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Purification of two low-molecular-mass serine proteinase inhibitors from chicken liver

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Abstract

Two serine proteinase inhibitors, designated clTI-1 and clTI-2 were purified from livers of chickens to apparent homogeneity by a combination of ethanol-acetone fractionation, gel filtration and ion-exchange chromatography on CM-cellulose and Mono S columns. The inhibitor clTI-1 is a single polypeptide chain, low-molecular-mass protein (M_r about 6200), very stable to heat and ethanol. It inhibits chicken, porcine and bovine trypsins as well as human plasmin. The second protein, lcTI-2 of M_r 17 000 was shown to be a very effective inhibitor of both trypsins and human cathepsin G. Since both inhibitors are sensitive to arginine modification with phenylglyoxal it is assumed that this amino acid residue is present at the P_1 position of the reactive site peptide bond. The N-terminal amino acid sequence of 28 residues of clTI-2 (SVDVSKYP-STVSKDGRTLVACPRILSPV) revealed a high homology of this protein to the third domain of the chicken ovoinhibitor, whereas, the clTI-1 (APPAAEKYYSLPPGAPRYYSPVV) has some sequence identity to a fragment of the human inter-α-trypsin inhibitor.

Keywords: Enzyme inhibitors; Proteins

1. Introduction

An ongoing interest in the proteinase inhibitors is stemming from the fact that these substances are regarded as very important factors in controlling proteolysis. The biological functions of many members of this group of proteins have been examined in details and described in a number of reviews. They were found to be present in biological fluids and numerous tissues of animal and plant origin. In animal organisms they control, for example, a variety of critical events associated with blood coagulation, complement activation, cell migration, hormone transportation, tumor suppression, prohormone convertion, fibrynolysis, and inflammatory reaction [1,2]. The physiological significance of proteinase inhibitors is however, much broader and is not limited to the cited functions. Some studies indicate, for instance, that a rapid clearance of tissue-type plasminogen activator (t-PA) in the liver, especially via parenchymal cells, is mediated by plasminogen activator inhibitor type 1 (PAI-1). In human HepG2 hepatoma cells t-PA first reacts with PAI, forming a complex, which in turn binds to the cell and is internalized and degraded [3]. This was confirmed in an isolated perfused rat liver system where the complexes of t-PA-PAI-1 were cleared much faster than the enzyme alone [4]. This suggests that serine proteinase inhibitors function as the receptors that facilitate internalization and degradation of unwanted proteolytic enzymes.

To study this problem we have isolated and

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partially characterized two different, low-molecular-mass proteinase inhibitors from chicken liver. One of them is active against trypsin and human plasmin and the other against trypsin and human cathepsin G. None of them inhibit bovine and chicken chymotrypsin, human thrombin and leukocyte elastase, and pancreatic elastase of chicken and porcine origin.

2. Experimental

2.1. Materials

Fresh chicken livers were obtained from a slaughterhouse and stored frozen at −20°C. Chemicals were purchased as follows: bovine β-trypsin and α-chymotrypsin, porcine pancreatic elastase (PPE), streptokinase, *N*-α-benzoyl–DL-arginine *p*-nitroanilide (BAPNA), *N*-succinyl–Ala–Ala–Pro–Phe–*p*-nitroanilide, *N*-(1-naphthyl)-ethylenediamine dihydrochloride, *N*-succinyl–Ala–Ala–Ala–*p*-nitroanilide, and *N*-methoxysuccinyl–Ala–Ala–Pro–Val–*p*-nitroanilide, from Sigma (St. Louis, MO, USA); Sephadex G-75, DEAE-Sephadex A-25, and molecular-mass markers for sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) from Pharmacia LKB Biotechnology (Uppsala, Sweden); *N,N′*-metylenobisacrylamide and *N,N,N′,N′*-tetramethylethylenediamine (TEMED), and 4-nitrophenyl-4-guanidinobenzoate hydrochloride (NPGB), from Fluka (Switzerland); basic pancreatic trypsin inhibitor (BPTI) Trascolan, from Pharmaceutical Co. Jelfa (Jelenia Góra, Poland). Human plasminogen was a gift from Athens Research and Technology, Athens, GA, USA Human leukocyte cathepsin G (catG) and elastase (HLE) were purified by the method of Wątorek et al. [5]. Chicken trypsin was purified according to Guyonnet et al. [6]. Squash inhibitor CMTI I was separated after Otlewski et al. [7]. All other reagents were of analytical grade.

2.2. Protein assay

Protein content was determined either by the bicinchonic acid method [8] with bovine serum albumin as standard or spectrophotometrically by measuring the absorbance at 235 and 280 nm and calculating the concentration using equation: $C(\mu g/ml)=(A_{235}-A_{280}):2.51$ [9].

