Purification and Physical Properties of Sweet-Almond α -Galactosidase

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1. α -Galactosidase from sweet almonds was purified about 2000-fold through eight steps. 2. The enzyme preparation was free from other related enzymes known to occur in sweet almonds, and behaved as a homogeneous protein on filtration through Sephadex G-75. 3. A molecular weight of about 33 000 was determined from the gel-filtration data. 4. The ultraviolet-absorption spectrum and thermal inactivation of the enzyme are described. 5. The purified enzyme hydrolysed *p*-nitrophenyl α -D-galactoside at a much faster rate than melibiose. 6. The pH optimum was at 5.5–5.7. 7. Besides hydrolysis, it also catalysed transfer of galactosyl residues, chain elongation of melibiose and the synthesis of oligosaccharides from galactose.

α -D-Galactoside galactohydrolases (α -galactosidases; EC 3.2.1.22) are very widely distributed in Nature (Wallenfels & Malhotra, 1961) and may be important in the metabolism of various galactose-containing compounds in the animal and plant kingdoms. However, all the studies reported so far have been carried out with only partially purified enzyme preparations (cf. Anagnostopoulos, Courtois & Petek, 1955; Lechevallier, 1962; Ahmed & Cook, 1964) and therefore might present difficulties in interpretation of the results. Sweet almonds are a rich source of several glycosidases (Helferich & Appel, 1932; Helferich, Gootz & Sparmberg, 1932; Helferich & Winkler, 1932; Helferich, Winkler, Gootz, Peters & Günther, 1932; Helferich & Lampert, 1935), including α -galactosidase, which was reportedly obtained by Zechmeister, Toth & Balint (1938) in a form free from accompanying β -glucosidase (EC 3.2.1.21) and chitinase (EC 3.2.1.14). However, their procedure did not work in our hands.

The present work deals mainly with a procedure to obtain an essentially homogeneous preparation of α -galactosidase from sweet almonds. Some of the physical properties of the enzyme are also described. A preliminary report on this work has been given (Malhotra & Dey, 1964).

EXPERIMENTAL

Materials

Seeds of sweet almonds, procured from the market, were crushed before use in a laboratory grinder. All the chemicals

for buffers and other reagents were of A.R. grade and the solvents used were purified in the laboratory. Only double-distilled water from an all-glass (Pyrex) assembly was used. *p*-Nitrophenyl α -D-galactoside (for m.p. see Westphal & Feier, 1956), *p*-nitrophenyl α -D-glucoside and *p*-nitrophenyl β -D-glucoside were prepared by the method of Montgomery, Richtmyer & Hudson (1942), and *o*-nitrophenyl β -D-galactoside was prepared by that of Seidman & Link (1950). Melibiose was obtained from British Drug Houses Ltd. (Poole, Dorset).

Alcohol dehydrogenase (EC 1.1.1.1), lactate dehydrogenase (EC 1.1.1.27), pyruvate kinase (EC 2.7.1.40), trypsin (EC 3.4.4.4) and chymotrypsin (EC 3.4.4.5) were obtained from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, W. Germany), cytochrome *c* (type II) was from Sigma Chemical Co. (St Louis, Mo., U.S.A.) and crystalline bovine serum albumin and CM-cellulose were from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.). Sephadex G-75 (lot no. To5360) was from Pharmacia (Uppsala, Sweden).

Methods

Enzyme assays. The chromogenic substrate *p*-nitrophenyl α -D-galactoside was chosen for general assay of α -galactosidase instead of melibiose (which is one of the commonly available natural substrates) because the former had a higher $V_{\max}$ and lower K_m value (Malhotra & Dey, 1964) and the assay procedure was also easier. The enzyme was assayed by following the initial rate of *p*-nitrophenyl α -D-galactoside hydrolysis. The appropriately diluted enzyme solution (0.2 ml.) in 0.1 M-acetate buffer, pH 5.6, was incubated at 30° with 0.2 ml. of 10 mM-substrate solution in the same buffer for 15 min. The reaction was stopped by adding 5.0 ml. of 0.1 M- Na_2CO_3 and the yellow colour of *p*-nitrophenol thus developed was measured at 405 m μ (light-path 1 cm.) on an Eppendorf photometer. Blank experiments with the inactive enzyme (boiled) were run concurrently and the appropriate correction was applied.

The specified conditions gave a linear relationship between

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product formation and the amount of enzyme or time of incubation provided that the extinction of the final test solution did not exceed 0.9. The pH chosen was the optimum for this enzyme-substrate system (Malhotra & Dey, 1964).

