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Enzymes from *Sulfolobus shibatae* for the production of trehalose and glucose from starch

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Abstract Enzymes that convert starch and dextrins to α,α-trehalose and glucose were found in cell homogenates of the hyperthermophilic acidophilic archaeon *Sulfolobus shibatae* DMS 5389. Three enzymes were purified and characterized. The first, the *S. shibatae* trehalosyl dextrin-forming enzyme (SsTDFE), transformed starch and dextrins to the corresponding trehalosyl derivatives with an intramolecular transglycosylation process that converted the glucosidic linkage at the reducing end from α-1,4 to α-1,1. The second, the *S. shibatae* trehalose-forming enzyme (SsTFE), hydrolyzed the α-1,4 linkage adjacent to the α-1,1 bond of trehalosyl dextrins, forming trehalose and lower molecular weight dextrins. These two enzymes had molecular masses of 80 kDa and 65 kDa, respectively, and showed the highest activities at pH 4.5. The apparent optimal temperature for activity was 70°C for SsTDFE and 85°C for SsTFE. The third enzyme identified was an α-glycosidase (SsαGly), which catalyzed the hydrolysis of the α-1,4 glucosidic linkages in starch and dextrins, releasing glucose in a stepwise manner from the nonreducing end of the polysaccharide chain. The enzyme had a molecular mass of 313 kDa and showed the highest activity at pH 5.5 and at 85°C.

Key words Extremophiles · *Sulfolobus shibatae* · Starch · Trehalose enzymes · α-Glucosidase

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Introduction

In recent years, enzymes isolated from thermophilic microrganisms have gained great attention for their thermostability, thermoactivity, resistance and activity in the presence of organic solvents, and, more generally, for the possibility to use them in industrial operative conditions that are not compatible with conventional enzymes. We have recently focused our attention on amylolytic activities from the hyperthermophile *Sulfolobus shibatae* that convert polysaccharides, as starch and dextrins, in α,α-trehalose and glucose.

Trehalose, a nonreducing disaccharide widely distributed in nature, has a multiple role because it acts as energy source in the blood of insects and protects some plants and organisms from damage caused by freezing and desiccation. Trehalose is a highly stable and nonhygroscopic disaccharide that does not caramelize and does not undergo Maillard reactions. It is used to stabilize enzymes, antibodies, vaccines, hormones, etc., and for the production of new types of foods in which it maintains the properties and aroma of the fresh product (Roser 1991). Because various applications are foreseen for trehalose and the extraction from baker's yeast is too expensive, interest in its industrial production has generated a search for organisms having biosynthetic pathways for its biosynthesis or that produce enzymes which could be used for its production from suitable substrates.

Lama et al. (1990, 1991) first demonstrated that the hyperthermophilic organism *Sulfolobus solfataricus* was able to produce trehalose from starch, and more recent reports by Nakada et al. (1996a,b), Kato et al. (1996a,b), and Di Lernia et al. (1996) described two enzymatic activities involved in the production of trehalose from starch in different hyperthermophilic organisms. In this paper, we describe the purification and characterization of a trehalosyl dextrin-forming enzyme (SsTDFE), of a trehalose-forming enzyme (SsTFE), and of an α-glycosidase from *Sulfolobus shibatae*. This latter enzyme, in addition to the other two, is also of industrial interest because a

thermostable and thermophilic α-glycosidase could be used to improve glucose production from starch hydrolysates and for the innovative production of glucose-trehalose syrups starting from low molecular weight trehalosyldextrins.

Materials and methods

Materials

Glucose/GOD-Perid method kit was purchased from Boehringer Mannheim (Milan, Italy), and MonoQ, PBE 94, and polybuffer 74 for fast performance liquid chromatography (FPLC) from Pharmacia Biotech (Milan, Italy). Other chemicals were from Sigma-Aldrich (Milan, Italy). Trehalosylglucose and a series of trehalosylmaltodextrins – trehalosylmaltose, trehalosylmaltotriose, trehalosylmaltotetraose, and trehalosylmaltopentaose – were prepared in our laboratory using SsTFE.

Microorganism and cultivation

Sulfolobus shibate strain DSM 5389 was grown at 87°C in a 100-l fermenter with an air flow of 20 l/min. The standard culture medium contained KH_2PO_4 (3.00 g l^{-1}), $(NH_4)_2SO_4$ (2.25 g l^{-1}), $MgSO_4 \cdot 7H_2O$ (0.20 g l^{-1}), $CaCl_2 \cdot 2H_2O$ (0.25 g l^{-1}), $MnCl_2 \cdot 4H_2O$ (1.8 mg l^{-1}), $Na_2B_4O_7 \cdot 10H_2O$ (4.5 mg l^{-1}), $ZnSO_4 \cdot 7H_2O$ (0.22 mg l^{-1}), $CuCl_2 \cdot 2H_2O$ (0.05 mg l^{-1}), $Na_2MoO_4 \cdot 2H_2O$ (0.03 mg l^{-1}), $VOSO_4 \cdot 2H_2O$ (0.03 mg l^{-1}), $CoSO_4 \cdot 7H_2O$ (0.01 mg l^{-1}); yeast extract (1 g l^{-1}), and casaminoacids (1 g l^{-1}). The pH was adjusted to 3.5 with 0.1 M H_2SO_4.

