Biochemistry (Moscow), Vol. 68, No. 9, 2003, pp. 1012-1019. Translated from Biokhimiya, Vol. 68, No. 9, 2003, pp. 1238-1246.

Expression, Isolation, Purification, and Biochemical Properties of Trehalose-6-phosphate Hydrolase from Thermoresistant Strain *Bacillus* sp. GP16

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Received July 24, 2002
Revision received October 29, 2002

Abstract—Here we describe cloning, expression, and purification of the enzyme trehalose-6-phosphate hydrolase from thermoresistant strain *Bacillus* sp. GP16. Principal biochemical properties of the enzyme at different pH and temperature values were determined. Entropy and enthalpy of activation of the enzyme for substrates trehalose-6-phosphate and *p*-nitrophenyl glucoside were calculated, and the dependence of the kinetic parameters from ionic strength was established.

Key words: trehalose-6-phosphate hydrolase, cloning of a gene, gene expression, catalysis, temperature dependence, determination of Michaelis constants

Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside), a non-reducing disaccharide, is present in various organisms including bacteria, fungi, plants, and mammals [1]. Trehalose is found in spores, in resting cells, and in cells subjected to different forms of stress. This universality is related to the unique ability of this sugar to maintain the native state of membranes in conditions of high ionic strength, dehydration, elevated temperature [1-5]. Many organisms can also use trehalose as an exogenous source of carbon.

Certain gram-positive and gram-negative bacteria including *Escherichia coli* can grow using trehalose as the only source of carbon at both low and high osmolarity, when the sugar is synthesized and accumulated inside a cell. It seems to be paradoxical: trehalose is cleaved as a source of carbon and simultaneously it is synthesized as an osmoprotector. For realization of these two pathways bacteria have developed two different systems of trehalose metabolism, one for high and the other one for low osmolarity. The scheme of trehalose metabolism has been studied in detail for *E. coli* [6]. Trehalose diffuses to the periplasm through LamB pores. Under conditions of low osmolarity, trehalose is transported through periplasm membrane and simultaneously phosphorylated by $EIICB^{Tre}$ (TreB) enzyme of the phosphotransferase system (PTS) using EIIA of glucose PTS ($EIIA^{Glc}$) as phosphoryl donor. In cytoplasm, trehalose-6-phosphate is hydrolyzed to glucose and glucose-6-phosphate by the enzyme trehalose-6-phosphate hydrolase (TreC). Free glucose is phosphorylated by glucokinase, and glucose-6-phosphate is subjected to glycolysis. At high osmolarity both TreB and TreC are repressed. Trehalose is hydrolyzed in periplasm to two glucose molecules by periplasmic trehalase (TreA). The latter is activated in turn at high osmolarity. Glucose is transported to the cytoplasm through the phosphotransferase system of glucose. On the other hand, trehalose is synthesized at high osmolarity by the enzymes trehalose-6-phosphate synthase (OtsA) and trehalose-6-phosphate phosphatase (OtsB) using glucose-6-phosphate and UDP-glucose as the substrates.

The objective of this study was cloning of the gene encoding the enzyme trehalose-6-phosphate hydrolase from the thermophilic bacterium *Bacillus* sp. GP16, and study of the principal enzymatic properties of this protein. Here we determined the Michaelis–Menten constant as functions of pH, temperature, and ionic strength. It should be noted that this enzyme is poorly studied biochemically, and the kinetic data in the literature are controversial. The expression and purification of the enzyme from a thermophilic source was carried out for the first time in this work.

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MATERIALS AND METHODS

Reagents and equipment. Absorbance and spectral analysis were studied with an Ultrospec 2000 UV spectrophotometer (Pharmacia Biotech, Sweden). The following reagents were used for study of biochemical characteristics: *p*-nitrophenyl-α-D-glucopyranoside (Fluka, Germany); trehalose-6-phosphate, glucose-6-phosphate dehydrogenase, *o*-dianisidine, peroxidase, glucose oxidase (Sigma, USA); glycerophosphate (Baker Chemical Co, USA); citric acid (Russian supplier).

For genetic engineering we used the enzymes from Fermentas (Lithuania) and Promega (USA); all other reagents were from Sigma.

Bacterial strains. Chromosomal DNA was isolated from *Bacillus* sp. GP16. *E. coli* Dh5α was used for isolation and transformation of plasmid DNA. The gene of trehalose-6-phosphate hydrolase cloned in pET21b vector under the control of T7 promoter was expressed in *E. coli* BL21(DE).

