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PURIFICATION OF COFFEE BEAN α-GALACTOSIDASE BY AFFINITY CHROMATOGRAPHY

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SUMMARY

The preparation and properties of a specific adsorbent for the rapid and complete purification of α-D-galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22) from green coffee beans by affinity chromatography is described. The adsorbent consists of *N*-ε-aminocaproyl-*N*-ε-aminocaproyl-α-D-galactopyranosylamine coupled to Sepharose. α-Galactosidase was adsorbed from a partially purified preparation obtained from a coffee bean extract, and eluted with either D-galactose or *p*-nitrophenyl α-D-galactopyranoside. The specific activity of the purified enzyme, tested with *p*-nitrophenyl α-D-galactopyranoside as substrate, was 94.5 units/mg protein, representing an 8600-fold purification of the original crude extract. Polyacrylamide gel electrophoresis at pH 8.3 revealed three protein bands, all of which possessed enzymic activity.

INTRODUCTION

Procedures for the partial purification of coffee bean α-galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22) involving alumina gel chromatography [1] and Sephadex gel filtration [2] have been described, and the catalytic properties of the crude preparations studied [3–8]. It is one of the few known α-galactosidases capable of catalyzing the hydrolysis of the non-reducing terminal α-D-galactopyranosyl residues of blood group B antigens [8]. As part of our studies on the structure of these antigens in human erythrocytes (ref. 9 and manuscript in preparation) we have synthesized a specific adsorbent for the convenient purification of the enzyme by affinity chromatography in high yield.

The procedure used was similar to that recently developed for the binding of β-L-fucopyranosylamine [10], of β-D-galactopyranosylamine [11], and of 2-acetamido-2-deoxy-β-D-glucopyranosylamine [12] to Sepharose. *N*-ε-Aminocaproyl-*N*-ε-aminocaproyl-α-D-galactopyranosylamine was prepared by sequential reaction of α-D-galactopyranosylamine [13] with two molecules of *N*-benzyloxycarbonyl-ε-aminocaproic acid [14], the *N*-benzyloxycarbonyl groups being removed at each step by hydrogenolysis. The product was coupled to Sepharose by the CNBr procedure [15]. α-Galactosidase was adsorbed on a column of the Sepharose conjugate from a par-

tially purified preparation, obtained from an extract of green coffee beans, and was eluted with either D-galactose or *p*-nitrophenyl α-D-galactopyranoside.

While this manuscript was in preparation the purification of other α-galactosidases by the principle of affinity chromatography was reported by Mapes and Sweeley [16], who adsorbed the enzymes from human sera on a column of *p*-aminophenyl melibioside conjugated with Sepharose. Elution of the α-galactosidases required the use of a non-ionic detergent, resulting in the separation of several enzyme species.

MATERIALS AND METHODS

Green "Santos" coffee beans were purchased locally. D-Galactose was obtained from B.D.H., $(NH_4)_2SO_4$ from Frutarom, Sepharose 4B from Pharmacia, CNBr from Fluka, *p*-nitrophenyl α-D-galactopyranoside from Koch–Light, bovine serum albumin from Sigma, and Coomassie Brilliant Blue G-250 from Serva. All other reagents were commercial preparations of the highest purity available.

Melting points were determined with a Fischer–Johns apparatus. Rotations were measured with a Perkin–Elmer No. 141 polarimeter, using 1-dm semimicro tubes. Infrared spectra were obtained with a Perkin–Elmer Model 237 spectrophotometer, and NMR spectra with a Varian T-60 spectrometer in deuterated dimethylsulfoxide with tetramethylsilane as internal standard. Evaporations were performed in vacuo in a rotatory evaporator with the bath temperature below 50 °C. Homogeneity of compounds was tested by thin-layer chromatography on microslides coated with silica gel G (Merck), and on precoated cellulose plates (Reidel–de Häen) and spots were detected by charring with H_2SO_4 or by spraying with ninhydrin. Microanalyses were performed by the Microanalytical Laboratory of the Weizmann Institute of Science.

