Gene Cloning, Expression, and Characterization of a Novel β-Mannanase from *Bacillus circulans* CGMCC 1416

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A DNA fragment containing 2,079 base pairs from *Bacillus circulans* CGMCC 1416 was cloned using degenerate PCR and inverse PCR. An open reading frame containing 981 bp was identified that encoding 326 amino acids residues, including a putative signal peptide of 31 residues. The deduced amino acid sequence showed the highest identity (68.1%) with endo-β-1,4-D-mannanase from *Bacillus circulans* strain K-1 of the glycoside hydrolase family 5 (GH5). The sequence encoding the mature protein was cloned into the pET-22b(+) vector and expressed in *Escherichia coli* as a recombinant fusion protein containing an N-terminal hexahistidine sequence. The fusion protein was purified by Ni^{2+} affinity chromatography and its hexahistidine tag cleaved to yield a 31-kDa β-mannanase having a specific activity of 481.55 U/mg. The optimal activity of the purified protein, MANB48, was at 58°C and pH 7.6. The hydrolysis product on substrate locust bean gum included a monosaccharide and mainly oligosaccharides. The recombinant MANB48 may be of potential use in the feed industry.

Keywords: β-Mannanase, *Bacillus circulans*, gene expression, characterization

β-Mannan and its heteropolysaccharides have a backbone chain of β-1,4-linked mannose units. These polysaccharides are the main component of hemicelluloses in hardwoods, softwoods, and gums extracted from the endosperm of primarily leguminous seeds [14]. Endo-1,4-β-D-mannanases (E.C. 3.2.1.78) catalyze the random hydrolysis of β-1,4-mannosidic linkages in the main chain of mannan,

galactomannan, glucomannan, and galactoglucomannan, releasing short- and long-chain oligomannosides that can be further hydrolyzed to mannose by β-mannosidases (E.C. 3.2.1.25). Moreover, the side-chain sugars of heteropolysaccharides can be completely hydrolyzed by α-galactosidase, β-glucosidase, and acetylmannan esterase [22].

β-Mannanase can be used to reduce the amount of chlorine-containing chemicals used during the bleaching of pulp, hence reducing the cost of chemical waste disposal [6]. The enzyme also can be used to reduce the viscosity of instant coffee and to clarify fruit juices and wines [31]. Thus, β-mannanases have wide commercial applications in industries such as the paper and pulp industry, foodstuff and feed industry, pharmaceutical industry, and energy industry [15, 19, 26].

Previous studies have focused on the isolation and characterization of β-mannanases from bacteria, fungi, plants, and animals [1, 7, 21]. In the past decade, gene cloning and sequencing of β-mannanases from different organisms have been reported [5, 10, 17, 32, 34]. These β-mannanases are classified as glycosyl hydrolase (GH) family 5 or 26, based on amino acid sequence similarity [11]. Enzymes from microorganisms, such as *Bacillus* bacteria and fungi, have the advantages of high activity and convenient isolation and thus have been used in research and industry [1]. However, β-mannanases with advantageous properties should be further isolated and efficiently expressed to satisfy the ever increasing requirements for their use in industrial processes.

We detected β-mannanase activity in cultures of *Bacillus circulans* B48 CGMCC 1416. Here, the gene *manB48*, encoding a novel β-mannanase, was cloned and efficiently expressed in *Escherichia coli*. We describe the purification and characterization of the recombinant protein, MANB48.

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MATERIALS AND METHODS

Materials

Bacillus circulans B48 CGMCC 1416 was isolated by our laboratory and deposited with the China General Microbiological Culture Collection Center (CGMCC). The plasmid pGEM-T easy and pET-22b(+), and *E. coli* JM109 and BL21, used for gene cloning and expression, respectively, were purchased from Novagen (U.S.A.). T_4 DNA ligase, *Taq* polymerase, and restriction enzymes were from TaKaRa (Japan). Locust bean gum (LBG) from *Ceratonia siliqua* seeds, guar gum, and bovine serum albumin were from Sigma (U.S.A.). Factor Xa was purchased from New England Biolabs (U.S.A). Other chemicals used were of reagent grade and available commercially.

