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Two forms of trehalase in rabbit enterocyte: Purification and chemical modification

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Abstract. Trehalase found to be associated with the brush border membrane vesicles and the Ca^{2+} aggregated basolateral membrane vesicles were purified to homogeneity. They were found to differ in their molecular weight, subunit structure, heal stability, N-terminal residues, amino acid composition and also the active site residues. Chemical modification showed the presence of a histidine and tyrosine at the active site of brush border membrane vesicle trehalase and two histidines at the active site of basolateral membrane vesicle.

Keywords. Trehalase; brush border membrane; basolateral membrane; chemical modification; rabbit.

Introduction

Trehalase has been purified from rabbit intestinal and renal brush border previously (Galand, 1984; Nakano and Sacktor, 1985; Yokota *et al.,* 1986). These reports are conflicting especially regarding the number of molecular forms, their molecular weights and subunit structure.

While, Galand (1984) reported the presence of only one form of trehalase from the rabbit intestinal· and renal brush border, exhibiting identical properties, Nakano and Sactor (1985) purified 4 forms of trehalase from the rabbit kidney. In the purification procedure reported by Galand (1984) only 45 % of total trehalase activity in the crude homogenate was recovered in the brush border membrane vesicles (BBMV). This led us to reinvestigate the behaviour of trehalase in the rabbit small intestine.

Preliminery experiments revealed the presence of two forms of trehalase, one associated with the brush border and the other with the Ca^{2+} aggregated basolateral membrane vesicles (BLMV). The enzymes purified seperately differed in many functional and structural properties.

Materials and methods

Trehalose, sucrose, starch, O-dianisidine, glucose oxidase, type VII, horse radish peroxidase type II, Triton X-100, Papain type VII, Tris (hydroxymethyl)

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Abbreviations used: BBMV, Brush border membrane vesicles; BLMV, basolateral and microsomal membrane vesicles; PMSF, phenylmethyl sulphonylfluoride; SDS, sodium dodecyl sulphate; NAI, N-acetyl-imidazole; M_n molecular weight; DEPC, diethylpyrocarbonate; TNM, tetranitromethane.

aminomethane, bovine serum albumin, phenylmethylsulfonylfluoride (PMSF), acrylamide, methelene bis acrylamide, sodium dodecyl sulphate (SDS) molecular weight markers (MW-SDS-Blue), octyl-agarose, *p*-nitrobenzene sulphonyl fluoride (NBSF), N-acetylimidazole (NAI), N-bromosuccinimide, 2-hydroxy-5-nitrobenzyl bromide, diethylpyrocarbonate, l-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide, N-ethylmaleimide, *p*-chloromercuri-benzoate, glycine methyl ester, pyridoxal-5-phosphate, trinitrobenzene sulphonic acid, hydroxylamine, dansylchloride and dansyl amino acids were purchased from Sigma Chemical Co., St. Louis, Missouri USA. DE-52 (DEAE cellulose) was obtained from Whatman Ltd., England. 2-Mercaptoethanol and formic acid were from BDH, England. Folins reagent ammonium sulphate (enzyme grade) and hydroxyapatite were obtained from Sisco Research Laboratories, Bombay. Tetranitromethane was obtained from Spectrochem Ltd., Bombay.

Assay of trehalase

Trehalase was assayed using Tris-glucose oxidase procedure of Dahlqvist (1964) Sucrose and glucoamylase were assayed in a similar manner using sucrose and starch as substrate. Protein was estimated by the procedure of Wang and Smith (1975), a modification of the procedure of Lowry *et al.,* (1951).

Purification of trehalase in the BBMV suspension

Brush border membranes were prepared according to the procedure of Ganapathy *et al.* (1981).

The membrane suspension (l·44mg/ml protein) was incubated at 37°C with 1% Triton X-100 (v/v) with 1 mM P M S F and 2·5 mM EDTA for 60 min. At the end of the time it was centrifuged at 38,000 g for 4 h, in a Sorvall Rc-5B refrigerated centrifuge.

The clear Triton X-100 supernatant containing about 85% trehalase activity was applied to a column of octyl-agarose (1·2 × 2·3 cm, bed volume 2·5 ml) equilibrated with 10 mM sodium phosphate buffer pH 6·8 containing 0·1% Triton X-100.

