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Properties of recombinant trehalose synthase from *Deinococcus radiodurans* expressed in *Escherichia coli**

Paweł Filipkowski✉, Olga Pietrow, Anna Panek and Józef Synowiecki

Department of Food Chemistry, Technology and Biotechnology, Faculty of Chemistry, Gdansk University of Technology, Gdańsk, Poland

A trehalose synthase gene from *Deinococcus radiodurans* (DSMZ 20539) containing 1659 bp reading frame encoding 552 amino acids was amplified using PCR. The gene was finally ligated into pET30Ek/LIC vector and expressed after isopropyl β-D-thiogalactopyranoside induction in *Escherichia coli* (DE3) Rosetta p*Lys*S. The recombinant trehalose synthase (*DraTreS*) containing a His_6-tag at the C-terminus was purified by metal affinity chromatography and characterized. The expressed enzyme is a homodimer with molecular mass of 126.9 kDa and exhibits the highest activity of 11.35 U/mg at pH 7.6 and at 30°C. *DraTreS* activity was almost unchanged after 2 h preincubation at 45°C and pH 7.6, and retained about 56% of maximal value after 8 h incubation at 50°C. The *DraTreS* was strongly inhibited by Cu^{2+}, Hg^{2+}, Zn^{2+}, Al^{3+} and 10 mM Tris. The K_m value of maltose conversion was 290.7 mM.

Key words: *Deinococcus radiodurans,* trehalose, trehalose synthase, gene expression, *Escherichia coli*

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INTRODUCTION

D. radiodurans is a red pigmented, Gram-positive, non-pathogenic mesophile that propagates in a temperature range from 30°C to 37°C. This bacteria is extremely resistant to ionizing radiation, ultraviolet light and desiccation (Lewis, 1971). *D. radiodurans* can be a potential source of an enzyme producing trehalose (α-D-glucopyranosyl-α-D-glucopyranoside). This stable, non-reducing disaccharide contains α,α-1,1-glucoside linkage between the α-glucose moieties (Elbein *et al.*, 2003). Trehalose can be used in the cosmetics, food, medical and biotechnological industries. The low sweetness of trehalose, good solubility in water, high water retention capabilities, stability, reduction of water activity, depression of freezing point and protein protection properties make it a valuable food ingredient (Teramoto *et al.*, 2008). There are ideas for the stabilization of vaccines, enzymes, antibodies, hormones, pharmaceutical preparations and organs for transplantation (Richards *et al.*, 2002; Higashiyama, 2002). One of the reasons for trehalose applications is participation in stabilization of proteins and/or biological membranes. Probably trehalose can form amorphous glass which entraps biological molecules without essential changes to their native structure during desiccation, freezing, heating or oxidation (Crove & Crove, 2000; Roser, 1991; Benaroudi, 2001; Lenart *et al.*, 1995). According to an alternate hypothesis trehalose can substitute water around biomolecules that provide hydrogen bonding network maintaining the structure of the protein. Trehalose is resistant to hydrolysis at low pH values and does not caramelize and does not participate in Maillard reactions. This compound has been accepted as a food additive and declared as GRAS (Generally Recognized As Safe) in the U.S. and at the EU respectively by FDA (Food and Drug Administration) and EFSA (European Food Safety Authority).

Enzymes involved in different pathways of trehalose biosynthesis were extensively studied (Schiraldi *et al.,* 2002; Woo *et al.,* 2010). There are known microorganisms which have only a single pathway, but some of them have two and even three trehalose biosynthesis pathways (Avonce, 2006). The most common biosynthesis pathway involves trehalose-6-phosphate synthase (TPS) which catalyses the transfer of glucose from uridine-diphosphate-glucose to glucose-6-phosphate. Afterwards, a trehalose-phosphate phosphatase (TPP) liberates phosphate forming trehalose. This pathway was described for *Pyrococcus horikoshii* and *Saccharomycopsis fibuligera* (Ryu *et al.*, 2005, Liang *et al.*, 2006). Another pathway, which involves two enzymes, was found in some hyperthermophilic archaeons, e.g.: *Sulfolobus solfataricus*, synthesising: maltooligosyltrehalose synthase (MTSase), and maltooligosyltrehalose trehalohydrolase (MTHase) to produce trehalose and oligosaccharide as intermediate (Kato, 1999). Recently, Pan *et al.* (2008), reported also a two step enzymatic production of trehalose from glycogen using MTSase and MTHase from *Mycobacterium smegmatis*. The third pathway, catalyzed by trehalose glycosyltransferring synthase (TreT), involves the conversion of nucleoside diphosphate glucose and glucose, instead of glucose-6-phosphate, into trehalose (Ryu *et al.,* 2005; Empadinhas *et al.,* 2006). On the other hand, production of trehalose in fungi such as *Pleurotus ostreatus* involves glucose-1-phosphate and glucose as substrates and trehalose phosphorylase (TreP) (Schwarz *et al.,* 2007).