Measurement of glucose-6-phosphate. The method is based on the enzymatic oxidation of glucose-6-phosphate to gluconolactone-6-phosphate by the enzyme glucose-6-phosphate dehydrogenase with simultaneous reduction $NADP^+$ to NADPH. The increase in glucose-6-phosphate was registered by measuring absorbance at 340 nm that indicates the quantity of generated NADPH. Eighty microliters of solution of 50 mM Tris, pH 7.8, containing 3.75 mM $MgCl_2$ and glucose-6-phosphate dehydrogenase (0.42 U/ml) and 10 μl of solution of 10 mM imidazole, pH 6.8, with 9 mM $NADP^+$-Na_2 were added to 10 μl of the studied solution, and the mixture was incubated for 5 min at 37°C. The mixture was then transferred to a cuvette and absorbance at 340 nm was measured against the control mixture that contained H_2O instead of the studied solution. The quantity of glucose-6-phosphate (in millimoles) was calculated using the formula $C = (A_{340}/0.622)$.

Measurement of α-glucose. The method is based on the enzymatic oxidation of α-glucose by glucose oxidase. Oxidation of α-glucose leads to the formation of an equimolar quantity of hydrogen peroxide. The latter oxidizes *o*-dianisidine in the presence of peroxidase. One hundred microliters of solution containing 100 mM sodium acetate, pH 5.0, peroxidase (0.1 mg/ml), glucose oxidase (0.1 mg/ml), and *o*-dianisidine (50 μg/ml) were added to 10 μl of the studied solution. The mixture was transferred to a cuvette of the spectrophotometer, and absorbance at 450 nm was measured at 37°C. The mixture was incubated for 30 min.

Measurement of the enzymatic activity. Enzymatic activity of trehalose-6-phosphate hydrolase was measured by two methods depending on the substrate. Hydrolysis of trehalose-6-phosphate was assessed by the increase in glucose-6-phosphate concentration. Activity on *p*-nitrophenyl-α-D-glucoside (pNPG) was measured in 50 mM glycine buffer, pH 9.5, using the formula $C = A_{400}/18.3$, where C is concentration of *p*-nitrophenol in solution in mM, and A_{400} is the absorbance of the solution at 400 nm. To measure the velocity of the reaction at other pH values we monitored the dependence of absorbance of *p*-nitrophenol on pH.

RESULTS

Cloning of the trehalose-6-phosphate hydrolase gene. Cloning and expression of the enzyme in *E. coli* was carried out as follows. The consensus sequence blocks were chosen in the primary amino acid sequences of bacterial α-glucosidases according to the data from the literature [7, 8]. They were used for design of degenerative primers taking into account the table of usage of codons in bacteria of *Bacillus* genus: A1 (5′-GT(GT) AGT (GT)CA (AG)(AG)T (AGCT)TA (CT)) and B3a (5′-CC(GT) (CT)(GT)(GT) T(CT)(AGCT) A(AG)C CA). These primers were used for amplification by PCR of the region of trehalose-6-phosphate hydrolase gene (Table 1).

The 545 nucleotide-long fragment of the gene encoding the N-terminus of the enzyme (approximately 1/3 of the gene) was cloned and sequenced. Chromosomal DNA of the studied strain was used as the template for amplification of this fragment. Comparison of the obtained amino acid sequence of the N-terminus of the enzyme with the sequences of the known α-glucosidases allowed for attribution of this fragment to the gene that codes α-glucosidase with 60-75% homology with three types of α-glucosidases: exo-α-1,4-glucosidase, oligo-1,6-glucosidase, and trehalose-6-phosphate hydrolase.

The obtained sequence was used for design of primers A25 (5′-CAG GTG TAT CCG AAA AGC TTC CGG G) and B21 (5′-GTT TTC CCA ATT CAA ATC GGC) directed inwards the cloned fragment and primer A20 (5′-CAT GAT GCA TTT TTG GCT AA) directed outside the cloned fragment towards the stop codon of the gene.

We constructed the genome library of the chromosomal DNA of *Bacillus* sp. GP16 using restriction endonucleases *Xho*I and *Hind*III and the plasmid pUC19.

Southern hybridization [9] showed that the cloned earlier fragment of the gene is located within a 7000-bp-long region in the *Xho*I library and within 4500-bp-long fragment in the *Hind*III library. The product of single-primer reamplification with A25 primer was used as radioactive probe, and the cloned earlier gene fragment was used as the template.

The *Xho*I library was used as the template in PCR for amplification of the 5′-region of the gene of α-glucosidase including the start codon of the gene. The reaction was carried out with primers B21 and direct sequencing primer Dir that anneals on pUC19 plasmid close to the

