DOI:10.1021/jf9006642

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

# Characterization and High-Pressure Microfluidization-Induced Activation of Polyphenoloxidase from Chinese Pear (*Pyrus pyrifolia* Nakai)

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Polyphenoloxidase (PPO) from Chinese pear (*Pyrus pyrifolia* Nakai) was characterized using catechol as a substrate. PPO had a $V_{max}$ of 289.2 units/min and a $K_m$ of 3.8 mmol/L, which indicates that *P. pyrifolia* Nakai PPO has a great affinity for catechol. The catalyzing reaction velocity was proportional to the PPO concentration. The optimum pH and temperature for PPO activity were 4.5 and 45 °C, respectively. In addition, an investigation was made on the effect of high-pressure microfluidization of treatment pressure, treatment pass, and enzyme solution temperature on *P. pyrifolia* Nakai PPO. As the treatment pressure increased, the PPO relative activity was elevated from 100% untreated to 182.57% treated at 180 MPa. PPO relative activity was enhanced as the treatment pass increased. PPO solution temperature (25, 35, and 45 °C) had a significant effect on PPO relative activity when treated at 120 and 140 MPa.

**KEYWORDS: Polyphenoloxidase; *Pyrus pyrifolia* Nakai; catechol; characterization; high-pressure microfluidization; activation**

## INTRODUCTION

Chinese pear (*Pyrus pyrifolia* Nakai) is widely cultivated and accounts for a considerable majority of the fruit market in the south of China. It is well-known for its health functions, such as relieving cough, moistening the lung, eliminating phlegm, prevention and cure of gout, rheumatic arthritis, etc., and its efficacy had been recorded in the materia medica of past dynasties in Chinese history. These functions entail a good quality within not only crude pears but also various pear products.

On the other hand, polyphenoloxidases (PPOs, EC 1.14.18.1) are widely distributed in nature and possess the six histidine residues that ligate the two copper ions of the active site (*1*−*3*). These enzymes are responsible for the browning of damaged fruits and vegetables by catalyzing hydroxylation of monophenols to *o*-diphenols and dehydrogenation of *o*-diphenols to *o*-quinones in the presence of oxygen (*4*). Because the enzymatic browning causes deterioration of sensory and nutritional quality, it is necessary to characterize the PPO to develop more effective methods for controlling browning in *P. pyrifolia* Nakai.

Characterization of PPO has been widely studied in various fruits, such as strawberry (*5*), grape (*6*), banana (*7*), apple (*8*), litchi (*9*), peach (*10*), etc. Besides, PPO has also been researched in pears, such as *Pyrus communis* L. (*11*), *Pyrus elaegrifolia* (*12*), and *Pyrus serotina* Reld (*13*). However, very little research has been reported on the characterization of PPO in *P. pyrifolia* Nakai.

High-pressure microfluidization is an emerging technology, which uses the combined forces of high-velocity impact, high-frequency vibration, instantaneous pressure drop, intense shear, cavitation, and ultra-high pressures up to 200 MPa (*14*, *15*) 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 1500 MPa (*16*, *17*) and between 300 and 700 MPa in commercial systems (*18*). High-pressure microfluidization has been applied to microbial reduction (*19*), preparation of nanoemulsions (*20*), improvement of dietary fiber (*21*), etc. However, few researchers have devoted its application into fruit juice processing. This "cold" treatment technology would be a potential fruit-juice-processing candidate with no exogenous chemicals, few flavor compounds and nutritional components loss, short treatment time, and continuous operation.

In 2002, a monograph named *Ultra High Pressure Treatments of Foods* edited by Marc E. G. Hendrickx and Dietrich Knorr summarized HHP treatment on PPO related to various fruits (*22*). Under most circumstances, when subjected to HHP treatment, PPO exhibited a slight increase in activity at a relatively low pressure (around 200−400 MPa) and PPO activity then decreased at high pressure (around 700 MPa or more). For avocado PPO, the inactivation of the enzyme can be achieved at 800−900 MPa and there was a pressure resistance below 250 MPa and temperatures exceeding 62.5 °C. Our previous work found that high-pressure microfluidization could lead mushroom PPO to activation (*23*).

