Gene Cloning, Prokaryotic Expression, and Biochemical Characterization of a Soluble Trehalase in *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae)

Dong Ai,1 Shenhang Cheng,1 Hetan Chang,2 Ting Yang,2 Guirong Wang,2 and Caihong Yu1,3

1School of Chemical and Environmental Engineering, China University of Mining and Technology (Beijing), Beijing 100083, China, 2State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China, and 3Corresponding author, e-mail: caihongyu2013@126.com

Received 8 January 2018; Editorial decision 17 May 2018

Abstract

Trehalase is an indispensable component of insect hemolymph that plays important role in energy metabolism and stress resistance. In this study, we cloned and expressed the gene encoding soluble trehalase (HaTreh-1) of *Helicoverpa armigera* (cotton bollworm) and characterized the enzyme. HaTreh-1 had a full-length open reading frame encoding a protein of 571 amino acids. Sequence comparison indicated that HaTreh-1 was similar to some known insect trehalases. Two essential active sites (D321 and E519) and three essential residues (R168, R221, and R286) were conserved in HaTreh-1. The recombinant trehalase was expressed in *Escherichia coli* and purified by nickel exchange chromatography. Molecular weight of the recombinant protein was about 71 kDa, and the optimum HaTreh-1 enzyme activity is at 55°C with pH 6.0. Enzymatic assays showed a $K_m$ value of 72.8 mmol/liter and a $V_{max}$ value of 0.608 mmol/liter-min. Inhibition assays in vitro indicated that castanospermine, a polyhydroxylated alkaloid, was an effective competitive inhibitor of trehalase with a $K_i$ value of 6.7 μmol/liter. The inhibitor action of castanospermine was linked to its modification effect on trehalase structure. The circular dichroism spectrum showed that the percentage of $α$-helix increased under the presence of castanospermine. Results of our study will aid in developing effective trehalase inhibitors for controlling *H. armigera* in the future.

Key words: castanospermine, *Helicoverpa armigera*, inhibitor, prokaryotic expression, soluble trehalase

Trehalase is a nonreducing dimer of glucose widespread in almost all tissues and organs of insects. It is synthesized mainly in the fat body, secreted into the hemolymph, and used as an energy source in insects (Asano 2003, Tang et al. 2008). In addition, the functions of trehalase are to protect insect protein and cell plasma membrane integrity when insects are exposed to adverse environments such as high temperature, freezing, high osmosis, dehydration, drought stresses, and pesticides (Elbein et al. 2003, Thompson 2003, Mitsumasu et al. 2010).

Trehalase (EC.3.2.1.28) is an anomer-inverting $α$-glucoside hydrolase (Lee et al. 2007) that converts trehalose ($α$-$d$-glucopyranosyl-$α$-$d$-glucopyranoside) to glucose in fungi, insects, and mammals. Insects have two kinds of trehalases, i.e., soluble trehalase (Treh1) and membrane-bound trehalase (Treh2; Tan et al. 2014). Treh1 is derived from Treh2 through loss of the C-terminal transmembrane loop (Gomez et al. 2013). The soluble trehalase gene in insects is mainly expressed in the midgut, Malpighian tubules, and ovary, while the membrane-bound trehalase gene is primarily expressed in the fat body, midgut, and Malpighian tubules (Zhang 2011). The expression level and activities of trehalase are higher in organs related to energy metabolism like the midgut and Malpighian tubules (Tang et al. 2008, Xie et al. 2013, Tan et al. 2014).

The similarity between Treh1 and Treh2 is very low, but most include a signal peptide, and both types have unique properties. For example, *Spodoptera exigua* Treh2 has a transmembrane domain of 20 amino acids, but *S. exigua* Treh1 does not have this domain (Tang et al. 2008). *Laodelphax striatellus* Treh2 has two different transmembrane domains (Zhang et al. 2012).

