Immobilized preparation of cold-adapted and halotolerant Antarctic β-galactosidase as a highly stable catalyst in lactose hydrolysis

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Abstract

A cold-active β-galactosidase of Antarctic marine bacterium *Pseudoalteromonas* sp. 22b was synthesized by an *Escherichia coli* transformant harboring its gene and immobilized on glutaraldehyde-treated chitosan beads. Unlike the soluble enzyme the immobilized preparation was not inhibited by glucose, its apparent optimum temperature for activity was 10 °C higher (50 vs. 40 °C, respectively), optimum pH range was wider (pH 6–9 and 6–8, respectively) and stability at 50 °C was increased whilst its pH-stability remained unchanged. Soluble and immobilized preparations of Antarctic β-galactosidase were active and stable in a broad range of NaCl concentrations (up to 3 M) and affected neither by calcium ions nor by galactose. The activity of immobilized β-galactosidase was maintained for at least 40 days of continuous lactose hydrolysis at 15 °C and its shelf life at 4 °C exceeded 12 months. Lactose content in milk was reduced by more than 90% over a temperature range of 4–30 °C in continuous and batch systems employing the immobilized enzyme.

Introduction

Polar regions provide terrestrial and marine habitats for psychrophilic and psychrotrophic microorganisms. Cold-adapted enzymes produced by these microbiota have good potential for biotechnological applications because running processes at low temperatures saves energy, protects thermosensitive substances and reduces the risk of contamination by mesophilic microbial communities. Enzymes that are efficient catalysts at about 0 °C are characterized by a lower conformational stability of molecules and a higher flexibility of catalytic domains in comparison with their mesophilic counterparts (Georlette *et al.*, 2004). The profound thermolability of cold-adapted proteins favors their easy and selective thermal inactivation, which is advantageous for certain uses.

Cold-active β-galactosidases have recently been the subject of extensive studies because of potential applications in dairy industry and biotechnology. β-galactosidases (EC 3.2.1.23) catalyse hydrolytic cleavage of lactose, one of the components of milk [*c.* 4.8% (w/v) in cows' milk]. Lactose intolerance, manifested as intestinal disorders such as flatulence and diarrhea resulting from ingestion of this disaccharide, is a relatively common condition. Dairy products for those with lactose intolerance should be deprived of lactose and this can be achieved by enzymatic treatment with microbial β-galactosidases. Their commercial preparations have hitherto been derived from mesophilic microorganisms, mainly from the yeast *Kluyveromyces lactis*, and therefore they are optimally active at elevated temperatures, although this process should be carried out in the cold, during shipping and storage of dairy products. Cold-active β-galactosidases are also attractive candidates for digestion of dairy sewage at temperatures below 20 °C. Interest in the production of cold-adapted β-galactosidases has fuelled recent research into psychrophilic and psychrotrophic microorganisms populating permanently cold environments, such as Antarctic ecosystems (Sheridan *et al.*, 2000; Hoyoux *et al.*, 2001).

Our earlier study (Turkiewicz *et al.*, 2003a) showed that β-galactosidase from the psychro- and halotolerant Antarctic marine bacterium *Pseudoalteromonas* sp. 22b displayed 11–35% of its maximum activity (155 U mg^{-1} homogeneous protein at 40 °C) at 0–20 °C and degraded lactose in refrigerated milk and its products. It was not inhibited by calcium ions and was activated by sodium ions (40% activation by 5 mM Na^{+}). The gene encoding *Pseudoalteromonas* sp. 22b β-galactosidase was identified as described elsewhere (Cieśliński *et al.*, 2005; GenBank accession number AY 498873). The three-dimensional structure of the

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enzyme was modeled *in silico* on the basis of gene sequence analysis, and possible determinants of kinetic adaptation to cold were identified (Tkaczuk *et al.*, 2005). Because the maximum biosynthetic yield of this β-galactosidase by the Antarctic bacterium reached only 0.3 $mg\,L^{-1}$ of culture medium (achieved at 6 °C), its gene was isolated and expressed in a mesophilic host (*Escsherichia coli* ER2566; our method) (Cieśliński *et al.*, 2005). The latter grew much faster and produced 3–4 mg of Antarctic β-galactosidase per liter of culture medium. The properties of wild-type and recombinant β-galactosidases were identical.

This work focused on characterization of the immobilized recombinant enzyme and its application for hydrolysis of lactose at 4, 15 and 30 °C in continuous and batch systems. Binding of enzymes to solid matrices is a convenient method of production of stable catalysts, which can be used many times and easily separated from reaction product(s). In addition, β-galactosidases were coupled to various matrices, such as cellulose (Roy & Gupta, 2003), glycophase-coated porous glass (Manjon *et al.*, 1985), silicon membranes and PCV (Bakken *et al.*, 1992),glutaraldehyde-treated chitosan (Sheu *et al.*, 1998), Ca-alginate (Beccera *et al.*, 2001), nylon (Portaccio *et al.*, 1998) and Sepharose CL-6B (Fernandes *et al.*, 2002).

