ISOLATION, CHARACTERIZATION AND IMMOBILIZATION OF POLYPHENOL OXIDASES FROM MULBERRY (*Morus alba*) LEAF TISSUES

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Approval of the Graduate School of Natural and Applied Sciences.

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This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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ABSTRACT

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In this study, the aim was to find an economical plant source for polyphenol oxidase (PPO) production as an alternative to mushroom and possible application areas by characterization and immobilization of the PPOs. For this purpose, tissues of various plants of no commercial value were screened for their PPO activities. Mulberry leaf tissues showed the highest PPO activity against 4-methyl catechol which was comparable to that of mushroom. Average K_m and V_{max} values of free mulberry leaf PPOs were found as 7 mM and 218 U/ml, respectively. Mulberry leaf PPOs were immobilized in a polypyrole matrix and the K_m and V_{max} values of immobilized PPOs were calculated as 35 mM and 3 U/ml, respectively. Mulberry leaf PPO was the most active at 45°C and pH 7. By using electrophoretic analysis,

laccase and catechol oxidase type activities of PPOs and in addition, peroxidase activity were detected. Molecular weights of laccase, peroxidase and catechol oxidase were found to be about 62, 64 and 62-64 kDa, with pI values of 8.0-8.5, 4.5 and 10, sequentially.

Keywords: Enzyme isolation, polyphenol oxidase, laccase, peroxidase, catechol oxidase, tyrosinase, immobilization, biochemical characterization.

ÖZ

DUT (*Morus alba*) YAPRAK DOKULARINDAN POLİFENOL OKSİDAZ İZOLASYONU, KARAKTERİZASYONU VE İMMOBİLİZASYONU

Sutay, Didem
Yüksek Lisans, Kimya Mühendisliği Bölümü
Tez Yöneticisi: Prof. Dr. Ufuk Bakır

Temmuz 2003, 98 sayfa

Bu çalışmanın amacı, mantara alternatif olabilecek ekonomik bir bitkisel PPO kaynağının bulunması ve enzimin olası uygulama alanlarının bulunması için karakterizasyonu ve tutuklanmasıdır. Bu amaçla, ticari değeri olmayan değişik bitkisel dokular PPO aktiviteleri açısından taranmıştır. Dut yapraklarının 4-metil katekol substratı ile mantarla karşılaştırılabilecek düzeyde yüksek PPO aktivitesine sahip olduğu saptanmıştır. Serbest dut yaprağı PPOlarının ortalama K_m ve V_{max} değerleri 7 mM ve 218 U/ml olarak bulunmuştur. Dut yaprağı PPOları polipirol matris içine tutuklanmış, K_m ve V_{max} değerleri 35 mM ve 3 U/ml olarak tespit edilmiştir. Dut yaprağı PPOlarının en yüksek aktivite gösterdikleri sıcaklık ve pH sırasıyla, 45°C ve pH 7 dir. Aktivite boyaması ile lakkaz, peroksidaz ve katekol

oksidaz aktiviteleri tespit edilmiştir. Lakkazın, peroksidazın ve katekol oksidazın moleküler ağırlıkları sırasıyla yaklaşık 62, 64 ve 62-64 kDa olarak tespit edilmiştir. İzoelektrik nokta incelemesiyle de lakkaz, peroksidaz ve katekol oksidazın izoelektrik noktaları (pI) sırasıyla yaklaşık 8.0-8.5, 4.5 ve 10 olarak bulunmuştur.

Anahtar Sözcükler: Enzim izolasyonu, polifenol oksidaz, lakkaz, peroksidaz, katekol oksidaz, tirosinaz, tutuklama, biyokimyasal karakterizasyon.

To My Parents

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LIST OF ABBREVIATIONS

ADA : 4-amino-*N*,*N*-diethylaniline

APS : Ammonium persulfate

BSA : Bovine serum albumin

CaA : Calcium aluminosilicate

CMC : Carboxymethylcellulose

CPE : Carbon paste electrode

CPO : Chloroperoxidase

FMN : Riboflavin-5-phosphate

H_2O_2 : Hydrogen peroxide

HRP : Horseradish peroxidase

ITO : Indium titanium oxide

L-DOPA : L-3,4-dihydroxyphenylalanine

LiP : Lignin peroxidase

MC : 4-methyl catechol

MM : Menthyl monomer

MnP : Manganese peroxidase

NaA : Sodium aluminosilicate

PCS : Poly(carbamoylsulfonate)

PPO : Polyphenol oxidase

PPy : Polypyrole

PVA-SbQ : Poly(vinyl alcohol) bearing strylpyridinium groups

PVPP : Polyvinyl polypyrolidone

Py : Pyrole

R_f : Relative mobility

SDS : Sodium dodecyl sulphate

SDS-PAGE : Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis

SGE : Solid graphite electrode

SPCE : Screen printed carbon electrodes

*t*BC : 4-tert-butyl catechol

TEMED : *N*,*N*-tetramethylene-ethylenediamine

TPMMA : Thiopene capped poly(methyl methacrylate)

TX : Triton X

U : Enzyme activity unit

CHAPTER 1

INTRODUCTION

Polyphenol oxidases (PPOs) can be divided into two subclasses; tyrosinase and laccase. Tyrosinase (E.C. 1.14.18.1, monophenol monooxygenase and E.C. 1.10.3.1 *o*-diphenol oxidoreductase or catechol oxidase), is an enzyme which catalyses two reactions, the hydroxylation of monophenols to *o*-diphenols (Figure 1.1) and the oxidation of *o*-diphenols to *o*-quinones (Figure 1.2) utilizing molecular oxygen (Durán and Esposito, 2000).

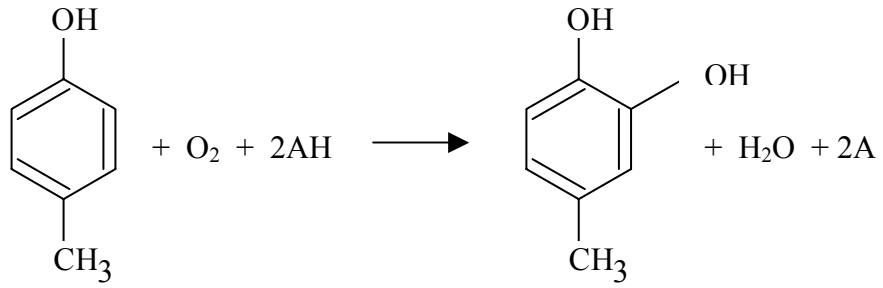

Figure 1.1 : Hydroxylation of monophenols to *o*-diphenols by PPO activity

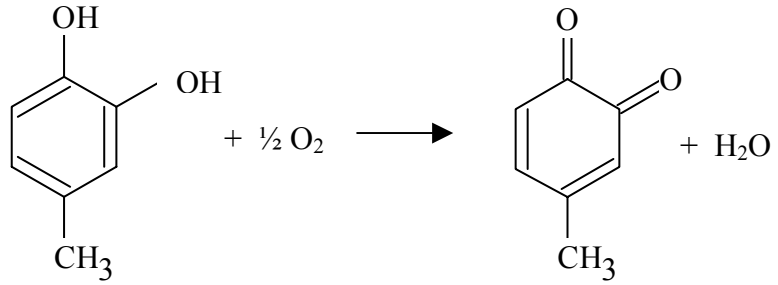

Figure 1.2 : Oxidation of *o*-diphenols to *o*-quinones by PPO activity

Laccase (E.C. 1.10.3.2, *p*-benzenediol: oxygen oxidoreductase) is also referred as PPO but it catalyses generally *p*-phenols. Because the end product, melanin, has a brown color, these reactions are called as browning reactions. Although PPOs are widely distributed in nature, almost in all plants, animals and in many microorganisms, mainly mushroom is used for commercial PPO production. The main function of PPOs in most cases is pigmentation however, they have a protective function in plants. Browning reactions make PPOs detrimental enzymes in food industry since a rapid tissue browning and loss of nutritional quality are consequences of bruises and cuts of fruits and vegetables. However, PPO activity is essential for color formation of many plant based food products like tea, coffee, cocoa, raisins and prunes (Tomás-Barberán and Espin, 2001).

1.1. Phenols

Phenolic compounds are plant secondary metabolites, having no apparent role in the growth and development of the plant, are biosynthesized through the shikimic acid pathway, from which they are produced using intermediates of carbohydrate metabolism (Tomás-Barberán and Espin, 2001).

Phenols are shown with the general formula ArOH, where Ar is phenyl, substituted phenyl, or one of the other aryl groups, such as naphthyl. Phenols are generally named as derivatives of the simplest member of the family, phenol. The methyl phenols are given the special name of cresols. Simplest structures of phenol and the structure of a simple phenolic, 4-methyl catechol, were shown in Figures 1.3 and 1.4, respectively. However, phenolics can be very large in size, such as condensed tannins. Different phenolics having relevant roles in food products include oleuropein, hydroxyl benzoic acid derivatives, cinnamates, isoflavones, lignans, stilbenes, anthocyanins, flavanones, chalcones and dihydrochalcones,

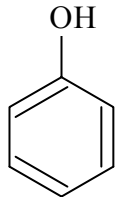

Figure 1.3 : Simplest structure of phenol

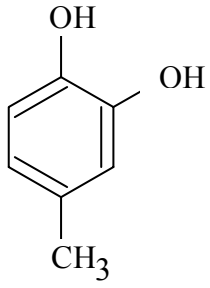

Figure1.4 : Structure of 4-methyl catechol

flovanols, flavones, flavonols, proanthocyanidins, tannin like compounds, ellagitannins and miscellaneous phenols. These phenolic compounds having different chemical structures have different biological functions. Of particular note are their antioxidant, anti-inflammatory, antitumorial and oestrogenic activities, which might suggest their potential in the prevention of coronary heart disease and cancer. Phenolic compounds play an important role in food visual appereance. Anthocyanin pigments are responsible for most of the blue, purple, red and intermediate hues of plant-derived foods and appear black in some commodities. Phenolic compounds are also relevant in terms of food flavour, as they can play a role in the bitter, sweet, pungent or astringent taste of some products and can also contribute to aroma (Tomás-Barberán and Espin, 2001).

As shown in Table 1.1 the two most prevalent naturally occurring substances which could potentially be PPO substrate in plant based foods are tyrosine and the chlorogenic acids (Schwimmer, 1981).

Although the phenolic content of plants depends both quantitatively and qualitatively on their genetic information, the environmental conditions like water availability (irrigation), soil composition (mineral and organic nutrients), post-harvest factors and processing conditions also affect the phenolic composition of plants (Tomás-Barberán and Espin, 2001).

Table 1.1 : Important phenolic compounds present in plant-based foods

Food	Substrate
Mushroom	Tyrosine
Apple	Chlorogenic acid (flesh), *o*-catechin (peel)
Cocoa	*epi*-Catechins
Coffee bean	Chlorogenic and caffeic acids
Date	Caffeoyl shikimic acid
Eggplant	Caffeic, coumaric, cinnamic acid derivatives
Fava bean	Dihydroxyphenylalanine (DOPA)
Lettuce	Tyrosine
Banana	3,4-Dihydroxyphenylethylamine (Dopamine)
Olive	Urushiol
Pear	Chlorogenic acid
Potato	Tyrosine, chlorogenic acids, flovanols
Quince	Chlorogenic acid, catechins, flovanols, leucoanthocyanidins
Sweet potato	Chlorogenic acid
Tea	Flovanols, catechins, tannins

Phenolics are present in wastes from several industrial processes, as coal conversion, olive oil production, petroleum refining, paper and pulp production, production of organic chemicals, etc. These compounds are harmful to the

environment, animals and humans and in addition, they give an undesirable taste and odor to drinking water, even in very low concentrations (Russell and Burton, 1999).

1.2. Phenol Oxidative Enzymes

There are different types of phenol oxidative enzymes in nature. Two major groups of these enzymes are peroxidases and PPOs.

1.2.1. Peroxidases

Peroxidases (E.C. 1.11.1.7) are hemoproteins, produced mainly by a number of micro-organisms and plant sources, which catalyze reactions in the presence of hydrogen peroxide (Durán and Esposito, 2000).

1.2.1.1. Horseradish Peroxidase (HRP)

HRP can catalyze the oxidation of phenols, biphenols, anilines, benzidines and related heteroaromatic compounds. HRP is suitable for wastewater treatment because it retains its activity over a broad pH and temperature range (Durán and Esposito, 2000).

1.2.1.2. Chloroperoxidase (CPO)

CPO from the fungus *Caldoriomyces fumago* has been reported to oxidize several phenolic compounds and in addition, oxidation of ethanol to aldehyde and oxidation of chloride ions occurred (Durán and Esposito, 2000).

CPO is capable of oxidizing chloride, bromide and iodide ions. Fluoride is not a substrate for chloroperoxidase but is an inhibitor of the halogenation reaction. Fluoride ions compete for both the hydrogen peroxide and the halogen anion binding sites of chloroperoxidase.

1.2.1.3. Lignin Peroxidase (LiP)

LiP's are quite well known, especially those coming from the basidiomycete as *Phana-erochaete chrysosporium* and few in ascomycetes. LiP from different sources was shown to mineralize a variety of recalcitrant aromatic compounds and to oxidize a number of polycyclic aromatic and phenolic compounds (Durán and Esposito, 2000).

1.2.1.4. Manganese Peroxidase (MnP)

MnP catalyzes the oxidation of several monoaromatic phenols and aromatic dyes, but depends on both divalent manganese and certain types of buffers. The enzyme requirement for high concentrations of Mn (III) makes its feasibility for wastewater treatment applications. In the free form, MnP acts on phenols and dyes (Durán and Esposito, 2000).

1.2.2. Polyphenol Oxidases

PPOs are oxidoreductases that catalyze oxidation of phenolic compounds. They are subdivided into two subclasses, tyrosinases and laccases, and both groups react with oxygen and no cofactors are needed (Durán and Esposito, 2000).They

catalyze the transformation of a large number of phenolic and non-phenolic aromatic compounds (Durán *et al.*, 2002).

1.2.2.1. Tyrosinase

Tyrosinase (E.C. 1.14.18.1, monophenol monooxygenase and E.C. 1.10.3.1, *o*-diphenol oxidoreductase or catechol oxidase) is widely distributed throughout the phylogenetic scale from bacteria to mammals and even present different characteristics in different organs of the same organisms, such as in roots and leaves of higher plants. It is well known that tyrosinase catalyses two different oxygen-dependent reactions that occur consequently: the *o*-hydroxylation of monophenols to yield *o*-diphenols (cresolase activity) and the subsequent oxidation of *o*-diphenols to *o*-quinones (catecholase activity). Chemical and spectroscopic studies of tyrosinase have shown that the active site contains a coupled binuclear copper complex (Durán *et al*, 2002).

1.2.2.2. Laccase

Laccase (E.C. 1.10.3.2, *p*-benzenediol: oxygen oxidoreductase) is a cuproprotein belonging to a small group of enzymes denominated blue oxidases. Laccase catalyzes the oxidation of various aromatic compounds; phenolic dyes, phenols, chlorophenols, lignin-related diphenylmethanes, benzopyrenes, N-substituted *p*-phenylenediamines, organophosphorus and non-phenolic beta-*O*-lignin model dimer with the concomitant reduction of oxygen to water. In general, laccases exhibit four copper atoms, which play an important role in the enzyme catalytic mechanism. Copper atoms are distributed in different binding sites and are classified in three types, according to specific spectroscopic and functional characteristics (Durán *et al*, 2002).

In a typical laccase reaction, a phenolic substrate is subjected to a one-electron oxidation giving rise to an aryloxyradical. This active species can be converted to a quinone in the second stage of oxidation. The quinone as well as the free radical product undergo non-enzymatic coupling reactions leading to polymerization (Durán *et al.*, 2002).

1.3. Plant Polyphenol Oxidases

Since PPOs are widely distributed in plants, there are many studies in the literature about plant PPOs.

PPOs were isolated from dates (Hasegawa and Maier, 1980), grape (Wissemann and Lee, 1980 and Sánchez-Ferrer *et al.*, 1988), banana (Galeazzi *et al*., 1981), wild potato (Ryan *et al*., 1982), air potato (Anosike and Ayaebene, 1982), avocado (Kahn, 1983 and Lelyveld *et al*., 1984), apple (Goodenough *et al*., 1983), pear (Smith and Montgomerry, 1985), spinach (Sánchez-Ferrer *et al.*, 1989, Hind *et al.*, 1995 and Sheptovitsky and Brudvig, 1996), strawberry (Wesche-Ebeling and Montgomery, 1990), mung bean leaf (Shin *et al*., 1997), highbush blueberry fruit (Kader *et al*.,1997), tobacco (Richardson and McDougall, 1997), apple (Murata *et al.*, 1997), mango kernel (Arogba *et al*., 1998), iceberg lettuce (Chazarra *et al*., 1999), apple leaf (Ridgway and Tucker, 1999), litchi fruit (Jiang *et al*., 1999), raspberry fruits (González *et al*., 1999), desert truffle (Pérez-Gilabert *et al*., 2001), apricot fruit (Chevalier *et al*., 1999), potato (Partington *et al.*, 1996 and 1999), coffee (Mazzafera and Robinson, 2000), *Duranta plumieri* (Roy *et al*., 2002), rubber tree seeds (Wititsuwannakul *et al*., 2002), medlar fruit (Dincer *et al.*, 2002).

