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Peroxidase: Purification from Soybean Seeds

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Abstract: Peroxidase, extracted from soybean seeds and was partially purified by precipitating with ammonium sulfate and 85 % saturation resulted in an increase in the activity of peroxidase up to 1.21 fold. Purification was carried out by diethyl amminoethyl cellulose chromatography and purification fold obtained was 2.62. Then, the enzyme was subjected to 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis which resulted in a marked decrease in unwanted proteins.

Key words: Peroxidase, soybean, DEAE-cellulose, SDS-PAGE

Introduction

Peroxidase an iron porphyrin organic catalyst, belongs to class oxidoreducatase, which occurs naturally in nearly all plants, animals and microorganism (Reed, 1975). It generally catalyzes a redox reaction by hydrogen peroxide as an electron acceptor and many kinds of substrates by means of oxygen liberation (Brill, 1966). There are different substrates for the enzyme but hydrogen peroxide is considered as best one (Laidler, 1954). It is present in radish, soybean (Ambreen *et al.*, 2000), tomato (Zia *et al.*, 2001), potato, turnip, carrot, wheat, peans, apricot, bananas, dates and strawberry etc. (Reed, 1975) but horseradish is the rich source as well as soybean. It is rare in animal kingdom, however is found in spleen, lungs, mammy and thyroid glands, bone marrow and intestine (Harris and Loew, 1996).

Peroxidase has wide applications in health sciences as diagnostic tool. A variety of enzymes are being utilized in ELISA (enzyme linked immunosorbent assay) among which peroxidase is widely used to prepare antibody enzyme or antispecies-antibodies enzyme conjugates due to its high turnover rate, rapid availability and better sensitivity (Zia *et al.*, 2001). The use of highly specific, sensitive and very stable peroxidase with a chromogenic donor has proven very useful for the assay system, providing H_2O_2 in determination of glucose by glucose oxidase (Hames and Hooper, 2001).

So, there is a great need for the production and purification of the said enzyme at local level to meet our requirements. The objective of this study was to isolate and purify peroxidase from soybean seeds by using ammonium sulfate precipitation and DEAE-cellulose chromatography techniques.

Materials and Methods

The present study was conducted at Department of Chemistry (Biochemistry) University of Agriculture, Faisalabad, which was completed in four months.

Preparation of enzyme extract: Soybean seeds were obtained from the market, washed with distilled water and kept immersed overnight. One hundred grams of seeds and 400 mL distilled water were homogenized for 10 min in waring blender. Contents were centrifuged at 10,000 rpm for 15 min at 4°C and supernatants were separated which were passed through filter paper. After getting the enzyme extract, it was heated at 65 EC for 3 minutes in water bath and then cooled promptly by placing in ice bucket as to selectively inactivate the traces of catalase (Zia *et al.*, 2001).

Enzyme assay: Peroxidase activity was measured at 470 nm wavelength using phosphate buffer of pH 6.5, hydrogen peroxide as substrate and guaiacol as chromogen. Then 0.02 mL of each enzyme sample was added to 1 mL of phosphate buffer and optical density (OD) was noted at 470 nm after 3 min of reaction period. (Civello *et al.*, 1995).

Estimation of protein: Protein contents of each sample were determined by Biuret reagent after plotting the standard curve of bovine serum albumin (Zia, 2002).

Isolation of peroxidase: Solid ammoimum sulfate was added to the crude extract until it becomes 50 % saturated. The minute was centrifuged at 10,000 rpm for 15 min. at 4 EC. Supernatant of 50 % saturation was adjusted to 85 % and again centrifuged at 10,000 for 15 min. at 4 EC after the incubation of 4 hr. at 4 EC. Then sample was dialyzed against distilled water to desalt the enzyme for 6 hr. at 4 EC (Zia, 2002)

Purification by DEAE-cellulose chromatography: A column of DEAE (diethyl aminoethyl)-cellulose was prepared by following method described by Cooper (1977). Exchanger was rapidly swollen by heating the slurry at 90 EC for 5 hr. using a water bath. Appropriate volume of the slurry was poured into the column in order to fill completely the required column bed height (5 cm). The column was washed with 15 mL of 0.5N HCI and with distilled water until pH became 7. The column was then washed with 15 mL of 0.5N NaOH followed by distilled water untill the effluent pH became 7. Then, it was equilibrated, overnight with phosphate buffer of pH 6.5. The desalted sample of 1 mL was poured with a fine pipette and it was eluted with phosphate buffer (pH 6.5) in 20 fractions of 2 mL each.

Electrophoresis: SDS-PAGE was applied after DEAE-cellulose chromatography to check the purity and homogeneity of the purified enzyme (Laemmli, 1970).

Results and Discussion

Peroxidase was extracted by blending the source for 15 min. with short intervals which was due to avoid the heating of material. Enzyme activity and specific activity obtained for crude extract was 2.67 U/mL and 0.714 U/mg respectively (Table 1). Crude extract was subjected to ammonium sulfate precipitation (Fig. 1) that is the most commonly used reagent for salting out of proteins due to its high solubility (Zia *et al.*, 2001). The supernatant of 50 % saturated with salt showed the activity of 0.530 U/mL that was increased in 85 % sadiments (1.39 U/mL). Desalt is the phenomenon to remove the contaminating salt present in sediments so after this process activity and specific activity values were increased upto 1.75 U/ml and 0.865 U/mg. Results obtained by Zia *et al.* (2001) showed the specific activity 0.546 U/mg from tomato peroxidase. So the difference in these values is due to the difference of source.

