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Isolation of a ribonuclease from sanchi ginseng (*Panax pseudoginseng*) flowers distinct from other ginseng ribonucleases

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Abstract

A single-chained ribonuclease was isolated from the aqueous extract of sanchi ginseng (*Panax pseudoginseng*) flowers. It exhibited a molecular mass of 23 kDa, an N-terminal sequence with some similarity to other enzymes involved in RNA metabolism but different from known ribonucleases, and considerably higher activity toward poly U than poly C and only slight activity toward poly A and poly G. The purification protocol entailed ion exchange chromatography on diethylaminoethyl (DEAE)-cellulose, affinity chromatography on Affi-gel blue gel, ion exchange chromatography on carboxymethyl (CM)-cellulose, and gel filtration on Superdex 75. The ribonuclease was unadsorbed on DEAE-cellulose and adsorbed on Affi-gel blue gel and CM-cellulose. Maximal activity of the ribonuclease was attained at pH 7. On either side of this pH the enzyme activity underwent a drastic decline. The enzyme activity was at its highest at 50 °C and dropped to about 20% of the maximal activity when the temperature was decreased to 20 °C or elevated to 80 °C. The characteristics of sanchi ginseng flower ribonuclease were different from those of the ribonucleases previously purified from sanchi ginseng and Chinese ginseng roots including ribonuclease from Chinese ginseng flowers which are morphologically very similar to sanchi ginseng flowers.

Keywords: Sanchi ginseng; Ribonuclease; Flowers; Isolation; *Panax pseudoginseng*

Various mammalian organs including the brain, kidney, pancreas, and liver [1], plants, fungi, and bacteria [1] elaborate ribonucleases. Ribonucleases exhibit different activities including antitumor, immunosuppressive, and antiviral activities [1].

The roots of the Chinese ginseng *Panax ginseng* produce a ribonuclease with two subunits, each with a molecular mass of 26 kDa [2]. A homodimeric ribonuclease with molecular mass, sequence, and biological activities similar to those of the Chinese ginseng ribonuclease is found in American ginseng (*Panax quinquefolia*) roots [3]. Both proteins exhibit antifungal and translation-inhibiting activities [2,3] and are distinctly different in N-terminal sequence compared with that of the previously isolated ribonucleases from ginseng calluses [4]. The roots of the sanchi ginseng produce a heterodimeric ribonuclease with antifungal, translation-inhibiting, and antiproliferative activities [5]. While the roots of the sanchi ginseng have well-known pharmacologic activities on the cardiovascular system [6], its flowers may be used to make tea, and can be used to treat hypotension, dizziness, and acute inflammation of the throat and pharynx [7].

A ribonuclease has been isolated from the flowers of the Chinese ginseng *P. ginseng* [8]. The purpose of this investigation was to isolate a ribonuclease from sanchi ginseng flowers and compare its characteristics with those of ribonuclease from Chinese ginseng flower and ribonucleases, from Chinese ginseng roots, Amercian ginseng roots, and sanchi ginseng roots. The study was deemed worthwhile

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in view of the striking morphological resemblance between sanchi ginseng flowers and Chinese ginseng flowers despite marked differences in appearance between their roots. The results indicate that sanchi ginseng flower RNase can be distinguished from Chinese ginseng flower ribonuclease in N-terminal sequence and from ribonucleases originating from sanchi ginseng roots, Chinese ginseng roots, and ginseng calluses in various characteristics.

Materials and methods

Dried sanchi ginseng (*Panax pseudoginseng* var. *notoginseng*) flowers (800 g) from Mainland China were used. A voucher has been deposited at Laboratory 203, Department of Biochemistry, The Chinese University of Hong Kong (03037). The flowers were homogenized in 2500 ml distilled water using a Waring blender. To the supernatant obtained (1600 ml) after centrifugation of the extract at 12,000 rpm for 30 min, 1 M Tris–HCl buffer (pH 7.3) was added until the concentration of Tris reached 10 mM. The supernatant was then applied on a 5×20 cm column of diethylaminoethyl (DEAE)-cellulose (Sigma) in 10 mM Tris–HCl (pH 7.3). The unadsorbed fraction (D1) containing ribonuclease activity was subjected to affinity chromatography on a 2.5×20 cm column of Affi-gel blue gel (Bio-Rad) in 10 mM Tris–HCl buffer (pH 7.3) while the adsorbed proteins were eluted as fraction D2 using 0.8 M NaCl in the 10 mM Tris–HCl buffer. After elution of the unbound fraction (B1), bound proteins were eluted from Affi-gel blue gel with a linear gradient of NaCl (0–1 M) in the 10 mM Tris–HCl buffer. The first adsorbed peak (B2) with ribonuclease activity was dialyzed and then loaded on a 2.5×20 cm column of carboxymethyl (CM)-cellulose (Sigma) in 10 mM NH_4OAc buffer (pH 4.5). After removal of unadsorbed proteins in fraction CM1, the column was eluted with a 0–1 M NaCl concentration gradient in the NH_4OAc buffer. The second adsorbed peak (CM3) was then gel filtered on a Superdex 75 HR 10/30 fast protein liquid chromatography (FPLC) column (Amersham Biosciences) in 0.2 M NH_4HCO_3 (pH 8.5). The second peak (SU2) represented purified sanchi ginseng flower ribonuclease.

