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EXTRACTION AND PURIFICATION OF PEROXIDASE FROM SOYBEAN SEEDS

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Peroxidase, extracted from soybean seeds was isolated by ammonium sulfate precipitation technique and purified by ion exchange and gel filtration chromatography. The crude enzyme having 17.29 U/mL activity and 1.586 U/mg specific activity was subjected to ammonium sulfate precipitation technique for partial purification and the resulted activity and specific activity were 12.85 U/mL and 5.68 U/mg respectively. After ion exchange chromatography through DEAE-cellulose, fraction No. 43 exhibited maximum activity of 18 U/mL and specific activity of 9.5 U/mg. This fraction was then applied to sephadex G-75 column and after elution, the activity and specific activity was enhanced to 16.04 U/mL and 14.948 U/mg respectively.

Key Words: Soybean seeds, peroxidase purification, ion exchange chromatography, gel filtration

INTRODUCTION

Peroxidase (EC. 1.11.1.7), an oxidoreductase, has iron porphyrin ring generally and catalyzes a redox reaction between H_2O_2 as an electron acceptor and many kinds of substrates by means of oxygen liberation from HzO_z (Brill, 1996).

$$2H_2O_z \xrightarrow{\text{Peroxidase}} 2H_2O + O_2$$

Peroxidase is usually intracellular, as are the other oxidoreductases (Reed, 1975). The enzyme occurs in plants like radish, soybean (Ambreen *et al., 2000),* tomato (Zia *et al.,* 2001), potato, turnip, carrot, wheat, pear, apricot, banana, dates (Reed, 1975), strawberry (Jen *et al.,* 1980) and horseradish (Rehman *et al.,* 1999). The separation and isolation of peroxidase from leukocyte by Agner (1943) assured its presence in animal tissue as well. It has also been found in spleen, lungs, mammary and thyroid glands, bone marrow and intestine (Harris and Loew, 1996). Reed (1975) purified this enzyme from *Streptococcus faecalis.*

Peroxidase is a heat stable enzyme, having wide range of applications in health sciences as a diagnostic tool (Kwak *et al.,* 1995). It is preferred for preparing Enzyme conjugated antibodies due to it's high specificity towards certain substances, and hence widely used in Enzyme Linked Immunosorbant Assays and other sensitive analytical techniques (Barnes *et al.,* 1993). The oxidative properties of peroxidase have been employed usefully in assay systems to detect metabolites by the formation of their respective oxidases (Eisenstaedt, 1939; Trinder, 1969).

Regardless of its wide use in quality control laboratories of food, pharmaceutical industry and in enzymological research purposes, no appreciable work has so far been done in Pakistan to produce commercial peroxidase. Peroxidase and it's kits especially for glucose estimation are therefore, being imported at a very high cost involving a huge amount of foreign exchange. Thus there is a need to commercialize and standardize these enzymes and kits depending upon our own sources and technology to reduce burden on our economy. The present project was, therefore, designed to purify the enzyme peroxidase from soybean seeds and to standardize the conditions for glucose estimation from/in serum.

MATERIALS AND METHODS

Preparation of Crude Extract

Soy bean seeds (100 g) were soaked overnight in distilled water and then blended with 400 mL distilled water for 15 min. It was centrifuged at 10,000 rpm for 15 min at 4°C and the supernatant was passed through filter paper (Ambreen *et al.,* 2000; Zia, 2002). It was heated in water bath at 65°C for 3 min to inactivate catalase present in the extract and cooled promptly by placing it in ice bucket for 10 min. (Zia, 2002). This extract was subjected to enzyme assay and protein estimation.

Purification of Peroxidase

a. Ammonium sulfate precipitation

The enzyme extract was subjected to $(NH_4)_zS04$ precipitation by the method of Evans (1968) for salting out of enzyme.

b. Ion exchange chromatography

A column of DEAE-cellulose was prepared by the method of Jakoby (1971) with some modifications (Zia, 2002). Exchanger was rapidly swollen by heating the slurry in a water bath at 95°C for 5 hours. It was then poured into the column and left undisturbed until the layer of slurry separated out. After packing, the column was washed with 0.5 M NaOH and 0.5 N HCl and equilibrated with phosphate buffer of pH 6.5. The sample was then applied on its top with the help of pipette and gradient elution was carried out with buffers of different pH ranging from 9 to 5. A total of 60 fractions of 2 mL each were collected at constant drop rate which were separately subjected to. enzyme assay.

Table 1. Summary of soybean peroxidase purification.

