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Synthesis of a novel prebiotic trisaccharide by a type I α-glucosidase from *B. licheniformis* strain TH4-2

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ABSTRACT

The use of α-glucosidase from *Bacillus licheniformis* TH4-2 in the glucosyl transfer reaction for the synthesis of oligosaccharides (OS) was evaluated. The conditions for α-glucosidase production were optimized. The enzyme was 112-fold purified with a 28% yield. The molecular mass was 64 kDa, and the optimum pH and temperature were 6.0 and 45 °C. The highest hydrolytic activity was observed towards *p*-nitrophenyl α-D-glucopyranoside followed by isomaltose, sucrose and maltose, supporting that it is a type I α-glucosidase. The enzyme could synthesize OS by transglucosylation from sucrose donor using various saccharides as acceptor. Melibiose, an α-galactoside, was selected as an efficient and interesting acceptor. TLC and HPLC analysis of the products revealed that the optimum condition for OS production was pH 6.0 with 15% melibiose, 5% sucrose and 5 U/ml enzyme at 45 °C for 24 h. Under these conditions two product peaks were observed in the HPLC profile with yields of 17.2% and 3.3%. The main product was isolated by Sephadex LH-20 column and analyzed by MS and NMR as the novel 504 Daltons trisaccharide, α-D-glucopyranosyl-(1 → 6)-α-D-galactopyranosyl-(1 → 6)-D-glucopyranose. The prebiotic nature of this product was suggested from its resistance to hydrolysis by rat intestinal acetone powder containing digestible enzymes of rat intestine.

1. Introduction

α-Glucosidase [EC 3.2.1.20; α-D-glucoside glucohydrolase] catalyzes the liberation of α-D-glucose from the non-reducing end of a substrate, including maltooligosaccharides, aryl- and alkyl-α-D-glucopyranosides. Besides this hydrolase activity, the enzyme also displays a transferase activity (transglycosylation reaction), which results in the formation of various α-glucosylated compounds [1–3]. α-Glucosidases are classified according to their primary structure into two families (I and II), which belong to the glycoside hydrolase (GH) families 13 and 31, respectively [1,4]. In addition, on the basis of substrate specificity, the enzymes are classified into three types (I, II and III). Type I α-glucosidase enzymes hydrolyze heterogeneous substrates, such as aryl-glucosides and sucrose, more efficiently than maltose. Type II enzymes show a higher activity on maltose and isomaltose as substrates with a low activity towards aryl glucosides. Type III enzymes exhibit the same specificity as type II enzymes, but also hydrolyze polysaccharides like amylose and starch [5,6].

Non-digestible oligosaccharides (NDOs) are widely known as functional food ingredients. In addition to providing useful modifications to the physicochemical properties of foods, NDOs have various physiological functions, such as the improvement of intestinal microflora based on the selective proliferation of bifidobacteria, stimulation of mineral absorption, anticarcinogenicity, immunomodulation and the improvement of both plasma cholesterol and blood glucose levels [7]. Enzymatic synthesis from simple sugars (e.g. maltose, sucrose and lactose) of oligosaccharides (OS) and their derivatives can be considered as very promising alternatives to chemical synthesis due to their high specificity, efficiency and environmental friendly status [8,9]. α-Glucosidases are one such type of enzyme with a high potential for the synthesis of functional OS [1]. However, the profile of the products obtained depends in part on the enzyme characteristics. The donor/acceptor specificity and the specificity of the linkage type are the main factors that govern the type of product formed. For example, the prebiotic 6-O-α-glucosyl maltotriose and 6-O-α-isomaltosyl maltose were synthesized by the α-glucosidase obtained from *Xanthophyllomyces dendrorhous* with maltose as a single substrate [10]. Using only sucrose as a substrate, the α-glucosidase from *Bacillus* sp. SAM 1606 synthesized 6-O-glucosylsucrose while the enzyme from the digestive juice of the snail *Archachatina ventricosa* yielded

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polyglucosylfructosides ranging from DP3 to DP8, with DP3 (maltosylfructose, α-D-Glc*p*-(1 → 4)-α-D-Glc*p*-(1 → 2)-β-D-Fru*f*) as the main product [1,11]. In view of these findings, the search for an enzyme with new properties for the synthesis of novel NDOs is of prime concern.

Bacillus licheniformis is a Gram-positive, spore-forming soil bacterium that is commonly used in the biotechnology industry to manufacture enzymes, antibiotics, biochemicals and consumer products [12]. The thermotolerant *B. licheniformis* strain TH4-2 was previously isolated from a soil sample in Thailand and screened for the production of thermoactive levansucrase enzyme [13]. In this work, we investigated the α-glucosidase activity of this bacterial strain and explored its potential for use in transglucosylation reactions for the synthesis of novel prebiotic OS.

2. Materials and methods

2.1. Chemicals

p-Nitrophenyl α-D-glucopyranoside (pNPGlc), *p*-nitrophenyl β-D-glucopyranoside, *o*-nitrophenyl β-D-galactopyranoside, D-fructose, D-glucose, lactose, isomaltose, maltooligosaccharides (G2 to G7), melibiose, cellobiose, palatinose, α-amylase from *Aspergillus oryzae*, glucoamylase from *Aspergillus niger* and rat intestinal acetone powder were obtained from Sigma (USA). Lactulose was from Fluka (Switzerland) and sucrose from Bio Basic Inc. (Canada). Raffinose pentahydrate was purchased from Nacalai Tesque, Inc. (Japan). Glucose oxidase kit was a product of Human Biochemical and Diagnostics mbH (Germany). Acetonitrile was from LAB-SCAN Analytical Science (Thailand). Other chemicals and solvents used were of analytical grade.

2.2. Strains and culturing condition

B. licheniformis TH4-2, a thermotolerant bacteria previously isolated from a soil sample in Thailand [13], was used in this study. The medium used for α-glucosidase production consisted of 1% (w/v) beef extract, 1% (w/v) peptone, 0.4% (w/v) ovalbumin, 0.5% (w/v) NaCl and 5% (w/v) soluble cassava starch at a pH of 6.5, except where modified as indicated. Cultivation was performed at 45 °C with shaking at 250 rpm for 42 h.

2.3. Identification of the Bacillus species

Bacillus sp. TH4-2 was identified by (i) morphological analysis and standard biochemical tests (API 20C AUX system) and (ii) comparison of sequence similarity and phylogenetic analysis of the existing species in the GenBank database using a partial fragment (1544 bp) of the 16S rRNA gene.

2.4. Enzyme and protein assay

2.4.1. Enzyme (hydrolysis) activity

α-Glucosidase activity was assayed by the hydrolysis of pNPGlc, with monitoring of the level of the released *p*-nitrophenol. Twenty-five microliters of 5 mM pNPGlc was incubated with 5 μl enzyme solution in 20 mM sodium acetate buffer (pH 6.0) in a total volume of 100 μl at 45 °C for 10 min. The reaction was stopped by adding 200 μl of 1 M sodium carbonate, and the absorbance at 405 nm was measured [1]. One unit was defined as the amount of enzyme that produced 1 μmol of *p*-nitrophenol/min.

