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PURIFICATION AND CHARACTERIZATION OF PEROXIDASE EXTRACTED FROM WHEAT BRAN

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SUMMARY

This study was aimed to purify peroxidase extracted from wheat bran and studying its biochemical characteristics. Wheat grains samples were collected from wheat crop field, and milled then wheat bran was collected by sieving using flour sieve. Peroxidase was extracted from wheat bran by using different extraction buffer solutions, then peroxidase was purified by three steps of purification includes precipitation with ammonium sulfate in a saturation ratio of 70 %, ion exchange chromatography through DEAE-Cellulose, and gel filtration chromatography throughout sephadex G-100. Results showed that the specific activity of purified enzyme was 510 u/mg, with a yield of 18% and purification fold of 5.1. Results of characterization of the purified enzyme indicates that enzyme molecular weight was 52000 dalton by using Gel filtration technique and the optimum pH for enzyme activity and stability was 7 , and the optimal temperature of the enzyme activity was 50°c and 45°c respectively. The activation energy for conversion of substrate to product was 10.18 Kcal / mol.

INTRODUCTION

Peroxidases (E.C.1.11.1.7) belong to the oxidoreductases class have heme III, protoporphyrin IX and ferriprotoporphyrin IX as prosthetic group.[1] Members of these groups catalyze the oxidation number of substances in the presence of hydrogen peroxide.[2] Peroxidases exist in very wide range of the plants especially at vacuoles, tonoplast, plasmalemma and inside and outside the cell wall.[3] In addition present in another living organisms such as animal and micro-organisms.[4] Different physiological functions of these enzymes reflect the presence as isoenzymes in plant cell organelles. They are include plant hormone regulation[5], participated

in lignifications and suberization[6], protection toward H2O2 and other oxidants[7], defense properties against pathogenic causes[8], and have role in tolerance of drought in plant[9] According to previous studies peroxidases used in varied scientific fields involve biotechnology, biochemistry, clinical and industrial purposes. It used in diagnosis and biosensors for recombination and expression of recombinant protein and protein engineering.[10] Immunochemistry and ELASA.[11] Manufacturing of many aromatic complexes, elimination of phenolics complexes from waste water and peroxides from foods, beverage and industrial wastes.[12] According to the importance of peroxidase, this study was aimed to find new source for peroxidase utilized in different fields of applications, purifying the enzyme from this source and studying its biochemical characteristics.

MATERIALS AND METHODS

Extraction of peroxidase

Peroxidase was extracted from wheat bran by mixing 200g of wheat bran with 0.2 M sodium acetate buffer solution (pH5) in a ratio of 3:1(weight: volume) with continuous stirring for 15 minutes at 4 °C. Mixture was then filtered throughout layers of cheese cloths, then filtrate was centrifuged at 10000 rpm for 15 minutes. Protein concentration, activity and specific activity of peroxidase were determined.

Protein Concentration

Protein concentration in peroxidase extracts was determined according to Bradford[13] by using Coomassie blue G-250 and Bovine serum albumin standard solution.

Enzyme assay

Peroxidase activity was assayed according to Bernhard and Whitaker.[14] Enzyme activity was defined as the amount of the enzyme oxidizing 1 µmol of glycol in one minute under the experiment conditions.

Purification of enzyme

Three steps of purification for peroxidase extracted from wheat bran include precipitation by ammonium sulfate, Ion exchange chromatography and gel filtration chromatography.

Ammonium sulfate precipitation

Peroxidase in crude extracts was precipitated by using different saturation ratios of ammonium sulfate ranged between 30% and 80%. Enzyme activity was determined after

precipitation with each saturation ratio. Precipitated enzyme was dissolved in distilled water and dialyzed against distilled water for 24 hours at 4 °C with three increments of substitutions. Then protein concentration, activity and specific activity of peroxidase were determined.

Ion exchange chromatography

Ion exchange chromatography was used to purify peroxidase obtained from precipitation step was purified throughout DEAE – cellulose. Washing step was achieved by using phosphate buffer (5mM, pH=7),while elution step was achieved by using gradient concentrations of sodium chloride (0.1-1 M). Fractions were collected at flow rate of 20ml/hour. Absorbance (280nm) and enzyme activity was measured in each fraction.

