FEBS Letters 362 (1995) 266–270 FEBS 15309

Limited proteolysis of cytochrome *c* in trifluoroethanol

Angelo Fontana*, Marcello Zambonin, Vincenzo De Filippis, Manuela Bosco, Patrizia Polverino de Laureto

CRIBI Biotechnology Centre, University of Padua, Via Trieste 75, 35121 Padua, Italy

Received 23 January 1995; revised version received 28 February 1995

Abstract Horse heart cytochrome *c* is cleaved by thermolysin in 50% aqueous TFE (v/v) at neutral pH (25°C, 24 h) at the Gly^{56}–Ile^{57} peptide bond of the 104-residue chain of the protein. Additional, but anyway minor, fragmentation at the Gly^{45}–Phe^{46} and Met^{80}–Ile^{81} peptide bonds is also observed. On the other hand, in buffer only and in the absence of TFE, cytochrome *c* is digested by thermolysin to numerous small peptides. Considering the broad substrate specificity of the TFE-resistant thermolysin, clearly the conformational state of the protein substrate dictates the observed selective proteolysis. It is proposed that the highly helical secondary structure acquired by cytochrome *c* when dissolved in aqueous TFE hampers binding and adaptation of the protein substrate at the active site of the protease and that peptide bond fission occurs at flexible chain segments characterized by a low α-helix propensity.

Key words: Cytochrome *c*; Limited proteolysis; Protein fragment; Trifluoroethanol; Circular dichroism

1. Introduction

Trifluoroethanol (TFE) was often used as an agent for inducing and stabilizing the secondary structure (helix) of peptides [1–3]. The mechanism of helix-induction by TFE is not fully understood and is debated in current literature [4–6]. More recently, the effect of TFE on the structure of intact proteins was investigated using circular dichroism (CD) and proton nuclear magnetic resonance (NMR) spectroscopy [7–10]. The general observation emerged from these studies is that the TFE-induced state of a protein is characterized by a significant content of helical secondary structure, but lacking the specific tertiary interactions of the native protein.

In previous studies, we have shown that the specific or preferential fission of peptide bond(s) by limited proteolysis [11,12] is a useful procedure to probe structural and dynamic aspects of globular proteins [13–17]. A detailed analysis of the structural characteristics of the sites of specific cleavages in proteins allowed us to establish that native globular proteins are cleaved at exposed and flexible loops only and never at chain segments of regular secondary structure (helices) [13,16]. In particular, we have emphasized that the sites of limited proteolysis in globular proteins are characterized by enhanced chain flexibility, since a correlation exists between sites of proteolysis and sites of high segmental mobility, these last determined crystallographically [13].

Here we show that horse heart cytochrome *c* in the presence of aqueous TFE is cleaved by the TFE-resistant proteolytic enzyme thermolysin [18] to few fragments only, whereas in aqueous solution and in the absence of alcohol many small peptides are formed. The preferential proteolysis was interpreted on the basis of the conformational properties of cytochrome *c* dissolved in aqueous TFE, as deduced from circular dichroism (CD) and fluorescence emission measurements.

2. Materials and methods

Horse heart cytochrome *c* (type VI) and thermolysin were obtained from Sigma. Trifluoroethanol (TFE) (special grade), trifluoroacetic acid (TFA) and cyanogen bromide (BrCN) were purchased from Fluka and the materials used for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) were from Bio-Rad. Solvents and reagents used for peptide/protein sequence analysis were obtained from Applied Biosystems.

SDS-PAGE was carried out in a vertical slab gel apparatus (Miniprotean II, Bio-Rad) using the polyacrylamide system of Shägger and von Jagow [19]. The gels were stained with Coomassie brilliant blue R-250. Protein/peptide samples were hydrolyzed for 1 h at 150°C in 6 N HCl containing 0.1% phenol using the Millipore-Waters Pico-Tag workstation. The amino acid mixture was derivatized with phenylisothiocyanate and analyzed by reverse phase HPLC using the Pico-Tag column (3.9 × 150 mm) [20]. N-terminal sequence analysis was performed with an Applied Biosystems pulsed-liquid phase sequencer (model 477A) equipped with on-line analyzer (model 120A) of phenylthiohydantoin-derivatives of amino acids.

