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Purification and biochemical properties of a galactooligosaccharide producing β-galactosidase from *Bullera singularis*

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Abstract

A β-galactosidase, catalyzing lactose hydrolysis and galactooligosaccharide (GalOS) synthesis from lactose, was extracted from the yeast, *Bullera singularis* KCTC 7534. The crude enzyme had a high transgalactosylation activity resulting in the oligosaccharide conversion of over 34% using pure lactose and cheese whey permeate as substrates. The enzyme was purified by two chromatographic steps giving 96-fold purification with a yield of 16%. The molecular weight of the purified enzyme (specific activity of 56 U mg^{-1}) was approx. 53 000 Da. The hydrolytic activity was the highest at pH 5 and 50 °C, and was stable to 45 °C for 2 h. Enzyme activity was inhibited by 10 mM Ag^{3+} and 10 mM SDS. The K_m for lactose hydrolysis was 0.58 M and the maximum reaction velocity (V_{max}) was 4 mM min^{-1}. GalOS, including tri- and tetra-saccharides were produced with a conversion yield of 50%, corresponding to 90 g GalOS l^{-1} from 180 g lactose l^{-1} by the purified enzyme.

Introduction

β-Galactosidases (EC 3.2.1.23) are present in a wide variety of sources including plants, animals, and microorganisms (Wallenfels & Weil 1972, Prenosil *et al.* 1987), and are known to catalyze transgalactosylation as well as hydrolysis reactions to produce galactooligosaccharides (GalOS) (Prenosil *et al.* 1987). The hydrolysis of lactose occurs predominantly at low lactose concentrations, while oligosaccharide production by the transgalactosidase reaction increases with increasing concentrations of lactose.

β-Galactosidases have been used in the dairy industry for the functional improvement of milk and cheese whey (Gekas & Lepz-Leiva 1985). GalOS can promote the growth of the intestinal bacterial flora of which bifidobacteria in the large intestine help to maintain human health by preventing of diarrhea and constipation, protecting of liver functions, reducing of serum cholesterol, and producing anticancer effects (Ohtsuka *et al.* 1989, Hughes & Hoover 1991). The enzymatic synthesis of GalOS from lactose and cheese whey using various crude and purified β-galactosidases has been reported by many investigators (Burvall *et al.* 1979, Toba & Adachi 1978, Toba *et al.* 1985, Mozaffer *et al.* 1984). Shin & Yang (1998) reported the culture conditions of *B. singularis* and reaction conditions for partially purified β-galactosidase giving the optimum production of GalOS. In the current study, we describe the purification and properties of the β-galactosidase from *Bullera singularis* and the increased yield of GalOS production obtained using purified *Bullera* β-galactosidase.

Materials and methods

Materials

The two FPLC columns (Hiprep 16/10 DEAE FF and Hiprep 16/10 Q FF) were purchased from Pharmacia. Molecular weight markers for gel electrophoresis

were obtained from Biorad. Lactose and other carbohydrates were from Sigma. All other chemicals were of analytical grade. *Bullera singularis* KCTC 7534 (originally ATCC 24193) was obtained from Korean Collection for Type Cultures (KCTC). Cheese whey was ultrafiltration permeate from skim milk derived from a process used to make milk protein concentrate, which was supported from Kraft Foods USA.

Preparation of cell-free extract

Bullera singularis was subcultured in medium (50 ml in a 250 ml flask) containing 5% (w/v) lactose, 0.75% (w/v) yeast extract with incubation at 25 °C for 48 h on a rotary shaker at 250 rpm. The main culture for the production of enzyme was carried out in a 5-l fermenter at 25 °C for 87 h. The culture broth was centrifuged at 7000 × *g* for 20 min, and the resulting cell paste was disintegrated by sonication, then unbroken cells and cell debris were removed by centrifugation at 10 000 × *g* for 20 min. The enzyme was purified from the supernatant.

