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**Extraction, purification and characterization of polyphenoloxidase from peel and pulp of tomato**

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**KEYWORDS**

Polyphenoloxidase;  
enzyme activity;  
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**A B S T R A C T**

Crude Polyphenoloxidase (PPO) from tomatoes peel and pulp was extracted by using potassium phosphate buffer and partially purified through Ammonium Sulphate precipitation and dialysis. The enzyme activity of PPO was analyzed in each step by spectrophotometric assay method using Pyrogallol substrate. Protein estimation was carried out by Biuret method. The specific activity and the enzyme yield for the peel was 321.42U/mL and for the pulp was 458.33U/mL. The optimum pH for PPO activity was found to be 6.5. Biuret method has been used for the protein estimation. The graph has been plotted to see the protein estimation more clearly.

**Introduction**

Enzymes are biological molecules responsible for thousands of metabolic processes that sustain life. They are highly selective catalysts, greatly accelerating both the rate and specificity of metabolic reactions.

Polyphenoloxidase are enzymes found abundantly in fruits and vegetables. Polyphenoloxidase catalyses the o-hydroxylation of monophenols to o-diphenols. They can further catalyze the oxidation of o-diphenols to produce o-

quinones. It is the rapid polymerization of o-quinones to produce black, brown or red pigments, that is the cause of fruit browning. Hence, many vegetables and fruits become discolored during storage. These highly reactive quinines polymerize with other quinines, amino acids and proteins to produce colored compounds, and nutrient quality and attractiveness is reduced. PPO is synthesized early in tissue developments and is stored in chloroplasts. It is often undesirable and responsible for unpleasant sensory qualities as well as

losses in nutrient quality. This is the main reason why phenolic content and PPO activity have been considered determinants of quality in fruits and vegetables (Lee and Whitaker 1995). Leaves and fruits of many plant species have been the major target tissues to investigate the occurrence of PPO because of its key role in the food quality of fresh and processed plant commodities (Vámos-Vigyázó 1981; Mayer 1987; Lee and Whitaker 1995). The levels of PPO are linked to species, cultivar, maturity and age (Amiot *et al.* 1995). In intact tissues, PPO and its polyphenolic substrates reside in separate compartments, plastids and vacuoles, respectively (Mayer 1987), thus no reaction occurs. The disorganization of the cell integrity that occurs naturally during senescence and as a result of physical damage of plant tissues bring PPO into contact with its substrates producing the so-called enzymatic browning observed in overripe fruits, bruised tissues, freshly cut fruits and vegetables and also in tissues affected by diseases and physiopathies. Tomato fruits are not prone to undergo enzymatic browning because of senescence or physical damage; instead these changes are linked to bacterial or viral infections and disorders such as blossom-end rot (BER).

The partial purification and characterization of PPO was done to determine the involvement of the enzyme in the above processes. Although the enzyme has been isolated from many sources and thus characterized, to the best of our knowledge, there is no report concerning such goals on tomato fruit PPO. In this sense, Czapski and Saniewski (1988) determined PPO activity in crude extracts and studied the influence of methyl jasmonate on PPO levels, and Hobson (1967) measured the phenolase activity of acetone powder preparations, studied its occurrence during

growth and ripening and determined its levels in BER-affected tomato fruits. In contrast to the limited knowledge of PPO enzyme in tomato fruits, the knowledge is large on tomato PPO genes and their spatial and temporal expression. PPOs are encoded by a seven-member (PPO A, A $\epsilon$ , B, C, D, E and F) multigene family (Newman *et al.* 1993) whose translations possess similar plastidic transit peptides to direct the preprotein to the thylakoid lumen (Sommer *et al.* 1994). Some of them possess hydrophobic, potentially membrane-spanning C-terminal domains (Newman *et al.* 1993; Sommer *et al.* 1994; Thipyapong *et al.* 1997). Immunogold localization and *in situ* hybridization have shown the presence of PPO protein and mRNA in several tissues of young (up to 7 days postanthesis) fruits and especially in specific solitary parenchyma cells called idioblasts that accumulate very high levels of PPO (Murata *et al.* 1997). The lack of knowledge on the expressed protein in fruits is largely caused by the absence of procedures for suitable extraction of PPO from this tissue, which contrasts with preparative and enzymatic studies of PPO from tomato leaves and trichomes (Yu *et al.* 1992). Extraction, purification and characterization of polyphenoloxidase from peel and pulp of tomato. This work is an attempt to cover the gap of knowledge on the PPOs present in tomato fruits, their characteristics, as a first step in our research program on PPO in tomato fruits. To that end, we have partially purified the enzyme from young tomato fruits, set up a new protocol and hence have carried out extraction, purification and characterization of the enzyme.

