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Quinqueginsin, a Novel Protein with Anti-Human Immunodeficiency Virus, Antifungal, Ribonuclease and Cell-Free Translation-Inhibitory Activities from American Ginseng Roots

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A homodimeric protein designated quinqueginsin, with a molecular weight of 53 kDa, has been isolated from the roots of American ginseng *Panax quinquefolium*. It was unadsorbed on DEAE cellulose in low ionic strength and neutral pH, and adsorbed on Affigel blue gel and SP-Sepharose under similar conditions. Its N-terminal sequence bore similarity to those of plant ribosome inactivating proteins and fungal ribonucleases. The protein displayed a variety of biological activities. It possessed ribonucleolytic activity toward yeast tRNA and specific activity toward poly C. It inhibited cell-free translation in a rabbit reticulocyte lysate system with an IC_{50} of 0.26 nM, and exerted antifungal action against *Fusarium oxysporum, Rhizoctonia solani,* and *Coprinus comatus.* An inhibitory action was expressed toward human immunodeficiency virus-1 reverse transcriptase. This action was potentiated after chemical modification with succinic anhydride.

Key Words: **ginseng; protein; ribonuclease; antifungal.**

Kitts *et al.* (1) have reported that a standardized North American ginseng extract which contained known ginsenosides, demonstrated strong free radical scavenging activity, effectively stopped non-site-specific DNA strand breakage caused by Fenton agents, and inhibited the Fenton agents and Fenton-induced oxidation of a 66-kDa soluble protein obtained from the mouse liver. The extract also suppressed metal-induced lipid peroxidation. Hu and Kitts (2) demonstrated that North American ginseng was more potent than Korean and Chinese ginseng in prolonging the initiation time of peroxidation induced by a thermolabile peroxyl radical generator 2,2′-azobis(2-amidionpropane) dihydrochloride and in preventing copper ion-induced oxidation of human low density lipoprotein (LDL). Li *et al.* (3) also showed that North American ginseng saponins diminished conversion of phosphatidylcholine to lysophosphatidylcholine in oxidized human LDL, reduced lipid peroxide levels, and inhibited reduction of endothelium-dependent relaxation after exposure to oxidized LDL. Other than the aforementioned antioxidant activities of American ginseng and beneficial effects on the cardiovascular system (4), very little is known about the proteins in American ginseng. We report herein the finding of a protein with potent ribonuclease and cell-free translation-inhibitory activity in American ginseng. In addition it exhibited antifungal and anti-human immunodeficiency virus activities.

MATERIALS AND METHODS

The roots of American ginseng (*Panax quinquefolium*) were purchased from a wholesaler. After homogenizing in 0.15 M NaCl at 4°C, the homogenate was stirred overnight at 4°C. To the supernatant obtained after centrifugation was added $(NH_4)_2SO_4$ to 80% saturation. Dialysis was carried out to remove $(NH_4)_2SO_4$ before chromatography on DEAE-cellulose which had previously been equilibrated with and then eluted with 10 mM Tris-HCl buffer (pH 7.4). Following removal of unadsorbed material, adsorbed proteins were eluted by increasing the NaCl concentration in the buffer. The unbound peak was then chromatographed on Affi-gel blue gel in 10 mM Tris-HCl buffer (pH 7.4). After unadsorbed proteins had been eluted, adsorbed proteins were desorbed with 1.5 M NaCl, dialyzed and applied to SP-Sepharose in 10 mM NH_4OAc buffer (pH 5.4). Unadsorbed materials came off in the same buffer while adsorbed materials were eluted with a linear NaCl gradient. The second adsorbed peak, D1B2SP3, represents the purified protein of interest and was designated quinqueginsin. The homogeneity and molecular weight of quinqueginsin were estimated by FPLC of 3.2 mg protein in 0.2 ml, on Superose 12 (Pharmacia Biotech) in 0.1 M NH_4HCO_3 (pH 8.8), with a flow rate of 0.4 ml/min and a fraction size of 0.8 ml. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of quinqueginsin was conducted as described by Laemmli and Favre (5). Molecular weight was determined by comparison of electrophoretic mobility with molecular weight marker proteins from Pharmacia Biotech. The N-terminal amino acid sequence of quinquegin-

