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Biocatalytic characterization of an endo-β-1,4-mannanase produced by *Paenibacillus* sp. strain HY-8

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Abstract

Objectives To evaluate the biocatalytic characteristics of a new endo-β-1,4-D-mannan-degrading enzyme (ManP) from *Paenibacillus* sp. strain HY-8, a gut bacterium of the longicorn beetle *Moechotypa diphysis.*

Results Purified ManP (32 kDa) with an *N*-terminal amino acid sequence of APSFAVGADFSYVPG displayed the greatest degree of biocatalytic activity toward locust bean gum (LBG) at 55 °C and pH 7.0. The enzyme degraded LBG, guar gum, ivory nut mannan, and mannooligosaccharides (M_2–M_5), but did not exhibit any hydrolytic activity against structurally unrelated substrates. The biocatalytic activity of ManP against LBG and guar gum was 695 and 450 U mg^{-1}, respectively. Especially, enzymatic hydrolysis of mannobiose yielded a mixture of mannose (16.6 %) and mannobiose (83.4 %), although the degree of mannobiose degradation by ManP with was relatively limited.

Conclusion The present results suggest that ManP is an endo-β-1,4-mannanase and is distinct from various other characterized endo-β-1,4-mannanases.

Keywords Endo-β-1,4-mannanase · Gut bacterium · Longicorn beetle · *Moechotypa diphysis* · *Paenibacillus* sp. strain HY-8

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Introduction

In ecosystems, biological recycling of β-1,4-D-mannan polysaccharides, which are one of the main hemicellulosic components of plant biomass, is mediated by the cooperative action of diverse β-1,4-mannanase-producing bacterial and/or fungal species. These microorganisms generally produce various exo- and endo-type mannolytic enzymes with different biocatalytic functions for the complete degradation of β-1,4-D-mannans (Moreira and Filho 2008). Most β-1,4-mannanases are key endo-acting enzymes responsible for depolymerization of β-1,4-D-mannan polymers. They are affiliated with retaining glycoside hydrolase (GH) families 5, 26, 123, and 134 based on primary structural similarities (http://www.cazy.org/

Glycoside-Hydrolases.html). β-1,4-Mannanases have drawn attention because they can be exploited as eco-friendly biocatalysts in various industrial processes, such as bleaching of softwood pulps, hydrolysis of coffee mannan, removal of mannan-containing stains in the detergent industry, production of poultry feed additives and prebiotic mannooligosaccharides, and degradation of galactomannan thickeners (van Zyl et al. 2010).

Similar to ruminants, including cattle and goats (Palackal et al. 2007), herbivorous insects contain diverse exo-symbiotic microorganisms in their digestive tracts that produce novel cellulolytic and/or hemicellulolytic enzymes with industrially valuable biocatalytic properties as well as unique molecular structures (Brune 2014). The longicorn beetle, *Moechotypa diphysis,* is a wood-feeding insect that has many fibrolytic bacteria in its gut to facilitate the intestinal digestion of plants (Park et al. 2007). Accordingly, we previously isolated a xylanolytic bacterium, *Paenibacillus* sp. HY-8 KCTC 10896BP, from the gut of *M. diphysis* and investigated the genetic and biocatalytic characteristics of its GH11 β-1,4-xylanase (Heo et al. 2006). Here, we describe the purification and biochemical properties of an extracellular endo-β-1,4-mannanase (ManP) produced by strain HY-8. ManP is the first mannan-degrading enzyme isolated and characterized from a gut bacterium of the longicorn beetle *M. diphysis*.

Materials and methods

Chemicals

A series of β-1,4-D-mannooligomers (M_2–M_5) and ivory nut mannan were purchased from Megazyme International Ireland Ltd. (Wicklow, Ireland). Carbohydrate polymers, such as chitosan, curdlan, and pectin, were obtained from USB Co. (Cleveland, USA). Poly(3-hydroxybutyrate) granules were microbially produced (Kim et al. 2011b). All other substrate compounds used in this study were provided by Sigma-Aldrich (St. Louis, USA).

