J Comp Physiol B (1988) 157:765–770

Journal of Comparative Physiology B Biochemical, Systemic, and Environmental Physiology

Trehalase from the bean-shaped accessory glands and the spermatophore of the male mealworm beetle, *Tenebrio molitor*

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Accepted October 16, 1987

Summary. In *Tenebrio molitor*, male adults transfer sperm to the female via a spermatophore or sperm sac. The spermatophore is formed from secretions of the bean-shaped accessory glands (BAGs) and the tubular accessory glands (TAGs) of the male beetle. Trehalase is found in the adult BAGs. During the pupal stage, the activity in the BAGs was very low. After adult ecdysis, the total activity increased 100-fold from 0 days to 6 days and reached maximum levels at 9 days. The specific activity increased 20-fold from the time of ecdysis to 6 days thereafter. In the 10 day adult, trehalase levels in testes, seminal vesicles, vas deferens, TAGs, or ejaculatory ducts, were lower by two orders of magnitude than in the BAGs. However, the specific activity in the spermatophore was similar to that in the BAGs. Trehalases in the BAGs and the spermatophores showed very similar properties (soluble, optimum pH of 5.75 and K_m value of 5.4 mM for trehalose). Thus trehalase appears to be secreted from the BAGs and becomes incorporated into the spermatophores.

Introduction

In many invertebrates, sperm is passed from male to female within a spermatophore or sperm sac (Mann 1984). In the mealworm beetle, *Tenebrio molitor*, the spermatophore is formed from the secretions of two pairs of accessory glands, the bean-shaped accessory glands (BAGs) and the tubular accessory glands (TAGs) (Happ 1984). The eight secretory cell types of the BAGs produce a semi-solid plug that is a precursor to the wall of the spermatophore (Dailey et al. 1980; Grimnes and Happ 1986; Grimnes et al. 1986; Shinbo et al. 1987).

BAGs arise from a larval mesodermal pouch (Huet 1966) that grows markedly in size by cell division during the 9 days of pupal development (Happ et al. 1982; Happ et al. 1985; Yaginuma et al., to be published). After adult ecdysis, there is rapid accumulation of proteins involved in the formation of the spermatophore (Happ et al. 1982; Shinbo et al. 1987). One would expect reasonably high metabolic rates to sustain the high rates of production of secretory proteins. One major source of energy might be trehalose in the blood (Wyatt 1967). If such is the case, BAGs might be expected to contain trehalase (α,α-glucoside-1-glucohydrolase: E.C. 3.2.1.28). In fact, high trehalase activity has been reported from the accessory glands of male American cockroaches (Ogiso and Takahashi 1984; Ogiso et al. 1985).

In this study, we show that trehalase activity is high in the mature BAGs. Trehalase activity increases dramatically as the glands mature in the post-ecdysial adult. Moreover, trehalase activity was found in the spermatophores.

Materials and methods

Animals. Larvae of *Tenebrio molitor* were purchased from a commercial supplier and reared on Purina Chick labchow at 25 °C. Male white pupae were collected after larval-pupal ecdy-

Abbreviations: BAG bean-shaped accessory gland; *TAG* tubular accessory gland

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sis and allowed to develop at 25 °C. Male adults were maintained on the same diet (Shinbo et al. 1987).

Enzyme preparation. BAGs were dissected from pupae and adults in ice-cold phosphate-buffered saline (PBS; 8.2 m*M* Na_2HPO_4, 1.5 m*M* K_2HPO_4, 137 m*M* NaCl and 26 m*M* KCl, pH 7.4) and blotted on filter paper (Shinbo et al. 1987). The glands were homogenized in 200 volumes of 25 m*M* NaH_2PO_4–Na_2HPO_4 buffer (pH 6.8 phosphate buffer) using a small glass homogenizer. This crude homogenate was used as an enzyme source.

