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Extraction, partial purification and characterization of acidic peroxidase from cabbage leaves (*Brasicca olearacea var. capitata*)

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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 *Km* 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. Thus, rapid and effective optimizing methodologies for the extraction and subsequent purification steps are of great importance in the process development (Wheelwright et al. 1987). In any bio-molecule purification process, several unit operations are involved and optimizing each unit operation is the best way to present a process which ultimately proves to be cost effective with required specified 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; Kwanele 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 oxido-reductase) 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:

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

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

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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 (H_2O_2) 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 (λ_{max} 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 H_2O_2 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:

$$4\text{Guaiacol}+2H_2O_2 \xrightarrow{\text{peroxidase}} \text{Tetraguaiacol}+8H_2O \quad (4)$$

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 thoroughly 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 effect of 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)

$$G\ (gL^{-1}) = 533\ (S_2-S_1)\ /\ 100-(0.3\times S_2) \qquad (5)$$

Where, G= amount of ammonium sulphate at respective S_2
S_2= desired percentage of ammonium sulphate
S_1= 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 thiosulphate (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 (McLellan et al. 1987). 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% H_2O_2 (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 (approximately 1 mg/ml) and the enzyme units (approximately 1.65 U/ml) (Fig.1).

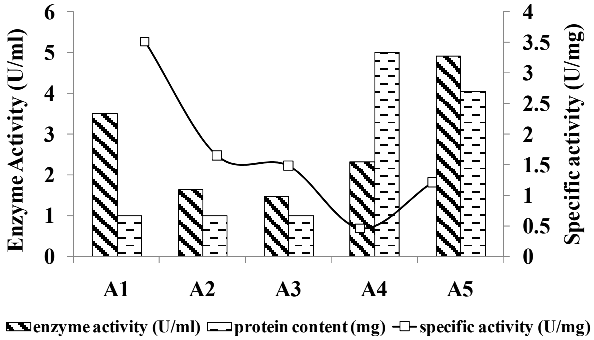


Figure 1: Presence of peroxidase enzyme at various parts of the cabbages

Though the overall protein content was found to be higher in Chinese cabbage, however, specific activity i.e. a ratio of total units of enzyme (U) to total proteins (mg) was found to be highest in the case of whole green cabbage (A1). Thus, as specified earlier, all subsequent experiments were carried out with green cabbage leaves.

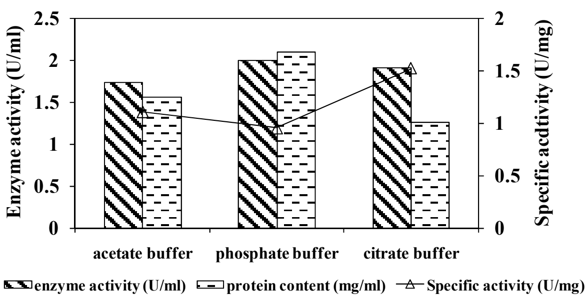


Figure 2: Effect of different buffers on protein extraction and enzyme activity from green cabbage per milliliter of cabbage juice.

Stability and Effect of different buffers on peroxidase extraction

As shown in Fig.2, we observed that, the total protein extracted with the citrate buffer (125 mg) is less than the total protein extracted with the acetate buffer (150 mg). Also, when the protein extraction was carried out with the help of phosphate buffer, maximum protein (2 mg/ml) was extracted out with higher enzyme activity (approximately 2 U/ml) than observed with acetate and citrate buffer. It can be also observed from Fig. 3 that, citrate buffer (1.5 U/mg) extracts lesser protein but it gives marginal higher specific activity than acetate (1.25 U/mg) and phosphate buffers (1.0 U/mg). As shown in Fig.4, in the case of phosphate and citrate buffer, the rate of decrease of the enzyme activity was about 10% to 30% of the original activity for first two days of storage. After this initial stage, the rate of decrease in the enzyme activity in the case of citrate buffer was much faster i.e. (only 45 % of original activity was retained) than that of phosphate buffer (75 % of the original activity was retained) system.

