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Short communication

Purification and characterization of a chitinase (sAMC) with antifungal activity from seeds of *Astragalus membranaceus*

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ABSTRACT

A chitinase (sAMC) was purified from the seeds of *Astragalus membranaceus* (Fisch.) Bunge, using a combination of 20–60% ammonium sulfate precipitation, regenerated chitin affinity column and Sephadex G-75. Purified chitinase was a monomer with a molecular mass of 35.5 kDa on SDS–PAGE. Based on the homology search of amino acid sequences of four internal peptides, sAMC belongs to glycosyl hydrolase (GH) family 19 chitinases which are mostly endochitinases. The optimal pH of sAMC was 5.0, and it was stable over a broad range of pH 4.0–8.0. sAMC exhibited an optimal temperature of 60 °C and it was stable up to 60 °C. sAMC hydrolyzed colloidal chitin into chitotriose, chitobiose and *N*-acetyl D-glucosamine suggesting its role in conversion of chitin wastes into useful products. It exhibited antifungal activity against *Trichoderma viride, Botrytis cinerea, Fusarium oxysporum* and *Fusarium solani*. This is the first report on chitinases from *Astragalus* sp.

1. Introduction

Chitin is a polysaccharide formed from β-1,4-links of *N*-acetyl-D-glucosamine (GlcNAc), which is widely distributed in fungi, crustaceans, molluscs, coelenterates, protozoan, and green algae [1–4]. Chitinases (EC. 3.2.1.14) are enzymes that randomly hydrolyze the β-1,4 glycosidic bonds of chitin producing *N*-acetylchitooligosaccharides $(GlcNAc)_n$ [1,2,4,5]. Chitinases are wide-spread in nature occurring in plants, animals, viruses, bacteria, fungi and insects, and are presumed to play a role in various functions including defense, nutrient digestion, morphogenesis, and pathogenesis [3,4,6]. They have been classified into two GH (glycosyl hydrolases) families, family 18 and 19 based on the amino acid sequence homology and their catalytic mechanisms [7–9]. Chitinases from microbes, animals, plants and other organisms belong to family 18 [1,3,7] whereas family 19 chitinases exist mainly in higher-order plants and are reported to have strong antibacterial properties [10].

Although plants lack endogenous chitin, they express chitinases that hydrolyze chitin. The function of plant chitinases appears to be defense against attack by chitin containing fungal pathogens and insect pests [2,4,10,12]. Due to their potential role in plant defense, chitinases have received wide research attention. Till date, a number of chitinases have been reported from different parts of plants. Plant chitinases have mostly been reported from different cereals, especially from *adenanthera pavonina* L. seeds [2], Canadian cranberry beans [4], soy bean [5], and oats [12]. There are very few reports about chitinases from medicinal herbs [1,6,11,13].

Radix Astragali, under the traditional name Huangqi in China has been used since a long time as antiperspirant, adiuretic and atonic. Earlier researchers have reported about lectin and PR-10 (pathogenesis related) protein from roots of *Astragalus* species [14,15]. However, there are no reports of any bioactive protein from the seeds of *Astragalus* sp. The present study describes the purification, characterization and antifungal activity of a novel chitinase from the seeds of Chinese medicinal herb *Astragalus membranaceus* (Fisch.) Bunge.

2. Materials and methods

2.1. Materials

Astragalus membranaceus seeds were obtained from Beijing Tongrentang Group, China. Sephadex G-75 and Sephacryl S-200 were purchased from GE Life sciences, USA. Fluorescent brightener 28 (Calcofluor white M2R), chitin, chitosan and glycol chitosan were purchased from Sigma Chemical Co. (USA). TLC silica gel plates were

Abbreviations: sAMC, a chitinase from seeds of *Astragalus membranaceus*; BSA, bovine serum albumin; GH, glycosyl hydrolase; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.

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purchased from E. Merck (Germany). All other chemicals and reagents used were of analytical grade unless otherwise stated.

