooled, concentrated and stored at 4 ^{0}C .

3.1.2 SDS-PAGE of α–galactosidase I

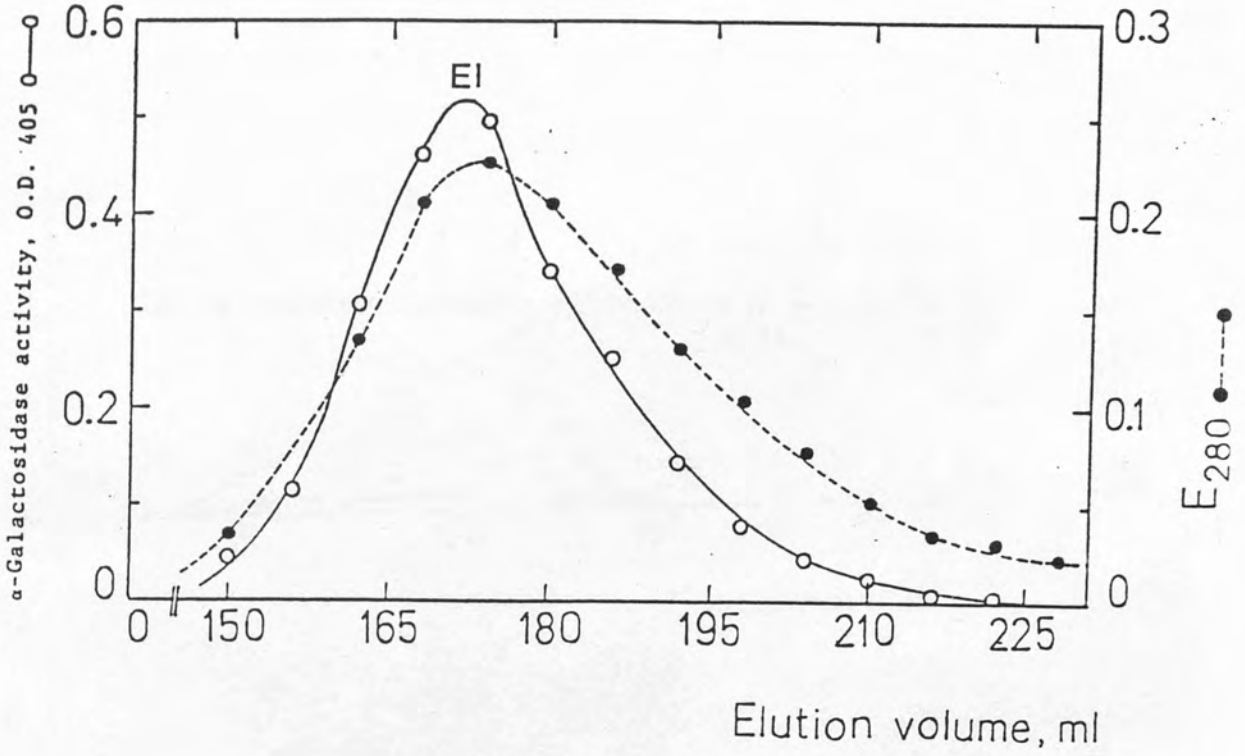
The homogeneity of EI (from Stage 7; Fig. 4) was investigated by SDS-PAGE (Fig. 10). EI migrated as a single band with M_r 44,500 corresponding to the monomer. The M_r values for the favin subunits are 20,700, 18,900 and 5771 and there was no indication that any of these were present in the purified EI preparation.

Figure 7

(a) Sephadex G-100 gel filtration of EI (Stage 5; Table 1)

(b) Co-elution of catalytic and lectin activities of EI (Stage 5; Table 1)

Experimental details are given in Section 2.4.1



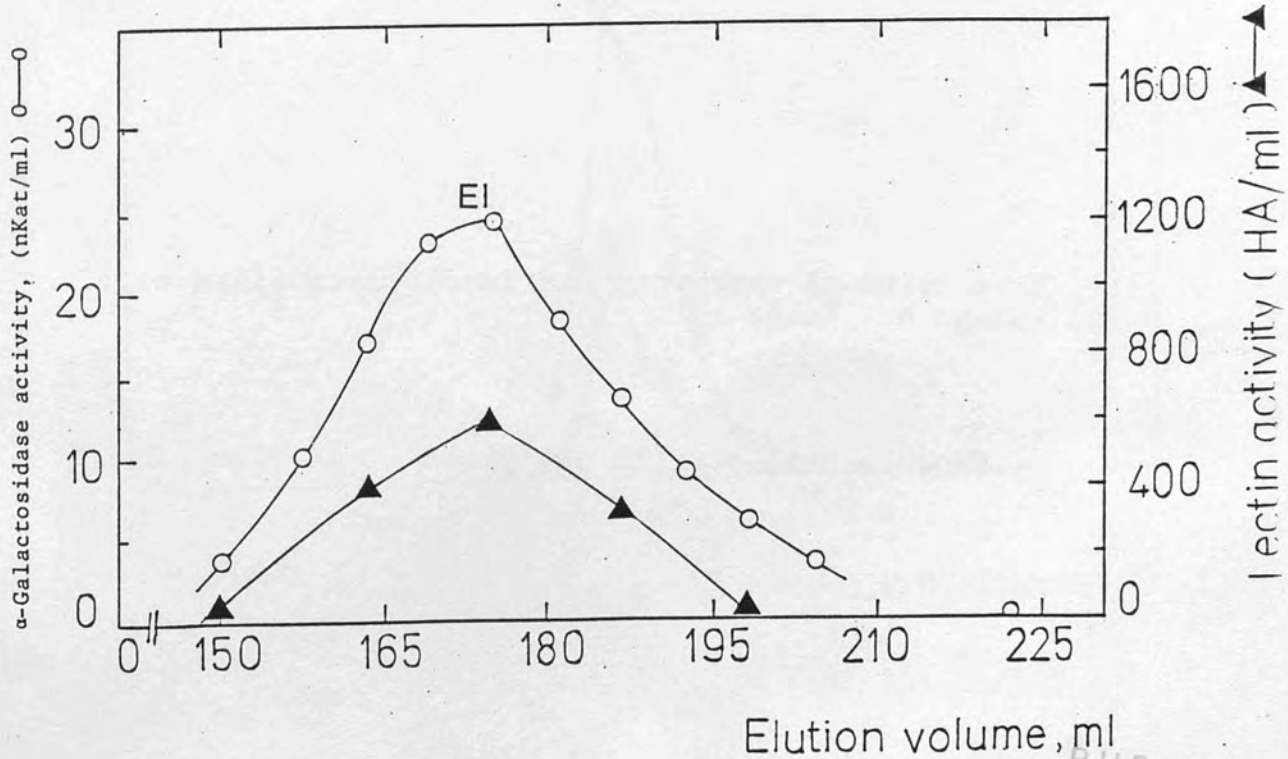
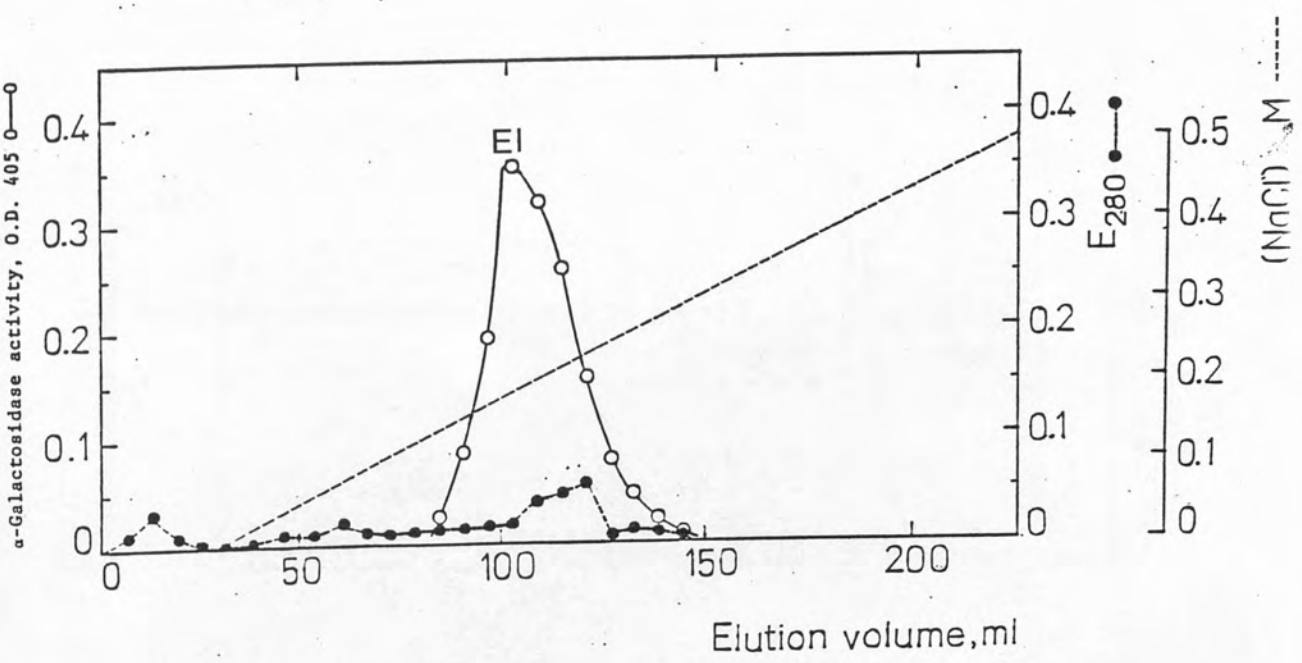


Figure 8

(a) CM-Cellulose ion-exchange chromatography of EI (Stage 6; Table 1)

(b) Co-elution of catalytic and lectin activities of EI (Stage 6; Table 1)

Experimental details are given in Section 2.4.2


α-Galactosidase activity, O.D. 405
EI
E280
(NaCl) M
Elution volume, ml

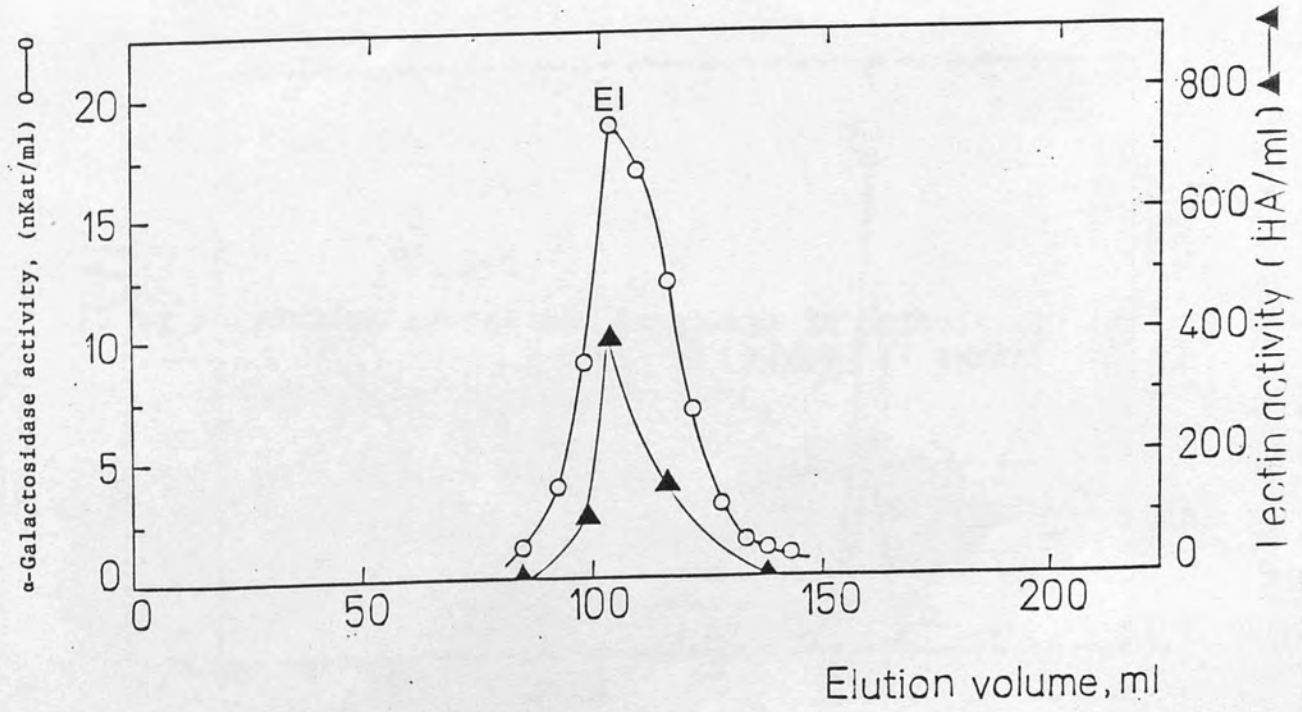
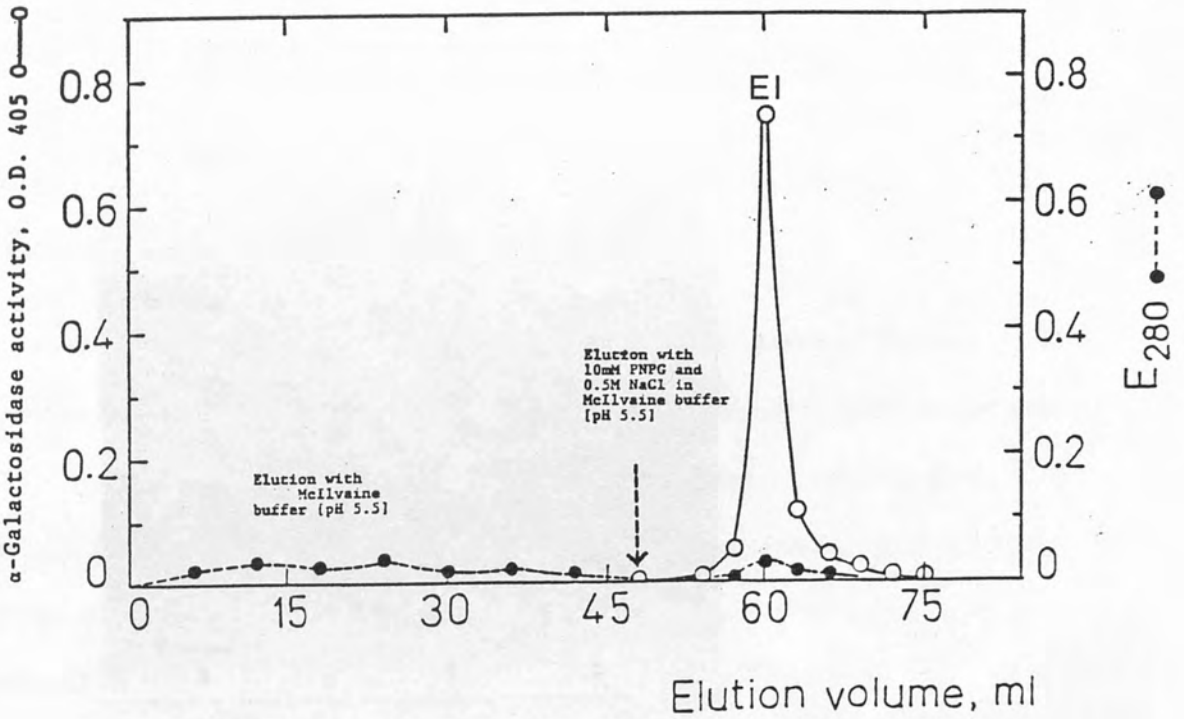

α-Galactosidase activity, (nKat/ml)
EI
Lectin activity (HA/ml)
Elution volume, ml

