Regular Paper

Enzymatic Degradation of Lactose in Milk

Acidophilic β-Galactosidase from *Aspergillus niger* AHU7120 with Lactose Hydrolytic Activity Under Simulated Gastric Conditions

Wataru Saburi,[1,*] Hiroshi M. Ueno,[2] Hirokazu Matsui[1] and Haruhide Mori[1]

[1]*Laboratory of Biochemistry, Research Faculty of Agriculture, Hokkaido University (Kita-9, Nishi-9, Sapporo 060-8589, Japan)*

[2]*Milk Science Research Institute, Megmilk Snow Brand Co., Ltd. (1-1-2 Minamidai, Kawagoe, Saitama 350-1165, Japan)*

*Corresponding author (Tel. & Fax. +81-11-706-2508; E-mail: saburiw@chem.agr.hokudai.ac.jp).

Abbreviations: LPH, lactase-phlorizin hydrolase; *p*NPGal, *p*-nitrophenyl β-D-galactopyranoside; AnBGal, β-galactosidase from *A. niger* AHU7120; PCR, polymerase chain reaction; rAnBGal, recombinant AnBGal

Acidophilic β-galactosidase is a useful enzyme as digestive supplement used to alleviate symptoms of lactose intolerance. *Aspergilli* are the source of several acidophilic β-galactosidases that retain enzymatic activity under gastric conditions. In this study, we investigated the extracellular acidophilic β-galactosidase activity of six *Aspergillus niger* strains, AHU7104, AHU7120, AHU7217, AHU7294, AHU7295, and AHU7296; *A. niger* AHU7120 was selected as an enzyme source. β-Galactosidase from *A. niger* AHU7120 (AnBGal) was purified from culture supernatant. Its N-terminal sequence was identical to that of An01g12150, which belongs to the glycoside hydrolase family 35, from *A. niger* CBS 513.88. The DNA sequence of *AnBGal* was identical to *An01g12150*. Recombinant AnBGal (rAnBGal) harboring yeast α-factor signal sequence was expressed in *Pichia pastoris*, and 21.9 mg of purified rAnBGal with 129 U/mg of enzyme activity was isolated from 200 mL of culture supernatant. Native and recombinant AnBGal enzymes showed similar pH optima, pH stability, and kinetics for *p*-nitrophenyl β-D-galactopyranoside and lactose; rAnBGal showed slightly lower thermal stability than the native enzyme. Lactose in milk was rapidly degraded by rAnBGal at higher pH values (range, 2.0–3.5), consistent with the pH optimum of AnBGal. We estimated that 3.5 μM AnBGal may degrade ≥66% of lactose before gastric half-emptying of ingested milk. These data indicate that AnBGal may help alleviate symptoms of lactose intolerance.

Key words: β-galactosidase, lactose intolerance, *Aspergillus niger*, milk, lactose

Lactose is a disaccharide found in the milk of mammals. *In vivo*, lactose is hydrolyzed to D-glucose and D-galactose by intestinal lactase (lactase-phlorizin hydrolase [LPH]), a β-galactosidase expressed exclusively on the brush border of small intestine epithelial cells. Lactase activity is high during the newborn period when milk is the sole nutrient, but declines markedly after weaning to a low adult level. As a consequence of this natural decline in enzyme level, some human adults may present with LPH deficiency.[1)] In these individuals, ingestion of lactose causes mild to severe gastrointestinal symptoms, including abdominal pain, diarrhea, and flatulence.[2)] Deficiency of LPH may result in insufficient consumption of milk and other lactose containing products.

β-Galactosidases originating from microorganisms such as bacteria and fungi have been widely used to hydrolyze lactose in milk and related products. These enzymes render dairy products consumable for lactose-intolerant individuals. In fact, digestive supplementation with β-galactosidase may alleviate symptoms of lactose intolerance. Acidophilic β-galactosidases are especially beneficial as they are active under gastric conditions. Several acidophilic enzymes have been isolated from *Aspergilli*.[3-6)] O'Connell and Walsh showed that β-galactosidases from *Aspergillus carbonarius* ATCC6276 and *Aspergillus niger* van Tiegh retain their activity under gastric conditions, and that these enzymes are useful for digestive supplementation;[4,6)] lactose hydrolytic activity of *A. niger* van Tiegh β-galactosidase was demonstrated under simulated gastric conditions.[6)] However, degradation of lactose in milk with these enzymes have not been fully investigated. In this study, we screened acidophilic β-galactosidases from six *Aspergillus niger* strains to develop an enzyme supplement ingested with milk and related products. We selected one strain and investigated the

characteristics of its β-galactosidase and degradation of lactose in milk with this enzyme.

