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Biochemical characterization of a recombinant thermostable β-mannosidase from *Thermotoga maritima* with transglycosidase activity

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ABSTRACT

A β-mannosidase gene (TM1624) from *Thermotoga maritima* MSB8, the hyperthermophilic bacterium was expressed as a soluble C-terminal His-tagged protein in *E. coli*. Heat treatment of cell lysate followed by metal affinity- and anion-exchange chromatographic techniques the recombinant β-mannosidase was purified to apparent homogeneity. The recovery of the purified protein from the crude lysate was 23%. Results of SDS-PAGE analysis (96.8 kDa) and gel permeation chromatography (93.2 kDa) indicated monomeric nature of the β-mannosidase protein. The enzyme displayed its maximal activity at pH 7.0 with pH stability over a range of pH 5.0–9.0. Similarly, the optimum temperature for maximal activity was found to be 95 °C and thermostability of up to 85 °C. The substrate specificity and kinetics of the enzyme was studied using different mannooligosaccharides and *p*NP-β-D-mannopyranoside. The K_m value of the purified enzyme for *p*NPM was 0.49 mM. Different mannooligosaccharides tested as enzyme substrates were hydrolysed in an exo-wise manner when checked by thin-layer chromatography (TLC). The enzyme also exhibited transglycosidase activity when the reaction was carried out with 10% (w/v) of mannobiose in the presence of alcohols or galactose. Because of extreme thermostability and transglycosylation properties of β-mannosidase from *T. maritima*, the enzyme may be of industrial applications in future. This is the first report on the purification and characterization of a β-mannosidase from *T. maritima*.

1. Introduction

β-Mannosidase (β-D-mannoside mannohydrolase, EC 3.2.1.25) is an exo-acting enzyme, which catalyzes the successive removal of D-mannose residues from the nonreducing end of various β-1,4-linked mannooligosaccharides. It is essential for the complete hydrolysis of plant polysaccharides such as galacto(gluco)mannan and mannan, and readily converts the mannooligosaccharides produced by β-mannanase to mannose [1–3]. Most of the reported β-mannosidases are classified under family 2 of glycosidase hydrolases with the exception of two β-mannosidases from *Pyrococcus* species, which are placed under family 1 [4–9]. β-Mannosidases, together with β-mannanases, are widely used for industrial production of various chemicals, fuels and bio-bleaching process [3]. In addition to this, β-mannosidases and related enzymes are being used in the synthesis of oligosaccharides or alkyl β-mannosides for medical and other purposes [10–12].

Purification and characterization of β-mannosidases from different microorganisms such as *Aspergillus niger*, *A. awamori*, *Aureobasidium pullulan*, *Cellulomonas fimi*, *Pyrococcus furiosus*, *Sclerotium rolfsii*, *Thermotoga neapolitana*, *Trichoderma reesei*, *Thermobifida fusca*, and *Thermoascus aurantiacus* have been reported [2,5,6,11,13–18]. Thermostable β-mannosidases have significant advantages in enzymatic bleaching of softwood pulp in the paper and pulp industries due to their higher thermostability than β-mannosidases derived from mesophilic ones [8,13,16–20].

Thermotoga maritima MSB8 is a hyperthermophilic bacterium which grows at an optimum temperature of around 80 °C [21]. In the *T. maritima* genome sequence data, three open reading frames (ORF) designated as TM1851, TM1227 and TM1624 have been annotated as α-mannosidase, endo-1,4-β-mannosidase and β-mannosidase, respectively [22,23]. The ORF TM1851 annotated as α-mannosidase has already been cloned and expressed in *E. coli*. This recombinant α-mannosidase exhibits its maximal enzymatic activity at pH 6.0

Abbreviations: BSA, bovine serum albumin; CAPS, (cyclohexylamino)-1-propanesulphonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethane sulphonic acid; IPTG, isopropyl β-D-thiogalactopyranoside; LB, Luria–Bertani broth; M1, mannose; M2, mannobiose; M3, mannotriose; M4, mannotetraose; M5, mannopentaose; MOPS, 3-(N-morpholino)-propane sulphonic acid; NBS, N-bromosuccinimide; ORF, open reading frame; PCR, polymerase chain reaction; *p*NP, *p*-nitrophenol; *p*NPM, *p*NP-β-D-mannopyranoside; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.