Table 1. Conservative blocks in amino acid sequences of α-glucosidases

Enzyme	Region				
	1	2	3	4	5
Family 1					
Saccharomyces cerevisiae AG	106-**DLVINH**	210-**GFRIDTAGL**	276-**EVAH**	344-YIE**NHD**	
Bacillus sp. (SAM1606) AG	113-**DLVANH**	210-**GFRMDVINA**	271-**ETGG**	340-YWT**NHD**	
Candida albicans AG*	98-**DLVINH**	202-**GFRIDTAGM**	263-**EVGH**	333-FIE**NHD**	
Pediococcus pentosaceus AG*	100-**DLVVNH**	197-**GFRMDVINQ**	256-**ETHG**	327-FWN**NHD**	
Thermus caldophilus AG*	96-**DLVPNH**	194-**GFRVDVLWL**	245-**EMRQ**	322-VLG**NHD**	
Streptococcus mutans DG*	98-**DLVVNH**	190-**GFRMDVIDM**	236-**ETWG**	308-FWN**NHD**	
Escherichia coli TPH*	100-**DMVFNH**	196-**GLRLDVVNL**	251-**EMSS**	320-FWC**NHD**	
Bacillus subtilis TPH*	101-**DLVVNH**	198-**GFRLDVINL**	253-**EMSS**	324-FWC**NHD**	
Bacillus halodurans TPH*	98-**DIVVNH**	195-**GFRLDVINL**	254-**EMSS**	324-FWC**NHD**	
Vibrio cholerae TPH*	103-**DIVVNH**	200-**GFRLDVINL**	253-**EMSS**	324-FWC**NHD**	
Pseudomonas fluorescens TPH*	96-**DIVVNH**	188-**GFRLDVINL**	242-**EMSS**	312-FWC**NHD**	
Yersinia pestis TPH*	100-**DMVFNH**	198-**GLRLDVINL**	254-**EMSS**	322-FWC**NHD**	
Salmonella typhimurium TPH*	99-**DMVFNH**	195-**GLRLDVVNL**	250-**EMSS**	319-FWC**NHD**	
***Bacillus* sp. (GP-16)** TPH*	101-**DMVVNH**	197-**GFRLDVINL**	256-**EMSS**	326-FWC**NHD**	
Bacillis thermoglucosidasius OG*	98-**DLVVNH**	195-**GFRMDVINM**	256-**ETPG**	325-YLN**NHD**	
Bacillis coagulans OG*	97-**DLVVNH**	195-**GWRMDVIGS**	255-**EAIG**	327-YFE**NHD**	
Bacillis cereus OG	98-**DLVVNH**	195-**GFRMDVINF**	255-**EMPG**	324-YWN**NHD**	
Bacillus sp. OG*	97-**DLVVNH**	194-**GWRMDVIGS**	254-**EAGG**	326-YFE**NHD**	
Bee AG	119-**DFVPNH**	219-**GFRVDALPY**	286-**EAYT**	343-VPG**NHD**	
Mosquito AG′	114-**DFVPNH**	215-**GFRIDAVPY**	290-**EGYT**	351-VLG**NHD**	
Drosophila AG′ (protein H)	115-**DFVPNH**	217-**GFRIDAVPY**	297-**EAYT**	358-VLG**NHD**	
Drosophila AG′ (protein D)	120-**DFVPNH**	222-**GFRIDAVPH**	298-**EAYS**	359-VVG**NHD**	
Drosophila AG′ (protein L)	116-**DFVPNH**	216-**GFRIDAVPH**	293-**EAYS**	354-VFG**NHD**	
Family 2					
Aspergillus niger AG		220-**GVWYDMSEV**	255-**EPGD**	316-**YVINHD**	421-**GADTCGF**
Aspergillus oryzae AG		488-**GVWYDMAEV**	523-**EPGN**	583-**YVINHV**	687-**GVDTCGF**
Emericella nidulans AG*		496-**GVWYDMSEV**	531-**EPGN**	593-**YVINHV**	697-**GVDTCGF**
Mucor javanicus AG		426-**GLWIDMNEP**			594-**GADICGF**
Candida tsukubaensis AG		522-**GIWLDMNEP**			769-**GADICGF**
Schwanniomyces occidentalis AG		466-**GIWADMNEV**			665-**GADVCGF**
Human AG, lysosomal acid		514-**GMWIDMNEP**			643-**GADVCGF**
Human IM (intestine)		501-**GLWIDMNEV**			631-**GADICGF**
Rabbit IM (intestine)		501-**GLWIDMNEV**			631-**GADICGF**
Rabbit SA (intestine)		1390-**GLWIDMNEP**			1527-**GADICGF**
Porcine IM (intestine)*		487-**GLWIDMNEV**			617-**GADICGF**
Porcine SA (intestine)*		1396-**GLWIDMNEP**			1513-**GADICGF**
Rat IM (intestine)		510-**GLWIDMNEV**			642-**GADSCGF**
Rat SA (intestine)		1395-**GLWIDMNEP**			1539-**GADICGF**
Shrew IM (intestine)*		487-**GLWIDMNEV**			617-**GADICGF**
Shrew SA (intestine)*		1394-**GLWIDMNEP**			1513-**GADICGF**
Sugar beet AG		465-**GIWIDMNEA**			595-**GADICGF**
Spinach AG		461-**GLWIDMNEI**			591-**GADICGF**
Barley AG		433-**GLWIDMNEI**			561-**GADICGF**
Buckwheat AG		451-**GLWIDMNEV**			581-**GADICGF**
α-Glucosidases without conservative blocks					
Sulfolobus solfataricus AG					
Thermotoga maritima AG					

Note: AG) α-glucosidase; AG′) enzyme homologous to α-glucosidase; DG) dextran glucosidase; TPH) trehalose-6-phosphate hydrolase (α,α-phosphotrehalase); OG) oligo-1,6-glucosidase; IM) isomaltase; SA) β-fructofuranosidase (saccharase). Conservative amino acids are indicated with bold letters; amino acids that are directly involved in catalysis are underlined; enzymes indicated with asterisk are added by the authors of this study.

polylinker. The resulting 2500-bp-long PCR product was cloned in pCR21 plasmid and sequenced.

