

Chapter 32

High-Pressure Fluorescence Spectroscopy

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Abstract The combination of fluorescence and pressure perturbation is a widely used technique to study the effect of pressure on a protein system to obtain thermodynamic, structural and kinetic information on proteins. However, we often encounter the situation where the available pressure range up to 400 MPa of most commercial high-pressure fluorescence spectrometers is insufficient for studying highly pressure-stable proteins like inhibitors and allergenic proteins. To overcome the difficulty, we have recently developed a new high-pressure fluorescence system that allows fluorescence measurements up to 700 MPa. Here we describe the basic design of the apparatus and its application to study structural and thermodynamic properties of a couple of highly stable allergenic proteins, hen lysozyme and ovomucoid, using Tryptophan and Tyrosine/Tyrosinate fluorescence, respectively. Finally, we discuss the utility and the limitation of Trp and Tyr fluorescence. We discuss pitfalls of fluorescence technique and importance of simultaneous use of other high-pressure spectroscopy, particularly high-pressure NMR spectroscopy.

Keywords Fluorescence spectroscopy • Highly stable protein • Intermediate conformer • Pressure • p - T phase diagram • Tryptophan • Tyrosine/Tyrosinate

32.1 Introduction

Most proteins have several fluorescent amino acid residues that are tryptophan, tyrosine and phenylalanine. These fluorophores provide the intrinsic emission

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spectrum of a protein by excitation at different absorption wavelengths. As an advantage, intrinsic protein fluorescence is quite sensitive to chemical environment of fluorophores and can be acquired very quickly in nanosecond time range, though it only gives limited information on protein conformation based on the change in Trp environment. Therefore fluorescence spectroscopy has been widely used as a convenient approach to study conformational transition, folding, self-association and dissociation mechanism of proteins, but normally limited up to 400 MPa (Royer 2002, 2006; Munishkina and Fink 2007; Shah et al. 2012). Pressure shifts equilibria within various conformational states of a protein in solution through volume differences (ΔV) in contrast to temperature perturbing conformational equilibria through heat capacity differences (ΔC_p), which cause both enthalpy and entropy changes. Therefore we can utilize pressure perturbation to investigate the intrinsic conformational fluctuation of a protein by increasing the population of lower volume conformers or sub-states by increasing pressure among the multiple conformational sub-states with different partial molar volumes, provided that the multiple sub-states exist in marginal balance of stability and that the volume difference (ΔV) among them are sufficiently large. For some proteins in nature, these conditions are rather hard to realize, in which case pressure denaturation cannot be realized in the pressure range of 0.1–400 MPa, which is the range of pressure for most commercial high-pressure fluorescence spectrometers. We, therefore, constructed a high-pressure fluorescence spectrometer system that spans a pressure range of 0.1–700 MPa.

In this review, we illustrate the use of high-pressure fluorescence spectrometer for analyzing a thermodynamic stability of a protein (a phase diagram) on pressure (p)-temperature (T) axes and for studying a highly stable conformational intermediate. Here, we choose two proteins, hen egg white lysozyme and ovomucoid, both being well-known as strong allergenic proteins in hen egg. As probes, we adopt the maximum wavelength of fluorescence emission from naturally occurring Trp residues for hen lysozyme, but the maximum wavelength of fluorescence emission from tyrosine and tyrosinate for ovomucoid, which lacks Trp residue in its amino acid sequence.

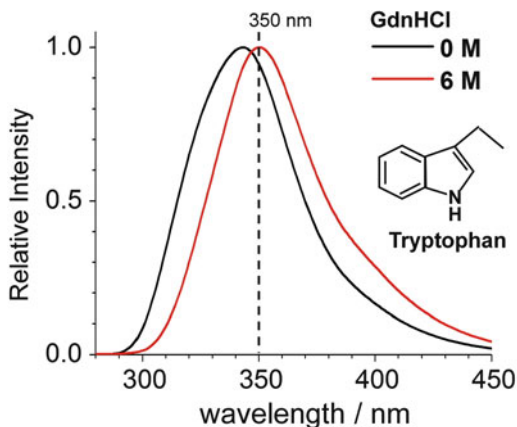
32.2 Intrinsic Fluorophores in a Protein

32.2.1 Tryptophan Fluorescence

Aromatic amino acid residues that are contained in most proteins work as inherent fluorophores: tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe). Among these, Trp becomes dominant probe in a protein because its extinction coefficient is by far larger than those of Tyr and Phe, though the quantum yields of Trp and Tyr are approximately the same ($\Phi = \sim 0.14$) in the neutral solution at room temperature (Chen 1967). In the case of Phe, since both the absorption coefficient

Fig. 32.1 The fluorescence spectra of tryptophan residues in the protein (hen lysozyme) at pH 7.0 at 25 °C.

Tryptophan fluorescence sensitively reflects the polarity around the ring. The maximum wavelength of tryptophan emission ($\lambda_{\max}$) shows *red*-shift, depending upon the exposure to the solvent. The degree of protein-unfolding induced by chemical denaturant such as guanidine hydrochloride (Gdn HCl)



and the quantum yield are both quite small, the emission from this residue is rarely observed in a protein sample. Another advantage of using Trp fluorescence is that most globular proteins have a relatively restricted number (a few to several) of Trp residues, and therefore, site-specific conformational information on the local environment may be obtained from a selective excitation of Trp residues at approximately 295 nm. Because, in the excited state, the dipole moment of the Trp ring is quite large (Pierce and Boxer 1995), the emission energy is highly sensitive to the change in polarity. Therefore, the shift in the maximum wavelength ($\lambda_{\max}$) of Trp emission is considered to represent sensitively the change in the microenvironment of the Trp ring (Royer 2006). The blue-shifted wavelength ($\lambda_{\max} \sim 330$ nm) shows that the Trp ring is located in the non-polar environment or buried in the hydrophobic core of the protein, while the red-shifted wavelength ($\lambda_{\max} \sim 350$ – 355 nm) indicates that the Trp ring is located in the polar environment or exposed to the aqueous environment (Fig. 32.1) (Lakowicz 2006). Based on the variation of the emission wavelength induced by a perturbation, we can utilize Trp fluorescence as a probe of conformational transitions from the folded state to the intermediate or to the globally unfolded state in many globular proteins.

