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Extraction ،Purification and Characterization Of Peroxidase From cabbage (*Brassica oleracea Var*) .

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Abstract

The activity of peroxidase (POD) in cabbage was evaluated using spectrophotometric method. The enzyme was extracted from the cabbage leaves with 0.1 M phosphate buffer solution pH 7. 0 . POD activity was determined using (O-dianisidine) as a substrate. The effects of the amounts of enzyme extract, substrate concentration, pH and temperature were investigated. The highest activity of POD was recored at 2 mg/ml. The highest activity of POD was optimized with 16 mM O-dianisidine, The optimum pH was 7.0 for POD , The optimum temperature was 30°C for POD. These optimum conditions were used to determined the enzyme activities in cabbage sample. Acetone fractionated peroxidase from crude extract of *Brassica oleracea leaves* (Cabbage) was purified on DEAE-Cellulose chromatographic columns. The specific activity of purified POD is 103.70 (U/mg) ،which is 5.37 times more than the crude extract with 28.72% recovery. Maximum pH, thermal activity and stability of this purified enzyme are also determined were 40°C .

Keywords: Peroxidase; Optimization; Cabbage; Purification.

استخلاص وتنقية وتوصيف انزيم البيروكسديز من اللهانة (*Brassica oleracea Var*)

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الخلاصة

أستخلص انزيم البيروكسديز من اوراق اللهانة ، باضافة دارىء فوسفات الصوديوم بتركيز مولار 0.1 برقم هيدروجيني 7. 0 وتم تقدير الفعالية الانزيمية باستعمال المطياف الضوئي .قدرت الظروف المثلى لتفاعل الانزيم ، باستخدام تراكيز من الانزيم ، وتركيزالمادة الاساس (اورثوداي انسيدين) ، اضافة الى الرقم الهيدروجيني ودرجة الحرارة . سجلت اعلى فعالية لانزيم البيروكسديز عند تركيز 2 ملغم / مل ، اما تركيز المادة الاساس اورثوداي انسيدين فقد سجلت اعلى فعالية عند تركيز 16ملي مولار وسجلت اعلى فعالية لانزيم البيروكسديز عند الرقم الهيدروجيني 7.0 ولوحظت اعلى فعالية لهذا الانزيم عند درجة الحرارة 30 °م .نقي أنزيم البيروكسديز من مستخلص اوراق اللهانة باستخدام الترسيب بالاسيتون ومن ثم باستخدام تقنية التبادل الايوني وسجلت الفعالية النوعية 70 .103 (وحدة / ملغم) مع عدد مرات التنقية 5.37 و حصيلة 28.72% ، وكانت اعلى فعالية مسجلة للانزيم عند الرقم الهيدروجيني الامثل والثبات عند القيم 5.0 و 6.0 على التوالي. في حين اعلى فعالية مثلى وثبات عند درجة الحرارة المثلى والثبات عند 40 °م .

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Introduction

Peroxidase (donor: H_2O_2 oxidoreductase, EC. 1.11.1.7) is distributed in a wide range of plant species, in multiple molecular forms. Many researchers have worked on different aspects of peroxidase. Peroxidase has been implicated in metabolic processes such as ethylene biogenesis, cell development and membrane integrity [1]. Its properties and physiological roles in fruits and vegetables have been reviewed by several authors. Many studies have been done on amino-acid sequencing and heme structure of peroxidases [2,3].