Protein determination. Protein concentration was determined with the method of Lowry et al. [9] using bovine serum albumin as standard.

Molecular mass determination by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and by FPLC-gel filtration. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out in accordance with the procedure of Laemmli and Favre [10], using a 12% resolving gel and a 5% stacking gel. At the end of electrophoresis, the gel was stained with Coomassie brilliant blue. FPLC-gel filtration was carried out using a Superdex 75 column which had been calibrated with molecular mass standards (Amersham Biosciences).

Analysis of N-terminal amino acid sequence. Amino acid sequence analysis was carried out using an HP Gl000A Edman degradation unit and an HP1000 HPLC system [11].

Activity of sanchi ginseng flower ribonuclease. The activity of sanchi ginseng flower ribonuclease (RNase) toward yeast transfer ribonucleic acid (tRNA) was assayed by determining the generation of acid-soluble, UV-absorbing species with the method of Mock et al. [12]. The RNase was incubated with 200 μg tRNA in 150 μg of 100 mM (Mes) (pH 6.0) at 37 °C for 1 h. The reaction was terminated by introduction of 350 μl of ice-cold 3.4% perchloric acid. After leaving on ice for 15 min, the sample was centrifuged (15,000*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 enzyme that brings about an increase of one in OD260 per min in the acid-soluble fraction per milliliter of reaction mixture under the specified condition.

Activity of sanchi ginseng flower RNase toward polyhomoribonucleotides. The ribonucleolytic activity of sanchi ginseng flower RNase toward polyhomoribonucleotides was determined with a modification of the method of Zimmerman and Sandeen [13]. Incubation of sanchi ginseng flower RNase with 100 μg of poly(A), poly(C), poly(G) or poly(U) in 250 μl of 100 mM sodium acetate (pH 5.0) was carried out at 37 °C for 1 h, prior to addition of 250 μl of ice-cold 1.2 N perchloric acid containing 20 mM lanthanum nitrate to terminate the reaction. After standing on ice for 15 min, the sample was centrifuged at 15,000*g* for 15 min at 4 °C. The absorbance of the supernatant, after appropriate dilution, and was read at 260 nm (in case of poly A, poly G, and poly U) or at 280 nm (in case of poly C).

Assay of Chinese ginseng flower RNase for antifungal activity and inhibitory activity toward HIV-1 reverse transcriptase. The assay was conducted as described in [2].

Results

Ribonuclease activity was located in the fraction of sanchi ginseng flower extract that was unadsorbed on DEAE-cellulose (D1) (Table 1). D1 was resolved on Affi-gel blue gel into an unadsorbed peak B1 without ribonuclease activity, an adsorbed peak B2 with activity, and another adsorbed peak B3 with residual activity (Table 1). Fraction B2 was separated, upon ion exchange chromatography on CM-cellulose, into a very small unadsorbed peak CM1 and an adsorbed peak CM2 without much ribonuclease activity, and an adsorbed peak CM3 with activity (Table 1 and Fig. 1). Peak CM3 yielded, upon gel filtration on Superdex 75, two peaks of similar size, and SU1 and SU2 (Fig. 2). Ribonuclease activity was concentrated in SU2. SU2 represented purified sanchi ginseng ribonuclease. It appeared as a single band with a molecular mass of 23 kDa in SDS–PAGE (Fig. 3). The N-terminal sequence of the ribonuclease resembled a part of the sequence in some enzymes that

Table 1
Yields and activities of ribonuclease-enriched chromatographic fractions obtained during purification of sanchi ginseng flower ribonuclease

