Reprint from
Cysteine Proteinases and their Inhibitors
Editor: V. Turk

PROPERTIES AND STRUCTURE OF HUMAN SPLEEN STEFIN B – A LOW MOLECULAR WEIGHT PROTEIN INHIBITOR OF CYSTEINE PROTEINASES

B.Lenarčič, A.Ritonja, A.Šali, M.Kotnik, V.Turk

Department of Biochemistry, J.Stefan Institute, Jamova 39, 61000 Ljubljana, Yugoslavia

W.Machleidt

Institut für Physiologische Chemie der Universität München, Goethestrasse 33, D-8000 München 2, FRG

Introduction

Low-M_r protein inhibitors of cysteine proteinases have been purified and characterized from many mammalian tissues and body fluids including human (1-3). These inhibitors can be found both intracellularly and extracellularly. The human inhibitors studied are from epidermis (4, 5), various squamous epithelia (6, 7, 8), liver (9), spleen (10), blood polymorphonuclear granulocytes (11, 12), serum (13-16), urine (17), amiloid fibrils (18), saliva (19-21) and amniotic fluid (22). Although these proteins share similar M_r of about 12,000 Da, they differ in their pI values and their immunological and kinetic properties. The amino acid sequences of human stefin A, previously called human stefin (12), human cystatin B (23), human cystatin C (16, 17, 24, 25), human cystatin S or SAP-1 (19), as well as of chicken cystatin (24, 26), rat liver TPI (27), rat epidermal TPI (28) and bovine colostrum CPI (29) have confirmed our earlier suggestion that these proteins can be grouped into two families, the stefin and cystatin families (1). Moreover, it has also been shown recently that these two families, along with the newly discovered kininogen family of cysteine proteinase inhibitors, belong to the same superfamily of cystatins (30, W.Müller et al., G.Salvesen et al., in this book).

We present here the purification, some properties and structural data of the

cysteine proteinase inhibitor from human spleen. This inhibitor occurs in two isoelectric variants with pI values of 5.9 and 6.5 and in an active monomer form as well as an inactive dimer form. The inactive dimer can be converted to the inhibitory active monomer form by reduction. The amino acid sequence of the N-terminal 65 amino acids is identical to that of human liver cystatin B (23). On the basis of amino acid sequence homology, the spleen inhibitor is shown to belong to the stefin family of cysteine proteinase inhibitors . It differs from the originally described stefin inhibitor found in polymorphonuclear granulocytes (12) and is therefore called stefin B.

Materials and methods

Materials. Cm-papain Sepharose 4 B was prepared as described previously (31). Human kidney cathepsin H was prepared by the modified method developed for bovine spleen (32). All other chemicals used were of analytical grade.

Methods

Purification procedure. Frozen human spleens (1100 g) were thawed and freed of fat and large blood vessels. The tissues were cut into small pieces, washed with 0.9 % NaCl (1 : 1.5, w/v) and centrifuged at 4200 g for 90 min. The supernatant was adjusted to pH 10.5 with 5 M NaOH and after 1 hr to pH 7.0 by 3 M HCl. After centrifugation at 4200 g for 30 min acetone was added to the supernatant. The 50-75 % acetone precipitate, dissolved in and dialysed against 10 mM Tris/HCl, pH 7.8, containing 0.1 % Brij and 1 M NaCl was applied to an affinity column of Cm-papain Sepharose 4 B, equilibrated with the same buffer. The column was washed with the starting buffer and the bound proteins eluted with 10 mM NaOH, pH 11.0. Fractions with inhibitory activity were collected, adjusted to pH 8.0 with 3 M HCl and concentrated on an ultrafiltration membrane YM-5 (Amicon). The bulked sample was dialysed against 10 mM Tris/HCl buffer, pH 7.8, containing 100 mM NaCl and then applied to a Sephadex G-75 column, previously equilibrated with the same buffer. Three peaks of the inhibitory activity were obtained. The last two peaks corresponding to M_r of about 24,000 and 12,000 Da were further concentrated, dialysed against 10 mM Tris/

HCl buffer, pH 9.3, and submitted to FPLC (Pharmacia) on a Mono Q column equilibrated with the same buffer. The inhibitor was eluted by increasing the concentration of NaCl (0 - 0.1 M) in the buffer.

