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FURTHER CHARACTERIZATION OF α-GALACTOSIDASE I-GLYCOPROTEIN LECTIN FROM *VICIA FABA* SEEDS

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

Abstract—The nature of the glucose/mannose specific lectin activity of α-galactosidase I from *Vicia faba* seeds has been examined. Gel filtration in the presence of high concentrations of glucose and SDS-PAGE failed to detect favin, a classical lectin which also occurs in the seed. A comparison of the haemagglutinating activities of the α-galactosidases from *Vigna radiata* and *V. faba* seeds strongly suggests that the catalytic site of the *Vigna* enzyme is also responsible for its agglutinating activity and that the catalytic and lectin sites are at different loci in the case of *V. faba* α-galactosidase I. The latter conclusion is supported by an investigation of the effects of glucose, mannose and galactose on the catalytic and lectin activities and by results obtained by demetallization of the *V. faba* enzyme. A single galactose-binding site and two mannose binding sites per subunit of enzyme I were detected by the method of equilibrium dialysis and the association constants for these monosaccharides measured. Mannose did not appear to affect the binding of galactose to the enzyme or *vice versa*. The removal of glycan chains from α-galactosidase I with endo-β-*N*-acetylglucosaminidase H released an active dimeric form of α-galactosidase. The possible involvement of lectin–glycoprotein interactions in the stabilization of the tetrameric form of the enzyme is considered.

INTRODUCTION

Following the report of two forms of α-galactosidase in coffee beans by Courtois and co-workers [1], multimolecular forms of this enzyme were shown to occur in *Vicia faba* seeds [2] and in several other plant tissues [3]. The enzymic activity from *V. faba* seeds has been resolved into three forms, I, II^1 and II^2 with M_r values of 160 400 ± 2850, 45 730 ± 3073 and 43 390 ± 1409, respectively [4]. Their kinetic properties have also been studied [3–5]. Evidence that the three forms are closely related was obtained from immunological studies and it is believed that I is a tetramer of II^2 [6]. All three forms of the enzyme are unique in that they display lectin activity with glucose/mannose specificities [7; *cf.* 8–10] and are also mannose-containing glycoproteins [6]. Recent collaborative studies (unpublished) with Dr. A. Dell (Imperial College, University of London) where glycopeptides obtained from α-galactosidase I by proteolytic digestion were examined by fast atom bombardment mass spectroscopy (FAB-MS) indicate that glycan chains are 'mannose-rich'.

There is little doubt that seed α-galactosidases play an important role in the early stages of germination by hydrolysing galactose-containing oligosaccharides and, hence, provide metabolites for the developing seedling [4, 11–14]. It is, however, not known how the enzymes and their natural substrates are prevented from interacting during the maturation of seeds. The glycoprotein/lectin nature of the enzyme offers various possibilities of *in vivo* compartmentation/inactivation of the enzymes [3, 6, 15–17] and the carbohydrate chains may be involved in the transport of these enzymes between compartments [*cf.* 18, 19]. Thus far little is known about the nature and function of glycan chains of plant enzymes [20, 21].

The final goal of our studies is to understand the full importance of α-galactosidase-glycoprotein lectin in the germination process and we now describe further work on the nature of form I of the enzyme.

RESULTS AND DISCUSSION

Examination of α-galactosidase I-lectin from V. faba *for favin contamination*

α-Galactosidase I was purified by a 7-step procedure [7], the salient features of which were acid treatment (pH 3.0), Sephadex G-100 gel-filtration (pH 5.5), CM-cellulose chromatography (pH 5.5) and melibiose-Sepharose affinity chromatography. All these stages would be likely to resolve a mixture of favin and α-galactosidase I.

