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The Effect of Dynamic High Pressure Microfluidization on the Activity of Papain*

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Abstract: The effect of dynamic high pressure microfluidization (DHPM) on the activity of papain had been investigated, and the molecular conformation changes of papain had been detected by fluorescence spectra. The results showed that after DHPM (120～180 MPa) treatment, the enzyme activity decreased in different degrees. The papain activity reduced to 90.04% after 180 MPa treatment. With the increasing of treatment pressure, the fluorescence emission peaks of papain, Tyr and Trp residues gradually red shifted from 334, 285 and 277.5 nm for untreated sample to 335.5, 285.5 and 278.5 nm for 140 MPa treated sample, and then returned to 334, 285 and 278 nm for 180 MPa treated sample, respectively. After 24 h placing at 0～4 ℃, papain activity further decreased and the fluorescence intensity of papain and Tyr residue showed oscillate (down, up and down), which indicated that DHPM treatment (120～180 MPa) changed the molecular conformation of papain visibly with low stability of the new formed conformation.

Key words: dynamic high pressure microfluidization (DHPM); fluorescence spectra; papain; molecular conformation

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1 Introduction

Dynamic high pressure microfluidization (DHPM) is an emerging high pressure treatment technology in China and abroad, which is currently the research focus in the fields of biological macromolecule modification[1-2], auxiliary sterilization[3], emulsification[4], liposome preparation[5], improvement of dietary fiber[6], etc. Its working principle is to bring changes to the structures and properties of materials through exposing the fluid material to the mechanical actions of high velocity impact, high frequency oscillation, transient pressure-decreasing, high-speed shearing, cavitation effect in interactive chamber with a short treatment time (less than 5 s) and continuous operation. Therefore, it differs from high hydrostatic pressure (HHP) which only uses ultra-high pressures from 100 to 1 500 MPa[7-8] and between 300 and 700 MPa in commercial systems[9].

Papain is a kind of sulfhydryl monomer protease, and it is composed of 212 amino acid residues comprising 19 Tyr residues and 5 Trp residues and 4 Phe residues[10]. Papain possesses relatively broad

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substrate specificity, which could hydrolyze the internal peptide chain —CO—NH— of protein molecule producing polypeptides of relatively small molecular weight; hence, papain has wide application in the fields of biological medicines and food chemistry with its enzymatic properties and structural modification always being the major issue.

In recent years, much research has been dedicated to the effect of high pressure treatment on the enzymatic properties and structures[11-12]. Gomes *et al*. (1997) studied the influence of high hydrostatic pressure on the activity and structure of papain, and the results showed that the enzyme activity gradually decreased with increasing pressure, but the size and conformation of enzyme didn't change[13]. Huang *et al*. (2005, 2007) investigated the effect of high hydrostatic pressure treatment[14] and ultra high pressure-enzyme inhibitor combined treatment[15] on the peroxidase and pectin methyl esterase in litchi pulp. Zeng *et al*. (2005) studied the effect of high hydrostatic pressure treatment on the activity of horseradish peroxidase with the relationship between secondary structure and activity of enzyme being analyzed[16]. However, so far there are not many reports on enzymes treated by DHPM.

Our research group has investigated the effect of DHPM on the enzyme activity of polyphenol oxidase in pear[1,17-18] and the molecular conformation of polyphenol oxidase from mushroom[19]. Based on these, we continued to investigate the effect of DHPM on papain activity and reveal the molecular conformation changes of papain by fluorescence spectra, which could help us preliminarily elucidating the mechanism of DHPM-induced changes of papain.

2 Materials and Method

2.1 Materials

Papain, enzyme activity $\geqslant$ 6 000 USP-U/mg (casein, pH 6.0, 40 ℃), purchased from Ourchem Co., Ltd. Reaction substrate is casein. All chemical reagents are analytic grade.

2.2 DHPM Treatment

Using cysteine (0.05 mol/L), EDTA disodium salt (0.035 mol/L) and phosphate buffer (0.05 mol/L, pH 6.0) to prepare the enzyme solution (1 mg/mL). Utilizing NCJJ-0.007/200 nano-ultrahigh pressure homogenizer from Langfang Universal Machinery Limited Company, feeding began under room temperature (20 $\pm$ 3)℃ and the treatment pressure was 120, 140, 160, and 180 MPa with one-pass treatment for each pressure. Then a 50 mL sample was taken under each treatment pressure for detection within 10 min, and another 50 mL sample was storage at 0~4 ℃ for further measurement.

