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ISOLATION AND PURIFICATION OF A KRINGLE 5 FROM HUMAN PLASMINOGEN USING AH-SEPHAROSE

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Our aim was to develop a method for isolation of human plasminogen kringle 5 possessing functional activity. The proposed method includes the following steps: hydrolysis of plasminogen with elastase, separation of mini-plasminogen from kringle fragments 1–3 and 4 on Lys-Sepharose, mini-plasminogen hydrolysis with pepsin, affinity chromatography on AH-Sepharose and polyacrilamide gel electrophoresis.

We obtained the electrophoretically pure fragment of human plasminogen kringle 5 showing functional activity towards the ligands with high and low molecular mass. Weight yield was 3.8% that corresponds to 25.3% of the theoretically possible.

It was established that affinity chromatography on AH-Sepharose was the sufficient step to isolate kringle 5 from mini-plasminogen hydrolysate with pepsin. This approach does not require additional purification steps while the ability of kringle 5 to bind specifically to AH-Sepharose demonstrates the functional activity of the kringle.

Key words: plasminogen, fragments of plasminogen, kringle 5, angiostatins.

Plasminogen (Pg)/plasmin (fibrinilytic) system is responsible for fibrin clot dissolution and involved into cell migration, angiogenesis, inflammation, commissure formation, pathogen invasiveness, etc. There are a number of enzymes in the organism which lead to the PG/plasmin degradation and as a result kringle-containing fragments are formed. These fragments are known as angiostatins because of their angiogenic effects [1, 2]. Angiogenesis inhibition is used as a therapeutic approach for the treatment of the cancer patients and patients with retina neovascularization, in particular in case of diabetic and sickle cell retinopathy [3]. The effect of kringle domains (K1–3, K2–3, K1–4, K1–3 and K5) on the endothelial cell proliferation was also studied. It is shown that K5 possesses the highest inhibitory activity [4]. Antiproliferative effect of K5 is in several times higher as compared with that one of K 1–4 (IC_{50} was 40 nM and 160 nM respectively) or any individual kringle domains. It has been suggested that different kringle domains may implement their effects through various mechanisms. Thus, there is a specific receptor for kringle 5 on the surface of human endothelial cells, the voltage-depended anion channel (VDAC 1) [5], while ATP-synthase (3.6.3.14) and integrin αVβ3 can be considered as the receptors for other angiostatins [6].

The effects of K5 are not limited to the process of inhibition of endothelial cell proliferation. Thus, recombinant K5 induces apoptosis of endothelial and tumor cells [7]. As it is shown on glioma cells the expression of kringle 5 inhibits the migration of tumor-associated macrophages and suppresses tumor vascularization and progression [8]. The investigation carried out on Muller's retinal cells shows that imbalance levels of K5 and vascular endothelial growth factor may be the cause of vascularization of the retina in case of diabetic retinopathy [9].

It is important to note that K5 affects only proliferating endothelial cells and therefore is non-toxic towards other cell types, including normal endothelial cells [10]. Furthermore, Pg is an endogenous protein of human tissue and therefore K5 doesn't cause an immune

response. Being a polypeptide with a small molecular weight and a stable protein as well, kringle 5 can be used in pharmacokinetic system when the agent has to be targeted and slowly released. The prospect of K5 successful usage as a therapeutic agent in case of tumor growth and angiogenesis disorder will require the development of the methods for K5 isolation from preparations of human plasma Pg.

Our aim was to develop a method of K5 isolation from a by-product of the human plasma fractionation process.

Materials and Methods

Plasminogen was purified in a form of Lys-Pg (here and after Pg) from Cohn fraction $III_{2,3}$ paste (supplied by Kyiv municipal blood centre, Ukraine). The paste was loaded onto Lys-Sepharose in the presence of kontrikal (AWD, Germany) as it was described in [11]. Mini-Pg was obtained by the limited hydrolysis of Pg with pancreatic elastase (3.4.21.36) (Sigma, USA) according to [12]. Degradation of mini-Pg was performed by pepsin (3.4.23.1), the proteolytic hydrolase from pig gastric mucosa (Sigma, USA). All used chemical reagents were qualified not less than "chemical pure".

