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T. Yaginuma · T. Mizuno · C. Mizuno · M. Ikeda
T. Wada · K. Hattori · O. Yamashita · G. M. Happ

Trehalase in the spermatophore from the bean-shaped accessory gland of the male mealworm beetle, *Tenebrio molitor*: purification, kinetic properties and localization of the enzyme

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Abstract Trehalase from the bean-shaped accessory glands of the male mealworm beetle, *Tenebrio molitor*, was purified by acid treatment, with subsequent chromatography on columns of DEAE-cellulofine and Sephacryl S-300. The molecular masses of the native and the denatured forms were estimated to be 43 and 62 kDa by gel filtration and SDS-PAGE, respectively, an indication that the trehalase may be composed of a single polypeptide. The optimum pH of the reaction catalyzed by trehalase was 5.6–5.8. The K_m for trehalose was 4.4 mmol·l^{-1}. Immunohistochemical experiments with trehalase-specific antiserum showed that the enzyme was localized in one specific type of secretory cell in the bean-shaped accessory gland epithelium and within the semisolid secretory mass that was a precursor to the wall of spermatophore. SDS-PAGE and immunoblotting analysis revealed the presence of a polypeptide of about 62 kDa in the spermatophore. Immunohistochemical observations showed that the trehalase was located at the outgrowth in the anterior portion of the spermatophore. When a fresh spermatophore was immersed in phosphate-buffered saline it discharged sperm in the same manner as in the bursa copulatrix of the female. Before the rupture of the expanded bulb of the spermatophore, almost all of the trehalase had dissolved in the phosphate-buffered saline. The addition of validoxylamine A to the saline, a specific inhibitor of trehalase, did not affect the expansion and evacuation of the spermatophore. These results demonstrate that trehalase, synthesized by a specific type of secretory cell in the bean-shaped accessory gland epithelium, is actively passed into the lumen of the bean-shaped accessory gland and then incorporated into the spermatophore. Trehalase appears to be one of the structural proteins of the spermatophore, although the possibility can not yet be completely ruled out that the trehalase-trehalose system functions for the nourishment and/or activation of the sperm in the bursa copulatrix of the female.

Key words Trehalase · Bean-shaped accessory gland · Spermatophore · Male mealworm beetle · *Tenebrio molitor*

Abbreviations *BAG* bean-shaped accessory gland(s) · *DEAE* diethylaminoethyl · *Kpi buffer* K_2HPO_4/KH_2PO_4 buffer (pH 7.0) · *PAGE* polyacrylamide gel electrophoresis · *PBS* phosphate-buffered saline · *SDS* sodium dodecy sulphate · *Spph* spermatophore(s) · *TAG* tubular accessory gland(s)

T. Yaginuma(✉) · T. Mizuno · C. Mizuno · M. Ikeda
O. Yamashita
Laboratory of Sericulture and Entomoresources, School of Agricultural Sciences, Nagoya University, Chikusa, Nagoya 464-01, Japan

T. Wada · K. Hattori
Laboratory of Plant Genetics and Breeding, School of Agricultural Sciences, Nagoya University, Chikusa, Nagoya 464-01, Japan

G. M. Happ
Department of Biology, Marsh Life Science Building, University of Vermont, Burlington, Vermont 05405, USA

Introduction

In many invertebrates, including insects, but in only a very few vertebrates, sperm is passed from the male to the female via a sperm sac or spermatophore (Spph). Enclosure in the Spph is thought to increase the chances of survival of sperm in the female genital tract (Mann 1984; Happ 1984, 1992). In the mealworm beetle, *Tenebrio molitor*, the Spph is formed from the secretory proteins of male reproductive accessory glands, the BAG and the TAG (Happ 1984, 1992). The eight types of cell in the BAG epithelium synthesize specific proteins and release them into the lumen of the BAG where they form a semisolid plug, a precursor to the wall of the Spph (Dailey and Happ 1983; Happ

1984). These secretory proteins are synthesized in the BAG after adult ecdysis (Grimnes and Happ 1986; Grimnes et al. 1986; Shinbo et al. 1987; Happ 1992). High activity of trehalase (α,α-trehalose glucohydrolase; EC 3.2.1.28) can be detected in the BAG a few days after adult ecdysis, but not in other reproductive organs of the male (Yaginuma and Happ 1988, 1989). High activity of trehalase is also found in the Spph. Therefore, the trehalase is thought to be secreted from the BAG and to become incorporated into the Spph (Yaginuma and Happ 1988).

In insects, as distinct from mammals, trehalose (α,α-glucoside-1-glucose) is known to be a major blood sugar (Wyatt 1967). Trehalase catalyzes conversion of 1 mol trehalose into 2 mol glucose, which is then utilized through glycolysis. Thus, for the uptake or utilization of trehalose in the blood, trehalase is an essential enzyme in insects and it is thought to be located on the cell membrane close to the blood or within the cells (Wyatt 1967; Azuma and Yamashita 1985a, b; Valaitis et al. 1993). In the midgut from larvae and in ovaries from pharate adults of the silkworm, *Bombyx mori*, trehalase binds, respectively, to the basolateral plasma membrane of the midgut epithelial cells and to the plasma membrane of the oocyte. It has been suggested that the function of the trehalase is to facilitate the uptake and utilization of trehalose from the blood (Azuma and Yamashita 1985a, b; Su et al. 1993, 1994). In vertebrate systems, trehalase binds at high levels to the apical surface of the brush-border membranes of the small intestine (Forstner et al. 1968; Ruf et al. 1991) and in the kidney proximal tubules (George and Kenny 1973; Takesue et al. 1986) but trehalose is not detectable at significant levels. In the case of the small intestine, trehalase is thought to be involved in the absorption of sugars in food (Ruf et al. 1991).

