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Thermostable β-glucosidase with a broad substrate specifity suitable for processing of lactose-containing products

Sylwia Wołosowska, Józef Synowiecki*

Department of Food Chemistry and Technology, Faculty of Chemistry, Gdańsk University of Technology, ul Gabriela Narutowicza 11/12, 80-952 Gdańsk, Poland

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Abstract

Sulfolobus shibatae produces intracellular β-glucosidase with β-galactosidase activity. The final yield of the wet cells after 72 h of growth at 75 °C (pH 3.5) was about 3.4 g per litre of the medium. Addition of 1% of lactose to the medium enhanced the enzyme activity by 40%. The cell-free extract of *Sulfolobus shibatae* had specific activity towards GlcβpNp (0.81 U mg^{-1}), GalβoNp (1.85 U mg^{-1}), cellobiose (0.47 U mg^{-1}) and lactose (0.29 U mg^{-1}). The enzyme was purified about 17-fold by ammonium sulphate precipitation, ion-exchange chromatography and gel filtration. The obtained preparation exhibited highest specific activity for GalβoNp hydrolysis (29.5 U mg^{-1}) at pH 5.5 and 98 °C. After 5 h of preincubation at 80 °C in acetate buffer (pH 5.5), the enzyme retained 67% initial activity. The β-glucosidase and β-galactosidase activities of the enzyme was eliminated by Hg^{2+} and Zn^{2+} ions. Among thiol reagents tested, only 4-chloromercuribenzoate exhibited a strong inhibitory effect on the investigated enzyme.

Keywords: β-glucosidase; Thermostable enzymes; Lactose hydrolysis

1. Introduction

β-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) catalyses the release of D-glucose residues from cellobiose in the last step of cellulose degradation. This reaction is important for effective cellulose hydrolysis, because cellobiose is a strong inhibitor of endo-β-1,4-glucanase and exo-β-1,4-glucanase (cellobiohydrolase) in the cellulases complex (Walker & Wilson, 1991). β-Glucosidases, produced intracellularly by many microorganisms, usually show a broad substrate specifity and also transferase activity (Sunna, Moracci, Rosi, & Antranikian, 1997). Such properties make them suitable, e.g., for lactose hydrolysis or for formation of galactooligosaccharides used as prebiotic food ingredients (Crittenden & Playne, 1996; Matsumoto et al., 1993). Hydrolysis of lactose at elevated temperatures by thermostable β-galactosidases or β-glucosidases, which often show β-galactosidase activity, minimizes undesired microbial contamination of the immobilized enzyme system. Compared with β-glucosidases from mesophilic sources, application of their thermostable counterparts assures higher reaction velocity, longer half-life of enzyme activity, and decreased viscosity of the substrate solution. Moreover, reduced activity of the thermostable enzymes at low temperature makes it possible to control the reaction by cooling. In our previous article the β-galactosidase activity of the cell extract from hyperthermophilic, obligate aerobic archaeon *Sulfolobus shibatae* was reported (Wołosowska & Synowiecki, 2003). However, strains of related *Sulfolobus solfataricus* contain β-galactosidase as well as β-glucosidase activity, which reside in the same enzyme (Kengen & Stams, 1994; Petzelbauer, Nidetzky, Haltrich, & Kulbe, 1999; Pisani et al., 1990; Pouwels et al., 2000). Further characterization of the investigated enzyme presented in this paper shows a broad substrate specifity of the same protein, e.g., toward lactose and cellobiose. Moreover, the reported data suggest suitability of *Sulfolobus shibatae* as a good source of β-glucosidase for processing of cellulose or lactose-containing by-products.

2. Materials and methods

2.1. Microorganism and culture conditions

Sulfolobus shibatae (DSM 5389) was cultivated on media composed of 0.4% of yeast extract (Difco

* Corresponding author.

