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FACTORS AFFECTING THE EXTRACTION AND THE RELATIVE PROPORTIONS OF MULTIPLE FORMS OF PLANT α-GALACTOSIDASES

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

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

INTRODUCTION

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

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

Whilst attempting to accurately determine the proportions (high and low MW forms) of α-galactosidases in resting *V. faba* seeds, a number of procedures which are commonly used for the extraction and purification of enzymes were examined to see if they affected the ratio of the multiple forms and the enzyme activity. Detailed information of this kind is rarely reported in the literature (however, see e.g. refs. [11] and [12]).

RESULTS AND DISCUSSION

Effect of salt concentration on enzyme extraction

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

Analysis of extracted fractions

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

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

Extraction medium	Total enzyme activity (nkat)	Total protein (mg)	Sp. act. (nkat/mg protein)
H_2O	1561	1356	0.11
NaCl			
0.05 M	213.2	2574	0.08
0.1 M	199.0	2555	0.08
0.5 M	270.4	2145	0.13
1.0 M	270.4	1417	0.19
Triton (w/v)			
0.1%	251.0	1742	0.14
0.5%	211.2	1513	0.14
1.0%	198.0	1200	0.16
5.0%	111.3	1950	0.06
Acetate buffer (pH 5.5)			
0.05 M	191.3	1162	0.16
0.1 M	218.8	1250	0.17
0.5 M	245.7	1586	0.15
1.0 M	244.8	1440	0.17
McIlvaine buffer (pH 5.5)			
×0.5 concn	210.6	1547	0.14
×1 concn	227.5	1560	0.15
×5 concn	298.8	1440	0.21
× 10 concn	314.4	1500	0.21

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

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

Returning to the problem of identifying the multiple forms of α-galactosidase occurring *in vivo*, both the extractability of the different forms and the formation of oligomers *in vitro* complicate any interpretation. However, a comparison of Figs. 1(a) and 1(c) shows that little conversion of II to I occurs during the processing time and hence most of enzyme I in Fig. 1(b) must presumably have been derived from the bean tissues and high sodium chloride levels were required for its extraction (cf. Figs. 1a and 1b). Again, a concentrated acetate buffer would appear to be required for extraction of α-galactosidase I (Fig. 3b). McIlvaine buffer at normal concentrations does appear to extract some α-galactosidase I, but the amount extracted with buffer of five times this ionic strength removes significantly more of enzyme I from the seed tissues. The intermediary form of α-galactosidase occurring with the concentrated sodium chloride solution (Figs. 1b and 1c) and McIlvaine buffer (Fig. 2b) may be a dimer.

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

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

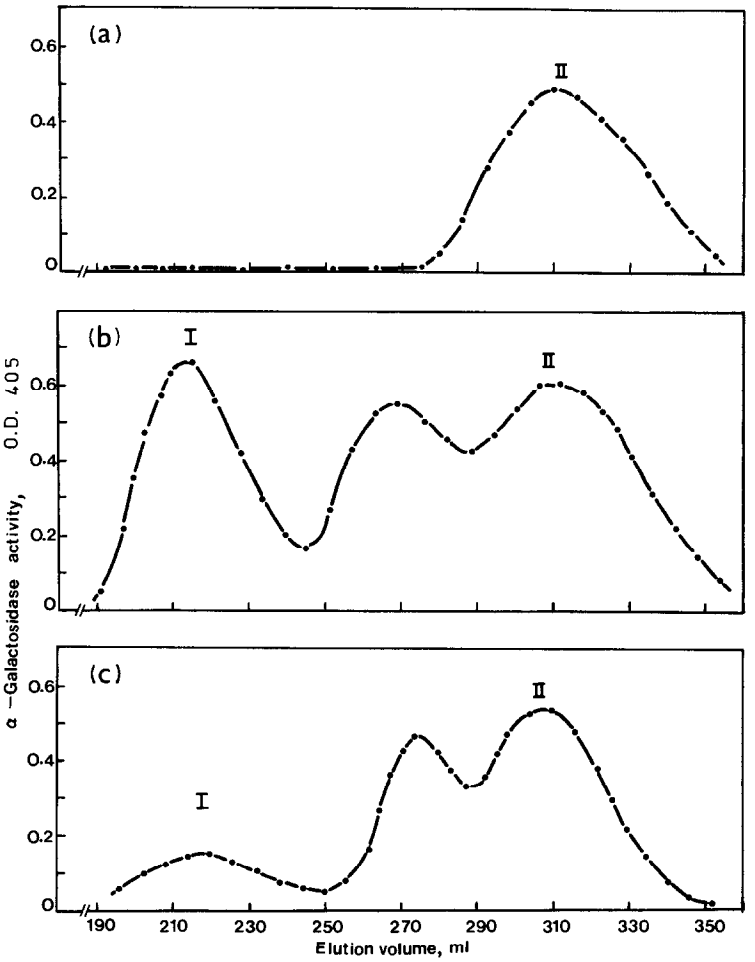

Fig. 1. Gel-filtration of crude α-galactosidase preparations in varying sodium chloride concentrations on Sephadex G-100. Extraction and elution were carried out as specified in the Experimental. a, 0.1 M NaCl; b, 0.5 M NaCl; c, 0.1 M NaCl extraction followed by 0.5 M NaCl elution.