Circular dichroism (CD) measurements were made at 25°C on a Jasco J-710 spectropolarimeter equipped with a thermostatically controlled cell holder. A 1-mm and 10-mm path-length cell was used for measurements in the far- and near-ultraviolet region, respectively. The mean residue ellipticity $[\theta]$ is expressed in units of deg·cm^2·dmol^{-1}. Fluorescence emission measurements were carried out on a Perkin-Elmer LS-50 spectrofluorimeter.

Digestion of horse heart cytochrome *c* was performed by incubation of the protein dissolved (1.0 mg/ml) in 20 mM Tris-HCl buffer, pH 7.8, containing 10 mM $CaCl_2$, with thermolysin (protein substrate : protease ratio 1 : 50, by mass) at 25°C. The reaction was conducted in the absence or presence of TFE up to 50% (by volume) concentration. At intervals, the proteolysis was stopped by removing 10-μl aliquots from the reaction mixture and mixing with 20 μl of 0.1% aqueous TFA. Samples were concentrated using the Speed-Vac system (Savant) and then analyzed by SDS-PAGE and reverse-phase HPLC utilizing a Vydac C_4 column (4.6 × 150 mm) purchased from The Separations Group. In order to produce protein fragments in sufficient quantity for their analytical characterization, a digestion mixture of cytochrome *c*, as obtained after proteolysis in 50% TFE (v/v) for 24 h at 25°C, was applied to a gel filtration Sephadex G-50 superfine column (2.6 × 95 cm) equilibrated and eluted with 5% (v/v) aqueous formic acid. The eluted fragments were further purified by micropreparative HPLC and analyzed for their amino acid composition after acid hydrolysis and N-terminal sequence.

*Corresponding author. Fax: (39) (49) 828 6659.

Abbreviations: TFE, trifluoroethanol; TFA, trifluoroacetic acid; CD, circular dichroism; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Gdn·HCl, guanidine hydrochloride.

SSDI 0014-5793(95)00237-5

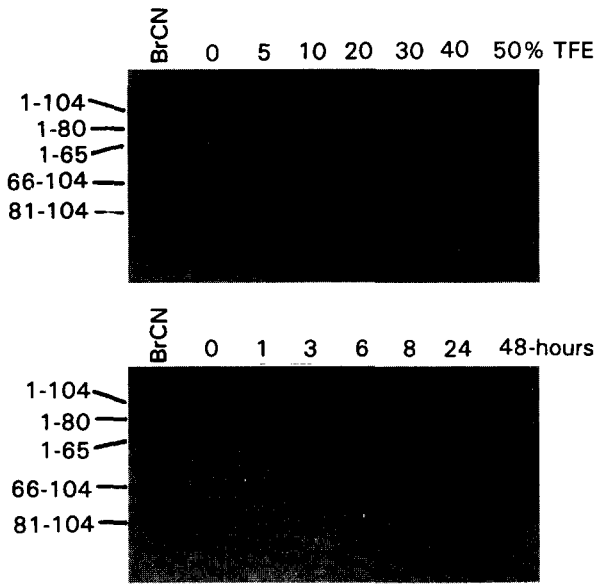

Fig. 1. Analysis by SDS-PAGE and HPLC of the proteolysis of horse heart cytochrome *c* digested with thermolysin in aqueous TFE. The protein was digested at a substrate to protease ratio of 1:50 (by mass) in the presence of 0–50% TFE (v/v) (see section 2). After 6 h reaction at 25°C, an aliquot of the proteolysis mixture was analyzed by SDS-PAGE (A). The time-course analysis of the proteolysis in 50% TFE (v/v) is also shown (B). In the lane indicated by BrCN was applied a sample of a limited BrCN-digest of cytochrome *c* producing protein fragments by chemical cleavage at methionine residues in position 65 and 80 of the chain; numbers near the electrophoretic bands indicate the BrCN-fragments of cytochrome *c* [21].