2.3. Protease activity and inhibitor measurements

Enzyme activities were measured spectrophotometrically at 22°C in a final volume of 1 ml, using chromogenic turn-over substrates. *N*-α-benzoyl–DL-arginine *p*-nitroanilide (BAPNA) for trypsin and plasmin, *N*-succinyl–Ala–Ala–Pro–Phe–*p*-NA for chymotrypsin and catG, *N*-methoxysuccinyl–Ala–Ala–Pro–Val–*p*-NA for HLE, and *N*-succinyl–Ala–Ala–Ala–*p*-NA for PPE. All substrate stock solutions were prepared in dimethyl sulfoxide. A suitable amount of inhibitor was allowed to complex with trypsin ($2.0\cdot10^{-7}$ *M*), plasmin ($2.7\cdot10^{-7}$ *M*) or chymotrypsin ($1.7\cdot10^{-7}$ *M*) in 50 m*M* Tris–HCl, 20 m*M* $CaCl_2$, 0.005% Triton X-100 buffer, pH 8.3 or with catG ($3\cdot10^{-7}$ *M*) in 50 m*M* Tris–HCl, 0.5 *M* NaCl, 20 m*M* $CaCl_2$, 0.005% Triton X-100 buffer, pH 8.3 in a final volume of 1.0 ml. The residual activity of the enzyme present at equilibrium was measured by addition of the respective substrates, BAPNA ($2\cdot10^{-4}$ *M*) or *N*-succinyl–Ala–Ala–Pro–Phe–*p*-NA ($3\cdot10^{-4}$ *M*). After 20 min incubation at 25°C the reaction was terminated by the addition of 50 μl of glacial acetic acid solution and the release of 4-nitroaniline was measured at 412 nm. A control reaction, in the absence of an inhibitor, was carried out under the same conditions. One unit of inhibitory activity was defined as the amount of protein needed to inhibit 2 μg of an enzyme by 50%.

2.4. Standardization of enzymes and inhibitors

The concentration of active enzymes in a stock solution of trypsin and plasmin (dissolved in 1 m*M* HCl, 20 m*M* $CaCl_2$), was determined by spectrophotometric titration with *p*-nitrophenyl *p*-guanidinobenzoate [10]. The standardized trypsin solution was used to titrate CMTI-1 which served as secondary standard for determining the activity of catG.

2.5. Electrophoresis

SDS–PAGE was performed by the method of

Laemmli [11] using 7–20% separating gel and 4% stacking gel with 0.1% SDS, pH 8.9. Coomassie Brilliant Blue G staining was used to visualize protein bands. Apparent M_r values of the purified inhibitors were calculated from semilog plots of standard protein M_r vs. migration distances. Low-molecular-mass standards were phosphorylase *b* (M_r 97 000), bovine serum albumin (66 000), ovoalbumin (43 000), carbonic anhydrase (29 000), soybean trypsin inhibitor (21 000), *Heliothis zea* elastase inhibitor (18 300) and BPTI (6500).

2.6. Measurements of equilibrium association constants

The equilibrium association constants were estimated using the method described by Empie and Laskowski [12]. The reactions were conducted in a polystyrene cuvette in the final volume of 2.0 ml of 0.1 *M* Tris–HCl buffer, pH 8.3 with 0.005% Triton X-100 which in case of cathepsin G and elastase was supplemented with 0.5 *M* NaCl. The enzyme concentrations used $[E_o]$ complied with the equation: $2<[E_o]\cdot K_a<50$ and the concentration of inhibitor $[I_o]$ ranged from 0 to $2\times[E_o]$. A constant amount of an enzyme was reacting with increasing amounts of inhibitor at 22°C for a predetermined period of time, to reach an equilibrium, followed by addition of a small volume of a stock solution of substrate whose final concentration in the reaction medium did not exceed $0.2\cdot K_m$. The hydrolysis of *p*-nitroanilides was monitored at 412 nm for 120 s and free enzyme was calculated.

2.7. Chemical modifications

Modification of arginine residues was performed by the addition of a 1000-fold molar excess of *p*-hydroxyphenylglyoxal to the inhibitor sample [13]. The protein (50 nmol) was incubated with 50 μmol of reagent in 0.1 *M* triethanolamine–sodium borate buffer, pH 7.8, in the dark at room temperature. Aliquots were removed at given time intervals, and residual activity measured against porcine trypsin.

