

Smilaxin, a novel protein with immunostimulatory, antiproliferative, and HIV-1-reverse transcriptase inhibitory activities from fresh *Smilax glabra* rhizomes

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Abstract

A protein, with a novel N-terminal amino acid sequence and a molecular mass of 30 kDa, was purified from fresh *Smilax glabra* rhizomes by adsorption on DEAE-cellulose, CM-cellulose, Con A-Sepharose, and Mono S, and by fast protein liquid chromatography-gel filtration on Superdex 75. The protein, designated as smilaxin, stimulated uptake of [methyl-³H]thymidine by murine splenocytes, peritoneal macrophages, and bone marrow cells, and production of nitric oxide by peritoneal macrophages. It inhibited uptake of [methyl-³H]thymidine by MBL2 and PU5 tumor cells but not uptake by S180 and L1210 cells. Smilaxin augmented glucose uptake into rat adipose tissue. It attenuated the activity of HIV-1-reverse transcriptase with an IC₅₀ of 5.6 μM. However, it did not display hemagglutinating, antifungal or translation-inhibitory activities, indicating that it is not a lectin, an antifungal protein, or a ribosome-inactivating protein.

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Smilax glabra Rox B. (Family Liliaceae) is the Chinese medicinal herb called tufuling. It is a perennial growing on dry slopes. It has antipyretic, detoxifying, and diuretic actions. It may be beneficial in treatments for brucellosis, syphilis, furunculosis, eczema, dermatitis, nephritis, cystitis, and mercury and silver poisoning [1].

From the rhizome of *S. glabra*, a new flavanone (smilatilbin) was isolated, together with engeletin, astilbin, dihydroquercetin, eurythrin, resveratrol, and 5-*O*-caffeoylshikimic acid [2]. The flavanonal glucosides isoengeletin, isoastilbin, and astilbin have also been isolated [3]. The hypoglycemic effect of the methanol extract of the rhizomes of *S. glabra* is produced by enhancing insulin sensitivity [4]. Immunomodulatory [5], free radical scavenging [6], and antioxidant enzyme fortifying [6] activities have also been demonstrated in *S. glabra* rhizome extract [5] and *S. china* root extract [6].

Mannose-binding lectins have been isolated and characterized from various monocot families including Alliaceae, Araceae Liliaceae, Orchidaceae, and Amaryllidaceae [7–16]. Antifungal proteins have been purified from Alliaceae [17–20], Liliaceae [21], and Amaryllidaceae [22]. The isolation of a novel bioactive protein from *S. glabra* is presented herein.

Materials and methods

Fresh *Smilax glabra* rhizomes were purchased from a local vendor of Chinese medicinal materials. The rhizomes were cut into small pieces and homogenized in distilled water (3 ml/g) using a Waring blender. The homogenate was centrifuged (14,000g, 30 min, 4 °C), and 1 M Tris-HCl buffer (pH 7.4) was added to the resulting supernatant until the concentration of Tris reached 10 mM. The supernatant was then passed through a 5 × 15 cm column of DEAE-cellulose (Sigma) which had previously been equilibrated with and was then eluted with 10 mM Tris-HCl buffer (pH 7.4). After removal of the unadsorbed fraction, adsorbed proteins were desorbed by addition of 1 M NaCl to the Tris-HCl buffer. The adsorbed fraction was dialyzed, lyophilized, and then chromatographed on a 2.5 × 18 cm column of CM-cellulose (Sigma) in 10 mM NH₄OAc

