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AN ANIONIC TRYPSIN-LIKE ENZYME FROM *STREPTOMYCES ERYTHREUS*: CHARACTERIZATION

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SUMMARY

Homogeneity of a purified anionic trypsin-like enzyme from *Streptomyces erythreus* was demonstrated by a simple band on polyacrylamide disc electrophoresis, a single symmetric peak in the sedimentation schlieren pattern and a sharp elution peak on gel filtration, and by equilibrium sedimentation experiment.

The molecular weight of the enzyme was investigated by sedimentation equilibrium, combination of sedimentation and diffusion, gel filtration, and amino acid analysis; the results were in excellent agreement, giving a molecular weight of 21 400 on average. Evaluation of the molecular size and shape from a sedimentation coefficient of 2.57 S and a diffusion coefficient of $10.7 \cdot 10^{-7}$ cm^2/s indicates an ellipsoid close to a sphere of radius 2.0 nm, with hydration of 0.2–0.24 g per g of the enzyme.

The circular dichroism spectrum of the enzyme was compared with those of bovine pancreatic trypsin (EC 3.4.4.4) and the trypsin-like enzymes from *Streptomyces fradiae* and *Streptomyces griseus*. It is suggested that trypsin and the trypsin-like enzymes investigated contain small amounts of α helix or β structure, the remainder of the molecules being unordered.

INTRODUCTION

Among the many esterolytic or proteolytic enzymes described as trypsin-like enzymes[1], two such enzymes were recently isolated from strains of *Streptomyces*, *S. fradiae*[2] and *S. griseus*[3], respectively. In a preliminary communication[4] we reported the isolation of a third trypsin-like enzyme from a microbial strain, *S. erythreus*. It would seem of great interest to compare the secondary and tertiary structures of the trypsin-like enzymes from *Streptomyces* strains with those of bovine pancreatic trypsin (EC 3.4.4.4) in relation to biological activity.

This paper describes determination of the size and shape of the protease from *S. erythreus*, and comparison of its CD spectrum with those of trypsin and the other trypsin-like enzymes from *Streptomyces* strains.

MATERIALS AND METHODS

Bovine pancreatic trypsin and trypsin-like enzymes from *S. erythreus*, *S. fradiae* and *S. griseus* are the same as used in the preceding work[4].

Disc electrophoresis

Polyacrylamide gel electrophoresis was carried out as previously described[5] but using a 7.5% cross-linked gel instead of a 15% one.

Ultracentrifugation

Sedimentation and diffusion measurements were done in a Hitachi ultracentrifuge UCA-1 equipped with a temperature control and measurement unit. Protein solutions for ultracentrifugal analyses were prepared with a 0.05 M phosphate buffer (pH 7.0) including 0.15 M NaCl, and were dialyzed against the buffer before ultracentrifugation. The dialysate was used as reference solvent.

Sedimentation velocity experiments were made at about 40 000 rev./min at 20.0 ± 0.1 °C in a double-sector synthetic boundary cell of a capillary type. Sedimentation coefficients were corrected to values in water at 20 °C ($s_{20,w}$).

Diffusion coefficients were computed from the schlieren patterns of the sedimentation velocity experiments according to Kawahara's[6] approximation of Fujita's[7] equation.

The low-speed sedimentation equilibrium experiments were performed with interference optics in a 12-mm double-sector cell at 14 400 rev./min and 20.0 ± 0.1 °C using the 3-mm column height technique of Van Holde and Baldwin[8], using protein solutions at concentrations between 2.52 and 0.50 mg/ml.

The apparent molecular weight M_{app} at the solute concentration $(c_a + c_b)/2$ is given by the equation[9]

$$M_{app} = \frac{2RT}{(1 - \bar{v}\varrho)} \frac{c_b - c_a}{c_0(r_b^2 - r_a^2)} \tag{1}$$

where R is the gas constant, T is the absolute temperature, $\bar{v}$ is the partial specific volume of the solute, ϱ is the density of the solvent, c is the solute concentration, r is the radial distance, and the subscripts a and b mean the meniscus and the bottom of the solution column, respectively. The molecular weight M of the solute is related to M_{app} by the equation[9]

$$\frac{1}{M_{app}} = \frac{1}{M} + \left(B + \frac{\bar{v}}{M}\right)\left(\frac{c_a + c_b}{2}\right) \tag{2}$$

where B is the second virial coefficient. Plotting $1/M_{app}$ *versus* $(c_a + c_b)/2$ gives a straight line, and the intercept of the plot at the limit of $(c_a + c_b) = 0$ yields the molecular weight.

