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VICIA FABA α-GALACTOSIDASE WITH LECTIN ACTIVITY

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

Abstract—The purification of α-galactosidase II^1 from mature *Vicia faba* seeds is reported. The enzyme (M_r 45 730 ± 3070) did not appear to be contaminated with the classical lectin favin: but possessed D-glucose/D-mannose-specific lectin activity. Four D-mannose residues/mole enzyme bind with a K_A of $2.18 \times 10^3\ M^{-1}$ and this binding is not inhibited by D-galactose. These observations, together with heat inactivation and pH studies, suggest that II^1 has different loci associated with its enzymic and lectin properties.

INTRODUCTION

Three forms of α-galactosidases (α-D-galactoside galactohydrolase: EC 3.2.1.22), I, II^1 and II^2 with apparent M_rs (sedimentation equilibrium method) of 160 400 ± 2850, 45 730 ± 3073 and 43 390 ± 1409, respectively have been shown to occur in the extracts of resting *Vicia faba* seeds [1, 2]. Purified samples of the three forms contain carbohydrate [1] which is presumed to be covalently linked to enzyme protein as all forms bind to ConA-Sepharose and can be subsequently eluted with aqueous methyl α-D-mannoside [2]. Furthermore, in the case of α-galactosidase I, a recent FAB-MS study (unpublished) indicates that this enzyme is a 'high-mannose' glycoprotein. Immunological evidence suggests that the three α-galactosidases are structurally similar, although I and II^2 are more closely related than II^1 with either of these two forms. SDS-PAGE indicates that enzyme I is a tertramer of II^2 [3].

Detailed studies withe α-galactosidase I from *V. faba* have shown that this form of the enzyme displays D-glucose/D-mannose specific lectin activity. Preliminary studies indicate that the monomeric forms II^1 and II^2 have a similar lectin specificity. This paper describes further characterization of α-galactosidase II^1.

RESULTS AND DISCUSSION

Purification

α-Galactosidase II^1 was purified up to the CM-cellulose chromatography stage according to a multi-step procedure described earlier [3]. This yields a fraction with coincident lectin and enzyme activities (cf. [3]). An outline of the purification scheme is shown in Fig. 1. The use of a Con A-Sepharose column was avoided in order to prevent contamination of the enzyme with lectin from the column matrix. However, an immobilized melibiose affinity column was used as the final step in the purification which increased the specific activity of the enzyme from 122 to 165 nkat/mg. The lectin specific activity of the preparation was 4400 HU/mg. On SDS-PAGE, the enzyme preparation showed one major protein band [cf. 2] and

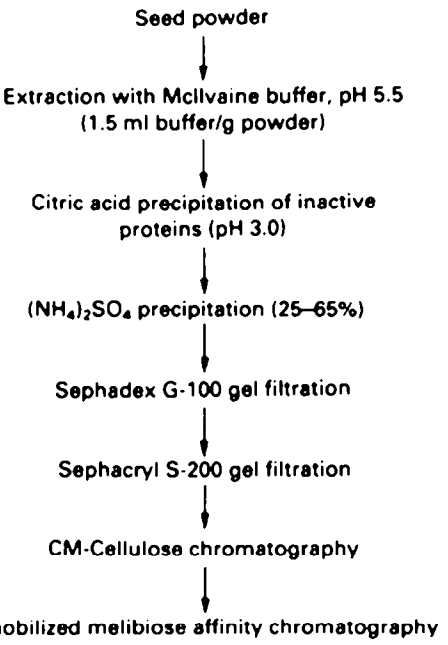

Fig. 1. Purification procedure for α-galactosidase II^1 from *V. faba*.

there was no evidence of contamination by the endogenous classical lectin, favin [4, 5]. This lectin is D-glucose/D-mannose specific and if present in a preparation would be eluted unretarded through the melibiose affinity column. Moreover, the specific lectin activity of favin reported in the literature [4] is 2800 HU/mg, thus the high specific activity of our preparation cannot be due to a small contamination with favin. The purification protocol as shown in Fig. 1 was successfully used earlier [3] for the removal of favin.

