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An Acidophilic β-Galactosidase from *Bispora* sp. MEY-1 with High Lactose Hydrolytic Activity under Simulated Gastric Conditions

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BgalA, a full-length gene (3,009 bp) that encodes a β-galactosidase, was cloned from the meso-acidophilic fungus *Bispora* sp. MEY-1 and expressed in *Pichia pastoris*. The deduced amino acid sequence of *BgalA* shares highest identity (55.5%) with the β-galactosidase from *Aspergillus phoenicis*, which belongs to the glycoside hydrolyase family 35. Purified recombinant BgalA is acidophilic, exhibiting maximum activity at pH 1.5, which is lower than that reported for other β-galactosidases. The enzyme has high pH and thermal stability and is resistant to proteases and cations found in milk. The K_m and V_{max} of BgalA for 2-nitrophenyl-β-D-galactopyranoside and lactose are 5.22 mM and 120.8 μmol/(min·mg), and 0.31 mM and 137.3 μmol/(min·mg), respectively. Under simulated gastric conditions, BgalA has greater stability (~100%) and hydrolysis ratio (>80%) toward milk lactose than the commercially available β-galactosidase from *Aspergillus oryzae* (ATCC 20423). Thus, BgalA may be a better digestive supplement for alleviating symptoms associated with lactase deficiency.

KEYWORDS: Acidophilic; *Bispora* sp. MEY-1; β-galactosidase; simulated gastric fluid

INTRODUCTION

Lactose, a natural disaccharide in the milk of most mammals, must be hydrolyzed by lactase for efficient absorption. However, in humans and other mammals, lactase phlorizin-hydrolase activity declines after weaning (*1*). Thus, greater than 70% of the world's population suffers from lactase deficiency, which limits consumption of lactose-containing dairy products. Lactose-intolerant individuals, who consume milk or other dairy products, even at reduced quantities, have gastroenterological complications related to the uptake of calcium and certain other nutrients (*2*).

β-Galactosidase (EC 3.2.1.23), which is commonly known as lactase, is a hydrolase that converts lactose into glucose and galactose. On the basis of the amino acid sequence in the carbohydrate-active enzyme database (CAZy; http://www.cazy.org), β-galactosidases are generally grouped into glycoside hydrolase (GH) families 1, 2, 35, and 42. Many microorganisms, animals, and plants produce β-galactosidases (*3*). Most of the β-galactosidases currently used as digestive supplements in dairy products are from fungi generally recognized as safe, such as *Aspergillus niger* and *Aspergillus oryzae* (*4*−*6*). These products, which enhance gastrointestinal digestion of lactose and alleviate the symptoms caused by lactase deficiency, are widely available on the market in the form of caplets, chewable tablets, or soft gel capsules (*7*).

The efficacy of lactase preparations in the gastrointestinal tract depends on the degradation of the carriers and residual enzyme activity under physiological conditions (*7*). Most supplemental lactases are coated to prevent gastric inactivation and become activated in the intestine (*8*). These enzymes have a pH optimum of 2.5−5.5. Because of their instability at the typical stomach pH of ~2.0, these supplemental lactases are not ideal for lactose hydrolysis in vivo. Furthermore, the activity of these enzymes are inhibited somewhat by proteases and the pH (~7) in the small intestine (*9*). In order to hydrolyze lactose in the stomach, the ideal lactase must resist denaturation by acid and proteases and must retain high activity at physiological temperature and low pH.

The purpose of this study was to obtain a β-galactosidase that functions well along the gastric tract. An acidophilic β-galactosidase, BgalA, was isolated from the meso-acidophilic fungus, *Bispora* sp. MEY-1 (*10*). BgalA was highly stable under acid conditions or in simulated gastric fluid (SGF). It had greater stability and hydrolytic activity against dietary lactose than a commercially available β-galactosidase, suggesting that it has potential as a digestive supplement for the treatment of lactase deficiency.

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MATERIALS AND METHODS

Strains, Media, Vectors, and Chemicals. To induce β-galactosidase production, *Bispora* sp. MEY-1 was cultivated at 30 °C in the inducing medium, which contained 5 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.2 g/L $CaCl_2$, 0.01 g/L $FeSO_4 \cdot 7H_2O$, 3 g/L wheat bran, and 3 g/L soybean meal. *Escherichia coli* JM109 (TaKaRa, Otsu, Japan) was cultivated at 37 °C in Luria−Bertani medium and used as the host for gene cloning. *Pichia pastoris* GS115 (Invitrogen, Carlsbad, CA, USA) cultivated at 30 °C in yeast extract peptone dextrose medium was used for gene expression. The plasmids pGEM-T-Easy (Promega, Madison, WI, USA) and pPIC9 (Invitrogen) were used as the cloning and expression vectors, respectively. Buffered glycerol complex medium, buffered methanol complex medium, regeneration dextrose base medium, minimal dextrose medium, and minimal methanol medium were prepared as described in the manual of the *Pichia* expression kit (Invitrogen).

