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Effects of the polyhistidine tag on kinetics and other properties of trehalose synthase from *Deinococcus geothermalis*

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Two recombinant trehalose synthases from *Deinococcus geothermalis* (DSMZ 11300) were compared. A significant influence of the artificial polyhistidine tag was observed in protein constitution. The recombinant trehalose synthase from *D. geothermalis* with His_6-tag has a higher K_m value of 254 mM, in comparison with the wild-type trehalose synthase (K_m 170 mM), and displayed a lower activity of maltose conversion when compared to the wild type. Moreover, differences in properties like temperature, pH, thermal- and pH-stability were observed. Presence of the histidine tag caused a decrease of thermal resistance in case of trehalose synthase with His_6-tag. These data confirmed a suggestion that the introduction of the histidine domain produces in some seldom cases undesirable changes in the protein.

Key words: *Deinococcus geothermalis*, trehalose synthase, influence of His_6-tag, trehalose, gene expression, *Escherichia coli*

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INTRODUCTION

Trehalose(α-D-glucopyranosyl-1,1-α-D-glucopyranoside) is a stable non-reducing disaccharide, which can be used in food, cosmetics, medical, and biotechnological industries, as well as for the stabilization of vaccines, enzymes, antibodies, hormones, pharmaceutical preparations, and organs for transplantations (Richards *et al.,* 1991; Higashiyama 2002; Berendsen 2002). Trehalose can create hydrogen bonds with protein and lipids, which stabilize their molecules and biological membranes (Lins *et al.,* 2004; Jain and Roy, 2008). Moreover, the formation of amorphous glass protects biological molecules during desiccation, freezing, heating, and oxidation (Crove and Crove, 2002; Banaroudi *et al*., 2001).

Enzymes responsible for the one-step production of trehalose from maltose are trehalose synthases, EC 5.4.99.16. This process employs an inexpensive substrate derived from starch. Trehalose synthases are also characterized by amylase activity that converts glycogen into trehalose (Pan *et al.,* 2008). Trehalose synthases have been identified as sources of such bacteria as for instance the *Pseudomonas putida*, *Meiothermus ruber*, *Thermus thermophilus*, *Deinococcus radiodurans* and *Arthobacter aurescens* (Ma *et al*., 2006; Zdziebło & Synowiecki, 2006; Xiuli *et al.,* 2009; Filipkowski *et al*., 2012; Zhu *et al*., 2008).

In our previous work, as a new source of trehalose synthase, we used the extremophilic bacteria, *D. geothermalis* (Filipkowski *et al.,* 2012). This microorganism is a red-pigmented, Gram-positive, non-pathogenic, moderate thermophile extremely resistant to ionizing radiation, ultraviolet light, and desiccation (Ferreira *et al*., 1997). In our study, the gene encoding the trehalose synthase from *D. geothermalis* was cloned and expressed in *Escherichia coli*. This recombinant enzyme contains the His_6 tag, which simplified the enzyme purification using immobilized metal affinity chromatography (IMAC). The method for puryfing proteins with histidine residues was first described by Hochuli *et al.* (1987). IMAC is based on the interaction between transitional metal ions, eg., Cu^{2+}, Co^{2+}, or Ni^{2+}, immobilized on a matrix in a gelling agent, such as agarose or derivatized silica gel and proteins. The main amino acid in proteins that interacts with the immobilized transitional metal ions is histidine. Nowadays, more than 100 structures of His-tagged proteins have been deposited in the Protein Data Bank. In some seldom cases the affinity tag may interfere with protein activity (Wu & Filutowicz, 1999; Terpe, 2003). Usually, the fusion enzymes are as active as in case of wild proteins. However, in some cases the V_{max} for the tagged enzymes is 50% of the wild-type. Lowered activities are caused by insoluble inclusion bodies, misfolded conformation when expressed in *E. coli,* or different dimerization/oligomerization properties (Halliwell *et al.,* 2001; Pietzsch *et al.,* 2000; Wu & Filutowicz, 1999).

