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Characterization of mannanase S1 from *Klebsiella oxytoca* KUB-CW2-3 and its application in copra mannan hydrolysis

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ABSTRACT: The mannanase S1 from *Klebsiella oxytoca* KUB-CW2-3 was purified by a single anion exchange chromatography to electrophoretic homogeneity. S1 is a protein with a molecular mass of approximately 165 kDa and a pI value of 3.5. The optimum pH and temperature of mannanase activity were 4.0 and 40 °C, respectively. The enzyme exhibited good stability over the broad pH range of 3–6. The mannanase S1 exhibited specific activity for different mannan substrates including galactomannan from locust bean gum (LBG), copra meal, and glucomannan from konjac, while neither xylanase nor cellulase activity were detectable. The Michaelis-Menten constants (K_m), maximum velocity (V_{max}), and the catalytic constant (k_{cat}) values of S1 against LBG and konjac mannan were 1.038–1.056 mg/ml, 6.149–6.183 μU ml^{-1} min^{-1}, and 0.047 s^{-1}, respectively. In addition, mannanase activity was activated by Co^{2+} (129%) but completely inhibited by EDTA and Zn^{2+}. The N-terminal amino acid sequence (GRVGEAGPHGPHGPH) of mannanase S1 is different from the N-terminal region of other bacterial mannanases belonging to the glycoside hydrolase family GH5. The degradation products of mannanase S1 from LBG hydrolysis were galactose and unknown oligosaccharides with a different molecular structure to mannobiose, triose, and tetraose indicating the cleavages of α-1,6-galactosidic and β-1,4-mannosidic linkages. Its hydrolysis of 100 mM $CoCl_2$-treated copra mannan enhanced the growth of *Lactobacillus reuteri* KUB-AC5 but inhibited that of *Salmonella* serovar Enteritidis S003, indicating potential prebiotic properties by the action of mannanase from *K. oxytoca*.

KEYWORDS: β-mannanase, copra meal, prebiotics

INTRODUCTION

Copra meal, a cheap agricultural by-product from the coconut industry in tropical areas, has recently gained significant attraction for the production of prebiotic manno-oligosaccharides[1–4]. Copra meal typically consists of 65% galactomannan, 5% lignin, 21% protein, and nearly 10% fat[5,6]. The abundance of heterogalactomannan found in this agricultural by-product can be conveniently used to produce manno-oligosaccharides (together with small amounts of mannose and galactose) by employing mannan endo-1,4-β-mannosidase, 1,4-β-D-mannanase (EC 3.2.1.78), or commonly named β-mannanase[7–9]. These mannan endo-1,4-β-mannosidases produced by various species of bacteria, fungi, plants, and invertebrates have also a number of other applications in different sectors of food, feed, pharmaceutical, and pulp/paper industries[8].

Klebsiella oxytoca KUB-CW2-3 was isolated from fermented coconut waste and was shown to actively hydrolyse both locust bean gum and copra mannan[10]. Its β-mannanase yields were lower than those reported for some other bacterial and fungal sources[11–13]. However, copra hydrolysates produced by *K. oxytoca* KUB-CW2-3 clearly showed growth promoting properties for *Lactobacillus reuteri* but growth inhibitory effects for *Salmonella* Enteritidis growth, indicating valuable prebiotic properties of this copra hydrolysates[10]. So far, there has not been

report on the property of the native enzyme and the action of mannan endo-1,4-β-mannosidase from *K. oxytoca* KUB-CW2-3. In this study, purification of the β-mannanase by chromatography and its subsequently characterization is shown. The hydrolytic activity of purified β-mannanase, and its degradation products together with potentially prebiotic properties are also presented.

MATERIALS AND METHODS

Chemicals

Locust bean gum (LBG; a galactomannan from *Ceratonia siliqua*), α-mannan from *Saccharomyces cerevisiae*, and xylan from oat spelts were obtained from Sigma (St. Louis, MO, USA). Ivory nut mannan (a β-mannan from *Phytelephas macrocarpa*), konjac mannan (a glucomannan from *Amorphophallus konjac*), mannobiose, mannotriose, mannotetraose, mannoheptaose, and mannose-7-butyrate were purchased from Megazyme (Bray, Ireland). Carboxymethylcellulose (CMC) and Avicel cellulose were obtained from BDH Laboratory (Poole, England) and Merck (Darmstadt, Germany), respectively. Glucose, maltose, galactose, and mannose were purchased from Fluka (Buchs, Switzerland). All other chemicals were analytical grade.