There is a possibility to use maltose-α-D-glucosyl transferase, EC 5.4.99.16 (trehalose synthase, TreS) in industrial applications. This enzyme converted maltose into α,α-trehalose and released a small amount of glucose and α,β-trehalose as by-products (Nishimoto *et al.,* 1996a; Nishimoto *et al.,* 1996b; Koh *et al.,* 2003). According to

✉e-mail: pawel.filipkowski@pg.gda.pl
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Abbreviations: GRAS, Generally Recognized As Safe; FDA, Food And Drug Administration; EFSA, European Food Safety Authority; *Dra*TreS, trehalose synthase from *D. radiodurans*; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen; 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; NCBI, National Center For Biotechnology Information

many authors trehalose synthase is suitable for industrial manufacture of trehalose (Chen *et al.*, 2006) because it allows one-step formation of trehalose and an inexpensive substrate, maltose derived from starch is employed. Wu *et al.* noticed in 2011 that this process is more promising than the bi-enzyme process currently used in industry. Maltose and glucose residues can be easily removed using a chromatographic process similar to purification of fructose manufactured from glucose. The fermentation route in yeast is too expensive because extraction and purification of trehalose require different reagents.

Up to now, trehalose synthases have been isolated from a variety of bacteria species, e.g., *Pseudomonas putida*, *Thermus thermophilus*, *Meiothermus ruber* and *Arthrobacter aurescens* (Ma *et al.,* 2006; Zdzieblo & Synowiecki, 2006; Zhu *et al.*, 2008; Xiuli *et al.,* 2009). In the present work the gene encoding trehalose synthase from *D. radiodurans* (*DraTreS*) was cloned and expressed in *E. coli*. This is not the first report on *D. radiodurans* trehalose synthase. Wang *et al.* (2007) tried to use a part of *dratreS* to determine the role of the C-terminus fragment of trehalose synthase.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions. *D. radiodurans* (DSMZ 20539, 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 30°C (pH 7.2) in a modified medium recommended by DSMZ as described previously (Kur *et al.,* 2005). The *E. coli* TOP10F' (Invitrogen, Carlsbad, CA) and Rosetta (DE3) p*Lys*S (Promega, Madison, WI) strains were used as a cloning host and expression host, respectively. The *E. coli* cells with plasmids were cultured aerobically at 37°C to OD_{600} of 0.3–0.5 in LB medium supplemented with tetracycline (1.25 μg/ml) or chloramphenicol (34 μg/ml) and kanamycin (20 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 construction of an expression system. Restriction enzymes were purchased from Fermentas, Lithuania.

Amplification of trehalose synthase (*DraTreS*) gene and construction of expression vector. The deoxyribonucleic acid (DNA) from *D. radiodurans* isolated using a genomic DNA preparation kit (A&A Biotechnology, Gdańsk, Poland) was used for amplification of *DraTreS* gene in polymerase chain reaction (PCR) with two primers: 5' aa cat ATG ACC CAG GCA CAC CCG GA 3' and 5' aa ctc gag ATT CAA CCG CAG CCA GTA ATA GTC 3' containing underlined recognition sites for restriction endonucleases NdeI and XhoI. 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 at 95°C for 1 min, 63.5°C for 1 min and 72°C for 2 min, with a final step of 5 min at 72°C. The DNA fragment encoding *DraTreS* was obtained and cloned into pJET vector according to the producer's manual (CloneJet Kit). The competent *E. coli* TOP10F' cells suspended in 1 ml 100 mM $CaCl_2$ were transformed by the ligation mixture for 1 h at 8°C and after heat shock (1.5 min at 42°C, then 1.5 min at 4°C) incubated for 1 h at 37°C in 400 μl SOC medium (Invitrogen). The transformed *E. coli* cells were plated on LB-agar tetracycline/ampicillin plates and incubated at 37°C for 16–18 h. The obtained colonies were examined for the presence of the trehalose synthase gene of *D. radiodurans* by PCR amplification and restriction analysis. The presence of the *DraTreS* gene in transformed clones was confirmed by sequencing of the amplified DNA fragment. Then the appropriate sequence was digested with NdeI and XhoI restriction endonucleases and subcloned into a pET30Ek/LIC vector. The digestion product slightly smaller than 1700 bp was isolated from agarose gel bands using the Gel-Out kit (A&A Biotechnology). The obtained construct, designated as pET30Ek/LIC-*DraTreS*, was isolated and selected by electrophoresis on a 1% agarose gel and then used for transformation of *E. coli* Rosetta (DE3) p*Lys*S cells.

Expression of the His_6-tagged 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 OD_{600} reached the value of about 0.3–0.5, the cell suspension was transferred to 2 l of LB medium containing kanamycin/chloramphenicol. The cultures were grown up to OD_{600} of 0.3–0.5 at conditions described above (37°C), and then *DraTreS* expression in *E. coli* Rosetta (DE3) p*Lys*S was induced at 25°C with isopropyl β-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM. The cells were harvested 18 h after induction by centrifugation and the pellet washed with distilled water was centrifuged and stored at –20°C until use.

