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Purification of Peroxidase from Horseradish (*Armoracia rusticana*) Roots

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Peroxidase (EC 1.11.1.7) from horseradish (*Armoracia rusticana*) roots was purified using a simple, rapid, three-step procedure: ultrasonication, ammonium sulfate salt precipitation, and hydrophobic interaction chromatography on phenyl Sepharose CL-4B. The preparation gave an overall yield of 71%, 291-fold purification, and a high specific activity of 772 U mg^{-1} protein. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis revealed that the purified enzyme was homogeneous and had a molecular weight of approximately 40 kDa. The isolated enzyme had an isoelectric point of 8.8 and a Reinheitszahl value of 3.39 and was stable when stored in the presence of glycerol at −20 °C, with >95% retention of original enzyme activity for at least 6 months. Maximal activity of purified horseradish peroxidase (HRP) was obtained under different optimized conditions: substrate (guaiacol and H_2O_2) concentrations (0.5 and 0.3 mM, respectively), type of buffer (50 mM phosphate buffer), pH (7.0), time (1.0 min), and temperature of incubation (30 °C). In addition, the effect of HRP and H_2O_2 in a neutral-buffered aqueous solution for the oxidation of phenol and 2-chlorophenol substrates was also studied. Different conditions including concentrations of phenol/2-chlorophenol, H_2O_2, and enzyme, time, pH, and temperature were standardized for the maximal activity of HRP with these substrates; under these optimal conditions 89.6 and 91.4% oxidations of phenol and 2-chlorophenol were obtained, respectively. The data generated from this work could have direct implications in studies on the commercial production of this biotechnologically important enzyme and its stability in different media.

KEYWORDS: Horseradish; peroxidase; enzyme; purification; characterization and stability of peroxidase; phenol and 2-chlorophenol oxidation

INTRODUCTION

Peroxidases (EC 1.11.1.7) are a class of heme-containing enzymes that can be used to reduce hydrogen peroxide while oxidizing a second substrate (*1*). Peroxidase catalyzes the following redox reaction:

$$H_2O_2 + \text{substrate}_{\text{reduced}} \leftarrow \text{peroxidase} \rightarrow 2H_2O + \text{substrate}_{\text{oxidized}}$$

Classified as an oxidoreductase, peroxidase has been extensively used as an important component of reagents for clinical diagnoses and various laboratory experiments. Some of the physiological roles that have been assigned to peroxidase include indole-3-acetic acid metabolism, lignification, cross-linking of cell wall polymers, suberin formation, and resistance to infection (*1*, *2*). There are many novel applications of peroxidase; the most recent include treatment of wastewater containing phenolic compounds (*3*−*7*), decolorization of waste (*8*), and removal of peroxide from foodstuffs and industrial effluents (*9*).

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Peroxidases have been purified from many sources (*10*−*14*), the major source being horseradish (*11*−*14*). Most of the existing procedures for purification of horseradish peroxidase (HRP) are expensive and time-consuming traditional methods of protein purification with resultant low specific activities, yields, and fold purification. With the increasing use of HRP for clinical and industrial applications, there is a need for the development of a simple, rapid, and cost-effective purification method that can be easily scaled up; we report here the results of such a study.

The sources of raw drinking water such as underground water and river and lake water could be contaminated with many toxic aromatic and aliphatic compounds; among these, phenol and phenolic compounds are the most common aromatic pollutants (*3*−*7*). They represent a potential danger to human health because almost all are toxic, and many of them are known or suspected carcinogens. These aromatics are not easily biodegradable in conventional water treatment facilities. Several chemical, microbiological, or enzymatic methods have been developed for the removal of phenolics from wastewaters, sludges, industrial effluents, and soils (*3*−*7*, *15*−*17*). Chemical methods, such as ozone treatment (*15*), are very expensive, and chlorine oxidation (*16*) may