3. Results

3.1. Proteolysis of cytochrome c

Horse heart cytochrome *c* was digested with thermolysin in Tris buffer, pH 7.8, under different experimental conditions of enzyme to substrate ratio, temperature and time of incubation and in the presence of increasing concentrations of TFE. The SDS-PAGE analysis of the proteolysis mixture after 6 h reaction at 25°C (Fig. 1A) reveals that, in the absence of TFE, cytochrome *c* is partly digested by thermolysin to small peptides not stained by Coomassie, since the intensity of the protein band in the gel is weaker than that of the unreacted protein. In the presence of TFE, cytochrome *c* is digested to relatively few and rather large protein fragments. After 24–48 h in 50% (v/v) TFE, the protein is completely digested to two major fragments, which show electrophoretic mobilities similar to those of the BrCN-fragments 1–65 and 66–104 of cytochrome *c* [21] (Fig. 1B). Since the sum of the molecular masses (about 7 and 5 kDa) of the two fragments, as estimated by SDS-PAGE, roughly match that of the intact protein (~12 kDa), it appears that the 104-residue chain of cytochrome *c* dissolved in 50% (v/v) TFE is preferentially cleaved by thermolysin at a single peptide bond. When an aliquot of the total proteolytic mixture of cytochrome *c* (24 h reaction), without prior purification, was subjected to N-terminal sequence analysis, 2–3 amino acid residues could be identified at each cycle of the sequential degradation. However, the major sequence observed (~80%) was Ile-Thr-Trp-Lys, which corresponds to residues 57–60 of cytochrome *c* [22]. Thus, since the α-amino group of cytochrome *c* is acetylated, the major proteolytic site along the protein chain is at the amino-side of Ile[57]. Other minor sequences (5–10% level) could not be identified firmly at this stage and required sequencing data obtained from a purified proteolytic mixture (see below). When the proteolysis of cytochrome *c* was performed for 8 h, the peptide bond fission at Ile[57] was more selective, but some intact protein remained in the proteolytic mixture (see Fig. 1B).

The results of the SDS-PAGE analysis were complemented by those obtained by reverse-phase HPLC. The proteolytic mixture of cytochrome *c* reacted in 50% (v/v) TFE (25°C, 24 h) gave two major peaks of peptide material in the HPLC chromatogram (not shown), while that obtained in buffer only gave numerous peaks (at least 30).

3.2. Isolation and characterization of thermolytic fragments

The thermolytic fragments of cytochrome *c* produced when proteolysis is conducted in 50% (v/v) TFE for 24 h at 25°C were isolated to homogeneity and in sufficient quantity for their analytical characterization after gel filtration chromatography and reverse-phase HPLC. The two major fragments of the proteolytic mixture were thus isolated, as well as the minor components which can be detected by a closer inspection of the SDS-PAGE gels shown in Fig. 1B. The various fragments thus isolated were analyzed for their amino acid composition (Table 1) and N-terminal sequence (not shown). A comparison of these data with the known amino acid sequence of horse cytochrome *c* [22] allowed us to identify fragments 1–56 and 57–104 as the major proteolytic products and fragments 1–45, 57–80 and 81–104 as the minor ones. Of note, fragment 1–45 is eluted

Table 1
Amino acid composition of fragments of horse heart cytochrome *c* produced by limited proteolysis with thermolysin in 50% (v/v) aqueous TFE

