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Reverse micellar extraction procedure for continuous isolation of peroxidase form plant source

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ABSTRACT

Horseradish peroxidase (HRP) is the group of enzymes that catalyze the oxidation of a compound by peroxide. Horseradish peroxidase is the universal choice of reagent in molecular level experiment, diagnostics, sewage treatment and in Biosensor. Horseradish peroxidase is separated through traditional down streaming method, which usually results in low yield and high cost. Liquid-liquid extraction (LLE) using organic/aqueous phase is not the choice of separation in biotechnology mainly due solubility and protein denaturation problems. Aqueous Two Phase System (ATPS) adapts the extraction through phase separation and is considered as single step isolation protocol. Triton X-100 and Tween 20 were used as surfactants, and efficiency were calculated. A forward extraction yield of 72% and activity 890 U/mg was obtained for Triton X-100/ Toluene at pH 6, salt concentration 0.15M, phase ratio 1:1 and surfactant concentration 15mM. In Tween 20/ toluene system there was only 58% yield in 10µl surfactant concentration. For back extraction a maximum yield of 13% and activity of 648 U/mg obtained in Triton X-100/ Toluene system. ATPS was found to be superior to traditional methods both in terms of yield and continuous process. Isolated peroxidase was checked for its purity using SDS-PAGE, which clearly displays 3 bands correspond to the standard marker and commercial peroxidase. Gas chromatography analysis conforms the presence of peroxidase however 11 more peaks indicating presence of other proteins and enzymes. It is suggested to optimize the techniques for continues separation and concentration of the peroxidase.
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KEYWORDS

Horse radish peroxidase;
Hydrogen peroxide oxidoreductase;
Down streaming process;
Aqueous two phase extraction;
Liquid-liquid extraction procedure.

INTRODUCTION

Peroxidase (E.C. 1.11.1.7) has become indispensable reagents for diagnosis and various laboratory experiments like immunology, organic synthesis, and biosensors. Peroxidases are widely distributed in nature and can be easily extracted from plant cells and from animal organs and tissues[2,7,19]. Peroxidase can oxidise various substrates due to different type of reactions: peroxidative oxidation, oxidative, catalytic and hydroxylation[18]. Peroxidase is a secretory enzyme that catalyze the reaction,

Donor + H_2O_2 → Oxidized donor + $2H_2O$

Horseradish (*Armoracia rusticana L.*) roots contain peroxidases currently used for commercial applications[12]. Horseradish peroxidase have been highly purified and characterized in terms of their amino acid and carbohydrate compositions[20]. HRP-C dominates quantitatively among the isoperoxidase of horse radish root and has an isoelectric point close to 0. It consist of a hemin prosthetic group, two calcium ions and 308 amino acids residues including four disulphide bridges in a single poly peptide chain that carries eight neutral carbohydrate side chain. HRP is a globular glycoprotein with a mass of 42000, of which the protein moiety is approximately 34000, the rest of the molecular weight being accounted for by the prosthetic group, two calcium ions and some surface bound glycans[6].

Peroxidase occurs as a large family of isoenzymes. Using DEAE cellulose chromatography, Morita isolated and characterized five neutral (B1, B2, B3, C1 and C2) and six basic isoenzymes (E1, E3, E3, E4, E5 and E6)[3]. Their main difference appears to be either behavior in pI focusing gels.

HRP based biosensors can be used to control and monitor the peroxides in pharmaceutical, environment and diary industries[16]. HRP is widely used as an enzyme label in medical diagnostics and research applications. HRP system offers a wide range of amplifying possibilities for ultra-sensitive detection in immunoassay and cytochemical application[11]. Universal covalent conjugate of proteins, antibodies and other molecules with HRP offer a wide range of amplifying possibilities. The availability of substrate for colorimetric, flurometric and chemilumiesent assay provides numerous detection options including western blotting[5]. HRP permits precise cell/tissue location in light and electron microscopy, identification and qualitative estimation of hormone receptors as prognostic markers in tumors evaluation and in the diagnosis of viral and other infection agents[15]. Several novel applications of this enzyme has been suggested viz. treatment of waste water containing phenolics, the synthesis of various aromatic chemicals etc[21]. New applications of peroxidases are being explored

HRP is present as complex mixture of contaminating enzymes, proteins and other biological substances. Separation of HRP is frequently complicated by the liability of the desired products and by the stringent purity specification of the final product. As a result, in recent year there has been an increased interest in the development of efficient methods for isolation, concentration and purification of biological products[4].

