

## **Orange Wastes as a Natural Source of Peroxidase: Extraction and Purification**

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### **Summary**

A variety of orange wastes obtained from citrus processing factories in Northern Iran demonstrated considerable variations in peroxidase content. Using the newly collected wastes of orange juice factories as a source, the peroxidase content was extracted and characterized. It was observed that the specific activity of peroxidase was increased at various stages of purification process. However, in all cases it was found that the specific activity and other characteristics were almost similar to peroxidases obtained from other sources. The optimum pH and temperature were slightly different from those reported for peroxidases from whole orange species examined in our previous research.

**Key words:** Peroxidase, optimum pH, optimum temperature.

### **Introduction**

Peroxidases (PODs, E.C. 1.11.1.7) are haemoproteins that are widely distributed in the plant kingdom [1], microorganisms and animal tissues. Moreover, many heme-containing proteins like haemoglobin, catalase and cytochrome P-450 also possess peroxidase activity to some extent. Peroxidases can catalyze the oxidation of a wide variety of substances through a reaction with hydrogen peroxide [2, 3].

The major source of commercially available peroxidase is the roots of horseradish (*Armoracia rusticana*) (HRP). However, availability of peroxidases with higher stability and different specificity may allow the improvement of its industrial applications. Some investigations are carried out with the aim of peroxidase production from other plant sources [4, 5]. Considering the wide variety of citrus species grown in North of Iran and many factories for processing and juicing, the main purpose of this study was to extract and characterize the peroxidase from wastes of mixed citrus wastes. It is suggested that the wastes obtained from citrus processing factories could be considered as an economic peroxidase source to replace the commercial HRP.

## Materials and Methods

### Material

Newly collected citrus, mostly orange, wastes were obtained from juice factories located in North of Iran near the Caspian Sea. Horseradish peroxidase, 1-phenyl-2,3-dimethyl-4-amino pyrazolan (AAP), folin-ciocateus, acrylamide, N,N-methylene-bis-acrylamide, ammonium persulphate, bromophenol blue, hydrogen peroxide and 2-mercaptoethanol were purchased from Merck Chemical Company, glycine from BDH and protein molecular weight marker from MBI.

### Extraction of peroxidase

100 g of orange wastes were homogenized in a porcelain mortar in the presence of liquid nitrogen. 15 ml of 0.25 M phosphate buffer solution (pH 7.0) containing 0.1mM EDTA (to prevent protease reaction), 1 mM mercaptoethanol (preventing peroxidase oxidation) and 0.1 M NaCl was added and the resulting solution centrifuged at 5°C (7500 rpm at for 35 min).

### Total protein and enzyme assay

Total protein content was measured in the supernatant [6]. Peroxidase activity was determined spectrophotometrically [7]. In a typical reaction mixture, 40 µl of enzyme solution was added to 300 µl of 0.2 M phosphate buffer containing substrates (2.5 mM 1-phenyl-2, 3-dimethyl-4-amino antipyrine and 1.7 mM hydrogen peroxide). Progress of the reaction was followed by measuring the change in absorbance of mixture at 510 nm (25°C).

### Enzyme purification

The homogenate was incubated for about an hour at room temperature. The extract was then fractionated by addition of ammonium sulphate, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> to 85% (at 4°C and controlled pH). The sample was then mixed for another 30 minutes followed by centrifugation at 5000 rpm at 5°C for 30 minutes. 2 ml of 0.2 M phosphate buffer was added to the precipitate and mixed well. The mixture was centrifuged again under the same conditions for 10 minutes in order to eliminate ammonium salts. The resulting peroxidase solution was applied to a DEAE-Sepharose column (1 × 50 cm) equilibrated with the same buffer. The elution was carried out with 10 mM tris-HCl buffer (pH 8.3) at a flow rate of 25 ml/h. The fraction containing active peroxidase was stored at 5°C.

### Optimum temperature

Thermal stability of a  $3 \times 10^{-5}$  M of purified peroxidase was measured at temperatures of 10-80 °C. The enzyme was incubated at the desired temperature for about 20 minutes and the change in its absorbance was then recorded.

### pH stability

To measure the optimum pH of the extracted enzyme, 2.6 mM 1-phenyl-2, 3-dimethyl-4-amino antipyrine was used and 1.8 mM hydrogen peroxide as electron acceptor. The following buffers were used: glycine/HCl (pH 3.0), sodium acetate (pH 4.0 and 5.0), phosphate (pH 6.0 and 7.0), tris/HCl (pH 8.0) and glycine/NaOH (pH 9.0).

### Results and Discussion

The extracts of wastes were purified to homogeneity using the following procedure. One typical purification route is summarized in Table I. The extract was first purified using ammonium sulphate. Sephadex G-75 was then used for further purification in the next stage. Finally, the gel filtration was carried out on Sepharyl S100 resulting in specific activities of 2562.3 U/mg protein.