Gel filtration

To separate fragments obtained after proteolytic degradation of Pg we used TOYOPEARL HW-50 Fine Grade (TOYOSODA Manufacturing Co., Ltd, Japan).

Affinity chromatography

Plasminogen fragments containing Lys-binding sites obtained after zymogen proteolytic degradation were isolated and purified by affinity chromatography on Lys-Sepharose. L-Lysine coupling to CNBr-activated Sepharose 4B (Amersham Biosciences, Sweden) was performed according to [13].

We used affinity chromatography on aminohexyl (AH)-sepharose (Amersham Biosciences, Sweden) to isolate K5 fragment from mini-Pg hydrolysate with pepsin.

Ion-exchange chromatography

To isolate K5 fragment from mini-Pg hydrolysate with pepsin ion-exchange chromatography on DEAE-Sephadex A-25 (Amersham Biosciences, Sweden) was used. All chromatografic procedures were carried out at 4 °C.

Ultrafiltration

The dialysis of protein solutions and their simultaneous concentrating were carried out by ultrafiltration on membranes with the nominal molecular weight limit (NMWL) of 3,10 and 30 kDA using Ultrafree –15 micro-concentrators (Millipore, USA) at 4 °C.

Determination of protein concentration

The protein concentration was determined as the difference of the solution optical density at 280 nm and 320 nm. To calculate protein concentration we used the following values of the extinction coefficient (E_{280}, 1%, 1 cm) and molecular mass respectively: Pg (Lys-form)-17 and 84 kDa [14], mini-Pg-14 and 38 kDa, kringle 1-3-18.6 and 32 kDa, kringle 4–25 and 12 kDa [12], kringle 5–12 and 14 kDa, the proteinase domain-15 and 27 kDa calculated in accordance with [15].

Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS)

The purity of the obtained proteins was assessed by PAGE (10% gel) with 0.1% SDS [16]. We used as an anode buffer 0.2 M Tris-HCl (pH 8.9), as a catode buffer 0.1 M Tris (pH 8.25), 0.1 M tricine (Sigma, USA), 0.1% SDS, as a gel buffer 2 M Tris-HCl (pH 8.45), 0.1% SDS. Tris was used as Trizma® base (Sigma, USA). Acrylamide and bis-acrylamide were used as 30% solution of Acryl/Bis in the ratio 37.5:1 (Sigma, USA).

The study was carried out in the apparatus Mini-PROTEAN-II, (Bio Rad, USA), glass plates 10×10 cm with 0.75 mm spacers. Resolution of the method is 1 mg of the protein per zone. Coomassi brilliant blue R-250 (Sigma, USA) staining was used to visualize the protein bands on the gel. As the standard proteins we used Spectra Multicolor Broad Range Protein Ladder, 10×260 kDa (Fermentas, Lithuania).

Statistical analysis

Data were analysed with the use of Prism 5 (Graph Pad Software Inc., USA). The permissible error did not exceed 5% ($P < 0.05$). The presented curves are typical for a set of repeated experiments (at least three repeats for each experiment). The presented electrophoretic patterns are also typical (at least three replicates for each experiment).

Results and Discussion

The native human Pg is a single chain 92 kDa glycoprotein containing 2% of carbohydrates [17]. Pg molecule consists of the NH2-terminal domain, five kringle domains and the proteinase domain. Mini-Pg is able to bind fibrin but it hasn't the Pg ability to bind Lys or Lys analogs [18]. After activating mini-Pg shows its catalytic activity [19].During elastolysis Pg is cleaved with the formation

of the following fragments: the NH_2-terminal fragment, K 1–3, K 4 and mini-Pg (Val 442-Pg) which contains K5 and proteinase domain [12].

