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Purification of Peroxidase from Chinese Cabbage Roots by the Reverse Micelle System

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Abstract The basic and optimum conditions for the extraction of peroxidase from Chinese cabbage root applying the reverse micelle system were investigated. In order to purify Peroxidase (POX) from crude extract of Chinese cabbage roots, isooctane containing 110 mM Aerosol OT (AOT) was well mixed with the same volume of crude extracts containing 50 mM NaCl and 30 mM Tris-HCl buffer of pH 8.0. After centrifugation, AOT reverse micelle containing the isooctane phase were mixed with 80 mM Tris-HCl buffer at pH 7.0 containing 1 M KCl. From these operations, POX was purified 20-fold with a 60% yield. For further purification, DEAE-Toyopearl column chromatography was applied, and it showed a single protein band on SDS-polyacrylamide gel electrophoresis. The resulting POX showed 93-fold purification with a 40% yield.

Key words: Purification, peroxidase, Chinese cabbage, reverse micelle

Peroxidase (hydrogen peroxide oxidoreductase, EC 1.11.1.7) (POX) is a ubiquitous enzyme that is widely present in animal, plant, and microorganism kingdoms. It can be used as reagents for chemical diagnosis, synthesis of organic substances, and removal of toxic aromatic compounds such as phenol from waste water [1, 8, 9, 10, 11]. The major source of commercially available POXs for the above applications is horseradish roots [19]. So far, many trials have been made for characterizing the catalytic feature of POX mostly from microorganisms in order to improve industrial applicability [2, 5, 14, 20, 21]. However, it is not easy to secure horseradish as a raw material in Korea, and it requires the high cost of cultivation in the case of the microorganism. Through a series of studies for the search of an alternative source of POX and for developing a domestic manufacturing process of POXs, we discovered that a substantial amount of POX was distributed in Chinese cabbage roots [16, 17]. It was found that the industrial application of POX would be very feasible because POX from Chinese cabbage roots showed good stability in a wide pH range and also had relatively good thermal stability [8, 16, 17].

The liquid-liquid extraction method has been used extensively in the antibiotics industry, but it has found only limited application in other sectors of biotechnology. This methodology has been found to be attractive and several methods based on liquid-liquid extraction have been investigated by a number of research groups [13, 18]. Recently, a process using AOT reverse micelle has been widely investigated. A reverse micelle is composed of amphiphilic molecules in organic solvent with a nanometer scale droplet which has the ability to solubilize hydrophilic macromolecules, such as proteins, in an organic environment without denaturation. As different proteins possess different propensities to be solubilized in the micellar phase, it is promising that this would allow the separation of proteins by selective solubilization in the micellar phase [3, 4, 6, 7, 13].

Chinese cabbage root has great economic potential and feasibility as a POX source since the production cost for Chinese cabbage root is almost negligible. The objective of this work is to establish optimum conditions for the mass production of POX from Chinese cabbage root by using the reverse micelle system.

MATERIALS AND METHODS

Reagents

Aerosol-OT (AOT) and 2,2'-azino-bis(-3-ethylbenzenethiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma

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Chemicals (St. Louis, MO, USA). DEAE-Toyopearl was purchased from Toyo Soda (Tokyo, Japan). All other chemicals used were reagent grade.

Preparation of Chinese Cabbage Root

Chinese cabbage roots cultivated for about 70 days in the summer of 1996 in Kangwon-Do, Korea, were used as enzyme sources. After harvesting, Chinese cabbage roots were washed with tap water, cut into ca. 0.5 cm pieces, and crushed with a juicer (Altran, Green Power Co., Korea) after well-mixing. The crude extract obtained was stored at -20°C.

Peroxidase Activity

The peroxidase activity was assayed at 37°C by using ABTS as a substrate. The reaction mixture contained 0.3 ml of 20 mM ABTS, 0.2 ml of 1 M Tris-HCl buffer (pH 7.0), 0.8 ml of distilled water, and 0.4 ml of sample. The reaction was initiated by the addition of 0.3 ml of 20 mM hydrogen peroxide. After 5 min, the reaction was terminated by adding 2.0 ml of absolute ethanol and absorbance was measured at 407 nm. One unit of peroxidase activity is defined as the amount of enzyme which catalyzes the formation of one μmole of oxidized ABTS per min.

