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High-level production, purification and characterization of a thermostable β-mannanase from the newly isolated *Bacillus subtilis* WY34

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Abstract

A newly isolated *Bacillus subtilis* WY34 produced high level of mannanase (1105 U/ml) when grown on konjac powder as the carbon source at 50 °C. The β-mannanase was purified 5.4-fold to homogeneity with a final recovery of 20.3% and a specificity of 8302.4 U/mg protein. The purified mannanase appeared as a single protein band on SDS–PAGE gel with a molecular mass of approx. 39.6 kDa. It was identified as a glycoprotein by periodic acid-Schiff staining with a carbohydrate content of 13.1%. The first 10 N-terminal amino acid sequence (HTVSPVNPNA) of the purified enzyme showed high homology (90% identity) with the N-terminal region of β-mannanase from *B. subtilis* NM-39. The optimal temperature and pH for mannanase activity was 65 °C and pH 6.0, respectively. The mannanase activity was stable within pH 5.5–10.1. It was stable up to 60 °C at pH 6.0. The mannanase was highly specific towards locust bean gum, but exhibited very low activity towards starch, CMC and birchwood xylan. Apparent K_m values of the mannanase for locust bean gum, guar gum and konjac powder were 7.6, 10.5 and 27.4 mg ml^{-1}, respectively. Copra mannan was degraded mainly to mannotetraose, mannobiose and mannotriose when incubated with the mannanase, suggesting that the mannanase is an endomannanase and is suitable for mannooligosaccharide production. These unique properties of the purified β-mannanase from *B. subtilis* WY34 make this enzyme attractive for biotechnological applications.

Keywords: *Bacillus subtilis*; Characterization; Glycoprotein; High-level production; Hydrolysis property; Mannanase; Purification

1. Introduction

The hemicelluloses are the second most abundant heteropolymers present in nature (Viikari et al., 1992). Depending on their sugar backbone composition, they are classified as either xylans, mannans, arabinogalactans or arabinans. The two most important and representative hemicelluloses are the hetero-1,4,-β-D-xylans and the hetero-1,4,-β-D-mannans. *endo*-β-D-Mannanase (EC 3.2.1.78, mannan *endo*-1,4-β-D-mannosidase) cleaves randomly within the 1,4-β-D-mannan main chain of galactomannan, glucomannanan, galacto glucomannanan and mannan (McCleary, 1988). Various mannanases from *Streptomyces* sp. (Takahashi et al., 1984), *Bacillus subtilis* (Mendoza, Arai, Kawaguchi, Yoshida, & Joson, 1994a; Zakaria, Yamamoto, & Yagi, 1998), *Sclerotium* (*Athelia*) *rolfsii* (Sachslehner & Haltrich, 1999), *Bacillus stearothermophilus* (Zhang, He, & Hu, 2000), *Aspergillus awamori* (Kurakake & Komaki, 2001) and *Trichoderma harzianum* (Ferreira & Filho, 2004) have been purified and characterized, as well as some genes from *B. subtilis* and *Bacillus stearothermophilus* encoding mannanases were also cloned, sequenced and expressed (Ethier, Talbot, & Sygusch, 1998; Mendoza et al., 1995).

Mannanases can be useful in several processes in the food, feed, as well as in the pulp and paper industries (Ademark et al., 1998; Kobayashi, Echigen, Mada, & Mutai, 1987; McCleary, 1988). Despite having high practical potentialities, the use of mannanase is still limited due

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to low yields and high-production costs (Zhang et al., 2000). Various microorganisms have been reported as mannanases producers (McCleary, 1988). Among those is *B. subtilis* which is recommended because of its safety, fast growth and ability to secrete a high level of mannanase into the medium (Mendoza et al., 1994b; Zakaria et al., 1998). In this study, we purified and characterized a thermostable mannanase from a newly isolated strain of *B. subtilis* WY34. This strain has been found as an outstanding producer of mannanase. Comparative studies of β-mannanases isolated from *B. subtilis* strains so far show that the quantitative production, optimal temperature, glycoprotein nature as well as the hydrolysis property are obviously different.

2. Materials and methods

2.1. Materials and chemicals

Locust bean gum (LBG), guar gum, birchwood xylan and carboxymethyl cellulose (sodium salt, low viscosity) were purchased from Sigma (St. Louis, MO, USA). Konjac powder was obtained from Dazhou Wufeng Co. Ltd. (Sichuan province, China). Glucomannan made up 90% (w/w) of total carbohydrate in konjac powder. Copra meal was from Yefeng Co. Ltd. (Hainan province, China). Copra mannan was prepared by the method of Takahashi et al. (1984). Superdex™ 75 was purchased from Amersham Biosciences AB (Uppsala, Sweden). Q-Sepharose Fast Flow was purchased from Pharmacia (Pharmacia, Uppsala, Sweden). All other chemicals were of analytical grade.