The electrophoretic patterns of the plasminogen fragments obtained after elastase digestion are presented at Fig.1. The elastase hydrolysis was carried out for 5 hours under the following conditions: 10 mg/ml Pg, elastase (enzyme/substrate ratio 1:50 w/w) and 0.05 M tris-HCl (pH 8.5) with 0.1 M NaCl, at 25 °C. It was found that during elastase digestion the maximum quantity of K1–3, K4 and mini-Pg was observed after 5 hour incubation. Preparative hydrolysis was carried out under the same conditions. The reaction was stopped by adding to the incubation medium *p*-nitrophenylguanidinebenzoate (final concentration 1×10^{-4} M). Excess of the inhibitor was removed by overnight dialysis against hydrolysis buffer.

The hydrolysate components were separated on the hydrophilic vinyl polymer TOYOPEARL HW-50. We obtained the mixture of mini-Pg and K1-3 and the fractions containing K4 (Fig. 2, *A*). The mixture was loaded onto the column with Lys-Sepharose. As mini-Pg doesn't have ability to bind Lys, this fragment was eluted in the outer volume of the column (V_0), while K1-3 was retained on the column (Fig. 2, *B*) and was eluted with 0.15 M ε-aminocaproic acid (ε-ACA) in 0.05 M Na-phosphate buffer (pH 7.4).

The obtained fragments were dialysed against 0.05 M Tris-HCl buffer (pH 8.5) with 0.1 M NaCl and simultaneously concentrated on ultrafiltration membranes with NMWL of 3 kDa. The purity of the obtained fragments was tested by SDS PAGE (Fig. 3).

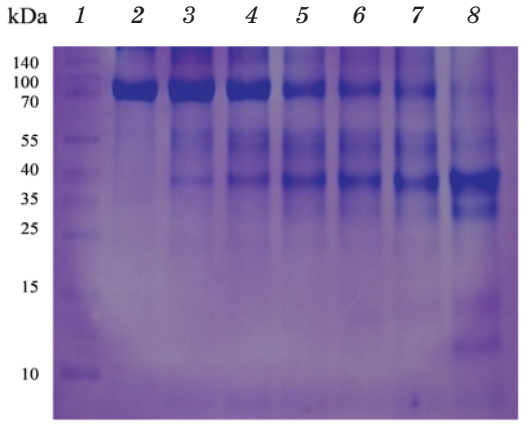

Fig. 1. **SDS-PAGE of a timed digest of human Pg incubated with elastase. For lines 3–8 the time of digestion in hours is given:** *1* — protein standards; *2* — Pg (Lys-form); *3* — 0.5 hr; *4* — 1 hr; *5* — 2 hrs; *6* — 3 hrs; *7* — 4 hrs; *8* — 5 hrs

Alternatively, we carried out the separation of hydrolysate fragments by another way. At first, we performed the chromatography on Lys-Sepharose as previously described. As a result two protein peaks were obtained: the first one contained mini-Pg and the peptides formed by the discrete action of the enzyme, the other one contained kringles 1–3 and kringle 4 which have affinity to Lys (Fig. 4). The fractions containing mini-Pg and the mixture of K1–3 and K4 were separately subjected to ultrafiltration on membranes (NMWL of 30kDa) for dialysis against 0.05 M Tris-HCl buffer (pH 8.5) with 0.1 M NaCl and concentrated.

