α-Glucosidase and β-Glucosidase from Psychrotrophic Strain *Arthrobacter* sp. C2-2

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Abstract

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In this work six psychophilic and psychrotrophic bacterial strains were screened for the presence of different glycosidase activities (α-galactosidase, α-glucosidase, β-glucosidase, α-mannosidase and β-glucuronidase). Nine enzymes were found and their elementary characteristics were measured ($t_{optimum}$, $pH_{optimum}$, K_m, V_{lim}). Two enzymes with the highest activities at low temperatures were chosen for the next study, i.e. α-glucosidase and β-glucosidase from the psychrotrophic strain *Arthrobacter* sp. C2-2. These enzymes were purified by ammonium sulphate precipitation, by chromatography with hydrophobic interaction, and by ion-exchange chromatography. Their molecular weights (α-glucosidase – 76 kDa, β-glucosidase – 93 kDa) were determined by gel chromatography. In addition to this, it was verified that both of these enzymes are able to catalyse the transglycosylation reaction with the saccharidic donor and acceptor.

Keywords: cold-active enzyme; glycosidase; transglycosylation

A big part of our planet lays in cold territories where the temperature is usually not higher than 5°C, for example oceans, deep lake waters and polar regions. In many other territories, the average year temperature is lower than 20°C – in ground waters, springs, caves or high mountain regions (Brenchley 1996). In these territories live many organisms (prokaryotic or eukaryotic) which are adapted to low temperatures. These organisms are called psychrophiles – optimal growth temperature is about 15°C or lower, maximal growth temperature is lower than 20°C, and minimal growth temperatures are 0°C or lower – and psychrotolerants or psychrotrophes – with optimal growth temperature higher than 20°C and the ability to grow at 0°C (Morita 1975). All these organisms may produce cold-active enzymes which are able to catalyse reactions at low temperatures. Cold-active enzymes have two typical properties: a high specific activity at low temperatures and thermosensibility (Feller *et al.* 1996). The application of such enzymes in biotechnological processes can be very useful because it may decrease the cost of the heating steps, prevent microbial contamination, and increase the yields of thermo-labile compounds. The possibility to stop the reaction catalysed by cold-active enzymes by heating to mediate temperatures is of importance in the food industry where the modification of the used substrates and product is unacceptable (Huston *et al.* 2000; Cavicchioli *et al.* 2002).

Glycosidases are naturally occurring enzymes which cleave the glycosidic bonds in oligosaccharides and polysaccharides or the bonds between

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saccharide and aglycon. Some of them are also able to catalyse the transglycosylation reaction (Blanchard & Wither 2001). There are two mechanisms of the cleavage of glycosidic bond. In the first of them, which is more common, the product of the reaction has the same configuration as the substrate. In the other one, the product has the opposite configuration (Heightman & Vasella 1999). Glycosidases are essential for the life of every organism but their application can be also very useful in medicine and the pharmaceutical industry, in the textile, paper and food industries, or in agriculture (Heightman & Vasella 1999). For example, the enzymes with the ability to catalyse the transport of saccharidic residues from the donor (saccharidic or nonsaccharidic) to the acceptor (also saccharidic or nonsaccharidic) are very important in the enzymatic syntheses of oligosaccharides or other compounds with saccharidic constituents (Scigelova *et al.* 1999). The interest in the enzymatic syntheses of these compounds has been increasing in the past few years because it has been found that glycoconjugates are important for the cell life, for example in the cell-cell interaction, and precise biochemical studies can give important information for the therapies of many serious diseases (Rastall & Bucke 1992).

In our laboratory, glycosidases from psychrophilic and psychrotrophic microorganisms have been studied, for example β-galactosidase from *Arthrobacter* sp. C2-2 and α-glucosidase from *Arthrobacter* sp. C1-1. Our aim has been to screen five chosen glycosidase activities in all our psychrophilic and psychrotrophic strains and to test their ability to catalyse the transglycosylation reaction at low temperatures.