Fraction	Total protein (weight in mg)	Total RNase activity (U)	Specific RNase activity (U/mg)	Recovery of RNase activity (%)
Extract	4010	4.81×10^5	120	100
D1	1220	3.64×10^5	298	75.7
D2	1620		<0.1	
B1	462		<0.1	
B2	293	2.59×10^5	915	53.8
B3	130		17	
CM1	31		<0.1	
CM2	71		42	
CM3	120	1.85×10^5	1541	38.4
SU1	40		48	
SU2	40	1.25×10^5	2992	26

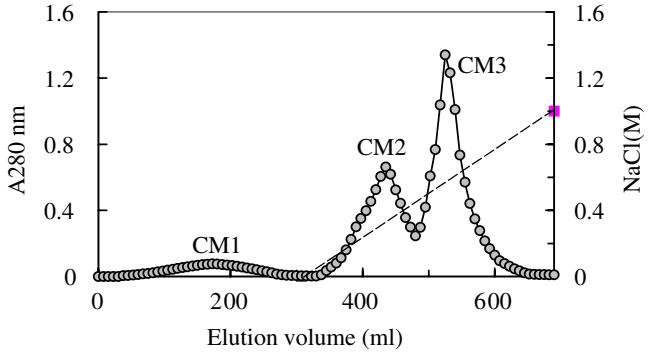

Fig. 1. Ion exchange chromatography on a column of CM-cellulose (2.5 × 20 cm). Sample: Fraction of *P. pseudoginseng* flower extract unadsorbed on DEAE-cellulose and subsequently adsorbed on Affi-gel blue gel and eluted as first adsorbed peak (peak B2). Eluents: 10 mM NH_4OAc (pH 4.5) for fraction CM1, and linear NaCl gradient (0–1 M) for fractions CM2 and CM3. Fraction size: 7.5 ml. Flow rate = 1.5 ml/min. Fractions with elution volume between 501 and 650 ml were pooled.

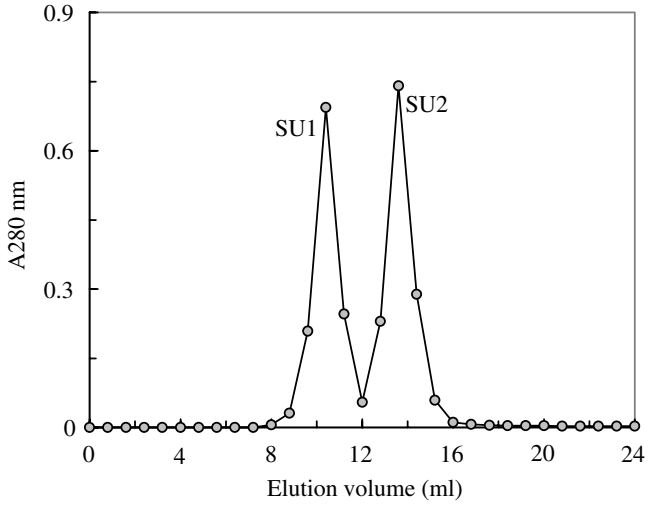

Fig. 2. Fast protein liquid chromatography of an aliquot of fraction CM3 on a Superdex 75 HR 10/30 column. Eluent: 0.2 M NH_4HCO_3 (pH 8.5). Flow rate: 0.4 ml/min. Fraction size: 0.8 ml. Fractions with elution volume between 12.8 and 14.4 ml were pooled.

use RNA or ribonucleotides as substrates and alkaline phosphatase (Table 2). There was little similarity to ribonucleases from Amercian ginseng roots, Chinese ginseng roots, sanchi ginseng roots, and Chinese ginseng flowers. Neither was there similarity to ribonucleases (RNases 1 and 2) from ginseng calluses (Table 2). The activity of the purified ribonuclease toward poly U was the highest (1173 U/mg), followed by poly C (350.6 U/mg), poly A (27.4 U/mg), and poly G (4.0 U/mg). The activity of the ribonuclease rose when the pH was raised from 3 to 7, and then fell rapidly to about 33% and only residual levels of the original activity when the pH was further increased to pH 8 and 9, respectively (Fig. 4). The activity of the enzyme at 50 °C was 4.5-fold higher than that at 20 °C. Beyond 50 °C the enzyme activity started to decline. At 80 °C the ribonuclease activity was reduced to about 20% of that at 50 °C. At 100 °C all ribonuclease activity disappeared (Fig. 5). The ribonuclease did not exhibit any inhibitory effect on HIV-1 reverse transcriptase at a concentration of 10 μM or antifungal activity (data not shown).