Information about secretory trehalases, such as the trehalase in the Spph of *Tenebrio*, is limited, although there are a few reports of secretory trehalases from insects, e.g. from the salivary glands of *Chironomus thummi* (Laufer et al. 1963) and in the cocoon floss of *B. mori* (Shimada et al. 1980). In this study we examined the cellular origin and the physiological role of the trehalase from the BAG by purifying trehalase from the BAG, determining its kinetic properties, and, by using a specific antiserum defined its localization in the BAG and the Spph.

Materials and methods

Animals

Mealworm larvae (*Tenebrio molitor*) were purchased from a commercial supplier and reared on wheat flour and fresh potatoes at 25 °C. Pupae were sexed and then kept at 25 °C. Adults were maintained on the same diet at 25 °C (Yaginuma and Happ 1988, 1989; Takiguchi et al. 1992).

Assay of trehalase activity and quantification of protein

The assay of trehalase activity was carried out as described by Yaginuma and Happ (1988, 1989). We used two procedures to determine levels of glucose: the Somogyi-Nelson method (Yaginuma and Happ 1988) and the glucose oxidase-peroxidase method (Sumida and Yamashita 1983). Trehalase activity was expressed as nmoles or μmoles glucose released per min.

Concentrations of protein were determined by the method of Lowry et al. (1951) or Bradford (1976) with a protein assay kit from Bio-rad, with bovine serum albumin as the standard protein.

Purification of trehalase

Step 1

All steps were carried out at 4 °C. BAG were dissected from 2- to 10-day-old male adults. They were kept at −70 °C until use. BAG (5.4 g fresh weight, about 1600 pairs of BAG) were homogenized in an agate mortar with ten volumes of 20 mmol·l^{-1} Kpi buffer (pH 7.0). The homogenate was centrifuged at 20000 *g* for 20 min. The pellet was homogenized with Kpi buffer, and the homogenate was centrifuged. The supernatants were combined.

Step 2

A solution of 1 mol·l^{-1} CH_3COOH was added to the combined supernatants until the pH had fallen to 4.8. The precipitate formed was collected by centrifugation at 25000 *g* for 20 min.

Step 3

The precipitate was homogenized with 7.5 ml of 20 mmol·l^{-1} Kpi buffer. The homogenate was centrifuged at 20000 *g* for 20 min. The pellet was homogenized with 20 mmol·l^{-1} Kpi buffer and, after centrifugation, the supernatants were combined and applied to a column (1.7 cm i.d. × 26 cm) of DEAE-cellulofine A-800 (Chisso Co., Tokyo) that had been equilibrated with 20 mmol·l^{-1} Kpi buffer. No trehalase activity was found in fractions eluted with 20 mmol·l^{-1} Kpi buffer. Adsorbed proteins were eluted with a 500-ml linear gradient of 0–0.4 mol·l^{-1} NaCl in 20 mmol·l^{-1} Kpi buffer. The fractions containing trehalase activity were eluted at around 0.18 mol·l^{-1} NaCl. The fractions containing trehalase activity were pooled and dialyzed against distilled water plus 0.5% (v/v) glycerol.

Step 4

The dialyzed sample was concentrated in a Centrifuge-Evaporator (EC-57; Sakuma, Tokyo). The resultant sample (0.5 ml) was applied to a column (1.6 cm i.d. × 90 cm) of Sephacryl S-300 (Pharmacia) that had been equilibrated with 20 mmol·l^{-1} Kpi buffer that contained 200 mmol·l^{-1} NaCl. Fractions with trehalase activity were pooled and dialyzed against distilled water plus 0.5% (v/v) glycerol.

Detection of proteins by silver staining on native and denaturing gels after PAGE

Proteins on each gel were stained according to the protocol provided with reagents in a silver-staining kit (2D-silver strain-II "Daiichi") from Daiichi Pure Chemicals Co., Tokyo.

Table 1 Purification of trehalase from bean-shaped accessory glands of male adult mealworm beetles. About 5 g of BAG were used as starting material. See Materials and methods for details

	Protein (mg)	Total activity ($\mu mol \cdot min^{-1}$)	Specific activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein)	Purification (-fold)	Yield (%)
Enzyme source	160.0	226.8	1.4	1	100
Acid treatment	51.6	208.3	4.0	2.9	91.8
DEAE-cellulose	3.9	195.9	50.1	35.8	86.4
Sephacryl S-300	0.08	41.7	521.3	372.3	18.4

Detection of trehalase activity after PAGE on a non-denaturing gel

The band corresponding to trehalase activity on the gel was visualized by the method of Talbot and Huber (1975) with slight modifications (Sumida and Yamashita 1983).