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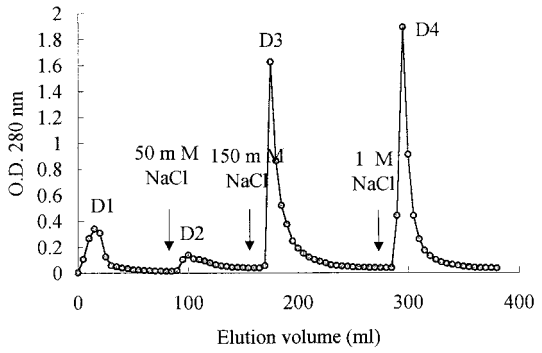

FIG. 1. Ion exchange chromatography of a crude extract of American ginseng roots on a DEAE-cellulose column (0.5 × 5 cm). The starting buffer was 10 mM Tris–HCl buffer (pH 7.4). Arrows indicate the points at which NaCl was added to the starting buffer.

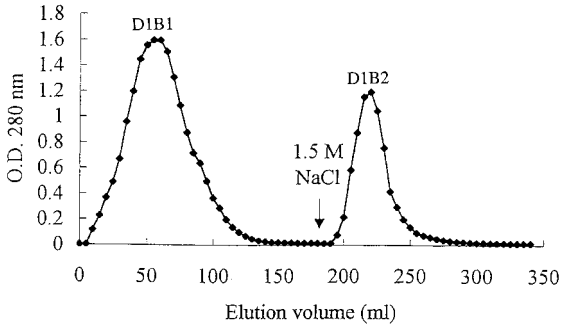

FIG. 2. Affinity chromatography of fraction D1 from DEAE-cellulose column on an Affigel blue gel column (1.5 × 10 cm). The starting buffer was 10 mM Tris–HCl buffer (pH 7.4). Arrow indicates the point at which NaCl was added to the starting buffer.

sin was determined by means of automated Edman degradation in a Hewlett Packard 1000A protein sequencer.

The assay for ribonuclease (RNase) activity toward yeast tRNA, a modified procedure of (6), was performed as follows. After incubation of quinqueginsin with 200 μg tRNA in 150 μl 100 mM Tris-HCl buffer (pH 7.5) at 37°C for 15 min, 350 μl ice-cold 3.4% perchloric acid was added to terminate the reaction. Centrifugation (15,000 g, 15 min at 4°C) of the reaction mixture to obtain the supernatant was carried out after it had been allowed to stand on ice for 15 min. The absorbance of the supernatant was read at 260 nm after suitable dilution. One unit of RNase activity is defined as the amount of enzyme which produces an absorbance increase of one per minute in the acid-soluble supernatant per ml of reaction mixture under the specified conditions.

The pH dependence of the RNase activity of quinqueginsin was assessed using 0.1 M NH_4OAc buffer at pH 4.5 and 5.5, 0.1 M phosphate buffer at pH 5.5, 6.5 and 7.5, and 0.1 M Tris-HCl buffer at pH 7.5, 8.5 and 9.5.

The specificity of quinqueginsin toward different polyhomoribonucleotides was determined by incubating with 100 μg poly A, poly U, poly G and poly C (Sigma) in 0.1 M Tris-HCl (pH 7.5) at 37°C for 15 min. The reaction was brought to an end by addition of 250 μl ice-cold 1.2 N perchloric acid containing 20 mM lanthanum nitratc. The rest of the procedure was identical to that detailed above using tRNA as substrate.

The assay of quinqueginsin for the ability to inhibit protein synthesis from ^{3}H-leucine in a cell-free rabbit reticulocyte lysate system was conducted as previously described (7, 8). The assay for N-glycosidase activity was performed as described (9). A positive result is indicated by presence of an Endo's band. These two assays measure activities characteristic of ribosome inactivating proteins.

