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Characterization and Immobilization of Peroxidase Extracted from Horse Radish and Decolorization of Some Dyes

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A B S T R A C T

The present work was undertaken to obtain crude Peroxidase extract from radish to characterize it in terms of pH, temperature, number of times use and decolrization of some dyes. The results shows that peroxidase from radish gave highest specific activity 4391 U/mg, and potassium phosphate buffer (0.1 M, pH 7) was best extraction buffer for peroxidase extraction from radish with specific activity 4489 U/mg, while The highest specific activity was measured for crude extract at 1:2 ratio, it was 4479 U/mg protein. Entrapment of peroxidase enzyme by agarose was best method for immobilization. The optimum pH of free and immobilized peroxidase enzyme activity was 6.0, while the pH stability of free peroxidase enzyme from radish was 6.0, and the pH stability of immobilized peroxidase enzyme was range from 4.0-8.0. The optimum temperature for free peroxidase activity from radish was 35 ^{O}C, while the free enzyme was stable in temperature 35-60 ^{o}C, then the activity begun to decrease and was completely lost in 65-70^{o}C. The best temperature for immobilized peroxidase activity was 35 ^{o}C, while the results showed that the immobilized enzyme was stable in temperatures between 35-45 ^{o}C. The activity of the immobilized enzyme which incubated many times with substrate was decreased after eighth time using. Giemsa stain and acridine orange, were removed and make change in their absorbance after incubation with immobilized eighth enzyme for period of time, while no degradation of the other dyes occur.

Introduction

Peroxidase enzymes (donor: H_2O_2 oxidoreductase, Ec. 1. 1 1.1.7 are widely distributed in plants, animal tissue and micro-organisms. Peroxidase was first found in the fig tree in 1936. In 1941 the enzyme was isolated and characterized from horseradish (HRP) (Andrew *et al.*, 2013). During the years of 1942 to 1959, isolation of the enzyme was reported from various sources such as yeast, potato, beans, Japanese radish and wheat.

Peroxidase enzyme is widely found in the nature, their diversity can be seen in the plant kingdom and within the same plant species also became as an indicator of genetic discrimination in many plants to detection of stress in plants as well as follow-up and study the mechanism of the effect of pathogens on

plant families (Cheung and Tai, 2007). Peroxidases became important industrially and medically, peroxidase were gained (hours radish peroxidase HRP) great economic importance through its uses in the number of diagnostic and analytical varied because of his qualities kinetic and physical and chemical suitable for such applications and perhaps the most important number is the number of ELISA (Enzyme - Linked Immuno Sorbent Assay) in labeling antibodies or antigens in the immune reactions by attaching these enzymes on solid surfaces as it is the specifications that qualify for that one of these properties the qualities high affinity toward the material basis (Yazdi *et al.,* 2002), ease of detection effectiveness configures outputs of color, do not need a process to measure the steps separating from substrates, high persistence during storage, low costs for preparation and purification as well as its importance in the analysis of the stigma blot assays and in pigmentation tissue it is also used in biochemical analysis to estimate the hydrogen peroxide generated by some systems, such as the oxidation of glucose, amino acids and cholesterol … etc. (Passardi *et al.,* 2007). Also reflected the importance of these enzymes in the processing of fruits and vegetables by evaluating the content extracts of food stuff from antioxidants such as ascorbic acid, phenols, flavonoids and tannins, which working on multiple modifications during the manufacturing process and storage (Fatima *et al.,* 2007). Enzyme immobilization can endow enzymes with some additional advantageous properties. The immobilized enzymes can be used repeatedly or continuously in a variety of reactors for the efficient recovery of costly enzymes, and be easily separated from reaction systems for reuse, which make the work-up simple and the protein of the final product uncontaminated. Furthermore, it is reported that immobilized enzymes may exhibit higher selectivity and specificity (Kiralp *et al.,* 2003).

Materials and Methods

Chemical materials

Guaiacol, phosphate buffer, Tris-HCl, Coomassie brilliant blue, bovine serum albumin were obtained from Sigma Co. other chemicals were supplied by BDH Chemicals.

