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Enzymatic properties and transglycosylation of α-galactosidase from *Penicillium oxalicum* SO

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ABSTRACT

Penicillium oxalicum SO α-galactosidase demonstrated weak hydrolysing activity but a high rate of transglycosylation in the reaction with melibiose, where the major product was 6-α-galactosyl melibiose. The transfer ratio was 83.6% and was maintained over a long reaction time of 80 h. The molecular weight was estimated to be 124,000 by SDS–PAGE. The optimal pH was ~3 and a stable pH, with a range of 2.4–9.5, was found. The optimal temperature was ~60 °C and the activity was stable below 60 °C. With respect to acceptor specificity, mono-alcohols, sugar alcohols and sugars were poor acceptors, but the di-alcohol ethylene glycol and the tri-alcohol glycerin were good acceptors. The percentage of transglycosylation to glycerin increased up to 41.7%, as that to melibiose decreased, with the initial glycerin concentration of 40%. The production of α-D-galactosylglycerol was 293 mg for each gram of melibiose used by the enzymatic reaction.

1. Introduction

α-Galactosidase (EC 3.2.1.22) is widely distributed in microorganisms and plants, and its enzymatic properties have been reported (Dey & Campillo, 1984). α-Galactosidase hydrolyses the α-1,6-linkage of galactosyl residues on saccharides, such as melibiose and raffinose. The non-reducing end of the galactosyl residue is recognised and released in the active site of the enzyme. On application of the enzyme, improvements in the rheological properties of guar gum (*Cyamopsis tetragonolobus*), used as a natural food additive, have been noted. Guar galactomannan consists of a β-1,4-linked D-mannose backbone and α-1,6-linked D-galactose side chains (galactose 38%, mannose 62%). Some α-galactosidases are able to partially release galactosyl residues from guar gum, improving its rheological properties such that it behaves similar to that of the more expensive locust bean gum (*Ceratonia siliqua*) (Bupin, Gidley, & Jeffcoat, 1990; McCleary, Amado, Waibel, & Neukom, 1981). In the production of beet sugar, α-galactosidase is used to release galactosyl residues from raffinose, which prevents sucrose crystallisation during the industrial process (Yamane, 1971).

Some α-galactosidases have a transglycosylation action, hence syntheses of new products, with α-1,6 linkages, have been studied. These products are expected to have medical applications since the galactosyl residues of biomolecules often have important roles in organisms (Arya et al., 1999; Coetzee et al., 1996; Poulos & Beckman, 1978). For example, Fabry's disease is caused by a lack of α-galactosidase in the body, leading to a buildup of trihexosylceramide with terminal α-galactosyl residue in the blood vessels, impairing their ability to function. Trihexosylceramide is also a cell-surface ligand of verotoxin that is produced by *Escherichia coli* (VTEC). Hence, the physiological function of the α-galactosyl residue is a significant target in the medical field.

Transglycosylation has been performed using either melibiose or *p*NP-α-D-galactopyranoside as the donor, resulting in the synthesis of various oligosaccharides and alkyl galactosides (Hashimoto, Katayama, Goto, Okinaga, & Kitahata, 1995; Hinz et al., 2005; Spangenberg et al., 2000; Van Laere et al., 1999). In particular, the enzyme from *Candida guilliermondrii* has demonstrated broad acceptor specificity to monosaccharides, disaccharides and alcohols (Hashimoto et al., 1995). In gene technology, the development of recombinant enzymes, to improve the transglycosylation activity, has been studied (Mi et al., 2007).

A guar gum-hydrolysing strain, *Penicillium oxalicum* SO, was separated from soil using a medium plate with 0.5% guar gum and 1.5% agar. This strain produced mainly β-mannanase with high hydrolysing activity that rapidly hydrolysed both guar gum and locust bean gum (Kurakake, Sumida, Masuda, Oonishi, & Komaki, 2006). It was found that α-galactosidases of the minor product had low hydrolysing activity, but showed high transglycosylation in the reaction with melibiose. In the present study, we investigated the enzymatic properties of α-galactosidase, such as acceptor specificity, in the transglycosylation.