Determination of molecular mass

The molecular mass of the purified trehalase was determined by gel filtration on the column of Sephacryl S-300 in 20 $mmol \cdot l^{-1}$ Kpi buffer that contained 500 $mmol \cdot l^{-1}$ NaCl and 3% (v/v) glycerol, or on a GF-250 column (0.94 cm i.d. × 25 cm; Zorbax Bio-Series, Du Pont) in 200 $mmol \cdot l^{-1}$ Kpi buffer that was part of an HPLC system (Trirotar-III, Jasco, Tokyo), with the following proteins (from Pharmacia) as standards: aldolase (153 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and chymotrypsinogen (25 kDa). The molecular mass of the denatured form of the purified trehalase was determined by SDS-PAGE using standards; phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and carbonic anhydrase (30 kDa) from Pharmacia, or β-galactosidase (116 kDa), albumin (66 kDa), aldolase (42 kDa) and carbonic anhydrase (30 kDa) from Daiichi.

Preparation of trehalase-specific antiserum

The purified enzyme was subjected to SDS-PAGE on a 10% polyacrylamide gel. After light staining with Coomassie brilliant blue R-250 the band was cut from the gel. The piece of gel was homogenized in PBS. The homogenate (about 8 μg protein) was mixed with an equal volume of Adjuvant Peptide (Wako Pure Chemical Co., Tokyo). The mixture was injected six times into a mouse at 1-week intervals. Two weeks after the last injection the blood was collected. The supernatant after centrifugation at 16000 *g* for 5 min was used as trehalase-specific antiserum.

Immunoblotting

After SDS-PAGE proteins were transferred electrophoretically to a nitrocellulose membrane which was probed with trehalase-specific antiserum. The immunoreactive band was visualized with the peroxidase-conjugated anti-mouse IgG serum (Zymed Laboratories) and an immunostaining HRP kit (IS-50B) from Konica (Tokyo).

Immunohistochemical staining

The staining was carried out by the method of Ikeda et al. (1991) with slight modifications. Male accessory glands and Spph were fixed in Carnoy's solution (ethanol:chloroform:acetic acid = 6:3:1, v/v) for 6 h. They were dehydrated through an ethanol series and an ethanol plus *n*-butanol series, embedded in paraffin (Histoprep; Wako Pure Chemical Co., Tokyo) and sectioned at a thickness of 8 μm. The sections were mounted on glass slides, deparaffinized, hydrated through xylene-ethanol and ethanol-water series, and finally washed with PBS. After treatment with 3% (v/v) hydrogen peroxide, the sections were incubated with 10% (v/v) normal rabbit serum to block non-specific staining. The sections were incubated with the trehalase-specific antiserum that had been diluted to 1:1000 with 0.1% (v/v) normal rabbit serum for 3 h and then washed with PBS. The antigen with bound primary antibodies was visualized with a Histofine SPO kit and a Histofine DAB Substrate kit (Nichirei Co., Tokyo). The sections were dehydrated through an ethanol-xylene series, and mounted in Entellan (Merck). The sections were observed under a light microscope.

Expansion and rupture of the Spph in vitro

Each Spph was collected from a male gonopore about 30 s after the start of copulation with a female. Spphs were placed in PBS and over the next 7 min they changed shape and burst to liberate the sperm as they do in the bursa of the female (Gadzama and Happ 1974). These events were observed under a binocular microscope at room temperature.

Results

Purification

The purification of trehalase from the BAG is summarized in Table 1. The enzyme was precipitated by shifting the pH of the solution to 4.8, even though the soluble trehalases from the male accessory gland of *Periplaneta americana* (Ogiso et al. 1982, 1985) and from the midgut of pharate adults of *B. mori* (Sumida and Yamashita 1983) are not precipitated by such acid treatment. When the purified trehalase was stained after electrophoresis with silver-staining reagents the site of the major band corresponded to that of the trehalase activity after PAGE on a non-denaturing gel (Fig. 1A, B).

Some properties of the enzyme

The molecular mass of the purified trehalase was estimated to be 43 kDa by gel filtration on Sephacryl S-300 and GF-250 (HPLC) (Table 2). The denatured enzyme gave a single band after SDS-PAGE (Fig. 1C), which corresponded to a molecular mass of 62 kDa (Table 2).

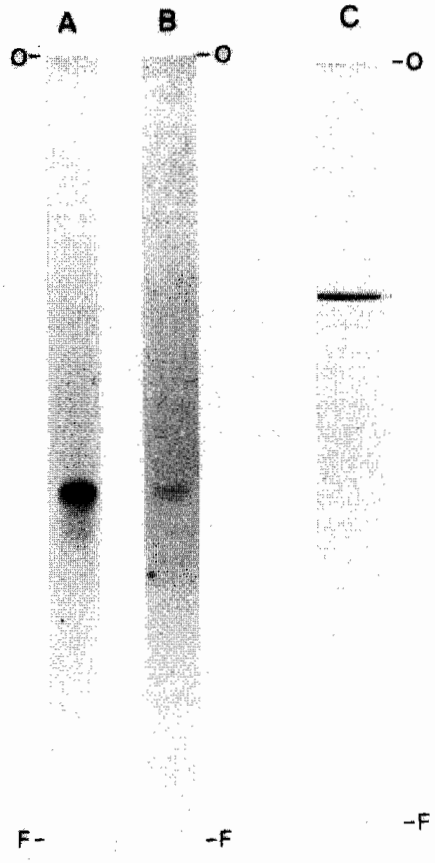

Fig. 1A–C Results of non-denaturing PAGE (**A** and **B**) and SDS-PAGE (**C**) of the purified trehalase. **A** and **C** staining for trehalase by the silver-staining method. **B** Detection of trehalase activity on the gel. Purified protein (1 µg) was loaded onto a 7% polyacrylamide gel (**A** and **B**) and a 10% polyacrylamide gel with SDS (**C**). *O* Origin; *F* front

