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Production of trehalose by intramolecular transglucosylation of maltose catalysed by a new enzyme from *Thermus thermophilus* HB-8

Anna Zdziebło, Józef Synowiecki *

Department of Food Chemistry, Technology and Biotechnology, Faculty of Chemistry, Gdansk University of Technology, ul. Gabriela Narutowicza 11/12, 80-952 Gdańsk, Poland

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Abstract

Thermus thermophilus HB-8 is a source of trehalose synthase (GTase), which catalyses conversion of maltose into trehalose. Specific activity of maltose transglucosylation by cell-free extracts of the bacteria was about 0.1 U mg^{-1} protein and precipitation at 28% saturation of ammonium sulphate caused 3.5-fold enzyme purification. The optimum temperature for conversion of maltose into trehalose was 65 °C with about 27% of maximum activity at 85 °C. The highest GTase productivity was achieved at cultivation temperature over 60 °C and at NaCl concentration range of 0.1–0.5% (w/v). However, larger concentrations of sodium chloride in the growth media caused a remarkable decrease of GTase synthesis. The results, of ammonium sulphate fractionation and activity towards maltotriose (0.028 U mg^{-1}), maltotetraose (0.16 U mg^{-1}) and GlcαpNp (0.27 U mg^{-1}), show that trehalose synthase and α-glucosidase activities reside in separate protein fractions of cell-free extracts from *T. thermophilus* cells.
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Keywords: Trehalose synthase; α-Glucosidase; *Thermus thermophilus*; Intramolecular transglucosylation

1. Introduction

Trehalose (α-D-glucopyranosyl, α-D-glucopyranoside) is non-reducing disaccharide containing 1,1 glycosidic linkages between glucose molecules. This carbohydrate is widespread in yeast, thermophilic microorganisms, some plants and insects. Trehalose is stable under hot and acidic conditions and has affinity for lipids in biological membranes and proteins. It forms hydrogen bonds involved in protection of biological structures during freezing, desiccation or heating (Roser, 1991; Richards et al., 2002). In addition, amorphous glasses of trehalose hold trapped biological molecules without essential changes of their native structure and in consequence limit damage to biological materials during desiccation (Crove & Crove, 2000). Furthermore, this glass is permeable to water and not permeable to hydrophobic, aromatic esters. It minimizes undesired loss of hydrophobic flavour compounds and therefore allows production of dried food with aroma similar to the fresh product. Trehalose can be used in the food, cosmetics, medical and biotechnological industries, as well as for stabilization of vaccines, enzymes, antibodies, hormones, pharmaceutical preparations and organs for transplantation. Account for trehalose application in food processing is its mild sweetness, low cariogenicity, good solubility in water, stability under low pH conditions, reduction of water activity, low hygroscopicity, depression of freezing point, high glass transition temperature and ability to protect proteins. This

* Corresponding author. Tel.: +48 58 347 13 95; fax: +48 58 347 26 94.
E-mail address: synowiec@chem.pg.gda.pl (J. Synowiecki).

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multi-functional food ingredient, that does not caramelise and does not undergo Maillard reactions, is safe for human consumption and has been accepted by the European regulation system (Richards et al., 2002; Schiraldi, Di Lernia, & De Rosa, 2002). Trehalose may be used in many products including beverages, chocolate and sugar confectionery, bakery, dairy and fruit products and as a cryoprotectant for surimi and other frozen foods.

The methods of trehalose extraction from yeast or other natural sources are expensive and the cost of the obtained product is not acceptable for the food industry. Recently, many trehalose synthesizing enzyme systems have been discovered in microorganisms (Di Lernia et al., 1998; Lama et al., 1990; Nakada et al., 1996). The maltooligosyl trehalose synthase and maltooligosyl trehalose trehalohydrolase derived from *Sulfolobus shibatae*, *Arthrobacter* sp. Q36 and several other bacteria are involved in production of trehalose from starch and dextrins (Di Lernia et al., 1998). Furthermore, very useful for industrial application seems to be trehalose synthase catalysing conversion of maltose into trehalose by intramolecular transglucosylation (Koh, Shin, Kim, Lee, & Lee, 1998; Nishimoto et al., 1996a, 1996b; Ohguchi et al., 1997). In this article we describe some properties of a new trehalose synthase from thermophilic bacteria *Thermus thermophilus* HB-8. Considering substrate availability, this enzyme is suitable for industrial production of trehalose from maltose.

