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Molecular Cloning and Expression of a Novel Protease-resistant GH-36 α-Galactosidase from *Rhizopus* sp. F78 ACCC 30795

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A 2,172-bp full-length gene (*aga-F78*), encoding a protease-resistant α-galactosidase, was cloned from *Rhizopus* sp. F78 and expressed in *Escherichia coli*. The deduced amino acid sequence shared highest identity (45.0%) with an α-galactosidase of glycoside hydrolase family 36 from *Absidia corymbifera*. After one-step purification with a Ni–NTA chelating column, the recombinant Aga-F78 migrated as a single band of ~82 and ~210 kDa on SDS–PAGE and nondenaturing gradient PAGE, respectively, indicating that the native structure of the recombinant Aga-F78 was a trimer. Exhibiting the similar properties as the authentic protein, purified recombinant Aga-F78 was optimally active at 50°C and pH 4.8, highly pH stable over the pH range 5.0–10.0, more resistant to some cations and proteases, and had wide substrate specificity (*p*NPG, melidiose, raffinose, and stachyose). The recombinant enzyme also showed good hydrolytic ability to soybean meal, releasing galactose of 415.58 μg/g soybean meal. When combined with trypsin, the enzyme retained over 90% degradability to soybean meal. These favorable properties make Aga-F78 a potential candidate for applications in the food and feed industries.

Keywords: α-Galactosidase, molecular cloning, *Rhizopus* sp. F78, protease-resistant

α-Galactosidases (α-D-galactoside galactohydrolase; E.C. 3.2.1.22) are widely distributed in microorganisms [1, 13], plants [14, 22], and mammals including human [3]. The enzyme catalyzes the removal of α-linked terminal nonreducing galactose residues in different substrates [1], thus having potential applications in various industrial processes such as sugar-producing [15], pulp and paper [7], food and feed additives [9, 19, 23], and medical treatment [25, 28]. On the basis of amino acid sequence similarity, α-galactosidases have been classified into glycoside hydrolase (GH) families 4, 27, 36, and 57 in the CAZy database (http://www.cazy.org/fam/acc_GH.html). The majority of the known α-galactosidases belong to GH-27 and GH-36, and they share a conserved consensus pattern of [LIVMFY]-x(2)-[LIVMFY]-x-[LIVM]-D-[DS]-x-[WY] [8]. To date, only a few GH-36 eukaryotic α-galactosidases have been reported [2, 18].

α-D-Galactosides (mainly raffinose and stachyose) are present in soybean and legume seeds as antinutritional factors and cause flatulence [20, 27]. To eliminate these antinutritional factors, improve digestibility, and make protein-rich materials more edible with higher nutritional value, α-galactosidase and/or protease are often supplemented in food and animal feed as an additive [9, 11, 19, 20, 23, 27]. Thus, the positive effect of both enzymes is very important in utilization of nutrition. In our previous study, a novel GH-36 α-galactosidase, named Aga-F78, was isolated from *Rhizopus* sp. F78 and showed some superior properties [5]. However, the hydrolytic ability of this enzyme to α-galacto-oligosaccharides in soybean meal in the presence of protease was not further studied. In this study, the gene encoding Aga-F78 was cloned and expressed in *Escherichia coli*. The recombinant enzyme exhibited similar characteristics to the authentic protein and retained most of the degradability to soybean meal when combined with trypsin. All of these features suggest that this α-galactosidase may have great potential in various biotechnological applications.

