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RESEARCH PAPER

A Novel Low-molecular Weight Alkaline Mannanase from *Streptomyces tendae*

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Abstract A novel, low-molecular weight, alkaline mannanase from *Streptomyces tendae* (MnSt) was purified to homogeneity and biochemically characterized. The extracellular mannanase was purified with 26.3% yield using a Sepharose Cl-6B column. The molecular mass of MnSt was approximately 24 kDa. MnSt was stable over a broad pH range (5 ~ 12.5), was thermally stable at 60°C, and functioned optimally at 50°C and a pH of 12.0. MnSt had K_m and V_{max} values of 0.05 ± 1 mg/mL and 439 ± 0.5 mmol/min, respectively, using bean gum galactomannan as a substrate. The N-terminal sequence of MnSt was GWSVDAPYIAXQPFS. Thin layer chromatography (TLC) analysis of the MnSt hydrolysis products suggested that the major oligosaccharide produced was mannobiose. MnSt activity was remarkably affected by metal ions, modulators, chelators, and detergents. MnSt was simple to purify, had high thermal stability, was stable over a broad pH range, and produced mannooligosaccharides. MnSt has high potential for use as an industrial biocatalyst, particularly as a bio-bleaching agent or for oligosaccharide production.

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1. Introduction

As the world population has expanded and more countries have become industrialized, global energy consumption has steadily increased. The increased energy demand has pressured limited fossil fuel reserves and threatens to surpass fossil fuel availability. Given the current consumption rate, the annual global oil production will decline from 25 billion barrels (2002) to approximately 5 billion barrels by 2050 [1]. Therefore, there is tremendous motivation to explore alternative energy sources. Plant biomass produced as industrial and agricultural waste can be used for energy production, as well as for starting points for the synthesis of value-added products such as oligosaccharides. Plant waste material generates pollution and unnecessarily occupies space; plant waste management is an expensive and challenging task. Extensive research has focused on the conversion of lignocellulosic biomass to ethanol, a second-generation biofuel [2]. The conversion includes two steps: hydrolysis of lignocellulosic biomass to fermentable sugars and fermentation of sugars to ethanol [1,3]. The first hydrolytic step is associated with a high processing cost and low yield, and has remained a major challenge to industrial ethanol production, whereas the fermentation step is inexpensively carried out by yeast and bacteria. Increasing attention has focused on improving the hydrolytic step by pre-treating lignocellulosic materials and using more efficient biocatalysts [1]. Structural analysis of lignocellulosic biomass had revealed three major classes of polysaccharides, including cellulose, hemicelluloses, and lignin [4]. Mannan and heteromannans are both hemicelluloses found in plant cell walls. β-Mannanase (1, 4-β-D-mannan mannanohydrolase,

EC 3.2.1.78) catalyzes the random hydrolysis of β-1, 4-mannosidic linkages in β-1, 4-mannan, glucomannan, and galactomannan. For the complete hydrolysis of softwood mannan, the synergistic actions of endo-1, 4-β-mannanases, β-mannosidases, β-glucosidases, α-galactosidases, and acetyl mannan esterases are required [5].

Broad application of mannanase has been explored in the food and pharmaceutical industries for the production of fruit juices, degradation of plant material, and extraction of coffee [5,6]. Furthermore, mannanases are used in combination with xylanase in the pulp and paper industries to partially breakdown mannan and xylan in softwood pulps, which decreases the use of environmentally damaging bleaching agents [6]. Mannanase occurs naturally in a broad range of microorganisms, including *Bacillus* sp. N16-5 [7], *Aspergillus niger* [8], *Vibrio* sp. strain MA-138 [9], *Sclerotium rolfsii* [10], and *Paenibacillus* sp. DZ3 [11]. But very few research on mannanase from *Streptomyces* has been explored, such as *Streptomyces lividans* 66 [12], *Streptomyces galbus* NR [13], *Streptomyces ipomoea* CECT3341 [14], *Streptomyces* sp. [15], *Streptomyces thermolilacinus* [16], and *Streptomyces* sp. S27 [17]. In this work, we describe the screening and isolation of a potent mannanase from *Streptomyces tendae* (MnSt). Biochemical characterization of the purified MnSt suggests that it is suitable for use as an industrial biocatalyst.

2. Materials and Methods

2.1. Materials

Locust bean gum galactomannan and mannoses were purchased from Sigma-Aldrich (St. Louis, MO, USA). Thin layer chromatography (TLC) silica gel plates were purchased from Merck (Darmstadt, Germany). Mannobiose and mannotriose were purchased from Megazyme (Ireland). Sepharose Cl-6B was purchased from Amersham Bioscience (Uppsala, Sweden). All the reagents used were of the highest grade available.