Enzyme fractions	Activity (Ulmg)	Protein contents (mg/mL)	Specific activity (U/mg)	Fold Purification
Crude	17.29	10.9	1.586	1
(NH4hS04 desalted	12.85	2.26	5.68	3.58
After ion exchange chromatography	18.0	1.9	9.5	5.99
After gel filtration chromatography	16.04	1.073	14.948	9.43

c. Gel filtration chromatography

A column of sephadex G-75 was prepared by the method of Jakoby (1971). The dry sephadex was dissolved in distilled water and heated in a water bath for 3 hours at 95°C without drying the slurry. It was then poured into the column and left undisturbed until distinct layers of gel and water separated out. The sample (eluant from ion exchange column) was applied with the help of pipette and elution were carried out by distilled water at a constant drop rate. A total of 25 fractions of 2 mL each were individually collected which were subjected to enzyme assay and protein estimation.

Enzyme Assay and Protein Estimation

Peroxidase assay was carried out in a reaction mixture containing 46 mL phosphate buffer of pH 6.5, 0.320 mL of H_2O_2 (Substrate) and 0.850 mL guaiacol (chromogen). The absorbance of the coloured complex was read on spectrophotometer at 470 nm wavelength after 3 min. of reaction interval (Ambreen *et al., 2000).* Protein contents of the enzyme extract at all steps were measured by biuret method (Gornall *et al., 1949).*

RESULTS AND DISCUSSI0f'! .I

The enzyme peroxidase was extracted from soybean by blending it for 15 min with short intermissions. These intermissions were to avoid heating up of blended material.. The activity and specific activity of crude extract obtained were 17.29 U/mL and 1.586 U/mg respectively (Table 1).

To purify the enzyme, the crude extract was subjected to 50-85% saturation with (NH4)S04 to remove unwanted proteins. The results of our findings are presented in Table 2 which shows 3.58 fold purification. This is the most commonly used reagent for salting out of proteins because its high solubility permits the achievement of solution with high ionic strength (Voet *et al.,* 1999). Evans (1968) standardized the saturation values of (NH4hS04 as 50-85%, while Rehman *et al.,* (1999) purified peroxidase from different seeds with 35-90% precipitation. The finding of Ambreen *et al.,* (2000) was 1.21 fold purification for soybean peroxidase using the same (85%) saturation values where as Rehman *et al.,* (1999) reported the degree of purification as 1.93 in horseradish peroxidase. This difference may be due to grade of salt used for purification or the concentration of salt and duration of saturation for purification.

Table 2. Soybean peroxidase purification by (NH4hS04 precipitation technique

Enzyme fractions	Absorbance (OD)	Activity (UlmL)	Protein contents (mg/mL)	Specific activity (Ulmg)
50% supernatant	2.358	29.55	6.36	4.65
50% sediment	3.0	37.59	9.81	3.83
85% supernatant	0.835	10.46	3.77	2.77
85% sediment	1.001	12.54	3.71	3.38
Desalted	1.025	12.85	2.26	5.68

The degree of purification of desalted enzyme was increased by applying it to DEAE-cellulose column for ion exchange chromatography. Peroxidase exists in many isozymic forms and majority of them are anionic (Evan, 1968). The most often used cellulosic anion exchanger is DEAE-cellulose (Voet and Voet, 1990). A number of other ion exchange resins i.e. DEAE-sephadex, carboxy methyl cellulose etc. are also available. But ion exchange cellulose is preferred because the finer particle size and higher density of the microgranular cellulose result in more compact adsorbent bed which provides higher resolution (Jakoby,1971).

