

Research article

Characterization of a pathogenesis-related class 10 protein (PR-10) from *Astragalus mongholicus* with ribonuclease activity

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Abstract

A pathogenesis-related (PR) class 10 protein (designated AmPR-10) was first isolated from the Chinese medicinal material *Astragalus mongholicus* using a combination of affinity chromatography on Zn-chelate Agarose 4B, ion exchange chromatography on QAE Sephadex A-25 and gel filtration on Sephadex G50. The purified AmPR-10 showed a single band with a molecular mass of 17.2 kDa in SDS-PAGE. The molecular mass of intact AmPR-10 was determined to be 32.8 kDa by gel filtration. Thus, AmPR-10 is a dimeric protein composed of two identical subunits. AmPR-10 was a glycoprotein detected by periodic acid-Schiff (PAS) staining and its neutral carbohydrate content was 13.7%. The carbohydrate was mainly composed of 73.0% (w/w) arabinose, 15.0% (w/w) glucose and 4.8% (w/w) fructose on the basis of high-performance anion exchange chromatography (HPAEC) analysis. Its N-terminal sequence of 15 amino acid residues was determined as GVISFNEETISTVAP, and showed significant sequence homology to some pathogenesis-related (PR) class 10 proteins. This sequence had 80% identity with the PR-10 protein LIPR10.1C from *Lupinus luteus* (yellow lupine) followed by 73.3% identity with the PR-10 protein PR10.2 from *Medicago sativa* (alfalfa), suggesting it is a new member of PR-10 proteins. AmPR-10 exhibited ribonuclease (RNase) activity as do some other PR-10 proteins. The optimal pH and temperature for RNase activity were pH 6.0 and 60 °C, respectively. The RNase activity was stable within pH 5.0–11.0. It was stable up to 60 °C at pH 6.0. The purification and characterization of AmPR-10 in this investigation furnish additional data to the relatively scanty literature pertaining to *Astragalus* radix proteins.

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Keywords: *Astragalus mongholicus*; Glycoprotein; Purification; Pathogenesis-related (PR) class 10 protein; Ribonuclease activity

1. Introduction

Pathogenesis-related (PR) proteins are part of the battery of plant inducible defenses [1]. PR proteins are currently

classified as 14 families (PR-1 to PR-14) based on their primary structure, serological relationships, and biological activities [2,3]. PR-10 proteins described as intracellular pathogenesis-related proteins are a family of small homologous, primarily acidic proteins which are homologous to a large family of food and tree pollen allergens [3,4]. Many studies suggest that PR-10 proteins are functionally involved in plant defense mechanisms as well as in plant development [5–8].

A number of cDNAs encoding PR-10 proteins were obtained. So far, there are many reports of cloning, expression and characterization of multiple PR-10 or PR-10-like proteins from plants. Furthermore, more than 100 different proteins from PR-10 family have already been identified, their biological function has not yet been cleared [8–12]. Because of

Abbreviations: AmPR-10, a pathogenesis-related class 10 protein from *Astragalus mongholicus*; CHES, 2-(cyclohexylamino)ethanesulfonic acid; HPAEC, high-performance anion exchange chromatography; MES, 2-(N-morpholino)ethanesulfonic acid; PAS, periodic acid-Schiff; PR, pathogenesis-related; PVDF, polyvinylidene difluoride; RNase, ribonuclease; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

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amino acid sequence similarity, PR-10 proteins have been classified as ribonuclease-like PR proteins [2]. Two PR-10 proteins from ginseng were first isolated as plant ribonucleases [13,14]. Also, the ribonuclease (RNase) activity was found in major birch-pollen allergen, Bet v 1, which was known to have homology to PR-10 proteins [15,16]. It was reported that a PR-10-like protein from *Lupinus albus* (white lupine) roots had ribonucleolytic activity [17]. Recently, Park et al. demonstrated that the induction and subsequent phosphorylation of CaPR-10 increased its ribonucleolytic activity to cleave invading viral RNAs [11]. Moreover, PR-10 proteins may have functions other than ribonuclease. Some PR-10 proteins (i.e. SsPR10 and CaPR-10) have shown antifungal or antibacterial activity and the level of such activities varied among species of the target microorganism [8,11,12]. CaPR-10 could inhibit hyphal growth of *Phytophthora capsici* [11]. The recombinant protein SsPR10 inhibited the hyphal growth of *Pyricularia oryzae*, but did not inhibit the hyphal growth of *Sclerotinia sclerotiorum* and *Gibberella zeae* [8].