Reduction and S-β-pirydylethylation of cystein was performed essentially according to Friedman and coworkers [14]. The inhibitor samples (about 10 nmol for clTI-1 and 6 nmol for clTI-2) were dissolved in 100 μl of 6 *M* guanidine–HCl, 0.25 *M* Tris–HCl, 1 m*M* EDTA, 2.5% (v/v) β-mercaptoethanol, pH 8.0 and incubated in dark under argon at room temperature for 2 h. After addition of 20 μl 4-vinylpyridine the incubation was continued for the next 2 h. The reaction was terminated with 25 μl of glacial acidic acid, then the samples were dialyzed (M_r cut off, MWCO 1000) against 0.01 *M* acetic acid.

2.8. Amino acid sequence determination

Automated sequence analysis of inhibitors (320 pmol) was carried out in a Pro-Sequencer model 6600 (Milli-Gen) and PTH analyzer using the program provided by the manufacturer and the amino acid sequence of the inhibitors was compared with other proteins stored in the Protein Sequence Swiss-Prot database.

2.9. Isolation of inhibitors

Frozen chicken livers (5.8 kg) was partially thawed and homogenized in a 2-fold volume (w/v) of 70% ethanol in 1% acetic acid with a Waring-blender. The homogenate was stirred in cold room for 30 min and centrifuged at 10 000 *g* for 20 min at 4°C with the lipid layer on the top being removed by filtration through miracloth. An equal volume of cold (−20°C) acetone was gradually added to the supernatant under stirring, and left overnight at 0–4°C. The precipitate was collected by centrifugation, suspended in water (60 ml/kg of tissue) and centrifuged again. The obtained brown solution was heated in the water bath at 65°C until the temperature reached 55°C. After one min the mixture was immediately cooled in the ice bath. This produced precipitate which was discarded by centrifugation and the clear supernatant was dialyzed (MWCO 3500) against 50 m*M* acetate buffer, pH 4.6 (buffer A), then applied to a CM-cellulose cation exchange column pre-equilibrated with buffer A, and eluted with a linear salt gradient. Elution was monitored by absorbance at 280 nm and fractions of eluate (15.8 ml) were collected and assayed for antitrypsin activity. Inhibitory peak fractions 1–4 (Fig. 1) were pooled, concentrated on an Amicon concentrator with YM-3 membrane and dialyzed (MWCO 3500)

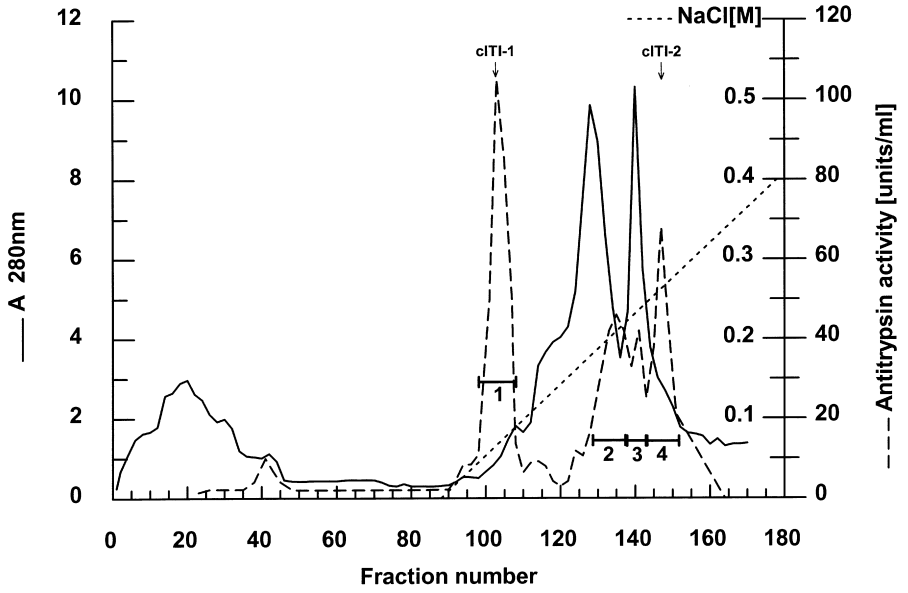

Fig. 1. CM cation-exchange chromatography of chicken liver trypsin inhibitors. The proteins obtained from the ethanol extract of whole chicken liver (5.8 kg) after acetone fractionation and heat treatment were applied to a CM-cellulose column (27.0×4.0 cm) previously equilibrated with 50 m*M* acetate buffer, pH 4.6. The proteins were eluted using a linear gradient of NaCl (-----) from 0–0.5 *M* in the same buffer at a flow rate of 140 ml h^{-1}. The fractions containing trypsin inhibitory activity (– – –) were pooled as indicated by the horizontal bars. The effluent was monitored at 280 nm (———).

against buffer A. Only preparation 1 and 4 as having the highest specific activity have been chosen for further purification.