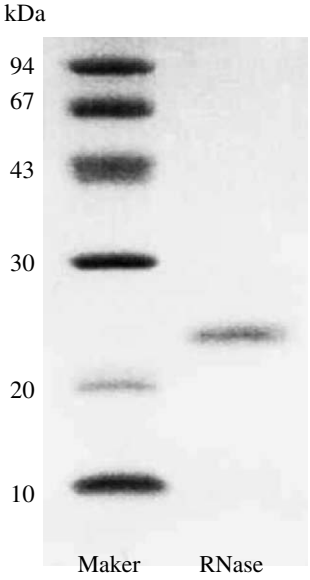

Fig. 3. SDS–SPAGE of sanchi ginseng flower ribonuclease (fraction SU2 from Superdex 75 column). Right lane: purified ribonuclease (10 mg). Left lane: Amersham Biosciences molecular mass markers. From top downward: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14.4 kDa).

Discussion

The ribonuclease reported in this study from sanchi ginseng flowers and the ribonuclease previously isolated from the roots of the same plant are distinct entities [2,3]. The two ribonucleases exhibit a number of differences. The root ribonuclease is heterodimeric with a molecular mass of about 60 kDa while the flower ribonuclease is monomeric with a molecular mass of 23 kDa. The flower and root ribonucleases are relatively specific for poly U and poly C, respectively. The root ribonuclease exhibits antifungal activity and inhibitory activity toward HIV-1 reverse transcriptase [5]. The flower ribonuclease manifests neither of these activities, however. The N-terminal sequences of the two ribonucleases are dissimilar. Structurally similar proteins in some cases, and structurally dissimilar proteins in other cases, may be synthesized in different parts of the same organism. For instance, the same lectin is elaborated by mycelia and fruiting bodies of the straw mushroom [14]. However, distinct lectins are found in the mycelia and fruiting bodies of the mushroom *Tricholoma mongolicum* [15,16]. Structurally disparate ribonucleases are found in different mammalian tissues [1]. It is thus not surprising that different ribonucleases are produced by sanchi ginseng roots and flowers. The two ribonucleases from sanchi ginseng resemble each other in some aspects, however. Both are adsorbed on

Table 2
N-terminal sequence of sanchi ginseng flower ribonuclease in comparison with other ribonucleases (results of BLAST search included)

	Residue number		Residue number	Total number of amino acid residues in protein
Sanchi ginseng flower RNase	1	KSHENIADSY	10	~200
RNA polymerase EcF-type Sigma factor (*Bacillus cereus*)	100	--HENIAD--	105	228
Ribonucleotide reductase (Halovirus HF2)	692	-THDDVADAY	700	805
Glutamyl-tRNA reductase (*Campylobacter jejuni*)	36	-STMENI----	41	432
Alkaline phosphatase (*Bacillus cereus* and *B. anthracis*)	96	--HENITDS-	102	461
Alkaline phosphatase (*Lactobacillus delbruekii* subsp. *Bulgaricus*)	106	--HENIADS	112	471
Sanchi ginseng flower RNase (23 kDa)	1	KS-HEN---IADSY	10	~200
TmRNA-binding small protein B (*Mycoplasma penetrans*)	32	KSIHINNANISD SY	45	146
Sanchi ginseng root RNase 29 kDa subunit	1	FVQQWPPAVN	10	From [5]
Sanchi ginseng root RNase 27 kDa subunit	1	SVCQINIYIF	10	From [5]
Panax ginseng flower RNase (23 kDa)	1	APNADGFR	8	From [8]
Panax ginseng root RNase (52 kDa)	1	GAHGARVYNIFRAAL	15	From [2]
American ginseng root RNase (52 kDa)	1	GAHGARVYNIDRNDV	15	From [3]
Ginseng callus RNase 1	1	GVQKTEVEATSTVPAQKLYA	20	From [4]
Ginseng callus RNase 2	1	GVQKTETQAISPVPAEKLFK	20	From [4]

---, Space introduced to maximize sequence similarity. Identical residues are underlined.

