Purification of Cowpea (*Vigna unguiculata*) Peroxidase with Single-Step Affinity Chromatography and, Characterization Study

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Abstract

In this report, novel peroxidase enzyme was isolated and characterized from fresh peels of cowpea (*Vigna unguiculata*). Four different amino benzohydrazide derivatives were used as ligand in affinity chromatography and with 4-amino 3-bromo 2-methylbenzohydrazide, 53.6% purification yield (91.6 fold) was achieved in a single-step. The molecular weight of the purified cowpea peroxidase (POD) showing single band at SDS-PAGE was calculated as 39 kDa. For evaluating the cowpea PODs affinity to ligands, IC_{50} and K_i values were determined. The 4-amino 3-bromo 5-chlorobenzohydrazide molecule showed highest inhibition with the IC_{50} value of 78 µM. In the characterization assays, optimum parameters were determined for purified cowpea POD as pH= 6.0, ionic strength= 0.3 M and temperature= 40 ºC. In addition, substrate specificity tests were carried out with guaiacol, H_2O_2, 4-methylcatechol and ABTS substrates. According to the obtained data, the K_M values of these substrates were calculated as 7.21 mM, 17.03 mM, 7.28 mM and 49.21 mM, respectively.

Keywords: Peroxidase, Cowpea, Purification, Affinity chromatography, Characterization

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1. Introduction

Peroxidases (PODs) are a superfamily of enzymes that were encountered in various forms in nature. These enzymes take part in many cellular processes such as the formation of stress-induced responses, defense mechanisms, biosynthesis of some biomolecules and degradation of various biological compounds. PODs use hydrogen peroxide to catalyze the oxidation of their substrates, and their catalytic mechanism is sustained by the redox cycle of the heme-iron center (Kohler et al. 2020).

In many industrial and clinic processes, PODs are widely used in the biocatalyzed redox processes like immunoassay tests (Craig and Hartz 1989), decolorization of toxic dyes (Kohler et al. 2020), removal of aromatic hydrocarbons (Pokora and Cyrus 1990) and hair lightening (Belinky et al. 2014).

Due to the wide range usage of PODs in industrial processes, these enzymes come to be commercial importance and studies on plant PODs are increasing day by day. There are many studies in the literature about purification of various type of PODs from different source including papaya, banana, horseradish (Singh et al. 2019), runner bean (Oztekin and Tasbasi 2020), black radish and turnip (Oztekin et al. 2019). However, according to our knowledge, there is a no report about purification of cowpea POD enzyme.

In purification steps of PODs, researchers encounter a wide range of challenges. Increasing the isolation steps causes both efficiency and time loss. Additionally, the small sample size is a limiting factor. To cope with these difficulties, affinity chromatography offers some advantages and this technique is a highly preferred type of chromatography providing high yield and purity at the end of the purification process (Janson 1984). Moreover, purification of PODs in one-step using the

affinity technique not only simplifies the isolation of these enzymes but also significantly reduces costs.

Nowadays, the use of 4-aminobenzohydrazide-derived molecules as ligands in the purification of plant POD enzymes continues to attract the attention of many researchers (Oztekin et al. 2021). In the literature, 4-amino 3-bromo 2-methylbenzohydrazide, 4-amino 3-bromo 5-fluorobenzohydrazide and 4-amino 3-bromo 5-chlorobenzohydrazide molecules have been effectively used for POD purification from various sources (Oztekin and Tasbasi 2020, Öztekin 2020, Oztekin et al. 2021). In our study, we used the above mentioned molecules and 5-amino isophthalohydrazide containing double hydrazine group. With the help of these ligands, POD enzyme was successfully purified for the first time from the cowpea plant in a single step affinity chromatography. In addition, the biochemical properties of the purified cowpea POD enzyme were determined in detail.

2. Materials and Methods

***Chemicals*:**4-amino 3-bromo 5-chloro methylbenzoate (%96), 4-amino 3-bromo 5-floromethylbenzoate (%97), 4-amino 3-bromo 2-methymethylbenzoate (%98), Dimethyl 5-amino isophthalate (%98) were purchased from Fluorochem (Derbyshire, UK), CNBr-Sepharose-4B and guaiacol (%98) were obtained from Merck KGaA (Darmstadt, Germany) and Protein standards (Lot:26616) was obtained from Thermo Fisher Scientific (MA, USA). All other chemicals not mentioned were of analytical grade.

