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Applications of β-gal-III isozyme from *Bacillus coagulans* RCS3, in lactose hydrolysis

Navneet Batra [a,*], Jagtar Singh [b], Amit Joshi [c], Sonu Bhatia [b]

[a] Dept. of Biotechnology, GGDSD College, Chandigarh, India
[b] Dept. of Biotechnology, Panjab University, Chandigarh, India
[c] Dept. of Biotechnology, SGGS College, Chandigarh, India

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ABSTRACT

Bacillus coagulans RCS3 isolated from hot water springs secreted five isozymes *i.e.* β-gal I–V of β-galactosidase. β-gal III isozyme was purified using DEAE cellulose and Sephadex G 100 column chromatography. Its molecular weight characterization showed a single band at 315 kD in Native PAGE, while two subunits of 50.1 and 53.7 kD in SDS PAGE. β-Gal III had pH optima in the range of 6–7 and temperature optima at 65 °C. It preferred nitro-aryl-β-D-galactoside as substrate having K_m of 4.16 mM with ONPG. More than 85% and 80% hydrolysis of lactose (1–5%, w/v) was recorded within 48 h of incubation at 55 °C and 50 °C respectively and pH range of 6–7. About 78–86% hydrolysis of lactose in various brands of standardized milk was recorded at incubation temperature of 50 °C. These results marked the applications of β-gal III in processing of milk/whey industry.

1. Introduction

β-Galactosidase (EC 3.2.2.23) is a commercially important enzyme used in the dairy industry for the functional improvement of milk and cheese whey [1–3]. It can alleviate the problems associated with whey disposal, lactose crystallization in frozen concentrated deserts and milk consumption by lactose-intolerant individuals [4]. β-Galactosidase from *Escherichia coli*, *Bacillus circulans*, *Kluyveromyces lactis*, *Aspergillus oryzae*, or *Aspergillus niger* were shown to have transgalactosylation reaction; lactose serves as galactosyl donor and an acceptor to form di to higher galacto-oligosaccharides [5–7]. Galacto-oligosaccharides are added to nutritional foods such as infant formula and yogurt in order to promote the growth and the establishment of beneficial bacteria bifidobacteria in the intestine [8,9]. They have a beneficiary effect on the human host. They prevent diarrhoea and constipation, protect liver functions, reduce serum cholesterol, and has anticancer effects [10]. Some thermostable β-galactosidases are extracted and characterized from *Sulfolobus sulfataricus* and *Pyrococcus furiosus* [11], archaeon *Pyrococcus sp.* [12], *Thermotoga maritima* [13]. Various studies reported the hydrolysis of lactose in milk and whey using free enzymes or immobilized cell systems [14–16]. However, only few workers reported the use of thermostable β-galacosidase. The usage of thermostable enzymes reduces the reaction time and the risk of contamination [17,18]. Five different isozymes of β-galacosidase were observed in *Bacillus coagulans* RCS 3 and numbered as β-gal I–V. Since β-gal III isozyme is present in high concentration, the present study involves the purification and characterization of this thermostable enzyme from *B. coagulans* RCS 3 to exploit its potential in processing of milk and whey.

2. Materials and methods

2.1. Chemicals

Acrylamide and N,N,β-methylene bisacrylamide were purchased from Boehringer-Mannheim (Mannheim, Germany). ONPG, DEAE-cellulose, Sephadex G 100, Riboflavin, dinitrosalicylic acid (DNS), β-mercaptoethanol, ammonium persulphate, Coomassie brilliant blue R 250, X-Gal, Glucose (Trinder) Kit No. 135–100 and 4-methylumbelliferyl-β-D-galactopyranoside, Native PAGE Kit (Tech. Bull. MKR137), SDS PAGE Protein markers (Kit No. MW-SDS-200) were obtained from Sigma Chemicals (St. Louis, MO, USA). All media components were of bacteriological grade and purchased from HiMedia (Mumbai, India). Ammonium sulphate was purchased from E Merck (Mumbai, India). All other chemicals were of

Abbreviations: ONPG, ortho nitro phenyl β-D-galactopyranoside; PAGE, poly acrylamide gel electrophoresis.

* Corresponding author at: PG Department of Biotechnology, GGDSD College, Sector 32-C, Chandigarh 160 030, India. Tel.: +91 172 2600090; fax: +91 172 2613656.
E-mail address: batranavneet@gmail.com (N. Batra).

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Table 1
Summary of purification of β-galactosidase from *B. coagulans* RCS3.

Purification step	Total protein (mg)	Total enzyme (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude enzyme	596.3	28,460.2	47.7	100	1
Ammonium sulphate (30–80% saturation)	49.7	26,156.6	526.3	91.9	11
DEAE cellulose column chromatography	18.3	19,253.0	1052.0	67.6	22
Sephadex G 100 column chromatography	11.7	16,723.3	1429.3	58.7	30

analytical grade and procured from Qualigens Fine Chemicals (Mumbai, India) and SD fine Chemicals (Mumbai, India).

2.2. Production of β-galactosidase

B. coagulans RCS3 isolated from hot water springs of Himachal Pradesh, India was used for the production of β-galactosidase. The selected strain was grown in the medium having lactose (1%), yeast extract (0.5%), $MgSO_4 \cdot 7H_2O$ (0.05%) and K_2HPO_4 (0.1%) at temperature of 40 °C and pH 7. The cell free supernatant obtained after growth was used as crude enzyme.