Peroxidase persists in many isozymic forms, majority of which are an ionic (Evans, 1968). So, the most often used cellulosic anion exchanger is diethyl amino ethyl (DEAE)-cellulose.

Due to its better efficiency to settle down and to form a compact mass it is preferred over other resins (Zia, 2002). The specific activity (2.034 U/mg) obtained after this treatment indicates a high degree of purity as compared to prior method (Table 1 and Fig. 2). The pattern of specific activity of proteins obtained in results of ion exchange chromatography shows the mode of separation of different components. Specific activity was zero in 1st protein and then its goes to increase gradually untill

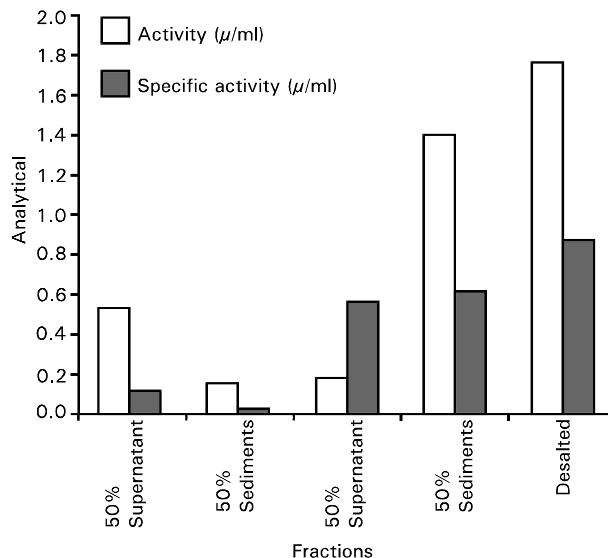

Fig. 1: Analysis of peroxidase isolation by ammonium sulfate precipitation

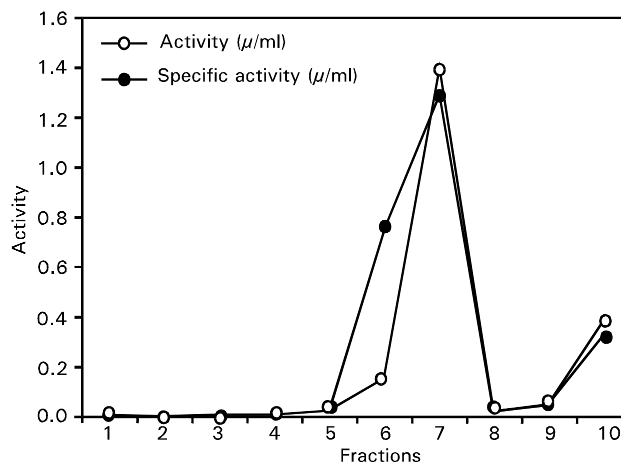

Fig. 2: Ion exchange chromatography of peroxidase

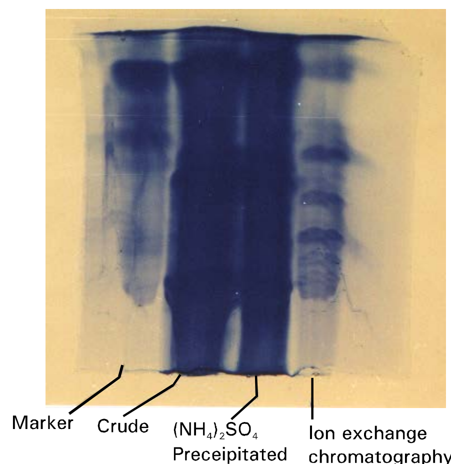

Fig. 3: SDS-PAGE of Soybean peroxidase

Table 1: Peroxidase purification from soybean seeds

Sample	Crude extract	$(NH_4)_2SO_4$ precipitation	Ion exchange chromatography
Activity (U/mL)	2.67	1.75	1.404
Protein Contents (mg/mL)	3.74	2.02	0.75
Specific activity (U/mg)	0.714	0.865	2.034
Degree of purification	1	1.21	2.62
R_z value	1.32	1.72	1.77
%age recovery	100	65	52

highest value (2.034 U/mg) is obtained fraction 7th and then it gradually decreases. The same pattern of results is reported by Zia *et al.* (2001) from tomato peroxidase as maximum specific activity of 1.844 U/mg in 5th fraction. Rehman *et al.* (1999) found is as 15.40 U/mg after in exchange chromatography of horseradish peroxidase.

To seek the purity of soybean peroxidase, then it was subjected to SDS-PAGE (electrophoresis) that was subjected to horizontal gel. It was showed that the said enzyme is free from even the contaminating agents and proteins and proved to be satisfactory (Fig. 3). The absorbance of enzyme was recorded at 470 nm after 3 min. of standard reaction period. It was seen that there is a gradual increase in activity within an increase of time. These are in accordance to Rehman *et al.* (1999); Ambreen *et al.* (2000) and Zia *et al.* (2001) who found a constant trend of increase in absorbance with our findings.

Rz value is the index of purification, as Rehman *et al.* (1999) reported an increase in Rz value from 0.782 to 1.82 during horseradish peroxidase purification and these observations are in line of our results that it is increased from 1.32 of crude to 1.77 of purified enzyme.

In brief it is further suggested that there is a great need to utilize the natural sources for the exploitation for enzymology through which we can fulfill our local requirements.

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