Previous literature reported that catechol is one of the simple phenols widely occurring in pears (*24*, *25*). In the present work, we studied partial characterization of crude PPO from *P. pyrifolia* Nakai using catechol as a substrate, and on the basis of this, we processed *P. pyrifolia* Nakai juice using high-pressure microfluidization and determined PPO activity.

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 Published on Web 05/21/2009 

## MATERIALS AND METHODS

**Materials and Reagents.** Fresh pears (*P. pyrifolia* Nakai) were obtained from the local market. They were washed with running water and then distilled water, dried in air, and then stored at 0−4 °C until use. The reaction substrate was catechol. All of the chemicals used were of analytical grade.

**Exraction of PPO from *P. pyrifolia* Nakai.** One pear was peeled and cut into small pieces. An aliquot of 50 g of prepared pear was weighed and then immediately put into a juicer (type JYZ-10, Joyoung Co., Ltd., from Hangzhou, China). Pear juice was mixed with 100 mL of 0.2 mol/L phosphate buffer (pH 6.8, 4 °C) containing 5 mmol/L ascorbic acid, 0.35 mol/L KCl, 0.5% Triton X-100, and 0.1 g/1 g of polyvininylpolyrrolidone (PVPP). Then, it was filtered through four layers of 250 mesh filter cloth and then centrifuged at 8000 rpm for 15 min at 4 °C in a refrigerated centrifuge (type Sorvall D-37520, Thermo Electron Co., from Germany). The supernatant was collected for use of characterization of *P. pyrifolia* Nakai. Pear juice for high-pressure microfluidization treatment was prepared using an aliquot of 50 kg of prepared pear (0−4 °C for 6 h) and then filtered through four layers of 250 mesh filter cloth. The prepared pear juice for the high-pressure microfluidization treatment was about 46 L. The consequent crude PPO solution was immediately stored at 0−4 °C.

**Determination of PPO Activity.** To determine PPO activity, a UV spectrophotometer (Puxi Tongyong Co., from Beijing, China) was employed throughout. One unit of enzyme activity was defined as the amount of enzyme causing a change of 0.001 in absorbance at 420 nm per minute (1 cm light path) at fixed temperature. The reaction mixture included 0.2 mL of 0.5 mol/L catechol and 2.6 mL of 0.2 mol/L phosphate buffer at pH 6.8, which were incubated at 30 °C. Then, 0.2 mL of enzyme solution was added to the reaction mixture to initiate the enzyme reaction. The blank mixture contained only the catechol and phosphate buffer. The mixed solution was allowed to stand for 5 min at 30 °C. The absorbance of the mixture at 420 nm was monitored immediately at room temperature (25 ± 1 °C) (*26*). Determination of PPO activity after high-pressure microfluidization treatment was carried out using the same method with different reaction systems, i.e., 0.2 mL of 0.02 mol/L catechol, 2.7 mL of 0.2 mol/L phosphate buffer at pH 6.8, and 0.1 mL of enzyme solution. The pre-experiments validated that the increase of absorbance was linear with time within 5 min. The specific and relative activities were calculated with the following equations: specific activity = $A_{420\,nm}\,min^{-1}$ (0.1 mL of undiluted enzyme solution)$^{-1}$; relative activity = specific activity of treated PPO/specific activity of untreated PPO.

**Enzyme Kinetics Using Catechol as a Substrate.** PPO activity was assayed using catechol as a substrate at concentrations of 0.001, 0.005, 0.01, 0.05, 0.1, 0.3, 0.5, 0.7, and 1 mol/L. The reaction system was 0.2 mL of various concentrations of catechol, 2.6 mL of 0.2 mol/L phosphate buffer at pH 6.8, and 0.2 mL of enzyme solution. To obtain the Michaelis constant ($K_m$) and maximum velocity ($V_{max}$), the kinetic data were plotted as the reciprocal of PPO activity versus the reciprocal of substrate concentration according to the method of Lineweaver and Burk (*27*).

