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# A Rapid and Efficient Method for Purification of Peroxidase from *Opuntia-Ficus Indica* Stem with Decolorization Effect

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## I. Introduction

The purification of proteins and enzymes is a complex procedure. Development of a new process requires the method increases purity and yield, decrease the time spent. Three-phase partitioning (TPP) is a simple and economical method for purification of enzymes and separation of oils. The technique is based on the hydrophobicity of proteins. Cosmotropy, electrostatic forces, protein hydration shifts and conformation tightening have been proposed of the physicochemical base for the protein precipitation. TPP uses ammonium sulfate and *t*-butanol to precipitate proteins in convenient aqueous solutions. The solution is separated into three phases after the centrifugation. These phases are an aqueous phase, *t*-butanol phase and intermediate precipitate phase. *t*-Butanol stabilizes protein structure extraordinarily. It also inhibits the enzyme activities and protein/protein interactions. The solubility of protein depends on the salt concentration of the solution. The salt used in TPP keeps the charged groups on a protein molecule at low concentrations. Then the protein is pushed into the solution and solubilized. This is "salting-in" mechanism. In addition to this, when the salt concentration is increased, it is reached to maximum solubility point of protein. The water available is finished by increasing the salt concentration. Finally, protein begins to precipitate due to not sufficient interaction between water and protein molecules. This precipitation in the presence of excess salt is called as "salting-out". Several salt types have been employed to increase separation and purification of protein by salting-out mechanism (Harde and Singhal 2012).

*Opuntia ficus-indica* (L.) Miller, called prickly pear, is a cactus species largely used in semi-arid areas as a fruit and forage crop. Cactus pear fruits and stems were described as a source of nutrients and vitamins (Saenz 2000). It is a kind of plant which can be adapted to the climate condition in different regions of the world. It is quite rich in vitamins, minerals, waters, sugars, and fibers about many plants. The plant has been used in the treatment of a wound, common cold, hyperlipidemia, obesity, ulcer, allergic reaction and prostate hypertrophies (Teixeira et al. 2000). Fruit ovoid, 5-10 cm long, red, yellow or purplish, bearing glochids in the areoles but no spines. In Turkey, *O. ficus-indica* is cultivated locally in South and West Anatolia (Köyceğiz, Antalya, Alanya, Anamur, Mersin, İskenderun for its fruit (prickly pear, in Turkish "frenk inciri") and sometimes used as a hedge plant. It is easily propagated vegetatively using joints (Matthews, 1972).

The enzyme peroxidase (E.C. 1.11.1.7) is widely distributed in nature. They are used for determination of pH, DNA, zinc, copper. They can use an indicator for reactive oxygen species in food processes as well as a catalyzer for the synthesis of resin (Aghelan and Shariat 2015). The peroxidase enzyme is used in the field of health, immune assay kits and clinical diagnosis of laboratory experiments. Also, peroxidases function as free radical scavengers in biotherapy. Furthermore, it is used as a detergent additive recently (Humaira and Qayyum, 2010).

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The demand is increasing in the industrial area of cosmetic, paper, textile and pharmaceutics for synthetic dyes. But these dyes lead to serious pollution on the environment, and also most of them have toxic, carcinogenic and mutagenic effects. The recent studies in this field are namely the enzymatic treatment of wastewater and soil. The advantages of the enzymatic decolorization consist of the application of non-soluble materials, process on high and low waste concentrations at a large pH, temperature and amount of the salt. Recently, utility of enzymes attracts considerable attention in the removal of synthetic dyes from aqueous solutions. Since enzymes interact with recalcitrant pollutants and remove them to another product through precipitation or transformation (Duran and Esposito 2000).

The present study includes purification and recovery of peroxidase from the cactus stem by using a rapid and cheap method called TPP which not requires the use of any column. Several parameters such as a concentration of *t*-butanol, salt, pH and temperature were optimized beside values of $K_M$, and $V_{max}$ was calculated. Furthermore, decolorization of some synthetic dyes has been performed with purified cactus stem peroxidase. The method used is a single step and promising for application in obtaining enzyme at industrial scale.

