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ORIGINAL PAPER

Purification and biochemical characterization of a native invertase from the hydrogen-producing *Thermotoga neapolitana* (DSM 4359)

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Abstract This is the first report describing the purification and enzymatic properties of a native invertase (β-D-fructosidase) in *Thermotogales*. The invertase of the hydrogen-producing thermophilic bacterium *Thermotoga neapolitana* DSM 4359 (hereby named *Tn*i) was a monomer of about 47 kDa having an amino acid sequence quite different from other invertases studied up to now. Its properties and substrates specificity let us classify this protein as a solute-binding protein with invertase activity. *Tn*i was specific for the fructose moiety and the enzyme released fructose from sucrose and raffinose and the fructose polymer inulin was hydrolyzed in an endo-type fashion. *Tn*i had an optimum temperature of 85°C at pH 6.0. At temperatures of 80–85°C, the enzyme retained at least 50% of its initial activity during a 6 h preincubation period. *Tn*i had a K_m and k_{cat}/K_m values (at 85°C and pH 6.0) of about 14 mM and 5.2×10^8 M^{-1} s^{-1}, respectively.

Keywords *Thermotogales* · *Thermotoga neapolitana* · Thermophilic · Invertase · Solute-binding protein

Dedicated to the memory of Prof. R. A. Nicolaus, founder of the Institute (1968).

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Abbreviations
*Tn*i *Thermotoga neapolitana* invertase
*Tm*i *Thermotoga maritima* invertase

Introduction

Several studies have already shown the ability of various microorganisms to produce hydrogen from sugars (also contained in organic wastewaters). For example, thermophilic *Bacteria* and *Archaea*, growing at temperatures above 60°C, often show higher values of hydrogen yield as compared to those of mesophilic bacteria. Moreover, thermophilic microorganisms can be readily inactivated and reactivated after storage at room temperature for several months, while monocultures of mesophilic bacteria can be easily contaminated. Members of *Thermotogales* order are thermophilic, rod-shaped, gram-negative, anaerobic and obligately heterotrophic bacteria, widely distributed in geothermal environments and playing an important role as decomposers of organic matter. Owing to their biotechnological potential, many research groups have focused their attention on these anaerobes that are able to utilize monomeric and polymeric carbohydrates for hydrogen production.

In studies carried out by us under an Italian framework project and focused to develop methodologies to obtain biohydrogen from thermophilic microorganisms, *Thermotoga neapolitana* DSM 4359 has been studied and its ability to produce hydrogen was described in a patent (Van Ooteghem 2005) reporting high hydrogen yields on mono- and disaccharides. In fact, glucose and sucrose have been extensively studied as carbon substrate for biohydrogen production and as model substrates for research purposes

due to their easy biodegradability and their presence in different carbohydrate-rich wastewaters and agricultural wastes.

Principally, there are two types of enzymes that can hydrolyze sucrose: the typical invertases, which are β-fructosidases (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26—GH32 & GH68, clan GH-J, and GH100) and some oligo-α-1,6-glucosidases (EC 3.2.1.10—GH13, clan GH-H) and α-1,4-glucosidases (α-D-glucoside glucohydrolase, EC 3.2.1.20—GH4, GH13 (clan GH-H), GH31 (clan GH-D), GH63 (clan GH-G) and GH97) with a broad substrate specificity. Moreover, other two kinds of enzymes hydrolyze sucrose: amylosucrases (EC 2.4.1.4), glucansucrose enzymes belonging to glycoside-hydrolase (GH) family 13, which catalyze de novo synthesis of an amylose-like polymer from sucrose (Potocki de Montalk et al. 2000) and sucrases (EC 3.2.1.48—GH31, clan GH-D), which hydrolyze sucrose and maltose by an α-D-glucosidase-type action.

Invertases are one of the earliest discovered enzymes and their name was coined because these enzymes produce "invert" sugars, a 1:1 mixture of dextrarotatory D-glucose and levorotatory D-fructose (Alberto et al. 2004). This type of enzyme catalyzes the release of β-fructose from non-reducing termini of various β-D-fructofuranosidase substrates. The transfructosylation activity of invertase indicated that the enzyme operates with a molecular mechanism leading to overall retention of anomeric configuration (Alberto et al. 2004).

In *Thermotogales* studies on enzymes able to degrade oligo- and polysaccharides have been already reported (Liebl et al. 1998 and references therein), but little is known about the enzymology of di- and trisaccharide utilization by these thermophiles. Nothing is known about thermophilic invertases, with the exception of the cloned one (EC 3.2.1.26—GH32, clan GH-J) from the hyperthermophilic *T. maritima* (Liebl et al. 1998; Alberto et al. 2004, 2006).

In addition to the invertase, *T. maritima* produces also a β-glucosidase with a broad substrate specificity, which is active against various β-glucosides and β-galactosides (Gabelsberger et al. 1993). Until now, an invertase has not been described in *T. neapolitana*, while the β-glucosidase B was expressed in *Escherichia coli* and used as an efficient catalyst for the synthesis of alkyl glucosides by transglycosylation (Park et al. 2005; Turner et al. 2007). In fact, the crude homogenate from *T. neapolitana,* strain DSM 4539, was used by Tramice et al. (2007) for performing a series of enzymatic transglycosylation reactions.