Astragali radix (under the name of Huang-qi in China) is the dried root of *Astragalus membranaceus* (Fisch.) Bunge var. *mongholicus* (Fisch.) Hsiao or *A. membranaceus* (Fisch.) Bunge (family Leguminosae). Much attention has been given to indigenous compounds in this medicinal herb, particularly such as polysaccharide, triterpene saponins, isoflavonoids, γ -aminobutyric acid, and various trace elements [18,19]. However, very few proteins have previously been isolated and characterized from this economically important herb. Recently, we have found a novel homodimeric lectin from *Astragalus mongholicus* with antifungal activity [20].

To further investigate the biological activities of proteins from Astragali radix, herein we describe the purification and characterization of a PR-10 protein (designated as AmPR-10) from roots of *A. mongholicus*. N-terminal sequence analysis of 15 amino acid residues revealed that it shared 80% of sequence homology with the PR-10 protein LIPR10.1C from *Lupinus luteus* (yellow lupine). Also, its ribonuclease activity was further characterized. This is the first report of a PR-10 protein isolated from Astragali radix.

2. Results

2.1. Purification of the protein

Protein yield and ribonuclease activity of the AmPR-10 at each stage of purification are presented in Table 1. Crude extract after dialysis (7.49 U mg^{-1}) was fractionated on Zn-chelate Agarose 4B chromatography into an unadsorbed peak (A1) with high RNase activity (12.57 U mg^{-1}) and an adsorbed peak (A2) with low RNase activity (1.88 U mg^{-1}). The concentrated A1 was chromatographed on QAE Sephadex A-25 column which had been pre-equilibrated with 20 mM sodium phosphate buffer (pH 6.4). As shown in Fig. 1A, four peaks (Q1–Q4) were obtained by the chromatography of A1. Adsorbed proteins were eluted with a step gradient of 400 mM NaCl to yield two peaks Q3 and Q4, which were enriched in RNase activity (41.18 U mg^{-1}). Subsequently,

Table 1
Purification summary of AmPR-10

Purification step	Total activity ^a (U)	Protein ^b (mg)	Specific activity (U mg ⁻¹)	Purification factor (fold)	Recovery (%)
Crude extract	1062	141.87	7.49	1	100
Zn-chelate Agarose 4B	545.34	43.37	12.57	1.68	51.35
QAE Sephadex A-25	429.98	10.44	41.18	5.50	40.49
Sephadex G50	310.59	4.19	74.11	9.90	29.25

^a Activity was measured in 50 mM MES (pH 6.0) at 56 °C using yeast tRNA as the substrate.

^b The protein was measured using the method of Lowry et al. [31], using BSA as the standard.

gel filtration of Q3 and Q4 on the Sephadex G50 yielded two peaks G1 and G2 (Fig. 1B). Peak G2 (74.11 U mg^{-1}) had much higher RNase activity than peak G1 (7.78 U mg^{-1}). The purified protein designated as AmPR-10 was

