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Effects of high hydrostatic pressure on *Rhizopus chinensis* lipase: II. Intermediate states during unfolding

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ABSTRACT

High hydrostatic pressure (HHP) is currently considered a well-established technology for processing food and biological materials and there is an interest in investigating the changes in the structural and functional properties of these materials after high pressure treatment. Therefore, the changes in the structure of *Rhizopus chinensis* lipase (RCL) after high hydrostatic pressure treatment were investigated. Far-UV circular dichroism (CD) spectra showed that the secondary structure of RCL is maintained at pressures below 400 MPa and becomes gradually disordered after higher pressures are applied. Near-UV CD spectra showed that the RCL begins to lose its tertiary structure at pressure over 400 MPa. Fluorescence quenching and the binding of 1-anilinonaphthalene-8-sulfonate confirmed that a partially unfolded intermediate, with loosely compacted conformation and hydrophobic regions, is formed at a pressure of 600 MPa. These results also suggest that RCL maintains a native-like state at pressures below 400 MPa. Above 500 MPa RCL molecules showed characteristics of being in a molten globule state. Dynamic light scattering (DLS) and atomic force microscopy (AFM) measurements indicated that RCL molecules at these pressures are aggregating. The addition of $(NH4)_2SO_4$ to the protein solution could prevent the aggregation, and at 600 MPa the molecule had a hydrodynamic radius approximately 8% larger than that observed for the control sample, which was regarded as being in the molten globule state. The observations suggest that at increasing pressures, the unfolding mechanism of RCL follows well-defined steps from a native state via a native-like structure ending in molten globular state or molecular aggregation.

1. Introduction

Proteins are bio-macromolecules with unique conformations and distinctive functional properties. The folded stability of native proteins is crucial to determine many of their functional properties, especially their catalytic ability. The transformation from a folded state to a completely unfolded state in proteins is a complex process involving different possible conformations and paths (Foguel & Silva, 2004). To explain the mechanism of protein unfolding, the classical two-state model was first proposed (Pfeil & Privalov, 1976). However, protein folding was later shown to occur in a stepwise mechanism involving populations of structural intermediates. Recently, it has been confirmed that thermodynamically stable intermediates may exist during the unfolding process (Uversky & Ptitsyn, 1996).

In 1983, the concept of molten globule state (MG) was introduced by Ohgushi and Wada (1983). The MG state was then characterized by Kuwajima (1989) as a state in which (1) secondary structure is significantly maintained, (2) there is a lack of a precise tertiary interaction due to the tight packing of the side chains to the polypeptide, (3) there is a loosely packed hydrophobic core that increases the hydrophobic surface accessible to the solvent, and (4) the compactness of the protein molecule is characterized by a radius of gyration that is 10–30% larger than that of the protein in the native state. After the conformation of the MG state was found for globular protein, it was reported that under appropriate conditions, many proteins can form a specific, compact and denatured conformation between the molten state and an unfolded state called the pre-molten globule state (PMG) whose features were detailed by Khan, Rahaman, and Ahmad (2011). The features of this state include a less native secondary (about 50%) structure than that of the native state and the absence of a rigid tertiary structure. This state also has a hydrodynamic volume several times larger than that of the protein in the native state and exhibits much weaker 8-anilino-1-naphthalene-sulphonic acid (ANS) binding compared to that observed when the protein is in the MG state (Ohgushi & Wada, 1983).

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Both MG and PMG are intermediates of partially unfolded states with special characteristics, promoting the further understanding of protein folding.

From a thermodynamic standpoint the process promoting the unfolding of proteins involves conformational changes, energy equilibrium and the formation and/or disruption of bonds. Chemical, physical and physiological conditions such as pH (Ahmad, Fatima, Khan, & Khan, 2010), and salts (Record, Guinn, Pegram, & Capp, 2013) are able to change the folding of proteins and to produce relevant intermediate states. The improvement of folding stability by salts and non-electrolytes can prevent aggregation, in contrast with the disruption of protein stability which is mainly associated with the exposure of buried amino acid residues that leads to aggregation under some conditions (Devaraneni, Mishra, & Bhat, 2012).

Applications of HHP in biochemistry have been developing in recent decades with applications that transform macromolecular structures, modify of enzyme activity, and alter the metabolism of microorganisms (Marchal et al., 2003; Silva et al., 2014). The pressure-induced unfolding free energy of proteins was found to be distinct from that induced by heat (Torrent et al., 2003). In proteins, high pressure may induce changes that range from small conformational to complete unfolding. The combination of temperature and pressure can also generate enzyme *P-T* phase graphs (Somkuti, Mártonfalvi, Kellermayer, & Smeller, 2013), which are somewhat different than the *P-T* phase graphs associated with enzyme stability.

