

Partial Purification of Mulberry (*Morus rotundifolia*) Peroxidase Using Aqueous Two-Phase Extraction Coupling with Ion-exchange and Gel-filtration Chromatography

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ABSTRACT

The third to fifth leaves of mulberry plant were selected for peroxidase extraction due to its high specific activity comparing to other sets of leaf. The initial enzyme isolation included homogenization and extraction of phenolic compound using aqueous two-phase extraction system consisting of 20/8.9 % (w/v) PEG/(NH)₂SO₄. This system gave a satisfactorily less partition coefficient (0.018) and also less peroxidase volume (34% of total volume). The purification was performed on a DEAE-Cellulose column and double chromatography of Sephadex G-75. The overall result of mulberry peroxidase purification gave 157.4-folds with 43.4% recovery. The native molecular weight of mulberry calculated from the relative fraction of the standard curve from gel filtration was found to be 29 kDa as which agreed with one band of the molecular weight identified by SDS-PAGE.

Key words: aqueous two-phase, mulberry leaf peroxidase, purification

INTRODUCTION

Peroxidase (E.C.1.11.1.7) is a group of haemoprotein whose main function is to oxidize various electron donor substrates at the expense of H₂O₂. Peroxidase has often served as a parameter to indicate the metabolic activity during growth alteration. It is also one of the key enzymes controlling plant differentiation and development. This enzyme plays a role in the construction and rigidification of cell wall by contributing to the formation of lignin and cross-links between cell wall protein, as well as protecting it from damage and infection by pathogen. It is also an indicator of plant stress (Seigel, 1993).

Peroxidase has been isolated and purified from a number of organisms including fungi, bacteria and higher plants. Horseradish (*Amaracia rusticana*) root tubers are commonly employed for commercial peroxidase production (Wilinder, 1992). However, peroxidase from different origins especially of locally grown plants is being sought after. Although palm and some tropical plants seem to be a good primary source of peroxidase (Sakharov *et al.*, 2001), the leaves of mulberry, *Morus sp.*, is another potential source which has not been purified for peroxidase before. Mulberry plants are commonly found all over the country and readily available to use.

As for enzyme purification, aqueous two-

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phase extraction (ATPE) is an interesting method to be used. This system provides a gentle environment for biomolecule and offers the advantages of high activity yield, high capacity and easy to scale up. The conventional initial purification step using ammonium precipitation is laborious and time-consuming. ATPE uses less time and low energy and is known to give biocompatible environment to enzyme because a lot of water in extraction system is involved. Besides, the chemical materials used in this ATPE system are inexpensive while the use of an aqueous polymer and a lyotropic salt as ammonium sulfate is preferred on an industrial scale. This was the first attempt to use an aqueous two-phase extraction for partial purification of peroxidase from mulberry leaves and subjected the enzyme to DEAE-Cellulose and double gel filtration chromatography.

MATERIALS AND METHODS

Mulberry leaves selection

The shoots, third to fifth, sixth to tenth and eleventh to fifteenth leaves of mulberry plant (*Morus rotundifolia* Koidz.) from Udon Thani province grown at the Department of Genetics, Faculty of Science, Kasetsart University, Thailand, were collected. They were separately subjected to enzyme extraction. The specific activity of each group of leaf was determined to select the highest enzyme concentration to be used for enzyme purification.

Preparation of enzyme extract

Crude enzyme extract was prepared by grinding fresh mulberry leaves in 150mM sodium phosphate buffer (pH 6.0) using a mortar and pestle. The homogenate was filtered through a linin cloth to remove suspended solid particle and then centrifuged at 10,000 g for 10 min at 4°C. The supernatant was collected and kept at 4°C for further analysis.

Aqueous two-phase (ATP) system preparation for the enzyme extraction

Aqueous two-phase (ATP) system was prepared by mixing polyethylene glycol (PEG M.W. 8,000) and ammonium sulfate with the enzyme extract. The amount of PEG/(NH₄)₂SO₄ was set at 15/10.1 (% w/v) and 20/8.9 (% w/v). The entire mixture was stirred on ice and then poured into a separatory funnel. The phases were allowed to come to equilibrium as indicated by the yellow color of the top phase and the green color of the bottom phase. The top and bottom phases were collected separately. Aliquots of each phase were analyzed for enzyme activity and protein concentration. The partition coefficient (*m*) of the enzyme was calculated as the ratio of enzyme concentration in the top phase to that of the bottom phase by the equation: $m = C_t/C_b$ where C_t and C_b were the enzyme concentrations in the top phase and bottom phase, respectively (Srinivas *et al.*, 1999).