2.2. Extraction and purification of a chitinase

Seeds from *A. membranaceus* (25 g) were crushed into powder using a semi industrial grinder, added to a 6-fold volume (150 mL) of 0.9% NaCl and extracted for 6 h at 4 °C with constant stirring. The extract was centrifuged at 10,000 × *g* for 30 min. The supernatant was subjected to 20–60% ammonium sulfate precipitation. The precipitated proteins were dialyzed against 20 mM sodium carbonate buffer (pH 8.4) for 16 h at 4 °C. The dialyzed sample was loaded on to a regenerated chitin column (1.5 × 20 cm), which was pre-equilibrated with 20 mM sodium carbonate buffer (pH 8.4) at a flow rate of 1.0 mL/min. Regenerated chitin column was prepared from acetylation of chitosan as described earlier researchers [16]. The bound proteins were eluted with 20 mM acetic acid (pH 3.2). Eluted protein was concentrated by 10,000 Da cut off membrane, using Amicon stirred cell, and applied on to a Sephadex G-75 column, which was pre-equilibrated with 50 mM sodium citrate buffer (pH 6.0). Fractions exhibiting chitinase activity and single homogenous band on SDS–PAGE were pooled and used as purified chitinase for further characterization.

2.3. Chitinase assay, carbohydrate and protein quantification

Chitinase activity was determined by measuring the reducing end group *N*-acetyl D-glucosamine (GlcNAc) produced from colloidal chitin as described by Miller [17], with slight modifications. Colloidal chitin was prepared in our lab from chitin according to the method published in earlier study [16]. The standard reaction mixture consisting of 0.1 mL of enzyme solution (in 50 mM sodium citrate, pH 5.0) and 0.1 mL of 1% (w/v) colloidal chitin was incubated at 50 °C for 30 min. After incubation, the reaction was terminated by adding 0.3 mL of dinitrosalicylic acid (DNS) reagent and heating in boiling water for 5 min. After boiling, samples were rapidly cooled to room temperature and centrifuged at 4500 × *g* for 10 min. The activity of the supernatant was analyzed by measuring the absorbance at 540 nm. One unit of chitinase activity was defined as the amount of enzyme that liberated 1 μmol *N*-acetylglucosamine per minute at pH 5.0 and 50 °C.

Carbohydrate content of the purified chitinase was determined by the phenol sulfuric acid method as described by Dubois et al. [18] using D-glucose as standard. Protein concentration was determined by the method of Lowry et al. [19] using BSA as a standard.

2.4. SDS–PAGE and zymogram

SDS-polyacrylamide gel electrophoresis (SDS–PAGE) was performed using 12.5% (w/v) acrylamide in gels as described by Laemmli [20]. Protein bands were visualized by Coomassie brilliant blue R-250. The activity staining of purified chitinase was carried out according to the method of Trudel and Asselin [21].

2.5. Molecular mass determination by size exclusion chromatography and analysis of internal peptide sequences

Native molecular mass of the purified chitinase was analyzed by size exclusion chromatography using a calibrated Sephacryl S-200 column (1.0 × 40.0 cm). The column was equilibrated with 50 mM sodium citrate buffer (pH 6.0) containing 0.1 M NaCl at a flow rate of 0.35 mL/min. To determine the partial amino acid sequences, purified chitinase was subjected to SDS–PAGE. The band was excised from the gel and submitted for amino acid sequencing using high performance liquid Chromatography–electrospray tandem mass spectrometry (HPLC–ESI–MS/MS) at National Center of Biomedical Analysis (China). Mass spectral sequencing was performed using a Q-TOF II mass analyzer (Micromass Ltd., UK).

2.6. Biochemical characterization of purified chitinase

To investigate the optimum pH of the purified chitinase, activity was carried out in 50 mM different buffers in the range of pH 2.5–10.0. To determine the pH stability, purified chitinase was incubated in 50 mM different buffers at 50 °C for 30 min, and the residual activities were measured by the standard enzymatic procedure as described earlier.

The optimum temperature of the purified chitinase was analyzed by incubating the enzyme in 50 mM sodium citrate buffer (pH 5.0) at different temperatures, ranging from 40 to 80 °C. For determination of thermal stability, purified chitinase was incubated in 50 mM sodium citrate buffer (pH 5.0) at different temperatures for 30 min, and kept on ice for cooling. After 30 min of cooling, the residual activities were measured by the standard procedure as mentioned earlier.