Isolation and purification of recombinant trehalose synthase. Transformed *E. coli* cells, harvested by centrifugation (6 000 × *g*, 15 min, 15°C) from 2 l of induced culture, were homogenized and sonicated at 20 kHz (three times for 30 s with 30 s intervals) in 80 ml 50 mM phosphate buffer (pH 7.6) containing 1 M EDTA, 100 mM $CaCl_2$, 0.1 mg lysozyme and 1 mg RNase, using Brenson Ultrasonic Sonifier II W250D (Geneve, Switzerland). The resulting suspension was centrifuged (8°C) at 9 000 × *g* for 40 min. Total cell lysate was incubated at 56°C for 10 min and precipitated host proteins were removed by centrifugation at 12 000 × *g* for 40 min. The obtained supernatant was then applied to Co^{2+}-IDA-agarose column (His×Bind®Resin, Novagen) equilibrated with 50 ml of 0.1 M phosphate buffer (pH 7.6) containing NaCl and imidazole at concentrations 0.5 M and 5 mM, respectively (buffer A). After loading, the column was washed three times with 50 ml of the same buffer. The recombinant *DraTreS* was then washed twice with 20 ml of buffers A, A', B, C, D containing imidazole concentrations 5, 25, 50, 80, 100 mM, respectively. The protein of interest was eluted with two portions of 20 ml elution buffer E containing imidazole at concentration of 0.5 M. The eluted fraction was desalted and concentrated on a Centrifugal Filter Device (Amicon®Ultra-15 30 000 MWCO Carrightwohill, Cork, Ireland) and used as a final preparation of the enzyme.

Enzyme assay. The activity of *DraTreS* was determined by measuring the trehalose produced from maltose. The assays were initiated by the addition of 0.1 ml of *DraTreS* solution to 1.0 ml of 0.3 M maltose solution in a 0.1 M phosphate citrate buffer (pH 7.6). The reaction at 30°C was terminated after sample heating at 100°C for 10 min. The samples purified by centrifugation (10 000 × *g*, 10 min) and filtration on a

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                 *        20         *        40         *        60         *
Drad : MTQAHP-EWYKSAVFYELSVRTFQDGNGDGKGDFPGLTSRLDYLKNLGVDCLWLLPWFPSPLRDDGYDVA :  69
Dgob : MTQTSAPEWYKSAVFYELSVRTFADGNGDGKGDFPGLTSHLDYLKNLGVDCLWLLPFFPSPLRDDGYDVA :  70
Ddes : MIQTTPPEWYKSAVFYELSVRTFADGNGDGKGDFPGLTSKLDYLKNLGVDCLWLLPWYPSPLRDDGYDVA :  70
Dgeo : MTQTSTSEWYKSAVFYELSVRTYADGNGDGKGDFPGLTGKLDYLKNLGVDCLWLLPFYPSPLRDDGYDVA :  70
Dmar : MIHP---DWYKRAVFYELSVRTFRDGNGDGKGDFPGLTSKLDYLKSLGVDCLWLLPFYPSPLRDDGYDVA :  67

                80         *       100         *       120         *       140
Drad : DYRGIHPDLGTLDDFKVFLREAHARGLRVIGDLVTNHTSSDHPWFQAARRGPTLPDGSPNEYHDYYVWSD : 139
Dgob : DYTNIHPDLGTLDDFKVFLREAHARGLRVIADLVTNHTSSDHPWFQAARRGPTLPDGSPNEYHDYYVWSE : 140
Ddes : DYVDIHPDLGTLEDFKVFLREAHARGLRVIGDLVTNHTSSDHPWFQAARRGPTLPDGSPNEFYNYYVWSD : 140
Dgeo : DYTDIHPDLGTLDDFKVFLREAHVRGLRVIGDLVTNHTSSDHPWFQAARRGPTLPDGSPNEYFDYYVWSD : 140
Dmar : HYTDVHPDLGTLEDFRVFLREAHARGLRVIGDLVTNHTSSDHPWFQEARKGKTV-DGQPNPYHDYYVWSD : 136

                 *       160         *       180         *       200         *
Drad : EGKEYADTRIIFTDTEVSNWTLDEQAGKYYWHRFFASQPDLNYDNPKVVEELHGAARFWLDLGLDGFRVD : 209
Dgob : TGTEYAGARIIFTDTETSNWTYDDQVGKYFWHRFFSSQPDLNYDNPRVVEELLAAARFWLDIGVDGFRVD : 210
Ddes : TGEEYPGARIIFTDTETSNWTFDEQTGQYYWHRFFSSQPDLNFDNPAVVEELLAAARFWLDLGVDGFRVD : 210
Dgeo : TGTEYADARIIFTDTETSNWTFDPMAGKYYWHRFFSSQPDLNYDNPRVQEELLNVLRFWLDLGLDGFRVD : 210
Dmar : TGTEYAGARIIFTDTETSNWTYDEQAGRYYWHRFFSSQPDLNFDHPPVKEELKNAMRFWLDLGLDGFRVD : 206

