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Subtilisin enzymes: a note on time-resolved fluorescence and circular dichroism properties

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This note briefly corrects previous information about the time-resolved fluorescence properties of preparations of subtilisin *Carlsberg* and subtilisin BPN'. We confirm the observation of segmental motion of the single tryptophan in subtilisin *Carlsberg* by analysis of the time-resolved fluorescence anisotropy, and present circular dichroism and spectroscopic data on the two proteins. Near-UV properties clearly differentiate between the two proteins. Far-UV circular dichroism confirms that the two subtilisins have closely similar secondary structure in solution; the multi-component analysis is consistent with the established X-ray conformations, but the quantitative agreement is still somewhat imperfect.

Subtilisin; Tryptophan; Fluorescence lifetime; Anisotropy; Segmental motion; CD

1. INTRODUCTION

The subtilisins (EC3.4.21.14) are a group of microbial proteinases. Two principal variants are recognised, namely subtilisin *Carlsberg* and subtilisin BPN' (also known as subtilisin Novo or nagarse). These two proteins have about 70% sequence identity [1]. X-ray crystal structures [2–5] show that the two proteins have very similar structure: the a-carbon backbone conformations differ only to an r.m.s. deviation of 0.53 Å for 274 residues, [4]. Most of the 84 sequence substitutions involve surface residues. These proteins therefore represent a pair of natural mutants suitable for comparing the effects of substitution of specific residues. Extensive molecular genetic replacement has also been performed using protein engineering techniques (see [6,7]).

Our interest is in the spectroscopic properties of the aromatic amino acids. Subtilisin *Carlsberg* contains one Trp (no. 112) whereas subtilisin BPN' contains three (nos 106,133,241). The fluorescence emission of the single Trp of subtilisin *Carlsberg* is known to be highly quenched, [8,9]. We have previously reported that it shows a complex fluorescence decay and a multiple fluorescence anisotropy decay which we attributed to a fast segmental motion <300 ps, [10,11]. This is superimposed on the Brownian global motion, (~ 12 ns for the 30 kDa protein; cf. [12]).

Some confusion currently exists on the authenticity of some commercial samples of subtilisins [7]. In the course of an extension of the original time-resolved fluorescence studies, we have now established unequivocally that the proteins used in the original studies [10,11] were in fact both subtilisin *Carlsberg*. The samples were obtained from Sigma (before May 1986) as products P-5380 (protease type VIII: Carlsberg: lots 35F–0206 and 84F–0750) and P-5255 (protease type VII: nagarse: lot 64F–0051) and P-8038* (protease type XXIV: nagarse: lot 125F–06731). As will be demonstrated in this paper, neither of these last two samples is subtilisin BPN'. Product P-8038 is now redesignated in the current Sigma catalogue as pro-

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tease type XXIV and is stated to be consistent with subtilisin *Carlsberg*. Since the same product number has been retained, we refer to the suspect sample (lot 125F-06731) as P-8038*.

2. EXPERIMENTAL

Protein preparation: preparative FPLC gel-filtration chromatography on a Superose-12 column (Pharmacia) with 0.1 M phosphate buffer (pH 7.0) at 5°C showed that the two samples (P-5380 and P-8038*) differed significantly in terms of the amounts of extraneous, UV-absorbing low molecular mass material present. This is probably related to the extraction procedures and the proteolytic activity of the protein. Loading 0.5 ml of a solution of subtilisin (20 mg) allowed separation of the major column fraction (2 ml; approx. 7 mg protein by UV absorbance) which corresponds in molecular mass to the native enzyme. Aliquots of samples were frozen and stored at -20°C. Samples of subtilisin inhibited by PMSF were also prepared by the method of Polgar and Bender [13] : 1.5 - 2-fold molar excess of PMSF (concentrated solution in dioxan) was reacted for 30 min at room temperature with 20 mg protein in 0.1 M phosphate buffer (pH 7.0), and the subtilison fraction isolated by FPLC as above. Protein concentrations were evaluated from UV absorbance using values of $A^{1\%}$ (277.5 nm, 1 cm) of 11.7 (BPN') and 9.6 (*Carlsberg*) respectively [14].

