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High concentrations of sodium chloride facilitate the use of immobilized chymotrypsin for separating virgin forms of specific trypsin inhibitors

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Abstract

It has been shown that specific trypsin inhibitors exhibit also antichymotrypsin activity in the presence of high NaCl concentrations. Taking advantage of this phenomenon a simple procedure of separation of the virgin forms of trypsin inhibitors from squash seeds and porcine pancreas (Kazal) was elaborated. In a typical experiment the inhibitor sample was loaded onto immobilized chymotrypsin equilibrated with 5 *M* NaCl at pH 8. After washing out unadsorbed material the virgin forms of inhibitors could be eluted either with water, buffer pH 8.0 or 0.02 *M* citrate buffer pH 2.6 containing no NaCl.

Keywords: Immobilized chymotrypsin; Sodium chloride; Trypsin inhibitors; Proteins

1. Introduction

Immobilized trypsin has been known to provide an efficient means for the purification of its inhibitors. However, the immobilized enzyme is prone to hydrolyse the peptide bond of the inhibitor molecule being separated, causing e.g. the inactivation of Kazal-type temporary inhibitors [1] or increasing the number of inhibitor molecular forms [2]. To avoid this inconvenience the catalytically inactive anhydrotrypsin [3] which retained its ability to form stoichiometric complexes with trypsin inhibitors, has been used as a ligand in affinity chromatography for isolation of their virgin forms [4]. However, the preparation of anhydrotrypsin is rather time consuming and includes a conversion of the active site serine residue to dehydroalanine, purification of anhydrotrypsin by affinity chromatography followed by additional inactivation of the residual trypsin activity by phenylmethylsulfonyl fluoride (PMSF).

It has been known that activity of some proteases towards both natural and synthetic substrates depends on ionic strength. The human leukocyte elastase activity when measured on N-succinyl-$(Ala)_3$-*p*NA increases 20-fold in the prersence of 5 *M* NaCl [5]. Kahana and Shalitin [6] have shown that in high NaCl concentrations chymotrypsin not only exhibits enhanced activity towards its specific substrates but also degrades polylysine, the specific substrate for trypsin.

In our studies we have shown that selective trypsin inhibitors from the squash family [7] and porcine pancreatic secretory trypsin inhibitor (Kazal) under the standard conditions have no antichymotrypsin

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activity but in a presence of high salt concentrations become good inhibitors of this enzyme. In the paper we applied this salt effect for the purification of these inhibitors by affinity chromatography on immobilized chymotrypsin in the presence of 5 *M* NaCl.

2. Experimental

2.1. Materials

Fresh porcine pancreas were obtained from a slaughterhouse and stored frozen at −15°C. The seeds of figleaf gourd (*Cucurbita ficifolia)* and summer squash (*Cucurbita pepo)* were obtained from the Garden Seed Company, Wrocław, Poland.

Trypsin (E.C. 3.4.21.4) was prepared according to Wilimowska-Pelc and Mejbaum-Katzenellenbogen [8], chymotrypsin Aα (E.C. 3.4.21.1), bovine serum albumin, turkey ovomucoid, *N*-succinyl-L-Phe-*p*NA, *N*α-benzoyl-DL-Arg-*p*-nitroanilide (BAPNA), divinyl sulfone, low-molecular-mass markers for sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (myoglobin fragments and glucagon) and Coomassie Brilliant Blue G-250 were from Sigma (St. Louis, MO, USA), DEAE-Sephadex A-25, Sepharose 4B was from Pharmacia LKB Biotechnology (Uppsala, Sweden); Bio-Gel P2 was from Bio-Rad Labs. (Richmond, CA, USA); *p*-nitrophenyl *p*-guanidinobenzoate·HCl (NPGB) was from Merck (Darmstadt. Germany); YM 1 membrane filter from Amicon (Danvers, MA, USA). All other reagents were of the analytical or HPLC-grade purity.

2.2. Immobilization of chymotrypsin

Chymotrypsin Aα (1g) was immobilized on divinylsulfone-activated Sepharose 4B (70 ml) according to Pepper [9].

