

Short communication

## Purification of human tissue prokallikrein excreted from insect cells by liquid chromatography

Qing Yang<sup>a</sup>, Zhen Liang<sup>a</sup>, Lihua Zhang<sup>a</sup>, Xiaodan Zhang<sup>a</sup>,  
Jiuyang Zhao<sup>b</sup>, Weibing Zhang<sup>a</sup>, Yukui Zhang<sup>a,\*</sup>

<sup>a</sup> National Chromatographic Research & Analysis Center, Dalian Institute of Chemical Physics,  
Chinese Academy of Sciences, 457 Zhongshan Road, Dalian 116023, China

<sup>b</sup> Nephrology Department, Dalian Medical University, Dalian 116023, China

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### Abstract

Tissue kallikrein, generally existing in living bodies as prokallikrein, is a serine proteinase that has proven of great significance to treat hypertension, cardiopathy and nephropathy. Although the extraction of tissue kallikrein from human urine is the most commonly used method to obtain such a protein, not only the yield is very little, but also the procedure is rather complex. Furthermore, the biological safety is uncertain. Therefore, the preparation of such a protein by genetic engineering method, including gene expression, cell culture, separation and purification, is very important. In this paper, a new method to obtain purified tissue prokallikrein excreted from insect cells by liquid chromatography has been proposed. In contrast to the previously published papers, the purification procedure is simplified to only three steps with the final yield of 57% and the purity of 95%, which is not only convenient, but also low-cost and suitable for the large-scale preparation of such a protein. The purified protein is further validated as prokallikrein by high performance liquid chromatography-mass spectrometry and amino acid sequencing.

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### 1. Introduction

Tissue kallikrein, commonly existing in living bodies as prokallikrein, belongs to a closely related subfamily of trypsin-like glycoproteins that has been found in pancreas, colon, kidney, urine and saliva, etc. [1–3]. Up till now, kallikrein has been proven to have many biological functions, such as attenuating myocardial infarction, inhibiting apoptosis, and renal protection and repairing [4–6]. Therefore, it has been regarded as a potent medicine in clinical treatment.

With the consideration of medicament safety, the separation and purification of kallikrein is of great significance. Up till now, the extraction of kallikrein from human urine is the most commonly used method. However, the quantity of

such a protein in urine is quite little. Generally, only 17 mg protein could be obtained from 1000 L urine. Furthermore, the extraction procedure is very complex, and the obtained protein might be suspected of hepatitis and even AIDS virus. In addition, kallikrein extracted from the pancreas of animals is another source. Nevertheless, the difference in kallikrein structure between animals and humans decreases the curative effects of kallikrein. In recent years, the rapid development of biotechniques brings new solution to obtain kallikrein safely, effectively and conveniently.

In our recent work, we have successfully constructed virus with human tissue prokallikrein gene, and carried out the following amplification and expression in insect cells. Subsequently, a liquid chromatography based three-step method for the purification of prokallikrein has been proposed. The prokallikrein purified by this method could be further activated to kallikrein by plasmin, themolysin and trypsin before

\* Corresponding author. Tel.: +86 411 84379560; fax: +86 411 84379560.  
E-mail address: [ykzhang1@online.ln.cn](mailto:ykzhang1@online.ln.cn) (Y. Zhang).

usage [7,8]. The purified product is further validated by liquid chromatography–mass spectrometry (LC–MS) and amino acid sequencing. Such a method has shown advantages of low-cost, simple and high yields, which is quite suitable for large-scale production of prokallikrein.

## 2. Materials and methods

### 2.1. Materials

Chromatography matrices, DEAE-Sepharose FF, Phenyl-Sepharose 6B and Sephacryl S-200 were purchased from Amersham Pharmacia Biotech AB (Uppsala, Sweden), and were packed in the columns of 26 mm × 40 cm, 26 mm × 20 cm and 26 mm × 80 cm, respectively, in our laboratory. Centriprep Plus-80 (10 kDa cut off) was obtained from Millipore Company (Billerica, MA, USA). Fetal bovine serum, peroxidase-avidin, Tween-20 and 2-mercaptoethanol were brought from Sigma Company (St. Louis, MO, USA). HyQ CCM<sub>3</sub> medium was purchased from Hyclone Company (Logan, UT, USA). PEG20,000, formic acid solution and acetic acid were from Beijing Chemical Reagent Company (Beijing, China). TMB was obtained from Pierce Company (Rockford, IL, USA). Polyclonal anti-HUK antibodies (HUK-IgG) was kindly offered by Prof. L. Chao (Medical University of South Carolina, USA). Water used in experiments was purified with a Milli-Q system (Millipore, Molsheim, France). All other chemicals and solvents were of analytical or HPLC grade.