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give rise to certain toxic chloro-organic compounds, which in turn are more dangerous than phenol itself. Microbiological treatment (*17*) of drinking water is not safe and is normally not in common practice (because this may result in the introduction of undesirable disease-producing bacteria and other microbes). Therefore, enzymatic methods have become considerably significant as new and good options to remove trace toxic aromatic compounds present in drinking water sources. Recently, peroxidase enzymes have gained tremendous momentum with reference to the removal of toxic organic and recalcitrant compounds from drinking water sources and industrial effluents (*3*−*9*). Isolation of peroxidase from horseradish and other plant sources is much cheaper than its production from microbial sources; therefore, plant peroxidases are used for many commercial applications including treatment of water (*3*−*7*). In tandem with purifying HRP to homogeneity, we have also standardized conditions for the maximal oxidation of phenol and 2-chlorophenol substrates using purified enzyme specimen. HRP purified using the method described in the present study could be a rich enzyme source for potential clinical and industrial applications.

MATERIALS AND METHODS

Enzyme Source. Horseradish (*Armoracia rusticana*) roots were purchased locally.

Chemicals. Authentic HRP enzyme (HRPF), catalase enzyme, guaiacol, hydrogen peroxide, potassium phosphate (dibasic), potassium phosphate (monobasic), boric acid, Tris, glycerol, acrylamide, bisacrylamide, ammonium persulfate, glycine, silver nitrate, formaldehyde, TEMED, potassium ferricyanide, 4-aminoantipyrine, phenol, and 2-chlorophenol were purchased from Fisher Scientific Co. (Fair Lawn, NJ). Bradford reagent, bovine serum albumin (BSA), and ethylenediaminetetraacetic acid (EDTA) were procured from Sigma-Aldrich Co. (Oakville, ON, Canada). Phenyl Sepharose CL-4B gel was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Coomassie brilliant blue, bromophenol blue, precision plus protein standards, and Econo column were products of Bio-Rad Laboratories (Hercules, CA). Commonly used inorganic chemicals and organic solvents of the highest analytical grade were obtained commercially and used without any further treatment.

Peroxidase Assay. Peroxidase activity was monitored spectrophotometrically by following the oxidation of guaiacol (2-methoxyphenol) to tetraguaiacol at 470 nm (*18*). The reaction mixture contained 3 mL of 50 mM phosphate buffer (pH 7.5), 0.5 mM guaiacol, 0.3 mM H_2O_2, and enough enzyme to give appreciable change in absorbance at 470 nm between 0 and 1 min. Reaction mixture without the enzyme served as a control. The enzyme activity is expressed in terms of units; 1 unit of peroxidase activity is defined as the amount of enzyme catalyzing the oxidation of 1 μmol of guaiacol per minute at 30 °C.

Protein Determination. Protein at each step of the purification procedure was determined according to the Bradford method (*19*). The amount of protein (mg) was obtained from A_{280nm} values by using a calibrated curve constructed using BSA as the standard ($y = 0.801x + 0.0011$).

Purification of HRP. All operations were carried out at 0−4 °C unless otherwise stated, and the working buffer was 50 mM phosphate buffer (pH 7.5). All centrifugations were carried out at 7500*g* for 20 min.

Preparation of Crude Extract. Diced horseradish roots (100 g) were suspended in 50 mL of working buffer, blended, and filtered using Whatman no. 4 filter paper by suction filtration. The volume was made up to 100 mL with working buffer, and this extract was sonicated (40 kHz) intermittently for three 15 min periods in ice. After three sonications, the solution was centrifuged (although there were no visible solid impurities in the sonic extract, the solution was turbid, so centrifugation was carried out instead of suction filtration). The residue containing negligible peroxidase activity was discarded, and the resultant transparent supernatant fluid rich in HRP activity was collected. This preparation (~100 mL) is referred to as the crude extract.

Ammonium Sulfate Fractionation. The crude extract (99 mL) was brought to 30% $(NH_4)_2SO_4$ saturation by slowly adding powdered $(NH_4)_2SO_4$ with stirring. The solution was allowed to stand for 1 h, and the precipitate formed was removed by centrifugation. The supernatant fluid was then brought to 65% $(NH_4)_2SO_4$ saturation, and the precipitate, which contained enzyme activity, was dissolved in 10 mL of 50 mM phosphate buffer (pH 7.5) containing 1.0 M $(NH_4)_2SO_4$ (buffer A).