The level of sucrose hydrolysis was determined by measuring the amount of reducing sugar produced by Somogyi–Nelson's method using glucose as standard [14]. The reaction mixture, containing 500 μl of 20% (w/v) sucrose in 20 mM sodium acetate buffer (pH 6.0), and 10 μl of enzyme in 490 μl of the same buffer, was incubated at 45 °C for 10 min, and then the absorbance at 520 nm was measured. One unit was defined as the amount of enzyme that produced 1 μmol of reducing sugar/min.

2.4.2. Transglucosylation activity

The transglucosylation activity of α-glucosidase was determined by incubating 25 μl of the enzyme preparation (0.5 units) with 475 μl of 5% (w/v) sucrose in the absence or presence of 5% (w/v) of the indicated saccharide acceptors in 20 mM sodium acetate buffer pH 6.0. The reaction was incubated for 24 h at 45 °C, and then the transglucosylation activity was evaluated by analyzing the products by HPLC.

2.4.3. Protein assay

Protein was quantified according to the Bradford method [15], using bovine serum albumin as a standard. The fractions eluted from all chromatographic runs were monitored for protein by measuring the absorbance at 280 nm.

2.5. Purification of α-glucosidase

Two liters of culture was prepared as above (Section 2.2), and then the bacterial cells were removed by centrifugation at 3800 × *g*, 4 °C for 30 min. The supernatant fraction was subjected to 30–60% saturation ammonium sulfate precipitation. The precipitate was then dissolved in 20 mM sodium acetate buffer (pH 6.0), dialyzed and loaded onto a DEAE-cellulose column (1.5 cm × 27 cm) in the same buffer. The column was run at a flow rate of 0.75 ml/min and the enzyme eluted by linearly increasing the NaCl gradient from 0 to 0.3 M (250 + 250 ml), collecting 5 ml fractions. The fractions positive for α-glucosidase (sucrose hydrolysis activity) were pooled and concentrated by ultrafiltration, then further purified by loading on a Sephadex G-100 column (1.9 cm × 90 cm) in 20 mM sodium acetate buffer with a flow rate of 0.33 ml/min and collecting 2 ml fractions. The fractions positive for α-glucosidase (sucrose hydrolysis activity) were pooled and concentrated as the final purified preparation and used for further analysis.

2.6. Characterization of α-glucosidase

2.6.1. SDS- and native-PAGE resolution

For both SDS- and native-PAGE, 7.5% (w/v) resolving gels were used. Enzyme purity was followed by native-PAGE with protein staining by Coomassie Blue R-250 and enzyme activity staining. Zymogram staining of the sucrose hydrolysis activity was performed using the 2, 3, 5-triphenyltetrazolium chloride (TTC) method as described [16]. The molecular weight of the purified enzyme was estimated by SDS-PAGE.

2.6.2. Effect of pH and temperature on sucrose hydrolysis

The effect of pH was determined at 45 °C. The buffers (all 20 mM) used were sodium acetate (pH 5.0–6.0), phosphate (pH 6.0–8.0) and borate (pH 8.0–9.0). The effect of temperature was determined at the above determined optimum pH with the temperature varied from 30 °C to 60 °C. The reaction was performed by incubation of 10% (w/v) sucrose with 0.2 unit/ml enzyme for 10 min at variable pH or temperature.

2.6.3. Substrate specificity

The hydrolytic activity towards pNPGlc, *p*-nitrophenyl β-D-glucopyranoside and *o*-nitrophenyl β-D-galactopyranoside, each at 5 mM, was determined by measuring the amount of nitrophenol released. The activity towards sucrose, isomaltose, maltose, maltotriose, cellobiose, lactose, melibiose, lactulose, palatinose and raffinose (all at 50 mM) was evaluated by measuring the amount of glucose released by the glucose oxidase-peroxidase method (GOD-POD) [17] using a glucose oxidase-based kit (Human Biochemical and Diagnostics mbH, Germany). The enzyme's hydrolytic activity towards starch, amylose and amylopectin as substrates, all at 1% (w/v), was followed by measuring the amount of glucose released using the method of Somogyi–Nelson [14].

2.7. Determination of kinetic parameters

2.7.1. Determination of K_m and V_{max} for pNPGlc

Hydrolysis of pNPGlc substrate was varied in the concentration range of 0.02–5 mM. The reaction was incubated with enzyme (0.15 unit/ml, pNPGlc hydrolysis activity) in 20 mM sodium acetate buffer (pH 6.0) at 45 °C for 10 min. The activity was determined as described under Section 2.4.1.

2.7.2. Determination of K_m and V_{max} for sucrose and maltose

Sucrose substrate was varied in the concentration range of 2.5–100 mM while maltose was varied in the concentration range of 5–400 mM. The reaction was incubated with enzyme (0.2 unit/ml, sucrose hydrolysis activity) in 20 mM sodium acetate buffer (pH 6.0) at 45 °C for 10 min. The activity was determined by the glucose oxidase-peroxidase method as in Section 2.6.3.

2.7.3. Determination of K_m and V_{max} for melibiose acceptor

At the fixed concentration of 150 mM sucrose, melibiose acceptor was varied in the range of 100–500 mM. The reaction was incubated with enzyme (0.5 unit/ml, sucrose hydrolysis activity) in 20 mM sodium acetate buffer (pH 6.0) at 45 °C for 180 min. The transglucosylation activity was evaluated by analyzing the products by HPLC as described in Section 2.8.

2.8. Transglucosylation reaction of α-glucosidase and analysis of the products

The transglucosylation reaction was followed by investigating the transfer products formed. The purified α-glucosidase (0.5 unit/ml, sucrose hydrolysis activity) was incubated in 20 mM sodium acetate buffer, pH 6.0, with either 5% (w/v) sucrose as a single substrate or with 5% (w/v) sucrose as the glucosyl donor and 5% (w/v) of one of the various acceptors (lactose, melibiose, cellobiose, raffinose, palatinose and lactulose), as indicated, at 45 °C for 24 h. Then, the reaction mixture was boiled and analyzed by TLC using a silica gel 60 plate (Merck), with a 7:1:2 (v/v/v) mixture of n-propanol: ethyl acetate: water as the mobile phase solvent. After running, the spots were detected by spraying with a 1:9 (v/v) mixture of concentrated sulfuric acid: ethanol, followed by heating at 110 °C for 15 min.

Product separation, and the determination of product yield, were performed by HPLC using a Shimadzu LC-3A HPLC machine equipped with Luna-NH2 column (250 mm × 4.6 mm) and a Shimadzu RID-3A refractometer. The reaction mixture was eluted with a 7:3 (v/v) ratio of acetonitrile:water at a flow rate of 1 ml/min. The yield of the product was calculated from the following equation:

$$\text{product yield } (\%) = \frac{\text{peak area of product}}{\text{peak area of acceptor at } t_o} \times 100$$

2.9. Isolation and characterization of transglucosylation products

The transfer products were purified on a Sephadex LH-20 column (1.2 cm × 120 cm). Elution from the column was in 70% (v/v) n-propanol at a flow rate of 0.6 ml/min collecting 1 ml fractions. The fractions were followed for the amount of glucose by the Somogyi–Nelson assay and positive fractions were then checked for their constituents by HPLC. The peak of the main product was collected for further characterization.

2.9.1. Mass spectrometry (MS)

The Electrospray Ionization-Time of flight Mass Spectrometry (ESI-TOF MS) profile was recorded on a micrOTOF at the Biological Service Unit of the National Center for Genetic Engineering and Biotechnology, Thailand. A 1:1 (v/v) mixture of methanol:H_2O was used as the solvent.