Over-the-counter lactase digestive supplement (lactase enzyme), the commercial β-galactosidase from *A. oryzae* ATCC 20423, was purchased from Vitamin World (Bohemia, NY, USA). The substrate, 2-nitrophenyl-β-D-galactopyranoside (ONPG), was purchased from Sigma (St. Louis, MO, USA). The DNA purification kit, Genome Walking kit, and LA Taq DNA polymerase were purchased from TaKaRa. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs (Hertfordshire, UK). All other chemicals were of analytical grade and were commercially available.

Cloning of the β-Galctosidase Gene from *Bispora* sp. MEY-1. A total of 67 amino acid sequences of GH family 35 β-galctosidases from subphylum *Ascomycota* were obtained from Pfam (release 18.0) (http://www.sanger.ac.uk/ Software/Pfam/) and aligned using ClustalX software (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/) (*11*). On the basis of the alignment, two conserved motifs, Y(F)GGTNWG and TGNLGGE, were identified, and a degenerate primer set was designed accordingly. Genomic DNA isolated from *Bispora* sp. MEY-1 was used as a template for PCR amplification. The PCR conditions were as follows: denaturing at 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, 48 °C for 30 s, and 72 °C for 1 min. The PCR product was cloned into pGEM-T-Easy vector and sequenced. The 5′ and 3′ flanking regions of the conserved region were obtained by thermal asymmetric interlaced (TAIL)-PCR (*12*) using nested insertion-specific primers (**Table 1**). TAIL-PCR was performed using the Genome Walking kit according to the manufacturer's instructions. The amplified upstream and downstream products were sequenced and assembled to obtain a full-length gene.

After 3 days of growth at 30 °C in the inducing medium, mycelia of *Bispora* sp. MEY-1 were collected and immediately ground to a fine powder in liquid nitrogen. Total RNA was isolated and purified using the Total RNA Isolation System (Qiagen, Hilden, Germany). Full-length β-galactosidase cDNA, *BgalA*, was obtained from the total RNA by RT-PCR amplification. Reverse transcription was performed using a reverse transcription kit (Invitrogen). PCR amplification was performed with the specific primers BgalA F and BgalA R (**Table 1**) at an annealing temperature of 60 °C.

Sequence Analysis. The signal peptide was predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP/). Homology searches in GenBank were performed using the BLAST server. Multiple alignments of protein sequences were accomplished using the ClustalW program (http://www.ebi.ac.uk/clustalW) and GeneDoc software. Putative *N*-glycosylation sites were predicted by the NetNGlyc 1.0 program (http://www.cbs.dtu.dk/services/NetNGlyc).

β-Galactosidase Activity Assay. β-Galactosidase activity was determined by measuring the amount of *o*-nitrophenol released from ONPG. Reactions contained 100 μL of diluted enzyme solution, 250 μL of 5 mM ONPG, and 150 μL of glycine−HCl buffer (pH 1.5). After incubation at 37 °C for 5 min, the reaction was terminated by adding 1.5 mL of 1 M sodium carbonate. Released *o*-nitrophenol was quantified by measuring the absorption at 420 nm. One unit of β-galactosidase activity was defined as the amount of enzyme that produced 1 μmol of *o*-nitrophenol per minute. Each reaction and its control were run in triplicate.

Expression of BgalA in *P. pastoris*. To construct the expression vector in *P. pastoris*, the cDNA fragment of *BgalA* without the signal peptide coding sequence was amplified using the P-BgalF and P-BgalR primers (**Table 1**). The PCR product was digested with *SnaB*I and *Not*I and cloned into pPIC9 in-frame with the α-factor signal peptide. The recombinant plasmid, pPIC9-*Bgal*A, was linearized by *Bgl*II and electroporated into *P. pastoris* GS115 competent cells. Transformed cells were plated on regeneration dextrose base medium plates and incubated at 30 °C for 2 to 3 days until colonies appeared. His^+ transformants were then transferred to minimal methanol and minimal dextrose plates and grown for 1 to 2 days at 30 °C until single colonies formed. These colonies were transferred and grown in 3 mL of buffered glycerol complex medium at 30 °C for 2 days. The cells were pelleted by centrifugation (5,000*g*, 5 min) and resuspended in 1 mL of buffered methanol complex medium containing 0.5% methanol for induction at 30 °C. After 2 days of induction, the culture supernatant was collected by centrifugation (12,000*g*, 3 min) to measure β-galactosidase activity assay. The transformant with the highest β-galactosidase activity was used for large-scale expression and cultivation in a 1,000-mL conical flask containing 400 mL of buffered glycerol complex medium at 30 °C with agitation (220 rpm) for 2 days. Cells were then harvested by centrifugation (5,000*g*, 10 min) and resuspended in 200 mL of buffered methanol complex medium at 30 °C followed by induction with 0.5% methanol every 24 h until the enzyme concentration in the medium started to level off. β-Galactosidase activity in the culture supernatant was assessed at 12-h intervals during induction/expression.