The initial acidification of the crude seed extract, for example, removes much of the favin and other storage proteins [see ref. 7]; a similar approach has been followed for the removal of lectin activity from *Lens culinaris* extracts [21, 22]. The optimum pH for activity for both α-galactosidase I-lectin and favin is approximately 7; hence, at pH 5.5 affinity binding to Sephadex would be expected to be weak. Estimation of the molecular size of the enzyme by gel-filtration [4] supports this belief and in the case of favin, purification is often achieved by absorption onto Sephadex at pH 7 [23]. Hence, in the absence of significant lectin binding a clear separation by molecular sieving of α-galactosidase I-lectin and favin would be expected. We have observed loss of lectin activity at this step [7] which is presumably due to separation of favin. Favin and α-galactosidase I-lectin possess very different ionic properties which can be demonstrated by polyacrylamide gel electrophoresis (compare refs [6] and [24]). As a result of these differences the two proteins should also have been separated at the CM-cellulose stage

of purification. Finally, the galactose-specific column (melibiose-Sepharose) would have no affinity for free favin, hence allowing its separation from column-bound α-galactosidase I-lectin [7]. However, as the enzyme is a mannose-rich glycoprotein [6], it is possible that it exists as a complex with favin which would bind to the affinity column but such a complex would not be expected to be stable at the acidic pH used in the preceding CM-cellulose step.

To substantiate the claim that the *V. faba* enzyme is a unique example of a protein possessing separate catalytic and lectin sites it is important to present further evidence to exclude contamination by favin. Hence, purified α-galactosidase was incubated with 0.5 M glucose (a hapten to dissociate possible enzyme–favin complexes) in 0.1 M potassium phosphate buffer, pH 7.2, for 22 hr at 4° followed by gel-filtration on a Sepharcryl S-200 column which had previously been equilibrated with the same medium. The elution profile (Fig. 1) was found to be identical to that obtained with the untreated enzyme indicating no apparent change in its molecular weight. Moreover, the recovery of lectin activity associated with the enzyme peak was 96% of that applied to the column and no lectin activity could be detected at an elution volume corresponding to that of favin. This study therefore suggests that no significant amounts of favin-α-galactosidase I complex were present in the purified enzyme preparation. However, it is possible that favin binds very strongly to α-galactosidase I and the complex is not dissociated under these conditions.

A search was also made for the presence of favin sub-units (β, M_r 20000, major; β', M_r 18700, minor, α, M_r 5571, minor) [25] in the enzyme protein by SDS-PAGE. In Fig. 2 the electrophoretic pattern obtained with favin (*cf.* ref. [25]) is shown alongside that for α-galactosidase I. The latter exhibited a single band (M_r 44000) and there were no detectable protein bands corresponding to the favin sub-units.

Comparison of haemagglutinin activities of α-galactosidases from Vigna radiata *and* Vicia faba *seeds*

Unlike α-galactosidase I from *V. faba*, the haem-agglutinin activities of α-galactosidases from some other legume seeds such as *V. radiata* [8] and *Glycine max* [9, 26] are reported to be galactose-specific. The question therefore arises whether these latter enzymes possess one locus which is responsible for both haemagglutinating and catalytic activities. Hankins and Shannon [8] reported that the tetrameric α-galactosidase I (M_r 160000) from *V. radiata* agglutinated rabbit erythrocytes at pH 7.0 and that on longer incubation the 'clot' dissolved. Galactose inhibited the agglutination reaction. The monomeric form, II, of the enzyme (M_r 40000) possessed no agglutination activity. These properties of the *V. radiata* enzyme were reinvestigated and compared with those of *V. faba* α-galactosidase I (see Fig. 3).

Rabbit erythrocytes that had received a separate preliminary treatment (2 hr) with α-galactosidases I and II from *V. radiata* (see Experimental for details) were washed and both cell preparations reincubated with enzymes I from *V. faba* and from *V. radiata*. The results presented in Fig. 3 show that erythrocytes which had been treated initially with the *V. radiata* enzymes and reisolated could not be agglutinated by further addition of *V. radiata* enzyme I. However, agglutination did occur in both cases in the presence of *V. faba* α-galactosidase I.

