

Limited proteolysis of lysozyme in trifluoroethanol Isolation and characterization of a partially active enzyme derivative

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Proteolysis of hen egg-white lysozyme by thermolysin in 50% aqueous trifluoroethanol for 6–24 h at 40–52°C produces a 'nicked' protein species which was purified to homogeneity by reverse-phase HPLC and characterized. Protein chemistry analytical methods were used to establish that thermolysin cleaves the 129-residue chain of lysozyme at peptide bond Lys97-Ile98. Nicked lysozyme, which is therefore constituted by fragments 1–97 and 98–129 cross-linked by disulfide bonds, was approximately 20% and 60% active towards *Micrococcus luteus* cells in respect to native intact lysozyme when assayed at 25°C or 5°C, respectively. Circular dichroic measurements provided evidence that nicked lysozyme in aqueous buffer at low temperature maintains the secondary structure content of native lysozyme, whereas the microenvironment of the aromatic chromophores, in particular of tryptophan residue(s), was somewhat perturbed. The stability to heat and urea denaturation of nicked lysozyme was dramatically reduced with respect to that of the intact protein. For example, the t_m of the nicked species was 28°C in comparison with 73°C for the unmodified enzyme, both at pH 7.0. Inspection of the X-ray structure of hen lysozyme reveals that thermolysin cleaves at the C-terminus of α -helix C (residues 88–98) located at the interface of the two structural domains of the protein, thus destabilizing the helix dipole and disrupting important tertiary interactions of the native enzyme. These results were interpreted considering that lysozyme in 50% aqueous trifluoroethanol is an expanded and flexible protein species largely maintaining native-like secondary structure, but lacking tertiary interactions [Buck, M., Radford, S. E. & Dobson, C. M. (1993) *Biochemistry* 32, 669–678]. Thus, whereas native lysozyme in its well-packed and rigid structure is quite resistant to proteolysis and only upon thermal unfolding is degraded to many small peptides in an all-or-none process, lysozyme in the trifluoroethanol state is sufficiently flexible to act as a substrate for the protease, but maintains significant secondary structure (helix) precluding extensive proteolytic degradation.

Keywords. Lysozyme; thermolysin; trifluoroethanol; limited proteolysis.

Trifluoroethanol has been widely used as a structure-inducing cosolvent for peptides and protein fragments that are otherwise unstructured in aqueous solution (Tamburro et al., 1968). Trifluoroethanol is known as a helix-inducing solvent, but this effect of trifluoroethanol does not occur independently of the amino acid sequence of the polypeptide chain, since peptides and protein fragments corresponding to helical regions of proteins in their native state are stabilized preferentially (Nelson and Kallenbach, 1986; Sönnichsen et al., 1992; Lehrman et al., 1990; Segawa et al., 1991). Indeed, a strong correlation has been found between the trifluoroethanol induced helicity and the predicted helical propensity using the Chou and Fasman (1978) algorithm (Lehrman et al., 1990). However, evidence has been provided that trifluoroethanol does not stabilize native-like secondary structures only, since a β -sheet \rightarrow helix conformational transition has been observed with some proteins dissolved in aqueous trifluoroethanol (Fan et al., 1993). The precise physical mechanism of structure induction by trifluoroethanol is not fully understood and debatable (Sönnichsen et al., 1992; Thomas and Dill, 1993; Jasanoff and Fersht, 1994; Blanco et al., 1994).

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Abbreviations. Nicked lysozyme, protein species with the peptide bond Lys97-Ile98 hydrolyzed; $[\theta]$, mean residue ellipticity.

Enzymes. Lysozyme (EC 3.2.1.17); thermolysin (EC 3.4.24.27).

In a recent study, the effect of addition of trifluoroethanol to an aqueous solution of hen egg-white lysozyme was studied by circular dichroism (CD) and proton NMR spectroscopy (Buck et al., 1993). Trifluoroethanol causes a conformational transition of lysozyme into a new stable conformational state characterized by a high degree of helical secondary structure and loss of the well-defined tertiary structure of the native protein. The midpoint of this transition is near 20% (by vol.) trifluoroethanol and is complete by 50% (by vol.) trifluoroethanol at room temperature. Overall, the data were interpreted as indicating that trifluoroethanol stabilizes the protein secondary structure, but destabilizes the hydrophobic core of the native lysozyme, leading to a trifluoroethanol state of the protein resembling that of the 'molten globule', i.e. a more expanded and flexible conformational state characterized by largely native-like secondary structure, but lacking specific tertiary interactions (Kuwajima, 1989; Pitsyn, 1992). The interest in this trifluoroethanol state is in that it offers the possibility to study partially folded protein species that probably represent intermediates in the protein folding process (Buck et al., 1993; Alexandrescu et al., 1994).

The present study was undertaken to contribute to the analysis of the trifluoroethanol-induced conformational state of proteins and peptides by using proteolytic enzymes as probes of protein structure (Neurath, 1980; Mihalyi, 1978). In previous

studies, we have emphasized that limited proteolysis of a globular protein, in its native state, occurs at exposed and flexible loops and, in particular, never at helical chain segments (Fontana et al., 1986, 1993; Signor et al., 1990; Poverino de Laureto et al., 1994, 1995). We wished to observe whether and how the proteolytic enzyme thermolysin (Matthews, 1988) in aqueous trifluoroethanol could attack and cleave the 129-residue chain of lysozyme at specific sites leading to nicked protein species, which could eventually be isolated and characterized. Thermolysin was used for this study, since this thermophilic protease exhibits noteworthy stability under harsh environments, including non-aqueous solvents (Welinder, 1988; Kitaguchi and Klibanov, 1989), and broad substrate specificity (Heinrikson, 1977; Keil, 1982). Thus, it was anticipated that by using thermolysin as the attacking protease, peptide bond fissions would occur at sites dictated by the stereochemistry and flexibility of the polypeptide substrate (lysozyme in its trifluoroethanol state) and not by the specificity of the protease (Fontana et al., 1986, 1993). In this study, we report the preparation and characterization of a partially active lysozyme species with the Lys97-Ile98 peptide bond cleaved. It is observed that this single peptide bond fission has no major effect on the three-dimensional structure of lysozyme in aqueous buffer at low temperature, but that the stability of the enzyme is dramatically decreased. The results obtained are discussed on the basis of the structural and dynamic features of the trifluoroethanol state of lysozyme, as deduced by proton NMR (Buck et al., 1993). This study represents a continuation of our efforts to demonstrate that proteolytic enzymes can be used as reliable probes of protein structure and dynamics (Fontana et al., 1986, 1993; Fontana, 1989; Signor et al., 1990; Poverino de Laureto et al., 1994, 1995).