Hydrophobic Interaction Chromatography (*HIC*). The $(NH_4)_2SO_4$ fraction (9.9 mL) was loaded onto phenyl Sepharose CL-4B gel contained in the Econo column (1.0 × 25 cm, total bed volume = 35 mL) preequilibrated with buffer A. The column was washed with 20 mL of buffer A at a constant flow rate of 60 mL h^{-1} using a Cole-Parmer Masterflex peristaltic pump (pressure maintained at 12 psi). HRP was then eluted with a gradient of decreasing $(NH_4)_2SO_4$ concentration (1.0−0.0 M) in 50 mM phosphate buffer (pH 7.5), and 24 5-mL fractions were collected (peroxidase activity and protein for each of the fractions were determined in triplicate). The HRP-active fractions were pooled, concentrated by $(NH_4)_2SO_4$, and redissolved in 5 mL of 50 mM phosphate buffer (pH 7.5) containing 20% glycerol. The purified enzyme specimen (5 mL) was stored at −20 °C. Dialysis was carried out to remove glycerol from the enzyme samples on the day of use.

Characterization of Purified Enzyme. Different conditions including enzyme and substrate (guaiacol and H_2O_2) concentrations, type of buffer, pH, time, and temperature of incubation were standardized for maximal peroxidase activity of the purified enzyme. The isoelectric point (p*I*) and Reinheitszahl (RZ) value of the final preparation (HIC fraction) were determined by using methods reported in the literature (*20*, *21*).

Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) of the native enzyme on an 8.0% gel was performed at 2 °C according to a procedure published earlier (*22*). Gel staining was carried out according to the silver staining method of Chevallet et al. (*23*). The approximate molecular weight of purified enzyme was determined by running aliquots of the isolated HRP in tandem with HRPF and molecular weight markers (precision plus protein standards from Bio-Rad).

Oxidation of Phenol and 2-Chlorophenol Using HRP. *Specimen Preparation*. All reactions were carried out in 50 mM phosphate buffer, pH 7.0 (buffer B), at room temperature. Studies involving the oxidation of phenol and 2-chlorophenol included the use of both isolated enzyme (purified in our laboratory, HRP) and authentic commercial enzyme (from Fisher Scientific Co., HRPF). Initially, the reaction mixture (2.5 mL) was prepared by adding 24 mM phenol/2-chlorophenol and 24 mM H_2O_2 to 2.45 mL of buffer B (in the case of blanks, H_2O_2 was eliminated from the reaction mixture). Fifty microliters of HRP (1.28 mg/mL) or HRPF (1.31 mg/mL) was then added to the reaction mixture. After an incubation period of 15 min at room temperature, 50 μL of catalase (2200 U/mL) was added to terminate the reaction by decomposing residual peroxide. Optimal conditions of phenol and 2-chlorophenol concentrations, HRP and HRPF concentrations, time, pH, and temperature for peroxidase-catalyzed oxidation of phenol and 2-chlorophenol were standardized.

Analysis. Visualization of the reaction was performed by adding 0.3 mL of potassium ferricyanide (83.4 mM in 0.25 M sodium bicarbonate solution) and 0.3 mL of 4-aminoantipyrine (20.8 mM in 0.25 M sodium bicarbonate solution) to the blank and experiment. The concentrations of phenol/2-chlorophenol were determined spectrophotometrically at 505 nm against buffer B.

Calculations for Peroxidase-Catalyzed Oxidation of Phenolics. Because the blanks (Bk) do not contain H_2O_2, it is assumed that there is no oxidation of phenolics (phenol and 2-chlorophenol in the present study); therefore, blank specimens should contain 100% phenolics (starting amount). In the experimentals (Exp), peroxidase brings about oxidation of phenolic compounds in the presence of H_2O_2; therefore, experimental samples would show depleted amounts of phenolics. The percentage of phenol or 2-chlorophenol is determined using the following formula:

$$\% \text{ phenolic} = (1 - \text{Exp}/\text{Bk}) \times 100$$

Data Analysis. The values reported are the mean of at least three independent determinations.