In order to improve hydrogen production, *T. neapolitana* DSM 4359 was grown on sucrose and, under this experimental condition, the microorganism produced a significant amount of hydrogen. In order to understand the metabolism, enzyme(s) involved in sucrose catabolism was also studied. It was found that the sugar was hydrolyzed by invertase while no α-glucosidase activity was evidenced. In this paper we describe for the first time the purification and properties of a native invertase in *Thermotogales*.

Materials and methods

Bacterial strain and culture conditions

Thermotoga neapolitana (DSM 4359^T) was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany).

The anaerobic cultivation technique employed was that described by Hungate (1969) and then modified by Balch and Wolfe (1976).

The bacterium was grown in the following medium (modified from ATCC 1977 medium) (in g/l): NaCl 10.0; KCl 0.1; $MgCl_2{\cdot}6H_2O$ 0.2; NH_4Cl 1.0; K_2HPO_4 0.3; KH_2PO_4 0.3; $CaCl_2{\cdot}2H_2O$ 0.1; cysteine-HCl 1.0; yeast extract 2.0; tryptone 2.0; glucose 5.0; vitamin solution (DSM medium 141) 10.0 ml; trace element solution (DSM medium 141) 10.0 ml; distilled water 1,000 ml. Also resazurin 0.0001% was added to the medium.

Vitamin solution contained (in mg/l): pyridoxine–HCl 10.0; thiamine–HCl 5.0; riboflavin 5.0; nicotinic acid 5.0; calcium D-(+)-pantothenate 5.0; *p*-aminobenzoic acid 5.0; lipoic acid 5.0; biotin 2.0; folic acid 2.0; vitamin B_{12} 0.1; distilled water 1,000 ml.

Trace element solution contained (in g/l): nitrilotriacetic acid 1.5; $MgSO_4{\cdot}7H_2O$ 3.0; $MnSO_4{\cdot}2H_2O$ 0.5; NaCl 1.0; $FeSO_4{\cdot}7H_2O$ 0.1; $CoSO_4{\cdot}7H_2O$ 0.18; $CaCl_2{\cdot}2H_2O$ 0.1; $ZnSO_4{\cdot}7H_2O$ 0.18; $CuSO_4{\cdot}5H_2O$ 0.01; $KAl(SO_4)_2{\cdot}12\ H_2O$ 0.02; H_3BO_3 0.01; $Na_2MoO_4{\cdot}2H_2O$ 0.01; $NiCl_2{\cdot}6H_2O$ 0.025; $Na_2SeO_3{\cdot}5H_2O$ 0.0003; distilled water 1,000 ml. The medium was always prepared aerobically and the pH was adjusted to 8.0 with 1 M NaOH at room temperature. Oxygen was removed by heating the batch reactors until the solution was colorless and then flushing their contents under a stream of O_2-free N_2 gas. The vessels were immediately sealed with aluminum crimps seals and autoclaved. The same medium was prepared by replacing glucose with sucrose (6 g/l).

A stock bacterial inoculum solution was transferred to the fresh medium and the serum bottles were incubated without shaking at 80°C for 24 h.

Purification of the enzyme

Batch cultures of *T. neapolitana* were used as inoculum for cultivation in a fermenter (Biostat-D, B. Braun Biotech International) at a working volume of 25 l: the inoculum

size was 8% of cultures in the exponential growth phase. The experiments were conducted at 80°C without stirring and under anoxic conditions by sparging N_2; the pH was controlled at 7.0 (measured at room temperature) by addition of 1 M NaOH.

At regular intervals, samples were drawn for the determination of growth as optical density at 540 nm and after 24 h of incubation, cells were harvested by centrifugation (Alfa Laval). Wet cells (15 g) were suspended in 50 mM sodium phosphate buffer pH 6.0 containing 5 mM phenylmethylsulfonyl fluoride (PMSF) and disrupted by threefold passage through a French pressure cell (Thermo Electron Corporation) at 1,500 psi for 2 min. Crude extract was gently stirred for 30 min at 37°C and then centrifuged at 10,000 rpm for 20 min at 4°C to remove unbroken cells. The resulting supernatant was recentrifuged at 10,000 rpm for 1 h at 4°C to obtain the soluble fraction (47 mg/ml), which was used for the following purification steps.

The sample was loaded onto a Q-Sepharose Fast Flow column (1.6 × 23 cm, Pharmacia) equilibrated with 300 ml of 50 mM sodium phosphate buffer pH 6.0 (buffer A) at a flow rate of 3 ml/min and the same flow rate was used also for the run. Bound proteins were eluted by applying a linear NaCl gradient (0–1 M) in buffer A and fractions of 12 ml were collected. The active fractions were pooled, concentrated and dialyzed against buffer A by ultrafiltration on Amicon, YM 10 filters (Millipore, Billerica, MA, USA).

Finally, aliquots of the active pulled fractions (100 μg of protein) were further purified by reverse-phase HPLC on a Vydac C18 column (25 × 0.46 cm, 5 μm) using a Waters HPLC instrument and an elution system consisting of 0.1% TFA (solvent A) and 95% acetonitrile (ACN) in 0.07% TFA (solvent B). Protein separation was achieved by means of a linear gradient from 10 to 70% solvent B over 60 min. Elution was monitored at 220 nm. Four main fractions were manually collected, dried in a Speed-Vac centrifuge (Savant) and stored at −20°C.