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Laboratories, USA), 0.8% peptone bio-Trypcase (bio Merieux, France), 1% (v/v) of mineral salts solution prepared according to Brock, Brock, Belly, and Weis (1972), and water. The cultures were grown in 200-ml Erlenmeyer flasks containing 100 ml of liquid medium (pH 3.5), autoclaved for 30 min at 121 °C. The flasks were inoculated with 6 ml of a *Sulfolobus shibatae* cell suspension ($OD_{600} > 0.8$) and stirred in a water bath at 150 rpm at 75 °C for 72 h without additional aeration. After the desired growth time, the cells were harvested by centrifugation at 12,000×*g* for 15 min. The pellet was washed with 5 mM Na_2HPO_4 solution, centrifuged, and stored at −18 °C until use.

2.2. Partial purification of β-glucosidase

Frozen cells (10 g) were disrupted for 15 min in a refrigerated mortar with 20 g of Alumina A-5 (Sigma) and 70 ml of 0.01 M sodium phosphate buffer (pH 7.5), which was gradually added during extraction. The resulting suspension was centrifuged at 8000×*g* for 15 min. Next, nucleic acids were removed by precipitation (30 min, 4 °C) at 2.5% (w/v) of streptomycin sulphate concentration and subsequent centrifugation (8000×*g*) for 15 min. Solid $(NH_4)_2SO_4$ was slowly added to the supernatant up to 50% of saturation. The mixture was left overnight at 4 °C and centrifuged at 8000×*g* for 15 min. Obtained precipitate was redissolved in approximately 20 ml of 0.1 M phosphate citrate buffer (pH 5.5) and concentrated by ultrafiltration on a Centriplus Centrifugal Filter Device with Ultracel-YM membrane 30-kDa cutoff (Millipore Corporation). The concentrated solution was applied to a DEAE Sepharose CL-6B column (2.6×38 cm) previously equilibrated with 50 mM Tris–HCl buffer (pH 7.5). The column was eluted with 0.25 M NaCl in the same buffer at a flow rate of 80 ml/h and 4 ml fractions were collected for determination of enzyme activity and protein content. Combined fractions with β-glucosidase activity were concentrated to a volume of 4 ml on a Centriplus Centrifugal Filter Device (30 kDa cutoff) and the resulting sample was passed through a column (2.6×38 cm of Sephadex G-200, pre-equilibrated with 50 mM Tris–HCl buffer (pH 7.5), at a flow rate of 12 ml/h. Single peaks with absorbance at 280 nm and enzyme activity were obtained.

2.3. Determination of enzyme activity

The activities of β-glucosidase and β-galactosidase were determined according to Legin, Copinet, and Duchiron (1998) by release of *p*-nitrophenol from 5 mM solution *p*-nitrophenyl-β-D-glucopyranoside (GlcβpNp) or *o*-nitrophenyl-β-D-galactopyranoside (GalβoNp) in 0.1 M phosphate/citrate buffer (pH 5.5), respectively. The assays were initiated by addition of 0.5 ml of enzyme solution to 2.5 ml of substrate, preincubated for 2 min at 70 °C. The reaction at 70 °C was terminated after 2 min, or after the desired time, by the addition of 1 ml of 1 M Na_2CO_3 solution. A blank, containing buffer instead of enzyme solution, was used to correct the thermal hydrolysis of GlcβpNp or GalβoNp. Absorbances at 405 nm were converted to *para*- or *ortho*-nitrophenol concentrations, using molar absorption coefficients given by Legin et al. (1998) and Craven, Steers, and Anfinsen (1965). One unit of β-glucosidase or β-galactosidase activity is defined as the amount of enzyme required to liberate 1 μmol of nitrophenol from GlcβpNp or GalβoNp, respectively, per minute under the described conditions. Specific activity is expressed as units per mg of protein, determined by the method of Bradford (1976).

