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RESEARCH COMMUNICATION

Trehalase from male accessory gland of an insect, *Tenebrio molitor*

cDNA sequencing and developmental profile of the gene expression

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A cDNA of αα-trehalase (EC 3.2.1.28) from a cDNA library of male bean-shaped accessory gland of the mealworm beetle, *Tenebrio molitor*, has been isolated by the homology screening approach. Sequence analysis of the cDNA (1830 bp) revealed that the cDNA encoded a protein of 555 amino acids with a calculated M_r of 64457. The deduced amino acid sequence had significant similarities to rabbit small intestine and *Escherichia coli* trehalases. Northern blotting and semi-quantitative PCR analyses revealed that a trehalase transcript with about 2.0 kb was abundant in bean-shaped accessory glands. In the glands, the amount of trehalase transcript increased from 1 to 2 days after adult ecdysis. These tissue- and stage-specific gene expressions of trehalase corresponded to the tissue- and stage-specificity of trehalase activity.

INTRODUCTION

In many insects, the male adults transfer the sperm to females via a sperm sac, the spermatophore [1]. In the mealworm beetle, *Tenebrio molitor*, the spermatophore is formed from the secretory proteins of two pairs of accessory glands, the bean-shaped accessory glands (BAGs) and the tubular accessory glands (TAGs) [2–4]. The eight secretory cell types of BAGs synthesize the corresponding secretory proteins and release them into the lumen of BAGs, in which a semi-solid plug, a precursor to the wall of the spermatophore, is formed [5–8]. In BAGs, these secretory proteins are actively synthesized a few days after adult ecdysis. An extremely high activity of trehalase (αα-trehalose glucohydrolase, EC 3.2.1.28) was found in BAGs, but not in other reproductive organs of the male. The trehalase is incorporated into the wall of the spermatophore [9].

In insects, trehalose is the main blood sugar, unlike in mammalian systems [10]. Trehalase is generally thought to be localized in the plasma membrane of the cell or within the cell to facilitate the uptake and utilization of blood trehalose. In the larval midgut or ovary of the silkworm, *Bombyx mori*, trehalase is bound to the plasma membrane at the basal surface of the midgut epithelial cell, instead of to the microvilli at the apical surface of the epithelial cell [11,12] or to the plasma membrane of the oocyte, close to the blood [13]. By contrast, trehalase is found in cocoon floss of *B. mori* and is thought to be secreted from the silk gland [14]. What then is the physiological function of trehalase secreted to the outside of the insect body? An actively secreted trehalase in *Tenebrio* BAGs is a good material for study of the secretory type of trehalase in insects. The cellular localization of trehalase is thought to be dependent on the protein structure. No primary sequence for any insect trehalase has yet been reported, although trehalase is an important key enzyme in carbohydrate metabolism of insects. In this study, the primary structure of *Tenebrio* BAG trehalase was deduced from the nucleotide sequence of the cDNA.

MATERIALS AND METHODS

Animals

T. molitor larvae were purchased and reared on wheat flour and fresh potatoes at 25 °C. The pupae were sexed, and the male adults were reared on the same diets at 25 °C [15]. On specified days, BAGs and other tissues were isolated in cold phosphate-buffered saline and kept at −70 °C until used.

Synthesis of oligonucleotide as a probe for screening

An oligonucleotide, 5′-AC(T/G)CC(G/A)TTGGTCCA-GCC(G/A)AA(G/C)CC-3′, complementary to the nucleotide sequence based on an amino acid sequence (GFGWTNGV in Fig. 2) conserved between trehalases of *Escherichia coli* and rabbit small intestine [16,17], was synthesized.

Isolation and sequencing of trehalase cDNA

Total RNAs were extracted from BAGs of 2–10-day-old male adults by the phenol method [18]. Poly(A)$^+$ RNA was isolated by using Oligotex-dT30 (Hoffmann–La Roche) and used for construction of a cDNA library by using a cDNA Synthesis Kit (Pharmacia LKB). Double-stranded cDNA was ligated to an *Eco*RI adapter and cloned into λgt11 (Stratagene). By using a synthetic oligonucleotide (23-mer) labelled with [γ-^{32}P]ATP, a cDNA library was screened, with final wash conditions 0.1 × SSPE/0.1 % SDS at 65 °C, and positive clones were obtained. The *Not*I insert was subcloned into the *Not*I site of pBluescript KS(+) (Stratagene), and serial deletions of the inserts were generated by exonuclease III [19]. Sequence analysis was carried out by the dideoxy chain-termination method [20].