MATERIAL AND METHODS

Microorganisms and culture condition. All six bacterial strains (*Arthrobacter* sp. C1-1, *Arthrobacter* sp. C2-2, *Arthrobacter* sp. C1-2a, CH07, CO85 and GY-26) originate from the territory Jane Cal, Signy Island, and were kindly granted by professor N. J. Russell MA, PhD (Camb) from the Imperial College at Wye, University of London. All cultures were cultivated on a platform shaker at 15°C and at 150 rpm in the liquid BHI (Brain Heart Infusion) medium for 2 days.

Disintegration of bacterial cells. For the disintegration of bacterial strains, lysozyme (5 mg/ml), natrium deoxycholate (the final dilution was 0.1%), DNase (30 U/ml) and sonication (3 × 30 s, 20 W) were used. After the disintegration, the homogenate was centrifuged at 40 000 g for 20 min at 4°C.

Glucosidase screening. For the glucosidase activity screening, chromogenic substrates (*p*-nitrophenyl-α-D-glucopyranoside, *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-α-D-galactopyranoside, *p*-nitrophenyl-α-D-mannopyranoside, *p*-nitrophenyl-β-D-glucuronide) were used. The reaction mixture contained 35.7 µl chromogenic substrate in dimethylformamide (10% w/v), 35.7 µl enzyme in 0.1M phosphate buffer, pH 7 (for the samples from the strains C2-2 and GY-26), or 100 µl enzyme in 0.1M phosphate buffer, pH 7 (for the samples from the strains CH07, C1-2a and C1-1), and was made up to 321 µl with 0.1M phosphate buffer, pH 7. The reaction mixture was incubated for 15 min at 15°C. The reaction was terminated with 178 µl 10% (w/v) Na_2CO_3 and the amount of the released *p*-nitrophenol was determined at 420 nm.

Enzyme assays. The glycosidase activity was assayed using the chromogenic substrate or disaccharidic substrate.

The measurement with the use of the chromogenic substrate was the same as that used for the glucosidase screening.

Disaccharides as substrates were used only for the enzymatic assay of α-glucosidase and β-glucosidase. The reaction mixture contained 10 µl 0.2M maltose or 0.1M cellobiose and 10 µl of the enzyme sample and was incubated for 15 min at 15°C. The reaction was terminated by heating for 3 min. The amount of the glucose released was determined with the commercial kit OXOCHROME-GLUCOSE (Lachema, Czech Republic).

Ammonium sulphate precipitation. Solid ammonium sulphate was slowly added to the stirred cytosolic extract to 35% saturation after the cell disintegration at 4°C. After 45 min, the precipitate was centrifuged (20 000 g, 15 min, 4°C) and more ammonium sulphate was added to the supernatant, i.e. up to 55% saturation. After 45 min and centrifugation (20 000 g, 15 min, 4°C), the pellete containing the precipitated proteins was dissolved in a small volume of 0.02M phosphate buffer, pH 7, using 1M ammonium sulphate.

Chromatography with hydrophobic interaction. The enzyme solution after ammonium sulphate precipitation was applied to the column Butyl-Sepharose (Pharmacia, Sweden) which was equlibrated with 15 ml of 0.02M phosphate buffer, pH 7, with

1M ammonium sulphate, at a flow rate 1 ml/min. For the elution a linear gradient of ammonium sulphate (from 1M to 0M) in phosphate buffer, pH 7, at a flow rate of 0.5 ml/min was used. The fractions possessing α-glucosidase/β-glucosidase activity were pooled together and applied to ion-exchange chromatography column.

Ion exchange chromatography. Due to the high concentration of ammonium sulphate in the pooled fractions after chromatography with hydrophobic interactions, these fractions were applied to the column PD10 (Pharmacia, Sweden). The fractions from the column PD10 were applied to the ion-exchange column Hi Trap QFF (Pharmacia, Sweden), which was equlibrated with 10 ml 0.01M phosphate buffer, pH 8 (buffer A), 10 ml 0.01M phosphate buffer, pH 8, with 1M NaCl (buffer B), and 10 ml 0.01M phosphate buffer, pH 8, at a flow rate of 1 ml/min. For the elution the linear gradient of NaCl with the following steps was used: 5 ml buffer A, 3 ml (0–20%) buffer B, 12 ml (20–38%) buffer B, 10 ml (38%) buffer B, 15 ml (38–60%) buffer B, 3 ml (60–100%) buffer B, and 5 ml (100%) buffer B at a flow rate of 0.5 ml/ml. The fractions containing glucosidase activity were used to test their ability to catalyse the transglycosylation reaction.