The amount of glucose released during carbohydrate hydrolysis was determined by the method of Hugget and Nixon (1955), using the GOPOD reagent containing 125 mg of glucose oxidase, 5 mg peroxidase, 0.5 ml of 1% solution of *o*-dianisidine in 96% ethanol and 0.5 M phosphate buffer (pH 7.0), added to a final volume of 100 ml. The assays were initiated by addition 0.5 ml of enzyme solution to 0.5 ml of 0.25% solution of lactose or cellobiose in 0.1 M citrate–phosphate buffer (pH 5.5). The reaction at 70 °C was stopped by addition of 3 ml of GOPOD reagent and incubation was continued for 30 min at 40 °C. The absorbance was measured at 415 nm and the amount of liberated glucose was calculated from the regression equation of the standard curve, determined at glucose concentratins up to 0.12 μmol ml^{-1}.

2.4. The pH profile, optimal temperature and enzyme thermostability

The effect of pH on β-glucosidase or β-galactosidase activity was determined at 70 °C using 5 mM GlcβpNp or GalβoNp solution in 0.1 M citrate/phosphate buffers, which covered the range between pH 2.5 and pH 8.0 adjusted at 70 °C. The temperature dependence of enzyme activity was assayed in the range 55–105 °C using the same substrate solutions at pH 5.5. The β-glucosidase and β-galactosidase thermostabilities were determined by subjecting the enzyme solution in 0.1 M phosphate (pH 7.5) or acetate (pH 5.5) buffers in sealed tubes to temperatures between 70 and 98 °C. Samples were withdrawn at various times, cooled in ice and assayed for remaining activity at 70 °C and pH 5.5 using the appropriate substrate. The enzyme half-life values were calculated from the graph of the log residual activity versus time.

2.5. Effect of various compounds on enzyme activity

Partially purified enzyme was preincubated 15 min at 70 °C in 0.1 M acetate buffer (pH 5.5) containing 1.0 mM of various cations in the form of chlorides or 5.0 mM of inhibitors or other compounds listed in Table 3

(see later). The activities towards GlcβpNp and GalβoNp were determined using the standard assay method and expressed as percentages of that of control. In the control, each compound was added after termination of the enzymatic reaction.

2.6. End-products analysis

The products formed during hydrolysis of 30 mM lactose or cellobiose solution in 0.1 M citrate/phosphate buffer (pH 5.5) at 70 °C were determined by HPLC using Polyspher® CHPB column (Merck) and refractive index detector (La Chrom L-7490, Merck). The samples, purified by thermal precipitation (110 °C) of enzyme, adsorption on charcoal, centrifugation (8000×*g*) for 15 min and filtration on a 0.2-μm Puradisc™ filter (Whatman), were passed through a column, using water as the mobile phase at a flow rate of 0.3 ml/min. The column temperature was 75 °C. Cellobiose (Cel), lactose (Lac), glucose (Glc) and galactose (Gal) were used as standards at concentrations of 10 mg/ml.

2.7. Electrophoresis and detection of enzyme activity in gel

Polyacrylamide gel electrophoresis was carried out at room temperature according to the method of Laemmli (1970) with 12% polyacrylamide gel, using the Bio-Rad Mini Protean system. After electrophoresis, the gel was incubated at 70 °C in a 5 mM solution of GlcβpNp in 0.1 M citrate/phosphate buffer (pH 5.5) until a yellow band appeared. The piece of gel having enzyme activity was cut out, washed twice with water and divided into two equal parts. Each part of the gel was placed in a separate test-tube containing 3.0 ml of 5 mM GlcβpNp or GalβoNp solution in 0.1 M citrate/phosphate buffer (pH 5.5), respectively. The reaction at 70 °C was stopped after 7.5 min by the addition of 1 ml of 1 M Na_2CO_3 solution. The absorbances of the both samples were measured at 405 nm against a blank heated at the same temperature without gel addition. The hydrolyses of both substrates confirm that the same protein has β-glucosidase as well as β-galactosidase activity.

