APPLICATION OF PLANT POLYPHENOXIDE IN DETERMINATION OF SODIUM BENZOATE

PROJECT REPORT

Submitted by

N. NAGAJOTHI

Register No: 1020203009

in partial fulfillment for the award of the degree

of

MASTER OF TECHNOLOGY

in

BIOTECHNOLOGY

KUMARAGURU COLLEGE OF TECHNOLOGY
(An Autonomous Institution Affiliated to Anna University of Technology, Coimbatore)

COIMBATORE -641 049

APRIL 2012

KUMARAGURU COLLEGE OF TECHNOLOGY

COIMBATORE-641049

DEPARTMENT OF BIOTECHNOLOGY

PROJECT WORK

PHASE II

APRIL 2012

This is to certify that the project entitiled

APPLICATION OF PLANT POLYPHENOL OXIDASE IN DETERMINATION OF SODIUM BENZOATE

is the bonafide record of project work done by

N. NAGAJOTHI

Register No: 1020203009

of M.Tech. (Biotechnology) during the year 2011-2012

…… ……..

Project Guide **Head of the Department**

Submitted for the Project Viva-Voce examination held on .....

…….. ……

Internal Examiner External Examiner

DECLARATION

I affirm that the project work titled "**APPLICATION OF PLANT POLYPHENOL OXIDASE IN DETERMINATION OF SODIUM BENZOATE**" being submitted in partial fulfilment for the award of M.Tech (Biotechnology) is the original work carried out by me. It has not formed the part of any other project work submitted for award of any degree or diploma, either in this or any other university.

N.NAGAJOTHI

Reg.No:1020203009

I certify that the declaration made above by the candidate is true.

Dr.VINOHAR STEPHEN RAPHEAL
Associate Professor
Department of Biotechnology
Kumaraguru College of Technology
Coimbatore - 641 049

ACKNOWLEDGEMENT

I wish to express my sincere thanks to **Dr.S.Ramachandran**, Principal, Kumaraguru College of Technology, Coimbatore, **Dr. A. Manickam** Head of the Department, Biotechnology, Kumaraguru College of Technology. Coimbatore.

I wish to express my warm and sincere gratitude to my guide **Dr.Vinohar Stephen Rapheal**, Associate Professor, Department of Biotechnology, whose encouragement, guidance and support from the initial to the final level enabled me to develop an understanding of the subject.

I would like to extend my special thanks to **Dr.J.Aravind**, Assistant Professor (SRG), **Dr.T.Sathish Kumar,** Assistant Professor (SRG), **Mr.M.Shanmuga Prakash**, Assistant Professor (SRG), **Dr.P.Ramalingam** Associate Professor, Department of Biotechnology, for their expertise, understanding and patience, and for the assistance they provided at all levels of the research project.

I am grateful to the teaching and non-teaching staff members of the Department of Biotechnology for their kind and patient help throughout the project.

Above all, I am thankful to the Almighty for his blessings without which nothing would have been possible.

(N.NAGAJOTHI)

TABLE OF CONTENTS

CHAPTER NUMBER	CONTENTS	PAGE NUMBER
	Abstract	i
	List Of Tables	ii
	List Of Figures	iii
	List Of Abbrevations	iv
1.	**INTRODUCTION**	1
	1.1 Polyphenol oxidases	
	1.1.1 Cresol oxidase	1
	1.1.2 Catechol oxidase	2
	1.1.3 Laccases	2
	1.2 Enzyme specificity	2
	1.3 Purification	
	1.3.1 Ammonium sulphate precipitation	4
	1.3.2 Ion exchange chromatography	4
	1.4 Enzyme Kinetics	4-5
	1.5 Preservatives	
	1.5.1 Benzoate	5
	1.5.2 Ascorbic acid	
	1.6 Detection techniques	7
	1.6.1 HPLC	
	1.7 Application of PPO	7
	OBJECTIVES	9
	LITERATURE REVIEW	10
	2.1 Polyphenols	10
	2.2 Sources	11
	2.2.1 Guava	11
	2.2.2 Mushroom	11
	2.2.3 Potato	12
	2.2.4 Banana	12
	2.2.5 Mango	13
	2.2.6 Papaya	13
	2.3 Oxidation	13
	2.4 Characterization	14
	2.4.1 Yam	14
	2.4.2 Banana	14
	2.4.3 Potato and Mushroom	15
	2.5 Ion exchange chromatography	16
	2.6 Inhibitors	17
	2.7 Preservatives	18
	2.8 Sodium benzoate	19
	2.8.1 General	19
	2.8.2 Preservatives	19
	2.8.3 Food application	19
	2.8.4 Pharmaceutical application	20
	2.8.5 Beverages	20
	2.8.6 Cider	20
	2.8.7 Margarine	20
	2.8.8 Syrups	20
	2.8.9 Fruits, Juices, and Salads	21
	2.8.10 Other applications	21
	2.8.11 Safety	21
	2.8.12 Solubility	21
	2.9 Benzoic acid determination	22
	2.9.1 HPLC	22
	2.9.2 Spectrophotometric method	24
	2.9.3 Isotachophoresis	25
	2.10 Ascorbic acid determination	25
	MATERIALS AND METHODS	27
	3.1 Materials	27
	3.1.1 Chemicals	27
	3.1.2 Samples	27
	3.1.3 Equipments	27
	3.2 Methods	27
	3.2.1 Isolation	27
	3.2.2 Ammonium Sulphate Precipitation	28
	3.2.3 Ion exchange Chromatography	28
	3.2.4 Enzyme specificity	28
	3.2.5 Assay and protein estimation	29
	3.2.6 Kinetic characterization	29
	3.2.7 Application	29
	3.2.8 HPLC	30
	RESULTS AND DISCUSSIONS	31
	4.1 Isolation of PPO	31
	4.2 Purification	35
	4.3 Kinetic characterization of purified	36
	4.4 Inhibition studies	39
	4.5 Percent inhiibition in food matrices	41
	4.6 Application	44
	4.7 HPLC results	45
	CONCLUSIONS	48
	APPENDICES	
	REFERENCES	

ABSTRACT

Quality and safety of the commercial products depends on the quantity of phenolics present in them and it needs to be detected. .Previously, detection of phenolics was mostly done by HPLC technique which is costly, requires skilled labours and method development for each component is a time consuming procedure. Thus, a simple enzymatic method was developed where the phenolics present in products can be detected using a spectrophotometer. Ascorbic acid, benzoic acid and benzoate are being used as preservatives in food products. Sodium benzoate with high solubility is used in most products. These compounds inhibit PPO and therefore the enzyme can be used in detection of total phenolics. Polyphenol oxidase (PPO) is a copper containing metalloenzyme. PPO occurs in wide range organisms from bacteria to plants and mammals. In the present study, PPOs from apple, banana, mango, guava, potato, brinjal were screened. Banana peel PPO showed highest activity and was further purified by ion exchange chromatography. Kinetic characterisation of enzyme with varying substrate concentration was observed to have a K_m value of 24.39mM. Inhibition of enzyme with phenolics such as ascorbic acid, benzoic acid and sodium benzoate was found to be dose dependent manner. Result obtained in this method was compared with that of HPLC and was found to be similar.

Keywords:
PPO, purification, kinetics, inhibition, benzoate, benzoic acid, ascorbic acid.

LIST OF TABLES

TABLE NO.	TABLE TITLE	PAGE NUMBER
4.1	Comparison of tyrosinase activity from various sources	31
4.2	Activity of tyrosinase in ammonium sulphate precipitated fractions	32
4.3	Specificity of banana peel polyphenol oxidase	33
4.4	Purification of enzyme in each stage	35
4.5	kinetic characteristics	37
4.6	Inhibition percentage of sodium benzoate in different food matrices	40
4.7	Inhibition percentage of benzoic acid in different food matrices	40
4.8	Inhibition percentage of ascorbic acid in different food matrices	41
4.9	Concentration of sodium benzoate obtained from standard graph.	43
4.10	HPLC chromatogram data	46
4.11	Detection of phenolics using enzymatic method-validation	47

LIST OF FIGURES

Figure No.	Title of Figure	Page No.
1.1	Tyrosinase – active site containing copper ions	3
1.2	The mechanism of melanin synthesis.	3
1.3	Structure of sodium benzoate and ascorbic acid	6
4.1	Ion exchage chromatography	34
4.2	LB plot showing enzyme kinetics	36
4.3	LB plot showing inhibition kinetics for sodium benzoate	36
4.4	LB plot showing inhibition kinetics for ascorbic acid	37
4.5	standard graph for sodium benzoate	38
4.6	standard graph for benzoic acid	39
4.7	standard graph for ascorbic acid	39
4.8	Dose dependent inhibition by benzoic acid in food matrix	41
4.9	Dose dependent inhibition by ascorbic acid in food matrix	42
4.10	Dose dependent inhibition by sodium benzoate in food matrix	42
4.11	Percentage inhibition of enzyme by samples	43
4.12	HPLC standard graph for sodium benzoate - 20µg/ml	44
4.13	HPLC chromatogram samples – jam	45
4.14	HPLC chromatogram samples – sauce	45
4.15	HPLC chromatogram samples – pickle	45
4.16	HPLC chromatogram samples – soft drink	46
4.17	Linear curve HPLC standard	47

ABBREVATIONS

BSA	Bovine Serum Albumin
$CuSO_4$	Copper sulphate
DEAE	Diethyl Amino Ethyl
[E]	Enzyme concentration
EA	Enzyme activity
FIG	Figure
HPLC	High Pressure Liquid Chromatography
I	Inhibition
K_m	Michaelis Menton constant
PPO	Polyphenol oxidase
PVPP	Polyvinyl pyrollidone
[S]	Substrate concentration
SA	Specific activity
U	Enzyme Unit
V_{max}	maximum rate of enzyme activity

CHAPTER 1
INTRODUCTION

1.1 POLYPHENOL OXIDASES (PPO)

Phenol oxidative enzymes include peroxidases and PPOs. Polyphenoloxidase is a group of copper containing metalloenzyme that includes cresol oxidase (E.C. 1.14.18.1, monophenol mono-oxygenase), catechol oxidase (E.C. 1.10.3.1, di-phenol di oxygenase), and laccase (E.C. 1.10.3.2, *p*-benzenediol: oxygen oxidoreductase). PPO catalyses the oxidation of para-monophenol and ortho-diphenols to insoluble polyphenols through two reaction steps known as cresolase and catecholase. This reaction is very important in maturation and ripening process of fruits and vegetables. PPO has been implicated in the formation of pigments, oxygen scavenging and defense mechanism against plant pathogens, and herbivorous insects. It is known that plant PPOs are synthesized as preproteins and contain putative plastid transit peptides at the N-terminal region, which target the enzyme into chloroplast and thykaloid lumen. The enzyme is responsible for browning of damaged or injured fruits and vegetables (McCaig *et al* 1999). On rupture, PPO from plastids leaks and reacts with phenolic compounds from vacuoles resulting in quinone formation. The mechanism of action depends on its capacity to oxidize phenolic compounds.PPO has a wide range of substrate specificity and are able to oxidize mono, di, and poly phenols. Now a day's the PPO is used as biosensor as well as a key enzyme in the clinical studies, detection, determination and detoxification of phenolic compounds from industrial wastewater (Sung et al., 2003; Allison et al., 2008). In this present study, we report that PPO's can be used in detection total phenolics present in food.

1.1.1 CRESOL OXIDASE

Cresol oxidase is an enzyme that acts as one of the biocatalysts in the synthesis of melanin and other pigments in various fruits and animals. It contains two copper atoms in its active structure for the binding of oxygen molecules forming intermediates that binds with the substrate (selinheimo *et al* 2008). Monophenols such as tyrosine is the substrate with which the enzyme reacts to form a di-phenol (cresolase activity) followed by its enzymatic oxidation to quinone (catecholase activity). Quinone through non-enzymatic reactions produces melanin and the mechanism is shown in fig 1.2. It is an anti-microbial agent present naturally in fruits and vegetables. It has its application in waste water treatment to degrade phenolic compounds, for colour formation in food products such as coffee, tea, etc.