Enzyme assays

The three enzymes were assayed under the same standard conditions at 75°C in 50 mM citrate phosphate buffer, pH 5.5, by using the different substrates at a concentration of 0.67 mM. SsTDFE activity was determined by incubating maltohexaose in the standard mixture with 0.3–2.0 U/ml of enzyme for 30 min. The reaction, linear for at least 2 h, was stopped in a ice-water bath and the product formed, trehalosylmaltotetraose, was determined by HPLC. One unit was defined as the amount of enzyme that produces 1 μmole/min of trehalosylmaltotetraose. SsTFE activity was determined by incubating trehalosylmaltotetraose in the standard mixture with 0.2–1.5 U/ml of enzyme for 30 min. The reaction, linear for at least 2 h, was stopped ina ice-water bath and the products formed, maltotetraose and trehalose, were determined by HPLC. One unit was defined as the amount of enzyme that produces 1 μmole/min of maltotetraose. SsαGly activity was determined by incubating maltohexaose in the standard mixture with 0.1–1.5 U/ml of enzyme for 30 min. The reaction, linear for at least 2 h, was stopped in a ice-water bath and the glucose produced was determined by the glucose/GOD-Perid method. One unit was defined as the amount of enzyme that produces 1 μmole/min of glucose.

Protein assay

The protein concentration was determined using Bradford's method (1976) with bovine serum albumin (BSA) as the protein standard. Absorbance at 280 nm was used to monitor protein concentration in column eluates.

Determination of enzyme molecular mass

Molecular weight estimation of the native SsTDFE and SsTFE was performed with Sephacryl S-200 gel filtration column chromatography (1.5 × 60 cm), equilibrated with 10 mM Tris-HCl buffer, pH 8.0, containing 0.2 M NaCl and using 6 and 4 units, respectively. The calibration standards were β-amylase (sweet potato, 200 kDa), aldolase (rabbit muscle, 158 kDa), alcohol dehydrogenase (yeast, 150 kDa), BSA (66 kDa), and ovalbumin (hen egg, 43 kDa). Molecular weight estimation of native α-glycosidase was performed on a FPLC Superdex 200 26/60 MR (Pharmacia Biotech) in the same buffer using 5 U of enzyme. The calibration standards were ferritin (horse spleen, 440 kDa), *Sulfolobus solfataricus* β-glycosidase (240 kDa), β-amylase (sweet potato, 200 kDa), and aldolase (rabbit muscle, 158 kDa).

Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed by the method of Davis (1964). The molecular mass of the enzyme was estimated on 10% SDS-PAGE by the method of Laemmli (1970). Carbonic anhydrase (bovine erythrocyte, 29 kDa), egg albumin (hen egg, 45 kDa), BSA (66 kDa), phosphorylase b (rabbit muscle, 97.4 kDa), β-galactosidase (*E. coli*, 116 kDa), and myosin (porcine muscle, 205 kDa) were used as standard proteins.

Isoelectric point

The isoelectric point of the enzymes was determined by FPLC isochromatofocusing, using a PBE 94 column with a eightfold diluted gradient of Polybuffer 74.

High pressure liquid chromatography

The quantitative determinations of the substrate and products in the reaction mixtures were performed by a Dionex Chromatograph, equipped with an electrochemical detector, using a Carbopac PA 1 column (Dionex, Rome, Italy). The elution was carried out with the following gradient: NaOH 160 mM (buffer A) and, sodium acetate 300 mM (buffer B) (t = 0 min, 0% buffer B; t = 6 min, 0% buffer B; t = 36 min, 60% buffer B).

Identification of reaction products

Trehalose was identified by HPLC. Trehalosylmaltotetraose was identified by NMR spectra according to the

procedure of Kato et al. (1996c), using a Brüker WH-500 (Rheinstetten, Germany).

Purification of the enzymes

Step 1: Extraction

Wet cells (100 g), harvested in stationary growth phase, were suspended in 45 ml of 10 mM Tris-HCl pH 7.5 and ground in a mortar with 100 g glass beads (100–200 μm). After centrifugation at 1.600 × *g* for 10 min, the supernatant was treated in a Parr cell disruptor (Parr Instrument, Moline, IL, USA) (20 min at 2200 psi). Cell debris was removed by centrifugation at 35000 × *g* for 1 h, and the supernatant was exhaustively dialyzed against sodium phosphate buffer 10 mM, pH 6.0 (buffer A).

