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A novel thermophilic fusion enzyme for trehalose production

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Abstract In recent years a number of hyperthermophilic micro-organisms of *Sulfolobales* have been found to produce trehalose from starch and dextrins. In our laboratory genes encoding the trehalosyl dextrin forming enzyme (TDFE) and the trehalose forming enzyme (TFE) of *S. solfataricus* MT4 have been cloned and expressed in *E. coli* (Rb791). Here we report the construction of a new protein obtained by fusion of TFE and TDFE coding sequences which is able to produce trehalose from dextrins at high temperature by sequential enzymatic steps. We demonstrate that the bifunctional fusion enzyme is able to produce trehalose starting from malto-oligosaccharides at 75°C. Furthermore we partially purified the recombinant fusion protein from bacterial cell free extracts and from insoluble fractions in which the fusion protein was also found as aggregate in inclusion bodies.

Key words Trehalosyl dextrin forming enzyme · Trehalose forming enzyme · Trehalose · *Sulfolobus solfataricus* · Fusion protein · Thermophilic enzymes

Introduction

Trehalose is a nonreducing disaccharide consisting of two α-1,1 linked glucose moieties. In living organisms trehalose serves as a carbohydrate energy source as well as a protecting agent against physical stress (Arguelles 2000). Trehalose protects membrane fluidity and preserves protein structure in dry conditions, due to its high water-holding activity. In the meantime, due to the absence of reducing activity, in the presence of trehalose chemical modification of biological molecules is avoided (Simola et al. 2000; Singer and Lindquist 1998; Xie and Timasheff 1997). The physicochemical properties of trehalose make this sugar valuable for industrial use as a component of a variety of commercial products such as food, pharmaceutical, and cosmetics (Paiva and Panek 1996; Portmann and Birch 1995; Roser 1991). Baker's yeast has been exploited as trehalose source; however, the cost of the extraction procedure has seriously limited its commercial use. To overcome this problem various strategies for industrial production of trehalose, mostly based on recombinant enzymes, are being developed. It has been found that hyperthermophilic micro-organisms of *Sulfolobales* are able to produce trehalose from starch and dextrins (Kato et al. 1996a, 1996b; Mukai et al. 1997; Nakada et al. 1996). We isolated and characterized trehalosyl dextrin forming enzyme (TDFE) and trehalose forming enzyme (TFE) from *S. shibatae* (Di Lernia et al. 1998), and subsequently the gene encoding these proteins in *S. solfataricus* strain MT4 were cloned in *Escherichia coli* (de Pascale et al. 2001).

Trehalose is formed by the coupled reaction of the two thermophilic enzymes; therefore we investigated the possibility of designing a fusion protein containing both enzymatic activities. Proximity of enzymes catalyzing sequential reactions might increase the overall reaction rate by facilitating the transfer of intermediate products (Béguin 1999). A number of techniques have been developed to establish enzyme proximity, including coimmobilization and chemical cross-linking. An attractive alternative approach is to fuse enzymes by ligating their structural genes (Lindbladh et al. 1992). This strategy mimics the evolution of naturally occurring multienzyme systems, such as the pyruvate dehydrogenase complex and the fatty acyl coenzyme A synthase complex (Nikolau et al. 2000; Rangan et al. 1998). Fused enzymes usually display better performance than mixtures of the individual enzymes catalyzing the same sequential reactions. Kinetic parameters governing the proximity

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effect have not been conclusively defined, nonetheless gene fusion appears to have a great potential in industrial enzyme technology (Bulow and Mosbach 1991).

Recently the production of two bifunctional enzyme for trehalose synthesis constructed by fusing the genes for trehalose-6-phosphate synthetase and trehalose-6-phosphate phosphatase from *E. coli* (Seo et al. 2000) or the malto-oligosyltrehalose synthase and malto-oligosyltrehalose trehalohydrolase from the nonpathogenic bacterium *Brevibacterium helvolum* (Kim et al. 2000), has been reported. These fusion enzymes catalyze the sequential reactions more efficiently than an equimolar mixture of individual enzymes due to a proximity effect on the catalytic sites. The use of enzymes derived from mesophilic organisms in industrial processes requiring high temperatures, however, may be hampered by their limited thermal resistance.

