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## A trehalose 6-phosphate synthase gene of the hemocytes of the blue crab, *Callinectes sapidus*: cloning, the expression, its enzyme activity and relationship to hemolymph trehalose levels

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### Abstract

Trehalose in ectoderms functions in energy metabolism and protection in extreme environmental conditions. We structurally characterized trehalose 6-phosphate synthase (TPS) from hemocytes of the blue crab, *Callinectes sapidus*. *C. sapidus* Hemo TPS (CasHemoTPS), like insect TPS, encodes both TPS and trehalose phosphate phosphatase domains. Trehalose seems to be a major sugar, as it shows higher levels than does glucose in hemocytes and hemolymph. Increases in HemoTPS expression, TPS enzyme activity in hemocytes, and hemolymph trehalose levels were determined 24 h after lipopolysaccharide challenge, suggesting that both TPS and TPP domains of CasHemoTPS are active and functional. The TPS gene has a wide tissue distribution in *C. sapidus*, suggesting multiple biosynthetic sites. A correlation between TPS activity in hemocytes and hemolymph trehalose levels was found during the molt cycle. The current study provides the first evidence of presence of trehalose in hemocytes and TPS in tissues of *C. sapidus* and implicates its functional role in energy metabolism and physiological adaptation.

### Background

Trehalose, a non-reducing disaccharide is a primary energy source in prokaryotes, yeasts, plants, and invertebrates. The accumulation of trehalose in anhydrobioses of artemia, nematodes, and chironomids [1-3] implies a role in physiological and biochemical adaptations in extreme environmental conditions.

In insects, trehalose is the major hemolymph sugar that is exclusively synthesized in the fat body in which hypertrehalosemic hormone (HTH) positively regulates its production. In addition, flight, feeding, and parasitic infections in insects have been shown to produce hypertrehalosemia, i.e. an increase in trehalose in hemolymph [4-6]. These findings further support trehalose as an

energy source and its involvement in physiological adaptation in insects.

Trehalose 6-phosphate synthase (TPS) is noted in insects as a fused gene that codes two functional domains in tandem: TPS, a homolog of Ost A of *Escherichia coli*, and trehalose 6-phosphate phosphatase (TPP), a homolog of Ost B of *E. coli*. *Drosophila* TPS introduced into human HEK-293 cells increased hypoxia tolerance by which elevated trehalose reduced protein aggregation under hypoxia [2,7]. This result indicates two domains of TPS and TPP are active. However, a relationship between the level of TPS expression and TPS enzyme activity resulting in the increase in trehalose production has not been described in insects.

In contrast to hypertrehalosemic response under stress and during flight activity in insects, the increase in glucose level in hemolymph (i.e. hyperglycemia) of crustaceans has been described during their initial physiological adaptation to stressful environments [8-15]. Lipopolysaccharide (LPS) injection, an accepted method for mimicking a pathogen infection, also induced hyperglycemia through modulating the level of crustacean hyperglycemic hormone [13]. The glycogen present in many crustacean tissues, including hemocytes, is tacitly accepted as the source of this hyperglycemia. Previous reports of the involvement of trehalose in osmoregulation and cold adaptation in crustaceans [16,17] and the ubiquitous abundance of trehalose in insect hemolymph as an energy source and its protective roles under stress emphasize the importance of this molecule in invertebrates. Therefore, we investigated the presence of *TPS* gene and trehalose in the blue crab, *Callinectes sapidus*, the population of which has been drastically declining in the Chesapeake Bay [18], in order to better understand the role of this sugar in energy metabolism during molt cycles and physiological adaptation under stressful conditions. Particularly, in an attempt to define an adaptive role of trehalose in a different physiological status of *C. sapidus*, we challenged animals with LPS that generally induced the response of a pathogen infection as well as the stress response of hyperglycemia in crustaceans [13,15]. We demonstrated hypertrehalosemic and hyperglycemic responses by LPS injection into the animal that was accompanied by increases in *TPS* expression and *TPS* enzyme activity in hemocytes. A relationship between *TPS* activity in hemocytes and the level of hemolymph trehalose during a molt cycle was established.

