Journal of Molecular Catalysis B: Enzymatic 60 (2009) 36–44

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Purification and characterization of black gram (*Vigna mungo*) husk peroxidase

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ARTICLE INFO

Article history:
Received 11 August 2008
Received in revised form 12 March 2009
Accepted 17 March 2009
Available online 1 April 2009

Keywords:
Peroxidase
Black gram husk
Enzyme purification

ABSTRACT

Black gram is one of the important pulse crops in India and a variety of food products are made with its dhal (splits). During milling of black gram into dhal about 25% is a by-product. Currently, it is used as cattle feed or wasted and, therefore, it does not have any commercial value. In the present study, the milled by-product was fractionated into husk, germ, plumule and aleurone layer rich husk fractions. Few oxidative and hydrolytic enzyme activities such as peroxidase, polyphenol oxidase, protease, amylase and xylanase were determined in these fractions. All the fractions exhibited more peroxidase activity compared to other enzyme activities and aleurone layer rich husk fraction showed maximum peroxidase activity (1155×10^3 units/g husk; 275×10^2 μmol/min/mg protein). Peroxidase is an important enzyme having a variety of applications in analytical chemistry, biochemistry and food processing. Peroxidase from aleurone layer rich husk fraction (BGHP) was purified using two steps, ion-exchange chromatography followed by gel filtration. The purity was determined from the high specific activity, purification fold (44), by the single peak obtained by HPLC and capillary electrophoresis, and a single band in native PAGE and SDS-PAGE. The molecular weight of the enzyme was found to be around 35 kDa. *o*-Dianisidine was found to be the good substrate for the enzyme compared to other hydrogen donor substrates. Dithiothretol and sodium azide were found to be non-competitive inhibitors for the enzyme. The K_m value of the enzyme for hydrogen peroxide and *o*-dianisidine was found to be 43.5 mM and 4.7 mM, respectively. Optimum pH of the enzyme was 5.5. The half-life of the enzyme at 50 °C was found to be 5 h and 15 min. Black gram husk, by-product of milling industry, is a rich source for a peroxidase.

1. Introduction

Peroxidase (EC 1.11.1.7; donor:hydrogen-peroxide oxidoreductase) is an enzyme that catalyzes the oxidation of a variety of substrates using H_2O_2. They are ubiquitous in nature and have diverse physiological functions, which include removal of H_2O_2, biosynthesis and degradation of lignin in cell walls [1], auxin metabolism and defensive responses to wound healing [2] and defense against pathogen or insect [3]. Peroxidase has wide substrate specificity and these characteristics make useful in a number of industrial, analytical and biomedical applications. Peroxidases are used commercially as a catalyst for phenolic resin synthesis [4] and as components of kit for medical diagnosis [5]. They are also used in the treatment of waste water containing phenolic compounds and aromatic amines [6,7], as labeling enzyme in immunochemistry and characterization of disease status in experimental pathology [8], as reagent for organic synthesis and bio-transformation as well as in coupled enzyme assays, chemiluminiscent assays and immuno assays [9,10]. Peroxidase is reported to have a role in wheat dough improvement due to the formation of protein–protein cross-links [11,12].

Roots of horseradish represent the traditional source of commercial production of peroxidase. Studies are being done for an alternative source of peroxidase with increased availability, higher stability and different substrate specificity. Isolation of peroxidase from soybean hulls, an inexpensive food industry by-product, was reported by Gillikin and Graham [13]. Black gram (*Vigna mungo*) or urd, belonging to leguminaceae family, is one of the important pulse crops in India, and it supplies a major share of protein requirement of vegetarian population. It is consumed in the form of split pulse as well as whole pulse and has a potential for diversified applications in legume based foods. During milling of black gram, about 25% of the material obtained as waste by-product, and currently, it is used for cattle feed. The present paper deals with the isolation, purification and characterization of peroxidase from black gram husk.

2. Materials and methods

2.1. Preparation of black gram milled fractions

Black gram (Thiram variety) was purchased from National Seed Corporation. Beej Bhavan, Pusa, New Delhi, India. It was milled in

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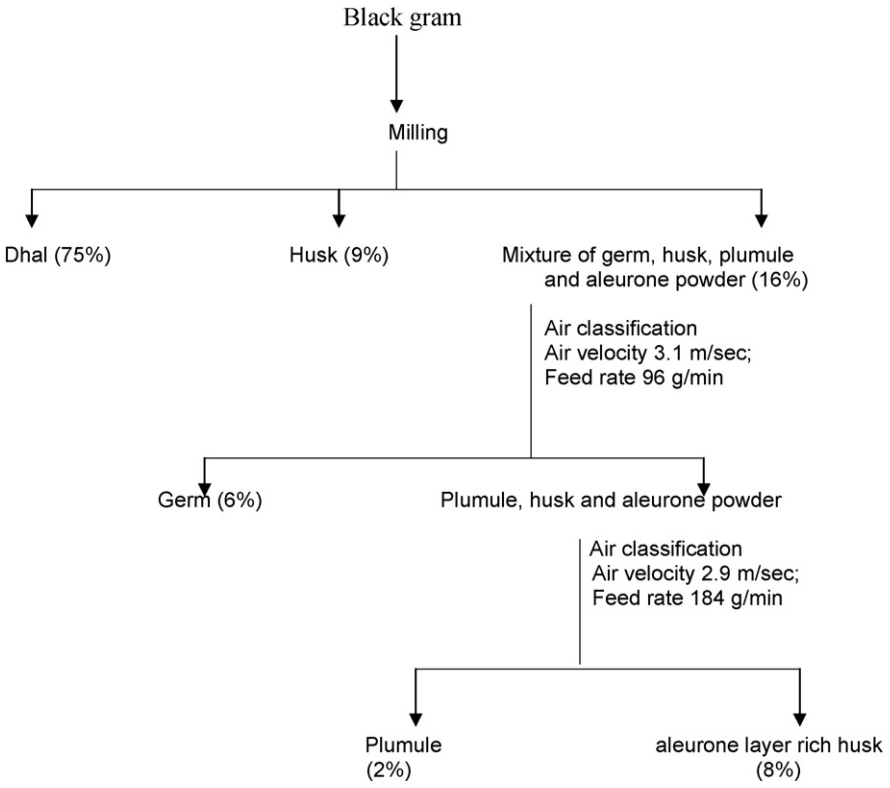
doi:10.1016/j.molcatb.2009.03.014



Scheme 1. Scheme for separation of black gram milled by-products.

Mini Dhal Mill at Central Food Technological Research Institute, Mysore, India. As shown in Scheme 1, during milling of black gram, various fractions viz., dhal, husk, germ rich factions, plumule, aleurone layer rich husk were obtained.