2.8. Statistical analysis

Analysis of variance and Tukey·s student *t*-test (Snedecor & Cochran, 1980) were used to determine differences in mean values of the reported data. Significance was determined at 95% of probability.

3. Results and discussion

Our preliminary investigations show that, during expotential growth of *Sulfolobus shibatae* without inducers on a medium containing peptone, yeast extract and mineral salts mixture, the cell density was highly correlated ($r = 0.98$) with total β-glucosidase activity of the culture (Wołosowska & Synowiecki, 2003). The final yield of the wet cells reached after 72 h of cultivation, was up to 3.4 g per litre of the growth medium. Cell fractionation by centrifugation showed that, like β-glucosidase from *Pyrococcus furiosus*, the investigated enzyme is located in the cytoplasm (Kengen, Luesink, Stams, & Zehnder, 1993).

Specific activities of β-glucosidase and β-galactosidase, determined in the cell free extract of *Sulfolobus shibatae* cells using GlcβpNp and GalβoNp as substrate, were 0.81 and 1.85 U/mg protein, respectively. Moreover, the activity changes measured towards both substrates, after each step of purification, were very similar (Table 1). Ion-exchange chromatography of the redissolved proteins, precipitated previously at 50% of the ammonium sulphate saturation, show that β-glucosidase, as well as β-galactosidase activities, were located exactly in the same fractions separated on a DEAE Sepharose CL-6B column (Fig. 1). Subsequent gel filtration on Sephadex G-200 showed only a single protein band, indicating the activity of both enzymes (Fig. 2). This suggests that the β-glucosidase and β-galactosidase activities reside in the same protein. This hypothesis was verified by polyacrylamide gel electrophoresis. The gel slice containing a single protein band was able to catalyse hydrolysis of GlcβpNp and GalβoNp with reaction rates of 0.08 and 0.13 μmol min^{-1}, respectively. This confirmed that *Sulfolobus shibatae*, like the related archaeon *Sulfolobus solfataricus*, produces β-glucosidase with a broad substrate specifity (Kengen & Stams, 1994). This enzyme was also active towards lactose and cellobiose (Table 2). However, in this case the reaction rate did not exceed 25.4 and 15.7% of the value determined for GalβoNp hydrolysis. The crude extract from *Sulfolobus shibatae* cells has relatively high specific

Table 1
Changes of specific activity during purification of β-glucosidase from *Sulfolobus shibatae* determined at 70 °C using GlcβpNp or GalβoNp as substrate[a]

Purification steps	Specific activity (U mg^{-1} protein) against		Purification (fold) for activity	
	GlcβpNp	GalβoNp	β-glucosidase	β-galactosidase
Cell extract	0.81	1.85	1.00	1.00
Precipitation of nucleic acids	0.97	2.25	1.20	1.21
$(NH_4)_2SO_4$ precipitation	1.21	2.83	1.49	1.53
Ultrafiltration (Ultracell YM)	1.24	2.92	1.53	1.58
DEAE Sepharose CL-6B	8.51	20.00	10.5	10.8
Sephadex G-200	14.2	29.5	17.5	15.9

[a] The results are mean values of two purification procedures. Standard deviations did not exceed 7% of the recorded values.