In general, glycoproteins tend to bind to resins used for gel filtration. Sugar moieties have been reported to be included in trehalases (Sumida and Yamashita 1983), and potential sites for glycosylation have been found in the amino-acid sequence deduced from the cDNA for trehalase from *Tenebrio* BAG (Takiguchi et al. 1992). Therefore, the apparent molecular mass of the native form of purified trehalase is probably lower than that of the denatured form because of retarded elution from the gel-filtration column due to the presence of the putative sugar moieties.

The purified trehalase was maximally active at around pH 5.6–5.8 (Table 2). The purified trehalase hydrolyzed only trehalose among various disaccharides tested (Table 3). The K_m for trehalose was about 4 mmol·l^{-1} (Table 2). The trehalase activity was strongly inhibited by validoxylamine A, a compound isolated from *Streptomyces* and known to be a specific competitive inhibitor of trehalases (Asano et al. 1987, 1990; Kameda et al. 1987; Takeda et al. 1988; Kono et al. 1994). The activity of the purified trehalase was

Table 2 Properties of the purified trehalase. Each valuc is the mean of results from three determinations with SEM

Molecular mass	
native (kDa)	43 ± 1^{ab}
denatured (kDa)	62 ± 2^{c}
pH optimum	5.6 – 5.8
K_m for trehalose (mmol·l^{-1})	4.4 ± 0.3
Activation energy (kJ·mol^{-1})	41.5 ± 1.5

[a] Estimated by column chromatography on Sephacryl S-300
[b] estimated by HPLC on GF-250 column
[c] estimated by SDS-PAGE

Table 3 Substrate specificity of the purified trehalase from bean-shaped accessory glands. The enzymatic activity was assayed in the presence of 66.6 mmol·l^{-1} citrate/Na_2HPO_4 buffer (pH 5.6) and each substrate (33.3 mmol·l^{-1}). Each value was estimated from three determinations. SEMs were within 6%. N.D. = not detected

Activity as % of control	
Trehalose (control)	100
Sucrose	0.1
Maltose	N.D.
Lactose	1.4
Melibiose	N.D.
Cellobiose	0.7
Raffinose	2.2

halved by validoxylamine A at $4.5 \cdot 10^{-9}$ mol·l^{-1}. Since V_{max} in the presence of the inhibitor was the same as that without the inhibitor, the inhibitor appeared to act as a competitor against trehalose in this case also. Using $4.5 \cdot 10^{-9}$ mol·l^{-1} validoxylamine A, we estimated the K_i value to be $5.9 \pm 0.4 \cdot 10^{-13}$ mol·l^{-1} ($n = 3$; mean ± SEM). The activity of the purified trehalase increased linearly with increases in the incubation temperature from 20 to 50 °C. From 60 to 70 °C the activity decreased. Arrhenius plots indicated that the activation energy was about 40 kJ·mol^{-1} (Table 2).

Western blotting analysis

Western blotting after SDS-PAGE with trehalase-specific antiserum showed that the purified trehalase was recognized by the antiserum (Fig. 2A). Tissues from male adults 6 days after ecdysis were homogenized and extracts were examined by Western blotting (Fig. 2B). The antiserum recognized bands of proteins of 62 and 58 kDa from BAG and Spph. A band of a 42-kDa protein was also found in analyses of TAG, BAG and Spph (Fig. 2A, B). The tissue-specific localization of the 62-kDa polypeptide corresponded closely to the tissue distribution of trehalase activity and of trehalase mRNA (Yaginuma and Happ 1988; Takiguchi et al. 1992). Although it is still unclear whether the polypeptides of 58 and 42 kDa were degraded forms of the

