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PURIFICATION AND PROPERTIES OF α,α-TREHALASE FROM THE MUCOSA OF RAT SMALL INTESTINE

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Summary

α,α-Trehalase (EC 3.2.1.28, α,α-trehalose glucohydrolase) was solubilized from the microvillous membrane of the intestinal mucosa of rats with Triton X-100 and butanol. It was purified 6350-fold by gel filtration on Sephadex G-150 and chromatography on DE-52 and hydroxyapatite. The purified enzyme, with a specific activity of about 127 units per mg of protein, showed almost a single band of protein and activity on polyacrylamide gel electrophoresis. Its molecular weight was estimated to be 96 000 on Sephadex G-150 and 90 000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Its pH optimum was 5.5—5.7 and its K_m value for trehalose was 5.4 mM. Its activity was inhibited 30 and 100% by 1 mM *p*-chloromercuribenzoate and 0.1 mM $HgCl_2$, respectively and 30% by 1 mM $MgCl_2$. Moreover, its activity was inhibited completely by 10 mM tris(hydroxymethyl)aminomethane and about 60% by 10 mM sucrose and cellobiose. The enzyme showed a high specificity for trehalose.

Introduction

Trehalase, which hydrolyzes trehalose to two glucose molecules, is widespread in plants and animals. The purification and properties of trehalases from insects have been reported [1—4]. Trehalase has also been found in the intestinal mucosa of some mammals, where it is associated with the membranes of microvilli and thought to be involved in glucose transport [5]. Borgström and Dahlqvist reported the intracellular distribution of trehalase in hog intestine and the characterization of a partially purified preparation [6,7]. The distribution of trehalase along the small intestine and the effect of diet on its activity have also been investigated [8,9]. However, intestinal trehalase has not been purified completely.

This paper reports the purification of trehalase to homogeneity from rat small intestine, and some of its properties.

Materials and Methods

Chemicals. Glucose oxidase was purchased from Nagase Sangyo Co., Osaka, Japan. Peroxidase was obtained from Boehringer Mannheim GmbH, Mannheim, Germany. *o*-Dianisidine and α,α-trehalose were products of Sigma Chemical Co., St. Louis, Missouri, U.S.A. Triton X-100 was a product of Wako Pure Chemical Industries Ltd, Osaka, Japan. Sephadex G-150 and blue dextran 2000 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden and DEAE-cellulose (DE-52) from Whatman, Kent, U.K. Hydroxyapatite was a product of Bio-Rad, Richmond, California, U.S.A. All other materials of reagent grade were commercial preparations.

Enzyme assays. α,α-Trehalase (EC 3.2.1.28, α,α-trehalose glucohydrolase) activity was determined by the one-step method of Messer and Dahlqvist [10]. The reaction mixture contained in 1.1 ml: 0.1 ml of enzyme solution, 0.5 ml of 62 mM trehalose and 0.5 ml of phosphate-glucose oxidase reagent containing 1 mg of glucose oxidase, 0.02 mg of peroxidase and 0.1 mg of *o*-dianisidine in 100 mM citrate phosphate buffer (pH 5.7). This mixture was incubated at 37°C for 60 min. The reaction was stopped by addition of 0.5 ml of 50% H_2SO_4 and the absorbance was measured at 530 nm. The enzyme activity catalyzing the hydrolysis of 1 μmol of trehalose per min was defined as one unit. For determination of the pH optimum, a two-step method was employed [11]. The initial reaction mixture, containing 0.1 ml of enzyme and 0.5 ml of 62 mM trehalose in 10 mM citrate phosphate buffer (pH 3.3—6.9), was incubated at 37°C for 60 min. Then, 1.5 ml of Tris/glucose oxidase reagent, containing the same concentrations of glucose oxidase, peroxidase and *o*-dianisidine in 500 mM Tris buffer (pH 7.5) was added to the initial reaction mixture. The mixture was then incubated at 37°C for 60 min. Following procedures were the same as in the one-step method.

Sucrase (EC 3.2.1.26, β-D-fructofuranoside fructohydrolase) activity was determined by the same method using 62 mM sucrose as substrate instead of trehalose [10]. Alkaline phosphatase (EC 3.1.3.1, orthophosphoric-monoester phosphohydrolase (alkaline optimum)) was measured by the method of Lowry et al. using *p*-nitrophenyl phosphate as substrate [12].