MATERIALS AND METHODS

Fungal strains and media. Six *A. niger* strains, AHU7104, AHU7120, AHU7217, AHU7294, AHU7295, and AHU7296, were supplied by the Japan Society for Culture Collection (Gifu, Japan). Potato dextrose medium (Becton Dickinson, Sparks, MD, USA) was used for preculture of each *A. niger* strain. Wheat bran medium, prepared by suspending wheat bran in water, was used for production of extracellular β-galactosidase. Wheat bran was a gift from Showa Sangyo Co., Ltd (Tokyo, Japan).

Screening for a source of acidophilic β-galactosidase. Each *A. niger* strain was cultured in 50 mL of potato dextrose broth at 30°C for 4 d. The culture medium (0.5 mL) was inoculated with 17.5 g of wheat bran medium (15% wheat bran) and incubated with occasional mixing at 30°C for 5 d. Resulting wheat bran was suspended in 10 mL of water, and supernatant was collected as extracellular enzymes by centrifugation (14,000×g, 4°C, 20 min). β-Galactosidase enzyme activity of the extracts was measured.

Purification of AnBGal. AnBGal was produced by cultivation of *A. niger* AHU7120 in 600 mL of 7.5% wheat bran medium at 30°C for 5 d. Following filtration through gauze and successive centrifugation (14,000×g, 4°C, 20 min), culture supernatant was dialyzed against 10 mM sodium phosphate buffer (pH 6.3). Sample was applied to a Q Sepharose Fast Flow column (column size, 2.5 cm I.D. × 32 cm; GE Healthcare, Uppsala, Sweden) equilibrated with 10 mM sodium phosphate buffer (pH 6.3). After

washing with the same buffer, adsorbed protein was eluted with a linear gradient of NaCl (0 to 0.5 M; total elution volume, 1 L). Active fractions were pooled, concentrated to 4 mL using a Vivaspin 20 (nominal molecular weight limit 30,000; Sartorius, Göttingen, Germany), and subjected to Sephacryl S-100 HR column chromatography (GE Healthcare) under the following conditions: column size, 1.6 cm I.D. × 60 cm; buffer, 10 mM sodium phosphate buffer (pH 6.3) containing 0.1 M NaCl; flow rate, 0.2 mL/min. Active fractions were applied to a Resource Q column (1 mL; GE Healthcare) equilibrated with 10 mM sodium phosphate buffer (pH 6.7). Adsorbed protein was eluted with a pH gradient (range, pH 6.7 to 3.4; 10 mM sodium acetate buffer [pH 3.4] was used for acidic buffer). Total elution volume was 30 mL. Highly purified fractions, confirmed by SDS-PAGE, were used in further analyses.

N-terminal sequence analysis of AnBGal. Purified AnBGal (1.6 μg) was separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane by electroblotting in a semidry blotting apparatus. The AnBGal band was excised from the membrane and subjected to N-terminal sequence analysis with a Procise 492 protein sequencer (Perkin Elmer, Waltham, MA, USA).

Construction of AnBGal expression plasmid. *AnBGal* was amplified by polymerase chain reaction (PCR) using Primestar HS DNA polymerase (Takara Bio, Otsu, Japan), *A. niger* AHU7120 genomic DNA as template, and the following primers: 5′-ATGAAGCTTTCCTCCGCTTGTGCTA-3′ (sense) and 5′-CTAGTATGCACCCTTCCGCTTCTTG-3′ (antisense). Amplified DNA fragments were cloned into the *Eco*RV site of the pBluescript II SK (+) vector (Stratagene, La Jolla, CA, USA) and sequenced with an Applied Biosystems 3130 Genetic Analyzer

(Life Technologies, Carlsbad, CA). Plasmid DNA was used as the template for amplification of each exon of *AnBGal*. The exons amplified were connected by overlap extension PCR,[7] cloned into the *Eco*RV site of pBluescript II SK (+), and used for construction of an expression plasmid. DNA fragments encoding mature AnBGal (Glu40-Tyr1007) were cloned into *Kpn*I and *Xba*I sites of the pPICZαA vector (Invitrogen, Carlsbad, CA, USA). The following primers were used to amplify a partial DNA sequence of *AnBGal*: 5′-AAAAAGGTACCGAACTGTTGCAGAAATACG-3′ (sense, *Kpn*I site underlined) and 5′-AAATCTAGACTAGTATGCACCCTTCCGCTT-3′ (antisense, *Xba*I site underlined).