Peroxidase Assay: In the POD activity measurement, chromogenic guaiacol reactant was used as substrate and increase in absorbance was observed spectrophotometrically. 3 mL final volume was consisted of 33 mM KH_2PO_4 (pH 6), 15 mM guaiacol and 10 mM H_2O_2. The reaction was started by adding 0.01 mL of enzyme to the prepared mixture, and the increase in absorbance at 470 nm for 3 minutes was monitored in 30-second intervals in the UV-Vis spectrophotometer (Lavery et al. 2010, Oztekin et al. 2019).

Homogenate preparation: 20 g of cowpea was mechanically crushed. Then, it was powdered using liquid nitrogen and 80 mL of the prepared homogenate buffer (0.3 M NaH_2PO_4) was added to it and mixed in a magnetic stirrer for 3 min. The resulting mixture was fully break into pieces at 2,400 rpm with ultra turrax, and the plant cells fragments were homogenized. The mixture was taken into centrifuge tubes and centrifuged at 12,000 xg for 40 minutes. At the end of centrifugation, the supernatant was separated from the precipitate and used at further steps.

Purification of cowpea POD: Ligand molecules needed to affinity purification, namely, 4-amino 3-bromo 2-methylbenzohydrazide, 4-amino 3-bromo 5-fluorobenzohydrazide, 4-amino 3-bromo 5-chlorobenzohydrazide and 5-amino isophthalohydrazide were synthesized according to procedure previously described (Oztekin and Tasbasi 2020). Initially, 4 g of CNBr activated Sepharose-4B was attached to L-tyrosine. Then, 20 mg of each ligand were diazolated and separately coupled to average 2 g of Sepharose 4B-L-tyrosine matrix. After reactions, obtained affinity gels were loaded to 1*10 cm column. Finally, supernatant (homogenate) was loaded into the affinity column equilibrated with 10 mM KH_2PO_4 (pH 6.8). Unbound molecules and other components were washed with 25 mM of the same buffer and cowpea POD enzyme was eluted with KH_2PO_4 buffer containing 1 M NaCl.

Electrophoresis: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method was conducted to calculate the molecular weight of the cowpea POD and to determine whether the enzyme was obtained in pure form. 3% polyacrylamide concentrations were applied for stacking gel and 10% for separation gel. The samples were migrated under electric current for 2 hours. Finally, protein bands were made visible by silver staining method (Chevallet et al. 2006).

***Determination of inhibition capacity of ligands*:** The activity limiting effects of 4-amino 3-bromo-2-methylbenzohydrazide, 4-amino 3-bromo-5-fluorobenzohydrazide, 4-amino 3-bromo-5-chlorobenzohydrazide and 5-amino isophthalohydrazide on cowpea POD were calculated to the clarify enzyme-ligands interaction. POD activity tests were carried out in the presence of five different concentrations of each compounds for defining the IC_{50} values. For estimation of the K_i values, three different inhibitor and five different substrate (guaiacol) concentrations were used. K_i values and the inhibition types were determinated

from Lineweaver–Burk graphs (Lineweaver and Burk 1934).

Determination of optimum parameters for cowpea POD: To determine the optimum pH of cowpea POD, 0.1 M sodium acetate buffer from pH 4.0 to 5.5, 0.1 M potassium phosphate buffer pH 6.0 to 7.5 and Tris-HCl buffer pH 8.0 to 9.0 were prepared. Then, activity measurements separately were performed in the each buffer at room temperature. Potassium phosphate between 0.1 M and 1 M were used to analyze the ionic strength effects on the cowpea POD activity. Throughout the tests, guaiacol was used as a substrate and activity measurements were made at room temperature. For analyze the temperature effect on activity of cowpea POD, enzyme activity test were carried out from 5 to 70°C. During the monitoring activity, all other parameters kept optimum.

Determination of cowpea POD affinity to substrates: For analyzing the cowpea PODs affinity to substrates, namely guaiacol, H_2O_2, 4-methlycatechol and ABTS, activity assays were conducted at different concentrations, while the other parameters were kept constant. After assays, 1/[V] versus 1/[S] plots were drawn. K_M and V_{max} values of each substrate were determined from these graphs.