Proteins present in these two preparations, designated clTI-1 and clTI-2, were rechromatographed on CM-cellulose column under essentially the same conditions to those of the first chromatography (Figs. 2, 4 respectively). Fractions with the highest antitryptic activity were pooled and concentrated on the Amicon concentrator with YM3 membrane. The concentrated solution of each inhibitor was then 4-fold diluted with water, reconcentrated to decrease NaCl content, and separately subjected to the ion-exchange fast protein liquid chromatography (FPLC) on a Mono S column which had been equilibrated with buffer A. The column was first washed with 5 column volumes of equilibrating buffer at 1.0 ml/min followed by a linear gradient of NaCl to elute bound proteins.

The antitrypsin activity of clTI-1 failed to bind to the column, therefore, the flow-through fractions containing all antitrypsin activity were lyophilized, then dissolved in 1.0 ml of water and applied to a Sephadex G-75 column calibrated with gel filtration molecular weight markers (Fig. 3). The peak of the inhibitor was pooled, concentrated as described above, desalted on a Sephadex G-10 column (180×5 mm I.D.) and its purity was checked by electrophoresis on SDS-PAGE and N-terminal amino acid sequence analysis.

The proteins from the preparation clTI-2 adsorbed to the Mono S column were eluted with a linear gradient of NaCl in the equilibrating buffer (Fig. 5A). Fractions containing the highest inhibitory activity were combined, then diluted 4-fold with buffer A and rechromatographed under the same conditions using the eluting gradient of NaCl as shown in Fig. 5B. Two fractions from the center of the protein peak showing identical specific activity, that appeared to be homogenous when analyzed by SDS–PAGE (Fig. 6, lane 3), were pooled and desalted on a Sephadex G-10 column.

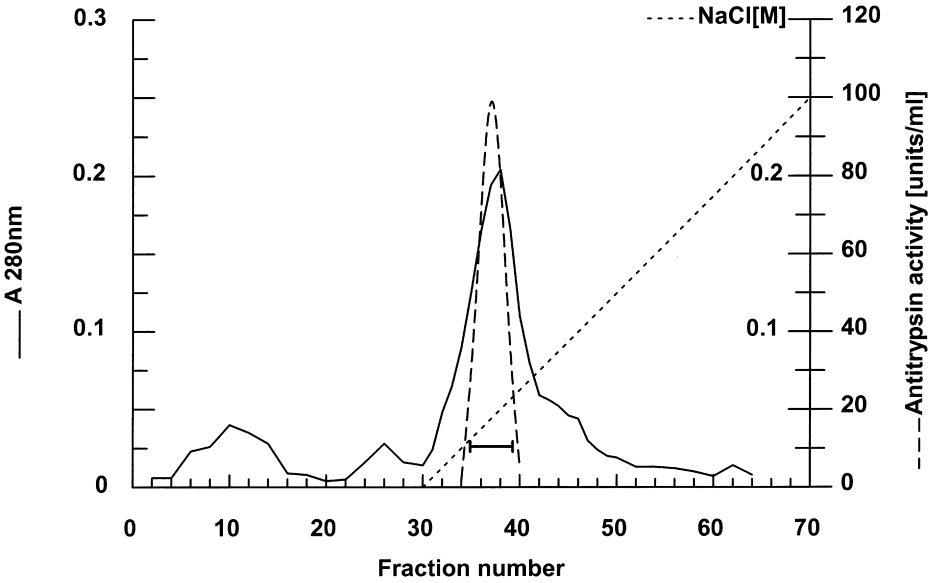

Fig. 2. Rechromatography of the inhibitor preparation clTI-1. Pooled peak fractions 1 from Fig. 1, see for details the Experimental section, were applied to a CM-cellulose column (15.0×2.0 cm) equilibrated with 50 m*M* acetate buffer pH 4.6. The column was washed and eluted using a linear gradient of NaCl (-----) from 0–0.25 *M*. Fractions (12.3 ml) were collected at a flow rate of 75 ml h^{-1} and assayed for antitrypsin activity (----) and absorbance at 280 nm (———). Fractions containing the inhibitory activity, were lyophilized.

