International Journal of Biological Macromolecules 104 (2017) 576–583

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Isolation of a protease-resistant and pH-stable α-galactosidase displaying hydrolytic efficacy toward raffinose family oligosaccharides from the button mushroom *Agaricus bisporus*

Yujing Hu[a,b], Mengjuan Zhu[c], Guoting Tian[d], Liyan Zhao[e], Hexiang Wang[b,*], Tzi Bun Ng[f,*]

[a] Department of Plant Science and Techonology, Beijing University of Agriculture, Beijing 102206, China
[b] State Key Laboratory for Agrobiotechnology and Department of Microbiology, China Agricultural University, Beijing 100193, China
[c] Department of Fungal Resource, Shandong Agricultural University, 61 Daizong Street, Tai'an, Shandong 271018, China
[d] Institute of Biotechnology and Germplasmic Resource, Yunnan Academy of Agricultural Science, Kunming 650223, China
[e] College of Food Science and Technology, Nanjing Agricultural University, Weigang, Nanjing 210095, China
[f] School of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China

ARTICLE INFO

Article history:
Received 19 October 2016
Received in revised form 16 June 2017
Accepted 16 June 2017
Available online 19 June 2017

Keywords:
α-galactosidase
Agaricus bisporus
Protease-resistant
Raffinose family oligosaccharides

ABSTRACT

A 45-kDa monomeric acidic α-galactosidase with a specific activity of 193.12 units/mg was isolated from the fruiting bodies of *Agaricus bisporus*. Blast search of internal peptide sequences suggested that it is a member of GH family 27 and it is most similar to hypothetical protein AGABI2DRAFT_70106. The enzyme displayed maximal activity at pH 4.0 and 60 °C, respectively. The enzyme remained stable within the pH range 2.0–9.0 but its activity was markedly suppressed in the presence of Cu^{2+}, Hg^{2+}, Fe^{3+} and Ag^{+} ions. It displayed resistance to α-chymotrypsin and neutral protease. Moreover, it manifested degradative activity toward both oligosaccharides and polysaccharides. The enzyme manifested Km values of 0.30 mM, 10.65 mM and 19.21 mM, toward pNPGal, stachyose and raffinose respectively. These results suggest that *Agaricus bisporus* α-galactosidase is a promising candidate for elimination of raffinose oligosaccharides (RFOs) in biotechnological applications.

1. Introduction

α-Galactosidases (melibiase; α-D-galactoside galactohydrolases; EC 3.2.1.22) are involved in catalytically removing α-1,6-linked α-galactopyranosyl residues from oligosaccharides such as melibiose, raffinose, and stachyose, or from polysaccharides like guar gum and locust bean gum [1]. They find biotechnological applications in food and feed industry [2], sugar-production industry [3], paper industry [4], structural analysis of biomacromolecules, and applications in clinical medicine encompassing blood group transformation, treatment of Fabry's disease and xenotransplantation [5–7]. In addition, α-galactosidases can also catalyze the synthesis of α-galactosides by transglycosylation at higher substrate concentrations [8] and reverse hydrolysis reactions [9].

Raffinose-type galactose oligosaccharides, mainly raffinose and stachyose, in soybean seeds and other legumes are responsible for flatulence and intestinal disturbance in α-galactosidase-deficient humans and monogastric animals. α-galactosidases could hydrolyze these oligosaccharides to improve the nutritional value of soybean milk and eliminate undesirable side effects. In the past few years, more and more microbial α-galactosidases have been reported, especially fungal a-galactosidases with the advantages of extracellular localization, acidic optimum pH and fine stability for biotechnological applications. At the same time, the popularity of using edible fungi as functional foods has been escalating because of their safety and absence of side effects. If edible fungi are employed as the food source of α-galactosidases, there would be advantages compared with α-galactosidases from other sources or industrial production. *Agaricus bisporus* is widely cultivated and consumed in most countries. It has been reported to have anti-inflammatory and antioxidant activities. The research of Huang et al. indicated that polysaccharides isolated from *A. bisporus* industrial wastewater had protective effects on acute hepatic injury induced by CCl4 in mice [10]. Li et al. evaluated its antioxidant activities *in vitro* and anti-hypoxic activity of its polysaccharide *in vivo* [11]. Ditamo et al. assessed the immunomodulatory effect of *A. bisporus* lectin

* Corresponding authors.
E-mail addresses: huyujing1216@126.com (Y. Hu), mengfan7777@163.com (M. Zhu), tiangt@aliyun.com (G. Tian), zhlychen@njau.edu.cn (L. Zhao), hxwang@cau.edu.cn (H. Wang), b021770@mailserv.cuhk.edu.hk (T.B. Ng).

http://dx.doi.org/10.1016/j.ijbiomac.2017.06.077
0141-8130/

in vivo [12]. So far, α-galactosidases have not been reported from *A. bisporus*.

In this study, we are the first group to report the isolation and physicochemical properties of an *A. bisporus* α-galactosidase, which was denoted as ABGI. The ability of ABGI to catalyze the hydrolysis of the oligosaccharides and polysachharides RFOs, was also evaluated to explore and analyze its latent applications.