The Bimolecular separation is still performed by techniques such as salt precipitation, column chromatography, and electrophoresis where the yield is low yield , contamination and high cost[9]. There fore designing efficient scalable alternative bioseperation process with potential for continuous operation is of great demand.

Liquid-liquid extraction (LLE) is a traditional chemical engineering unit operation used in chemical, petrochemical and hydro-metallurgical industries and in antibiotics industries[17]. LLE using organic/aqueous phase has been employed in many chemical industries however this technique fails to find its application in Biotechnology mainly due solubility and protein denaturation problems[1]. In recent years LLE using Aqueous Two Phase System (ATPS) has been recognized as superior and versatile technique used for downstream processing of biomolecules[13]. ATPS adapts the extraction through phase separation and is achieved by gravity, there by eliminating the need for centrifuges and other down streaming process. In ATPS physical properties such as density, viscosity and interfacial tension, determine the phase separation time and also contribute to the biomolecule partition behavior. Purification of Biomolecule is carried out in Single step when compared to the number of steps such as filtration, concentration and purification. The advantages of ATPS are high capacity, biocompatible environment, low interfacial tension, high yields, and lower process time energy. Extraction of enzymes/proteins by organic solvents using reverse micelles (RMs) is relatively a new concept. LLE using reversed micelles, as extractant to separate and purify protein/enzymes in highly effective and very selective. RME has been successfully used for the purification of various proteins like BSA, α-amylase, etc. Survey of literature indicates most of the RME technique uses ionic surfactants with isooctane or hexane as solvents. The ionic surfactants have shown

very good extraction efficiency. However due to strong electro static interaction, they tend to denature the biomolecules which is non desirable. A few studies are reported that non ionic surfactants are less interactive with biomolecule and do not denature them. Hence the present study was designed to compare the various methods of Isolation and purification of peroxidase from radish extracts.

MATERIALS AND METHODS

For the RME of peroxidase, the crude extract was obtained by crushing of radish mixed with Phosphate buffer (0.1M, pH 7.0) at 1:2 ratio (root: buffer), filtered, salting -out with 70% saturated ammonium Sulfate. The extract after centrifugation has been dialyzed for two days using 10kda molecular weight cutoff dialysis membrane. The dialyzed sample was lyophilized and stored at 4°C in cooled room.

The RME was carried out with the crude extract under two steps procedure, forward and backward transfer.

RME studies

The lyophilized crude was subjected to reverse micellar extraction to obtain maximum yield of peroxidase, which has its activity either retained or enhanced compared to crude extract. The two non-ionic surfactants Triton X-100 (isooctyl phenoxy polyehoxy ethanol) and Tween- 20 (polyoxyethylene sorbitan mononucleate), were used for the extraction.

Forward transfer

The initial aqueous phase contained crude extract; to the crude extract sodium chloride of 0.15 M salt concentration was added to adjust the ionic strength. The surfactant was added to the organic phase containing toluene (phase ratio 1:1). Both the phase was mixed at 500 rpm for 15 minutes. After centrifugation at 5000 rpm for 15 minutes, both the phases were separated and aqueous phase was assayed for enzyme content using Lowry method.

Back transfer

Mixing the organic phase of forward extraction with freshly prepared aqueous phase of known concentration and pH carried out the back transfer. The ionic strength of the aqueous phase is adjusted by adding known concentration of potassium chloride. Both phase are mixed for 30 minutes at 500 rpm and centrifuged at 5000 rpm for 15 minutes.