Amino acid	Cyto-chrome *c*	Fragments				
	1–104	1–56	57–104	1–45	57–80	81–104
Asx	8.0(8)	5.1(5)	2.9(3)	2.3(2)	1.1(1)	2.1(2)
Thr	10.5(10)	5.0(5)	4.8(5)	3.2(3)	3.0(3)	2.1(2)
Glx	12.5(12)	5.2(5)	7.0(7)	5.1(5)	4.5(4)	3.2(3)
Pro	4.3(4)	2.0(2)	1.8(2)	2.0(2)	2.1(2)	–
Gly	12.3(12)	10.4(10)	2.0(2)	8.9(9)	1.1(1)	1.1(1)
Ala	6.0(6)	3.0(3)	2.9(3)	2.1(2)	–	3.2(3)
Val	2.8(3)	3.0(3)	–	2.9(3)	–	–
Met	1.4(2)	–	1.7(2)	–	1.9(2)	–
Ile	5.9(6)	1.0(1)	4.6(5)	0.9(1)	1.7(2)	3.1(3)
Leu	5.9(6)	2.0(2)	3.8(4)	2.0(2)	2.0(2)	2.1(2)
Tyr	3.8(4)	0.8(1)	2.7(3)	–	2.0(2)	1.0(1)
Phe	4.2(4)	3.0(3)	1.0(1)	2.0(2)	–	1.0(1)
Lys	19.6(19)	9.5(10)	8.9(9)	8.3(8)	3.9(4)	4.9(5)
His	2.7(3)	3.0(3)	–	2.9(3)	–	–
Arg	2.0(2)	1.0(1)	1.0(1)	1.1(1)	–	1.0(1)
Trp	n.d.(1)	–	n.d.(1)	–	n.d.(1)	–
Cys	n.d.(2)	n.d.(2)	–	n.d.(2)	–	–
No. of residues	104	56	48	45	24	24

Cytochrome *c* fragments were purified to homogeneity by reverse-phase HPLC as described in the text. Experimental figures are the results of amino acid analyses after acid hydrolysis conducted in triplicate (see section 2). The amino acid composition of intact cytochrome *c* (1–104) is also included as a reference. The values for Asx and Glx are the sum of Asp and Asn and Glu and Gln calculated from the sequence of cytochrome *c*. n.d., not determined.