β -Glucosidase (EC 3.2.1.21), α -glucosidase (EC 3.2.1.22) and β -galactosidase (EC 3.2.1.23) activities were determined under the same conditions as described for α -galactosidase, with their corresponding substrates, namely *p*-nitrophenyl β -D-glucoside, *p*-nitrophenyl α -D-glucoside and *o*-nitrophenyl β -D-galactoside respectively.

Invertase was assayed by the method of Hestrin, Feingold & Schramm (1955), trehalase by that of Avigad, Ziv & Neufeld (1965) and α -amylase by the starch-iodine method of Markovitz & Klein (1955).

Unit of enzyme activity. One unit of activity was defined as the amount of enzyme required to hydrolyse 1 μ mole of the substrate/min. under the conditions of quantitative assay described above. This unit, however, proved to be too big, and therefore milliunits were more commonly used. The specific activity has been expressed as enzyme milliunits/mg. of protein.

Protein determination. Protein was estimated as described by Lowry, Rosebrough, Farr & Randall (1951) with the Folin-Ciocalteu reagent, calibrated with crystalline bovine serum albumin.

CM-cellulose chromatography. Washed CM-cellulose (2g.) was dispersed in 0.05M-acetate buffer, pH4.7, thoroughly equilibrated and poured into a glass column (1.5 cm. diam.) plugged with glass wool near the bottom. The column was packed to a height of 15 cm. Elution was done with the same buffer and the flow rate adjusted to about 0.5 ml./min. The column was maintained at 2-4°.

Sephadex-gel filtration. A column (2.4 cm. $\times$ 50 cm.) was prepared from the bead form of Sephadex G-75 according to the method of Andrews (1964) in 0.05M-tris-acetic acid buffer, pH6.0, containing KCl (0.1M). The same buffer was used for elution at room temperature and 4-4.5 ml. fractions were collected with an automatic fraction collector at about 0.5 ml./min. Enzyme activity and protein content were estimated in each fraction. After use the column was washed each time with 300-400 ml. of buffer.

Hydrolysis of melibiose. Substrate and enzyme were

incubated in buffered solutions at 30° for 15 min., the test volume being 0.25 ml. The reaction was stopped by adding 0.1 ml. of 0.3N-trichloroacetic acid and glucose was estimated by the method of Huggett & Nixon (1957) at 436 m μ .

Paper chromatography. Descending paper chromatography was performed with Whatman no. 1 filter paper and butanol-pyridine-water (6:4:3, by vol.) solvent. After drying, the chromatograms were sprayed with aniline-phthalate reagent (0.97 g. of aniline, 1.66 g. of phthalic acid and 5.0 g. of trichloroacetic acid dissolved in 100 ml. of water-saturated butanol) and heated at 120-130° for 3 min.

RESULTS

Purification of α -galactosidase from sweet almonds. The following operations were carried out at 0-4° unless stated otherwise; a summary of the purification procedure for a single batch is given in Table 1.

Step 1: extraction of the enzyme. Crushed almond seeds (500 g.) were dispersed in 300 ml. of 0.1M-acetate buffer, pH5.6, held overnight and the supernatant solution collected by centrifugation. The residue was similarly extracted twice with 200 and 100 ml. more of buffer respectively. All extracts were combined. When the rates of liberation of nitrophenols from different glycosides were compared under similar test conditions, the proportions of α -galactosidase: β -glucosidase: α -glucosidase: β -galactosidase activities were found to be 1:106:10:28.

Step 2: manganese sulphate treatment. The above extract (500 ml.) was treated with 16.7 ml. (i.e. one-thirtieth of the extract volume) of M-manganese sulphate and left to stand for 24 hr. The supernatant solution obtained after centrifugation did not give any further precipitate with Mn²⁺ or protamine (salmine) sulphate, indicating more or less complete removal of nucleic acids. Without this step further purification could not be achieved

Table 1. Purification of α -galactosidase from sweet almonds

The starting material was 500 g. of almond seeds. Recovery was calculated with respect to the preceding step. Overall recovery and purification were 5.81% and 1937-fold respectively.