RESULTS

Purification of HRP to Homogeneity. HRP enzyme has several applications including treatment of water and food (*3*−*9*). The availability of purified enzyme with a high specific activity and

Table 1. Purification of Peroxidase from Horseradish Roots[a]

purification step	total activity (U)	total protein (mg)	specific activity (U mg^{-1} of protein)	yield (%)	purification (fold)
sonication	9640	3638	2.65	100	1.0
30 − 65% $(NH_4)_2SO_4$ fractionation	8146	375	21.72	84.5	8.2
HIC on phenyl Sepharose CL-4B	6873	8.9	772.25	71.3	291

[a] Data presented are for 100 g of fresh weight of horseradish roots. Values given are a mean of three independent determinations. One enzyme unit is defined as the amount required to transform 1 μmol of guaiacol per minute at 30 °C. Amount of protein (mg) was obtained from A_{280nm} values by using a calibrated curve constructed using BSA as the standard ($y = 0.801x + 0.0011$).

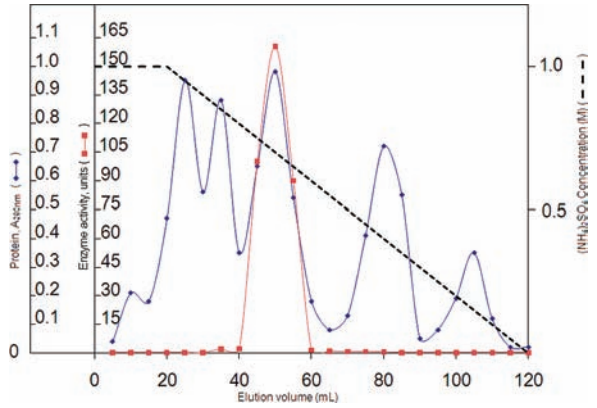

Figure 1. Hydrophobic interaction chromatography of horseradish (*Armoracia rusticana*) peroxidase on phenyl Sepharose CL-4B. Details are given under Materials and Methods. HRP activity (red area) is reported in terms of units; one enzyme unit is defined as the amount oxidizing 1 μmol of guaiacol per minute at 30 °C. For HIC fractions 9, 10, and 11 only (40−55 mL elution volume), the enzyme activity units represent values for 20-fold diluted fractions. Amount of protein (mg) was obtained from A_{280nm} values (blue area) by using a calibrated curve constructed using BSA as the standard ($y = 0.801x + 0.0011$).

good yield is a prerequisite for a biocatalyst in commercial applications. A profile of the simple and rapid three-step procedure for the purification of HRP developed in the current study is presented in **Table 1**. Ammonium sulfate precipitation of the crude extract followed by HIC on phenyl Sepharose CL-4B gel gave a homogeneous enzyme specimen. The final preparation (HIC fraction) gave an overall yield of 71%, 291-fold purification, and a high specific activity of 772 U mg^{-1} of protein. It is evident from **Table 1** that most of the unwanted protein was removed at the HIC step. HRP adsorbed on phenyl Sepharose gel was successfully eluted by employing a gradient and the enzyme eluted at a concentration of 0.74−0.66 M $(NH_4)_2SO_4$ (**Figure 1**).

The isolated HRP specimen was purified to homogeneity; a single protein band was obtained on SDS-PAGE (**Figure 2**). The respective R_f values for the molecular weight markers and enzyme specimens are as follows: protein standard, 37 kDa (0.44); protein standard, 50 kDa (0.33); HRPF, 40 kDa (0.42); and purified HRP (0.41). This indicates that the isolated enzyme has a molecular weight of approximately 40 kDa. Because single protein bands were obtained on SDS-PAGE in the presence of 2-mercaptoethanol, it can be concluded that the isolated HRP enzyme protein is a monomer. A RZ value of 3.39 obtained for the HIC fraction indicates a very high level of purity. This purified enzyme specimen had an isoelectric point of 8.8 and retained >95% of its original activity for at least 6 months when stored in the presence of glycerol at −20 °C. Freezing the enzyme in the absence of

Figure 2. SDS-PAGE of purified horseradish peroxidase (HRP), authentic horseradish peroxidase (HRPF), and molecular weight markers. Direction of migration is from top to bottom. Gel stained by silver staining procedure (*23*). Lanes are numbered from left to right. Lane 3, 4 μL of precision plus protein standards (Bio-Rad Laboratories); lane 5, 4 μL of HRPF (commercially obtained from Fisher Scientific Co.); lane 8, 4 μL of HRP. The respective R_f values for the molecular weight markers and enzyme specimens are as follows: protein standard, 37 kDa (0.44); protein standard, 50 kDa (0.33); HRPF, 40 kDa (0.42); purified HRP (0.41).