1.1.2 CATECHOL OXIDASE

Catechol oxidase is one of the biocatalysts in the melanin synthesis. L-DOPA acts as the substrate which on catalysis with the enzyme produces quinone and dopachrome (Aniszewski *et al* 2008). Thus, the enzyme is specific for diphenolic substrates and is also called diphenol dioxygenase (catecholase activity). Cresolase and catecholase are collectively called tyrosinases.

1.1.3 LACCASES

Laccase is a cupro-protein belonging to a small group of enzymes denominated blue oxidases. In general, laccases exhibit four copper atoms, which play an important role in the enzyme catalytic mechanisms. Laccases are characterized by low substrate specificity and their catalytic competence varies widely depending on the source. For majority of laccases guaiacol and 2,6-dimethoxyphenol generally are better substrates (Duran *et al* 2002).

1.2 ENZYME SPECIFICITY

Enzymes are sensitive to the existing conditions such as pH and temperature. Enzymes have ionic groups in their active site relevant to their function. Variations in the pH of the medium will alter the ionic form of the enzyme and its active site. This will affect the maximum reaction rate, activity and changes the secondary structure of the enzyme, thus reducing the affinity of substrate towards the active site. Similarly, increase in temperature will increase the activity to some extent. Above a certain range increase in temperature will decrease the enzyme activity by denaturing them. Thus, enzymes optimum temperature should be maintained for better activity. Also, substrate should be specific based on the active site and its optimum concentration should be maintained.

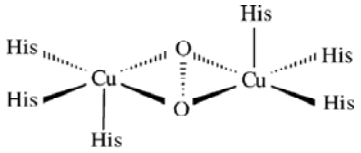


Fig 1.1 Tyrosinase – active site containing copper ions

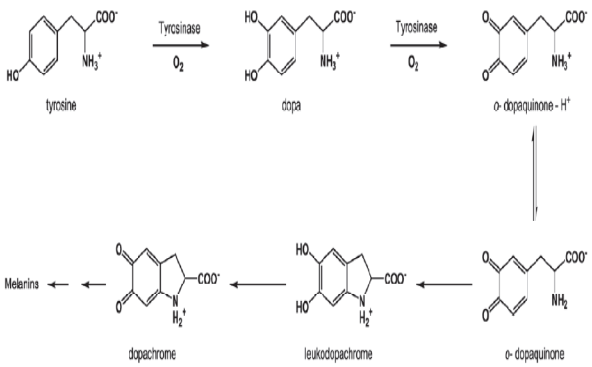


Fig 1.2 The mechanism of melanin synthesis

1.3 PURIFICATION OF PPO

1.3.1 AMMONIUM SULPHATE PRECIPITATION

Precipitation of protein may be done using organic solvents, salts, metal ions, etc. Salts used should contain polyvalent ions, so that the salting-out constant is higher. Ammonium sulphate is mostly used for salting out proteins due to the reasons as follows: it is more easily soluble, its effect is less dependent on temperature, protein in ammonium sulphate precipitate are stable for a long period (kong *et al* 1998), and the cost of salt is cheaper. Protein was separated from other cell debris and some protiens by ammonium sulphate precipitation technique. Thus, ammonium sulphate precipitation technique is used as a preliminary purification step. But, the salt being toxic and other undesirable effects it could cause, the salt should be removed by dialysis.

1.3.2 ION EXCHANGE CHROMATOGRAPHY

Separating the desired protein from a homogenate is the biggest challenge in enzyme technology. Some extent of purification is achieved in ammonium sulphate precipitation based on solubility thus, highly soluble proteins and other cell debris are removed there. The basic principle behind ion exchange includes: Proteins are charged molecules. At specific pH, it can exist in anionic (-), cationic (+) or zwitterions (no net charge) stage. In Ion exchange chromatography separation is based on the charges carried by the protein molecules. Some of the sugars, polysaccharides, amino acids are removed and protein isomers were separated in ion exchange chromatography.

1.4 ENZYME KINETICS

Michaelis and Menten postulated that the first step in an enzymatic reaction is the formation of a complex between substrate and enzyme, the ES complex Michaelis-Menten equation:

$$V_0 = V_{max}[S]/K_m + [S]$$

Where, V_0 = the initial reaction velocity

V_{max} = the maximal velocity, obtained when all of E is in the ES complex

[S] = the substrate concentration

K_m = the Michaelis constant = $(k_2 + k_{-1})/k_1$

Enzyme activity can be measured by following the change in concentration of any substrate or product. Ideally, this is done in a direct manner, is very sensitive, and involves a change in absorbance in the UV-Visble range. One can then measure the reaction velocity at different substrate concentrations and make a plot of V_0 vs [S] to verify that the enzyme follows Michaelis-Menten kinetics. The kinetic parameters V_{max} and K_m can be obtained by a fit of the data to the Michaelis-Menten equation, or they can be derived from a double reciprocal plot. Taking the reciprocal of both sides of the Michaelis-Menten equation yields the Lineweaver-Burk relationship:

$$1/V_0 = (K_m / V_{max}*[S]) + 1/ V_{max}$$

Tyrosinase catalyzes the oxidation of L–DOPA to dopachrome, using molecular oxygen (O_2) as the second substrate. Mushroom tyrosinase is a tetrameric enzyme with a total molecular weight of 128,000. Each active enzyme binds four molecules of Cu^+, which interact with the bound oxygen.

To monitor the enzyme activity we will follow oxidation of DOPA at 475 nm. The extinction coefficient of dopachrome is 3600 $M^{-1}cm^{-1}$. To convert the raw data (change in absorbance per minute) to moles of product formed per minute per liter, each ΔA/min is divided by 3600. This is then being converted to μmoles/min.mg by making the appropriate conversions.

1.5 PRESERVATIVES

Food preservation is the procedure of treating and handling food to prevent or greatly slow spoilage caused or accelerated by micro-organisms. Preservatives, just like any other food additive, can be divided into two broad categories: Natural Preservatives and Artificial Preservative the roles of food preservatives**:**

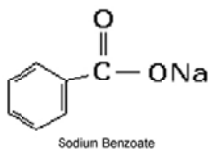
- To increase the shelf value of food for storage
- To preserve the natural characteristics of food
- To preserve the appearance of food. Chemical food preservatives are also being employed for fairly some time now. They seem to be the very best as well as the most efficient for a longer shelf life.

Examples of chemical food preservatives are**:** 1 Sulphites (such as sulphur dioxide) that may lead to acute asthmatic effects, 2 Sorbates (such as sodium sorbate, potassium sorbate, 3 Benzoates (such as sodium benzoate, benzoic acid), 4 Nitrites (such as sodium nitrite. Nitrates are more dangerous additives that may result in reduced oxygen carrying capacity of red blood cells. Over one billion pounds of chemical additives are consumed every year. Liver is in charge of detoxifying this garbage. The liver is the major organ that has the job of breaking down and disposing of all this material. Thus, the level of phenolics added should be monitored and controlled.

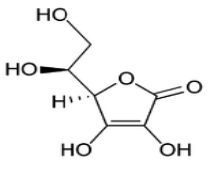
1.5.1 BENZOATE

Benzoate is an aromatic compound that is highly soluble in water. Its major applications include food, cosmetics and pharmaceutical industries as a preservative. Sodium benzoate (C_6H_5COONa) is the most used form, produced by neutralization of benzoic acid with sodium hydroxide. It is 200 times more soluble than benzoic acid and is generally regarded as a safe component to be used as preservative. The concentration of benzoate has to be around 0.1%. Excess concentration is not advisable for human consumption or use.

FIG 1.3 Structure of (a) sodium benzoate and (b) ascorbic acid:



Sodium Benzoate



1.5.2 ASCORBIC ACID

Ascorbic acid is a crystalline solid, also known as vitamin C that is essential for life. Chemically synthesized ascorbic acid may be harmful if inhaled, swallowed, absorbed through skin and also may cause eye irritation. They are stable at a pH around 2.2 and are completely soluble in water at room temperature.

1.6 DETECTION TECHNIQUES

1.6.1 HPLC

HPLC separates mixture of compounds on the basis of polarity. It is used to analyze, identify, purify and quantify compounds. It has a mobile phase, a stationary phase, and detector. The mobile phase is continuously pumped at a fixed flow rate through the system and mixed by the pump. The injector is used to introduce a plug of a sample into the mobile phase without having to stop the mobile phase flow, & without introducing air into the system. The mixture of components is carried in a narrow band to the top of the column. Some compounds in the sample mixture will have greater preference for stationary phase than the mobile phase and will be retained in the column longer. HPLC is used for qualifying and quantifying the phenolic content in any commercial product.

1.7 APPLICATIONS OF PPO

Level of phenolics in various products has to be checked before use, in order to avoid any adverse effects. Various techniques such as gas chromatography, high pressure liquid chromatography, modified ion exchange chromatography, isotachophoresis are used in benzoate detection. These methods are time consuming, costly and require skilled labors and are also totally a chemical dependent process. As a modification enzyme such as PPO can be used in benzoate detection. The basis of detection is the extent of its inhibition by phenolic compounds. It may also lead to hyper- pigmentation problem (Flurkey *et al* 2008).

In present investigation, plant sources such as apple, banana, brinjal, guava, mushroom, papaya and potato were screened for the presence of polyphenol oxidases. Enzyme activity and specific activity of crude enzyme isolated from each source was tabulated and compared. The isolated enzyme was partially purified using ammonium sulphate precipitation technique and the

activities were compared. Source with highest activity was selected for further studies. Banana peel showed the highest activity and it was further purified using positive ions containing DEAE sephacel column. The bound enzyme was subjected to gradient elution using NaCl and a total of 85 fractions were collected. Each fraction was observed at 260nm and 280nm to find the concentration of enzyme present and also was checked for enzyme activity. Both enzyme activity and concentration of protein were plotted against the fraction number and the fractions containing highest activity were pooled together. The enzyme kinetics and inhibition kinetics were studied. Application of enzyme in determining the total phenolic content of food was tested with the view to develop a simple laboratory scale method.

1.8 OBJECTIVES

- To isolate polyphenol oxidase from suitable plant source.
- To purify the enzyme and study its kinetic characteristics.
- To develop a method for detecting the presence of phenolics using the enzyme.

CHAPTER 2

LITERATURE REVIEW

2.1 POLYPHENOLS

Babendure *et al.,* 2011 had developed a protocol to study the enzyme substrate protocol. The browning of mushrooms, fruits and vegetables is the result of the oxidation of phenolic compounds by tyrosinase when cells are broken or damaged. In fungi such as mushrooms, the role of tyrosinase and subsequent melanin production is related to reproductive organ differentiation and spore formation as well as protection of tissues after injury. Tyrosinase is a copper-containing enzyme whose activity may be inhibited by a variety of natural and synthetic compounds. Natural inhibitors of mushroom tyrosinase include ascorbic acid, Green Tea, Aloe vera, and some sulfur compounds, to name a few. Other synthetic sources have been identified as well. There is much interest in tyrosinase inhibitors within the food and restaurant industries they are used widely to prevent unwanted discoloration of fruits and vegetables. The cosmetic industry has recently invested in research and applied as creams and other substances containing a tyrosinase inhibitor, marketing to consumers within cultures that desire lighter skin. Examples of tyrosinase inhibitors are sodium benzoate and 4-hexylresorcinol.

