Extraction, Purification and Characterization of Peroxidase from Okra (*Abelmoschus esculentus)*

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Extraction, Purification and Characterization of Peroxidase from Okra (*Abelmoschus esculentus)*

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Abstract. This study was aimed to extract peroxidase enzyme from the okra plant. The enzyme was extracted by using sodium phosphate buffer (pH 6.5, 0.2 M). The crude extract then precipitated at the saturation range between 20 – 70 % and dialyzed. Further purified carried out through ion exchange chromatography using DEAE-Cellulose column (2.5 × 40 cm). The purified peroxidase had a molecular weight of 43.651KDa, and optimum pH and temperature for enzyme activity about 6.5 and 55◦C using pyrogallol and H_2O_2 as substrate. The specific activity, fold of purification and yield of purified peroxidase were 87.5U/mg, 6.57 and 33.30% respectively.

Keywords. Peroxidase activity, Ion exchange, Molecular weight.

1. Introduction

Enzymes are vital catalysts that accelerate the reactions in which they participate with high degrees of specialization when the appropriate conditions for their work are available. Additionally, the enzymes work to increase the quality of products, reduce production costs, and less impact on the environment, and consequently, the use of enzymes increased in the applied fields.

Bacteria, fungi, algae, plants, and animals all include peroxidases (EC1.11.1.7), one of the principal enzymatic molecules that catalyze redox reactions of a variety of phenolic and non-phenolic substrates. With hydrogen peroxide as an electron acceptor, peroxidases catalyze the oxidation of many substances [1,2].

Class III peroxidases, of which plant peroxidases are members, have been implicated in a wide range of crucial plant growth and development processes throughout the plant's life cycle, including cell wall metabolism, lignification, suberization, reactive oxygen species (ROS) metabolism, auxin metabolism, fruit growth and ripening, defense against pathogens, and more [3,4].

Bioremediation, synthetic dye decolorization, polymer synthesis, wastewater and drinking water degradation, and detergent manufacture are just a few of the many industrial sectors that benefit from POD's capacity to catalyze numerous redox reactions for a wide range of substrates. POD is also significant as a browning enzyme that contributes to the darkening of fruit and vegetable goods during processing and storage, and it is one of the key quality deterioration indicators due to its role in flavor loss and various biodegradation processes [5].

Dough containing peroxidase, which triggers the oxidative crosslinking of pentosan. To prevent the dough from becoming sticky, oxidative enzymes like peroxidase are added. Larger aggregates of cross-linked feruloylated arabinoxylans are responsible for the beneficial effect of peroxidases on breading. These are superior in their capacity to absorb and transport water, making them ideal for use in dough. In addition, peroxidase can modify the gluten network by cross-linking the gluten proteins or by introducing arabinoxylans to gluten proteins [6,7].
Research on peroxidase enzymes from a wide variety of plants suggested that the enzyme's physical and kinetic properties, as well as its substrate choice, may vary greatly, even within a single source [8,9]. Furthermore, it is well believed that peroxidase activity and related enzyme patterns fluctuate with changes in plant development. This investigation was conducted to extract and purify the enzyme from the okra plant and characterize some of its features because of its multifaceted usefulness.

2. Materials and Methods

2.1. Extraction of the Enzyme

The okra plant were collected from a local market at Baghdad city. the okra plant were blended with 0.2 M sodium phosphate buffer (pH 6.5) in a mixing ratio (1:3), homogenized for 15 minutes using Magnetic stirrer, large parts filtered by Watt man filter No.1 and centrifuged at 2800 xg for 15 minutes at 4 C to remove the coarse parts. The supernatant was designated as crude extract, then its volume, enzymatic activity, and protein concentration were determined and the specific activity was calculated

2.2. Enzyme Activity Assay

The enzymatic activity was estimated according to the [10], method using pyrogallol and H_2O_2 as a substrate.

2.3. Protein Concentration

Protein concentration was calculated using the Bradford method using Bovine Serum Albumin (BSA) as the reference [11].

