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MULTIPLE FORMS OF *VICIA FABA* α-GALACTOSIDASES AND THEIR RELATIONSHIPS

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

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

INTRODUCTION

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

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

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

RESULTS AND DISCUSSION

Enzyme purification

Stages 1 and 2 (see Table 1) in the purification of the α-galactosidases from resting *V. faba* seeds were essentially those described by Dey and Pridham [4]. In the present work this was followed by ammonium sulphate fractionation and Sephadex G-100 chromatography. The resolved forms, I and II, were then passed separately through columns of Con A-Sepharose on which they were largely retained. In both cases, however, significant proportions of the activities applied (17% of I and 20% of II) appeared to be unbound as they were eluted from the columns with phosphate buffer (pH 7).

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

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

Table 1. Purification of *V. faba* α-galactosidases

Stage	Volume (ml)	Activity (nkat/ml)	Protein (mg/ml)	Specific activity (nkat/mg)	Recovery* (%)
1. Crude extract	1010	16.0	88	0.2	—
2. Citric acid pptn	1438	9.1	9.2	1	81
3. $(NH_4)_2SO_4$, 25–60%	68	124.4	98	1	52
4. Sephadex G-100					
α-Galactosidase I	9	253.7	6.1	42	14
α-Galactosidase II	11	165.3	62.9	3	11
5. Sephadex G-100, recycle					
I	3	426.6	5.4	79	8
II	4	229.5	37	6	6
6. Con A-Sepharose					
I	5	184	0.8	185	4.5
II	5	114.4	3.1	36	3.5
7. CM-Cellulose chromatography					
I	1.0	307	0.24	1278	2
II^1	2.5	43.6	1.08	40	0.7
II^2	2.5	48.4	0.56	86	0.56
8. CM-Cellulose chromatography, recycle					
I	1.0	180.0	0.14	1286	1.1
II^1	1.0	60.0	0.78	76	0.37
II^2	1.0	72.0	0.63	113	0.44

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

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

Structural relationships between the enzyme forms

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

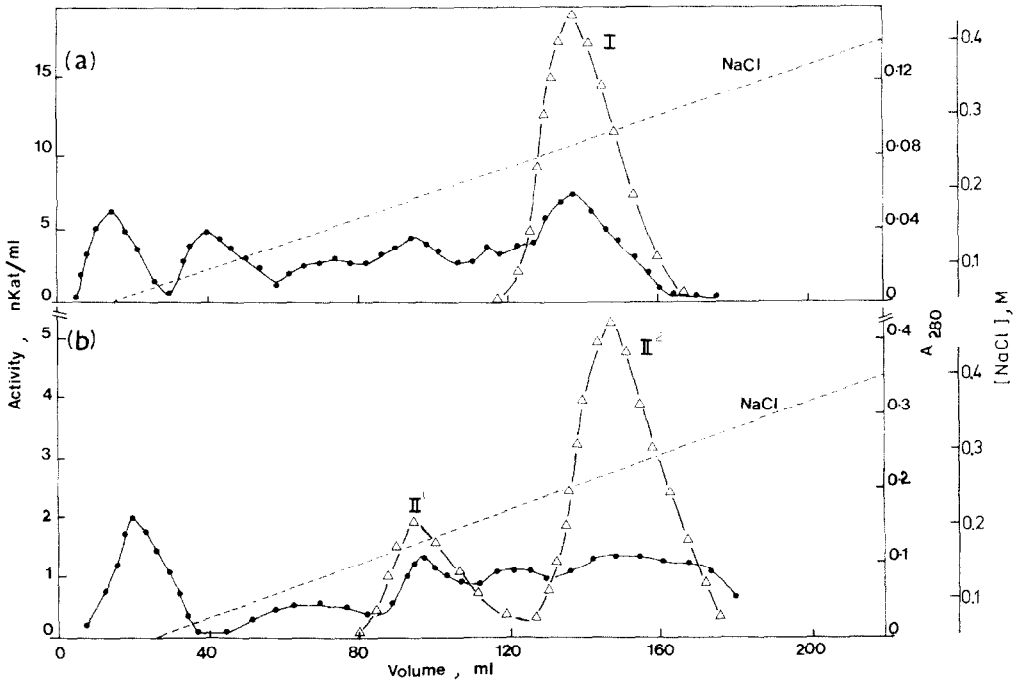

Fig. 1. Elution profiles of enzymes I, II^1 and II^2 on CM-cellulose columns. Elution procedure as described in the Experimental. (a) α-Galactosidase I; (b) α-galactosidases II^1 and II^2; (●) protein (A_{280}); (△) enzyme activity.