2.3. Activity measurement

Trypsin activity was measured on BAPNA, as substrate, in 0.05 *M* Tris–HCl buffer, pH 8.0, with 20 m*M* $CaCl_2$ [10]. One unit of antitrypsin activity is defined as that amount of inhibitor which reduced by half the activity of 2 mg of trypsin. Chymotrypsin activity was measured on *N*-succinyl-L-Phe-*p*-NA, as a substrate, in 0.1 *M* Tris–HCl buffer, pH 7.5 [11]. The inhibitory activity was estimated from the residual enzymatic activity after 10 min preincubation of the enzyme with an inhibitor solution.

2.4. Protein determination

The protein in the inhibitor preparations was determined by the method of Goa [12] with bovine serum albumin as a standard. The concentration of trypsin was determined by active-site titration with trypsin-specific burst titrant *p*-nitrophenyl *p*-guanidinobenzoate [13]. The standardized trypsin solution was used to titrate turkey ovomucoid and chymotrypsin Aα was in turn titrated with the standarized ovomucoid solution.

2.5. Electrophoresis procedure

Native gel electrophoresis in 7.5% polyacrylamide gel, pH 8.6, was run according to Davis [14] and SDS–PAGE was carried out according to Schägger and von Jagow [15]. Gels were stained for protein with Coomassie Brilliant Blue G-250.

2.6. Preparation of crude trypsin inhibitor from porcine pancreas

Pancreas were partially thawed at room temperature and homogenized in Waring-blender homogenizer with 3 volumes (w/v) of 80% methanol in 0.3 *M* HCl. The homogenate was left standing for 20 h and centrifuged. The supernatant was decanted, the sediment was re-extracted for 2 h with 1.5 volumes of the same solvent with mechanical stirring and clarified by centrifugation. The two exctracts were pooled and 7 volumes of cold acetone were added. After 20 h the precipitate formed was collected by centrifugation and dried under vacuum. The aceton powder was solubilized in 0.01 *M* HCl (150 ml/kg of fresh tissue) and 1.8 *M* $HClO_4$ was added to a final concentration of 0.45 *M*. After 1 h the denatured proteins were removed by centrifugation. To remove perchloric acid, the extract was neutralized with 10 *M* KOH, left at 4°C overnight and crystals of potassium perchlorate were filtered off. From 1 kg of pancreas 7.2 g of protein with 130 antitrypsin units were obtained.

2.7. Preparation of crude inhibitors from figleaf gourd (CFTI-Cucurbira ficifolia trypsin inhibitor) and summer squash(CPTI-Cucurbita pepo trypsin inhibitor)

The crude inhibitor preparations were isolated from figleaf gourd and summer squash seeds following previously published procedure [16]. The ground seeds were extracted with 5 volumes (w/v) of 0.05 *M* acetate buffer, pH 4.7. The suspensions were stirred for 1 h at room temperature, then clarified by centrifugation and protein from the exctracts were salted out with ammonium sulphate at 0.9 saturation. After 24 h the proteins sedimented were collected by centrifugation and dissolved in water then methanol was added to 80% (v/v) concentration, and the mixture was stirred for 30 min. The precipitate of ammonium sulfate and denatured proteins were filtered off and methanol was evaporated from the filtrate in the stream of air. After this step of purification from 300 g of summer squash seeds, 480 mg of protein with 90 antitrypsin units and from 100 g of figleaf gourd seeds, 210 mg of protein with 240 antitrypsin units, were obtained.

2.8. Amino acid sequence analysis

The amino acid sequences of purified inhibitors from figleaf gourd and summer squash seeds (100 *p*moles) were determined with a ProSequencer model 6600 (MilliGen) and phenylthiohydantoin (PTH)-analyser using the program provided by the manufacturer. Sequences of inhibitors were determined in their disulfide bridges form, therefore no signal was detected at cysteine and assignment of this residue was based on homology with other squash inhibitors [7].

