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Characterization of the *Bacillus subtilis* WL-3 Mannanase from a Recombinant *Escherichia coli*

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A mannanase was purified from a cell-free extract of the recombinant *Escherichia coli* carrying a *Bacillus subtilis* WL-3 mannanase gene. The molecular mass of the purified mannanase was 38 kDa as estimated by SDS-PAGE. Optimal conditions for the purified enzyme occurred at pH 6.0 and 60°C. The specific activity of the purified mannanase was 5,900 U/mg on locust bean gum (LBG) galactomannan at pH 6.0 and 50°C. The activity of the enzyme was slightly inhibited by Mg^{2+}, Ca^{2+}, EDTA and SDS, and noticeably enhanced by Fe^{2+}. When the enzyme was incubated at 4°C for one day in the presence of 3 mM Fe^{2+}, no residual activity of the mannanase was observed. The enzyme showed higher activity on LBG and konjac glucomannan than on guar gum galactomannan. Furthermore, it could hydrolyze xylans such as arabinoxylan, birchwood xylan and oat spelt xylan, while it did not exhibit any activities towards carboxymethylcellulose and *para*-nitrophenyl-β-mannopyranoside. The predominant products resulting from the mannanase hydrolysis were mannose, mannobiose and mannotriose for LBG or mannooligosaccharides including mannotriose, mannotetraose, mannopentaose and mannohexaose. The enzyme could hydrolyze mannooligosaccharides larger than mannobiose.

***Keywords*:** *Bacillus subtilis* WL-3, characterization, mannanase, recombinant *Escherichia coli*

Mannan-based polysaccharides, which are composed of β-1,4-linked linear backbone of mannose residues that carry other carbohydrates or acid substitutions, are widely distributed in the cell walls of higher plants. There are three known enzymes that participate in the complete decomposition and conversion of mannan: endo-1,4-β-mannanase (mannanase), exo-1,4-β-mannanase, and β-mannosidase. Mannanase catalyzes the random hydrolysis of the β-D-1,4-mannopyranosyl linkages within the backbone of various mannans including mannan, glucomannan, galactomannan, and galactoglucomannan. The interest in mannanase is due to its application to the food, feed, and pulp industries (Dhawan and Kaur, 2007). The most important activity of mannanase, which is characterized as a hemicellulase together with xylanase and glucanase, is its activity to saccharify hemicellulose, a recyclable plant resource, into a carbon source that is readily metabolized by living organisms. For instance, galactomannans of leguminous seeds are regarded as anti-nutritional factors because monogastric animals are unable to digest them rapidly enough to obtain the edible sugars. Therefore, mannanase has been used as a feed additive enzyme to increase the nutritional value of major components of animal feed such as soybean meal, corn, and guar meal.

Due to a recent increase in oil prices, many countries over the world are eagerly looking for alternative energy sources. Bio-energy is definitely one of the alternatives, and lots of grains are currently being used as carbon sources for the microbial production of bio-energy. As a result, the prices of feed grains have escalated, and at the same time, grains with low qualities have been used as feeds. Hence, much attention has been paid to feed additive enzymes for breeding animals.

Though mannanases are widely distributed in microorganisms, higher plants, as well as animals, much attention has currently been focused on the microbial mannanases for their industrial applications. Bacterial mannanases have been reported from various strains in 20 different genera, and purified from *Bacillus circulans* (Yosida *et al*., 1997), *B. stearothermophilus* (Talbot and Sygusch, 1990), *B. subtilis* WY34 (Jiang *et al*., 2006), *Caldicellulosiruptor* sp. (Gibbs *et al*., 1996), *Cellulomonas fimi* (Stoll *et al*., 2000), *Caldibacillus cellulovorans* (Sunna *et al*., 1999), *C. tertium* (Kataoka and Tokiwa, 1998), *C. thermocellum* (Kurokawa *et al*., 2001), *Pseudomonas* sp. (Yamaura *et al*., 1990), *Enterococcus casseliflavus* (Oda *et al*., 1993), *Bacteroides ovatus* (Gherardini and Salyers, 1987), *Dictyoglomus thermophilum* (Gibbs *et al*., 1999), and several stains of *Bacillus* sp. (Akino *et al*., 1988; Hossain *et al*., 1996; Ma *et al*., 2004). These mannanases have been shown to belong to either glycosyl hydrolase (GH) family 5 or GH family 26 on the basis of their amino acid similarities. While numerous types of mannanases and their respective genes have been identified from several microorganisms, research on mannanases has been limited in comparison to other carbohydrases such as cellulase, xylanase and amylase.