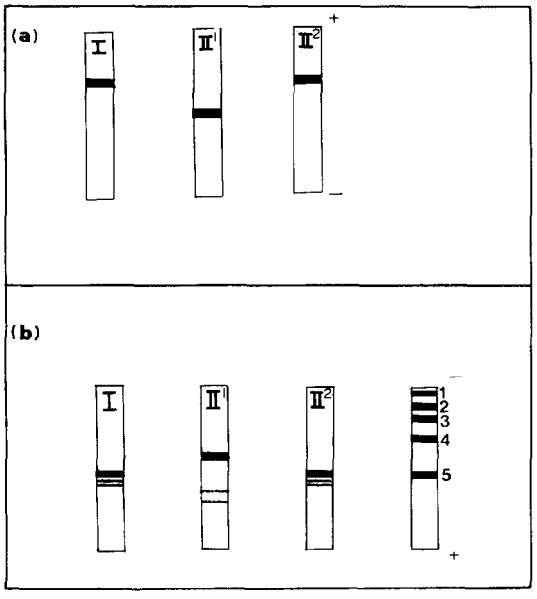

Fig. 2. (a) Polyacrylamide disc gel electrophoresis of α-galactosidases I, II¹ and II². (b) SDS-polyacrylamide gel electrophoresis of enzymes I, II¹ and II². The standard MW markers were 1, myosin, MW 200 000; 2, β-galactosidase, MW 130 000; 3, phosphorylase *b* MW 94 000; 4, BSA, MW 68 000; 5, ovalbumin, MW 43 000.

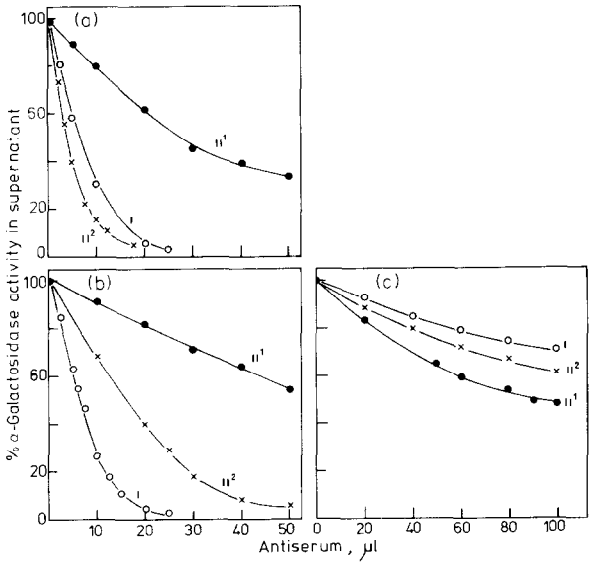

Fig. 3. Immunoprecipitation curves with α-galactosidases I, II¹ and II² using: (a) antiserum-II²; (b) antiserum-I; (c) antiserum-II¹.

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

Figure 2(b) shows the SDS-PAGE mobilities of α-galactosidases I, II¹ and II². In the case of forms I and II², major identical protein bands (apparent MW 41 300 ± 1200) were observed together with two minor components with higher mobilities. (In the case of enzyme I there was clear evidence of microheterogeneity in the major band.) This MW value considered together with the MWs for I and II² obtained by ultracentrifugation, suggests that I is a tetramer of II². α-Galactosidase II¹ exhibits an apparent MW of 42 650 ± 950 on SDS gels and the sedimentation equilibrium method also indicates that the MW of II¹ is greater than that of II². The electrophoretic pattern for II¹ (Fig. 2b) also showed the presence of minor polypeptide bands.

Rabbit antisera were raised against the three purified *V. faba* α-galactosidases and each serum was titrated against the enzymes by assaying changes in the activities in the supernatants (Fig. 3). In all cases immunoprecipitation occurred although the reaction was relatively poor in the case of the anti-II¹ serum. The immunoprecipitation titration curves for anti-I indicate homology amongst the three enzyme forms, however the slopes of the curves show that I and II² are structurally more closely related than I and II¹. Similarly both the anti-II¹ and -II² sera reacted with all of the α-galactosidases and again showed the close relationship between I and II². Examination of these interactions by the Ouchterlony double diffusion method [11] confirmed the above findings.