Medium and Culture Conditions

Bacillus circulans B48 CGMCC 1416 was cultivated at 37°C for 12–16 h in medium consisting of 1% tryptone, 0.3% beef extract, and 0.5% NaCl, pH 7.0. For enzyme production, inoculums (1%, v/v) of the cell suspension were incubated on an orbital shaker at 30°C, 240 rpm, for 3 d, using the enzyme-production medium including 2% LBG, 2% yeast extract, 0.3% NH₄Cl, 0.03% KH₂PO₄, 0.3% CaCl₂, 0.06% MgCl₂·6H₂O, and 0.35% Na₂CO₃, pH 7.0. *E. coli* JM109 and *E. coli* BL21 (DE3) were grown at 37°C in Luria-Bertani medium containing 0.1 mg/ml ampicillin.

Cloning of the Gene *manB48*

The genomic DNA of *B. circulans* B48 CGMCC 1416, was prepared by standard methods as described by Wang *et al.* [30], and used as a template for degenerate PCR. The degenerate primers P1 (degenerate nucleotides are indicated inside parentheses), 5'-ATGCA(C/G)A(C)AG(C)CAA(G)ACA(G)ACAA(G)A-3', and P2, 5'-CCACCTA(G)AACCAC(T)TCA(G/T)CA(C)-3', were designed based on the conserved sequences of *Bacillus* β-mannanases. The PCR amplification conditions were initial denaturation at 95°C for 3 min followed by 30 cycles of 94°C for 40 s, 52°C for 30 s, and 72°C for 40 s. The PCR product was cloned in pGEM-T easy vector for sequencing.

Two additional primers, P3, 5'-ACAAGACGACTCGTACGGTA-TTGGCTCCCG-3', and P4, 5'-TGGAAATTGGAACGGCAGCGG-ATGGGCCG-3', designed according to the sequence of amplified conserved fragment, were used for inverse PCR (IPCR) as previously described [23]. Shortly, after independent digestion of genomic DNA with HindIII, the DNA fragments were self-ligated and used as templates for IPCR. The PCR amplification was carried out as follows: 30 cycles of denaturation for 30 s at 94°C, annealing and extension for 4 min at 68°C. The fragment generated by IPCR was cloned in pGEM-T easy vector and sequenced. A complete sequence was determined by assembling the sequences obtained by degenerate PCR and IPCR.

Expression of β-Mannanase in *E. coli*

Once the complete sequence of the *manB48* gene was obtained, primers P5 (NcoI site underlined, 5'-CG**CCATGG**CAGCCACAGG-ATTTTATG-3'), P6 (HindIII site underlined, 5'-CG**AAGCTT**TAA-AAGATTCCCGCTTTTTGG-3'), and P6H (XhoI site and factor Xa recognition site underlined and double underlined, respectively) 5'-ATCTCGAGGCGACCTTCGATATAAATGCCCGCTTTCTTGG-3') were designed to amplify the open reading frames by PCR using the bacterial genomic DNA as a template. PCR was performed with 32 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 80 s. The amplified DNA fragments, *manB48* and *manB48H*, were digested and ligated into vector pET22b(+), which encoded a C-terminal hexahistidine tag sequence for affinity purification. The recombinant plasmids were transformed into *E. coli* BL21 (DE3), and positive transformants were cultured in 100 ml of Luria-Bertani medium containing ampicillin (0.1 mg/ml) at 37°C with shaking to an optical density at 600 nm of 0.5. After addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mmol/l and incubation for 12 h at 18°C, the culture was analyzed by SDS-PAGE and for mannanase activity.

Mannanase Activity Assay

β-Mannanase activity was estimated by the Somogyi-Nelson method as previously described [27]. The standard assay contained 0.4 ml of 0.3% (w/v) LBG in Tricine-NaOH buffer (pH 7.6) and 0.1 ml of diluted enzyme. The reaction was incubated at 58°C for 10 min. One unit of mannanase activity was defined as the amount of enzyme required to liberate 1 μmol of mannose per min at a given assay temperature.

Purification of Recombinant Mannanase

The periplasmic protein from *E. coli* was firstly extracted by methods previously described [13]. The culture pellet was obtained by centrifugation (10,000 ×g, 5 min, 4°C), exposed to osmotic shock by suspension in ice-cold 40% sucrose in 50 mmol/l Tris-HCl, pH 7.5, and lysozyme (600 μg/g cells) was added. Following 20 min incubation on ice, the spheroplasts were pelleted (12,000 ×g, 15 min, 4°C) and resuspended in the same volume of ice-cold 50 mmol/l Tris-HCl, 2 mmol/l MgSO₄, pH 7.5. After centrifugation as above, the supernatant was stored at 4°C for subsequent mannanase purification.

