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# Inactivation of human cystatin C and kininogen by human cathepsin D

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A papain inhibitor of 22 kDa was isolated from human placenta and shown to be identical to residues Cys246-Leu373 of the third domain of human kininogen. This kininogen domain and recombinant human cystatin C were inactivated by peptide bond cleavages at hydrophobic amino acid residues due to the action of cathepsin D. These results further support the proposed role of cathepsin D in the regulation of cysteine proteinase activity.

Cystatin C; Kininogen; Cathepsin D; Proteinase inhibitor inactivation

## 1. INTRODUCTION

Cathepsin D (EC 3.4.23.5) is an aspartic proteinase widely distributed in all mammalian tissues (reviewed in [1]). The enzyme is located intracellularly, in lysosomes, but has also been detected extracellularly under a variety of pathological conditions [2–5]. Cathepsin D plays an important role in the degradation of proteins through limited proteolysis. Thus, cathepsin D is responsible for the specific cleavage of the myelin basic protein [6] and other brain proteins [7], for the conversion of procollagen to collagen [8], parathyroid hormone degradation [9], the release of T-kinin from rat T-kininogen [10,11] and inactivation of human stefin A and stefin B, intracellular protein inhibitors of cysteine proteinases [12].

In this report we show that cathepsin D is able to degrade human cystatin C and the third domain of kininogen, both extracellular protein inhibitors of cysteine proteinases. This finding might be important for elucidation of additional extracellular actions of cathepsin D.

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

Recombinant human cystatin C was prepared as described [13]. Human cathepsin D was isolated from spleen [14]. Papain (type III) (EC 3.4.22.2) and substrate BANA were from Sigma. Bovine haemoglobin was prepared as described in [15]. Pepstatin was from Peptide Research Foundation (Osaka, Japan). Acetonitrile and trifluoroacetic acid were from Merck and BDH Chemicals, respectively. All other chemicals were of analytical grade.

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### 2.2. *Isolation of the third domain of kininogen*

The isolation was carried out following the procedure as described in [16] with some modifications. Briefly, human placenta (1000 g) was homogenized in 0.9% NaCl (1:1.5, w/v) solution and centrifuged for 60 min at 14 000 × *g*. The supernatant was adjusted to pH 10.5 with 5 M NaOH, and after standing 1 h at room temperature, to pH 7.5 with 3 M HCl. After centrifugation the supernatant was applied to an affinity column (7.5 × 5.0 cm) of Cm-papain-Sepharose 4B prepared as in [17], equilibrated with 10 mM Tris-HCl buffer (pH 7.5). The column was washed with the same buffer and the bound proteins eluted then with 10 mM NaOH (pH 11.0). Papain-inhibiting fractions were pooled, adjusted to pH 8.0 with 3 M HCl and concentrated by ultrafiltration on an YM-5 membrane (Amicon). The concentrated sample was dialyzed against 10 mM Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl and applied to a column (120 × 5 cm) of Sephadex G-75 equilibrated in the same buffer. Papain-inhibiting fractions were resolved as three peaks corresponding to $M_r$ of about 12, 25 and 65 kDa. The 25 kDa-$M_r$ protein peak was dialyzed against 10 mM Tris-HCl buffer (pH 8.5) and subsequently chromatographed on DEAE-Sephacel equilibrated with the same buffer. The inhibitor was eluted in a single symmetrical peak with linear gradient of NaCl (0.05–0.15 M) in the same buffer. The sample was concentrated, assayed for its purity by SDS-PAGE and amino acid sequence analysis, and stored at −25°C until use.

### 2.3. *Assays of enzymes and inhibitors*

The inhibitory activity of cystatin C and the third domain of kininogen was determined by measuring the inhibition of papain using BANA as substrate [18]. The cathepsin D activity was measured using haemoglobin as substrate [14] and checked by active-site titration with pepstatin [19]. Protein concentrations were determined by the method of Lowry [20]. One unit of the inhibitor activity was defined as the amount which inhibited 1 μg of papain completely.

