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Purification and Characterization of Trehalase from *Artemia* Embryos and Larvae

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ABSTRACT—A soluble alkaline trehalase was purified from embryos and larvae of the brine shrimp, *Artemia*, by acetone treatment, chromatography on columns of DEAE-Sepharose Fast Flow, Con A-Sepharose and TSKgel AF-Chelate TOYOPEARL 650M, and preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The purified enzyme subjected to SDS-PAGE showed a single protein band, suggesting a molecular mass of 70,000 Da. The enzyme exhibited an apparent molecular mass of 58,000 Da on gel filtration. Endoglycosidase H digestion of the enzyme did not affect the activity of the trehalase, and resulted in a molecular mass of 66,000 Da on SDS-PAGE. The isoelectric point of the enzyme was estimated by gel electrofocusing to be approximately 4.7~4.8. The catalytic activity showed a maximum at pH 8.0, and a specific activity of 140 μmoles glucose liberated from α,α-trehalose $min^{-1} \times mg^{-1}$ was observed at 30°C. The Km value for α,α-trehalose was estimated to be 8.4 mM. Among the eleven oligosaccharides and two α-glucoside derivatives studied, the enzyme hydrolyzed only α,α-trehalose . The enzyme was maximally active at 55°C and had an activation energy of 55.8 kJ × mol^{-1}. The enzymatic reaction was completely inhibited by 0.1 mM $HgCl_2$. The activity of the purified enzyme was inhibited by 1 mM EDTA in the presence of 50 mM phosphate buffer, and the additions of appropriate amounts of $MnCl_2$, $MgCl_2$ and $CaCl_2$ to the reaction mixture each protected the activity.

INTRODUCTION

Trehalose, a nonreducing disaccharide (α-D-glucopyranosyl (1→1)-α-D-glucopyranoside) has been found in diverse organisms including crustaceans (Elbein, 1974). Trehalose is known to be a main carbohydrate in encysted dry embryos of the brine shrimp, *Artemia*, which are arrested at the gastrula stage and in a state of dormancy (Dutrieu, 1960; Clegg, 1962). This major carbohydrate, which is not supplied by the parent but is synthesized by the embryo itself entering dormancy (Clegg, 1965), accounts for about 15% of the dry weight of the cysts (Clegg, 1962).

The dehydrated encysted embryos resume development upon rehydration and aerobic incubation with an adequate salinity, coinciding with a decrease in the trehalose level of the cysts accompanied by a corresponding increase in the contents of glycogen and glycerol. Thus, this disaccharide is used as a substrate of respiration, providing a large portion of the cellular energy required for the further development of the embryo (Clegg, 1964; Ewing and Clegg, 1969).

The early change in the metabolic levels mentioned above suggests a marked fluctuation of trehalase activity during the early development of *Artemia*. The activity of trehalase (α,α-trehalose glucohydrolase, EC 3.2.1.28), which hydrolyzes α,α-trehalose into two glucose moieties, has been observed in the embryos and larvae of *Artemia*. However, Boulton and Huggins (1977) found no increase of trehalase activity measured at pH 7 during *Artemia* development. Ballario *et al.* (1978) reported that encysted dry embryos of *Artemia* contained a trehalase that was optimally active at pH 5.6, present in insoluble form and which could be solubilized by deoxycholate treatment at high ionic strength and sonication. The reported enzyme hydrolyzed not only trehalose but also cellobiose and lactose, suggesting low specificity for trehalose or non-homogeneity. A study of a partially purified trehalase of the hydrated embryos of *Artemia* (Hand and Carpenter, 1986) found that this enzyme was soluble and in two active forms that interconverted when exposed to physiological transitions in pH. Vallejo (1989) showed the change of trehalase activity during the development of *Artemia* as well as the subcellular localization of the enzyme. The enzyme was thought to be associated with yolk granules and could be solubilized by 1% Triton X-100. The highest activity of the enzyme was detected in late nauplius.

However, most of the above authors used crude enzyme solution. Reliable methods for the detection and purification of trehalase have not yet been established, nor has anyone detected peak activity of the trehalase in the early stage of the development, although a decrease in the trehalose level of *Artemia* in the early stage of the development was reported by Clegg (1964), Ewing and Clegg (1969), Boulton and

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Huggins (1977), and Vallejo (1989).

To elucidate the dynamic activity and molecular characteristics of the trehalase in *Artemia* development, we purified the soluble alkaline trehalase from *Artemia* embryos and larvae, and investigated the trehalase activity during the development of *Artemia*. This report describes the purification and the properties of the trehalase from *Artemia* embryos and larvae as well as the developmental changes of the trehalase activity of *Artemia*.

MATERIALS AND METHODS

Reagents

DEAE-Sepharose Fast Flow, Con A-Sepharose, and the HiTrap™ Desalting column, Pharmalyte 3-10 and its broad pI calibration kit (pH 3-10) were purchased from Pharmacia Biotech. (Uppsala, Sweden). TSKgel AF-Chelate TOYOPEARL 650 M and the TSKgel G3000SW column were obtained from TOSOH (Tokyo, Japan). Endoglycosidase H (*Streptomyces plicatus*) was from Seikagaku Kogyo (Tokyo). Marker proteins for molecular weight calibration were from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade and purchased from Nacalai Tesque (Kyoto, Japan).

Animals

Dehydrated cysts of *Artemia* were purchased from Japan Pet Drugs Co. (Tokyo, and Los Angeles, CA). *Artemia* cysts were from the Great Salt Lake in Utah, USA. The dry cysts (10 g) were treated with 100 ml of 7% antiformin for 1.5 hr at 4°C (Nakanishi *et al.*, 1962). The treated cysts were washed with ice-cold distilled water, and immersed in the cold distilled water for more than 2.5 hr. The *Artemia* cysts thus prepared were incubated in 1,000 ml of 2% NaCl containing 0.01% each of penicillin G and streptomycin sulfate for up to 30 hr at 30°C under illumination and appropriate aeration, resulting in living nauplii of about 90%.