2.3 Determination of Papain Activities

Activity of papain was determined by using casein as substrate[20]. First, 1 mL papain solution, 5 mL PBS for pH 7.5, and 1 mL casein solutions of 10 g/L were added to a 100 mL conical flask. After enzymatic reaction at 40 ℃ for 15 min with gentle stirring, 2 mL CCl_3COOH of 0.4 mol/L was added into the conical flask. The reaction mixture was statically equilibrated at room temperature for 10 min and then centrifuged. After that, 5 mL of 0.4 mol/L Na_2CO_3 solution and 1 mL of Folin solution were added to a 50 mL beaker including 1 mL of the filtrate. The beaker was incubated at 40 ℃ for 20 min. The absorbance of the mixture was measured at 680 nm (T60 UV-Vis spectrophotometer from Beijing Purkingje General Instrument Co., Ltd. was used). One unit of enzyme activity was defined as the tyrosine content formed per minute at 40 ℃ and pH 7.5. Relative enzyme activity is defined as follows

$$\text{Relative enzyme activity} = \frac{\text{Activity of treated papain}}{\text{Activity of untreated papain}} \times 100\%$$

2.4 Fluorescence Spectra Analysis of Papain

Fluorescence spectra of papain were determined by F-2500 fluorescence spectrophotometer

(Hitachi Ltd,Japan). Scanning speed:1 500 nm/min,delay:0 s,EX slit:10.0 nm,EM slit:10.0 nm.

2.4.1 Fluorescence Spectra

Using 280 nm as excitation wavelength,the emission spectra within the range of 300～400 nm were recorded.

2.4.2 Synchronous Fluorescence Spectra

When $\Delta\lambda=20$ nm,scanning with excitation wavelength in the range of 240～360 nm to obtain the synchronous fluorescence spectra of Tyr residues;when $\Delta\lambda=60$ nm,scanning with excitation wavelength in the range of 240～360 nm to obtain the synchronous fluorescence spectra of Trp residues (1～5 in each graph respectively stand for control group,120,140,160,and 180 MPa treated group).

2.5 Statistical Analysis

Analysis of variance (ANOVA) was performed to compare the effect of pressure level at 5% confidence level. All of the determinations of papain activity were triplicated. The results were represented as mean plus or minus standard deviation.

3 Results and Analysis

3.1 The Effect of DHPM on the Papain Activity

The effect of DHPM on papain activity was studied. After DHPM treatment under 120,140,160,and 180 MPa,the relative enzyme activity of papain were 96.04%,95.27%,93.44% and 90.04% respectively,which showed that the papain activity gradually decreased with the increasing of treatment pressure. Placing papain solution of each group in refrigerator for 24 h at 0～4 ℃,the enzyme activity all decreased with higher degrees for 160 and 180 MPa (as shown in Fig. 1),which could probably be ascribed to the stronger effect of DHPM on the papain conformation stability under relative higher pressures.

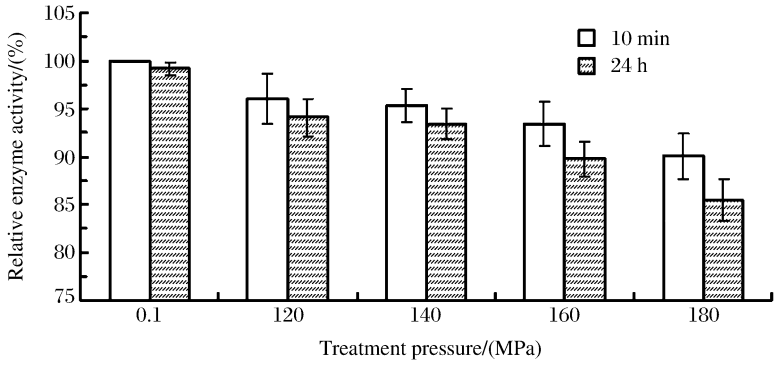


Fig. 1　Effect of DHPM treatment on the papain activity

In a certain level the result is similar to that of some HHP-related experiments. For instance, Gomes (1997) reported that papain was progressively inactivated by increasing HHP treatment pressures in both phosphate (pH 5.0 and 6.8) and Tris (pH 6.8) buffer,but in all systems the effect at pressures up to 600 MPa was minimal,and significant losses were found at 800 MPa[13]. Katsaros (2009) investigated the inactivation kinetics of papain for static ultrahigh pressure/temperature (500～900 MPa at 50～80 ℃) process conditions,and reported that at high pressures,at each process temperature,the increase of pressure increases the inactivation rate[21].

