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RESEARCH ARTICLE

EXTRACTION PARTIAL PURIFICATION AND CHARACTERIZATION OF POLYPHENOL OXIDASE FROM TOMATO

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Abstract

Tomato is one of the most important horticulture crops in India; it is a good sourse of polyphenol oxidase. Polyphenol oxidases play an important role in the resistance of plants to microbial and viral infections and to adverse climatic conditions. Crude polyphenol oxidase was extracted with sodium phosphate buffer from two different varaities of tomatoes and partially purified through ammonium sulphate precipitation and dialysis. The enzyme activity of PPO was determined by spectrophometric assay method using pyrogallol substrate. The specific activity of desi tomato of crude sample was 1.8 U/mg and dialyzed sample was 34 U/mg whereas hybrid tomato (Hybrid 6, 4-4) had the specific activity of 0.81 U/mg in crude sample and 20.09 U/mg in dialyzed sample. Protein concentration of polyphenol oxidase was higher at pH 7 i.e 2.85 µ/mg. The enzyme showed high activity over a broad pH range of 4 - 8. The optimal temperature for enzyme activity was 50-60 °C, respectively.

Key words: PPO, sodium phosphate buffer, specific activity, protein concentration, optimum pH.

INTRODUCTION

Enzymes are macromolecular biological catalysts. They are responsible for thousands of metabolic processes that sustain life. Enzymes are highly selective catalysts accelerating both the rate and specificity of metabolic chemical reactions, from the digestion of food to the synthesis of DNA. Poyphenol oxidase is a class of enzymes, first discovered in mushrooms and are widely distributed in nature. They appear to reside in the plastids and chloroplasts of plant, although freely existing in the cytoplasm of ripening plants. Polyphenol oxidases play an important role in the resistance of plants to microbial and viral infections and to adverse climatic conditions. Polyphenol oxidase also present in animals and increases disease resistance in insects and crustaceans.

MATERIAL AND METHOD

Material

Instruments Used

The following instruments have been used for carrying out the experiment:
Weighing balance, Cooling centrifuge, Spectrophotometer, Magnetic stirrer, Water bath, pH meter, Shaker, Test-tube, Pipette, Beaker, Measuring cylinder, Centrifuge tube.

Chemicals

Chemicals used in the study were samples of fresh tomato pulp were taken from the local market of Allahabad for the investigation. 0.1M 100mL pH 6.5 Sodium Phosphate buffer(0.78g Sodium Dihydrogen orthophosphate dehydrate LR [NaH2PO4.2H2O] + 0.89g Disodium Hydrogen Phosphate 2-Hydrate GR [Na2HPO4.2H2O]), distilled water, pyrogallol, colorimeter.

Methods

Enzyme Extraction

Two varities of tomato were used, one was desi tomato and another one was a hybrid species namely: Hybrid6,4-4. 25g of fresh tomatoes were homogenized in the blender with 0.1M 30 mL Sodium Phosphate buffer (pH 6.5) each. The homogenate was filtered through a filter paper. The filtrates were combined and centrifuged at 10000 RPM for 20 minute at 4^0C. The supernatant, referred as crude soluble PPO fraction, was stored for further use.

Partial purification of Polyphenol Oxidase

The crude enzyme was subjected to ammonium sulphate precipitation for partial purification of enzyme. Ammonium sulphate precipitation was carried out by the step wise addition of salt. The addition of salt removes the layer of water molecules surrounding hydrophobic groups on the protein surface, which allows the hydrophobic groups to cause protein aggregation and its precipitation.

Procedure

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 on ice bags to maintain the temperature and 45% Ammonium Sulphate were added to both pinch by pinch and kept for whole night at 4^0C and then centrifuged at 10000 RPM for 20 minutes. The resulting precipitate was removed by precipitation. Rest of supernatant of both were kept in the refrigerator for further use (reagent should be kept under cool conditions to avoid the growth of bacteria) **(Shanti *et al*., 2014).**

Dialysis

5mLof the crude extract containing Ammonium Sulphate of both the species was kept in dialysis bags separately using a plastic pipette. The bag was sealed with the help of the clip from one end. Excess air was removed from the bag and at least 25% of extra space was left for volume expansion. The other end of the bag was cliped. The dialysis was done against buffer for at least 3-6 hours. It was ensure that the dialysate was gently stirred throughout the procedure. Maximum dialysis time depends on sample stability and should not exceed 48 hours. Upon completion of dialysis, dried the outside of the bag using tissue and removed the clips by taking the sample out using a plastic pipette.

