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# Purification and Properties of Trehalase from Hemolymph of the Pupae of Blowfly, *Aldrichina grahami*

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## Abstract

Trehalase (E.C.3.2.1.28) has been isolated from hemolymph of the pupae of blowfly, *Aldrichina grahami*, and purified approximately 550-fold by chromatography with CM-cellulose, DEAE-cellulose, Sephadex G-150 and ConA-Sepharose. The molecular weight estimated from Sephadex G-200 chromatograpy was 80,000. The enzyme was specific for trehalose. The purified enzyme showed 1.7 mM as *Km* for trehalose, 5.6 for optimum pH and 12.6 kcal/moles as activation energy. The enzyme was inhibited by divalent cation such as $Cu^{2+}$, $Mn^{2+}$, $Zn^{2+}$ and $Mg^{2+}$. The enzyme was inhibited by sucrose, fructose and mannose.

## Introduction

The presence of the enzyme, trehalase (α, α′-trehalose-1-glucohydrolase) which hydrolyzes trehalose to 2 molecules of glucose, has been found in microorganisms, plants, and animals[1-3]. In many cases, the location and the probable function of the enzyme may correlate well with those of the "common" hydrolytic enzymes, but on the other hand it is also found in different place, such as mammalian kidney[4], plant pollen[5], adult blowfly muscle mitochondria[6], and adult blowfly blood[7].

Biochemical properties of trehalase have been reported for many of insect species[8-12]. The recent works on this enzyme from insects have been focussed on two separate but somewhat related problemes: the regulation of trehalase activity in relation to the physiological utilization of the disaccharide; the presence, distribution, and function of trehalase isozymes in insect tissues. Two different types of trehalase have been identified in insects: (1) a soluble enzyme, mainly located in intestinal tissues, with *Km* value of $10^{-4}$ M and optimum pH at 5.5 – 5.7; and (2) a muscle membrane-bound enzyme with *Km* value of about $10^{-3}$ M and optimum pH 6 – 6.5[13]. Both types occur in a single insect[14, 15]. Trehalase of adult blowfly has been studied by several workers.[6, 7, 13, 16, 17]. However, there has been no report on the enzyme from larval blowfly. This paper describes the purification and the properties of the trehalase from pupal hemolymph of the blowfly, *Aldrichina grahami*.

## Materials and Methods

*Insect*

Blowflies, *Aldrichina grahami*, were reared aseptically on semi-synthetic diets at 25°C as described by Miura et al.[18]. The pupariation of larva occurred on the seventh day after hatching. Pupae 8-days old were used.

*Chemicals*

Standard proteins for the estimation of molecular weight were obtained from Boehringer/Mannheim. DEAE-cellulose, CM-cellulose, Sephadex G-150, Sephadex G-200 and ConA-Sepharose were purchased from Pharmacia Fine Chemicals.

*Assay of enzyme*

Trehalase activity was measured by determinating glucose production when trehalose was hydrolyzed in the presence of the enzyme[19, 20]. The glucose contents were determined by the method of Nelson and Somogyi. The assay mixture contained 0.05 ml of 20 mM acetate buffer (pH 5.6), 0.1 ml of 20mM trehalose, and 0.05 – 0.1 ml of enzyme solution. Distilled water was added to make the total volume to be 0.5 ml. The mixture was incubated for 15 min at 30°C, and the reaction was stopped by the addition of Somogyi reagent.

One unit of enzyme is defined as the amount of enzyme which liberates 1 μmole of glucose from trehalose per min under the standard assay conditions. The specific activity is the number of units of enzymes per mg of protein.

*Determination of protein*

Protein contents were estimated according to the method of Lowry et al.[21] with bovine serum albumin as the standard.

*Molecular weight of the enzyme*

The molecular weight of trehalase was estimated by using a Sephadex G-200 column (1.5 × 85 cm) calibrated with following globular protein standards: Ferritin (450,000), catalase (240,000), bovin serum albumin (68,000), hen egg albumin (45,000), chymotrypsinogen A (25,000) and cytochrome C (12,500).

