Neutral maltase/glucoamylase from rabbit renal cortex

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Maltase activity (EC 3.2.1.20) was solubilized from rabbit kidney brush-border membrane by using 1.0% Triton X-100 and purified 230-fold with an overall recovery of 30%. The purification procedure makes use of heat precipitation, chromatography on DE-52 DEAE-cellulose and gel filtration on Sephacryl S-300. Rabbit kidney brush border exhibited glucoamylase activity with a maltase/glucoamylase ratio of 1.5:1 to 2.0:1. During purification the maltase and glucoamylase activities behaved identically. The M_r of the complex is 590 000, and it appears to be composed of eight identical subunits linked by disulphide bridges.

INTRODUCTION

The ultrastructures of the brush-border membrane of the intestine and kidney have a striking similarity. Both have a battery of hydrolases for peptides and carbohydrates (Kenny & Booth, 1978). The peptidases from kidney and carbohydrases from intestine have been the subject of extensive investigation. The carbohydrases from kidney that have been purified so far are trehalase from rabbit (Galand, 1984; Nakano & Sacktor, 1985; Yoneyama, 1987) and neutral maltase from the horse (Guidicelli *et al.*, 1980, 1985) and rat (Reiss & Sacktor, 1981, 1982). The physiological need for the carbohydrases in the kidney is still unclear. In the present paper we report a method for the purification of detergent-solubilized maltase from rabbit kidney cortex and some of its properties. The results are compared with those obtained with the enzyme from rabbit small intestine (Sankaran *et al.*, 1983).

MATERIALS AND METHODS

Chemicals

The following chemicals and reagents were purchased as indicated. Glucose oxidase, horseradish peroxidase, Tris, maltose, malto-oligosaccharides, Triton X-100 and acrylamide were from Sigma Chemical Co., St. Louis, MO, U.S.A. *NN'*-Bisacrylamide, *NNN'*-tetramethylethylenediamine and Folin-Ciocalteu reagent were from Sisco Research Laboratories, Bombay, India. The M_r marker kit was purchased from Pharmacia, Uppsala, Sweden. All other chemicals were of the highest analytical grade available.

Assay of enzyme activities

Maltase and glucoamylase activities were assayed by measurement of the glucose released by the Tris/glucose oxidase/peroxidase procedure of Dahlqvist (1964), as described previously (Sivakami & Radhakrishnan, 1976).

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. The modification described by Wang & Smith (1975) was used for samples in presence of Triton X-100.

Enzyme units

One unit of enzyme activity is defined as the amount of enzyme required to liberate 1 μ mol of glucose/min at 37 °C under standard assay conditions. Concentration of starch is expressed as mol of glycosidic bonds/l (Sivakami & Radhakrishnan, 1976).

Purification of maltase

A summary of the purification is given in Table 1. Adult rabbits of either sex were killed, the kidneys were removed and decapsulated, and the cortex was minced and washed in 0.01 M-sodium/potassium phosphate buffer, pH 7.0, and weighed. The tissue was homogenized, in a blender for 30 s, in ice-cold 0.01 M-sodium/potassium phosphate buffer, pH 7.0, containing 1 mM-phenylmethanesulphonyl fluoride, to give a 20% (w/v) homogenate. The homogenate was centrifuged at 9000 *g* for 30 min in a Sorvall model RC 5B refrigerated centrifuge. The pellet was suspended in 0.5 vol. of 0.01 M-sodium/potassium phosphate buffer, pH 7.0, containing 1 mM-phenylmethanesulphonyl fluoride. To it Triton X-100 was added to a final concentration of 1.0% (w/v), maintaining 1 mM-phenylmethanesulphonyl fluoride, and the mixture was left for 60 min at 37 °C with periodic shaking. At the end of 60 min the membranes were centrifuged at 20000 *g* for 120 min. The supernatant, containing soluble maltase activity, was subjected to heat precipitation at 55 °C for 30 min and centrifuged at 20000 *g* for 60 min. The supernatant, containing maltase activity, was passed through a column of DE-52 DEAE-cellulose equilibrated with 0.01 M-sodium/potassium phosphate buffer, pH 7.0. The column was washed with 5 bed volumes of the equilibration buffer and eluted with a linear gradient of 0.025–0.25 M-sodium/potassium phosphate buffer, pH 7.0, formed by using a Pharmacia GM-1 gradient maker. Both maltase and glucoamylase activities were eluted as a single symmetrical peak at the same position. The active fractions were pooled and dialysed against 0.01 M-sodium/potassium phosphate buffer, pH 7.0, and applied to a second column of DE-52 DEAE-cellulose equilibrated with the same buffer as above. In this case the elution was carried out with

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Table 1. Purification of maltase/glucoamylase from rabbit renal cortex

For experimental details see the text. Abbreviations: M, maltase activity; G, glucoamylase activity.