Spermatophores were obtained fresh after interruption of copulation or collected from the diet. When males do not copulate with females for a day or more, they spontaneously eject spermatophores. These deposited spermatophores dry quickly and do not burst to liberate sperm. The dried spermatophores stick to particles of the Purina labchow, and can be retrieved after careful inspection of the diet in cultures of male beetles. For studies of enzyme kinetics, about 200 spermatophores were homogenized in 4.7 ml phosphate buffer (pH 6.8). The crude homogenate was used as an enzyme source.

Protein determination was by the method of Lowry et al. (1951) with bovine serum albumin as the standard. TCA-insoluble material from a homogenate was washed twice with diethyl ether: ethanol (1:1) and once with pure diethyl ether. These dried materials were dissolved in 1N NaOH and used for the Lowry assay.

Assay of trehalase activity. Conditions for routine enzyme assays were established from kinetic experiments, as suggested by Sumida and Yamashita (1983). The standard reaction mixture contained: 1) 100 µl enzyme source (*ca.* 30–70 µg protein, and 2) 200 µl substrate solution (50 m*M* trehalose, 100 m*M* citrate-Na_2HPO_4 buffer, pH 5.6). The final reaction mixture (300 µl) was at pH 5.75. The reaction was allowed to proceed at 30 °C for 20 min and then stopped by boiling for 5 min. After cooling, 0.5 ml of distilled water was added and the solution spun at 1000 *g* for 15 min.

An aliquot of the supernatant was used for determination of the glucose released from trehalose. Glucose content was determined by the Somogyi-Nelson (Nelson 1944) or glucose oxidase (Hugget and Nixon 1957) methods. Enzyme activity was expressed as µmole or nmole of glucose formed per minute.

Determination of sugars in spermatophores. Four hundred spermatophores were homogenized in 2.0 ml of 80% ethanol using a glass homogenizer. The homogenate was centrifuged at $1000 \times g$ and the ethanolic supernatant saved. The precipitate was reextracted twice with 1.0 ml of 80% ethanol and the ethanolic extracts combined. The combined extract was dried under reduced pressure and then dissolved in 0.5 ml of distilled water.

For *qualitative analysis*, an aliquot of this solution was spotted at the origin of a thin layer chromatography plate, precoated with silica gel (20 cm × 20 cm, Sigma). These plates were developed using two solvent systems: 1) butanol:acetic acid:ethanol:distilled water (9:6:3:1) and 2) ethyl acetate:isopropanol:distilled water (65:17.5:12). Each sugar was visualized by spraying the chromatogram with 1% $KMnO_4$ in 1N NaOH (Yaginuma and Yamashita 1978). Each sugar in the spermatophore extract was identified by comparison of the RF values with those of authentic co-migrating sugars.

For *quantitative analysis*, 0.1 ml of the aqueous solution (above) was used. Glucose content was determined as indicated above. To determine trehalose content, the aqueous extract was incubated with trehalase from the BAGs which had been partially purified using DEAE-Trisacryl M (LKB) column chromatography. Trehalose content was determined from the difference between glucose content before and after incubation with trehalase.

Results

Changes in the wet weight and protein content of BAGs during pupal-adult development

Immediately after larval-pupal ecdysis, the wet weight of one pair of BAGs (including the small outgrowths that will become the TAGs) was about 0.34 mg. Wet weight increased gradually during the pupal stage and linearly from adult ecdysis to six days later when it reached a plateau value of slightly over 3 mg (Fig. 1 A). Protein content increased in a similar manner (Fig. 1 B), confirming the earlier result of Happ et al. (1982).

Changes in trehalase activity of the BAGs during pupal-adult development

Trehalase activity increased during the maturation of the BAGs. During the pupal stage, trehalase activities in the BAGs were very low – around 0.1 to 0.8 nmol/min·BAG pair (Fig. 1 A). After adult ecdysis, the trehalase activity in BAG homogenates increased in an almost linear manner – from ca. 4 nmol/min·BAG pair on the day of adult ecdysis to 380 nmol/min·BAG pair on day 6 and finally reaching the maximum level of 430 nmol/min·BAG pair on day 9 (Fig. 1 A). Specific activity followed a similar pattern, as it increased about 20-fold from adult ecdysis to the sixth day (Fig. 1 B).