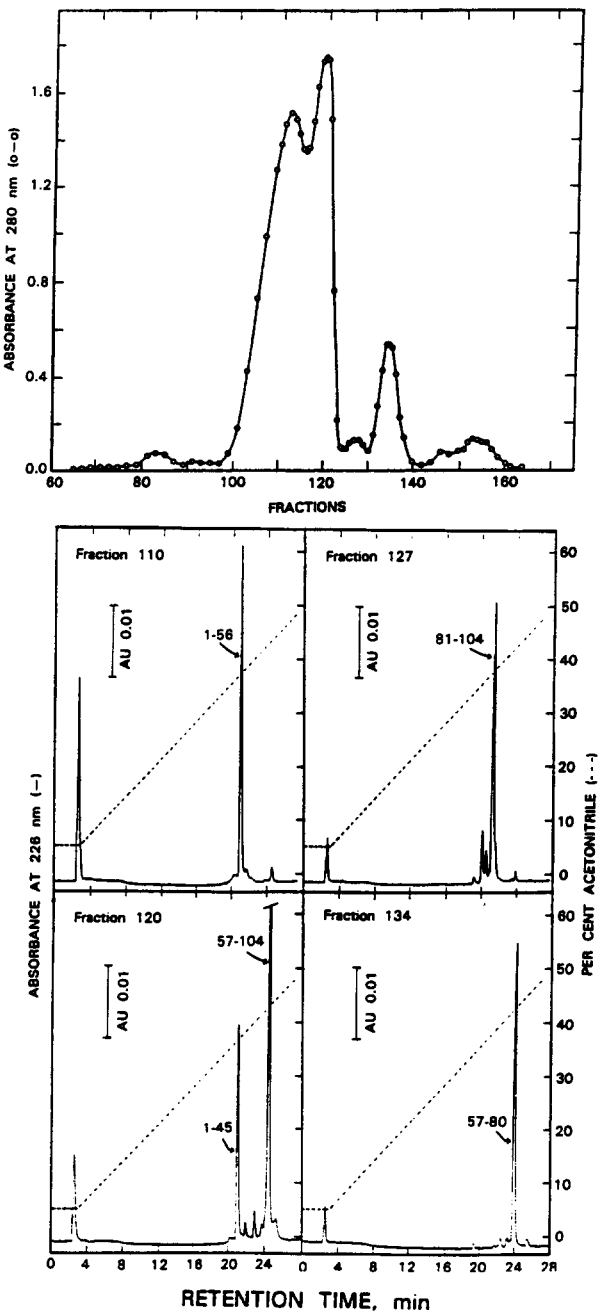

Fig. 2. Purification of proteolytic fragments of cytochrome *c*. *Top:* a proteolytic mixture obtained by thermolytic digestion in 50% (v/v) TFE for 24 h at 25°C was applied to a Sephadex G-50 superfine column (2.6 × 95 cm) eluted with 5% (v/v) aqueous formic acid at a flow rate of 6 ml/h. The effluent was monitored at 280 nm. Fractions of 3 ml were collected. *Bottom:* reverse-phase HPLC analysis of the peptide material of fractions of the effluent from the gel filtration column. A Vydac C_4 column (4.6 × 150 mm) was employed and eluted at a flow rate of 1.0 ml/min with a gradient of acetonitrile (- - -) in 0.05% aqueous TFA. The effluent from the column was monitored at 226 nm (——). Numbers near the chromatographic peaks refer to the identity of the fragments eluted from the column (see text).

from the gel filtration column together with fragment 57–104, whereas fragments 57–80 and 81–104 are eluted well separated from each other despite their identity in size. It could be well that conformational and/or hydrophobic effects play a role in the observed separation in the Sephadex G-50 column. Fragment 57–80 contains the single Trp^{59} of cytochrome *c*, explaining its much higher absorption at 280 nm in respect to fragment 81–104 (see Fig. 2, top).

3.3. Spectroscopic measurements

Fig. 3A shows the far-ultraviolet circular dichroism (CD) spectra of horse heart cytochrome *c* in Tris buffer, pH 7.0, in the absence and presence of 50% (v/v) TFE. In aqueous TFE, the far-ultraviolet CD spectrum of the protein shows, in respect to that of the protein in buffer only, a substantial increase in negative ellipticity at 208 and 222 nm wavelengths, thus signifying that cytochrome *c* in 50% TFE acquires a new conformational state characterized by enhanced helical secondary structure [23]. Quantitative analysis of the far-ultraviolet CD spectrum of cytochrome *c* in TFE in terms of secondary structure [24] allowed us to estimate that the TFE-induced state is characterized by a ~65% helix content, compared with a 45% content of the native protein in the crystal state [25].

In the near-ultraviolet region, the negative ellipticity of cytochrome *c* in 50% (v/v) aqueous TFE is substantially reduced in respect to that of the native protein and, in particular, the two negative CD bands at 283 and 290 nm of the aromatic chromophores (tyrosine, tryptophan) [26] are absent (not shown). This signifies that the specific side-chain interactions of the tertiary structure of the native protein are lost and that the aromatic amino acid residues have no fixed conformation in the TFE-state [26].

The fluorescence emission of the single tryptophan, as well as of the four tyrosine residues, in native cytochrome *c* is efficiently quenched by fluorescence energy transfer to the heme moiety [27]. When dissolved in 50% (v/v) TFE, cytochrome *c* exhibits both tyrosine and tryptophan fluorescence, as given by the emission maxima near 305 and 355 nm, respectively (Fig. 3B). Of interest, the fluorescence emission spectrum of the protein in TFE is quite similar to that observed under strong protein denaturing conditions such as 6 M guanidine hydrochloride (Gdn·HCl). The lower fluorescence intensity in TFE in respect to that in 6 M Gdn·HCl is due to solvent effects, as demonstrated by comparing fluorescence spectra of a 1:4 mixture of *N*-acetyl-tryptophanamide and *N*-acetyl-tyrosinamide in 50% (v/v) TFE and 6 M Gdn·HCl (not shown). Of note, the tyrosine fluorescence emission at 300–310 nm is seen when cytochrome *c* is dissolved in both TFE or 6 M Gdn·HCl. This implies an expanded conformational state of the protein in TFE, considering that with native globular proteins containing both tryptophan and tyrosine residues usually there is quenching of the tyrosine fluorescence by energy transfer, due to the proximity of tyrosine and tryptophan residues in the folded protein [28]. Therefore, we can conclude that the TFE-state of cytochrome *c* is a non-compact state with tryptophan and tyrosine residues well separated from each other and from the heme group.