RNA analysis

RNA was prepared as described above [18]. For Northern hybridization analysis, each RNA sample (25 μg) was electrophoresed in a 1.0 %-agarose gel containing formaldehyde [21] and blotted on to Hybond N$^+$ membrane (Amersham). Hybridiz-

Abbreviations used: BAGs, bean-shaped accessory glands; TAGs, tubular accessory glands; SSPE, saline/sodium phosphate/EDTA.

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The nucleotide sequence data reported will appear in the EMBL/GenBank and DDBJ sequence data banks under the accession number D11338.

ation was performed in the presence of 50% formamide at 42 °C, with final wash conditions 0.1 × SSPE/0.1% SDS at 65 °C. The pBluescript KS(+) containing trehalase cDNA (1830 bp) was made linear by cutting with *Xba*I restriction enzyme. The sense-stranded trehalase cDNA was asymmetrically amplified by the PCR method by using a T_7 primer and then purified by electrophoresis in low-gelling-temperature agarose. The antisense-stranded cDNA was radiolabelled with [α-^{32}P]dCTP [22] and used as a probe.

Semi-quantitative PCR for trehalase mRNA

Semi-quantitative PCR was performed by the method of Dallman & Porter [23]. From 1 μg of each RNA sample, first-stranded cDNA was synthesized by RNAase H$^-$ reverse transcriptase (Superscript; GIBCO BRL), and one-fourth of the cDNA was further used for PCR amplification. As primers, two oligonucleotides, 5′-GAAGAATGTTCTTCCCTTCG-3′ and 5′-TGGTTTATAAATTCTAAAAC-3′ (underlined with hyphens in Fig. 1), were synthesized. Amplified 426 bp fragment was analysed by agarose-gel electrophoresis followed by Southern blotting and then hybridized to the ^{32}P-labelled internal oligonucleotide 5′-AC(T/G)CC(G/A)TTGGTCCAGCC(G/A)AA-(G/C)CC-3′ (underlined doubly in Fig. 1). The radioactivity of hybridized PCR product was made visible and quantified with an imaging analyser (BAS 2000; Fujifilm, Tokyo, Japan). The amount of PCR product increased linearly until 27 cycles, and here 25 cycles was adopted for quantification.

Computer analysis

Sequence analysis was carried out with software from NBRF (National Biomedical Research Foundation) and from SWISS-PROT protein library by the FAST program [24] by using the GENETYX fast homology search (SDS Software Development Co., Tokyo, Japan).

RESULTS AND DISCUSSION

Cloning, sequencing and cDNA characterization

When amino acid sequences were compared between rabbit small intestine and *E. coli* trehalases which had been previously reported [16,17], several segments were found to match in amino acid sequence (Fig. 2). For this study, an oligonucleotide complimentary to the nucleotide sequence corresponding to a segment (GFGWTNGV; Fig. 2) near the *C*-terminal regions of both kinds of trehalases was synthesized to isolate trehalase cDNA from a *Tenebrio* BAG cDNA library in λgt11. A total of 6×10^5 plaques was screened, and four hybridized with the probe. A clone containing the largest insert cDNA was sequenced on both strands. It covered 1830 bp, encompassing a 49-bp 5′-untranslated region, the 1665-bp coding region and 116-bp 3′-untranslated region (Fig. 1); 20 nucleotides in 23-mer underlined double in Fig. 1 matched those of the hybridization probe (23-mer) used in this experiment. An in-frame TAA stop codon appeared at position 1715, and one hexamer corresponding to the polyadenylation signal, AATAAA, was found at position 1818 in the 3′-non-coding region (underlined in Fig. 1).