Estimation of Protein

Protein contents of the enzyme extracts was determined according to **Lowry *et al*., method**. **(1984)**. According to this method first, make a reagent (A) (2% sodium carbonate anhydrous in 100 ml of 0.1 N NaOH). Reagent (B) (0.5% copper sulphate in 1% sodium potassium tartrate) and reagent (C) (50ml of reagent (A) dissolved in 1ml of reagent (B), just prior to use. Stock standard protein from BSA was prepared by dissolving, 50mg BSA in 50 ml of distilled water. Diluted 10 ml of stock standard solution to 50 ml of distilled water to obtain 200 mg protein/ml. After this pipetted out sample in 0.1 and 0.2 conc. made up 1 ml with distill water. Add 5 ml of reagent (C) to each and allowed to incubate at room temperature for 10 minutes. Then add 0.5 ml of folin & Ciocalteu's reagent. Colour was allowed to develop for 30 minutes at room temperature in the dark and the absorbance measured at 550 and blanked on the water. Although in this experiment the plates were read immediately. The reaction was stable up to an hour. This procedure was done on both, before and after dialysis and for different pH also.

Assay of Polyphenol oxidase 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 to3.0mL 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 was 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.

Partial Characterization

pH Optimum and Stability

PPO activity as a function of pH was determined using pyrogallol as substrate. Phosphate buffer, ranging from pH 4.0 to 8.0 was used at the assays. The pH stability was determined by incubating the enzyme in the above buffer (pH 4.0 to 8.0) for 30 min and at the end of the incubation period, sample were taken and assayed under standard condition. PPO activity was calculated in the form of unit per mg protein at the optimum pH.

Effect of Temperature

To determine the optimum temperature for PPO, the activity of the enzyme was measured at different temperature ($20\text{-}70^0$) using 0.2 conc. Of enzyme, 2.5 ml of pyrogallol as a substrate and completed to 3 ml with 0.1 M sodium phosphate buffer (pH 6.5). Controls were run under the same tested temperature. The tubes were pre-heated to the selected temperature to prevent temperature lag before the addition of a 0.2 ml aliquot of enzyme solution. The enzyme sample was removed from water bath after pre-set times and were immediately transferred to ice bath to stop thermal inactivation. After the sample was cooled in ice bath, the residual activity was determined spectrophotometrically using the standard reaction mixture. A non- heated enzyme sample was used as blank.

RESULT AND DISCUSSION

Standard curve of BSA For the determination of protein concentration a standard curve of BSA was prepared by following the Lowery method of protein determination. **(Lowry *et al.*, 1984).**

Table 1.1: Optical Density of standard BSA solution at 660 nm

Concentration	Absorbance
0.0	0
0.2	0.234
0.4	0.43
0.6	0.71
0.8	0.838
1.0	1.25
1.2	1.495