Circular dichroism

CD spectra were measured from 320 to 200 nm at room temperature and 2 °C on a JASCO ORD/UV/CD-6 spectropolarimeter. The instrument was calibrated with (+)-10-camphorsulfonic acid[10]. The mean residue ellipticity $[\theta]$ (degrees·cm²/dmole) was calculated from the equation

$$[\theta] = 3300\ (\varepsilon_L - \varepsilon_R) \quad (3)$$

where $(\varepsilon_L - \varepsilon_R)$ is the difference between molar extinction coefficients for left and right circularly polarized light. The follwoing mean residue weights were employed[4]: trypsin, 105; the protease from *S. erythreus*, 101; from *S. fradiae*, 102; from *S. griseus*, 101.

Concentrations of the proteases were determined from their absorbances at 280 nm, measured by a spectrophotometer attached to the CD spectrometer, using molar extinction coefficients of $2.5 \cdot 10^4$, $3.6 \cdot 10^4$, $3.1 \cdot 10^4$, and $3.9 \cdot 10^4$ $mole^{-1} \cdot cm^{-1}$ for the trypsin-like enzymes from *S. erythreus*, *S. fradiae* and *S. griseus*, and trypsin[11], respectively.

Gel filtration for molecular weight determination

The method of Whitaker[12] was applied to the purified protease from *S. erythreus*. Details of the experimental procedure and the marker proteins have been previously described[5].

RESULTS

Disc electrophoretic patterns

The disc electrophoretic patterns illustrated in Fig. 1 represent the protein bands for the enzyme fraction obtained from DEAE-Sephadex (A) and QAE-Sephadex (B). The latter was the final preparation with a single band as shown in Fig. 1B.

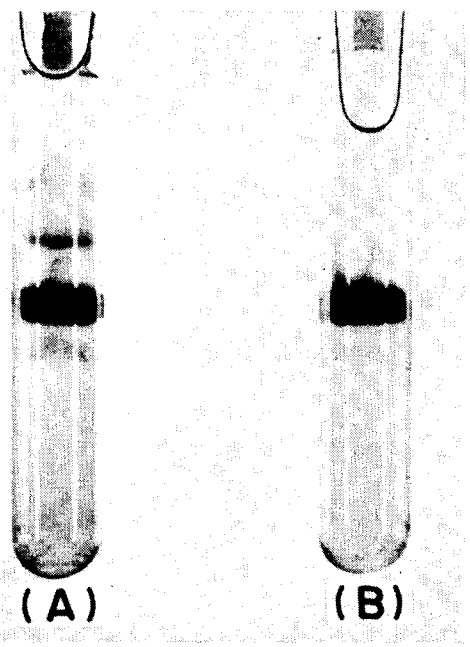

Fig. 1. Polyacrylamide gel electrophoretic patterns obtained during the isolation of the trypsin-like enzyme from *S. erythreus*. 7.5% gel, running at pH 8.0, 2.0 mA per gel for 1 h. (A) The active fraction from the DEAE-Sephadex chromatography step. (B) The final product after the QAE-Sephadex chromatography step.

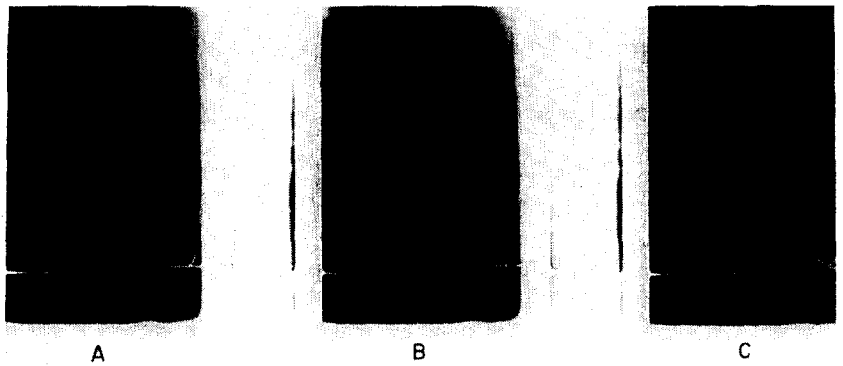

Fig. 2. Ultracentrifuge patterns for sedimentation velocity experiment of the trypsin-like enzyme from *S. erythreus* in a synthetic boundary cell at 40 220 rev./min and 20.0 °C. The enzyme concentration is 14.05 mg/ml in 0.05 M phosphate buffer (pH 7.0) including 0.15 M NaCl. The pictures were taken 20 (A), 30 (B), and 40 (C) min after reaching the final speed. Sedimentation is from left to right.