The Forward Transfer System

The forward transfer experiments were performed by mixing 5 ml extract at a pH range between 3.0 and 10.0, which was adjusted to the ionic strength of 50 mM by various salts, with an equal volume of 110 mM AOT dissolved in isooctane solution. Mixing was made by agitation with a magnetic stirring bar in a 100 ml Erlenmeyer flask for 10 min at 25°C. Then, the final dispersion was centrifuged at 10,000 g, at 25°C for 20 min to obtain a distinct phase boundary. Both phases were separated, dialyzed at 4°C overnight and assayed for POX activity.

The Backward Transfer System

For the backward transfer experiments, 5 ml of the upper phase from the forward transfer experiments was taken and mixed with an equal volume of a new aqueous phase of the desired pH and ionic strength. Backward transfer was accomplished by the same methods of centrifugation as mentioned above, and both phases were separated and dialyzed at 4°C overnight against the 10 mM Tris-HCl buffer (pH 8.0). The dialyzed enzyme solution was loaded on a DEAE-Toyopearl column (2×14 cm) preequilibrated with the same buffer. The adsorbed proteins were eluted with a linear gradient of 0 to 0.5 M NaCl in the same buffer, and each fraction was assayed for POX activity.

Protein Assay

Protein concentrations were routinely assessed by the spectrophotometric assay of Lowry *et al.* [15] using bovine serum albumin as a standard.

Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out using the methods of Laemmli *et al.* [12]. Gels were stained with Coomassie brilliant blue R-250.

Results and Discussion

In order to determine the optimum operation condition, we investigated the effects of AOT concentration, the pH of the aqueous phase, ionic strength and type of salt on the transfer efficiency of POX during forward and backward transfers.

Effect of pH on Reverse Micelles of POX

POX was transferred from the aqueous phase (30 mM buffer) to the organic phase (80 mM buffer) by varying the pH (pH 3 to 5, Citrate; pH 5 to 7, Potassium Phosphate; pH 6 to 8, Tris-HCl; pH 8 to 10, Glycine-NaOH). Figure 1 shows the effect of the pH on the extraction efficiency of POX in the aqueous and the oil phase. It showed a maximum value of 93% transfer efficiency at 30 mM

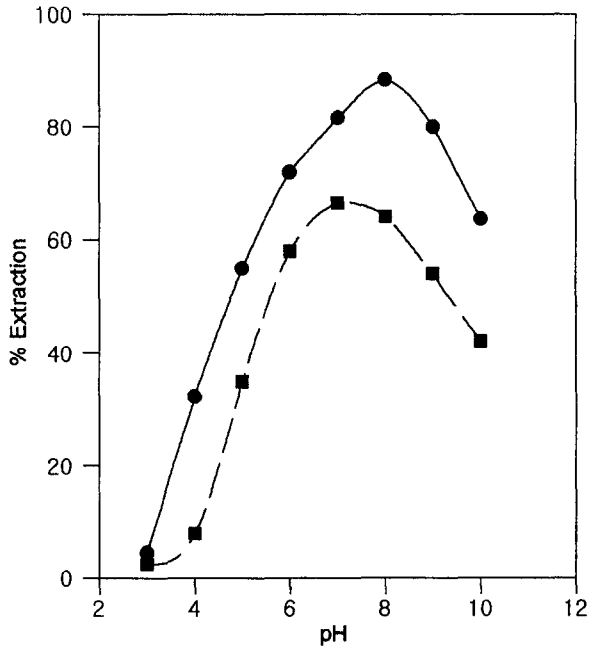

Fig. 1. pH dependence on forward transfer and backward transfer of peroxidase.

Forward transfer (●): 30 mM buffer systems, containing 50 mM NaCl. Backward transfer (■): 80 mM buffer systems, containing 1 M KCl. *Buffer system: pH 3 to 5, Citrate; pH 6 to 7, Potassium phosphate; pH 6 to 8, Tris-HCl; pH 9 to 10, Glycine-NaOH.

