

Chemical, photochemical and spectroscopic characterization of an alkaline proteinase from *Bacillus subtilis* variant DY

A comparison with other subtilisins

Nicolay GENOV,* Maria SHOPOVA,* Raina BOTEVA,* Giulio JORI† and Fernanda RICCHELLI†‡

**Institute of Organic Chemistry, Bulgarian Academy of Science, Sofia 1113, Bulgaria, and †Institute of Animal Biology, C.N.R. Centre for Haemocyanins and other Metalloproteins, University of Padova, Padova 35100, Italy*

(Received 2 March 1982/Accepted 20 July 1982)

Circular-dichroism and fluorescence studies indicate that the 5-dimethylaminonaphthalene-1-sulphonyl and phenylmethanesulphonyl derivatives of subtilisin DY have three-dimensional structure closely similar to that of native enzyme. The single tryptophan residue is largely accessible to the aqueous solvent, and is not directly involved in the enzyme–substrate interactions, since its photochemical modification causes only a partial inhibition of the enzyme activity. It appears very likely that the location of the single tryptophan residue in the three-dimensional structure of subtilisin DY is similar to that of the single tryptophan residue in subtilisin Carlsberg. Fluorescence-quenching experiments further indicate that the 14 tyrosine residues are also largely accessible to the aqueous solvent, and probably interact with hydrated peptide carbonyl groups. The charge environment for tryptophan and tyrosine residues in subtilisin DY, as deduced by quenching experiments with ionic species, is also discussed. In general, subtilisin DY displays strong similarities to subtilisin Carlsberg, as suggested by a comparative analysis of the amino acid composition and fluorescence properties.

Subtilisins are a group of serine proteinases originating from strains of *Bacillus subtilis* or related bacteria. Several of these proteinases have been characterized with regard to amino acid composition, enzymic activity and immunological properties (Ottesen & Svendsen, 1970; Svendsen, 1976). The structural properties of subtilisin Novo (identical with subtilisin BPN') and subtilisin Carlsberg have been intensively investigated (Schlessinger *et al.*, 1975; Svendsen, 1976; Brown *et al.*, 1977), and the three-dimensional structure of subtilisins BPN' and Novo in the crystalline state have been determined (Wright *et al.*, 1969; Hol, 1971).

An alkaline proteinase of the subtilisin type has been previously isolated from *Bacillus subtilis* var. DY. The enzyme is stoichiometrically inhibited by

Pms-F, which suggests the involvement of one serine residue in the catalytic site, and it displays optimum pH about 10 for the proteolytic activity (Nedkov *et al.*, 1976).

In the present work we have investigated some physicochemical and conformational properties of this protein. Our studies have been extended to some derivatives of subtilisin DY specifically blocked at the active site to avoid controversial results arising from the presence of autolytic products of the protein (Brown & Schleich, 1975). The results obtained are compared with the available information on the conformational properties of subtilisin Carlsberg, which appears to possess several characteristics similar to those of subtilisin DY (Ricchelli *et al.*, 1981).

Materials and methods

The crude enzyme preparation, obtained from the culture medium of *Bacillus subtilis* var. DY (Pharmachim, Botevgrad, Bulgaria), was fractionated by

Abbreviations used: Dns, 5-dimethylaminonaphthalene-1-sulphonyl; Pms, phenylmethanesulphonyl; AcTrpNH₂, *N*-acetyl-L-tryptophan amide; AcTyrNH₂, *N*-acetyl-L-tyrosine amide.

‡ To whom all correspondence should be addressed.

chromatography on a CM-cellulose column (5.5 cm × 23 cm) eluted with 3 mM-Tris/HCl buffer, pH 7.2, containing a linear 0–0.3 M-NaCl gradient at 4°C (Fig. 1). Peak IV, which contains subtilisin DY, was re-chromatographed under the same conditions. The freeze-dried product retains almost full proteolytic activity for at least 6 months on storage in a refrigerator.

Pms-subtilisin DY was prepared by the procedure described by Genov & Shopova (1978).

Dns-subtilisin DY was prepared by adding 5.1 mg of subtilisin DY in 4.7 ml of 0.1 M-potassium phosphate buffer, pH 7, containing 0.15 M-NaCl to 0.3 ml of 0.17% Dns-F in propan-2-ol and by stirring the mixture for 6 h at room temperature. At various intervals, samples were withdrawn for measurements of activity with casein as substrate. Preparative amounts of the modified enzyme were obtained in the presence of a 10-fold molar excess of Dns-F. Excess of Dns-F was removed by using a Sephadex G-75 column (2.6 cm × 80 cm) equilibrated with 10 mM-potassium phosphate buffer, pH 7.0, containing 0.2 M-KCl.