2.2. Microorganisms and culture conditions

Bacillus subtilis WY34, a highly active β-mannanase producer used in this investigation, was newly isolated from the soil samples of Henan Province, China. The strain was identified by the Institute of Microbiology, Chinese Academy of Sciences (IMCAS).

The medium for mannanase production consisted of the following ingredients (w/v) in distilled water: 0.5% NaH_2PO_4, 0.06% $MgSO_4 \cdot 7H_2O$, 0.4% yeast extract, 0.8% beef peptone and 2.0% konjac powder. After four days of growth at 50 °C and 200 rpm, cells were removed by centrifugation (10,000*g*). The resulted supernatant was used as crude enzyme preparation.

2.3. Enzyme assays, protein and carbohydrate determination

Mannanase activity was assayed by mixing 0.1 ml of an appropriately diluted enzyme solution with 0.9 ml of 0.5% locust bean gum (LBG) in 50 mM citrate buffer (pH 6.0) at 50 °C for 10 min. The reducing sugar released was determined by the dinitrosalicylic acid (DNS) method using mannose as standard (Miller, 1959). One unit (U) of enzyme activity was defined as the amount of enzyme producing 1 μmol of mannose per minute under the assay conditions. CMCase activity and xylanase activity were measured in a similar way using 1.0% (w/v) solution of carboxymethyl cellulose and birchwood xylan as the substrates, respectively.

The concentration of soluble proteins was determined according to the method of Lowry, Rosebrough, Farr, and Randall (1951) using bovine serum albumin (BSA) as the standard. The carbohydrate content of the purified enzyme was determined using the phenol sulfuric acid method (Dubois, Gillis, Hamilton, Rebers, & Smith, 1956), with D-glucose as standard.

2.4. Purification of the mannanase

All purification steps were performed at 4 °C unless stated otherwise. The crude extracellular mannanase was obtained by centrifuging the culture broth at 10,000*g* for 10 min at 4 °C. The crude supernatant was subjected to 40–80% ammonium sulfate saturation. The precipitated protein was collected by centrifugation (10,000*g*), and dissolved in 20 mM Tris–HCl (pH 8.0) buffer. Further purification was done by gel filtration followed by ion exchange chromatography. A 1.0 ml sample from ammonium sulfate precipitation was loaded on a Superdex™ 75 column ($35 \times 1.0\ cm^2$) and equilibrated with 20 mM Tris–HCl buffer, pH 8.0, and the protein was eluted at a flow rate of 0.3 ml/min. The active fractions were combined. A 8.0 ml solution was applied to a Q-Sepharose Fast Flow column ($10 \times 1.0\ cm^2$) equilibrated with 20 mM buffer, pH 8.5. The bound mannanase was eluted with 0–0.5 M NaCl gradient at a flow rate of 1.0 ml/min. The active fractions were combined and dialyzed against the same buffer and the dialyzate was used in further experiments as the purified mannanase.

2.5. Polyacrylamide gel electrophoresis (PAGE) and zymograms

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed in a 12.5% (w/v) polyacrylamide gel by the method of Laemmli (1970). Protein bands were visualized by Coomassie brilliant blue R-250 staining. The molecular weight standard used was the low molecular weight calibration kit (Amersham): phosphorylase b (97.0 kDa), albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa). Glycoproteins were detected by Periodic acid Schiff (PAS) staining of the gel after the SDS–PAGE (Zacharius, Zell, Morrison, & Woodlock, 1959).

Zymograms were obtained by co-polymerizing 0.2% (w/v) LBG with 12.5% (w/v) polyacrylamide. After electrophoresis, the gel was 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 at 4 °C in 50 mM citrate buffer (pH 6.5) for 30 min. After a further incubation for 45 min at 40 °C, zymograms were

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 were observed as clear colourless areas.

2.6. Effect of pH, temperature and various reagents on enzyme activity

The optimal pH of mannanase activity was examined at pH 3.0–11.0 under standard assay conditions using 50 mM of various buffers: citrate buffer (pH 3.0–6.5), phosphate buffer (pH 6.7–8.2) and CHES [2-(cyclohexylamino)-ethanesulfonic acid] (pH 8.7–11.0). The effect of pH on mannanase stability was determined using the same buffer system in the range of pH 3.0–11.0. After incubation of the enzyme solution at various pH values for 30 min at 50 °C without the substrate, the remaining enzyme activity was measured.

The effect of temperature on mannanase activity was determined by incubating the purified enzyme with the substrate at temperatures ranging from 30 to 90 °C in 50 mM citrate buffer at pH 6.0. Thermal stability of the enzyme was determined by assaying for residual enzyme activity after incubation at various temperatures for 30 min in 50 mM citrate buffer (pH 6.0).

