AN ENDOGENOUS INHIBITOR OF CYSTEINE AND SERINE PROTEINASES FROM SPLEEN

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1. Introduction

Much attention has been paid to endogenous inhibitors of proteinases which have the property of inhibiting 2 different types of proteinases, such as cysteine proteinases, e.g., cathepsin B, H, L, S, papain [1–6,9] and the serine intracellular proteinases of chymotrypsin type [2,7–9]. We have studied the inhibitory mechanism of this inhibitor, based on an active sulfhydryl group of the inhibitor which may interact with a disulphide bond of the inhibited proteinase. According to this property we named it sericystatin, indicating its inhibitory property against serine and cysteine proteinases [10]. Multiple molecular forms exist of this type of intracellular inhibitor [4,9]. Their M_r-values range from 11 000–13 000 and, they differ in pI and intracellular location, and in inhibitory activity against various proteinases.

This paper describes the techniques used for the purification of the cysteine cytosolic inhibitors from bovine spleen, as well as some of their characteristics. In a purification procedure we have taken advantage of high stability of this type of inhibitor in alkaline solutions.

2. Materials and methods

Spleen cytosolic inhibitors were prepared from fresh bovine spleen (100 g) which was cut up and cells squeezed out to avoid excessive damage. The cells were suspended in 400 ml cold Hank's buffer, followed by centrifugation at 400 × *g* for 5 min. This procedure was repeated 3 times to remove serum proteins. The rinsed cells were resuspended with a 1:3 (v/v) ratio of Hank's solution and homogenized with a Teflon homogenizer under ice-cooling. The homogenate was centrifuged (4°C) at 5000 × *g* for 15 min. The sediment was discarded and the supernatant further centrifuged at 50 000 × *g* for 45 min. This supernatant, cytosol (freed of nuclei and granules) was used in our experiments.

Purified samples of cathepsin B, H and S were prepared from bovine spleen by covalent coupling to commercial thiol–Sepharose [11]. Papain was supplied by Sigma (St Louis) and trypsin from NBC (Ohio). Sepharose 4B, thiol–Sepharose, Sephadex G-75 and G-50 and DEAE-Sephacell were supplied by Pharmacia (Sweden).

2.1. *Assays*

The activities of cathepsin B and papain were determined using Bz Arg Nap (Sigma) as substrate, and the activity of cathepsin H was determined with Leu Nap (Sigma) as substrate, according to [12]. Cathepsin S was assayed with azocasein as substrate, following [11]. Trypsin activity was measured with Bz Arg Na as substrate following [13].

Inhibitor activity was assayed under the same conditions as those used for proteinase activities, but with inhibitor sample in place of some of the buffer. The enzyme was maintained at a constant level (10–25 μl) in 0.2 N phosphate buffer at pH 6.5 with the addition of variable quantities of inhibitor (25–200 μl). The total volume of buffer, enzyme and inhibitor was 0.25 ml. After 10 min preincubation at room temperature, the samples were assayed on substrates for the determination of the residual enzyme activity.

2.2. *Other methods*

The M_r of the inhibitor was estimated by gel filtration on a Sephadex G-50 Superfine column (1 × 60 cm). Standards were ovalbumin, trypsinogen,

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cytochrome *c* and pancreatic trypsin inhibitor (Sigma).

Disc gel electrophoresis of inhibitor samples was done as in [14] on 6 cm cylindrical gels of 7% acrylamide in 25 mM Tris, 0.2 M glycine (pH 8.4). Gels were stained for protein with Coomassie blue.

The isoelectric point of the inhibitor samples was determined on an apparatus used for analytical isoelectric focusing (Desaga), using 5% polyacrylamide plates of 9 × 16 cm, with carrier ampholines of pH 2–8 and by isoelectric focusing. In this case, pH 3.5–10.0 carrier ampholite in an LKB 8101 column in sucrose density gradient were used.

Protein was assayed as in [15]. The dissociation constant of the inhibitor, K_i, isolated from papain Sepharose, was determined from Dixon plots [16].