Table 1. Primers Used in This Study

primers	primer sequence (5′→3′)[a]	size (bases)
usp1	GCACGGTGAGCATGTAACTCGTAGATGC	28
usp2	CGGATAAAGTAGAAATTGGTCCCGTACTCTCC	32
usp3	CGCGTCACGGTCCGGTCTTCTTTG	24
dsp1	GAAACCACATTCTTCATCGAGACAGAAGGTGG	32
dsp2	GGCCACTCCGTCTGGCTCAATAGC	24
dsp3	CATTCCCGACGACGCCATGAAGCAAC	26
BgalA F	ATGTTATTGTCACGCTCTTTCGCGGCCGGTGTAGTTGGC	39
BgalA R	CGAGCGGCCGCTCAATACGCCCCAGGCCGTGGGCTATAC	39
P-BgalF	GTCTACGTAATGTTGTTGTCACGCTCTTTCGCGGCCGGT	39
P-BgalR	CGAGCGGCCGCTCAATACGCCCCAGGCCGTGGGCTATAC	39

[a] Underlined sequences indicate restriction sites incorporated into the primers.

Purification of Recombinant BgalA (r-BgalA). All of the purification steps were carried out at 4 °C unless otherwise specified. The cell-free culture supernatant was obtained by centrifugation at 12,000*g* at 4 °C for 10 min, concentrated by ultrafiltration using a 6-kDa cutoff membrane, and dialyzed against 20 mM Tris-HCl buffer (pH 7.0). After removing the undissolved materials by centrifugation at 12,000*g* for 10 min at 4 °C, the clear supernatant was loaded onto a HiTrap Q Sepharose XL FPLC column (Amersham Pharmacia Biotech, Uppsala, Sweden). Proteins were eluted with a step-gradient of NaCl (0−1.0 M) at a flow rate of 5.0 mL/min. Fractions with enzyme activity were pooled and concentrated by ultrafiltration at 4,000*g* for 20 min using a 10-kDa molecular weight cutoff filter (Millipore, Billerica, MA, USA). Protein concentration was determined using the Bradford assay (*13*) with bovine serine albumin as the standard.

Electrophoresis and Glycosylation Analysis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was carried out using a 10% gel and a vertical electrophoretic system as described by Laemmli (*14*). Protein bands were visualized by staining with Coomassie brilliant blue R-250.

Purified r-BgalA (~5 μg) was deglycosylated using 250 U of endo-β-*N*-acetylglucosaminidase H (Endo H) for 2 h at 37 °C according to the manufacturer's instructions (New England Biolabs). Deglycosylated and untreated r-BgalA were analyzed by SDS−PAGE.

Characterization of Purified r-BgalA. To characterize the properties of r-BgalA, 0.2 μg purified r-BgalA was used in each individual reaction system. The pH versus activity profile was determined by measuring enzyme activity in the following buffers: 0.1 M glycine−HCl (pH 0.5−2.2) and 0.1 M citric acid−Na_2HPO_4 (pH 2.2−8.0). The effect of pH on r-BgalA stability was estimated by measuring the residual activity under standard conditions (pH 1.5, 37 °C, 5 min) after incubating the purified enzyme in buffers of differing pH for 60 min.

The temperature versus activity profile was determined by measuring β-galactosidase activity over the range 30−90 °C in 0.1 M glycine−HCl (pH 1.5). The thermal stability was determined by measuring residual β-galactosidase activity (by the standard procedure) after incubating the purified enzyme (1 mg/mL) in 0.1 M glycine−HCl at 60 °C, 70 or 80 °C for 2, 5, 10, 15, 20, 30, or 60 min.

The effect of various metal ions and chemical reagents on enzyme activity was determined by measuring the residual activity after incubation with 2, 5, or 10 mM of Na^+, K^+, Ca^{2+}, Li^+, Co^{2+}, Cr^{3+}, Ni^{2+}, Cu^{2+}, Mg^{2+}, Fe^{3+}, Mn^{2+}, Zn^{2+}, Pb^{2+}, Ag^+, Hg^{2+}, EDTA, SDS, or β-mercaptoethanol in 0.1 M glycine−HCl (pH 1.5) at 37 °C for 5 min.

To examine the resistance of purified r-BgalA (1 mg/mL) to proteolysis, it was incubated with either pepsin (dissolved in 0.1 M glycine−HCl, pH 2.0) or trypsin (dissolved in 0.1 M Tris-HCl, pH 7.0) at 37 °C for different periods, using a protease/β-galactosidase (w/w) ratio of 0.1:1. Protease resistance was assessed by measuring the residual β-galactosidase activity under standard conditions following protease treatment. As a control, the recombinant enzyme was incubated under the same conditions in the absence of protease.

Kinetic parameters, K_m and V_{max}, were determined in 0.1 M glycine−HCl (pH 1.5) containing 0.5−4.0 mM ONPG or 5−50 mM lactose. The concentration of glucose was determined using a glucose oxidase−peroxidase assay kit (Biosino, Beijing, China) after hydrolysis at 37 °C for 5 min. Lineweaver−Burk plots were constructed to determine kinetic parameters. Three independent experiments were averaged, and each experiment included three replicates.

