Copra meal hydrolysis by the recombinant β -mannanase KMAN-3 and MAN 6.7 expressed in *Escherichia coli*

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Abstract

Hydrolysis products of defatted copra meal (DCM) hydrolysis were investigated with either recombinant β -mannanases from *Klebsiella oxytoca* KUB-CW2-3 (KMAN-3) or *Bacillus circulans* NT 6.7 (MAN 6.7). Morphological changes and functional groups of solid residues were also determined by scanning electron microscopy (SEM) and Fourier transform infrared (FTIR) spectroscopy. Results revealed that the Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) values of KMAN-3 on DCM were 2.4 mg/ml and 5.4 U/mg, respectively, while MAN 6.7 recorded K_m and V_{max} at 2.0 mg/ml and 4.3 U/mg, respectively. Both enzymes efficiently randomly hydrolysed DCM and produced a range of different manno-oligosaccharides (MOS). The profile of hydrolysis products was different for each enzyme used. Main products from hydrolysis of DCM by KMAN-3 and MAN 6.7 were various MOS including mannobiose (M2), mannotriose (M3), mannotetraose (M4), and mannose, whereas mannopentaose (M5) was only found from KMAN-3. Amount of M3 produced by KMAN-3 was about three times higher than from MAN 6.7. Total MOS yield for KMAN-3 was 1.5-folds higher than for MAN 6.7. SEM analysis showed that enzymatic hydrolysis with KMAN-3 and MAN 6.7 resulted in deconstruction of the DCM structure which generated a variety of MOS products. FTIR spectra revealed that the properties of both hydrolysed solids were not significantly different compared to the original DCM. Results suggested that KMAN-3 was a promising candidate for production of high MOS content from copra meal.

Keywords Manno-oligosaccharides · Copra meal · Recombinant β -mannanase · Enzymatic hydrolysis

Introduction

Copra meal is abundantly available as the main by-product from coconut milk extraction and Thailand is the world's ninth largest producer of coconuts (Burton 2018). In 2016, Thai coconut production was 1231 million tonnes, generating 123 million tonnes of copra meal (Thailand Department of Agricultural Extension 2018). Copra meal cannot be efficiently utilised as an animal feed ingredient because it contains high levels of non-starch polysaccharides, especially galactomannans (61%) and mannans (26%). Therefore, bioconversion of copra meal has been intensively studied to

produce value-added biological products such as manno-oligosaccharides and galactosylmanno-oligosaccharides (Chaikaew et al. 2017).

Manno-oligosaccharides (MOS) as non-digestible oligosaccharides have recently attracted significant interest due to their beneficial effects in stimulating intestinal bacteria, mainly bifidobacteria and lactobacilli and reduction in dietary fat absorption. Copra meal is a rich source of MOS and contains large amounts of galactomannan and mannan. The galactomannan in copra meal is composed of repeating β -(1 $\rightarrow$ 4)-mannose units and a few α -(1 $\rightarrow$ 6)-galactose units attached to a β -(1 $\rightarrow$ 4)-mannose backbone (Ghosh et al. 2015). MOS is produced from copra meal by β -mannanase enzymatic hydrolysis. β -mannanase exhibits high specific mannan, glucomannans, and galactomannan which are produced by various microorganisms including fungi, yeast, and bacteria. Several studies have investigated MOS production by copra meal hydrolysis using bacteria to produce mannanase. Ariandi and Meryandini (2015) hydrolysed copra meal with β -mannanase from *Streptomyces* sp. BF3.1. The

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hydrolysis reaction resulted in mannobiose (M2), mannotriose (M3), mannotetraose (M4), mannopentaose (M5), and mannohexaose (M6). Pangsi et al. (2015) produced MOS from copra meal using β -mannanase from *Bacillus circulans* NT 6.7 resulting in MOS, M2–M6.