Authentic samples of subtilisin *Carlsberg*, product P-8038 (protease type XXIV: lot 28F-0598) and nagarse (subtilisin BPN'), product P-4789 (protease type XXVII: lot 97F-0218), have now been supplied by Sigma. These are validated by amino acid composition and electrophoretic properties. The proteins have been further purified by FPLC as above, as both native and PMSF-inhibited proteins.

The purified proteins have been examined using standard methods of absorption spectroscopy (Cary 118 spectrometer), fluorescence spectra (SLM-8000) and CD (Jasco J-600). All CD spectra are given as ΔE in units $M^{-1} \cdot cm^{-1}$ evaluated on a per residue basis; i.e. molar residue concentration, [M] = (mg/ml)/110 where 110 = mean residue M_r. Protein concentration [c] = (mg/ml) M_r, where M_r = 275 × 110 for 275 residues. The spectra are original spectra, averaging 6-8 scans at time constant 0.5 s, with baseline subtraction.

Time-resolved fluorescence measurements: fluorescence decays were measured by time-correlated single photon counting techniques. A cavity-dumped dye laser (Coherent 701-3CD) using rhodamine 6G was synchronously pumped by the frequency-doubled output of a mode-locked cw Nd:YAG laser (Coherent Antares 76-S) providing a 3.8 MHz train of pulses (FWHM < 10 ps). The dye laser output was frequency doubled with an angle-tuned KDP crystal, providing vertically polarised 295 nm UV radiation. The system will be described in detail elsewhere (Janot et al., unpublished). The fluorescence emission was isolated using a UV interference filter centred at 340 nm (bandwidth 10 nm), and detected via an analyser prism with a Hamamatsu R1564U-01 micro-channel plate photomultiplier, and photon counting electronics.

Each decay curve is calculated from the sum of the vertically and horizontally polarised components as $I(t) = I_v(t) + 2gI_h(t)$, where each component is counted at 13 kHz (maximum rate) to greater than 100 000 peak counts. Decay curves were analysed as the sum of n exponential functions, using a new combined non-linear least-squares and regression method, which gives rapid analysis programmed in Borland Turbo Pascal 4.0 on an IBM-AT computer with 80287 coprocessor. The value of n was increased progressively to obtain statistically acceptable fits, as judged by values of χ^2 and the autocorrelation of the residuals. χ^2 is determined for fitting from the maximum of the decay curve for data over at least 400 channels at 21.9 ps/channel. DW is the Durbin-Watson statistical parameter applied to the residuals. Lifetimes are quoted with standard deviations evaluated from parameters reported by the Marquardt algorithm [15].

Correlation times were calculated by simultaneous analysis of $I_v(t)$ and $I_h(t)$ using the fitted lifetimes to represent the decay. The number of counts is the maximum of the difference curve $I_v(t) - gI_h(t)$. χ^2 and DW are calculated over both decay curves $I_v(t)$ *and* $I_h(t)$. The correlation times are reported as ϕ_1 and ϕ_2 corresponding to segmental and global motion, respectively. The anisotropy decay [16] is then given by:

$$r(t) = [r_1 \cdot \exp(-t/\phi_1) + r_2] \cdot \exp(-t/\phi_2)$$

and

$$r_0 = r_1 + r_2.$$

The standard deviations on ϕ_2 reflect the smallness of the amplitude r_2 relative to r_0.

3. RESULTS AND DISCUSSION

The identity of the subtilisin prepared from samples P-5380 and P-8083* as subtilisin *Carlsberg* has been confirmed using UV absorbance, near- and far-UV CD, and fluorescence excitation and emission spectra, and time-resolved fluorescence. The fluorescence lifetime and anisotropy of subtilisin *Carlsberg* (Janot et al., unpublished) have been studied in detail and will be reported elsewhere. Typical results are given in table 1. The results indicate the identity of samples P-8038* and P-5380 in both lifetime and anisotropy properties. Subtilisin *Carlsberg* shows complex fluorescence decay of the heavily quenched single tryptophan, with 3–4 exponential components, (dependent on emission wavelength), required to fit the total fluorescence decay curve. The quality of fitting (χ^2 criteria) is notable for a decay curve made by summation of the polarised components.