The molecular weight of subtilisin DY was calculated from the amino acid composition. The N-terminal residue was determined as described by Ivanov & Vladovska-Yukhnovska (1972).

Subtilisin Carlsberg was a gift from Professor I. Svendsen (Carlsberg Laboratories, Copenhagen, Denmark). Pms-F was purchased from Merck (Darmstadt, West Germany). Dns-F was synthesized from Dns-Cl (BDH Chemicals, Poole, Dorset, U.K.) by the method of Vaz & Schoellman (1976). CM-cellulose and casein were obtained from Reanal (Budapest, Hungary). Proflavine sulphate was

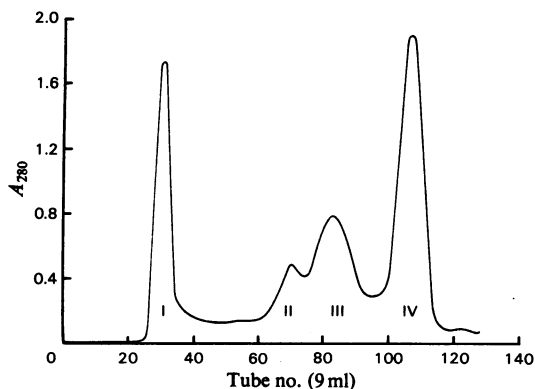


Fig. 1. Chromatography of the enzyme preparation isolated from the culture medium of *Bacillus subtilis* var. DY. Chromatography was on a CM-cellulose column (5.5 cm × 23 cm) with 3 mM-Tris/HCl buffer, pH 7.2, containing a linear 0–0.3 M-NaCl gradient (tubes). For details see the text.

purchased from Fluka (Buchs, Switzerland) and Sephadex G-75 from Pharmacia (Uppsala, Sweden). AcTyrNH₂, L-tyrosine, AcTrpNH₂ and L-tryptophan (Sigma Chemical Co., St. Louis, MO, U.S.A.) were recrystallized from 100% ethanol. All other reagents used were of analytical grade.

The amino acid composition of Dns-subtilisin DY was calculated by using an automatic analyser (model AAA 881; Michrotechna, Prague, Czechoslovakia) after 24 h, 48 h and 72 h of hydrolysis in 6 M-HCl in evacuated sealed tubes at 110°C.

The photo-oxidation experiments were performed at 21°C by exposing 3 ml of solution containing native or Pms-subtilisin DY (0.05 mg/ml) and 6 μM-proflavine in 0.1 M-potassium phosphate buffer, pH 7.5, to four 250 W tungsten lamps (Cozzani & Jori, 1980). At various times, samples were removed and assayed for caseinolytic activity. The photo-oxidized subtilisin DY was purified by gel filtration on a column (1.6 cm × 30 cm) of Sephadex G-75 equilibrated with 50 mM-potassium phosphate buffer, pH 7.0.

Absorption spectra were recorded with a Perkin-Elmer model 576 spectrophotometer and c.d. spectra with a Cary 61 Dichrograph. C.d. data are expressed in terms of mean residue ellipticity:

$$[\theta] = \frac{\nu}{10} \cdot \frac{M_r}{l \cdot c} \text{ (degree} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}\text{)}$$

where ν is the ellipticity measured in degrees, M_r is the molecular weight per mean residue (i.e. 101 as calculated from the amino acid analysis), l is the optical path of the solution in cm and c is the protein concentration in g/ml. The α -helix content of the protein was calculated by the procedure of Chen *et al.* (1974). The data were treated by the method of least squares.

Fluorescence measurements were performed with a Perkin-Elmer model 650–40 spectrofluorimeter, microprocessor-controlled and equipped with a thermostatically controlled assembly. Spectra were recorded in the ratio mode to minimize the errors due to lamp fluctuations, but they were not corrected for the wavelength-dependence of the photomultiplier response. For tyrosine-to-tryptophan energy-transfer experiments the correction was performed by reference to the corrected emission spectrum of indole (Berlman, 1965). The optical absorbance of the solutions was lower than 0.05 at the excitation wavelength to avoid inner filter effects. Fluorescence quantum yields (Q) were determined from the following equation:

$$Q_x = Q_s \cdot \frac{F_x}{A_x} \cdot \frac{A_s}{F_s}$$

where Q , F and A are the emission quantum yield, the area below emission spectra and the absorbance at the excitation wavelength respectively for the protein (x) and for the standard (s). As standards AcTrpNH₂ ($Q = 0.13$; Lehrer, 1971) and AcTyrNH₂ ($Q = 0.11$; Homer & Allsopp, 1976) were used. Tryptophan was excited at 300nm, where tyrosine absorption is negligible. Q_{Tyr} was calculated from the tyrosine pure emission spectrum, isolated as the difference spectrum between the total emission ($\lambda_{\text{exc.}} = 275$ nm) and the emission due to tryptophan alone ($\lambda_{\text{exc.}} = 300$ nm) (Longworth, 1971). Tyrosine emission was corrected on the basis of its fractional absorption in the protein at 275 nm.

In studies of fluorescence quenching by acrylamide, CsCl and KI, the results were plotted in accordance with the Stern–Volmer equation (Lehrer, 1971):

$$F_0/F = 1 + K_Q[X]$$

where F_0 and F are the fluorescence emission intensities in the absence and the presence of quencher respectively, $[X]$ is the quencher concentration and K_Q is the quenching constant. The Cs⁺ and I⁻ solutions were kept at constant ionic strength by additions of KCl. To avoid I₃⁻ formation a small amount of Na₂S₂O₃ was added to the I⁻ solutions. The inner filter effects due to the added acrylamide were corrected by the factor $X = 10^{(A_1 + A_2)/2}$, where A_1 and A_2 are the absorbances

at the excitation and emission wavelengths respectively.

The efficiency e of the tyrosine-to-tryptophan energy transfer was estimated (Eisinger, 1969) by using the relationship:

$$\phi_{\text{Pr}} = \phi_{\text{Trp}} \cdot [f_{\text{Trp}}(\lambda) + e \cdot f_{\text{Tyr}}(\lambda)]$$

where $f_{\text{Trp}}(\lambda)$ and $f_{\text{Tyr}}(\lambda)$ are the fractional absorptions of tryptophan and tyrosine respectively at the excitation wavelength λ , calculated from their molar ratio in the protein. ϕ_{Trp} is the tryptophan quantum yield in the protein excited at 300nm, and ϕ_{Pr} is the quantum yield of the protein as a function of the excitation wavelength. The above equation is valid if tryptophan is the only fluorescent species. For subtilisin DY, the tyrosine contribution to the fluorescence intensities at the wavelength used to record ϕ_{Pr} is very strong. Therefore ϕ_{Pr} values have been corrected as described by Eisinger (1969).

Results

From the amino acid composition, a molecular weight of 27700 is obtained for subtilisin DY. N-Terminal-group analysis shows the presence only of alanine, indicating that the protein consists of a single polypeptide chain and is molecularly homogeneous. The amino acid composition of Dns-subtilisin DY (Table 1) is in accord with that reported for the Pms derivative (Toushek *et al.*,

Table 1. Amino acid compositions of some subtilisins
For details see the text.

| Amino acid | Amino acid composition (residues/molecule) | | | | |
|------------|--|--|---|--|--|
| | Subtilisin DY (present work) | Subtilisin Carlsberg (Smith <i>et al.</i> , 1968) | Subtilisin BPN' (Markland & Smith, 1967) | Subtilisin amylosacchariticus (Kurihara <i>et al.</i> , 1972) | Mesenterico- peptidase (Genov & Jori, 1973) |
| Lys | 13 | 9 | 11 | 8 | 8 |
| His | 5 | 5 | 6 | 6 | 6 |
| Arg | 3 | 4 | 2 | 4 | 4 |
| Asx | 27 | 28 | 28 | 25 | 22 |
| Thr | 18 | 19 | 13 | 17 | 19 |
| Ser | 33 | 32 | 37 | 41 | 43 |
| Glx | 13 | 12 | 15 | 15 | 14 |
| Pro | 8 | 9 | 14 | 13 | 15 |
| Gly | 33 | 35 | 33 | 33 | 33 |
| Ala | 37 | 41 | 37 | 35 | 34 |
| Val | 28 | 31 | 30 | 25 | 22 |
| Met | 5 | 5 | 5 | 4 | 6 |
| Ile | 14 | 10 | 13 | 16 | 14 |
| Leu | 18 | 16 | 15 | 15 | 14 |
| Tyr | 14 | 13 | 10 | 12 | 13 |
| Phe | 4 | 4 | 3 | 3 | 3 |
| Trp | 1* | 1 | 3 | 3 | 3 |

* Determined by the method of Beaven & Holiday (1952).