In insects, trehalases play very important roles in chitin synthesis, development, energy metabolism, and stress tolerance (Tatun et al. 2008, Chen et al. 2014). Recent studies showed that trehalases could control insect molting by regulating chitin synthesis (Chen et al. 2010, Zhang et al. 2012). After silencing trehalase genes in *Spodoptera litura*, chitin could not be synthesized, and as a consequence, the insect could not survive (Chen et al. 2010). Similarly, the development of *S. exigua* was delayed and mortality was increased when its trehalase gene expression was inhibited by RNAi technology (Chen et al. 2010). Treh2 induced *Bombyx mori* to produce heterogenic eggs, helping eggs survive cold temperatures...
HarmTreh1-R x CCG
HarmTreh1-F CGC
Primer name Sequence (5′ → 3′)
Table 1. Primers for experiments

| Primer name      | Sequence (5′–3′)                  |
|------------------|-----------------------------------|
| HarmTreh1-F      | CCGGGATCCATGGGTGTCTTTTTGTAAGATGCGA(BamHI) |
| HarmTreh1-R x    | CCGCTCGAGTCATGCACATTATCGTAGTTAGAGTC(Xhol) |
added to the culture at a final concentration of 0.25 mmol/liter, and the culture was incubated at 25°C for 16 h. The resulting cells were harvested by centrifugation (8,000 rpm, 15 min) and dissolved with 1x phosphate-buffered saline (PBS). The suspension was sonicated on ice, and the resulting mixture was centrifuged at 12,000 rpm at 4°C for 15 min. The supernatant was purified using HisTrap affinity columns (GE Healthcare Biosciences, Uppsala, Sweden) and then dialyzed with 50 mmol/liter Tris–HCl (pH 7.4) overnight at 4°C. Finally, the protein was concentrated. The purity was checked by SDS–PAGE, and the concentration was calibrated by a standard curve.

**Trehalase Assay**

Trehalase activity was determined indirectly by the 3, 5-dinitrosaliicylic acid method (Lindsay 1973). The reaction mixture (1 ml) consisted of 10-µl purified protein (0.3 mg/ml), 200-mmol/liter trehalose (Sigma, St Louis, MO), and 940-µl PBS. The mixture was incubated at 4, 25, 37, 50, 55, and 60°C for 30 min, and the reaction was stopped in boiling water for 5 min. Coagulated protein was removed by centrifugation at 12,000 rpm for 10 min at 4°C. Trehalase activity was determined by measuring the content of glucose released during incubation. Similarly, the optimal pH value was measured by incubating the mixture at 55°C and varying pH from 3.0 to 9.0. To measure the kinetic parameters (K_m and V_max) of trehalase, 1, 2.5, 5, 7.5, 10, and 12.5 mmol/liter of trehalose were added to the reaction mixture and incubated at 55°C, pH 7.4 for 30 min. The trehalase activities were recorded with a microplate reader (Molecular Devices, Palo Alto, CA) at 540 nm in a 96-well plate. The kinetic parameters were calculated based on the results. One unit of enzyme (U) is defined as the amount that hydrolyzes 1 µmol of trehalose per minute.

**Assay of Trehalase Inhibitor**

For inhibitor studies, the trehalase inhibitor castanospermine was dissolved in 1× PBS (pH 7.4) at a gradient concentration of 0.5, 1, 2.5, 5, 10, and 20 µmol/liter. The reaction mixture contained 10-µl of purified trehalase, 50-µl of trehalose (200 mmol/liter), 100-µl of inhibitor, and 840-µl of 1x PBS (pH 7.4). The inhibitor was routinely preincubated with the enzyme at 55°C for 30 min. Other procedures were the same as the trehalase assays.

**Circular Dichroism Measurements**

Circular dichroism experiments were conducted on a Chirascan CD spectrometer (Applied Photophysics, United Kingdom). The reaction volume contained castanospermine with a final concentration of 0, 0.25, 2.5, 10, or 20 µmol/liter, and the concentration of purified trehalase was kept at 0.0075 mg/ml. The reaction mixture was incubated at room temperature under a nitrogen atmosphere. Data were obtained from 198 to 260 nm at 100 nm/min with 1-nm bandwidth using a 1-mm optical path length. Each spectrum shown is the average of three consecutive scans, and the absorbance of the 1x PBS buffer (pH 7.4) was subtracted from the spectra. The secondary structure contents were calculated from the measured spectra using CDNN CD spectra deconvolution software (Böhm et al. 1992).