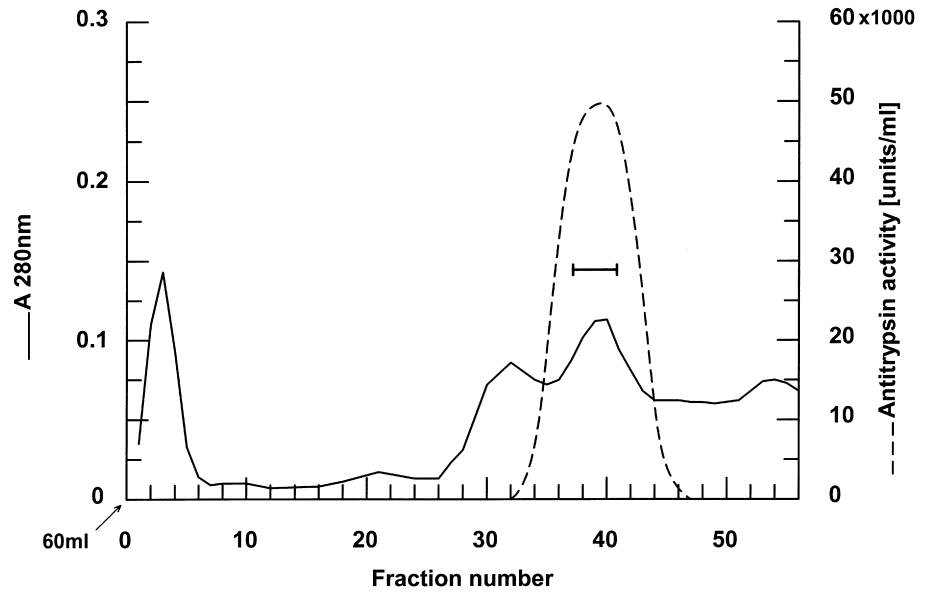

Fig. 3. Gel filtration chromatography of clTI-1 on Sephadex G-75 fine column. The concentrated flow-through fractions from a Mono S column were applied to a 106×1.4 cm column equilibrated with 50 m*M* Tris–HCl buffer, pH 8.0, containing 0.2 *M* NaCl. Fractions (1.1 ml) were collected at a flow rate of 3.3 ml h^{-1} and assayed for antytrypsin activity (----) and absorbance at 280 (———). The fractions collected are marked with a bar.

3. Results and discussion

3.1. Purification of inhibitors

The results of the stepwise purification of the trypsin inhibitors are summarized in Table 1. The use of an acidic 70% ethanol solution for the extraction of proteins from the chicken liver tissue enabled elimination of plasma proteins with antiproteolytic activity. The first step of inhibitor purification involved acetone precipitation and heat treatment of the precipitated proteins. This simple procedure significantly reduced the volume of the solution and effectively removed about 45% of the balast proteins with only limited loss of antitrypsin activity. The use of CM-cellulose column chromatography as a second step (Fig. 1) allowed separation of bound antitrypsin activity into four peak fractions. Peaks 2 and 3 having very low specific activity were excluded from further purification procedure.

The inhibitor present in peak 1 called clTI-1 (**c**hicken **l**iver **t**rypsin **i**nhibitor) upon rechromatography on a CM-cellulose column was eluted at approximately 30 m*M* NaCl as a single peak (Fig. 2); however, the level of its purity was found to be about 50%. When the collected inhibitory fractions were then applied to a Mono S column the active proteins failed to bind to the resin and all antitrypsin activity was found in the flow-through fractions. However, this step was helpful in removing some of the inactive proteins. The inhibitor present in the flow-through fractions was readily separated from the remaining balast proteins on a Sephadex G-75 column (Fig. 3). As a result, 1 mg of pure clTI-1 (Fig. 6, lane 4) exhibiting both antitrypsin and antiplasmin activity was obtained from 5.8 kg (fresh mass) of chicken livers with a 4300-fold increase of specific activity (1640 μ/mg) as compared to the crude extract.

When the proteins of clTI-2 preparation were rechromatographed on a column of CM-cellulose (Fig. 4), a broad protein peak and a single inhibitory peak with activity against trypsin and human catG was eluted at approx. 200 m*M* NaCl. Fractions containing the inhibitory activity were pooled, concentrated and, to decrease their ionic strength, diluted 4-fold with water followed by reconcentration. Then the proteins were fractionated by cation exchange chromatography on a Mono S FPLC column (Fig. 5A). The inhibitor was totally separated from inactive proteins by rechromatography using a different mode of NaCl gradient elution. Fig. 5B presents the elution profile of the last step of the purification which yielded over one mg of homogenous protein with a specific activity 930 u/mg and purification factor over 2400-fold

3.2. Characterization of the proteinase inhibitors

The purified inhibitors were homogenous by SDS–PAGE under reducing conditions (Fig. 6) and dose-dependently inhibited porcine and chicken trypsins. Inhibitor clTI-1 was also active against human plasmin whereas inhibitor clTI-2 exhibited anticathepsin G activity. None of them inhibited bovine and chicken chymotrypsins, human thrombin and

Table 1
Summary of purification of the trypsin inhibitors from 5.8 kg of chicken liver. One unit of inhibitory activity was defined as the amount of protein needed to inhibit by 50% 2 μg of an enzyme

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)
Ethanol extract	51 500	20 800	0.38	100
Acetone precipitation and heat treatment	28 387	18 000	0.63	86.5
CM-cellulose chromatography[a]	clTI-1	54.0	5160	95.5	25.6
	clTI-2	263.8	5970	22.6	29.7
Mono S FPLC Sephadex G-75	clTI-1	1.0	1640	1640	7.2
Mono S FPLC	clTI-2	1.018	946	930	4.7

[a] Assayed after concentration.

