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KINETIC STUDIES AND PARTIAL PURIFICATION OF PEROXIDASE IN SOYBEAN

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Peroxidase from soybean seeds was extracted and partially purified by ammonium sulfate precipitation technique and then by ion-exchange OEAE-cellulose chromatography. It was observed that conditions at which enzyme exhibited maximum activity did not change even after ammonium sulfate precipitation, while after OEAE-cellulose chromatography. specific activity of enzyme was increased. Various kinetic parameters were applied for peroxidase activity determination. The enzyme under discussion was found to be quite active up to noe with optimum temperature of 2~oC Optimum pH for the enzyme was 5.5. Thermal treatment of crude extract of soybean peroxidase was more stable at pH 3.0. It was found that enzyme followed the Michealis-Menton mechanism and 5.238 absorbance units/min and 2mM were the calculated values for Vmax and Km. The enzyme became reactivated when placed at _10°C for 4 hr after partial inactivation at 70°C for 3 min. It was found that the crude and partially purified extract possessed all appreciable enzyme activity. Soybean appears to be a rich and cheaper source of peroxidases from among potato. tomato and cauliflower.

Key words: kinetics, peroxidase, soybean

INTRODUCI10N

Peroxidases are iron-porphyrin ring containing enzymes, which belong to the class oxidoreductase. Peroxidases (EC. I. 11.1.77) generally catalyze a redox reaction between HP2 as electron accepter and many kinds of substrates by means of oxygen liberation from HP2 (Brill, 1996). The enzyme occurs naturally in nearly all plants, animals and micro-organisms (Burnette, 1977). It is found primarily in the roots ahd sprouts of higher plants (Tauber, 1949). The documented sources of peroxidase in plants are soybean, beat. horseradish, turnip, potato, tomato, carrot, wheat. dates, bananas etc. (Reed, 1975). Peroxidase has been reported to participate in late stages oflignin-forming process (Civello et al., 1(95). Peroxidase is the most heat-stable enzyme (Reed, 1(75). having a wide range of application in health sciences asa diagnostic tool (Kwak et al., 1995). Autoantibodies directed against the thyroid peroxidase are widely used to diagnose human auto immune thyroid disease (Nord, 1953). Peroxidase is also used to prepare antibody-enzyme and anti-antibody enzyme conjugates in enzyme linked immunosorbant assay (ELISA). In such a conjugation, it is preferred as having a high turnover rate and cheaper as compared to other enzymes (Kemeny and Challacombe, 1989: Bames et al.. 1993).

The objectives of this work included the kinetic studies, partial purification and characterization through ion-exchange chromatography from soybeanseeds.

MATERIALS AND METHODS

Preparation of Soy bean Extract: The peroxidase activity of soybeanseed was measured according to Jen et al. (1980). One hundred gram of soybeanseeds were added to 400 ml distilled water and thoroughly blended for 15 min. Sediments were discarded and supernatants were passed through filter paper. Total volume of prepared extract was 500 nil which was then heated at 65°e for 3 min in water bath to inactivate catalase present in the extract. The crude extract was subjected to partial purification or enzyme by using ammonium sulfate precipitation technique (Evans. 1%8)

Purification of Peroxidase by DEAE - Cellulose Chromatography: A column of OEAE - cellulose was prepared and partially purified sample of enzyme was applied to the column using a Pasteur pipette which was allowed to penetrate in column. bed. Buffer layer measuring I cm was poured on top of the column. Then 25.fractions were collected in about 7 hr by a constant drop rate.

Enzyme Assay and Protein Estimation: Phosphate buffer of pH 6.5 containing 0.320 ml HP2 and 2 ml guaiacol was used and 00 was noted at 470 nm for enzyme assay (Civello et al., 1995). Studies were conducted to determine the protein contents in enzyme extract before and after partial purification by biuret method (Gornall et al.. 19~9).

Effect of Various Kinetic Parameters on Crude and Pnrtially' Purified Peroxidase Activity

Enzyme Activity at Varying Time Intervals: The selected concentration of enzyme (0.30 ml) was added to 3 ml of buffer substrate and 00 was noted at ~70 run at 20 sec interval up to 280 sec. while partially purified extractswere used as such (Rehman, 1999).

