Identification of bovine stefin A, a novel protein inhibitor of cysteine proteinases

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Received 10 January 1995

Abstract For the first time, three different stefins, A, B and C, have been isolated from a single species. The complete amino acid sequence of bovine stefin A was determined. The inhibitor, with a calculated M_r of 11,123, consists of 98 amino acid residues. Although it exhibits considerable similarity to human and rat stefin A, some significant differences in inhibition kinetics were found. Bovine stefin A bound tightly and rapidly to cathepsin L ($k_{ass} = 9.6 \cdot 10^6$ $M^{-1} \cdot s^{-1}$, $K_i = 29$ pM). The binding to cathepsin H was also rapid ($k_{ass} = 2.1 \cdot 10^6$ $M^{-1} \cdot s^{-1}$), but weaker ($K_i = 0.4$ nM) due to a higher dissociation rate constant. In contrast, the binding to cathepsin B was much slower ($k_{ass} = 1.4 \cdot 10^5$ $M^{-1} \cdot s^{-1}$), but still tight ($K_i = 1.9$ nM).

Key words: Stefin; Cystatin; Cysteine proteinase; Cathepsin; Amino acid sequence

1. Introduction

The lysosomal papain-like cysteine proteinases, which play an important role in intracellular protein degradation [1], are controlled by their endogenous protein inhibitors, the cystatins [2,3]. On the basis of sequence similarity, the cystatins can be divided into three evolutionarily related families of proteins: the stefins, the cystatins and the kininogens. The stefins (also called Family I cystatins) are small, acidic proteins, consisting of about 100 amino acid residues and lacking disulphide bonds. The cystatins (Family II cystatins) are somewhat larger proteins than the stefins, consisting of about 115 amino acid residues and having two disulphide bridges. The best characterized representatives of the stefins are human stefins A and B [2,3]. Recently, we purified and characterized two members of the stefin family from bovine thymus, bovine stefins B and C [4,5].

Both the stefins and the cystatins are competitive, reversible inhibitors which form tight, equimolar complexes with their target proteinases [6–8]. On the basis of the X-ray crystal structures of chicken cystatin [9] and stefin B in complex with papain [10], a novel enzyme–inhibitor binding mechanism was proposed. Three regions of the inhibitor were shown to interact with the enzyme: two hairpin loops and the N-terminal part [9,10], which appears to be more important in the cystatins [11–13].

In this paper we describe the purification and characterization of stefin A from bovine skin. The complete amino acid sequence of the inhibitor was determined and compared with other known sequences of inhibitors from the stefin family. Moreover, the kinetics of interaction of the isolated inhibitor with mammalian cysteine proteinases were characterized to clarify further the role of cystatins as potent physiological regulators of cysteine proteinase activity.

2. Materials and methods

2.1. Enzymes

Papain (EC 3.4.22.2; 2× crystallized; Sigma), human cathepsin L (EC 3.4.22.15) and bovine cathepsins B (EC 3.4.22.1) and H (EC 3.4.22.16) were purified [6,14–16] and active site titrated with Ep-475 (Peptide Research Institute) [5].

2.2. Purification of the inhibitors

The inhibitors were purified from bovine skin by a method similar to that used for the purification of inhibitors from bovine thymus [5]. Following homogenization, alkaline treatment, affinity chromatography on Cm-papain-Sepharose and gel-filtration on a Sephadex G-50 (Pharmacia-Biotech) column, the dialysed inhibitory sample was applied to a DEAE-cellulose (Whatman) column (40 × 2 cm), equilibrated with 20 mM Tris buffer, pH 7.3. After thorough washing, the bound proteins were eluted with a linear gradient of NaCl (0–0.3 M) in the starting buffer. The inhibitory fractions were pooled and concentrated.

SDS-PAGE and analytical isoelectric focusing were performed with the PhastSystem (Pharmacia-Biotech), following the instructions of the manufacturer.

Protein concentrations were determined by absorption measurements at 280 nm with the use of absorption coefficients and molecular masses calculated from amino acid sequences with the Lasergene program (DNASTAR Inc.).

The active concentration of the inhibitor was determined by fluorescence titration with papain [6,7].