Materials and Methods

Strains, Plasmids, and Reagents

The fungal strain of *Rhizopus* sp. F78 ACCC 30795 was isolated from soil and conserved in our laboratory. *E. coli* BL21 (DE3) and

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vector pET-22b(+) were purchased from Novagen (Darmstadt, Germany). *E. coli* TOP10 and pEASY-T3 vector were obtained from TransGen Biotech (Beijing, China). Chemicals including *p*NPG (*p*-nitrophenyl-α-D-galactopyranoside), *p*NP (*p*-nitrophenol), 6-bromo-2-naphthyl-α-D-galactopyranoside, melibiose, stachyose, trypsin, α-chymotrypsin, subtilisin A, collagenase, proteinase K, and agents for SDS-PAGE were purchased from Sigma (St. Louis, MO, U.S.A.). The authentic Aga-F78 was purified from the culture of *Rhizopus* sp. F78 ACCC 30795 in our laboratory [5]. D-Galactose and raffinose from Amresco (Solon, OH, U.S.A.), isopropyl-α-D-thiogalactopyranoside (IPTG) from Calbiochem (Darmstadt, Germany), low and high molecular weight calibration kit from GE Healthcare (Piscataway, NJ, U.S.A.), proleather from Amano Enzyme Inc. (Nagoya, Japan), and alkaline protease (from *Bacillus pumilus* SMJ-P) were also purchased. Restriction endonucleases and *pfu* DNA polymerase from TaKaRa (Kyotanabe, Japan), and T4 DNA ligase from Invitrogen (Carlsbad, CA, U.S.A.) were also obtained. All other chemicals were of analytical grade.

Preparation of Genomic DNA and Total RNA

Potato dextrose broth (PDB) was used to cultivate *Rhizopus* sp. F78 at 30°C for 48 h. The CTAB method was used to extract the total genomic DNA after biomass collection [10]. The total RNA was isolated with the RNeasy Plant Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Cloning of α-Galactosidase Gene (*aga-F78*)

Based on molecular mass, GH-36 α-galactosidases can be classified into two subgroups: high molecular mass group (HMWG; 70–80 kDa) and low molecular mass group (LMWG; 50–60 kDa). The partial internal peptide sequence analysis of the authentic Aga-F78 indicated that Aga-F78 was a HMWG α-galactosidase [5]. Based on the conservative motifs specific for HMWG α-galactosidases, D-D-G-[AW]-[WF], [IL]-G-D-W, and F-G-[IL]-W-x-E-P-F-[MSN], a set of degenerate primers, P7 (5'-TYGTBMTKGAYGAYGGYTGG-3') and Pr8 (5'-GACCATYTCNGGYTCNAMCC-3') (Y stands for C or T, B for C or G or T, M for A or G, K for G or T, and N for A or T or G or C), was designed and used to amplify the partial fragment of the α-galactosidase gene. A touchdown PCR was performed as follows: 5 min at 95°C; 10 cycles of denaturation at 95°C for 30 s, annealing at 58 to 48°C (decreasing 1°C after each cycle) for 30 sec, and extension at 72°C for 1 min; followed by 25 cycles of 94°C for 30 sec, 48°C for 30 sec, and 72°C for 1 min; and a final extension at 72°C for 10 min. The amplified product with appropriate length was cloned into pEASY-T3 vector and sequenced. Based on the sequence of the partial fragment, six nested insertion-specific primers (data not shown) were designed and used to amplify the 5' and 3' flanking regions by thermal asymmetric interlaced (TAIL)-PCR [16]. The genomic DNA of *Rhizopus* sp. F78 was used as template for touchdown and TAIL-PCR amplification. The resulting amplified fragment was ligated into pEASY-T3 vector for sequencing and subjected to BLAST analysis. The putative open reading frame (ORF) of the α-galactosidase gene, *aga-F78*, was analyzed with Vector NTI and Genomescan (http://genes.mit.edu/genomescan.html).

α-Galactosidase cDNA was obtained by RT-PCR amplification with the total RNA as template. Reverse transcription reactions were performed using a reverse transcription kit (Invitrogen) according to the manufacturer's instructions. PCR amplification was performed using the specific primers F78up (5'-AATAATGAAACTACTTCAA ATTGGTATTTCTC-3') and F78down (5'-CTTCATATTTATTTCGG AATAAAATAATC-3'), designed according to the sequence of the putative ORF. The obtained fragment was cloned into pEASY-T3 and sequenced. Homology searches were carried out with NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Signal peptide analysis was performed with the SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/).