2.4. Enzyme Purification

Ammonium sulfate saturation (20, 40, 60 and 80) % were set up to precipitate the crude enzyme. The precipitation allowed to continue for about 4 hrs. Subsequently, the mix was centrifuged at 10,000 xg for 30 min, carefully the supernatant poured off and the pellets dissolved in appropriate volume of 0.2 M sodium phosphate buffer at pH 6.5. Whole process achieved in a cold room. The ammonium sulfate precipitate enzyme was dialyzed against sodium phosphate buffer (pH6.5, 0.2 M) for 24 hours with three changes of dialysis buffer. DEAE – Cellulose, column 2.5 × 40, was prepared according to GE Healthcare company instructions, where 4 gm of dry powder was suspended in sodium phosphate buffer (pH6.5, 0.2 M). The slurry was prepared with binding buffer in a ratio of 75% settled medium to 25% buffer. After column equilibration, concentrated enzyme was applied gently onto the surface of the Cellulose beads. The column was washed by sodium phosphate buffer to make unbinding proteins pass through. Fraction collector was set up to collect 3ml for each fraction at a flow rate of 30 ml / hr. Elution is achieved using a continuous increasing salt gradient to release the other proteins bounded to the column, NaCl (0-1M) dissolved in 0.2 M sodium phosphate buffer solution (pH 6.5) was used for this purpose. The absorbance and the enzymatic activity of each fraction were measured by using spectrophotometer at 280 nm and peroxidase activity assay respectively. Fractions with peroxidase activity were pooled and stored at 4°C.

2.5. Characterization of the Enzyme

Determination of the molecular weight of the peroxidase by gel filtration method Use a Sephadex G-100 gel column (1.8 x 70 cm). Estimate the void volume (Vo) of the column by passing the blue dextran solution (at a concentration of 5 mg / ml) and calculate the sum of the volumes of the separate fractions from the beginning of the passage of the dextran solution to its absorption peak at a wavelength of 600 nm and the recovery volumes (Ve) for the standard proteins (Bovine Serum

Albumin, 67000), (Pepsin 35000), (Trypsin, 23000) and (Lysozymes 14400) was estimated to collect the parts in the same way and read the optical absorption at a wavelength of 280 nm, then the linear relationship between the ratio of the recovery volume of each standard protein to the void volume (Ve / Vo) versus the logarithm of its Molecular weight.

2.6. Determination of the Optimum pH for the Peroxidase Activity

The optimum pH for the activity of the enzyme was determined using sodium acetate buffer solution of (0.2 M) with pH values ranging from 3.5-5.5, sodium phosphate buffer solution with pH value ranging between 6.0-7.5, and Tris-HCL buffer solution with a pH value (8-9) to prepare the substrate solution. The reaction carried out by incubating the substrate with enzyme for each pH (3.5-9.0) in test tubes for 15 minutes in a water bath at 37 ° C, then it was cooled directly in an ice bath to stop the enzymatic reaction and the enzymatic activity determined as explained previously and then the relationship between the enzyme activity and the pH values is plotted.

2.7. Determination of the Optimal pH for Enzyme Stability

A particular volume of the pure enzyme was incubated with an equal volume of buffer solutions with different pH values (3.5-9.0.) in test tubes for 15 minutes in water bath at 37 ° C, then cooled directly in an ice bath and the activity was measured. The remaining enzyme activity as a percentage of the activity out of the highest activity. The relationship between the remaining enzyme activity versus the pH values was plotted to determine the optimal pH for the enzyme stability.

2.8. Determination of the Optimum Temperature for Enzyme Activity

The activity of the enzyme was estimated at different temperatures ranging from 25-80 ° C at the optimum pH for the reaction.

2.9. Determination of the Thermal Stability of the Enzyme

The enzyme was incubated in a water bath at different temperatures ranging from 20-80 ° C for 15 minutes, then the tubes were cooled directly in an ice bath and the reaction solution was added to them (with the optimum pH). The residual enzymatic activity is estimated and the relationship between the remaining activity and different temperatures is drawn to determine the optimum temperature for the enzyme stability.

3. Results and Discussion

3.1. Extraction, Concentration and Purification of the Enzyme

The curd peroxidase extract from the okra plant using sodium phosphate buffer 0.2 M at pH 6.5 at 4 C, showed enzymatic activity of 2.8 units/ml, protein concentration 0.21 mg/ml, specific activity 13.3 units/mg, total activity 420 units and yield 100% (Table 1).