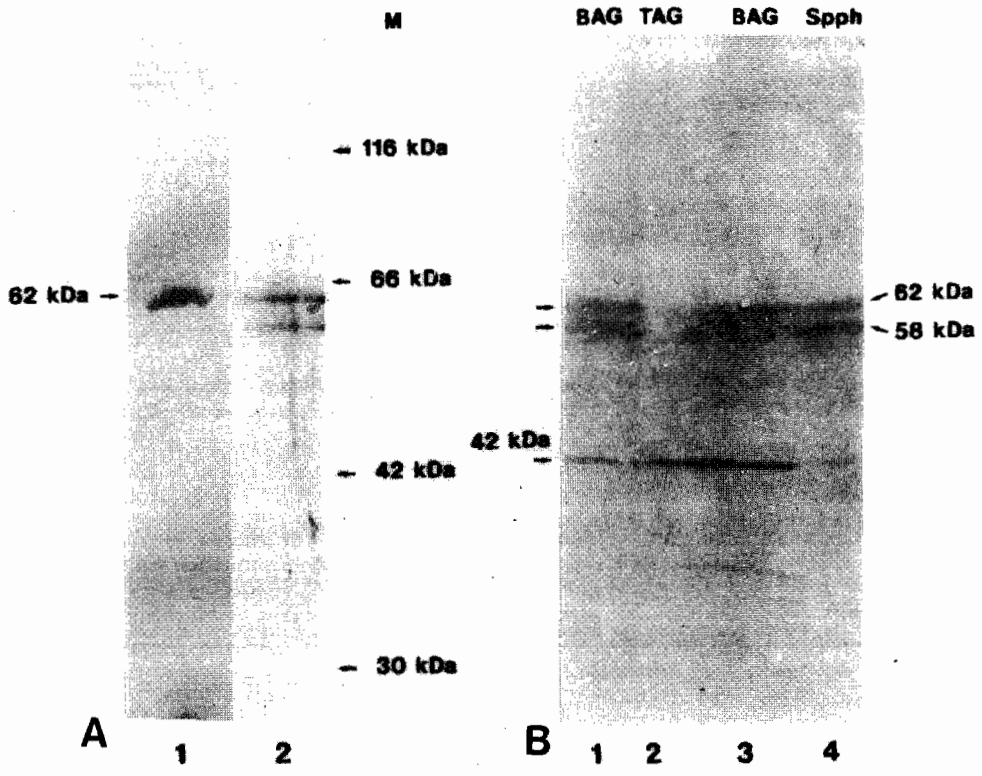

Fig. 2A, B Western blot after SDS-PAGE of purified trehalase and tissue homogenates. Each tissue was collected from males 6 days after adult ecdysis. Proteins were separated by SDS-PAGE on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane which was then incubated with trehalase-specific antiserum. **A** Purified trehalase (1 μg of protein, *lane 1*) and Spph (100 μg of protein, *lane 2*); *M* size markers; **B** BAG (100 μg of protein, *lanes 1 and 3*), TAG (100 μg of protein, *lane 2*) and Spph (100 μg of protein, *lane 4*)

mature trehalase protein, they clearly had common immunological determinants.

Immunohistochemical localization of trehalase in the BAG

To examine the localization of trehalase in the BAG, paraffin sections of BAG were treated with the trehalase-specific antiserum. Trehalase was confined to specific cells in the secretory epithelium of the BAG (Fig. 3A, B). Furthermore, products of reactions with the antiserum were found on the semisolid plug in the lumen of the BAG (Fig. 3B). No staining was observed in control sections that had been treated with the pre-immune serum.

Distribution of trehalase activity in various fractions during evacuation of Spph in vitro

To determine the location of trehalase in the Spph, trehalase activity in various fractions was investigated during and after evacuation of the Spph in PBS. As the Spph emerges from the male gonopore (Fig. 4A), the sperm are enclosed in a cavity between the outer wall and the inner core (Gadzama and Happ 1974; Paesen et al. 1992). The anterior portion of the Spph undergoes a two-stage elongation (Fig. 4B, C), forms a bulb (Fig. 4D) and finally bursts.

In the first experiment the Spph was placed in a small volume of PBS, allowed to burst, and then left in the same solution for 5 h so that the contents of the lumen and soluble molecules could diffuse into the PBS. The empty Spph contained very little trehalase activity; almost all of the enzymatic activity had been dissolved in the PBS (Fig. 5A).

In the second experiment the Spph was allowed to expand and burst. Just as the bulb ruptured the emerging sperm mass was collected with a microcapillary pipette. This sperm mass consisted mostly of the contents of the lumen from inside the Spph. This sample contained very little trehalase activity (Fig. 5B).

In the third experiment the freshly produced Spph was rinsed in PBS just after it had been collected from the male (Fig. 4A) and then it was transferred to a second container of PBS where it was allowed to expand to form a bulb (Fig. 4B, C, D). Before the bulb could rupture, the expanded Spph (Fig. 4D) was transferred to a third container of PBS where it subsequently burst to liberate the sperm. The third solution of PBS contained in a mixture of the luminal contents, the wall of the Spph and materials that had been washed off the wall. Significant trehalase activity was found in all three PBS solutions (Fig. 5C). Since trehalase activity was found in the first and second washes (before rupture of