Peroxidase activity guaiacol method

Peroxidase activity was determined at 30° C with a UV-visible spectro-photometer following the formation of tetraguaiacol (Amax = 470 nm) in a 3 ml reaction mixture containing 1 ml of 0.1M phosphate buffer pH 6.0; 1 ml of 15 mM 2-methoxyphenol (guaiacol); 1 ml of 3 mM H2O2 and 0.1 ml of enzyme extract.One unit of peroxidase activity represents the amount of enzyme catalyzing the oxidation of 1 μM of guaiacol in 1 minute.

$$\text{Activity (u/ml)} = \frac{\text{(Change in OD x volume of reaction mixture taken)}}{\text{(26.6 x volume of enzyme added)}}$$

Specific activity (U/mg)

Specific activity of peroxidase was calculated as the ratio of total activity of the enzyme to the total protein concentration. Protein content was measured by Bradford method

SDS-PAGE Separation and purity check

Crude and extracted enzymes were subjected to SDS-PAGE resolution with 5% Stack and 12% separating gel system. Commercial peroxidase and standard marker were resolved along with the sample. Gel staining was accomplished using the Silver staining method[14].

Gas chromatography

Gas chromatography analysis of sample were vaporized and injected into the head of the chromatographic column with the following specification.

Carrier	: nitrogen @ 30 ml / min.
Oxidant	: oxygen @ 300 ml/min.
Fuel	: hydrogen @ 30 ml/ min.
Column	: 1/8 inch ss, packing: ov-17
Injector temperature	: 300 ºC, detector temp: 300 ºC
Oven initial	: 80 ºC, oven final: 250 ºC,
oven rate	: 10
Detector used	: FID

The peaks were analyzed and compared with that of the commercial peroxidase.

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RESULTS AND DISCUSSION

Reverse micellar extraction of peroxidase was carried out from fresh radish crude extract and compared with the traditional isolation and purification procedures. 70 % saturated Ammonium sulphate precipitation exhibits high activity of peroxidase. Maximum peroxidase extraction was at a pH 7 .0. Temperature optimum is 47 °C which perfectly agrees with the available literature (Yuan and Jiang., 2003). Earlier studies by[8] indicated that the use of buffer having pH 7 and distilled water for the extraction of peroxidase did not vary crude concentration. The crude extract obtained from radish was stored in 4°C in a cold room and stability was measured everyday for 5 days. As can be observed from the TABLE 1 there was no major change in the specific activity of peroxidase during storage.

TABLE 1 : Stability study of peroxidase

S.NO.	Time (hrs)	Specific activity (U/mg)	Concentration (mg/ml)
1	0	816	0.248
2	24	780	0.268
3	48	820	0.257
4	72	838	0.253
5	96	798	0.248
6	20	830	0.250

In this extraction, different surfactants namely Triton X-100 and Tween 20, and efficiency were used. A forward extraction yield of 72% and activity 890 U/mg was obtained for Triton X-100/ Toluene at pH 6, salt concentration 0.15M, phase ratio 1:1 and surfactant concentration 15mM. In Tween 20/ toluene system there was only 58% yield in 10µl surfactant concentration. For back extraction a maximum yield of 13% and activity of 648 U/mg obtained in Triton X-100/ Toluene system.

The aqueous phase pH was varied from 3 to 7 during forward transfer. When the pH was increased from 3, the extraction remained almost same with a maximum of 72 % at pH 6 and activity of 890 U/mg at pH 7. However, further increase in pH decreases the extraction efficiency. It is well reported the extraction efficiency depends upon the aqueous phase pH and the electrostatic and hydrophobic interactions are the driving forces responsible for the extraction.

It can be seen from the Figure 2; the back extraction yield was very low in all cases. The extraction which was nearly 13% at pH 3, reduced further on increase in forward transfer pH. The specific activity also showed a considerable decrease (648 U/mg) in the range pH 3 and 5.

To study the effect of surfactant concentration extraction efficiency of RME, the addition of Tween - 20 to the organic phase was varied. 5, 7.5,10, 50 µl of Tween-20 was added during different runs. Increase in

