Interaction of human cathepsin C with chicken cystatin

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Abstract Cathepsin C was purified from human spleen by a rapid procedure, which included homogenization, ammonium sulfate precipitation, gel filtration on Sephacryl S-200 and finally affinity chromatography on chicken cystatin-Sepharose. The interaction between cathepsin C and chicken cystatin was further characterized. It was found to be accompanied by a maximum decrease in fluorescence emission intensity at 330 nm. Fluorescence titration showed that human cathepsin C can bind four chicken cystatin molecules. The 4:1 binding stoichiometry was confirmed by titration monitored by the loss of enzyme activity. A non-competitive-competitive type of inhibition was determined from a double-reciprocal Lineweaver-Burk plot with a K_i value of 0.22 nM for the non-competitive inhibition.

Key words: Cathepsin C; Dipeptidyl aminopeptidase I; Cysteine proteinase; Chicken cystatin; Binding stoichiometry

1. Introduction

Cathepsin C or dipeptidyl aminopeptidase I (EC 3.4.14.1) is a lysosomal cysteine proteinase and belongs to the papain family [1]. Like other cysteine proteinases cathepsin C is involved in intracellular protein degradation [2] and is found in a variety of human and rat tissues [3]. Cathepsin C is an oligomeric enzyme, consisting of four identical subunits, each consisting of a heavy and a light chain and a large proregion part, thus differing from other papain-like cysteine proteinases [4]. It is active in the pH range between 3.5 and 8.0 [5] and cleaves peptides and proteins with an unsubstituted amino terminus [6–11], as well as certain synthetic substrates with a blocked amino terminus [12].

Cathepsin C is inhibited by stefins A and B [13–15] and chicken cystatin [13,16], three protein inhibitors of cysteine proteinases from the cystatin superfamily [17]. All inhibitors were shown to be of the competitive, reversible type, with stefin A being the weakest by 2–3 orders of magnitude [13–16]. However, the mechanism of interaction between cathepsin C and cystatins is not known.

Herein we describe a new, efficient method for isolating cathepsin C, based on the interaction between the enzyme and chicken cystatin. A further goal was to determine the binding stoichiometry between these two proteins, since cathepsin C is an oligomeric enzyme.

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Abbreviations: 4MβNA, 4-methoxy-β-naphthylamide; -βNA, -β-naphthylamide.

2. Materials and methods

2.1. Materials

Sephacryl S-200, activated CH-Sepharose 4B and low molecular mass standards were from Pharmacia (Sweden), Ser-Tyr-βNA and Gly-Phe-4MβNA were from Bachem (Switzerland), and DTE and EDTA were from Serva (Germany). Stock solutions of substrates were prepared in dimethylsulfoxide (Merck, Germany). Chicken cystatin was purified [18] and titrated with active-site titrated papain [19]. Chicken cystatin-Sepharose 4B affinity chromatography gel was prepared using activated CH-Sepharose 4B following the manufacturer's instructions. Protein concentrations were determined by the Bio-Rad (USA) Protein Assay [20]. All other chemicals were of analytical grade. All the kinetic experiments were performed in 100 mM phosphate buffer, pH 6.0, containing 100 mM NaCl, 1 mM EDTA and 2 mM DTE, at 25°C.

2.2. Purification procedure

Human cathepsin C was purified from human spleen by a procedure modified from that reported previously for purification from human kidney, which included homogenization, ammonium sulfate precipitation, ion-exchange chromatography on CM Sephadex C-50 column, gel filtration on Sephacryl S-200 column, affinity chromatography on thiol-Sepharose 4B and ion-exchange chromatography on a Q-Sepharose FF column [4]. Following homogenization and ammonium sulfate precipitation, the sample was directly applied on a Sephacryl S-200 (Pharmacia, Sweden) column (3×140 cm). Cathepsin C-containing fractions were pooled, concentrated and then applied to a chicken cystatin-Sepharose column (1.5×10 cm), equilibrated with 100 mM piperazine buffer pH 5.0 containing 100 mM NaCl and 1 mM EDTA. After thorough washing of the column with the same buffer, cathepsin C was eluted with 5 mM citric acid, pH 3.5. 5-ml fractions were collected and the pH immediately adjusted to pH 6.0 with 1 M Bis-Tris buffer (pH 6.0). Fractions with $A_{280} > 0.05$ were pooled, concentrated and dialyzed against 100 mM acetate buffer, pH 5.0 containing 300 mM NaCl and 1 mM EDTA. Cathepsin C was stored at 4°C.

