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Extraction and Purification of *Ipomoea* Peroxidase Employing Three-phase Partitioning

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Abstract Three-phase partitioning (TPP) is a novel separation process used for the extraction and purification of biomolecules. The biomolecules are recovered in a purified form at the interface (precipitate), while the contaminants partition in *t*-butanol and aqueous phases. Peroxidase from the leaves of *Ipomoea palmata* was purified by using TPP. The ratio of the crude extract to *t*-butanol of 1:1 and 30% ammonium sulfate at 37 °C resulted in about 160% activity recovery and twofold purification in the aqueous phase of the first cycle of TPP. On subjecting the aqueous phase to the second cycle of TPP, a purification of 18-fold was achieved with about 81% activity recovery. The sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis showed substantial purification, and the molecular weight of peroxidase was found to be 20.1 KDa. The present study shows a higher degree of purification and activity yield as a primary purification process in comparison with existing literature values, thus demonstrating TPP as an attractive downstream process for the purification of peroxidase.

Keywords Three-phase partitioning · *Ipomoea palmata* · Peroxidase · Cross-partitioning · Aqueous two-phase extraction · Downstream process · *t*-Butanol · Ammonium sulfate

Introduction

Peroxidases [E.C.1.11.1.7] are hemoproteins widely distributed in nature, which are found in plant cells, animal organs, and tissues [1]. Plant peroxidases are found mainly in cell walls, vacuoles, transport organelles, and also on rough endoplasmic reticulum. They play an important role in plant physiological responses including auxin catabolism,

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modification of the cell wall, lignification, pathogen defense, and wound healing. Peroxidases are used widely in enzyme immunoassays, clinical biochemistry, treatment of wastewater containing phenolic compounds, and decolorization of dyes and also serve as catalysts in organic synthesis [2, 3]. Peroxidases have been divided into three superfamilies according to their source and mode of action as plant peroxidase, animal peroxidase, and catalases [1].

The separation and purification of biomolecules accounts for a major fraction (70–80%) of the total production cost [4]. In view of this, the present study has been undertaken for the extraction and purification of peroxidases from *Ipomoea palmata* leaves by using a process called "three-phase partitioning" (TPP). *I. palmata* is commonly known as a railway creeper and belongs to the family Convolvulaceae. TPP is a novel separation technique in which an organic/aqueous system such as *t*-butanol and ammonium sulfate are used to selectively extract the target biomolecule at the interface (in the form of precipitate) leaving the contaminants in the alcohol and aqueous phases [5, 6]. Plant peroxidase from *Ipomoea* is a good alternative source to the conventional horseradish peroxidase (HRP). TPP has been used to purify a number of biomolecules such as alkaline phosphatase from chicken intestine, amylase inhibitor from wheat germ, pectinase from tomato, xylanase from *Aspergillus niger*, green florescent protein from *Escherichia coli*, and protease inhibitor from ragi [7–12].

Literature review shows that peroxidase has been purified from different sources employing various primary and final purification processes. *Ipomoea cairica* was purified using ammonium sulfate precipitation, acetone fractionation, and gel filtration to result in a 51-fold purification with 84% activity recovery [13]. Peroxidase from *I. palmata* was extracted and purified by using a combination of aqueous two-phase extraction (ATPE) and gel filtration to result in 49-fold purification with 75% activity recovery [14]. The same group reported peroxidase purification of 5.9-fold with 76% activity recovery by using a combination of ATPE and ultrafiltration [15]. Shaffiqu et al. [16] have used ammonium sulfate precipitation, ion exchange chromatography, and gel filtration chromatography to result in 78% purification of *Ipomoea* peroxidase. Two groups used a combination of TPP and chromatographic techniques for the extraction and purification of peroxidase from horseradish and turnip, respectively [17, 18]. Szamos and Hoschke [17] reported 24-fold purification with 30% activity recovery by an integration of TPP and ion exchange chromatography. Singh and Singh [18] achieved 80.3-fold purification with 20% activity recovery by a combination of TPP and metal affinity chromatography. In all these studies, the aims of the authors were to recover the protein in a highly purified form by a primary purification process followed by a chromatography step.

In the present study, the potential of TPP alone was explored for the extraction and purification of peroxidase by optimizing the parameters such as concentrations of *t*-butanol and ammonium sulfate and the temperature of extraction.

Materials and Methods

Materials

I. palmata leaves were procured from the campus of Central Food Technological Research Institute, Mysore, Karnataka, India. *t*-Butanol and ammonium sulfate were pure grade and were procured from Merck, Germany, and Rankem, Mumbai, India, respectively.