2. Materials and methods

2.1. Plant materials and chemicals

Fresh fruiting bodies of *A. bisporus* were purchased from a local market in Beijing. CM-cellulose, Q-Sepharose, the substrates 4-nitrophenyl-α-D-galactopyranoside (pNPGal), *o*-nitrophenyl-α-D-galactoside (oNPαGal), 4-nitrophenyl-β-D-glucuronide, locust bean gum, guar gum, melibiose, galactose, lactose, maltose, sucrose, glucose, xylose, fructose, stachyose, raffinose, phenylmethanesulfonyl fluoride (PMSF), ethylene diamine tetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), and proteases such as acid protease, neutral protease, proteinase K, collagenase type-I, α-chymotrypsin, subtilisin and trypsin were purchased from Sigma Chemical Co., USA. Superdex G-75 HR 10/30 and AKTA Purifier were purchased from GE Healthcare, USA. All of the other reagents used were of analytical grade.

2.2. Assay of enzyme activity

α-Galactosidase activity was assayed according to the previous methods [13].

2.3. Protein estimation

Protein concentration was estimated using bovine serum albumin (BSA) as standard in accordance with the method of Lowry et al. [14].

2.4. Isolation and purification of ABGI

A. bisporus fruiting bodies (1254 g) were homogenized by using a Waring blender. Afterward, the homogenate was centrifuged at 10,000*g* for 10 min. The supernatant was applied to a column (5 cm × 20 cm) of Q-Sepharose equilibrated with 10 mM NaOAc-HOAc buffer (pH 5.6) in advance. The active fraction located in the 100 mM NaCl, was named as Q3.

After thorough dialysis, fraction Q3 was also subjected to anion exchange chromatography on a Q-Sepharose column (2.5 cm × 30 cm), pH 4.6. The active fraction (Q2) was eluted with a linear 0–150 mM NaCl gradient.

Fraction Q2 was injected after dialysis into a CM-cellulose (2.5 cm × 10 cm) cation-exchange chromatography column, pH 5.0. The active fraction (C2) was further purified by fast protein liquid chromatography (FPLC) on a Superdex 75 HR 10/30 gel filtration (GE-Healthcare, USA). The active fraction was collected separately, dialyzed, and freeze-dried for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS- PAGE).

2.5. Native molecular mass

Native molecular mass of the purified ABGI was determined by FPLC as described above. Marker proteins are standard proteins with known molecular masses and elution time, and are used for column calibration to estimate the quality of tested proteins. Marker proteins included bovine serum albumin (67-kDa), ovalbumin (43- kDa), ribonuclease A (13-kDa), aprotinin (6.5-kDa) and vitamin B12 (1.355-kDa).The activity peak was determined spectrophotometrically by the absorbance value at 405 nm using pNPGal as the substrate.

2.6. SDS-PAGE

Protein homogeneity was assessed by SDS-PAGE according to the method of Laemmli [15]. The proteins were stained with 0.25% G-250. The protein markers used range from 17-kDa to 175-kDa.

2.7. Analysis of amino acid sequence

There is no report on the amino acid sequence of ABGI in an available protein database. Therefore, the ratio of *m*/*z* values obtained from MALDI-TOF spectra corresponding to peptides of ABGI was matched with α-galactosidases from other sources and protein database of *Agaricus bisporus* using Mascot (www.matrixscience.com). Comparative analysis is based on parameters such as digestion with trypsin with 1 missed cleavage and the rest is by default.

2.8. Effects of pH and temperature on ABGI

pNPGal was used as the substrate, and the enzyme activity of ABGI was determined under different pH range of 1–12. The pH stability was analyzed by incubating ABGI in buffers at different pH values at 37 °C for 1 h and then determining its residual activity. The optimum temperature was determined over the temperature range of 4–80 °C at its optimum pH value. Thermostability was investigated by measuring the enzyme activity remaining after incubation for 40 min, 60 min, 80 min, 100 min, 120 min, and 140 min at 4–80 °C, respectively.

2.9. Effects of various metal ions, chemical reagents, and chemical modification reagents on ABGI

The effects of various metal ions, chemical reagents, and chemical modification reagents on purified ABGI were examined in accordance with the method mentioned in our previous reports [16].

2.10. Protease treatments

Purified ABGI (0.05 U mL^{-1}) was incubated at 37 °C for 1 h with 2 mg mL^{-1} acid protease (pH 4.0), subtilisin (pH 7.5), α-chymotrypsin (pH 7.0), trypsin (pH 7.0), neutral protease (pH 7.0), collagenase type I (pH 7.5) and proteinase K (pH 7.5), respectively. The remaining enzyme activity was determined similar to the previous methods [16].

2.11. Substrate specificity

For substrates such as melibiose, 4-nitro-phenyl-α-D-galactopyranoside (pNPGal), 2-nitrophenyl-α-D-galactopyranoside (oNPGal) and 4-nitrophenyl-β-D-glucuronide, the enzymatic activities were determined as previously described [16]. For natural and polymeric substrates, the reaction system consisted of enzyme and stachyose (50 mM), raffinose (50 mM), locust bean gum or guar gum solutions (1%) in a ratio of 1:1, respectively. The activities after incubation for an hour at 40 °C was determined using 3, 5-dinitrosalicylic acid (DNS) [17].

2.12. Determination of kinetic parameters

For all kinetic studies, dialyzed enzyme obtained after gel filtration chromatography and dialysis was used. Kinetic experiments were performed at 40 °C. The Michaelis-Menten constant (Km)