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

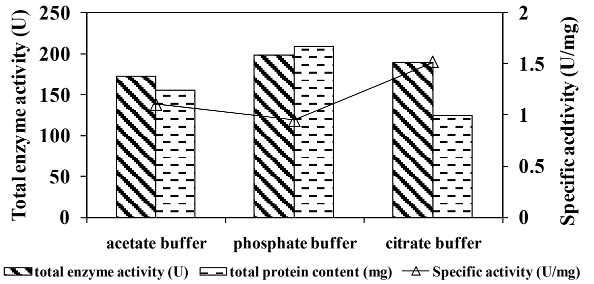


Figure 3: Total protein content and enzyme activity in different buffer systems

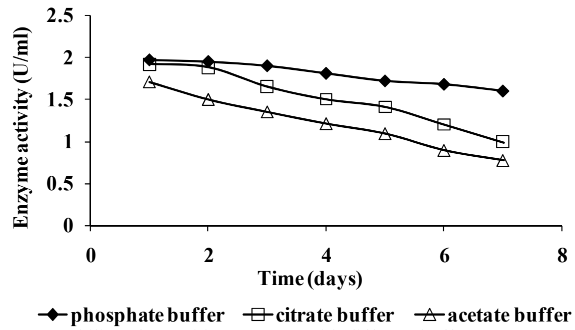


Figure 4: stability of peroxidase enzyme with different buffer systems

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

Strength of buffer (M)	Final pH after grinding	Total juice volume(ml)
0.1	6	215
0.2	5.8	215
0.5	5.5	212
1	5.2	212
2	5	210

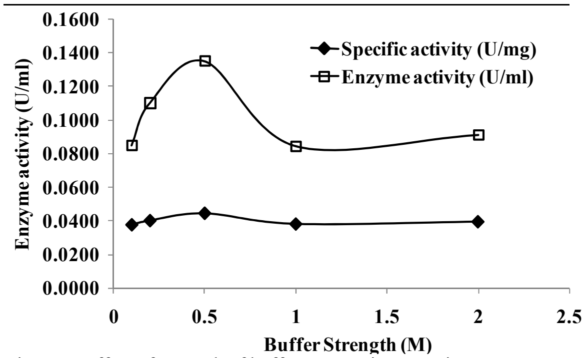


Figure 5: Effect of strength of buffer on protein extraction

and from wheat germ was 5.5-6.3 (Billaud et al. 1999). Lopez and Burgos (1995) reported that the release of heme group from the enzyme active site was pH dependent and occurred most rapidly at lower and higher pH and led to the loss in activity. The active site of the enzyme is mainly composed of ionic groups (prosthetic group) that must be in the proper ionic form in order to maintain the conformation of the active site of enzyme for substrate binding or reaction catalysis (Whitaker et al. 1995)

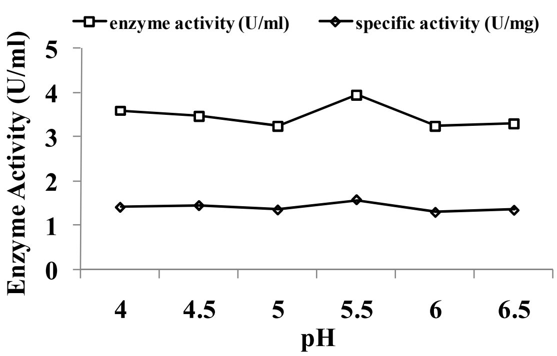


Figure 6: Effect of different pH on peroxidase enzyme activity

Effect of buffer volume on peroxidase extraction

As the buffer volume used for the peroxidase extraction increased from 50 ml to 150 ml, the extracted protein was found to be in more diluted form resulting into lower values of enzyme activity (U/ml). Fig.7&8 depicts the pattern of the extent of protein extraction with different buffer volume. It can be observed from fig. 7 that when buffer volumes of 50 ml, 75 ml, 100 ml, 125 ml and 150 ml were used, the observed protein content and enzyme activity were [3.22 (mg/ml), 2 (U/ml)], [2.48 (mg/ml), 1.75 (U/ml)], [2.38 (mg/ml), 1.75 (U/ml)], [2.38 (mg/ml), 1.5 (U/ml)], [2.38 (mg/ml),1.5 (U/ml)] respectively. Also, from Fig. 8, it can be seen that, there is a steady increase in the total protein content (mg).and total enzyme units (activity U/ml × volume) with an increase in the buffer volume used for protein extraction. Thus, for the further protein extraction studies, for each 100 gm of cabbage leaves, 150 ml of phosphate buffer has been used.