Recently, recombinant β -mannanase from *B. circulans* NT 6.7 (MAN 6.7) and *Klebsiella oxytoca* KUB-CW2-3 (KMAN-3) were reported to produce high β -mannanase activities (Piwpankaew et al. 2014; Pongsapipatana et al. 2016). The β -mannanase-producing strain *B. circulans* NT 6.7 was isolated from coconut factory soil in Thailand and the β -mannanase gene from *B. circulans* *circulans* NT 6.7 was successfully cloned and expressed by *E. coli* BL21 (DE3). Activity of recombinant MAN6.7 was about 20 times higher than the wild-type and exhibited high specificity for galactomannan. *K. oxytoca* KUB-CW2-3 is a Gram-negative bacterium isolated from fermented coconut waste. The mannanase gene from *K. oxytoca* KUB-CW2-3 was cloned and expressed in *E. coli* TOP10 resulting in 400 times higher β -mannanase activities than the wild-type. The gene exhibited higher activity to glucomannan but lower activity to linear mannan (Tuntrakool and Keawsompong 2018). Tuntrakool and Keawsompong (2018) reported Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) values of purified KMAN-3 against konjac glucomannan and DCM as 2.13×10^6 mg/ml, 37 mg/ml and 1.56×10^{10} U/mg, 3,930 U/mg, respectively. Moreover, both KMAN-3 and MAN 6.7 exhibited relatively high activity of mannanase (42–50 unit/ml), while xylanase, cellulase, and glucanase activities were relatively low (< 0.7 unit/ml) (Sathitkowitchai. 2018). As mentioned above, two β -mannanase recombinants showed potential for MOS production from agro-industrial biomass, especially copra meal. This is the first report comparing hydrolysis products and structural change of DCM after enzymatic hydrolysis by two recombinant β -mannanase enzymes. Here, products of DCM hydrolysis using two recombinant β -mannanases as KMAN-3 and MAN 6.7 were investigated. Morphological changes and functional groups of solid residues were also determined.

Materials and methods

Preparation of defatted copra meal

Dried copra meal was obtained from a coconut milk factory in Nakhon Pathom, Thailand. The copra meal was sieved to obtain 40–60 mesh fractions and Soxhlet-extracted with hexane for 4 h to remove lipids. The DCM was stored in plastic bags at room temperature until required for use.

Bacterial strains and culture conditions

Two recombinant β -mannanases were used as KMAN-3 and MAN 6.7. KMAN-3, a recombinant *E. coli* TOP10 harbouring plasmid pFlag-CTS/KMAN-3, constructed by Pongsapipatana et al. (2016), was cultured in Luria–Bertani broth (LB) medium containing 100 μ g/ml ampicillin and incubated at 37 °C with shaking at 200 rpm until the OD₆₀₀ reached 0.6. Isopropyl- β -D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.25 mM and incubated at 26 °C for 8 h. MAN 6.7, a recombinant *E. coli* BL21(DE3) harbouring plasmid pET-21d(+)/man 6.7, cloned and expressed by Piwpankaew et al. (2014), was cultivated in LB medium containing 100 μ g/ml of ampicillin. The culture was incubated at 37 °C and 200 rpm until the OD₆₀₀ reached 0.5 and then IPTG was added to a final concentration of 0.5 mM. Culture was carried out at 18 °C with 200 rpm for 18 h. The supernatant was obtained by centrifugation at 10,000 rpm for 10 min to remove bacterial cells and collected to determine β -mannanase activity.

Enzyme assay

β -Mannanase activity of KMAN-3 was determined by mixing 0.1 ml of an appropriately diluted enzyme solution with 0.1 ml of 1% (w/v) locust bean gum (LBG) in 50 mM citrate buffer (pH 4.0) at 40 °C for 30 min. Enzyme activity of MAN 6.7 was assayed using 0.1 ml of 1% (w/v) LBG in 50 mM potassium phosphate buffer (pH 6.0) and 0.1 ml of appropriately diluted enzyme solution. The reaction was conducted at 50 °C for 60 min. Amounts of reducing sugars released during the reaction were analysed by the 3,5-dinitrosalicylic acid (DNS) method using D-mannose as the standard. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μ mole of mannose per minute under assay conditions.

Enzymatic hydrolysis

Enzymatic hydrolysis was carried out at 5% (w/v) of DCM. The hydrolysis reaction of MAN 6.7 was incubated with 8 unit/ml enzyme in 50 mM potassium phosphate buffer pH 6.0 at 50 °C and 150 rpm. For KMAN-3, hydrolysis reaction of 8 unit/ml enzyme was performed at 50 mM sodium citrate buffer pH 4.0 at 40 °C, 150 rpm. After hydrolysis for 24 h, the reaction was stopped for 10 min by adding boiling water. Solid residues were separated using Whatman filter paper No.1, lyophilised for 48 h and analysed using scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR). The hydrolysate was analysed by

high-performance liquid chromatography (HPLC) to determine concentrations of MOS and mannose.

Protein assay

Protein concentration was determined by Bradford assay using Bradford reagent (Bio-Rad, USA). Bovine serum albumin (BSA) was used as a protein standard.