               220         *       240         *       260         *       280
Drad : AVPYLIEREGTSCENLPETHEILKGFRAMVDREYPGRLLIAEANQWPEEVVEYFGTEAEPEFHMCFNFPV : 279
Dgob : AVPYLIEREGTNCENLPETHDILKKMRRLVDEDYPGRLLIAEANQWPEEVVEYFGTEQDPEFHMCFNFPV : 280
Ddes : AVPYLIEREGTNCENLPETHAILKKMRQLVDQDYPGRLLIAEANQWPEEVVEYFGSETDPEFHMCFNFPV : 280
Dgeo : AVPYLIEREGTNCENLPETHAILQKLRRVVDEEYPGRLLIAEANQWPEEVVEYFGTETHPEFHMCFNFPV : 280
Dmar : AVPYLIEREGTNCENLPETHGILKELRALVDAEYPGRLLIAEANQWPEDVVEYFGTEEEPEFHMCFNFPV : 276

                 *       300         *       320         *       340         *
Drad : MPRLYMSLKREDTSSIREIMGRLRKIPSFGQWQTFLRNHDELTLEMVTDDERAFMYAAYAPDARMKINVG : 349
Dgob : MPRLYMSLKREDTTSIREIMDRLRAIPSFGQWATFLRNHDELTLEMVSDEERGFMYAAYSPDERMRINVG : 350
Ddes : MPRLYMSLKREDTSSIRDIMDRLRAIPSFGQWATFLRNHDELTLEMVTDDERAFMYASYAPDERMRINVG : 350
Dgeo : MPRLFMSLKREDTTSIREIMARLRKLPSFGQWATFLRNHDELTLEMVTEDERAFMYAAYAPDARMKINVG : 350
Dmar : MPRLYMSLKREDTSSIREIMGRLRQIPSFGQWATFLRNHDELTLEMVTEDERAFMYAAYAPDERMKINVG : 346

               360         *       380         *       400         *       420
Drad : IRRRIAPLLDNDRRRIELLNTVLLALPGSPILYYGDEIGMGDDLGLPDRNGVRTPMQWNAGTSGGFSTAQ : 419
Dgob : IRRRIAPLLDNDRRRVELLNTVLLALPGSPILYYGDEIGMGDDLGLADRNGVRTPMQWNAGTSGGFSTAA : 420
Ddes : IRRRIAPLLDNDRRRIELLNSVLLALPGSPILYYGDEIGMGDDLSLADRNGVRTPMQWNSSLSGGFSSAS : 420
Dgeo : IRRRIAPLLDNDRRRIELLTTVLLALPGSPILYYGDEIGMGDNLSLADRNGVRTPMQWNAGISGGFSTAL : 420
Dmar : IRRRIAPLLDNDRRRLELLNAVLLSLPGSPILYYGDELGMGDDLGLADRNGVRTPMQWNAGPNGGFSTAD : 416

                 *       440         *       460         *       480         *
Drad : PSDCFFPPIQDPVYGFGRVNVQSQLQDPSSLLKWTARQLELRRAHFAFAHGDLTFIETGNPAIIAFTRQY : 489
Dgob : PTDCFFPPIQDPVYGFARVNVQSQEQDPSSLLKWTARQLELRRRHFAFAHGELKFVDTSNPAVIAFTRTT : 490
Ddes : PEHCFYPPIEDPVYGFHRVNVQSQEQDPSSLLKWTARQLEMRRQHFAFAYGELNFVDSDNPAIIAFTRTT : 490
Dgeo : PEQCFYPPISDPVYGYQRVNVNSQEQDPSSLLKWVSRQLEVRRAHFAFAHGDLTFIETNNPAVIAFTRRY : 490
Dmar : PQDLFFPIIQDPVYGFQRVNVESADHDEGSLLTFTRRLLDLRRQNFAFATGTLTFIDTDNPAVIAYLREE : 486

               500         *       520         *       540         *
Drad : DGETLLIVSNFAGNAQAGLDLDAPFVG-RAPVTLSGASPLPVVTGNGQYPVVMGKYDYYWLRLN--- : 552
Dgob : PDETLLIVSNFASNAQSVTLDLSAYTH-RTPVTLSGASPFPAITDA-PYALTMGKYDYYWLRLN--- : 552
Ddes : PDETLLIVSNFAANAQACTLDLSAHHR-RTPVTLSGGSHLPPVQDE-RYPLVMGKYDYYWLRLN--- : 552
Dgeo : DNEVLLIVSNFAGNAQAVELDLFAYRG-CVPVTIAGGSHFPPVGDRGLYPLTLGKYDYYWLKLSGVR : 556
Dmar : DGQHLLVVANFAANAQAAHLHLGALCCGRTPVTLTGGASFPPITDA-PYPIAIGKHDWYWLKLN--- : 549
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Figure 1. Multiple amino acid sequence alignment of TreS proteins.
Description of similarity: white fonts on black boxes 100% identity; white fonts on grey boxes similarity < 80%; black fonts on grey boxes similarity < 60%. Dra, *D. radiodurans*; Dgob, *D. gobiensis*; Ddes, *D. deserti*; Dgeo, *D. geothermalis;* Dmar, *D. maricopensis.*

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 1.6 ml/min. The column temperature was 30°C (modified method from Drożdżyńska *et al.,* 2009). 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.