Molecular weight

Sedimentation–diffusion

A typical schlieren boundary of the enzyme in an ultracentrifugation is shown in Fig. 2. Enzyme solutions varying in concentration gave a single symmetrical peak, indicating homogeneity of the component in the ultracentrifugal sense.

The sedimentation coefficient of the protease changes linearly with concentration. The line $s_{20,w} = 2.57\ (1 - 0.0097c)$ was calculated through the five experimental points ranging from 2.68 to 14.05 mg/ml by the least squares method. As the concentration dependence of $s_{20,w}$ is linear, we can evaluate the diffusion coefficient D from the schlieren boundary curves of sedimentation velocity experiments[6]. In the present case the angular velocity (about 40 000 rev./min) was rather high. Nevertheless, the conditions under which D is derived were satisfied for any concentration investigated owing to the small concentration dependence of s and the rather short time of the sedimentation run (see Eqn 5 of ref. 6). The values of $D_{20,w}$ obtained are scattered in the range between $10.4 \cdot 10^{-7}$ and $11.1 \cdot 10^{-7}$ cm²/s, and no concentration dependence of $D_{20,w}$ is observed. Averaging these values yields $D^{\circ}_{20,w}$ of $10.7 \cdot 10^{-7}$ cm²/s.

The partial specific volume of the protease estimated from its amino acid composition[4,13] is 0.715.

On the basis of $s^{\circ}_{20,w} = 2.57$ S, $D^{\circ}_{20,w} = 10.7 \cdot 10^{-7}$ cm²/s, $\bar{v} = 0.715$ and $\varrho = 1.01$, a molecular weight of 21 000 was computed from the equation[14],

$$M = \frac{R\,T\,s^{0}}{D^{0}(1 - \bar{v}\varrho)} \tag{4}$$

Equilibrium sedimentation

Another method used for molecular weight determination is the sedimentation equilibrium. Plots of the logarithm of the fringe displacement, J, corresponding to the solute concentration, against the square of radical distance from the center of rotation showed straight lines in all cases investigated: a typical plot is represented

in Fig. 3. This linearity in the sedimentation equilibrium plots as well as the symmetric boundary shape in the sedimentation velocity patterns supports the homogeneity of the molecular weight of the enzyme. An apparent molecular weight at the concentration of $(c_a + c_b)/2$ is calculated from Eqn 1. A linear relationship was obtained between the reciprocals of the apparent molecular weights and the concentrations $(c_a + c_b)/2$. Therefore, the molecular weight of the protease was calculated to be 21 600 by Eqn 2.

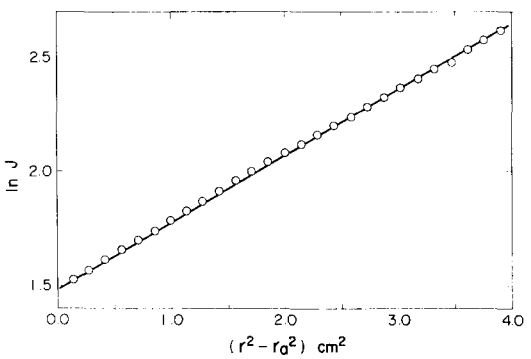

Fig. 3. Semi-logarithmic plot of the fringe displacement, ln J, as a function of $(r^2 - r_a^2)$, where r is the centrifugal radius and r_a is the distance of meniscus from the center of rotation. The solution of the trypsin-like enzyme from *S. erythreus* of the initial concentration 2.52 mg/ml was brought to sedimentation equilibrium after centrifugation for 24 h at 14 520 rev./min and at 20 °C.

Gel filtration

A sharp single peak was observed in the elution pattern of the purified protease: a ratio of the elution volume of the protease, V_e, to the void volume, V_0 was 1.76. Employing the linear relation of V_e/V_0 *versus* the logarithms of the molecular weights of the reference proteins (see Fig. 7 in ref. 5), the molecular weight of the protease was estimated to be 21 000.