Table 2. Optimal Parameters for the Assay of Purified HRP[a]

parameter	range/ parameter tested	optimal value/ parameter
purified HRP enzyme concentration (1.28 mg of protein mL^{-1})	2.0−25.0 μL	10.0 μL
guaiacol concentration	0.1−1.0 mM	0.5 mM
H_2O_2 concentration	0.1−1.0 mM	0.3 mM
50 mM buffer, pH 7.5	borate, phosphate, Tris-HCl	phosphate
pH	6.0−9.0	7.0
time	0.0−3.0 min	1.0 min
temperature	25, 28, 30, 37, 42 °C	30 °C

[a] Under these optimized conditions, specific activity of native enzyme was 772 U mg^{-1} of protein. One enzyme unit is defined as the amount required to transform 1 μmol of guaiacol per minute at 30°C.

glycerol resulted in inactivation, and storage at 0−2 °C gave a decrease in activity by about 40−45% within 48−72 h.

Optimal Parameters for the Assay of Purified Peroxidase. The various parameters that were optimized to obtain maximal HRP activity with purified enzyme specimen are shown in **Table 2**, and the isolated native enzyme gave a high specific activity of 772 units mg^{-1} of protein under these standardized conditions. During the monitoring of the peroxidase activity by following the oxidation of guaiacol (colorless) to tetraguaiacol (brown) in the presence of H_2O_2 at 470 nm, linear absorbance values were obtained for enzyme concentration up to 17.5 μL and an incubation time of 1.5 min. The concentrations of enzyme, guaiacol, and H_2O_2 exceeding the optimal values (10.0 μL, 0.5 mM, and 0.3 mM, respectively) did not show significant increase in HRP activity. Although peroxidase activity was comparable in the pH range of 6.0−7.5 (optimal at pH 7.0), significant decline in enzyme activity was observed when the pH was increased to ≥8.0.

Table 3. Effect of Substrate and Enzyme Concentrations and Incubation Time on the Oxidation of Phenol and 2-Chlorophenol[a]

parameter	range/ parameter tested	optimal value/ parameter
purified HRP enzyme (HRP) concentration (1.28 mg of protein mL^{-1})	2.0−25.0 μL	10.0 μL
authentic HRP enzyme (HRPF) concentration (1.13 mg of protein mL^{-1})	5.0−20.0 μL	7.5 μL
phenol concentration	0.1−1.0 mM	0.2 mM
2-chlorophenol concentration	0.1−1.0 mM	0.2 mM
H_2O_2 concentration	0.1 −1.0 mM	0.3 mM
time	5.0−60.0 min	15.0 min

[a] HRP, HRP purified in our laboratory; HRPF, HRP obtained commercially from Fisher Scientific Co.

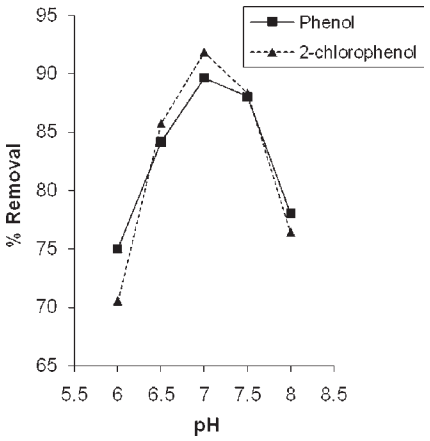

Figure 3. Effect of pH on the oxidation of phenol and 2-chlorophenol using HRP. At the optimum pH (7.0), 89.6% of phenol and 91.8% of 2-chlorophenol were oxidized, respectively. The calculations involved in determining the percentage of phenolics oxidized are explained under Materials and Methods.