The assay for antifungal activity essentially followed the procedure of Ye *et al.* (10) using petri plates containing potato dextrose agar. Sterile blank paper disks were placed around a central disk. Quinqueginsin was applied to a disk and the plate was incubated at 23°C for 72 h until mycelial growth from the central disk had enveloped peripheral disks containing the control (buffer) and had produced crescents of inhibition around disks containing samples with antifungal activity.

The assay of quinqueginsin for ability to inhibit human immunodeficiency virus (HIV) reverse transcriptase activity was the same as that described by Collins *et al.* (11) using a nonradioactive ELISA kit. The assay for glycohydrolase (α-glucosidase, β-glucosidase and β-glucuronidase)-inhibitory activity was also the same as that detailed by Collins *et al.* (12). The enzymes and their corresponding *p*-nitrophenyl glycoside substrates were used to set up the enzymatic reaction at the appropriate pH in 96-well microplates. Quinqueginsin was allowed to react with the enzyme (0.2, 0.01 and 1000 units/well for α-glucosidase, β-glucosidase and β-glucuronidase respectively) for 5 min before reaction (at pH 6.5, 5.5 and 5.6 for α-glucosidase, β-glucosidase and β-glucuronidase respectively) was initiated by addition of substrate. After an incubation period of 15 min glycine-NaOH at pH 10 was added to stop the reaction and absorbance was read at 405 nm.

Quinqueginsin was also subjected to chemical modification with succinic anhydride and then tested for ability to inhibit HIV reverse transcriptase and glycohydrolases as mentioned above. The reaction conditions were similar to those described in the procedure of (13). Succinic anhydride (10 mg) was added to a solution of 10 mg of the protein in 10 ml 0.2 M K_2HPO_4 (pH 8) and the solution was stirred until all anhydride dissolved. The pH was maintained at 8–8.5 using 3 M NaOH. The reaction mixture was left at 25°C and in the dark for 1 h before centricon centrifugation was used to separate the chemically modified protein from succinic anhydride.

RESULTS

Ion exchange chromatography of the crude extract of American ginseng root on DEAE-cellulose resulted in a small unadsorbed fraction D1, another small but adsorbed fraction D2 eluted by 50 mM NaCl, and two large adsorbed fractions (D3 and D4) of approximately equal size eluted by 150 mM and 1 M NaCl respectively

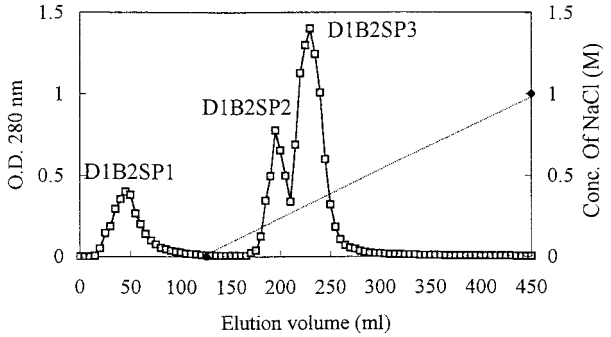

FIG. 3. Ion exchange chromatography of fraction D1B2 from the Affi-gel blue gel column on an SP-Sepharose column (1.5 × 20 cm). The starting buffer was 10 mM NH_4OAc (pH 5.4). The fine dotted line indicates application of a linear NaCl gradient (0–1 M) in the starting buffer.

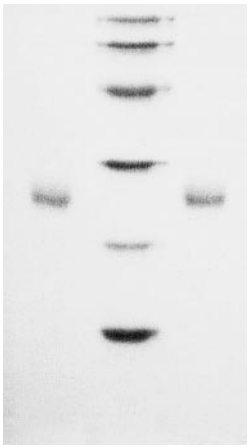

FIG. 4. SDS-PAGE of quinqueginsin. Left and right lanes: quinqueginsin. Middle lane: Pharmacia molecular weight markers, from top downward, phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and lactalbumin (14.4 kDa).