Enzyme assay

Trehalase activity was assayed at 30°C for 30 min by incubating an appropriate amount of enzyme in 0.5 ml of 50 mM sodium phosphate buffer (pH 7.5) and 133 mM α,α-trehalose. If the apparent optimum pH of the enzyme changed as the purification was in progress, the buffer was exchanged to 50 mM HEPES buffer (pH 7.2) or 20 mM Tricine buffer (pH 8.0) as described below. The reaction was stopped by heating the mixture in a boiling bath for 4 min. The liberated glucose was enzymatically determined as described by Dahlqvist (1964). One unit of the enzyme was defined as the amount of enzyme that produced 1 μmol of glucose per min at 30°C and optimum pH in each purification step.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Electrophoresis

SDS-PAGE was carried out on a BIORAD Mini Protean II electrophoresis apparatus according to the method of Laemmli (1970) using an 8% polyacrylamide slab gel. Gels were stained for protein with Coomassie Brilliant Blue R-250. The activity of trehalase in the gel was assayed after the electrophoresis, if necessary, by incubating a non-stained sliced gel in 0.5 ml of the enzyme assay mixture as described above in the Enzyme assay section, at 37°C for 40~60 min. After the supernatant of the reaction mixture was boiled, the glucose liberated in the supernatant was enzymatically assayed by the method of Dahlqvist (1964). For the detection of the enzyme activity and preparation of the enzyme, the electrophoresis was carried out in a cold room, and the heating of the sample before electrophoresis was omitted.

Isoelectric focusing

Analytical isoelectric focusing was performed with a 5% polyacrylamide slab gel in a cold room as described by Killick (1983). The activity of trehalase in the gel was assayed after the isoelectric focusing, if necessary, as described in the Electrophoresis section above.

Estimation of molecular mass

The purified enzyme was applied to a TSKgel G3000SW column (0.75 × 60 cm) attached to a high performance liquid chromatograph, 600E (Waters, Tokyo), equilibrated with 10 mM HEPES buffer, pH 7.0, containing 0.1 M Na_2SO_4, 1 mM DTT and 0.0005% leupeptin. The flow rate was 1 ml/min. The positions of blue dextran and marker proteins were determined by measuring the absorption at 280 nm. The position of the trehalase was estimated by measuring the enzymatic activity at pH 8.0.

Endoglycosidase H digestion

The trehalase was treated with 3 mU of endoglycosidase H in 50 mM acetate buffer, pH 5.0, containing 10 mM EDTA and 0.02% SDS for 5 hr at 37°C. The reaction mixture was lyophilized and subjected to SDS-PAGE.

Preparation of the crude enzyme

All of the operations described below were performed at 4°C unless noted.

After a 15-hr incubation, the embryos and nauplii derived from 10 g of dry cysts were collected on a nylon-mesh (40 μm), washed and homogenized in 100 ml of Buffer A (50 mM sodium phosphate buffer, pH 7.5, 1 mM EDTA, 5 mM dithiothreitol (DTT), 0.001% soybean trypsin inhibitor (STI), and 0.0005% leupeptin) by a high speed homogenizer, Physcotron (NITION, Tokyo). The supernatant with floating broken cysts resulting from centrifugation at 1,200 × g for 10 min was filtered and used as a crude enzyme solution, and is hereafter referred to as the post-nuclear fraction. The solution was further centrifuged at 40,000 × g for 10 min. The supernatant and the floating orange pigment layer were pooled and homogenized , and named the post-mitochondrial fraction.

Purification of trehalase from *Artemia* embryos and larvae

Step 1. Acetone treatment. A volume of acetone cooled at -20°C, equal to 8/3 of the volume of the post-mitochondrial fraction, was added to the post-mitochondrial fraction, mixed and allowed to stand in a freezer for 1 hr. The precipitate resulting from centrifugation at 1,200 × g for 10 min was washed with an appropriate volume of acetone cooled to -20°C and dried. The resulting white precipitate was suspended in 100 ml of Buffer A, sonicated for 5 min and extracted for 30 min. The extract obtained from centrifugation at 15,000 × g for 10 min was dialyzed against Solution A (1 mM EDTA, 5 mM 6-amino-hexanoic acid (AHA) and 1 mM DTT) and lyophilized.

Step 2. DEAE-Sepharose Fast Flow anion exchange column chromatography. The freeze-dried extracts obtained from two preparations were pooled and dissolved in 40 ml of 0.1 M NaCl in Buffer B (50 mM sodium phosphate buffer, pH 8.0, 1 mM EDTA, 5 mM AHA, 0.001% STI, 0.0005% leupeptin and 1 mM DTT) and applied to a DEAE-Sepharose Fast Flow column (2.5 × 40.0 cm) equilibrated with 0.1 M NaCl in Buffer B. The column was washed with 320 ml of 0.1 M NaCl in Buffer B. Subsequently, the enzyme was eluted with 0.25 M NaCl in Buffer B at a flow rate of 2.7 ml/min. Fractions rich in the enzyme activity determined at pH 7.5 were pooled, added with 0.01% *p*-toluenesulfonyl-L-phenylalanine chloromethyl ketone (TPCK), dialyzed against Solution A, and freeze-dried.

Step 3. Con A-Sepharose affinity chromatography. The freeze-dried material was dissolved in 7 ml of Buffer C (5 mM HEPES buffer, pH 7.2, 0.25 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 10 μM $HgCl_2$)

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and applied to a Con A-Sepharose column (1.45 × 7.0 cm) equilibrated with Buffer C. The column was washed with Buffer C, and the enzyme was eluted with 0.3 M methyl α-D-mannoside in Buffer C at a flow rate of 1.4 ml/min. Fractions rich in the enzyme activity determined at pH 7.2 were pooled, added with 0.01% TPCK , dialyzed against Solution A, and lyophilized.

Step 4. TSKgel AF-Chelate TOYOPEARL 650 M metal chelate affinity chromatography. The lyophilized dialyzate was dissolved in 6 ml of distilled water, and dialyzed against Buffer D (20 mM HEPES buffer, pH 8.0, and 0.5 M NaCl) for 4 hr. The dialyzate was applied to a TSKgel AF-Chelate TOYOPEARL 650 M column (1.4 × 7 cm) pretreated with $ZnCl_2$ and equilibrated with Buffer D. The column was washed with Buffer D, and the enzyme was eluted with 15 mM glycine in Buffer D at a flow rate of 1.7 ml/min. The active fractions determined at pH 8.0 were pooled, dialyzed against Solution A and lyophilized.