The far- and near-ultraviolet CD spectra of thermolysin in 50% (v/v) aqueous TFE were essentially identical to those of the enzyme dissolved in Tris buffer (not shown), thus demonstrating the stability of both secondary and tertiary structure of the thermophilic protease to the action of TFE at high concentrations. The resistance of thermolysin to organic solvents has been previously noted [29]. Indeed, the crystallization

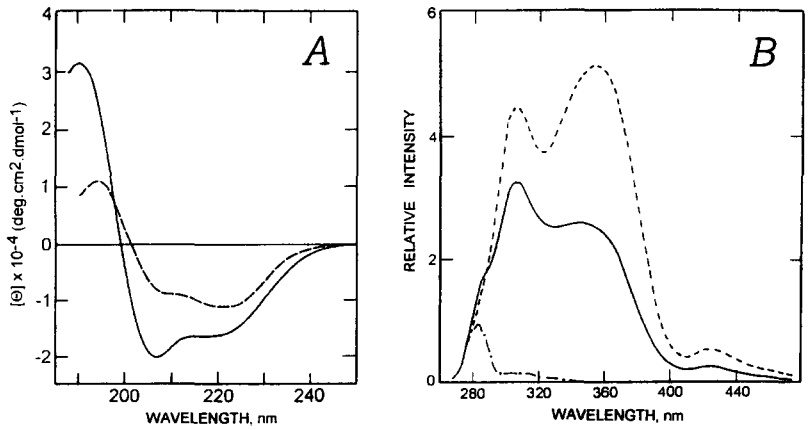

Fig. 3. *Left:* far-ultraviolet CD spectra of cytochrome *c* in aqueous buffer solution (- - - - -) and in the presence of 50% (v/v) TFE (———). *Right:* fluorescence emission spectra of cytochrome *c* in Tris buffer upon excitation at 280 nm. The buffer employed was 10 mM Tris-HCl buffer, pH 7.0, and measurements were performed at 25°C. · · ·, Tris buffer; ——, 50% TFE (v/v) in Tris buffer; - - -, 6 M Gdn·HCl in Tris buffer.

of thermolysin has been achieved utilizing an enzyme solution in 70% (v/v) aqueous dimethylsulfoxide [30].

4. Discussion

Initial thermolytic cleavage of horse cytochrome *c* in 50% (v/v) TFE occurs at Gly^{56}–Ile^{57}, followed by minor cleavages at Gly^{45}–Phe^{46} and Met^{80}–Ile^{81} (Fig. 4). Since thermolysin cleaves peptide bonds with poor specificity, even if with some preference at the N-terminus of hydrophobic and bulky amino acid residues (Ile, Leu, Phe) [18,31], clearly the selective proteolysis herewith observed is dictated by the specific structural and dynamic features of the protein substrate dissolved in aqueous TFE. In previous studies, we have emphasized that the site of limited proteolysis in globular proteins are exposed and flexible loops and that, in particular, helices are not prone to proteolytic attack [13–17]. These generalizations of the limited proteolysis phenomenon appear to hold also in the present case of cytochrome *c* in its TFE-state. First of all, CD and fluorescence emission measurements provide evidence that the TFE-state is an expanded, highly helical conformational state and devoid of specific tertiary interactions, likely containing at least the helical segments of native cytochrome *c*, as previously demonstrated for other proteins [7,8]. Indeed, inspection of the three-dimensional model of horse cytochrome *c* [25] reveals that the thermolytic cleavages occur at chain loops, outside the helical segments of the native protein, as shown in Fig. 4. Moreover, these sites of proteolysis are also among those of higher flexibility in native cytochrome *c*, as given by measuring the relative protection factor for hydrogen-deuterium exchange by proton NMR measurements [32].