### 2.2. Instruments

CO<sub>2</sub> incubator and Megafuge 1.0R centrifuge were bought from Kendro Laboratory Products (Hanau, Germany). Mini-plus 3 auto-control pump was obtained from Gilson company (Villiers-le-Bel, France). An HPLC system (P200 II) with UV detector (UV200 II) was purchased from Dalian Elite Scientific Instruments Company (Dalian, China). Mini Protein 3 Cell Gel system was bought from Bio-Rad Laboratories (Hercules, CA, USA). DYY-2 Electrophoresis instrument was from Beijing Liuyi Instruments Company (Beijing, China). ELISA plate reader (Multiskan Mk3) and well washer (Wellwash 4 Mk2) were purchased from Thermo Company (Vantaa, Finland). LCQ<sup>DOU</sup> mass spectrometer was from Finnigan (San Jose, CA, USA). Total ion chromatograms and mass spectra were recorded on a personal computer with the Xcalibur software Version 1.2 (Thermo Finnigan).

### 2.3. Amplification of recombinant baculovirus

Sf-9 cells were cultivated in a spinner flask (250 mL). After reaching a density of 10<sup>7</sup> cells/mL, the cells were infected with recombinant baculovirus at a rate of 0.1 plaque forming units/cell. After three days, the cell super-

natant was harvested by centrifugation at 2000 × g for 20 min. The infectious potency of recombinant baculovirus was determined by virus plaque assay. This procedure was repeated until the titer of recombinant baculovirus reached 10<sup>8</sup> pfu/mL. The supernatant was stored at 4 °C for further use.

### 2.4. Expression of recombinant human prokallikrein

Hi-5 cells were cultivated in a spinner flask (500 mL) to reach a density of 3 × 10<sup>6</sup> to 5 × 10<sup>6</sup> cells/mL. Infection with recombinant virus occurred at a rate of 10 plaque forming units/cell. After four days' infection, the supernatant was harvested by centrifugation at 2000 × g for 20 min, and the supernatant was stored at 4 °C for further use.

### 2.5. SDS-PAGE experiments

SDS-PAGE was performed on 12% (w/v) polyacrylamide slab gels under reducing conditions, using discontinuous Tris–glycine buffer system. Samples were mixed at a ratio of 5:1 (v/v) with sample buffer (60 mM Tris–HCl, pH 6.8) containing 20% glycerol, 2% SDS, 5% 2-mercaptoethanol and 0.05% bromophenol blue. Fifteen microliters of each sample was loaded into each well, and run at 160 V in accumulate gel and 200 V in separation gel. Proteins were visualized by Coomassie blue in acetic acid/ethanol/water (1:45:45, v/v/v), and destained in acetic acid/ethanol/water (15:10:175, v/v/v).

### 2.6. ELISA experiments

In this assay, microtiter plates (Nunc-Immuno Plate, Maxisorp, Nunc Inc., Naperville, IL, USA) were coated over night with 100 μL/well of 1 μg/mL polyclonal HUK IgG in PBS at 4 °C. The plates were washed three times with 200 μL of PBS containing 0.1% Tween-20 and blocked with 200 μL 10% BSA solution in PBS for 2 h. One hundred microliters of each standard HUK and test sample at appropriate dilution was added to triplicate wells and incubated for 2 h. Then, 100 μL Biotin-HUK IgG was added to each well and the plate was incubated for 1 h. The plates were further incubated with peroxidase-avidin for 30 min. After washing the plate, a substrate solution containing 0.01% TMB in 0.1 M citrate buffer, pH 5.0, with 0.03% H<sub>2</sub>O<sub>2</sub> was added to each well. The reaction was stopped by adding 100 μL of 1N H<sub>2</sub>SO<sub>4</sub>, and the intensity of color was measured at 450 nm in an ELISA plate reader. The assay was calibrated with purified HUK and a linear correlation was obtained in the range of 0.4–25 ng/mL HUK.

