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# Purification and Characterization of Polyphenoloxidase from Guava Infected with Fruit-rot Disease

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**Abstract:** Polyphenoloxidase (PPO), an important oxidative enzyme which played a vital role in the brewing of guava after infection with fruit-rot disease was purified from disease-affected guava. The method was accomplished by ion exchange chromatography of crude enzyme extract on DEAE-cellulose followed by CM-cellulose. The purified enzyme was found to be homogeneous as judged by polyacrylamide disc gel electrophoresis. Molecular weight of the enzyme was estimated to be 178 kDa and 180.5 kDa by gel filtration and SDS-PAGE respectively. The enzyme appeared to be a single polypeptide chain as revealed by SDS-PAGE either in presence or in absence of reducing agent. Optimum activity of the enzyme was at 48°C and pH around 8. Activity was destroyed completely in presence of EDTA, a metal chellator. Purified PPO showed an estimated $k_m$ value of 4.4 mM for catechol as substrate.

**Key words:** Polyphenoloxidase, fruit-rot disease-affected guava

## Introduction

Polyphenoloxidase catalyses the oxidation of monophenol and ortho-diphenols to insoluble polyphenols. This reaction is very important in maturation and ripening process of fruits and vegetables since it removes astringency by converting soluble phenolics into insoluble ones through oxidation and polymerization process. Mowlah and Itoo (1982) reported that PPO activity in immature and mature guava was significantly low whereas in ripen stage, the activity was quiet high. They correlated this high PPO activity with gradual disappearance of astringency in ripe guava. Several reports indicated that PPO and other oxidases also have a significant link to disease resistance in fruits and vegetables (Dong *et al*., 1990; Okey *et al*., 1997; Nema, 1991). Moore and Stone (1972) reported that the activity of these enzymes is usually increased in the cells surrounding the lesions where localization of the pathogen occurs. It was found in our laboratory that the activity of oxidase enzymes such as PPO, catalase, and peroxidase increased remarkably in the flesh of guava after infection with fruit-rot disease (Razzaque, 1997) which is quiet in harmony with the above report. This paper describes the purification and some characteristics of polyphenoloxidase from fruit-rot disease-affected guava.

## Materials and Methods

Fruit-rot disease infected guava was collected from Terokhadia, Rajshahi, Bangladesh in June-September. Sephadex G-150, DEAEcellulose, and CM-cellulose were the products of Sigma Chemical Co. USA. All other reagents were of analytical grade.

**Preparation of enzyme:** Unless mentioned otherwise, all the operations were performed at 4°C. Guava pulp (200 gm) were cut into pieces and ground into paste in a mortar with 80 ml of cold 0.1 M phosphate buffer, pH 6.0, and finally homogenized into a slurry using a tissue homogenizer. The slurry was filtered through double layer of cheese cloth and the filtrate was further clarified by centrifugation at 6,000 rpm for 15 minutes. The clear supernatant was concentrated to 1/8 of its original volume by commercial sucrose. The concentrate was then dialyzed against 5 mM phosphate buffer, pH 7.8 for 24 hours. The dialysate was then centrifuged at 7,000 rpm for 6 minutes and the clear supernatant was used as crude enzyme extract.

**DEAE-cellulose chromatography:** The crude enzyme preparation was loaded onto a DEAE-cellulose column pre-equilibrated with 5 mM phosphate buffer, pH 7.8 and the protein was eluted from the column using the same buffer with step wisely increasing concentration of NaCl. Enzyme activity and protein concentration were monitored at intervals of one fraction (3 ml).

**CM-cellulose chromatography:** The active fractions obtained from DEAE-cellulose chromatography were collected and dialyzed against 5 mM phosphate buffer, pH 6.4 for 24 hours. After centrifugation, the clear supernatant was loaded onto a CM-cellulose column pre-equilibrated with the same buffer. The separation was achieved by stepwise elution of protein from the column with increasing concentration of NaCl in the same buffer. Enzyme activity and protein concentration were monitored at intervals of one fraction (3 ml).

