Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 340 (2006) 118–124
BBRC
www.elsevier.com/locate/ybbrc

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

K.T. Chu, T.B. Ng *

Department of Biochemistry, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China

Received 11 November 2005
Available online 12 December 2005

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-^{3}H]thymidine by murine splenocytes, peritoneal macrophages, and bone marrow cells, and production of nitric oxide by peritoneal macrophages. It inhibited uptake of [methyl-^{3}H]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 IC_{50} 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.

Keywords: *Smilax glabra*; Rhizomes; Glycoprotein; Antiproliferative; Immunostimulatory; Isolation; Protein

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 (smilitilbin) was isolated, together with engeletin, astilbin, dihydroquercetin, eurryphin, resveratrol, and 5-*O*-caffeoylshikimic acid [2]. The flavanonal glucosides isoengetitin, isoastilbin, and astilibin 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 (14,000*g*, 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 NH_4OAc

* Corresponding author.
E-mail address: b400486@mailserv.cuhk.edu.hk (T.B. Ng).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.12.010

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-^{3}H]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-^{3}H]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 (ELSIA) 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 collegenase. 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 μg/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-^{3}H]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