**Effect of the Enzyme Concentration.** PPO activity was determined using catechol as a substrate at various enzyme concentrations. The reaction system was 0.2 mL of 0.5 mol/L catechol, 50, 100, 150, 200, 250, 300, 400, and 600 μL of enzyme solution, respectively, and corresponding concentrations of 0.2 mol/L phosphate buffer at pH 6.8 (the total volume of catechol, enzyme solution, and phosphate buffer was 3 mL).

**Effect of pH.** PPO activity as a function of pH was determined using catechol as a substrate. The reaction system was 0.2 mL of 0.5 mol/L catechol, 0.2 mL of enzyme solution, and 2.6 mL of 0.2 mol/L phosphate buffer at various pH values of 2, 3, 4, 4.5, 5, 6, 7, 8, 9, 10, and 11, adjusted with 0.1 mol/L citric acid and sodium carbonate.

**Effect of the Temperature.** PPO activity influenced by the temperature was determined using catechol as a substrate at different temperatures of 5, 15, 25, 35, 45, 55, 65, 75, 85, and 95 °C. The reaction system was 0.2 mL of 0.5 mol/L catechol, 0.2 mL of enzyme solution, and 2.6 mL of 0.2 mol/L phosphate buffer at pH 6.8.

**High-Pressure Microfluidization System.** Microfluidizer M-7125 (Microfluidics Co., Newton, MA) was used. Equipped with a single acting intensifier pump that amplifies the hydraulic pressure at the simple turn of a knob, the Microfluidizer M-7125 is able to drive the product stream through the interaction chamber, which uses diamond construction. Its pressure range, flow rate, and feed temperature are as follows: pressure range up to 40 000 psi, flow rate up to 3.6 gallon/min, and feed temperature maximum at 75 °C.

**Effect of the Treatment Pressure.** A total of 10 L of prepared pear juice was gently poured into the product inlet reservoir at room temperature and ambient pressure (0.1 MPa). The solution was treated under the pressures of 80, 100, 120, 140, 160, and 180 MPa one pass, respectively, and it was immediately cooled by running water at room temperature after treatment. A total of 50 mL of different samples was collected and then stored immediately at the temperature of 0−4 °C for 6 h. Untreated pear juice as a control was circulated in a high-pressure microfluidization system under the same processing conditions without high-pressure microfluidization treatment.

**Effect of the Treatment Pass.** A total of 6 L of each pear juice (18 L divided into 3 parts) was filled into the product inlet reservoir at room temperature and ambient pressure (0.1 MPa) and treated under the pressures of 120, 140, and 160 MPa for 1, 2, and 3 passes, respectively. A total of 50 mL of each sample was collected, and subsequent procedures were the same as the effect of the treatment pressure.

**Effect of the PPO Solution Temperature.** Before filled into the product inlet reservoir at room temperature and ambient pressure (0.1 MPa), 6 L of each pear juice (18 L divided into 3 parts) was incubated for 30 min with a water bath at 25, 35, and 45 °C, respectively. They were treated under the pressures of 120, 140, and 160 MPa for one pass, respectively. A total of 50 mL of each sample was pooled, and subsequent procedures were the same as the effect of the treatment pressure.

**Statistical Analysis.** Analysis of variance (ANOVA) was performed to compare the effects of treatment pressure and treatment pass at 5% confidence level. All of the determinations of PPO activity were triplicated. The results were represented as mean ± standard deviation.

## RESULTS AND DISCUSSION

**PPO Kinetics Using Catechol as a Substrate.** The Michaelis constant ($K_m$) and maximum reaction velocity ($V_{max}$) were determined using catechol as a substrate at various concentrations, and the results were shown in **Figure 1**. On the basis of the equation in the inset of **Figure 1**, $K_m$ and $V_{max}$ were 3.8 mmol/L and 289.2 units/min, respectively. The $K_m$ was considerably smaller than the values reported using catechol as a substrate on other pear species, including Bartlett pears (48 mmol/L) (*28*), *P. communis* L. CV. Bosc (22.7 mmol/L) and Red (16.2 mmol/L) (*11*), and d'Anjou pears (10 mmol/L) (*29*), and close to the value of two avocado varieties (7.3 and 2.4 mmol/L) (*30*). Besides, the $K_m$ value from *P. pyrifolia* Nakai was much smaller than that of other fruits using catechol as a substrate, such as Amsya apples (34 mmol/L) (*31*), and nearly close to clingstone peaches (4.2 mmol/L) (*32*). The diverse varieties of $K_m$ values of PPO reported may be due to different reasons: different assay methods used, different varieties, different origins of the same variety, different values of pH of extraction (*33*), and different purification grades. The relatively small value of $K_m$ found in *P. pyrifolia* Nakai demonstrated that the PPO has a considerably stronger affinity for catechol.

