Purification of two species of exudate cysteine-proteinase inhibitors that are acute-phase reactants in the carrageenin-induced inflammation in rats

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Two species of cysteine-proteinase inhibitors (CPIs) have been purified to homogeneity from exudate in the carrageenin-induced inflammation in rats. The exudate CPIs were separated into two forms (named CPI-1 and -2) in affinity chromatography on *S*-carboxymethyl-papain-Sepharose, the final stage of purification. CPI-1 and -2 gave different mobilities in polyacrylamide gel electrophoresis (PAGE), probably because of different isoelectric points (p*I* 4.47 for CPI-1 and p*I* 4.21 for CPI-2). Both CPI-1 and -2 showed immunological identity in double immunodiffusion and same molecular mass of 68 kDa when analysed by SDS-PAGE. These results indicate that CPI-1 and -2 are very similar but distinct CPIs. CPI-1 and -2 are acute-phase reactants and probably represent two species of T-kininogens having inhibitory activity toward cysteine proteinases.

Acute-phase reactant *Cysteine-proteinase inhibitor* *α_1 Acute-phase protein* *T-kininogen* *Inflammation*

1. INTRODUCTION

Plasma concentration of several proteins markedly increases in inflammation, connective tissue disorders and cancer [1–3]. One of these acute-phase reactants in rats is α_1MAP that is identical to α_1CPI [4,5]. This CPI has been identified as T-kininogen which contains a T-kinin sequence (Ile-Ser-bradykinin) [6,7]. Recently, Furuto-Kato et al. [6] reported the close resemblance in nucleotide sequence between the α_1MAP mRNA and the T-prekininogen mRNAs by the cloning and sequence analysis of cDNAs for rat low-molecular-mass prekininogens including K-prekininogen and two types of T-prekininogens. The increase in plasma CPIs is due to the increase in T-kininogens that are able to inhibit cysteine proteinases [8,9]. We report here the purification of two types of exudate CPIs that are acute-phase reactants in the carrageenin-induced inflammation in rats.

Abbreviations: CPI(s), cysteine-proteinase inhibitor(s); α_1 MAP, α_1 major acute-phase protein; PAGE, polyacrylamide gel electrophoresis; Cm, *S*-carboxymethyl; BAPNA, N^{α}-benzoyl-DL-arginine *p*-nitroanilide

2. MATERIALS AND METHODS

2.1. *Materials*

DEAE-Sephacel, Sephadex G-150 and CNBr-activated Sepharose 4B were purchased from Pharmacia; Ampholine (pH 4–6) was from LKB Instruments; papain (type IV) and BAPNA from Sigma; gelatin and Freund's complete adjuvant from Difco and carrageenin (Seakem 202) was from Marine Colloid Inc. All other reagents were of the highest purity available.

2.2. *Collection of exudate from inflamed rats*

Inflammation was induced by a subcutaneous injection of 4 ml of a 2% (w/v) solution of carrageenin into a preformed air-pouch on the back

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of rats (male Wistar rats weighing 150–180 g) [10]. Inflammatory fluid was collected from the carrageenin-air pouch on day 7 after the carrageenin injection and centrifuged at 100 000 × *g* for 2 h at 4°C. The supernatant (exudate) was stored at −30°C until use.

2.3. *Purification of CPIs*

All purification procedures were carried out at 4°C. The exudate (total 650 ml) was thawed and dialyzed against 0.02 M Tris-HCl buffer (pH 7.8) containing 2 mM $CaCl_2$ and 0.02% NaN_3. The dialyzed exudate was chromatographed on a DEAE-Sephacel column (2.6 × 36 cm) with a linear gradient of NaCl from 0 to 0.4 M. Papain-inhibiting fractions from the DEAE-Sephacel column were pooled and fractionated by 70% saturation with $(NH_4)_2SO_4$. The resulting precipitate was dissolved and dialyzed against 0.05 M Tris-HCl buffer (pH 7.5) containing 1 M NaCl, 5 mM $CaCl_2$ and 0.02% NaN_3. The dialyzed sample was applied to the Sephadex G-150 column (3.2 × 85 cm) that had been equilibrated in the same buffer.