2.3. Sequence determination and amino acid analysis

10 nmol of inhibitor (bovine stefin A or bovine stefin C) were dissolved in 70% formic acid. Crystalline CNBr (Pierce) was added in two portions in a 1:1 weight ratio immediately and after 24 h of incubation at room temperature in the dark. The reaction was terminated 12 h later by freeze-drying.

Native stefin A (10 nmol) was digested with endoproteinase Lys-C (EC 3.4.21.50; Boehringer) at a 1:25 enzyme-to-substrate weight ratio for 24 h at room temperature in 0.3 M Tris buffer, pH 8.6, containing 0.1 mM $CaCl_2$ and 5.0 M urea. The reaction was stopped by acidification to pH 3.0 with trifluoroacetic acid.

Clostripain (EC 3.4.22.8; Sigma) digestion of stefin A was done in 20 mM Tris buffer, pH 7.0, containing 10 mM $CaCl_2$, 0.005% (w/v) Brij

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Abbreviations: -AMC, 4-methyl-7-coumarylamide; Brij 35, polyoxyethylenelaurylether; Bz-, benzoyl; CNBr, cyanogen bromide; Cm-, carboxymethyl-; CPI, cysteine proteinase inhibitor; Ep-475, L-3-carboxy-trans-2,3-epoxypropyl-leucylamido-4-(guanidino) butane; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel-electrophoresis; Z-, benzyloxycarbonyl-.

The amino acid sequence data reported in this paper will appear in the EMBL Database under the accession number P80416 Stefin A from Bovin.

SSDI 0014-5793(95)00060-7

35 and 2.0 M urea at a proteinase-to-substrate weight ratio of 1:50. After 3 h at 40°C, the peptides were immediately separated by HPLC.

Peptide mixtures were separated by HPLC (Milton Roy) on a reverse-phase ChromSep C8 column (Chrompack), equilibrated with 0.1% trifluoroacetic acid in water. The peptides were eluted with an acetonitrile gradient (0–70%) at a flow rate of 1 ml/min. The absorbance was continuously monitored at 215 nm.

Amino acid analyses of hydrolyzed samples (24 h in 6.0 M HCl at 110°C) were performed on a 421 Amino Acid Analyzer (Applied Biosystems) with pre-column phenylisothiocyanate derivatization.

An Applied Biosystems 475A liquid-pulse sequencer, connected to a 120A phenylthiohydantoin-amino acid analyzer from the same manufacturer, was used for automated sequence analyses of the proteins.

2.4. Kinetic measurements

Papain was routinely assayed with Bz-DL-Arg-2-naphtylamide (Sigma) [17] as substrate in 100 mM phosphate buffer, pH 6.0, containing 1.5 mM EDTA and 2 mM dithiothreitol. The activity of the inhibitors against papain throughout the course of the purification was determined under the same conditions.

The kinetics of inhibition of cysteine proteinases were analysed at 25°C as described for the thymus inhibitors [5]. All experiments were done under pseudo first-order conditions with at least tenfold molar excess of the inhibitor. The following buffers were used: 100 mM phosphate buffer, pH 6.0, for cathepsin B; 100 mM phosphate buffer, pH 6.8, for cathepsin H; and 50 mM acetate buffer, pH 5.5, containing 100 mM NaCl, for cathepsin L. All buffers also contained 1.5 mM EDTA and dimethylsulfoxide at a concentration of 2.5% (v/v). Cathepsins B and L were assayed with 5 μM Z-Phe-Arg-AMC (Peptide Research Institute) as substrate, whereas cathepsin H was assayed with 10 μM Arg-AMC (Bachem). Substrate consumption was less than 3% in all experiments.

3. Results

3.1. Purification of inhibitors from skin

A mixture of cysteine proteinase inhibitors from bovine skin was isolated by affinity chromatography on immobilized papain and subsequently separated on an ion-exchange column. Seven papain-inhibiting peaks were obtained (not shown), of which four were identified as described below. The first peak, which did not bind to the column, was bovine cystatin C. Stefin B (peak 2) eluted at 0.06 M NaCl, stefin C (peak 5) at 0.18 M NaCl and stefin A (peak 6) at 0.20 M NaCl. The other peaks were not characterized. Additional attempts to separate the two isoforms of stefin A (see below) on a Mono Q column (Pharmacia-Biotech) were unsuccessful; all the material bound to the column and was eluted as a symmetrical peak. From 1 kg of starting material we obtained 1.5 mg of stefin A.

