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CHARACTERIZATION OF GUAIACOL PEROXIDASE ENZYME FROM CARAMBOLA FRUIT

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Abstract: Carambola is a fruit grown in tropical and subtropical regions of the world. Natural antioxidants including vitamin C, carotenoids, and certain phenolic substances are abundant in carambola fruit. As antioxidants support health by acting as nutraceutical and functional food additives, they help preserve food by preventing oxidation processes. The oxidation of various organic or inorganic substrates by hydrogen peroxide or organic peroxides as terminal oxidants is a process in which peroxidase, which is abundantly present in fruits and vegetables, participates. In this study, guaiacol peroxidase enzyme from carambola fruit was partially purified and characterized. Purification procedure made up the homogenate preparation, ammonium sulfate precipitation, and dialysis. After purification, optimum ionic strength, pH and substrate concentration were investigated. These values were determined as 200 mM Tris, pH: 7.5, 7.5 mM H_2O_2 and 15 mM guaiacol for carambola fruit guaiacol peroxidase enzyme, respectively.

Keywords: Carambola, Enzyme, Characterization, Peroxidase, Purification

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1. Introduction

Nutritional content of fruits is one of the most important factors affecting the customer demand for the product and the development of the market value of these products. The promotion and living campaign for a healthy lifestyle by health institutions has increased the awareness of the significance of customers' dietary needs (O'Dougherty et al., 2006; Barrett et al., 2010; Lampila et al., 2009). Fruits and vegetables, which have important health benefits, play an important role in reducing the incidence of various diseases due to these properties (Park et al., 2003; Marnewick et al., 2011; Franzini et al., 2012; Rautiainen et al., 2012).

Carambola (*Averrhoa carambola* L.) is a tropical and subtropical fruit that is widely grown (Muthu et al., 2016). *Averrhoa carambola* (Figure 1) is a woody plant from the Oxalidaceae family that is native to India, Indonesia, the Philippines, Malaysia, Vietnam, Sri Lanka, and Bangladesh (Manda et al., 2012). Its fruits and leaves are frequently utilized in Ayurvedic medicine to treat a variety of diseases (Dasgupta et al., 2013). Because of its unusual form, it is historically known as "kamrakh" and more popularly known as star fruit. Malaysia is a significant producer of carambola in the globe (Zainudin et al, 2014). Carambola has both antioxidant capacity and high nutritional value (Leong and Shui, 2002; Shui et al., 2004; Isabelle et al., 2010; Shofian et al., 2011). The fruit contains proanthocyanins, which mainly act as antioxidants and play an important role for the immune system in defending against cancer, reactive oxygen species (ROS) damage and lipid peroxidation, as well as helping to remove toxins from the body (Ikram et al., 2009). In studies on different fruit varieties of Carambola, it was determined that the antioxidant potential changed (Zainudin et al., 2013). In addition, it has been found that different fruits such as olive, orange, tomato and pear jujube show different antioxidant properties among different fruits with varying antioxidant properties (Huang et al., 2007; Castrejón et al., 2008; Ilahy et al., 2011; Wu et al., 2012).

Figure 1. Carambola (*Averrhoa carambola* L.) fruit (Herath et al., 2021).

ROS, an essential component of aerobic life, are formed when photosynthetic organisms release molecular oxygen directly into the atmosphere (Gupta et al., 2017; Taverne et al., 2018). Although reactive oxygen species are a natural component of a cell metabolism, when the

ratio of ROS created to ROS scavenged is out of balance, the detrimental effects of these species (Olson and Straub, 2015; Sewelam et al., 2016; Sengul et al., 2021).

Endogenous antioxidant enzymes can scavenge reactive oxygen species (ROS). Antioxidant enzymes like as superoxide dismutase, catalase, and peroxidases are well recognized for preventing intracellular ROS production and lipid peroxidation (Gelen et al., 2021).