Figure 1 shows the enzyme activity through ammonium sulfate precipitation of crude enzyme extract. It has been noticed that the enzyme precipitated out between 20 - 80% of ammonium sulfate saturation and the highest activity for the precipitated fraction were achieved at 70% saturation. It was clear that as the enzyme activity increased in precipitated fraction, it is decreased in supernatant fractions. The enzyme units and the specific activity in this step was 21 U/ml and 30.8 U/mg respectively. [12], reported that the peroxidase can be isolated in the 30-80% saturation using fractional precipitation with ammonium sulfate. Another study demonstrated that peroxidase precipitated out between 30-90% of ammonium sulfate saturation [13].

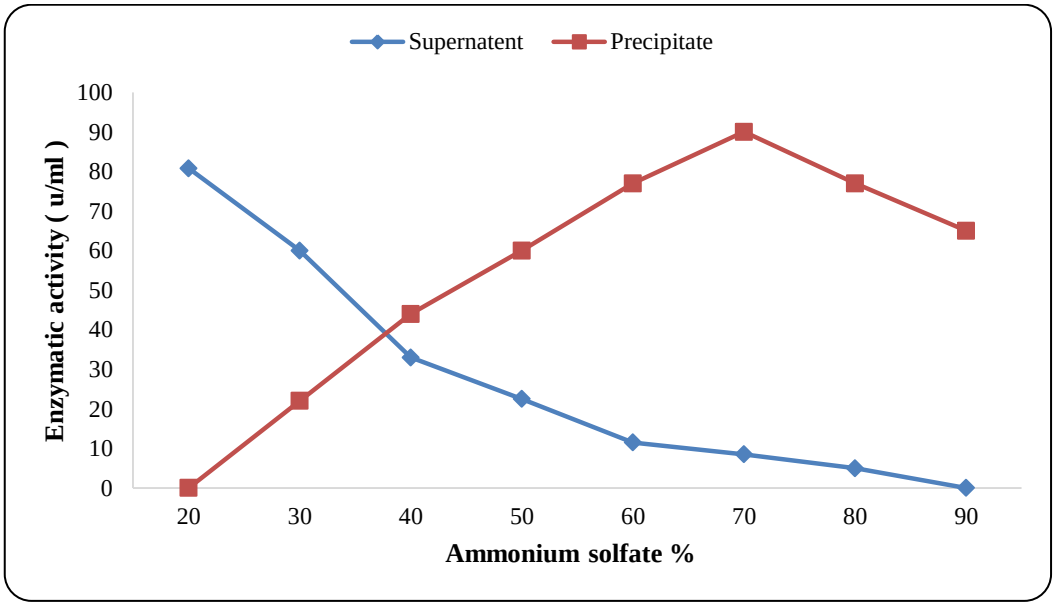

Figure 1. Ammonium sulfate salt Precipitation of peroxidase.

The results in Figure 2 show a single peak in elution step (major peak) at 0.7 mM gradient. the peaks had peroxidase activity, the major peak fractions were pooled and dialyzed against phosphate buffer and concentrated using polyethylene glycol. The specific activity, enzyme activity and the yield after DEAE chromatography, were 87.5 U/mg, 14 U/ml and 33.3% respectively (Table 1). [14],Peroxidase activity was isolated from Peganum harmala seeds using the DEAE -Cellulose column, which had been pre-equilibrated with 0.2 M phosphate buffer pH 5. The enzyme's specific activity was 343 U/mg, the purification fold was 2.4, and the yield was 23%. [13], who stated that the purified peroxidase from apple seed was eluted with phosphate buffer (0.2 M and pH 6) containing (0.1-1) M NaCl.

Table 1. Peroxidase purification summary.