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and 80 °C [24]. But, the putative ORF TM1227 annotated as endo-1,4-β-mannosidase has been characterized as a β-mannanase [19]. Previously, in our laboratory the β-mannosidase encoded by the ORF TM1624 of *T. maritima* MSB8 was expressed in *E. coli* as His-tagged fusion protein as a soluble protein [25]. According to the homology search of amino acid sequences using the BLAST database search, the recombinant β-mannosidase shows 80% identity to the β-mannosidase Man2 of *T. neapolitana* (accession No. AAK52304.1) and has less than 53% identity to the β-mannosidases from other species. Among *Thermotoga* species, the gene *man2* encoding β-mannosidase from *T. neapolitana* was only isolated, cloned, and expressed in *E. coli*. The recombinant Man2 from *T. neapolitana* has been further characterized and is active at an optimum temperature of approx. 90 °C and pH 7 [19]. Purification and the detailed biochemical characteristics of the recombinant β-mannosidase have been studied in the present investigation and reported here.

2. Experimental

2.1. Chemicals

The *p*-nitrophenol (*p*NP) substrates *viz.*, *p*NP-β-mannopyranoside (*p*NPM), *p*NP-α-D-mannopyranoside, *p*NP-β-D-glucopyranoside, *p*NP-α-L-arabinofuranoside, *p*NP-β-D-xylopyranoside, *p*NP-β-D-fucopyranoside, *p*NP-β-D-galactopyranoside, locust bean gum, guar gum, birchwood xylan, and carboxymethylcellulose (low viscosity) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Chromatographic media DEAE-52 and Ni-NTA agarose beads were obtained from Whatman Inc. (Fairfield, NJ, USA) and Qiagen Co. (Hilden, Germany), respectively. All other chemicals used were of analytical grade quality unless and otherwise stated.

2.2. Cloning and expression of the gene in E. coli

The gene TM1624 (accession number AAD36691.1) of *T. maritima* MSB8 was PCR amplified using gene specific forward and reverse primers, cloned in the pET28a (+) vector and transformed to *E. coli* BL21(DE3) competent cells [25]. All molecular biology techniques followed were in accordance with Sambrook and Russell [26]. Seed culture (10 mL) of *E. coli* BL21 harboring β-mannosidase gene in pET28a vector was prepared by growing cells on a rotary shaker (200 rpm) for about 14 h at 37 °C. Thus prepared seed culture (OD_{600} = 3.74) was used to inoculate LB medium (100 mL) containing kanamycin (50 μg/mL) which was cultured at 37 °C with a rotary shaker (200 rpm) until the optimal density at 600 nm reached 0.5–0.6. IPTG (isopropyl β-D-thiogalactopyranoside) was added to a final concentration of 1 mM, and incubation was then continued for 12 h.

2.3. Purification of the recombinant β-mannosidase

Briefly, the cells were harvested by centrifugation at 10 000 × *g* for 15 min at 4 °C, and suspended in 50 mM sodium phosphate buffer (pH 8.0) and disrupted by sonication. After centrifuging at 12 000 × *g* for 10 min at 4 °C, the crude β-mannosidase enzyme in the supernatant was heated for 10 min at 70 °C to remove most of the *E. coli* proteins. The clear supernatant was collected by centrifuging at 12 000 × *g* for 10 min at 4 °C, and further purified by Ni-affinity chromatography using Ni-NTA beads. Thus purified protein was dialyzed over night against 20 mM phosphate buffer (pH 7.2) and was applied to a DEAE 52 column (1 cm × 8 cm) pre-equilibrated with 20 mM phosphate buffer (pH 7.2). The bound proteins were eluted with a NaCl gradient (100–300 mM) at a flow rate of 1.0 mL/min. The protein eluted as a single peak and used as a purified β-mannosidase for enzyme characterization. The homogeneity of the purified enzyme was monitored by SDS-PAGE.

2.4. Enzyme assay and protein determination

The β-mannosidase activity was determined by measuring the release of *p*-nitrophenol from *p*NPM at 70 °C for 10 min. The assay mixture consisted of 225 μL of 5 mM *p*NPM in 50 mM phosphate buffer (pH 7.0). Appropriately diluted enzyme (25 μL) in the above buffer was added to the mixture to a final volume of 250 μL. To stop the reaction 750 μL of 2 M Na_2CO_3 was added and the absorbance at 410 nm was measured. One unit of the enzyme activity was defined as the amount of enzyme required to catalyze the release of 1 μmol *p*NP per min under the assay conditions. Protein concentrations were measured by the Lowry method [27] with BSA (bovine serum albumin) as the standard. Specific activity was expressed as U per mg of protein. All assays were performed in triplicate.

2.5. SDS-PAGE and molecular mass of the enzyme

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of protein samples was performed using 7.5% (w/v) acrylamide gel according to the method described by Laemmli [28]. Protein bands were stained with Coomassie brilliant blue R-250. The molecular mass standard used was the high molecular mass calibration kit for SDS electrophoresis (Sigma): myosin (220 kDa), α_2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa), and glutamic dehydrogenase (53 kDa).