**Effect of the PPO Concentration.** The velocity of the PPO catalyzed reaction was proportional to the enzyme concentration (**Figure 2**). When the PPO concentration was increased from 50 to 600 μL/mL, the reaction velocity was gradually enhanced from 20.8 to 152 units/min. The result was in a similar agreement to that of PPO from other sources, such as kiwifruit (*34*) and Airen grapes (*35*).

**Effect of pH.** The effect of pH ranging from 2 to 11 on PPO activity using catechol as a substrate is shown in **Figure 3**. Starting with the lowest reaction velocity at pH 2, PPO then displayed the highest activity (105.8 units/min), with the increase of pH up to 4.5, from which the PPO reaction velocity rapidly dropped to 16.4 units/min at pH 6. Subsequently, PPO exhibited a gradual decline in activity, with a slight recovery at pH 10. Although catechol as a

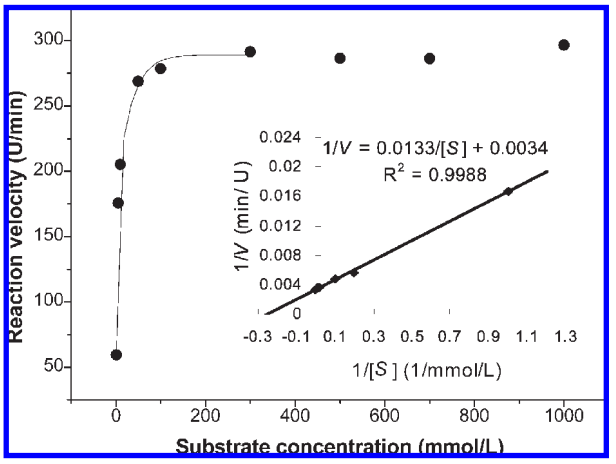


**Figure 1.** Effect of the catechol concentration on the reaction velocity of *P. pyrifolia* Nakai PPO. Reaction system: 0.2 mL of catechol at different concentrations (0.001, 0.005, 0.01, 0.05, 0.1, 0.3, 0.5, 0.7, and 1 mol/L), 2.6 mL of 0.2 mol/L phosphate buffer at pH 6.8, and 0.2 mL of enzyme solution. (Inset) Lineweaver−Burk plot for evaluating catechol. *V* and [*S*] represent the reaction velocity and catechol concentration, respectively.

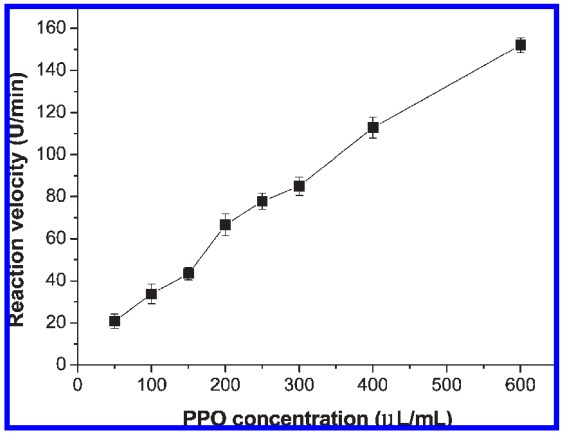


**Figure 2.** Effect of the PPO concentration on the reaction vilocity of *P. pyrifolia* Nakai PPO. Reaction system: 0.2 mL of 0.5 mol/L catechol, 50, 100, 150, 200, 250, 300, 400, and 600 $\mu$L of enzyme solution, and corresponding volumes of 0.2 mol/L phosphate buffer at pH 6.8.