The effect of various reagents on mannanase activity was determined by assaying for residual activity after incubating the enzyme with 1 mM of various reagents dissolved in 50 mM citrate buffer (pH 6.0) for 30 min at 50 °C.

2.7. Substrate specificity and kinetic parameters

The substrate specificity of the mannanase was determined by incubating the enzyme with 5 mg ml^{-1} of each substrate in 50 mM citrate buffer (pH 6.0) at 50 °C for 10 min. Amount of reducing sugars produced was estimated using DNS method as described above.

For the kinetic experiments, 50 mM citrate buffer (pH 6.0) with substrates (i.e., locust beam gum, guar gum and konjac powder) were used at concentration of 2–20 mg ml^{-1}, and incubated with the purified mannanase at 50 °C for 5 min. The K_m and k_{cat} values were calculated from the kinetics data using the "GraFit" software (Leatherbarrow, 1999).

2.8. Hydrolysis properties

Locust bean gum or copra mannan was prepared at a concentration of 1.0% (w/v) in 50 mM sodium citrate buffer, pH 6.0. After addition of the purified mannanase (5 U/ml), the solution was incubated at 50 °C for 36 h. Aliquots were removed at various times, and heated at 100 °C for 5 min.

In order to further determine the mode of action of mannanase, 25 mM of different mannooligosaccharides (mannobiose to mannopentose) were separately incubated at 50 °C for 36 h with 4 U of mannanase in a reaction volume of 1.0 ml containing 50 mM citrate buffer (pH 6.0). Samples were analyzed for hydrolyzed products by the thin-layer chromatography (TLC) using silica gel plates 60 F 254 (E. Merck, Germany) according to the method of Jiang et al. (2004). A mannooligosaccharide mixture consisted of mannose to mannopentose was used as the standard.

2.9. Amino acid analysis and N-terminal sequence

The purified mannanase was hydrolyzed in a PicoTag workstation using HCl vapour (6 M) at 110 °C for 24 h in the presence of 0.01% phenol and 0.003% 2-mercaptoethanol. Amino acids were analyzed using a HP 1100 amino acid analyzer. Tryptophan residue was not determined.

The N-terminal amino acid sequence was determined by automated Edman degradation using a PROCISE amino acid sequencer (Applied Biosystems, USA) at Peking University, Beijing. N-terminal sequence homology was analyzed using the BLAST database.

3. Results and discussion

3.1. Production of the mannanase

The newly isolated *B. subtilis* WY34 produced a high level of extracellular mannanase during growth on 2% konjac powder at 50 °C (Fig. 1A). Maximum level of mannanase (1105 U/ml) was reached after four days. Under the above culture conditions, it produced extremely high mannanase activities with only low concomitantly formed xylanase (32.4 U/ml) and cellulase (3.6 U/ml) activities. SDS–PAGE (Fig. 1B) and zymogram analysis (Fig. 1C) of the crude supernatants showed that a single clear band of β-mannanase activity corresponding to a strong protein band of 39.6 kDa was detected at the incubation period of 2–6 days, it was subsequently identified as β-mannanase.

To our knowledge, this is the first report on the high-level production of β-mannanase from *B. subtilis*, which produce a mannanase at 50 °C. Other *B. subtilis* strains isolated so far produced maximum mannanases less and below 50 °C. Mannanase production has been reported to be induced with locust bean gum by *B. subtilis* NM-39 at 37 °C (Mendoza et al., 1994b). Some *B. subtilis* strains can produce mannanases up to 45 °C (Khanongnuch et al., 1998; Zakaria et al., 1998). Frequently, microbial mannanase appear extracellularly in multiple forms (Gübitz, Hayn, Urbanz, & Steiner, 1996; Hossain, Abe, & Hizukuri, 1996; Stålbrand, Siika-aho, Tenkanen, & Viikari, 1993). *Sclerotium rolfsii* secrets at least five β-mannanases when cultivated in the glucomannan (Gübitz et al., 1996). *Bacillus* sp. KK01 produces four β-mannanases in the culture medium (Hossain et al., 1996). However, *B. subtilis* WY34 only produce one β-mannanase.