32.2.2 Tyrosine Fluorescence

We occasionally encounter proteins containing no tryptophan residue in their primary sequence. In this case, tyrosine (Tyr) is available as the dominant fluorescence probe in proteins. Figure 32.2a is the schematic view of the electronic states of a molecule and the transitions between them, called *Jablonski diagram*, drawn for the absorption and the emission of tyrosine. On the ground state S_0 , under acidic or neutral pH ($\text{pH} < 10.4 : \text{p}K_a$), Tyr is dominantly excited at 280 nm and gives intrinsic emission at ~ 304 nm (Fig. 32.2a, transition *a*). For the pH of the solution

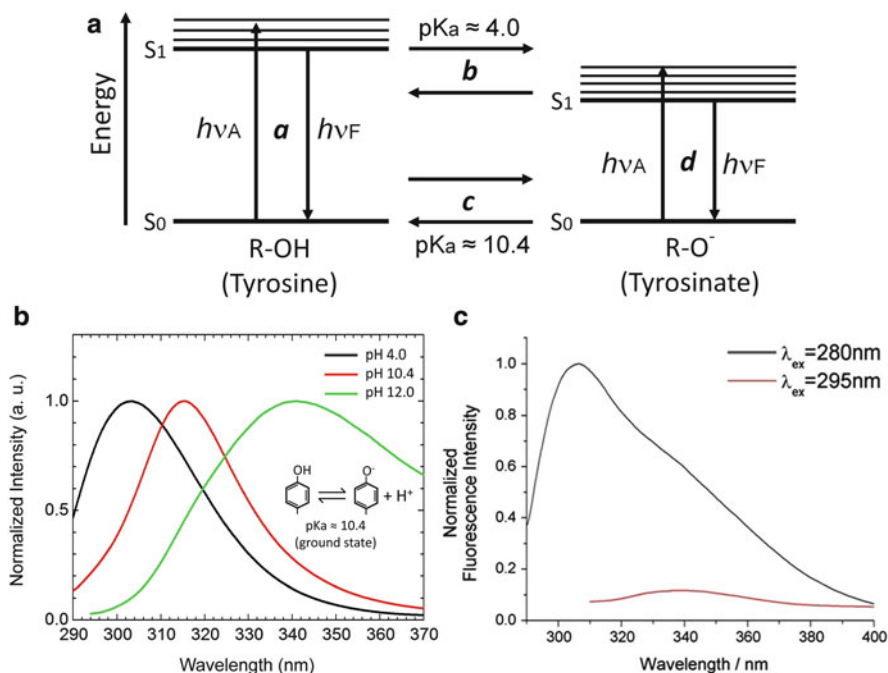


Fig. 32.2 The feature of tyrosine (Tyr) fluorescence. (a) Schematic view of energy-level diagram for tyrosine and tyrosinate fluorescence. (b) The fluorescence spectra of amino acid tyrosine at three pH values, at 0.1 MPa at 25 °C. All the excitations were made at 280 nm and the emission intensity is normalized to unity at its maximum. The emission with $\lambda_{\max}$ at ~ 304 nm is assignable to tyrosine, the emission with $\lambda_{\max}$ at ~ 350 nm to tyrosinate, and the emission with $\lambda_{\max}$ at ~ 315 nm to a tyrosine/tyrosinate mixture. (c) The emission spectra of tyrosine (black line) and tyrosinate (red line) in the presence of proton acceptor. Tyrosine fluorescence excited at 280 nm shows the emission with a maximum at ~ 304 nm and a shoulder at approximately 340 nm, while that of tyrosinate excited at 295 nm gives lower emission with maximum wavelength ($\lambda_{\max}$) at 340 nm

is larger than the $pK_a(10.4)$, the side-chain turns from tyrosine to tyrosinate due to deprotonation in the ground state (Fig. 32.2b, inset), and the ionized tyrosinate gives emission at ~ 340 nm by excitation at 280 nm (Fig. 32.2b, green) (O'neil and Hofmann 1987; Ross et al. 1992) (Fig. 32.2a, reaction c and d).

Interestingly, the pK_a value of the Tyr residue decreases from 10.4 to approximately 4.0 in the excited state (Fig. 32.2a, reaction b), provided that its life time is long. However, since the transition (emission) a is fast, only a fraction of the excited state of tyrosine goes to the excited state of tyrosinate (Fig. 32.2a, reaction b) (O'neil and Hofmann 1987). This means that intrinsic tyrosinate emission at 340 nm is difficult to observe under neutral pH conditions. Therefore, when the pH of the solution is below its $pK_a(10.4)$, the fluorescence emission of Tyr occurs at $\lambda_{\max} \sim 304$ nm by excitation at 280 nm (Fig. 32.2b, black) (Martin et al. 1958).

As a result, the intrinsic emission of tyrosine is insensitive to the polarity around the Tyr ring under pH conditions below its pK_a (10.4), namely $\lambda_{\max}$ does not vary irrespective of whether Tyr residue is buried in the interior of the protein or exposed to the solvent. On the other hand, the presence of proton-acceptor (e.g. acetic acid, phosphate, aspartic acid and glutamic acid) accelerates the deprotonation of phenolic hydroxyl group of tyrosine in the excited state (Rayner et al. 1978; Szabo et al. 1978). When the Trp-lacking protein coexists with the sufficient amount of proton acceptors, tyrosinate emission excited at 280 nm is often observed as a shoulder at near 340 nm even under neutral pH (Fig. 32.2c, *black line*) (Rayner et al. 1978). Alternatively, we can obtain tyrosinate emission independently by excitation at 295 nm (Fig. 32.2c, *red line*). The combination of tyrosine and tyrosinate fluorescence will be useful as probes for studying thermodynamics, conformational transition and kinetic reaction in Trp-lacking proteins.

32.3 High Pressure Fluorescence Apparatus Operating at 3–700 MPa: Innovated Design of Pressure-Resistive Optical Vessel and Inner Cell

The combination of fluorescence spectroscopy and pressure perturbation has been utilized as a convenient means to trap and characterize structures and thermodynamic properties of folding intermediates (Royer 2006; Mitra et al. 2007), and to discuss the contributions of volume changes of conformational transition among protein structures (Maeno et al. 2009), as well as to study pressure-induced dissociation of amyloid fibrils (Abdul Latif et al. 2007; Shah et al. 2012). While many proteins respond sensitively to pressure and often unfold below 400 MPa, some proteins are exceptionally stable and need higher pressures to denature. A class of such proteins is commonly found in proteinase inhibitors (Hiromi et al. 1985) and allergenic proteins (Astwood et al. 1996). In order to resolve such experimental difficulty, we have recently developed a high-pressure fluorescence spectrometer, for which the pressure limit is extended from 400 to 700 MPa. This methodological advance allows pressure-resistive protein to become a target of investigation. Here, we show some points of improvements in our high-pressure fluorescence system, the detail of which will be published elsewhere.