The periplasmic extract was dialyzed against buffer A (10 mmol/l imidazole, 10 mmol/l NaH₂PO₄, 10 mmol/l Na₂HPO₄, 0.5 mol/l NaCl, pH 7.4) and applied to a nickel-charged affinity resin packed into a fast protein liquid chromatography column (Pharmacia Biotech) equilibrated with buffer A. The recombinant mannanase, MANB48, became bound to the column by virtue of the N-terminal hexahistidine tag and was eluted with a linear gradient of imidazole (10–500 mmol/l). The fractions containing enzyme activity were pooled, dialyzed extensively against buffer B (20 mmol/l Tris-HCl, 100 mmol/l NaCl, 2 mmol/l CaCl₂, pH 8.0), and concentrated with a 10-kDa cutoff ultrafiltration tube (Pall). The recombinant protein was cleaved of the hexahistidine tag with 0.2 μg factor Xa in 1 ml buffer B overnight at 23°C. The reaction was applied to a nickel-affinity chromatography column again as above, and the unbound protein, MANB48, was pooled and concentrated as above for characterization.

Characterization of the Recombinant Mannanase

The influence of pH on MANB48 activity was examined in 0.1 mol/l citric acid-Na₂HPO₄ buffer (pH 2.2–7.5), Tricine-NaOH buffer (pH 7.5–9.0), and glycine-NaOH (pH 9.0–11.0). The pH stability of MANB48 was followed over the pH range 2.2–11.0 of the buffers used above. The purified enzyme solution was added to buffers of different pH and incubated at 37°C for 30 min. The enzyme activity remaining was assayed as described above.

The influence of temperature on MANB48 activity was detected over the temperature range of 10–90°C in Tricine-NaOH (pH 7.6).

Thermal stability of the enzyme was measured after the enzyme was treated at 50°C or 60°C up to 60 min. The enzyme activity remaining was assayed as described above.

The effect of different chemicals on MANB48 activity was measured by adding each chemical to the reaction at a final concentration of 1 mmol/l. Enzyme activity was measured as described above.

To measure resistance of MANB48 to proteolysis, MANB48 was mixed with pepsin (pH 2.0) or trypsin (pH 7.0) at a 1:10 ratio (enzyme:protease, w/w). After incubation for 30 min at 37°C, MANB48 activity was assayed.

For analysis of the hydrolysis product by MANB48, 0.6% (w/v) LBG was used as the substrate to be hydrolyzed by the purified MANB48 at 58°C for 6 h, which made the substrate LBG be hydrolyzed totally. The hydrolysis reaction was assayed by high-performance anion-exchange chromatography (HPAEC) with a model 2500 HPAEC system (Dionex, U.S.A.) [33].

Nucleotide Sequence Accession Number

The nucleotide sequence reported here had been deposited in the GenBank under Accession No. AY907668.

RESULTS

Cloning and Analysis of the *manB48* Gene

Two conserved regions were identified by nucleotide sequence alignment of *Bacillus* β-mannanases, including *B. subtilis* Z-2 (AY827489), *B. subtilis* (D37964), *B. subtilis* (AY601725), *B.* sp. (AB016163), *B.* sp. (AB005755), and *B.* sp. (M31797). Based on this information, degenerate primers, P1 and P2, were designed for degenerate PCR using *B. circulans* B48 genomic DNA as template. The expected 500-bp fragment was amplified and sequenced. To further obtain the upstream and downstream sequences, primers P3 and P4 were designed based on the sequence and used for IPCR. Finally, the sequences of the two DNA fragments were assembled.

The resulted gene, *manB48* (AY907668), consisted of 2,079 bp and included an open reading frame (ORF) of 981 bp that encoded a protein of 327 amino acid residues, including a predicted 31-residue signal peptide. The coding sequence of the mature protein, MANB48, showed the highest identity (68.1%) with the protein encoded by the *Bacillus circulans* K-1 β-mannanase gene [34], suggesting that *manB48* encodes a new β-mannanase.