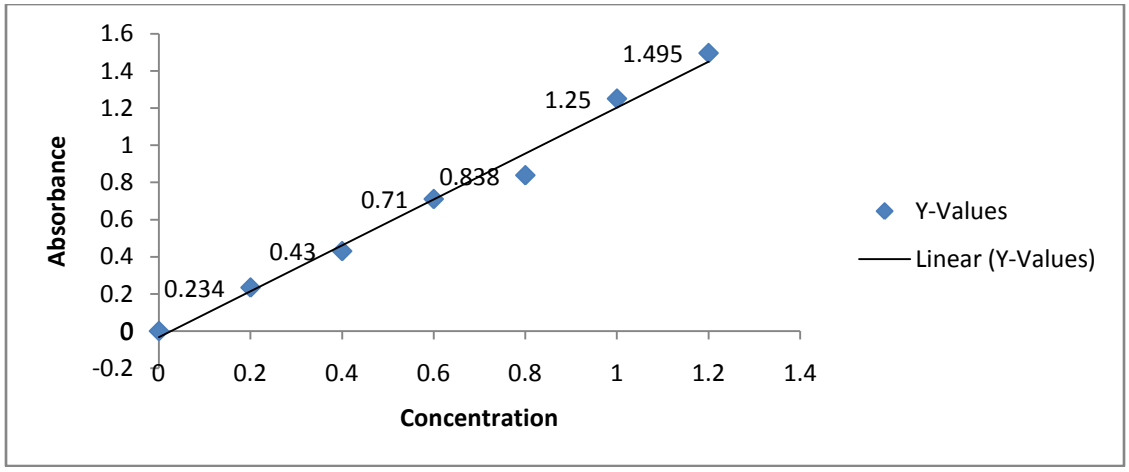

Fig : 1.1 Calibration curve of standard BSA at 660 nm

Table 1.2: Specific activity (U/mg) of protein sample obtained from two varaities at 420 nm

S.No.	Type of Sample	Crude Sample (U/mg)	Dialyzed Sample (U/mg)
1.	Desi tomato	1.8	34
2.	Hybrid Tomato	0.81	20.09

Purification of Polyphenol Oxidase

To purify the desired enzyme, extract was subjected to a 45% saturation with $(NH_4)_2SO_4$. which is the most commonly used reagent for salting out of the protein because of its high solubility permits the achievement of solution with high ionic strength

Table 1.3: Purification table for Desi tomato

Sample	Protein concentration	Specific activity (U/mg)	Fold purification	% Recovery
Crude extract	8.6	1.8	1	100
Ammonified extract	7.5	2.16	1.2	95.5
Before dialysis	4.7	13.4	7.44	24.4
Dialysed sample	2.75	34	18.8	16.86

Polyphenol oxidase was purified partially from Desi tomato using two steps, which was, ammonification of polyphenol oxidase and dialysis of ammonified sample. Dialyzed sample exhibited maximum specific activity of 34 U/mg which was 18.8 fold higher with 16.86% recovery. The specific activity of an enzyme is another common unit. This is the activity of an enzyme per milligram of total protein (expressed in μmol $min^{-1}mg^{-1}$). Specific activity gives a measurement of enzyme purity in the mixture.

Table 1.4: Purification table for Hybrid Tomato (Hybrid 6-4, 4,)

Sample	Protein contentration	Specific activity (U/mg)	Fold purification	% Recovery
Crude Extract	8.6	0.81	1	100
Ammonified Extract	7.5	2.9	3.5	31.5
Before dialysis	4.7	8.76	10.8	15.43
Dialyzed sample	2.75	20.09	25.80	12.23

Polyphenol oxidase was purified partially from Hybrid tomato (Hybrid 6-4, 4) using two steps which are ammonification of polyphenol oxidase and dialysis of ammonified sample. Dialyzed sample exhibited maximum specific activity of 20.09 U/mg which was 25.08 fold higher with 12.23% recovery.

Partial characterization of PPO from tomato

Table 1.5 Protein concentration of PPO at different pH

S.No.	pH	Protein concentration (µ/mg)
1	4	1.15
2	5	2.05
3	6	2.55
4	**7**	**2.85**
5	8	2.2