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2. Materials and methods

2.1. Materials

Guar gum and locust bean gum were purchased from Sigma–Aldrich (St. Louis, MO, USA). The sugar compositions were 38% galactose/62% mannose and 23% galactose/77% mannose, respectively. Melibiose, raffinose and stachyose were purchased from Wako Pure Chemical Industries Limited (Osaka, Japan). All other chemicals were of reagent grade.

2.2. Production and purification of enzymes

P. oxalicum SO was cultivated in liquid medium containing 1% guar gum, 0.5% yeast extract and 0.2% $Na_2HPO_412H_2O$ (100 ml) in a 500 ml flask at 150 rpm and 30 °C for 4 days. The spores of the SO strain were inoculated. The inoculum was grown in a plate medium (0.5% guar gum and 1.5% agar). The supernatant of the culture broth, after centrifugation at 1500*g* for 10 min, was used to determine enzyme activity. The sediment strain was washed with distilled water, after centrifugation, and homogenised. Enzyme activity of the strain suspension was determined as either intracellular or binding enzyme.

The culture filtrate (360 ml) was desalted by saturation in 80% ammonium sulphate. After filtration, the precipitate was dissolved in 100 mM sodium acetate buffer (pH 5). Cold acetone was added to the enzyme solution (3 × volume). After incubation at −20 °C for 30 min, precipitated proteins were separated by centrifugation at 1500*g* for 10 min. The precipitate was dissolved in 100 mM sodium acetate buffer (pH 5). The crude α-galactosidase was purified by the following chromatography step. The crude enzyme solution (0.5 ml) was subjected to gel filtration on preparative high-performance liquid chromatography (HPLC), with a 60 × 2.15 cm i.d. TSKgel G3000SW column (Tosoh Limited, Tokyo, Japan.), pre-equilibrated with 50 mM phosphate buffer (pH 6.8) containing 0.3 M NaCl. Proteins were eluted at a flow rate of 1.6 ml/min and fractions were collected at 1 min intervals. Proteins were detected by their absorbance at 280 nm in a UV detector (SPD-7A, Shimadzu Co. Limited, Kyoto, Japan). Each purification step was carried out at 4 °C.

2.3. Determination of enzyme activity

p-Nitrophenyl α-D-galactopyranoside (1 mM) was incubated with an enzyme sample in 0.1 M acetate buffer (pH 5) at 40 °C for 10 min (working volume of 1 ml). The reaction was stopped by adding 0.5 ml of 1 M Na_2CO_3 and the absorbance of the released *p*-nitrophenol at 400 nm determined. One unit was defined as the amount of enzyme that could produce 1 μmol *p*-nitrophenol/min.

2.4. Enzymatic reaction for melibiose

One gram of melibiose was dissolved with 0.1 M acetate buffer (pH 5) and an enzyme solution (2 ml) added. The mixture (8.3% melibiose and 0.027 U/ml enzyme) was incubated at pH 5 and 40 °C for 24–80 h. The aliquot was sampled at the given time and the enzyme deactivated by heating in boiling water for 5 min. After centrifugation at 1500*g* for 10 min, the supernatant was filtered using a 0.22-μm membrane filter and analysed by HPLC. The transfer ratio [(Glc − Gal)/Glc × 100(%)] was calculated from the concentrations of galactose (Gal) and glucose (Glc).

2.5. Acceptor specificity

Twenty percent of melibiose (40 μl), 20% of acceptor (40 μl) and 0.16 U/ml of enzyme (20 μl) were mixed, giving final concentrations of 8% melibiose, 8% acceptor and 0.032 U/ml enzyme, and incubated at pH 5 and 40 °C for 24 h. The reaction mixture was deactivated by boiling and the resulting sugars were analysed as described above. The acceptors used were alcohols (methanol, ethanol, 1-propanol, 2-propanol, *n*-butanol, 3-methyl-1-butanol, ethylene glycol (1,2-ethanediol), and glycerin), sugar alcohols (erythritol, xylitol, myo-inositol, sorbitol, mannitol, and lactitol), sugars (L-arabinose, D-xylose, D-ribose, D-glucose, D-galactose, D-mannose, D-tagatose, D-fructose, L-sorbose, *N*-acetylglucosamine, palatinose, trehalose, isomalto-oligosaccharides, and α-cyclodextrin) and other agents (hesperidin, L-ascorbic acid, L-tyrosine, and L-serine). The transglycosylation to the acceptor was evaluated by the peak area of the transferred product shown on the HPLC chromatogram, whereby ++++ denoted >150,000 (μVs), +++ denoted 120,000–150,000, ++ denoted 50,000–120,000, + denoted <50,000, and – denoted no peak.