Gel filtration chromatography

Enzyme solution produced from Ion exchange step was concentrated and purified by gel filtration chromatography throughout Sephadex G-100 and eluted with potassium phosphate buffer solution (0.2M, pH 7), at flow rate of 20ml/ hour. Absorbance (280nm) and enzyme activity was measured in each fraction.

Characterization of purified peroxidase

Characterization of purified peroxidase was achieved by determining the optimum pH and temperature for enzyme activity and stability, and the activation energy according to Segel (15), while the molecular weight of peroxidase was determined by gel filtration chromatography technique using Sephadex G-100 according to Andrews.[16]

RESULTS AND DISCUSSION

Peroxidase was extracted from wheat bran by using sodium acetate buffer solution. Results showed that enzyme specific activity was 181 u/mg in crude extracts. Crude enzyme was purified first by precipitation with ammonium sulfate in different saturation ratios. Results showed that peroxidase was precipitated efficiently with ammonium sulfate in saturation ratio of 70%. Enzyme specific activity reached to 240.1 U/mg, with a purification fold 2.4, and yield of 51.7% as shown in table (1). The ammonium sulfate was used in enzyme precipitation because it high soluble and cheap compared with the others salts, unaffected in pH and enzyme stability. The concentration by ammonium sulfate depending on equilibrate the charges found in protein surface and disrupt of the water layer surrounding it, that leads to protein precipitation as it was mentioned by Schmander.[17]

Ion exchange chromatography

Dialyzed enzyme produced from ammonium sulfate precipitation step was more purified throughout DEAE-Cellulose ion exchanger. Results illustrated in figure (1) showed that one peak of proteins was appeared in washing step represents enzyme activity. This indicates that peroxidase carry positive charge similar to the charge of ion exchange under the experimental conditions. In this step of purification, enzyme specific activity was 343.3 U/mg with a purification fold 3.4 and yield reach to 32.8% as shown in table (1). While in the elution step illustrated in figure (1), four protein peaks were eluted by gradient salt concentrations not represents enzyme activity. In another study, Zia et al.[18] purify peoxidase extracted from apple seeds, and he was found that the specific activity of peroxidase after this step of purification was 7.53 U/mg, with purification fold of 6.82 and 42.89% recovery.

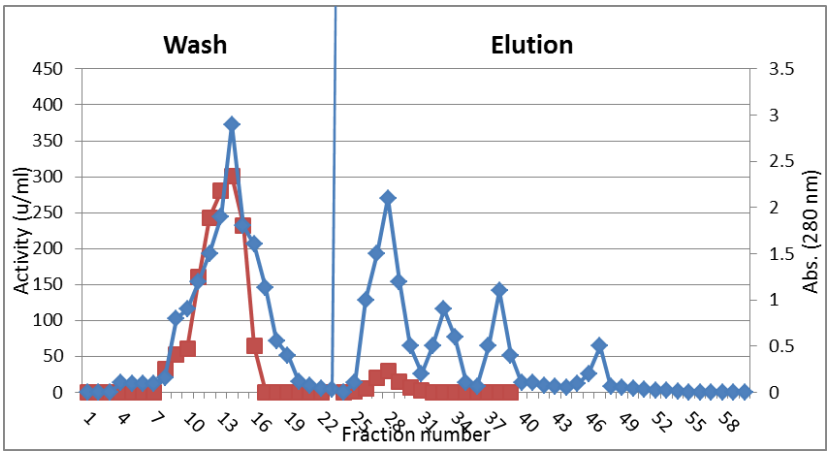
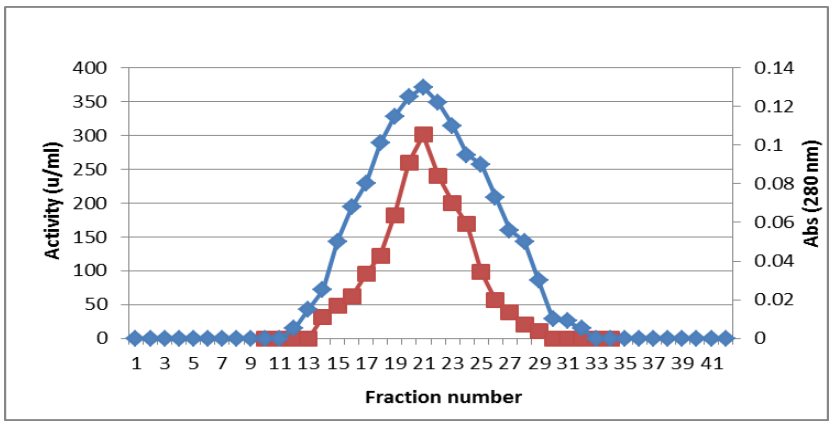


