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RESEARCH PAPER

Isolation, partial purification and characterization of peroxidase enzyme from orange seed

SHAILJA* AND SAIMA H. KHAN
Department of Chemistry, Sam Higginbottom Institute of Agriculture, Technology and Sciences, ALLAHABAD (U.P.) INDIA

ABSTRACT

The aim of this research was the isolation, partial purification and characterization of peroxidase from orange seed. The guaiacol is used as substrate in the detection of enzymatic activity of peroxidase. The optimization of extraction process was done by controlling the type and concentration of buffer, pH of the buffer used. The phosphate buffer with 0.1M and pH 6.5 was found to be the best buffer for extraction of peroxidase. Peroxidase activity in crude extract of orange seeds was measured by recording a spectrophotometric value. Partial purification of crude enzyme extract was done by ammonium sulphate precipitation. It was observed that after partial purification, the enzyme activity was increased as compared to crude enzyme extract. It is more evident that peroxidase is the most heat stable enzyme, therefore, it is concluded that it may be potentially useful for industrial purposes. Characterization results demonstrated that, the optimal pH for activity and stability was 7 and 7-7.5, respectively, the optimal temperature for activity and stability was 45 and 30-40°C, respectively.

Key Words : Orange seeds, Extraction, Peroxidase, Purification, Characterization

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Peroxidase is a ubiquitous enzyme which belongs to the oxidoreductase class of enzyme and generally catalyzes a reaction between H_2O_2 as electron acceptor and many kinds of substrates by means of O_2 liberation from H_2O_2 (Zia *et al.*, 2011). Peroxidases are divided into three classes which differ in molecular weight and in absorption spectra including: (i) Ferriprotoporphyrin peroxidases which are brown in nature and their main sources are plants, animals and microorganisms, (ii) Verdoperoxidases which are green in nature and are gotten from animals and (iii) flavoprotein peroxidases having FAD as prosthetic group and found in animals and microorganisms (Burnette, 1977). Plants are the rich sources of peroxidases and primarily found in roots and sprouts of higher plants. The rich plant sources of peroxidases are potato tuber, horse radish and beet, while it is also present in higher plants like turnip, sweet potato, tomato, sour lime, soybean, carrot, wheat, pears, apricot, bananas, date and sap of fig tree (Reed, 1975).

*** Author for correspondence**
Shailja, Department of Chemistry, Sam Higginbottom Institute of Agriculture, Technology and Sciences, ALLAHABAD (U.P.) INDIA (Email: shailjachaudhary0@gmail.com)

Peroxidase is widely used in the health sciences, food industry and for diagnostic purposes (Kawak *et al*., 1995). Chitinase and peroxidase which were isolated from soybean were considered to be involved in the defense of plant against pathogens (Staehelin *et al*., 1992). Being the most heat resistant enzyme, it is used in food industry as an index of blanching procedures (Reed, 1975).

RESEARCH METHODOLOGY

Enzyme extraction :

Peroxidase was extracted from orange seeds by the method of Geng *et al*.(2001) with minor modifications. Orange seeds were separated from fruits, dried and soaked in 200 ml of 0.1 M phosphate buffer of pH 6.0 over night and thoroughly homogenized by blending for 15 to 20 min. The contents were centrifuged at 10,000 g for 15 min to remove cell debris. The supernatant was removed carefully from the sediments and filtered through whatman number 1 filter paper to get more clarity of the crude enzyme extracted.

Thermal treatment :

To selectively inactivate the contaminating traces of the catalase moieties, crude enzyme extract was heated at 65°C for 3 min in a water bath and cooled promptly by placing it in ice bucket for 30 min (Wang *et al.,* 1999). After thermal inactivation, the final extract was preserved at 4°C until further use.

Enzyme activity and protein contration :

The activity of the enzyme was determined using a UV–Vis spectrophotometer at the wavelength of 470 nm according to the method of Rad *et al*. (2007) with minor modifications. A mixture of pyrocatechol (170 mM) and aniline (2.5 mM) was prepared in 0.2 M phosphate buffer solution of pH 6.5. To each blank and sample cuvette, 500 µl of the earlier mentioned mixture solution and 500 µl of hydrogen peroxide (35%) was pipette and incubated at 25°C for 3 to 4 min. Then, 50 µl of crude enzyme extract and 50 µl phosphate buffer solution was added to the sample and blank cuvettes, respectively. Increase in absorbance was recorded for 4 to 5 min interval. Protein was determined by the method of Lowry *et al*. (1951) using bovine serum albumin as standard.

Partial purification and dialysis of peroxidase enzyme :

Ammonium sulfate was added to the crude enzyme extract until itwas 50 per cent saturated and kept for 4 to 6 h at 4°C. The result ingprecipitate was collected by centrifugation at 10,000 g for 15 min at 4°C. The pellet of precipitated proteins was discarded. In the supernatant, more crystals of ammonium sulfate were added to attain 85 per cent saturation at 0°C. It was again kept for 4 to 6 h at 4°C and centrifuged as described previously. After centrifugation, the supernatant was kept separate and sediments were dissolved in small amount of buffer in which the enzyme was originally extracted. The solution was kept in a dialysis bag after sealing securely, and dialyzed against distilled water for a few hours with 4 regular change of the water after every 6 h.

Characterization of peroxidase :

Optimal pH of enzyme activity :

Prepared phosphate buffers have ionic power of 0.1M and pH range of (4.5- 9).The buffers above were used to prepare the substrate that used to estimate the enzyme activity for peroxidase by replacing the buffer with different pH and the relation between pH and enzyme activity was drawn to determine the optimum pH of peroxidase activity.