Molecular weight determination

The molecular weight of the protein was measured using SDS-PAGE according to Laemmli (1970) on a 12% acrylamide gel. BLUitra Prestained Protein Ladder (GeneDireX) was used as the protein standard. The gel was stained using Coomassie Brilliant Blue R-250 (Thermo Fisher Scientific, USA).

Determination of kinetic parameters

Both K_m and V_{max} of KMAN-3 and MAN 6.7 were determined using DCM as a substrate. Reaction of KMAN-3 was performed at 50 mM sodium citrate buffer pH 4.0 at 40 °C, while reaction of MAN 6.7 was carried out at 50 mM potassium phosphate buffer pH 6.0 at 50 °C. Substrate concentration was studied using values of 0.1% to 1% (w/v). Both K_m and V_{max} of each enzyme were calculated using the Kaleidagraph program version 3.5.

Scanning electron microscopy (SEM)

Structure and surface characteristics of the enzyme-hydrolysed solids were observed by SEM. A SU8020 (Hitachi Co., Tokyo, Japan) instrument operated at 15 kV was used. Before SEM observation, samples were fixed with osmium tetroxide and sputter-coated with a thin layer of gold to prevent degradation and render fibre conductivity.

Fourier transform infrared spectroscopy (FTIR)

FTIR spectroscopy (Bruker, USA) was used to reveal changes of the functional groups in solid residues. Samples were ground with potassium bromide (KBr) powder and then pressed into pellets for FTIR analysis. Data were recorded within a range of 374–4000 cm^{-1} , resolution 4 cm^{-1} and 16 scans per sample.

Analysis of hydrolysis products

To determine the concentration of MOS in enzymatic hydrolysis, hydrolysates of DCM were analysed by HPLC with a refractive index detector (Waters; Milford, MA, USA). Separations were performed on an Aminex HPX-42C

column (Bio-Rad, USA) using deionised water (Milli-Q, Millipore Corp; Bedford, MA, USA) as the mobile phase at 70 °C with a flow rate of 0.40 ml/min. Mannose (M1), mannobiose (M2), mannotriose (M3), mannotetraose (M4), mannopentaose (M5), and mannohexaose (M6) (Megazyme, Ireland) were used as standards.

Yield of each MOS was calculated using the equation below:

$$\text{Yield of MOS (g/100 g dry DCM)} = \frac{\text{Mass of sugar in the hydrolysate (g)}}{\text{Total weight of dry DCM (g)}} \times 100.$$

Raw material analyses

Total protein content was determined according to the Kjeldahl method. Acid-insoluble lignin was determined following the method of the Technical Association of Pulp and Paper Industry (1996). Carbohydrate composition of DCM was analysed by the National Renewable Energies Laboratory (NREL) method according to Sluiter et al. (2018). Monosaccharide concentrations (glucose, mannose, galactose, arabinose, and xylose) were determined using an HPLC system equipped with an Asahipak NH₂P-50 4E column (4.6 mm × 250 mm, Shodex, Japan) and refractive index detector (Shimadzu, Japan). Column temperature was operated at 30 °C with a flow rate of 1.0 ml/min using a mobile phase of water and acetonitrile (25:75 v/v).

Results and discussion

Chemical composition of copra meal

Table 1 shows the chemical composition of copra meal with 54.3% (w/w of dry weight) mannose as the major sugar followed by 7.1% glucose, 1.5% xylose and 1.1% arabinose. These results are interesting from a prebiotics context because copra meal with high mannan content is

Table 1 Chemical composition of copra meal

Component	% (w/w) dry weight
Protein	7.9
Acid-insoluble lignin	3.2
Monosaccharides	
Mannose	54.3
Glucose	7.1
Xylose	1.5
Arabinose	1.1
Galactose	Not detected

the preferred substrate for MOS production. Saittagaroon et al. (1983) reported the composition of copra meal as 72% mannose, 25% glucose, 3% galactose and trace amounts of xylose and arabinose.

Characterisation of recombinant β -mannanases KMAN-3 and MAN 6.7

To identify the recombinant β -mannanases enzyme from KMAN-3 and MAN 6.7, each enzyme was concentrated and analysed by SDS-PAGE to estimate protein molecular mass (Fig. 1). Apparent molecular masses of β -mannanase from MAN 6.7 and KMAN-3 were estimated at 40 and 43 kDa, respectively. The molecular mass of β -mannanase from MAN 6.7 concurred with Pangsri et al. (2015) who revealed that mannanase from wild-type *B. circulans* NT 6.7 had a molecular mass of 40 kDa. The molecular mass of β -mannanase from KMAN-3 was lower than results obtained from wild-type *K. oxytoca* KUB-CW2-3 (Chantorna et al. 2013). Nevertheless, the molecular mass of β -mannanase from KMAN-3 was similar to some bacterial and fungal mannanases in the range of 32–46 kDa (Chantorna et al. 2013; Pangsri et al. 2015).