Step 5. Preparative SDS-PAGE. The freeze-dried material was divided into 6 parts, and each part was applied to a preparative SDS-polyacrylamide mini slab gel with a thickness of 1.5 mm, respectively. After electrophoresis, a portion of the gel which corresponded to the enzyme activity determined at pH 8.0 was excised, and the enzyme was extracted in distilled water by the Physcotron homogenizer. The supernatant of the extract resulting from centrifugation at 1,200 × g for 10 min was desalted by a HiTrap™ Desalting column and lyophilized. This material was successively subjected to the second preparative SDS-PAGE.

RESULTS

The activity of the trehalase in the post-nuclear fraction from developing *Artemia* at different times was determined at pH 7.5, and the data obtained are shown in Fig. 1. The trehalase activity was found to be unchanged until the emergence of the stage E-1 prenauplius, followed by a 10-fold increase in its activity coinciding with the peak of the emergence of the stage E-2 prenauplius. Stage E-1 and E-2 prenauplius were defined by Nakanishi *et al.* (1962) (Fig. 1c). The level of the activity decreased after the hatching of the nauplius, and the activity remained at a relatively high level in the nauplii incubated for 30 hr.

Most of the enzyme activity was present in soluble form; the remaining part was recovered in a floating pigment layer after centrifugation. The trehalase activity in the insoluble fraction was negligible when the fraction was obtained from cysts incubated for more than 9 hr. The purification procedure and representative results are summarized in Table 1. An approximately 2,500-fold purification with a yield of 5% was obtained.

The purified enzyme exhibited one protein band on SDS-PAGE corresponding to a molecular mass of approximately 70,000 Da, and a peak of the activity of the purified trehalase coincided with the protein band (Fig. 2). SDS-PAGE of the enzyme under reducing and non-reducing conditions revealed the same molecular mass. The purified enzyme was applied to high performance gel filtration using a TSKgel G3000SW column. A molecular mass of 58,000 Da was calculated. These results indicate that the enzyme is a monomer lacking intramolecular disulfide bonds. After treatment of the purified enzyme with endoglycosidase H, the molecular mass of 66,000 Da was estimated on SDS-PAGE and its activity was unchanged.

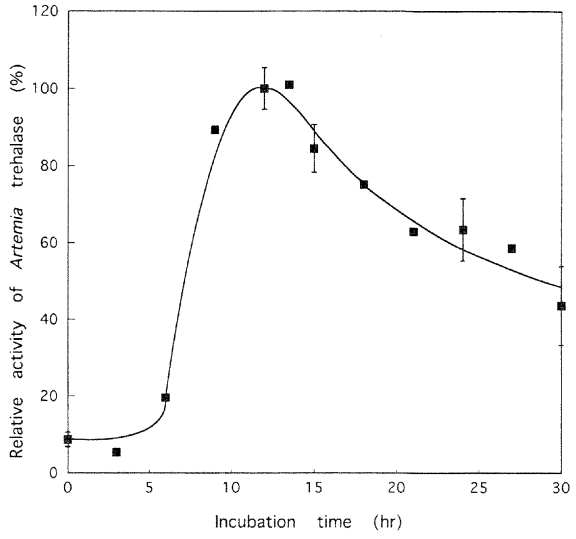

Fig. 1a. The activity of the trehalase obtained from developing *Artemia*. Five grams of dry cysts of *Artemia* were incubated at 30°C in 1,000 ml of 2% NaCl-0.01% penicillin G-0.01% streptomycin sulfate, and an aliquot of 20 ml was drawn from the incubation medium at indicated times. Developing cysts in the aliquot were homogenized as described in the Materials and Methods section. The trehalase activity in the post-nuclear fraction was determined at pH 7.5. The highest activity was observed at 12 hr and was found to be 16.1 ± 0.9 units/g dry cysts (arithmetic mean ± SD, n = 4). Data with longitudinal bars represent arithmetic mean ± SD (n = 3~6, n: number of incubations).

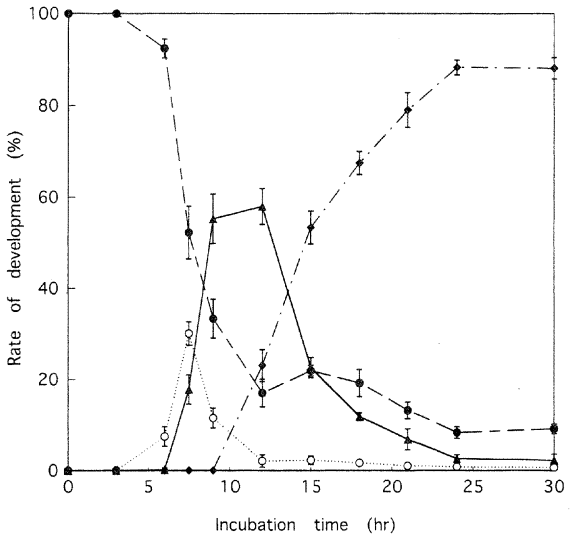

Fig. 1b. Development of *Artemia* embryos and larvae. Dry cysts of *Artemia* (5 g) were incubated at 30°C in 1,000 ml of 2% NaCl-0.01% of penicillin G-0.01% streptomycin sulfate, and an aliquot of the incubation medium was drawn at the indicated times. The numbers of unruptured cysts, emerged stage E-1 prenauplii, emerged stage E-2 prenauplii, and hatched nauplii were counted under a stereoscopic microscope. The numbers of embryos and larvae were counted three times at each point; the total number was at least 400. Data with longitudinal bars represent arithmetic mean ± SD (n = 3~6, n: number of incubations). ●: unruptured cyst, ○: stage E-1 prenauplius, ▲: stage E-2 prenauplius, ◆: nauplius.