Ion-exchange chromatography on DEAE-Cellulose

The lower part of aqueous two-phase extract was collected and dialyzed against 50mM phosphate buffer (pH 6.0). The dialyzed sample (25.26mg of total protein) was loaded onto a DEAE-Cellulose column (0.5×10 cm) which has been equilibrated with the same buffer. Bound proteins were sequentially eluted with 50mM phosphate buffer (pH 6.0) containing different concentrations of NaCl, i.e., 0.5M NaCl, 1.0M NaCl and 1.5M NaCl. Each fraction of 2.0 ml was collected at a flow rate of 30 ml/hr. Protein concentration was detected using spectrophotometer at the absorbency of 280 nm. Peroxidase activity was further determined and the fractions containing peroxidase activity were pooled and concentrated using aquasorb before subjecting to the following purification steps.

Gel filtration chromatography on Sephadex G-75

The pooled enzyme (12.88 mg of total protein) from the previous step was dialyzed against 50mM phosphate buffer (pH 6.0) containing 20mM NaCl. The enzyme was loaded onto a Sephadex G-75 column (0.25×30 cm) and eluted with the same buffer at a flow rate of 30 ml/hr. The fractions recovered were of 1.5 ml each. The enzyme and its activity were determined as previously described.

Rechromatography of gel filtration on Sephadex G-75

The pooled peroxidase (1.965 mg of total protein) from Sephadex G-75 chromatography was loaded onto the same size of column (0.25×30cm). The purification procedure was repeated as described above. In addition, molecular weight of mulberry leaf peroxidase could be estimated from the standard curve having molecular weight markers: alcohol dehydrogenase from yeast (150 kDa), BSA (66 kDa), carbonic anhydrase from bovine erythrocytes (29 kDa) and cytochrome C from horse heart (12.4 kDa) eluted from the same column.

Electrophoresis

SDS electrophoresis (SDS-PAGE) was carried out on a 5% acrylamide gel (stacking gel) and a 12.5% acrylamide gel (separating gel) in the presence of 0.1% SDS according to Laemmli (1970). The gel was stained with silver stain (Amersham Pharmacia Biotech). The molecular weight marker used was a standard kit (Sigma) containing BSA (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase from rabbit (36 kDa), carbonic anhydrase (29 kDa), trypsinogen from bovine pancreas (24 kDa), trypsin inhibitor (20.1 kDa) and α - lactoalbumin from bovine milk (14.2 kDa). Commercial horseradish peroxidase was used as a standard peroxidase. The samples from the fraction of each purification step were loaded on the gel at 20 μ g protein/well.

Peroxidase assay and protein concentration determination

Peroxidase activity was determined spectrophotometrically at 25°C by following the formation of tetraguaiacol ($A_{\text{max}} = 470 \text{ nm}$ and $\epsilon = 26.6 \text{ mM}^{-1} \text{ cm}^{-1}$) (Srinivas *et al.*, 1999). The assay mixture contained 330 μ l of 150 mM sodium phosphate buffer (pH 6.0), 330 μ l of 150 mM guaiacol and 30 μ l of enzyme sample. The reaction was initiated by the addition of 330 μ l of 17 mM H_2O_2 , and the absorbance at 470 nm was recorded within 0-5 min using Jenway 6400 spectrophotometer. One unit of peroxidase activity (U) represents the amount of enzyme catalyzing the oxidation of 1 μmol guaiacol in one minute. Protein concentration was determined by Lowry's method (Lowry *et al.*, 1951).

RESULTS AND DISCUSSION

To determine the suitable materials to be used for peroxidase assay, the shoots, third to fifth leaves, sixth to tenth leaves and eleventh to fifteenth leaves the youngest to the oldest leaves were tested. It was found that the third to fifth leaves gave the best specific activity as seen in Figure 1. They were, then, selected for peroxidase extraction. Interestingly, these third to fifth leaves are also the leaves generally collected to use as feed for silkworm (Aruga, 1994).