Many *Bacillus* strains, which produce valuable hydrolytic

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enzymes, have been isolated from Korean fermented foods. In a previous study, we cloned the mannanase genes from *B. subtilis* WL-3 (Yoon and Lim, 2007) and WL-7 (Kweun *et al*., 2004), which had been isolated from Korean fermented soybean. The WL-3 mannanase showed a sequence identity of 99% with *B. subtilis* NM-39 (Mendoza *et al*., 1995), and approximately 75% with *B. subtilis* 168 (GenBank accession no. CAB12407), *B. subtilis* Z-2 (GenBank accession no. AAV84100), *Bacillus* sp. 5H (Khanongnuch *et al*., 1999), and *B. subtilis* WL-7 (Kweun *et al*., 2004). These mannanases have a catalytic module belonging to GH family 26. To compare the biochemical and reactional properties of WL-3 mannanase with others, the WL-3 mannanase was purified and characterized from a recombinant *Escherichia coli* carrying the *B. subtilis* WL-3 mannanase gene in the present work.

Materials and Methods

Chemicals, enzymes, bacterial strain, and media

Locust bean gum (LBG), guar gum, CMC, oat spelt xylan, birchwood xylan and the chromogenic substrates were purchased from Sigma Chemical Co. (USA). The mannooligosaccharides and arabinoxylan were obtained from Megazyme (Ireland), the konjak powder from Daeshinmulsan Co. (Korea), and the bacterial medium from Difco (USA). The restriction endonucleases, protease, and RNase A was obtained from Boehringer Mannheim (Germany), and T4 DNA ligase from Solgent Co. (Korea). They were all used as recommended by their manufacturers. *E. coli* XL-1 blue (*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*46 *relA*1 *thi lac*$^-$ F'[*proAB*$^+$ *lacI*^{q} *lacZ* M15 Tn10(*tet*^{r})]) was used as a host for recombinant plasmid. A plasmid pUC19 was used as a vector for constructing a recombinant plasmid pNA15 carrying a *B. subtilis* WL-3 mannanase gene.

Mannanase purification

A recombinant *E. coli* (pNA15) was used for producing the mannanase of *B. subtilis* WL-3. The recombinant strain was grown overnight at 37°C in LB medium supplemented with ampicillin (50 μg/ml). The cells were collected by centrifugation at 6,000×g for 15 min. After the collected cells were washed one time and suspended in 50 mM Tris-HCl buffer (pH 8.0), they were disrupted by sonication with a Branson Sonifier. The resulting cell debris was then removed by centrifugation at 10,000×g for 30 min. The cell-free extract was precipitated with ammonium sulfate (30∼70% saturation) and then dialyzed against the same buffer. The crude samples were run through a DEAE-Sepharose column equilibrated with the same buffer, and the proteins were eluted with a linear NaCl gradient (0 to 1.0 M). The active fractions were concentrated by ultrafiltration, and dialyzed against 50 mM Tris-HCl buffer (pH 8.0) containing 1.0 M $(NH_4)_2SO_4$. The dialyzed sample was run through a phenyl-Sepharose CL-4B column, and the proteins were eluted with a linear $(NH_4)_2SO_4$ gradient (1.0 to 0 M). The active fractions were concentrated, and then dialyzed against 50 mM Tris-HCl buffer (pH 8.0). The enzyme solution was again loaded into the DEAE-Sepharose column, and proteins were eluted with a linear NaCl gradient (0 to 0.4 M). The active fractions were pooled and concentrated by ultrafiltration. Proteins were analyzed by SDS-PAGE.