The *Hind*III library was used as the template in single-primer PCR with A20 primer for amplification of the 3′-region of the α-glucosidase gene including the stop codon. The resulting 1700-bp-long PCR product was cloned in plasmid pCR21 and sequenced.

The resulting nucleotide sequences of the 5′- and 3′-regions of the gene were used for design of the primers Start (5′-ATC CAT ATG TCG ACA ACT CCA) and StopXho (5′-TGC TCG AGC ATC ACC GTC TGT A) for amplification of the full-length gene and its cloning in the expressing vector pET21b. Primer Start corresponds to the 5′-region (starting from ATG-codon) of the gene and contains the *Nde*I restriction site; primer StopXho corresponds to the 3′-region (terminating with Stop-codon) of the gene and contains the *Xho*I restriction site.

Sequencing of both strands of two independently obtained plasmids pET21b with insertion of α-glucosidase gene obtained in two independent PCR experiments provided similar results.

Reading frame of the α-glucosidase gene from *Bacillus* sp. GP16 consists of 1689 bp. This length corresponds to a 563-amino acid-long protein with molecular weight 66,262 daltons (Table 2). The theoretically calculated p*I* is approximately 5.67, and the molecular coefficient of absorption ε is 139,090 $M^{-1}\cdot cm^{-1}$ at 280 nm.

Preparative induction in *E. coli* BL21(DE) strain. Twenty milliliters of the overnight culture of BL21(TreC+) cell culture and 2 ml of ampicillin solution (100 mg/ml) were added to 2 liters of LB medium and incubated at 37°C until A_{600} was equal to 1. Then isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the cell culture to the final concentration 0.5 mM and cells were incubated for 2 h. Cells were collected by centrifugation for 15 min at 4000*g*. Thus, 4 g of cells were obtained. Figure 1 (lane *1*) shows the expression of the cloned gene in *E. coli*.

Isolation and purification of trehalose-6-phosphate hydrolase. Five milliliters of buffer A (125 mM imidazole, pH 7.0, 50 mM KCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 10 mM DTT) and 15 ml of H_2O were added to 4 g of BL21(TreC+). The cell suspension was treated with ultrasound 40 times for 10 sec at 0°C. Then the solution was centrifuged for 30 min at 40,000*g*. Twenty milliliters of the cell extract were supplemented with 80 ml of 100% ammonium sulfate and incubated overnight at 4°C. The solution was centrifuged for 30 min at 20,000*g*. The pellet was resuspended in 7 ml of 20 mM Tris, pH 7.5. The protein solution was dialyzed overnight against 1 liter of 20 mM Tris, pH 7.5, at 4°C. After dialysis the solution was incubated for 30 min at 60°C and then centrifuged for 30 min at 20,000*g*.

The supernatant was applied on a chromatographic column (10 × 50 mm) with DEAE-Sepharose Cl-6B equilibrated with 20 mM Tris, pH 7.5. The column was washed with 20 ml of 20 mM Tris, pH 7.5 (10 ml/h). The protein was eluted with 200 ml of a linear NaCl gradient (0-0.5 M, 10 ml/h). Absorption at 260 and 280 nm was measured in fractions (V = 2 ml) together with enzymatic activity on pNPG. Fractions with maximal activity (36-39) were pooled, dialyzed against 20 mM Tris, pH 7.5, and concentrated on a Centricon-30 to concentration 30 mg/ml. Distribution of absorbance at 260 and 280 nm and of the enzymatic activity between fractions is represented in Fig. 2. Protein purity during purification procedures was assessed by SDS-PAGE (Fig. 1, lanes *2* and *3*). The purification steps are characterized in Table 3. Total protein concentration was measured by the method of Lowry. The initial velocity of formation of *p*-nitrophenol (mmol/min) in 1 ml of 20 mM Tris, pH 7.5, at 55°C and final pNPG concentration 5 mM was taken as the unit of enzymatic activity.