Peroxidase source

Different sources were used for extraction of peroxidase. These sources were radish, cabbage, potato, green onion top, white onion, green onion leaves, white bean, apricot seeds, red bean, and soybean. All sources were washed with tab water then extraction of the enzyme. The enzyme activity and concentration of peroxidase were determination.

Determination of peroxidase activity

The activity of peroxidase is measured according to Silva and Koblitz (2010). The substrate solution was prepared by mixing the following volumes according to each ratio, guaiacol: Hydrogen peroxide solution: Sodium acetate solution: Distilled water [1: 1: 1: 7 (v: v: v: v)]. The reaction solution contained 2.9 mL of substrate solution (pH 6.0) and 0.1 ml of the enzyme. 3 mL of substrate solution was used as blank sample.

The oxidation of guaiacol was detected by measuring the absorbance increase at 470 nm after 3 min using a spectrophotometer. One unit of peroxidase activity was defined as the amount of enzyme that caused an increase in absorbance of 0.001/min. Calculation of peroxidase activity was used the following equation:

Activity of peroxidase ($U.ml^{-1}$) = [(A2 sample – A1 sample) – (A2 blank – A1 blank)]/ (0.001× t)

A2 sample: is the final absorbance of the sample, A1sample: is the initial absorbance of the sample; A2 blank: is the final absorbance of the control, A1 blank: is the initial absorbance of the control, and t is the reaction time in minutes (3 minutes).

Protein concentration determination

Protein concentration was determined according to the dye-binding method of Bradford using bovine serum albumin as a standard (Bradford, 1976).

Type of extraction buffer

Radish was homogenized with different types of buffers for peroxidase extraction.

These buffers are sodium acetate at concentration 0.1M at pH 5.0 and 6.0, sodium citrate 0.1M at pH 4.0, potassium phosphate 0.1M at pH 7.0 and Tris-HCl 0.1M at pH 8.0., also used water tap and distilled water for extraction of peroxidase enzyme from the radish. The activity and concentration estimated in each treatment (Silva and Koblitz, 2010).

Extraction ratio

A twenty five grams of radish were homogenized in different volumes of buffer extract (0.1 M phosphate buffer pH 7.0) for extraction of peroxidase enzyme. The extraction ratios were 1:0.5, 1:0.75, 1:1, 1:1.5, 1:2, 1:3 and 1:4 (v:w). The enzyme activity and protein concentration was then determined.

Immobilization of peroxidase

Entrapment by agarose

The entrapment on agarose done by dissolved 3g from agarose in 100ml distil water under heat condition then cooling and mixing with peroxidase in volume 1:0.25(v/v) then pour it in plate until solid and cut it a square pieces (2x2cm) and store in sodium acetate buffer (0.02M pH 6.0).

The activity of the immobilized enzyme was calculated (Kiralp *et al.*, 2003).

Entrapment by Ca-alginate

It was prepared by dissolving 5 g of Na-alginate in 100ml distilled water under heat condition then cooling it and mixing it with peroxidase in volume 1:0.25(v/v) then add the mixture as drop by syringe in 2 M $CaCl_2$ solution, then beads was washed with 0.02M sodium acetate buffer (2M, pH 6.0).

The activity of the immobilized enzyme was calculated, then the remaining activity % was measured (Duran *et al.*, 2002).

Characterization of free and immobilized enzyme

The effect of pH on enzyme activity

Different types of buffer were used (1M sodium acetate pH range between 3 to 6, 0.1M phosphate buffer pH7, and 0.1M Tris-HCl pH8 and 9) in the preparation of reacting substrate then we incubate free and immobilized enzyme with substrate and measure the activity (Mizobutsi *et al.*, 2010).

The effect of pH on enzyme stability

Different types of buffer (0.1M sodium acetate pH 3-6, 0.1M phosphate buffer pH 7.0 and 0.1M Tris-HCl pH8-9} were prepared to incubate with the free and immobilized enzyme at a ratio (1:1 v:v) for 30 min., then the remaining activity % for each enzyme was calculated (Saeidian, 2013).