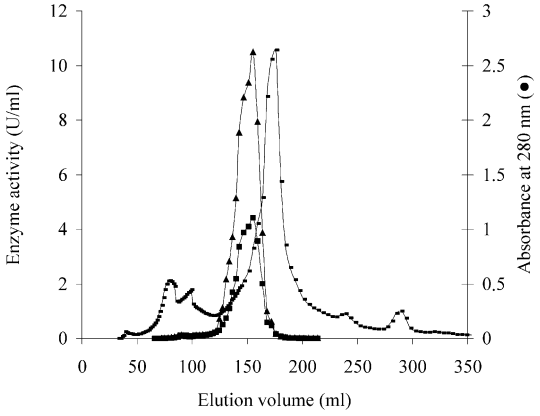

Fig. 1. Ion-exchange chromatography of a protein fraction with β-glucosidase (■) and β-galactosidase (▲) activity. Chromatography was carried out using a column (2.8×38 cm) of DEAE Sepharose CL-6B. The proteins were eluted with 0.25 M NaCl solution in 50 mM Tris–HCl (pH 7.5) at a flow rate of 80 ml/h. Absorbances at 280 nm (●) represent changes in protein content between collected fractions (4 ml).

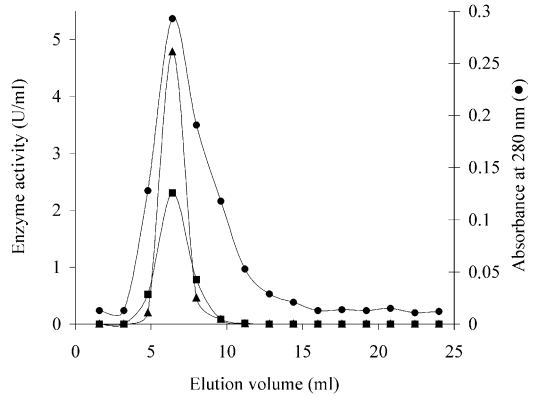

Fig. 2. Gel filtration of a protein fraction with β-glucosidase (■) and β-galactosidase (▲) activity. Chromatography was carried out using a column (2.6×38 cm) of Sephadex G-200. The enzyme was eluted with 50 mM Tris–HCl (pH 7.5) at a flow rate of 12 ml/h. Absorbances at 280 nm (●) represent changes in protein content.

Table 2
Substrate specifity of β-glucosidase from *Sulfolobus shibatae*[a]

Substrate	Rate of hydrolysis (μmol min^{-1})	Relative rate of hydrolysis (%)
GalβoNp	1.85	100
GlcβpNp	0.81	43.8
Lactose	0.29	15.7
Cellobiose	0.47	25.4

[a] The results are mean values of three replicates. Standard deviations did not exceed 5% of the recorded values.

activities towards GlcβpNp and GalβoNp (Table 1). For comparison, the specific activity of GalβoNp hydrolysis, catalysed by enzyme from *Sulfolobus solfataricus*, was only 0.12 U/mg protein (Pisani et al., 1990). The yield of β-glucosidase extracted from *Sulfolobus shibatae* cells reached the satisfactory level of about 190

Table 3
Effects of various cations on the activity of partially purified β-glucosidase from *Sulfolobus shibatae* determined using GlcβpNp or GalβoNp as substrates[a]

Cation	Residual activity (%) against substrate	
	GlcβpNp	GalβoNp
K^+	99.7±2.5a	95.9±2.3a
Ba^{2+}	93.8±2.0a	95.2±1.8a
Mg^{2+}	101.3±2.9a	100.2±2.5a
Co^{2+}	98.1±3.5a	99.2±4.6a
Cu^{2+}	64.4±2.1a	19.2±1.4b
Ca^{2+}	94.4±2.4a	95.4±4.4a
Cd^{2+}	87.9±3.3a	100.7±5.3b
Mn^{2+}	101.0±1.2a	101.0±1.2a
Fe^{2+}	83.7±2.2a	99.1±3.8b
Zn^{2+}	0.0	0.0
Hg^{2+}	0.0	0.0

[a] Means represent three determinations±standard deviations. Values in each row with the same letter are not significantly different ($P>0.05$) from one another. The cation concentrations are 1 mM.

U/g of the wet cells and was slightly lower than that reported for *Pyrococcus furiosus* (264 U/g wet cells) by Kengen et al. (1993). However, after addition of 1.0% lactose to the growth medium of *Sulfolobus shibatae*, the β-glucosidase production was increased by about 40%. The productivity of β-glucosidase in *Sulfolobus solfataricus* cells calculated according to data reported by Pisani et al. (1990), did not exceed 15 U per gramme of the cells.