Measurement of the molecular mass and N-terminal amino acid sequencing

The apparent molecular mass of the native enzyme was determined by gel filtration chromatography on a prepacked Sephacryl S-100 16/60 (Pharmacia) adapted to an AKTA Fast Protein Liquid Chromatography system using a standard gel filtration calibration kit (670–1.35 kDa). The column was equilibrated with 50 mM sodium phosphate buffer pH 6.0, 0.15 M NaCl at a flow rate of 1 ml/min; the elution was made with the same buffer, but with a flow rate of 0.5 ml/min and fractions (2 ml for each one) were assayed for invertase activity.

Native polyacrylamide gel electrophoresis was performed at an alkaline pH as described by Davis (1964) and an electrophoresis calibration kit (LMW, 97–14.4 kDa) was used.

The apparent molecular mass of the enzyme and its purity were confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using Mini Protean II apparatus (Bio-Rad).

The electrophoresis was carried out as described by Laemmli (1970) using 10% acrylamide in the resolving gel, 5% acrylamide in the stacking gel and Tris-glycine buffer, pH 8.3 containing SDS 1 g/l as running buffer. Markers for molecular mass determination were perfect protein markers (LMW, 97–14.4 kDa). Protein bands were stained with 0.1% Coomassie brilliant blue R-250 and destained with a mixture of distilled water/methanol/acetic acid (50:40:10).

MALDI-TOF-MS analyses of the HPLC fractions were carried out on aliquots containing about 5 pmol of protein samples, mixed with 1 μl of matrix (10 mg/ml CHAC in 50% ACN), deposited onto MALDI sample probe and dried under ambient conditions. Mass spectra were generated on a MALDI-TOF mass spectrometer Voyager DE™ PRO (Applied Biosystems, Foster City, CA, USA), operating in linear delay extraction and positive-ion mode. The laser intensity (N2, 337 nm) was set just above the ion generation threshold and mass spectra were acquired by accumulating 100 laser shots from each sample. Mass spectra were acquired in the m/z range 10,000–80,000 and externally calibrated using the average doubly and singly charged peaks originated from bovine serum albumin (m/z 33,216 and 66,431, respectively).

For N-terminal amino acid sequencing, the blotting condition was that indicated by a Bio-Rad instruction for protein sequencing by Sequi-Blot PVDF membrane (Matsudaira 1987). Sequencing was performed using an Applied Biosystems Procise 494 automatic sequencer, equipped with on-line detection of phenylthiohydantoin amino acids. Protein sequence databases were searched using the FASTA and BLAST software at the National Center for Biotechnology Information server (NCBI, URL address: http://www.ncbi.nlm.nih.gov).

Protein identification by peptide mass fingerprint strategy

Coomassie-stained protein bands were manually excised from gels and destained with 50% acetonitrile (ACN) in 100 mM ammonium bicarbonate and dehydrated in ACN. Proteins contained in gel pieces were treated with 10 mM dithiothreitol in 100 mM ammonium bicarbonate at 56°C for 1 h to reduce disulfide bridges and alkylation of the cysteine residues was carried out with 50 mM iodoacetamide in

100 mM ammonium bicarbonate at room temperature in the dark for 30 min.

In-gel tryptic digestion and mass spectrometric analyses were carried out as already reported by Tosco et al. (2005).

Protein identification was achieved using the MALDI mass spectral data for searches against the NCBInr database using the MASCOT search algorithm (http://www.matrixscience.com) (Pappin et al. 1993). Parameters for the searches were as follows: no taxonomic category was specified, trypsin as enzyme, carbamidomethyl as fixed modification for cysteine residues and methionine oxidation as variable modification, up to one missed cleavage allowed and 30 ppm as mass tolerance for the monoisotopic peptide masses.

Enzyme assay and protein determination

Invertase activity was measured by the increase of reducing groups with the dinitrosalicylic acid method (Bernfeld 1955). Standard assay mixtures (500 µl) contained 50 mM sodium phosphate buffer pH 6.0, 1.5% sucrose and appropriately diluted enzyme solution. After 30 min at 85°C the reaction was stopped on ice and 500 µl of 1% dinitrosalicylic acid solution was added. The samples were boiled at 100°C for 5 min and then immediately cooled on ice. The absorbance was measured at 546 nm against 50 mM sodium phosphate buffer pH 6.0. The dinitrosalicylic acid color reaction was calibrated under the assay conditions using an equimolar mixture of glucose and fructose.

One unit of invertase activity was defined as the amount of enzyme that liberates 1 µM fructose/min.

The protein concentration was determined using Bio-Rad protein assay kit with bovine serum albumin as a standard protein according to Bradford (1976) method.

Effects of pH and temperature on invertase activity

The dependence of invertase activity on the pH was monitored at 85°C in the range 3.0–10.0 with the following buffers (50 mM): glycine–HCl (pH 3.0), acetate buffer (pH 3.5–5.5), Na_2HPO_4–NaH_2PO_4 (pH 6.0–8.0) and glycine–NaOH (pH 9.0–10.0).

The influence of temperature on sucrose hydrolysis was studied over the range 50–100°C in 50 mM sodium phosphate buffer pH 6.0. In both cases, the relative activity was measured by the standard assay.