Enzyme Activity Assay

α-Galactosidase activity was assayed with the *p*NPG method as reported previously [5]. One unit of α-galactosidase activity was defined as the amount of enzyme that released 1 μmole of *p*NP from *p*NPG per minute at 37°C. The GOD-POD method was also used to assay the α-galactosidase activity according to Varbanets *et al.* [26] with a GOD-POD kit (Biosino Bio-Technology and Science Inc., Beijing, China). One unit of α-galactosidase activity was defined as the amount of enzyme that released 1 μmole of glucose per minute at 37°C. Specific activity was defined as units per milligram of protein with *p*NPG as substrate. Except for special cases, all enzyme assays in this paper were carried out using the *p*NPG method.

Expression and Purification of Recombinant Aga-F78

To construct the expression vector in *E. coli*, the gene fragment encoding the mature protein (Aga-F78) was amplified using primers F78AG3 (EcoRI) (5'-CCCGAATTCGATGAAACTACTTCAAATT GGTATTTCTC-3') and F78AG42 (NotI) (5'-ATAGCGGCCGCTCT ACCTTCTATAGCTTTCGTCAACAGGAC-3') (restriction sites are underlined). The PCR product was digested with EcoRI and NotI, and cloned into pET-22b(+). The resulting construct, pET-22b(+)/*aga-F78*, was then transformed into *E. coli* BL21 (DE3). The positive transformants were grown in Luria-Bertani medium (LB) at 37°C to OD_{600} of ~0.6 and induced with 1 mM IPTG at 18°C for 12 h by shaking at 180 rpm [12].

To purify the recombinant Aga-F78, the induced cells were harvested, resuspended in buffer A (20 mM Tris-HCl, 500 mM NaCl, and 10% glycerol, pH 7.6), and sonicated. Five ml of the supernatant, so-called crude enzyme, was applied into a 1 ml Ni-NTA chelating column (Qiagen), which was previously equilibrated with buffer A for 10 column volumes. The crude enzyme was eluted with 5 ml of each buffer containing 20 mM Tris-HCl, 500 mM NaCl, 10% glycerol, and 20, 40, 60, 80, 100, 200, or 300 mM imidazole, pH 7.6. The fraction with enzyme activity was collected and concentrated with Nanosep centrifugal devices (3 kDa) (Pall Corp., East Hills, NY, U.S.A.). The protein concentration of each step was measured by the Bradford method with bovine serine albumin as a standard [4].

Electrophoretic Analysis and Biochemical Characterization

The purified recombinant Aga-F78 was analyzed by 12% (w/v) SDS-PAGE with low molecular mass marker. Native protein electrophoresis was performed using a nondenaturing gradient PAGE (4–12%, w/v) and analyzed with high molecular mass marker. The nondenaturing gel was separated into two parts; one part was stained with Coomassie brilliant blue R250, and the other part was reacted with 6-bromo-2-naphthyl-α-D-galactopyranoside and then stained with Fast blue B salt (Fluka, Buchs, Switzerland) [5].

The optimum pH of purified recombinant Aga-F78 was determined at 37°C in 0.1 M McIlvaine buffer (pH 2.0–8.0) and 0.1 M glycine-NaOH (pH 9.0–11.0). To determine pH stability, the enzyme residual

activity was measured after pre-incubation at 37°C for 30 min in the same buffer systems as described above, at the ratio of 1:19 (v/v). Optimum temperature was determined in 0.1 M McIlvaine buffer (pH 4.8) at 0 to 70°C. Thermal stability of the enzyme was determined after the enzyme was pre-incubated at 50 or 60°C for 2, 5, 10, 15, 20, and 30 min, respectively. The effects of different metal ions and chemicals (including NaCl, KCl, $CaCl_2$, LiCl, $CoCl_2$, $CrCl_3$, $NiSO_4$, $CuSO_4$, $MgSO_4$, $FeCl_3$, $MnSO_4$, $ZnCl_2$, $PbCl_2$, $AgNO_3$, $HgCl_2$, β-mercaptoethanol, SDS, EDTA, CTAB, or Triton X-100) on recombinant Aga-F78 activity were measured by adding each chemical to the reaction system at a final concentration of 1 mM. The control was performed under the same conditions without addition of any chemicals.