Enzyme and protein assay

The β-galactosidase activity was assayed by the addition of 50 μl enzyme solution, appropriately diluted with 50 mM sodium phosphate buffer (pH 6), to 5 mM PNPG (*p*-nitrophenyl β-D-galactopyranoside). After 30 min incubation, reaction was stopped by addition of 0.9 ml 0.2 M Na_2CO_3. The *p*-nitrophenol produced was determined by measuring the absorbancy at 400 nm. One unit of β-galactosidase activity was defined as the amount of enzyme producing 1 μmol *p*-nitrophenol or hydrolyzing 1 μmol lactose per min at 37 °C and pH 6. When measuring the activity using lactose as substrate, 0.5 ml enzyme solution with the assay buffer, was combined with 50 g lactose l^{-1}. The reaction was terminated by heating at 100 °C for 10 min.

Proteins were measured by the bicinchoninic acid method using bovine serum albumin as the standard (Pierce, USA). SDS-PAGE was performed at a concentration of 8% (w/v) polyacrylamide according to the method of Laemmli. The protein bands were revealed by silver staining after staining gels with Coomassie Brilliant Blue R-250.

Enzyme reaction and GalOS assay

Batch-wise enzyme reactions were carried out in a thermo-stat water bath at 37 °C. The reaction was started by the addition of 5.4 U g^{-1} lactose of enzyme dosage with 200 g lactose l^{-1}, appropriately diluted with 50 mM sodium phosphate buffer (pH 6). The reaction was terminated by heating the reaction mixtures in a boiling water bath for 10 min.

Table 1. Comparison of reaction products produced from pure lactose and cheese whey by crude β-galactosidase from *Bullera singularis*.

Carbohydrates	Product composition (%, w/w)[a]	
	Cheese whey	Pure lactose
Lactose	39	35
Monosaccharides	27	24
DP3[b]	22	18
DP4	8	15
DP5	4	8
Total GalOS	34	41

[a]Compositions were given at the reaction time at which a maximum oligosaccharide yield was reached.
[b]DP means degree of polymerization. P3: trisaccharides, DP4: tetrasaccharides and DP5: pentosaccharides.
[c]Enzyme dosage: 5.4 U g^{-1} lactose.
[d]Initial substrate concentration: 200 g l^{-1}.
[e]Reaction pH: 6, temperature: 37 °C.

The products of enzymatic reaction were analyzed by Bio-LC (Dionex) system using a CarboPac PA-1 (Dionex, 250 × 4 mm), and guard column (Dionex, 50 × 4 mm) with an electrochemical detector (ED40) at room temperature. The mobile phase used was a gradient of 200 mM NaOH, 300 mM sodium acetate + 100 mM NaOH, 1 M sodium acetate + 200 mM NaOH at a flow rate of 0.3 ml min^{-1}.

Results and discussion

Enzymatic reaction using crude β-galactosidase with pure lactose and cheese whey

Reaction aspects of the crude enzyme from *B. singularis* on pure lactose and cheese whey are summarized in Table 1. Lactose in cheese whey and pure lactose were decreased to around 39% and 35%, respectively, while the total GalOS content increased to about 34% and 41% (of total sugar), respectively, after 27 h. The main products were DP (degree of polymerization) 3 and DP4 using pure lactose as substrate, while DP3 was major product on cheese whey as substrate. The GalOS conversion (%) and reaction rate compared to cheese whey as substrate were slightly higher when pure lactose was used as substrate.

Table 2. Summary of purification of β-galactosidase from *Bullera singularis*[a].