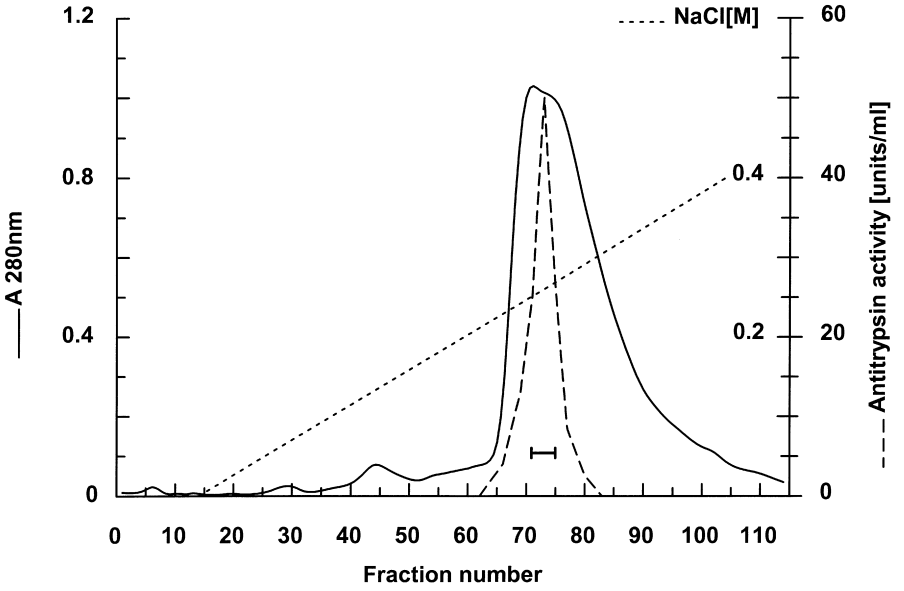

Fig. 4. Rechromatography of the inhibitor preparation clTI-2. Pooled peak fractions 4 from Fig. 1, see for details the Experimental section, were applied to a CM-cellulose column (15.0×2.0 cm). Elution was done with a linear gradient of 0–0.5 *M* NaCl in the equilibrating buffer. Remaining conditions are described in Fig. 2.

leukocyte and pancreatic elastases of both porcine and chicken origin.

The molecular mass of the clTI-1 as estimated by Sephadex G-75 gel chromatography was shown to be 6300. This was in agreement with the value determined by SDS–PAGE (M_r 6200) indicating that the native molecules are monomers (Fig. 6 lane 4). The inhibitor is resistant to acetone, ethanol and heat denaturation. Its antitrypsin activity remained unchanged after treatment of the purified preparation at 100°C for 15 min at neutral pH. Automated Edman degradation of the native protein yielded the NH_2-

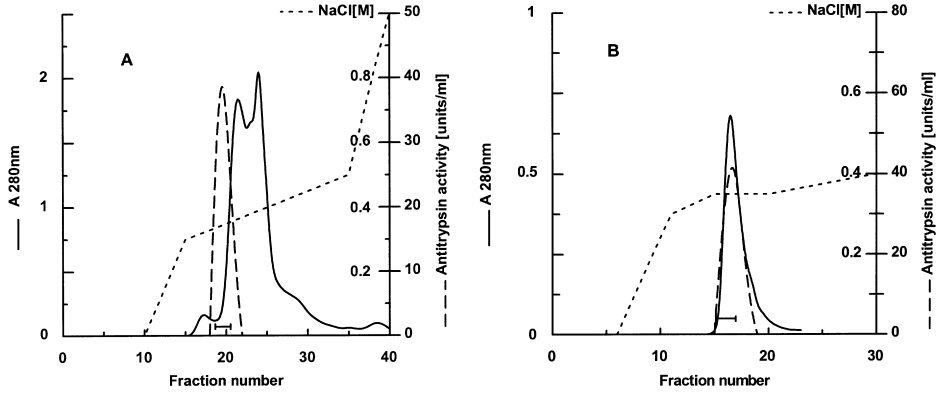

Fig. 5. Cation-exchange chromatography of clTI-2 on a Mono S FPLC column. (A) Inhibitory fractions obtained after CM-cellulose column rechromatography (Fig. 4) were applied to a Mono S column equilibrated with buffer A and eluted with a linear gradient of NaCl (0–1.0 *M*). One-ml fractions were collected at 1 ml/min. (——), absorbance; (– – – –), activity; (------), NaCl. (B) Fractions from (A) containing the highest antitrypsin activity were rechromatographed on a Mono S FPLC column under the same conditions using eluting gradient of NaCl as shown in the figure.