2.3. *Inactivation of inhibitor*

The effect of alkaline treatment on the activity of inhibitor samples was tested by preincubating the inhibitor samples at 37°C in 0.2 M Tris buffer (pH 12.0) for 10 min. After the incubation the mixtures were readjusted to pH 6.5 with 5 M HCl. Heat treatment was performed at the same concentration of inhibitor samples, for the same time (10 min) in 0.2 N phosphate buffer (pH 6.5). Residual inhibitory activity was tested against papain, cathepsin B and cathepsin H.

2.4. *Gel filtration, ion-exchange chromatography and affinity chromatography*

The concentrated cytosol (alkaline activated) was dialyzed against 20 vol. 0.01 M Tris–HCl, containing 0.3 M NaCl (pH 7.5) and gel filtered on Sephadex G-75 column (8 × 100 cm). Pooled fractions of the low M_r inhibitory protein were concentrated by ultrafiltration through a UM-10 membrane and dialyzed against 20 vol. 2 mM Tris–HCl (pH 7.5). The dialysand was divided in half and chromatographed twice on a DEAE-Sephacel column (3 × 30 cm). Inhibitory proteins eluted with the starting buffer and with a linear gradient of NaCl (0.00–0.15 M), total vol. 1 litre.

Papain coupled to Sepharose 4B (1.5 g) was prepared following the Pharmacia pamphlet. Papain was dissolved in 0.1 M sodium bicarbonate (pH 8.5) at 6 mg protein/ml. The washed gel was suspended in 5 ml ligand solution and shaken for 24 h at 4°C. The yield of papain bound to Sepharose was ~70% (mg protein) and 75% of enzyme activity. Papain–Sepharose was finally washed with 2 mM Tris buffer (pH 7.5).

3. Results

Cytosol was adjusted to pH 12 with 5 M NaOH at room temperature. After 10 min it was readjusted to pH 7.5 with 5 M HCl and the precipitate formed was removed by centrifugation at 5000 × *g* for 15 min. This final supernatant was used in purification. Cytosol alkalization resulted in ~17-fold increase (release) of inhibitory activity, from 300 IU to 5200 IU (table 1A) and therefore we term it the 'alkaline activating step of cytosol'.

Table 1
Purification of sericystatin from spleen

Stage	Vol. (ml)	Protein (mg/ml)	Act. (IU)	Spec. act. (IU/mg)	Yield (%)
A: Cytosol	300	20	300	0.05	
Cytosol pH 12-treated	260	7.2	5200	2.9	100
Sephadex G-75	119	0.6	2100	29.1	40
DEAE-Sephacel (7 peaks)	60	0.3	1500	83.3[a]	29
B: Peak 4 from DEAE-Sephacel	6.2	0.3	228	123	100
Affinity chrom.	7.6	0.04	172	570	77

[a] Average specific activity of 7 eluted inhibitory peaks from DEAE-Sephacel; the values vary from 52–135 IU/mg

IU, inhibitory units based on papain activity toward Bz Arg Nap; 1 inhibitory unit = decrease of ΔA_{520} of 0.1/10 min

The results of the purification procedure are shown in table 1A,B. By ultrafiltration (UM-10) concentrated cytosol was dialyzed, and then chromatographed on a Sephadex G-75 column. Papain inhibitory proteins eluted in 2 peaks. The fractions from both peaks were also tested on trypsin and a smaller inhibitory activity was noted in fractions of ~5000 M_r and 8000 M_r. The small amount (~10%) of high M_r papain inhibitory proteins was discarded and the low M_r inhibitory fractions, from the major peak with ~15 000 M_r were concentrated (UM-10), and applied on a DEAE-Sephacel column (fig.1). A minor part (15–20%) of the inhibitory activity eluted with non-adsorbed material in peaks 1 and 2. After addition of a NaCl gradient, the main part of the inhibitory activity eluted in peaks 3–7. The total yield of all 7 inhibitory peaks from the DEAE-Sephacel column was ~30%, in comparison to the total inhibitory activity of the alkaline activation step, (table 1A). The eluted fractions of inhibitory peaks 1–4 and 7 were tested on cathepsin B, H, S and papain. The inhibitors of separated peaks differ considerably in their inhibitory activity against the enzymes tested (fig.2, table 2).

