Abstract

The present work deals with extraction of cabbage peroxidase (CP) from fresh cabbage leaves and subsequent purification using ammonium sulphate (80% w/v) precipitation. The peroxidase extraction has been carried out by screening two different cabbage and then different parameters like different buffer systems, strength of buffers, buffer volumes, grinding time and cabbage leaves weight ratio to buffer volumes were optimized. The purified peroxidase showed maximum activity at pH 5.5 and at temperature 55 °C. The enzyme action followed the Michelis–Menton kinetics and gave a $K_m$ of 0.7018 mg/ml for Guaiacol oxidation over different concentrations (0 – 10 mg/ml) at pH 5.0 and Vmax was obtained as 0.6498 mg/min.ml. The molecular weight of the partially purified enzyme was found to be about 67,000 Daltons using SDS-PAGE and zymogram method.

Keywords: Enzyme activity, cabbage peroxidase, protein recovery, ammonium sulphate precipitation, kinetic parameters, optimization

Introduction

Extraction and purification of biomolecules is one of the critical steps in the field of bio-processing and based on the requirements of the biomolecules, many processing steps are involved for maximizing the recovery from the source and the required degree of purification (Desai et al. 2000; Liu et al. 1977). The goal of any process design is to obtain maximum recovery at lowest cost, or in other words, an effective optimization approach is required to analyze all the operating parameters with an objective of maximizing the recovery of bio-molecule of required purity. The current work deals with development of an inexpensive extraction procedure for peroxidase from the easily available agricultural source, cabbage with subsequent purification and characterization. An effort has been made to optimize each unit operation required in extraction, such as use of different buffers, buffer volume, grinding time etc. in the extraction process followed by partial purification using ammonium sulphate precipitation and characterization of the recovered peroxidase.

Although peroxidases are ubiquitous in the plant kingdom, at present the major source of commercially available peroxidase is the roots of horseradish (Wei et al. 2003). The extraction and purification of the peroxidase enzyme has been reported from various sources like apples (Angela et al. 2011; Kwanle et al. 2005), turnip roots (Ragaa et al. 1998), sweet tuber potato (Castillo et al. 2002) and Cynara scolymus flowers (Dorotea et al. 2003). Peroxidase (EC 1.11.1.7; donor: hydrogen-peroxide oxidoreductase) catalyses the oxidation reaction of various electron donor substrates (e.g. phenols, aromatic amines) and hydrogen peroxide. The general reactions can be expressed as follows:

$$E + H_2O_2 \rightarrow \text{Cpd.I} + H_2O \quad (1)$$
$$\text{Cpd.I} + AH_2 \rightarrow \text{Cpd.II} + AH^* \quad (2)$$
$$\text{Cpd.II} + AH_2 \rightarrow E + AH^* + H_2O \quad (3)$$

where E is the peroxidase enzyme, Cpd I and Cpd II are Compound I and Compound II, the oxidized intermediates of peroxidase; AH$_2$ and AH$^*$ are the electron donor substrate and the radical product of single electron oxidation, respectively. It is also known that peroxidase participates in the construction, rigidification and eventual lignification of cell walls; in the biosynthesis of $H_2O_2$; in the protection of plant tissues from damage and infection by pathogenic microorganisms and finally in wound healing (Tijssen et al. 1985). In vitro, this enzyme is widely employed in microanalysis as a diagnostic tool (Krell et al. 1991; Tamura et al. 1975). Currently, peroxidases are also used in organic synthesis for the production of polymers and for the biotransformation of various drugs and chemicals (Egorov et al. 2000; Wu et al. 2000).

Although we are the first one to report the extraction and purification of peroxidase from abundantly available renewable indigenous source i.e. cabbage, trying to give alternative to the
horseradish peroxidase (Valderrama et al. 2004), artichoke (Angel et al. 2011), Schizophyllum fungal peroxidase (Cheng et al. 2007), Marula fruit peroxidase (Kwanele et al. 2005) etc., we also presented optimized extraction protocol along with the necessary engineering information required for scale up. Thus obtained partially purified cabbage peroxidase is ready for immobilization by various methods and further its applications in azo dye degradation (Kim G et al. 2005), phenol degradation (Cheng J et al. 2006) and can be possibly useful in blood glucose determination kits or in making of bi-enzyme electrode (D. Mackey et al. 2007).