Papain-inhibiting fractions obtained from gel filtration were pooled and dialyzed against 0.01 M phosphate buffer (pH 7.2) containing 0.145 M NaCl and 0.02% NaN_3 (phosphate buffered saline, PBS). A portion (50 ml) of the dialyzed solution was applied to a Cm-papain-Sepharose 4B column (1.2 × 25 cm) which had been prepared according to Anastasi et al. [11]. The binding proteins were eluted stepwise from the column with the following buffer [12]; (i) PBS, (ii) PBS containing 2 M KSCN, (iii) PBS containing 4 mM KSCN and (iv) 0.02 M phosphate buffer (pH 12.1) containing 0.1 M NaCl and 0.02% NaN_3. Papain-inhibiting fractions obtained from the Cm-papain-Sepharose column were dialyzed against 0.02 M Tris-HCl buffer (pH 7.2) containing 0.05 M NaCl and 0.02% NaN_3, and stored at −80°C.

During the course of purification CPI was followed by measuring its papain-inhibiting capacity using Barrett's method [13].

2.4. *Detection of CPIs by gelatin-containing PAGE*

CPI was detected by electrophoresis on polyacrylamide gel copolymerized with gelatin (a substrate of papain) according to Hanspal et al. [14] and Heussen and Dowdle [15]. CPI protects gelatin from papain digestion and is visualized as a band of undigested gelatin.

2.5. *PAGE and SDS-PAGE*

Two CPIs were separated by PAGE following the method of Davis [16], using 7% acrylamide. The molecular masses of CPIs were measured by SDS-PAGE according to Laemmli [17], using 7% acrylamide. Samples were reduced with 23-mercaptoethanol just before SDS-PAGE.

3. RESULTS

2.3. *Purification of CPIs*

Two CPIs were purified from day-7 exudate in three steps; DEAE-Sephacel chromatography, Sephadex G-150 gel filtration and affinity chromatography on Cm-papain-Sepharose. In the final stage of purification, two CPIs were successively eluted with KSCN and phosphate buffer (pH 12.1) from Cm-papain-Sepharose column (fig.1). The two CPIs differed in mobility in PAGE (fig.2) and are referred to as 'CPI-1' and 'CPI-2', in order of elution from Cm-papain-Sepharose column. CPI fractions eluted with 2 M

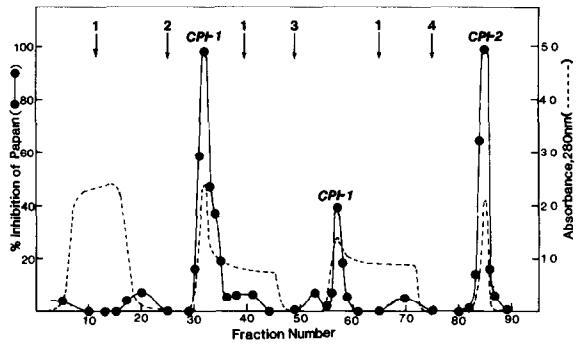

Fig.1. Cm-papain-Sepharose affinity chromatography. Exudate CPI was partially purified by DEAE-Sephacel column chromatography and Sephadex G-150 gel filtration. The partially purified CPI fraction was applied to Cm-papain-Sepharose column. At the points indicated by arrows, PBS (1), 2 M KSCN (2), 4 M KSCN (3) and 0.02 M phosphate buffer (pH 12.1)–0.1 M NaCl (4) were applied stepwise at a flow rate of 42 ml/h. Effluent fractions of 5 ml were collected and papain-inhibiting activity of each fraction was determined. CPI-1 (fraction nos. 31–34 and no. 56) and CPI-2 (fraction nos 84 and 85) were pooled separately.