To determine resistance to neutral and alkaline proteases, purified recombinant Aga-F78 was incubated with trypsin, α-chymotrypsin, proteinase K, subtilisin A, collagenase, and proleather at a ratio of 10:1 (Aga-F78: protease, w/w) at 37°C for 30 min, respectively, and then subjected to residual activity assay. The control was performed under the same conditions without protease addition [5].

Kinetic studies were performed using substrates of *p*NPG (0.0125–1 mM) and melibiose (1–10 mM) with the *p*NPG and GOD-POD methods, respectively. The apparent Michaelis constant (K_m) and V_{max} were calculated from the Lineweaver–Burk plot.

Hydrolytic Ability of Recombinant Aga-F78

The hydrolytic ability of purified recombinant Aga-F78 on natural substrates was performed in 0.1 M McIlvaine buffer (pH 4.8) containing 1 mg/ml melibiose, raffinose, or stachyose and 1 or 3 U Aga-F78 at 37°C for 24 h [17]. The high-performance anion-exchange chromatography (HPAEC) was used to determine the amount of released galactose in samples using a Dionex system equipped with a CARBOPAC PA10 on the column (4 mm×250 mm) (Dionex Corp., Sunnyvale, CA, U.S.A.) with 25 μl sample volume in 18 mM NaOH, 10 ng/ml borate solution. The buffer flow rate was 1 ml/min. The percentage of galactose hydrolyzed by Aga-F78 was calculated. The control was performed under the same conditions with boiling-inactivated enzyme.

The hydrolytic ability of Aga-F78 to α-galactooligosaccharides in soybean meal was determined in a reaction system containing 0.1 M McIlvaine buffer (pH 4.8), 5% (w/v) soybean meal, 1 U/ml Aga-F78, and no or 1.77 U/ml trypsin (α-galactosidase:trypsin=10:1, w/w) at 37°C for 24 h. The released amount of galactose was analyzed with HPAEC as described above.

RESULTS AND DISCUSSION

Gene Cloning and Sequence Analysis

Gene fragments of 160–180 bp were amplified from genomic DNA of *Rhizopus* sp. F78 with the degenerate primers P7 and Pr8. BLASTX analysis showed that the amino acid sequence of this fragment had 66% identity with the corresponding part of an α-galactosidase from *Absidia corymbifera* (Genbank Accession No. ABB43117). The primers also successfully cloned α-galactosidase gene fragments from other microorganisms (data not shown). Thus, the degenerate primers together with the touchdown PCR technique are very useful to clone other novel GH-36 α-galactosidase genes.

The putative ORF of *aga-F78*, acquired with TAIL–PCR and RT–PCR, was in full length of 2,172 bp without intron, encoding 724 amino acids with a calculated molecular mass of 82 kDa and an isoelectric point (pI) of 5.44. The nucleotide sequence of *aga-F78* was deposited in the GenBank database under Accession No. FJ159431. No signal peptide was predicted in Aga-F78. BLASTP analysis showed that the identities between Aga-F78 and α-galactosidases from *A. corymbifera* (GenBank ABB43117), *Streptomyces avermitilis* MA-4680 (GenBank NP_822257), *Clostridium perfringens* E str. JGS1987 (GenBank ZP_02630450.1), and *Penicillium* sp. F63 (GenBank ABC70181) were 45%, 37%, 31%, and 23%, respectively. A high homology region was found at 300–500 amino acids, and the N-terminal and C-terminal sequences showed lower similarity. These results suggested that Aga-F78 was a novel α-galactosidase. The known peptide sequences of authentic Aga-F78 were found at the corresponding positions of the *aga-F78* encoding amino acid sequence, suggesting that the cloned *aga-F78* encoded the authentic Aga-F78, as reported in the original study [5].