Thermal denaturation of isolated HRP occurred at temperatures >42 °C, and the enzyme activity decreased rapidly.

Oxidation of Phenol and 2-Chlorophenol Using HRP. Recently, enzymes including peroxidases have shown promise for removing trace aromatic compounds in groundwater and wastewater sources. Enzymatic polymerizations offer the advantages of low process energy requirements and low solubility of polymerized product (*3*). With this objective in mind, we have conducted a systematic study to investigate the reaction of HRP with phenol and 2-chlorophenol substrates.

Effect of Enzyme and Substrate Concentrations and Time of Reaction. It was observed that for both the maximal activity of HRP (**Table 2**) and its reaction with phenolics (**Table 3**), the optimal concentrations of the enzyme and H_2O_2 were identical, that is, 10 μL of HRP (1.28 mg of protein mL^{-1}) and 0.3 mM, respectively. However, the optimal concentrations of phenol and 2-chlorophenol (0.2 mM) were substantially lower than the optimal concentration of guaiacol (0.5 mM) for maximal HRP activity. Linear absorbance values (at 505 nm) were obtained for enzyme concentration up to 17.5 μL (for both HRP and HRPF) when the oxidation of phenol/2-chlorophenol was determined. No significant enhancement in peroxidase enzyme activity was observed when phenol and 2-chlorophenol were substituted as substrates in place of guaiacol. Oxidation of phenol/2-chlorophenol on reaction with HRP in the presence of H_2O_2 reached a plateau at about 90% within 15 min.

Effect of pH and Temperature. The effects of pH and temperature on HRP-catalyzed oxidation of phenol and 2-chlorophenol

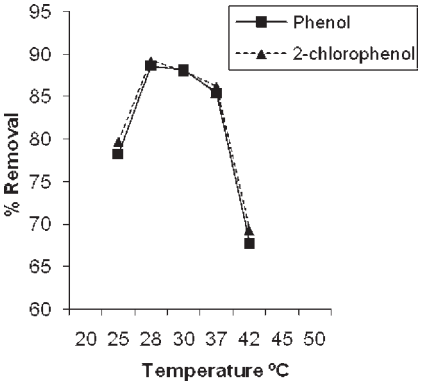

Figure 4. Effect of temperature on the oxidation of phenol and 2-chlorophenol using HRP. At the optimum temperature (28 °C), 88.6% of phenol and 89.2% of 2-chlorophenol were oxidized, respectively. The calculations involved in determining the percentage of phenolics oxidized are explained under Materials and Methods.

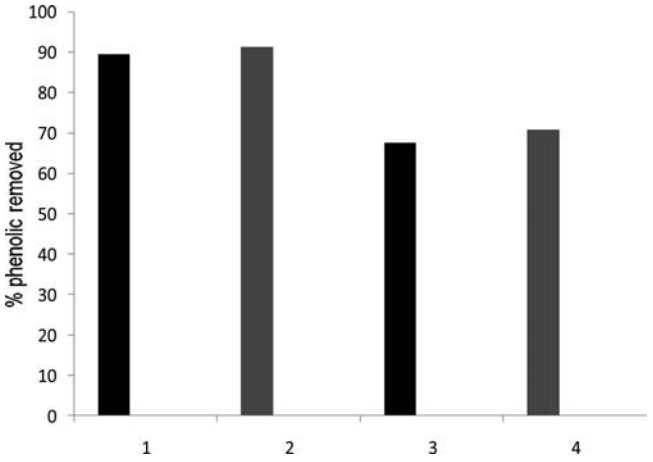

Figure 5. Efficiency of HRP and HRPF in the oxidation of phenol and 2-chlorophenol. Columns: 1, % phenol oxidized with HRP; 2, % 2-chlorophenol oxidized with HRP; 3, % phenol oxidized with HRPF; 4, % 2-chlorophenol oxidized with HRPF. The efficiency of oxidizing phenol (solid black filling) and 2-chlorophenol (solid gray filling) with HRP was about 89.6 and 91.4%, respectively; and with HRPF was approximately 67.5 and 70.8%, respectively. The calculations involved in determining the percentage of phenolics oxidized are explained under Materials and Methods.