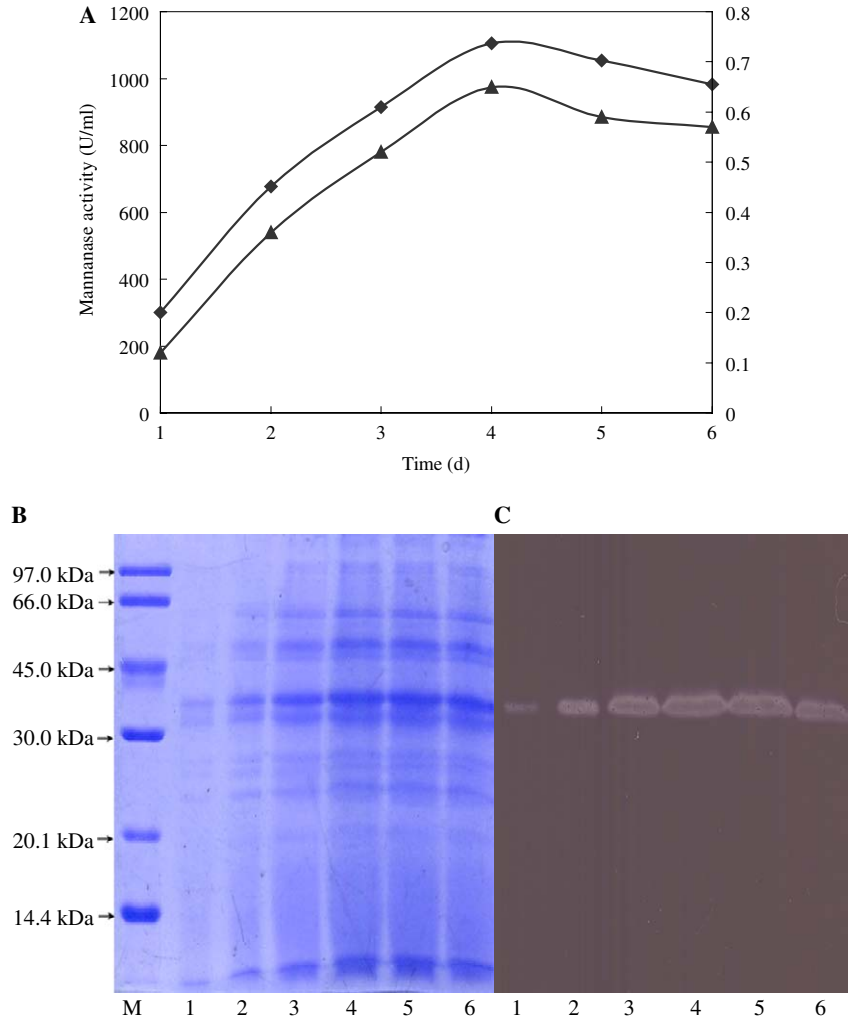

Fig. 1. Time course of mannanase production by the newly isolated *Bacillus subtilis* WY34 at 50 °C for six days: mannanase activity (A) and SDS–PAGE (B) as well as zymogram (C). Means ± standard deviation, $n = 3$. (▲), mannanase activity; (♦), protein content. Lane M, low molecular weight calibration kit; lane 1, 1 day and lanes 2–6, 2–6 days, respectively.

3.2. Purification of the mannanase

The purification is summarized in Table 1. An overall recovery of 20.3% and a 5.4-fold purification of the mannanase were obtained (Fig. 2A). The specific activity determined using locust bean gum as substrate was 8302.4 U/mg protein. The enzyme was also proved to be a glycoprotein by PAS staining followed by SDS–PAGE (Fig. 2B). The carbohydrate content of the enzyme measured with phenol sulfuric acid method was determined to be 13.1%.

The specific activity of the purified mannanase was much higher than those of bacterial mannanases, which is almost two times the value (4341 U/mg) from *Bacillus licheniformis* (Zhang et al., 2000). The apparent molecular mass of mannanase (39.6 kDa) from *B. subtilis* WY34 is similar to the values of 40.0 kDa for *Bacillus* sp. (Ooi & Kikuchi, 1995) and 39.0 kDa for *B. subtilis* KU-1 (Zakaria et al., 1998), and higher than 38.0 kDa for *B. subtilis* NM-39 (Mendoza et al., 1994a) and 37.0 kDa for *B. subtilis* 5H (Khanongnuch et al., 1998). Many mannanases,

Table 1
Summary of mannanase purification from the newly isolated *Bacillus subtilis* WY34

Purification step	Total activity[a] (U)	Protein[b] (mg)	Specific activity (U/mg)	Purification factor (-fold)	Recovery (%)
Crude supernatant	31114.8	20.2	1543.4	1.00	100.0
40–80% Ammonium sulfate precipitation	14935.5	4.0	3729.2	2.4	48
Superdex™ 75	9096.9	1.8	5025.9	3.3	30
Q-Sepharose fast flow	6301.5	0.8	8302.4	5.4	20.3

[a] Activity was measured in 50 mM citrate buffer (pH 6.0) at 50 °C using 0.5% (w/v) locust bean gum as substrate by the DNS method.
[b] The protein was measured using the method of Lowry et al. (1951), using BSA (bovine serum albumin) as the standard.