Protein was determined by the method of Lowry et al. [13].

Enzyme purification. Male Wistar rats, weighing 250—300 g, were killed by decapitation. Their small intestines, from the duodenum to the jejunum, were removed and everted after removing the contents by washing with cold saline. The mucosa was scraped off with a razor blade. For the usual purification of trehalase, about 200 g of mucosa were collected from 100 rats . The mucosa was homogenized with a Polytron (Kinematica, GmbH, Luzern, Switzerland) operating at full speed for 4 min in 9 vol. of 10 mM sodium-potassium phosphate buffer (pH 6.8). The homogenate was centrifugated at 850 × *g* for 10 min and the resulting supernatant was again centrifuged at 27 000 × *g* for 30 min. The precipitate obtained was homogenized in 2 vol. of the same buffer containing 3% Triton X-100 in a Potter-Elvehjem type homogenizer with a

teflon pestle. After centrifugation at 75 000 × *g* for 90 min, the supernatant was collected and shaken with the same volume of water-saturated butanol at 4° C for 1 min. The water layer was separated by centrifugation at 10 000 × *g* for 15 min, and solid ammonium sulfate was added to 40% saturation. The mixture was centrifuged and solid ammonium sulfate was added to the resulting supernatant to give 60% saturation. The precipitate obtained was dissolved in 5 ml of 10 mM sodium-potassium phosphate buffer (pH 6.8) and dialyzed against the same buffer. The dialyzed solution was centrifuged to remove insoluble materials and the clear supernatant was applied to a Sephadex G-150 column (2.6 × 90 cm) and eluted with the same buffer. Fractions containing trehalase activity were pooled and applied to a DE-52 column (1 × 3 cm) equilibrated with 10 mM sodium-potassium phosphate buffer (pH 6.8). Trehalase was eluted with 100 ml of a linear gradient of 0—300 mM NaCl in the same buffer. The fractions containing trehalase activity were dialyzed and rechromatographed on DE-52 under the same conditions. The fractions of eluate containing enzyme were dialyzed against 10 mM sodium-potassium phosphate buffer (pH 6.8) and applied to a hydroxyapatite column (1 × 3 cm) equilibrated with the same buffer. Trehalase was eluted with 50 ml of a linear gradient of 10—200 mM sodium-potassium phosphate buffer (pH 6.8).

Polyacrylamide gel electrophoresis. About 40 μg of purified enzyme protein were applied to two 7.5% polyacrylamide gels by the method of Davis [14]. Electrophoresis was performed at 4 mA per gel for 40 min in 5 mM Tris/glycine buffer (pH 9.3). After electrophoresis, the enzyme protein on one gel was stained with Coomassie Brilliant Blue. The other gel was sliced into 1 mm sections and each section was eluted with 1 ml of 10 mM sodium-potassium phosphate buffer (pH 6.8) and assayed for trehalase activity. Sodium dodecyl sulfate-gel electrophoresis was performed by the procedures of Shapiro et al. [15]. For molecular weight determination, bovine serum albumin, hen ovalbumin, whale myogloblin and β-galactosidase from *Escherichia coli* were used as molecular size markers for dodecyl sulfate-polyacrylamide gel electrophoresis.

Results

Enzyme purification. The results of the purification are summarised in Table I. At the final stage of purification, the specific activity of trehalase was 127 units per mg of protein, representing 6350-fold purification over the homogenate. When stored at −10° C for a week, the purified enzyme lost 20—30% of its activity. On centrifugation of the crude homogenate at 27 000 × *g*, 70% of the trehalase activity was recovered in the precipitate and 70% of the activity in this fraction was solubilized with 3% Triton X-100. Sucrase activity was also solubilized by treatment with 3% Triton X-100, but most of the alkaline phosphatase was not solubilized by this treatment (data not shown). On treatment of the solubilized enzyme with butanol, about 60% of the trehalase activity was recovered in the water layer and the specific activity increased 2-fold. However, trehalase was rapidly inactivated during butanol treatment at temperatures above 4° C or for periods of more than 3 min at 4° C. Fig. 1 shows the gel-filtration pattern of the enzyme from the Sephadex G-150 column. The