MATERIALS AND METHODS

Materials. Hen egg-white lysozyme and thermolysin from *Bacillus thermoproteolyticus* were purchased from Sigma. Iodoacetamide, dithiothreitol, and all the solvents for spectroscopic purposes were obtained from Fluka. Reagents used for the protein/peptide sequence analysis were from Applied Biosystems and those used for amino acid analysis were from Waters (Millipore). The reagents used for SDS/PAGE were obtained from Bio-Rad. All other chemicals were reagent-grade quality.

Proteolysis of lysozyme. Limited digestion of lysozyme with thermolysin was carried out in closed vials in 50 mM Tris/HCl, pH 7.0, containing 5 mM CaCl₂ in the presence of increasing concentrations of trifluoroethanol, with an enzyme/protein ratio of 1:20 (by mass) for 6–24 h at 40, 52, and 60°C. The reaction products were separated by reverse-phase HPLC using a Vydac C₄ column and an acetonitrile/water gradient containing 0.05% CF₃COOH.

Reduction and S-alkylation of nicked lysozyme. The reduction of disulfide bonds of nicked lysozyme was performed with dithiothreitol in 0.5 M Tris/HCl, pH 8.0, containing 6 M guanidinium chloride and 2 mM EDTA. The reaction mixture was kept at 37°C for 3 h under nitrogen atmosphere and 50 mol iodoacetamide were added to the reaction mixture. After 1 h under nitrogen atmosphere and in the dark, the removal of excess dithiothreitol and reaction by-products was carried out by reverse-phase HPLC on a Vydac C₄ column using the same acetonitrile/water gradient employed for the purification of nicked lysozyme.

Electrophoresis. SDS/PAGE was performed under reducing conditions utilizing the Tricine buffer system (Shägger and von Jagow, 1987). The gels were stained with Coomassie brilliant

blue R-250 for protein detection. Capillary zone electrophoresis was carried out by using a Bio-Rad HPE-100 system with coated capillaries (20 cm×25 μm). The samples were dissolved in 0.1 M sodium phosphate buffer, pH 2.5. The analysis was run at 8 kV and protein elution was monitored at 200 nm.

Amino acid analysis and sequencing. Protein/peptide samples (50–300 pmol), contained in heat-treated borosilicate tubes (4 mm×50 mm), were hydrolyzed *in vacuo* at 150°C for 1 h with 6 M HCl containing 0.1% (by mass) phenol on the Pico-Tag workstation (Waters). The amino acids were derivatized with phenylisothiocyanate (Heinrikson and Meredith, 1984) and the resulting phenylthiocarbamoyl-derivatives were analyzed by HPLC using the Pico-Tag column (150 mm×3.9 mm; Waters).

N-Terminal sequence analysis was performed with an Applied Biosystem pulsed liquid-phase sequencer (model 477A) equipped with an on-line analyzer (model 120A) of phenylthiohydantoin-derivatives of amino acids.

Mass determination. Mass spectra were recorded using a time-of-flight matrix-assisted laser desorption/ionization mass spectrometer (Reflex, Bruker). A mixture of 5 μl 10 μM analyte solution and 5 μl 0.01 mM sinapinic acid in 20% acetonitrile was applied to the metallic probe tip and dried *in vacuo*. A pulsed laser beam (337 nm, 3 ns) was focused on the sample and the ions were accelerated with a linear potential of 30 kV. The mass spectrometer was calibrated with horse heart myoglobin utilized as an internal standard. The raw data were analyzed and stored by X-Mass software provided by Bruker.

Circular dichroic measurements. Circular dichroic (CD) spectra were recorded on a Jasco (model J-710) spectropolarimeter fitted with a thermostated cell holder and interfaced with a Neslab RTE-110 water bath. The results are expressed as the mean residue ellipticity, $[\theta] = (\theta_{\text{obs}}/10) \cdot (\text{mean residue mass}/l \cdot c)$, where θ_{obs} is the observed ellipticity in degrees, l is the path-length in cm, c the protein concentration expressed as g/ml. The mean residue molecular mass for intact and nicked lysozyme, calculated on the basis of the amino acid sequence (Jollès et al., 1963), was 111 Da. CD spectra were recorded in 20 mM sodium phosphate, pH 7.0, containing 50 mM NaCl, in 0.1-mm and 0.5-cm cylindrical quartz cells in the far-ultraviolet and near-ultraviolet regions, respectively.

Urea gradient electrophoresis. Urea-induced unfolding of intact and nicked lysozyme was monitored by urea gradient gel electrophoresis, essentially as described by Creighton (1979). Slab gels (15.5 cm×8.5 cm) were prepared containing a transverse gradient of 0–8 M urea with an inverse gradient of 15–11% acrylamide in order to compensate for the electrophoretic effects of the urea. The buffer was 0.05 M Tris acetate, pH 4.0, and the electrophoresis of proteins (50 μg) was run at 15 mA constant current for 2 h towards the cathode at 15°C. Urea gradient electrophoresis was also performed in a slab gel containing a transverse gradient of 0–4 M urea with an inverse gradient of 15–13% acrylamide in the same conditions. Protein detection in the gel was obtained with Coomassie brilliant blue dissolved in 10% (mass/vol.) trichloroacetic acid and 10% (mass/vol.) sulfosalicylic acid (Goldenberg and Creighton, 1984).

Enzymic activity. Bacteriolytic activities of intact and nicked lysozyme were evaluated as the decrease of absorbance at 540 nm of *Micrococcus luteus* cell suspension in 0.1 M potassium phosphate, pH 6.2, monitored in the presence of 0.5 μg of protein at 5°C and 25°C (Locquet et al., 1968).