On chromatofocusing the purified enzyme, one major activity peak (pI 8.4) was observed; however, four minor

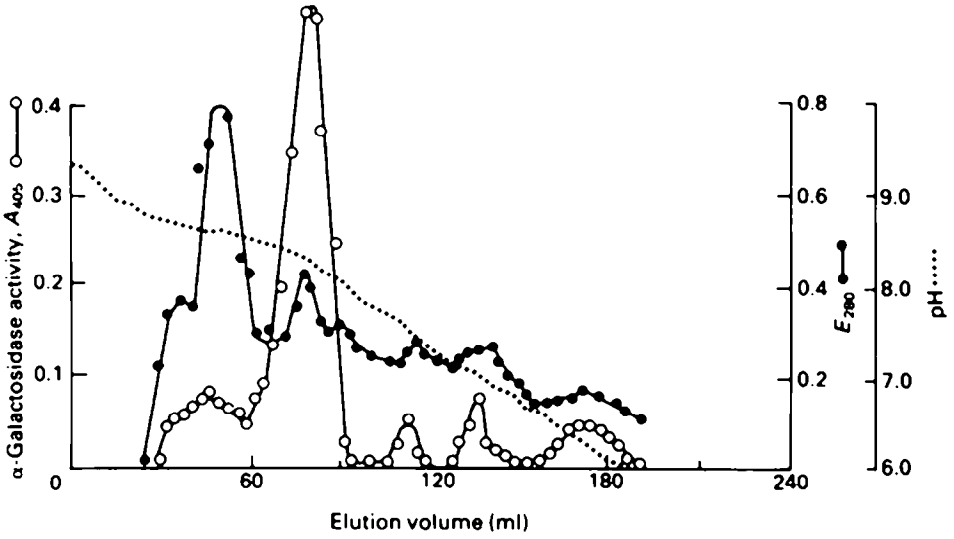

Fig. 2. Chromatofocusing of α-galactosidase II[1] from *V. faba*. Details are given in the Experimental section.

peaks were also present (Fig. 2). It is difficult to ascertain whether the minor peaks are due to post-translational changes in the protein moiety resulting during the enzyme purification process or are an indication of multiple glycoprotein forms with different carbohydrate compositions. Complex isoelectric focusing patterns of crystalline Con A have been reported [6].

Inhibition by carbohydrates

The carbohydrate specificity of the affinity-purified enzyme-lectin was studied by hapten inhibition of haemagglutination [7]. The results presented in Table 1 clearly indiate that D-mannose and to a lesser extent D-glucose, together with their low M_r derivatives, are significantly more effective inhibitors of haemagglutination than D-galactose and its derivatives. Methyl α-D-mannoside is the most potent inhibitor, and of the two anomers of methyl D-glucoside, the α-form is approximately six times more effective than the β-form, suggesting α-specificity, as has previously been observed with several D-mannose-specific lectins [4, 8]. α-Specificity is also evident from the hapten inhibition studies with glycogen, starch and yeast mannan when compared with galactomannans. In the latter case the observed inhibition was presumably due to β-linked D-mannosyl residues of the polymer 'backbone'. The interaction of the enzyme with solutions of yeast mannan, glycogen or soluble starch resulted in the formation of an insoluble complex.

With yeast mannan a reciprocal relationship was observed between turbidity and enzymic activity in the supernatant; this could be fully accounted for by changes in the amounts of the enzyme bound to mannan: a bell shaped curve was obtained with increasing concentrations of the polysaccharide (Fig. 3). Such characteristic behaviour is typical of lectin–polysaccharide interactions [9]. As expected from the hapten inhibition studies (Table 1) the formation of turbidity with mannan was prevented by including D-mannose (0.1 M) in the incubation mixture (see also ref. [10]). Both D-mannose and D-glucose were relatively poor non-competitive inhibitors

Table 1. Inhibition of lectin activity of *Vicia faba* α-galactosidase II[1] by carbohydrates