The molecular mass of the enzyme in non-denaturing condition was also determined by gel permeation chromatography on Sephacryl-200 column (1 cm × 40 cm, Pharmacia) after pre-equilibrated the column with 50 mM citrate buffer (pH 6.0). The column was eluted with the same buffer at a flow rate of 0.3 mL/min. Alcohol dehydrogenase (150 kDa), fetuin from fetal calm serum (68 kDa), albumin from egg chicken white (45 kDa), and α-chymotrypsinogen A (Type II from bovine pancreas, 25.7 kDa) were used as the standards.

2.6. Effect of pH and temperature on enzyme activity

The optimum pH for the β-mannosidase was determined at 70 °C by measuring the activities at pH values ranging from pH 2.5 to pH 11.0 in 50 mM of various buffers *viz.*, citrate (pH 2.5–6.0), MOPS [3-(N-morpholino)-propane sulphonic acid] (pH 6.5–9.0), phosphate (pH 6.0–8.5), HEPES [N-2-hydroxyethylpiperazine-N′-2-ethane sulphonic acid] (pH 6.0–9.0), CAPS [(cyclohexylamino)-1-propanesuhinic acid] (pH 9.0–11.0). The pH stability was investigated by incubating the purified enzyme in the above mentioned buffers for 30 min at 70 °C. After cooling the treated enzymes on ice for 30 min, the residual activities of these treated enzymes were determined at optimum pH by the standard assay protocol.

The optimum temperature for maximal enzyme activity was determined by incubating the reaction mixture containing enzyme at temperatures ranging from 30 °C to 100 °C in 50 mM MOPS buffer (pH 7.0). For estimating the thermostability, the enzyme was pre-incubated in 50 mM MOPS buffer (pH 7.0) in the temperature range of 30–100 °C. Further, the thermostability of the enzyme at 80 °C, 85 °C, 90 °C, and 95 °C for a longer duration was also studied. After 30 min the samples were rapidly cooled on ice-water bath for 30 min and the activity of the samples were checked.

The effect of various metal ions and reagents on β-mannosidase activity was determined by incubating the enzyme with the individual reagent at a final concentration of 1 mM in 50 mM MOPS

buffer (pH 7.0) at 70 °C for 30 min. Activities were then measured at 70 °C in the presence of the metal ions or reagents. The activity assayed in the absence of metal ions or reagents was considered to be 100%. The results presented are the average of three trials.

2.7. Substrate specificity and analysis of kinetic parameters

Substrate derivatives of *p*-nitrophenol at 5 mM in 50 mM MOPS buffer, pH 7.0 were used to check the enzyme activity at 70 °C for 10 min by measuring the release of *p*-nitrophenol at 410 nm. The β-mannosidase activity towards polysaccharides (0.5%, w/v) and mannooligosaccharides (1.0%, w/v) was determined by monitoring the liberation of mannose using Somogyi–Nelson method [29]. In brief, 0.5 mL of reaction mixture was diluted to 5 mL with distilled water and to this, 5 mL of the Somogyi reagent was added and boiled for 15 min. After cooling to room temperature, 2 mL of 2% KI and 1.5 mL of 1 M H_2SO_4 were added and mixed thoroughly before analyzing the sugar concentrations by titration with 5 mM $Na_2S_2O_3$.

The Michaelis–Menten constant (K_m) and the maximal reaction velocity (V_{max}) for *p*NPM were determined at 70 °C for 3 min in 50 mM MOPS buffer (pH 7.0). The initial hydrolysis rates were determined at six different concentrations ranging from approximately 0.5 to 2.0 times of the K_m value. K_m and k_{cat} and their standard errors were calculated using a statistical software program "Grafit".

2.8. Hydrolysis of mannooligosaccharides by β-mannosidase

Hydrolysis of different mannooligosaccharides (1%, w/v) by 0.5 U/mL of the purified β-mannosidase was studied in 50 mM MOPS buffer (pH 7.0) at 70 °C for 24 h. Samples were withdrawn at different time intervals and boiled for 10 min to inactivate the enzyme. Products of enzymatic hydrolyses were analyzed by TLC (thin-layer chromatography) on Kieselgel 60 plates (Merck). The plates were developed with two runs in a 1-butanol–acetic acid–water (2:1:1, v/v) solvent system followed by spraying the plates with a methanol–sulfuric acid mixture (95:5, v/v) and heating for few seconds at 130 °C in an oven. A mixture of mannose (M1), mannobiose (M2), mannotriose (M3), mannotetraose (M4), and mannopentaose (M5) was used as the standards.