2.2. Enzyme extraction from black gram husk and its milled fractions

To 1 g of black gram powder or milled fractions, 0.5 g of acid washed sand was added and was ground into paste using 5 ml of 50 mM sodium phosphate buffer (pH 7.5) containing 0.01% Tween-20 using mortar and pestle at 4 °C. To the resultant paste, 20 ml of buffer was added, stirred for 1 h and was centrifuged at 8000 × *g* for 15 min at 4 °C. The supernatant obtained was used to estimate the protein content and to assay peroxidase, polyphenol oxidase, xylanase, protease and amylase activities as described below.

2.3. Enzyme assays

2.3.1. Peroxidase assay

Peroxidase activity was determined from the increase in the optical density ($\lambda_{460\,nm}$) at 27 °C by measuring the initial rate of oxidation of *o*-dianisidine–HCl by hydrogen peroxide. In brief, the enzyme activity was determined using 1 ml of reaction mixture containing varying quantities of appropriately diluted enzyme, 100 μl of 1% hydrogen peroxide (292 mM) and 100 μl of 7.9 mM *o*-dianisidine–HCl as substrates in 50 mM sodium acetate buffer (pH 5.5). One unit of the enzyme is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of *o*-dianisidine–HCl per minute at 27 °C into colored product ($\varepsilon_{460\,nm}$ = 30,000 M^{-1} cm^{-1}) [14].

2.3.2. Polyphenol oxidase assay

Polyphenol oxidase (PPO) activity was assayed using 100 μl of 0.5 M catechol as substrate in 1 ml of reaction mixture at its optimum pH 7.0, in 50 mM sodium phosphate buffer at 27 °C [15]. One unit of PPO activity was defined as that amount of enzyme, which produces an increase in absorbance of one per minute at 420 nm in 1 ml reaction mixture.

2.3.3. Protease assay

Protease content was estimated in 50 mM Tris–HCl buffer (pH 8.0) using azocasein (25 mg/ml buffer) as substrate at 27 °C [16]. An increase in absorbance of one per minute at 440 nm was regarded as one unit of enzyme activity in 1 ml reaction mixture.

2.3.4. Amylase and xylanase assays

Amylase activity was assayed using gelatinized soluble starch solution as substrate, which was dissolved in sodium acetate buffer (50 mM, pH 4.6). To 1 ml of 1% starch solution, 50 μl of appropriately diluted enzyme extract was added and the reaction mixture was incubated at 45 °C for 30 min as per the procedure described by Bernfeld [17]. Xylanase activity was determined using xylan as substrate, which was dissolved in sodium acetate buffer (100 mM, pH 4.8). To 1 ml of 0.5% xylan solution, 100 μl of appropriately diluted enzyme extract was added at 50 °C for 60 min. The enzyme reactions were stopped by boiling the reaction mixture for 10 min after the addition of 1 ml of 3,5,dinitrosalicilic acid (DNS) reagent and the reducing sugar was estimated by the DNS method as described by Miller [18]. One unit of amylase activity was defined as 1 μmol maltose equivalents released per minute. For xylanase activity, one unit of xylanase activity was defined as the amount of enzyme required to release 1 μmol of xylose per minute.

2.4. Protein estimation

Protein content in different extracts was determined using the dye-binding method as described by Bradford [19]. Bovine serum albumin was used as a standard.

2.5. Determination of optimum peroxidase extraction

Extraction was done with different solutions by soaking, grinding or by homogenizing to obtain maximum activity of peroxidase from black gram aleurone layer rich husk fraction. Water, 0.05 M sodium phosphate buffer (pH 7.5) and also buffer containing 0.01% Tween-20 was used for extracting peroxidase.

2.5.1. Soaking method

Aleurone layer rich husk fraction (1 g) was soaked in 25 ml of water or 0.05 M sodium phosphate buffer (pH 7.5) or buffer containing 0.01% Tween-20, and kept for different time intervals of 2 h and 24 h. The solution obtained was centrifuged at 8000 × *g* for 15 min at 4 °C. The protein content and the peroxidase activity in the supernatants were determined as described in sections 2.4 and 2.3.1, respectively.

2.5.2. Homogenization method

Aleurone layer rich husk fraction (1 g) was homogenized using a homogenizer with 25 ml of water or 0.05 M sodium phosphate buffer (pH 7.5) or buffer containing 0.01% Tween-20. The resultant homogenate was centrifuged at 8000 × *g* for 15 min at 4 °C. The protein content and the peroxidase activity in the supernatants were determined as described earlier.

2.5.3. Grinding method

To aleurone layer rich husk fraction (1 g), 0.5 g of acid washed sand was added and was ground into paste using 5 ml of water or 0.05 M sodium phosphate buffer (pH 7.5) or buffer containing 0.01% Tween-20. To the resultant paste, 20 ml of corresponding aqueous medium was added, stirred for 1 h and centrifuged at 8000 × *g* for 15 min at 4 °C. The protein content and the peroxidase activity in the supernatants were determined as described earlier.

2.6. Ion-exchange chromatography on DEAE-Sephacel

Extract of aleurone layer rich husk fraction obtained by grinding method and buffer containing 0.01% Tween-20 was loaded onto DEAE-Sephacel column (2.5 × 18 cm) and the unbound proteins were eluted with 0.05 M Tris–HCl buffer (pH 8.0). The bound enzyme was eluted with a gradient of 0–0.5 M sodium chloride in 0.05 M Tris–HCl buffer (pH 8.0). Fractions (2 ml) were collected with a flow rate of 12 ml/h and monitored for protein by determining the absorbance at 280 nm. The protein fractions were assayed for peroxidase activity as described earlier. The active enzyme fractions were pooled and used for further purification by gel filtration.

2.7. Gel filtration chromatography on Sephadex G-100

The active enzyme fraction obtained from ion-exchange chromatography was concentrated by lyophilization and chromatographed on Sephadex G-100 column (1.2 × 140 cm) using 0.05 M Tris–HCl buffer (pH 8.0) as eluent. Fractions (2 ml) were collected with a flow rate of 12 ml/h and monitored for protein by determining the absorbance at 280 nm. The protein fractions were assayed for peroxidase activity. The active enzyme fractions were pooled and concentrated by lyophilization and used as enzyme for further studies.