## Results and discussion

*Changes of trehalase activity*

The trehalase activites in whole insect, gut and hemolymph were investigated. The results 4, 6 and 8 days after hatching were shown in Table 1. The activities in hemolymph appeared at 6 days and reached its maximum 8 days after hatching, which is just after pupation.

Table 1. Trehalase activity in tissue of larvae

| Tissue | Activity (units/insect) | | |
|---|---|---|---|
| | 4 days | 6 days | 8 days |
| Whole insect | 0.005 | 0.078 | 0.324 |
| Hemolymph | – | 0.042 | 0.117 |
| Gut | 0.009 | 0.029 | 0.025 |

*Purification of trehalase*

All steps were conducted at 1 – 5°C. Pupae some 12 – 24 hr after larval-pupal

ecdysis were washed in distilled water, loosely wiped off on filter paper, and excised with a razor in a 2/3 vol of cold saline solution containing 120 mM NaCl, 5 mM KCl, 0.5 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.2 mM $NaHCO_3$, 0.13 mM $NaH_2PO_4$, and 5 mM MES buffer, pH 6.6. The saline containing hemolymph centrifuged at 25,000 × g for 20 min. The supernatant was further centrifuged at 105,000 × g for 30 min to remove any cell organella.

The supernatant was fractionated with ammonium sulfate. The precipitate between 25 and 45 % saturation of ammonium sulfate was collected by centrifugation at 15,000 × g for 20 min. The precipitate was dissolved in a small volume of 10 mM acetate buffer, pH 5.6, containing 0.1 mM mercaptoethanol and dialyzed against the same buffer overnight at 4°C.

The dialysate was centrifuged at 20,000 × g for 15 min. The supernatant was applied to a CM-cellulose column (3.5 × 35 cm) equilibrated with the same buffer. The elution was carried out with a linear gradiedt of potassium chloride (0–0.3 N) in the same buffer. The active enzyme fraction was pooled and concentrated with ultrafiltration. The concentrated enzyme was dialyzed against 10 mM Tris-HCl buffer, pH 8.0, containing 0.1 mM mercaptoethanol overnight at 4°C.

The enzyme solution was applied to a DEAE-cllulose column (3 × 35 cm) equilibrated with 10 mM Tris-HCl buffer, pH 8.0, containing 0.1 mM mercaptoethanol and eluted with a linear gradient of potassium chloride (0 – 0.4 N) in the same buffer. Active fraction was pooled and concentrated with ultrafiltration, and then chromatographed on a Sephadex G-150 column eqilibrated with 0.5 mM potassium phosphate buffer, pH 6.8.

For the final purification the dialysis and concentration steps were repeated. The active fraction was applied to a ConA-Sepharose column (0.5 × 5.7 cm) eqilibrated with the buffer. The enzyme was not absorbed. The active fraction was pooled, concentrated and used for the characterization of the enzyme.

The results of the purification are summarized in Table 2. At the final stage of purification, the specific activity of trehalase was 16.5 units/mg protein, representing 550-fold purification.

Table 2. Purification of trehalase from *Aldrichna grahami*

| Fraction | Total Protein (mg) | Total Activity (units) | Specific Activity (units/mg) | Recovery (%) | Fold |
|---|---|---|---|---|---|
| Hemolymph | 4800 | 145 | 0.03 | 100 | 1 |
| $(NH_4)_2SO_4$ | 714 | 78.4 | 0.11 | 55 | 4 |
| CM-Cellulose | 80 | 52.7 | 0.66 | 35 | 23 |
| DEAE-Cellulose | 6.8 | 29.1 | 4.28 | 20 | 148 |
| Sephadex G-150 | 1.1 | 16.9 | 14.9 | 11 | 514 |
| ConA-Sepharose | 0.8 | 13.2 | 16.5 | 9 | 550 |

*Properties of trehalase*

*Effect of the hydrolysis time and the enzyme concentration*: The hydrolysis of trehalase was linear with respect to the hydrolysis time and protein concentration.