Preparation of copra meal

The residual coconut cake bought from Pakkret Market (Nonthaburi, Thailand) was dried at 60 °C for 4 h, blended, and milled by a hammer mill (Janke & Kunkel IKA Labortechnik) to obtain a particle size of 0.5 mm. This preparation is designated as copra meal (CM). It was used as a substrate both for enzyme production and the determination of enzyme activity.

Microorganisms and culture condition

K. oxytoca KUB-CW2-3[10] was cultivated in nutrient broth (NB; Pronadisa, Madrid, Spain) at 37 °C for 12–15 h with shaking at 150 rpm. *L. reuteri* KUB-AC5, *Escherichia coli* E010, and *Salmonella* serovar Enteritidis S003 obtained from chicken intestine[14] were used as target strains to determine prebiotic properties of the oligosaccharide preparations. They all were kept at the culture collection of the Department of Biotechnology, Kasetsart University, Thailand. *L. reuteri* KUB-AC5 was grown in de Man Rogosa Sharpe (MRS) broth (Merck) at 37 °C for 12–15 h, while *E. coli* E010 and *S*. Enteritidis S003 were cultivated in NB under the same conditions with shaking at 150 rpm.

Crude enzyme preparation

One millilitre of a preculture of *K. oxytoca* KUB-CW2-3, grown in 20 ml NB medium in a 100-ml Erlenmeyer flask with shaking at 150 rpm under condition mentioned elsewhere[10], was transferred into 100 ml of enzyme production medium by modified from Mohammad et al[15] consisting of 1% copra meal, 2% KH_2PO_4, 3% polypeptone, 0.06% $MgSO_4$, and 2% corn steep liquor in a 250-ml Erlenmeyer flask. After 24 h incubation at 37 °C, cells were removed by centrifugation at 4 °C, 10 000g for 20 min. The supernatant was collected and stored at −20 °C for further studies.

Determination of mannanase activity

Mannanase activity was determined by mixing 0.5 ml of 0.5% copra meal in 50 mM K_3PO_4 buffer pH 7.0 with 0.5 ml of appropriately diluted enzyme sample for 30 min at 50 °C otherwise mentioned elsewhere. The reducing sugars released were measured by the dinitrosalicylic acid method[16] against a standard curve of mannose. One unit of enzyme activity was defined as the amount of enzyme that gave rise to reducing sugar end groups corresponding to 1 μmol of mannose per minute under the selected experimental conditions.

Protein determination

Protein concentration was determined by the method of Lowry et al[17]. Bovine serum albumin was used as a protein standard. All chromatographic runs were monitored by measuring the absorbance at 280 nm.

Purification procedure

All purification steps were carried out at room temperature using an AKTA FPLC chromatographic system (GE Healthcare, Uppsala, Sweden). Starting from 1000 ml of crude culture supernatant, mannanase was purified by adding ammonium sulphate to 70% saturation, collecting the precipitate by centrifugation at 4 °C, 7000g for 30 min, dissolving this precipitate in 100 ml of 10 mM bis-Tris buffer pH 7.0, and dialysis against the same buffer. The dialysed enzyme solution (2 ml) was applied to a Q Sepharose column (1.6 × 20 cm; GE Healthcare) preequilibrated with 10 mM bis-Tris buffer, pH 7.0. Bound protein was eluted with a linear gradient of NaCl (0–1 M) at a constant flow rate of 2 ml/min and active fractions were collected, pooled, and concentrated approximately 10-fold by ultrafiltration using a 10 000-Da molecular-weight-cutoff membrane on a 180-ml stirring cell (Amicon, Beverly, Mass). This preparation was then reloaded

to the same column and repurified by performing the identical procedure.

Electrophoresis

Molecular mass of the purified enzyme was determined by SDS-PAGE according to Laemmli[18] using a 10% acrylamide separating gel and staining with the silver stain plus kit (Bio-Rad, USA). Isoelectric focusing was performed on Ampholine PAGplate pH 3.5–9.5 (GE Healthcare) according to the instruction of the manufacturer. The isoelectric point was evaluated by staining with the silver stain plus kit and comparison with the broad range pI calibration kit (GE Healthcare).