Trehalase activities of reproductive organs of adult males

We determined the level of trehalase activity in the various reproductive organs of male adults. For testes, seminal vesicles/vasa deferentia, the ejaculatory ducts, and most notably, the TAGs, trehalase activity was lower by two orders of magnitude than for the BAGs (Table 1). To determine whether the trehalase of the BAGs was secreted into the lumen and incorporated into the spermatophore, dried spermatophores (collected from the beetle culture boxes) were homogenized and assayed for trehalase. A significant amount of trehalase was found in the spermatophores, and the specific activity was quite similar to that in BAGs from 10-day adult males. This spermatophore fraction was inevitably contaminated with particles of

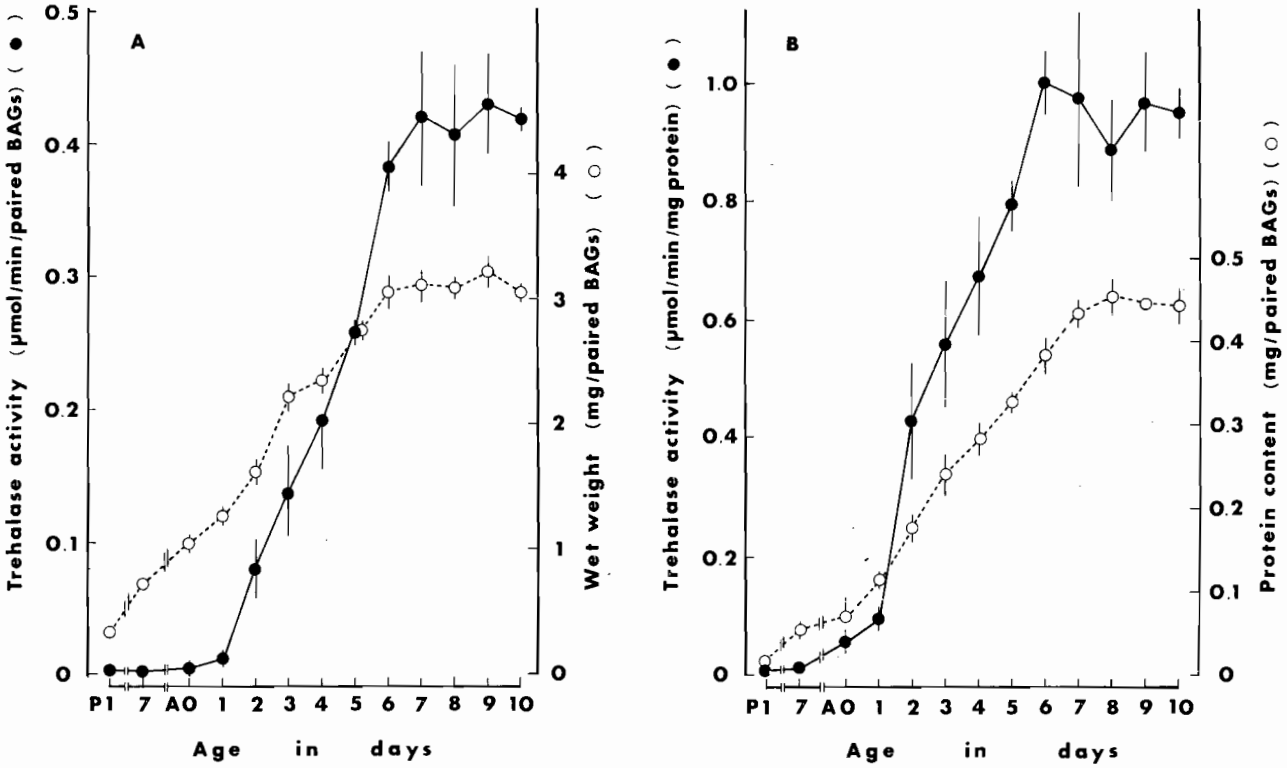

Fig. 1 A, B. Changes in trehalase activity, wet weight and protein content of BAGs during pupal-adult development. **A** Changes in trehalase activity and wet weight of BAGs per animal. **B** Changes in specific activity of trehalase of BAGs and protein content of BAGs per animal. *P* pupal stage; *A* adult stage. For the 1 day pupa, 40 TAG/BAG complexes were used. For 7 day pupa, 0 day adult, and 1 day adult, 25, 14, and 10 BAG pairs were used, respectively. For 2 day to 10 day adults, 5 BAG pairs were used. Trehalase activity was determined as in Materials and Methods and was expressed as μmoles of glucose released per minute. Each point represents a mean of two experiments for pupal stages and three experiments for adult stages ±SEM (vertical bars)

Table 1. Trehalase activities in reproductive system of 10 day male adults and spermatophore of *Tenebrio molitor*