The simple procedure used for β-glucosidase purification consists of nucleic acids precipitation, fractionation of proteins at 50% saturation of ammonium sulphate, ultrafiltration, ion-exchange chromatography on DEAE Sepharose CL-6B and gel filtration on Sephadex G-200. Approximately 17-fold purification, to a specific activity of 14.2 U per mg protein and 16-fold purification up to 29.5 U/mg protein, were obtained after the overall process for β-glucosidase and β-galactosidase activities, respectively. Achieved purification efficiency was similar to that (18-fold increase of specific activity) obtained by Venturi, De Lourdes-Polizeli, Terenzi, Furriel, and Jorge (2002) during separation of β-glucosidase from *Chaetomium thermophilum* on DEAE cellulose.

The investigated enzyme displayed a maximum activity at pH 5.5, similar to that determined by Kengen et al. (1993) and Pouwels et al. (2000) for β-glucosidase from *Sulfolobus solfataricus* (pH 5.4) and *Pyrococcus furiosus* (pH 5.0). A higher pH optimum (6.5) was reported for β-galactosidase from *Thermus thermophilus* (Maciuńska, Czyz-Grzybowska, & Synowiecki, 1998). The retention of about 80% of maximal activity over a wide pH range of 4.2–6.9 suggests that β-glucosidase from *Sulfolobus shibatae* may be suitable for processing of different dairy products.

A remarkable feature of β-glucosidase from *Sulfolobus shibatae* was its activity and stability at high temperatures.

The optimum temperature for the GlcβpNp and GalβoNp hydrolysis was 98 °C with about 68% of maximal activity at 105 °C. The presented optimal temperature for *Sulfolobus shibatae* β-glucosidase activity is slightly lower than that (103 °C) determined for an analogous enzyme isolated from *Pyrococcus furiosus* and higher than that of α-glucosidase (95 °C) from *Sulfolobus solfataricus* (Kengen & Stams, 1994; Rolfsmeier & Blum, 1995). To examine the stability of *Sulfolobus shibatae* β-glucosidase, the enzyme was preincubated with different buffers, temperatures and time intervals before determination of activity. Results show that the investigated β-glucosidase retained 50% of the initial activity towards GlcβpNp and GalβoNp after 146 and 127 h of incubation at 70 °C in phosphate (pH 7.2) or acetate (pH 5.5) buffers. However, the half-lifes of the enzyme activity at this temperature were decreased after preincubation in acetate buffer (pH 5.5) up to 32 and 27 h, respectively. The activity differences toward both substrates were almost not observed in the case of incubation at 98 °C and the half-lives of the enzyme at pH values of 7.2 or 5.5 were 0.9 and 0.6 h. Furthermore, the measurement of *Sulfolobus shibatae* β-glucosidse thermostability as a function of time and temperature showed that, after 5 h of preincubation at 80 °C in 0.1 M acetate buffer (pH 5.5), the enzyme retained about 67% of the initial activity (Fig. 3). For comparison, a loss of 50% of *Sulfolobus solfataricus* β-glucosidase activity was observed after 10 h of enzyme incubation (pH 6.5) at 80 °C (Pisani et al., 1990).