2.9.2. Nuclear magnetic resonance (NMR)

The structure of the main product was elucidated using a combination of ^1H, ^{13}C NMR and 2D-NMR (HSQC and HMBC). Each spectrum was determined using a Varian Gemini 400 MHz spectrometer, performed at 400 MHz for protons and 100 MHz for carbons. The chemical shifts are expressed in ppm downfield from the signal of tetramethylsilane (TMS), which was used as an internal standard.

3. Results and discussion

3.1. Species identification

The standard API 20 AUX biochemical test and morphological analysis (data not shown), combined with a molecular phylogenetic approach suggested the identification of *Bacillus* sp. TH4-2, a thermotolerant bacteria isolated from soil in Thailand [13], as that of *B. licheniformis*. The 16S rRNA gene sequence of the strain showed a similarity of 99% to that of *B. licheniformis* 14580.

3.2. Enzyme purification

B. licheniformis TH4-2 was used for the production of a specific type of α-glucosidase, namely one with invertase/sucrase activity. The optimized cultivation conditions for high enzyme production were found to be in medium containing 5% (w/v) soluble cassava starch, 1% (w/v) peptone, 1% (w/v) beef extract, 0.5% (w/v) NaCl and 0.4% (w/v) ovalbumin at pH 6.5, 45 °C for 42 h. The α-glucosidase is produced as an extracellular enzyme in the stationary phase of growth. From these optimal culture conditions, the crude enzyme was purified from the culture medium by ammonium sulfate precipitation at 30–60% saturation followed by two column chromatography steps, the anionic DEAE-Cellulose and the gel filtration Sephadex G100 columns (Table 1). Overall the enzyme specific activity was enriched 112-fold (from 1.32 to 1.37 unit/mg protein after purification) to apparent homogeneity at a yield of 28%.

The success of α-glucosidase enzyme purification was judged by the apparent homogeneity of the protein band observed in the final enrichment fraction under both native- and SDS-PAGE (Fig. 1). A single red band in the zymogen (enzyme activity stained) native-PAGE gel (Fig. 1A), which is based on the reduction of TTC to keto sugars [16] confirmed that the purified protein has invertase/sucrase activity. However, investigation of the complete genome of *B. licheniformis* revealed the presence of no glucansucrase genes, whereas at least two α-glucosidase genes were reported [12]. The possibility of this enriched enzyme being a levansucrase is also excluded since this enzyme could not hydrolyze

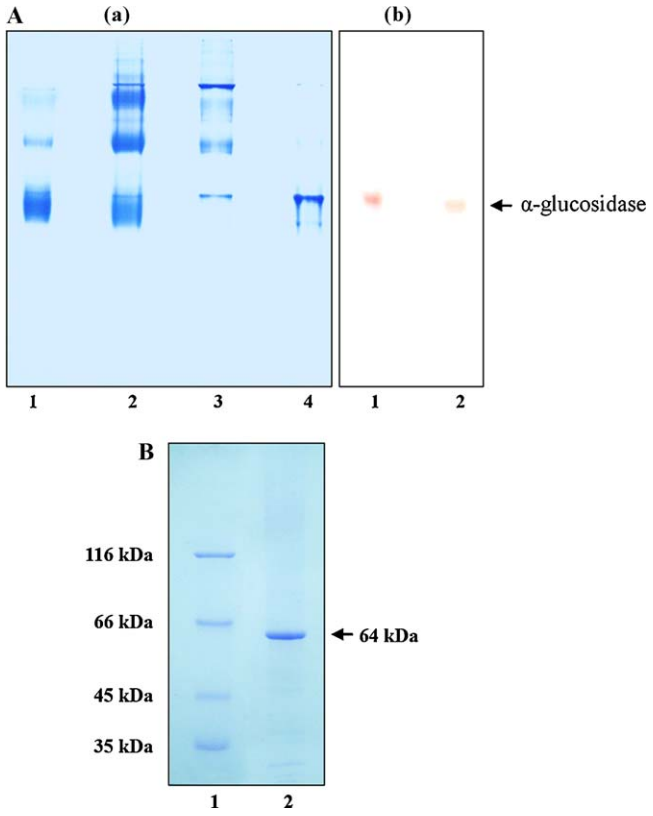

Fig. 1. Native- and SDS-PAGE of the α-glucosidase from *B. licheniformis* strain TH4-2. (A) Native-PAGE (a) *Protein staining*, lane 1: crude enzyme; lane 2: proteins after ammonium sulfate precipitate; lane 3: proteins after DEAE-cellulose column; lane 4: purified α-glucosidase after Sephadex G-100 column. (b) *Zymogram* (*enzyme activity*) *staining*, 3 units (sucrose hydrolysis activity) loaded; lane 1: after DEAE-cellulose column; lane 2: after Sephadex G-100 column. (B) SDS-PAGE, lane 1: protein molecular weight markers; lane 2: purified α-glucosidase after Sephadex G-100 column.

raffinose. Rather the observed hydrolysis of pNPGlc, and the identification of the transglucosylation product (see later), supports the notion that it is an α-glucosidase. From previous works, α-glucosidases from various sources, such as animals, plants and microorganisms, can be classified into three types based on their substrate specificities [5,18]. We propose that the enzyme purified in this study was a type I α-glucosidase, since it can release glucose from sucrose in addition to the hydrolysis of pNPGlc. Most α-glucosidases reported are classified as type II, because the enzymes cannot hydrolyze sucrose but are specific for the hydrolysis of the α-(1 → 4) glucosidic bonds of maltose and maltooligosaccharides

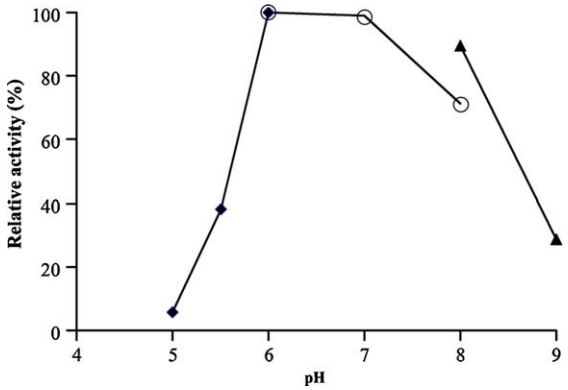

Fig. 2. Effect of pH on enzyme activity at 45 °C: acetate buffer pH 5.0–6.0 (♦); phosphate buffer pH 6.0–8.0 (○) and borate buffer pH 8.0–9.0 (▲).

Table 1
Purification of the extracellular α-glucosidase from *B. licheniformis* TH4-2.

Purification step	Total volume (ml)	Protein		Activity			Fold	Yield (%)
		Concentration (mg/ml)	Total protein (mg)	Activity (U/ml)	Total activity ($\times 10^3$ U)	Specific activity (U/mg protein)		
Crude enzyme	1940	2.10	4074	2.50	4.89	1.23	1	100
Ammonium sulfate 30–60% saturation	350	3.70	1295	12.5	4.39	3.35	2.7	90
DEAE—cellulose	300	0.240	72.0	10.8	3.22	46.0	37	66
Sephadex G-100	75	0.130	9.80	18.2	1.37	137	112	28

[2,4,6,10,19–22]. Type I α-glucosidases have been reported in the related *Bacillus subtilis* [23] and *Bacillus stearothermophilus* [9] as well as within *B. licheniformis* [24,25]. In addition, they have also recently been reported in the digestive juice of the snail *A. ventricosa* [1] and the insect *Quesada gigas* [26].