2.3. SDS-PAGE

SDS-PAGE was performed on a PhastSystem (Pharmacia, Sweden) using an 8–25% gradient gel following the manufacturer's instructions.

2.4. Fluorescence measurements

Fluorescence measurements were performed in a Perkin-Elmer LS-50 spectrofluorimeter (UK) using an excitation wavelength of 280 nm. Cells with 1 cm path lengths were used. The excitation and emission bandwidths were 5 and 10 nm, respectively. Titration of cathepsin C with chicken cystatin was performed as described previously for other cysteine proteinase-cystatin interactions [21,22] by monitoring the decrease in fluorescence emission intensity accompanying the interaction at 330 nm, where the maximum fluorescence change was observed (see Section 3). The final concentration of cathepsin C was 11.5 nM. The binding curve was analysed by non-linear regression analysis to the equilibrium binding equation [23].

2.5. Titration of cathepsin C by the loss of enzymatic activity

Enzymatic titrations of cathepsin C with chicken cystatin were performed similarly to the procedure described earlier [19]. Cathepsin C (11.5 nM final concentration) was added to 200 μl of reaction buffer, followed 5 min later by the addition of 300 μl of chicken cystatin (various concentrations) in the same buffer. After 30 min of incubation at 25°C, 100 μl of 150 μM Ser-Tyr-βNA in the same buffer were added. The release of product was monitored with a Perkin-Elmer

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spectrofluorimeter. Residual activities were determined by linear regression analysis and the binding stoichiometry was then determined by non-linear regression analysis of the data to the theoretical binding equation [24].

In order to determine inhibition constants and type of inhibition, experiments were performed essentially as described above, except that cathepsin C and chicken cystatin were used at much lower concentrations (0.01 and 0–0.088 nM final concentrations, respectively). In addition, substrate concentration was varied (1–120 μM final concentration).

3. Results and discussion

Cathepsin C was purified to homogeneity from human spleen by a simple and rapid method based on affinity chromatography on immobilized chicken cystatin. After solubilization of lysosomal enzymes, the high-molecular mass cathepsin C (200 kDa) was separated from other lower molecular mass proteins. In the final step active cathepsin C was separated by affinity chromatography. A product gave a pattern on SDS-PAGE (Fig. 1) which was identical with that obtained for the enzyme prepared by using the earlier method [4], indicating that the isolated enzyme was pure. Cathepsin C is an oligomeric enzyme, each subunit consisting from three different polypeptide chains [4]. From 500 g of spleen we obtained 8 mg of cathepsin C. This is a much higher yield than those obtained by other methods, which included several chromatography steps [4,25,26]. The main advantage of this procedure, however, is its rapidity, which allows larger quantities of the enzyme to be purified in a short time. Since chicken cystatin is known as a good inhibitor of various cysteine proteinases, this method may well serve as a tool for the rapid purification of other papain-like cysteine proteinases.

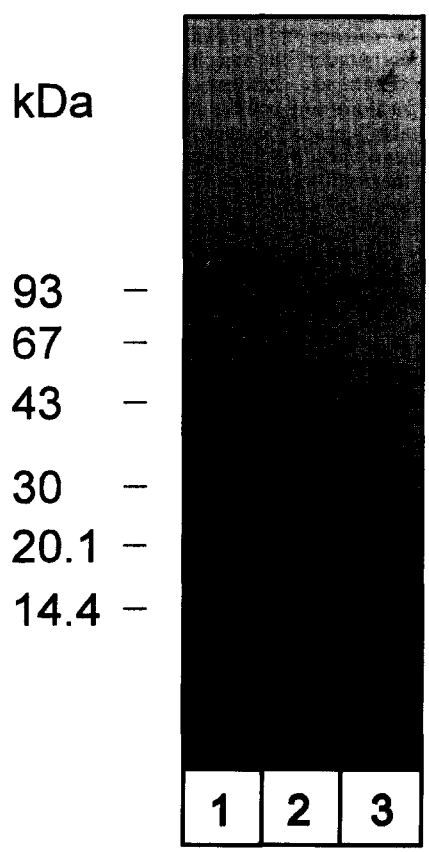

Fig. 1. SDS-PAGE of human cathepsin C after the affinity chromatography step. Lanes: 1, standards; 2, human cathepsin C under reducing conditions; 3, human cathepsin C under nonreducing conditions.