The anisotropy analysis shows a fast depolarisation process in the range ϕ_1 = 100–200 ps. The anisotropy cannot be fitted with a single rotational correlation coefficient, irrespective of whether 3 or 4 components are used to represent the fluore-

Table 1

Fluorescence lifetime and anisotropy analysis of subtilisin *Carlsberg* samples (see section 2)

No.	Sample P-8038*		Sample P-5380	
	Lifetime (ps) (SD)	Amplitude (%)	Lifetime (ps) (SD)	Amplitude (%)
1	69 (40)	53.5	71 (20)	60.6
2	266 (200)	14.0	231 (60)	17.7
3	1671 (340)	15.1	1623 (100)	12.4
4	5186 (420)	17.4	4652 (200)	9.4
χ^2	1.36		1.19	
DW	1.59		1.54	
ps/ch	21.9		21.9	
No.	Correlation time			
	ϕ_i (ps) (SD)	r_i	ϕ_i (ps) (SD)	r_i
1	133 (84)	0.189	204 (84)	0.230
2	10952 (7028)	0.064	11248 (19124)	0.031
r_0		0.253		0.261
χ^2	1.21		1.15	
DW	1.58		1.47	
Counts	41300		50856	

scence decay. This fast process accounts for 70–90% of the amplitude, as seen by the value of r_1/r_0. Thus, the results on the authentic, purified protein subtilisin *Carlsberg* confirm the earlier observations [11] and are consistent with a dynamic process such as an extensive fast segmental motion of the single tryptophan residue of this protein. It is interesting to note that this Trp residue is apparently both physically mobile and highly quenched; inspection of the crystal structure using computer graphics, FRODO (courtesy of Dr I.J. Tickel, Birkbeck College, London), indicates an exposed location for Trp-112, with substantial space for segmental motion relative to adjoining residues.

Samples of the purified proteins prepared from P-4789 (BPN') and P-8038 (*Carlsberg*) show differences in absorption spectra consistent with the reported amino acid compositions (subtilisin *Carlsberg*: 1 Trp, 13 Tyr, 4 Phe; subtilisin BPN': 3 Trp, 10 Tyr, 3 Phe) [1]. Fluorescence emission spectra (uncorrected, but after subtraction of solvent Raman scatter) show typically maxima at 331 nm (*Carlsberg*) and 344 nm (BPN') for excitation at 300 nm (excitation and emission bandpass 2 nm), with the *Carlsberg* spectrum at least one order of magnitude weaker on a molar basis [8,17–19]. Willis and Szabo [19] have recently reported that subtilisin BPN' shows a decay at approx. 8 ns as a major component, clearly distinguishing this protein from subtilisin *Carlsberg*.

Because of their protease activity, native subtilisins are susceptible to autolytic degradation, though this is relatively slow at neutral pH. The stability of various samples was examined with time for the column-purified samples. It would be expected that such autolysis would be a strong function of protein concentration. At 10 μM (~ 0.3 mg/ml) the fluorescence intensity showed little change over a period of 20 h at 20°C for either subtilisin *Carlsberg* or BPN', and the samples retained their characteristic differences in maxima. Consistent with these findings, the far UV-CD spectra, generally recorded at 3–6 μM (0.1–0.2 mg/ml; 1 mm path length) were constant with time over extended periods of measurement. The concentrations used for near-UV CD are 100 μM (3 mg/ml) for 2 mm path length. The near-UV CD spectrum of the purified native (uninhibited) protein was reproducible for fresh samples taken from storage at −20°C. The intensity decreased progressively with time, losing 30–35% signal over 5 h at 20°C. By contrast, the PMSF-inhibited proteins decreased less than 5% in the same period. Thus, the degree of instability will depend on the protein concentration used, and appears negligible at values less than 10 μM. It may be avoided by prompt measurement, or alternatively by PMSF inhibition. Willis and Szabo [18] have also drawn attention to the potential problem of autolysis in subtilisin samples.