Scalbert *et al.,* 2000 had reviewed the dietary intake and bioavailability of polyphenols. Polyphenols include phenolic acids (benzoic acid, cinnamic acid and their derivatives) and flavonoids (flavanols). The main polyphenol sources include fruits, vegetables, beverages, legumes and cereals. Thus, phenolics account for a total intake of ~1 g/day.

Manach *et al.,* 2004 had reviewed the nature and contents of various polyphenols present in food sources and the influence of agricultural practices and industrial processes. Finally, bioavailability appears to differ greatly between the various polyphenols, and the most abundant polyphenols in our diet are not necessarily those that have the best bioavailability profile. A thorough knowledge of the bioavailability of the hundreds of dietary polyphenols will help us to identify those that are most likely to exert protective health effects.

Palmer *et al.,* 1963 had worked on Banana polyphenol oxidases. Polyphenoloxidase has been shown to occur in the pulp and peel of the banana fruit. It can be readily extracted from the pulp and rendered soluble in buffered detergent solution. These preparations were essentially colorless, and had little or no endogenous activity. The enzyme was further purified by acetone precipitation and chromatography on DEAE-cellulose.

2.2 SOURCES

2.2.1 GUAVA

Razzaque *et al.,* 2000 studied the polyphenol oxidases present in guava fruit. In guava, the proteins of crude enzyme extract were eluted from DEAE- cellulose column as one major peak and three minor peaks designated as F-1, F-2, F-3, F-4 respectively. PPO activity was eluted as the major peak by the initial buffer while the other fractions were eluted by the same buffer with the step wise increasing concentration of NaCl. The peak F-1 was saved and subjected to polyacrylamide gel electrophoresis

2.2.2 MUSHROOM

Bouchilloux *et al.,* 1962 had extracted tyrosinase from mushroom. Multiple forms of mushroom tyrosinase had been obtained in homogeneous state by a process involving preparative electrophoresis and chromatography on hydroxylapatite. Four enzymes, α-, β-, y-, and & tyrosinase, were obtained, the last three essentially pure. Although these enzymes possessed partially different activities towards mono- and o-diphenols, the three homogeneous ones had very similar amino acid composition. Both cuprous and cupric copper were present in each enzyme. Apparent dissociation into subunits with retention of activity occurs in the presence of sodium dodecyl sulfate.

Makino *et al.,* 1973 worked with mushroom.The mushroom enzyme contains 4 g atom of copper per mole of the tetrameric protein (mol wt about 120,000); most of the copper is electron paramagnetic resonance-undetectable even under aerobic conditions. The reaction mechanism of the enzyme, including the role of the copper ions, is unknown. It has recently been reported that in the presence.The structure of the T" chromophorc is equivalent to CU(I)~O~, and that of resting enzyme T, is equivalent to Cu(II),, it appears likely that dipllenol call act as a rcductant in the reaction:

$$Cu(II)_2 + \text{diphenol} \rightleftharpoons Cu(I)_2 + \text{quinone} + 2H^+$$

followed by the oxygenation

$$Cu(I)_2 + O_2 \rightleftarrows Cu(I)_2O_2$$

Seo *et al.,* 2003 had reviewed the recent prospects of mushroom tyrosinase. Tyrosinase from *A. bisporus* was reported to be a heterotetramer comprising two heavy (H) and light (L) chains with a molecular mass of 120 kDa and 48kDa respectively. This review also highlights the application part of tyrosinase in cosmetics and their role in cancer.

2.2.3 POTATO:

Kwon *et al.,* 1996 found that, one of the problems for the purification of polyphenol oxidase from potato tuber was the relative abundance of patatin, which is the major potato storage protein. The reason for only potato tuber periderm being used in this experiment is that the ratio of polyphenol oxidase to patatin in tuber periderm is higher than that in the rest of the tuber. Another thing to consider in the purification of polyphenol oxidase in plants is to remove phenolics because these compounds are changed to quinones by PPO, which could react covalently with the enzyme. Glycosylated PPO was purified from potato by ammonium sulphate fractionization,Sephadex G-100, and concanavalin A Sepharose column Chromatography.

2.2.4 BANANA:

Nematpour *et al.,* 2008 had studied the PPO activity in banana fruit. In banana pulp, the crude extract was subjected to a two-stage precipitation. Ammonium sulphate was added to the supernatant from the former step to make a 30% saturated solution. The resulting solution was stirred in an ice bucket for 30 min, then, centrifuged for 30 min at 20000xg and 4ºC (Bouhilloux *et al.,* 1963). After removing the precipitate, the supernatant was saturated to 65% ammonium sulphate.The solution was left in ice stirring for 2 hours followed by centrifugation at 20000xg for 30min. After discarding the supernatant, the resulting precipitate was dissolved in PBS and dialysed against similar PBS at 4ºC overnight. The dialysed protein solution was loaded onto an ion exchange DE-52 column, 2.5x45 cm. The proteins were eluted from the column by a gradient of NaCl solution, 0 to 200 mM, with the flow rate of approximately 1ml min^{-1} controlled by a peristaltic pump. The output of the column was monitored spectrometrically at 280nm

2.2.5 MANGO

Prabha *et al.,* 1982 had worked with mango peel to study the presence of PPO. Polyphenoloxidase from mango (Mangifera indica) peel was purified to homogeneity by ammonium sulphate fractionation, chromatography on DEAE-Sephadex and gel filtration of Sephadex G-200. The enzyme had an apparent molecular weight of 136,000. Its pH and temperature optimum were 5.4 and 50°C, respectively. The enzyme possessed catecholase activity and was specific to o dihydroxy phenols. The enzyme also exhibited peroxidases activity. Some non-oxidizable phenolic compounds inhibited the enzyme competitively. High inhibitory effects were also shown by some metal chelators and reducing agents, Mango peel polyphenol oxidase when immobilized onto DEAE Sephadex showed slightly higher Km for catechol and lower pH and temperature optima.

2.2.6 PAPAYA

Shaw *et al.,* 1991 analysed the presence of PPO isoenzymes in root, leaf and stem of papaya plant. The high specific activity of PPO was involved in auxin biosynthesis (Gordon and Paleg, 1961). Therefore, it could be related to the growth and differentiation of papaya. This needs to be further investigated.

2.3 OXIDATION

The reactivity of the *ortho*-quinone products of tyrosinase oxidation of catechols prompted the suggestion that the inactivation was the result of covalent binding to the enzyme and binding to tyrosinase has been demonstrated using radiolabelled substrates. However, the *ortho*-quinone product is also generated by oxidation of monohydric phenols and inactivation is not a feature of cresolase activity. Therefore a direct attack by the quinone product does not explain the inactivation of the enzyme (Land *et al.,* 2008). The browning of mushrooms, fruits and vegetables is the result of the oxidation of phenolic compounds by tyrosinase when cells are broken or damaged. In fungi such as mushrooms, the role of tyrosinase and subsequent melanin production is related to reproductive organ differentiation and spore formation as well as protection of tissues after injury (Parmesha *et al.,* 2011).

2.4 CHARACTERISATION

2.4.1 YAM

Paranjpe 2003 had worked with *Amorphophalus campanulantus* and studied its kinetic characterization.PPO activity in the corm extract was highest with L- DOPA as a substrate. The apparent K_m value of 1.05 mM obtained from the Lineweaver-Burk double reciprocal plots of initial velocity against the substrate concentrations is similar to that reported for tyrosinases from other plant sources. The optimum pH for tyrosinase was observed to be around 6.0 although stable activity was evident in the pH range of 6.0 to 7.0. The optimum temperature was found to be 40ºC.

2.4.2 BANANA:

Wong *et al.,* 1971 worked with banana source. In banana, PPO activity was assay using the diphenols catechol, Dcatechin, 4-methylcatechol, chlorogenic acid, caffeic acid, L-DOPA and dopamine and the monophenols p-cresol and L-tyrosine as substrates. The affinity of PPO (lowest Km) was highest for dopamine, followed by D-catechin. The ratio Vmax/ Km indicates the efficiency of substrate conversion by the enzyme.

Wuyts *et al.,* 2006 had studied the affinity characteristics of PPO from banana root. D-Catechin was the most efficient phenolic substrate, followed by dopamine. L-Dopa and catechol were poor substrates. PPO showed good affinity for the hydroxycinnamic acids caffeic acid and chlorogenic acid, but the reaction was slow which made it less efficient. Affinity for dopamine and efficiency of the reaction of banana root PPO was comparable to this of pulp PPO (K_m = 0.17 mM; V_{max}/K_m = 118 U.mM−1.min−1). D-catechin, on the other hand, is not a good substrate for pulp PPO. Monophenolase activity was detected with p-cresol as substrate, but the reaction did not follow Michaelis-Menten kinetics. L-Tyrosine was not a substrate for banana root PPO.

Sathyanarayana *et al.,* 2011 had found that in banana, the K_m values for catechol, L-DOPA, L-tyrosine, and p-Cresol are 4.12 × 10-3, 16.4 × 10-3, 6.12 × 10-3, and 5.01 × 10-3, respectively. Catechol was the best suited substrate, having the lowest K_m value and highest V_{max} value when compared to other 3 substrates.

2.4.3 Potato and Mushroom

Duckworth *et al.,* 1970 had done a comparative study on potato and mushroom PPOs. When potato and four edible fungi were compared the enzyme followed the Michaelis- Menten kinetics, with the lowest K_m of 0.60mM by mushroom tyrosinase and the highest K_m of 12.6 mM by potato tyrosinase.

Yang *et al.,* 2006 had worked with mushroom tyrosinase. Although the previous studies showed a K_m of 0.26 for mushroom, the difference may be related to the difference in pH used for enzyme assay. Higher K_m represents its lower affinity for substrate.

Altunkaya *et al.,* 2011 had characterized the purified polyphenoloxidase, peroxidase, and lipoxygenase A total of four PPO (PPO1, PPO2, PPO3 and PPO4), one POD and two LOX (LOX1 and LOX2) isoenzymes were obtained by using ion-exchange column chromatography with pH gradient. Isoenzymes having the maximum activities (PPO1, PPO4, POD1, POD2, LOX1 and LOX2) were characterized in terms of pH and temperature optima, kinetic parameters, substrate specificity (for PPO), and the effects of different inhibitors (for PPO and LOX). The pH activity profiles were determined in 0.067 M phosphate buffers at different pH values ranging from pH=4.0 to 9.0. At optimum pH for each enzyme, activities were also determined as a function of temperature ranging from 10 to 70 °C. Substrate specificities and kinetic parameters of PPO were determined for five substrates including catechol, catechin, chlorogenic acid, caffeic acid and gallic acid by means of Michaelis-Menten plots.

2.5 ION EXCHANGE CHROMATOGRAPHY

Haghbeen *et al.,* 2004 had developed a simple preparatory method for purification of tyrosinase from edible mushroom. The frozen mushroom was homogenized in Tris-HCl buffer (50mM, pH 5.8) containing 1 mM of PMSF (Phenyl methyl sulphonyl fluoride). The suspension was stirred for 30 min at room temperature and filtered through a cotton mesh. The resulting supernatant was separated and subjected to ammonium sulfate precipitation. In order to desalt the obtained protein solution from the previous stage, it was chromatographed on a G-50 column (2.6×45cm, Pharmacia) using Tris-HCl buffer (50 mM, pH 5.8) as the mobile phase and

dialysed. The dialyzed protein solution from the previous step was loaded onto ion exchange (DE-52) column and passed through by Tris- HCl buffer (50 mM, pH 6.8). Then, the proteins were eluted from the column using NaCl solution. The collected protein fractions at 150 mM salt eluent were pooled and concentrated by lyophylization. It was redissolved in PBS (Phosphate buffer solution) and was further purified using Sephadex G-200 column.