In the first case mini-Pg (38 kDa) was obtained without any additional peptides, in the second one we got K 1-3(35.5–37.5 kDa) and K4 separately.The purity of the obtained fragments was assessed by SDS PAGE (Fig. 3). We founded that this method was more convenient as we excluded the gel-filtration step and the quality

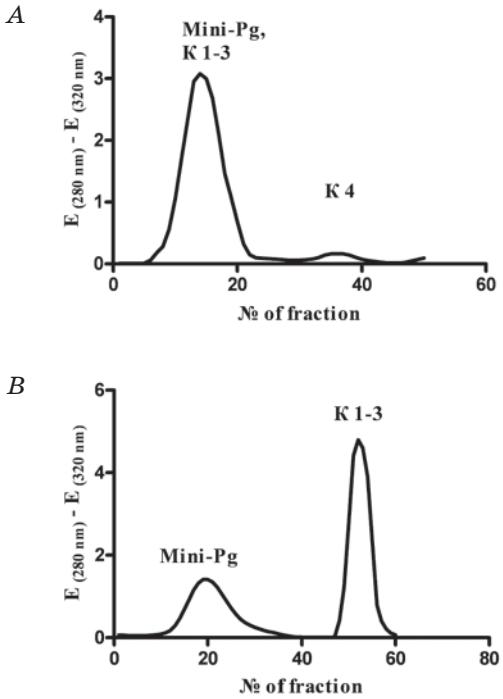

Fig. 2. A — The averaged (n = 3) elution profile on Toyopearl HW-50 of Pg elastase hydrolysate. Column (2×65 cm) was equilibrated with 0.05 M Na-phosphate buffer (pH 8.5) with 0.1 M NaCl, elution rate of 54 ml/hr, fractions volume of 3.8 ml.

B — The averaged (n = 3) elution profile on Lys-Sepharose of mini-Pg and K 1–3 mixture. Column (1.7×25 cm) was equilibrated with 0.05 M Na-phosphate buffer (pH 7.4), elution rate of 30 ml/hr, fractions volume of 2 ml. The vertical axis represents the difference of values of the optical density of the solutions indicated for undiluted fractions were diluted as necessary to their performance not higher than 0.4 at λ = 280 nm

of the protein preparations wasn't lost. The exclusion of the separate dialysis step can also be considered as a positive factor as in this case the yield was increased.

According to [20] pepsin digestion of mini-Pg leads to formation of two unequal fragments with molecular mass 27 and 12 kDa. These fragments have more or less cooperative structure and possess the resistance towards further pepsin proteolysis. The separation of these fragments on mono Q column by FLPC shows that 12 kDa fragment doesn't bind to an anion exchanger, while the bigger fragment (27 kDa) binds to this sorbent. Besides, the bigger fragment reveals proteolytic activity after its activation by streptokinase. The authors made a conclusion that 12 kDa fragment corresponds to K5, while the bigger fragment is the proteinase domain.

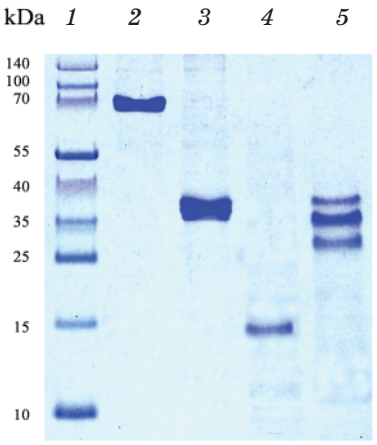

Fig. 3. **SDS-PAGE of Pg and its fragments:** *1* — protein standards; *2* — Pg (Lys-form); *3* — mini-Pg; *4* — kringle K4; *5* — kringle K1–3

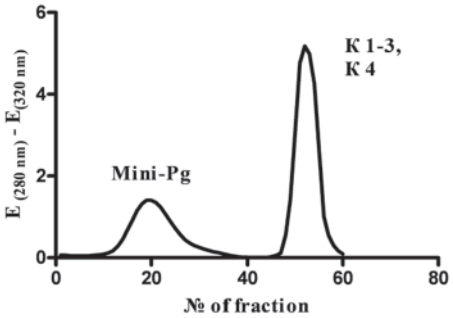

Fig. 4. **The averaged (n = 3) elution profile on Lys-Sepharose of Pg elastase hydrolysate. Column (1.7–25 cm) was equilibrated with 0.05 M Na-phosphate buffer (pH 7.4), elution rate of 30 ml/hr, fractions volume of 2 ml.** The vertical axis represents the difference of values of the optical density of the solutions indicated for undiluted fractions were diluted as necessary to their performance not higher than 0.4 at λ = 280 nm