Peroxidase (POD) is an enzyme commonly found in vegetables which bind to hydrogen peroxide and produce an activated complex that can react with a wide range of donor molecules and cause off-flavors and colors in raw and unblanched frozen vegetables [4, 5]. Inhibition of the enzyme activity in fruits and vegetables is generally achieved using physical or chemical treatments such as heating (blanching), lowering pH and/or aw or adding chemical additives. Several studies to extend shelf-life of minimally processed fruits and vegetables have been focused on methods such as using of acidulants, reducing and chelating agents, and inorganic salts, however, due to the consumer market demands which is concerned about the use of chemicals in such products, more attention has been given to the search for alternative anti browning compounds [6, 7]

Peroxidase (POD) is an oxidoreductase that is directly involved in many plant functions such as hormone regulation, defence mechanisms, indolacetic degradation and lignin biosynthesis [8] . It catalyzes a reaction in which hydrogen peroxide acts as the acceptor and another compound acts as the donor of hydrogen atom. POD involved in enzymatic browning since diphenols may function as reducing substrate in this reaction [9 ,8] . The involvement of POD in browning is limited by the availability of electron acceptor compounds such as superoxide radicals, hydrogen peroxide and lipid peroxidase. Enzymatic browning is a significant problem in a number of fruits and vegetables such as strawberry[9] , grape [10] , potato [11], and lettuce [12] .The discoloration in fruits and vegetables by enzymatic browning, resulting from conversion of phenolic compounds to o-quinones which subsequently polymerize to be a brown or dark pigment, The enzyme involved these processes is POD [13] . Because PPO and POD are the main enzymes involved the phenolic oxidation of many fruits and vegetables, their activities have attracted much attention.

Peroxidases, notably horseradish and turnip, have been studied in great detail during the past two decades [14]. Studies in our laboratories and by other workers on peroxidase enzymes from many different plants indicated that physical and kinetic properties and substrate preference of this peroxidase even from a single source might vary significantly [15 , 16]. In addition, it is generally accepted that peroxidase activity and its enzyme patterns alter with changes in plant development.

Material and Methods

Extraction of Crude Enzyme

Cabbage leaves *(Brassica oleracea capitata L.)* was purchased at local markets in baghdad, in winter. All stepes and purification procedures were carried out at 20 -25°C. The leaves were washed, cut into small pieces and frozen at −20°C for 24 h. After thawing, leaves were homogenized with 1/2 (W/V) K_2HPO_4 (0.1 M) for 5 min. The homogenate was filtered through chesse clothe and then centrifuged at 10,000 rpm with cooling Centrifuge for 10 min, to remove debris. The supernatant was designated as crude enzyme.

Peroxidase and Protein Assay

Enzyme assays

POD activity: was assayed spectrophotometrically at 470 nm using O-dianisidine as a substrate to measure peroxidase activity (Shannon method) [17]. The reaction mixture contained 0.15 mL of 16.3 mM O-dianisidine, 0.15 mL of 1% (v/v) H_2O_2, 2.66 mL of 0.1 M phosphate buffer pH 7.0 and 40 µL of the enzyme extract at 30°C. The blank sample contained the same mixture solution without the enzyme extract. One unit of activity was defined as the amount of enzyme that causes an increase of absorbance per min .

$$\text{Activity(U/ml)} = \frac{(\text{V ml of mix})}{\text{Volume of enzyme} * 1 * 6.4} * \text{slope}$$

V: volume of mix.

Protein concentration was measured according to Bradford method [18].

Effect of amounts of enzyme extract on enzyme activity

The activity of POD as a function of amounts of enzyme extract was investigated. POD activity was performed using the amounts of the enzyme extract from (1 , 1.5 ,2 ,2.5 ,3 ,3.5) mg/ ml . The solution of the reaction mixture contained 0.1 mL of 1% (v/v) H_2O_2 , 0.1 mL of 16.3 mM O-dianisidine and 2.78 mL of 0.1 M phosphate buffer pH 7.0 [19] .

Effect of substrate concentration on enzyme activity

POD activity was performed using the substrate concentrations (10 ,12 ,14, 16 ,18) mM , POD activity was observed by using the mixture containing 40 µL of the enzyme extract, 0.1 mL of 1%(v/v) H_2O_2, 16.3 mM O-dianisidine and 2.78 mL 0.1 M phosphate buffer pH 7.0 at a selected volume. the final volume of the reaction solution in a quartz cuvette was 3 mL [19].