2.2. Bacterial strain selection, enzyme production, and purification

Twenty-five different bacterial strains, collected from different provinces of Korea, were cultivated in locust bean gum galactomannan (LBG) medium (pH 6.5) composed of 1.25% locust bean gum galactomannan (LBG), 0.5% yeast extract, 1% tryptone, 0.07% KH_2PO_4, 0.03% K_2HPO_4, and 0.05% $MgSO_4$. These strains were cultivated in 250 mL Erlenmeyer flasks containing 50 mL LBG medium at 28°C for 2 days, with shaking at 130 rpm. Cultures were centrifuged at 10,000 × *g* for 15 min, and the supernatant was collected for enzyme activity measurements. Extracellular mannanase production for each strain was assayed using the agar plate method in addition to the enzyme activity assay. Agar plates containing 1.25% locust bean gum galactomannan (LBG), 0.5% yeast extract, 1% tryptone, 0.07% KH_2PO_4, 0.03% K_2HPO_4, 0.05% $MgSO_4$, and 1.5% agar were inoculated with spots of enzyme extract or buffer, as a control, and incubated at 37°C for 12 h [18]. The plates were then stained with 0.01% Calcofluor white M2R in 0.5 M Tris-HCL, pH 8.0, for 30 min, and then destained with water. The lytic zones were imaged using a UV transilluminator. *S. tendae* exhibited the highest mannanase activity among all the strains tested, according to both the enzyme assay and the agar plate assay, and was selected for the further study.

All purification procedures were carried out at 4°C unless stated otherwise. After the selection of the bacterial strain *S. tendae*, this strain was cultivated in 1 L baffled flasks containing 200 mL of LBG medium at 28°C for 2 days, with shaking at 130 rpm. Cell-free supernatants were collected by centrifugation at 10,000 × *g* for 15 min. Ammonium sulfate was added to the supernatant at 30 ~ 80% saturation, and the mixture was incubated overnight. The precipitate was centrifuged at 10,000 × *g* for 45 min. The pellets were re-suspended in 10 mM Tris-HCl (pH 7.5), dialyzed against the same buffer overnight, and concentrated with a 10 kDa ultra-filtration centrifugal device (Millipore Corp, Darmstadt, Germany). The dialyzed enzyme solution was loaded onto a Sepharose CL-6B column (85 cm × 1.7 cm) pre-equilibrated with 10 mM Tris/HCl, pH 7.5. Proteins were eluted at 30 mL/h (3 mL/fraction). Mannanase active fractions were pooled, concentrated, and analyzed for purity. Further characterizations were carried out using the pure enzyme.

2.3. Enzyme activity assays and protein estimation

The protein concentrations were determined at 595 nm using the Bradford method [19] using bovine serum albumin as standard.

Mannanase activities were determined at 50°C by using locust bean gum galactomannan as the substrate. Briefly, 0.1 mL enzyme (appropriately diluted in 50 mM KCl/NaOH, pH 12.5) was added to 0.1 mL substrate solution containing 0.5% LBG prepared in 50 mM KCl/NaOH (pH 12.5) was incubated for 30 min. A parallel reaction was carried out with all the reagents at 4°C. Released reducing sugars were quantified using the DNS method [20] at 540 nm. A mannose standard curve was constructed to calculate enzyme activities. One unit of mannanase activity was defined as the amount of enzyme that released 1 μmol of mannose per min under standard assay conditions. Mannanase activities with substrates including xylan, chitin, pectin, and cellulose were evaluated under standard assay conditions.

2.4. Polyacrylamide gel electrophoresis

The molecular weight of the purified enzyme was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% (w/v) polyacrylamide gel, as described by Laemmli [21]. Following electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 and then destained with a solution containing methanol, glacial acetic acid, and distilled water at a volumetric ratio of 1:1:8. Molecular weight was estimated by comparison with the relative mobility of reference proteins (MBI, Fermentas). Galactomannan activity was analyzed using zymography, as described previously by Z. Jiang *et al.* [22]. Briefly, zymography (activity staining) was performed by electrophoresing the enzyme through a 12% (w/v) polyacrylamide gel containing 0.25% (w/v) of substrate LBG. After electrophoresis, the gel was washed twice with distilled water (10 min per wash) and soaked in 25% (v/v) isopropanol with gentle shaking to remove the SDS and renature the proteins in the gel. The gel was then washed four times (10 min per wash) at 4°C in 50 mM KCl/NaOH (pH 12.5). After further incubation for 60 min at 37°C, the zymogram was stained for residual carbohydrates with Congo red solution (0.1%, w/v), destained with 1 M NaCl and fixed with 0.5% (v/v) acetic acid. The activity bands appeared as clear colorless regions and were photographed.

