Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 859 (2007) 111–118
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Affinity purification of serine proteinase from *Deinagkistrodon acutus* venom

Yu Xin [a], Dexian Dong [a], Ting Wang [b], Rongxiu Li [a,*]

[a] *Key Laboratory of Microbial Metabolism of Ministry of Education, Shanghai Jiao Tong University, College of Life Science and Biotechnology, 800 Dongchuan Road, Shanghai 200240, China*
[b] *Shanghai Nisheng Biotechnology Co. Ltd., Box 619-1, 680 Guiping Road, Shanghai 200000, China*

Received 13 April 2007; accepted 10 September 2007
Available online 14 September 2007

Abstract

An affinity protocol was developed for the preparation of the main serine proteinase from *Deinagkistrodon acutus* venom on industrial scales. As affinity ligand, L-arginine was composed to medium and its structure was confirmed by ESI-MS analysis. The purification process consisted of one major affinity chromatography step to remove more than 95% of other proteins, and a polishing step of DEAE ion-exchange chromatography for removal of minor contaminants. The serine proteinase was 100% pure analyzed on HPLC Vydac C4 column, 99.4% on TSK G3000SW column, and 97.7% with SDS-PAGE analysis. The yield of the main serine proteinase was 3.6% of crude venom protein, the recoveries of typical fibrinogen (Fg) clotting activity and arginine esterase activity of serine proteinase were 82.2% and 84%, higher than those of other reported traditional protocols, the proteinase also showed arginine amidase activity. Reducing SDS-PAGE analysis showed that the arginine esterase was a single polypeptide with the mass of ~40 kDa, while MALDI-TOF-TOF-MS analysis showed that the purified proteinase should be a ~34 kDa glycoprotein. The desorption constant K_d and the theoretical maximum absorption Q_{max} on the affinity medium were 9.93×10^{-5} and 38.1 mg/g medium in absorption analysis.

Keywords: Affinity chromatography; Serine proteinases; *Deinagkistrodon acutus* venom

1. Introduction

Serine proteinases from snake venoms act on different steps of blood coagulation cascade [1,2]. Some hydrolyze fibrinogen and release fibrinopeptide A or fibrinopeptide B or both [1,3–5], and have been used as anti-thrombotics drugs to prevent arterial embolism [6]. *Deinagkistrodon acutus* (*Agkistrodon acutus* or hundred-pace snake) is a monotypic pit viper existed in east and south part of China. Several serine proteinases have been characterized in *D. acutus*, designated as acutobin (acutin or acutase), Dav-KN and Dav-PA [7–10]. Major serine proteinase in this venom has been used in clinical treatment of thrombolysis in China. There exists many serine proteinases in snake venoms, and purification of any single one is always a challenging task. The purification protocol usually consists of three or more steps of chromatography, *i.e.* one approach with two ion-exchange chromatographies on DEAE-Sepharose, one on MonoQ, and one gel-filtration on Superose 12 [7], the other approach with one ion-exchange chromatography on DEAE-Trisacryl, one gel-filtration on TSK G2000SW, and one reverse-phase chromatography on Vydac RP-C_4 [9] or other combination of gel-filtration chromatographies and ion-exchange chromatography [12,14]. Improvement has been made with agmatine and benzamidine affinity chromatography incorporated along with gel-filtration chromatography [11,13]. However, the sample capacity and inefficiency of gel-filtration chromatography step made the protocol time consuming and productivity low in production scale [7,9,12,14]. Another affinity chromatography protocol was also reported [15], but the recovery of activity was low.

Here reported is an affinity preparation protocol of the main serine proteinase from *D. acutus* venom on industrial scales.

2. Materials and methods

2.1. Materials

Venom powder of *D. acutus* venom was from Huang Shan Institute of Snake (Anhui, China). Bz-DL-Arg-pNA (DL-BAPA) and Nα-benzoyl-L-arginine ethyl ester (BAEE) were from

* Corresponding author. Tel.: +86 2134204207.
E-mail address: rxli@sjtu.edu.cn (R. Li).

1570-0232/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2007.09.010

Sigma–Aldrich Chemical Co., USA. Protein molecular mass markers were from Promega Co., USA. Toyopearl DEAE-650 M, SuperQ-650 M, and Sp-650 M were from Toyopearl Co., Japan. Sepharose 4B was from Pharmacia Biotech, Sweden. Human fibrinogen was from ShangHai Raas-Corp Co., China. All other chemicals were of analytical reagent grade from local companies.

2.2. Synthesis and analysis of affinity medium

Serine proteinases with arginine esterase and arginine amidase activities specifically acted on substrates containing arginine-amide as DL-BAPA or arginine-ester such as BAEE, therefore, arginine was chosen as affinity ligand. Sepharose was derivatized to amino-Sepharose according to the procedure described by Matsumoto et al. [16] and L-arginine and amino-Sepharose were linked through cyanuric chloride according to Li et al. [17].

To confirm the conformation of ligand on the medium, 80 mg dried affinity medium was incubated with 6 M hydrochloric acid at 100 °C for 24 h, and hydrochloric acid was removed by vacuum evaporation. The hydrolyzed chemical was first applied onto column of Toyopearl Sp-650 M (0.5 cm × 3 cm) at pH 3.0 and eluted with 10 mM NaOH, then applied onto column of Toyopearl SuperQ-650 M (0.5 cm × 3 cm) and eluted with 10 mM HCl. After the HCl was removed under vacuum, the purified chemical was analyzed with ESI-MS (HP1100LC MSD, Agilent, USA).

