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A rare protein fluorescence behavior where the emission is dominated by tyrosine: case of the 33-kDa protein from spinach photosystem II

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Abstract

An abnormal fluorescence emission of protein was observed in the 33-kDa protein which is one component of the three extrinsic proteins in spinach photosystem II particle (PS II). This protein contains one tryptophan and eight tyrosine residues, belonging to a "B type protein". It was found that the 33-kDa protein fluorescence is very different from most B type proteins containing both tryptophan and tyrosine residues. For most B type proteins studied so far, the fluorescence emission is dominated by the tryptophan emission, with the tyrosine emission hardly being detected when excited at 280 nm. However, for the present 33-kDa protein, both tyrosine and tryptophan fluorescence emissions were observed, the fluorescence emission being dominated by the tyrosine residue emission upon a 280 nm excitation. The maximum emission wavelength of the 33-kDa protein tryptophan fluorescence was at 317 nm, indicating that the single tryptophan residue is buried in a very strong hydrophobic region. Such a strong hydrophobic environment is rarely observed in proteins when using tryptophan fluorescence experiments. All parameters of the protein tryptophan fluorescence such as quantum yield, fluorescence decay, and absorption spectrum including the fourth derivative spectrum were explored both in the native and pressure-denatured forms.

Keywords: Fluorescence; Tyrosine and tryptophan fluorescence; Hydrophobicity; High pressure denaturation

The fluorescence spectroscopy of proteins is a powerful tool to study their structure, function, and interaction. Many achievements have been obtained in thousands of research articles on protein intrinsic or extrinsic fluorescence [1–6]. Even if the mechanism of protein fluorescence is being understood more and more, some questions still remained and need further exploration. The relationship between tryptophan and tyrosine fluorescence emission is one of them. For most native proteins containing both tryptophan and tyrosine residues (termed as "B type proteins") their fluorescence emission excited at 280 nm is dominated by the tryptophan fluorescence where the band maxima vary in wavelength between 328 and 350 nm, as observed for the 21 globular proteins studied by Teale [7]. The tyrosine fluorescence emission was hardly to detect in spite of both the high absorption coefficient at 280 nm and the high fluorescence quantum yield of free tyrosine in aqueous solution. A typical example of this phenomenon is given by the fluorescence emission of human serum albumin. This protein contains one tryptophan and 17 tyrosine residues. Interestingly, it is only the tryptophan fluorescence of the protein that can be detected,

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irrespective of the wavelength of the exciting light, even if the tyrosine residues predominate in absorption [1,7,8]. However, in our recent study on the unfolding of the 33-kDa protein from spinach photosystem II particle induced by high hydrostatic pressure, it was observed that, in its native form, the fluorescence emission, upon an excitation at 280 nm, did not predominate in the wavelength range between 328 and 350 nm, as generally observed in proteins containing tryptophan [7], but at a shorter wavelength (around 305 nm) [9]. The 33-kDa protein is one component of the three extrinsic proteins in photosystem II particle (PS II), playing an important role in stabilizing manganese cluster and in maintaining high oxygen-evolving activity of PS II [10–12]. The protein consists of 248 amino acid residues with a single tryptophan residue (Trp241) and eight tyrosine residues [13]. It was reported that the protein has a very low free energy for chemical denaturation [14] and that a relatively low pressure of about 180 MPa can totally unfold the protein, which is rarely observed in a pressure-unfolding study [9]. This indicates that physical–chemical parameters can largely modulate the protein conformation which could be due to an unusual intramolecular topology. Regarding the presence of one tryptophan residue, it was expected that the protein fluorescence excited at 280 nm should not have any detection for tyrosine emission as observed for human serum albumin [7]. However, we found that in such a condition, i.e., upon excitation at 280 nm, the fluorescence was dominated by the emission of the tyrosine residues. Its fluorescence emission spectrum is obviously different from that excited at 295 nm, an unusual observation compared with the behavior of the "B type proteins" studied by Teale [9].

Materials and methods

Purification of the 33-kDa protein. The 33-kDa protein was isolated and purified from spinach chloroplast photosystem II according to the methods of Kuwabara and Murata [15] and Xu and Briker [16] with minor modifications. The purified protein was dialyzed against 10 mM NH_4HCO_3 and then lyophilized. The protein concentrations were determined as described in the Xu and Briker report [16]. The protein was dissolved in 0.1 M, pH 6.0 Mes buffer. All other reagents were of A.R. grade. Distilled water was further purified by a Millipore system to a resistance of 18 MΩ.