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buffer (pH 4.6). The adsorbed proteins were eluted with the same buffer. The adsorbed proteins were desorbed with a NaCl concentration gradient, dialyzed lyophilized, and then chromatographed on a 2.5×8 cm column of Con A–Sepharose (Amersham Biosciences) in 20 mM Tris–HCl buffer (pH 7.4) containing 0.5 M NaCl, 1 mM CaCl_2 , and 1 mM MgCl_2 . Unadsorbed proteins were eluted with the starting buffer before adsorbed proteins were desorbed by 0.5 M α -methyl-D-glucoside added to the starting buffer. The adsorbed fraction was dialyzed, lyophilized, and then subjected to ion-exchange chromatography by fast protein liquid chromatography on a 0.5×5 cm 1-ml Mono S column (Amersham Biosciences) in 10 mM NH_4OAc buffer (pH 4.5). After elution of unadsorbed proteins, the column was eluted with a linear NaCl (0–0.5 M) gradient in 10 mM NH_4OAc buffer (pH 4.5). The single adsorbed peak was lyophilized and then further purified by fast protein liquid chromatography on a gel filtration Superdex 75 HR 10/30 column (Amersham Biosciences) using an AKTA Purifier (Amersham Biosciences). The purified protein was designated as smilaxin.

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 [23], 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 HR 10/30 column that 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 G1000A Edman degradation unit and an HP 1000 HPLC system [24].

Assay of mitogenic activity. Four C57BL/6 mice (20–25 g) were sacrificed by cervical dislocation and the spleens were aseptically removed. Splenocytes were isolated by pressing the tissue through a sterilized 100-mesh stainless steel sieve and resuspended to 5×10^6 cells/ml in RPMI 1640 culture medium supplemented with 10% fetal bovine serum, 100 units penicillin/ml, and 100 μg streptomycin/ml. The splenocytes (7×10^5 cells/100 μl /well) were seeded into a 96-well culture plate and serial concentrations of smilaxin in 100 μl medium were added. Following incubation of the splenocytes at 37°C in a humidified atmosphere of 5% CO_2 in the presence or absence of smilaxin for 24 h, 10 μl [methyl- ^3H]thymidine (0.25 μCi , Amersham Biosciences) was added, and the splenocytes were incubated for another 6 h under the same conditions. The splenocytes were then harvested with an automated cell harvester onto a glass filter, and the radioactivity was measured with a Beckman model LS 6000SC scintillation counter. All reported values are means of triplicate samples [25].

Assay of nitric oxide production by murine peritoneal macrophages. The assay for the ability to stimulate production of nitric oxide by mouse macrophages was conducted as described by Chu and Ng [40]. Peritoneal macrophages were collected from mice 3 days after eliciting by 3% thioglycollate medium (Becton Dickinson, Le Pont de Claix, France). The cells were washed, counted, and resuspended in RPMI medium without phenol red, 10% fetal bovine serum, 100 IU/ml penicillin, and 100 mg/ml streptomycin. Cells (2×10^5 cells/well per 200 μl) were allowed to adhere onto the surface of the wells of a 96-well culture plate for 1 h before incubation with the antifungal protein for 24 h. The amount of nitric oxide in the culture medium was determined by a colorimetric method using sodium nitrite (NaNO_2) as the standard [40]. In the assay, a 100- μl aliquot of cell-free culture medium from each culture well was allowed to react with 50 μl of Griess reagent (1% sulfanilamide in 5% H_3PO_4 /0.1% naphthalene-ethylenediamine dihydrochloride) for 10 min before the absorbance was read at 540 nm using a microplate reader (Bio-Rad 3550). Lipopolysaccharide from *Escherichia coli* (Sigma) was used as a positive control in this assay.

Stimulatory activity on proliferation of mouse bone marrow cells. The activity was determined as described above for splenocytes. The bone marrow cells were collected from femurs. Bone marrow cells were seeded into culture plates at a cell density of 2×10^5 cells/well, respectively. Con A was used as a positive control. All reported values are means of triplicate samples.

Assay of antiproliferative activity on tumor cell lines. The antiproliferative activity of smilaxin was determined as follows. The cell lines L1210 S180, PU5, and PBL2 were purchased from American Tissue Culture Collection. The cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 mg/L streptomycin, and 100 IU/ml penicillin at 37°C in a humidified atmosphere of 5% CO_2 . Cells (1×10^4) in their exponential growth phase were seeded into each well of a 96-well culture plate (Nunc, Denmark) and incubated for 3 h before addition of smilaxin. Incubation was carried out for another 48 h. Radioactive precursor, 1 μCi ([methyl- ^3H]thymidine, from Amersham Biosciences), was then added to each well and incubated for 6 h. The cultures were then harvested by a cell harvester. The incorporated radioactivity was determined by liquid scintillation counting [26].