2.3. Purification of serine proteinases

4.0 g of *D. acutus* venom powder was dissolved in 40 ml equilibrating buffer (10 mM sodium phosphate/1 mM EDTA/0.05 M NaCl, pH 6.0, 8 ms/cm) and centrifuged at 12,000 rpm for 30 min. The equilibrated affinity column (25 mm × 80 mm) was loaded all the supernatant (total proteins from 4 g venom powder, ∼208 mg) at 5 ml/min, washed with equilibrating buffer until $A_{280\,nm}$ base line, and further washed with 10 mM sodium phosphate/1 mM EDTA (pH 6.0) until $A_{280\,nm}$ base line, then eluted with 10 mM glycine (pH 9.0). Fraction of 75 ml (∼153 μg/ml) was collected during chromatography operation and tested for clotting activity, arginine esterase activity and protein concentration. Active fractions containing clotting and arginine esterase activities were pooled, adjusted to pH 6.8 and applied onto a DEAE-650 M column (25 mm × 40 mm) equilibrated with 5 mM sodium phosphate (pH 6.8) at 2.5 ml/min. The column was eluted with gradient of NaCl from 0 to 0.2 M and fraction of 97.5 ml was collected and tested for clotting activity, esterase activity and protein concentration. The chromatography process was monitored at 280 nm during the whole purification. The purified proteinase fractions were pooled and stored at −70 °C. The concentrations of crude and purified proteinases were determined with Lowry Method, referenced with bovine serum albumin. The purified proteinase was further analyzed with SDS-PAGE (12.5%) under reducing conditions.

2.4. SDS-PAGE analysis

Reducing SDS-PAGE (12.5%) analysis was carried out on a Miniprotean II system from Bio-Rad (Hercules, CA, USA). For gel electrophoresis 10 μl samples were mixed with 10 μl loading buffer (2% SDS, 350 mM DTT, 25% (v/v) glycerol, 0.01% Bromophenol Blue in 62.4 mM Tris–HCl, pH 6.8) and incubated at 95 °C for 5 min before loading. Proteins were separated on a 12.5% SDS-polyacrylamide gel. Gels were stained with Coomassie® Brilliant Blue R, destained, and imaged on Gelpro Analyzer 3.0 software (Media Cybernetics, Inc.) for analysis of purity and molecular mass. The purity with reducing SDS-PAGE (12.5%) analysis was calculated by integration of the lane darkness.

2.5. Mass spectrometry

ESI-MS analysis was performed using HP 1100LC MSD (Agilent, USA). The chemical solution hydrolyzed from affinity medium was introduced into the mass spectrometer with a syringe. Nitrogen was used as nebulizer/drying gas (13 l/min, 350 °C). Capillary voltage was set at 4000 V, two sampling cone voltages at 70 and 170 V were selected. Scanning was performed from *m/z* 100 to 400 in 10 s, and several scans were summed to obtain the final spectrum.

MALDI-TOF-TOF-MS analysis was performed using AutoFlex MALDI-TOF-TOF-MS (Bruker, Germany). The purified proteinases was mixed with matrix (saturated sinapinic acid in acetoritrile/H_2O, containing 0.1% TFA, 1:1, v/v) to promote desorption and ionisation. An N_2 laser at 337 nm was used to desorb the solute molecules from the sample disc and a voltage of 20 kV were established in the source region. Data were analyzed by flex analysis (Bruker Daltonics).

2.6. Adsorption analysis

The adsorption capacity of the affinity medium was analyzed according to the method reported [18]. In a total volume of 1 ml varying amounts of purified serine proteinase solution (0.3, 0.6, 0.9, 1.2, 1.5, 1.8, 2.1, 2.4, and 2.7 nM), previously dialyzed against 10 mM sodium phosphate/1 mM EDTA/0.05 M NaCl (pH 6.0), were mixed with 2 mg of affinity medium. The suspensions were shaken for 4 h at 25 °C, in order for the system to reach equilibrium. The mixtures were then centrifuged (2000 rpm, 5 min) and the supernatants were assayed for Fg clotting activity. For each experiment, a control was carried out to ensure that there was no loss of activity under these conditions. The data were analyzed according to the Scatchard method, Q_{max} (the theoretical maximum adsorption capacity) and K_d (the constant of desorption) were determined.

2.7. HPLC analysis

The proteinase sample isolated from *D. acutus* venom was analyzed at Knauer system of K501 pumps and K2501 UV-monitor (Germany). The samples were injected into a Vydac C4 column (4.6 mm × 250 mm) at 1 ml/min and eluted with a

gradient of acetonitrile/0.1% TFA from 5% to 95% in 20 min in 0.1% TFA. In addition, the isolated proteinase was injected into a gel-filtration column (TSK G3000SW, 4.5 mm × 250 mm) equilibrated and eluted with 0.2 M sodium phosphate/1% isopropyl alcohol at the speed of 1 ml/min. The purity of proteinase sample was calculated as the percentage of main peak integration.

2.8. Enzymatic activity

The amidase activity of the serine proteinase with DL-BAPA was assayed in vitro, by the absorbance at 410 nm at 37 °C of a reaction containing 0.5 ml of 4.35 mg DL-BAPA/ml of 10% dimethyl sulfoxide solution and 0.5 ml of enzyme (50 μg) dissolved in 50 mM potassium phosphate buffer (pH 7.5) [11,19].

The esterase activity of the serine proteinase with BAEE was assayed in vitro, following the increase in absorbance at 253 nm at 25 °C. The substrate was dissolved in 50 mM Tris–HCl buffer (pH 8.0). The calibration curve was built by a series of the concentration of hydrolyzed BAEE and the changes in value of absorbance at 253 nm according to the method previously reported [20,21]. 0.2 ml purified enzyme solution (0.05 mg/ml) was mixed with 3 ml BAEE (250 nmol/ml) in 50 mM Tris–HCl buffer (pH 8.0), and the changes in value of absorbance at 253 nm was measured in 3 min. The initial reaction speed of the esterase activity could be calculated [8,20,21].

Fg clotting activity was assayed according to the method previously reported [22,23], and standard batroxobin was used to build the calibration curve of clotting activity versus initial coagulant time. Clotting activity was measured by mixing appropriate concentration of the sample 200 μl with 200 μl Fg (0.4%) in 50 mM Tris–HCl buffer (pH 7.4), containing 0.9% NaCl at 37 °C. One unit of coagulant activity was considered to be equivalent to one batroxobin unit. Every sample was tested for at least five times. One hundred batroxobin units are equivalent to 17.5 NIH thrombin units [24].