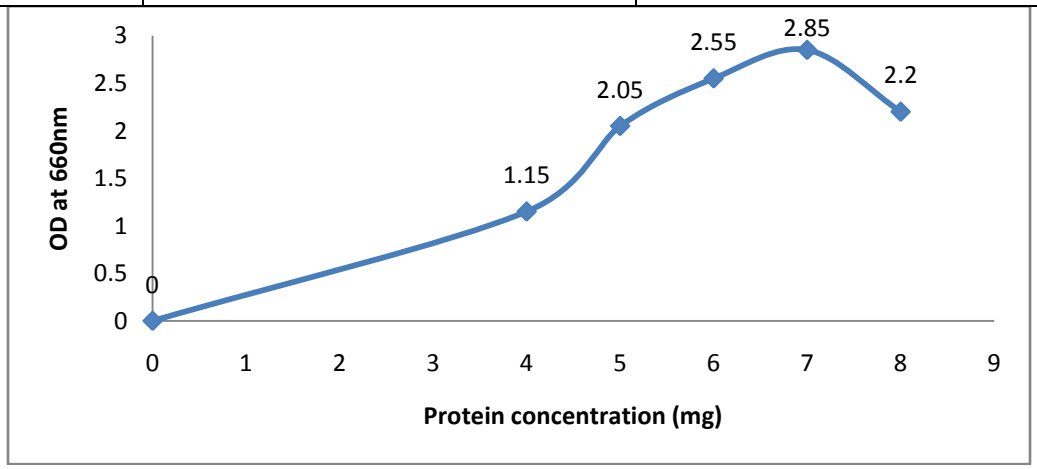

Fig 1.5 Protein concentration of Polyphenol oxidase

Fig 1.5 shows that there was a continuous increase in the protein concentration starting at 4 pH which showed 1.15 U/mg, at pH 5, 2.05 U/mg was seen, at pH 6 2.55 U/mg was seen, pH 7 shows the highest activity, that is 2.85 U/mg but at pH 8 decrease in protein concentration, this could be due to the changes in pH affecting the chemistry of amino acid residues leading to denaturation. Hydrogen bonding often involves these side changes. Protonation of the amino acid residues ie, when an acidic proton H^+ attaches to a lone pair of electrons on a nitrogen, lone pair of electrons doesn't participate in hydrogen bonding, so a change in the pH denatures a protein.

1.6 Specific activity of tomato at different pH

The specific activity of PPO enzyme from tomato at different pH is shown higher at pH 7.

S.No.	pH	Specific Activity (U/mg)
1	4	5.5

2	5	13.25
3	6	14.96
4	**7**	**19.89**
5	8	18

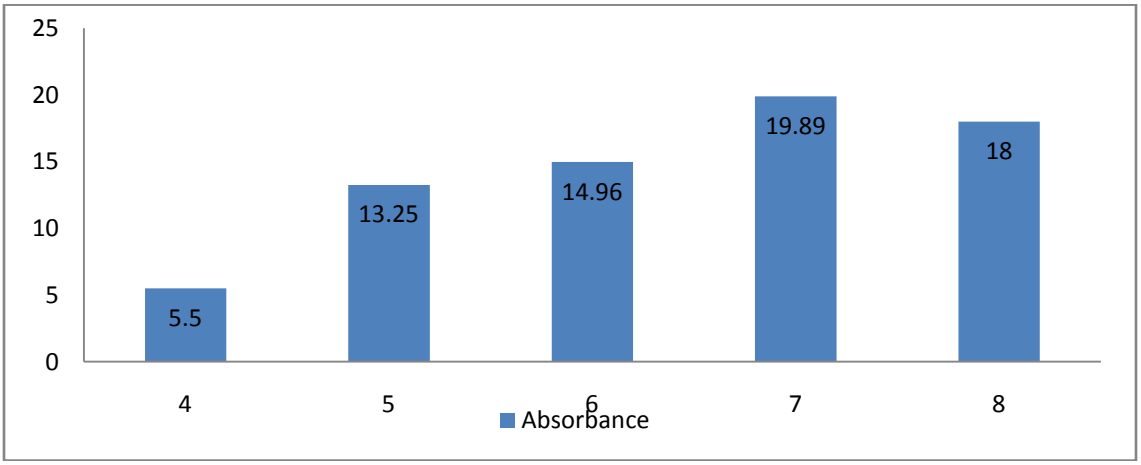

Fig 1.6 : Specific activity of PPO at different pH

The figure (1.6) showed that specific activity of the enzyme increased with increase in the pH values ranging from pH 4-7, i.e at 4 pH, 5.5 U/ml, at pH 5, 13.25 U/ml, at pH 6, 14.95 U/ml, at pH 7 it showed the maximum specific activity ie, 19.9U/ml, using pyragallol as a substrate for the enzyme, but at pH value of 8 the specific activity gradually decreased to 18 U/ml.

(Lourenço *et al.*, 1990; Gonzalez *et al.*, 2000; Kavrayan and Aydemir, 2001; Dogan *et al.*, 2004; Rapeanu *et al.*, 2006) reported differences in optimum pH for PPO using distinct substrates for the enzyme. However, pH optimum for PPO activity in presence of catechol and pyrogallol in tomato were same, when enzyme was exposed to 60°C, along with that 100 % residual activity was registered for 10 minute. **(Rapeanu *et al.*, 2006, Lourenco *et al.*, 1992, Dogan *et al.*, 2004)** showed that tomato - PPO was a heat-stable enzyme at 40 - 60°C and in comparison to PPO in peppermint, tomato-PPO was more resistant to heating.