Expression of MANB48 in *E. coli*

Two coding region fragments, *manB48H* and *manB48*, were amplified using the primers P6H and P6, respectively. The vectors, pET22-*manB48H* and pET22-*manB48*, were then constructed, in which the sequence encoding MANB48 was fused or not to a sequence encoding a hexahistidine tag, respectively (Fig. 1), and transformed into *E. coli* BL21 (DE3) for gene expression. After culture and induction by IPTG, β-mannanase activity in cell lysates was analyzed.
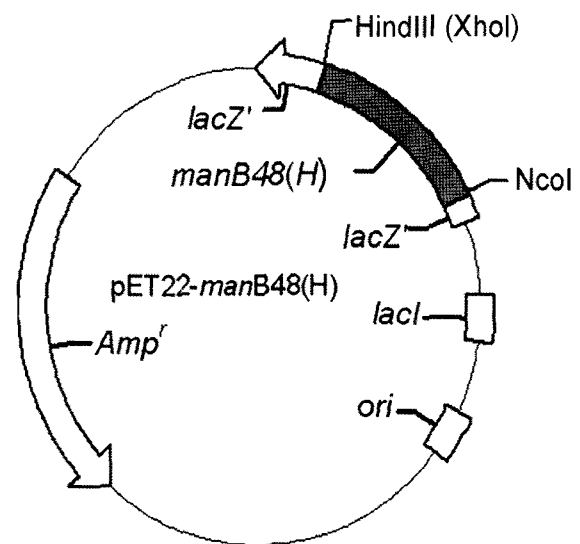

Fig. 1. Diagram for the construction of the vectors pET22-*manB48* and pET22-*manB48H*.

The coding sequence *manB48* was ligated to the NcoI and HindIII sites. The fragment, *manB48H*, and XhoI site used in pET22-*manB48H* construction, in which the gene was fused to His-tag in pET22b(+), is shown in brackets. The gene was under the control of the T7 promoter and an N-terminal *pelB* signal sequence.

No activity was detected from a lysate of the control strain, transformed by pET22b(+), whereas activities of 15.84 and 13.64 U/ml were detected in lysates of cells transformed with pET22-*manB48* and pET22-*manB48H*, respectively. Furthermore, enzyme activities of 8.75 U/ml and 0.52 U/ml were detected in the periplasm extract and culture supernatant, respectively, of cells transformed with pET22-*manB48H*. The recombinant protein was accumulated mainly in periplasmic space by the *pelB* signal sequence in
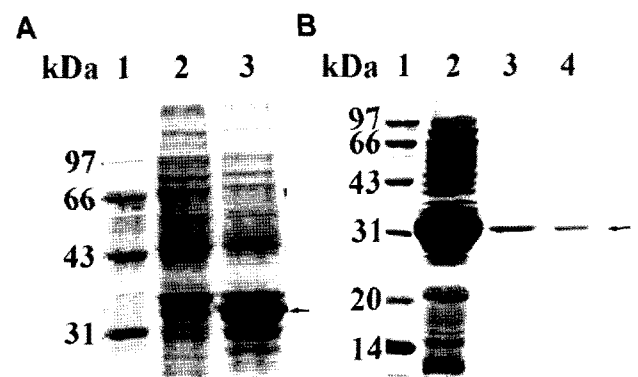

Fig. 2. A. SDS-PAGE analysis of the mannanase MANB48 expressed in *E. coli*. Lane 1, standard protein molecular mass markers; lane 2, *E. coli* transformed with plasmid pET-22b(+); lane 3, *E. coli* transformed with recombinant pET22-*manB48*. **B.** SDS-PAGE analysis of purified MANB48 using nickel chromatography. Lane 1, standard protein molecular mass markers; lane 2, periplasmic protein with expressed MANB48; lane 3, MANB48E-H after nickel chelate chromatography; lane 4, MANB48 after cleavage by factor Xa.

Table 1. Purification of recombinant MANB48 expressed in *E. coli*.

Purification steps	Protein concentration (μg/ml)	Volume (ml)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Periplasmic extract	95	100	875	92.1	1.0	100
Affinity chromatography	708.6	1.0	341.3	481.6	5.2	39

the vector. The expressed protein in the cell lysate had an apparent molecular mass of ~32 kDa (Fig. 2A).

Expression of active MANB48 in the periplasmic space of *E. coli* was optimized by varying the temperature and time of induction. Several induction conditions, including temperatures of 18, 24, and 30°C and induction times of 3, 6, and 12 h, were investigated. Optimal periplasmic enzyme activity (8.75 U/ml) was obtained after induction for 12 h at 18°C, and therefore these induction conditions were used for bulk expression and protein purification.