DraTreS 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 in the range 0–55°C. The pH stability of *DraTreS* was determined by measuring the residual activities after 2 h incubation (30°C) in 50 mM Britton-Robinson buffers at various pH values. The thermostability of trehalose synthase was investigated by incubation of enzyme solution in 0.1 M phosphate citrate buffer (pH 7.6) at 10–60°C for 2 hours. Protein concentrations were determined by the Lowry method (Lowry *et al.,* 1952). The effects of metal ions and chemicals were determined according to standard procedure with 0.3 M maltose solution in water containing a final concentration of cations or chemicals given in Table 2.

Protein sequence analysis. The amino acid sequence of *DraTreS* was analyzed using standard protein–protein BLAST and RPS-BLAST. Multiple sequence alignment was generated by using the program ClustalX. The results were prepared using the editor program Gendoc (copyright Karl Nicholas).

Molecular mass determination of recombinant *DraTreS*. The purified *DraTreS* was applied on a Superdex 200 HR 10/30 column (Amersham Bioscence AB, Uppsala, Sweden) equilibrated with 150 mM NaCl and 10 mM EDTA in a phosphate buffer (pH 7.5) and then was eluted with the same buffer. Absorbance at 280 nm was measured to monitor the elution profile. The elution patterns of recombinant *DraTreS* proteins were then compared with those of standard proteins: carbonic anhydrase (29 kDa), ovoalbumin (43 kDa), monomer of bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), β-amylase (200 kDa), apoferritin (443 kDa) and thyroglobulin (669 kDa).

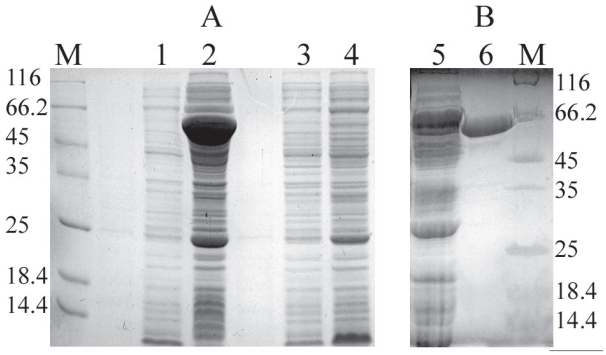

Figure 2. SDS/PAGE of protein in the fraction during purification steps of recombinant *DraTreS*.
Lanes: M — protein marker (Fermentas SM0431, size of proteins at kDa), lane A1 — recombinant *E. coli* strain with vector before induction, lane A2 — cell extract after 18 h of protein expression induced by IPTG, lane A3 — control *E. coli* strain with vector pET only before induction, lane A4 — control *E. coli* strain with vector pET only after 18 h of IPTG induction, lane B5 — heat treatment at 56°C for 10 min, lane B6 — purified *DraTreS* after Co^{2+} — affinity chromatography.

The molecular mass of the enzyme subunit was determined by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS/PAGE) using 12% (w/v) polyacrylamide gel in a Tris-glycine buffer (pH 8.3) (Laemmli 1970). The samples (20 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 staining with Coomassie Brillant Blue R250. The molecular mass was determined using a molecular mass marker kit (Fermentas). Purity and molecular mass were determined from SDS/PAGE gels with Quantity One Software (BioRad, CA, USA).

RESULTS AND DISCUSSION

Cloning of *DraTreS*

This study concerns the previously cloned recombinant trehalose synthase from *D. radiodurans*. The cloning experiments were performed using the sequence annotated as a putative trehalose synthase in GenBank (locus DR-2036, accession no. AE000513 GI: 6459827). The CAZy database shows that trehalose synthase share common structural features with the members of the glucoside hydrolase family (GH13) (Cantarel *et al.,* 2009).

Our experiments confirm that the wild-type region of the *D. radiodurans* gene amplified by PCR had a sequence consisted of 1659 nucleotides encoding 552 amino acid residues (Fig. 1) and that with oligohistidine tags has two additional amino acids: Leu and Glu and consisted of 1683 nucleotides and 560 amino acid residues. These two extra residues are connected with construction of the gene encoding *DraTreS* in vector pET30Ek/LIC. Theoretical values of molecular mass (M_r) and isoelectric point of the native enzyme were calculated to be 62.71 kDa and pI 4.94, respectively (data for recombinant protein containing the His_6 tag at the C-terminus preceded by two residues were 64.69 kDa and pI 5.13). The M_r value deduced from the amino acid sequence of the enzyme was confirmed by SDS/PAGE (Fig. 2). To determine the oligomeric state of this protein, it was analyzed by gel filtration chromatography, which showed that native *DraTreS* has the molecular mass of 126.9 kDa (Fig. 3). Data obtained from this experiment suggest that *DraTreS* exists as homodimer.