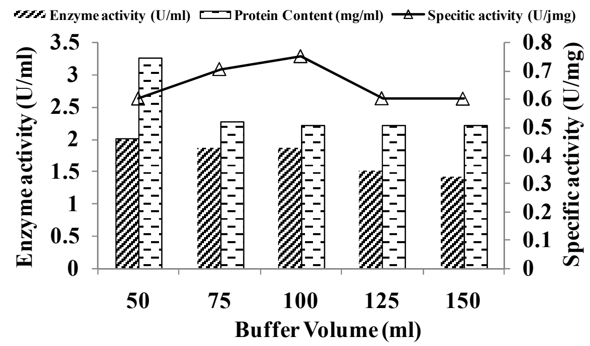


Figure 7: Effect of different buffer volumes on protein extraction and enzyme activity from green cabbage per milliliter of cabbage juice.

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 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).

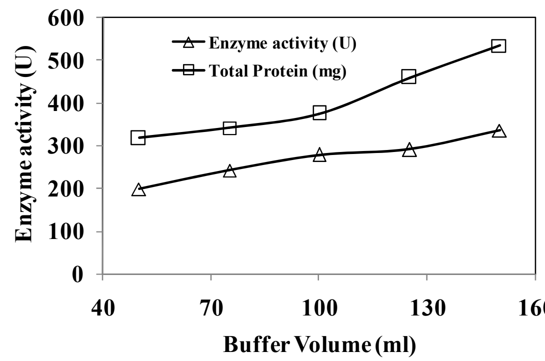


Figure 8: Effect of buffer volume on total protein content and enzyme activity in different buffer volumes

In the case of 5 min. (fig.12D) as the grinding time, it was observed that there is an increase in the local temperature of the system. As a result the proteins are likely to get denatured resulting into a net loss When the specific activity was calculated, it was observed that at 2 min of grinding time, the specific activity was found to be maximum (approximately 2.7 U/mg).

The variation in the enzyme activity, protein content and specific activity with grinding time has been shown in Fig. 9 and it has been observed that, at 2 min of grinding time, the protein content, enzyme activity and specific activity values were found to be the maximum.