2.5. Effects of pH and temperature

The optimum pH for enzyme activity was determined by carrying out enzyme assays at 50°C and varying various pH buffer pH from 2.0 to 13.6, using buffers including citric acid and sodium phosphate (pH 2 ~ 7.0), Tris-HCl (pH 7.0 ~ 9.5), sodium bicarbonate-NaOH (pH 9.5 ~ 11), and KCl-NaOH (pH 11 ~ 13.6). Temperature was optimized at pH 12.5 for temperatures ranging from 40 to 80°C. To evaluate stability with respect to pH, 100 mM enzyme was incubated in buffers of varying pH at 4°C for 24 h, and then residual enzyme activity was measured under standard assay conditions. To determine thermal stability, enzyme samples were pre-incubated at various temperatures (up to 80°C) for 60 min, and residual enzyme activity was measured under standard assay conditions.

2.6. Effects of metal ions, detergents, and modulators

The effects of various additives such as metal ions, detergents, and reducing and oxidizing agents on the activity of MnSt were examined. To characterize the effects of metal ions on MnSt activity, the purified enzyme was incubated with 1 mM monovalent (K^+ or Na^+) or divalent (Ca^{2+}, Mg^{2+}, Cu^{2+}, Co^{2+}, Zn^{2+}, Mn^{2+}, or Fe^{2+}) metal ions. The effects of oxidizing and reducing agents including hydrogen peroxide, sodium perborate, β-mercaptoethanol, and 1, 4-dithiothreitol on MnSt activity were evaluated. The chelating agents investigated included ethylenediaminetetraacetic acid (EDTA) and ethylene glycol tetra-acetic acid (EGTA). Detergents investigated included Triton X-100, Tween-20, Tween-80, polyoxyethylene-4-laurylether, deoxycholic acid (DCA), and sodium dodecyl sulfate (SDS), all at a concentration of 0.25%. For all conditions tested, relative enzyme activities were measured under standard assay conditions and normalized to enzyme activity in the absence of additives.

2.7. N-terminal amino acids

The N-terminal amino acid sequence of MnSt was determined by Edman degradation, using a Procise Model 492 protein sequencer (Applied Biosystems, CA, USA).

2.8. Substrate specificity and kinetics assays

To evaluate the substrate specificity of the purified enzyme, MnSt was incubated with 0.5% (w/v) substrate, which included locust bean gum galactomannan, beech wood xylan, birch wood xylan, colloidal chitin, sigma cell cellulose, carboxymethyl cellulose, avicel, paranitrophenyl D-cellobioside (pNPC), or paranitrophenyl-β-D-glycopyranoside (pNPG). Kinetic assays were performed using five concentrations of LBG, from 0.6 to 10 mg/mL, and constant enzyme concentration (0.01 mg). Assays were performed under the optimized standard assay conditions. The Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) were determined from a Line weaver-Burk plot.

2.9. Enzymatic hydrolysis and oligosaccharide production

MnSt enzymatic hydrolysis products were evaluated using thin layer chromatography (TLC). TLC was analyzed as described previously [23]. Briefly, purified MnSt (0.4 mg/mL) was incubated with locust bean gum galactomannan (5 mg/mL) in 50 mM KCl/NaOH buffer (pH 12.5) at 37°C. The reaction was stopped by boiling for 20 min and then samples were spotted on the silica gel plates (60F 254, E. Merck, Germany). The plates were developed using a chloroform, acetic acid, and water solvent system (6:10:2, v: v: v). Plates were sprayed with a methanol and sulfuric acid mixture (95:5, v: v), and then heated for few minutes at 150°C. A mixture of mannose oligosaccharides consisting of mannose (Mn_1), mannobiose (Mn_2), and mannotriose (Mn_3) (10 mg/mL) were used as standards.