Enzyme assay

The mannanase activity was determined by measuring the amount of reducing sugars liberated during the hydrolysis of LBG by the dinitrosalicylic acid method (Miller *et al*., 1960). The standard assay reaction mixture consisted of 0.5% (w/v) of polysaccharide substrates supplemented with 50 mM sodium citrate buffer (pH 6.0) and enzyme to make a final volume of 0.3 ml. The reaction mixture was incubated at 50°C for 15 min. One unit of the enzyme activity was defined as the amount of enzyme to produce 1 μmol of reducing sugar per min.

Effects of pH, temperature, and various reagents on enzyme activity

The effect of pH on the reaction rate was determined by measuring mannanase activity at different pH values under the condition of 50°C, using 50 mM of sodium citrate (pH 3.5 to 6.0), sodium phosphate (pH 6.0 to 8.0) and KCl-borate (pH 8.0 to 9.0) buffers. The activity of the purified enzyme was also assayed with 50 mM sodium citrate buffer (pH 6.0) at various temperatures, ranging from 30°C to 70°C. The thermostability of mannanase was also examined by measuring the residual activity after preincubating at various temperatures (40 to 70°C) without the substrate. To investigate the effect of chemicals on enzyme activity, the mannanse activity was measured by adding several different reagents into the standard reaction mixture to make final concentration of 5 mM. In addition, the effect of chemicals on the enzyme stability was also found by preincubating the enzyme in a solution of various reagents dissolved in 50 mM sodium citrate buffer (pH 6.0) at 4°C for 24 h. After the preincubated enzyme were diluted with 50 mM citrate buffer, the residual activity was then measured by using the standard assay condition.

Analysis of the hydrolysis products

Reaction mixtures containing mannooligosaccharides or mannans were incubated at 50°C for a long time to ensure the complete hydrolysis. The reaction mixtures were then boiled for 10 min and centrifuged. The hydrolysis products in the supernatant were analyzed by the thin layer chromatography. The supernatant was spotted on silica gel-precoated thin layer plate (Merck, USA), and developed at room temperature with 1-propanol, nitromethane, and water (7:2:1, v/v). For detection of carbohydrates, a mixture consisting of 0.5 ml of *p*-anisaldehyde, 0.5 ml of concentrated H_2SO_4, and a few drops of glacial acetate in 9 ml of 95% ethanol was used as a spray reagent, and incubated at 121°C for 5 min.

Results and Discussion

Optimal reaction conditions of the purified mannanase

In the previous work, a mannanase gene of *B. subtilis* WL-3 was cloned and sequenced. The *manA* gene was strongly expressed in *B. subtilis* 168 by cloning the gene downstream of a strong *B. subtilis* promoter. Since the mannanase enzyme produced by the *B. subtilis* 168 showed a 74% sequence

identity with the WL-3 mannanase and the mannanase of *B. subtilis* 168 had a molecular mass identical to the WL-3 mannanase, the WL-3 mannanase was purified by using a recombinant *E. coli* carrying the *manA* gene. The *Nae*I-generated 1.5 kb DNA fragment of plasmid pM3C1, which included the WL-3 *manA* gene, was introduced into the *Sma*I site of pUC19 to create a recombinant plasmid, pNA15. The plasmid was transformed into *E. coli* XL-1 blue.

From the cell-free extract of recombinant *E. coli* cells (pNA15), the mannanase was purified by means of column chromatography on DEAE, and Phenyl-Sepharose, as described in the 'Materials and Methods' section. The purified enzyme gave a single protein band corresponding to molecular mass of 38 kDa according to the results from the SDS-PAGE (Fig. 1). The molecular mass of the purified enzyme agrees with that of the mature mannanase predicted by the nucleotide sequence of the *B. subtilis* WL-3 *manA*. Its molecular mass was similar to those of mature mannanases from *D. thermophilum* (40 kDa) (Gibbs *et al*., 1999), and several strains of *B. subtilis* (37~39 kDa) (Mendoza *et al*., 1994; Khanongnuch *et al*., 1998; Kweun *et al*., 2004). These mannanases had a catalytic module, while many GH26 mannanases of high molecular mass such as those produced from alkaliphilic *Bacillus* sp. JAMB-750 (Hatada *et al*., 2005), *B. stearothermophilus* (Talbot and Sygusch, 1990), *C. thermocellum* (Kurokawa *et al*., 2001), *Caldicellulosiruptor* sp. Rt8.B4 (Gibbs *et al*., 1996), *C. fimi* (Stoll *et al*., 2000), *C. cellulovorans* (Sunna *et al*., 1999), *E. casseliflavus* (Oda *et al*., 1993), and *B. ovatus* (Gherardini and Salyers, 1987) were composed of at least two modules.