## II. Materials And Methods

*a) Plant and chemicals*

*Opuntia ficus-indica* stems were collected from Antalya, Turkey. The stems stored -80°C deep freezer until they were used. The chemicals, ammonium sulfate, t-butanol, Coomassie Brillant Blue G-250, Bovine Serum Albumin, phenol, pyrogallol, hydrogen peroxide glucose diagnostic kit and horseradish peroxidase were purchased from Sigma-Aldrich (St. Louis, MO, USA).

*b) Preparation of Homogenate*

10 g of the cactus stem were weighed, ground in a mortar until completely crushed. It was homogenized in 100 mL phosphate buffer (100 mM, pH 7) using a magnetic stirrer. The obtained homogenate was centrifuged at 20.000×g for 20 min. The resulting precipitate and supernatant were separated. The studies of peroxidase activity and protein content were performed on the supernatant.

*c) Three-phase Partitioning of Peroxidase*

The TPP was employed with a slight modification of the method of Roy and Gupta (2002). The cactus homogenate adding to *t*-butanol at ratios of 1:0.5, 1:1, 1:1.5, and 1:2 (v/v) was studied in a glass reactor. 4 g of ammonium sulfate was gradually added over 10 ml of homogenate at room temperature. The ammonium sulfate was completely dissolved in water and then mixed with *t*-butanol. The reaction mixture was stirred at 200 rpm for 60 min.; it was then centrifuged at 10.000×g for 20 min. The upper phase was discarded. The intermediate protein precipitate and lower aqueous phase were separated carefully. Protein precipitate in intermediate phase was dissolved in 100 mM phosphate buffer of pH 7. The obtained intermediate and bottom phases of TPP were dialyzed for 12 h against 100 mM phosphate buffer at pH 7 and protein content, and peroxidase activity was measured of both dialyzers.

The ammonium sulfate concentrations were defined according to Roy and Gupta 2002. At the concentrations of 20, 30, 40, and 50% (w/v) were evaluated by using 1:0.5 (v/v) ratio of homogenate:*t*-butanol on intermediate phase. The lower aqueous phase and the intermediate protein precipitate were separated carefully and dialyzed 12 h against 100 mM pH 7, phosphate buffer at 4°C. After the dialysis, the assays of peroxidase activity and protein content were applied to obtained fractions. The phases obtained from partitioning systems were checked against by gel electrophoresis to determine the best combination for yielding the highest peroxidase recovery and purification fold.

*d) Protein Content*

Protein concentration was analyzed according to the method developed by Bradford. In the method, Coomassie Blue G-250 main reagent and Bovine Serum Albumin (BSA) were used as standard (Bradford 1976). The mechanism of this reaction is that Coomassie Blue G-250, which has a negative charge, binds to positively charged proteins.

*e) Peroxidase Activity Assay*

The activity of peroxidase enzyme was determined with a colorimetric assay, depends on the absorbance at 420 nm (Ebiloma et al. 2011). The method based on forming the purpurogallin from pyrogallol in the presence of hydrogen peroxide ($H_20_2$) by the catalysis peroxidase at $25^0$C.

*f) SDS-PAGE Analysis*

SDS-PAGE analysis of the homogenate and purified enzyme was performed according to the method developed by Laemlii (1970).

*g) Determination of Optimum pH, Optimum Temperature, $K_M$ and $V_{max}$ Values*

Optimum pH studies were carried out from pH 3 to 10. The different buffer solutions which are acetate buffer, citrate buffer, phosphate buffer and tris/HCl buffer were used. The enzyme activities of the samples at each pH were measured at 420 nm, and a figure was drawn from obtained results. For optimum temperature, the obtained peroxidase has incubated at 0, 10, 20, 30, 40, 50, 60, 70 and 80°C about 10 minutes. The peroxidase activities of the samples at each temperature were measured at 420 nm, and a figure was drawn from obtained results. Pyrogallol was used a as substrate for

the determination of $K_M$ and $V_{max}$ values in the presence of $H_2O_2$. Substrate solutions were prepared at different concentration (50-500 $\mu$moles). Then the peroxidase activities of each sample were measured at 420 nm, and a figure was drawn about results.