RESULTS

Proteolysis of lysozyme by thermolysin in the presence of trifluoroethanol. The proteolysis of lysozyme by thermolysin

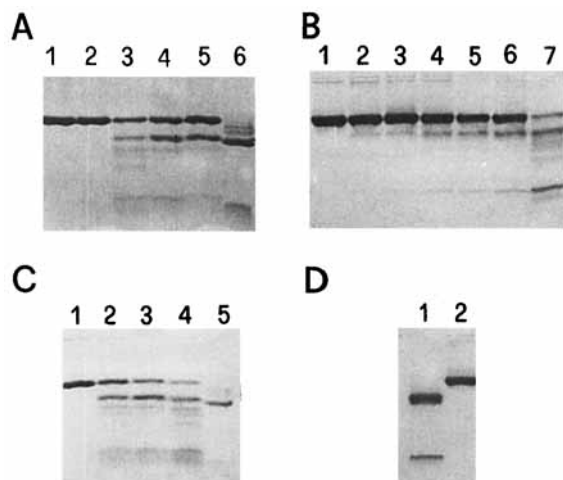


Fig. 1. SDS/PAGE analysis (reducing conditions) of the proteolysis of lysozyme by thermolysin. (A) Lysozyme was incubated at 40°C for 6 h with thermolysin (1:20, by mass) in 50 mM Tris/HCl, pH 7.0, containing 5 mM CaCl₂ and different concentrations of trifluoroethanol: 0, 20, 30, 40 and 50% (by vol.) trifluoroethanol, lanes 1–5. (B) Time course of the proteolysis of lysozyme by thermolysin in Tris/HCl, pH 7.0, containing 50% (by vol.) trifluoroethanol at 40°C: 0, 1, 2, 3, 4, 6 and 24 h, lanes 1–7. (C) Proteolysis of lysozyme with thermolysin for 6 h in the absence of trifluoroethanol (lane 1) and in the presence of 50% (by vol.) trifluoroethanol at 40°C (lane 2), 52°C (lane 3) and 60°C (lane 4). (D). SDS/PAGE analysis of purified nicked (lane 1) and intact (lane 2) lysozyme. An aliquot of lysozyme reacted with CNBr was loaded in (A) lane 6 and C-lane 5; the major protein band observed in the SDS/PAGE gels corresponds to that of fragment 13–106 (\approx 11 kDa) (see text).

was monitored by SDS/PAGE using the Tricine buffer system (Shägger and von Jagow, 1987). In the absence of trifluoroethanol, lysozyme was resistant to proteolysis by thermolysin (5%, by mass) when incubated in buffer, pH 7.0, at 20–37°C for a few hours, since only the band of intact lysozyme (\approx 14 kDa) was observed in the stained gel. At higher temperatures (40–55°C) and as the time of incubation increased (up to 24 h), there was some gradual disappearance of the band corresponding to the intact protein. When aliquots of the proteolysis mixture were analyzed by reverse-phase HPLC, it was found that the intact lysozyme was eluted from the column together with minor amounts of several peptides of low molecular mass (data not shown). SDS/PAGE and reverse-phase HPLC analyses indicate that thermolysin degrades lysozyme at 40–55°C in an all-or-none process leading to the formation of relatively small peptides (not stained in the SDS/PAGE gels) and no intermediate digestion products, implying that the native-denatured conformational transition of lysozyme dictates the rate of protein degradation and that the unfolded protein only is digested by thermolysin to small peptides.

The proteolysis of lysozyme by thermolysin in the presence of trifluoroethanol was studied by varying the alcohol concentration, time, and temperature of incubation. The results of the SDS/PAGE analyses of aliquots taken from the various reaction mixtures show that in the presence of trifluoroethanol lysozyme can be cleaved by thermolysin in a time-dependent manner to generate only a few fragments and that, depending upon the experimental conditions of proteolysis, essentially only two bands of peptide material are observed in the gels (Fig. 1). A cyanogen bromide (CNBr) (Gross, 1967) digest of lysozyme at the two methionine residues in positions 12 and 106 of the polypeptide chain (Jollès et al., 1963), leading mostly to fragment 13–106 (94 residues, \approx 11 kDa) served as an approximate marker of the

size of the obtained proteolytic fragments. The most abundant fragment appearing in the gels is the one of electrophoretic mobility similar to that of the CNBr-fragment 13–106 (Fig. 1A and C). The other fragment of much higher mobility is apparent in the gels of Fig. 1. SDS/PAGE analyses using proteins and protein fragments of known molecular masses as markers (data not shown), allowed us to evaluate the molecular mass of the fragment of higher electrophoretic mobility as 3.5 kDa. Considering that the sum of the molecular masses of the two more abundant proteolytic fragments of lysozyme observed in the gels roughly matches that of the intact protein (\approx 14 kDa), it can be concluded that thermolysin preferentially cleaves lysozyme in its trifluoroethanol state into two pieces only, i.e. selectively at a single peptide bond.

The data of Fig. 1A show that the digestion of lysozyme by thermolysin occurs at trifluoroethanol concentrations exceeding approximately 20% (by vol.), indicating that only above these concentrations of trifluoroethanol lysozyme acquire a new conformational and dynamic state prone to attack by thermolysin. This is in agreement with the results of a previous study showing that the conformation of lysozyme is marginally affected by approximately 15% (by vol.) trifluoroethanol (Buck et al., 1993; see also below). The majority of the proteolysis experiments were conducted in the presence of 50% (by vol.) trifluoroethanol, since under these solvent conditions highly specific proteolysis of lysozyme can be obtained. Moreover, the trifluoroethanol state of lysozyme induced by 50% (by vol.) alcohol has been thoroughly characterized by CD and proton NMR measurements (Buck et al., 1993), thus allowing an easier interpretation of the proteolysis data in terms of structure and dynamics of the protein substrate (see Discussion).

Substantial lysozyme fragmentation can be obtained after a 24-h reaction at 40°C (Fig. 1B, lane 7) or a 6 h reaction at 60°C (Fig. 1C, lane 4). Under these experimental conditions, the two main bands of peptide/protein material observed in the gel, resulting from a single nick of the protein chain (see above), are accompanied by other minor ones of higher mobility. Thus, the results of the SDS/PAGE analyses of the various proteolytic mixtures (Fig. 1) indicate that heating and longer reaction times enhance the yields of protein fragmentation, but somewhat reduce the selectivity of the proteolytic event.

Isolation and characterization of nicked lysozyme. In order to isolate to homogeneity nicked lysozyme in sufficient quantity for further characterization, an aliquot (\approx 10 μ g protein) of a proteolytic mixture obtained after a 5 h reaction at 52°C in 50% (by vol.) trifluoroethanol was applied to a Vydac C₄ column eluted with a gradient of acetonitrile. The chromatogram of Fig. 2 shows that a major peak of protein material is eluted from the column at approximately 37% acetonitrile, followed by a minor one at about 47%. It was found by SDS/PAGE that the protein material of the major peak was constituted by two fragments of molecular masses of about 11 kDa and 3.5 kDa (Fig. 1D) and thus was identified as nicked lysozyme (see above), whereas that of the last eluted peak was shown to be intact lysozyme. Analysis by SDS/PAGE of the protein material eluted in front of nicked lysozyme established that this material contained most of the protein fragments that appear as faint bands in the SDS/PAGE gels shown in Fig. 1 (Fig. 1B, lane 7; Fig. 1C, lane 4). Further purification to homogeneity and analysis of these lysozyme fragments were conducted using the methods described below (data not shown; see also Discussion).