Step no.	Purification step	Vol. (ml.)	Enzyme activity (milliunits/ml.)	Protein (mg./ml.)	Specific activity (milliunits/mg. of protein)	Recovery (%)
1	Original extract	500	344	45.0	7.64	—
2	MnSO ₄ treatment	488	264	20.0	13.2	75.1
3	Acetone fractionation	18.3	4650	71.0	65.5	65.8
4	Change of pH	50.0	1020	21.6	47.2	59.9
5	(NH ₄) ₂ SO ₄ fractionation	8.0	5420	15.7	345.2	85.0
6	First CM-cellulose chromatography and concentration	2.5	11 500	4.16	2764	66.3
7	Second CM-cellulose chromatography and concentration	1.1	16 300	1.28	12 730	62.3
8	Sephadex G-75 gel filtration and concentration	1.2	8330	0.564	14 800	55.7

so efficiently. The proportions of the different enzyme activities did not change in this step.

Step 3: acetone fractionation. The above solution (cooled to -10°) was treated with 0.5 vol. of acetone (-15°) with constant stirring (final acetone concn. 33%, v/v). The mixture was kept at -15° for 45 min. and centrifuged, and the bulky residue rejected. The acetone concentration in the supernatant solution was raised to 66% (v/v) by the addition of more precooled acetone and kept at -15° for 10 min. The precipitate thus formed was centrifuged down and suspended in 15 ml. of 0.1 M-acetate buffer, pH 5.6. The pH of the suspension, which was nearly 7, was adjusted to 5.6 with 0.1 N-acetic acid. On centrifugation it gave a clear enzymically active supernatant (18.3 ml.). At this stage β -glucosidase, α -glucosidase and β -galactosidase activities were respectively 48-, 4.5- and 13-fold the α -galactosidase activity.

Step 4: change of pH. The pH of the above solution was brought to nearly 2.0 by the gradual addition of precooled (-5°) 2 M-citric acid (18.3 ml., i.e. equal in volume to the enzyme solution, was required). The solution was held at this pH for 24 hr. and centrifuged and the supernatant solution adjusted to pH 4.7 by the dropwise addition of N-sodium hydroxide (25 ml.) at -5° with vigorous stirring. It was centrifuged after another 24 hr. The supernatant (50 ml.) showed an α -galactosidase: β -glucosidase activity ratio 1:0.0015; the other two activities could not be detected.

Step 5: ammonium sulphate fractionation. The above enzyme solution (50 ml., containing 1.08 g. of protein) was brought to 23% saturation with ammonium sulphate by slow addition of 6.5 g. of the salt and the stirring continued for 2 hr. The precipitate that formed was discarded, and the ammonium sulphate concentration in the supernatant solution (55 ml.) raised to 45% saturation by the further addition of 10 g. of the salt and stirring for 2 hr. The precipitate thus formed was collected by centrifugation, dissolved in 0.05 M-acetate buffer, pH 4.7, and dialysed against the same buffer to get an NH_4^+ -free enzyme solution (8 ml.). There was no change in the proportions of different enzyme activities.

Step 6: first CM-cellulose chromatography. The above solution was chromatographed on a 1.5 cm. $\times$ 15 cm. column (see the Methods section) that was thoroughly equilibrated with 0.05 M-acetate buffer, pH 4.7. Enzymically active fractions were pooled together (53 ml.) and concentrated by precipitating with ammonium sulphate (70% saturation) and dissolving the residue in a small volume of the 0.05 M-acetate buffer, pH 4.7. The solution was dialysed against several changes of the same buffer to get an NH_4^+ -free enzyme solution (2.5 ml.).

Step 7: second CM-cellulose chromatography.

The above solution was filtered through a fresh CM-cellulose column (1.5 cm. $\times$ 10 cm.) and subjected to the rest of the procedure of step 6, and finally an NH_4^+ -free enzyme solution (1.1 ml.) was obtained.

Step 8: Sephadex-gel filtration. On gel filtration of the above solution through a Sephadex G-75 column (see the Methods section), some fractions showed a higher specific activity than that of the material applied. The enzyme activity was eluted in a single and symmetrical peak. The fractions eluted from eluate volume 140.5–164 ml. showed 10–20% higher specific activity and were pooled together (23.5 ml.). The entire protein in it was precipitated by 75% saturation with ammonium sulphate, dissolved in a small volume of the gel-filtration buffer and dialysed to give an NH_4^+ -free enzyme solution (1.2 ml.) that had no trace of β -glucosidase activity.