We have developed a high-pressure fluorescence spectrometer system in collaboration with Syn-Corporation. Co. Ltd. (Kyoto, Japan) (Fig. 32.3). The system consists of a pressure-resistive optical vessel (Fig. 32.3b-2 and 32.3c) and the inner cell parts (Fig. 32.3d), and was added to a conventional high-pressure fluorescence system (FP-6500, JASCO Inc., Japan) (Fig. 32.3a). Though the detailed design of the body of pressure-resistive optical vessel is not shown here, the material of the body part is stainless steel (JIS SUS630). This vessel has three sapphire windows for excitation light or emission light to penetrate, each attached to an optical plug.

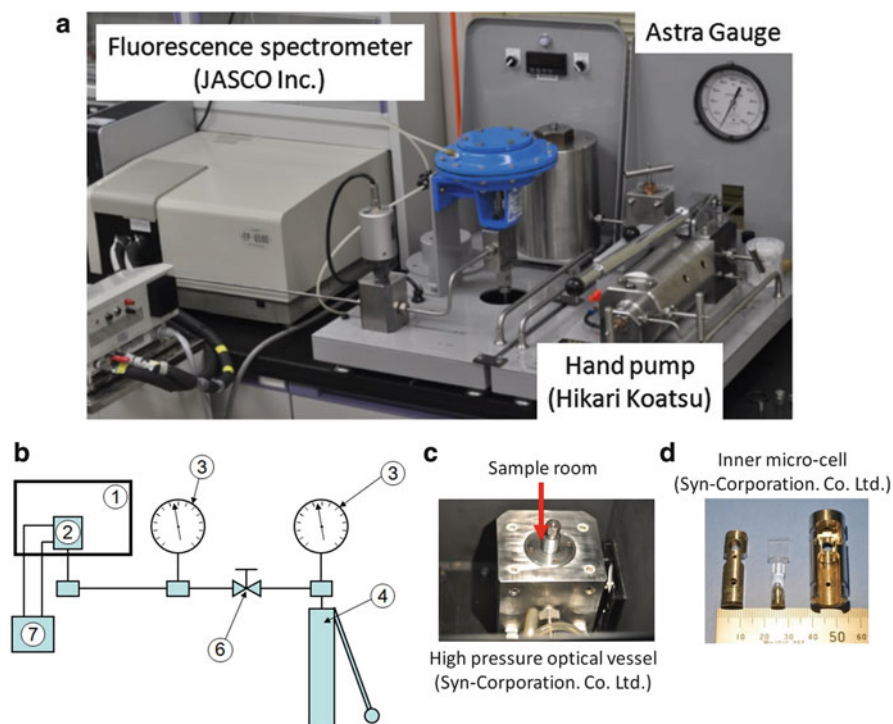


Fig. 32.3 (a) and (b) Overview of our improved high-pressure fluorescence system available between 3 and 700 MPa. (c) High pressure optical vessel made by SUS 630 and (d) the optical inner micro-cell made by quartz (*center*) and its holder parts (*right and left*)

When the internal pressure of vessel reaches 700 MPa, these optical plugs are sustained by using three grand nuts since considerable force is applied to each plug. Upper portion plug is the lid of the high-pressure optical vessel, and the holder to fix an inner optical cell is attached to this plug in the vessel (arrow in Fig. 32.3c). The optical inner micro-cell corresponding to sample chamber consists of two parts. One is an optical cell made of doped fused silica glass and another is a diaphragm which was made of polytetrafluoroethylene (PTFE) (Fig. 32.3d, center part). The total volume of sample chamber is about 0.2 cm^3 . When the inner optical cell is compressed by hydrostatic pressure, the cylinder of a diaphragm of inner cell will make a dent. Therefore, the wall of this cylinder is made very thin, the thickness of which is about 0.25 mm. Importantly, in order to maintain the reproducibility of the cell attachment position, this inner optical cell is tightly secured in an inner cell holder (Syn-Corporation. Co. Ltd., Japan) (Fig. 32.3d, left and right metal parts).

32.4 Application to Highly Stable Proteins

Knowledge on thermodynamic stability and conformational state of a globular protein forms a basis for understanding its function, folding as well as misfolding into amyloid fibrils (Moriarty and Colon 2005). In addition to the characterization of them on the temperature axis (Privalov 1989, 1990), the characterization of the same on the pressure axis is increasingly important in basic protein science (Zipp and Kauzmann 1973; Nash and Jonas 1997; Akasaka 2006) as well as in the applied protein science (Hayashi 2002). Here, we introduce the methodology to characterize thermodynamic property and/or high-energy intermediary state of highly stable allergenic proteins in hen egg white, using tryptophan and tyrosine/tyrosinate fluorescence spectroscopy in the pressure range from 3 to 700 MPa. Fluorescence spectroscopy up to 700 MPa was pioneered by Hoa et al. (1982, Hoa and Marden 1982) to study heme proteins. Hen egg is well known as one of food allergen, and its allergenicity usually arises because of the excessive immune response to “allergenic protein” as antigen. As common feature, they have quite stable folded conformers so that its allergenic potency remains even after perturbations of heat, pressure and chemical denaturants. Therefore, so far, basic information such as thermodynamic and conformational stability in these proteins has rarely been reported.

32.4.1 *p-T Phase Diagram of Hen Lysozyme at pH 2 Using Trp Fluorescence*

Hen egg white lysozyme (HEWL), consisting of 129 amino acid residues (approximately 14.4 kDa), is one of the most well characterized globular proteins as a model system both for structure and function, and is also known as a source of allergenicity in egg white. There are six tryptophan residues in the molecule, two of which (Trp62 and Trp63) are found in the β domain and the rest are found in the α domain, of which Trp28, Trp108 and Trp111 are close to the large water-containing cavity (navy spheres in Fig. 32.4c, left). Therefore, we can utilize tryptophan fluorescence averaged from them as a probe of thermodynamic analysis for global unfolding. However, in the previous study, the global unfolding of HEWL has been hardly attainable even at acidic pH and at 400 MPa which is the highest pressure available in a traditional high pressure fluorescence system, because of the high pressure-resistance of this protein. With the improved fluorescence system, the study of the pressure-induced unfolding equilibrium of HEWL was carried out at pH 2 in the pressure range of 3–700 MPa. To depict a rather complete free energy landscape of HEWL on the pressure-temperature plane, temperature was widely varied between -10°C and 50°C .