Analysis of Hydrolysis Products. Reactions containing 1 mg of purified r-BgalA (100 U) and 500 μg of lactose in 500 μL of 0.1 M glycine−HCl (pH 1.5) were incubated at 60 °C for 12 h. A reaction in which r-BgalA was omitted was used as a control. After hydrolysis, the enzyme was removed using the Nanosep Centrifugal 10 kDa Device (Pall, Chicago, IL, USA). The remaining products were analyzed using high-performance anion-exchange chromatography with a model 2500 system from Dionex (Sunnyvale, CA, USA). Glucose and galactose were used as standards.

Enzyme Stability under Simulated Gastric Conditions. The stability of r-BgalA in SGF was examined using a modified method of Huang et al. (*15*−*17*). Briefly, SGF was prepared as follows: 0.2 M glycine−HCl or 0.2 M sodium acetate−acetic acid containing 2.0 mg/mL NaCl and 3.2 mg/mL pepsin was adjusted with HCl or sodium acetate, respectively, to pH 1.5, 2.0, 3.0, and 4.0 or pH 5.0 and 6.0, respectively. Purified r-BgalA (10 μg) was incubated in SGF (pH 1.5−6.0) at 37 °C for 60 min. Residual β-galactosidase activity was determined by the standard procedure. As a control, purified β-galactosidase was incubated in buffer (0.2 M glycine−HCl or 0.2 M sodium acetate−acetic acid) or SGF without pepsin (SGF-NP) under the same conditions. The results from three individual experiments were averaged; each experiment included three replicates.

Enzyme stability of the commercial β-galactosidase from *A. oryzae* (2 μg), which showed the same activity with the r-BgalA, was tested using the same procedure and compared with r-BgalA. All experiments were conducted in triplicate.

Ability to Hydrolyze Milk Lactose in Simulated Gastric Conditions. The stomach digestion phase for milk lactose hydrolysis was simulated according to the model described by Minekus et al. (*18*) with some modifications. Reactions containing 2.0 mg/mL NaCl and 0.2 M glycine in 10 mL of milk were initially adjusted to pH 1.9 with HCl on ice, and then 50 U or 100 U purified r-BgalA was added to the mixture. The enzyme was kept in the mixture for 20 min. Then the pH gradient was adjusted with NaOH or HCl on ice according to the following protocol: pH 5.5, 10 min; pH 4.6, 10 min; pH 3.8, 10 min; pH 2.8, 20 min; and pH 1.9, 40 min (*16*). Pepsin (0.5 mg/mL) was added to the reactions at the beginning of each pH step during the entire experiment. All procedures were conducted in a rotary incubator at 37 °C and 220 rpm. Samples (500 μL) were collected at the end of each phase and transferred to a 10 mL volumetric flask, followed by the addition of 250 μL of 20% $Pb(CH_3CO)_2$ and 250 μL of $K_2C_2O_4/Na_2HPO_4$ solution (16 mM potassium oxalate and 20 mM disodium hydrogen phosphate) to precipitate protein. The volumetric flask was filled with water and placed in the dark for 30 min to ensure that the protein was precipitated completely. Glucose content in the supernatant was tested using the glucose oxidase−peroxidase assay kit (Biosino). A reaction without r-BgalA was tested as a negative control.

Nucleotide Sequence Accession Number. The nucleotide sequence for the *Bispora* sp. MEY-1 β-galactosidase gene (*BgalA*) was deposited in the GenBank database under accession number FJ472925.

RESULTS

Cloning of the β-Galactosidase Gene and Sequence Analysis. On the basis of the conserved motifs of GH family 35 β-galactosidases from Ascomycota, a degenerate primer set (BgalAP1, 5′-TWYGGNGGNACNAAYTGGGG-3′ and BgalAP2, 5′-TCNCCNCCNAGRTTNCCNGT-3′, in which W, Y, N, and R represent A/T, C/T, A/C/T/G, and A/G, respectively) was designed to amplify the partial core region of the β-galactosidase gene. Using these primers, a 1494-bp fragment was amplified from the genomic DNA of *Bispora* sp. MEY-1. The 5′ and 3′ flanking regions were amplified by TAIL-PCR and assembled with the known gene fragment. The resulting 3,774-bp DNA sequence contained one complete chromosomal gene of 3,181 bp. The full-length cDNA sequence (3,009 bp) of the β-galactosidase was obtained through RT-PCR using the specific primers P-BgalA F and P-BgalA R. Three introns of 64, 53, and 55 bp interrupted the coding sequence (**Figure 1**).

SignalP analysis indicated that BgalA had no N-terminal signal peptide. The mature protein contained 1,002 amino acid residues with a calculated molecular mass of ∼110 kDa. Database searches and alignment of the gene and its deduced amino acid sequence with known β-galactosidases were performed. Sequence comparison classified BgalA as a member of the GH 35 family. The amino acid sequence of BgalA shared the highest identity (55.5%) with a GH 35 β-galactosidase from *Aspergillus phoenicis*, followed by the β-galactosidases from *A. niger* (55.1%), *A. candidus* (54.8%), and *Talaromyces emersonii* (54.7%). Structure analysis using the Swiss-pdb viewer (http://www.expasy.ch/spdbv) indicated that two catalytic glutamates in BgalA, Glu (195) and Glu (295), act as the proton donor and acceptor, respectively, in the catalytic domain.