The β-glucosidase and β-galactosidase activities of the investigated enzyme were completely eliminated by Hg^{2+} and Zn^{2+} (Table 3). A strong inhibitory effect was also observed at 1 mM concentration of Cu^{2+} ions, while other cations only slightly changed the enzyme activity. A very low influence of Ca^{2+} and other cations, present in milk products, on activity of the investigated β-glucosidase is beneficial for its possible application in the dairy industry. None of the tested salts increased the β-glucosidase activity. This finding shows that the enzyme from *Sulfolobus shibatae* may not require metal ions for activity. This was also confirmed by the lack of inhibitory effect of EDTA or 1,10-phenanthroline, which chelates cations essential for catalytic properties of metalloenzymes. Thermostable enzymes are often resistant towards chemical treatment. Sulphydryl inhibitors, such as monoiodoacetate or

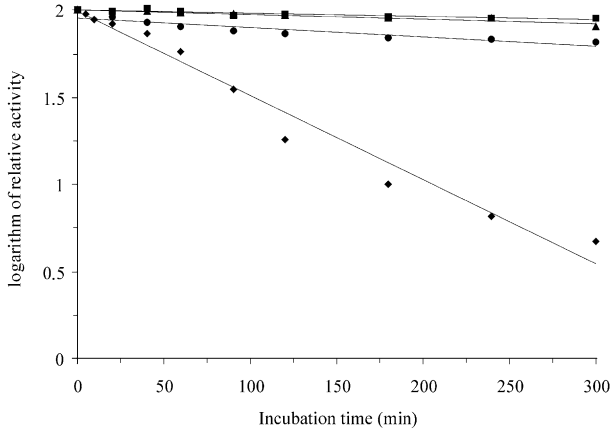

Fig. 3. Thermal stability of β-glucosidase from *Sulfolobus shibatae* incubated in 0.1 M acetate buffer (pH 5.5) at 70 °C (■), 80 °C (▲), 90 °C (●) and 98 °C (◆). The results are mean values of three replicates. Standard deviations did not exceed 5% of the recorded values.

Table 4
Effects of various inhibitors on the activity of β-glucosidase from *Sulfolobus shibatae*[a] determined using GlcβpNp or GalβoNp as substrate

Compound	Residual activity (%) against	
	GlcβpNp	GalβoNp
Phenylmethanesulphonyl fluoride	111.4±1.9[a]	113.1±4.1[a]
Monoiodoacetate	100.4±2.1[a]	97.0±1.5[a]
4-chloromercuribenzoate	9.6±1.4[a]	6.4±1.8[a]
Iodoacetamide	102.4±1.5[a]	99.5±3.2[a]
1,10-phenanthroline	94.9±2.8[a]	98.6±3.0[a]

[a] Means represent three determination±standard deviation. Values in each row with the same letter are not significantly different ($P > 0.05$) from one another. The inhibitor concentrations are 5 mM.

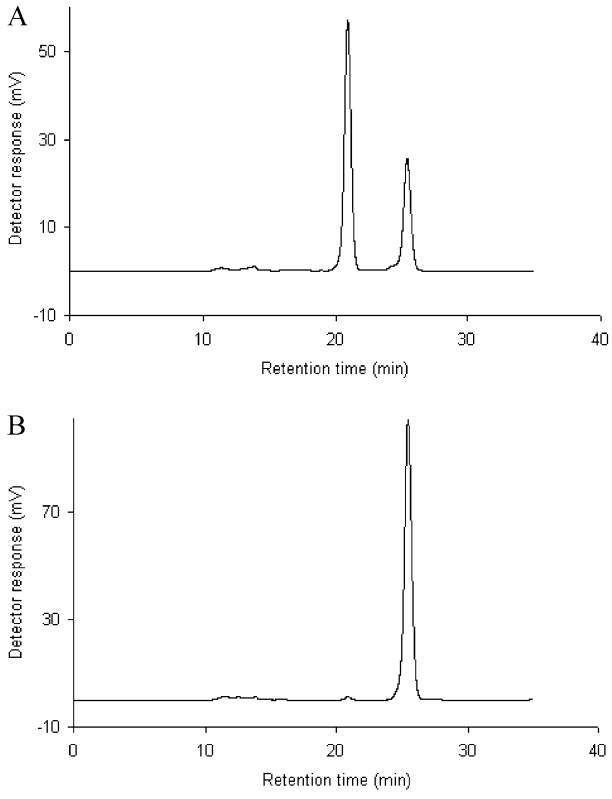

Fig. 4. Spectrum of the products (glucose-Glc) obtained after 5 min (A) and 60 min (B) of hydrolysis of 30 mM cellobiose (Cel) solution in 0.1 M phosphate/citrate buffer (pH 5.5) at 70 °C. The products were determined according to Section 2 using Polyspher® CHPB column and refractive index detector (LaChrom L-7490, Merck).