3. Results and discussion

3.1. The effect of NaCl concentration on the inhibition of chymotrypsin by specific trypsin inhibitor

The 29 amino acid residue inhibitors of serine proteinase of squash family form very strong complexes with bovine β trypsin ($K_a \cong 10^{11}$–$10^{12} M^{-1}$) [7] but they have no activity against bovine chymo-

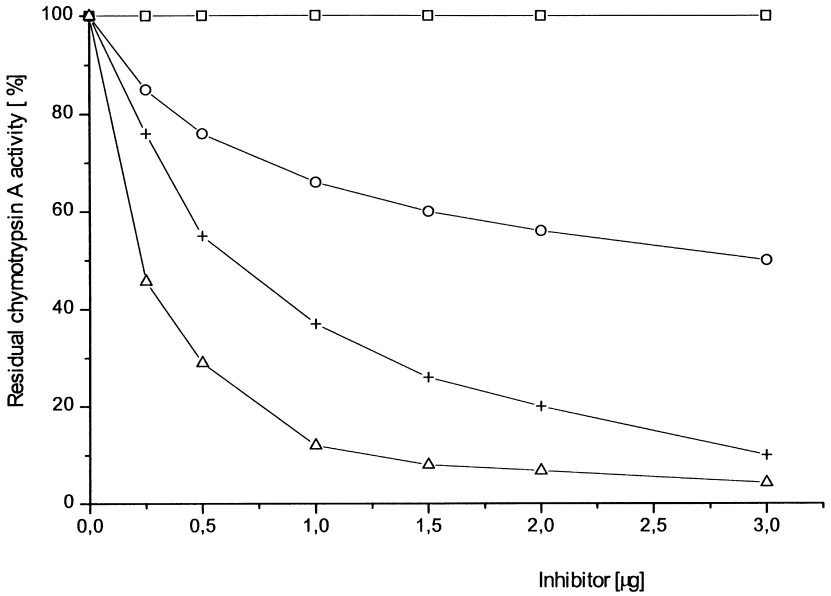

Fig. 1. Influence of various NaCl concentration on inhibition of chymotrypsin Aα activity by porcine pancreatic trypsin inhibitor using *N*-succinyl-L-Phe-*p*NA as a substrate. □–□ without NaCl; ○–○ 2 *M* NaCl; +–+ 3 *M* NaCl; ◁–◁ 4.5 *M* NaCl.

