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Purification, Characterization and Effect of Physico-chemical Agents on Stability of Polyphenoloxidase from Sajna (*Moringa oleifere* L.) Leaves at Mature Stage

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Abstract: Polyphenoloxidase (PPO), a highly active oxidative enzyme in Sajna leaves at mature stage was purified by ion exchange chromatography on DEAE-cellulose followed by CM-cellulose. The purified enzyme was monomer in nature and its molecular weight was estimated to be 56,000 and 55,500 by gelfiltration and SDS-PAGE respectively. The enzyme appeared to be a single polypeptide chain as revealed by SDS-PAGE either in presence or absence of reducing agent. The enzyme has following characteristics : optimum pH 6.2, optimum temperature 32°C, Km value 0.047M against catechol as substrate and maximum absorption 279 nm. The activity of the enzyme was enhanced by Ca^{2+} and Cu^{2+} but its activity lost completely in presence of ascorbic acid, EDTA, KCN and $NaHSO_3$.

Key words: Polyphenoloxidase, Sajna leaves, SDS-PAGE

Introduction

Polyphenoloxidase (PPO) is a copper-containing enzyme which catalyzes the hydroxylation of monophenols to o-diphenols and the oxidations of o-dihydroxyphenol to o-quinones utilizing molecular oxygen. These quinones are highly reactive, electrophilic molecules which covalently modify and cross link to a variety of cellular constituents. The reaction is very important in maturation and ripening process of fruits and vegetables since it removes astringency by converting soluble phenolic compounds into insoluble ones through oxidation and polymerization process. It is present in almost all the plant species either as active or latent. Polyphenoloxidase has been purified from mushrooms (Smith and Krueger, 1962; Bouchillous *et al.*, 1963) and potato tuber (Joanna and Paul, 1996) and Bartlett pears (Tate *et al.*, 1964). It was found in our laboratory that large sized Sajna leaves at mature stage contained the highest amount of PPO activity (Khatun, 1999). The present paper describes the purification, characterization and effect of physico-chemical agents on the stability of PPO from Sajna leaves at mature stage.

Materials and Methods

Freshly harvested large sized Sajna leaves at mature stage were collected from the selected Sajna (*Moringa oleifera* L.) tree at Kazla, Rajshahi, Bangladesh. Catechol, DEAE-cellulose and CM-cellulose were obtained from Pharmacia Fine Chemicals Co. Sweden. Trypsin inhibitor, BSA, α-amylase and SDS were purchased from Sigma Chemicals Co. USA. All other reagents used were of analytical grade.

Measurement of enzyme activity : Procedure was followed as described in Methods of Physiological Plant Pathology (Mahadevan & Sridhar, 1982) using catechol as substrate.

Purification of Enzyme:

Preparation of crude enzyme extract: All the operations were performed at 4°C. Sajna leaves (155 gm) were cut into small pieces and ground into paste with cold 0.1M phosphate buffer, pH-6.0 and finally homogenized well into a uniform slurry using a tissue homogenizer. The slurry was filtered through double layer of cheese cloth and the filtrate was further clarified by centrifugation at 6000 rpm for 15 min and the clear supernatant was concentrated to about $1/8^{th}$ of its original column. The concentrate was then dialyzed against 10mM phosphate buffer, pH 7.5 overnight. The dialyzate was then centrifuged at 7000 rpm for 8 min and the clear supernatant was used as crude enzyme extract.

DEAE Cellulose Column Chromatography: The crude enzyme extract was loaded onto a DEAE-cellulose column, which was previously equilibrated with 10 mM phosphate buffer, pH 7.5, and the protein, was eluted from the column by the same buffer with step wise increasing concentrations of NaCl. Enzyme activity and protein concentration were measured at intervals of one fraction (3 ml / tube).

CM cellulose column chromatography: The enzyme active fraction, obtained from DEAE-cellulose column, was collected and dialyzed against 10mM sodium phosphate buffer; PH 6.5 for 24 hours. After centrifugation the clear supernatant was loaded onto a CM-cellulose column. The separation was achieved by stepwise elution of protein from the column with increasing concentrations of NaCl in the same buffer. Enzyme activity and protein concentration were monitored at intervals of one fraction (3 ml/tube).