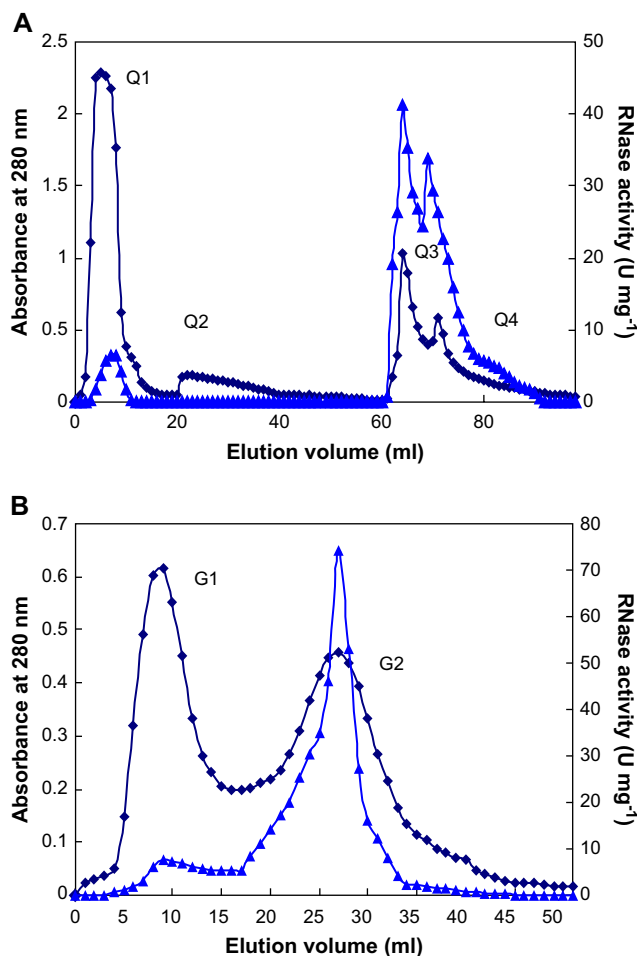


Fig. 1. QAE Sephadex A-25 ion exchange chromatography (A) and Sephadex G50 gel filtration chromatography (B) of *Astragalus mongholicus* AmPR-10. The elution profiles were monitored at 280 nm (◆). Fractions (1.0 ml/tube) were collected. For QAE Sephadex A-25 chromatography, the column was eluted with two-step gradient of 50 mM NaCl (from tube no. 19) and 400 mM NaCl (from tube no. 58) in 20 mM sodium phosphate buffer (pH 6.4), respectively. The active fractions were detected by RNase activity assay (▲). Conditions are described in Section 4.

present in the second peak (G2). AmPR-10 appeared as a single band with a molecular mass of 17.2 kDa in SDS-PAGE (Fig. 2). It exerted a specific RNase activity of 74.11 U mg^{-1} protein toward yeast tRNA. An overall recovery of 29.25% and a 9.9-fold purification of the AmPR-10 were obtained (Table 1). A total of 4.19 mg of AmPR-10 was purified from 10 g of *Astragali radix* roots.

2.2. Molecular mass determination and glycoprotein nature

AmPR-10 had an apparent molecular mass of 17.2 kDa, as determined by SDS-PAGE under nonreducing and reducing conditions (lanes 6 and 7 in Fig. 2). Furthermore, the relative molecular mass of the intact AmPR-10 was estimated by gel filtration on a calibrated Superdex 75 column. A relative molecular mass of 32.8 kDa was calculated for AmPR-10 by using $V_e/V_0 = 1.4$ (data not shown). The results suggest that AmPR-10 is a dimer composed of two identical polypeptides.

Periodic acid-Schiff (PAS) staining suggested that AmPR-10 was a glycoprotein (lane 5 in Fig. 2). The purified AmPR-10 when estimated for the presence of sugar moiety showed the presence of $137 \mu\text{g}$ of sugar mg^{-1} of protein. Thus, the carbohydrate content of AmPR-10 is 13.7%. Acid hydrolysis of AmPR-10 with TFA was performed at 100°C for 2 h. The resulting monosaccharides were further analysed by the high-performance anion exchange chromatography (HPAEC), combined with pulsed amperometric detection (Fig. 3). The monosaccharide composition was found to mainly consist of 73.0% (w/w) arabinose, 15.0% (w/w) glucose and 4.8% (w/w) fructose detected by HPAEC analysis.

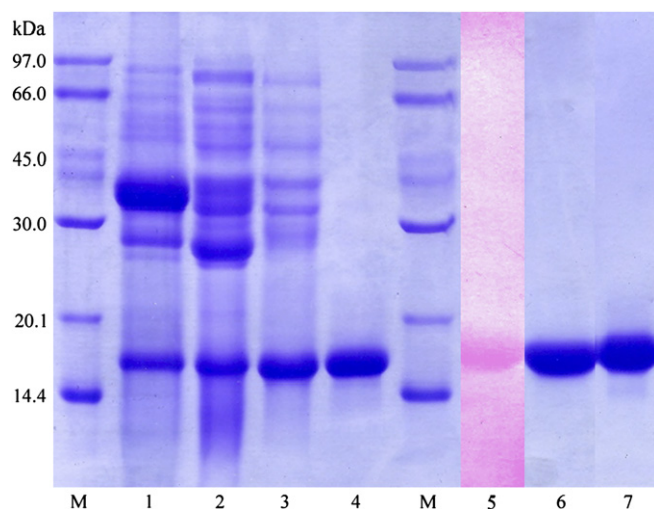


Fig. 2. SDS-PAGE of the protein from each purification step under reducing conditions. Protein bands were stained by Coomassie brilliant blue R-250. Lane M, molecular weight marks; lane 1, crude extract; lane 2, fraction (unadsorbed peak A1) after Zn-chelate Agarose 4B; lane 3, fraction (peaks Q3 + Q4) after QAE Sephadex A-25; lane 4, fraction (peak G2) after Sephadex G50; lane 5, PAS staining of the purified protein; lane 6, SDS-PAGE of the purified protein with β -mercaptoethanol and lane 7, SDS-PAGE of the purified protein without β -mercaptoethanol.