Some lipases exhibit higher activity and stability after being treated under HHP, and these changes have been highly correlated with their structural change (Eisenmenger & Reyes-De-Corcuera, 2009). However, to the best of our knowledge, the unfolding path of lipases induced by HHP treatment is seldom studied. Recently, it was observed that the MG state formed during the unfolding of *Rhizopus niveus* induced by chemical reagents (Rabbani, Ahmad, Zaidi, Fatima, & Khan, 2012). Additionally, HHP treatment has the capacity of inducing the formation of MG state of proteins. On the other hand, it was found that the activity of RCL which is highly homologous to *Rhizopus niveus* lipase and belongs to globular protein, increased after HHP treatment. Thus in this work, RCL was selected as a model lipase to investigate the process of lipase unfolding caused by HHP. This study is not only relevant because it provides the knowledge of transient conformations of proteins under various high pressure conditions, their conformational information and mechanisms of folding but also because it promotes the use of HHP technology to modify enzymes.

2. Experimental

2.1. Materials

Lipase from *Rhizopus chinensis CCTCC M201021* was kindly donated by Yiming Biotechnology Company (Taixing, China). 1-Anilinonaphthalene-8-sulfonate (ANS) was purchased from Sigma Chemical Co. (St. Louis, MO). Olive oil, ethanol, polyvinyl alcohol and other chemicals were of analytical grade and purchased from Sinopharm Company (Shanghai, China).

2.2. Purification of RCL

Lipase was purified using the method described by Zhu, Li, Yu, and Xu (2013). The crude lipase was dissolved into 20 mM potassium phosphate buffer at pH 5.5. Then the lipase solution was loaded onto a HiTrap SP FF column (Pharmacia, 5 × 5 ml) and eluted with 0–0.8 M NaCl. Fractions of lipase activities were dialyzed and chromatographed on a HiTrap Phenyl HP (Pharmacia, 5 × 5 ml) column. Lipases were then eluted using the same buffer with an ammonium sulfate concentration gradient from 1.6 to 0 M. Protein fractions with lipases activities were finally dialyzed against phosphate buffer at pH 7.5.

2.3. Lipolytic activity assay

The lipolytic activity was determined using the olive oil-polyvinyl alcohol method (Arima, Liu, & Beppu, 1972; Yang, Chen, Du, Miao, & Feng, 2016). One unit (U) was defined as the quantity of enzyme that liberated 1 μmol of free fatty acid per minute under the assay conditions. All enzyme activity determinations were replicated at least three times. The residual relative activity was calculated by Eq. (1) as:

$$A/A_0(\%) = \frac{\text{lipolytic activity after HHP treatment}}{\text{lipolytic activity before HHP treatment}} \times 100 \tag{1}$$

2.4. Effect of HHP treatment on RCL catalytic ability

The HHP treatment was implemented in a high pressure apparatus (MICRO FOODLAB FPG5740, Stansted Fluid Power Ltd., UK) equipped with a temperature control system. The lipase powder was diluted in 20 mM potassium phosphate buffer (pH 7.5), and the formed solution was placed in a 5 ml plastic tube with a screw lid. The lipase solution was loaded into the chamber of the HHP processing equipment and treated for 10 min. After the treatment, the activity was determined immediately.

2.5. Circular dichroic (CD) measurement

The secondary structure changes of RCL after HHP were investigated by CD spectrometry (Bio-Logic MOS-450, France) using a scan rate of 300 nm/min. For the far-UV CD determination the spectra were detected in a cell of 1 mm path length with a protein concentration of 0.1 mg/ml. The near-UV CD spectra were measured with protein concentration of 1 mg/ml in a cell with a 5 mm path length. Each spectrum was the average of three scans.

The CD result was expressed as mean residue ellipticity (MRE) (McCabe, Rodger, & Taylor, 2005):

$$\text{MRE} = \frac{\theta_{\text{obs}}}{10 \times n \times C \times l} \tag{2}$$

where θ_{obs} is the CD measurement in millidegrees, n is the number of amino acid residues, l is the path length of the cell (cm), and C is the molar concentration of the protein (M). The α-helix content was calculated using MRE at 222 nm ($\text{MRE}_{222\ \text{nm}}$) according to the following equation (Rabbani et al., 2012):

$$\%(\alpha\text{-helix}) = \left(\frac{\text{MRE}_{222\ \text{nm}} - 2340}{30300}\right) \times 100 \tag{3}$$

Meanwhile, the β-strand content was estimated using the BeStSel algorithm that reliably distinguishes the features of β-sheets in protein according to the twisting angles between β-strands and that predicts the responsible content by CD spectroscopy and the reference spectra (András et al., 2015).

2.6. Fluorescence quenching

Fluorescence quenching experiments were performed on a F-7000 spectro-fluorimeter (HITACHI, Japan) at 25 °C with a 1 cm path length cell. In the experiments, aliquots of 2 M quencher stock solution of KI were added to protein solutions (4 μM) to achieve the desired range of quencher concentration (0.1–1 M). To prevent the formation of I_3^-, the KI stock solutions contained 0.1 M sodium thiosulfate. Excitation was set at 295 nm to excite tryptophan residues only. The excitation and emission slits were set at 2.5 and 5 nm respectively. The emission spectrum was recorded in the range from 300 nm to 400 nm. The decrease in fluorescence intensity at λ_m was analysed by the Stern-Volmer equation (Eq. (4)):

$$F_0/F = 1 + K_{sv}\, Q \tag{4}$$

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