3.2. SDS-PAGE and analytical isoelectric focusing

All the inhibitors migrated as single bands with M_r 12,000–14,000 in SDS-PAGE without reduction (not shown). In analytical isoelectric focusing, cystatin C was shown to be the only basic inhibitor with a pI value of 8.2. Stefins B and C showed single bands with pI values of 5.6 and 4.8, respectively, whereas stefin A appeared in two isoforms with pI values 4.7 and 4.95, similar to the human and rat variants [2]. All other inhibitory proteins were acidic with pI values ranging from 4.5 to 6.5 (Fig. 1).

3.3. N-Terminal sequencing and amino acid analyses

The N-terminal sequence of the first inhibitory peak from DEAE-cellulose, LGGLMEADV, together with amino acid analysis, identified the protein as a truncated form of bovine cystatin C [18]. The protein in the second peak was N-terminally blocked; however, amino acid analysis showed that it was identical to bovine stefin B from thymus [4]. The N-terminus of the fifth inhibitory peak was also blocked, but sequence analysis of the second CNBr fragment of this protein identified it as full-length stefin C [5]. The sixth inhibitory peak was identified as bovine stefin A by total sequence determination, as described below.

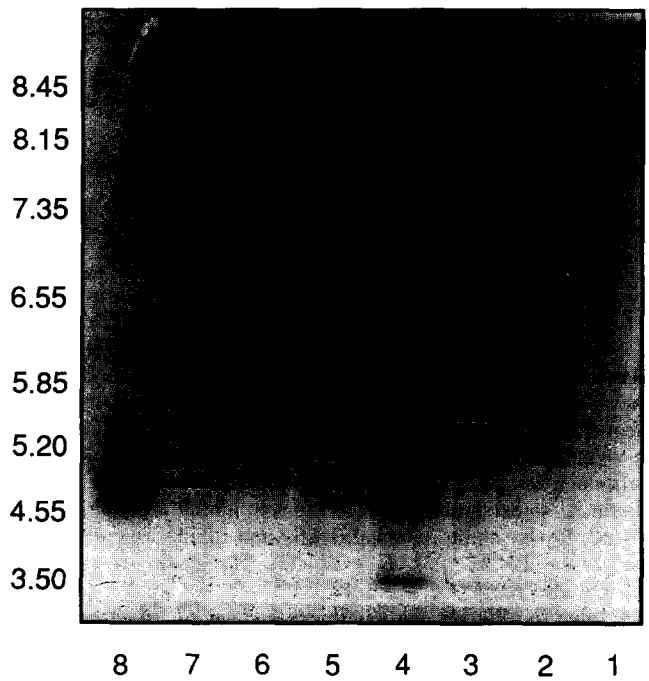

Fig. 1. Isoelectric focusing of the purified inhibitors after ion-exchange chromatography. Lane 1, peak 1 (cystatin C); lane 2, peak 2 (stefin B); lane 3, peak 3; lane 4, standards; lane 5, peak 4; lane 6, peak 5 (stefin C); lane 7, peak 6 (stefin A); lane 8, peak 7.

3.4. Sequence determination of stefin A

N-Terminal sequence analysis of native stefin A gave a single sequence up to residue 32 (uncleaved molecule in Fig. 2). A very high initial yield of the run (>95%) indicated that the α-amino group of the inhibitor was not blocked. The sequence revealed a Met residue at position 22, and therefore the first set of peptides was prepared by CNBr cleavage (CN peptides in Fig. 2). The resulting two peptides were sequenced, and the CN-1 peptide was found to correspond to the N-terminal part of the inhibitor, whereas the CN-2 peptide corresponded to the stefin A sequence from Val-23 onwards.

Two other sets of peptides were prepared from stefin A by endoproteinase Lys-C (K-peptides in Fig. 2) and clostripain (R-peptides in Fig. 2) digestions. The sequence of the C-terminal part of the inhibitor was established from the K-7 (residues 72–89) and K-8 (residues 90–98) peptides. The K-8 peptide ended with Phe, which is not a cleavage site of the highly specific Lys-C endoproteinase, indicating that the C-terminal residue is Phe, as in all other inhibitors of the stefin family (Fig. 2). The sequence of the R-2 peptide (residues 64–93) provided the overlap between the K-6, K-7 and K-8 peptides.