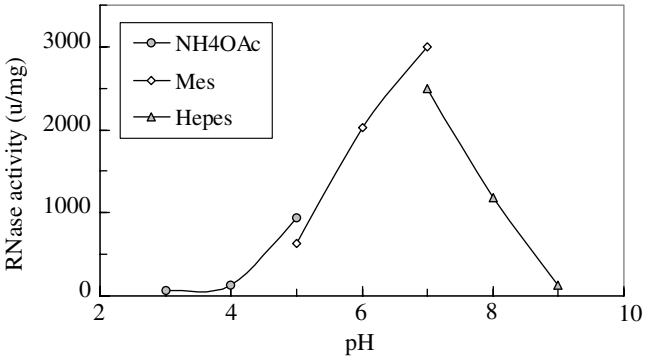

Fig. 4. pH dependence of ribonuclease activity of sanchi ginseng flower ribonuclease.

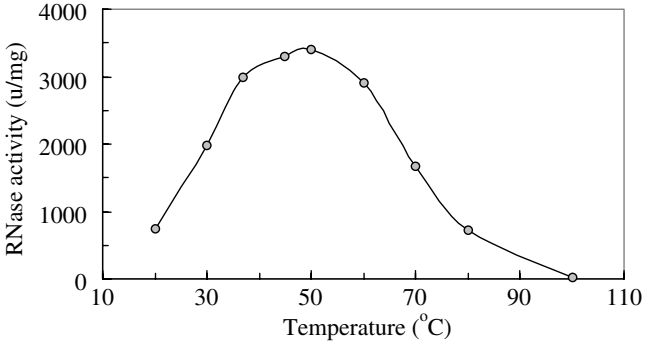

Fig. 5. Temperature dependence of ribonuclease activity of sanchi ginseng flower ribonuclease.

Affi-gel blue gel and CM-cellulose. For both ribonucleases a pH of 6.5–7, is required for maximal activity. The optimum temperature is 60 °C for the root ribonuclease and 50 °C for the flower ribonuclease.

The chromatographic behavior of sanchi ginseng flower ribonuclease on ion exchangers and Affi-gel blue gel, and its pH optimum are similar to those of ribonucleases from American ginseng roots and Chinese ginseng roots. However, the two root ribonucleases are homodimeric with two 26 kDa subunits and possess HIV-1 reverse transcriptase inhibitory and antifungal activities [2,3], unlike sanchi ginseng flower ribonuclease.

Sanchi ginseng flowers are morphologically very similar to Chinese ginseng flowers. A ribonuclease with a molecular mass of 23 kDa, a pH optimum at pH 7.0, and a temperature optimum at 50 °C has been isolated from Chinese ginseng (*P. ginseng*) flowers [8]. The RNase was unadsorbed on DEAE-cellulose and adsorbed on Affi-gel blue gel and CM-cellulose. The aforementioned characteristics are similar to those of sanchi ginseng flower RNase. The activity of Chinese ginseng flower RNase toward poly U, poly C, poly A, and poly G was 206.9, 327.5, 5.4, and 0.5 U/mg, respectively. The activity of sanchi ginseng flower RNase toward the various polyhomoribonucleotides was in general much more potent than that of Chinese ginseng flower RNase, but the two RNases are similar in specificity. Sanchi ginseng flower RNase exhibited a specific RNase activity of 2992 U/mg toward yeast tRNA (418 U/mg). The N-terminal sequence of Chinese ginseng flower RNase is distinctly different from that of sanchi ginseng flower RNase (Table 2) but is similar to a segment of the RNase sequence in *Xanthomas* species [8]. It is noteworthy that despite morphological resemblance, sanchi ginseng, and Chinese ginseng flowers can be distinguished by the N-terminal sequences of their RNases.

Some of the characteristics of sanchi ginseng flower ribonuclease are comparable to those of some non-ginseng ribonucleases. Its chromatographic behavior on ion exchangers and Affi-gel blue gel is similar to other ribonucleases [17–19]. Its optimum pH and its high activity toward poly(U) and poly(C) are analogous to the corresponding characteris-

tics of *Pleurotus ostreatus* ribonuclease [20]. However, its thermostability characteristics and molecular mass are distinctly different from those of *P. ostreatus* ribonuclease [20].

A chitinase-like antifungal protein [21], a ribonuclease [5], and a xylanase [22] have been isolated from sanchi ginseng roots. Antifungal proteins are not present in sanchi ginseng flowers but a ribonuclease can be isolated from the flowers in this study. This investigation also disclosed that sanchi ginseng flowers and Chinese ginseng flowers, both used as traditional Chinese medicinal materials, can be distinguished based on the N-terminal amino acid sequences of their ribonucleases in spite of pronounced morphological likeness. Sanchi ginseng flower ribonuclease possesses an N-terminal sequence not found in known ribonucleases.

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