Expression and Purification of r-BgalA. The gene encoding the mature BgalA was cloned into vector pPIC9 and linearized by *Bgl*II digestion. The recombinant plasmid was transformed into *P. pastoris* GS115 by electroporation. Thirty of the 200 transformants showed β-galactosidase activity in the culture supernatant, confirming that *BgalA* encodes a functional β-galactosidase. The transformant with the highest β-galactosidase activity was subjected to large-scale expression. After 48 h of induction with methanol, the enzyme activity reached 0.08 U/mL.

r-BgalA was purified by a one-step anion exchange chromatography procedure, which yielded 1.2-fold purification and a specific activity of 99.5 U/mg. SDS−PAGE analysis of both the crude and purified enzyme yielded only one band with an approximate molecular mass of 130 kDa (**Figure 2a**), which is higher than the calculated value of ∼110 kDa. However, after deglycosylation with Endo H, which removes the glycan portion of *N*-glycoproteins, the purified r-BgalA migrated as a single band of ∼110 kDa, matching that calculated for the mature protein (**Figure 2b**).

Characterization of r-BgalA. Purified r-BgalA had measurable enzymatic activity over a pH range of 0.5 through 8.0 (measured at 37 °C) with an optimum pH of 1.5 (**Figure 3a**). The activity was substantially lower when assayed at pH < 1.0 or pH ≥ 4.0. In the pH stability assay, the enzyme retained greater than 50% of its initial activity after incubation at pH 0.5 through 12.0 for 60 min (**Figure 3b**).

Purified r-BgalA showed maximum activity at 60 °C and retained greater than 40% of maximum activity between 37 and 80 °C (**Figure 3c**). The thermostability of r-BgalA was determined at 60 and 70 °C. No activity was lost after incubation at 60 °C for

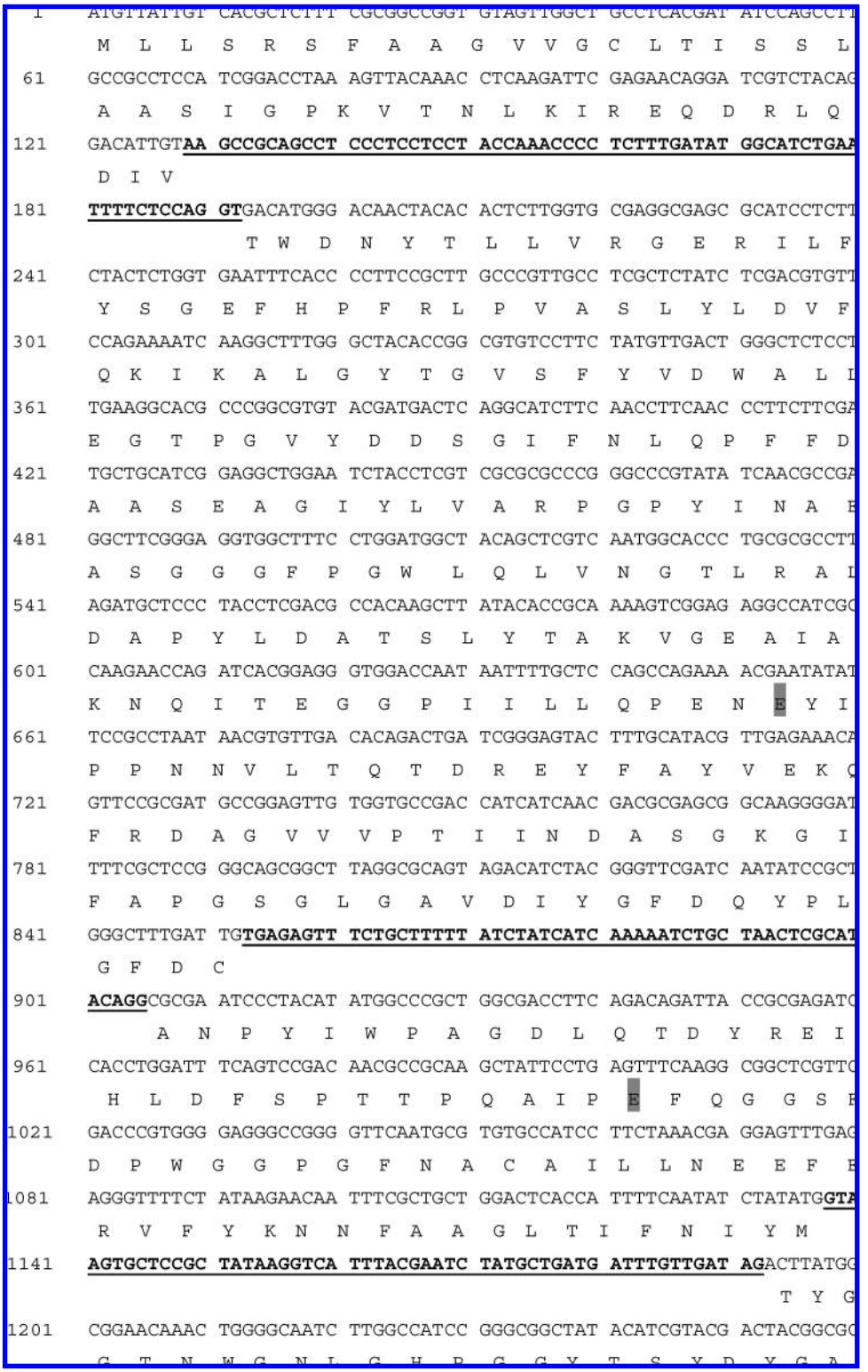

Figure 1. Partial amino acid sequence of the β-galactosidase from *Bispora* sp. MEY-1. Three introns are underlined and in bold. The catalytic glutamate residues are indicated with gray boxes.