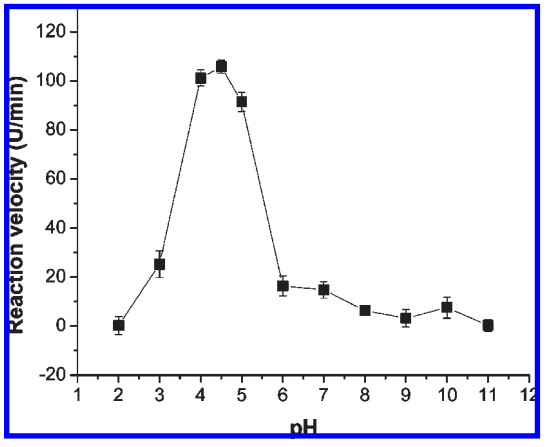


**Figure 3.** Effect of pH on the reaction vilocity of *P. pyrifolia* Nakai PPO. Reaction system: 0.2 mL of 0.5 mol/L catechol, 0.2 mL of enzyme solution, and 2.6 mL of 0.2 mol/L phosphate buffer at different pH (2, 3, 4, 4.5, 5, 6, 7, 8, 9, 10, and 11).

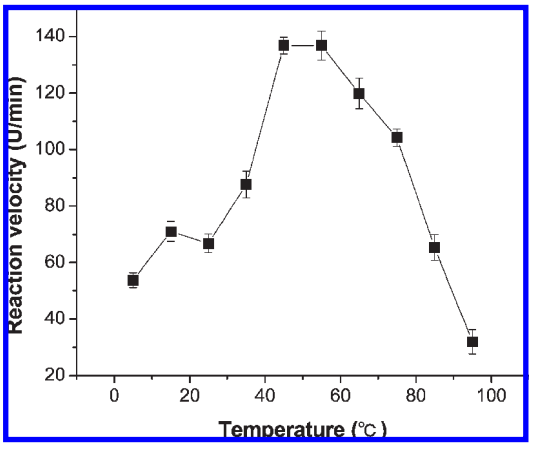


**Figure 4.** Effect of the temperature on the reaction vilocity of *P. pyrifolia* Nakai PPO. Reaction system: 0.2 mL of 0.5 mol/L catechol, 0.2 mL of enzyme solution, and 2.6 mL of 0.2 mol/L phosphate buffer at pH 6.8. The reaction velocity was determined at temperatures of 5, 15, 25, 35, 45, 55, 65, 75, 85, and 95 °C.

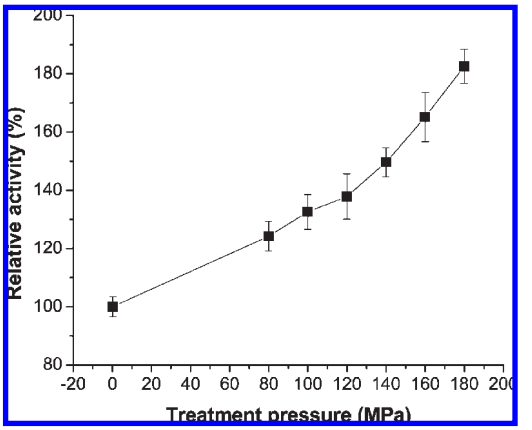


**Figure 5.** Effect of the high-pressure microfluidization treatment pressure (80, 100, 120, 140, 160, and 180 MPa for one pass, respectively) on *P. pyrifolia* Nakai PPO activity. Reaction system: 0.2 mL of 0.5 mol/L catechol, 0.2 mL of enzyme solution, and 2.6 mL of 0.2 mol/L phosphate buffer at pH 6.8.

substrate was employed, the optimum pH of PPO varies with the source of enzyme, so that a range of maximum PPO activity has been reported in the literature between pH 4.0 and 7.0 for PPO from various sources. It is reported that the optimum pH values of PPO using catechol as a substrate are 5.0 and 5.5 for *P. communis* L. CV. Bosc and Red (*11*), 6.2 for Bartlett pears (*28*), and 7.0 for d'Anjou pears (*29*) and avocados (*36*). The optimum pH for *P. pyrifolia* Nakai PPO was relatively low.