2. Materials and methods

2.1. Microorganism and culture conditions

T. thermophilus HB-8 (ATCC 27634) was cultivated in a media composed of 0.4% of yeast extract (Difco Laboratories, USA), 0.8% peptone bio-Trypcase (bio Merieux, France), 0.2% NaCl and water. The effect of sodium chloride in the growth medium was investigated at NaCl concentrations ranging 0 –2.5% (w/v). The cultures were grown in 200 ml Erlenmeyer flasks containing 100 ml of liquid medium (pH 7.5) autoclaved for 30 min at 121 °C. The flasks were inoculated with 1 ml of a *T. thermophilus* cell suspension (OD_{600} 1.2) and stirred in a water bath at 150 rpm at 70 °C without additional aeration. At the end of expotential growth the cells were harvested by centrifugation at 12,000×*g* for 15 min. The pellet was washed with 5 mM Na_2HPO_4 solution, centrifuged, and stored at −18 °C until use.

2.2. Preparation of crude enzyme

Frozen cells (10 g) were disrupted for 15 min in a refrigerated mortar with 20 g of Alumina A-5 (Sigma) and 70 ml of 0.1 M sodium phosphate/citrate buffer (pH 6.2) which was gradually added during extraction. The resulting suspension was centrifuged at 12,000×*g* and proteins from cell-free extract were fractionated by addition of solid $(NH_4)_2SO_4$. The proteins precipitated successively at 28%, 45% or 60% of ammonium sulphate saturation were collected by centrifugation at 12,000×*g* during 15 min and dissolved in 0.1 M phosphate/citrate buffer (pH 6.2). The fraction with α-glucosidase activity was applied to a column (2.1 × 86 cm) of Sephadex G-200, equilibrated with 0.1 M Tris/HCl buffer containing 1 mM dithiotreitol and 1 mM EDTA (pH 7.5) and was eluted at a flow rate of 0.2 ml min^{-1} with the same buffer.

2.3. Enzyme assays

The activity of trehalose synthase was determined by measuring of trehalose produced from maltose. The assays were initiated by addition of 5 ml of crude enzyme preparation to 10 ml of 30 mM maltose solution in 0.1 M phosphate/citrate buffer (pH 6.2). The reaction at 70 °C was terminated after the desired time, by cooling in ice. The temperature dependence of enzyme activity was assayed in the range 35–90 °C. Amounts of the products formed during conversion of maltose were calculated from the area of the peaks obtained after sample separation by HPLC using Polyspher® CHPB column (Merck) and refractive index detector (La Chrom L-7490, Merck). The samples purified by thermal precipitation (110 °C) of enzyme, adsorption on charcoal, centrifugation (8000×*g*) for 15 min and filtration on a 0.2 μm Puradisc™ *serif* filter (Whatman), were passed through a column, using water as the mobile phase at a flow rate of 0.3 ml min^{-1}. The column temperature was 75 °C. Trehalose (Tre), maltose (Mal) and glucose (Glc) were used as standards at concentrations of 10 mg ml^{-1}. Trehalose synthase activity was expressed as the amount of enzyme that produces 1 μmol of trehalose per minute under described conditions.

α-Glucosidase activity was determined by release of *p*-nitrophenol from 5 mM *p*-nitrophenyl-α-D-glucopyranoside (GlcαpNp) solution from Sigma in 0.1 M phosphate/citrate buffer (pH 6.2 adjusted at 70 °C) using the procedure of Legin, Copinet, and Duchiron (1998). The assays were initiated by addition of 0.5 ml of enzyme solution to 2.5 ml of substrate, incubated for 2 min at 70 °C. The reaction at 70 °C was terminated after 2 min by the addition of 1 ml of 1 M Na_2CO_3 solution. A blank containing buffer instead of enzyme solution was used to correct the thermal hydrolysis of GlcαpNp. Absorbances at 405 nm were converted to *p*-nitrophenol concentration using a molar absorption coefficient of 16.86×10^3 M^{-1}. One unit of α-glucosidase activity is defined as the amount of enzyme required to liberate 1 μmol of *p*-nitrophenol from GlcαpNp per minute under the described conditions. Specific activity is α-glucosidase units

per milligrams of protein determined by Lowry, Rosenbrough, Farr, and Randall (1951), using bovine serum albumin as a standard.