Materials and methods

Chemicals

Disodium hydrogen orthophosphate, sodium dihydrogen orthophosphate di-hydrate, ammonium sulphate, Triton X-100, methanol, sodium dodecyl sulfate, Silver Nitrate and sodium chloride were procured from Sisco Research Laboratories (Mumbai, India). Comassie brilliant blue G-250, Guaiacol, hydrogen peroxide (H2O2) were obtained from Biorad (USA). Sodium thiosulphate, Sodium carbonate, formaldehyde, glacial acetic acid, acrylamide, TEMED were procured from Himedia, India. All these chemicals were of analytical grade and used as received from the supplier. Fresh cabbage leaves were obtained from the local market.

Enzyme (Peroxidase) assay

Peroxidase activity was determined at room temperature using a spectrophotometer (Schimadzu, UV-1800) based on the quantification of Tetraguaiacol (λmax, 436 nm) formation in a 3-ml reaction mixture containing 2.8 ml of 0.1 M phosphate buffer at operating pH of 7.0, 0.05 ml of 15 mM 2-methoxyphenol (Guaiacol), 0.05 ml of 3 mM H2O2 and 0.1 ml of enzyme extract. The reaction was carried out for 6 minutes. One unit of peroxidase activity (U) represents the amount of enzyme which catalyses the conversion of one micromole of hydrogen peroxide per minute at 25 °C as per the following reaction:

\[
4\text{Guaiacol}+2\text{H}_2\text{O}_2 \xrightarrow{\text{peroxidase}} 4\text{Tetraguaiacol}+8\text{H}_2\text{O} \quad (4)
\]

Protein concentration estimation

Protein was determined by Bradford’s method, using bovine serum albumin as standard (Bradford et al. 1976)

One factor variation method

Extraction parameters were optimized by one factor at a time method which involves changing one of independent variables and maintaining all the other operating parameters at constant levels.

Preparation of crude extract

Fresh cabbage leaves were washed with distilled water at room temperature. The leaves were cut into pieces and homogenized with various buffer systems like phosphate, acetate and citrate buffer systems using a lab grade mixer grinder. Also, homogenization time was varied ranging from 1 min to 5 min. The homogenization speed was kept constant for all the experiments. The extract was filtered using a four layered cheese cloth to remove suspended fibrous solid particles. The clear filtrate was subjected to centrifugation (15000 × g, 20 min.) The clear supernatant obtained after centrifugation was used for further analysis and enzyme purification.

Screening of cabbage (selection of cabbage leaves for enzyme extraction)

To check the abundance of peroxidase content on the variety of cabbage leaves, two types of cabbage were tried for the extraction process as given below:

1. Green cabbage (Brassica oleracea L.var.capitata L. forma alba)
2. Chinese cabbage (Brassica oleracea L.var.capitata L. forma rubra)

Presence of peroxidase enzyme

During any extraction run, initially the whole cabbage is peeled off and first four to five layers (outer layers) and the most of the innermost tender leaves were homogenized separately. Analysis was carried out for the protein estimation and enzyme activity (peroxidase activity) separately. In any experimental run, 100 g of cabbage leaves were analyzed each time.

Effect of different buffers on peroxidase extraction

In this study, various buffers screened for the protein extraction include phosphate buffer (pH 5.0, 0.5 M, 100 ml), citrate buffer (pH 4.0, 0.5 M, 100 ml) and acetate buffer (pH 4.0, 0.5 M, 100 ml). The type of buffer was investigated keeping other factors like grinding time (3 min.), grinder speed, buffer volume and temperature of buffer (4 °C) constant.

Stability of enzyme in various buffer systems

The enzyme was extracted in various buffer systems as described in section 2.3.4 and each extract was analyzed for enzyme activity after every 24 hrs for a period of 7 days to determine the loss of enzyme activity.

Effect of strength of buffer

To study the effect of strength of the buffer on the peroxidase extraction from cabbage, various strength of phosphate buffer (pH 5.0, 150 ml, per 100 gm of cabbage leaves) were used over the range of 0.1 M to 2 M.

Effect of pH on enzyme activity

In order to study the effect of initial pH of the phosphate buffer (0.5 M, 100 ml), protein extraction was carried out at different buffer pH as 4, 4.5, 5, 5.5, 6 and 6.5. The other parameters like grinding time (3 min.), grinder speed, buffer volume, and temperature of buffer (4 °C) were kept constant.