1.4. General Properties of Polyphenol Oxidases from Different Plant Sources

PPOs from different plant sources were investigated in terms of optimum pH, optimum temperature, molecular weight, isoelectric point and kinetic properties in literature.

Ridgway and Tucker (1999) partially purified apple PPO by using a method suitable for commercial application. Concentration of polyvinyl pyrrolidine (PVP) in enzyme extraction was optimized. The yield of PPO extracted from leaf tissue was found to be greater than that from fruit and apple leaves were observed as a suitable PPO source for commercial application. PPO was purified 50-fold by the use of DEAE-Sephadex and ultrafiltration. Using 4-methyl catechol as substrate partially purified PPO had a specific activity of 4.9 μkat mg^{-1} and K_m value of 3.6 mM.

Arogba *et al.* (1998) investigated PPO in mango (*Mangifera indica*) kernel. The enzyme was most active at pH 6.0 and 25°C. Michaelis Menten constant, K_m, was found to be 24.6 mM by using catechol as substrate.

Galeazzi *et al*. (1981) purified PPO from banana. 38.8-fold purification by acetone precipitation was achieved. The purified enzyme with four isozymes gave a polypeptide molecular weight (SDS-PAGE) of 31 ± 1 kDa. The isoelectric point determined by isoelectric focusing on polyacrylamide gel was 5.2.

Shin *et al*. (1997) isolated and characterized PPO from mung bean leaf. A mature form of PPO, purified from leaf chloroplasts, contained two proteins with subunit M_r values of 65 and 59 kDa, respectively. The purified enzyme had a pH optimum of 6.0 and a pI of 5.1. K_m for L-DOPA was 24 mM.

Chevalier *et al*. (1999) studied molecular cloning and characterization of apricot fruit PPO. They calculated PPO molecular weight as 67.1 kDa and the isoelectric point of 6.84 where the mature protein had a predicted molecular mass of 56.2 kDa and an isoelectric point of 5.84.

Hasegawa and Maier (1980) purified PPO from dates by ammonium sulphate precipitation, followed by two successive DEAE-cellulose columns, which resulted in a 510-fold increase in specific activity. The enzyme had a maximum activity over a wide pH range, 4.5-6.5, and was relatively heat stable.

Chazarra *et al*. (1999) characterized monophenolase activity of PPO from iceberg lettuce by using (*p*-hydroxyphenyl) propionic acid with 3-methyl-2-benzothiazolinone hydrazone. Monophenolase activity toward (*p*-hydroxyphenyl) propionic acid showed a maximum at pH 5. Kinetic parameters, K_m and V_{max} were found to be 6.3 mM and 11.9 µM/min, respectively.

Jiang *et al*. (1999) purified PPO and investigated the browning control of litchi fruit by glutathione and citric acid. PPO was purified from litchi peel yielding a single protein with a molecular weight of about 75.6 kDa by Sephadex G-100 filtration and a 108-fold purification of PPO was achieved.

Kader *et al*. (1997) partially purified and characterized blueberry fruit PPO. They achieved 19-fold purification by ultrafiltration, ammonium sulphate precipitation and hydrophobic chromatography. Native-PAGE of the purified fraction revealed the presence of two isoforms. PPO activity showed a maximum at pH 4.

Kahn (1983) studied avocado PPO. PPO molecular weights were determined by Sephacryl S-300 gel filtration in 4 fractions with molecular weights of 87.5 kDa-500 kDa. Their kinetic parameters, K_m and V_{max}, determined with 4-methyl catechol were in the range of 1.0-2.2 mM and 0.09-1.33 ΔOD/min per 20µl, respectively.

Wititsuwannakul *et al*. (2002) investigated purification and characterization PPO from latex of *Hevea brasiliensis*. Acetone precipitation and CM-Sepharose chromatography were performed, affording two PPOs having molecular weights of 32 and 34 kDa, respectively. Both PPOs possessed the same pI (9.2), optimum pH 7 and optimum temperature 35-45°C. The K_m values of PPO-I for dopamine, L-DOPA and catechol as substrates were 2.08, 8.33 and 9.09 mM, while those for PPO-II were 2.12, 4.76 and 7.14 mM, sequentially.

Pérez-Gilabert *et al*. (2001) studied partial purification, characterization and histochemical localization of fully latent desert truffle (*Terfezia claveryi* Chatin) PPO. The enzyme was partially purified by using phase partitioning in Triton X-114 (TX-114) and 2-fold purification was achieved from a crude extract with a 66% recovery of activity.

Paul and Gowda (2000) purified and characterized PPO from the seeds of field bean (*Dolichos lablab*). A combination of ammonium sulphate precipitation, DEAE-Sephacel chromatography, phenyl agarose chromatography and Sephadex G-200 gel filtration were used. The purified enzyme had a molecular weight of 120 ± 3 kDa and is a tetramer of 30 ± 1.5 kDa. Native-PAGE of the purified enzyme revealed the presence of a single isoform with an observed pH optimum of 4.0. 4-methyl catechol was the best substrate, followed by catechol and L-3,4-dihydroxyphenylalanine.

Richardson and McDougall (1997) isolated a laccase-type PPO from lignifying xylem of tobacco. A molecular weight of 100 kDa with non-denaturing PAGE was observed. Ion-exchange chromatography on DEAE-Sepharose retained this 100 kDa laccase activity and resulted in a 10-fold purification and a 6-fold increase in the recovery of oxidase activity. In contrast, hydrophobic interaction chromatography was unsuccessful.

Wissemann and Lee (1980) purified grape PPO with hydrophobic chromatography. The largest portion of PPO contained in one peak was clearly separated from the bulk of the other material. Finally, 252-fold purification was achieved.

Partington and Bolwell (1996) purified PPO free of the storage protein patatin from potato tuber by hydrophobic chromatography on octyl-Sepharose. The purified PPO was found to be a doublet of M_r 60 kDa and 69 kDa when analyzed by SDS-PAGE with a K_m 4.3 ± 0.3 mM for L-DOPA.

1.5. General Properties of Polyphenol Oxidases from Mushroom

Rescigno *et al*. (1997) performed a single polyacrylamide gel electrophoresis for detection of laccase, peroxidase and catechol oxidase by using commercial enzymes and crude mushroom extracts. The identification of one particular activity in the presence of other enzymes was often difficult as enzymes can oxidize the same substrates. Laccase, peroxidase and catechol oxidase activities were predicted on the gel and by using a PPO inhibitor, salicylhydroxamic acid, it was proved that catechol was oxidized by catechol oxidase, not only by laccase.

Zhang and Flurkey (1997) studied the PPOs in *Portabella* (mushroom). Two tyrosinase (R_fs 0.23 and 0.27) and three laccase isoforms (R_fs 0.72, 0.76 and 0.81) after native electrophoresis were observed and at least 10 tyrosinase isoforms after isoelectric focusing, ranging from pI 4.45 to 5.9 were predicted.

Zhang *et al*. (1999) characterized tyrosinase from the cap flesh of *Portabella* (mushroom). A native molecular weight of 41 kDa for the enzyme was obtained by size exclusion chromatography, whereas SDS-PAGE indicated that the enzyme contained a single subunit with a size of 48 kDa. Isolelectric focusing demonstrated

that the enzyme preparation, apparently homogeneous by electrophoresis, still contained three isoforms of pI 5.1, 5.2 and 5.3.

Rescigno *et al*. (1997) investigated diafiltration in the presence of ascorbate in the purification of mushroom tyrosinase. During the extraction of tyrosinase, the oxidation of mushroom phenolics was avoided, keeping phenolic compounds in their reduced form throughout the extraction step. Their complete removal by means of a diafiltration apparatus was achieved. The method described was found to be a useful way to obtain an enzymic solution of tyrosinase, devoid phenolics and melanic compounds.

1.6. Industrial Applications of Polyphenol Oxidases

PPOs have different applications in industrial processes like food and medicine. These enzymes were also used to remove phenolics from waste waters. Horticultural products suffer losses of quality and value between harvest and consumption due to oxidation of the endogenous phenolic compounds resulting in an undesired enzymatic browning. Determination of PPO activity has, therefore, been of central importance for determining the quality of food products and their shelf-life and hence a rapid, reliable and sensitive method estimating the PPO activity in the horticulture products is desirable. Biosensors are ideally suited for this purpose. A careful review of literature indicates that there have been a few studies dealing with immobilised tyrosinase enzymes and their use in estimating corresponding substrates such as phenol, catechol, p-cresol and tert-butylcatechol. In such applications the enzyme is generally immobilised into a matrix such as carbon paste, nafion membrane, hydrogel, conducting polymers and natural material such as chitosan (Dutta *et al.*, 2001). PPOs were also used in food industry for the improvement of flavor in tee, cocoa and coffee production (Motoda, 1979).

The selective recognition and oxidation of phenols by the enzyme polyphenol oxidase to produce reactive o-quinones can be successfully exploited as a waste minimization strategy for effluent treatment. However, although polyphenol oxidase is effective in converting phenol and a number of associated derivatives to their corresponding o-quinones, these o-quinones and the low molecular weight polymers formed from them remain in the treated effluent. At low concentrations the remaining colored products do not achieve high enough degrees of polymerization to effectively precipitate from solution. Thus, the goal of reducing the toxicity of the phenol-containing effluent is achieved, but the resultant treated stream is highly colored, which is unacceptable for discharge purposes. Therefore, to complete the wastewater treatment process, it is necessary to remove the polyphenol oxidase-generated polymerization products from the bioreactor permeate stream (Edwards *et al.*, 1999).

1.7. Immobilization of Polyphenol Oxidases

The term "immobilized enzyme" has been used to describe an enzyme that has been chemically or physically attached to a water-insoluble matrix, polymerized into a water-insoluble gel or entrapped within a water-insoluble gel matrix or water-insoluble microcapsule. In all these cases, the localization of the enzyme was achieved by producing water-insoluble, enzyme containing material (Zaborsky, 1973). After immobilization, some changes were observed in the enzymatic activity, optimum pH and temperature, affinity to substrate and stability of enzymes (Arica, 2000).

Several methods; adsorption, ionic binding, covalent binding, cross-linking, matrix entrapment, membrane confinement or the combination of two or more of these methods can be used for immobilization of enzymes (Hartmeier, 1988).

Among various techniques, the electrochemical polymerization (matrix entrapment) appears as a simple and attractive avenue for fabricating biosensors (Besombes *et al.*, 1997).

Besombes *et al*. (1997) immobilized PPO in poly (amphiphilic pyrole) enzyme electrodes via the incorporation of synthetic laponite-clay-nanoparticles, where the presence of incorporated laponite particles within the electrogenated polymer induced a strong improvement of the analytical performances of amperometric biosensors based on polyphenol oxidase. It was found that the presence of laponite allows to keep a higher specific activity for immobilized PPO.

Boshoff *et al*. (1998) applied the combination of cross-linking, adsorption and membrane confinement methods for PPO. Commercial PPO was immobilized on the nylon membrane by using gluteraldehyde as cross-linking agent and on the polyether-sulfone membrane by adsorption. The intermediate product of the PPO reaction, 4-methyl catechol, was detected when the enzyme was immobilized on the nylon membranes or was not immobilized, but they detected only the *o*-quinone final product when polyethersulphone was used as the immobilization matrix.

The immobilization of tyrosinase on the graphite electrodes was studied by Nistor *et al*. (1999). The operational and storage stability of tyrosinase biosensors for different tyrosinase modified electrodes, i.e., plain bulk modified carbon paste electrodes (CPEs), surface modified by simple adsorption to solid graphite electrodes (SGEs), and surface modified by the immobilization in Eastman AQ, a polyester-sulphonic acid cation exchanger, and Nafion, a perfluorinated-sulphonated ionomer, on the surface of both CP and SGEs were investigated. As a result, after about 42 days 80% and 75% of the original response for the Eastman and Nafion modified tyrosinase electrodes remained, respectively, whereas the tyrosinase bulk-modified CPE and adsorbed tyrosinase SGEs had lost virtually 100% of the original response.

Covalent binding method was studied by Arica (2000). PPO was covalently immobilized onto carboxymethylcellulose (CMC) hydrogel beads. As a result, it was found that immobilization onto CMC hydrogel beads made PPO more stable to heat and storage, implying that the covalent immobilization imparted higher conformational stability to the enzyme.

Dutta *et al*. (2001) immobilized tyrosinase by the incorporation of tiron as a substrate into a polypyrole film deposited on indium titanium oxide (ITO) glass. It was concluded that the present sensor offers a good and reliable means for the detection of monophenolase activity in food or horticulture products.

Seetharam and Saville (2002) immobilized PPO on zeolite. The production of L-3,4-dihydroxyphenylalanine (L-DOPA) from commercial mushroom tyrosinase immobilized (by using gluteraldehyde) on chemically modified supports, sodium aluminosilicate (NaA) and calcium aluminosilicate (CaA) (two separate forms of zeolite) was studied. No loss of activity of the immobilized enzyme during 40-48 h of repeated batch operations was observed.

Chang *et al*. (2002) made a disposable tyrosinase-peroxidase bi-enzyme sensor system for amperometric detection of phenols. The phenol sensor uses horseradish peroxidase modified screen-printed carbon electrodes (HRP-SPCEs) coupled with immobilized tyrosinase prepared using poly(carbamoylsulfonate) (PCS) hydrogels or a poly(vinyl alcohol) bearing strylpyridinium groups (PVA-SbQ) matrix. As a result, they found that the comparison of the electrode responses indicated the feasibility of the disposable sensor system for sensitive determination of phenols.

Kiralp *et al.* (2003) immobilized PPO in copolymers of thiophene functionalized menthyl monomer (MM) with pyrrole. Immobilization of PPO was performed via entrapment in conducting copolymers during electrochemical

polymerization of pyrole. Optimum temperature of free PPO was found as 40°C where by using only pyrole, optimum temperature remained constant. By using MM with pyrole, optimum temperature was increased to 60°C. After 10 assays, remaining activity of electrodes were 60%. Kinetic parameters, K_m and V_{max} values of free PPO, immobilized in PPy and immobilized in MM/Polypyrole PPOs were determined as 4 mM and 11.2 μmol/min.mg protein, 100 mM and 0.11μmol/min.electrode, 200 mM and 0.10μmol/min.electrode

1.8. Immobilization of Different Enzymes in Polypyrole Matrix

Selampinar *et al*. (1997) immobilized invertase by electrochemical polymerization of pyrole. The preparation and characterization of polypyrole/invertase and polyamide/polypyrole/invertase electrodes under conditions compatible with the enzyme were investigated. Results showed that conducting polymers can be used successfully for the immobilization of invertase.

Erginer *et al*. (2000) immobilized invertase in functionalized copolymer electrodes constructed with pyrole-capped polyazotetrahydrofuran-block-polystyrene copolymer matrices. Sodium dodecyl sulphate was found to be the best supporting electrolyte. Results showed that conducting polymers can be used successfully in invertase immobilization.

Alkan *et al*. (2001) immobilize urease in conducting polypyrole (PPy) and block copolymers of thiopene-capped poly(methyl methacrylate) (TPMMA) matrices by electropolymerization. As a result, TPMMA/PPy graft films showed better properties in terms of enzymatic activity, amount of immobilized protein and kinetic properties than PPy films.

1.9. Scope of the Study

The aim of this study was to find an economical plant source for PPO production, characterization and immobilization of the PPOs for the ultimate purpose of PPO electrode and/or immobilized PPO bioreactor development. For this purpose, different plant tissues of no commercial value like horse chestnut fruits and leaves of many fruit trees were screened for PPO activities. Since mulberry leaves were found to have the highest PPO activity, PPO was partially purified from this source by ultrafiltration and characterized in terms of kinetic parameters, temperature and pH dependencies, PPO types (activity staining), molecular weights and isoelectric points. The enzyme was next immobilized in polypyrole matrix, characterized in terms of stabilities and kinetic parameters.

CHAPTER 2

MATERIALS AND METHODS

2.1. Chemicals

Acrylamide and bisacrylamide were purchased from Appli Chem Ltd. Isoelectric focusing marker was obtained from SERVA Ltd. Other electrophoresis and isoelectric focusing chemicals were purchased from BIO-RAD Lab. All other chemicals were analytical grade and obtained from SIGMA Ltd. or MERCK Ltd. companies.

2.2. Plants Screened

Horse chesnut fruit shells and leaves, mushroom, leaves of mulberry, pear, sourcherry, apricot, cherry, apple, grape, quince and green shells of hazelnut were screened for PPO activity. Mushroom was purchased from local market. Other plant materials were collected from trees and collection dates were recorded. They were stored at -20°C.