3.5. Amino acid sequence comparison

Alignment of the amino acid sequence of bovine stefin A with those of other stefins (Fig. 3) showed that the sequence identity

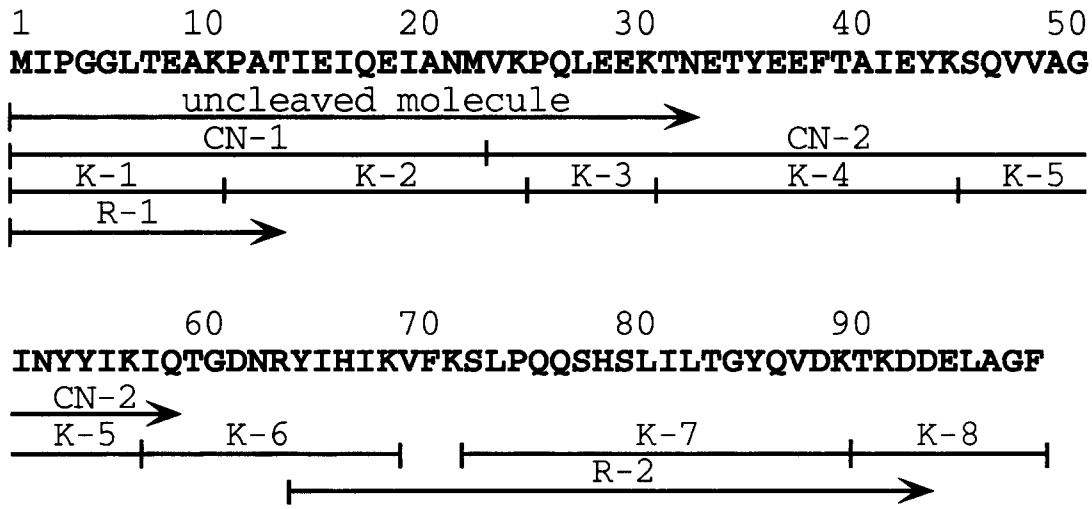

Fig. 2. Complete primary structure of bovine stefin A. CN, peptides obtained by CNBr cleavage; K, peptides from endoproteinase Lys-C digestion; R, peptides from clostripain digestion.

with porcine leukocyte cysteine proteinase inhibitor and human stefin A is 72%, with rat stefin A 55%, with bovine stefin B 49%, with human stefin B 48% and with rat stefin B and bovine stefin C 47%.

3.6. Inhibition kinetics

The interaction kinetics of bovine stefin A with the cysteine proteinases cathepsins B, H and L were characterized under pseudo first-order conditions by continuous fluorimetric assays. All progress curves showed an exponential approach to a final linear rate and were analyzed by the least-squares fitting of the appropriate integrated rate equation [19] to the experimental data. The linearity of product formation in separate experiments without inhibitors verified that the enzymes were stable during the experiments. A linear dependence of the observed pseudo first-order rate constant on inhibitor concentration was observed for all enzyme–inhibitor pairs investigated (Fig. 4), consistent with a simple, competitive inhibition mechanism [8,19]. Apparent second-order association rate constants, k_{ass}, were obtained from the slopes of these plots. No correction for substrate concentration was necessary for cathepsins B and H, as the substrate concentrations were appreciably lower than the K_m values for the enzymes, i.e. 150 μM for cathepsin B [20] and 115 ± 20 μM, determined in this work, for cathepsin H. However, the k_{ass} values for cathepsin L were corrected with the use of a K_m of 2 μM [21]. Most dissociation rate constants, k_{diss}, could not be obtained from these measurements, as the intercept on the ordinate was indistinguishable from zero. However, the k_{diss} value for the interaction with cathepsin H, estimated from this intercept, was in good agreement with the value calculated as described below.