Estimation of protein concentration: Protein concentration was routinely analyzed by the absorbance at 280nm and by the method of Lowry *et al.* (1951).

Polyacrylamide disc gel electrophoresis: Purity of the enzyme was checked by polyacrylamide disc gel electrophoresis following the method of Ornstein (1964).

Molecular weight determination: Molecular weight of PPO under non-denaturing conditions was determined by gel filtration on Sephadex G-150 column (0.9 × 90 cm^2) 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 proteins. Furthermore, the molecular weight under denaturing condition as well as the sub-unit structure was determined by SDS-PAGE (Weber and Osborn, 1969).

Ultraviolet absorption spectrum: It was recorded in aqueous solution with Shimadzu UV-180 double beam spectrophotometer at room temperature.

Determination of pH-optimum: The activity of PPO was determined at different pH-values ranging from pH-3.0 to 9.5 (pH : 3.0 – 4.5, Na-acetate buffer; pH 5.0 – 7.0, phosphate buffer, pH 7.5 – 9.5, and tris HCl buffer) at 32 °C following the procedure as described earlier (Mahadevan & Sridhar, 1982).

Determination of temperature optimum: The activity of PPO was measured at different temperature, ranging from 5 to

70°C, using 50 mM phosphate buffer pH 6.2. The enzyme solution was incubated for 20 min at respective temperature and after cooling the activity was measured following the procedure of Mahadevan & Sridhar (1982).

Determination of activity of PPO in presence of different chemicals and metallic salts: To the PPO solution (O.D : 0.25–0.30 at 280 nm) (0.5 ml), were added chemicals and metallic salts of different concentrations and incubated for 10 min at 20 °C and enzymatic activity was measured following the procedure as described by Mahadevan & Sridhar (1982).

Results

Purification of enzyme: As shown in Fig. 1 the proteins of the crude enzyme extract were eluted from the DEAE column in two major peaks, F-1 and F-4, and four other minor peaks, (F-2, F-3, F-5 and F-6). The major peak, F-1 was eluted from the column by the buffer only while the five other fractions were eluted from the column stepwise with increasing concentration of NaCl. Of these fractions, it was found that only the major fraction F-4 contained the PPO activity. The fraction F-4 as indicated by solid bar was collected and its purity was checked. It was found to contain more than one band on the gel (Fig. 3).

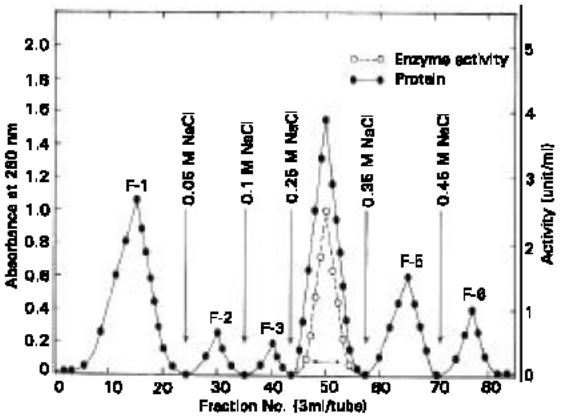

Fig. 1: Stepwise elution profile of PPO from Sajna leaves on DEAE – cellulose column. Crude enzyme extract (155 mg protein) was applied to a column (1.5 × 28 cm^2) pre-equilibrated with 10mM phosphate buffer, pH 7.5 and the protein were eluted from the column step wisely with the same buffer containing different concentration of NaCl with a flow rate of 25 ml / hour.

So for further purification, this fraction was dialyzed against 10 mM sodium phosphate buffer, pH 6.5 for 24 hours and applied to a CM – cellulose column. As shown in Fig-2, the components of F-4 fraction were separated into one major peak (F-4a) and two other minor peaks, (F-4b and F-4c). Of these fractions, the major fraction, F-4a, which was eluted by the buffer only, while the other fractions, F-4b and F-4c were eluted by the buffer containing 0.05 M and 0.1M NaCl respectively. Of these three fractions only F-4a contained the PPO activity. The fraction, F-4a, as indicated by solid bar was pooled and its purity was checked. From Fig. 3 it might be concluded that the fraction F-4a contained pure Polyphenoloxidase since it gave single band on the gel.