TABLE I

PURIFICATION OF TREHALASE FROM RAT SMALL INTESTINE

Procedure	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Purification (fold)	Yield (%)
Homogenate	348.0	15300	0.02	1.0	100
27000 × *g* precipitate	255.0	5100	0.05	2.5	73
75000 × *g* supernatant	182.0	2300	0.08	4.0	52
Butanol treatment water layer	113.0	816	0.14	7.0	32
40–60% ammonium sulfate precipitate	80.5	94.6	0.85	42.5	23
Sephadex G-150	36.0	7.50	4.80	240	10
DE-52	26.0	1.08	24.1	1200	7.5
Second DE-52	19.5	0.62	31.5	1580	5.6
Hydroxyapatite	14.0	0.11	127.0	6350	4.0

trehalase was eluted as a single peak after a large peak of protein. Sucrase and the slight amount of alkaline phosphatase solubilized were eluted in the void volume. The elution position of trehalase corresponded to a molecular weight of around 96 000, and this value was also confirmed with the final purified enzyme. Traces of sucrase and alkaline phosphatase were detected in the enzyme preparation eluted from the second DE-52 column but the final preparation from the hydroxyapatite column, shown in Fig. 2, was entirely free from these two enzymes. Characterization of trehalase as described below was performed using the enzyme eluted from the hydroxyapatite column.

Characteristics of the purified enzyme. As shown in Fig. 3, on polyacrylamide gel electrophoresis the protein band and the activity completely coincided.

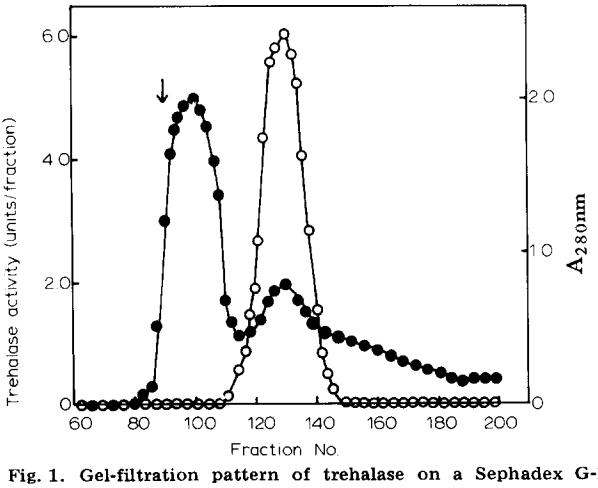


Fig. 1. Gel-filtration pattern of trehalase on a Sephadex G-150 column (2.6 × 90 cm). Elution was performed with 10 mM sodium-potassium phosphate buffer (pH 6.8) at a flow rate of 15 ml/h and the fraction size was 2 ml. ○———○ trehalase activity, ●———● absorbance at 280 nm. The arrow indicates the position of void volume.

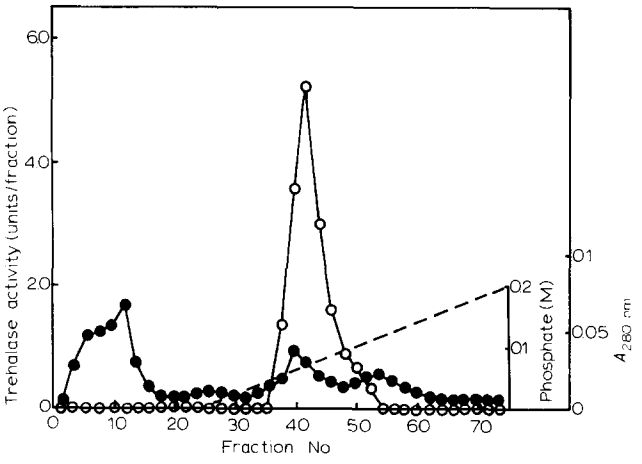


Fig. 2. Hydroxyapatite column chromatography of trehalase. A column (1 X 3 cm) was eluted with 50 ml of a linear gradient of 10—200 mM sodium-potassium phosphate buffer (pH 6.8) and fractions of 1 ml were collected. o———o trehalase activity, •———• absorbance at 280 nm.