To estimate the concentration of ligand in solution, aliquots were applied to the short column of a Beckman–Spinco amino acid analyzer, eluted at pH 5.25, 50 °C, and reacted with ninhydrin. The color yield was calibrated with known amounts of the compound.

α-Galactosidase was assayed by addition of 50 μl of 5 mM *p*-nitrophenyl α-D-galactopyranoside to a solution containing 50 μl of McIlvaine phosphate–citrate buffer, pH 6.0, 50 μl of bovine serum albumin (1 mg/ml), and 50 μl of enzyme solution. Incubation was at 37 °C for appropriate time intervals (usually 2–10 min). The reaction was terminated by addition of 0.8 ml of 0.5 M Na_2CO_3, and the absorbance at 405 nm measured spectrophotometrically. A control blank was prepared by incubation of the reaction mixture in the presence of Na_2CO_3. Under these conditions, the assay was linear up to an absorbance of about 1.2. One unit of α-galactosidase is defined as the amount of enzyme catalyzing the hydrolysis of 1 μmole of *p*-nitrophenyl α-D-galactopyranoside per min under the conditions used. The molar extinction coefficient of *p*-nitrophenol (ε = 18 300) was determined by measuring the absorbance of a solution of known concentration in 0.5 M Na_2CO_3. Protein was determined according to Lowry et al. [17], using bovine serum albumin as standard.

Electrophoresis on 6% polyacrylamide gels at pH 8.3 was performed as described by Maizel [18]. Proteins were stained with Coomassie Brilliant Blue G-250 according to Diezel et al. [19]. Enzymically active bands were located by slicing the unfixed polyacrylamide gel into slices 2-mm thick and immersing them in 0.5 ml of 12.5 mM *p*-nitrophenyl α-D-galactopyranoside in 0.5 M phosphate–citrate buffer, pH

6.0, for 2 h. Upon the addition of 2 ml of 0.5 M Na_2CO_3 followed by incubation at 4 °C for 0.5 h, the absorbance of the supernatant at 405 nm was measured.

RESULTS

Synthesis of the specific adsorbent

*N-(N-benzyloxycarbonyl-ε-aminocaproyl)-α-*D*-galactopyranosylamine (Compound 1).* Isobutyl chloroformate (3.9 ml, 30 mmoles) and triethylamine (4.1 ml, 30 mmoles) were added simultaneously to a solution of *N*-benzyloxycarbonyl-ε-aminocaproic acid [14] (7.95 g, 30 mmoles) in dry dimethylformamide (50 ml), kept at −5 °C in an ice-salt bath. The mixture was stirred for 20 min at −5 °C, filtered and the filtrate added to a stirred suspension of α-D-galactopyranosylamine–ammonia complex, prepared according to Frush and Isbell [13] (2.45 g, 12.5 mmoles; $[\alpha]_D^{23}$, 138°; m.p. 107–109 °C) in dry dimethylformamide (50 ml) at room temperature. Stirring was continued until the solution became clear (0.5 h), and the solution was kept at room temperature for 16 h. After evaporation to dryness, the solid residue was dissolved in a minimal volume of chloroform–methanol–water (30:10:1, by vol.) and chromatographed on a column of 800 g of silica gel (Merck, 0.05–0.2 mm, 70–235 mesh) pre-equilibrated with the same solvent. Aliquots of the eluate were analyzed by thin-layer chromatography on silica gel G using the same solvent. Impurities were eluted first (mainly *N*-benzyloxycarbonyl-ε-aminocaproylamide, R_F 0.8, and traces of an *O*-acylated sugar derivative, R_F 0.6), followed by Compound 1, R_F 0.5. The peak containing the product was evaporated in vacuo to give a syrup which could not be crystallized. A single spot was also obtained upon thin-layer chromatography on silica gel G developed with acetone–methanol (9:1, v/v, R_F 0.44) and ethyl acetate–methanol (5:1, v/v, R_F 0.58). Yield, 4.85 g (91%); $[\alpha]_D^{23}$, 88.8° (*c*, 2.7, methanol); infrared data: $\nu_{max}^{chloroform}$ 1520 cm^{-1} (peptide amide 1), 1655 (CONH), 1700 (benzyloxycarbonyl C=O); NMR data: τ 2.65 (5 H, phenyl), 3.55 (*d*, 1 H, NH) 4.40 (*d*, 1 H, $J_{1,2}$ 2.5, anomeric proton), 4.90 (2 H, benzyloxycarbonyl CH_2). Found: C, 55.12; H, 7.55; N, 6.60. $C_{20}H_{30}O_8N_2 \cdot 1/2\ H_2O$ requires: C, 55.16; H, 7.17; N, 6.43.