**Effect of the Temperature. Figure 4** shows the effect of the temperature on the activity of PPO using catechol as a substrate. After a slight decline in reaction velocity at 25 °C, PPO activity soared and stopped with a maximum activity at 45 °C. Then, the reaction velocity decreased gradually with increasing temperatures. Using catechol as a substrate, the optimum temperatures of PPO are 15 °C for Amasya apples (*31*), 20 and 23 °C for *P. communis* L. CV. Bosc and Red (*11*), and between 25 and 45 °C for Airen grapes (*35*). Obviously, the optimum temperature of *P. pyrifolia* Nakai PPO was higher than that previously reported. The reasons seem to be different PPO sources and different assay methods used.

**Effect of High-Pressure Microfluidization.** The effect of the treatment pressure ranging from 80 to 180 MPa on PPO is shown in **Figure 5**. With the increase of the treatment pressure, PPO exhibited an enhancement in relative activity. The higher the

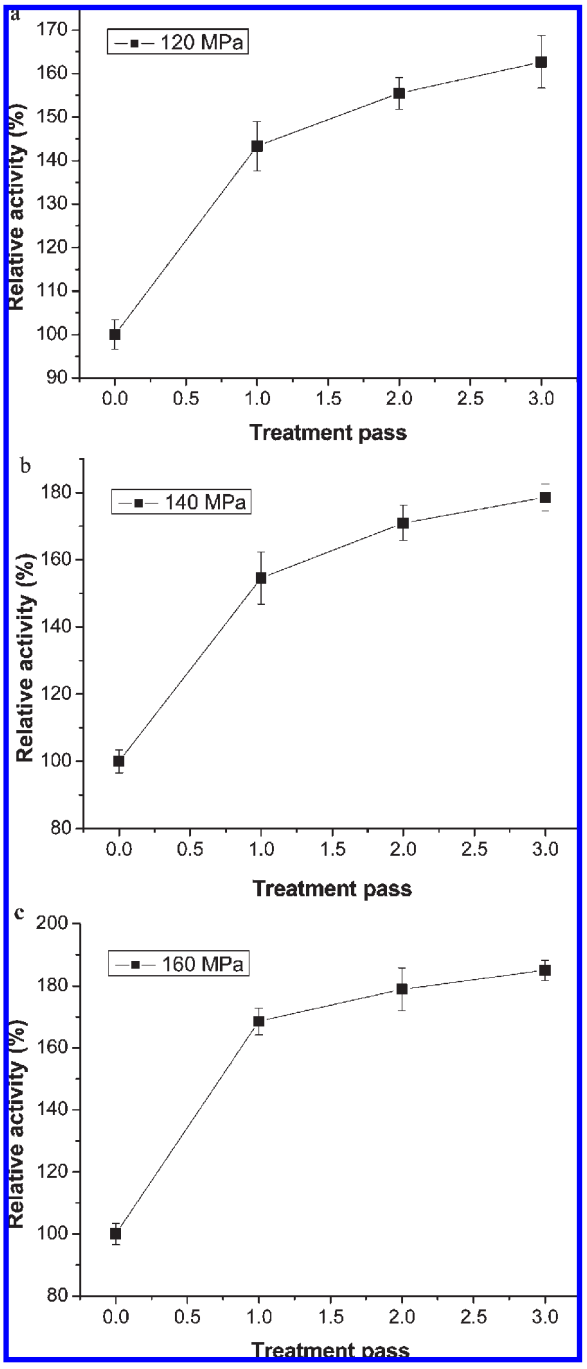


**Figure 6.** Effect of the high-pressure microfluidization treatment pass (1, 2, and 3 passes) on *P. pyrifolia* Nakai PPO activity: (a) 120 MPa, (b) 140 MPa, and (c) 160 MPa. Reaction system: 0.2 mL of 0.5 mol/L catechol, 0.2 mL of enzyme solution, and 2.6 mL of 0.2 mol/L phosphate buffer at pH 6.8.

treatment pressure, the greater the increase of PPO relative activity. PPO relative activity was enhanced from 100% untreated to 182.57% treated at 180 MPa. The treatment pressure had a significant effect on the relative activity of PPO ($p < 5\%$).