2.2.1. Optimization of Extraction Conditions for Screening

2.2.1.1. Optimization of pH

To determine the optimum pH, 10 g wet plant material was homogenized in 200 ml sodium phosphate buffer at pHs 6-8 at 4°C in a blender (ARCELIK Rollo K-1350) for 4 x 15 sec. Extracts were centrifuged at 10,000xg for 10 minutes (SIGMA) and filtered. The supernatants were used as the crude extracts at different pHs. By comparing the enzyme activities in these extracts, optimum extraction pH was determined.

2.2.1.2. Optimization of PVPP Ratio

Extractions were performed at the optimum pH (pH 7) by using polyvinyl polypyrolidone (PVPP) at different concentrations in a range of 5-75 mg/ml to precipitate phenols which can react with oxygen in the presence of PPOs.

2.3. Analytical Methods

2.3.1. Polyphenol Oxidase Assay

PPO activity was assayed by using 4-methyl catechol as substrate at the observed optimum conditions. Crude extract was diluted 5 times by using sodium phosphate buffer, pH 7. Reaction mixture contained 80 mg/ml substrate (1ml) and 5 times-diluted extract (1 ml). Absorbance data were collected with a time interval of 5 seconds for 30 seconds at room temperature at 410 nm in a spectrophotometer (Heλios) (Jiang *et al.*, 1999). Initial reaction rates were calculated from initial linear part of the absorbance vs. time graphs. In PPO assay experiments, extractions were

repeated 2 times and 2 samples were taken from each extract. Results were calculated by averaging these 4 data and error bars were inserted by calculating the deviation from the average value.

One unit of PPO activity (U) was defined as 0.01 change in absorbance at 410 nm under given reaction conditions per minute.

2.3.2. Protein Analysis

Protein concentration was determined by using Bradford Method (Bradford, 1976). Bovine serum albumin (BSA) was used as the standard protein. Composition of reagents, procedure and standard curve are given in Appendices A, B and C, sequentially.

2.4. Enrichment of Polyphenol Oxidase

Ultrafiltration and membrane concentration methods were used for enrichment of PPO.

2.4.1. Preparation of Crude Extract for Partial Purification

30 g plant material was homogenized in 200 ml pH 7 sodium phosphate buffer at 4°C in a blender (ARCELIK Rollo K-1350) for 15 sec x 4. In order to avoid the reaction of PPOs with phenols present in the extract, polyvinyl polypyrolidone (PVPP) was added at a concentration of 12.5 mg/ml. Extract was centrifuged at 10000xg for 10 minutes (SIGMA) and filtered. The supernatant was used as the crude extract.

2.4.2. Ultrafiltration

Ultrafiltration was carried out in two steps in a 50 ml (Amicon) stirred cell at room temperature. First, 70 ml crude extract was ultrafiltrated by using 0.22 μm pored cellulose membrane to 50 ml to remove the coarse particles. Then, extract was concentrated and enriched by using a 30 kDa cut-off cellulose membrane until 20 ml extract remains in the ultrafiltration cell. Pressure applied was 1.5 bar in each step.

After each step, specific activities, yields and enrichment folds were calculated with using following equations:

$$\text{Specific Activity} = \frac{\text{Total activity of concentrated enzyme (U)}}{\text{Total protein (mg)}}$$

$$\text{Yield (\%)} = \frac{\text{Total activity of concentrated enzyme (U)}}{\text{Total activity of crude enzyme (U)}} \times 100$$

$$\text{Enrichment Fold} = \frac{\text{Specific activity of concentrated enzyme (U/mg)}}{\text{Specific activity of crude enzyme (U/mg)}}$$

2.4.3. Concentration with Membrane Concentrator

5 ml of ultrafiltrated crude extract was further concentrated to 0.8 ml by using a membrane concentrator (Vivapore) with a molecular weight cut-off of 7.5 kDa.

2.5. Characterization of Polyphenol Oxidase

2.5.1. Kinetic Analysis of Free Polyphenol Oxidases

To perform the kinetic analysis of mulberry leaf and mushroom PPOs, activities at different concentrations of 4-methyl catechol (substrate) ranging from 0 to 75 mM were measured. By plotting PPO activity vs. time, Michaelis- Menten type curve was obtained. K_m and V_{max} values were calculated from Lineweaver-Burk Plot, non-linear regression analysis (Sigma Plot) and substrate inhibition model (EZ-FIT, Perrella, 1988).

2.5.2. Temperature Dependency of Polyphenol Oxidase Activity

To determine the temperature dependency of PPOs, activities at different temperatures ranging from 4 to 60°C were measured by using 5 times-diluted crude extract and 80 mg/ml 4-methyl catechol in the reaction mixture.

2.5.3. pH Dependency of Polyphenol Oxidase Activity

To determine the pH dependency of PPOs, activities at different pHs ranging from 4 to 9 were measured by using 5 times-diluted crude extract and 80 mg/ml 4-methyl catechol in the reaction mixture. For pH 4 and 5 citrate phosphate buffer, for pH 6 citrate phosphate and sodium phosphate buffer, for pH 7 sodium phosphate buffer, for pH 8 tris buffer, for pH 9 glycine-sodiumhydroxide buffer was used. All buffers were at a concentration of 100 mM.

2.5.4. Electrophoretic Analysis

Electrophoretic analysis involved activity staining and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) studies. Experimental setup, Bio-Rad Electrophoresis Equipment, was shown in Figure 2.1.

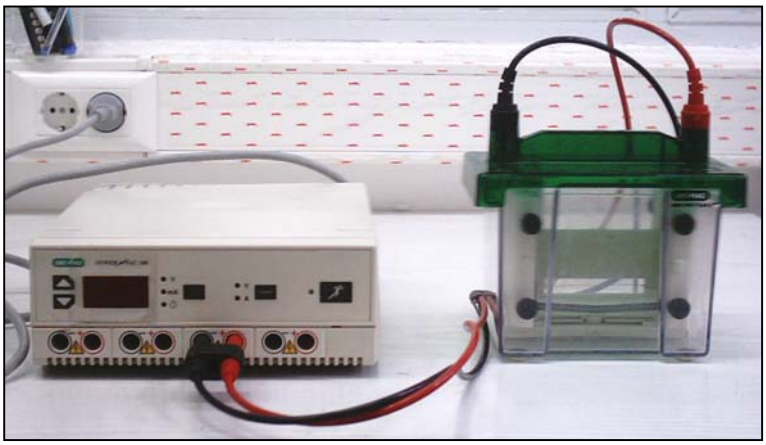

Figure 2.1 : Electrophoresis equipment

2.5.4.1. Activity Staining

Concentrated mulberry leaf extract by ultrafiltration was run in polyacrylamide gel to separate proteins. For that purpose, electrophoresis was carried out by using 4% polyacrylamide stacking gel and 5% polyacrylamide separating gels containing no anionic detergent, SDS (Appendix D). 0.5 ml protein sample was mixed with 1.0 ml sample buffer (Appendix D). Sample was loaded on the gel in one well without denaturation. Separation was performed at a constant current of 40 mA both in stacking and separating gels.

2.5.4.1.1. Gel Processing of Activity Staining

After electrophoretic run, two small portions of the gel was cut and stained for different phenol oxidase activities depending on the modified procedure of Rescigno *et al.* (1997). The procedure consisted of 4 steps including staining with 4-amino-*N*,*N*-diethylaniline (ADA) for laccase activity, with hydrogen peroxide (H_2O_2) for peroxidase activity and with two substrates, 4-tert-butyl catechol (*t*BC) and 4-methyl catechol, (MC) for catechol oxidase (PPO) activity with and without salicylhydroxamic acid, a catechol oxidase inhibitor.

Preparation of reagents and procedure was given in Appendix E. After taking a photograph of the gel, it was used for isolation of PPOs for molecular weight determination (SDS-PAGE).

2.5.4.1.2. Enzyme Elution from Polyacrylamide Gel

After staining a small portion of the gel, thin parts corresponding to active bands were cut from the remaining gel. Active bands were cut into pieces and incubated in 0.75 ml pH 7 sodium phosphate buffer at 4°C in an eppendorf tube overnight. Then, they were centrifuged and supernatant was taken as the enriched sample.

2.5.4.2. Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis in the presence of anionic detergent (SDS) was carried out to check the purity of the isolated enzymes and to predict their molecular weights. Electrophoresis was performed with 4% stacking gel and 12% separating gel. Samples were mixed with sample buffer by a volume ratio of

1:2 and were kept in boiling water for 5 minutes for denaturation. 20 µl of samples and 5µl of molecular weight markers (Appendix F) were loaded on the gel.

Electrophoresis was performed at a constant current of 40 mA in stacking and 50 mA in separating gels.

2.5.4.2.1. Gel Staining of SDS-PAGE with Silver Staining Method

Gels were stained with silver staining method after electrophoretic run was completed using the procedure of Blum *et al*. (1987). Silver staining method was performed in 6 steps including fixing, washing with 50% ethanol, pretreatment, impregnation, developing and stopping. Preparation of reagents and the procedure of silver staining method were given in Appendix G.

After staining, the gel was photographed. The molecular weights of enzymes were determined by measuring the migration distance and by comparing them with molecular weight markers.

The relative mobility (R_f) of each protein was determined by dividing its migration distance from the top of gel to the center of the protein band by the migration distance of the tracking dye from the top of the gel.

The equation of relative mobility (R_f) was given as:

$$R_f = \frac{\text{Distance migrated by protein}}{\text{Distance migrated by tracking dye}}$$

2.5.4.3. Isoelectric Focusing

To determine the isoelectric points of PPOs present in mulberry leaves, isoelectric focusing was carried out. For that purpose, concentrated crude extract, prepared for activity staining was used. Preparation of reagents and the procedure of isoelectric focusing were given in Appendix H.

Four µl of crude sample and pI markers were loaded on the gel. Gel was run 15 minutes at 100V, 15 minutes at 200 V and 30 minutes at 300 V. After completing focusing, the part containing the marker was stained with quick coomassie blue staining method (Appendix I) and sample containing part of the gel was subjected to activity staining (Appendix E). Then they were matched to determine the isoelectric points.

Experimental setup for isoelectric focusing, Bio-Rad Isoelectric Focusing Equipment, was shown in Figure 2.2.

Figure 2.2 : Isoelectric focusing equipment

2.5.5. Polyphenol Oxidase Immobilization in Polypyrole Matrix

PPOs isolated from mulberry leaves were immobilized in polypyrole matrix. Mushroom PPOs were also immobilized and used as a reference.

2.5.5.1. Crude Extract Preparation for Immobilization

In order to obtain a more concentrated extract, 50 g plant material was used in extraction. The extraction procedure was the same as in Sec. 2.4.1.

2.5.5.2. Immobilization of Polyphenol Oxidases in Polypyrole Matrix

Immobilization of PPOs was achieved by electrochemical polymerization of pyrole on Pt electrodes. For that purpose, immobilization was performed in a typical 3 electrode cell containing Pt working and counter electrodes and Ag reference electrode by constant potential electrolysis (at +1.0 V) at room temperature. Experimental setup for immobilization was shown in Figure 2.3. Immobilization solution contained 2 mg/ml SDS as supporting electrolyte and 5 µl/ml pyrole as monomer in 20 ml of crude extract.

Figure 2.3 : Immobilization equipment

The pH chosen for an enzyme assay system must be near the optimum value. Since enzymes are very sensitive molecules, a change in pH or temperature might cause denaturation. During immobilization, protons were released into electrolysis media during electropolymerization of pyrole which causes a decrease in pH of the medium (Erginer *et al*., 2000). In order to prevent this, immobilization solution were prepared with pH 7 sodium phosphate buffer.

After 30 minutes immobilization, enzyme entrapped electrode was removed and washed with distilled water and sodium phosphate buffer, pH 7, to remove the supporting electrolyte and unbound enzymes from the electrode surface. Electrodes were stored in 10 ml of sodium phosphate buffer at pH 7 until use.

2.5.5.3. Kinetic Analysis of Immobilized Polyphenol Oxidases

To perform the kinetic analysis of the electrodes, activities at different concentrations of 4-methyl catechol ranging from 0 to 300 mM were measured. Electrodes were inserted into the reaction mixture in a test tube containing 5 ml of 4-methyl catechol at different concentrations. Spectrophotometric data at 410 nm were recorded with a time interval of 2.5 minutes for 10 minutes. After observing the Michaelis-Menten kinetics, K_m and V_{max} values were calculated by using Lineweaver-Burk plot.

2.5.5.4. Stability of Immobilized Polyphenol Oxidases

Storage stability of immobilized mushroom PPO and operational stabilities of immobilized mulberry leaf and mushroom PPOs were determined by using 5 ml of 100 mM 4-methyl catechol. Absorbance data were collected at 410 nm with a time interval of 2.5 minutes for 10 minutes.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Screening Tissues of Different Plants for Polyphenol Oxidase Activity

Phenolics, the substrates of PPOs, are found in all plant cells in varying quantities and forms. Phenolic concentration in some food products like wine affects the product quality excessively. Therefore a practical and rapid measurement of phenolic concentration during the process is very important and a simple PPO electrode can be developed and used for this purpose. On the other hand, waste streams of many chemical and food production plants, like olive oil and paper and pulp industries contain high concentrations of phenolics. Since phenolics are detrimental to the environment, they should be removed before waste disposal. Water-soluble phenolics can be removed from the waste stream by using an immobilized PPO membrane bioreactor which converts the phenolics into bulky water-insoluble molecules.

Although PPOs are widely distributed in nature, almost all plants, animals and in many microorganisms, mushroom is used for commercial production of these enzymes. Therefore, the aim of this study was to find an economical plant source for PPO production as an alternative to mushroom and to characterize the enzyme.

3.1.1. Optimization of Polyphenol Oxidase Screening Conditions

In this study, different plant tissues of no commercial value like fruits of horse chestnut and leaves of many fruit trees, were screened in terms of PPO activities. In the studies, mushroom was used as a reference which is a commercial PPO source. Since horse chestnut and fruit trees are widely distributed in Turkey, they could be a good and cheap source for PPO production. Therefore, screening conditions were optimized by using horse chestnut tissues, which was our primary target PPO source, and mushroom. Sour cherry, apple, pear and mulberry leaf tissues were also used for pH optimization.

3.1.1.1. Optimization of PPO Extraction Conditions

The first step was the optimization of extraction pH. For this purpose, fruit shell of horse chestnut, mushroom and leaves of sour cherry, pear and cherry were used. Since the results of this experiment would be used in screening of other plant tissues, no extreme pHs were tested. Experiments were carried out around neutral pHs, 6-8, with sodium phosphate buffer. Results were given in Table 3.1

Table 3.1 : pH optimization results for PPO extraction in screening experiments

	PPO Activity (U/ml)		
PPO Source	pH		
	6	7	8
Mushroom	120	304	64
Horse chestnut fruit shell	11	36	30
Mulberry leaf	56	213	129
Pear leaf	234	165	151
Sour cherry leaf	149	55	0
Apple leaf	18	45	22

Maximum PPO activities were observed at pH 7 except sour cherry and pear leaf tissues. So, further screening experiments were carried out with using sodium phosphate buffer at pH 7. Since sour cherry and pear leaf tissues showed high PPO activities at pH 6 in the tested pH range, pH 6-8, they can be investigated for their PPO activities at more acidic pHs for possible use in wine industry.

Second step was the optimization of PVPP ratio in extraction. PVPP has been used during plant enzyme extractions due to its ability to hydrogen bond to phenolics and prevent phenol-PPO interaction (Smith and Montgomery, 1985). For this purpose, chestnut fruit shells were used as PPO source and PVPP was added at different concentrations between 5-75 mg PVPP/ml. As observed in Table 3.2, 12.5 mg PVPP/ml seemed a suitable concentration to remove phenolics.

Table 3.2. : PVPP optimization results for PPO extraction in screening experiments

PVPP Concentration (mg/ml)	PPO Activity (U/ml)
5.0	42 ± 12
12.5	50 ± 16
25	51 ± 22
37.5	53 ± 9
50.0	42 ± 11
62.5	40 ± 7
75.0	50 ± 11

3.1.2. Screening Results of Plant Tissues for Polyphenol Oxidase Activity

Fruit shells and leaves of horse chestnut, mushroom, leaves of mulberry, pear, sour cherry, apricot, cherry, apple, grape, quince and hazelnut green shell were screened for their PPO activities.

Results were shown in Figure 3.1. More or less, all plant materials showed PPO activity. Mulberry leaves showed the highest PPO activity which was even slightly higher than mushroom. Pear, sour cherry and apricot leaves were also good PPO sources. Other plant materials; cherry, chestnut, apple, grape, quince leaves, hazelnut fruit shell and horse chestnut fruit showed low PPO activity. At the beginning of the studies, our purpose was to use leaves or fruit shells of horse chestnut as PPO source. However, mulberry leaf tissues were selected instead of horse chestnut and characterization-immobilization studies were performed by mulberry leaf tissues. Mushroom was also studied as a reference.

