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Extraction and Evaluation of Peroxidases from Various Vegetable Sources

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ABSTRACT

Peroxidase, widely distributed in the plant kingdom, was extracted and comparatively evaluated from turnip, tomato, radish, horseradish legumes (HRL) and horseradish roots (HRR). Effect of varying time intervals, pH, temperature and NaCl were studied. All the sources differed significantly from one another for peroxidase activity. The optimum pH was 6.0 for radish and 6.5 for turnip, HRL and HRR. The enzyme was found thermostable showing 60 to 80% activity at 50°C. Peroxidase was purified with diethyleaminoethyle cellulose chromatography after ammonium sulphate precipitation and the degree of purification was 14 folds.

Key Words: Peroxidase; Vegetables; Thermostable; DEAE-cellulose chromatography

INTRODUCTION

A variety of enzymes including Horseradish peroxidase (POD), Alkaline phosphatase, ß-D-Galactosidase etc. have been used in ELISA (Kemeny & Challacombe, 1989). Among these, POD is widely distributed in the plant kingdom (Evans, 1968), used in living systems (Michel *et al.*, 1990; Ito *et al.*, 1991) and many other clinical procedures (Park *et al.*, 1990; Sandusky & Wightman, 1985). Horseradish POD (EC 1.11.1.7) having a high turnover rate, is relatively cheaper and readily available. A large number of chromogens giving readily visible colours with this enzyme are commercially available. Moreover, carbohydrate portion of POD serves as a bridge for conjugation to immunoglobulins (Kemeny & Challacombe, 1989).

Nonetheless, no remarkable work on this enzyme has so far been done in Pakistan. The commercial POD is imported for use either by the quality control laboratories of canned food industry or for experimental research institutions for specific enzymological research purposes. Inexpensive vegetables, grown in Pakistan may be explored for POD yield. This paper comprises extraction and characterization of POD from turnip, tomato, radish, horseradish legume (HRL) and horseradish roots (HRR). Partial purification of horseradish POD by ammonium sulphate precipitation and Diethyleamineoethyle (DEAE) cellulose chromatography is also reported.

MATERIALS AND METHODS

Extraction of POD. Crude POD enzyme from turnip, tomato, radish, horseradish legumes and roots was extracted, monitored and evaluated following the methods of Theorell (1942), Evans (1968), Civello *et al.* (1995) and Talat (1996) with some frivolous modifications.

Preparation of enzyme extract. 100 g of the chopped pieces of fresh vegetables were added to 400 ml of distilled water and then thoroughly blended for 15 minutes. The contents were centrifuged at 6,000 rpm for 15 minutes and supernatant was passed through filter paper. The volume was made upto 450 ml. The extract was heated at 65°C for 3 min. to inactivate any catalase present in extract. Four dilutions of the crude enzyme i.e. 1:5, 1:10, 1:15 and 1:20 were made.

Effect of various parameters on enzyme activity

Measurement of enzyme activity at varying intervals of time. The selected enzyme concentration (0.10 ml) was added to 1 ml of the buffered substrate and the respective absorbance values were recorded at 20 seconds interval upto 300 seconds.

Effect of pH on POD activity. A set of test tubes was taken and numbered sequentially from 1 to 11. Enzyme extract of 5 ml was taken in each test tube and pH was adjusted to 3.0, 4.0, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 9.0 and 10.0 by the supplementation of 1M HCl or 1M NaOH. Each sample was incubated at 30°C for 20 minutes at 3 to 10 pH (Civello *et al.*, 1995). After this treatment, enzyme activity in terms of OD was evaluated.

Effect of temperature. The crude enzyme extract was incubated for 20 minutes in six test tubes containing 1.0 ml each, to variable temperature like, 40, 50, 60, 70, 80 and 90°C. The absorbance value was noted at three minutes reaction interval.

Effect of sodium chloride. Different concentrations of sodium chloride (0.2, 0.4, 0.6, 0.8 and 1.0 M) were made in a set of five tubes using the constant volume (5 ml) of the enzyme extract. For this purpose, NaCl was added in corresponding tubes in varying concentrations as 0.06, 0.12, 0.18, 0.24 and 0.3 g.

Partial purification of POD by ammonium sulphate

Solid reagent $(NH_4)_2SO_4$ was added to the crude extract until it became 50% saturated. The mixture was centrifuged at 10,000 rpm. The supernatant of 50% saturation was adjusted to 85% saturation by adding more ammonium sulphate. The mixture was centrifuged then again at 10,000 rpm for 15 minutes and sample was dialyzed to desalt the enzyme.