The near-UV CD properties are particularly sensitive to local tertiary structure in the vicinity of the aromatic residues. The spectra are generally an excellent qualitative indicator of the native structure of a given protein, and useful for comparing small differences introduced by chemical modification, ligand binding [20] or, by extension, site-directed, or as in the case of these subtilisins, natural mutagenesis. Near-UV CD spectra are given in fig.1 showing the characteristic differences of the two proteins. Subtilisin *Carlsberg* is overall

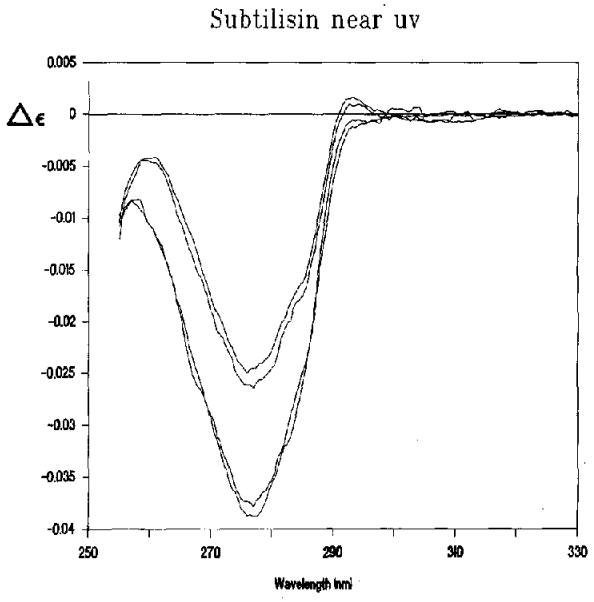

Fig.1. Near-UV CD of samples of purified subtilisins. (Upper) Subtilisin *Carlsberg*, (lower) subtilisin BPN′. Each pair of curves shows the native and inhibited enzyme. Units of $\Delta\epsilon$ are $M^{-1} \cdot cm^{-1}$.

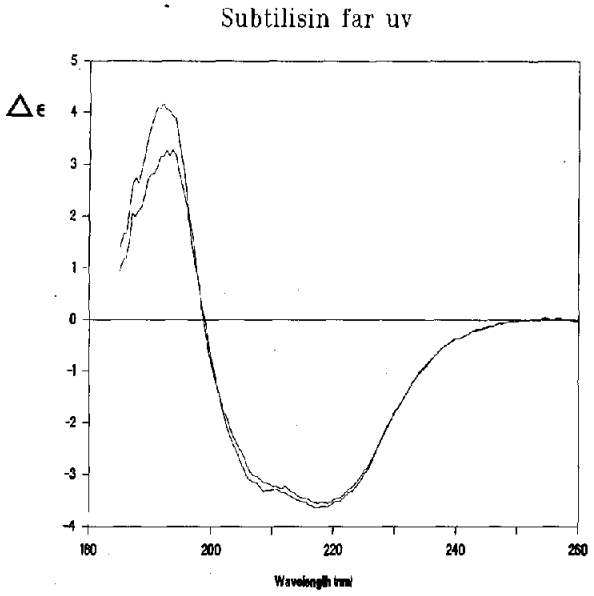

Fig.2. Far-UV CD of samples of purified subtilisins *Carlsberg* and subtilisin BPN′. (The latter is less intense at 197 nm.) Units of $\Delta\epsilon$ are $M^{-1} \cdot cm^{-1}$.