The sample of nicked lysozyme purified by HPLC was subjected to further analyses in order to establish its homogeneity and identity. First, capillary electrophoresis established the high purity of nicked lysozyme, since this protein is eluted from the

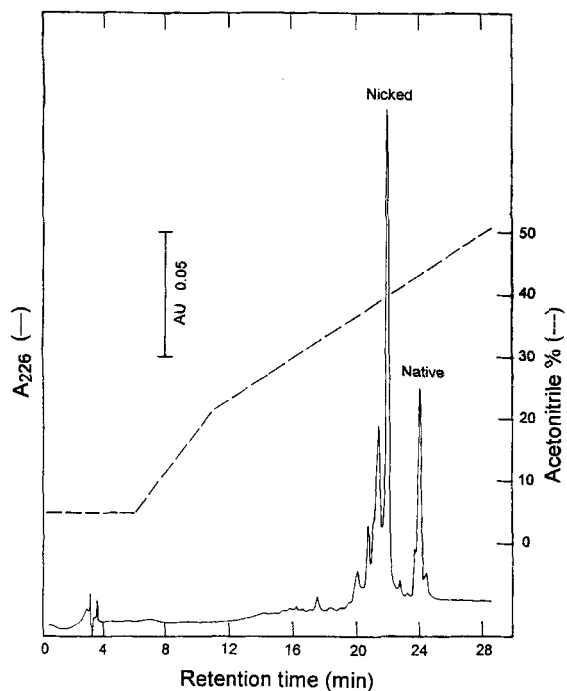


Fig. 2. Reverse-phase HPLC analysis of the proteolytic mixture of lysozyme reacted with thermolysin. Lysozyme was treated at 52°C with thermolysin at a 1:20 ratio (by mass) of protease to lysozyme in 50 mM Tris/HCl, pH 7.0, containing 5 mM CaCl₂ and 50% (by vol.) trifluoroethanol. An aliquot (≈10 µg protein) of the reaction mixture was analyzed on a Vydac C₄ column (4.6 mm×150 mm), eluted at a flow rate of 0.6 ml/min with a gradient of acetonitrile in 0.05% (by vol.) CF₃COOH. The effluent from the column was monitored at 226 nm.

capillary as a highly homogeneous species, well separated from the intact species (data not shown). N-terminal sequence analysis (five Edman degradation cycles) of a nicked lysozyme sample gave two sequences, the N-terminal Lys-Val-Phe-Gly-Arg and Ile-Val-Ser-Asp-Gly. Comparison of these sequence data with the known amino acid sequence of lysozyme (Jollès et al., 1963; Canfield, 1963) allowed us to infer that protein nicking occurs at the N-terminus of Ile98. The amino acid composition of nicked lysozyme, after acid hydrolysis, is essentially the same as that of the intact protein (Table 1). A sample of nicked lysozyme reduced with excess thiol and *S*-carboxamidomethylated (see Materials and Methods) eluted from the Vydac C₄ column in two peaks (data not shown). The amino acid compositions after acid hydrolysis of the two *S*-carboxamidomethylated fragments constituting nicked lysozyme were in agreement with those calculated for fragments 1–97 and 98–129 (Table 1). Finally, it was clearly demonstrated that nicked lysozyme is a protein species with just a single cleaved peptide bond by determining its molecular mass using time-of-flight mass spectrometry. The experimental value for the difference in molecular masses between nicked (14327 Da) and intact lysozyme (14314 Da) was 13 Da; considering the actual accuracy of the mass measurements, this value is in agreement with the fact that hydrolysis of a single peptide bond in lysozyme is expected to enhance the molecular mass of the protein by 18 Da (the calculated mass for the intact protein is 14313 Da).

Circular dichroic studies. As shown in Fig. 3, intact lysozyme dissolved in sodium phosphate, pH 7.0, in the presence of 50% (by vol.) trifluoroethanol acquires a significantly higher proportion of α -helix with respect to the protein in buffer alone. The negative ellipticity in the 200–250 nm region of lysozyme in

Table 1. Amino acid composition of nicked lysozyme and its *S*-carboxamidomethylated fragments 1–97 and 98–129. Amino acid compositions are reported as amino acid residues/molecule. Expected values are given in parentheses and were calculated from the amino acid sequence of hen egg-white lysozyme (Jollès et al., 1963). The values of Asx and Glx are the sum of Asp and Asn, and of Glu and Gln calculated from the sequence of lysozyme. The values given are average values obtained from three separate amino acid analyses. Samples of nicked lysozyme and of *S*-carboxamidomethylated fragments 1–97 and 98–129 were prepared and isolated to homogeneity by micropreparative reverse-phase HPLC as described in the text. n.d., not determined.

Amino acid	Lysozyme		Fragment (residue positions)	
	intact	nicked	1–97	98–129
Asx	19.9 (21)	18.9 (21)	15.5 (16)	4.9 (5)
Thr	6.6 (7)	6.7 (7)	5.7 (6)	1.3 (1)
Ser	9.7 (10)	9.8 (10)	8.1 (9)	1.0 (1)
Glx	4.5 (5)	4.6 (5)	4.1 (4)	1.2 (1)
Pro	2.1 (2)	2.2 (2)	2.1 (2)	
Gly	12.2 (12)	11.8 (12)	6.3 (8)	3.9 (4)
Ala	11.6 (12)	11.5 (12)	8.3 (9)	3.1 (3)
Val	5.6 (6)	6.1 (6)	3.0 (3)	2.9 (3)
Met	2.2 (2)	2.3 (2)	1.3 (1)	1.2 (1)
Ile	6.0 (6)	6.0 (6)	3.9 (4)	1.7 (2)
Leu	7.6 (8)	7.6 (8)	7.1 (7)	1.3 (1)
Tyr	2.9 (3)	3.0 (3)	2.7 (3)	
Phe	2.6 (3)	3.3 (3)	3.3 (3)	
Lys	6.3 (6)	5.7 (6)	5.0 (5)	1.2 (1)
His	1.1 (1)	1.0 (1)	1.0 (1)	
Arg	10.6 (11)	10.6 (11)	7.2 (7)	4.2 (4)
Trp	n.d. (6)	n.d. (6)	n.d. (3)	n.d. (3)
¹ / ₂ -Cys	n.d. (8)	n.d. (8)	n.d. (6)	n.d. (2)
No. residues	129	129	97	32