2.8. Determination of purity of black gram husk peroxidase (BGHP)

2.8.1. Polyacrylamide gel electrophoresis (native PAGE)

The purified protein was dissolved in sample buffer, centrifuged at 8000 × *g* and was loaded on 10% gel. The electrophoresis was carried out at 50 V and continued till the tracking dye was about 0.5 cm above the lower end of the gel. The gel composition, sample buffer compositions, protein staining and destaining were followed according to the method described by Laemmli [20] without SDS and β-mercaptoethanol. After the electrophoresis the gel was cut into different parts and each part was subjected to protein staining, glycoprotein staining and peroxidase staining.

2.8.2. Peroxidase staining

Peroxidase staining was done according to the method described by Hoffman et al. [21]. After electrophoresis, the gel was washed twice (for 5 min each) in 0.05 mM sodium acetate buffer, pH 5.5. The gel was transferred into the freshly prepared solution containing 1 mg/ml diaminobenzidene in the above buffer. The color reaction was started by the addition of 0.05 ml of 6% H_2O_2. The gel was allowed to remain in the solution till the bands were visible. The reaction was stopped by the addition of 5% acetic acid and washed several times with water to remove traces of substrate. The gel was stored in 5% ethanol.

2.8.3. HPLC of purified BGHP

Reverse phase HPLC of the purified protein was carried out using a C18 column (25 cm × 5 mm, 100 Å) on LC-10 A system (Shimadzu LC) using a gradient of two solvents: Solvent A – 0.01% trifluroacetic acid (TFA) in water; and solvent B – 0.01% TFA in 70% acetonitrile. The gradient for separation consists of 1% B traversing to 100% in 60 min at a flow rate of 1 ml/min. The sample detection was carried out using diode array detector.

2.8.4. Capillary electrophoresis

Capillary electrophoresis of purified enzyme was carried out on a Prince Capillary Electrophoresis (Prince 550, Prince Technologies, The Netherlands) using fused silica capillary (id—100 μm, length—100 cm) connected to an UV detector (220 nm) at 10 kV, 100 mbar, 26 ± 10 °C. Before the run, the capillary was rinsed with 50 mM Tris–192 mM glycine buffer (pH 8). The sample (1 mg/ml) was dissolved in tris–glycine buffer and was introduced in the capillary using a low pressure (0.5 psi) hydrodynamic injection of 20 s. The data acquisition and control were performed on DAx software.

2.8.5. Determination of molecular weight by gel filtration

The molecular weight of the purified enzyme was determined by gel filtration chromatography on Sephadex G-100 using 0.05 M Tris–HCl buffer (pH 8.0) as eluent. It was calibrated with alcohol dehydrogenase (150 kDa), β-galactosidase (116 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and cytochrome C (12.4 kDa). The void volume (V_o) was determined using blue dextran. The log molecular weight of each standard protein was plotted against its V_e/V_o (V_e elution volume of protein) and molecular weight of peroxidase enzyme calculated from the calibration graph.

2.8.6. Determination of molecular weight by SDS-polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis of purified fractions was performed using 10% gel at 50 V according to the method of Laemmli [20]. Enzyme sample loaded in each lane was 10 μg. Samples containing a mixture of molecular weight markers containing bovine serum albumin (66 kDa), ovalbumin (45 kDa), pepsin (36 kDa), trypsin (24 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.3 kDa) as well as the enzyme sample prepared in buffer containing 1% SDS and 5% mercaptoethanol were kept in boiling water bath for 5 min. After the electrophoresis, proteins were stained with Coomassie brilliant blue for 5 h and destained with solution containing 10% methanol and 7.5% acetic acid.

2.8.7. Mass spectrometry analysis of purified BGHP by MALDI-TOF

Molecular mass of black gram husk peroxidase was confirmed by Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS). The enzyme was subjected to MS on a MALDI-TOF Ultima (Waters Corporation, MA, USA) with nitrogen laser at 280 nm wavelength and 5 ns pulse width. The laser beam was focused onto the sample at an angle of 45° to the surface normal. Typical spot size ranged from 10–30 μM. Ions were accelerated to energy of 3 kV before entering the spectrophotometer. At the detector, ions were post accelerated to a maximum kinetic energy up to 30 kV for more efficient detector. The matrix was prepared by dissolving α-cyano-4-hydroxy cinnamic acid in 10% ethanol. Protein (100 μg/ml) was dissolved in triple distilled water and then diluted with matrix. Aliquots of resulting mixture (1 μl) were placed on a piece of silver plate; solvent removed by air-drying and the sample matrix mixture was transferred into a vacuum chamber of the mass spectrophotometer. Bovine serum albumin was used for external calibration.

2.9. Properties of peroxidase

2.9.1. Substrate specificity

Substrates such as *o*-dianisidine ($\varepsilon_{460\,nm}$ = 30,000 M^{-1} cm^{-1}), guaiacol ($\varepsilon_{470\,nm}$ = 26,600 M^{-1} cm^{-1}), ferulic acid ($\varepsilon_{318\,nm}$ = 31,100 M^{-1} cm^{-1}), pyrogallol ($\varepsilon_{430\,nm}$ = 2470 M^{-1} cm^{-1} and coumaric acid ($\varepsilon_{240\,nm}$ = 17,900 M^{-1} cm^{-1}) were tested for substrate specificity for peroxidase. Enzyme activity units were defined in μmol/min. 1 μmol/min was defined as the conversion of 1 mol of substrate into product for second.

2.9.2. Determination of K_m value (Michaelis constant)

Peroxidase activities at varying concentration of *o*-dianisidine and hydrogen peroxide were determined. Double reciprocal plots were plotted against 1/*V* versus 1/[*S*] and K_m values were calculated [22].

2.9.3. Effect of inhibitors on peroxidase activity

The effect of varying concentrations of inhibitors on the peroxidase activity was determined. The compounds tested were sodium azide, hydrazine, citric acid, oxalic acid, thiourea, dithiothretol (DTT), EDTA, and CTAB. With the exception of hydrazine, which was alcohol soluble, all the compounds were soluble in aqueous media. Appropriately diluted enzyme (0.1 ml) was incubated with 0.1 ml of varying concentrations of inhibitors in 0.6 ml of 50 mM sodium acetate buffer (pH 5.5) at room temperature (27 °C) for 5 min and the peroxidase activity was determined as described earlier.

2.9.4. Determination of inhibition kinetic analysis

Peroxidase activity was carried out with 0.1 ml of 1% H_2O_2 in the presence of inhibitors (DTT or sodium azide) at different concentrations of inhibitors and *o*-dianisidine. The type of inhibition was determined from the Lineweaver Burk plot of 1/*V* versus 1/[*S*]. The K_i value was determined from the slope of Lineweaver Burk plot against the concentration of corresponding inhibitor as described by Segel [23].