Fluorescence and absorption measurements. The fluorescence measurements were carried out using either an Aminco Bowman Series 2 (AB2) fluorospectrophotometer or a 48000 SLM fluorospectrophotometer (SLM Instruments). The slits of both excitation and emission were 2 nm. The quantum yields of tryptophan in the protein were determined according to the relative method of Chen [17].

The absorption spectra of the protein between 260 and 300 nm were measured using a Cary-3 absorption spectrophotometer. The fourth derivative absorption spectra were calculated from the absorption spectrum according to Lange et al. [18,19]. The measurements of both the protein fluorescence spectra and the fourth derivative absorption spectra under high hydrostatic pressure were achieved as previously described [9].

Results and discussion

The intrinsic fluorescence characteristics of the 33-kDa protein

Fig. 1 shows the fluorescence emission spectra of the 33-kDa protein excited either at 295 nm (A) or at 280 nm (B). The 295-nm wavelength excites only the tryptophan residue; meanwhile, the 280 nm excites both tryptophan and tyrosine residues. In A, the maximum fluorescence emission is at about 317 nm, indicating that the single tryptophan residue (Trp241) is located in a very hydrophobic environment, a consistent observation with the results reported by Tanaka and co-workers [13]. The emission spectrum (B) is significantly different in comparison with the spectrum A. Its maximum emission is at about 306 nm, a shorter value than the 317 nm maximum emission observed in A, but a little longer than the fluorescence spectrum of free tyrosine in the same Mes buffer (303 nm, spectrum D). These observations show that the 33-kDa fluorescence excited at 280 nm cannot be attributed only as coming from the tryptophan residue, with the tyrosine residue also being involved. A detailed analysis of the intensities of spectra A and B indicates that the tryptophan residue is responsible of about 30% of the total fluorescence, clearly showing that the fluorescence emission excited at 280 nm is mainly dominated by the tyrosine residues rather than the tryptophan one. Such a phenomenon is rare to be observed. In the Teale study [7], most "B type" globular proteins have only a tryptophan detectable fluorescence when excited at 280 nm. It was also worth indicating that the maximum emission wavelength of tryptophan in 33-kDa

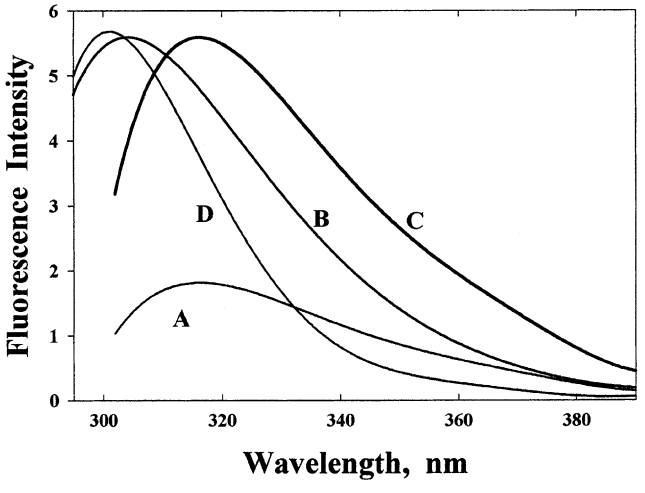

Fig. 1. Fluorescence emission spectra of the 33-kDa protein and free tyrosine. A and B, fluorescence spectra of the 33-kDa protein excited at 295 and 280 nm, respectively; C, normalized fluorescence emission spectrum A to B; D, normalized fluorescence spectrum of free tyrosine to spectrum B excited at 280 nm. Protein concentration: 0.1 mg/mL in 0.1 M, pH 6.0 Mes buffer. Free tyrosine concentration: 2.0×10^{-6} M in the same buffer. Temperature, 20 °C.