PPO from different plant tissues shows substrate specificities and degrees of inhibition. Hence, characterization of peroxidase will suggest us more methods to

control the browning of the plants and plant products. Our purpose was to characterize PPO from tomato pulp and pericarp. PPO can be released from latency or activated by various treatments or agents, including urea (3), polyamines (4), anionic detergents such as SDS (5), proteases (6) and fatty acids (7). Control of the enzymatic PPO activity in fruits and vegetables has become a separate research area because of its relevance in the food industry (Almeida and Nogueira 1995). A wide range of reagents and chemicals such as acidulants, halides, copper-chelating compounds, reducing agents and antioxidants have the capacity to prevent enzymatic browning in fruits and other plant commodities (Vámos-Vigyázó 1981), but their well-known function as PPO inhibitors is, in many cases, not matched with industrial manufacturing or food industry because some of them are toxic or harmful (Taylor and Bush 1986). Four families of inhibitory compounds, described as plant-PPO inhibitors, have been assayed to monitor tomato fruit PPO inhibition. These are reducing agents (ascorbic acid, sodium pyrosulfite and thiolic compounds), structural analogs (tropolone), halide salts (NaCl, NaF) and the copper chelator sodium diethyl dithiocarbamate (DEDTC). Table shows the effect of different inhibitors on both PPO fractions using TBC as substrate tested at three different concentrations. Reducing agents, structural analogs and copper chelators were strong inhibitors even at low concentrations, while halide salts inhibited moderately. The particulate PPO was more sensitive to the natural reducing thiol group in glutathione, while the soluble was more sensitive to the synthetic reducing thiol dithiotreitol. The ability of sulfhydryl groups to inhibit PPO is well known (Sanada *et al.* 1972; Golan-Goldhirsh and Whitaker 1984; Sayavedra-Soto and Montgomery 1986; Dudley and Hotchkiss

1989; Richard-Forget *et al.* 1992; Friedman 1994; Negishi and Ozawa 2000), but the inhibitory effect strongly depends on the structure of the sulfhydryl-bearing molecule and the position of the sulfhydryl group in the molecule (Friedman 1994). Both fractions were similarly sensitive to the strong reducing agents sodium pyrosulfite and ascorbic acid that act as antioxidants. These reduce the quinonoid products to their initial phenolic form, thus avoiding secondary reactions to form brown polymers and causing apparently a strong inhibition (Harper *et al.* 1969; Hus *et al.* 1988). Pyrosulfite has been suggested to follow a mixed inhibition mechanism acting both as an antioxidant and as a true inhibitor forming enzyme-inhibitor complexes (Valero *et al.* 1992). Sodium fluoride clearly inhibits more strongly than sodium chloride, with the particulate PPO being more sensitive than the 396 J. CASADO-VELA, S. SELLÉS and R. BRU soluble to both anions. Eventually, the particulate PPO activity was more sensitive than the soluble to the copper-chelator DEDTC, which could suggest that binding to the catalytic site could be stronger for the soluble PPO. The structural analog tropolone showed a stronger inhibition effect on the soluble PPO compared to that in the particulate. From these experiments, it can be appreciated that soluble and particulate PPO fractions have quantitatively different sensitivities to the inhibitors that interact with the enzyme to form enzyme-inhibitor complexes. Thus, this inhibitor study brings about a new evidence of the presence of different PPO isoenzymes in tomato fruits.

Partial purification of enzymes is a series of processes intended to isolate a single type of enzyme or protein from a complex mixture. Enzyme purification is vital for the characterization of the function,

structure and interactions of the enzyme of the interest. The various steps in purification processes may free the protein or enzyme from a matrix that confines it, separate the protein and non-protein parts of the mixture, and finally separates the desired proteins from all the other proteins.

Dialysis is the process of separating molecules in solution by the difference in their rates of diffusion through a semi-permeable membrane, such as dialysis tubing. Diffusion is random, thermal movement of molecules in solution that leads to the net movement of molecules from an area of higher concentration to the area of lower concentration until equilibrium is reached. Due to the pore size of the membrane, large molecules in the sample cannot pass through the membrane, thereby restricting their diffusion from the sample chamber. By contrast, small molecules will freely diffuse across the membrane and obtain equilibrium across the entire solution volume, thereby changing the overall concentration of these molecules in the sample and dialysate (buffer solution). Once equilibrium is reached, the final concentration of molecules is dependent on the volumes of the solutions involved, and if the equilibrated dialysate is replaced (or exchanged) with fresh dialysate, diffusion will further reduce the concentration of the small molecules in the sample. Dialysis can be used to either introduce or remove small molecules from a sample, because small molecules move freely across the membrane in both directions. This makes dialysis a useful technique for a variety of applications.