Purification step	Enzymatic activity (U/ml)	Volume (ml)	Protein conc. (mg/ml)	Specific activity (U/mg)	Total activity (U)	No. of folds	Yield %
Crude extract	2.8	150	0.21	13.3	420	1	100
Ammonium Sulfate 20-80%	21	15	0.68	30.8	315	2.31	75
Ion exchange DEAE-Sephadex	14	10	0.16	87.5	140	6.57	33.3

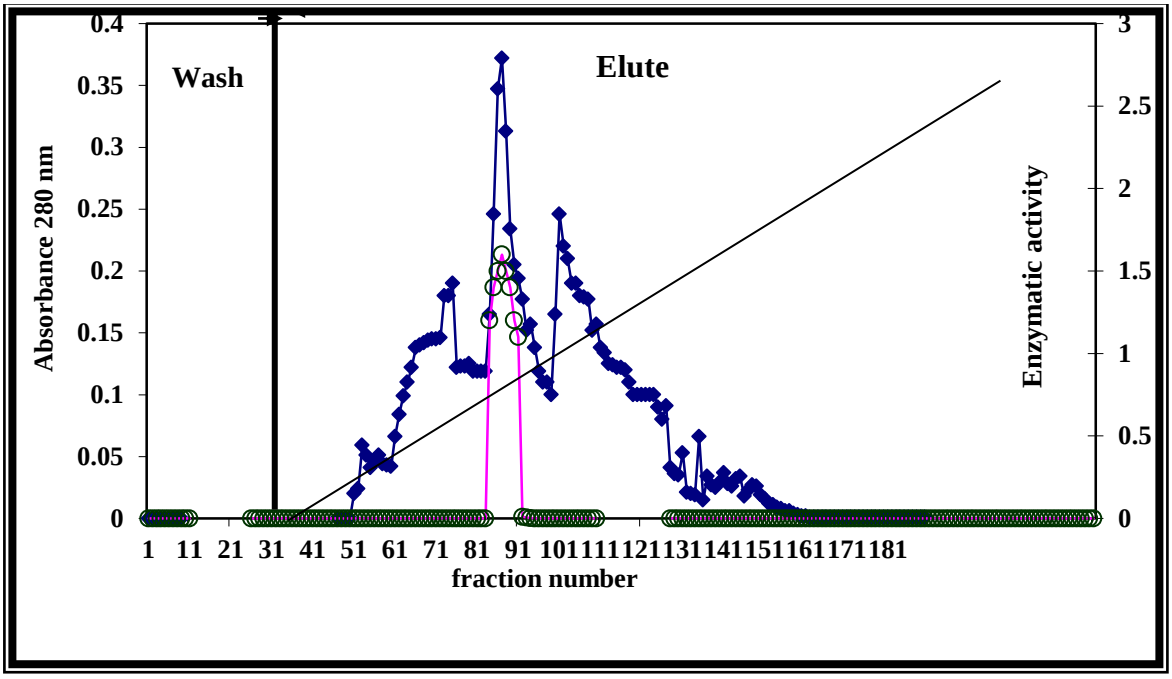

Figure 2. Ion- exchange Chromatography using DEAE Sephadex A-50. The column (2.5 × 40) was equilibrated with 0.2 M phosphate buffer (pH 6.5). The gradient elution was done as indicated and fractions of 3 ml were collected at flow rate 30 ml/hr.

3.2. Characterization of the Enzyme

3.2.1. Molecular Weight

Fig (3) indicates that the molecular weight of the purified peroxidase was 43651 Dalton. This finding was close to that found by [15], the molecular weight of the purified peroxidase from *Moringa oleifera* leaves was 43,000 Dalton using PAGE -SDS. [16], found the molecular weight of the purified peroxidase from buckwheat seed was 46,000 Dalton. These findings were different from the result recorded by [17], that the molecular weight of the purified peroxidase from Leaves of Black Blueberry (*Morus nigra*) was 62340 Dalton. These variations in molecular weight of peroxidase could be due the source of enzyme and the methods of determination (2).