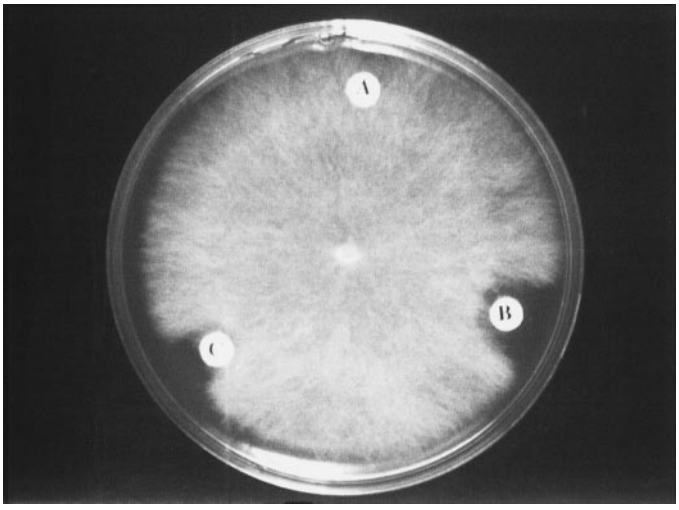

FIG. 6. Inhibitory activity of quinqueginsin toward *Coprinus comatus*. (A) 0.1 M Tris–HCl, pH 7.4, (B) 60 μg quinqueginsin, and (C) 12 μg quinqueginsin.

(Fig. 1). Chromatography of D1 on Affi-gel blue gel resulted in a large unadsorbed peak D1B1 and an adsorbed peak (fraction D1B2) of about two-thirds the size of D1B1 (Fig. 2). Fraction D1B2 was fractionated on SP-Sepharose into a small unadsorbed peak (D1B2 SP1) and two adjacent adsorbed peaks (D1B2 SP2 and D1B2 SP3). D1B2 SP3 was the largest of the three peaks (Fig. 3). It represents purified protein designated quinqueginsin. It yielded a single peak with a molecular weight of 53 kDa in FPLC on Superose 12 (data not shown). Its molecular weight was 26 kDa according to SDS-PAGE results (Fig. 4). The antifungal effects of quinqueginsin are presented in Figs. 5–7.

Table 1 presents the yields and RNase activities of the various chromatographic fractions throughout the purification stages. Table 2 reports the cell-free translation-inhibitory activity (IC_{50} = 0.26 nM) of quinqueginsin, the purified protein which had an RNase activity of 1450 u/mg (Table 1). It was specific toward poly C: the RNase activity toward poly A, poly G, poly U and poly C being 32.66, 0.21, 29.91 and 548.57 u/mg respectively. The N-glycosidase activity of quinqueginsin was not obvious as revealed by the lack of a distinct Endo's band (data not shown). The effect of pH on the RNase activity of quinqueginsin is illustrated in Fig. 8. The pH optimum was 6.5. Quinqueginsin exhibited chromatographic behavior on DEAE-cellulose, SP-

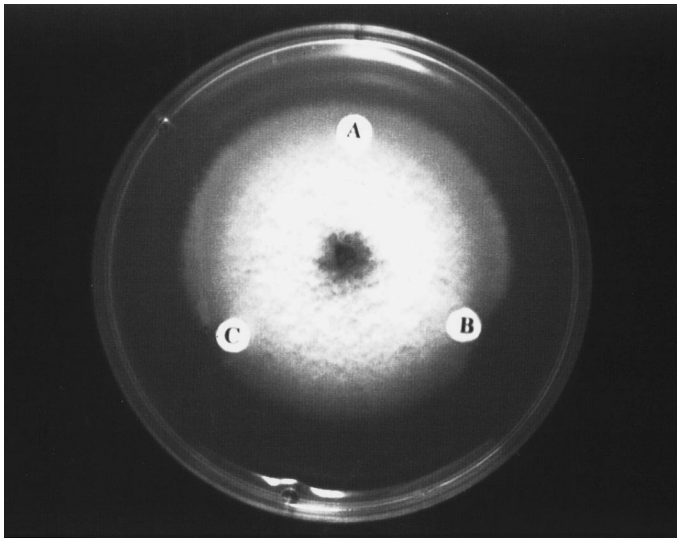

FIG. 5. Inhibitory activity of quinqueginsin toward *Fusarium oxysporum*. (A) 0.1 M Tris–HCl, pH 7.4, (B) 60 μg quinqueginsin, and (C) 12 μg quinqueginsin.