## **Materials and Methods**

### **Materials**

Samples of fresh tomato pulp and pericarp were taken for the investigation. 0.1M

100mL pH 6.5 Sodium Phosphate buffer(0.78g Sodium Dihydrogen orthophosphate dehydrate LR [NaH<sub>2</sub>PO<sub>4</sub>.2H<sub>2</sub>O] + 0.89g Disodium Hydrogen Phosphate 2-Hydrate GR [Na<sub>2</sub>HPO<sub>4</sub>.2H<sub>2</sub>O]), distilled water, pyrogallol, colorimeter, biurette reagent, 45% ammonium sulphate. Sodium Phosphate Buffer is prepared by adding the aforementioned salts to 100mL water. Magnetic stirrer is needed for the process of dialysis.

### **Enzyme extraction**

Fully grown green or ripe tomatoes were picked. 20g of fresh tomato pulp and peel were homogenized separately in the blender in the presence of 0.1M 25mL Sodium Phosphate buffer(pH6.5) each. The homogenate was filtered through a filter paper. The filtrates (29mL) were combined and centrifuged at 30000g for 20 minute at 4C. The supernatant, to be referred to as crude soluble PPO fraction, was stored to further use.

### **Ammonium Sulphate Precipitation**

Ammonium Sulphate precipitation was used as a purification step for the crude soluble PPO. 20mL of the supernatants of both the extracts were taken in a beaker and kept over ice bags to maintain the temperature and 45% Ammonium Sulphate is added to both pinch by pinch. The resulting precipitate was removed by precipitation. Rest 9mL of supernatant of both are kept in the refrigerator for further use (reagent should be kept under cool conditions to avoid the growth of bacteria).

### **Dialysis**

5mL of the crude extract containing Ammonium Sulphate of both pulp and peel

were kept in the dialysis bags separately using a plastic pipette. The bag was sealed with the help of the clip from one end. Remove excess air from the bag and leave at least 25% extra space for volume expansion. Clip the other end of the bag. Commence dialysis against water for at least 3 hours. Ensure that the dialysate is gently stirring throughout the procedure. Maximum dialysis time depends on sample stability and should not exceed 48 hours. Upon completion of dialysis, dry the outside of the bag using tissue and remove the clips and take the sample out using a plastic pipette. Measure sample volume post dialysis.

### **Protein determination**

Protein contents of the enzyme extracts were determined according to Biuret method using biuret reagent. The sample(0.5mL) of crude and ammonified peel and pulp was taken in test tubes separately. To that, 1.5mL of distilled water and 3mL of biuret reagent were added. The samples were incubated for 10 minutes at room temperature and OD values were observed at 520nm. The same process was repeated with the dialysed sample of the peel and pulp at 520nm.

### **Assay of polyphenoloxidase activity**

PPO activity was determined by measuring the absorbance at 420nm using a colorimeter. To determine the best concentration of enzyme preparation corresponding to the highest enzyme activity, the activity was assayed in 3mL of reaction mixture consisting of 2.5mL of substrate(0.02M pyrogallol + distilled water) and different concentrations(0.1-0.3 mL) of the enzyme preparation(1mg/mL). This mixture was topped up to 3.0mL with the phosphate buffer(pH 6.5) in a 1cm light

path quartz cuvette. The blank consisted of 3.0mL 0.1M phosphate buffer(pH 6.5). A control was prepared in which the cuvette contained 2.5mL substrate(pyrogallol solution) and 0.5mL buffer solution. Absorbance value of the blank is subtracted from that of the sample. PPO activity was calculated from the linear portion of the curve. The initial rate of PPO catalysed oxidation reaction was calculated from the slope of absorbance-time curve. One unit of PPO activity was defined as the amount of enzyme that produces 1mM of quinone per minute. PPO activity was done for the extract of pulp and peroxidase, containing ammonium sulphate and the ones without ammonium sulphate, separately. Assays were carried out at RT and results are the averages of at least three assays and the mean and standard deviations were plotted.

### **Extraction of PPO**

#### **From Peel**

The crude polyphenoloxidase was extracted by crushing the peel using 0.1M 25mL Sodium Phosphate buffer having pH 6.5.

#### **From Pulp**

The crude polyphenoloxidase was extracted by crushing the pulp using 0.1M 25mL Sodium Phosphate buffer having pH 6.5.