2.9.5. Determination of optimum pH

The enzyme activity was determined at pH values ranging from 4 to 6 (0.05 M sodium acetate buffer), 6 to 8 (0.05 M sodium phosphate buffer) and 8 to 9 (0.05 M Tris–HCl buffer) at 27 °C.

2.9.6. Determination of temperature stability

The purified enzyme was incubated at room temperature, 40 °C, 50 °C, 60 °C, 70 °C, 80 °C and 90 °C for 10 min and assayed for enzyme activity using acetate buffer (pH 5.5) under standard conditions as described earlier. The temperature stability of peroxidase enzyme at 50 °C was determined by incubating the enzyme at different time intervals such as 30 min, 1 h, 2 h, 6 h, 10 h, 12 h, 20 h, 24 h, 36 h and the activity of the enzyme was determined under standard assay conditions at pH 5.5. The half-life of the enzyme was calculated.

2.9.7. Effect of metal ions

The effects of various metal ions such as Fe^{2+}, Ca^{2+}, Cu^{2+}, Al^{3+}, Mg^{2+}, Zn^{2+}, Li^{+}, Ba^{2+}, Na^{+}, K^{+}, Cd^{2+}, Mn^{2+} were determined by pre-incubating peroxidase with the individual metal ions at 5 mM concentration in 0.05 M sodium acetate buffer at pH 5.5 for 10 min at 27 °C. The activity of the enzyme was determined under standard assay conditions in the presence and absence of metal. Residual activity was calculated taking activity of control as 100%.

3. Results and discussion

3.1. Enzyme activities in different milled fractions

During milling of black gram into dhal about 25% was obtained as by-product. This by-product was separated into four fractions viz., husk, germ rich fraction, plumule and aleurone layer rich husk fraction using air classification. These fractions and whole black gram flour were analyzed for few oxidative and hydrolytic enzymes such as polyphenol oxidase, peroxidase, protease, amylase and xylanase and the results are shown in Table 1. As peroxidases are important enzymes having a variety of applications in analytical chemistry, biochemistry and food processing, further studies were focused on peroxidase from aleurone husk as this fraction had the highest peroxidase activity (1155 × 10^3 units/g husk; 275 × 10^2 μmol/min/mg protein) compared to other fractions.

The native PAGE of the enzyme extracts from whole black gram and different milled fractions were carried out and subjected for peroxidase staining. Peroxidase enzyme staining clearly showed that black gram contains two isoenzymes, one major and one minor form and the minor band has less mobility on the gel (figure not shown). Aleurone layer rich husk fraction showed only one form of the peroxidase enzyme (major form) and all other fractions showed both major and minor form of the enzymes. Since the aleurone layer contained significant portion of the peroxidase activity of black

Table 1
Enzyme activities in different black gram milled fractions.

Fraction	Content (%)	Protein (mg/g)	Peroxidase (U/g)	Polyphenol oxidase (U/g)	Protease (U/g)	Amylase (U/g)	Xylanase (U/g)
Whole gram	–	96 ± 8^{d}	105600 ± 3366^{d}	30 ± 8^{a}	2,124 ± 82^{d}	16 ± 1.2^{c}	1.3 ± 0.1^{c}
Dhal	75	145 ± 12^{e}	16500 ± 1782^{a}	54 ± 6^{b}	1,823 ± 48^{c}	14 ± 1.8^{c}	1.7 ± 0.1^{d}
Germ	6	95 ± 10^{d}	96030 ± 528^{c}	172 ± 12^{c}	2,014 ± 82^{d}	8 ± 0.8^{b}	1.0 ± 0.1^{b}
Plumule rich	2	64 ± 5^{c}	18876 ± 472^{a}	24 ± 4^{a}	102 ± 10^{a}	4 ± 0.5^{a}	0.4 ± .0.1^{a}
Aleurone rich husk	8	42 ± 3^{b}	1155000 ± 4620^{e}	160 ± 15^{c}	2,020 ± 56^{d}	12 ± 2^{c}	2.0 ± 0.1^{e}
Husk	9	10 ± 1^{a}	59400 ± 759^{b}	32 ± 4^{a}	828 ± 14^{b}	6 ± 1.2^{b}	0.5 ± 0.1^{a}

All data are the mean ± SD of three replicates. Mean value followed by different letters in the same column differs significantly ($P \leq 0.05$).

Table 2
Different methods of extraction of peroxidase from aleurone layerrich black gram husk fraction.

Extraction medium	Protein (mg/g)	Activity (U/g)	Specific activity (U/mg protein)
Soaking			
Water			
2 h	1.19 ± 0.20[a]	121671 ± 1353[a]	102244 ± 396[j]
24 h	1.22 ± 0.10[a]	117480 ± 1155[b]	96295 ± 594[i]
Buffer*			
2 h	2.12 ± 0.20[b]	139623 ± 792[d]	65860 ± 695[h]
24h	2.48 ± 0.30[b]	137940 ± 660[c]	55621 ± 396[g]
Buffer* + Tween-20**			
2 h	4.43 ± 0.15[c]	245190 ± 924[g]	55348 ± 462[g]
24 h	5.93 ± 0.15[d]	215424 ± 2178[f]	36328 ± 561[f]
Homogenization			
Water	12.00 ± 1.20[e]	288288 ± 462[h]	24024 ± 660[c]
Buffer*	21.00. ± 1.40[f]	422730 ± 792[i]	20130 ± 1353[b]
Buffer* + Tween-20**	39.7 ± 1.37[h]	841632 ± 357[j]	21200 ± 462[b]
Grinding			
Water	12.45 ± 1.4[e]	193710 ± 1056[e]	15559 ± 759[a]
Buffer*	32.18 ± 1.5[g]	862620 ± 693[k]	26806 ± 396[d]
Buffer* + Tween-20**	42.00 ± 3.0[h]	1155000 ± 4620[l]	27500 ± 396[e]

All data are the mean ± SD of three replicates. Mean value followed by different letters in the same column differs significantly ($P \leq 0.05$).
* Sodium phosphate buffer (50 mM, pH 7.5)
** 0.01% Tween-20.

gram and has only one isoform of the enzyme, this fraction has been taken up for further studies.