Thermal stability of PPO at different temperature

Table 1.7: Specific activity of Polyphenol oxidase at different temperature at pH 4

Temperature	**Activity of PPO (U/mg)**
20	4.6
30	6.2
40	7.8
50	**13.17**

60	9.39
70	8.2

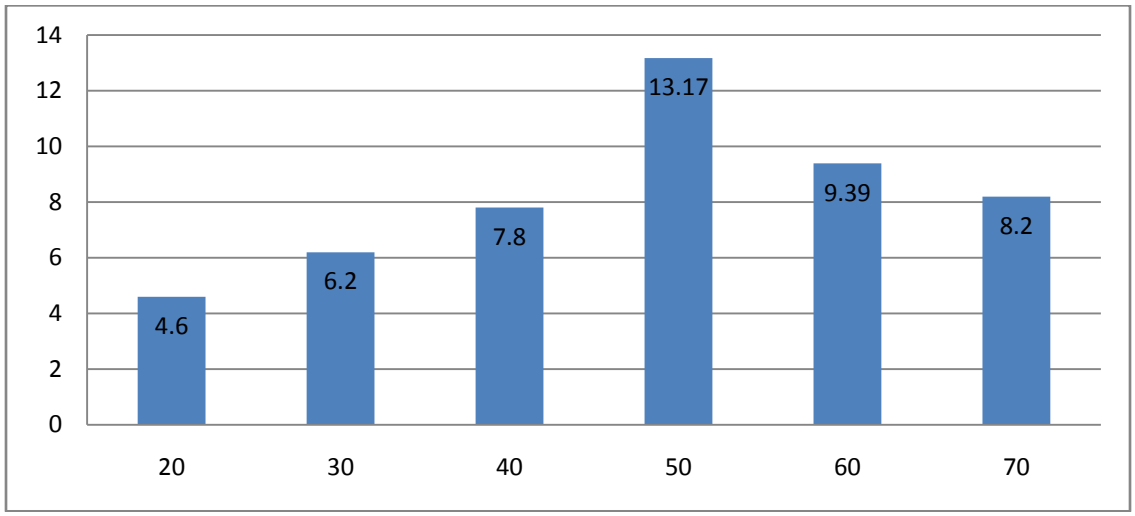

Fig. 1.7: Specific activities of Polyphenol oxidase at different temperature at pH 4

As shown in fig. 1.7, Specific activity of the polyphenol oxidase PPO in tomato using pyragallol as a substrate, at optimum temperature of 50° C and pH 4, was 13.17 U/mg.

The optimum pH for the PPO was 7.5 on substrates catechol and DL-dopa. Heat inactivation studies showed temperature 40 °C resulted in loss of enzyme activity. Heating for 30 min at 40 °C did not cause a significant loss of enzymatic activity.

Table 1.8: Specific activity of Polyphenol oxidase at different temperature at pH 5

Temperature	Activity of PPO (U/mg)
20	3.5
30	6.1
40	7.9
50	**14.08**
60	10.2
70	6.9