Production and purification of recombinant AnBGal (rAnBGal). The expression plasmid of AnBGal was linearized by *Sac*I digestion and introduced into *Pichia pastoris* X-33 by electroporation in a Gene Pulser (Bio-Rad, Richmond, CA). Four colonies grown on a YPDSZ plate (10 mg/mL yeast extract, 20 mg/mL peptone, 20 mg/mL D-glucose, 1 M sorbitol, 20 mg/mL agar, and 100 μg/mL zeocin) were selected. Transformed yeast cells were cultured in 10 mL of BMGY medium (10 mg/mL yeast extract, 20 mg/mL peptone, 13.4 mg/mL yeast nitrogen base, 4 μg/mL D-biotin, 10 mg/mL glycerol, and 0.1 M potassium phosphate buffer, pH 6.0) at 30°C for 24 h, harvested by centrifugation (400×g, 4°C, 2 min), resuspended in 10 mL of BMMY medium (10 mg/mL glycerol in BMGY was changed to 0.5% methanol), and cultured at 30°C for 96 h to induce expression of the recombinant enzyme. Protein production was maintained by the addition of methanol at a final concentration of 0.5% every 24 h. Enzyme activity of the culture supernatant was measured by β-galactosidase enzyme activity assay. The transformant with the highest activity was chosen for large scale

production of rAnBGal.

The *P. pastoris* transformant selected was cultured in 200 mL of BMGY medium at 30°C for 24 h. Subsequently, yeast cells were transferred to 200 mL of BMMY medium supplemented with 5 mM EDTA and incubated with vigorous shaking at 20°C for 96 h. The culture supernatant was harvested by centrifugation (4200×g, 4°C, 10 min), and solid ammonium sulfate was added up to 90% saturation. After incubation at 4°C for 24 h, precipitated protein was collected by centrifugation (31,000×g, 4°C, 10 min). The precipitate was dissolved in 50 mL of 10 mM sodium phosphate buffer (pH 6.3), and the solution was dialyzed against the same buffer. A sample was applied to a DEAE Sepharose CL-6B column (column size, 2.5 cm I.D. × 26 cm; Amersham Biosciences, Uppsala, Sweden) equilibrated with 10 mM sodium phosphate buffer (pH 6.3). Non-adsorbed protein was eluted with the same buffer, and adsorbed protein was eluted with a linear gradient of NaCl (0 to 0.5 M; total elution volume, 0.5 L). Active fractions were pooled, concentrated to 7 mL as previously described, and applied to a Sephacryl S-300 HR column (column size, 1.6 cm I.D. × 60 cm; GE Healthcare) equilibrated with 10 mM sodium phosphate buffer (pH 6.3) containing 0.1 M NaCl. Highly purified fractions were dialyzed against 10 mM sodium phosphate buffer (pH 6.3).

Protein assay. The protein concentration of the *A. niger* AHU7120 culture supernatant was determined by the Bradford method[8)] using bovine serum albumin (Nakalai Tesque, Kyoto, Japan) as the standard. Protein concentrations of column chromatography fractions were determined by UV (absorbance at 280 nm),[9)] assuming an extinction coefficient of 1 mg/mL of protein is equal to 1.00. Purified enzyme concentrations were determined assuming an extinction coefficient of 1 mg/mL of

enzyme is equal to 1.98. This value was calculated based on the amino acid concentrations that resulted from complete acid hydrolysis of purified rAnBGal. Amino acid concentrations were measured by the ninhydrin colorimetric method using JLC-500/V (JEOL, Tokyo, Japan).[10)]