3. Results

3.1. Affinity medium preparation and confirmation

Serine proteinases have arginine-ester and arginine-amide hydrolase activities, they could specifically recognize arginine-related substrates. In the preparation of the affinity medium, L-arginine was composed to amino-Sepharose through cyanuric chloride. To confirm the ligand structure, the medium was hydrolyzed with 6 M HCl, and then the resultant with molecular formula of $C_{12}N_8O_4H_{21}Cl$ and molecular mass of 376.5 (Fig. 1A) was purified with two ion-exchange chromatography. The chemical was then analyzed with ESI-MS (Fig. 2). Since chlorine on triazine ring was unstable in either alkaline or acidic condition [17], the hydrolysis with 6 M HCl would replace the chlorine on the ligand with a hydroxyl group, thus the theoretical structure of the purified ligand should be with a molecular formula of $C_{12}N_8O_5H_{22}$ and molecular mass of 358.4. About 540 μg of this chemical was isolated from 80 mg affinity medium; the ligand density was about 18.9 μmol/g medium. At cone voltage of 70 V (Fig. 2A), $C_{12}N_8O_5H_{23}$ requires $[M+H]^+$, 359.3; *m/z* 343.1 $[M+H-O]^+$, 299.1 $[M+H-O-CO_2]^+$, 149.1 $[M+2H-O-CO_2-2H]^{2+}$. At cone voltage of 170 V, the ligand was broken into fragments and showed several smaller peaks (Fig. 2B). $C_{12}N_8O_5H_{23}$ requires $[M+H]^+$, 359.3; *m/z* 343.1 $[M+H-O]^+$, 365.1 $[M+Na-O]^+$, 239.1 $[M+H-CN_2H_4-C_3H_6O_2-2H]^+$, 200.1 $[M+H-C_3H_6O_2-C_3N_3H_7]^+$, 155.1 $[M+2H-3O-2H]^{2+}$. The possible structures of chemicals in principal peaks are shown in Fig. 2.

3.2. Purification of the proteinase

The supernatant of crude venom was first loaded onto the equilibrated affinity column and washed with 10 mM sodium phosphate/1 mM EDTA/0.05 M NaCl (pH 6.0, 8 ms/cm) then washed with 10 mM sodium phosphate/1 mM EDTA (pH 6.0), finally eluted with 10 mM glycine (pH 9.0). In the affinity

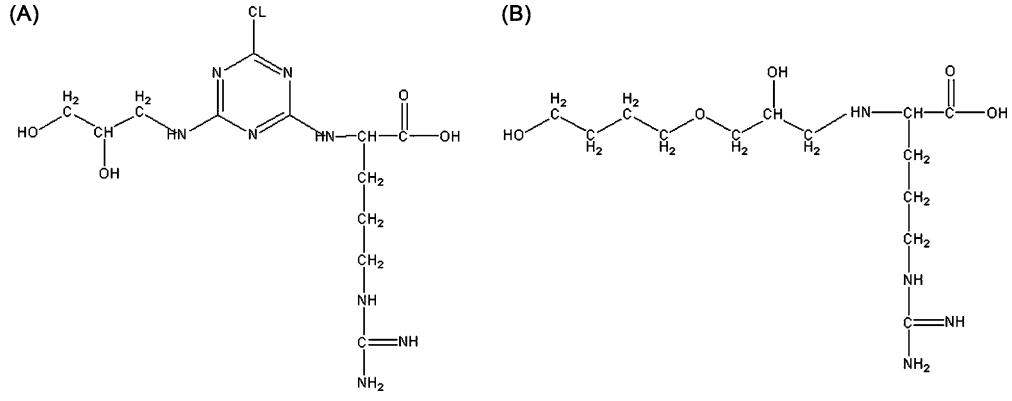

Fig. 1. The theoretical ligand of the affinity medium. (A) L-Arginine is composed to Sepharose 4B through cyanuric chloride and 3-amino-1,2-propanediol with molecular formula of $C_{12}N_8O_4H_{21}Cl$ and molecular mass of 376.5. The hydrolysis with 6 M HCl would replace the chlorine on the ligand with a hydroxyl group; it should be with a molecular formula of $C_{12}N_8O_5H_{22}$ and molecular mass of 358.4. (B) The ligand of arginine Sepharose 4B according to the description of Pharmacia Biotech. L-Arginine is immobilized to Sepharose 4B via a long hydrophilic spacer and a stable ether and alkylamine bonds, its spacer may be even longer than that in this picture.