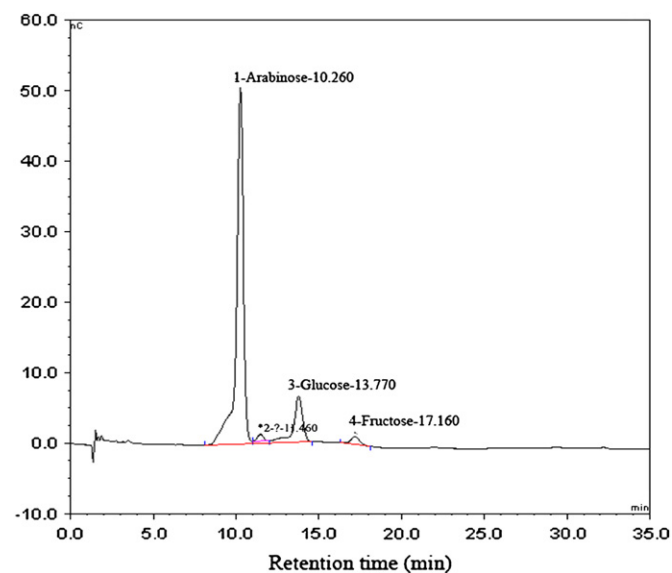


Fig. 3. Chromatogram of the acid hydrolysis products of AmPR-10 analysed by HPAEC. Peak 2 marked with asterisk belongs to unidentified compound.

2.3. N-terminal sequence analysis

N-terminal sequence of 15 amino acid residues of AmPR-10 was determined as GVISFNEETISTVAP by automated Edman degradation. By comparing its N-terminal sequence with those available in data banks, we found that it was not identical to any other proteins published so far. The N-terminal sequence of the protein is compared with other proteins in Table 2. The above sequence exhibited 80% identity with the PR-10 protein LIPR10.1C from *L. luteus* (yellow lupine) followed by 73.3% identity with the PR-10 protein PR10.2 from *Medicago sativa* (alfalfa) [21,22]. Besides, the N-terminal sequence of AmPR-10 had less than 66.7% similarity with the other reported proteins. These results suggest that AmPR-10 is a new member of the PR-10 family.

2.4. Characterization of RNase activity of the purified AmPR-10

The RNase activity of AmPR-10 reached a maximum at pH 6.0 (Fig. 4A). The optimal temperature for RNase activity was 60°C . It increased gradually with the temperature reaching 60°C , but significantly decreased when the temperature was improved further (Fig. 4B). In addition, it was stable within pH 5.0–10.0 and was stable up to 60°C at pH 6.0 (data not shown). The RNase activity of AmPR-10 was inhibited slightly by Ca^{2+} , Mg^{2+} and EDTA (ethylenediaminetetracetic acid) (Table 3). But it was strongly inhibited by Cu^{2+} , Ag^{+} and SDS.

3. Discussion

In this report, we have purified and identified a novel PR-10 protein (i.e. AmPR-10) from *Astragali radix* with RNase

Table 2
Comparison of N-terminal sequence of AmPR-10 with the other proteins: results of a BLAST search