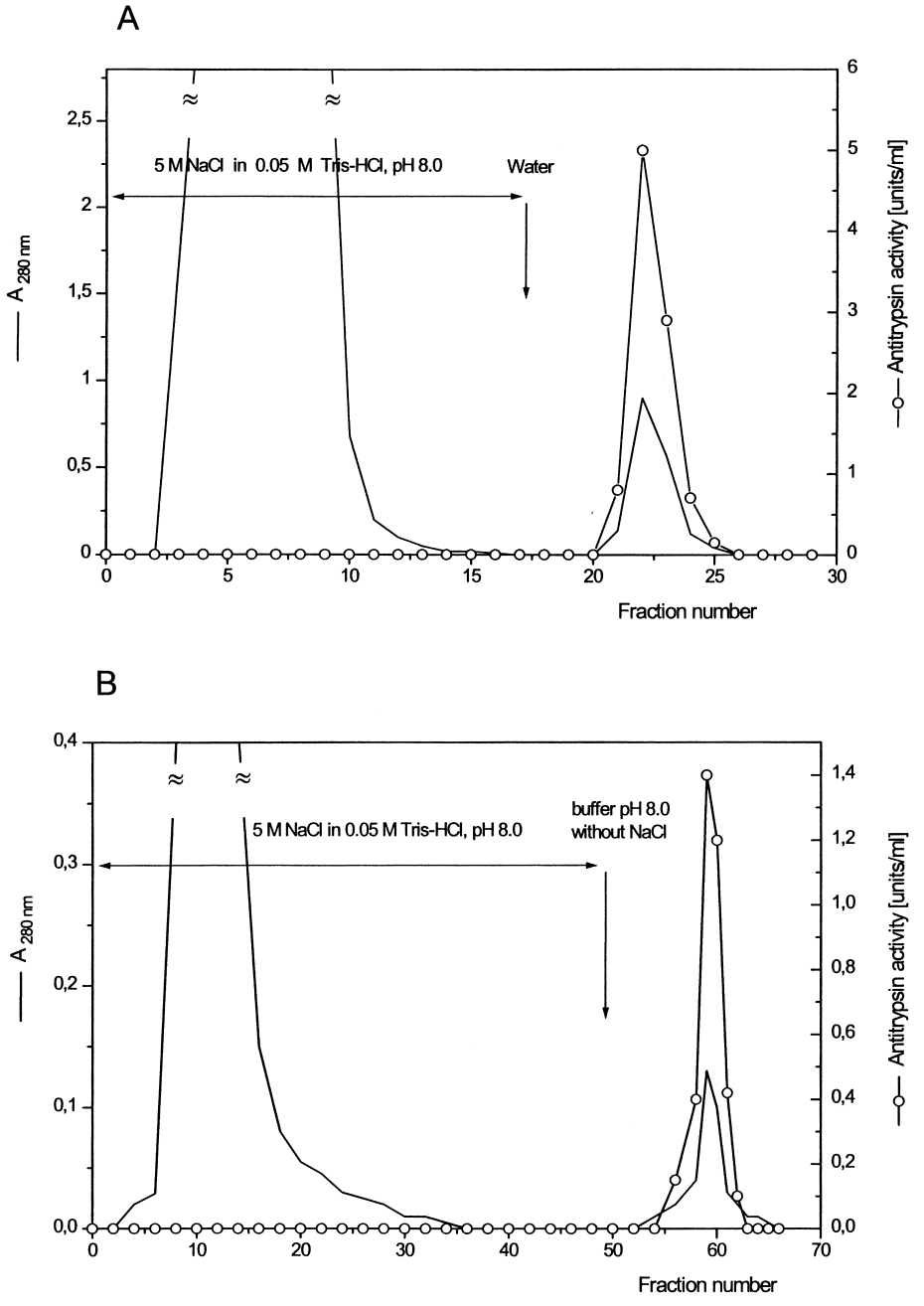

Fig. 2. Affinity chromatography of trypsin inhibitors from: porcine pancreas (A), seeds of *Cucurbita pepo* (B) and *Cucurbita ficifolia* (C) on immobilized chymotrypsin. Samples of 35 to 45 ml of crude inhibitor preparations containing 5 *M* NaCl at pH 8.0, were loaded on the chymotrypsin-Sepharose column (20×160 mm I.D.), equilibrated with 0.05 *M* Tris–HCl buffer, pH 8.0, 5 *M* NaCl. All columns were washed intensively with equilibrating buffer containing 5 *M* NaCl to remove unadsorbed material until A_{280} dropped below 0.02. Finaly inhibitors were eluted with: water in (A), 0.05 *M* Tris–HCl, pH 8.0 in (B) or 0.02 *M* citrate buffer, pH 2.6 in (C). 11 ml fractions at a flow-rate of 80 ml/h were collected.

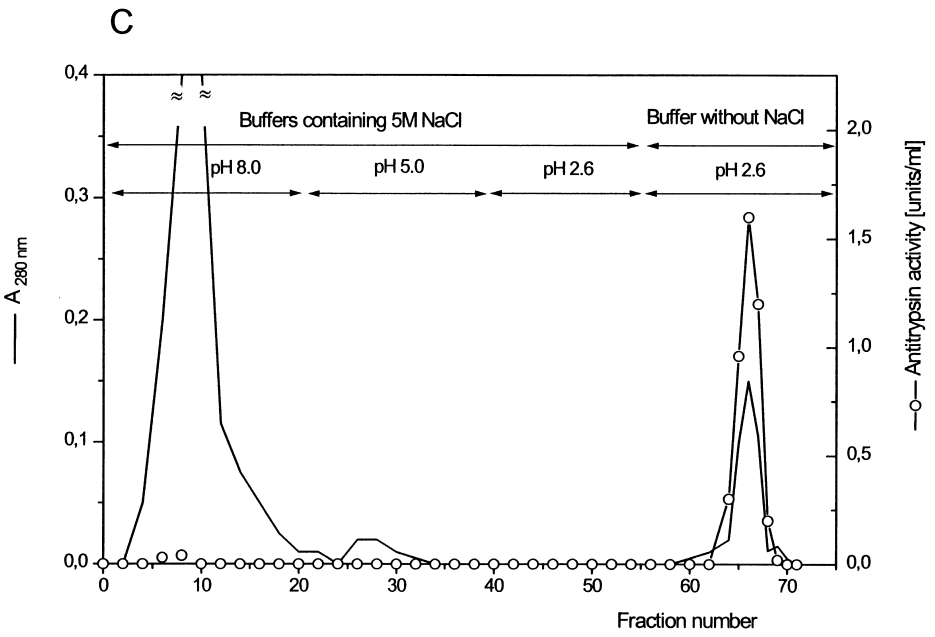

Fig. 2. (*continued*)