β-Galactosidase enzyme activity assay. A reaction mixture (100 μL) containing enzyme, 4 mM *p*-nitrophenyl β-D-galactopyranoside (*p*NPGal; Nacalai Tesque), and 40 mM sodium acetate buffer (pH 3.7) was incubated at 37°C for 10 min. The enzyme reaction was terminated by the addition of 200 μL of Na_2CO_3. The concentration of *p*-nitrophenol was determined from absorbance at 400 nm; 1 U enzyme activity was defined as the amount of enzyme that produces 1 μmol of *p*-nitrophenol from *p*NPGal in 1 min under these conditions.

pH optima and pH and temperature stability. pH optima were determined by measuring enzyme activities at different pH values. Reaction pH was adjusted with 100 mM Britton-Robinson buffer (pH 2.3–6.2). For pH stability, 20 μL of enzyme solution was incubated over a range of pH values at 4°C for 24 h. The pH was adjusted with 100 mM Britton-Robinson buffer for pH 2.3–11.1 and KCl-HCl buffer for pH 1.5. For temperature stability, 60 μL of mixture containing enzyme and 67 mM sodium acetate buffer (pH 3.7) were incubated at 30–75°C for 15 min. The enzyme was considered stable when it retained more than 95% of its initial activity.

Kinetic parameters. Reaction velocities with 10– 200 mM lactose (Nacalai Tesque) or 0.4–4 mM *p*NPGal as substrate were measured using the β-galactosidase enzyme activity assay. Results were fitted to the Michaelis-Menten equation using the Grafit

version 7.0.2 program (Erithacus Software, East Grinstead, UK). The reaction with lactose was terminated by the addition of 200 μL of 2 M Tris-HCl buffer (pH 7.0). The concentration of D-glucose was measured with the Glucose CII Test (Wako Pure Chemical Industries, Osaka, Japan).

Degradation of lactose under gastric conditions. The pH of 200 μL of commercial ultra high temperature sterilized milk was adjusted to 2.0–3.5 with 6 M HCl. The sample was mixed with 20 μL of 35 μM rAnBGal and incubated at 37°C for 2 h. Forty μL of the reaction mixture were heated at 100°C for 5 min to terminate the reaction. The mixture was diluted 5-fold with water and centrifuged (13,000×g, 4°C, 10 min) to obtain the supernatant. The sample was desalted with Amberlite MB4 (Roam and Haas, Philadelphia, PA). One μL was subjected to thin layer chromatography with a developing solvent of 2-propanol/1-butanol/water (2:2:1, v/v/v). The chromatogram was visualized with a detection reagent (acetic acid/sulfuric acid/anisaldehyde, 100:2:1, v/v/v) and heating at 120°C.

The degradation rate of lactose was measured. A reaction mixture (0.2 mL) containing 3.5 μM rAnBGal, 0.1 M sodium acetate buffer (pH 3.5), and 50 mg/mL (146 mM) of lactose was incubated at 37°C for 2 h. Ten μL of the reaction mixture were mixed with 490 μL of water and heated at 100°C for 5 min. The concentration of D-glucose was measured as described above.

RESULTS AND DISCUSSION

Purification and gene cloning of AnBGal.

Six *A. niger* strains were cultured in wheat bran medium, and extracellular β-galactosidase activities were compared (Table 1). *A. niger* AHU7120 produced the

highest β-galactosidase activity and was selected as the source of enzyme for further investigations; 114 U of enzyme activity was obtained from the supernatant of 600 mL of culture broth. AnBGal (96.4 μg) with enzyme activity of 140 U/mg was purified by Q Sepharose, Sephacryl S-100, and Resource Q column chromatography (Table 2). SDS-PAGE analysis of purified AnBGal revealed a single band of 142 kDa (Fig. 1A).

The N-terminal amino acid sequence of AnBGal was Glu-Leu-Leu-Gln-Lys-Tyr-Val-Thr-Trp-Asp-Asp-Lys. This is identical to a putative β-galactosidase (An01g12150), belonging to the glycoside hydrolase family 35, from *A. niger* CBS 513.88. *AnBGal* was amplified by PCR using genomic DNA of *A. niger* AHU7120 as the template. The DNA fragment of interest included 8 introns, and the sequence was identical to *An01g12150* of *A. niger* CBS 513.88. Sequencing analysis of the tryptic peptide of the extracellular β-galactosidase isolated by O'Connell and Walsh from *A. niger* van Tiegh indicated that this enzyme is also encoded by the gene corresponding to *An01g12150* of *A. niger* CBS 513.88.[6)]

Production and purification of rAnBGal.