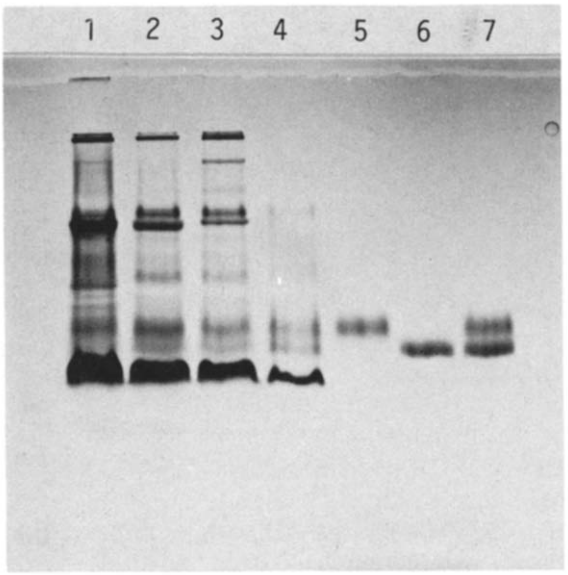

Fig.2. PAGE at various stages in the purification of exudate CPIs. The samples were taken after (1) day 7; (2) DEAE-Sephacel chromatography; (3) $(NH_4)_2SO_4$ precipitation; (4) Sephadex G-150 gel filtration; Cm-papain-chromatography of (5) CPI-1, (6) CPI-2 and (7) mixture of CPI-1 and CPI-2.

KSCN had the same properties as those eluted with 4 M KSCN and, therefore, both the fractions were pooled as CPI-1. CPI-1 and -2 showed reactions of complete immunological identity when run in double immunodiffusion against anti-CPI-1 rabbit serum (not shown), suggesting that CPI-1 and -2 are very similar. Each of CPI-1 and -2 gave a single band on PAGE (fig.2), SDS-PAGE and gelatin-containing PAGE (fig.3). These results indicate that each of CPI-1 and -2 is a purified preparation. Table 1 summarizes the purification of exudate CPIs. From 650 ml of day-7 exudate we obtained 543 mg of CPIs in the two separated species, a recovery of 37% (table 1).

3.2. *Properties of CPIs*

Exudate CPIs are purified into two forms, CPI-1 and -2, that have different mobilities in PAGE (fig.2). These different mobilities in PAGE are due to different isoelectric points; isoelectric points of the CPIs were p*I* 4.47 for CPI-1 and p*I* 4.21 for CPI-2 when measured by sucrose density gradient isoelectric focusing in pH 4–6 Ampholine. In SDS-PAGE the mobilities of CPI-1 and -2 were indistinguishable, corresponding to M_r 68 000 ± 1000 for both CPI-1 and -2. The inhibition of papain by CPI-1 and -2 is non-competitive. K_i values of CPI-1 and -2 were 2.7×10^{-7} M and 4.6×10^{-7} M, respectively, when papain was assayed with BAPNA at pH 6.0 in the presence of CPI-1 or -2. No inhibition of serine proteinases such as trypsin and chymotrypsin by CPIs was found.

As shown in fig.3, both CPI-1 and -2 are present in normal rat plasma in a low concentration, whereas plasma and exudate levels of CPI-1 and -2 markedly increased during the inflammation. On day 2 after carrageenin injection plasma concentrations of CPI-1 and -2 in the inflamed rats were about 14-times higher than those in normal rats when the amounts of CPI-1 and -2 were determined by measuring color intensity of CPI-derived bands in gels after gelatin-containing PAGE. Similarly, on day 7 plasma and exudate concentrations of CPIs in the inflamed rats were about