trypsin [17]. Similarly, for pancreatic secretory trypsin inhibitors (Kazal type), no inhibition was detected with chymotrypsin. Nevetherless, we have observed that these specific trypsin inhibitors both with Arg (*Cucurbita maxima* trypsin inhibitor-CMTI I) and Lys (*Cucurbita pepo* trypsin inhibitor-CPTI II and porcine trypsin inhibitor) at P_1 position of the reactive site are able to inhibit chymotrypsin Aα in the presence of high NaCl concentrations. The example of the inhibition of chymotrypsin Aα by porcine pancreatic trypsin inhibitor as a function of NaCl concentration is illustrated in Fig. 1. The association equilibrium constant for bovine chymotrypsin Aα and CMTI I increased from $4.1 \cdot 10^4 M^{-1}$ when reaction was carried out in the absence of NaCl, to $8.5 \cdot 10^8 M^{-1}$ when the concentration of NaCl in the reaction mixture reached 5*M* [18].

3.2. Chromatography of trypsin inhibitors on chymotrypsin-Sepharose 4B

The advantageous effect of high salt concentrations on the inhibition of chymotrypsin by the specific trypsin inhibitors we turned to account for improving the isolation of these inhibitors on immobilized chymotrypsin.

For this purpose the crude inhibitor preparations were brought to pH 8.0 with 1 *M* Tris, NaCl was added to a final concentration of 5 *M* then applied to the column with immobilized chymotrypsin equillibrated with 0.05 *M* Tris–HCl buffer, pH 8.0, containing 5 *M* NaCl. The columns were washed exhaustively with the same buffer containing 5 *M* NaCl until A_{280} dropped below 0.02. Though the inhibitors used in this experiment were specific only against trypsin, under these conditions all of them have adsorbed to chymotrypsin-Sepharose. The adsorbed inhibitors were eluted either with water, 0.05 *M* Tris–HCl buffer, pH 8.0 or 0.02 *M* citrate buffer, pH 2.6, containing no NaCl. Fig. 2 A–C shows chromatograms of the separation of porcine pancreatic trypsin inhibitor (A), CFTI (B) and CPTI (C).

It is worth noting to say that adsorbed trypsin-specific inhibitors on chymotrypsin-Sepharose could not be desorbed even at pH 2.6 in the presence of 5 *M* NaCl whereas they can be eluted very easely with water or any solution containing no sodium chloride.

In the previous work [19], we described the effect of NaCl on the desorption of bovine trypsin and two

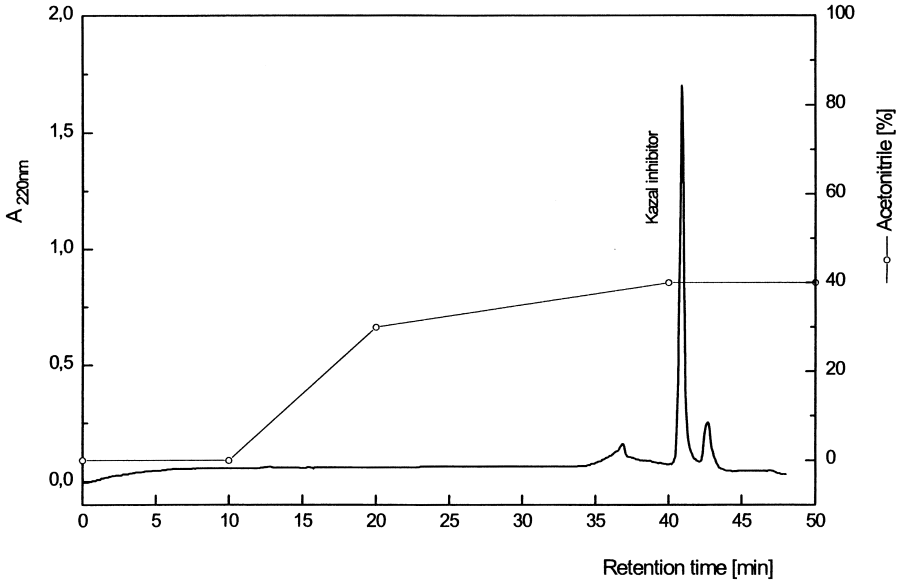

Fig. 3. Reversed-phase HPLC of porcine pancreatic trypsin inhibitor. Aliquots (50 μl) of the material obtained after affinity chromatography and desalted on Bio-Gel P2 were loaded onto a Nucleosil-100 C_{18}, 10 μm (250×8 mm I.D.) reversed-phase column and eluted with an acetonitrile/water gradient in 0.1% trifluoroacetic acid. Acetonitrile was removed from eluted fractions by evaporation.