Step 2: CM 50

The dialyzed cell-free extract (154 ml, 9.5 mg protein/ml) was added to 100 ml 4% CM 50 suspension, equilibrated in the same buffer. After 2 h mixing and centrifugation for 10 min at 1600 × *g*, the supernatant (160 ml, 4.05 mg protein/ml), containing the three amylolytic activities, was dialyzed against 21 10 mM Tris-HCl buffer, pH 8.0 (buffer B).

Step 3: DEAE fast flow column chromatography

The sample (160 ml, 4.05 mg protein/ml) was applied to a column (2.0 × 20 cm) of DEAE Fast Flow equilibrated with buffer B. After exhaustive washing the enzymes were eluted with a linear gradient from 0 to 0.5 M NaCl in the same buffer (170 ml each) (see Fig. 1 for the separation of the three activities). From this point on each enzyme was purified to homogeneity in a different way, using the following purification steps.

SsTDFE purification

Step 4: phenyl Sepharose column chromatography

The active fractions containing SsTDFE (70 ml, 2.17 mg protein/ml), pooled from DEAE fast flow column chromatography, were supplemented with solid $(NH_4)_2SO_4$ to 1 M and filtered through a column (1.5 × 50 cm) of phenyl Sepharose equilibrated with buffer B containing 1 M $(NH_4)_2SO_4$. After exhaustive washing, bound proteins were eluted with a linear gradient from 1.0 to 0 M $(NH_4)_2SO_4$ in buffer B (400 ml), followed by a linear gradient from 0% to 40% ethanediol in buffer B (400 ml). The active fractions (20 ml, 0.55 mg protein/ml), derived from elution peak at 15% ethanediol, were dialyzed against buffer B and concentrated to 2 ml on a UF module (PM-10; Amicon (Millipore, Milan, Italy)).

Step 5: Sephacryl S-200 column chromatography

The concentrated enzyme solution was filtered onto a column (1.5 × 56 cm) of Sephacryl S-200 equilibrated and eluted with 0.2 M NaCl in buffer B. The active fractions (5 ml, 0.26 mg protein/ml) were dialyzed against buffer B.

Step 6: MonoQ FPLC

The enzyme solution was adsorbed onto a column of MonoQ HR 5/5 (1 ml) equilibrated with buffer B. After exhaustive washing, bound proteins were eluted with a linear gradient from 0 to 0.5 NaCl in the same buffer, and fractions having activity were combined (2 ml, 0.10 mg protein/ml). This constituted the purified SsTDFE.

SsTFE purification

Step 4: phenyl Sepharose column chromatography

The active fractions containing SsTFE activity (100 ml, 0.67 mg protein/ml), pooled from DEAE fast flow column chromatography, were supplemented with solid $(NH_4)_2SO_4$ to 1 M and filtered onto a column (1.5 × 50 cm) of phenyl Sepharose equilibrated with buffer B containing 1 M $(NH_4)_2SO_4$. After exhaustive washing, bound proteins were eluted with a linear gradient from 1.0 to 0 M $(NH_4)_2SO_4$ (400 ml) in buffer B, followed by a linear gradient from 0% to 40% ethanediol (400 ml). The active fractions (8 ml, 0.74 mg protein/ml), eluted by 35% ethanediol, were dialyzed against buffer B and concentrated to 2 ml on a UF module (PM-10; Amicon).

Step 5: Sephacryl S-200 column chromatography

The concentrated enzyme solution was filtered onto a column (1.5 × 56 cm) of Sephacryl S-200 equilibrated and eluted with 0.2 M NaCl in buffer B. The active fractions (5 ml, 0.145 mg protein/ml) were dialyzed against buffer B.

Step 6: MonoQ FPLC

The enzyme solution from the previous step was adsorbed onto a column of MonoQ HR 5/5 (1 ml) equilibrated with buffer B. After exhaustive washing, bound proteins were eluted with a linear gradient from 0 to 0.5 NaCl in the same buffer, and fractions having activity were combined (2 ml, 0.061 mg protein/ml). This constituted the purified SsTFE.

SsαGly purification

Step 4: phenyl Sepharose column chromatography

The active fractions containing SsαGly activity (100 ml, 2.2 mg protein/ml), pooled from DEAE fast flow column chromatography, were supplemented with solid $(NH_4)_2SO_4$ to 1 M and filtered onto a column (1.5 × 50 cm) of phenyl Sepharose equilibrated with buffer B containing 1 M $(NH_4)_2SO_4$. After exhaustive washing, bound proteins were eluted with a linear gradient from 1.0 to 0 M $(NH_4)_2SO_4$

(400 ml) in buffer B, followed by a linear gradient from 0% to 40% ethanediol in buffer B (400 ml). The active fractions (24 ml, 1.04 mg protein/ml), derived from the elution peak at 25% ethanediol, were dialyzed against buffer B and concentrated to 2 ml on a UF module (PM-10; Amicon).

Step 5: Sephacryl S-200 column chromatography

The concentrated enzyme solution was filtered through a column (1.5 × 56 cm) of Sephacryl S-200 equilibrated and eluted with 0.2 M NaCl in buffer B. The active fractions (5 ml, 1.8 mg protein/ml) were dialyzed against buffer B.