*N-ε-Aminocaproyl-α-*D*-galactopyranosylamine (Compound 2).* Compound 1 (2.72 g, 6.36 mmoles) in 80% aqueous methanol (100 ml) was hydrogenolyzed over 10% palladium-on-charcoal (100 mg) for 8 h at atmospheric pressure. After filtration, the solution was evaporated in vacuo, and Compound 2 was isolated as a syrup. It gave a single spot on thin-layer chromatography on silica gel G developed with isopropanol–acetic acid–water (3:1:1, by vol., R_F 0.4), and on cellulose developed with ethyl acetate–pyridine–water (8:3:1, by vol., developed three times, R_F 0.15). Yield, 1.84 g (99%); $[\alpha]_D^{23}$, 127.8° (*c*, 2.5, methanol); infrared data: ν_{max}^{KBr} 1520 cm^{-1} (peptide amide 1), 1655 (CONH). Found: C, 48.12; H, 8.64; N, 9.55. $C_{12}H_{24}O_6N_2 \cdot 1/2\ H_2O$ requires: C, 47.99; H, 8.39; N, 9.33.

*N-(N-benzyloxycarbonyl-ε-aminocaproyl-N-ε-aminocaproyl)-α-*D*-Galactopyranosylamine (Compound 3).* Reaction of Compound 2 (292 mg, 1.0 mmole) with *N*-benzyloxycarbonyl-ε-aminocaproic acid (318 mg, 1.2 mmoles) followed by chromatographic purification on a column of silica gel (20 g) as described for Compound 1, gave Compound 3, which was isolated after evaporation in vacuo as a syrup. Thin-layer chromatography gave a single spot on silica gel G developed with chloroform–methanol–water (30:10:1, by vol., R_F 0.50) and with acetone–methanol (9:1, v/v, R_F

0.66). Yield, 528 mg (95%); $[\alpha]_D^{23}$ 63.1° (*c*, 3.0, methanol); infrared data: $\nu_{max}^{chloroform}$ 1520 cm^{-1} (amide), 1655 (CONH), 1700 (benzyloxycarbonyl C=O); NMR data: τ 2.65 (5 H, phenyl), 4.40 (*d*, 1 H, anomeric proton, $J_{1,2}$ 2.5), 4.90 (2 H, benzyloxycarbonyl CH_2). Found: C, 56.87; H, 7.94; N, 7.60. $C_{26}H_{41}O_9N_3 \cdot 1/2\ H_2O$ requires: C, 56.92; H, 7.72; N, 7.66.

N-ε-Aminocaproyl-N-ε-aminocaproyl-α-D-galactopyranosylamine (Compound 4). A solution of Compound 3 (485 mg, 0.9 mmole) was hydrogenated as described for Compound 2. Isolation of Compound 4, carried out as described for 2, afforded a syrup, which gave a single spot on thin-layer chromatography on silica gel G developed with isopropanol–acetic acid–water (3:1:1, by vol., R_F 0.4) and on cellulose developed with ethyl acetate–pyridine–water (8:3:1, by vol., developed three times, R_F 0.3). Yield, 345 mg (95%); $[\alpha_D^{23}]$ 73.1° (*c*, 1.8 methanol); infrared data: ν_{max}^{KBr} 1520 cm^{-1} (amide), 1655 (CONH). Found: C, 48.49; H, 8.58; N, 9.46. $C_{18}H_{35}O_7N_3 \cdot 2\ H_2O$ requires: C, 48.99; H, 8.90; N, 9.52.

