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Purification and Properties of a Novel Thermostable Galacto-Oligosaccharide-Producing β-Galactosidase from *Sterigmatomyces elviae* CBS8119

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A thermostable β-galactosidase which catalyzed the production of galacto-oligosaccharide from lactose was solubilized from a cell wall preparation of *Sterigmatomyces elviae* CBS8119. The enzyme was purified to homogeneity by means of chromatography on DEAE-Toyopearl, Butyl-Toyopearl, Chromatofocusing, and *p*-aminobenzyl 1-thio-β-D-galactopyranoside agarose columns. The molecular weight of the purified enzyme was estimated to be about 170,000 by gel filtration with a Highload-Superdex 200pg column and 86,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Its isoelectric point, determined by polyacrylamide gel electrofocusing, was 4.1. The optimal temperature for enzyme activity was 85°C. It was stable at temperatures up to 80°C for 1 h. The optimal pH range for the enzyme was 4.5 to 5.0, it was stable at pH 2.5 to 7.0, and its activity was inhibited by Hg^{2+}. The K_m values for *o*-nitrophenyl-β-D-galactopyranoside and lactose were 9.5 and 2.4 mM, respectively, and the maximum velocities for these substrates were 96 and 240 μmol/min per mg of protein, respectively. In addition, this enzyme possessed a high level of transgalactosylation activity. Galacto-oligosaccharides, including tri- and tetrasaccharides, were produced with a yield, by weight, of 39% from 200-mg/ml lactose.

β-Galactosidase (EC 3.2.1.23) catalyzes not only hydrolysis of β-D-galactopyranosides, such as lactose, but also a transgalactosylation reaction that produces galacto-oligosaccharides (Gal-OS) (23). The enzyme is very useful in the dairy industry and has been used widely for lactose-free milk production. Recently, Gal-OS, enzymatic transgalactosylation reaction products from lactose, have become of interest for human health as, collectively, Gal-OS have been recognized to be a growth-promoting factor of intestinal bifidobacteria (19, 29), which have been studied extensively and found to be helpful for the maintenance of human health (11, 16). On the basis of these findings, development of an efficient and inexpensive Gal-OS production method is highly desirable. Although many β-galactosidases are known to possess transgalactosylation activities, their Gal-OS productivities were rather low (3, 10, 17, 30).

In a previous paper (21), we described screening for β-galactosidase-producing microorganisms that possess transgalactosylation activity to find those which could produce high yields of Gal-OS and found that *Sterigmatomyces elviae* CBS8119 produced a large amount of Gal-OS from lactose. The main product of the Gal-OS produced was identified as *O*-β-D-galactopyranosyl-(1→4)-*O*-β-D-galactopyranosyl-(1→4)-D-glucopyranoside by ^{13}C nuclear magnetic resonance spectroscopy (21). It was noted that the optimal temperature for the transgalactosylation reaction with toluene-treated resting cells was very high, 80°C, even though *S. elviae* CBS8119 is mesophilic and does not grow at temperatures above 40°C (21). In addition to its high level of transgalactosylation activity, the high level of thermal stability of the β-galactosidase from the mesophilic yeast *S. elviae* CBS8119 is particularly interesting and merits further study.

In this paper, we describe the purification and properties of a novel thermostable Gal-OS-producing β-galactosidase from *S. elviae* CBS8119.

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MATERIALS AND METHODS

Chemicals. *o*-Nitrophenyl-β-D-galactopyranoside (ONPG) and *p*-aminobenzyl 1-thio-β-D-galactopyranoside (PATG) agarose were purchased from Sigma Chemical Co. (St. Louis, Mo.), lactose was purchased from Junsei Kagaku Co., Ltd. (Tokyo, Japan), Usukizyme was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan), DEAE-Toyopearl 650M and Butyl-Toyopearl 650M were obtained from Tosoh Corp. (Tokyo, Japan), and Chromatofocusing PBE94 gel media, Polybuffer 74, and Highload-Superdex 200pg columns were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). All the other chemicals used in this study were commercially available and of analytical grade.

Microorganisms and cultivation. *S. elviae* CBS8119, which we selected in our accompanying study (21) and is a thermostable β-galactosidase-producing strain, was used. A loopful of *S. elviae* CBS8119 cells subcultured on a potato-dextrose agar slope was inoculated into 5 ml of medium II (21) in a test tube and cultivated aerobically at 30°C for 48 h. Then, 1 ml of the culture was transferred to 80 ml of fresh medium (medium II) in a 500-ml flask and cultivated aerobically at 30°C for 60 h on a reciprocal shaker.