Sources	Accession no.	N-terminal sequence ^a	Residue no.	Identity (%)	Sequence length ^b
AmPR-10		GVISFNEETISTVAP	1–15		
LIPR10.1C [<i>Lupinus luteus</i> (yellow lupine)]	AAD55099.1	<i>GVFSFEEETISIVAP</i>	2–16	80	156
Class 10 PR protein [PR10.2] [<i>Medicago sativa</i> (alfalfa)]	CAC37691.1	<i>GVINFEETTSIVAP</i>	2–16	73.3	158
Pprg2 protein [pprg2] [<i>Medicago sativa</i> (alfalfa)]	CAC86467.1	<i>GVFTFNDENVSTVAP</i>	2–16	66.7	157
Drought-induced protein RPR-10 [<i>Retama raetam</i>]	AAL32031.2	<i>GVFTFKEENVSPVAP</i>	2–16	60	157
PR-10 protein [<i>Lupinus albus</i>]	CAA03926.1	<i>GIFTFEDESTSTVAP</i>	2–16	53.3	158
18 kDa winter accumulating protein A [<i>Morus bombycis</i>]	AAV33670.1	<i>GVFTFDDEFTSTVAP</i>	2–16	60	157
Disease resistance response protein DRRG49-C [<i>Pisum sativum</i> (pea)]	AAA33663.1	<i>GVFNFEETTSIVAP</i>	2–16	60	158

^a Identical corresponding amino acid residues are italicized.
^b Amino acid numbers of the protein.

activity. An efficient three-step purification protocol, consisting of affinity, anion exchange and gel filtration chromatographies, resulted in an apparently electrophoretic homogeneity preparation with 9.9-fold purification in a yield of 29.25% (Table 1). AmPR-10 also has an RNase activity and is constitutively expressed at high level in roots like a Sanchi RNase [23]. The purified AmPR-10 exhibited a molecular mass of 17.2 kDa in SDS-PAGE, which is similar to most of PR-10 proteins [6,8–11,14]. Analytical gel filtration in Superdex 75 revealed the molecular mass of the native protein to be 32.8 kDa. Hence, AmPR-10 is a homodimeric protein. A major birch-pollen allergen Bet v 1 has previously been reported as either a monomer or a dimer [15]. Also, a 16 kDa PR-10 protein SPE-16 from the seeds of *Pachyrrhizus erosus* is a homodimer in solution, consisting of two 16 kDa monomers [24]. Besides, the Ni-NTA purified PR-10c-His is observed as a dimer of PR-10c-His [25]. No other reported PR-10 family members have been observed as dimers. The purified protein showed 13.7% of carbohydrate content with positive PAS, confirming that AmPR-10 is a glycoprotein. So far, no other studies have been reported on the existence of glycosylated PR-10 proteins [8–17,24–26]. Whether this dimer formation and glycosylation are functionally significant is under investigation.

The purified AmPR-10 was subjected to sequence analysis and its N-terminal amino acid sequence was determined. Table 2 shows the sequence alignment of AmPR-10 and other proteins. Results from the computer search for similarity with

other protein sequences reveal that AmPR-10 has high level of similarity to the reported PR-10 proteins. Sequence analysis of N-terminal 15 amino acid residues of AmPR-10 exhibits 80% identity with PR-10 protein LIPR10.1C from *L. luteus* (yellow lupine), indicates that AmPR-10 is a novel member of PR-10 family. Sikorski et al. [7] have identified in yellow lupine two gene families encoding PR-10 proteins of LIPR10.1 subclass (155 amino acid residues, three homologues: LIPR10.1A, B, and C) and LIPR10.2 subclass (157 amino acid residues, five homologues: LIPR10.2A, B, C, D, and E). Within each subclass the sequence identity is about 75–91%, while across the subclasses it is only 59–60% [7]. Although the biological function of PR-10 proteins is little known yet, the sequence homology between a ginseng ribonuclease and PR-10 proteins suggested a ribonuclease activity for PR-10 family [13]. However, only some PR-10 proteins have been reported to possess RNase activity in vitro: SsPR10 [8], GaPR-10 [9], SPE-16 [10], CaPR-10 [11], Bet v 1 [15,16], LaPR-10 [17], birch PR-10c [25], and LIPR10.1B [26]. LIPR10.1A, LIPR10.1C, PR10.1C, ABR17, PR10.2A, PmPR10-1.10 and MSPR10-1 did not have the correspondent RNase activity reports [12,26]. The specific activity of AmPR-10 determined using yeast tRNA as the substrate is 74.11 U mg^{−1} protein.