*h) Decolorization Activity Assay*

Partitioned cactus peroxidase was used individually for decolorization of synthetic dyes congo red, methyl red, indigo carmine, crystal violet and ponceau red. The activity of decolorization was determined by a slight modification of Kumar et al. (2012). A calibration curve prepared at different concentrations of dye solutions at 497 nm, 520 nm, 610 nm, 592 nm and 510 nm, respectively. Dye concentrations determined according to this curve. All experiments were carried out in 3 ml cuvettes and 2 ml final reaction volume. Reaction mixture included 100 mM phosphate buffer at pH 7, 100 ppm dye concentration and 20 U/ml purified peroxidase. When the partitioned peroxidase was added, the reaction started, and it was incubated at 30°C. Finally, the absorbance of dyes was measured using UV-Vis spectrophotometer at 10 min on appropriate wavelengths for each of them. Blanks included all components of the reaction mixture except the dyes.

## III. Results And Discussion

There is an increasing demand for new methods for purification of the enzyme including peroxidases, with the purpose more economically and efficiently uses of them. The TPP is an effective method for purification of proteins, where significant recovery of the various enzymes reported (Azmi et al. 1998, Shaffiqu et al. 2002). In the present study, we have used TPP for single-step purification of peroxidase from the cactus stem and the purification results showed in Table 1. TPP can be affected by various conditions, such as salt concentrations, solvent ratio, and pH. Therefore, different ratios of *t*-butanol, different concentrations of salt and various pH values were performed in this study.

*a) Effect of Homogenate to t-Butanol Ratio on TPP*

Correlation between *t*-butanol and ammonium sulfate was used for concentration of enzymes at an interface. *t*-Butanol has selected because of various advantages over the other organic solvents. It has not lead to denaturation of the purified enzyme due to its high molecular size and no ability to penetrate of the three-dimensional structure of a protein. The ratio of homogenate: *t*-butanol was studied in the range of 0.5 to 2 (v/v) in the presence of 40% ammonium sulfate. Purity and activity recovery of obtained peroxidase increased from 0.5 to 2 (v/v) homogenate: *t*-butanol ratio as shown in Figure 1. As a result, maximum purity and recovery of cactus peroxidase were obtained as 2, 25 and 106, 64%, respectively.

*b) Effect of Ammonium Sulfates Concentration on TPP*

Ammonium sulfate has an important role in TPP system due to its relation with protein-protein interaction and precipitation. It uses mechanism of salting out for precipitation of protein at an interface. The salt ions affect water molecules using strong protein-protein interactions, and the protein molecules are coagulated by hydrophobic interactions (Roy and Gupta 2002). Ammonium sulfate saturations of 20%, 30%, 40% and 50% were studied with 1:0.5 homogenate to *t*-butanol ratio. 40% ammonium sulfate showed the best activity recovery result as can be seen in Figure 2.

*c) Effect of pH on Partitioned Peroxidase*

The pH is an important parameter ionization of amino acids which are acidic or basic. The effect of pH on partitioned peroxidase was investigated on values from 3 to 10 about purity. Different pH values (3-10) was investigated for 60 min, at 40% ammonium sulfate concentration with 1:0.5 (v/v) homogenate:*t*-butanol ratio at 25°C, as seen in Figure 3. In this study, the optimal pH value of cactus peroxidase was found like 8.

*d) Effect of Temperature on Partitioned Peroxidase*

Temperature is a significant factor which affects the configuration and stability of the enzyme. The temperature optimization assay was performed between 0°C and 80°C while keeping all other parameters constant. Figure 4 showed that the temperature effect on a characteristic of peroxidase in the TPP system. The degree of purity of the enzyme has increased to about 30°C. After this temperature, the activity started to decrease. This decrease in activity at high temperature can result from the thermal deactivation of the enzyme. The possible reason behind this may be the *t*-butanol has important cosmotropic and aggregation effects at 20-30°C. Consequently, the optimal temperature of partitioned peroxidase was found at 30°C. Vetal and Rathod (2015) found the similar result in their work for peroxidase enzyme from orange peel.