*Specificity of the enzyme*: The following compounds were assayed for trehalase specificity; maltose, sucrose, lactose, cellulose, raffinose, mellibiose, stachyose, methyl-D-glucose, maltotriose. The enzyme appears fairly specific for trehalose, and shows no hydrolytic activity for any other carbohydrates tested.

*Effect of pH*: The effect of pH upon the hydrolysis of trehalose was studied in acetate, phosphate and Tris-HCl buffer in the range of 4 – 9 (Fig. 1). The optimum pH of the trehalase was 5.6 in acetate or phosphate buffer. This value is close to that of trehalase from blowfly (adult)[16], fruitfly (adult)[8], wax moth (larval stage)[22], fungi[23, 24], yeast[25], rat (intestine)[26] and mice (kindney)[27].

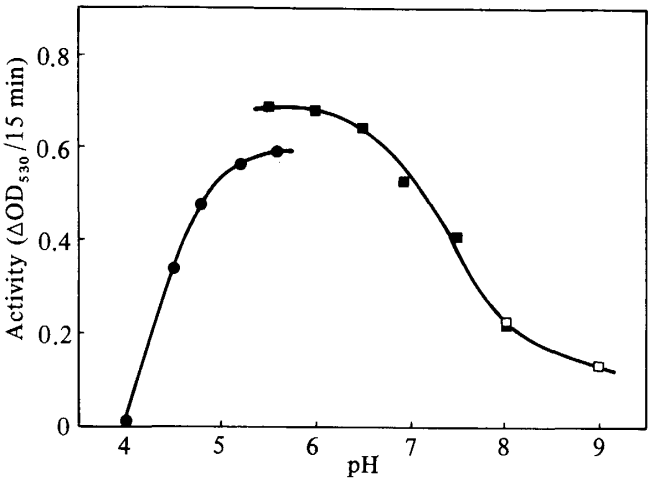


Fig. 1. Effect of pH on trehalase activity
—●— Acetate, —■— Phosphate, —□— Tris-HCl

*Effect of temperature*: The effect of temperature on the rate of hydrolysis of trehalose was determined in the range of 25 – 50°C (Fig. 2). The optimum temperature for catalysis was 45°C. Inactivation of the enzyme proceeded rapidly above this temperature. Arrhenius plots for the trehalase activity was shown in Fig.3. Activation