Purification of β-1,4-mannanase

Extracellular β-1,4-mannanase (ManP) from *Paenibacillus* sp. HY-8 KCTC 10896BP (Heo et al. 2006)

was produced by culturing the microorganism on a rotary shaker (200 rpm) for 48 h at 37 °C in two 2 l baffle flasks containing 500 ml LYM9 medium [5 g locust bean gum (LBG), 5 g yeast extract, 6.78 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 1 ml of 1 M $MgSO_4$, and 1 ml 0.1 M $CaCl_2$ per liter of distilled water]. Following cultivation, the culture supernatant, which showed β-1,4-mannanase activity against LBG, was collected by centrifugation ($5000 \times g$) for 20 min at 4 °C. Purification of the enzyme was accomplished by $(NH_4)_2SO_4$ precipitation (80 % saturation) and two consecutive chromatographies using HiPrep CM FF and HiLoad 26/60 Superdex 200 PG columns (GE Healthcare, Sweden) attached to a FPLC system (Amersham Pharmacia Biotech), according to the method described by Kim et al. (2011a) with minor modifications.

Protein analysis

To determine the relative molecular mass of purified ManP, SDS-PAGE of the denatured proteins was performed using a 12 % gel. The separated protein bands were visualized by staining the gel with Coomassie Brilliant Blue R-250. The *N*-terminal amino acid sequence of ManP was analyzed by automated Edman degradation of the peptides using a sequencer as described elsewhere (Kim et al. 2011a). The protein concentrations were measured using the Bradford reagent with bovine serum albumin as a standard.

Enzyme assays

β-1,4-Mannanase activity was assayed by determining the amount of reducing sugars released from LBG using 3,5-dinitrosalicylic acid reagent and mannose as a standard. The standard assay mixture (0.5 ml) was made up of 0.5 % LBG and appropriately diluted enzyme solution (0.05 ml) in 50 mM sodium phosphate buffer (pH 7). The biocatalytic reaction was routinely conducted at 55 °C for 15 min. One unit (U) of ManP activity for LBG and guar gums was defined as the amount of enzyme required to release 1 μmol reducing sugar per min under standard assay conditions.

Effects of pH, temperature, and chemicals on the β-1,4-mannanase activity

β-1,4-Mannanase activity of purified ManP was assessed from pH 3.5 to 10.5 at 50 °C for 15 min

using the following buffer systems (50 mM): sodium citrate (pH 3.5–5.5), sodium phosphate (pH 5.5–7.5), Tris/HCl (pH 7.5–8.5), and glycine/NaOH (pH 8.5–11.0). β-1,4-Mannanase activity was also evaluated from 30 to 65 °C under the standard assay conditions. Thermal stability of ManP was assessed by determining its residual biocatalytic activity after holding at various temperatures for 15, 30, and 60 min, as previously described (Kim et al. 2011b). The stimulatory or inhibitory effects of various chemical compounds (1 or 5 mM) on the β-1,4-mannanase activity were ascertained after pre-incubation of ManP at 55 °C for 10 min in 50 mM sodium phosphate buffer (pH 7) with the compound of interest.

Analysis of the hydrolysis products

Biocatalytic hydrolysis of LBG (1.5 mg), ivory nut mannan (1.5 mg), and mannooligomers (M_2–M_5, each 1 mg) was performed using the purified ManP (5 μg) in 100 μl 50 mM sodium phosphate buffer (pH 7) for 12 h at 30 °C, during which time the enzyme retained more than 90 % of its original β-1,4-mannanase activity. After the reaction was completed, the degradation products were examined by LC–MS/MS, as previously described by Kim et al. (2011b).