**Results**

**Gene Cloning and Sequence Analysis of HaTreh-1**

Through homology searching in our transcriptional sets, the entire sequence of HaTreh-1 of about 1,716 bp was obtained. The sequence contained a 1,693-bp-long open reading frame that encodes 571 amino acids. Molecular weight of this protein was 65.61 kDa, and its theoretical isoelectric value was 5.02. As an extracellular enzyme, HaTreh-1 also had a N-terminal signal peptide with 23 amino acids. Multiple sequence alignment showed that HaTreh-1 shares a significant sequence similarity with other lepidopteran insect Treh-1 proteins (Fig. 1). The sequence alignment also indicated that HaTreh-1 had two signature motifs, PGGRFKEIYYWD and QWDFPNVWPPE. In addition, we observed a glycine-rich region (GGGGEY) that was highly conserved among insect species.

**Fig. 1.** Multiple alignment of amino acid sequences of soluble trehalase in lepidopteran insects. The sequences used in the alignment were as follows: Bombyx mori (BmTre1, GenBank BAA13042.1), Spodoptera litura (SlitTre1, GenBank ADA68384.1), Spodoptera exigua (SexiTre1, GenBank ABY60218.1), and Spodoptera frugiperda (SfruTre1, GenBank ABE27185.1). Red lines represent the two signature motifs. Identical and similar motifs are shown in black and gray. Red circles represent three essential residues. The arrowhead represents the glycine-rich region.
Three-Dimensional Structure Modeling for HaTreh-1

A three-dimensional model of HaTreh-1 was predicted using the SWISS-MODEL server based on the resolved crystal structure of periplasmic trehalase of *E. coli* (PDB ID 2WYN; Fig. 2). Reliability of the homology model was verified via the Procheck server and a Ramachandran plot (Fig. 3). The predicted α-toroidal structure of HaTreh-1 was very similar to that of the template protein, and the model structure loses a β-sheet at position 1, 2 separately (Fig. 2). The principal structure consisted of 25 α-helixes and 12 β-sheets. In the periplasmic trehalase of *E. coli*, Asp312 and Glu496 are two catalytic residues (Gibson et al. 2007). Accordingly, it could be presumed that Asp 321 and Glu 519 were the essential catalytic residues in HaTreh-1 model, but their positions in the model structure were slightly different from those in the template structure.

Recombinant Protein Expression

After being expressed in *E. coli* and purified by HisTrap affinity columns, the final concentration of the recombinant trehalase protein was 0.3 mg/ml. SDS–PAGE (Fig. 4) showed that the molecular weight of trehalase protein was about 71 kDa. The purified recombinant protein was used in later trehalase assays.

Enzymatic Assays

The purpose of the trehalase assays was to determine the optimal reaction conditions. As shown in Fig. 5, the optimal pH value of the purified trehalase 1 was 6.0 and the temperature optimum was 55°C. The enzyme activities at pH 6.0 and 55°C were 1.62 and 0.41 U/mg, respectively. This assay suggested that HaTreh-1 have higher activities at 45–60°C, pH 4.0–6.0. HaTreh-1 lost its enzymatic activity at extreme acidic or alkaline condition.

We also examined the kinetic parameters (Km and Vmax) of HaTreh-1. According to the Lineweaver–Burk plot, the Km value of HaTreh-1 was 72.8 mmol/liter and the Vmax value of HaTreh-1 was 0.608 mmol/(liter·min).

Inhibition Effect of Castanospermine on Trehalase

The inhibition assays showed that castanospermine was an effective inhibitor of HaTreh-1. Enzymatic activity of HaTreh-1 was completely inhibited by 1-µM castanospermine. In castanospermine dose-dependent inhibition assay, the median inhibitory concentration (IC50) of HaTreh-1 was 0.36 µM/liter. More importantly, the Lineweaver–Burk plots showed that the inhibition constant (Ki) was 6.7 µmol as analyzed by the Dixon plots (Fig. 6).

Circular Dichroism Spectra of Trehalase

The circular dichroism spectra of trehalase are shown in Fig. 7. According to CDNN CD spectra deconvolution, the secondary structure consisted of 46.2% α-helix, 11.4% β-sheet, 15% β-turn, and 22.3% of random coils (Table 2). Such structures shared high similarities with the three-dimensional model predicted by SWISS-MODEL.