The inhibitor eluted in peak 1 acted most efficiently against all 4 tested enzymes, whereas the inhibitors of all other peaks did not inhibit cathepsin B. Inhibitor eluted in peak 2 showed only a weak inhibition against cathepsin H and papain.

The isoelectric point of the proteins eluted in the 7 inhibitory peaks, determined by analytical isoelectric focusing were: 7.0–6.8 (peak 1); 6.7–6.5 (peak 2); 5.8–5.2 (peaks 3–6); and 4.8–4.6 (peak 7). The estimated M_r values of these isoinhibitors were ~13 000.

Table 2 shows the alkaline (pH 12) and heat-stability of the inhibitors of peaks 1 and 4. Both inhibitors were relatively stable at pH 12, but they differ in heat-stability. The inhibitor of peak 1 showed thermal instability; significant loss of activity was noted against all 3 tested enzymes.

The protein inhibitor of peak 4 from DEAE-Sephacel was further purified on papain–Sepharose (fig.3). Non-specific proteins were eluted with starting buffer, containing 3 M NaCl. The inhibitor was eluted with 0.1 M Tris (pH 10.5) in fractions 45–52. Each eluted fraction was immediately readjusted from pH 10.5 to pH 6.5 with 5 M HCl. The specific activity in this purification step increased 4.7-fold (table 1B), from 123 IU/mg to 570 IU/mg, and the yield of ~77%, is rather high. The gel electropherogram of this inhibitor showed 2 well-defined protein bands (fig.3) and their pI-values were 5.2–5.3. Increase of specific activity from alkaline-activated cytosol