3.2 The Effect of DHPM on the Fluorescence Spectra of Papain

In protein molecules the intrinsic fluorescence of protein mainly comes from Trp and Tyr residues,which henceforth are commonly regarded as the intrinsic fluorescence probe to study the confor-

mation of protein in solution state. Papain has 19 Tyr residues and 5 Trp residues[8]. When protein is excited at 280 nm, where both Trp and Tyr residues of the protein are affected, tertiary structure of protein is reflected[22]. Fig. 2(a) and Fig. 2(b) respectively represent the changes of fluorescence spectra of papain detected within 10 min immediately after DHPM treatment and detected after 24 h storage.

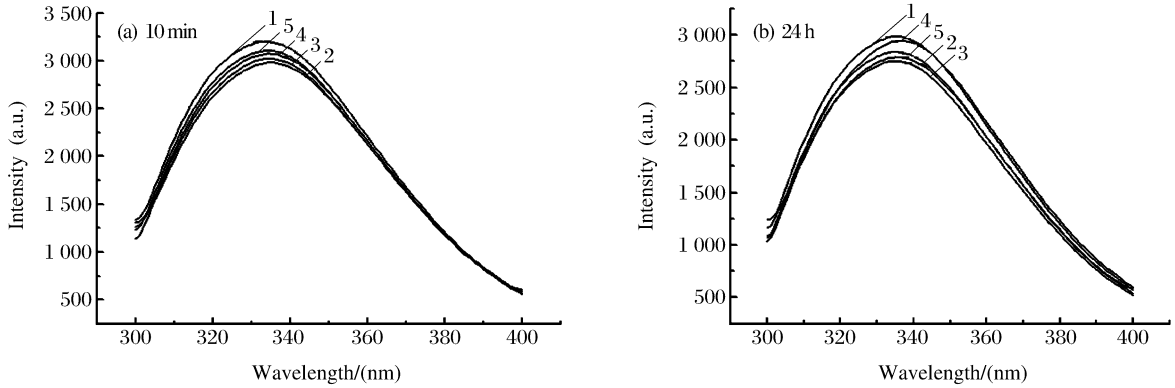


Fig. 2 Effect of DHPM treatment on the fluorescence spectra of papain

Being immediately detected after 120～180 MPa treatment, the fluorescence intensity of papain all showed decreasing in different degrees with the highest decreasing at 120 MPa. As the pressure went up, the fluorescence intensity gradually increased and the location of fluorescence emission peak gradually red shifted from 334 nm for untreated sample to 335.5 nm for 140 MPa treated sample and then returned to 334 nm for 180 MPa treated sample, as shown in Fig. 2(a). After DHPM treatment, the samples were stored for 24 h at 0～4 ℃, and the fluorescence intensity of papain fluctuated (down, up and down) with increasing pressure, as shown in Fig. 2(b). It might indicated that after DHPM treatment in pressure ranges of 120～180 MPa the structure stability of papain decreased and its molecular conformation remarkably changed with time.

3.3 The Effect of DHPM on the Fluorescence Spectra of Tyr

The fluorescence intensity of Tyr in papain all decreased after 120～180 MPa DHPM treatment, but with increasing treatment pressure the fluorescence intensity gradually increased and the fluorescence emission peak red shifted to 285.5 nm for 140 MPa treated sample and then returned to 285 nm for 180 MPa treated sample, as shown in Fig. 3(a). After 24 h placing at 0～4 ℃ in fridge, the fluorescence intensity of Tyr in papain samples of all treatment groups were still lower than that of comparison group, but with pressure going up, the fluorescence intensity of Tyr fluctuated (down, up and down), as shown in Fig. 3(b), which indicated that the influence of DHPM treatment in pressure ranges of 120～180 MPa on the conformation of Tyr residues in papain molecule were relatively strong with its conformation stability decreased.