Trehalase migrated as a single protein band when subjected to dodecyl sulfate-polyacrylamide gel electrophoresis. Its molecular weight was calculated to be 90 000 (Fig. 4). Its optimum pH was estimated to be 5.5—5.7 by the two-step method, as shown in Fig. 5. Fig. 6 shows Lineweaver-Burk plots obtained using purified trehalase with trehalose as substrate. The K_m value for trehalose was 5.4 mM in 100 mM citrate phosphate buffer (pH 5.7). Tables II and III show the effects of various inhibitors on trehalase activity. Trehalase was inhibited completely by 1 mM $HgCl_2$ and about 30% by $MgCl_2$ at 1 mM. $ZnSO_4$, $CaCl_2$ at concentration of 1 mM had little or no effect on the trehalase activity. *p*-Chloromercuribenzoate at a concentration of 1 mM inhibited the activity about 30%. Tris-(hydroxymethyl)aminomethane, sucrose and cello-

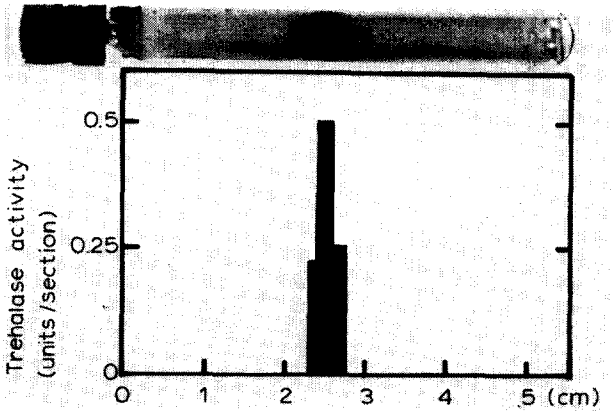
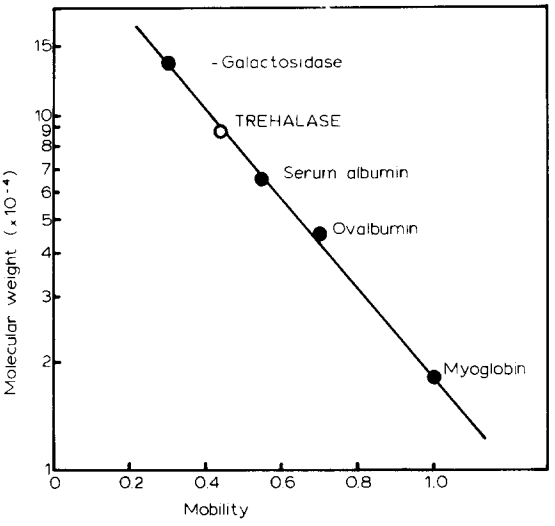


Fig. 3. Polyacrylamide gel electrophoretic pattern of the purified enzyme. After electrophoresis, one gel was stained for protein with Coomassie Brilliant Blue and another gel was cut into 1 mm sections. Each section was eluted with 1 ml of 10 mM sodium-potassium phosphate buffer (pH 6.8) and assayed for trehalase activity.



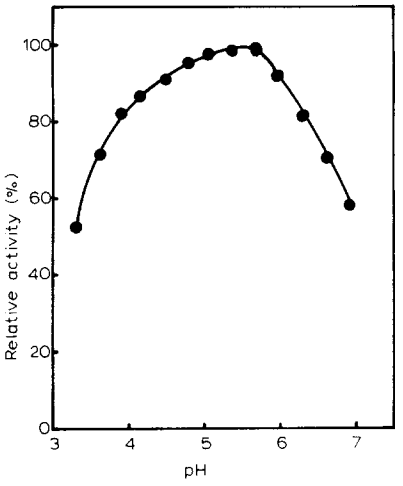


Fig. 4. Estimation of the molecular weight of trehalase by dodecyl sulfate polyacrylamide gel electrophoresis. Gels and samples were prepared as indicated by Shapiro et al. [15]. As molecular size markers β-galactosidase from *E. coli*, bovine serum albumin, hen ovalbumin and whale myoglobin were used.

Fig. 5. pH-Dependence of trehalase activity. Purified enzyme was assayed by the two-step method as described in the text. The highest activity is expressed as 100%.

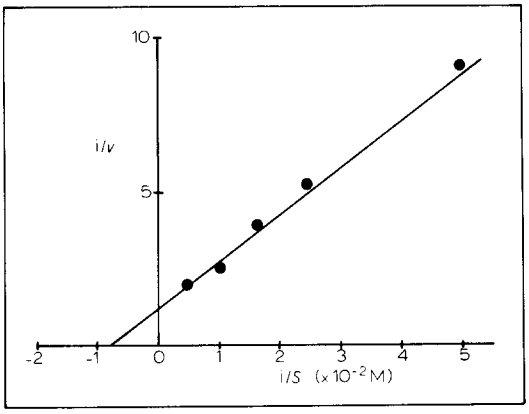


Fig. 6. Lineweaver-Burk plots of purified trehalase against various concentrations of trehalose.