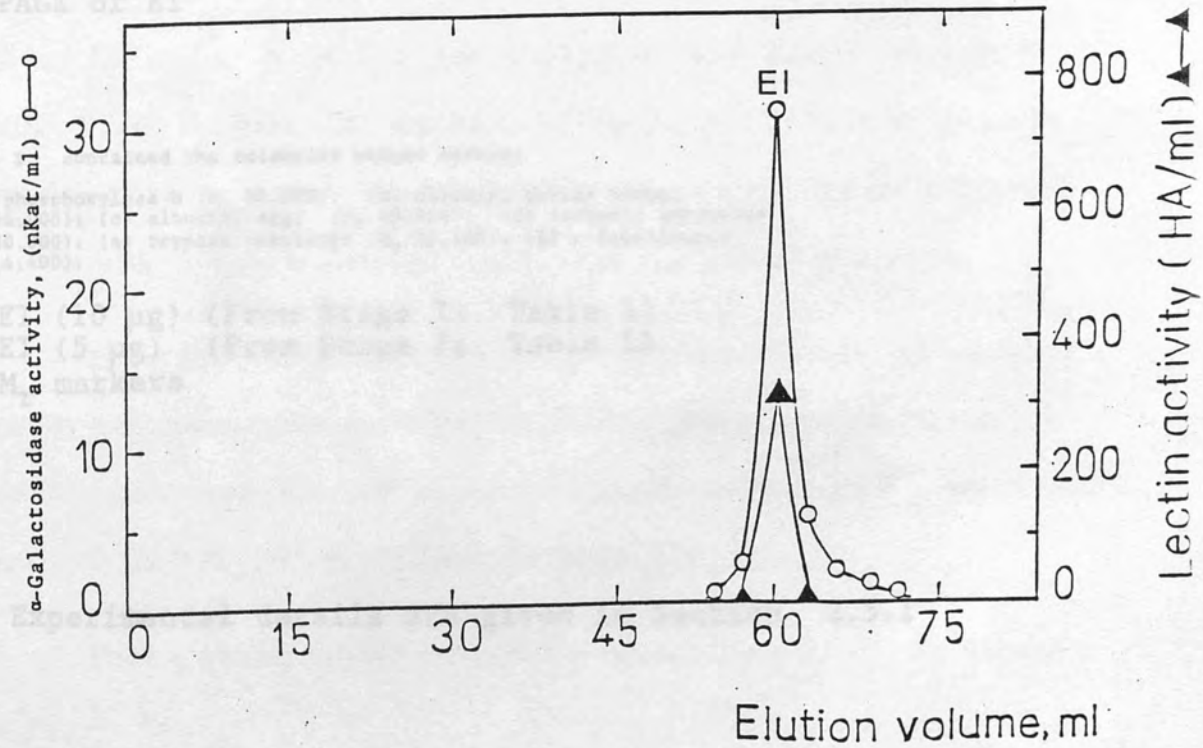
Figure 9

(a) Immobilised melibiose affinity chromatography of EI (Stage 7; Table 1)

(b) Co-elution of catalytic and lectin activities of EI (Stage 7; Table 1)

Experimental details are given in Section 2.4.3(i)


α-Galactosidase activity, O.D. 405
0.8
0.6
0.4
0.2
0
EI
Elution with McIlvaine buffer (pH 5.5)
Elution with 10mM PNPG and 0.5M NaCl in McIlvaine buffer (pH 5.5)
E_{280}
0
15
30
45
60
75
Elution volume, ml


α-Galactosidase activity, (nKat/ml)
30
20
10
0
EI
800
600
400
200
0
Lectin activity (HA/ml)
0
15
30
45
60
75
Elution volume, ml

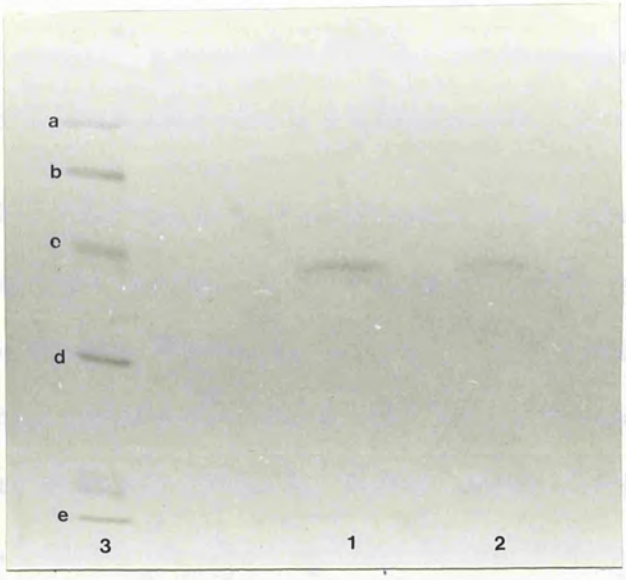


Figure 10

SDS-PAGE of EI

Lane 3 contained the molecular weight markers

(a) phosphorylase b (M_r 94,000); (b) albumin, bovine serum, (M_r 66,000); (c) albumin, egg; (M_r 45,000); (d) carbonic anhydrase (M_r 30,000); (e) trypsin inhibitor (M_r 21,100); (f) α-lactalbumin (M_r 14,400).

1. EI (10 µg) (From Stage 7; Table 1)
2. EI (5 µg) (From Stage 7; Table 1)
3. M_r markers

Experimental details are given in Section 2.5.1

3.1.3 Chromatofocusing of α–Galactosidase I

Studies with EI by Dey and co-workers [147] showed that the purified enzyme was composed of microheterogeneous catalytically-active forms although they were not examined for lectin activity. The chromatofocusing study was therefore repeated to re-examine the microheterogeneity of the enzyme and to see if the various forms also possessed lectin activity.

The reliability of chromatofocusing for the separation and purification of proteins and glycoproteins has been demonstrated by various workers in the past [166]. For example Massuda and colleagues [167] working on vitamin D binding protein, managed to isolate three forms from partially purified human γ–globulin by the use of chromatofocusing. This separation could not be achieved by gel-filtration, owing to the similarity in the molecular sizes of the forms.

The pattern of peaks obtained with EI in the present study was different to that observed by Dey and co-workers. This was probably due to the use of different batches of *V. faba* seeds in the two studies. Large differences in the EI and EII content of different seed samples have been observed (Dey, P. M., Palan, D. and Pridham, J.B., unpublished results) and it would therefore not be surprising if varing degrees of post-translational changes had occurred in different samples with different histories, eg. due to dissimilar harvesting methods.

Peaks A, B, C, D, E and F (Fig. 11(a)) which all possessed α–galactosidase activity were concentrated separately by a centrifugation technique using semipermiable membranes with 'cut-off' values of M_r 30,000. The fractions were dissolved separately, in PBS (pH 7.2) and tested for haemagglutinin activity.

Peak A which possessed the highest α–galactosidase activity also showed

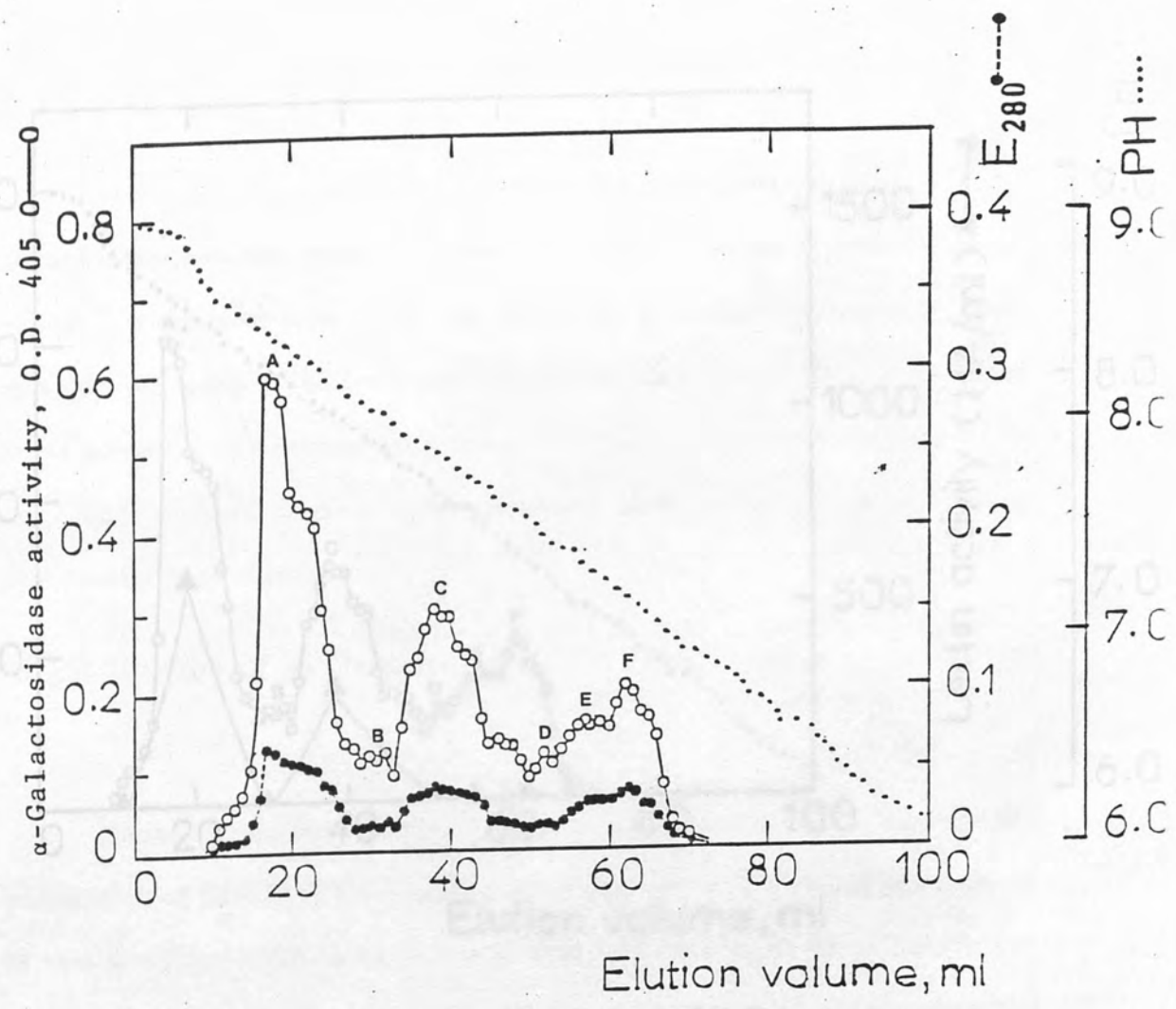


Figure 11 a

Separation of isomeric forms of EI using Chromatofocusing

Experimental details are given in Section 2.7.1

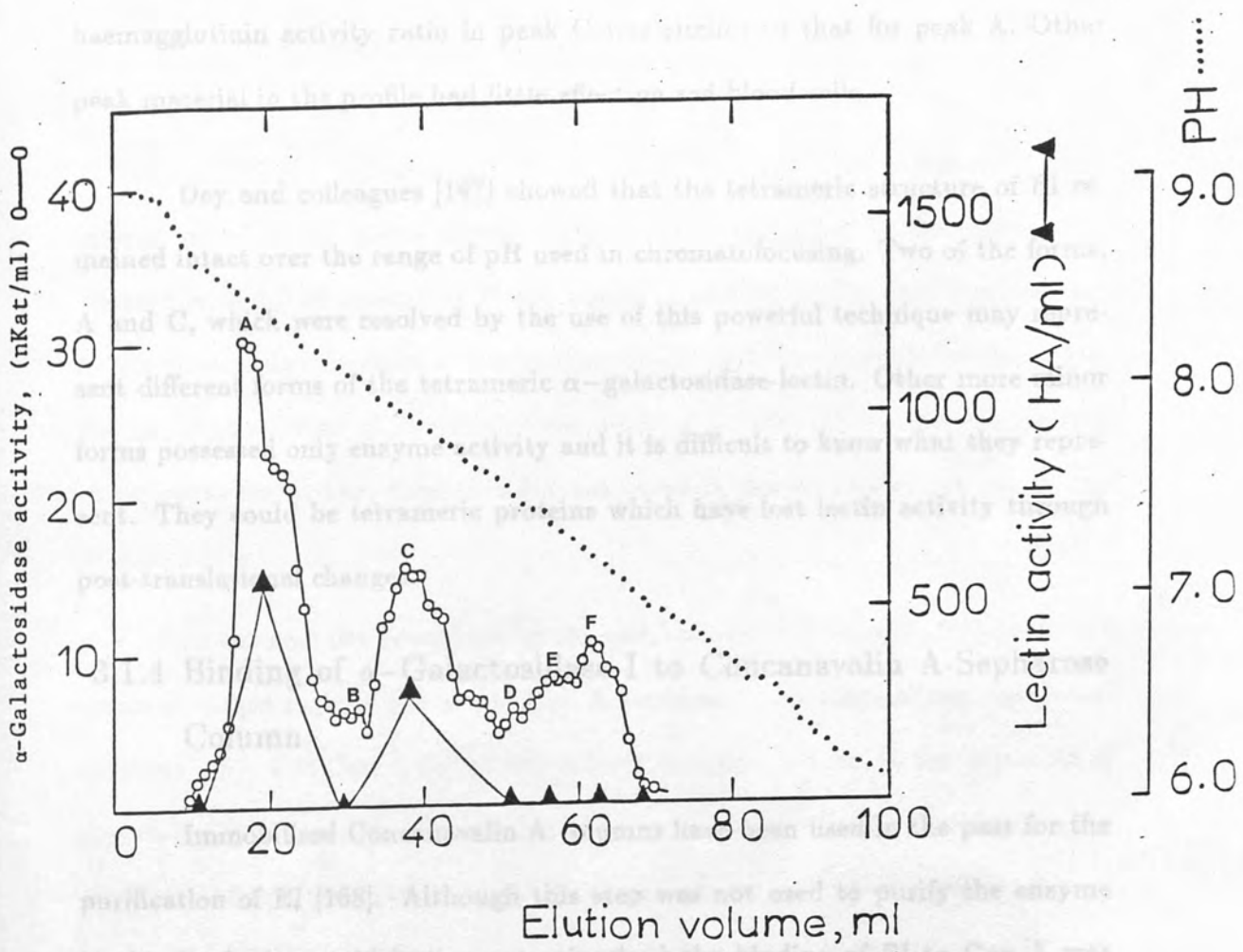


Figure 11b

Co-elution of catalytic and lectin activities of isomeric forms of EI separated using Chromatofocusing

a high titre (Fig. 11(b)) when tested for agglutination and there was also haemagglutinin activity associated with the material in peak C. The α−galactosidase/haemagglutinin activity ratio in peak C was similar to that for peak A. Other peak material in the profile had little effect on red blood cells.

Dey and colleagues [147] showed that the tetrameric structure of EI remained intact over the range of pH used in chromatofocusing. Two of the forms, A and C, which were resolved by the use of this powerful technique may represent different forms of the tetrameric α−galactosidase-lectin. Other more minor forms possessed only enzyme activity and it is difficult to know what they represent. They could be tetrameric proteins which have lost lectin activity through post-translational changes.

3.1.4 Binding of α−Galactosidase I to Concanavalin A-Sepharose Column

Immobilised Concanavalin A columns have been used in the past for the purification of EI [168]. Although this step was not used to purify the enzyme in this study (to avoid lectin contamination) the binding of EI to Con A was re-examined, as it had been claimed [168] that a fraction of EI was not absorbed by the lectin.

Concanavalin A is a 'classical' glucose/mannose-specific lectin obtained from jack bean seeds which when coupled to an immobilised matrix functions as an affinity column. It is highly specific in binding 'mannose-rich' glycoproteins, such as EI, and elution of the bound glycoproteins is achieved by introducing carbohydrate (a hapten) into the eluting buffer that competes for the lectin site of Concanavalin A thereby displacing the bound glycoproteins. The effectivness

of Con A-Sepharose affinity chromatography for the purification of glycoproteins has been well established by many workers in the past [169-170].

EI (from Stage 6; Fig 4) was applied to a Con A-Sepharose column (Fig. 12). The fractions which eluted with acetate buffer (pH 5.5) showed some absorbance at 280 nm indicating the presence of proteins, but there was no enzyme activity present. All of the enzyme activity that was applied to the column was released with 0.2 M methyl $\alpha-$D-mannoside in acetate buffer: this was contrary to the observation made in a previous study. If, as believed in the earlier work, the unbound fraction of EI was a consequence of *in vitro* deglycosylation (during the enzyme extraction) then this did not occur in preparing the enzyme in the present study.

In the past the procedure for the purification of EI from *V. faba* seeds has involved stages such as the use of Con A-Sepharose and 'immobilised melibiose' columns [25]. The Con A-Sepharose affinity matrix operates on the principle of glycan-lectin interactions whilst substrate-enzyme interactions are involved in the operation of the 'melibiose column'. Both procedures presented problems during EI purification. In the former case it was the danger of contaminating EI with Con A and in the latter, the low capacity of the column. Hence, in the earlier part of this study a number of alternative methods for the purification of EI were investigated before the final set of procedures was adopted. Some of this work is reported below.