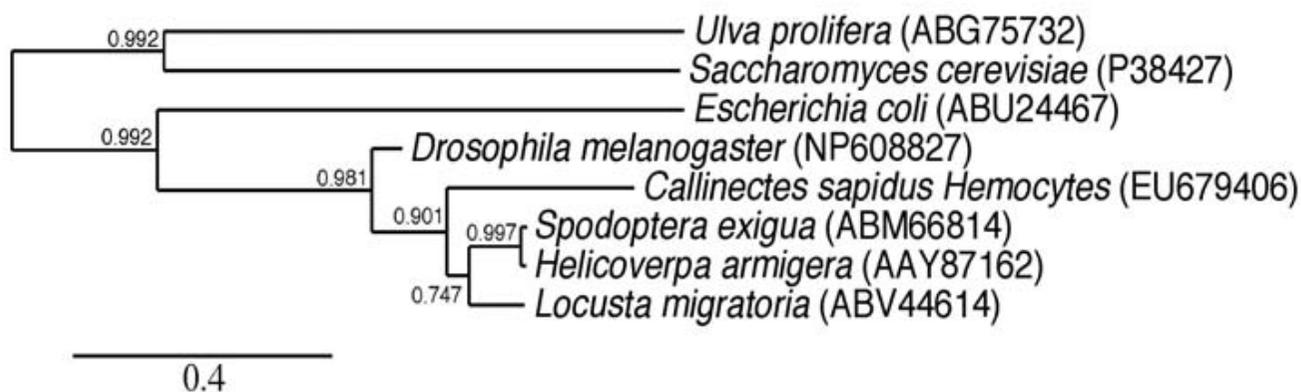
## Results and discussion

### Phylogenetic tree analysis of multiple sequence alignments of *TPS* gene

*CasHemoTPS* (GenBank accession no. [EU679406](#)) consisting of 755 amino acid encodes a putative *TPS* and a *TPP* domain in tandem. Phylogenetic tree analysis of multiple sequence alignments of *TPS* gene revealed that *C. sapidus* Hemocytes *TPS* (*CasHemoTPS*) is closely related to those of insects, forming a separate group from *E. coli*, *Saccharomyces cerevisiae*, and *Ulva prolifera* (Fig. 1) [19]. The *TPS* gene in arthropods appears to be a fused gene of a homolog of *Ost A* and *Ost B* in *E. coli*.

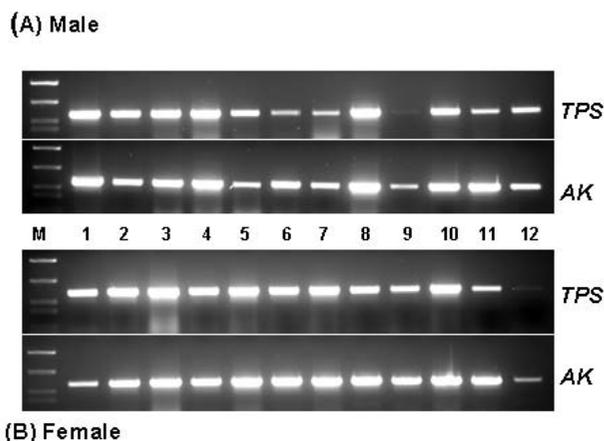
### Spatial distribution of *TPS* gene expression in various tissues of *C. sapidus*

cDNAs of various tissues prepared from an adult male and female *C. sapidus* were tested for the *TPS* expression. As shown in Fig. 2, *TPS* expression was ubiquitous in all the tissues of both sexes of adult crabs, indicating that all these tissues could produce trehalose. It appears that multiple isoforms of *TPS* genes are present in tissues of the blue crab, as three of these, coding both *TPS* and *TPP*, have already been identified (unpublished observation). This wide distribution of *TPS* gene in crab tissues is surprising in contrast to what has been described in insects. In insects, the fat body is known as the exclusive biosynthetic site of trehalose [4,20,21]. After synthesis in the fat body, trehalose is released into hemolymph and serves as a major hemolymph sugar for energy required during flight.