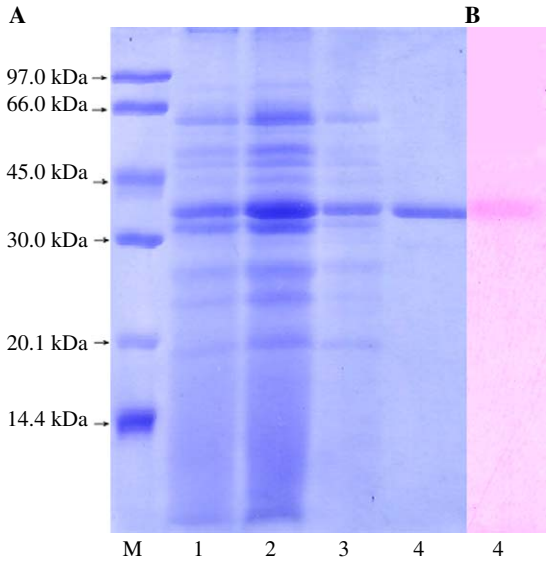

Fig. 2. Purification of mannanase (A) and PAS staining of the purified xylanase (B) from *Bacillus subtilis* WY34. Lane M, low molecular weight calibration kit; lane 1, crude extract; lane 2, fraction of 40–80% ammonium sulfate precipitation; lane 3, fraction of Superdex™ 75 Column and lane 4, fraction of Q-Sepharose Fast Flow Column.

especially from fungi are glycoproteins. A thermostable acidic *endo*-β-1,4-D-mannanase from *Sclerotium rolfsii* contained 15% carbohydrate (Sachslehner & Haltrich, 1999).

The β-mannanase sequence was determined by Edman degradation. N-terminal amino acid sequence of β-mannanases from *B. subtilis* WY34, was H-T-V-S-P-V-N-P-N-A. The first 10 amino acid sequence at the N-terminal shared 90% identity with that (H-T-V-Y-P-V-N-P-N-A) from *B. subtilis* NM-39 (Mendoza et al., 1995). The amino acid composition of the purified β-mannanase is shown in Table 2. The β-mannanase was rich in Gly (16.0%), Asp (10.5%), Glu (12.3%) and Ala (9.4%) and contained low levels of Met (1.2%), Lys (1.5%) and His (2.1%). Cys was not detected. The amino acid composition of the β-mannanase showed a similar pattern of amino acid composition as mannanase from *B. subtilis* NM-39 (Mendoza et al., 1994a, 1995).

Table 2
Amino acid compositions of *Bacillus subtilis* WY34 mannanase

Amino acids	*Bacillus subtilis* WY34 mannanase (mol/100 of all amino acids)
Asp	10.5
Thr	6.5
Ser	6.8
Glu	12.3
Pro	3.5
Gly	16.0
Ala	9.4
Cys	NF[a]
Val	5.7
Met	1.2
Ile	4.6
Leu	8.1
Tyr	3.8
Phe	3.5
His	2.1
Lys	1.5
Arg	4.5

[a] NF, not found.

3.3. Enzyme properties

The optimal pH for β-mannanase activity was pH 6.0 (Fig. 3A). The enzyme demonstrated broad pH stability within a pH range of 5.5–10.1 (Fig. 3B). The optimal temperature for β-mannanase activity was 65 °C (Fig. 4A). The enzyme was stable up to 60 °C, but about 90% of its activity was lost at 75 °C after 0.5 h of incubation (Fig. 4B).

Several mannanases of *B. subtilis* strains were characterized, although their molecular masses are similar to that of WY34, enzymatic properties are obviously different. The optimal pH for the mannanase was pH 6.0 compared to pH 5.0 from *B. subtilis* NM-39 (Mendoza et al., 1994a) and pH 7.0 from *B. subtilis* KU-1 (Zakaria et al., 1998) and *Bacillus* sp. (Ooi & Kikuchi, 1995). Like mannanases isolated from some *B. subtilis* strains, the mannanase was also stable over a wide pH range (Mendoza et al., 1994a; Zakaria et al., 1998). The mannanase was optimally active at 65–70 °C compared to 50–55 °C from *B. subtilis* strains NM-39 (Mendoza et al., 1994a), KU-1 (Zakaria et al., 1998) and 5H (Khanongnuch et al., 1998), and 60 °C from *Bacillus licheniformis* (Zhang et al., 2000). This mannanase in present investigation was stable up to 60 °C and retained 75% of its activity at 65 °C after 0.5 h of incubation. This thermostability is comparable to that reported for several mannanases from the thermophilic fungus and significantly higher than that of other *B. subtilis* strains (Khanongnuch et al., 1998; Mendoza et al., 1994a; Ooi & Kikuchi, 1995; Puchart et al., 2004; Zakaria et al., 1998). The thermal stability of the purified mannanase makes this enzyme attractive for use in industrial applications.