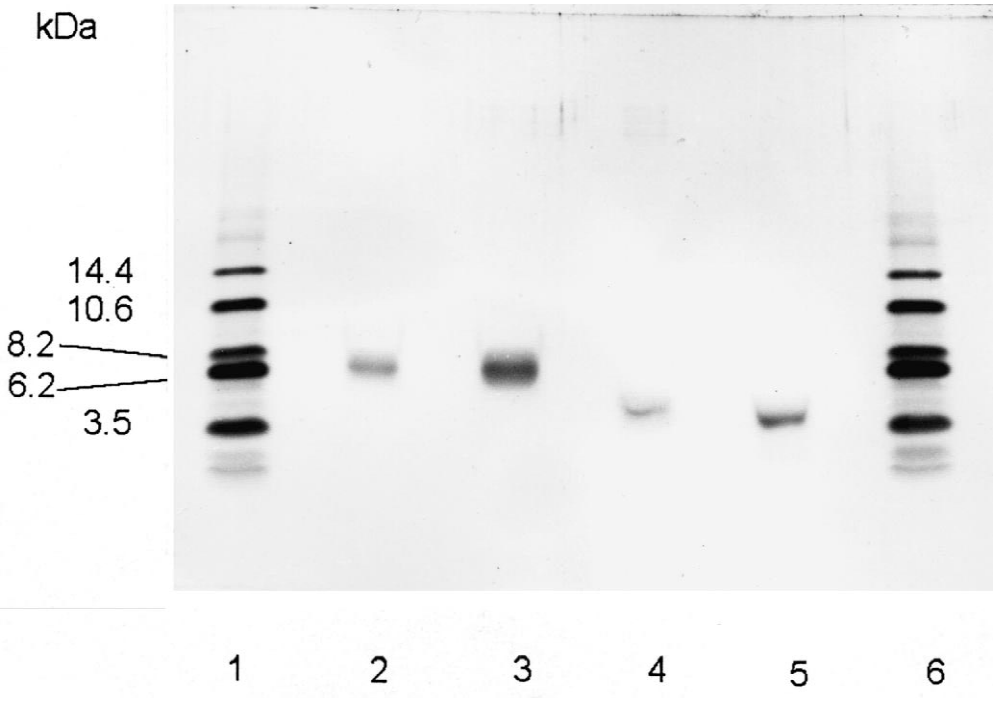

Fig. 4. SDS–PAGE of purified inhibitors. Lanes: 1 and 6: protein size markers; 2 and 3: pancreatic trypsin inhibitor (2 and 10 μg, respectively); 4: CFTI (2 μg); 5: CPTI (5 μg), kDa=kilodaltons.