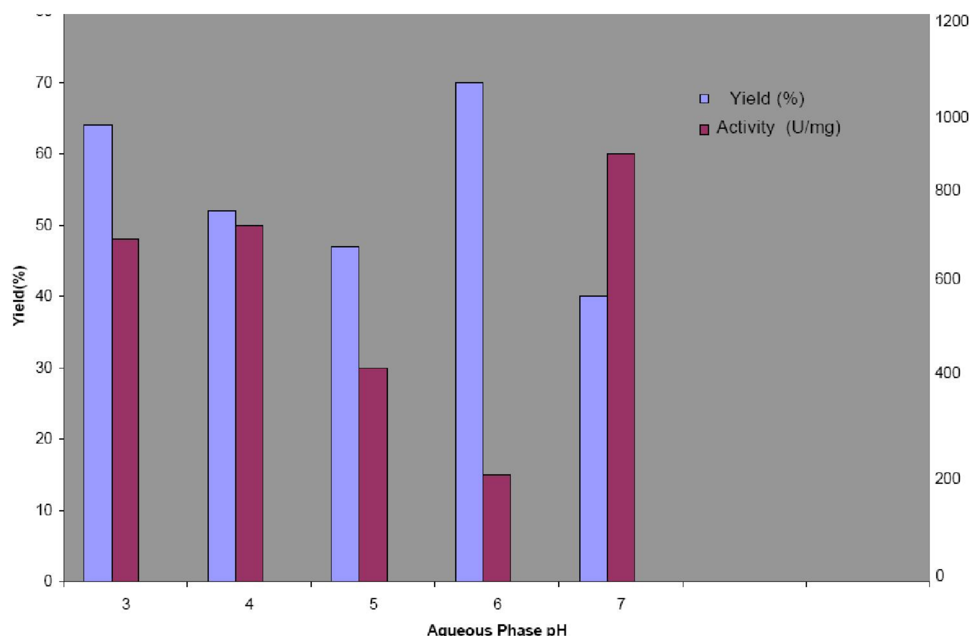

Figure 1 : Effect of pH on forward transfer Triton X-100

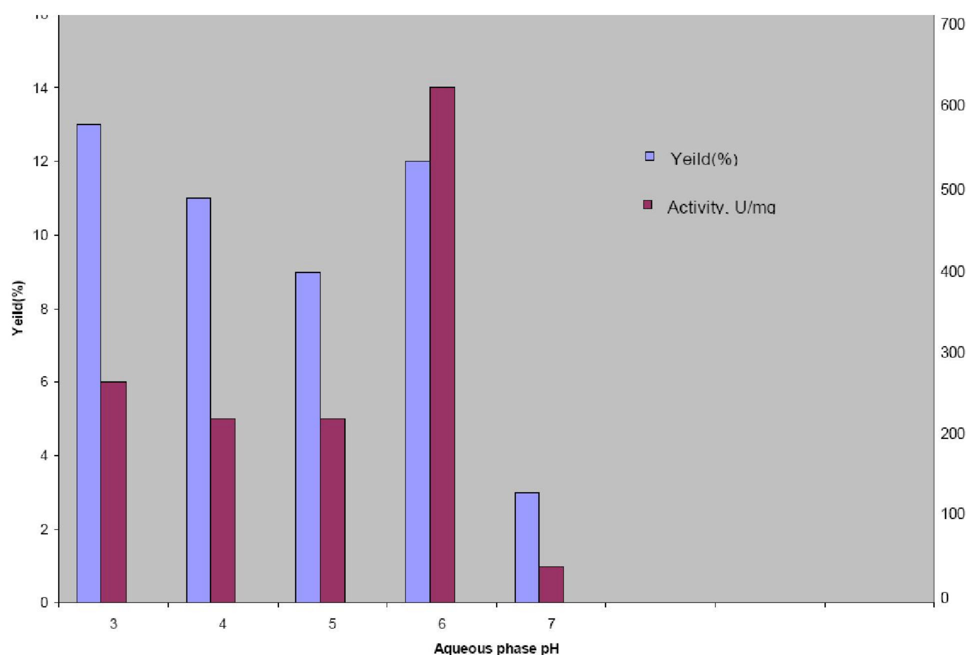

Figure 2 : Effect of pH on backward transfer Triton X-100

surfactant concentration increased the overall yield with maximum extraction of 56% at 10µl concentration. With the further increase in surfactant concentration the extraction was reduced. The activity showed a slight decrease with increased concentration up to 10µl (Figure 3)

The extraction efficiency could not be estimated for forward extraction as Tween-20 in aqueous phase was forming a gel.

The banding pattern in SDS-PAGE gel was analyzed. Lane 1 showed the banding pattern of enzyme marker, Ovalbumin of low molecular weight 43000 Da. Lane 2 showed the banding pattern of antibody conjugated HRP. A total of 3 polypeptide bands were observed in lane 2 among which two bands were the heavy and light chains of antibody and the other band was of HRP. One of the band seen in the lane 3 was specific to that of the marker and the conjugated HRP.