Effect of pH on enzyme activity

The activity of POD were determined at pH values of 3, 4, 5, 6, 7 and 8 using 0.1 M citrate buffer (pH 3- 5) and phosphate buffer (pH 6 - 8). The optimum pH for POD was obtained by using O-dianisidine as a substrate in these buffers . The effect of pH on POD activity was observed by using the reaction mixture contained 0.15 mL of 16.3 mM O-dianisidine , 0.15 mL of 1% (v/v) H_2O_2, 2.66 mL of 0.1 M buffer solution and 40 µL of the enzyme extract [19] .

Effect of temperature on enzyme activity

POD activity was determined at 20, 30, 40, 50, 60 and 70°C. The substrate and buffer solutions were incubated for 5 min at various temperatures from 20 to 70°C before adding of the enzyme extract. Spectrophotometric measurement for 5 min was carried out at 25°C. The activity of POD under optimum temperature was determined by adding 0.15 mL of 16.3 mM O-dianisidine , 0.15 mL of 1% (v/v) H_2O_2 , 2.66 mL of 0.1 M phosphate buffer pH 7 .0 and 40 µL of the enzyme extract [19].

Acetone Fractionation

The crude enzyme was treated with 1:1 volume cold (−20°C) acetone and left for 6 h at refrigerator (−20°C),with agitatation then centrifuged for 10 min at 10,000 rpm. The supernatant was removed and another volume of cold (−20°C) acetone was added to it and kept at 4°C over night. The precipitated proteins were collected by centrifugation at 10,000 rpm for 10 min and dissolved in acetate buffer pH 5.4 , 10 mM [20].

DEAE-Cellulose Chromatography

It was done on Pharmacia DEAE-Cellulose in a column (1.5 x 8 cm) with 10 mM tris buffer pH 8.5. Absorbed proteins were eluted with stepwise gradient of 10 ml of the same buffer consisting 1, 2, 3…15 mM ,0.3 ,0.5, 0.6 M NaCl at flow rate of 18 ml h−1. Three ml_Fractions were collected [20].

Activity of POD in Different pHs and Temperatures

Activity of purified peroxidase (POD) was measured in pHs (3 - 9), using the substrate O-dianisidine in these buffers 0.05 M buffers of sodium acetate for pH ranging from 3.5 - 6.5 and Tris-base for pH ranging from 7 – 9 [17]. Activity of POD in different temperatures (30-70°C) was estimated as the enzyme assay [17].

Stability of POD in Different pHs and Temperatures

Determination of pH stability of POD was incubated for 4 h in different pH(3 - 9), and then remaining activity for enzyme was measured as Shannon method [17]. For measuring of thermal stability of POD, enzyme in acetate buffer (50 mM, pH 5.4) was incubated for 30 min in different temperatures, then stoped the reaction using ice bath , Enzyme assay was performed as Shannon method [17].

Results

Optimization conditions for enzyme activity measurements POD was oxidative enzyme which catalyze the oxidation of phenolic substrate mainly due to enzymatic browning [21]. It was catalyze the oxidation of phenolic compounds to *o*-quinone which polymerize to form undesirable pigments [9]. The enzymatic oxidation of O-dianisidine by POD/ H_2O_2 changed the substrate into orange-pink products with a maximum amount of enzme at 2 mg/ml . The substrate oxidation was found to be dependent on the amounts of the enzyme extract. (Figure-1)