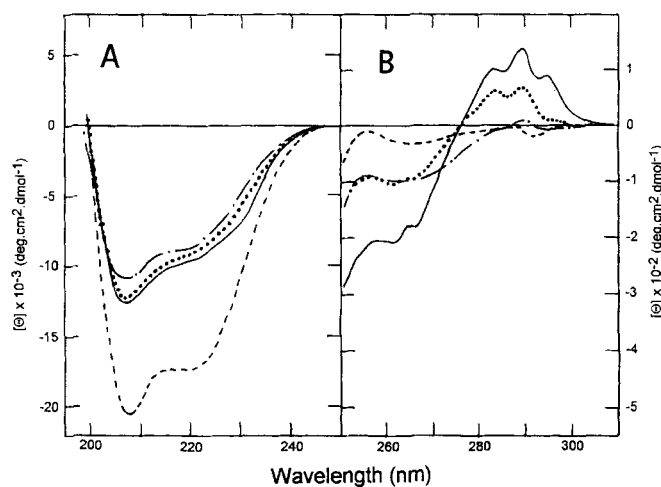


Fig. 3. Far-ultraviolet (A) and near-ultraviolet (B) CD spectra of nicked and intact hen lysozyme. Spectra were obtained in 20 mM sodium phosphate, 50 mM NaCl, pH 7.0, with or without 50% (by vol.) trifluoroethanol. Protein concentrations were about 0.1 mg/ml and 0.8 mg/ml in the far-ultraviolet and near-ultraviolet regions, respectively. (—), intact lysozyme in sodium phosphate buffer; (---) intact lysozyme in 50% (by vol.) trifluoroethanol; (---) nicked lysozyme in sodium phosphate buffer at 25°C; (·····) nicked lysozyme in sodium phosphate buffer at -5°C.

the trifluoroethanol state is much greater than that of the native enzyme in buffer and shows the characteristics typical of helical polypeptides with minima of ellipticity near 208 nm and 222 nm (Greenfield and Fasman, 1969). In contrast, the near-ultraviolet CD spectrum of lysozyme in the presence of trifluoroethanol shows a marked reduction of the positive ellipticity in the 275–300 nm region exhibited by the native enzyme, indicating that the microenvironments of the tryptophan and tyrosine (at least) chromophores are significantly perturbed and located in a less-packed or unfolded protein structure. These CD data are in agreement with those of previous studies (Galat, 1985; Buck et al., 1993) and indicate that the trifluoroethanol state of lysozyme is characterized by enhanced α -helical content and loss of tertiary structure, thus resembling a molten globule state (Kuwajima, 1989; Ptitsyn, 1992). In contrast, both the secondary and tertiary structures of thermolysin are not affected by the presence of trifluoroethanol up to 50% (by vol.), since the far-ultraviolet and near-ultraviolet CD spectra of the protease dissolved in 50 mM Tris/HCl, pH 7.0, containing 5 mM CaCl₂, are identical in shape and ellipticity values to those measured in buffer alone (data not shown).

The far-ultraviolet CD spectrum of nicked lysozyme at 25°C indicates that this protein maintains an overall secondary structure comparable to that of the native species (Fig. 3). This similarity of structure between nicked and intact lysozyme appears to be even greater, if the CD spectrum of the nicked species is measured at -5°C. In contrast, the near-ultraviolet CD spectrum of nicked lysozyme at 25°C shows a marked reduction of ellipticity in the 275–300 nm region, indicating substantial differences in tertiary structure between the nicked and native protein. However, the near-ultraviolet CD spectrum of nicked lysozyme at -5°C is very similar to that of intact lysozyme, including positive bands at about 283 nm and 290 nm. The positive band at 294 nm of native lysozyme is essentially not observed with the nicked species. Since the CD spectra in the 250–310-nm region are sensitive and reliable probes of the microenvironments of aromatic residues and disulfide bonds (Strickland, 1974), the features of the near-ultraviolet CD spectrum of nicked lysozyme at -5°C indicate a strong similarity of tertiary structure between the nicked and intact protein at low temperature.

Thermal and urea-induced unfolding transitions. The thermal denaturation profiles of intact and nicked lysozyme were measured by following the decrease of the CD signal at 222 nm upon increasing, at a uniform rate, the temperature of the protein solution in the cuvette. CD melting curves and the corresponding derivative curves for the nicked and native protein species are shown in Fig. 4. The values of t_m , defined by a peak in the differentiation of the melting curve, are 73°C and 28°C for intact and nicked lysozyme, respectively. Thus, a single nick of the polypeptide chain of lysozyme leads to a dramatic protein destabilization.

The stability of nicked lysozyme with respect to the intact protein was also evaluated by the urea gradient gel electrophoresis technique (Creighton, 1979; Goldenberg and Creighton, 1984). As shown in Fig. 5, the electrophoretic mobility of intact lysozyme is only slightly affected by increasing the urea concentration up to about 8 M, thus signifying that lysozyme is a rather stable protein resistant to high concentrations of denaturant (Creighton, 1979; Ahmad and Bigelow, 1982). In contrast, nicked lysozyme shows a continuous, sigmoidal curve for its electrophoretic mobility in the urea gradient gel, which is characteristic of a protein that undergoes an unfolding transition from a compact to an expanded state. The data of Fig. 5 allow us to evaluate the midpoint for the urea-mediated unfolding of nicked lysozyme as approximately 2 M urea. Thus, the nicked

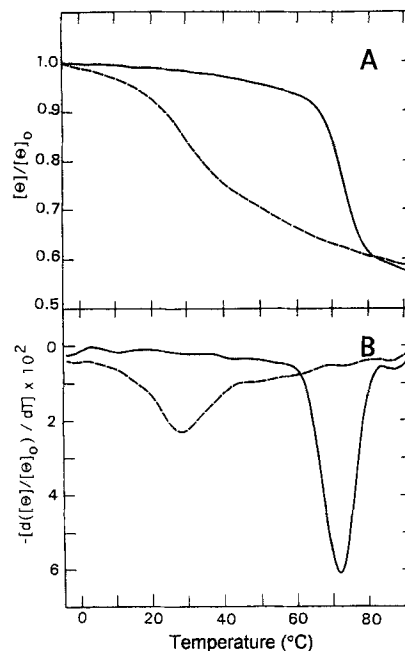


Fig. 4. Temperature dependence of the mean residue ellipticity, $[\theta]$ at 222 nm of intact (—) and nicked (---) lysozyme. Melting curves (A) were recorded in 20 mM sodium phosphate, pH 7.0, containing 50 mM NaCl. The derivatives for the corresponding curves are shown in B.