Step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification fold	Yield (%)
Crude enzyme	361	209	0.6	1	100
$(NH_4)_2SO_4$	264	185	0.7	1.2	88
(40–60%) DEAE FF	21	159	8	13	76
Q FF	0.6	34	56	96	16

[a]The crude enzyme solution was concentrated by ammonium sulfate precipitation (40–60% saturation) followed by desalting using centriprep YM-30 (Amicon, USA) and then applied on a Hiprep 16/10 dietylamino ethyl (DEAE) Sepharose Fast Flow (FF) column (1.6 × 10 cm, Pharmacia, Sweden) by FPLC AKTA system (Pharmacia) pre-equilibrated with 50 mM sodium phosphate buffer (pH 6). Adsorbed proteins were eluted in salt gradients with 420 ml of 0 to 1 M KCl at 3 ml min^{-1}. The next chromatographic step was performed on a column of Hiprep 16/10 quaternary ammonium (Q) Fast Flow (FF) (1.6 × 10 cm, Pharmacia, Sweden) pre-equilibrated with 50 mM sodium phosphate buffer (pH 6). Proteins were eluted with linear gradient of 0–1 M KCl at 4 ml min^{-1}.

Table 3. Comparison of reaction products produced from pure lactose by crude and purified β-galactosidase.

Carbohydrates	Product composition (%, w/w)[a]	
	Crude enzyme	Purified enzyme
Lactose	26	29
Monosaccharides	30	21
DP3[b]	19	21
DP4	16	20
DP5	9	9
Total GalOS	44	50

[a]Compositions were given at the reaction time at which a maximum oligosaccharide yield was reached.
[b]DP3: trisaccharides, DP4: tetrasaccharides, DP5: pentosaccharides.
[c]Enzyme dosage: 19 U g^{-1} lactose.
[d]Initial substrate concentration: 0.5 M.
[e]Reaction pH: 5, temperature: 50 °C.

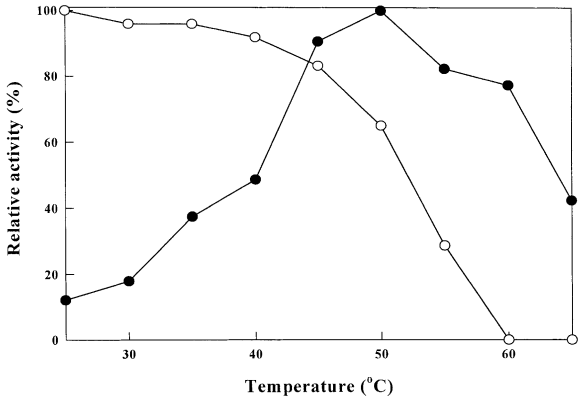

Fig. 1. Effect of temperature (●) and thermal stability (○) on *Bullera singlularis* β-galactosidase activity. The effect of temperature stability was studied by keeping the enzymes for 2 h at various temperatures (25–65 °C). The actual value of 100% of the relative activity was 0.6 units mg^{-1}.

Purification of β-galactosidase

The results of the purification procedure are reported in Table 2. SDS-PAGE revealed a single protein band of molecular mass 53 kDa (data not shown). Many investigators have reported molecular weights of microbial β-galactosidases: for example, the molecular mass of β-galactosidases from *Bacillus circulans* were 240 and 160 kDa, whereas those of the other microbial galactosidase were around 86 and 70 kDa from *Sterigmatomyces elviae* and *Penicillium* sp., respectively (Mozaffer *et al.* 1984, Onishi & Tanaka 1995, O *et al.* 1981).

Effect of pH and temperature on the hydrolytic activity of β-galactosidase

β-galactosidase had maximal activity at pH 5. The pH stability of the β-galactosidase was determined by incubating the enzyme in 0.1 M buffer of different pH values for 24 h and then determining remaining activities. The enzyme was stable at a narrow pH range of 4.5–5.5. The enzyme activity was easily inactivated at neutral and alkaline pH values. The optimum temperature for β-galactosidase activity was 50 °C (data not shown). The effect of temperature on stability was studied by keeping the enzymes, for 2 h, at various temperatures (25–65 °C). The remaining activities were then assayed (see Figure 1). β-Galactosidase was stable below 45 °C, retaining over 80% of its original activity. O *et al.* (1981) and Onishi & Tanaka (1995)

Table 4. Comparison of galactooligosaccharides production by transgalactosylation of purified β-galactosidases from various microorganisms.