Our earlier study revealed that chitosan modified with-glutaraldehyde was superior to other carriers used for immobilization of the *Pseudoalteromonas* sp. 22b β-galactosidase (Turkiewicz *et al.*, 2003b). Coupling to other matrices, such as porous glass, Ca-alginate, Whatman cellulose and polyvinyl alcohol, gave poorer results (low recovery of activity, leakage of enzyme from the matrix, low stability of the preparation). Chitosan [poly-β-(1-4)-2-amino-2-deoxy-D-glucose] is a cheap, chemically inert, hydrophilic and biocompatible material, frequently used as a matrix for immobilization of enzymes (Bodalo *et al.*, 1998; Cetinus & Oztop, 2003).

Materials and methods

Crude preparation of β-galactosidase

The gene encoding *Pseudoalteromonas* sp. 22b β-galactosidase was expressed in an *E. coli* ER2566 host (Cieśliński *et al.*, 2005). The recombinant *E. coli* strain was cultured at 20 °C until it reached a cell density ($A_{600\,nm}$) of 1.5–1.8 in liquid LB culture medium (1% peptone, 0.5% yeast extract, 1% NaCl) supplemented with ampicillin (100 $\mu g\,mL^{-1}$). Biosynthesis of β-galactosidase was induced by 1 mM IPTG (isopropyl-β-D-thiogalactoside) added to the culture medium when $A_{600\,nm}$ reached 0.6.

Cells were pelleted by centrifugation (45 min, 10 000 ***g***, 4 °C), washed with 0.05 M sodium phosphate buffer (pH 7.6, supplemented with 200 mM Mg^{2+} and 15 mM EDTA), suspended in this buffer (1 g of wet mass per 2 mL of the buffer) and sonicated (Vibrocell 72840, Bioblock; twice for 2.5 min at 0 °C). The homogenate was centrifuged (45 min, 10 000 ***g***, 4 °C) and the supernatant was used as a crude preparation of β-galactosidase (the pellet was discarded).

β-galactosidase assay

β-galactosidase activity in hydrolysis of ONPG (*o*-nitrophenyl-β-D-galactopyranoside; 10 mM) and lactose was determined at 30 °C and pH 7.6 (Turkiewicz *et al.*, 2003a). One unit of enzyme activity (1 U) represented one micromole of product (*o*-nitrophenol or glucose) released from the respective substrate in 1 min under standard reaction conditions. Specific activity of soluble and immobilized enzyme was expressed in $U\,mg^{-1}$ of protein, and activity of immobilized β-galactosidase was expressed in $U\,g^{-1}$ of the carrier.

Immobilization

Chitosan purchased from the Marine Fishery Institute (Gdynia, Poland) was used as a matrix for enzyme immobilization. A 2% chitosan solution in 2% acetic acid was extruded as small drops and stirred into a 10% pentasodium triphosphate solution (pH 8.9). The beads (1–1.5 mm in diameter) were hardened in the latter solution (gentle stirring, 24 h, 4 °C), separated by filtration through nylon membrane, suspended in a 0.1% solution of glutaraldehyde (Sigma) and incubated for 2 h to activate amino groups of chitosan. The beads were then separated by filtration, washed five times with distilled water, suspended in β-galactosidase solution (cell-free extract from the *E. coli* transformant), incubated for 24 h at 4 °C (gentle stirring) and separated by filtration. The residual β-galactosidase activity in ONPG hydrolysis and soluble protein concentration (Lowry *et al.*, 1951) were determined in the filtrate. The immobilized preparation was washed several times with 0.05 M potassium phosphate buffer, pH 7.6, and kept in this buffer at 4 °C.

Chitosan beads with increased porosity were formed as described above from the solution of chitosan supplemented with another polymer [2% polyvinyl alcohol (PVA) or polyvinylpyrrolidone (PVP)], treated with glutaraldehyde, washed with distilled water for 48 h and used for enzyme immobilization as described above.

Estimation of carrier porosity

Enzyme-bearing chitosan beads were treated with 1% glutaraldehyde (for 2 h at room temperature), washed with potassium phosphate buffer, pH 8.0, fixed with 1% osmic acid and stained with a solution of uranyl acetate in ethanol. They were then suspended in Epon/Spurr resin and entrapped in wells. On completion of polymerization, the

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beads were sliced with a Reichart ultratome. The slices were stained with 2% uranyl acetate and analysed using a Jeol 1010 electron microscope at 80 kV.