protein is 317 nm. Such a short maximum emission wavelength is not often observed in protein fluorescence. The shortest maximum emission of tryptophan in wild proteins studied so far was at 308 nm, as found in azurin [1,20]. Other shorter values reported were 324 nm for RNase T_1 and 325 nm for aldolase, respectively [20]. Generally, the shorter maximum emission corresponds to the more hydrophobic environment around the tryptophan residue. Therefore the 317 nm maximum emission observed implies that the single tryptophan (Trp241) is located in a very strong hydrophobic environment, which is not the case for most wild proteins studied by fluorescence. The 33-kDa protein is fairly hydrophilic, consisting of 64 charged amino acid residues [11]. The primary structure shows three regions rich in the charged amino acids and Trp241 is located near the third one (Asp224–Glu238) [11]. Trp241 is very close to the C-terminal amino acid residues from 239 to 248: G–V–W–Y–A–Q–L–E–Q–Q. There are few hydrophobic amino acid residues near the Trp241 according to the sequence. Therefore the questions are: (1) what are the amino acid residues located around Trp241 in the tertiary structure of the protein that are able to form so a high hydrophobic environment, and what is the role of this conformation in the protein function? For the moment, the answers are still open.

Fluorescence of the 33-kDa protein-denatured form by pressure

To further prove that both tryptophan and tyrosine residues are responsible for the protein fluorescence excited at 280 nm, the fluorescence variation induced by pressure-unfolding was followed. In bio-macromolecule high pressure studies, fluorescence spectroscopy introduced by Weber is a suitable method in which the fluorescence spectrum, the fluorescence intensity, the fluorescence polarization, and the lifetime of the intrinsic and extrinsic fluorophores can be used to detect the pressure effect on oligomeric protein dissociation, conformational changes or folding–unfolding [21–29]. The 33-kDa protein can be totally unfolded by a pressure of 200 MPa [9]. Fig. 2 shows the tryptophan fluorescence spectra of the protein excited at 295 nm either at an atmospheric pressure (0.1 MPa) at 200 MPa, indicating that the spectrum of pressure-denatured protein had a huge 35 nm red shift from 317 nm to 352 nm for the native and pressure-denatured proteins, respectively. The latter wavelength is the characteristic fluorescence emission for free tryptophan in aqueous solution. This implies that the single tryptophan residue Trp241 in the pressure-denatured protein is totally exposed to the solvent. Fig. 3 shows the emission spectra of the protein excited at 280 nm, at 0.1 and 200 MPa, respectively. The comparison of these two spectra indicates that the fluorescence in the region

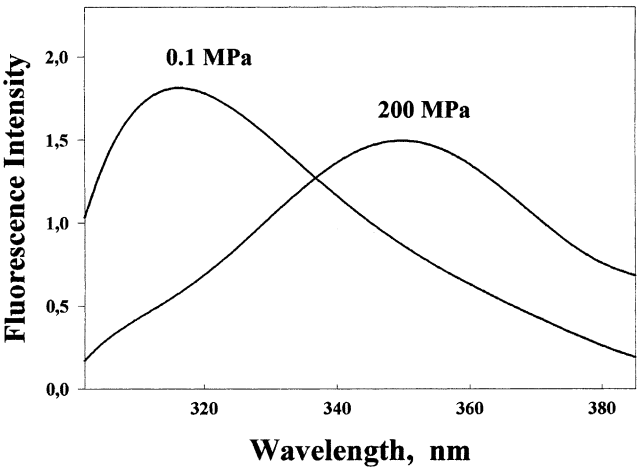

Fig. 2. Fluorescence spectra of the 33-kDa protein at 0.1 and 200 MPa. Excitation wavelength: 295 nm. The protein concentration was the same as in Fig. 1.

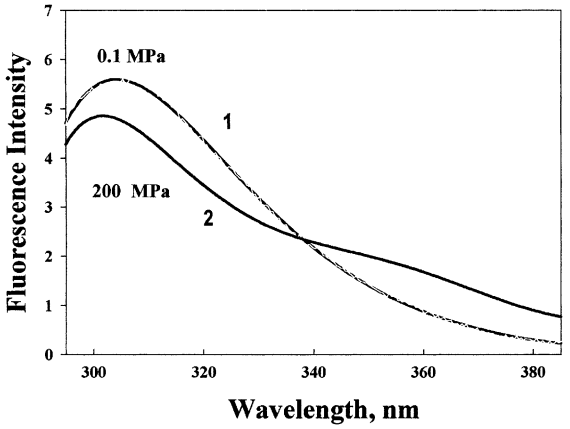

Fig. 3. Fluorescence spectra of the 33-kDa protein at 0.1 and 200 MPa. Excitation wavelength: 280 nm. The protein concentration was the same as in Fig. 1.