Tissue[a]	Total activity (nmol/ min·animal)	Specific activity (nmol/ min·mg protein)
Testes	2.14± 0.39	7.05± 2.02
Seminal vesicles and vasa deferentia	1.87± 0.17	21.99± 1.98
TAGs	0.12± 0.00	1.17± 0.01
BAGs	517.25±42.37	1156.87±36.28
Ejaculatory duct	1.35± 0.24	30.78± 2.46
Spermatophores[b]	12.94± 0.57[c]	1648.46±80.88

[a] Each tissue pooled from 10 animals was used for trehalase assay

[b] Spermatophores were deposited on diet by male adults and then collected. 200 spermatophores were used for the assay

[c] The activity was expressed as per one spermatophore. Each value represents a mean of three experiments with ±SEM. Trehalase activity was examined in the reaction mixture containing 66.7 m*M* citrate-NaOH buffer (pH 5.75) and 33.3 m*M* trehalose and was expressed as nmoles of glucose formed per min

the diet but no trehalase activity was detected in the diet itself (Table 1).

The kinetics of the trehalase enzymes in BAGs and spermatophores were compared. For trehalases from both sources, most of the enzyme activity was recovered in the supernatant fractions after centrifugation at 25000 *g* for 30 min (pellet washed twice and the three supernatants pooled). Optimum pH and K_m values for trehalose were very similar in the BAGs and the spermatophores (Fig. 2). Since the specific activities are high in the BAGs but not in other male organs, and since the kinetic parameters are so similar between the BAGs and the spermatophores, we conclude that the trehalase in the spermatophores originated from the BAGs.

Glucose and trehalose in spermatophores

To determine whether a spermatophore contained trehalose as a substrate for trehalase, the sugar components in an ethanolic extract of spermato-