**Figure 1**

Phylogenetic tree analysis of deduced amino acids of *TPS* including *Callinectes sapidus* hemocytes ([EU679406](#)), *Spodoptera exigua* ([ABM66814](#)), *Helicoverpa armigera* ([AAY87162](#)), *Locusta migratoria* ([ABV44614](#)), *Drosophila melanogaster* ([NP608827](#)), *Escherichia coli* ([ABU24467](#)), *Saccharomyces cerevisiae* ([P38427](#)), and *Ulva prolifera* ([ABG75732](#)). The neighbor-joining tree was constructed and bootstrapped (1000 iterations) using Robust Phylogenetic Analysis for the Non-Specialist [19]. Bootstrap values are noted on the branch and the scale bar (= 0.4) represents fixed mutations per amino acid position.



**Figure 2**

Spatial distribution of *TPS* gene in cDNAs of various tissues of adult male and female of *C. sapidus*. *TPSF2* and *TPSR1* primers as listed in Table 1 was used for PCR amplification with 12.5 ng of total RNA equivalent of each tissue. Arginine kinase served for a reference gene. Lane 1: eyestalk, 2: brain, 3: thoracic ganglion, 4: antennal gland, 5: gill, 6: hindgut, 7: heart, 8: chelae muscle, 9: hypodermis, 10: testis (A) and ovary (B), 11: hepatopancreas, and 12: Y-organ.

#### The effect of LPS on the expression of *TPS*, *TPS* activity and trehalose levels

Animals were challenged by the injection of 1  $\mu$ g LPS to test the response of trehalose. The resting level of trehalose in hemocytes was higher than in hemolymph:  $3.5 \pm 0.3$  mg (n = 6) (Fig. 3A) and  $1.1 \pm 0.1$  mg/ml (n = 6), respectively. In contrast, the level of glucose was higher in hemolymph than in hemocytes:  $180 \pm 14.6$   $\mu$ g/ml (n = 6) and  $70 \pm 10$   $\mu$ g/mg protein in hemocyte extracts (n = 6), respectively (Fig. 3B). Overall, the concentration of trehalose was higher than glucose in both hemolymph and hemocytes: 6 and 50 fold, respectively, suggesting that trehalose is a major sugar in crab hemolymph as in insects [4,21]. The intracellular level of trehalose was increased  $\sim 2.5$  fold in response to the LPS challenge, while a modest 1.5 fold elevation of glucose was found. LPS injection after 24 h did not cause general hypertrehalosemia or hyperglycemia in hemolymph in *C. sapidus*, although it was reported that a much higher dose of LPS induced hyperglycemia after 2 h in other crustacean species [13,15]. LPS induced a significant 2.5 fold increase in HemoTPS mRNA, a three fold increment of TPS activity, compared to those of the controls (Figs. 3C and 3D). This could be responsible for the increase in trehalose levels in Fig. 3A. A slight change (130%) in the level of *trehalase* (*Treh*) mRNA that breaks down trehalose into two glucose molecules is responsible for the modest rise (1.5 fold) in intracellular glucose. The basal level of *TPS* mRNA in hemocytes was  $\sim 100$  fold less than that of *Treh*.