3. Results and Discussion

3.1. Bacterial strain selection, enzyme production, and purification

All twenty-five bacterial strains collected from different provinces of Korea were cultivated in galactomannan medium at 28°C for 2 days, with shaking at 130 rpm. Among them,

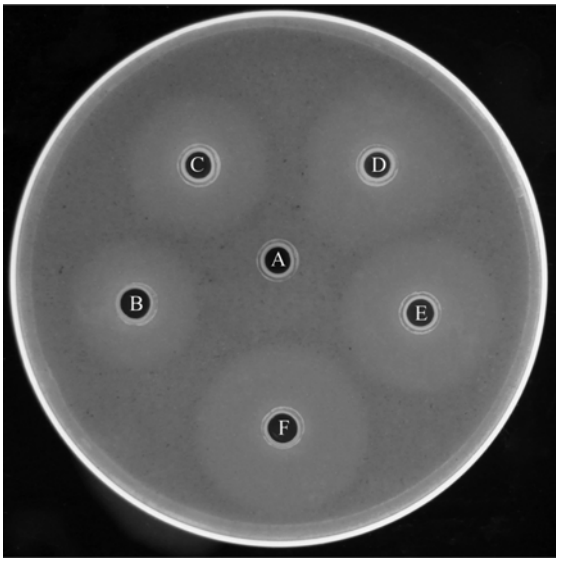

Fig. 1. The plate showing the mannan hydrolysis by MnSt. The plates contained 1.25% LBG, spots inoculated with different concentration of enzyme and incubated at 37ºC for 12 h and then stained with 0.01% Caucofluor white M2R in 0.5 M Tris/HCl, pH 8.0 for 30 min, and destain with water. The lytic zones were photographed under the UV-transilluminator. The hole contains (A) control, (B) 10 U, (C) 20 U, (D) 50 U, (E) 100 U, and (F) 200 U of purified MnSt.

strain *S.* sp. CS656 had strong mannan degrading activity was selected for further study. Previously, our group identified this strain as *Streptomyces tendae* based on morphological data, biochemical characterization, and 16S rRNA sequencing [23]. Further, when MnSt (10 ~ 200 U) was spotted onto mannan agar plates and incubated at 37°C for 48 h, the lytic zone, a measure of mannan hydrolysis, increased with increasing enzyme concentration, while the control produced no clear lytic zone (Fig. 1). The presence of clear lytic zones showed the mannan hydrolysis. In other words, mannan was utilized by the bacterial extracellular enzyme for mannan degradation.

MnSt was purified from the *S. tendae* culture by centrifugation to separate the cells from the supernatant containing the secreted mannanase, followed by ammonium sulfate precipitation of the supernatant, and finally, Sepharose Cl-6B column chromatography. A summary of the mannanase purification procedure is illustrated in Table 1. Mannanase was purified 9.6-fold, with a recovery yield of 26.3%. Active fractions were pooled, concentrated, and analyzed

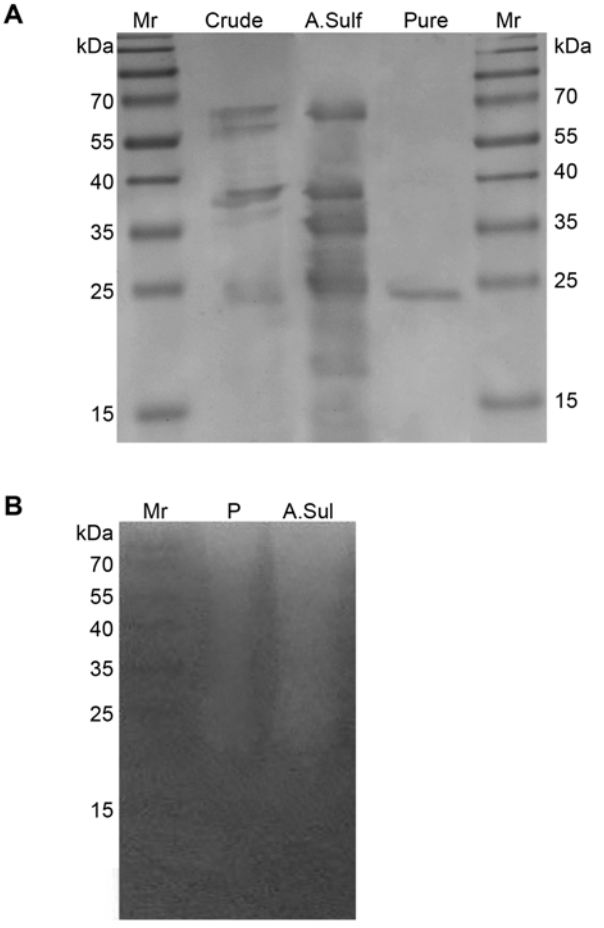

Fig. 2. 12% (w/v) SDS-PAGE (A) and Zymography (B) of purified mannanase from *S. tendae*. Lanes Mr, protein molecular weight marker (Fermentas); lane Crude, Crude extract; lane A. Sul, 30 ~ 80% ammonium sulfate precipitation fraction; lane Pure, purified mannanase after Sepharose Cl-6B column chromatography.

for purity. The pooled active fractions appeared as single band when analyzed by SDS-PAGE, indicating purity. Mannanases from *Streptomyces* sp. have been purified using multiple column chromatographic steps, as described previously [14,15].