Assay for HIV–reverse transcriptase inhibitory activity. The assay of smilaxin for ability to inhibit HIV-1–reverse transcriptase was carried out by using an enzyme-linked immunosorbent assay kit from Boehringer Mannheim (Germany) as described by Ye and Ng [27]. The assay takes advantage of the ability of reverse transcriptase to synthesize DNA, starting from the template/primer hybrid poly(A) · oligo (dT) 15. Instead of radio-labeled nucleotide, digoxigenin- and biotin-labeled nucleotides in an optimized ratio are incorporated into one and the same DNA molecule, which is freshly synthesized by the reverse transcriptase (RT). The detection and quantification of synthesized DNA as a parameter for RT activity follows a sandwich enzyme-linked immunosorbent assay protocol. Biotin-labeled DNA binds to the surface of microtiter plate modules that have been precoated with streptavidin. In the next step, an antibody to digoxigenin, conjugated to peroxidase (anti-FIG-POD), binds to the digoxigenin-labeled DNA. In the final step, the peroxidase substrate is added. The peroxidase enzyme catalyzes the cleavage of the substrate, producing a colored reaction product. The absorbance of the samples at 405 nm can be read using a microtiter plate (ELISA) reader and is directly proportional to the level of RT activity. A fixed amount (4–6 ng) of recombinant HIV-1–reverse transcriptase was used. The inhibitory activity of smilaxin was calculated as percent inhibition as compared to a control without the protein.

Assay of protein concentration. Protein concentrations were determined using the Bradford Reagent (Bio-Rad). Bovine serum albumin was used as protein standard.

Stimulation of glucose uptake by rat adipose tissue. Male rats weighing approximately 200 g were sacrificed by cervical dislocation. Epididymal fat pads were removed and washed twice with Krebs–Ringer bicarbonate buffer (KRB). Approximately 0.8 g fat was added to a 50 ml containing 5 ml KRB-containing bovine serum albumin and was then equilibrated at 37°C . Smilaxin was added to the tube with rat fat pads and incubated at 37°C for 20 min. Two hundred microliters of 2-deoxy-D-[U- ^{14}C]glucose (Amersham Bioscience) was added to the reaction mixture and incubation was continued for 1 h. At the end of the incubation, the fat pads were washed with phosphate-buffered saline and the tissue was digested with collagenase. The cell suspension was lysed by scintillation fluid. The uptake of 2-deoxy-D-[U- ^{14}C]glucose into the adipose tissue was determined by measuring the radioactivity of cell lysate using a Packard Tri-Carb 2900 TR low-activity liquid scintillation counter. Insulin (2 $\mu\text{g}/\text{ml}$) was added instead of smilaxin and used as a positive control, and cytochalasin B was used to determine the non-specific binding of radioactive glucose.

Assay for cell-free translation-inhibitory activity. An assay based on the rabbit reticulocyte lysate system was used [25]. Smilaxin (10 μl) was added to 10 μl of a radioactive mixture (500 mM KCl, 5 mM MgCl_2 , 130 mM creatine phosphate, and 1 μCi [4,5- ^3H]leucine) and 30 μl working rabbit reticulocyte lysate containing 0.1 mM hemin and 5 μl creatine kinase. Incubation proceeded at 37°C for 30 min before addition of 330 μl of 1 M NaOH and 1.2% H_2O_2 . Further incubation for 10 min allowed decolorization and tRNA digestion. An equal volume of the reaction mixture was then added to 40% trichloroacetic acid with 2% casein hydrolyzate in a 96-well plate to precipitate the radioactively labeled protein. The precipitate was collected on a glass fiber Whatman GF/A filter, washed, and dried with absolute alcohol passing through a cell harvester attached to a vacuum pump. The filter was suspended in 2 ml scintillant and counted in a

Packard Tri-Carb 2900TR low-activity liquid scintillation counter. Agrostin, a type 1 ribosome-inactivating protein (RIP) isolated from the seeds of *Agrostemma githago*, was used as a positive control

$$\% \text{ inhibition} = \frac{\text{CPM}(\text{Smilaxin}) - \text{CPM}(\text{NSB})}{\text{CPM}(\text{negative control}) - \text{CPM}(\text{NSB})} \times 100\%,$$

where NSB is non-specific binding and CPM is radioactivity in counts per minute.