Figure (1): Ion exchange chromatography for purification of peroxidase extracted from wheat bran using DEAE-Cellulose column (2.5×16) with flow rate of 20 ml/hour



Figure(2): Gel filtration chromatography for purification of peroxidase extracted from wheat bran using sephadex G-100 (36×1.5 cm) equilibrated with potassium phosphate buffer solution (0.2M, pH 7), at flow rate of 20ml/ hour

Gel filtration chromatography

Enzyme solution obtained from ion exchange chromatography purification step was more purified by Gel filtration chromatography technique throughout sephadex G-100. One protein peak represents enzyme activity was appeared (figure (2)). Enzyme specific activity was reached 510 U/mg, with a purification fold 5.1 and enzyme yield of 18% as shown in table (1).

Table (1): The purification steps of peroxidase extracted from wheat bran

Purification step	Volume (ml)	Enzyme activity (U/ml	Protein concentration (mg/ml)	Specific activity (U/mg)	Total activity (U\ml)	Purification fold	Yield (%)
Crude extract	91	310	3.1	100	28210	1	100
Ammonium sulfate precipitation	20	730	3.04	240.1	14600	2.4	51.7
Ion exchange chromatography	15	618	1.8	343.3	9270	3.4	32.8
Gel filtration Chromatography	20	255	0.5	510	5100	5.1	18

Characterization of purified peroxidase

Optimum pH for enzyme activity

Results illustrated in figure (3) showed an increase in enzyme activity with increasing pH value until reach the maximum activity (311U/ml) in pH7 using guaiacol as an enzyme substrate, and then it began to decrease at higher pH values. It was found that peroxidase was active in neutral pH, and the activity was decreased at acidic and alkaline pH. The differences in peroxidase activity at different pH values may be mainly due to the changes occurred in ionic state of enzyme and substrate molecules. In another study, Al-Badri[19] mentioned that the optimum pH for activity of peroxidase purified from *Malva neglecta* was pH7.