**Polyphenoloxidase activity:** Activity of polyphenoloxidase was measured according to the procedure as described in Methods in Physiological Plant Pathology (Mahadevan and Sridhar, 1982) using catechol as substrate. One unit of PPO activity was defined as a change in absorbance of 0.001 unit per min per gm of guava flesh.

**Determination of protein concentration:** Protein concentration was routinely determined by the absorbance at 280 nm or by the method of Lowry *et al.* (1951) using BSA as standard.

**Polyacrylamide disc gel electrophoresis:** Purity of the enzyme at every step of purification was monitored by polyacrylamide disc gel electrophoresis following the method as described by Ornstein (1964) on 7.5 percent gel at pH 8.3.

**Molecular weight determination:** Molecular weight of the purified polyphenoloxidase under non-denaturing condition was determined by gel filtration on Sephadex G-150 column (0.9 x 90 cm) as described by Andrews (1965). Trypsin (20 kDa), egg albumin (45 kDa), bovine serum albumin (67 kDa), β-galactosidase

(160 kDa), and β-amylase (200 kDa) were used as marker protein. Furthermore, molecular weight under denaturing condition as well as the sub-unit structure was determined by SDS-PAGE by the method of Weber and Osborn (1969). Before electrophoresis, the sample was treated with 0.1 percent SDS in presence or absence of 1 percent 13-mercaptoethanol and heated at 100°C for 3 minutes. Molecular weight markers used were the same as those for gel filtration. Commassie brilliant blue was used as staining reagent.

## Results

**Purification of enzyme:** As shown in Fig. 1, the proteins of crude enzyme extract were eluted from DEAE-cellulose column as one major peak (F-1) and three minor peaks (F-2, F-3, and F-4). Polyphenoloxidase activity was eluted as the major peak by the initial buffer while the other fractions were eluted by the same buffer with step wisely increasing concentrations of NaCI. The peak F-1 was saved and subjected to polyacrylamide gel electrophoresis (PAGE). As shown in Fig. 3a, the pooled fraction contained more than one protein band. Therefore, it was dialyzed against 5 mM sodium phosphate buffer, pH 6.4 for 24 hours and applied to a CM-cellulose column for further purification. As shown in Fig. 2, the active fraction was bound to the column and was eluted as F-lb with step wisely increasing concentrations of NaCI (10 mM) in the same buffer. The other fractions (F-1a and F-1c) did not contain any further polyphenaloxidase activity. The fraction F-1b was saved and its homogeneity was determined by PAGE. Fig. 3b shows that F-1b must contain pure enzyme since it gave single band on the gel.

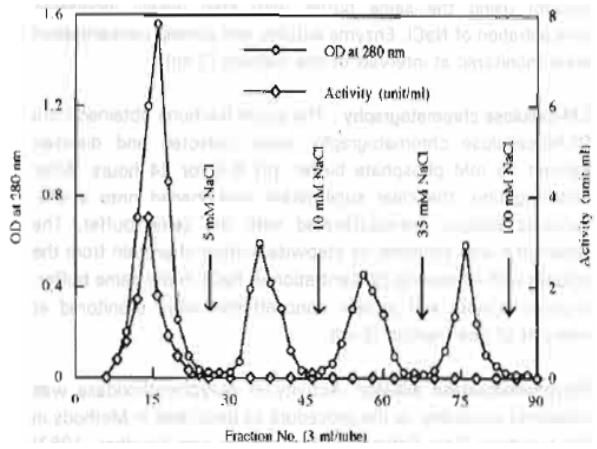


Fig. 1: Stepwise elution profile of polyphenoloxidase from fruit-disease infected guava flesh on DEAE-cellulose column. Crude enzyme extract (153 mg protein) was applied to a column (1.6 x 36 cm) pre-equilibrated with 5 mM phosphate buffer, pH 7.8 and eluted with the same buffer containing different concentration of NaCI with a flow rate of 30 ml/hour

A brief summary of purification steps of polyphenoloxidase are given in Table 1. As shown in the Table 1, the specific activity of the enzyme increased in every purification step. Although the yield was only 27 percent and over 95 percent of the extracted protein was removed during purification, the enzyme was purified up to 28 fold. The decrease in yield might be resulted from denaturation of the enzyme during the course of purification probably due to phenolic content. The yield could have been improved by inactivating phenolics during extraction by adding PVP which we could not use in our study. Mowlah and Itoo (1982) reported that addition of PVP to the extraction buffer resulted in about 20 fold increase in specific activity of the enzyme.