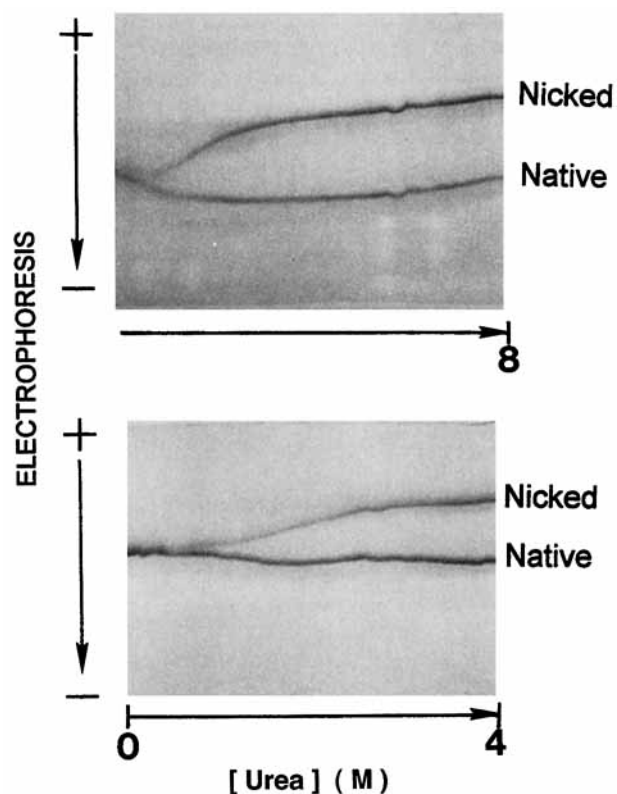


Fig. 5. Urea gradient electrophoresis of nicked and native lysozyme. The buffer was 0.05 M Tris acetate, pH 4.0. The linear gradient of 0–8 M urea (top) was superimposed on an inverse linear gradient of 15–11% acrylamide. The gradient of 0–4 M urea (bottom) was superimposed on an inverse linear gradient of 15–13% acrylamide. Electrophoresis towards the cathode at 15°C was carried out for 2 h.

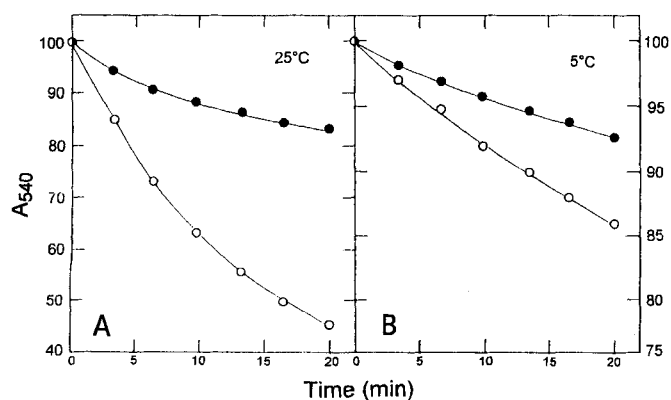


Fig. 6. Enzymic assays of nicked (●) and native (○) lysozyme at 25°C (A) and 5°C (B). The decrease of absorbance at 540 nm of *M. luteus* cell suspension in 0.1 M potassium phosphate, pH 6.2, was monitored in the presence of 0.5 μg enzyme.

protein shows a marginal stability to urea denaturation with respect to the stability exhibited by several of other globular proteins previously studied either with the urea gradient electrophoresis technique (Goldenberg and Creighton, 1984) or in solution utilizing spectroscopic techniques (Pace, 1975, 1986). Interestingly, electrophoretic mobilities of both nicked and intact lysozyme are identical in the absence of urea (Fig. 5), signifying that both protein species share the same charge and overall hydrodynamic volume in the absence of urea.

Enzymic activity of nicked lysozyme. The catalytic activity of nicked lysozyme was evaluated by following the lysis of *M. luteus* cells (Locquet et al., 1968). The data of Fig. 6 show that the nicked species exhibits a lower catalytic activity with respect to that of the intact lysozyme. Moreover, it is observed that the relative reduction in catalytic power of the nicked species is higher at 25°C with respect to that at 5°C. The specific catalytic activity of nicked lysozyme, evaluated from initial velocity of the lysis of *M. luteus* cells, was diminished to about 60% and 20% of the activity of intact lysozyme when assayed at 5°C and 25°C, respectively.

DISCUSSION

The results presented in this study show that thermolysin cleaves selectively the 129-residue chain of hen lysozyme in its trifluoroethanol state (Buck et al., 1993) at the peptide bond Lys97-Ile98, leading to a nicked protein species constituted by fragments 1–97 and 98–129 covalently linked by four disulfide bridges. The specificity of peptide bond fission is striking, if one considers the broad substrate specificity of thermolysin which cleaves mostly at the amino side of hydrophobic amino acid residues such as Leu, Ile, Phe, Val, and Tyr, but at other residues as well [Heinrikson, 1977; Morihara and Suzuki, 1970; see Keil (1982), for a compilation of the relative rates of peptide bond hydrolysis by thermolysin]. The observed peptide bond cleavage is dictated by the stereochemistry and dynamics of the protein substrate (lysozyme in its trifluoroethanol state) and not by the specificity of the attacking protease. For example, there are plenty of hydrophobic residues in the lysozyme polypeptide chain as possible sites of cleavage by thermolysin and, in particular, there are six Xaa-Ile, eight Xaa-Leu and three Xaa-Phe peptide bonds (Fig. 7). Upon prolonged proteolysis and at high temperature, such as 24 h at 40°C or 6 h at 60°C, additional but few cleavages of the protein chain occur. Isolation to homo-