Purification of MANB48 in *E. coli*

The recombinant enzyme from the periplasm extract was subjected to (NH₄)₂SO₄ precipitation and nickel affinity chromatography, and MANB48H was purified to apparent homogeneity as a single band on SDS-PAGE (Fig. 2B). After cleavage of the hexahistidine tag with factor Xa, the apparent molecular mass of the purified MANB48 was

estimated as ~31 kDa, slightly smaller than the noncleaved recombinant MANB48 and in agreement with the value calculated from the amino acid sequence. The specific β-mannanase activity of the purified MANB48 was 481.55 U/mg after 5.2-fold purification with a 39% yield (Table 1). The cleaved enzyme was used for further enzymatic characterization.

Characterization of MANB48

The β-mannanase activity of MANB48 was measured at various pHs. Optimal activity was at pH 7.6, and more than 75% of maximal activity was retained over the pH range 6.8 to 8.0. MANB48 had no activity below pH 4.0 (Fig. 3A), but the enzyme was alkaline-tolerant, with more than 75% of maximum activity retained after incubation at 37°C for 60 min in buffers ranging from pH 7.0 to 9.0. Moreover, MANB48 retained 31% of maximum activity after treatment at pH 11.0 (Fig. 3B).
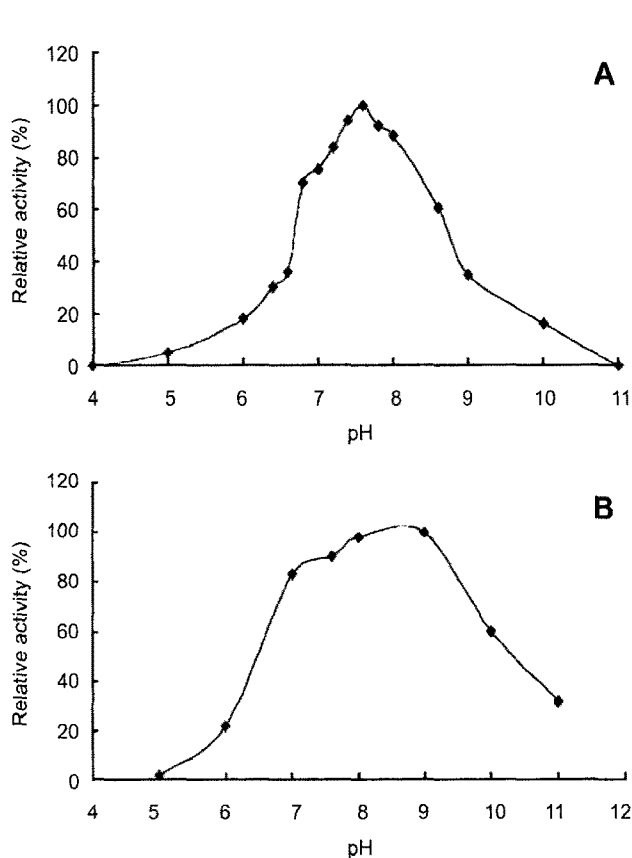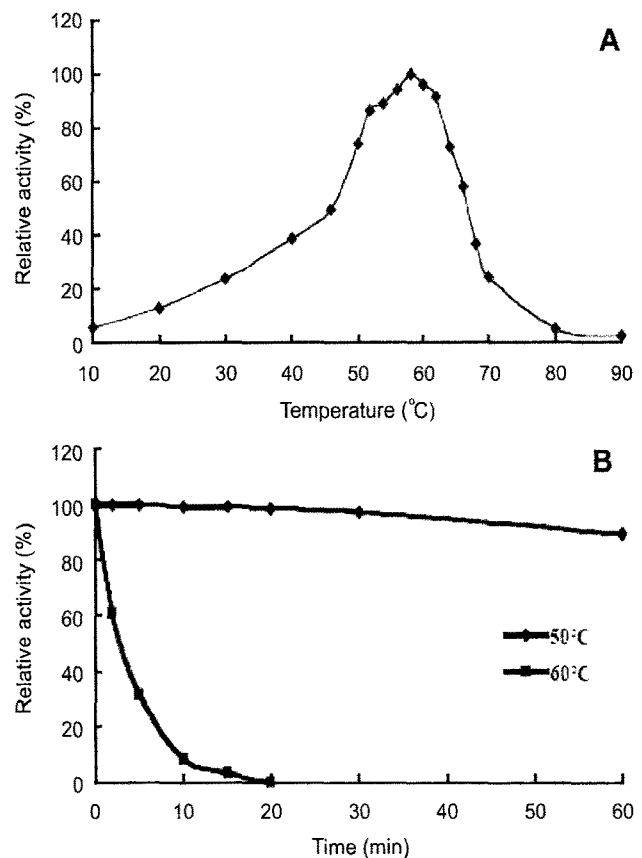

Fig. 3. Determination of the optimum pH (A) and pH stability (B) of MANB48.