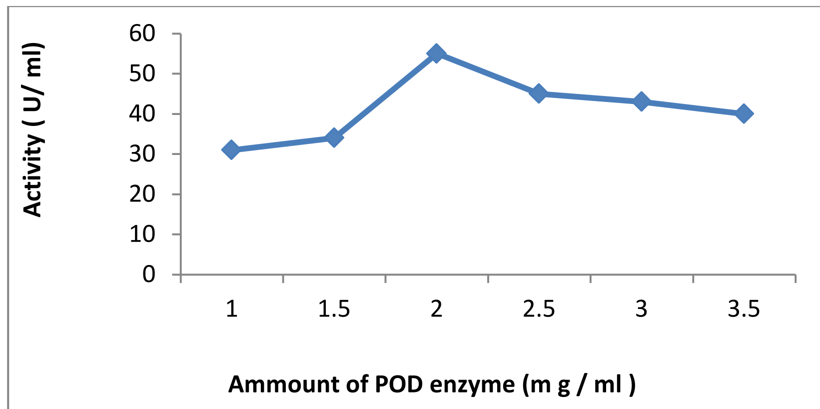

Figure 1- Effect of amounts of the enzyme extract on POD activity.

As shown in Figure -2, the oxidation of O-dianisidine by cabbage POD generated products which had absorbance maximum at 470 nm. Thus, the optimal O-dianisidine concentration was determined by measuring the increase in absorbance at 470 nm, using different amounts of the substrate. As expected, an increase in the substrate concentration resulted in an increase in pigment formation. The rate of which stayed practically constant at saturating O-dianisidine concentration. Therefore, the concentration of 16 mM O-dianisidine was routinely chosen because at higher concentrations of the substrate did not significantly affect the formation of the *o*-quinone intermediate [19].

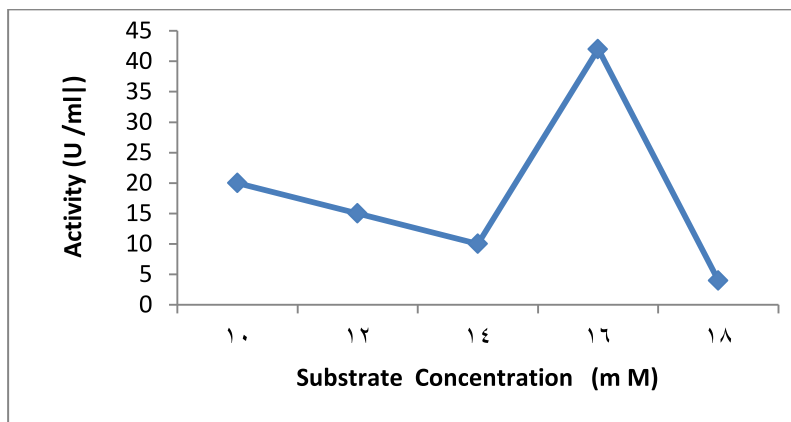

Figure 2- Effect of O-dianisidine concentrations on the cabbage POD activity.

The activity of POD was measured at different pH values using O-dianisidine as substrate . As shown in Figure -3 the optimum pH 7.0 of enzyme POD was obtained. It is known that the optimum pH for any enzymes depends on plant materials and substrate in the activity assay. In general, most plants show maximum enzyme activity at or near neutral pH. Different optimum pH values for both enzymes obtained from various sources and substrates used have been reported.The optimum pH 6.0-8.5 for kiwifruit POD using p-phenyllenediamine as substrate [22] and pH 6.0 for spring cabbage POD using guaiacolas substrate [23] .pH values are 6.8 and 5.5 for butter lettuce PPO using 4-methycatechol and catechol as substrates, respectively [12], pH 6.5 for longan fruit PPO using 4-methycatechol as substrate [13].

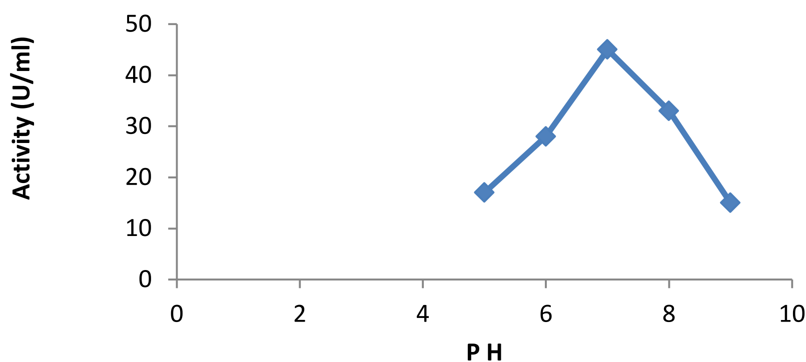

Figure 3- Effect of pH on the cabbage POD activity.