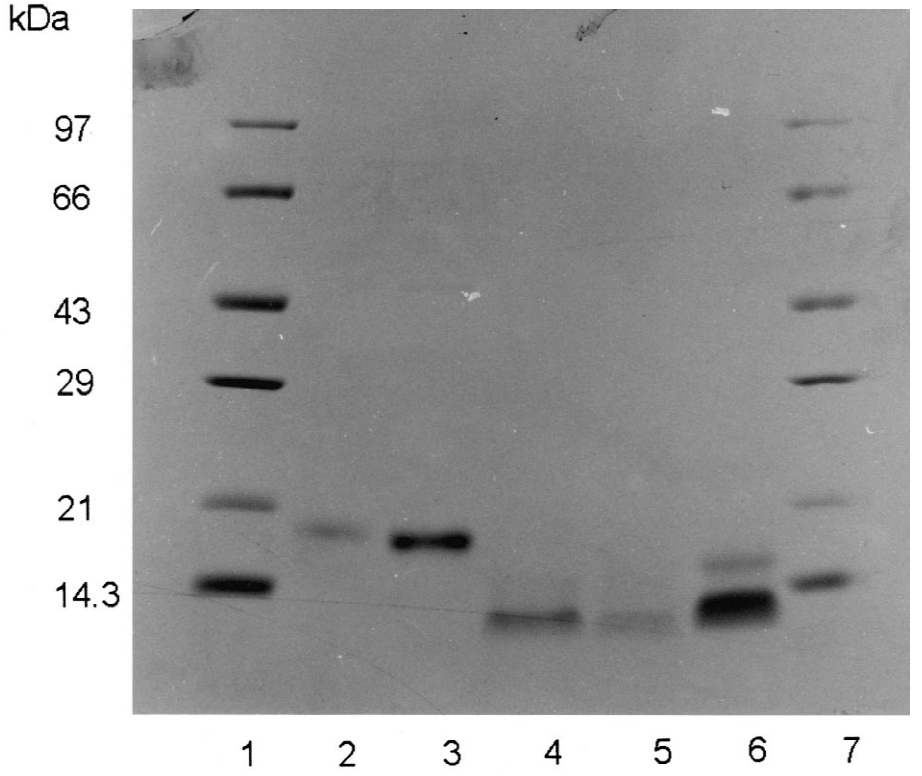

Fig. 6. Polyacrylamide gel electrophoresis of inhibitors in 10–20% gradient gel. Lanes 1 and 7, molecular mass markers; lane 2 and 6, reference proteins: *Heliothis zea* elastase inhibitor (M_r 18 300) and BPTI (M_r 6500); lane 3 clTI-2 (10 μg); lane 4 and 5, clTI-1 (10 and 5 μg respectively); The molecular mass markers are phosphorylase *b* (M_r 97 400), bovine albumin (M_r 66 000), egg albumin (M_r 43 000), carbonic anhydrase (M_r 29 000), soybean trypsin inhibitor (M_r 21 000), chicken egg lysozyme (M_r 14 300). Electrophoresis was carried out as described in the Experimental section kDa=kilodalton..

terminal sequence of 23 amino acid residues (Fig. 8). This sequence showed some identity to a fragment of the human inter-α-trypsin inhibitor [15]. The equilibrium association constants (K_a) for the interaction of purified inhibitors with selected enzymes are presented in Table 2. The association constants for the binding of trypsin to purified inhibitor were high and nearly identical for the porcine and chicken enzymes ($2.5\cdot10^9$ M^{-1} and $1.1\cdot10^9$ M^{-1} respectively). The inhibition of plasmin was significantly lower and appeared to be $1.2\cdot10^7$ M^{-1}. The data presented in Fig. 7 show that the antitrypsin activity of the inhibitor is very sensitive to arginine modification, indicating that this amino acid residue is located in the trypsin binding sites of the protein.

Table 2
Association equilibrium constants (M^{-1}) for the interaction of chicken liver inhibitors with some serine proteinases. The association constants were determined in 0.1 *M* Tris–HCl buffer, pH 8.3 with 0.005% Triton X-100 which in case of cathepsin G was supplemented with 0.5 *M* NaCl

Inhibitor	Trypsin (porcine)	Trypsin (chicken)	Cathepsin G (human)	Plasmin (human)
clTI-1	$2.5\cdot10^9$	$1.1\cdot10^9$	NI[a]	$1.2\cdot10^7$
clTI-2	$4.1\cdot10^8$	$4.8\cdot10^8$	$7.1\cdot10^7$	NI[a]

[a] NI – no inhibition.