The pH optimum for RNase activity of AmPR-10 is 6.0 and is insensitive to EDTA, similar to most reported PR-10 proteins with RNase activity [9,12,13,17]. The RNase activity of some PR-10 proteins may be considered as class II ribonucleases according to the general biochemical classification [27]. The RNase activity of the 17 kDa protein (LaPR-10) isolated from 10-day-old healthy root tissue of white lupine (*L. albus*) was insensitive to EDTA, had no requirement for the divalent cations Ca²⁺, Mg²⁺ or Mn²⁺, and was completely inhibited by DTT. Furthermore, the enzyme reaction could be catalyzed in water or Tris–HCl buffer, with an optimal activity at pH 6.0–6.5 and pH 7.5, respectively [17]. For LIPR10.1B, the optimum pH was 6–8. The activity did not require divalent cations and was not sensitive to EDTA [26]. Hence, it is suggested that both LaPR-10 and LIPR10.1B belong to the class II ribonucleases. However, CaPR-10 cleaves all types of RNA tested with a pH optimum

Table 3
Effects of metal ions and reagents on the RNase activity

Metal ions and reagents (at 1 mM)	Relative activity ^a (%)
Control	100 ± 1.2
Ca ²⁺	92.56 ± 1.31
Mg ²⁺	87.44 ± 0.60
Cu ²⁺	24.08 ± 0.98
Ag ⁺	10.47 ± 0.42
EDTA	90.63 ± 1.4
SDS	31.41 ± 0.9

^a At least three determinations were made and results were expressed as mean ± SD.

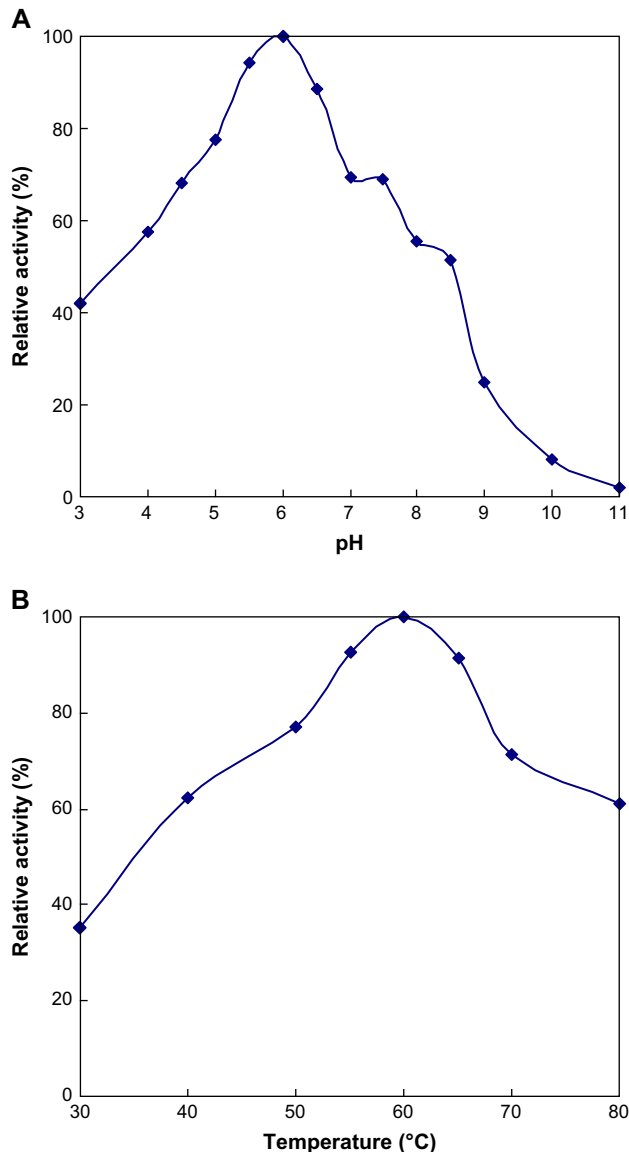


Fig. 4. Effects of pH (A) and temperature (B) on RNase activity of AmPR-10. The influence of pH on RNase activity was determined at 40 °C using 50 mM of different buffers: citrate buffer (pH 3.0–4.0), MES buffer (pH 4.5–6.5), Tris–HCl buffer (pH 7.0–8.5) and CHES buffer (pH 9.0–11.0). The maximum activity was measured at different temperatures.

of 4.5–5.5, is insensitive to EDTA [11]. Recently, it has been found that several PR-10 proteins have antibacterial and antifungal activities [8,11,12]. Assay of antifungal activity of AmPR-10 in the present study was performed as described by Yan et al. [20]. It did not exhibit antifungal activity toward the commonly encountered agronomically harmful fungi *Botrytis cinerea*, *Colletotrichum* sp., and *Drechslera turcia*, *Fusarium oxysporum*, *Mycosphaerella arachidicola* and *Rhizoctonia solani* at the concentration of 100 µg well⁻¹.