3.3. Characterization of the purified α-glucosidase

3.3.1. Determination of molecular weight

The molecular weight of the purified enzyme was estimated to be 64 kDa by its mobility in reducing SDS-PAGE (Fig. 1B). This size is in broad agreement with that reported for monomeric α-glucosidases in other strains of *B. licheniformis*, such as 66 and 63 kDa for *B. licheniformis* strains NCIB 8549 [24] and NCIB 6346 [25], respectively. However, α-glucosidases are fairly diverse in size, with that for *Geobacillus thermodenitrificans* HRO10 and *Entamoeba histolytica* being 45 and 55 kDa, respectively [27,28], whilst a homodimer of 130 kDa was reported for *Geobacillus* sp. HTA-462 [21]. In addition, a homohexameric form from *B. thermoamyloliquefaciens* has been reported [29]. Eukaryotic α-glucosidases are mostly monomeric, such as the 61 kDa form from the insect *Q. gigas* [26], 105 kDa for the shrimp *Penaeus vannamei* [30] and 80–86 kDa for the germinating millet seeds *Panicum miliaceum* L. [19].

3.3.2. Effect of pH and temperature on sucrose hydrolysis

When the effect of pH on the sucrose hydrolysis activity of the purified α-glucosidase was investigated, the optimum activity was found at pH 6.0–7.0 (Fig. 2). This is a little less acidophilic than expected since most α-glucosidases have a reported optimum pH in the range of 4.0–7.0, including that of pH 4.5 for *Oryza sativa*

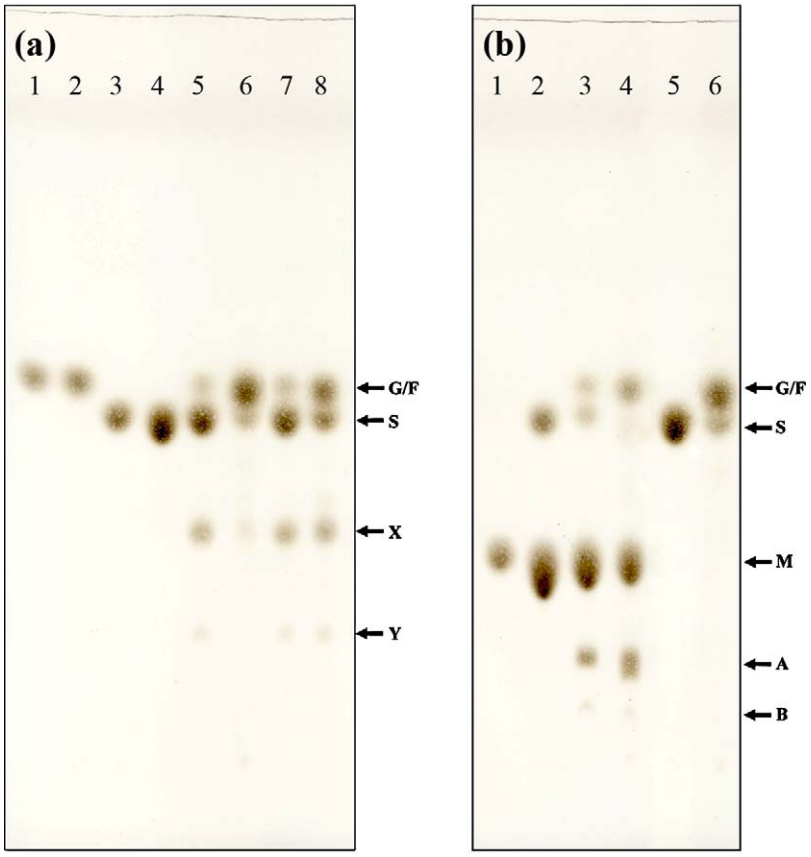

Fig. 3. TLC chromatogram of the reaction products. (a) From using sucrose as a single substrate; lanes 1–3: standards: glucose (G), fructose (F) and sucrose (S), each at 20 mg; lanes 4–8: products (X, Y) with sucrose as the substrate after incubation for 0 (lane 4) and 24 h (lanes 5–8) without subsequent treatment (lane 5) or after subsequent treatment for 12 h at 37 °C with rat intestinal acetone powder (lane 6), for 12 h at 40 °C with α-amylase (lane 7) or glucoamylase (lane 8). (b) From using sucrose as the donor and melibiose as the acceptor, lane 1: standards: melibiose (M) 20 mg; lanes 2–4: products (A, B) with sucrose (S) as the donor and melibiose (M) as acceptor after 0 (lane 2) and 24 h (lanes 3 and 4) incubation without treatment (lane 3) or after treatment with rat intestinal acetone powder as in (A) (lane 4); lanes 5 and 6: sucrose (S) without treatment (lane 5) or after treatment for 12 h at 37 °C with rat intestinal acetone powder (lane 6).

Table 2
Substrate specificity of the (type I) α-glucosidase enriched from *B. licheniformis* strain TH4-2.

Substrate[a]	Glycosidic linkage	Activity (unit/ml)
pNPGlc (5 mM)	–	3.51[b]
*p*NP β-D-glucopyranoside (5 mM)	–	–
*o*NP β-D-galactopyranoside (5 mM)	–	–
Sucrose (50 mM)	Glc-α-(1 → 2)-Fru	2.50[c]
Maltose (50 mM)	Glc-α-(1 → 4)-Glc	1.25[c]
Maltotriose (50 mM)	Glc-[α-1 → 4]-Glc-α-(1 → 4)-Glc	0.69[c]
Maltotetraose (50 mM)	Glc-$[\alpha\text{-}1 \rightarrow 4]_3$-Glc	0.64[c]
Maltopentaose (50 mM)	Glc-$[\alpha\text{-}1 \rightarrow 4]_4$-Glc	0.20[c]
Maltohexaose (50 mM)	Glc-$[\alpha\text{-}1 \rightarrow 4]_5$-Glc	0.16[c]
Maltoheptaose (50 mM)	Glc-$[\alpha\text{-}1 \rightarrow 4]_6$-Glc	–
Isomaltose (50 mM)	Glc-α-(1 → 6)-Glc	3.05[c]

The activity is expressed as the hydrolytic activity of the substrate (5 mM for all aryl glucosides or 50 mM for oligosaccharides). The enzyme of 0.2 unit/ml (sucrose hydrolysis activity) in 20 mM acetate buffer (pH 6.0) was incubated with the substrates at 45 °C for 10 min. (–) no detectable activity.

[a] Cellobiose, lactose, lactulose, melibiose, palatinose, raffinose, starch, amylose and amylopectin were unable to act as a substrate.

[b] Released glucose measured as a function of the amount of *p*-nitrophenol released from pNPGlc.

[c] Released glucose measured as a function of the glucose oxidase-peroxidase enzymatic assay system.

cv. [20], pH 6.0 for *P. vannamei* [30], pH 6.5 for *E. histolytica* [28] and pH 7.0 for *Chaetomium thermophilum* [6]. Indeed, the type I enzymes that can hydrolyze sucrose have an optimum activity in the pH range of 5.5–6.0, such as pH 5.5 for *A. ventricosa* [1], and pH 6.0 for *Bacillus* sp. SAM1606, *B. licheniformis* NCIB 8549 [11,24] and the insect *Q. gigas* [26].