Expression and Purification of Recombinant Aga-F78

The gene encoding the mature Aga-F78 was cloned into pET-22b(+) and then transformed into *E. coli* BL21 (DE3). Induction at 18°C with 1 mM IPTG was performed in this study, and the recombinant Aga-F78 reached its highest enzyme activity of 1.69 U/ml at 12 h, which was much shorter than that of other α-galactosidases induced at low temperatures [13, 14]. Although low temperature may slow down the growth rate of cells, induction at lower temperatures may improve the expression of a soluble target protein as well as reduce proteolytic degradation and heat-shock-like responses in the cell [24]. The recombinant Aga-F78 was purified about 2,461.3-fold with a yield of 19.64%. The specific activity against *p*NPG was 56.61 U/mg.

Electrophoretic Analysis and Characterization of Recombinant Aga-F78

The recombinant Aga-F78 migrated as a single band of ~82 kDa when subjected to SDS–PAGE analysis (Fig. 1A). Electrophoresis of the recombinant Aga-F78 using nondenaturing gradient PAGE showed one band of ~210 kDa with α-galactosidase activity (Fig. 1B). The zymogram suggested that the native structure of recombinant Aga-F78 might be a trimer, the same as the authentic protein [5]. It has been reported that native α-galactosidases from different sources were often in multiple forms [2, 6] and heterologous expression did not influence this multimer property [2, 18].

The optimal conditions for the activity of purified recombinant Aga-F78 were 50°C and pH 4.8, which were very similar to that of authentic Aga-F78 from *Rhizopus* sp. F78 and other fungal GH-36 α-galactosidases [5, 18].

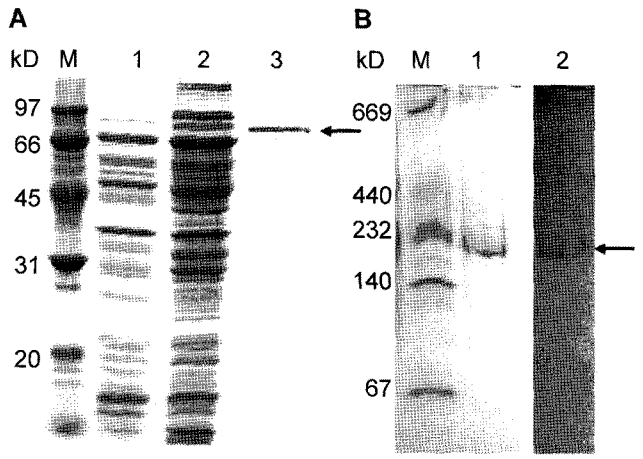

Fig. 1. Electrophoretic analysis of recombinant Aga-F78 from *E. coli* BL21 (DE3).
A. SDS-PAGE analysis of recombinant Aga-F78 expressed in *E. coli*. Lane M, low molecular mass markers; lane 1, cell extract of *E. coli* pET-22b(+) with IPTG induction; lane 2, crude enzyme of pET-22b(+)/*aga-F78* induced with IPTG; lane 3, purified recombinant Aga-F78. **B.** Nondenaturing gradient PAGE of native recombinant Aga-F78. Lane M, high molecular mass markers; lane 1, nondenaturing purified native recombinant Aga-F78 stained with Coomassie brilliant blue; lane 2, nondenaturing purified recombinant Aga-F78-hydrolyzed 6-bromo-2-naphthyl-α-D-galactopyranoside was stained by Fast blue B salt.