### 2.4. *The interaction of CPIs with cathepsin D*

The experiment was performed essentially as in [12]. Solutions of cystatin C or the third domain of kininogen (2 μM) were mixed with an equal volume of cathepsin D (0.5 μM) in 100 μM sodium acetate buffer (pH 3.5) and incubated at 37°C. At various times 100 μl samples were removed and the cathepsin D activity was stopped by acidifying the samples to pH 2.0 with 3 M HCl. As a control, pepstatin (1 μM) was added to cathepsin D prior to incubation. The cleavage products were separated by RP-HPLC on Vydac $C_{18}$-column (250 × 4.6 mm) (10 μm) in a LDC Milton-Roy HPLC using a linear gradient of 5–80% acetonitrile in 0.1% trifluoracetic acid. Flow rate

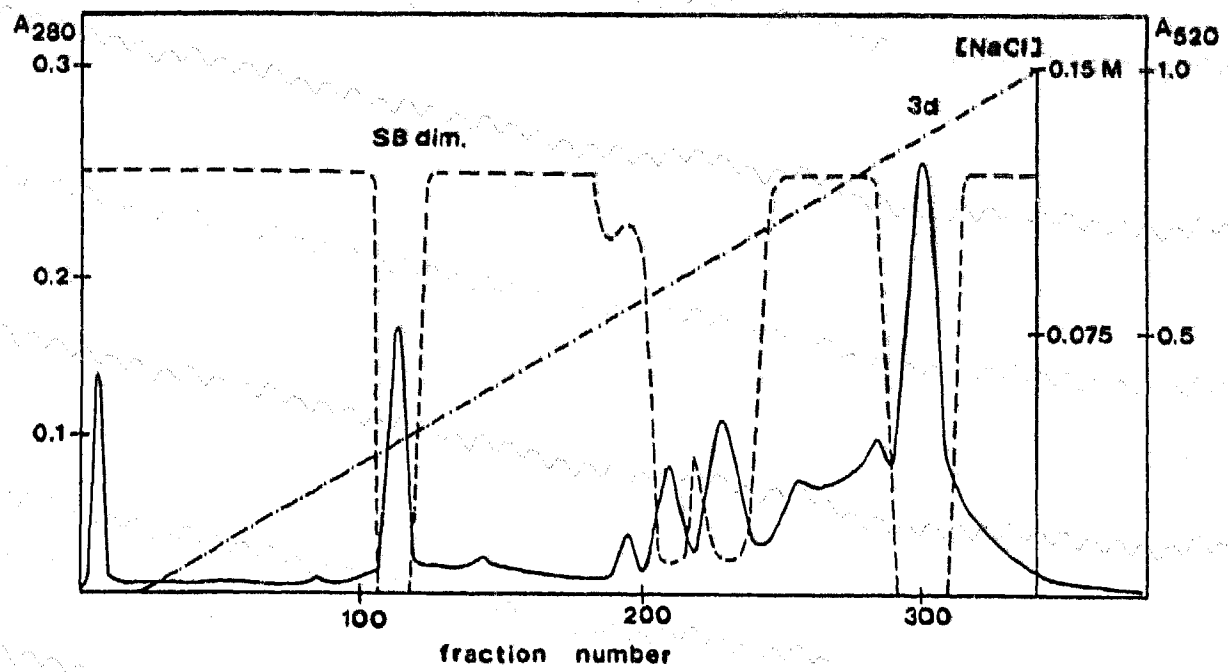


Fig. 1. DEAE-Sephacel chromatography of the 25 kDa-$M_r$ protein peak eluted from Sephadex G-75. (—) protein $A_{280}$; (····) inhibitory activity $A_{520}$; (-·-·-) gradient NaCl; SB dim = dimer form of stefin B; 3d = third domain of kininogen. Fractions of 6.4 ml were collected at a flow rate of 18 ml/min.

was 1.0 ml/min. Peptides were detected by UV-absorption at 220 nm, collected and dried on a Speed Vac (Savant).

2.5. *Amino acid sequence analysis*

Sequence analysis was performed by automatic gas-phase Edman degradation on an Applied Biosystems sequenator 470 A [21], with on-line detection of the released phenylthiohydantoin derivatives of amino acids by an Applied Biosystems amino acid analyser 120A [22].