Total activities of KMAN-3 and MAN 6.7 were investigated using LBG as a substrate and found to be 21,400 and 16,000 units, respectively. Kinetic parameters of KMAN-3 and MAN 6.7 on DCM were investigated using 0.1% to 1% (w/v) DCM. The K_m and V_{max} values of KMAN-3 were

observed as 2.4 mg/ml and 5.4 U/mg, respectively, while those of MAN 6.7 were 2.0 mg/ml and 4.3 U/mg, respectively, and almost identical. In addition, the activities of two crude recombinant β -mannanase enzymes has been investigated by Sathitkowitzchai (2018). The results showed that both KMAN-3 and MAN 6.7 exhibited high activity of mannanase (42–50 unit/ml), while xylanase, cellulase, and glucanase activities were relatively low (<0.7 unit/ml). It confirmed that the hydrolysis reactions were solely caused by the recombinant β -mannanase and MOS products obtained were the result of the copra meal hydrolysis by recombinant mannanase enzymes.

Enzymatic hydrolysis of DCM with recombinant KMAN-3 and MAN 6.7

To investigate potential use of recombinant β -mannanase as an enzyme for MOS production from DCM, each enzyme was separately incubated with DCM and hydrolysis products were monitored by HPLC. Hydrolysis products with KMAN-3 and MAN 6.7 at 24 h of hydrolysis time are illustrated in Table 2. Results revealed that KMAN-3 and MAN 6.7 efficiently randomly hydrolyzed DCM and generated a range of different MOS; however, amounts of hydrolysis products were significantly different. The main products of DCM hydrolysis by KMAN-3 in descending order were M3, M2, and M4 while M5 and M1 were found only in negligible amounts. Meanwhile, products of DCM hydrolysis by MAN 6.7 were M4 and M3 with smaller amounts of M2 and M1. Interestingly, the amount of M3 produced by KMAN-3 was about three times higher than from MAN 6.7. KMAN-3 produced high quality MOS from DCM, especially M3 with negligible mannose, suggesting potential in industrial prebiotics preparations without the need for separation of monomers and oligosaccharides. Several authors reported DCM hydrolysis products with other bacterial mannanases. Pangsri et al. (2015) demonstrated that hydrolysis products from DCM by purified wild-type *B. circulans* NT 6.7 enzyme were M3, M4, M5, and M6,

Fig. 1 SDS-PAGE analysis of recombinant β -mannanase KMAN-3 and MAN 6.7. Lane M molecular marker, (BLUltra Prestained Protein Ladder, Genedirex); Lane 2, KMAN-3; Lane 3, MAN 6.7

Table 2 Products of DCM hydrolysis with either recombinant β -mannanase KMAN-3 or MAN 6.7 at 24 h hydrolysis

MOS and mannose (g/100 g dry DCM)	Recombinant β -mannanase	
	KMAN-3	MAN 6.7
Mannohexaose (M6)	–	–
Mannopentaose (M5)	1.2	–
Mannotetraose (M4)	3.2	6.1
Mannotriose (M3)	13.4	4.3
Mannobiose (M2)	3.7	2.3
Mannose	1.2	2.7
Total MOS	22.7	15.4

while Piwpankaew (2014) reported that products of DCM hydrolysis by purified recombinant β -mannanase from *B. circulans* NT 6.7 consisted of M5, M3, and M2. Ghosh et al. (2015) demonstrated that copra meal hydrolysis by recombinant endo- β -(1 $\rightarrow$ 4)-mannanase of *Clostridium thermocellum* (CtManf) produced MOS including M3, M2, and mannose. Pongsapipatana et al. (2016) found that hydrolysis of copra meal by purified recombinant β -mannanase from *K. oxytoca* KUB-CW2-3 in *E. coli* yielded M5, M4, M3, M2, and mannose, while Intaratkul et al. (2017) reported the main products of purified recombinant β -mannanase from *B. circulans* NT 6.7 in *Lactobacillus platarum* catalysed hydrolysis of copra meal as M4, M3, and M2 with trace amounts of M1 and M6.

