Purification and Properties of α -Galactosidases from *Vicia faba* Seeds

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Two forms of α -galactosidase, I and II, exist in *Vicia faba* seeds and these have been purified 3660- and 337-fold respectively. They behaved as homogeneous preparations when examined by ultracentrifugation, disc electrophoresis and gel filtration. The apparent molecular weights of enzymes I and II, as determined by gel filtration, were 209 000 and 38 000 respectively. The carbohydrate contents of enzymes I and II were 25% and 2.8% respectively, and the enzymes differed in their aromatic amino acid compositions. Enzyme I was split into six inactive subunits in the presence of 6M-urea. α -Galactosidases I and II showed different pH optima and K_m and V_{max} values with *p*-nitrophenyl α -D-galactoside and raffinose as substrates, and also differed in their thermal stabilities.

α -D-Galactosyl derivatives of sucrose, such as raffinose and stachyose, are known to occur in many seeds (French, 1954) including the broad bean, *Vicia faba* (Pridham, 1958). These oligosaccharides are reserve substances that are broken down during germination, probably by the concerted action of α -galactosidase (α -D-galactoside galactohydrolase, EC 3.2.1.22), β -fructofuranosidase (β -D-fructofuranoside fructohydrolase, EC 3.2.1.26) and sucrose-UDP glucosyltransferase (EC 2.4.1.13) (e.g. see Pazur, Shadaksharaswamy & Meidell, 1962; Pridham, Walter & Worth, 1969). α -Galactosidase activity in *Vicia faba* seeds has been recognized for several years (Pridham & Walter, 1964), but the enzyme has not been purified. The isolation and partial purification of this enzyme from various other sources have been reported (Helferich & Vorsatz, 1935; Zechmeister, Toth & Balint, 1938; Ahmed & Cook, 1964; Malhotra & Dey, 1964, 1967; Agrawal & Bahl, 1968) and, in particular, Petek & To Dong (1961) isolated two α -galactosidases from coffee beans by fractionation on an alumina column.

As a preliminary stage in the study of the metabolism of the raffinose family of oligosaccharides during germination of *Vicia faba* seeds, it was necessary to look in more detail at the postulated primary hydrolytic reaction. This paper reports the isolation of two protein fractions with high α -galactosidase activity. A preliminary account of this work has been published (Dey & Pridham, 1968).

MATERIALS AND METHODS

Buffer reagents were of A.R. grade, and only deionized water was used. *p*-Nitrophenyl α -D-galactoside (Dey, 1967), *p*-nitrophenyl α -D- and β -D-glucopyranosides (Montgomery, Richtmyer & Hudson, 1942) and *p*-nitrophenyl β -D-galactoside (Seidman & Link, 1950) were synthesized in the laboratory.

Enzyme assay. α -Galactosidase was assayed by addition of enzyme solution (0.1 ml.) to a mixture of McIlvaine buffer, pH 5.5 (0.25 ml.), and 12 mM-*p*-nitrophenyl α -D-galactoside (0.05 ml.) preheated to 30°. This temperature was maintained for 15 min. and the reaction was then stopped by the addition of 0.1 M-Na₂CO₃ solution (5 ml.). The release of *p*-nitrophenol was measured at 405 nm. α -Glucosidase (α -D-glucoside glucohydrolase, EC 3.2.1.20), β -glucosidase (β -D-glucoside glucohydrolase, EC 3.2.1.21) and β -galactosidase (β -D-galactoside galactohydrolase, EC 3.2.1.23) were assayed with the corresponding *p*-nitrophenyl glycosides as substrates under conditions identical with those used for α -galactosidase. Similarly, 'alkaline' invertase activity was determined at pH 7.8 with sucrose as substrate (Pridham & Walter, 1964).

A unit of enzyme activity is defined as the amount that hydrolyses 1 μ mole of substrate/min. under the conditions specified above. Specific activities are expressed as enzyme units/mg. of protein. The protein determinations were made by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine serum albumin as a standard.

Sephadex-gel filtration. Sephadex G-100 and G-200 columns (2.4 cm. $\times$ 50 cm.) were prepared as described by Andrews (1964). Columns were eluted with McIlvaine buffer, pH 5.5, containing 0.1 M-KCl, at a flow rate of 30 ml./hr., and fractions (4 ml.) were collected. The columns

were calibrated by determination of the elution volumes of a number of reference proteins of known molecular weight. Sephadex G-100 columns were calibrated at 20° and Sephadex G-200 columns at 4°. The results were expressed in terms of $R_{S,A}$ values (i.e. elution volume of bovine serum albumin/elution volume of the protein ratio).