3.2. Determination of optimum extraction for peroxidase

Different extraction methods were employed to extract maximum peroxidase enzyme activity and the results are shown in Table 2. Soaking the aleurone layer rich husk fraction in water, sodium phosphate buffer (0.05 M, pH 7.5) and the same buffer containing 0.01% Tween-20 extracted the enzyme with an activities of 121,671, 139,623 and 245,190 U/g, respectively. When the aleurone rich husk fraction was homogenized with water, phosphate buffer (0.05 mM, pH 7.5) and buffer containing Tween-20 the enzyme activity obtained was more than the soaking method (288,288–841,632 U/g). Maximum enzyme was extracted by grinding with acid washed sand using mortar and pestle with an activity of 1,155,000 U/g. In all the cases, buffer containing Tween-20 extracted maximum enzyme. Earlier, Gonzalez et al. [24] reported that 0.5% Triton X-100 was found to enhance the extractability of peroxidase from black berry fruits.

3.3. Purification of peroxidase enzyme

Peroxidase was extracted from the aleurone layer husk rich fraction by grinding method using sodium phosphate buffer (0.05 M, pH 7.5) containing 0.01% Tween-20. This extract was subjected to DEAE-Sephacel chromatography and the unbound fraction did not show peroxidase activity. As shown in Fig. 1, bound proteins were eluted by sodium chloride gradient from 0 to 0.5 M. Peroxidase was eluted just before the major protein peak. The fractions having peroxidase activity were pooled and concentrated by lyophilization. The elution profile suggested that peroxidase enzyme is an anionic protein, which possesses negative charge, which allows them to adsorb onto positively charged surface such as DEAE-Sephacel. Peroxidase purification by ion-exchange chromatography yielded 63% activity with 26-fold purification (Table 3).

The concentrated enzyme fraction obtained after ion-exchange chromatography was subjected to gel filtration chromatography. Fig. 2 shows the elution profile of peroxidase on Sephadex G-100. At

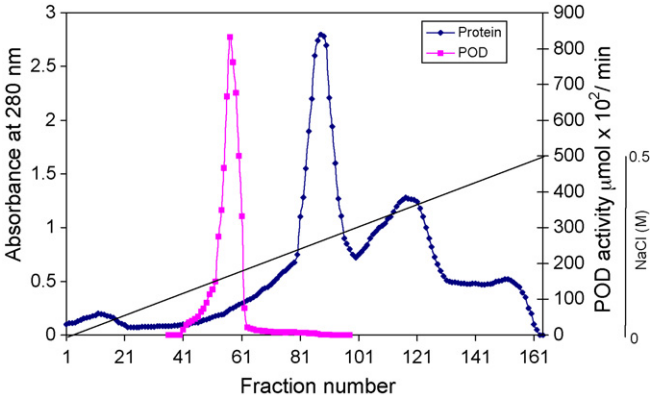


Fig. 1. Elution profile of black gram peroxidase from DEAE-Sephacel.

this step of purification, enzyme was purified to 44-fold with 44% recovery as shown in Table 3. Active fractions containing peroxidase activity were pooled and concentrated by lyophilization. This concentrated enzyme was used for further studies.

3.4. Purity of peroxidase

The purity of the enzyme was determined by native PAGE, HPLC and capillary electrophoresis. Single protein band was obtained on native PAGE (Fig. 3), which showed peroxidase activity on zymogram studies. The purified enzyme was chromatographed on reverse phase HPLC column and it was eluted as a single peak around 40 min (Fig. 4). The purified enzyme also gave a single peak on capillary electrophoresis (Fig. 5).

3.5. Properties of peroxidase

3.5.1. Spectral characteristics

The purified enzyme showed absorbance at 280 nm and 408 nm (Soret band), which are characteristics of protein and in particular heme protein. RZ (Reinheitszahl) value, which is a measure of hemin content of the peroxidase, was found to be 1.9 (figure not shown). RZ value indicates the presence of heme moiety as well as purity of peroxidase. However, there was no fixed value reported in the literature for a purified peroxidase from the same source or different sources. RZ values for three purified isoperoxidases of wheat kernel ranged from 2.8 to 3 [25], for wheat germ peroxidase isozymes C1, C2 and C3 the RZ value was reported as 4.1, 3.7 and 2.9 [26], respectively, for HRP-C, it was 3.2 and for pure type II HRP it was reported as 1.9 [27]. It has been reported that variations in RZ

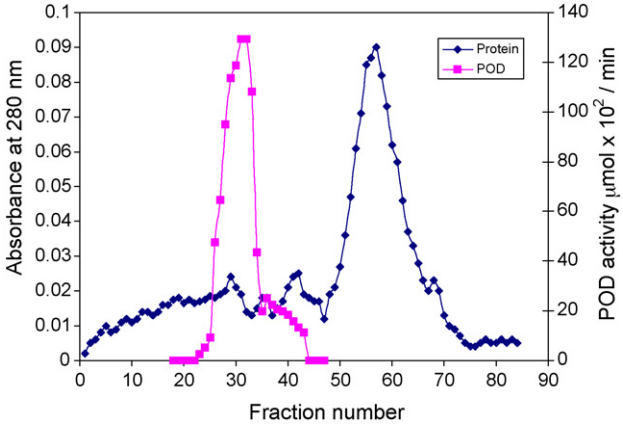


Fig. 2. Elution profile of black gram peroxidase from Sephadex G-100.

Table 3
Purification of peroxidase.

Fractions	Total POD activity (U)	Protein (mg)	Specific activity (U/mg protein)	Fold purification	Yield (%)
Crude	608256 ± 892	22.2 ± 0.12	27390 ± 41	–	100
DEAE-Sephacel fraction	385506 ± 674	0.54 ± 0.08	713881 ± 1247	26	63
Sephadex G-100 fraction	267366 ± 298	0.22 ± 0.01	1215291 ± 2402	44	44

All data are the mean ± SD of three replicates.

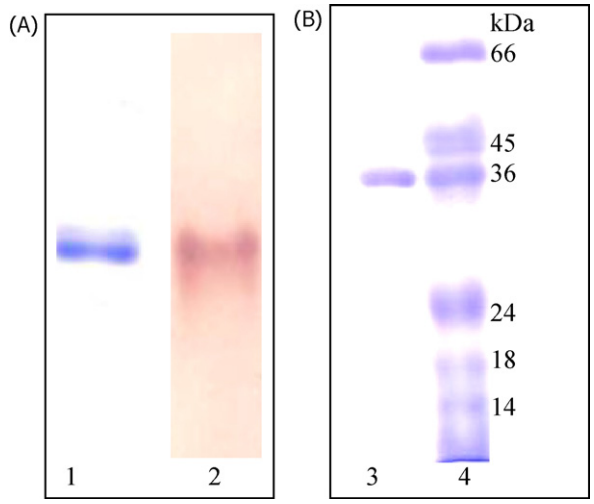


Fig. 3. Electrophoresis of purified peroxidase. (A) Native PAGE: lanes 1, Coomassie staining; 2, peroxidase staining; (B) SDS-PAGE: lanes 3, purified enzyme; 4, molecular weight markers.