The amount of glucose released during carbohydrate hydrolysis was determined according to method of Huggett and Nixon (1955), using the GOPOD reagent containing 125 mg of glucose oxidase, 5 mg peroxidase, 0.5 ml of 1% solution of *o*-dianisidine in 96% ethanol and 0.5 M phosphate buffer (pH 7.0) added to a final volume of 100 ml. The assays were initiated by addition 0.5 ml of α-glucosidase solution to 0.5 ml of 0.25% solution of maltose, maltotriose, maltotetraose, maltopentaose or maltohexaose in 0.1 M phosphate/citrate buffer (pH 6.2). The reaction at 70 °C was stopped by addition of 3 ml of GOPOD reagent and incubation was continued for 30 min at 40 °C. The absorbance was measured at 415 nm and the amount of liberated glucose was calculated from the regression equation of the standard curve, determined at glucose concentrations up to 0.12 μmoles ml^{-1}.

3. Results and discussion

In a previous article we reported that enzyme originated from *T. thermophilus* HB-8 strain catalyses cleavage of the α-1,4-glucosidic linkages in *p*-nitrophenyl-α-D-glucopyranoside (GlcαpNp), maltose and other investigated oligosaccharides (Zdziebło & Synowiecki, 2002). The highest α-glucosidase activity was achieved at pH 6.2 and 85 °C. However, the HPLC identification of the products of maltose hydrolysis catalysed by cell-free extracts of *T. thermophilus* show significant amounts of trehalose in the reaction mixture (Fig. 1). Trehalose synthesising enzymes performed different substrate specificity. Maltooligosyl trehalose synthases, MTSase's (EC 5.4.99.15) from hyperthermophilic archaeon *S. shibatae* and *Arthrobacter* sp. Q36 were active towards maltooligosaccharides except for maltose (Di Lernia et al., 1998). By contrast, trehalose synthases (EC 5.4.99.16) existing in *Pimelobacter* sp. R48, *Pseudomonas putida* H262 and several other bacteria are active only on maltose (Nishimoto et al., 1996a, 1996b; Schiraldi et al., 2002). The activity towards maltose leads to conclusion that the enzyme involved in trehalose production in *T. thermophilus* can be identified as trehalose synthase (GTase). All GTase's catalyse conversion of maltose into trehalose by intramolecular transglucosylation and trehalose into maltose in reverse reaction. During both reactions a small amount of glucose is released (Nishimoto et al., 1996a). From this reason, the first purpose of the presented study was to establish whether the formation of glucose in the presence of cell-free extracts of *T. thermophilus* was caused by separate proteins or GTase and α-glucosidase activities reside in the same enzyme. Ammonium sulphate fractionation indicated