Step 6: MonoQ FPLC

The enzyme solution was adsorbed on a column of MonoQ HR 5/5 (1 ml) equilibrated with buffer B. After exhaustive washing, bound proteins were eluted with a linear gradient from 0 to 0.5 NaCl in buffer B and active fractions were combined (4 ml, 0.375 mg protein/ml). This constituted the purified α-glycosidase.

Results and discussion

A number of thermophilic and hyperthermophilic microorganisms have been found to produce very thermostable enzymes, capable of hydrolyzing natural polymers such as starch, cellulose, and xylan (Leusher and Antranikan 1995; Antranikan et al. 1995; Sunna et al. 1997). During our investigation on amylolytic activities in *Sulfolobales* we reported (Lama et al. 1990, 1991) that these Archaea were able to produce trehalose from starch, and recently several groups have reported data on the isolation and characterization, from *S. solfataricus* and *S. acidocaldarius*, of two enzymes responsible for the synthesis of trehalose from starch (Nakada et al. 1996a,b; Kato et al. 1996a,b; Di Lernia et al. 1996). However, in addition to these enzymes we have purified and characterized, from *S. shibatae*, an α-glycosidase with interesting properties.

Purification of SsTDFE, SsTFE, and SsαGly

SsTDFE, SsTFE, and SsαGly activities were purified to homogeneity from the cell-free extract of *S. shibatae* as described in Materials and methods. Tables 1 and 2 summarize the results of the purification procedures of the three enzymes.

The simultaneous presence of both SsTDFE and SsTFE prevented the measurement of their individual activities in the homogenate and CM 50 supernatant fractions. The DEAE cellulose step (Fig. 1) separated the three enzymatic activities that were subsequently independently purified by using hydrophobic chromatography (phenyl Sepharose), gel filtration (Sephacryl S-200), and ion-exchange chromatography (MonoQ). The purification factor was about 110 fold for both SsTDFE and SsTFE and about 200 fold for SsαGly, with a recovery of 15% for SsTDFE and about 20% for SsTFE and SsαGly. All three enzyme preparations were homogenous because each gave a single protein band on SDS-PAGE (not shown), and the specific activity was 90 units/mg of protein for SsTDFE, 550 units/mg protein for SsTFE, and 2.8 units/mg protein for SsαGly.

Characterization and properties of SsTDFE

The effects of pH and temperature on SsTDFE activity and stability are shown in Fig. 2. The enzyme had a pH optimum of 4.5, was stable in the range of pH between 4.5 and 9.5 (Fig. 2a), had an apparent optimal activity temperature at

Table 1. Purification procedure of TDFE and TFE from *Sulfolobus shibatae* DMS 5389

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification factor	Yield (%)
Crude extract	–[a]	1463	–	–	–
CM 50 batch	–[a]	648	–	–	–
DEAE fast flow	122[b] 348[c]	152[b] 67[c]	0.8[b] 5.0[c]	1	100
Phenyl Sepharose	55[b] 181[c]	11[b] 6[c]	5[b] 30[c]	6[b] 5[c]	45[b] 52[c]
S-200 gel filtration	23[b] 87[c]	1.30[b] 0.72[c]	18[b] 120[c]	22[b] 24[c]	19[b] 25[c]
Mono Q FPLC	18[b] 66[c]	0.20[b] 0.12[c]	90[b] 550[c]	112[b] 110[c]	15[b] 19[c]

[a] TDFE and TFE activities could not be measured individually (see Results)
[b] TDFE, trehalosyl dextrin-forming enzyme
[c] TFE, trehalose-forming enzyme

Table 2. Purification procedure of α-glycosidase from *Sulfolobus shibatae* DMS 5389

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification factor	Yield (%)
Crude extract	20	1463	0.014	1	100
CM 50 batch	18.56	648	0.029	2	93
DEAE fast flow	11.14	221	0.05	3.6	56
Phenyl Sepharose	7.00	25	0.28	20	35
S-200 gel filtration	6.00	9	0.67	48	30
Mono Q FPLC	4.20	1.5	2.8	200	21