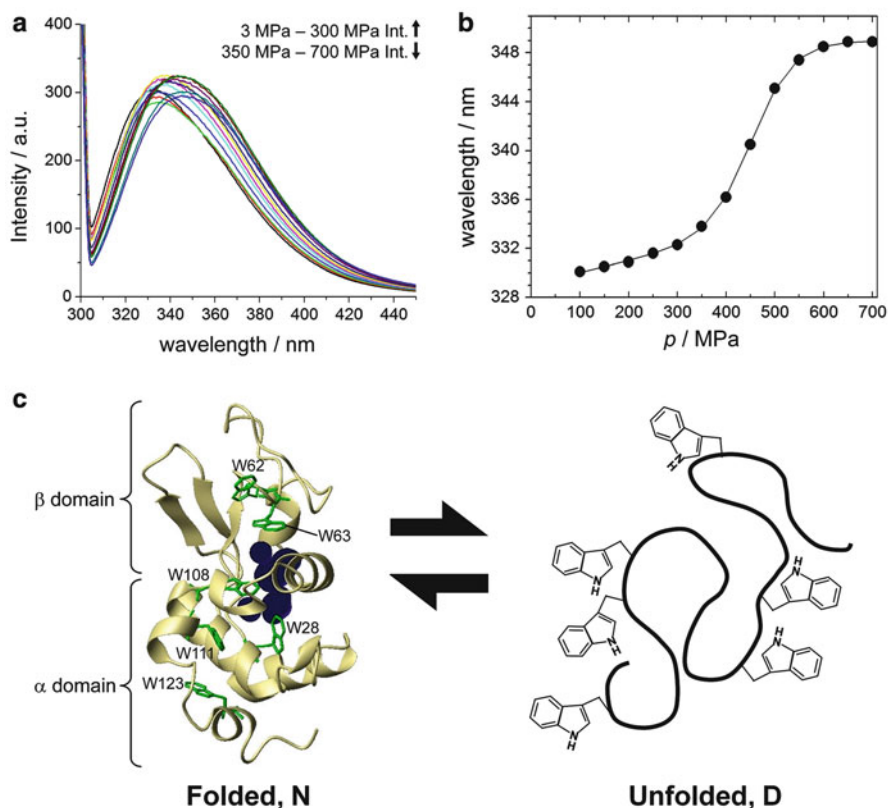


Fig. 32.4 Tryptophan fluorescence on hen egg white lysozyme at pH 2. (a) Trp fluorescence changes as a function of pressure at 15 °C. The upward and downward *arrows* indicate whether the fluorescence intensity is increased or decreased with increasing pressure in the pressure range indicated. (b) Plots of the wavelength of maximum fluorescence intensity ($\lambda_{\max}$) as a function of pressure at 15 °C. The *solid curves* are the best-fit of eq. 32.2 to $\lambda_{\max}$, giving ΔG_0 and ΔV (Eq. 32.3) values. Obtained thermodynamic parameters at various temperatures (–10 °C to 50 °C) are listed in Table 32.1. (c) (*Left*) The folded structure N of hen lysozyme in crystal (PDB ID; 135 L), which consists of two domains, α and β . The six tryptophan residues are colored *green* and the water-accessible internal cavity is shown by navy-spheres. (*Right*) The expected fully unfolded structure D of hen lysozyme including 6 Trp residues which exposed to solvent

32.4.1.1 Pressure-Induced Changes in Trp Fluorescence Spectra of HEWL at 0.1–700 MPa

Figure 32.4a gives the representative averaged fluorescence spectra obtained from six Trp residues contained in HEWL as a function of pressure from 3 to 700 MPa at 15 °C, and the rest were measured at –5 °C, –10 °C, 5 °C, 25 °C, 40 °C and 50 °C, respectively. For details of pressure-induced spectral changes at respective temperatures, see a reference of Maeno et al. (2009). At all measured-temperatures,

the fluorescence spectral changes in $\lambda_{\max}$ are fully reversible with pressure, but the intensity of emission shows less reversibility ($\sim 80\%$). The lack of full reversibility is often encountered in high-pressure fluorescence experiments owing to some technical reasons. The shift in $\lambda_{\max}$ of Trp fluorescence is considered to represent correctly the change in the microenvironment of the tryptophan ring, which has already described in Sect. 32.2.1. From the plot of $\lambda_{\max}$ against pressure (Fig. 32.4b), we note that $\lambda_{\max}$ stayed initially within 330–335 nm at all temperatures ($-10\text{ }^{\circ}\text{C}$ to $50\text{ }^{\circ}\text{C}$), showing that Trp residues are almost fully buried in the folded conformer of the protein. Finally at 700 MPa, $\lambda_{\max}$ shifted to 349–350 nm, indicating that all the six Trp residues become exposed to the solvent. Therefore, we conclude that this protein is totally unfolded at 700 MPa at all measured temperature points. These results presumably represent pressure-induced two-state equilibrium transitions of HEWL from the folded conformer (N; “native” conformer) to the unfolded conformer (D; denatured conformer) (Fig. 32.4c).