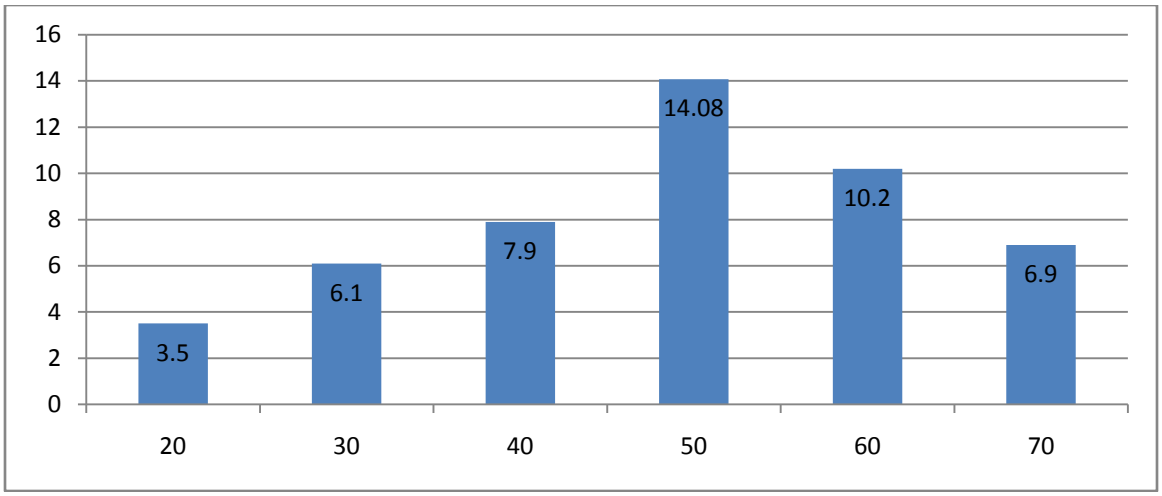

Fig. 1.8: Specific activities of polyphenol oxidase at different temperature at pH 5

As shown in fig. 1.8, Specific activity of the polyphenol oxidase PPO in tomato using pyragallol as a substrate, at optimum temperature of 50° C and pH 5, was 14.08 U/mg.

Table. 1.9: Specific activity of Polyphenol oxidase at different temperature at pH 6

Temperature	Activity of PPO (U/mg)
20	6.35
30	7.05
40	10.5
50	**18.03**
60	14.11
70	12.7

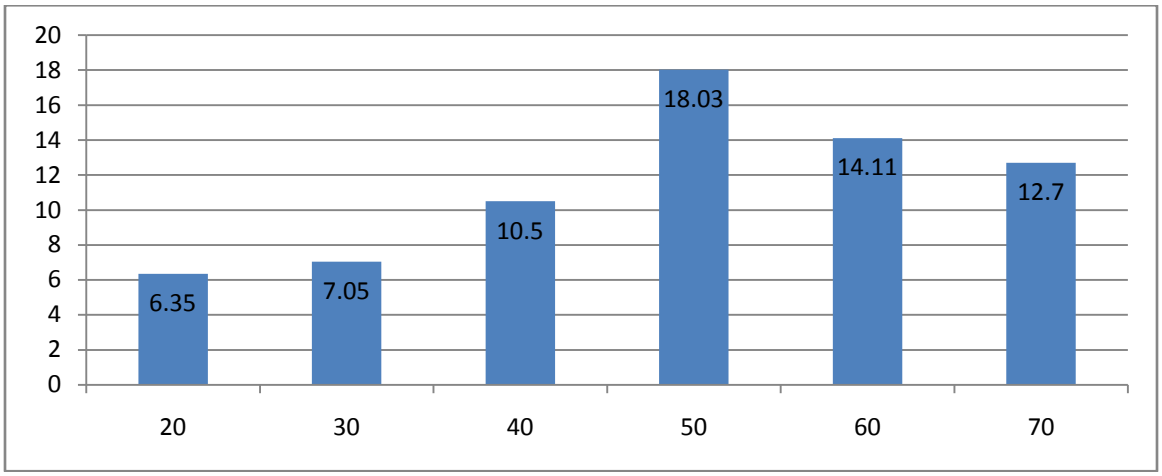

Fig: 1.9 : Specific activity of Polyphenol oxidase at different temperature at pH 6

As shown in fig. 1.9, Specific activity of the polyphenol oxidase PPO in tomato using pyragallol as a substrate, at optimum temperature of 50° C and pH 6, was 18.03 U/mg.

Similar results were reported by **(Dogan. and Dogan, M. (2004)** polyphenol oxidase (PPO) of several *Ferula* species was extracted and purified through $(NH_4)2SO_4$ precipitation, dialysis, and gel filtration chromatography The most effective inhibitor was sodium diethyl dithiocarbamate for leaf samples and sodium metabisulphite for stem samples. Both inhibitors indicated competitive reactions. PPO showed irreversible denaturation after 40 min at 60 °C.