Total MOS yield was determined as the summation of M2-M6 found in the enzymatic hydrolysate. Total MOS yield for KMAN-3 (22.7 g/g dry DCM) was 1.5-folds higher than for MAN 6.7 (15.4 g/g dry DCM). Total MOS yield in this study was higher than yields from other mannan-rich biomasses such as palm kernel (19.7 g/100 g dry biomass) and coffee (12.51 g/100 g dry raw material) (Chiyanzu et al. 2014; Li et al. 2018). Therefore, our results suggested that KMAN-3 could be a promising candidate for production of MOS content from copra meal.

SEM analysis

Structures of original DCM and solid residues after 24 h hydrolysis with either KMAN-3 (DCMKMAN-3) or KMAN

6.7 (DCMMAN 6.7) were observed by SEM to investigate the effect of enzymatic hydrolysis on morphology (Fig. 2). Results showed that the DCM surface was very compact, smooth, and non-porous (Fig. 2a). By contrast, morphologies of both solid residues (DCMKMAN-3 and DCMMAN 6.7) were significantly altered. Outer surface layers of the two solid residues were destroyed with some damage to the inner structure (Fig. 2b, c). This result indicated that both KMAN-3 and MAN 6.7 enzymes could efficiently destroy the structure of DCM to produce a variety of MOS products.

FTIR analysis

FTIR spectra of original and solid residues after 24 h hydrolysis with either KMAN-3 or KMAN 6.7 are illustrated in Fig. 3. Spectra of both solid residues showed no significant change compared to original DCM. No new peaks occurred suggesting no chemical reaction in the solids after enzymatic hydrolysis. The peak between 3600 cm^{-1} and 3000 cm^{-1} was attributed to hydroxyl stretching vibration. Compared with the original DCM, peaks of both hydrolysed solids shifted to higher wavenumbers (from 3283 to 3377 cm^{-1} and 3415 cm^{-1}), indicating reduction in hydrogen bonds of the hydrolysed solid and suggesting that hydrogen bonds of the cellulose chain were destroyed after enzymatic hydrolysis with recombinant β -mannanases. The peak at 2925 cm^{-1} was assigned to C-H stretching vibration of the methyl group, while the peak at 1746 cm^{-1} exhibited vibration of acetyl and uronic acid ester groups of hemicellulose or ester

Fig. 2 SEM micrographs of samples at 24 h hydrolysis. **a** DCM, **b** solid after hydrolysis with KMAN-3, and **c** solid after hydrolysis with MAN 6.7 (DCMMAN)

Fig. 3 FTIR spectra of DCM (a) and solid DCM after 24 h hydrolysis with either recombinant β -mannanase KMAN-3 (b) or KMAN 6.7 (c)

linkage of carboxylic groups of ferulic acid and p-coumaric acid of lignin. The peak at 1648 cm^{-1} was generated from absorbed water molecules via hydrogen bonding in amorphous regions of cellulose (Wang et al. 2012). The peak at 1459 cm^{-1} was associated with C–H deformation in methyl and methylene, while the peak at 1155 cm^{-1} indicated anti-symmetric bridge stretching of C–O–C groups. In the anomeric region ($950\text{--}700\text{ cm}^{-1}$), both hydrolysed solids exhibited peaks at 872 and 813 cm^{-1} , suggesting the existence of mannose as reported by Ghosh et al. (2015). The peak at 872 cm^{-1} revealed beta-glycosidic bonding in both hydrolysed solids.

Conclusions

Our results demonstrated that the kinetic parameters (K_m and V_{max}) of KMAN-3 and MAN 6.7 on DCM hydrolysis were similar. Recombinant β -mannanases KMAN-3 and MAN 6.7 efficiently randomly hydrolysed DCM and generated a range of different MOS; however, amounts of hydrolysis products were significantly different. Hydrolysis products of DCM by KMAN-3 and MAN 6.7 were MOS including M2, M3, M4, M5, and mannose. M5 was only detected from KMAN-3. Total MOS generated by KMAN-3 was approximately 1.5-folds higher than from MAN 6.7. For residual solids, both enzymes exhibited high efficiency in destroying the structure of copra meal, producing high amounts of MOS. FTIR spectra revealed that the physiochemical properties of both hydrolysed solids showed no significant change compared to the original DCM. Therefore, KMAN-3 was proposed as a good candidate for commercial production of high quality MOS from copra meal with promising application in the animal feed industry.

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Author contributions NP and SK conceived and designed experiments and wrote the manuscript. SU and SK gave some valuable advices about structure of manuscript. NP performed the experiments. All authors participated, read, and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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