**Table I. Extraction and purification steps of peroxides from orange wastes.**

Procedure	Volume (ml)	Protein (mg)	Specific activity (U/mg protein)	Total activity (U)	Yield (%)	Purification fold
Homogenate	6.378	4.8	734.1	330	100	1
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.983	0.366	323.4	116	36	5
Sephadex G75	0.622	0.054	1852.5	101.5	30	29
Sepharyl S100	0.01	0.021	2562.3	56.4	18.1	44

The results showed this newly extracted peroxidase was as active as horseradish peroxidase towards its most common substrate, 1-phenyl-2,3-dimethyl-4-amino antipyrine. It is known that peroxidase activity does not depend on the plant source [9-11] and it can be predicted that the peroxidase extracted from the wastes may fairly be similar to peroxidase of plant itself.

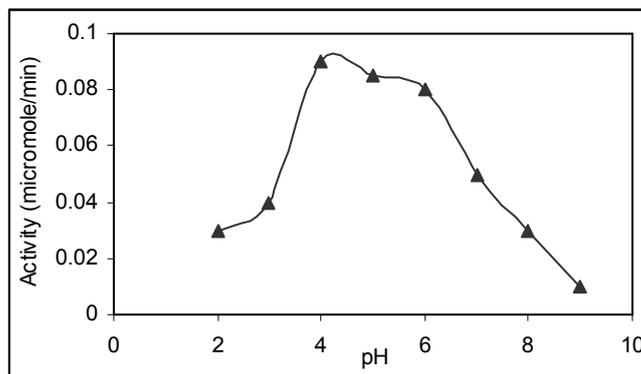
We had previously found that the specific activity of *Orange Limon* is about 10 times higher than that of *Orange nobilis* in the starting homogenate. This difference reduced during purification so that their specific activities are close after the last stage of purification showing the higher proportion of other enzymes in *Orange nobilis* compared to *Orange Limon*.

Using a step by step purification process, peroxidase was isolated almost specifically with a purification fold of 44. Lawry protein assay was performed to measure the protein concentration using purified enzyme instead of bovine serum albumin as standard. The yields were 12.5% after 55 fold. The yield of later is fairly high compared to the values obtained for other peroxidases [10].

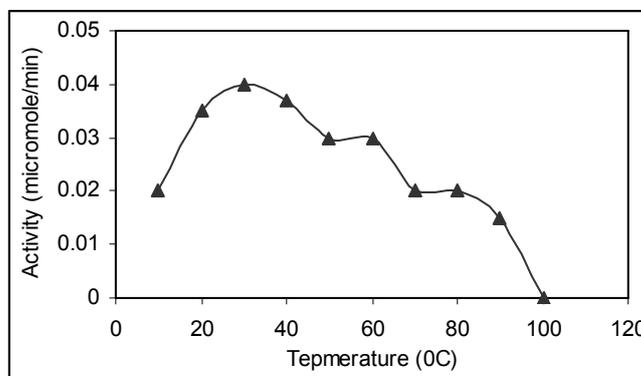
The typical Soret maximum at 403 nm for all plant peroxidases [10-12] was observed for the novel peroxidase. The purity index (RZ value) calculated from the product of the absorbance at 403 and 280 was 3.48.

$$RZ = A_{403}/A_{280}$$

It is well known that the optimal conditions for catalysis by different peroxidases are not identical [13-14]. We, therefore, measured the optimum pH and temperature for peroxidase extracted from orange wastes (Figures 1 and 2 respectively). The optimum pH for this peroxidase was 4-6, while the temperature at which the highest activity was observed varied from 25 to 40°C.



**Fig. 1: The effect of pH on the activity of peroxidase extracted from orange wastes.**



**Fig. 2: Optimum temperature obtained for orange waste peroxidase.**

It was found that Orange waste peroxidase is less stable to changes in pH compared to peroxidase extracted from orange. Measuring thermal stability of orange waste peroxidase, showed that a fair activity over a wide range of temperature (25-40°C). A similar thermal stability has also been observed for peroxidase from leaves of *lipomoea palmetto* [15] and soybean peroxidase [16].

The molecular weight of this novel peroxidase was estimated from SDS-electrophoresis and its value was about 40 kDa.

### Conclusions

Based on the results obtained from this research the following conclusions were made:

1. Specific activity of peroxidase extracted from orange waste is about 8 times higher than that of *Orange nobilis* in the starting homogenate.
2. The molecular weight was estimated from migration in sodium dodecyl sulphate gel electrophoresis (PAGE) and it was 40 kDa.
3. The optimum pH was obtained within 2 to 4 and optimum temperature 25-40 °C. This type of pH and temperature stability is similar to peroxidase from other sources.

4. Electrophoresis of purified enzymes under different buffer conditions showed three isozymes for (in acidic, basic and neutral pH values).
5. The yield of purified peroxidase was 15.5% after 39 folds purification
6. Orange peroxidase could be suggested as a replacement candidate for HRP, especially in Northern Iran where orange wastes could be obtained cheap and fairly fresh.

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