The rates of thermoinactivation were determined by preincubating the enzyme (2.3 µg/ml) in 50 mM sodium phosphate buffer pH 6.0 at various temperatures (80–100°C). Aliquots were drawn at defined intervals (15 min to 6 h) and the residual activity was measured under the standard assay conditions described above.

Effects of metal ions on invertase activity

For determining the influence of metal ions, the invertase activity was assayed in the standard conditions in the presence of 2 and 5 mM of various metal ions. The activity assayed without metal ions was considered to be the reference value (100%).

Effects of denaturing agents on invertase activity

The enzyme solution was preincubated at 85°C for 30 min in the presence of various chemical reagents and then the residual invertase activity was measured under the standard assay conditions. Invertase activity assayed without denaturing agents was considered to be 100%.

Substrate specificity and determination of kinetic constants

All natural substrates, chromogenic aryl-glycosides and dinitrosalicylic acid were purchased from Sigma.

Invertase activity was assayed in the standard conditions with the following natural substrates (1.5%): raffinose, inulin (from dahlia tubers), maltose, lactose, trehalose, melibiose, cellobiose, laminarin (from *Laminaria digitata*), starch (from potato) and xylan (from birchwood).

To evaluate the hydrolysis of chromogenic aryl-glycosides the enzyme was incubated in the standard assay condition using 5 mM of aryl-substrates, instead of sucrose. The reaction was stopped on ice and 500 µl of 1 M Na_2CO_3 was added. The absorbance was measured at 420 nm against 50 mM sodium phosphate buffer pH 6.0.

The kinetic parameters for sucrose hydrolysis were estimated from Eadie–Hofstee plots of the cleavage velocities measured under standard assay conditions at substrate concentrations of 0.01–3.5% (0.3–103 mM).

Sugar analysis

The hydrolysis of mono- and oligosaccharides was monitored by thin layer chromatography (TLC) on 0.2-mm silica-gel-coated aluminum sheets (type 60; Merck, Germany) developed with butanol/acetic acid/distilled water (6:2:2). Spots were detected by a specific reagent for carbohydrates (α-naphtol).

Results

Purification of invertase

The invertase was expressed in a same amount when the microorganism was grown in the presence of glucose or

sucrose. The *T. neapolitana* invertase was purified as described in "Materials and methods" and purification steps are summarized in Table 1. The specific activity of the enzyme increased by 469.5-fold to 51833.5 U/mg protein with a 25.8% yield.

Molecular properties, N-terminal amino acid sequence and protein identification by peptide mass fingerprint (PMF) strategy

SDS-PAGE analysis of the HPLC purified invertase resulted in a single protein band with a mobility corresponding to a molecular mass of about 47 kDa (Fig. 1a), based on the comparison with the mobility of the marker proteins. The same molecular mass was obtained by native gel analysis (Fig. 1b) and by gel filtration separation by FPLC thus indicating that the invertase from *T. neapolitana* is a monomeric protein.

Noteworthy, the MALDI-TOF-MS analysis of the HPLC fraction exhibiting invertase activity (Fig. 1c) showed the presence of a single strong peak at m/z 44,298 (other peaks at m/z 22,157, 14,772 and 11,070 were originated from doubly, triply and quadruply charged ions of the same protein), thus definitively ruling out the possibility that other minor protein contaminations could be responsible for the enzymatic activity.

The N-terminal sequence of the purified enzyme was VKITMTSGGVGKELEVLKKQ. On the basis of this N-terminal sequence and of the peptide mixture, obtained by reduction, alkylation and in-gel tryptic digestion of the purified enzyme and analyzed by MALDI-TOF-MS, no proteins with similar invertase activity were retrieved by database searches using BLAST or FASTA programs. Surprisingly, this sequence had a 100% alignment with extracellular solute-binding proteins, family 1 of *Thermotoga* sp. RQ2, *T. petrofila* RKU-1 and *Thermosipho melanesiensis* BI429. In particular, the protein identified by MALDI-TOF-MS had a high Mascot score of 145 (scores greater than 80 indicate reliable identification, $P < 0.05$) and 27% sequence coverage with an extracellular solute-binding protein, family 1 from *Thermotoga petrophila* RKU-1 (accession number gil148270088, MW 46229 Da).

The theoretical molecular weight of the identified protein is in good agreement with the experimental one, taking into account that a peptide signal 1–20 (theoretical molecular weight 2,195 Da) is cleaved during the maturation process and other amino acidic variations and/or post-translational modifications could be also present.

Protein identification was also confirmed verifying the sequence of six of the matched peptides by means of tandem mass spectrometric experiments carried out on a hybrid quadrupole-TOF instrument equipped with a static nano-electrospray source (data not shown).

These results, which confirm the results of the BLAST search, demonstrate that the purified enzyme shares a high sequence homology with the extracellular solute-binding protein from *T. petrophila* RKU-1, although an invertase activity has not been reported for this protein.

Moreover *Tn*i had 82, 75 and 72%, respectively, alignment with extracellular solute-binding proteins, family 1 of *Fervidobacterium nodosum* Rt17-B1, *Marinomonas* sp. MWYL1 and *Petrotoga mobilis* SJ95. In addition the N-terminal sequence of the purified *T. neapolitana* invertase corresponded to 100% to a *T. maritima* uncharacterized protein in bglA 3′ region (ORF2): this truncated sequence seems to codify for an exported protein (Liebl et al. 1994).