Here we report the construction of a fused protein derived from the trehalosyl dextrins forming enzyme and the trehalose forming enzyme of the extreme thermophilic archaeon *S. solfataricus* MT4. This new fusion enzyme is able to produce trehalose from dextrins at high temperature in sequential enzymatic steps.

Materials and methods

Materials

Molecular mass standards for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Gibco BRL, Pharmacia Biotech. International. pTrc99A and pUC18 *Sma*/BAP plasmids were from Pharmacia Biotech International. Radioactive materials were obtained from Amersham International. Deoxynucleotides and enzymes for DNA restriction and modification were purchased from Roche. *E. coli* strain Rb791 (W3110 lacI^q L8) was kindly provided by Prof. G. Sannia (Dipartimento di Chimica Organica, Università di Napoli Federico II, Naples, Italy). Gene amplification experiments were carried out with Expand High Fidelity TAQ system supplied by Roche. All synthetic oligonucleotides were purchased from PRIMM (Milan, Italy). Maltohexaose (M6) was from Sigma-Aldrich. Other chemicals were from Sigma-Aldrich. Protein concentration was determined using the Bradford's method, using Bio-Rad Protein Assay.

Analytical methods for DNA

DNA electrophoresis on 1% (w/v) agarose gel was performed in buffer of 90 mM Tris/borate/20 mM EDTA. Plasmid transformation of *E. coli* cells was carried out by electroporation using a Gene Pulser purchased from Bio-Rad. DNA sequencing was carried out by the dideoxy chain termination method with [^{35}S]dATP, using the Sequenase version 2.0 sequencing kit (Amersham) on alkali-denatured double-stranded templates and the universal primer or specific synthetic oligonucleotides. More than 90% of the DNA sequences were determined in duplicate on both strands. Gene sequence analysis was performed using PC-GENE software (Intelligenetics, Mountain View, Calif., USA).

Construction of expression plasmid pTrcTFE*

The complete sequence encoding TFE, in which the translational stop codon was corrected to a Gly (named TFE*), was amplified by PCR using 200 ng pTrcTFE, described by de Pascale et al. (2001), as template and a downstream primer introducing a mutant codon, as described below.

The following oligonucleotides derived from the nucleotide sequence of TFE sequence from *S. solfataricus* MT4 were used:

- oligonucleotide AAA-N: 5′-GCGTTCATGACGTTTG CTTATAAAATAG-3′ containing the recognition site for RcaI endonuclease that encode the first Met residue, followed by sequence encoding the N-terminus of S. solfataricus TFE
- oligonucleotide AAA-C NSC: 5′-GCTTTATATAAACT TTCGGGATCCAAGCTTCG-3′ containing the mutagenized stop transcription codon from TAG to TCG, the recognition site for BamHI endonuclease, the recognition site for HindIII endonuclease and sequence complementary to the one encoding C-terminus of TFE.

PCR was carried out in a 100 µl (total volume) reaction mixture containing 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, deoxynucleotide triphosphate at a concentration of 0.2 mM each, 100 ng of template DNA, 150 ng of each primer, and 2.5 U *Taq* DNA polymerase. The mixture was heated for 5 min at 94°C and then subjected to the following thermal profile: 15 s at 94°C, 30 s at 45°C, 1 min and 30 s at 68°C, extension 20 s/cycle for 30 cycles. The amplified TFE* DNA was made blunt-end by filling in with the Klenow fragment of DNA polymerase, in the presence of deoxyribonucleoside triphosphates and then digested with *Rca*I and *Hin*dIII endonucleases and purified by preparative 1% agarose gel. TFE* was cloned in pTrc99A, previously digested with *Nco*I and *Hin*dIII enzymes. The recombinant expression vector was designed pTrcTFE*.