Disc electrophoresis. Electrophoresis on polyacrylamide columns was carried out by the method of Steward, Lyndon & Barber (1965) with a Shandon apparatus. Reservoir buffer (tris-glycine, pH 8.3) was prepared as described by Davis (1964). Enzyme preparations (25 μ g. of protein) in 4% (w/v) sucrose solution were applied to the polyacrylamide columns and electrophoresis was carried out for 90 min. at 100 v and 3 mA/tube.

Ultracentrifugation study. Sedimentation-velocity experiments were carried out by Dr S. P. Spragg of the University of Birmingham with a Spinco model E ultracentrifuge.

RESULTS

Purification of the enzyme. Unless specified, all operations were carried out at approx. 4°. Activities of β -galactosidase, β -glucosidase, α -glucosidase and 'alkaline' invertase in addition to that of α -galactosidase were determined at each stage of purification.

Step 1. Enzyme extraction. In a typical experiment 500g. of testa-free dormant broad beans was powdered in a mechanical grinder and extracted three times with McIlvaine buffer, pH 4.8 (300ml.).

In the extract, the α -glucosidase: β -galactosidase: 'alkaline' invertase: β -glucosidase: α -galactosidase activity proportions were 1.0:2.0:2.1:3.0:5.0.

Step 2. Variation of pH. The pH of the enzyme solution was lowered to approx. 3.0 with 1M-citric acid. Efficient stirring was essential at this step. The mixture was kept overnight at 4° and this produced a heavy precipitate of inactive proteins, which were removed by centrifugation at 1000g for 20 min. The pH of the supernatant solution was adjusted to 4.8 with 1M-disodium hydrogen phosphate solution. Glycosidase activities other than α -galactosidase were absent.

Step 3. Protamine sulphate treatment. Protamine sulphate was added to the enzyme solution (final concn. 10mg./ml.) and any nucleic acid precipitated over 24hr. was rejected. There was no marked increase in the specific activity of the enzyme, but this step proved helpful in achieving further purification.

Step 4. Acetone precipitation. Throughout this step, the temperature was held between -10° and -5°. Acetone at -20° was gradually added to the enzyme solution (at -5°) with stirring, up to a final concentration of 30% (v/v). After 45 min., the precipitated inactive protein was removed by centrifugation at 1000g for 5 min. The acetone concentration of the supernatant solution was increased to 60% (v/v) and the resulting active

Fig. 1. Separation of two α -galactosidases (I and II) by Sephadex G-100 gel filtration. Enzyme (5000 milliunits) from purification step 5 (see Table 1) was applied to the column. Experimental conditions are given in the text. $\circ$, α -Galactosidase activity; $\times$, protein; Δ , α -galactosidase specific activity.

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

Recovery was calculated on the basis of the total enzyme activity from the preceding step. The overall recoveries of enzymes I and II were 8.75% and 7.25% and the overall purifications were 3660- and 337-fold respectively.

Step no.	Purification step	Volume (ml.)	Enzyme activity (milliunits/ml.)		Protein (mg./ml.)		Sp. activity (milliunits/mg. of protein)		Recovery (%)		
1	Extraction	750	450		37.50		12		—		
2	Variation of pH	800	274		4.00		68.5		65		
3	Protamine sulphate treatment	810	270		3.55		76		100		
4	Acetone fractionation	20	6900		42.00		168		63		
5	Dialysis	5	16560		19.48		850		60		
<hr/>											
	Enzyme form ...		I	II	I	II	I	II	I	II	
6	First Sephadex G-100 gel filtration and concentration	2	2	18837	15318	0.43	5.67	43800	2700	45	37
7	Second Sephadex G-100 gel filtration and concentration	2	2	14768	12250	0.335	3.02	44000	4050	78	80

Fig. 2. Determination of the molecular weights of α -galactosidases I and II by Sephadex G-100 (a) and Sephadex G-200 (b) gel filtration. The procedure is described, and $R_{S.A.}$ is defined, in the text. Proteins used for calibration were: 1, cytochrome c; 2, ribonuclease; 3, α -chymotrypsin; 4, pepsin; 5, ovalbumin; 6, haemoglobin; 7, bovine serum albumin; 8, alcohol dehydrogenase; 9, hexokinase; 10, lactate dehydrogenase; 11, pyruvate kinase.

precipitate was centrifuged down and dissolved in McIlvaine buffer, pH 4.8.

Step 5. Dialysis. The enzyme solution was dialysed against water (11.) and then against McIlvaine buffer, pH 3.5 (diluted 1:5 with water), for 4 days. During this period the buffer (11.) was changed daily. An active precipitate was formed and this was isolated and dissolved in McIlvaine

buffer, pH 4.8. The recovery of activity at this stage was variable with different preparations. The highest recovery was 60%.