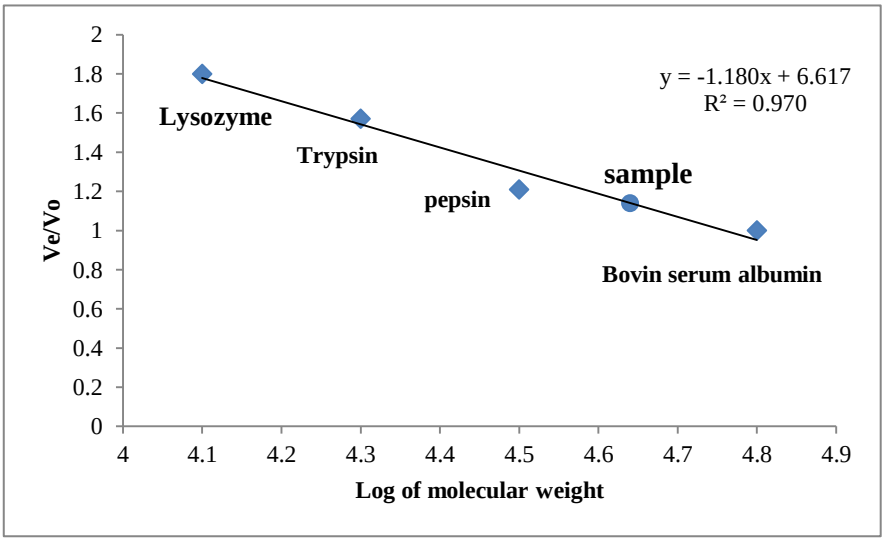

Figure 3. Standard curve for estimating the molecular weight of the peroxidase enzyme by gel filtration method through Sephadex G-100 column with dimensions (1.8 x 70) cm.

3.2.2. The Optimum pH for Peroxidase Activity

Figure (4) shows the pH profile of the peroxidase activity (pH range 3.5 – 9). It has been oticed that the optimum pH for the activity of the enzyme was (6.5). The enzymatic activity decrease remarkably at extreme values of pH (below 4 and above 8). This may be due to the effect of the pH changes on the ionic properties of the enzyme molecule or the matrix and other components of the reaction medium. As a result, there is a change in the composition of the three-fold stacked shape to a more random composition, meaning a change in the natural state of the enzyme occurs. This may weaken the activity of the enzyme to the extent that the enzyme completely loses its effectiveness. The effectiveness of the enzyme may also decrease due to a change in the shape, or arrangement of the molecules of the base material. As a result, the tendency of the enzyme to interact with the base material will decrease, which leads to a decrease in the enzymatic activity [18,19]. The results of the current study were close to the results of other related studies about peroxidase from different sources. It was mentioned [7,20], that the optimum pH for the activity of peroxidase extracted from ginger (Zingiber officinale) and medicinal plant *Artocarpus lakoocha* was 6. Whereas, [21], mentioned that the optimal pH for the turnip roots (Brassica rapa var. rapa Barkant) peroxidase was 4.

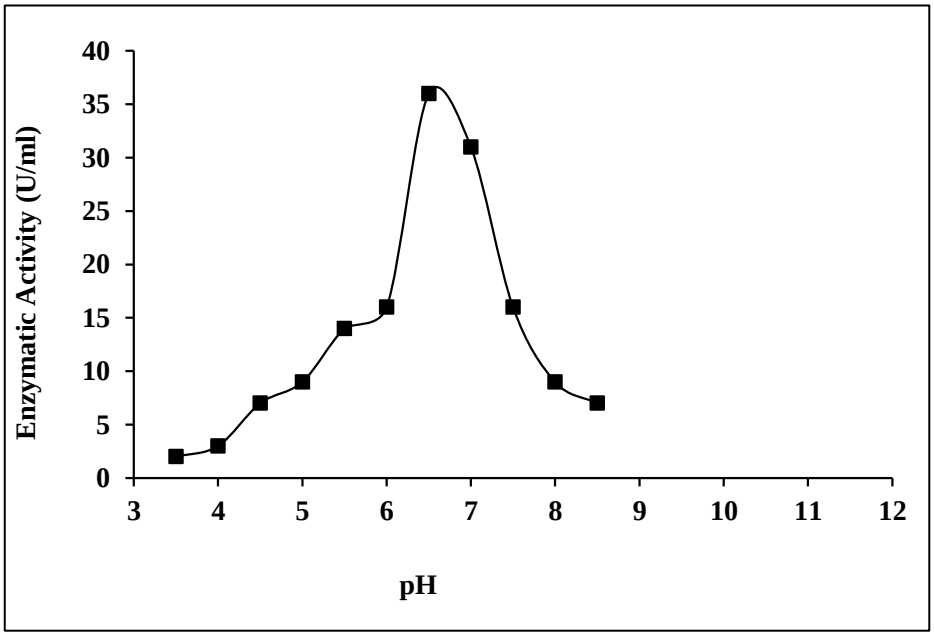

Figure 4. The optimal pH for the okra plant peroxidase activity.