Lactose hydrolysis

Lactose hydrolysis by immobilized Antarctic β-galactosidase was carried out at 4, 15 and 30 °C in a column reactor (with substrate recycling), as shown in Fig. 1. The enzyme/substrate ratio was 30 U of β-galactosidase activity (in ONPG hydrolysis) per gram of lactose. Milk processed on the column was supplemented with 0.02% sodium azide to prevent microbial contamination and the rate of its flow through the bed was maintained at 1 mL min^{-1}. Progress of lactose hydrolysis was followed by determination of glucose concentration in periodically withdrawn samples of eluate (1 mL) from the column. The samples were deproteinated with 5% trichloroacetic acid and glucose was measured through conversion with glucose oxidase, which was coupled to reduction of concomitantly formed hydrogen peroxide and oxidation of 4-amino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one (4-aminoantipyrine) by peroxidase (a commercial diagnostic kit, POCh). In a second variant of the hydrolysis conditions, chitosan beads with immobilized β-galactosidase were placed in a nylon bag and immersed in milk. Lactose hydrolysis was conducted at 4 °C (in a refrigerator) under stationary conditions using 144 U of enzyme activity per 1000 mL of milk.

Stability of the immobilized preparation

To evaluate the storage stability of immobilized β-galactosidase, its activity (measured as hydrolysis of ONPG and lactose) was assayed immediately after coupling to the matrix and after maintaining the preparation at 4 °C for 12 months.

The operational stability of the immobilized preparation was assayed as follows:

(1) A portion of immobilized preparation was applied for 15 successive 10-min processes of ONPG (10 mM) hydrolysis carried out at 30 °C and pH 7.6. On completion of 10 min of the reaction, the beads were separated from hydrolysis products by filtration through the nylon membrane, washed three times with the buffer, pH 7.6, and reused. The operational stability was determined on the basis of a decrease in ONPG concentration within 10 min.

(2) The process of continuous hydrolysis of lactose in milk was carried out at 15 °C for 40 days in a column reactor packed with β-galactosidase immobilized on glutaraldehyde-treated chitosan beads (conditions as described above). The operational stability was evaluated on the basis of glucose concentration in samples of eluate from the column.

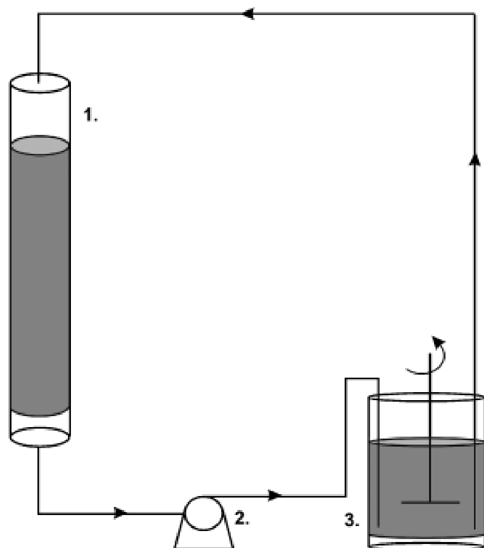

Fig. 1. The system with substrate recycling used for continuous hydrolysis of lactose in milk. (1) Column packed with immobilized β-galactosidase; (2) peristaltic pump; (3) tank with milk.

Enzyme characterization

The temperature optimum of β-galactosidase (in ONPG hydrolysis) was determined by measuring its activity over a temperature range between 0 and 70 °C at pH 7.6. The pH optimum for enzyme activity was estimated on the basis of assays carried out at 30 °C and over a pH range of 4–11. Thermostability of β-galactosidase was evaluated by enzyme incubation for 60 min at temperatures of 0–70 °C followed by assays of residual activity under standard conditions (pH 7.6, 30 °C, 10 mM ONPG). The pH stability of the enzyme was determined by measuring residual activity (30 °C, pH 7.6, 50 mM sodium phosphate buffer) after preincubating (for 60 min at 4 °C) in 10 mM Britton–Robinson buffer solutions, pH 4–11. The effect of NaCl on stability of β-galactosidase was estimated by incubation with 0.5–6 M NaCl for 24 h at 4 °C, followed by assays of residual activity under standard conditions. Values of the Michaelis–Menten constant (K_m) and maximum reaction velocity (V_{max}) were determined for ONPG concentrations of 0.05–50 mM under standard reaction conditions (30 °C, pH 7.6). Effects of glucose and galactose on activity of β-galactosidase were evaluated for concentrations of these sugars ranging up to 150 mM. The concentration of 150 mM was equivalent to the sum of glucose and galactose released by complete hydrolysis of 4.8% lactose. Samples of the soluble (cell-free extract) and immobilized enzyme were incubated for 24 h at 4 °C with solutions of these two sugars (containing one or both of them) and the residual activity in ONPG hydrolysis was estimated under standard conditions. To determine the type of inhibition of the soluble β-galactosidase by glucose, enzyme activity was assayed for 0.05–50 mM ONPG and 150 mM glucose.

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Statistical analysis

All measurements and/or experiments were conducted in triplicate. Results are presented as mean ± SD. Data were analysed using one-way ANOVA to test for significant differences at the significance level (P) of 0.05 between batches of the immobilized and soluble β-galactosidase.