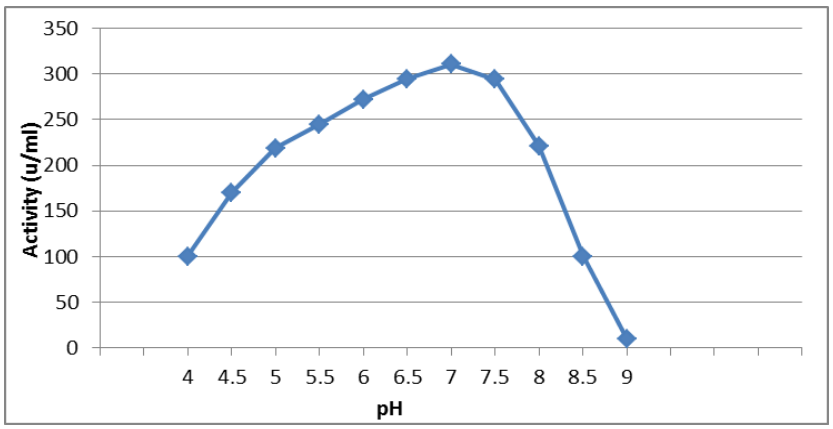


Figure (3): Effect of pH on peroxidase activity purified from wheat bran

The optimum pH of enzyme stability

To study the effect of optimum pH for enzyme stability the peroxidase solution was incubated with different buffers pH values ranging between (4-9.0) for 30minute at 35˚C, then measured the remaining activity. The figure (4) show the optimum pH of stability (7) and this stability was decreased in extreme acidic and alkaline pH. The enzyme was kept 87% of its activity in pH 8 while kept88% of activity in pH 6, the activity was decline in pH 4and 9 to 54% and 0% respectively. The effect at pH on the enzyme stability is belong to the effect of pH in enzyme structure lead to denaturation of enzyme molecule or change the ionic state of active site, also it effect on secondary and tertiary of enzyme structure lead to losing the activity in buffers that far away from optimum pH (Segel, 1976). Silva and Valdir (1990) found that the optimum pH of peroxidase purified from papaya fruits ranging between (6.0- 9.0) when incubation the enzyme with buffer at pH 3.0.

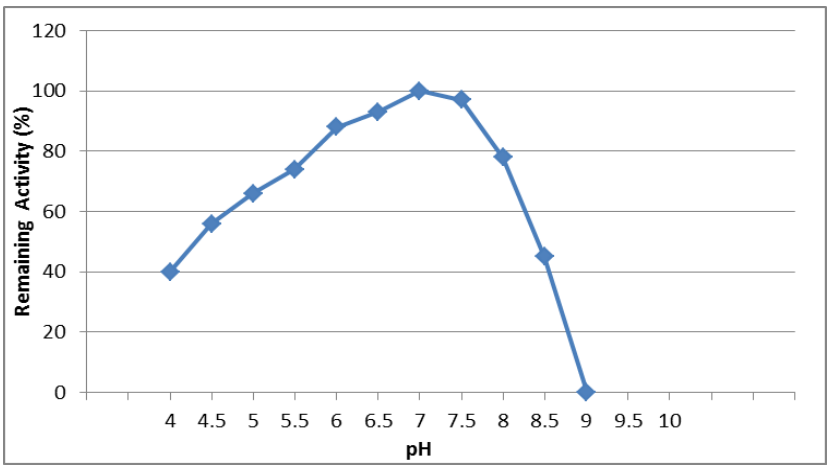


Figure (4): Optimum pH for stability of peroxidase purified from wheat bran

Optimum temperature of enzyme activity

To determine the optimum temperature for peroxidase activity purified from wheat bran, enzyme reaction was done in different range of temperature (30-80 ˚C). Results illustrated in figure (5) showed an increase in peroxidase activity with the increase of temperature, and reached the maximum (100%) at 50˚C, then it begin to decline with increasing temperature until reached 40% of its activity at 70˚C. This study was agreed with Gisele et al.[20] who found that highest activity of peroxidase purified from litchi pericarp was reached at 40-50˚C for 10 minute, after this temperature the activity of peroxidase started to reduce until reach to 58.8 and 76.6% at 60˚C and 70˚C respectively.

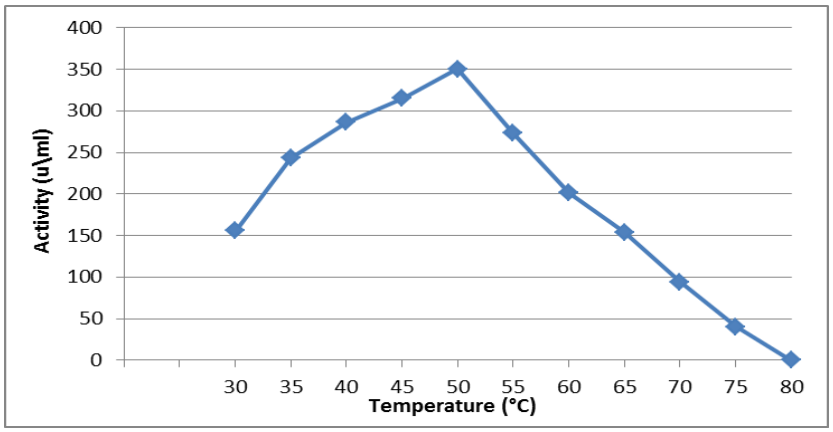


Figure (5): Effect of temperature on the activity of peroxidase purified from wheat bran