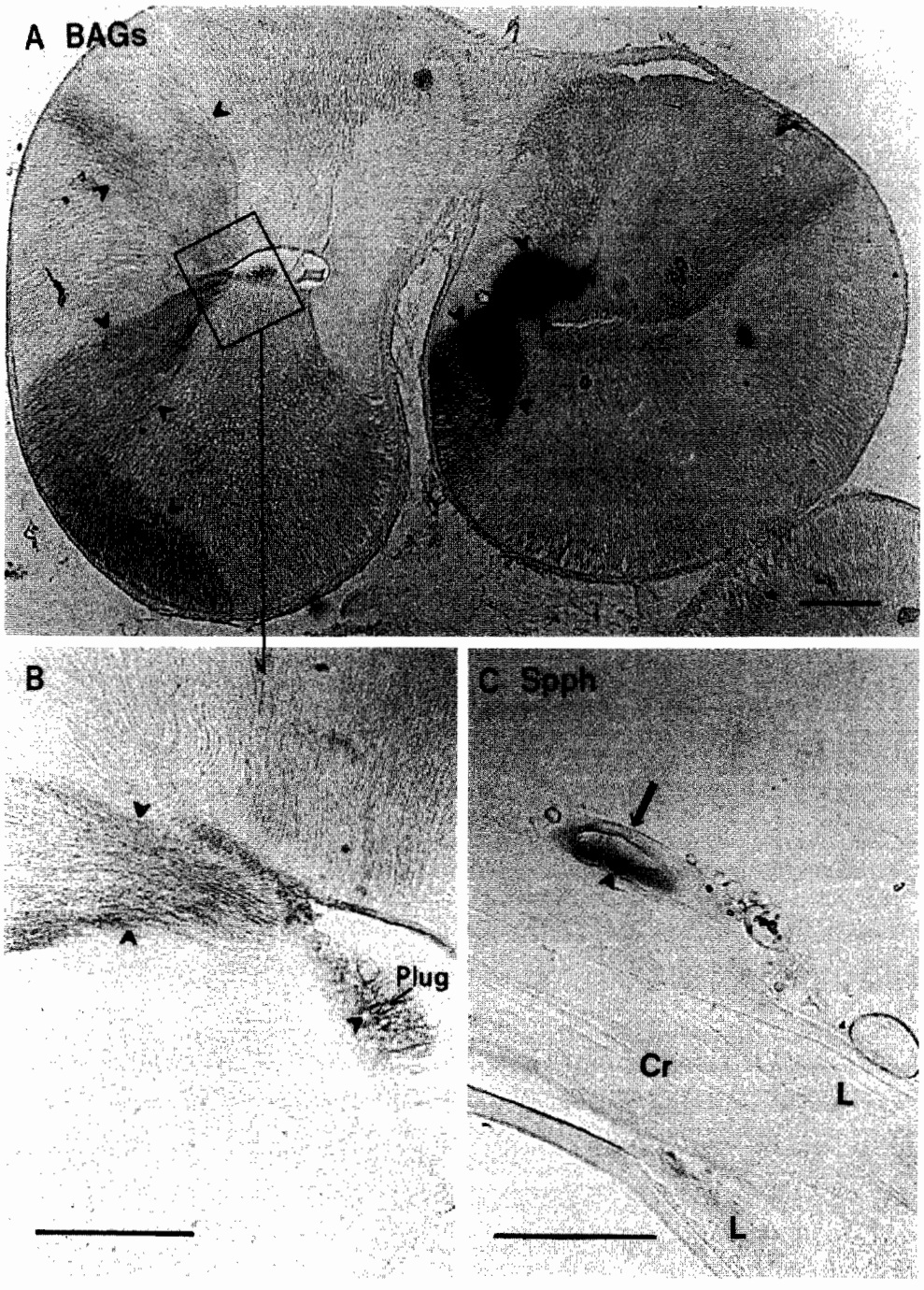

Fig. 3A–C Immunohistochemical localization of trehalase in the BAG and the Spph. Histological sections of BAGs from 6-day-old adults and of a Spph were stained with trehalase-specific antiserum. **A** and **B** BAG; **C** Spph. *Plug* semisolid plug, namely, a precursor to the wall of the Spph; *Cr* core; *L* lumen. Each *bar* represents 100 μm. Dark areas (*arrowheads*) and the *arrow* indicate specific staining. No staining was observed in control sections treated with the pre-immune serum

the wall), much of the enzyme must have been readily released from the outer surface of the Spph and, perhaps, also from the core material that flows forward during expansion of the Spph (Fig. 4).

From these three experiments we concluded that the trehalase in the wall of the Spph was soluble, that it was not present in the sperm mass or in the contents of the lumen, and that it was loosely associated with the outer wall and/or the multilayered core in the anterior portion of the newly formed Spph (Fig. 4).

Immunohistochemical localization of trehalase in the Spph

To confirm our conclusions the localization of trehalase in the Spph was determined by an immunohistochemical method. Immunohistochemical observations indicated that trehalase was located at the evaginating core, the outgrowth on the wall of the Spph (arrow and arrowhead in Fig. 3C; asterisk in Fig. 4B). No staining was found in sections that had been treated with pre-immune serum.