Assay of β-galactosidase. The β-galactosidase activity was determined by measuring the hydrolysis of ONPG. The incubation mixture comprised 1 mM ONPG, 100 mM sodium acetate buffer (pH 5.0), and appropriately diluted enzyme solution in a total volume of 1 ml. The reaction was carried out at 60°C for 10 min and then stopped by adding 0.2 ml of 1.0 M Na_2CO_3. The amount of *o*-nitrophenol released was determined spectrophotometrically by measuring the A_{420} of the solution. The molar extinction coefficient used for *o*-nitrophenol was 4,900. One unit of enzyme activity was defined as the amount of enzyme that hydrolyzed 1 μmol of substrate per min under the assay conditions.

In order to determine the lactose hydrolytic activity of the enzyme, the reaction was carried out at 60°C for 10 min and stopped by boiling for 5 min. The amount of glucose formed in the incubation mixture was determined by high-performance liquid chromatography with a Shodex ION Pak S-801 column (4.6 by 500 mm; Showa Denko Co., Tokyo, Japan) with distilled water as the eluent at a flow rate of 0.6 ml/min. The saccharides formed were detected by measuring the refractive index of each fraction and identified and quantitated by comparing their retention times with those of authentic samples.

Formation of Gal-OS. The incubation mixture comprising 2 g of lactose, 100 mM sodium acetate buffer (pH 5.0), and 0.25 U of enzyme in a total volume of 10 ml was incubated at 60°C, and the reaction was stopped by boiling for 5 min. The amount of Gal-OS formed in the reaction mixture was determined by high-performance liquid chromatography, as described previously (21).

Enzyme purification. The following six procedures were performed at 4°C.

(i) Crude extract. The cells (wet weight, 600 g) from 8,000 ml of culture were harvested by centrifugation (10 min, 10,000 × *g*) and suspended in 2,000 ml of 50

mM potassium phosphate buffer (pH 7.2). The cell suspension was mixed with an equal volume of glass beads (0.75 mm in diameter), and the cells were disrupted in a bead beater (Biospec, Bartlesville, Okla.) for 5 min. The resulting disrupted cell suspension was decanted and centrifuged at 10,000 × *g* for 10 min. The enzyme activity was only observed in the precipitate consisting mainly of cell wall. The precipitate was resuspended in 3,000 ml of 50 mM potassium phosphate buffer (pH 7.2) containing 30 ml of toluene, treated with 0.2% (wt/vol) Usukizyme (a cell wall-lytic enzyme derived from *Trichoderma* sp. and containing β-1,3-glucanase, chitinase, and cellulase activities) at 37°C for 72 h, and then centrifuged for 20 min, at 10,000 × *g*. The supernatant was dialyzed against the same buffer and used as the crude extract.

(ii) Heat treatment. The crude extract (2,300 ml) was incubated at 60°C for 1 h and then centrifuged at 10,000 × *g* for 10 min to remove the denatured proteins.

(iii) DEAE-Toyopearl chromatography. The supernatant was applied to a column (25 by 500 mm) of DEAE-Toyopearl 650M preequilibrated with 20 mM potassium phosphate buffer (pH 7.2). After the column was washed with 1,000 ml of this buffer, the enzyme was eluted with a linear gradient of 0 to 600 mM NaCl in this buffer. The active fractions (1,120 ml) were eluted at an NaCl concentration of 50 to 150 mM and used for further purification.

(iv) Butyl-Toyopearl chromatography. Ammonium sulfate was added to the resulting enzyme solution to produce 40% (wt/vol) saturation. This mixture was applied to a Butyl-Toyopearl 650M column (25 by 500 mm) preequilibrated with 20 mM potassium phosphate buffer (pH 7.2) containing a 40% (wt/vol) saturation of $(NH_4)_2SO_4$. After the column was washed with 500 ml of this buffer, the enzyme was eluted with 20 mM potassium phosphate buffer (pH 7.2) with a decreasing gradient of a 40 to 0% (wt/vol) saturation of $(NH_4)_2SO_4$ at a flow rate of 150 ml/h. The active fractions (660 ml) were eluted with a 20% (wt/vol) saturation of $(NH_4)_2SO_4$.