Carbohydrates	Minimum concentration (mM) causing 50% inhibition of six haemagglutinin units*
Methyl α-D-mannoside	2.00
D-Mannose	4.68
D-Glucose	8.36
3-*O*-Methyl D-glucose	8.36
N-Acetylglucosamine	8.36
Methyl α-D-glucoside	16.72
Methyl β-D-glucoside	100
L-Arabinose	125
D-Xylose	100
D-Galactose	1000
Methyl α-D-galactoside	1000
Melibiose	62.50
Raffinose	62.50
Stachyose	62.50
Starch (soluble)	0.02 mg/ml
Glycogen	0.02 mg/ml
Yeast mannan	0.03 mg/ml
Guar galactomannan	0.05 mg/ml
Clover galactomannan	0.06 mg/ml

*See Experimental section for details.

(*ca* 55% inhibition at 500 mM concentrations) of the catalytic activity of II[1].

Thus the lectin specificity of *V. faba* α-galactosidase II[1] is dissimilar to the 'so-called' α-galactosidase-haemagglutinins from *Vigna radiata* [12] and *Glycine max* [13] which are reported to be D-galactose-specific and also display 'clot dissolution'. It has recently been suggested [14] that 'clot dissolving' enzymes should not be classified as lectins; their haemagglutinating properties are believed to be due to the formation of short-lived stable

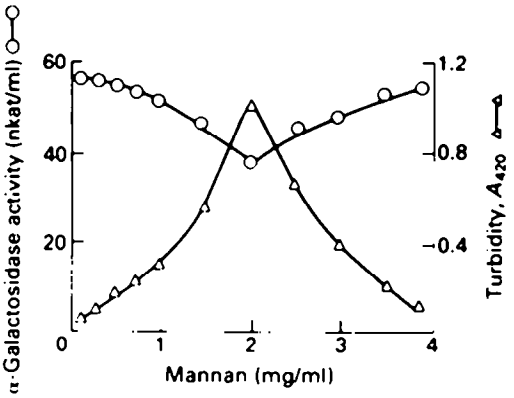

Fig. 3. Interaction of *V. faba* α-galactosidase II[1] with yeast α-mannan. The affinity purified enzyme (0.1 mg/ml) was dialysed against 0.1 M acetate buffer (pH 6.1) and 200 μl samples were added to various concentrations of yeast mannan solutions (200 μl) in the same buffer. After incubation at 25° for 10 min, the turbidity of each mixture was measured at 420 nm. This was followed by centrifugation and measurement of enzymic activity in the supernatant.

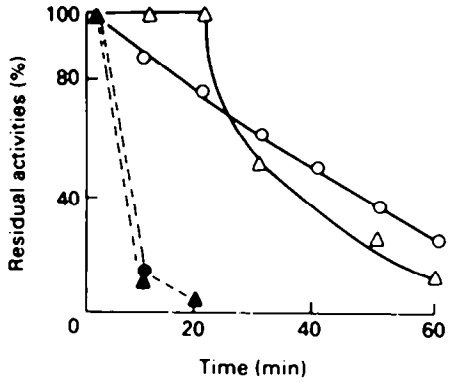

Fig. 5. Protection of catalytic and lectin activities of *V. faba* α-galactosidase II[1] at 75° by monosaccharides. ○——○, Catalytic activity in the presence of D-galactose (0.1 M); ●- - - -●, catalytic activity in the absence of D-galactose or, in the presence of D-mannose (0.1 M); △——△, lectin activity in the presence of D-mannose (0.1 M); ▲ - - - ▲, lectin activity in the absence of D-mannose or, in the presence of D-galactose (0.1 M). Details are given in the Experimental section.

complexes between the catalytic sites of the tetrameric enzymes with the α-D-galactosyl moieties of the erythrocyte cell-surfaces. Subsequent catalytic hydrolysis destroys these complexes. In contrast to enzyme II[1], the low M_r (monomeric forms) of *V. radiata* and *G. max* α-galactosidases do not display haemagglutinating property [13, 15].