32.4.1.2 Thermodynamic Analysis: Volume, Expansivity, Stability and Heat Capacity Changes

We assume the two-state transition between N and D, $N \leftrightarrow D$, and best-fit the observed changes in the wavelength of maximum fluorescence intensity ($\lambda_{\max}$) in Fig. 32.4b with Eq. 32.2, which gives ΔV and ΔG^0 values listed in Table 32.1, together with pressures at the midpoint of transition p_m (Eq. 32.4). (For the detail of thermodynamic analysis, see a reference of Maeno et al. (2009)).

$$\Delta G = G_D - G_N = -RT \ln K \quad (32.1)$$

$$\lambda_{\max} = \frac{\lambda_{\max} + \lambda_{\max} \exp[-\{\Delta G^0 + \Delta V^0(p - p^0) + \Delta\alpha(p - p^0)(T - T^0)\} / RT]}{1 + \exp[-\{\Delta G^0 + \Delta V^0(p - p^0) + \Delta\alpha(p - p^0)(T - T^0)\} / RT]} \quad (32.2)$$

Table 32.1 Thermodynamic parameters for unfolding of hen lysozyme determined from Trp fluorescence experiments (Maeno et al. 2009)

T ($^{\circ}\text{C}$)	ΔG^0 (kJ mol $^{-1}$) ^a	ΔV (ml mol $^{-1}$) ^b	p_m (MPa) ^c
−10	31.5 ± 3.8	-85.9 ± 3.8	366.7
−5	22.1 ± 2.6	-58.2 ± 6.7	379.7
5	23.1 ± 1.5	-51.0 ± 3.5	452.9
15	25.7 ± 1.1	-56.2 ± 2.4	357.3
25	9.5 ± 3.4	-22.4 ± 9.3	424.1
40	7.6 ± 2.8	-23.4 ± 8.2	324.8
50	4.2 ± 3.1	-16.8 ± 11.8	250.0

^aGibbs free energy change at 0.1 MPa calculated with Eq. 32.2

^bPartial molar volume change ($\Delta V = \Delta V^0 + \Delta\alpha(T - T^0)$) calculated with Eq. 32.2

^cThe denatured pressure

$$\Delta V = V_D - V_N = \Delta V^0 + \Delta\alpha (T - T^0) \quad (32.3)$$

$$p_m = \Delta G^0 / \Delta V \quad (32.4)$$

$$\Delta G = \Delta H_m \left(1 - \frac{T}{T_m}\right) + \Delta C_p \left\{T - T_m - T \ln \left(\frac{T}{T_m}\right)\right\} \quad (32.5)$$

$$\Delta S_m = \frac{\Delta H_m}{T_m} \quad (32.6)$$

where ΔG is the Gibbs free energy difference between N and D conformer, K is the equilibrium constant, ΔV is the change in partial molar volume of conformer D relative to N and $\Delta\alpha$ is the change in expansivity, respectively. In addition, T_m , p_m , ΔH_m , ΔS_m and ΔC_p represent the temperature and pressure at the midpoint of transition between N and D state, the unfolding enthalpy change at T_m , the unfolding entropy change at T_m and the unfolding heat capacity change, respectively.

In Table 32.1, we note that p_m ranges from 250 (50 °C) to 453 MPa (5 °C), revealing a relatively high stability of HEWL against pressure unfolding even at low pH. The stability extrapolated to 0.1 MPa (ΔG^0) shows an increasing trend by lowering temperature, but the values may not be as reliable as ΔV in the present case, because 0.1 MPa is far from p_m and no reliable data points are available to 0.1 MPa. Figure 32.5a gives plots of ΔV against temperature (Eq. 32.3), which are all negative within the temperature range from −10 °C to 50 °C, but with a significant temperature dependence. From the slope of the dashed line in Fig. 32.5a, we estimate the expansivity change upon unfolding $\Delta\alpha$ ($=1.07 \text{ ml mol}^{-1} \text{ deg}^{-1}$) in Eq. 32.2. This value is comparable to those in staphylococcal nuclease ($1.33 \text{ ml mol}^{-1} \text{ deg}^{-1}$) (Lassalle et al. 2000) as well as in metmyoglobin ($1.8 \text{ ml mol}^{-1} \text{ deg}^{-1}$) (Zipp and Kauzmann 1973) and in ribonuclease A ($1.32 \text{ ml mol}^{-1} \text{ deg}^{-1}$) (Brandts et al. 1970). The positive value of $\Delta\alpha$ is taken to indicate the increased thermal volume due to the increased exposure of the polypeptide chain upon unfolding (Royer 2006). Figure 32.5b shows the plot of the experimentally determined values of ΔG (Eq. 32.1) against temperature at constant pressures, which depict concave features at all pressures studied. Data are limited to above 200 MPa, as no significant fraction unfolds in the lower pressure range to give sufficiently reliable ΔG values below 200 MPa. The plots were best fitted to Eqs. 32.5 and 32.6, giving parameters of ΔC_p , T_s (the temperature of maximum stability: the temperature for $\Delta S = 0$), T_m , ΔH_m and ΔS_m (for both heat and cold denaturations) as summarized in Table 32.2. The ΔG values are fitted reasonably well with a single positive ΔC_p value at each pressure, covering both the cold denaturation and heat denaturation ranges. In general, a positive ΔC_p upon unfolding is accepted as due to the exposure of nonpolar amino acid groups to the solvent water (Royer 2006). In accordance with this, ΔH_m for heat denaturation increases with increasing T_m . Interestingly, while the stability is found to decrease with pressure, T_s increases with increasing pressure.