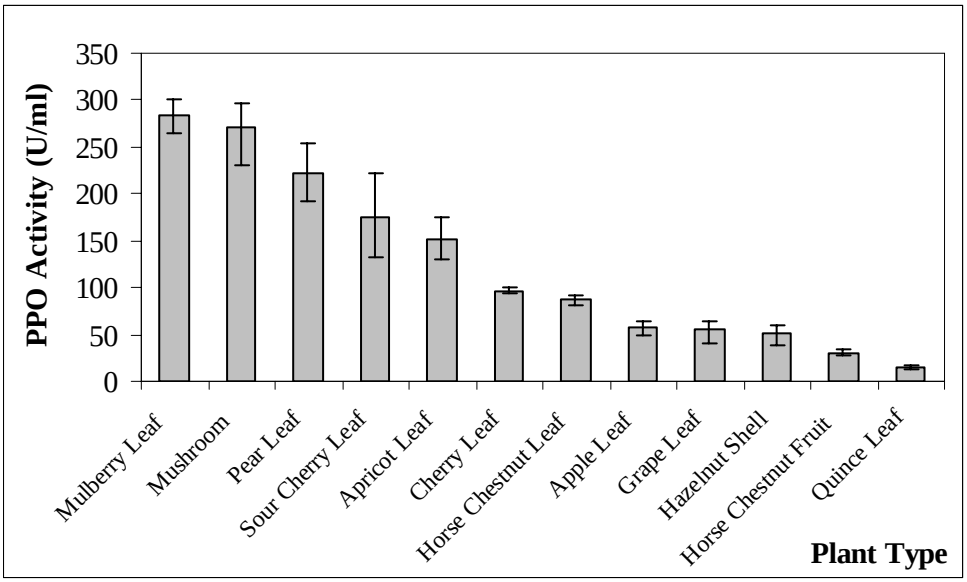

Figure 3.1 : Screening results for PPO activity

PPO activity of mulberry leaf tissues were also tested according to harvest time by using the leaves collected in august, september, october and november. As observed in Figure 3.2, PPO activities of mulberry leaf tissues increased with harvesting time and no activity loss was detected after 8 months storage at -20°C. Therefore, leaf tissues can be harvested at the beginning of the winter and used as PPO source.

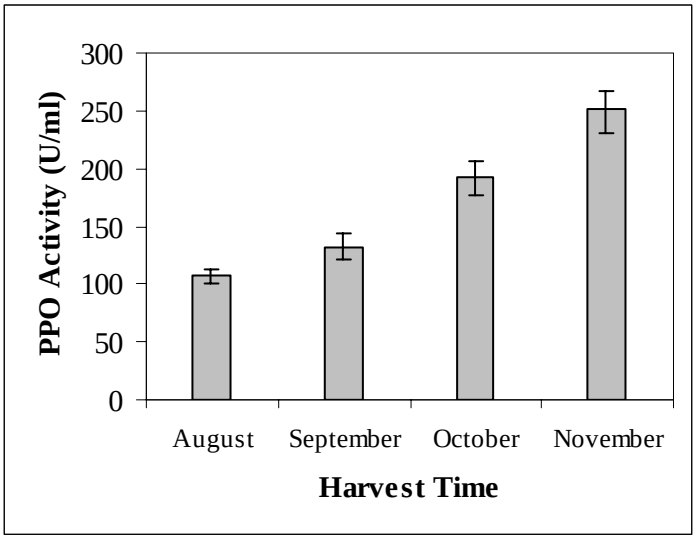

Figure 3.2 : The effect of harvest time on PPO activity of mulberry leaf tissues

3.2. Enrichment of Polyphenol Oxidase

Enrichment of mulberry leaf and mushroom PPOs were achieved by two step ultrafiltration by using 0.22 µm pored cellulose and 30 kDa cut-off cellulose membranes. In the first step, by using 0.22 µm pored membrane, coarse particles from the crude extracts were removed. In the second step, the filtrate from the first step was concentrated and enriched by using 30 kDa cut-off membrane. The enrichment steps, enzyme activities, specific activities, yield and enrichment fold at each step were given in Table 3.3 for mulberry leaf PPO and in Table 3.4 for mushroom PPO.

By two step ultrafiltration, 2.2 fold enrichment was achieved for mulberry leaf PPO and 1.7 fold enrichment was observed for mushroom PPO.

3.3. Biochemical Characterization of Enriched Mulberry Leaf Polyphenol Oxidases

Mulberry leaf PPOs were characterized in terms of kinetic parameters, pH and temperature dependencies of activity, PPO types by activity staining, molecular weight and isoelectric points. As a reference, mushroom PPO was also characterized in terms of kinetic parameters.

3.3.1. Kinetic Analysis of Free and Immobilized Polyphenol Oxidases

3.3.1.1. Kinetic Analysis of Free Polyphenol Oxidases

To determine the kinetic parameters of free mulberry leaf and mushroom PPOs, initial reaction rates at different 4-methyl catechol concentrations, ranging from 0 to 75 mM were measured. Results obeyed Michaelis- Menten kinetics. There was a substrate inhibition of mushroom PPOs. Therefore, for Lineweaver-Burk plot, last 3 data points corresponding to substrate inhibition were neglected.

Data points were also fitted by non-linear regression method (Sigma Plot) to Michaelis-Menten Equation;

$$V = V_{max} [S] / (K_m + [S])$$

Table 3.3 : Enrichment table of mulberry leaf PPO

Step	Volume (ml)	Enzyme Activity (U/ml)	Total Activity (U)	Protein Concentration (mg/ml)	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Enrichment Fold
Crude Extract	70	28	1960	0.17	11.9	165	100	1.0
Filtrate (0.22 µm)	50	36	1800	0.11	5.5	327	92	2.0
Retentate (30 kDa)	20	89	1780	0.25	5.0	356	90	2.2

Table 3.4 : Enrichment table of mushroom PPO

Step	Volume (ml)	Enzyme Activity (U/ml)	Total Activity (U)	Protein Concentration (mg/ml)	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Enrichment Fold
Crude Extract	70	29	2030	0.029	2.0	1015	100	1.0
Filtrate (0.22 µm)	50	28	1400	0.018	1.0	1400	69	1.5
Retentate (30 kDa)	20	42	840	0.026	0.5	1680	41	1.7

Since mushroom PPOs were inhibited at high concentrations of 4-methyl catechol, data points were fitted to substrate inhibition model (EZ-FIT) (Perrella, 1988);

$$V = V_{max} / (1 + (K_m/[S]) + ([S]/K_{si}))$$

where K_{si} was the substrate inhibition coefficient.

From Lineweaver-Burk plots shown in Figures 3.3 and 3.4, K_m and V_{max} values of free mulberry leaf and mushroom PPOs were found as 6 mM and 204 U/ml, and, 2 mM and 313 U/ml, respectively.

Kinetic parameters of mulberry leaf and mushroom PPOs were also calculated by fitting data to Michaelis-Menten Equation by using non-linear regression (Sigma Plot).

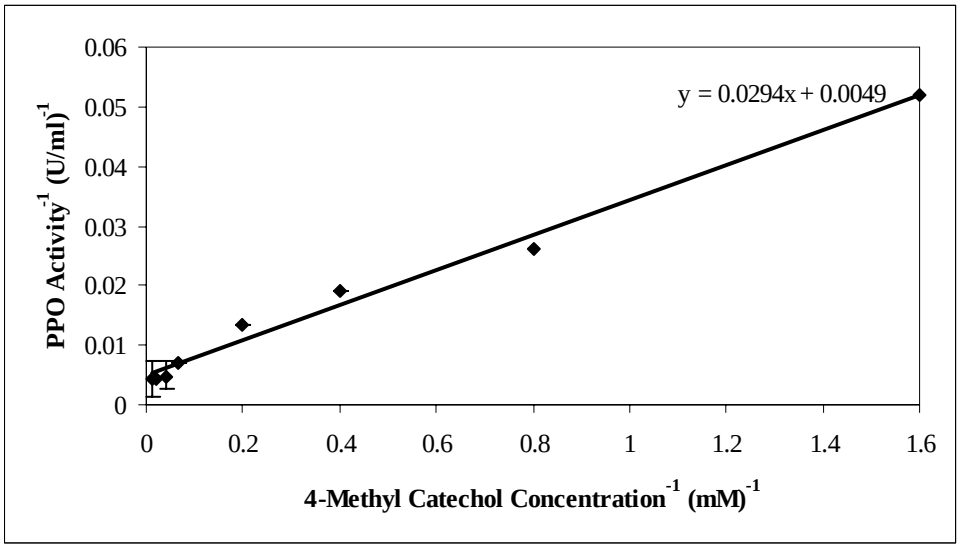

Figure 3.3 : Lineweaver-Burk Plot for free mulberry leaf PPO

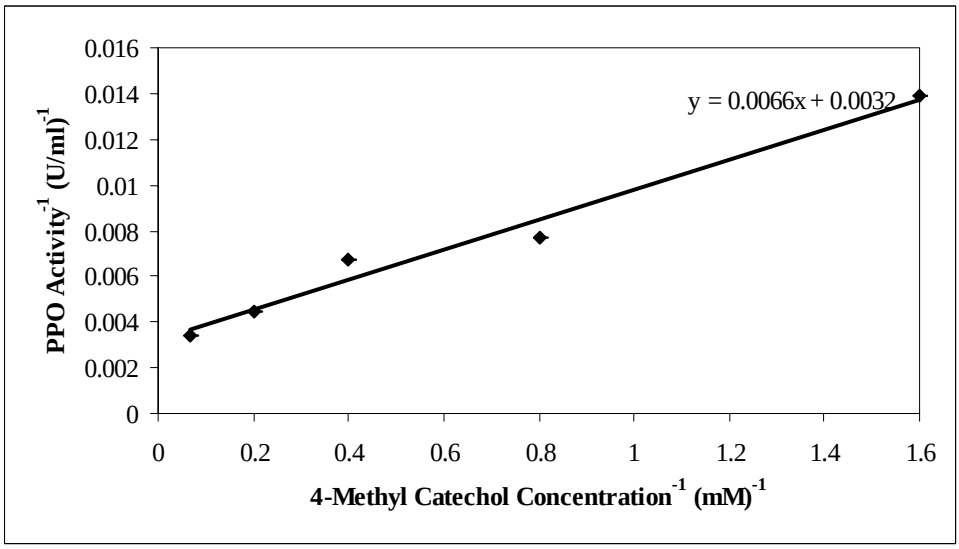

Figure 3.4 : Lineweaver-Burk Plot for free mushroom PPO

For free mulberry leaf and mushroom PPOs, by neglecting the last three data points for mushroom, the fitted Michaelis-Menten Curves were plotted as shown in Figures 3.5 and 3.6.

K_m and V_{max} values of free mulberry leaf and mushroom PPOs were found as 7 mM and 231 U/ml, and, 3 mM and 217 U/ml, respectively, by using non-linear regression method.

Since mushroom PPOs were inhibited at high 4-methyl catechol concentrations, K_m ,V_{max} and K_{si} values were also calculated by using substrate inhibition fit model (Perrella, 1988) as 3 mM, 372 U/ml and 130 mM, sequentially, as shown in Figure 3.7. Substrate concentration, $[S]=(K_m\ K_{si})^{1/2}$, where dV/d[S] becomes zero. [S] was found with using calculated K_m and K_{si} as 19 mM, where it has a close value, 15 mM, in Figure 3.7.

Kinetic parameters calculated by different methods of freemulberry leaf and mushroom PPOs were summarized in Table 3.5. As observed, Lineweaver-

Burk, non-linear regression models for mulberry leaf and mushroom PPOs and in addition, substrate inhibition model for mushroom PPOs predicted similar results.

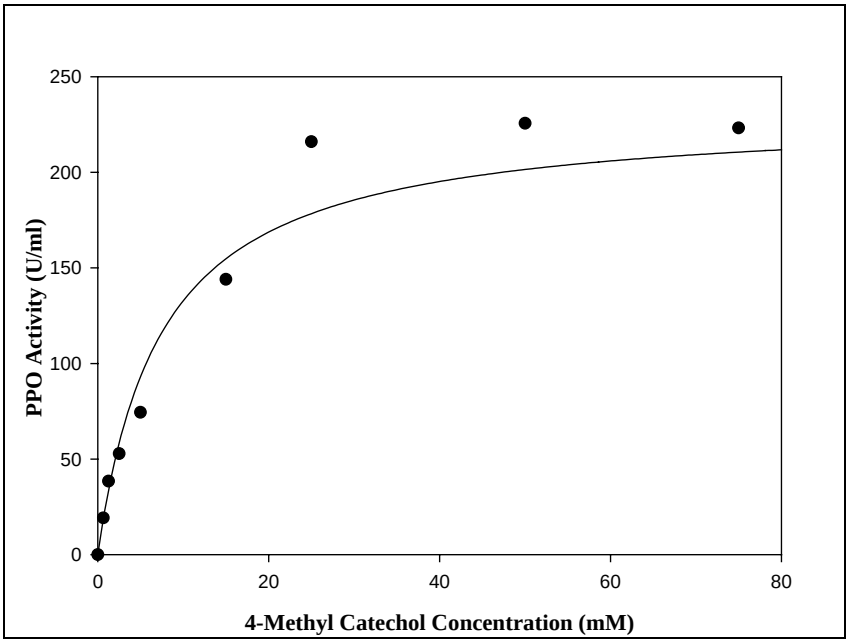

Figure 3.5 : Fitted Michaelis-Menten Curve of free mulberry leaf PPO

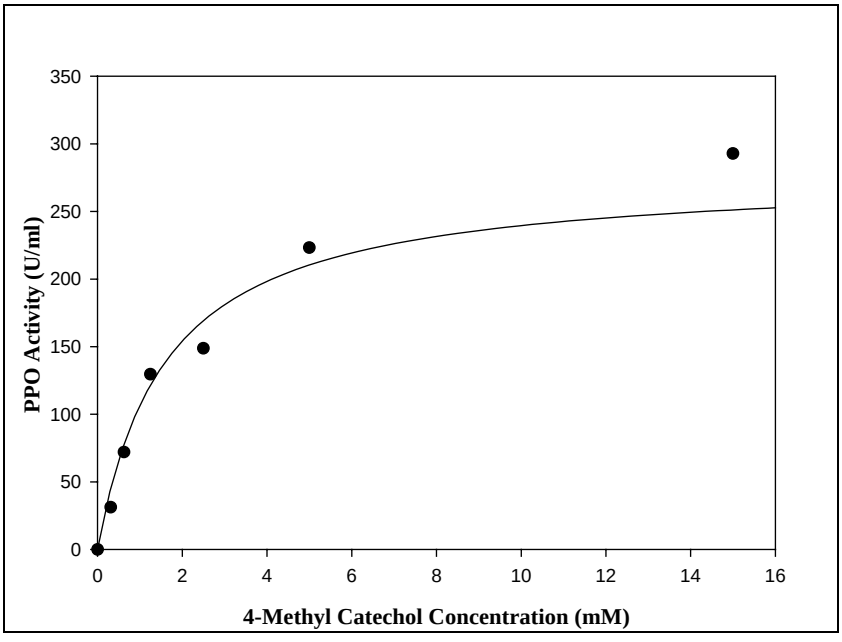

Figure 3.6 : Fitted Michaelis-Menten Curve of free mushroom PPO

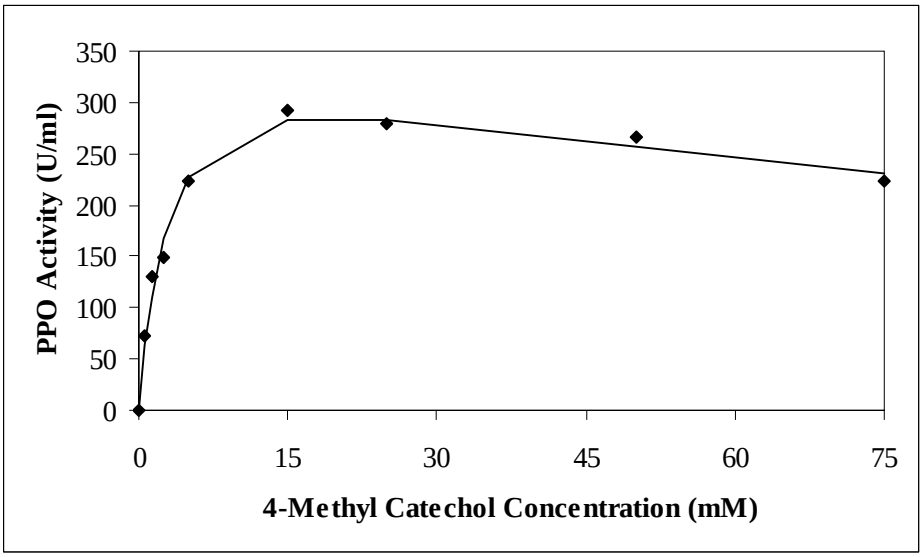

Figure 3.7 : Fitted Substrate Inhibition Model of free mushroom PPO

In Table 3.6, several examples were given from literature about kinetic parameters of PPOs from different sources and mushroom. K_m values found by using 4-methyl catechol as substrate, were in the range of 0.6-9 mM. For mushroom, K_m was found as 2.1 and 2.36 mM where our result was 2-3 mM. Mulberry leaf K_m value was also in the K_m range reported in literature. In table 3.6, V_{max} values were also given but since the enzyme activity unit definitions were different, they could not be compared.