The optimum temperature for enzyme activity usually depends on experimental conditions. Generally, the reaction rate decreases because of thermal denaturation when the temperature is increased. This situation is similar for most enzymes.

Temperature dependence in the enzyme activity is presented in Figure -4. It was found that the highest activity of POD was obtained at 30°C. The POD activity increased when the temperature was increased from 20 to 30°C, and then decreased probably due to denaturation of the enzyme at higher temperatures. From previous studied, the temperature at POD, the enzyme was highly active up to 40°C and lost its activity at higher temperatures [23- 25]. From the obtained results, the optimum temperature of the enzyme was found at 30°C. Thus, we determined the enzyme activity of cabbage sample at ambient temperature (30 ± 3 °C).

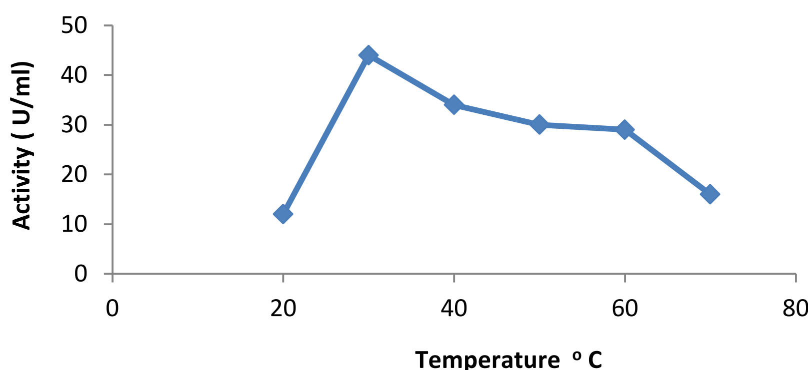

Figure 4- Effect of temperature on the cabbage POD activity.

Enzyme Purification

A summary of the purification procedure appears in Figure -5 and Table- 1. Extracted solution of 300 g cabbage leaves was fractionated by cold acetone (−20°C). Precipitation of two volume acetone was dissolved in 28 ml acetate buffer pH 4.5, 10 mM. This solution was first dialyzed against 10 mM tris buffer (pH 8.5) and fractionated on a DEAE-Cellulose column (1.5 x 8 cm) equilibrated and washed with 10 mM tris buffer pH 8.5 and eluted with 10 mM tris buffer (pH8.5) buffer and 0 – 600 mM NaCl gradient, at a flow rate (18 ml/h). Fractionated by cold acetone (−20°C) helped to improve peroxidase purification and concentrate the crude extract. The specific activity and purification- fold was recored 29.62 (U/mg) and 1.50 fold respectivly . After anion exchange chromatography, peroxidase was distributed into two peaks, the first of which was eluted during the washing step and the second eluted with the salt gradient Figure -5. The specific activity of the pooled bound protein fractions was recored 103.70 (U/mg).

DEAE-Cellulose resulted in an increase of 5.37 times. This relatively small increase in specific activity may be associated with the large amount of absorbing materials eluted along with the enzyme. Peroxidases are secretory proteins localized mainly in the plant cell walls, cytoplasm, and vacuole, depending on the nature of the cell and its development. Plant POD is present as multiple isoenzymes

differing in molecular and catalytic properties [26] .POD are believed to be responsible for the final enzymatic step in lignificaton [27].

