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Thermostable *Pyrococcus woesei* β-D-galactosidase — high level expression, purification and biochemical properties

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The gene encoding β-D-galactosidase from *Pyrococcus woesei* was PCR amplified, cloned, expressed in *Escherichia coli* under the control of an inducible T7 promoter, purified and characterized. The expression system was developed by the construction of recombinant plasmid, based on the high copy number pUET1 vector, giving four times more efficient expression of *P. woesei* β-D-galactosidase (20 mg of enzyme from 1 liter of culture) than that obtained from a previously constructed one. The recombinant enzymes were purified in a two-step procedure: double heat-denaturation of *E. coli* cell proteins and affinity chromatography on *p*-aminobenzyl 1-thio-β-D-galactopyranoside–agarose. To achieve efficient purification of *P. woesei* β-D-galactosidase by immobilized metal-ion affinity chromatography (IMAC), a His-tag was placed either at the N- or the C-terminal of the coding sequence. The obtained fusion proteins revealed the same specific activity of approximately 5400 U/mg, which was 10 times lower than the wild-type β-D-galactosidase (51100 U/mg). The activity of *P. woesei* β-D-galactosidase was enhanced by thiol compounds, Mg^{2+} ions and D-galactose, and was inhibited by heavy metal ions and D-glucose, while Ca^{2+} ions had no effect.

Keywords: β-D-galactosidase, *Pyrococcus woesei*, *Escherichia coli*, expression system, purification

β-Galactosidases (EC 3.2.1.23) catalyze the hydrolysis of β-1,4-D-galactosidic linkages. These enzymes are widely distributed in nature, being found in numerous microorganisms, plant and animal tissues (Shukla, 1975). The most commonly found natural substrate for β-galactosidases is lactose, the main sugar of milk and dairy products. A large number of people suffer from intestinal dysfunctions after consumption of milk (Rosado *et al*., 1987). Moreover, lactose is poorly soluble and with a low sweetness compared to its hydrolysis products: glucose and galactose. Some of β-galactosidases are able to disrupt the β-1,4 bond and form other covalent bonds, as β-1,6, β-1,3, and β-1,2 (Mahoney, 1998), and then, during the hydrolysis of lactose by such β-galactosidases, some galactosyl-oligosaccharides are formed. These compounds are indigestible, acting as dietary fibre, and they promote the growth of bifidobacteria in the intestine, with the subsequent health-enhancing effect in the intestine and the liver (Crittenden & Playne, 1996; Mahoney, 1998; Sako *et al*., 1999). Commercial β-galactosidases are produced by GRAS (generally recognized as safe by the FDA) microorganisms (yeasts of the genus *Kluyveromyces* and fungis of the genus *Aspergillus*). The enzymes from yeast are active at neutral pH and, thus, employed in milk and sweet whey. The enzymes from fungi are active at acidic pH and are used to hydrolyse lactose in acidic whey (Gekas & Lopez Leiva, 1985). The activity of these enzymes has been well characterized, both when they are employed for the hydrolysis of lactose (Papayannakos *et al*., 1993; Portaccio *et al*., 1998; Santos *et al*., 1998; Ladero *et al*., 2000; Jurado *et al*., 2002) and for the synthesis of oligosaccharides (Lopez Leiva & Guzman, 1995; Mahoney, 1998; Reuter *et al*., 1999; Boon *et al*., 2000). The main drawback that hinders the utilization of enzymes as industrial catalysts is their low stability. To overcome this problem, several approaches have been proposed, including immobilization of the enzyme, and the screening for enzymes from extremophilic microorganisms. A

Abbreviations: FDA, Food and Drug Administration; GRAS, generally recognized as safe by the FDA; IMAC, immobilized metal-ion affinity chromatography; IPTG, isopropyl-β-D-thiogalctopyranoside; ONPG, *o*-nitrophenyl-β-D-galactopyranoside.

continuous-flow reactor with immobilized enzyme assures more efficient lactose hydrolysis compared to batch treatments with free enzyme, because in an immobilized enzyme system the hydrolysis products are removed from the reactor operating at a higher substrate concentration and a lower level of products inhibition. However, the long operating time of the immobilized enzyme system causes a hazard of undesired microbial contamination. This problem can be solved using thermostable β-galactosidases, with activity at temperatures eliminating the growth of mesophilic microorganisms. *Pyrococcus woesei*, an extremely thermophilic archaeon, is able to produce a thermostable β-galactosidase, which may be potentially useful for whey utilization, for the preparation of low-lactose milk and dairy products or for oligosaccharide synthesis.