The effect of temperature on enzyme activity

The free and immobilized enzyme were incubated with substrate for 3 min in a water bath at different temperature (35, 40, 45, 50, 55, 60, 65 and 70°C), then the activity was measured for each temperature (Mizobutsi *et al.*, 2010).

The effect of temperature on enzyme stability

One ml and 1 gm from free and immobilized enzyme respectively were incubated in water bath at different temperature (35, 40, 45, 50, 55, 60, 65 and 70 °C) for 30 min then transfer it directly to cold water and the remaining activity % was measured for each temperature (Saeidian, 2013).

The effect of number of times for using the immobilized enzyme

The experiment of number of times for using the immobilized enzyme was done by weighting 1gm from immobilized enzyme and incubate with substrate then the enzyme activity was measured, then the immobilized enzyme was washed with 0.1M potassium phosphate buffer (pH 7) and repeated incubation of enzyme with new substrate and measure enzyme activity then the activity was repeated for many time, until less the enzyme activity to calculate the number of times for using the immobilized enzyme (Guisan, 2014).

Decolorization of some dyes by immobilized peroxidase enzyme

Stock solutions of 7 type of dyes (brilliant green, bromophenol blue, Giemsa stain, toluidine blue, neutral red, acridine orange and indigo carmine) were prepared in sterilized distilled water and diluted to 25 mg/L. The optical density of each dye was measured depending on its λ-max using a spectrophotometer. The reaction mixture was prepared by adding 1 gm of immobilized peroxidase enzyme to 3 ml of the dye solution, then measure the change of absorbance for each dye depend on their λ-max. A control sample was prepared for each dye without immobilized enzyme and treated under the same condition. Decolorization efficiency of immobilized peroxidase was assessed by monitoring the decrease in absorbance under maximum wavelength of the dye (Guisan, 2014).

Results and Discussion

Effect different sources on peroxidase activity

Different specific peroxidase activities were shown for various plant sources as in figure 1. This figure shows that peroxidase from radish gave highest specific activity 4391 U/mg, while cabbage, potato, green onion top, green onion leaves, soybean and red bean were 1796, 140, 255, 455, 8 and 21 U/mg, respectively. White onion, white bean, and apricot seeds didn't give any activity. Radish was used as a source of peroxidase because it's available, and inexpensive.

A study by (Sariri *et al.*, 2003), was illustrated that the specific activity of peroxidase extracted from soybean seed coat was 37.7U/mg protein. Other study by (Rudrappa *et al.*, 2005), was shown the specific peroxidase activity from hairy root cultures of red beet was 600 U/mg proteins.

Type of extraction buffer

The specific peroxidase activity was estimated after extraction the enzyme with different buffer, as shown in figure 2. These results show that potassium phosphate buffer (0.1 M,

pH 7) was best extraction buffer with specific activity reached to 4489 U/mg, while other buffers were given low specific activity.

pH effect on enzymatic activity and stability can be explain by the fact that depending on influence the structure of enzyme by changing the pH degree that may lead to differences ionization state of the various amino acid residues (Mizobutsi *et al.,* 2010). The effect of pH on peroxidase activity was shown in several ways. First, each enzyme has its own optimum pH, for maximum enzyme activity, but the enzyme is stable within certain limits under and above the optimum pH thus any changing in the degree of pH lead to denature the enzyme structure .Third, any change in the pH may cause dissociate of substrate-enzyme complex (Guisan, 2014). (Harco *et al.,* 1999) were shown the optimum pH value for extraction of peroxidase from *Nicotiana tabacum* by potassium phosphate buffer 50mM was pH= 7.5 adding to its 1mM Corbett, DTT, EDAT and 3% Triton X-100 and 0.8M NaCl in extraction ratio 1:4 (w:v).

Entrapment by agarose

After the preparation of immobilized enzyme by agarose and cutting it to squares, the activity of immobilization enzyme was 153 U/gm, and the amount of the enzyme which immobilized in this process was 64 %.

Entrapment by Ca-alginate

Entrapment of peroxidase enzyme by Ca-alginate was the best immobilized enzyme method. It was shown that immobilized peroxidase activity was 109 U/gm. The entrapment method is based on the occlusion of an enzyme within a polymeric network that allows both substrate and product to pass through the polymer but retains the enzyme. This method differs from the other coupling methods, in that the enzyme is not bound to the matrix or membrane. There are different approaches to entrap enzymes such as gel or fiber entrapping and microencapsulation (Kiralp *et al.,* 2003).