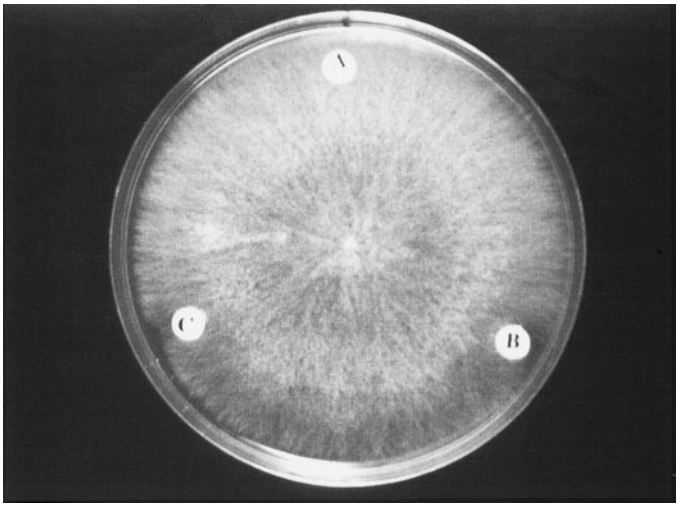

FIG. 7. Inhibitory activity of quinqueginsin toward *Rhizoctonia solani*. (A) 0.1 M Tris–HCl, pH 7.4, (B) 60 μg quinqueginsin, and (C) 12 μg quinqueginsin.

TABLE 1

Yields and Ribonuclease Activities of Various American Ginseng Chromatographic Fractions toward Yeast tRNA

Chromatographic fraction	Yield from 1 kg ginseng root (mg)	RNase activity (U/mg)
Crude powder	3314	5.29
D1	321	36.88
D2	125	12.11
D3	1038	1.12
D4	1182	0.46
D1B1	135	2.89
D1B2	105	163.26
D1B2SP1	15.6	1.21
D1B2SP2	14.9	168.11
D1B2SP3 (quinqueginsin)	48.1	1449.7

Sepharose and Affi-gel blue gel identical to that of ribosome inactivating proteins (RIPs).

The N-terminal sequence (the first 15 residues) of quinqueginsin demonstrates sites of similarity to those of plant ribosome inactivating proteins (14, 15). There are 8 residues identical to those of α-momorcharin, and 5–6 residues identical to those of trichosanthin, bryodin, momorcochin-S and abrin-A chain (Table 3). The N-terminal sequence of quinqueginsin also manifests similarity to segments of the sequences of fungal RNases (16) to some extent (Table 4) although little homology to the previously reported ginseng callus RNases (17) is discernible.

Quinqueginsin induced a dose-dependent inhibition of HIV-1 reverse transcriptase which was augmented after succinylation (Table 5). It also exhibited a slight suppressive action on α- and β-glucosidases.

DISCUSSION

Quinqueginsin, the protein purified from American ginseng roots in the present study, was marked by potent ribonuclease and cell-free translation-inhibitory activities. The former activity was high compared with the plant RIPs α- and β-momorcharins (6) while the latter activity was within the range reported for RIPs (14). Quinqueginsin suppressed fungal growth in several species including *Fusarium oxysporum*, *Rhizoctonia solani* and *Coprinus comatus*. It also notably exhibited anti-human immunodeficiency virus-1 transcriptase inhibitory activity. All of the aforementioned biological activities of quinqueginsin are also characteristics of plant RIPs. Mock *et al.* (6) have demonstrated that plant RIPs possess intrinsic RNase activity. Leah *et al.* (18) have furnished evidence for the antifungal activity of barley RIP. The inhibition of plant RIPs on HIV-1 replication in acutely and chronically infected cells of lymphocyte and mononuclear phagocyte lineage (19) and on HIV-1 integrase activity (20) has been observed. A comparison of the profile of biological activities of quinqueginsin, with that of RIPs and its N-terminal sequence with those of RIPs lends credence to the suggestion that the lack of activity of quinqueginsin, in the assay for N-glycosidase activity in which the activity typical of RIPs is evidenced by the presence of Endo's band, may be caused by the high ribonuclease activity of the protein which degrades the