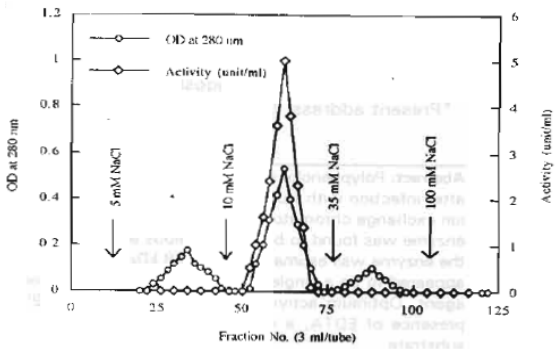


Fig. 2: Stepwise elution profile of F-1 fraction on CM-cellulose column. Protein (3.45 mg) was applied to a column (0.5 × 15 cm) pre-equilibrated with 5 mM phosphate buffer, pH 6.4 and eluted with the same buffer containing different concentration of NaCi with a flow rate of 20 ml/hour

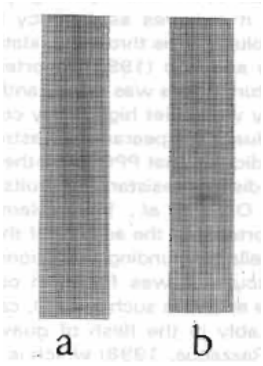


Fig.3: Polyacrylamide disc gel electrophoretic pattern of the protein on 7.5% gel
a: F-1 fraction (after DEAE cellulose chromatography)
b:F-1b fraction (Pure polyphenoloxidase)

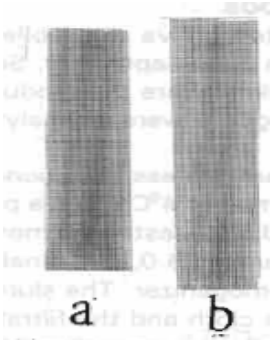


Fig. 4: SDS-polyacrylamide disc gel electrophoresis of polyphenoloxidase under reducing and non-reducing conditions on 7.5 percent gel
a : Absence of β-mercaptoethanol
b : Presence of β-mercaptoethanol

Table 1. Summary of purification of polyphenoloxidase from guava infected with fruit-rot disease

| Steps | Total protein (mg) | Total activity (units) | Specific activity (unit/mg) | Yield (%) | Purification fold |
|---|---|---|---|---|---|
| Crude extract | 172.00 | 110.0 | 0.64 | 100.0 | 1.0 |
| DEAE-cellulose | 5.75 | 60.4 | 10.50 | 54.9 | 16.4 |
| CM-cellulose | 1.64 | 29.4 | 17.90 | 26.7 | 28.0 |