The column was sequentially washed with 10 bed volumes each, of the equilibrating buffer containing 5% Triton X-100, 50% ethylene glycol and 1 M NaCl. The bound trehalase activity was then eluted with 50 mM Tris-HCI buffer, pH 7 containing 0·1% Triton X-100. The fractions containing trehalase activity was dialysed against 1 mM sodium phosphate buffer pH 6·8 for 24 h with several changes.

The Tris eluate after dialysis was applied to a 1 ml column of DE-52 equilibrated with 10 mM sodium phosphate buffer pH 6·8 containing 0·1% Triton X-l00 Trehalase activity which was held on the column was directly eluted with 200 mM KCl in the equilibrating buffer. The eluate was dialysed and used for further studies.

Purification of trehalase from ELMV fraction

The Ca^{2+} aggregated membranes (BLMV) was prepared according to the method of Danielsen *et al.* (1981) using mucosa as starting material. The membrane fraction

was solubilised with 1% Triton X-100 (v/v) at 10-15 mg protein concentration. The membranes were incubated with the detergent at 37°C for 60 min and centrifuged at 38,000 g for 4 h in Sorvall RC-5B centrifuge.

The clear supernatant was raised to 40% ammonium sulphate concentration. The precipitate was pelleted down at 13,000 *g* for 45 min. The supernatant was raised to 80% ammonium sulphate. The resultant precipitate was pelleted at 13,000 *g,* dissolved in 20 ml of 10 mM sodium phosphate buffer, pH 6·8, containing 0·1% Triton X-100 and dialysed against 1 mM sodium phosphate buffer, pH 6·8, with 4 changes.

The 40-80% precipitate containing the trehalase activity was applied to a column of DE-52 (4×1cm) equilibrated with 10 mM sodium phosphate buffer, pH 6·8, containing 0·1% Triton X-100. The column was washed with 5 bed volumes of the equilibrating buffer and eluted with a linear gradient of 20–200 mM KCl in the equilibrating buffer. The fractions showing trehalase activity were pooled and dialysed against 1 mM sodium phosphate buffer pH 6·8.

The dialysate from above was applied to a column of hydroxyapatite (1·6 × 1·0 cm) equilibrated with 1 mM sodium phosphate buffer, pH 6·8, containing 0·1% Triton X-100. After washing the column with the equilibrating buffer (5 bed volumes), the column was eluted with a linear gradient from 1–50 mM sodium phosphate, pH 6·8, containing 0·1% Triton X-100. The fraction showing trehalase activity were pooled, dialysed against 1 mM sodium phosphate buffer and used for further studies.

Polyacrylamide gel electrophoresis in presence of SDS

Electrophoresis was carried out according to the method of Laemmli (1970) with 12% separating gel. The gels were run at 100 volts till the tracking dye entered the separating gel. The voltage was then increased to 150. The gels were stained with silver according to the procedure of Sammons *et al.* (1981). Prestained molecular weight (M_r) markers used were α_2-macroglobulin (180,000), *ß*-galactosidase (116,000), fructose-6-phosphate kinase (84,000), pyruvate kinase (58,000), fumarase (48,500), lactic dehydrogenase (38,500) and triose phosphate isomerase (26,600).

Polyacrylamide gel electrophoresis

Electrophoresis was carried out by the method of Laemlli (1970), using 9% running and 4% stacking gels at a constant voltage of 150 volts. The gels as well as electrode buffer contained 0·1 % Triton X-100.

Triton X-100 extracts of crude membrane, BBMV and BLMV were applied separately and the run was continued till the tracking dye (bromophenol blue) was run out of gel. After the run, the gels were soaked in 20 mM trehalose in 100 mM sodium phosphate buffer, pH 5·6 and incubated at 37°C for 30 min. The gels were stained for trehalase activity using triphenyltetrazolium chloride in 0·5 N NaOH at 100°C for 10 min (Bhavsar *et al.,* 1983).

N-terminal analysis

The N-terminal amino acid was identified using dansyl chloride by the method of Hartley (1970).

Amino acid composition

Amino acid composition was determined after total hydrolysis of the protein on a Beckman automatic amino acid analyser.