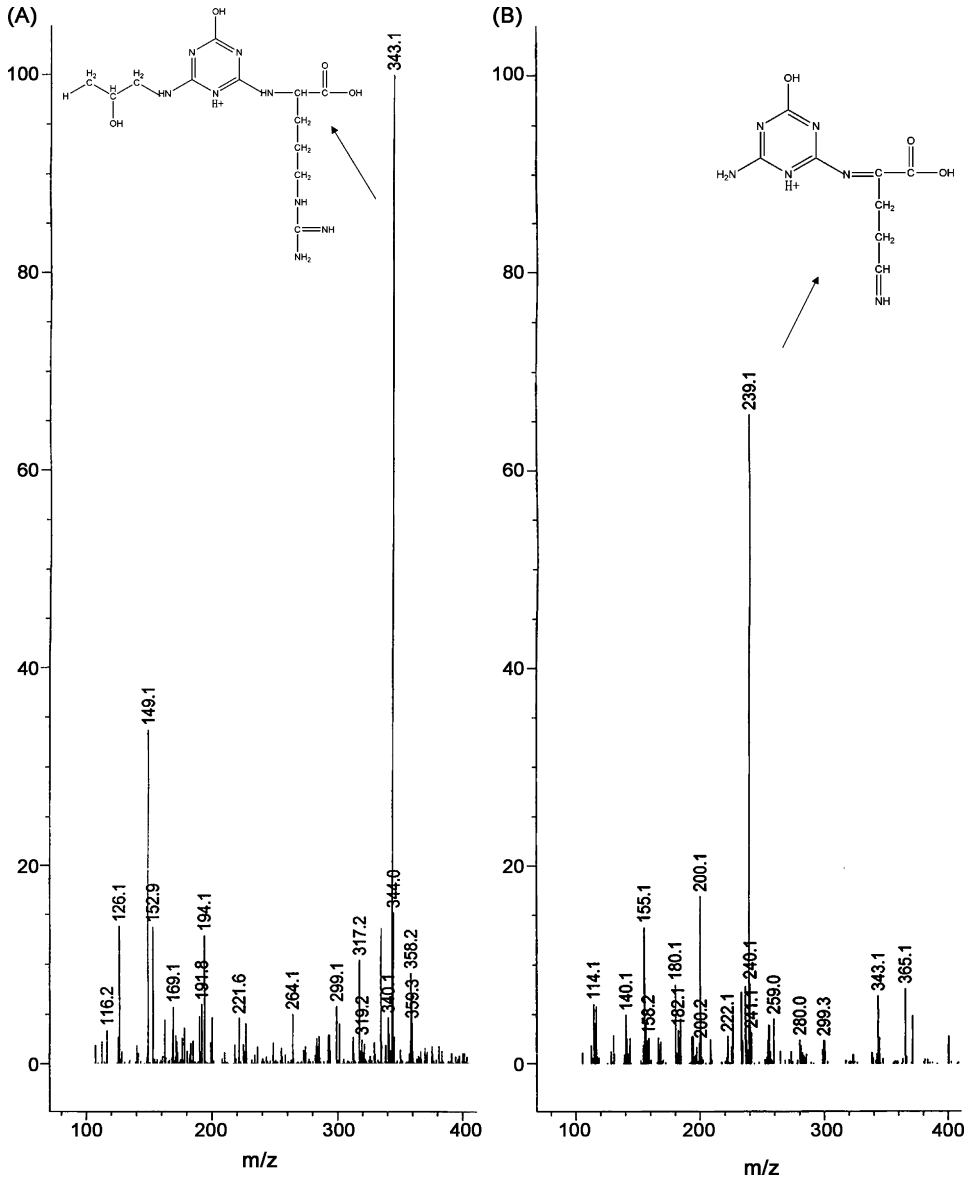

Fig. 2. The ESI-MS analysis of the affinity ligand. The possible structures of the chemicals in principal peaks are shown. (A) With an ESI-MS cone voltage of 70 V, the principal peak $[M+H-O]^+$ was at 343.1; other peaks: $[M+H]^+$ at 359.3, $[M+H-O-CO_2]^+$ at 299.1, $[M+2H-O-CO_2-2H]^{2+}$ at 149.1. (B) With an ESI-MS cone voltage of 170 V, the ligand was broken into fragments. The principal peak $[M+H-CN_2H_4-C_3H_6O_2-2H]^+$ was at 239.1; other peaks: $[M+Na-O]^+$ at 365.1, $[M+H-O]^+$ at 343.1, $[M+H-C_3H_6O_2-C_3N_3H_7]^+$ at 200.1, and $[M+2H-3O-2H]^{2+}$ at 155.1.

chromatography, two peaks having absorbance at 280 nm were obtained as shown in Fig. 3A. Proteins in flow through peak at 18 min could not bind onto the affinity column. High arginine-ester and Fg clotting activities were detected in peak eluted at 67 min. Start with 208 mg of protein, 10.3 mg of protein was obtained in peak eluted at 67 min, with a yield of 5.0% (Table 1).

The clotting active fractions were pooled (10.3 mg), adjusted to pH 6.8 and applied onto a DEAE-650 M column equilibrated with 5 mM sodium phosphate (pH 6.8). The column was then eluted with gradient of NaCl from 0 to 0.2 M. The chromatograms are shown in Fig. 3B and the results are summarized in Table 1. Only one principal peak of absorbance at 280 nm was obtained as shown in Fig. 3B, high arginine esterase and Fg clotting activities were detected in peak eluted at 99 min. 7.5 mg of active protein was obtained with a total yield of 3.6% from crude venom protein (Table 1), it is the main serine proteinase in *D. acutus* venom. The whole process of affinity chromatography and ion-exchange chromatography only takes less than 4 h. The serine proteinase protein yield of this affinity protocol 3.6%, compared with serine proteinase protein yield of traditional methods, as 1.6% of Wang et al. [9], 0.8% of Oyama and Takahashi [14], 2.1% of Magalhaes et al. [13], 3.4% of Magalhaes et al. [11], is higher.

3.3. Affinity adsorption

Equilibrium adsorption studies were employed to characterize the interaction of the enzyme and the affinity medium. The approach provides a relationship between the concentration of the enzyme in the solution and the amount of enzyme absorbed