3.2.3. Optimum pH for Peroxidase Stability

Figure.5 represents the stability of the experimental peroxidase at different pH values. The enzyme stability was noticed at pH ranges from 5 – 7, as the enzyme retained 100% of its original activity when incubated at above pH values for 15 minutes at 37ºC. The enzymatic activity remarkably decreased at pH value above 8 and below 5. These results were in agreement with [15], findings that leaves of the *Moringa oleifera* plant peroxidase was at pH values ranged between (5-7).

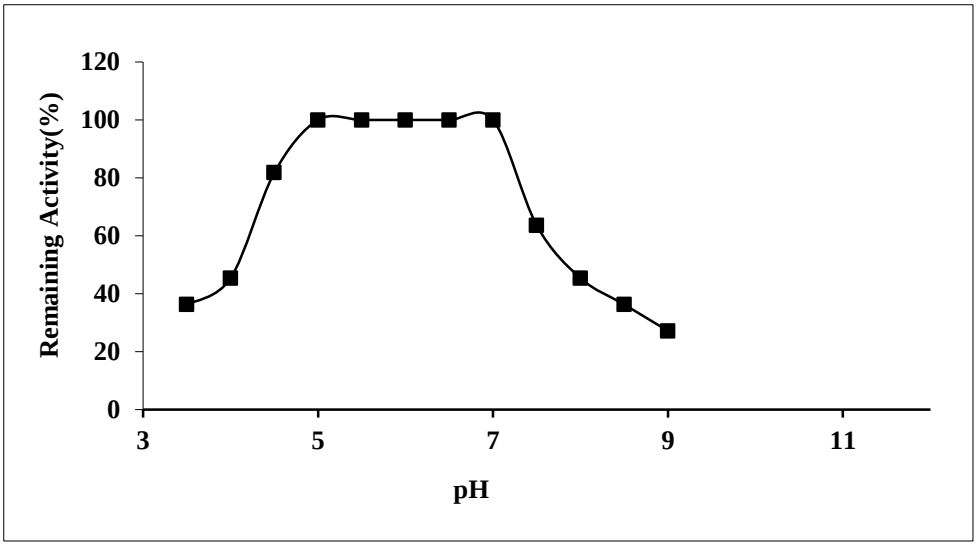

Figure 5. The optimal pH for the okra plant peroxidase stability.

3.2.4. Optimum Temperature for Peroxidase Activity

Figure (6) shows the effect of temperature on the activity of the peroxidase. The enzymatic reaction carried out at different temperature ranging between (25-80 °C) with a difference of 5 degrees between one treatment and another. The results showed that the enzyme activity increased as the reaction temperature increased up to 55 °C when the activity reached the highest value. Then it gradually decreased when the temperature increased to above 55°C. This could be attributed to the increase in contact between the enzyme molecules and the substrate due to the increase in the kinetic energy of the molecules [19]. The enzymatic activity decreases due to a change in the nature of the enzyme or the substrate or both, which in turn leads to a decrease in enzyme activity and reaction rate. reaction decreases, and the heat may affect the base material itself and become not subject to the enzyme [20-22].

The results of this study were in accordance with the results of [23], who found the optimum temperature for activity of the peroxidase extracted from the Ziziphus jujuba fruit, was at 55 ° C. In contrast to [24] results, they found the optimum temperature of peroxidase purified from Plum (*Prunus domestica*) was 25 °C.