K_i values were calculated by linear regression analysis of the

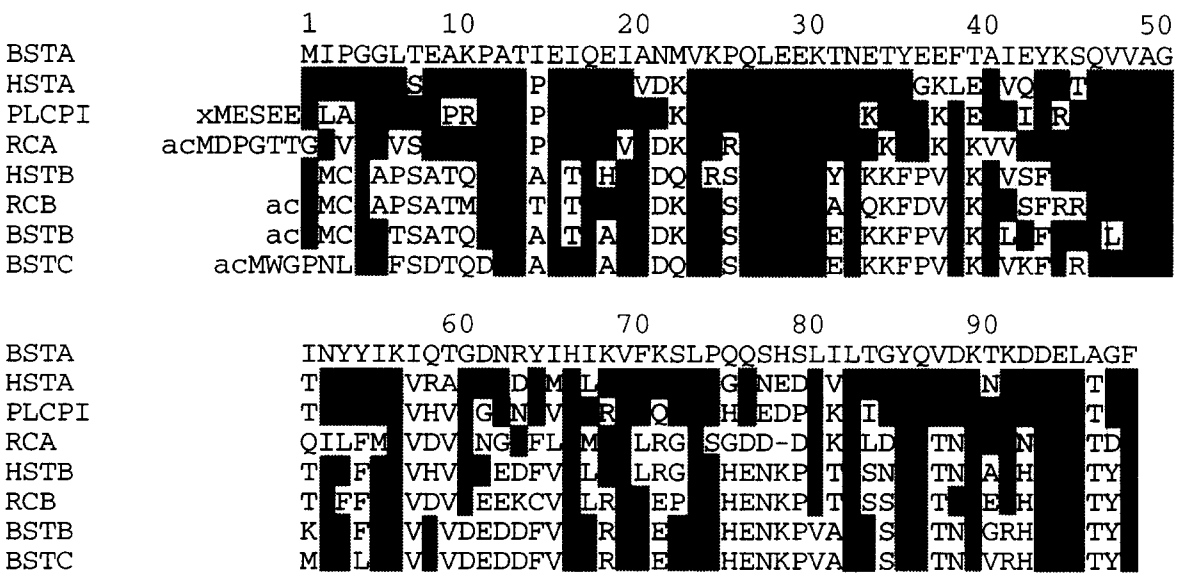

Fig. 3. Alignment of the amino acid sequence of bovine stefin A with those of other stefins. BSTA, bovine stefin A; HSTA, human stefin A; PLCPI, porcine leukocyte CPI [28]; RCA, rat cystatin α; HSTB, human stefin B; RCB, rat cystatin β; BSTB, bovine stefin B [4]; BSTC, bovine stefin C [5]. Residues identical to those in bovine stefin A are marked with a filled square; the numbering is that of bovine stefin A. Other references are listed in Turk and Bode [3].

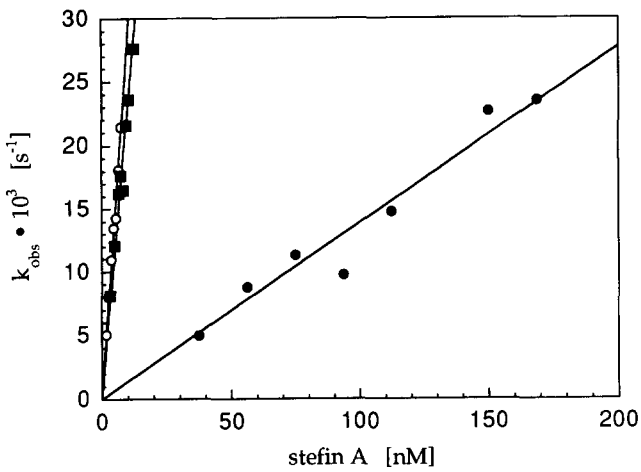

Fig. 4. Dependence of the observed pseudo first-order rate constant (k_{obs}) on the inhibitor concentration for the interaction between bovine stefin A and cathepsins B, H and L. (●) Cathepsin B; (■) cathepsin H; (○) cathepsin L. Experimental conditions are described in section 2. The solid lines were generated using the best estimates for apparent k_{ass} and k_{diss} obtained by linear regression analysis according to Morrison [19].

initial (v_z) and steady-state (v_s) rates of substrate hydrolysis obtained at different inhibitor concentrations according to Morrison [19]. All plots of $(v_z/v_s - 1)$ vs. $[I_o]$ gave straight lines. Again, corrections for substrate competition were necessary only for cathepsin L. The k_{diss} values were calculated from K_i and k_{ass} values ($k_{diss} = K_i \cdot k_{ass}$). All kinetic and equilibrium constants are given in Table 1.