Altunkaya *et al.,* 2011 had purified polyphenol oxidases from lettuce. DEAE ion-exchange chromatography was selected to remove the contaminating proteins and to purify different oxidative enzymes in lettuce. Purification of enzymes was carried out by contacting an impure liquid enzyme preparation containing enzyme and soluble impurities (hinder the recovery of the enzymes and strongly lower the yield) with DEAE in a column. Thus, the soluble impurities are preferentially adsorbed by DEAE and the adsorbed enzyme is displaced from the DEAE to produce a purified liquid enzyme preparation containing higher enzyme activity than before purification. Enzyme isolation by any of the precipitation methods is normally followed by chromatographic separation. Denaturation or loss in activity of enzymes during extraction could occur. Therefore, adsorption to ion exchanger could be appropriate and can achieve adequate concentration of diluted protein solution.

Bull *et al.,* 1973 worked with *Aspergillus nidulans* and isolated tyrosinase, had studied its kinetics and molecular properties. Removal of polyphenols with 5 % (w/v) polyvinylpyrrolidone (PVP, a soluble preparation of mol. wt 25 000) (Sanderson, 1965) failed to produce activity. In retrospect this latter result was not surprising because the *Aspergillus* phenol oxidase is severely inhibited by PVP. Finally, extracts were passed through beds of dextran gels in an attempt to remove presumed inhibitors. Chromatography of the extract on Sephadex G-25 gel did not result in enzyme recovery in the eluant, however, when a G-200 gel was used, successful production of activity, albeit slight (0.54 u/mg protein), was obtained reproducibly. The enzyme was eluted immediately after the void volume of eluting buffer had passed through the column, which indicated a mol. wt of the order of 500000. Ion-exchange chromatography ultimately proved to be the most effective means of obtaining the phenol oxidase, and the use of anionic exchangers resulted in a considerable one-step purification of the enzyme. The separation achieved on columns of DEAE-cellulose showed the enzyme was absorbed very strongly at pH 8.2 and was eluted only when the sodium chloride gradient approached 500 mM.

2.6 INHIBITORS

Khan 2007 had reviewed the novel tyrosinase inhibitors. Various dermatological disorders, such as melasma, age spots, and sites of actinic damage, arise from the accumulation of an excessive level of epidermal pigmentation. The exploration and characterization of new inhibitors are not only useful for the medicinal purposes, but their potential applications in improving food quality and nutritional value, controlling insect pests, etc., are also important. So it is very important to discover novel and potent inhibitors of the enzyme tyrosinase. It was also recommend preclinical studies, such as in vitro toxicity studies for short- and long-term effects, as well as pharmacokinetic and pharmacodynamic studies, which are important for the development of improved clinically active inhibitors. Toxicity studies were also important to be considered while improving food quality.

Kubo *et al.,* 2003 had studied the tyrosinase inhibition of anisic acid. The inhibition kinetics of tyrosinase by anisic acid was investigated. The oxidation of L-DOPA catalyzed by tyrosinase follows Michaelis-Menten kinetics. The kinetic parameters for this enzyme obtained from a Lineweaver-Burk plot show that *K*m is equal to 0.645 mm and *V*m is equal to 138 mm/min. It should be noted however that tyrosinase catalyzes a reaction between two substrates, a phenolic compound and oxygen, but the assay was carried out in air-saturated aqueous solutions. Therefore, *K*m and *V*m values determined in this condition were only apparent, and the effect of oxygen concentration on these parameters is unknown. The kinetic behavior of the oxidation of l-DOPA by mushroom tyrosinase at different concentrations of anisic acid, indicates that anisic acid is a classical noncompetitive inhibitor to the enzyme. It can decrease the apparent value of *V*m with no effect on that of *K*m. This behavior is observed that anisic acid can combine with both free enzyme and the enzyme-substrate complex, and there are same binding intensity between anisic acid and both of the enzyme forms.

2.7 PRESERVATIVES

Sharma *et al.,* 2010 had reviewed the focuses on natural preservatives. Preservative is a natural or synthetic substance that is added to products such as foods, pharmaceuticals, paints, biological samples etc. to retard spoilage due to whether, microbial growth or undesirable chemical changes. There are basically three types of preservatives used in foods: (i) Antimicrobials (ii) Antioxidants (iii)Antibrowning agents. Phenolic compounds include monophenols (e.g., p cresol), diphenols (e.g. hydroquinone) and triphenols (e.g. gallic acid). Practical use of simple phenols for preservation is found in the application of wood smoke. Their action involves interaction of the cytoplasmic membrane and the activity is selectively increased against gram-positive bacteria and fungi. Hop bitter acids inhibit growth of beer spoilage bacteria by dissipating trans-membrane pH gradient.

Brul *et al.,* 1999 had reviewed the preservative agents in foods and their mode of action. Preservative agents are required to ensure that manufactured foods remain safe and unspoiled. The most common classical preservative agents are the weak organic acids, for example acetic, lactic, benzoic and sorbic acid. These molecules inhibit the outgrowth of both bacterial and fungal cells and sorbic acid is also reported to inhibit the germination and outgrowth of bacterial spores.

2.8 SODIUM BENZOATE

2.8.1 GENERAL:

Wibbertmann *et al.,* 2005 Although undissociated benzoic acid is the more effective antimicrobial agent for preservation purposes, sodium benzoate is used preferably, as it is about 200 times more soluble than benzoic acid. About 0.1% is usually sufficient to preserve a product that has been properly prepared and adjusted to pH 4.5 or below.

2.8.2 PRESERVATIVES

There are basically four generally accepted methods for preserving foodstuffs and these methods rely on one or the other methods of microorganism control. 1) Sterilization by heat or radiation destroys the microorganisms; 2) refrigeration reduces or stops the activity of microorganisms; 3) drying reduces or stops the activity of the microorganism by removing essential water; and 4) chemical preservatives reduce or inhibit the activity of the microorganisms. The addition of chemical preservatives to food is not new and been practiced for centuries.

Some of the most familiar preservation methods, those of brining, pickling with vinegar, smoking, and preserving with sugar solutions, depend upon chemical preservatives. These methods inhibit microorganism activity and retard microorganism growth and multiplication. These methods act in one of two generalized ways: 1) by physically increasing the density of the microorganisms environment (raising the osmotic pressure) or 2) chemically, by a direct inhibiting action on the microorganism themselves. Consequently, chemical preservatives which perform by a direct inhibiting action on the microorganisms themselves are not new. Sodium benzoate is a chemical preservative which in very low concentration inhibits the activity of the microorganisms themselves.

2.8.3 FOOD APPLICATION

A major market for sodium benzoate is as a preservative in the soft drink industry, as a result of the demand for high-fructose corn syrup in carbonated beverages. Sodium benzoate is also widely used as a preservative in pickles, sauces, and fruit juices (Srour, 1998). Benzoic acid and sodium benzoate are used as antimicrobial agents in edible coatings (Baldwin *et al.,* 1995).

2.8.4 PHARMACEUTICAL APPLICATION

Sodium benzoate is also used in pharmaceuticals for preservation purposes (up to 1.0% in liquid medicines) and for therapeutic regimens in the treatment of patients with urea cycle enzymopathies.

2.8.5 BEVERAGES

Sodium benzoate is the standard preservative used in carbonated beverages. Typically 0.03 to 0.08 % is used for the finished products. Sodium benzoate is often used to preserve the flavor syrup prior to the addition of the beverage acidulant. Non-carbonated beverages normally require somewhat higher concentrations of 0.05 to 0.1 % sodium benzoate in the finished products.

2.8.6 CIDER

The shelf life of non-pasteurized cider can be greatly extended by using sodium benzoate and it should be added as soon as the juice is pressed. A slight tang, which many tasters apparently prefer, may be imparted to the cider by concentrations of sodium benzoate as low as 0.04 %.

2.8.7 MARGARINE

Margarine is regulated by standards of identity described in 21 CFR 166.110. sodium benzoate is allowed as a preservative up to 0.1 %. Special attention to the preservation may be required for low salt or salt free margarine, as salt may exhibit a synergistic effect with sodium benzoate.

2.8.8 SYRUPS

Concentrated sugar solutions are somewhat resistant to fermentation under ideal conditions but may be subject to quality deterioration in non-ideal circumstances. Sodium benzoate may be used to inhibit microbial growth in these syrups at levels of about 0.1 % at pH values below 4.5. In chocolate syrups and other fountain syrups with pH values above 4.5, sodium benzoate may be used in conjunction with other preservatives that are more effective in that pH range.

2.8.9 FRUITS, JUICES, AND SALADS

Typically sodium benzoate will be used at levels of 0.05 to 0.1 % to preserve these products. Maraschino cherries are preserved with 0 .05 to 0.1 % sodium benzoate. The shelf life of chilled citrus salads is materially improved by using 0.03 to 0.08% sodium benzoate in the syrup.

2.8.10 OTHER APPLICATIONS

Possibly the largest use of sodium benzoate, accounting for 30–35% of the total demand (about 15 000 tonnes of benzoic acid), is as an anticorrosive, particularly as an additive to automotive engine antifreeze coolants and in other waterborne systems (Scholz & Kortmann, 1991; Srour, 1998). A new use is the formulation of sodium benzoate into plastics such as polypropylene, to improve strength and clarity. Sodium benzoate is used as a stabilizer in photographic baths/processing.

2.8.11 SAFETY

In the United States, benzoic acid and sodium benzoate are on the FDA list of substances that are generally recognized as safe (GRAS). Both may be used as antimicrobial agents, flavouring agents and as adjuvants with a current maximum level of 0.1% in food. The FDA has not determined whether significantly different conditions of use would be GRAS. The FDA has sought fully up-to-date toxicology information (Scientific committee on consumer products 2005).

2.8.12 SOLUBILITY

Hydrotropy refers to the ability of a concentrated solution of a chemical compound to increase the aqueous solubility of another compound. Sodium benzoate, sodium salicylate, sodium acetate, sodium ascorbate, niacinamide and sodium citrate are the most popular examples of hydrotropic agents which have been used to solubilize a large number of poorly water-soluble compounds (Maheshwari *et al.,* 2009).

2.9 BENZOIC ACID AND SODIUM BENZOATE DETERMINATION

Ahmad *et al.,* 2009 had development an one step spectrophotometric method for analysis of benzoic acid and sorbic acid in ointments. Assay was performed in ethanol extract. Recovery percentage was observed from the synthetic mixture and thus, the method was validated.

2.9.1 HPLC

Abdollahpour *et al.,* 2009 developed a simple and sensitive method for detection of sodium benzoate, methylparaben and propylparaben in nystatin suspensions. Nystatin is an antibiotic obtained from actinomycete species *Streptomyces noursei*. In pharmaceutical industries, certain drugs are preserved using sodium benzoate to prevent the microbial contamination on long time storage. Overdose of these preservatives have possible side- effects on humans. Thus, quantification of preservatives in finished products had received close attention. The method used here was reversed phase high performance liquid chromatography with C18 column and a photodiode detector. To optimize the chromatographic separation conditions, various mixtures of acetonitrile and acetate buffer of pH 4.4 were used. The results showed that an increase in the amount of acetonitrile in the solvent mixture decreases the analytes retention times. A binary mixture with an acetonitrile/acetate buffer ratio of 35:65 v/v was then selected as the best practical mobile phase. The flow rate of mobile phase was optimized at 1 ml min-1. The experiments were performed at ambient temperature and under isocratic elution conditions. Under the optimized experimental conditions, separation of the preservatives was achieved in less than 20 min. The limits of quantifications (LOQs) and the linear dynamic ranges (LDRs) of sodium benzoate, methylparaben and propylparaben were 0.3 and 50-1000 µg ml-1, 0.5 and 50-600 µg ml-1 and 0.3 and 50-900 µg ml-1, respectively; the respective precisions (%RSD) at 500 µg ml-1 level were 0.72%, 0.73% and 0.51% (n = 6). The average recoveries of sodium benzoate, methylparaben and propylparaben for spiked nystatin samples were obtained as 98%, 97% and 98%, respectively.