3.2. Polyacrylamide gel electrophoresis

SDS-PAGE and mannan zymography of the purified enzyme were performed to analyze enzyme purity and estimate the molecular mass of the enzymes (Figs. 2A and 2B). The molecular mass of MnSt was estimated to be 24 kDa and was observed on gels as a single band. Consistent

Table 1. Purification summary

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purity fold
Crude broth	272.8	46,362.6	170	100.0	1.0
A. sulfate (30 ~ 80%) fractionation	89	32,443	364.5	70	2.1
Sepharose CL-6B	7.5	12,181.0	1,624.1	26.3	9.6

Table 2. Comparison of MnSt with other mannanase from *Streptomyces*

Stain name		Optimum		Stability		Reference
	Molecular weight	pH	Temperature	pH	Temperature	
S. galbus NR	-	6.5	40	5 ~ 8	50	[13]
S. ipomoea CECT3341	40	7.5	55	7.5 ~ 8	80	[14]
S. sp.	42.9	6.8	57	5.8 ~ 8.4	45 ~ 70	[15]
S. thermolilacinus	-	7	55	6 ~ 8	30 ~ 80	[16]
S. sp S27	37.2	7	65	5 ~ 9	50	[17]
S. lividans 66	36	6.8	58	5.5 ~ 8.0	35 ~ 70	[24]
Streptomyces tendae	24	12.0	50	5 ~ 12.5	60	Current study

with SDS-PAGE results, the purified enzyme produced a clear zymography band, confirming that it was a mannanase. These results indicate that the enzyme is a monomeric protein and is different from the other *Streptomyces* mannanases. The other *Streptomyces* mannanases have molecular masses of approximately 36 ~ 42.9 kDa [15,24]. Among purified *Streptomyces* mannanases reported, *S. tendae* mannanase has the lowest molecular weight (Table 2).

3.3. Effect of pH and temperature on the activity and stability of the purified mannanase

Enzyme activity and stability were markedly affected by pH and temperature. The effects of pH and temperature on the activity and stability of MnSt in comparison to other mannanases from *Streptomyces* are shown in Table 2. The mannanase from *S. tendae* was active from pH 11.0 to 12.0, with the highest activity at pH 12 (Fig. 3A). MnSt was stable over a broad pH range (5 ~ 12.5), which is the broadest range reported for *Streptomyces* mannanases [13,16] (Fig. 3A, Table 2). MnSt stability may be due to reversible protein denaturation, which would render incubation at varying pH levels ineffective at permanently altering protein structure. MnSt produced maximal activity at 50°C (Fig. 3B), retained maximal activity after a 1 h incubation at 60°C, and retained almost 75% of its activity when incubated at 65°C. MnSt is more stable than mannanases from *S. galbus* NR [13] or *S.* sp. S27 [17] (Table 2). Many industrial processes operate at extreme pH values and elevated temperatures; thus, the enzyme must withstand these harsh conditions for prolonged periods. Since reaction rates double with a 10°C rise in temperature, an enzyme with higher thermal stability could be used in smaller amounts or in a process with a reduced conversion time. Carrying out conversions at increased temperatures (above 60 ~ 65°C) significantly reduces microbial infection of the material being processed [25] and increases the solubility of polymeric substrates such as carbohydrates, thereby improving the ease of mechanical handling and rendering them more amenable to enzymatic attack. MnSt