When the initial incubations of erythrocytes with *V. radiata* enzymes I and II were repeated in the presence of 3 mM galactose (an inhibitor of α-galactosidase activity) no haemagglutination was observed. The resultant cells after washing were, however, agglutinable by enzymes I from both *V. radiata* and *V. faba*. These observations suggest that *V. radiata* α-galactosidase I and II both removed 'receptors' from the red blood cell-surface (hence preventing subsequent binding and agglutination by *V. radiata* enzyme I) but were without action on the 'receptors' for the *V. faba* enzyme I which is a glucose/mannose specific lectin. Thus, agglutination and 'clot-dissolution' by *V. radiata* enzyme I can be explained in terms of multiple galactose-binding catalytic sites on the enzyme which first combine with the erythrocyte galactose-containing 'receptors' producing agglutination and then slowly hydrolyse the galactosyl-linkage. Agglutination of the red blood cells followed by 'clot-dissolution' has also been described in the case of a fungal D-galactose oxidase [27]. This enzyme presumably combines via its catalytic site to cell-surface galactose

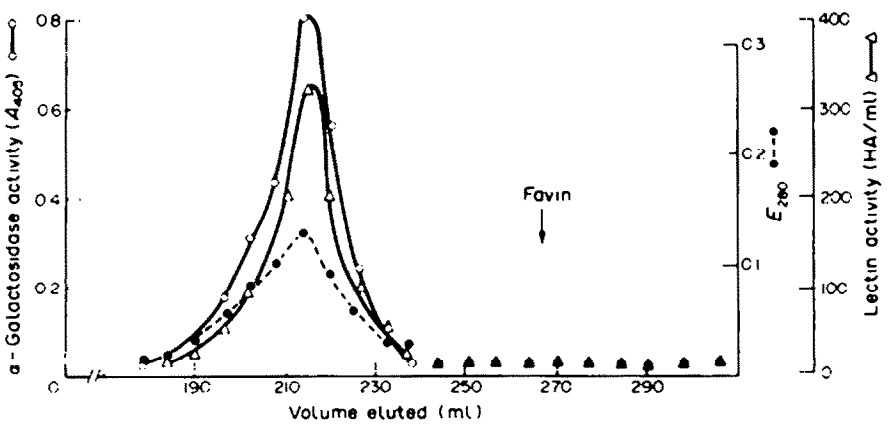

Fig. 1. Sephacryl S-200 gel-filtration profile of purified *V. faba* α-galactosidase I in the presence of 0.5 M D-glucose. Arrow indicates the elution volume of favin. Details are given in the Experimental section.

Fig. 2. SDS-PAGE of purified α-galactosidase I and favin from *V. faba* seeds. 1, α-galactosidase I (10 μg); 2, favin (15 μg); β,β' and α are the sub-units of favin (see text).

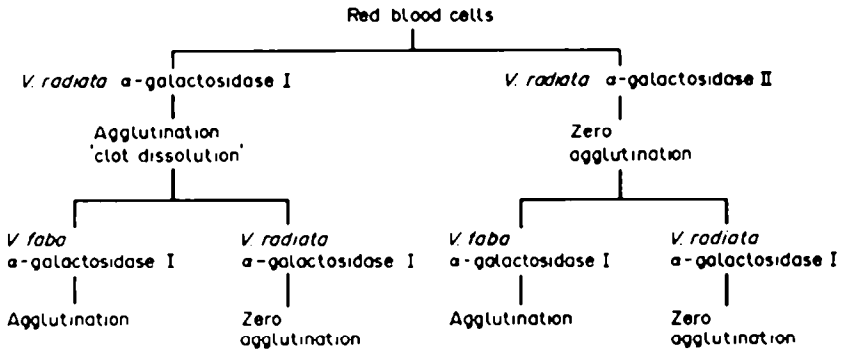

Fig. 3. Treatment of rabbit red blood cells with α-galactosidases from *V. radiata* and *V. faba* (see text).

residues causing 'clot' formation, then after oxidation of the hydroxyl group at C-6, 'product' and enzyme separate and the 'clot' dissolves.

It is not known to what extent the *V. faba* enzyme-lectin removes galactose from the erythrocyte surface. This may be minimal because of rapid aggregation of the cells caused by glucose/mannose-specific lectin cross-links which would presumably result in steric hindrance of the catalytic site.