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

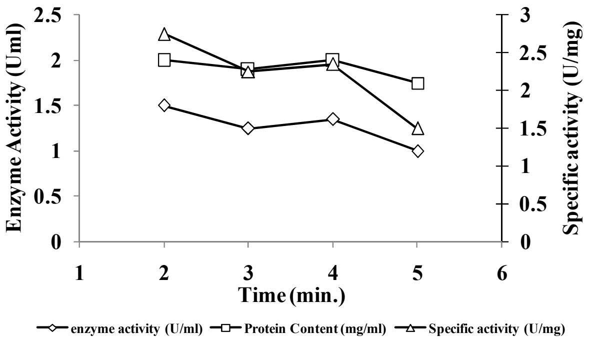


Figure 9: Effect of different grinding time on enzyme activity

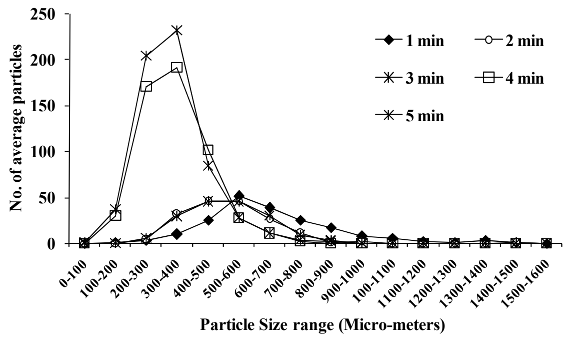


Figure 10: Particle size distribution for various grinding times

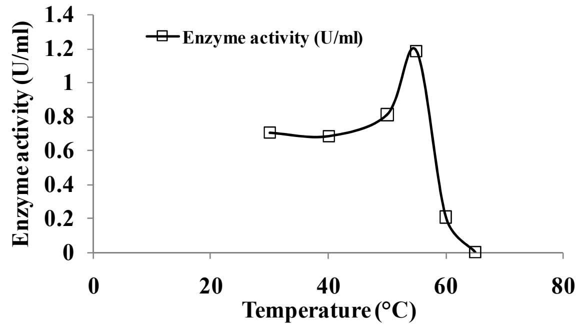


Figure 11: Effect of different temperatures on enzyme activity

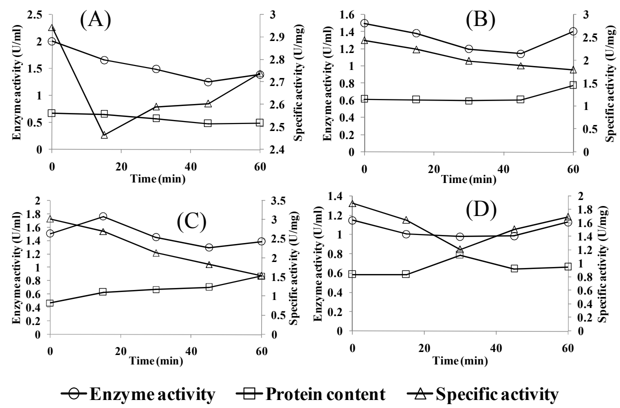


Figure 12: Effect of different grinding time on protein extraction (from A-D)

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 was optimum.

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.

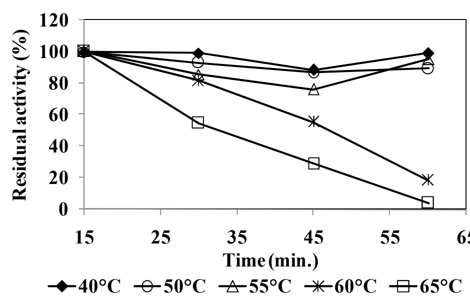


Figure 13: Thermal stability study of peroxidase at various temperatures and at different time intervals

Also, most of the peroxidase protein precipitates out when 80 % w/v of ammonium sulphate concentration is used. The maximum specific activity (14 U/mg) is obtained at 60 % w/v of final concentration of ammonium sulphate.

However, from fig. 15, it is clear that, for the partial purification of peroxidase, we can use the precipitated pool in between 40%-80% w/v of ammonium sulphate for maximum recovery of peroxidase.

Table 2: Final observations for single and multistage extraction

	Single Stage Extraction	Multistage Extraction					cumulative
		Stage I	Stage II	Stage III	Stage IV	Stage V	
Total volume obtained (ml)	210	150	100	100	100	100	550
Enzyme activity (U/ml)	2.57	3.55	0.499	0.096	0	0	-
Total activity (U)	540.26	533.6	49.97	9.6	0	0	592.57
Total protein (mg)	359.56	343.95	147.5	102.4	87.2	89.2	770.85
Specific activity (U/mg)	1.50	1.551	0.33	0.094	0	0	1.98

Enzyme Kinetics and Data Analysis

The oxidation of Guaiacol (coefficient of extinction, ε = 25.5 $cm^2/\mu mol$) 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 H_2O_2 (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 3: Final Findings for the Single and Multistage Batch Extraction

	Single stage extraction	Multistage extraction
Total protein (mg)	359.56	770.25
Total activity (U)	540.26	593.22
Specific activity (U/mg)	1.502	1.984

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:

$$1/V_0 = (K_M/V_{max}) \times (1/[S]) + (1/V_{max}) \quad (6)$$

Where,: K_M Michaelis menten constant for substrate (mg/ml), V0= Initial velocity at t = 0 (mg/min.ml), Vmax = Maximum rate of reaction obtained at specific concentration of substrate, pH and temperature (mg/min.ml) and [S] = concentration of substrate (mg/ml).