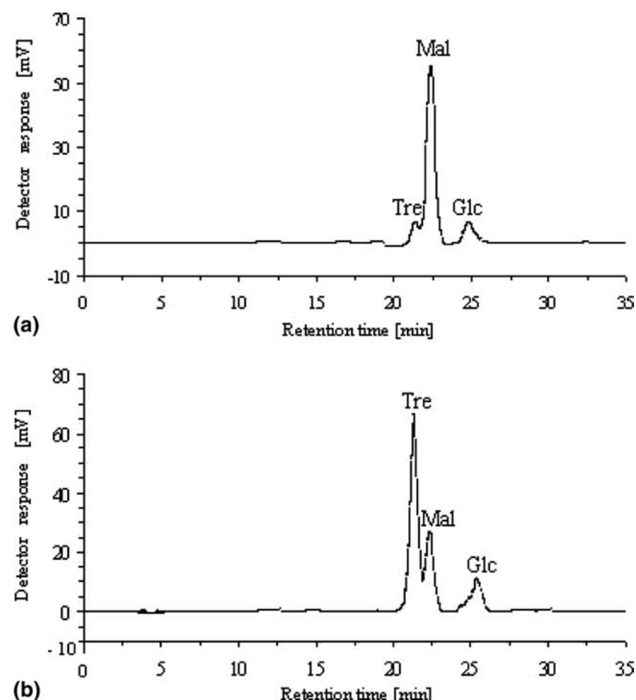

Fig. 1. Spectrum of the products (glucose-Glc, trehalose-Tre) obtained after 10 min (a) and 400 min (b) of conversion 30 mM maltose (Mal) in 0.1 M phosphate/citrate buffer (pH 6.2) at 70 °C. The products were determined according to Section 2 using Polyspher® CHPB column and refractive index detector (LaChrom L-7490, Merck).

that proteins with α-glucosidase and GTase activities were precipitated at different $(NH_4)_2SO_4$ saturation (Table 1). Subsequent gel filtration on Sephadex G-200 of the dissolved proteins precipitated previously at 45% of the ammonium sulphate saturation, show that α-glucosidase was located in protein bands not indicating GTase activity (Fig. 2). Furthermore, the proteins precipitated at 28% of $(NH_4)_2SO_4$ saturation have about 74% of total GTase activity in cell-free extract of *T. thermophilus* and approximately 3.5-fold enzyme purification to a specific activity of 0.37 U mg^{-1} protein was achieved. Existence of α-glucosidase as a separate enzyme was also confirmed by activity of the crude extract from *T. thermophilus* cells towards maltotriose, maltotetraose, maltopentaose and maltohexaose (Table 2), because GTase's isolated from *Pimelobacter* sp. R48 and *Thermus aquaticus* were inactive on these substrates (Nishimoto et al., 1996a, 1996b).

Optimum temperature (65 °C) for conversion of maltose into trehalose by Gtase from *T. thermophilus* is similar to that determined for analogous enzymes from *Thermus aquaticus* (Nishimoto et al., 1996b). By contrast, the highest activity of α-glucosidase from *T. thermophilus* was observed at 85 °C (Zdziebło & Synowiecki, 2002). The remarkable differences between optimal temperatures were also reported for MTSase (70 °C) and α-glucosidase (85 °C) from *S. shibatae*

Table 1
Effect of ammonium sulphate precipitation and gel filtration (Sephadex G-200) on the activity of α-glucosidase and trehalose synthase (GTase) from *Thermus thermophilus* (HB-8)[a]

Sample	α-Glucosidase		GTase	
	U mg^{-1}	Purification	U mg^{-1}	Purification
Cell extract	0.368 ± 0.004	1.00	0.104 ± 0.001	1.00
Precipitation at $(NH_4)_2SO_4$				
Saturation of:				
28%	0.171 ± 0.003		0.366 ± 0.002	3.52
45%	0.542 ± 0.004	1.47	0.064 ± 0.002	
60%	0.174 ± 0.002		0.003 ± 0.001	
Gel filtration of the proteins Precipitated at 45% of $(NH_4)_2SO_4$ saturation	1.773 ± 0.082	4.81	0.0[b]	

[a] The results are the mean values of data from three determinations ± standard deviations.
[b] The GTase activity in the fraction, which after gel filtration show highest α-glucosidase activity.

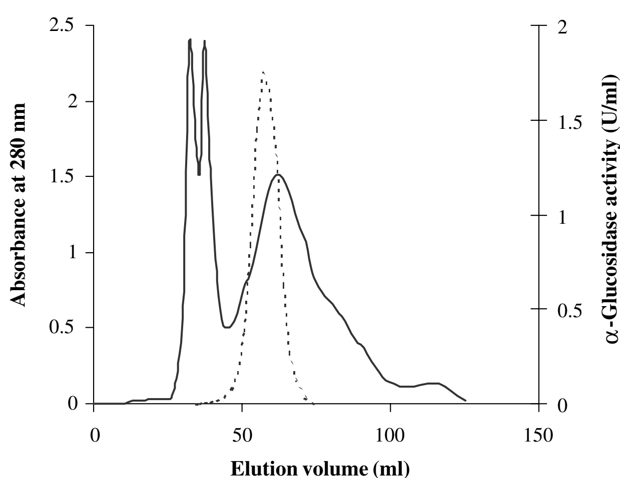

Fig. 2. Gel filtration of a protein fraction with α-glucosidase (···) activity. Chromatography was carried out using a column (2.1 × 86 cm) of Sephadex G-200. The enzyme was eluted with 0.1 M Tris–HCl (pH 7.5) at a flow rate of 0.2 ml min^{-1}. Absorbances at 280 nm (---) represents changes in protein content.