3. Results and discussion

Nowadays, the use of biocatalysts in various processes has come to the fore as an environmentally friendly approach. In order for enzymes to find practice area in various industrial processes, enzymes can meet the necessary basic parameters. Therefore, it is important to purify and characterize new enzymes with desired properties. Accordingly, cowpea POD enzyme was isolated with single-step affinity technique for the first time in this study. This technique allowed to reach up to specific activity of 837.5 EU mg^{-1} (with 94.1-fold) with the 4-amino 3-bromo 5-chlorobenzohydrazide affinity ligand and 53.6% of yield was achieved with the 4-amino 3-bromo 2-methylbenzohydrazide ligand. Purification summary are presented in Table 1. In recent studies, the authors reported isolation of PODs from various sources such as 253-fold purification of runner bean POD with a yield of 56.2% using 4-amino 3-bromo 2-methylbenzohydrazide ligand (Oztekin and Tasbasi 2020), 1151-fold purification of horseradish peroxidase (HRP) with a yield of 8.5% by 4-amino-3-bromo benzohydrazide (Almaz et al. 2021) and similarly, 677-fold purification of BR-POD with a yield of 31.4% (Oztekin et al. 2019).

SDS-PAGE is a one of the steps to confirm the success of purification. In this regard, molecular weight and the purity of the isolated cowpea POD were examined. As can be seen in Figure 1, purification of the enzyme was confirmed by observing a single band at 39 kDa. Other authors reported that molecular weight of POD isolated from jackfruit was 104 kDa (Tao et al. 2018) and the molecular weight of the ginger POD was 42 kDa (El-Khonezy et al. 2020).

Table 1. Detailed purification profile of cowpea POD with affinity chromatography

***Source*/Ligand**	**Total Volume (mL)**	**Activity (EU/mL)**	**Protein (mg/mL)**	**Specific activity (EU/mg)**	**Yield (%)**	**Fold**
Homogenate	6	12.2	1.37	8.9	100	1
4-amino 3-bromo 2-methylbenzohydrazide	4	9.8	0.012	816.6	53.6	91.6
4-amino 3-bromo 5-fluorobenzohydrazide	4	4.6	0.011	418.2	25.1	47
4-amino 3-bromo 5-chlorobenzohydrazide	4	6.7	0.008	837.5	36.6	94.1
5-amino isophthalohydrazide	4	3.6	0.009	400	19.7	44.9

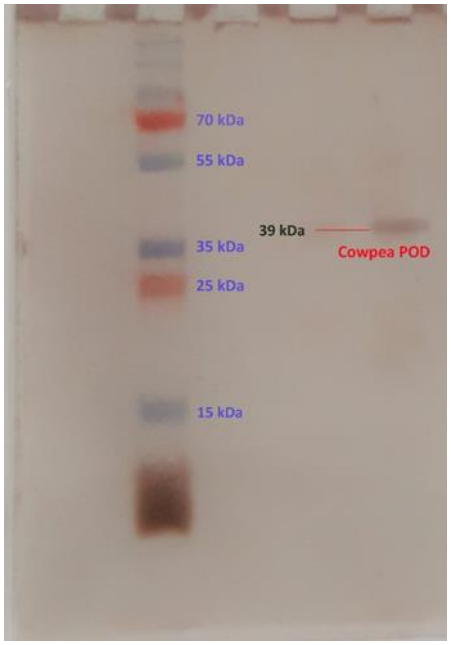

Figure 1. SDS page image of purified cowpea POD with 4-amino 3-bromo 5-chlorobenzohydrazide ligand