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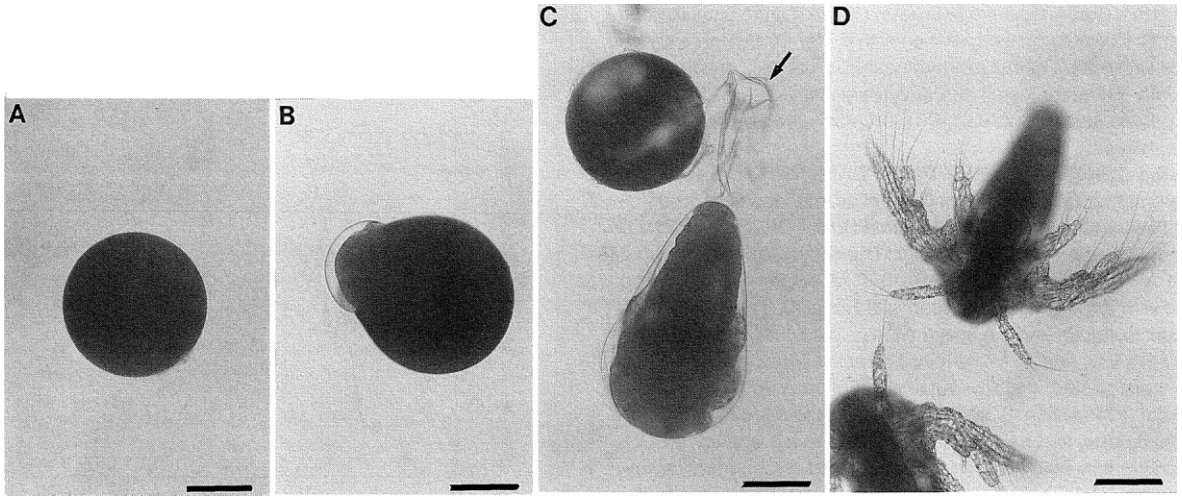

Fig. 1c. Developmental stages of *Artemia* embryo and larva. **A**: a cyst resuming development. **B**: a prenauplius at stage E-1, emerged halfway from the shell of the cyst. **C**: a prenauplius at stage E-2, emerged from the shell and still enclosed by an inner membrane (hatching membrane). An arrow shows cast-off outer membrane. **D**: a nauplius swimming freely. Scale bar = 0.1 mm.

Table 1. Purification of alkaline trehalase from the developing embryos and larvae of *Artemia*

Step	Total activity units	Specific activity units/mg	Purification fold	Yield %
Post-nuclear fraction	146	0.0562	1	100
Post-mitochondrial fraction	146	0.0653	1.2	100
Acetone treatment	163	0.171	3	112
DEAE-Sepharose Fast Flow chromatography	188	0.472	8.4	129
Con A-Sepharose chromatography	139	6.62	118	95.2
TSKgel AF-Chelate TOYOPEARL 650M chromatography	56.8	26.9*	479	38.9
Preparative SDS-PAGE	7.56	140	2491	5.2

The purification was performed as described in the Materials and Methods section. Starting with 20 g of dry cysts incubated for 15 hr, about 50 μg of the purified trehalase was obtained.
*Because of the presence of glycine in the elution buffer, the amount of protein could not be determined. The protein concentration was roughly estimated from UV absorption at 280 nm.

The isoelectric point of the purified enzyme was determined by isoelectric focusing on a 5% polyacrylamide gel in a pH range of 3~10 (Fig. 3). An isoelectric point of 4.7~4.8 was estimated by measuring the enzyme activity at pH 8.0 and by staining the protein. The trehalase activity had one peak, which corresponded with one protein band.

The dependence of the activity of the purified enzyme on the pH was investigated in a mixed buffer of 20 mM each of HEPES, MES and acetate (pH 4~8) and in 20 mM Tricine buffer (pH 7~9) adjusted to various pH values with NaOH. The maximum activity was observed at pH 8.0 in both buffers, and the activity measured in the latter buffer was 1.3 times higher than that in the former.

From a Lineweaver-Burk plot, 8.4 mM α,α-trehalose was calculated for the apparent Michaelis constant of the purified enzyme measured at pH 8.0. The enzyme was assayed for substrate specificity using 34.5 mM β,β-trehalose, 24 mM trehalose 6-phosphate, 20 mM each of methyl α-D-glucoside, *p*-nitrophenyl α-D-glucoside, sucrose, maltose, isomaltose, cellobiose, β-gentiobiose, melibiose, lactose, and raffinose. The enzyme showed a very high specificity for α,α-trehalose, and exhibited no hydrolytic action on the other substrates.

The dependence of the activity of the purified enzyme on the temperature was examined at temperatures between 25~60°C at pH 8.0. The activity nearly doubled when the temperature rose 10°C between 25~40°C, followed by a peak of activity at 55°C. The activation energy of the enzyme was calculated to be 55.8 kJ/mol from an Arrhenius plot of the results obtained between 25~40°C.

Various compounds were examined for their effects

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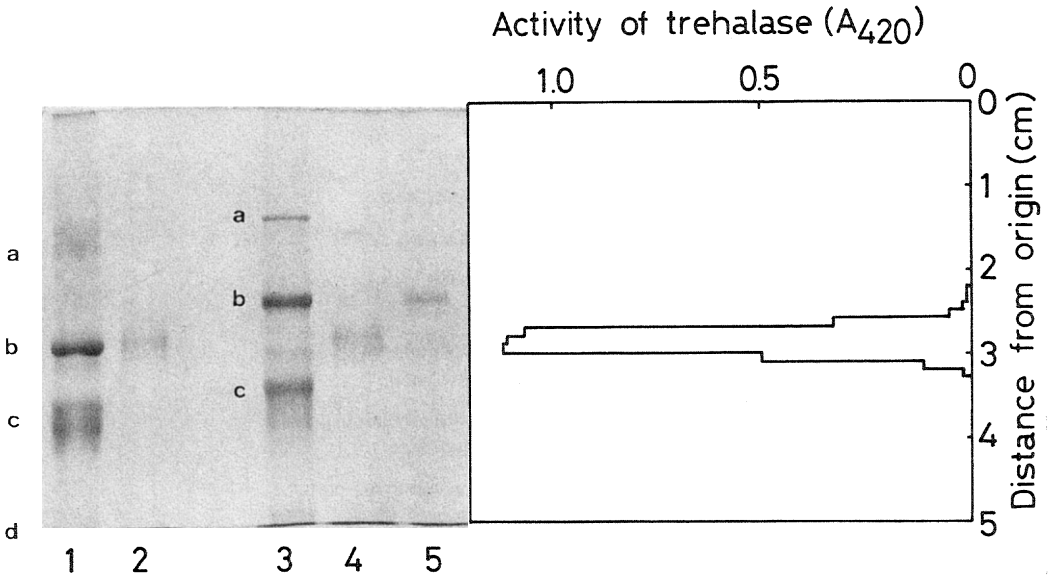

