Further Purification and Characterization of the Acid α -Glucosidase

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1. Centrifugation of rat liver acid glucosidase, which had been purified by adsorption on dextran gel, on a density gradient of sucrose showed the enzyme to be impure. 2. Preliminary purification of the enzyme before the gel filtration improved the final degree of purity of this preparation. Disc gel electrophoresis of this preparation showed a single band of protein. 3. The sedimentation coefficient and the molecular weight determined on a sucrose gradient were 4.9–5.1s and 76000–83000 respectively for the rat liver enzyme, and 5.6s and 97000 for the acid α -glucosidase purified by means of the same procedure from the human kidney. 4. The Michaelis constants of rat liver and human kidney enzyme were $4.7 \times 10^{-3}M$ and $13.6 \times 10^{-3}M$ respectively with maltose as substrate. 5. The enzyme from both tissues was inhibited by tris and by erythritol. The inhibition of the rat liver acid glucosidase by erythritol was competitive.

There is a lack of acid α -(1 $\rightarrow$ 4)-glucosidase in tissues of children affected by glycogenosis type II (Hers, 1963). Nevertheless it is not clear whether the absence of this enzyme could by itself explain the pathogenic character of this type of glycogen storage disease (Brown & Zellweger, 1966; Cardiff, 1966), especially since the physiological role of this enzyme, which is already active in the 6-month-old human foetus (Bruni, Auricchio, Sica & Liguori, 1967), is still rather obscure. These facts have prompted many investigations on this enzyme (Baudhuin, Hers & Loeb, 1964; Torres & Olavarría, 1964; Brown & Brown, 1965; Auricchio & Sica, 1967; Auricchio, Bruni, Sica & Parisi, 1967).

As previously reported, an acid glucosidase from rat liver and human kidney was purified by means of dextran-gel filtration (Auricchio & Bruni, 1967). The enzymes from both tissues were similar and on the basis of their inhibition by turanose and the pH-dependence of their activities it was concluded that they were lysosomal acid α -(1 $\rightarrow$ 4)-glucosidases. Enzyme purified in this way, and also by other methods (Hers, 1963; Rosenfeld, 1964; Torres & Olavarría, 1964), is able to hydrolyse both maltose and glycogen, whereas the neutral α -glucosidase from human liver cannot hydrolyse glycogen (Brown & Brown, 1965).

The present paper deals with the further purification and characterization of this enzyme from rat liver and human kidney.

MATERIALS AND METHODS

Sephadex G-100 (lot no. 1395; particle size 40–120 μ) was obtained from Pharmacia, Uppsala, Sweden. Bio-Gel P60 was obtained from Bio-Rad Laboratories, Richmond,

Calif., U.S.A. All other reagents were of analytical grade.

The α -glucosidase activity was determined by measuring the rate of formation of glucose from maltose (maltase activity) and from glycogen (glucoamylase activity) as described by Auricchio & Bruni (1967). A unit of glucosidase activity is defined as the amount of enzyme that catalyses the hydrolysis of 1 μ mole of maltose/min. (Torres & Olavarría, 1964). The specific activity is referred to as units of glucosidase activity/mg. of protein. Protein concentration was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) as modified by Eggstein & Kreutz (1955) or by the biuret method (Weichselbaum, 1946). Crystalline bovine plasma albumin was used as a standard in both cases.

Rat liver. The purification procedure of the acid α -glucosidase described by Auricchio & Bruni (1967) was modified as follows: 20–40 male Wistar rats were starved for 30 hr. and then killed. The livers were homogenized for 3 min. in an MSE homogenizer with a solution (1:1, w/v) of 1 mM-EDTA containing 25 mM-NaCl, adjusted with 2 N-NaOH to pH 6.7. The homogenate was frozen and thawed and then centrifuged at 105000g for 60 min. at 4°. The supernatant was acidified to pH 4.2 with N-acetic acid and then centrifuged at 12000g for 20 min. The supernatant was immediately neutralized with aq. 1 N-NH₃ solution and then centrifuged at 12000g for 20 min. The clear supernatant was fractionated with solid (NH₄)₂SO₄ between 30% and 60% saturation. The proteins precipitated were collected by centrifugation at 25000g for 30 min., 10 min. after the addition of (NH₄)₂SO₄. The precipitate was dissolved in a small volume (10–15 ml.) of EDTA-NaCl solution, centrifuged to remove some insoluble proteins, and finally applied to the top of the dextran-gel columns. All these steps were performed at 3°.