4. Discussion

Several low-M_r inhibitors of cysteine proteinases, including cystatin C, stefin A, stefin B and stefin C, were isolated from bovine skin. This is the first time that three different inhibitors of the stefin family have been isolated from a single species. Stefin A was found to be the most abundant inhibitor, in agreement with previous studies with rat and human stefin A, showing a high level of this inhibitor in skin [2].

The sequence of bovine stefin A showed considerable similarity with those of other stefins, especially human stefin A and porcine leukocyte CPI. The sequence identity with human stefin A is high in the N-terminal part (86% in the first 35 residues) and in the C-terminal part (88% in the last 17 residues), whereas it is lower in the middle part of the molecule. The central QVVAG region is conserved in bovine stefin A, as in other stefins, except bovine stefin B, in which the first Val is replaced by Leu [4].

Table 1
Kinetic data for the interaction between cathepsins B, H and L and bovine stefin A

Enzyme	$10^{-5} \times k_{ass}$ ($M^{-1} \cdot s^{-1}$)	K_i (nM)	$10^4 \times k_{diss}$ (s^{-1})
Cathepsin B	1.4 ± 0.15 (7)	1.79 ± 0.10 (6)	2.5
Cathepsin H	21.0 ± 1.0 (8)	0.44 ± 0.03 (7)	9.2
Cathepsin L	96.5 ± 5.3 (7)	0.029 ± 0.003 (7)	2.8

The experimental conditions are described in section 2. The best estimates for the association rate constants (k_{ass}) and equilibrium constants (K_i), together with their standard errors, were calculated as described in section 3. The dissociation rate constants (k_{diss}) were calculated from K_i and k_{ass} values. The number of measurements is given in parentheses.

Kinetic and equilibrium studies revealed that bovine stefin A binds rapidly and tightly to cathepsin L, with a K_i value substantially lower than that reported earlier for human stefin A [22]. The rate and equilibrium constants for the other two enzymes are in good agreement with previously reported values for the human inibitor [22,23]. However, the inhibitory properties of bovine stefin A differ from those of bovine stefins B and C, especially with regard to the tight inhibition of cathepsin B [5].

The kinetic data indicate that the mechanism of inhibition of cysteine proteinases by stefin A is different for different proteinases. The mechanism of cathepsin L inhibition resembles most closely that of papain, the only enzyme for which the mechanism is known [9,10]. The complex with cathepsin H was formed rapidly as well, but was more unstable, partly due to a higher dissociation rate constant. An octapeptide of the proregion remains bound to mature cathepsin H via a disulphide bond in the vicinity of the active site at the S2 subsite [24]. This octapeptide may substantially influence or even prevent binding of N-terminal residues of inhibitors to cathepsin H, consistent with the high dissociation rate constant. The rate of association of stefin A with cathepsin B was appreciably lower than that with the other enzymes. The crystal structure of human cathepsin B shows an additional loop, not present in papain, of about 20 amino acid residues, which partially occludes the active site. This loop was proposed to interfere with inhibitor binding [25], a suggestion supported by observations that the association rate constants for the interactions of inhibitors with cathepsin B are markedly lower than those for the binding to other enzymes [11,12,23].

A proteinase inhibitor can be of physiological importance if inhibition is achieved at physiological concentrations of the inhibitor in a sufficiently short time with negligible dissociation of the complex [26]. Stefin A entirely fulfils these conditions for all three enzymes investigated and may thus be involved in the control of endogenous cysteine proteinases. It may also play a protective role against invasive organisms that use cysteine proteinases for penetration [27].

Acknowledgements: This work was supported by the Ministry of Science and Technology of the Republic of Slovenia and by the Swedish Council for Forestry and Agricultural Research. We thank A. Sali for the critical reading of the manuscript. When finishing the work, B.T. was supported by a long-term FEBS Fellowship.

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