The purified mannanase had specific activity of a 5,900 U/mg toward LBG at reaction conditions of pH 6.0 and 50°C. Its specific activity was higher than those of *D. thermophilum* Rt46B.1 mannanase (3,590 U/mg), *B. licheniformis* mannanase (4,341 U/mg) (Zhang *et al*., 2000), *B. stearothermophilus* (455 U/mg), *C. thermocellum* (135 U/mg). Recently, mannanases of *B. subtilis* WY34 (8,302 U/mg) and *B. subtilis* WL-7 (10,080 U/mg) were reported to have higher specific activities than the WL-3 mannanase. From these reports, it can be assumed that mannanases from *B. subtilis* show higher specific acitivities on LBG than mannanases from other strains. The purified mannanase had the highest activity on LBG at pH 6.0 and 60°C with greater than 80% activity at temperatures between 45°C and 65°C (Fig. 2). It showed more than 95% of maximal activity at a pH range from 5.0 to 6.5. Many purified mannanases of *Bacillus* strains were reported to have optimal temperatures between 50°C to

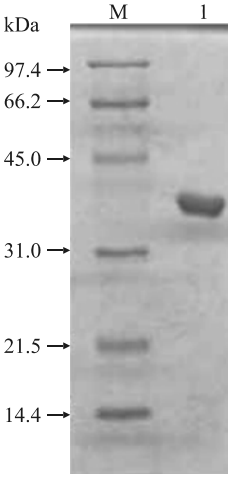

Fig. 1. SDS-PAGE of the mannanase purified from recombinant *E. coli*. Lane 1, the molecular weight markers; lane 2, the purified enzyme. Molecular size is shown in kilodaltons on the left side of the gel.

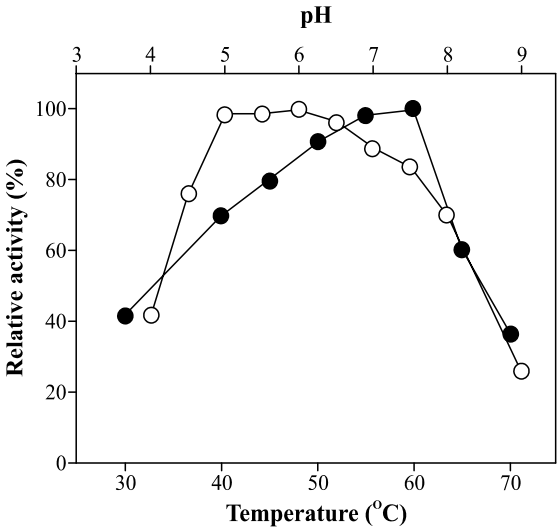

Fig. 2. Effects of reaction temperature and pH on the mannanase activity. Temperature profile (●) was obtained by measuring the mannanase activities at pH 6.0 and different temperatures. The pH profile (○) was obtained by measuring the mannanase activities at various pH's and at a constant temperature of 50°C. Buffers (50 mM) used were as follows: sodium citrate (pH 3~6), sodium phosphate (pH 6~8), and KCl-borate (pH 8~9).

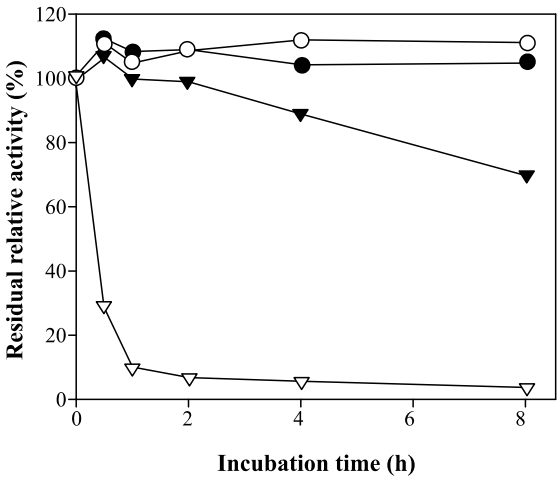

Fig. 3. Thermostability of the purified mannanase. Thermostability was determined by measuring the residual activities after pre-incubation at different temperatures accordingly: 40°C (●), 50°C (○), 60°C (▼), 70°C (▽).