2.9. Transglycosylation of mannobiose and alcohols (or galactose) by β-mannosidase

To demonstrate whether alcohols or galactose act as acceptors of glycosyl moieties, various alcohols (1 M) or galactose at different concentrations were used as an acceptor and mixed with mannobiose (10%, w/v) as a substrate and incubated with 5 U/mL of the purified β-mannosidase in 50 mM MOPS buffer (pH 7.0) at 70 °C for 2 h. The transfer products were analyzed by TLC as described above.

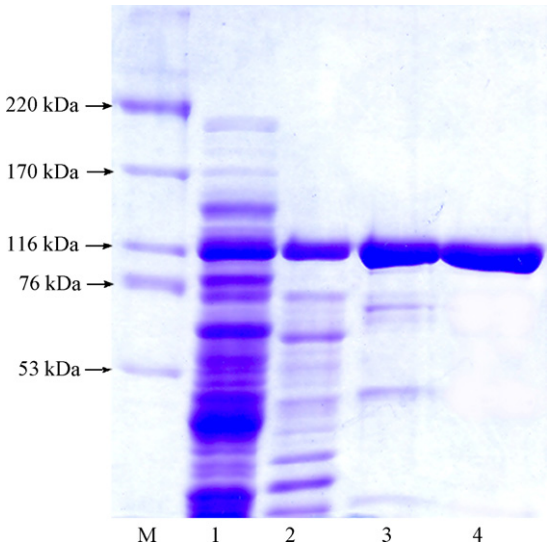

Fig. 1. SDS-PAGE of a purified β-mannosidase from *T. maritima*. Lane M, high MW standards; lane 1, crude enzyme; lane 2, heat treatment; lane 3, Ni-NTA step; lane 4, DEAE-52 step (purified β-mannosidase).

3. Results and discussion

3.1. Purification of the β-mannosidase

The crude recombinant β-mannosidase enzyme was purified to homogeneity by heat treatment, affinity chromatography and anion-exchange chromatographic methods (Table 1). Purification level of 11.8-fold and a final protein recovery yield of 23% was achieved using the above steps. Under denaturing condition the purified enzyme moved as a single protein band at a molecular mass of 96.8 kDa on SDS-PAGE gel (Fig. 1). By gel permeation chromatography the molecular mass of β-mannosidase was found to be about 93.2 kDa (data not shown).

The gene TM1624 coding for a β-mannosidase from *T. maritima* MSB8 was cloned and expressed as 6× His-tagged fusion protein in *E. coli* as a soluble recombinant enzyme [25]. In the present study, β-mannosidase was purified to homogeneity with a recovery yield of 23% (Table 1 and Fig. 1). Its molecular mass was estimated to be 96.8 kDa and 93.2 kDa by SDS-PAGE and gel filtration, respectively, suggesting that it is a monomeric protein. Molecular masses ranging from 90 kDa to 105 kDa have been reported for β-mannosidases from *T. reesei* [15], *T. fusca* [17], *T. aurantiacus* [18] and *T. neapolitana* [19]. However, existence of multimeric form of β-mannosidases has also been reported [5,11,13,30].

3.2. Effect of pH and temperature on enzyme activity

Optimum pH for maximal activity of the recombinant β-mannosidase was found to be around pH 7.0 with the retention of 80% of maximal activity between the range of pH 5.0 and 8.0 (Fig. 2A). β-Mannosidase also exhibited a high pH stability within

Table 1
Summary of β-mannosidase purification.

Purification step	Total activity[a] (U)	Total protein[b] (mg)	Specific activity (U/mg)	Purification factor (-fold)	Recovery (%)
Crude enzyme	5850	1860	3.2	1	100
Heat treatment	4780	452	10.6	3.4	82
Ni-NTA	2850	109	26.2	8.3	49
DEAE-52	1340	36	37.2	11.8	23

[a] Activity was measured in 50 mM phosphate buffer (pH 7.0) at 70 °C using 5 mM *p*NPM as substrate.
[b] The protein was measured by the Lowry method [27], using BSA as the standard.

the range of pH 5.0–9.0 at 70 °C (Fig. 2B). At 95 °C, the maximal hydrolysis of *p*NPM by β-mannosidase was recorded (Fig. 3A). Even at 100 °C the enzyme showed about 68% of its maximal activity. The thermostability of the purified β-mannosidase was further investigated in the temperature range between 80 °C and 95 °C (Fig. 3C). At 80 °C, the enzyme had a half-life of 828 min. The enzyme was stable up to 85 °C with a half-life of 213 min. The half-lives of the purified enzyme at 90 °C and 95 °C were 189 min and 28 min, respectively. The above results indicate the hyperthermal-stability and hyper-activity of the recombinant β-mannosidase.