2.6. *Electrophoresis*

SDS gradient PAGE (8-25%) was carried out according to Laemmli [23].

## 3. RESULTS AND DISCUSSION

### 3.1. *Isolation of the third domain of kininogen*

Ion exchange chromatography of the 25 kDa protein fractions, obtained from gel chromatography on Sephadex G-75, resulted in two major inhibitory peaks eluting at 0.04 M NaCl and 0.13 M NaCl, respectively (Fig. 1). The purity of both inhibitory peaks was checked by SDS-PAGE (not shown). The first peak showed an apparent $M_r$ of 25 kDa without prior reduction by 2-mercaptoethanol whereas an apparent $M_r$ of 13 kDa was obtained after reduction (not shown). This behaviour corresponds to human stefin B which forms an inactive dimer in the absence of reducing agents [16]. The second peak (Fig. 1, 3d) was eluted as a homogenous protein with $M_r$ of about 22 kDa. The N-terminal amino acid sequence analysis of the first 15 residues revealed that the isolated inhibitor is pure. This protein corresponds to the third domain of the kininogen molecule [24]. This fragment starting with Cys-246 (kininogen numbering), was generated by cleavage at Ile-245–Cys-246 due to an unknown proteinase. Similarly, the isolated and sequenced human kininogen fragment of 20 kDa from human synovial fluid [25] resulted from the cleavage at position Thr-243–Lys-244 of the kininogen. These two fragments derived from the junction of the second and the third cystatin-like domain, constituting the kininogen heavy chain. This junction represents one of the proteinase-sensitive regions in the kininogen molecule [24,26].

### 3.2. *Fragmentation of human cystatin C and the third domain of kininogen by cathepsin D*

Incubation of cystatin C and the kininogen domain with cathepsin D resulted in rapid fragmentation of both protein inhibitors. Prolonged incubation up to 45 min resulted in additional fragments. These fragments were separated by RP-HPLC (Fig. 2) and subjected to $NH_2$-terminal sequence analysis. Points of cleavages for human cystatin C and the third domain of kininogen are presented in Fig. 3. Cathepsin D preferentially cleaved hydrophobic residues, one often being aromatic. When digestion was performed at pH 5.0, otherwise under identical experimental conditions the same fragments were obtained (data not shown), although the rate was significantly slower. This pH corresponds to that of lysosomes [27]. Degradation of both protein inhibitors was inhibited completely when