2.6. HPLC analysis of sugars

The sugars formed by the enzymatic reaction were analysed by HPLC under the following conditions (Kurakake et al., 2006): (1) column, 250 × 7 mm i.d. GL-C610 (Hitachi Kasei Limited, Tokyo, Japan); mobile phase, water; column temperature, 60 °C; flow rate, 1.0 ml/min; and detector, L-3300 differential refractive index monitor (Hitachi High-Technologies Limited, Tokyo, Japan); or (2) column, 250 × 4.6 mm i.d. NH2P-50 (Asahi Kasei Co. Limited, Tokyo, Japan); mobile phase, water:acetonitrile = 25:75; column temperature, 25 °C; and detector, RI 8020 differential refractive index monitor (Tosoh Limited, Tokyo. Japan).

2.7. Identification of transfer product

Transfer products were separated by HPLC (column, 250 × 10 mm i.d. NH2P-50; mobile phase, water:acetonitrile = 20:80 or 25:75) and identified by ^{13}C NMR analysis. ^{13}C NMR spectra were taken on a JEOL JMN-LA 500 (500 MHz for ^{1}H and 125 MHz for ^{13}C) spectrometer (JEOL Limited, Tokyo, Japan). Each sample (1%) was dissolved in deuterium oxide and the chemical shifts were calibrated using the signal acetone as an internal standard.

3. Results and discussion

3.1. Purification and properties of SO α-galactosidase

When used in a liquid culture in a 500-ml flask for 4 days, *P. oxalicum* SO produced a maximum of 0.038 U/ml α-galactosidase. The enzyme was either intracellular or was bound to the cell wall at an early period, but was released from the cell during culture; 40% of the enzyme was released in 4 days (0.015 U/ml; supernatant). When *p*NP-galactopyranoside was used as the substrate, the activity of α-galactosidase was very low. When melibiose was used as the substrate; however, the enzyme demonstrated high transglycosylation.

A crude enzyme solution was prepared by desalting in a saturated 80% ammonium sulphate solution, followed by precipitation in cold acetone and gel filtration on preparative HPLC with a TSKgel G3000SW column (data not shown). Eluted proteins were detected by their absorbance at 280 nm. The first small peak corresponded to α-galactosidase activity. On SDS–PAGE, only one band was detected and the molecular weight was estimated to be 124,000 (Fig. 1). Fig. 2 shows the effects of pH and temperature on the activity of purified α-galactosidase. The optimal pH, at 40 °C, was found to be approximately 3 and the enzyme was stable over a wide pH range (2.4–9.5), as shown in Fig. 2A. The optimal

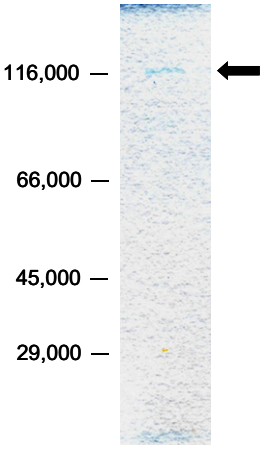

Fig. 1. Analysis of SO α-galactosidase on SDS–PAGE.

temperature was approximately 60 °C at pH 5, and the activity was stable at temperatures of up to 60 °C (Fig. 2B). There are few studies of α-galactosidase from *Penicillium*. The molecular weight and enzymatic properties of *Penicillium purpurogenum* and *simplicissimum* enzymes were reported to be different from those of *P. oxalicum* SO enzyme (Luonteri, Alatalo, Siika-Aho, Penttila, & Tenkanen, 1998; Shibuya et al., 1995).