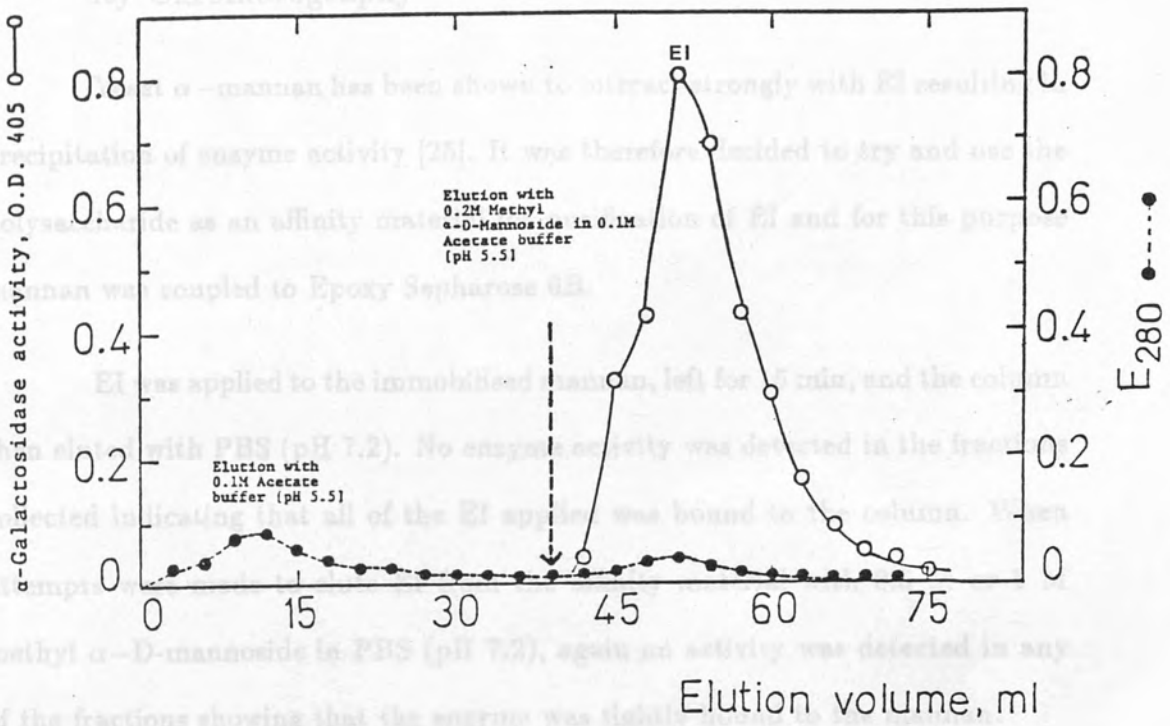


Figure 12

Concanavalin A-Sepharose affinity chromatography of EI (From Stage 6; Fig. 4)

Experimental details are given in Section 2.4.3(ii)

3.2 Attempts to Develop an Alternative Method for the Purification of α–galactosidase I from *V. faba*

3.2.1 Attempted Mannan-Sepharose and Mannose-Sepharose Affinity Chromatography

Yeast α–mannan has been shown to interact strongly with EI resulting in precipitation of enzyme activity [25]. It was therefore decided to try and use the polysaccharide as an affinity material for purification of EI and for this purpose mannan was coupled to Epoxy Sepharose 6B.

EI was applied to the immobilised mannan, left for 15 min, and the column then eluted with PBS (pH 7.2). No enzyme activity was detected in the fractions collected indicating that all of the EI applied was bound to the column. When attempts were made to elute EI from the affinity material with 0.5 M or 1 M methyl α–D-mannoside in PBS (pH 7.2), again no activity was detected in any of the fractions showing that the enzyme was tightly bound to the mannan.

At this stage it was decided that immobilised mannose would be likely to bind less strongly to EI than mannan-Sepharose [25]. Mannose was, therefore, coupled to Epoxy Sepharose 6B and the gel packed into a 'syringe column'. EI was applied and the column eluted with PBS (pH 7.2). No enzyme activity was detected in the resulting fractions (Fig. 13(a)). As with mannan-Sepharose, when attempts were made to elute the bound EI with 0.5 M or 1 M methyl α–D-mannoside in PBS (pH 7.2) no enzyme activity was released.

Studies of the lectin sites of EI by Dey and colleagues [25] have shown that EI interactions with mannose residues are at their weakest at acidic pH values. Hence, the pH of the PBS was changed to 5.5 and attempts were again made to

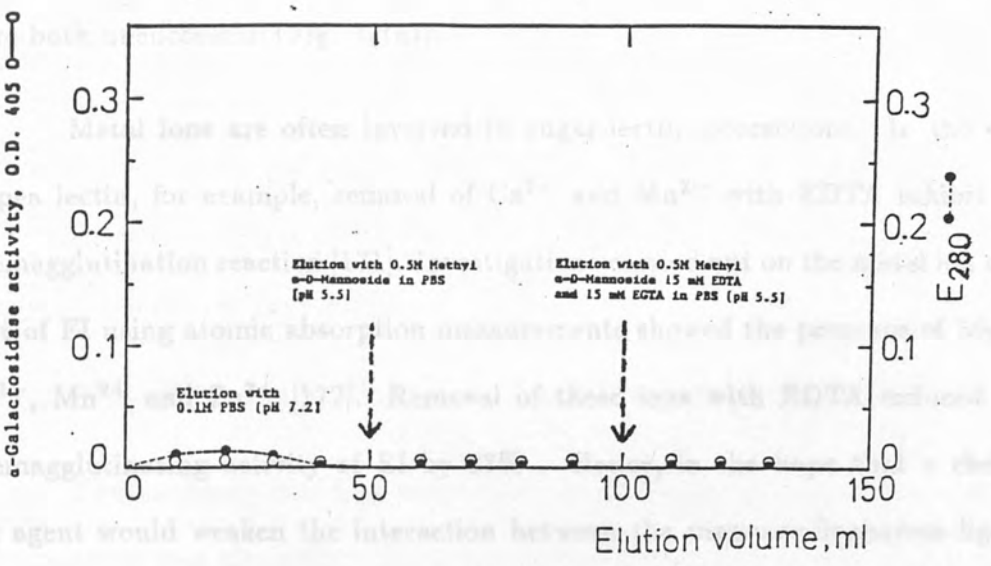
Figure 13

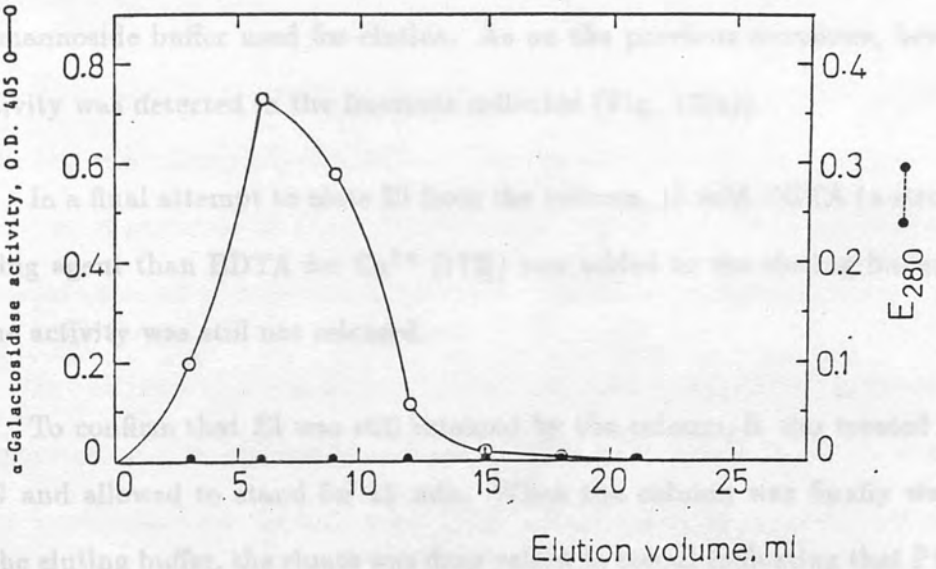
(a) Attempted elution of EI from Mannose-Sepharose affinity column

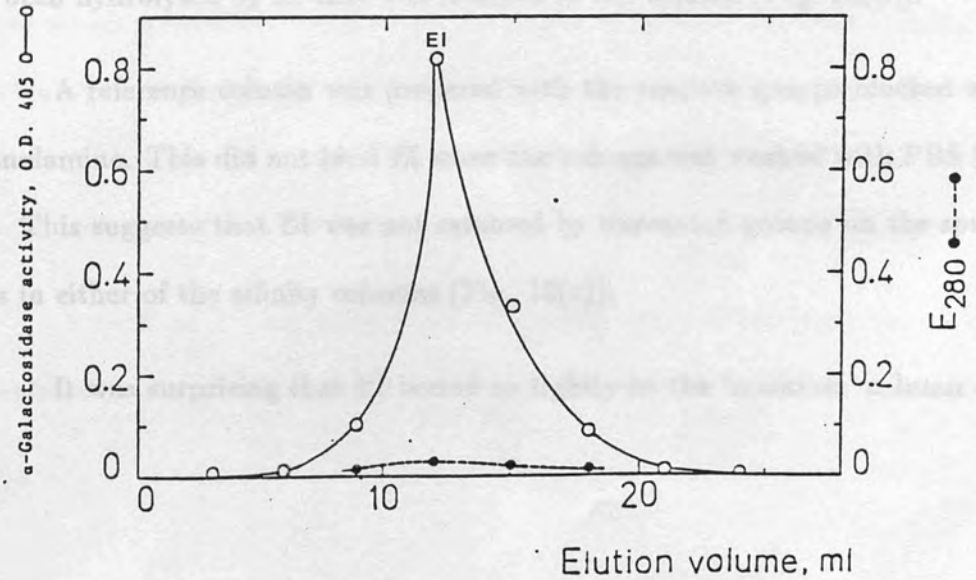
(b) Testing for the presence of EI activity on the Mannose-Sepharose affinity column

(c) Testing for the binding affinity of EI on an Ethanolamine treated Sepharose column

Experimental details are given in Section 2.4.3(iv)


α-Galactosidase activity, O.D. 405
E280
Elution with 0.5M Methyl α-D-Mannoside in PBS (pH 5.5)
Elution with 0.5M Methyl α-D-Mannoside 15 mM EDTA and 15 mM EGTA in PBS (pH 5.5)
Elution with 0.1M PBS (pH 7.2)
Elution volume, ml


α-Galactosidase activity, O.D. 405
E280
Elution volume, ml


EI
α-Galactosidase activity, O.D. 405
E280
Elution volume, ml

elute EI from the column with 0.5 M and 1 M methyl α−D-mannoside. These were both unsuccessful (Fig. 13(a)).

Metal ions are often involved in sugar-lectin interactions. In the case of pea lectin, for example, removal of Ca^{2+} and Mn^{2+} with EDTA inhibit the haemagglutination reaction [171]. Investigations carried out on the metal ion content of EI using atomic absorption measurements showed the presence of Mg^{2+}, Ca^{2+}, Mn^{2+} and Zn^{2+} [172]. Removal of these ions with EDTA reduced the haemagglutinating activity of EI by 87% . Hence, in the hope that a chelating agent would weaken the interaction between the mannose-Sepharose ligand groups and the enzyme, 15 mM EDTA was added to the PBS-0.5 M methyl α−D-mannoside buffer used for elution. As on the previous occasions, however no activity was detected in the fractions collected (Fig. 13(a)).

In a final attempt to elute EI from the column, 15 mM EGTA (a stronger chelating agent than EDTA for Ca^{2+} [173]) was added to the eluting buffer but enzyme activity was still not released.

To confirm that EI was still retained by the column, it was treated with PNPG and allowed to stand for 15 min. When the column was finally washed with the eluting buffer, the eluate was deep yellow in colour indicating that PNPG had been hydrolysed by EI that was retained in the column (Fig. 13(b)).

A reference column was prepared with the reactive groups blocked with ethanolamine. This did not bind EI when the column was washed with PBS (pH 7.2). This suggests that EI was not retained by unreacted groups on the spacer arms in either of the affinity columns (Fig. 13(c)).

It was surprising that EI bound so tightly to the 'mannose' column and

it is presumed that more than one type of interaction was involved. Hydrophobic and non-specific interactions have often been encountered in affinity chromatography [174]. However, in the present study, no attempts were made to elute with chaotropic or hydrophobic agents (eg. $MgCl_2$ or ethylene glycol) as the aim was to try and develop a simple and a more efficient technique compared to the existing methods for the purification of EI.

3.2.2 Use of an α–Galactosidase I antibody-Sepharose Column

The antibody that had been raised to EI to probe the structure of this enzyme-lectin was also utilised in an attempt to develop a new and an improved purification stage for EI.

Since the development of coupling gels (eg. CNBr-activated Sepharose 4B) the use of antibody affinity columns has become increasingly popular for the purification of proteins. In particular, the high capacity of these columns allows substantial amounts of materials to be purified within a short period of time [175].

In the present study, EI antiserum was initially applied to a Protein A-Sepharose column which was then washed with PBS (pH 7.2). This removed protein impurities from the antiserum (Fig. 14(a)) which were monitored at 280 nm in the column fractions. (Protein A is a glycoprotein from *Staphylococcus aureus* which specifically binds to the F_c region of antibodies). The column was next eluted with glycine-HCl (pH 2.2) and the fractions exhibiting high absorbance readings at 280 nm were pooled and dialysed against PBS (pH 7.2) to prevent acid proteolysis of the EI antibodies.

A total of 1.1 mg of purified antibodies were eluted from the Protein A-Sepharose column. Their purity was then tested by SDS-PAGE. Two protein

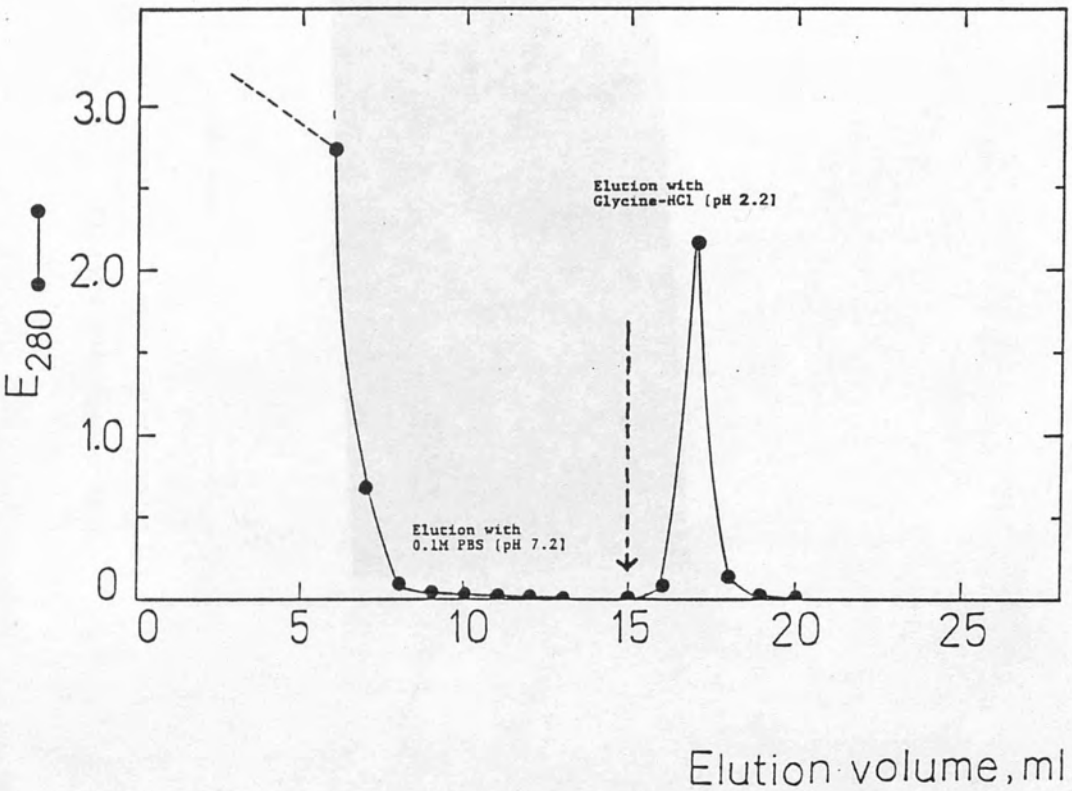


Figure 14a

Purification of EI antibodies using a Protein A-Sepharose column

Experimental details are given in Section 2.4.3(v)

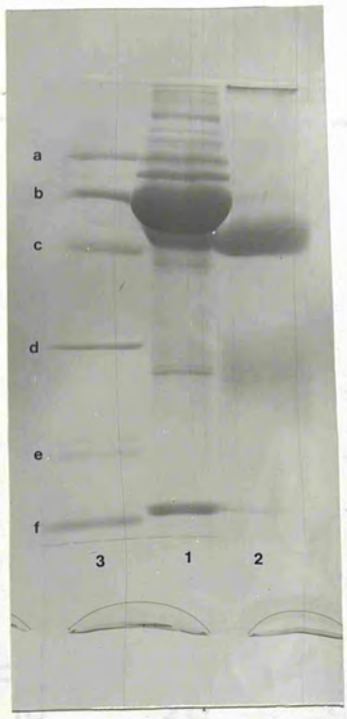


Figure 14b

SDS-PAGE of crude and purified EI antibodies

Lane 3 contained the molecular weight markers
(b) albumin, bovine serum, (M_r 66,000); (c) albumin, egg, (M_r 45,000);
(d) carbonic anhydrase, (M_r 29,000);
(e) trypsin inhibitor, (M_r 20,100);
(f) α-lactalbumin, (M_r 14,400); (a) phosphorylase b, (M_r 94,000).