The extent of purification, recovery, and yield of enzyme at each step are summarized in Table 1.

The specific activity of the enzyme increased in each of the purification step. Although the yield was only about 25% and over 95% of the extracted protein was removed during

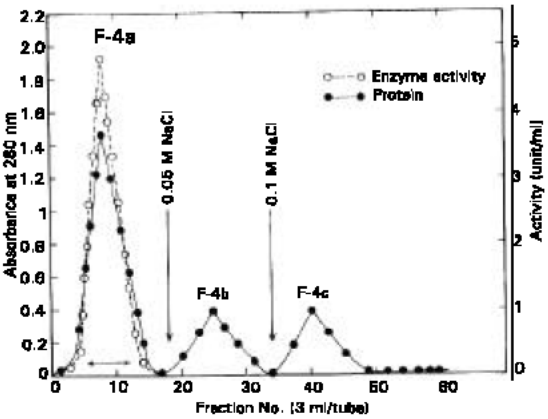

Fig. 2: Stepwise elution profile of F-4 fraction on CM-cellulose column. Protein (4.25 mg) was applied to a column (0.5 × 15 cm^2) pre-equilibrated with 10 mM phosphate buffer, pH 6.5 and eluted with the same buffer containing different concentration of NaCl with a flow rate of 20 ml / hour.

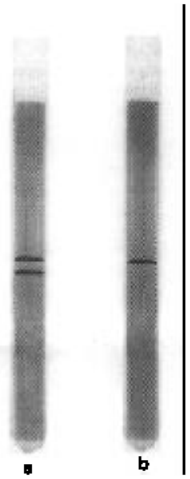

Fig. 3: Polyacrylamide disc gel electrophoretic pattern of the protein at room temp. pH 8.3 on 7.5% polyacrylamide gel.
a : F-4 fraction (after DEAE-cellulose chromatography)
b : F-4a fraction (pure Polyphenoloxidase).

purification steps but the enzyme was purified with an increase in purification fold of about 28.

Characterization of enzyme: The MW of PPO was calculated to be about 56,000 under de-naturing conditions. On the other hand, the MW of PPO by SDS-PAGE under denaturing condition was found to be 55,500 (Fig. 4). 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 non-denaturing condition indicating that the enzyme consisted of a single polypeptide chain.

The purified PPO in aqueous solution gave absorption maximum at 279 nm and minimum around 238 nm (Fig. 5).

As shown in Fig. 6 the activity of PPO was greatly affected with changes in pH and the pH-activity profile showed a characteristic bell-shaped curve. It was found from the curve that the enzyme gave about 80% activity at pH 5.0 and at pH

Table 1: Summary of purification of PPO enzyme

Steps	Total protein (mg)	Total activity units/ml	Specific activity (unit/mg)	Activity yield (%)	Purification fold
Crude extract	155	109	0.703	100	1
DEAE-cellulose ion exchange chromatography	4.25	56.5	13.29	51.83	18.90
CM- cellulose ion exchange chromatography	1.4	27.2	19.4	24.95	27.59

Table 2: Effect of Ascorbic acid, KCN, EDTA & Sodium bisulfite on the activities of PPO.

Concentration of Ascorbic acid (M)	0	0.002	0.005	0.01	0.05	0.1	0.2	0.3	0.4
Relative activities (%) of PPO	100	92.2	80.3	49.22	28.10	10.0	5.0	1.3	0
Concentration of KCN (M)	0	0.001	0.002	0.005	0.01				
Relative activities (%) of PPO	100	85.5	40.0	16.12	0				
Concentration of EDTA(M)	0	0.0005	0.001	0.002	0.003	0.004			
Relative activities (%) of PPO	100	91	70.1	42.2	17.4	0			
Concentration of $NaHSO_3$	0	0.001	0.002	0.004	0.006	0.007	0.008	0.01	
Relative activities (%) of PPO	100	92.5	82.2	69.1	55.5	35.9	8.5	0	

Table 3: Effect of metallic salts on PPO activity.