### 2.7. HPLC–MS experiments

HPLC was directly coupled to mass spectrometry without stream splitting. The mobile phase consisted of (A) acetonitrile/water (95:5, v/v) containing 0.6% (v/v) formic acid

and (B) acetonitrile/water (5:95, v/v) containing 0.6% (v/v) acetic acid, using a linear gradient of 26–30% (v/v) A in 5 min and then 30–70% (v/v) A in 15 min. The flow rate was 0.25 mL/min. The ESI–MS was performed on a Finnigan LCQ<sup>DUO</sup> Ion Trap Mass Spectrometer (Finnigan MAT, San Jose, CA, USA). The mass spectrometer was equipped with an electrospray ion source. Online ESI–MS was performed in the positive-ion full-scan mode and selected-ion-monitoring scan mode. Spray voltage of 4.5 kV was employed. The temperature of the heated transfer capillary was set to 230 °C. The flow rates of sheath gas and auxiliary gas were, respectively, set at 40 and 10 mL/min. Data were recorded on a Xcalibur workstation with Core data system software, Version 1.2 (Finnigan).

### 3. Result and discussion

In our experiments, a three-step liquid chromatography based purification method was developed. In each step, the existence of our target protein was confirmed by gel electrophoresis.

#### 3.1. Purification procedure for rHUK

In the first step, DEAE-Sepharose FF column was equilibrated with 10 mM Tris–HCl–0.15 M NaCl (pH 7.2) buffer. After Hi-5 cell supernatant (250 mL) was loaded, the column was washed with the same buffer, and the eluate was monitored at UV 280 nm. When the absorbance was less than 0.05 mV, stepwise elution with 10 mM Tris–HCl (pH 7.2) containing 0.3 M NaCl was used, and the eluent was collected (as shown in Fig. 1A). Later on, the column was regenerated by the same buffer with 2 M NaCl.

Since prokallikrein has a rather big hydrophobic group, in the second step, Phenyl-Sepharose 6B column was selected. With NaCl added, the final concentration of NaCl in the fraction collected from the first step was increased to 3 M. Then, the sample was loaded on the column, which had been equilibrated with a buffer of 10 mM Tris–HCl–3 M NaCl (pH 7.2). When  $A_{280}$  of the eluate was less than 0.05, a linear gradient elution with 10 mM Tris–HCl containing 3 M to that with 0 M NaCl was carried out in 60 min at the flow rate of 2.2 mL/min, as shown in Fig. 1B. By gel electrophoresis experiments, the asterisked peak was found of high concentration prokallikrein, and collected for the further purification.

In the third step, the fraction collected from step 2 was put into the dialysis bag with molecular weight cut off 10,000, which was suspended in 5 mM phosphate buffer (pH 7.2) containing 750 mM NaCl at 4 °C overnight. Then, it was put into PEG20,000 for the further concentration of proteins. Finally, the concentrated sample solution was loaded onto a Sephacryl S-200 column, which had been equilibrated with 5 mM sodium phosphate (pH 7.2) containing 750 mM NaCl. After loading, the column was washed