*e) Effect of Kinetic Constants ($K_M$ and $V_{max}$)*

Influence of substrate concentration on peroxidase activity was performed with the beginning concentration of pyrogallol ranging from 1 to 6 mM at pH 8 and 30°C. Values of $K_M$ and $V_{max}$ for pyrogallol were calculated from Lineweaver-Burk plot as 12.5 mM and 9.2 U/ml.min, respectively. The plot was composed of 1/V against 1/[S] for system determining the Michaelis-Menten equation. Results were indicated in Figure 5. $K_M$ value of peroxidase obtained in this study which is in agreement with those presented in the literature (Triplett et al. 1992).

*f) SDS-PAGE Analysis*

Molecular weight and purity of partitioned peroxidase were estimated by SDS-PAGE. Molecular mass was found to be nearly 28 kDa which was similar to the previous results by Vetal and Rathod (2015)

purified peroxidase from orange peel, and they found molecular weight as 26 kDa. Figure 6 showed the bands of the homogenate and purified peroxidase with TPP. Minor stain on the peroxidase bands indicated the purity of the purified enzyme. Many major bands of the homogenate were not seen in the TPP fractions. SDS-PAGE showed a substantial purification of peroxidase from the cactus stem.

*g) Decolorization Effect of Partitioned Peroxidase*

Effect of decolorization of various synthetic dyes was analyzed using partitioned peroxidase from the cactus stem. Synthetic dyes are recalcitrant compounds (Azmi et al. 1998). The cactus peroxidase decolorized more than 80% synthetic dyes within a short time. These dyes have electron donating methyl and methoxy groups. Thanks to these groups they could bleach fast and efficiently. As follows from Figure 7 indigo carmine (84.12%) was the most sufficiently decolorized through enzymatic treatment, indicating the exact removal of color in the treated samples. Following indigo carmine, congo red and ponceau red were decolorized at the ratio of 35.16% and 30.93%, respectively. While crystal violet (22.23%) was less decolorized compared to the above three dyes. Methyl red (17.4%) had the least decolorization effect among treated samples. Results in the present study were in agreement with the literature (Shaffiqu et al. 2002). When the work investigated from an industrial point, it could be easier to add a solution of peroxidase partitioned with the TPP directly into the wastewater. Consequently, TPP peroxidase obtained from cactus stem should be used for decolorization of synthetic dyes present in the industrial wastewater.

## IV. Conclusions

Recovery activity of peroxidase purified from the cactus stem by using the TPP was found to be higher than the other plant peroxidases studied. The main advantages of the purified enzyme are to be stable for several days having optimal pH and temperature values, one step and cheap purification procedure with high recovery activity which make this technique good choice for several industrial applications. Also, the purified peroxidase showed a significant decolorization activity on the synthetic dyes without any redox mediators. It is noticeable that cactus peroxidase enzyme is fairly effective in removing textile wastewaters.

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*Declaration of interest*

The authors declare that they have no conflicts of interest to disclose.

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*Table 1:* Three-phase partitioning of peroxidase from the stem of cactus.

| Samples | Total activity (U) | Total protein (mg) | Spesific activity (U/mg) | Purification fold | Activity recovery (%) |
|---|---|---|---|---|---|
| Homogenate | 262,61 | 0,141 | 1863,71 | 1 | 100 |
| TPP intermediate phase | 280,04 | 0,067 | 4200,61 | 2,25 | 106,64 |
| TPP bottom phase | 1,62 | 0,021 | 77,76 | 0,04 | 0,62 |

**The experiments were carried out in triplicate for purification steps and the difference in the readings was less than ±5%.*