The first step of the mulberry leaf peroxidase purification, using aqueous two-phase extraction, was performed to eliminate the phenolics that were present in mulberry leaves. The results of the peroxidase extraction at various phase compositions are shown in Table 1. When peroxidase extraction was partitioned in aqueous two-phase system having $\text{PEG}/(\text{NH}_2\text{SO}_4$ at 15/10.1% and 20/8.9 % (w/v), the high activity of peroxidase was found in the salt rich phase (bottom phase). At the partition equilibrium, the color of the top phase was yellowish brown while the color of the bottom phase was green. It was anticipated

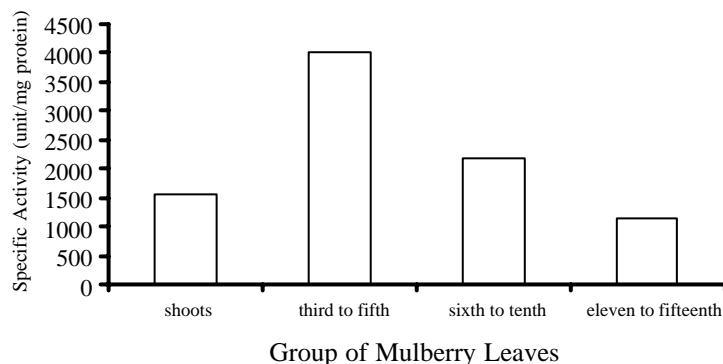


Figure 1 Specific activity of crude extract peroxidase from different sets of mulberry leaves.

Table 1 Aqueous two-phase extraction of mulberry leaf peroxidase with PEG/(NH₄)₂SO₄ system.

Phase ^a composition (%w/v)	Volume of top/bottom Phase (ml)	Partition coefficient (<i>m</i>)	Enzyme recovery (%)	Total enzyme activity (U)	Specific ^b activity (U/mg)	Purification (fold)
15/10.1	2.8/2.2	0.02	99.11	47,819.50	5,560.40	2.06
20/8.9	3.3/1.7	0.018	99.22	47,869.70	6,759.60	2.48

Note : ^a Total volume of phase composition was 5 ml.

^b Initial specific activity of crude enzyme was 2,699 U/mg protein.

that the yellowish brown color seen in the top phase could be the phenolic compound contained in the mulberry leaves as also reported by Nomura (1999). It is, therefore, beneficial to have this contaminant separated from the remaining of the peroxidase solution (Figure 2) in the early step of extraction.

The partition coefficient (*m*) of the enzyme was calculated as the ratio of enzyme concentration in the top phase to that of the bottom phase by the equation: $m = C_t/C_b$ where C_t and C_b were the enzyme concentrations in the top phase and bottom phase, respectively. The less partition coefficient value indicated the preferred condition of having higher concentration of enzyme in the bottom phase than in the top phase. Not only the peroxidase from 20/8.9% (w/v) (PEG/(NH₄)₂SO₄) phase gave a less partition coefficient value than that of the 15/

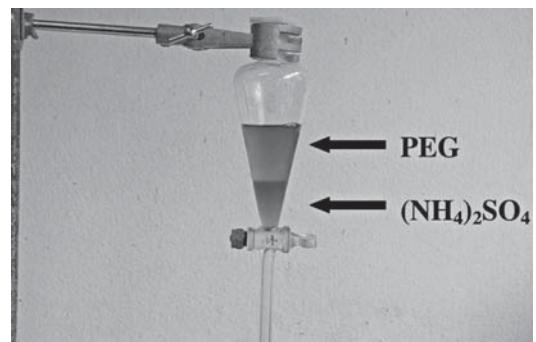


Figure 2 Separation of mulberry leaf peroxidase using aqueous two-phase system (ATPs) with the ratio of 20/8.9 PEG/(NH₄)₂SO₄ % (w/v).

10.1 % (w/v) (PEG/(NH₄)₂SO₄) phase but it also gave less bottom phase volume. Therefore, the 20/8.9 % (w/v) (PEG/(NH₄)₂SO₄) was proven to be

better combination of aqueous two-phase extraction system and was chosen for further purification processing. The settlement of mulberry leaf peroxidase found at the bottom phase was similar to that of Impomea peroxidase as reported by Srinivas *et al.* (2002). However, the partition coefficient of Impomea peroxidase was as high as 0.175 while enzyme recovery was only 76.9% and the total volume was 38%.