1. Crude EI antibodies (35 µg)
2. Purified EI antibodies (25 µg)
3. M_r markers

Experimental details are given in Section 2.4.3(v)

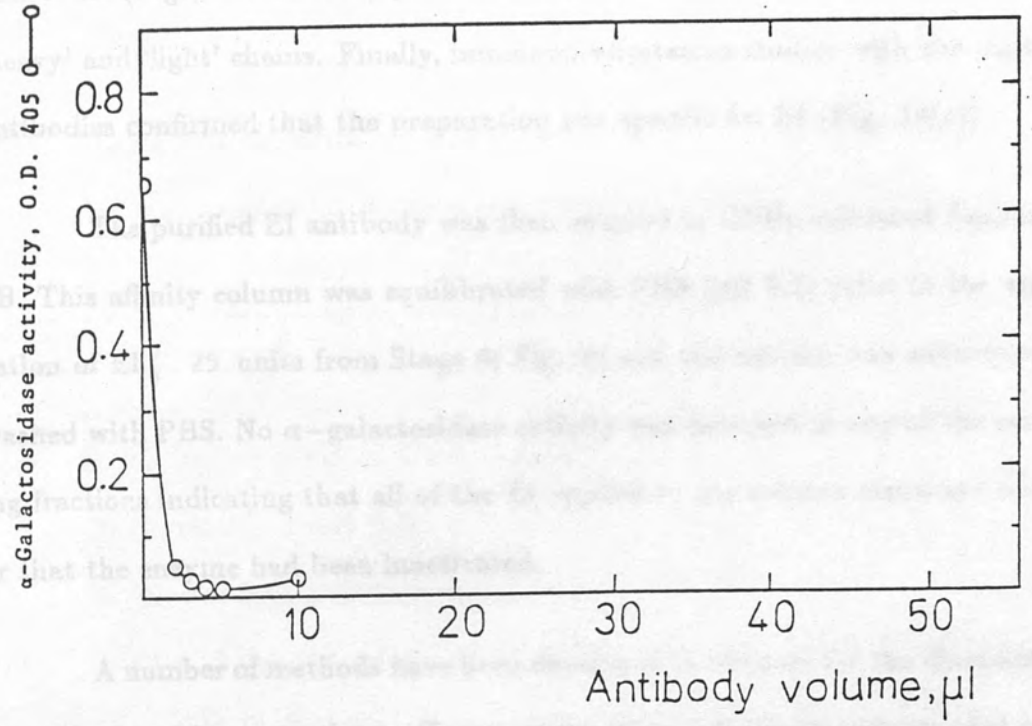


Figure 14c

Immunoprecipitation of EI using purified EI antibodies

Experimental details are given in Section 2.10.4(ii)

components corresponding to the 'heavy' (M_r 51,000) and 'light' (M_r 23,000) chains of the EI antibodies were observed in the purified preparation (Fig. 14(b); Lane 2): no other bands were detected on this lane. In contrast, the crude antiserum (Fig. 14; Lane 1) yielded many bands in addition to the antibody 'heavy' and 'light' chains. Finally, immunoprecipitation studies with the purified antibodies confirmed that the preparation was specific for EI (Fig. 14(c)).

The purified EI antibody was then coupled to CNBr-activated Sepharose 4B. This affinity column was equilibrated with PBS (pH 7.2) prior to the application of EI (25 units from Stage 6; Fig. 4) and the column was subsequently washed with PBS. No $\alpha-$galactosidase activity was detected in any of the resulting fractions indicating that all of the EI applied to the column remained bound or that the enzyme had been inactivated.

A number of methods have been developed in the past for the dissociation of antibody-antigen complexes. Comprehensive lists of solvent systems that have been used for this purpose have been published [176-177]. It is clear from previous work that quite different conditions may be needed to dissociate different antigen-antibody complexes. These conditions may have to be extreme (eg. with respect to pH) in which case it would be difficult to use the procedure for obtaining active enzymes. Hence the selection of the solvent system for removing enzymes from 'antibody-columns' is a crucial step in developing the method.

In the present study the stability of EI was tested against a range of buffers and chaotropic agents prior to selecting the suitable eluent for the EI antibody affinity column (see Table 2).

Aqueous ethylene glycol has been used to reduce hydrophobic interactions

TABLE 2 Testing for the stability of α–galactosidase I in different solvent systems prior to using them on the α–galactosidase I antibody-Sepharose column.

Solvent system	Absorbance (405 nm)
1. McIlvaine buffer (pH 5.5; control)	0.377
2. Glycine-HCl (pH 2.2)	0.258
3. Glycine-HCl-Ethylene glycol	0.077
4. 50 mM Diethylamine (pH 10.75)	0.088
5. 50 mM Diethylamine-Ethylene glycol	0.111
6. 1 M Propionic acid (pH 2.3)	0.045
7. 3 M $MgCl_2$	0.041

α- galactosidase I activity was assayed in the various solvent systems listed below, according to the procedure described in section 2.1.1, except that McIlvaine buffer (pH 5.5) was replaced with the solvents listed. For the assay in 3M $MgCl_2$, Na_2CO_3 could not be added at the end of the reaction because $MgCO_3$ would precipitate out. There is therefore considerably uncertainty in the measurement of EI activity in 3M $MgCl_2$.

between antigens and antibodies. However, when stability of EI in 50% (v/v) ethylene glycol in PBS (pH 7.2) was tested, almost 70% of the activity was lost after 15 min at 30 ^{0}C suggesting that some intramolecular hydrophobic interactions are responsible for the preservation of the stable tetrameric structure of EI.

Chaotropic ions which disrupt the structure of water molecules have been used to break down hydrophobic bonding between protein molecules. Some of the commonly used ions in immunoaffinity chromatography are Cl^-, SCN^-, and CCl_3COO^- . The stability of EI in the presence of 3 M $MgCl_2$ in neutral solution was very low (Table 2): almost 80% of activity was lost during 15 min at 30 ^{0}C. It is assumed that the salt behaved similarly to the ethylene glycol.

The sharp fall in the binding constants of proteins to antibodies at pH values below 4 and above 10, allows dissociation of antigen-antibody complexes to be achieved under conditions of extreme pH. This was observed for example in the case of an alkaline phosphatase-phosphatase antibody complex using a high pH buffer [176] whilst the dissociation of a cellulase-cellulase antibody complex could only be achieved at low pH [176]. Protein denaturation is one disadvantage in using highly acidic or alkaline conditions. However, in view of the fact that these conditions are very effective in disrupting strong hydrophobic bonding, which may be resistant to other agents and solvent systems, the tendency to cause some denaturation is often tolerated. In the present study three different systems representing a range of pH values were examined as potential eluents for the column: 1. glycine-HCl (pH 2.2); 2. 1 M propionic acid (pH 2.3); 3. 50 mM diethylamine (pH 10.75). Low pH glycine-HCl buffers had been used previously [178] to dissociate $\beta-$glucosidase-$\gamma-$globulin complexes via a stepwise elution

process. When EI was treated with glycine-HCl buffer (pH 2.2; Table 2) about 80% of the activity remained showing that the acidic conditions had no drastic effect on the stability of the enzyme. However, EI lost about 90% of its activity in the presence of 1 M propionic acid (pH 2.3). This must be a function of the high molar concentration of the organic acid and not the hydrogen ion concentration. Treatment with 50 mM diethylamine (pH 10.75) also resulted in an unacceptable loss of enzyme activity.

Thus on the basis of the above results glycine-HCl (pH 2.2) was selected as the suitable solvent for the attempted elution of bound EI from the EI antibody affinity column.

However, only 20% of the EI activity originally applied to the column was eluted by this buffer (Fig. 15).

To test whether active enzyme still remained bound to the antibody 10 mM PNPG was applied to the column. No yellow colour appeared in the eluate thus indicating the absence of any active bound EI. The antibody affinity column procedure was therefore, abandoned as a method for the purification of EI as a high loss of activity was noticed.

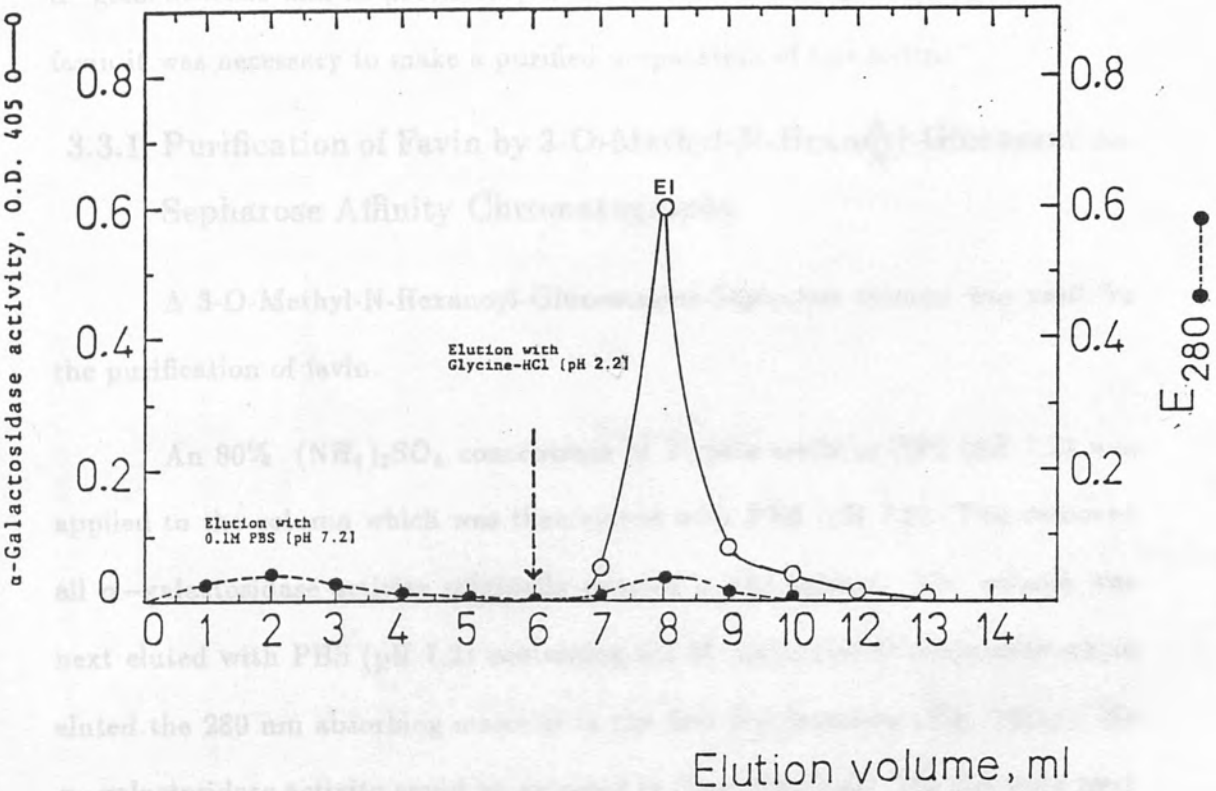


Figure 15

Purification of EI (From Stage 6; Table1) using an EI antibody-Sepharose column.

Experimental details are given in Section 2.4.3(vi)

3.3 Purification of Favin from *V. faba* Seeds

In order to discover more about the haemagglutinating properties of $\alpha-$galactosidase and in particular, to see if there was any contamination with favin it was necessary to make a purified preparation of this lectin.

3.3.1 Purification of Favin by 3-O-Methyl-N-Hexanoyl-Glucosamine-Sepharose Affinity Chromatography

A 3-O-Methyl-N-Hexanoyl-Glucosamine-Sepharose column was used for the purification of favin.

An 80% $(NH_4)_2SO_4$ concentrate of *V. faba* seeds in PBS (pH 7.2) was applied to the column which was then eluted with PBS (pH 7.2). This removed all $\alpha-$galactosidase activity originally present in the extract. The column was next eluted with PBS (pH 7.2) containing 0.2 M methyl-$\alpha-$D-mannoside which eluted the 280 nm absorbing material in the first few fractions (Fig. 16(a)). No $\alpha-$galactosidase activity could be detected in these fractions. The fractions were dialysed against PBS (pH 7.2) and tested for haemagglutinin activity. High titres were obtained indicative of the presence of favin. This was confirmed by SDS-PAGE which showed polypeptide bands corresponding to the $\beta-$ and β'-subunits of favin. No polypeptide contaminants appeared to be present (Fig. 16 (b)).

Although this method yielded pure favin, multiple use of the column was required to provide sufficient quantities of the lectin. This was because of the low capacity of the column material. Hence while this method was being used other methods were tried in an attempt to find an alternative higher yield procedure.