TABLE II

EFFECTS OF METAL IONS ON TREHALASE

	Concentration (M)	Remaining activity (%)
$HgCl_2$	10^{-4}	0
	10^{-5}	42
$MgCl_2$	10^{-3}	70
	10^{-4}	90
	10^{-5}	100
$CaCl_2$	10^{-3}	100
$MnCl_2$	10^{-3}	100
$ZnCl_2$	10^{-3}	100

TABLE III

EFFECTS OF INHIBITORS ON TREHALASE

	Concentration (M)	Remaining activity (%)
p-Chloromercuribenzoate	10^{-3}	70
	10^{-4}	100
Tris(hydroxymethyl)aminomethane	10^{-2}	0
	10^{-3}	66
	10^{-4}	95
Sucrose	10^{-2}	44
	10^{-3}	70
	10^{-4}	81
Cellobiose	10^{-2}	45
	10^{-3}	60
	10^{-4}	76

biose were also inhibitors of trehalase. The purified enzyme had no hydrolytic activity on other disaccharides such as sucrose, maltose, cellobiose and melibiose, the rates of hydrolysis for these disaccharides being less than 1/1000 of that for trehalose.

Discussion

The purification of intestinal trehalase from mammals to homogeneity has not previously been reported. This is probably because it is difficult to solubilize trehalase from the membranes of microvilli and to separate it from other disaccharidases. However, in this work we succeeded in solubilizing and purifying the enzyme and the homogeneity of the final preparation was confirmed by polyacrylamide gel electrophoresis. Our purification procedure was different from those reported previously in several respects. We utilized the detergent Triton X-100 to solubilize the enzyme under mild conditions. Treatment of the 27 000 × *g* precipitate of the homogenate with 1% Triton X-100 only solubilized about 40% of the trehalase activity but solubilized 80% of the sucrase activity. This suggests that trehalase is attached to the microvillous membrane more firmly than sucrase. Using 3% Triton X-100, about 70% of the trehalase was solubilized from the membrane. Scarcely any alkaline phosphatase was solubilized even with 3% Triton X-100, so that in this way trehalase was separated from most of the alkaline phosphatase activity. When subsequent butanol treatment was omitted, half the trehalase activity solubilized by Triton X-100 was precipitated by 40% saturation of ammonium sulfate and this precipitate was insoluble, suggesting that the enzyme solubilized by Triton X-100 may still be in a large molecular form, possibly bound to membrane components. Huber and Lefebre reported the purification of trehalase from *Drosophila melanogaster* using gel filtration on Sephadex G-200 and DEAE-cellulose column chromatography [4]. However, their preparation obtained by rechromatography on DEAE-cellulose still contained other proteins so that they finally purified the enzyme by preparative gel electrophoresis. As seen in Fig. 2, in our work after

the second DE-52 column chromatography, a trace of contaminating protein was still present, although the specific activity had increased 1200-fold. Hydroxyapatite column chromatography with a linear gradient of sodium-potassium phosphate buffer effectively removed this impurity with a 4-fold increase in the specific activity of trehalase. The molecular weight of trehalase was estimated to be 96 000 on Sephadex G-150 and 90 000 by dodecyl sulfate-polyacrylamide gel electrophoresis. The identity of the molecular weight obtained on denaturation with that obtained under native conditions indicates that trehalase consists of a single polypeptide chain in the native state. Its molecular weight is comparable to those of trehalases purified from insects [3,4]. The substrate specificity of our purified preparation is consistent with that reported for hog intestinal trehalase by Dahlqvist [7]. It should be pointed out that the enzymatic properties of the rat intestinal trehalase which we examined are very similar to those of the intestinal enzyme from insects and hog [3,4,6]. The relatively low inhibitory effect of *p*-chloromercuribenzoate on trehalase suggests that a sulfhydryl group may not be directly involved in the active site of this enzyme, as reported by Huber and Lefebre [4]. The inhibition of disaccharidases by tris(hydroxymethyl)aminomethane was also confirmed with the trehalase from rat intestine. The mechanism of this inhibition was reported to be competitive [4,16,17,18].

Acknowledgements

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