Table 2. Carbohydrate content of *V. faba* α-galactosidases

Sample	Sugars present (mol/mol)			
	Xylose	Mannose	Glucose	Glucosamine
Galactosidase I	2.7	6.7	13	2.1
Galactosidase II¹	0.9	2.3	1.5	0.7
Galactosidase II²	1.9	3.2	5.1	0.94

V. faba α-galactosidase I, therefore, is probably a tetramer composed of II² glycoprotein units and in this respect it would appear to be similar to α-galactosidases occurring in a number of other leguminous plants [12–15]. Although *V. faba* seeds possess two forms of α-galactosidase, II¹ and II², of similar molecular size only the latter appears to aggregate to produce the high MW enzyme I. The situation is similar in the case of *Vigna radiata* (mung bean) seeds where examination of the high MW form (160 000) by SDS-PAGE yielded a single subunit of MW 45 000 [13]. However, there was no report of a second low MW enzyme equivalent to II¹ in these seeds. It should perhaps be noted that Hankins and Shannon [13] examined the α-galactosidases in mung bean seeds that had imbibed water whereas in the present study resting seeds were used. The pattern of multiple forms of α-galactosidase are known to change when seeds germinate [2]. Del Campillo [16], however, has reported that soybean seeds possess a high MW (160 000) α-galactosidase which in the presence of SDS dissociates into two subunits with MWs of 38 000 and 40 000. Regarding enzyme II¹ in *V. faba* seeds, it appears from the immunological studies that it is structurally related to the smaller α-galactosidase II² and hence may be the precursor of II² via post-translational modification.

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

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

Conclusions

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

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

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

EXPERIMENTAL

Enzyme assay. α-Galactosidase activity was determined using *p*-nitrophenyl-α-D-galactopyranoside as described earlier [4]. For detection of enzyme activity on polyacrylamide gels, 6-bromo-2-naphthyl-α-D-galactoside was used [20]. Protein was assayed, after pptn with 10% TCA, by the method of ref. [21]. BSA was used as standard.

Enzyme isolation and purification. Testa free, dormant beans were ground in a mechanical grinder. The resultant bean meal (1 kg) was suspended in 1.5 l. of McIlvaine buffer pH 5.5, stirred and left for 1 hr at 4°. The slurry was subsequently centrifuged at 10 000 *g* for 40 min and the residue discarded. The pH of the supernatant was lowered to 3.2 by gradual addition of 1 M citric acid with constant stirring. The ppted proteins were discarded by centrifuging at 10 000 *g* and the pH of the supernatant raised to 5.5 with a satd soln of Na_2HPO_4. This fraction was then made 25% satd with $(NH_4)_2SO_4$ and the ppted protein centrifuged and discarded. The resultant supernatant was made 60% satd with $(NH_4)_2SO_4$ and stirred for 3 hr at 4°. The ppted protein was collected by centrifugation and suspended in McIlvaine buffer (pH 5.5) and dialysed against the same buffer (2 l.) overnight. This dialysed fraction was applied to a 5 × 90 cm, Sephadex G-100 column prepared according to the method of ref. [22]. McIlvaine buffer containing 0.1 M KCl was used to elute the enzyme from the column. Two peaks of α-galactosidase activity I and II were obtained which were pooled and concentrated separately. From this stage onwards enzymes I and II were purified separately. α-Galactosidases I and II were recycled through the Sephadex column. The active fractions were dialysed against KPi buffer (0.1 M, pH 7) and applied in 2 ml aliquots to Con A-Sepharose columns (2 × 20 cm) equilibrated with KPi buffer (pH 7) containing 0.5 M NaCl. The column was washed with buffer until all the unbound proteins had been eluted. The bound enzyme was then eluted with 0.5 M α-D-methyl mannoside in the same buffer. Fractions (2 ml) were collected and the active fractions pooled and concentrated. The enzymes were then dialysed against McIlvaine buffer (pH 3.5) and the dialysed samples applied to 1.0 × 14 cm, CM-cellulose columns equilibrated with McIlvaine buffer, pH 3.5. The flow rate was 15 ml/hr. Following sample application, the column was washed with the buffer and elution of protein carried out in a linear NaCl gradient of 0.05–0.4 M in the same buffer. α-Galactosidase II was further resolved into II^1 and II^2. All three enzymes I, II^1 and II^2 were recycled through the CM-cellulose column. The purified enzymes were stored at 4° with thymol added as preservative.

Electrophoresis. PAGE was carried out under cationic conditions according to an earlier method [23] with a slight modification. Electrophoresis was performed in 7.5 × 0.5 cm gel tubes, the separating gel being 6.5 cm and the stacking gel 0.5 cm. The gels were run at pH 3.5 in β-alanine–acetate buffer diluted 1 : 1 with H_2O. For SDS-PAGE, samples were heated to 100° in buffer containing 1.25% SDS and subjected to electrophoresis through 5% acrylamide stacking gels and 8% acrylamide separating gels [24]. Gels were stained with Coomassie brilliant blue-R250 [0.2% (w/v)] in 50% TCA for 15–30 min at 60° and then destained with a soln containing 7.5% (v/v) HOAc and 12.5% (v/v) *iso*-PrOH. The protein standards used were obtained as a kit from Bio-Rad.

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

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

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

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

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

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