Effect of the pH value on activity and stability

The optimum pH of mannanase activity was examined at pH values of 3.0–10.0 under otherwise standard assay conditions. An LBG solution (0.5%, w/v) in the appropriate buffers, each at 10 mM—citrate (pH 3.0–6.0), phosphate (pH 6.0–8.0), and glycine-NaOH (pH 8.0–10.0)—was used to determine mannanase activity at 50 °C for 30 min. Enzyme stability was determined using the same buffer systems in the range of 3.0–10.0 by incubating an enzyme solution in the various buffer solutions at 4 °C for 24 h. Then, the remaining enzyme activity was measured under standard assay conditions.

Effect of the temperature on activity and stability

The temperature optimum of mannanase activity was determined by performing the standard activity assay at temperatures ranging from 30–60 °C in 10 mM buffer at pH 4.0 for 30 min. For the determination of thermal stability, an enzyme solution was incubated in 10 mM buffer at pH 4.0 and at various temperatures for 30 min. Thereafter, the remaining enzyme activity was measured.

Effect of various metal ions and chemicals on mannanases

The effects of EDTA and mercaptoethanol as well as various metal ions (Li^+, Ca^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Ni^{2+}, and Co^{2+}), each at a concentration of 1 mM, on mannanase activity were determined under standard assay conditions.

Determination of kinetic parameters

The Michaelis-Menten kinetic parameters, K_m, V_{max}, and k_{cat} were determined by using LBG and konjac mannan as substrates. These reactions were performed at 50 °C and pH 4.0 with various substrate concentrations ranging from 0.1–1% (w/v). Samples were taken every 5 min for a total of 30 min and only the linear part of the reaction curve was used for the determination of activity. The K_m and V_{max} were calculated from Lineweaver-Burk plots.

Determination of substrate specificity

Various substrates including locust bean galactomannan, yeast α-mannan, ivory nut mannan, konjac glucomannan, xylan (from oat spelts), carboxymethylcellulose (CMC), avicel, and copra meal, each at a concentration of 0.5% (w/v), were used for the determination of mannanase activity under standard assay conditions.

Determination of amino acid sequence

The N-terminal amino acid sequence of the purified mannanase was determined by Edman degradation at Research and Research Training Services, University of Newcastle, Australia. Sequence homology was analysed by using the BLAST database.

Hydrolysis experiments

The purified mannanase (0.05 U/ml) was incubated with 0.5% (w/v) LBG in 10 mM citrate buffer pH 4.0. The reaction mixture was incubated at 40 °C for 4 h. Aliquots were removed at 1 h intervals, and the reaction was stopped by heating at 70 °C for 10 min. The hydrolysates were analysed by thin-layer chromatography (TLC) according to a modified method of Sachslehner et al[19]. The solvent used as mobile phase was composed of butanol:isopropanol:ethanol:deionized water in the ratio of 2:3:3:2, respectively. Two microlitres of sample was applied onto Kieselgel 60 plates (Merck) and developed for 90 min in developing solvent. The TLC plates were then developed by dipping in 0.2% (w/v) orcinol in 10% (v/v) H_2SO_4 in ethanol at 100 °C for 15 min, and sugars appeared as brown spots.

Evaluation of prebiotic properties

The copra meal hydrolysate (CM-hydrolysate) obtained by mannanase S1 treatment was used to determine potential prebiotic properties according to the method of Titapoka et al[10]. *L. reuteri* KUB-AC5, *S*. Enteritidis S003, and *E. coli* E010 were used as the target strains to determine possible growth effects of CM-hydrolysate. Briefly, each culture solution of the target strains was adjusted to absorbance of 0.5 at 600 nm. Culture solution of each target strain (1%) and 1% of the CM-hydrolysate or enzyme reaction buffer (control) were transferred into 5 ml of MRS medium for *L. reuteri* and NB medium for *S*. Enteritidis and *E. coli*. The mixture was incubated at 37 °C

Table 1 Purification of mannanase from *K. oxytoca* KUB-CW2-3.

Steps	Total volume (ml)	Total activity (Units)	Total protein (mg)	Sp. activity (U/mg protein)	Purification fold	Yield (%)
Crude	1000	515	1962	0.26	1	100.00
Precipitate + dialysis	100	44.3	288.4	0.15	0.58	8.60
Q Sepharose I + UF	20	7.8	0.92	8.48	32.62	1.51
Q Sepharose II + UF	20	4	0.12	33.33	128.19	0.77

for 4 h and then cell number was determined by the standard plate count assay overnight at 37 °C using either MRS or NB medium containing 2% agar. The prebiotic properties of CM-hydrolysate were defined as an enhancing activity of *L. reuteri* growth and inhibitory activity of *S*. Enteritidis and *E. coli* growth according to the following equations:

$$\text{Enhancing activity} = \text{SF} - \text{CF},$$

$$\text{Inhibition activity} = \text{CF} - \text{SF},$$

where SF and CF were the cell number obtained in the growth experiments containing CM-hydrolysate and the cell number in control experiments, respectively (log cfu/ml).