Fig. 4. Determination of the optimum temperature (A) and thermal stability (B) of MANB48.

Table 2. Effects of various chemicals on MANB48.

Chemical*	$CaCl_2$	$CoCl_2$	LiCl	NaCl	KCl	$MgCl_2$	$FeCl_3$	$AlCl_3$
Relative activity (%)	124	118	112	100	100	100	98	88
Chemical	$MnSO_4$	$ZnSO_4$	$CuSO_4$	$HgCl_2$	$AgNO_3$	EDTA	SDS	CK
Relative activity (%)	62	57	12	8	2	54	ND	100

*Each chemical was at 1 mM final concentration.

The optimal temperature for MANB48 activity was 58°C (Fig. 4A). The thermal stability of MANB48 was measured by incubating the enzyme at 50°C or 60°C for different times, after which the residual activity was measured at 58°C and pH 7.6. MANB48 retained 90% of its activity after incubation at 50°C for 60 min, but no activity remained after incubation at 60°C for 20 min (Fig. 4B).

MANB48 was substantially inhibited by 1 mM Hg^{2+}, Ag^+, Cu^{2+}, or SDS and was inhibited to a lesser degree by Mn^{2+}, Zn^{2+}, or EDTA. Other metal ions, however, had little effect (Table 2).

About 90% and 31% of maximum activity was retained after MANB48 was treated with pepsin and trypsin, respectively, for 30 min at 37°C.

Substrate Specificity and Analysis of Hydrolysis Product

The substrate specificity of the enzyme was assayed by hydrolyzing 0.3% LBG, guar gum, soluble starch, or methylcellulose. MANB48 was most effective at hydrolyzing LBG and guar gum, with relative hydrolysis percentages of 100 and 26, respectively, but MANB48 had no activity for soluble starch or methylcellulose (Table 3). Although LBG and guar gum are galactomannans with the same linkage, guar gum is more highly galacto-substituted than LBG, suggesting that MANB48 prefers mannan substrates with a low level of galactose substitution.

We next examined the predominant products of MANB48 hydrolysis of LGB. Following hydrolysis of LBG, MANB48 was separated from the reaction products by ultrafiltration through 10-kDa-cutoff tubes. The reaction products, which flowed through the tubes, were analyzed by HPAEC using standard oligosaccharides for comparison. As shown in Fig. 5, together with 4% mannose (monosaccharide), the main products were oligosaccharides, including 14% mannobiose, 11% mannotriose, 9% mannotetraose, 10% mannopentaose, and other oligosaccharides that were

**Table 3.** Substrate specificity of MANB48.

Substrate (0.3%, w/v)	Relative activity (%)
Locust bean gum	100
Guar gum	26
Soluble starch	0
Methylcellulose	0

detected but not identified because no standard was available for comparison. These oligosaccharide products could potentially be used in the food industry.

DISCUSSION

In this study, the Somogyi-Nelson method [27] was used to measure β-mannanase activity, and the specific activity of purified MANB48 was 481.55 U/mg. When assayed by the dinitrosalicylic (DNS) method [25, 33], the specific activity of MANB48 was 842 U/mg. The two methods are widely used for enzymatic activity assay. The DNS method is easier to manipulate, but the reproducibility is poorer and the activity value consistently higher compared with the Somogyi-Nelson method.