Fig. 2. SDS-polyacrylamide gel electrophoresis of the *Artemia* trehalase. The purified *Artemia* trehalase weighing 3.3 μg was subjected to SDS-PAGE as described in the Materials and Methods section. Lane 1; standard proteins under non-reducing conditions. The proteins were not boiled before SDS-PAGE. a: β-galactosidase (116 kDa), b: bovine serum albumin (66 kDa), c: ovalbumin (45 kDa), d: bromphenol blue. Lane 2; purified *Artemia* trehalase under non-reducing conditions and without boiling before SDS-PAGE. In this case, the enzyme was active after SDS-PAGE. Lane 3; standard proteins (a,b,c) under reducing conditions; the proteins were boiled before SDS-PAGE. Lane 4; purified *Artemia* trehalase under reducing conditions. The enzyme was not boiled before SDS-PAGE. In this case, the enzyme was active after SDS-PAGE. Lane 5; purified *Artemia* trehalase under reducing conditions; the enzyme was boiled before SDS-PAGE. A lane containing 0.5 μg of the purified trehalase was subjected to SDS-PAGE under reducing conditions and without boiling; the lane was sliced, and incubated for 40 min, and the trehalase activity was determined.

on the activity of the enzyme (Table 2). Tris, sucrose, cellobiose and the aromatic derivatives of α- and β-glucosides all inhibited the activity at a concentration of 10~20 mM. Phlorizin at 2 mM decreased the activity. The enzyme was sensitive to sulfhydryl reagents (30% and 100% inhibition by 1 mM *p*-hydroxymercuribenzoate (PHMB) and 0.1 mM $HgCl_2$, respectively). Heavy metal ions such as Zn^{2+}, Cd^{2+} and Cu^{2+} were also potent inhibitors of the trehalase. The effect of EDTA on the activity of the enzyme was examined in several conditions. EDTA at 5 mM in 50 mM HEPES buffer did not affect the activity, but it in 50 mM each of Tricine and phosphate buffer decreased the activity by 13% and 42%, respectively. The enzyme was also sensitive to 100 mM sodium phosphate buffer , resulting in a 64% inhibition of the activity. The enzyme stored in 50 mM phosphate buffer was very sensitive to EDTA, and in that condition the activity sometimes decreased to 30% by 1 mM EDTA. However, the activity was protected by the addition of appropriate amounts of Mn^{2+}, Mg^{2+}, and Ca^{2+}, respectively, to the reaction mixture (Fig. 4).

DISCUSSION

In this paper we describe a novel trehalase from *Artemia* embryos and larvae, which is present in soluble form and optimally active at pH 8.0, although the exact subcellular localization of the enzyme was not determined. The nature of this purified enzyme is quite different from that of the *Artemia* dry cyst trehalase reported earlier by Ballario *et al.* (1978). The latter enzyme was insoluble and active at pH 5.6. Equally important, a similarity between our *Artemia* alkaline trehalase and an *Artemia* hydrated embryo trehalase (Hand and Carpenter, 1986) should be noted. The latter authors reported that *Artemia* hydrated embryos contained a trehalase in soluble form which might mediate a pH-induced metabolic transition. The respiratory significance of the pH optimum of our *Artemia* trehalase at the resumption of *Artemia* development after long dormancy could be considered as follows: *Artemia* embryos may enter a metabolically active state through an optimization of the trehalase activity by cellular alkalinization at the resumption of development. The cellular alkalinization was previously suggested by Busa *et al.* (1982). We investigated this idea by elucidating the properties of the *Artemia* trehalase purified to homogeneity.

The coincidence of the increase in the trehalase activity with the emergence of the stage E-2 prenauplius, a phenomenon first reported here, suggests the significance of the enzyme in providing glucose to produce glycogen and glycerol, which are major respiratory substrates in the early development of *Artemia* (Clegg, 1964; Ewing and Clegg, 1969). This also suggests that glycerol was involved in the emergence of the prenauplius. Hygroscopic glycerol was reported to have a possible osmotic role in the rupture of the shell and the outer membrane of the *Artemia* cyst, i.e. the emergence (Clegg, 1964). The emerged prenauplius, still enclosed by the inner membrane (hatching membrane), protrudes from the cyst. We observed that the prenauplius

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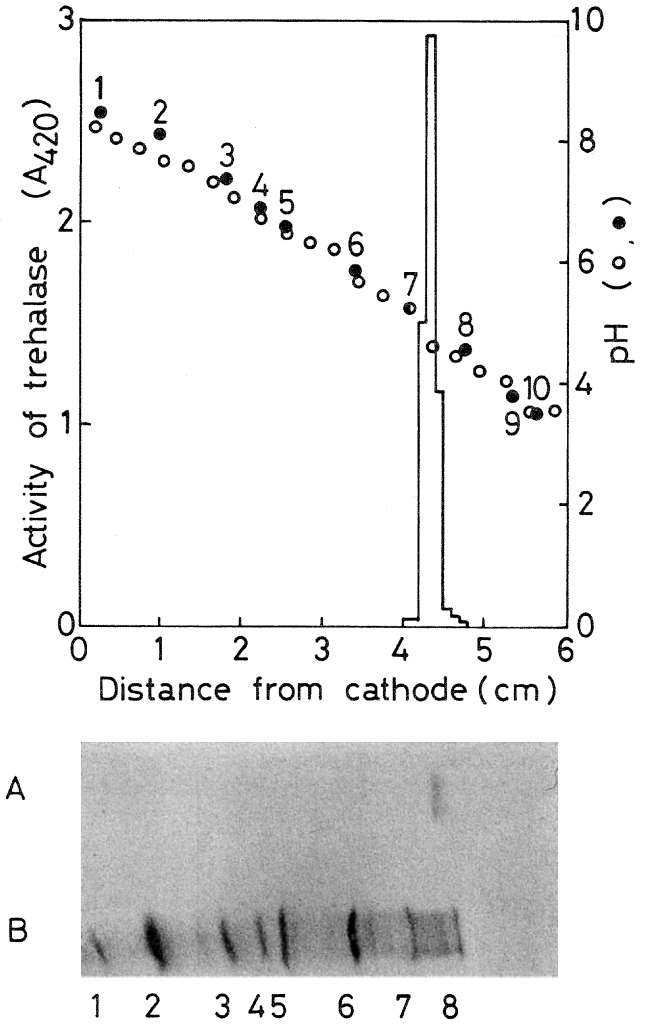