The Lineweaver–Burk equation for kinetic study for peroxidase with varying concentration of Guaiacol as a substrate with constant

H_2O_2 concentration is as follows:

$$1/V_0 = (K_M{}^G/V_{max}) \times (1/ [\text{Guaiacol}]) + (1/V_{max}) \quad (7)$$

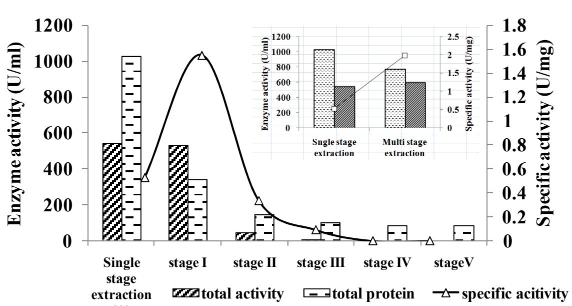


Figure 14: Effect of single and multistage batch extraction on protein extraction

where, $K_M{}^G$: Michelis menten constant for Guaiacol as a substrate (mg/ml), V_{max}: the maximum rate of reaction (mg/min ml) attained at a specific enzyme concentration (U/ml) and substrate concentration (mg/ml) and V_0: initial velocity at t=0 (mg/min.ml)

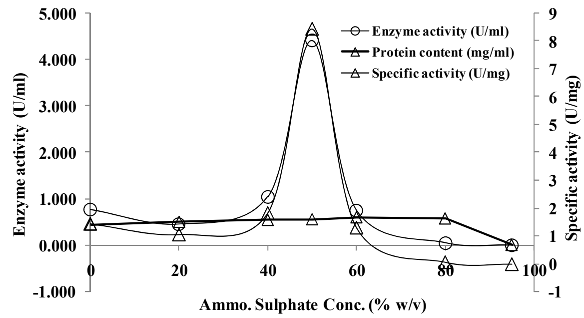
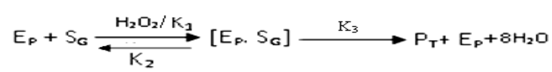


Figure 15: Partial purification by Ammonium sulphate precipitation (20%-95% w/v)

Table 4 reports the estimated kinetic 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.

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.



Where, E_P: peroxidase enzyme, S_G: Guaiacol as a substrate, $[E_P.\ S_G]$: Peroxidase and Guaiacol substrate complex, K_1, K_2& K_3: reaction rate constants, P_T: Tetraguaiacol as a product, Scheme 1: reaction mechanism proposed for peroxidase enzyme using Guaiacol as a substrate.

From table 4, we can observe that, the Km values for peroxidases like Horseradish peroxidase (571 mg/ml) (Putter et al. 1983), Ipomoea peroxidase (2259.5 mg/ml) (Srinivas et al. 1999), and peroxidase obtained from oil palm leaves (491.6 mg/ml) (Deepa et al. 2002) are much higher than the Km value of cabbage peroxidase (0.70 mg/ml), which indicates that, cabbage peroxidase obtained in this work has much higher affinities for Guaiacol as a substrate than the enzymes obtained from other sources.

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

Table 4: Kinetic parameters for oxidation of Guaiacol, H_2O_2, 2, 2'-azino-bis (3-ethyl-benzo thiazoline -6-sulfonic acid) (ABTS), 2, 6-dimethoxyphenol (DMP), catechin, 4-methylcatechol, catechol, 3, 4-Dihydroxyphenylpropanoic Acid (DHPPA) by various peroxidases