60 min, and more than 60% of the initial activity was retained after incubation at 70 °C for 60 min (**Figure 3d**). Most of the enzymatic activity was lost within 2 min at 80 °C (data not shown).

The effects of different metal ions and chemical reagents on r-BgalA activity are summarized in **Table 2**. β-Mercaptoethanol and SDS strongly inhibited r-BgalA activity, whereas EDTA and 10 mM Pb^{2+} partially inhibited the activity. Ten millimolar Co^{2+}, Cr^{3+}, Cu^{2+}, Ca^{2+}, and Li^{+} moderately activated r-BgalA activity. Other metal ions and reagents had little effect on activity at any of the concentrations tested.

After treatment with pepsin or trypsin at 37 °C for 60 min, r-BgalA retained 103.6% and 97.4% of its initial activity, respectively, indicating that the enzyme is strongly resistant to pepsin and trypsin digestion. Kinetic constants (K_m and V_{max}) were calculated from a Lineweaver−Burk plot. The K_m and V_{max} values were 5.22 mM and 120.8 μmol/(min·mg), respectively, for ONPG and 0.31 mM and 137.3 μmol/(min·mg), respectively, for lactose.

High-performance anion-exchange chromatography revealed that the main products of lactose hydrolysis by r-BgalA were 19.5% galactose, 72.5% glucose, and 8.0% oligosaccharide.

Stability of r-BgalA Activity in SGF. After incubation in SGF at 37 °C for 60 min, r-BgalA retained almost all of its activity at the pH values encountered at the whole stomach stage with or without the presence of pepsin (**Figure 4**), whereas the commercial *A. oryzae* β-galactosidase lost all of its activity at pH 1.5 through 3.0, mainly due to the low pH.

Hydrolysis of Milk in the Simulated Gastric Phase. Under conditions that simulate the five stages of the gastric phase, a total of 85.8% and 53.1% of lactose in milk was hydrolyzed by 100 U and 50 U, respectively, of r-BgalA (**Figure 5**), suggesting that r-BgalA efficiently hydrolyzes milk over a wide range of pH. By comparison, *A. oryzae* β-galactosidase (100 U) only hydrolyzed 3.5% of milk lactose during the whole procedure (data not shown), suggesting that it is inactivated at low pH.

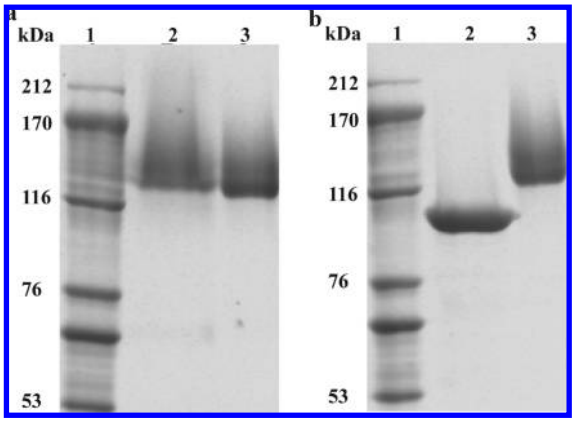

Figure 2. SDS−PAGE analysis of r-BgalA. (**a**) SDS−PAGE analysis of *BgalA* expressed in *P. pastoris*. Lanes: 1, molecular mass standard; 2, culture supernatant from the induced transformant; 3, r-BgalA purified by anion exchange chromatography. (**b**) SDS−PAGE analysis of purified r-BgalA before and after deglycosylation with Endo H. Lanes: 1, molecular mass standard; 2, purified r-BgalA after deglycosylation with Endo H; 3, purified r-BgalA.

DISCUSSION

In this study, a β-galactosidase gene, *BgalA*, was isolated from the meso-acidophilic fungus *Bispora* sp. MEY-1 and expressed in *P. pastoris*. To our knowledge, this is the first report of cloning and expression of a β-galactosidase from the genus *Bispora*. The deduced amino acid sequence of *BgalA* shares highest identity (~56%) with the β-galactosidases from *Aspergillus* and *T. emersonii*. Purified r-BgalA was optimally active under acidic conditions, exhibited excellent thermal and pH stability, was highly