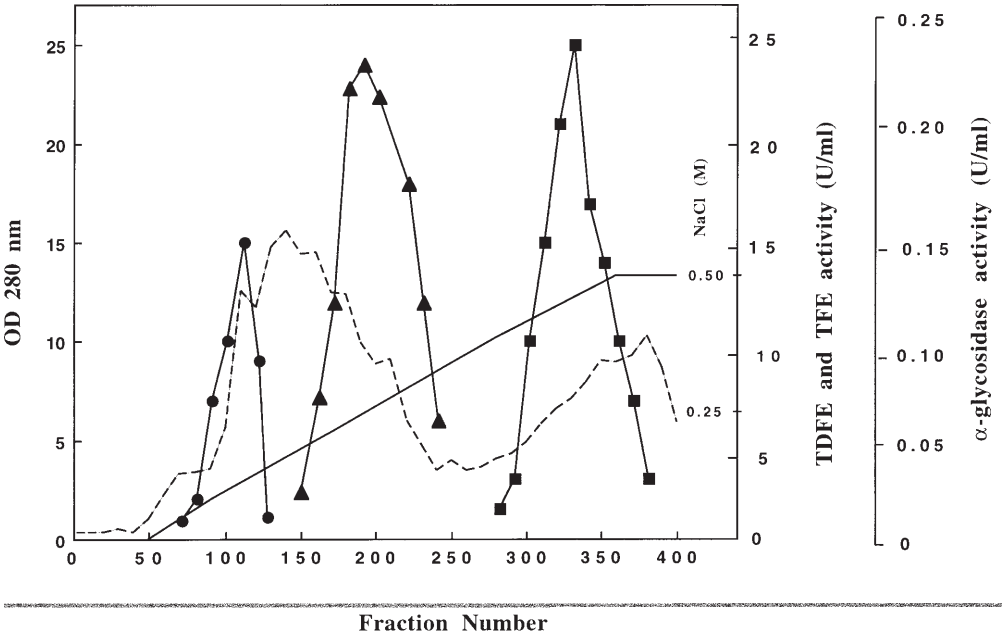


Fig. 1. Separation of the three amylolytic activities by DEAE fast flow Column Chromatography. *Dashed line*, Absorbance at 280 nm; *solid line*, NaCl concentration; *circles*, trehalosyl dextrin-forming enzyme (TDFE) activity; *triangles*, α-glycosidase (αGly) activity; *squares*, trehalose-forming enzyme (TFE) activity

70°C, and was quite stable at temperatures up to 85°C (Fig. 2b). The pI, determined by FPLC chromatofocusing, was 5.0. The molecular mass, determined by S-200 gel filtration chromatography and by SDS-PAGE (not shown), was 80 kDa, indicating that the native enzyme is monomeric. SsTDFE, using maltohexaose as substrate, showed a turnover number of 33.0 s^{-1} and a K_m of 2 mM, as calculated from a Lineweaver-Burk plot (1934); by contrast, the K_m of the enzyme from *S. acidocaldarius* was 5.7 mM.

The corresponding enzymes isolated from *S. acidocaldarius* and *S. solfataricus* had, respectively, a molecular mass of 74 and 76 kDa and a pI of 5.0 and 6.1. Other parameters such as optimal pH, activity as function of temperature, and thermostability were quite similar for the enzymes from the three sources.

Characterization and properties of SsTFE

The effects of pH and temperature on SsTFE activity and stability are shown in Fig. 3. The enzyme had a pH optimum at 4.5, was stable in the range of pH between 4.5 and 9.5 (Fig. 3a), had an apparent optimal temperature at 85°C, and was quite stable at temperatures up to 85°C (Fig. 3b). The pI, determined by FPLC chromatofocusing, was 5.0. The molecular mass, determined by S-200 gel filtration chromatography and by SDS-PAGE (not shown), was 65 kDa, indicating that the native enzyme is monomeric. SsTFE, using trehalosylmaltotetraose as substrate, showed a turnover number of 83 s^{-1} and a K_m of 1 mM; the K_m of the enzyme from *S. acidocaldarius* was 3.7 mM.

The corresponding enzymes from *S. acidocaldarius* and *S. solfataricus* had a molecular mass of 62 and 61 kDa, respectively, and similar isoelectric points, activity as function of temperature, and thermostability.

Combined action of SsTDFE and SsTFE on maltodextrins

The concerted action of SsTDFE and SsTFE using maltodextrins as substrate allowed a cyclic process in which the terminal trehalose molecules were removed from the nonreducing end, converting the whole glycosidic chains into trehalose. For maltodextrins having an odd number of glucose residues, the end product was a maltotriose, whereas maltose was obtained from maltodextrins with an even number of glucose residues.

Analysis of the reaction kinetics was performed using maltoheptaose as substrate (Fig. 4) for the two enzymes. During the reaction, trehalosylmaltopentaose, the first product, was the most abundant compound after 5 min and maltopentaose was still present after 20 min. After 2 h, the final products of the enzymatic process, maltotriose and trehalose, were present as expected in a 1:2 molar ratio.

From the results shown here, it is clear that these two proteins are of great interest for studying the structure–function relationships of the enzymes involved in starch degradation and for the production of trehalose and nonreducing maltodextrins.

Characterization and properties of SsαGly

SsαGly is an intracellular enzyme active on starch, amylose, and a series of maltodextrins with a degree of polymerization from 2 to 7. These data indicate a difference from the homologous enzyme purified from *S. solfataricus*, which is not active on starch. The K_m and the turnover number values for maltose were, respectively, 8 mM and 45.5 s^{-1}, as calculated from a Lineweaver-Burk plot.