Preparation of crude membrane fraction

Rabbit intestinal mucosa was scraped using a blunt knife, after washing with 1·15% KCl. A 20% homogenate was prepared using Potter-Elvehjam type homogeniser in 10 mM sodium phosphate buffer pH 6·8. The homogenate was centrifuged at 13,000 *g* for 90 min. The pellet was resuspended in the same buffer as above using a waring blender operating at maximum speed.

Extraction with n-butanol

The crude membrane suspension was diluted with buffer to give a protein concentration of 6mg/ml. Equal volume of ice cold n-butanol was added and vortexed for 30 s and cetrifuged at 38,000 *g* for 4 h. The aqueous layer was carefully removed and dialysed against 1 mM sodium phosphate buffer, pH 6·8. Trehalase and sucrose were assayed after dialysis.

Butanol extraction was carried out with Triton X-100 extract of BBMV and BLMV by a similar procedure at protein levels of 0·3 mg/ml and 2·5 mg/ml respectively.

Gel permeation chromatography

This was performed on a column of Sephacryl s-200 (0-8×100 cm, bed volume 200 ml) equilibrated with 10 mM sodium phosphate buffer, pH 6·8, containing 0·1% Triton X-100. The column was calibrated with the following markers: chyrao-trypsinogen A (25,000), albumin (egg) (43,000), albumin (bovine) (66,000), aldolase (158,000) and *ß*-amylase (sweet potato) (200,000).

Heat inactivation

Crude membrane suspension, BBMV and BLMV suspension were kept in constant temperature water bath maintained at 55° and 60°C. Aliquots withdrawn at 20 min intervals were chilled in ice immediately and assayed for trehalase activity.

Immunological studies

Male wistar strain rats were immunised with 100 μg of purified BLMV trehalase with equal volume of Freund's complete adjuvant. After 3 injections at weekly intervals, a booster dose of 200 μg protein was given. Serum was collected by heart puncture 4 days after the booster dose.

Immunodiffusion

Double immunodiffusion (Ouchterlony, 1970) was carried out in 1% agarose

polymerised in 10 mM sodium phosphate buffer pH 7, coated on a microscopic slide. Fifteen μl of antigen (Triton X-100 extracts of BBMV and BLMV) and 15 μl of 1:5 dilution antiserum were applied to the wells and allowed to diffuse at 37°C for 8 h.

Chemical modification using group specific reagents

Trehalase was incubated with the respective reagent at 1 mM concentration for 30 min. The reaction was stopped by addition of excess of free amino acids and chilled on ice. The residual activity was determined immediately.

Protection of modification

Trehalose, the substrate and Tris a competitive inhibitor were used for these studies. The enzyme was incubated under identical conditions as for modification with 40 mM trehalose or Tris for 5 min prior to the addition of the reagent. The incubation was continued for 30 min and then dialysed against 1 mM sodium phosphate buffer, pH 6·8, overnight. Trehalase was assayed for after dialysis.

Reversal of diethylpyrocarbonate modification with hydroxylamine

The enzyme was modified with diethylpyrocarbonate (DEPC) (1 mM) and L-histidine (20 mM) was added in equal volume to stop the reaction. Neutralised hydroxylamine (100 mM) (final concentration 50 mM) was added and the incubation continued at 22°C for 30 min. At the end of the time it was dialysed against 1 mM sodium phosphate buffer, pH 6·8 and assayed for trehalase activity.

First order reaction kinetics of DEPC and tetranitromethane modification

The enzyme (5–10 μg protein) was incubated with varying concentration of respective reagents under conditions described earlier. Aliquots of 20 μl were withdrawn at specific intervals were mixed with equal volume of respective amino acid, and chilled on ice. The residual activity was determined immediately. The theoretical basis for the calculation of the first and the second order rate constants are as described in Levy *et al.* (1963).

Results and discussion

The results summarised in table 1 provide evidence for the presence of two forms of trehalase in the rabbit enterocyte, one sedimenting with the brush border membrane and the other with the Ca^{2+} aggregated basolateral membrane vesicles. Of the total activity in the crude homogenate one half is present each in BBMV and BLMV. Sucrose assayed as a marker for brush border (Semenza, 1986) was almost completely recovered in the BBMV fraction. Ouabain sensitive $Na^{+}K^{+}$ ATPase an established marker for basolateral membrane (Fujita *et al.,* 1972) was found to be present only in the BLMV fraction.