Effect of buffer volume on protein extraction

The effect of buffer volume used during extraction was investigated by using different volumes as 50 ml, 75 ml, 100 ml, 125 ml and 150 ml) of phosphate buffer (0.5 M, pH 5.0) for the extraction of 100 gm of fresh cabbage leaves.

Effect of grinding time on particle size distribution

In the present study, the particle size distribution measurements were performed to investigate the dependency on the grinding time. Particle size analysis of cabbage leaves after grinding for various time intervals like 1 min, 2 min, 3 min, 4 min and 5 min using lab grinder was performed. After the grinding with phosphate buffer,
the slurry was filtered through the four layered muslin cloth with gentle squeezing. The cell debris from the slurry was subjected for particle size analysis and muslin cloth filtered cabbage juice was subjected for analysis of enzyme activity and protein determination. The particle size analysis was performed using optical microscope and Biovis image analysis software.

Effect of grinding time on extent of extraction

To study the effect of grinding time on the extent of protein extraction from the cabbage leaves, the cabbage leaves were ground for different time periods (1 min to 5 min) at the same grinding speed with phosphate buffer (0.5 M, pH 5.0, 100 ml, 4 °C) followed by agitated for 60 minutes using magnetic stirrer. Two identical sets of experiments were performed simultaneously to check the reproducibility. The supernatant obtained after solid filtration and centrifugation (15000 ×g, 20 min.) were analyzed for enzyme activity and protein content.

Thermal stability studies of enzyme

To study the effect of temperature on peroxidase activity in the cabbage juice, the extracted cabbage juice was exposed to various temperatures (30 °C, 40 °C, 50 °C, 55 °C, 60 °C and 65 °C) for 15 minutes.

Single stage and multistage extraction

To see the effect of protein extraction in single and multistage extraction, the proteins from fresh cabbage leaves (100 gm) were extracted in single stage as well as in a multistage extraction operation. In a single stage protein extraction, the phosphate buffer (0.5 M, pH 5.0, 4 °C, 150 ml) was used for the extraction of protein from freshly ground cabbage leaves (100 gm). In the case of multistage extraction, the proteins were extracted in five consecutive stages of extraction using phosphate buffer (0.5 M, pH 5.0, 4 °C) using 100 ml of buffer in each stage subjecting the treated leaves again and again. Other parameters like grinding time (2 min.), grinder speed, and temperature were kept constant.

Purification of protein by precipitation

To achieve the partial purification by ammonium sulphate precipitation, various concentrations of ammonium sulphate like 20 %, 40 %, 50 %, 60 %, 80 % and 95 % (w/v) have been investigated. The quantity of powdered ammonium sulphate to be added to obtain the desired concentration was estimated using the expression given below (Eq.5)

\[ G (\text{gl}^{-1}) = 533 (S_2-S_1) / 100-(0.3×S_2) \]  

Where,  

- \( G \): amount of ammonium sulphate at respective \( S \)  
- \( S_1 \): desired percentage of ammonium sulphate  
- \( S_2 \): initial concentration of ammonium sulphate in phosphate buffer

In the purification stage, the pre-filtered and clarified cabbage juice was subjected to ammonium sulphate addition. The salt addition was performed in an ice bath with constant stirring and the solution was allowed to stand at 4°C for 8h. After the complete sedimentation, the cabbage juice was centrifuged (15000×g, 20 min.) and the obtained precipitate obtained was again dissolved in 15 ml of phosphate buffer (0.5 M, pH 5.0, 4 °C) and again centrifuged (10,000×g, 10 min). The supernatant so obtained was dialyzed using dialysis membrane–60 (Hi media, India) against phosphate buffer (0.5 M, pH 5.0, 4 °C) for the removal of excess salt and analyzed for enzyme activity

Determination of Molecular weight of peroxidase by SDS-PAGE:

The purified peroxidase along with molecular marker standards (GENEI, Bangalore, India) was analyzed by SDS-PAGE (12 % w/v). SDS-PAGE electrophoresis (Biorad, USA) was carried out in the presence of 2-mercaptoethanol (1% w/v) and 0.5M Tris-HCl at pH 8.8 and detected with silver staining method. Pre-stained molecular weight markers in the range of 10– 170 kDa (Thermo, Fermentas) were used. After running the peroxidase enzyme sample against 200 V for approximately 30 min, the band of denatured protein on gel slab was fixed in methanol (40 % v/v) and formaldehyde (13.2 % v/v) solution for about 10 min. After 10 min, the gel slab was washed twice with deionized water for approximately 5 min each. The gel slab thus obtained was exposed to sodium thioulsulphate (0.2 mg/ml) solution followed by silver nitrate (1.0 mg/ml) solution. This pretreated gel slab was then treated with the developing solution which contains sodium carbonate (30 mg/ml), 125 µl of formaldehyde and 200 µl of sodium thiosulphate (0.2 mg/ml). Finally, the development of band was stopped with the help of glacial acetic acid (10 % v/v).

Zymogram

4ml of the purified enzyme was mixed with sample loading buffer (2ml) and analysed on 12.5% SDS–PAGE without prior boiling (McElvan et al. 1997). Gel was run at 200V for 1 h and soaked in 10% Triton X-100 for the displacement of SDS. Gel was washed with water to remove Triton X-100 and incubated for 10min in phosphate buffer (0.5M, pH 5.0), containing 20% H2O2 (v/v). For colour development, another gel slab which contains Guaiacol (50mg/10ml of separating gel) was prepared. The gel from Phosphate buffer was then superimposed on the gel slab containing Guaiacol and some hold time was allowed for the reaction of active peroxidase with Guaiacol, which gives pinkish red colour (Tetraguaiacol is formed)

Results and discussion

Screening of cabbages (selection of cabbage leaves for enzyme extraction)

Two varieties of cabbages i.e. green cabbage and Chinese cabbage were screened for the extraction of peroxidase enzyme. From Fig 1, it has been observed that the Chinese cabbage is more enriched in the protein content (5 mg/ml) than the green cabbage leaves (approximately 1 mg/ml). However, the presence of peroxidase enzyme in green cabbage is much higher (5.2 U/mg) than the Chinese cabbage (1 U/mg). Thus, green cabbage leaves were chosen for the extraction of cabbage peroxidase (CP). Extensive investigations of several peroxidases of different origins have been recently reported (Cinthia et al. 2006; Forsyth et al. 1999).

As stated above, we have examined the peroxidase activity from two varieties of cabbage source which is widely cultivated commercially as a vegetable in most of the Asian countries. However, the peroxidase enzyme can be present in different parts of the vegetable like per carp of fruits (Cheng et al. 2007), leaves of some plants (Srinivas et al. 1999), roots (Lopez et al. 1995), peel of sweet tuber potato (Castillo et al. 2002) etc.

Also, the quantity of peroxidase enzyme is found to be different at different locations of the same source. When the quantity of peroxidase enzyme was checked in outer leaves and inner leaves of both the cabbage varieties, it was found that, in the case of green cabbage, the outer leaves of green cabbage (A2) and inner leaves of green cabbage (A3) contains the same protein content
In the case of acetate buffer system, the enzyme activity decreases linearly (42% of the original activity was retained) over the period of 7 days. From the above studies we conclude that, phosphate buffer gives maximum protein extraction as well as good enzyme activity and stability (Fig. 4). Thus, for the further set of experiments phosphate buffer was used for protein extraction.

Effect of buffer strength on protein extraction

Conformational instability refers not only to unfolding, aggregation or denaturation but also to subtle changes in the localized protein domains and the alteration in enzyme catalytic properties (Farrell et al. 1989). These changes may result from the buffer-component binding, proton transfer and metal or substrate binding effects directly or indirectly mediated by the buffers themselves acting as pseudo-substrates. With this background the effect of buffer strength on enzyme activity and protein release has been investigated. When the fresh cabbage leaves (100 gm) were ground with phosphate buffer (pH 5.0, 150 ml) of different strengths, the resultant cabbage juice had a varying pH as shown the in Table 1. Also as shown in Fig 5, when the cabbage leaves were extracted with 0.5M phosphate buffer (pH 5.0, 150ml), it was found that the enzyme activity increased nearly 1.5 times than that obtained in the case of extraction with 1M phosphate buffer (pH 5.0, 150ml).