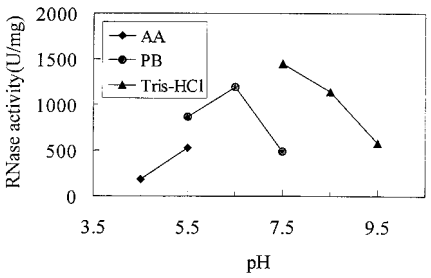

FIG. 8. Effect of pH on ribonuclease activity of quinqueginsin toward yeast tRNA. AA, 0.1 M ammonium acetate buffer; PB, 0.1 M phosphate buffer; Tris–HCl, 0.1 M Tris–HCl buffer.

TABLE 2

Cell-Free Translation-Inhibitory Activity of Quinqueginsin

Concentration (μg/ml)	% inhibition of [^{3}H]leucine incorporation into protein in rabbit reticulocyte lysate (mean ± SD, n = 3)
0.003	32.75 ± 1.38
0.03	55.40 ± 1.26
0.3	68.64 ± 1.14
3	81.84 ± 0.89
30	94.52 ± 2.65
300	98.09 ± 1.39

Note. IC_{50} = 0.26 nM.

TABLE 3

Comparison of the N-Terminal Sequence of Quinqueginsin with Those of Type 1 Plant Ribosome-Inactivating Proteins (RIPs) and the A Chains of Type 2 RIPs Abrin and Ricin (14, 15)

Quinqueginsin	GAHGA··RVY··NI·D·RNDV
α-Momorcharin	DVSFRLSGADPRSYGMFIKDLRNAL
Trichosanthin	DVSFRLSGATSSSYGVFISNLRKAL
Luffin-1	DVRFSLSGSSSTSYSKFIGDL
Bryodin	DVSFRLSGATTTSYGVFIKNLREAL
Momorcochin-S	DVTFSLLGANTKSYAAFITNFRKDV
α- and β-kirilowin	DNIIFRLSGA
Trichokirin	DVSFSLSGGGTASYE
Abrin-A chain	IINFTTAGATSQSYKQFIEALRERL
Ricin-A chain	KFSTEGATVQSYTNFIRAVRGRL

Note. Residues identical to those of quinqueginsin are underlined. All proteins mentioned in the table except quinqueginsin, abrin, and ricin are type 1 RIPs. Abrin and ricin are both made up of an A chain (RIP) and a B chain (lectin). Ricin (14, 15) refers to references 14 and 15 in reference list.

TABLE 4

Comparison of N-Terminal Sequence of Quinqueginsin with Sections of the Sequence of Fungal RNases Beginning Respectively at the 70th Residue and 87th Residue (16) and with Those of Ribonucleases Previously Reported from Ginseng Calluses (17)

Quinqueginsin	GAHGA·RV·YNIDRNDV	Quinqueginsin	GA··H·GARVYNIDRNDV
U1 (70)	GSPGADRV·YYDSNDGT	U1 (87)	GAITHTGASGNNFVQCSY
P0 (70)	GSPGADRVIYDQQSGRF	F1 (87)	GAITHTGASGNNFVGCSG
U2 (70)	VSPGPDRVIYQTNTGEF	THL (87)	GIITHTGASGDAFVACGG
F1 (70)	GSPGADVVINTNCEYAG	MS (87)	GVITHTGASGDDFVACSS
Th1 (70)	GSPGADRVVINGNCSIA	T1 (87)	GVITHTGASGNNFVECT
MS (70)	GSPGADRVIFNGDDELA	C2 (87)	GLITHTGASGDGFVACY
T1 (70)	GSPGADRVVFNENNQLA	Ap1 (87)	GLITHTGASGNGFVACG
C2 (70)	GSPGADRVVFNDNDELA	Pch1 (87)	GVITHTGASGNNFVACD
AP1 (70)	GSPGADRVVFNNNDELA	Pb1 (87)	GVITHTGASGNNFVACT
Pch1 (70)	NSPGADRVVFNGNDQLA	N1 (87)	MLITHNGASGNNFVACN
		Ginseng RNase	1GVQKT•EV•EATSTVPA
		Ginseng RNase	2GVQKT•ET•QAISPVPA