The sequence studies were repeated on three different preparations and the results obtained were the same already described.

Effects of pH and temperature on invertase activity

Enzymatic activity assayed in standard conditions over a broad pH range from pH 3.0 to pH 10.0 showed an optimum at pH 6.0 in 50 mM sodium phosphate buffer and more than 50% of the maximum activity was measured between pH 4.0 and 7.0: this optimum pH is typical for

Table 1 Purification steps of *Thermotoga neapolitana* invertase

Purification step	Volume (ml)	Total protein (mg)	U/ml	Total activity (U)	Specific activity (U/mg)	Purification factor (-fold)	Yield (%)
Crude extract	12	564	5,190.5	62,286	110.4	1	100
Q-Sepharose F.F.	2	129	21,666.7	43,333.4	335.9	3	69.6
Sephacryl S-100	3.6	3.24	11,506.7	41,424.1	12,785.2	115.8	66.5
HPLC	0.6	0.31	26,780.7	16,068.4	51,833.5	469.5	25.8

Assays of specific activity were carried out under the standard conditions at 85°C at pH 6.0. One unit was defined as the amount of enzyme that liberates 1 μM fructose/min under these conditions

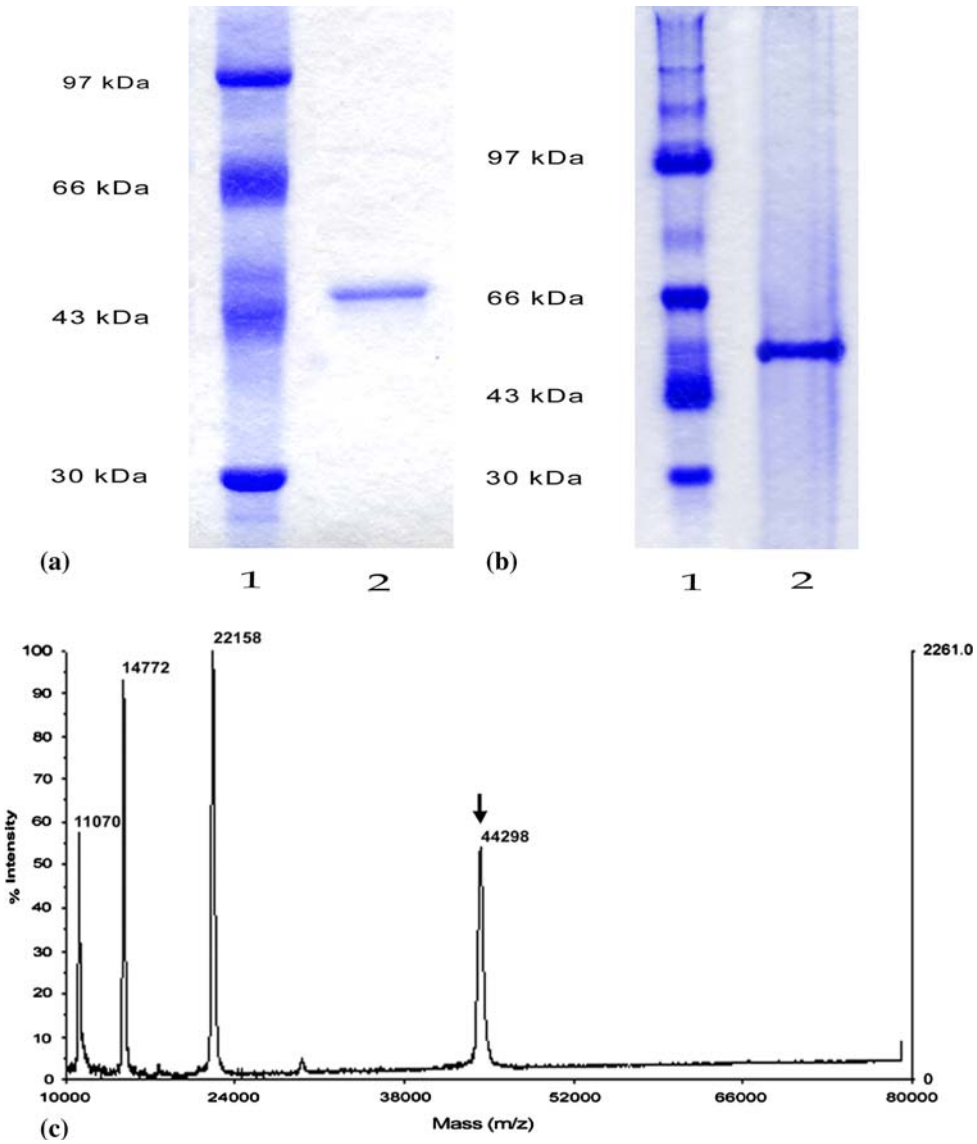

Fig. 1 **a**, **b** Sodium dodecyl sulfate and native polyacrylamide gel electrophoresis (10%), respectively, of the *T. neapolitana* invertase. *Lanes 1* low molecular mass markers (the sizes of the marker proteins are indicated; *2* purified invertase by HPLC; **c** MALDI-TOF-MS analysis of the HPLC purified enzyme

invertases (Fig. 2a). Moreover, this enzyme showed the highest activity at 85°C (Fig. 2b) under the standard assay conditions at the optimal pH.