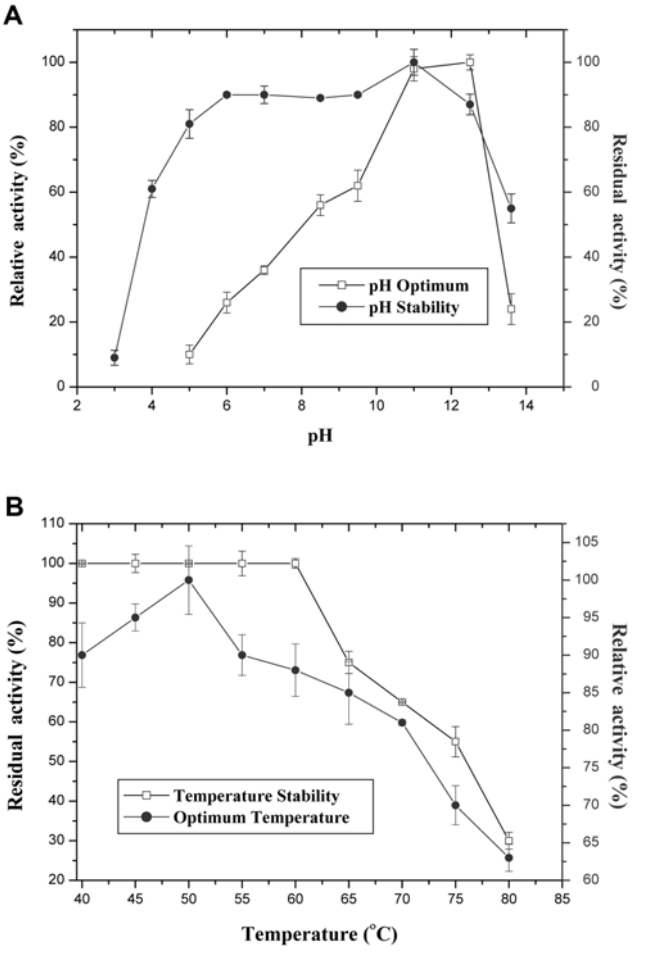

Fig. 3. (A) (□) optimal pH, (●) pH stability of mannanase from *S. tendae*. To determine pH optimum, the activities of mannanase were determined at 50ºC using different pH buffers. To examine mannanase stability, mannanase were incubated with different pH buffers (2 ~ 13.6) at 4ºC for 24 h and relative mannanase activity were evaluated under standard assay conditions. Each point represents as mean (n = 3). (B) (□) optimal temperature, (●) thermal stability of mannanase from *S. tendae*. To determine optimum temperature, the reactions were performed at different temperatures at optimum pH (12), whereas to determine mannanase stability purified MnSt were stored at different temperatures for one hour. Activities were assayed under standard assay conditions. Each point represents as mean (n = 3).

Table 3. Effects of detergents, modulators, and metal ions on the activities of mannanase from *S. tendae*

Reagents	Concentration	Type of ion	Relative activity* (%)
Triton X - 100	0.25%	Non - ionic	107 ± 0.15
Tween - 20	0.25%	Non - ionic	110 ± 0.39
Tween - 80	0.25%	Non - ionic	111 ± 0.4
Polyoxylethylene-4-laurylether	0.25%	Non - ionic	92 + 0.93
Deooxycholic Acid	0.25%	Anionic	94 ± 1.6
Sodium Dodecyl Sulfate	0.25%	Anionic	62 ± 0.26
CHAPS	0.25%	Zwitter ionic	56 ± 2.5
Hydrogen peroxide	5 mM		3 ± 2.7
Sodium perborate	5 mM		1 ± 0.4
β- mercaptoethanol	5 mM		97 ± 0.7
EDTA	1 mM		2 ± 1.7
EGTA	1 mM		1 ± 1.7
Ca^{2+}	1 mM		203 ± 0.47
Mg^{2+}	1 mM		118 ± 4.6
Cu^{2+}	1 mM		1 ± 3.8
Co^{2+}	1 mM		163 ± 3.6
Zn^{2+}	1 mM		20 ± 0.43
K^{+}	1 mM		148 ± 2.9
Na^{+}	1 mM		119 ± 4.2
Mn^{2+}	1 mM		104 ± 0.27
Fe^{2+}	1 mM		103 ± 2.9
None	-		100 ± 1.2

*The results presented are the averages of three separate determinations (n = 3) ± standard deviations.

is novel among *Streptomyces* mannanases, considering it's alkaline in nature, tolerance to a broad pH range, a high optimum temperature, and high thermal stability.