The optimum ratio of peroxidase extraction

Seven ratios were chosen (1:0.5, 1:0.75, 1:1, 1:1.5, 1:2, 1:3 and 1:4 (v:w) to determine the best ratio for peroxidase extraction by using potassium phosphate 0.1M (pH= 7.0). The highest specific activity was measured for crude extract at 1:2 ratio, it was 4479 U/mg protein, while other ratio were 1:0.5, 1:0.75, 1:1, 1:1.5, 1:3 and 1:4 gave the following specific activities 2183, 2526, 2227, 294, 2950 and 3315 U/mg protein respectively (Figure 3). Nidadavolu *et al.,* (2010) were found that the best extraction ratio for peroxidase from discarded mushroom beds was 1:1 and 1:2. While (Tsujimura *et al.,* 1994), shown the best ratio was 1:5 for peroxidase extraction from *Arthromyces ramosus.s*

Immobilization of peroxidase

Enzyme characterization

Determination of the optimum pH of free peroxidase activity

The effect of pH on peroxidase activity was studied. The results showed that the pH 6.0 is the optimum pH for peroxidase activity, while the activity decreased in pH below and above 6.0.

Figure 4 shows the effect of different pH on free enzyme activity. Mader *et al.,* (1977) found that the optimum pH of peroxidase from Tobacco leaves was 5.5- 6.0, while (Padiglia *et al.,* 1995), pointed to the maximum activity of peroxidase from Rice seedlings was in the pH equal to 5.7, also the optimum pH for peroxidase purified from strawberry fruits was 6.0 (Civello *et al.,* 1995).

Fig.1 Effect different sources on peroxidase activity

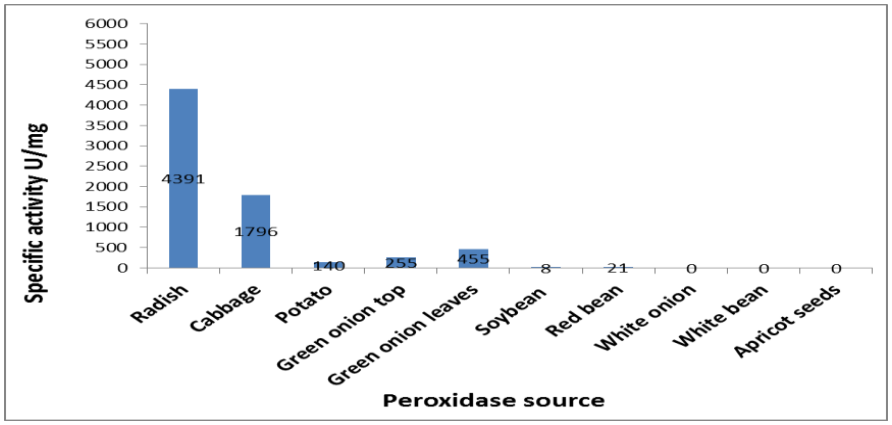
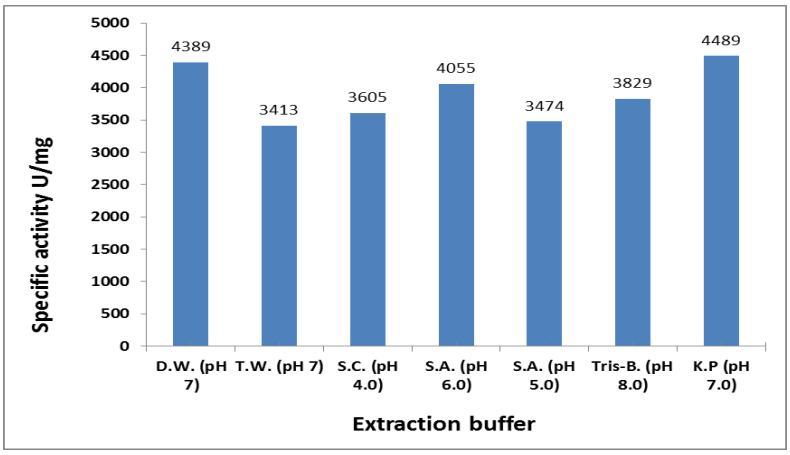


Fig.2 The effect the type of extraction buffer for peroxidase extraction from the radish



S.A. = Sodium acetate, Tris-B. = Tris-base, K.P. = Potassium phosphate, S.C. = Sodium citrate, D.W. = Distilled water, T.W. = Tap water.