Construction of the plasmid pTrcTFE*/TDFE

The complete coding sequence of the TDFE gene was obtained by restriction from the pTrcTDFE previously constructed (de Pascale et al. 2001). The pTrcTDFE was digested with *Nco*I and then was made blunt-end by filling in with the Klenow fragment. The 2186-bp fragment was obtained by restriction with *Hin*dIII endonuclease; the TDFE sequence was purified by preparative 1% agarose gel. The pTrcTFE* was digested with *Bam*HI endonuclease, made blunt-end by filling in with the Klenow fragment, and then digested with *Hin*dIII endonuclease. The TDFE fragment was inserted and ligated into *Bam*HI/*Hin*dIII pTrcTFE* sites, with the following sequence TCGGGATC CGGA linking the ORFs. The recombinant plasmid was designed as pTrcTFE*/TDFE.

Micro-organism and cultivation

E. coli Rb791 competent cells were transformed with pTrcTFE*/TDFE expression vector and grown at 37°C to different densities in shake flasks containing 500 ml Luria-Bertani medium. The production of biomass was scaled up employing a 100 l-fermenter. All experiments were performed without addition of isopropyl-D-thiogalactopyranoside since induction did not greatly increase enzyme yield.

Enzyme assay

TFE*/TDFE fusion enzyme activity was assayed under standard conditions at 75°C, in 50 mM sodium acetate buffer pH 5.5 using maltohexaose as substrate at a concentration of 0.67 mM. The activity was determined by incubating the substrate in the standard mixture with 2–10 µg partially purified enzyme. The reaction, linear for at least 1 h, was stopped in an ice-water bath, and the amount of products formed were determined by high-performance anion exchange chromatography (HPAEC). One unit was defined as the amount of enzyme which produce 1 µmol/min of trehalose.

High-performance anion exchange chromatography

The quantitative determinations of the substrate and products in the reaction mixtures were performed by a Dionex Chromatograph, equipped with pulsed amperometric detector (PAD). Baseline separation of carbohydrates was achieved within 10 min using Carbopac PA-100 guard and analytical column. As mobile phase was used a linear gradient employing 160 mM NaOH (buffer A) and 300 mM sodium acetate (buffer B). The elution was carried out as follows: t = 0 min 0% buffer B; t = 6 min 0% buffer B; t = 30 min 100% buffer B. The sample loop volume was 50 µl, and the eluent flow rate was 1.0 ml/min. Detection was by triple-pulsed amperometry using a gold electrode. The analysis was performed using an internal standard method. A 1 mg/ml internal standard solution was prepared by dissolving maltoheptaose in water. An appropriate volume of internal standard solution was added to each sample to give a final concentration of 11.5 µg/ml maltoheptaose. Calibration graphs were plotted based on the linear regression analysis of the peak-area ratios.

Partial purification of the recombinant TFE*/TDFE enzyme from *E. coli* extract

Crude extracts were treated by progressive thermoprecipitations in order to separate the recombinant enzyme from *E. coli* mesophilic proteins.

Step 1: Extraction

Wet cells (25 g) were suspended in 75 ml 50 mM sodium acetate buffer pH 5.5 and treated in a French press disruptor (2000 ψ). Cell debris were removed by centrifugation at 10,000/*g* for 1 h.

Step 2: Heat treatments

The cell-free extract (50 ml; 8.7 mg/ml; 1.25 U_{tot}) was heated at 50°C for 30 min under stirring in a water bath and centrifuged at 10,000/*g* at 4°C for 30 min. The supernatant (47.5 ml; 8 mg/ml; 1.25 U_{tot}) was heated at 60°C for 30 min. The enzyme preparation was then centrifuged at 10,000/*g* at 4°C for 30 min. The supernatant (47 ml; 4.7 mg/ml; 1.25 U_{tot}) was heated at 70°C for 30 min and centrifuged at 10,000/*g* at 4°C for 30 min. The supernatant (45 ml; 2.7 mg/ml; 1.08 U_{tot}) was heated at 75°C overnight (about 17 h) and then centrifuged at 10,000/*g* at 4°C for 30 min.