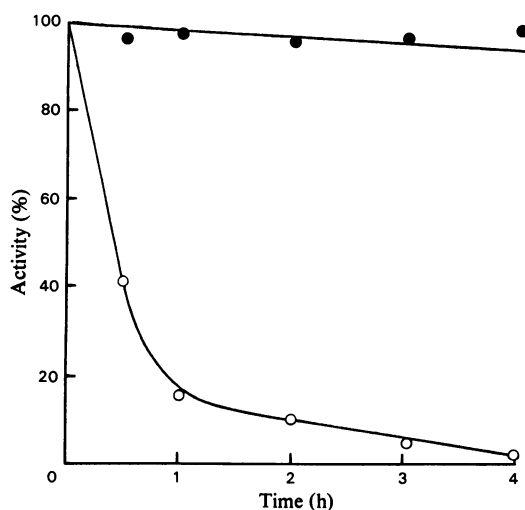


Fig. 2. Changes in the enzymic activity of subtilisin DY as a function of incubation time with Dns-F

○, Incubation of subtilisin DY in the presence of Dns-F; ●, control incubation in the absence of Dns-F. Casein was used as the substrate in the assay of proteolytic activity. For details see the text.

1982). Only one tryptophan residue is present in subtilisin DY, as estimated spectrophotometrically (Beaven & Holiday, 1952).

On the basis of the content of aromatic amino acids, the expected molar absorbance at 280nm of native subtilisin DY should be $2.42 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$. This value was calculated by using $\epsilon_{\text{M},280} = 5600 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for tryptophan and $\epsilon_{\text{M},280} = 1330 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for tyrosine (Beaven & Holiday, 1952). We find $\epsilon_{\text{M},280} = 2.5 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ by measuring the absorbance at 280nm of a protein solution whose concentration had been determined by amino acid analysis.

The caseinolytic activity of subtilisin DY (Fig. 2) is totally lost after 4h incubation with Dns-F, at which time one Dns group is bound per protein molecule. The dansylated protein shows a molar absorbance of $4.1 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 342nm, where only Dns group absorbs, in agreement with that of Dns-Et ester, i.e. $4.2 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Vaz & Schoellman, 1976). The Dns group is likely to be located at the protein active site, since the competitive inhibitor boric acid exerts a protective effect. The experimental conditions used in the absence of Dns-F do not influence the enzymic activity of subtilisin DY, as shown by control experiments (Fig. 2). Similar results are obtained on reaction of Pms-F with subtilisin DY, indicating the specific introduction of one Pms group at the enzyme active site.

The c.d. spectra of native, Pms- and Dns-subtilisin DY in the far-u.v. spectral range (Fig. 3) are

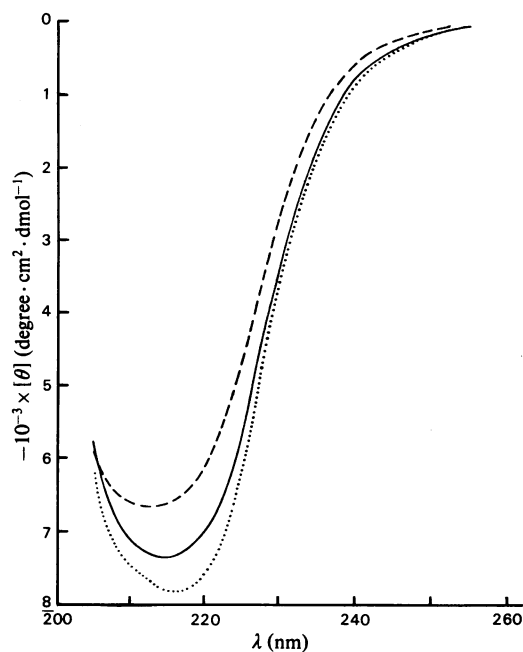


Fig. 3. Far-u.v. c.d. spectra of native subtilisin DY (—), Pms-subtilisin DY (---) and Dns-subtilisin DY (.....)

Spectra were recorded at 21°C, with solutions containing 0.4mg of protein/ml in 0.1M-phosphate buffer, pH 7.0, containing 0.2M-KCl. For details see the text.

very similar. The α -helix content is 21%, 18% and 22% respectively.