3.4. Effects of metal ions

The influences of metal ions on the activities of mannanase obtained from *S. tendae* were studied and presented in Table 3. The *S. tendae* mannanase activity was enhanced by the presence of metal ions like Ca^{2+}, Mg^{2+}, Co^{2+}, K^{+}, and Na^{+}, similar to the mannanases from *Bacillus* sp. N16-5 [7], *Cellulosimicrobium* sp. strain HY-13 [26], *Vibrio* sp. strain MA-138 [27] (Table 3) but different from mannanases from *Paenibacillus* sp. DZ3 [11] and *Bacillus subtilis* WY34 [28]. MnSt activity was inhibited by $Zn^{2+}$and Cu^{2+}, similar to the mannanases from *S.* sp. [15], *Cellulosimicrobium* sp. strain HY-13 [26], *Vibrio* sp. strain MA-138 [27], and *Bacillus subtilis* KU-1 [29] but different from the mannanases from *Bacillus* sp. N16-5 [7], *S.* sp. S27 [17], and *Bacillus subtilis* WY34 [28]. MnSt enzyme activity was unaffected by the presence of Fe^{2+} and Mn^{2+}, similar to the mannanases from *S.* sp. S27 [17], *Cellulosimicrobium* sp. strain HY-13 [26], and *Bacillus subtilis* WY34 [28], but different from the mannanase from *Bacillus* sp. N16-5 [7], *Vibrio* sp. strain MA-138 [27], *Bacillus subtilis* WY34 [28], *Bacillus subtilis* KU-1[29]. These results should guide the selection of metals to be included or excluded for industrial applications.

3.5. Effects of detergents and modulators

In case of detergents, MnSt activity increased with TritonX-100, Tween-20, and Tween-80, similar to the mannanase from *Cellulosimicrobium* sp. strain HY-13 [26]. MnSt activity was slightly affected by polyoxylethylene-4-laurylether and deoxycholic acid, but the activities were reduced for CHAPS and SDS, similar to the mannanase from *Bacillus subtilis* WY34 [28], but not the mannanase from *Bacillus* sp. N16-5 [7]. Hydrogen peroxide and sodium perborate, but not β-mercaptoethanol, had an inhibitory effect on MnSt, similar to the mannanases from *S.* sp S27 [17] and *Bacillus subtilis* WY34 [28]. Further, the mannanase activities were highly inhibited by EDTA and EGTA, similar to mannanases from *S. thermolilacinus* [16], *Cellulosimicrobium* sp. strain HY-13 [26], and *Bacillus subtilis* WY34 [28].

3.6. N-terminal amino acids

The last 15 N-terminal amino acids of MnSt were GWSVDAPYIAXQPFS. This sequence was compared with available sequences in the National Center for Biotechnology Information (NCBI) protein database by using BLAST (basic local alignment search tool; http://blast.ncbi.nlm.nih.gov/Blast.cgi). The BLAST search did not suggest homology with other mannanase sequences, but showed a high degree of homology with β- mannanase from *Streptomyces* sp. Tu6071.

3.7. Substrate specificity and kinetics assays

Purified MnSt was assayed using different substrates. Locust bean gum galactomannan was the most effective substrate under the standard assay conditions. MnSt was not active against beech wood and birch wood xylan, colloidal chitin, sigma cell cellulose, carboxymethyl cellulose, avicel, paranitrophenyl D-cellobioside (pNPC), or paranitrophenyl-β-D glycopyranoside (pNPG). An ideal mannanase, useful in bio-bleaching, must be active and stable at high temperatures and alkaline conditions, and it must not impair pulp quality by attacking cellulose fibers. MnSt is a low-molecular weight mannanase and can more easily penetrate the re-precipitated galactomannan constituents. MnSt is an excellent candidate for use in enzymatic bleaching of softwood pulps particles [30]. MnSt mitigates the problem of a mannan barrier on the surface of lignin-containing pulp during subsequent chemical bleaching steps. Although many microorganisms have been known to produce mannanase, *S. tendae* produces cellulase-free mannanase, which is stable over a broad pH range and high temperatures, meaning that it could be used for the degradation of agricultural waste and for bio-bleaching in the paper and pulp industries.

The kinetic constants K_m and V_{max} of MnSt, determined using a Line weaver-Burk Plot, were 0.5 ± 1 mg/mL and 439 ± 0.5 μmol/(min mg), respectively. The K_m value was lower than for most other mannanases, which have the following K_m values: 1.05 mg/mL, *Paenibacillus* sp. DZ3 [11]; 3.4 mg/mL, *S. ipomoea* CECT 3341 [14]; and 7.6 mg/mL, *Bacillus subtilis* WY34 [28], but higher than S. sp S27 (0.16 mg/mL) [16]. The V_{max} value for *S. tendae* mannanase was comparable to the V_{max} for the mannanase from *S. thermolilacinus* (114 μmol/(min mg)) [16], but very low in comparison to the mannanase from *S.* sp S27 (3739 μmol/(min mg)) [16]. These findings suggest that substrate affinity and the catalytic efficiency of MnSt is different from the mannanases from other microorganisms.

