Volume 298, number 2,3, 237–239 FEBS 10753 February 1992

The complete primary structure of bovine stefin B

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Received 15 November 1991; revised version received 27 December 1991

A new stefin B-type low-M_r CPI was isolated from bovine thymus and subjected to structural analysis. The inhibitor consisted of 98 amino acids and its M_r was calculated to be 11,178. The NH_2-terminal amino acid residue was blocked. The sequence was determined by automated sequencing of peptides derived by cleavage with cyanogen bromide and fragments of the inhibitor resulting from enzymatic digestion with β-trypsin and *Staphylococcus aureus* V-8 proteinase. The NH_2-terminal blocking group was established with mass spectrometry. The inhibitor exhibits considerable sequence homology with inhibitors from the stefin family. Furthermore, a highly conserved QVVAG region within the stefin family is for the first time replaced by the QLVAG sequence.

Stefin B; Cystatin; Cysteine proteinase inhibitor; Primary structure

1. INTRODUCTION

The inhibitors from the cystatin superfamily are reversible, tight-binding inhibitors of the cysteine proteinases from the papain superfamily [1]. On the basis of sequence homology, the cystatin superfamily is divided into three families: low-M_r stefins, cystatins and high-M_r kininogens. Low-M_r inhibitors are structurally different. The stefins with M_r 11,000 lack disulfide bonds, while the cystatins with M_r 13,000 have two intramolecular disulfide bonds (reviewed in [1–3]). The central QVVAG region is more conserved in the stefins than in the cystatins (reviewed in [4]). They also differ in their tertiary structure, the shorter helix nearer to COOH-terminus being present only in the cystatins [5,6].

The mammalian stefins have been isolated and characterized from human [7–13] and rat [14–18] origins, but not from bovine origin. The only well-characterized bovine low-M_r CPI is bovine colostrum cystatin [19,20], which belongs to the cystatin family.

Recently, we isolated a new low-M_r CPI from bovine thymus. Herein we present the complete amino acid sequence of bovine stefin B. It indicates homology to other known stefins, especially to human stefin B.

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Abbreviations: CPI, cysteine proteinase inhibitor; CM, carboxymethyl; PE, pyridylethyl; TFA, trifluoroacetic acid; PP IV, papaya proteinase IV; HPLC, high pressure liquid chromatography; PTH, phenyl-thio-hydantoin; FAB, fast atom bombardment.

2. MATERIALS AND METHODS

CM-papain Sepharose was prepared as described [21]. Bovine stefin B was purified from bovine thymus using a procedure modified from that previously described [5]. Acetone precipitation was excluded and Sephadex G-50 (Pharmacia, Sweden) was used instead of Sephadex G-75. In the final step, the third inhibitory peak obtained by separation on Sephadex G-50 was concentrated, dialyzed against 20 mM Tris buffer (pH 7.3) and chromatographed on DEAE-cellulose (Whatmann, USA) equilibrated with the same buffer. Inhibitors were eluted with a linear gradient of NaCl (0–0.3 M NaCl) in the same buffer. 1.1 mg of bovine stefin B and 2.5 mg of dimeric form of bovine stefin B was obtained. Inhibitor was sequentially pure.

The isolated protein was reduced with β-mercaptoethanol and cysteine residues alkylated with 4-vinylpyridine (Fluka, Switzerland) as described [22].

CNBr cleavage of PE-bovine stefin B was made in 80% formic acid, with a 50-fold molar excess of the reagent (Pierce, USA), under reducing conditions. The reaction, which took place at room temperature in the dark for 24 h, was quenched with a 10-fold dilution of the reaction mixture with distilled water, and freeze dried.

For enzymatic fragmentations of PE-bovine stefin B, β-trypsin (EC 3.4.21.1), prepared according to Štrop and Čechova [23] and *Staphylococcus aureus* strain V-8 proteinase (EC 3.4.21.19) from Miles (UK) were used.

Hydrolysis by β-trypsin was made on PE-bovine stefin B and on maleylated PE-bovine stefin B. Inhibitor was maleylated according to the procedure of Butler and Hartley [24]. The cleavage conditions in both cases were 0.1 M *N*-methyl-morpholine acetate buffer, pH 8.12, at 37°C, and the final enzyme-to-substrate ratio was 1:50. Proteinase was added in two equal portions, at the beginning and after 30 min of incubation.