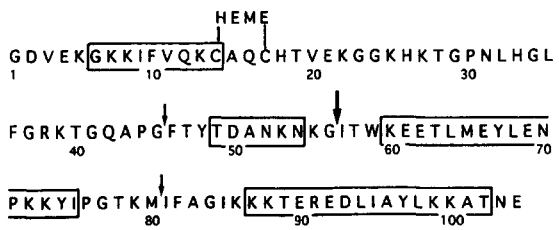

Fig. 4. Location of the thermolytic cleavages along the polypeptide chain of cytochrome *c*. Minor cleavages (see text) are indicated by smaller arrows. The chain segments in helical secondary structure in the native protein [25] are boxed.

The proteolysis of cytochrome *c* in its TFE-state by thermolysin requires relatively more drastic conditions than those usually employed in limited proteolysis experiments on other proteins [11]. This slow proteolysis can be due to several reasons, besides the fact that the highly helical TFE-state of the protein substrate hampers proteolysis (see above). First at all, in the presence of organic solvents proteolytic enzymes, including thermolysin [33], can catalyze the reverse reaction, i.e. the synthesis instead of the hydrolysis of the peptide bond [34]. Thermolysin is also less active in the presence of aqueous organic solvents (unpublished data). The detailed mechanism of inhibition is not known, but may involve binding of TFE at the hydrophobic active site of the enzyme, in analogy to the action of 2-propanol [35]. Finally, even if both secondary and tertiary structure of thermolysin are maintained in 50% (v/v) TFE (see section 3), it could be well that the addition of TFE causes an enhancement of protein rigidity [36] and consequently a reduction of its catalytic power, since some chain motility is required for catalysis [37].

In summary, the results of this study show that the TFE-resistant thermolysin can be used to achieve selective fragmentation of proteins in their TFE-induced helical state. Considering that recently in our laboratory a specific proteolysis of the type here described for cytochrome *c* has been achieved also using hen egg-white lysozyme, bovine pancreatic ribonuclease A, bovine α-lactalbumin and horse myoglobin (to be published), limited proteolysis by thermolysin in 50% (v/v) TFE can be considered a novel procedure to produce from globular proteins relatively large protein fragments useful for the purposes of protein sequencing [38], as well as for biophysical and functional studies.

Acknowledgements: This work was supported by the Italian National Council of Research. The authors thank Mrs. Anna Mocavero for the expert typing of the manuscript.