Electrophoresis

The molecular mass of the enzyme was estimated on 8% SDS-PAGE (Laemmli 1970). Trypsin inhibitor (soybean 201 kDa), carbonic anhydrase (bovine erythrocyte, 29 kDa), egg albumin (hen egg, 45 kDa), bovine serum albumin (66 kDa), phosphorylase b (rabbit muscle, 97.4 kDa), and a raibow molecular weight markers were used as standards.

Analysis of N-terminal sequence

The analysis of N-terminal sequence was performed to identify the fusion protein. For this purpose the homogenate was subjected to 8% SDS-PAGE analysis (Maxi Protean, Bio-Rad) and after the run the gel was electroblotted onto a polyvinylidene fluoride membrane. After staining with red Ponceau a band of about 145 kDa was cut, and the N-terminal sequence was performed by Edman degradation.

Preparation of inclusion bodies

E. coli (10 g) containing pTrcTFE*/TDFE plasmid was suspended in 40 ml (4 ml/g wet cells) of lysis buffer (100 mM Tris-HCl pH 8.0, 5 mM EDTA, 5 mM DTT, 5 mM benzamidine-HCl), at 0°C for 20 min. Cells were disrupted by ultrasound sonication by bursts of 30 s followed by cooling until the solution clears. The lysed cell suspension was centrifuged at 30,000/*g* for 1 h at 4°C, to pellet unbroken cells, large cellular debris, and inclusion bodies. The pellet was resuspended in 10 ml/g washing buffer (100 mM Tris-HCl pH 7.0, 5 mM EDTA, 5 mM DTT, 2 M urea, 2% Triton X-100), sonicated, and again centrifuged. The pellet was then resuspended with washing buffer, containing DNase I (20 mg/l) for 1 h at 37°C. Inclusion bodies were then sedimented by centrifugation at 30,000/*g* for 30 min at 4°C. The pellet was solubilized in 10 ml (1 ml/g cells) of guanidine buffer, 50 mM Tris-HCl pH 7.0, 5 mM EDTA, 8 M guanidine HCl, 5 mM DTT, and left for 1 h at 4°C. Insoluble material was removed by centrifugation at 100,000/*g* for 10 min.

Partial purification of the recombinant TFE*/TDFE from inclusion bodies

Inclusion bodies (about 15 mg) solubilized in guanidine buffer, containing more than 50% of recombinant protein, were used as starting material for purification of the recombinant protein by gel-filtration chromatography on Sephacryl S-300 (Amersham Pharmacia Biotech) packed into a column (100×1.5 cm). The column was equilibrated at 4°C with gel-filtration buffer (50 mM Tris-HCl pH 7.5, 4 M guanidine HCl, 5 mM DTT). The sample was loaded onto the column and the chromatography was performed for 18 h at flow rate of 25 ml/h. Fractions (about 6 mg total proteins) containing TFE*/TDFE were pooled and precipitated with 90% ethanol to eliminate guanidine HCl.

Protein refolding

The pooled fractions were subjected to a refolding procedure to obtain the active fusion protein; they were diluted to about 1 mg/ml protein using 4 M guanidine HCl, 5 mM DTT. Of the diluted solution 6 ml was slowly mixed to 60 ml 50 mM Tris HCl pH 7.5, 0.5 M NaCl, 2 mM DTT using a peristaltic pump at a flow rate of about 3 ml/h, while stirring gently with a magnetic stirrer. The diluted protein solution was then concentrated and ultrafiltered to about 1 mg/ml using a stirred cell on a UF module (PM-10; Amicon).