Table 1. Biochemical properties exhibited by the two forms of trehalase.

Experiment	BBMV trehalase	BLMV trehalase
Localization	Brush border membrane	Probably basolateral membrane
n-Butanol	inactivated	Solubilised in active form
$(NH_4)_2SO_4$ fractionation	0–40% saturation	40–80% saturation
M_r on SDS electrophoresis	165,000	97,000
M_r on Sephacryl S-200 gel filtration	165,000	97,000
Stokes radius	50 Å	39 Å
Octyl-agarose hydrophobic chromatography	Bound eluted with 100 mM Tris/HCl, pH 7	Excluded
Heat inactivation at 55°C, 60 min	90% inactivation	25% inactivation
Immunological cross reactivity	Cross reaction	Cross reaction
N-Terminal residues	Tyrosine	Arginine

Electrophoresis of the crude membrane fraction homogenised and solubilised in the presence of PMSF distinguished two well separated bands of trehalase activity.

The BBMV trehalase has been purified to homogenity using a single chromatography step on octyl-agarose. Other purification procedures reported involve 6-8 steps with low final recoveries (Galand, 1984; Yokota *et al.,* 1986). The strong affinity showed by the BBMV trehalase for octyl-agarose appears to be a highly hydrophobic protein. The mechanism by which Tris elutes the bound enzyme is not clear, although it appears that Tris probably acts as a 'deformer' and brings about a conformational change (Shaltiel, 1984), The purified enzyme showing a specific activity of 66·66 was purified 45-fold with respect to brush border vesicles (table 2).

BLMV trehalase was purified using conventional protein purification procedures.

Table 2. Purification of trehalase from the BBMV and BLMV of the rabbit small intestine.

Fraction	Activity (μ/ml)	Total units	Protein (mg/ml)	Total protein (mg)	Specific activity	Fold purification
BBMV						
BBMV suspension	2·1	210	1·44	144	1·46	0
Triton X-100 supernatant	1·0	180	0·481	88	2·08	1·42
Octyl-agarose Tris eluate	1·2	120	0·018	1·8	66·66	45·78
BLMV						
BLMV suspension	1·96	196	24·4	2440	0·077	0
Triton X-100 supernatant	1·10	176	1·30	208	0·84	10·9
40–80% $(NH_4)_2SO_4$ precipitate	6·2	155	3·05	76·2	2·02	26·2
DE-52 eluate	3·9	105·3	0·381	10·3	10·2	133·8
Hydroxypatite eluate	2·34	58·5	0·035	0·87	65·36	822·8

The preparation showed a specific activity of 65·36 and was 822-fold purified over the Ca^{2+} aggregated membranes (table 2). The enzyme was completely excluded from octyl-agarose, the activity emerging in the flow through.

Both the purified proteins migrated as a single band in the presence of SDS. There was a marked difference in the electrophoretic pattern of the two enzymes. Where as the BBMV trehalase appears to be a homodimer of M_r 78,000 daltons, the BLMV trehalase was found to be a monomer of M_r 97,000 daltons. The M_r and subunit structure of BBMV trehalase correlated well with already reported values (Galand, 1984; Yokota *et* α/., 1986).

Amino acid composition showed a higher amount of hydrophobic amino acids in BBMV trehalase than BLMV enzyme (table 3). This may explain the affinity

Table 3. Amino acid composition of BBMV and BLMV trehalase (residues/mol[a].)

Amino acid	BBMV trehalase	BLMV trehalase
Aspartate	155	77
Threonine	101	68
Serine	107	60
Glutamate	144	73
Proline	75	51
Glycine	170	78
Alanine	154	78
Cystine	14	8
Valine	109	92
Methionine	28	16
Isoleucine	67	41
Leucine	152	70
Tyrosine	50	34
Phenylalanine	66	35
Histidine	12	9
Lysine	92	45
Arginine	57	76
Hydrophobic amino acids[b]	548	316

[a]Amino acids, residues/mol with respect to, histidine.
[b]Phenylalanine, leucine, isoleucine, valine and alanine are taken as hydrophobic amino acids.

exhibited by the enzyme for octyl-agarose. The two trehalases differ in sensitivity to heat (figure 1) and n-butanol. Double immunodiffusion showed a single continuous precipitin line indicating that the proteins are immunologically identical.