Determination of anomerity of generated glucose. Anomerity of glucose was assessed using the glucose oxidase system at pH 5.5 [10]. α- and β-glucoses were used as standards. pNPG and trehalose-6-phosphate substrates were hydrolyzed at 25°C in 0.1 M NaCl, 50 mM citrate-glycerophosphate buffer, pH 5.5. Substrate concentrations were chosen to be of the order $5K_m$. After incubation for 30 sec, 10 μl of the reaction mixture were mixed with 90 μl of glucose oxidase system, and kinetics of β-glucose oxidation was monitored spectrophotomet-

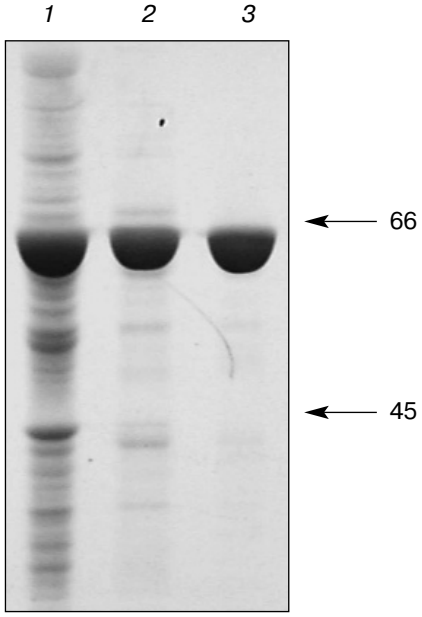

Fig. 1. Expression of the cloned gene of trehalose-6-phosphate hydrolase in *E. coli*: *1*) total protein after destruction of cells by ultrasound; *2*) after thermal treatment (60°C, 30 min); *3*) after chromatography on DEAE-Sepharose Cl-6B. Molecular weights of the marker proteins are indicated on the right: BSA (66 kD) and ovalbumin (45 kD).