Optimum temperature for peroxidase stability

Results illustrated in figure (6) showed that peroxidase maintained its activity when it was incubated at 45 ˚C, while it keeps 95% and 43% of its activity when the enzyme was incubated at 50˚C and 70˚C respectively. The differences of thermostability of the enzyme depend on the type of substrate, ionic strength of buffer solution and enzyme molecular weight.[21,22] Other study referred that the optimum temperature for stability of peroxidase purified from litchi pericarp was 40˚C for 10 minute. After that, the activity of peroxidase started to decrease at higher temperatures.[20]

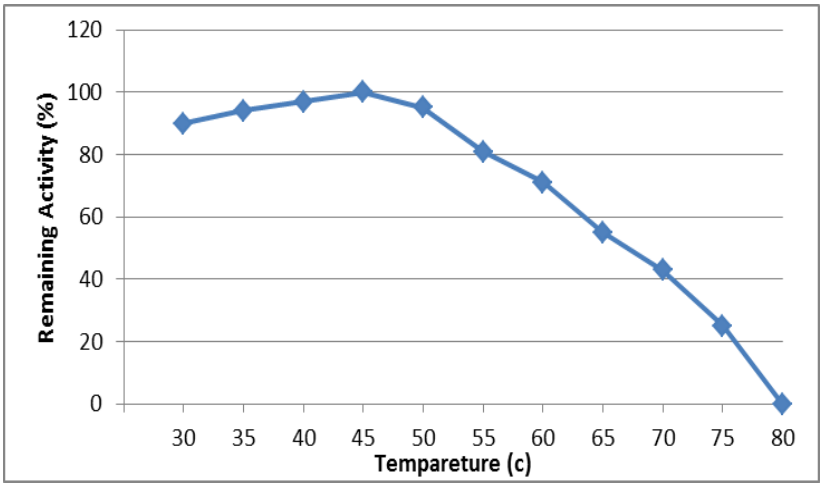


Figure (6): Optimum temperature for stability of peroxidase purified from wheat bran

Molecular mass

The molecular weight of peroxidase purified from wheat bran was determined by using gel filtration chromatography throughout sephadex G-100 in presence of five standard proteins as a size indicators and plotting the relationship between elution volume/ void volume against log molecular weight. Results illustrated in figure (7) showed that the molecular weight of

peroxidase was 52000 Dalton. Gel filtration chromatography was used to determine molecular mass of plant peroxidases where it was ranged between 28-60 kDa as it was mentioned by Hiraga et al.[23] In another study, it was found that the molecular weight of peroxidase purified from *Brassica Oleracea* was 95 kDa.[24]

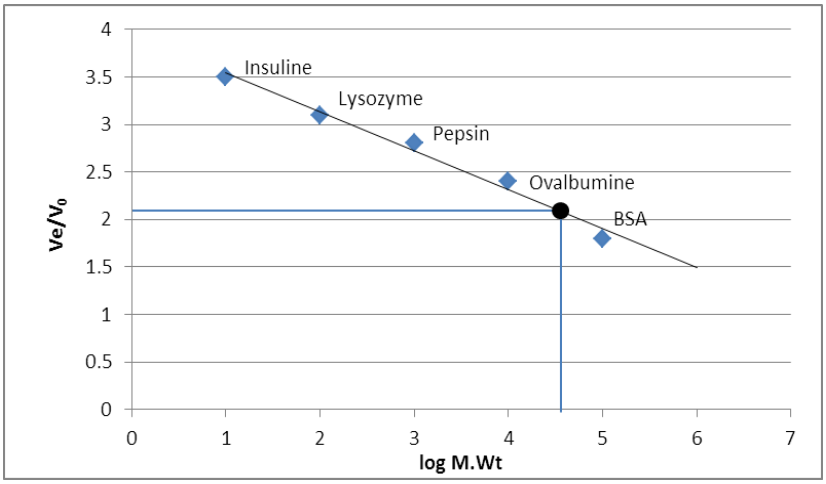


Figure (7): Molecular weight of wheat bran peroxidase.

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