Results and discussion

Comparison of properties of soluble and immobilized Antarctic β-galactosidase

As mentioned in the Introduction, the yield of β-galactosidase synthesis by *Pseudoalteromonas* sp. 22b was very low and *E. coli* ER2566 was used to produce this enzyme. Activity of β-galactosidase in ONPG hydrolysis in cell-free extracts from the *E. coli* transformant was higher ($126\,U\,mL^{-1}$, $12.4\,U\,mg^{-1}$ protein) than in cell-free extracts from *Pseudoalteromonas* sp. 22b ($3.4\,U\,mL^{-1}$, $5.44\,U\,mg^{-1}$ protein). The immobilized preparation of the latter protein was *c*. 10-fold less active than that of the recombinant enzyme (1.1 and $12.0\,U\,g^{-1}$ chitosan, respectively). The specific activity of the immobilized β-galactosidase from the *E. coli* transformant ($3.7\,U\,mg^{-1}$ protein) was more than threefold lower than that of the soluble enzyme. The recovery of enzyme activity in the immobilized preparation was close to 36% and *c*. 30% of the original activity was unbound to the matrix and retained in the filtrate after separation of the beads. No statistically significant differences (at $P = 0.05$) were observed between the examined batches of soluble and immobilized β-galactosidase. Results of the presented assays aimed at characterization of both these preparations differed by only 4–5% (shown using error bars in the following figures).

The retention of Antarctic β-galactosidase activity approached *c*. 36% irrespective of the porosity of chitosan beads used as a matrix for immobilization (beads with increased porosity were produced by supplementing chitosan with PVA or PVP). The mechanical properties of the latter beads were much poorer and they were easily deformed or even fractured, particularly when lactose hydrolysis was carried out under conditions of agitation. Recovery of activity for other immobilized β-galactosidases was reported as 60–70% (Fernandes *et al*., 2002), *c*. 55% (Sheu *et al*., 1998; Albayrak & Yang, 2002; Tanriseven & Dogan, 2002), 16–80% (Bodalo *et al*., 1998) and *c*. 90% (Szczodrak, 2000). The incomplete recovery of enzyme activity can be ascribed either to disadvantageous conformational changes in protein molecules caused by formation of covalent bonds between enzyme and carrier or to diffusional limitations between the solid immobilized enzyme preparation and the substrate. Electron microscope images of sliced chitosan beads bearing Antarctic β-galactosidase revealed that the size of matrix pores was three orders of magnitude larger (0.6–1.3 μm) than the diameter of *E. coli* β-galactosidase molecules (0.15×0.05 nm) (Manjon *et al*., 1985). The internal diameter of matrix pores providing free diffusion of lactose to the latter enzyme is *c*. 15 nm (Manjon *et al*., 1985). Because three-dimensional structures of *Pseudoalteromonas* sp. 22b and *E. coli* β-galactosidases are similar (Tkaczuk *et al*., 2005) it appears that the decrease in activity caused by immobilization of the Antarctic enzyme was not a result of the limited substrate diffusion. Most probably, it was brought about by changes in conformation of a part of the enzyme molecules linked to the matrix.

This assumption was confirmed by the more than sixfold lower affinity of the immobilized Antarctic β-galactosidase for ONPG relative to the soluble protein (K_m^{-1} of 1.12 and $7.14\,mM^{-1}$, respectively). Immobilization frequently changes the affinity of enzyme for substrate. For instance, binding of *Thermus* sp. T2 β-galactosidase to epoxy-Sepabeads brought about a 2.5-fold increase in K_m (from 3.1 to 7.7 mM) for ONPG (Pessela *et al*., 2003). However, molecules of this thermophilic enzyme are more rigid and tightly packed, and presumably less susceptible to deformation caused by binding to a carrier as compared with the psychrophilic protein. It is of note that the soluble *Pseudoalteromonas* sp. 22b β-galactosidase displays more than 20-fold higher affinity for ONPG at optimum temperature for activity (K_m of 0.14 mM at 40 °C) than *Thermus* sp. T2 enzyme (K_m of 3.1 mM at 70 °C). Although this difference is reduced for their immobilized preparations, the K_m for ONPG of Antarctic β-galactosidase is ninefold smaller (0.89 and 7.7 mM, respectively). The significant decrease in affinity for ONPG was also caused by immobilization of *E. coli* β-galactosidase on ethylenediamine-coated porous glass and calcium alginate (K_m increased from 0.2 to 1.64 and 0.44 mM, respectively) (Manjon *et al*., 1985). However, when the latter enzyme was covalently bound to silanized porous glass its affinity for ONPG was almost unchanged.

Another result of *Pseudoalteromonas* sp. 22b β-galactosidase immobilization was an increase of 10 °C (from 40 to 50 °C) in the apparent optimum temperature for activity (Fig. 2a) whereas the profile of activity at 0–20 °C remained unaltered. The upshift in the apparent optimum temperature for activity (from 35 to 50 °C) was also observed for *Kluyveromyces fragilis* β-galactosidase covalently immobilized on silanized glass (Szczodrak, 2000). By contrast, the optimum temperature for activity of *E. coli* β-galactosidase immobilized on glycophase-coated glass was 10 °C lower than that of the soluble enzyme (45 and 55 °C, respectively) whereas its relative activity at 25–45 °C was increased (Manjon *et al*., 1985).