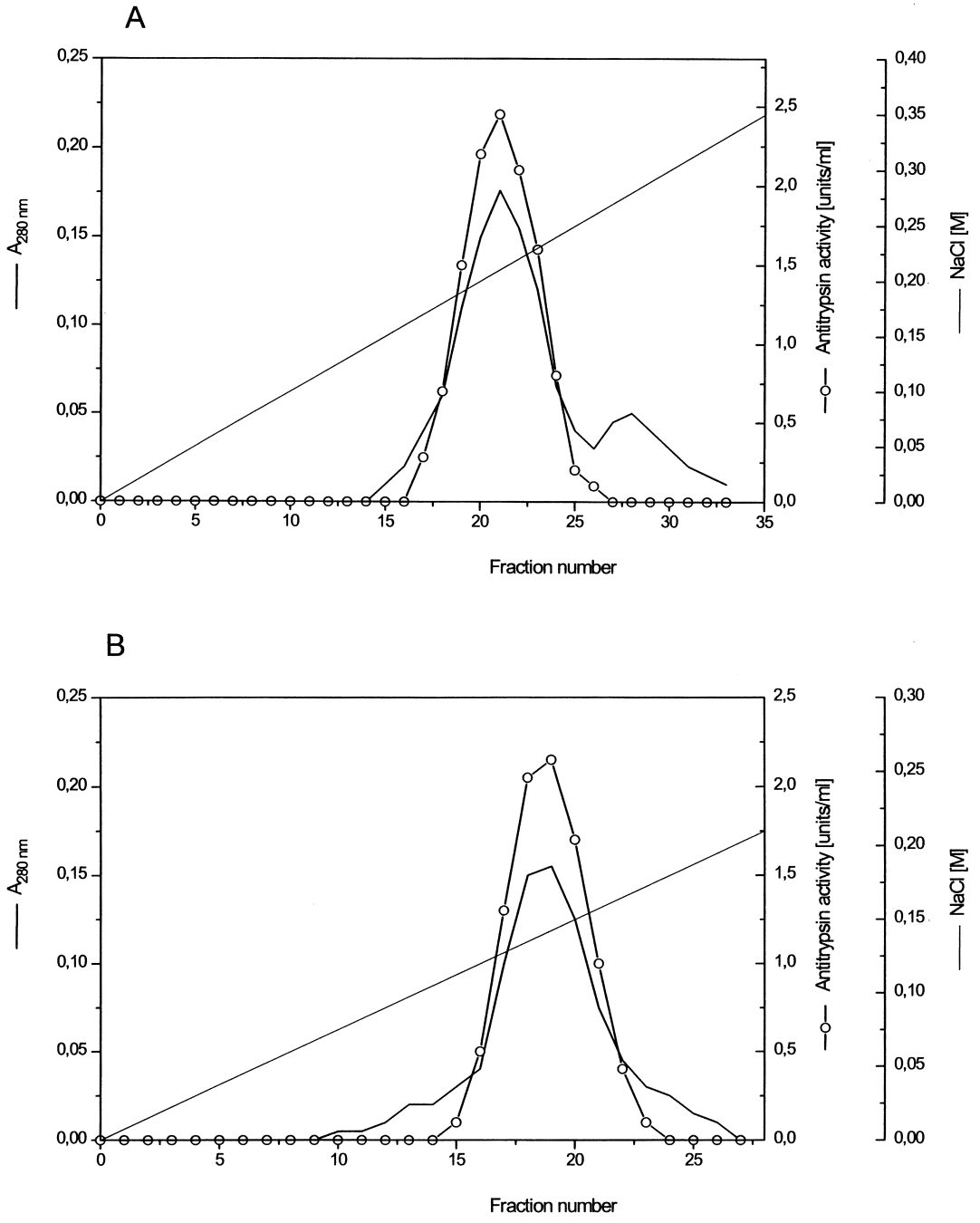

Fig. 5. An anion-exchange chromatography of CPTI II (A) and CFTI I (B). The inhibitor preparations obtained after chymotrypsin-Sepharose were loaded on a DEAE-Sephadex A-25 columns (17×160 mm I.D.) equilibrated with 0.05 *M* Tris–HCl buffer, pH 8.0. Elution was performed by a linear gradient 0.0–0.4 *M* NaCl in the same buffer. Fractions of 10 ml were collected at a flow rate of 60 ml/h.

chymotrypsins (A_α and B) during chromatography on immobilized Bowman-Birk inhibitor. Bound trypsin and chymotrypsin A_α were eluted with $10^{-2}M$ HCl containing 0.5 *M* NaCl. In contrast, chymotrypsin B appeared to be retarded and could be eluted with $10^{-2}M$ HCl containing no sodium chloride.

Upon affinity chromatography pancreatic trypsin inhibitor, CFTI and CPTI were purified 180-, 23- and 7-fold with a yield of 68%, 72% and 75%, respectively. Low purification factors were consequences of removing most of impurities after applying 80% methanol either for extraction of Kazal inhibitor or at the early stage of purification procedure of CFTI and CPTI.

3.3. Final purification of trypsin inhibitors

Porcine pancreatic trypsin inhibitor were obtained after affinity chromatography was lyophilized, dissolved in 0.1 *M* acetic acid, centrifuged to remove insoluble impurieties, desalted on Bio-Gel P2 column equilibrated with 0.1 *M* acetic acid and finally chromatographed on C_{18} column in RP-HPLC (Fig. 3). From 1 kg of pancreas 16 mg of inhibitor preparation were obtained with the recovery around 40% and approximately 270-fold purification over the HCl–methanol extract. Compared to the other methods, the use of 80% methanol in 0.3 *M* HCl essentially simplified the purification procedure and increased the yield of Kazal trypsin inhibitor from pancreatic tissue. Inhibitor preparation showed in native polyacrylamide gel electrophoresis the presence of 3 active bands (not shown), as described by Menegatti et al. [20]. In SDS–PAGE one diffused band of these three forms slightly differed in M_r, was observed (Fig. 4).