Results

Smilaxin was adsorbed on DEAE-cellulose, CM-cellulose, Con A-Sepharose, and Mono S (Figs. 1–4). The adsorbed fractions from DEAE-cellulose and CM-cellulose were only about half as big as the corresponding unadsorbed fractions. The fraction adsorbed on ConA-Sepharose was much smaller than the unadsorbed fraction. The fraction adsorbed on Mono S was slightly smaller than the unadsorbed fraction (Figs. 1–4). In both gel filtration and SDS-PAGE, the purified protein from the Mono S column designated as smilaxin exhibited a molecular mass of 30 kDa (Fig. 5). From 700 g rhizomes were obtained 482 mg crude extract, 114 g DE2, 63 mg CM2, 7.5 mg CA2, and 3 mg MS. Smilaxin stimulated uptake of [methyl-³H]thymidine by both murine peritoneal macrophages and murine splenocytes (Fig. 6) with a potency lower than that of Con A. It also augmented nitric oxide production by murine peritoneal macrophages in a dose-dependent manner (Fig. 7). It inhibited [methyl-³H]thymidine uptake by a variety of tumor cells including MBL2 and PU5 (Fig. 8). However, there was no effect on S180 and L1210 tumor cells (data not shown). The N-terminal sequence of smilaxin demonstrates homology to very few proteins (Table 1). The protein was devoid of hemagglutinating and antifungal activities. Smilax stimulated glucose uptake into adipose tissue (Fig. 9).

Discussion

Smilaxin is an interesting protein. It resembles other plant proteins such as lectins [28–32], ribosome-inactivat-

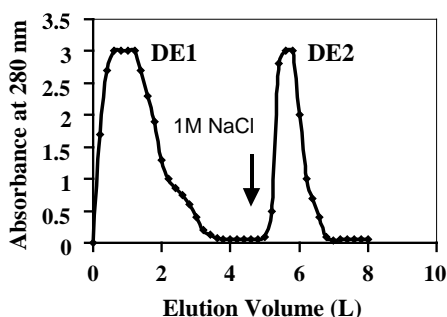


Fig. 1. Anion-exchange chromatography on a DEAE-cellulose column. The crude extract of *S. glabra* rhizomes was chromatographed on a 5 × 15 cm DEAE-cellulose column at a flow rate of 5.3 ml/min. The unadsorbed fraction was eluted with 10 mM Tris-HCl buffer (pH 7.4), and the adsorbed fraction with 1 M NaCl added to the buffer as indicated by the arrow. Smilaxin was present in the adsorbed fraction DE2.

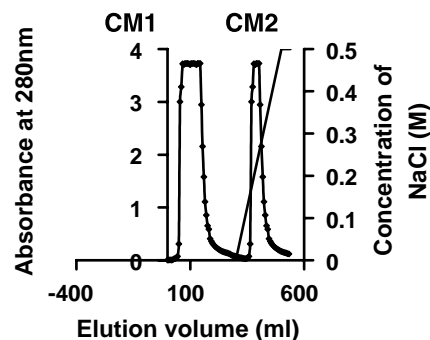


Fig. 2. Cation-exchange chromatography on a CM-cellulose column. The unadsorbed fraction DE1 from the DEAE-cellulose column, after dialysis against 10 mM NH₄OAc buffer (pH 4.6), was applied to a 2.5 × 18 cm CM-cellulose column at a flow rate of 3.4 ml/min. The unbound fraction was washed off with 10 mM NH₄OAc buffer. Smilaxin was eluted in fraction CM2 with the 0–0.5 M NaCl gradient as indicated by the slanting line across the right half of the chromatograph.