We performed the limited proteolysis of mini-Pg with pepsin (3.4.23.1). The steps of the proteolysis were controlled by PAGE. Mini-Pg (9.0 mg/ml) was incubated with pepsin in 0.05 M glycine buffer (pH 2.5) at 27 °C (enzyme/substrate ratio 1:200 w/w). At the regular time intervals the reaction was stopped by pH changing in the medium (till weak alcaline value). The result of successive mini-Pg digestion was seen on the electrophoretic patterns: 27 kDa fragment and a doublet band with molecular mass around 15 kDa. We also noted the appearance of 10 kDa fragment when mini-Pg was not completely hydrolysed. Further hydrolysis was accompanied by the complete degradation of 27 kDa fragment. As a result the fragment with molecular mass around 10 kDa was accumulated while the above-mentioned 15 kDa polypeptide was resistant towards pepsin action (Fig. 5).

Taking into consideration that K5 has a compact structure with the distinct globular type of hydrophobic core and reveals the resistance towards proteolytic hydrolysis we suggested that 15 kDa fragments in our experiments corresponded to K5 and the observed peptides with low molecular mass are the fragments of the proteinase domain. Our suggestion is in accordance with the earlier published data [4, 21].

K5 isolated by Sao et al. [4] from mini-Pg hydrolysate with pepsin has molecular mass about 15 kDa. Amino acid analysis of the NH_2-terminal region of K5 revealed two elastase cleavage sites located between Val 448 and Val 449, and between Leu 451 and Pro 452, and

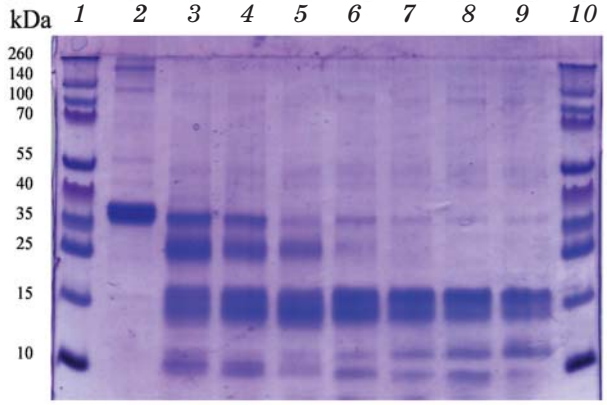

Fig. 5. **SDS-PAGE of a timed digest of mini-Pg incubated with pepsin. For lines 3–9 the time of digestion in hours is given:** *1* — protein standards; *2* — mini-Pg; *3* — 0.5 hr; *4* —1 hr; *5* — 1.5 hrs; *6* — 2 hrs; *7* — 2.5 hrs; *8* — 3 hrs; *9* — 3.5 hrs; 10 — protein standards

pepsin cleavage site at the position between Phe 547 and Asp 548 (Fig. 6).

According to the result of the electrophoresis K5 is shown as two bands with molecular mass of 14.9 and 15.7 kDa correspondingly K5 Pro 452-Phe 547 and K5 Val 449-Phe 547. The K5 structure is confirmed by 1H-NMR spectroscopy [4, 21]. To isolate and purify K5 the gel-filtration and ion-exchange chromatography were used [4, 20 and 21].

Investigations of Pg ligand specificity found that K5 may be involved in zymogen interaction with benzamidine-[22], homoarginine-[23] and AH-Sepharose [24] because this fragment reveals affinity to the positively charged groups located at the end of the alkyl chain[25,26]. Therefore, for further separation of the fragments obtained after 3 hours of mini-Pg hydrolysis with pepsin (in accordance with the data presented at Fig.5) the ion-exchange chromatography on DEAE-Sephadex A-25 and affinity chromatography on AH-Sepharose were used.