Substrate specificity is shown in Table 1, with the enzymatic activity presented as a value relative to melibiose. The enzymes ability to recognise an α-1,6 linkage was shown by the lack of hydrolysis of both *p*NP-β-D-galactopyranoside and lactose. Enzyme activity towards melibiose was much higher than that with *p*NP-α-D-galactopyranoside, and the relative activity increased with molecular size (raffinose and stachyose). This specificity differs from that of the enzymes from *Bacillus stearothermophilus*, *Trichoderma reesei*, and *Aspergillus fumigatus*, which have high activity to *p*NP-α-D-galactopyranoside (Puchart & Biely, 2005; Shabalin, Kulminskaya, Savel'ev, Shishlyannikov, & Neustroev, 2002; Spangenberg et al., 2000). The SO enzyme has no activity with the α-1,6 galactosyl side chains in the galactomannan of either guar gum or locust bean gum.

3.2. Transgalactosylation of SO α-galactosidase

Fig. 3 shows the chromatogram of the reaction mixture in 8.3% melibiose and 0.027 U/ml α-galactosidase. In a 24-h reaction, glucose and galactose were released from melibiose; however, the galactose peak was very small. Many galactosyl residues were transferred to melibiose, forming trisaccharides as transfer products. Nuclear magnetic resonance analysis (NMR) showed that the trisaccharide resulted from the formation of an α-1,6 linkage between the galactosyl residue and the C6 position of the galactosyl residue of the melibiose molecule. This self-transglycosylation to melibiose has also been shown by the α-galactosidases of *C. guilliermondrii*, *Bifidobacterium adolescentis*, and *Lactobacillus reuteri* (Hashimoto et al., 1995; Tzortzis, Jay, Baillon, Gibson, & Rastall, 2003; Van Laere et al., 1999). As the reaction progressed, a small amount of tetrasaccharide was formed by transglycosylation to the trisaccharide.

Table 1
Substrate specificity of SO α-galactosidase.

Substrate	Relative activity (%)
Methyl-α-D-galactopyranoside	0
*p*NP-α-D-galactopyranoside	18
*p*NP-β-D-galactopyranoside	0
Melibiose	100
Lactose	0
Raffinose	105
Stachyose	112
Guar gum	0
Locust bean gum	0

Table 2 shows the sugar composition over a long reaction time (33, 56, and 80 h). In the 80-h reaction, the transglycosylation reached an equilibrium with an oligosaccharide composition of 46.8% (34.4% trisaccharide and 12.4% tetrasaccharide). The small decrease in the transfer ratio, in comparison with that at 33 h, is attributable to the hydrolysis of the tetrasaccharide to galactose and trisaccharide. The transfer ratio was maintained at a high level of 83.6% at 80 h. As has been reported for α-galactosidases from other origins, the hydrolysis reaction of oligosaccharides proceeds gradually, therefore, a long reaction time will not necessarily increase the transfer ratio (Hashimoto et al., 1995; Van Laere et al., 1999). SO α-galactosidase has the unique characteristic of low hydrolysing activity.

The relationship between transglycosylation and the melibiose concentration was investigated. Fig. 4 shows the transglycosylation reaction where the melibiose concentration ranged from 0.4% to 8%. The concentration of glucose released increased with substrate concentration. The concentration of galactose released, however, was relatively constant. The transfer ratio showed that transglycosylation was efficiently enhanced at >1% melibiose. In general, transglycosylation by glycosidase (hydrolase) can be carried out with a high substrate concentration, for example a concentration of 10%. The SO enzyme also has high transferring activity at relatively low substrate concentrations. It would be expected, therefore, that a high production of α-1,6-linked oligosaccharides would occur during transglycosylation.