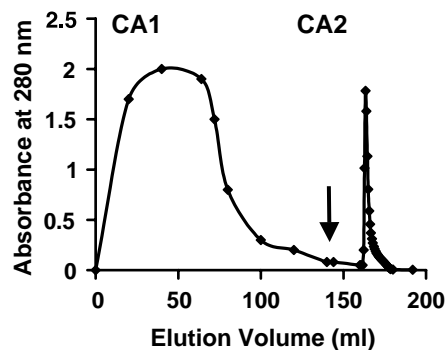


Fig. 3. Affinity chromatography on a Con A-Sepharose column. The fraction CM2 from the CM-cellulose column was subjected to affinity chromatography on a Con A-Sepharose column (column size: 2.5 × 8 cm) at a flow rate of 3.4 ml/min. After washing with binding buffer (20 mM Tris-HCl (pH 7.4) containing 0.5 M NaCl, 1 mM MnCl₂, and 1 mM CaCl₂), the adsorbed fraction CA2 containing smilaxin was eluted with the binding buffer supplemented with 0.5 M methyl-α-D-glucopyranoside as indicated by the arrow.

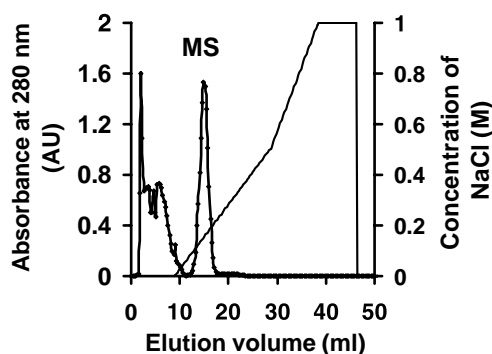


Fig. 4. Cation-exchange chromatography on an FPLC-Mono S column. The adsorbed fraction CA2 from the Con A-Sepharose column was applied to an FPLC-Mono S column (column size: 0.5 × 5 cm, flow rate: 1 ml/min). Smilaxin was bound to the column and eluted in fraction MS with the 0–0.5 M NaCl gradient.

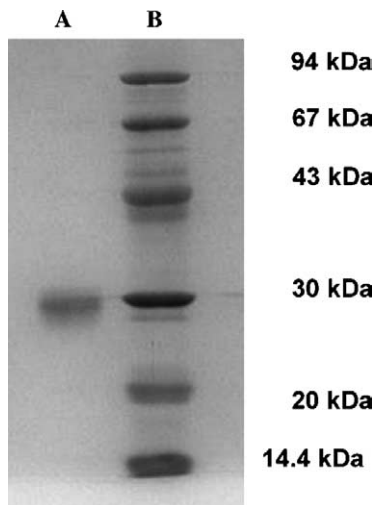
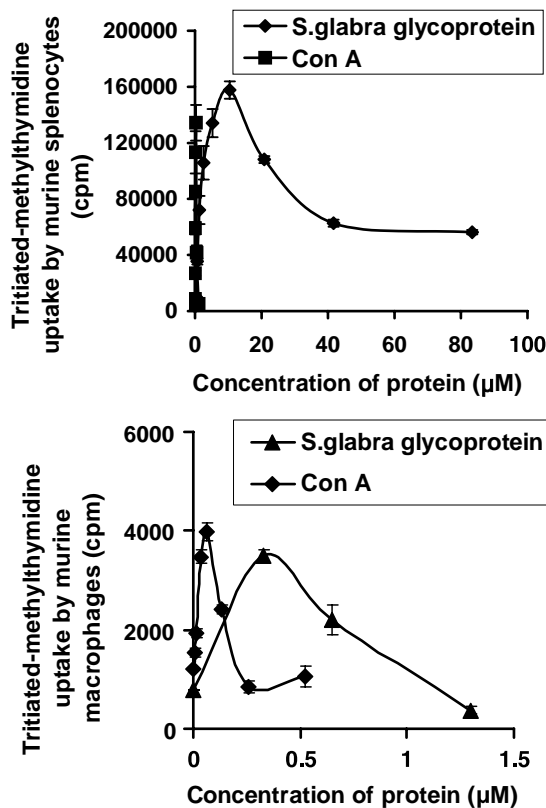
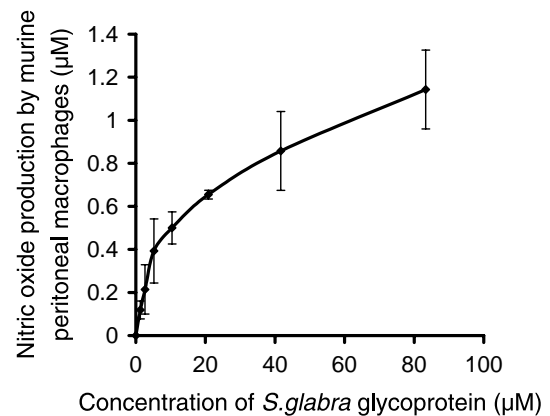
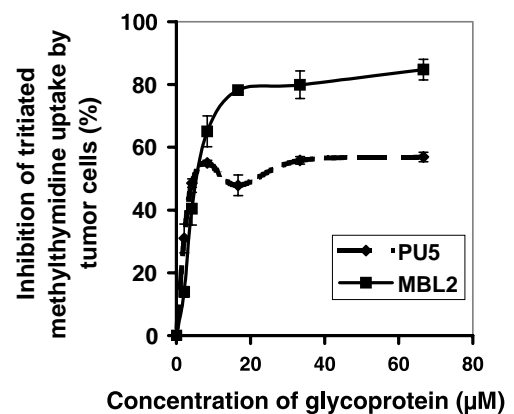