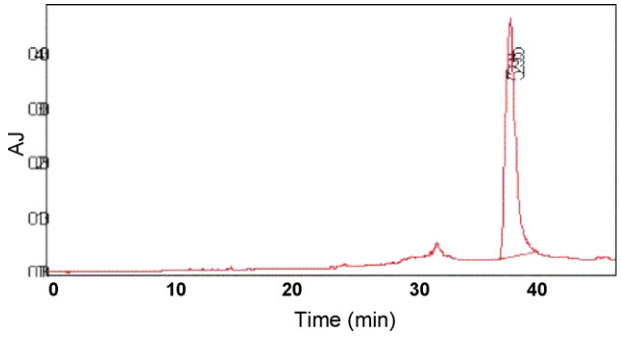


Fig. 4. HPLC of purified peroxidase on RP-C18 column. Solvent A – 0.01% trifluroacetic acid (TFA) in water; Solvent B – 0.01% TFA in 70% acetonitrile. The gradient for separation consists of 1% B traversing to 100% in 60 min at a flow rate of 1 ml/min.

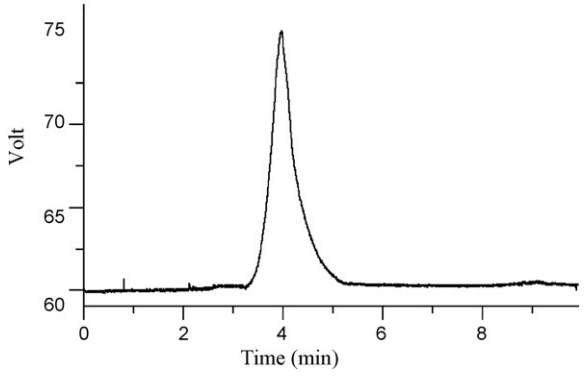


Fig. 5. Capillary zone electrophoresis of purified peroxidase. Volts: 10 kV; pressure: 100 mbar. Enzyme concentration: 1 mg/ml.

values may be due to the conformational changes in the vicinity of the heme [28].

3.5.2. Molecular weight determination

The molecular weight of the peroxidase enzyme was determined by gel filtration, SDS-PAGE and MALDI-TOF-MS. The purified enzyme was chromatographed on Sephadex G-100 column, which was calibrated with standard proteins. The molecular weight of the enzyme was calculated from the plot of V_e/V_o versus log of molecular weight and was found to be 38 kDa. The purified enzyme showed a single protein band on SDS-PAGE by Coomassie staining (Fig. 3B) and the molecular weight was found to be 36 kDa. The molecular mass of the enzyme determined by MALDI-TOF-MS was found to be 35,035.00 Da. The molecular weights of different plant peroxidases were reported to vary from 35 to 50 kDa. The molecular weight of black gram husk peroxidase was found to be in the same range those reported for peroxidase from wheat germ with molecular mass of 35 kDa [26] and from tobacco with 36 kDa [29]. However, it was different from the molecular weight of turnip peroxidase and horseradish peroxidases, which were reported, have molecular weights of 49 kDa and 40–46 kDa, respectively [30].

3.5.3. Substrate specificity of peroxidase

Different compounds were tested as hydrogen donor substrates for the enzyme. The enzyme showed activity with different substrates such as *o*-dianisidine, guaiacol, *p*-coumaric acid, progallol and ferulic acid. *o*-Dianisidine was found to be the best substrate for the enzyme followed by guaiacol (Table 4). As the enzyme showed wide substrate specificity BGHP may belong to class III of the plant peroxidase superfamily with EC 1.11.1.7 (donor:hydrogen peroxide oxidoreductase), similar to horseradish peroxidase or seed coat soy protein [31,32]. However, amino acid sequence and/or gene sequence of BGHP is required to establish the progenity of the enzyme.

3.5.4. Effect of substrate concentration and K_m value for the peroxidase

The effect of substrate concentration on purified peroxidase was studied by assaying the activity at different concentration of substrates. The peroxidase activity increased with increasing concentration of substrate and reached maximum at 1.25 mM for *o*-dianisidine and 29.4 mM for hydrogen peroxide. Beyond these substrate concentrations, the enzyme activity decreased. The V_{max} value for *o*-dianisidine was 200 μM and for hydrogen peroxide, it was 120 μM. The K_m value was calculated from the Lineweaver-Burk plot and was found to be 4.7 mM for *o*-dianisidine and 43.5 mM

Table 4
Effect of different substrates on the activity of peroxidase.

Substrate	Concentration (mM)	Specific Activity (μmol/min/mg enzyme)
o-Dianisidine	1.0	1288000
Guaiacol	1.0	247000
Pyrogallol	1.0	231000
Ferulic acid	1.0	46000
p-Coumaric acid	1.0	84000

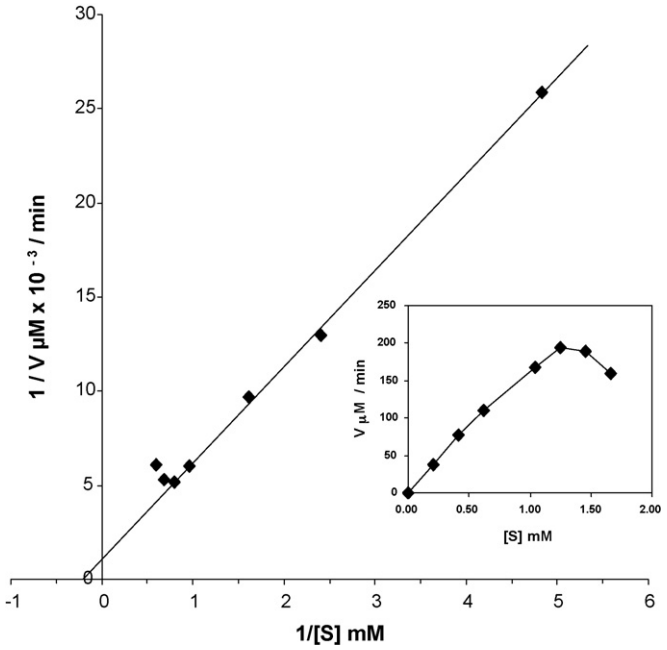


Fig. 6. Lineweaver Burk double reciprocal plot of the effect of concentration of *o*-dianisidine on the initial velocity of peroxidase from black gram husk; Inset: Michaelis–Menten curve.

for hydrogen peroxide (Figs. 6 and 7). The very low K_m value of the enzyme towards *o*-dianisidine shows its higher affinity towards this substrate. The peroxidase enzyme displayed Michaelis–Menten kinetics within a concentration of 0.2 to 1.25 mM for *o*-dianisidine and 9.8–29.4 mM for hydrogen peroxide and above these concentrations enzyme activity decreased. Inhibition of peroxidase activity by hydrogen peroxide at higher concentrations was reported by several workers [33–35]. It may be due to substrate inhibition combined with oxidation of the iron at the heme group most likely forming oxyperoxidase [35]. Hydrogen peroxidase is an oxidative substrate for peroxidase and it causes inactivation of plant and fungal peroxidase at higher concentration. Therefore, this substrate is known as suicide substrate [36].