RESULTS

Purification of mannanase

One litre of culture supernatant containing mannanase activity was purified by ammonium sulphate precipitation and anion exchange chromatography. Twenty millilitres of the active fraction after 70% ammonium sulphate precipitation were dialysed and subsequently loaded onto a Q Sepharose column twice in succession to obtain a single peak of activity with the purification efficiency and yield of 128-fold and 0.77%, respectively. The final mannanase preparation thus obtained was electrophoretically homogeneous as judged by SDS-PAGE and was designated as mannanase S1. The results of the purification are summarized in Table 1. The molecular mass and pI value of mannanase S1 were 165 kDa and 3.5, respectively (Fig. 1).

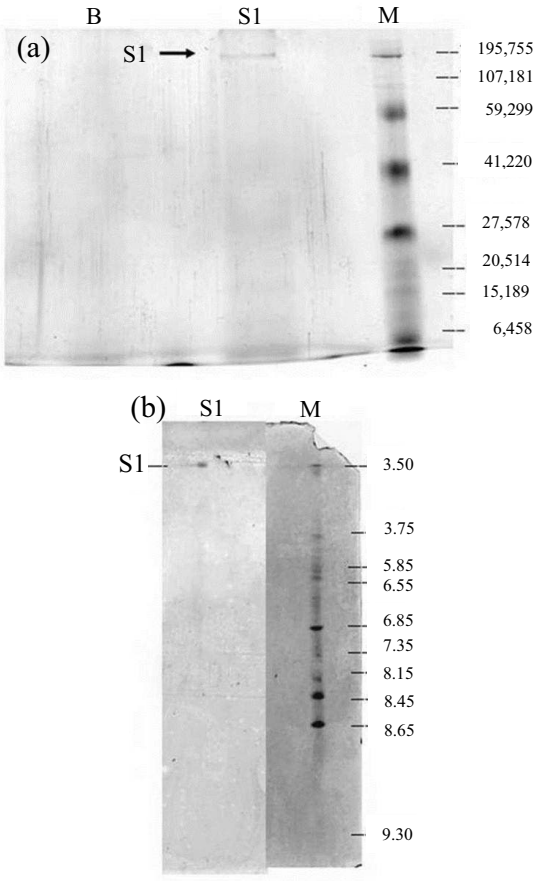

Fig. 1 Electrophoretic analysis of purified S1. (a) SDS-PAGE of purified S1: lane B, sample buffer; lane S1, purified S1; lane M, prestained marker (Bio-Rad). (b) Isoelectric focusing electrophoresis: lane S1, purified S1; lane M, broad pI calibration kit (Amersham).

Effects of pH and temperature on mannanase S1

The optimum pH of mannanase S1 activity was shown to be at pH 4.0 (Fig. 2a). The enzyme was stable over a broad pH range of 3–6, retaining more than 60% of its activity when incubating the enzyme at the respective pH and 4 °C for 24 h, while the activity rapidly decreased at pH 7.0 and 8.0 (Fig. 2a) and completely lost at pH 9.0 under these conditions.

The optimum temperature of mannanase S1 was at 40 °C for standard assay conditions (Fig. 2b). The half-life times of stabilities of mannanase S1 at 40, 50, 60, and 70 °C were 4, 3, 3, and 1 h, respectively.

Effect of chemicals

The effects of various metal ions and chemical reagents on mannanase activity are shown in Table 2. Mannanase activities is completely inhibited by EDTA and Zn^{2+}. The enzyme showed strong inhibi-