The previously obtained recombinant trehalose synthase from the *D. geothermalis* containing the polyhistidine domain had decreased the affinity to maltose. The purpose of this study was to produce a trehalose synthase destitute of histidine residues. Kinetic parameters between the wild-type enzyme and the recombinant protein with the polyhistidine tag were examined to compare their properties.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *D. geothermalis* (DSMZ 11300, Braunschweig, Germany) was used as a source of the trehalose synthase gene. Bacterial cells were cultivated aerobically on a rotary shaker (Forma Orbital, Thermo Scientific, Marietta, OH, US) at 45°C (pH 7.2) in a modified medium recommended by the DSMZ as described previ-

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Abbreviations: K_m, Michaelis constant, V_{max}, maximum rate, *Dgeo*TreS, trehalose synthase from *D. geothermalis* with His_6-tag, *Dgeo*TreSW, trehalose synthase from *D. geothermalis* wild type, DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, CD, Circular dichroism spectroscopy OD_{600}, optical density at λ=600 nm, PCR, polymerase chain reaction, DNA, deoxyribonucleic acid, EDTA, ethylenediaminetetraacetic acid, HPLC, high-performance liquid chromatography, SDS/PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

ously (Filipkowski *et al.,* 2006, Ferreira *et al.,* 1997). The *E. coli* TOP10F' (Invitrogen, Carlsbad, CA) and Rosetta (DE3)p*Lys*S (Novagen, Nottingham, United Kingdom) strains were used as a cloning host and an expression host, respectively. The *E. coli* cells with plasmids were cultured aerobically at 37°C to OD_{600} of 0.6 in an LB medium supplemented with tetracycline (12.5 μg/mL) or chloramphenicol (34 μg/mL), respectively. The cells were harvested by centrifugation at 3 000 × *g* for 10 min. The pJET (Fermentas UAB, Vilnius, Lithuania) and pET30Ek/LIC (Novagen, Darmstadt, Germany) plasmids were used for the construction of the expression system. Restriction enzymes were purchased from Fermentas.

Amplification of trehalose synthase (DgeoTreSW) gene. DNA from *D. geothermalis* isolated using a genomic DNA preparation kit (A&A Biotechnology, Gdańsk, Poland) was used for polymerase chain reaction (PCR) amplification with the following two primers:

5' aaaaCATATGACGCAAACCTCCACCTCCGAGT 3', and
5' tatCTCGAGTTACCGCACCCCCGACAGC 3'

containing underlined recognition sites for restriction endonucleases, *Nde*I and *Xho*I (Fermentas, Lithuania). The reaction was performed using 250 ng of DNA, 10 pmoles of each primer, 12 μmoles of dNTPs, 12.5 μL 2 × PCR buffer (5 mM $MgCl_2$,100 mM Tris, pH 9.0, 40 mM $(NH_4)_2SO_4$, 10 mM DMSO) and 0.5 μL Marathon DNA polymerase (*Pwo* & *Taq* polymerase mixture). After 1 min of preliminary heating at 95°C in a thermal cycler (EpGradient S, Eppendorf, Hamburg, Germany), each of 30 cycles was conducted 5-times at 95°C for 1 min, at 59°C for 1 min and at 72°C for 2 min, then 25-times at 95°C for 1 min, at 67°C for 1 min and at 72°C for 1.5 min, with a final step of 5 min at 72°C. The DNA fragment encoding *Dgeo*TreSW was obtained and cloned into pJET vector following the steps described by the producer of the CloneJet kit (Fermentas). After sequencing and confirming the proper sequence, the DNA construct was digested with *Nde*I and *Xho*I restriction endonucleases and resubcloned into a pET30Ek/LIC vector. The digestion product, approximately below 1,700 bp, was isolated from an agarose gel bands using the Gel-Out kit (A&A Biotechnology).

Construction of expression vector. The competent cells *E. coli* TOP10F' suspended in 1 mL 100 mM $CaCl_2$ were transformed by the ligation mixture for 1 h at 8°C, and after a heat shock (1.5 min at 42°C, then 1.5 min at 4°C) were incubated for 1 h at 37°C in 400 μL SOC medium (Invitrogen). The transformed *E. coli* cells plated on LB-agar tetracycline/kanamycin (tetracycline as described previously, kanamycin 20 μg/mL) plates were incubated at 37°C. After 16–18 h of growth, the obtained colonies were examined for the presence of trehalose synthase gene of *D. geothermalis* by PCR amplification and restriction analysis. The authenticity of the clone was ascertained by sequencing of the complete DNA fragment. The obtained construction, designated as pET30Ek/LIC*Dgeo*TreSW, was isolated and selected by electrophoresis on 1% agarose gel and then transformed into *E. coli* Rosetta (DE3)p*Lys*S cells.