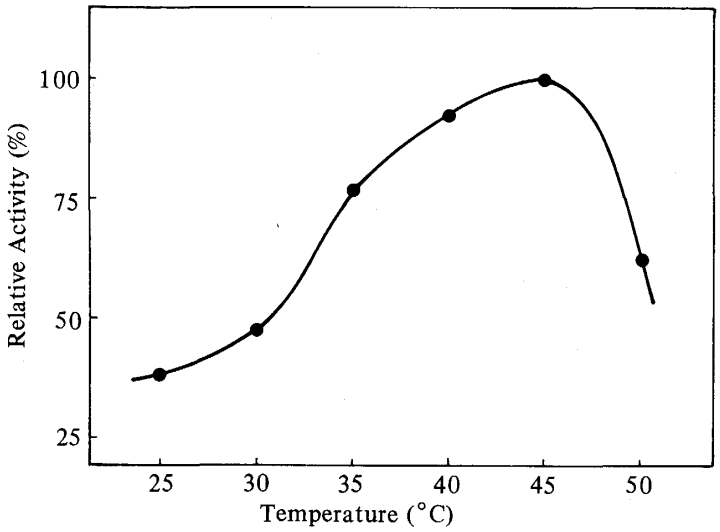


Fig. 2. Effect of temperature on trehalase activity

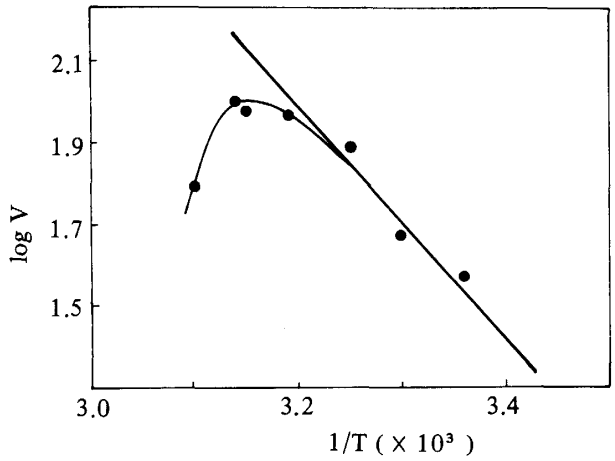


Fig. 3. Arrenius plot for trehalase

energy calculated from the plots was 12.6 kcal/mol. Values of activation energy of the enzyme from other sources have been known from 7 to 18 kcal/mol[28].

The purified enzyme preparation was stable at 0 – 5 °C for over 3 months and showed no loss of activity.

*Effect of substrate concentration*: The effect of substrate concentrations on the catalytic rate of trehalase was determined at pH 5.6 and a typical Michaelis-Menten relationship was obtained. The *Km* value calculated from a Lineweaver-Burk plot was found to be 1.7 mM (Fig. 4). The *Km* values for the trehalase from blowfly[17], fruit-fly[8], honeybee[29], silkworm[30] and cockroach[31], are similar to that of the trehalase of pupal hemolymph.

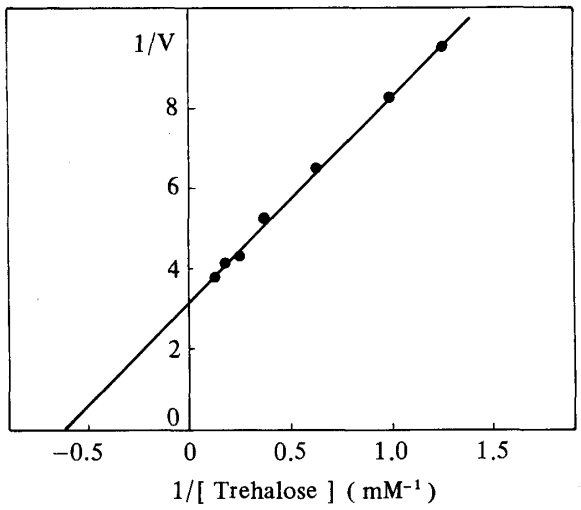


Fig. 4 Lineweaver Burk plot of trehalase

*Effect of divalent cation*: The trehalase solubilized from *Mycobacterium smegmatis* membrane shows a requirement for $Mg^{2+}$, with the optimal concentration of 2.5

mM. The effect of some cations on trehalase activity is illustrated in Table 3. Divalent cations, especially $Hg^{2+}$, $Cu^{2+}$, $Mn^{2+}$, $Zn^{2+}$ and $Mg^{2+}$, showed some inhibitory effects. It is similar to the fact that the enzyme of silkworm was inhibited by divalent cation such as $Mn^{2+}$, $Cu^{2+}$ and $Zn^{2+}$.[30]

Table 3. Effects of various cations on trehalase activity
Compuonds were added to the assay mixture in the concentrations indicated

| Compound | concn. (mM) | Activity (%) |
|---|---|---|
| $CuSO_4$ | 2.0 | 15 |
| $ZnSO_4$ | 2.0 | 59 |
| $CaSO_4$ | 2.0 | 94 |
| $MgSO_4$ | 2.0 | 73 |
| $MnCl_2$ | 2.0 | 54 |
| $Li_2SO_4$ | 2.0 | 94 |
| $(NH_4)_2SO_4$ | 2.0 | 91 |
| $HgCl_2$ | 0.2 | 0 |

*Effect of saccharides*: The effects of several saccharides and their derivertives on trehalase activity were estimated. As shown in Table 4, the activities were inhibited by sucrose and fructose. The inhibition by sucrose was competitive (Fig. 5). Sucrose inhibitions have been reported on the trehalase of silkmoth[14], honeybee[29], cockroach[31], ant[32]. The significance of these inhibitions in terms of the metabolism of trehalose is not clear.