Effect of temperature

Heat inactivation of the lectin and catalytic activities of α-galactosidase II[1] is shown in Fig. 4. At 65° the lectin activity is more stable than the catalytic function. However, at 75° both activities decay very rapidly at a similar rate (Fig. 5). On the other hand, the two activities respond differently at 75° in the presence of D-mannose and D-galactose (Fig. 5): D-galactose protects the catalytic function but not the lectin activity, whereas D-mannose has the opposite effect. Thus it seems that different loci are responsible for the two activities of the protein. Here it is interesting to note that the activity of the 'classical' D-glucose/D-mannose-specific lectin from *Lens culinaris* is protected at 72° by D-glucose (0.1 M) but not by D-galactose. However, neither of the monosaccharides could afford protection at 75°.

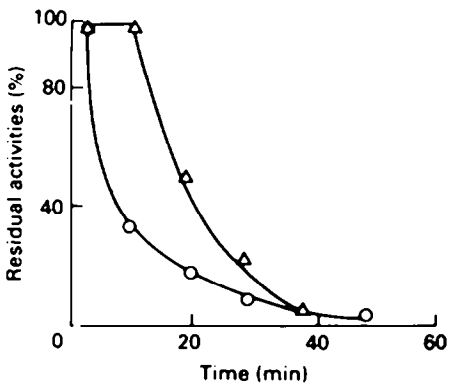

Fig.4. Stability of catalytic and lectin activities of *V. faba* α-galactosidase II[1] at 65°. ○——○, Catalytic activity; △——△, lectin activity. Details are given in the Experimental section.

Other properties

The pH profiles of the catalytic and lectin activities of the enzyme (Fig. 6) show distinct optima at pH 5.7 and 7.0, respectively. An additional optimum for the catalytic activity (67% of that at pH 5.7 was also observed) at pH 3.5, whereas no other optimum for lectin activity was seen in the acidic pH range. These results further indicate the involvement of different sites on the protein for the two activities. The number of lectin sites was determined by the equilibrium dialysis method using [^{14}C]-D-mannose analysing according to the method of Scatchard [16] (Fig. 7). Four D-mannose residues ($K_A = 2.18 \times 10^3\ M^{-1}$) were found to bind per molecule of the enzyme. The binding of [^{14}C]-D-mannose was also studied in the presence of varying amounts of unlabelled D-galactose and the Scatchard plot in this case was

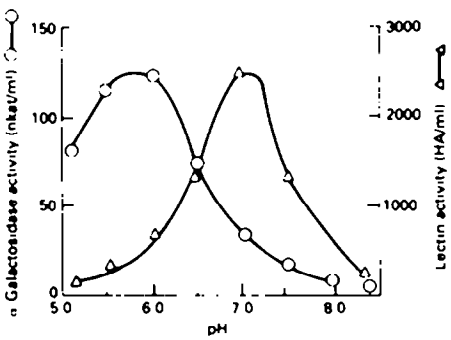

Fig. 6. Effect of pH (0.1 M KPi buffer) on the catalytic and lectin activities of *V. faba* α-galactosidase II[1].

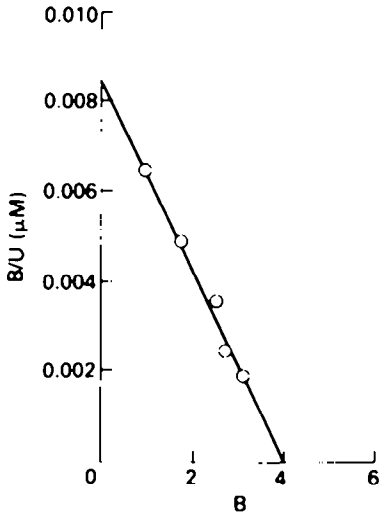

Fig. 7. Scatchard plot for the binding of D-mannose to *V. faba* α-galactosidase II[1]. B, Number of D-mannose molecules bound per molecule of protein; U, concentration of free sugar. Details are given in the Experimental section.

identical to that shown in Fig. 7. Thus, D-galactose did not alter either the affinity of the enzyme for D-mannose or the number of molecules of this hexose bound to the enzyme. The lectin activity of enzyme II[1] is therefore unlikely to be due to the presence of favin, which has only two D-mannose binding sites/molecule [5], unless there was heavy contamination which was not indicated by the SDS-PAGE study.