3.4 The Effect of DHPM on the Fluorescence Spectra of Trp

After DHPM treatment in pressure ranges of 120～180 MPa, the fluorescence intensity of Trp in papain all decreased. With pressure going up, the fluorescence intensity of Trp in papain had a changing process of decreasing, increasing and decreasing again, and the location of fluorescence emission peak of Trp gradually red shifted form 277.5 nm for untreated sample to 278.5 nm for 140 MPa treated sample and then returned to 278 nm for 180 MPa treated sample, as shown in Fig. 4(a). After 24 h storage under 0～4 ℃ in fridge, the fluorescence intensity of Trp in papain solution of all treatment groups were still lower than that of comparison group and basically maintained the changing rule at

10 min, as shown in Fig. 4(b), which indicated that after DHPM treatment in pressure ranges of 120～180 MPa, the conformation of Trp residues in molecule were relatively stable with time.

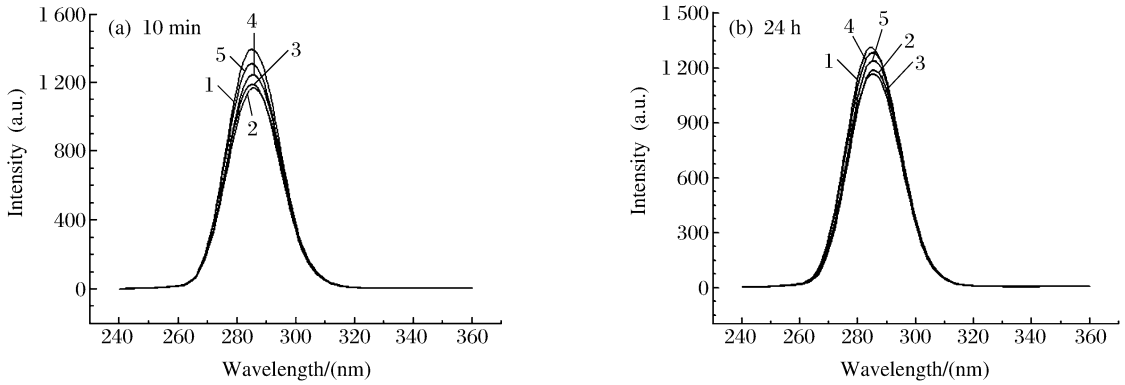


Fig. 3　Effect of DHPM treatment on the synchronous fluorescence spectra of Tyr ($\Delta\lambda$=20 nm)

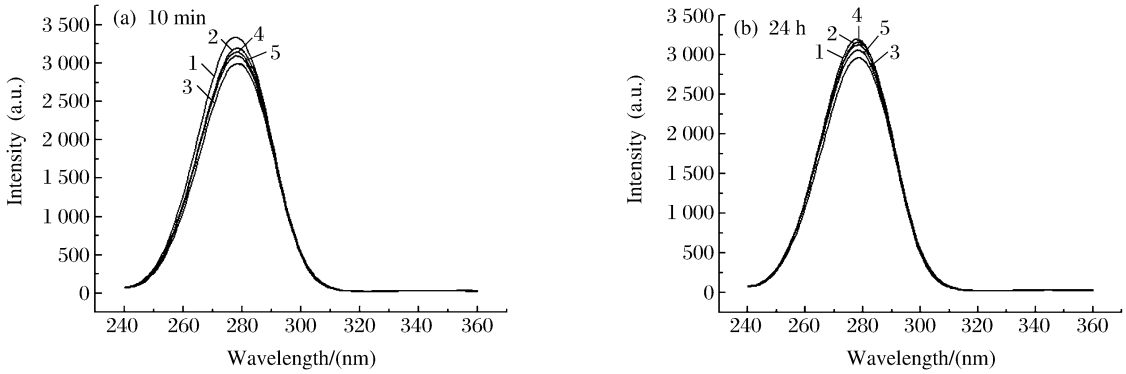


Fig. 4　Effect of DHPM treatment on the synchronous fluorescence spectra of Trp ($\Delta\lambda$=60 nm)