Table 2. Nucleotide and amino acid sequences of trehalose-6-phosphate hydrolase from *Bacillus* sp. GP16

```
     M  S  T  T  P  W  W  K  K  A  V  V  Y  Q  I  Y  P  K  S  F
   1 atgtcgacaactccatggtggaaaaaagcagtggtgtatcaaatttatccgaaaagcttc
     R  D  T  N  G  D  G  I  G  D  L  P  G  I  I  E  K  L  D  Y
  61 cgggatacgaacggagatggcatcggcgatttacccggaatcatcgaaaagctcgattat
     L  K  E  L  G  V  D  V  I  W  L  T  P  I  Y  A  S  P  Q  R
 121 ttgaaagaacttggcgttgacgtgatttggctgacgccgatttatgcgtcgccgcagcgc
     D  N  G  Y  D  I  S  D  Y  F  A  I  H  H  A  Y  G  T  M  A
 181 gacaacggctatgacatcagcgattatttcgccatccatcatgcatacgggacgatggct
     D  F  D  R  L  L  D  E  V  H  A  R  G  M  K  L  V  M  D  M
 241 gactttgaccgcctgctcgatgaggtgcatgcgcgcgggatgaagctcgtcatggatatg
     V  V  N  H  T  S  T  D  H  E  W  F  K  Q  A  S  A  S  K  T
 301 gtcgtcaaccacacgtcaaccgaccatgaatggttcaaacaggcgagcgcatcgaaaacg
     N  P  Y  R  Q  F  Y  I  W  R  D  P  K  P  D  G  S  A  P  N
 361 aacccgtaccgccagttttacatttggcgcgatccgaagccggacggcagcgccccgaac
     N  W  Q  S  K  F  G  G  S  A  W  E  Y  D  E  Q  T  G  Q  Y
 421 aactggcagtcgaaattcggcggttcggcgtgggagtatgatgaacaaaccggacaatat
     Y  L  H  L  F  D  V  T  Q  A  D  L  N  W  E  N  E  E  L  R
 481 tatttgcatttgttcgatgtgacgcaagccgatttgaattgggaaaacgaagagctgcgc
     R  R  I  Y  D  M  M  H  F  W  L  K  K  G  V  D  G  F  R  L
 541 cgccgtatttatgacatgatgcatttttggctgaaaaaaggcgtggatggcttccggttg
     D  V  I  N  L  L  S  K  D  Q  R  F  P  D  D  D  G  S  I  P
 601 gatgtcatcaatttgctgtccaaagaccagcgctttccggacgatgacggatcgattccg
     P  G  D  G  R  R  F  Y  T  D  G  P  R  I  H  E  F  L  Q  E
 661 ccgggggacgggcgccgcttttatacggacgggccgcgcatccacgaatttttgcaagag
     M  N  R  E  V  F  S  K  Y  D  V  M  T  V  G  E  M  S  S  T
 721 atgaaccgcgaagtattctcgaaatacgatgtgatgaccgtcggggaaatgtcgtcgacg
     T  I  D  H  C  I  R  Y  T  N  P  E  N  R  E  L  N  M  T  F
 781 acgatcgaccactgcatccgttacacaaacccggaaaaccgggagctcaatatgacgttt
     N  F  H  H  L  K  V  D  Y  P  N  G  E  K  W  A  V  A  P  F
 841 aacttccatcacttgaaagtcgattatccgaatggggaaaaatgggccgtcgctccgttt
     D  F  L  A  L  K  R  I  L  S  E  W  Q  V  R  M  H  E  G  G
 901 gattttctcgcgttaaaacgcattttgtcggagtggcaagtgcgaatgcacgaaggcggg
     G  W  N  A  L  F  W  C  N  H  D  Q  P  R  I  V  S  R  Y  G
 961 ggatggaatgcgctcttttggtgcaaccatgatcaaccgcgcatcgtctcgcgctacggc
     D  D  G  T  Y  W  K  E  S  A  K  M  L  A  T  A  I  H  L  M
1021 gatgacgggacgtattggaaagagtcggcgaaaatgctagcgacggccatccatctgatg
     Q  G  T  P  Y  I  Y  Q  G  E  E  I  G  M  T  D  P  K  F  T
1081 caagggacgccatacatttaccaaggcgaagaaatcggcatgacagatccgaaattcacc
     D  I  R  D  Y  R  D  V  E  S  L  N  M  Y  R  I  L  L  G  Q
1141 gatatccgcgattaccgcgacgtcgagtcgctcaatatgtaccggattttgcttggacaa
     G  K  S  E  Q  E  V  L  D  I  L  Q  R  K  S  R  D  N  S  R
1201 ggcaaaagtgagcaagaggtgttggacattttgcagcggaagtcgcgcgacaattcgcgc
     T  P  M  Q  W  D  D  S  A  H  A  G  F  T  S  G  T  P  W  I
1261 acgccgatgcaatgggacgacagcgctcatgccggcttcacatctgggacgccgtggatt
     R  V  A  N  N  Y  R  R  I  N  V  K  Q  A  L  A  D  P  D  S
1321 cgcgtcgcgaacaattaccggcgcattaatgtgaaacaggcgcttgccgatcccgattca
     I  F  Y  H  Y  K  R  L  I  S  L  R  K  Q  Y  D  I  I  T  T
1381 atttttttaccattataagcggttaatttcattgcgcaagcaatacgacatcatcacaacc
     G  R  Y  E  L  L  L  E  D  D  P  H  I  F  A  Y  M  R  H  G
1441 gggcgctacgaactgcttttggaggacgatccgcacattttcgcttacatgcgccatgga
     E  K  E  K  L  L  V  V  N  N  F  Y  P  V  K  T  T  F  V  L
1501 gagaaggagaagctgcttgtcgtcaacaacttttatccggtcaaaacgacgttcgtgctg
     P  K  E  A  E  M  D  G  W  K  G  E  R  L  L  S  N  Y  P  D
1561 ccaaaagaagcggaaatggacggttggaagggagagcggctgctgtccaactatccggat
     S  P  D  D  F  R  R  I  D  L  R  P  Y  E  S  V  V  Y  L  L
1621 tcaccggacgattttcgccgcatcgacctgcgtccgtacgaatcagtcgtttatcttttg
     L  R  P  &
1681 ctccgtccgtga
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Table 3. Purification procedure for trehalose-6-phosphate hydrolase

Stage of purification	Activity, U/mg protein		Protein quantity, mg	Specific activity, mmol/min per mg protein	Total activity, mmol/min	Yield, %	Purification degree
	at 260 nm	at 280 nm					
Destruction of cells by ultrasound, centrifugation	6.9	4.9	650	0.46	300	100	1