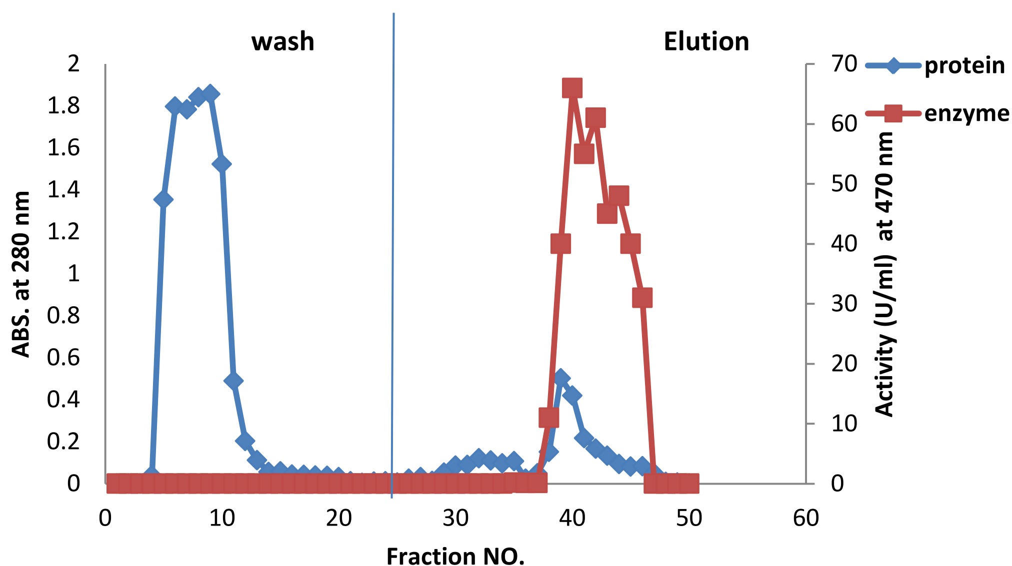

Figure 5- Ion exchange chromatography for peroxidase extracted from cabbage leaves. DEAE-cellulose column (1.5 x 8 cm) equilibrated and washed with 10 mM tris buffer pH 8.5 and eluted with [10 mM tris buffer pH8.5 buffer and 0 – 600 mM Nacl gradient], at a flow rate (3ml/5min).

Table 1- Fractionation protocol of cabbage peroxidase *(Brassica oleracea capitata L.)*

Purification stepes	Vol. (ml)	Activity (U/ml)	Protein (mg/ml)	Specific activity(U/mg)	Total activity(U)	Recovery (%)	Purification fold
Crude extract	100	39	1.98	19.69	3900	100	1
Acetone precipitation	28	48	1.62	29.62	1344	34.46	1.50
DEAE-Cellulose	20	56	0.54	103.70	1120	28.72	5.37

Data concerning effect of various pHs on POD activity was examined at 37°C between pH 3- 9 using 0.05 M buffers. Figure -6. The optimum pH for POD in this condition was about 5.0 in acetate buffer. The optimum pH for acidic turnip PODs was reported to be between 5 and 5.5 with ABTS as H donor [28]. The POD enzyme from tomato juice was reported to have an optimum pH of 5.5 with guaiacol as H donor [29]. The optimum pH for strawberry POD was found to be at pH 6.0 [30] , and the optimum for POD from potato sprouts and tubers was 4-4.5 [31].

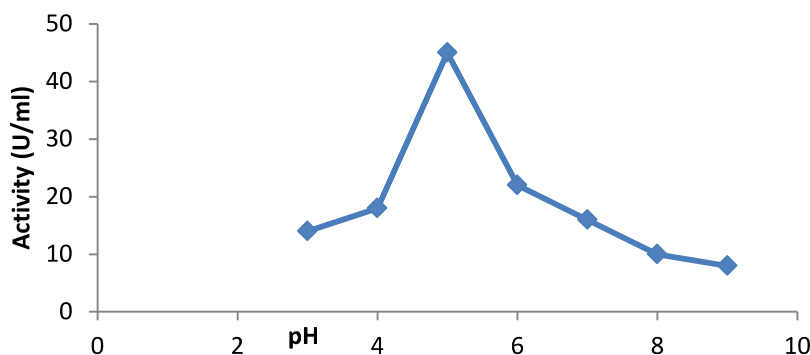

Figure 6- Effect of different pHs on the activity of POD.