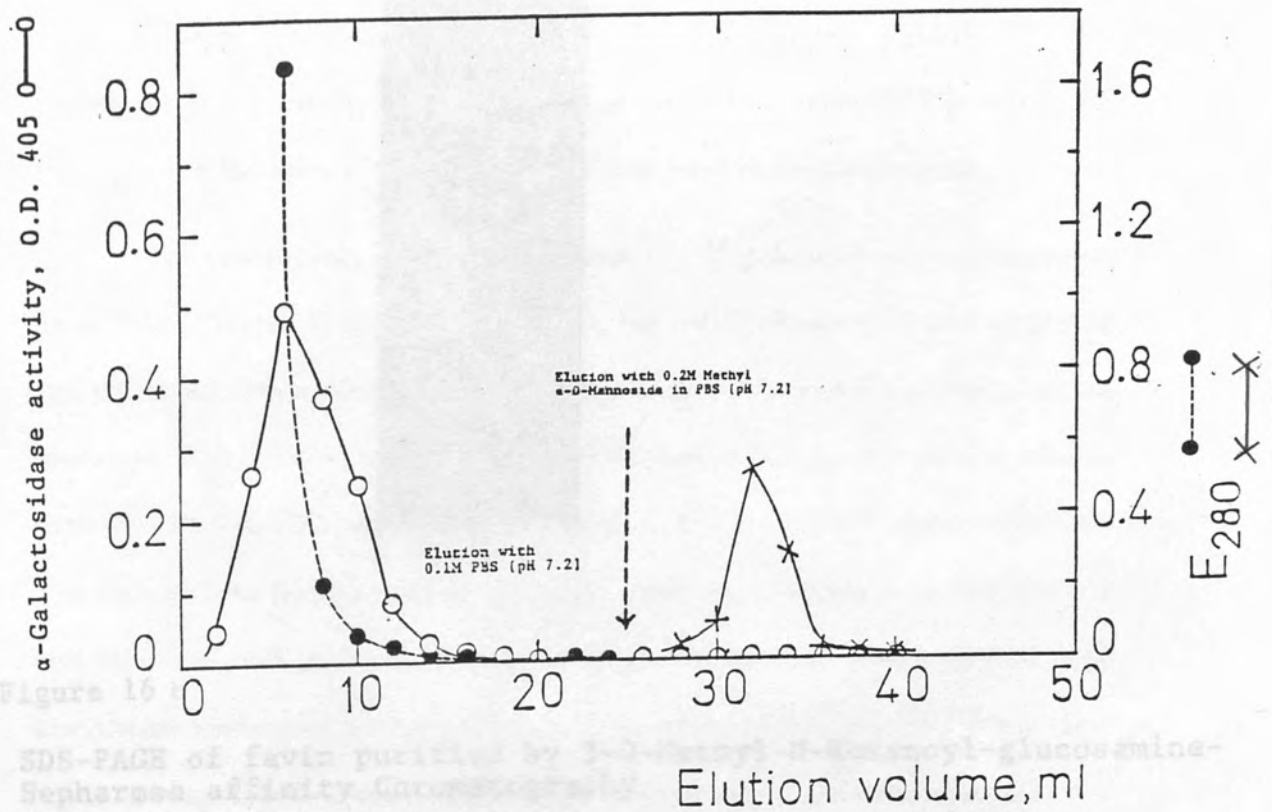


Figure 16 a

3-0-Methyl-N-Hexanoyl-glucosamine-Sepharose affinity Chromatography for the purification of favin

Experimental details are given in Section 2.4.3(vii)

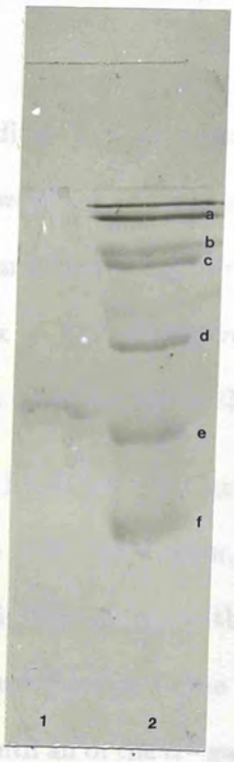


Figure 16 b

SDS-PAGE of favin purified by 3-0-Methyl-N-Hexanoyl-glucosamine-Sepharose affinity Chromatography

Lane 2 contained the molecular weight markers

(a) phosphorylase b (M_r 94,000); (b) albumin, bovine serum, (M_r 66,000); (c) albumin, egg; (M_r 45,000); (d) carbonic anhydrase (M_r 30,000); (e) trypsin inhibitor (M_r 21,100); (f) α-lactalbumin (M_r 14,400).

1. Favin (10 μg)
2. M_r markers

Experimental details are given in Section 2.4.3(vii)

3.3.2 Purification of Favin by Sephadex G-100 Affinity Chromatography

Because of the difficulties encountered with the 3-O-methyl ... column other affinity materials were tested to see if they were suitable for favin purification. Sephadex G-75 and G-150 had previously been used [140,141] so in the present study a Sephadex G-100 column was tested (see Table 3). The conditions for operating the column were similar to those used in the earlier work.

The protein in a PBS (pH 7.2) extract of *V. faba* seeds was concentrated by adding $(NH_4)_2SO_4$ to 80% saturation, the pellet obtained by centrifugation was extracted with a minimum volume of the same buffer and the extract dialysed overnight. The solution was applied to the Sephadex column and elution effected with with PBS (pH 7.2) until all of the $\alpha-$galactosidase activity had been removed. The column was further washed with PBS until the absorbance at 280 nm was less than 0.01 and then eluted with 0.5 M glucose in PBS. Fractions with high absorbance readings at 280 nm possessed no $\alpha-$galactosidase activity. When these fractions were dialysed and tested for agglutination a high titre was observed (Fig. 17 (a)). These fractions were pooled, concentrated and subjected to SDS-PAGE. Protein bands with M_r's corresponding to the $\beta-(M_r$ 20,700) and β'- (M_r 18,900) subunits were detected (Fig. 17 (b)) on the gel thereby confirming that the isolated material was favin. No other bands were present.

TABLE 3 Purification of favin from *V. faba* seeds.†

Stage	Total activity (haem-agglutinin units;HA)	Vol (ml)	Protein (mg/ml)	Specific Activity (HA/mg)	Recovery
1. PBS (pH 7.2) extract	64,000	70	160	5.7	100
2. 80% $(NH_4)_2SO_4$ precipitate	51,200	15	1050	3.25	80
3. Fractions from the Sephadex G-100 affinity column	42,240	1.0	90	469.3	66

† Recovery has been calculated relative to stage 1. Weight of the seed powder=100 g.

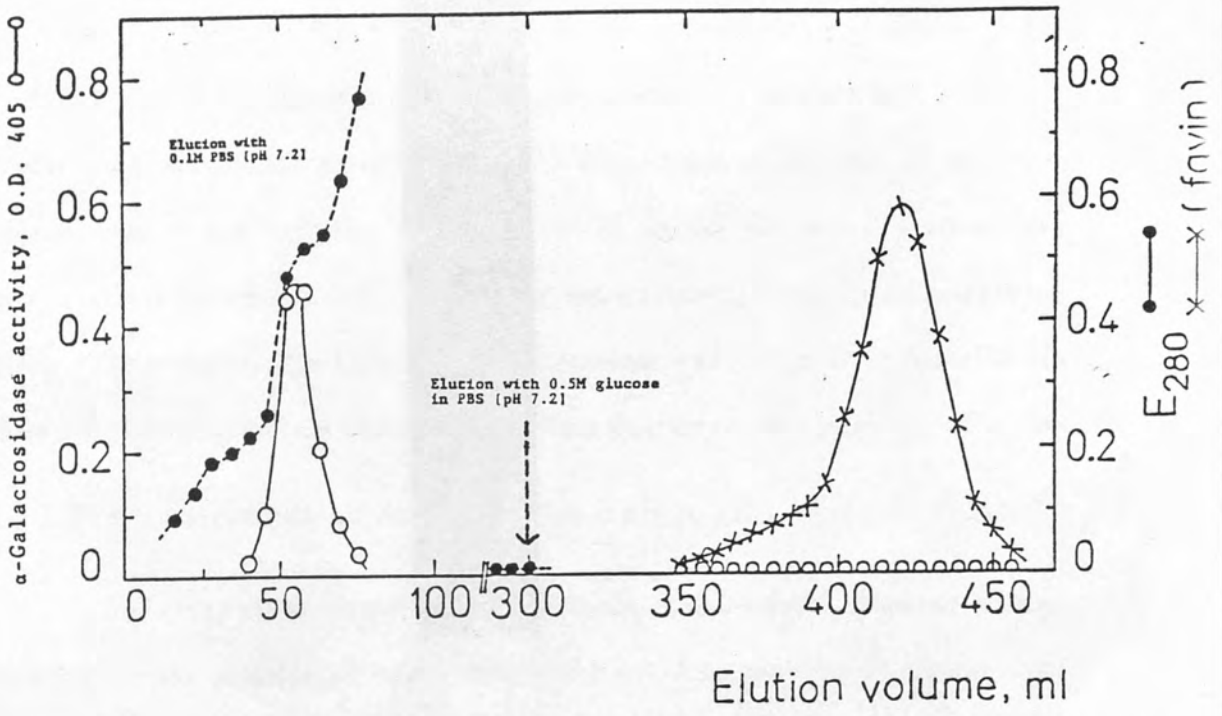


Figure 17a

Sephadex G-100 affinity chromatography for the purification of favin

Experimental details are given in Section 2.4.3(viii)

Figure 17 b

SDS-PAGE of favin purified by Sephadex G-100 affinity chromatography

Lane 2 contained the molecular weight markers
(a) albumin, bovine serum, (M_r 66,000); (b) albumin, egg, (M_r 45,000); (c) glyceraldehyde-3-phosphate dehydrogenase, (M_r 36,000); (d) carbonic anhydrase, (M_r 29,000); (e) trypsinogen, (M_r 24,000); (f) trypsin inhibitor, (M_r 20,100); (g) α-lactalbumin, (M_r 14,400).

1. Favin (20 μg) (From Stage 3; Table 3)
2. M_r markers

Experimental details are given in Section 2.4.3(viii)

3.4 Simultaneous Purification of EI, EII and Favin.

In view of the above results, an attempt was made to purify simultaneously EI, EII and favin on a Sephadex G-100 column. The initial procedure for the purification of EI and EII (Stage 1-3) was carried out as in Fig. 4 with elution of EI and EII from the Sephadex G-100 column (Stage 4) using McIlvaine buffer (pH 5.5) containing 0.1 M KCl. The column was then washed with the same buffer until the column eluate gave a zero absorbance at 280 nm. It was next eluted with McIlvaine buffer containing 0.5 M glucose which produced protein rich fractions with haemagglutinin activity but no detectable $\alpha-$galactosidase activity. The presence of favin in the pooled fractions was confirmed by SDS-PAGE. The complete purification of the three protein fractions is summarised in Fig. 18.

3.5 An Investigation of the Lectin Nature of $\alpha-$Galactosidase I

Following the preparation of EI and favin, and observations on some properties of the two proteins, attempts were made to find out more about the reported lectin nature of EI and, in particular, to see if this could possibly be due to the presence of contaminating favin or whether the lectin site was, in fact, an integral part of the EI protein.

3.5.1 Isoelectric Focusing of EI and Favin

Isoelectric focusing has been extensively used as a tool for the separation and identification of proteins and glycoproteins [179-180]. A good example is the use of the technique for the separation and analysis of various forms of normal and modified human haemaglobins [181].

Isoelectric focusing of EI showed protein components with pH values from 7.5 to 8.6 (Fig. 19) thus confirming the results obtained with chromatofocusing

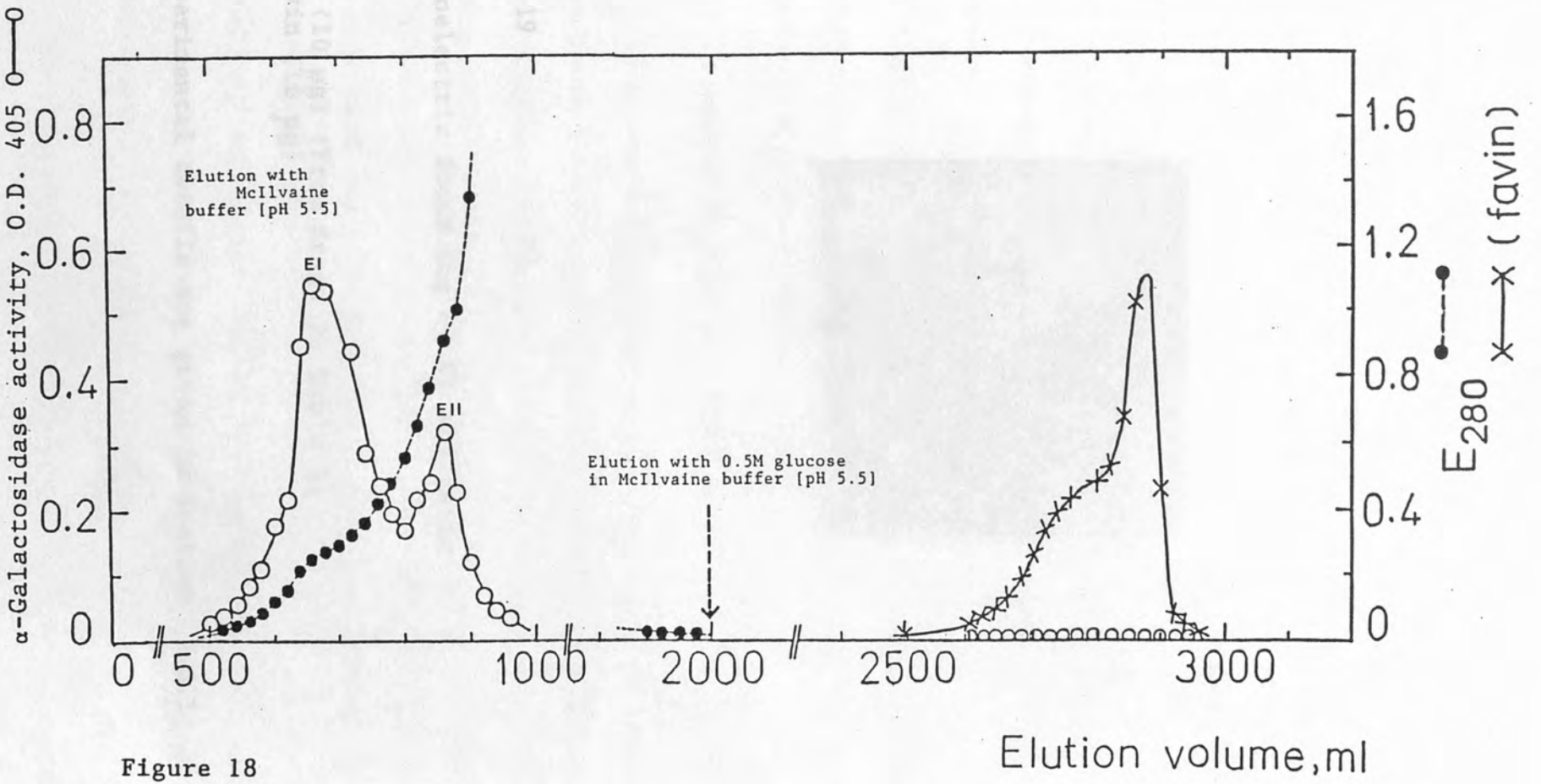


Figure 18

Simultaneous purification of EI, EII and favin on Sephadex G-100.

Experimental details are given in Section 2.4.4

Figure 19

Isoelectric focus ing of EI and favin

1. EI (10 µg) (From Stage 7; Table 1)
2. Favin (10 µg)

Experimental details are given in Section 2.8.1

and the microheterogeneous nature of EI.

Favin showed the presence of a number of forms with a range of pI values from 5.2 to 5.7. The existence of different forms of favin (in a purified extract) has been observed in the past [143]. Although immunologically related [143], the high degree of focusing and distinct pI values suggest that each form of favin has different surface characteristics.

There were no bands in the range 5.2 to 5.7 in the lane that contained the EI, indicating the absence of any loosely bound favin contaminants in the enzyme preparation.

IEF, therefore, suggests that the EI preparation was not contaminated with favin unless the lectin was present tightly bound perhaps by ionic interactions in the interior of the undenatured forms of EI.

3.5.2 Proteolysis of α–galactosidase I and Favin

V8 Protease from *Staphylococcus aureus* is an endo acid protease which cleaves peptide bonds at aspartic and glutamic acid residues. Hence partial hydrolysis of proteins can be achieved and structures compared by examining the resulting peptide 'maps'. When EI was treated with V8 protease and then examined by SDS-PAGE (Fig. 20) one major peptide band (M_r 20,000) together with three minor bands between M_r 20,000 and M_r 14,500 were observed. The electrophoretic mobility of the major band corresponded very closely to the β–subunit of favin. The β–subunit of favin itself was hydrolysed when the lectin was treated with protease and low M_r peptides were produced. The latter were dissimilar to the minor peptides formed by the proteolysis of EI.

The release of the M_r 20,000 fragment from the EI subunit suggests the

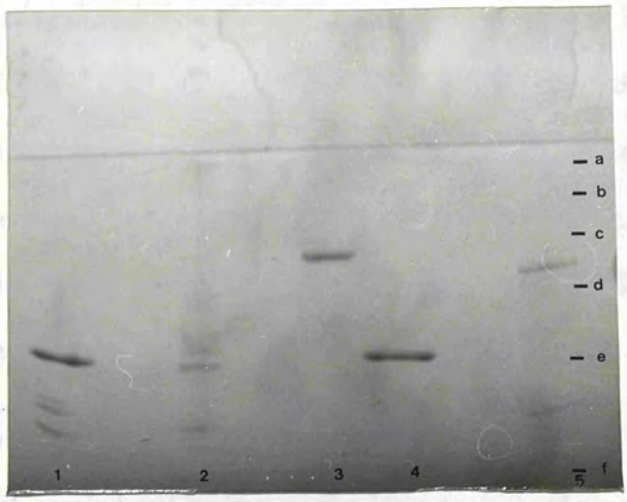


Figure 20

One dimensional peptide mapping of EI and favin using V8 protease from Staphylococcus aureus

Lane 5 contained the molecular weight markers

(a) phosphorylase b (M_r 94,000); (b) albumin, bovine serum, (M_r 66,000); (c) albumin, egg; (M_r 45,000); (d) carbonic anhydrase (M_r 30,000); (e) trypsin inhibitor (M_r 21,100); (f) α-lactalbumin (M_r 14,400).