70°C. Additionally, they were known to exhibit maximal activities in the pH ranges of 5.0~6.5 (Mendoza *et al*., 1994; Jiang *et al*., 2006) or 9.0~10.0 (Ma *et al*., 2004; Hatada *et al*., 2005). Mendoza *et al*. (1994) reported that the *B. subtilis* mannanase NM-39 showing the highest identity (99%) of amino acid sequence to WL-3 mannanase exhibited maximal activity at pH 5.0 and 55°C. To examine thermostability, the purified enzyme was incubated for 8 h at various temperatures and the residual activities were then assayed. The enzyme was stable up to 50°C for 8 h, but its stability rapidly decreased at temperature above 70°C (Fig. 3). The enzyme retained 70% of its activity at 60°C after 8 h of incubation. This thermostability is comparable to that reported for *B. subtilis* WY34.

Effects of metal ions and other reagents on enzyme activity

The effects of various reagents, including metal salts, EDTA, dithiothreitol and SDS on the WL-3 mannanase activity were investigated (Table 1). Microbial mannanases were differently affected by metal ions. When the enzyme activity of the WL-3 mannanase was measured in the presence of the reagents, the activity was noticeably enhanced by Fe^{2+} as in the case of *B. subtilis* WY34, while ferrous ions inhibited the mannanase activity of both *D. thermophilum* Rt46B.1 and *Streptomyces galbus* (Kansoh and Nagieb, 2004). In addition, the mannanases of *B. subtils* WL-7 and NM-39 were not affected by Fe^{2+}. Like Fe^{2+}, the effect of Cu^{2+} on mannanases from different strains also varied. While the activity of the mannanase from strains such as *Pseudomonas* sp. PT-5 (Yamaura *et al*., 1990), *S. galbus*, *D. thermophilum* Rt46B.1, and *B. subtilis* WY34 was inhibited by Cu^{2+}, that of WL-3 mannanase was slightly increased. The acitivity of WL-3 mannanase was inhibited by 5 mM of SDS, Mg^{2+}, and EDTA.

After treating the enzyme with chemicals for 24 h in the absence of substrate (LBG), residual activity of the enzyme was determined at the standard reaction condition without the reagents (Table 1). The enzyme activity was decreased to 60~93% of its original level by chemicals such as SDS, EDTA, Ca^{2+}, Na^{+}, and K^{+}. It is worth noting that the enzyme treated with 5 mM Fe^{2+} had no residual activity. Because the Fe^{2+} ion could be oxidized to Fe^{3+} in ambient conditions, it was expected that the enzyme activity was lost by Fe^{3+}. To protect Fe^{2+} from the oxidation during preincubation of the enzyme, the enzyme was treated with Fe^{2+} and dithiothreitol (DTT) together. When the enzyme was treated with Fe^{2+} in the presence of DTT, the enzyme activity maintained stably for 24 h (Table 2).

Substrate specificity

The purified mannanase was assayed with various substrates to investigate its substrate specificity. When polysaccharides were used as substrates, the activity was determined by measuring the amount of the released reducing sugars, and the synthetic substrate derivatives were assayed by measuring the release of *para*-nitrophenol (pNP). As shown in Table 3, when LBG galactomannan (mannose/galactose ratio, 4:1) was used as the substrate, the largest quantity of reducing sugars was liberated. Konjac glucomannan (mannose/glucose ratio, 1.5:1) used as the substrate showed comparable results with LBG, while replacement of LBG by the guar gum galactomannan (mannose/galactose ratio, 2:1) resulted in an activity reduced by 90%. The lower activity towards guar gum than LBG supports the hypothesis that the enzyme ac-

Table 1. Effects of metal ions and other reagents on the activity and stability of mannanase