Thermal treatment (60°C, 30 min), centrifugation	43.7	23.7	80	3.33	266	80	7.2
Chromatography (DEAE Sepharose Cl-6B)	1.3	2.5	60	3.88	233	72	8.4

rically (curves *3* and *4*, Fig. 3). In the control experiments solutions of α- and β-glucose were added instead of the reaction mixture. Under these conditions, mutarotation slows down significantly during the experiment (compare curves *1* and *2*). Here we also show the curve of mutarotation for equilibrated by weight under experimental conditions mixture of α- and β-glucoses (curve *5*). The experiment confirms that α-glucose is the product of trehalose-6-phosphate hydrolase reaction.

Measurement of K_m and k_{cat} of trehalose-6-phosphate hydrolase for substrates trehalose-6-phosphate and pNPG at different pH values. The reactions were carried out at 37°C in 0.1 M NaCl, 50 mM citrate glycerophosphate buffer (pH varied from 4.0 to 7.5). At pH < 4 the enzyme quickly denatures, while at pH > 7.5 the Michaelis constants become too high. Figures 4a and 4b present dependences of K_m and k_{cat} on pH for substrates trehalose-6-phosphate and pNPG. In both cases k_{cat} is relatively independent of pH.

Measurement of K_m and V_{max} of the enzyme trehalose-6-phosphate hydrolase for substrate pNPG at different concentrations of NaCl. To study the influence of ionic strength on the enzymatic activity, we measured K_m and k_{cat} of the substrate pNPG at different values of ionic strength. When NaCl concentration increases in the range from 0 to 1 M, k_{cat} decreases twofold (from 4000 to 2000 min^{-1}), while K_m decreases from 25 to 5 mM. The reactions were carried out at 37°C in 50 mM sodium acetate buffer, pH 5.5.

Determination of thermodynamic parameters of the reaction catalyzed by trehalose-6-phosphate hydrolase for substrates trehalose-6-phosphate and pNPG. The initial velocities of the reactions catalyzed by trehalose-6-phosphate hydrolase at different concentrations of substrates

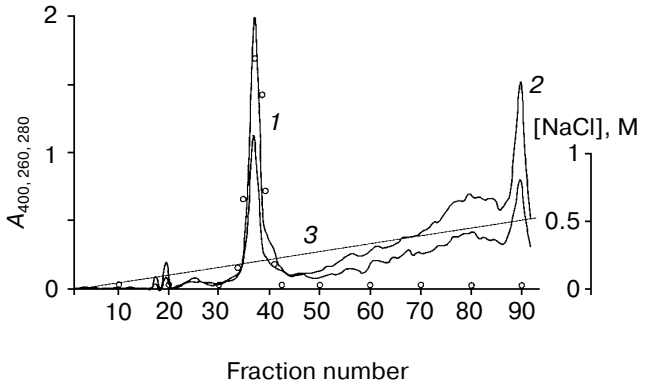

Fig. 2. Chromatography of the protein on DEAE-Sepharose Cl-6B in NaCl gradient: *1*) A_{280}; *2*) A_{260}; *3*) NaCl gradient. Circles correspond to A_{400} for 1 min when 5 mM pNPG, 20 mM Tris, pH 7.5 (37°C) were added to the fraction.

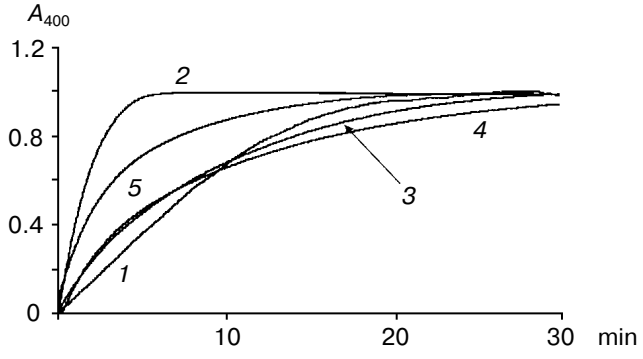

Fig. 3. Determination of glucose anomerity using the glucose oxidase system: *1*, *2*) calibration of the system with α- (*1*) and β-glucose (*2*); *3*, *4*) enzymatic hydrolysis of trehalose-6-phosphate (*3*) and pNPG (*4*); *5*) calibration of the system with the mixture of 50% α-glucose and 50% β-glucose.

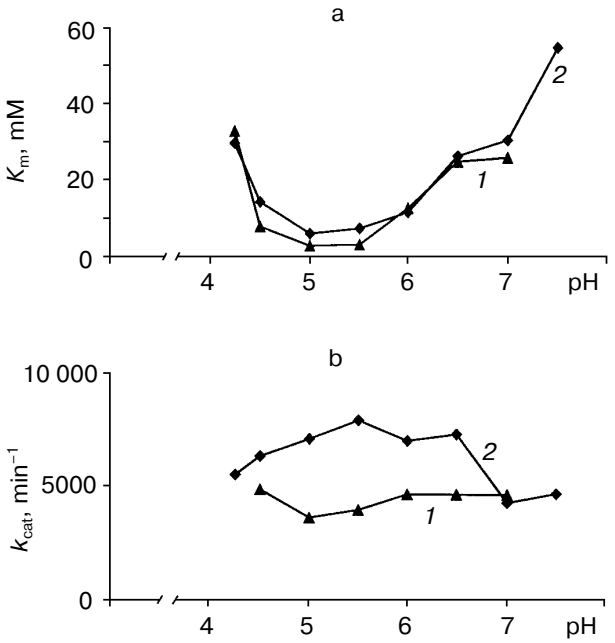

Fig. 4. Dependence of K_m and k_{cat} on pH: a) K_m for trehalose-6-phosphate (*1*) and pNPG (*2*); b) k_{cat} for trehalose-6-phosphate (*1*) and pNPG (*2*).

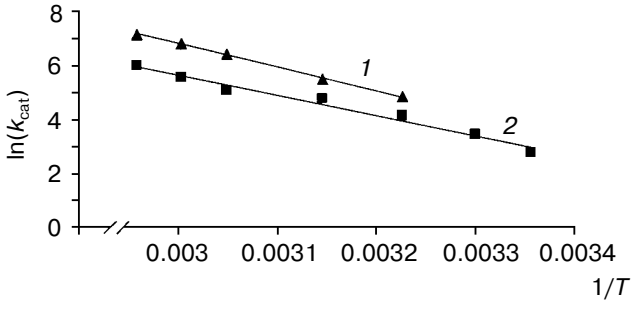

Fig. 5. Dependence of ln(k_{cat}) on inverse temperature for pNPG (*1*) and trehalose-6-phosphate (*2*).

were measured in the temperature range from 37 to 65°C. The reactions were carried out in 50 mM citrate-glycerophosphate buffer, pH 5.5, 0.1 M NaCl. Figure 5 presents the curves of dependence of logarithm of k_{cat} on inverse temperature for trehalose-6-phosphate and pNPG. The graphs allow for calculation of enthalpy and entropy of activation of the enzymatic reaction. For trehalose-6-phosphate $\Delta H^{\neq}$ = 16.9 kcal/mol, $\Delta S^{\neq}$ = 0.28 cal/mol·deg; for pNPG $\Delta H^{\neq}$ = 17.1 kcal/mol, $\Delta S^{\neq}$ = 2.55 cal/mol·deg. It is evident that the thermodynamic parameters are comparable, although almost zero activation entropy for trehalose-6-phosphate indicates structural similarity of the transitional complex with the substrate, and not with the reaction product.

DISCUSSION