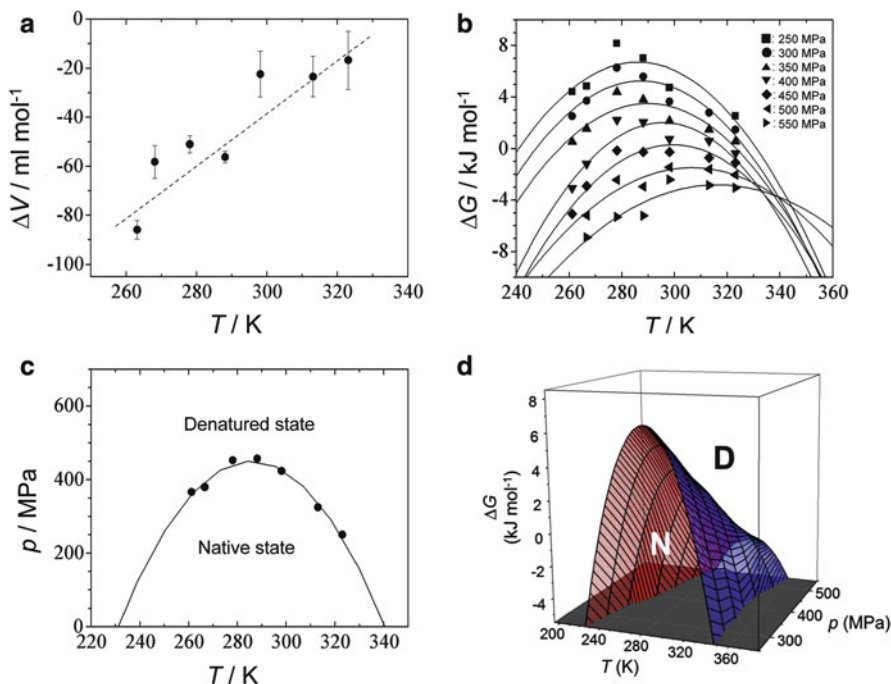


Fig. 32.5 Thermodynamic analysis of HEWL at pH 2. (a) Plot of the change in partial molar volume ΔV on unfolding against temperature. Best-fit to Eq. 32.3 gives a change in expansivity $\Delta\alpha$ on unfolding (260–320 K, pH 2) to be $1.07 \text{ ml mol}^{-1} \text{ deg}^{-1}$. (b) Plots of the change in free energy ΔG on unfolding at various pressures. The solid lines show best-fit of eq. 32.5 to the experimental points, with melting temperature (T_m), enthalpy change at T_m (ΔH_m) and heat capacity change on unfolding (ΔC_p) as fitting parameters listed in Table 32.2. (c) The phase diagram of hen lysozyme at pH 2 as obtained by fluorescence measurements. Plot of p_m (pressure at the midpoint of transition between N and U or half-denaturation) obtained from Eq. 32.4 against T_m . The solid curve represents the best-fit of Eq. 32.5 for $\Delta G = 0$ to the experimental points. (d) Three-dimensional free-energy landscape of hen lysozyme at pH 2 in the range of pressure from 250 to 550 MPa and temperature from 200 to 380 K, drawn as overlay at various pressures

32.4.1.3 Pressure-Temperature Phase Diagram and Free Energy Landscape

Based on the experimentally obtained thermodynamic parameters, we depicted the phase diagram (for $\Delta G = 0$) of HEWL for the first time in aqueous environment on the pressure-temperature plane (Fig. 32.5c). So far, the thermodynamic stability on the temperature-pressure plane in aqueous environment has been reported for a limited number of proteins, including metmyoglobin (Zipp and Kauzmann 1973), ribonuclease A (Brandts et al. 1970), chymotrypsinogen (Hawley 1971) and staphylococcal nuclease (Lassalle et al. 2000; Panick et al. 1998; Panick et al. 1999). In these proteins, except for chymotrypsino-

Table 32.2 Thermodynamic parameters for heat and cold denaturation of hen lysozyme (Maeno et al. 2009)

P (MPa)		ΔH_m (kJ mol ⁻¹) ^a	ΔS_m (J mol ⁻¹ K ⁻¹) ^b	ΔC_p (kJ mol ⁻¹ K ⁻¹) ^c	T_m (K) ^d	T_s (K) ^e
250	Heat	95.1 ± 14.2	287.0 ± 14.5	1.9 ± 0.8	331.4 ± 3.0	286
	Cold	-77.4 ± 10.6	-319.4 ± 14.3		242.3 ± 9.6	
300	Heat	83.9 ± 7.5	256.3 ± 8.6	1.9 ± 0.6	327.4 ± 4.2	287
	Cold	-70.2 ± 8.3	-282.8 ± 8.5		248.2 ± 2.0	
350	Heat	66.3 ± 5.6	204.7 ± 6.0	1.8 ± 0.5	323.9 ± 2.2	290
	Cold	-56.4 ± 13.0	-219.6 ± 13.8		256.8 ± 4.5	
400	Heat	54.2 ± 14.0	170.0 ± 14.6	1.8 ± 0.6	318.8 ± 4.1	296
	Cold	-48.5 ± 6.0	-178.0 ± 6.3		272.5 ± 1.8	

^aUnfolding enthalpy at T_m ^bUnfolding entropy at T_m ^cUnfolding heat capacity at T_m ^dDenaturation temperature^eTemperature for maximum stability ($(\partial \Delta G / \partial T) = \Delta S = 0$)

gen which clearly gives a region of pressure-induced folding at elevated temperature (Hawley 1971), an ellipsoid type pattern like that in Fig. 32.5c with no regions of pressure-induced folding has been commonly observed, although the individual pattern is characteristic of each protein (Ravindra and Winter 2003). Finally, we have been successful in drawing the energy landscape of hen lysozyme at low pH on pressure and temperature axes on the approximation of null isothermal compressibility change ($\Delta\kappa = 0$) within the pressure range studied. The knowledge and the method presented here will serve as a basis for studying dynamics and folding of HEWL as a whole, but will also provide useful information as to the condition for the formation of amyloid fibrils or insoluble aggregates in a wider perspective (Abdul Latif et al. 2007; Shah et al. 2012).

32.4.2 Characterization of an Intermediate Conformer of Chicken Ovomucoid at pH 12 Using Tyrosine/Tyrosinate Fluorescence

Ovomucoid (OVM) from chicken egg white, consisting of 186 amino acid residues (approximately 28 kDa), is well known as an allergenic glycoprotein (Kato et al. 1976) as well as a trypsin inhibitor (Feeney et al. 1963; Hiromi et al. 1985). This protein consists of three sub-domains (domain 1 – domain 3), each having three intra-domain disulfide bonds and two Tyr residues, but no Trp residue is included in the primary sequence (Beeley 1976; Kato et al. 1987). Investigation of its thermodynamic stability is important from the practical viewpoint, as it consists of

11 % of the total protein content and is the most potent allergen in egg white. Though the unfolding of OVM that proceeds in two distinctive steps with a intermediary formation in between them has been shown using urea and guanidine hydrochloride (Waheed et al. 1977; Mustafa et al. 2011), studies on pressure axis, which can give the structural as well as thermodynamic information on semi-stable high energy conformers of this protein located higher on the folding funnel (Akasaka 2006), have not been carried out. Such information would be crucial for understanding the inhibitory function, enzymatic degradability, allergenicity and gel formation (Akasaka 2010). We show the results of high-pressure fluorescence measurements on OVM using improved high-pressure fluorescence spectrometer that can operate in the pressure range up to 700 MPa. To detect unfolding transitions of OVM, we utilize the change in fluorescence emission from six Tyr residues containing two each per domain. A highly alkaline solution condition, pH 12.0, was chosen to ensure that a Tyr residue of OVM will automatically give distinct tyrosinate fluorescence due to the side-chain ionization of tyrosine upon exposure to the alkaline environment.