Methods

Preparation of Crude Extract

Fresh leaves (*I. palmata*) were washed thoroughly with distilled water and cut into pieces. The leaves were then homogenized with 10 mM phosphate buffer (pH 6). The ratio of quantity of leaves taken to that of buffer was maintained constant at 1:1 (*w*/*v*). The crude extract was filtered through cheese cloth and centrifuged to remove traces of fibrous particles and cell debris. The supernatant was stored at 4 °C and used as stock solution for further experiments.

Isoelectric Point Determination by Aqueous Two-phase Extraction (PEG/salt System)

Predetermined quantities of polyethylene glycol (PEG) 1500, potassium phosphate (pH 5–11), and distilled water/crude extract were mixed with known concentrations of NaCl and Na_2SO_4 separately (two sets of experiments). The phase compositions were selected based on our previous work [14]. The components were mixed and equilibrated using a magnetic stirrer for 30 min and kept for phase separation overnight (about 12 h). After complete phase separation, the top and bottom phases were separated and analyzed, and the partition coefficient, *K* (ratio of the concentration of the enzyme partitioned in the top phase to that at the bottom phase), was calculated. A plot of pH versus *K* for two salts gives two different curves intersecting or crossing at a point called the cross-point [19]. The pH corresponding to the cross-point gives the isoelectric point of the enzyme.

Three-phase Partitioning

Predetermined quantities of ammonium sulfate and crude extract were mixed thoroughly for 30 min in a magnetic stirrer, and the pH was adjusted to 9 (p*I* of the protein) followed by the addition of *t*-butanol. After mixing again, the phase system was equilibrated in a water bath at 25 °C for 30 min for three-phase formation. Once complete phase separation was observed, the top, bottom and interfaces were separated. The bottom phase and interface were analyzed for enzyme activity and total protein content using a spectrophotometer (Shimadzu, model UV 160A) by comparing with a blank system (without enzyme). The blank system was prepared containing ammonium sulfate, distilled water, and *t*-butanol in the similar manner as for the crude extract. After the first cycle of TPP, it was found that most of the enzyme partitioned to the bottom phase; hence, a second cycle of TPP similar to the first cycle was carried out for the possible recovery of the peroxidase at the interface.

All the experiments were performed in duplicates, and the difference in reading was within ±5%.

Estimation of Enzyme and Total Protein Content

The activity of the peroxidase was determined at 25 °C by a spectrophotometer following the formation of tetraguaiacol [20]. A 3-ml reaction mixture containing 1 ml of 15 mM 2-methoxyphenol (guaiacol), 1 ml of 3 mM H_2O_2, and 50 μl enzyme extract were mixed, and the absorbance at 470 nm was taken at intervals of 1 min for a total of 3–5 min (ε [extinction coefficient]=26.6 mM^{-1} cm^{-1}). One unit of peroxidase activity represents the amount of enzyme catalyzing the oxidation of 1 μmol of guaiacol in 1 min. The protein assay was carried out by Bradford's method, using bovine serum albumin as the standard [21].

Measurement of Interfacial Tension of the System

Interfacial tensions were measured by spinning drop tensiometer (SITE100 no. 20002806, Kruss, Germany) according to the method described in [22]. The denser phase (ammonium sulfate) was filled in a glass tube, and a small drop of the less dense phase (*t*-butanol) was injected and rotated about its axis of symmetry. This caused the drop to migrate to the center of the tube. At a higher rotational speed, the drop becomes elongated. The interfacial tension was calculated as the mean of rotation at 5,000–10,000 rpm for ten counts at 25±2 °C using the drop shape analysis software (DSA-II).

Results and Discussion

Determination of Isoelectric Point of the Protein by Cross-partitioning

Cross-partitioning is a method employing ATPE by which the isoelectric point of a protein close to or similar to that of electrophoretic means (isoelectric focusing) can be obtained [19]. In the present study, PEG 1500 and potassium phosphate were used in which the effect of salts (NaCl and Na_2SO_4, each 1% *w*/*w* added) on the partitioning of peroxidase was evaluated by a plot of *K* vs pH (Fig. 1). The pH corresponding to the point where the two curves cross (p*I* of the protein) was found to be 9. Experiments for the optimization of parameters were carried at pH 9 since p*I* is the optimum pH for TPP [5].