Assay of inhibitor. Papain was assayed using Bz-Arg-NNap as the substrate (33). Cathepsin H activity was determined using Leu-NNap as substrate according to Barrett (33) in the presence or absence of cysteine as reducing agent. In inhibition assays , the enzyme and the inhibitor were preincubated together at 37° C for 5 min before the addition of the substrate. One unit of inhibitory activity was defined as the amount which inhibited 1 μg of papain completely.

Amino acid sequence analysis. Reduction, S-carboxymethylation with iodo-(3 H) acetic acid (Amersham) and cyanogen bromide cleavage were performed according to standard protocols. Fragments used for sequence analysis were purified by gel chromatography on a column (0.6 x 150 cm) of Sephacryl S-200 superfine and eluted (0.84 ml/hr) with 80 % formic acid (v/v) at room temperature. Amino acid analysis was done with a Kontron Liquimat II analyser using fluorescence detection after post-column reaction with o-phtalaldehyde. Amino acid sequences were determined by automated solid-phase Edman degradation (34). The polypeptides to be sequenced were coupled via lysine side chains to diisothiocyanate-activated aminopropyl glass and via carboxyl groups to aminopropyl glass after hydroxybenzotriazole-catalysed carbodiimide activation as detailed in (35). The degradations were performed in a non-commercial solid-phase sequencer with on-line detection of the released amino acid phenylthiohydantoin derivatives (PTH) by quantitative high performance liquid chromatography (35). Cysteine was identified as S-(3 H)carboxymethylcysteine by radioactivity.

Comparisons of amino acid sequences. The physical parametric approach (36) to protein sequence comparison was used to derive the maximal summed cross correlation coefficients with a lag value between -20 and +20 for all possible pairs of known sequences of stefins and cystatins. Once all the homologues had been compared in this way, a different matrix was constructed and systemic clustering according to the method of weighted pair-group with arithmetic averaging was undertaken (37).

Other methods. Polyacrylamide gel electrophoresis (PAGE) and SDS-PAGE were performed as described in (38) and (39), respectively. Analytical gel isoelectric focusing (pH range from 2 to 10) was performed following the manufacturer's instructions (Pharmacia). Protein was assayed by the method of Lowry et al. (40).

Results

Stefin B was isolated from human spleen by affinity chromatography on Cm-papain Sepharose 4 B which is the essential purification step for the isolatation of cysteine proteinase inhibitors. Gel chromatography on Sephadex G-75 separated three inhibitory fractions (a, b, c) with M_r of about 65,000, 24,000 and 12,000 Da, respectively (Fig.1). Fraction according to its M_r value corresponds to low-M_r

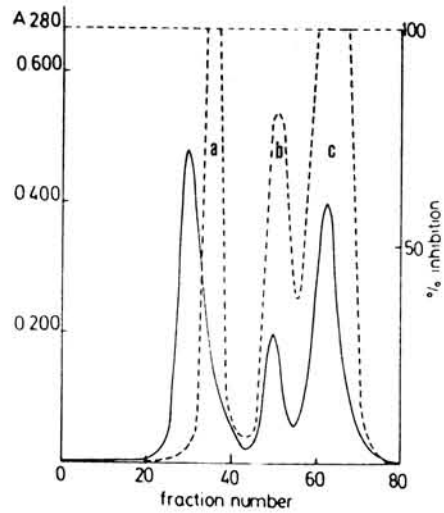

Fig.1. Gel chromatography of CPIs on Sephadex G-75.
(——) protein; (----) % inhibition; fractions 6.5 ml

kininogen and was not further investigated. Both fractions b and c were further purified by FPLC (Fig. 2A and B). Whereas fraction b (M_r of about 24,000 Da) yielded a single sharp inhibitory peak (Fig. 2A), fraction c (M_r of about

12,000 Da) was separated into several peaks with inhibitory activity (Fig. 2B). It can be seen that the elution profile of the middle peak with highest inhibitory activity corresponds to the inhibitor from Fig. 2A.