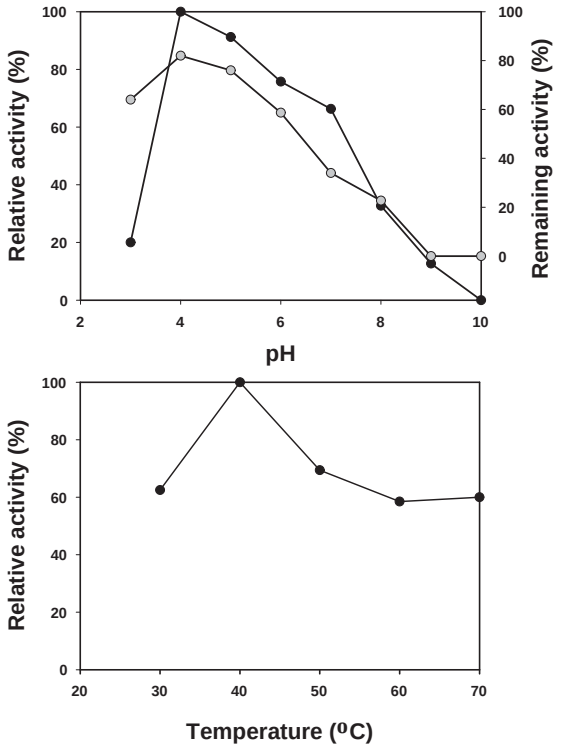

Fig. 2 Effects of (a) pH and (b) temperature on the activity of mannanase S1 from *K. oxytoca* KUB-CW2-3. Black circles: relative activity; grey circles: remaining activity.

Table 2 Effect of various salts/metal ions and other chemical compounds (each at a concentration of 1 mM) on the activity of mannanase S1 from *K. oxytoca* KUB-CW2-3.

Ion	Relative activity (%)
Control	100
EDTA	0
$ZnSO_4$	0
$CaCl_2$	13
$CuSO_4$	16
$FeSO_4$	16
$MnSO_4$	23
$NiSO_4$	32
$MgSO_4$	36
LiCl	45
Mercaptoethanol	84
$CoCl_2$	13

tion of more than 70% by Ca^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+} when present at 1 mM. Mannanase activity was, however, activated by Co^{2+} with the highest activity of 0.055 U/ml observed at 100 mM Co^{2+} ion.

Table 3 Substrate specificity of mannanase S1 from *K. oxytoca* KUB-CW2-3 towards various polysaccharide substrates.

Substrate	Relative activity (%)
Locust bean gum (galactomannan)	100
Ivory nut mannan (mannan)	60
Konjak glucomannan (glucomannan)	54
Alpha-mannan (yeast)	< 1
Xylan (from oat spelts)	< 1
Carboxymethylcellulose	< 1
Avicel	< 1
Copra meal	20

Substrate specificity

Table 3 shows the relative activities of mannanase S1 for various polysaccharide substrates. Mannanase S1 exhibited the highest activity against LBG, which is taken as 100% relative activity. It also hydrolysed ivory nut mannan, konjac glucomannan, and copra meal with lower relative activities of 60, 54, and 20%, respectively. On the other hand, no hydrolytic activity against yeast α-mannan, xylan (from oat spelts), CMC, and avicel was observed.

Kinetic properties of mannanase S1

The K_{m}, $V_{\max}$, and k_{cat} values of mannanase S1 for its substrate locust bean gum galactomannan were determined to be 1.06 mg/ml, 6.15 μU ml^{-1} min^{-1}, and 0.047 s^{-1}, respectively, while the ones on konjac glucomannan were 1.038 mg/ml, 6.183 μU ml^{-1} min^{-1}, and 0.047 s^{-1}, respectively. This could be concluded that the kinetic values for LBG and konjac mannan were nearly identical.

Amino acid sequence

The sequence of 15 N-terminal amino acids obtained was "GRVGEAGPHGPHGPH". This showed no matching with the N-terminal region of any bacterial mannanase by Protein-Protein BLAST (blastp) analysis (http://www.ncbi.nlm.nih.gov).

Degradation pattern

Both copra mannan and LBG are galactomannans. However, the activity of mannanase S1 against copra mannan is lower than against LBG as concluded from the previous results. Hence LBG was selected as a substrate for further experiments, in which the hydrolysis of galactomannan by mannanase S1 and the corresponding hydrolysis products were studied. Samples of LBG degradation by mannanase S1 were taken at 0, 1, 2, 3, and 4 h and analysed for the