Binding assay

Structurally different insoluble polymers, Avicel PH-101, chitin, chitosan, ivory nut mannan, curdlan, insoluble oat spelts xylan, lignin, and poly(3-hydroxybutyrate) granules, were employed to assess the substrate binding ability of ManP. To remove any water-soluble carbohydrates, the polymers were washed five times in 50 mM sodium phosphate buffer (pH 7) prior to the binding assay. The binding affinity of the enzyme to insoluble materials was then investigated by determining the residual β-1,4-mannanase activity and protein concentration in the supernatant collected from the reaction mixtures, as described elsewhere (Kim et al. 2011b).

Results and discussion

Purification and analysis of ManP

Similar to many cellulolytic and xylanolytic enzymes (Collins et al. 2005; Kim et al. 2016), most of microbial β-1,4-mannanases have been identified as acidic proteins (Moreira and Filho 2008). However, our preliminary experiments clearly showed that ManP did not bind to anion exchange resins such as DEAE-Sepharose, suggesting that the enzyme is a basic protein, analogous to an endo-β-1,4-mannanase from the blue mussel *Mytilus edulis* (Xu et al. 2002). Thus, purification of the enzyme was performed by $(NH_4)_2SO_4$ precipitation (80 % saturation), cation exchange chromatography using CM-Sepharose resin, and gel permeation chromatography using Superdex 200 resin. After the three step purification procedure, ManP was purified 51-fold with an overall yield of 66 %.

SDS-PAGE analysis revealed that the relative molecular mass of ManP, which was isolated in an electrophoretically homogeneous state, was approx. 32 kDa (Fig. 1). The molecular size (32 kDa) of ManP on SDS-PAGE was similar to that (32.5 kDa) of a β-1,4-mannanase from *Trichoderma harzianum* T4 (Ferreira and Filho 2004), but smaller than those (>40 kDa) of other microbial multi-domain β-1,4-mannanases (Kim et al. 2011b; Zang et al. 2015). The N-terminal amino acid sequence of purified ManP was APSFAVGADFSYVPG, which did not share any obvious identity with other characterized β-1,4-mannanases available in the NCBI database.
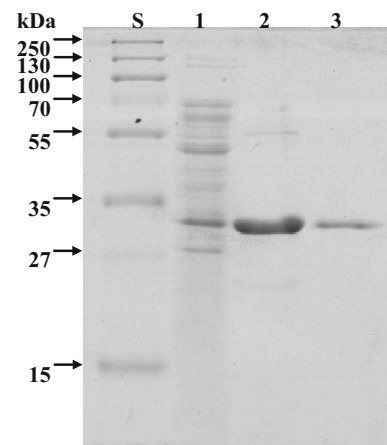

Fig. 1 SDS-PAGE of the purified ManP. *Lane S* standard marker proteins, *lane 1* proteins after 80 % ammonium sulfate precipitation, *lane 2* concentrated proteins after cation exchange chromatography on HiPrep™ CM FF 16/10, *lane 3* concentrated proteins after gel permeation chromatography on HiLoad™ 26/60 Superdex™ 200 pg

Enzymatic characterization of ManP

ManP had activity against LBG at 55 °C in 50 mM sodium phosphate buffer (pH 7) (Fig. 2a, b). It maintained over 85 % of its original biocatalytic activity toward LBG after pre-incubation for 1 h from pH 4.5 to 9.5 (Fig. 2c). Under the optimal reaction conditions, the half-life of ManP was approx. 25 min (Fig. 2d), which is similar to the thermal properties of diverse mesophilic hemicellulases from invertebrate-symbiotic bacteria (Heo et al. 2006; Kim et al. 2011b).

