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Purification and properties of trehalase from monkey small intestine

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Abstract. Brush border membrane trehalase was purified from monkey small intestine by a procedure which includes solubilisation by Triton X-100, ammonium sulphate fractionation, and chromatography on DE-52 and hydroxyapatite. The purified enzyme had a specific activity of 11 units/mg protein and was purified 140-fold. The enzyme showed a single protein band on Polyacrylamide gel electrophoresis. It had a K_m value of 17·4 mM for trehalose and a V_{max} of 1·33 units. Sucrose and Tris acted as competitive inhibitors of the enzyme.

Keywords. Trehalase; intestinal brush border membrane; hydrophobic protein.

Introduction

Trehalase (EC 3·2·1·28) is a disaccharidase present in the intestinal brush border membrane along with sucrase, maltase, glucoamylase and lactase. Though the enzymic properties and membrane organisation of sucrase and glucoamylase have been studied in detail, there is relatively little information on trehalase. The precise function of trehalase in the brush border membrane is not known. A possible role in glucose transport is suggested (Sacktor, 1968). Purification of trehalase from the intestine of rat and rabbit has been reported (Sasajima *et al.,* 1975; Galand, 1984; Yokota *et al.,* 1986). Trehalase from the brush border membrane of the kidney has been studied in greater detail than the intestinal enzyme where it appears to exist in multiple molecular forms (Nakano and Sacktor, 1985). In this paper, we report a method for the purification of trehalase from the brush border membrane of the monkey small intestine and some of the properties of the enzyme.

Materials and methods

Assay of enzyme

Trehalase activity was measured by the estimation of glucose formed by the Tris glucose oxidase peroxidase method of Dahlquist (1964). Sucrase was assayed by a similar method but using sucrose instead of trehalose. Protein was estimated by the method of Lowry *et al.* (1951), using bovine serum albumin as standard. The modified method of Wang and Smith (1975) was used for samples containing Triton X-100. One unit of enzyme activity was defined as the amount of enzyme required to hydrolyse 1 μmol of substrate per min at 37°C.

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Abbreviations used: PAGE, Polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; PCMB, *p*-chloromercuribenzoate; M_r, molecular weight.

Polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Laemmli (1970) using a 9% gel. The gels were stained for protein using Coomassie brilliant blue. PAGE in the presence of sodium dodecyl sulphate (SDS) was performed according to the method of Weber and Osborn (1969).

Heat Inactivation

The crude brush border membrane was diluted with buffer, to a final protein concentration of 3 mg/ml and kept in a constant temperature bath maintained at 55°C. Aliquots were withdrawn every 20 min and assayed for trehalase activity.

Purification of the enzyme

A summary of the purification procedure is given in table 1. Adult animals of either sex were used. The small intestines were washed with 1·15% (w/v) KCl, slit open longitudinally, and the mucosa scraped with a blunt knife. The scrapings were homogenised in 0·01 M potassium phosphate buffer, pH 7, in a Waring blender for 30 s in the cold. The homogenate was centrifuged at 13,000 *g* for 20 min in a Sorvall RCG5B refrigerated centrifuge. The pellet was suspended in half volume of 0·01 M potassium phosphate buffer, pH 7, and homogenised using a teflon pestle. The crude membrane fraction thus obtained was diluted 1:1 with the same buffer as above and treated with 1% (w/v) Triton X-100 at 37°C for 60 min, with occasional stirring. At the end of this time, the suspension was centrifuged at 38,000 *g* for 4 h. The supernatant which contained over 90 % of the trehalase and sucrase activities was raised to 30% saturation with ammonium sulphate, and centrifuged at 13,000 *g* for 30 min. The precipitate which contained no trehalase activity was discarded. To the supernatant, Triton X-100 was added to a final concentration of 1 % and the solution dialysed against 1 mM sodium phosphate buffer to remove the salt. The dialysed supernatant was applied to a column of DE-52 (4 × 1 cm, bed volume 10 ml) equilibrated with 10 mM sodium phosphate buffer, pH 5·6, containing 1 % Triton X-100. Trehalase was located in the flow through and washings. The fractions containing trehalase activity were pooled and dialysed against 1 mM sodium phosphate buffer, pH 6·8, and applied to a column of hydroxyapatite (1·6 × 1 cm, bed volume 5 ml) equilibrated with 1 mM sodium phosphate buffer, pH 6·8 containing

Table 1. Purification of trehalase from monkey small intestine.

Fraction	Activity (units/ml)	Total units	Protein (mg/ml)	Total protein (mg)	Specific activity	Recovery (%)
Pellet	2·5	125	32	1600	0·078	100
Triton X-100 supernatant	1·33	120	4·2	378	0·316	96
30% Ammonium sulphate supernatant	1·00	120	1·5	180	0·666	96
DE-52 flow through	0·75	90	0·46	54·6	1·64*	72
Hydroxyapatite I	2·2	66	0·47	14·07	4·7*	53
Hydroxyapatite II	2·4	24	0·22	2·28	10·9*	19

*Protein could not be estimated accurately due to interference by Triton X-100.