This enzyme from *B. licheniformis* strain TH4-2 reported here was found to be most active at 45 °C (data not shown), which is in accordance with that of most α-glucosidases that have been reported to be in the range of 40–60 °C, such as 45 °C for *E. histolytica* [28] and the snail *A. ventricosa* [1], 55 °C for *G. thermodenitrificans* HRO10 [27], 50–60 °C for *P. miliaceum* L. seeds [19] and 60 °C for *Geobacillus* sp. HTA-462 [21].

3.3.3. Substrate specificity

When the specificity of the purified α-glucosidase on the hydrolysis reaction of a single substrate was evaluated, the highest activity was found with pNPGlc, followed by isomaltose, sucrose, maltose and maltotriose (Table 2). However, no detectable activity was found with *p*-nitrophenyl β-D-glucopyranoside, *o*-nitrophenyl β-D-galactopyranoside, maltoheptaose, cellobiose, lactose, lactulose, melibiose, palatinose, raffinose, starch, amylose and amylopectin as substrates. The hydrolysis activity, of sucrose was twofold higher than that of maltose, respectively. The enzyme was able to cleave longer maltodextrins with α-(1 → 4) glycosidic bonds up to maltohexaose, with a decreasing activity as the maltodextrin chain became longer, and with no detectable activity on maltoheptaose. The fact that the enzyme could hydrolyze sucrose in addition to the α-glucosidic linkages in the appropriate sized maltooligosacharides supports the identification of this enzyme as an α-glucosidase type I, as mentioned before. Among the natural substrates, a high activity towards isomaltose, sucrose and maltose was observed. The specificity of this enzyme was similar to the α-glucosidase from *B. licheniformis* strains NCIB 8549 and NCIB 6346 [24,25], these enzymes can hydrolyze α-(1 → 6) in addition to α-(1 → 4) glucosidic linkage. α-Glucosidase is an exo-acting enzyme that catalyzes the release of glucosyl residues from the non-reducing end of a substrate [9]. Thus, melibiose and raffinose, which are α-linked but with galactose at the non-reducing end, could not be hydrolyzed. Some α-glucosidases are known to also act on glucosidic linkage of trehalose (α-Glc 1 ↔ 1 α-Glc), kojibiose (α-Glc 1 → 2 Glc) and nigerose (α-Glc 1 → 3 Glc) [4,28,31], but these substrates were not evaluated here.

Table 3
Kinetic parameters of the (type I) α-glucosidase enriched from *B. licheniformis* strain TH4-2.

Substrate	K_m (mM)	V_{max} (μmol/min)	V_{max}/K_m (μmol/min)/mM
Hydrolysis reaction			
pNPGlc	0.23	1.3×10^{-3}	5.6×10^{-3}
Sucrose	17.0	9.8×10^{-3}	5.8×10^{-4}
Maltose	11.5	2.4×10^{-3}	2.1×10^{-4}
Transglucosylation reaction			
Melibiose	148	0.072	4.8×10^{-4}

The activities were measured in 20 mM acetate buffer (pH 6.0) at 45 °C, as described in Section 2. Variable ranges of substrates used were: pNPGlc, 0.02–5 mM; sucrose, 2.5–100 mM; maltose 5–400 mM. For the transglucosylation reaction, sucrose was fixed at 150 mM while melibiose was varied from 100 to 500 mM. The reaction time was 180 min.

The apparent size, substrate specificity, pH and temperature optimum of the α-glucosidase from *B. licheniformis* strain TH4-2 reported here is very similar to those enzymes purified from *B. licheniformis* strains NCIB 8549 [24] and NCIB 6346 [25]. A transglucosylation activity was also reported for the enzyme from NCIB 8549, but with isomaltose and maltotriose as substrates [24].

3.4. Kinetic parameters

The K_m, V_{max} and V_{max}/K_m values for the hydrolysis of pNPGlc and sucrose, and for melibiose as an acceptor in the transglucosylation reaction, were determined. The results (Table 3) demonstrate that this α-glucosidase hydrolyzes the aryl-glucoside ten and twenty times more effectively than sucrose and maltose, respectively. In addition to hydrolysis, this enzyme also catalyzes transglucosylation reaction with similar efficiency, as shown in the

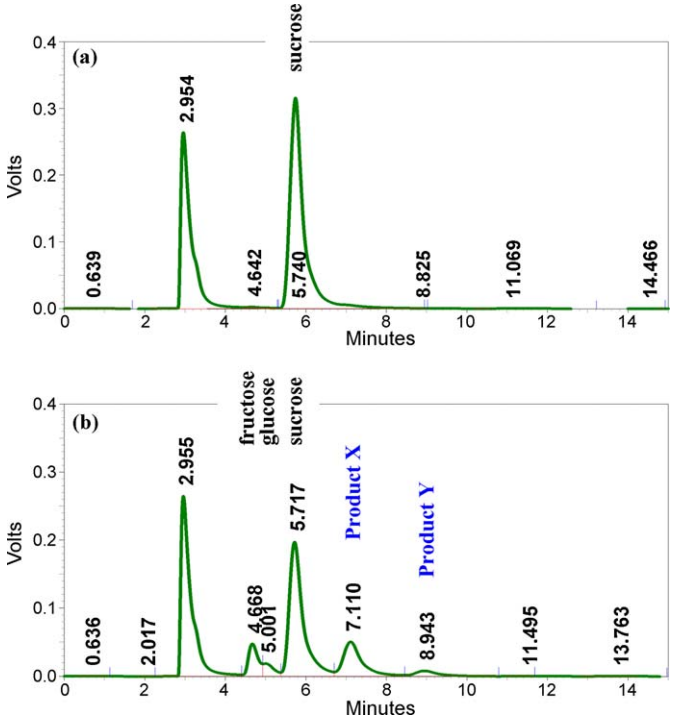

Fig. 4. HPLC chromatogram of the reaction products formed from sucrose after 0 (a) and 24 h (b) of incubation with the purified α-glucosidase from *B. licheniformis* strain TH4-2.

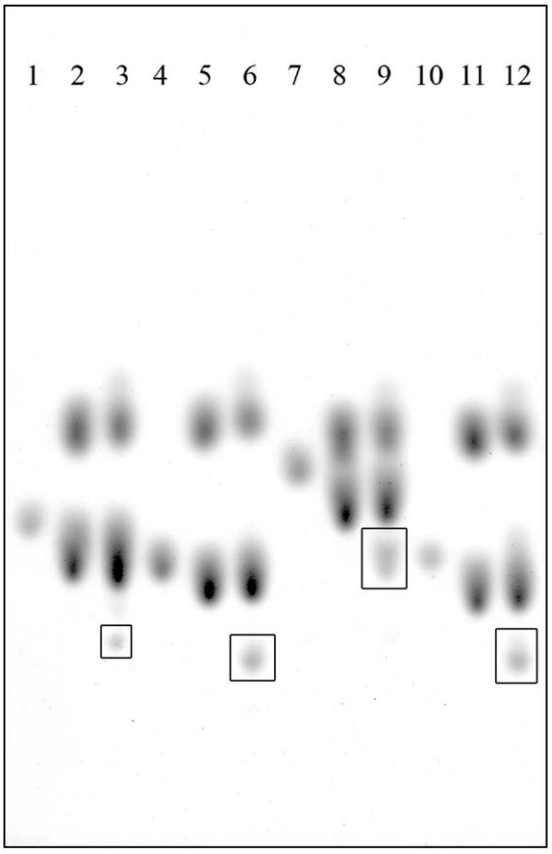

Fig. 5. TLC chromatogram of the reaction products of α-glucosidase incubated with sucrose and various acceptors (lactose, melibiose, cellobiose and raffinose) for 24 h, lane 1: standard lactose (20 μg); lanes 2 and 3: lactose as acceptor, 0 and 24 h; lane 4: standard melibiose (20 μg); lanes 5 and 6: melibiose as acceptor, 0 and 24 h; lane 7: standard cellobiose (20 μg); lanes 8 and 9: cellobiose as acceptor, 0 and 24 h; lane 10: standard raffinose (20 μg); lanes 11 and 12: raffinose as acceptor, 0 and 24 h. Glucosylated products are shown in boxes.