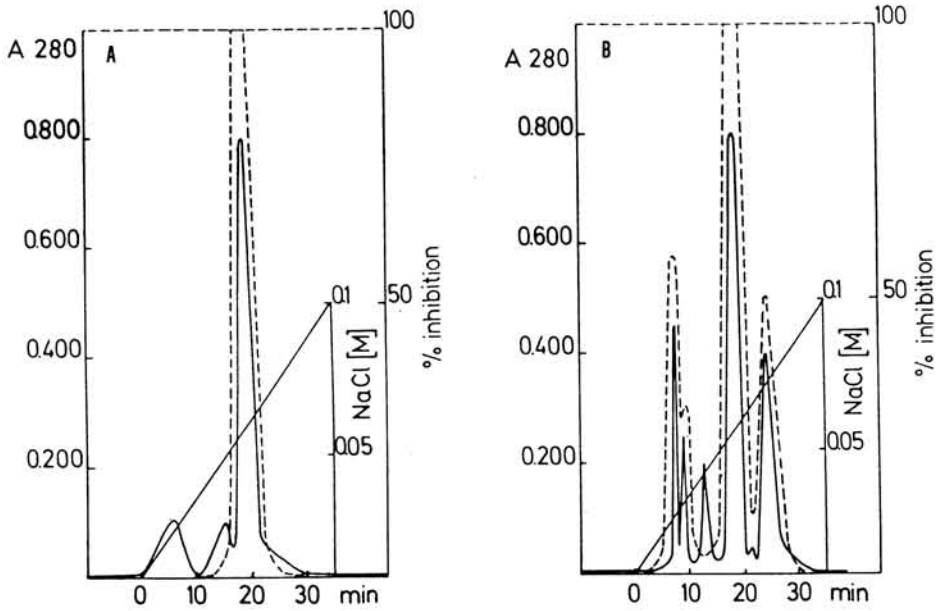

Fig.2. Fast protein liquid chromatography on Mono Q anion exchange resin.
A : fraction b from gel chromatography
B : fraction c from gel chromatography
(——) protein ; (----) % inhibition

Therefore both inhibitory active peaks were subjected to isoelectric focusing (Fig.3). It is evident that both peaks are composed of two forms, focused at pH 5.9 and 6.5. Other inhibitor peaks from Fig. 2B showed pI values of pI 4.65 (stefin A), 5.5 and above 7.0 (not shown) and were not further investigated. Both inhibitory peaks from Fig.2 A and B with M_r of 24,000 and 12,000 Da obtained by gel chromatography were analysed by SDS-PAGE (Fig.4). The M_r of both peaks under reducing conditions was 12,000 Da, whereas the inhibitor from Fig. 2A showed two bands under non-reducing conditions at 24,000 and 12,000 Da. These results indicate that fraction b (with M_r of about 24,000 Da obtained by gel

chromatography)represents a dimeric form of the inhibitor from Fig. 2 B.

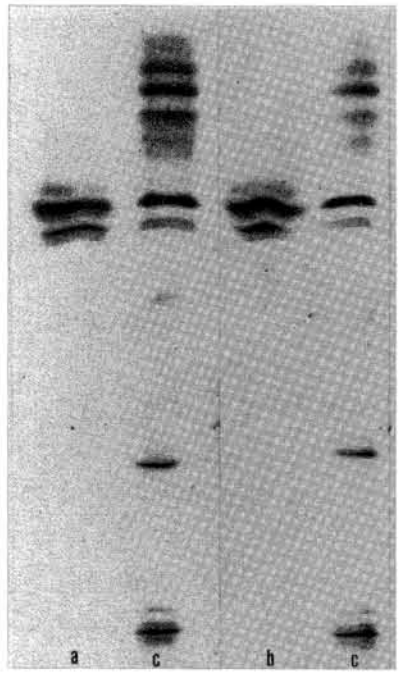

Fig.3. Isoelectric focusing of stefin B
a) monomer form (20 μg)
b) dimer form (23 μg)
c) standards

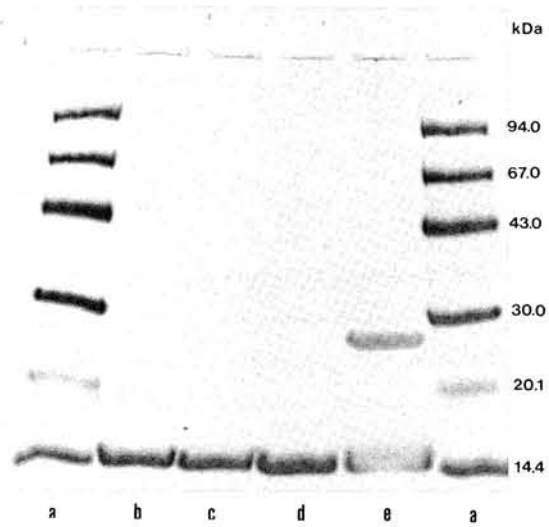

Fig.4. SDS-polyacrylamide gel electrophoresis of stefin B (25 μg)
a) standards, b) monomer form (reduced), c) monomer form (non-reduced), d) dimer form (reduced), e) dimer form (non-reduced).