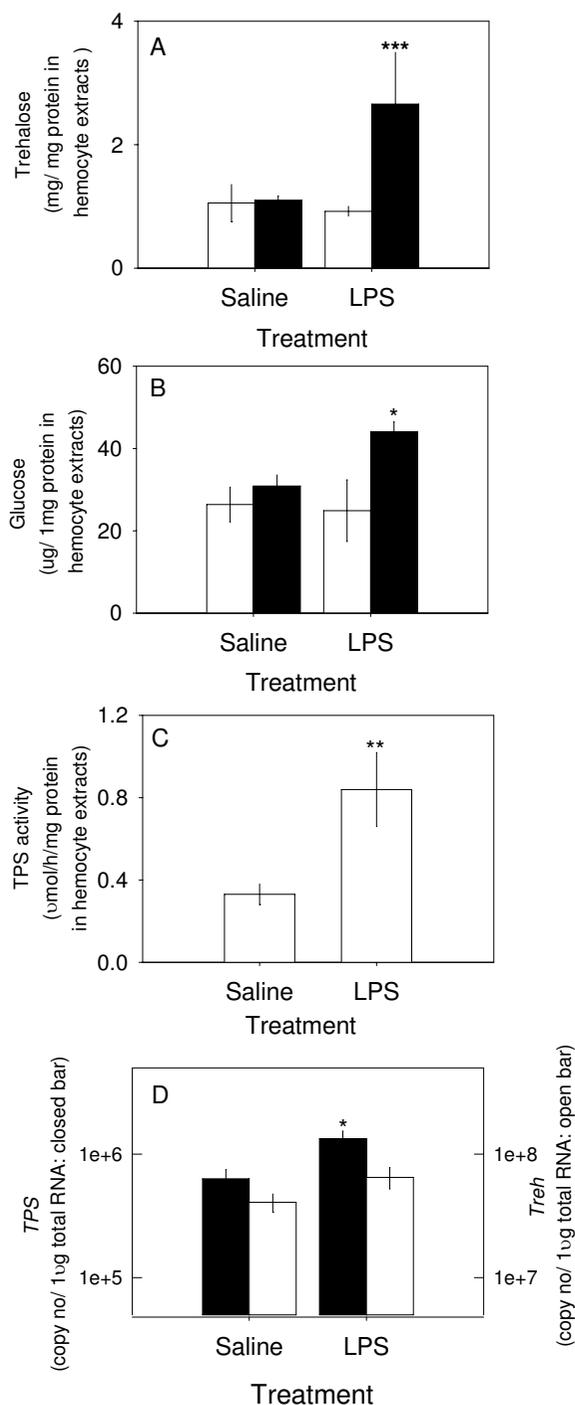
Our result demonstrates that hemocytes possess *TPS* and *Treh* for the synthesis and metabolism of trehalose. More importantly, they modulate cellular trehalose levels for physiological and biochemical adaptation under LPS challenge, through the dynamic regulation of the expression of *TPS* and *TPS* enzyme activity. Furthermore, our data indicate that *C. sapidus* expresses *TPS* in multiple tissues, in contrast to insects where the fat body is considered the exclusive biosynthesis site of this sugar. Considering trehalose is the major blood sugar, it is also likely to be involved in crustacean hyperglycemia. We anticipate its ubiquitous presence in most if not all crustacean hemolymph with similar functions as those found in insects.

#### Levels of *TPS* activity in hemocytes and trehalose in hemolymph during a molt cycle

Concentrations of trehalose in hemolymph of *C. sapidus* showed a bimodal pattern that exhibited two peaks during molt cycle, at early ecdysis and post ecdysis  $C_{1-3}$  (Fig. 4). The lowest level of trehalose ( $0.65 \pm 0.05$  mg/ml hemolymph, n = 8) was measured at stage A during and after the occurrence of the largest water intake occurred [22,23]. The fluctuation of *TPS* activity in hemocytes was also noted during the molt cycle from the lowest at intermolt to the highest at postmolt stage B:  $0.3 \pm 0.08$   $\mu$ mol/h/mg protein in hemocyte extracts (n = 12) and  $1.98 \pm 0.74$   $\mu$ mol/h/mg protein in hemocyte extracts (n = 7), respectively. *TPS* enzyme activity of HemoTPS was determined only at intermolt by measuring [Pi] in the same samples that were prepared for *TPS* activity. The activity of *TPS* was slightly high:  $0.78 \pm 0.41$   $\mu$ mol [Pi]/h/mg protein in hemocyte extracts (n = 5), however, this value was not significantly different from that of *TPS* activity. In general, *TPS* activity was elevated at premolt and peaked at stage B, which correlates with the highest concentration of trehalose noted at stage  $C_{1-3}$ . The level of trehalose and *TPS* activity at the postmolt stage imply a possible involvement of this sugar in chitin synthesis, as found in insects [24]. Chitin synthesis is required for cuticle hardening and the calcification process in the exoskeleton of animals after ecdysis.