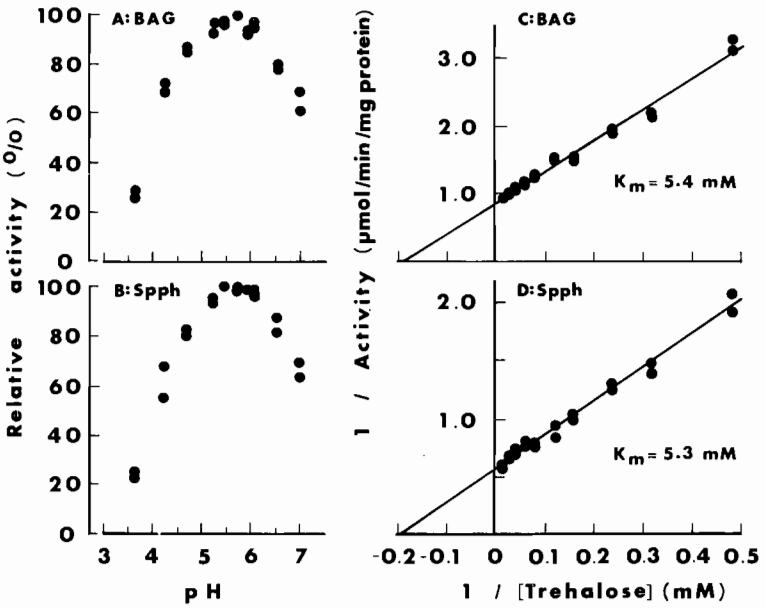

Fig. 2A–D. Kinetic properties of trehalase in BAGs and spermatophores. **A** and **B** Effects of pH on trehalase activities in BAGs and spermatophores, respectively. The pH was adjusted by 200 m*M* citrate-NaOH buffer. The concentration of trehalose was 33.3 m*M*. **C** and **D** Lineweaver-Burk plots on the effects of trehalose concentrations on trehalase activities in BAGs and spermatophores, respectively. pH was constant at 5.75. Trehalase activities in BAGs and spermatophores showed typical Michaelis-Menten saturation curves. *BAGs* bean-shaped accessory glands; *Spph* spermatophores. Enzyme sources were prepared from 15 BAG pairs (10-day adult males) or from 200 dried, deposited spermatophores. Each point represents the value from separate duplicate experiments

Table 2. Thin layer-chromatographic identification of sugars in spermatophores of *Tenebrio molitor*

	Rf values of chromatograms developed with			
	I		II	
Fructose	0.56		0.18	
Glucose	0.57		0.13	
Mannose	0.59		0.20	
Sucrose	0.46		0.04	
Trehalose	0.37		0.02	
Sorbitol	0.48		0.10	
Glycerol	0.69		0.45	
Spermatophores	0.36	0.10	0.02	0.05
	0.56	0.95	0.12	0.91

(I) n-Butanol:acetic acid:ethanol:water = 9:6:3:1; (II) Ethyl acetate:isopropanol:water = 65:17.5:12. Sugar fraction was extracted from 400 of deposited, dried spermatophores with 80% ethanol and spotted on Silica gel TLC plates. After development with solvents and drying, each compound was detected by spraying with 1% $KMnO_4$ in 1N NaOH

Table 3. Contents of reduced sugars and trehalose in spermatophores of *Tenebrio molitor*

	Reduced sugars[a] (nmol as glucose equivalents)	Glucose[b] (nmol)	Trehalose[a,b] (nmol)
Diet			
per weight of 13.8 mg	259.4 ± 30.3	89.9 ± 10.5	10.9 ± 6.2
Spermatophores			
per weight of 13.8 mg	2773.0 ± 424.2	2079.1 ± 350.3	3228.3 ± 329.9
per one spermatophore	6.9 ± 1.1	5.2 ± 0.9	8.1 ± 0.8
per mg protein	937.9 ± 143.5	703.8 ± 119.1	1092.2 ± 111.0

400 of deposited, dried spermatophores (13.8 ± 0.8 mg) were collected and the sugar fraction extracted from them. Sugar content was determined by

[a] the Somogyi-Nelson method and

[b] the glucose oxidase-peroxidase method. Each value represents a mean of six experiments with ± SEM

phores were determined. After applying the extracts to silica gel plates, developing with two solvent systems, and spraying with 1% $KMnO_4$, two major and two minor spots were observed on the chromatograms. The two major spots showed Rf values of 0.36 and 0.56 in solvent system I and 0.02 and 0.12 in solvent system II. By comparison with the migration of authentic sugars applied to parallel lanes on the same plates, the sugars from the ethanolic extract were identified as trehalose and glucose (Table 2). The minor spots were not identified.

The amounts of glucose and trehalose were determined in dried, deposited spermatophores. In four hundred such spermatophores, we found 2080 nmoles of glucose and 3230 nmoles of trehalose (Table 3). In the same amount of the diet, there were 90 nmoles of glucose and 11 nmoles of trehalose. Even if the surfaces of the spermatophores were contaminated with particles of the

diet, diet contribution to the sugar content of the spermatophores was not significant.

Discussion

In pupal *T. molitor*, BAGs grow by increasing the cell number about 3.5-fold (Happ et al. 1982, 1985) and in the adult stage, the glands grow by increases in cell volume, which result from the accumulation of proteins and other components (Fig. 1 of this paper; Happ et al. 1982). Trehalase activity also rises as the BAGs grow in the post-ecdysial adult (Fig. 1). The increase in trehalase activity may be related in part to glucose production from blood trehalose for energy production to support the active protein synthesis and in addition, the trehalase protein from the BAG may be exported to the spermatophore. This conclusion is supported by the fact that the spermatophore has a high amount of trehalase which has the same kinetic properties as the trehalase from the BAGs (Fig. 2).