Fraction	Total activity (units)		Total protein (mg)	Specific activity (units/mg)		Purification (fold)		Yield (%)		M/G ratio
	M	G		M	G	M	G	M	G	
Pellet	1000	797	3420	0.3	0.23	1.0	1.0	100	100	1.3
Triton X-100 supernatant	924	570	1344	0.7	0.43	2.3	1.9	93	72	1.6
Heat supernatant	710	360	700	1.01	0.51	3.4	2.2	71	45	2.0
1st DE-52 DEAE-cellulose chromatography	515	277	70.4	7.3	4.0	24	17	52	35	1.8
2nd DE-52 DEAE-cellulose chromatography	409	170	7.3	56	23	186	100	41	21	2.4
Sephacryl S-300 gel filtration	304	140	4.4	69	32	230	139	30	18	2.2

a linear gradient of 0.025–0.30 M-sodium/potassium phosphate buffer, pH 7.0. The two activities were eluted as a single sharp peak at the same position. The active fractions were pooled and freeze-dried and loaded on a Sephacryl S-300 column (100 cm $\times$ 1.6 cm; bed volume 200 ml) equilibrated and eluted with 0.01 M-sodium/potassium phosphate buffer, pH 7.0, containing 0.1% Triton X-100. The flow rate was maintained at 40 ml/h with the aid of a Pharmacia P-1 pump. Both the activities were eluted as a single sharp symmetrical peak at the same position. The active fractions were pooled, freeze-dried and used for further experiments. The M_r and Stokes radius of the enzyme were found by calibrating the Sephacryl S-300 column with the following markers: thyroglobulin (M_r 669 000), ferritin (M_r 440 000), catalase (M_r 232 000), fructose-bisphosphate aldolase (M_r 158 000) and bovine serum albumin (M_r 67 000). All M_r markers were run in the presence of 0.01 M-sodium/potassium phosphate buffer, pH 7.0, containing 0.1% Triton X-100.

Polyacrylamide-disc-gel electrophoresis

This was performed on the native protein by the method of Laemmli (1970) with a 5% gel. The gels were stained for protein with Coomassie Brilliant Blue as well as AgNO_3 . Electrophoresis in the presence of SDS and 2-mercaptoethanol was performed on 9% gels along with the following markers: myosin (M_r 205 000), β -galactosidase (M_r 116 000), phosphorylase *b* subunit (M_r 97 400), bovine plasma albumin (M_r 66 000), ovalbumin (M_r 45 000) and carbonic anhydrase (M_r 29 000).

RESULTS

Purity of the enzyme

The enzyme migrates as a single protein band during polyacrylamide-disc-gel electrophoresis in the native state as well as in the presence of SDS (Fig. 1). On native gels the position corresponding to the protein band was eluted and assayed for maltase and glucoamylase activities. Both activities were exhibited by the same protein band.

M_r of the enzyme

The native enzyme appears to have an M_r of $590\,000 \pm 7920$ during gel filtration (Fig. 2) and electrophoresis in the presence of SDS without reducing agents (Fig. 3). Upon reduction with 2-mercaptoethanol in the presence of SDS it gives a single protein band with an M_r of $79\,000 \pm 525$ (Fig. 4). This shows that the protein is made up of eight identical subunits of M_r 79 000 linked by disulphide linkages.

Fig. 1. Polyacrylamide-gel electrophoresis of purified maltase/glucoamylase

The gels were stained with Coomassie Brilliant Blue. (a) 5% gel: left, native; right, SDS-treated. (b) 9% gel: centre, SDS-treated; left, SDS/2-mercaptoethanol-treated; right, standard markers [from the top, myosin (M_r 205 000), β -galactosidase (M_r 116 000), phosphorylase *b* (M_r 97 400), bovine plasma albumin (M_r 66 000), ovalbumin (M_r 45 000) and carbonic anhydrase (M_r 29 000)].

Fig. 2. Determination of the M_r of maltase/glucoamylase by gel filtration on Sephacryl S-300

The standard markers used were thyroglobulin (THY; M_r 669 000; Stokes radius 8.5 nm), ferritin (FER; M_r 440 000; Stokes radius 6.1 nm), catalase (CAT; M_r 232 000; Stokes radius 5.22 nm), fructose-bisphosphate aldolase (ALD; M_r 158 000; Stokes radius 4.8 nm) and bovine serum albumin (BSA; M_r 67 000; Stokes radius 3.55 nm). MAL indicates the position of maltase/glucoamylase.

Kinetic properties of the enzyme

The enzyme exhibits a broad optimum pH, with maltase activity remaining constant from pH 6 to 7 and glucoamylase activity remaining constant from pH 6 to 8. The variation of K_m and V_{max} values with pH indicates the participation of a group or groups with pK values in

Fig. 3. Determination of the M_r of maltase/glucoamylase by SDS/polyacrylamide-gel electrophoresis

The standard markers used were thyroglobulin (THY; M_r 669 000), ferritin (FER; M_r 440 000), catalase (CAT; M_r 232 000), myosin (MYO; M_r 205 000), β -galactosidase (GAL; M_r 116 000) and phosphorylase b (PHO; M_r 97 400). MAL indicates the position of maltase/glucoamylase.