Purification of POD by DEAE chromatography

A column of DEAE (diethylaminoethyl) cellulose was prepared by following the methods described by Cooper (1977), Ausubel *et al.* (1989). Exchanger was rapidly swollen by heating the slurry at 90°C for 5 hours using a water bath. Appropriate volume of the slurry was poured into the column in order to fill completely the required column bed height. After the column was packed to the desired bed height (i.e. 5 cm) a layer of buffer was poured on its top with the help of a pipette. For 5 cm bed volume, the column was washed with 15 ml of 0.5 N HCl. The column was then washed with distilled water until the effluent pH was 7. The column was washed with 15 ml 0.5 N NaOH. The column was then again washed with distilled water until the effluent was of pH 7.

RESULTS AND DISCUSSION

Preparation of extract. Enzyme was extracted by blending the vegetable sources for 15-20 minutes with short intermission (2-3 min.). This intermission was to avoid heating up of the blended material. Blending is quite efficient procedure for breaking plant and animal tissues (Cooper, 1977) and centrifugation at 6000 rpm for 15 minutes can remove particulate matter and any intact nuclei from solution.

POD activity and comparison from different sources. Enzyme activity determination is based upon the rate of utilization of substrate or formation of product per unit time under controlled conditions. The enzyme activity in terms of chromogenic reaction development was quantified spectrophotometrically. With the increase in reaction period, there was found a constant trend of increase in the absorbance values in POD in all sources.

The mean decrease in absorbance of crude extract of turnip, radish, HRL and HRR was 0.224, 0.166, 0.214 and 0.304, respectively with the increase of two fold dilution from 1:5 to 1:10. The difference in OD of the remaining dilutions was less as dilutions grew higher and higher. That particular enzyme dilution showing absorbance value in the range of 0.2 to 0.6 at 3 to 4 minutes reaction time was considered as an index of the optimum enzyme activity and was selected for further studies. Data revealed that 1:5 dilution of the crude extract of turnip, radish and HRL and 1:10 dilution of the crude extract of HRR was the optimum dilution for subsequent analysis. The observed POD activity was minimum in the tomato under the adopted conditions. So this source was excluded from the subsequent studies.

LSD value 0.02028 at $\alpha 0.050$ in DMR test revealed that all the vegetable sources differed from one another significantly. Similarly activity of POD at various dilutions and at different time interval varied significantly. This remarkable difference between the OD values was attributed to the variation in the POD activity in plants in relation with anatomical locations, age of tissue/plant, physiological activity and state of being fresh or stocked (Evans & Alldridge, 1965). The difference in peroxidative activity in relation to anatomical locations, HRL and HRR were in line with findings of Rahayuningsih (1990) who found higher POD activity in roots than in stems and leaves of papper plants.

Enzyme activity at different time intervals. Absorbance values of crude extract of turnip, radish, HRL and HRR with constant volume of 0.1 ml of dilution 1:5, 1:5, 1:5 and 1:10, respectively were recorded. The rise in OD in all the cases was recorded with uniform interval of 20 second upto 300 seconds. The results revealed that with the passage of time, the rate of reaction was in a decreasing order (Fig. 1). Initially the rate of reaction increased rapidly as POD has high turnover rate (Kemeny & Challacombe, 1989). When substrate is going to be exhausted, the peroxidatic activity with the passage of time is decreased. Yet beyond 220 sec reaction period, the comparative differences in respective densities became fairly diminutive. These findings are further corroborated as the inferences drawn by other workers are referred. Theorell (1940) during his studies on kinetics of

Fig. 1. Activity of Peroxidases from different sources at various time intervals

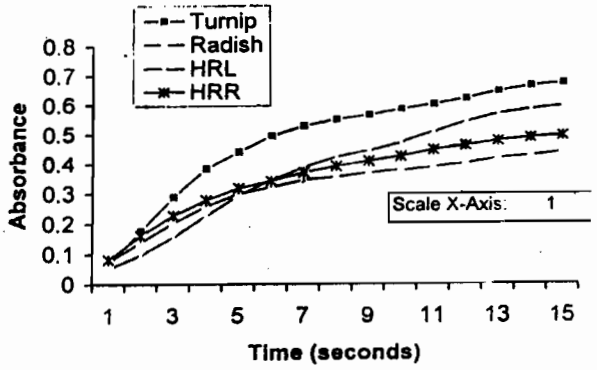


Fig. 2. Activity of Peroxidases from different sources at different pH

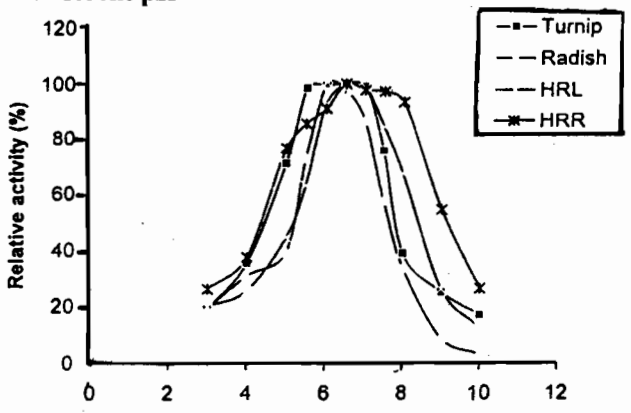


Fig. 3. Activity of Peroxidases from different sources at various NaCl concentrations