Further evidence of separate catalytic and lectin sites on V. faba *α-galactosidase I-lectin*

Protection of catalytic and lectin activities. The pH profile and the heat stability of both enzymic and haemagglutinin activities of *V. faba* α-galactosidase I described earlier [7] suggest the presence of separate sites for these activities. We have now examined the protection of these sites against heat inactivation at 75° by monosaccharides and the results (Fig. 4) show that D-galactose is effective in preserving the catalytic function but not the haemagglutinin activity, whereas D-mannose has the

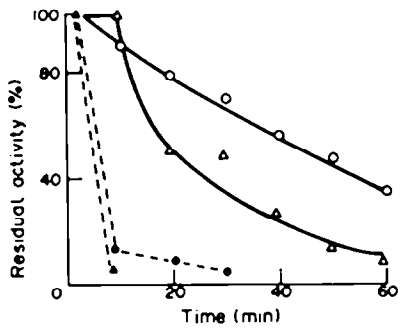

Fig. 4. Protection of catalytic and lectin activities at 75°C of purified α-galactosidase I from *V. faba* seeds in the presence of D-galactose and D-mannose. O——O, Catalytic activity remaining in the presence of 0.1 M D-galactose; ●——●, catalytic activity remaining in the absence of D-galactose or, in the presence of 0.1 M D-mannose; △— -△, lectin activity remaining in the presence of 0.1 M D-mannose, ▲——▲, lectin activity remaining in the absence of D-mannose or, in the presence of 0.1 M D-galactose.

opposite effect. These effects are most marked up to 10 min of incubation but lessen with increasing incubation times when presumably there is a general denaturation of the protein. In this connection Howard and Sage [22] reported that D-glucose (0.1 M) afforded strong protection to the glucose/mannose specific activity of *Lens culinaris* lectin for 30 min at 72° but none at 75°.

Enzyme kinetic studies using D-glucose, D-mannose and D-galactose showed that only D-galactose significantly inhibited the hydrolysis of *p*-nitrophenyl α-D-galactoside by the enzyme (Table 1); the inhibition was competitive (results not shown). D-Glucose and D-mannose at 31 and 16 mM concentrations, respectively resulted in 50% inhibition of haemagglutinin activity, whereas 1 M D-galactose was required to achieve this level of inhibition. These two studies, therefore, again suggest that separate catalytic and lectin sites exist.

Metal ion content and the effect of demetallization. The metal ion content of purified *V. faba* α-galactosidase I was determined by atomic absorption spectrometry. Calcium was found to be the major metal component (Table 2). Taking the molecular weight of the enzyme as 160000, approximately 1.2 mol of Ca^{2+} would appear to be bound per sub-unit (M_r 40000). Magnesium, manganese and zinc ions were also detected but at a much lower level. On demetallization with EDTA all of the Mn^{2+} and Zn^{2+} were removed but Ca^{2+} (representing approximately 1 mol/sub-unit) and some Mg^{2+} remained bound. Manganese ions can often be readily removed from lectins by EDTA–acetic acid treatment, but this is not always the case with the alkaline earth metals [28]. *Arachis hypogea* lectin, for example, contains 0.98 mol of Ca^{2+} and 0.78 mol of Mg^{2+} per sub-unit, none of which is lost on dialysis against the chelating medium [29]; however, no acid was used here.

Demetallization resulted in a 60% increase in catalytic activity but 87% decrease in haemagglutinating activity (Table 2). This reciprocal effect further supports the notion that separate sites on the protein are involved in the two functions of *V. faba* α-galactosidase I.

Table 1. Inhibition of enzymic and lectin activities of *V. faba* α-galactosidase I

Monosaccharide	Concentration (mM)	Inhibition of enzymic activity (%)	Inhibition of lectin activity (%)
D-Galactose	5	70	0
	25	90	0
	100	100	0
	1000	100	50
D-Glucose	25	10	nd
	31	nd	50
	100	40	nd
	500	58	nd
D-Mannose	16	nd	50
	25	10	nd
	100	38	nd
	500	60	nd

Details are given in the Experimental section. nd, Not determined.