Step 6. First Sephadex G-100 gel filtration. The enzyme solution from step 5 was dialysed against McIlvaine buffer, pH 5.5, containing 0.1M-potassium chloride, and then passed through the Sephadex column. Two protein fractions with α -galactosidase activity were resolved (peaks I and II; Fig. 1). The fractions of peaks I and II with high specific activity were pooled separately and concentrated by using Aquacide (Calbiochem, Los Angeles, Calif., U.S.A.).

Step 7. Second Sephadex G-100 gel filtration. Enzyme fractions I and II were recycled separately through a Sephadex G-100 column and the fractions of high specific activity were again pooled and concentrated.

A summary of the purification procedure is given in Table 1.

Molecular-weight determination by Sephadex-gel filtration. The method of Andrews (1964), which is based on the elution volumes of proteins of known molecular weight, was followed with both Sephadex G-100 and Sephadex G-200 columns. Calibration curves are shown in Fig. 2 and, assuming that the molecular shape of the α -galactosidases is similar to those of the marker proteins, enzymes I and II have apparent mol.wt. 209 000 and 38 000 respectively.

Carbohydrate contents of enzyme preparations. These were measured by the phenol-sulphuric acid method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956) and expressed as glucose equivalents; they

Fig. 3. Sephadex G-200 gel filtration of purified α -galactosidases I (a) and II (b). The procedure and the conditions were the same as those described for Sephadex G-100 gel filtration. The recoveries of enzymes I and II were 97% and 95% respectively. $\circ$, α -Galactosidase activity; $\times$, protein; Δ , α -galactosidase specific activity.

Fig. 4. Schlieren patterns of purified α -galactosidase I (top) and α -galactosidase II (bottom) sedimenting from left to right at 20° . The photographs were taken 24, 64, 80 and 112 min. after the rotor had attained maximum speed (50740 rev./min.). Enzymes were examined in Mollvaine buffer, pH 5.5, containing 0.1 M-KCl, and the concentrations of enzymes I and II were 0.27% and 0.31% respectively.

were 25% and 2.8% respectively for α -galactosidases I and II.

Homogeneity of the enzyme preparations. When the final fractions of α -galactosidases I and II were rechromatographed on Sephadex G-200, protein and activity peaks coincided exactly and were symmetrical (Fig. 3) and the specific activities of the preparations applied to the columns were completely recoverable.

In the ultracentrifuge, α -galactosidases I and II

both sedimented as single components and gave symmetrical schlieren peaks (Fig. 4). The sedimentation coefficients were 5.56s and 3.49s respectively. The two fractions also gave single bands when examined by polyacrylamide-gel electrophoresis.

Ultraviolet spectrum. α -Galactosidase I showed an absorption maximum at 280 nm. and an inflexion at 291 nm. The $E_{280}^{1\%}$ was 18 and the E_{280}/E_{260} ratio was 1.4. α -Galactosidase II had an absorption maximum

Fig. 5. Gel filtration of α -galactosidase I on a Sephadex G-100 column in the presence of 6M-urea. The procedure is described in the text.

Fig. 7. Effect of substrate concentration on the initial rate of hydrolysis of raffinose. α -Galactosidase I (0.62 μ g.) (●) and α -galactosidase II (5.5 μ g.) (○) were incubated with various amounts of the substrate; conditions were as in Fig. 6. Liberated galactose was determined by a microcuprimetric method (Nelson, 1944) with arsenomolybdate reagents (Hestrin, Feingold & Schramm, 1955). A Lineweaver-Burk double-reciprocal plot is given as an inset.

Fig. 6. Effect of substrate concentration on the initial rate of hydrolysis of *p*-nitrophenyl α -D-galactoside in McIlvaine buffer, pH 4.0. α -Galactosidase I (0.5 μ g.) (●) and α -galactosidase II (5.0 μ g.) (○) were incubated under standard conditions with various amounts of substrate and the extent of hydrolysis was measured as described in the Materials and Methods section. A Lineweaver-Burk double-reciprocal plot is given as an inset.

at 278 nm. with an inflexion at 284 nm. The $E_{278}^{1\%}$ and $E_{280}^{1\%}$ were 20 and 19 respectively, and the E_{280}/E_{260} ratio was 1.3.

Tyrosine and tryptophan residues were determined spectrophotometrically by the method of Bencze & Schmid (1957), and the respective values were 51 and 47 moles/mole of α -galactosidase I, and 13 and 9 moles/mole of α -galactosidase II.