The stimulatory or inhibitory effect of divalent cations (each 1 mM) including Ni^{2+}, Zn^{2+}, and Sn^{2+} on the LBG-degrading activity of ManP was negligible (data not shown). However, the activity was increased by approx. 1.3-fold by adding 1 mM Mn^{2+} or Co^{2+}, even though two thermostable β-1,4-mannanases from *Bacillus subtilis* WY34 (Jiang et al. 2006) and *Bacillus* sp. N16-5 (Ma et al. 2004) were significantly inactivated by 1 mM Mn^{2+}. In addition, β-1,4-mannanase activity of ManP slightly increased when it was reacted with the substrate in the presence
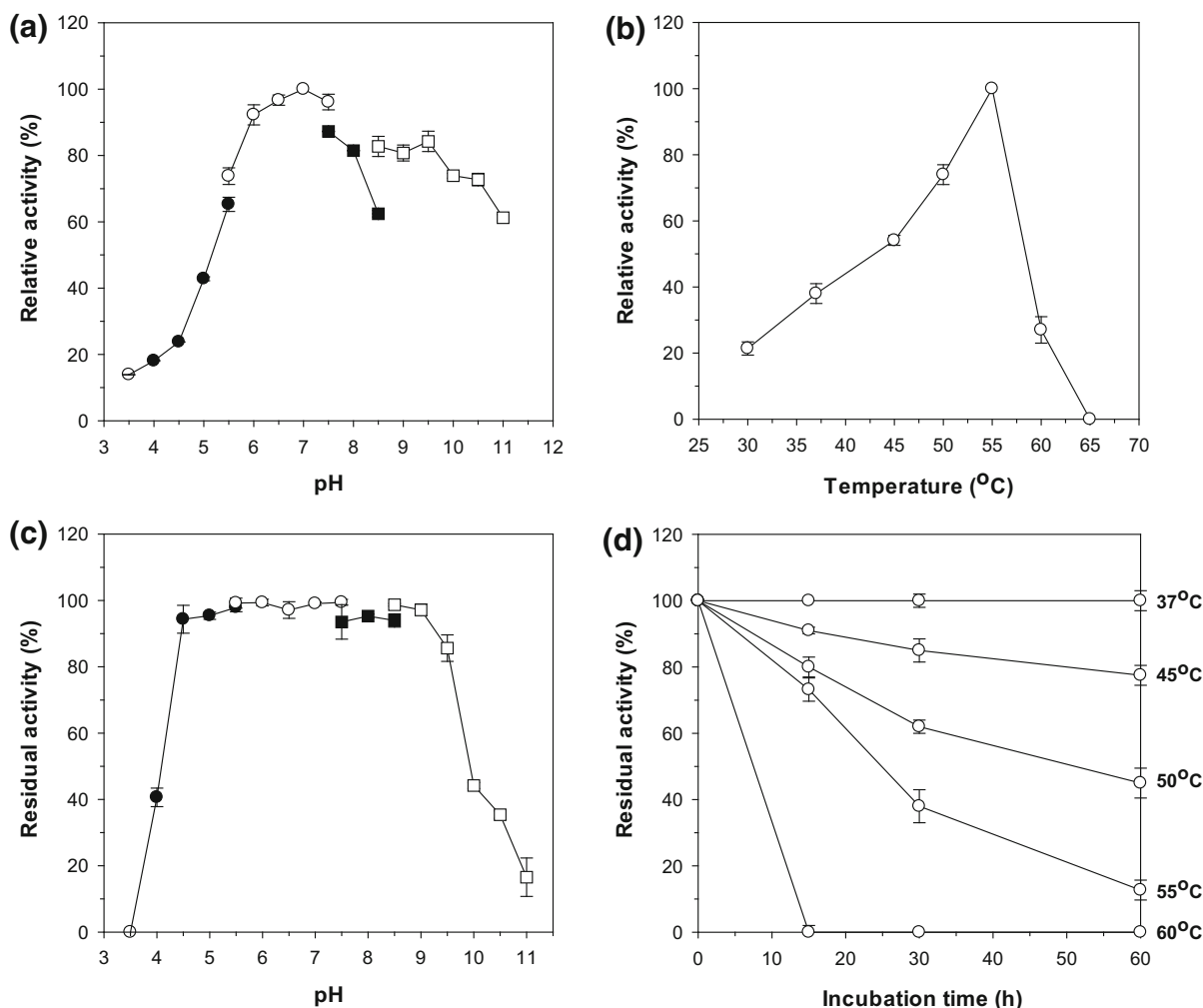