Table 2. Effect of Metal Ions and Chemical Reagents on the β-Galactosidase Activity of Purified r-BgalA

	relative β-galactosidase activity (%)[a]		
reagent added	2 mM	5 mM	10 mM
none	100.0	100.0	100.0
Hg^{2+}	110.4 ± 4.1	96.8 ± 2.8	94.3 ± 1.2
Li^{+}	107.1 ± 4.0	101.1 ± 3.0	110.1 ± 4.1
K^{+}	106.6 ± 7.8	102.4 ± 1.7	107.7 ± 5.2
Pb^{2+}	105.6 ± 7.9	91.7 ± 3.2	84.6 ± 3.7
Na^{+}	105.3 ± 4.7	102.6 ± 0.8	102.4 ± 5.8
Co^{2+}	105.2 ± 4.1	103.0 ± 1.6	109.1 ± 4.4
Cr^{3+}	105.2 ± 3.2	106.3 ± 1.4	115.0 ± 3.2
Ni^{2+}	103.4 ± 4.3	101.9 ± 2.0	99.4 ± 5.4
Mg^{2+}	101.7 ± 3.0	100.6 ± 1.7	100.7 ± 8.6
Zn^{2+}	101.3 ± 5.2	102.9 ± 2.3	105.2 ± 5.6
Cu^{2+}	99.3 ± 2.5	99.2 ± 4.9	110.1 ± 6.6
Ca^{2+}	98.3 ± 9.2	101.9 ± 1.2	108.7 ± 6.7
Fe^{3+}	95.6 ± 7.5	96.8 ± 4.9	104.4 ± 2.1
Mn^{2+}	94.9 ± 6.9	90.1 ± 7.7	ND[b]
EDTA	97.0 ± 3.6	92.1 ± 8.4	86.2 ± 4.0
β-mercaptoethanol	79.9 ± 2.5	53.7 ± 5.7	41.5 ± 0.8
SDS	38.1 ± 4.1	7.2 ± 4.6	ND[b]

[a] Values represent the mean ± SD (n = 3) relative to untreated control samples. [b] ND, not determined.

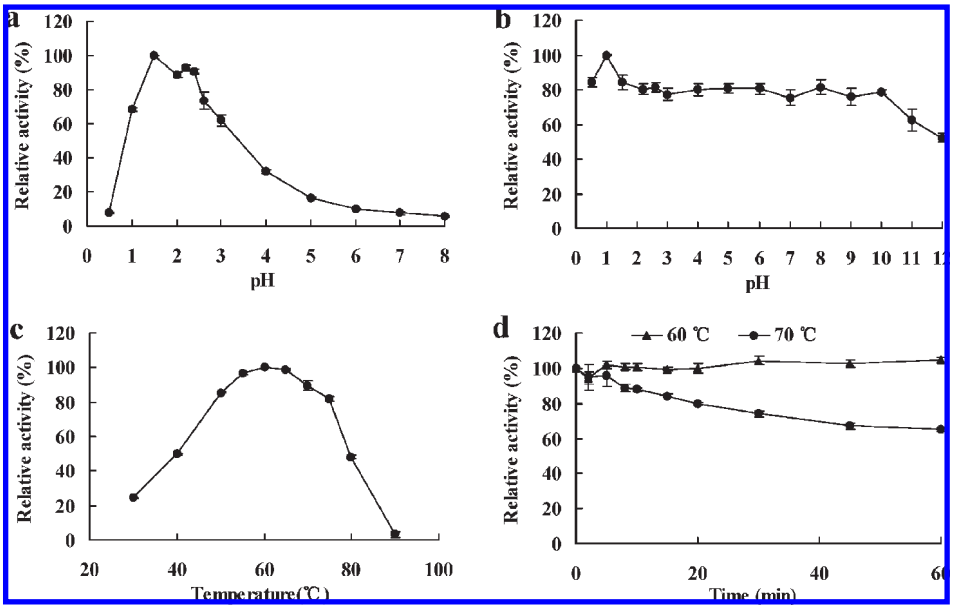

Figure 3. Characterization of purified r-BgalA activity. (**a**) Effect of pH on β-galactosidase activity. The r-BgalA activity assay was performed at 37 °C in buffers ranging from pH 0.5 through 8.0. (**b**) Stability of β-galactosidase activity over pH 0.5 to 12.0. After incubation at 37 °C for 60 min in buffers of varying pH, β-galactosidase activity was measured in 0.1 M glycine−HCl buffer (pH 1.5) at 37 °C. (**c**) Effect of temperature on β-galactosidase activity measured in 0.1 M glycine−HCl (pH 1.5). (**d**) Thermostability of r-BgalA measured following preincubation at 60 or 70 °C in 0.1 M glycine−HCl (pH 1.5). Aliquots were removed at specific time points to measure residual activity at 37 °C. Each value represents the mean ± SD (n = 3).

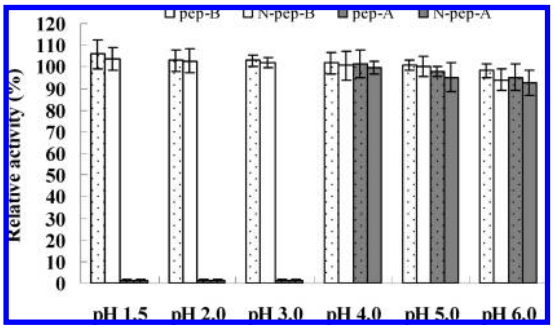

Figure 4. Stability of r-BgalA (white background) and *A. oryzae* (gray background) β-galactosidase activities under simulated gastric conditions. Dots, enzymes incubated with simulated gastric fluid (SGF); blanks, enzymes incubated with SGF without pepsin. The activity of each enzyme incubated in 0.2 M glycine−HCl buffer at the different pH values was taken as 100%. Each value represents the mean ± SD (n = 3).