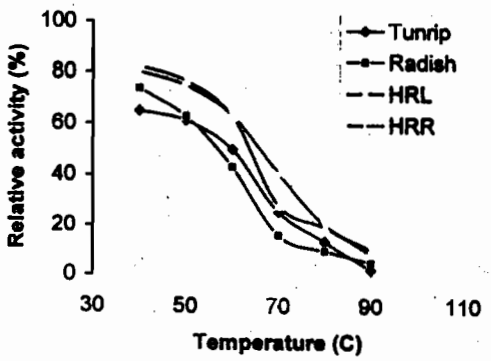
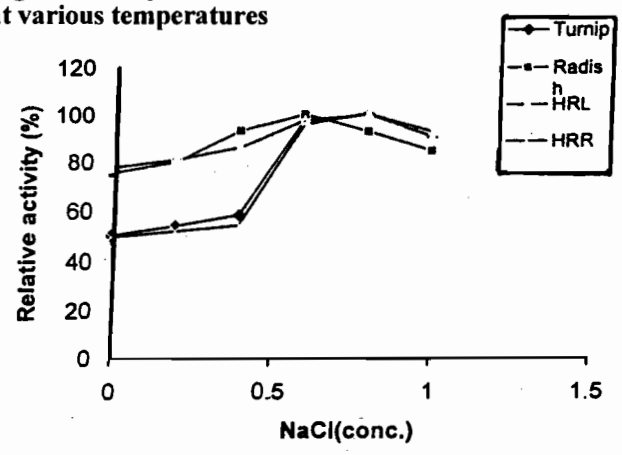


Fig. 4. Activity of Peroxidases from different sources at various temperatures



horseradish POD, recommended a suitable reaction interval between 180 to 240 seconds. During purification and characterization of POD isozymes from green peas three minutes reaction period was used by Halpin *et al.* (1989).

Effect of pH on the activity of POD. The optimum pH for radish was found to be 6.0, while for turnip, HRL and HRR, it was recorded as 6.5 (Fig. 2). The results were in accordance with the findings of Civello *et al.*(1995), Halpin *et al.* (1989), Talat (1996) and Rehman *et al.* (1997) who reported optimum pH 6.0 to 6.5 for PODs from different vegetable sources.

Effect of temperature on the activity of enzyme. Effect of temperature on the enzyme activity was studied by heating the extracts over a range of temperatures from 40°C to 90°C for three minutes in water bath. Results of fall of peroxidatic activity of all the studied sources are shown in Fig. 3. The observed activity in terms of OD was maximum at room temperature and decreased with rise of temperature and approached minimum at 90°C. POD was thought to be the most heat stable enzyme in plants (Burnette, 1977). The fall in enzymatic activity was not so pronounced by increasing temperature from 40 to 50°C, while during teatment beyond this temperature, the fall in the activity was steep. These findings are similar to the studies of Civello *et al.* (1995) who reported maximum enzyme activity at 30°C.

Effect of NaCl on enzyme activity. The activity of the enzyme POD of radish was maximum at molar concentration of NaCl 0.6. Without supplementation of salt, the activity of the enzyme was about 76% of the maximum activity with 0.6 M salt concentration. Peroxidatic activity of all other remaining sources was at the maximum in the presence of 0.8 M NaCl concentration (Fig. 4). In absence of salt, the activity was 51.78 and 54% of the maximum activity of turnip, HRL and HRR sources, respectively. It is quite evident that a small amount of salt concentration is required to maintain the three dimensional structure and provide natural milieu.

Enzyme purification. Horseradish POD was partially purified by ammonium sulphate fractionation and DEAE cellulose chromatography. To purify the enzyme POD from horseradish, 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 sulphate precipitation was found 1.93. Ammonium sulphate is the most commonly used reagent for salting out proteins because its high solubility permits the achievement of solutions with high ionic strength (Voet & Voet, 1990). Kermasha and Metche (1988) purified POD three folds from seeds with 35–90% $(NH_4)_2SO_4$ precipitation. The difference might be due to the concentration of ammonium sulphate used for this purification. Horseradish POD exist in many isozymic forms (Dange & Reddy, 1984), majority of which are anionic (Evan, 1968). The most often used cellulosic anion exchanger is DEAE cellulose (Voet & Voet, 1990). Degree of purification of horseradish POD was 14.08 fold with DEAE cellulose chromatography. Rz value (absorbance at 403/275), the index of enzyme purification (Sesto & Huystee, 1989), was increased from 0.783 to 1.82 during POD purification. Specific activity of HRP in crude extract was 1.080 and it increased during the process of purification to 15.21 (Table I).