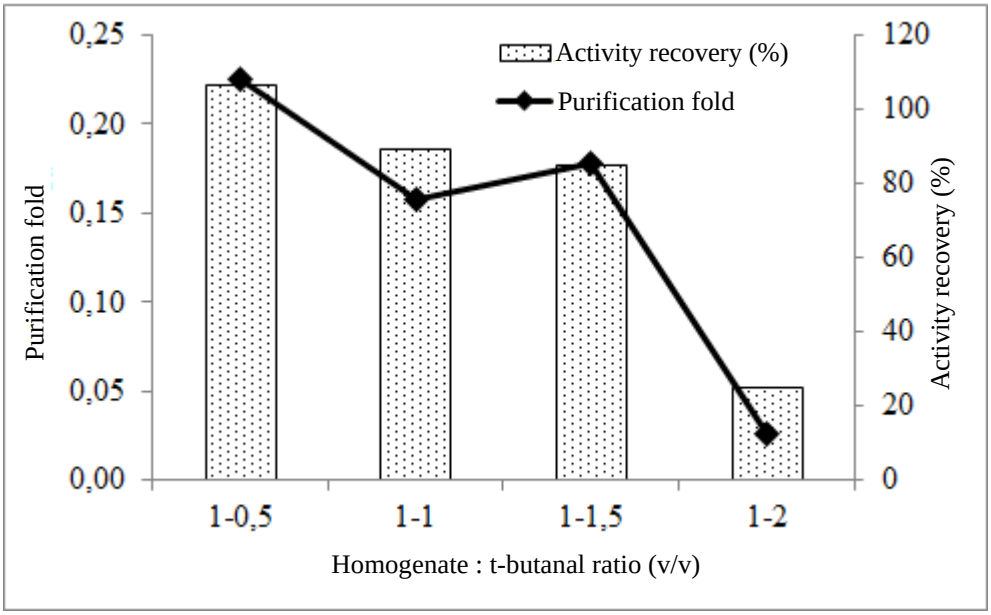


*Figure 1:* Effect of homogenate: *t*-butanol ratio (v/v) on purification fold and activity recovery of peroxidase from the stem of cactus.

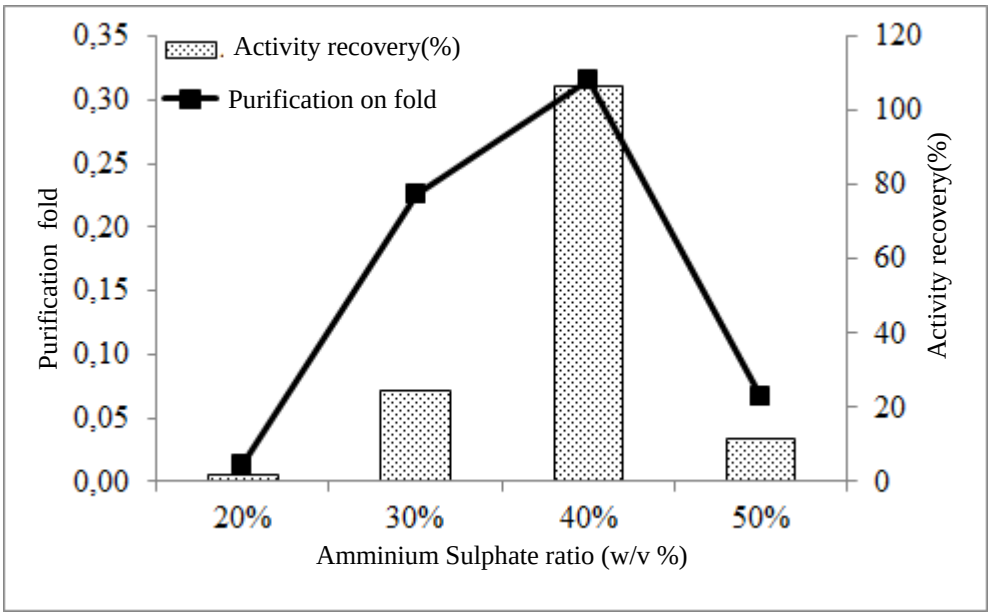


*Figure 2:* Effect of the concentration of ammonium sulfate (%, w/v) on purification fold and activity recovery of peroxidase from the stem of cactus.