1. Favin (20 µg; Stage 3, Table 3) (V8 protease treated)
2. EI (10 µg; Stage 7; Table 1) (V8 protease treated)
3. EI (5 µg; Stage 7; Table 1) (V8 protease untreated)
4. Favin (10 µg; Stage 3; Table 3) (V8 protease untreated)
5. M_r markers

Experimental details are given in Section 2.9.1

possibility that either; (1) there were no more aspartic acid or glutamic acid residues in this fragment or (2) the time period of 30 min for hydrolysis was not sufficient for the V8 protease to cleave the remaining -COOH terminals of aspartic and glutamic acid residues present in this M_r 20,000 peptide from the EI subunit. If the situation is (1) then it is unlikely that there is significant homology between the M_r 20,000 fragment of the EI subunit and the β−subunit of favin, as the latter was digested by the protease and, hence, must have contained aspartic and glutamic acid residues. On the other hand if (2) was the reason, it would not rule out the possibility of some degree of homology between the EI subunit and the β−subunit of favin.

Time course experiments to optimise the digestion of EI subunit by V8 protease were not carried out owing to the long periods of time required for the purification of the substantial amounts of EI which would have been needed for this purpose. Instead, other approaches based on immunological methods requiring relatively small amounts of purified EI were employed to probe homology between the EI subunit and the β−subunit of favin.

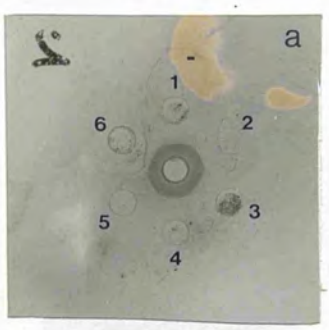
3.6 Immunological Studies

Following the observation that the main proteolytic product of EI and the β−subunit of favin had similar electrophoretic mobilities and noting that EI preparations and favin both displayed lectin activity with similar ligand specificities, further work was undertaken to investigate the possible existence of other similarities between EI and favin. For this purpose antibodies against EI, favin and β−subunit of favin were raised with the intention of handling the problem through immunological methods.

3.6.1 Testing for the Presence of α−galactosidase I Antibodies using Immunodiffusion

In preparing the EI antibodies, although the rabbit was injected successesively with purified EI (from Stage 7; Fig. 4) the production of specific antibodies remained uncertain, as this depended on a number of factors including the antigenicity of the injected protein and the antigenic response by the rabbit to the antigen of interest. Hence the serum was tested using Ouchterlony double immunodiffusion, where precipitin lines are formed as a result of cross-reactivity between the antigen and the serum if the latter contains antibodies corresponding to the antigen.

The centre well of plate [a] (Fig. 21) contained the antiserum and the six surrounding wells different concentrations of purified EI: plate [b] contained the antiserum in the centre well and five surrounding wells contained the antigen. The centre well of plate [c] contained control serum from an uninjected rabbit and, again, the six surrounding wells contained different concentrations of purified EI. The plates were observed for the appearance of precipitin lines after 48 hr.



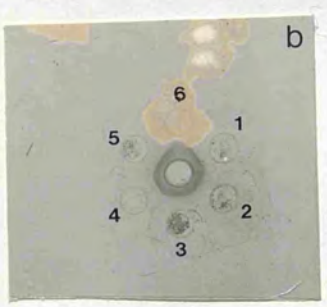


Figure 21a,b

Testing for the presence of EI antibodies using Ouchterlony double immunodiffusion

Plate [a] Wells 1-6 contained 2, 4, 6, 8, 10 and 12 µgs of EI respectively

Plate [b] Wells 1-5 contained 2, 4, 6, 8 and 10 µgs of EI respectively

Plate [c] Wells 1-6 contained 2, 4, 6, 8, 10 and 12 µgs of EI respectively

Centre wells of Plates [a] and [b] contained 20 µls of EI antiserum while [c] contained 20 µl of the serum prior to the rabbit being injected with EI.

Experimental details are given in Section 2.10.1(ii)

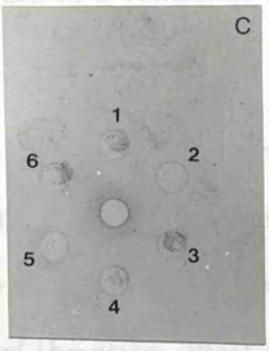


Figure 21c

Testing for the presence of EI antibodies using Ouchterlony double immunodiffusion

Plate [a] Wells 1-6 contained 2, 4, 6, 8, 10 and 12 µgs of EI respectively
Plate [b] Wells 1-5 contained 2, 4, 6, 8 and 10 µgs of EI respectively
Plate [c] Wells 1-6 contained 2, 4, 6, 8, 10 and 12 µgs of EI respectively

Centre wells of Plates [a] and [b] contained 20 µls of EI antiserum while [c] contained 20 µl of the serum prior to the rabbit being injected with EI.

Experimental details are given in Section 2.10.1(ii)

Plate [a] contained distinct white precipitin lines, plate [b] a horse-shoe shaped arc and plate [c] no precipitin lines.

When the plates were stained with Coomassie Brilliant Blue G, plate [a] showed a sharp blue ring in a colourless background, while plate [b] showed a horse-shoe shaped blue arc in a colourless background. Plate [c] showed no precipitin lines around the centre well.

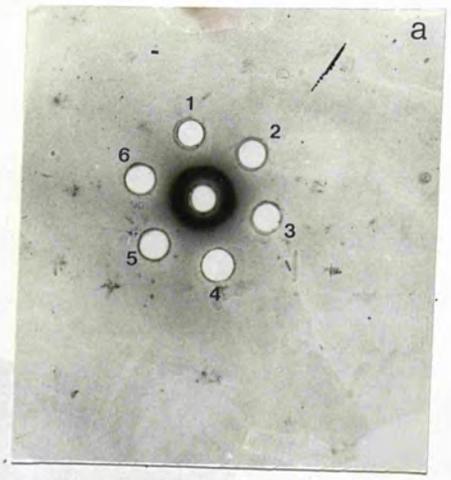
Plates [a] and [b], therefore confirm the presence of EI antibodies in the tested antiserum. The formation of an arc rather than a ring in the case of plate [b] was due to the absence of any antigen in well 6 so that a precipitin line was not formed in this region of the plate. Plate [c] was not expected to show any precipitin lines since the centre well of this plate contained control serum taken from the rabbit before innoculation.

3.6.2 Testing for the Presence of Favin Antibodies using Immunodiffusion

As in the case with EI antibodies, the presence of antibodies corresponding to favin in the antisera was probed using Ouchterlony double immunodiffusion.

The centre well of plate [a] (Fig. 22) contained antiserum from the rabbit that had been injected with purified favin while the six surrounding wells contained different concentrations of purified favin. Plate [b] was similar to plate [a] except only five wells contained favin. The centre well of plate [c] contained control serum from an uninjected rabbit and the surrounding wells, again, contained different concentrations of purified favin.

The plates were observed for the appearance of precipitin lines after 48 hr. Plate [a] contained distinct white precipitin lines. Plate [b] showed a horse-shoe



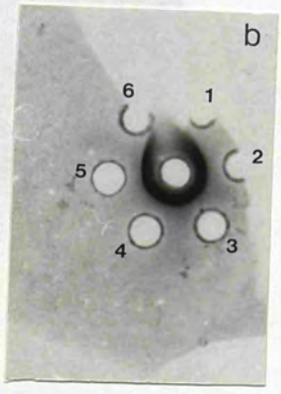


Figure 22a,b

Testing for the presence of favin antibodies using Ouchterlony double immunodiffusion

Plate [a] Wells 1-6 contained 3, 6, 9, 12, 15 and 18 µgs of favin respectively

Plate [b] Wells 1-5 contained 3, 6, 9, 12 and 15 µgs of favin respectively

Plate [c] Wells 1-6 contained 3, 6, 9, 12, 15 and 18 µgs of favin respectively.

Centre wells of plates [a] and [b] contained 20 µl of favin antiserum while [c] contained 20 µl of the serum prior to the rabbit being injected with favin.

Experimental details are given in Section 2.10.2(ii)

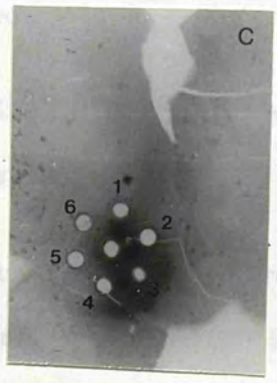


Figure 22c

Testing for the presence of favin antibodies using Ouchterlony double immunodiffusion

Plate [a] Wells 1-6 contained 3, 6, 9, 12, 15 and 18 µgs of favin respectively

Plate [b] Wells 1-5 contained 3, 6, 9, 12 and 15 µgs of favin respectively

Plate [c] Wells 1-6 contained 3, 6, 9, 12, 15 and 18 µgs of favin respectively.

Centre wells of plates [a] and [b] contained 20 µl of favin antiserum while [c] contained 20 µl of the serum prior to the rabbit being injected with favin.

Experimental details are given in Section 2.10.2(ii)

shaped arc; no precipitin lines were found in plate [c].

When the plates were stained with Coomassie Brilliant Blue G, plate [a] showed a sharp blue ring in a colourless background, while plate [b] showed a horse-shoe shaped blue arc in a colourless background. Plate [c] showed no precipitin lines around the centre well. Plates [a] and [b] therefore confirm the presence of favin antibodies in the antiserum.

3.6.3 Testing for the Presence of Favin β–subunit Antibodies using Western Blotting

Following the transfer of proteins from SDS-PAG to a nitrocellulose sheet (Fig. 23) one half (Section A; Lane 1-2) of it was stained for proteins with amido black while the other (Section B; Lane 3) was incubated with the antiserum.

Section A, Lane 1 which contained the purified favin shows two bands at M_r 20,000 and 18,900. They correspond to the $\beta-$ and the β'-subunits of favin, respectively. Lane 2 contained the M_r markers.

Section B, Lane 3 which contained the purified favin shows peroxidase positive bands at M_r's 28,000, 20,000 and 14,000 respectively. These bands correspond to the M_r's of the β–subunit precursor, the β–subunit and the hydrolysed β–subunit, respectively. This confirmed that the antiserum contained favin β–subunit antibodies.

3.6.4 Immunoprecipitation of α–galactosidase-I using α–galactosidase I Antibodies and Favin Antibodies

Immunological techniques such as immunoprecipitation, are commonly employed to study structural relationships between protein surfaces. In the present

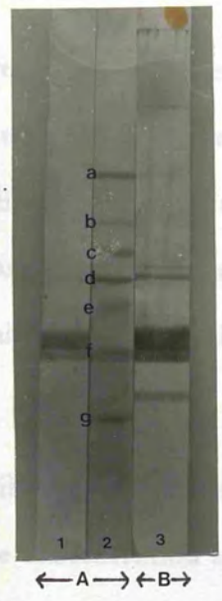


Figure 23

Testing for the presence of favin β-subunit antibodies using Western blotting.

Lane 2 contained the molecular weight markers (a) albumin, bovine serum, (M_r 66,000); (b) albumin, egg; (M_r 45,000); (d) carbonic anhydrase (M_r 29,000); (f) trypsin inhibitor (M_r 21,100); (g) α-lactalbumin (M_r 14,400); (c) glyceraldehyde-3-phosphate dehydrogenase (M_r 36,000); (e) trypsinogen (M_r 24,000).

Section A stained with 0.1% Amido Black (see experimental 2.2.4)

1. Favin (20 µg) (Stage 3; Table 3)
2. M_r markers

Section B stained for peroxidase (see experimental 2.1.3)

3. Favin (20 µg) (Stage 3; Table 3)

Experimental details are given in Section 2.10.3(ii)

study the interactions of EI with favin antibodies and EI antibodies were investigated.

Immunoprecipitation reactions were performed in the presence of 0.2 M methyl α−D-mannoside in an endeavour to prevent any lectin type interactions between antigens and antibodies. This was also considered to be a precaution against the interaction of antibodies produced against 'mannose-rich' glycan chains with the the glycan chains of the tested protein (both EI and favin are 'mannose-rich' glycoproteins).

The volumes of the antibody solutions used were kept constant at all antibody concentrations whilst the concentration and the volume of the EI solution were the same in each tube. When EI antibodies were titrated against EI, all enzyme activity was precipitated (Fig. 24) as expected. However, when favin antibodies were used, only part of the EI activity (30%) was precipitated from the solution (Fig. 25). This suggests that 30% of the surface structure of EI is homologous with favin.

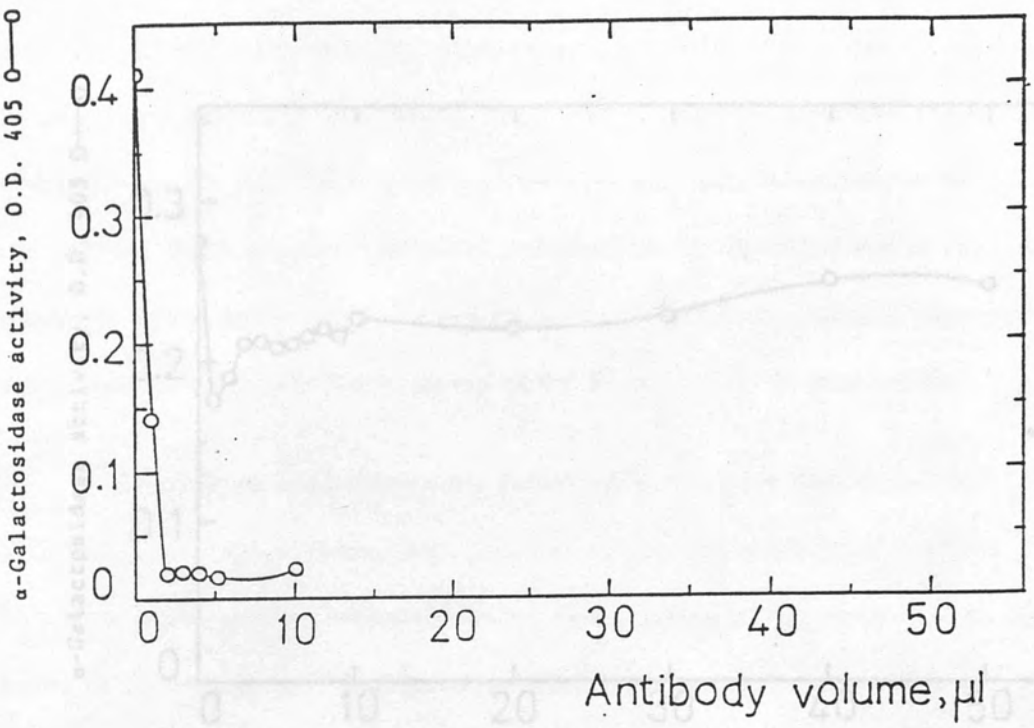


Figure 24

Immunoprecipitation of EI using EI antibodies

Experimental details are given in Section 2.10.4(i)

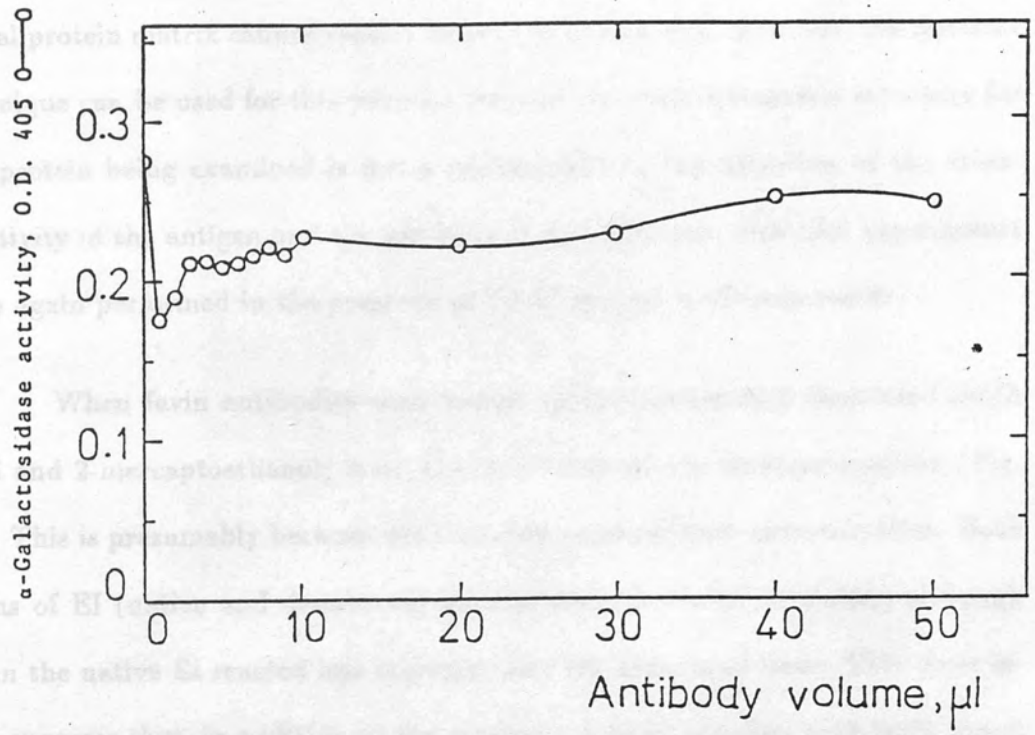


Figure 25

Immunoprecipitation of EI using favin antibodies

Experimental details are given in Section 2.10.4(i)

3.6.5 Dot-Blot Experiments with α–galactosidase I and Favin using Favin Antibodies

Although the surface characteristics of proteins can be studied via immunoprecipitation experiments, peptide structures buried in the three dimensional protein matrix cannot readily be probed in this way. However, the dot-blot technique can be used for this purpose because an intact tetrameric structure for the protein being examined is not a pre-requisite in the detection of the cross-reactivity of the antigen and the antibody in this instance. Dot-blot experiments were again performed in the presence of 0.2 M methyl α–D-mannoside.