Gel chromatography. The pooled most active fractions obtained from ion-exchange chromatography were concentrated by ultracentrifugation and then applied to the column Sephacryl S-300 (Pharmacia, Sweden) which was equlibrated with 400 ml 0.01M phosphate buffer, pH 8, with 100mM NaCl, at a flow rate of 0.5 ml/min. For the elution the same buffer was used. Due to the low purification effect, gel chromatography was used only for the determination of the molecular weight.

Transglycosylation reaction. The ability to catalyse the transglycosylation reaction was tested with the purified α-glucosidase and β-glucosidase. The pooled most active fractions resulting after chromatography with hydophobic interactions and ion-exchange chromatography were used. The samples were incubated with saccharidic substrates, α-glucosidase with maltose (0.2M in reaction mixture) and β-glucosidase with cellobiose (0.1M in reaction mixture) in 0.01M phosphate buffer, pH 8, at the laboratory temperature. At various time intervals samples were taken from the reaction mixture, the internal standart was added (galactose – to the final concentracion 0.06M) and the samples were heated for 3 minutes at 95°C to terminate the reaction. Before HPLC analysis, the samples were filtered using Centricon YM-100 (Amicon, USA).

High-performance liquid chromatography (HPLC). For the separation of saccharides after the transglycosylation reaction by HPLC, the column Supelcogel Ca (300 × 7.8 mm) was used. The experiment was carried out at 80°C at a flow rate of 0.5 ml/ml. 40 µl of each sample were applied to the column and their elution with deionised water was monitored with the refractometer RIDK 101 (Laboratorní přístroje, Czech Republic).

RESULTS

Six psychrophilic and psychrotrophic bacterial strains were tested for five glycosidase activities (α-galactosidase, α-glucosidase, β-glucosidase, α-mannosidase and β-glucuronidase). The activities were measured in the presence of chromogenic substrates after the disintegration of bacterial cells. We found 12 positive reactions (Table 1) and in

Table 1. Glycosidase activities determined in cytosolic extracts of six psychrophilic and psychrotrophic bacterial strains in presence of chromogenic substrate

Enzyme	Bacterial strain					
	CH07	CO-85	C2-1a	C1-1	GY-26	C2-2
α-Galactosidase	++	–	+	+	–	++
α-Mannosidase	–	+	–	–		++
α-Glucosidase	++	–	++	++	–	+++
β-Glucuronidase	–	–	–	–	–	
β-Glucosidase	–	–	–		++	+++

+++ = high activity, ++ = intermediate activity, + = low activity, – = no activity

Table 2. The dependence of enzyme activities at temperature determined in presence of chromogenic substrate

Enzyme and strain	% of activity		$t_{optimum}$
	at 4°C	at 15°C	
α-Galactosidase CH07	9	25	35
α-Glucosidase CH07	11	42	30
α-Glucosidasc C2-1a	10	24	35
α-Glucosidase C1-1	12	33	35
β-Glucosidase GY-26	7	18	45
β-Glucosidase C2-2	17	37	35
α-Glucosidase C2-2	25	64	25
α-Mannosidase C2-2	10	23	45
α-Galactosidase C2-2	5	18	30

9 cases the glycosidase activity was high enough to measure the elementary characteristics ($t_{optimum}$, $pH_{optimum}$, K_m, V_{lim}). The temperature profiles for all nine enzymes are presented in Table 2.

The lowest $t_{optimum}$ was determined with the enzyme α-glucosidase from *Arthrobacter* sp. C2-2 which had also the highest ratio of the activity at 4°C and the activity at $t_{optimum}$. The second highest ratio of activities was found with β-glucosidase from *Arthrobacter* sp. C2-2. These two enzymes were chosen for further experiments. The criterion for the choice was the activity at low temperatures and the ability to cleave disaccharidic substrates (maltose and cellobiose) which was necessary for testing the ability to catalyse transglycosylation reaction. In addition to this, both enzymes were stable during storage.