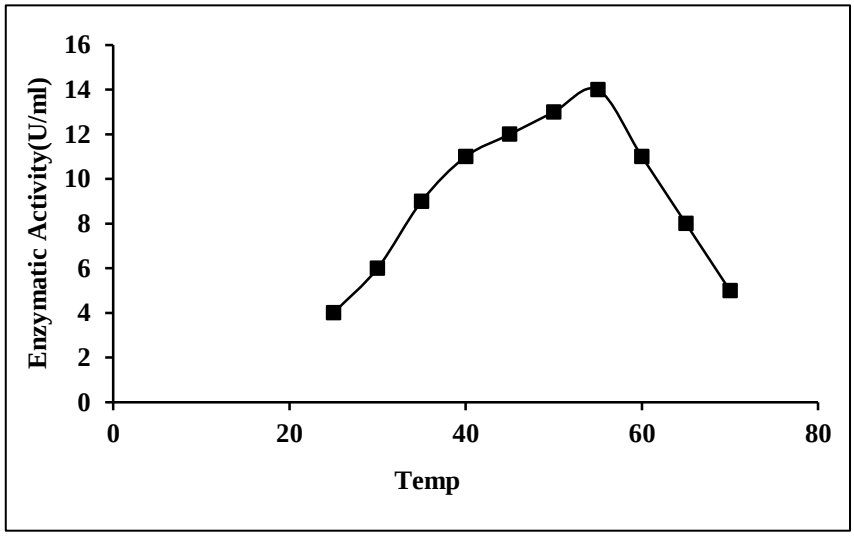

Figure 6. Optimal temperature for peroxidase activity extracted from okra plant.

3.2.5. Thermal Stability of the Peroxidase from Okra Plant

Figure (7) shows the thermal stability of the purified peroxidase when incubated for 15 minutes at temperatures ranging between (25-80) ^{o}C, with a difference of 5 degrees between each treatment. The enzyme retained its entire activity when incubated at temperatures ranging between (25- 55 ^{o}C) for 15 minutes, then the activity started to decrease as the temperature increased above 55C. The enzyme retained approximately 21% of its activity at 70 ^{o}C. This could be due to the effect of heat on the triple structure of protein, which may lead to deformation of the protein and loss of its activity.

The results of this study were similar to [15], they found the purified peroxidase from from *Moringa oleifera* leaves was stable at temperatures ranging between 20-50 ° C. Whereas [25], found the peroxidase enzyme extracted from turnip (Brassica napus var.okapi)was stable at temperatures ranging from 40 - 60 ° C.

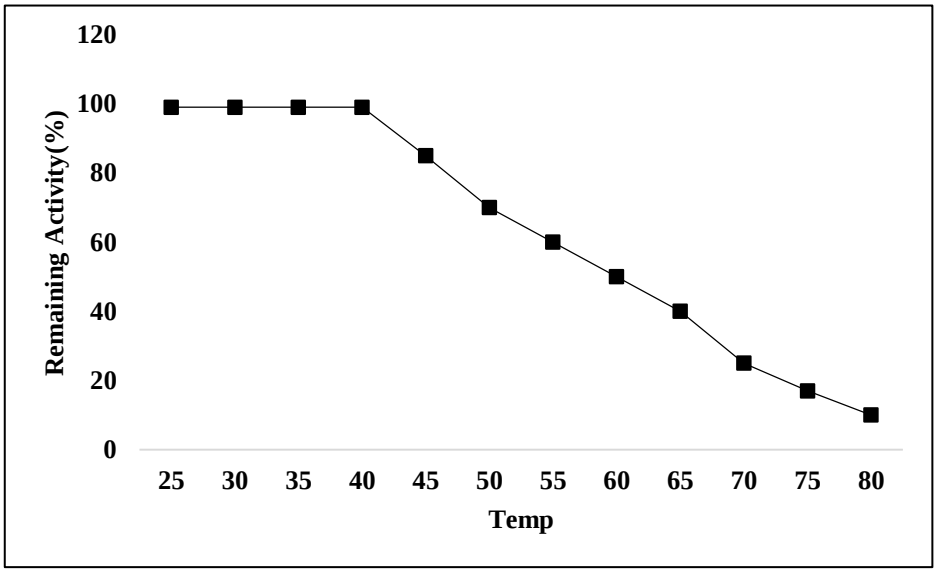

Figure 7. Thermal stability curve of peroxidase extracted from okra plant.

Conclusion

Peroxidase is one of most important industrial enzymes with application in catalyzing redox reaction for wide range of substrate. In this study it can be concluded that okra it's a promising, low cost source for peroxidase and it could be extract and purified easily by ammonium sulfate purification and ion-exchange chromatography with good percentage of yield.

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