Extraction of peroxidase from apple peel waste mediated by tween 80 reverse micelle system

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Abstract. Plants peroxidase is an important class of oxidoreductases. It exists widely in plants, animals and microorganisms, capable of catalyzing the oxidation of H₂O₂ phenols, vitamin C, nitrite, colorless dyes, indole and inorganic ion reaction. The reverse micelles extraction operation process is simple, continuous-operation, recyclable solvent, maintaining the native conformation of the protein. And it will not produce secondary pollution to the environment so that it has been gradually applied in the extraction and purification of the enzyme.

Experimental operation of this paper was divided into the following two aspects: on the one hand, the selection of extraction technique and methods of detecting enzyme content and activity. We selected coomassie blue staining to determine enzyme content, while using ultraviolet spectrophotometry to resveratrol as a substrate for peroxidase activity assessment. On the other hand, the main aspect was how single factor affected reverse micelles extraction efficiency, including the aqueous phase pH, surfactant concentration and extraction time. By comparing difference of the recovery and purification of peroxidase between multiple different variables under same factors, we could get the optimum conditions: Tween 80 at a concentration of 0.30 mol/L, pH = 4.5, oscillation time of 20 min.

1. Introduction

Peroxidases in plants are an important class of oxidoreductases[1, 2]. It is found in a wide range of plants, animals, and microorganisms, and can catalyze the oxidation of phenolic compounds such as H₂O₂, strontium, and inorganic ions. Plant peroxidase has been gradually applied to pollution prevention and control, biomedicine, chemical detection and water treatment of phenol-containing waste water[3, 4]. Plant peroxidase is a bio-secreting macromolecule protein that has a high redox potential and a strong oxidizing ability for phenolic substances or aromatic hydrocarbons[5, 6]. Taking lignin peroxidase (Lip) as an example, it can directly oxidize methyl oxygen and non-phenolic aromatic compounds[7, 8].

Peroxidase is essentially a biological protein macromolecule[9]. The extraction and purification process follows the law of protein separation. Due to the wide variety of proteins, high activity requirements, and high propensity for decomposition, the best purification methods and conditions must be selected. Several used separation methods are mainly ultrafiltration membrane separation technology[10] and salting precipitation separation technology[11]. However, target protein molecules...
have different diameters, low molecular cuts, which are difficult to clean and expensive using ultrafiltration membrane separation technology. And salting precipitation separation technology has not significant effect. Reverse micelle technology is an extensive application in the biological field liquid-liquid extraction of plant enzyme proteins[12, 13]. Reversed micelle extraction technology has ability to maintain the natural conformation of proteins, high extraction rates. The extraction process has the following characteristic: (1) the thermodynamic properties of the extraction system are stable and the optical transparency is high; (2) the activity of the enzyme is similar to the activity of the enzyme in the aqueous solution in the reverse micelle system. Mie's equation can be used; (3) it has a wide range of extraction and can extract water-soluble polar substances and fat-soluble substances; the extraction process is simple. The continuous operation can be performed, and the solvent can be recycled.

When the reverse micelle solution is contacted with the aqueous protein solution, the protein can be solubilized in this pool. This process is known as front extraction[14]. The reversed-micelle solution containing the protein is contacted with another aqueous phase and the protein is separated by changing the conditions to transfer the protein from the reversed micelle to the aqueous phase[15]. This process is called back extraction. Due to the protection of the aqueous layer and polar head, the extent of protein inactivation is very small[16]. Non-ionic surfactants dissolve by forming hydrogen bonds with water. They do not form ions in water, which is different from both anionic and cationic surfactants. Because of this non-ionizing nature in water, it determines superiority in certain aspects: high stability, not easily affected by acid and alkali, good compatibility with other surfactants, excellent solubility and more convenience[17, 18]. Tween 80 (Sorbitan monooleate polyoxyethylene ether) has a good wetting, solubilizing, and protective colloidal effect[19]. Therefore, we chose Tween 80 as a non-ionic surfactant prepared from the reverse micelle system.