It is of note that the immobilized β-galactosidase displayed low but evident activity at 70 °C, unlike the soluble enzyme, which was completely inactivated at this

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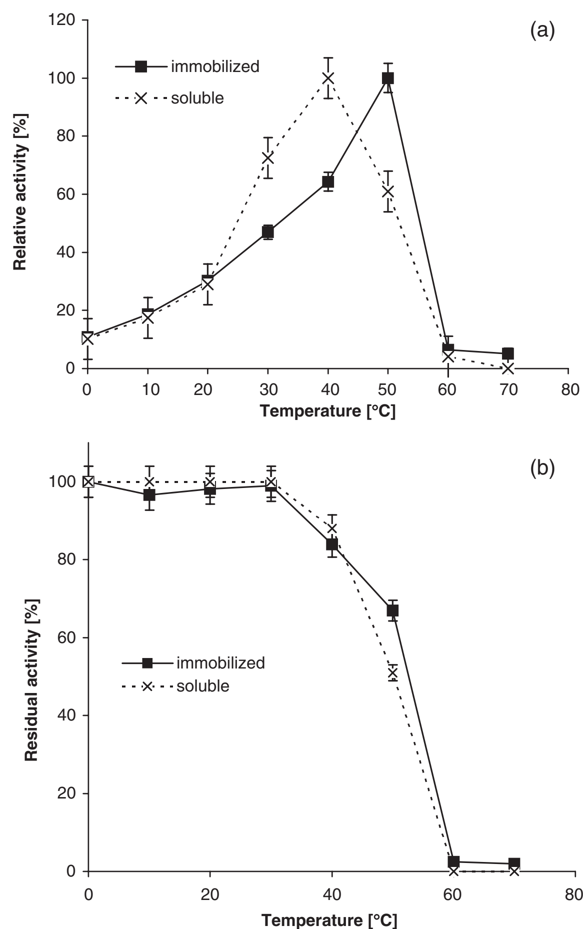

Fig. 2. Thermodependence of activity (a) and stability (b) of the immobilized and soluble Antarctic β-galactosidase.

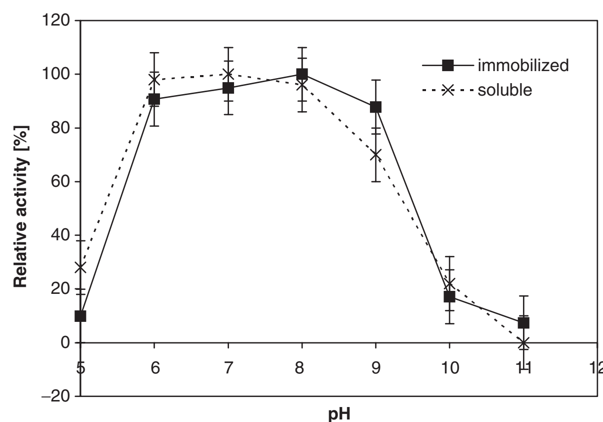

Fig. 3. Activity of soluble and immobilized Antarctic β-galactosidase as a function of pH.

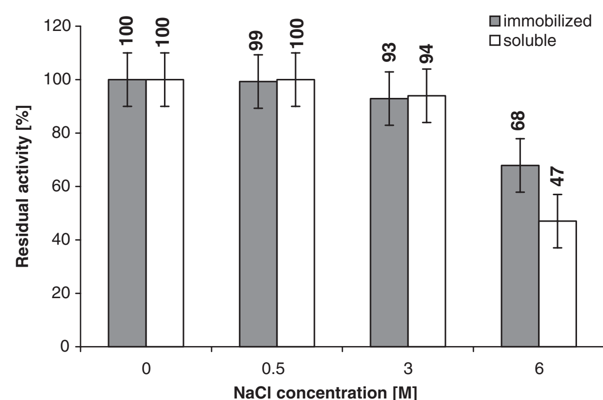

Fig. 4. The effect of NaCl on activity of immobilized and soluble Antarctic β-galactosidase.

temperature. Thermostability of the Antarctic β-galactosidase was also slightly enhanced by immobilization. Both preparations were completely stable up to 30 °C (Fig. 2b). However, the immobilized enzyme displayed *c.* 70% of the initial activity after 1 h incubation at 50 °C whereas this was only 50% for the soluble enzyme. The range of optimum pH for activity was slightly wider after immobilization, i.e. pH 6–8 for the soluble enzyme vs. pH 6–9 for the immobilized preparation (Fig. 3). Their pH stability was the same (data not shown). The immobilized Antarctic β-galactosidase was more stable in concentrated solutions of NaCl: it retained *c.* 68% of the initial activity after 24 h incubation in 6 M NaCl whereas the soluble enzyme retained only around 47% (Fig. 4).