Table 4. Effect of various saccharides and derivertives on trehalase activity
Compounds were added to the assay mixture in the final concentration of 3mM.

| Saccharide | Activity (%) | Derivertive | Activity (%) |
|---|---|---|---|
| Glucose | 97 | $CH_3$-Glu | 104 |
| Galactose | 99 | $CH_3$-Man | 100 |
| Mannose | 99 | Glu-$NH_2$ | 102 |
| Fructose | 82 | Sorbitol | 103 |
| Arabinose | 96 | Inositol | 97 |
| Xylose | 96 | Mannitol | 89 |
| Sucrose | 75 | G-6-P | 109 |
| Maltose | 105 | G-1-P | 99 |
| Lactose | 105 | F-6-P | 105 |
| Cellobiose | 108 | F-1, 6-P | 89 |
| Stachyose | 106 | UDPG | 101 |

$CH_3$-Glu: Methylglucose, $CH_3$-Man : Methylmannose
Glu-$NH_2$ : Glucosamine, G-1-P : Glucose-1-phosphate
G-6-P : Glucose-6-phosphate,
F-6-P : Fructose-6-phosphate
F-1, 6-P : Fructose-1, 6-diphosphate
UDPG : Uridine diphosphate glucose

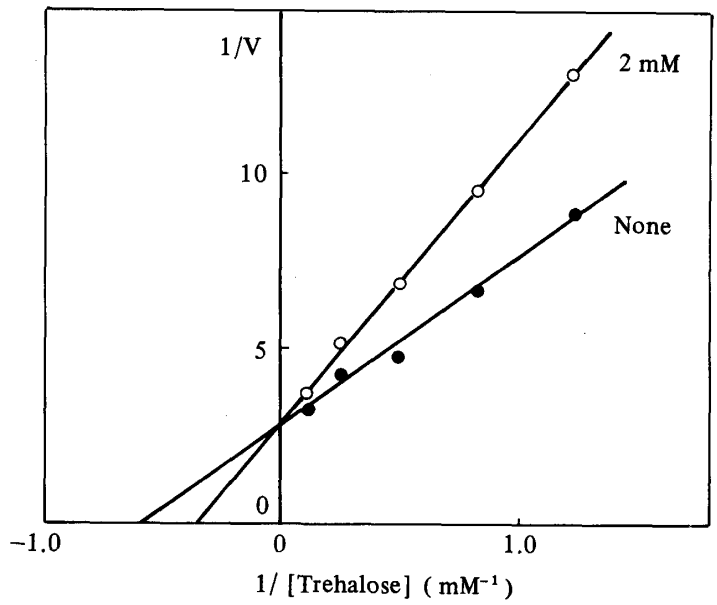


Fig. 5. Effect of Sucrose on trehalase activity

*Effect of SH-blocking reagent*: The SH-blocking reagents such as *p*-CMB and $HgCl_2$ partially inhibited the trehalase activity, and the inhibition was of noncompetitive type. Mercaptoethanol completely protected this inhibition (Table 5). Thus, it seems possible that SH groups are important for the activity and the retention of conformation of the enzyme molecule.