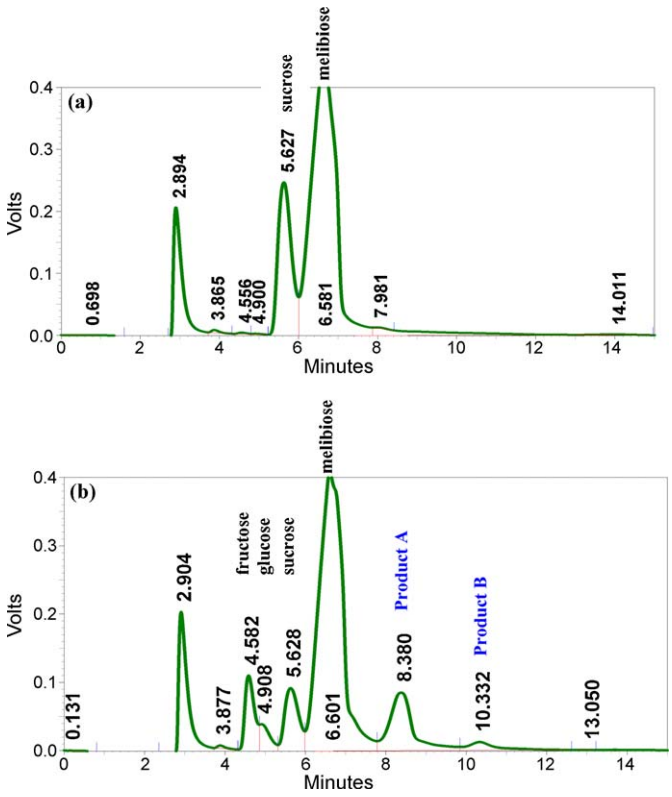

Fig. 6. HPLC chromatogram of the reaction products of the optimized α-glucosidase reaction, with melibiose as the acceptor and sucrose as the donor, after incubation for 0 (a) or 24 h (b).

case of sucrose hydrolysis and transglucosylation from sucrose substrate. The transglucosylation activity of this enzyme is worthwhile to be explored due to the advantage of producing useful OS for food and health benefits.

3.5. Transglucosylation reaction of α-glucosidase

Many α-glucosidases that are primarily known to catalyze the hydrolysis of OS have also been found to catalyze the transglycosylation reaction to yield α-glucosylated transfer products [1,9]. Since the α-glucosidase from *B. licheniformis* strain TH4-2 was found to be able to hydrolyze sucrose very well (Table 2), then sucrose was selected for use as a single substrate or as a glucosyl donor with saccharide acceptors in the transglucosylation reaction of α-glucosidase, especially since sucrose is a cheap, renewable and commonly available resource.

3.5.1. Transglucosylation products with sucrose as a single substrate

After incubating α-glucosidase (0.5 unit/ml, sucrose hydrolysis activity) with 5% (w/v) sucrose in 20 mM sodium acetate buffer pH 6.0 at 45 °C for 24 h, two main products were observed by TLC (X and Y in Fig. 3a) and HPLC (X and Y with Rt of 7.1 and 8.9 min, respectively in Fig. 4). From comparison with the retention times of standard maltooligosaccharides and sucrose, we propose that the unidentified products X and Y were a tri- and tetra-saccharide of the oligoglucosyl fructosides family. A recent report on the transglycosylation activity of a novel α-glucosidase from the digestive juice of the snail, *A. ventricosa*, when using sucrose as single substrate, identified two major polyglucosylfructoside products as α-D-Glc*p*-(1 → 4)-α-D-Glc*p*-(1 → 2)-β-D-Fru*f* and α-D-Glc*p*-(1 → 4)-α-D-Glc*p*-(1 → 4)-α-D-Glc*p*-(1 → 2)-β-D-Fru*f* [1]. However, the products X and Y here could not be hydrolyzed by α-amylase and glucoamylase, which are known to be specific for α-(1 → 4) glucosidic linkage (Fig. 3a), suggesting that the glucosyl residues at the non-reducing end of sucrose were not α-(1 → 4) linked. Thus, the products X and Y here are likely to be different from the products from the α-glucosidase of the snail digestive juice [1]. However, the product X and Y can be hydrolyzed by rat intestinal acetone powder containing

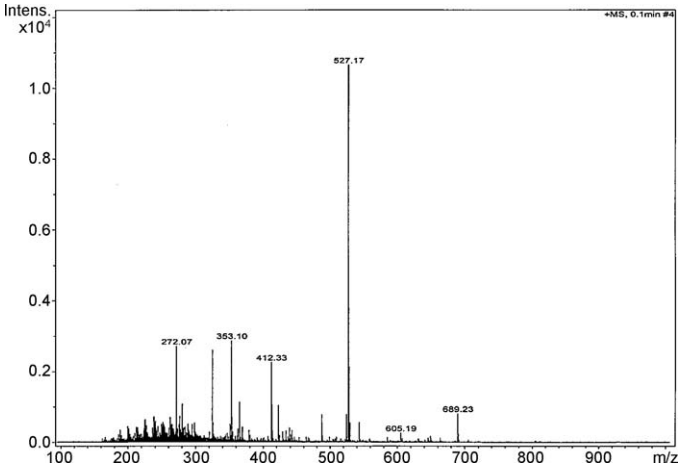

Fig. 7. ESI-TOF mass spectrum of the glucosylated melibiose (product A).