### **Partial purification and enzymatic activity**

PPO was purified from tomato pulp and peel using Ammonium Sulphate precipitation and dialysis. Firstly Ammonium Sulphate was added to the crude extract of pulp and peel and then stirred in the magnetic stirrer for dialysis. And then enzymatic activity was performed

in which absorbance was taken from the dialysed extract in which pyrogallol and sodium Phostphate buffer with pH 6.5 was added. A summary of the purification and assay is given in the following table:

Units of peroxidase activity were calculated according to the empirical formula:

Units/mL= OD\* dilution factor \* 1000÷ mL of enzyme used in assay.

### Protein Estimation

Protein estimation was done using biurett reagent from tomato peel and pulp. Protein estimation is being summarized below and the plot is being made:

SAMPLES	PROTEIN ESTIMATION
CRUDE PEEL	8.6
CRUDE PULP	7.8
AMMONIFIED PEEL	7.4
AMMONIFIED PULP	6.6
DIALYZED AMMONIUM PEEL	5.6
DIALYZED AMMONIUM PULP	4.8

### Calculations

#### Protein Estimation

Crude Peel:-0.5- 0.22 - 4300 – 8.6  
 Crude Pulp:-0.5 - 0.20 - 3900 - 7.8  
 Ammonia Peel:-0.5 - 0.19 - 3700 – 7.4  
 Ammonia Pulp:-0.5 - 0.17 -3300 – 6.6

#### Enzyme Activity

Crude Peel:-0.5 – 0.4-800  
 Crude Pulp:-0.5 -0.5-1000  
 Ammonia Peel:-0.5 –0.6-1200

Ammonia Pulp:-0.5 -0.8-1600

### Specific Activity

Crude Peel:-units/mg – 800/8.6 =93.2  
 Crude Pulp:-units/mg – 1000/7.8=128.2  
 Ammonia Peel:-units/mg – 1200/7.4 =162.16  
 Ammonia Pulp:-units/mg – 1600/6.6 =242.42

### Dialysis

#### Protein Estimation

Dialysied Ammonia Peel:-0.5 – 0.15- 2800 – 5.6  
 Dialysied Ammonia Pulp:-0.5 -0.13 - 2400 – 4.8

#### Enzyme Activity

Dialysied Ammonia Peel:-0.5 –0.9-1800  
 Dialysied Ammonia Pulp:-0.5 – 1.1 - 2200

#### Specific Activity

Dialysied Ammonia Peel:-units/mg - 1800/5.6 =321.42  
 Dialysied Ammonia Pulp:-units/mg - 2200/4.8 =458.33

### Conclusion

PPO is an enzyme that is responsible for the browning of the fruits and vegetables. It is present in nearly all plants. In this journal, we have tried to extract the enzyme polyphenoloxidase from the pulp and peel of tomato and also to know the amount of the enzyme present in both. The amount of the PPO was determined by taking down the absorbance by protein estimation using the biurett reagent with the help of the colorimeter. PPO was extracted from the pulp with the help of sodium phosphate

**Table.1** Crude polyphenoloxidase

S.no.	Sample	Vol. of sample	Vol.of distilled water	Biurett reagent	Protein estimation	Enzyme activity
1.	crude peel	0.5ml	1.5ml	3ml	0.22	0.4
2.	crude pulp	0.5ml	1.5ml	3ml	0.20	0.5
3.	ammonia peel	0.5ml	1.5ml	3ml	0.19	0.6
4.	ammonia pulp	0.5ml	1.5ml	3ml	0.17	0.8
5.	dialyzed ammonium peel	0.5ml	1.5ml	3ml	0.15	0.9
6.	dialyzed ammonium pulp	0.5ml	1.5ml	3ml	0.13	0.4

**Table.2** Partial purification and enzymatic activity

Samples	Enzyme activity	Specific enzyme activity
crude peel	800	93.02
crude pulp	1000	128.2
ammonified peel	1200	162.16
ammonified pulp	1600	242.42
dialyzed ammonium peel	1800	321.42
dialyzed ammonium pulp	2200	458.33

**Table.3** Protein Estimation

Samples	Protein estimation
crude peel	8.6
crude pulp	7.8
ammonified peel	7.4
ammonified pulp	6.6
dialyzed ammonium peel	5.6
dialyzed ammonium pulp	4.8

buffer as it gets precipitated and then separated out completely with the help of centrifugation method. It was partially purified by ammonium sulphate precipitation and dialysis (a process to isolate a protein from a complex mixture). With these two processes, we got the PPO extracted and purified. And also we got to know the amount of PPO present in the pulp and peel of tomato i.e., 4.8µg/ml & 5.6µg/ml respectively. The specific activity of the pulp and peel is 458.33U/ml & 321U/ml respectively.

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