In a previous work, pET30LIC expression vector was used for production of β-D-galactosidase from *P. woesei* in native form (Dąbrowski *et al*., 1998), and to enable efficient purification of *P. woesei* β-galactosidase by immobilized metal-ion affinity chromatography (IMAC), a His-tag was placed at the N-terminal (Dąbrowski *et al*., 2000). The His-tagged recombinant enzyme exhibited a lower activity than the unmodified β-galactosidase. In this study, the expression system was improved by the construction of a recombinant plasmid based on the high copy number pUET1 vector and a His-tag was placed at the C-terminal of the coding sequence.

MATERIALS AND METHODS

Bacterial strains, plasmids, enzymes, and reagents. The *Escherichia coli* TOP10F′ strain (Invitrogen, USA) was used for preparation of plasmids and for cloning, and *E. coli* BL21(DE3) pLysS (Novagen, UK) cells were used for expression of recombinant β-galactosidases. The pGal2 plasmid (Dąbrowski *et al*., 1998) was used for PCR amplification of the *P. woesei* β-galactosidase gene. The pET30LIC (Novagen, UK) and pUET1 (DNA-Gdańsk II s.c., Poland) (Dąbrowski & Kur, 1999) plasmids were used for the construction of the expression systems. Plasmids pET30β-Gal and pUET1β-Gal (coding for *P. woesei* β-galactosidase with His-tag placed at the N-terminal of the enzyme) were described previously (Dąbrowski *et al*., 2000).

Restriction and modification enzymes were purchased from Fermentas (Lithuania). The reagents for PCR were obtained from DNA-Gdańsk II s.c. (Poland). The His•Bind resin for protein purification was purchased from Novagen (UK). *p*-Aminobenzyl 1-thio-β-D-galactopyranoside–agarose and all other reagents were analytical grade and supplied by Sigma-Aldrich.

PCR amplification. The *P. woesei* β-galactosidase gene was amplified using the pGal2 plasmid (Dąbrowski *et al*., 1998) as a source of DNA, *Pwo* DNA polymerase (DNA-Gdańsk II s.c., Poland) and two primers: forward Pyrogal1 — 5′ GCGGGTAC-CAT**ATG**TTCCCTGAAAAGTTCCTTTGG 3′ (35 nt, containing *Kpn*I and *Nde*I recognition sites), and reverse Pyrogal3 — 5′ GCGCGAAGCTT**TCCCCT-CAGCAATTCCTCTTCA** 3′ (33 nt, containing *Hin*dIII recognition site). The boldface parts of primer sequences are complementary to the nucleotide sequences of the *P. woesei* β-D-galactosidase gene, whereas the recognition sites for restriction endonucleases are underlined. The reaction solution consisted of: 0.2 μg pGal2 plasmid DNA, 1 μl (10 μM) of each primer, 5 μl (10 mM) dNTPs, 5 μl 10x PCR buffer (100 mM Tris/HCl, pH 8.8, 500 mM KCl, 25 mM $MgCl_2$, 1% Triton X-100) and 2 U of thermostable *Pwo* DNA polymerase. Each of the 35 cycles was performed with a temperature profile: 30 s at 93°C, 30 s at 54°C, and 30 s at 72°C in a GeneAmp PCR System 2400 thermal cycler (Applied Biosystems, USA). The amplification products were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. A specific 1552 bp (based on nucleotide sequence) PCR product was obtained.