In conclusion, AmPR-10 as a pathogenesis-related class 10 protein from *Astragali radix* with ribonuclease activity was first purified and characterized in this study. Further information on the properties of AmPR-10 will come from studies on gene structure and expression.

4. Materials and methods

4.1. Materials

Astragali radix was obtained from Beijing Tongrentang Group Co. Ltd and identified as *A. membranaceus* (Fisch.) Bunge var. *mongholicus* (Bunge) P.K. Hsiao (i.e. *A. mongholicus* Bunge) [28]. QAE Sephadex A-25, Sephadex G50 and Superdex™ 75 were purchased from Pharmacia (Pharmacia, Uppsala, Sweden). Yeast tRNA was purchased from Sigma. Agarose 4B was obtained from Shanghai Donglian Pharmaceuticals & Functional Food Co. Ltd (Shanghai, China). All other chemicals used were analytical grade reagents unless otherwise stated.

4.2. Preparation of Zn-chelate Agarose 4B

Epoxide agarose supports were prepared by a modification of the procedures described by Jerker and Birgit [29]. Activation was performed with epichlorohydrin. Agarose 4B (20 g) was washed thoroughly with distilled water and the moist gel was suspended in 25 ml of 2.0 M NaOH containing 12.5 ml of NaBH₄ and 12.5 ml of acetonitrile. The suspension in a flask was shaken (150 rpm) for 24 h at 30 °C and finally washed thoroughly with distilled water. Furthermore, the activated Agarose 4B was suspended in 40 ml sodium carbonate–iminodiacetic acid disodium salt monohydrate (IDA) buffer (sodium carbonate, 2 M; IDA, 0.1 M). The flask was gently shaken (150 rpm) in a water-bath shaker at 60 °C for 24 h. The IDA gels were filtered and washed with distilled water, and then loaded into a column (10 mm × 18 cm). The column was equilibrated with 1 mg ml⁻¹ ZnCl₂. Finally, the supports were washed thoroughly with distilled water and used as Zn-chelate Agarose 4B column chromatography.

4.3. Extraction and purification of the protein in *A. mongholicus* Bunge

Astragali radix (*A. mongholicus* Bunge) was broken into pieces and added to a sixfold volume of 20 mM phosphate buffer, pH 6.0. It was extracted at 4 °C for 10 h and was passed through four layers of cheesecloth to remove large particles. The supernatant was obtained by centrifugation and was dialyzed against 2 l of 20 mM sodium phosphate buffer (pH 7.0) overnight at 4 °C. The dialyzed sample was then applied to a Zn-chelate Agarose 4B column (10 mm × 18 cm) pre-equilibrated with 20 mM sodium phosphate buffer (pH 7.0). The unbound fractions were collected and concentrated by ultrafiltration using a 10 kDa membrane (Stirred Cell Model 8050, Millipore). The concentrated sample was then applied to a QAE Sephadex A-25 column (10 mm × 8 cm) pre-equilibrated with 20 mM sodium phosphate buffer (pH 6.4). The column was washed and then eluted with a step gradient of 50 mM NaCl in 20 mM sodium phosphate buffer (pH 6.4). After the absorbance of the elute had reached the baseline, the column was further eluted with another step gradient of 400 mM NaCl in 20 mM sodium phosphate buffer (pH 6.4).

The eluted fractions with high ribonuclease activity were pooled, and then concentrated to 1 ml. The concentrated sample was then subjected to a Sephadex G50 column (10 mm × 95 cm) pre-equilibrated with 20 mM sodium phosphate buffer (pH 6.2). The elution occurred with a flow rate of 5 ml h⁻¹. The purified protein (designated AmPR-10) was used in the following experiments. Absorbance at 280 nm was used to monitor the protein content in the chromatographic fractions. Also, the chromatographic fractions at various stages of purification were tested for ribonuclease activity.