Comparison of the amino acid sequence with primary amino acid sequences of the known α-glucosidases showed that the cloned gene codes α-glucosidase with 50-80% of homology with trehalose-6-phosphate hydrolase (EC 3.2.1.93), 50-60% with oligo-1,6-glucosidase and 40-50% with exo-α-1,4- and exo-α-1,6-glucosidase. Watanabe et al. [11] compared known to that time sequences of oligo-1,6-glucosidases from *Bacillus cereus* [12] and *Bacillus thermoglucosidiasus* [11] with sequences of α-amylase from *Aspergillus oryzae* [13] and α-amylase from porcine pancreas [14], for which tertiary structures have been determined. They demonstrated close similarity of the predicted from sequence tertiary structure for oligo-1,6-glucosidase with the structure of α-amylase. Therefore, they attributed several conservative amino acid residues to the active centers of glucosidases. High homology between sequences of oligo-1,6-glucosidases and trehalose-6-phosphate hydrolase from *Bacillus* sp. GP16 suggests that these regions are involved in the formation of the active center of hydrolases. These data were confirmed when the tertiary structure of oligo-1,6-glucosidase from *Bacillus cereus* was estimated [15]. The critical residues are His103, Asp200, Glu255, His329, and Asp330.

Trehalose-6-phosphate hydrolase has been purified to homogeneity from two sources: *E. coli* [6] and *Bacillus subtilis* 168 [16]; however, the enzymes have been biochemically characterized only qualitatively. Since we did not find in the literature the experimental evidence that this enzyme is an α-glucosidase, i.e., that one of the reaction products is α-glucose, we performed an experiment to determine the anomerity of the generated glucose. Our data confirm that trehalose-6-phosphate hydrolase from *Bacillus* sp. GP16 is an α-glucosidase.

Figure 4a presents the dependence of K_m on pH for trehalose-6-phosphate and pNPG. In the pH range from 4 to 7 the affinities of both substrates are almost identical, although the aglycone parts differ significantly (hydrophilic negatively charged glucose-6-phosphate and hydrophobic not charged *p*-nitrophenol). Possibly, the affinity for this enzyme is mainly determined by the glycone residue, α-glucose.

The dependence ln(k_{cat}/K_m) on pH suggests that two carboxyl groups are located in the active center of the enzyme, one in protonated form with pK ~ 5.5, and the other one in deprotonated form with pK ~ 4.5. Apparently, the first group plays a role of a common acid catalyst, and the second one of a common base (or it stabilizes the possible carbocation of the transition state). Table 1 suggests that these residues are Asp200, Glu255, and Asp330.

Data on activation of the enzymatic activity by salt are discussed in the literature [6, 16]. Our results do not confirm these observations. In both studies, dependences of velocity on salt concentration are presented without indication what velocity was exactly measured in the

reaction, V_{max} or arbitrary velocity. According to our data, the presence of NaCl decreases the maximal velocity and increases the affinity to the substrate. Consequently, at intermediate substrate concentrations, when the measured constant is equal to V_{max}/K_m, the apparent activation of the reaction can be observed.

Actually, the mechanism of action of α-glucosidases is well established [8], and it appears that trehalose-6-phosphate hydrolase acts through this mechanism, although it has not a standard type of glucoside bond. This mechanism suggests two subsequent kinetic reactions—formation of transitional β-glucosyl enzymatic intermediate followed by hydrolysis of the intermediate with formation of α-glucose. We attempted to determine the limiting stage of the reaction. One may suggest [17] that for "good" releasing groups (low pK in the case of substituted phenols) hydrolysis of the intermediate is the limiting step, while for "bad" releasing groups (phenols with high pK, sugar residues) the reaction is limited by formation of the intermediate. In this case we expect that addition of the nucleophilic agent (DTT, methanol) to the reaction mixture with the substrate with "good" releasing group should lead to an increase in the velocity of the reaction, and this effect should be absent for a "bad" releasing group. We carried out an experiment on pNPG hydrolysis (pK 7.2) in the presence of 0.05 and 0.1 M DTT and 0.1 M methanol. In all cases we did not observe any acceleration of the reaction. This observation may possibly be explained by the fact that in the case of pNPG the limiting stage of the reaction is formation of the intermediate.

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