EXPERIMENTAL

Enzyme assay. α-Galactosidase was assayed at 30° by following the initial rate of hydrolysis of *p*-nitrophenyl α-D-galactoside (PNPG) in McIlvaine buffer [17], pH 5.5 as described earlier [11, 18]. Inhibition of catalytic activity by monosaccharides was measured by preincubating the enzyme/carbohydrate mixture at 30° for 10 min prior to assaying activity. Protein was assayed, after pptn with 10% TCA, by the method of ref. [19] using BSA as standard.

Haemagglutination (HA) assay. The method of ref. [20] was used. A serial dilution of lectin was made into KPi-buffered saline, pH 7.2 and lectin soln (100 μl) was added to a 1.5% rabbit erythrocyte suspension (200 μl). The mixtures in tubes were held at room temp. for 2 hr and the extent of agglutination was visually assayed on a scale of 0 to + + + + . One HA unit (HU) is the amount of lectin required to cause half-maximal (+ +) agglutination. To determine the 50% inhibition values for various carbohydrates, the method of ref. [7] was used.

Effect of temperature. Purified enzyme samples (0.23 mg/ml; sp. act., 165 nkat/mg) in 0.1 M KPi buffer, pH 7 or in McIlvaine buffer, pH 5.5 were incubated either at 65° or 75° both in the absence and presence of D-mannose or D-galactose. At specified time intervals aliquots were withdrawn into pre-cooled test tubes and then dialysed for 8 hr against appropriate buffers prior to lectin or enzymic assay.

Enzyme purification. The scheme for purification is shown in Fig. 1 and the experimental details of the various steps, except affinity chromatography, have been described [2]. For the immobilized melibiose (Pierce, U.K.) affinity chromatography a 5 ml plastic syringe (without the piston) fitted with a porous disc as a gel support was used as a column. The packed column was equilibrated with McIlvaine buffer, pH 5.5, the enzyme sample was applied and the column washed (15–20 ml/hr) with the same buffer until the A_{280} was nil. The bound enzyme was eluted with 50 mM PNPG soln in the equilibrating buffer and fractions (2 ml) collected until 100 ml soln had passed through the column. Active fractions which were detected by their yellow colouration, were dialysed and assayed for lectin and enzymic activities.

Chromatofocusing. The manufacturer's (Pharmacia) instructions for packing and running the column were followed. The packed column (1 × 15 cm) was equilibrated with 0.02 M ethanolamine–HOAc buffer, pH 9.4. Polybuffer 96 (5 ml, pH 6) was passed through the column prior to application of the affinity purified enzyme sample. The column was then eluted with Polybuffer 96 (250 ml) at a flow rate of 30–40 ml/hr and 2 ml fractions were collected. Each fraction was monitored for pH, A_{280} and enzymic activity.

Electrophoresis. SDS-PAGE of α-galactosidase II[1] was carried out as described earlier [2].

Equilibrium dialysis. Experiments were carried out using Dianorm equilibrium dialyser (Diachema AG, Zürich) fitted with 250 μl dialysis cells. Solns containing a constant amount of [^{14}C]-D-mannose (1.3 μCi; 200–300 mCi/mmol) to which had been added varying amounts of unlabelled D-mannose (0–0.12 mg) were placed in the cell compartments. On the opposite side of each compartment, separated by dialysis membrane, was placed enzyme soln (0.31 μg protein). The total vol. of each compartment was made up to 200 μl using 0.1 M KPi buffer, pH 7. The D-mannose concn in the compartments was calculated from the specific radioactivity. These D-mannose binding experiments were also repeated in the presence of varying amounts (0–0.12 mg) of unlabelled D-galactose. In all cases the dialysis cells were rotated for 24 hr at 25° to establish equilibrium. Samples (100 μl) were then drawn from each compartment, mixed with NCS tissue solubilizer (500 μl) in scintillation vials and incubated for 2 hr at 60°. After cooling, HOAc (20 μl) and toluene–PPO (10 ml; 5 g PPO/l. toluene) was added to each vial and radioactivity measured in a Beckman LS7500 scintillation counter.

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