Table 2
Substrate specifity of the protein fraction from *Thermus thermophilus* (HB-8), which show α-glucosidase activity [a]

Carbohydrate	Rate of hydrolysis (10^{-3}U mg^{-1})	Relative rate of hydrolysis (%)
Maltose	28.0 ± 0.95	100.0
Maltotriose	27.8 ± 0.23	99.1
Maltotetraose	15.9 ± 0.13	56.8
Maltopentaose	8.5 ± 0.10	30.5
Maltohexaose	5.7 ± 0.23	20.3

[a] The results are the mean values of data from three determinations ± standard deviation.

(Di Lernia et al., 1998). Investigated GTase has high thermostability and at 85 °C retained about 27% of the maximum activity (Fig. 3).

Incubation of the proteins extracted from *T. thermophilus* cells with Cu^{2+} added to a final concentration of 1 mM did not influence GTase activity. However, analogous enzymes from *Pimelobacter* sp. R48 and *Thermus aquaticus* were strongly inhibited at the same concentration of copper ions and retained only 8% and 7% of initial activity, respectively.

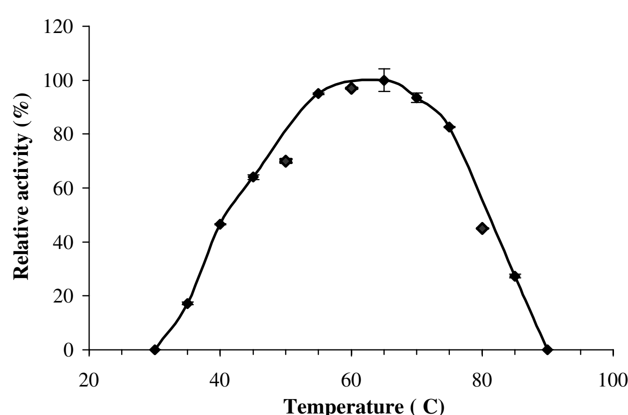

Fig. 3. Effect of temperature at pH 6.2 on activity of trehalose synthase from *Thermus thermophilus* (HB-8) on maltose. The results are mean values of three replicates.

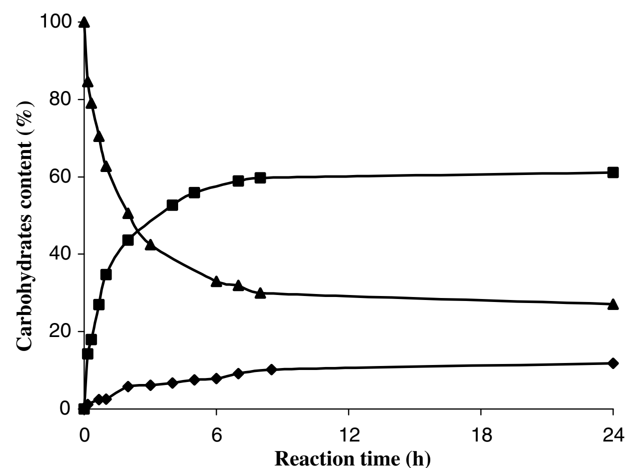

Fig. 4. Effect of reaction time at 70 °C on the trehalose (■) and glucose (◆) formation in 1% solution of maltose (▲) in phosphate/citrate buffer (pH 6.2).