are illustrated in **Figures 3** and **4**. When 50 mM phosphate buffer at pH 7.0 (optimum pH) was used, 89.6% oxidation of phenol and 91.8% oxidation of 2-chlorophenol were obtained (**Figure 3**). The optimum temperature for maximal enzyme reactivity with phenolics was 28 °C; this was comparable to the optimal temperature of 30 °C for maximal peroxidase activity of purified enzyme (**Table 2**). At this temperature (28 °C), HRP oxidations of phenol and 2-chlorophenol were 88.6 and 89.2%, respectively (**Figure 4**). The isolated enzyme was probably denatured at temperatures exceeding 42 °C, resulting in negligible activity with the phenolics. This trend was also observed at extremes of pH; that is, the enzyme gave appreciable effect only in the pH range of 6.0−8.0.

HRP and HRPF Activity with Phenolics. Oxidation of phenol and 2-chlorophenol substrates was tested with both HRP and HRPF (**Figure 5**). The reactivities of HRP with phenol and 2-chlorophenol were about 89.6 and 91.4%, respectively. This preparation gave significantly higher values than those obtained with HRPF (67.5 and 70.8%, respectively).

Table 4. Peroxidase Purification by Different Workers

organism	no. of steps employed	purification (fold)	yield (%)	specific activity (U mg^{-1})	ref
Armoracia rusticana (horseradish)	3	13.5	90.0	4.8	*12*
Armoracia rusticana (horseradish)	3	23.6	29.5	N/A[a]	*24*
Ipomoea palmata (morning glory)	4	48.6	75.3	349.8	*25*
Brassica napobrasica (turnip)	3	80.3	20.3	512.0	*26*
Armoracia rusticana (horseradish)	4	80.0	46.0	86.0	*27*
Armoracia rusticana (horseradish)	3	41.0	28.0	N/A	*28*
Armoracia rusticana (horseradish)	3	291.0	71.3	772	present study

[a] N/A, data not available.

DISCUSSION

There has been an increased interest in the use of peroxidase for water remediation and food treatment (*3*−*9*). Several methods have been developed for the isolation and purification of peroxidase from different sources (*10*−*14*, *24*−*28*). The present study extends and improves further the earlier work on purification of peroxidase (**Table 4**). We have obtained a highly purified HRP enzyme (291-fold purification), and the level of purity is significantly higher than the values reported by other workers (*12*, *24*−*28*). In addition to obtaining a yield (71% recovery) that is higher than or comparable to most reported methods, the specific activity reported here (772 U mg^{-1} of protein) is considerably higher than the values of 4.8 and 86 for HRP-purified specimens reported by Miranda et al. (*12*) and Regalado et al. (*27*), respectively, and is 1.5−2.2-fold greater than the specific activities of 349.8 and 512.0 U mg^{-1} reported by Srinivas et al. (*25*) and Singh and Singh (*26*). Production of a homogeneous enzyme specimen (**Figure 2**) with very high specific activity and fold purification has several economical advantages including use of only microliter volumes of the enzyme and substrates making up the reaction milieu. This cuts down operational costs considerably.

In their study on HRP purification by reversed micellar extraction, Huang and Lee (*29*) have reported that the specific activity increases to 1100 U mg^{-1} of protein using 55 U mg^{-1} of crude protein (20-fold). In our study, the specific activity increase from the crude extraction step to the salt precipitation step is 2.65−21.7 (about 8-fold) and by subjecting the salt-precipitated sample to HIC is from 21.7 to 772 (about 36-fold), to give a final purification of 291-fold. Therefore, the level of enzyme purity obtained in the present work as compared to that of Huang and Lee is greater by a factor of about 15-fold. It is difficult to strictly interpret their data in light of the present work because they have reported HRP activity in terms of micromoles of H_2O_2 decomposed rather than the norm of reporting HRP activity in terms of micromoles of guaiacol oxidized to tetraguaiacol, and there are no data on peroxidase inactivation over time. It is pertinent to mention here that for potential peroxidase enzyme applications in water remediation and food treatment, high yield and stability are considered issues with high priority. To address the issue of enzyme inactivation with time, we have successfully demonstrated the stability of purified HRP when stored in the presence of glycerol at −20 °C (95% of original enzyme activity was retained for at least 6 months). Glycerol is known to stabilize enzymes by preferential hydration of the protein (*30*), and the polyhydric alcohol may be stabilizing HRP active conformation in a similar manner. Our immediate future goal is to use stabilizing additives, including metal ions, to maintain enzyme activity over long-time storage.