Tris-HCl buffer (pH 8.0) in the case of forward transfer. For backward transfer, the maximum POX extraction efficiency was 68% at 80 mM Tris-HCl buffer (pH 7.0). From the above results, POXs from Chinese cabbage root have several different isoenzymes, with isoelectric points covering a wide pH range. The chemical nature of the buffer seemed to be very important in obtaining a high POX extraction efficiency, because different buffer systems produced different peak heights even at pH 8.0 (Glycine-NaOH buffer) in forward transfer and at pH 7.0 (potassium phosphate buffer) in backward transfer. This may be due to physicochemical interactions among the proteins, buffer components, and surfactant molecules at the reverse micellar wall, resulting in incomplete protein release.

Effect of Ionic Strength in Forward Transfer

The effects of ionic strength in forward transfer were investigated. Figure 2 shows the effects of ionic strength for the forward transfer with 30 mM Tris-HCl (pH 8.0). As a matter of convenience, the ionic strength was varied from 0 to 600 mM NaCl, while other ions remain constant. As shown in Fig. 2, NaCl concentrations between 50 and 100 mM were most suitable for the aqueous phase. However, the transfer efficiency of POX decreased rapidly beyond 150 mM of NaCl. This is probably because the concentration up to 150 mM NaCl is less electrostatically favorable such that the size of the reverse micelles is no longer enough to host the POX. Therefore it would be desirable to use 50 mM of NaCl in order to obtain the optimum ionic strength in forward transfer.

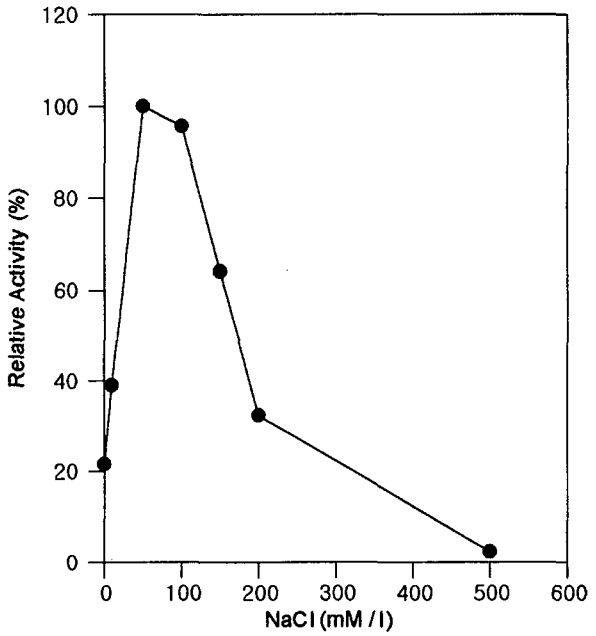

Fig. 2. Effect of ionic strength change in forward transfer.
System: 30 mM Tris-HCl (pH 8.0) containing various concentrations of NaCl.

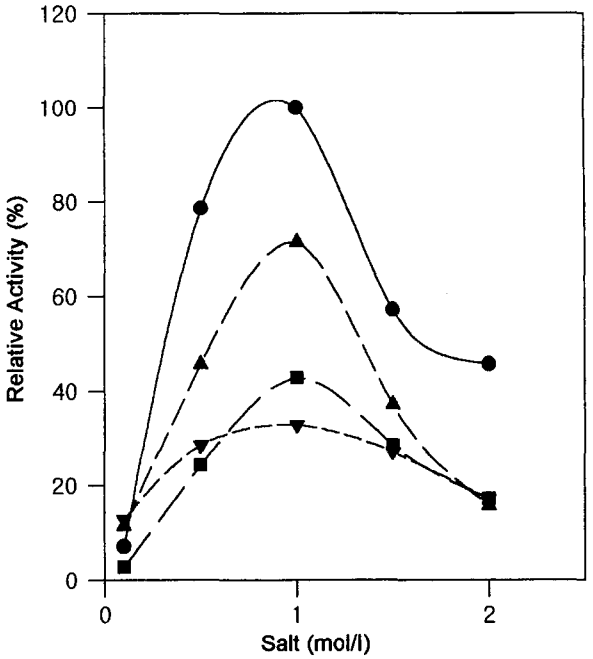

Fig. 3. Effect of ionic strength change in backward transfer.
System: 80 mM Tris-HCl (pH 7.0) containing various concentrations of salts. Symbol: ●, NaCl; ■, KCl; ▲, $CaCl_2$; ▼, $MgCl_2$.