Fig. 3. Isoelectric focusing of the *Artemia* trehalase. The purified trehalase from *Artemia* was subjected to isoelectric focusing using a 5% polyacrylamide slab gel with pH 3-10 ampholites as described in the Materials and Methods section, and stained by the method described in the calibration kit manual for pI-determinations. Lane A; 4.5 μg of purified trehalase, Lane B; pI markers, 1: lentil lectin-middle band (pI 8.45), 2: lentil lectin-acidic band (pI 8.15), 3 : horse myoglobin-basic band (pI 7.35), 4 : horse myoglobin-acidic band (pI 6.85), 5 : human carbonic anhydrase B (pI 6.55), 6 : bovine carbonic anhydrase B (pI 5.85), 7 : β-lactoglobulin A (pI 5.20), 8 : soybean trypsin inhibitor (pI 4.55), 9 : methyl red (pI 3.75), 10 : amyloglucosidase (pI 3.50). Methyl red disappeared during staining. Amyloglucosidase was scarcely stained. After isoelectric focusing, a lane containing 0.2 μg of the purified trehalase was sliced, and incubated for 60 min, and the trehalase activity was determined. Following the isoelectric focusing, four lanes without specimens were sectioned into 3 mm slices and homogenized in 2 ml of 10 mM KCl. The pH of each homogenized slice was determined at 25°C.

began to move its antennae and mandibles within the inner membrane shortly after the emergence, piercing and tearing the inner membrane of the cyst by chance. This mechanical movement leads to the hatching of the nauplius. The moving requires much energy. Cysts incubated in distilled water (i.e., 0% NaCl) at 30°C did emerge, but the stage E-2 prenauplii

Table 2. Inhibition of the trehalase activity by various compounds

Compounds tested	(mM)	Inhibition (%)
Tris	10	48
Sucrose	10	19
Cellobiose	20	23
Phlorizin	2	34
p-Nitrophenyl α-D-glucoside	20	49
p-Nitrophenyl β-D-glucoside	10	55
p-Aminophenyl β-D-glucoside	20	17
p-Hydroxymercuri-benzoate	1	30
$HgCl_2$	0.1	100
$ZnSO_4$	0.1	54
$CdCl_2$	0.1	60
$CuSO_4$	0.1	84
EDTA	1 or 5	see text

The activity of the enzyme was assayed as described in Materials and Methods in the presence or absence of each compound to be tested, and the rate of the inhibition of the trehalase activity was determined.

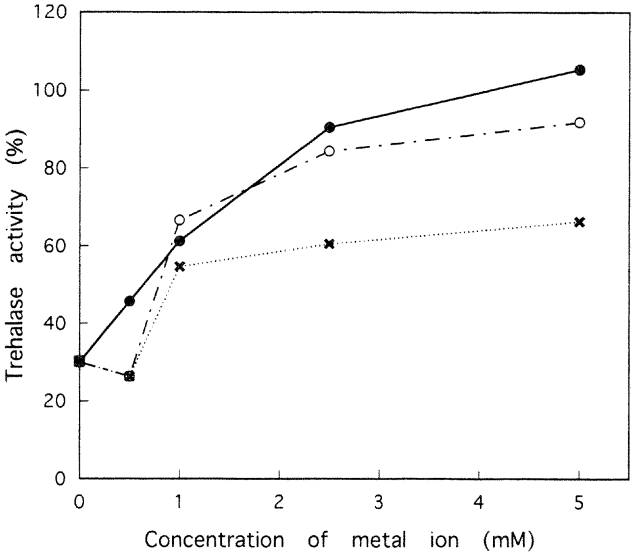

Fig. 4. Inhibition of the *Artemia* trehalase activity by EDTA and protection by metal ions from the inhibition. Trehalase activity was assayed in 50 mM sodium phosphate buffer (pH 7.5) in the presence of 1 mM EDTA and various concentrations of metal ions. The control experiment was carried out in the absence of EDTA and metal ions, and the activity of trehalase in the control experiment was represented as 100%. ■; with 1 mM EDTA and without metal ions, ●; with 1 mM EDTA and 0.5~5 mM $MnCl_2$, ○; with 1 mM EDTA and 0.5~5 mM $MgCl_2$, ✖; with 1 mM EDTA and 0.5~5 mM $CaCl_2$. In the case of $MnCl_2$ and $CaCl_2$, the precipitation of phosphate was observed during the incubation of the enzyme. The supernatant of the reaction mixture was used for the determination of the trehalase activity.

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did not move their antennae and mandibles, resulting in no hatching. Their trehalase activity was observed to be comparable to that of cysts ordinarily incubated in 2% NaCl (data not shown). These results indicate the significance of the enzyme in the early stage of *Artemia* development. Boulton and Huggins (1977) and Vallejo (1989) did not find an increase in the activity of the trehalase during the early stage of *Artemia* development. Differences of the extraction method of the enzyme, and of the assay method of the enzyme activity as well as of the hatchability and of the degree of synchronism in the developing *Artemia* from various sources might account for this discrepancy.

Considerable trehalase activity was also observed in the present study during the development of the nauplii which swam freely but did not take food. This is in parallel with the presence of a considerable amount of trehalose in nauplius (Vallejo, 1989).