The second inhibitor (clTI-2) appeared to be a single chain protein of molecular mass 17 000 as judged from the elution volume on a Sephadex G-75 column and SDS–PAGE. Its activity remained stable during 15 min treatment up to 80°C at neutral pH. At higher temperatures the protein was rapidly inactivated. The N-terminal amino acid sequence of 28 residues (Fig. 8) revealed high homology of this

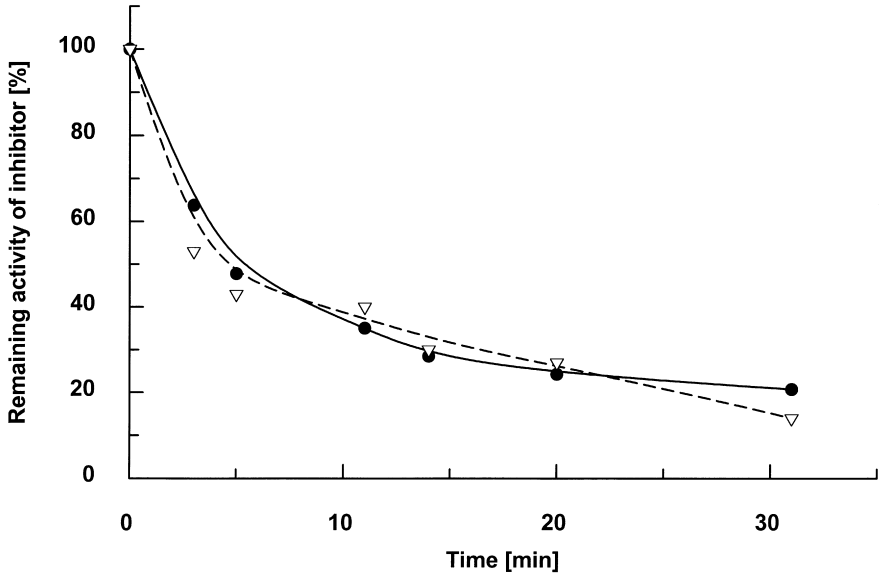

Fig. 7. Effect of *p*-hydroxyphenylglyoxal on inhibitory activity of clTI-1 and clTI-2. Inhibitor (50 nmol) and *p*-hydroxyphenylglyoxal (50 μmol) were incubated for given time periods in 0.1 *M* triethanolamine–sodium borate buffer, pH 7.8, in dark at room temperature and aliquots were removed and assayed for residual activity towards porcine trypsin. ●—●; ▽-----▽, clTI-2.

inhibitor to the third domain of chicken ovoinhibitor [16]. The inhibitor strongly inhibited both porcine (K_a=4.1·10^8 M^{-1}) and chicken (K_a=4.8·10^8 M^{-1}) trypsins as well as human cathepsin G (K_a=7.1·10^7 M^{-1}) but did not affect the activity of human plasmin, thrombin and elastase from human

	1	10	20
clTI-1	**A P P** A A E K Y Y **S** L P P **G A** P R **Y Y** - **S** P **V V**		
inter-α-trypsin inhibitor	**A P P** - - - - - - **S** C C S **G A** L - **Y Y** G **S** K **V V**		
	379		398
	1	10	20
clTI-2	**S V D** V **S K Y P S T V S K D G R T L V A C P R I L S P V**		
chicken ovoinhibitor	**S V D** C **S K Y P S T V S K D G R T L V A C P R I L S P V**		
	156		183

Fig. 8. Alignment of N-terminal sequence of clTI-1 and clTI-2 with a fragment of human inter-α-trypsin inhibitor and the third domain of chicken ovoinhibitor, respectively. Automated sequence analysis was performed on 320 pmol aliquots of each preparation.

leukocytes as well as the chicken pancreatic elastase. In contrast to ovoinhibitor [17], clTI-2 had no antichymotrypsin activity. Since blocking of arginine residue with *p*-hydroxylphenylglyoxal, alike to clTI-1, resulted in a rapid decrease of activity towards trypsin (Fig. 7) it is assumed that this residue is involved in the binding site for this enzyme.

To date only a few inhibitors of serine proteinases, other than serpins, have been found in rat and bovine livers [18,19]. The inhibitors purified in this study are members of either Kazal or Kunitz family and to our knowledge are the first serine proteinase inhibitors separated from the liver of chicken.

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