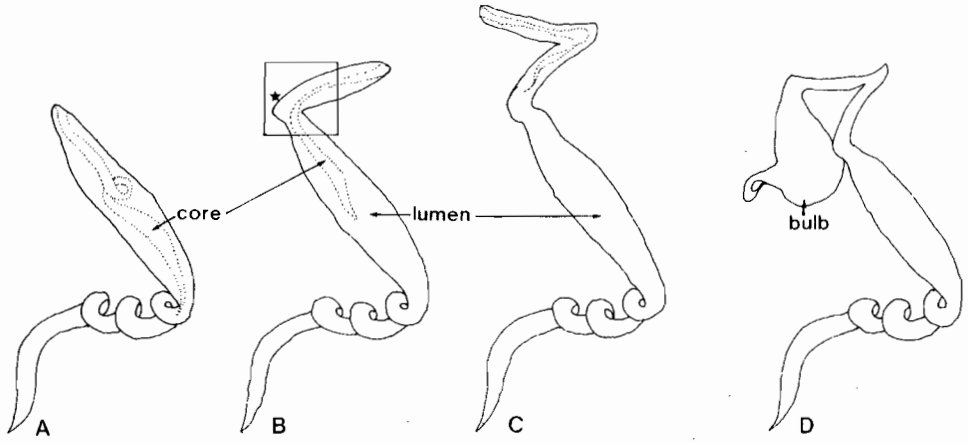

Fig. 4A–D Diagram showing the details of evacuation of the Spph in PBS. The Spph undergoes a two-stage elongation, forms a bulb and then bursts to release the contents of the lumen which include sperm and semen. The elongations are accompanied by changes in the distribution of the core contents (Gadzama and Happ 1974). **A** Spph just after isolation from the gonophore of an adult male; **B** The first elongation; the region in a box corresponds to the section in Fig. 3C. An *asterisk* indicates the region that was stained with the antiserum; **C** The second elongation; **D** Formation of the bulb

Effect of an inhibitor specific to trehalase on the evacuation of the Spph in vitro

The driving forces underlying the expansion of the Spph and expulsion of sperm are not well understood in any species of insects (Happ 1984). In the cricket, *Acheta domesticus*, osmotic flow of water has been invoked to explain the expulsion of sperm (Khalifa 1949), and in the tick production of gaseous CO_2 apparently accounts for the movement of sperm into the bursa (Feldman-Muhsam et al. 1973). Neither explanation seems sufficient to explain the programmed sequence of evaginations that leads to the rupture of the Spph of *T. molitor* (Gadzama and Happ 1974). In the giant octopus, osmotic differences have been shown to act as the driving force for the spermatophoric reaction in vitro. However, highly active glycosidases in the Spph appear to play an important role in the spermatophoric reaction, most likely by facilitating degradation of those structural mucoproteins that might otherwise have constituted a mechanical barrier to the advancement of the sperm rope and evagination of the ejaculatory apparatus (Mann 1984). Thus, to determine whether trehalase activity is required for evacuation of the Spph of *Tenebrio*, newly formed Spphs were placed in PBS with an inhibitor, validoxylamine A, that is specific to trehalase. In control experiments, the elongation and formation of the bulb that had ruptured took place within about 7 min (Table 4). Neither significant retardation of the expansion nor inhibition of the rupture was observed in PBS that contained validoxylamine A (Table 4).

Fig. 5A–C Distribution of trehalase activity in various fractions of the Spph. Spph were collected from gonophores of male adults about 30 s after the start of copulation and transferred to PBS. **A** In the first batch of PBS, Spph finished releasing the contents of the lumen that included sperm and semen, and empty Spph were obtained after 5 h; **B** In the first batch of PBS, Spph expanded (Fig. 4) and released the luminal contents. Using microcaps (Drumond) we collected the luminal contents including the sperm mass, and empty Spph were also collected; **C** Spph were just rinsed in the first batch of PBS (Fig. 4A) and then they were rapidly transferred to the second batch of PBS where they expanded but did not burst (Fig. 4 B, C, D). In the third batch of PBS the Spph finally burst and released their contents. PBS plus luminal contents and the empty Spph in **A**; PBS, the luminal contents and the empty Spphs in **B**; and the first batch of PBS, the second batch of PBS and the third batch of PBS plus luminal contents plus the empty Spphs in **C**, were used as sources of enzyme. The assays of enzymatic activity were carried out at 30 °C for 60 min. Each reaction mixture contained 66 mmol·l^{-1} citrate/Na_2HPO_4 buffer (pH 5.6) and 33 mmol·l^{-1} trehalose (Yaginuma and Happ 1988). Each percentage represents the mean of results from eight experiments (SEMs were within 8%). Trehalase activity in one fresh Spph was 16.8 ± 1.1 nmol·min^{-1}

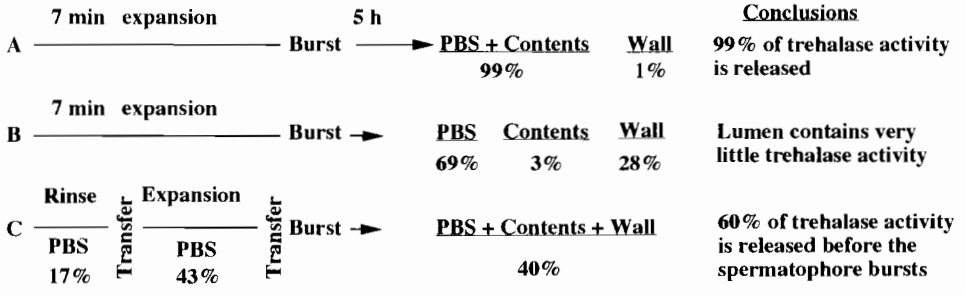

Table 4 Effect of validoxylamine A on the expansion and bursting of the fresh spermatophores. Spph were immersed into PBS without or with validoxylamine A at a concentration of 8.6 μmol·l^{-1} at which it inhibited more than 99% of the trehalase activity. The expansion and evacuation of Spph were observed under a binocular microscope at room temperature