The effects of various metal ions and chemical reagents on mannanase activities are shown in Table 3. The mannanase activity was strongly inhibited by Mn^{2+} (21.8%), EDTA (37.3%), Mg^{2+} (53%) and Ag^{+} (53.6%), and was slightly inhibited by Zn^{2+} (90.5%) and Na^{+} (94.5%). The mannanase activity was activated by Fe^{2+} (124.2%), Co^{2+} (126.4%) and was not affected by Fe^{3+}. The chemical reagent *N*-bromosuccinimide (NBS) completely inhibited mannanase activity. SDS and β-mercaptoethanol inhibited the activity by 44.5% and 8.7%, respectively. NBS was a strong inhibitor of mannanase activity from *T. harzianum* strain T4 (Ferreira & Filho, 2004). Mannanases from *Streptomyces* sp. (Takahashi et al., 1984), *Pomacea insularus* (Yamaura & Matsumoto, 1993) and *Vibrio* sp. strain MA-138 (Tamaru, Araki, Amagoi, Mori, & Morishita, 1995) were completely inhibited by *N*-bromosuccinimide. The mannanase from *B. subtilis* WY34 was also completely inhibited by *N*-bromosuccinimide. This result may suggest that the tryptophan residue(s) play an important part at the

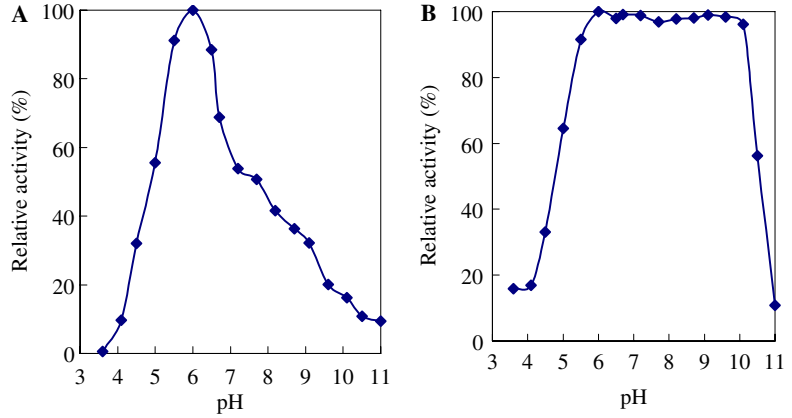

Fig. 3. Effect of pH on mannanase activity (A) and stability (B). The influence of pH on mannanase activity was determined at 50 °C using 50 mM of different buffers. The remaining activity was measured after incubation for 30 min at 50 °C over various pH.

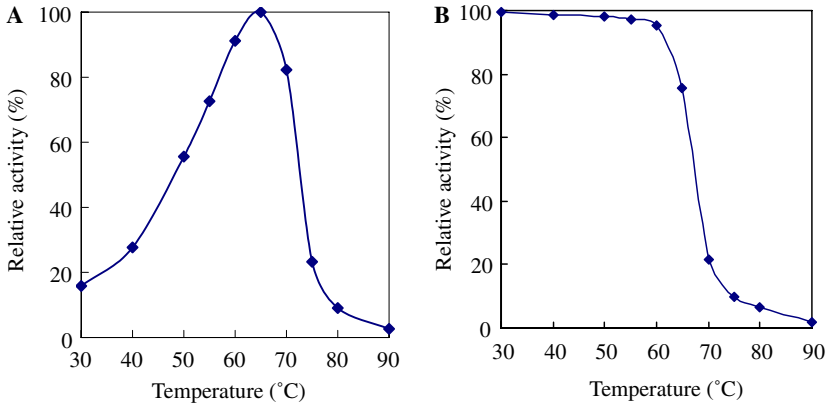

Fig. 4. Effect of temperature on mannanase activity (A) and stability (B). The optimal temperature was measured at different temperatures. For determination of thermal stability, the residual activity of the treated mannanase was measured after 30 min pre-incubation at different temperatures at pH 6.0.

Table 3
Effects of metal ions and reagents on the activity mannanase

Metal ions and reagents (at 1 mM)	Relative activity (%)
Control	100
EDTA	37.3
Ag^{+}	53.6
Li^{+}	79.6
K^{+}	72.6
Na^{+}	94.5
Ca^{2+}	86.0
Co^{2+}	126.4
Cu^{2+}	71.0
Fe^{2+}	124.2
Fe^{3+}	99.8
Mg^{2+}	53
Mn^{2+}	21.8
Ni^{+}	60.9
Sr^{2+}	84.4
Zn^{2+}	90.0
β-Mercaptoethanol	91.3
NBS	3.2
SDS	55.5

site or the substrate binding site of mannanase. However, the mannanase from *B. subtilis* NM-39 was strongly inhibited by Ca^{2+} and slightly by Ag^{+}, and was not affected by most metal ions (Mendoza et al., 1994a). The mannanase from *B. subtilis* KU-1 was strongly inhibited by Hg^{2+}, Cr^{2+}, Ag^{+}, Mn^{2+} and Cu^{2+} (Zakaria et al., 1998). The mannanase from *B. subtilis* 5H was strongly inhibited by Ag^{+}, Hg^{2+} and Fe^{3+} while other metals did not inhibit the activity (Khanongnuch et al., 1998).