Next, we investigated whether the inhibitor, castanospermine, can affect the structure of HaTreh-1. Our results showed that the CD spectra of trehalase had an increase in band intensity when more castanospermine was added, suggesting that castanospermine can alter the secondary structure of HaTreh-1. Typically, the content of α-helix increased dramatically while others were reduced, and...
a minor modification in concentration of castanospermine led to a larger change in the content of α-helix.

Discussion

Trehalases are involved in a variety of physiological activities such as glucose metabolism and chitin biosynthesis. Insects have soluble and membrane-bound trehalases (Takiguchi et al. 1992, Mitsumasu et al. 2005). Previous studies have shown that the soluble trehalase have the majority of total enzyme activity in vivo (Tatun et al. 2008, Tan et al. 2014). In this study, we focused on the soluble trehalase of H. armigera (HaTreh-1). Heterologous expression is an increasingly important way to study trehalases. At present, trehalase has been isolated and heterologously expressed successfully in Apis mellifera (Lee et al. 2007), Spodoptera frugiperda (Silva et al. 2009), B. mori (Ujita et al. 2011), and Chironomus riparius (Forcella et al. 2012). However, heterologous expression of trehalase has not been studied in H. armigera.

Trehalase contains some conserved regions including two signature motifs, three Arg residues, five conserved motifs, and one glycine-rich region (Mitsumasu et al. 2005, Forcella et al. 2012). According to the crystal structure of periplasmic trehalase from E. coli, D312 and E496 are the acid and base catalysts, respectively (Gibson et al. 2007). Similar results were also found in the trehalase of S. frugiperda (Silva et al. 2010) and T. molitor (Gomez et al. 2013). Our analysis of the amino acid sequence of HaTreh-1 found a high identity to some known insect trehalases. The overall HaTreh-1 sequence shared the highest identity with those of S. frugiperda (74% identity, GenBank ABE27189.1), followed by S. litura (73% identity, ADA63846.1), S. exigua (68% identity, ABY86218.1), B. mori (60% identity, BAA13042.1), Operophtera brumata (60% identity, KOB73697.1), and Ostrinia furnacalis (59% identity, ANY30160.1). Due to the amino acid similarity of soluble trehalase in S. frugiperda and H. armigera, we proposed that Asp 321 and Glu 519 are the active sites. In addition, three Arg residues (R168,
R221, and R286) located in conserved motifs were necessary for trehalase activity. Our results with HaTreh-1 were consistent with those reported in other insect trehalases (Silva et al. 2009, Zou et al. 2013). The primary three-dimensional structure of HaTreh-1 was similar to the periplasmic trehalase of E. coli. Both structures mainly consisted of α-helixes and were surrounded by α-toroidal structures. This structure was also observed in the trehalase of other insects (Forcella et al. 2012, Gomez et al. 2013). Two active sites (Asp and Glu) and three Arg residues in HaTreh-1 were located at the same secondary structure as in template protein. Therefore, they were considered as functional motifs of HaTreh-1.

Some variations were observed between model protein and template protein. For instance, the SOPMA server determined that 44.48% of all the amino acids were involved in α-helixes, but that the model structure lost two β-sheets at position 1 (Fig. 2). In addition, the β-sheet at the C-terminal domain of the template was replaced by an α-helix at position 2 (Fig. 2); this may be caused by a replacement from asparagine to tyrosine and the asparagine could damage the α-helix according to the Chou–Fasman method. Two key conserved catalytic residues existed in the template protein, and Asp 312 and Glu 496 determined its enzymatic properties (Aleshin et al. 1993).

Although HaTreh-1 is similar to other Lepidoptera trehalases, there are still some important differences. In two conserved motifs, threonine was replaced by lysine in BmTreh-1 and HaTreh-1, while alanine was replaced with valine in BmTreh-1 (Fig. 1). However, these amino acid replacements did not change trehalase activity.