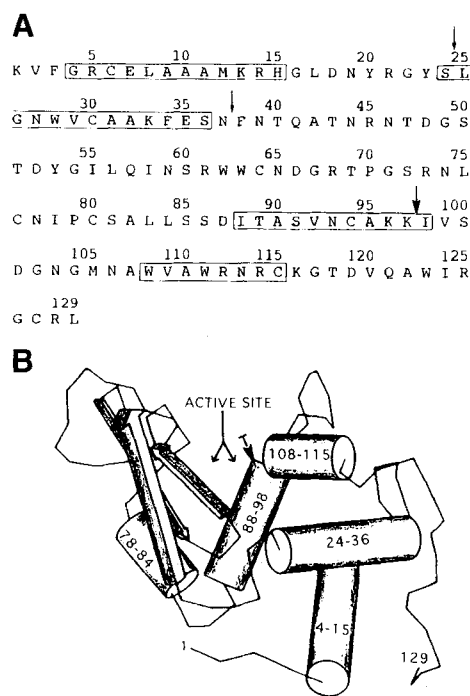


Fig. 7. Amino acid sequence and schematic three-dimensional structure of hen egg-white lysozyme. (A) The four major α -helical segments along the polypeptide chain are boxed (helix A, 4–15; B, 24–36; C, 88–98; D, 108–115). Lysozyme contains four disulfide bridges (6–127, 30–115, 64–80, and 76–94) (Jollès et al., 1963; Canfield, 1963). The site of peptide bond fission in nicked lysozyme is indicated (↓). The sites of proteolytic cleavage observed under more drastic conditions of reaction (see text) are also shown (↓). (B) Regions of secondary structure and the two structural domains of native lysozyme. Besides the four helices of the α -domain, the 3_{10} -helix of the β -domain is also shown by a cylinder (residues 78–84). The active site cleft at the interface between the domains is also shown (adapted from Johnson et al., 1988). The site of cleavage by thermolysin is indicated (↓).

geneity and analytical characterization of most of these additional fragments of lysozyme (data not shown) allowed us to identify Ser24-Leu25 and Asn37-Phe38 as the peptide bonds cleaved more slowly by thermolysin after the initial cleavage at Lys97-Ile98. Thus, it seems that in the presence of aqueous trifluoroethanol thermolysin displays enhanced substrate selectivity by cleaving at the amino side of Ile, Leu, and Phe residues only.

The major result from a previous study on the structure and dynamics of the trifluoroethanol state of lysozyme (pH 2.0, 50% trifluoroethanol) was that trifluoroethanol stabilizes predominantly native-like secondary structure (Buck et al., 1993). Significant protection (up to 200-fold) from H/D exchange occurs mostly at chain regions that are helical in the native enzyme. However, the trifluoroethanol state of lysozyme is much more flexible than the native one, since corresponding protection factors are 10^5 – 10^7 higher (Radford et al., 1992). Indeed, the rigidity of native lysozyme is reflected by its resistance to proteolytic degradation by thermolysin (this study) and by other proteases (Imoto et al., 1974, 1986). Only upon thermal unfolding is the denatured, flexible protein degraded to small peptides in an all-or-none type of process (Imoto et al., 1974, 1986). Nevertheless, even if the trifluoroethanol state of lysozyme is characterized by a much higher chain flexibility than the native state, proteolysis by thermolysin requires relatively high temperature and longer reaction times.

Accepting the view that stable helical chain segments are not cleaved by the proteolytic probe (Fontana et al., 1986, 1993), the relatively slow proteolytic digestion of lysozyme in its trifluoroethanol state is consistent with the fact that this state is characterized by a high content of helical secondary structure (Fig. 3; Buck et al., 1993). Even under drastic conditions of proteolysis (such as 24 h at 40°C), very few peptide bonds are cleaved and these are located essentially outside the helical segments of native lysozyme (Fig. 7). This is in agreement with the fact that the far-ultraviolet CD spectrum of lysozyme in 50% (by vol.) trifluoroethanol at pH 7.0 (this study) or at pH 2.0 (Buck et al., 1993) indicates a higher helical content than that of the native state. The actual significance of this finding is not known and has been discussed by Buck et al. (1993). Nevertheless, only the chain segments that are helical in native lysozyme show significant protection of amides from H/D exchange, implying that the additional helices of the trifluoroethanol state (if present) are much more dynamic entities with respect to those of the native state.

The slow proteolysis of hen lysozyme by thermolysin results both from the structural and dynamic features of the protein substrate dissolved in aqueous trifluoroethanol (see above), and from the fact that thermolysin is much less active in the presence of trifluoroethanol. In this respect, it should be mentioned that proteolysis of a protein substrate in the presence of organic solvents (e.g. acetonitrile, methanol, 2-propanol) (Welinder, 1988), and other protein denaturing agents (SDS, urea, guanidinium chloride) (Rivière et al., 1991), has been studied previously with the aim of developing useful techniques for PAGE peptide mapping (Cleveland et al., 1977) and for producing protein fragments suitable for sequence analysis. Both organic solvents (Welinder, 1988; Houen and Sando, 1991; Iyer and Acharya, 1987; Acharya et al., 1992) and SDS (Cleveland et al., 1977) appear to control and restrict the degree of proteolysis of a protein substrate. However, the reduced catalytic activity of thermolysin in the presence of 50% (by vol.) trifluoroethanol can also be explained by considering that proteolytic enzymes in the presence of nonaqueous solvents can catalyze the reverse reaction, e.g. the synthesis instead of hydrolysis of peptide bonds (Fruton, 1982). Indeed, the use of proteases, including thermolysin, in the presence of aqueous glycerol, 2-propanol, acetonitrile, dimethylformamide, or dimethylsulfoxide is an established procedure for the synthesis and semi-synthesis of peptides and proteins (Chaiken, 1981; Kullman, 1987; Kitaguchi and Klibanov, 1989; De Filippis and Fontana, 1990). Moreover, it may be possible that trifluoroethanol inhibits (partly) thermolysin by preferential binding at its hydrophobic binding site (Matthews, 1988), in analogy to 2-propanol (van der Burg et al., 1989). In this respect, it is worth mentioning that organic solvents (acetonitrile) appear to bind preferentially at the hydrophobic active site of subtilisin (Fitzpatrick et al., 1993). Finally, in the presence of trifluoroethanol, thermolysin may become more rigid and thus less active, if one accepts the view that in the presence of organic solvents protein flexibility is reduced (Affleck et al., 1992; Hartsough and Merz, 1992) and that some chain mobility is required for catalysis (Welch, 1986).