3.4. Substrate specificity and kinetic parameters

The relative activity of the purified mannanase on various substrates is shown in Table 4. The mannanase exhibited high activity on locust bean gum galactomannan (100%) followed by konjac powder (56.8%), but considerably weaker activity on copra mannan (20.4%) and guar gum galactomannan (10.7%). It also showed low activity towards starch (2.8%), CMC (2.7%) and birchwood xylan (0.5%).

The Michaelis–Menten constants were determined for locust bean gum, konjac powder and guar gum (Table 5). The K_m and k_{cat} values were 7.6 mg ml^{-1} and 640.4 s^{-1} for locust bean gum, 10.5 mg ml^{-1} and 367.2 s^{-1} for konjac powder and 27.4 mg ml^{-1} and 287.3 s^{-1} for guar gum. The determined K_m for locust bean gum and guar gum were similar to the value of *B. subtilis* KU-1, and were higher

Table 4
Substrate specificity of the purified mannanase

Substrate	Relative activity (%)[a]
Locust bean gum	100.0
Konjac powder	56.8
Copra mannan	20.4
Guar gum	10.7
Starch	2.8
CMC	2.7
Birchwood xylan	0.5

[a] The activity for locust bean gum was defined as 100%.

than those of other mannanases (Emi, Fukumoto, & Yamamoto, 1972; Ferreira & Filho, 2004; Sachslehner & Haltrich, 1999; Zakaria et al., 1998).

3.5. Hydrolysis properties

The purified mannanase produced mainly mannotetraose, mannotriose and mannobiose in the hydrolysis of copra mannan and it hydrolyzed locust bean gum to give various sizes of oligosaccharides (Fig. 5). This indicates that the mannanase is an endomannanase. The action of the enzyme on mannooligosaccharides was also analyzed by TLC. As shown in Fig. 6, the enzyme hardly hydrolyzed mannotriose and mannobiose. Mannotetraose was however hydrolyzed very slowly by the enzyme. When mannopentose were incubated with the enzyme, mannotetraose, mannotriose and mannobiose were produced.

Many mannanases hydrolyze ivory nut mannan to yield mainly mannotriose and mannobiose (Ademark et al., 1998; Sachslehner & Haltrich, 1999; Stålbrand et al., 1993). Also, the four mannanases from *Bacillus* sp. KK01 hydrolyzed copra mannan to mainly mannotriose and mannobiose (Hossain et al., 1996). The hydrolysis property of present mannanase is similar to the mannanase from a mud snail, *P. insularus* (Yamaura & Matsumoto, 1993). It hydrolyzed copra mannan to give mainly mannotetraose, mannotriose and mannobiose, and did not produce mannose in the hydrolysis of locust bean gum. However, mannose was observed in the hydrolysis of locust bean gum by the mannanases from *B. subtilis* NM-39 and 5H (Khanongnuch et al., 1998; Mendoza et al., 1994a). The mannanase from *Bacillus* sp. hydrolyzed locust bean gum better than konjac glucomannan and mainly produced mannose and mannobiose (Ooi & Kikuchi, 1995). Thus, the mannanase is suitable to produce mannooligosaccharide, which are used as functional food additives for selective growth of human-beneficial intestinal microflora (Kobayashi et al., 1987). The ability of mannanases to degrade mannotriose was shown by Takahashi et al.

Table 5
Kinetic parameters for the purified mannanase[a]

Substrate	V_{max} (μmol min^{-1} mg^{-1})	K_m (mg ml^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (mg^{-1} s^{-1} ml)
Locust bean gum	970.3 ± 10.3	7.6 ± 0.2	640.4	84.3
Konjac powder	556.4 ± 15.2	10.5 ± 0.6	367.2	35.0
Guar gum	435.3 ± 29.1	27.4 ± 2.5	287.3	10.5

[a] Enzymatic reactions were carried out for 5 min at 50 °C in 50 mM citrate buffer (pH 6.0).