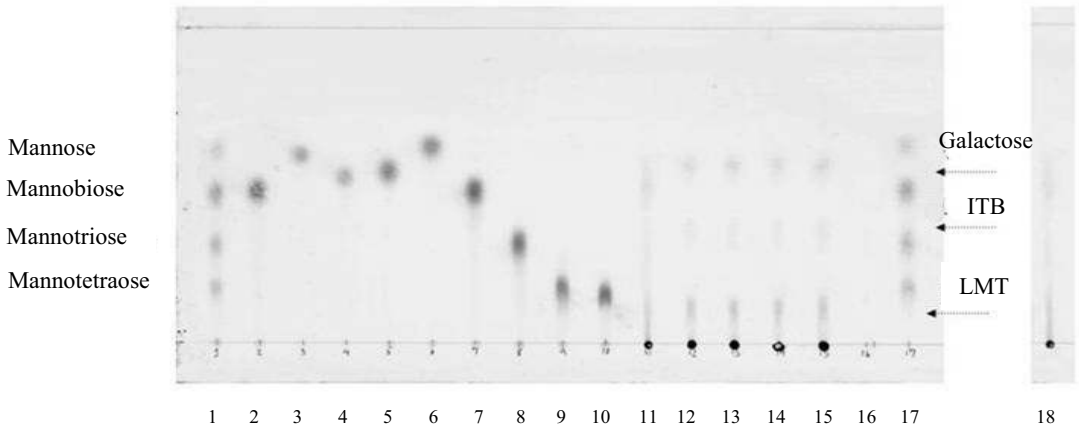

Fig. 3 Locust bean gum hydrolysis by mannanase S1 from *K. oxytoca* KUB-CW2-3 after reaction times of 0, 1, 2, 3, and 4 h. Lane 1: mannooligosaccharide standard. Lane 2: mannose-7-butyrate. Lane 3: glucose. Lane 4: maltose. Lane 5: galactose. Lane 6: mannose. Lane 7: mannobiose. Lane 8: mannotriose. Lane 9: mannotetraose. Lane 10: mannoheptaose. Lane 11: 0 h. Lane 12: 1 h. Lane 13: 2 h. Lane 14: 3 h. Lane 15: 4 h of reaction time. Lane 16: S1 mannanase preparation. Lane 17: standard mannooligosaccharides. Lane 18: locust bean gum.

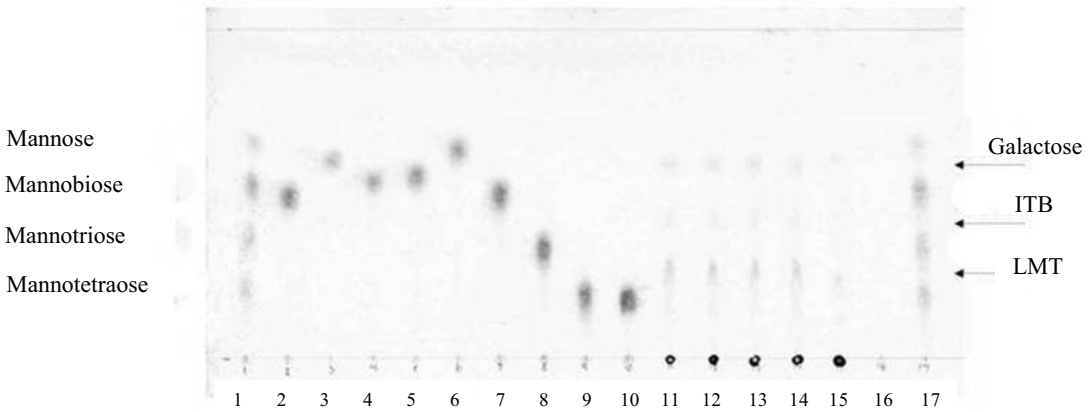

Fig. 4 Locust bean gum degradation by S1 with 100 mM $CoCl_2$ at various times of 0, 1, 2, 3, and 4 h. Lane 1: standard mannooligosaccharides. Lane 2: mannose-7-butyrate, Lane 3: Glucose. Lane 4: Maltose. Lane 5: galactose. Lane 6: mannose. Lane 7: mannobiose. Lane 8: mannotriose. Lane 9: mannotetraose. Lane 10: mannoheptaose. Lane 11: 0 h. Lane 12: 1 h. Lane 13: 2 h. Lane 14: 3 h. Lane 15: 4 h of reaction time. Lane 16: S1 mannanase preparation. Lane 17: standard mannooligosaccharides.

oligomeric reaction products by thin layer chromatography. Glucose, galactose, maltose, mannose, mannobiose, mannotriose, mannotetraose, mannoheptaose, and mannose-7-butyrate were used as standard markers (Fig. 3). For the reaction with purified mannanase S1, the three main products that were identified during the hydrolysis reaction were galactose, an unknown compound having an intermediate R_f between mannotriose and mannobiose, designated as ITB, and unknown molecules larger than mannotetraose, designated as LMT (Fig. 3). Both galactose and ITB were still detected as shown in Fig. 4 when 100 mM $CoCl_2$ was added in the hydrolysis reaction as an effector of mannanase activity. However, a new compound with R_f between mannotriose and mannotetraose, designated as ITT, appeared. Interestingly, no unsubstituted mannooligosaccharides such as mannobiose or mannotriose could be detected by TLC. The unidentified oligosaccharides ITB and ITT could be mannooligosaccharides substituted with galactosyl side chains.