CD spectra of trypsin-like enzymes and trypsin

We have compared the CD spectrum of the trypsin-like enzyme from *S. erythreus* with those of the trypsin-like enzymes from *S. fradiae* and from *S. griseus*, and of bovine pancreatic trypsin in Fig. 4. The spectra were measured in 0.1 M Tris–HCl buffer (pH 7.0) at room temperature and at 2 °C. Little differences in ellipticity and extremum wavelength were observed between the CD spectra at the two temperatures for each enzyme, so we will discuss only the CD spectra obtained at room temperature. The CD spectrum of trypsin at pH 7.0 resembles that at pH 2.2[15] although ellipticity in the near-ultraviolet region is intensified at pH 7.0 to some extent and a shoulder appears at 220 nm in the neutral solution.

In general the CD bands of the trypsin-like proteases investigated are weak in comparison with those of a globular protein having substantially an ordered structure. The far-ultraviolet CD spectra of trypsin and the trypsin-like proteases presumably suggest the presence of small amounts of an α helix or β structure. This subject is examined in Discussion.

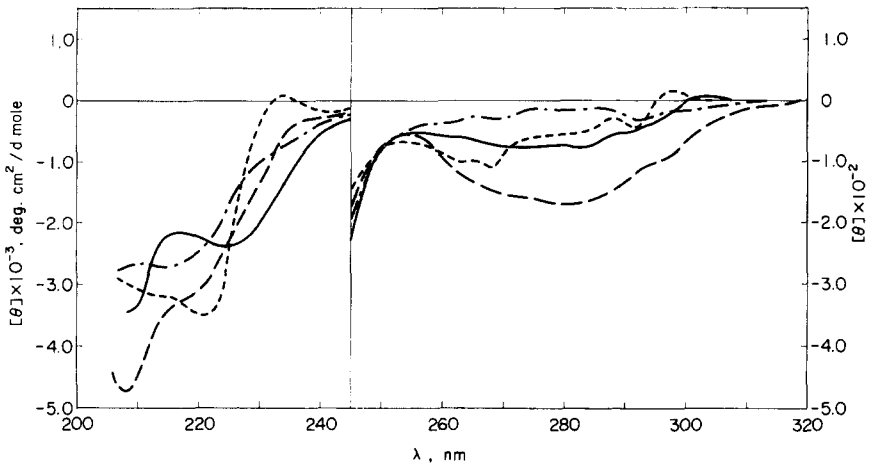

Fig. 4. Comparison of CD spectra between trypsin (— — —), and the trypsin-like enzymes from *S. erythreus* (———), from *S. fradiae* (---------) and from *S. griseus* (— · —). The spectra were measured in 0.1 M Tris–HCl buffer (pH 7.0) at room temperature.

Bovine trypsin exhibits a larger negative CD spectrum in the near-ultraviolet region, where the side-chain chromophores of proteins contribute to the CD spectra. Each trypsin-like protease shows a small negative extremum at 292 nm, corresponding to a shoulder of the near-ultraviolet absorption spectrum at the same wavelength. This negative band is assigned to a tryptophan residue[16,17].

In order to discern the contribution of side-chain chromophores to the CD spectra, alkaline denaturation was performed on the three trypsin-like enzyme so-