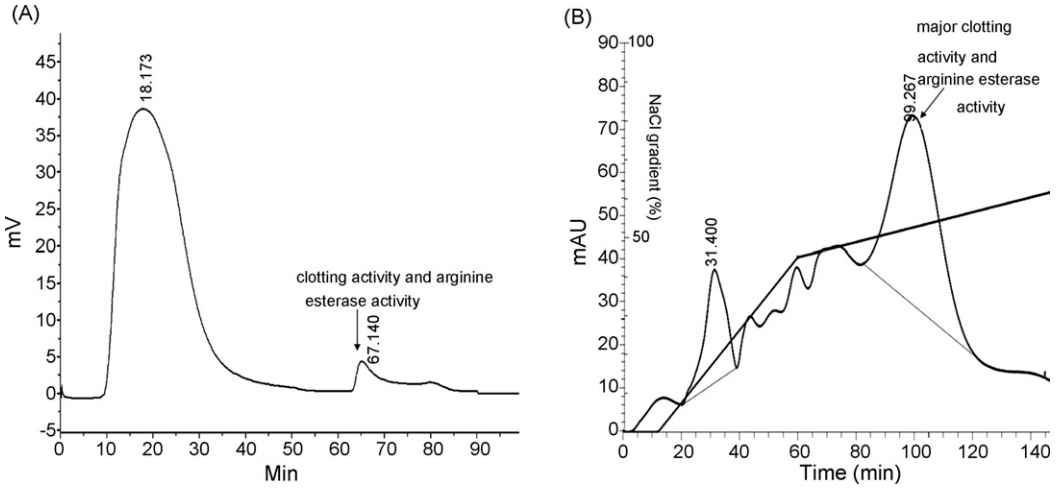

Fig. 3. The purification of the main serine proteinase from *D. acutus* venom. (A) Affinity purification of the main serine proteinase from *D. acutus* venom. 40 ml crude venom solution (~5.2 mg/ml) in 10 mM sodium phosphate (pH 6.0)/1 mM EDTA/0.1 M sodium chloride was applied onto equilibrated affinity column (25 mm × 80 mm) with the equilibrating buffer. The column was washed by 10 mM sodium phosphate/1 mM EDTA (pH 6.0) until $A_{280\,nm}$ base line, and then eluted with 10 mM glycine (pH 9.0), at 5 ml/min. The peak eluted at 67 min was collected and further purified. (B) DEAE ion-exchange purification of the collections in affinity chromatography. About 75 ml active fractions (~137 μg/ml) were further purified on a DEAE-650 M column (25 mm × 40 mm). Elution buffers A and B were 10 mM sodium phosphate (pH 6.8) and 10 mM sodium phosphate (pH 6.8)/0.2 M sodium chloride. After the fraction was loaded, elution was performed at a flow rate of 2.5 ml/min with a linear NaCl gradient (from 0 to 0.2 M). The major serine proteinase peak was eluted at about 99 min.

on affinity medium. According to the Scatchard method [25], the data should fit to:

$$Q = \frac{Q_{max}[C^*]}{K_d + [C^*]} \quad (1)$$

In Eq. (1), Q is the enzyme absorbed to the medium, Q_{max} is the theoretical maximum absorption of serine proteinase to the medium, $[C^*]$ is the concentration of the enzyme in solution, and K_d is the desorption constant. Eq. (1) could be transformed to:

$$\frac{Q}{[C^*]} = \frac{Q_{max}}{K_d} - \frac{Q}{K_d} \quad (2)$$

According to the Scatchard method, a plot of Q and $Q/[C^*]$ should yield a straight line. The batch adsorption of the purified enzyme on affinity medium is shown in Fig. 4. The respective correlation coefficient R^2 was 0.994, indicating that the model fits the data well. From Fig. 4, the desorption constant K_d was 9.93×10^{-5} mg/g medium, and the theoretical maximum absorption Q_{max} was 38.1 mg/g medium.

3.4. Characterization of purified enzyme

On Vydac C4 column (4.6 mm × 250 mm), the samples were loaded at 1 ml/min and eluted with a gradient of acetonitrile/0.1% TFA from 5% to 95% in 20 min in 0.1% TFA. On gel-filtration column (TSK G3000SW, 4.5 mm × 250 mm) the samples were loaded and eluted with 0.2 M sodium phosphate/1% isopropyl alcohol at the speed of 1 ml/min. The purities from peak integration of gel-filtration and reverse-phase HPLC were 100% and 99.4% according to the software EuroChrom2000, respectively (Fig. 5). Reducing SDS-PAGE (12.5%) analysis showed that the enzyme was a single polypeptide with the mass of ~40 kDa, and the purity was about 97.7% (Fig. 6). Although MALDI-TOF-TOF-MS analysis showed that the molecular mass should be ~34 kDa (Fig. 7), the molecular mass on SDS-PAGE gel was ~40 kDa, the same as the main part of serine proteinases in *D. acutus* venom [7,9]. In all the purification steps it could be demonstrated that arginine-ester and Fg clotting activities super-imposed each other.

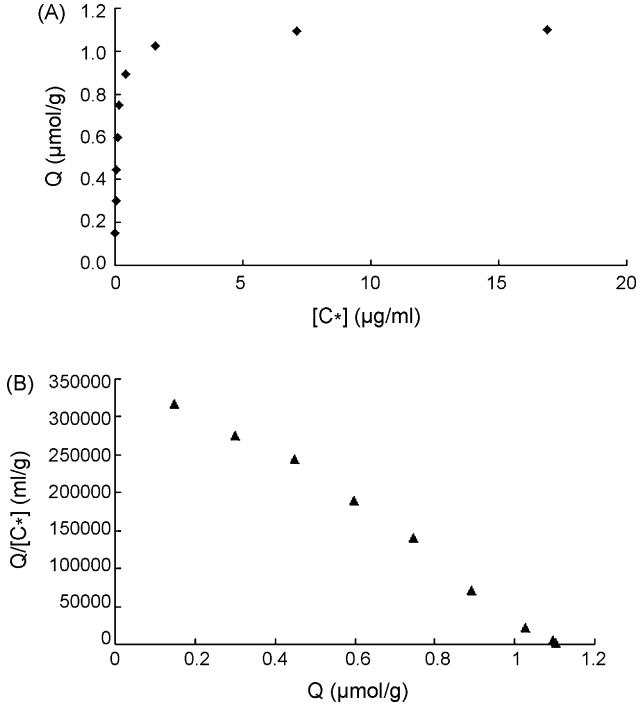

Fig. 4. Adsorption analysis. (A) Equilibrium adsorption of the serine proteinase on affinity medium in a batch system (pH 6.0, 25 °C). (B) A plot describing the equilibrium of the absorption on medium and the enzyme concentration in liquid phase. The desorption constant K_d was 9.93×10^{-5} mg/g medium, and the theoretical maximum absorption Q_{max} was 38.1 mg/g medium.

Table 1
The recovery of the purification

Step	Protein		Clotting activity			Arginine esterase activity			Arginine amidase activity		
	mg	Yield (%)	Total activity (NIH unit)	Specific activity (NIH unit/mg)	Yield (%)	Total activity (μmol/min)	Specific activity (μmol/min/mg)	Yield (%)	Total activity (μmol/min)	Specific activity (μmol/min/mg)	Yield (%)
Crude sample	208 ± 1.5	100	18385 ± 4.16	88.4 ± 0.02	100	18.5 ± 0.13	0.09 ± 0.001	100	–	–	–
Affinity medium	10.3 ± 0.74	5.0	16830 ± 4.6	1634 ± 0.4	91.5	17.8 ± 0.1	1.72 ± 0.01	96	12.6 ± 0.68	1.22 ± 0.07	–
DEAE-650M	7.5 ± 0.45	3.6	15104 ± 18.8	2025 ± 2.55	82.2	15.6 ± 0.07	2.09 ± 0.01	84	10.0 ± 0.24	1.34 ± 0.03	–

Arginine esterase activity was determined using BAEE as substrate. Arginine amidase activity was determined using DL-BAPA as substrate. Clotting activity was determined using Fg as substrate. Values of protein, arginine esterase activity and arginine amidase activity are the means of at least three determinations, and values of clotting activity are the means of at least five determinations.