Time prior to expansion of the bulb[a] (min)	
In the absence of the inhibitor	7.1 ± 0.7[b]
In the presence of the inhibitor	7.3 ± 0.5[b]

[a] Rupture took place just after the formation of the bulb. Each value represents the mean of results from six experiments with SEM
[b] No significant difference between results, as determined by Student's *t*-test

Discussion

In this study, trehalase was purified from the BAG of *T. molitor* by acid treatment and column chromatography on DEAE-cellulofine and Sephacryl S-300. The kinetic properties of the purified trehalase were similar to those of the enzyme in a crude preparation from BAG (Yaginuma and Happ 1988) and were mostly consistent with the properties reported for other trehalases purified from various organisms including insects (Gilby et al. 1967; Lefevre and Huber 1970; Friedman 1975; Talbot and Huber 1975; Talbot et al. 1975; Terra et al. 1978; Nakano 1980; Sumida and Yamashita 1983; Ogiso et al. 1985; Terra et al. 1985; Vaandrager et al. 1989; Jahagirdar et al. 1990; Valaitis et al. 1993), except as described below. From the specific activity of the purified trehalase (Table 1) the trehalase protein is estimated to account for about 0.3% of the total proteins in the BAG or Spph of *T. molitor*.

Although the molecular mass (62 kDa) of the denatured form of the purified trehalase from BAG was larger than that (43 kDa) of the native form, the trehalase from BAG appears to consist of a single polypeptide of 62 kDa. In fact, a cDNA was isolated from a cDNA library generated from poly(A)$^+$ RNA prepared from the BAG of mealworm beetles of the same age. The cDNA encoded a putative protein of 64457 Da (Takiguchi et al. 1992). After removal of the signal peptide, the molecular mass of the mature trehalase was estimated to be 62669 Da (Takiguchi et al. 1992). This molecular mass is consistent with the value of 62 ± 2 kDa determined by SDS-PAGE in this study. The elution of native trehalase might perhaps have been retarded by sugar moieties that bound to the gel-filtration column. This property was useful in that it allowed us to separate the enzyme from other proteins by the gel filtration.

As described previously, the male reproductive accessory glands of *T. molitor* consist of two pairs of accessory glands, the TAG and the BAG (Happ 1984, 1992). They originate from a mesodermal pouch near the ninth sternite of the last larval instar (Huet 1966) and grow as a result of increases in cell number through cell division during the 9-day pupal stage (Happ et al. 1985; Yaginuma et al. 1988). During the period of cell division, the many cells that will eventually give rise to the secretory epithelium of the mature BAG are morphologically similar to one another. From the end of the pupal stage to 2 days after adult ecdysis, the eight cell types that are characteristic of the mature glands become clearly defined (Dailey et al. 1980; Dailey and Happ 1983; Happ 1984). When the definitive adult morphology of the BAG has been established, rapid synthesis and accumulation occur of adult-specific proteins (Grimnes and Happ 1986; Grimnes et al. 1986; Shinbo et al. 1987). Cell-specific secretory proteins synthesized by each type of cell are incorporated first into the semisolid plug and then they become organized into specific layers of the Spph (Happ 1984, 1992). To sustain the high rates of production of the proteins that are involved in the formation of the Spph, high rates of energy production must be required in the BAG. Since one major source of energy might be trehalose in the blood (Wyatt 1967), BAG might be expected to contain trehalase. In fact, extremely high-level activity of trehalase is found in the BAG. It is, however, surprising that the Spph also has high trehalase activity (Yaginuma and Happ 1988).

The immunohistochemical experiments in this study clearly demonstrate that trehalase protein is abundant in one type of cell that is restricted to a specific region within the BAG epithelium and is then secreted into the lumen to form the semisolid secretory plug. Finally, the trehalase protein is located in the outgrowth on the wall of the Spph. The trehalase protein from the mature BAG is an adult-specific protein. It has been shown that an ecdysteroid hormone acts on the pupal BAG and renders it competent to synthesize trehalase in the adult BAG (Yaginuma and Happ 1989; Takiguchi et al. 1992).

What is the physiological function of trehalase in the Spph? Since the Spph has been shown to contain a significant amount of trehalose, the substrate for trehalase (Yaginuma and Happ 1988), the trehalase-trehalose system seems likely to play a physiological role. However, addition of the specific inhibitor of trehalase, validoxylamine A, did not affect the evacuation of the Spph in vitro. Therefore, trehalase activity seems not to be required for evacuation of the Spph. The results suggest that trehalase might function as one of the structural proteins of the Spph. However, in the course of the evacuation of the Spph in vitro, trehalase in the Spph is almost all dissolved in the surrounding medium before the rupture of the sperm sac. Since trehalose is thought to be located in the lumen of the Spph before the rupture of the Spph, the trehalase on the outside and/or within the core is unlikely to come into contact with trehalose as a substrate. The enzyme and its substrate might gain access to each other when the sperm package bursts in the bursa of the female and the

seminal fluids rush out to mix with trehalase released from the wall. In addition, the trehalase on the wall of the Spph can act on the trehalose in the fluid within the bursa of the female if this substrate is present in the fluid of the bursa. Thus, the possibility can not yet be completely ruled out that the glucose liberated at this site might provide the energy required for the activation and/or viability of the sperm.

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