Thermoinactivation of invertase

Thermostability data were obtained by preincubating purified invertase at a concentration of 2.3 μg/ml in 50 mM sodium phosphate pH 6.0 without any stabilizing additives at various temperatures and then measuring residual sucrase activity under the standard assay conditions. At temperatures of 80–85°C, the enzyme retained at least 50% of its initial activity during a 6 h preincubation period, but a loss of activity was observed at temperatures up to 90°C after 4 h of preincubation (Fig. 2c).

Effects of metal ions and denaturing agents on invertase activity

Various metal ions (Na^+, Mg^{2+}, Ba^{2+}, Ca^{2+}, Mn^{2+} and K^+) added as chloride salts at 2 mM had neither stimulatory or inhibitory effects on enzyme. Instead, the addition of 2 mM $CuSO_4$, $ZnSO_4$, $FeCl_2$ and $HgCl_2$ completely inhibited enzyme activity, suggesting the presence of thiolic groups in the catalytic site of the enzyme (Table 2), while EDTA (5–10 mM) had no significant effect on enzyme activity.

The presence of dithiothreitol and β-mercaptoethanol (1–5 mM) had no effect on sucrose hydrolysis by invertase and this suggested that disulfide-like bonds are not involved in preserving the enzymatic structure.

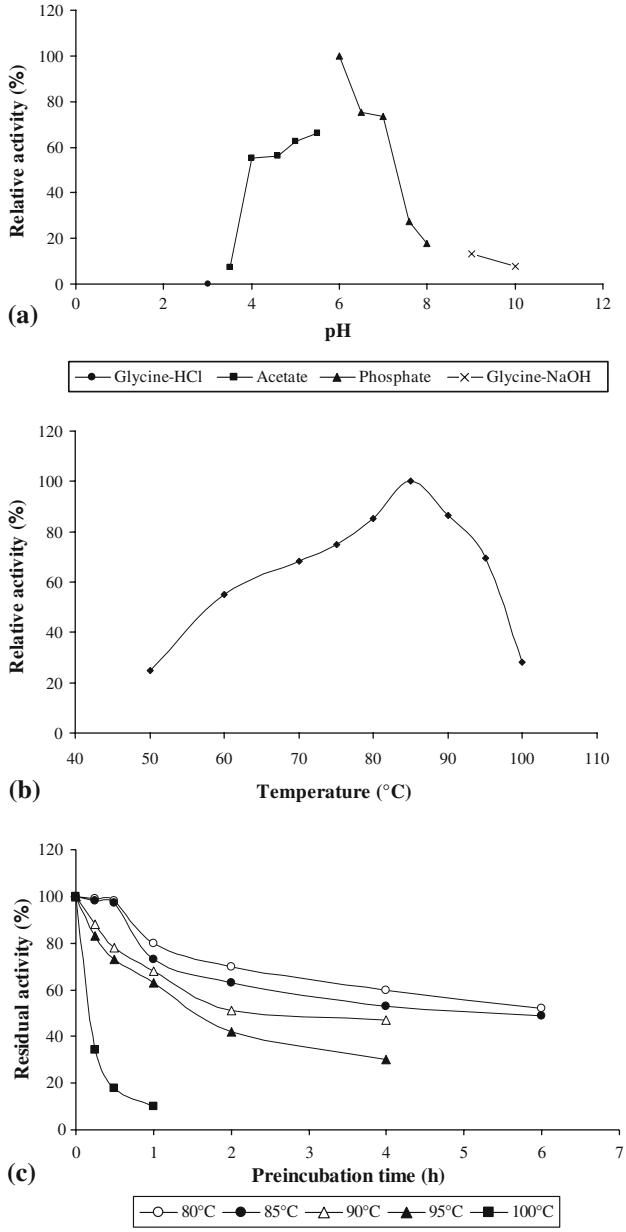

Fig. 2 Dependence from **a** pH, **b** temperature and **c** thermoinactivation profile

Urea and guanidine-HCl (2–4 M) inhibited enzyme activity, indicating that hydrogen bonds play an important role in protein stabilization. Moreover, the invertase produced by *T. neapolitana* was sensitive to tensioactives, as SDS and DBS (Table 3).

Substrate specificity and determination of kinetic constants

TLC and liquid assays analyses revealed that the *T. neapolitana* invertase liberated fructose from the following carbohydrates: sucrose [α-D-glucopyranosyl-(1 → 2)-β-D-fructofuranose], raffinose [α-D-galactopyranosyl-(1 → 6)-α-D-glucopyranosyl-(1 → 2)-β-D-fructofuranose] and inulin [a linear β-(2 → 1)-linked fructose polymer with a terminal β-(2 → 1)-linked glucose unit] (Figs. 3, 4). The fructose polymer was degraded with the appearance of oligomeric intermediates, probably indicating an endo-type action. A variety of other naturally occurring di-, tri- and polysaccharides were not attacked: maltose [α-D-glucopyranosyl-(1 → 4)-D-glucopyranose], trehalose [α-D-glucopyranosyl-(1 → 1)-α-D-glucopyranose], melibiose [α-D-galactopyranosyl-(1 → 6)-D-glucopyranose], lactose [β-D-galactopyranosyl-(1 → 4)-D-glucopyranose], cellobiose [β-D-glucopyranosyl-(1 → 4)-D-glucopyranose] and the polysaccharides laminarin [poly β(1 → 3)-glucan with some β-(1 → 6) interstrand linkages and branch points], starch and xylan.