The effect of various cations, reducing agents and other chemical compounds at 1 mM concentration was tested on the activity of β-mannosidase (Table 2). At 1 mM concentration β-mannosidase activity was strongly inhibited by Cu^{2+} (28%) and was inhibited by Co^{2+} (88%), Hg^{2+} (1.8%) and Ag^{+} (2.4%). However, it was activated by Ca^{2+} (108%), β-mercaptoethanol (112%), Ni^{2+} (144%), SDS (148%), DTT [dithiothreitol] (154%), NBS [N-bromosuccinimide] (177%), Na^{+} (180%), Li^{+} (187%), K^{+} (193%) and Sr^{2+} (203%). EDTA [ethylenediaminetetraacetic acid] (102%), Zn^{2+} (99.8%), Mg^{2+} (96%) and Mn^{2+} (96%) at 1.0 mM affected the activity marginally.

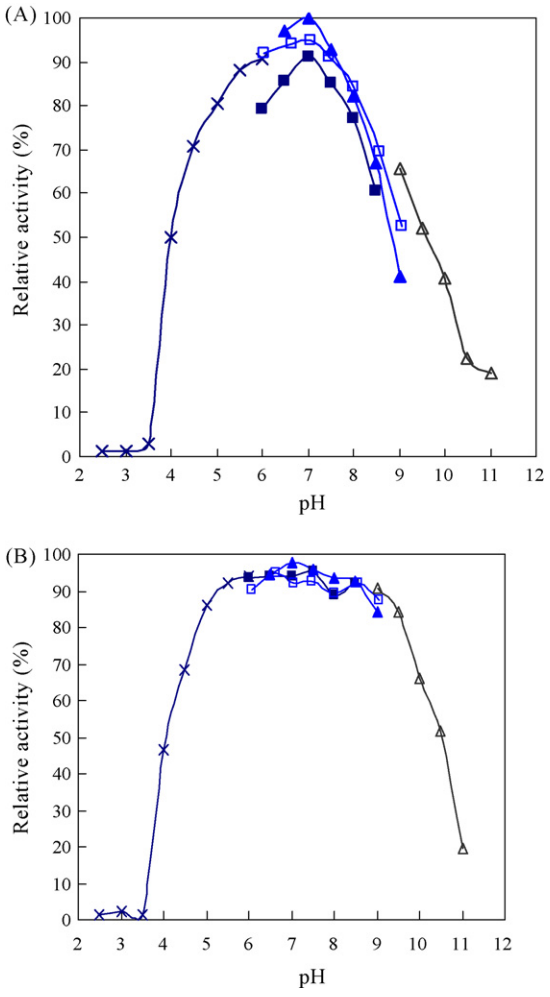

Fig. 2. Optimal pH (A) for maximal activity and pH stability (B) of the purified β-mannosidase from *T. maritima*. The influence of pH on β-mannosidase activity was determined at 70 °C using 50 mM of different buffers. To determine pH stability, the remaining activity was measured after incubation for 30 min at 70 °C over various pH ranges. Buffers used: citrate (×), phosphate (■), MOPS (▲), HEPES (□), CAPS (△). The highest specific activity of 47.6 U/mg was taken as 100%. Values are means of three different experiments.

Table 2
Effect of various reagents on the enzyme activity of purified β-mannosidase.

Reagents (1 mM)	Specific activity (U/mg)	Relative activity[a] (%)
Control	47.6 ± 0.9	100
Fe^{2+}	50.2 ± 1.8	105
Cu^{2+}	13.1 ± 0.6	28
Ca^{2+}	51.3 ± 2.3	108
Mg^{2+}	45.8 ± 1.9	96
Mn^{2+}	45.5 ± 2.1	96
Co^{2+}	41.8 ± 1.5	88
Zn^{2+}	47.5 ± 1.9	99.8
Hg^{2+}	0.8 ± 0.04	1.8
Sr^{2+}	96.8 ± 3.2	203
Ni^{2+}	68.3 ± 1.8	144
Ag^{+}	1.2 ± 0.03	2.4
K^{+}	91.7 ± 2.7	193
Li^{+}	89.1 ± 2.8	187
Na^{+}	85.8 ± 3.6	180
DTT	73.4 ± 2.1	154
SDS	70.3 ± 1.3	148
EDTA	48.5 ± 1.8	102
β-Mercaptoethanol	53.1 ± 2.4	112
NBS	84.1 ± 2.2	177

[a] Values are means of three different experiments.