In order to examine the inhibitory effect of the molecules used as a ligand for the purification of cowpea POD, the enzyme activity was measured at different concentrations of these molecules. Using the obtained data in the assays, Activity/concentration graphs were drawn (Figure 2) and The IC_{50} values for 4-amino 3-bromo 2-methylbenzohydrazide, 4-amino 3-bromo 5-fluorobenzohydrazide, 4-amino 3-bromo 5-chlorobenzohydrazide, and 5-amino isophthalohydrazide were 970 µM, 166 µM, 78 µM, and 295 µM, respectively. In addition, activity measurements were made at five different substrates and three different inhibitor concentrations, and K_i values were calculated by drawing Lineweaver-Burk graphs (Figure 3). K_i values for 4-amino 3-bromo 2-methylbenzohydrazide, 4-amino 3-bromo 5-fluorobenzohydrazide, 4-amino 3-bromo 5-chlorobenzohydrazide and 5-aminoisophthalohydrazide molecules were calculated as 970±130 µM, 338±176 µM, 97±7,8 µM and 249±24 µM respectively. In the inhibition studies, it was determined that all of the ligand inhibition types was noncompetitive. When the K_i values and purification coefficients were examined, it was found that the 4-amino 3-bromo 5-chlorobenzohydrazide molecule had a high affinity for the enzyme. Correspondingly, the highest purification coefficient was achieved with using this molecule.