As for the second purification step, both DEAE-Cellulose (anion exchange chromatography) and CM-Cellulose (cation exchange chromatography) were used at the optimum pH of mulberry leaf peroxidase (pH 6.0). It was interesting to find that DEAE-Cellulose gave a better elution of mulberry leaf peroxidase than using CM-Cellulose (result not shown) while most of the contaminants were also adsorbed to the beads (Figure 3). The common practice of using DEAE-Cellulose was in the pH range of 5-9 and it was found that the optimum pH of 6.0 not only gave a considerably high peroxidase recovery (Table 2)

but also kept the enzyme in the best condition. The DEAE-Cellulose, therefore, was selected against CM-Cellulose as a second purification column.

A major activity peak (fractions 10 to 12) was obtained after peroxidase was passed through a Sephadex G-75 column in the third step of purification. The main peroxidase was eluted in a narrow peak while most of the protein was eluted as a broad peak (fractions 6 to 21) showing a better elimination of the contaminated protein from peroxidase in this step (Figure 4). However, passing the elute through a Sephadex G-75 for the second time resulted in having peroxidase activity in fractions 10 and 11 (Figure 5). The results of different steps of peroxidase purification are summarized in Table 2.

The purification of peroxidase (7.3-folds) obtained by using aqueous two-phase extraction on mulberry leaves was proven to be better than the one did by using acetone precipitation on barley leaves (5.8-folds) (Saeki *et al.*, 1986) or by using ammonium precipitation on *Opuntia ficus*

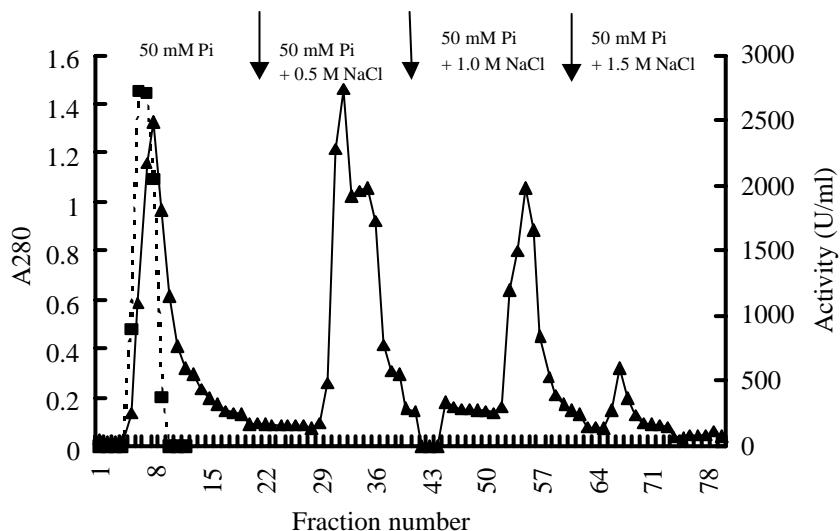


Figure 3 Elution profile of mulberry leaf peroxidase obtained from DEAE-Cellulose column. The total protein of 25.26 mg from aqueous two-phase extraction fraction was loaded onto DEAE-Cellulose column. The column (size: 0.5 × 10 cm) was eluted with phosphate buffer (pH 6.0) at 30 min/hr. Fractions No. 6 - 10 were pooled to determine the peroxidase activity. (—▲—) protein profile, (---■---) peroxidase activity.

indica (1.2-folds) (Padiglia *et al.*, 1995). However, it was similar to ammonium precipitation on royal palm leaves (7.7-folds) (Sakharov *et al.*, 2001). Yet, enzyme recovery from using aqueous two-

phase extraction of mulberry leaf peroxidase was better (144%) than that of royal palm leaves (82%).

Considering the second purification step, a better mulberry peroxidase purification (34.9-