This paper studied the extraction of peroxidase from reversed micelles in order to obtain valuable application information. The experiment used Tween 80 as a non-ionic surfactant for building reverse micelle system. Plant peroxidase was used as target protein product. To obtain higher enzyme activity recovery and purification factor from apple peel extract, the effect of various key factors were studied on backward extraction process, including the extraction phase pH, Tween 80 concentration, and oscillating time were studied.

2. Materials and methods

2.1. Materials
Fresh red apple peels in the preservation period (2 days) were selected as experimental materials. Tween 80 was acquired from Tianjin Fuchen Chemical Reagent Factory (Tianjin, China). Bovine albumin and coomassie blue were supplied by Sinopharm Group Chemical Reagent Co., Ltd. Resveratrol was supplied by Sigma Company, USA. All other chemicals of AR grade were used for the experiments and analyses.

2.2. Preparation of crude extract
The mixed solution was prepared by taking 5 g of juice of apple peel, adding 0.05 mol/L, pH=6.0 phosphate buffer solution (about 18 mL). The filtrate was stirred for 20 minutes and centrifuged. And then the filtrate was centrifuged at 6000 r/min for 15 minutes to obtain the crude enzyme extraction solution.

2.3. Reversed micelle extraction of peroxidase
A certain amount of Tween 80 surfactant was weighed into a PP tube, and an equal volume of isooctane was mixed thoroughly. The mixed solution was subjected to ultrasonic oscillating to form a stable homogenous transparent reversed micelle system. 5 mL reverse micelle solution and 5 mL crude enzyme solution were mixed, which was shaken for a certain period of time to fully mix them. The mixture was centrifuged at 1000 r/min for 10 min. And then the organic phase was retained after
phase separation of mixed solution. The organic phase after liquid separation is the peroxidase-encapsulated reverse micelle solution. The pre-extraction process has been completed. Then, the organic phase was mixed with an equal volume of citric acid buffer solution, shaken for 30 minutes, centrifuged at 5000 r/min for 10 minutes. The aqueous phase is retained after phase separation of mixed solution, which is a peroxidase-enveloping solution. This completes the back extraction process. The reversed micelle extracted solution was stored at room temperature in the dark.

2.4. Peroxidase activity and Content
The activity of protein was determined by ultraviolet spectrophotometry. Resveratrol was used as an enzyme substrate to produce verataldehyde by the oxidation of the enzyme. The UV absorbance method was used to determine the change in the absorbance of the product verataldehyde at 310 nm that was catalyzed. The amount of enzyme required for the oxidation of resveratrol to produce 1 umol verataldehyde per minute was defined as an enzyme activity unit (U). The activity of protein in the aqueous phase was determined by ultraviolet spectrophotometry, and the following steps were performed: 0.6 mL 10 mmol/L resveratrol, 1.8 mL 250 mmol/L tartrate buffer (pH=3.0) and 0.6 mL distilled water were added to the cuvette. The light absorption value was measured at a wavelength of 310 nm, and the light absorption value A310nm was recorded as a blank group; 0.6 mL 10 mmol/L resveratrol solution, 1.8 mL 250 mmol/L tartrate buffer (pH=3.0) and 0.6 mL test enzyme solution were mixed to another cuvette. The oxidation reaction of resveratrol was activated by adding 0.03 mL 40 mmol/L H2O2 (reaction at 25 °C for 3 min), and measured absorbance at 310 nm wavelength A310 nm. The obtained average absorbance difference \( \Delta A_{310} \) was used in the calculation formula of POD vitality unit:

\[
POD \text{ vitality unit } (\text{umol L}^{-1}) = \frac{\Delta A_{310} \text{ nm} \cdot t \cdot V}{\varepsilon \cdot L} \]

- \( V \): The volume of the reaction solution (mL), the volume of the reaction solution in this experiment was 3 mL;
- \( \varepsilon \): The molar extinction coefficient of the product verataldehyde was 9.3 mL/umol·cm;
- \( L \): Cuvette light path (1cm);