Fig. 2 Effects of pH (**a**) and temperature (**b**) on the biocatalytic activity of ManP and effects of pH (**c**) and temperature (**d**) on the stability of ManP. The optimal pH of the enzyme was evaluated using the following buffers (50 mM): sodium citrate (*filled circle*), sodium phosphate (*open circle*), Tris/HCl (*filled square*), and glycine/NaOH (*open square*). The pH stability of

ManP was determined after pre-incubation of the enzyme for 1 h at a broad pH range of 3.5–11.0. The optimal temperature and thermal stability of ManP were assessed using 50 mM sodium phosphate buffer (pH 7.0), which was used to determine the pH stability of the enzyme

of Ca^{2+}, Ba^{2+}, Triton X-100, and sulfhydryl reagents such as sodium azide and *N*-ethylmaleimide. Conversely, the enzyme was completely inhibited by Trp-specific oxidizing reagents [Hg^{2+} (1 mM) and *N*-bromosuccinimide (5 mM)], similar to other functional homologues that contain Trp residues closely related to the enzyme-substrate interaction in their catalytic domains (Zolotnitsky et al. 2004). EDTA (5 mM) and Cu^{2+} (1 mM) inhibited the activity of ManP by approx. 28 %.

Substrate specificity

ManP readily decomposed two galactomannans (LBG and guar gum) under the optimized reaction conditions (55 °C, pH 7), although its ability to degrade galactomannans was less efficient than that of some other β-1,4-mannanases with a high specific activity (Jiang et al. 2006; Kim et al. 2011a, b). The specific activities of ManP toward LBG and guar gum were 695 and 450 U mg^{-1}, respectively. However, like the β-1,4-mannanase produced by *Cellulosimicrobium* sp. HY-13 (Kim et al. 2011b), ManP did not show any detectable biocatalytic activity toward structurally unrelated polysaccharides such as carboxymethylcellulose, soluble starch, birchwood xylan, and pectin. The LBG-degrading activity of the enzyme assessed in this study was approx. 2- and 5.5-fold higher than that of an endo-β-1,4-mannanase from *Trichoderma virens* UKM1 (Chai et al. 2016) and that of a β-1,4-mannanase from *Bacillus subtilis* TJ-102 (Wang et al. 2013), respectively. In addition, the guar gum-degrading activity of ManP was approx. 1.7-fold higher than that of an endo-β-1,4-mannanase from *T. virens* UKM1 (Chai et al. 2016). Mannooligosaccharides of mannobiose (M_2) to mannopentaose (M_5) and β-1,4-D-mannan polysaccharides could be efficiently hydrolyzed by ManP, as determined by HPLC (Table 1). The biocatalytic depolymerization of LBG yielded a mixture of hydrolysis products of mannose (M_1) to mannohexaose (M_6), which mainly contained 54.1 % M_5 and 31.8 % M_6. In particular, the enzymatic degradation of M_2 gave rise to the production of only 16.6 % M_1 and the reaction proceeded very slowly. The formation of M_1 as a hydrolysis product was also observed at below 4.5 %, when β-1,4-D-mannosidic materials were subjected to ManP-mediated biocatalytic degradation.