Substrate		Guaiacol	H_2O_2	ABTS	DMP	CATECHIN	4-METHYL CATECHOL	CATECHOL	DHPPA
Cabbage peroxidase	V_{max}	0.649	-	-	-	-	-	-	-
	K_m	0.7	-	-	-	-	-	-	-
Schizophyllum fungal peroxidase (Cheng et al. 2007)	V_{max}	0.032	0.137	0.025	0.006	-	-	-	-
	K_m	0.013	0.007	0.047	0.122	-	-	-	-
Marula fruit peroxidase (Kwanele et al. 2005)	K_m	-	60.18	-	-	409.3	177.5	549.4	619.9
Horseradish peroxidase (Valderrama et al. 2004)	V_{max}	14.6	-	-	-	-	-	-	-
	K_m	571	-	2459.8	-	-	-	-	-
Ipomoea palmetto Peroxidase (Srinivas et al. 1999)	V_{max}	3.364	-	-	-	-	-	-	-
	K_m	2259.5	-	-	-	-	-	-	-
Artichoke peroxidase (Angela et al. 2011)	V_{max}	0.073	-	-	-	-	-	-	-
	K_m	645.5	-	-	-	-	-	-	-
Oil palm leaf peroxidase (Deepa et al. 2002)	K_m	491.6	-	514.6	-	-	-	92.5	-

Note: V_{max} in mg/ (min.ml) and K_m in mg/ml

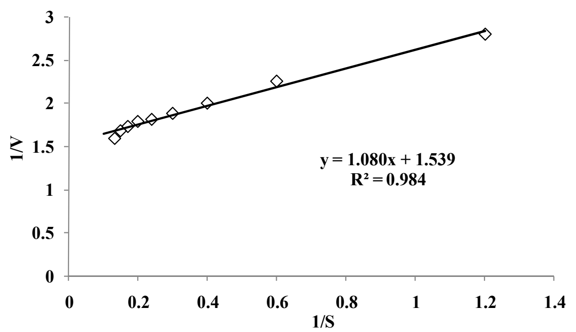


Figure 16: Lineweaver-Burk plot for cabbage peroxidase (CP) for Guaiacol as substrate at pH 5.0

found to be about 67 kDa which is of a similar order of magnitude.

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 *Km* of 0.7018 mg/ml for Guaiacol oxidation at pH 5.5 with Vmax of 0.6498 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 the one of the very important enzymes in the diagnostic field.

Table 5: Summary of the enzyme kinetics which gives an idea about initial steady-state rates at different concentrations of Guaiacol (0-10 mg/ml) catalyzed by cabbage peroxidase

Substrate conc. (mg/ml)	Velocity (mg/min ml)	1/s	1/v
0	0	-	-
0.833	0.357	1.20	2.796
1.667	0.443	0.599	2.252
2.500	0.500	0.400	1.998
3.330	0.531	0.300	1.880
4.167	0.552	0.239	1.811
5.000	0.559	0.200	1.787
5.833	0.578	0.171	1.728
6.667	0.595	0.149	1.679
7.500	0.628	0.133	1.590
8.333	0.704	0.120	2.796

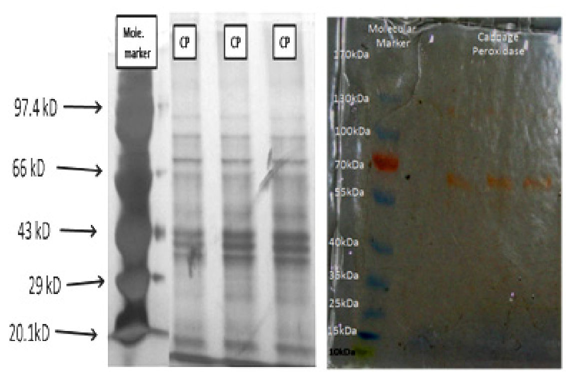


Figure 17: SDS-PAGE gel electrophoresis pattern for peroxidase enzyme stained by silver staining method, CP: samples of cabbage peroxidase and zymogram

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
$AH^{\bullet}$: the radical product of its one electron oxidation
S_2= desired percentage of ammonium Sulphate

S_I= initial concentration of ammo. Sulphate in phosphate buffer
E_P: peroxidase enzyme
S_G: Guaiacol as a substrate
$[E_P. S_G]$: Peroxidase and Guaiacol substrate complex
K_1, K_2& K_3: reaction rate constants
P_T: Tetraguaiacol as a product
Km^G: Michelis menten constant for Guaiacol as a substrate
Vmax: the maximum velocity attained at a specific enzyme concentration and substrate concentration and
V_O: initial velocity at t=0

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