Moreover, invertase was incubated with a number of nitrophenyl saccharides, but none of the tested chromogenic substrates (*p*Np-α-D-glucopyranoside, *p*Np-α-D-galactopyranoside, *p*Np-β-D-galactopyranoside, *p*Np-α-D-maltoside, *p*Np-β-D-maltoside, *p*Np-β-D-xylopyranoside, *p*Np-β-D-

Table 2 Effects of metal ions on invertase activity: the relative activity was assayed under the standard assay conditions in the presence of various metal ions

Metal ions	Concentration (mM)	Relative activity (%)
None	–	100
NaCl	2	102
	5	97
$MgCl_2 \cdot 6H_2O$	2	102
	5	96
$BaCl_2 \cdot 2H_2O$	2	102
	5	55
$CaCl_2 \cdot 2H_2O$	2	91
	5	83
$MnCl_2 \cdot 4H_2O$	2	86
	5	14
KCl	2	79
	5	56
$CoCl_2 \cdot 6H_2O$	2	64
	5	7
$CuSO_4 \cdot 5H_2O$	2	5
	5	0
$ZnSO_4 \cdot 7H_2O$	2	0.8
	5	0
$FeCl_2 \cdot 3H_2O$	2	0.8
	5	0
$HgCl_2$	2	0
	5	0

Table 3 Effects of denaturing agents on invertase activity: the relative activity was assayed under the standard assay conditions in the presence of various chemical reagents

Reagent	Concentration	Residual activity (%)
None	–	100
DL-dithiothreitol (DTT)	1 mM	134
	5 mM	116
β-mercaptoethanol	1 mM	106
	5 mM	99
N-bromosuccinimide (NBS)	1 mM	2
	5 mM	2
Ethylendiaminetetracetic acid (EDTA)	5 mM	112
	10 mM	91
Triton X-100	0.1%	91
	0.2%	75
Sodium dodecyl sulfate (SDS)	0.1%	48
	0.2%	25
Sodium dodecyl benzenesulfonate (DBS)	0.2%	8
	0.4%	5
Urea	2 M	29
	4 M	6
Guanidine-HCl	2 M	2
	4 M	0

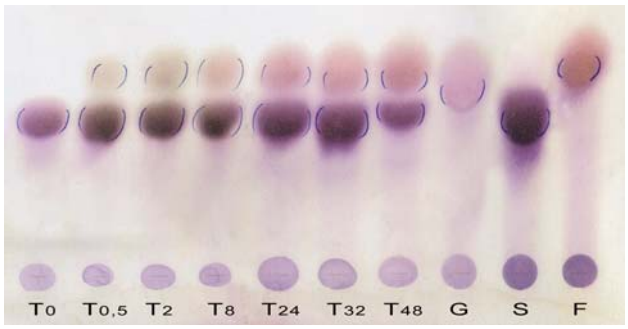

Fig. 3 Thin-layer chromatography (TLC) analysis of sucrose cleavage by purified invertase. Enzyme diluted with 50 mM sodium phosphate buffer pH 6.0 (final concentration 2.3 μg/ml) was incubated at 85°C with 1.5% sucrose. After 30 min, 2, 8, 24, 32 and 48 h, samples were subjected to TLC separation. *Lanes 1–7* samples at various incubation times; *8–10* sugar standards glucose, sucrose and fructose, respectively. The *line* of sample application is drawn at the *bottom*

lactopyranoside, *p*Np-α-L-arabinopyranoside, *p*Np-β-L-arabinopyranoside) was hydrolyzed.

The kinetic parameters for sucrose hydrolysis were estimated from Eadie-Hofstee plots of the cleavage velocities measured under standard assay conditions at substrate concentrations of 0.01–3.5% (0.3–103 mM). The results are summarized in Table 4.

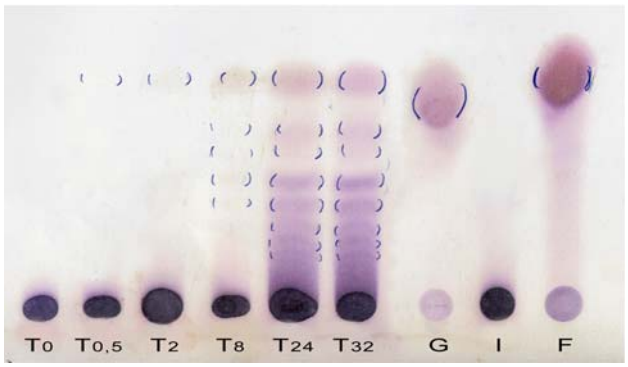

Fig. 4 TLC analysis of inulin cleavage by purified invertase. Enzyme diluted with 50 mM sodium phosphate buffer pH 6.0 (final concentration 2.3 μg/ml) was incubated at 85°C with 1.5% inulin. Then, after 30 min, 2, 8, 24 and 32 h, aliquots were drawn for TLC analysis. *Lanes*: *1–6* samples at various incubation times; *7–9* sugar standards glucose, inulin and fructose, respectively. The *line* of sample application is drawn at the *bottom*