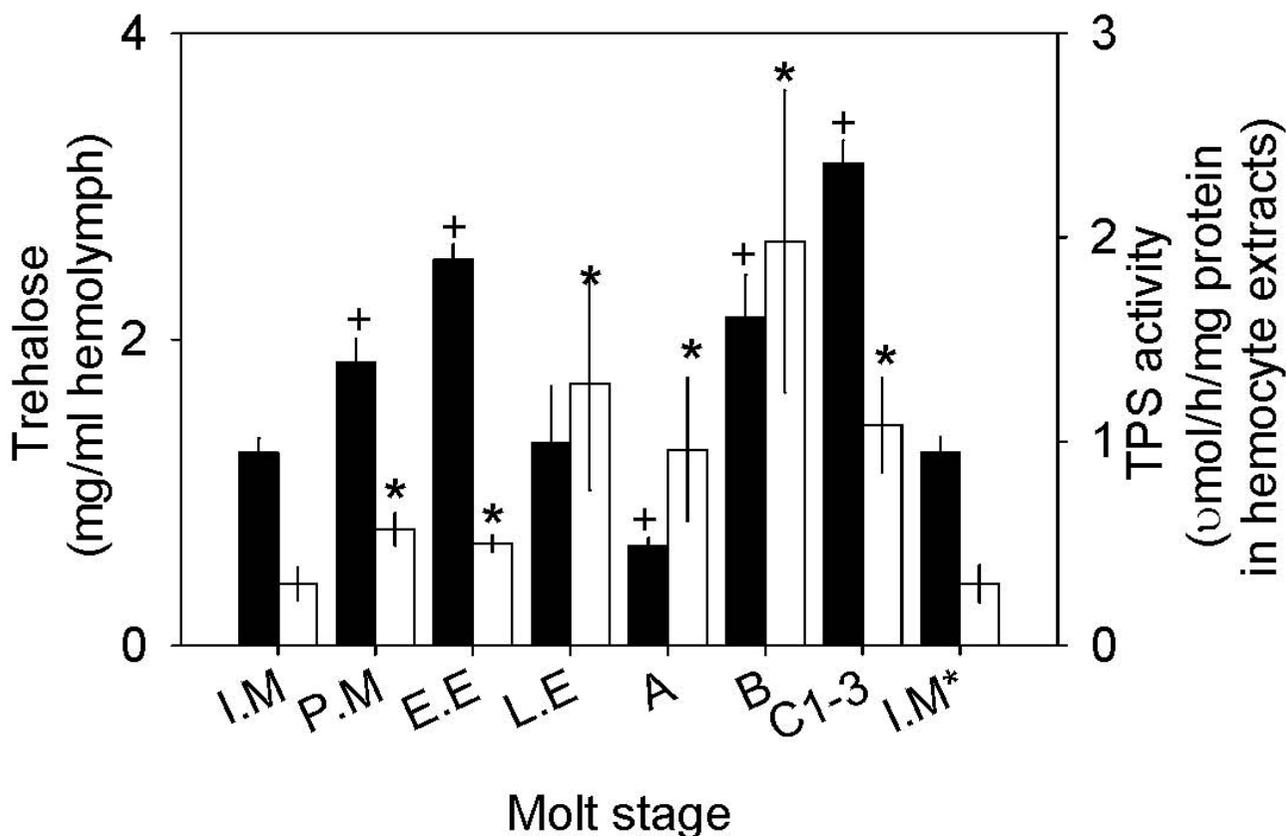
#### Conclusion

We isolated, for the first time in crustaceans, the cDNA sequence of the *TPS* gene coding functional and active domains of *TPS* and *TPS* in hemocytes of *C. sapidus* where its expression was widespread in most tissues. LPS injection into animals, mimicking the induction of internal stress, stimulated the expression and enzyme activity of *TPS* in hemocytes, resulting in the increase in intracellular trehalose in hemocytes. Our results provide evidence of the presence and a possible adaptive function of trehalose in energy metabolism and stress response of decapod crustaceans.



### Figure 3

The effects of LPS injection after 24 h on the levels of intracellular glucose, trehalose, TPS enzyme activity, and the expressions of *TPS* and *Treh* in hemocytes. A) Trehalose: open bar at t = 0 h, solid bar at 24 h; B) Glucose: open bar at t = 0 h, solid bar at 24 h; C) TPS enzyme activity at 24 h; and D) expressions of *TPS* and *Treh* at 24 h: solid bar = *TPS*; open bar = *Treh*. Data is presented as mean  $\pm$  1 SE (n = 5–8) of trehalose in mg/mg protein in hemocyte extracts, of glucose in  $\mu$ g/mg protein in hemocyte extracts, of TPS enzyme activity in  $\mu$ mol/h/mg protein in hemocyte extracts and *TPS* and *Treh* expressions in copy number/ $\mu$ g total RNA. Statistical significance at P < 0.05 = \*, at P < 0.05 = \*\*, at P < 0.001 = \*\*\*.