(v) Chromatofocusing chromatography. The active fractions were pooled and dialyzed against 20 mM piperazine-HCl buffer (pH 5.5). The dialysate (920 ml) was applied to a Chromatofocusing PBE94 column (25 by 200 mm) preequilibrated with this buffer. After the column was washed with 200 ml of this buffer, the enzyme was eluted with 10-fold-diluted Polybuffer 74 (adjusted to pH 2.8 with HCl) at a flow rate of 50 ml/h. The active fractions (180 ml) were eluted at pHs between 4.3 and 3.8 and then were pooled.

(vi) PATG chromatography. The enzyme fractions obtained from step v were dialyzed against 20 mM piperazine-HCl buffer (pH 5.5), the dialysate was applied to a PATG column (15 by 200 mm) preequilibrated with this buffer, and the enzyme was eluted with a decreasing pH gradient from pH 5.5 (20 mM piperazine-HCl buffer) to pH 2.8 (20 mM glycine-HCl buffer). The active fractions (60 ml) were eluted at pH 4.0 and then pooled. The purified enzyme recovered after these six steps was used for further studies to characterize the enzyme.

Protein determination. The protein concentration of the purified enzyme preparation was determined by the method of Bradford (1), with bovine serum albumin as the standard.

SDS-PAGE. The samples (15 μg) were boiled in the presence of 1% (wt/vol) sodium dodecyl sulfate (SDS) and 2% (vol/vol) 2-mercaptoethanol and subjected to SDS-polyacrylamide gel electrophoresis (PAGE), which was carried out with precast 10 to 20% gradient gel (model, SDS-PAG Plate 10/20; Daiichi Pure Chemicals Co., LTD. Tokyo, Japan) according to the supplier's instructions conformed to the method of Laemmli (13). The proteins were stained with Coomassie brilliant blue R-250 to detect protein bands and with periodic acid-Schiff reagent to detect glycoprotein bands (26). The standard molecular weight samples used were phosphorylase *b* (M_r = 94,000), bovine serum albumin (M_r = 67,000), ovalbumin (M_r = 43,000), carbonic anhydrase (M_r = 30,000), soybean trypsin inhibitor (M_r = 20,100), and β-lactoalbumin (M_r = 14,400).

Isoelectric focusing. Isoelectric focusing was performed at 4°C using precast polyacrylamide gel (model, IEF-PAGE mini; TEFCO Co., Nagano, Japan) with ampholine (pH 2.5 to 7.0), by the method of Garfin (6). The gel was stained with Coomassie brilliant blue R-250 to determine the location of the protein bands, and the isoelectric point of the enzyme was estimated by comparing its mobility with those of the following standard proteins: pepsinogen (pH 2.80), amyloglucosidase (pH 3.50), glucose oxidase (pH 4.15), soybean trypsin inhibitor (pH 4.55), β-lactoglobulin (pH 5.20), bovine carbonic anhydrase B (pH 5.85), and human carbonic anhydrase B (pH 6.55).

Estimation of molecular weight. The molecular weight of the enzyme was determined by the following two methods. (i) The purified enzyme together with the standard proteins was subjected to SDS-PAGE, and its molecular weight was estimated by comparing its mobility with those of the standard proteins. (ii) The purified enzyme was applied to a Highload-Superdex 200pg gel-filtration column (16 by 600 mm) preequilibrated with 100 mM potassium phosphate buffer (pH 7.2), gel filtration was performed by high-performance liquid chromatography with the same buffer as the eluent at a flow rate of 1.5 ml/min, and the elution patterns were compared with those of the standard proteins. The standard proteins used were thyroglobulin (M_r = 669,000), glutamine synthetase (M_r = 510,000), ferritin (M_r = 440,000), pyruvate kinase (M_r = 260,000), alanine dehydrogenase (M_r = 230,000), aldolase (M_r = 158,000), glyceraldehyde-3-phosphate dehydrogenase (M_r = 140,000), and glucose isomerase (M_r = 95,000).