Table 3.5 : Kinetic parameters of free mulberry leaf and mushroom PPOs determined by different methods

	Mulberry Leaf PPOs			Mushroom PPOs		
Method	K_m (mM)	V_{max} (U/ml)	K_{si} (mM)	K_m (mM)	V_{max} (U/ml)	K_{si} (mM)
Lineweaver- Burk Plot	6	204	-	2	313	-
Non-linear Regression	7	231	-	3	217	-
Substrate Inhibition	-	-	-	3	372	130
AVERAGE	7	218	-	3	300	-

Table 3.6 : Kinetic parameters of free PPOs from different plant sources and mushroom

Source	K_m (mM)	V_{max}	Substrate	Reference
Apple Fruit	3.2 0.2	- -	4-methyl catechol 3-hydroxyphloridzin	Goodenough *et al*. (1983)
Avocado fruit	1-2.2 (4 fractions)	0.09-1.33 OD/ minper20µl	4-methyl catechol	Lelyveld *et al*. (1984)
Spinach	4.2	244 µmol/min	4-methyl catechol	Sánchez-Ferrer *et al.* (1989)
Potato	4.3±0.3	-	L-Hydroxy phenyl alanine	Partington and Bolwell (1996)
Mung bean leaf	4 24	0.11 U/min 0.13 U/min	4-methyl catechol L-DOPA	Shin *et al*. (1997)
Mushroom (cap skin)	2.1	0.15 U	Catechol	Zhang and Flurkey (1997)
Mango Kernel	24.6	2.14 U/g mango kernel	Catechol	Arogba *et al.* (1998)
Iceberg Lettuce	6.3	11.9 µM/min	(p-hydroxyphenyl) propionic acid	Chazarra *et al*. (1999)
Apple Leaf	0.6 3.6	- -	4-methyl catechol Phloridzin	Ridgway and Tucker (1999)
Raspberry	663.9 84.2	4.47 OD/min/g fw 1.28 OD/min/g fw	Catechol Catechol	González *et al*. (1999)
Mushroom (cap flesh)	5 9	- -	Catechol L-DOPA	Zhang *et al.* (1999)
Coffee	0.88 and 2.27 (2 isoforms)	-	Chlorogenic acid	Mazzafera and Robinson (2000)
Field bean	10.5 4.0	- -	Catechol 4-methyl catechol	Paul and Gowda (2000)
Grape	9	-	4-methyl catechol	Sánchez-Ferrer *et al.* (1988)
Tobacco	6.8	-	Catechol	Shi *et al.* (2001)
Mushroom	0.7±0.04 2.36±0.4	- -	Monophenol 4-methyl catechol	Fenoll *et al*. (2002)
Mulberry leaf	7	218 U/ml	4-methyl catechol	This study

Since phenol oxidation is a two substrate reaction, phenol and oxygen, oxygen concentration should be high enough to perform kinetic analysis for phenolic substrate. In one study, K_m values for 4-methyl catechol and O_2 were investigated. As shown in Table 3.7, since K_{mO2} values were about thousand times smaller than K_m values, it can be concluded that 4-methyl catechol was the limiting substrate. Because, to reach 10 K_m in the reaction medium, a common substrate concentration, low concentrations of oxygen was enough. Therefore it was reasonable that kinetic parameters were determined only for 4-methyl catechol as substrate in our study. Besides, a control study was performed by vortexing the reaction mixture much more to increase dissolved oxygen concentration in the reaction mixture, however the results have not changed.

Table 3.7 : K_m values of mushroom PPO for 4-methyl catechol and oxygen

	Monophenolase activity		Diphenolase Activity		Reference
Source	K_m (mM)	K_{mO2} (mM)	K_m (mM)	K_{mO2} (mM)	Fenoll *et al*. (2002)
Mushroom	0.7 ± 0.04	$8.3x10^{-4}$ ± $1.8x10^{-4}$	2.36 ± 0.4	$3.7x10^{-2}$ ± $1.1x10^{-2}$	

The results of two reports in which substrate inhibiton kinetic model was used were given in Table 3.8. In our study, a relatively high value as compared to documented substrate inhibition constants, 130 mM, was found for mushroom PPO.

Table 3.8 : Substrate inhibition coefficients (K_{si}) of PPOs from different plant sources

Source	Substrate	K_{si} (mM)	Reference
Spinach	4-methyl catechol	104	Sánchez-Ferrer *et al.* (1989)
Desert truffle	4-methyl catechol	6.35	Pérez-Gilabert *et al.* (2001)
Mulberry leaf	4-methyl catechol	7	This study

3.3.1.2. Kinetic Analysis of Immobilized Polyphenol Oxidases

To determine the kinetic parameters of immobilized mulberry leaf and mushroom PPOs, initial reaction rates at different 4-methyl catechol concentrations, ranging from 0 to 300 mM were measured. Results obeyed the Michaelis- Menten Curve and K_m and V_{max} values for mulberry leaf and mushroom PPOs were calculated from Lineweaver-Burk plot as shown in Figures 3.8 and 3.9 as, 35 mM and 3 U/ml, and, 20 mM and 5 U/ml, respectively.

Kinetic parameters calculation results of immobilized mulberry leaf and mushroom PPOs were summarized in Table 3.9.

Table 3.9 : Kinetic parameters of immobilized mulberry leaf and mushroom PPOs determined by Lineweaver-Burk Plot

	Mulberry Leaf PPOs		Mushroom PPOs	
Method	K_m (mM)	V_{max} (U/ml)	K_m (mM)	V_{max} (U/ml)
Lineweaver- Burk Plot	35	3	20	5

Results in Table 3.9 showed that after immobilization K_m values of mulberry leaf and mushroom PPOs increased about 7 fold as compared with free PPOs because of diffusional resistance. V_{max} values can not be compared with literature data since the quantity of immobilized PPO was not known. In Table 3.10, studies were given which resulted in K_m increase and V_{max} decrease after immobilization.

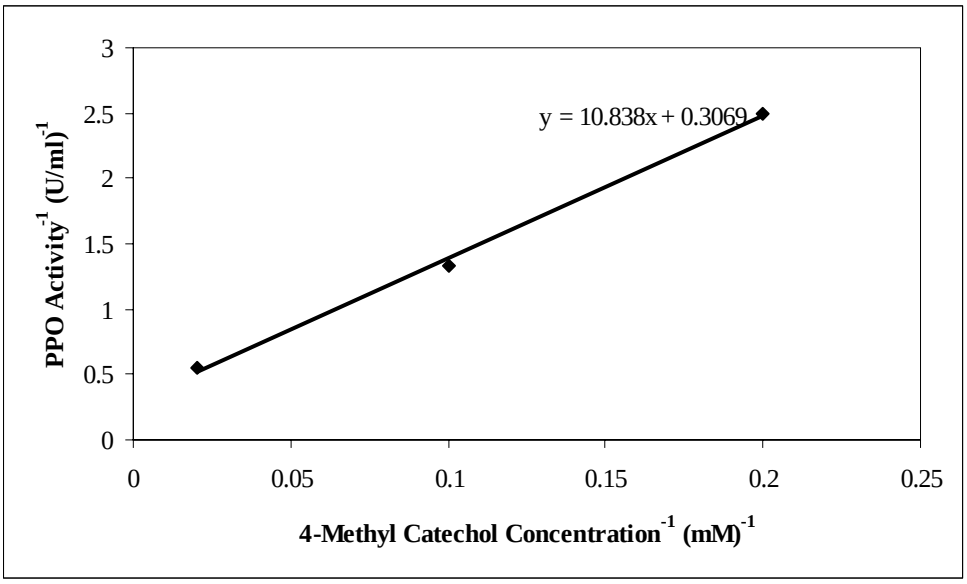

Figure 3.8 : Lineweaver-Burk Plot for immobilized mulberry leaf PPO

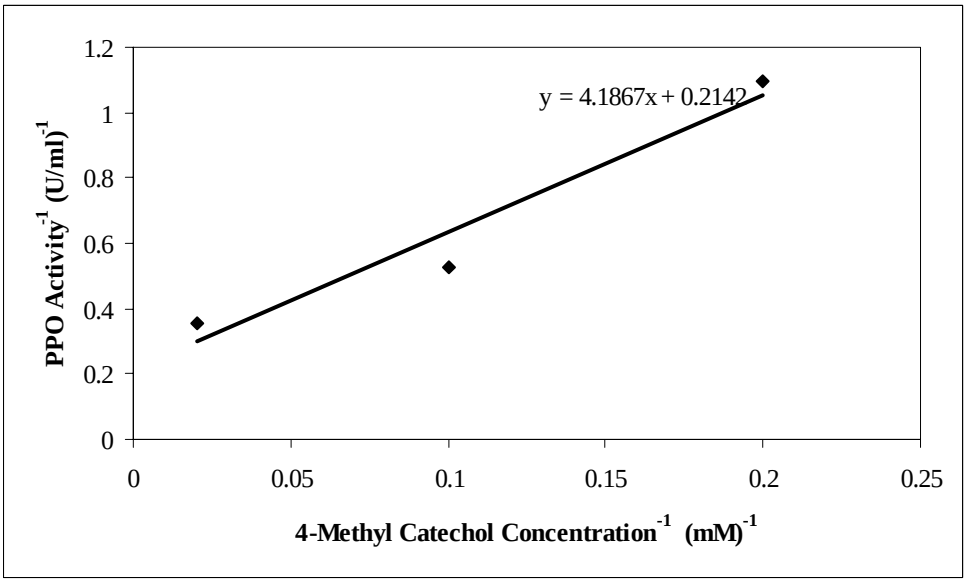

Figure 3.9 : Lineweaver-Burk Plot for immobilized mushroom PPO

Table 3.10 : Kinetic parameters of immobilized PPOs and other enzymes

Enzyme	Free K_m (mM)	Imm. K_m (mM)	Increase fold in K_m	Free V_{max}	Imm. V_{max}	Decrease fold in V_{max}	Reference
PPO	0.65	0.87	1.3	1890 U/mg	760 U/mg	2.5	Arica (2000)
PPO	4	100 200	25 50	11.2µmol/min.mg protein	0.11 and 0.10µmol/min.electrode	Not given	Kiralp *et al.* (2003)
Urease	2.82	16.7	5.9	7.31 U/elec	1.74 U/elec	4.2	Alkan *et al.* (2001)
Invertase	24.3	41.8	1.7	82.3 µmol/min	0.65 µmol/min	127	Erginer *et al.* (2000)
Invertase	30.2	63	2.1	98 µmol/min	3.7 µmol/min	94.3	Selampinar *et al.* (1997)

3.3.2. Stability of Immobilized Polyphenol Oxidases

Stability of immobilized mushroom PPO at 4°C was investigated for 8 months. Results were shown in Figure 3.10. It was stable for first 6 monts but after that, in 2 months, PPO totally lost its activity.

Operational stabilities of mulberry leaf and mushroom PPOs were also determined. Results were given in Figure 3.11. Immobilized mulberry leaf PPO showed better operational stability than mushroom PPO. After 5. usage PPO activity of both electrodes decreased to 20-30% of the initial activity. Similar result was observed by Kiralp *et al.* (2003). In the study, 40% activity loss after 10 usages was detected by immobilization of commercial PPO in PPy matrix. Since our PPO source was partially purified extract, it was difficult to perform a successful immobilization. To reach high concentations of PPO, high amounts of plant

materials were used but in this case, extracts became viscous. Because of this viscousity, polymerization of pyrole was a problem. Therefore, by performing extra purification steps, less viscous extracts and better operational stabilities could be achieved.

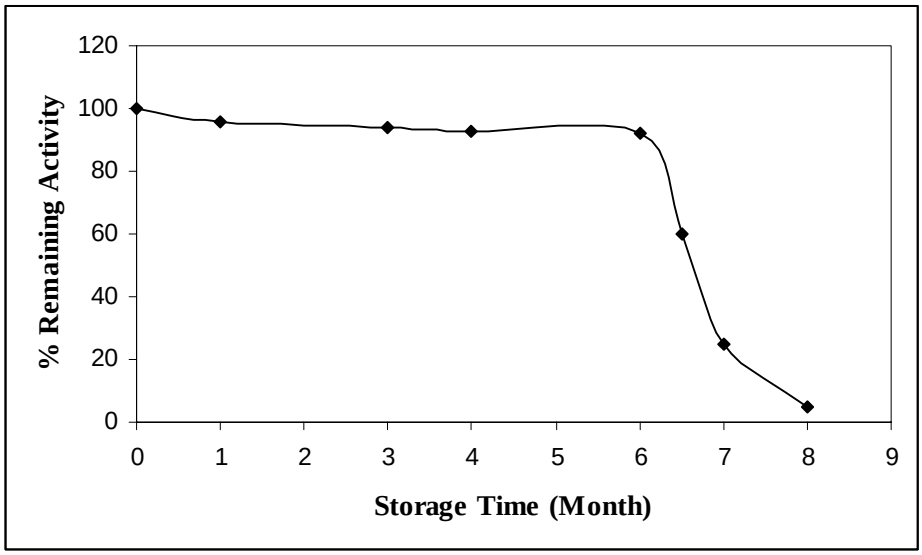

Figure 3.10 : Storage stability of immobilized mushroom PPO

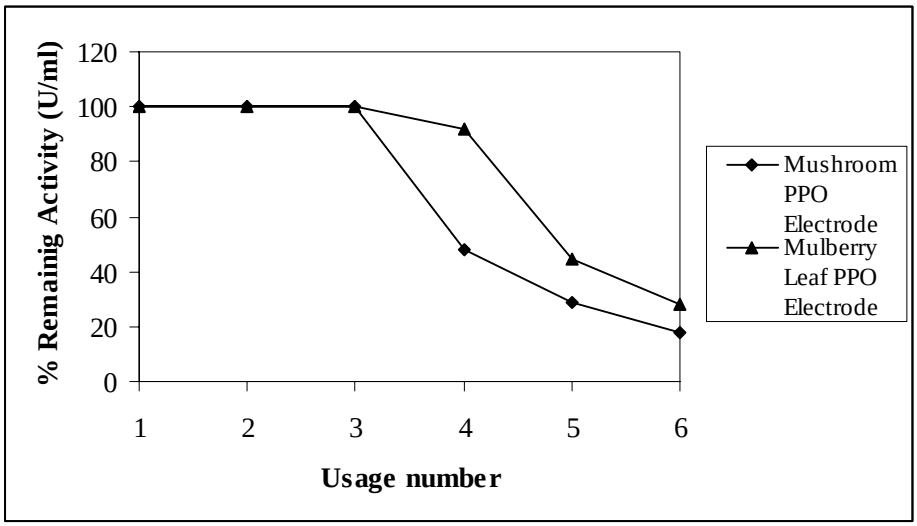

Figure 3.11 : Operational stability of immobilized mulberry leaf and mushroom PPOs

3.3.3. Temperature Dependency of Polyphenol Oxidase Activity

To determine the temperature dependency of mulberry leaf PPO activity, activities at different temperatures ranging from 4 to 60°C were measured. Extracts and substrate solutions were equilibriated to the required temperatures for 10 minutes before activity assay. The maximum activity of mulberry leaf PPO was observed at 45°C as shown in Figure 3.12. This result for optimum temperature was reasonable according to results from literature given in Table 3.11.