The peroxidase content was determined using the staining properties of the Coomassie brilliant blue stain in this paper[20]. Preparation of Coomassie brilliant blue solution: weigh 0.1 mg Coomassie brilliant blue G-250, dissolved 50 mL 90% ethanol, added 100 mL 85% phosphoric acid, distilled water to 1 L, stored in a brown bottle. The solution was mixed by 1 mL extracted enzyme solution and 5 mL Coomassie Brilliant Blue solution. The absorbance of mixed solution was measured at 595 nm wavelength with an UV spectrophotometer to obtain the protein content value by standard curve. The protein content of the crude enzyme solution and the post-extraction solution were measured, and the enzyme activity recovery rate (AR) and the purification factor (PF) were calculated. The activity recovery (AR) and the purification factor (PF) were estimated as shown below:

\[
\text{Activity Recovery (\%)} = \frac{\text{Peroxidase activity in aqueous phase after back extraction}}{\text{Peroxidase activity in the feed}} \times 100
\]

\[
\text{Purification Factor (\%)} = \frac{\text{Specific activity in aqueous phase after back extraction}}{\text{Specific activity in the feed}} \times 100
\]

3. Results and discussion

3.1. The standard linear equation
A standard bovine albumin standard curve was plotted. The abscissa was the concentration of the protein solution. And the ordinate was the absorbance of the solution. As shown in Figure 1, the standard curve showed a good linear relationship with a linear equation of \( y = 0.4815x + 0.0753 \) and a linear correlation coefficient of \( R^2 = 0.98181 \).




3.2 Determination of Tween 80 CMC in Isooctane
The critical micelle concentration of Tween 80 was determined by the principle of UV absorption spectrophotometry. The UV absorption wavelength $\lambda_{\text{max}}$ at different concentration was measured. The $\lambda_{\text{max}}$~c was plotted. The concentration at the curve inflection point was the surfactant CMC value. By measuring the maximum absorption wavelength of the solution at different concentrations of surfactant concentration, the graph corresponding to $\lambda_{\text{max}}$~c could be obtained.

As shown in Figure 2a, the curve had a turning point when the Tween 80 surfactant concentration was 0.01 mol/L. The photometric probe was maximally solubilized in the core of the micelles, resulting in a drastic decrease in the photometric absorption and reflected as a vertex on the curve [21]. Therefore, the UV-Vis spectrophotometric determination of the critical micelle concentration of Tween 80 in isooctane solution was 0.01 mol/L.

3.3 Effect of Aqueous Phase pH
Electrostatic attraction is one of the forces between micelles and proteins during the extraction of reverse micelles[22, 23]. The pH value affected the dissociation degree of the charged groups of the surfactant and the enzyme protein. Thus, it affected the extraction efficiency. The effect of peroxidase extraction was studied on the reversed micellar system by adjusting the pH (3.0, 3.5, 4.0, 4.5 and 5.0) of the aqueous phase. The experimental results were shown in Figure 2b.

The experimental results showed that the enzyme recovery rate and the purification factor of peroxidase had an upward trend when the pH=3.0-4.5. The values of the enzyme recovery rate and the purification factor reached the maximum When the pH=4.5. When the pH value continued to increase, both showed a downward trend, and the recovery rate of the enzyme decreased more than the purification factor. Studies had shown that the driving force of reverse micelle extraction process was mainly electrostatic interaction[24]. The same charge groups repelled each other, and the different charge groups attracted each other. The value of pH in the aqueous phase could change the surface
charge distribution of the protein. Thus, the pH value affected the extraction effect of reverse micelle system. Tween 80 was a non-ionic surfactant. The reverse micellar system with isoctane showed a negative state while the enzyme protein molecule shows a positive state[25, 26]. When the pH of the aqueous phase was less than 4.5, the degree of binding of H+ to the reverse micelle group in the aqueous phase was greater than the degree of binding of the enzyme protein to the reverse micelle group, resulting in a low extraction rate; When the pH value of the aqueous phase was equal to 4.5, the interaction between the enzyme protein and the reverse micelle group were the strongest. The extraction effect was significant. Therefore, the pH of the aqueous phase most suitable for the extraction of peroxidase was about 4.5 under Tween 80 reverse micelle system.