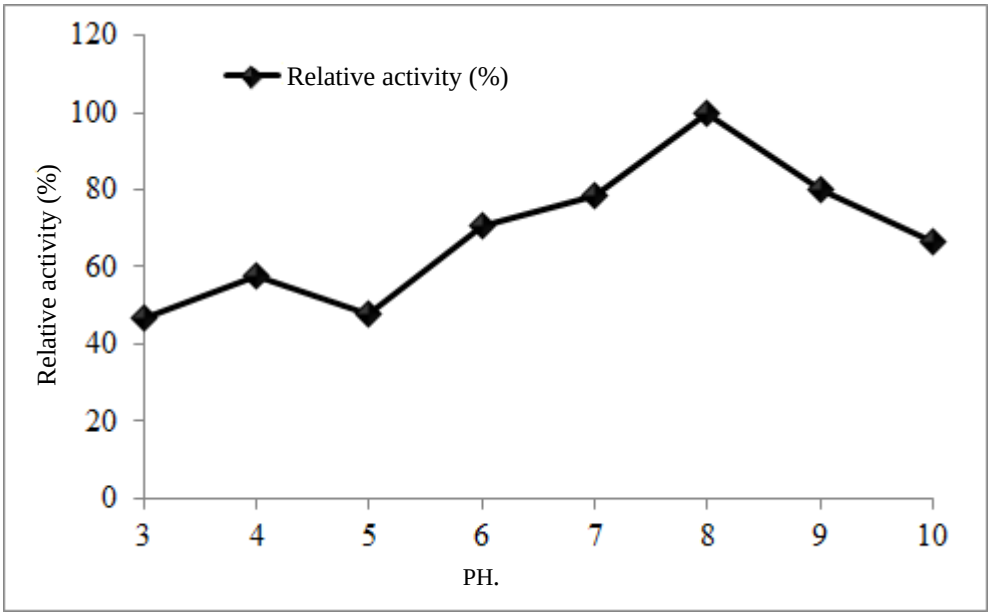


*Figure 3:* Effect of pH on the activity of peroxidase from the stem of cactus.

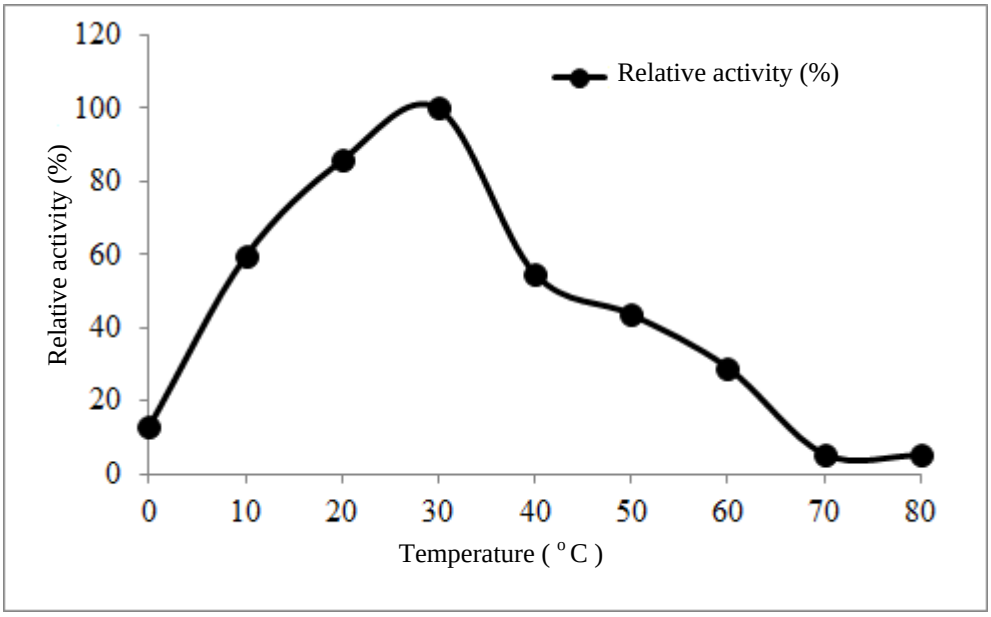


*Figure 4:* Effect of temperature on the activity of peroxidase from the stem of cactus.