32.4.2.1 Fluorescence of OVM by Excitation of Tyrosines

Figure 32.6a shows an overlay of the fluorescence spectra from six Tyr residues of OVM in the solution condition of pH 12.0 measured at pressures from 5 to 700 MPa at 25 °C by excitation of tyrosine absorption at 280 nm. At 5 MPa, the fluorescence emission is dominated by the emission with λ_{max} at 306 nm (Fig. 32.6a) assignable to tyrosine (R-OH) (cf. Fig. 32.2b), showing that even at 25 °C in the solution of pH 12.0 the effective $\text{p}K_{\text{a}}$ values of all six Tyr of OVM are larger than 12. This finding indicates, in turn, that all the three domains of OVM are surely folded and that the phenolic OH groups of the Tyr residues are largely protected from the solvent, even at pH 12.0 at 25 °C. With increasing pressure, the decrease of the emission at 306 nm and a concomitant increase in the emission around 350 nm (assignable to tyrosinate (R-O⁻), see inset) were observed, with a clear isoemissive point at 335 nm (red circle in Fig. 32.6a). This result strongly suggests that a two-state transition takes place between the tyrosine (R-OH) and tyrosinate (R-O⁻) forms of Tyr residues of OVM in the wide pressure range from 5 to 700 MPa (Fig. 32.6b). Quantitatively, the fraction ($47/75 \approx 2/3$) of the original emission at 306 nm at 5 MPa is decreased at 700 MPa, while a significant fraction ($28/75 \approx 1/3$) of the original emission still remains even after the transition is nearly complete at 700 MPa (Fig. 32.6a). These results indicate that 4 Tyr residues out of six corresponding to two domains have transformed into tyrosinate (R-O⁻), while two Tyr residues corresponding to one domain remain as tyrosine (R-OH) even at 700 MPa, strongly suggesting that the folding state of one domain of OVM is strongly resistive against denaturation, even to the extreme condition of pH 12.0 at 700 MPa.

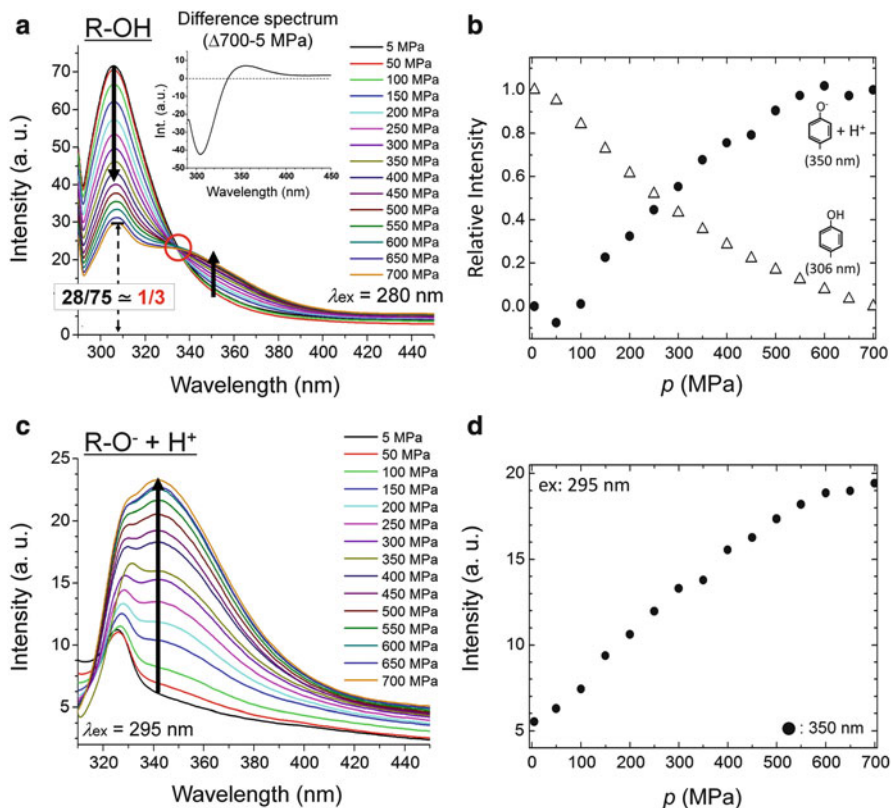


Fig. 32.6 Pressure-dependent changes of the fluorescence emission spectra of OVM at pH 12.0 at 25 °C between 5 and 700 MPa. (a) Spectral changes by excitation at 280 nm (tyrosine absorption). An isoemissive point is observed at 335 nm. The inset shows the difference spectrum obtained by subtracting the spectrum at 700 MPa from that at 5 MPa. (b) Plot of emission intensities at 306 nm (tyrosine) and at 350 nm (tyrosinate) (from panel A), after correcting the measured intensity by taking the solvent contraction into account (for example, $\sim 9\%$ by 300 MPa at 25 °C) (Bridgman 1912). Open triangle: 306 nm; filled circle: 350 nm. (c) Spectral changes by excitation at 295 nm (tyrosinate absorption). (d) Plot of emission intensity at 350 nm (tyrosinate) (from panel C) (Adapted from Fig. 2 in Maeno et al. (2013) by permission of Elsevier Inc.)

32.4.2.2 Fluorescence of OVM by Excitation of Tyrosinate

To confirm the production of tyrosinates (R-O^-) in ground state of OVM at high pressure, next we excite directly the tyrosinate absorption at 295 nm to observe its fluorescence at pH 12.0 both at 25 °C (Fig. 32.6c). Initially, we observe emission with λ_{max} around 325 nm at 5 MPa (black line in the spectrum). By increasing pressure, the fluorescence emission at $\lambda_{\text{max}} \sim 350 \text{ nm}$, assignable to tyrosinates (R-O^-) (cf. Fig. 32.2b), increased dramatically to the final value at 700 MPa (Fig. 32.6c). Thus, we have at least two fluorescent species, one giving the emission

with $\lambda_{\max}$ around 325 nm existent already at 5 MPa and another giving a broad emission with $\lambda_{\max}$ at ~ 350 nm that increases with pressure. First, the species giving the emission with $\lambda_{\max}$ at around 350 nm can be identified with fully exposed tyrosinates (R-O^-), and the result of Fig. 32.6d indicates that the fraction of tyrosinates (R-O^-) increases dramatically with the increase in pressure. These results confirm those from Fig. 32.6a that a majority of tyrosine (R-OH) residues turn into tyrosinates (R-O^-) with increasing pressure. Figure 32.6d shows plots of the emission intensities at 350 nm at various pressures at 25 °C, obtained from Fig. 32.6c. Second, the relatively weak emission with $\lambda_{\max}$ around 325 nm needs attention (Fig. 32.6c). This emission appears already at 5 MPa when OVM is in the basic folded state N and stays as shoulder even at 700 MPa when OVM is in the intermediate state I in which only the most stable domain is folded. Therefore, the most reasonable interpretation seems that this emission belongs to a Tyr residue in the most stable domain.