Plasmid construction. In order to obtain plasmids encoding β-galactosidase with a His-tag placed at the C-terminal, the obtained amplification product digested with *Kpn*I and *Hin*dIII was ligated into pET30LIC *Kpn*I–*Hin*dIII sites (pET30*Kpn*I–β-gal–*Hin*dIII, Fig. 1A). Next, the 116 bp *Nde*I fragment was cut out from pET30*Kpn*I–β-gal–*Hin*dIII. The resulting expression plasmid was named pET30β-gal-His-C (Fig. 1B). The fragment containing the β-galactosidase gene (1576 bp) was cut out from the pET30β-gal-His-C recombinant plasmid with *Xba*I and *Hin*dIII, purified from an agarose gel band using DNA Gel-Out kit (A&A Biotechnology, Poland), and cloned directionally into pUET1 *Xba*I–*Hin*dIII sites. The resulting plasmid was named pUET1β-gal-His-C (Fig. 1C). The nucleotide sequence of the pUET1β-gal-His-C plasmid near the site of the *P. woesei* β-galactosidase gene insertion is the same as that of the pET30β-gal-His-C plasmid.

In the previous work (Dąbrowski *et al*., 1998), the low copy-number pET30LIC vector was used for production of *P. woesei* β-D-galactosidase in native form. In this study, in order to obtain more effective expression of native *P. woesei* β-D-galactosidase, the high copy number pUET1 vector was used. The DNA fragment digested with *Xba*I and *Hin*dIII of the pGal2 DNA plasmid (Dąbrowski *et al*., 1998), containing the β-galactosidase gene (1578 bp), was isolated from agarose gel using Gel-Out kit (A&A Biotechnology, Poland) and was ligated into pUET1

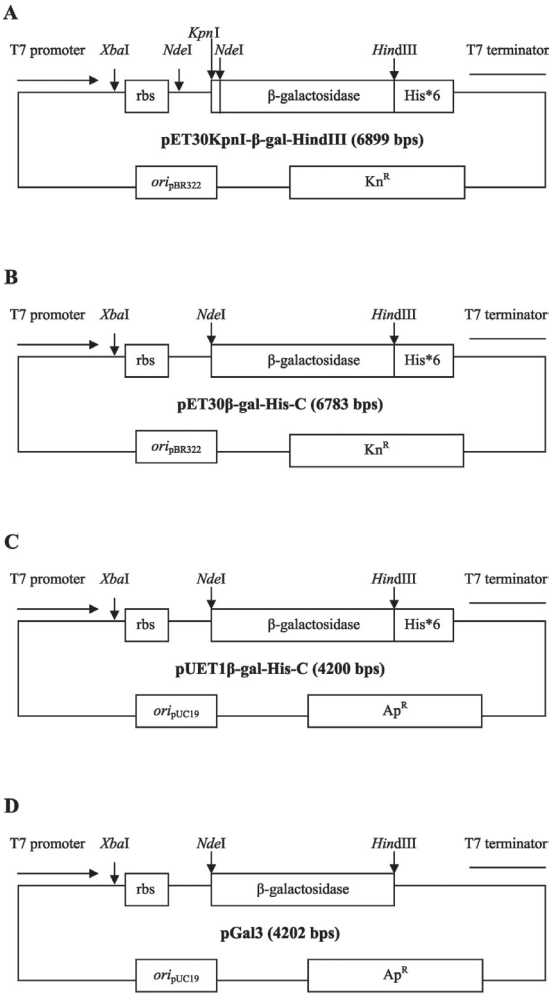

Figure 1. Scheme of genetic constructs used in this study.
pET30*Kpn*I–β-gal–*Hin*dIII (A), pET30β-gal-His-C (B), pUET1β-gal-His-C (C) and pGal3 (D). Constructs: pET30-β-Gal (Dąbrowski *et al*., 2000), pUET1β-Gal (Dąbrowski *et al*., 2000), and pGal2 (Dąbrowski *et al*., 1998) were previously described.

between the *Xba*I-*Hin*dIII sites. The resulting plasmid was named pGal3 (Fig. 1D).