4.4. Activity of ribonuclease

The activity of ribonuclease (RNase) toward yeast tRNA was assayed according to the method of Zhou et al. [9]. The sample was incubated with 200 µg of yeast tRNA in 400 µl of 100 mM MES (2-(*N*-morpholino)ethanesulfonic acid) (pH 6.0) at 56 °C for 30 min. The reaction was terminated by introducing 1 ml of 4 M LiCl. After leaving at 4 °C for 3 h, the sample was centrifuged (12,000 × g, 15 min) at 4 °C. The OD₂₆₀ of the supernatant was read after appropriate dilution. One unit of enzymatic activity is defined as the amount of protein that brings about an increase in OD₂₆₀ of 1.0 after 30 min of incubation in the acid-soluble fraction per milliliter of reaction mixture under the specified condition.

4.5. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and protein determination

SDS-PAGE was performed using 12.5% (w/v) acrylamide in gels as described by Laemmli [30]. Protein bands were stained by Coomassie brilliant blue R-250. The molecular weight standard used was the low molecular weight calibration kit (Amersham): phosphorylase *b* (97.0 kDa), albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). The protein concentration was determined according to the method of Lowry et al. [31] using BSA (bovine serum albumin, fraction V) as the standard.

Glycoprotein was detected by the periodic acid-Schiff (PAS) staining of gel [32]. After SDS-PAGE, the gel was soaked in 7.5% acetic acid for 1 h at 25 °C and then transferred to 1% (w/v) aqueous periodic acid and 3% acetic acid. After incubating at 4 °C for 60 min, the gel was washed several times with sodium metabisulfite to minimize the background. It was placed in Schiff's reagent for 60 min at 4 °C in dark.

4.6. Molecular mass determination by gel filtration

Molecular mass of the intact AmPR-10 was measured by gel filtration on a Superdex 75 column (1.0 cm × 30.0 cm) previously equilibrated with 10 mM phosphate buffer (pH 7.0). The protein was eluted (0.25 ml min⁻¹) with the same buffer. Molecular standards from Sigma were used to calibrate the column, they were albumin bovine V (68.0 kDa), albumin (45.0 kDa), chymotrypsinogen A (25.7 kDa) and cytochrome *c* (12.3 kDa).

4.7. Neutral sugar content determination and monosaccharide composition

An aliquot (0.4 ml) of AmPR-10 (3.6 mg ml⁻¹) was mixed with 0.1 ml TFA (trifluoroacetic acid) and was boiled for 2 h. The resulting solution was dried in vacuum and was again dissolved in 0.2 ml distilled water before sugar analysis. Estimation of the carbohydrate content of the protein samples was done by the phenol sulphuric acid method [33], with D-glucose as the standard.

The resulting monosaccharides were analysed by high-performance anion exchange chromatography (HPAEC), combined with pulsed amperometric detection. Hydrolysates (25 µl) were applied onto a Dionex BioLC system (Model 2500) fitted with a Dionex CarboPac PA10 analytical column (4 mm × 250 mm) (Dionex Corp.) combined with a CarboPac PA10 precolumn (4 mm × 50 mm). A mixture of arabinose, galactose, glucose, xylose, and fructose was used as the standard.

4.8. N-terminal sequence

Following SDS-PAGE, the protein was transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, USA) that was stained with Coomassie brilliant blue R-250, and the band corresponding to the protein was excised from the membrane. N-terminal sequence was determined by automated Edman degradation using a PROCISE amino acid sequencer (Applied Biosystems, USA) at Peking University, Beijing. N-terminal sequence homology was analysed using the BLAST database search.

4.9. Effect of pH, temperature and various reagents on the ribonuclease activity

The optimal pH of RNase activity was determined at pH 3.0–11.0 under standard assay conditions using 50 mM of various buffers: citrate buffer (pH 3.0–4.0), MES buffer (pH 4.5–6.5), Tris–HCl buffer (pH 7.0–8.5) and CHES (2-(cyclohexylamino)ethanesulfonic acid) buffer (pH 9.0–11.0). The effect of pH on RNase stability was determined using the above buffer system in the range of pH 3.0–11.0. After incubation of the protein solution at various pH values for 30 min at 40 °C, the remaining enzyme activity was measured.

The effect of temperature on RNase activity was determined by incubating the purified protein with the substrate at temperatures ranging from 30 °C to 80 °C in 50 mM MES buffer at pH 6.0. Thermal stability of the protein was determined by assaying for residual activity of the protein after incubation at various temperatures for 30 min in 50 mM MES buffer (pH 6.0).

The effect of various reagents on RNase activity was determined by assaying for residual activity after incubating the protein with 1 mM of various reagents dissolved in 50 mM MES buffer (pH 6.0) for 30 min at 40 °C.

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