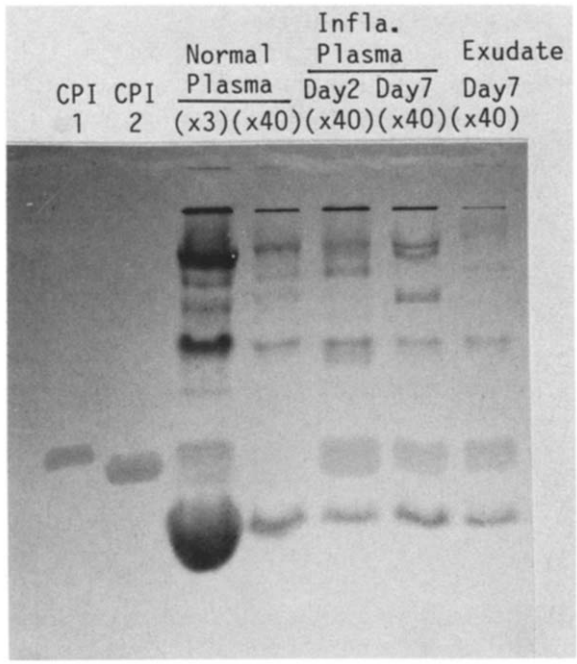

Fig.3. Gelatin-containing PAGE of plasma and exudate. Plasma and exudate were obtained from inflamed rats on day 2 or 7 after carrageenin injection. Normal plasma was obtained from 7-week-old rats that were about the same age as the inflamed rats. Dilution factors of samples are shown in parentheses. Purified CPI-1 and -2 were run simultaneously.

Table 1

Purification of exudate CPIs

Stage	Total volume (ml)	Total protein (mg)	Total activity (units)	Recovery (%)	Spec. act. (units/mg protein)	Purification factor
Day-7 exudate	650	28535	3380	100	0.119	1.0
DEAE-Sephacel	360	9479	2524	75	0.266	2.3
Sephadex G-150	413	4766	1830	54	0.384	3.2
Cm-papain-Sepharose						
CPI-1	184	322	767	23	2.383	20.1
CPI-2	108	221	455	14	2.059	17.4

Units of CPI are defined as mg of papain inhibited in the assay system. Protein was measured by the method of Lowry et al. [19], using bovine serum albumin as a standard

9-times higher than plasma concentration of CPIs in normal rats. These results indicate that both CPI-1 and -2 are acute-phase reactants in the carrageenin-induced inflammation in rats.

4. DISCUSSION

Plasma concentration of CPI-1 and -2 markedly increased in the acute phase and a high concentration of exudate CPIs was found in the chronic phase of the carrageenin-induced inflammation in rats (fig.3). Esnard and Gauthier [5] demonstrated that rat α_1MAP is identical to α_1CPI. They also found that the α_1-CPI gave two electrophoretically distinct components on PAGE but a single band of M_r 68000 on SDS-PAGE. On the basis of molecular mass and properties on PAGE, these two distinct components in acute-phase rat serum are probably identical to CPI-1 and -2 that we have purified from rat exudate in this study. It has been shown by Bauer et al. [18] that molecular masses of rat α_1MAP are 68 kDa for a secreted form and 63.5 kDa for an intracellular unglycosylated form when determined by SDS-PAGE. Recently, Furuto-Kato et al. [6] demonstrated that rat low-molecular-mass prekininogens are encoded by at least three very similar but distinct mRNAs (K-prekininogen, T-prekininogen I and II mRNAs) and indicated a close similarity between mRNA of rat α_1MAP and each mRNA of two species of rat T-prekininogens. In addition, Kageyama et al. [9] found that the T-prekininogen I and II mRNAs increase about 10- and 13-fold over their normal levels after induction of inflammation. A high plasma concentration of CPIs in rats having adjuvant arthritis is almost solely due to the increase in T-kininogen [8]. These findings strongly suggest that CPI-1 and -2 correspond to two species of T-kininogens that have the ability to inhibit cysteine proteinases and markedly increase in an acute phase of inflammation in rats [4–8,18].

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