Lowest enzyme activity was obtained when the enzyme was extracted with 2M phosphate buffer. Cecil et al. (1959) and Liu et al. (1977) have reported that, as the thiolate anion (rather than the thiol) is a reactive species in the thiol–disulfide interchange, it is possible that the phosphate anion prevents the nucleophilic attack of the thiolate anion on the disulfide linkage and hence an increase in the buffer concentration decreases the extent of thiol–disulfide interchange (Wu et al. 2000) resulting into good enzyme activity. It should be also noted here that the specific activity was found to be approximately constant throughout the experimentation.

Effect of pH on enzyme activity

pH is a determining factor in deciding the enzyme activity as it alters the ionization state of amino acid chains or the ionization of the substrate. The effect of pH on the enzyme activity has been shown in fig 6 and it can be seen that the enzyme showed maximum enzyme activity at pH 5.5 and it decreased sharply with an increase in the pH. Similar results have been reported in the literature and it has been reported that the optimum pH for maximum activity of peroxidase from grape was 5.4, from banana was 4.5-5.0, from pineapple was 4.2, from HRP was 4.6-5.8, from potato was 5.0-5.4
Table 1: Effect of buffer strength on protein extraction and enzyme activity

<table>
<thead>
<tr>
<th>Strength of buffer (M)</th>
<th>Final pH after grinding</th>
<th>Total juice volume (ml)</th>
</tr>
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<tr>
<td>0.1</td>
<td>6</td>
<td>215</td>
</tr>
<tr>
<td>0.2</td>
<td>5.8</td>
<td>215</td>
</tr>
<tr>
<td>0.5</td>
<td>5.5</td>
<td>212</td>
</tr>
<tr>
<td>1</td>
<td>5.2</td>
<td>212</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>210</td>
</tr>
</tbody>
</table>

Figure 5: Effect of strength of buffer on protein extraction

Figure 7: Effect of different pH on peroxidase enzyme activity

Effect of grinding time on extraction

The obtained results for the effect of grinding time has been given in Fig. 9 & 12 and it can be observed that the total protein content for different grinding times was approximately the same (i.e., 0.61 mg/ml). In the case of 2 min grinding time (Fig. 12A), the enzyme activity is about 2 U/ml and it marginally decreases to about 1.5 U/ml for the higher grinding times as 3 min. (Fig. 12B) and 4 min. (Fig. 12C) of enzyme activity (the new activity was 1.15 U/ml).

Figure 8: Effect of buffer volume on total protein content and enzyme activity in different buffer volumes

Thermal stability studies of enzyme

In order to determine the thermal stability of the enzyme, aliquots of the enzyme were incubated at different temperatures varying from 30°C (room temperature) to 65°C for 15 min. The studies indicated that peroxidase activity slightly increased with an increase in temperature when incubated at 50°C and it increased rapidly at a temperature of 55°C. Above 55°C, a drastic loss of activity was observed as depicted in the fig. 13. The thermal stability and residual activity studies of the peroxidase at temperatures like 40°C, 50°C, 55°C, 60°C and 65°C have been performed by monitoring the
enzyme activity periodically at intervals of 15 min till a maximum time of 1 hr. It can be seen from Fig.13 that, the CP shows good stability at temperatures like 40°C, 50°C and 55°C for 1 hr. However, when CP was exposed to temperatures at 60°C and 65°C, CP rapidly degrades and after 1 hr of exposure, it nearly gets fully deactivated. The peroxidase has high thermal stability attributed to the presence of sugar in their structure (Mellon et al. 1991). However, this thermo-stability cannot be extended to all peroxidase due to the existence of isoenzymes with different resistance to Temperature (Valderrama et al. 2004). The main process found to be involved in the thermal denaturation of peroxidase was due to the dissociation of prosthetic groups from the holoenzyme, a conformation change in the apoenzyme and modification or degradation of the prosthetic group (Marcel et al. 1993).

**Effect of grinding time on particle size distribution**

Fig. 11 shows particle size distribution for various grinding times. It can be seen that when the cabbage leaves were ground for 4 or 5 min, most of the particles lie in the range i.e. 100µm-600 µm whereas for the grinding time of 1 min, 2 min, and 3 min, most of the particles lie in the range of 400 µm-1000 µm. The earlier extraction studies have indicated that 2 min of grinding time is adequate to completely extract the required protein and enzyme in the subsequent period of slow agitation.

Thus, 400 to 1000 µm particles as observed after 2 min of grinding time is adequate to completely extract the required protein and enzyme in the subsequent period of slow agitation.