Table 5. Effect of SH-reagent on trehalase activity

| SH-reagent | Activity (%) |
|---|---|
| 0.10 mM Mercaptoethanol | 133 |
| 0.10 mM p-CMB | 42 |
| 0.02 mM p-CMB | 48 |
| 0.02 mM p-CMB + 0.10 mM Mercaptoethanol | 134 |

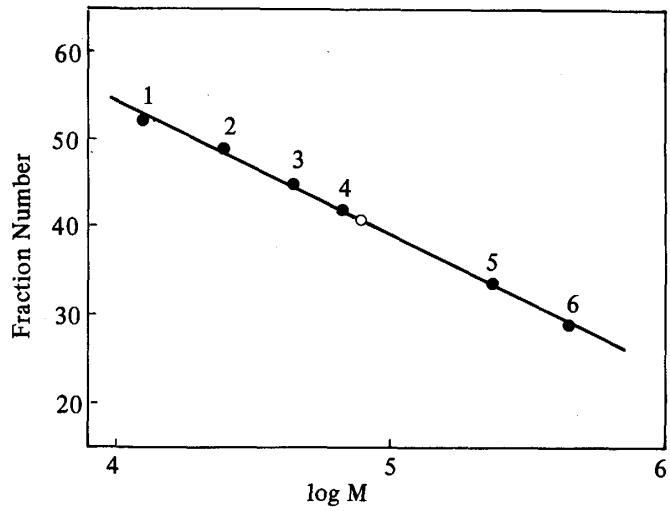


Fig. 6 Estimation of molecular weight of trehalase by gel filtration on Sephadex G-200
1. Cytochrome C 2. Chymotrypsinogen 3. Hen egg albumin
4. Bovin serum albumin 5. Catalase 6. Ferritin

*Estimation of molecular weight*: Fig.6 shows the molecular weight of trehalase as determined by gel filtration on a calibrated Sephadex G-200 column (1.5 × 85 cm). From this result, the estimated molecular weight is 80,000. This value is comparable to those repoted for other insect trehalase.[15, 17, 31]

## References


1) WYATT, G. R. (1967). Biochemistry of sugars and polysaccharides in insects. *Adv. Insect Physiol.*, **4**, 287-360.
2) ELBEIN, A. D. (1974). The metabolism of α, α′-trehalose. *Adv. Carbohydr. Chem. Biochem.*, **30**, 227-256.
3) THEVELEIN, J. M. (1984). Regulation of trehalase mobilization in fungi., *Microbiological. Rev.*, **48**, 42-59.
4) SACKTOR, B. (1968). Trehalase and the transport of glucose in the mammalian kidney and intestine. *Proc. natl. Acad. Sci. U.S.A.*, **60**, 1007-1014.
5) GUSSIN, A. E. S., MCCORMACK, J. H., WAUNG, L. Y-L. and GLUCKIN, D. S. (1969). Trehalase: A new pollen enzyme. *Plant Physiol.*, **44**, 1163-1168.
6) REED, W. D. and SACKTOR, B. (1971). Localization of trehalase in flight muscle of the blowfly, *Phormia regina. Arch. Biochem. Biophys.*, **145**, 392-401.
7) FRIEDMAN, S. (1961). Inhibition of trehalase activity in hemolymph of *Phormia regina. Arch. Biochem. Biophys.*, **93**, 550-554.
8) MARZLUF, G. A. (1969). Studies of trehalase and sucrase of *Drosophila melanogaster. Arch. Biochem, Biophys.*, **134**, 8-18.
9) HUBER, R. E. and LEFFBVRE, Y. A. (1971). The purification and some properties of soluble trehalase and sucrase from *Drosophila melanogaster. Can J. Biochem.*, **49**, 1155-1164.
10) DAHLMANN, D. L. (1971). Purification and properties of trehalase from tobacco hornworm larvae. *J. Insect Physiol.*, **17**, 1677-1687.
11) TOLBOT. B. G. and HUBER, R. E. (1975). An electrophoretic and pH comparison of soluble trehalases of several insect species. *Comp. Biochem. Physiol.*, **53B**, 367-369.
12) BARGILLO, T. A., and GROSSFIELD, J. (1979). An electrophretic and biochemical comparison of soluble trehalase from *Drosophila melanogaster* and *D. simulans. Insect Biochem.*, **9**, 323-329.
13) DUVE, H. (1972). Purification and properties of trehalase isolated from the blowfly *caliphora erythrocephala. Insect Biochem.*, **2**, 445-450.
14) GUSSIN, E. S. and WYATT, G. R. (1965). Membrane-bound trehalase from cecropia silkmoth muscle. *Arch. Biochem. Biophys.*, **112**, 626-634.
15) YANAGAWA, H. A. (1971). Purification and properties of trehalase from larval muscle and midgut of the silkworm, *Bombyx mori. Insect Biochem.*, **1**, 102-112.
16) FRIEDMAN, S. (1960). The purification and properties of trehalase isolated from *Phormia regina. Arch. Biochem. Biophys.*, **87**, 252-258.
17) FRIEDMAN, S. (1975). Multiple forms of trehalase in *Phormina regina.* Partial purification, tissue, specificities, and some kinetic properties of adult enzyme. *Insect Biochem.*, **5**, 151-164.
18) MIURA, K., TAKAYA, T. and KOSHIBA, K. (1967). The effect of biotin deficiency on the biosynthesis of fatty acids in blowfly, *Aldrichina grahami,* during metamor-