from 295 to 340 nm has both a decrease in intensity and a small spectral blue shift for the pressure-denatured protein. Meanwhile, in the region from about 340 nm to 380 nm the fluorescence intensity increases. Considering the spectral red shift of the pressure-unfolding protein observed in Fig. 2, the fluorescence increasing in the region 340–380 nm is obviously attributed to the red spectral shift of the tryptophan fluorescence. On the other hand, the reasonable explanation for the change in the region from 295 to 340 nm is as follows. The spectrum 1 could have consisted of a spectral component from both tyrosine and tryptophan residues. The tyrosine fluorescence maximum emission wavelength is at about 303 nm and is not significantly influenced by the environmental conditions. Therefore, when the tryptophan emission component in spectrum 1 moved to the red direction upon pressure-denaturation, the

remaining fluorescence spectrum in this region should mainly come from the tyrosine residues. As shown in the spectrum 2, both a decrease in intensity and a blue shift are observed. The spectral changes shown in Figs. 2 and 3 strongly supported the conclusion that both the tyrosine and tryptophan residues are involved in the 33-kDa fluorescence when the protein is excited at 280 nm. Fig. 3 also indicates that the tyrosine contribution to the protein fluorescence emission is larger than the tryptophan one.

As mentioned in the introduction, the 280 nm excitation induced fluorescence emission of the native "B type" proteins, often dominated by tryptophan fluorescence [9]. For native protein, with the tyrosine emission is frequently undetectable, the tyrosine emission being observed only in the denaturation state [1]. The reasons for this phenomenon have been generally attributed either to the energy transfer from tyrosine to tryptophan or to the tyrosine fluorescence quenching by the nearby charged amino groups and by neutral carboxylate groups [1,7]. However, the puzzle of precise mechanism for some particular proteins is not yet solved. The 33-kDa protein contains one tryptophan and eight tyrosine residues, the ratio 8/1 being much smaller than that in human serum albumin (16/1) [7]. However, an expected tyrosine fluorescence was observed with an excitation at 280 nm.

Complementary experiments: fourth derivative UV spectroscopy, fluorescence quantum yield, and fluorescence decay

To strengthen the above conclusions, the fourth derivative UV absorption spectra (4thD), the fluorescence quantum yield, and the tryptophan fluorescence decay were explored. Fig. 4 shows both the absorption spectra (zero order) and the fourth derivative UV absorption spectra of the protein at pH 6.0 and 20 °C. The advantage of the 4thD is that the corresponding spectral contributions of tyrosine and tryptophan residues in protein can be identified. According to Lange et al. [18,19], the spectra near 283 and 290 nm originate mainly from the contributions of tyrosine and tryptophan residues, respectively. A detailed analysis of the spectra indicated that no significant difference was observed when comparing the 33-kDa protein with other proteins we previously studied (for example, with the thermolysin pressure-denaturation, see [30]). On increasing the pressure, the fourth derivative spectra were blue-shifted in both regions, suggesting, as observed in fluorescence, that the mean polarities of both tyrosine and tryptophan residues were increased by pressure, i.e., more exposed to the solvent. The change in their environments can be approximately estimated from the shift of maximum amplitude of the derived spectrum [18]. For example, the maximum for tyrosine residues corresponds to an increase of the dielectric constant from about 25 to 65. The fluorescence quantum yield (0.04) (see Materials and methods) of the protein tryptophan is rather low (the tryptophan quantum yield in protein is mostly about 0.1) and the fluorescence decay showed multiple components. For the main components it was 3.3 ns and for the minor components 0.7 and 7.45 ns, usual values for most B proteins. It seems that these parameters cannot provide a clue to explain the phenomenon described. To solve all the questions mentioned above, we thought that further explorations using various methods are needed. A detailed analysis of the fluorescence of various 33-kDa protein mutants in which eight tyrosine residues are orderly substituted by site-directed mutation together with a study on the three-dimensional structure of the protein by X-ray crystallography or NMR could provide definitive conclusions. However, there is no doubt that the 33-kDa protein from spinach photosystem II is an ideal model to solve the puzzle why only tryptophan fluorescence emission can be observed in B type proteins upon a 280 nm excitation.