Effect of Ionic Strength in Backward Transfer

To transfer POX from the AOT phase into another aqueous phase, the composition of the forward transfer solution was kept constant in 30 mM Tris-HCl buffer (pH 8.0) containing 50 mM NaCl and 100 mM AOT. Figure 3 represents the effects of different types of salts and ionic strengths on the desolubilization of POX from the AOT phase to the aqueous phase. The backward transfer solution was another aqueous phase containing 30 mM Tris-HCl buffer (pH 7.0) and various salts. From this result, 1.0 M of NaCl solution was optimum for desolubilizing POX. However, in the cases of KCl and $MgCl_2$, both phases became turbid when aqueous phase mixed with AOT phase. And particularly, in the case of $MgCl_2$, Mg^{2+} may have interacted with AOT, which resulted in the occurence of soluble precipitates. In both cases, it is speculated that the salt and surfactant might have caused aggregation of POXs which resulted in the denaturation of proteins. Although POX transfer was nearly constant for up to 1 M NaCl, the curve shown in Fig. 3 indicated that there was a decreasing trend in backward POX transfer. Hydrophobic interactions between the POX and the surfactant layer of the reverse micelle with increasing ionic strength could be also involved. Thus, POX transfer during backward transfer monotonously decreases with increasing ionic strength.

Effect of AOT Concentration in Forward Transfer

The effects of surfactant concentrations in forward transfer were investigated. The forward transfer solution contained

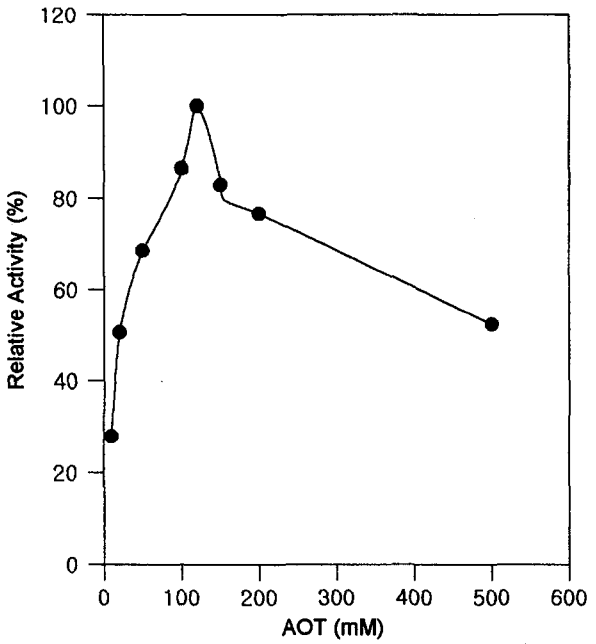

Fig. 4. Effect of AOT surfactant concentration on forward transfer.

System: 30 mM Tris-HCl (pH 8.0) containing various concentrations of AOT surfactant.

30 mM Tris-HCl buffer (pH 7.0), 50 mM NaCl, crude extract, and various concentrations of AOT. As shown in Fig. 4, transfer efficiency increased as AOT concentration increased up to 110 mM and then decreased abruptly as AOT concentration increased further, which shows that an AOT concentration of 110 mM would be optimum for efficient transfer.

In summary, the following optimal condition was established for the purification of POX from Chinese cabbage roots by using the AOT-reverse micelles system. In the forward transfer system, it is recommended that an aqueous phase composed of 30 mM Tris-HCl buffer (pH 8.0) and 50 mM NaCl should be mixed with 110 mM AOT dissolved in isooctane. The optimum aqueous phase for backward transfer should contain 80 mM Tris-HCl buffer (pH 7.0) and 1 M NaCl. Moreover, among the various phase ratios tried in forward and backward transfers, the 1:1 ratio showed the highest average efficiency (data not shown).