As the yield of acidophilic β-galactosidase from the culture supernatant of *A. niger* AHU7120 is low, we expressed sufficient rAnBGal to examine the availability of AnBGal for lactose degradation under gastric conditions. The enzyme activity of the culture supernatant of *P. pastoris* expressing rAnBGal harboring the yeast α-factor signal sequence was 12.2 ± 2.5 U/mL (four transformants were analyzed).

For large scale production of rAnBGal, induction culture was carried out at 20°C in the presence of 5 mM EDTA to enhance expression of recombinant enzyme as previously described;[11)] 6,440 U of rAnBGal were obtained (32.2 U/mL) from 200 mL of culture supernatant. The concentration of rAnBGal in the culture broth was

estimated at 0.25 mg/mL according to the specific activity of the purified enzyme. The recombinant enzyme was purified by ammonium sulfate precipitation (90% saturation), DEAE Sepharose CL-6B, and Sephacryl S-300 HR column chromatography, yielding 21.9 mg of purified rAnBGal with an activity of 129 U/mg (Table 3). The specific activity of rAnBGal was close to that of the native enzyme. In contrast to the native enzyme, purified rAnBGal showed a broad band on SDS-PAGE (Fig. 1B). Dragosits *et al.* reported that this smear band results from heterogeneous *N*-glycosylation of recombinant protein.[12)]

Comparison of enzymatic properties of native and recombinant AnBGal.

Only limited publication data are available for comparisons of the characteristics of native and recombinant AnBGals. Therefore, we compared the enzymatic properties of native and recombinant AnBGals (Table 4). The pH optima, pH stability, and kinetic parameters for *p*NPGal and lactose were similar for the two enzymes. Thermal stability of rAnBGal was slightly lower than the native enzyme; the reason for the lower thermostability of rAnBGal remains unclear. The K_m for lactose was 56-fold higher than for *p*NPGal. However, as the lactose concentration of milk is approximately 5% (146 mM) and 2-fold higher than the K_m for lactose, our data suggest that AnBGal can rapidly degrade lactose in milk.

Degradation of lactose with rAnBGal under gastric conditions.

Commercial milk was incubated at pH 2.0–3.5 in the presence of rAnBGal, and the digestion of lactose was monitored (Fig. 2A). Lactose in milk was more rapidly degraded at pH 3.5 than pH 2.0. These observations are consistent with the pH profile of rAnBGal and indicate that rAnBGal will effectively degrade lactose in the stomach,

as gastric pH remains around 3 for 3 h following the ingestion of milk.[13] Hydrolysis of 50 mg/mL of lactose, a concentration similar to that of milk, was monitored at pH 3.5 (Fig. 2B). 66% of lactose was degraded at 15 min, and the degradation rate reached 90% at 2 h. Gastric half-emptying time for milk is 25 min, and >90% of ingested milk is emptied from the stomach at 2 h following ingestion.[14] Taken together, these observations suggest that AnBGal may degrade more than 66% of lactose before gastric half-emptying of ingested milk. These data indicate that AnBGal may help alleviate symptoms of lactose intolerance, and this enzyme may be useful for an enzyme supplement to degrade lactose in the gastric condition.

ACKNOWLEDGMENTS

We are grateful to Tomohiro Hirose of the Instrumental Analysis Division, Equipment Management Center, Creative Research Institute, Hokkaido University for amino acid analysis and N-terminal amino acid sequence analysis. We thank the staff of the DNA Sequencing Facility of the Research Faculty of Agriculture, Hokkaido University for assistance with DNA sequence analyses.

REFERENCES

1) E.E. Sterchi, P.R. Mills, J.A. Fransen, H.P. Hauri, M.J. Lentze, H.Y. Naim, L. Ginsel, J. Bond: Biogenesis of intestinal lactase-phlorizin hydrolase in adults with lactose intolerance. Evidence for reduced biosynthesis and slowed-down maturation in enterocytes. *J. Clin. Invest.*, **86**, 1329-1337 (1990).
2) N.S. Scrimshaw and E.B. Murray: The acceptability of milk and milk products in populations with a high prevalence of lactose intolerance. *Am. J. Clin. Nutri.*, **48**, 1083-1159 (1988).