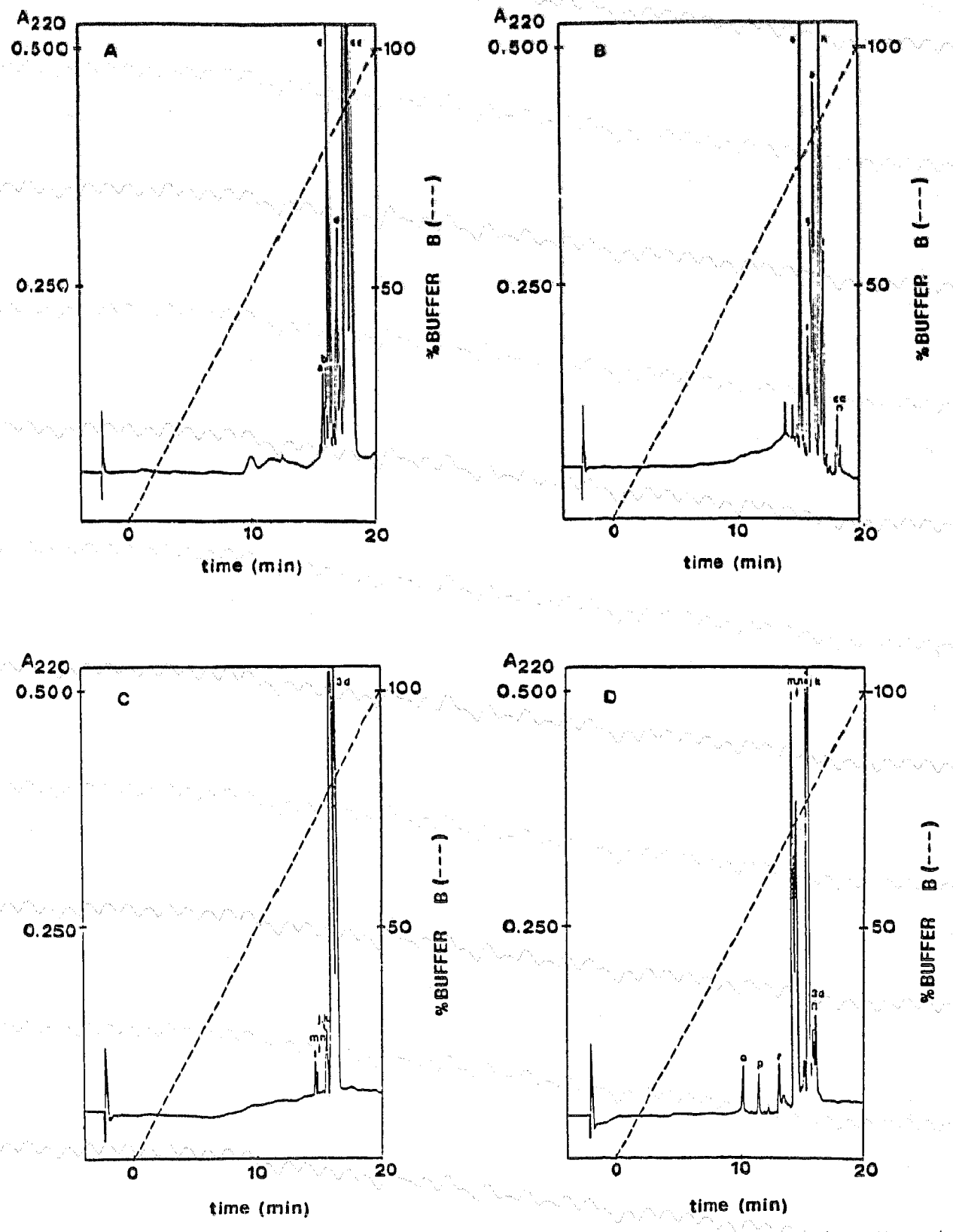


Fig. 2. RP-HPLC of peptides released from cystatin C and the third domain of kininogen after incubation with cathepsin D. (A) cystatin C (cc) after 1 min of incubation; (B) cystatin C (cc) after 40 min of incubation; (C) third domain of kininogen (3d) after 30 s of incubation; (D) third domain of kininogen (3d) after 40 min of incubation; (—) absorbancy $A_{220}$; (- - -) acetonitrile. Volume of 100 μl of reaction mixture was injected into the reverse-phase column. Major peaks are marked and their positions in the sequences are indicated in Fig. 3.