Characteristics of the protein

The cDNA sequence encoded a protein of 555 amino acids with a calculated M_r of 64457. An arrow in Fig. 1 indicates the predicted site of signal cleavage [25]. If this is the case, the M_r of mature protein is estimated to be 62669. This value almost agrees with the M_r (62000 ± 2000) estimated by SDS/PAGE for trehalase purified from BAGs of *T. molitor* (T. Yaginuma, T. Mizuno, C. Mizuno & G. M. Happ, unpublished work). Five potential *N*-glycosylation sites, Asn-Xaa-Ser/Thr, were found (boxed in Fig. 1). In insects as well as mammals, trehalase is generally known to be a glycoprotein [17,26,27].

The amino acid sequences of *Tenebrio* BAG, rabbit small intestine and *E. coli* trehalases are aligned in Fig. 2. There were 42.5 and 31.4% identical residues with rabbit small intestine and *E. coli* trehalases respectively, but no significant similarity to any

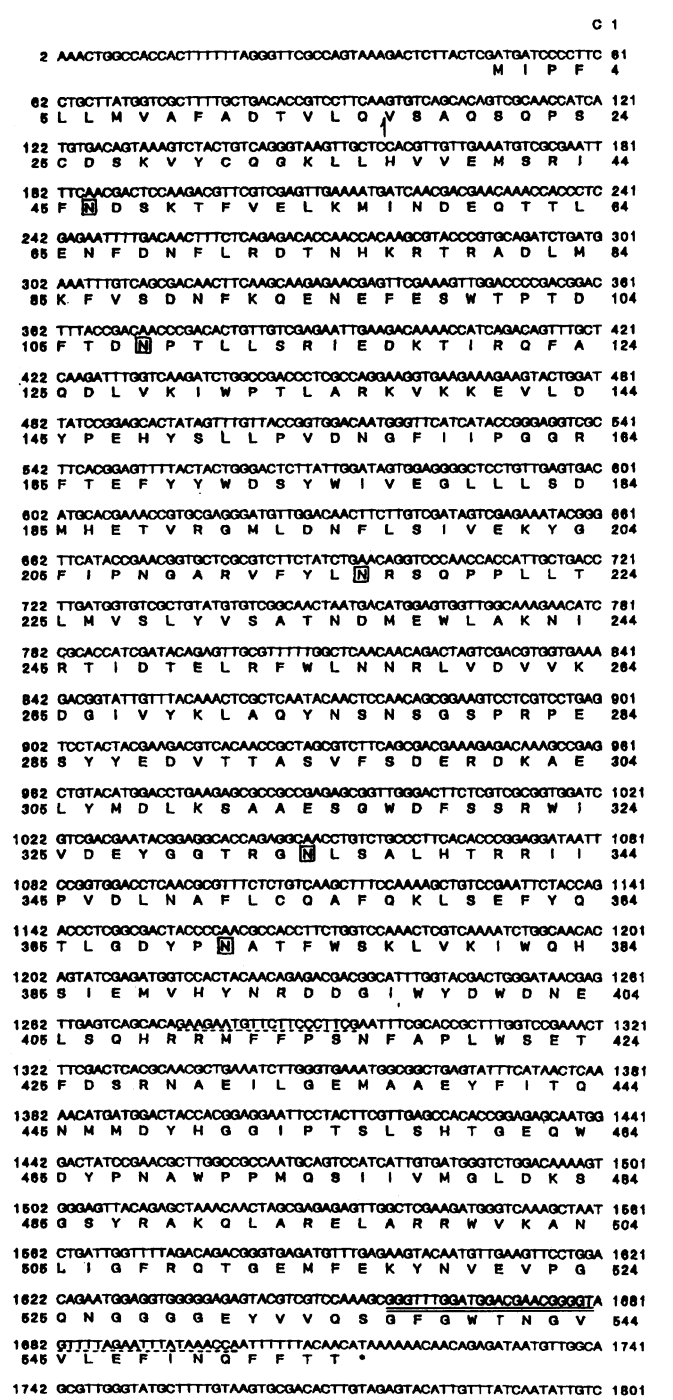

Fig. 1. Nucleotide sequence of *Tenebrio* BAG trehalase cDNA and the deduced amino acid sequence

Asterisk denotes the termination codon. The presumed polyadenylation signal is underlined singly. The region which seemed to hybridize with an oligonucleotide probe is underlined doubly. Arrow shows putative signal cleavage site [25]. Sequences underlined with hyphens were used as primers for the semi-quantitative PCR analysis. Potential *N*-glycosylation sites are boxed.