Test salts	Concentration (mM)	PPO activity (%)
None	-	100
	0.001	118
	0.002	107
$CuCl_2$	0.001	125
	0.002	117
$MgCl_2$	0.001	55
	0.002	35
$HgCl_2$	0.001	85
	0.002	80
$FeCl_2$	0.001	80
	0.002	74

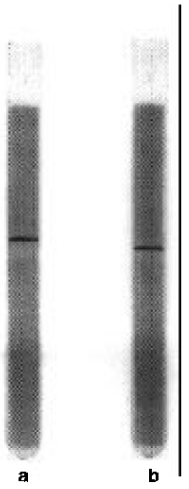

Fig. 4: SDS-Polyacrylamide disc gel electrophoresis of PPO under reducing and non-reducing conditions on 10% gel.
a : Absence of β-mercaptoethanol
b : Presence of β-mercaptoethanol

7.5 with an optimum pH value of 6.2. Beyond these pH ranges, both at the acidic as well as alkaline sides the activity of the enzyme decreased sharply and the enzyme lost more than 80% of its activity at pH 3.0 and at pH 9.5.

The activity of PPO was also found to be remarkably affected by the changes in temperature. As shown in Fig. 7, the activity of PPO increased gradually with rise in temperature and the enzyme gave maximum activity at 32 °C. With further rise in temperature the activity decreased rapidly and more than 95% of its activity was lost at 68 °C.

The Km value of the PPO against catechol as substrate,

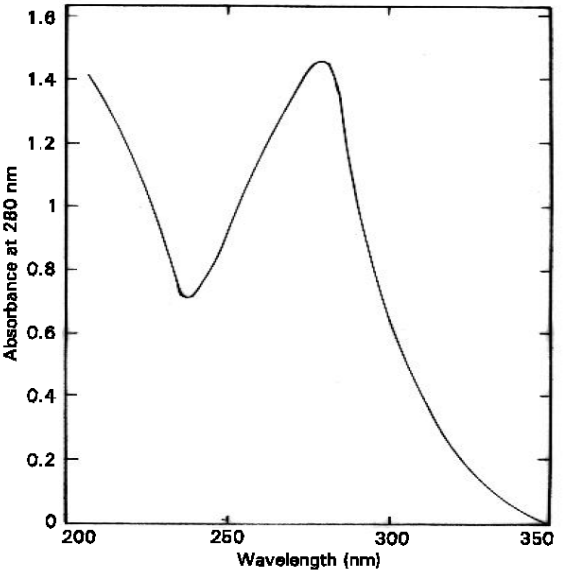

Fig. 5: Ultraviolet absorption spectrum of the purified PPO enzyme.

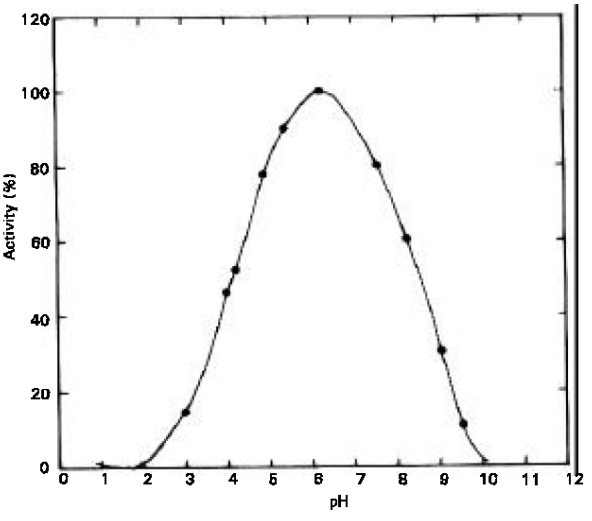

Fig. 6: Effect of pH on Sajna leaves PPO activity.