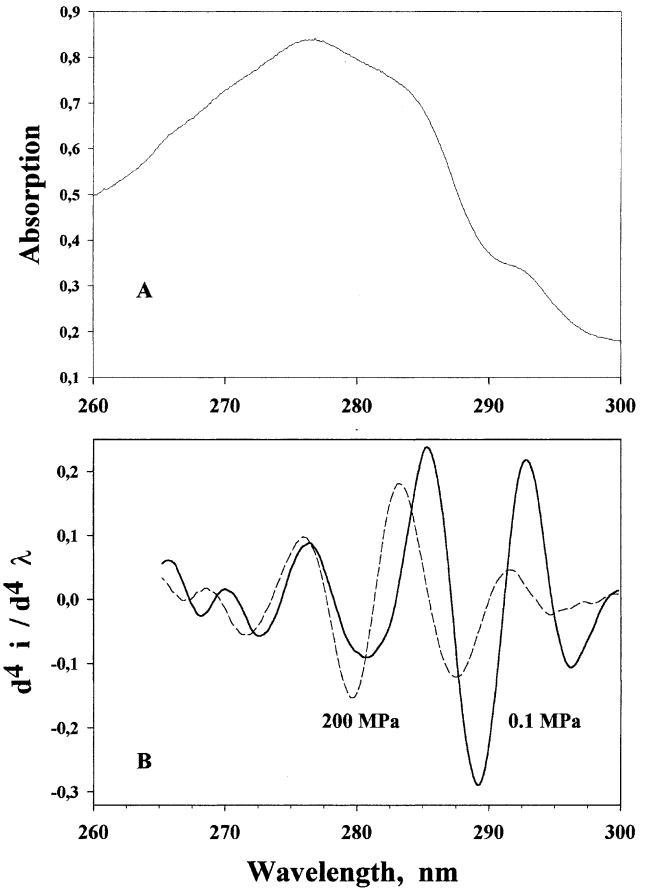

Fig. 4. UV absorption spectra of the 33-kDa protein. A, zero-order absorption spectrum at 0.1 MPa; B, fourth derivative spectra at 0.1 and 200 MPa. Protein concentration: 0.8 mg/mL in 0.1 M, pH 6.0 Mes buffer. Temperature, 20 °C.