2.2.1. Enzyme assay

The enzyme activity was studied as described by Batra et al. [19] using ONPG as substrate. One millilitre of enzyme solution was used with 2 ml ONPG (5 mM in 0.1 M sodium phosphate buffer; pH 7) and incubated at 60 °C (or otherwise stated) for 10 min. After incubation, 2 ml of 0.5 mM chilled sodium carbonate was added. The absorbance was measured at 420 nm with appropriate blanks. Ortho nitrophenol (ONP) was used as standard.

One unit (1 U) of β-galactosidase is the amount that produced 1 μmol of ONP/min/ml at pH 7.0 and 60 °C.

2.2.2. Estimation of lactose

Glucose concentration in lactose hydrolysis broth was measured by Glucose (Trinder) kit 135–100 (Sigma Chemicals, USA). To 5 μl of the sample, 1 ml of the reagent solution (from kit) was added. The reaction mixture was incubated for 15 min at 37 °C. Absorbance was measured at 505 nm. Glucose (0–7.5 mg/ml) solution was used as standard. From the glucose concentration, residual lactose level during hydrolysis was calculated according to the stochiometry of the reaction.

2.2.3. Estimation of protein

Extra cellular protein concentration was estimated by the method of Lowry et al. [20].

2.3. Purification of β-galactosidase

All the purification procedures were carried out at 4 °C. 10 ml cell free supernatant was precipitated with 30–80% saturation with ammonium sulphate and centrifuged at 15,000 × *g* for 30 min. The resulting precipitates were dialyzed against phosphate buffer (pH 7.0). The dialyzed enzyme was applied to DEAE cellulose column of 1.5 cm × 15 cm using Bradford Collector system followed by estimation of protein and enzymes using Beckman DU640B spectrophotometer The samples were eluted at a flow rate of 15 ml h^{-1} and 2 ml fractions were collected using gradient of NaCl (0.1–2 M). The fractions containing β-galactosidase activity were pooled and concentrated using Centrisart filter (Sartorius AG, Germany) followed by column chromatography (2.5 cm × 75 cm) using Sephadex G-100 [21].

2.3.1. Non denaturing PAGE

Native PAGE (4.5–10%) was performed as described in Manufacturer's Instructions manual (MKR 137, Sigma Chemicals). Urease (272 kDa, 545 kDa), BSA (66 kDa, 132 kDa), chicken egg albumin (45 kDa), carbonic anhydrase (29 kDa) and β-lactalbumin (14.2 kDa) were used as standard molecular mass markers.

2.3.2. SDS PAGE

Molecular weight determination of purified protein subunits was carried out in SDS PAGE gels (7%). Standard protein markers: myosin, rabbit (205 kDa); β-galactosidase (116 kDa); phosphorylase B, rabbit (97.4 kDa); albumin, bovine (66 kDa); ovalbumin, chicken (45 kDa) and carbonic anhydrase, bovine (29 kDa) were used to prepare calibration curve.

2.3.3. Staining and de-staining of gels

Gels were stained for 2 h in the fixative solution (methanol:acetic acid:water 4:0.7:5.3) followed by overnight incubation in staining solution containing coomassie brilliant blue R 250 (0.1% in fixative solution). De-staining of gel was done against several changes in fixative solution.

For activity staining pattern, gel with β-galactosidase was incubated in chromogenic X-gal solution followed by detection of blue bands.

2.3.4. Molecular weight determination using Native PAGE

The migration distances of tracking dye and proteins from the start of separating gels of different concentrations were recorded. Relative mobility (R_f) of each protein band was determined. 100[log($R_f \times 100$)] values were plotted against the gel concentration percent on standard graph for each protein. The negative (−ve) slopes of these graphs were plotted against the known molecular weight standards and molecular weight of unknown protein was determined.

2.4. Properties of β-gal III

2.4.1. Substrate specificity

Various para- and ortho nitrophenyl glycosides including o-nitrophenyl-β-D-galactopyranoside, p-nitrophenyl-β-D-galactopyranoside, p-nitrophenyl-β-D-glucopyranoside, p-nitrophenyl-α-D-fucopyranoside (Table 2) were compared for the hydrolytic activity of soluble β-gal III from *B. coagulans* RCS3 at pH 7 and temperature 60 °C.

2.4.2. Kinetic properties

For hydrolytic activity, kinetic constants were determined with its natural substrate and artificial substrate lactose and ONPG,

Table 2
Specificity of β-galactosidase III towards different substrates.

Substrate	Relative activity (%)
o-Nitrophenyl-β-D-galactopyranoside	100
o-Nitrophenyl-α-D-galactopyranoside	0
p-Nitrophenyl-β-D-galactopyranoside	87
p-Nitrophenyl-β-D-glucopyranoside	0
Methyl-β-D-galactopyranoside	69
Phenyl-β-D-galactopyranoside	13
Glucose-β-D-galactopyranoside	83
p-Nitrophenyl-α-D-galactopyranoside	0
p-Nitrophenyl-α-D-mannopyranoside	0
p-Nitrophenyl-α-D-fucopyranoside	0