The initial reaction speed of amidase activity with DL-BAPA was calculated with absorbance at 410 nm, with $K_m = 68.5 \times 10^3 \pm 5.6 \times 10^3$ μM, and $V_{max} = 49 \times 10^{-2} \pm 0.07 \times 10^{-2}$ μM/min. The initial reaction speed of esterase activity with BAEE was calculated with absorbance at 253 nm. The recoveries at the two steps were 96% and 84%, respectively (Table 1), with $K_m = 403 \pm 26$ μM, and $V_{max} = 1.56 \pm 0.07$ μM/s. And the esterase activity could be completely inhibited by PMSF (2 mM) or AEBSF (2 mM). Compared with esterase activity recoveries of traditional methods, as 58% of Araujo et al. [12], 7.8% of Oyama and Takahashi [14], the esterase activity recovery of this affinity protocol is higher.

Fg clotting activity was calculated with the initial coagulant time, and the recoveries at the two steps were 91.5% and 82.2%, respectively (Table 1), much higher than that of other preparation protocols reported.

4. Discussion

Purification protocols of serine proteinases from snake venoms usually consist of three or more steps of gel-filtration and ion-exchange chromatography [7,9,13,15], and agmatine affinity chromatography was incorporated along with gel-filtration chromatography [14,16,21]. However, the protein yield and activity recoveries were low. In order to found a preparation protocol with great efficiency and large capacity, L-arginine was composed to medium as affinity ligand in different ways, and the most appropriate one was chosen during experiments. Benzamidine-modified Sepharose was also used as affinity medium control; however, it could not bind any protein from *D. acutus* venom (unpublished). For serine proteinases' recognition and hydrolyzation abilities on arginine-esters and arginine-amides, as affinity ligand, arginine is more suitable than serine proteinase inhibitors, such as benzamidine and agmatine.

Arginine Sepharose 4B from Pharmacia Biotech is also an affinity medium based on arginine, and it has been used to isolate or remove a number of different serine proteinases, *e.g.* prekallikrein, clostripain, plasminogen activator, prothrombin, and maturation promoting factor [26–30]. However, it has not been reported in the preparation of serine proteinases from snake venoms, and in our experiments it did not bind any protein from venom of *D. acutus* either. The medium is L-arginine immobilized to Sepharose 4B via a long hydrophilic spacer and a stable ether and alkylamine bonds, the L-arginine structure is similar to the affinity medium we composed (Fig. 1B). Although the long hydrophilic spacer could avoid nonspecific hydrophobic adsorption, the folding and twist between these soft long spacers could prevent the ligands from binding with target proteins, which may increase nonspecific binding ratio [31]. In our method, L-arginine was composed to medium through a rigid frame-work spacer of triazine. Its length and high stiffness could avoid the mutual interference of the spacers and provide more affinity binding opportunities. In order to reduce the hydrophobic nonspecific adsorption, in the affinity step, the electrical conductivity of equilibrating buffer was raised to 8 ms/cm with a little higher concentration of NaCl.

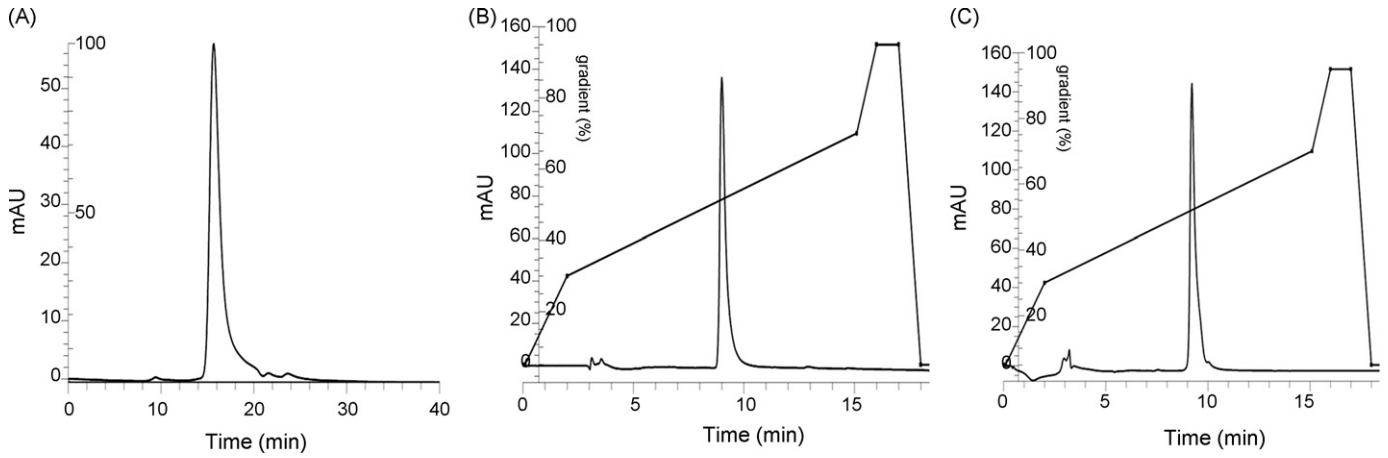

Fig. 5. HPLC analysis of the proteinase. (A) Gel-filtration analysis of protease. The purified proteinase was injected into a TSK G3000SW column (4.5 mm × 250 mm) in 0.2 M sodium phosphate (pH 7.0)/1% isopropyl alcohol. The purity of the principal peak is about 99.4% according to the software EuroChrom2000. (B) RP-HPLC analysis of the serine proteinase we isolated. The purified proteinase was injected into a Vydac C4 column (4.6 mm × 250 mm) and eluted with a gradient of acetonitrile/0.1% TFA from 5% to 95% in 20 min in 0.1% TFA. The purity of the principal peak is 100% according to the software EuroChrom2000. (C) RP-HPLC analysis of an anti-thrombotics drug "defibrase injection". The protein powder was dissolved in 0.1% TFA and injected into a Vydac C4 column (4.6 mm × 250 mm), eluted with a gradient of acetonitrile/0.1% TFA from 5% to 95% in 20 min in 0.1% TFA. It showed a similar peak as that of the isolated serine proteinase.