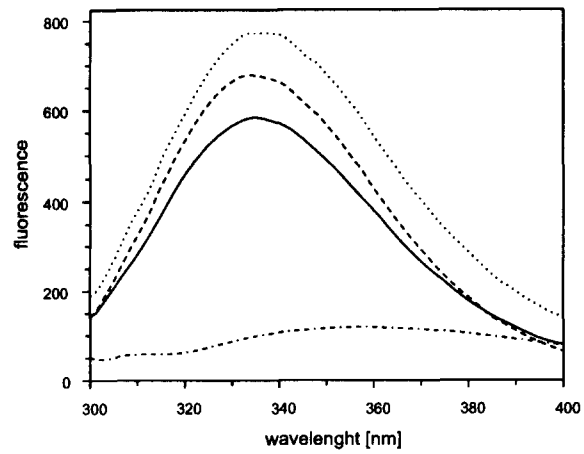

Fig. 2. Fluorescence emission spectra of chicken cystatin (· - ·), human cathepsin C (- - -), the complex between cathepsin C and cystatin (——), and the sum of spectra for cathepsin C and cystatin (· · ·). The concentrations of cathepsin C and cystatin were 11.5 and 46 nM, respectively.

We have studied the mechanism of interaction between chicken cystatin and cathepsin C by various methods. Binding of chicken cystatin to cathepsin C resulted in a decrease of fluorescence emission intensity (Fig. 2). The largest decrease of intensity due to complex formation was observed between 330 and 340 nm. This suggests that some of the Trp residues of enzyme and inhibitor are involved in the interaction. From previous studies it is known that Trp^{104} of chicken cystatin and Trp^{26}, Trp^{69} and Trp^{177} of papain [21,27,28] are involved in the enzyme-inhibitor interaction. However, the fluorescence intensity accompanying the interaction decreased only by 28% (at 330 nm), which is substantially less than for the chicken cystatin/papain interaction [21]. Nevertheless, the value is comparable to those of other chicken cystatin/cathepsin interactions (B. Turk, unpublished results). This difference could be explained by Trp^{26}Tyr and Trp^{69}Tyr substitutions (papain numbering) in cathepsin C [29].

Fluorescence titration of cathepsin C with chicken cystatin, standardized with highly active papain, gave an apparent chicken cystatin/cathepsin C binding stoichiometry of 3.4 ± 0.1 (Fig. 3a). The result thus indicates four inhibitor binding sites on each cathepsin C molecule, in agreement with the previous study [4], which showed four substrate binding sites on each cathepsin C molecule. The observed binding stoichiometry most likely reflects the presence of some inactive material in the cathepsin C preparation. Despite the low protein concentrations used, it was impossible to determine the K_d values with good precision. Nevertheless, from the shape of the curve it can be concluded that the K_d values are ≤ 2 nM, although it is impossible to distinguish between the K_d values for individual binding sites.

To confirm the above results, titration of cathepsin C with chicken cystatin was monitored by the loss of enzymatic activity against synthetic substrates. As can be seen in Fig. 3b,

3.5 ± 0.1 mol of inhibitor were needed to saturate 1 mol of cathepsin C, in agreement with the results on fluorescence titration.