Chemical modification of BBMV trehalase

BBMV trehalase was inactivated by DEPC, a histidine modification reagent (Burstein *et al.,* 1974; Miles, 1977; Daron and Aull, 1982) and the 3 tyrosine modification reagents [tetranitromethene (TNM), *p*-nitrobenzene sulphonyl fluoride (NBSF) and N-acetylimidazole (NAI)] (Riordan and Vallee, 1972a, b; Glazer *et al.,* 1976; Bunning *et al.,* 1978; Liao, 1982). The other reagents had no effect on activity.

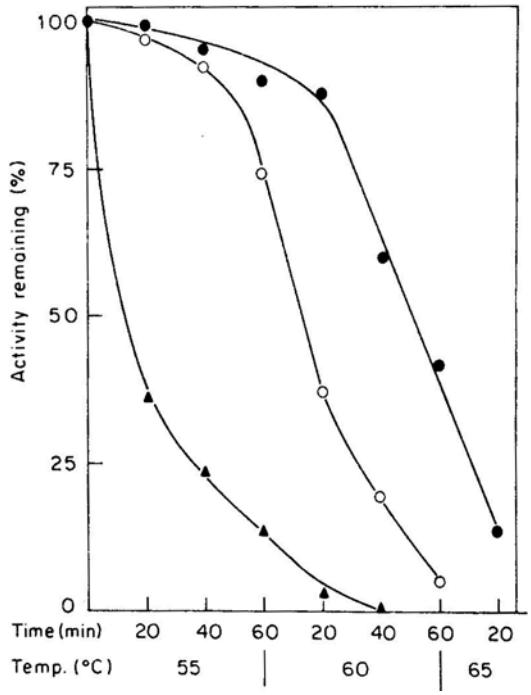

Figure 1. Heat inactivation profiles of crude membrane fraction (●), BLMV suspension (O) and BBMV suspension (▲),

Inactivation of BBMV trehalase by DEPC

A first order plot was constructed from the inactivation curves obtained at different concentrations of DEPC (300–500 μM). The plot of log K_{app} as a function of log (DEPC) concentration gave a slope of 1·04, indicating that reaction with a single molecule of DEPC was probably sufficient to inactivate the enzyme (figure 2). The second order rate constant was calculated to be 0·076 min^{-1} m M^{-1} .

Trehalose the substrate, protected the enzyme from inactivation at 40 mM concentration. The modification by DEPC was reversed by treating the modified enzyme with hydroxylamine (Boopathy and Balasubramanian, 1985; Burstein *et al.,* 1974). A nonsepecific modification of tyrosine or lysine is not reversed by hydroxylamine (Burstein *et al.,* 1974; Boopathy and Balasubramanian, 1985; Baskaran and Balasubramanian, 1987). Reversal of DEPC modification of BBMV trehalase was to the extent of 79%.

Carbethoxy histidine shows a specific absorption at 242 nm. Absorption spectra of the modified enzyme could not be recorded due to the presence of Triton X-100 in the enzyme. Keeping the enzyme in 0·1% Triton X-100 was absolutely necessary as the enzyme rapidly lost activity in the absence of the detergent.