Effect of *t*-Butanol Concentration

The amount of ammonium sulfate was fixed to 30% (*w*/*w*), and the ratio of the crude extract to *t*-butanol varied from 1:0.3 to 1:1 (Fig. 2). It was found that during the first cycle of TPP, activity yield of enzyme partitioned in the aqueous phase was 12–47%; hence, a second cycle of TPP was carried out similar to the first cycle for possible recovery of the remaining protein at the interface. In the second cycle of TPP, for 1:0.75 and 1:1 crude extract to *t*-butanol ratios, the content of ammonium sulfate was decreased to 20% and 15%, respectively. The decrease in ammonium sulfate content was because at concentrations greater than 15% and 20%, most of the ammonium sulfate remained insoluble (in presence

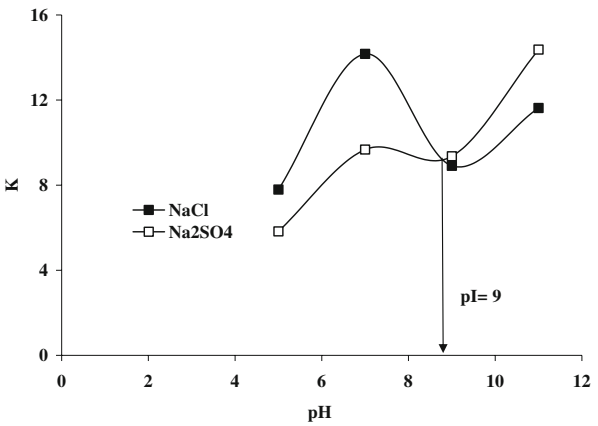

Fig. 1 Cross-partitioning of peroxidase from *I. palmata* employing the PEG 1500/ potassium phosphate system

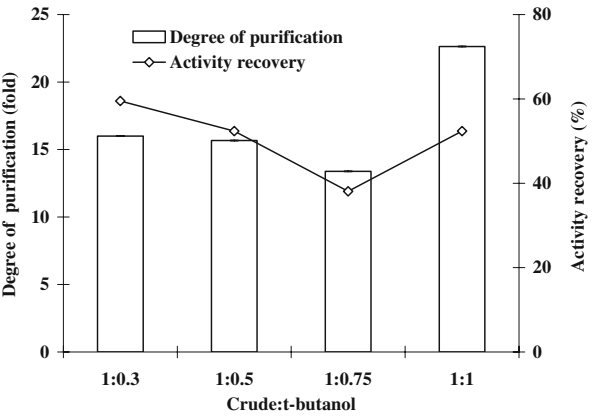

Fig. 2 Effect of *t*-butanol concentration on the degree of purification and activity recovery of peroxidase in the aqueous phase

of *t*-butanol) indicating that its solubility limit has been crossed (visual observations). The decrease in ammonium sulfate in turn resulted in decrease in activity yield by 27% for the 1:0.75 ratio, but for the 1:1 ratio, an increase in yield by 38% is observed. The decrease in yield at the 1:0.75 ratio may be attributed to the synergistic effects of the increase in concentration of *t*-butanol (from 1:0.5 to 1: 0.75) and decrease in concentration of ammonium sulfate. An increase in yield at the 1:1 ratio may be due to the synergistic effects of the decrease in ammonium sulfate concentration by 15% (unlike the 1:0.75 ratio where the decrease was by 20%) and increase in concentration of *t*-butanol (from 1:0.75 to 1:1). The second cycle of TPP also resulted in high purification and activity yield in the aqueous phase (supernatant) than in the precipitate similar to the first cycle. Equal volumes of the crude extract to *t*-butanol (ratio of 1:1) resulted in 22-fold purification with 52% activity recovery (Fig. 2). Previous studies in TPP using HRP as the source has also shown the purification to be higher in the bottom (aqueous) phase [17].

The observed optimum ratio of the crude extract to *t*-butanol of 1:1 may be because of two factors. If the *t*-butanol content is less, the synergistic effect of ammonium sulfate required to recover the protein at the interface is less [8]. If the *t*-butanol content is high

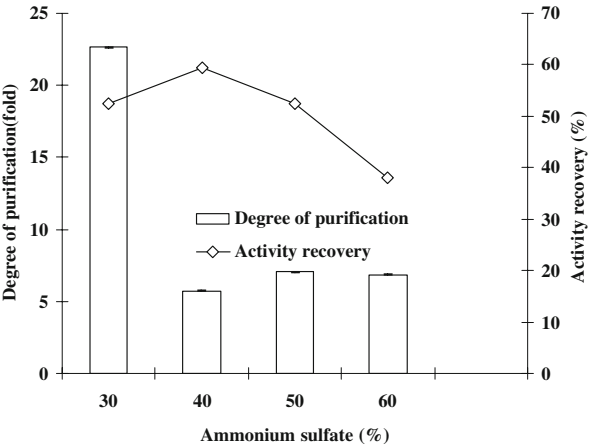

Fig. 3 Effect of ammonium sulfate concentration on the degree of purification and activity recovery of peroxidase

Table 1 Effect of concentration of *t*-butanol on the interfacial tension of the system.