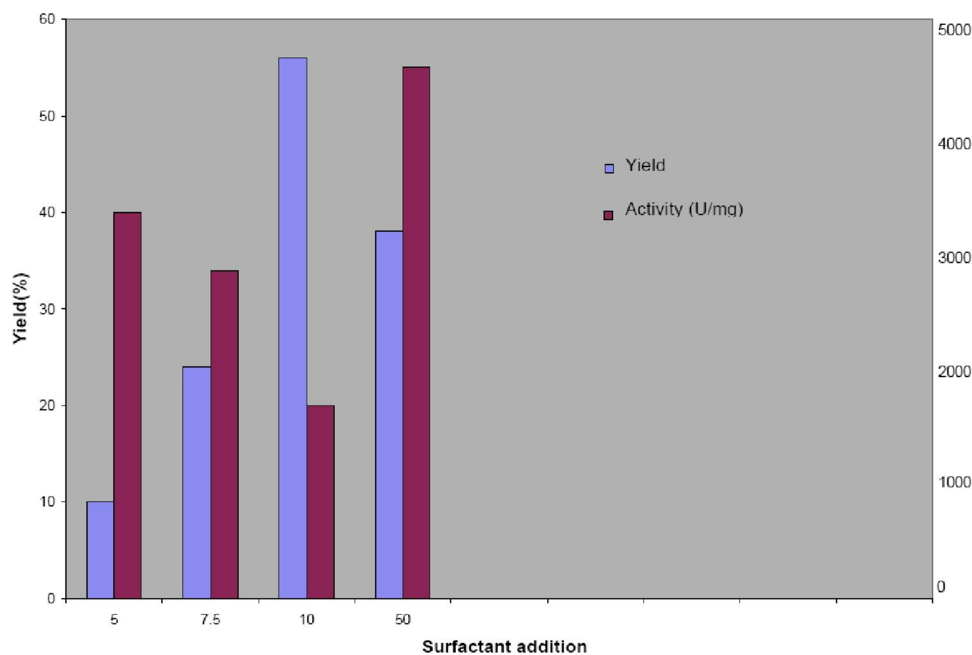

Figure 3 : Effect of surfactant addition on yield and specific activity, Tween-20.

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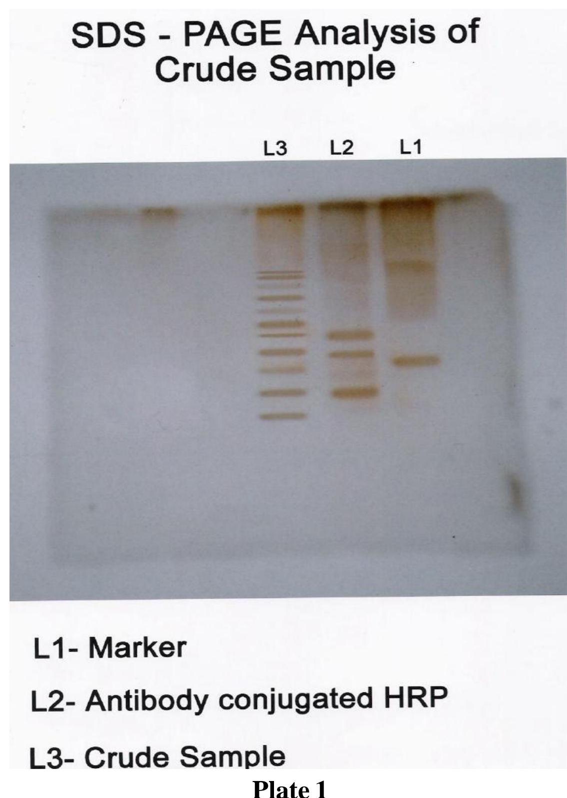

Plate 1

Thus, the presence of peroxidase enzyme in the crude sample was confirmed (Plate No: 01).

Gas chromatography analysis peroxidase was carried out in separate column for Standard peroxidase and crude sample. The data processed using specific software given 12 separate peaks. The peak obtained at retention time of 00.57 second confirmed the presence of peroxidase in the crude sample on comparing with the standard's peak. Other peaks were not relevant with this study.

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