Results and discussion

The aim of this study was the production of a bifunctional fusion enzyme able to form trehalose at high temperatures starting from starch and dextrins. In many sequential enzymatic reaction the proximity of enzymes may provide highly attractive advantages for the whole process. Enzyme proximity allows the transfer of a reaction intermediate directly to the active site of the next enzyme in sequential reactions, and therefore the overall reaction rate of whole enzymatic process could be increased by preventing diffusion of intermediate (Béguin 1999). The fusion protein was partially purified from *E. coli* crude extract and its capability to produce trehalose at high temperatures was analyzed. In addition, we partially purified the fusion protein from inclusion bodies.

Construction of plasmid pTrcTFE*/TDFE

To test the effect of the physical proximity of the two enzymes catalyzing trehalose production from dextrins the *S. solfataricus* genes encoding TFE and TDFE were fused and the recombinant bifunctional enzyme (TFE*/TDFE) was expressed in *E. coli*. Gene fusion was carried out so that the C-terminus of TFE* was in frame with N-terminus of TDFE. To improve the expression of the fusion protein we placed at the N-terminus the TFE enzyme, since our previous studies demonstrated that the expression level of this protein is higher than that detected for TDFE. To obtain the fusion enzyme the coding sequence of TFE was amplified by PCR as described above. The TFE* and the TDFE coding sequence were sequentially cloned into pTrc99A, previously made end-compatible, and the recombinant plasmid was named pTrcTFE*/TDFE. The linker was designed to facilitate the recombinant DNA construction without deleting any amino acid of either protein moiety, resulting in the insertion of the four amino acid Ser-Gly-Ser-Gly.

Purification of recombinant TFE*/TDFE fusion enzyme

The enzyme was partially purified from the cell-free extract of *E. coli*, as described above, although most of the recombinant protein was found in the insoluble fraction. The soluble fraction (435 mg protein with a specific activity of 0.0028 U/mg) was heat-treated (see above) to denature mesophilic host proteins, as reported in our previous work (de Pascale et al. 2001). The purification factor was sixfold, with an activity recovery of about 80%.

Production of trehalose from malto-oligosaccharides

Enzymatic activity of the bifunctional protein was analyzed by HPAEC using malto-oligosaccharides as substrates at 75°C. In particular, analysis of the reaction kinetic was performed using maltohexaose as substrate (Fig. 1). The recombinant fusion enzyme converts maltohexaose into trehalose at high temperature, as does the equimolar mixture of TDFE and TFE. The rate of trehalose formation, however, is higher in the presence of the fusion enzyme, giving a yield of 13% of trehalose, relative to the complete substrate transformation, in 15 min (see Fig. 1), while an equimolar mixture of the individual enzymes gives only 5% in the same experimental conditions. In agreement with this observation, the intermediate product formed by TDFE activity, trehalosyl-maltotetraose, is easily detected in reactions with the two separate enzymes, while it is absent in reactions with the fusion protein, suggesting that it is immediately hydrolyzed to produce trehalose and maltotetraose (data not shown and de Pascale et al. 2001). Thermostabilities of the individual recombinant enzymes and of the recombinant fusion protein resulted similar, in fact the fusion enzyme retained 70% of its initial activity at 75°C for 6 h (data not shown). As shown in Fig. 1, after 3 h maltohexaose was completely transformed in an equimolar mixture of trehalose and maltotetraose. In this reaction maltohexaose has been first converted into trehalosyl-maltotetraose (not detected) that was then hydrolyzed to produce trehalose and maltotetraose. The conversion rate of maltotetraose into trehalosyl-maltose and then into trehalose is slower than the rate of its hydrolysis into smaller oligosaccharides (maltotriose, maltose, and glucose). This observation can be explained assuming that maltotetraose could not be a suitable substrate for the fusion enzyme. Figure 1 shows that small amounts of these malto-oligosaccharides can be formed starting from both maltotet-