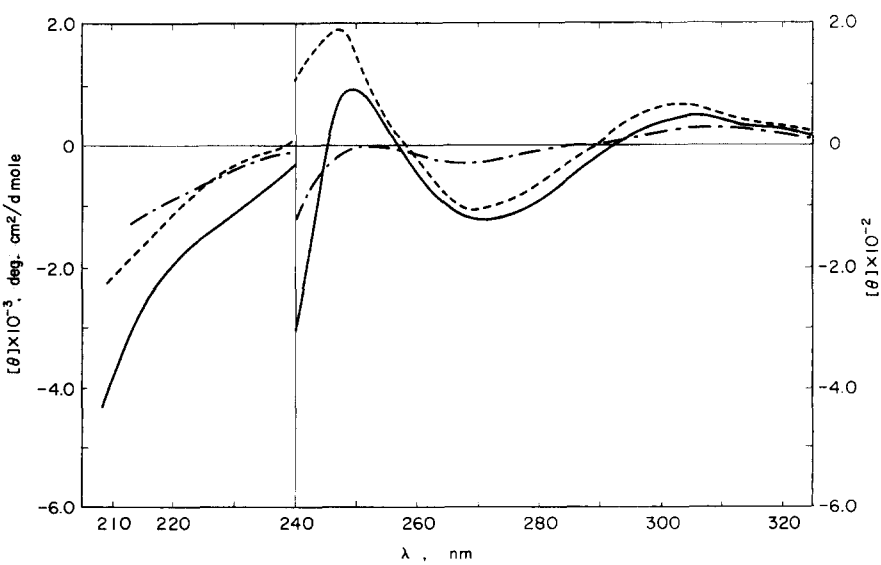

Fig. 5. Effect of alkaline denaturation on CD spectra of the trypsin-like enzymes from *S. erythreus* (———), from *S. fradiae* (---------), and from *S. griseus* (— · —). The spectra were obtained after keeping solutions at pH 12 for 24 h.

lutions. Fig. 5 shows the CD spectrum changes in the alkaline denatured proteases obtained after keeping the solutions at pH 12 for 24 h. Interpretation of the changes is rather difficult because the contributions of several aromatic and disulfide side-chains complicate the CD spectra in this region. However, positive extrema centered at 300–305 nm and at 247 nm are probably associated with tyrosines accessible for alkali titration[18]. Addition of alkali induced disappearance of the trough in the far-ultraviolet CD spectra, indicating decrease in the ordered α helix or β structure to a negligible amount. This does not mean that the protease molecule is in the freely random-coiled conformation. The alkaline denaturation leaves the disulfide bridges unbroken, so the polypeptide chain is constrained in the region of these bridges at least[19].

DISCUSSION

Homogeneity and molecular weight

Homogeneity of the purified trypsin-like protease from *S. erythreus* was investigated by several physicochemical techniques; polyacrylamide gel electrophoresis (Fig. 1), sedimentation velocity (Fig. 2), sedimentation equilibrium (Fig. 3), and gel filtration. All the methods indicate that the protease is quite homogeneous.

The results of the four independent methods for determination of molecular weight are: sedimentation equilibrium 21 600; sedimentation-diffusion 21 000; gel filtration 21 000; and amino acid analysis 21 800. The first three methods were performed over a wide range of the protease concentration (see Results). The values obtained are in excellent agreement, giving a molecular weight of 21 400. This leads to the inference that the protease molecule dissolves in the monomer state and that there is little association to dimer, trimer, *etc.*, or dissociation to subunits in aqueous neutral solutions.

Molecular shape and size

The sedimentation and diffusion coefficients were obtained for the protease from *S. erythreus* using an ultracentrifuge. The two coefficients make it possible to estimate the hydrodynamic shape and size of the protease molecule. According to hydrodynamic theory, the ratio of the actual frictional coefficient of the particle, f, to the minimum possible frictional coefficient of the hypothetical unhydrated sphere, f_{min}, is related to the diffusion and sedimentation coefficients[20],

$$\frac{f}{f_{min}} = \frac{R\,T}{6\pi\,\eta\,N\,D^0}\left\{\frac{4\pi\,N\,D^0(1-\bar{v}_2\,\varrho)}{3\bar{v}_2\,R\,T\,s^0}\right\}^{1/3} \tag{5}$$

where N is Avogadro's number, and $\bar{v}_2$ is the partial specific volume of the particle.

Protein molecules in aqueous solution possess a certain amount of water which will travel with the same velocity as the host protein molecule in hydrodynamic experiments. Then the ratio f/f_{min} depends on two factors; the shape and the degree of hydration of the protein, *i.e.*

$$\frac{f}{f_{min}} = \frac{f}{f_0}\left(\frac{\bar{v}_2 + \delta_1\,v_1^0}{\bar{v}_2}\right)^{1/3} \tag{6}$$

where f/f_0 is the deviation of the shape of the hydrodynamic protein particle from a