Homogeneity of the enzyme preparation. The purified enzyme sample as obtained above was devoid of other related enzyme activities, namely β -glucosidase, β -galactosidase, α -glucosidase, invertase (EC 3.2.1.26), trehalase (EC 3.2.1.28) and α -amylase (EC 3.2.1.1). When the enzyme solution was subjected to another gel filtration through the same Sephadex column, the enzyme activity and the protein were eluted in a single and almost symmetrical peak, parallel to each other (Fig. 1). The

Fig. 1. Gel filtration of sweet-almond α -galactosidase through a Sephadex G-75 column. Enzyme (2 mg.; specific activity 14.8×10^3 milliunits/mg. of protein) was applied on the top of the column by layering beneath the buffer layer and eluted with 0.05 M-tris-acetic acid buffer, pH 6.0, containing KCl (0.1 M), at room temperature. Enzyme activity (O) and protein (Δ) in individual fractions are plotted against volume of eluate. V_e , Elution volume.

individual fractions had almost the same specific activity ($\pm 3.8\%$) as that of the solution applied. The eluate volume of the peak active fraction, termed the 'elution volume', was also the same in both cases.

Determination of molecular weight from gel-filtration data. The Sephadex column was calibrated with several proteins of known molecular weights and elution volumes were determined for each. The results were expressed in terms of the ratio:

$$\frac{\text{Elution volume of bovine serum albumin}}{\text{Elution volume of the protein}}$$

(R_{SA}) rather than as elution volumes. The term R_{SA} was introduced by Andrews (1964) and has been shown to be superior to elution volume for calibration purposes (Malhotra & Philip, 1966). A plot of R_{SA} versus $\log$ (molecular weight) is shown in Fig. 2. For R_{SA} values 0.627–1.071, corresponding to molecular weight 12400–88000, the plot was linear. The R_{SA} of sweet-almond α -galactosidase (0.848) (broken horizontal line in Fig. 2) lay within the linear portion of the curve and corresponded to a molecular weight of nearly 33000.

Ultraviolet-absorption spectrum. The absorption maximum of the purified enzyme sample was at 276 m μ . The extinction coefficients at 276 and 280 m μ were 2.55 and 2.43 cm.²/mg. of protein respectively and the E_{280}/E_{276} ratio was 1.25.

Thermal inactivation. Sweet-almond α -galactosidase is a fairly stable enzyme, and its solution (1.0 mg./ml.) at pH 5.6 lost no activity even after storage for 3 months at 4°. The kinetics of its

thermal inactivation were studied at 50°, 55°, 60°, 62° and 65° (Fig. 3). At all the temperatures first-order kinetics were obeyed and the inactivation was irreversible. An Arrhenius plot of the data gave a straight line (Fig. 4), the slope of which corresponded to an energy of activation of 69.4 kcal./mole of active site for the thermal inactivation of the enzyme. Such high energies of activation are generally observed for thermal denaturation of proteins.

Fig. 3. Thermal inactivation of sweet-almond α -galactosidase. Enzyme of specific activity 12.71×10^3 milliunits/mg. of protein was taken in 0.05 M-phosphate buffer, pH 6.0 (10 μ g./ml.), and maintained at the following temperatures: $\square$, 50°; $\blacktriangle$, 55°; $\triangle$, 60°; $\bullet$, 62°; $\circ$, 65°. Samples were withdrawn at different time-intervals, chilled and tested as usual with *p*-nitrophenyl α -D-galactoside.

Fig. 2. Calibration of Sephadex G-75 column and determination of the molecular weight of sweet-almond α -galactosidase. Procedure and conditions were as given in Fig. 1. Points refer to cytochrome *c* (1), trypsin (2), α -chymotrypsin (3), bovine serum albumin (4), horse-liver alcohol dehydrogenase (5), lactate dehydrogenase (6) and pyruvate kinase (7). The broken horizontal line indicates the position of sweet-almond α -galactosidase.

Fig. 4. Arrhenius plot for thermal inactivation of sweet-almond α -galactosidase (data calculated from Fig. 3).

Fig. 5. Influence of substrate concentration on the enzymic hydrolysis of melibiose (○) and *p*-nitrophenyl α-D-galactoside (Δ) in citrate-phosphate buffer, pH 5.5, at 30°. Hydrolyses of nitrophenyl galactoside (0.5 μg. of enzyme/0.4 ml. test volume) and of melibiose (3.0 μg. of enzyme/0.25 ml. test volume) were followed as described in the Methods section. Velocities, *v*, are expressed as extinctions at the appropriate wavelengths.