The presence of the 12,000 Da band can be explained by the presence of monomer which was not well separated from dimer during gel filtration (Fig.1). The dimerization of the inhibitor can most probably be explained by the formation of S-S bond between two free cysteins.

In order to understand in more detail the nature of the monomer and dimer forms of the inhibitor the complex with cathepsin H was prepared under reducing (in the presence of 2 mM dithiothreitol) and non-reducing conditions. The samples were tested for mobility on nondenaturating PAGE and for cathepsin H activity. From Fig.5 it is evident that under non-reducing conditions the monomer exhibits two bands and the dimer only one band. Both monomer and dimer forms of the inhibitor can bind cathepsin H under reducing conditions (Fig.5e and i) and as shown by inhibitory activity (Table I). However, only the monomer form

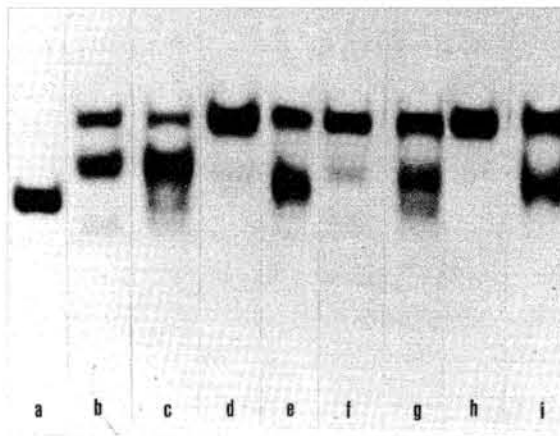

Fig.5. Polyacrylamide gel electrophoresis of stefin B (monomer and dimer form) and its interaction with dithiothreitol and cathepsin H
a) cathepsin H, b) monomer form , c) monomer form and cathepsin H, d) monomer form in the presence of DTT, e) monomer form in the presence of DTT and cathepsin H, f) dimer form, g) dimer form and cathepsin H, h) dimer form in the presence of DTT, i) dimer form in the presence of DTT and cathepsin H
The content of reactants : monomer and dimer form of stefin B (18 μg), cathepsin H (30 μg), 10 mM dithiothreitol (10 μl).

of the inhibitor can inhibit cathepsin H activity under non-reducing conditions. The dimer must be converted to monomer in order to be able to inhibit cathepsin H.

Table I. The effect of monomer and dimer form of stefin B on cathepsin H activity (The contents of reactant : cathepsin H 3.1 μg, stefin B -monomer and dimer form 2.2 μg, dithiothreitol 2 mM.)

	residual enzyme activity (%)
Cathepsin H	100
Cathepsin H + dithiothreitol	100
Cathepsin H + monomer	50
Cathepsin H + dithiothreitol + monomer	0
Cathepsin H + dimer	100
Cathepsin H + dithiothreitol + dimer	0

To test whether the cysteine residue is involved in activity the inhibitor was S-carboxymethylated. The inhibitory activity was not affected indicating that the single cysteine in stefin B is not essential for formation of the enzyme-inhibitor complex.

To summarize these results , the monomer contains a slow band of the inhibitor and a faster band ascribed by Katunuma et al. (41) for the rat inhibitor to a mixed disulphide with glutathione. The product of S-carboxymethylation runs in the same position as the mixed disulphide, having the same charge. The dimer runs in the same position as the reduced monomer as expected for the disulphide bonded dimer which cannot therefore bind glutathione. It is of interest that, while the cysteine of the monomer is shown not to be involved in inhibitory activity, the dimer is inactive. This suggests that the binding site is located not far from the cysteine.