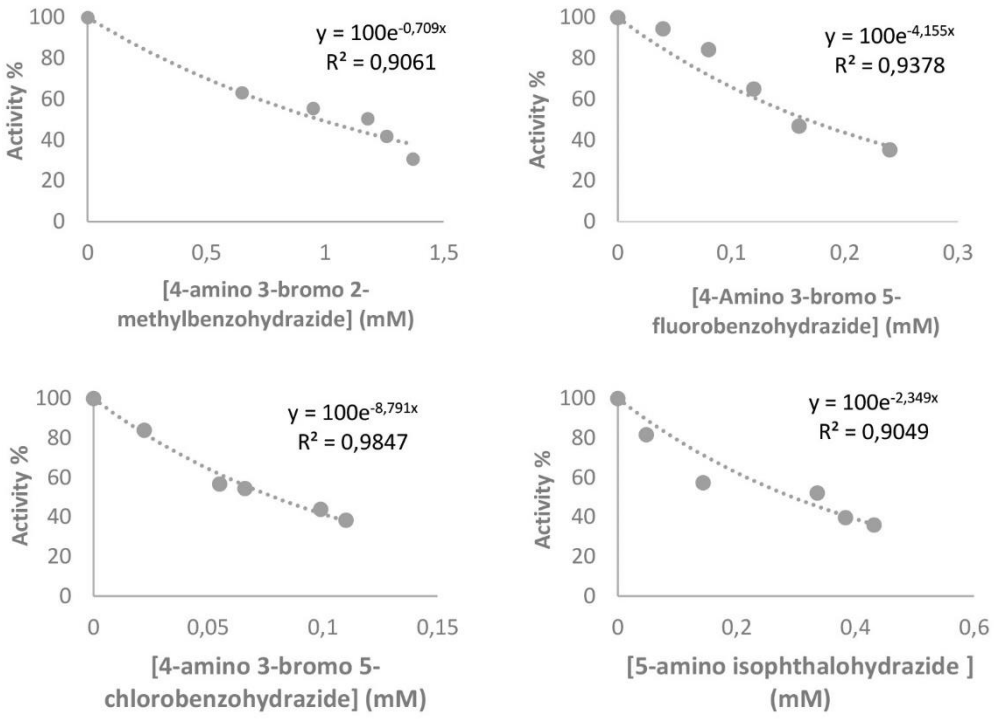

Figure 2. IC_{50} assay graphs of cowpea POD with ligand molecules

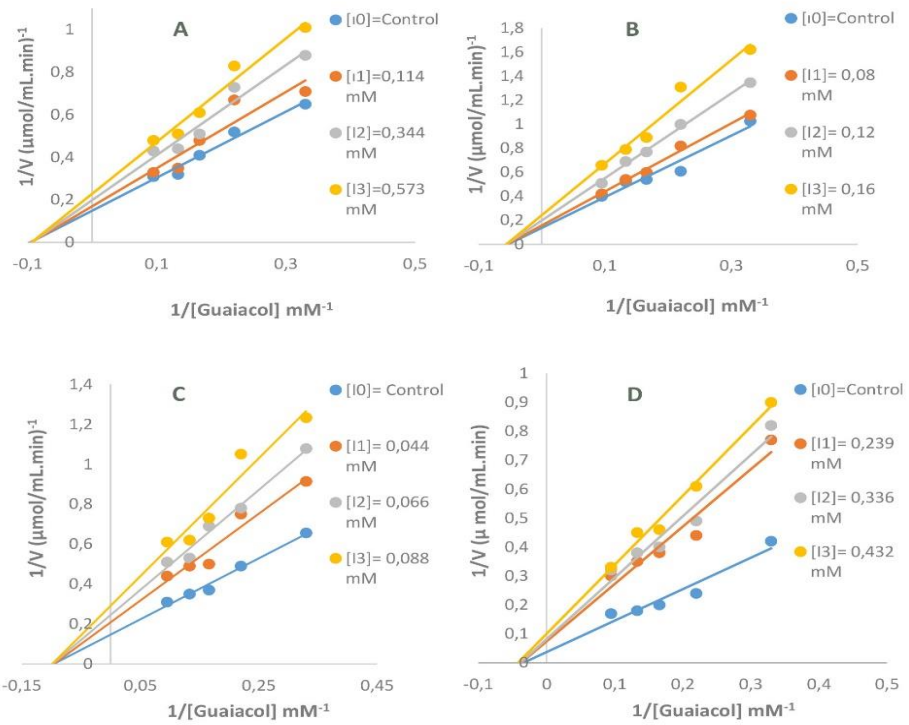

Figure 3. K_i assay graphs of cowpea POD with ligand molecules, **A**: 4-amino 3-bromo 2-methylbenzohydrazide, **B**: 4-amino 3-bromo 5-fluorobenzohydrazide, **C**: 4-amino 3-bromo 5-chlorobenzohydrazide, **D**: 5-aminoisophthalohydrazide

The pH, ionic strength and temperature factors changing the enzyme's catalyst capacity were plotted versus relative activities and graphs were shown in Figure 4. The cowpea POD exhibited maximum catalyzing activity at pH 6. Comparing optimum pH results of cowpea to the palm leaves POD, both study are in accordance and similar to cowpea POD, this enzyme presents maximum activity at pH 5.5 (Al-Senaidy and Ismael 2011). Optimum ionic strength tests were performed in the different KH_2PO_4 concentrations from 0.1 to 1 M to determine the maximum relative activity of cowpea POD. In the assays, enzyme reached maximum activity in the 0,3 M of ionic strength. Similarly, Koksal et al. reported that sweet gourd POD showed high activity at the 0.4 M of ionic strength (Koksal et al. 2012). In order to determine the optimum temperature of the cowpea POD, relative activity tests were conducted at different temperatures ranged from 5 to 70 ºC and the optimum temperature for the enzyme was determined as 40 ºC. In comply with this result, Lavery et. al. revealed that the HRP exhibits optimum catalyzing activity at 30°C (Lavery et al. 2010).

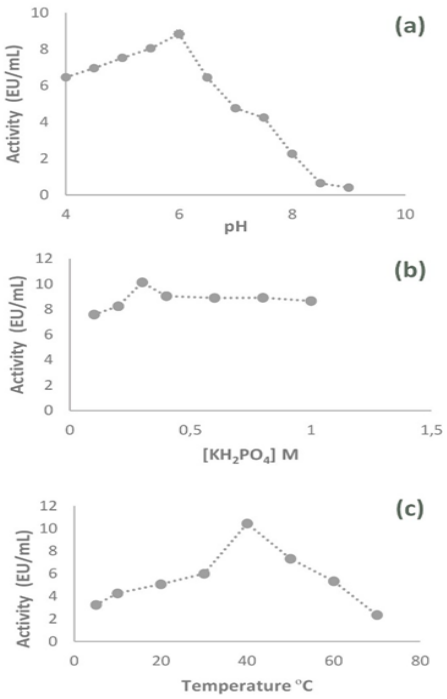

Figure 4. Optimum parameters for Cowpea POD. Activity-pH (a), Activity İonic-strength (b) and Activity-temperature (c) graphs