Fig.3 The effect of extraction ratio on peroxidase extraction from radish

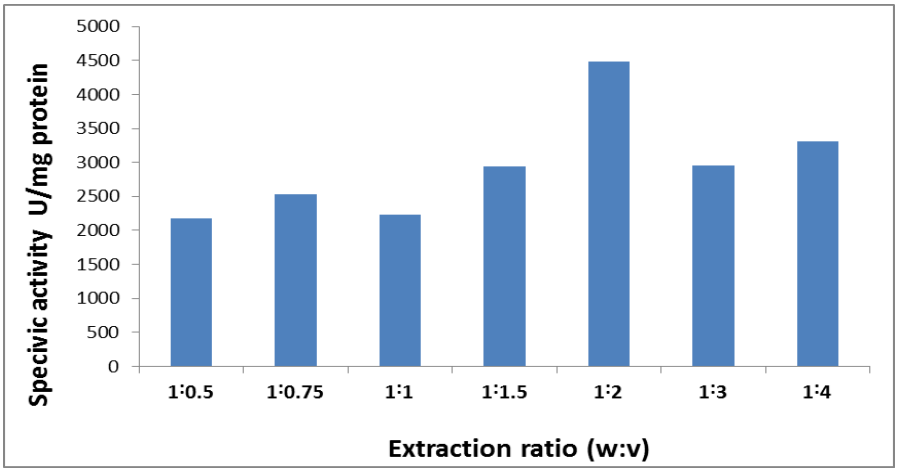


Fig.4 Optimum pH for free peroxidase activity from the radish

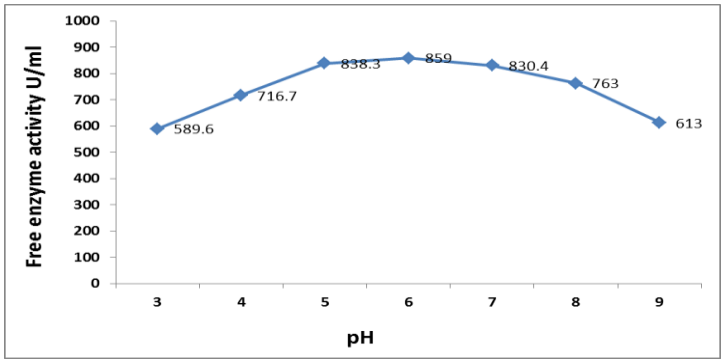


Fig.5 Effect of pH on the stability of free peroxidase from radish

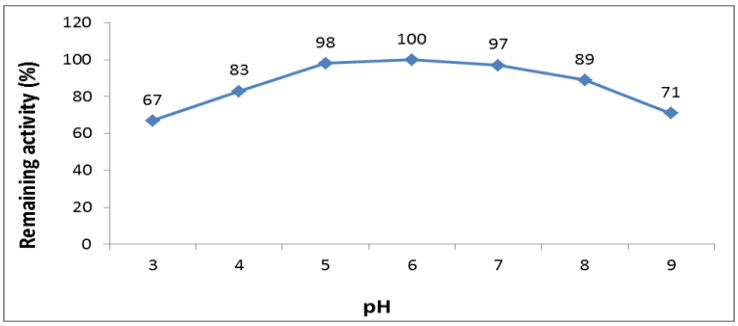


Fig.6 Optimum pH for immobilized peroxidase activity from radish

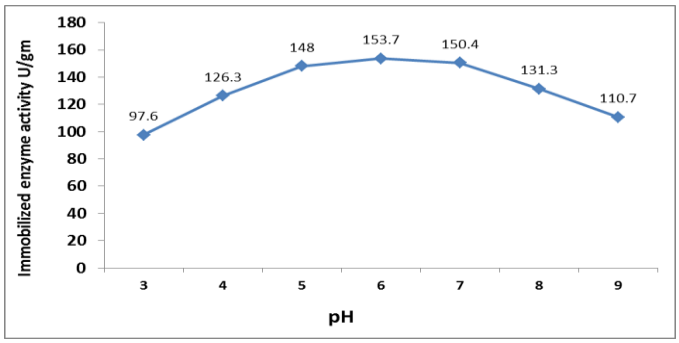


Fig.7 Effect of pH on the stability of immobilized peroxidase enzyme from the radish