3) S. Ogushi, T. Yoshimoto, and D. Tsuru: Purification and comparison of two types of β-galactosidases from *Aspergillus oryzae*. *J. Ferment. Technol.*, **58**, 115-122 (1980).
4) S. O'Connell and G. Walsh: Application relevant studies of fungal β-galactosidases with potential application in the alleviation of lactose intolerance. *Appl. Biochem. Biotechnol.*, **149**, 129-138 (2008).
5) X. Hu, S. Robin, S. O'Connell, G. Walsh, and J.G. Wall: Engineering of a fungal β-galactosidase to remove product inhibition by galactose. *Appl. Microbiol. Biotechnol.*, **87**, 1773-1782 (2010).
6) S. O'Connell and G. Walsh: A novel acid-stable, acid-active β-galactosidase potentially suited to the alleviation of lactose intolerance. *Appl. Microbiol. Biotechnol.*, **86**, 517-524 (2010).
7) S.N. Ho, H.D. Hunt, R.M. Horton, J.K. Pullen, and L.R. Pease: Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene*, **77**, 51-59 (1989).
8) M.M. Bradford: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248-254 (1976).
9) M.O. Dayhoff, G.E. Perlmann and D.A. MacInnes: The partial specific volumes, in aqueous solution, of three proteins. *J. Am. Chem. Soc.*, **74**, 2515-2517 (1952).
10) S. Moore and W.H. Stein: Photometric ninhydrin method for use in the chromatography of amino acids. *J. Biol. Chem.*, **176**, 367-388 (1948).
11) N. Himeno, W. Saburi, S. Wakuta, R. Takeda, H. Matsuura, K. Nabeta, S. Sansenya, J.R. Ketudat Cairns, H. Mori, R. Imai, and H. Matsui: Identification of

rice β-glucosidase with high hydrolytic activity towards salicylic acid β-D-glucoside. *Biosci. Biotechnol. Biochem.*, **77**, 934-939 (2013).

12) M. Dragosits, S. Pflügl, S. Kurz, E. Razzazi-Fazeli, I.B.H. Wilson, and D. Rendic: Recombinant Aspergillus β-galactosidases as a robust glycomic and biotechnological tool. *Appl. Microbiol. Biotechnol.*, in press, doi: 10.1007/s00253-013-5192-3.

13) M.C. Martini, G.L. Bollweg, M.D. Levitt, and D.A. Savaiano: Lactose digestion by yogurt β-galactosidase: influence of pH and microbial cell integrity. *Am. J. Clin. Nutr.*, **45**, 432-436 (1987).

14) S. Mahé, J.F. Huneau, P. Marteau, F. Thuillier, and D. Tomé: Gastroileal nitrogen and electrolyte movements after bovine milk ingestion in humans. *Am. J. Clin. Nutr.*, **56**, 410-416 (1992).

Fig. 1. SDS-PAGE analysis of purified native and recombinant AnBGals.

A, Native AnBGal (0.5 μg). B, rAnBGal (2 μg). Molecular masses of standard proteins are indicated on the left. The proteins were visualized using Coomassie Brilliant Blue.

Fig. 2. Digestion of lactose in milk with rAnBGal.

A, TLC analysis. Lactose in milk was digested with rAnBGal at various pH values. Reaction time (min) is shown below the figure. B, Time course of lactose hydrolysis.

Table 1. Comparison of *A. niger* extracellular β-galactosidase activities

Strain	Activity (U/mL)[a]
AHU7104	0.442
AHU7120	0.764
AHU7217	0.455
AHU7294	0.282
AHU7295	0.279
AHU7296	0.375

[a]Activity of extract from wheat bran medium after culture at 30°C for 5 d.

Table 2. Purification of AnBGal

Procedure	Activity (U)	Protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Culture supernatant	114	44.1[a]	2.59	100	1
Q Sepharose	69.0	30.9[b]	2.23	60.5	0.86
Sephacryl S-100	18.0	0.988[b]	18.2	15.8	7.0
Resource Q	13.5	0.0964[c]	140	11.8	54

[a]Determined by the Bradford method. [b]Determined by the UV method, assuming the extinction coefficient of 1 mg/mL of protein was 1.00. [c]Calculated assuming the extinction coefficient of 1 mg/mL of purified AnBGal was1.98.