TABLE 1. Purification of β-galactosidase from *S. elviae* CBS8119

Purification step	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Yield (%)
Solubilization	2,145	1,364	0.64	100
Heat treatment	763	1,352	1.78	99
DEAE-Toyopearl 650M	392	1,243	3.18	91
Butyl-Toyopearl 650M	102	1,150	11.3	84
Chromatofocusing	54	884	16.4	65
PATG agarose	36	727	20.2	53

RESULTS

Purification. β-Galactosidase was solubilized from the cell wall preparation of *S. elviae* CBS8119 produced by treatment with Usukizyme and purified by a six-step procedure, as described in Materials and Methods. A summary of the purification procedure for the enzyme is presented in Table 1. The enzyme was purified about 32-fold and the recovered yield was 53% after solubilization. The purified enzyme showed a single band each of protein and glycoprotein after SDS-PAGE (Fig. 1).

Properties of the purified enzyme. (i) Molecular weight. The molecular weight of the denatured enzyme was estimated to be 86,000 by SDS-PAGE after the sample had been boiled in the presence of SDS and 2-mercaptoethanol. The molecular weight of the native enzyme was estimated to be about 170,000 by gel-filtration with a Highload-Superdex 200pg column in the presence of internal standard proteins, and semilogarithmic values of the elution volumes versus the molecular weights were plotted. The results indicated that the enzyme consisted of two similar or identical subunits.

(ii) Isoelectric point. The isoelectric point of the enzyme was determined by isoelectric focusing and was about 4.1.

Effects of pH on enzyme activity and stability. The optimal pH for enzyme activity was between 4.5 and 5.0, and 50% activity was seen at pH 3.5 and 5.5. It was stable over the wide pH range of 2.5 to 7.0.

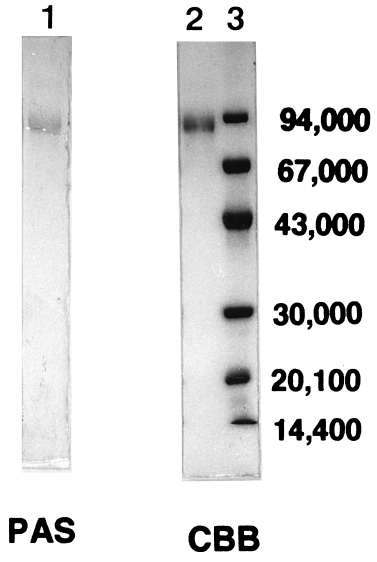

FIG. 1. SDS-PAGE of the purified β-galactosidase from *S. elviae* CBS8119. Lane 1, purified enzyme stained with periodic acid-Schiff reagent; lane 2, purified enzyme stained with Coomassie brilliant blue R-250; lane 3, standard proteins.

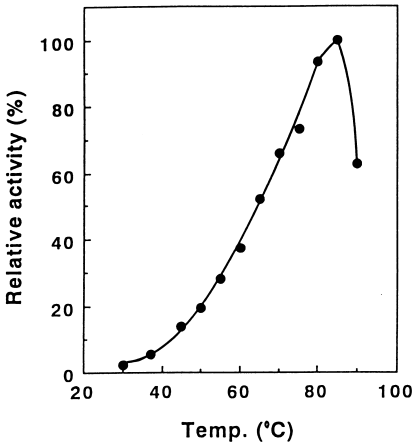

FIG. 2. Effect of temperature on activity of the purified *S. elviae* β-galactosidase. A reaction mixture containing 0.1 ml of 10 mM ONPG, 0.1 ml of enzyme solution, and 0.8 ml of 100 mM sodium acetate buffer (pH 5.0) was incubated at various temperatures for 10 min.

Effects of temperature on enzyme activity and stability. As shown in Fig. 2, the enzyme showed a high level of activity at high temperatures and the maximal activity was observed at 85°C with a 5-min reaction at pH 5.0. Furthermore, the enzyme showed a high level of thermostability and retained 90 and 62% of its activity after treatments at 80 and 85°C, respectively, for 1 h.

Effects of divalent cations and enzyme inhibitors on enzyme activity. The requirements of various divalent cations for and the effects of enzyme inhibitors on enzyme activity were examined. The divalent cations tested were $FeSO_4 \cdot 7H_2O$, $ZnCl_2$, $MnCl_2 \cdot 4H_2O$, $CoCl_2$, $CuSO_4 \cdot 5H_2O$, $BaCl_2$, $CaCl_2$, $PbCl_2$, $CdCl_2$, and $HgCl_2$, and the enzyme inhibitors examined were EDTA disodium salt, phenylmethylsulfonyl fluoride, iodoacetic acid, *N*-ethylmaleimide, *o*-phenanthroline, *p*-chloromercuribenzoic acid, α,α′-dipyridyl, dithiothreitol, and NH_2OH. Divalent cations and enzyme inhibitors were each added at 1 mM (final concentration) to aliquots of the enzyme solution. After incubating at 40°C for 1 h, the residual activities were assayed under standard reaction conditions. Hg^{2+} and Pb^{2+} inhibited 54 and 21% of the enzyme activity, respectively, whereas thiogroup-specific inhibitors such as *p*-chloromercuribenzoic and iodoacetic acids did not. EDTA disodium salt also did not. Therefore, no divalent cations were required for enzyme activity.