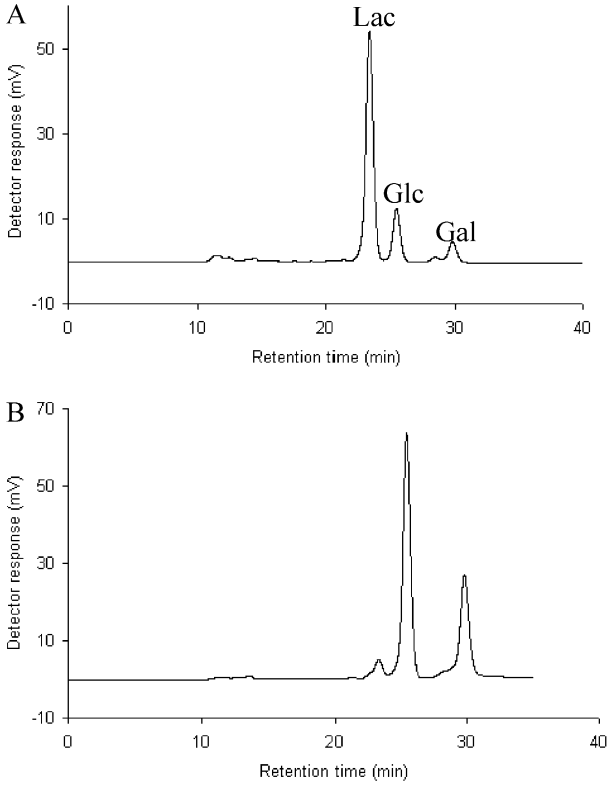

Fig. 5. Spectrum of the products (glucose-Glc, galactose-Gal) obtained after 10 min (A) and 60 min (B) of hydrolysis of lactose (Lac) solution in 0.1 M phosphate/citrate buffer (pH 5.5) at 70 °C. The HPLC was performed according to Section 2 using Polyspher® CHPB column and refractive index detector (LaChrom L-7490, Merck).

iodoacetamide, show almost no influence on the activity of β-glucosidase from *Sulfolobus shibatae* (Table 4). However, strong inhibitory effect was caused by 4-chloromercuribenzoate or Hg^{2+} (Tables 3 and 4). Observed differences may be explained by the unusual tetrameric structure of the thermostable protein, which makes sulphydryl groups not accessible for some thiol reagents. For example, the reaction of 5,5′-dithiobis(2-nitrobenzoic) acid (Elman's reagent) with thiol groups in β-glucosidase from *Sulfolobus solfataricus* was possible only after unfolding of the enzyme by SDS treatment to expose the cysteine side chains, which otherwise are not reactive (Pisani et al., 1990). Phenylmethanesulphonyl fluoride, which acts as an efficient inhibitor of enzymes with histidine and serine residues in the active site, did not significantly affect the activity of *Sulfolobus shibatae* β-glucosidase (Table 4).

The hydrolysis products obtained during action of the crude β-glucosidase on lactose or cellobiose were identified by HPLC (Figs. 4 and 5). This enzyme hydrolysed lactose to generate glucose and galactose as the main end products. Furthermore, in the case of cellobiose hydrolysis, only glucose was formed. The absence of oligosaccharides formed during condensation reactions or by reason of transferase activity of the enzyme could be explained by relatively low concentrations of the substrates used for hydrolysis.

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