No amino acid sequence was obtained when the intact inhibitor was directly subjected to automated Edman degradation indicating that the molecule is N-terminally blocked. The blocking group could not be removed by incubation under deformylating conditions (42). Cyanogen bromide cleavage followed by gel chromatography generated two fragments of approximate apparent M_r of 23,000 Da and 11,000 Da showing identical amino acid compositions and containing no methio-

nine, and a low-M_r fraction containing only homoserine lactone. As the intact inhibitor has only two methionine residues, these findings provide indirect evidence for an N-terminal Met-Met sequence which is N-acylated, most probably N-acetylated.

Automated solid-phase Edman degradation of the 11,000 Da fragment yielded a continuous sequence of 65 residues which, except for one single residue, was identified unambiguously (Fig.6). An identical sequence was obtained for 60

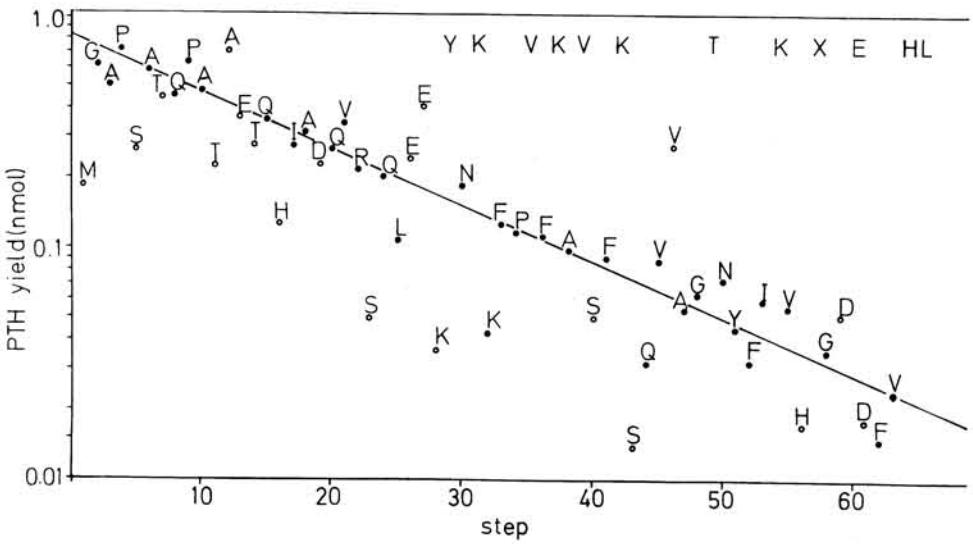

Fig.6. Solid-phase Edman degradation of the 11,000 Da cyanogen bromide fragment of the inhibitor.
The semilogarithmic plot shows the yields of amino acid phenylthiohydantoin derivatives (PTH) released in the individual degradation steps as determined by high performance liquid chromatography. Residues indicated along the top of the panel were identified, but not quantitated. X stands for a residue which could not be identified in this degradation. Position 1 was found to contain cysteine in a different experiment. The methionine found in step 1 belongs to a minor sequence resulting from incomplete cleavage of the N-terminal Met-Met bond of the inhibitor (cf. text). The straight line represents the theoretical yields corresponding to a mean repetitive yield of 94.5 %.

steps of the 23,000 Da fragment (not shown) suggesting that this is a dimer of the 11,000 Da molecule. The non-identified residue at position 57 was found to

be valine. The first residue of both the monomeric and dimeric fragment was shown to be a cysteine in separated degradations of the S-(3H) carboxymethylated peptides. An observed minor sequence (less than 20%) starting with methionine and running one residue behind the frame of the main sequence (not shown) would be due to incomplete cyanogen bromide cleavage of the Met-Met bond thus confirming the proposed acylated Met-Met sequence and aligning it with the sequence of the large cyanogen bromide fragments.

The amino acid sequence of human liver cystatin B, an inhibitor with similar or identical properties to that described here from human spleen, has recently been determined (23). Amino acid sequences of both inhibitors in the first 65 amino acid residues in the N-terminal part of the molecule are identical thus strongly suggesting that both inhibitors are structuraly identical proteins (Fig.7).