Photo-oxidation of native and Pms-subtilisin DY decreases the tryptophan content in accordance with first-order kinetics (Fig. 4). The first-order rate constants of the photoprocess, as calculated from the slopes of the semi-logarithmic plots, are $3.57 \times 10^{-3} \text{ s}^{-1}$ and $2.67 \times 10^{-3} \text{ s}^{-1}$ for native and Pms-enzyme respectively, i.e. very close to that typical of the proflavine-sensitized photo-oxidation of exposed tryptophan residues (Cozzani & Jori, 1980). The modification of the single tryptophan residue of the native protein causes an approx. 50% decrease in the caseinolytic activity.

The 275nm-excited fluorescence spectrum of native subtilisin DY (Fig. 5) shows a predominant contribution of the tyrosine fluorophores (emission $\lambda_{\text{max.}} = 305 \text{ nm}$). In this connection, subtilisin DY shows an anomalous behaviour in comparison with most class B proteins, i.e. proteins containing both tyrosine and tryptophan residues (Teale, 1960).

Closely similar emission spectra are given by Dns- and Pms-subtilisin DY. The main fluorescence properties of the three proteins are shown in Table 2,

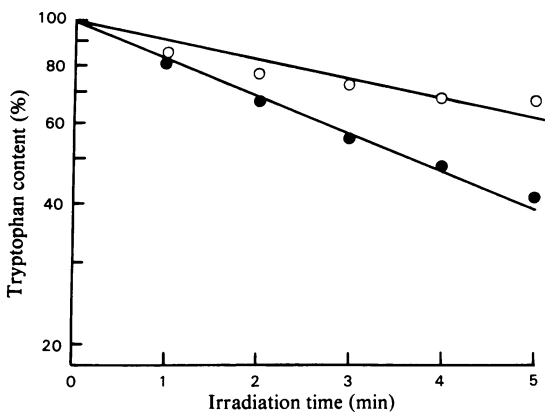


Fig. 4. Decrease in the tryptophan content of native subtilisin DY (●) and Pms-subtilisin DY (○) as a function of the time of visible-light irradiation of the protein samples in the presence of proflavine

The experiments were done at 21°C in 0.1M-phosphate buffer, pH 7.0, containing 0.2M-KCl. For details see the text.

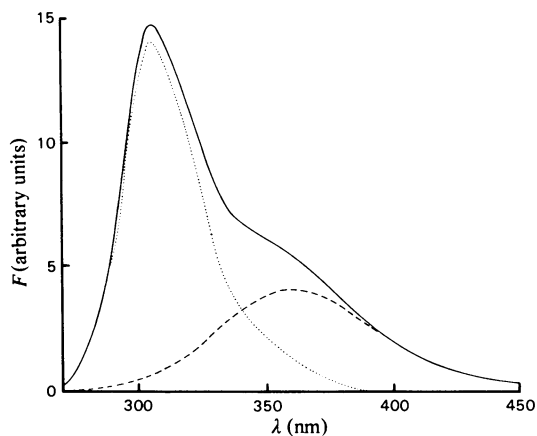


Fig. 5. Fluorescence emission spectra of native subtilisin DY on 275 nm (—) and 300 nm (----) excitation

The tyrosine emission spectrum (.....) was obtained as the difference spectrum between the 400nm-normalized spectra cited above. Experimental conditions were as indicated in Fig. 3 legend.

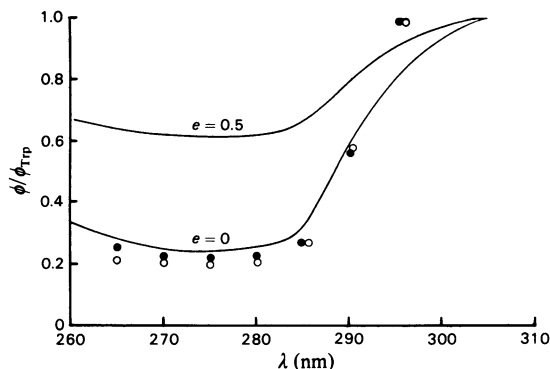


Fig. 6. Tyrosine-to-tryptophan energy-transfer efficiency in native subtilisin DY (●) and Pms-subtilisin DY (○) The continuous curves are theoretical and are obtained for different values of the transfer efficiency e . All theoretical curves and experimental results are normalized at 300nm. For details see the text.

together with the corresponding properties of subtilisin Carlsberg. Q_{Tyr} and Q_{Trp} of Dns-subtilisin DY have not been estimated owing to uncertainties in assessing the contribution of the Dns moiety to the absorption of the exciting 275 nm or 300 nm radiation. Tyrosine residues exhibit particularly high emission quantum yield, in agreement with the lack of tyrosine-to-tryptophan energy transfer (Fig. 6).