When favin antibodies were tested against native and denatured (with SDS and 2-mercaptoethanol) favin, the latter showed the stronger reaction (Fig. 26). This is presumably because denaturation exposes more antigenic sites. Both forms of EI (native and denatured) reacted with the favin antibodies although again the native EI reacted less strongly than the denatured form. This observation suggests that, in addition to the antigenic sites in common with favin found on the surface of EI, more antigenic sites cross-reacting with favin antibodies are present within the three-dimensional structure of the enzyme. These results could provide an explanation for the relatively mild cross-reactivity observed between EI and favin antibodies in the immunoprecipitation study.

3.6.6 Study of the Antigenic Similarity between Favin and α–galactosidase-I using Western Blotting with Favin Antibodies.

In order to study further the antigenic similarity between EI and favin, these proteins together with a crude extract of *V. faba* were subjected to SDS-PAGE. The gel was then Western blotted and probed with antibodies raised

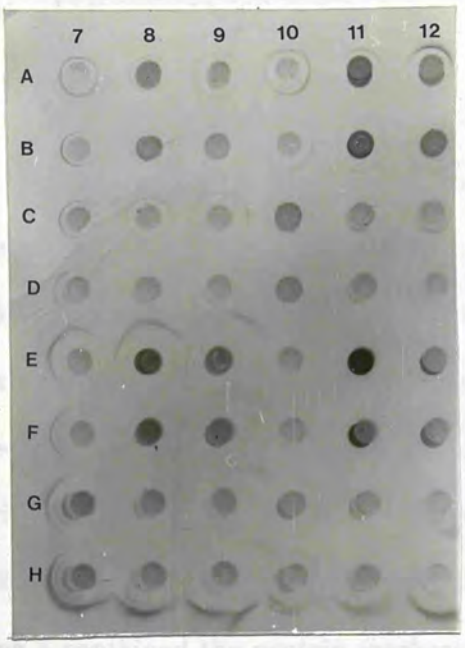


Figure 26

Dot-Blot experiments of EI and favin using favin antibodies

Wells 7-9 and A-D consisted of 14 µg undenatured EI
Wells 10-12 and A-D consisted of 14 µg denatured EI

Wells 7-9 and E-H consisted of 14 µg undenatured favin
Wells 10-12 and E-H consisted of 14 µg denatured favin

Wells 8: A,B; 9: A,B; 7: C,D; 8: C,D and 9: C,D contained 100 µl (each) of favin antibody at dilutions 1:500, 1:1000, 1:2000, 1:4000 and 1:8000 respectively.
Wells 7: A,B remained without any antibodies.

Wells 11: A,B; 12: A,B; 10: C,D; 11: C,D and 12: C,D contained 100 µl (each) of favin antibody at dilutions 1:500, 1:1000, 1:2000, 1:4000 and 1:8000 respectively.
Wells 10: A,B remained without any antibodies

Wells 8: E,F; 9: E,F; 7: G,H; 8: G,H and 9: G,H contained 100 µl (each) of favin antibody at dilutions 1:500, 1:1000, 1:2000, 1:4000 and 1:8000 respectively.
Wells 7: E,F remained without any antibodies

Wells 11: E,F; 12: E,F; 10: G,H; 11: G,H and 12: G,H contained 100 µl (each) of favin antibody at dilutions 1:500, 1:1000, 1:2000, 1:4000 and 1:8000 respectively.
Wells 10: E,F remained without any antibodies.

Experimental details are given in Section 2.10.5

against favin. These results are shown in Fig. 27. Section A (Lanes 1-4) was stained with amido black, while Section B (Lanes 5-7) was stained with the substrate for goat anti-rabbit peroxidase conjugate.

Section A, Lane 1 shows the large number of protein-staining bands that are present along with EI and favin subunits in the crude extract of *V. faba.* Lane 2 which contained purified favin shows a strong and a weak band at M_r's 20,000 and 18,500, which correspond to the $\beta-$ and β'-subunits of favin. (The latter is a degradation product of favin which was first reported by Cunningham and co-workers (see Section 1.6.3 for more details about the subunit structure and composition of favin). Lane 3 which contained the purified EI shows a single band at about M_r 40,000 and it is clearly noticeable that there are no bands in the M_r 20,000 region. Lane 4 contained the protein markers: trypsin inhibitor has been degraded on storage to produce a diffuse band at Mr 21,800.

Section B, Lane 5 which contained the crude extract shows bands at two places which had reacted with the peroxidase conjugate. The top band corresponds to a $M_r \approx 40{,}000$ whilst the bottom two bands have M_r values of 20,000 and 18,500. The M_r 40,000 band corresponds to the subunit M_r of EI. The M_r 20,000 band corresponds to the $\beta-$subunit of favin and the M_r 18,500 band to the β'-subunit. Lane 6 which contained purified favin shows two very strong peroxidase-positive bands at M_r 20,000 and M_r 18,500. They conform to the Mr values of the $\beta-$ and β'-subunits of favin. Reasonably strong peroxidase-positive bands are also seen at M_r's between 27,000 - 30,000. These bands correspond to the M_r values for the $\beta-$subunit precursors which have been observed by Cunningham [143]. Lane 7 which contained purified EI shows a relatively strong peroxidase reaction in the region of M_r 40,000 against the favin antibodies, thereby indicat-

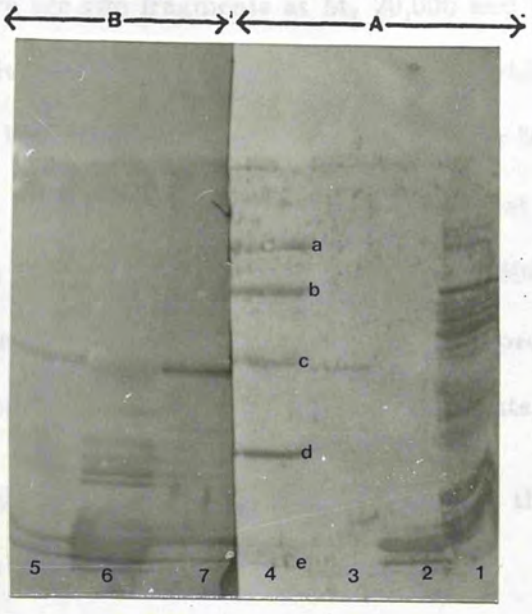


Figure 27

Studies of antigenic similarity between favin and EI using Western blotting with favin antibodies

Lane 4 contained the molecular weight markers

(a) phosphorylase b (M_r 94,000); (b) albumin, bovine serum, (M_r 66,000); (c) albumin, egg; (M_r 45,000); (d) carbonic anhydrase (M_r 30,000); (e) trypsin inhibitor (M_r 21,100);

Section A stained with 0.1% Amido Black (see experimental 2.2.4)

1. Crude extract (25 µg) (Stage 1; Table 1)
2. Favin (25 µg) (Stage 3; Table 3)
3. EI (10 µg) (Stage 7; Table 1)
4. M_r markers as given below

Section B stained for peroxidase (see experimental 2.1.3)

5. Crude extract (25 µg) (Stage 1; Table 1)
6. Favin (25 µg) (Stage 3; Table 3)
7. EI (10 µg) (Stage 7; Table 1)

Experimental details are given in Section 2.10.6

ing the existence of some homology between EI monomer and favin. However, in the same lane there are two fragments at M_r 20,000 and M_r 18,500 which show a peroxidase positive reaction. Lane 3 (see Section A) which contained the same amount of EI as in lane 7 shows no bands at M_r 20,000 or M_r 18,500 when stained with amido black. Hence, the above results suggest that the EI preparation is contaminated with two fragments of M_r 20,000 and 18,500 which show a strong affinity for favin antibodies but represent low levels of protein. There are three possible explanations for the origin of these two fragments.

(1) They are $\beta-$ and β'-subunits of favin linked to the native EI by sugar-lectin type interaction.

(2) They are $\beta-$ and β'-subunits of favin, linked to the EI subunit through ionic and/ or disulphide bonds produced by oxidation of -SH groups.

(3) They are *in vivo* or *in vitro* proteolytic products of the M_r 40,000 EI subunit.

Assuming the normal situation, that the glycan receptors of EI are present at the surface of the native enzyme and not within the protein, it seems rather unlikely that the two putative favin fragments are attached to the surface of the EI molecule through sugar-lectin type bonds for the following reasons:

If the putative favin fragments were attached to the surface of native EI by sugar-lectin interactions then they would render the native EI more immunoprecipitable by favin antibodies than what was observed. The extent of immunoprecipitability of enzyme activity (in native EI) by favin antibodies was about 30% (see Section 3.6.4). This value is not high enough for the observed precipitation to have been caused by putative favin fragments on the surface of

EI.

In addition, when an attempt was made to purify EI using Sephadex G-100 as an affinity matrix (unpublished results) the enzyme preparation, unlike favin, showed no affinity for the Sephadex under various conditions which included the use of PBS (pH 7.2), PBS (pH 5.5), McIlvaine buffer (pH 7.2) and McIlvaine buffer (pH 5.5). This observation again indicates the absence of any $\beta-$ and β'-fragments on the surface of the native EI, as they would allow binding of EI to the column, as occurs with favin.

3.6.7 Study of the Antigenic Similarity between $\alpha-$galactosidase I and Favin using Western Blotting with EI Antibodies

To complement the Western blotting study with favin antibody, EI, favin and a crude extract of *V. faba* was also probed with EI antibodies. The results are shown in Fig. 28. Section A (Lanes 1-4) was stained with amido black while Section B (Lanes 5-7) was treated with peroxidase substrate.

Section A, Lane 1 shows various proteins present along with EI subunit and favin subunits in the crude extract. Lane 2 which contained purified favin shows two bands ($\beta-$ and β'-subunits) at *ca.* M_r 20,000 and M_r 18,500 while two minor fragments, believed to be favin $\beta-$subunit precursors [143], are present at about M_r 28,000. Lane 3 which contained purified EI shows the M_r 40,000 subunit and, in addition, the presence of some minor impurities. However, no bands corresponding to the M_r's of favin subunits are apparent. Lane 4 contained the M_r markers.

Section B, Lane 5 which contained the crude extract shows strong peroxidase-positive bands at M_r values *ca.* 20,000 and 40,000. However in this lane, the

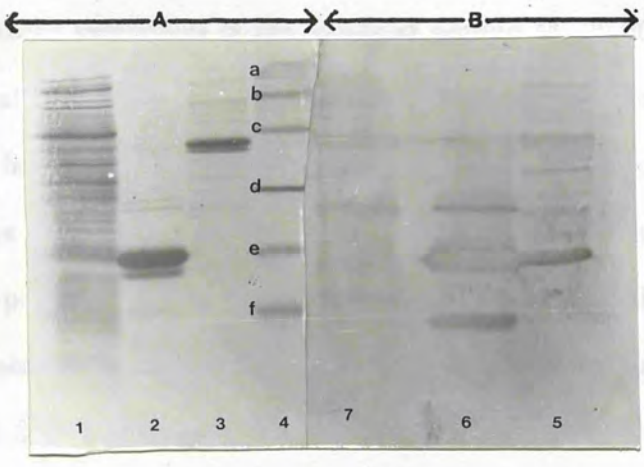


Figure 28

Studies of antigenic similarity between favin and EI using Western blotting with EI antibodies

Lane 4 contained the molecular weight markers

(a) phosphorylase b (M_r 94,000); (b) albumin, bovine serum, (M_r 66,000); (c) albumin, egg; (M_r 45,000); (d) carbonic anhydrase (M_r 30,000); (e) trypsin inhibitor (M_r 21,100); (f) α-lactalbumin (M_r 14,400).

Section A was stained with 0.1% Amido Black (see experimental 2.2.4)

1. Crude extract (25 µg)(Stage 1; Table 1)
2. Favin (20 µg)(Stage 3; Table 3)
3. EI (10 µg)(Stage 7, Table 1)
4. M_r markers

Section B was stained for Peroxidase (see experimental 2.1.3)

5. Crude extract (25 µg)(Stage 1; Table 1)
6. Favin (20 µg)(Stage 3: Table 3)
7. EI (20 µg)(Stage 7; Table 1)

Experimental details are given in Section 2.10.7

presence of a number of minor bands over a M_r range of 20,000 to 40,000 are also noticeable. Lane 6 which contained purified favin shows very strong cross-reactivity with EI antibodies in the bands at around M_r 20,000 and a reasonably strong band at M_r 28,000. A band at M_r 14,000 is also visible which was not observed when favin was 'blotted' with favin antibody: this could be a breakdown product of the β−subunit of favin. Lane 7 which contained the purified EI shows a peroxidase-positive band at M_r 40,000. The intensity is rather weak because it has diffused across the nitrocellulose sheet as a result of the micro-heterogeneous nature of EI. There is no band in the EI lane at M_r 20,000 in contrast to the first Western blot in which the purified EI was exposed to the favin antibodies (Fig. 27). This suggests that EI in this instance was free of any contaminating β−subunit of favin. However, one band (M_r 28,000) which has a similar M_r value to a precursor of the favin β−subunit [143] is observed in the EI lane [Lane 7].

A comparision of the favin antibody and the EI antibody blotting studies with EI presents rather confusing results. Assuming that EI was contaminated with favin, then the contaminants, M_r 20,000 and M_r 18,500, should have been seen on both blots as the EI preparation used was the same. The fact that the two Western blots exhibited two conflicting Mr values and both of these low M_r fragments cross-reacted with the respective antibodies, and they in return with EI, indicated the possibility that these fragments might have originated as a result of some form of proteolytic action of EI during the preparation of EI.

3.6.8 Use of Proteolytic Inhibitors to Study Fragments Present in the α–galactosidase-I Preparation

In an attempt to decide whether the fragments detected in EI using favin and EI antibodies were the *in vitro* proteolytic products of EI, *V. faba* extracts were made in the presence of protease inhibitors and then subjected to Western blotting.

Although plant proteases have been isolated and analysed, much of the information regarding this family of enzymes has come from detailed studies of a few animal and microbial proteases [182]. However, the relative abundance and high activity of plant proteases suggests that they play an important role in the processing of polypeptides during different stages of plant development. During the first 3 weeks of seed development in plants, the rate of protein turnover reaches a peak level. The newly formed polypeptides are digested to produce other polypeptides and this process continues until the seeds reach maturity when the action of proteases dwindles to a negligible rate [182].