Fig. 5. SDS-PAGE results.

Fig. 6. Proliferative response of murine splenocytes (upper panel) macrophages (lower panel) to smilaxin. Results represent means \pm SD ($n = 3$).

ing proteins [33–36], antifungal proteins [37–42] and trypsin inhibitors [43–45] in that it exerts immunomodulatory, antiproliferative and HIV-1-reverse transcriptase activities and yet it lacks lectin and antifungal activities. It is devoid of translation inhibitory activity in the rabbit reticulocyte lysate system which is characteristic of ribosome-inactivating proteins [33–36], napin-like polypeptides [46,47], and antifungal proteins [25,34], indicating that it is not one

Fig. 7. Nitric oxide production by murine peritoneal macrophages in response to smilaxin (*S. glabra* glycoprotein). Results represent means \pm SD ($n = 3$).Fig. 8. Anti-proliferative effect of smilaxin (*S. glabra* glycoprotein) on PU5 and MBL2 tumor cells. No significant inhibition of proliferation of tumor cells L1210 and S180 by the glycoprotein was noted.

of these proteins. This suggests that it is a novel protein. This is also borne out by the fact that its N-terminal sequence exhibits some similarity to only plant lipoxygenases which are much larger proteins ($MW \approx 100$ kDa) than smilaxin and to oxygen-evolving enhancer protein from rice that has a molecular mass similar to smilaxin. Plant lipoxygenases are induced by pathogen infection [48] and have been implicated in potato tuber development [49]. Pathogen infection brought about, in addition to an elevated lipoxygenase activity, increased lipid peroxidation and production of reactive oxygen species [50]. However, smilaxin does not exhibit antifungal or translation-inhibiting activity characteristic of the defense proteins, ribosome-inactivating proteins [33–35], and antifungal proteins [25].