4　Conclusions

The effects of DHPM on the enzyme activity and conformation of papain were studied. The results showed that after DHPM treatment under 120, 140, 160, and 180 MPa, the relative enzyme activity of papain went down to 96.04%, 95.27%, 93.44% and 90.04% respectively, and the fluorescence intensity of papain, Tyr and Trp residues all decreased in different degrees. But with the pressure going up, the fluorescence intensity of papain and Tyr residue gradually increased, and the fluorescence emission peaks of papain, Tyr and Trp residues gradually red shifted from 334, 285 and 277.5 nm for untreated sample to 335.5, 285.5 and 278.5 nm for 140 MPa treated sample, and then returned to 334, 285 and 278 nm for 180 MPa treated sample, respectively. All these facts above suggested that after DHPM treatment the polarity of micro environment of Tyr and Trp residues in papain gradually increased. With high velocity impact, high-speed shearing and other intense mechanical actions of DHPM treatment, the former tight protein macromolecule unraveled into looser state or formed smaller protein fragments resulting in the exposure of Tyr and Trp residues in hydrophobic environment, meanwhile the fluorescence emission groups rapidly contacted with solvent (water) producing fluorescence quenching. However, as the treatment pressure went up, the quenching degree of papain gradually decreased and the fluorescence intensity increased. When the pressure was above 140 MPa, the loosed and unraveled molecule would probably aggregate resulting in the violet shift of fluorescence emission peak. In a certain level this is similar to the results of our research on mushroom PPO treated by

HPM[19]. After 24 h placing at 0～4 ℃, the fluorescence intensity of Trp residues maintained the changing rule at 10 min, but the fluorescence intensity of papain and Tyr residues fluctuated and the enzyme activity of papain further decreased, which indicated that the effect of DHPM in pressure ranges of 120～180 MPa on changing the molecular conformation of papain was relatively obvious with the formed new conformation having a low stability.

References:

[1] Liu W, Liu J H, Xie M Y, et al. Characterization and High Pressure Microfluidization-Induced Activation of Polyphenoloxidase from Chinese Pear (Pyrus Pyrifolia Nakai) [J]. J Agric Food Chem, 2009, 57(12): 5376-5380.

[2] Iordache M, Jelen P. High Pressure Microfluidization Treatment of Heat Denatured Whey Proteins for Improved Functionality [J]. Innovat Food Sci Emerg Tech, 2003, 4(4): 367-376.

[3] Giancarlo F, Marco F, Maria E G. Effect of High Hydrostatic Pressure and High Pressure Homogenization on the Enantioselectivity of Microbial Reductions [J]. Tetrahedron: Asymmetry, 1996, 7(10): 2879-2887.

[4] Jafari S M, He Y H, Bhandari B. Optimization of Nano-Emulsions Production by Microfluidization [J]. Eur Food Res Technol, 2007, 255(5-6): 733-741.

[5] Thompson A K, Singh H. Preparation of Liposomes from Milk Fat Globule Membrane Phospholipids Using a Microfluidizer [J]. J Dairy Sci, 2006, 89: 410-419.

[6] Liu C M, Liu W, Xiong H W, et al. Effect of Instantaneous High Pressure (IHP) Treatment on Solubility and Rheologic Properties of Soybean Dietary Fiber [A]//The 232nd ACS National Meeting [C]. San Francisco, America, 2006: 33.

[7] Smelt J P P M. Recent Advances in the Microbiology of High Pressure Processing [J]. Trends Food Sci Tech, 1998, 9(4): 152-158.

[8] Liu W, Zhang Z Q, Xie M Y, et al. Ability and Stability Change of Trypsin Treated by Dynamic High-Pressure Microfuidization [A]//International Conference on Non-Thermal Processing Technologies [C]. Beijing, 2009: 1.

[9] Wallerstein L. Prevention the Clouding of Beer on Chilling by Adding Papain: US, 995: 825 [P]. 1911-6-20.

[10] Drenth J, Jansonius J N, Koekoek R, et al. Structure of Papain [J]. Nature, 1968, 218: 929-932.

[11] Zeng Q M, Pan J, Xie H M, et al. Effect of High Pressure (HP) Treatments on Polyphenoloxidase (PPO) Activity in Pear Juices [J]. Chinese Journal of High Pressure Physics, 2004, 18(2): 144-148. (in Chinese)
曾庆梅, 潘 见, 谢慧明, 等. 超高压处理对多酚氧化酶活性的影响 [J]. 高压物理学报, 2004, 18(2): 144-148.

[12] Xie H M, Huang X D, Pan J, et al. Effect of High Pressure on Exoproteinase of Bacillus Subtilis [J]. Chinese Journal of High Pressure Physics, 2007, 21(1): 95-102. (in Chinese)
谢慧明, 黄训端, 潘 见, 等. 超高压对枯草杆菌胞外蛋白酶的影响 [J]. 高压物理学报, 2007, 21(1): 95-102.