Large Scale Purification of POX Using the Optimal Condition of the Reverse Micelle System

Based on the above results, large scale purification of POX was tried. Five kg of roots were crushed with a juicer, producing 3100 ml of crude extract. Forward transfer was performed by mixing a 500 ml sample of crude extracts including 250 ml distilled water, 50 ml of 1 M NaCl, and 200 ml of 1 M Tris-HCl (pH 8.0) with 1000 ml of 110 mM AOT dissolved in isooctane. Mixing was done by agitating at 750 rpm with a magnetic bar for 30 min. Then the resulting dispersion was centrifuged at 10,000 g for 60 minutes to obtain a distinct phase boundary. Backward transfer was accomplished by mixing 960 ml of upper aqueous phase with 960 ml of 80 mM Tris-HCl (pH 7.0) containing an additional 1 M KCl. Mixing and centrifugation were performed in the same way as in the forward transfer experiments. After centrifugation, 950 ml of enzyme solution was obtained from the new aqueous solution.

Production of Highly Purified POX by Chromatography Methods

Table 1 shows the result of POX purification from Chinese cabbage roots by the AOT reverse micelle system. POX was purified 20-fold with a 60% yield from the AOT reverse micelle system, showing that this method would be an efficient separation technique. However, in order to increase the extent of purification, partially purified POX from the AOT reverse micelle system was chromatographed on an ion exchange column. The partially purified POX solution (100 ml) was dialyzed overnight and then chromatographed on the DEAE-Toyopearl column (2× 14 cm) in 10 mM Tris-HCl buffer (pH 8.0) at 4°C, and was eluted with a 100 ml-linear gradient from 0 to 0.5 M NaCl. As shown in Table 1, the POX was purified 93-fold with a 40% yield from the purification using both the AOT reverse micelle system and the chromatography method.

Every sample of each purification step was applied to SDS-PAGE and the results are shown in Fig. 5. Lane B was the crude extract. Lane D was purified POX using reverse micellar system. Lane C was the purified POX using DEAE-Toyopearl column chromatography which showed the highly purified form.

From these above results, it can be concluded that the new process combining both the AOT reverse micelle system and chromatography would be a suitable purification method for selective extraction of POX from Chinese cabbage roots within 24 hours. Chinese cabbage is one of the most cultivated vegetables in Korea with a total production rate of about 4 million tons per year. Its root

Table 1. Purification of peroxidase from Chinese cabbage root using the AOT reverse micelle system.

Step	Protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Yield (%)	Purification folds
Crude extracts	1300	2236	1.72	100	1
AOT reverse micelle	40	1349	33.7	60.3	19.6
DEAE-Toyopearl	5.5	894	160	40	93

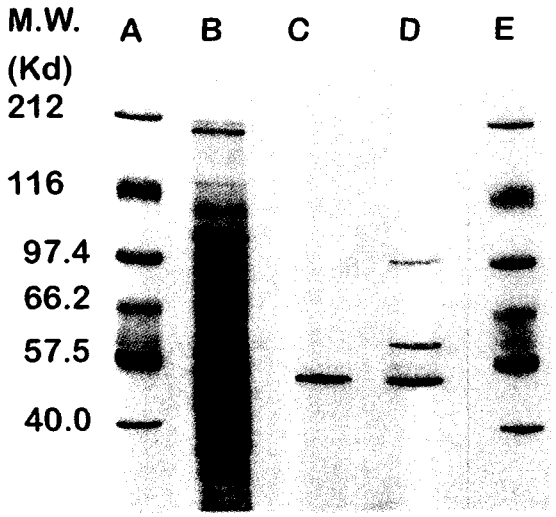

Fig. 5. SDS-Polyacrylamide gel electrophoresis of each purification step.

Lanes A, E, marker proteins; lane B, crude extract; lane C, DEAE-Toyopearl column chromatography; lane D, backward transfer.

has great economic potential and feasibility as a POX source since its production cost is almost negligible.

In this study, a possible practical process for the mass production of POX was developed and systematized so that highly valuable products like POX can be produced from agricultural wastes such as Chinese cabbage roots. The separation technique established in this study would be efficient and allow for continuous operation and easy scale-up.

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