Literature review shows that HRP is a multifunctional, heme-containing enzyme made up of many different isoenzymes (*31*,*32*), with isozyme C (HRP-C) being the most abundant and important. HRP-C is a single peptide chain with a molar mass of about 44 kDa and an isoelectric point in the range of 8.5−9.0 (*32*). The presence of a distinct single band for our purified HRP specimen (**Figure 2**) confirms that our preparation contains a monomeric protein. Although we have not carried out isolectric focusing to conclusively determine the isozyme nature, we have obtained an isolectric point of 8.8 for the soluble enzyme preparation using the method of Jaffe (*21*). In addition, a RZ value of 3.39 obtained indicates isolation of highly pure enzyme.

Phenolic compounds contaminate many industrial wastewater streams, particularly those from the oil-refining, polymer-processing, and fiber industries (*3*−*7*). Because conventional treatment methods such as chemical, physical, and biological processes (*15*−*17*) are not always suitable, enzyme-based treatment methods have been developed (*3*−*9*). Such methods have several advantages because they act with specificity and are very efficient in removing targeted compounds and the enzymes are easy to handle and store. In addition to purifying HRP to homogeneity, the present study involved substantial biochemical analyses of purified HRP specimen for its effectiveness in transforming phenol and 2-chlorophenol to free radicals that precipitate easily. In their study involving phthalic anhydride-modified horseradish peroxidase treatment of aqueous chlorophenols, Song et al. (*3*) have reported removal of 67% of phenol and 67.1% of 2-chlorophenol. Lai and Lin (*4*) have used immobilized horseradish peroxidase to remove 4-cholorophenol from wastewaters (they were able to obtain 25% removal at pH 7.5), and Nicell et al. (*33*) obtained 25% removal of 4-chlorophenol at pH 7.5. The results obtained in the present study are a significant improvement over several methods reported earlier in that we have successfully obtained removal of 89.6% of phenol and 91.4% of 2-chlorophenol by employing the conditions optimized in our laboratory (**Table 3**; **Figures 3** and **4**). Although our results are comparable to the 95% removal of phenol reported by Caza et al. (*34*) and the 80% removal of 4-chlorophenol reported by Siddique et al. (*35*), it is pertinent to mention here that we have developed a rapid procedure (15 min reaction time) in contrast to the 3 h polymerization times.

The optimal pH for maximal removal of phenol and 2-chlorophenol in the current study was found to be 7.0 (**Figure 3**). These results are in good agreement with data reported by other workers (*4*,*6*,*33*,*35*). In line with earlier findings on the removal of toxic phenolics from water, the enzyme purified in our laboratory gave appreciable effect only in the pH range of 6.0−8.0. Song et al. (*3*) have reported that there is a progressive decrease in HRP-catalyzed removal of chlorophenols above a temperature of 30 °C. We have also obtained a similar trend when studying the effect of temperature (**Figure 4**). In fact, HRP activity was negligible at temperatures >42 °C (data not shown). From **Figure 5**, it can be seen that we have obtained about 20% higher removal of toxic phenolics with HRP (about 90% removal) as compared to HRPF (about 70% removal). Therefore, the HRP preparation obtained in our laboratory has potential for commercialization.

In this study we have demonstrated a simple protocol for the purification of HRP and its feasibility in oxidizing toxic phenol and 2-chlorophenol. Our future studies are aimed at stabilizing HRP (most likely by immobilization techniques and by using stabilizing additives) for continuous use in removing toxic phenolic compounds and peroxide and scaling up the process for bulk enzyme production.

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