Expression of the wild-type trehalose synthase. A single colony of transformed *E. coli* from LB-agar plates was inoculated in a 50 mL liquid LB medium, supplemented with kanamycin/chloramphenicol, and cultivated at 37°C. When the OD_{600} reached the value of about 0.6, the cell suspension was transferred to 2 L of LB medium containing kanamycin/chloramphenicol. The cultures were grown up to OD_{600} of 0.6 at conditions described above. Then, the D*geo*TreS expression in *E. coli* Rosetta (DE3)p*Lys*S was induced at 37°C with isopropyl β-D-thiogalactopyranoside (IPTG) at the final concentration of 1 mM. The cells were harvested 3 h after induction by centrifugation, and the pellet washed with distilled water was centrifuged and stored at –20°C until use. SDS/PAGE separation of proteins was performed according to the method of Leammli (1970). The samples (10 μL) denatured by β-mercaptoethanol as a reducing agent and SDS were layered on the gel and separated using a voltage gradient of 15 V/cm. Protein bands were located by stained with Coomassie Brillant Blue R250.

Isolation and purification of recombinant trehalose synthase. The transformed *E. coli* cells, harvested from 2 L of induced culture, were sonicated at 20 kHz (three times for 10 s in 30 s intervals) in 80 mL 10 mM phosphate buffer (pH 7.6) containing 1M EDTA, 100 mM $CaCl_2$, 0.1 mg lysozyme, and 1 mg RNase, using the Brenson Ultrasonic Sonifier II W250D (Geneve, Switzerland). The resulting mixture was centrifuged at 9 000 × *g* for 40 min to give a clear supernatant. The protein fraction with trehalose synthase activity was precipitated by $(NH_4)_2SO_4$ to a final concentration of 20% (w/v). The centrifuged precipitate (9 000 × *g*, 15 min) was dissolved in a small volume of citrate-phosphate buffer pH 7.2. Then, this fraction was purified, concentrated on the Centrifugal Filter Device (Amicon®Ultra-15 50 000 MWCO Carrighwohill, Cork, Ireland), and after changing the buffer to physiological salt, used as a final preparation of the enzyme.

Enzyme assay. The activity of *Dgeo*TreSW was determined by measuring the trehalose produced from maltose. The assays were initiated by the addition of 0.25 mL of *Dgeo*TreSW solution to 1.0 mL of 0.3 M maltose solution in a 50 mM Britton-Robinson buffer (pH 7.6). The reaction at 40°C was terminated after heating the sample at 100°C for 10 min. The samples purified by centrifugation (9 000 × *g* for 10 min) and filtration on a 0.2 μm Chromafil® PEF 20/25 filter (Machery-Nagel GmbH, Duren, Germany) were passed through a APS-2 HYPERSIL column (Thermo Electron Corporation, Dreiech, Germany) using acetonitrile/methanol/water (78:11:11, v/v/v) as mobile phase at a flow rate of 1.8 mL/min. The column temperature was 30°C. The amounts of the products formed during conversion of maltose were calculated from the area of the peaks obtained after sample separation by HPLC using a refractive index detector (La Chrom-7490, Merck, Hitachi, Tokyo, Japan). Trehalose, maltose, and glucose were used as standards at concentrations of 10 mg/mL.

The *Dgeo*TreSW activity was expressed as the amount of enzyme that produces 1 μmol of trehalose per minute under described conditions. The relative enzyme activity (%) was defined as the percentage of enzyme activity in the control.

The temperature dependence of enzyme activity was assayed within the range of 5–70°C. The thermal stability of trehalose synthase was investigated by incubation of the enzyme solution in 50 mM Britton-Robinson buffer (pH 7.6) at 40°C, 55°C, and 60°C for different periods (up to 8 h). Protein concentrations (mg/mL) were determined by the measurement of absorbance at 280 using a Nano-Drop spectrophotometer (Thermo Fisher Scientific Inc. Wilmington, DE, US).

RESULTS AND DISCUSSION

Molecular cloning and characterization of His-tag free *Dgeo*TreSW

Our previous experiments indicate that the recombinant trehalose synthase from *D. geothermalis* possessing His_6-tag (*Dgeo*TreS) had decreased the affinity to maltose, and the K_m value was determined at 256 mM (Filipkowski *et al.*, 2012). It suggests an influence of this domain on enzyme activity. To examine the effect of the polyhistidine tag has on activity and kinetics of this protein we compared their properties to the wild-type enzyme (*Dgeo*TreSW) from *D. geothermalis* expressed in *E. coli* Rosetta (DE3)pLysS. The region of *D. geothermalis* gene amplified by PCR had a sequence consisting of 1668 b.p. encoding 556 amino acid residues. Theoretical values of the molecular mass and isoelectric point of the wild-type enzyme were calculated to be 63.63 kDa and pI 4.92, respectively. These values estimated for the *Dgeo*TreSW are slightly lower than those for *Dgeo*TreS, due to the fact that the wild-type enzyme does not contain the His_6-tag preceded by Leu (L) and Glu (E) residues.