The effects of pH and temperature on α-glycosidase activity and stability are shown in Fig. 5. The enzyme showed

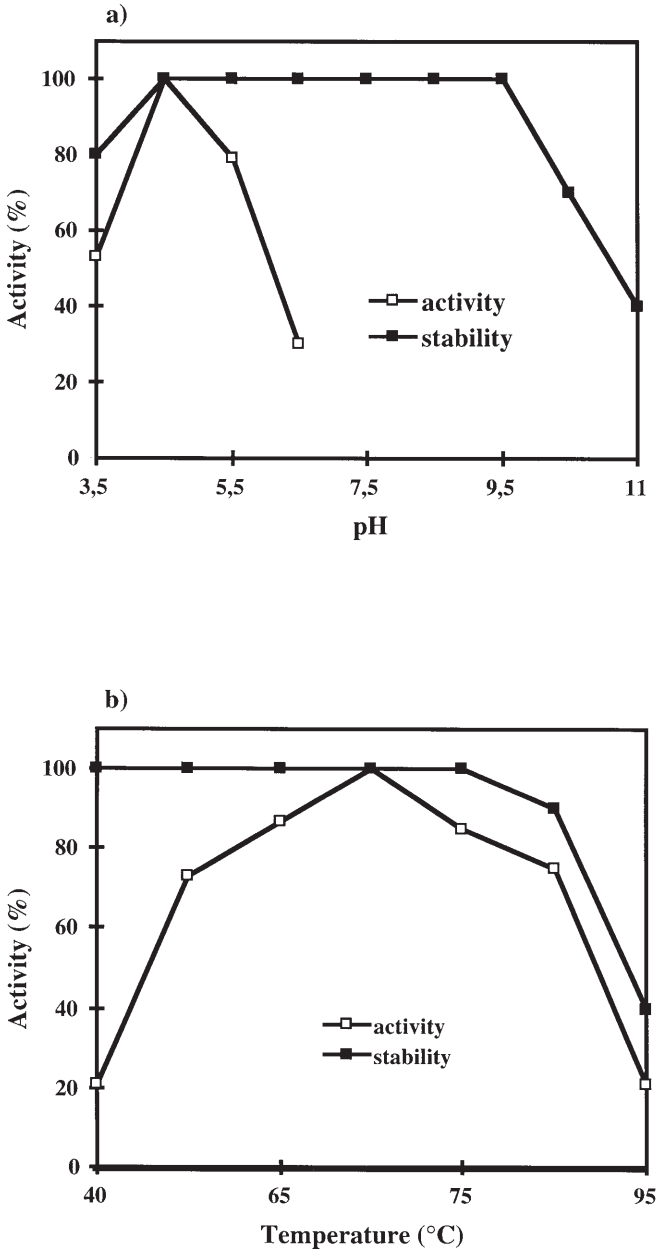


Fig. 2a,b. Effects of pH and temperature on the activity (*open squares*) and stability (*solid squares*) of SsTDFE (*S. shibatae* TDFE). **a** Effects of pH. The enzyme (0.9 U/ml) was assayed under standard conditions using the following buffers: 50 mM citrate-phosphate buffer (pH 3.5–6.5), 50 mM Tris-HCl (pH 7.5–8.5), and 50 mM Na_2CO_3 (pH 9.5–11). To determine pH stability, the enzyme (2.7 U/ml) was incubated in the appropriate buffer at 4°C for 24 h and residual activity was measured under standard conditions. **b** Effects of temperature. The enzyme (0.9 U/ml) was incubated at different temperatures under standard conditions. Thermostability was determined by incubating the enzyme (1.5 U/ml) at pH 4.5 for 120 min at different temperatures and measuring the residual activity at 75°C