Table 3
Effect of NaCl concentration in the growth media of *Thermus thermophilus* (HB-8) on the cells yield and α-glucosidase and GTase activity

NaCl concentration (%)	Wet cells (g l^{-1} of medium)	α-Glucosidase activity (U g^{-1} of wet cells)	α-Glucosidase yield (U l^{-1} of medium)	Specific activity (U mg^{-1}protein)	Relative activity of GTase (% activity of control sample)
0.0	10.3 ± 1.7	9.52 ± 1.69	98.5	0.21 ± 0.1	100.0
0.1	9.1 ± 0.5	11.58 ± 0.15	105.1	0.28 ± 0.1	104.5
0.5	8.1 ± 1.4	13.30 ± 1.33	108.0	0.30 ± 0.0	112.5
1.0	6.8 ± 1.3	18.61 ± 1.60	127.3	0.37 ± 0.1	74.5
1.5	5.9 ± 0.9	21.41 ± 0.28	127.8	0.54 ± 0.0	68.0
2.0	4.4 ± 0.1	23.20 ± 1.08	102.3	0.60 ± 0.1	62.5
2.5	2.8 ± 0.0	28.19 ± 0.38	78.9	0.68 ± 0.0	65.0

The results are mean values of three determinations ± standard deviation.

Fig. 4 shows the course of trehalose and glucose formation by trehalose synthase determined at various reaction times using a 1% solution of maltose in phosphate/citrate buffer (pH 6.2) as substrate. The conversion of maltose was almost terminated after 8 h and the reaction mixture contained trehalose (59.8%), glucose (10.2%) and a residue of maltose (30.0%). Further increase of reaction time up to 48 h enhanced the trehalose and glucose contents to 61.6% and 12.2%, respectively. The fact that even at prolonged reaction time 26.2% of substrate remains unconverted suggests the existence of equilibrium between maltose transglucosylation and reverse reaction producing maltose from trehalose. The study on the enzyme kinetics of trehalose synthase is in progress and will appear elsewhere.

Sodium chloride influenced the weight of the cells obtained from culture broth, as well as the productivity of α-glucosidase and GTase activities (Table 3). The maximum GTase specific activity measured at the end of the expotential growth phase of *Thermus thermohilus* was obtained at NaCl concentrations ranging from 0.1% to 0.5% w/v. The cell-free extract from the bacteria growing in a standard medium containing 0.2% of sodium chloride had a specific activity of 0.10 ± 0.01 U mg^{-1} protein, similar to that reported for *Pimelobacter* sp. R48 (0.13 mg^{-1} protein) by Nishimoto et al. (1996a). At NaCl concentrations higher than 0.5%, a large decrease of GTase productivity was observed. However, the α-glucosidase activity was enhanced over the whole examined range (0–2.5% w/v) of NaCl concentrations (Table 3). It can be explained by progress of energy consumption for maintenance of a constant level of Na^+ and Cl^- ions inside cells when the NaCl concentration in the cultivation broth was increased. It stimulated the synthesis of α-glucosidase involved in cell metabolism. The study on this area is now in progress and will be reported elsewhere.

The trehalose forming-activity of the cell-free extract from *T. thermophilus* depended also on the cultivation temperature. It was confirmed by observed changes in participation of trehalose in products of maltose conversion catalysed by enzymes isolated from the bacteria cultivated at different temperatures. The amount of GTase in the cells growing at 60 °C was enhanced by about 52% of the value obtained during microorganism cultivation at 55 °C. Higher growth temperatures (up to 80 °C) had almost no influence on the enzyme productivity. However, the large decrease of biomass yield (from 9.2 to 2.2 g of the wet cells) at growth temperatures ranged from 75 to 80 °C reduced the total GTase activity from a maximum level of 53.6 U l^{-1} of the growth medium to about 12.7 U l^{-1}, respectively.

The information presented above indicate that *T. thermophilus* (HB-8) is useful as a source of enzyme for industrial production of trehalose from maltose in a simple one-step process. Observed high thermal stability of GTase from these bacteria allow maltose conversion at temperatures that minimizes undesired microbial contamination of the reaction mixture. Considering substrate availability and process simplicity the GTases are more suitable for trehalose production than maltooligosyl trehalose synthase (MTSase) and maltooligosyl trehalose trehalohydrolase (MTHase) systems derived from *S. shibatae* or soil microbe designated as *Arthrobacter ramosus* (Di Lernia et al., 1998; Richards et al., 2002). The process based on MTSase and MTHase is more complicated because, starch used as a substrate should be previously gelatinized and liquefied by α-amylase at increased temperature and then cooled for further processing.

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