The identities of the recombinant plasmids were confirmed by restriction analysis and the *P. woesei* β-D-galactosidase gene was sequenced using a Perkin-Elmer ABI Prism 377 automatic sequencing system.

Expression and purification. The T7 expression system using *E. coli* BL21(DE3) pLysS is one of the most powerful systems developed for cloning and expression of recombinant proteins in *E. coli.* In this system the target gene is cloned under the control of the T7 promoter, which is not recognized by *E. coli* RNA polymerase. Plasmids are transferred into expression hosts containing a chromosomal copy of the T7 RNA polymerase gene under *lacUV5* control. The pLysS plasmid encodes T7 lysozyme, which is a natural inhibitor of T7 RNA polymerase, and thus reduces its ability to transcribe target genes in uninduced cells.

E. coli BL21(DE3) pLysS cells were transformed with recombinant plasmids containing the recombinant β-D-galactosidase gene under the control of the T7 promoter. One liter of LB medium (10 g pepton K, 5 g yeast extract, 10 g NaCl) supplemented with chloramphenicol (0.034 mg/ml) and kanamycin (0.020 mg/ml) or ampicilin (0.1 mg/ml) was inoculated with 20 ml of overnight culture. The inoculated culture was grown with agitation at 37°C to OD_{600} of 0.5, induced with isopropyl-β-D-thiogalactopyranoside (IPTG) at 1 mM, and grown at 30°C for 12 h. The pellet (10 g of cell mass) harvested by centrifuging (10 min, 5000 × *g*, 4°C) was suspended in 20 ml of buffer A (0.01 M sodium phosphate buffer, pH 6.6) or buffer B (20 mM Tris/HCl, pH 7.9, 500 mM NaCl, 5 mM imidazole). The cells were disrupted by sonication (4 cycles of 30 s), and the insoluble debris was removed by centrifugation (10 min, 5000 × *g*). About 20 ml of cell extract was obtained.

For the purification of N-His-tagged β-galactosidase and C-His-tagged β-galactosidase, the cell extract was incubated in a water bath for 30 min at 75°C and then for 30 min at 85°C (double heat-treatment), cooled on ice and centrifuged (20 min, 12000 × *g* at 4°C). A 20 ml sample of clarified supernatant (in buffer B) was applied onto a column with 15 ml of Ni^{2+}-His•Bind resin (Novagen, UK) equilibrated with 60 ml of buffer B. After loading, the column was washed four times with 20 ml of buffer B, and two times with 20 ml of each W1, W2, and W3 buffers (buffer B containing 20, 40, 60 mM imidazole, respectively). The enzyme was eluted two times with 15 ml of elution buffer E (buffer B containing 0.5 M imidazole). The eluted fractions were pooled and dialyzed against 3 liters of 0.1 M sodium phosphate buffer (pH 6.6).

For purification of recombinant β-galactosidase in its native form, 20 ml of clarified supernatant (in buffer A) was applied onto 10 ml of affinity matrix of agarose coupled with *p*-aminobenzyl 1-thio-β-D-galactopyranoside (Sigma-Aldrich, USA) equilibrated with buffer A. The column was washed with 30 ml of buffer A, and 30 ml of each A1, A2, A3 and A4 buffers (buffer A containing 10, 20, 40, 60 mM NaCl, respectively). β-Galactosidase was eluted three times with 10 ml of 0.05 M sodium borate (pH 10.0) buffer at a flow rate of 0.5 ml/min.

The purity of the proteins was examined by SDS/PAGE (12%) and their concentration was determined by the Bradford (1976) method using bovine serum albumin (BSA) as a standard.

β-D-Galactosidase activity assays. *o*-Nitrophenol released from 0.8 mg/ml *o*-nitrophenyl-β-D-galactopyranoside (ONPG) solution in 0.1 M sodium phosphate buffer, pH 6.6, by β-D-galactosidase was measured at 420 nm using a modified Craven *et al*.