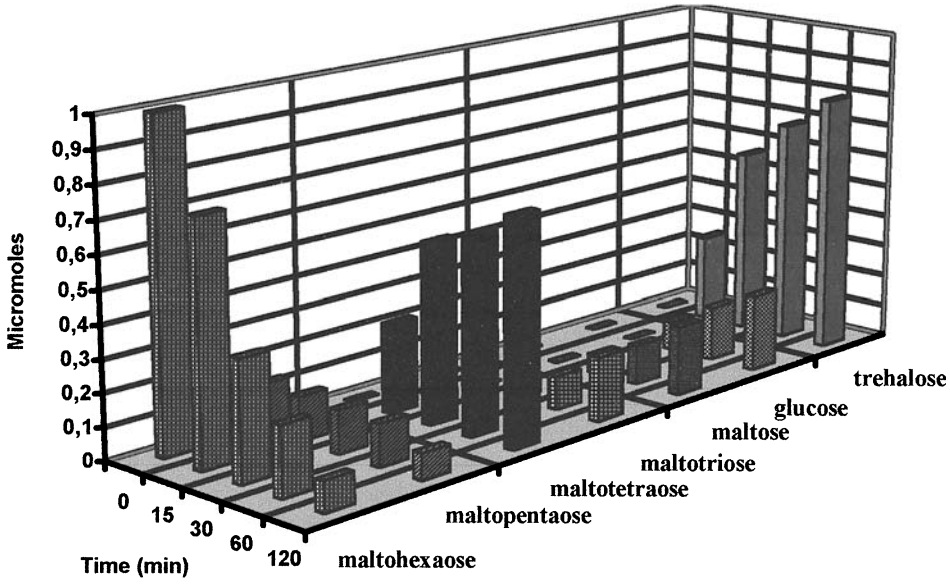

Fig. 1. Kinetic analysis of the reaction catalyzed by TFE*/TDFE fusion enzyme. The reaction mixture (1.5 ml) containing 1 mg maltohexaose and 15 mU enzyme was incubated at 75°C, pH 5.5, for 3 h. Samples were withdrawn at different times and reaction products were analyzed by HPAEC

raose and maltopentaose; the latter sugar is present as impurity (5–10%) of the substrate (M6). It has been reported that these side products are formed through parasite reactions catalyzed by both enzymes (Gueguen et al. 2001; Kato et al. 1996a, 1996b).

Recently Kim et al. (2000) reported the construction of a mesophilic fusion protein produces trehalose at 40°C. The bifunctional enzyme obtained in our experiments from *S. solfataricus* would be much more efficient for industrial trehalose production due to the possibility of operating at the high temperature required to reduce the risk of contamination and medium viscosity. Moreover, we propose an easier technique for the preparation of the recombinant fusion enzyme responsible of trehalose production, as described above.

Recovery of TFE*/TDFE fusion enzyme from inclusion bodies

The crude extract of *E. coli* recombinant cells revealed a poor level of the protein of interest; thus it would be useful to recover the enzyme from the insoluble fractions. SDS-PAGE analysis of extracts from recombinant bacteria (Fig. 2) shows a band corresponding to the expected molecular weight for the fusion enzyme.

Overexpression of cloned or synthetic genes in *E. coli* often results in the formation of insoluble proteins in inclusion bodies. Within the past decade specific methods and strategies have been developed to obtain active recombinant proteins from inclusion bodies (De Bernardez Clark 1998), which can be easily separated from other cell components by centrifugation, and then solubilized by denaturants such as guanidine hydrochloride (Gdn-HCl) or urea. We used inclusion bodies as starting material for a partial purification of TFE*/TDFE by gel filtration chromatography (Sephacryl S-300) performed in denaturant conditions.