Peroxidases (EC 1.11.1.7) are heme-containing enzymes that may decrease hydrogen peroxide while also oxidizing another substrate (Manu et al., 2009). Peroxidase (POD), an oxidoreductase, has been widely employed as a component of reagents for clinical diagnosis and numerous scientific investigations. Peroxidase has been attributed physiological tasks such as indole-3-acetic acid metabolism, lignification, cross-linking of cell wall polymers, suberin production, and infection resistance (Veitch, 2004). Peroxidases are present in a wide range of organisms, including mammals, bacteria, fungus, and the majority of green plants (Sakharov et al, 2000). Plant peroxidases play crucial physiological roles, such as defending against pathogens and stress (Twala et al., 2020), regulating cell wall damage (Wakamatsu and Takahama, 1993), taking part in the removal of hydrogen peroxide, and protecting against unwanted discoloration (Ashie et al, 1996; López-Serrano and Barceló, 1996).

In order to reveal the importance of POD enzyme in plant defense system, we aimed, for the first time, to partially purify the enzyme from carambola fruit, to determine new potential natural antioxidant sources and optimum ionic strength, pH and substrate amount.

2. Material and Methods

2.1. Chemicals

The chemicals used in the purification process were supplied by Sigma-Aldrich (St. Louis, Mo, USA). The other chemicals used were supplied by Merck (Darmstadt, Germany).

2.2. Homogenate Preparation and Enzyme Analysis

The fruit was obtained from the plant *Averrhoa carambola*. It was brought in small pieces and homogenized through crushing with liquid nitrogen (approximately -196°C). The powdered sample was mixed with 100 mM Tris (2-Amino-2-(hydroxymethyl)-1, 3-propanediol) buffer and then centrifuged at 4°C, 15.000 xg. After centrifugation, the supernatant was filtered, and the enzyme's activity was evaluated. The activity was measured with a Shimadzu UV-1800 spectrophotometer at 470 nm (Şişecioğlu et al., 2010).

Hydrogen peroxide (H_2O_2) and guaiacol was used as the substrate for POD. 100 µl of H_2O_2 and 100 µl guaiacol in 0.1 M Tris buffer (pH 7.0) was added in the cuvette. In order to start the enzymatic reaction, 100 µl of supernatant containing POD enzyme was added to the cuvette. The remaining 1 ml of the solution was then filled with distilled water. The reaction's absorbance value was measured in a spectrophotometer for 3 minutes at 470 nm.

2.3. Ammonium Sulfate Precipitation and Dialysis

The obtained homogenate was precipitated with ammonium sulfate. The homogenate was then tested at various solid ammonium sulfate at regular intervals ranging from 0 to 100% salt content. The precipitate was sufficiently dissolved with 0.1 M Tris. Dialysis was applied to remove salts from the protein content. The obtained sample was placed in the dialysis bag, passed through the appropriate buffer and mixed slowly. As tiny molecules flowed through the membrane during this application, the buffer outside the membrane was altered several times until the osmotic pressure was controlled (Smith et al., 1988).

2.4. Kinetic Parameters for The Enzyme's Characterization

In the enzyme characterization study, pH and substrate in different ranges, in addition to, ionic strength parameters in different buffers and ranges were investigated.

3. Result and Discussion

In this study, POD enzyme from the fruit of the carambola plant was partially purified and characterized. The high antioxidant content of carambola fruit and the positive effects of its use in human health reveal the importance of our study. Peroxidase has been found in a variety of species, including bacteria, fungus, and higher plants. It has also been isolated, sequenced, and described (Passardi et al., 2007; Welinder, 1992).

Characterization study is important for determination and selection of optimum values for POD enzyme and antioxidant properties obtained from carambola fruit. Many plant peroxidases, especially those from horseradish (*Armoracia sp*.), are well recognized for their structures, substrate specificities, and kinetic characteristics (Al-Senaidy and Ismael, 2011). In this study, it is of great importance to determine the optimum values for the importance of the POD enzyme obtained from the carambola plant and its antioxidant properties. In addition to the importance of the antioxidant content of the Carambola fruit, POD characterisation is important for the enzyme and the plant.