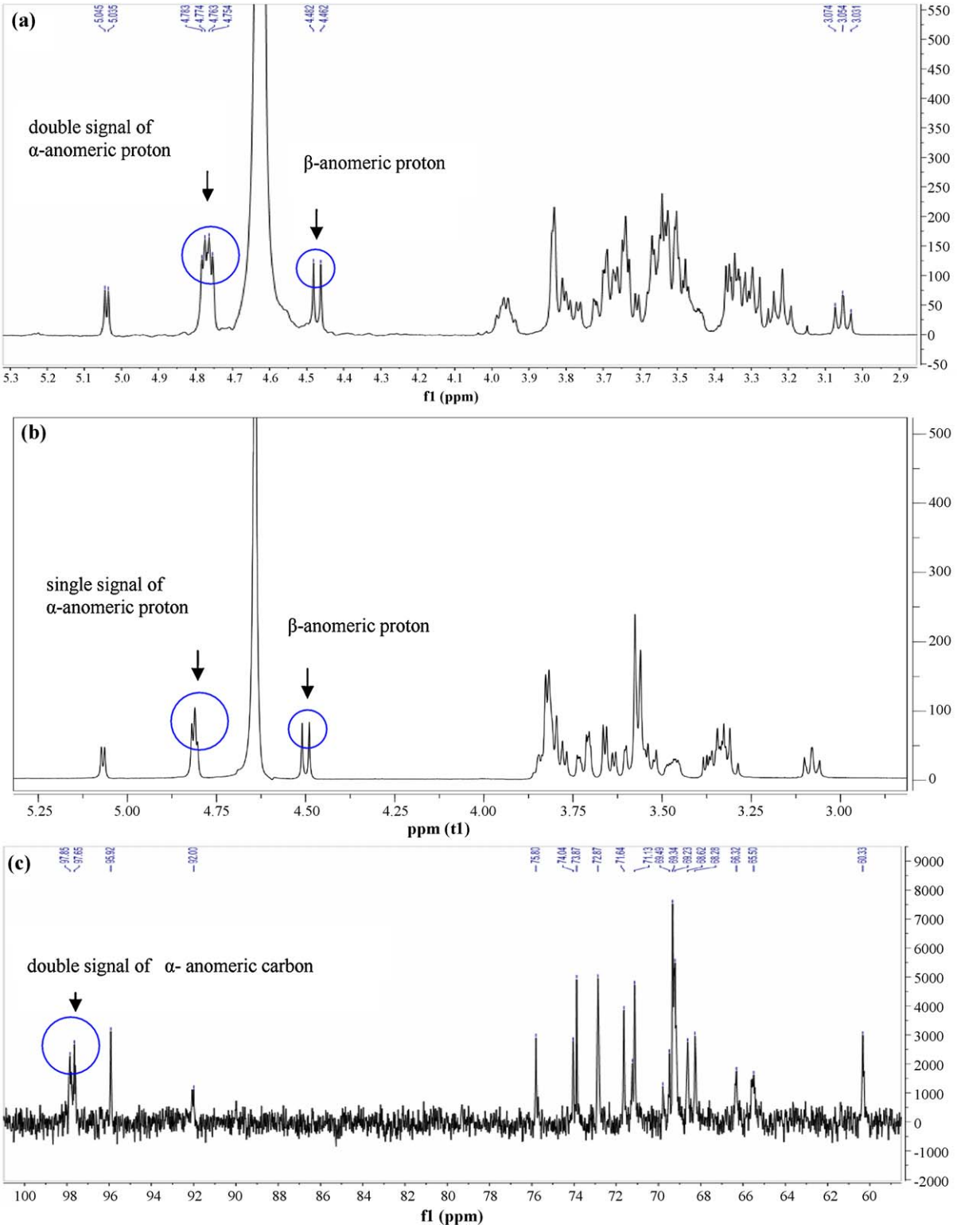

Fig. 8. One dimension NMR spectrum of: (a) ^{1}H-spectrum of product A, (b) ^{1}H-spectrum of standard melibiose and (c) ^{13}C-spectrum of product A.

sucrase–isomaltase and maltase–glucoamylase complexes thus, the enzyme mixture can hydrolyze α-(1 → 2) glycosidic linkage of sucrose and α-(1 → 4)/α-(1 → 6) glucosidic linkages (Fig. 3a). When comparing with previous report, the α-glucosidase from *Bacillus* sp. SAM 1606 can catalvze the transglucosylation of sucrose to produce three regioisomers of the glucosylsucrose, with theanderose (6-O^{G}-glucosylsucrose; α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 2)-β-D-Fru*f*) as main product [32]. It is thus likely that the product X is a trisaccharide theanderose.

3.5.2. *Transglucosylation products with sucrose as the donor and various saccharides as the acceptor*

In this study, the acceptor specificity was determined by using sucrose as the donor and six different di/trisaccharides (lactose,

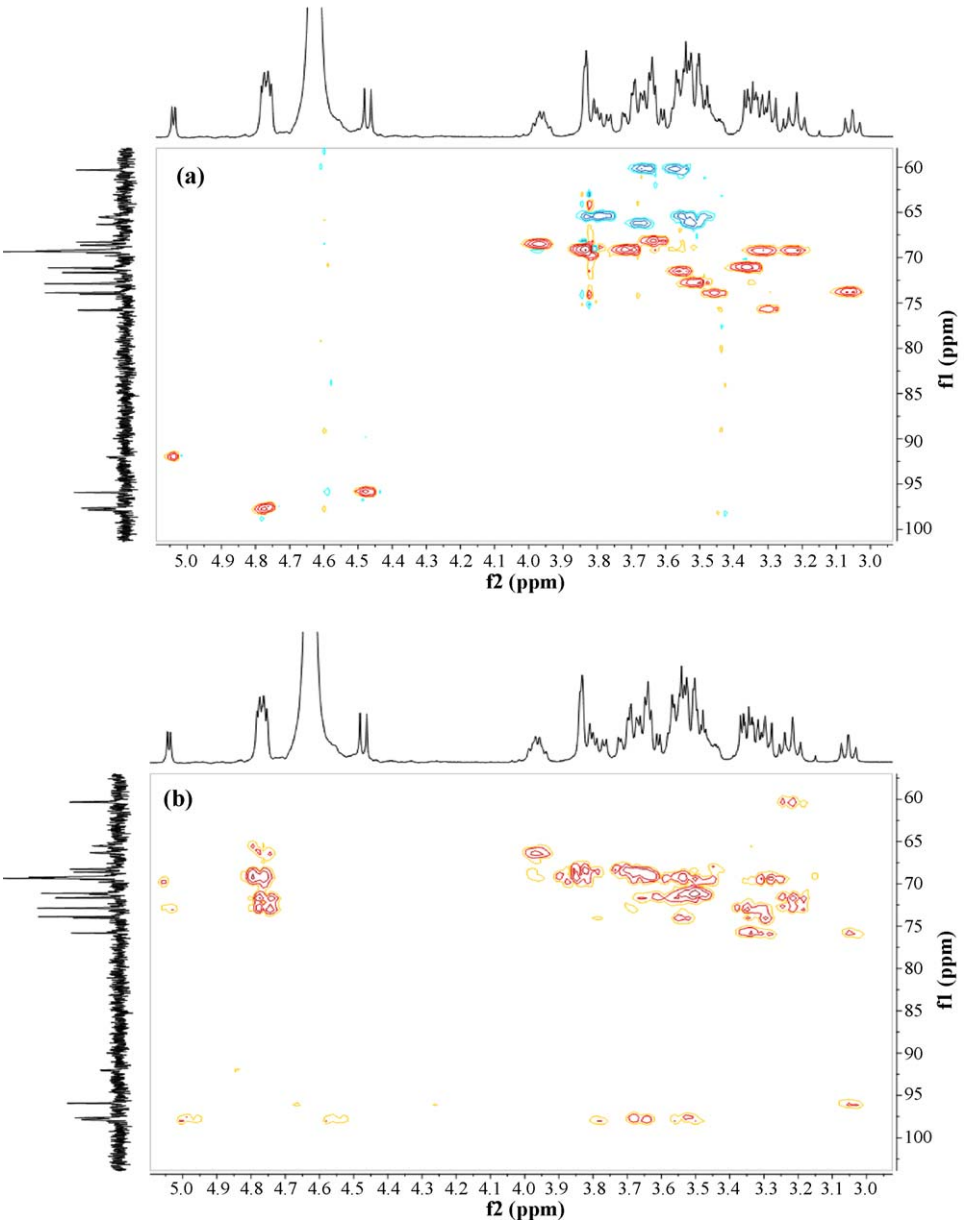

Fig. 9. Two-dimension NMR: (a) HSQC and (b) HMBC spectrum of product A.