The column with DEAE-Sephadex A-25 was equilibrated with 0.05M Tris-HCl buffer (pH 8.5). After hydrolysate application the column was consecutively washed with the working buffer and the working buffer with 0.3M NaCl until absorbance at 280 nm for collected fractions was close to baseline (E_{280nm} 0.01).

Protein fractions obtained during the washing (outer volume of the column) with the working buffer and the buffer containing 0.3 M NaCl were separately collected as two peaks respectively (Fig. 7, *A*). These fractions were dialysed against 0.05 M Tris-HCl buffer (pH 8.5) and concentrated using ultrafiltration membranes with NMWL of 3 kDa. To analyse the protein composition of the fractions SDS PAGE was used (Fig. 8).

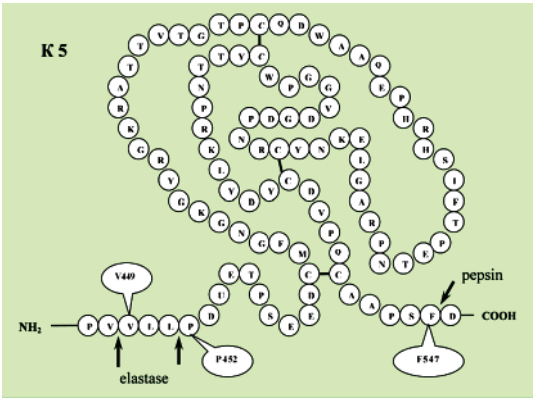

Fig. 6. **Schematic structure of human Pg kringle 5, the places of elastase and pepsin attacks are indicated by relevant arrows, reproduced similarly to the scheme in [4]**

The fraction which doesn't bind to anion exchanger contains a 15 kDa fragment. This fragment probably corresponds to K5 as it has total positive charge. Another fragment which was eluted in the outer volume has molecular mass below 10 kDa (Fig. 8, track *5*). The fraction eluted from the column with 0.3 M NaCl contains other 10 kDa fragments (Fig. 8, track *4*). These polypeptides possess negative charge as they are bound to the column at pH 8.5. We can suggest that these peptide fragments derived from mini-Pg proteinase domain, as it is known that the isoelectric point of the plasmin light chain is at pH range 5.8–6.4 [14]. So, at pH 8.5 the net charge on these fragments is negative. Moreover, pepsin, which was also present in the reaction medium, was able to bind to DEAE-Sephadex A-25 [27] but this enzyme was not detected in the gel due to the small amount of the sample. Further purification of 15 kDa fragment was

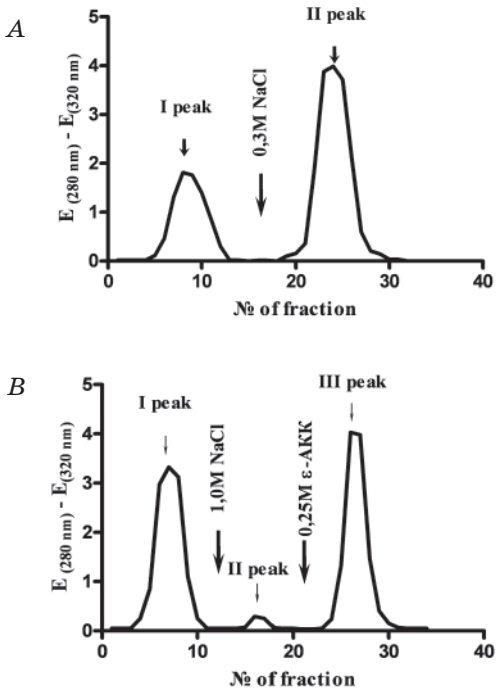

Fig. 7. **The averaged (n = 3) elution profiles of the human mini-Pg pepsin hydrolysate:**
A — DEAE Sephadex A-25, column (1.7–7.4 cm) was equilibrated with 0.05 M Tris-HCl buffer (pH 8.5), elution rate of 30 ml/hr, fractions volume of 1.4 ml; *B* — AH-Sepharose, column (1.4–6.2 cm) was equilibrated with 0.1 M Na-phosphate buffer (pH 7.4) with 0.2 M NaCl, elution rate of 30 ml/hr, fractions volume of 1.2 ml. The vertical axis represents the difference of values of the optical density of the solutions indicated for undiluted fractions were diluted as necessary to their performance not higher than 0.4 at λ = 280 nm