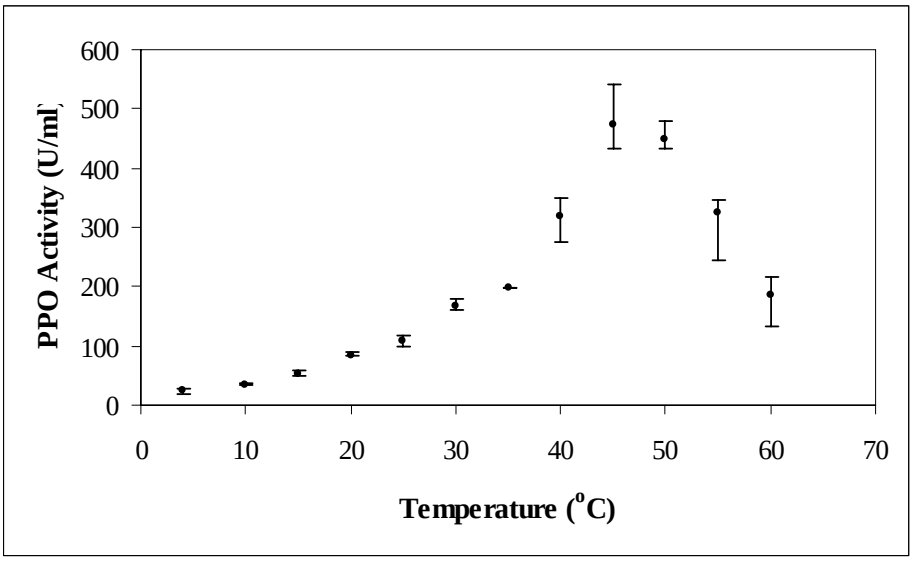

Figure 3.12 : Temperature dependency of mulberry leaf PPO activity

Table 3.11 : Optimum temperatures of PPOs from different plant sources

Source	Optimum T (°C)	Reference
Grape	20-40	Sánchez-Ferrer *et al.* (1988)
Tobacco	40	Shi *et al.* (2001)
Rubber tree seeds	35-45	Wititsuwannakul *et al.* (2002)
Medlar fruit	35	Dincer *et al.* (2002)
Mulberry leaf	45	This study

3.3.4. pH Dependency of Polyphenol Oxidase Activity

To determine the pH dependency of mulberry leaf PPO, activities in a pH range of 4-9 were measured. The maximum activity of mulberry leaf PPO was observed at pH 7 as shown in Figure 3.13. Optimum pH values of PPOs from different plant tissues and mushroom were given in Table 3.12.

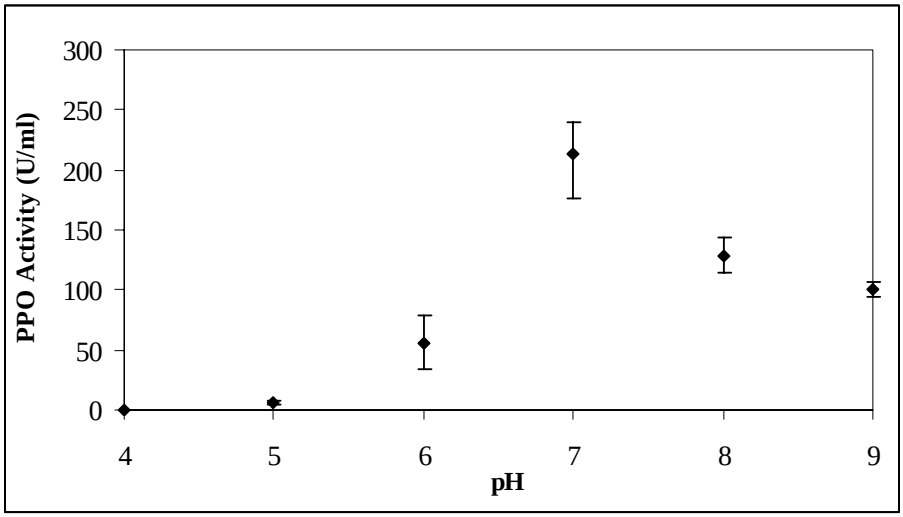

Figure 3.13 : pH dependency of mulberry leaf PPO activity

3.3.5. Determination of the Type of Polyphenol Oxidase(s) by Activity Staining on Polyacrylamide Gel

After PAGE without SDS, mulberry extract was electrophorised and the gel was stained for different types of PPO activities using the modified procedure of Rescigno *et al.* (1997) (Appendix E). The procedure consists of 4 steps including staining with 4-amino-*N*,*N*-diethylaniline (ADA) for laccase activity, with hydrogen peroxide (H_2O_2) for peroxidase activity and with 4-tert-butyl catechol (*t*BC) and

Table 3.12 : pH Dependencies of PPOs from different plant sources and mushroom

Source	Optimum pH	Substrate	Reference
Table beet	7	Catechol	Lee and Smith (1979)
Date	4.5-6.5	Chlorogenic acid	Hasegawa and Maier (1980)
Pear	5.1	Catechol and chlorogenic acid	Smith and Montgomery (1985)
Strawberry	4.5 5.5	4-methyl catechol Catechol	Wesche-Ebeling and Montgomery (1990)
Mung bean leaf	6	Catechol	Shin *et al*. (1997)
Mushroom	7	Catechol	Zhang anf Flurkey (1997)
Table beet leaves	4.5-5	4-*tert*-butyl catechol	Escribano et al. (1997)
Mango kernel	6	Catechol	Arogba *et al.* (1998)
Iceberg lettuce	5	(p-hydroxyphenyl) propionic acid	Chazarra *et al*. (1999)
Raspberry	7	Catechol and catechin	González *et al*. (1999)
Coffee	6-7	Chlorogenic acid	Mazzafera and Robinson (2000)
Field bean	4	Catechol and 4-methyl catechol	Paul and Gowda (2000)
Grape	3.5-5	4-methyl catechol	Sánchez-Ferrer *et al.* (1988)
Tobacco	7	Catechol	Shi *et al.* (2001)
Medlar fruit	6.5	4-methyl catechol	Dincer *et al.* (2002)
Mulberry leaf	7	4-methyl catechol	This study

4-methyl catechol (MC) for catechol oxidase (PPO) activity with and without catechol oxidase inhibitor using small portions of the gel, sequentially.

After staining with ADA, a slightly visible pink band appeared. Since ADA was a specific substrate for laccase, it showed the presence of laccase activity. By

adding H_2O_2, the band corresponding to laccase activity became visible and another thick pink band analogous to peroxidase activity was observed as shown in Figure 3.14.

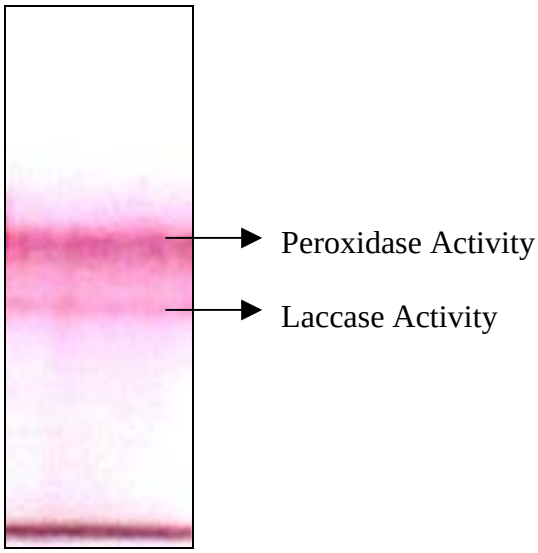

Figure 3.14: Activity staining of mulberry leaf sample on slab gel with ADA and H_2O_2

No additional band was detected by staining the gel further with *t*BC, however laccase and peroxidase activity bands became dark blue as shown in Figure 3.15. This means that either there was no catechol oxidase activity for 4-tertbutyl catechol or the catechol oxidases were located at the same position with laccase and peroxidase bands, so they showed activity without any additional band. In order to solve this dilemma, without staining with H_2O_2, the gel was stained only with ADA and *t*BC after electrophoresis. Because in the presence of catechol oxidase, *t*BC is oxidized to the corresponding yellow o-quinone, which in turn quickly reacts with the coupling agent, ADA, leading to a blue adduct. However also in this case no additional band was observed. According to this result, it can be

concluded that mulberry leaf extract did not contain any catechol oxidase which was specific to *t*BC or its concentration was low in the extract. Another reason could be the high isoelectric point of catechol oxidase (specific for *t*BC) which causes migration of these enzymes in the gel in the opposite direction, so, they can not be separated from the crude extract by the electrophoretic method used.

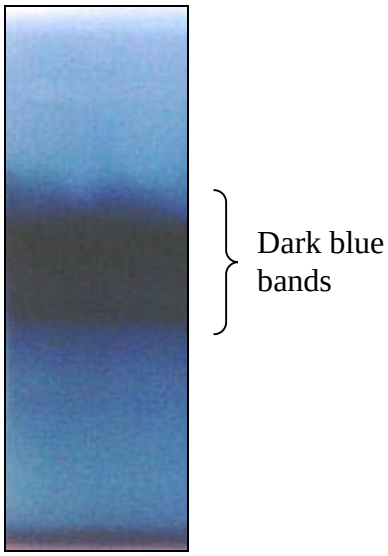

Figure 3.15 : Activity staining of mulberry leaf sample on slab gel with *t*BC

Since, in PPO assay experiments, 4-methyl catechol (MC) was used as substrate, it was predicted that mulberry leaf extract contains catechol oxidase. To see this activity on the gel, gel was stained with MC and two orange-brown bands were observed corresponding to PPO activity (catechol oxidase activity specific for MC) where laccase and peroxidase activities were also detected as shown in Figure 3.16.

To be sure that MC was oxidized by catechol oxidase, not by laccase, another gel was soaked in salicylhydroxamic acid which is a catechol oxidase inhibitor. After inhibiton of catechol oxidase, gel was stained with MC and very

slight bands were observed. In Figure 3.17, part (a) shows catechol oxidase activity in the presence of inhibitor and part (b) shows catechol oxidation by laccase in the absence of catechol oxidase inhibitor.

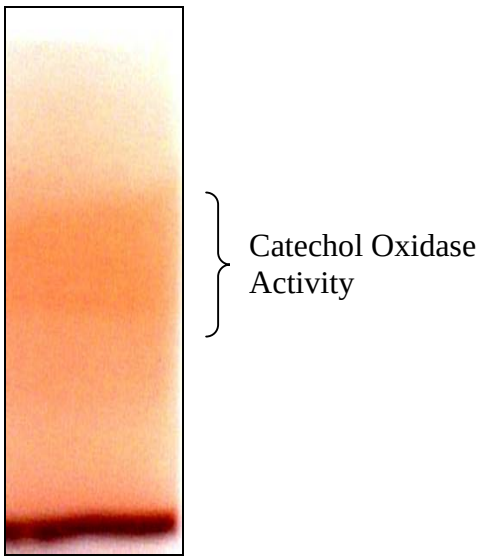

Figure 3.16 : Activity staining of mulberry leaf sample on slab gel with MC

As a result, laccase, peroxidase and PPO activities (catechol oxidase activity specific for MC) of mulberry leaf extract were all observed on the polyacrylamide gel. But, catechol oxidase activity specific for *t*BC could not be detected with this method. By using catechol oxidase inhibitor, it was proved that MC was oxidized by catechol oxidase, not only by laccase.

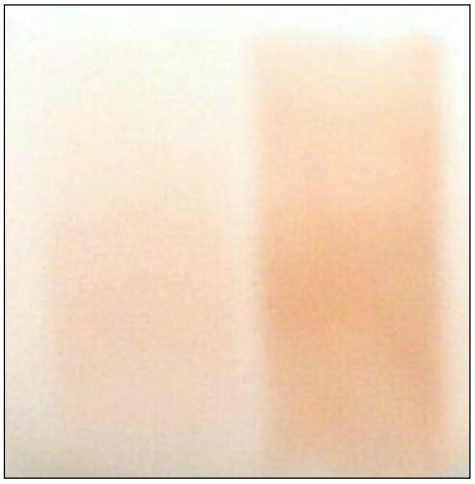

Figure 3.17 : Catechol oxidase activity with (a) and without (b) salicylhydroxamic acid (inhibitor)

3.3.6. Molecular Weight Analysis of Mulberry Leaf Polyphenol Oxidases

To determine the molecular weight of these separated enzymes, polyacrylamide gel electrophoresis in the presence of anionic detergent (SDS) was carried out. Electrophoresis was done with 4% stacking gel and 12% separating gel.

After proteins were eluted from 2 active bands (one thick and one thin bands) on the polyacrylamide gel, they were further concentrated with (vivapore) membrane concentrator. Molecular weight markers, crude and concentated proteins were loaded on the gel and SDS-PAGE was performed. At the end of the run, proteins were stained with silver staining method (Appendix G). Resulting gel was shown in the Figure 3.18.

After SDS polyacrylamide gel electrophoresis, molecular weight of laccase was estimated as about 62 kDa and molecular weight of peroxidase was found as about 64 kDa. Since these two bands showed catechol oxidase activity, molecular weight of catechol oxidase was estimated as around 63 kDa.

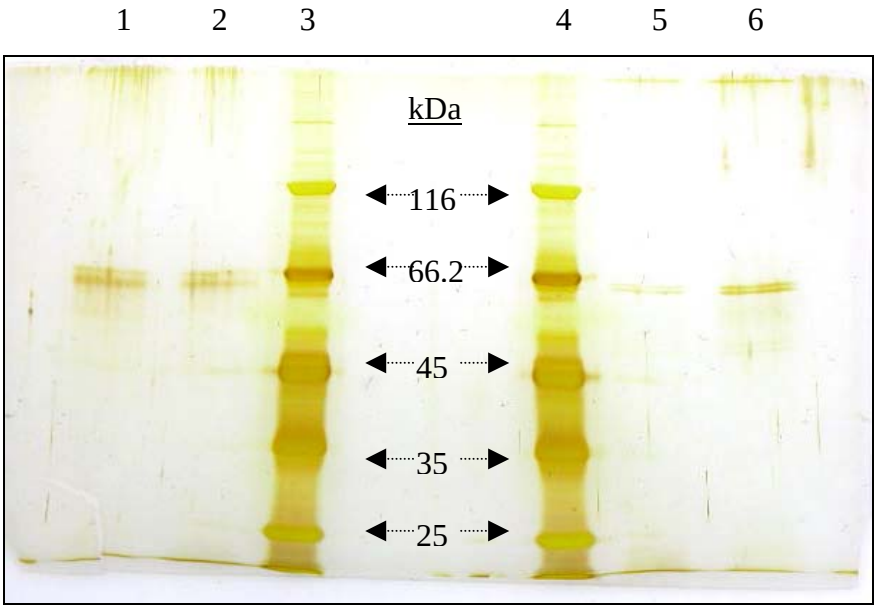

Figure 3.18 : Silver stained SDS-PAGE profile of PPO

(Lanes; 1 and 2: Concentrated thick band, 3 and 4: Molecular weight markers, 5 and 6: Concentrated thin band)

Studies about molecular weights of different plant-based PPOs from literature were given in Table 3.13. Molecular weights of PPOs from different plants and mushroom were given in the range of 30-120 kDa and our results were in this range.

Table 3.13 : Molecular weights of PPOs from different plant sources and mushroom

Source	Molecular weight (kDa)	Reference
Banana	65-70	Galeazzi *et al*. (1981)
Air potato	115±2 (4 subunits)	Anosike and Ayaebene (1982)
Avocado fruit	500-87.5 (5 isoforms)	Lelyveld *et al*. (1984)
Strawberry	34.5 and 111 (2 isoforms)	Wesche-Ebeling and Montgomery (1990)
Spinach	64	Hind *et al*. (1995)
Potato	60 and 69 (2 isoforms)	Partingtan and Bolwell (1996)
Mung bean leaf	65 (major) and 59 (minor)	Shin *et al*. (1997)
Tobacco	100	Richardson and McDougall (1997)
Apricot fruit	67.1 (calculated) 56.2 (predicted)	Chevalier (1999)
Litchi peel	75.6 (gel filtration) with 2 subunits of 25 and 45 (by SDS-PAGE)	Jiang *et al*. (1999)
Field bean	120±3 (4 subunits)	Paul and Gowda (2000)
Tobacco	35.7	Shi *et al.* (2001)
Rubber tree seeds	32 and 34 (2 isoforms)	Wititsuwannakul *et al.* (2002)
Mulberry leaf	62-64	This study

By plotting relative mobilities vs. logarithms of molecular weights of marker proteins a linear profile was observed (Figure 3.19). This result showed a good separation of marker proteins in the gel.

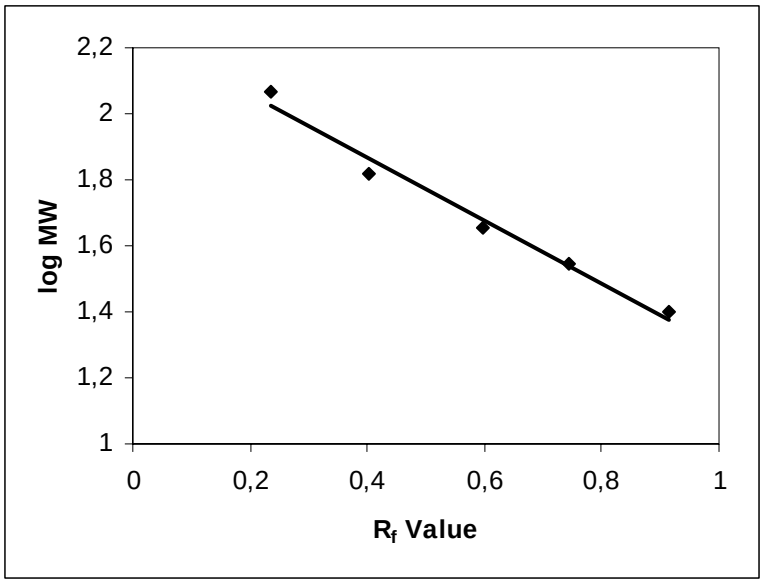

Figure 3.19: Standard Curve for SDS-PAGE

3.3.7. Isoelectric Focusing Analysis of Mulberry Leaf Polyphenol Oxidases

Isoelectric focusing was performed to determine the isoelectric points of different mulberry leaf PPOs which showed activities in the same bands on polyacrylamide gel. By loading the isoelectric point markers and ultrafiltrated and (vivapore) concentrated mulberry leaf extract on the gel, isoelectric focusing was carried out.