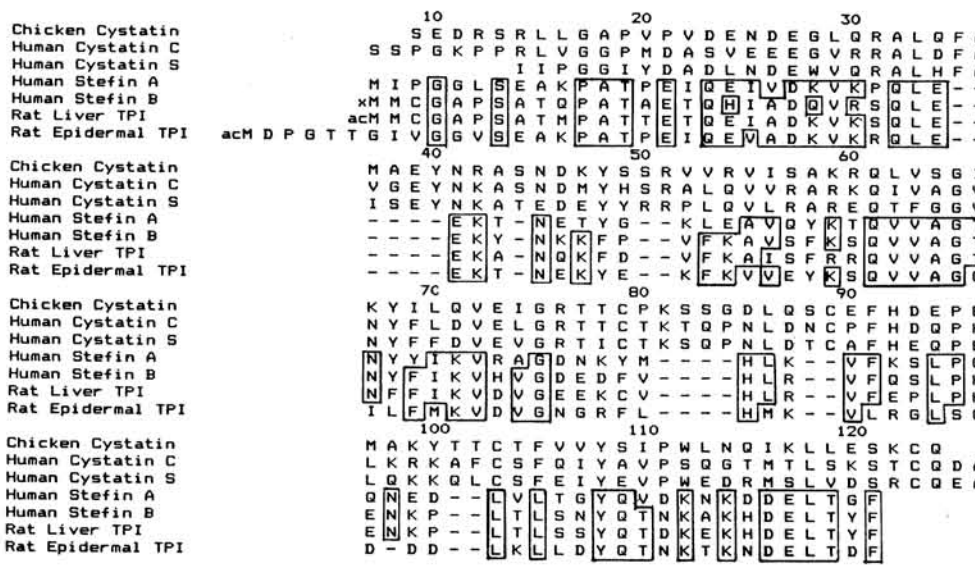

Fig.7. Comparison of the amino acid sequence of human stefin B with other known sequences of the members of the stefin and cystatin families (for sequences see references).

Therefore the human spleen inhibitor would also consist of 98 amino acid residues (cofirmed by amino acid composition - not shown). It is evident from Fig.7 that the human spleen inhibitor shows 53 % identity with human stefin A (12) and 79 % identity with rat liver TPI (27). Sequence homologies with other known cystatins show that they are only distantly related in terms of sequences.

The method of Argos et al. (36) has been used to make a structural comparison based on amino acid sequences between the inhibitors whose sequences are known. The comparison is based on the physical characteristics thought to determine the three dimensional folding of a given polypeptide sequence and is therefore capable of following the conservation or change of a conformation even where sequence homology is not detectable. This provides a more effective way of assessing evolutionary relation between different proteins. Such a "phylogenetic" tree is shown in Fig.8. This shows the developments by divergent evolution of two families of inhibitor, stefins and cystatins. On the basis of the pronounced similarity between rat liver TPI and human spleen inhibitor or liver cystatin B we propose the name human stefin B for human spleen inhibitor.

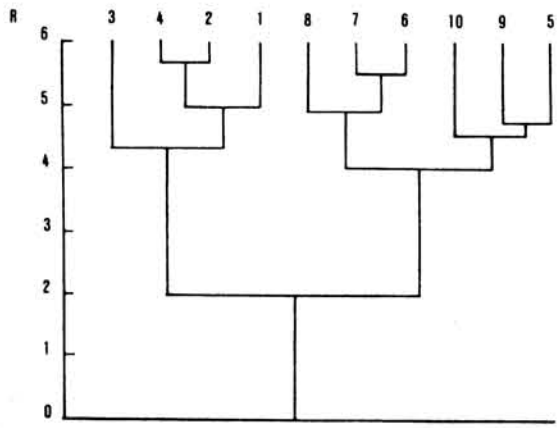

Fig.8. Dendrogram constructed from the difference matrix. Cophenetic correlation coefficient : 0.9634. R stands for maximal summed cross correlation coefficient. 1) human stefin A, 2) human stefin B, 3) rat epidermal TPI, 4) rat liver TPI , 5) human cystatin C, 6) human cystatin S 7, 7) human cystatin S 5, 8) human cystatin S 1, 9) bovine colostrum cystatin, 10) chicken cystatin.

Discussion

We have isolated from human spleen an inhibitor of cysteine proteinases comprising two components with isoelectric points 5.9 and 6.5 which are similar to the neutral type of isoelectric variants (10) and cystatin B (9) described earlier. Attempts to separate the two forms have not so far been successful. Using gel chromatography we have isolated in almost equal amounts monomer and dimer forms of the inhibitor with M_r of about 12,000 Da and 24,000 Da, respectively. In some preparations from human liver (9) and spleen (10) the dimer form appeared as a minor component. When subjected to SDS-PAGE under reducing conditions the dimer form was converted to the monomer form, indicating that the dimerization is the result of disulphide bond formation . Similarly, the inactive dimer form was converted into inhibitory active monomer after the addition of dithiothreitol. The carboxymethylated monomer form of the inhibitor retains its inhibitory activity, clearly showing that the thiol group is not essential for the activity. These results are consistent with similar behaviour of the monomer and the dimer form of rat liver TPI (41).