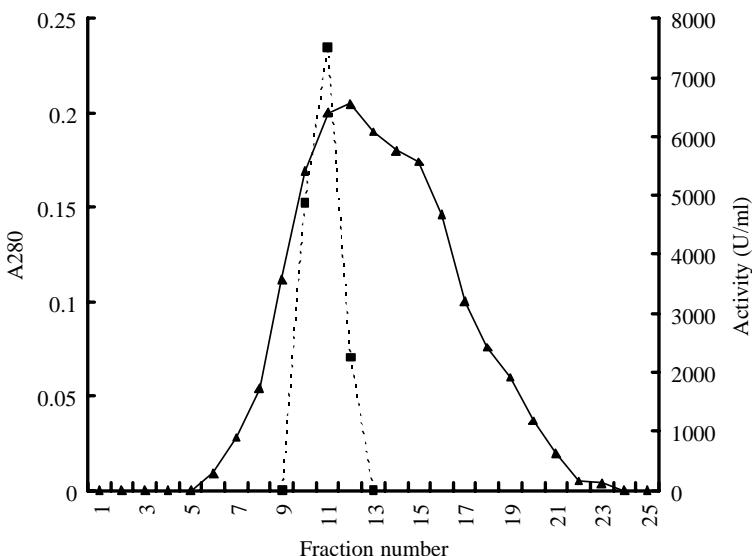


Figure 4 Elution profile of mulberry leaf peroxidase from Sephadex G-75. Protein of pooled active fractions No. 6 – 10 from DEAE-Cellulose column (12.88 mg of total protein) was loaded onto the Sephadex G-75. The column (size: 0.25 × 30 cm) was eluted with phosphate buffer (pH 6.0) at 30 min/hr. Fractions No. 10-12 were pooled to determine the peroxidase activity. (—▲—) protein profile, (---■---) peroxidase activity.

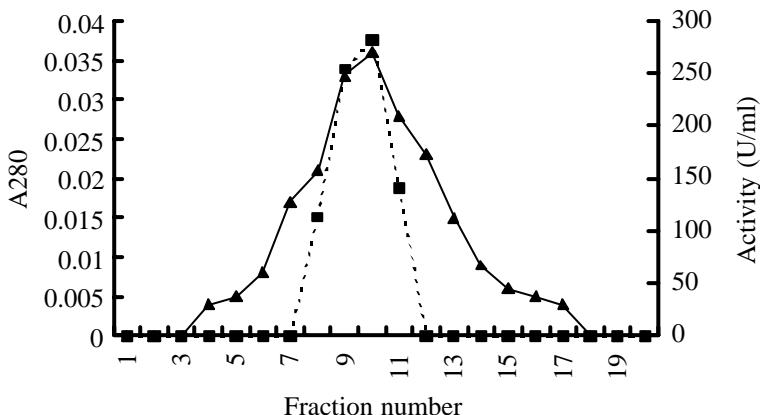


Figure 5 The repeated elution profile of mulberry leaf peroxidase on Sephadex G-75. The total protein of 1.965 mg was loaded onto the column (size: 0.5 × 10 cm) and was eluted with phosphate buffer (pH 6.0) at 30 min/hr. Fractions No. 10 - 11 were pooled to determine the peroxidase activity. (—▲—) protein profile, (---■---) peroxidase activity.

Table 2 Overall results of different steps of mulberry leaf peroxidase purification.

Fraction	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude extract	100	5,740,500.0	5,740.5	1	100.0
ATPE (PEG/(NH ₄) ₂ SO ₄)	60.3	2,525,820.0	41,905.6	7.3	144.0
DEAE-Cellulose	22.9	4,580,919.0	200,343.5	34.9	79.8
Sephadex G-75	9.48	3,058,686.5	322,616.1	56.2	53.3
Rechromatography on Sephadex G-75	2.75	2,491,377.0	903,554.7	157.4	43.4

folds) was achieved using DEAE-Cellulose column than from using CM-Cellulose column on barley leaves (17-folds) (Saeki *et al.*, 1986) and also from using DEAE-Cellulose column on *Opuntia ficus indica* (3.8-folds) (Padiglia *et al.*, 1995). However, purification folds of mulberry leaf peroxidase at this step was similar to that of royal palm leaves using DEAE-Tyrosearl column (30-folds) (Sakharov *et al.*, 2001).

Finally, our overall purification of mulberry leaf peroxidase using four steps obtained 157.4-

folds with 43.4% recovery. It was reported in *Opuntia ficus indica* (82-folds with 47% recovery) (Padiglia *et al.*, 1995) and in royal palm leaves using six steps (73-folds with 12.5% recovery) (Sakharov *et al.*, 2001).