Optimal temperature of enzyme activity :

The different ranges of temperatures including (30, 35, 40, 45, 50, 55, 60, 65, 70) °C were used to estimate the optimal temperature of enzyme activity, the substrate that prepared was incubated in temperature ranges above for 10 minutes, added 100µl peroxidase solution and incubated for 3 minutes on these temperatures, then the activity was estimated, the relation was drawn between enzyme activity and temperature.

RESULTS AND REMONSTRATION

The results obtained from the present investigation as well as relevant discussion have been summarised under following heads:

Extraction of peroxidase :

Crude peroxidase was extracted from orange seeds using distilled water as solvent at room temperature by blending the seeds for 15 min with short intervals and centrifuged at 10,000 g for 15 min at 4°C to remove particulate matter and any intact nuclei from solution. Peroxidase values obtained after enzyme assay of blended seeds, supernatant (crude enzyme) and

sediments of orange seeds are shown in Table 1.

Partial peroxidase purification :

For the partial purification of peroxidase, crude extract was precipitated by using solid ammonium sulfate that was added gradually to the extract until they were 85 per cent saturated. During precipitation of peroxidase by ammonium sulfate, the absorbance values of 50 per cent sediments, 50 per cent supernatant, 85 per cent sediments and 85 per cent supernatant recorded in Table 2.

Table 2 showed that the sediments of 85 per cent saturation were the richest source of peroxidase. So, this rich form of peroxidase was subjected to dialysis to remove an extra salt and dialyzed against distilled water for several times. Bruemmer *et al.* (1976) partially purified the peroxidase from fresh orange juices by using ammonium sulphate precipitation which increases activities to different fold in each variety.

The Fig. 1 shown the increasing of activity by increasing the pH value until reach to maximum activity 1.15U/ml in pH=7 using guaiacol as a substrate of enzyme, then it began to decreased in a higher pH values. It observed that the peroxidase active in neutral pH and lowering of activity nearby acidic and alkaline pH. The differences in peroxidase activity with the different pH values return mainly to changes that occur in ionic state of enzyme molecule and substrate with other compound of activation mixture, it is necessary to known that the different of pH values of activity may change according to substrate uses (Wilder, 1962).

The results come closer to Lee and Dickson (1984) when they found that the optimum pH of peroxidase purified from *Broccoli* spp. Mader *et al.* (1977) was found that the optimum pH of peroxidase purified from Tobacco leaves was 5.5- 6.0.

Optimum temperature of enzyme activity :

To determine the optimum temperature of peroxidase activity purified from orange seed the enzyme reaction was done in different range of temperature (30-70) °C and the results shown in Fig. 2 increasing in

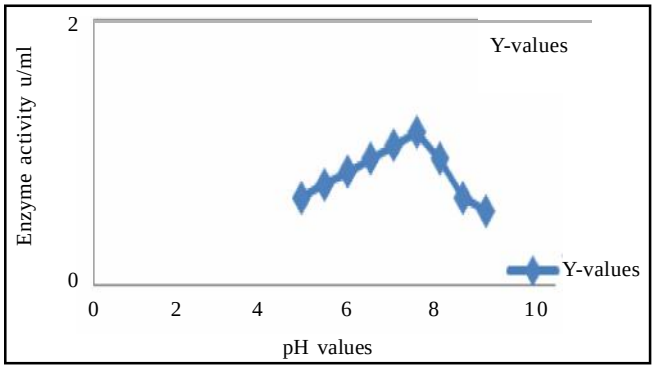

Fig. 1 : Effect of pH value on the peroxidase activity purified from orange seed

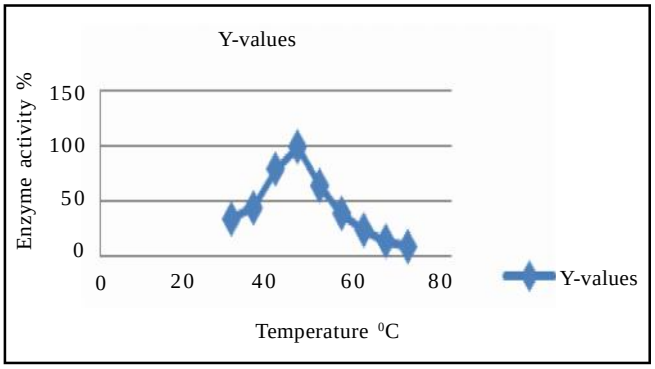

Fig. 2 : Effect of different temperature (30-70) °C on peroxidase activity purified from orange seed

Table 1: Peroxidase activities of orange seeds extracts		
Sr.No.	Sample	Peroxidase activity (U/ml)
1.	Blended seeds	3.38
2.	Sediments	0.75
3.	Supernatant (crude)	4.21

Table 2 : Peroxidase activities of orange seeds extracts after ammonium sulphate precipitation		
Sr. No.	Saturation with $(NH_4)_2SO_4$	Peroxidase activity (U/ml)
1.	50% sediments	1.20
2.	50% supernatant	1.71
3.	85% supernatant	0.65
4.	85% sediments	4.71
5.	After dialysis	5.43

peroxidase activity by increasing the temperature when it reached to maximum 100 per cent in 45°C, then it begin to decline with increasing temperature until reached to 10 per cent in 70°C.

This study was agreed with Gisele *et al.* (2010) when he shown the highest activity of peroxidase purified from *litchi pericarp* was in 40°C for 10 minute, after this temperature the activity of peroxidase started to reduce until reach to 58.8 and 76.6 per cent at 60°C and 70°C, respectively.

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