(1965) procedure. The substrate (2.5 ml) was preincubated at 85°C for 2 min, and the reaction was initiated by addition of 0.5 ml of enzyme sample. The reaction was stopped after 15 min with 1 ml of cold 1 M sodium carbonate solution. A blank, containing 0.5 ml of 0.1 M sodium phosphate buffer instead of enzyme solution, was used to correct for the thermal hydrolysis of ONPG. One unit of activity is defined as the amount of enzyme releasing 1 nmole of *o*-nitrophenol per minute under described conditions.

Influence of metal ions, thiol compounds and sugars on β-galactosidase activity. The hydrolytic activity of *P. woesei* β-D-galactosidases (His-tagged or native) was measured in the presence of dithiothreitol (10 mM), 2-mercaptoethanol (10 mM), glutathione (10 mM), cysteine (10 mM), metal ions (Mg^{2+}, Ca^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+} and Cu^{2+}; each at 1 mM), and sugars (D-glucose or D-galactose; each at 10, 50 or 100 mM).

Gel electrophoresis. SDS/PAGE was performed in 12% (w/v) gels by the method of Laemmli (1970). SigmaMarker Wide Range purchased from Sigma-Aldrich was used as a molecular mass standard (205, 116, 97, 84, 66, 55, 45, 36, 29, 24, 20, 14.2 and 6.5 kDa), and the proteins were detected by staining with Coomassie Brilliant Blue.

Estimation of molecular mass. The molecular weight of native *P. woesei* β-D-galactosidase was estimated by HPLC gel filtration on a Superdex 200 HR 10/30 column (Amersham Biosciences AB, Sweden). Elution was carried out with 0.1 M sodium phosphate buffer, pH 6.6, with a velocity of 0.5 ml/min. The elution pattern of the enzyme was then compared with those of standard proteins: carbonic anhydrase from bovine erythrocytes (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase from yeast (150 kDa), β-amylase from sweet potato (200 kDa), and apoferritin from horse spleen (443 kDa), all purchased from Sigma-Aldrich.

RESULTS

Expression and purification of recombinant *P. woesei* β-galactosidases

In the previous work, the pET30LIC expression vector was used for production of *Pyrococcus woesei* β-D-galactosidase in native form (Dąbrowski *et al*., 1998), and to achieve efficient purification of *P. woesei* β-galactosidase by immobilized metal-ion affinity chromatography (IMAC), a His-tag was placed at the N-terminal (Dąbrowski *et al*., 2000). The His-tagged recombinant enzyme exhibited lower activity than unmodified β-galactosidase. In the present study, the expression system was further developed by the construction of recombinant plasmids with a His-tag placed at the C-terminal and using the high copy number pUET1 vector. It was revealed that the pUET1 plasmid copy number per cell is much higher than that for pET30LIC (not shown). The use of the pUET1 vector for cloning and expression of recombinant proteins should increase the yield of the target protein. The constructed pET30β-gal-His-C and pUET1β-gal-His-C plasmids encode the same protein consisting of *P. woesei* β-galactosidase and 13 additional amino-acid residues at the C-terminus with a cluster of six histidine residues necessary for the purification by IMAC. The pGal3 plasmid constructed here contains the same nucleotide sequence at the site of the *P. woesei* β-galactosidase gene insertion as that of pGal2, previously described (Dąbrowski *et al*., 1998).

Six *E. coli* expression constructs were tested for efficiency of expression and purification of *P. woesei* β-galactosidase. The *E. coli* BL21(DE3) pLysS strain was transformed with: pET30β-Gal or pUET1β-Gal (His-tag was placed at the N-terminal of the coding sequence; Dąbrowski *et al*., 2000); pET30β-gal-His-C or pUET1β-gal-His-C (His-tag at the C-terminal of the coding sequence; this study); and pGal2 (Dąbrowski *et al*., 1998) or pGal3 (this study) encoding the unmodified enzyme. For optimizing expression, *E. coli* cells with plasmids were cultured aerobically at 37°C in 50 ml of LB medium (containing appropriate antibiotics) to OD_{600} of 0.1, 0.2, 0.3, 0.4, 0.5 or 0.6. IPTG was then added to a final concentration of 1 mM, and the cells were further cultured at 37 or 30°C. After 3, 4, 5, 6 or 12 h of cultivation a 10 ml sample was transferred to a centrifuge tube and cell extract was obtained as described in Materials and Methods. β-Galactosidase activity was measured in those extracts. The highest activity was obtained from cells cultured to OD_{600} of 0.5 prior to IPTG induction, followed by 12 h of cultivation at 30°C for each expression system. These optimized conditions (described in Materials and Methods) were used for production of β-galactosidases for comparison experiments.