32.4.2.3 Characterization of Intermediate Conformer of OVM

With chicken ovomucoid at pH 12.0 as target, we found a distinct intermediate state I at 700 MPa, in which two domains are unfolded, leaving one domain intact. So far, we have not specifically identified the intermediate conformer, under our specific experimental conditions, as to which one of the three domains of chicken OVM is the domain that remains folded even at 700 MPa at 25 °C. However, previous urea titration experiments have been carried out on isolated domains or domain combinations of chicken OVM at pH 7.0, and showed that domain 3 has by far the highest stability ($[\text{urea}] \ 1/2 \approx 6.4 \text{ M}$) compared to two other domains ($[\text{urea}] \ 1/2 \approx 2.7 \text{ M}$) (Das et al. 1991). Based on this result, it appears rather certain that the single domain remaining intact at an extreme condition of pH 12.0 and 700 MPa be assigned to domain 3, giving the conformational equilibrium of OVM as depicted in Fig. 32.7. In this regard, we plan to carry on an independent assignment of domain 3 using high-pressure NMR in later studies. The outstanding stability of domain 3 is considered responsible for the strong allergenicity of this protein. In the present work, we have restricted our solution condition to a highly alkaline pH, pH 12.0, to study conformational fluctuations of a Trp-lacking protein by utilizing the strong tyrosinate fluorescence at high pH. The experiment has been successful in monitoring differential stabilities of domains and to find a locally unfolded intermediate state of OVM in a clear-cut manner (Fig. 32.7). This work can be extended to the study of conformational fluctuations in Trp-lacking proteins in the neutral pH region by using tyrosine/tyrosinate fluorescence in combination with various proton acceptors to facilitate tyrosinate production (O'neil and Hofmann 1987). We have already carried out same experiments in OVM at neutral pH, and the thermodynamic feature of this allergenic protein will be reported elsewhere. Thermodynamic properties of the intermediate state disclosed here are expected to be a key to establish a new strategy to eliminate an allergenic potency from allergens.

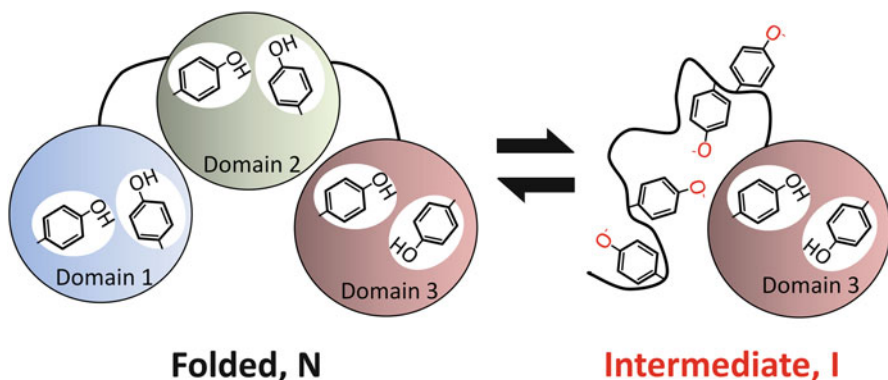


Fig. 32.7 Schematic view of conformational fluctuation of OVM between the folded conformer N and the intermediate conformer I deduced from the experiments at pH 12.0 at 25 °C. All the tyrosine side-chains of OVM are buried at 0.1 MPa at 25 °C at pH 12.0, but those in domains 1 and 2 become exposed to the solvent in the form of tyrosinate at 700 MPa, while those in domain 3 remain buried (Adapted from Fig. 6 in Maeno et al. (2013) by permission of Elsevier Inc.)

32.5 Caution in the Use of High-Pressure Fluorescence Spectroscopy for Studying Proteins

As we have shown here, fluorescence is very sensitive but provides only the limited information around the ring of inherent fluorophores (tryptophan and tyrosine) in a protein. Therefore, we recommend the combination of high-pressure fluorescence spectroscopy and other high-pressure methodologies that can compensate mutually, particularly high-pressure NMR spectroscopy (Maeno et al. 2009; Kitahara et al. 2012; Sunilkumar et al. 2012), when we study pressure-induced local and/or global unfolding of a protein. Otherwise we may erroneously take the site-specific internal hydration around the intrinsic fluorophores with the global unfolding of the protein. For instance, there is a case such that, while the λ_{max} value of Trp fluorescence tells that the protein is extensively hydrated, the high-pressure NMR spectrum shows that the protein is still folded (Sunilkumar et al. 2012). This could arise because the Trp fluorescence reports essentially the change in the local environment of the Trp residue, which does not necessarily coincide with the change in the local environment of other parts of the protein structure. This problem can be avoided if one monitors pressure-induced conformational changes also by NMR, preferably at least at some points of measurement. When the extra-high pressure experiment (e.g. up to 700 MPa) is to be carried out below the freezing temperature of the buffer, we should pay attention to the phase diagram of water. Otherwise, during the experiment at high pressure and low temperature, the buffer might be iced, and then the inner-cell might get a crack due to the volume expansion of the iced water. For instance, the pressure limit under freezing temperature is 200 MPa at -20 °C and 630 MPa at 0 °C.

32.6 Conclusion

The fluorescence spectroscopy combined with pressure perturbation is a well-established technique, for studying conformational transition, folding, dynamics of proteins and amyloid-forming systems. Use of the natural fluorescent probe Trp is particularly convenient, but caution is also necessary to differentiate the local structural change from the overall structural change. The improved high-pressure fluorescence system (0.1–700 MPa) introduced here is particularly; useful to extend the technique to cover a wide range of pressure-resistant proteins such as highly stable allergenic proteins for characterizing their thermodynamic stability and high-energy intermediates. The advancement would provide critical information and strategy for decreasing allergenicity, finding efficient pasteurization and improving food quality and other utility of pressure.

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