From the amino acid analysis and partial amino acid sequence of the spleen inhibitor, it is apparent that the human spleen inhibitor, stefin B, and human liver cystatin B (23) are identical proteins and highly homologous to rat liver TPI (27). Human stefin B contains only one cysteine, at position 3. In contrast, rat liver TPI contains two free cysteine residues, at position 3 and 64 (27). The Cys-64 of rat liver TPI is replaced by Phe in human stefin B, a further indication that neither of two cysteine residues is essential for the inhibitory activity. This hypothesis is supported by the finding that in rat liver TPI Cys-3 is exposed on the surface of the molecule whereas Cys-64 is buried (41).

From inspection of the known sequences of the cysteine proteinase inhibitors it is evident that human stefin B also contains a highly conserved region between residues 44 and 57 which might be responsible for the inhibitory activity. It has been suggested that the stefin family consists of two subfamilies characterized by the presence or absence of cysteine residues (1). Human stefin A and rat epidermal TPI do not contain cysteine (stefin subfamily 1), whereas rat liver TPI contains two and human stefin B only one cysteine (stefin subfamily 2). It

is now recognized that conformation is a more important basis for evolutionary and functional similarity than sequence homology. Comparisons on this basis are shown to be in support of the fundamental structural resemblance of individual inhibitors of both stefins and cystatins (1, 30), and their division into two families that have diverged during evolution. The most closely related proteins so far sequenced are human stefin B and rat liver TPI. These results clearly indicate that human stefin B belongs to the stefin family , and not to the cystatin family.

References

1. Turk,V., J.Brzin, B.Lenarčič, P.Ločnikar, T.Popovič, A.Ritonja, J.Babnik, W.Bode, W.Machleidt. 1985. In: Intracellular Protein Catabolism (E.A. Khairallah, J.S.Bond and J.W.C.Bird, eds.). A.R.Liss, New York, p.91.
2. Barrett, A.J. 1985. In: Intracellular Protein Catabolism (E.A.Khairallah, J.S.Bond, J.W.C.Bird, eds.). A.R.Liss, New York, p.105.
3. Turk,V., J.Brzin, M.Kopitar, M.Kotnik, B.Lenarčič, T.Popovič, A.Ritonja, M.Trstenjak, B.Rozman, W.Machleidt. 1986. In: Proteinases in Inflammation and Tumor Invasion (H.Tschesche, ed.). Walter de Gruyter, Berlin (in press).
4. Järvinen,M. 1978. J.Invest. Dermatol. 71, 114.
5. Ohtani,O., K.Fukuyama, W.L.Epstein. 1982. Biochim.Biophys.Acta 707, 21.
6. Hopsu-Havu,V.K., K.Kalimo, M.Järvinen. 1981. Br.J.Dermatol. 104, 621.
7. Hopsu-Havu,V.K., J.A.Joronen, M.Järvinen, A.Rinne. 1983. Arch.Dermatol.Res. 275, 305.
8. Ohtani,O., K.Fukuyama, W.L.Epstein. 1982. J.Invest.Dermatol. 82, 280.
9. Green,G.D.J., A.A.Kembhavi, M.E.Davies, A.J.Barrett. 1984. Biochem. J. 218, 939.
10. Järvinen,M., A.Rinne. 1982. Biochim.Biophys.Acta 708, 210.
11. Brzin,J., M.Kopitar, V.Turk, W.Machleidt. 1983. Hoppe-Seyler´s Z. Physiol.Chem. 364, 1475.
12. Machleidt,W., U.Borchart, H.Fritz, J.Brzin, A.Ritonja, V.Turk. 1983. Hoppe-Seyler´s Z.Physiol.Chem. 364, 1481.
13. Lenney,J.F., J.R.Tolan, W.J.Sugai, A.G.Lee. 1979. Eur.J.Biochem. 101, 153.