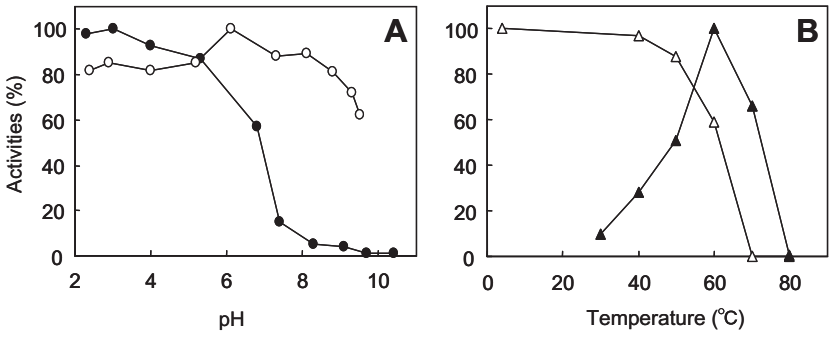

Fig. 2. Effects of pH and temperature on SO α-galactosidase activity. A and B shows effects of pH and temperature, respectively. Symbols; A: optimal pH (●), stable pH (○), and B: optimal temperature (▲), stable temperature (△).

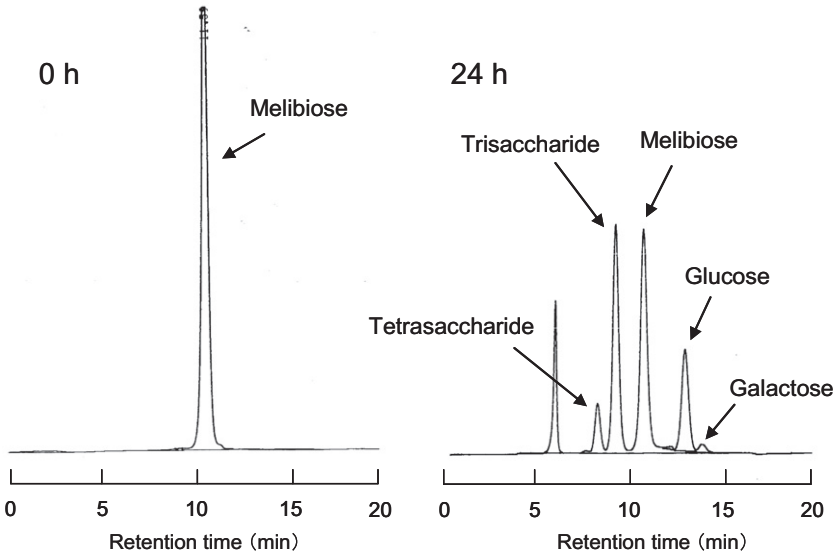

Fig. 3. HPLC charts of reaction mixture in transglycosylation by SO α-galactosidase. Melibiose (8.3%) and α-galactosidase (0.027 U/ml) were incubated at pH 5 and 40 °C for 24 h.

Table 2
Transgalactosylation with melibiose by SO α-galactosidase.

Time (h)	Sugar composition (%)					Transfer ratio (%)	Hydrolysis ratio (%)
	Glucose	Galactose	Melibiose	Trisaccharide	Tetrasaccharide		
33	17.2	2.3	37.3	36.4	6.8	86.5	13.5
56	20.5	3.3	29.5	36.5	10.2	83.8	16.2
80	22.9	3.7	26.6	34.4	12.4	83.6	16.4

Melibiose (8.3%) and α-galactosidase (0.027 U/ml) were incubated at pH 5 and 40 °C.

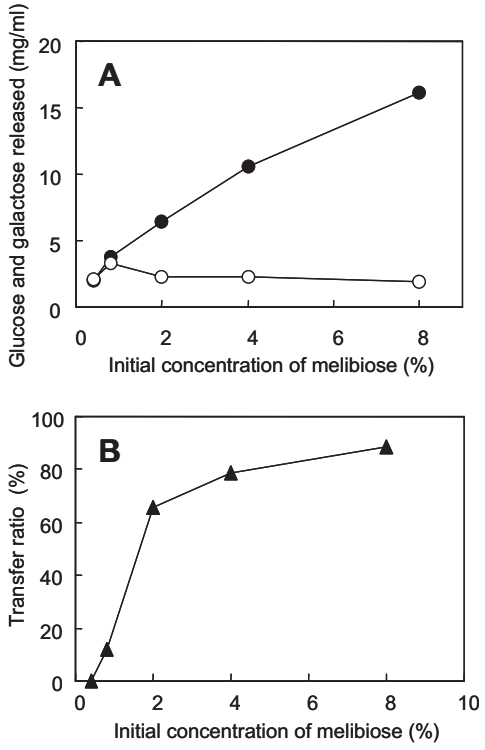

Fig. 4. Effect of melibiose concentration on transglycosylation reaction of SO α-galactosidase Melibiose (0–8%) and α-galactosidase (0.027 U/ml) were incubated at pH 5 and 40 °C for 24 h. Symbols; A: glucose (●), galactose (○), and B: transfer ratio (▲).