Based on preliminary purification studies of *P. woesei* β-galactosidases, a two-step procedure, including double heat-treatment and IMAC for N-His-tagged and C-His-tagged β-galactosidases or affinity chromatography on *p*-aminobenzyl 1-thio-β-D-galactopyranoside–agarose for the unmodified enzyme, was used. The purity of the proteins was examined by SDS/PAGE (Fig. 2). A band corresponding to a protein of about 61 kDa was observed in clarified lysate after heat treatment from the *E. coli* BL21(DE3) pLysS + pUET1β-gal-His-C strain (Fig. 2, lanes 1 and 2) and from *E. coli* BL21(DE3) pLysS + pUET1β-Gal (Fig. 2, lanes 4, 5). The recombinant C-His-tagged β-galactosidase purified by IMAC (Ni^{2+}-affinity chromatography) gave a single band on SDS/PAGE (Fig. 2, lane 3). The same result was obtained for the

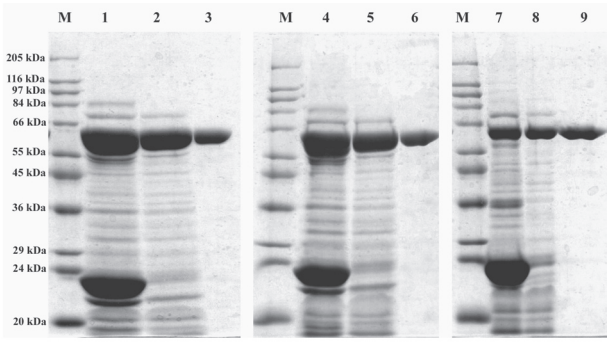

Figure 2. SDS electrophoresis in 12% polyacrylamide gel of fractions obtained during purification of recombinant *P. woesei* β-D-galactosidase.
Lane M, molecular mass marker (SigmaMarker Wide Range, Sigma-Aldrich); lane 1, 20 μl of clarified lysate of *E. coli* pUET1β-gal-His-C after first heat treatment (75°C); lane 2, 20 μl of clarified lysate after second heat treatment (85°C); lane 3, 20 μl of purified enzyme obtained by Ni^{2+}-affinity chromatography; lane 4, 20 μl of clarified lysate of *E. coli* pUET1β-gal after first heat treatment (75°C); lane 5, 20 μl of clarified lysate after second heat treatment (85°C); lane 6, 20 μl of purified enzyme obtained by Ni^{2+}-affinity chromatography; lane 7, 20 μl of clarified lysate from *E. coli* pGal3 after first heat treatment (75°C); lane 8, 20 μl of clarified lysate after second heat treatment (85°C); lane 9, 20 μl of purified enzyme obtained by affinity chromatography. Representative results are shown.