**Single stage and multistage extraction**

From the Fig. 14 and the data given in Table 2, it is clear that multistage extraction mode gives beneficial results for the protein extraction as compared to the single stage extraction. In single stage extraction with phosphate buffer (0.5 M, pH 5.0, 4°C, 150 ml), it is observed that approximately 540.26 Units of peroxidase enzyme are extracted. However, in the case of multistage extraction with phosphate buffer (0.5 M, pH 5.0, 4°C), after three stages (each of 100 ml), 593.22 Units of peroxidase enzyme (Table 3) was extracted out. Thus, as shown in Table 2, multistage extraction gives higher extent of extraction as compared to the single stage extraction.

**Purification of protein by precipitation**

From the Fig.15, it can be seen that as the ammonium sulphate salt concentration increases from 40 % w/v to 80 % w/v, the hydrophobicity of peroxidase protein increases and it precipitates out. Thus, as shown in Table 2, multistage extraction gives higher extent of extraction as compared to the single stage extraction.
Enzyme Kinetics and Data Analysis

The oxidation of Guaiacol (coefficient of extinction, ε = 25.5 cm²/μmol) was measured at wavelength of 436 nm (Cheng et al. 2007) to assess the kinetics of enzyme action. The kinetics of reactions with different concentration of substrate was quantified in distilled water. The reaction mixture (3 ml) contained different concentrations of Guaiacol as a substrate (50 µl), 0.73 mM H₂O₂ (50 µl), 100 mM phosphate buffer (2.8 ml) and peroxidase enzyme (100 µl) was added lastly to start the reaction. Reaction was performed at room temperature (28±2 °C). The kinetic data were analyzed using double-reciprocal plot of the rate of the reaction versus substrate concentrations.

Table 2: Final observations for single and multistage extraction

<table>
<thead>
<tr>
<th>Stage</th>
<th>Single Stage Extraction</th>
<th>Multistage Extraction</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Stage I</td>
<td>Stage II</td>
</tr>
<tr>
<td>Total volume obtained (ml)</td>
<td>210</td>
<td>150</td>
</tr>
<tr>
<td>Enzyme activity (U/ml)</td>
<td>2.57</td>
<td>3.55</td>
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<tr>
<td>Total activity (U)</td>
<td>540.26</td>
<td>533.6</td>
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<tr>
<td>Total protein (mg)</td>
<td>359.56</td>
<td>343.95</td>
</tr>
<tr>
<td>Specific activity (U/mg)</td>
<td>1.50</td>
<td>1.551</td>
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</table>

Steady-state kinetic constants were obtained by measuring the initial rates of Guaiacol oxidation at 25 °C in 0.5 M sodium phosphate buffer of pH 5.0 for about 10 min. Initial steady-state rates were determined using a chronometric method, in which absorbance change at the wavelength of 436 nm were measured at different initial Guaiacol (0.0–10 mg/ml) concentrations.

The Lineweaver–Burk equation is as follows:

\[
\frac{1}{V_0} = \frac{(K_{\text{M}}/V_{\text{max}})}{1/[S]} + \frac{1}{V_{\text{max}}} \tag{6}
\]

Where: Kₘ: Michaelis menten constant for substrate (mg/ml), V₀: initial velocity at t = 0 (mg/min.ml), Vₘₐₓ: the maximum rate of reaction (mg/min.ml) attained at a specific enzyme concentration (U/ml) and substrate concentration (mg/ml) and Vₘₐₓ: initial velocity at t = 0 (mg/min.ml)

Data was fitted by linear regression to Eq. (7) which was obtained by a complete kinetic analysis of the mechanism depicted below in scheme 1.

\[
\frac{1}{V_0} = \frac{(K_{\text{M}}^C)}{V_{\text{max}}} \times \left( \frac{1}{[\text{Guaiacol}]} \right) + \frac{1}{V_{\text{max}}} \tag{7}
\]

where, Kₘ⁰: Michaelis menten constant for Guaiacol as a substrate (mg/ml), Vₘₐₓ: the maximum rate of reaction (mg/min.ml) attained at a specific enzyme concentration (U/ml) and substrate concentration (mg/ml) and Vₘₐₓ: initial velocity at t = 0 (mg/min.ml)

Figure 15: Partial purification by Ammonium sulphate precipitation (20%-95% w/v)

Table 4 reports the estimated kine tic parameters for different peroxidases from various sources) reported in the literature along with the parameters obtained in this work. The Lineweaver-Burk plot (fig.16) constructed using data presented in Table 5, gives Km of 0.7018 mg/ml for Guaiacol at pH 5.0 and Vmax was 0.6498 mg/min.ml.