1% Triton X-100. The column was washed with the equilibrating buffer and eluted with a linear gradient of 20-200 mM sodium phosphate buffer, pH 6·8, using a Pharmacia GM-1 gradient maker. Trehalase eluted as a single sharp symmetrical peak. The fractions showing trehalase activity were pooled and dialysed against 1 mM sodium phosphate buffer pH 6·8 and applied to a second column of hydroxy-apatite (bed volume 1 ml) equilibrated with 1 mM sodium phosphate buffer pH 6·8. The column was washed with the equilibrating buffer and eluted with a linear gradient from 20–100 mM sodium phosphate buffer pH 6·8. The fractions showing trehalase activity were pooled and used for subsequent experiments.

Results

The purified enzyme was free of maltase and sucrase activities. The enzyme was assayed at different pH values from pH 3-7 using citrate phosphate buffer in a typical assay mixture. Trehalase activity exhibited a sharp peak at pH 5·8. The enzyme showed a K_m of 17·4 mM for trehalose and a V_{max} value of 1·33 units at pH 5·6 with sodium phosphate buffer. The variation of K_m with pH was studied using citrate phosphate buffer at different pH values. The plot of pK_m versus pH seems to indicate the involvement of two groups with pK values 4·5 and 6 (figure 1). Sucrose inhibits the enzyme competitively with a K_i value of 22·6 mM (Dixon and Webb, 1979) (figure 2). Tris was found to be a competitive inhibitor of the enzyme with a K_i value of 19·5 mM (figure 3). *p*-Chloromercuribenzoate (PCMB) inhibits trehalase activity. About 50% of the activity is inhibited by 3·33 mM PCMB and 85% by 10 mM PCMB. Mercuric chloride (0·4 mM) totally abolishes enzyme activity. At 55°C, about 70% of the enzyme activity was lost at the end of 1 h. The residual activity when maintained at 60°C, was totally denatured at the end of 40 min. The crude membrane fraction as well as the partially purified fraction behaved identically.

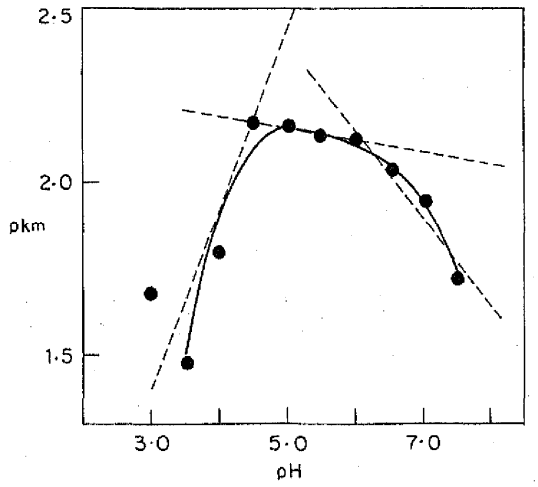

Figure 1. Variation of K_m with pH using trehalose as substrate.

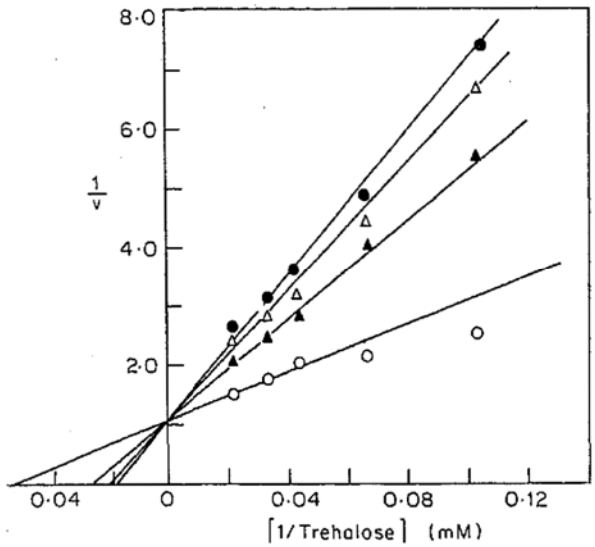

Figure 2. Inhibition of trehalase activity by sucrose. Line-weaver-Burk plots of velocities in the absence of sucrose (O) and in the presence of 20 mM sucrose (▲), 40 mM sucrose (Δ) and 60 mM sucrose (●).