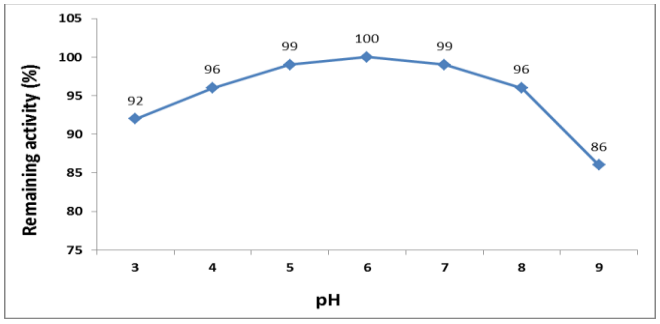


Fig.8 Optimum temperature for free peroxidase activity from the radish

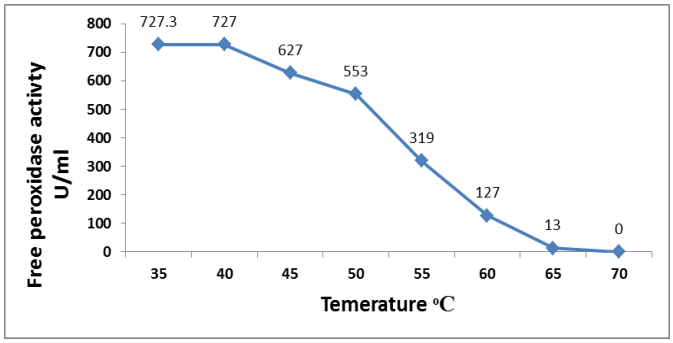


Fig.9 Thermal stability of free peroxidase from the radish

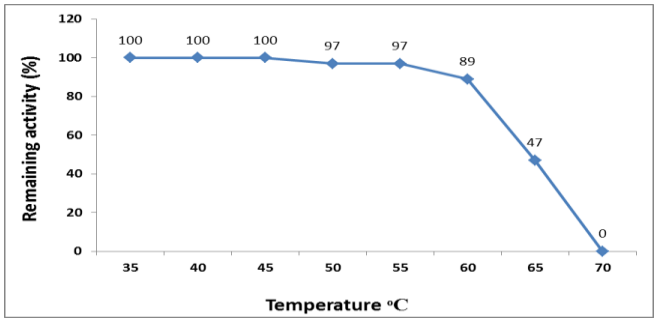


Fig.10 Optimum temperature for immobilized peroxidase activity from the radish

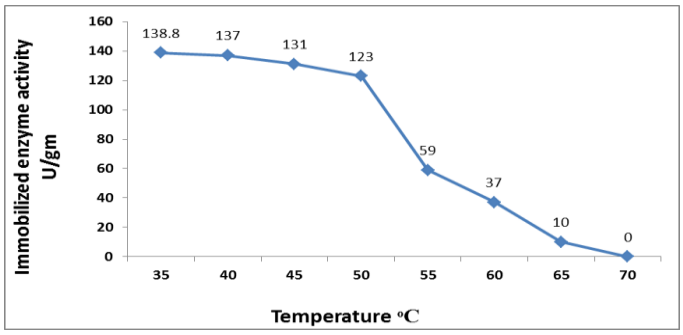


Fig.11 Thermal stability of immobilized peroxidase from the radish

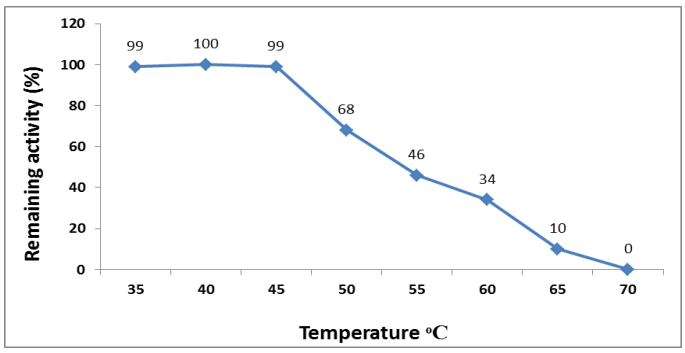


Fig.12 Number of times for using the immobilized peroxidase from the radish

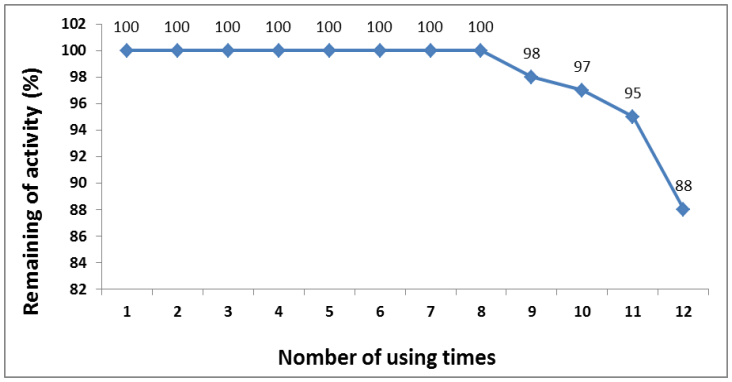


Table.1 Decolorization of some dyes by immobilized peroxidase enzyme

Dye / Time	ʎ-max (nm)	Ab.at zero time (nm)	3 min	6 min	9 min	12 min	15 min	18 min	21 min	24 min	27 min
Brilliant green	628	0.262	0.262	0.262	0.262	0.262	0.262	0.262	0.262	0.262	0.262
Bromophenol blue	590	1.817	1.817	1.817	1.817	1.817	1.817	1.817	1.817	1.817	1.817
Giemsa stain	520	0.164	0.142	0.134	0.121	0.117	0.101	0.092	0.073	0.073	0.073
Toluidine blue	626	1.542	1.542	1.542	1.542	1.542	1.542	1.542	1.542	1.542	1.542
Neutral red	540	1.47	1.587	1.468	1.231	1.162	1.034	0.872	0.691	0.627	0.573
Acridine orange	490	0.347	0.319	0.239	0.111	0.096	0.073	0.058	0.041	0.054	0.067
Indigo carmin	490	0.896	0.896	0.896	0.896	0.896	0.896	0.896	0.896	0.896	0.896

Determination of pH for free peroxidase stability

The pH of enzyme stability was studied because it is an important criteria in determining the optimum conditions for purification and enzyme storage. The results (Figure 5) were demonstrated o the range of peroxidase stability was from 4.0 - 8.0, while the activity was low at 3.0 and 9. pH values. The lowering in enzymatic activity in extreme acidic and extreme basic conditions may be due to changes in secondary and tertiary structure of the enzymatic residues as well as changes in ionic state of the active site of the enzyme (Lehmacher and Bisswanger, 1990). (Yoon *et al.*, 2003), showed that the optimum pH of peroxidase stability from *Helianthus tuberosus* ranging between 5.0 and 6.0, while (Silva and Valdir, 2000), found that the optimum pH of peroxidase purified from papaya fruits ranging between 6.0 and 9.0 when incubation the enzyme with buffer at pH 3.0.