Efficient overexpression of the gene was observed in *E. coli* Rosetta (DE3) pLysS (which can recognize rare codons) at 25°C. After analysis of Rare Codon Usage with software from www.kazusa.org.jp we can say that in the *DraTreS* gene there are 22 rare codons recognized by the *E. coli* Rosetta strain: 1 × Gly, 1 × Arg, 20 × Pro. Problems with efficient overexpression of genes containing rare codons were also noticed for example by Yue *et al.,* (2009).

Characterization of *DraTreS*

The comparison of molecular mass (M_r) and some other properties of *DraTreS* and that for trehalose synthases from other bacteria is shown in Table 1. Similar M_r values were reported for subunits of trehalose synthases from *Pseudomonas* sp. F1 (Ohguchi M *et al.,* 1997), *D.* radiodurans (Wang *et al.,* 2007), and *Pimelobacter* sp. R48 (Nishimoto *et al.,* 1996a). In comparison with enzymes from these sources, a higher M_r value was detect-

Table 1. Properties of different trehalose synthases.

Trehalose synthase from:	Molecular mass (kDa)	Optimal temperature (°C)	Optima pH	Thermostability	pH stability	References
Deinococcus radiodurans	64*(His6)	30	7.6	Below 40°C (2 h)	5.5–9.5 (2 h)	This study
Deinococcus radiodurans	61	15	6.5	Below 40°C (0.5 h	5.5–8.0 (2 h)	Wang *et al.,* 2007
Pimelobacter sp. R48	62	20	7.5	Below 30°C (2 h)	6.0–9.0 (1 h)	Nishimoto *et al.,* 1996
Pseudomonas sp. F1	67	45	7.0	Below 55°C (2 h)	7.0–9.0 (1 h)	Ohguchi *et al.,* 1997
Thermus aquaticus	105	65	6.5	Below 80°C (2 h)	5.5–9.5 (1 h)	Nishimoto *et al.,* 1996
Thermus thermophilus	106	65	6.5	Below 80°C (2 h)	6.0–9.0 (2 h)	Wang *et al.,* 2007

ed for *T. aquaticus* and *T. thermophilus* (Nishimoto *et al.,* 1996b; Zdzieblo & Synowiecki 2006).

The analysis of the primary structures revealed the presence of several regions common to previously reported proteins of the Deinococcus/Thermus phylum (Wang *et al.* 2007). The comparison of *DraTreS* sequences and those for trehalose synthases from *D. gopiensis, D. desertii, D. geothermalis, D. maricopensis* shows that even within the Deinococcus phylum there are significant differences. The sequence of *DraTreS* shares 83 to 84% identity and 89 to 90% similarity with *D. gopiensis, D. desertii, D. geothermalis* and 78% identity and 89% similarity to *D. maricopensis.* More similar fragments are located on the N-terminus of the protein molecules (Fig. 1). Further comparison using Blast tools (NCBI) indicated that *Corallococcus coralloides, Thermobaculum terrenum, Streptomyces hygroscopicus subsp. Jinggangensis* TreS sequences have 56%, 55%, 46% identity and 71%, 70%, 69% similarity, respectively to *DraTreS* (data not shown on Fig 1.).

Activity and kinetics of maltose conversion

Single step purification of the enzyme by metal affinity chromatography allowed 17% of protein recovery. The obtained results showed that the enzyme could convert maltose to trehalose accompanied by a low level of glucose as a by-product. The small amount of glucose usually released in most of the reactions catalyzed by trehalose synthases is generated under entry of water molecules into the catalytic site prior to isomerization of the glycosidic linkage (Koh *at al.,* 2003). This weak hydrolytic action of trehalose synthases depends on the enzyme origin and increases with temperature of reaction (Wei *et al.* 2004).

The temperature optimum for maltose conversion was found to be 30°C (Fig. 4) and this value was higher than that for the trehalose synthase of *D. radiodurans* reported previously by Wang *et al.,* (2007).

Furthermore in the case of other trehalose synthases produced by mesophilic bacteria, the optimal temperature is higher by about few degrees than the growth temperature of the bacteria and never lower. Optimal temperature for trehalose synthase from *P. putida*, *Corynebacterium glutamicum* and *Enterobacter hormaechei* is 35°C, 35°C and 37°C, respectively (Ma *et al.,* 2006; Tae-Kyun *et al.,* 2010; Yue *et al.,* 2009). However, growth of *D. radiodurans* was not observed at temperature 10°C or at 45°C (Lewis 1971).

The thermostability of *DraTreS* reported in this article was not as high as that of the enzyme from the genus Thermus (Table 1). The half-life of *DraTreS* (examined in this work) activity at 40°C (pH 7.6) was 28.5 h, while previously described trehalose synthase from *D. radiodurans* showed similar activity after incubating at the same temperature for 30 min (Wang *et al.,* 2007). When the thermostability of *DraTreS* was examined at 50°C and 55°C the enzyme retained 50% of its maximal activity after 9.5 h and 0.5 h of incubation, respectively.