Chemical (5 mM)	Relative activity (%) after pre-incubation for	
	0 h[a]	24 h[b]
None	100.0	100.0
NaCl	93.0	85.0
KCl	95.0	93.5
$MgCl_2$	87.0	82.1
$MnCl_2$	96.6	101.8
$CaCl_2$	91.1	79.0
$CuCl_2$	117.7	87.2
$FeCl_2$	151.2	0
EDTA	86.0	70.2
SDS	68.9	61.3
Dithiothreitol	120.3	102.5

[a] The mannanase activity was measured in the presence of the chemicals. [b] Residual activity of the mannanase was measured in the absence of the chemicals after treating the enzyme with chemicals for 24 h.

Table 2. Effects of ferrous ion on the mannanase stability in the presence of dithiothreitol

Chemical	Residual relative activity (%) after pre-incubation for	
	12 h	24 h
None	100.0	100.0
$FeCl_2$ (3 mM)	36.4	0
DTT (15 mM)	104.9	104.5
$FeCl_2$ (3 mM) and DTT (15 mM)	102.9	99.9

Table 3. Substrate specificity of the purified mannanase

Substrates	Relative activity (%)
Locust bean gum galactomannan	100.0
Konjac glucomannan	97.0
Guar gum galactomannan	11.3
Oat spelt xylan	0.1
Birchwood xylan	0.1
Arabinoxylan	0.3
Carboxymethylcellulose	ND
pNP-β-Mannoside	ND
pNP-β-Cellobioside	ND
pNP-β-Xyloside	ND

ND, not detected

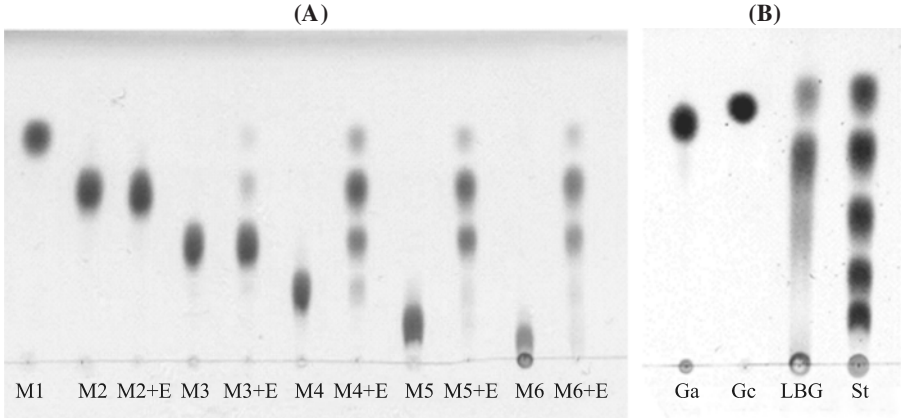

Fig. 4. Thin-layer chromatogram of hydrolysis products of β-1,4-linked manno-oligosaccharides (A) and LBG galactomannan (B) with purified mannanase. The reaction mixtures containing the purified mannanase and mannooligosaccharides or LBG in 50 mM sodium citrate buffer (pH 6.0) were incubated for 4 h at 50°C. (A) M1 to M6 represent mannose to mannohexaose; E, purified mannanase. (B) St, LBG, Ga, and Gc stands for standard mannooligosaccharides with a degree of polymerization 1-5, LBG hydrolyzate, galactose and glucose, respectively.

tivity is limited by a number of branched α-galactose residues. Several mannanases from *B. stearothermophilus* (15%), *B. circulans* (9.8%), *B. subtilis* WY34 (10.7%), and *B. subtilis* WL-7 (12.8%) exhibited less activity on guar gum galactomannan than LBG.