Table 2. Metal ion content of V. *faba* α-galactosidase I

Metal content and enzyme/lectin activities	Native	Demetallized
Ca^{2+} (g ion/mol enzyme)	5.60	3.20
Mg^{2+} (g ion/mol enzyme)	0.40	0.30
Mn^{2+} (g ion/mol enzyme)	0.15	Nil
Zn^{2+} (g ion/mol enzyme)	0.16	Nil
Enzymic activity (nkat/ml)	5000	8000
Lectin activity (HA/ml)	320	40

Determination of the number of carbohydrate-binding sites

The number of lectin and catalytic sites and the association constants for carbohydrate binding were determined by the equilibrium dialysis method; the degree of binding was also examined by a spectrophotometric method.

(a) *Equilibrium dialysis.* Samples of enzyme I-lectin were dialysed against solutions (0.1 M potassium phosphate buffer, pH 7.0) containing a fixed amount of [^{14}C] monosaccharide (D-galactose or D-mannose) together with varying amounts of unlabelled monosaccharides. The data were evaluated according to the method of Scatchard [30] (Fig. 5). The enzyme combined with D-galactose with an association constant of 1.67×10^4 M^{-1}. The abscissa in Fig. 5a indicates that four molecules of this hexose were bound to the tetrameric enzyme I. D-Galactose is a competitive inhibitor of the enzymic activity [5]. For D-mannose, the association constant was found to be 1.37×10^4 M^{-1} and in this case the tetramer bound eight molecules of the hexose (Fig. 5b). Further equilibrium dialyses showed that the estimated number of D-galactose-binding sites was not affected by the presence of D-mannose or *vice versa* and yielded Scatchard plots similar to those in Fig. 5a.

(b) *Spectrophotometric method.* Changes in the ultraviolet difference spectra produced by adding specific sugars to lectin solutions can be used to determine binding constants [29, 31]. In this way, *Arachis hypogea* lectin with lactose exhibited two tyrosine peaks at 285 and 279 nm whereas lectins from *Lens culinaris*, *Sophora japonica*, *Solanum tuberosum* and wheat germ all showed two peaks, characteristic of tryptophanyl residues, at 292 and 284–287 nm, when treated with methyl α-D-mannoside, lactose, *N*,*N*'-diacetylchitobiose or *N*,*N*',*N*''-triacetylchitotriose [31], respectively.

The difference spectrum of *V. faba* α-galactosidase I induced by D-mannose showed a single broad peak (tyrosinyl) between 282.5 and 279 nm. The titration curve obtained from the difference spectra at varying D-mannose concentrations is shown in Fig. 6. The association constant was determined from the free hexose concentration at 50% saturation using the relationship: $K = 1/[S_{free}]$ where $[S_{free}] = [S_{total}] - 0.5\,nP$ and nP represents protein sub-unit concentration. The value of the

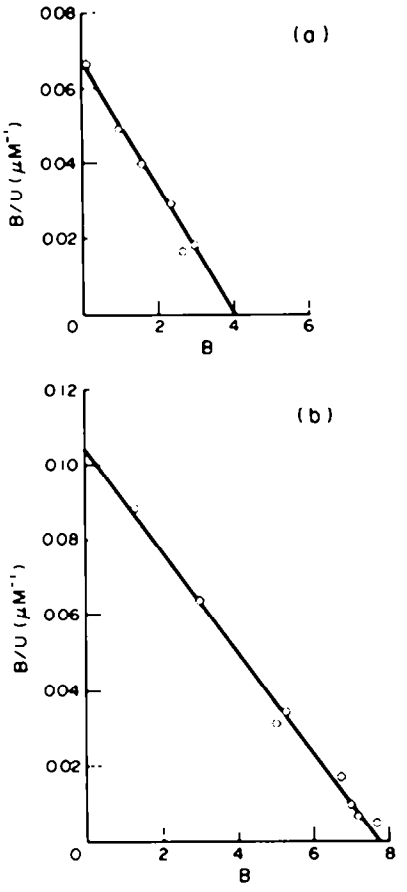

Fig. 5. Determination of the number of binding sites of α-galactosidase I from *V. faba* seeds. (a) Binding of D-galactose to the catalytic sites; (b) binding of D-mannose to the lectin sites; B, number of molecules of monosaccharide bound per molecule of protein (*M*, 160000); U, concentration of free sugar.