Ammonium sulfate (% *w*/*v*)	Crude extract/*t*-butanol (*v*/*v*)	Interfacial tension (mN/M)	Protein concentration at interface (mg/ml)
30	1:0.3	21.2	0
30	1:0.5	25.6	1.40±0.10
30	1:0.75	29	2.04±0.14
30	1:1	33.8	1.99±0.01

(>1:1), the denaturation of the protein is more likely, in addition to an increase in the viscosity of the top phase (*t*-butanol).

Effect of Ammonium Sulfate Concentration

The effect of ammonium sulfate was studied by maintaining the ratio of the crude extract to *t*-butanol constant (1:1) and varying the concentration of ammonium sulfate from 30% to 60% *w*/*w*. During first cycle of TPP, about 13–140% activity yield of peroxidase was observed to partition in the aqueous phase; hence, a second cycle of TPP was thought desirable to be carried out similar to the first cycle as explained in the previous section ("Effect of *t*-Butanol Concentration"). With an increase in concentration of ammonium sulfate, the degree of purification (purification factor) decreased significantly above 30% *w*/*w* and remained practically constant (from 40% to 60% ammonium sulfate), while the activity recovery initially increased to 60% (at 40% *w*/*w*) and then decreased to 38% (at 60% *w*/*w*; Fig. 3).

Sulfate ion and *t*-butanol are known to be excellent protein structure makers (kosmotropes) [5]. The principle of sulfate ion for salting out protein has been viewed in five different ways namely, ionic strength effects, kosmotropy, cavity surface tension enhancement osmotic stressor (dehydration), exclusion crowding agent, and the binding of SO_4^{2-} to cationic sites of protein [5]. TPP depends upon the concentration of ammonium sulfate and the p*I* of the protein. It may be noted that all the experiments were carried out at the p*I* of the protein, i.e., 9. At concentrations of ammonium sulfate greater than 40% *w*/*w*, the activity yield decreased significantly (about 36%) due to the precipitation of protein and may possibly cause its irreversible denaturation. In addition to the decrease in percent activity recovery, the high concentration of ammonium sulfate also reduces the selectivity of extraction (>30% *w*/*w* ammonium sulfate), thus reducing the degree of purification.

Effect of the Phase Composition

The effect of phase composition on interfacial tension was studied for the same compositions as that for the effects of *t*-butanol and ammonium sulfate. With an increase

Table 2 Effect of concentration of ammonium sulfate on the interfacial tension of the system.

Crude extract/t-butanol (*v*/*v*)	Ammonium sulfate (% *w*/*v*)	Interfacial tension (mN/M)	Protein concentration at interface (mg/ml)
1:1	30	33.8	1.99±0.01
1:1	40	51.3	2.96±0.17
1:1	50	74.9	2.96±0.15

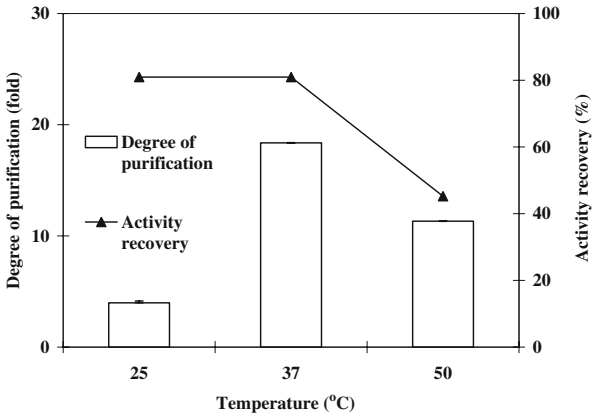

Fig. 4 Effect of temperature on the degree of purification and activity recovery of peroxidase

in *t*-butanol or ammonium sulfate composition, the surface tension of the system increased, and thus the interfacial tension also increased. The increase in interfacial tension in turn increased the partitioning of peroxidase at the interface (Tables 1 and 2). Higher interfacial tension values signify higher density differences and also higher polarity differences between the phases [23]. In Table 1, it is observed that at concentrations of the crude extract to *t*-butanol of 1:0.75 and higher and in Table 2, at concentrations of ammonium sulfate of 40% *w*/*w* and higher, the protein that partitioned at the interface remains constant.