Table: 1.10: Specific activity of Polyphenol oxidase at different temperature at pH 7

Temperature	Activity of PPO (U/mg)
20	9.4
30	11.36
40	15.7
50	**18.35**
60	17.03
70	14.52

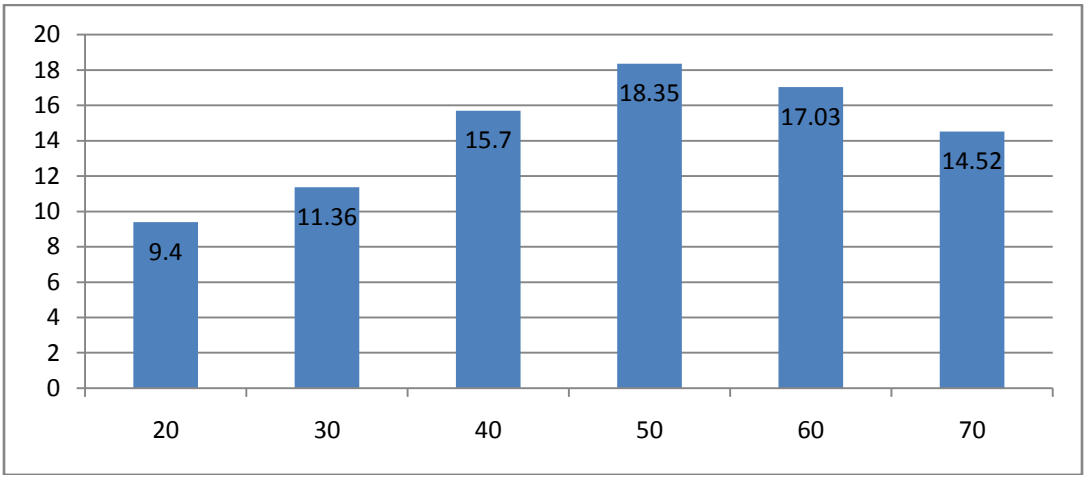

Fig. 1.10: Specific activity of Polyphenol oxidase at different temperature at pH 7

As shown in fig. 1.10, Specific activity of the polyphenol oxidase PPO in tomato using pyragallol as a substrate, at optimum temperature of 50° C and pH 7, was 18.35 U/mg.

Polyphenoloxidase (PPO) of peppermint leaves *(Mentha piperita)* and isolated polyphenol by $(NH_4)_2SO_4$ precipitation and dialysis. Its pH and temperature optima were 7.0 and 30°C, respectively. On heat-inactivation, half of the activity was lost after 6.5 and 1.5 min of treatment at 70 and 80°C, respectively.

(Bosch et al., 2014) also repoted similar results while working on crude extracts prepared from medlar fruits (Mespilus germanica L., *Rosaceae)* which possess a diphenolase activity toward catechol, 4-methyl catechol, L-3,4-dihydroxyphenylalanine, epicatechin and 3-(3,4-dihydroxyphenyl)propionic acid. The pH-activity was optimum for the enzyme, in the presence of this substrate, was 6.5 and the pH-stability profile for the enzyme showed that 80% of the PPO activity was retained at physiological pH values. The temperature-activity was optimum, for the enzyme in the presence 4-methyl catechol, was 35 °C. The enzyme was stable for 30 min at its optimum temperature and moderately stable at 60 °C. At higher temperatures, heat denaturation of the enzyme occurred after 10 min of

incubation. Thermal inactivation parameters indicated that the medlar enzyme was very heat-labile. Moreover, the medlar PPO activity was very sensitive to some common PPO inhibitors, especially to cysteine and metabisulfite.

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Table 1.11: Specific activity at different temperature at pH 8

Temperature	Activity of PPO (U/mg)
20	4.09
30	5.72
40	8.18
50	**12.7**
60	10.6
70	9.8