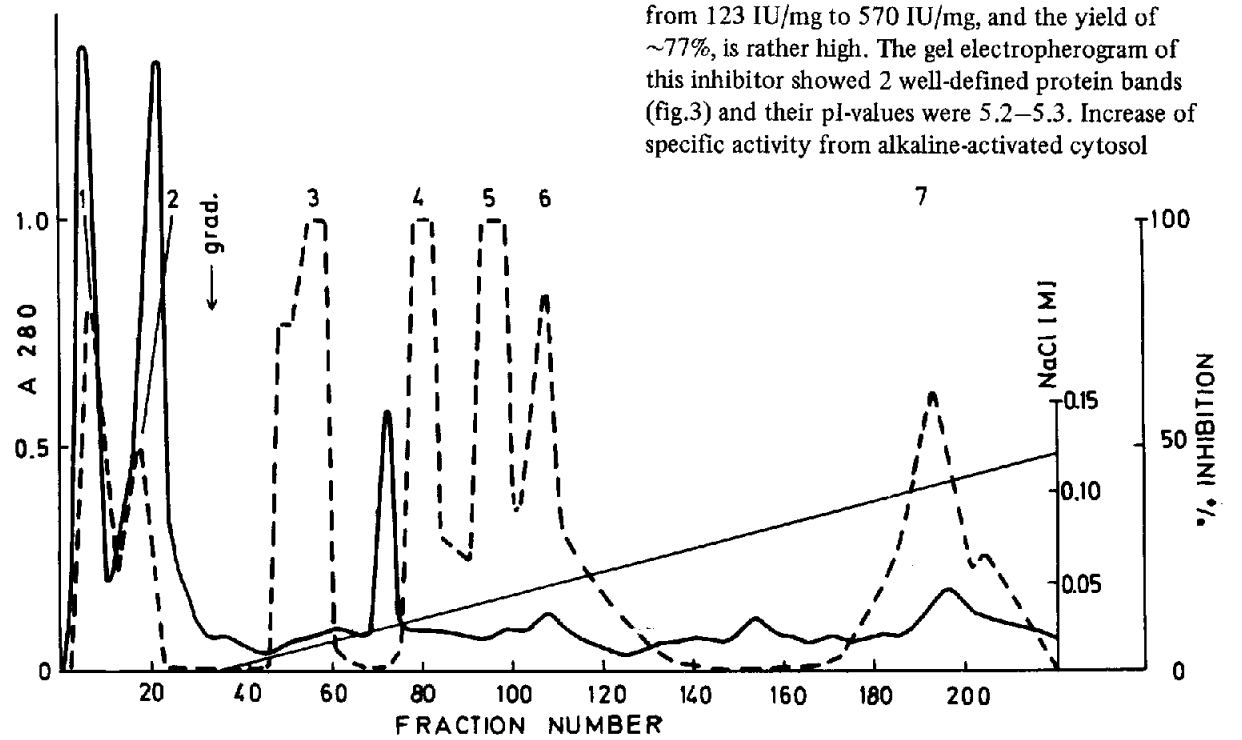

Fig.1. DEAE-Sephacel chromatography of pooled and concentrated inhibitory fractions from Sephadex G-75: 0.1 ml fraction was assayed against 3 μg papain for inhibitory activity; (——) protein; (– – –) % inhibition.

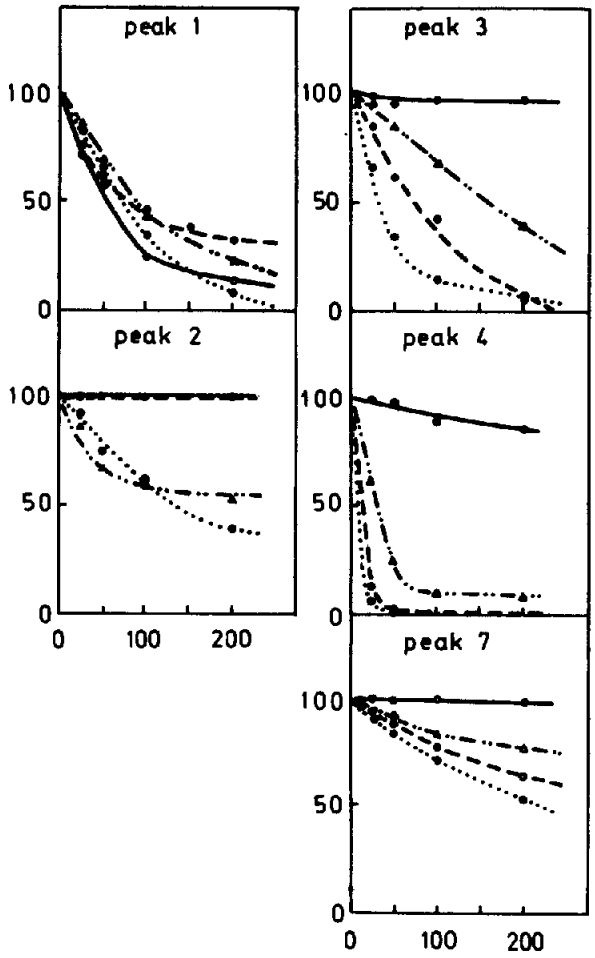

Fig.2. Inhibition of cathepsin B (——), cathepsin H (·—·—·), cathepsin S (– – –) and papain (·····) with increasing amounts of inhibitors from peaks 1–4 and 7, eluted from DEAE-Sephacel: ordinate, residual activity (% of control); abscissa, amount of inhibitor added (μl).