The recombinant Aga-F78 had no significant difference from authentic protein in pH stability, but showed higher activity after incubation at pH 4.0 for 30 min (Fig. 2A). The recombinant enzyme showed worse thermostability of losing about 60% of the initial activity after incubation at 50°C for 30 min and was completely inactivated at 60°C for 5 min (Fig. 2B). The activity of recombinant Aga-F78 was significantly enhanced in the presence of Pb^{2+}, Fe^{3+}, Cr^{3+}, and EDTA, and strongly inhibited by Ag^{+}, Hg^{2+}, Mn^{2+}, and SDS. The addition of other metal ions or chemicals had little or no effect on the activity. Compared with the authentic enzyme, recombinant Aga-F78 was more resistant to some metal ions, especially to some heavy-metal ions (*e.g.*, Cr^{3+}, Cu^{2+}, Fe^{3+}, Hg^{2+}, and Ag^{+}). Prokaryotic expression systems, such as *E. coli*, are generally useful for producing heterologous proteins from cloned eukaryotic DNA. In some cases, however, such recombinant eukaryotic proteins are either unstable or lack biological activity, since a number of modifications to eukaryotic proteins occur at the post-translational stage [21]. Therefore, when the recombinant Aga-F78 was expressed in *E. coli*, some post-translational modifications may not be performed appropriately, and may lead to some different properties from the authentic one, such as decreasing of pH stability and thermostability and activity promoted by EDTA. In the proteolytic experiment, recombinant Aga-F78 showed excellent protease resistance after incubation with several proteases (α-chymotrypsin, trypsin, proteinase K, subtilisin A, collagenase, proleather, and alkaline protease) and retained more than 70% of the initial activity. Moreover, the residual activity increased about 24%, 20%, and 50% after treatment with trypsin, α-chymotrypsin, and collagenase, respectively (Fig. 3). The reason for the enhanced activity after protease treatment is unclear, and need further investigation. The K_m and V_{max} values were 0.61 mM and 74.63 μM/min for substrate *p*NPG, and 27.24 mM and 188.68 μM/min for substrate melibiose, respectively. By comparison with the kinetic parameters of the authentic enzyme from *Rhizopus* sp. F78 [5], K_m values of the recombinant enzyme decreased, and V_{max} values increased for both substrates. Although many properties of recombinant Aga-F78 were similar to the authentic protein, there were still some differences, such as thermostability and resistance to metal ions and protease.

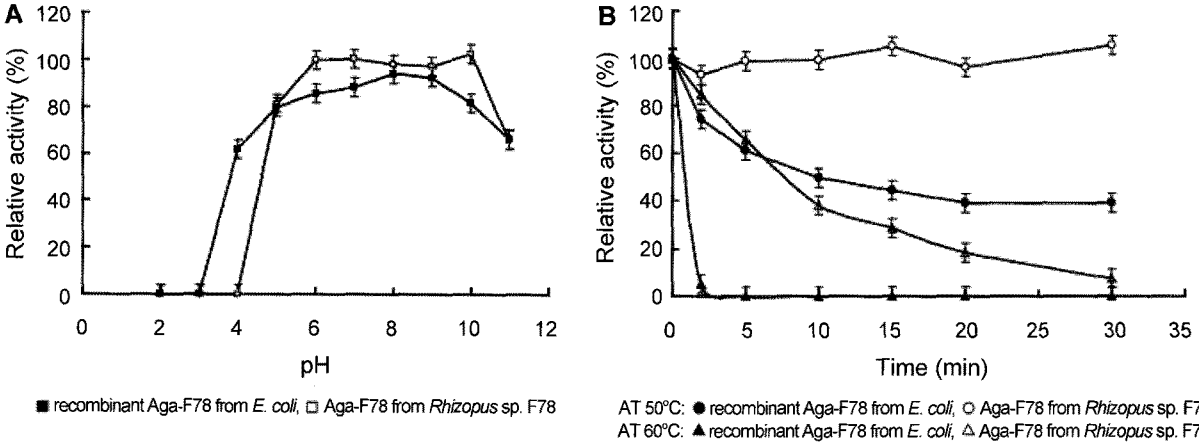

Fig. 2. pH and temperature stabilities of purified recombinant and authentic Aga-F78.
A. pH stability of enzyme activity. After incubation in buffers of pH 2.0–11.0 at a ratio of 1:19 (w/w) at 37°C for 30 min, the enzyme activity was determined in 0.1 M McIlvaine buffer (pH 4.8) at 37°C. **B.** Thermostability of enzyme. Aga-F78 was pre-incubated at 50°C or 60°C in 0.1 M McIlvaine buffer (pH 4.8). Aliquots were removed at specific time points for the measurement of residual activity at 37°C. Each value in the panel represents mean±SD of triplicate experiments.