Both neutral compounds, such as acrylamide, and ionic species, such as CS^+ and I^- , quench the tyrosine and tryptophan emission in subtilisin DY. The quenching process fits a linear Stern-Volmer plot, as exemplified in Fig. 7. The K_Q values are summarized in Table 3, together with those obtained for subtilisin Carlsberg and model compounds.

To investigate the charge environments of the fluorophores, the ratio of the Stern-Volmer constants of I^- and CS^+ (K_{Q-}/K_{Q+}) was calculated, as suggested by Homer & Allsopp (1976) (Table 3). From studies on selected tyrosine derivatives, these authors concluded that this ratio defines an electrostatic parameter (E) sensitive to the charge of the fluorophore, evaluable without knowledge of the

Table 2. Fluorescence properties of native subtilisins DY and Carlsberg and of subtilisin DY derivatives For details see the text.

| Property | Native subtilisin DY | Pms-subtilisin DY | Dns-subtilisin DY | Native subtilisin Carlsberg |
|----------------------------------|----------------------|-------------------|-------------------|-----------------------------|
| λ_{max} (exc. at 275 nm) | 305 nm | 305 nm | 305 nm | 305 nm |
| λ_{max} (exc. at 300 nm) | 360 nm | 360 nm | 360 nm | 360 nm |
| Q_{Trp} | 0.020 | 0.026 | | 0.017 |
| Q_{Tyr} | 0.045 | 0.060 | | 0.14 |

Table 3. Quenching constants (M^{-1}) of the tryptophan and tyrosine emission and electrostatic parameters E ($E = K_Q/K_{Q^-}$) in subtilisins DY and Carlsberg and in model compounds

For details see the text:

| Sample | Quencher ... Acrylamide | CS^+ | | I^- | | E_{Trp} | E_{Tyr} |
|-----------------------|-------------------------|--------------|--------------|--------------|--------------|-----------|-----------|
| | | $K_{Q(Trp)}$ | $K_{Q(Tyr)}$ | $K_{Q(Trp)}$ | $K_{Q(Tyr)}$ | | |
| Subtilisin DY | 14.40 | 2.10 | 0.62 | 6.10 | 2.96 | 2.90 | 4.8 |
| Pms-subtilisin DY | 15.00 | 2.00 | 0.60 | 6.24 | 2.80 | 3.12 | 4.7 |
| Dns-subtilisin DY | 14.60 | 2.00 | 0.64 | 6.00 | 2.90 | 3.00 | 4.5 |
| Subtilisin Carlsberg | 14.00 | 1.83 | | 5.54 | | 3.03 | |
| AcTrpNH ₂ | 16.33 | 1.88 | | 11.70 | | 6.20 | |
| L-Tryptophan, pH 7.5 | | 2.80 | | 11.60 | | 4.13 | |
| L-Tryptophan, pH 2.1 | | 1.55 | | 6.94 | | 4.50 | |
| L-Tryptophan, pH 11.2 | | 2.84 | | 8.48 | | 2.98 | |
| Ac-TyrNH ₂ | | | 1.54 | | 7.88 | | 5.1 |

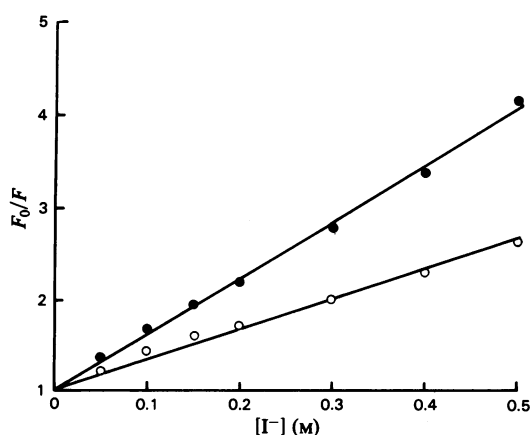


Fig. 7. Quenching of the fluorescence emission of the tryptophan (●) and tyrosine (○) residues of native subtilisin DY by I^-

The experiments were done in 0.1M-phosphate buffer, pH 7.5. For details see the text.

fluorescence life-times, if ions of similar size (e.g. CS^+ and I^-) are used. By comparing the electrostatic parameter E obtained from model compounds with that obtained from proteins, the charge environment for the fluorophores in macromolecular structures can be deduced.