Compounds 2 and 4 behaved as competitive inhibitors of α-galactosidase as determined by Lineweaver–Burk plots with 1.25 mM *p*-nitrophenyl α-D-galactopyranoside as substrate. K_i values for both compounds were about $3 \cdot 10^{-4}$ M at pH 4.5, and $9 \cdot 10^{-4}$ M at pH 6.0. Overnight incubation of these compounds (5 mM) in the presence of purified α-galactosidase (2.5 units/ml) at 37 °C, pH 4.5 and 6.0, showed that less than 1% of the compound was hydrolyzed, as judged by the absence of galactose upon analysis of aliquots of the incubation mixtures by descending paper chromatography (ethyl acetate–pyridine–water (8:3:1, by vol.)) and staining with $AgNO_3$. Under the same conditions *p*-nitrophenyl α-D-galactopyranoside was completely hydrolyzed within several minutes.

Coupling of the ligand to Sepharose

Sepharose 4B (10 ml), activated with CNBr (1 g) [15], was suspended in a stirred solution of Compound 4 (approx. 120 mg, 0.3 mmole) in 0.1 M $B(ONa)_3$–NaOH buffer, pH 9.5 (10 ml), and stirring was continued overnight at 4 °C. After thorough washing of the Sepharose conjugate with distilled water on a sintered-glass funnel, it was resuspended in 5 vol. of 0.1 M ethanolamine and stirred for 3 h at room temperature to block any residual active groups, then filtered and washed again until the washings were neutral. The Sepharose conjugate was resuspended in McIlvaine phosphate–citrate buffer, pH 4.5, at roomtemperature, and poured into a small column (15 cm × 5.5 cm) fitted with a sintered-glass bottom.

The amount of ligand bound to Sepharose was estimated by determining the amount of ligand in solution before the addition of activated Sepharose and in the combined washings after the coupling step. Typical results gave 13–15 μmoles/ml Sepharose.

In earlier experiments it was found that *N*-ε-aminocaproyl-α-D-galactopyranosylamine–Sepharose conjugate was a satisfactory adsorbent at relatively high ligand concentrations (13–15 μmoles/ml Sepharose), but less satisfactory at intermediate concentrations (9 μmoles/ml Sepharose). However, *N*-ε-aminocaproyl-*N*-ε-aminocaproyl-α-D-galactopyranosylamine proved effective even at 7 μmoles/ml Sepharose.

Purification of α-galactosidase

Preliminary fractionation. Green "Santos" coffee beans were ground, extracted

with benzene, dried, and 100 g of the resulting meal extracted overnight with 500 ml of normal saline at 4 °C, as described [1]. The suspension was centrifuged at 25 000 × *g* for 0.5 h at 4 °C, and the supernatant fluid brought to 35% $(NH_4)_2SO_4$ saturation at room temperature by dropwise addition of a saturated $(NH_4)_2SO_4$ solution with rapid stirring. Following removal of the precipitate by centrifugation at 15 000 × *g* for 15 min at room temperature, the supernatant fluid was adjusted to 50% $(NH_4)_2SO_4$ saturation in a similar manner. The precipitate was isolated by centrifugation as before, suspended in approx. 45 ml of distilled water, and chilled at 4 °C for 1 h. The pH was adjusted to 4.5 by dropwise addition of cold citric acid (0.1 M) and the resultant precipitate removed by centrifugation at 30 000 × *g* for 0.5 h at 4 °C. The supernatant fluid (approx. 50 ml, 14 mg/ml protein; see Table I) was used directly for affinity chromatography.

Affinity chromatography. The solution of crude enzyme obtained as above was applied to the affinity column at room temperature. The column was washed extensively with McIlvaine phosphate–citrate buffer, pH 4.5, until no more protein was eluted. A 50 mM solution of *p*-nitrophenyl α-D-galactopyranoside in the same buffer (3 ml) was then applied and washing continued as before. A sharp, yellow band, due to enzymically liberated *p*-nitrophenolate, descended through the column, indicating the location of the α-galactosidase. The yellow fractions were pooled and freed from low molecular weight materials by rapid passage through a column (3.5 cm × 60 cm) of Sephadex G-25 equilibrated with distilled water. Enzymically active fractions, eluted at void volume, were pooled.