Activation of high-pressure-microfluidization-treated PPO as a function of the treatment pass at treatment pressures of 120, 140, and 160 MPa is shown in panels a−c of **Figure 6**. As the treatment pass increased, PPO exhibited an increase in relative activity at three treatment pressure levels. Obviously, PPO relative activity was enhanced by a larger extent after treatment of one pass than treatment of two or three passes. After treatment at 120, 140, and 160 MPa for one pass, the relative activity was

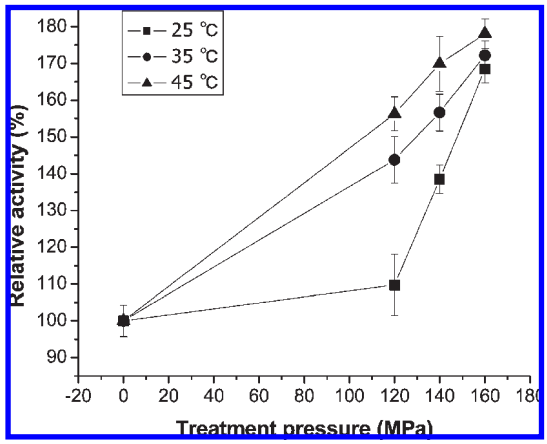


**Figure 7.** Effect of the PPO solution temperature on *P. pyrifolia* Nakai PPO activity after high-pressure microfluidization treatment (120, 140, and 160 MPa for one pass, respectively). Reaction system: 0.2 mL of 0.5 mol/L catechol, 0.2 mL of enzyme solution, and 2.6 mL of 0.2 mol/L phosphate buffer at pH 6.8.

increased by 43.35, 54.58, and 68.57%, respectively, while it was elevated by 55.46, 70.99, and 78.88% after treatment of two passes and 62.69, 78.61, and 85.01% after treatment of three passes. The treatment pass had a significant influence on the relative activity of PPO ($p < 5\%$).

**Figure 7** demonstrates the enhancement of PPO relative activity after high-pressure microfluidization treatment at different PPO solution temperatures under the pressures of 120, 140, and 160 MPa for one pass, respectively. At 120 MPa, the temperature had a more prominent effect on PPO relative activity than that treated at 140 and 160 MPa. The PPO relative activity was elevated from 109.74% at 25 °C to 156.28% at 45 °C under the pressure of 120 MPa, while it was increased from 168.43% at 25 °C to 178.05% at 45 °C under the pressure of 160 MPa. At 120 and 140 MPa, the PPO solution temperature had a significant effect on PPO relative activity ($p < 5\%$), while no significant difference could be seen at 160 MPa ($p > 5\%$).

Activation of PPO in fruits by HHP treatment at relatively low pressure (no more than 400 MPa) has been reported (*37*−*39*). PPO from different fruits possesses different pressure sensitivity. PPO from apricot, strawberry, and grape could be activated by HHP at pressures not exceeding 100, 400, and 600 MPa, respectively (*40*, *41*). On the other hand, high-pressure microfluidization is an newly developed technology with a short treatment time (less than 5 s) and low pressure (60−200 MPa). After high-pressure microfluidization treatment, the activation effects could be attributed to conformational changes on enzyme and/or substrate molecules (*42*), a possible PPO regeneration (*43*), and different kinds and numbers of isoenzymes (*44*).

Because high-pressure microfluidization induces activation in *P. pyrifolia* Nakai PPO, extraordinary attention should be paid to the consequent aggravation in enzymatic browning of *P. pyrifolia* Nakai juice. The browning was obviously obtained by visual inspection, and hence, this undesirable result should be avoided in pear juice processing. After controlling this enzymatic browning using other methods, such as blanching, high-pressure microfluidization can be employed to sterilization at higher treatment pressure and with more treatment passes (*15*). The future work will be focused on purification of *P. pyrifolia* Nakai, characterization using different substrates, and mechanism of activation induced by high-pressure microfluidization.

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**Received February 26, 2009. Revised manuscript received May 8, 2009. This study was supported financially by the National High Technology Research and Development Program of China (863 Program) (2007AA100403), the Research Program of Sate Key Laboratory of Food Science and Technology, Nanchang University (SKLF-MB-200808), and Discipline Leaders Programme of Jiangxi Province (050006).**