weaker, and has a small but real positive component at 300 nm (cf. [9], which reports the dissymetry factor $\Delta\epsilon/\epsilon$ as a function of wavelength, and [21]). The near-UV band is otherwise relatively featureless, deriving from the superposition of a potentially large number of effects. The spectra retain a shoulder at 283 nm, consistent with the predominant intensity deriving from the overall substantial number of Tyr residues. Fine-structural contributions from Phe residues at 250–270 nm or Trp ^1L_b transitions from 280–290 nm are not obviously resolved (cf. [22]). The latter are characteristic of highly immobilised Trp residues, and the absence of such effects is consistent with the observed mobility of Trp-112 in subtilisin *Carlsberg*. By contrast, the Tyr near-UV CD is relatively strong (assuming the spectra to derive exclusively from the 13 (or 10) Tyr residues in either protein) compared with model compounds in rigid and viscous media at low temperatures [22]. The intensity is consistent with a compact structure in which Tyr residues are relatively non-mobile, and this may be related to the known stability of the subtilisins to conventional denaturing agents such as concentrated urea or guanidinium hydrochloride [23]. The spectra (of either enzyme) are effectively identical in the native or inhibited form, the slight enhancement in the inhibited state (fig.1) being within experimental error. This is consistent with the minor nature of the PMSF modification of the active-site serine residue.

The far-UV CD (fig.2) shows that the spectra, normalised on a molar basis using the reported absorption coefficients, are very similar in intensity and shape down to 195 nm, below which subtilisin BPN′ is significantly weaker. This identity in intensity throughout the greater part of the spectrum also indicates an internal consistency of both CD and absorption spectral values as used here. The overall intensity suggests a protein of medium α-helical content (the unfractionated proteins showed spectra of similar line shape, but lower intensity).

The determination of subtilisin conformation from far-UV CD spectra has been problematical. Other workers have presented far-UV CD data for both proteins, generally used without additional purification [24–27]. These spectra differ in intensity and line shape to such an extent as to make any comparison unreliable. The uncertainty in the nature of the starting material, although a con-

tributing factor, cannot account for all these discrepancies. We therefore believe that the spectra of fig.2 may be taken as authentic spectra of the two proteins.

Even with this degree of assurance on the authenticity of the samples, problems remain in the analysis of the far-UV spectra. The spectrum of subtilisin BPN′ (from [24]) was excluded from the set of reference spectra in the definitive analytical study of Provencher and Glockner [28], since it appeared to be inconsistent with the rest of the set of well-characterised globular proteins used in the analysis. Analysis according to the method of Provencher and Glockner gives the result as in table 2. As expected the two proteins are very similar in secondary structural analysis, consistent with the X-ray structures. However, table 2 shows that the best-fit analyses differ significantly from the components deduced by X-ray analysis; it appears that the CD underestimates the α-helical content, overestimates the β-sheet content and underestimates the turn content relative to the X-ray structures as cited [28]. Possible causes of this discrepancy are currently under investigation.

In conclusion, this paper corrects the previous assignment of spectral data to subtilisin BPN′ [11], caused by an error in identification of the original samples. The basic finding of this earlier work on the fast depolarisation of fluorescence (and hence likely segmental mobility) of the single Trp of subtilisin *Carlsberg* is confirmed. Spectroscopic data are presented which allow rapid validation of purified samples. These are used to establish the near- and far-UV CD properties of the two proteins; however, the analysis of secondary-tertiary structure of subtilisin BPN' and subtilisin *Carlsberg* structure by CD requires further detailed quantitation.

Table 2

Analysis of the far-UV CD of subtilisin *Carlsberg* and subtilisin BPN′ by the method of Provencher and Glockner [28]

Samples	Range (nm)	f_α	f_β	f_t	f_r	F
BPN′	190–240	0.20	0.43	0.00	0.37	1.094
	200–240	0.25	0.41	0.00	0.35	1.044
Carlsberg	190–240	0.21	0.55	0.00	0.24	1.032
	200–240	0.23	0.38	0.10	0.37	1.045
Both	X-ray	0.31	0.10	0.22	0.37	–

The analysis shows the fractions of α-helix, β-sheet, turn (t) and remainder (r) structure, normalised by scaling factor F, which give the best fit to the far-UV CD spectra of authentic samples of the two proteins, for two data ranges. The assignment of the X-ray structure of the subtilisins is as quoted by Provencher and Glockner [28].

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