D-Galactose (0.1 M, 3 ml) could substitute for *p*-nitrophenyl α-D-galactopyranoside in the elution step; the eluted protein was located by measuring the absorbance at 280 nm (Fig. 1).

Elution could also be effected by extensive washing (approx. 120 ml) with phosphate–citrate buffer, pH 6.0. Two separate enzyme peaks were eluted, the nature of which is under investigation. No enzyme was eluted using *p*-nitrophenyl β-D-galactopyranoside (50 mM) as eluent at pH 4.5.

Purity of α-galactosidase

The purified enzyme preparation showed no activity towards the *p*-nitrophenyl glycopyranosides of β-D-galactose, α- and β-D-glucose, α-L-fucose, α-D-mannose, *N*-acetyl-β-D-glucosamine and *N*-acetyl-β-D-galactosamine, when incubated at pH 5.0 and 6.0 for 2 h with these compounds under conditions similar to the assay conditions described previously. α-Galactosidase concentration in the solutions tested was 2.5 units/ml, and McIlvaine buffers of pH 5.0 and 6.0 were used.

Polyacrylamide gel electrophoresis of the purified enzyme showed three protein bands of about equal intensity (Fig. 2A). Slight "tailing" of the fastest-moving band was observed. All three species were enzymically active in parallel unfixed gels, and were identical in their migration rates with three enzymically active bands found upon polyacrylamide gel electrophoresis of the original crude extract (Fig. 2B).

Stability of α-galactosidase

Purified α-galactosidase stored in solution at 4 °C in polyethylene containers was stable for at least several weeks under these conditions. Several drops of toluene were added to prevent bacterial contamination. Storage in glass resulted in rapid

TABLE I

PURIFICATION OF COFFEE BEAN α-GALACTOSIDASE BY AFFINITY CHROMATOGRAPHY

Procedure	Volume (ml)	Enzyme concn (U/ml)*	Total enzyme (U)	Protein concn (mg/ml)**	Total protein (mg)	Specific activity (U/mg)*	Total yield (%)	Step yield (%)	Total purification	Step purification
Crude extract	315	1.14	360	33	1040	0.011	100	100	1	1
35–50% $(NH_4)_2SO_4$	45.5	7.00	318	55	251	0.117	88	88	10.5	10.5
Supernatant from acid (pH 4.5) precipitation	52.4	5.59	293	14	73	0.40	81	92	36.2	3.1
Affinity chromatography	26.8	10.4	279	0.11	2.95	94.5	78	95	8 600	237

* 1 unit (U) = 1 μmole/min *p*-nitrophenol liberated in standard assay (see text).

** Protein measured by method of Lowry et al. [17], using bovine serum albumin as standard.

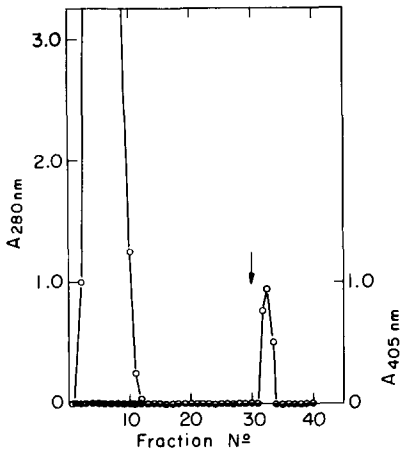

Fig. 1. Purification of α-galactosidase on a column of *N*-ε-aminocaproyl-*N*-ε-aminocaproyl-α-D-galactopyranosylamine–Sepharose conjugate. After sample application, the column was washed with McIlvaine phosphate–citrate buffer, pH 4.5, and the enzyme was eluted with 3 ml of 0.1 M D-galactose in the same buffer (arrow) followed by continued washing as before. In each fraction (volume, 5 ml), protein was monitored by absorbance at 280 nm (○—○). α-Galactosidase activity was assayed with *p*-nitrophenyl α-D-galactopyranoside by absorbance at 405 nm (●—●). Fractions 1–31 were assayed directly. The fractions of the second protein peak (Fractions 32–34) were pooled and assayed for α-galactosidase activity after removal of D-galactose by gel filtration on Sephadex G-25. This peak contained 95% of the applied enzyme activity (specific activity 94.5 units/mg, see Table I).