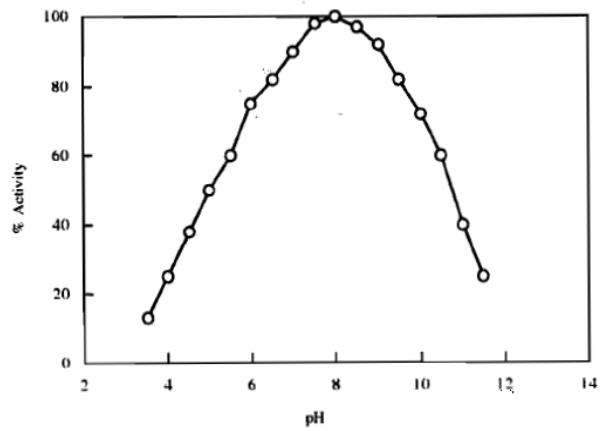


Fig. 5: Effect of pH on polyphenoloxidase activity

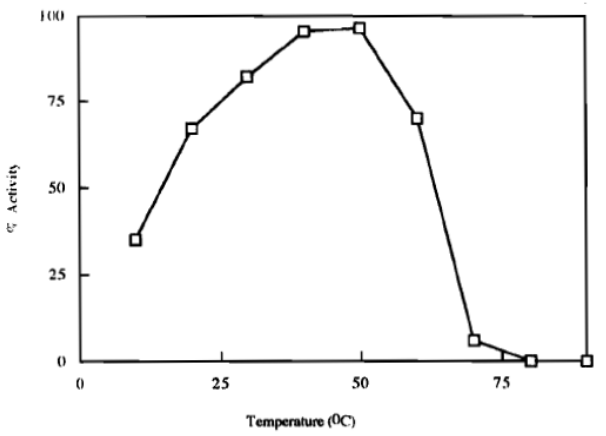


Fig. 6: Effect of temperature on polyphenoloxidase activity

**Characterization of enzyme:** The purified polyphenoloxidase completely lost its activity when incubated with 35 mM EDTA, a metal chellating agent, suggesting that a metal ion is necessary for the activity of the enzyme. Molecular weight of the enzyme determined by gel filtration and SDS-PAGE were found to be 178 and 180.5 kDa respectively. This small discrepancy in MW might be within the error range of SDS-PAGE. Furthermore, the MW obtained in denaturing and reducing conditions was the same as that found in denaturing condition only, indicating that the enzyme consisted of a single polypeptide chain (Fig. 4).

Changes in pH had great influence on the activity of polyphenoloxidase isolated from diseased guava. As it is obvious from Fig. 5, the pH-activity profile showed a characteristic bell shaped curve. The optimum pH for the enzyme activity was found to be around 8. Beyond this point, the activity decreased gradually in both side of the pH scale.

The activity of PPO was also found to be remarkably affected by the changes in temperature. As shown in Fig. 6, activity increased gradually with increase in temperature and maximum activity was observed at 48°C. Further increase in temperature resulted in a rapid decrease in activity and more than 95% of the activity was lost at around 70°C.

The $K_m$ and $V_{max}$ of the polyphenoloxidase against catechol as substrate, calculated from Lineweaver-Burk double reciprocal plot were 4.4 mM and $1.66 \times 10^{-2}$ unit $mg^{-1}$ protein of guava respectively.

## Discussion

There are more than 25 reports on the purification and characterization of polyphenoloxidase from different sources. To our knowledge, this study reports for the first time the purification and characterization of polyphenoloxidase from fruit-rot disease infected guava that might play an important role during browning of light green healthy guava. Most of the PPO have been shown to have several isoforms having MW within the range of 35-116 kDa (Owusu-Ansah, 1989; Ganesa *et al*., 1992). The molecular weight of PPO from guava infected with fruit-rot disease was very high, similar to that of latent PPO from pre-pupae of housefly, *Musca domestica* (Tsukamoto *et al.,* 1986). But we did not investigate whether this enzyme has any isoform. However, optimum pH for the activity of guava PPO showed uniqueness with those isolated from many other sources (Raymond *et al.,* 1993; Owusu-Ansah, 1989).

In the study, guava PPO was observed to lose activity very rapidly beyond the optimum temperature (48°C) and showed lability to high temperature around 70°C. This rapid decrease in activity might be due to involvement of disulfide bond in the active site or in three dimensional conformation of the enzyme. There are several other reports that described high-temperature lability of PPO from other sources to the same temperature range (Fujita and Tono, 1988; Lourenco *et al*., 1990).

The kinetic parameters of guava PPO was studied against a single substrate, but the result showed a unique similarity with other reports (Gauillard and Richard-Forget, 1997). Though we did not investigate the effect of metal ion on the activity of the enzyme, inactivation in presence of EDTA is a clear indication of metal ion dependence of the enzyme. Carrot PPO has been reported to be activated by $Ca^{2+}$ ion (Soderhall, 1995) that indirectly supports our assumption.

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