N-His-tagged enzyme (Fig. 2, lane 6). We obtained approx. 35 mg of purified enzyme (C-His-tagged β-galactosidase) from 1 liter of *E. coli* BL21(DE3) pLysS + pUET1β-gal-His-C culture and approx. 29 mg of purified N-His-tagged β-galactosidase from 1 liter of *E. coli* BL21(DE3) pLysS + pUET1β-Gal culture (Table 1). The specific activity was determined as 5430 U/mg and 5414 U/mg for the C-His-tagged and the N-His-tagged β-galactosidase, respectively. As shown in Table 1, the total β-galactosidase activity was about three to four times higher for the pUET1 expression system than that achieved with the pET30LIC system. That result is probably the consequence of the high pUET1 copy number and it was supported by the result obtained with the new *E. coli* BL21(DE3) pLysS + pGal3 expression system (pUET1 vector) giving four times more efficient expression of untagged *P. woesei* β-galactosidase (20 mg of purified β-galactosidase from 1 liter of culture) than that obtained from the previously constructed system. A band corresponding to a protein of about 60 kDa was observed in SDS/PAGE of clarified lysate from *E. coli* BL21(DE3) pLysS transformed with the pGal3 plasmid after heat treatment (Fig. 2, lanes 7, 8), and in the eluted fraction from affinity chromatography (Fig. 2, lane 9). The double heat-treatment during purification was necessary because of difficulties in removing a protein of about 23 kDa which was very strongly expressed in *E. coli* BL21(DE3) pLysS cells and was tightly adsorbed to the agarose resin (see Fig. 2, lines 1, 4 and 7). The specific activity of untagged *P. woesei* β-galactosidase was 51100 U/mg, about ten times higher than for the His-tagged β-galactosidases.

Influence of metal ions, thiol compounds and sugars on β-galactosidase activity

The activity of *P. woesei* β-D-galactosidase (His-tagged or unmodified) was enhanced slightly by Mg^{2+} (by about 7%), whereas Ca^{2+} had no impact. The enzyme activity was inhibited by Zn^{2+} (by 65%), Cu^{2+} (72%), Ni^{2+} (85%) and Co^{2+} (92%) in comparison

Table 1. Summary of the purification of *P. woesei* recombinant β–galactosidases obtained from 1 liter of *E. coli* BL21(DE3) pLysS culture

Expression system	Purification step	Total protein (mg)	Total activity (kU)	Specific activity (U/mg)	Purification (fold)	Recovery of protein (%)
pET30β-Gal	Lysate	300	–	–	–	100
	DHT [a]	82	38.3	467	1	27
	IMAC [b]	7	37.8	5400	11	2
pUET1β-Gal	Lysate	325	–	–	–	100
	DHT [a]	95	158.0	1663	1	29
	IMAC [b]	29	157.0	5414	3	9
pET30β-gal-His-C	Lysate	300	–	–	–	100
	DHT [a]	90	68.6	762	1	30
	IMAC [b]	12.5	68.0	5440	7	4
pUET1β-gal-His-C	Lysate	340	–	–	–	100
	DHT [a]	100	191.0	1910	1	29
	IMAC [b]	35	190.0	5430	3	10
pGal2	Lysate	290	–	–	–	100
	DHT	80	375.0	4687	1	27
	AC [c]	5.6	286.0	51070	11	2
pGal3	Lysate	320	–	–	–	100
	DHT	85	1 150.0	13530	1	26
	AC [c]	20	1 022.0	51100	4	6

[a]DHT, double heat treatment; [b]IMAC, immobilized metal-ion affinity chromatography; [c]AC, affinity chromatography on *p*-aminobenzyl 1-thio-β-D-galactopyranoside–agarose.

Table 2. Influence of metal ions and thiol compounds on β-galactosidase activity (representative results for native β-galactosidase-pGal3 expression system)

Metal ion[a]	Activity of β-galactosidase (%)	Reagent[b]	Activity of β-galactosidase (%)
None	100	None	100
Mg^{2+}	107	2-Mercaptoethanol	100
Ca^{2+}	99	Dithiothreitol	148
Co^{2+}	92	Glutathione	113
Ni^{2+}	85	Cysteine	111
Cu^{2+}	72		
Zn^{2+}	65		

[a]Metal chlorides were used in all cases except $CuSO_4$; [b]Thiol compounds were added to the ONPG solution.

with the activity of the enzyme in the absence of cations (Table 2). Glutathione and cysteine caused a slight activation (by about 10%), dithiothreitol was a strong activator, and 2-mercaptoethanol had no impact on the enzyme activity (Table 2). The beneficial influence of the thiol compounds (particularly dithiothreitol) suggests that free thiol groups are essential for *P. woesei* β-galactosidase activity.