Inactivation of BBM V trehalase by tyrosine specific reagents

All 3 tyrosine modification reagents [TNM (1 mM), NBSF (1 mM) and NAI

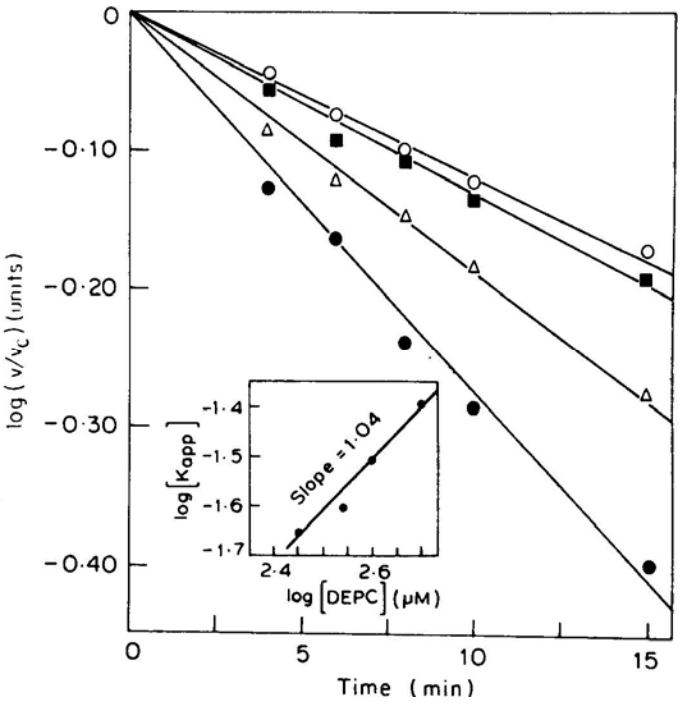

Figure 2. First order plots of inactivation of trehalase associated with BBMV by DEPC. Inactivation was carried out at different concentrations of DEPC (O, 300 μM; ■, 350 μM; Δ, 400 μM; ●, 500 μM).v/v_c the ratio of remaining activity to activity of uninhibited enzyme. Inset: Second order plot of the pseudo first order rates (K_{app}) of inactivation of different concentrations of DEPC (300–500 μM).

(10 mM)] inactivated the enzyme (Riordan and Vallee, 1972; Glazer *et al.,* 1976; Liao, 1982).

TNM was used to study the kinetics of inactivation due to its high specificity for tyrosine at pH 8 (Riordan and Vallee, 1972a, b). The rate of inactivation, at different concentrations of TNM (2–10 mM) was determined as described. The plot of log K_{app} against log (TNM) concentration gave a straight line with a slope of 0·9, indicating that interaction with a single molecule of TNM resulted in inactivation of the enzyme (figure 3). Trehalose, the substrate and Tris a competitive inhibitor protected the enzyme from inactivation at 40 mM concentration.

Spectral studies showed a λ_{max} at 350 nm. Although nitroformate ion, a byproduct of TNM, absorbs at 350 nm, appearance of a 350 nm peak can serve as a qualitative gauge for TNM modification (Riordan and Vallee, 1972a, b). The second order rate constant for TNM modification of BBMV trehalase was 0·00526 mM^{-1} min^{-1}.

The above results indicate the possible presence of one histidine and one tyrosine at the active site of BBMV trehalase.

Chemical modification of BLMV trehalase

BLMV trehalase showed loss of activity upon modification by DEPC alone. All other reagents failed to inactivate the enzyme.