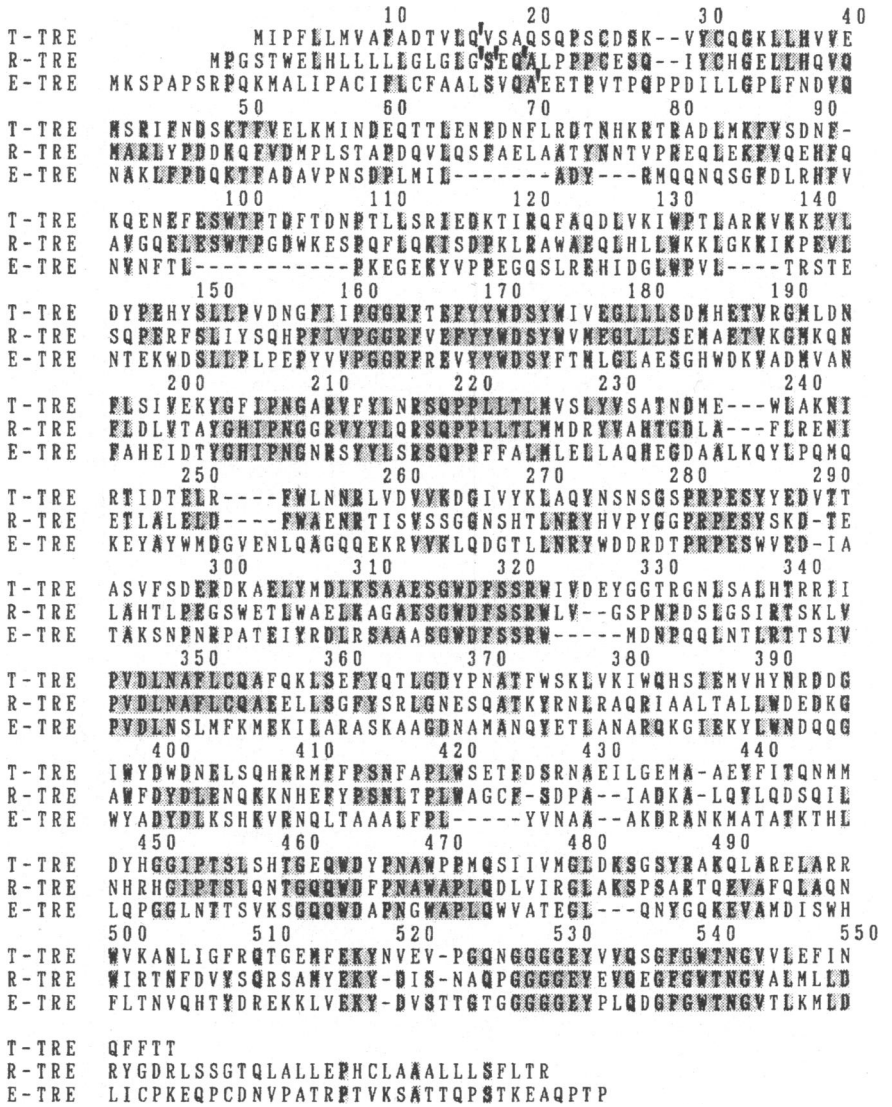

Fig. 2. Comparison of the primary structures of trehalases from *Tenebrio* BAG (T-TRE), rabbit small intestine (R-TRE [17]) and *E. coli* (E-TRE [16])

Numbers refer to the *Tenebrio* BAG trehalase. The identical amino acids are bold and shaded. Arrowheads indicate the predicted sites of signal cleavage for T-TRE and R-TRE [25] and the experimentally determined cleavage site for E-TRE.

other proteins. These results indicate that this cDNA encodes the trehalase of *Tenebrio* BAGs. It is especially interesting for us that *Tenebrio* BAG trehalase does not include the hydrophobic sequence in the *C*-terminal region, presumed to act in rabbit small-intestine trehalase as a temporary membrane anchor which then is replaced by a glycosylphosphatidylinositol anchor [17].