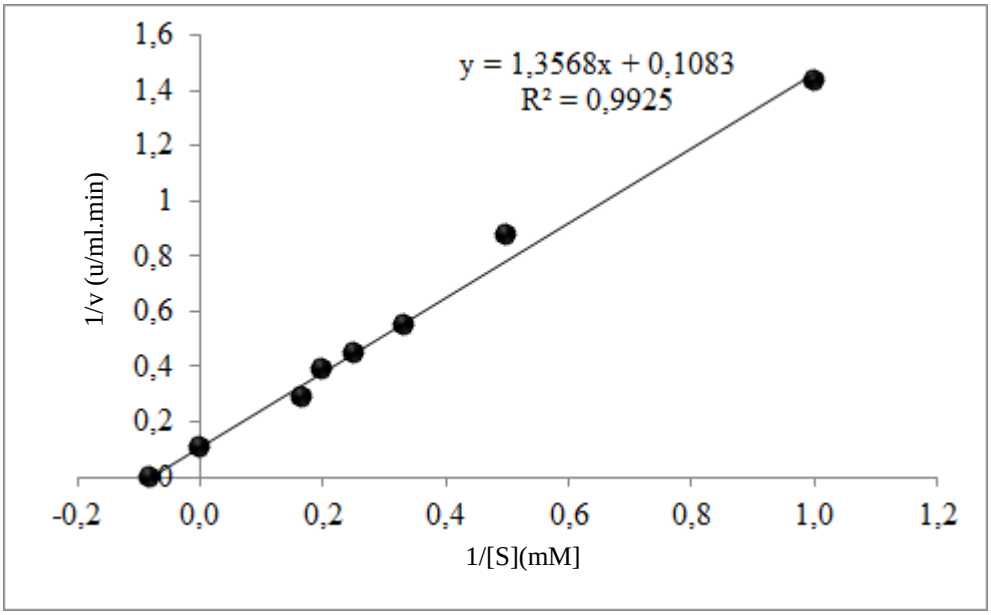


*Figure 5:* Lineweaver-Burk plot of substrate of peroxidase from the stem of cactus.