Pylypiw *et al.,* 2000 had presented a method for determination of sodium benzoate and potassium sorbate in various foods. In food, preservatives are used to prevent spoilage by inhibiting yeast, mold and bacterial growth. Under the provisions set forth by Food and Drug Administration (FDA) in the Code of Federal Regulations, food additives can be used if they are generally recognized as safe (GRAS) and are declared on the label.Level of preservatives should be within the prescribed limit, say 0.1% for sodium benzoate and 0.2% for potassium sorbate. The procedure utilizes high performance liquid chromatography (HPLC) followed by UV diode array detection for identification and quantitation of the two preservatives. Liquid samples were prepared by diluting 1 ml of the sample with 10 ml of an acetonitrile /ammonium acetate buffer solution. Samples of viscous or solid foods were prepared by blending the sample with the same buffer solution in a 1:5 ratio followed by a dilution identical to liquid samples. All samples were filtered to remove particulate matter prior to analysis. The HPLC determination of the preservatives was performed using a reversed-phase C column 18 and UV detection at 225 nm for sodium benzoate and 255 nm potassium sorbate. The percentage of preservative in the sample was calculated by external standard using authentic sodium benzoate and potassium sorbate. Apple juice, apple sauce, soy sauce, and peanut butter, spiked at 0.10 and 0.050% for both sodium benzoate and potassium sorbate, yielded recoveries ranging from 82 to 96%. The method can detect 0.0010% (10 mg/ l) of either preservative in a juice matrix.

Tfouni *et al.,* 2002 had determined the level of preservatives in commercial food products using HPLC method of detection. It involved a photodiode detector, C18 column and acetonitrile (HPLC grade)/ ammonium acetate buffered mobile phase. Each group of sample followed a different preparation steps.

Saad *et al.,* 2005 had determined benzoic acid and other preservatives in 67 food stuffs using HPLC method. The sample preparation involved methanol extraction and filtering. Benzoic acid was detected at a wavelength of 254nm using an UV-Visible spectrophotometric detector. C18 column and methanol-acetate buffer at pH 4.4 were used. The mobile phase used was methanol - acetate buffer (pH 4.4) (35:65, v/v) for 9 min, after which it was changed to methanol–acetate buffer (pH 4.4) (50:50, v/v). Peak identification of the preservatives in various foodstuffs was based on the comparison between the retention time of standard compounds and was confirmed by spiking known standard compounds to the sample. Quantification was based on the external standard method using calibration curves fitted by linear regression analysis.

2.9.2 SPECTROPHOTOMETRIC METHOD

The principal problem concerning the application of absorption spectroscopy to the analysis of tryptophan and tyrosine in proteins is the effect of the compact, globular form of most proteins on the absorption spectrum of the component amino acids which absorb in the near - ultraviolet region. The second problem is to select the appropriate model substances, the sum of whose absorption should agree with that of the protein. The problem of the protein structure may be largely resolved by destroying the structure in a concentrated guanidine hydrochloride solution. Numerous studies have shown that proteins are highly unfolded and solvated in solutions of 5-6 M guanidine hydrochloride (Edelhoch *et al.,* 1966, 1967; Palmer *et al.,* 1963). In choosing a model compound for the tryptophanyl chromophore in proteins, the effect of neighboring substituents must be considered. The absorption spectrum of tryptophan is much

more influenced by the charged state of the amino than that of the carboxyl group (Church *et al.,* 1982). Since the tryptophanyl chromophore in a protein will usually have two neighboring peptide groups, a tryptophan derivative whose a-amino and a-carboxyl groups are blocked by peptide linkages should be a suitable model. The a-amino group has almost no effect on the absorption of the tryptophanyl residue when it is separated from the indole chromophore by a glycyl residue as in glycyltryptophan.

Kumari *et al.,* 2010, had developed a spectrophotometric method for the analysis of RB in bulk and in pharmaceutical formulation. The D0 method is useful for tablet formulations where there is no interference of excipients in the absorbance of RB and method D1 and D2 can be utilized for formulations containing any interfering excipients. The developed methods were also validated and from the statistical data, it was found that methods were accurate, precise, reproducible and can be successfully applied to the pharmaceutical formulations without interference of excipients.

Haghbeen *et al.,* 2003 had developed a direct spectrophotometric assay of monooxygenase and oxidase of mushroom tyrosinase in the presence of natural and synthetic substrates.Although p coumaric and caffeic acid can be used for direct kinetic studies of the MT activities, the following reasons have convinced the authors to introduce the analogue pair of MeBAPh and MeBACat for the regular direct assay of the cresolase and catecholase activities of MT, respectively: (a) they are cheaply and easily prepared, (b) they have high extinction coefficients, (c) they have simple and not busy UV–Vis spectra even while reacting with the enzyme, (d) they have fairly fast enzymatic reactions, (e) they are stable solutions at pH 6.8, (f) except for the cosolvent, there is no need for any other substances to be added to the reaction mixtures, and (g) MT does not suffer serious inactivation in the presence of these substrates.

2.9.3 ISOTACHOPHORESIS

Benzoic acid is often used as a preservative in food products. Isotachophoresis was applied to identify and to quantify it simultaneously. The method proposed is characterized by high precision accuracy and simple sample pre-treatment (extraction and filtration). The method was used for determination of benzoic acid in ketchup, jam, marmalade, mustard, etc. If the matrix of the sample is simple, it is possible to used only one electrolyte system in both columns. In the case of the sample with a rather complicated matrix, it is recommended to use two different electrolyte systems for each column to avoid interferences. It is possible to analyse another organic acid (formic, citric, malic) simulteneously.

2.10 ASCORBIC ACID DETERMINATION:

Asami *et al.,* 2003 had studied and compared the total phenolics and ascorbic acid in various crops of different agricultural practises**.** Samples were homogenized for 1 min at maximum speed in a Waring blender. The homogenate (1-5 g) was added to 20 mL of 4.5% metaphosphoric acid and vortexed. Extracts were centrifuged at 1640*g* for 15 min at 20 □C. The supernatant was filtered through a Whatman no. 1 filter and diluted to 25 mL with the 4.5% metaphosphoric acid. AA concentrations were measured according to established methods with minor modifications (*20*). Analysis was performed using a Waters 515 HPLC pump equipped with a Waters 486 tunable absorbance detector (Waters, Milford, MA). Reverse-phase separation was attained using an Agilent (Palo Alto, CA) Zorbax 5 µm Eclipse XDB-C18 (4.6 _ 250 mm). The mobile phase was Nanopure water brought to pH 2.2 with sulfuric acid. The flow rate was 0.5 mL/min, and the detection wavelength was 245 nm. Sample aliquots were filtered through a 0.45 µm poly(tetrafluoroethylene) filter prior to injection. All samples were run in triplicate. The linearity range was determined from 0.005 to 0.04 µg/µL with a 20 µL injection volume (R^2 = 0.9997), yielding an absorbance range of 0.055-0.500 AU.

CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 CHEMICALS

Polyvinyl pyrollidone, Triton X 100, L- DOPA, Sodium Benzoate and other formal analytical chemicals purchased from Himedia.

3.1.2 SAMPLES

Fruits, vegetables and mushroom extracts for enzyme extraction.

Jam, pickle, sauce, and soft drink for testing the preservative concentration.

3.1.3 EQUIPMENTS

Spectrophotometer, pH meter, water bath, Ion exchange column, HPLC column

3.2 METHODS

3.2.1 ISOLATION

Polyphenol oxidase was isolated from 1g of each fresh plant source (apple, banana peel, banana pulp, brinjal, guava, mushroom, potato and papaya) using extraction buffer. The extraction buffer was 0.2M phosphate buffer containing 6% triton X 100 and 1% polyvinyl pyrollidone. The samples were homogenized in 1:10 ratio, filtered through 4-6 layers of cheese cloth and the crude enzyme obtained was stored at -26ºC as 1ml aliquots. The filtrate was then incubated at 4ºC for 10min and was centrifuged at 10,000rpm for 30min (Sanchez-Ferrer *et al.,* 1993). The pellet was discarded and the supernatant was subjected to temperature induced phase partitioning by increasing the triton X 100 concentration by an additional 4% at 4ºC and warming it to RT for 10min. After 10 min the solution became turbid due to aggregation and micelles formation in the detergent rich phase removing hydrophobic protein and phenolic compounds. The solution was again centrifuged at 10,000 rpm for 10min. The pellet was discarded; oily detergent layer in the supernatant was removed carefully and the clear aqueous layer was collected and further purified.

3.2.2 AMMONIUM SULPHATE PRECIPITATION

To the aqueous layer 30% ammonium sulphate was added and dissolved by gentle swirling. It was then centrifuged at 4ºC, 10, 000rpm for 10min. The pellet was dissolved in 5ml PO_4 buffer and was checked for enzyme activity and to the supernatant 60% ammonium sulphate was added mixed and centrifuged at 4ºC. Supenatant was discarded and pellet dissolved in buffer (Nematpour *et al.,* 2008). The pellet with better activity dialysed overnight against 20mM phosphate buffer to remove the protein bound ammonium salt.

3.2.3 ION EXCHANGE CHROMATOGRAPHY

- DEAE sephacel was mixed with water in the ration of 3:1 and is slowly and uniformly packed into the column.

- The column packed with DEAE sephacel was regenerated using buffers as follows: 1). 0.1M Tris HCl containing 0.5M NaCl pH 8 2). 0.1M Sodium acetate containing 0.5M NaCl pH 3.5. 3). 50mM Sodium phosphate buffer pH 6.7
- 1-5% column volume of sample (3ml) was added and is equilibrated using 50mM phosphate buffer. The equilibration buffer dropping out was collected as 3ml fractions and the protein concentration was checked until the values become negative.
- Gradient elution: Initially the column was eluted with 0.2M NaCl (50ml), followed by 0.4M, 0.6M, 0.8M, 1M NaCl (each 50ml) respectively and 85 fractions with 3ml each was collected. Each fraction was checked for protein concentration and enzyme activity.
- Fractions with highest activity were pooled together and it was used for further work.
- The column was washed with 50mM phosphate buffer (Flurkey *et al*., 1999).

3.2.4 ENZYME SPECIFICITY

PPO activity was assayed using five different substrates (20mM prepared to contain 6.6mM in reaction mixture) such as tyrosine, phenol, catechol, L-DOPA, and pyrogallol. Most of the plant PPOs shows catecholase activity compared to cresolase activity that is again proved here. Banana PPO showed its highest activity with L-DOPA and catechol that is , diphenols followed by triphenolase activity with pyrogallol but no activity was found with monophenols say phenol and tyrosine. Also, optimum pH of the enzyme was found to be between 6.5 to 7,based on the literatures and optimum temperature for storage is 4ºC. Enzyme was stable at temperatures below 54ºC but the shelf life decreased.

3.2.5 ASSAY AND PROTEIN ESTIMATION

Presence of polyphenol oxidase was confirmed through enzyme assay involving its specific substrate, L- DOPA (di-phenol oxygenase activity) from the rate of quinone formation. Quinone resulted in reddish brown colour development. 0.1ml of aliquot was added to the reaction mixture containing phosphate buffer, water and the substrate. Change in absorbance was read at 475nm for 2min.

The protein concentration was measured by lowry's method. Standard solutions of bovine serum albumin were prepared at different concentrations to obtain a calibration curve. To 0.1ml of aliquots reagent C was added and incubated for 10min, then followed by folin – ciocalteau reagent incubated for 30min. The occurrence of dark blue colour confirmed the presence of protein and the concentration was observed at 660nm.