The optimal pH for maltose conversion was 7.6 (in 0.1 M phosphate citrate buffer) as shown in Fig. 5. This pH value is similar to that reported for trehalose synthases

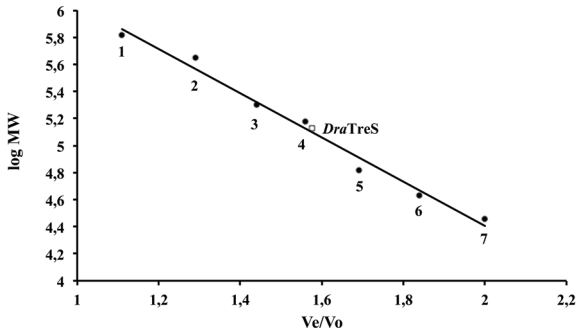

Figure 3. *DraTreS* molecular mass determination by gel filtration chromatography on Superdex 200 HR 10/30.
The molecular mass standards (●) are represented by a number from 1 to 7, respectively: thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), ovoalbumin (43 kDa) and carbonic anhydrase (29 kDa). Retention rate V_e/V_0, where: V_e elution volume of each protein, V_0 void volume of the column determined with blue dextran.

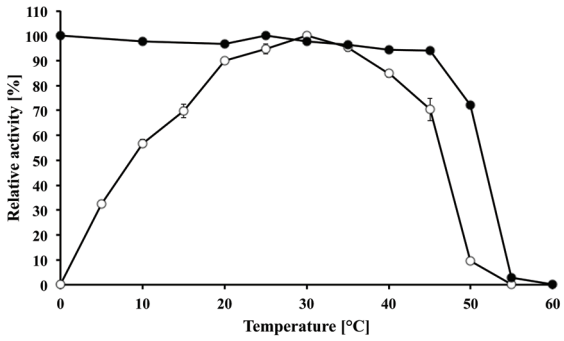

Figure 4. Optimal temperature and thermostability of *DraTreS*.
Activity (○) and stability (●) at pH 7.6. The results are mean values of three replicates.

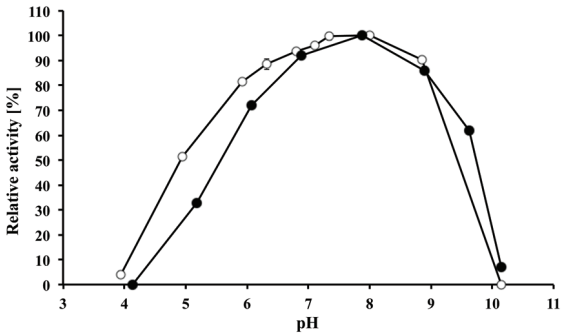

Figure 5. Optimal pH and pH-stability of *DraTreS*.
Activity (○) and stability (●).The pH stability was determined by measuring residual activity after 2 h incubation at 30°C in the same buffer as those used for the optimal pH determination.

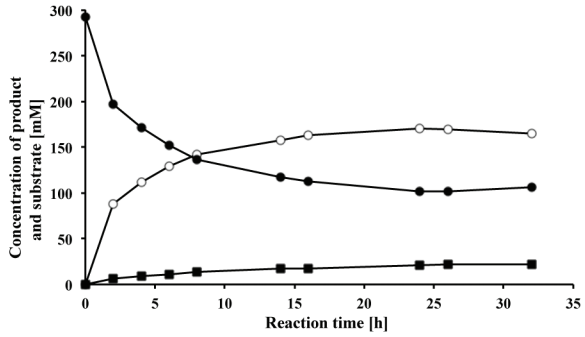

Figure 6. Effect of reaction time at 30°C on the trehalose (○) and glucose (■) formation in 0.3 M solution of maltose (●) in phosphate citrate buffer (pH 7.6).

from *Pimelobacter* sp. R48 (Nishimoto *et al.,* 1996a) and *T. aquaticus* (Nishimoto *et al.,* 1996b). The retention of about 50% of maximal activity of *DraTreS* was observed at a wide pH range of 5.5 to 9.5 (Fig. 5). This suggests that *DraTreS* might be useful for industrial trehalose production. Even if we used 10% maltose solution as a substrate no more than 7.5% of it was converted to glucose as a by-product after 32 h of the reaction (Fig. 6).

The obtained enzyme had the specific activity of 11.35 U/mg. The K_m values calculated using Lineweaver-Burk plot (Lineweaver & Burk 1934) for recombinant *DraTreS* were found to be 290.7 mM for maltose and 486.14 mM for trehalose. With regard to this result the recombinant *DraTreS* had a lower affinity to maltose than enzymes from *T. aquaticus* (K_m 34.5 mM) (Nishimoto *et al.,* 1997) and *E. hormaechei* (K_m 25 mM) (Yue *at al.,* 2009). Furthermore, the K_m value of trehalose-synthesizing enzyme from *Pseudomonas* sp. F1 was 1.1 mM (Ohguchi *et al.,* 1997). The turnover number (k_{cat}) values of recombinant *DraTreS* for maltose and trehalose were 23.6 s^{-1} and 17.5 s^{-1}, respectively. To compare trehalose synthase from *Meiothermus ruber* is characterized by k_{cat} equal 147.0 s^{-1} for maltose conversion and k_{cat} 68.9 s^{-1} for trehalose conversion (Zhu *et al.*, 2010). Moreover *DraTreS* had over twofold higher enzyme efficiency (k_{cat}/K_m) toward maltose (0.081 $mM^{-1} \times s^{-1}$) than trehalose (0.036 $mM^{-1} \times s^{-1}$) indicating maltose as the preferred substrate. A similar situation was noticed for trehalose synthase from *M. ruber* and *Picrophilus torridus* (Zhu *et al.*, 2010, Chen *et al.*, 2006).