3.8. Enzymatic hydrolysis and oligosaccharide production

The time course of enzymatic hydrolysis of locust bean gum galactomannan was analyzed by TLC (Fig. 4). Initially, mannooligosaccharides were the main oligosaccharides released upon hydrolysis, but with further hydrolysis, mannobiose (Mn_2), and mannotriose (Mn_3), were released. In this study, mannobiose and other mannooligosaccharides were produced by the cumulative action of MnSt, demonstrating that MnSt is an endo-type enzyme [7], which is similar to the mannanases from *Sclerotium (Athelia) rolfsii* [6] and *Aspergillus niger* [8]. MnSt hydrolyzed mannan to produce mainly mannobiose and mannotriose, but did not produce mannose by the hydrolysis of locust bean gum galactomannan. However, mannose was observed as a product of hydrolysis of locust bean gum galactomannan by the mannanase from *B. subtilis* 5H [31]. A study of endo-mannanases by Tamaru *et al.* [9] showed that endo-mannanase from *Vibrio* sp. could not hydrolyze mannotriose, while endo-mannanase from *Streptomyces* could hydrolyze mannotriose to form mannose and mannobiose [32]. Most of the available mannanases are endo β-mannanase (*e.g.,* gamanase and hemicell mannanase), and when this type of enzyme is included in the diet, we speculate that mannooligosaccharide, mannotriose, and mannobiose, as well as a small amount of mannose, are generated. The production of mannooligosaccharide improves chicken health [33], either by increasing the population of specific bacteria such as bifidiobacteria, which consume mannooligo-saccharides in the caeca and suppress pathogenic bacteria, or by flushing out the pathogenic bacteria that were attached to the mannooligosaccharides. The use of mannanase in poultry research has been widespread and successful [34]. Mannooligosaccharides produced by various microbes are used as food additives for humans to promote selective growth of beneficial intestinal microflora [28]. Further, mannanase may also be used for hydrolyzing galactomannan present in liquid coffee extract to inhibit gel formation during freeze-drying of instant coffee [10].

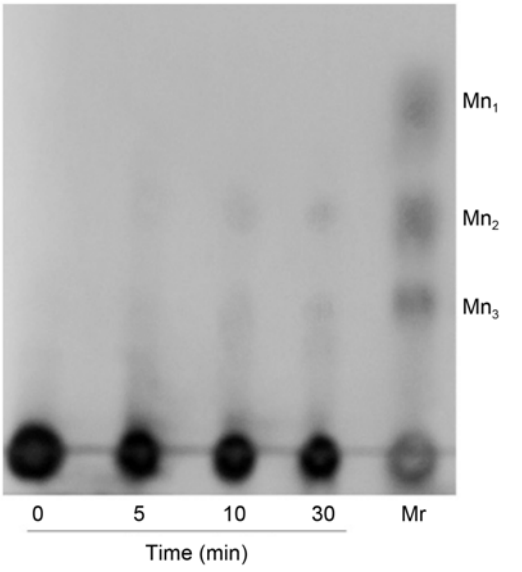

Fig. 4. Plot showing mannan degradation after treating with locust bean gum galactomannan at 37ºC and pH 12 with mannanase from *S. tendae*. Mr: mixture of Mannose (Mn), Mannobiose (Mn_2), and Mannotriose (Mn_3).

4. Conclusion

Streptomyces tendae isolated from Korean soil samples produced a low molecular weight (~ 24 kDa) mannanase (the lowest among the *Streptomyces* mannanases), which

was purified by single chromatography with a yield of 26.3%. MnSt works optimally at 50°C and a pH of 12, tolerates a broad pH range (pH 5 ~ 12.5), and has high thermostability. MnSt is the most alkaline tolerant mannanase ever reported from *Streptomyces.* Its N-terminal amino acid sequence was GWSVDAPYIAXQPFS. MnSt activity was highly influenced by Ca^{2+}, but inhibited by Cu^{2+}, Zn^{2+}, EDTA, EGTA, hydrogen peroxide, sodium perborate, CHAPS, and SDS. Deoxycholic acid, Triton X-100, Tween-20, Tween-80, β- mercaptoethanol, Fe^{2+}, and Mn^{2+} had no effect on MnSt activity. MnSt produced mainly mannobiose and mannotriose as major locust bean gum galactomannan hydrolysis products. MnSt could potentially be useful as a biocatalyst in the biofuel, food, and textile industries.

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