Discussion

Subtilisin DY closely resembles other subtilisins with regard to amino acid composition (see Table 1), N -terminal group and molecular weight. Three forms of subtilisin DY have been investigated in the present study. Clearly, the Dns and Pms derivatives have three-dimensional conformations closely similar to that of native enzyme, as shown by the shapes of the c.d. spectra in the far-u.v. range,

the almost equal values of the α -helix content and the fluorescence properties (Tables 2 and 3). The slight changes observed in Q_{Trp} and Q_{Tyr} for Pms-subtilisin DY probably reflect some local perturbations of the fluorophore environment due to the introduction of the serine-blocking group. This fact allows the use of the inactive derivatives as probes of the three-dimensional arrangement of subtilisin DY.

The single tryptophan residue of subtilisin DY is largely accessible to the aqueous solvent. The photo-oxidation rate constant for this residue is closely similar to that observed for tryptophan incorporated into peptides devoid of tertiary structure (Cozzani & Jori, 1980). In the presence of the Pms group the photo-oxidation rate constant is slower, possibly owing to the local perturbations of tryptophan environment caused by the modifying group. The photochemical modification of the tryptophan residue only partially inhibits the enzyme activity, and hence this residue is not directly involved in the enzyme-substrate interactions. Moreover, the low energy of the fluorescence emission maximum is typical of tryptophan side chains at the surface of a protein molecule (Burstein *et al.*, 1973). Finally, the quenching constants for both acrylamide and charged species are characteristic of exposed tryptophan residues (Kirby & Steiner, 1970; Lehrer, 1971). However, the Q_{Trp} is abnormally low for a water-accessible tryptophan residue, indicating a proximity to functional groups endowed with strong fluorescence-quenching ability [this point is discussed in more detail in the following paper (Ricchelli *et al.*, 1982)].

The observed high tyrosine quantum yield may arise from several concomitant circumstances, including the absence of disulphide bonds, which are efficient quenchers of tyrosine emission (Longworth, 1971), the low tryptophan quantum yield and the lack of tyrosine-to-tryptophan energy transfer. This last-mentioned process usually plays a

major role in depressing tyrosine emission of class B proteins. Some information on the properties of the tyrosine microenvironment can be obtained by the R_{Tyr} value, i.e. the ratio of the fluorescence emission of protein tyrosine residues and that of free tyrosine at pH 7.0, normalized to the same absorbance (Cowgill, 1976). In our case, R_{Tyr} is 0.26 and 0.35 for native and Pms-subtilisin DY respectively, typical of tyrosine residues exposed at the surface of protein molecules and quenched by hydrated peptide carbonyl groups (Cowgill, 1976).

A discrimination among the various tyrosine residues was attempted by fluorescence-quenching experiments. However, linear Stern-Volmer plots have been constantly obtained for quenching by both Cs^+ and I^- . This finding does not necessarily mean that all the 14 tyrosine residues have homogeneous microenvironment, since the same phenomenon may be caused by inter-tyrosine electronic energy transfer, which represents a generally very efficient process in proteins (Weinryb & Steiner, 1971).

However, some indications are available with regard to the local charge in the tyrosine microenvironment. The electrostatic parameter E , deduced from model tyrosine compounds (Homer & Allsopp, 1976), falls into the ranges of ≤ 2.1 for negative, 2.7–4 for neutral and ≥ 6.7 for positive tyrosine environments. In subtilisin DY at pH 7.5, E_{Tyr} is 4.5–4.8, and hence the quenchable tyrosine residues must have a neutral microenvironment.

The application of this procedure to tryptophan is less clear-cut. The E values for tryptophan residues bearing different overall charges are very close (Table 3). However, E_{Trp} values for subtilisin DY and its derivatives (2.9–3.1) approximate to that found for negatively charged tryptophan at pH 11.2. The presence of a negatively charged tryptophan microenvironment is supported also from K_Q values obtained with Cs^+ : K_Q values for fluorophores in peptides and proteins are lower than those for standard substrates in the absence of charge effects owing to the lower diffusion coefficient (Lehrer, 1971). The slightly higher K_Q for Cs^+ , as compared with K_Q for AcTrpNH_2 , might reflect an electrostatic attraction between Cs^+ and negative charges adjacent to the tryptophan residue. Moreover, the low tryptophan quantum yield, and hence the short fluorescence life-time in subtilisin DY, should cause a low K_Q value unless charge effects influence the interaction of the quencher with the fluorophore.