For the first time, POD enzyme from Carambola fruit was partly purified and characterized in this work. After the homogenized sample, the precipitate saturation of the enzyme with solid ammonium sulfate $(NH_4)_2SO_4$ was determined in the range of 60-80%. This result demonstrates that the functioning during the purification protocol period is consistent with previous research results and will serve as an example for future studies. In order to determine the optimum ionic strength optimization, both tris and potassium phosphate buffer optimization measurements were carried out.

Optimum ionic strength optimization experiments were evaluated in the range of 10 mM to 400 mM Tris buffer and determined as 200 mM Tris (Figure 2). In order to find the optimum pH, measurements were made between

pH 6.5 and 8.5 and the optimum pH was found to be 7.5 (Figure 3). Moreover, the optimal substrate concentration for H_2O_2 was determined to be 7.5 mM H_2O_2 after being evaluated between 2.5 and 15 mM (Figure 4). In addition, the optimum substrate concentration for the other substrate, guaiacol, was evaluated between 5 and 30 mM, and 15 mM guaiacol was found to be the optimal substrate concentration (Figure 5).

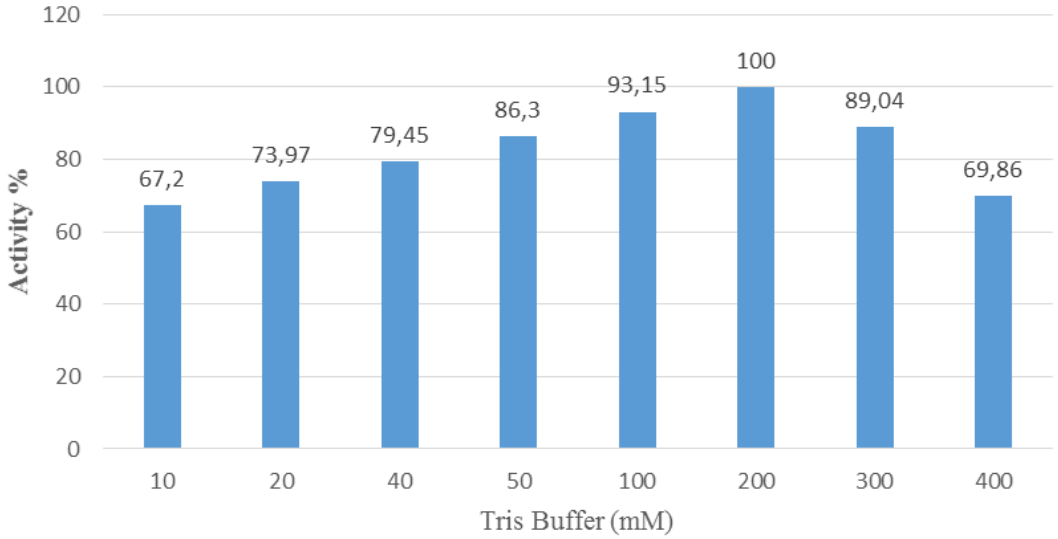

Figure 2. Activity measurements of carambola fruit guaiacol peroxidase enzyme optimal ionic strength TRIS buffer.