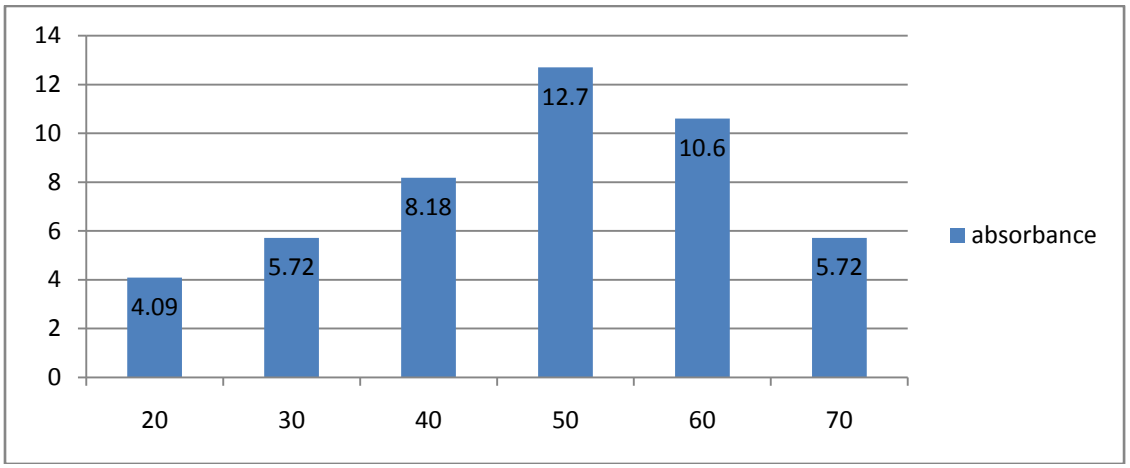

Fig. 1.11: Specific Activity of Polyphenol Oxidase at different temperature at pH 8

As shown in fig. 1.11, Specific activity of the polyphenol oxidase PPO in tomato using pyragallol as a substrate, at optimum temperature of 50° C and pH 8, was 12.7U/mg, which tells us that specific activity showed highest at pH 7 and optimum temperature of 50° C, but at reaching pH 8 at optimum temperature 50° C, specific activity decreased considerably by 12.7 U/mg as the specific activities of Polyphenol oxidase at different temperature of pH 4 was 14.08 U/mg, at pH 5 it was found to be 13.17 U/mg, at pH 6 it was 18.35 U/mg, at pH 7 it was 18.3 U/mg , and at pH 8 it was the lowest, 12.7 U/mg and 50^0 C became temperature was favorable for pyragallol substrate.

Similar results were given by **(Ziyan and Perkyardimci, 2004; Siddiq *et al*., (1992)** incubated the PPO enzyme of pear and plum at different temperatures (20–70^0C) for 30 min and, after cooling, the residual enzyme activity was measured. Consequently, the enzyme was stable at 50-60 ^{0}C, pear PPO was stable at 60 ^{0}C and plum PPO at 70^0 C. The decrease in percentage residual activity at higher temperatures was due to the unfolding of the tertiary structure.

Summary and Conclusion

In the present study, the enzyme polyphenol oxidase was extracted from tomato and determined in the given sample. The amount of the PPO was determined by taking down the absorbance of protein estimation using BSA with the help of the colorimeter. PPO was extracted from the pulp with the help of sodium phosphate buffer as it gets

precipitated and then separated completely with the help of centrifugation method. It was partially purified by ammonium sulphate precipitation and dialysis

The protein concentration present in desi tomato was 2.15 µg/ml and in hybrid 2.75µg/ml respectively. The specific activity in desi tomato was 2.61 U/mg and for hybrid 2.15 U/mg respectively. The specific activity of polyphenol oxidase was higher at pH 7 i.e 19.89 U/mg. Polyphenol oxidase was purified partially from Desi tomato followed by 2 steps- ammonification of polyphenol oxidase and dialysis of ammonified sample. Dialyzed sample exhibited maximum specific activity of 34 U/mg which was 18.8 fold higher with 16.86% recovery and from Hybrid tomato(Hybrid 6-4,4) followed by 2 steps- ammonification of polyphenol oxidase and dialysis of ammonified sample. Dialyzed sample exhibited maximum specific activity of 20.09 U/mg which was 25.08 fold higher with 12.23% recovery.

During partial characterization of polyphenol oxidase, sample was incubated at different temperature (20^0-70^0 C) and at different pH (4-8) find out its highest specific activity at a certain temperature and pH and the results showed that the highest specific activity was at pH 4 obtained 13.17 U/mg at 50^0 C , pH 5 obtained 14.08 U/mg at 50^0 C , pH 6 obtained 18.03 U/mg at 50^0 C , pH 7 obtained 18.35 U/mg at 50^0 C , pH 8 obtained 12.7 U/mg at 50^0C .

On the basis of above result it was found that desi tomato is better than hybrid tomato. Polyphenol causes discoloration in tomato during storage, so if the temperature was maintained below 50^0 C and pH 7, it prevents from discoloration and increases the value of plant protein.

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