Effect of Temperature

The thermal stability was studied by incubating the peroxidase subjected to TPP at 25, 37, and 50 °C for 1 h in a water bath. At a higher temperature (50 °C), the degree of purification and activity yield decreased by 44% and 38%, respectively, as compared to 37 °C (Fig. 4). This may be due to the synergistic effects of the phase components along with the effect of temperature coming into play, which decreases the selectivity of extraction, thus reducing both the activity yield and the degree of purification. The thermal stability of the present study is in good agreement with earlier reports, which have shown *Ipomoea* peroxidase to be stable at 60 °C for 5–6 h [13, 14].

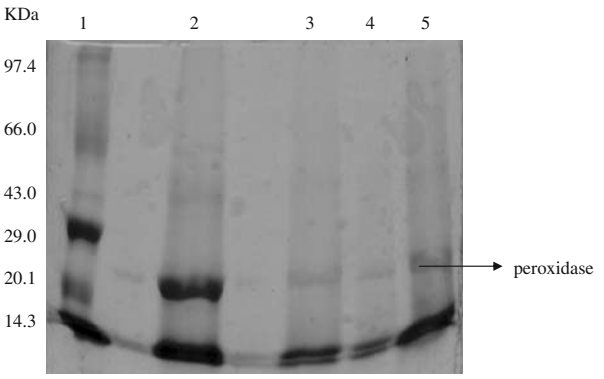

Fig. 5 SDS-PAGE of the purified peroxidase by TPP. *Lane 1*: molecular marker, *lane 2*: TPP 1(first cycle) precipitate, *lane 3*: TPP 1 (first cycle) aqueous phase, *lane 4*: TPP 2 (second cycle) aqueous phase, *lane 5*: TPP 2 (second cycle) precipitate

Table 3 Overall purification of peroxidase by TPP.

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Fold purification	Activity yield (%)
Crude extract	0.04	0.39	0.11	1	100
First TPP (aqueous phase)	0.07	0.313	0.214	1.98±0.01	159.52±0.01
First TPP (interphase)	0.41	1.02	0.40	3.70±0.05	976.19±0.05
Second TPP (aqueous phase)	0.03	0.017	1.98	18.36±3.29	80.95±3
Second TPP (interphase)	0.26	1.03	0.252	2.33±0	616.67±0

Overall Purification

The overall purification is given in Table 3, and the results of sodium dodecyl sulfate polyacrylamide gel elctrophoresis (SDS-PAGE) are shown in Fig. 5. The molecular weight of the peroxidase was found to be 20.1 KDa. SDS-PAGE shows a substantial purification of peroxidase (lane 4).The increase in enzyme activity to 160% (first TPP aqueous phase), 976% (first TPP interface), and 616% (second TPP interface) may be due to the existence of the enzyme in an excited state that results in its increased flexibility (Table 3).The unusual increase in enzyme activity in TPP has been reported previously [24].The authors

Table 4 Comparison of different processes from literature for peroxidase purification.

Sl number	Process	Source	In primary purification method		After chromatography		Reference
			Initial purification factor (fold)	Activity yield (%)	Overall purification factor (fold)	Activity yield (%)	
1	Ammonium sulfate precipitation + acetone fractionation + gel chromatography	*I. cairica*	1.25	77.7	51	84	[13]
2	ATPE + gel chromatography	*I. palmata*	2.18	91.5	48.6	75.3	[14]
3	ATPE + ultrafiltration	*I. palmata*	5.9	76	Not employed		[15]
4	Ammonium sulfate precipitation + ion exchange chromatography + gel chromatography	*I. palmata*	–	–	77.8	–	[16]
5	TPP + ion exchange chromatography	Horseradish	5.7	65.6	23.6	29.5	[17]
6	TPP + immobilized metal affinity chromatography	Turnip	6.8	36	80.3	20.3	[18]
7	TPP	*I. palmata*	18.36	80.95	Not employed		Present study

attributed the increase in enzyme activity due to a higher concentration of B-factor as observed by X-ray diffraction studies.

Comparison with Literature

The results of the present study were compared with the existing literature values (Table 4). The literature values show that in all the processes, a minimum of two steps are used with the second step invariably being chromatography. Though the overall purification is high in previous studies, the present study showed a high degree of purification and activity yields using TPP as a primary purification step.

Conclusion

TPP employed for the extraction and purification of peroxidase from *I. palmata* has shown potential to be an attractive process as the primary purification step. The high concentration of peroxidase partitioned at the interface during the first and second cycles of TPP was due to the high interfacial tension of the system. However, due to nonselective extraction, the contaminants also partitioned, thus decreasing the purification factor at the interface. The purification of the peroxidase by TPP was found high in the aqueous phase of the second cycle of TPP. The present study has shown high values of the purification factor and activity yield in a single step as compared to the primary purification step of existing literature methods.

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