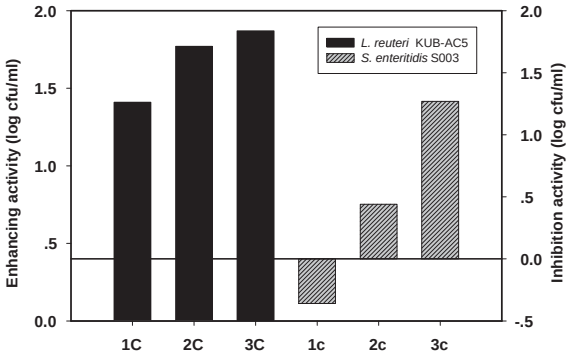

Fig. 5 Effects of Co-CM-hydrolysate on enhancing activity of *L. reuteri* KUB-AC5 and inhibition activity of *Salmonella* serovar Enteritidis S003.

Table 4 Effect of Co-CM-hydrolysate on growth of *L. reuteri* KUB-AC5 and *Salmonella* serovar Enteritidis S003.

Treatment	Survival cell (log cfu/ml)	
	KUB-AC5	S003
Control	8.20^{c}	12.39^{b}
0.31 mg/ml, 1c	9.61^{b}	12.75^{a}
0.62 mg/ml, 2c	9.97^{a}	11.95^{c}
0.93 mg/ml, 3c	10.07^{a}	11.12^{d}

Different letters in a column indicate statistical difference with $p < 0.05$.

Prebiotic properties

This hydryolysis was then tested for potential prebiotic properties, i.e., its effect on growth of *L. reuteri* KUB-AC5 as a positive probiotic strain, as well as on growth of *S*. Enteritidis S003 and *E. coli* E010 as examples of potentially pathogenic/undesirable strains. The hydrolysis product obtained from a hydryolysis reaction of 4 h contained 0.31 mg/ml of reducing sugar. The different reducing sugar concentration of 0.31, 0.62, and 0.93 mg/ml defined as 1c, 2c, and 3c, respectively, were tested for prebiotic properties (Table 4). All of the experiments containing Co-CM-hydrolysate showed an effect on growth of the bacterial strains tested ($p < 0.05$). The growth-enhancing activity on *L. reuteri* KUB-AC5 of 1.41–1.87 log cfu/ml (Fig. 5) increased when the amount of Co-CM-hydrolysate added to the growth medium was also increased. The experiment containing of 0.93 mg/ml of Co-CM-hydrolysate showed the highest enhancing and inhibition activity of 1.87 log cfu/ml and 1.27 log cfu/ml against *L. reuteri* KUB-AC5 and *S*. Enteritidis S003, respectively. Interestingly, no effect on the growth of *E. coli* E010 could be detected. These results indicate that Co-CM-hydrolysate contains yet unidentified oligosaccharides which have a potential prebiotic property of enhancing growth of *L. reuteri* while apparently inhibiting growth of *Salmonella* Enteritidis, which are representatives of desirable lactic acid bacteria and pathogenic bacteria, respectively. To understand the growth-affecting mechanism, identification of the main oligosaccharides obtained in this hydrolysate and further studies will be needed.

DISCUSSION

Only one single mannanase protein designated mannanase S1 was detected in the culture solution of *K. oxytoca* KUB-CW2-3 when grown on a medium containing copra mannan. It is a monomeric protein of 165 kDa with pI of 3.5. The molecular mass of mannanase S1 is somewhat higher than those obtained from other microbial mannanases. *Bacillus* spp. mannanases have a molecular mass in the range of 37–40 kDa[20–22]. Nevertheless, Takeda et al[23] reported that the purified mannanase from *Bacillus* sp. strain JAMB-750 has a molecular mass of 130 kDa. Fungal mannanases, however, seem to vary more significantly in their mass. *Aspergillus* mannanases range from 30–110 kDa[24] while those of *Trichoderma* range from 32–46 kDa[25,26].