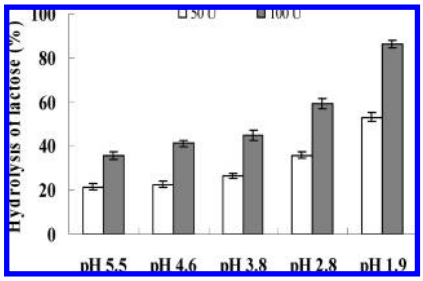

Figure 5. Ability of r-BgalA (50 U or 100 U) to hydrolyze milk lactose under simulated gastric conditions. Hydrolysis percentage of lactose was calculated as the amount of produced glucose against the content of lactose in milk. Each value in the panel represents the mean ± SD (n = 3).

resistant to proteases and most metal ions that exist in milk, and hydrolyzed milk well under simulated gastric conditions. All of these properties are critical for a β-galactosidase to act as a digestive supplement in the stomach.

r-BgalA is acidophilic, exhibiting maximum activity at pH 1.5. Greater than 60% of maximum activity is retained at pH 1.0 through 3.0. The β-galactosidase from *A. carbonarinus* ATCC6276 is active over the range of pH 2.0 through 5.5, displaying almost 80% of maximal activity at pH 2.0 (*19*). However, it is not stable at low pH and retains less than 20% activity after 2 h of incubation under simulated gastric conditions (pH 2.0 in the presence of the pepsin) (*20*). BgalA also has better thermostability than β-galactosidases from *A. candidus* (*21*), *A. niger* (*22*), *A. oryzae* (*6*, *23*), *Alternaria alternate* (*24*), and some yeasts (*25*), which have already been proposed as candidates to hydrolyze lactose during milk production. The thermostability advantage of BgalA makes it suitable for industrial processing because high temperature must be used to reduce microbial populations and may actually accelerate the rate at which BgalA could hydrolyze lactose in milk (*26*).

Before and after purification, r-BgalA produced smeared bands and had an apparent molecular weight of ~130 kDa that was much greater than the calculated molecular weight (110 kDa). Ten putative *N*-glycosylation sites (Asn-X-Ser/Thr, in which X represents any amino acid other than proline) (*17*) were identified in mature BgalA. Endo H treatment yielded a single protein band of lower apparent molecular weight (~110 kDa, **Figure 2b**), suggesting that r-BgalA is a glycoprotein.

Dairy products, especially milk, contain a variety of minerals, such as those containing Ca^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+}, K^{+}, Na^{+}, and Mg^{2+}, that are necessary for human health. A few β-galactosidases from *Candida pseudotropicalis*, *Kluyveromyces lactis*, and *Psychrotrophic pseudoalteromonas* are inhibited to some extent by Ca^{2+}, Cu^{2+}, and/or Zn^{2+}, thus limiting their potential application in the diary industry. r-BgalA activity is unaffected by these cations, and some of the cations even activated its activity slightly.

A series of in vitro studies that simulated gastric conditions indicated that BgalA was more stable and exhibited higher hydrolytic ratio toward milk lactose than the *A. oryzae* β-galactosidase at low pH in the presence of pepsin. *A. oryzae* is generally recognized as a safe fungus and has served as the source of lactose digestive supplements currently on the market. Most of these supplements are made into tablets or capsules with a special coating that is removed only when the pill enters the intestinal tract or the later phases of gastric digestion, where the pH varies between 4.0 and 5.5. The fast state of the stomach, where the initial pH is 1.9, has been omitted in previous studies in which the pH gradient in the gastric digestion phase begins at pH 5.5 (*18*). In this study, we kept the mixture at pH 1.9 for 20 min first and simulated the pH gradient under the gastric conditions over about 2 h. Under these conditions, noncoated *A. oryzae* β-galactosidase only hydrolyzed 3.5% of the lactose in milk due to enzyme inactivation by low pH. By contrast, r-BgalA hydrolyzed up to 86% of the lactose in milk. The high hydrolytic activity might be related to the high Ca^{2+} content in milk because high concentrations of Ca^{2+} activate r-BgalA activity. According to Gao (*25*), eliminating less than 50% of lactose in milk may completely alleviate the symptoms associated with lactose deficiency. Furthermore, most commercially available β-galactosidases are coated, which is both time-consuming and costly. Thus, the possibility that r-BgalA could be used without coating offers a significant advantage over currently used β-galactosidase diary supplements.

ABBREVIATIONS USED

GH, glycoside hydrolase; ONPG, 2-nitrophenyl-β-D-galactopyranoside; SGF, simulated gastric fluid; TAIL-PCR, thermal asymmetric interlaced-PCR.

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