Determination of the optimum pH for immobilized peroxidase activity

The effect of pH on immobilized peroxidase activity was studied; figure 6 shows results that the pH 6.0 is the optimum pH for peroxidase

activity, while the activity decreased in pH below and above 6.0. The pH of a solution can have several effects on the structure and activity of enzymes; pH can have an effect on the state of ionization of acidic or basic amino acids. Acidic amino acids have carboxyl functional groups in their side chains, basic amino acids have amine functional groups in their side chains. If the state of ionization of amino acids in a protein is altered then the ionic bonds that help to determine the 3-D shape of the protein can be altered. This can lead to altered protein recognition or an enzyme might become inactive. Changes in pH may not only affect the shape of an enzyme but it may also change the shape or the properties of the substrate so that either the substrate cannot bind to the active site or it cannot undergo catalysis (Chesworth *et al.*, 1998).

Determination of pH for immobilized peroxidase stability

The pH of immobilized enzyme stability was studied because it is an important criterion in determining the optimum conditions for the storage of the immobilized enzyme. Figure 7 shows the results of the study, where the range of immobilized peroxidase stability was from 4.0 - 8.0, while the activity was very low at acidic pH (below 3) and basic pH at 9 conditions. The lowering in enzymatic activity in low extreme acidic and extreme basic conditions may be due to changes in the secondary and tertiary structure of the enzymatic residues as well as changes in the ionic state of the active site of the enzyme and substrate (Lehmacher and Bisswanger, 1990). The pH of the environment of an enzyme may affect the activity of the enzyme in several ways. First, each enzyme has its pH optimum, at which the enzyme is most active, but the enzyme is stable within certain limits on each side of the optimum. Secondly, the environmental pH of the enzyme may influence its stability, and at extremes of acidity or alkalinity the enzyme may be denatured. Thirdly, the pH of the reaction mixture may cause dissociation of the substrate and so by its action on the substrate influence the character of the pH activity curve and the pH optimum. These factors have been discussed by (Moat *et al.*, 2002).

Optimum temperature of free peroxidase activity

Optimum temperature of free peroxidase activity was studied by incubating the enzyme with the substrate at different temperatures 35–70 ^{o}C for 3 min., the results showed that the optimum temperature for free peroxidase activity from radish was 35 ºC, where it gave the highest activity at this temperature 727.3 U/ml (Figure 8), and the activity decreases higher than this temperature and lost completely at 65 ºC.

Thermal stability of free peroxidase enzyme

Incubation of peroxidase from radish in different temperature degrees between 35 – 70 ^{o}C for 30 min, showed that the enzyme was stable in temperature 35-60 ^{o}C, then the activity began to decrease and was completely lost in 65-70^{o}C (Figure 9). This decrease in enzymatic activity may be due to the thermal effect on the enzyme structure then its denaturation. Where temperature may effect on the protein structure by breaking the bonds that stabilize the secondary and tertiary structure of the protein which results to denaturation (Chesworth *et al.*, 1998).

Optimum temperature of immobilized peroxidase activity

Optimum temperature of immobilized peroxidase activity was studied by incubating the enzyme with the substrate at different temperatures 35–70 ^{o}C for 3 min., the results showed that the best temperature for immobilized peroxidase activity from radish was 35 ºC, with activity 138.8 U/gm (Figure 10), and the activity decreases above than this temperature and lost completely at 65 and 70 ºC.

Thermal stability of immobilized peroxidase

The stability of horse radish peroxidase was measured after incubated the enzyme for 30 min at temperature degrees range of 35-45ºC, and the results show that the activity of enzyme began to decrease at 55ºC and was completely lost at 70ºC (Figure 11).

Number of times for using the immobilized peroxidase

The results of reuse immobilized enzyme with its substrate show that the activity of immobilized enzyme decrease after 12 time as in figure 12.

Whereas a study by Gar and Glu (2002), demonstrated that the activity of immobilized peroxidase from quince decrease after four times.

Dye decolorization

The immobilized peroxidase was used for the decolorization several types of dyes in order to demonstrate their ability in treatment of waste water .

The results show that only two dyes (Giemsa stain and Acridine orange) were reduced and make changes in their absorbance, while no degradation of the other dyes occurs as shown in the table 1.

The optimum pH of free and immobilized peroxidase enzyme activity was 6.0. The stability pH for free and immobilized peroxidase enzyme isolated from radish was 4.8 and 3.9 respectively.

The immobilized peroxidase had the ability to decolorization some dyes such as Giemsa stain and acridine orange.

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