3.2.6. KINETIC CHARACTERISATION

PPO activity was assayed using the diphenol L-DOPA with different concentrations ranging from 2mM to 20mM. The effect of substrate concentration in the enzyme was thus observed and Michaelis- Menten constant K_m, maximum velocity of reaction V_{max} were determined by plotting the activity data as a function of substrate concentration according to the method of Lineweaver and Burk (Wuyts *et al.*,. 2006; Duckworth *et al.*, 1970).

3.2.7. APPLICATION

Decrease in enzyme activity in a dose dependent manner was plotted as a standard. Change in inhibition dose when added to a food matrix was observed and a caliberation curve was plotted. Different samples such as jam, pickle, sauce and soft drink preserved using class II preservatives (mostly sodium benzoate) was taken and tested for its extent of enzyme inhibition (Chang, 2009). Extent of inhibition is directly proportional to the presence of total phenolics. Sample preparation involved mixing the sample with methanol, followed by vortexing, centrifugation and ultrasonication.

3.2.8 HPLC

Standard preparation

50mg of sodium benzoate was dissolved in 100ml of deionised water and is used as a stock. The working standards was prepared to have 5µg/ ml, 10 µg/ ml, 15 µg/ ml, 20µg/ml, 25µg/ ml respectively using mobile phase.

Sample preparation

1g of sample (jam, sauce, pickle and soft drink) was weighed. To this add 25ml of methanol was added and degassed in a sonicator. This was followed by vortexing, centrifugation and filtration through 2.5µm membrane filter.

Procedure

C18 column was prewashed using 100% methanol for 1day to obtain baseline correction. Then the column was again washed with mobile phase methanol: acetate (55:45) for10min. 25µl of each standard was injected to obtain a standard chromatogram followed by sample injection.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 ISOLATION OF PPO

Table 4.1 Comparison of PPO activity from various sources

S.NO	SPECIES	TOTAL ACTIVITY(U)	SPECIFIC ACTIVITY(U/mg)
1	Apple	10.50	8.30
2	Banana peel	221.55	169.11
3	Banana pulp	180.85	50.04
4	Brinjal	204.05	84.23
5	Guava	NA	NA
6	Mushroom	46.55	82.35
7	Papaya peel	144.85	14.32
8	Potato	454.00	166.49

Plant sources such as Apple, Banana, Brinjal, Guava, Papaya, Potato and a fungal source Mushroom were weighed to contain 10g of fresh weight each. Phosphate buffer containing 6% Triton X 100 and 1% PVPP was used as a extraction buffer. The extraction buffer was added to a sample (say apple) in the ratio of 1:10. Using mortar and pestle, the sample was finely ground. It was then filtered via 4-6 layers of cheese cloth and the volume of homogenate was measured. The same was repeated in all other sources. Enzyme activity was calculated using the formula given below:

$$E\ (U/ml) = \frac{OD/min \times Volume\ of\ reaction\ mixture\ (ml) \times dilution\ factor}{Extincition\ coefficient \times volume\ of\ enzyme\ in\ test\ sample\ (ml)}$$

Similarly, total activity and specific activity were calculated for each sample using the formula:

$$TA\ (U) = E \times volume\ of\ homogenate\ in\ ml$$

S.NO.	SPECIES	ACTIVITIES IN AMMONIUM SULPHATE PRECIPITATED FRACTIONS			
		TOTAL ACTIVITY (U)		SPECIFIC ACTIVITY (U/mg)	
		30%	60%	30%	60%

1	Apple	3.30	NA	NA	3.80
2	Banana peel	64.00	9.15	366.80	49.60
3	Banana pulp	43.33	28.33	73.17	68.18
4	Brinjal	87.50	3.50	96.89	52.15
5	Guava	2.65	NA	NA	1.43
6	Mushroom	14.00	6.65	24.54	12.77
7	Papaya peel	14.83	11.65	8.89	11.99
8	Potato	81.50	70.80	359.97	149.4

SA = E/(mg of protein)

Table 4.2 Activity of tyrosinase in ammonium sulphate precipitated fractions

The crude extract was then purified using Ammonium Sulphate precipitation method. Purification was done with 30% ammonium sulphate added to the crude and it was centrifuged at 10,000rpm for 10min. followed by 60% and 90% concentrations respectively. 90% pellet doesnot show any activity and so its results were not considered. Enzyme activity and specific activity for the 30% and 60% pellet dissolved in PO_4 buffer were calculated by the above formula. Banana peel showed the highest activity followed by potato, banana pulp, brinjal, mushroom and papaya peel respectively. Nearly no activity was found in apple and guava in the partially purified state. Source with highest activity was used in further proceedings.

Table 4.3 Specificity of banana peel polyphenol oxidase

S.NO	PHENOLIC COMPOUND	WAVELENGTH (nm)	ENZYME ACTIVITY *(U)
1	Catechol (diphenol)	420	9
2	L-DOPA (diphenol)	475	8
3	Pyrogallol (triphenol)	420	1.5
4	Tyrosine (monophenol)	420	0
5	Phenol (monophenol)	390	0

* phenolics were added at 20mM concentration (6.67mM in reaction mixture)

Substrate specificity of the enzyme based on activity was studied through the assay procedure. The activity is dependent on the substrate concentration and affinity of substrate towards the enzyme. The five substrates categorized to be monophenols such as L-Tyrosine, phenol., diphenols such as L-DOPA, catechol., and a triphenol pyrogallol were considered. Quinone formation per minute was observed for each substrate based on which enzyme activity was calculated. The efficiency of substrate conversion by enzyme was high with diphenols. Monophenolase activity was poor or nil and that of triphenol had a very minute activity and were not specific substrates for banana PPO.

Wuyts *et al.,* had studied the substrate specificity of enzyme through Michaelis- Menton kinetics with substrates such as L-cresol, L-tyrosine, D-catechin, dopamine, hydroxycinnamic acid, caffeic acid and chlorogenic acid. From this study we may find that banana PPO had highest affinity for diphenols and also, no monophenol specificity was found. Monophenols did not follow the enzyme kinetics. They also had concluded that L-DOPA and catechol were poor substrates. This may be due to the variations in isoenzymes of PPO in banana root and banana peel.

4.2 PURIFICATION OF PPO

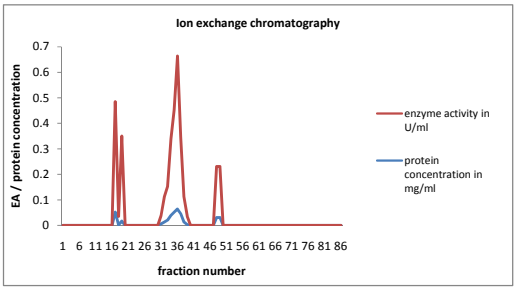


Fig 4.1 Purification chart for PPO

Anion exchange chromatography was done for further purification. The column packed with DEAE sephacel was regenerated, equilibrated with sample and the sample was eluted by using sodium chloride salt of different concentration ranging from 0.2- 1 M. Eluted samples were collected as 3ml per fraction and total number of fractions counted 84. The fig shows the DEAE-sephacel chromatographic pattern of banana peel PPO. Protein concentration was estimated from OD_{260nm} and OD_{280nm} values. Enzyme activity was assayed and observed at 475nm. 3ml fractions were collected. The linear buffer system used to elute the protein were: 1. 50mM PO_4 containing 0.2M NaCl 2. 50mM PO_4 containing 0.4M NaCl 3.50mM PO_4 containing 0.6M NaCl 4.50mM PO_4 containing 0.8M NaCl 4.50mM PO_4 containing 1M NaCl respectively. Three isoenzymes were obtained with 1st, 2nd and 3rd gradients of elution whereas, in the 4th and 5th gadients the fractions observed negative at 280nm. Each fraction was observed at 280nm and 260nm and were checked for Enzyme activity and specific activity. Protein concentration was found using the formula :

Concentration in mg/ml = (A280nm x 1.55) – (A260nm x 0.75).

Kong *et al.,* 1998 had purified PPO from pine needles using DEAE sephacel column chromatography. From the eluted fractions three isoenzymes were separated with 74, 50 and 59 fold increase in activity. This was because the dialysed protein were loaded on to the sephacryl S-200 and Sephadex G-75 for removing other protein impurities before being purified using DEAE-sephacel.

Table 4.4 Purification of enzyme in each stage

S.NO.	SAMPLE	TOTAL ACTIVITY (U)	SPECIFIC ACTIVITY (U/mg)	FOLD PURIFICATION
1	Crude	221.55	69.11	-
2	Ammonium sulphate precipitated fraction	65	89.60	1.29
3	Dialysed fraction	40	138	1.54
4	Ion exchange purification	34	224.2	1.6

Altunkaya *et al.,* 2009 purified PPO from lettuce using DEAE cellulose. Purification resulted in four isoenzymes with 1413, 392, 527, 767 fold purifications respectively. Razzaque *et al.,* 2000 had purified the enzyme using DEAE – cellulose with the fold purification of 16.4 followed by gel filtration. Change in fold purification may be due to lack of pretreatments, and enzyme source.

4.3 KINETIC CHARACTERIZATION OF PURIFIED ENZYME

Kinetic characteristics of enzyme based on substrate concentration ranging from 2mM to 20mM was performed and it varied with substrate and inhibitor.

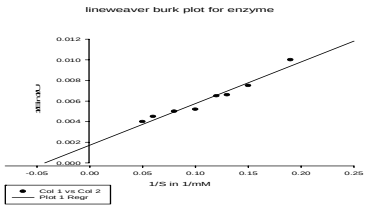


Fig 4.2: LB plot showing enzyme kinetics

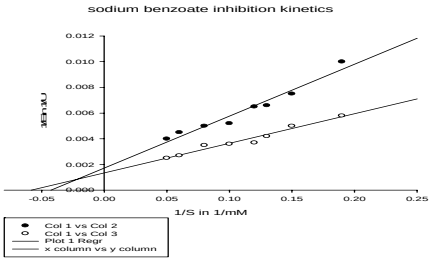


Fig 4.3 LB plot showing inhibition kinetics for sodium benzoate

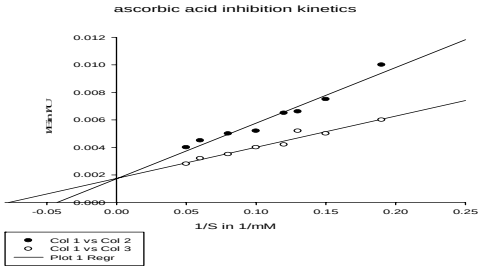


Fig 4.4: LB plot showing inhibition kinetics for ascorbic acid

Table 4.5 Kinetic characteristics

parameter	Enyme	Sodium benzoate	Ascorbic acid
V_{max} in U	588.23	625	588.23
K_m (or K_i) in Mm	24.39	16.39	13.39

PUBISHED LITERATURE OF PPO KINETIC CHARACTERISTICS

REFERENCES	Km in mM	pH	TEMPERATURE in ºC	SOURCE
Wong *et al.*, 1970	4isoenzymes: 6.6,4.2,7,36	6.8, 6.5, 7.2, 7	55	Peach with catechol as substrate
Duckworth and Coleman 1970	0.263	7.02	-	Mushroom with L-DOPA as substrate
Wuyts *et al.*, 2006	69 and 56.4	7	-	Banana with catechol and L-DOPA respectively
Sathyanarayana *et al.*, 2011	16.4 x 10^{-6}	6,5- 7	30- 40	Banana with L-DOPA as substrate
Altunkaya and Gokmen 2010	15	6	30	Lettuce with catechol as substrate