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

The four-stage purification was completed in *ca* 24 hr and the α-galactosidase pattern at Stage (b), which was reached in *ca* 2 hr, (Fig. 4b) can be compared with that obtained after storing the crude extract (see Fig. 4a) at 4° for 60 hr, over which there was a steady increase in specific activity which paralleled the accumulation of α-galactosidase I (Fig. 5). It can be concluded that the normal aggregation of the protein with time is enhanced by citric acid precipitation and that it goes beyond the 60 hr storage pattern (Fig. 5) at Stage (d) mainly as a result of the long period required for dialysis. The expected continuous change in pattern during the ammonium sulphate fractionation (Stage c) appears to be interrupted, possibly because of 'salting-out' effects. This contrasts with the enhancement of aggregation produced by high levels of salts (see Fig. 1b) but the 'salting-out' effect can vary significantly with the nature of the ionic species [15].

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

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

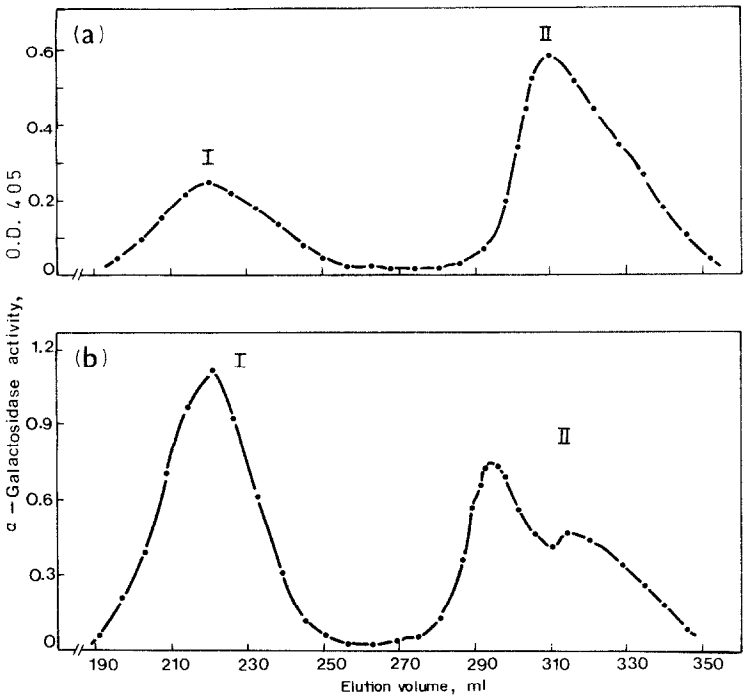

Fig. 2. Gel-filtration of crude α-galactosidase preparations in different concentrations of McIlvaine buffer, pH 5.5, on Sephadex G-100. a, Extraction and elution with buffer (normal concentration); b, extraction and elution with buffer (five-fold concentration).

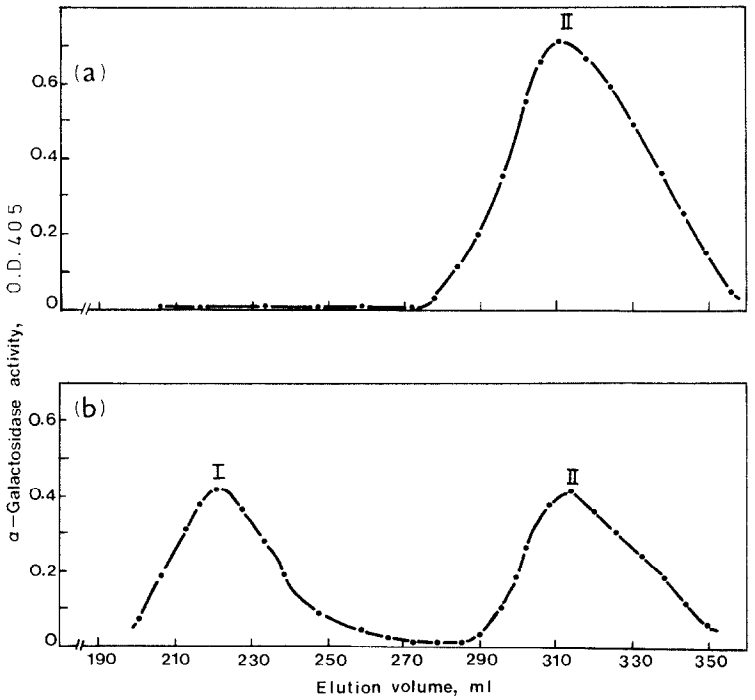

Fig. 3. Gel-filtration of crude α-galactosidase preparations in different concentrations of sodium acetate buffer, pH 5.5. a, Extraction and elution with 0.1 M buffer; b, extraction and elution with 0.5 M buffer.