An optimal expression was achieved at 30°C after 4 h of induction by IPTG (Fig. 1). The wild-type trehalose synthase has been preliminary purified by thermal precipitation of *Escherichia coli* proteins. This step removes 66.35% of native *Escherichia coli* proteins. What is more, specific activity of cell-free extract increases from 0.0159 up to 0.0416 μmol/min/mg. Solubilized recombinant trehalose synthase was precipitated at 20% ammonium sulphate saturation. Dissolved precipitate was purified on a centrifugal filter device (Amicon Ultra-15 30000 MWCO). SDS/PAGE (Fig. 2) confirmed high purity of DgeoTreSW and their band was localized at the same place as his-tagged trehalose synthase. Among very intensive enzyme band only one weak band of other protein was visible. Such small amounts of other protein cannot influence the K_m value and their changes are caused by the polyhistidine tag.

Effect of His_6-tag on kinetic of maltose conversion

Compared to the K_m of trehalose synthase with polyhistidine tag (256 mM), the wild-type enzyme expressed in *E. coli* has a lower K_m of 170 mM. It suggests higher affinity of *Dgeo*TreSW to maltose. Observed lower affinity of *Dgeo*TreS to substrate may be a result of possible changes of enzyme conformations caused by the His_6-tag. Recent reports also shown that His-tagged proteins are often expressed in *E. coli,* both in soluble form and insoluble inclusion bodies, limiting the amount of the enzyme accessible to the reaction (Halliwell *et al.,* 2001; Terpe, 2003; Khan *et al.,* 2012). According to the study of Khan *et al.* (2012) His-tag-free proteins have lower minima of CD spectrum at 208 and 222 nm, as compared with their His-tagged counterparts. It indicates a higher amount of α-helical content. Among the proteins investigated in our study, the catalytic efficiency (k_{cat}/K_m) value of *Dgeo*TreSW was higher than that of *Dgeo*TreS, indicating that *Dgeo*TreSW is a better catalyst than trehalose synthase with the polyhistidine tag. The location of the hexa-histidine tag on the protein N-terminus or C-terminus also can influence the protein activity. The study on *Dgeo*TreS with His_6-tag localized on the N-terminus is in progress and its results will appear elsewhere.

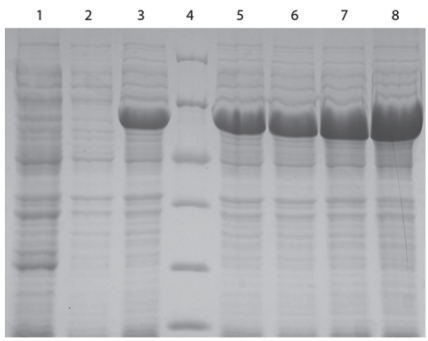

Figure 1. SDS/PAGE of protein in the fraction during the biosynthesis of the recombinant *Dgeo*TreSW.
Lane 1, control *E. coli* strain with vector pET before induction; lane 2, recombinant *E. coli* strain with vector before induction; lane 3, cell extract after 3 h of protein expression induced by IPTG; lane 4, protein marker (Fermentas #SM0431); lane 5, cell extract after 4 h of protein expression induced by IPTG; lane 6, cell extract after 5 h of protein expression induced by IPTG; lane 7, cell extract after 6 h of protein expression induced by IPTG; lane 8, cell extract after 18 h of protein expression induced by IPTG.

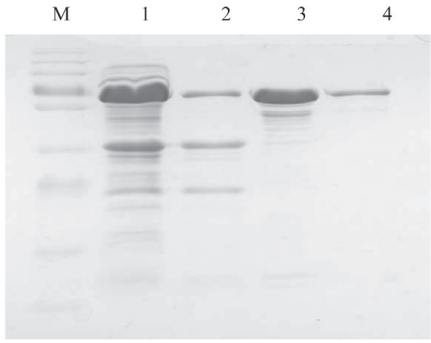

Figure 2. SDS/PAGE of proteins during the purification steps.
Lane M, protein marker (Page Ruler™ Plus (Thermo Scientific. Line 1, 10 μL cell free extract cells of *E. coli* Rosetta (DE3) D*geo*TreSW. Line 2, 10 μL of cell free extract after heat treatment at 65°C for 10 min. Line 3, 10 μL of protein fraction precipitated at 20% $(NH_4)_2SO_4$ and purified on Amicon®, cut-off 100 kDa. Line 4, 10 μL D*geo*TreS protein after metal affinity chromatography.