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References

[1] J.R. Lakowicz, Principle of Fluorescence Spectroscopy, second ed., Kluwer Academic/Plenum Publishers, New York, 1999.
[2] G. Weber, Protein Interactions, Chapman & Hall, London, 1992.
[3] R.F. Cheng, F. Edelhoch, R.F. Steiner, Fluorescence of protein, in: S.T. Leach (Ed.), Physical Principle and Techniques of Protein Chemistry, Academic Press, New York, 1969, pp. 171–245.
[4] G. Nemethy, J.W. Peer, H.A. Scherraga, Effect of protein–solvent interaction on protein conformation, Ann. Rev. Biophys. Bioeng. 10 (1981) 459–497.
[5] J.R. Lakowicz, Fluorescence studies of structural fluctuations in macromolecules as observed by fluorescence spectroscopy in time, lifetime and frequency domains, Meth. Enzymol. 131 (1986) 518–567.
[6] K. Ruan, C. Balny, High pressure static fluorescence to study macromolecular structure–function, Biochim. Biophys. Acta (2002) in press.
[7] F.W.J. Teale, The ultraviolet fluorescence of proteins in neutral solution, Biochem. J. 76 (1960) 381–388.
[8] M.V. Vladimirov, E.A. Burstein, Luminescent spectra of aromatic amino acids and proteins, Biophysika 5 (1960) 385–392.
[9] K. Ruan, C. Xu, Y. Yu, J. Li, R. Lange, N. Bec, C. Balny, Pressure-exploration of the 33-kDa protein from spinach photosystem II particle, Eur. J. Biochem. 268 (2001) 2742–2750.
[10] N. Murata, M. Miyao, Extrinsic membrane proteins in the photosyntheticoxygen-evolving complex, Trends Biochem. Sci. 10 (1985) 122–124.
[11] M. Miyao, N. Murata, Role of 33-kDa polypeptide in preserving Mn in the photosynthetic oxygen-evolution system and its replacement by chloride ions, FEBS Lett. 170 (1984) 350–354.
[12] T. Kuwabara, M. Miyao, T. Murata, N. Murata, The function of 33-kDa protein in the photosynthetic oxygen-evolution system studied by reconstitution experiments, Biochim. Biophys. Acta 806 (1985) 283–289.
[13] H. Oh-oka, S. Tanaka, K. Wada, T. Kuwabara, N. Murata, complete amino acid sequence of 33-kDa protein isolated from spinach photosystem II particles, FEBS Lett. 197 (1986) 63–66.
[14] S. Tanaka, Y. Kuwada, K. Wada, K. Hamaguchi, Extrinsic 33-kDa protein of spinach oxygen-evolving complexes: kinetic studies of folding and disulfide reduction, Biochemistry 28 (1989) 7188–7193.
[15] T. Kuwabara, N. Murata, Purification and characterization of 33 kilodalton protein of spinach chloroplasts, Biochim. Biophys. Acta 581 (1979) 228–236.
[16] Q. Xu, T.M. Briker, Structural organization of proteins on the oxidizing side of photosystem II, J. Biol. Chem. 267 (1992) 25816–25821.
[17] R.F. Chen, Fluorescence quantum yield measurements: vitamin B6 compounds, Science 150 (1965) 1593–1595.
[18] R. Lange, J. Frank, J.L. Saldana, C. Balny, Fourth derivative UV-spectroscopy of proteins under high pressure. I. Factors affecting the fourth derivative spectrum of the aromatic amino acids, Eur. Biophys. J. 24 (1996) 277–283.
[19] R. Lange, N. Bec, V.V. Mozhaev, J. Frank, Fourth derivative UV-spectroscopy of proteins under high pressure. II. Application to reversible structural changes, Eur. Biophys. J. 24 (1996) 284–292.
[20] M.R. Eftink, C.A. Ghiron, Exposure of tryptophanyl residues in proteins. Quantitative determination by fluorescence quenching studies, Biochemistry 15 (1976) 672–680.
[21] V.V. Mozhaev, K. Heremans, J. Frank, P. Masson, C. Balny, High pressure effects on protein structure and function, Proteins: Struct. Funct. Gene 24 (1996) 81–91.
[22] A.A. Paladini, G. Weber, Absolute measurements of fluorescence polarization at high pressure, Rev. Sci. Instrum. 52 (1981) 419–427.
[23] K. Ruan, G. Weber, Dissociation of yeast hexokinase by hydrostatic pressure, Biochemistry 27 (1988) 3295–3301.
[24] K. Ruan, G. Weber, Hysteresis and conformational drift of pressure-dissociated glyceraldehyde phosphate dehydrogenase, Biochemistry 28 (1989) 2144–2153.
[25] K. Ruan, R. Lange, N. Bec, C. Balny, A stable partly denatured state of trypsin induced by high pressure, Biochem. Biophys. Res. Commun. 239 (1997) 150–154.
[26] K. Ruan, R. Lange, Y. Zhou, C. Balny, Unusual effect of high hydrostatic pressure on basic phospholipase A_2 from venom of *Agkistrodon Halys* Pallas, Biochem. Biophys. Res. Commun. 249 (1998) 844–848.
[27] K. Ruan, S. Tian, R. Lange, C. Balny, Pressure effects on tryptophan and its derivatives, Biochem. Biophys. Res. Commun. 269 (2000) 681–686.
[28] O. Roitel, N. Bec, R. Lange, G. Branlant, Pressure denaturation of phosphorylating glyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearithermophilus*, Biochem. Biophys. Res. Commun. 283 (2001) 347–350.
[29] J.-M. Zhou, L. Zhu, C. Balny, S. Perrett, Pressure denaturation of the yeast prion protein Ure2, Biochem. Biophys. Res. Commun. 287 (2001) 147–152.
[30] S. Kunugi, M. Kitayaki, Y. Yanagi, N. Tanaka, R. Lange, C. Balny, The effect of high pressure on thermolysin, Eur. J. Biochem. 248 (1997) 567–574.