The peroxidase activity in different temperatures was measured by determination the enzyme activity in temperatures ranging from 30 - 70°C Figure 7. The best temperature for highest activity of POD at 40°C [20].

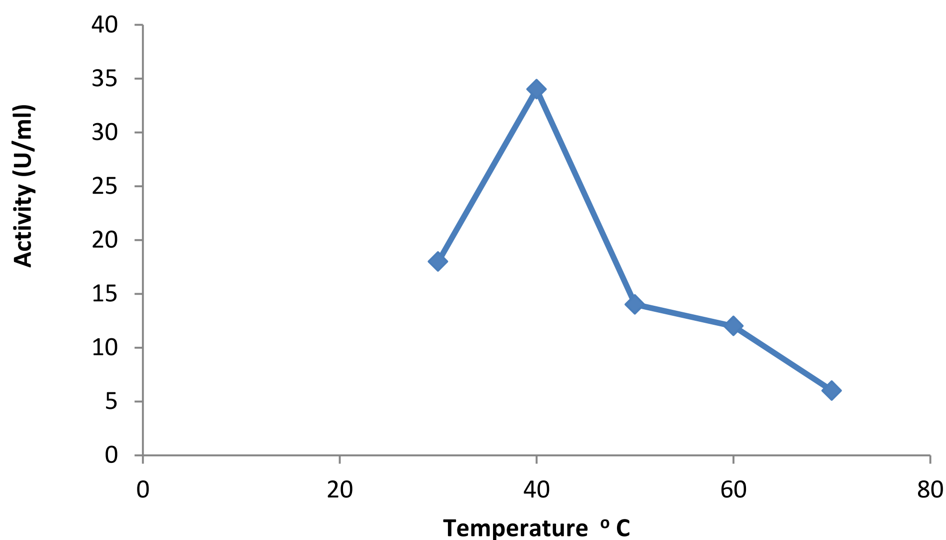

Figure 7- Effect of different Temp.on the peroxidase activity.

Figure -8 also show **pH stability** of POD in 0.05 M buffers with pHs between (3 -9) . After four hours of incubation of POD in mentioned pHs, the activity was assayed. The maximum stability for POD after four hours is in pH 6.0 . In pHs ranging (5 – 6) , POD loses of its activity in higher pHs than 6.0 [20] .

Thermal stability of POD at temperatures ranging from 30 - 60°C after 30 min was also measured. As it is shown in (Figure 9) , POD keep 100% of its activity at 40°C, but in higher temperatures it loses of its activity [20].

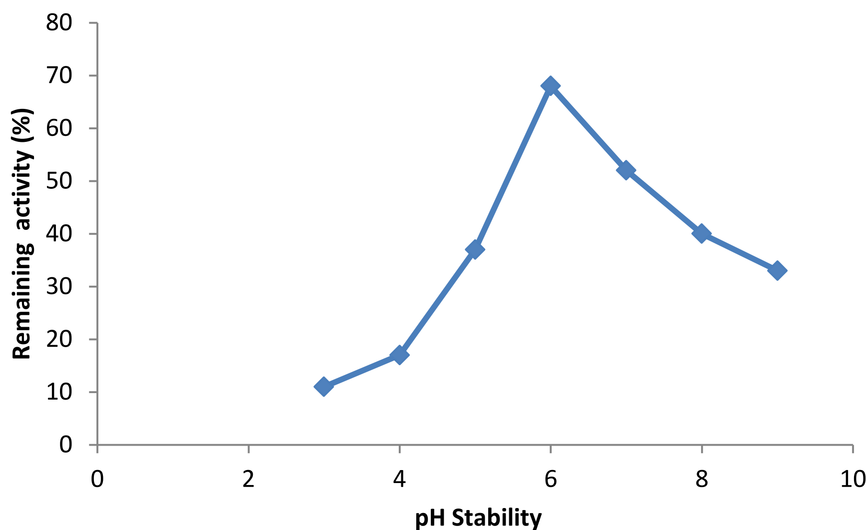

Figure 8- Effect of different pHs on on the stability of POD after four hours.