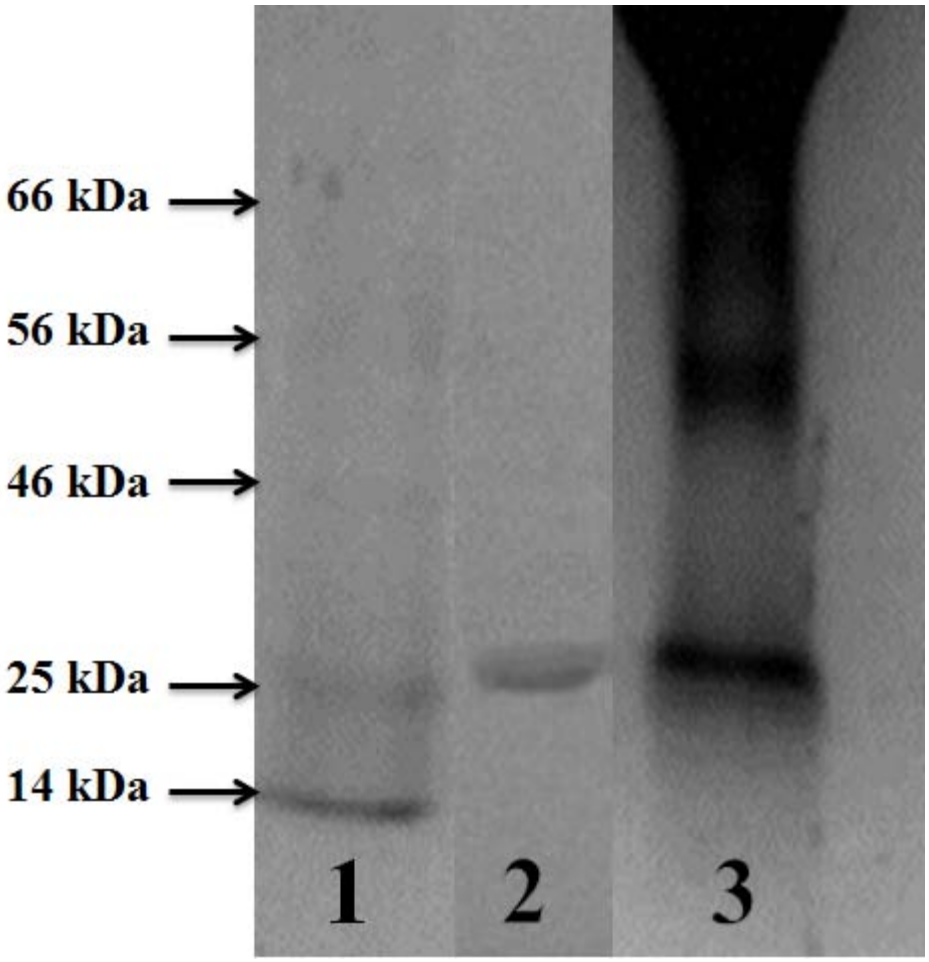


*Figure 6:* SDS-PAGE pattern of the purified peroxidase. Lane 1: Molecular weight marker, Lane 2: TPP intermediate phase, Lane 3: Homogenate.

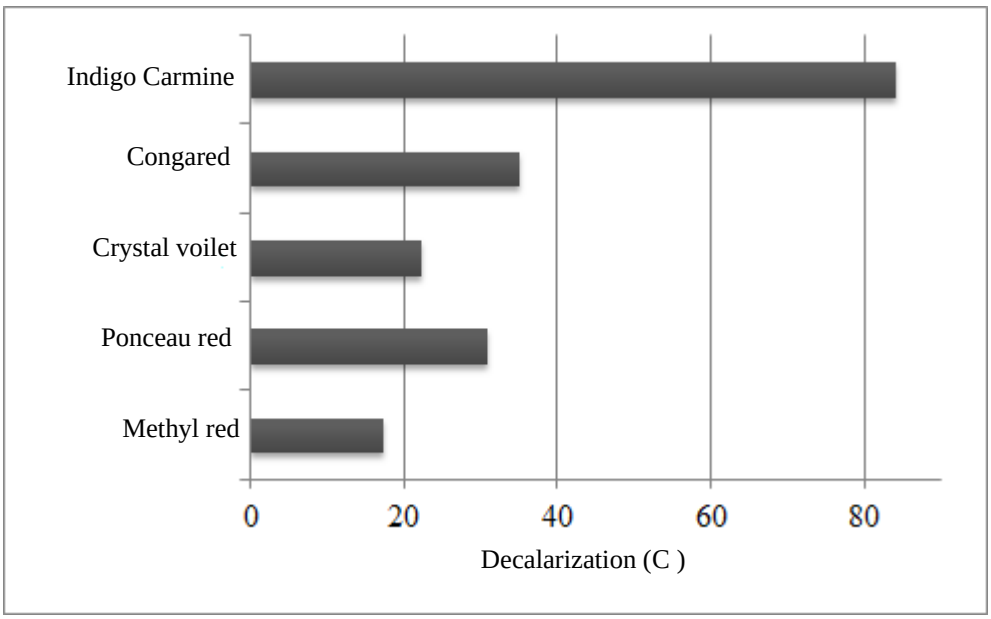


*Figure 7:* Decolorization activity of peroxidase from the stem of cactus.