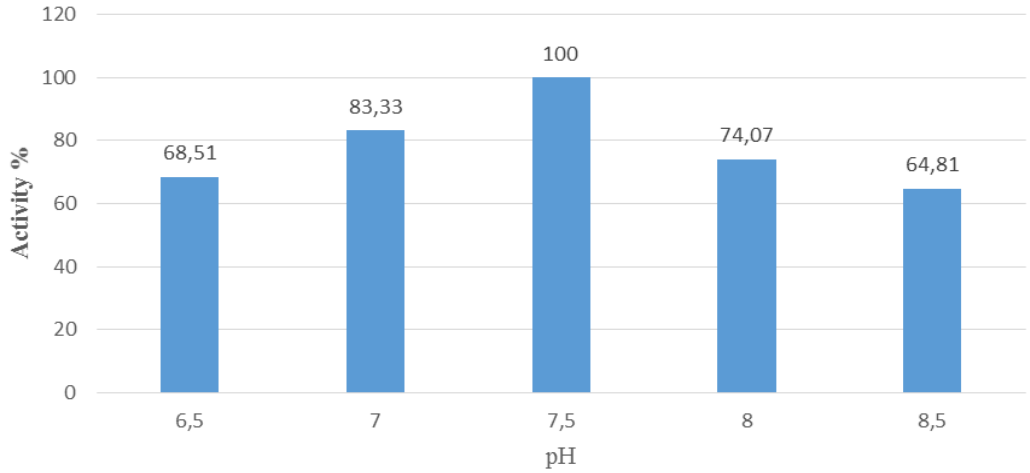

Figure 3. Activity measurements of carambola fruit guaiacol peroxidase enzyme optimal pH value Tris (200 mM) buffer

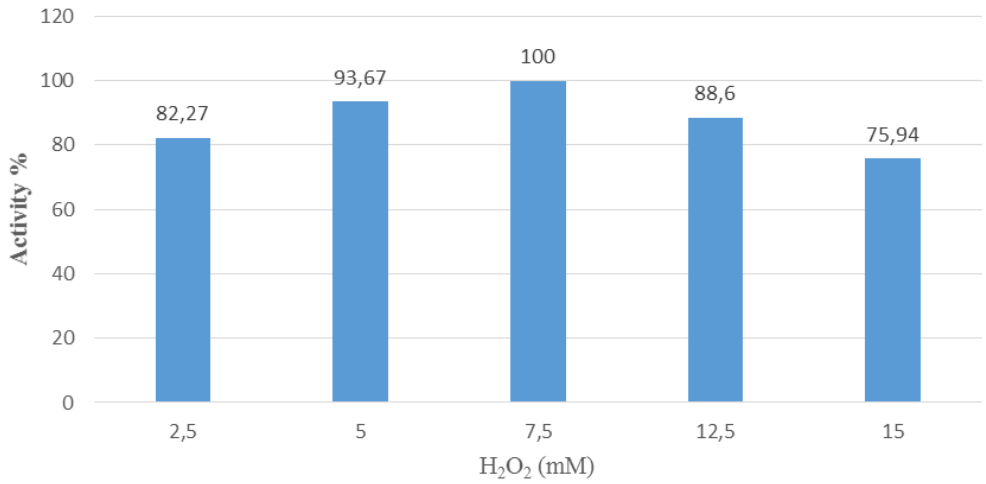

Figure 4. Carambola fruit guaiacol peroxidase enzyme activity measurements in buffer at 200 mM TRIS (pH=7.5) for optimum concentration of H_2O_2 substrate

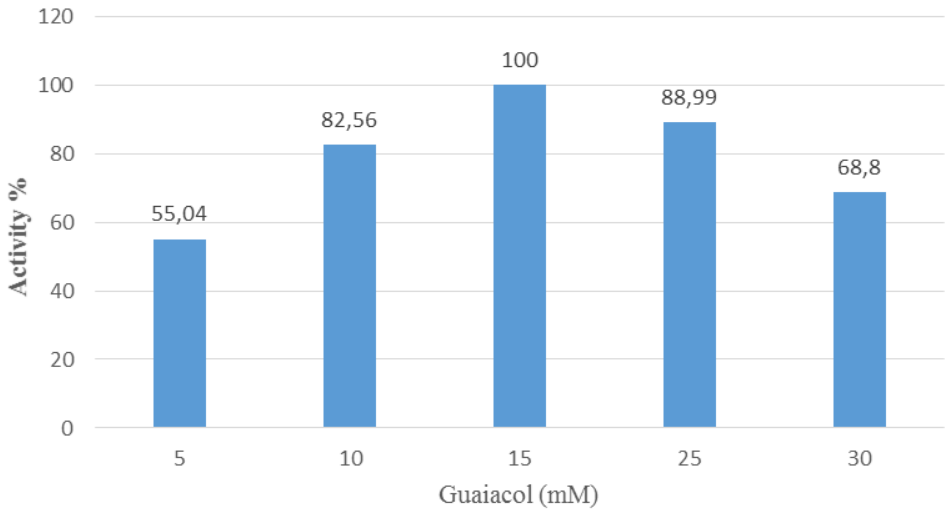

Figure 5. Carambola fruit guaiacol peroxidase enzyme activity measurements in buffer at 200 mM TRIS (pH=7.5) for optimum concentration of guaiacol substrate