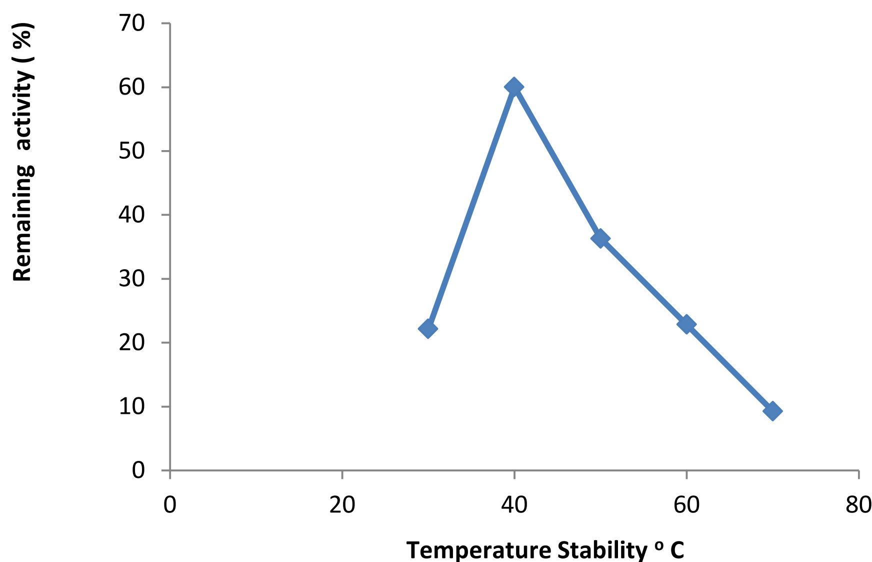

Figure 9- Effect of different temperatures on the thermal stability of POD after 30 min.

Discussion

Several authors have studied on peroxidase. Schoenbein (1855) was the first who searched vigorously on peroxidase [32]. Figure peroxidase was the first one isolated and investigated in 1936 by summer and Howell [33]. Peroxidases have been purified from such diverse sources as horseradish, yeast, sweet potato, turnip, and wheat during 1942-1956 [34].

One of the first successful methods in purification and crystallization of purified peroxidase was performed by Shannon and his colleagues in 1966 [17].

Ion exchange chromatography have already been used for purification of peroxidase from horseradish [35]. In this work, after fractionation by acetone, main cabbage peroxidase (POD) was successfully purified by ion exchange comparing with crude extract, the recovery of purified enzyme was not very high, but purity was good. Therefore this method may be useful for purification of peroxidase from sources with high protein content.

Peroxidases are mainly used in determination of metabolites with other enzymes, therefore it is required to have good thermal stability and activity in a wide range of pH. POD is more active and stable in acid pHs. Horseradish peroxidase is more active and stable in neutral pHs and therefore POD could be a suitable alternative whenever more stability and activity in acidic conditions are needed.

Optimum pH and temperature for cabbage peroxidase and its stability are comparable with other reports too [36, 37]. For example, optimum pH and temperature for strawberry peroxidase are reported to be 6.0 and 30°C respectively. This enzyme keeps almost its original activity after heat treatment up to 45°C for 20 min at pH 6.0 - 8.0 [38] .

Brassica *oleracea capitata L.* (cabbage) is a vegetable available almost in all seasons at very low price and large quantity in our country. Although peroxidase content of cabbage might not to be as high as horseradish peroxidase, most of its properties are similar to HRP and by this procedure it is possible to obtain highly purified peroxidase, suitable for diagnostic application.

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