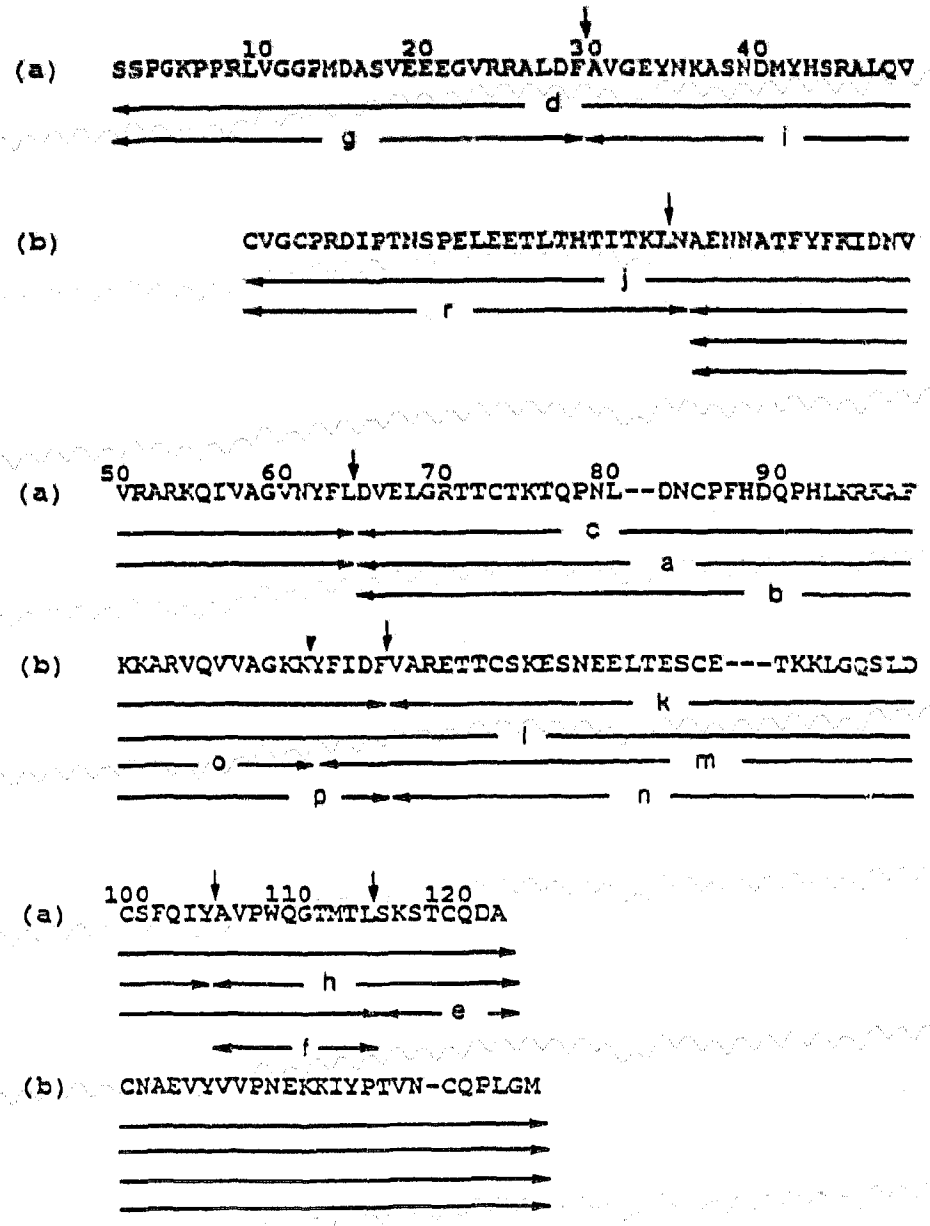


Fig. 3. Action of human cathepsin D on cystatin C (a) and the third domain of kininogen (b). (↓) Major sites of cleavage; (▼) additional cleavage sites. Continuous lines indicates regions of peptide sequenced in cystatin C (a, b, c, d, e, f, g, h, i) and in the third domain of kininogen (j, k, l, m, n, o, p, r).

pepstatin [2 μM] was added, prior to the inhibitors, to the incubation mixture (results not shown). This indicates that the cleavages were generated by cathepsin D. The proteolytic fragments obtained after interaction with cathepsin D did not show inhibitory activity on papain. The exception were both peptides containing residues 55–59 in cystatin C and the corresponding region in the kininogen domain. This region has been identified as important in the binding to proteinases [28,29]. $K_i$ values were above $10^{-6}$ M indicating a greatly decreased binding. However, a similar inhibitory peptide containing this regions with similar $K_i$s was obtained recently after CNBr cleavage of chicken cystatin [30]. Cleavage by cathepsin D inactivates most of the inhibitory activity of the cysteine proteinase inhibitors. Consequently, limited proteolysis of cystatins by this action indicates a possible physiological role for cathepsin D in the regulation of cysteine proteinase activities. Inactivation of CPIs by the aspartic proteinase cathepsin D is important since we would not expect the cysteine proteinases of lysosomes to inactivate them. Specifically, it is known that the interaction of CPIs with their target enzymes does not lead to peptide bond cleavage [29,31].

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