Changes of reaction conditions caused by His_6-tag

Figure 3 shows that the optimal temperature of maltose conversion into trehalose catalyzed by *Dgeo*TreS was 40°C and it was increased up to 45°C in case of the wild-type enzyme. The thermostability of *Dgeo*TreSW was higher in comparison with the enzyme containing the polyhistidine tag. After 8 h of heating at 55°C, the *Dgeo*TreS retained about 57% of its maximal activity, whereas the *Dgeo*TreSW was completely active (100%) after 8 h of incubation.

In comparison with the wild trehalose synthase indicating maximal activity at pH 7.1 in a 50 mM Britton-

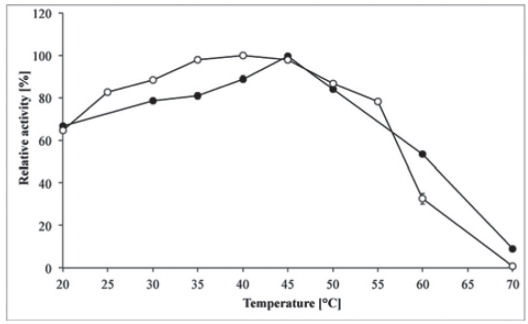

Figure 3. Optimal temperature of *Dgeo*TreS (○) and *Dgeo*TreSW (●) at pH 7.0.
The results are mean values of three replicates.

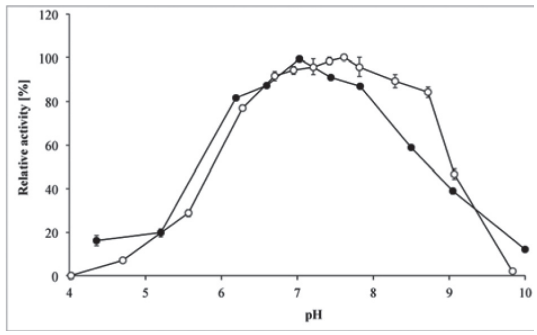

Figure 4. Optimal pH of *Dge*TreS (○) and *Dgeo*TreSW (●). The results are mean values of three replicates.

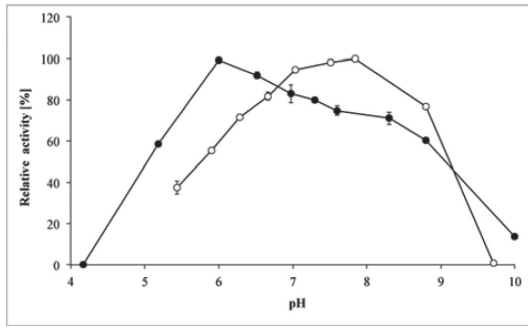

Figure 5. pH-stability of *Dgeo*TreS (○) and *Dgeo*TreSW (●). The pH stability was determined by measuring the residual activity after 1 h of incubation (40°C) in 50 mM Britton-Robinson buffers at various pH values. The results are mean values of three replicates.

Robinson buffer, the optimal pH for maltose conversion catalyzed by *Dgeo*TreS was 7.6. These pH values are similar to those reported for trehalose synthases from *Pimelobacter* sp. R48 (Nishimoto *et al*., 1996a) and *Thermus aquaticus* (Nishimoto *et al*., 1996b). The investigated enzymes retained about 50% of maximal activity at a pH range of 6.0 to 9.0 (*Dgeo*TreS), and 5.5 to 8.6 (*Dgeo*TreSW) (Fig. 4).

To determine the effect of pH level on the stability of trehalose synthases containing polyhistidine tag or enzyme without this domain, the samples were incubated for 1 h at different pH levels adjusted by Britton Robinson buffers. The *Dgeo*TreS had the highest remaining activity when incubated at pH 7.7, whereas the *Dgeo*TreSW displayed maximal activity at the pH level of 6.0. Similarly to the *Dgeo*TreS, the wild-type trehalose synthase was completely inactivated after the incubation at pH of about 4.2 and retained 85% of its maximal activity at the pH level of 7.0 (Fig. 5).

CONCLUSION

The present study leads to considerations about the influence of polyhistidine tags on kinetic parameters, and some properties of the recombinant trehalose synthase from *D. geothermalis*. The wild-type trehalose synthase exhibited higher activity when compared with his-tagged *Dgeo*TreS, which had been studied previously. This report sends an important message that proteins containing His-tags may differ from their wild-type counterparts as far as their activity and other kinetic properties are concerned.

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