The close similarity of K_Q and E_{Trp} values for subtilisins DY and Carlsberg strongly indicate a similar molecular environment of the single tryptophan residue in these proteinases. This conclusion is supported by the almost identical values for tryptophan quantum yield in both subtilisins and by the absence of tyrosine-to-tryptophan energy transfer;

the latter result was obtained for subtilisin Carlsberg by Brown *et al.* (1977).

References

- Beaven, G. H. & Holiday, E. R. (1952) *Adv. Protein Chem.* **7**, 319–325
- Berlman, I. B. (1965) in *Handbook of Fluorescence Spectra of Aromatic Molecules* (Berlman, I. B., ed.), p. 101, Academic Press, New York
- Brown, M. F. & Schleich, T. (1975) *Biochemistry* **14**, 3069–3074
- Brown, M. F., Omar, S., Raubach, R. A. & Schleich, T. (1977) *Biochemistry* **16**, 987–992
- Burstein, E. A., Vedenkina, N. S. & Ivkova, M. N. (1973) *Photochem. Photobiol.* **18**, 263–279
- Chen, Y. H., Yang, J. T. & Chai, K. H. (1974) *Biochemistry* **13**, 3350–3359
- Cowgill, R. W. (1976) in *Biochemical Fluorescence: Concepts* (Chen, R. F. & Edelhoch, H., eds.), vol. 2, pp. 441–486, Marcel Dekker, New York
- Cozzani, I. & Jori, G. (1980) *Biochim. Biophys. Acta* **623**, 84–88
- Eisinger, J. (1969) *Biochemistry* **8**, 3902–3907
- Genov, N. & Jori, G. (1973) *Int. J. Pept. Protein Res.* **5**, 127–133
- Genov, N. & Shopova, M. (1978) *Int. J. Pept. Protein Res.* **11**, 185–189
- Hol, W. G. J. (1971) Ph.D. Thesis, University of Groningen
- Homer, R. B. & Allsopp, S. R. (1976) *Biochim. Biophys. Acta* **434**, 297–310
- Ivanov, C. H. & Vladovska-Yukhnovska, Y. (1972) *J. Chromatogr.* **71**, 111–117
- Kirby, E. P. & Steiner, R. F. (1970) *J. Biol. Chem.* **245**, 6300–6306
- Kurihara, M., Markland, F. S. & Smith, E. L. (1972) *J. Biol. Chem.* **247**, 5619–5631
- Lehrer, S. S. (1971) *Biochemistry* **10**, 3254–3263
- Longworth, J. W. (1971) in *Excited States of Proteins and Nucleic Acids* (Steiner, R. F. & Weinryb, I., eds.), pp. 403–408, Plenum Press, New York
- Markland, F. S. & Smith, E. L. (1967) *J. Biol. Chem.* **242**, 5198–5211
- Nedkov, P., Bobatinov, M., Shopova, M. & Genov, N. (1976) *Izv. Otd. Khim. Nauki Bulg. Akad. Nauk* **9**, 443–450
- Ottesen, M. & Svendsen, I. (1970) *Methods Enzymol.* **19**, 199–215
- Ricchelli, F., Jori, G., Genov, N., Shopova, M. & Boteva, R. (1981) *Proc. Int. Conf. Chemistry and Biotechnology of Biologically Active Natural Products 1st, Varna*, vol. 3, pp. 194–198
- Ricchelli, F., Jori, G., Filippi, B., Boteva, R., Shopova, M. & Genov, N. (1982) *Biochem. J.* **207**, 201–205
- Schlessinger, J., Roche, R. S. & Steinberg, I. Z. (1975) *Biochemistry* **14**, 255–262
- Smith, E. L., De Lange, R. J., Evans, W. H., Landon, M. & Markland, F. S. (1968) *J. Biol. Chem.* **243**, 2184–2191
- Svendsen, I. (1976) *Carlsberg Res. Commun.* **41**, 237–291

Teale, F. W. J. (1960) *Biochem. J.* **76**, 381–388

Toushek, D., Nedkov, P., Genov, N. & Shopova, M.
(1982) *Dokl. Bolg. Akad. Nauk* in the press

Vaz, W. L. C. & Schoellman, G. (1976) *Biochim.
Biophys. Acta* **439**, 194–205

Weinryb, I. & Steiner, R. F. (1971) in *Excited States of
Proteins and Nucleic Acids* (Steiner, R. F. & Weinryb,
I., eds.), pp. 277–318, Plenum Press, New York

Wright, C. S., Alden, R. A. & Kraut, J. (1969) *Nature
(London)* **221**, 235–242