Human kidney. The kidneys of a patient with chronic glomerulonephritis were removed during a transplantation and immediately frozen. After thawing, the kidney was homogenized in a Waring Blendor, the homogenate was centrifuged at 105000g and then acidified, neutralized, fractionated with (NH₄)₂SO₄ and filtered through a

Sephadex-gel column in the same way as for the rat liver extract.

Sucrose gradients. Preparation, centrifugation and collection of sucrose density gradients were performed essentially as described by Martin & Ames (1961). Linear sucrose gradients (5–20%, w/v) were prepared in 0.02M-phosphate buffer, pH 6, containing 25mM-NaCl. The samples layered over the sucrose gradients contained 1% (w/v) or less of protein. The gradients were run for 12–14 hr. in a Spinco model L2 centrifuge at 4° in the SW 39 rotor at 38000 rev./min. Crystallized bovine serum albumin and crystallized glyceraldehyde 3-phosphate dehydrogenase were used as standard proteins.

Gel chromatography. Dextran-gel chromatography was performed as described by Auricchio & Bruni (1967).

Bio-Gel P60 was prepared as for the Sephadex gel with the exception that swelling was performed in water instead of 6M-urea. Columns 1 cm. diam. $\times$ 40 cm. were used. The flow rates were approx. 10 ml./hr. A solution of 1mM-EDTA-0.25M-NaCl, adjusted to pH 6.7 with 2N-NaOH, was used for the chromatographic development.

All the chromatographic experiments were performed at 3°.

Polycrylamide disc gel electrophoresis. Disc gel electrophoresis at low pH was performed essentially as described by Reisfeld, Lewis & Williams (1962). The degree of polymerization of the gel was 7.5%, and the electrophoresis was run for 4 hr. at 4° in acetic acid- β -alanine buffer, pH 5.3, with a current of 3 mA per tube. Gel was stained for protein with 0.2% Naphthalene Black 12B in 7% acetic acid.

RESULTS

The homogeneity of the acid α -glucosidase prepared as described by Auricchio & Bruni (1967) was examined by sucrose-density-gradient centrifugation. The supernatant from a rat liver homogenate that had been centrifuged at 105000g was concentrated by dialysis under reduced pressure to a protein concentration of 200mg./ml. A sample (100ml.) was layered on the top of a Sephadex G-100 column (3 cm. diam. $\times$ 133 cm.). The elution profile of the acid α -glucosidase activity of this preparation was very similar to that obtained when smaller quantities of protein were filtered through smaller columns (Auricchio & Bruni, 1967).

The fractions that were eluted after the total volume of the column and had no detectable extinction at 280m μ but a strong acid α -glucosidase activity were pooled and freeze-dried. The powder was resuspended in a small volume of water and exhaustively dialysed against 0.02M-phosphate buffer, pH 6, containing 25mM-sodium chloride. The non-diffusible material was freeze-dried, and then resuspended in 0.3 ml. of water and centrifuged. A sample of this supernatant (0.15 ml., 6.5 mg. of protein/ml.) was layered on the top of a sucrose density gradient. The fractions collected from the gradient were examined for their extinction at 280m μ and for acid maltase activity. The peak of enzymic activity did not coincide with that of extinction at 280m μ but was on the descending part of the protein profile.

To obtain a more highly purified acid α -glucosidase a number of purification steps were included before the gel filtration. Acidification and ammonium sulphate fractionation had been used by Torres & Olavarria (1964) for the purification of this enzyme from dog liver. The purification procedure and the results of a typical preparation are summarized in Table 1. Fig. 1(a) is the elution diagram from a dextran-gel column of a sample of the enzyme that had been prepared in this modified manner. The maltase activity at pH 7.5 and 3.6 (at the latter pH both in the absence and in the presence of 30mM-turanose) and glucoamylase activity at pH 3.6 were measured.