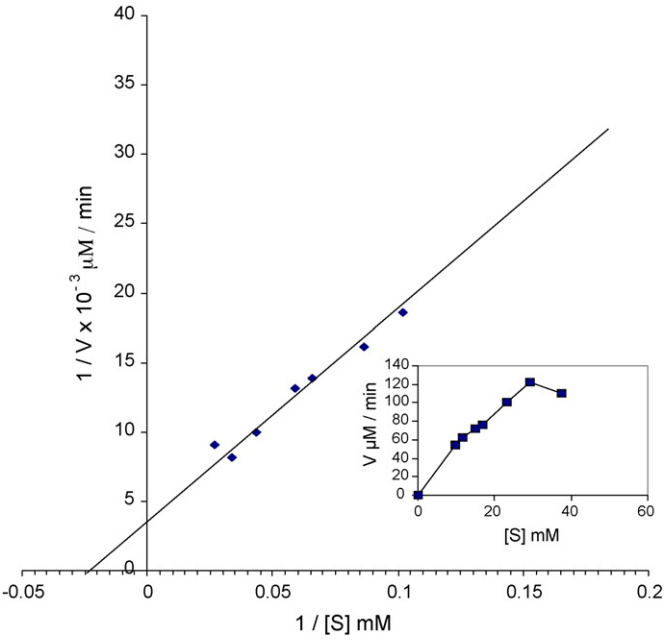


Fig. 7. Lineweaver Burk double reciprocal plot of the effect of concentration of H_2O_2 on the initial velocity of peroxidase from black gram husk. Inset: Michaelis–Menten curve.

Table 5
Effect of different inhibitors on peroxidase activity.

Inhibitors	Concentration (mM)	% Inhibition
Sodium azide	5	95
Hydrazine	5	97
DTT	5	95
Thiourea	5	92
CTAB	20	66
EDTA	20	33
Oxalic acid	10	93
Citric acid	10	73

3.5.5. *Effect of inhibitors and inhibition kinetics*

The inhibition of peroxidase activity by different compounds was determined. Inhibitors like thiourea, sodium azide, dithiotheretiol and hydrazine exhibited more than 90% inhibition at 5 mM, where as carboxylic acids such as oxalic acid and citric acid which are known to be a peroxidase inhibitors showed 93% and 73% inhibition respectively (Table 5). EDTA and CTAB at a concentration of 20 mM were found to be weak inhibitors with an enzyme inhibition of 33% and 66%, respectively. EDTA, metal chelator, was unable to fully combine with Fe^{2+} ions; consequently, the active site maintained its integrity [37].

The inhibition kinetics was determined following the procedure of Lineweaver Burk [8]. Non-competitive inhibition was observed for sodium azide and DTT (Figs. 8 and 9). Non-competitive inhibition was observed when the inhibitor combines with the enzyme substrate complex and prevents transformation of the substrate to products; the substrate and the inhibitors do not compete with each other in the binding to the enzyme active site [23]. The affinity of inhibitor (K_i) of black gram husk peroxidase by sodium azide and DTT were determined and they were found to be 2.08 mM and 2.00 mM, respectively. The result suggests that the inhibitor did not react with the free enzyme but it binds to the intermediate compound, thus preventing the formation of final product [23].

3.5.6. *Effect of pH on peroxidase*

pH is a determining factor in the expression of an enzyme activity as it alters the ionization state of amino acid chains or the ionization of the substrate. The effect of pH on the activity of enzyme was shown in Fig. 10, the enzyme showed maximum enzyme activity at pH 5.5 and it decreased sharply with increase in pH. The pH optima of peroxidase from grape were 5.4, banana

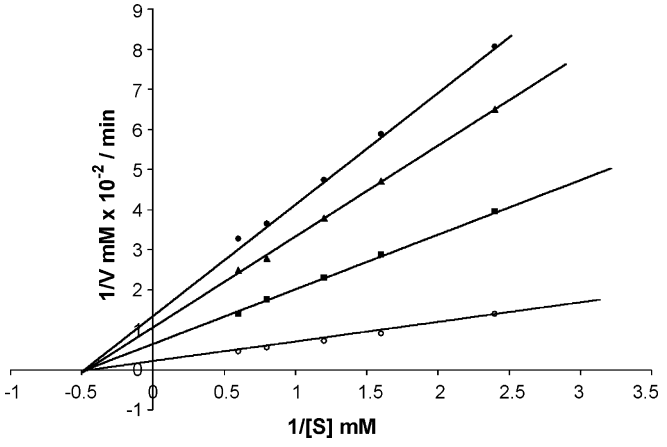


Fig. 8. Lineweaver Burk double reciprocal plot of $1/V$ versus $1/[S]$ in the presence of different concentrations of sodium azide as an inhibitor for peroxidase at different concentrations of *o*-dianisidine.

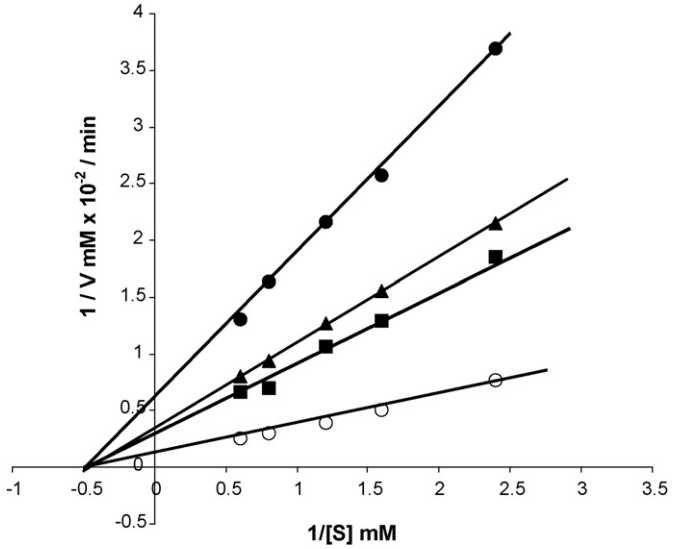


Fig. 9. Lineweaver Burk double reciprocal plot of 1/[*V*] versus 1[*S*] in the presence of different concentrations of DTT as an inhibitor for peroxidase at different concentrations of *o*-dianisidine.