Molecular weight determination

The molecular weight of cabbage peroxidase (CP) was determined by SDS-PAGE electrophoresis (fig17). The sample used for the molecular weight determination was the fraction obtained after ammonium sulphate precipitation (80 % w/v). According to Robinson (1991) the molecular weight for most plant peroxidase lies within the range of 40–50 kDa while Vamos-Vigyazo (1981) indicates a slightly wider range of 30–54 kDa. The peroxidase was
Conclusions

In the present study, an optimized protocol of a sequence of unit operations needed for the effective extraction of cabbage peroxidase from cabbage, which is commercially cultivated as a vegetable, has been proposed. The crude extract of cabbage containing CP was partially purified and characterized for some of its properties like thermal stability and molecular weight etc.

For the extraction of peroxidase enzyme from cabbage leaves, different buffer systems were investigated and the phosphate buffer system (0.5 M, pH 5.0) was proved to be the best. Phosphate buffer system not only extracts more peroxidase enzyme but also enhances the stability of peroxidase enzyme for a longer period. Also, for the maximum extraction of peroxidase enzyme, it was observed that the multistage extraction is better to extract more amount of peroxidase enzyme than a single stage extraction. The partially purified peroxidase showed maximum activity at optimum of pH 5.5 and temperature of 55°C. The molecular weight was confirmed to be around 67 kDa with the help of SDS-PAGE and Zymogram. The enzyme kinetic parameters were calculated as $K_m$ of 0.7018 mg/ml for Guaiacol oxidation at pH 5.5 with $V_{max}$ of 0.649 mg/min-ml. The present work has revealed the use of a completely new source for the extraction and purification of the peroxidase enzyme, which is one of the very important enzymes in the diagnostic field.

### Abbreviations

- CP: cabbage peroxidase
- ε: coefficient of extinction for Guaiacol
- Cpd I and Cpd II: oxidized intermediates of peroxidase
- AH$_2$: the electron donor substrate
- AH$: the radical product of its one electron oxidation
- S$_2$= desired percentage of ammonium Sulphate

### Tables

<table>
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<tr>
<th>Substrate</th>
<th>Guaiacol</th>
<th>$V_{max}$ (mg/min-ml)</th>
<th>$K_m$ (mg/ml)</th>
<th>H$_2$O$_2$</th>
<th>ABTS</th>
<th>DMP</th>
<th>CATECHIN</th>
<th>4-METHYL CATECHOL</th>
<th>CATECHOL</th>
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<td>0.649</td>
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<td>0.032</td>
<td>0.137</td>
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<td>(Cheng et al. 2007)</td>
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<td>Ipomoea palmasteh Peroxidase</td>
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<tr>
<td>(Srinivas et al. 1999)</td>
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<td>(Angela et al. 2011)</td>
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<td>(Deepa et al. 2002)</td>
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<th>Velocity (mg/min-ml)</th>
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Figure 16: Lineweaver-Burk plot for cabbage peroxidase (CP) for Guaiacol as substrate at pH 5.0

found to be about 67 kDa which is of a similar order of magnitude.
S<sub>i</sub> = initial concentration of ammo. Sulphate in phosphate buffer
E<sub>p</sub>: peroxidase enzyme
S<sub>0</sub>: Guaiacol as a substrate
[E<sub>p</sub>, S<sub>i</sub>]: Peroxidase and Guaiacol substrate complex
K<sub>i</sub>, K<sub>a</sub>& K<sub>3</sub>: reaction rate constants
P<sub>r</sub>: Tetraguaiacol as a product
K<sub>m</sub>: Michelis menten constant for Guaiacol as a substrate
V<sub>max</sub>: the maximum velocity attained at a specific enzyme concentration and substrate concentration and
V<sub>i</sub>: initial velocity at t=0

Acknowledgements
The authors are grateful to the Vice Chancellor, Institute of Chemical Technology (ICT) for providing the infrastructural facilities as well as constant encouragement. Support of fellowship to Pramod Kharatmol by University Grant Commission, Government of India is duly acknowledged.

References