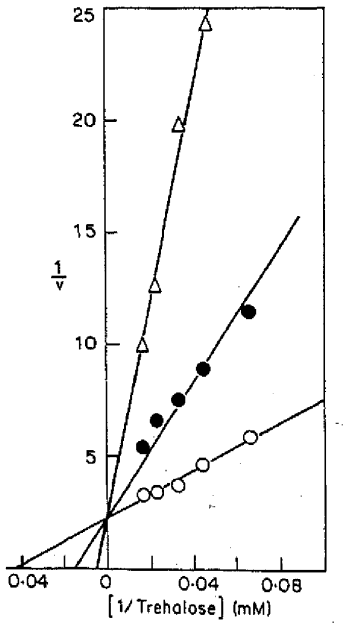

Figure 3. Inhibition of trehalase activity by Tris. Lineweaver-Burk plots of velocities in the absence of Tris (O) and in the presence of 50 mM Tris(●)and 100 mM Tris (Δ).

Molecular weight of the enzyme

The purified enzyme was apparently homogeneous since it moved as a single band after electrophoresis in nondenaturing gels. After electrophoresis in the presence of

SDS alone, one band, corresponding to a molecular weight (M_r) of 138,000, and a minor band, corresponding to a M_r of 28,000 were seen. However, upon reduction with β-mercaptoethanol, only one band was seen, with a M_r of 28,000; the heavier band of 138,000 was not seen after reduction (figure 4).

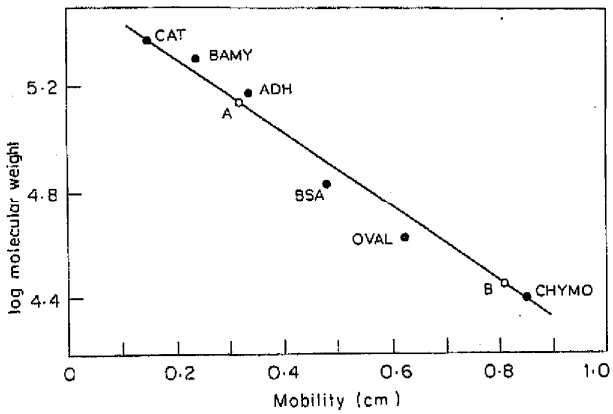

Figure 4. Estimation of the M_r of trehalase by SDS-PAGE. The standard markers used were catalase (243,000), alcohol dehydrogenase (150,000), β-amylase (200,000), bovine serum albumin (66,000), ovalbumin (42,000), and chymotrypsinogen (25,000). **A.** SDS treated. **B.** SDS/β-mercaptoethanol treated.

Discussion

Trehalase has been purified from the brush border membrane of the monkey small intestine by a relatively simple procedure after solubilisation with Triton X-100 with an overall recovery of 19%. Subsequent to solubilisation, it was necessary to maintain 1 % Triton X-100 throughout the purification process, in the absence of the detergent, trehalase was rapidly inactivated. Sasajima *et al.* (1975) have used butanol extraction to remove Triton X-100. We found that monkey trehalase activity was destroyed totally during extraction of Triton supernatant or crude membranes with *n*-butanol. Precipitation with 80% ammonium sulphate also results in denaturation of trehalase activity. Hence, in our procedure, some of the proteins were removed by precipitation with 30% ammonium sulphate and the supernatant containing soluble trehalase activity was passed through a DE-52 column in the presence of 1 % Triton X-100. The results seem to indicate that the trehalase of the monkey small intestine is a highly hydrophobic protein. Though trehalase has been purified from different sources, its molecular properties like size and quaternary structure are not well understood. Recently, Yokota *et al.* (1986) have isolated an amphiphilic trehalase from rabbit small intestine by solubilisation with Triton X-100 in the presence of EDTA. The presence of EDTA inhibits endogenous proteinases and hence the procedure results in trehalase carrying the hydrophobic tail which appears to be less than 5,000 in M_r. However, the hydrophobic anchor peptide corresponding to trehalase has not so far been isolated from any source.

The M_r of 138,000 for the native enzyme seems to be somewhat higher than the value of 75,000 to 95,000 reported for the intestine and kidney enzymes of other

animals. This might be due to the high hydrophobicity of the protein and consequent binding of higher amounts of Triton X-100. The enzyme appears to give products of M_r 28,000 upon reduction. Nakano *et al.* (1977) reported the presence of a polypeptide of M_r 30,000 in the rat small intestine. Recently, Yokota *et al.* (1986) observed that though their preparation of trehalase behaved as a single protein under nondenaturing conditions, it showed a protein band corresponding to a M_r of 30,000 on SDS gel electrophoresis. This was thought to be a proteolytic product but still associated with the protein of M_r 75,000. Such anomalies have long been known during SDS gel electrophoresis (Dreyer *et al.,* 1972; Wallach and Winzler, 1974). Hence it appears reasonable to conclude that the trehalase from the monkey intestine is a monomeric protein of M_r 138,000. Further studies are in progress to resolve the quaternary structure.

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