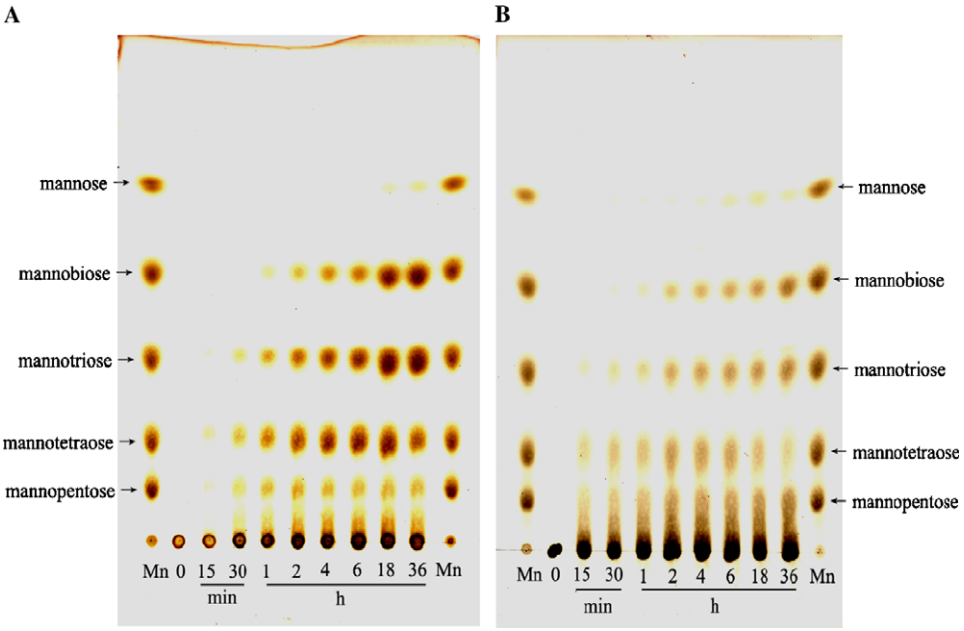

Fig. 5. Thin-layer chromatography of hydrolyzate of copra mannan (A) and locust bean gum (B) by the mannanase. Incubation times (h or min) are indicated. Lanes Mn, authentic standards, a mixture of mannose to mannopentose.

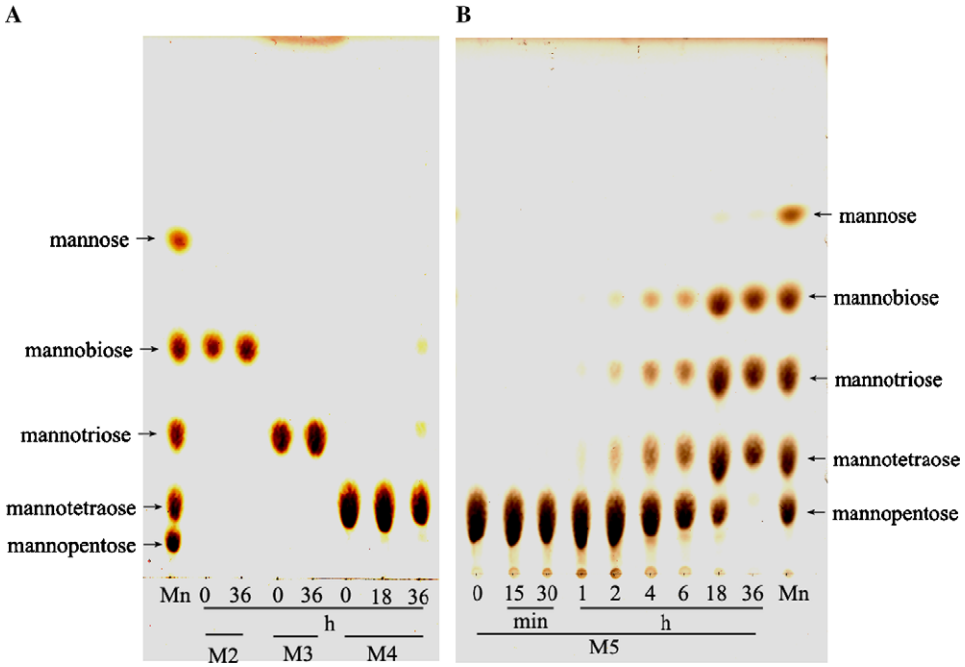

Fig. 6. Thin-layer chromatography of hydrolyzate of mannooligosaccharides (A and B) by the mannanase. Incubation times (h or min) are indicated. Lanes Mn, authentic standards. M2, mannobiose; M3, mannotriose; M4, mannotetraose and M5, mannopentose.

(1984). Some fungi mannanases can convert mannotetraose to mannotriose and mannobiose through the transglycosylation reaction (Puchart et al., 2004). For most mannanases, a degree of polymerization of at least four is required for a significant hydrolysis rate (Ademark et al., 1998; Gübitz et al., 1996; Sachslehner & Haltrich, 1999). In this study, mannotriose was not degraded at all, and mannotetraose was converted very slowly. Mannopentose was converted mannotetraose, mannotriose and mannobiose. This phenomenon is explained by a mechanism of transglycosylation reaction. Thus, together with other properties above, the hydrolysis properties obviously distinguished the present β-mannanase from other β-mannanases.

In conclusion, we describe the high-level production, purification and characterization of an extracellular β-mannanase from a newly isolated strain of *B. subtilis* WY34. The mannanase showed many differences from the mananases of other *B. subtilis* strains.

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