14. Lenney,J.F., J.R.Liao, S.L.Sugg, V.Gopalakrishnan, H.C.H.Wong, K.H.Ouye, P.W.H.Chan. 1982. Biochem.Biophys.Res.Commun. 108, 1581.

15. Gauthier,F., M.Pagano, F.Esnard, H.Mouray, R.Engler. 1983. Biochem. Biopyhs.Res.Commun. 110, 449.

16. Brzin,J. T.Popovič, V.Turk, U.Borchart, W.Machleidt. 1984. Biochem. Biophys.Res.Commun. 118, 103.

17. Grubb,A., H.Löfberg. 1982. Proc.Natl.Acad.Sci.USA 79, 3024.

18. Cohen,D.H., H.Feiner, O.Jensson, B.Frangione. 1983. J.Exp.Med. 158, 623.

19. Isemura,S., E.Saitoh, K.Sanada. 1984. J.Biochem. 96, 489.

20. Minakata, K., M.Asano. 1984. Hoppe-Selyer's Z.Physiol.Chem. 365, 399.

21. Isemura,S., E.Saitoh, M.Isemura, K.Sanada. 1984. J.Biochem. 96, 1311.

22. Rohrlich,S.T., H.Levy, D.B.Rifkin. 1985. Biol.Chem.Hoppe-Seyler 366, 147.

23. Ritonja,A., W.Machleidt, A.J.Barrett. 1985. Biochem.Biophys.Res.Commun. 131, 1187.

24. Turk,V., J.Brzin, M.Longer, A.Ritonja, M.Eropkin, U.Borchart, W.Machleidt . 1983. Hoppe-Seyler's Z.Physiol.Chem. 364, 1487.

25. Barrett,A.J.,M.E.Davies, A.Grubb. 1984. Biochem.Biophys.Res.Commun. 120, 631.

26. Schwabe,C., A.Anastasi, H.Crow, J.K.McDonald, A.J.Barrett. 1984. Biochem.J. 217, 813.

27. Takio,K., E.Kominami, N.Wakamatsu, N.Katunuma, K.Titani. 1983. Biochem.Biophys.Res.Commun. 115, 902.

28. Takio,K., E.Kominami, Y.Bando, N.Katunuma, K.Titani. 1984. Biochem. Biophys.Res.Commun. 121, 149.

29. Hirado,M., S.Tsunasawa, F.Sakiyama, M.Niinobe, S.Fujii. 1985. FEBS Lett. 186, 41.

30. Müller-Esterl,W., H.Fritz, J.Kellermann, F.Lottspeich, W.Machleidt, V.Turk. 1985. FEBS Lett. 191, 221.

31. Barrett,A.J. 1981. Methods Enzymol. 80, 771.

32. Zvonar,T., I.Kregar, V.Turk. 1979. Croat.Chem.Acta 52, 411.

33. Barrett,A.J. 1972. Anal.Biochem. 67, 280.

34. Laursen,R.A., W. Machleidt. 1980. Methods Biochem.Anal. 26, 201.

35. Machleidt, W. 1983. In: Modern Methods in Protein Chemistry (H.Tschesche, ed.). Walter de Gruyter, Berlin, p.267.

36. Argos,P., M.Hanei, J.M.Wilson, W.N.Kelley. 1983. J.Biol.Chem. 258, 6450.

37. Davis,J.C.,R.J.Sampson. 1973. In: Statistics and Data Analysis in Geo - logy.J.Willey, New York, p.456.

38. Davis,B.J. 1964.Ann.N.Y.Acad.Sci. 121, 404.

39. Laemmli,U.K. 1973. Nature 227, 680.

40. Lowry,O.H.,N.J.Rosebrough,A.L.Farr,R.J.Randall. 1951.J.Biol.Chem. 193, 265.

41. Wakamatsu,N.,E.Kominami,K.Takoi,N.Katunuma. 1984.J.Biol.Chem. 259, 13832.

42. Sheehan,J.C.,D.D.H.Yang.1958.J.A.Chem.Soc.80, 1154.

Acknowledgements

We acknowledge the excellent technical assistance of Stanka Košir, Andreja Burkeljc and Karin Wiedenmann. This work was supported by the Research Council of Slovenia, Yugoslavia, by the Sonderforschungsbereich 0207 of the University of Munich,and by the International Bureau of the Kernforschungsanlage Jülich GmbH. The investigations were supported also by the NSF Grant No. F7F030Y.