Table 3 shows the effects of sugars on *P. woesei* β-D-galactosidase (in its native form) activity with ONPG as the substrat. In the presence of 10 mM D-glucose or D-galactose, the activity of the enzyme was enhanced. Higher concentrations of D-glucose only moderately inhibited *P. woesei* β-D-galactosidase, whereas D-galactose was activatory also at higher concentrations. The same results were obtained for N- and C-His-tagged β-galactosidases. The apparent activation of *P. woesei* β-D-galactosidase by an exogenous nucleophile (D-galactose) is indicative of a reaction mechanism in which degalactosylation is the rate-limiting reaction step in the hydrolysis of ONPG. This result suggests also that the enzyme can catalyze galactosyl transfer to the monosaccharide products of lactose hydrolysis. Studies on the application of *P. woesei* β-D-galactosidase for the synthesis of galacto-oligosaccharides are in progress.

Molecular mass

The nucleotide sequence revealed that *P. woesei* β-D-galactosidase in its native form consists of 510 amino acids and has a predicted molecular mass of 59.056 kDa (Dąbrowski *et al*., 2000). The molecular mass of the denatured protein was estimated to be about 60 kDa by SDS/PAGE (Fig. 2, lane 9), whereas the molecular mass of the active form of the enzyme was estimated at 122.594 kDa by gel filtration. The obtained results indicate that the *P. woesei* β-D-galactosidase has a dimeric form in *E. coli*. The same results were obtained with N- and C-His-tagged β-galactosidases. Taking all the data into consideration, it seems that protein oligomerization is not responsible for the drastically decreased enzyme activity of the His-tagged β-galactosidases in comparison to untagged β-galactosidase.

Table 3. Influence of sugars on β-galactosidase activity (representative results for native β-galactosidase-pGal3 expression system)

Sugar[a]	Concentration (mM)	Activity of β-galactosidase (%)
None	0	100
D-Glucose	10	131
	50	94
	100	80
D-Galactose	10	105
	50	106
	100	131

[a]Sugars were added to the ONPG solution.

DISCUSSION

We hereby demonstrated expression and purification of recombinant thermostable *P. woesei* β-D-galactosidase. The β-D-galactosidase was efficiently expressed in the mesophilic *E. coli* and purified almost to homogeneity. The enzyme is a homodimer and has a molecular mass of 122.594 kDa. Three forms of the β-D-galactosidase were obtained: N-His-tagged, C-His-tagged and native (untagged) one. Two types of vectors were used to create *E. coli* expression systems: pET30LIC and pUET1. The systems based on the high copy number pUET1 vector gave a four times more efficient expression of *P. woesei* β-galactosidase than those with pET30LIC. The recombinant enzymes were purified in a two-step procedure: double heat-denatation of *E. coli* cell proteins and affinity chromatography on *p*-aminobenzyl 1-thio-β-D-galactopyranoside–agarose for the native enzyme or IMAC for the His-tagged β-galactosidases. Thermal precipitation greatly facilitated further purification, which required just one chromatographic step using affinity chromatography to obtain a single protein band. The fusion proteins (with the His-tag at either the N- or the C-terminal position) were obtained with the same specific activity, which was 10 times lower than for the untagged β-galactosidase. These results show that the presence of the His-tag, whether at the N- or the C-terminus, drastically decreases the enzyme activity. The activity of *P. woesei* β-galactosidase (native or tagged) was en-

hanced by thiol compounds, Mg^{2+} ions and D-galactose, and was inhibited by Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+} ions and D-glucose, while Ca^{2+} had no effect.

In summary, using an *E. coli* expression system, we were able to produce recombinant *P. woesei* β-galactosidase with a high yield. The described purification procedure is simple and rapid. The high activity of the enzyme in ONPG hydrolysis at 85°C, pH of 6.6 and in the presence of Ca^{2+} ions suggests potential usefulness of this enzyme in the hydrolysis of milk lactose. Studies on large-scale application of recombinant *P. woesei* β-D-galactosidase for degradation of this sugar are in progress.

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