Since several proteolytic activities were reported in developing *Artemia* (Osuna *et al.*, 1977; Nagainis and Warner, 1979; Garesse *et al.*, 1980; Perona and Vallejo, 1982) and neutral trehalase from yeast was described to be sensitive to proteinase (App and Holzer, 1989), many kinds of protease inhibitors were tested and introduced during the present isolation procedure. During the initial course of this work, we observed much loss of the enzyme activity when using mismatched inhibitors. The yields of the enzyme, obtained from the post-nuclear fraction through the Con A-Sepharose affinity chromatography, were improved by using the inhibitors described in Materials and Methods.

The enzyme was purified by preparative SDS-PAGE, because no inhibition of the crude enzyme activity by 0.1% SDS was observed. The activity of the purified enzyme was reduced by 50% in the presence of 0.1% SDS, and almost all of the activity was recovered after removing the SDS (data not shown).

The molecular mass of the purified trehalase from *Artemia* embryos and larvae was estimated to be 70 kDa by SDS-PAGE and 58 kDa by gel filtration. An earlier report (Hand and Carpenter, 1986) described the interconversion of *Artemia* hydrated embryo trehalase between 110 kDa at pH 8.6 and 235 kDa at pH 6.3 measured on a gel filtration column, whereas a molecular mass of 75 kDa was estimated by gel filtration for *Artemia* dry cyst trehalase (Ballario *et al.*, 1978). The reported molecular mass of trehalase from different species determined by SDS-PAGE is as follows: *E. coli*, 58 kDa (Boos *et al.*, 1987); yeast, 80 kDa (App and Holzer, 1989); rabbit, 66 kDa (Ruf *et al.*, 1990); silkworm, 70 kDa (Su *et al.*, 1993); and mealworm beetle, 62 kDa (Yaginuma *et al.*, 1996).

The sensitivity of the purified enzyme to endoglycosidase H and the ability of the enzyme to bind to a Con A-Sepharose column suggest that the enzyme is a glycoprotein. The finding that the trehalase activity was not decreased by the endoglycosidase H treatment suggests that the carbohydrate moiety of the trehalase does not participate in its activity. The enzyme also bound a column of *Lens culinalis* agglutinin (LCA, Seikagaku Kogyo) , and did not bind a column of wheat germ agglutinin (WGA, Seikagaku Kogyo) (data not shown). These results suggest that the sugar moiety of the trehalase is a high mannose type with fucose attached to the Asn-linked GlcNAc.

The isoelectric point of the purified enzyme was estimated to be 4.7~4.8. This value is similar to those of trehalase from different species: silkworm, pI 4.85 (Sumida and Yamashita, 1983), rabbit intestine, pI 4.6~4.8 (Galand, 1984), and yeast neutral trehalase, pI 4.7 (App and Holzer, 1989), but quite different from those of *Artemia* dry cyst trehalase (pI 6.2; Ballario *et al.*, 1978) and of the cellular slime mold (pI 7.2~7.3; Killick, 1983).

The calculated Km value of the purified trehalase for trehalose at pH 8.0, i.e., 8.4 mM, is smaller than those of the *Artemia* hydrated embryo trehalase (34.4 mM at pH 6.3 and 16.4 mM at pH 8.6; Hand and Carpenter, 1986). The Km of *Artemia* dry cyst trehalase was estimated to be 4.3 mM by Ballario *et al.* (1978). The moderate but specific affinity of our *Artemia* trehalase for trehalose is consistent with the high concentration of trehalose in *Artemia* embryos and larvae (Vallejo, 1989).

The activation energy calculated from the Arrhenius plots was 55.8 kJ/mol, which is similar to the reported values for the intestinal brush-border membrane trehalase from rabbit (46.76 kJ/mol, Galand, 1984) and for the trehalase from the cellular slime mold (50.2~54.4 kJ/mol, Killick, 1983).

Tris, sucrose and phlorizin are known to be competitive inhibitors for trehalases from different species (rat, Nakano *et al.*, 1977; rabbit, Galand, 1984; pig, Yoneyama, 1987) and the inhibition of the present purified *Artemia* trehalase by these compounds was also observed. The aromatic derivatives of α- and β-glucosides listed in Table 2 were found to be inhibitors of the *Artemia* trehalase. It was reported in the case of honey bee trehalase that β-glucosides were potent inhibitors of the trehalase, while α-glucosides were not (Talbot *et al.*, 1975). The trehalase from gypsy moth was insensitive to inhibition by high concentrations of Tris, sucrose, *p*-nitrophenyl-β-D-glucoside or phlorizin (Valaitis and Bowers, 1993).

The SH-blocking agents 1 mM PHMB and 0.1 mM $HgCl_2$ each inhibited the activity of the purified trehalase in the present study. These results are consistent with earlier reports of the effect of $HgCl_2$ on the trehalase activity of different species (rat, Nakano *et al.*, 1977; rabbit kidney, Nakano, 1982; cellular slime mold, Killick, 1983; pig, Yoneyama, 1987; yeast, App and Holzer, 1989). The activity of the purified trehalase was protected against the inhibitory effect of 0.1 mM $HgCl_2$ by the addition of 1 mM KI and 100 mM NaCl, respectively, to the reaction mixture (data not shown). These results are in good agreement with those reported by Nakano (1982) on kidney trehalase.

Zn^{2+}, Cd^{2+} and Cu^{2+} at 0.1 mM concentration were potent inhibitors of the activity of the purified trehalase. The effect of EDTA on the activity of the enzyme was somewhat complicated. An inhibitory effect of EDTA on the enzyme activity was shown in Tricine and phosphate buffers, which have the ability to bind metal ions. In contrast, EDTA had no

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effect on the activity of the trehalase in HEPES buffer which does not bind metal ions . A high concentration (100 mM) of sodium phosphate buffer by itself inhibited the enzyme activity. These results suggest that metal ion(s) are required for the enzyme activity. The experiment designed to protect the trehalase activity against the inhibitory effect of EDTA revealed the participation of metal ions such as Mn^{2+}, Mg^{2+} and Ca^{2+} in the activity of the purified trehalase.

Hand and Carpenter (1986) reported an inhibition of the trehalase activity of *Artemia* embryos by 0.1~1.6 mM ATP. Neither Ballario *et al.* (1978) nor we observed such an inhibition (data not shown).