References

[1] Tamburro, A.M., Scatturin, A., Rocchi, R., Marchiori, F., Borin, G. and Scoffone, E. (1968) FEBS Lett. 1, 298–300.
[2] Nelson, J.W. and Kallenbach, N.R. (1986) Proteins Struct. Funct. Genet. 1, 211–217.
[3] Lehrman, S.R., Tuls, J.L. and Lund, M. (1990) Biochemistry 29, 5590–5596.
[4] Thomas, P.D. and Dill, K.A. (1993) Protein Sci. 2, 2050–2065.
[5] Jasanoff, A. and Fersht, A.R. (1994) Biochemistry 33, 2129–2135.
[6] Blanco, F.J., Jiménez, M.A., Pineda, A., Rico, M., Santoro, J. and Nieto, J.L. (1994) Biochemistry 33, 6004–6014.
[7] Buck, M., Radford, S.E. and Dobson, C.M. (1993) Biochemistry 32, 669–678.
[8] Alexandrescu, A.T., Ng, Y.-L. and Dobson, C.M. (1994) J. Mol. Biol. 235, 587–599.
[9] Liu, Z.-P., Rizo, J. and Gierash, L.M. (1994) Biochemistry 33, 134–142.
[10] Shiraki, K., Nishikawa, K. and Goto, Y. (1995) J. Mol. Biol. 245, 180–194.
[11] Mihaly, E. (1978) Application of Proteolytic Enzymes to Protein Structure Studies, CRC, Boca Raton, FL, USA.
[12] Neurath, H. (1980) in: Protein Folding (Jaenicke, R. Ed.) pp. 501–504, Elsevier/North Holland Biomedical Press, Amsterdam.
[13] Fontana, A., Fassina, G., Vita, C., Dalzoppo, D., Zamai, M. and Zambonin, M. (1986) Biochemistry 25, 1847–1851.
[14] Signor, G., Vita, C., Fontana, A., Frigerio, R., Bolognesi, M., Toma, S., Gianna, R., De Gregoriis, F. and Grandi, G. (1990) Eur. J. Biochem. 189, 221–227.
[15] Polverino de Laureto, P., De Filippis, V., Bertolero, F., Orsini, G. and Fontana, A. (1994) in: Techniques in Protein Chemistry (Crabb, J.W. Ed.) Vol. V, pp. 381–388, Academic Press, New York.
[16] Fontana, A., Polverino de Laureto, P. and De Filippis, V. (1993) in: Stability and Stabilization of Enzymes (Van den Tweel, W.J.J., Harder, A. and Buitelaar, R.M., Eds.) pp. 101–110, Elsevier, Amsterdam.
[17] Polverino de Laureto, P., Toma, S., Tonon, G. and Fontana, A. (1995) Int. J. Peptide Protein Res., in press.
[18] Heinrikson, R.L. (1977) Methods Enzymol. 3, 561–568.
[19] Shägger, H. and von Jagow, G. (1987) Anal. Biochem. 166, 368–379.
[20] Heinrikson, R.L. and Meredith, S.C. (1984) Anal. Biochem. 136, 65–74.
[21] Toniolo, C., Fontana, A. and Scoffone, E. (1975) Eur. J. Biochem. 50, 367–374.
[22] Margoliash, E. and Schejter, A. (1966) Adv. Protein Chem. 21, 113–286.
[23] Greenfield, N. and Fasman, G.D. (1969) Biochemistry 8, 4108–4116.
[24] Yang, J.T., Wu, C.-S. and Martinez, H.M. (1986) Methods Enzymol. 130, 208–269.
[25] Bushnell, G.W., Louie, G.V. and Brayer, G.D. (1990) J. Mol. Biol. 214, 585–595.
[26] Strickland, E.H. (1974) CRC Crit. Rev. Biochem. 5, 113–175.
[27] Fisher, W.R., Taniuchi, H. and Anfinsen, C.B. (1973) J. Biol. Chem. 248, 3188–3195.
[28] Brand, L. and Witholt, B. (1967) Methods Enzymol. 11, 776–856.
[29] Welinder, K.G. (1988) Anal. Biochem. 174, 54–64.
[30] Holmes, M.A. and Matthews, B.W. (1982) J. Mol. Biol. 160, 623–639.
[31] Keil, B. (1982) in: Methods in Protein Sequence Analysis (Elzinga, M., Ed.) Vol. IV, pp. 291–304, Humana Press, Clifton, New York.
[32] Jeng, M.-F., Englander, S.W., Elöve, G.A., Wand, A.J. and Roder, H. (1990) Biochemistry 29, 10433–10437.
[33] Wayne, S.I. and Fruton, J.S. (1983) Proc. Natl. Acad. Sci. USA 80, 3241–3244.
[34] Kullman, W. (1987) Enzymatic Peptide Synthesis, CRC Press, Boca Raton, FL, USA.
[35] Van den Burg, B., Eijsink, V.G.H., Stulp, B.K. and Venema, G. (1989) J. Biochem. Biophys. Methods 18, 209–220.
[36] Affleck, R., Haynes, C.A. and Clark, D.S. (1992) Proc. Natl. Acad. Sci. USA 89, 5167–5170.
[37] Welch, G.R. (1986) The Fluctuating Enzyme, J. Wiley, New York.
[38] Walsh, K.A., Ericsson, L.H., Parmelee, D.C. and Titani, K. (1981) Annu. Rev. Biochem. 50, 261–284.