In the experiments that were performed with the rat liver acid α -glucosidase, portions of the eluate from several columns were used. Nevertheless, for simplicity and because the experiment shown in Fig. 1(a) is highly reproducible, we refer throughout to the various fractions by the effluent volumes shown in this Figure.

Some fractions eluted between 40 and 90 ml. were tested for acid and neutral maltase activity before and after heat-inactivation at 55° for 30 min. at pH 4.5. The acid activity was less sensitive than the neutral (60% and 90% inhibition respectively).

Table 1. *Purification of acid α -(1 $\rightarrow$ 4)-glucosidase from rat liver*

For definition of the units of glucosidase activity see the Materials and Methods section.

Purification step	Vol. (ml.)	Enzyme activity (units/ml.)	Protein (mg./ml.)	Sp. activity (units/mg.)	Recovery (%)
Homogenate	400	75	83	0.9	100
105000g supernatant	250	78	50	1.57	66
Acid precipitation	280	64	12.5	5.15	60
(NH ₄) ₂ SO ₄ fractionation (30–60% satd.)	11	850	121	7	31
Sephadex G-100 chromatography*	54	39	0.007	5520	7

* Enzyme eluted between 200 ml. and 254 ml. (Fig. 1a).

Fig. 1. Elution diagram of rat liver (a) and human kidney (b) α -glucosidase activities on a Sephadex G-100 column (1.5 cm. diam. $\times$ 67 cm.) equilibrated with a solution of 1 mM-EDTA containing 25 mM-NaCl, pH 6.7. Samples applied to the column were: (a) 11 ml. containing 121 mg. of protein/ml. and (b) 8 ml. containing 29.2 mg. of protein/ml. The eluate was examined for E_{280} (○), for maltase activity at pH 3.6 in the absence (▲) and in the presence (●) of 30 mM-turanose, and for maltase activity at pH 7.5 (△) and for glucoamylase activity at pH 3.6 (□). In Expt. (b) the maltase activity at pH 3.6 continued to be eluted until 548 ml.

Fractions eluted between 200 ml. and 220 ml. were tested for acid maltase and glucoamylase activity before and after heat-inactivation at pH 4.5. Both activities were diminished by 50%.

The eluates between 120–160 ml. and 160–200 ml. were pooled. The two solutions were freeze-dried, and the residues were dissolved in water and dialysed against phosphate buffer. The specific

activities were 220 and 5400 units/mg. of protein respectively. The samples were freeze-dried again, dissolved in a small volume of water and layered on the top of a sucrose density gradient. The extinction profiles at 210 m μ showed that the less pure preparation was the more heterogeneous (Fig. 2a). In the more purified sample (Fig. 2b), besides a peak of substances that sedimented slowly, there

Fig. 2. Sucrose-density-gradient sedimentation patterns of samples of: (a) rat liver α -glucosidase (0.1 ml. containing 10 mg. of protein/ml., specific activity 220 units/mg. of protein); (b) rat liver α -glucosidase (0.15 ml. containing 0.66 mg. of protein/ml., specific activity 5400 units/mg. of protein); (c) human kidney α -glucosidase (0.15 ml. containing 10 mg. of protein/ml., specific activity 300 units/mg. of protein). Fraction 1 is from the top of the gradient. The fractions were examined for E_{210} (○) and for maltase activity at pH 3.6 (▲). For further experimental details see the text.

was only one peak with higher sedimentation coefficient. It coincided with the single peak of enzymic activity.

The sedimentation coefficient and the molecular weight of these two enzymic preparations were determined by the method of Martin & Ames (1961). The preparation of acid α -glucosidase shown in

Fig. 2(b) had $S_{20,w}$ 4.9s and mol.wt. 81000 with albumin as standard ($S_{20,w}$ 4.3s, mol.wt. 66000) (Ghulman, 1953). With glyceraldehyde 3-phosphate dehydrogenase as standard ($S_{20,w}$ 7.01s, mol.wt. 120000) (Taylor & Lowry, 1956), the $S_{20,w}$ was 5.1s and the mol.wt. 76000. Values of $S_{20,w}$ 5.0s and mol.wt. 83000 were obtained for the

preparation shown in Fig. 2(a) with albumin as standard.