4.4 INHIBITION STUDIES

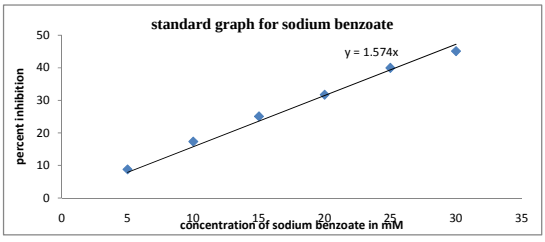


Fig 4.5 standard graph for sodium benzoate

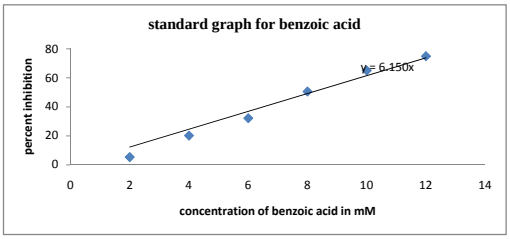


Fig 4.6: standard graph for benzoic acid

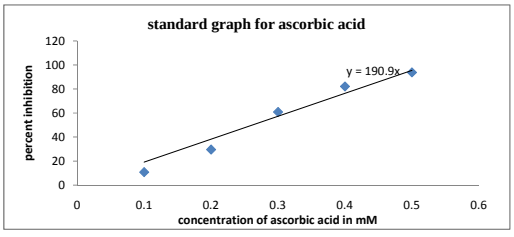


Fig: 4.7 standard graph for ascorbic acid

4.5 PERCENT INHIIBITION IN FOOD MATRICES

Table 4.6: Inhibition percentage of sodium benzoate in different food matrices

Concentration (mM)	%I	%I in Rice	%I in pea	%I in dal	%I in mixture
5	8.79	17.15	4.58	6.94	4.78
10	17.34	19.89	25	25	11.93
15	24.09	22.81	34.75	33.33	17.4
20	31.75	17.15	37.5	37.5	23.89
25	39.99	28.52	43.1	44.42	29.5
30	44.14	37.04	48.6	50	36.21

Table 4.7 Inhibition percentage of benzoic acid in different food matrices

Concentration (mM)	%I	%I in Rice	%I in pea	%I in dal
2	4.29	36.76	20.45	23.99
4	20.11	40.51	31.16	29.99
6	32.15	47.89	41.94	44.99
8	50.54	50.57	46.26	50.99
10	64.08	51.42	54.84	59.03
12	74.98	59.05	64.61	64.98

Table 4.8 Inhibition % of ascorbic acid in individual food matrices

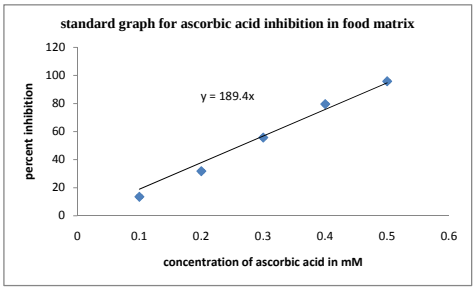


Fig 4.9: Dose dependent inhibition by ascorbic acid in food matrix

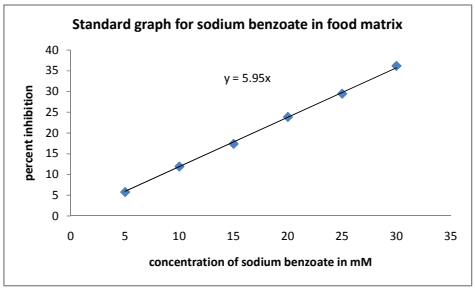


Fig 4.10 Dose dependent inhibition by sodium benzoate in food matrix

4.6 APPLICATION:

0.1	10.78	20.68	24.77	24.28
0.2	29.56	34.48	33.61	27.36
0.3	60.85	49.45	73.59	44.21
0.4	82.14	70.13	96	72.68
0.5	93.81	94.4	98.4	93.68

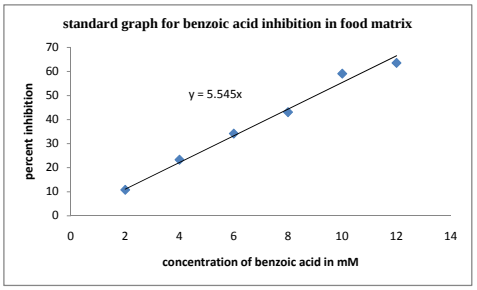


Fig 4.8 Dose dependent inhibition by benzoic acid in food matrix

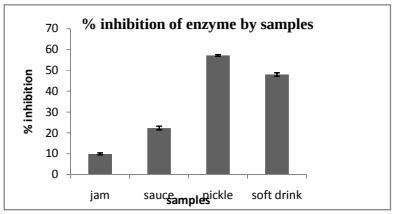


Fig 4.11 Percentage inhibition of enzyme by samples

Table 4.9 Concentration of sodium benzoate obtained from standard graph.

PRODUCTS	CONCENTRATION OF SODIUM BENZOATE	
	mM	mg/g
Jam	1.66	4.98
Sauce	3.74	13.5
Pickle	9.6	34.6
Soft drink	8.06	29.1

4.7 HPLC RESULTS

The C18 column was optimized for separation conditions using 100% methanol, followed by mobile phase. A binary mixture with a methanol: acetate buffer mixture of 55: 45 was used.

20µl of standard solution (5, 10, 15, 20 and 25µg/ml) was injected and the change in response area was monitored at 225nm. Ideal retention time for sodium benzoate was found to be around 17min with the flow rate of 0.5ml/min. Abdollahpour *et al.,* 2010 had separated preservatives including sodium benzoate using a mobile phase Acetonitrile: acetate with the buffer ratio of 35:65 within 16min with the flowrate of 1ml/min and the change was monitored at 254nm.

4.7.1 HPLC standard graph for sodium benzoate:

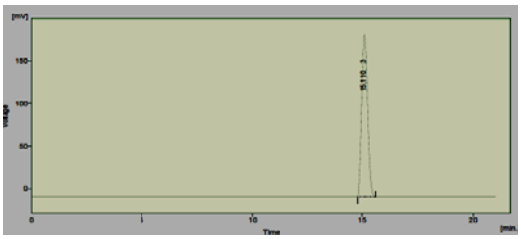


Fig 4.12 Sodium benzoate standard 20µg/m

4.7.2 HPLC chromatogram for samples

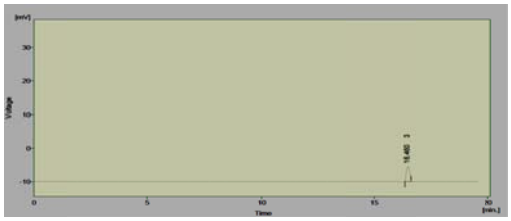


Fig 4.13 Sample jam

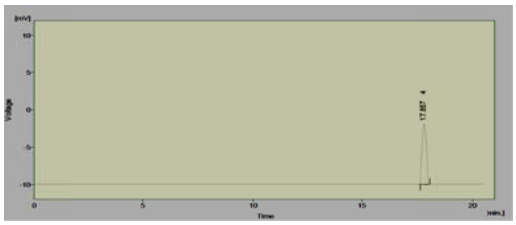


Fig 4.14 Sample sauce

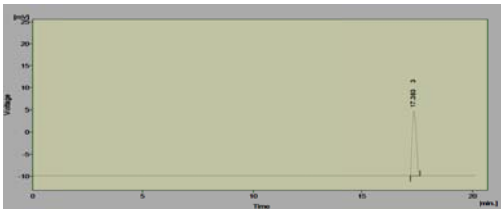


Fig 4.15 Sample pickle

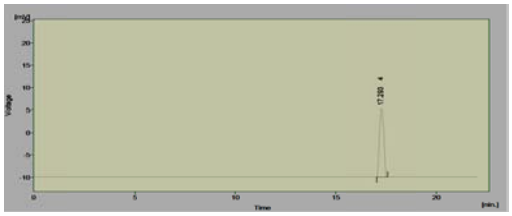


Fig 4.16 Sample soft drink

Table 4.10 HPLC chromatogram data

Standard (µg/ml)/ Sample	Area (mV.s)	Height (mV)	Area in %	Height in %	W05 (min)
5	1017.558	50.138	46.2	31.9	0.33
10	1684.21	93.467	62.5	48.9	.029
15	2646.223	136.2	71.3	58.9	0.32
20	3512.253	190.263	79.4	66.9	0.30
25	4684.855	238.959	84.5	73.3	0.31
Jam	44.919	4.435	6.2	6.3	0.18
Sauce	109.739	8.106	36.7	28.1	0.24
Pickle	227.448	14.257	33.2	24.9	0.26
Soft drink	210.898	14.710	31.8	24.4	0.25

From HPLC data area in mV.s was plotted against the concentration in µg/ml and was used as a standard graph to find the concentration sodium benzoate in unknown samples.

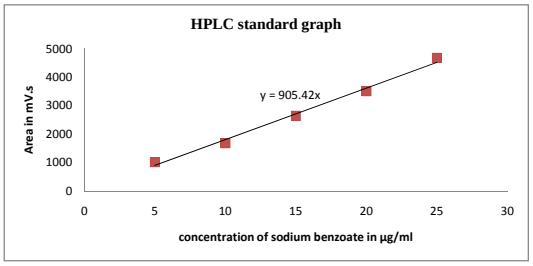


Fig 4.17 Linear curve – HPLC standard

Area of unknown sample was used to find the concentration of sodium benzoate in µg/ml and it was calculated for 1g of sample. Concentration of sodium benzoate in mg/g was compared between enzymatic method and HPLC technique.

Table 4.11 Detection of phenolics using enzymatic method – validation

Samples	**Concentration of sodium benzoate in mg/g**	
	HPLC method	**Enzymatic method**
Jam	4.1	4.98
Sauce	12	13.5
Pickle	25	34.6
Soft drink	23	29.1

CHAPTER 6
CONCLUSIONS

PPO activity was found to be highest in banana peel followed by potato. Thus, banana peel PPO was chosen for further studies. Banana peel PPO was purified by ammonium sulphate precipitation and ion exchange chromatography and resulted in 1.54 and 1.6 fold increase in activity respectively. Enzyme kinetics and inhibition kinetics for sodium benzoate and ascorbic acid with varying substrate concentrations were observed and found to have K_m =24.39mM, K_i = 16.39mM and K_i =13.39mM respectively. The type of inhibition was mixed for sodium benzoate and competitive for ascorbic acid. PPO inhibition with ascorbic acid, benzoic acid and sodium benzoate was dose dependent. In food matrices, the percent inhibition was comparatively less with respect to direct assay. Phenolic content present in commercial products such as jam, sauce, pickle and soft drink were determined using the enzyme PPO. Results obtained from HPLC and results obtained from enzymatic method were found to be similar and thus, the method is validated.

REFERENCES

Abdollahpour, A., Forouhi , M., Shamsipur, M. and Yamini, Y. (2010) 'High Performance Liquid Chromatographic Determination of Sodium Benzoate, Methylparaben and Propylparaben as Preservative Components in Nystatin Suspensions', J. Iran. Chem. Soc., Vol. 7, No. 2, pp:516-520.

Ahmad, I. and Hm-vaid, F. (2009) 'Determination of benzoic acid and salicylic acid in commercial benzoic and salicylic acids ointment by spectrophotometric method', Pak.J. Pharm. Sci., Vol.22, No.1, jan. 2009, pp.18-22

Altunkaya, A. and Gökmen, V. (2011) 'Purification and Characterization of Polyphenol Oxidase, Peroxidase and Lipoxygenase from Freshly Cut Lettuce (L. sativa)', Food Technol. Biotechnol., Vol 49 (2), pp: 249–256.

Aniszewski, T., Lieberei, R. and Gulewicz, K. (2008) 'Research on catecholases, laccases and cresolases in plants', Acta Biologica Cracoviensia series Botanica 50/1 pp: 1- 18.