carried out on the ultrafiltration membrane with NMWL of 10 kDa. This step was a suitable replace of gel filtration.

The AH-Sepharose column was equilibrated with 0.1M Na-phosphate buffer (pH 7.4) containing 0.2 M NaCl. After hydrolysate application the column was successively washed with the mentioned buffer and with 0.1 M Na-phosphate buffer (pH 7.4) containing 1.0 M NaCl to elute non-specifically sorbed proteins. The fragments of mini-Pg specifically bound to AH-Sepharose were eluted from the column with 0.25 M ε-ACA in 0.1 M Na-phosphate buffer (pH 7.4) (Fig. 7, *B*). Approximately 30% of the applied amount of polypeptide fragments was eluted in the outer volume of the column that was seen as the first peak. The fragments were not detected in the fractions collected during elution with 1.0 M NaCl. The basic amount of proteins was eluted with 0.25 M ε-ACA (the third peak). Protein fractions of the first and third peaks were collected separately then dialysed against 0.1 M Na-phosphate buffer (pH 7.4) and concentrated using ultrafiltration membranes with NMWL of 3 kDa. Protein composition of these fractions was determined by SDS PAGE. The results presented at Fig. 8 show that 10 kDa polypeptide fragments obtained from mini-Pg digested by pepsin and smaller fragments (Fig. 8, track *6*), which are thought to be derived from mini-Pg proteinase domain, don't bind to AH-Sepharose. It is known that mini-Pg proteinase domain hasn't the site providing its binding to AH-Sepharose.

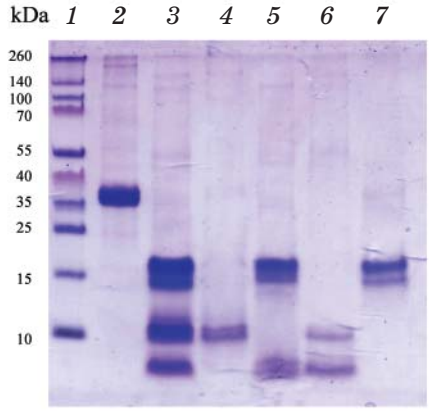

Fig. 8. **SDS-PAGE of mini-Pg, its pepsine hydrolysate and the fragments obtained after separation on DEAE-Sephadex A-25 and AH-Sepharose:** *1* — protein standards; *2* — mini-Pg; *3* — pepsine hydrolysate of mini-Pg; *4, 5* — the fragments which were retained or weren't retained on DEAE-Sephadex A-25 correspondingly; *6, 7* — the fragments which weren't retained on AH-Sepharose or were eluted with 0.25 M ε-ACA correspondingly

The protein fraction which was retained on AH-Sepharose and was eluted with 0.25 M ε-ACA contains the fragment with molecular mass of about 15 kDa. This fragment was seen as a doublet band on the electrophoresis gel (Fig. 8, track *7*). The high affinity of this fragment towards AH-Sepharose (it wasn't eluted even with 1 M NaCl) gives us the reason to think that this is K5.

The results of chromatography on AH-Sepharose showed that kringle 5, which was formed by mini-Pg digestion with pepsin, kept its structural and functional characteristics in particular it showed its specificity towards ligands with low molecular mass.

As we can conclude, the chromatography on AH-Sepharose is the sufficient step to obtain the isolated fragment K5 from mini-Pg hydrolysate with pepsin. It should be noted that this approach doesn't require any additional purification steps to verify the functional activity of K5 and can increase its output by 10% .