The effect of reaction time on the yield of trehalose by *DraTreS* was determined at 30°C using a 0.3 M solution of maltose in 0.1 M phosphate citrate buffer (pH 7.6). The reaction was almost terminated after 24 h and 65.27% of the substrate was converted into 58.16% of trehalose and 7.11% of glucose (Fig. 6). It indicates that the equilibrium between conversion of maltose into trehalose and reverse reaction was achieved. Maximum conversion of maltose into trehalose depends on enzyme sources. A similar value of conversion was for example reported by Chen *et al.* (2006) for the enzyme from *P. torridus*.

Table 2. Effect of some cations and chemicals on activity of trehalose synthase from *D. radiodurans* expressed in *E. coli*.

Cation/Chemical	Relative activity (%) at reagent concentration of		
	1.0 mM	5.0 mM	10.0 mM
none	100.0	100.0	100.0
Hg^{2+}	0.0	0.0	0.0
Ca^{2+}	90.5 ± 0.6	88.8 ± 0.1	86.6 ± 0.6
Co^{2+}	82.8 ± 0.4	79.5 ± 0.4	74.3 ± 0.1
Cu^{2+}	1.5 ± 1.2	1.5 ± 0.4	0.0
Mg^{2+}	91.4 ± 0.2	91.9 ± 0.4	91.5 ± 0.3
Mn^{2+}	80.7 ± 0.7	75.7 ± 0.1	70.7 ± 0.1
Ni^{2+}	71.5 ± 0.9	64.8 ± 0.6	55.3 ± 0.2
Zn^{2+}	1.1 ± 0.1	0.0	0.0
Al^{3+}	1.5 ± 0.3	1.5 ± 0.2	1.4 ± 0.4
Dithiotreitol	nd	91.1 ± 0.6	88.3 ± 0.2
EDTA	85.1 ± 0.2	76.1 ± 0.1	64.5 ± 0.7
SDS	nd	4.9 ± 0.6	3.2 ± 0.7
Tris	nd	42.2 ± 0.7	21.7 ± 0.9

Results are mean values of three determinations ± standard deviation. nd, not determined

Effect of some cations and chemicals on *DraTreS* activity

Data reported in Table 2 indicated that the enzyme was highly sensitive to Hg^{2+}, Al^{3+}, Zn^{2+} and Cu^{2+} while Ca^{2+} and Mg^{2+} did not significantly affect the catalytic activity of the enzyme at concentrations of 1 mM. However, at a concentration of 5 mM almost all examined cations decreased *DraTreS* activity. EDTA causes *DraTreS* inactivation when the concentration of this chelator was increased (Table 2). It is known that cations like Ca^{2+} or Zn^{2+} are required for enzyme activity analogical to other members of GH13 hydrolases, which have binding sites with different affinity to metal ions. Ca^{2+} stabilizes the linkage of the $(\alpha/\beta)_8$-barrel. Removal of this ion by EDTA causes enzyme inactivation. Attachment of bivalent cations to other metal binding sites is responsible for unfolding of the protein structure and enzyme inactivation at increased concentration of the cations (Linden *et al.,* 2003; Janecek 1997).

The results presented in Table 2 indicating strong inhibition of *DraTreS* by 10 mM Tris are rather surprising but that was also reported for trehalose synthases originating from *Pimelobacter* sp.48, *Arthrobacter aurescens*, *T. thermophilus* and *M. smegmatis* (Nishimoto *et al.,* 1996a; Xiuli *et al.,* 200; Wang *et al.,* 2007; Pan *et al.,* 2004, Wei *et al.,* 2000). It seems that this is connected with the ionic strength and the presence of Cl^-.

No effect on activity in the case of dithiothreitol, which acts as a suppressor of thiol group oxidation, suggests that reduction of disulfide bridges does not influence the active site and catalytic properties of *DraTreS*.

CONCLUSIONS

It is the purpose of the present study to obtain further evidence for the trehalose synthases. This work indicates the potential for the production of *D. radiodurans* strain trehalose synthase by an *E. coli* expression system. In our study we characterized the properties of the obtained recombinant enzyme and compared them with that for other trehalose synthases from other bacterial species. *DraTreS* reported here is fully active at moderate temperature (30°C) and their acid resistance is similar to that reported to many other trehalose synthases This report on the trehalose synthase from *D. radiodurans* allowed broadening our knowledge about the biodiversity of microbial sources of this enzyme.

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