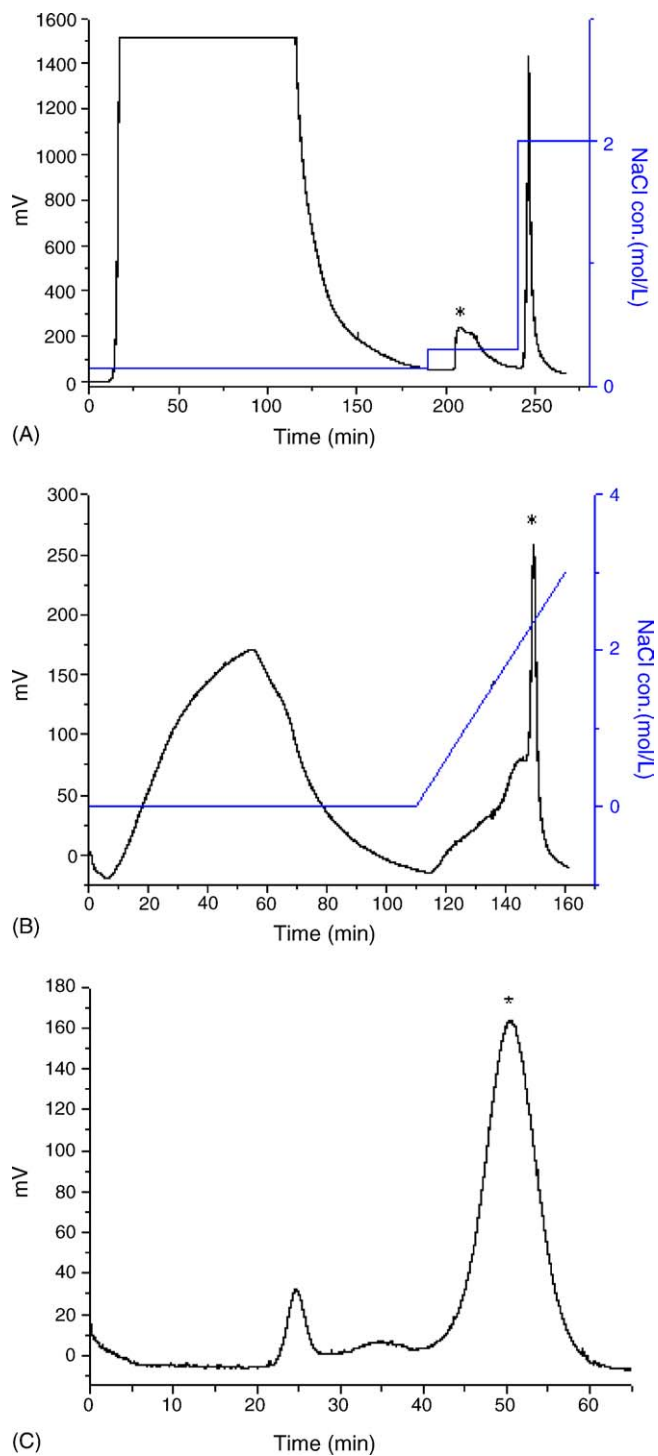


Fig. 1. Results of three-step purification procedure based on liquid chromatography on: (A) DEAE-Sepharose FF column; (B) Phenyl-Sepharose 6B column; (C) Sephacryl S-200 column. Other detailed experimental conditions as shown in Section 3.1. The asterisked peak is the target protein.

with the same buffer, and the eluent was collected, which was further concentrated by superfiltration with Centriprep Plus-80 (10 kDa cut off). After the buffer was changed to 50 mM PBS (pH 7.2), it was stored at –20 °C for the further usage.

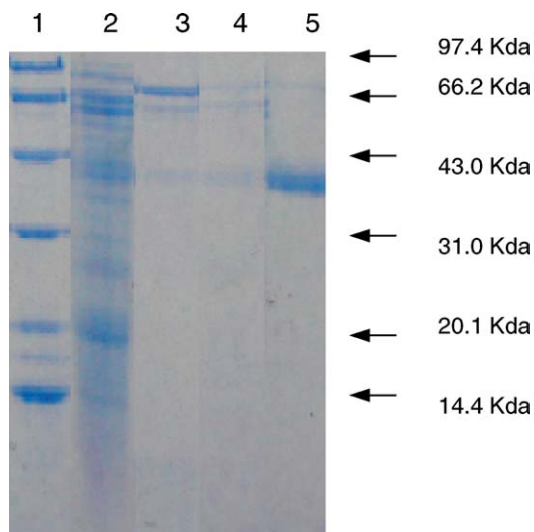


Fig. 2. Detection of recombinant human kallikrein after each purification step by SDS-PAGE. Lane 1: protein marker; lane 2: supernatant of Hi-5 cell culture; lane 3: fraction collected from DEAE-Sepharose FF column; lane 4: fraction collected from Phenyl-Sepharose 6B column; lane 5: recombinant human prokallikrein after Sephacryl S-200 column. Experimental conditions as shown in Section 2.5.

### 3.2. Characterization of the purified protein

In our experiment, since cells were maintained in HyQ CCM3 medium without fetal bovine serum, the chromatography based purification procedure could be simplified. The whole purification procedure took about three days, and gel electrophoresis was used to confirm the existence of prokallikrein in the collected fraction of each step, as shown in Fig. 2. From lane 2, we could see that the culture medium of Hi-5 cells contained recombinant human prokallikrein. By ion-exchange chromatography in the first step, most other proteins could be separated from prokallikrein (lane 3), and the recovery of recombinant prokallikrein in this step was found to be above 97% by ELISA method. Since prokallikrein has a large hydrophobic group, such a protein could be purified (lane 4) by hydrophobic chromatography in the second step. In the last step, by using Sephacryl S-200 column, the collected fractions could be further purified. After the whole procedure of three-step purification, high purity prokallikrein could be obtained with recovery about 57% (lane 5) (about 10 mg/L culture medium). Compared with other purification method, this method gives higher recovery by a simpler process [9,10]. It is not only convenient, but also low-cost, suitable for the large-scale preparation of such a protein. The purified recombinant prokallikrein could be kept for a long time at  $-20^{\circ}\text{C}$ .