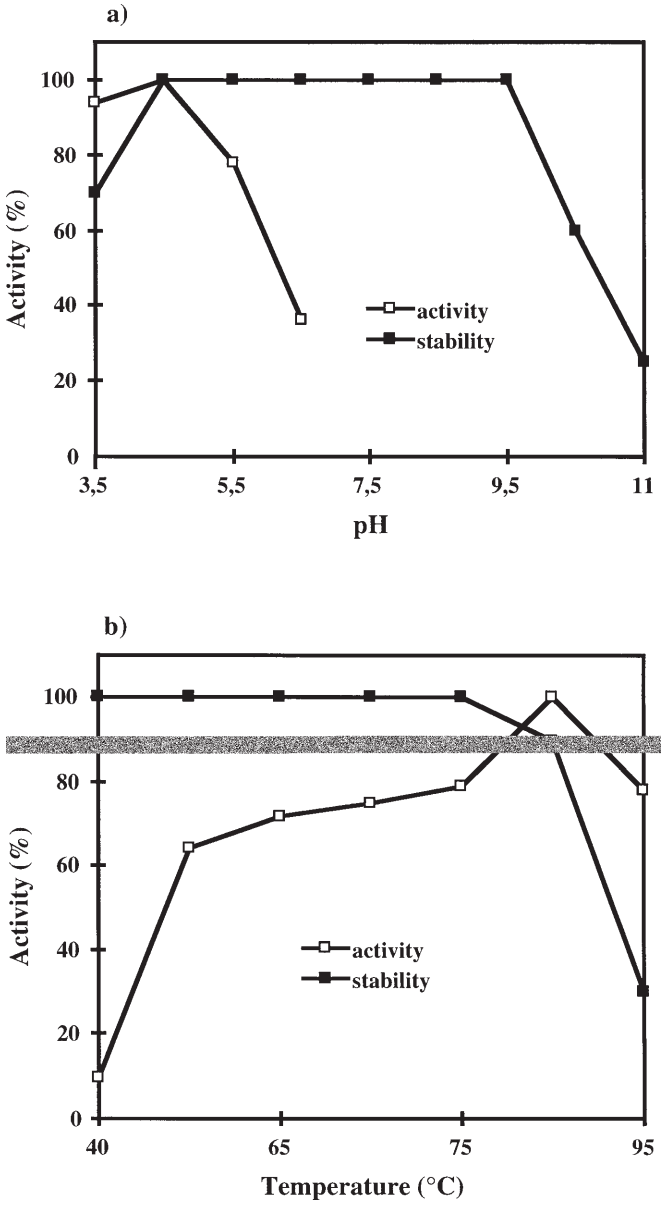


Fig. 3a,b. Effects of pH and temperature on the activity and stability of SsTFE. **a** Effects of pH. The enzyme (0.5 U/ml) was assayed under standard conditions using the following buffers: 50 mM citrate-phosphate buffer (pH 3.5–6.5), 50 mM Tris-HCl buffer (pH 7.5–8.5), and 50 mM $NaHCO_3$-Na_2CO_3 (pH 9.5–11). To determine pH stability, the enzyme (1.5 U/ml) was incubated in the appropriate buffer for 24 h at 4°C, and the residual activity was measured under standard conditions. **b** Effects of temperature. The enzyme (0.5 U/ml) was incubated under standard conditions. Thermostability was determined by incubating the enzyme (1.5 U/ml) at pH 4.5 for 120 min and measuring the residual activity at 75°C

a pH optimum at 5.5, was stable from pH 3.5 to 9.5 (Fig. 5a), had an apparent optimal activity at 85°C, and was stable at temperatures up to 105°C (Fig. 5b). The pI, determined by FPLC chromatofocusing, was 4.7. The molecular mass of the native enzyme as determined by Superdex 200 (Fig. 6) was 313 kDa and by SDS-PAGE was 80 kDa. Thus, the enzyme seems to be a tetramer composed of subunits similar or identical to the other intracellular α-glycosidase purified from *S. solfataricus* (Rolfs meier and Blum 1995). The intracellular α-glycosidase isolated from *Pyrococcus furiosus* is, on the other hand, a monomer with a molecular mass of 125 kDa. The N-terminal sequence was as follows: NH_2-Met-Gln-Thr-Ile-Lys-Ile-Tyr-Glu-Asn-Lys-Gly-Val-Tyr-Lys-Val-Val-Ile-Gly-Glu-Pro-Phe-Pro.

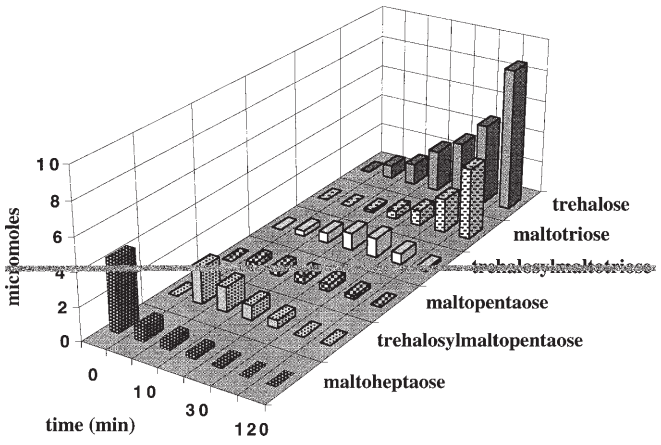
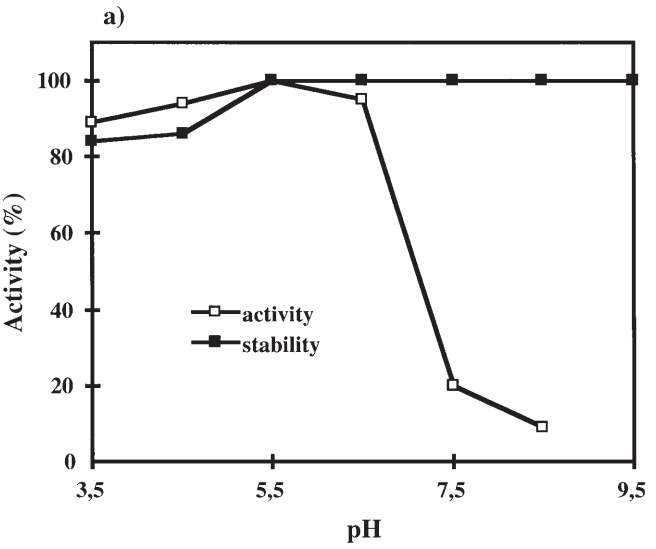