Effect of Substrate toncentration: Application of Michealis-Menton equation was confirmed by using the

partially purified extract of soybean contairung various concentrations of guaiacol.. Crude extract reaction mixtures having pH 1-10 were prepared and analyzed on spectro-photometer at ·no nm after 1 min of reaction (Theorell, 1942). One millilitre crude extract was heated for 10 min at various temperatures such as 20. 24. 28. 12. 16. 40. 44. 48..56. ()4. 72. 88 and noc and absorbance (OD) was recorded at 470 nm on spectrophotometer (Civcllo et al.. 1995). Again I ml of crude extract was subjected to a variable thermal treatment The samples were heated at 70°C for 2. 4. 8 and 10 min and were then placed at 4°C for 10 min. 1, 2 and 4 hr to examine the phenomena of enzyme inactivation and reactivation. The absorbance was noted at 470 run (Gibriel et al.., 1978). For determination of thermal activity of peroxidase. 5 ml partially purified samples were heated at variable temperatures and time durations. The OD was recorded at 470 nm (Civello et al., 1995).

RESULTS AND DISCUSSION

Crude peroxidase extract of soybean was obtained using the method of Civello et al. (1995) since it is simple to perform at room temperature and distilled water is used as a solvent. It was observed that centrifugation at 10.000 rpm gave better results than at 6.000 rpm.

The partial purification was done by using ammonium sulfate precipitation (Evans, 1968) and DEAE-cellulose chromatography. To purify the enzyme POD from soybean, firstly it was salted out with $(NH_4)_2SO_4$' The enzyme of interest was procured at precipitation from 50 to 85% saturation. Degree of purification after ammonium sulfate precipitation was found 1.21. Rehman et al. (1999) reported the degree of purification as 1.93 in horseradish peroxidase. whereas Civelloet al. (1995) reported 2.37 degree of purification from strawberry fruit using the same technique. The most often used cellulosic anion exchanger is DEAE-cellulose (Rehman et al., 1999). Degree of purification of soybeanPOD was 2.62 fold with DEAE-cellulose chromatography. Specific activity of soybean per-oxidase 'in crude extract was 0.714 and it increased during the process of purification to 1.872 (Table I).

Protein contents were estimated by biuret method. being rapid and accurate.

The absorbance values of crude and partially purified extracts were recorded at specific wavelength of 470 um after 3 min reaction period. The OD values with respect to time interval were noted (Fig. I). There was a persistent increase in enzyme activity with enhancement of reaction time. These results are in line with those of Rehman et al. (1999) who found a constant trend of increase in absorbance values with increasing time intervals. In the presen1 studies. guaiacol was used as a substrate in partially purified extract 10 see the effect of substrate concentration (Fig. '). In crude and partially purified extract., the optimum pH was 5.5 with a range 3-10. It was observed that activity of enzyme increased gradually with increasing pH with Its peak al pH 5.5 with guaiacol.. The optimum pH depends upon H' donor. It may be changed according to the substratc used (Halpin et al., 1989). Jen et al. (1980) also found pH 5.5 as optimum with guaiacol while purifying tomato peroxidase.

Crude and partially purified extract showed optimum activity at 24°C. With an Increase in temperature keeping the exposure time constant (3 min). both types exhibited a drop in activity as shown in Fig. 4. These results' tended to show that peroxidase is the most heat stable enzyme which shows some activity even at 92°e.. These findings coutormcd to those ofCivello et al. (1995) who reported maximum aclivil\ at 30°e.. According to Zoueil and Esselen (J ')'"». longer the duration of heat treatment at a particular tcmpcnuürc greater is the denaturation of peroxidase The renaturation of both crude and partially purified peroxidase at-\ onc storage for 10 min and I hr was also found mutually comparable with no significant gain in cnzy me activit~ The renaturation was significantly increased after 4 hr interval. These results also agreed with those of Gibricl (1'>78) who observed high ratio of enzyme reactivation in apricot.. spinach and carrot extracts. It may be stated that soybcan peroxidase showed high stability at 60°C. It kept almost its original activity after treatment at 45°C for 20 nun (Fig. 5).