Note. Residues identical to Those of Quinqueginsin Are Underlined. U1(70) and U1(87) refer to G being respectively the 70th and 87th residues in the amino acid sequence of U1. U1(70) and U1(87) refer to G being respectively the 70th and 87th residues in the amino acid sequence of U1. U1, RNase from *Ustilago sphaerogena;* P0, RNase from *Pleurotus ostreatus;* U2, RNase from *Ustilago sphaerogene;* FL, RNase from *Fusarium lateritium;* Th1, RNase from *Trichoderma harzianum;* MS, RNase from *Aspergillus saitos;* T1, RNase from *Aspergillus oryzae;* C2, RNase from *Aspergillus clavatus;* AP1, RNase from *Aspergillus pallidus;* Pch1, RNase from *Penicillium chrysogenum;* N1, RNase from *Neurospora crassa;* Pb1, RNase from *Penicillium breri-compactum*.

RNA used for the assay. RIPs with minimal ribonuclease activity like trichosanthin yield a distinct band in the N-glycosidase assay (21) whereas ribonucleases tend to produce a smear by breaking down RNA. The structural resemblance of quinqueginsin to fungal ribonucleases is noteworthy and may explain its high ribonucleolytic activity.

Another unique feature of quinqueginsin is its possession of two identical subunits. Type 1 RIPs consist of a single chain whereas type 2 RIPs are made up of an RIP subunit and a galactose-binding lectin subunit. Hence the American ginseng protein cannot be classified either as a type 1 RIP or as a type 2 RIP although its molecular weight falls within the range for RIPs. It may be considered as a dimeric type 1 RIP.

Thaumatin-like proteins (10) and chitinase (22, 23) also elicit antifungal effects. However, comparison of the sequences of these antifungal proteins with the N-terminal sequence of quinqueginsin does not disclose any similarity. Hence quinqueginsin is a novel antifungal protein.

The ability of quinqueginsin to inhibit HIV-1 reverse transcriptase was considerably augemented after chemical modification with succinic anhydride. However, succinylation did not enhance its activity in inhibiting α-glucosidase, β-glucosidase and β-glucoronidase. Previously it has been demonstrated that derivatization of epsilon-NH_2 groups of lysine residues with succinic anhydride introduces negative charges into a milk protein converting it into a polyanionic protein which may then acquire anti-HIV-1 activity (24, 25).

TABLE 5

Inhibitory Action of Quinqueginsin on HIV-1 Reverse Transcriptase and the Glycohydrolases α-Glucosidase, β-Glucosidase, and β-Glucuronidase

	5 mg/ml	0.5 mg/ml	50 μg/ml	5 μg/ml	500 ng/ml	50 ng/ml	5 ng/ml
HIV-1 reverse transcriptase							
Quinqueginsin	98.7 ± 2.6	52.8 ± 3.1	ND	ND	ND	ND	ND
Succinylated quinqueginsin	96.4 ± 7.3	90.8 ± 5.6*	75.7 ± 4.4*	60.5 ± 3.3*	50.9 ± 2.1*	29.6 ± 2.2*	1.8 ± 0.2*
α-Glucosidase							
Quinqueginsin	23.3 ± 1.7						
Succinylated quinqueginsin	9.1 ± 0.8						
β-Glucosidase							
Quinqueginsin	32.8 ± 2.7						
Succinylated quinqueginsin	1.4 ± 0.1						
β-Glucuronidase							
Quinqueginsin	1.9 ± 0.2						
Succinylated quinqueginsin							

Note. Results are mean ± SD (n = 2). ND, not determined. *, significant increase ($P < 0.001$ by Student's t test) compared with quinqueginsin at the same concentration.

Quinqueginsin is thus a protein with potentially exploitable activities.

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