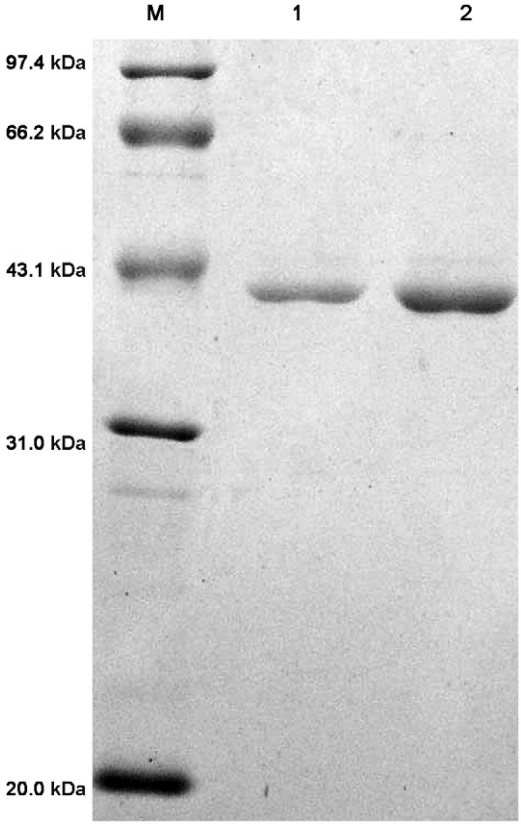

Fig. 6. SDS-PAGE analysis of the purified serine proteinase. Purified serine proteinase was applied to SDS-PAGE (12.5%). Lane M, the molecular mass standard protein marker (97.4, 66.2, 43.1, 31.0, 20.0 kDa); lane 1, an anti-thrombotics drug "defibrase injection" (2 μg) was loaded, the effective component is identified as the major fibrinogen coagulating proteinase (acutin or acutobin) in *D. acutus* venom [7,9]; lane 2, 5 μg of the main serine proteinase isolated from *D. acutus* venom was loaded. Analyzed by Gel-pro Analyzer 3.0 software, the band of the main serine proteinase was at ~40 kDa, the same as that of acutobin (acutin), and its purity was about 97.7%.

After the affinity chromatography, the admixture of several serine proteinases is eluted from the column. For their high corresponding in structure and function, to isolate each proteinase, an ion-exchange chromatography on DEAE is necessary. Almost all the proteins isolated in this step showed arginine-ester and arginine-amide activities. Several serine proteinases in the venoms have been isolated, and the characterizations are still in process.

In SDS-PAGE analysis, because the proteinase band could be stained by Alcian Blue 8GX (not shown), a dye for carbohydrate, there should be glycan chains in the proteinase. Additionally, in MALDI-TOF-TOF-MS analysis, maybe for the glycans, the protein could not be ionized with normal matrixes/TFA (0.1%) then sinapinic acid (matrix usually used for glycoprotein) was used instead [32,33]. According to MALDI-TOF-TOF-MS analysis, the molecular mass of the purified proteinase should be ~34 kDa. In SDS-PAGE analysis, SDS could only bind with amino acid residues but not carbohydrates, thus the glycan chains prevent SDS away from their binding sites in the protein. Therefore, compared with normal proteins, the proteinase is with fewer electrons in electrophoresis and moves much more slowly on gel, which shows a higher molecular mass band. In the articles reported [7,9], the main part of serine proteinases of *D. acutus* venom were not applied to MALDI-TOF-TOF-MS analysis, however, on SDS-PAGE gel, they showed same molecular mass with the proteinase we purified. Additionally the anti-thrombotics drug "defibrase injection", identified as the major fibrinogen coagulating proteinase (acutin or acutobin) in *D. acutus* venom [7,9], showed almost a same band on SDS-PAGE gel as the proteinase we isolated (Fig. 6). Also, it revealed a similar peak as the isolated serine proteinase in RP-HPLC (Fig. 5C). And for the high recoveries of arginine esterase activity and Fg clotting activity, the serine proteinase purified from *D. acutus* venom with the affinity protocol should be the main serine proteinase in this venom identified as acutin (acutobin).

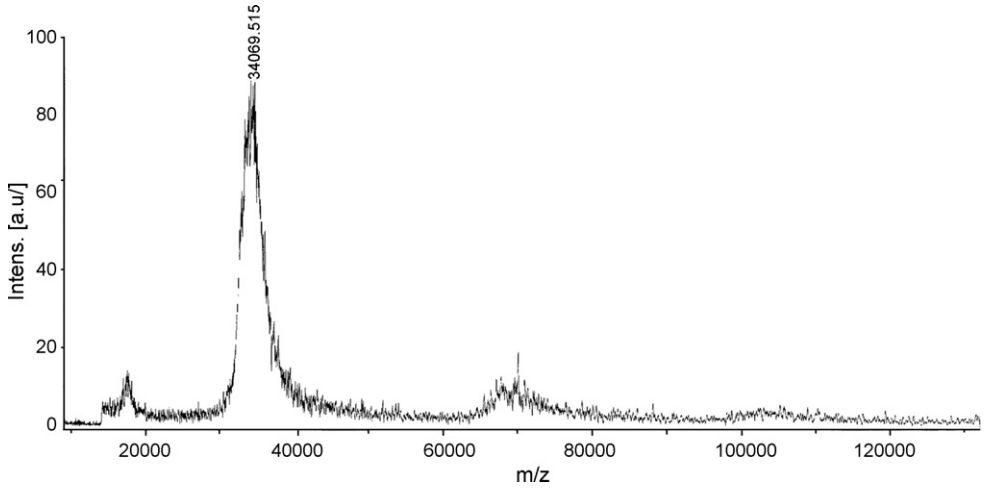

Fig. 7. MALDI-TOF-TOF-MS analysis. MALDI-TOF-TOF-MS analysis was performed using AutoFlex MALDI-TOF-TOF-MS (Bruker, Germany). The purified proteinases was mixed with matrix (saturated sinapinic acid in acetoritrile/H_2O, containing 0.1% TFA, 1:1, v/v) to promote desorption and ionisation. An N_2 laser at 337 nm was used to desorb the solute molecules from the sample disc and a voltage of 20 kV were established in the source region. The proteinase only showed a principal peak at ~34 kDa.