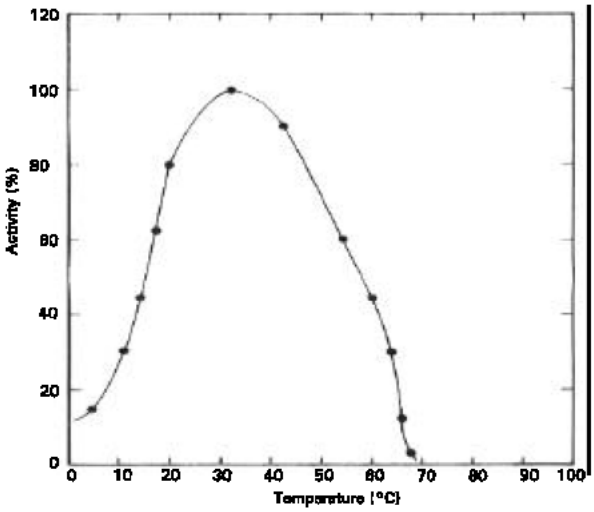

Fig. 7: Effect of temperature on PPO activity.

calculated from Linewveaver – Burk double reciprocal plot was 0.047M.

Effect of chemicals and metallic salts on the activity of PPO: As shown in Table-2 the activity of PPO decreased gradually with higher concentration of chemicals and the enzyme lost its activity completely at 0.004M, 0.4M, 0.01M and 0.01M EDTA, ascorbic acid, KCN and $NaHSO_3$ respectively. Further, metallic salts such as Cu^{2+} and Ca^{2+} increased the activity significantly, while Mg^{2+} salt decreased the activity remarkably. On the other hand Hg^{2+} and Fe^{2+} salt have moderate inhibitory effect on activity of PPO.

Discussion

Sajna leaves are the cheapest vegetable, available almost throughout the year in our country. Pressed juice of the leaves of this plant show strong antibacterial activity and in our laboratory PPO was found to be very much active in the leaf juice of mature stage. Many reports were published on the purification and characterization of PPO from different sources. Some reports have shown PPO as isomeric form having MW within the range of 35 – 116 kDa (Owusu – Ansah, 1989; Ganesa *et al.*, 1992) but we purified homogeneous form of PPO from Sajna leaves with MW of about 56,000, which was close to the MW of PPO isolated from potato tuber (Joanna and Paul, 1996). The yield of purified PPO from Sajna leaves was found to be only about 25%, which might be due to denaturation of protein during lengthy purification periods. Further, in our laboratory it was found also that Sajna leaves contained significant amount of phenolic compounds. It was reported that the yield of PPO from guava was improved about 20 fold by adding PVP to the extracting buffer (Mowlah and Itoo, 1982), but we could not use PVP in this study.

The activity of PPO was found to be decreased significantly at the acidic as well as alkaline pH-regions indicating the ionization of groups located in or near active sites at these pH-regions. The optimum pH of purified PPO showed uniqueness with that isolated from bartlett pears (Tate *et al.*, 1964).

The PPO, was found to be very labile to temperature as compared to that from other sources and gave maximum activity at 32°C. The rapid decrease in activity above high temperature might be due to changes in secondary and tertiary structure or destruction of active site of enzyme at higher temperature. The kinetic parameter, km of Sajna leaf PPO, obtained in present study showed a unique similarity with that of PPO from bartlett pears (Tate *et al.*, 1964).

The purified PPO lost its activity in the presence of 4 mM EDTA, suggesting the necessity of metal ions for the activity of enzyme which was also confirmed from the findings that the activity of PPO increased in the presence of metallic salts of Cu^{2+} and Ca^{2+}. The activity of PPO from carrot was also reported to be activated by Ca^{2+} ion (Soderhall, 1995). The activity of Sajna leaves PPO was abolished completely in presence of ascorbic acid, KCN, and $NaHSO_3$, suggesting that these chemicals may directly act on the active site of enzyme. Golan-Goldhirsh *et al.* (1992) suggested that ascorbic acid and copper ions catalyze a free radical reaction that oxidizes the imidazole group of the histidine residues that ligand the active site coppers, thereby inactivating the enzyme.

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