Table I. Summary of horseradish peroxidase purification

	CE	AS	DEAE
Protein (mg/ml)	1.810	1.910	0.324
Activity (U)	1.955	3.985	4.927
Specific gravity (U)	1.080	2.086	15.210
Degree of purification	1.000	1.930	14.080
R_z value	0.783	1.020	1.820

CE= Crude extract; AS= $(NH_4)_2SO_4$ precipitated; DEAE= After diethyleamoinoethyle cellulose chromatography

CONCLUSIONS

1. Horseradish roots (HRR) were found as the best source of POD among the studied vegetable sources.
2. The optimum pH for enzyme activity was measured as 6.0 for radish whereas 6.5 for turnip, HRL and HRR.
3. The enzyme was found stable even at temperature of 50°C showing relative activity from 60 to 80%.
4. The DEAE cellulose chromatography purified the enzyme 14 times.

REFERENCES

Ausubel, M., R. Brent, R. Kingston, D. Moore, G.J. Seidman, J.A. Smith and K. Struhl, 1989. *Current Protocols in Molecular Biology.* Greene Publishing Associates and Wiley Interscience, Johns Wiley and Sons, New York.

Burnette, F.S., 1977. Peroxidase and its relationship to food flavour and quality: A review. *J. Food Sci.*, 42: 1–6.

Civello, P.M., G.A. Martinez, A.R. Chaves and M.C. Anon, 1995. Peroxidase from strawberry fruit: Partial purification and determination of some properties. *J. Agri. Food Chem.*, 43: 2596–2601.

Cooper, T.G., 1977. *Tools of Biochemistry,* pp: 50–4. University of Pitts-brough, New York, Chichester, Brisbance, Singapore.

Dange, V. and G.M. Reddy. 1984. Substrate specificity of peroxi-dase isozymes in rice. *Indian J. Plant Phys.*, 27: 271–5.

Evans, J.J. and N.A. Alldridge, 1965. The distribution of peroxidases in extreme dwarf and normal tomato (*Lycopersicon esculentum* Mill). *Phytochem.*, 499: 499–503.

Evans, J.J., 1968. Peroxidases from the extreme dwarf tomato plant: Identification, isolation and partial purification. *Plant Physiol.*, 43: 1037–41.

Halpin, B., R. Pressey, J. Sen and N. Mondy, 1989. Purification and characterization of peroxidase isoenzymes from green peas (*Pisum sativum*). *J. Food Sci.*, 54: 644–9.

Ito, H., N. Hiraoka, A. Ohbayashi and Y. Ohashi, 1991. Purification and characterization of rice peroxidases. *Agri. Biol. Chem.*, 55: 2445–54.

Michel, F.C., E.A. Grulke and C.A. Reddy, 1990. Development of a stirred tank reactor system for the production of lignin peroxidases (ligninases) by Phanerochaete chrysosporium BKM-F-1767. *J. Indust. Microbiol.*, 5: 103–12.

Park, I., S. Kho and I. Nam, 1990. Application of cabbage peroxidase for glucose assay. J. Korean Soc. Food Nut., 19: 224–8.

Rahayuningsih, S.A., 1990. Peroxidase activities and their relationship to resistance of rapper plants to *Phytophotlra palminora. Indust. Crops Res.*, 3: 18–22.

Rehman, K.U., M. Yaqub, M.A. Usman and M. Arshad, 1997. Studies on comparative evaluation of peroxidase, extracted from tomatoes and Horseradish legume. *Proc. Ist Nat. Symp. Biotech. for Sustainable development,* p. 87. University of Agriculture, Faisalabad, Pakistan.

Sandusky, G.E. and K.A. Wightman, 1985. Application of the peroxidase-antiperoxidase procedure to the localization of pituitary hormones and calcitonin in various domestic animals and human beings. *American J. Vet. Res.*, 46: 739–41.

Sesto, P.A. and R.B.V. Huystee, 1989. Purification and yield of a cationic peroxidase from peanut suspension cell culture. *Plant Sci. Irish Republic,* 61: 163–8.

Talat, T., 1996. Studies on comparative evaluation of peroxidase extracted from turnip and radish. *M.Sc. Thesis,* Department of Chemistry, University of Agriculture, Faisalabad, Pakistan.

Theorell, H., 1940. *Chemistry and Methods of Enzymes,* 2nd ed., p. 207. Academic Press, New York.

Voet, D. and J. Voet, 1990. *Biochemistry.* John Wiley and Sons, New York.

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