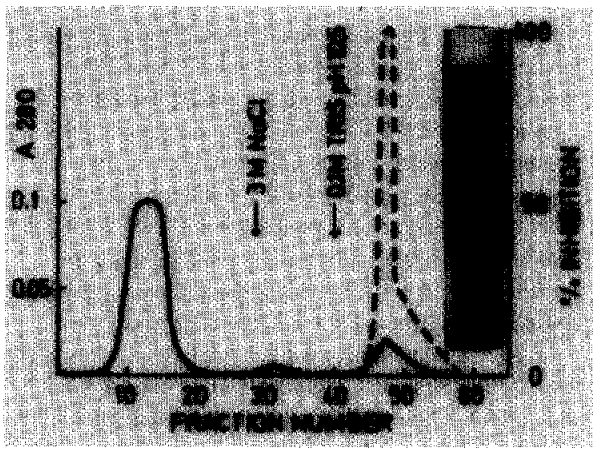

Fig.3. Profile of affinity chromatography on papain–Sepharose. Combined fraction 82 and 83 (peak 4) from DEAE-Sephacel were applied: (——) protein; (– – –) % inhibition of papain.

(2.9 IU/mg) to the final purification step of the inhibitor (570 IU/mg) was 197-fold, whereas from the starting cytosol (0.5 IU/mg) it was 11 400-fold.

The inhibitor, bound to papain–Sepharose, can be eluted also by 0.02 M NaOH (pH 12) or by GSSG (0.5 mg/ml in 0.2 N phosphate buffer (pH 7.0) In a former experiment, by pH 12 treatment (corrected back to pH 7.0) inhibitor protein possessed inhibitory activity, but the yield was much smaller (~40%). In case of GSSG treatment, the eluted inhibitor protein was inactive, because it was eluted in the form of an oxidized glutathione:inhibitor complex, as reported in [10].

The purified inhibitor non-competitively inhibited papain, tested on Bz Arg Nap substrate, and its K_i was 6.5×10^{-9} M.

Table 2
Effect of heat (90°C) and pH 12 treatments on the inhibitory activity of isoinhibitors isolated from DEAE-Sephacel

Sample	Treatment	Cathepsin B (3 μg)	Cathepsin H (5 μg)	Papain (3 μg)
Inhibitor	Control	79	73	76
(12 μg) from	pH 12	46	53	66
peak 1	90°C	14	25	36
Inhibitor	Control	0	87	94
(12 μg) from	pH 12	0	55	66
peak 4	90°C	0	85	93

The results are expressed as % of cysteine proteinase inhibition, by treated inhibitor samples, relative to control (C) inhibitor samples

Control = inhibitor sample preincubated for 10 min at room temperature in buffer solution of pH 6.5

4. Discussion

Leucocytes [2,7,9,10], spleen [2,8], liver [4,6], skin [1] and other tissues contain not only sericystatin with an acid pI, but also an isoform with a pI near neutrality [9,10]. It was already known that these protein isoinhibitors inhibit intracellular proteinases of cysteine and serine type. We had also found that cathepsin D does not inactivate (degrade) sericystatin, whereas it does inactivate other types of intracellular inhibitors, such as the inhibitors of urokinase [17,18] and elastase [17].

The experiments including gel filtration, anion exchange chromatography and affinity chromatography of the activated (treated to pH 12) spleen cytosol revealed the presence of 7 inhibitory proteins of ~13 000 M_r and pI-values from pH 4.8–7.0. These inhibitors differ not only in pI but also in their activity against various tested cysteine proteinases and in heat stability.

It is evident that the 'alkaline activating step of cytosol' (table 1A) is very important in the generation (release) of inhibitor activity. The total inhibitory activity increased ~17-fold. Thus the increase of inhibitory activity may be based on dissociation of enzyme–inhibitor complexes, and on inactivation (precipitation) of cysteine and chymotrypsin-like neutral proteinases. The dissociation of such complexes (cysteine proteinase–sericystatin) has been confirmed: cysteine proteinases can be replaced from their complexes by the action of chymotrypsin-like neutral proteinase or by oxidized glutathione [10].

A similar mechanism of enzyme–inhibitor complex dissociation has been found for collagenases [19,20], and a neutral proteinase [21]. Concerning the action of cysteine [22] and chymotrypsin-like neutral proteinase [23] in normal and pathological events it is likely that these inhibitors play a protective role in elimination of unwanted proteolysis. This type of intracellular inhibitor has the ability to inhibit the growth of metastases in vivo [24], as well as the growth of cells in colonies in vitro [9].

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