Both enzymes were purified by a three-stage purification process. The first step was the ammonium sulphate precipitation. Both enzymes precipitated together between 35% and 55% saturation of ammonium sulphate. The average yield of α-glucosidase was 74% (4.4 fold purification) and that of β-glucosidase 55% (3.8 fold purification). In the second step, chromatography with hydrophobic interactions was used which was also successful (α-glucosidase: yield – 58%, purification – 3.9 fold, and β-glucosidase: yield – 50%, purification – 3.4 fold), but even after this step both enzymes were contained in the same fractions. As the last purification step ion-exchange chromatography was used (α-glucosidase: yield – 71%, purification – 3.3 fold, and β-glucosidase: yield – 58%, purification – 3.6 fold). In this step α-glucosidase and β-glucosidase were separated.

Molecular weights of α-glucosidase (76 kDa) and β-glucosidase (93 kDa) were determined by gel chromatography. Due to a low purification effect, this step was not used in the purification process.

The ability of both enzymes to catalyse the transglycosylation reaction at the laboratory temperature was tested. For the reaction, purified enzymes after ion-exchange chromatography were used. Both enzymes were found to be able to transport the glycosyl residue from the saccharidic donor to the saccharidic acceptor (donor and acceptor for α-glucosidase: maltose, for β-glucosidase: cellobiose). The molar ratio of glucose and trisaccharides formed by α-glucosidase was 2:1, and by β-glucosidase 1:1.

DISCUSSION

Out of nine glycosidases detected in six psychrophilic and psychrotrophic bacterial strains, α-glucosidase and β-glucosidase from *Arthrobacter* sp. C2-2 proved to possess the most suitable properties for further studies and were chosen, consequently, for the isolation and purification.

For the purification process three steps were used. Neither with ammonium sulphate precipitation nor with chromatography with hydrophobic interaction was it possible to separate α-glucosidase and β-glucosidase, but the yields and the purification effects of both methods were good enough for them to be applied for the purificaton. The enzymes were separated by ion-exchange chromatography and the total yield after the purification process was 30.5% (56.6 fold purification) with α-glucosidase and 15.9% (46.4 fold purification) with β-glucosi-

dase. From the ability of the enzymes studied to bind to the ligand group of anex it is possible to assume that the isoeletric points of both enzymes are in the acidic part of the pH scale.

The molecular weights of both enzymes (α-glucosidase – 76 kDa, β-glucosidase – 93 kDa), as determined by gel chromatography, are comparable with the molecular weights of glucosidases from other sources presented in the database BRENDA (www.brenda.uni-koeln.de) (α-glucosidases: 27–1300 kDa, β-glucosidase: 15–450 kDa).

It was determined that both enzymes are able to catalyse the transglycosylation reaction with disaccharidic donors. The ratio of the glucose and trisaccharides formed is lower when using β-glucosidase than α-glucosidase, so β-glucosidase seems to be better for the application as the catalyser in the transglycosylation reaction.

The fact that both enzymes were able to catalyse the transport of the glycosyl residue from the saccharidic donor to the acceptor is very important for our further plans. Due to the temperature characteristics – low $t_{optimum}$ and high activity at low temperatures – it is possible to test even the thermosensitive compounds as acceptors for the glycosyl group. Our next goal is to find the genes for both enzymes, to insert them into the expression plasmide to increase their yields, and to test the ability to catalyse the transglycosylation reaction in the presence of other saccharidic and non-saccharidic acceptors.

CONCLUSION

- Five different glycosidase activities were screened in six psychrophilic and psychrotrophic bacterial strains.
- Elementary characteristics were measured for nine glycosidases with sufficiently high activities.
- Two enzymes (α-glucosidase and β-glucosidase) possessing high activities at low temperatures from the psychrotrophic strain *Arthrobacter* sp. C2-2 were chosen for further studies.
- Both enzymes were purified by ammonium sulphate precipitation, chromatography with hydrofobic interaction, and by ion-exchange chromatography.
- Molecular weights of both enzymes were estimated by gel chromatography (76 kDa with α-glucosidase and 93 kDa with β-glucosidase).
- The ability of both enzymes to catalyse the transglycosylation reaction was verified.

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