Considering that nicked lysozyme maintains the functional properties of the intact enzyme, even if the activity is lower ($\approx 60\%$ at 5°C), a similar overall three-dimensional structure between the nicked and intact species was anticipated. Indeed, the far-ultraviolet CD spectra of both proteins are very similar, especially at low temperature (Fig. 3). In the near-ultraviolet region, the CD spectrum of nicked lysozyme displays significant differences with respect to that of the intact species at 25°C and strong similarities at -5°C, implying that, in order to acquire the specific tertiary interactions of native lysozyme, some con-

formational freezing of the nicked protein is required. However, the positive dichroic band at 294 nm of intact lysozyme appears to be essentially missing in the spectrum of nicked lysozyme, even at -5°C. This band has been proposed to arise from contributions of Trp108 [Teichberg et al., 1970; see Strickland (1974), for a discussion of this proposal]. If this attribution is correct, the absence of the 294-nm band in nicked lysozyme would imply a different microenvironment of Trp108 in the nicked protein with respect to that in the intact protein, as one would expect from the fact that Trp108 is, together with Ile98, part of the hydrophobic core of native lysozyme (see above). Finally, the reduced catalytic activity of nicked lysozyme with respect to that of the native species can be explained by considering that the cleaved peptide bond is located in one lip of the active site cleft of the enzyme and near amino acid residues (e.g. Asp101) involved in substrate binding (Fig. 7).

A chain fission is expected to destabilize a protein by enhancing the conformational entropy of the unfolded chain, thus leading to effects that are opposite to those exhibited by covalent crosslinks (Poland and Scheraga, 1965; Johnson et al., 1979). Indeed, there are numerous examples of nicked proteins maintaining the structural and even functional features of the parent intact species, but showing reduced stability to heat or other common protein denaturants (Mihalyi, 1978). For example, thermolysin S, a nicked partially active derivative of thermolysin constituted by fragments 5-224(225) and 225(226)-316, is less stable to heat than the intact protein (Vita et al., 1985). Thus, the lower stability of nicked lysozyme with respect to the intact species is expected, but nevertheless appears to be striking. The t_m of nicked lysozyme is decreased by as much as 45°C and in 3 M urea, pH 4.0, the protein is unfolded, whereas intact lysozyme requires an urea concentration above 8 M for complete unfolding (Ahmad and Bigelow, 1992; Fig. 5). Similarly, a lysozyme derivative that has a nick at Gly104, obtained as a side-product in the nitration of the protein with tetranitromethane (Yamada et al., 1990), shows an approximately 20°C reduction in t_m with respect to that of the intact enzyme (Imoto et al., 1994). Conversely, an ester cross-link between the γ -carboxylate of Glu35 in the β -domain and oxindolylalanine at position 108 in the α -domain of lysozyme, obtained by selective oxidation of the protein by *N*-bromosuccinimide, increases the t_m of the protein by as much as 29°C (at pH 2.0; Johnson et al., 1979).

The results of numerous recent experiments using site-directed mutagenesis of proteins have clearly indicated that proteins tolerate extensive amino acid replacements at their solvent-exposed sites, whereas those located in the hydrophobic core at the protein interior are much more critical in affecting the structure, stability, and function of mutated proteins (Alber, 1989; Matthews, 1993; Fontana, 1991). On this basis, the dramatic destabilizing effect of the single peptide bond fission in nicked lysozyme is predicted to be located at the interior of native enzyme. Indeed, examination of the three-dimensional structure of hen lysozyme (Imoto et al., 1972; Diamond, 1974) reveals that the side-chain of Ile98 participates in the formation of a hydrophobic core of the protein constituted by the additional residues Ala31, Ile58, Trp63, Ala107 and Trp108 (see Fig. 9 in Hooke et al., 1994; Fig. 5 in Ueda et al., 1994, for the structural details of the hydrophobic cluster of hen lysozyme). This core is likely a key structural parameter dictating protein stability and is formed first in the folding pathway of hen lysozyme (Radford et al., 1992; Dobson et al., 1994). The charges produced by fission of the Lys97-Ile98 peptide bond are expected to greatly destabilize the protein hydrophobic core. A similar dramatic destabilizing effect has also been found in T_4 lysozyme mutants, where a Met \rightarrow Lys substitution at position 102 of the chain, in

the hydrophobic interior, reduces the protein t_m by 35°C (Dao-Pin et al., 1991).

In a recent study (Ueda et al., 1994), protein engineering experiments were used to show that helix C (residues 88–98) is essential for maintaining a thermodynamically stable, folded structure of hen lysozyme. This amphipathic helix is stabilized by the positive charges at Lys96 and Lys97 located at its C-terminus, thus explaining why the two positive charges at positions 96 and 97 are firmly conserved among C-type lysozymes. Indeed, the α -helix has a dipole moment with the positive pole at the N-terminus and the negative one at the C-terminus (Hol, 1985), and it has been often demonstrated that proteins can be stabilized by the appropriate introduction by site-directed mutagenesis of negative and positive charges at the N-terminus and C-terminus of a helix, respectively (Nicholson et al., 1988; Sali et al., 1988). On this basis, the proteolytic nicking of the lysozyme chain at the Lys97-Ile98 peptide bond leads to the introduction of a helix-destabilizing carboxylate group at the C-terminus of helix C. This amphipathic helix is located at the interface of the two structural domains of hen lysozyme (Fig. 7) and its hydrophobic side faces close to Trp63 of the β -domain (Ueda et al., 1994). Thus, it appears that nicking of the Lys97-Ile98 peptide bond leads to a major alteration of the forces and interactions stabilizing the two-domain architecture of hen lysozyme.

In summary, the results of this study indicate that the trifluoroethanol-resistant thermolysin can be used to obtain selective fission of peptide bonds in protein substrates dissolved in aqueous trifluoroethanol. This procedure seems to be useful for other proteins, since recently in our laboratory selective protein fragmentation of the type described in this study for hen lysozyme has also been achieved with bovine ribonuclease A, horse heart cytochrome *c*, and bovine α -lactalbumin. The results of these studies will be published elsewhere. Finally, considering that few proteins are as well known as hen lysozyme (Imoto et al., 1972) and that this protein is a prototype model protein used in several laboratories to address several problems of contemporary interest (Dobson et al., 1994), the nicked species of lysozyme described in this study, and its constituting fragments 1–97 and 98–129, can be used for additional biophysical studies.

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