Babendure, J., Bartzat, A., DeAngelis, M., Gastil, H., Lee, S. G., Liwanag, H. and Lyman, J. (2011)'Enzyme substrate reaction', Science bridge, pp: 08-12.

Bouhilloux, S., Mcmahill, P. and Mason H.S. (1963) 'The multiple forms of mushroom tyrosinase- purification and molecular properties of enzyme', J.biolog. chem., Vol.238.

Bull, A.T. and Carter, L.A. (1973) 'The Isolation of Tyrosinase from Aspergillus nidulans, Its Kinetic and Molecular Properties and Some Consideration of Its Activity in vivo', J. general Microbiol., pp: 61-73.

Chang,T.(2009) 'An Updated Review of Tyrosinase Inhibitors', International J. Molecular Sciences, pp: 2440-2475.

Church, F. C., Swaisgood, H. E., Porter, D. H. and Catignani, G. L. (1982) 'Spectrophotometric Assay Using o-Phthaldialdehyde for Determination of Proteolysis in milk and isolated milk proteins', J. Dairy Sci., Vol 66, pp:1219-1227.

Duckworth, H. W. and Coleman, J. E.,(1970) 'Physicochemical and kinetic properties of mushroom tyrosinase', J. Biol. Chem., Vol 245, pp:1613-1625.

Durán, N., Rosa, M. A., D'Annibale, A. and Gianfreda, L. (2002) 'Applications of laccases and tyrosinases (phenoloxidases) immobilized on different supports: a review', Enzyme and Microbial Technol., Vol 31, pp:907-931.

Edelhoch, H. (1967) 'Spectroscopic Determination of Tryptophan and Tyrosine in Proteins', Biochemistry Vol.6.

Flurkey, A., Cooksey, J., Reddy, A., Spoonmore, K., Rescigno, A., Inlow, J. and Flurkey, W. H. (2008)' Enzyme,Protein, Carbohydrate and Phenolic Contaminants in Commercial Tyrosinase Preparations: Potential Problems Affecting Tyrosinase Activity and Inhibition Studies', J. Agric. Food. Chem. Vol 56, pp: 4760-4768.

Gordon, S. A. and Paleg, L. G. (1961)'Formation of auxin from tryptophan through action of polyphenols', Plant physiol., Vol 36, pp: 838- 845.

Haghbeen, K., Jazii, F.R., Karkhane, A. and Borojerdi, S. (2004) 'Purification of Tyrosinase From Edible Mushroom', Iranian J. biotechnol, Vol. 2, No. 3.

Haghbeen, K. and Tan, E. (2003) 'Direct spectrophotometric assay of monooxygenase and oxidase activities of mushroom tyrosinase in the presence of synthetic and natural substrates', Analytical Biochem., Vol 312, pp:23-32.

Khan, M. T. H. (2007) 'Molecular design of tyrosinase inhibitors: A critical review of promising novel inhibitors from synthetic origins', Pure Application Chemistry., Vol. 79, No. 12, pp: 2277-2295.

Kong, K., Lee, J., Park, H. and Cho, S. (1998)' Purification and characterization of the tyrosinase isozymes of pine needles',J. Biochem. Mol. Biol. International., Vol 45 pp 717-724.

Kubo, I., Chen, Q., Nihei, K., Caldero´ n, J. S. and Ce´spedes, C. L. (2003)' Tyrosinase Inhibition Kinetics of Anisic Acid', Z. Naturforsch, Vol 58c, PP:713-718.

Kumari, A. S., Subhasish, S. and Kaushik, D. K. (2010) 'UV-Spectroscopic methods for estimation of Rizatriptan benzoate in pharmaceutical preparations', International J. Chem. Tech Research., pp: 653-659.

Kwon, D. and Kim, W. (1996) 'Purification of the glycosylated polyphenol oxidase from potato tuber', J. Biochem. Mol. Biol., Vol. 29, pp: 163-168.

Land, E. J., Ramsden, C. A., Riley, P. A. and Stratfordc, M. R. L. (2008) 'Studies of *para* quinomethane formation during the tyrosinase-catalyzed oxidation of 4-alkylcatechols', Arkivoc, pp: 258-267.

Razzaque, M.A., Saud, Z.A., Absar, N., Karim, M.R. and Hashinaga, F. (2000) 'Purification and characterization of polyphenoloxidase from guava infected with fruit-rot disease', Pakistan J. Biol. Sci. Vol 3, pp: 407-410.

Maheshwari, R. K., Singh, R. M., Vineet, M., Yamini, S. and Amit, G. (2009) 'Quantitative Estimation of Benzoic acid in bulk sample using Sodium Benzoate and Sodium Salicylate as Hydrotropic solubilizing agent', Pharma research, Vol 2, pp: 1-7.

Makino, N. and Masox, H. S. (1973) 'Reactivity of oxy-tyrosinase toward substrates', J. biol.

Chem., Vol 248, pp: 5731-5735.

Manach, C., Scalbert, A., Monard, C., Rémésy, C. and Jime´nez, L. (2004) 'Polyphenols: food sources and bioavailability', Am. J. Clin. Nutr. Vol. 79: pp727– 47.

McCaig T. N., Fenn D.Y. K. , Knox R. E., DePauw R. M.,Clarke J. M. and McLeod, J. G. (1999) 'Measuring polyphenol oxidase activity in a wheat breeding program', Canada J. Bioscience.

Minelli, A., Charteris, A. T., Voltattorni, B. C. and John, R. A. (1979) 'Reactions of DOPA (3,4-Dihydroxyphenylalanine) Decarboxylase with DOPA', J. Biochem., Vol 183, pp: 361-368.

Naish-Byfield, S. and Riley P.A. (1992) 'Oxidation of monohydric phenol substrates by tyrosinase- an oximetric study', Biochemistry journal, Vol 288, pp: 63-67.

Nematpour, F. S., Haghbeen, K., Babaei, M. K., Jazii, F. R., Nouraeen, M.B. and Yancheshmeh, M. B. (2008) 'The Banana pulp polyphenol oxidase is a tyrosinase', J. Biol. Sci.

Palmer, J. K. (1963) 'Banana Polyphenoloxidase. Preparation and Properties' , Plant physiology, Central Research Laboratories.

Paranjpe, P. S., Karve, M. S. and Padhye, S. B. (2002) 'Characterisation of tyrosinase and accompanying laccase from *Amorphophallus campanulatus',* Indian J. Biochem. Biophy., Vol 40, pp: 40-45

Pylypiw, H. M. and Grether, M. T. (2000) 'Rapid high-performance liquid chromatography method for the analysis of sodium benzoate and potassium sorbate in foods', J. Chromatog. A,, pp: 299–304.

Saad, B., Baria, F., Saleh, M. I., Ahmad, K. and Talib, M. K. (2005) 'Simultaneous determination of preservatives (benzoic acid, sorbic acid, methylparaben and propylparaben) in foodstuffs using high-performance liquid chromatography, J. Chromatogr. A, 1073 393–39.

Sanchez-Ferrer, A., Laveda, F. and Garcia-Carmona, F. (1993) 'Partial purification of soluble potato polyphenol oxidase by partitioning in an aqueous two-phase system', *J. Agric. Food Chem.,* Vol 41 (8), pp:1219–1224

Sanderson,G. (1965)'The action of polyphenolic compounds on enzymes'. Biochemical Journal, Vol 95, pp:2 4.

Sathyanarayana, R., Kumari, V., Ramesh, C.K., Parmesha, M., Habeebulla, M. and Khan, M. (2011) 'Characterization of Poly phenol oxidase in two in vitro regenerated cultivators of *Mucuna: Mucuna pruriens L. and Mucuna prurita H.',* Turkey Journal of Biology.

Scalbert, A. and Williamson, G. (2000) 'Dietary intake and Bioavailability of polyphenols', J. Nutr. Vol 130, pp: 2073S—2085S.

Selinheimo, E. (2008) 'Tyrosinase and laccase as novel crosslinking tools for food biopolymers', VTT Publications, Vol 693.

Seo, S., Sharma, V. K.,and Sharma, N. (2003)'Mushroom Tyrosinase: Recent Prospects', J. Agri. Food Chem., Vol 51, pp: 2837-2853.

Sezgintürk, M. K., Göktugw, T. and Dinçkaya, E. (2005) 'Detection of Benzoic Acid by an Inhibitor Biosensor', Food Technol., Biotechnology, Vol 43 (4), pp: 329–334.

Shaw, J., Chao, L. and Chen, M. (1991) 'Isozymes of papaya polyphenol oxidase', Bot. Bul. Academia Sinica, Vol 32, pp: 259-263.

Siegbahn, P. E. M. (2003)' The catalytic cycle of tyrosinase: peroxide attack on the phenolate ring followed by O-O bond cleavage', J. biochem. inorganic chem., pp: 567.

Smit, N., Vicanova, J. and Pavel, S. (2009) 'The Hunt for Natural Skin Whitening Agents',

International J. Molecular Sciences, pp: 5326-5349.

Sudershan, R. V., Pratima, R. and Polasa, K. (2009) 'Food safety research in India: a review', Asian J. Food and Agro-Industry, Vol 2(03), pp: 412-433.

Sugumaran, M., Dali, H. and Semensi, V. (1991) 'The mechanism of tyrosinase-catalysed oxidative decarboxylation of α-(3,4-dihydroxyphenyl)-lactic acid', J. Biochem., Vol 277, pp: 849-853.

Tfouni, S.A.V. and Toledo, M.C.F. (2002) ' Determination of benzoic and sorbic acids in Brazilian food', Food Control, Vol 13, pp: 117–123

Thygesen, P.T., Dry, L. B. and Robinson, S. P. (1995) 'Polyphenol Oxidase in Potato', Plant Physiology, Vol 109, pp: 525-531.

Tomita, Y., Hariu, A., Mizuno, C. and Seiji, M. (1980) 'Inactivation of tyrosinase by DOPA', J. Investigative Dermatology, Vol 75, pp: 379-382.

Wibbertmann, A., Kielhorn, J., Koennecker, G., Mangelsdorf,I. and Melber,C. (2005) 'Benzoic acid and Sodium Benzoate', Concise International Chemical Assessment Document no. 26.

Wittenberg, C. and Edward, L. T. (1985) 'A detergent activated tyrosinase from Xenopus laevis-Purification and partial characterization', J. biological chemistry Vol 280, pp: 12535-1241.

Wong, T. C., Luh, B.S. and Whitaker, J. R. (1971) 'Isolation and characterization of polyphenol oxidase, Isozymes of Clingstone peach', Plant Physiology, pp: 19-23

Wuyts, N., Waele, D. and Swennen, R. (2006) 'Extraction and partial characterization of polyphenol oxidase from banana (Musa acuminata Grande naine) roots', Plant Physiol. Biochem., Vol 44, pp: 308–314.

Yang, Z. and Wu, F., (2006) 'Catalytic properties of tyrosinase from potato and edible',

Biotechnology pp: 344-348.

CURRICULUM VITAE

NAME	:	N.Nagajothi
REGISTRATION No.	:	1020203009
E-MAIL	:	jothi.knv@gmail.com
MOBILE No.	:	9677719101
PERMANANT ADDRESS	:	No.3/161,Vellusamy nagar, Sambakudi via, Nagamalai, Madurai-625 019.
UNDERGRADUATION	:	B.Tech
Course	:	Biotechnology
College	:	Kamaraj College of Engineering and Technology, Virudhunagar
Year	:	2006-2010

PARTICIPATION IN

i) Conference/Seminar: Foodxplore'11, TNAU; NSEB 2012, Periyar University; Confluence 2012, Kumaraguru College of Technology.
ii) Workshop :

SIGNATURE