**Figure 4**

Changes in the levels of trehalose in hemolymph and TPS activity in hemocytes during molt cycle. Data is presented as mean  $\pm$  1 SE ( $n = 8-15$ ) of trehalose in mg/ml hemolymph (solid bar) and of TPS enzyme in  $\mu\text{mol/h/mg}$  protein in hemocyte extracts (open bar). Statistical significances at  $P < 0.05$  marked as + (trehalose) or \* (TPS activity) were determined using Student's  $t$  test by comparing the values at intermolt stage with each different molt stage. I.M = intermolt, P.M = premolt, E.E = early ecdysis, L.E = late ecdysis, A = within 3 h after ecdysis, B = 1-2 days after ecdysis, C<sub>1-3</sub> = 3-7 days after ecdysis, and I.M\* = same as I.M.

## Materials and methods

### Animals

Juvenile blue crabs, *C. sapidus* (20-30 mm carapace width), were received from the blue crab hatchery in the Aquaculture Research Center, Center of Marine Biotechnology (University of Maryland Biotechnology Institute, Baltimore, MD) and reared as described [25].

### 5', 3' RACEs of *C. sapidus* TPS gene

Hemocytes were harvested from 1 ml hemolymph withdrawn in a sterilized marine anticoagulant (filtered through 0.22  $\mu\text{m}$  membrane) at 1:1 ratio and immediately spun at 800 g for 10 min 4°C. After discarding the plasma, the pelleted cells were washed once in 100  $\mu\text{l}$  of anticoagulant and re-centrifuged as above. The washed hemocytes were homogenized and total RNA extraction and quantification were carried out by following the pro-

cedures as described [26]. Degenerate primers of *TPS* were generated based on the conserved region of insect genes listed in GenBank using a multiple alignment program, CLUSTALW <http://www.genome.jp>.