The purified β-mannosidase exhibited an optimal activity at pH 7.0 and 95 °C, respectively. It was stable in the pH range of 5.0–9.0 and up to 85 °C (Fig. 2 and Fig. 3). For most of the reported β-mannosidases, pH optima are in the range of pH 3.0–6.0 [8,11,16,18,30]. But β-mannosidases isolated from *P. furiosus* [5], *C. fimi* [6], *T. neapolitana* [13,19], and *T. fusca* [17] have been reported to show maximal activity at pH 7.0 or above. The enzyme's temperature optimum of 95 °C reported here is higher than that of the β-mannosidases from *T. neapolitana* [13,20]. This temperature optimum appeared distinctly higher than that (50–80 °C) reported for most of β-mannosidases [2,6,11,16–18]. Also, the enzyme showed good thermostability. The thermostability of β-mannosidase from *T. maritima* is comparable to the values of thermostable β-mannosidase from *T. neapolitana* [13,19]. The β-mannosidase described here may represent one of the most thermostable β-mannosidases yet reported, which is extremely thermostable and thermoactive as might be expected of enzymes from *Thermotoga* species [5,8,13,20]. Besides, similar observation of activation by Ca^{2+} of β-mannosidase from *T. reesei* has been previously reported [15]. Strong inhibition of the enzyme by Hg^{2+} could be due to the reaction of protein's sulfhydryl groups with histidine and tryptophan residues. As DTT was not inhibitory the requirement of disulphide bond/s may be non-essential for the β-mannosidase activity.

3.3. Substrate specificity and kinetic parameters

The enzyme was highly active towards *p*NPM, in addition to the hydrolysis of β-1,4-linked mannooligosaccharides (Table 3). The relative rates of reducing sugars released were 15%, 47%,

Table 3
Substrate specificity of the purified β-mannosidase.

Substrate	Specific activity (U/mg)	Relative activity (%)[a]
*p*NP-β-mannopyranoside	47.6 ± 0.9	100
Mannobiose	7.2 ± 0.24	15
Mannotriose	22.4 ± 0.6	47
Mannotetraose	15.2 ± 0.3	32

[a] Enzymatic reactions were carried out at 70 °C for 10 min in 50 mM MOPS buffer (pH 7.0). The activity for *p*NPM was defined as 100%. No activity was observed for other *p*NP derivatives and polysaccharides such as, locust bean gum, guar gum, konjac powder, copra mannan, birchwood xylan, and carboxymethylcellulose.