phosis under aseptic conditon. *Arch. Int. Physiol. Biochem.*, **75**, 65-76.
19) NELSON, N. (1944). A photometric adaptation of the Somogyi for the determination of glucose. *J. Biol. Chem.*, **193**, 265-275.
20) SOMOGYI, M. (1952). Notes on sugar determination. *J. Biol. Chem.*, **195**, 19-23.
21) LOWRY, O. H., ROSEBROUCH, N. J., FARR, A. L. and RANDALL. R. L. (1944). Protein measurment with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265-275.
22) KALL, G. F. and RIEDER, S. V. (1958). The purification and properties of trehalase. *J. Biol. Chim.*, **230**, 691-698.
23) CHANG, P. L. Y., and TREVITHICK, J. R. (1972). Release of wall-bound invertase and trehalase in *Neurospora crassa* by hydrolytic enzymes. *J. Gen. Microbiol.*, **70**, 13-22.
24) KILLICK, K. A. (1983). Trehalase from the cellular slime mold *Dictyostelium discoideum*: Purification and characterization of the homogeneous enzyme from *Myxamoebae. Arch. Biochem. Biophys.*, **222**, 561-567.
25) PANEK, A. and SOUZA, N. O. (1964). Purification and properties of Baker's yeast trehalase. *J. Biol. Chem.*, **239**, 1671-1673.
26) SASAJIMA, G., KAWACHI, T., SATO, S. and SUGIMURA, T. (1975). Purification and properties of $\alpha$, $\alpha'$-trehalase from the mucosa of rat small intestine. *Biochim. Biophys. Acta*, **403**, 139-146.
27) TENAN, M. N., OLIVEIRA, C. P. H. DH. and PANEK, A. D. (1979). Mouse kidney trehalase: Purification and properties. *An. Acad. brasil Cienc.*, **51**, 151-158.
28) KILLICK, K. A. (1980). Coupled, continuous and discontinuous fluorometric assays for trehalase activity. *Anal. Biochem.*, **105**, 291-298.
29) LEFEBVRE, Y. A. and HUBER, R. E. (1970). Solubilization, purification, and some properties of trehalase from honeybee (Apis mellifera). *Arch. Biochem. Biophys.*, **140**, 514-518.
30) SHIMADA, S., KAMADA, A. and ASANO, S. (1980). The cocoon trehalase of the silkworm, *Bombyx mori. Insect Biochem.*, **10**, 49-52.
31) GILBY, A. R., WYATT, S. S. and WYATT, G. R. (1967). Trehalase from the cockroach, *Blaberus discoidalis*: Activation, solubilization, and properties of the muscle enzyme and some properties of the intestine enzyme. *Acta Biochem. Pol.*, **14**, 83-100.
32) PAULSEN, R. (1971). Characterization of trehalase from labial gland of ants. *Arch. Biochem. Biophys.*, **142**, 170-176.