Many researches on the characterisation of POD and other antioxidant enzymes have been published in the literature, with comparable results to ours. In a study by Aghelan and Shariat (2015), POD characterization was performed from leaf tissue of Rosemary (*Rosmarinus officinalis L.*) plant. Optimum pH, temperature and ionic strength values were found in 6.0, 40 °C and 0.3 M sodium phosphate buffer, respectively.

Loukili et al. (1999) tomato plant internode POD characterization study, optimum pH and temperature were found to be 5.0 and 55 °C, respectively. Bursal et al. (2013) POD enzyme characterization study of chard leaves the optimum temperature, pH and ionic strength were found to be 40 °C, 5.5 and 25 mM, respectively. In another study conducted by Koksal et al. (2012) on the characterization of POD from sweet gourd, optimum pH, optimum ionic strength and optimum temperature measurements were made and the values were found as 7.2, 50 °C, 0.4 M, respectively.

In a study by Al-Senaidy and Ismael (2011), the optimum pH and temperature of the palm leaf POD enzyme were determined and the optimum values were found to be 5.5 and 55 °C, respectively. POD enzyme partial purification and characterization study by Mafulul et al. (2018) in the *Calotropis procera* leaves optimum pH and temperature values were found to be 6.0 and 50 °C, respectively. The range of POD enzyme ammonium sulfate was found to be 40-80% in lettuce stems by Hu et al. (2012). In addition, optimum temperature and pH values were found to be 5.0 and 45 °C (Hu et al., 2012).

Erdem et al. (2015) evaluated optimal pH, ionic strength, and temperature parameters in their POD characterisation research using white cabbage (*Brassica Oleracea var. capitata f. alba*). It was observed that the values were 6.5, 0.1 M KH_2PO_4, and 30 °C, respectively. Lavery et al. (2010) determined optimal pH, ionic strength, substrate, and temperature parameters in Horseradish (*Armoracia rusticana*) roots POD characterisation investigation. The results obtained are 7.0, 50 mM KH_2PO_4, 0.5 and 0.3 mM (guaiacol and H_2O_2) and 30 °C, respectively. In a study by Maciel et al. (2007) studies on optimum pH, substrate and temperature were carried out in the study of POD enzyme characterization from *Copaifera langsdorffii* leaves. The values obtained as a result of the measurements are 6.0, 0.04 and 0.39 mM (guaiacol and H_2O_2) and 35 °C, respectively.

POD enzyme was isolated from many tissues of both plant and mammalian animals and characterization tests were performed as observed in our study and other studies. We believe that the findings of our investigation will be useful in future plant and POD enzyme purification and characterisation studies.

4. Conclusions

As a consequence, POD enzyme was isolated from carambola fruit. This is the first study to demonstrate the characterization and partial purification of the POD enzyme of carambola in the fruit part, which is an important plant with high economic and antioxidant value. These findings will help encourage the consumption of the plant and its antioxidant benefits, as well as the preference for carambola fruit properties.

Author Contributions

The percentage of the author(s) contributions is presented below. All authors reviewed and approved the final version of the manuscript.

	Ö.T	M.Y	D.E
C	40	20	40
D	40	20	40
S	20	10	70
DCP	40	20	40
DAI	40	20	40
L	30	20	50
W	30	20	50
CR	30	10	60
SR	40	20	40
PM	20	20	60
FA	40	20	40

C=Concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

Ethics committee approval was not required for this study because of there was no study on animals or humans.

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