Microorganisms	Concentration (g l^{-1})		
	Initial lactose	Lactose conversion	Galactooligosaccharide (conversion yield, w/w %)
Rhodotorula minuta[a]	200	114	76 (38%)
Sterigmatomyces elviea[b]	200	128	78 (39%)
Penicillium sp. KFCC 10888[c]	400	292	160 (40%)
Bacillus circulans[d]	47	29	19 (41%)
Saccharopolyspora rectivirgular[e]	600	480	264 (44%)
Bullera singularis (this work)	180	128	90 (50%)

[a]Onishi & Tanaka (1996).
[b]Onishi & Tanaka (1995).
[c]In & Chae (1998).
[d]Mozaffer *et al.* (1984).
[e]Nakao *et al.* (1994).

reported similar properties for β-galactosidases from *Penicillium* sp. and *Sterigmatomyces elviea*.

Effect of metal ions and detergents on hydrolytic activity of β-galactosidase

The hydrolytic activity of the enzyme was measured with PNPG as the substrate in the presence of various metal ions and additives at 10 mM. $AgNO_3$ and SDS inhibited by 87 and 84% of its initial activity, respectively (0.54 unit mg^{-1} was 100%).

Determination of the kinetic parameters

Initial reaction rate was determined as the amount of lactose hydrolyzed (mM) per min against various substrate concentrations (pH 5 and 50 °C). The K_m value and maximal velocity for the reaction with lactose was calculated from a Lineweaver–Burk plot. The K_m value was 0.58 M, higher than the other β-galactosidases, and the maximal velocity was 4.13 mM min^{-1}. In & Jung (1998) reported that K_m values for lactose hydrolysis with β-galactosidases from *Kluyveromyces lactis, Aspergillus oryzae*, and *Bacillus* sp., were 0.5 M, 0.049 M, and 0.005 M, respectively.

Production of galactooligosaccharide using purified β-galactosidases

As shown in Table 3 and Figure 2, there was no significant difference in the product distribution from the reactions of crude and purified enzymes. The main

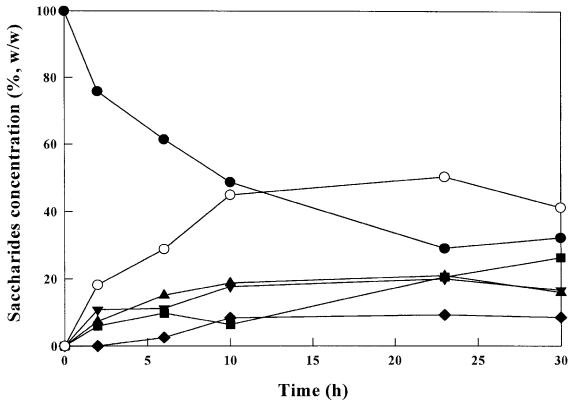

Fig. 2. Typical time profiles of reaction products produced from lactose by purified β-galactosidase. (■) Monosaccharides; (●) disaccharides; (▲) trisaccharides; (▼) tetrasaccharides; (♦) pentasaccharides and (○) total GalOS. Enzyme reactions were performed at 50 °C and 30 h. Initial saccharides concentration was 180 g l^{-1} (100% concentration).

products were GalOS with degree of polymerization (DP) of 3 and 4. The maximal content of GalOS produced was 90 g l^{-1} (50% of total sugar) after 23 h of incubation. The amount of GalOS formed in the reaction mixture decreased slightly after 23 h of incubation, resulting in an increase of disaccharide, while the monosaccharide content increased as the reaction proceeded (Figure 2). Table 4 shows that the GalOS conversion yields were 38, 39, 40, 41, and 44% of total sugar by the enzymes of *Rhodotorula minuta, Sterigmatomyces elviea, Penicillium* sp., *Bacillus circulans, Saccharopolyspora rectivirgular* (Onishi & Tanaka 1995, 1996, In & Chae 1998, Mozaffer *et al.*

1984, Nakao *et al.* 1994). The relatively high K_m of the β-galactosidase of *B. singularis* may explain its high transgalactosylation activity.

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