S. aureus V-8 proteinase cleavage was performed on maleylated PE-bovine stefin B in 0.1 M ammonium acetate buffer, pH 4.0, at 37°C for 24 h with 2% (w/w) of proteinase.

After enzymatic cleavages of maleylated PE-bovine stefin B, samples were demaleylated by acidification of the reaction mixtures to pH 3.5 with TFA and incubation at 37°C for another 48 h.

NH_2-terminal peptide for mass spectrometer analysis was obtained from the native bovine stefin B after the cleavage with PP IV, a generous gift of Dr. Allan J. Barrett. The incubation buffer was 0.1 M Na phosphate, 1 mM EDTA, 2 mM DTT, pH 6.51. Reaction

proceeded at room temperature for 24 h with the enzyme-to-substrate mass ratio 1:50.

Peptide purification was performed by HPLC (Milton Roy, USA) on Chrompack ChromSpher C8 and C18 columns (100 × 3 mm) equilibrated with 0.1% TFA in water, and eluted by linear gradients to 80% acetonitrile and 0.1% TFA in water. The flow rate was 1 ml/min. Absorbance was monitored at 215 nm.

Amino acid analyses of peptide hydrolysates, obtained with 6 M HCl at 110°C, were done with HPLC (Pharmacia LKB, Sweden), using a LKB SuperPac ODS2 column and pre-column *o*-phthalaldehyde derivatization.

Primary structure was determined with an Applied Biosystems (USA) liquid-pulsed sequencer 470A, on-line connected to a 120A PTH amino acid analyser of the same manufacturer.

Mass spectrometer analysis was made on an Autospec Q mass spectrometer (VG-Analytical, UK), using the FAB ionization method. Glycerol was used as matrix.

3. RESULTS AND DISCUSSION

When the native, intact bovine stefin B was applied to the sequence analysis no amino acid residue could be detected, indicating that the inhibitor was blocked at the NH_2-terminal. Attempts to remove the blocking group under the deformylating conditions (1.5 M HCl for 30 min at 25°C) were unsuccessful.

CNBr cleavage gave only one large peptide of virtually the same size as uncleaved PE-bovine stefin B but it was not blocked anymore, as was indicated by sequence analysis. It was sequenced to residue 43 (CN3 in Fig. 1) starting with the Cys amino acid residue. In the first sequencing cycle Met was also detected (about 5% with respect to Cys). As amino acid analysis of the native inhibitor gave a Met value of 2, the conclusion was that the additional Met resulted from the uncomplete CNBr cleavage following two successive Met residues located at the NH_2-terminal of the molecule. Met-Met sequence was NH_2-terminally blocked.

In order to establish the blocking group, NH_2-terminal peptide of appropriate length for mass spectrometer analysis was prepared with PP IV fragmentation of the native bovine stefin B (PP1 in Fig. 1). The PP1-peptide mass spectra gave the M_r+1 value of 483 Da, which together with its amino acid analysis revealed the *N*-acetylation as in the highly homologous rat cystatin β [18].

Twelve peptides were isolated from the cleavage of PE-bovine stefin B with β-trypsin (T-peptides) and characterized, but only those important for establishing the primary structure of the inhibitor are shown in Fig. 1. T10 peptide (residues 69–89) could not be completely sequenced because the reaction yield fell drastically after Pro[79]. Peptides T11 and T12 ended with Phe, and since the COOH-terminal Phe residue would not have been a site of cleavage by β-trypsin they correspond to the COOH-terminus of the molecule.

Maleylation of Lys amino acid residues in PE-bovine stefin B restricted the tryptic cleavage only after Arg amino acid residues. Three peptides were obtained (TR-peptides). Only TR2 was sequenced to provide the missing part of the structure (residues 80–89) and overlap with the COOH-terminal peptide T11 (residues 90–98) (Fig. 1).