For a comparative study, cysts of *Artemia* from China (Japan Pet Drugs Co.) were also studied in our laboratory. The hatchability of the Chinese cysts was much lower than that of the American cysts, about 60%. The trehalase from the Chinese cysts incubated for 40 hr was purified by the same method described in Materials and Methods, and characterized. The properties of the trehalase from the Chinese cysts, i.e., the molecular mass, pH dependency, pI, etc., were much the same as those from the Great Salt Lake, USA (data not shown).

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REFERENCES

App H, Holzer H (1989) Purification and characterization of neutral trehalase from the yeast ABYS1 mutant. J Biol Chem 264: 17583–17588

Ballario P, Bergami M, Cacace MG, Scala F, Silvestri L (1978) α,α-Trehalase from the brine shrimp *Artemia salina*. Purification and properties. Comp Biochem Physiol 61B: 265-269

Boos W, Ehmann U, Bremer E, Middendorf A, Postma P (1987) Trehalase of *Escherichia coli*. Mapping and cloning of its structural gene and identification of the enzyme as a periplasmic protein induced under high osmolarity growth conditions. J Biol Chem 262: 13212-13218

Boulton AP, Huggins AK (1977) Biochemical changes occurring during morphogenesis of the brine shrimp *Artemia salina* and the effect of alterations in salinity. Comp Biochem Physiol 57A: 17-22

Busa WB, Crowe JH, Matson GB (1982) Intracellular pH and the metabolic status of dormant and developing *Artemia* embryos. Arch Biochem Biophys 216: 711-718

Clegg JS (1962) Free glycerol in dormant cysts of the brine shrimp *Artemia salina*, and its disappearance during development. Biol Bull Woods Hole 123: 295-301

Clegg JS (1964) The control of emergence and metabolism by external osmotic pressure and the role of free glycerol in developing cysts of *Artemia salina*. J Exp Biol 41: 879-892

Clegg JS (1965) The origin of trehalose and its significance during the formation of encysted dormant embryos of *Artemia salina*. Comp Biochem Physiol 14: 135-143

Dahlqvist A (1964) Method for assay of intestinal disaccharidases. Anal Biochem 7: 18-25

Dutrieu J (1960) Observations biochimiques et physiologiques sur le développement d'*Artemia salina* Leach. Arch Zool exp gén 99: 1-133

Elbein AD (1974) The metabolism of α,α-trehalose. Advan Carbohyd Chem 30: 227-256

Ewing RD, Clegg JS (1969) Lactate dehydrogenase activity and anaerobic metabolism during embryonic development in *Artemia salina*. Comp Biochem Physiol 31: 297-307

Galand G (1984) Purification and characterization of kidney and intestinal brushborder membrane trehalases from the rabbit. Biochim Biophys Acta 789: 10-19

Garesse R, Perona R, Marco R, Vallejo CG (1980) The unmasking of proteolytic activity during the early development of *Artemia salina*. Identification of a precursor after hatching. Eur J Biochem 106: 225-231

Hand SC, Carpenter JF (1986) pH-Induced metabolic transitions in *Artemia* embryos mediated by a novel hysteretic trehalase. Science 232: 1535-1537

Killick KA (1983) Trehalase from the cellular slime mold *Dictyostelium discoideum*: Purification and characterization of the homogeneous enzyme from myxamoebae. Arch Biochem Biophys 222: 561-573

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-275

Nagainis PA, Warner AH (1979) Evidence for the presence of an acid protease and protease inhibitors in dormant embryos of *Artemia salina*. Develop Biol 68: 259-270

Nakanishi YH, Iwasaki T, Okigaki T, Kato H (1962) Cytological studies of *Artemia salina*. I. Embryonic development without cell multiplication after the blastula stage in encysted dry eggs. Annot Zool Jap 35: 223-228

Nakano M, Sumi Y, Miyakawa M (1977) Purification and properties of trehalase from rat intestinal mucosal cells. J Biochem (Tokyo) 81: 1041-1049

Nakano M (1982) Effect of inorganic anions on the inhibition of trehalase activity by mercuric chloride. Biochim Biophys Acta 707: 115-120

Osuna C, Olalla A, Sillero A, Günther Sillero MA, Sebastián J (1977) Induction of multiple proteases during the early larval development of *Artemia salina*. Develop Biol 61: 94-103

Perona R, Vallejo CG (1982) The lysosomal proteinase of *Artemia*. Purification and characterization. Eur J Biochem 124: 357-362

Ruf J, Wacker H, James P, Maffia M, Seiler P, Galand G, von Kieckebusch A, Semenza G, Mantei N (1990) Rabbit small intestinal trehalase. Purification, cDNA cloning, expression, and verification of glycosylphosphatidylinositol anchoring. J Biol Chem 265: 15034-15039

Su Z-H, Sato Y, Yamashita O (1993) Purification, cDNA cloning and Northern blot analysis of trehalase of pupal midgut of the silkworm, *Bombyx mori*. Biochim Biophys Acta 1173: 217-224

Sumida M, Yamashita O (1983) Purification and some properties of soluble trehalase from midgut of pharate adult of the silkworm, *Bombyx mori*. Insect Biochem 13: 257-265

Talbot BG, Muir JG, Huber RE (1975) Properties of a free and a solubilized form of bound α,α-trehalase purified from honey bee thorax. Can J Biochem 53: 1106-1117

Valaitis AP, Bowers DF (1993) Purification and properties of the soluble midgut trehalase from the gypsy moth, *Lymantria dispar*. Insect Biochem Molec Biol 23: 599-606

Vallejo CG (1989) *Artemia* trehalase: Regulation by factors that also control resumption of development. In "Cell and Molecular Biology of *Artemia* Development" Ed by AH Warner, TH MacRae, JC Bagshaw, NATO ASI Series, Series A: Life Sciences Vol 174, Plenum Press, NY, pp 173-189

Yaginuma T, Mizuno T, Mizuno C, Ikeda M, Wada T, Hattori K, Yamashita O, Happ GM (1996) Trehalase in the spermatophore from the bean-shaped accessory gland of the male mealworm

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beetle, *Tenebrio molitor*: purification, kinetic properties and localization of the enzyme. J Comp Physiol B 166: 1-10

Yoneyama Y (1987) Purification and properties of detergent-solubilized pig kidney trehalase. Arch Biochem Biophys 255: 168-175

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