The inhibitor preparations from the seeds of figleaf gourd and summer squash puriefied on immobilized chymotrypsin were concentrated and desalted on a YM 1 membrane filter and chromatographed on DEAE-Sephadex A-25 column, equilibrated with 0.05 *M* Tris–HCl buffer, pH 8.5. Adsorbed proteins were eluted with gradient of NaCl. In both experiments inactive material was well separated from inhibitors (Fig. 5A, B). Active fractions were pooled, lyophylized, desalted on a Bio-Gel P2 and chromatographed on Vydac C_{18} column using an HPLC system (Fig. 6A and B). From 300 g of summer squash seeds and 100 g of figleaf gourd seeds 9 mg of CPTI and 21 mg of CFTI were obtained, with a yield of 36% and 41% and 410- and 105-fold of purification factor, respectively. The described method did not essentially increase the yield or improve

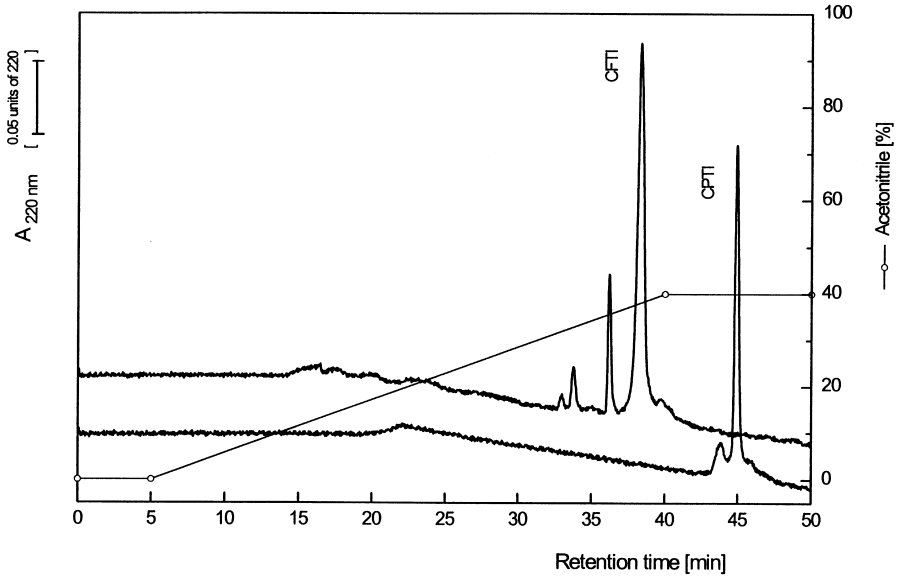

Fig. 6. Reversed-phase HPLC of CPTI II (A) and CFTI (B). For details see Fig. 3.

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                *↓
CFTI   R C V P R I L M E C K K D S D C L A E C V C L E H G Y C G
CPTI   R C V P K I L M E C K K D S D C L A E C I C L E H G Y C G
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Fig. 7. Amino-acid sequences of trypsin inhibitors from the seeds of figleaf gourd (CFTI) and summer squash (CPTI). *-P_1 residue of the reactive site; ↓-reactive site peptide bond.

the purification procedure but allowed to obtain in a simple way the virgin forms of the inhibitors.

3.4. Amino acid sequence of CFTI and CPTI

The amino acid sequences of CFTI and CPTI are presented in Fig. 7. Both inhibitors isolated by affinity chromatography on immobilized chymotrypsin shown only one N-terminus. It means that during affinity chromatography in applied conditions the immobilized enzyme did not split the reactive site peptide bond of the inhibitor molecules. They were remained in there virgin forms. The comparison of amino acid sequences of CPTI and CFTI with those of known squash family serine proteinase inhibitors revealed that the first one is identical to CPTI II and the latter with CMTI I-inhibitor isolated from *Cucurbita maxima* seeds.

Acknowledgements

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