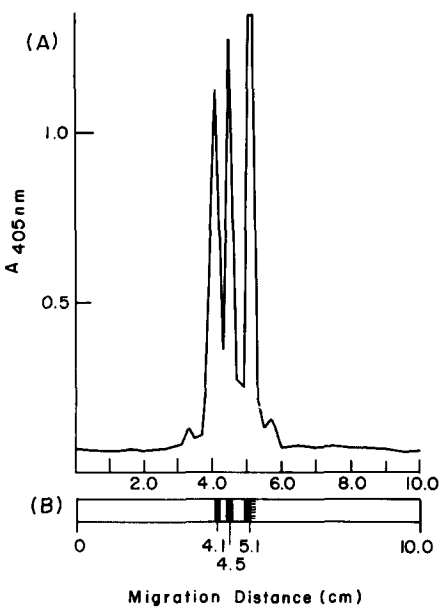

Fig. 2. Gel electrophoresis of α-galactosidase. (A) Electrophoresis of 250 μg of protein from a crude coffee bean extract was performed on 6% polyacrylamide gel at pH 8.3. The unfixed gel was cut into slices 2-mm thick and assayed for α-galactosidase activity (absorbance at 405 nm) by immersion in 12.5 mM *p*-nitrophenyl α-D-galactopyranoside as described in text. (B) Electrophoresis of 80 μg of purified α-galactosidase was performed under the same conditions. The gel was fixed and stained for protein with Coomassie Brilliant Blue G-250 according to Diezel et al. [19].

inactivation of enzymic activity (60% overnight) possibly because of ions leached from the glass surface; the addition of 250 μg of bovine serum albumin per ml of solution prevented inactivation, and could restore about 50% of activity lost prior to its addition. Lyophilization also required the presence of bovine serum albumin (1 mg/ml) which could be removed from the solution of the enzyme by passage through a column (2.5 cm $\times$ 30 cm) of Sephadex G-50.

DISCUSSION

Insolubilized derivatives of 1-amino sugars have thus far been successfully employed in the affinity chromatography of lectins [10–12]. Using *N*-ε-aminocaproyl-*N*-ε-aminocaproyl-α-D-galactopyranosylamine–Sepharose conjugate we have extended this approach to the purification of α-galactosidase from coffee beans.

The synthesis of this affinity adsorbent has been described. While the intermediate compounds were not crystallizable and were hygroscopic, their homogeneity and identity were verified by thin-layer chromatography, NMR and infrared spectroscopy and elemental analysis. Sepharose conjugates of the intermediate *N*-ε-aminocaproyl-α-D-galactopyranosylamine also provided satisfactory absorbents when the extent of substitution on the Sepharose was high, but were less effective than the longer-chain ligand at lower degrees of substitution, an effect which has been noted previously with other affinity adsorbents [20].

Evidence for the specificity of the adsorption of the enzyme to, and elution from, the affinity adsorbent has been presented. Its specific activity was more than two orders magnitude higher than that of previously described preparations [1], and was similar to those of other α-galactosidases purified to apparent homogeneity [21–24], although strict comparison is not possible because of the different assay conditions.

Polyacrylamide gel electrophoresis of both the original crude coffee meal extract and the preparation obtained by affinity chromatography (Fig. 2) revealed three α-galactosidase species. The separation of two α-galactosidase fractions from coffee beans has been reported previously [1]. These species apparently have similar molecular weights, being inseparable upon gel filtration [2]. Multimolecular forms of α-galactosidase have been reported in a variety of other sources (for review see ref. 25).

The large quantities of coffee bean α-galactosidase which can be isolated by the method we have described in this paper provide a convenient source of enzyme for studies where the pure enzyme is required. Further studies are in progress on the physical, chemical and enzymic properties of the multiple enzyme forms, as well as the ability of the enzyme to convert intact erythrocytes from type B to type O.

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