The synthesis of 3' RACE cDNA of total RNA of hemocytes was carried out using GeneRacer™ (Invitrogen), while 5' RACE cDNAs was produced using SMART cDNA synthesis kit (BD Biosciences). Touchdown PCR was employed for initial amplification of *TPS*: dF1 (5'TTYGAYTCYTAYTA YAA YGG3') and dR1 (5'TCDCCRGCDCCRG CRAAD GG3'). The cDNA was amplified with Advantage Taq polymerase (BD Biosciences) at the following PCR conditions: after initial denaturation for 2.5 min at 94°C, 3 cycles each step at annealing temperatures: 47°C, 45°C, and 43°C and the final step at 48°C for 25 cycles. The final amplification was achieved at annealing temperature

48°C. The touchdown PCR products served as templates for the nested PCR of TPS with a primer combination of dF2 (5' TTYTGCCNYNTTYCAYTCYATGCC 3') and dR2 (5' ATYT GRCARGCSACRAAYTC3') at 55°C annealing temperature. For the TPS gene, the cDNA from hemocytes produced a band with an expected size of 900 bp. The cloning and sequencing procedures were as stated [27]. Based on the obtained *C. sapidus* sequences of TPS, the following gene specific primers (listed in table 1) were made for the completion of 5', 3' RACE.

#### Spatial distribution of TPS in various tissues of *C. sapidus*

Tissues were collected from male and female crabs at intermolt stage after they were anesthetized on ice as follows: eyestalk, brain, thoracic ganglion, antennal gland, gill, hindgut, heart, chelae muscle, hypodermis, testis or ovary, hepatopancreas, and Y-organ. Total RNAs were extracted using TRIzol® (Invitrogen) and quantified with a NanoDrop 1000 (Thermo Scientific). After treatment with DNase I to eliminate genomic DNA contamination, one µg of total RNAs were used for the first cDNA synthesis with MMLV and random hexamers (Promega). Samples of cDNAs (each 12.5 ng) were amplified with a combination of primers: forward, 5' ATGTTGGTGGAAACACAATTC AAGGAC3' and reverse, 5' TACAGAAGAGTCTCGGTA-GAATGCA for TPS. Arginine kinase, a reference gene, was amplified using the same primers as described [27]. The PCR conditions were as follows: initial denaturation at 94°C for 2.5 min, 35 cycles at 94°C for 20 sec, 60°C for 20 sec, 70°C for 30 sec, and final step at 70°C for 5 min. PCR products were visualized by staining with ethidium bromide after electrophoresis on a 1.5% agarose gel.

#### Lipopolysaccharide challenge

Prior to the injection of LPS or saline, 100 µl of hemolymph was withdrawn from juvenile animals (70–90 mm, carapace width) as described [27] to establish the resting levels of glucose and trehalose in hemocytes. Animals in

the test group received 1 µg LPS (*E. coli* 0111:B4, Sigma) in 100 µl crustacean saline, while control animals received 100 µl saline alone. 24 h after injection, 500 µl of hemolymph were withdrawn in an anticoagulant at a ratio of 1:1 and immediately centrifuged as described above. The hemocytes were re-suspended in ice cold DEPC treated PBS or Tris buffered saline and homogenized. Half of the samples were dedicated for estimating glucose, trehalose, and TPS activity, while the rest were used for RNA extraction as described above. Hemocyte protein was determined using BioRad DC protein assay (BioRad).

#### Quantitative RT-PCR analysis (QRT-PCR)

The extraction and quantification procedures of total RNA of hemocytes and cDNA synthesis were stated in Chung and Zmora [27]. Standards for QRT-PCR were produced as described [25]. Sample cDNAs (12.5 – 25 ng) were analyzed for the estimation of the expressions of TPS using primers of QF: 5' ATGTTGGTGGAAACACAATTC AAGGAC3' and QR: 5' CTTTGTATAATCTAACCGATCCACTC3' and the data were calculated as copy number/µg of total RNA of hemocytes. The level of hemocyte trehalase (*Treh*, GenBank accession no. EU679407) was quantified using the following primers, QF: 5' GCAGAGAGTGGATGGG A3' and QR: 5' CCCTGACAGCAGCAAGCCCTCA3'. The expression levels of TPS and *Treh* were represented as copy number/µg total RNA as described [26].