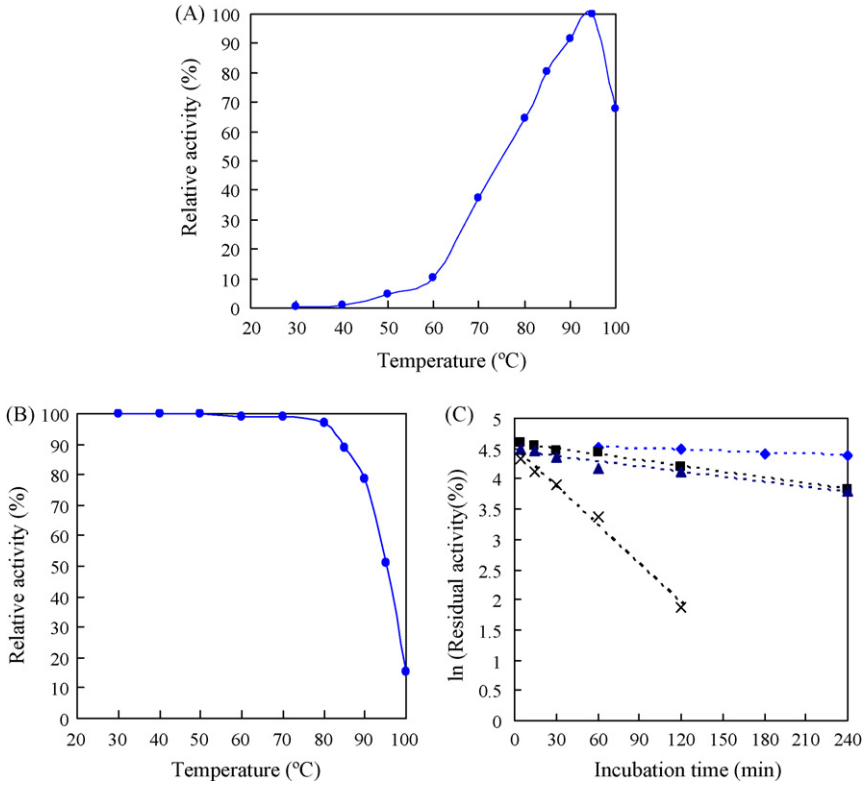

Fig. 3. Optimal temperature (A) for maximal activity and thermostability (B) of the purified β-mannosidase from *T. maritima*. The temperature profile was measured at different temperatures in 50 mM MOPS buffer (pH 7.0). For determination of thermostability, the residual activities of the treated enzymes were measured according to the standard assay after a 30 min pre-incubation at different temperatures. The highest specific activity of 127.9 U/mg was taken as 100%. (C) Thermostability of the purified β-mannosidase at temperatures from 80 °C to 95 °C. Purified enzyme was incubated at 80 °C (♦), 85 °C (■), 90 °C (▲), and 95 °C (×). 80 °C, $Y = -0.0008x + 4.5747$, $R^2 = 0.9727$; 85 °C, $Y = -0.0032x + 4.5933$, $R^2 = 0.9937$; 90 °C, $Y = -0.0029x + 4.4588$, $R^2 = 0.9534$; 95 °C, $Y = -0.0213x + 4.4982$, $R^2 = 0.9899$. Values are means of three different experiments.

and 32% for mannobiose (M2), mannotriose (M3), and mannotetraose (M4), respectively. This enzyme did not show any activity towards *p*NP-α-D-mannopyranoside, *p*NP-α-L-arabinofuranoside, *p*NP-β-D-glucopyranoside, *p*NP-β-D-xylopyranoside, *p*NP-β-D-fucopyranoside and *p*NP-β-D-galactopyranoside. Polysaccharides such as locust bean gum, guar gum, konjac powder, copra mannan, birchwood xylan, and carboxymethylcellulose also did not act as substrates for β-mannosidase of *T. maritima*.

The K_m and V_{max} values for the recombinant β-mannosidase of *T. maritima* MSB8 towards *p*NPM were determined to be 0.49 mM and 50.5 U/mg (data not shown). The enzyme turn over number (k_{cat}) was calculated to be 506 min^{-1} which resulted in a catalytic efficiency (k_{cat}/K_m) of 1033 mM^{-1} min^{-1}.

The purified β-mannosidase was highly specific for *p*NPM and showed no activity towards other *p*NP derivatives (Table 3). Activity towards *p*NPM only for β-mannosidases from *T. neapolitana* [13,19], *A. niger* [11,31], *T. fusca* [17], *Aplysia fasciata* [30] has been reported. The characteristics of hydrolysis of β-1,4-linked mannooligosaccharides by β-mannosidases have also been published [15,19,30,32,33]. However, few β-mannosidases could release mannose from both mannooligosaccharides and polymeric compounds, such as ivory nut mannan and galactomannan [2,11,31]. In contrast, several β-mannosidases exerted significant hydrolytic activity on other *p*NP derivatives (except *p*NPM), such as *p*NP-β-D-galactopyranoside, *p*NP-β-D-glucopyranoside and/or *p*NP-β-D-xylopyranoside [5,8,15]. The K_m value (0.49 mM) for *p*NPM is comparable to the values reported (0.1–0.5 mM) for the corresponding enzymes from *P. furiosus* [5], *C. fimi* [6], *Pyrococcus horikoshii* [8], *Bacteroides thetaiotaomicron* [9], *A. niger* [11], *T. reesei* [15], and *T. fusca* [17]. The K_m value (0.49 mM) of recombinant β-mannosidase of *T. maritima* is significantly lower than those of β-mannosidases from *T. neapolitana* [13], *T. aurantiacus* [18], *A. fasciata* [30], and *A. niger* [31]. The k_{cat}/K_m of β-mannosidase is comparable to the value reported for the β-mannosidases from *T. neapolitana* (1454 mM^{-1} min^{-1}) [13]. Thus, the enzyme displayed a strict specificity for the β-mannoside linkage and high catalytic efficiency compared to analogous enzymes isolated from other sources.

3.4. Hydrolytic properties of β-mannosidase

The hydrolysis of various mannooligosaccharides by the purified β-mannosidase was studied by analyzing the reaction products on TLC plates (Fig. 4). The results indicate that β-mannosidase catalyzed hydrolysis of mannooligosaccharides in an exo-wise manner as detected by TLC analysis of the hydrolysis products. β-Mannosidase released mannose from all substrates and the major products were mannose and an oligosaccharide with a

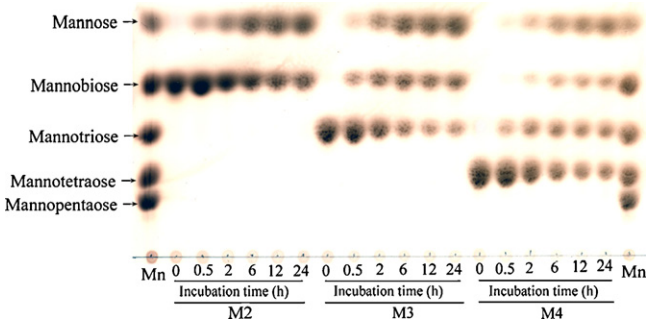

Fig. 4. TLC analysis of hydrolysis products from mannooligosaccharides by the purified β-mannosidase. Incubation times (h) and substrates are indicated. Lanes Mn, a mixture of mannose (M1) to mannopentaose (M5).