It has been observed with many plants that proteins are degraded by the action of proteases during the initial stages of extraction of soluble material. For this reason a 'cocktail' of protease inhibitors is often included in the media for protein extraction to prevent *in vitro* proteolytic activity and the loss of proteins of interest. Several synthetic inhibitors have proved to be effective for the irreversible inhibition of plant proteases [183]. PMSF, TLCK, PCMB, TPCK, EPNP, DBA and IA are some which are commonly used.

Although sulphhydryl proteases are the most abundant type found in plants, serine and acid proteases, and carboxyl and amino peptidases are also

known. In the family Leguminosae, sulphhydryl and serine proteases are most common. Thus a 'cocktail' of inhibitors (PMSF, EPNP and IA) which are specific for the irreversible inhibition of these enzymes was used in the present study. No mercury containing inhibitors (eg. PCMB) were used as it has been observed that mercury salts are strong inhibitors of $\alpha-$galactosidases from *V. faba* [45].

A crude extract of *V. faba* beans prepared in the presence of the three inhibitors was passed through a Sephadex G-l00 column to obtain an EI preparation (Stage 4, Fig. 4). Following SDS-PAGE, Western blotting using favin antibodies was carried out as before. The blot showed one very weak band at M_r 20,000 in addition to the M_r 40,000 band corresponding to EI monomer. The intensity of the former was the same as that in extracts prepared in the absence of protease inhibitors and assuming that the latter inhibit all the proteases during extraction then the M_r 20,00 band is not an *in vitro* proteolytic product of EI. In this experiment, the EI was not purified beyond stage 4 (Fig. 4) since it was assumed that the Sephadex G-100 column would remove all free, native favin (see Section 3.4). Any favin remaining would then be denatured favin, hence having lost its lectin activity, it therefore would be unable to bind to the column.

When the purity of EI was tested (after Stage 7, Fig. 4) using SDS-PAGE followed by Coomassie Blue staining, a single band at M_r 40,000 was observed (Fig. 10); but no bands at M_r 20,000 were visible. However the results described in Section 3.6.6 showed the presence of two bands (at M_r's ca. 20,000 and 18,500) in addition to the M_r 40,000 band when purified EI was probed with favin antibody indicating the presence of two proteins (in minor quantities) along with EI. On a w/w basis, the affinity of these two bands for the favin antibodies appeared to be much greater than the EI subunit. On the contrary, if these two bands were the

proteolytic products of EI, then on a w/w basis the M_r 40,000 EI subunit band should have shown a much greater colour intensity in the peroxidase reaction in comparison to the two minor bands. Since this was not the case, it appears to rule out the possibility that the M_r 20,000 fragments arose by *in vivo* processing of EI. We can therefore conclude that the M_r 20,000 fragments in EI do not arise by either *in vivo* or *in vitro* processing of EI, adding weight to the idea that they represent contaminating favin.

The results obtained so far, therefore, leave two possibilities as to the origin of the bands that were seen in the EI preparation through Western blotting. They are:

[1] contaminating traces of favin which were not removed in Stage 4 (Fig. 4) of the EI purification, possibly linked to EI by disulphide bonds and/or ionic interactions.

[2] contaminating traces of favin arising at some stage of the experimental procedure.

3.6.9 Cross-reactivity Studies on EI and Favin using Antibodies Raised to the β–subunit of Favin.

The Western blotting experiment with the favin antibodies showed that they cross-reacted with the EI protein. Attempts were therefore made to eliminate the possibility that the purified favin used for raising favin antibody was contaminated with trace quantities of EI (or EI breakdown products), to which EI antibodies could also have been produced.

Favin β–subunit was isolated by electro-eluting the M_r 20,000 β–subunit band from SDS-PAG of purified favin. The β–subunit was used to raise antibod-

ies. Purified favin and EI protein were then subjected to SDS-PAGE. Following the transfer of the proteins to the nitrocellulose sheet, (Fig. 29), one half of the sheet (Section A; Lanes 1-3) was stained for proteins with amido black whilst the other half (Section B; Lanes 4-5) was incubated with favin β−subunit antibody.

Section A, Lane 1 which contained EI shows a band at M_r 40,000. Lane 2 which contained purified favin shows two bands corresponding to the β−subunit and β'-subunit of favin and lane 3 contained the M_r markers.

Section B, Lane 4 which contained purified favin shows two strongly peroxidase positive bands (β− and β'-subunits) at M_r's *ca.* 20,000 and 18,500, respectively. In addition there are two bands (M_r's *ca.* 28,000 and 29,000) which appear to be the favin β−subunit precursors. The lack of α−galactosidase activity in the purified favin extract, and the absence of a peroxidase-positive band at M_r 40,000 (in the favin lane) indicate that the purified favin extract is free of any contaminating EI. Lane 5 which contained the purified EI shows a relatively strong cross-reactivity (at M_r 40,000) with the favin β−subunit antibodies. This eliminates any doubt that the cross-reactivity observed between favin antibodies and EI in Section 3.6.6 was due to the presence of contaminating EI antibodies in the favin antiserum. However, in Lane 5 (containing the purified EI), a minor band at M_r 20,000 (which is the M_r value for the β−subunit of favin) is observed. M_r 20,000 bands have thus been detected in Western blots of pure EI probed with antibodies to both favin and favin β−subunit, further suggesting that the EI is slightly contaminated with favin β−subunit.

It is important to establish the source of the favin contamination of EI as well as the identity of the M_r 28,000 protein contaminating EI, in order to answer questions about the lectin nature of EI and its antigenic similarity to favin.

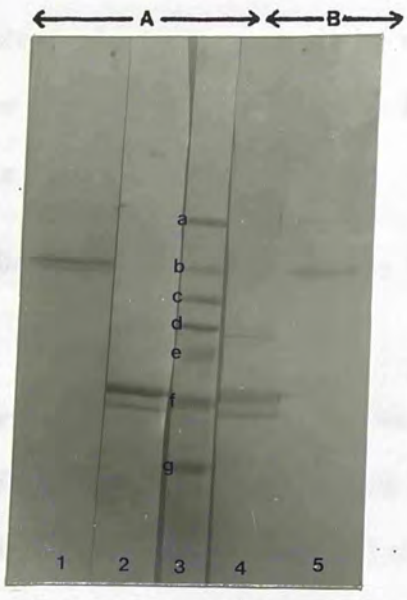


Figure 29

Studies of the antigenic similarity between favin and EI using Western blotting with favin β-subunit antibodies.

Lane 3 contained the molecular weight markers
(a) albumin, bovine serum, (M_r 66,000); (b) albumin, egg, (M_r 45,000); (c) glyceraldehyde-3-phosphate dehydrogenase; (M_r 36,000); (d) carbonic anhydrase, (M_r 29,000); (e) trypsinogen, (M_r 24,000); (f) trypsin inhibitor, (M_r 20,100); (g) α-lactalbumin, (M_r 14,400).

Section A stained with 0.1% Amido Black (see experimental 2.2.4)

1. EI (10 µg) (Stage 7; Table 1)
2. Favin (15 µg) (Stage 3; Table 3)
3. M_r markers

Section B stained for peroxidase (see experimental 2.1.3)

4. Favin (15 µg) (Stage 3; Table 3)
5. EI (10 µg) (Stage 7; Table 1)

Experimental details are given in Section 2.10.9(i)

When the results of the Western blots were carefully assessed two clear observations were made. (1) In each instance, irrespective of the antibody that was used, EI and favin always showed cross-reactivity with one another. (2) With all three antibodies a low M_r band (at M_r's 20,000 or 28,000) was detected in the lane which contained the EI.

Based on the above experimental results the following observations can be made.

Regarding observation (2), apart from the case of proteolytic inhibitor studies, other Western blots of EI were made with the same homogeneous preparation of EI. Therefore, if EI was truly contaminated with favin (β−subunit), the contaminant should have displayed a definitive trend in relation to EI when tested on a Western blot. One such observation included the intensity of the peroxidase band at M_r *ca.* 20,000 in relation to the EI subunit band at M_r 40,000. However, when compared visually, the ratio of the intensities of these two bands showed great variation in the two blots (Fig. 27 and Fig. 29) indicating great variations in the levels of the contaminating fragment, although the same amount of EI was used on both occasions.

In addition, if EI was truly contaminated with favin subunits (on the basis of the results of Sections 3.6.6 and 3.6.9) then the contaminant should have displayed a consistent M_r value in all three Western blots. For instance, for the Western blot where EI antibodies were used, a peroxidase-positive band should have been seen at M_r 20,000 (as EI antibodies showed a very strong cross-reaction with favin as seen on Fig. 28; Lane 6). However, the results for this blot were contrary to what was expected assuming that the β−subunit was the contaminant. Here a M_r 28,000 band (rather than M_r 20,000) was noticed along with EI.

These rather conflicting results lead to the suggestion that the observed favin contamination might have taken place during the experimental procedure.

Since the same batch of EI (Stage 7; Fig. 4) stored in the same microcentrifuge tube was used for all three Western blots, a variable contamination by favin could only arise during the gel electrophoresis procedure. All the glassware used in this project was washed thoroughly prior to each experiment, the chemicals were of 'Analar' type and the buffers freshly made. The Gilson tips and microcentrifuge tubes used were all new and not reused. Therefore, no contamination was likely from the above sources. Hence assuming that contamination of EI by favin did take place, the probability was that it occurred during the SDS-PAGE. As a relatively high level of favin had been placed in the well there was some likelihood that trace amounts of favin could have flowed across to the adjoining well which contained the EI (Fig. 27, 28, 29). It is also possible that an intensely coloured peroxidase positive band in the favin lane could have partly diffused across to either side thereby creating an artificial band in the M_r 20,000 region in the EI lanes.

To test these possibilities, a second Western blot was performed using β−subunit antibodies under identical conditions to the above except that a well was left empty between the EI and favin lanes. Fig. 30 shows the results. As before one half of the nitrocellulose sheet was stained for protein with amido black (Section A, Lane 1) and the other (Section B, Lanes 2-5) incubated with the β−subunit antibodies.

Section A, Lane 1 contains the M_r markers.

Section B, Lane 2 contained no proteins. Lane 3 which contained purified

EI shows a peroxidase-positive band at M_r 40,000 as before. However, there are no contaminating fragments of M_r 20,000, in contrast to the earlier observations. Moreover, Lane 4, which contained no protein, shows the presence of a band at M_r 20,000 which was observed in the earlier experiments. Hence, the appearance of this band resulted from the spillage of favin from the adjoining well (Lane 5) and/or the partial diffusion of the coloured peroxidase positive band from the favin lane to the blank lane (Lane 4).

When this experiment was repeated under the same conditions, identical results were obtained, ie, no band corresponding to M_r 20,000 (or M_r 28,000) was detected in the lane which contained the purified EI preparation.

This clearly demonstrates that the fragments of M_r 20,000 detected in the EI preparation in the earlier Western blots were not favin fragments contaminating the original EI preparation but appeared in the EI lanes as result of excessive favin flowing across to wells either side of the favin well.

In the lane which contained EI (Fig. 30; Lane 3) a peroxidase positive band is also seen at M_r 66,000 and traces of others at higher M_r values. These bands were also observed in the EI lane on the previous favin β−subunit Western blot (Fig. 29; Lane 5). Both of these Western blots were performed using an EI preparation from the same seed batch approximately one year after the studies with the favin and EI antibodies (see Sections 3.6.6, 3.6.7) were performed. Therefore, it is possible that due to the long period of storage the tetrameric EI protein had undergone some changes which eventually produced this M_r 66,000 fragment. Irreversible aggregation of proteins on storage, producing polymeric peptides which are stable in the presence of SDS and 2-mercaptoethanol has been observed in the past, as in the case with D-1 and D-2 protein from photosystem

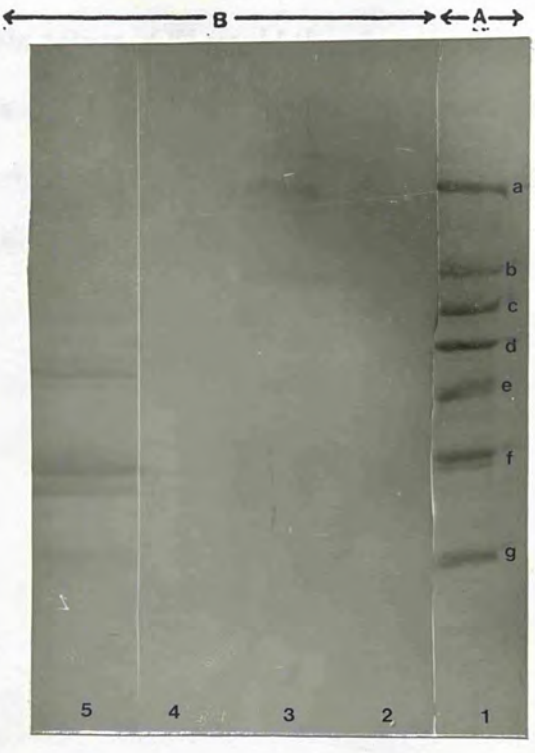


Figure 30

Studies of antigenic similarity between favin and EI using Western blotting with favin β-subunit antibodies.

Lane 1 contained the molecular weight markers
(a) albumin, bovine serum, (M_r 66,000); (b) albumin, egg, (M_r 45,000); (c) glyceraldehyde-3-phosphate dehydrogenase; (M_r 36,000); (d) carbonic anhydrase, (M_r 29,000); (e) trypsinogen, (M_r 24,000); (f) trypsin inhibitor, (M_r 20,100); (g) α-lactalbumin, (M_r 14,400).

Section A stained with 0.1% Amido Black (see experimental 2.2.4)

1. M_r markers

Section B stained for peroxidase (see experimental 2.1.3)

2. Blank
3. EI (From Stage 7; Table 1)
4. Blank
5. Favin

Experimental details are given in Section 2.10.9(ii)

II of spinach (184).

The lectin nature of EI would therefore appear to be an inherent property of the EI protein and not due to contaminating favin. This lectin property of EI may be due to a structural feature which is reponsible for the immunological cross-reactivity between EI and favin.

CONCLUSIONS

The major aim of this project was to obtain more information about the ability of EI protein to behave as a classical lectin with glucose/mannose specificity, as this was the first well-investigated example of a peptide structure with separate, but integral, catalytic and lectin sites. In particular, doubt had been expressed about the purity of EI and the possibility that it was contaminated with the lectin, favin, which is synthesised concurrently with α–galactosidase in the developing *V. faba* seeds. Favin is also located in the seed protein bodies, which are probably the main sites for the accumulation of α–galactosidase (although this has not been established unequivocally).

The 'macro' approach to this problem included the use of techniques such as isoelectric focusing, Cleveland peptide mapping and the behaviour of EI and favin on different affinity columns, comparing in all cases the differences and the similarities between favin and EI, and looking for evidence of favin contamination of EI. Whilst isoelectric focusing gave no evidence for the presence of loosely attached favin contaminants, one dimensional peptide mapping following protease hydrolysis of EI produced one major band with a similar M_r to that of the β–subunit of favin. The two proteins behaved differently on affinity columns. For example Sephadex G-100 and 3-O-methyl-D-glucosamine immobilised both showed affinity for favin but not for EI. Conversely, immobilised melibiose bound EI but did not bind favin.

A 'micro' approach essentially involved the use of immunological methods. Immunoprecipitation experiments with EI using favin antibodies showed that approximately 30% of EI activity could be precipitated, indicating some degree of homology between favin and EI. Dot-Blot experiments confirmed the above

recognition and also showed that more antigenic sites in common with favin were present in the three dimensional structure of the EI protein. Western blotting experiments using favin and EI antibodies confirmed the homology between the two proteins. However, in the former case (with favin antibodies) a peroxidase-positive band was also seen at M_r 20,000 in the EI lane while in the latter (with EI antibodies) a band was seen at M_r 28,000 in the EI lane. These M_r values correspond to those for the favin β−subunit and its precursor, respectively. When favin β−subunit antibodies were used for cross-reactivity studies using a Western blot, as in the earlier instances, EI protein was recognised by the antibody preparation. However, as in the case of the favin antibodies, a weak band was observed in the region of M_r 20,000.

Eventually the anomalous M_r 20,000 (and 28,000) seen on Western blots were traced to technical problems occurring during SDS-PAGE. When modified procedures were adopted no low M_r band was observed in the EI preparation.

Therefore, we can now conclude that the lectin properties of EI are not due to favin contamination but can probably be accounted for by a peptide structure which is an integral part of the EI protein molecule and homologous with the β−subunit of favin.

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