[13] Gomes M R A, Sumner I G, Ledward D A. Effects of High Pressure on Papain Activity and Structure [J]. J Sci Food Agr, 1997, 75(1): 67-72.

[14] Huang L, Sun Y M, Pan K, et al. Influence of Ultra High Pressure on Peroxidase Pectin Methyl Esterase and Soluble Protein in Litchi Fruit [J]. Chinese Journal of High Pressure Physics, 2005, 19(2): 179-182. (in Chinese)
黄 丽, 孙远明, 潘 科, 等. 超高压处理对荔枝果肉中两种酶和可溶性蛋白的影响 [J]. 高压物理学报, 2005, 19(2): 179-182.

[15] Huang L, Sun Y M, Chen B N, et al. Influence of Combined Treatment of Ultra High Pressure and Enzyme Inhibitors on Peroxidase and Pectin Methyl Esterase in Litchi Pulp [J]. Chinese Journal of High Pressure Physics, 2007, 21(1): 89-94. (in Chinese)
黄 丽, 孙远明, 陈柏暖, 等. 超高压和酶抑制剂联合处理对荔枝果肉中过氧化物酶和果胶甲基酯酶的影响 [J]. 高压物理学报, 2007, 21(1): 89-94.

[16] Zeng Q M, Pan J, Xie H M, et al. Effect of Ultra High Pressure (UHP) Treatment on the Secondary Structure and Activity of Horseradish Peroxidase (HRP) [J]. Food Science, 2005, 26(5): 29-33. (in Chinese)

曾庆梅,潘　见,谢慧明,等.超高压处理对辣根过氧化物酶二级结构及其活力的影响[J].食品科学,2005,26(5):29-33.

[17] Liu W,Liu C M,Zhong Y J,et al. Effect of Instantaneous High Pressure Process on Polyphenoloxidase Activities [J]. Food Science,2006,27(10):179-183. (in Chinese)
刘　伟,刘成梅,钟业俊,等.瞬时高压作用对多酚氧化酶活性的影响[J].食品科学,2006,27(10):179-183.

[18] Liu W,Xie M Y,Liu J H,et al. Effect of Instantaneous High Pressure Treatment on Activity of Polyphenol Oxidase from Pears [A]//The 14th World Congress of Food Science & Technology [C]. Shanghai,2008:538.

[19] Liu W,Liu J H,Liu C M. Activation and Conformational Changes of Mushroom Polyphenoloxidase by High Pressure Microfluidization Treatment [J]. Innovat Food Sci Emerg Tech,2009,10(2):142-147.

[20] Li F Y,Xing Y J,Ding X. Immobilization of Papain on Cotton Fabric by Sol-Gel Method [J]. Enzyme Microb Technol,2007,40(7):1692-1697.

[21] Katsaros G I,Katapodis P,Taoukis P S. High Hydrostatic Pressure Inactivation Kinetics of the Plant Proteases Ficin and Papain [J]. J Food Eng,2009,91(1):42-48.

[22] Sun N,Lee S,Song K B. Effect of High-Pressure Treatment on the Molecular Properties of Mushroom Polyphenoloxidase [J]. LWT-Food Sci Techol,2002,35(4):315-318.

动态高压微射流技术对木瓜蛋白酶活性的影响

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摘要:研究了动态高压微射流技术对木瓜蛋白酶活性的影响,并以荧光光谱为检测手段对木瓜蛋白酶的分子构象进行表征。结果显示,动态高压微射流处理(120～180 MPa)后,木瓜蛋白酶酶活降低。经 180 MPa 处理 1 次,木瓜蛋白酶相对酶活降至 90.04%。随着处理压力的增加,木瓜蛋白酶分子、酪氨酸残基、色氨酸残基的荧光发射峰位置分别从对照组的 334、285、277.5 nm 红移至 140 MPa 处理组的 335.5、285.5、278.5 nm,然后回移至 180 MPa 处理组的 334、285、278 nm。在 0～4 ℃放置 24 h 后,酶活进一步降低,木瓜蛋白酶和酪氨酸残基的荧光强度出现波动(降低、上升然后再降低),表明动态高压微射流处理(120～180 MPa)改变木瓜蛋白酶分子构象的效果较为明显,形成的新构象稳定性低。

关键词:动态高压微射流;荧光光谱;木瓜蛋白酶;分子构象

中图分类号:O521.9;O433.4　　**文献标识码**:A