The optimum pH of mannanase S1 was similar to those of some bacterial and fungal mannanases which are in the range of 3.0–6.0[22,26–29]. With regard to stability of mannanase S1, the results obtained suggest that mannanase S1 is quite stable at higher temperature with half life times of several hours in the temperature range of 50–70 °C.

Mannanases are glycoside hydrolases (GH) which degrade mannans and heteromannans. Glycosides hydrolases from various sources were classified into different families based on amino acid sequences similarities and hydrophobic cluster analysis[30]. These enzymes have been shown to belong to either GH family 5 or 26. The N-terminal sequence of mannanase S1 showed no match with the N-terminal region of any other bacterial mannanase belonging to these two families when analysed by Protein-Protein BLAST (blastp) (http://www.ncbi.nlm.nih.gov). In addition, no close similarity could be detected when aligning this N terminal sequence with other microbial mannanases by using the CLUSTALW program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Family GH5 was formerly known as cellulase family A and encompasses diverse enzymes[31]. Amino acid sequence alignments of members of family 5 rarely presented levels of sequence identity greater than 20%[32]. More-

over, typical mannanase domain sequences of family GH5 were rather distributed throughout the entire gene. Family GH26 typically shows a short conserved region rich in aromatic amino acid, WFWWG[33], which can be found in the N-terminal region but also in other parts of the protein[34]. The N-terminal sequence of mannanase S1 does not show this conserved region. Hence mannanase S1 might belong to family GH5. However, the complete protein sequence should be known for an exact classification.

Based on its rather broad substrate specificity, mannanase S1 can be applied for the hydrolysis of various mannans, that carry substituents to a varying degree. LBG and konjac mannan were employed as substrates for the determination of kinetic constants of the enzyme. Locust bean gum is a galactomannan consisting of a mannose backbone with galactose side groups, while konjac mannan is a glucomannan with both mannosyl and galactosyl moieties in its main chain but which is devoid of side chain substituents. The kinetic constants of mannanase S1 were rather similar for both substrates. As judged from studying the hydrolysis of LBG and the resulting reaction products, both galactose and various oligosaccharides were obtained, indicating that mannanase S1 can hydrolyse both the β-1,4-mannosidic linkage in the polymer main chain as well as the galactosyl side chain. Hydrolysis of locust bean gum by 100 mM $CoCl_2$ treated mannanase S1 yielded galactose and unknown compounds which moved further than mannotriose and mannotetraose on TLC. Sugiyama et al[35] reported that hydrolysis of mannan can result in various kinds of unknown oligosaccharides and unknown substances. These unknown compounds may be mannooligosaccharides carrying side chains that can result from transferase activity of the hydrolase.

In general, mannooligosaccharides (MOS) that are already available on the market are carbohydrates extracted from the outer wall of yeast cells. They are used because of their ability to reduce growth of pathogenic bacteria, such as *Salmonella* sp. and *E. coli*, in the digestive tract of animals. Few studies have found an effect of MOS to growth inhibition of *Salmonella* sp. Spring et al[36] determined the effect of MOS on caecal fermentation parameters, caecal microflora, and enteric pathogens in chicks. Caecal *S*. Typhimurium 29E concentrations decreased from 5.40 to 4.01 log cfu/g ($P < 0.05$) after receiving 4000 ppm dietary MOS. Moreover, *S*. Typhimurium-challenged turkeys fed by MOS had a decreased incidence of faecal contamination, whereas broilers fed by MOS reduced faecal counts of *S*. Dublin and *E. coli*[37]. The effect of these yeast mannans and mannooligosaccharides, however, is thought to be through their binding of certain adhesion sites on these bacteria. Our study indicates a different source of MOS, those derived from copra mannan. Co-CM-hydrolysate exhibited a potential growth inhibition of *Salmonella* Enteritidis S003 and was able to promote growth of *L. reuteri*, which is a well known probiotic organism. Typically, prebiotic properties should not only reduce growth of pathogens but must also promote growth of desirable and beneficial bacteria, such as *Lactobacillus*. This is the first paper dealing with MOS formation from copra mannan by using mannanase S1 from *K. oxytoca* KUB-CW2-3 and the elucidation of its prebiotic properties.

MOS are commonly used in animal feed to improve digestibility and support gastrointestinal health[36,38,39]. In addition, it may have a diverse prebiotic property showing an anti-obesity effect as proposed by Smith et al[4]. Its combination with an appropriate probiotic would lead to synbiotic application. Further studies relating to the properties of MOS, formed by mannanase S1-treated copra mannan will be carried out in the future to address some of these questions.

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