Subunits of α -galactosidase I. The enzyme, when subjected to Sephadex G-100 gel filtration in the presence of 6M-urea, showed six protein peaks, of

which two were major (*B* and *C* in Fig. 5). Peak *A* had an elution volume corresponding to that of the native enzyme. The apparent molecular weights of components *B* and *C* (cf. Olson & Liener, 1967) were 18200 and 14400 respectively. Peaks *D*, *E* and *F* seemed to be low-molecular-weight components and were not within the range of the calibration curve for this particular column. These estimates, based on gel-filtration data, should, however, be regarded as tentative, as the chromatographic behaviour of proteins on Sephadex gel can be appreciably altered in the presence of urea (de la Llosa, Tertrin & Jutisz, 1966). None of the above protein peaks showed enzymic activity, before or after dialysis against water. The enzyme activity was also not restored by pooling the fractions followed by dialysis and concentration. This was expected, as earlier observations showed that inactivation of this enzyme with 6M-urea was irreversible (P. M. Dey & J. B. Fridham, unpublished work).

When the enzyme was examined by disc electrophoresis at pH 8.3 in the presence of 6M-urea, three distinct and three diffused zones were observed.

Effect of substrate concentration. *p*-Nitrophenyl α -D-galactoside was inhibitory at higher concentrations with both of the α -galactosidases (Fig. 6).

Fig. 8. Effect of pH on the activity of α -galactosidases I and II. (a) Hydrolysis of *p*-nitrophenyl α -D-galactoside (1.5 mM) measured spectrophotometrically at 405 nm.; (b) hydrolysis of raffinose (5 mM) followed by determination of the liberated galactose as described in Fig. 7. McIlvaine buffer with a pH range of pH 2.5–8 was used.

Fig. 9. Thermal inactivation of α -galactosidases I and II. Enzyme I (50 μ g./ml.) and enzyme II (600 μ g./ml.), both in McIlvaine buffer, pH 4.0, were maintained for 15 min. at the given temperature and suitable samples were withdrawn and assayed spectrophotometrically with *p*-nitrophenyl α -D-galactoside as substrate.

The K_m values determined from the uninhibited linear parts of the curves were 3.8×10^{-4} M and 4.5×10^{-4} M for enzymes I and II respectively. The corresponding V_{max} values were 25.53 and 2.39 μ moles/min./mg. of enzyme protein.

No substrate inhibition occurred with raffinose, and classical Michaelis–Menten kinetics were followed (Fig. 7). The K_m values for enzymes I and II with raffinose were 4×10^{-3} M and 5×10^{-3} M respectively, and the corresponding V_{max} values were 28.4 and 4.18 μ moles/min./mg. of enzyme protein.

Effect of pH on enzyme activity. Fig. 8(a) shows the pH–activity profile of the α -galactosidases with *p*-nitrophenyl α -D-galactoside as substrate. The activity patterns of the enzymes were different when raffinose was used as substrate (Fig. 8b).

Thermal inactivation. The patterns of thermal inactivation of α -galactosidases I and II are shown in Fig. 9. Enzyme I is heat-resistant over a wider range of temperature than is enzyme II, but with the former there was an initial 15% loss of activity at 35°, whereas enzyme II appeared to be very stable within the range 35–45°.

DISCUSSION

This study has produced the first reported highly purified preparations of α -galactosidase. The two forms of α -galactosidase that were prepared from coffee beans by Petek & To Dong (1961) were impure and obtained in low yields; one form possessed both α - and β -galactosidase activities and both forms had identical specificities towards α -galactosides. The two enzymes exhibited a difference in pH optima (5.3 and 6.3) with phenyl α -D-galactoside as substrate and the K_m values with this substrate were also different. Transfer of galactose to sucrose by the enzymes selectively produced raffinose.

The purification procedure used with the broad-bean enzymes eliminated β -galactosidase, α - and β -glucosidase and 'alkaline' invertase in the acidification stage of step 2. The final, apparently homogeneous preparations of α -galactosidases I and II appeared to be chemically distinct on the basis of aromatic amino acid composition and carbohydrate content. The glycoprotein nature of enzyme I could account for the thermal stability of this enzyme in comparison with enzyme II. The presence of a high proportion of carbohydrate in enzyme I could conceivably interfere with the molecular-weight determination by gel filtration. Further differences in the two forms of the enzyme are apparent on inspection of the pH optima and K_m values obtained with *p*-nitrophenyl α -D-galactoside and raffinose as substrates. In addition, kinetic studies with a variety of other substrates indicate different catalytic properties (P. M. Dey & J. B. Pridham, unpublished work). Treatment of enzyme I with urea produces dissociation of the protein, as shown by gel filtration and disc electrophoresis, and hence enzyme I appears to be com-

posed of subunits. The molecular relationship between enzymes I and II, if any, is at present not apparent: the presence of carbohydrate in enzyme I complicates the issue. Enzymes I and II do not appear to be artifacts of the purification procedure, as both fractions are found when gel filtration of a crude buffered extract of beans is carried out (P. M. Dey & J. B. Pridham, unpublished work).

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