3.3. Acceptor specificity in the transgalactosylation of SO α-galactosidase

The acceptor specificity of the transglycosylation reaction of SO α-galactosidase was investigated using melibiose as a substrate (Table 3). No transgalactosylation was observed when mono-alcohols were used as acceptors. However, di- and tri-alcohols, such as ethylene glycol and glycerin, demonstrated good transgalactosylation activity. Enzymes from *Aspergillus niger*, *Candida guilliermondii*, and *Taramyces flavus* efficiently transferred galactosyl residues to mono-alcohols, producing alkyl galactopyranosides (Hashimoto et al., 1995; Simerska et al., 2006; Tzortzis et al., 2003). The high degree of transglycosylation to both the di-alcohol ethylene glycol and the tri-alcohol glycerin is a unique characteristic of SO α-galactosidase.

For sugars and sugar alcohols, acceptor specificity was very low. The transfer product peaks for xylitol, mannose, and tagatose were clearly detectable; however, the peak size was very small. Mannose and tagatose are structurally similar; the only difference being the position of the hydroxymethylene group (C6 for mannose and C1 for tagatose). As is the case in ethylene glycol (C2) and glycerin (C3), the sugar alcohols have a hydroxyl group bound to each carbon atom. The low acceptor specificity can therefore be attributed to the increase in molecular size (>C3), since the smallest sugar alcohol, erythritol (C4), did not demonstrate any acceptor activity. This research shows that the acceptor specificity of SO α-galactosidase, from *P. oxalicum*, is in contrast to that of α-galactosidase extracted from other enzymes. For example, it has been reported that *B. adolescentis* and *A. fumigatus* enzymes have acceptor specificity to oligosaccharides such as malto-oligosaccharides

Table 3
Acceptor specificity of transgalactosylation by SO α-galactosidase.

Acceptors	Transfer product	Acceptors	Transfer product
Alcohols		*Sugars*	
Methanol	–	L-Arabinose	+
Ethanol	–	D-Xylose	–
1-Propanol	–	D-Ribose	–
2-Propanol	–	D-Glucose	–
n-Butanol	–	D-Galactose	–
3-Methyl-1-butanol	–	D-Mannose	++
Ethylene glycol	++++	D-Tagatose	++
Glycerin	++++	D-Fructose	–
Sugar alcohols		L-Sorbose	–
Erythritol	+	*N*-Acetylglucosamine	–
Xylitol	++	Palatinose	–
myo-Inositol	–	Trehalose	–
Solbitol	–	Isomalto-oligosaccharides	–
Mannitol	+	α-Cyclodextrin	–
Lactitol	–	*Others*	
		Hesperidin	+
		L-Ascrorubic acid	–
		L-Tyrosine	–
		L-Serine	–

Melibiose (8%), acceptor (8%) and α-galactosidase (0.032 U/ml) were incubated at pH 5 and 40 °C for 24 h.

(Hinz et al., 2005; Puchart & Biely, 2005). Furthermore, α-galactosidase from *C. guilliermondii* has broad specificity and efficiently transfers galactosyl residues to monosaccharides such as glucose and galactose (Hashimoto et al., 1995). Other substances, such as amino acids and L-ascorbic acid, were also incubated with SO α-galactosidase from *P. oxalicum*, but were not found to act as acceptors.

SO α-galactosidase demonstrated high acceptor specificity to ethylene glycol and glycerin. The conditions for the transglycosylation reaction to glycerin were investigated as it is expected that this reaction would lead to the synthesis of a new glycolipid, as a result of esterification between the transfer product (α-D-galactosylglycerol) and fatty acids.