After the run was finished, pI markers (Figure 3.20) were stained with quick coomassie blue staining method (Appendix I) and sample containing part was stained with using the modified procedure of Rescigno *et al.* (1997) (Appendix E). The procedure consists of 4 steps including staining with 4-amino-*N*,*N*-diethylaniline (ADA) for laccase activity, with hydrogen peroxide (H_2O_2) for peroxidase activity, with 4-tert-butyl catechol (*t*BC) for catechol oxidase

(polyphenol oxidase) activity and with 4-methyl catechol (MC) for catechol oxidase (polyphenol oxidase) activity.

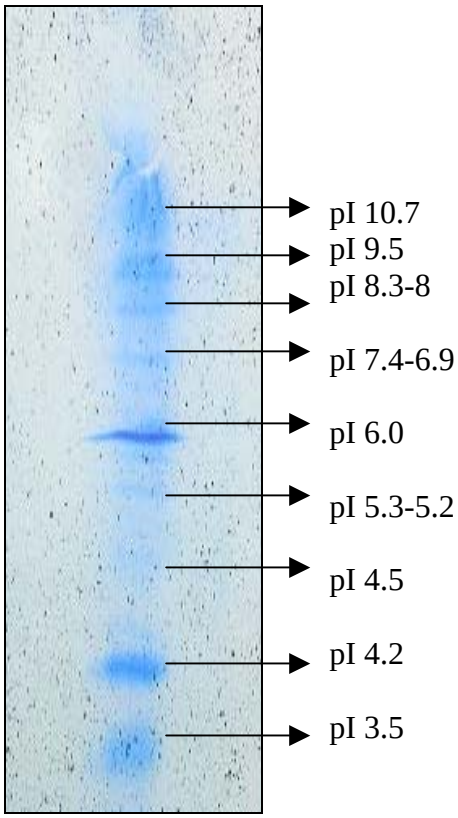

Figure 3.20 : Coomassie blue stained isoelectric focusing markers

After staining with ADA, indistinctly pink spot appeared corresponding to laccase activity around pH 8-8.5 as shown in Figure 3.21.

By adding H_2O_2 a thick pink band analogous to peroxidase activity at around pH 4.5 was observed as shown in Figure 3.22.

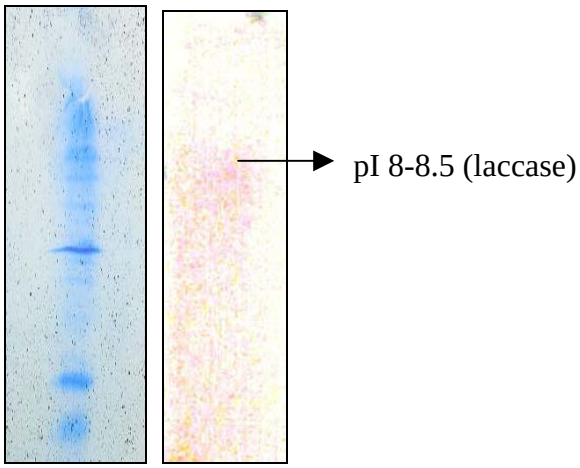

Figure 3.21: Activity staining of mulberry leaf sample with ADA on isoelectric focusing gel

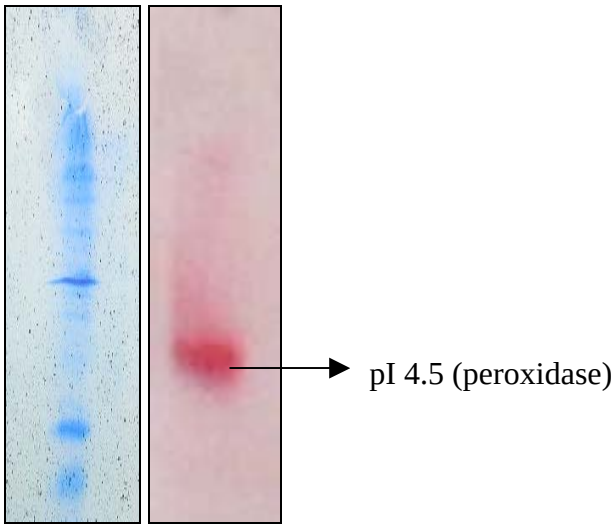

Figure 3.22: Activity staining of mulberry leaf sample with H_2O_2 on isoelectric focusing gel

No additional band was detected by staining the H_2O_2 stained gel with *t*BC, band corresponding to laccase and peroxidase activities became dark blue (Figure

3.23). This means that there was no catechol oxidase activity for 4-tertbutyl catechol. Same result was observed with activity staining on polyacrylamide gel.

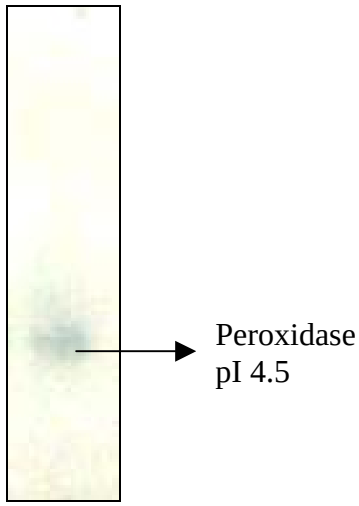

Figure 3.23 : Activity staining of mulberry leaf PPO with *t*BC on isoelectric focusing gel

On the other gel, stained with MC, a clear yellow-orange band was observed corresponding to PPO activity (catechol oxidase activity) around pH 10. Since the gel was cream-colored, slight yellow-orange band can not be shown with a photograph.

As a result, isoelectric points of laccase, peroxidase and catechol oxidase were found as 8-8.5, 4.5, 10, sequentially. These results were reasonable according to results from literature given in Table 3.14.

Table 3.14 : Isoelectric points of phenol oxidative enzymes from different plant sources and mushroom

Source	pI	Reference
Banana PPO	5.2	Galeazzi *et al*. (1981)
Mung bean leaf PPO	5.1	Shin *et al*. (1997)
Mushroom (cap skin) PPO	Between 4.45 – 5.9 (10 isoforms)	Zhang and Flurkey (1997)
Apricot fruit PPO	6.84 (calculated) 5.84 (predicted)	Chevalier *et al.* (1999)
Mushroom (cap flesh) PPO	5.1, 5.2, 5.3 (3 isoforms)	Zhang *et al*. (1999)
Melon peroxidase	3.7	Rodríguez-López *et al*. (2000)
Rubber tree seed PPO	9.2	Wititsuwannakul *et al.* (2002)
Mulberry leaf peroxidase	4.5	This study
Mulberry leaf laccase	8.0-8.5	This study
Mulberry leaf catechol oxidase	10	This study

CHAPTER 4

CONCLUSIONS AND RECOMMENDATIONS

The aim of this study was to find an economical plant source for PPO production, characterization and immobilization of the PPOs for the ultimate purpose of PPO electrode and/or immobilized PPO bioreactor development. For this purpose, different plant tissues of no commercial value like horse chestnut fruits and leaves of many fruit trees, were screened for PPO activities. Since mulberry leaf tissues showed the highest PPO activity against 4-methyl catechol, PPOs from mulberry leaf tissues were immobilized and characterized. Pear, sour cherry and apricot leaves were also good PPO sources. Other plant materials; cherry, chestnut, apple, grape, quince leaves, hazelnut fruit shell and horse chestnut fruit showed lower PPO activities.

Mulberry leaf tissue PPOs were found to be suitable for phenolic determination and/or removal from neutral systems without dilution because optimum pH was found as 7. Especially for phenolic determination in wine, having an acidic pH, mulberry leaf PPOs could be used after diluting the sample in order to increase pH values. For waste waters at high pHs, like paper and pulp industry waste water, mulberry leaf PPOs seemed to be suitable for phenolics removal. Since sour cherry and pear leaf tissue PPOs showed acidic optimum pH, they can be more suitable for acidic systems, like wine.

Laccase, peroxidase and catechol oxidase activities were detected in mulberry leaf tissues with a very close molecular weight of 63 kDa and isoelectric points of 8.0-8.5, 4.5 and 10, sequentially. Since three different types of phenol oxidative enzymes were found in mulberry leaf tissues, it could be advantageous to use them together in phenolic concentration determination within an electrode and phenolic removal from waste waters because of the existence of a wide substrate range. It could be also possible to separate these three types of enzymes and used them for specific purposes.

Average K_m and V_{max} values of free mulberry leaf and mushroom PPOs were found as 7 mM, 218 U/ml and 3 mM, 300 U/ml, respectively. No substrate inhibition was observed for mulberry leaf PPOs in the tested substrate concentration range, where mushroom PPOs showed substrate inhibition with a K_{si} value of 130 mM. Therefore at high substrate concentrations it can be advantageous to use mulberry leaf PPOs.

After immobilization of PPOs in polypyrole matrix, average K_m and V_{max} values of mulberry leaf and mushroom PPOs were found as 35 mM, 3 U/ml and 20 mM, 5 U/ml, respectively. Results showed that K_m values increased after immobilization because of the diffusional resistance where as V_{max} values can not be compared since the quantity of immobilized enzyme was not known. To overcome diffusional resistance, instead of matrix entrapment, PPOs can be immobilized on a surface. Since our PPO source was dilute and partially purified, it was difficult to perform a successful immobilization. By purification and concentration of the enzyme immobilization can be improved.

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APPENDIX A

PREPARATION OF BRADFORD REAGENT

Chemicals used to prepare concentrated stock reagent solution (5x stock) were given in Table A.1.

Table A.1 : Bradford reagent preparation procedure

Chemicals	Amount used
85% Ortho-phosphoric acid	500 ml
95% Ethanol	250 ml
Brillant Blue G-250 dye	500 mg

These chemicals were mixed and diluted to 1 L with distilled water to prepare 5x concentrated stock reagent solution.

The stock solution was stored at 4°C. To prepare diluted (1x) reagent solution 1 volume concentrate was mixed with 4 volumes of distilled water. This solution was well mixed and filtered.

Bradford reagent should wait at least 24 hours at room temperature before use.

APPENDIX B

PREPARATION OF PROTEIN STANDARD FOR BRADFORD METHOD

The Bradford assay is very fast and uses about the same amount of protein as the Lowry assay. It is fairly accurate and samples that are out of range can be retested within minutes. The Bradford is recommended for general use, especially for determining protein content of cell fractions and assesing protein concentrations for gel electrophoresis.

The assay was based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. The assay was useful since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range.

Bovine serum albumin (BSA) was used as protein standard. To prepare 1 mg/ml stock BSA solution, 25 mg BSA was dissolved in 25 ml of pH 7 sodium phosphate buffer. This stock solution was diluted at different ratios given in Table B.1.

Table B.1 : BSA dilution ratios for Bradford Method

Protein (mg/ml)	0	0.01	0.02	0.03	0.04	0.05
BSA stock (ml)	0	0.1	0.2	0.3	0.4	0.5
Buffer (ml)	10	9.9	9.8	9.7	9.6	9.5

After preparation of diluted BSA samples, 0.5 ml BSA sample and 5 ml of Bradford reagent were mixed in a glass test tube. Ten minutes later absorbance at 595 nm was measured by using a spectrophotometer.

APPENDIX C

BSA STANDARD CURVE FOR BRADFORD METHOD

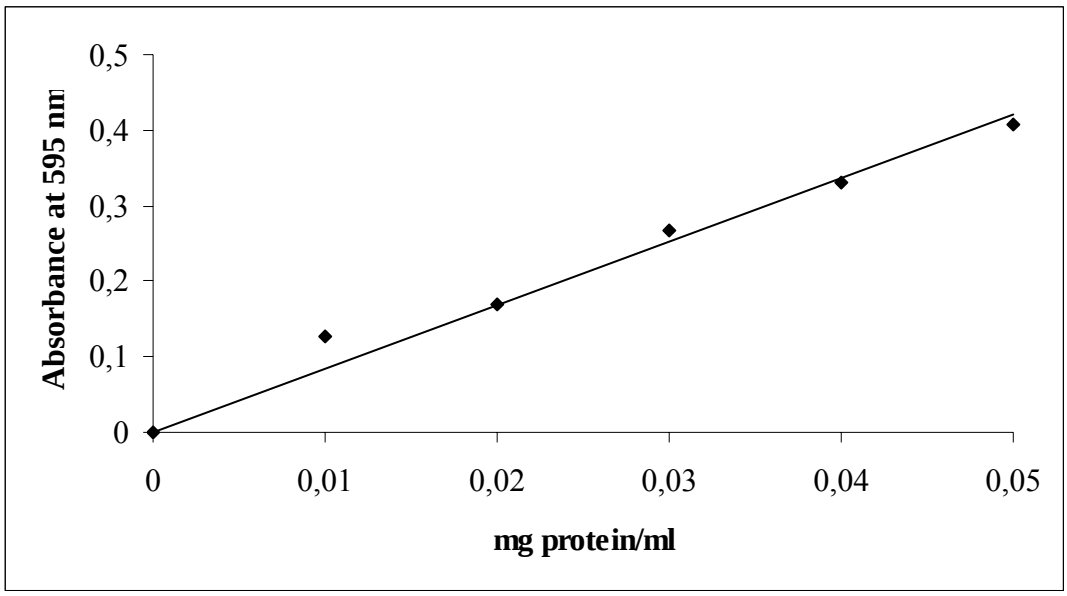

Figure C.1 : BSA standard curve for Bradford Method

APPENDIX D

REAGENTS AND GEL PREPARATION FOR POLYPHENOL OXIDASE ACTIVITY STAINING OF SLAB GEL

Stock Solutions

A. Acrylamide/bis (30% T, 2.67% C)

87.6 g acrylamide (29.2g/100 ml)

2.4 g N'N'-bis-methylene-acrylamide (0.8g/100 ml)

Make to 300 ml with distilled water. Filter and store at 4°C in the dark (30 days maximum). Since acrylamide is a neuro toxin, precautions should be taken by wearing gloves and mask during preparation of this solution.

B. 1.5 M Tris-HCl, pH 8.8

27.23 g Tris base

~80 ml distilled water

Adjust to pH 6.8 with 1N HCl. Make to 100 ml with distilled water and store at 4°C.

C. 0.5 M Tris-HCl, pH 6.8

6 g Tris base

~60 ml distilled water

Adjust to pH 6.8 with 1N HCl. Make to 100 ml with distilled water and store at 4°C.

D. Sample Buffer (SDS reducing buffer) (store at room temperature)

Distilled water	6.0 ml
0.5 M Tris-HCl, pH 6.8	1.0 ml
Glycerol	0.8 ml
0.05% (w/v) bromophenol blue	0.2 ml
	8.0 ml

E. 5X Electrode (Running) Buffer, pH 8.3 (enough for 10 runs)

Tris base	9.0 g
Glycine	43.2 g
SDS	3.0 g

Bring to 600 ml with distilled water. Store at 4°C. Warm to 37°C before use if precipitation occurs. Dilute 60 ml 5X stock with 240 ml distilled water for one electrophoretic run.

F. 10% Ammonium Persulfate (APS)

Dissolve 100 mg APS in 1 ml of distilled water in an eppendorf by vortexing. This solution should be prepared fresh daily.

G. TEMED (*N*,*N*-tetramethylene-ethylenediamine)

Use TEMED neat from the bottle.

Procedure

A. Preliminary Preparation

Clean the glasses and spacers with ethanol. Assemble the gel sandwich on a clean surface. Lay the longer rectangular glass plate down first, the place two

spacers of equal thickness along the short edges of rectangular plate. Next, place the shorter glass plate on top of the spacers. Install the clamps and fasten the screws. Transfer the clamp assembly to one of the casting stand. To check whether the glasses are properly sealed, pour distilled water between glasses. If water level does not decrease, glasses are properly sealed. Then, pour out the water and let the glasses to dry.

B. Preparation of Gel Solution

Separating Gel

Add the followings into a small beaker.

Table D.1 : Preparation of %5 separating gel for activity staining

Monomer Concentration (30% T, 2.67% C)	%5
Acryamide/bis (30% T, 2.67% C Stock)	1.67 ml
Distilled water	5.78 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml
0.5 M Tris-HCl, pH 6.8	-
10% Ammonium persulfate (fresh)	50 µl
TEMED (*N*,*N*-tetramethylene-ethylenediamine)	5 µl

Prepare the monomer solution by combining all reagents except ammonium persulfate and TEMED. Dearate the solution under vacuum for at least 15 minutes. Add the two catalysts just prior to casting the gels.