The native molecular weight of mulberry leaf peroxidase was found to be 29 kDa as calculated from the relative fraction of the standard curve from gel filtration (Figure 6). It was found that molecular weights of peroxidase in other organisms vary greatly from 220 kDa of bacterium,

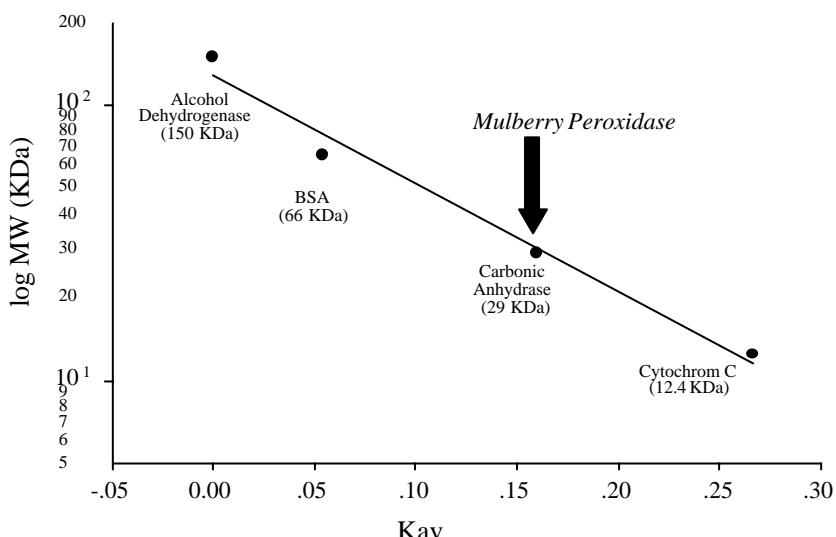


Figure 6 Gel filtration showing standard curve of molecular weight markers. The native molecular weight of mulberry leaf peroxidase is 29 kDa as indicated by the arrow.

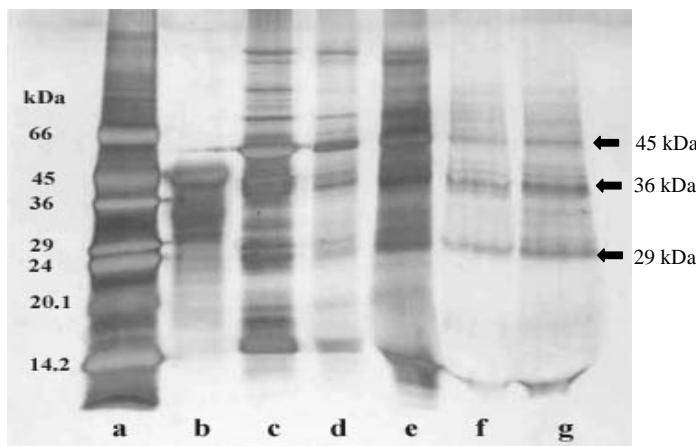


Figure 7 SDS-PAGE of partial purified mulberry leaf peroxidase. Lane a, molecular marker consisting of BSA (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase from rabbit (36 kDa), carbonic anhydrase (29 kDa), trypsinogen from bovine pancreas (24 kDa), α -lactoalbumin from bovine milk (14.2 kDa); lane b, Commercial HRP standard peroxidase; lane c, crude peroxidase extract; lane d, aqueous two-phase extraced peroxidase; lane e, DEAE-Cellulose fraction; lane f, Sephadex G-75 fraction; lane g, rechromatograph on Sephadex G-75 fraction. 20 μ g protein was loaded on each well, except 30mg protein of lane g. Proteins were separated on 12.5 % of SDS-PAGE and silver- stained.

Flavobacterium meningosepticum (Koga *et al.*, 1999) to 28 kDa of tobacco, *Nicotiana tabacum* (Kim *et al.*, 1980).

CONCLUSION

The third to fifth leaves of mulberry (*Morus rotundifolia*) was the most suitable source of peroxidase with highest specific activity of 4,000 U/mg protein comparing to the other groups of mulberry leaves. A 20/8.9% (w/v) of PEG/ $(\text{NH}_2)_2\text{SO}_4$ was the best phase composition for mulberry leaf peroxidase extraction giving 2.48-purification folds and 99.22% recovery. After having passed through aqueous two-phase extraction, DEAE-Cellulose and double chromatography on Sephadex G-75, the overall purification of mulberry leaf peroxidase gave 157.4-purification folds and 43.3% recovery. The native molecular weight of mulberry leaf

peroxidase was 29 kDa as calculated from gel filtration while the result from SDS-PAGE was not conclusive due to some contamination.

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