Table 2. Purification of α-galactosidase from mature *Vicia faba* seeds

Purification stage	Total activity (nkat)	Total protein (mg)	Sp. act. (nkat/mg protein)	Purification (fold)	Recovery* (%)
(a) Crude	833.3	3575	0.23	—	—
(b) Citric acid precipitation	1700.0	2500	0.68	3.8	268.4
(c) Ammonium sulphate precipitation (30–60%)	1676.0	289	5.8	32.8	98.6
(d) Dialysis	2845.0	297	9.6	54.2	169.7

Details as described in the Experimental.
*Expressed with respect to the preceding stage.

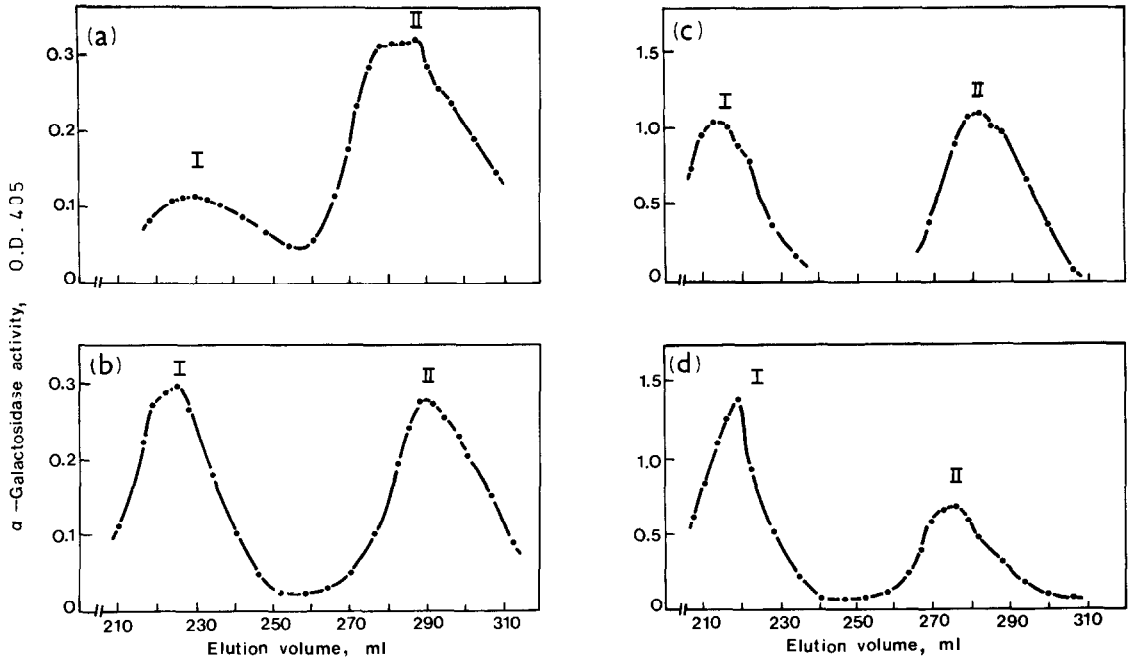

Fig. 4. Sephadex G-100 elution patterns of α-galactosidase fractions at various stages of purification (Table 2). McIlvaine buffer, pH 5.5, was used for elution. a, Stage (a), crude extract; b, Stage (b), citric acid precipitation; c, Stage (c), ammonium sulphate fractionation; d, Stage (d) dialysis.

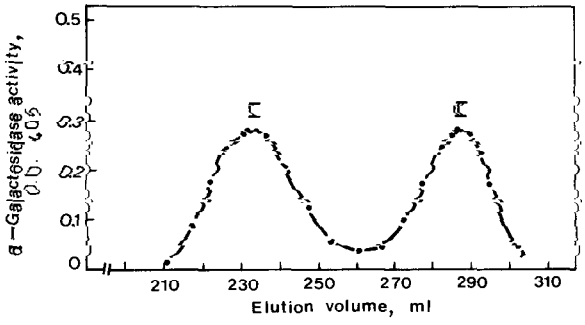

Fig. 5. Sephadex G-100 elution pattern of crude α-galactosidase preparation after storage at 4° for 60 hr.

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

EXPERIMENTAL

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

When purifying the enzyme, the pH of the supernatant was first lowered to 3.2 (Stage b) by slow addition of 1.0 M citric acid soln with constant stirring; stirring was maintained for a further 30 min after the required pH was reached. The resulting ppt was removed by centrifugation at 20 000 *g* for 20 min and the pH of the active supernatant readjusted to 5.5 with satd Na_2HPO_4 soln. The enzyme soln was then fractionated with $(NH_4)_2SO_4$ (Stage c) and the ppt obtained at 30–65% satn centrifuged down and dissolved in McIlvaine buffer (pH 5.5). This soln was then dialysed (Stage d) against McIlvaine buffer (pH 5.5, diluted 1:1 with deionized H_2O) for 16 hr.

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

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

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