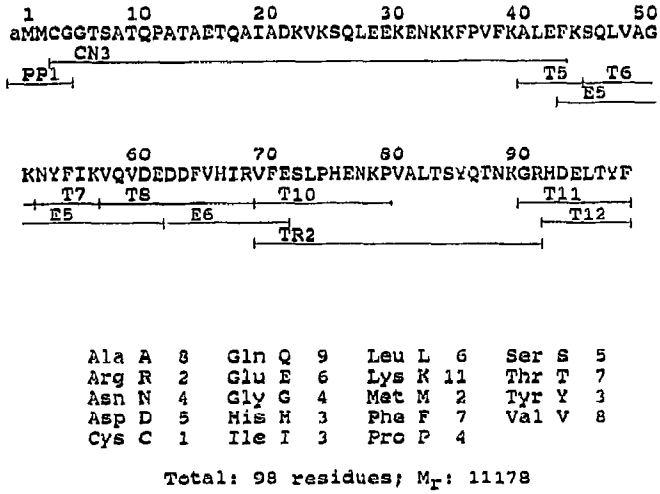

Fig. 1. The amino acid sequence and composition of bovine stefin B. CN-peptide is from CNBr cleavage, T-peptides from the β-tryptic fragmentation, TR-peptide from the limited β-tryptic hydrolysis, E-peptides from *S. aureus* proteinase cleavage and PP1-peptide from papaya proteinase IV digestion of the inhibitor.

Overlapping peptides for the central part of the molecule (E5 and E6 in Fig. 1) were obtained with the *S. aureus* proteinase. PE-bovine stefin B was maleylated before cleavage in order to achieve complete denaturation and therefore better access of the proteinase.

The results show that the inhibitor consists of 98 amino acid residues (Fig. 1) with M_r of 11,178. In Fig. 2 the amino acid sequence of bovine stefin B is aligned with some other sequences in the stefin family. The present sequence is 77.5% identical with that of human stefin B, 71.4% with that of rat cystatin β, and 48.9% with that of human stefin A. The sequence comparison indicates that it belongs to the stefin B subgroup of the stefin family. The most interesting feature in the bovine stefin B sequence is the difference in the well-conserved QVVAG region in the stefins of mammalian origin (Fig. 2): Val[54] (chicken cystatin numbering) is replaced with Leu[54]. From the determined X-ray crystal structures of chicken cystatin [5] and human stefin B–papain complex [6] it is evident that this region folds into an exposed 'first' hairpin loop which is flanked on both sides by the amino-terminal segment and the 'second' hairpin loop. The main hydrophobic interactions are provided by the 'first' hairpin loop. It was shown recently [25–27] that some exchanges of amino acids within this region in the recombinant stefins have only minor effect on the inhibitory activity indicating that the conserved QVVAG region is not essential. Our result shows that also natural inhibitor with a mutation in this otherwise strictly conserved region within the stefin family exists.

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                    1         10        20        30        40        50
BOV.STEF. B     aMMCGGTSATQPATAETQAIADKVKSQLEEKENKKFPVFKALEFKSQLVAG
HUM.STEF. B     xM***AP**********H***Q*R******Y**********VS****V***
RAT CYST. ß     aM***AP***M***T***E***********A*Q**D****IS*RR*V***
HUM.STEF. A      MIP**L*EAK***P*I*E*V****P*****T*ETYGKLE*VQY*T*V***

                         60        70        80        90
BOV.STEF. B      KNYFIKVQVDEDDFVHIRVFESLPHENKPVALTSYQTNKGRHDELTYF
HUM.STEF. B      T******H*GDE****L***Q********LT*SN*****AK*******
RAT CYST. ß      T*F****D*G*EKC**L****P*******LT*S****D*EK*******
HUM.STEF. A      T**Y***RAGDN*YM*LK**K***GQ*EDLV*TG**VD*NKD****G*
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Fig. 2. Alignment of amino acid sequences of bovine stefin B with those of human stefin B [12], rat cystatin β [18] and human stefin A [13]. Residues identical to those in bovine stefin B are marked with an asterisk (*), numbering is according to human stefin B.

Acknowledgements: We thank Dr. B. Kralj (Dept. of Spectroscopy, J. Stefan Institute, Slovenia) for mass spectrometer analysis as well as Dr. F.E. Russell (Dept. of Pharmacy, University of Arizona, USA) for critical reading of the manuscript. This work was supported by a grant from the Research Council of Slovenia.

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