These results clearly showed that the substrate specificity of ManP capable of hydrolyzing M_2 was significantly different from that of β-1,4-mannanases from *Cellulosimicrobium* sp. HY-13 (Kim et al. 2011a, b) *Thielavia arenaria* XZ7 (Lu et al. 2013), and *B. subtilis* WY34 (Jiang et al. 2006), which could not hydrolyze the same substrate to M_1. In contrast to ManP, *B. subtilis* WY34 β-1,4-mannanase is inactive with mannotriose (M_3) and mannotetraose (M_4) as well as M_2 (Jiang et al. 2006). Taken together, this evidence suggests that ManP is a new endo-β-1,4-mannanase that has unique hydrolysis patterns for β-1,4-D-mannosidic substrates, which are distinct from those of other microbial mannolytic enzymes. This endo-β-1,4-mannanase is expected to be useful as an animal feed additive because the viscosity caused by β-1,4-D-mannans during the ruminal decomposition of plant foods would be more efficiently reduced by ManP than other conventional endo-type β-1,4-mannanases. In particular, D-mannose and its mannooligosaccharides, which would be produced by ManP-mediated biocatalytic degradation of β-1,4-D-mannans, also inhibit *Salmonella typhimurium* and *E. coli* infections in the intestinal tracts of animals (Oyopo et al. 1989; Spring et al. 2000).

Binding ability of ManP to hydrophobic polymers

The binding ability of ManP in this study was assessed using diverse hydrophobic polymers with different microstructures: pentose- and hexose-based polysaccharides, PHB granules, and lignin. Table 2 shows that the enzyme strongly bound to Avicel and ivory nut mannan, but not to chitin, chitosan, curdlan, insoluble oat spelts xylan, or PHB granules. The binding capacities of ManP were comparable to those of other characterized functional homologues toward the same hydrophobic polymers. A β-1,4-mannanase from *M. edulis* (Xu et al. 2002) and a β-1,4-mannanase (Man26A) from *Cellulomonas fimi* (Stoll et al. 2000) did not bind to either cellulose or insoluble mannan. Although a β-1,4-mannanase (ManK) from *Cellulosimicrobium* sp. HY-13 bound strongly to Avicel, lignin, and PHB granules (Kim et al. 2011a). A β-1,4-mannanase (ManH) from *Cellulosimicrobium* sp. HY-13 showed strong binding affinities to chitosan and chitin as well as Avicel and ivory nut mannan (Kim et al. 2011b). The distinct binding affinities of ManP and the aforementioned β-1,4-mannanases to various hydrophobic polymers suggest that the molecular structure of the present enzyme is different from that of other β-1,4-mannanases.

Table 1 LC analysis of the hydrolysis products of mannosidic compounds by ManP

Substrate	Composition (%)[a] of products formed by hydrolysis reaction					
	M_1	M_2	M_3	M_4	M_5	M_6
M_2	16.6	83.4				
M_3	2.8	11.8	85.4			
M_4	2.4	9.5	62.3	25.8		
M_5	3.1	11.2	41.1	21.7	22.9	
Locust bean gum	0.5	1.5	2.5	9.6	54.1	31.8
Ivory nut mannan	4.4	8.3	46.7	29.3	3.6	7.7

[a] LC area %

Table 2 Binding of ManP to insoluble materials

Substrate	Residual ManP activity after binding (total U)
Control	0.50
Avicel	≤0.15
Chitin	0.45 ± 0.02
Chitosan	0.42 ± 0.03
Ivory nut mannan	≤0.05
Curdlan	0.47 ± 0.02
Insoluble oat spelt xylan	0.44 ± 0.02
Lignin	0.47 ± 0.03
Poly(3-hydroxybutyrate) granules	0.48 ± 0.02

Conclusion

The β-1,4-D-mannan-degrading enzyme (ManP) produced by *Paenibacillus* sp. strain HY-8 is the first endo-β-1,4-mannanase that has been isolated and biocatalytically characterized from mannolytic exo-symbionts resident in the gut of the longicorn beetle *M. diphysis*. The enzyme is distinguishable from other microbial β-1,4-mannanases by its *N*-terminal amino acid sequence, substrate specificities toward various mannose based-materials, and binding affinity to hydrophobic polymers. The results of this study demonstrate the ecological importance and contribution to the decomposition of plant biomass of the symbiotic gut bacteria of the longicorn beetle *M. diphysis*.

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