Table 3. Purification of rAnBGal

Procedure	Activity (U)	Protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Culture supernatant	6,440	3580	1.80	100	1
Ammonium sulfate precipitation (90% saturation)	5,450	850	6.41	84.6	3.56
DEAE Sepharose CL-6B	3,610	83.2	43.4	56.1	24.1
Sephacryl S-300 HR	2,830	21.9[a]	129	43.9	71.7

[a]This value was determined as 1.98 based on an extinction coefficient of 1 mg/mL of purified AnBGal. Other values were calculated assuming the extinction coefficient of 1 mg/mL of protein was 1.00.

Table 4. Comparison of enzymatic properties of native and recombinant AnBGals.

		Native	Recombinant
Optimum pH		3.7	3.7
pH stability[a]		2.3-8.9	2.3-8.9
Heat stability[b]		≤65°C	≤60°C
*p*NPGal	k_{cat} (s^{-1})	394 ± 4	418 ± 1
	K_m (mM)	1.33 ± 0.03	1.29 ± 0.02
	k_{cat}/K_m ($s^{-1}mM^{-1}$)	296	324
Lactose	k_{cat} (s^{-1})	556 ± 32	515 ± 9
	K_m (mM)	74.5 ± 9.3	72.0 ± 3.9
	k_{cat}/K_m ($s^{-1}mM^{-1}$)	7.46	7.15

[a]pH treatment was carried out at 4°C for 24 h. [b]Heat treatment was done at pH 3.7 for 15 min.

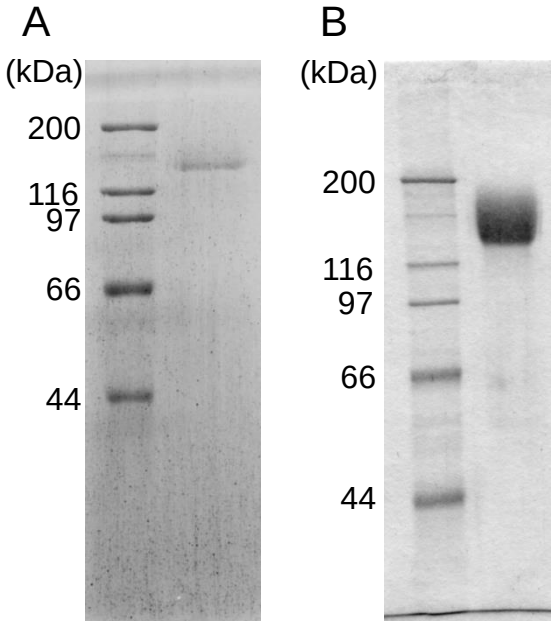
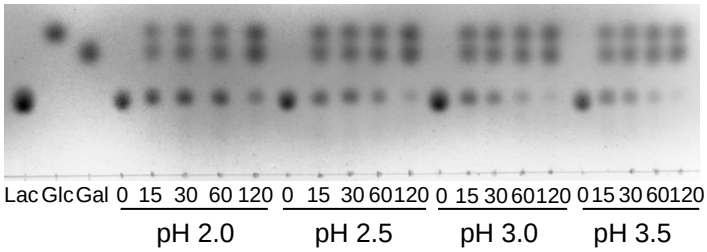


Fig. 1., Saburi et al.

A



B

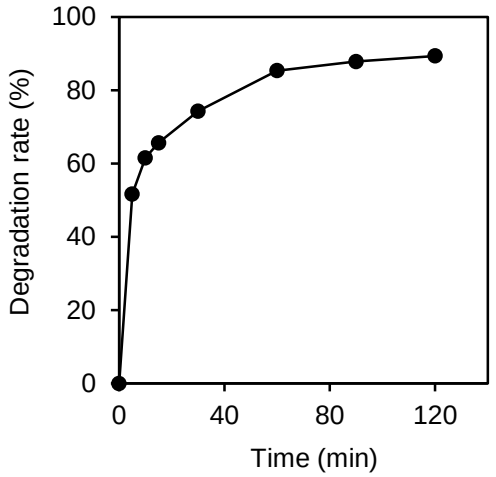


Fig. 2., Saburi et al.