Another portion of the sample with specific activity 220 units/mg. of protein was examined by chromatography on Bio-Gel P60. A separation of a small peak of extinction at 280m μ , corresponding with the peak of enzymic activity, from a second larger extinction peak, devoid of activity, was obtained (Fig. 3a). The specific activity of the acid α -glucosidase after Bio-Gel chromatography was improved to 2000.

The eluate between 200ml. and 250ml. was pooled, freeze-dried, dissolved in water, dialysed

against phosphate buffer and freeze-dried again. The specific activity of this sample (approx. 50 μ g.) was 2240, and it was used for a single disc gel electrophoresis run, which showed a single band of protein.

The purification procedure for the enzyme from human kidney is summarized in Table 2. Fig. 1(b) shows the elution diagram from a dextran-gel column of the maltase activity at pH 7.5 and 3.6. This latter activity was measured in the absence and presence of 30mm-turanose. The eluate between 85ml. and 160ml. was pooled and freeze-dried. The powder was dissolved in a small volume of water, dialysed against phosphate buffer, freeze-dried, again dialysed and centrifuged. The supernatant (1ml.) contained about 10mg. of protein/ml. The specific activity was 300. A portion of this solution was applied to the top of a sucrose density gradient, and another portion was used for chromatography on Bio-Gel. The sucrose gradient (Fig. 2c) showed the heterogeneity of the material with extinction at 210m μ . The $S_{20,w}$ and mol.wt. of the acid α -glucosidase, determined by the method of Martin & Ames (1961) with albumin only as standard, were 5.6s and 97 000 respectively.

The profile from Bio-Gel (Fig. 3b) showed three peaks of material with extinction at 280m μ . The enzymic activity was found in the first peak. When a sample of human kidney acid α -glucosidase with a specific activity of 4800 units/mg. of protein was chromatographed through a Bio-Gel P60 column the extinction at 280m μ showed a single peak coincident with the enzymic activity, and the specific activity was unchanged.

The effects of tris and erythritol on the maltase and glucoamylase activity at pH 4.5 of the rat liver and human kidney acid α -glucosidase are reported in Table 3. These substances are known to be inhibitors of a plant α -glucosidase (Kelemen & Whelan, 1966) and of barley malt α -glucosidase (Jørgensen & Jørgensen, 1967).

The maltase activity at pH 7.5 of the 105 000g supernatant from rat liver was also inhibited by erythritol and by tris (60% and 80% respectively).

Fig. 3. Elution diagram of samples of (a) rat liver α -glucosidase (0.9ml. containing 10mg. of protein/ml., specific activity 220 units/mg. of protein) and (b) human kidney α -glucosidase (0.85ml. containing 10mg. of protein/ml., specific activity 300 units/mg. of protein) on a Bio-Gel P60 column (1cm. diam. $\times$ 40cm.) equilibrated with 1mM-EDTA-0.25M-NaCl, pH 6.7. The fractions were examined for E_{280} (O) and for maltase activity at pH 3.6 ($\blacktriangle$).

Table 2. Purification of acid α -(1 $\rightarrow$ 4)-glucosidase from human kidney

For definition of the units of glucosidase activity see the Materials and Methods section.

Purification step	Vol. (ml.)	Enzyme activity (units/ml.)	Protein (mg./ml.)	Sp. activity (units/ml.)	Recovery (%)
Homogenate	250	179	8.5	21	100
105 000g supernatant	238	168	7.65	22	89
Acid precipitation	275	123	4.9	25	75
(NH ₄) ₂ SO ₄ fractionation (30–60% satd.)	8	3560	29.2	122	63
Sephadex G-100 chromatography*	388	22	0.0045	4800	19

* Enzyme eluted between 160ml. and 548ml. (Fig. 1b).