Smilaxin increases glucose uptake into adipose tissue. Thus, it has potential for antidiabetic therapy. In this regard it is noteworthy that an extract of *S. glabra* rhizomes exerts hypoglycemic activity in normal as well as diabetic mice [4]. The extract also demonstrates immunomodulatory activity in the later phase of adjuvant-induced

Table 1
Comparison of N-terminal sequence of smilaxin (*S. glabra* glycoprotein) with other proteins

Protein	Sequence	Length
Smilaxin (<i>S. glabra</i> glycoprotein) (1–16)	TEFDSFVLTL...LYYPGTL	
Putative oxygen-evolving enhancer protein 3–2, chloroplast precursor (OEF3) <i>Oryza sativa</i> (japonica)	TEFDRFVRLTPKVYESYLYYEKTL	225
Smilaxin (<i>S. glabra</i> glycoprotein) (2–11)	EFDSFVLTLY	
Lipoxygenase from <i>Nicotiana attenuata</i> (300–311)	EFDSFEDVLRLY	862
Lipoxygenase from <i>Solanum tuberosum</i> (301–312)	EFDSFEDVLRLY	862
Lipoxygenase from <i>Prunus dulcis</i> (302–313)	EPDSFEDVLRLY	862

Length = number of amino acid residues in protein.

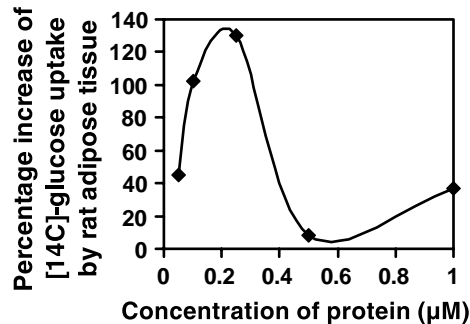


Fig. 9. Smilaxin-induced stimulation of [¹⁴C] glucose uptake by rat adipose tissue. Bovein insulin (33 pM) brought about 265% increase.

arthritis in rats [5]. In line with this finding, smilaxin is found in the present investigation to have immunostimulatory activity. In response to smilaxin treatment, macrophages produce a larger amount of nitric oxide, and the proliferation rate in splenocytes, macrophages, and bone marrow cells is increased. The maximal uptake of thymidine achieved by smilaxin is similar in magnitude to that induced by Con A although smilaxin has a lower potency compared with Con A. Smilaxin has selective antiproliferative activity toward MBL2 and PU5 transcriptase. This is reminiscent of the observation that ribosome-inactivating proteins display different antiproliferative potencies toward different tumor cell lines [51]. Thus, smilaxin appears to be an antitumor and antiviral protein (Fig. 10, Table 2).

In summary, smilaxin is a new protein with potentially exploitable activities.

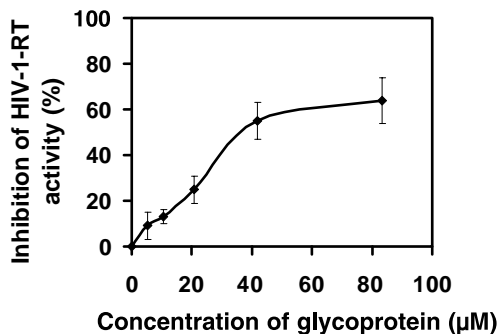


Fig. 10. Inhibitory effect of smilaxin (*S. glabra* glycoprotein) on the activity of HIV-1-reverse transcriptase. Results are means \pm SEM, $n = 3$, IC₅₀ value = 40 μ M.

Table 2
Proliferation of murine splenocytes and bone marrow cells stimulated by smilaxin

Concentration (μ M)	[Methyl- ³ H]thymidine uptake by	
	Splenocytes (cpm)	Bone marrow cells (cpm)
<i>Con A</i>		
0	5374 \pm 586	1125 \pm 188
0.26	26948 \pm 1780	4937 \pm 685
0.52	134340 \pm 12746	7391 \pm 835
1.04	5050 \pm 2265	5610 \pm 763
<i>Smilaxin</i>		
0	5374 \pm 586	1252 \pm 66
0.05	9938 \pm 340	1572 \pm 89
0.38	105779 \pm 3942	1822 \pm 127
1.56	157717 \pm 6204	3787 \pm 442
3.16	108139 \pm 2465	5308 \pm 336
6.24	62675 \pm 2555	7958 \pm 122
12.4	56278 \pm 1613	8299 \pm 287

Results are means \pm SEM ($n = 3$).

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