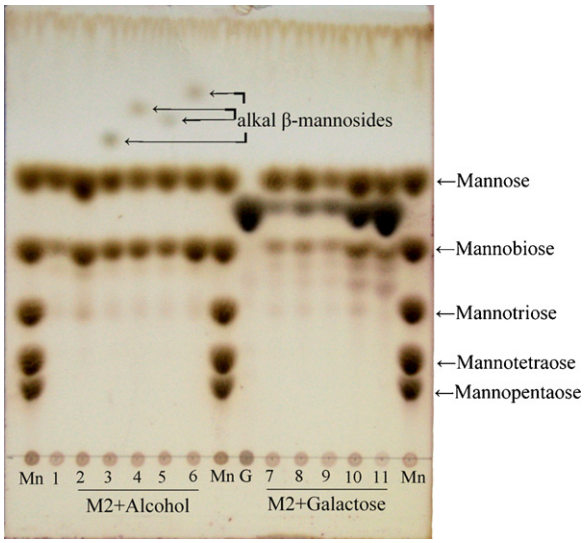

Fig. 5. Transglycosylation of various alcohols or galactose using the β-mannosidase (5 U/mL) incubated with mannobiose (10%, w/v) in 50 mM MOPS buffer (pH 7.0) at 70 °C for 2 h. TLC analysis of hydrolysis products from in the absence or presence of alcohols or galactose. Lanes Mn, a mixture of mannose to mannopentaose; lane G, galactose; lane 1, M2; lane 2, M2 + methanol; lane 3, M2 + ethanol; lane 4, M2 + 1-propanol; lane 5, M2 + iso-propanol; lane 6, M2 + 1-butanol; lane 7, M2 + 1% galactose; lane 8, M2 + 2% galactose; lane 9, M2 + 5% galactose; lane 10, M2 + 10% galactose; lane 11, M2 + 20% galactose. Alkyl β-mannosides spot on the TLC are marked.

degree of polymerization 1 unit smaller than the substrate (Fig. 4). This indicates that β-mannosidase appears to hydrolyse mannooligosaccharides in a stepwise manner, releasing one mannose unit at a time. Many β-mannosidases have been shown to hydrolyse mannooligosaccharides in a stepwise manner [2,11,30].

3.5. Transglycosylation by β-mannosidase

When β-mannosidase was incubated in the presence of M2 (10%, w/v), hydrolysis and self-transfer of the mannosyl group (higher oligosaccharides than substrates) were observed (lane 1 in Fig. 5). As indicated in Fig. 5, TLC analysis of the hydrolysis products indicated that β-mannosidase catalyzed hydrolysis and transglycosylation reactions simultaneously when M2 was hydrolysed in the presence of alcohols or galactose. When the reaction was performed in the presence of ethanol, 1-propanol, iso-propanol or 1-butanol, alkyl β-mannosides migrating ahead of mannose were formed (lanes 3–6 in Fig. 5). Also, when mannobiose was hydrolysed in the presence of galactose, some oligosaccharides higher than mannobiose but migrating ahead of mannotriose were observed in lines 8–11 of Fig. 5.

Transglycosylation activity of β-mannosidase has also been observed for β-mannosidases from *C. fimi* [6,12], *A. niger* [10], *A. awamori* [16], *T. fusca* [17], *T. aurantiacus* [18], and *A. fasciata* [30]. The β-mannosidase in this study exhibited transglycosidase activity when it hydrolysed mannobiose at 10% (w/v) of concentration in the presence of alcohols (Fig. 5). Several β-mannosidases have been shown to transglycosylate effectively using alcohols as acceptors [10,16]. Moreover, the enzyme had a transglycosylation action, transferring mannose residue to galactose (Fig. 5). This acceptor specificity is attractive for synthesis of a new hetero-mannooligosaccharides. Further investigations are required to evaluate the industrial applications of this highly thermostable β-mannosidase from *T. maritima* MSB8.

4. Conclusions

A β-mannosidase gene (TM1624) of *T. maritima* MSB8 was expressed in *E. coli* as a 6× His-tagged fusion protein. The purified enzyme exhibited an optimal activity at pH 7.0 and 95 °C, respectively. It was stable in the pH range of 5.0–9.0 and up to 85 °C. The enzyme displayed a strict specificity for the β-mannoside linkage and high catalytic efficiency compared to analogous enzymes isolated from other sources. It was verified that this is an exo-acting glycosyl hydrolytic behavior of the enzyme with transglycosylation activity.

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