Fig. 1 Specific activity of soybean peroxidase after ion exchange chromatography

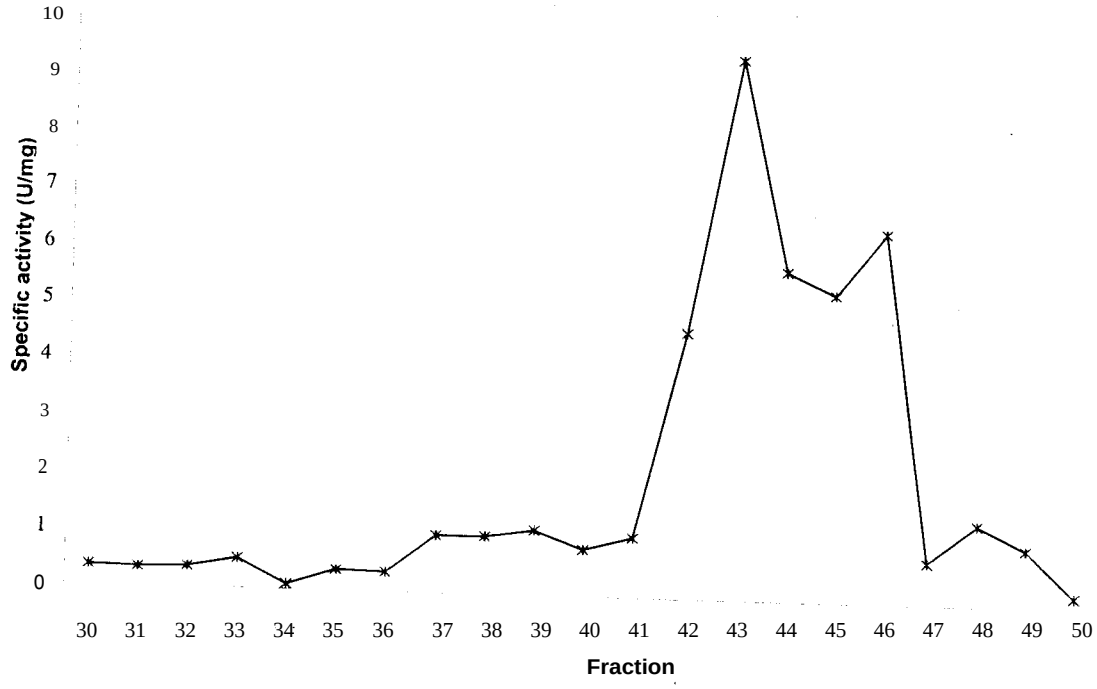
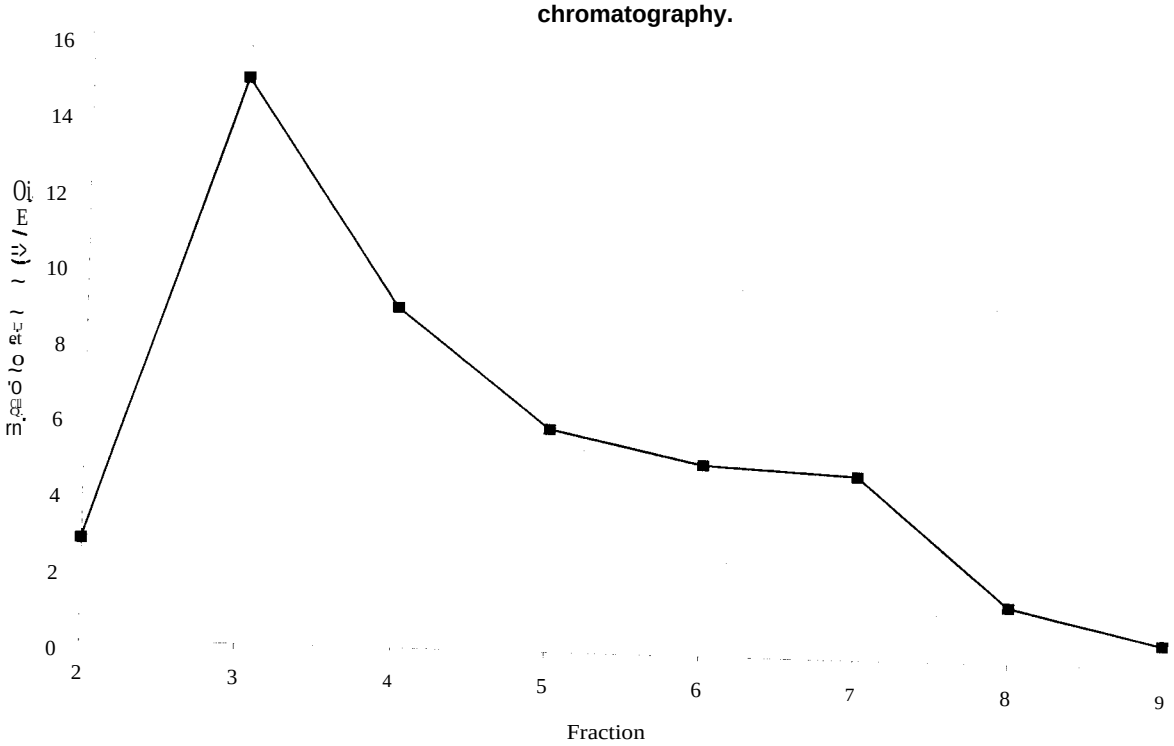


Fig. 2. **Specific activity of soybean peroxidase after gel filtration chromatography.**



It was noted that fraction no. 43 exhibited maximum specific activity of 9.5 *U/mg* which was 5.99 fold higher (Fig. 1). Ambreen *et al.,* (2000) applied soybean peroxidase to DEAE-cellulose and recorded 2.62 fold purification with specific activity of 1.872 *U/mg.* This increase in degree of purification might be due to gradient mode of elution in our case.

Gel filtration chromatography was applied by sephadex G-75 which proved very efficient as fraction 3rd showed 9.43 fold purification with maximum specific activity of 14.94 *U/mg* with highly reduced protein contents (Fig. 2). This technique represent higher degree of purification which appreciates this method and encourages its further use for purification purposes. Zia (2002) obtained 18.644 fold purification after applying horseradish peroxidase to gel filtration chromatography. This increased value of horseradish peroxidase might be due to difference in parent source of the enzyme. The following Table 1 shows summary of peroxidase purification from soybean.

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