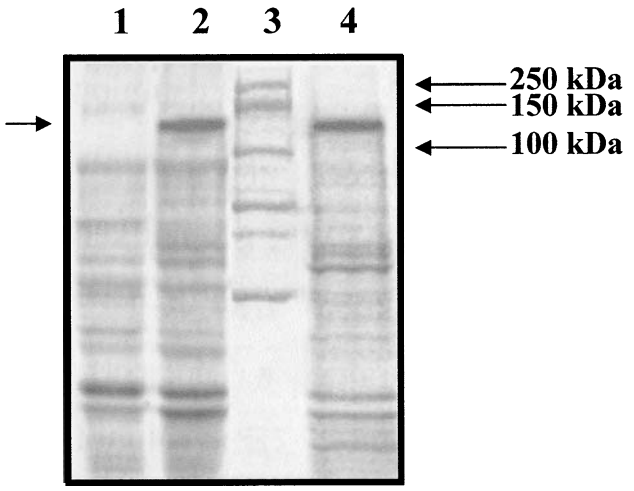

Fig. 2. SDS-PAGE analysis of recombinant *E. coli* (Rb791) extracts. *Lane 1* Insoluble fraction of bacteria transformed with pTrc99A; *lane 2* insoluble fraction of bacteria transformed with pTrcTFE*/TDFE; *lane 3* molecular mass standards; *lane 4* inclusion bodies prepared from bacteria transformed with pTrcTFE*/TDFE. Arrow Fusion protein

Fractions containing the protein (fractions 2–6, see Fig. 3), were collected and renatured by the dilution and dialysis refolding process (De Bernardez Clark 1998). Pooled fractions revealed a specific activity of 0.8 U/mg proteins. This purification/renaturation procedure seems to be much more efficient than the partial purification described for the cell-free extract, as the enzyme specific activity recovered from inclusion bodies, was about 280-fold higher than the corresponding one obtained from the crude extract.

In addition, N-terminal sequence analysis was performed on partially purified fractions (see above). The result of this determination confirms the presence of TFE N-terminal sequence, as expected. In fact, the following amino acids: M-T-F-A-Y-K-I were identified.

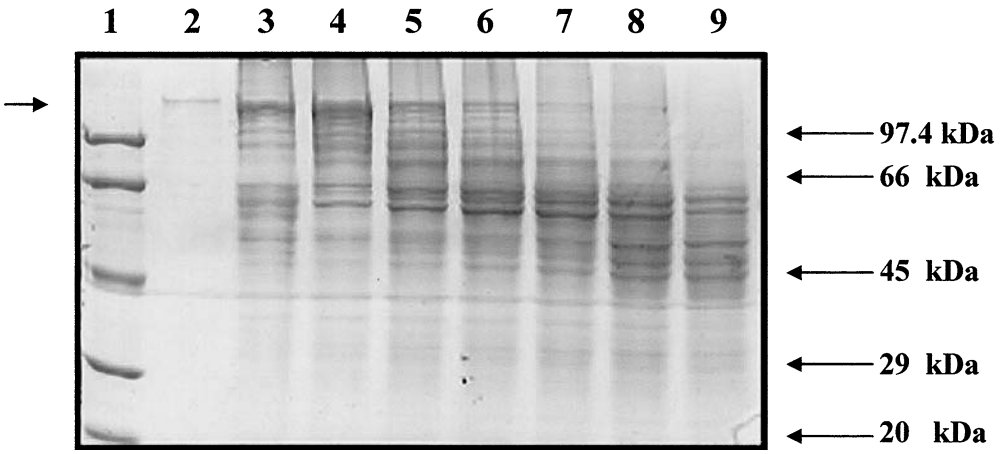

Fig. 3. SDS-PAGE analysis of inclusion bodies partially purified by S-300 chromatography. *Lane 1* Molecular mass standards; *lanes 2–9* fractions eluted from the column, containing TFE*/TDFE

For industrial application, it appears that the critical point for a convenient biotechnological production of trehalose is the complex catalytic mechanism of its biosynthesis. To overcome this complexity the expression of the fusion TFE*/TDFE protein might significantly improve trehalose production, increasing the reaction rate, due to the bifunctionality of one single polypeptide chain. Furthermore the thermophilic TFE*/TDFE fusion protein offers the possibility of operating at high temperatures, thus preventing microbial contamination and lowering medium viscosity, which usually severely affects carbohydrates industry.

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