3.4 Effect of Tween 80 Concentration

The influence of the surfactant on the extraction effect determines the electrostatic attraction between the reversed micelle and the enzyme protein in the unit solution[25, 27]. When the reverse micelle concentration was too lower, the force between the reversed micelle and the enzyme protein was insufficient to complete the extraction process. If the concentration of reverse micelles was too large, the resistance of the protein to the movement of the reversed micelles will increase, which will affect the extraction effect. This section discussed the effect of different concentrations of Tween 80/iso-octane surfactant solution (0.1, 0.2, 0.3 and 0.4 mol·L⁻¹) on extracting peroxidase. The experimental results were shown in Figure 3a.

![Figure 3a](image)

Figure 3. (a) The curve of AR and PF with different concentrations of Tween 80. (b) The curve of AR and PF with different shock time.

As shown in Figure 3a, when the Tween 80 concentration was lower 0.30 mol/L, the enzyme AR and the PF could increase in proportion to the concentration. The increase concentration of the surfactant in the solution increased the polarity of the organic phase[28]. This could increase the chance of protein entering the interior of the reversed micelles. When the Tween 80 concentration reached 30 times the CMC (0.30 mol/L), both the enzyme AR and the PF reached the maximum. When the concentration of Tween 80 continued to increase, the enzyme AR and PF showed a sharp decline. The increase in the density of surfactant in a unit volume of solution increased the internal resistance of the protein into the reversed micelle. The reversed micelles were deformed. The interior of the reverse micelles became a “water pool” due to the large number of reversed micelles. Therefore, the optimal surfactant concentration for the extraction of peroxidase was approximately 0.30 mol/L in the Tween 80 reverse micelle system.

3.5 Effect of oscillating time

The reverse micelle extraction process included the following processes: the enzyme protein was transferred into the reverse micelle; the reverse micelle encapsulating the enzyme protein was ruptured to release the enzyme protein; and the enzyme protein was diffused into the water and the like[29, 30]. The oscillating time had a certain degree of influence on the extraction effect. This section explored the effect of extraction of peroxidase by Tween 80/iso-octane surfactant solution on the oscillating time (10, 20, 30, 40, and 50 min). Figure 3b showed that the best oscillating time was 20 min. At this time, the enzyme AR was about 20%. However, with the increase of oscillating time, the recovery rate
of enzyme and the purification factor both showed a decreasing trend, and the extraction effect was not good.

Studies had shown that the solution was a non-Newtonian fluid during the shock process [31]. The shear force occurring during the oscillation was large and the conformation of the enzyme changes to varying degrees. Therefore, the prolongation of the shaking time will destroy the conformation of the enzyme and decrease the enzyme activity. In addition, violent shocks increased the number of non-target products in the solution, causing irreversible changes, resulting in reduced enzyme recovery [32]. Therefore, the most suitable oscillation time for the extraction of peroxidase in Tween 80 reverse micelle system was about 20 min.

4. Conclusions and Prospects
In this paper, the purposes were to study the process of extracting peroxidase from plants under Tween 80 reverse micelle system, and to explore the effects of single variable such as pH of aqueous phase, concentration of Tween 80, and oscillating time on extraction efficiency. By comparing the difference between the peroxidase recovery rate and the purification factor for the same factor and different variables, the best conditions were obtained. The results were as follows: (1) The optimum pH value was 4.5; (2) the critical micelle concentration of Tween 80 was 30 times (about 0.30 mol/L); (3) the best oscillating time for the peroxidase extraction of Tween 80 reverse micelle system was 20 min.

Even though this method has an excellent performance on extraction process, more study still need to be done to improve our experiments, such as the mechanism of action of the surfactant. Therefore, future research directions mainly focus on the following aspects: (1) the surfactant at the liquid-liquid interface and its mechanism of action with enzyme protein molecules should study; (2) the influence of factors, such as temperature, surfactant type, and solution ion concentration, should be influenced on extraction efficiency; (3) The surfactant complex system has a significant effect on the extraction of enzyme proteins. Therefore, the combination of Tween 80 and other anionic and cationic surfactants will be a feasible way to improve the extraction efficiency of plant peroxidase.

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