We studied the in vitro interaction between trehalase and an inhibitor using castanospermine as the enzyme inhibitor. Results showed that the assayed alkaloid was a competitive inhibitor of trehalase. Castanospermine is an effective inhibitor of HaTreh-1 with an IC50 of 0.36 μmol/liter. The Ki value of castanospermine was 10,800 times higher than that of HaTreh-1. It has been reported that trehazolin inhibits silkworm trehalase with an IC50 of 27 nM (Ando et al. 1995). Amygdalin inhibited housefly trehalase with an IC50 of 31.7 μM, and VA has an IC50 of 0.4 μM against housefly trehalase (Park et al. 2008). VAA, another validamycin compound, showed a strong inhibitory effect with a Ki value of 10.3 μM. Some kinds of toxic β-glucosides like prunasin inhibit trehalase from Tenebrio molitor and S. frugiperda at the mMol scale (Silva and Terra 2006). Castanospermine could also play a role in in vitro inhibition at the μMol scale. Previous studies in feeding bioassays demonstrated that castanospermine was a potent inhibitor toward trehalase of many insects in vivo (Campbell et al. 1987). Our results confirmed that castanospermine was also effective against HaTreh-1. To further decipher the mechanisms of its inhibitor action, we examined the secondary structure of HaTreh-1 and its modification induced by castanospermine using circular dichroism spectroscopy. Our results showed that the protein is mainly composed of α-helix structure. The percentage of α-helix structure in the protein was significantly increased under the action of the inhibitor. Thus, the presence of alkaloids changes the hydrogen bonds inside the protein, rearranges the peptide chains, and increases the hydrophobicity of the trehalase protein.

The structure of trehalase inhibitors is usually similar to trehalose, and some of them contain one glucose molecule and one cyclitol molecule. Many of these potent inhibitors also have a bridgehead nitrogen and another heterocyclic nitrogen (Kyosseva et al. 1995). At the molecular level, the hydroxide radical in the inhibitors could interact with different amino acid residues in trehalase (Gibson et al. 2007),

Table 2. Secondary structure of trehalase in the presence of castanospermine at different concentrations

| Secondary structure | Concentration of castanospermine (μM) |
|---------------------|---------------------------------------|
|                     | 0          | 0.25         | 2.5          | 10           | 20           |
| α-Helix             | 46.20%     | 67.30%       | 71.10%       | 74.00%       | 75.50%       |
| β-Sheet             | 11.40%     | 5.90%        | 5.10%        | 4.50%        | 4.40%        |
| β-Turn              | 15.00%     | 12.20%       | 11.70%       | 11.30%       | 11.00%       |
| Random col          | 22.30%     | 12.20%       | 10.80%       | 9.40%        | 9.50%        |

![Fig. 7. The CD spectra of 0.0075 mg/ml soluble trehalase from Helicoverpa armigera in the presence of castanospermine at different concentrations.](image)

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| β-Sheet             | 11.40%     | 5.90%        | 5.10%        | 4.50%        | 4.40%        |
| β-Turn              | 15.00%     | 12.20%       | 11.70%       | 11.30%       | 11.00%       |
| Random col          | 22.30%     | 12.20%       | 10.80%       | 9.40%        | 9.50%        |
which could explain the inhibitory effect of castanospermine. The in vivo and in vitro inhibition effects usually differ greatly. Some trehalase inhibitors had a high inhibition rate in vivo but low rate in vitro due to their poor penetration ability (UCHIDA et al. 1995, KNUESEL et al. 1998). Further study will be carried out to explore the inhibition capability of castanospermine in vivo.

The application of traditional chemical pesticides has led to serious environmental pollution, meaning it is important to develop environmentally friendly pesticides. Due to the high cost of field sex pheromones trapping application, other potential biological pesticides are getting increasing attention. Selection of trehalase as a pesticide target is not a threat to nontarget organisms because of the absence of endogenous trehalase in mammals (THOMPSON 2003). Plant-derived pesticides have various advantages including low residue and low pollution (ZHANG et al. 2015). Castanospermine, a kind of alkaloid, could affect insect carbohydrate metabolism by inhibiting hydrolyzing enzymes (CAMPBELL et al. 1987), but a study on pig kidney trehalase revealed inferior inhibition of castanospermine to mammal trehalase (KYOSEVA et al. 1995). This showed that this inhibitor (castanospermine) has some selectivities to insect trehalases, and it has the potential to be an environmental safe pesticide.

To date, some trehalase inhibitors have been subjected to molecular modification to improve their inhibitory effects (BINI et al. 2012). Castanospermine could be used as a prodrug to develop potent biological pesticide. Its specific trehalase inhibitory activity can be used for insect pest control in field. Our study confirmed the interaction between castanospermine and HaTreh-1.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (31471803, 31171912). All the authors declare that they have no conflicts of interest.

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