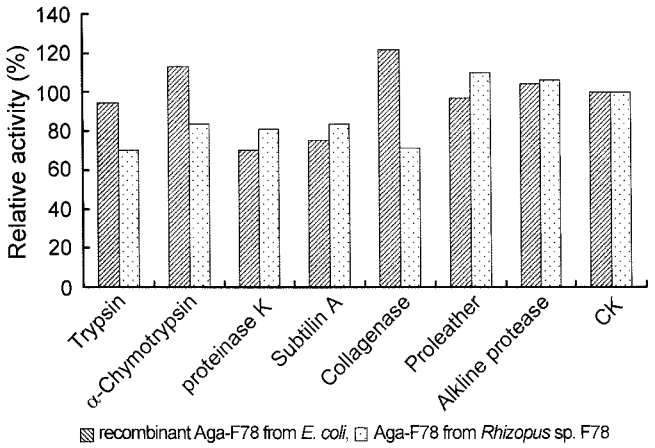

Fig.3. Effect of proteases on activity of purified recombinant and authentic Aga-F78.
The residual activity was determined after incubation with the proteases at a ratio of 10:1 (w/w) at 37°C for 30 min.

Hydrolytic Ability of Recombinant Aga-F78

α-Galactosidases are usually applied in food and feed to eliminate α-D-galactosides (mainly raffinose and stachyose). In this study, Aga-F78 could hydrolyze natural substrates effectively as authentic Aga-F78 did, and the hydrolytic ability of Aga-F78 to natural substrates was melibiose > stachyose > raffinose at both tested concentrations (1 and 3 U/ml) (Table 1). It means that recombinant Aga-F78 can hydrolyze α-galactooligosaccharides efficiently, and this hydrolytic ability was developed to decrease the level of raffinose-series oligosaccharides in soybean meal and other legume seeds.

The α-galactosidase from *Aspergillus niger* in combination with protease P1 or P3 gave different effects on true metabolizable energy and true nitrogen digestibility values [9]. The difference might be due to the different resistant ability of the *A. niger* α-galactosidase to P1 and P3. Protease exists widely in organisms (*e.g.*, in mammal digestive system) and also applies in the feed industry by combination with α-galactosidase. Thus, the protease-resistant characteristic would be favorable in applications. In this study, recombinant Aga-F78 showed resistance to neutral and alkaline proteases, even better than the authentic protein. In the hydrolytic ability test of Aga-F78 to α-galactooligosaccharides in soybean meal, 1 g of soybean could release 415.58 μg of galactose when treated with Aga-F78 alone. When trypsin was present, 380.83 μg of galactose was released, which was 91.63% of that of Aga-F78 alone.

Table 1. Hydrolytic ability of Aga-F78 on natural substrates.

Substrate	Hydrolytic ability (%)[a]	
	1 unit	3 units
Melibiose	21.45±0.30	32.73±0.69
Raffinose	4.66±0.64	10.39±0.51
Stachyose	12.94±0.21	20.65±0.31

[a]Percentage of galactose hydrolyzed by Aga-F78 from 1 mg/ml substrates in 0.1 M McIlvaine buffer (pH 4.8).

In conclusion, the gene encoding protease-resistant Aga-F78 was successfully cloned from *Rhizopus* sp. F78 and functionally expressed in *E. coli*. The purified recombinant Aga-F78 showed similar properties to the authentic one, was highly resistant to several proteases, and exhibited effective hydrolysis to α-galactooligosaccharides in soybean meal in the presence of trypsin. These results indicate that recombinant Aga-F78 has potential in food and animal feed applications.

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