Furthermore, HPLC and MS were coupled to obtain more detailed data for our purified protein, and the result is shown in Fig. 3A. The peak appeared at 1.16 min should be due to impurity, and so is peak 1 eluted at about 13 min. For peak 2, its mass spectrogram is shown in Fig. 3B. By the software of Biomass, the molecular weight is calcu-

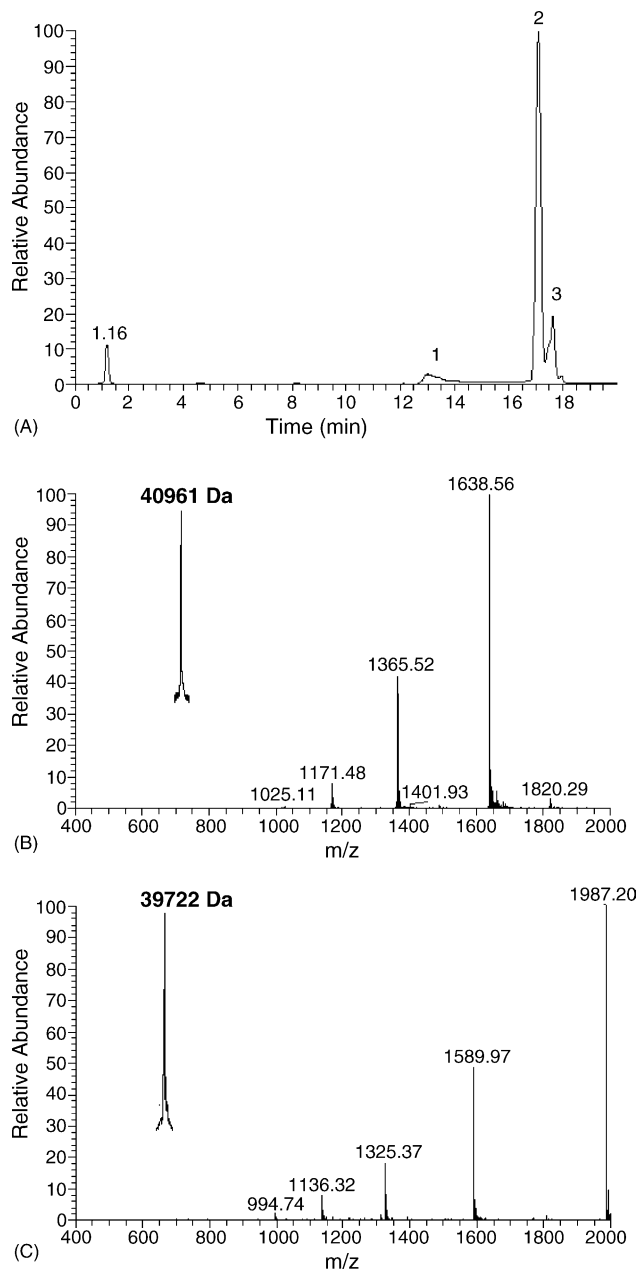


Fig. 3. Analysis of purified protein by HPLC–MS: (A) full-scan mass spectra; (B) identification of peak 2; (C) identification of peak 3. Experimental conditions as shown in Section 2.7.

lated to be 40,961 Da. For peak 3, based on the spectrogram shown in Fig. 3C, the molecular weight should be 39,722 Da. Therefore, we can deduce that these two peaks might, respectively, be the inactive and active human tissue kallikrein. Totally, the purity of our purified protein by three-step LC method is 95%, which is satisfactory for practical applications.

The purified protein was sent for the analysis of amino acid content and N-terminal amino acid sequence. The total amino acid content is illustrated in Table 1. The first 15 N-terminal amino acid sequence is

Table 1  
Total amino acid content of recombinant human tissue prokallikrein

Amino acids	No. of residues	Theoretical
Asx	24.2	26
Glx	28.7	28
Cys (Cm)	9.7	10
Ser	13.5	16
Gly	19.6	19
His	10.7	11
Thr	12.7	14
Ala	12.8	12
Pro	13.6	13
Arg	5.4	5
Tyr	5.1	5
Val	20.9	21
Met	3.3	4
1/2 Cys	10.0	10
Ile	8.2	8
Leu	22.2	22
Lys	8.4	8
Trp	7.7	8
Total		238

I–V–G–G–W–E–C–E–Q–H–S–Q–P–W–Q, which demonstrates that the purified protein is human tissue kallikrein. Through its further application in the model rats, the results show the safety and effectiveness of recombinant human prokallikrein purified by our proposed method [11]. The

present method can be easily modified, and might be a good choice in large-scale production of karrikrein.

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