sphere, δ_1 is the amount of bound water in g/g protein, and v_1° is the specific volume of water. The term in parentheses corresponds to the hydration factor. With the determined values of $D^\circ_{20,w}$ and $s^\circ_{20,w}$ the ratio f/f_{min} was calculated to be 1.10. Employing this value we shall consider the range of possible values which the two factors might possess. At one extreme the protein is unhydrated and only the asymmetry of shape contributes to the frictional ratio f/f_{min}. We can calculate the axial ratio, a/b, which an ellipsoid must have to produce the observed value of f/f_{min}[14]. The calculation leads to a maximum possible value of $a/b = 2.8$ for both prolate and oblate ellipsoids. At the other extreme the shape is spherical and the frictional ratio is ascribed only to hydration. Using Eqn 6 with $f/f_{min} = 1.10$ and $f/f_0 = 1.0$, the maximum possible degree of hydration is found to be 0.24 g per g protein. Calculation of the radius of this hypothetical sphere ,R_e, by the equation $R_e = RT/6\pi\eta N D^0$ results in a radius of 2.0 nm. It has been shown that the degree of hydration of certain proteins ranges mainly between 0.25 and 0.40 g per g protein[21]. It is not unreasonable, therefore, to approximate the protease molecule as an ellipsoid of axial ratio close to unity, and with the degree of hydration between 0.24 and 0.20 g per g protein.

Protein conformation

Attempts have recently been made to evaluate the conformation of proteins from ORD and CD spectra[22–25]. These treatments are based on the assumption that CD and ORD spectra of proteins can be represented by a linear combination of experimentally obtained curves for α helix, random coil and antiparallel pleated-sheet (β structure) conformations of poly-L-lysine. The protein conformations evaluated showed good agreement with those derived from X-ray diffraction studies, particularly with proteins having a high order of α helical structure; though agreement was poor for proteins with lesser degrees of α helical structure[26]. There are several possible origins of this discrepancy: for example, the distortion of long-range periodic structures by the constraint of the polypeptide chain backbone, contributions by side-chain chromophores and disulfide bonds to the far-ultraviolet CD spectra, and inadequacy of the polypeptide models of the three conformational types. The CD spectrum of randomly coiled poly-L-lysine at pH 7.7 may not be a realistic model for random or unordered* conformation within a protein[19,27]. In the present state of CD analysis it seems too early to define the secondary structure of a protein solely from its CD spectrum.

Taking into account the situation of the CD spectroscopy, we examined the CD spectra of the trypsin-like enzymes and trypsin. The n–π^* transition of an α-helical polypeptide amide occurs at 222 nm[29]. If a protein molecule has β structure in addition to α helical structure, the negative extremum at 222 nm is blue-shifted[23]. The protease from *S. erythreus* has a negative extremum at 225 nm in its far-ultraviolet CD spectrum. This extremum may be ascribed to the n–π^* transition of α helix that is red-shifted as a result of superposition of the optical activity of tryptophan, tyrosine or phenylalanine residues. The two extrema at around 300 nm in the near-ultraviolet CD spectrum suggest that at least one of the two tryptophan residues is buried in the hydrophobic region of the molecule[30].

* We use here the definition of Timasheff *et al.*[28] for the terms random and unordered structures.

The negative extremum at 221 nm in the trypsin-like protease from *S. fradiae* and that at 215 nm in the protease from *S. griseus* are probably interpretable as evidence for the presence of α-helical and β-structural conformations, respectively. However, the content of the periodic ordered structure is estimated as less than 10% from the ellipticity. The small positive peak of the former protease at 233 nm may be ascribed to a tyrosine residue[18] whose ellipticity is enhanced by interaction with the n–π^* transition of polypeptide in α-helical form. Alkaline denaturation at pH 12 results in a red-shift of the peak to 247 nm and a larger ellipticity[18]. From the negative extremum at 208 nm with the shoulder at 220 nm in its far-ultraviolet CD spectrum, bovine pancreatic trypsin may be described as having small amounts of β-structural and α-helical regions, the remainder being unordered. Pantaloni *et al.*[15] have inferred that the trypsin conformation at pH 2.2 has a small content of β structure and no α helix from the lack of the 220-nm shoulder in the CD spectrum. Alkaline denaturation of the three trypsin-like enzymes markedly reduced ordered structures if any of them are present. To sum up it seems safe to conclude that the trypsin-like enzymes and trypsin have small amounts of ordered structure such as α helix or β structure, while the majority of the polypeptide main-chains are folded into some aperiodic, unordered but rigid structure.

Assessment of side-chain transition contributions to the near-ultraviolet CD spectra of proteins is more difficult because there are many chromophores that exhibit optical active transition in this wavelength region. Before assignment of the near-ultraviolet CD spectra it is necessary to investigate the CD spectra of proteins of which a certain definite side-chain chromophores have been chemically modified. It is further preferable that such modification affects the secondary conformation of the polypeptide main-chain as little as possible. Alkaline denaturation alone is not sufficient for this purpose.

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