We got electrophoretically pure Pg fragment K5, which possesses the functional activity towards ligands with high and low molecular mass. Our way for K5 isolation includes the following steps:

- Pg purification from Cohn fraction $III_{2,3}$ paste,which is a by-product of the human plasma fractionation process;
- the limited hydrolysis of Pg with elastase;
- mini-Pg purification by the combination of affinity chromatography on Lys-Sepharose and ultrafiltration on membranes with NMWL of 30kDa;
- the limited hydrolysis of mini-Pg with pepsin;
- K5 purification on AH-Sepharose.

We also obtained other kringle-containing fragments of Pg during the purification procedure: K1–3, K4 and mini-Pg, the last one was used to obtain K5. Preparative yield (by weight) values of the fragments derived from human Pg are the following: 38% for mini-Pg, 29.5% for K1–3, 8.2% for K4 and 3.8% for K5. The data calculated on the assumption of theoretically possible yield with Pg as a protein source are the following: 92% for mini-Pg, 84.8% for K1–3, 62.9% for K4 and 25.3% for K5.

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ОДЕРЖАННЯ ТА ОЧИЩЕННЯ КРИНГЛА 5 ПЛАЗМІНОГЕНУ ЛЮДИНИ З ВИКОРИСТАННЯМ АН-СЕФАРОЗИ

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Метою роботи було розробити спосіб одержання функціонально активного фрагмента плазміногену людини кринґла 5. Запропонований спосіб включає такі етапи: гідроліз плазміногену еластазою, відокремлення міні-плазміногену від фрагментів кринґлів 1–3 та 4 на Lys-сефарозі, гідроліз міні-плазміногену пепсином, афінну хроматографію на АН-сефарозі, електрофорез у поліакриламідному гелі.

Одержано електрофоретично чистий фрагмент плазміногену людини кринґл 5, що виявляє функціональну активність стосовно високомолекулярних та низькомолекулярних лігандів. Вихід за масою становив 3,8%, що відповідає 25,3% від теоретично можливого.

Встановлено, що для одержання ізольованого фрагмента кринґла 5 із гідролізату міні-плазміногену пепсином достатньою є тільки афінна хроматографія на АН-сефарозі. Такий підхід не потребує додаткових етапів очищення, а здатність кринґла 5 специфічно зв'язуватись із АН-сефарозою є свідченням його функціональної активності.

Ключові слова: плазміноген, фрагменти плазміногену, кринґл 5, ангіостатини.

ПОЛУЧЕНИЕ И ОЧИСТКА КРИНГЛА 5 ПЛАЗМИНОГЕНА ЧЕЛОВЕКА С ИСПОЛЬЗОВАНИЕМ АН-СЕФАРОЗЫ

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Цель работы заключалась в разработке способа получения функционально активного фрагмента плазминогена человека кринґла 5. Предлагаемый метод включает следующие этапы: гидролиз плазминогена эластазой, отделение мини-плазминогена от фрагментов кринґлов 1–3 и 4 на Lys-сефарозе, гидролиз мини-плазминогена пепсином, аффинную хроматографию на АН-сефарозе, электрофорез в полиакриламидном геле.

Получен электрофоретически чистый фрагмент плазминогена человека кринґл 5, проявляющий функциональную активность по отношению к высокомолекулярным и низкомолекулярным лигандам. Выход по массе составил 3,8%, что соответствует 25,3% от теоретически возможного.

Установлено, что для выделения изолированного кринґла 5 из гидролизата мини-плазминогена пепсином проведение только аффинной хроматографии на АН-сефарозе является достаточным. Такой подход не требует дополнительных этапов очистки, а способность кринґла 5 специфически связываться с АН-сефарозой свидетельствует о его функциональной активности.

Ключевые слова: плазминоген, фрагменты плазминогена, кринґл 5, ангиостатины.