4.5–5.0, pineapple 4.2, HRP 4.6–5.8, potato 5.0–5.4 [38], wheat germ 5.5–6.3 [39]. Lopez and Burgos [40] 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 lead 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 [41].

3.5.7. Temperature stability of peroxidase

In order to determine the temperature stability of the enzyme, aliquots of enzyme was incubated at different temperatures varying from 27 °C (room temperature) to 90 °C, for 10 min as described in methods. Temperature stability studies indicated that peroxidase activity slightly increased with increase in temperature incubated up to 60 °C and the enzyme was stable up to 70 °C. Above 70 °C a drastic loss of activity was observed (Fig. 11).

The thermal stability of the peroxidase at 50 °C was determined by incubating the enzyme at 50 °C for different time intervals from 30 min to 24 h. The half-life of the enzyme at 50 °C was found to be 5 h and 15 min (Fig. 12). The enzyme showed 25% activity even after 24 h of incubation at 50 °C. The peroxidase has high thermal stability, attributed to the presence of sugar in their structure [42]. However, this thermostability cannot be extended to all peroxidases due to the existence of isoenzymes with different resistance to temperature [43]. 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 [44].

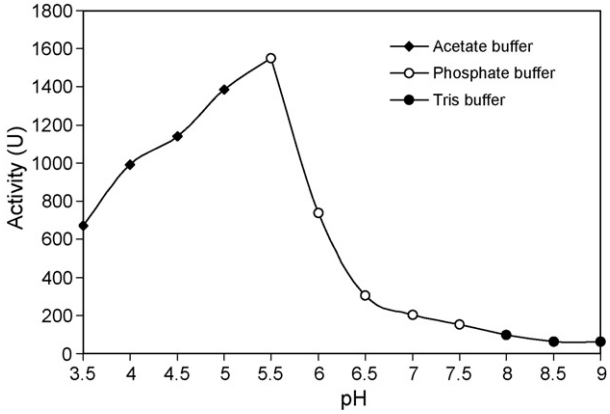


Fig. 10. Effect of pH on the activity of peroxidase enzyme.

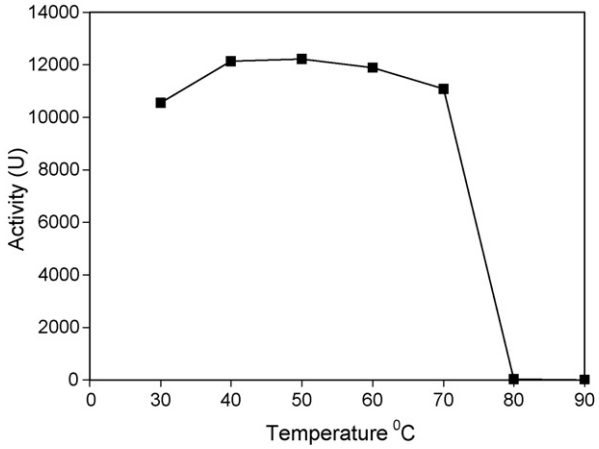


Fig. 11. Temperature stability of peroxidase.

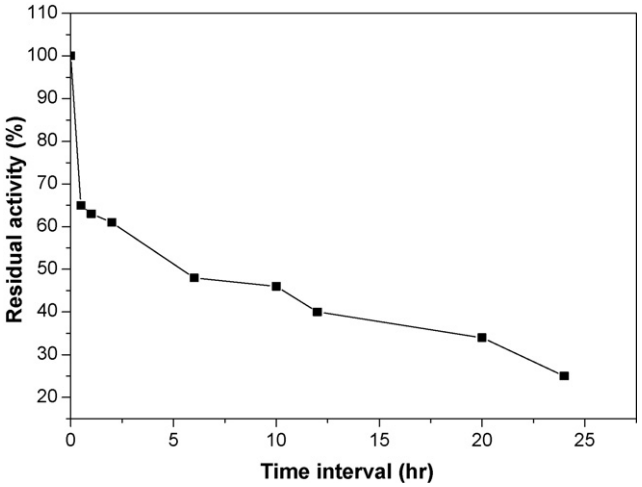


Fig. 12. Thermal stability of peroxidase at 50 °C at different time intervals.

3.5.8. Effect of metal ions on peroxidase

The activities of peroxidase from black gram husk were affected by the presence of different metal ions. Zn^{2+}, Li^{+}, Mg^{2+}, Ba^{2+}, Ca^{2+},

Table 6
Effect of different metals on peroxidase enzyme activity.

Compound	Concentration	% Activity
Control	0	100
$ZnCl_2$	5 mM	120
LiCl	5 mM	120
$MgCl_2$	5 mM	104
$BaCl_2$	5 mM	107
$MnCl_2$	5 mM	94
$CuCl_2$	5 mM	9
$CdCl_2$	5 mM	83
$CaCl_2$	5 mM	110
$FeCl_3$	5 mM	145
$AlCl_3$	5 mM	93
NaCl	5 mM	81
KCl	5 mM	98

Fe^{3+} stimulated the activity of peroxidase at 5 mM. On the other hand, Mn^{2+}, Cd^{2+}, Al^{3+}, Na^{+}, K^{+} moderately inhibited the enzyme activity at 5 mM, where as Cu^{2+} decreased the enzyme activity to 9% even at 5 mM (Table 6). At 5 mM concentration, Fe^{3+} was able to enhance the activity of peroxidase to 145%. Iron is considered essential for the activity of most of the plant peroxidases as it is involved in binding of H_2O_2 and formation of compound I [41]. Ca^{2+} also stimulated the enzyme activity to 110%. Ca^{2+} is a cofactor that serves to maintain the conformational integrity of the enzyme active site [45]. Activation by Ca^{2+} was reported for peroxidases of avocado, barely grain and wheat germ [39,46,47].

4. Conclusions

Black gram husk, which is a major waste by-product from milling industry, was found to be a rich source for a peroxidase enzyme. The enzyme showed high thermostability and half-life of the enzyme at 50 °C was found to be around 5 h. This new source will be a potential source for a peroxidase enzyme.

Acknowledgements

Authors thank Dr. S.G. Bhat for his valuable discussions during this study. C.M. Ajila thanks Council of Scientific and Industrial Research, New Delhi, India, for the award of Senior Research Fellowship.

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