Kinetic properties. The dependence of the reaction rate on the substrate concentration was assayed at pH 5.0 and 85°C. The Michaelis constant (K_m) and maximal velocity for the reactions with ONPG and lactose were calculated from Lineweaver-Burk plots. The K_m values for ONPG and lactose were 9.5 and 2.4 mM, respectively, and their maximal velocities were 96 and 240 μmol/min per mg of protein, respectively.

Substrate specificity. The relative hydrolytic activities with various *p*-nitrophenyl glycosides were compared by measuring ONPG hydrolysis (Table 2). The enzyme catalyzed not only the hydrolysis of *p*-nitrophenyl-β-D-galactoside but also those of *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-fucopyranoside, and *p*-nitrophenyl-β-L-arabinopyranoside. *p*-Nitrophenyl-β-D-fucopyranoside was the best substrate, being hydrolyzed 7.3-fold better than ONPG.

Production of Gal-OS. Figure 3 shows the time course of Gal-OS production from lactose (200 mg/ml) by the purified enzyme of *S. elviae* CBS8119 at pH 5.0 and 60°C. Aliquots of the reaction mixture were withdrawn periodically and analyzed by high-performance liquid chromatography. The maximal amount of Gal-OS produced was 78 mg/ml, comprising 68 mg of trisaccharide per ml and 10 mg of tetrasaccharide per ml, which was reached after incubation for 24 h. Few polysaccharides larger than tetramers were observed. The amount of Gal-OS formed in the reaction mixture hardly decreased after incubation for 48 h; the amount of glucose increased slightly as the reaction proceeded, but little galactose was formed. These findings suggest that the purified enzyme of *S. elviae* CBS8119 possesses a fairly high level of transgalactosylation activity but a low level of hydrolytic activity.

TABLE 2. Relative rates of hydrolysis of various substrates by purified *S. elviae* CBS8119 β-galactosidase

Substrate[a]	Relative rate of hydrolysis (%)[b]
ONPG	100
PNP-β-D-galactopyranoside	39
PNP-β-D-glucopyranoside	115
PNP-β-D-fucopyranoside	730
PNP-β-L-arabinopyranoside	51

[a] PNP, *p*-nitrophenyl. PNP-β-D-xylopyranoside, PNP-β-D-mannopyranoside, PNP-β-D-glucuronide, PNP-*N*-acetyl-β-D-galactosaminide, PNP-*N*-acetyl-β-D-glucosaminide, PNP-1-thio-β-D-galactopyranoside, PNP-β-L-fucopyranoside, and PNP-α-L-arabinopyranoside were also tested and were not hydrolyzed.

[b] Sufficient enzyme was added to cause a linear release of product during the first 10 min of the reaction at pH 5.0 and 60°C. For PNP glycoside, activity was measured as the release of PNP (420 nm). The relative initial rate of hydrolysis of PNP glycoside is expressed as a percentage of that obtained with ONPG.

DISCUSSION

A thermostable β-galactosidase with a high level of transgalactosylation activity was purified to homogeneity from a cell wall preparation of the yeast *S. elviae* CBS8119, and its properties were investigated. The enzyme was active at high temperatures up to 85°C and also showed a high level of thermostability. Extensive studies on thermostable β-galactosidases and β-glucosidases have been carried out previously (2, 4, 5, 7,