Table 4 Kinetic parameters for the hydrolysis of sucrose by *T. neapolitana* invertase

Substrate	K_m (mM)	V_{max} (μM min^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Sucrose	14	220	7.3×10^6	5.2×10^8

Kinetic parameters K_m and V_{max} were determined graphically from Eadie–Hofstee diagrams. k_{cat} value was calculated assuming a molecular mass of about 47 kDa for invertase

Discussion

Members of *Thermotogales* order possess an array of important hyperthermophilic glycosyl hydrolases (Henrissat and Coutinho 2001). This is the first study reporting the isolation and characterization of a native invertase (β-D-fructosidase) from *Thermotoga neapolitana* (DSM 4359) able to produce H_2 in a molar ratio 2.5 with glucose. Previous reports regarded a cloned invertase from *Thermotoga maritima* expressed in *E. coli*. The purified invertase from *T. neapolitana* was not inducible because the same amount was found when the microorganism was grown both on glucose and on sucrose medium.

The N-terminal amino acid sequence of invertase from *T. neapolitana* revealed a surprisingly high degree of similarity (100%) with extracellular solute-binding proteins, family 1 of *Thermotoga* sp.RQ2, *Thermotoga petrofila* RKU-1 and *Thermosipho melanesiensis* BI429. Moreover, the N-terminal sequence of the purified *T. neapolitana* invertase corresponded to 100% to a sequence of a *Thermotoga maritima* uncharacterized protein in bglA 3′ region (ORF2), which seemed to codify for an exported protein (Liebl et al. 1994).

These solute-binding proteins family 1 belong to the family of ATP-binding cassette (ABC) transporters, which use the energy of ATP hydrolysis to take up solutes. These ATP-linked primary transporters are ubiquitous among sequenced prokaryotic genomes (Paulsen et al. 2000), suggesting that they were among the first transport systems to evolve (Nanavati et al. 2002). Several papers were published on these transporters in *Thermotogales* (Galperin et al. 1996, 1997; Nelson et al. 1999; Nguyen et al. 2004; Noll et al. 2008) and the results supported the hypothesis that many transporters family are involved in carbohydrates transport and explained the observation that glycoside hydrolases are often colocalized with these genes (Conners et al. 2005).

In addition, biological function for the extracellular solute-binding protein, family 1 of *Thermotoga petrofila* RKU-1 (accession number ABQ46972) has been inferred by sequence homology and no direct evidence of the enzymatic activity of the purified protein has been reported up to now. Moreover, it is quite interesting that solute-binding proteins are involved in the uptake of carbohydrates and they have a sugar-binding domain, which could be also required in a protein exhibiting invertase activity.

The N-terminal sequence of the invertase from *T. neapolitana* had no similarity with invertase from *T. maritima* and also with β-glucosidase from *T. neapolitana.*

The 469.5-fold purification and 25.8% yield achieved in the procedure carried out for invertase from *T. neapolitana* were higher with respect to those obtained from cloned *T. maritima* invertase (hereby named *Tm*i) (76-fold and 14% yield). As these enzymes could be used for industrial applications, it is important to underline that the developed purification procedure assures good enzyme yields.

The optimal temperature and thermostability of *Tn*i and *Tm*i reflected the growth temperatures of the microorganisms, which were 85°C and 90–95°C, respectively.

The *Tn*i showed very similar features with those from *T. maritima*, e.g., molecular weights and pH, which were 47 kDa and pH 6.0, respectively, with respect to 50 kDa and pH 5.5.

Moreover, the substrates specificity and effects of metal ions and denaturing agents were quite superimposing, even if *Tn*i acted on *p*Np-β-D-glucopyranoside, but we had the same result also by using the invertase isolated from baker's yeast (Sigma) in its standard assay conditions (data not showed).

Moreover, the *Tn*i could be an endo-β-fructosidase because of the presence of oligomeric intermediates after polysaccharide (as inulin) hydrolysis in comparison with *Tm*i, which appeared to be an exo-type invertase (Liebl et al. 1998) and in fact only fructose was produced after polysaccharide hydrolysis.

Two catalytic activities of the *Tn*i may be of interest from a biotechnological point of view, i.e., the invertase activity and the inulinase activity. Invertase obtained from yeast has been used for many years in the confectionery industry and for the production of invert sugar. However, the heat lability of the yeast invertase is a major drawback for its application (Hasal et al. 1992; Badr et al. 1994). Instead a thermophilic enzyme like *T. neapolitana* invertase may be advantageous for industrial sucrose inversion. Also, its thermostable inulinase activity could be of considerable biotechnological value. Inulin, a natural storage polysaccharide found in plants such as Jerusalem artichoke, dandelion, chicory, *Vernonia herbacea* and other Compositae, is a linear polymer consisting of $\beta(2 \rightarrow 1)$-linked fructosyl units with a terminal glucose unit. The enzymatic hydrolysis of this renewable carbohydrate can be used for the production of high-fructose syrups (Vandamme and Derycke 1983; Allais et al. 1986).

Nevertheless, even if the N-terminal sequence of *Tn*i was different from other invertases studied up to now (Kunst et al. 1974; Schmid et al. 1982; Blatch and Woods 1993; Lee and Sturm 1996; Liebl et al. 1998), its properties and substrates specificity let us classify this protein as a solute-binding protein with invertase activity.

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