As a general rule, there are two types of trehalases in insects, a soluble 'gut' type and a particle-bound 'muscle' type which differ in their kinetic properties (Wyatt 1967). With an optimum pH of 5.75 and a K_m of 5.4 m*M* for trehalose, the trehalases of the BAG and spermatophore have intermediate kinetic properties. The trehalase of the male reproductive system of *T. molitor* is clearly different from the soluble trehalase reported from the midgut of larvae which has an optimum pH of 5.0 and a K_m value of 0.40 m*M* for trehalose (Terra et al. 1985). The trehalases from the BAG and the spermatophore also appear somewhat different from the purified enzyme within the male accessory glands of the American cockroach – a trehalase of the soluble type with an optimum pH of 5.2 and a K_m value of 0.98 m*M* (Ogiso et al. 1985). Other secretory trehalases have been reported from the salivary glands of *Chironomus thummi* (Laufer et al. 1963) and in the cocoon floss of *Bombyx mori* (Shimada et al. 1980). In the latter case, the trehalase is soluble and shows an optimum pH of 5.5 and a K_m value of 1.41 m*M*; its function is unknown.

Our evidence is consistent with the transfer of the trehalase from the male to the female at the time of copulation. In several other instances, enzymes are transferred from male to female via a spermatophore or seminal fluids. An esterase is transferred to female *Drosophila melanogaster* in an ejaculate from the male (Sheehan et al. 1979). In two crickets, *Acheta domesticus* (Destephano and Brady 1977) and *Teleogryllus commodus* (Loher et al. 1981), prostaglandin synthetase is passed to the female. In *T. commodus*, the prostaglandin synthetase originates from the testes (Tobe and Loher 1983). Both the enzyme system and its substrate (the prostaglandin precursor, arachidonic acid esterified to phospholipids) are transferred to the female via spermatophores (Stanley-Samuelson and Loher 1983). In the spermatheca of the female, prostaglandins are formed and they elicit egg-laying behavior (Loher et al. 1981). In *Bombyx mori*, arginase is passed from the male to the female as the spermatophore is formed within her bursa copulatrix (Osanai et al. 1986). In this spermatophore, the arginase converts arginine into urea and ornithine. Ornithine then reacts with pyruvate, derived from glycolysis, to produce alanine and 2-oxoglutarate. The latter is the preferred substrate for ATP production in the sperm (Osanai et al. 1987). As already noted, the male accessory glands of the American cockroach contain a trehalase of unknown function (Ogiso and Takahashi 1984).

As shown in the present work, both trehalase and its substrate are found in the spermatophore of *T. molitor*. Trehalose has been reported in the seminal fluids of many species. Semen contains several small sugars, notably fructose in mammals (Rodger 1976). In insects, the seminal sugars are often mixtures. For example, the penis bulb of the honey bee contains fructose, glucose, and trehalose (3.21, 3.02, and 0.51 μg/mg penis bulb tissue, respectively (Tauber 1977)) and the spermatophore of *T. molitor* contains glucose and trehalose (27.2 and 80.0 μg/mg per dried deposited spermatophore) but no fructose (Table 3).

What is the physiological function of this enzyme and its substrate? Two possibilities suggest themselves: either the trehalase-trehalose system is important for spermatophore evacuation (as described by Gadzama and Happ 1974) or it serves some role in sperm nourishment and activation. Preliminary evidence from our laboratory suggests that the trehalase of the spermatophore is soluble and is released into the surrounding medium before the rupture of the sperm sac.

Acknowledgements. We would like to thank Connie S. Bricker, Karin A. Grimnes, Hiroshi Shinbo, and Christine Y. Happ for fruitful discussions during the course of this work and Kelly Baillargeon for mealworm care in our laboratory. This work was supported by grants from the National Institutes of Health (AI-15662 and GM-26140).

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