Influence of substrate concentration. Rates of enzymic hydrolysis of melibiose and *p*-nitrophenyl α-D-galactoside at different substrate concentrations are shown in Fig. 5. Whereas simple Michaelis-Menten-type kinetics were observed with melibiose, inhibition by excess of substrate was observed with *p*-nitrophenyl α-D-galactoside. The K_m values for melibiose and *p*-nitrophenyl α-D-galactoside (calculated from the data at low substrate concentrations) in citrate-phosphate buffer, pH 5.5, at 30° were $2.4 \times 10^{-3} \text{M}$ and $7.6 \times 10^{-4} \text{M}$ respectively. The corresponding V_{max} values were 2.0 and 35.0 μmoles of substrate hydrolysed/min./mg. of enzyme. Similar results were obtained in 0.1 M-acetate buffer of the same pH. The values were $2.44 \times 10^{-3} \text{M}$ and $5.26 \times 10^{-4} \text{M}$ for K_m and 1.61 and 27.0 μmoles/min./mg. of enzyme for V_{max} of melibiose and *p*-nitrophenyl α-D-galactoside respectively.

Influence of pH. At 30°, α-galactosidase of sweet almonds showed a pH optimum at 5.5–5.7 (Fig. 6) with both the substrates in citrate-phosphate buffers.

Galactose-transfer activity. When glucose (1.0M) and *p*-nitrophenyl α-D-galactoside (0.05M) were incubated with the enzyme (0.16mg./ml.) in 0.1 M-acetate buffer, pH 5.5, at 30°, a disaccharide appeared in the reaction mixture. On paper chromatography in butanol-pyridine-water solvent it had R_{Glc} 0.38, which is similar to that of melibiose (0.38). When melibiose (1.0M) alone was incubated

Fig. 6. Influence of pH on the enzymic hydrolysis of melibiose (2.5 mM) (○) and *p*-nitrophenyl α-D-galactoside (1.5 mM) (Δ) in citrate-phosphate buffers at 30°. Velocities, *v*, are expressed as μmoles of substrate hydrolysed/min./mg. of enzyme.

with the enzyme (0.16mg./ml.), three new products (other than glucose and galactose) appeared on the chromatogram with R_{Glc} values 0.15, 0.19 and 0.24. Similarly, incubation of galactose (1.0M) with the enzyme (0.16mg./ml.) yielded two higher sugars (R_{Glc} values 0.30 and 0.37). In each case the test mixtures were examined at different time-intervals. The concentration of the products appeared to increase with time, pass through a maximum and then decrease as the incubation was prolonged.

DISCUSSION

Purification of sweet-almond α-galactosidase was mainly directed to remove the contaminating related enzyme activities. Several known methods for the removal of β-glucosidase, namely chromatography over bauxite (Zechmeister *et al.* 1938) and silver hydroxide treatment (Helferich & Winkler, 1932), failed; the failure of the latter method was perhaps due to the inactivation of α-galactosidase itself by Ag^+ (Malhotra & Dey, 1964). Success was finally achieved by lowering the pH of the enzyme solution (cf. Tauber, 1932), which almost completely inactivated the contaminating activities but destroyed only about 40% of the α-galactosidase activity and thus lowered the specific activity. This step also facilitated further purification of the enzyme. A single peak of enzymic activity was eventually obtained on Sephadex-gel filtration that coincided with the protein peak (Fig. 1). This indicated the presence of a single α-galactosidase in the preparation, unlike a coffee preparation (Petek & Dong, 1961) and *Plantago ovato* enzyme prepara-

tion (Courtois, Petek & Dong, 1963) in which two α -galactosidases have been reported. It is noteworthy that thermal denaturation of α -galactosidase activity followed first-order kinetics at all temperatures and the rate constants obeyed Arrhenius law, again indicative of a single catalytically active protein component.

The molecular weight of the enzyme as estimated from the gel-filtration data showed that it is a rather small protein. This method for determination of molecular weights has been used for several enzymes by earlier workers (Andrews, 1964; Auricchio & Bruni, 1964; Burges, Brammer & Coombes, 1965; Malhotra & Philip, 1966). Andrews (1964) showed that the values obtained by this method were within $\pm 10\%$ of those obtained by other methods.

The purified enzyme hydrolysed the natural substrate as well as an aryl galactoside. The latter was, however, a better substrate. The nature of the buffer seems to have a minor effect only on K_m and V_{max} values. Besides hydrolysis, the enzyme also catalysed the transfer of galactosyl residues and the formation of higher sugars from melibiose and galactose when these were incubated individually with the enzyme.

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