Based on the superiority of the affinity protocol, such as: less steps of chromatography, short time of the protein purification cycle, and higher purity, higher protein yield and higher activity recoveries of serine proteinase, industrial application of this method is of great potential.

Furthermore, different genera of snakes all over the world contain serine proteinases, with the similar arginine esterase active sites. The protocol could be used as the reference for the preparation of other genus of snake as well.

In experiments, some other serine proteinases, such as trypsin, prekallikrein, and other snake venom proteinases could be absorbed with the affinity medium.

Acknowledgements

The authors gratefully acknowledge the financial support by program for Natural Science Foundation of China (30660012) and National Basic Research Program of China (973 Program, 2007CB936004). The authors thank the Instrumental Analysis Center of Shanghai Jiao Tong University for supplying ESI-MS analysis, and we also thank Research Center for Proteome Analysis, Key Lab of proteomics, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Science, Chinese Academy of Science for supplying MALDI-TOF-TOF-MS analysis.

References

[1] F.S. Markland, Toxicon 36 (1998) 1749.
[2] K.F. Stocker, G.S. Bailey (Eds.), Enzymes from Snake Venom, Fort Collins, CO, Alaken, 1998, p. 705.
[3] H. Dekhil, A. Wisner, N. Marrakchi, M. Ayeb, E.C. Bon, H. Karoui, Biochemistry 42 (2003) 10609.
[4] M.W. Mosesson, J. Thromb. Haemost. 3 (8) (2005) 1894.
[5] J.W. Weisel, Adv. Protein Chem. 70 (2005) 247.
[6] P. Rojnuckarin, C. Muanpasitporn, L. Chanhome, J. Arpijuntarangkoon, T. Intragumtornchai, Toxicon 47 (3) (2006) 279.
[7] H. Pan, X.Y. Du, G.Z. Yang, Y. Zhou, X. Wu, Biochem. Biophys. Res. Commun. 255 (1999) 412.
[8] Q.Q. Huang, M.K. Teng, L.W. Liu, Toxicon 37 (1999) 999.
[9] Y.M. Wang, S.R. Wang, I.H. Tsai, Biochem. J. 354 (2001) 161.
[10] Y. Komori, T. Nikai, Y. Suzuki, H. Sugihara, J. Nat. Toxins 5 (1996) 375.
[11] A. Magalhaes, H.P.B. Magalhaes, M. Richardson, S. Gontijo, R.N. Ferreira, A.P. Almeida, E.F. Sanchez, Comp. Biochem. Physiol. A Mol. Integr. Physiol. 146 (4) (2007) 565.
[12] A.L.D. Araujo, J.L. Donato, C. Bon, Toxicon 36 (5) (1998) 745.
[13] A. Magalhaes, M.R. Montero, H.P.B. Magalhaes, M. Mares-Guia, E. Rogana, Toxicon 35 (10) (1997) 1549.
[14] E. Oyama, H. Takahashi, Toxicon 41 (2003) 559.
[15] S.G. De-Simone, C. Correa-Netto, O.C.A. Antunes, R.B. De-Alencastro, F.P. Silva Jr., J. Chromatogr. B 822 (2005) 1.
[16] I. Matsumoto, Y. Mizuno, N. Seno, J. Biochem. (Tokyo) 85 (4) (1979) 1091.
[17] R. Li, V. Dowd, D.J. Stewart, S.J. Burton, C.R. Lowe, Nat. Biotechnol. 16 (2) (1998) 190.
[18] N.E. Katsos, N.E. Labrou, Y.D. Clonis, J. Chromatogr. B 807 (2004) 277.
[19] R.J. Wallace, J. Kopecny, Appl. Environ. Microbiol. 45 (1) (1983) 212.
[20] F. Aragon-Qrtiz, F. Gubensek, Toxicon 31 (11) (1993) 1435.
[21] Y. Komori, R. Tatematsu, S. Tanida, T. Nikai, J. Nat. Toxins 10 (3) (2001) 239.
[22] K. Stocker, G.H. Barlow, Methods Enzymol. 45 (1976) 214.
[23] W.H. Holleman, L.J. Weiss, J. Biol. Chem. 251 (6) (1976) 1663.
[24] H. Banninger, B. Lammle, M. Furlan, Biochem. J. 298 (1994) 157.
[25] Y. Kong, Biophys. Chem. 81 (1) (1999) 7.
[26] Y. Tamura, S. Fujii, J. Biochem. (Tokyo) 80 (3) (1976) 507.
[27] T. Suzuki, H. Takahashi, Method Enzymol. 34 (1974) 432.
[28] H.M. Jiao, L.X. Yang, B. Lu, Y.Q. Wu, Y.C. Zhou, Acta Biochim. Biophys. Sin. (Shanghai) 37 (12) (2005) 835.
[29] P.L. Wang, S. SHirasu, M. Shinohara, M. Daito, T. Fujii, Y. Kowashi, K. Ohura, Eur. J. Oral Sci. 107 (2) (1999) 147.
[30] T. Abe, S. Ohtake, K. Tanaka, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 118 (1) (1997) 131.
[31] C.R. Lowe (Ed.), Y.X. Liu (translator), An Introduction to Affinity Chromatography, Science Press, Beijing, 1983, p. 38 (in Chinese).
[32] A.T. Kicman, M.C. Parkin, R.K. Iles, Mol. Cell. Endocrinol. 260–262 (2007) 212.
[33] P. Chaurand, R.M. Caprioli, Electrophoresis 23 (18) (2002) 3125.