Analysis of trehalase gene expression

As shown by Northern-blot analysis of total RNAs (Fig. 3), the probe hybridized to an RNA of about 2.0 kb found in BAGs, but not to RNAs in testes and TAGs. Further, the result obtained by using a semi-quantitative PCR method showed that the trehalase transcript was abundant in BAGs, but not in TAGs and testes (Fig. 4). In BAGs, the amount of trehalase transcript increased from 1 to 2 days after adult ecdysis, but decreased after 4 days. The tissue-specific gene expression and the changing pattern in the amount of trehalase transcript in BAGs corresponded well to the tissue distribution of trehalase activity and the changing pattern in trehalase activity respectively [9]. These results are consistent with our belief that the cDNA sequenced in this study encodes the secreted trehalase purified from BAGs of *Tenebrio*.

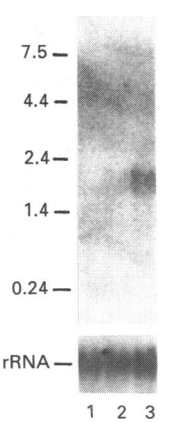

Fig. 3. Northern-blot analysis

Total RNAs (each 25 μg) extracted from TAGs (1), testes (2) and BAGs (3) of 2–10-day-old male adults of *T. molitor* were electrophoresed in agarose gel, blotted and then hybridized with radiolabelled anti-sense-stranded cDNA of BAG trehalase or rDNA. RNA size markers were co-migrated (kb).

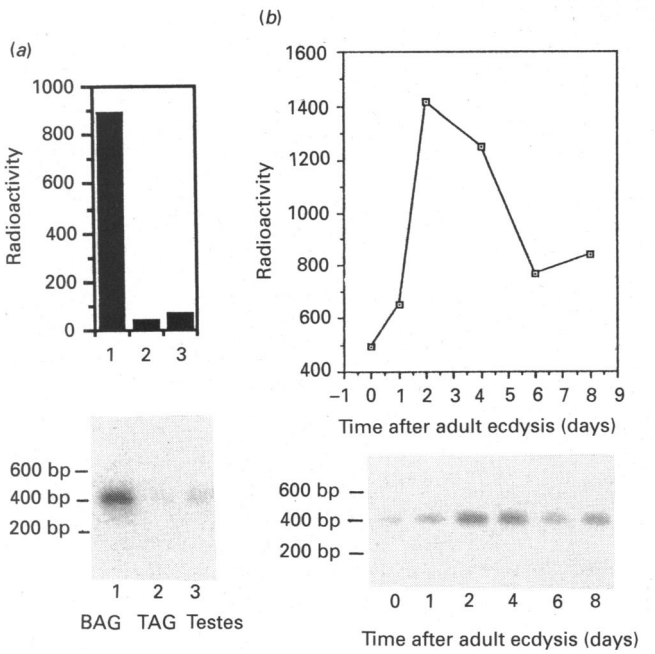

Fig. 4. Semi-quantitative PCR analysis

RNAs were extracted (*a*) from BAGs (1), TAGs (2) and testes (3) of 2–10-day-old male adults, and (*b*) from 0–8-day-old BAGs. By using each RNA sample (1 μg), the first-stranded cDNA was synthesized and used as a template for PCR. The PCR product was electrophoresed in agarose gel, blotted and hybridized with a radiolabelled internal oligonucleotide. The radioactivity was made visible and quantified by imaging analyser (BAS 2000; Fujifilm). The radioactivity (c.p.m.) on the ordinate is an arbitrary value.

Isolation of cDNAs corresponding to membrane-bound or soluble types of trehalase from other insect sources will provide further insight into the mechanisms for cellular localization of trehalase and the relationship between each type of trehalase and its tissue type.

In the BAGs of *T. molitor*, the active production of trehalase occurs a few days after adult ecdysis and was shown to be dependent on ecdysteroid hormone action during pupal stage [15]. 20-Hydroxyecdysone acts in the male pupa to commit BAGs toward trehalase production in the adult. Determination of the gene structure by using BAG trehalase cDNA will give us a tool to help to understand the relationship between pupal ecdysteroid hormone and the gene expression of trehalase in matured BAGs.

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