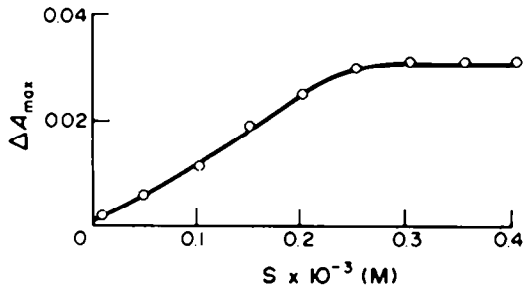

Fig. 6. Effect of D-mannose (S) on the difference spectrum (282.5–279 nm) of purified α-galactosidase I from *V. faba* (see text for details).

association constant was found to be $1.48 \times 10^4\ M^{-1}$ which is in good agreement with the results obtained by equilibrium dialysis. No significant change in the spectrum of the enzyme protein was observed in the presence of D-galactose, thus this technique could not be used for determining the binding constant for this sugar.

The foregoing studies all confirm our original suggestion [6, 7] that *V. faba* α-galactosidase I-lectin possesses separate carbohydrate binding sites associated with catalytic and lectin activities and, further, eliminates the possibility that the lectin nature of α-galactosidase I is due to contamination with non enzymic favin.

Endo-H digestion of α-galactosidase I

In view of the glycoprotein nature of the *V. faba* α-galactosidase the effect of modifying the glycan chains of the enzyme on its activity and molecular size was investigated. After incubation of α-galactosidase I with endo-H (endo-β-N-acetylglucosaminidase), the mixture was fractionated by Sephacryl S-200 gel filtration. Two enzymically active peaks (1 and 2; Fig. 7b) were obtained: (1) possessed an elution volume corresponding to the native enzyme (*M*, 160000) (Fig. 7a) and (2) a higher elution volume corresponding to *M*, 80000. On analysing the eluted fractions for carbohydrate with anthrone-H_2SO_4 reagent, a peak with an elution volume of 440 ml (i.e. beyond the inclusion range of the gel-filtration column) was observed and peaks (1) and (2) also contained carbohydrate. Furthermore, the enzymically active peaks were absorbed on Con A-Sepharose columns and could be desorbed with methyl α-D-mannoside solutions. This is a further indication that (1) and (2) still contained some residual carbohydrate after the endo-H treatment.

From the known specificity of endo-H, it would, therefore, appear that α-galactosidase I is a 'mannose-rich' glycoprotein with glycan chains linked to the protein moiety via asparagine residues. This confirms the FAB-MS study (unpublished). The formation of peak (2) (Fig. 7b) suggests that endo-H caused a dissociation of the tetrameric enzyme to an active dimeric form. This leads us to propose that the glycan chains are in some way involved

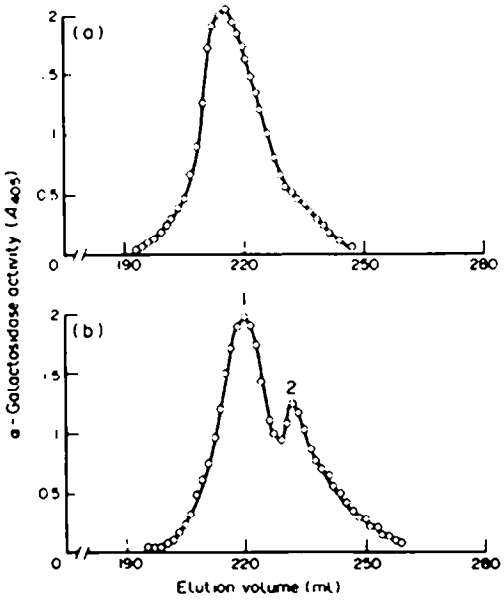

Fig. 7. Effect of endo-H action on the Sephacryl S-200 gel filtration profile of purified α-galactosidase I from *V. faba* seeds. (a) Native enzyme; (b) endo-H treated enzyme; 1 and 2 are the two enzymically active peaks.

in the formation/stabilization of the tetramer, perhaps via glycan-lectin linkages. However, treatment of enzyme I with 0.1 M methyl α-D-mannoside does not produce lower molecular weight forms. This is not entirely unexpected in the view of the high degree of binding (K_a = 10^6–10^7 M^{-1}) shown by some lectins for their natural receptors [32] and in addition other binding forces are, no doubt, involved in the quaternary structure.