2.7. Hydrolysis of colloidal chitin by purified chitinase

For enzymatic hydrolysis of colloidal chitin, reaction mixture containing 1 mL of 1% (w/v) colloidal chitin with 10 U of purified chitinase was incubated at 50 °C for 48 h. The aliquots were analyzed for hydrolysis products at different time intervals 0.5, 1, 2, 4, 8, 12, 36, and 48 h by TLC. TLC procedure was slightly modified as described by Dean [22]. The reaction mixtures were spotted on to a silica gel plate (Merck Silica Gel 60F 254, Germany), and developed twice in a solvent system containing butanol-1-ol/acetic acid/water (2:1:1, v/v/v). The plates were sprayed with Solution I [12.5% (w/v) KOH in ethanol and 1% (v/v) acetyl acetone in butanol-1-ol at 1/20] and dried. The hydrolysis products were detected by heating in an oven after spraying the plates with Solution II [3.33% (w/v) dimethyl aminobenzaldehyde in ethanol/HCl/butanol-1-ol at 6:6:1 (v/v/v)].

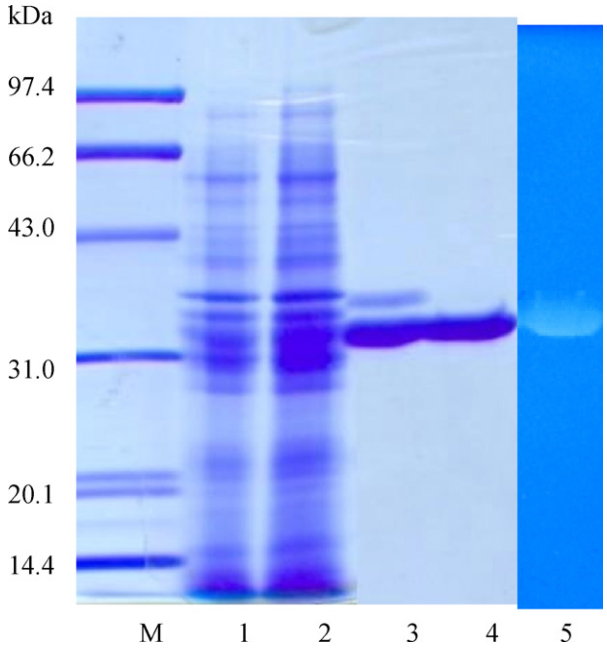

Fig. 1. SDS–PAGE of the purification steps of sAMC. Lane M, low molecular weight markers (in kDa); lane 1, crude extract; lane 2, ammonium sulfate precipitation (20–60%); lane 3, regenerated chitin affinity column elution; lane 4, Sephadex G-75 elution; lane 5, zymogram of purified sAMC.

2.8. Assay for antifungal activity

Antifungal activity of the purified chitinase was estimated by a hyphal extension-inhibition assay as described by Yan et al. [14]. The selected fungi were cultured in Petri dishes (100 × 15-mm) containing 10 mL of potato dextrose agar.

3. Results

3.1. Purification of a chitinase

The crude extract from seeds of *A. membranaceus* contained relatively high levels of chitinase activity. The purified protein exhibited chitinase activity, and appeared as a single homogenous band on SDS–PAGE (Fig. 1, lane 4). The carbohydrate content of the sAMC was found to be 61 μg of sugar per every mg of protein as estimated by phenol sulfuric acid method [18]. This indicated that sAMC is a glycoprotein with 6.1% neutral sugars. The zymogram of sAMC appeared as a single band (Fig. 1, lane 5). The purified sAMC was used for further characterization.

3.2. Molecular mass determination and sequence homology

The native molecular mass of the purified sAMC was 36.9 kDa as determined by size exclusion chromatography on a calibrated Sephacryl S-200 column (data not shown). However, sAMC possess a molecular weight of 35.5 kDa on SDS–PAGE as estimated from its electrophoretic mobility relative to those of molecular weight markers (Fig. 1). Thus, the results indicate that sAMC is a monomeric protein.

sAMC was subjected to tryptic digestion for identification of partial amino acid sequences and analyzed by Q-TOF2.