It was also reported that mannanases from *C. cellulovorans* (Sunna *et al*., 1999) and *C. tertium* (Kataoka and Tokiwa, 1998) could comparably hydrolyze galactomannan guar gum to LBG, but konjac poorly. The WL-3 mannanase was not active on either carboxymethyl cellulose (CMC) or synthetic substrate derivatives such as pNP-β-mannoside, pNP-β-cellobioside, and pNP-β-xyloside, indicating that the mannanase could not hydrolyze the β-1,4-cellulosic linkages and had no β-mannosidase activity. On the other hand, the enzyme hydrolyzed the xylan substrates low efficiency. Xylan was not hydrolyzed by mannanases from *B. stearothermophilus*, *C. cellulovorans*, and *Bacillus* sp. 5H. The activity of the mannanase produced by the extreme thermophile, *D. thermophilum* Rt46B.1, was weak on xylans, and absent on CMC. Among the mannanases belonging to GH26 family, xylanase activity was detected in two mannanases of both strains WL-3 and Rt46B.1. The purified mannanase from *B. subtilis* WY34, which had hydrolysis activities on broad substrates such as starch, CMC and xylan as well as mannans, has been reported (Jiang *et al*., 2006).

Hydrolysis patterns of mannan

The final hydrolyzates of LBG and mannooligosaccharides by the purified mannanase were analyzed by thin-layer chromatography (Fig. 4). The main products in each case were mannose, mannobiose, and mannotriose from LBG, mannotetraose, mannopentaose and mannohexaose. Many mannanases hydrolyzed LBG to yield mainly mannobiose and mannotriose. Depending on the origin of mannanase, mannose can be produced in the hydrolyzates of LBG. While mannanase from *B. subtilis* NM-39, 5H, and WL-7 produced mannose from LBG, those from *Bacillus* sp. KK01 (Hossain *et al*., 1996) and *B. subtilis* WY34 did not produce mannose.

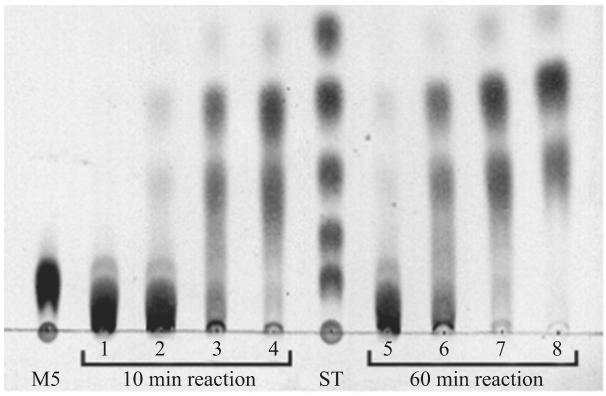

Fig. 5. Hydrolysis patterns of mannopentaose by various amounts of mannanase. 10-serial dilutions were used to dilute the enzyme for the hydrolysis reactions. Reactions were performed for 10 min and 60 min with the diluted enzyme. Mannopentaose was hydrolyzed by the diluted mannanase: lanes 1 and 5, 1000×; lanes 2 and 6, 100×; lanes 3 and 7, 10×. Non-diluted enzyme was used for lanes 4 and 8. ST represents standard mannooligosaccharides with a degree of polymerization 1-5; M5, mannopentaose.

The WL-3 mannanase showed lower hydrolyzing activity on mannotriose than mannooligosaccharides with a degree of polymerization (DP) of 4-6, and no activity on mannobiose. When mannotriose was treated for a long time with an excessive enzyme present, all three compounds of mannose, mannobiose and mannotriose were observed with similar amount in the reactant (data not shown). Mannanase from *B. subtilis* WL-7 and *Bacillus* sp. AM-001 (Akino *et al*., 1988) hydrolyzed mannooligosaccharides with 3 or more mannose units. However, mannanases from *B. subtilis* WY34 (Jiang *et al*., 2006), *E. casseliflavus* FL2121 (Oda *et al*., 1993), and *Pseudomonas* sp. strain PT-5 (Yamaura *et al*., 1990) could hydrolyzed only mannooligosaccharides with 4 or more mannose units to produce several oligosaccharides, indicating

that these mannanases could not hydrolyze mannotriose.

To investigate the intermediate materials produced during the hydrolysis reaction, mannopentaose was hydrolyzed with various amounts of the mannanase. When the substrate was treated for a short time by the enzyme with a low activity, the intermediate materials larger than mannopentaose were quantitatively detected on TLC (Fig. 5). This suggests that the mannanase has transglycosylation activity.

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