After adding two catalysts immediately pour the solution between glasses up to 5 cm below the upper edge of the small glass. In order to avoid air contact, pour distilled water onto gel. Allow to stand to complete the polymerization.

Stacking Gel

Add the followings into a small beaker.

Table D.2 : Preparation of %4 stacking gel for activity staining

Monomer Concentration(%T, 2.67% C)	%4
Acryamide/bis (30% T, 2.67% C Stock)	1.3 ml
Distilled water	6.2 ml
1.5 M Tris-HCl, pH 8.8	-
0.5 M Tris-HCl, pH 6.8	2.5 ml
10% Ammonium persulfate (fresh)	50 µl
TEMED	10 µl

Dry the area above the separating gel with filter paper before pouring the stacking gel. Immediately pour the gel solution between glasses. Place a comb in the gel sandwich and tilt it so that the teeth are at a slight (~10°) angle. This will prevent air from being trapped under the comb teeth while the monomer solutions are poured. Allow the gel to polymerize 30-45 minutes.

After polymerization is completed remove the corb by pulling it straight up slowly and gently and fill the wells with 1x loading buffer. Load the the samples (diluted 1:2 with sample buffer) into the wells in an order and keep note for them.

After the gels are cast, the clamp assemblies are snapped onto the inner cooling core to form the upper buffer chamber. The upper buffer is in direct contact with the inner glass plate of the gel sandwich to provide even heat distribution over the entire gel length, preventing thermal band distortion during electrophoretic separations. Fill the chamber with 1x loading buffer. Gently place the cooling core into the electrophoresis tank.

Place the lid on top of the buffer chamber to fully enclose the cell. Attach the electrical leads to a suitable power supply with the proper polarity. The recommended power condition for optimal resolution with minimal thermal band distortion is 200 volts, constant voltage setting. The usual run time is approximately 45 minutes. This electrophoresis cell is for rapid separation and is not recommended for runs over 60 minutes long. When run finishes, extrude gels very carefully. Carry out the modified activity staining method of Rescigno *et al.* (1997).

APPENDIX E

MODIFIED ACTIVITY STAINING METHOD OF RESCIGNO *et al.* (1997)

Reagents

A. 4-amino-*N*,*N*-diethylaniline (ADA) Solution

0.655 g 4-amino-*N*,*N*-diethylaniline was dissolved in 100 ml of distilled water. 98 µl HCl was also added. This solution should always be prepared freshly.

B. Hydrogen peroxide (H_2O_2) Solution

100 µl hydrogen peroxide was mixed with 100 ml of distilled water.

C. 4-tert-butyl catechol Solution (*t*BC) Solution

0.34 g 4-tert-butyl catechol was dissolved by stirring in 100 ml of distilled water. 57 µl acetic acid was also added.

D. 4-methyl catechol (MC) Solution

1.24 g 4-methyl catechol was dissolved in 100 ml of distilled water.

E. Sodium phosphate Buffer, pH 7

Stock Solutions

a: 0.2 M solution of monobasic sodium phosphate (27.8 g in 1000 ml of distilled water)

b: 0.2 M solution of dibasic sodium phosphate (53.65 g of $Na_2HPO_4.7H_2O$ or 71.7 g of $Na_2HPO_4.12H_2O$ in 1000 ml of distilled water)

39.0 ml of a + 61.0 ml of b are mixed and diluted to a total volume of 200 ml with distilled water.

Procedure

A. A small portion of the gel was stained with ADA, H_2O_2 and *t*-BC by the procedure given in the following table:

Table E.1 : Modified activity staining procedure of Rescigno *et al.* (1997)

Step	Reagent	Time	Comment
1	Sodium phosphate buffer, pH 7	5 min	Gel was first soaked in buffer at room temperature
2	ADA Solution	5 min	Buffer was poured off and ADA solution wad added. Indistinctly pink spot appeared corresponding to laccase activity
3	Sodium phosphate buffer, pH 7	5 min	ADA was poured off and gel was soaked in buffer
4	H_2O_2 Solution	30 sec	Buffer was poured off and H_2O_2 solution was added. Pink-red spots appeared immediately corresponding to peroxidase activity. Laccase band also becomes more visible.
5	Sodium phosphate buffer, pH 7	5 min	H_2O_2 solution was poured off and gel was soaked in buffer
6	*t*BC Solution	4-8 min	Buffer was poured off and *t*BC was added. Deep blue spots were observed corresponding to catechol oxidase (polyphenol oxidase activity)

B. A small portion of the gel was stained with MC by the procedure given in the following table.

Table E.2 : Activity staining procedure with MC

Step	Reagent	Time	Comment
1	Sodium phosphate buffer, pH 7	5 min	Gel was first soaked in buffer at room temperature
2	MC Solution	2-3 min	Buffer was poured off and MC was added. Orange-brown spots were observed corresponding to catechol oxidase (polyphenol oxidase activity)

APPENDIX F

REAGENTS AND GEL PREPARATION FOR SDS-PAGE SLAB GEL (LAEMMLI BUFFER SYSTEM)*

Stock Solutions

A. Acrylamide/bis (30% T, 2.67% C)

87.6 g acrylamide (29.2g/100 ml)

2.4 g N'N'-bis-methylene-acrylamide (0.8g/100 ml)

Make to 300 ml with distilled water. Filter and store at 4°C in the dark (30 days maximum). Since acrylamide is a neuro toxin, precautions should be taken by wearing gloves and mask during preparation of this solution.

B. 1.5 M Tris-HCl, pH 8.8

27.23 g Tris base

~80 ml distilled water

Adjust to pH 6.8 with 1N HCl. Make to 100 ml with distilled water and store at 4°C.

C. 0.5 M Tris-HCl, pH 6.8

6 g Tris base

~60 ml distilled water

Adjust to pH 6.8 with 1N HCl. Make to 100 ml with distilled water and store at 4°C.

D. 10% SDS

Dissolve 10 g SDS in distilled water with gentle stirring and bring to 100 ml with distilled water.

E. Sample Buffer (SDS reducing buffer) (store at room temperature)

Distilled water	4.0 ml
0.5 M Tris-HCl, pH 6.8	1.0 ml
Glycerol	0.8 ml
10% (w/v) SDS	1.6 ml
2-b-mercaptoethanol	0.4 ml
0.05% (w/v) bromophenol blue	0.2 ml
	8.0 ml

Dilute the sample at least 1:4 with sample buffer and heat 95°C for 4 minutes.

F. 5X Electrode (Running) Buffer, pH 8.3 (enough for 10 runs)

Tris base	9.0 g
Glycine	43.2 g
SDS	3.0 g

Bring to 600 ml with distilled water. Store at 4°C. Warm to 37°C before use if precipitation occurs. Dilute 60 ml 5X stock with 240 ml distilled water for one electrophoretic run.

G. 10% Ammonium Persulfate (APS)

Dissolve 100 mg APS in 1 ml of distilled water in an eppendorf by vortexing. This solution should be prepared fresh daily.

H. TEMED (*N*,*N*-tetramethylene-ethylenediamine)

Use TEMED neat from the bottle.

Procedure

A. Preliminary Preparation

Clean the glasses and spacers with ethanol. Assemble the gel sandwich on a clean surface. Lay the longer rectangular glass plate down first, the place two spacers of equal thickness along the short edges of rectangular plate. Next, place the shorter glass plate on top of the spacers. Install the clamps and fasten the screws. Transfer the clamp assembly to one of the casting stand. To check whether the glasses are properly sealed, pour distilled water between glasses. If water level does not decrease, glasses are properly sealed. Then, pour out the water and let the glasses to dry.

B. Preparation of SDS-PAGE Gel Solution

Separating Gel

Add the followings into a small beaker.

Table F.1 : Preparation of %12 SDS-PAGE separating gel

Monomer Concentration (30% T, 2.67% C)	%12
Acryamide/bis (30% T, 2.67% C Stock)	4.0 ml
Distilled water	3.35 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml
0.5 M Tris-HCl, pH 6.8	-
10% SDS	100 µl
10% Ammonium persulfate (fresh)	50 µl
TEMED (*N*,*N*-tetramethylene-ethylenediamine)	5 µl

Prepare the monomer solution by combining all reagents except ammonium persulfate and TEMED. Dearate the solution under vacuum for at least 15 minutes. Add the two catalysts just prior to casting the gels.

After adding two catalysts immediately pour the solution between glasses up to 5 cm below the upper edge of the small glass. In order to avoid air contact, pour distilled water onto gel.

Allow to stand to complete the polymerization.

Stacking Gel

Add the followings into a small beaker.

Table F.2 : Preparation of %4 SDS-PAGE separating gel

Monomer Concentration(%T, 2.67% C)	%4
Acryamide/bis (30% T, 2.67% C Stock)	1.3 ml
Distilled water	6.1 ml
1.5 M Tris-HCl, pH 8.8	-
0.5 M Tris-HCl, pH 6.8	2.5 ml
10% SDS	100 µl
10% Ammonium persulfate (fresh)	50 µl
TEMED	10 µl

Dry the area above the separating gel with filter paper before pouring the stacking gel. Immediately pour the gel solution between glasses. Place a comb in the gel sandwich and tilt it so that the teeth are at a slight (~10°) angle. This will prevent air from being trapped under the comb teeth while the monomer solutions are poured. Allow the gel to polymerize 30-45 minutes.

After polymerization is completed remove the corb by pulling it straight up slowly and gently and fill the wells with 1x loading buffer. Load the standard and the samples (diluted at least 1:4 with sample buffer and heated at 95°C for 5 minutes) into the wells in an order and keep note for them.

SDS-PAGE molecular weight marker was a mixture of proteins given in Table F.3.

Table F.3 : SDS-PAGE molecular weight markers

Protein	Source	Approx. MW (kDa)
β-galactosidase	*E.coli*	116.0
Bovine serum albumin	Bovine plasma	66.2
Ovalbumin	Chicken egg white	45.0
Lactate dehydrogenase	Porcine muscle	35.0
Restriction endonuclease *Bsp*981	*E.coli*	25.0
β-lactoglobulin	Bovine milk	18.4
Lysozyme	Chicken egg white	14.4

After the gels are cast, the clamp assemblies are snapped onto the inner cooling core to form the upper buffer chamber. The upper buffer is in direct contact with the inner glass plate of the gel sandwich to provide even heat distribution over the entire gel length, preventing thermal band distortion during electrophoretic separations. Fill the chamber with 1x loading buffer. Gently place the cooling core into the electrophoresis tank.

Place the lid on top of the buffer chamber to fully enclose the cell. Attach the electrical leads to a suitable power supply with the proper polarity. The recommended power condition for optimal resolution with minimal thermal band distortion is 200 volts, constant voltage setting. The usual run time is approximately 45 minutes. This electrophoresis cell is for rapid separation and is not recommended for runs over 60 minutes long.

When run finishes, extrude gels very carefully. Immerse gels in fixing solution containing 50% methanol, 12% acetic acid (300 ml in total) and 150µl 37% formaldehyde for at least 1 hr (overnight incubation is all right) in a shaker.

The gels were then silver stained using the procedure of Blum *et* al. (1987).

APPENDIX G

SILVER STAINING METHOD

Reagents

A. Fixer

Mix 150 ml methanol + 36 ml acetic acid + 150 µl 37% formaldehyde and complete to 300 ml with distilled water. This solution can be used several times.

B. 50% Ethanol

Mix 600 ml pure ethanol + 600 ml distilled water. This solution should always be prepared freshly.

C. Pretreatment Solution

Dissolve 0.08 g sodium thiosulphate ($Na_2S_2O_3.5H_2O$) in 400 ml distilled water by mixing with a glass rod. Take 8 ml and set aside for further use in developing solution preparation.

D. Silver Nitrate Solution

Dissolve 0.8 g silver nitrate in 400 ml distilled water and add 300 µl 37% formaldehyde.

E. Developing Solution

Dissolve 9 g potassium carbonate in 400 ml distilled water. Add 8 ml from pretreatment solution and 300 µl 37% formaldehyde.

F. Stop Solution

Mix 200 ml methanol + 48 ml acetic acid and complete to 400 ml with distilled water.

Procedure

Procedure followed was given in Table G.1.

Table G.1 : Silver staining procedure

	STEP	SOLUTION	TIME OF TREATMENT	COMMENTS
1	Fixing	Fixer	≥ 1 hr	Overnight incubation is all right
2	Washing	50% Ethanol	3 x 20 min	Should be fresh
3	Pre-treatment	Pretreatment Solution	1 min	Should be fresh Time should be exact
4	Rinse	Distilled water	3 x 20 sec	Time sholud be exact
5	Impregnate	Silver Nitrate Solution	20 min	
6	Rinse	Distilled water	2 x 20 sec	Time should be exact
7	Developing	Developing Solution	~ 10 min	After a few minutes add some distilled water to proceed the reaction slowly. Time should be determined by observation of color development.
8	Wash	Distilled water	2 x 2 min	
9	Stop	Stop Solution	≥ 10 min	The gels can be kept in this solution overnight".

APPENDIX H

ISOELECTRIC FOCUSING

Stock Solutions

A. Monomer Concentrate (25% T, 3%C)

24.25% (w/v) acrylamide

0.75% (w/v) bis (*N*,*N*-bis-methylene-acrylamide)

Dissolve 24.25 g acrylamide and 0.75 g bis in water, bring to a final volume of 100 ml and filter through a 0.45 µm filter. Store protected from light at 4°C. This solution may be stored up to 1 month.

B. 0.1% (w/v) Riboflavin-5-phosphate (FMN)

20 mg riboflavin-5-phosphate

20 ml distilled water

This solution may be stored up to 1 month at 4°C protected from light.

C. 10% (w/v) Ammonium persulfate (APS)

150 mg APS

1 ml distilled water

Prepare fresh daily. Make sure that the APS is completely dissolved before using.

D. 25% Glycerol (w/v)

Add 25 g glycerol to 50 ml distilled water. Dilute to 100 ml with distilled water.

E. TEMED (*N,N*-tetramethylene-ethylenediamine)

Use TEMED neat from the bottle. Use only pure, distilled TEMED. Store cool, dry and protected from light.

Reagents

A. Monomer-Ampholyte Solution

Distilled water	2.25 ml
Monomer Conc.(25%T, 3%C)	1.0 ml
25% (w/v) Glycerol	1.0 ml
Ampholyte	0.25 ml

B. Catalyst Solution

10% (w/v) APS	7.5 µl
0.1 (w/v) FMN	25.0 µl
TEMED (neat)	1.5 µl

Marker

Isoelectric focusing marker was purchased from SERVA. It was a mixture of proteins given in Table H.1 in the pH range of 3-10.

Table H.1 : Isoelectric focusing markers

Protein	pI
Cytochrome C	10.7
Ribonuclease A	9.5
Lectin	c. 8.3
Lectin	m. 8.0
Lectin	a. 7.8
Myoglobin	c. 7.4
Myoglobin	a. 6.9
Carb.anhydrase	6.0
β-Lactoglobin	c. 5.3
β-Lactoglobin	a. 5.2
Trypsin inhibitor	4.5
Glucose oxidase	4.2
Amyloglucosid	3.5

Procedure

A. Preliminary Preparation

Clean the isoelectric focusing glass with ethanol. Pipet a few drops of water onto the clean glass plate, wipe with filter paper and place the hydrophobic side of the light sensitive gel supporting film against the plate. Roll the gel support film flat with a test tube to force out excess water and air bubbles. Remove the paper from the film and let to dry and stick on the glass under light. After 15 minutes, turn the glass (film side should face the gel solution) on the casting tray.

B. Gel Preparation

Prepare the monomer-ampholyte solution (Reagent A) in a small beaker . Degas the solution for 5 minutes under vacuum. Prepare the catalyst solution (Reagent B) in an eppendorf. Mix and pipet them between the glass plate and casting ray. Be sure that there are no bubbles in gel solution. Let the gel to

polymerize under flourescent light for 2 hours. After polymerization remove the gel from the plate and turn upwards. Let the gel further polymerize for 30 minutes under flourescent light. Put the sample template on the gel and load samples. Let the samples diffuse into the gel approximately 15 minutes. Remove the sample template and run the gel at 100 V for 15 minutes, at 200 V for 15 minute and at 300 V for 30 minutes to 1 hour.

APPENDIX I

QUICK COOMASSIE BLUE STAINING METHOD FOR ISOELECTRIC FOCUSING

Stock Solution

A. Staining Solution

Dissolve 0.025 g Coomassie G-250 dye in 3.5 ml perchloric acid and 96.5 ml distilled water.

B. Intensification Solution

Mix 7 ml acetic acid with 93 ml distilled water.

Procedure

Procedure was given in Table I.1.

Table I.1 : Quick coomassie blue staining procedure

Step	Reagent	Time
1	Staining Solution	1 hr
2	Intensification Solution	∞