#### Estimation of glucose and trehalose in hemocytes

Glucose levels in hemocytes were determined using glucose oxidase/peroxidase assay (Sigma) as described [28]. Trehalose concentration in hemocytes was estimated by subtracting the amount of glucose from the values determined by anthrone assay, as this assay measures both sugars [29]. Trehalose (Sigma) was used for the standard of anthrone assay. The results were presented as µg glucose or mg trehalose/mg protein in hemocyte extracts.

#### Two-step TPS activity assay

TPS activity in hemocytes was estimated using a modified procedure that was previously described [30,31]. For the first step of the synthesis of trehalose 6-phosphate, 100 µg of extracts from hemocytes was incubated in 200 µl final volume of the first reaction mixture containing 50 mM HEPES buffer (pH 7.1), 5 mM UDP-glucose (UDPG), 10 mM glucose-6-phosphate, and 12.5 mM MgCl<sub>2</sub> at 35°C for 30 min. In controls, glucose-6-phosphate was omitted. The reactions were terminated with heat treatment at 100°C for 5 min and were centrifuged at 13,000 rpm for 5 min at room temperature. For the second step, the supernatants (150 µl) were further incubated at 35°C for 10 min in the following reaction mixture (150 mM HEPES buffer, pH 7.6, 2 mM phosphoenolpyruvate, 0.5 mM NADH, 5 U lactic dehydrogenase and 5 U pyruvate kinase). Samples were cooled on ice for 5 min and briefly

**Table 1: The list of primer sequences that was used for cloning of TPS gene and QRT-PCR**

	Primer sequences (5'-3')
TPS dR1	TCDCRCGDCRCRGCRAADGG
TPS dR2	ATYTGRCARGCSACRAAYTC
TPS 3dF1	TTYGAYTCYTAYTAYAAYGG
TPS 3dF2	TTYTGCCNYNTTYCAYTCYATGCC
TPSF1	ATGCCTGACAGAGCAACATTTTCAG
TPSF2(=QF1)	ATGTTGGTGGAAACACAATTC AAGGAC
TPSR1	TACAGAAGAGTCTCGGTAGAATGCA
TPSR2(=QR)	CTTTGTATAATCTAACCGATCCACTC
TPSR3	GCACGGAGTCTGGGTGGCTCTCA

'd' represents degenerate primers. Two forward primers of TPS F1 and F2 were used for 3' RACE and three of TPSR1, R2, and R3 were for 5'RACE. Primers of QF1 and QR1 were used for QRT-PCR analysis.

centrifuged for 13,000 rpm for 1 min. 100  $\mu$ l of the supernatant was placed into a 96 well plate, and the absorbance was measured at 340 nm (Spectra M5, Molecular Device). Known concentrations of UDP at 1000, 500, 250, 125, and 62.5 nmol were treated as above and served for a standard curve. TPS activity was calculated per  $\mu$ mol UDP/h/mg hemocyte protein.

#### Trehalose 6-phosphate phosphatase (TPP) assay

In order to test the functionality of TPP domain of CasHemoTPS, the hemocytes were extracted in Tris-buffered saline and TPP enzyme activity was measured by following the procedure described in Klutts *et al.* [32]. The activity was calculated as  $\mu$ mol [Pi]/h/mg hemocyte protein.

#### Estimation of TPS activity in hemocytes during molt cycle

Hemolymph samples were collected from animals at molt stages as described [33] and assayed as described above. Hemocytes homogenized in 200  $\mu$ l of ice cold PBS by sonication (Branson); the extracts were centrifuged at 14,000 rpm for 10 min at 4°C; and, the supernatants were collected for the estimation of protein concentration as described above.

#### Statistical analysis

Statistical significance was determined at  $P < 0.05$  using GraphPad InStat 3 program (GraphPad Software, Inc).

#### Abbreviations

TPS: trehalose 6-phosphate synthase gene; Treh: trehalase

#### Competing interests

The author declares that they have no competing interests.

#### Authors' contributions

JSC carried out the molecular cloning of TPS gene, TPS and TPP bioassays, and the bioassays.

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