Fig. 5 shows the relationship between the degree of transglycosylation to glycerin and the initial concentration of glycerin. Although the absolute transfer ratio from melibiose was reduced from 91% to 79%, with an increase in the initial concentration of

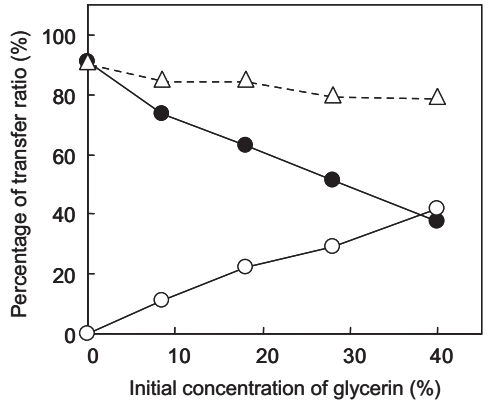

Fig. 5. Relationship between transglycosylation to glycerin and the initial concentration of glycerin. Melibiose (8%), glycerin (0–40%) and α-galactosidase (0.032 U/ml) were incubated at pH 5 and 40 °C for 24 h. Symbols; transfer ratio (△) and transglycosylation to melibiose (●) and to glycerin (○).

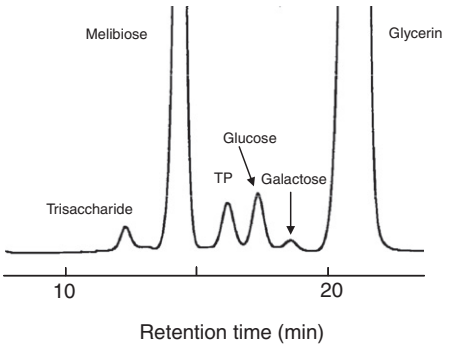

Fig. 6. HPLC analysis with GL C610 column of reaction mixture in transglycosylation to glycerin. Melibiose (8%), glycerin (40%) and α-galactosidase (0.027 U/ml) were incubated at pH 5 and 40 °C for 24 h. TP: transfer product.

glycerin from 0% to 40%, the transglycosylation to glycerin increased linearly as the rate of self-transglycosylation of melibiose decreased. Fig. 6 shows the chromatogram of the reaction mixture in 40% glycerin. The transfer product produced a high peak between those of melibiose and glucose, whereby the transfer ratio to glycerin was 41.7%.

In the ^{13}C NMR spectrum of the transfer product, signals of α-galactopyranoside were detected (C1 99.8, 99.4, 99.1, C2 69.5, C3 70.4, C4 70.2, C5 72.0, and C6 62.2 ppm) and the C1 and C2 signals of glycerin (C1 63.5, C2 73.0, and C3 63.5 ppm) were shifted to 72.1 and 79.7 ppm, respectively. Therefore, the transfer product, formed by the interaction of the OS enzyme, was identified with 1-O-α-D-galactosylglycerol and 2-O-α-D-galactosylglycerol. Although both β-D-galactosylglycerol and α-D-glucosylglycerol are well known transfer products of transglycosylation (Irazoqui et al., 2009; Nakano, Takenishi, & Watanabe, 1988; Takenaka & Uchiyama, 2000), very little is known of the product α-D-galactosylglycerol. This product may prove to be of great interest for the synthesis of glycolipids that have physiological activity.

The production of α-D-galactosylglycerol, in the reaction with 40% glycerin, was 293 mg in 24 h per 1 g melibiose used by the enzymatic reaction. The rate of production could be improved by altering the reaction time, the enzyme activity, and the concentration of glycerin. The synthesis of sugar lipids can be carried out readily, by the reverse reaction with fatty acids using lipase.

In conclusion, α-galactosidase from *P. oxalicum* SO efficiently transferred galactosyl residues to the melibiose substrate, despite a low hydrolysing activity of *p*NP-α-galactopyranoside. With respect to its acceptor specificity, SO α-galactosidase showed little transglycosylation to mono-alcohols, sugar alcohols, and sugars; however, the di-alcohol ethylene glycol and the tri-alcohol glycerin were shown to be good acceptors for the transglycosylation reaction.

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