In order to determine the type of inhibition of cathepsin C by chicken cystatin, their interaction was studied at different substrate and inhibitor concentrations. The rates of product formation are shown in double-reciprocal Lineweaver-Burk plots (Fig. 4). Lines 1–3 show non-competitive inhibition (intercept on the baseline) and lines 3–5 exhibit competitive inhibition (intercept on the vertical axis). For reasons of clarity all the data have not been presented in Fig. 4. All the plots of *1/v* vs. [I] (Dixon plot) exhibited a downward curvature at higher inhibitor concentrations (not shown). According to Dixon [30], such an inhibition type can be described as a non-competitive-competitive one, i.e. non-competitive at low and competitive at high ligand concentrations. Under non-competitive conditions, an approximate K_i value of 0.22 nM was determined for the human cathepsin C-chicken cystatin interaction from the plot of *1/v* vs. 1/[S] [30]. At higher substrate and inhibitor concentrations, however, the system becomes too complex to allow determination of individual K_d values for the other binding sites.

In conclusion, cathepsin C was purified from human spleen by a modified method, where the most important step is affinity chromatography on immobilized chicken cystatin. Various titrations showed that one molecule of human cathepsin C can simultaneously bind four chicken cystatin molecules. The type of inhibition was determined to be non-competitive-competitive.

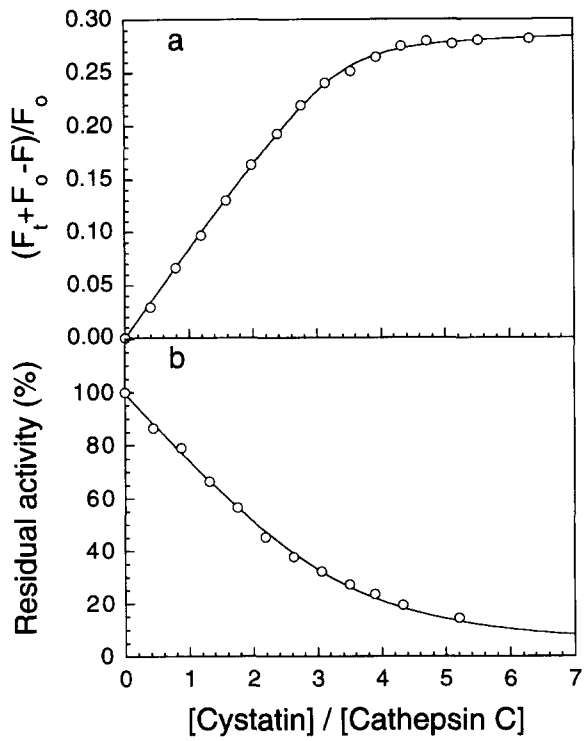

Fig. 3. Titration of human cathepsin C (11.5 nM) with chicken cystatin. (A) Monitored by measurements of tryptophan fluorescence. F_t, fluorescence of added chicken cystatin; F_0, fluorescence of cathepsin C; F, fluorescence of cystatin-cathepsin C mixture. Fitted curve was generated by nonlinear regression analysis [23]. (B) in the presence of substrate, monitored by measurements of the loss of enzyme activity. Fitted curve was generated by nonlinear regression analysis [24].

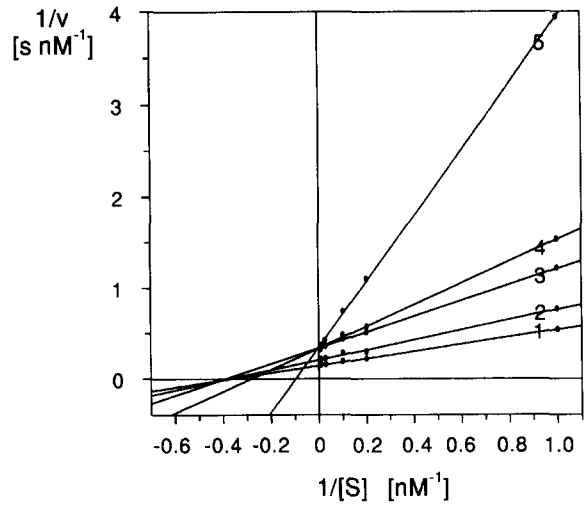

Fig. 4. Lineweaver-Burk plots for the inhibition of human cathepsin C with chicken cystatin. Line 1 represents cathepsin C activity (0.01 nM) in the absence of inhibitor. Lines 2–5 show the enzyme activity in the presence of 0.011, 0.022, 0.066, and 0.088 nM cystatin, respectively.

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