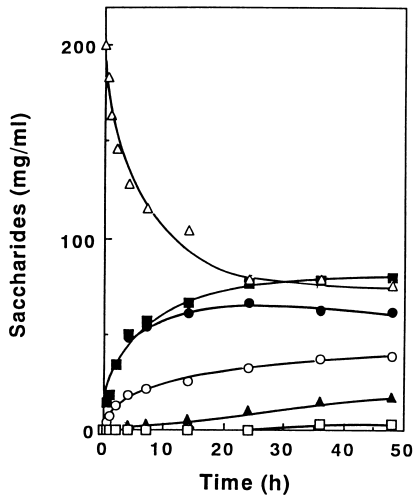

FIG. 3. Time course of Gal-OS formation by the purified *S. elviae* β-galactosidase. A mixture of 2 ml of enzyme solution (0.25 U) and 8 ml of 250-mg/ml lactose solution in 100 mM sodium acetate buffer (pH 5.0) was incubated at 60°C. Symbols: △, lactose; ○, glucose; □, galactose; ▲, Gal-OS (tetrasaccharide); ●, Gal-OS (trisaccharide); ■, total Gal-OS.

12, 14, 18, 22, 24, 25, 27, 28, 31). The thermostability of β-galactosidase from *S. elviae* was slightly lower at 85°C than those from *Sulfolobus solfataricus* (22) and *Thermotoga maritima* (4). As the *S. elviae* β-galactosidase retained 90% of its activity after preincubation at 80°C, it is comparable with β-galactosidases from *Thermus aquaticus* (31), *Bacillus stearothermophilus* (7), *Bacillus acidocaldarius* (12), *Thermoaerobacter* sp. (14), *Saccharopolyspora rectivirgula* (18), and the thermophilic anaerobe strain NA10 (24). Almost all the thermostable β-galactosidases have so far been found in thermophilic and thermotolerant microorganisms. Our present paper is the first report of thermostable β-galactosidase from a mesophile, *S. elviae.*

The β-galactosidase from *S. elviae* hydrolyzed not only the β-D-galactoside linkage but also the β-D-glucoside and β-D-fucoside linkages. It is known that several types of β-galactosidase catalyze the hydrolysis of β-glucoside and β-fucoside (4, 8, 9, 15, 27). With respect to substrate specificity, it was noted that the β-galactosidase of *S. elviae* catalyzed hydrolysis of *p*-nitrophenyl-β-D-fucopyranoside at a high velocity (7.3 times faster than that of ONPG and 6.3 times faster than that of *p*-nitrophenyl-β-D-glucopyranoside). Furthermore, the most remarkable feature of this enzyme is its high level of transgalactosylation activity. A thermostable β-galactosidase from a thermophilic actinomycete, *S. rectivirgula* (12), gave only about 30% (wt/wt) Gal-OS from 1.75 M lactose with the maximal yield. Also, the reaction yielded about 10% (wt/wt) galactose, an undesired by-product. On the other hand, the maximal yield of transgalactosylation products from 200-mg/ml (0.56 M) lactose with *S. elviae* β-galactosidase was about 40% (wt/wt) and little galactose was produced. In addition, the thermostability of β-galactosidase from *S. elviae* is far superior to that from *S. rectivirgula*, since the enzyme from *S. rectivirgula* was inactivated after incubation at temperatures higher than 60°C for 1 h.

Ohtsuka et al. (20) isolated a β-galactosidase with a high level of transgalactosylation activity from *Cryptococcus laurentii*, with the following properties: (i) the enzyme was bound in the cell wall and was solubilized by a cell wall-lytic enzyme; (ii) it had a molecular weight of about 200,000 and consisted of two subunits; (iii) its optimum pH was 4.3, and it was stable in the neutral-to-weak-acidic pH range; (iv) it was strongly inhibited by Hg^{2+} but not by *p*-chloromercuribenzoic acid; and (v) it possessed a high level of transgalactosylation activity, and the main product was *O*-β-D-galactopyranosyl-(1→4)-*O*-β-D-galactopyranosyl-(1→4)-D-glucopyranoside. Therefore, the β-galactosidases of *S. elviae* and *C. laurentii* are similar. However, the thermostability of *S. elviae* β-galactosidase was considerably greater than that of *C. laurentii*: *S. elviae* β-galactosidase retained 90% of its activity after preincubation at 80°C for 1 h, whereas that from *C. laurentii* was inactivated completely after treatment at 65°C for 10 min.

This study demonstrated that the β-galactosidase of *S. elviae* is the most thermostable enzyme with a high level of transgalactosylation activity that has been found so far. From the viewpoint of commercial production, the ease of the yeast culture, the high levels of enzyme production, and the enzyme's high levels of thermostability and transgalactosylation activity are particularly favorable for the bioconversion of lactose to Gal-OS.

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