Effects of lactose hydrolysis products on activity of Antarctic β-galactosidase

The soluble *Pseudoalteromonas* sp. 22b β-galactosidase was not inhibited by galactose, which is the competitive inhibitor of many microbial β-galactosidases, but it was inhibited by glucose (Fig. 5a). Its activity was reduced more than twofold after 24 h incubation at 4 °C in 150 mM glucose solution whereas in equimolar solution of glucose and galactose (sum of their concentrations was 150 mM) a decrease in enzyme activity of 35% was observed. Glucose was a noncompetitive inhibitor given that K_m for ONPG was unchanged (0.14 mM at 30 °C) in the presence of glucose in the reaction mixture while the maximum reaction velocity was reduced threefold (from 12.4 to 4.1 U mg^{-1} protein in cell-free extract of transformant *E. coli* cells; Fig. 6).

The immobilized Antarctic β-galactosidase was inhibited neither by glucose (Fig. 5b) nor by galactose. Arguably, the conformational changes resulting from binding to chitosan occur not only in the active site of Antarctic β-galactosidase but also in its allosteric center. The occurrence of the latter in *E. coli* β-galactosidase and its homologs from other microorganisms was postulated by Ladero *et al.* (2000). Therefore glucose, a strong noncompetitive inhibitor of the soluble Antarctic β-galactosidase (45–50% decrease in

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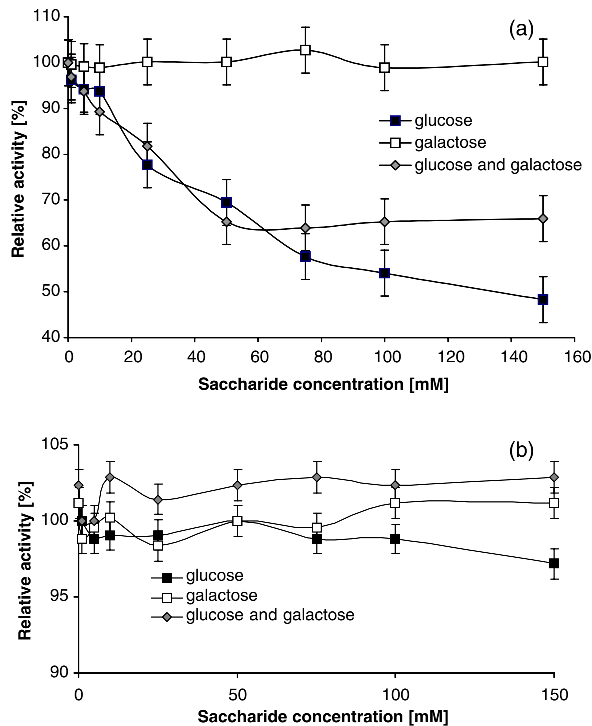

Fig. 5. Effects of glucose and galactose concentration on activity of soluble (a) and immobilized (b) Antarctic β-galactosidase.

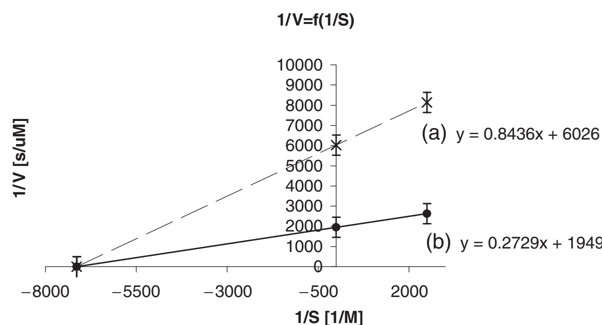

Fig. 6. Kinetics of ONPG hydrolysis by soluble Antarctic β-galactosidase in the presence (a) and absence (b) of glucose.

activity for 75–150 mM glucose), does not inhibit the immobilized preparation.

This advantageous change considerably increases the technological value of immobilized Antarctic β-galactosidase. A similar phenomenon was observed after immobilization of *Thermus* sp. T2 β-galactosidase on epoxy-Sepabeads, which abolished the noncompetitive inhibition by glucose and the competitive inhibition by galactose (the latter was not observed for our Antarctic enzyme) (Pessela *et al.*, 2003). In addition, covalent immobilization of *K. lactis* β-galactosidase almost completely abolished its inhibition by glucose (inhibition by galactose was not abolished; Mateo *et al.*, 2004). However, immobilization of this enzyme on silica-alumina gel had no effect on the character of inhibition by lactose-hydrolysis products, as in the case of the chimeric *E. coli* β-galactosidase bearing the choline-binding domain from *Pneumococcus pneumoniae* autolysin and immobilized on the same carrier (Ladero *et al.*, 2000).

Stability of the immobilized β-galactosidase

The shelf life at 4 °C of the Antarctic β-galactosidase immobilized on glutaraldehyde-treated chitosan exceeded 12 months. The operational stability of this preparation was also good. Its activity was maintained within 40 days of continuous lactose hydrolysis in milk, conducted at 15 °C in a column reactor.