EXPERIMENTAL

Broad beans (*Vicia faba*) and mung beans (*Vigna radiata*) were purchased locally. General laboratory chemicals of analytical grade were obtained from B.D.H. (Poole, U.K.) and Sigma (Kingston-upon-Thames, U.K.). Sephadex, Sephacryl, Concanavalin A-Sepharose were from Pharmacia (London, U.K.), CM-cellulose from Whatman (Maidstone, U.K.) and melibiose-Sepharose from Pierce Chemical Company (Rockford, U.S.A.). Endo-β-*N*-acetylglucosaminidase H (endo-H) was from Seikagaku Fine Biochemicals (Tokyo, Japan) and 3-O-methyl-*N*-hexanoylglucosamine-Sepharose was a gift from Dr. A. K. Allen (Charing Cross Hospital, London, U.K.).

α-Galactosidase isolation and assay. α-Galactosidase I from *V. faba* was purified according to a previous procedure [7] except that the step involving α-methyl mannoside-Agarose column was omitted and Sephacryl S-200 gel-filtration was incorporated after the Sephadex G-100 step. The enzymes I and II from mung beans were purified according to earlier methods [8, 13].

Enzyme activity was assayed by following the initial rate of hydrolysis of *p*-nitrophenyl α-D-galactoside using McIlvaine buffer [33] according to ref. [21]. The activity is expressed either as A_{405} or Katals (kat). Protein was estimated by the method of ref. [34] using crystalline bovine serum albumin as standard.

Favin isolation and lectin assay. Favin from broad beans was purified according to a published method [35] and haemagglutination assayed using a 1.5% suspension of rabbit blood cells in phosphate buffered saline, pH 7.2 [36].

Gel-filtration of D-glucose-treated α-galactosidase I. Vicia faba α-galactosidase I (395 nkat; 2 ml) was dialysed against KPi buffer, pH 7.2 containing 0.5 M D-glucose for 22 hr at 4° and applied to a pre-calibrated Sephacryl S-200 column (2.5 cm × 90 cm). The dialysis buffer was used for equilibration and elution. The fractions (3.0 ml) obtained were dialysed against three changes of KPi buffer, pH 7.2 for 22 hr and assayed for catalytic and lectin activities.

Polyacrylamide gel electrophoresis. Sodium dodecyl sulphate-polyacrylamide slab gel electrophoresis was carried out according to the method of ref. [37]. A 5% stacking gel was used and the separating gel was a 10–15% gradient. Gels were stained for protein with 0.2% Coomassie brilliant blue (R 250) in 50% TCA for 15–20 min at 60°. Destaining was at room temp. using a soln containing 7.5% HOAc and 12.5% *iso*-PrOH.

Treatment of rabbit erythrocytes with α-galactosidases. α-Galactosidases I and II from *V. radiata* were added to erythrocyte suspensions (1.5% in phosphate buffer saline, pH 7.2; ~ 200 nkat/ml suspension) and incubated for 2 hr at room temp. These experiments were repeated also in the presence of D-galactose (3 mM). The erythrocytes were finally washed × 3 with the buffer and used to test for agglutination with enzyme I of *V. faba* and enzyme I of *V. radiata*.

Heat inactivation and effect of monosaccharides. Vicia faba α-galactosidase I (0.4 mg/ml) samples were dialysed separately against 0.1 M KPi buffer, pH 7.2 and McIlvaine buffer, pH 5.5 for lectin and enzyme assays, respectively. The dialysed samples were incubated separately at 75° for various time intervals with 0.1 M D-galactose or without monosaccharides then immediately cooled in an ice-bath and dialysed against the appropriate buffer. The catalytic and lectin activities of all samples were then measured.