melibiose, cellobiose, raffinose, palatinose and lactulose) as the glucosyl acceptor. The reaction products were analyzed by TLC. All saccharides tested could act as an acceptor since a variety of OS products were observed on the TLC plates in each case (Fig. 5). Melibiose was one of the best glucosyl acceptors, as judged from a distinct and resolvable product spot. After optimization, two product spots were clearly observed (A and B in Fig. 3b). The spots expected to be the glucosylated acceptor were observed at a slightly lower position than their acceptor counterparts. In addition, melibiose is an interesting disaccharide because of its α-D-Gal*p*-(1 → 6)-D-Glc structure and, from our preliminary identification, the transfer products obtained were resistant to glucoamylase, which suggests a non-α-(1 → 4) glucosidic linkage. It has previously been reported that using sucrose as the donor and melibiose as the acceptor, α-D-Glc*p*-(1 → 4)-α-D-Gal*p*-(1 → 6)-D-Glc and α-D-Glc*p*-(1 → 3)-α-D-Gal*p*-(1 → 6)-D-Glc were the major transglucosylated products catalyzed by dextransucrase from *Leuconostoc mesenteroides* B-512F and alternansucrase from *L. mesenteroidea* NRRL B-1355, respectively [33]. Although most α-glucosidases are specific for the α-(1 → 4) linkage, those that have specificities to other linkages have been reported. The transglycosylation activity of a novel α-glucosidase from *Xantophyllomyces dendrorhous* using maltose as a single substrate could synthesize prebiotic OS with α-(1 → 2), α-(1 → 4) and α-(1 → 6) linkages, but the major products were 6-*O*-α-glucosyl-maltotriose and 6-*O*-α-isomaltosyl-maltose [10]. On the other hand, the membrane-bound α-glucosidase from the parasite *E. histolytica* was more specific for hydrolysis of the α-1,3 and α-1,2 linkages and was proposed to be involved in glycoprotein processing [28], while the soluble α-1,6-glucosidase is most probably involved in general carbohydrate metabolism [34]. However, these parasite enzymes have not been evaluated for their transglycosylation activities.

In order to produce a higher OS yield, the synthesis conditions were optimized. The optimum condition for OS production was found to be 15% (w/v) melibiose, 5% (w/v) sucrose and an enzyme concentration of 5 U/ml at pH 6.0, with the reaction proceeding at

45 °C for 24 h. Under these conditions, two products were observed in both the TLC and HPLC profiles (A and B in Figs. 3b and 6). From the HPLC profile, the main product (A at a Rt of 8.3 min) and the minor product (B at a Rt of 10.3 min) were obtained with yields relative to the melibiose acceptor of 17.2% and 3.3%, respectively. When the yields relative to sucrose donor were calculated similarly, the values were 58.5% and 11.3% for products A and B, respectively. It was observed that at a low concentration of melibiose, transfer products from sucrose (X and Y in Fig. 4) were formed at detectable levels, but as the melibiose concentration was increased to ≥ 12.5% (w/v), X and Y disappeared while A and B were significantly increased.

3.6. Isolation and characterization of transglucosylation products

The glucosyl melibiose products were isolated from the reaction mixture using Sephadex LH-20 column chromatography and eluted with 70% (v/v) aqueous n-propanol. Before loading, the reaction mixture was treated with invertase (20 unit/ml) at 37 °C for 3 h to hydrolyze any residual sucrose. The fractions were followed by Somogyi–Nelson's method and the sugars in the fraction were identified by HPLC. From this column, the major product (A) could be well separated from melibiose, glucose and fructose.

3.6.1. Mass spectrometry (MS)

The molecular weight of the main transfer product (A with a Rt of 8.3 min) was estimated to be 504 Daltons by ESI-TOF mass spectrometry with [M+Na]+ at *m*/*z* of 527 (Fig. 7). This corresponds to the size of a trisaccharide.

3.6.2. Nuclear magnetic resonance (NMR)

The structure of product A was determined by ^{1}H NMR, ^{13}C NMR, HSQC and HMBC analysis. In the ^{1}H NMR spectrum, the characteristic signal for the α-anomeric proton was split into two overlapping peaks at δ = 4.78 ppm (J = 3.6 Hz) and δ = 4.75 ppm (J = 3.6 Hz) (Fig. 8a). The coupling constant (J = 3.6 Hz) suggested that the glucosyl residue was joined to melibiose (α-D-Gal*p*-(1 → 6)-D-Glc) by an α-linkage. The ^{13}C NMR spectrum also showed a double signal at the chemical shift of α-anomeric carbon (Fig. 8c). The transferred glucose moiety is attached to the galactose residue at the non-reducing end of melibiose, as suggested by the retained characteristic signal for the β-anomeric proton at δ = 4.47 ppm of product A compared to melibiose (Fig. 8a and b), and with no coupling effect observed. This is expected since, in most OS synthesized by transglycosylation reactions, the moieties of the monosaccharides from the donor substrate are transferred to the non-reducing end of the saccharide acceptor [1,35–37].

Because the ^{1}H NMR and ^{13}CNMR of natural saccharides usually suffer from heavy overlaps of proton and carbon resonances, due to an inherent low dispersion of their chemical shifts [9], two-dimensional NMR was also performed in this study. The ^{13}C ^{1}H HSQC spectrum (Fig. 9a) indicates three methylene carbons at δ_c = 60.4, 65.5 and 66.3 ppm, while the ^{13}C ^{1}H HMBC spectrum displayed a characteristic correlation between the anomeric proton at the H1 position (δ = 4.78 ppm and δ = 4.75 ppm) and the methylene carbon (CH_2) at the C6 position (δ = 66.3 ppm and δ = 65.5 ppm), respectively (Fig. 9b), suggesting the glucosyl residue was attached to melibiose acceptor by a (1 → 6) linkage. From all the NMR results, the structure of product A was then identified as α-D-glucopyranosyl-(1 → 6)-α-D-galactopyranosyl-(1 → 6)-D-glucopyranose. This trisaccharide has never been reported before. The potential prebiotic nature of this novel trisaccharide is suggested by its resistance to hydrolysis by rat intestinal acetone powder (Fig. 3b, lane 4).

4. Conclusions

A type I α-glucosidase that hydrolyzes pNPGlc, isomaltose and sucrose more efficiently than maltose was purified from the stationary phase culture supernatant of *B. licheniformis* strain TH4-2. The transglucosylation activity was investigated using sucrose as a single substrate and also as a glucosyl donor with melibiose as the acceptor. The major products formed in both cases were a trisaccharide and a tetrasaccharide. Structural elucidation from NMR of the main product from sucrose and melibiose substrates revealed a novel trisaccharide of the structure α-D-Glc*p*-(1 → 6)-α-D-Gal*p*-(1 → 6)-D-Glc. This trisaccharide product potentially shows a prebiotic property.

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