Table 1, Summary of soybean peroxidase purification

	Crude extract	$(NH_4)_2SO_4$ treated	DEAE-cellulose
Protein (mg/ml)	3.740	2.023	0.750
Activity (µ)	534.000	350.000	280.800
Specific activity	0.714	0.865	1.872
Degree of purification	1.000	1.210	2.620
Percentage recovery	100.000	63.000	52.000

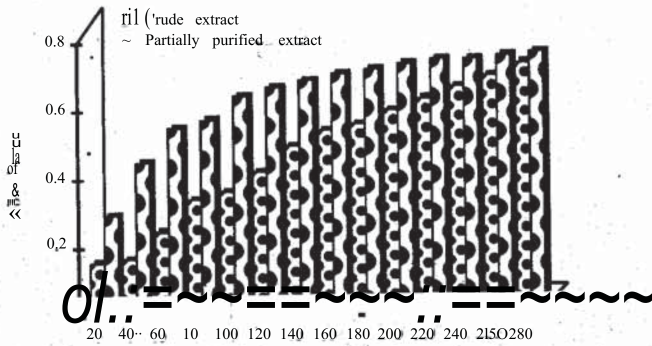

Fig. 1, Comparative absorbance values for the same volumes of crude and partially purified extracts of soybean at varying time intervals.

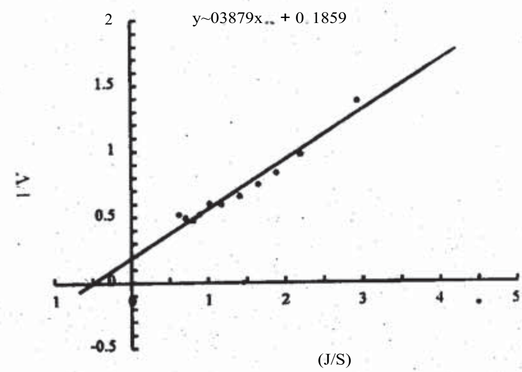

Fig. 2. Live weaver Burk Plot

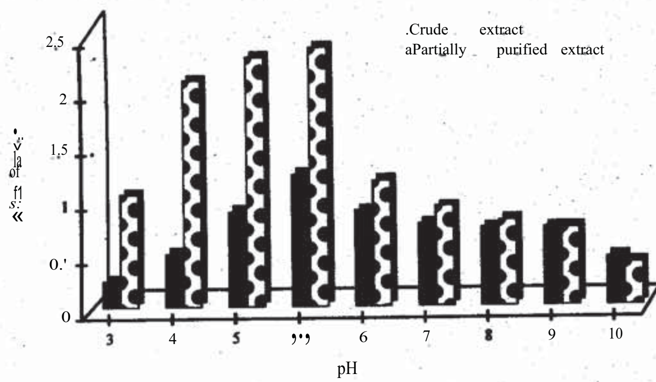

Fig. 3. Effect of pH on peroxidase activity in crude and partially purified extract of soybean.

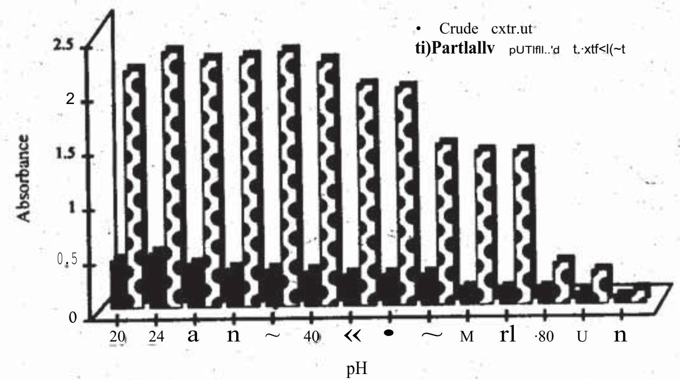

Fig. 4. Effect of temperature on peroxidase in crude and partially purified extract of soybean.

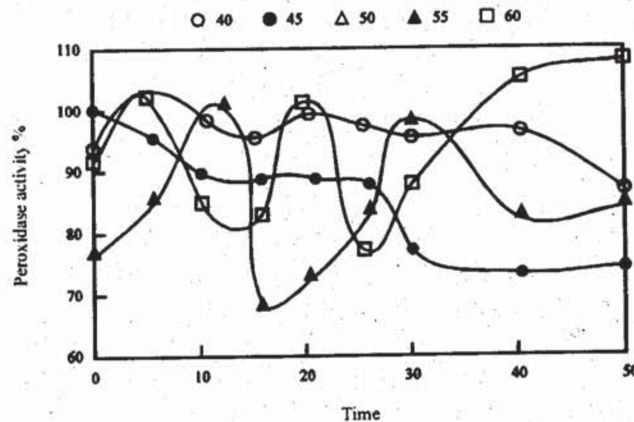

Fig. 5. Thermostability of soybean peroxidase.

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