Table 3. Inhibition of acid α -(1 $\rightarrow$ 4)-glucosidases, purified from rat liver and human kidney, by tris and erythritol (0.38M)

	Inhibition (%)			
	Maltase activity (pH 4.5)		Glucoamylase activity (pH 4.5)	
	Erythritol	Tris	Erythritol	Tris
Rat liver enzyme (sp. activity 5400 units/mg. of protein)	81	97	90	95
Human kidney enzyme (sp. activity 4800 units/mg. of protein)	83	97	90	98

Fig. 4. Inhibition of maltase activity of rat liver acid α -glucosidase by erythritol at pH 4.5 (O, nil; ●, 0.05M; □, 0.2M). Velocity (v) is expressed as μ moles of maltose hydrolysed/min./mg. of protein.

The Michaelis constants (K_m) of purified samples of rat liver and human kidney acid α -glucosidase were 4.7×10^{-3} M and 13.6×10^{-3} M respectively with maltose as substrate. The inhibition of the acid α -glucosidase activity from rat liver by erythritol was competitive (Fig. 4).

DISCUSSION

By means of dextran-gel filtration, it was possible to purify the acid α -glucosidase, because this enzyme was adsorbed by Sephadex and therefore eluted after the majority of the protein. Nevertheless several other molecular species appeared to behave similarly when filtered through dextran gel and hence were eluted with this enzyme. Therefore a preparation of acid α -glucosidase purified only by means of dextran-gel filtration contained heterogeneous material, as shown by the sucrose-density-gradient experiment. A preliminary purification of the sample applied to the dextran columns improved the final purification of the enzyme from rat liver.

The sucrose-density-gradient experiments performed with the two samples of the second peak of rat liver acid maltase activity collected from Sephadex columns at different effluent volumes showed that in both cases the sedimentation coefficient was the same. This fact showed that this peak of activity was due either to a single protein or to proteins with similar hydrodynamic characteristics.

In the sucrose-density-gradient experiment shown in Fig. 2(b) there was a single peak of heavier material showing extinction at $210m\mu$, which coincided with the enzymic activity. The disc gel electrophoresis experiment with rat liver enzyme also showed a single band of protein, which suggested that our preparation contained only one protein.

The acid α -glucosidase was retarded on Sephadex columns; this fact could be due to an interaction of the 'enzyme-substrate' type between this protein and the dextran matrix (Auricchio & Bruni, 1967). On Bio-Gel, a polyacrylamide gel, the acid α -glucosidase was not retarded and hence was separated from the small molecules with which it was eluted from the Sephadex columns. Therefore Bio-Gel filtration was a very good method to purify further that part of the rat liver enzyme eluted from Sephadex columns with lower specific activity. In

this way it was possible to improve the yield of highly purified enzyme.

Our results showed that erythritol and tris were powerful inhibitors at pH 4.5 of the acid enzyme. The inhibition by erythritol was competitive, suggesting that with mammalian acid α -glucosidase the substrate also interacts with the enzyme at C-3, C-4, C-5 and C-6. The erythritol could thus prevent enzyme-substrate interactions, as was suggested for a plant α -glucosidase by Kelemen & Whelan (1966). Tris and erythritol also inhibited the neutral maltase activity, and hence they seem to be general inhibitors of mammalian glucosidases.

Both the maltase and the glucoamylase activities were inhibited at acid pH by these substances and were also inactivated by heat to the same extent. These facts are additional proof that both activities depend on the same protein.

As previously reported, the acid α -glucosidases purified from human kidney and from rat liver were similar with regard to adsorption on dextran gel, pH optimum with maltose and glycogen as substrates, and inhibition by turanose. The enzymes had similar molecular weights and K_m values for maltose, and were inhibited to the same extent by erythritol and tris.

Two lines of evidence suggest that the first peak of rat liver acid maltase activity cannot have been due exclusively to the activity at acid pH of a neutral glucosidase. This peak was quantitatively similar to the single peak of the neutral enzyme, so that it is unlikely that it was due to the activity at acid pH of a neutral α -glucosidase. Further, the acid and neutral maltase activities of the first peak were inactivated in different measure by heat. It is possible that in the first peak there was an acid α -glucosidase besides a neutral α -glucosidase, and that this acid enzyme was different from that

present in the second peak. However, this point needs further experimentation.

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