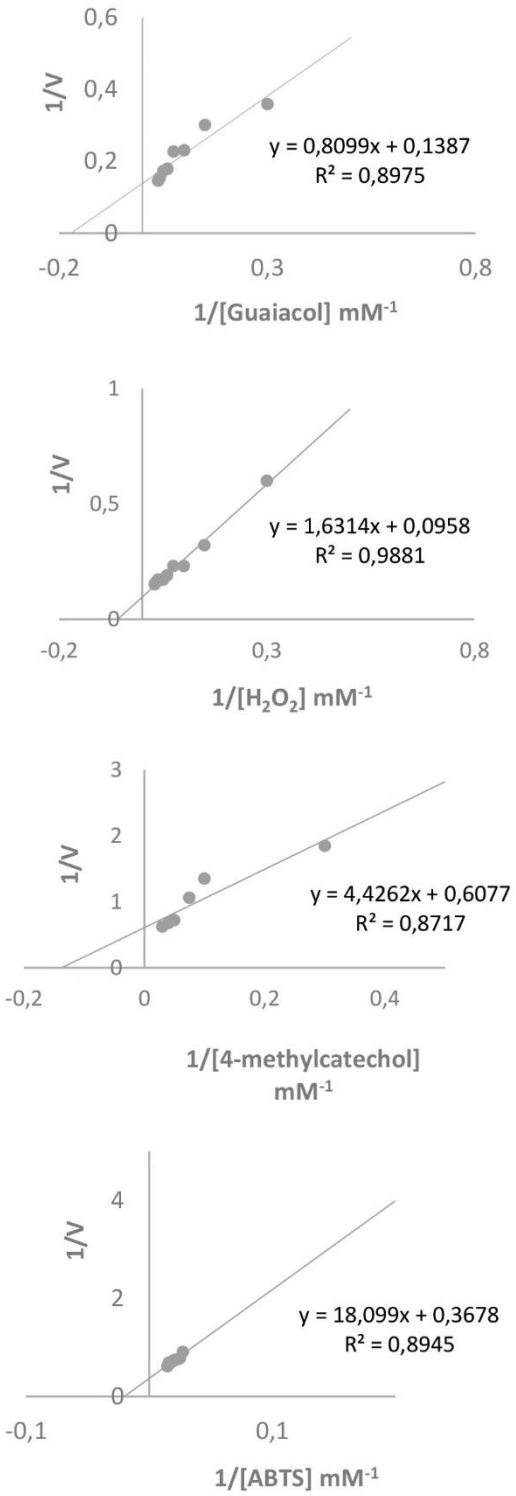

Figure 5. Specificity assay graphs of Cowpea POD for guaiacol, H_2O_2, 4-methylcatechol and ABTS substrates

PODs are enzymes with low substrate specificity and can catalyze the oxidation of many organic compounds. For this reason, kinetic tests were carried out to determine the affinity of the purified cowpea POD enzyme to guaiacol, H_2O_2, 4-methlycatechol and ABTS substrates. According to results obtained from 1/V versus 1/[S] graphs (Figure 5), K_M and V_{max} values were calculated as 7.21 mM and 5.83 EU/mL for guaiacol, 17.03 mM and 10.04 EU/mL for H_2O_2, 7.28 mM and 1.64 EU/mL for 4-methlycatechol and 49.21 mM and 2.72 EU/mL for ABTS, respectively. In previous studies, the K_M affinity of lettuce POD for guaiacol was 4.74 mM (Hu et al. 2012) while onion POD for guaiacol was 3.44 mM (Oztekin 2020). In the light of these data, it is clear that the relative affinity of cowpea POD to the guaiacol substrate is greater than the other substrates tested.

4. Conclusion

Enzyme-catalyzed process designs are widely used in today's industry. PODs are one of the commercial enzymes that can meet a wide variety of biocatalyst needs. In this study, to investigate the alternative PODs that have the potential to meet the increasing demand for redox-biocatalysts, POD enzyme was purified from cowpea using a single-step purification method with high yield (53.6%) for the first time. SDS-PAGE was performed to check the isolated cowpea POD's purity and to estimate its relative molecular weight, and a single band was observed at approximately 39 kDa. In the kinetic assays, 4-amino 3-bromo 5-chlorobenzohydrazide exhibited the highest affinity to enzyme with the K_i value of 97 µM and accordingly, the highest purification fold was reached with this molecule. In addition, in tests to determine the optimum conditions for the cowpea POD, enzyme showed maximum activity at pH 6.0 and 40 °C in the presence of 0.3 M ionic strength.

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