The effects of monosaccharides on the enzymic activity under assay conditions were determined as described earlier [38]. To determine the 50% inhibition of haemagglutination by various sugars, the method of ref. [39] was used.

Metal-ion estimation and demetallization. The metal-ion contents of native and demetallized enzyme samples were determined by atomic absorption spectroscopy, using a Unicam SP 90A, Series 2 spectrometer. The purified enzyme (1.5 mg/ml) used for metal analysis was thoroughly dialysed against deionized H_2O.

Demetallization of enzyme samples (1.5 mg/ml) was carried out by sequential dialysis against deionized H_2O, 0.1 M EDTA, deionized H_2O, 1 M HOAc and finally against H_2O. The samples were then examined for catalytic and lectin activities.

Equilibrium dialysis. These experiments were performed using a Dianorm (Diachema AG, Zurich) equilibrium dialyser fitted with 250 μl dialysis cells. The dialysis membranes were first boiled in 1% Na_2CO_3 and washed exhaustively with distilled H_2O before use. KPi buffer (0.1 M), pH 7.2 was used.

A constant amount of [^{14}C]-D-mannose (1.3 μCi) and varying amounts of unlabelled D-mannose (0–0.12 mg) and enzyme (0.13 mg) were placed in opposite compartments of the dialysis cells, the protein being separated from the sugar soln by the dialysis membrane. The total vol. was made up to 200 μl in each compartment. The cells were rotated for 24 hr at 25° to establish equilibrium. Samples (100 μl) were then withdrawn from each compartment and placed in scintillation vials. NCS tissue solubilizer (500 μl) was added to each vial and incubated for 2 hr at 60°. Glacial HOAc (20 μl) and toluene-PPO (10 ml; 5 g PPO/l. toluene) were added after cooling the vials to room temp. and the radioactivity determined in a Beckman LS 7500 liquid scintillation counter. In determining the concentration of monosaccharide in the cell compartments the changing specific radioactivity was taken into account (e.g. ref. [40]).

In the study of the number of catalytic sites, the same procedure was followed, except that McIlvaine buffer, pH 5.5, was used and D-mannose was replaced by D-galactose ([^{14}C]-D-galactose (1.3 μCi) and unlabelled D-galactose (0–0.12 mg)).

Spectrophotometric analysis. The method for determining the association constant for binding D-mannose to enzyme I was essentially the same as described in ref. [29]. The difference spectra were measured using a Perkin–Elmer 550S spectrophotometer, an instrument capable of reading absorbance to 0.001 unit.

Aliquots of enzyme solution (2 ml; 0.4 mg/ml) were dialysed against 0.1 M KPi buffer, pH 7.2, containing 0.1 M NaCl and added to sample and reference cells (quartz cuvettes, 1 cm light path). The baseline was recorded into the instrument measuring unit to be subtracted automatically from the subsequent spectra (300–250 nm). D-Mannose soln (1 M, 5 μl) was then added to the sample cuvette while the corresponding reference cuvette received the same volume of buffer. The difference spectrum was then recorded (282.5–279 nm). The amount of D-mannose soln used did not absorb between these wavelengths.

Digestion with endo-β-N-acetylglucosaminidase H. Endo-β-*N*-acetylglucosaminidase H (10 milliunits) was added to *V. faba* α-galactosidase I (136 μg in 1 ml of McIlvaine buffer, pH 5.5). A mixture (5 μl) was incubated at 37° for 15 hr and then applied to a Sephacryl S-200 column (2.5 × 90 cm). The fractions (3 ml) were eluted with the incubation buffer and assyed for α-galactosidase activity. Carbohydrate estimations were carried out by the anthrone method [41]. According to the manufacturer's instruction sheet for endo-H digestion no detergent was required in the incubation medium (see also ref. [42]). This was suitable for our

purposes as we wished to avoid effects of detergent on α-galactosidase activity.

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