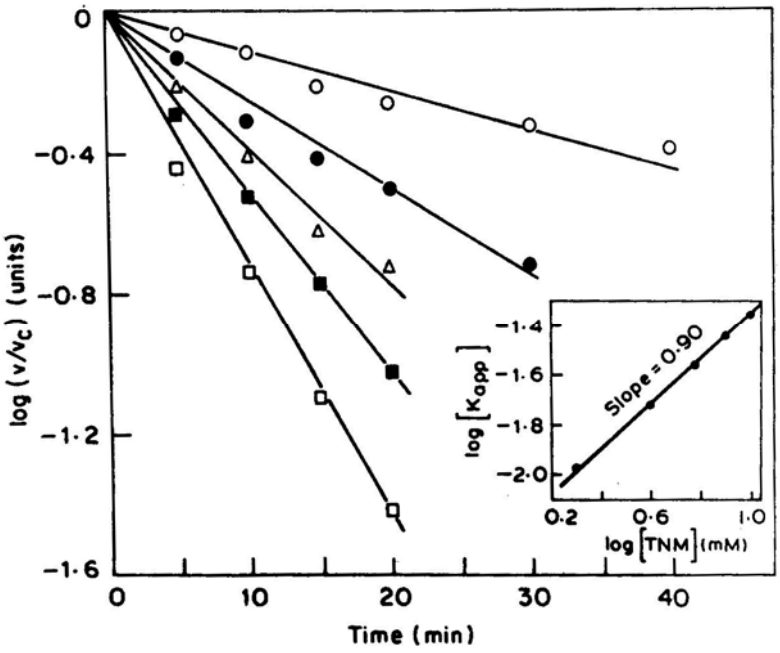

Figure 3. First order plots of inactivation of trehalase associated with BBMV by TNM Inactivation was carried out at different concentrations of TNM (O, 2 mM; ●, 4 mM; Δ, 6mM; ■, 8 mM; □, 10 mM). v/v_c is the ratio of remaining activity to activity of uninhibited enzyme. Inset: Second order plot of the pseudo first order rate (K_{app}) of inactivation at different concentrations of TNM (2–10 mM).

Inactivation of BLM V trehalase by DEPC

A first order plot was constructed from the inactivation curves obtained at different concentrations of DEPC (600 μM to 1 mM).

The plot of log Ka $_{PP}$ against log (DEPC) concentration gave a slop of 1·62 indicating the possible need for two molecules of DEPC to inactivate the enzyme (figure 4). The second order rate constant was found to be 0·03190 mM^{-1} min^{-1} Trehalose at 40 mM concentration protected the enzyme from modification by DEPC.

Since the modification was -reversed by treating the modified enzyme with hydroxylamine at neutral pH, it is not modifying a tyrosine or lysine residue non-specifically (Burstein *et al.,* 1974; Boopathy and Balasubramanian, 1985; Baskaran and Balasubramanian, 1987). The spectral studies could not be carried and due to the presence of Triton X-100 in the enzyme.

The results presented indicate that the active sites of the two forms of trehalase are possibly different. The two trehalases from rabbit small intestine showed a significant difference from trehalase reported from other sources, in terms of the absence of a sulphydryl group at the active site (Nakano and Sacktor, 1984; Chen, *et al.,* 1987; Sanker and Sivakami, 1988).

Sacktor (1968) had reported that trehalase is possibly involved in sugar transport across the intestinal and renal brush border. In the light of trehalose being a sugar

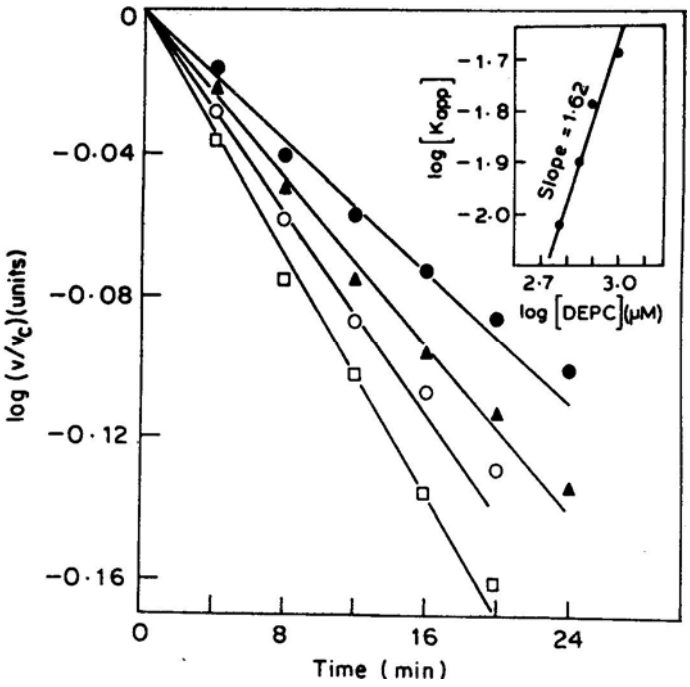

Figure 4. First order plots of inactivation of trehalase associated with BLMV by DEPC. Inactivation was carried out at different concentrations of DEPC (O, 600 μM; ▲, 700 μM; ●, 800 μM;□, 1000 μM). v/v_c is the ratio of remaining activity to activity of uninhibited enzyme. Inset: Second order plot of pseudo first order rates (K_{app}) of inactivation at different concentrations of DEPC (600–1000 μM).

of little dietary significance in mammals, it is possible that, trehalase in mammalian intestine is involved in other physiological functions. The difference in the active site residues between the two forms of trehalase may possibly be serving different functions at the brush border and the basolateral membrane.

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