Stability of other immobilized β-galactosidases was also relatively high. For example, *E. coli* β-galactosidase bound to glycophase-coated porous glass lost only 20% activity after 254 days of storage at 4 °C but its activity was halved after 20 days of lactose hydrolysis at 55 °C (Manjon *et al.*, 1985). The half-life of *Aspergillus oryzae* β-galactosidase coupled to tosylated cotton cloth was *c.* 50 days at 50 °C and more than 1 year at 40 °C (Albayrak & Yang, 2002).

Hydrolysis of lactose in milk by immobilized β-galactosidase

Over the temperature range 4–30 °C, the immobilized Antarctic β-galactosidase efficiently hydrolysed lactose in milk. The degree of lactose digestion reached 93% in a continuous system (column reactor) at an enzyme/substrate ratio of 30 U per gram of lactose within 8, 18 and 24 h at 30, 15 and 4 °C, respectively (Fig. 7). This result is comparable with that achieved for immobilized preparations of

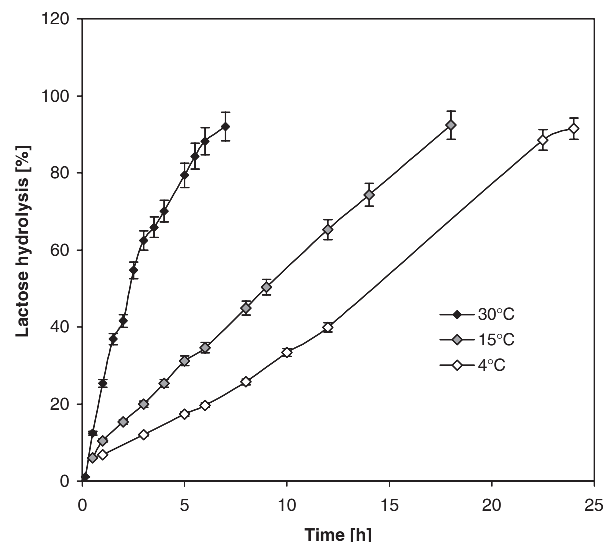

Fig. 7. Lactose hydrolysis progress during continuous milk treatment by the immobilized Antarctic β-galactosidase in a column reactor with substrate recycling (see Fig. 1).

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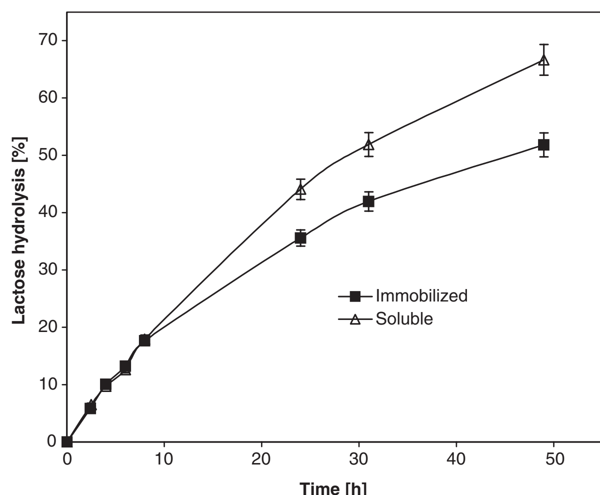

Fig. 8. Lactose hydrolysis progress during milk treatment by the immobilized and soluble Antarctic β-galactosidase at 4 °C under static conditions (see Materials and methods).

β-galactosidases of *Thermus* sp. T2 (Pessela *et al.*, 2003) and *K. fragilis* (Szczodrak, 2000) (lactose concentration was reduced by 99% and 90%, respectively). Immobilized β-galactosidases of *K. lactis* (Zhou & Chen, 2001) and *Pseudoalteromonas* sp. TAE 79 (cold-adapted) (Fernandes *et al.*, 2002) were less efficient in this respect (lactose content was reduced by 70% and 53%, respectively). Under static conditions, which are unfavorable for penetration of chitosan beads by substrate molecules and diffusion of hydrolysis products, the immobilized preparation of Antarctic β-galactosidase, when placed in a nylon bag, has proven to be excellent for lactose degradation in refrigerated milk. The relatively high (close to 53%) degree of lactose decomposition was achieved in 48 h at 4 °C (Fig. 8).

In summary, our results demonstrate that the cold-active *Pseudoalteromonas* sp. 22b β-galactosidase synthesized by a mesophilic host and immobilized on glutaraldehyde-treated chitosan beads can be applied for digestion of lactose in dairy products at low temperatures.

References

Albayrak N & Yang ST (2002) Immobilization of *Aspergillus oryzae* β-galactosidase on tosylated cotton cloth. *Enz Microb Technol* **31**: 371–383.

Bakken AP, Hill CG & Amundson CH (1992) Hydrolysis of lactose in skim milk by immobilized β-galactosidase (*Bacillus circulans*). *Biotechnol Bioeng* **39**: 408–417.

Beccera M, Baroli B, Fadda A, Blanco Mendez J & Gonzales MI (2001) Lactose bioconversion by calcium-alginate immobilization of *Kluyveromyces lactis* cells. *Enz Microb Technol* **29**: 506–512.