The coding sequence of the mature protein, MANB48, showed the highest identity (68.1%) with the domain I sequence of the Bacillus circulans K-1 β-mannanase, and 45.7% identity with the whole protein sequence [34]. The enzymes showed little difference in properties except for the pH optimum. However, no PT box and domain II sequence were found in MANB48, which existed in some mannanases from B. circulans K-1 and other microorganisms,
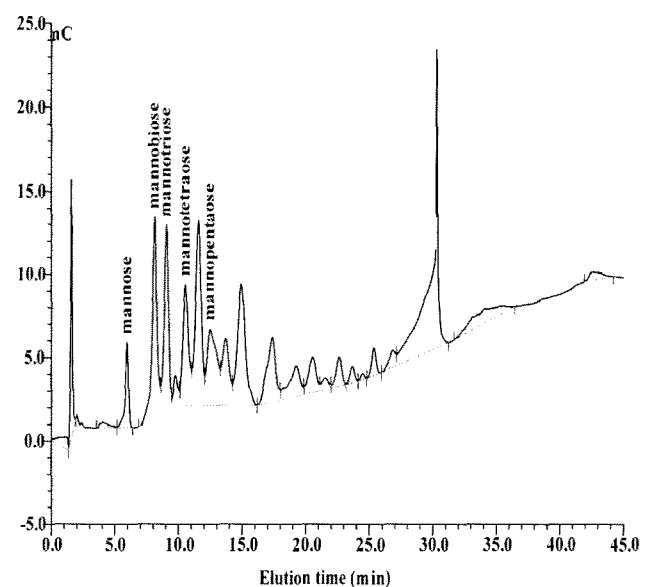
Fig. 5. HPAEC analysis of the reaction products resulting from MANB48 hydrolysis of LBG.

and the function of the domains was not determined [34]. The enzyme MANB48 seems to be a novel mannanase because of its sequence, structure, and enzyme properties, and it could be a candidate for research in function-structure relationships.

Over 60% of MANB48 protein was secreted to the periplasmic space using the *pelB* signal sequence of the pET22b(+) vector. This facilitated the purification of MANB48. Expression of active MANB48 in the periplasmic space of *E. coli* was optimized by varying the temperature and time of induction. Optimal activity was obtained in the periplasm at 18°C, suggesting that this relatively low temperature may enhance the solubility of the recombinant MANB48. This result is in accordance with the idea of Vasina and Baney [28] that low temperatures during induction/expression improve the solubility of heterologous proteins expressed in *E. coli*.

The enzymes of GH5 from different sources such as bacteria [2], fungi [24], and plants [5] are highly divergent at the amino acid level. Amino acid sequence alignments of GH5 members rarely reveal levels of sequence identity greater than 20%. As a result, GH5 members have been classified into eight subfamilies, A1 to A8 [12, 16]. The members of subfamily A8 exhibit more than 43% sequence identity. The low sequence similarity between different subfamilies has been attributed to evolution of distantly related ancestral proteins [5]. Our homology search using the deduced amino acid sequence of *manB48* as the query indicated that mature MANB48 β-mannanase has the highest sequence identity (68.1%) with that of *Bacillus circulans* K-1 [34], and has 62.6% and 60.6% sequence identity with the catalytic domains of two other *Bacillus circulans* strains (GenBank accession nos. AY540747, AY623903). These three β-mannanases of *Bacillus circulans* were all identified as GH5 members. The three highly conserved active-site residues Asn-156, Glu-157, and Glu-252 and the strictly conserved residues Arg-78, His-118, His-222, Tyr-224, and Trp-281, which are characteristic of all GH5 enzymes [3, 4, 9, 18, 29], were also found in MANB48. Conservation of these residues strongly suggests that MANB48 is a member of GH5.

Purified recombinant MANB48 had maximum activity at pH 7.6 and was stable over the pH range of 6.8 to 9.0; comparison with the pH range for β-mannanase from *Bacillus circulans* K-1, which is pH 6.0 to 7.0 [35], indicates that MANB48 is a more alkalophilic enzyme. This result indicates that MANB48 has potential for use in the pulp industry, in which the processes are generally carried out under alkaline conditions. Moreover, MANB48 has relatively greater potential for utilization in the production of fish feed additives, because of the neutral pH optimum, which is the case in the gastrointestinal tract of fishes.

Addition of β-mannanase to animal diets improves the growth efficiency of pigs and chickens [15, 20]. To our

knowledge, however, there are no reports on the use of β-mannanase as a fish feed additive. Certain freshwater fish, such as cyprinoids and grass carp, do not have a stomach, and thus trypsin and pepsin are secreted directly into the gastrointestinal tract with a neutral pH [8]. MANB48 has the optimum of pH 7.6, along with its pH stability and resistance to proteolysis, particularly to pepsin. Thus, it is suitable for utilization in the fish feed industry. However, the effect of MANB48 addition on animal growth, especially fishes, should be further determined.

Moreover, the products of MANB48 hydrolysis to LGB were mainly oligosaccharides. The enzyme can be used for oligosaccharides production, which are utilized widely in the food and feed industries [31].

Acknowledgments

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