Fig. 4. Kinetic analysis of reaction using SsTDFE and SsTFE. A reaction mixture (0.750 ml) containing 5 mg of maltoheptaose and 0.8 U of each enzyme was incubated at 75°C, pH 5.5, for 2 h. Samples were withdrawn at different times and reaction products were analyzed by HPLC



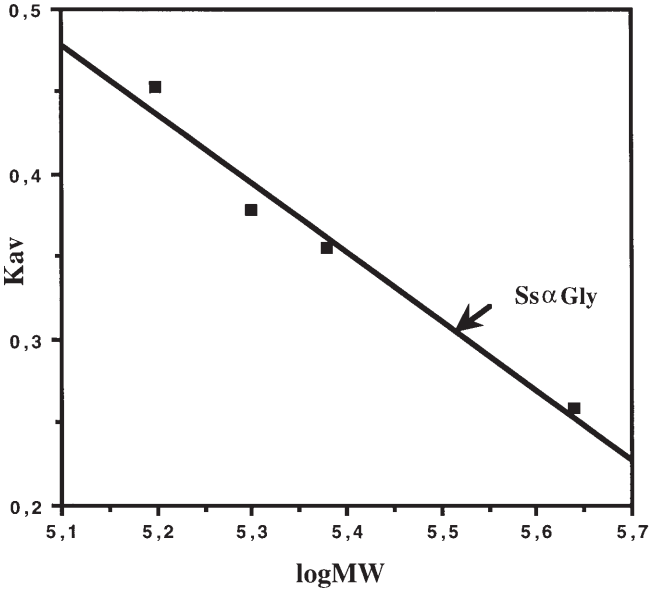


Fig. 6. Native molecular weight of SsαGly as determined with FPLC Superdex 200 (see Methods)

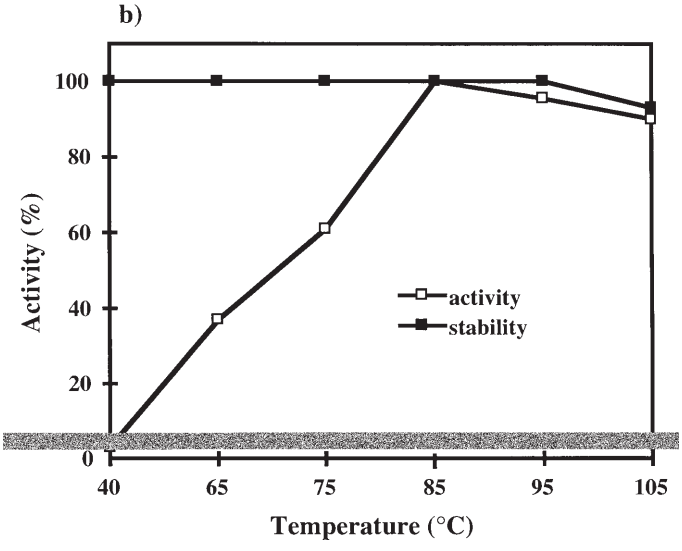


Fig. 5. Effects of pH and temperature on the activity and stability of SsαGly. **a** Effects of pH. The enzyme (0.2 U/ml) was assayed under standard conditions using the following buffers: 50 mM citrate-phosphate buffer (pH 3.5–6.5), 50 mM Tris-HCl buffer (pH 7.5–8.5), and 50 mM $NaHCO_3$-Na_2CO_3 (pH 9.5–11). To determine pH stability, the enzyme (0.8 U/ml) was incubated for 24 h at 4°C, and the residual activity was measured under standard conditions. **b** Effects of temperature. The enzyme (0.2 U/ml) was incubated at various temperatures. Thermostability was determined by incubating the enzyme (0.8 U/ml) at pH 5.5 at different temperatures for 120 min and measuring the residual activity at 75°C

The enzyme was inhibited almost 75% by 1 mM β-mercaptoethanol but not by glucose to 10 mM. Several metal ions (not shown) slightly activated the enzyme with a maximum of about 1.5 fold for $CuSO_4$ and $ZnSO_4$. However, incontrast to *Pyrococcus furiosus*, the enzyme was not inactivated by EDTA.

Conclusions

Three enzymes that convert starch and dextrines to trehalose and glucose have been isolated from *Sulfolobus shibatae*. The production of trehalose involves two enzymes, a trehalosyldextrin-forming enzyme and a trehalose-forming enzyme, that are quite similar to those isolated from *Sulfolobus acidocaldarius* and *Sulfolobus solfataricus*. The α-glycosidase seems to be a novel enzyme because its characteristics are different compared to those of the

enzymes isolated from *S. solfataricus* and *Pyrococcus furiosus*.

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