Bodalo A, Gomez E, Gomez JL, Bastida J, Maximo MF & Diaz F (1998) A comparison of different methods of β-galactosidase immobilization. *Proc Biochem* **26**: 349–353.

Cetinus SA & Oztop HN (2003) Immobilization of catalase into chemically crosslinked chitosan beads. *Enz Microb Technol* **32**: 889–894.

Cieśliński H, Kur J, Białkowska A, Baran I, Makowski K & Turkiewicz M (2005) Cloning, expression, and purification of a recombinant cold-adapted beta-galactosidase from Antarctic bacterium *Pseudoalteromonas* sp. 22b. *Protein Expr Purif* **39**: 27–39.

Fernandes S, Geueke B, Delgado O, Coleman J & Hatti-Kaul R (2002) β-galactosidase from a cold-adapted bacterium: purification, characterization and application for lactose hydrolysis. *Appl Microbiol Biotechnol* **58**: 313–321.

Georlette D, Blaise V, Collins T, D'Amico S, Gratia E, Hoyoux A, Marx J-C, Sonan G, Feller G & Gerday C (2004) Some like it cold: biocatalysis at low temperatures. *FEMS Microbiol Rev* **28**: 25–42.

Hoyoux IJ, Dubois P, Genicot S, Dubail F, François JM, Baise E, Feller G & Gerday C (2001) Cold-adapted β-galactosidase from the Antarctic psychrophile *Pseudoalteromonas haloplanktis*. *Appl Environ Microbiol* **67**: 1529–1535.

Ladero M, Santos A, Garcia JL & Garcia-Ochoa F (2000) Activity over lactose and ONPG of a genetically engineered β-galactosidase from *Escherichia coli* in solution and immobilized: kinetic modelling. *Enz Microb Technol* **29**: 181–193.

Lowry OH, Rosbrough NJ, Farr AL & Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275.

Manjon A, Llorca FI, Bonete MJ, Bastida J & Iborra JL (1985) Properties of β-galactosidase covalently immobilized to glycophase-coated porous glass. *Proc Biochem* **20**: 17–22.

Mateo C, Monti R, Pessela BC, Fuentes M, Torres R, Guisan JM & Fernandez-Lafuente R (2004) Immobilization of lactase from *Kluyveromyces lactis* greatly reduces inhibition promoted by glucose. Full hydrolysis of lactose in milk. *Biotechnol Prog* **20**: 1259–1262.

Pessela BC, Mateo C, Carrascosa AV, Vian A, Garcia JL, Rivas G, Alfonso C, Guisan JM & Fernandez-Lafuente R (2003) One-step purification, covalent immobilization, and additional stabilization of a thermophilic ply-His-tagged beta-galactosidase from *Thermus* sp. Strain T2 by using novel heterofunctional chelate-epoxy Sepabeads. *Biomacromolecules* **4**: 107–113.

Portaccio M, Stellato S, Rossi S, Bencivenga U, Mohy Eldin MS, Gaeta FS & Mita DG (1998) Galactose competitive inhibition of β-galactosidase (*Aspergillus oryzae*) immobilized on chitosan and nylon supports. *Enz Microb Technol* **23**: 101–106.

Roy I & Gupta MN (2003) Lactose hydrolysis by lactozym™ immobilized on cellulose beads in batch and fluized bed modes. *Proc Biochem* **39**: 325–332.

Sheridan P, Panasik N, Coombs J & Brenchley J (2000) Approaches to deciphering the structural basis of low

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temperature enzyme activity. *Biochem Biophys Acta* **1543**: 413–429.
Sheu DC, Li SY, Duan KJ & Chen CW (1998) Production of galactooligosaccharides by β-galactosidase immobilized on glutaraldehyde-treated chitosan beads. *Biotechnol Techn* **12**: 273–276.
Szczodrak J (2000) Hydrolysis of lactose in whey permeate by immobilized β-galactosidase from *Kluyveromyces fragilis*. *J Mol Cat B* **10**: 631–637.
Tanriseven A & Dogan S (2002) A novel method for the immobilization of β-galactosidase. *Proc Biochem* **38**: 27–30.
Tkaczuk KL, Bujnicki JM, Białkowska A, Bielecki S, Turkiewicz M, Cieśliński H & Kur J (2005) Molecular modelling of a psychrophilic beta-galactosidase. *Biocatal Biotransform* **23**: 201–209.
Turkiewicz M, Białkowska A, Kalinowska H & Bielecki S (2003a) Antarctic marine bacterium *Pseudoalteromonas* sp. 22b as a source of cold-adapted β-galactosidase. *Biomol Eng* **20**: 317–324.
Turkiewicz M, Makowski K, Białkowska A & Szczęsna-Antczak M (2003b) The immobilized Antarctic β-galactosidase and its application for lactose hydrolysis. *11th European Congress on Biotechnology, 24–29 August 2003*. Basel, Switzelrland. Abstracts, p. 710.
Zhou QZK & Chen XD (2001) Immobilization of β-galactosidase on graphite surface by glutaraldehyde. *J Food Eng* **48**: 69–74.

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