Fig. 4. Determination of the M_r of maltase/glucoamylase subunit by SDS/polyacrylamide-gel electrophoresis in the presence of 2-mercaptoethanol

The standard markers used were myosin (MYO; M_r 205 000), β -galactosidase (GAL; M_r 116 000), phosphorylase b (PHO; M_r 97 400), bovine plasma albumin (BSA; M_r 66 000), ovalbumin (OVA; M_r 45 000) and carbonic anhydrase (CAR; M_r 29 000). MAL indicates the position of maltase/glucoamylase subunit.

the range 6.0–7.5 during catalysis of maltose and starch hydrolysis (Fig. 5). Besides maltose and starch, the enzyme hydrolyses three malto-oligosaccharides tested, namely maltotriose, maltotetraose and maltopentaose, with K_m and V_{max} values as shown in Table 2.

Although maltose does not exhibit substrate inhibition, maltotriose, maltotetraose and maltopentaose show substrate inhibition at concentrations exceeding 0.4 mM. The K_i values of the substrate acting as inhibitor are shown in Table 2.

Mixed-substrate incubation studies were performed with various concentrations of maltose and fixed concentration of starch as inhibitor. The hydrolysis of maltose is non-competitively inhibited by starch (Fig. 6).

The enzyme is competitively inhibited by Tris with K_i values of 6.8 ± 0.5 mM and 9.1 ± 0.3 mM for maltose and starch respectively. Two thiol-blocking agents, *N*-ethylmaleimide and *p*-chloromercuribenzoate, inhibited the enzyme (Table 3). The activities were assayed after preincubation of the enzyme with these inhibitors for 30 min at 37 °C. Sucrose was an inhibitor for starch hydrolysis only, having no effect on maltose hydrolysis

Table 2. Kinetic constants of maltase/glucoamylase for some substrates tested

Substrate	K_m (mM)	V_{max} (units/ml)	V_{max}/K_m [(units/ml)/mM]
Maltose	0.67 ± 0.03	0.63 ± 0.07	0.94
Maltotriose*	0.30 ± 0.02 (K_i 1.5 mM)	0.31 ± 0.02	1.03
Maltotetraose*	0.29 ± 0.01 (K_i 2.4 mM)	0.23 ± 0.03	0.79
Maltopentaose*	0.22 ± 0.01 (K_i 7.0 mM)	0.14 ± 0.01	0.64
Starch	3.70 ± 0.10	0.40 ± 0.01	0.11

* Reaction velocities are expressed as μ mol of α -1,4-linkages hydrolysed/min.

Fig. 5. Variation with pH of K_m and $V_{\max}$ values of maltase/glucoamylase for maltose (a) and starch (b)

Table 3. Effect of some inhibitors and thiol-blocking reagents on maltase/glucoamylase activity

	Concn. (mM)	Residual maltase activity (%)	Residual glucoamylase activity (%)
Inhibitor			
Sucrose	2	100	73
	5	100	69
	10	100	66
Chloride	30	64	53
	50	54	57
Thiol-blocking reagent			
<i>N</i> -Ethylmaleimide	1	81	68
	5	79	56
	10	76	54
<i>p</i> -Chloromercuri- benzoate	1	30	0
	5	28	0

(Table 3). Both activities were inhibited by the Cl^- anion, to the extent shown in Table 3.

DISCUSSION

Rabbit kidney is endowed with trehalase, maltase and glucoamylase activities. Sucrase activity has not been detected in kidney brush-border membrane so far, from any animal. In rabbit kidney the maltase and glucoamylase exist as a complex similar to that of the intestine,

Fig. 6. Inhibition by starch of maltase activity of maltase/glucoamylase

The Figure shows Lineweaver-Burk plots of velocities in the absence of starch (○), in the presence of 1.2 mM-starch (●) and in the presence of 1.85 mM-starch (■).

since the two activities do not separate during conventional purification procedures. The M_r value of 590000 of the native enzyme is close to the value of 760000 of the intestinal enzyme complex (Sankaran *et al.*, 1983). The Stokes radius of the kidney enzyme as estimated by gel filtration is 7.4 nm, whereas that of the intestinal enzyme is 12 nm (Sankaran *et al.*, 1983).

However, the kidney enzyme is different from the intestinal enzyme in some of its kinetic properties. Whereas the intestinal enzyme was not inhibited by thiol-blocking reagents at all, the kidney enzyme is inhibited partially, indicating an indirect involvement of thiol groups during catalysis. Also, maltase and glucoamylase partial activities of the kidney are inhibited to different extents by thiol-blocking reagents. The most dramatic difference is in the inhibition by sucrose, which has no effect on maltose activity but inhibits glucoamylase activity significantly. This seems to suggest a spatial separation of the maltose-hydrolysing and starch-hydrolysing sites. The non-competitive inhibition of maltase by starch further strengthens this idea.

Though the physicochemical properties of the intestinal enzyme have been investigated in detail, the number and nature of the subunits and their interactions are not clear (Sankaran *et al.*, 1983). The kidney enzyme seems to possess a simpler quaternary structure, being made up of eight identical subunits linked by disulphide bridges.

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