

**Production of Commercially Suitable  
Pectin methylesterase and Polyphenol oxidase  
from Agro-industrial Wastes**

**By**

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## ABSTRACT

In this study, some simple and effective extraction and/or partial purification procedures were developed to obtain pectin methylesterase (PME) and polyphenol oxidase (PPO) enzymes from orange peels and mushroom stems, respectively. Also, some characteristics of enzymes were investigated and their stable preparations were obtained in liquid or lyophilized forms. Valencia orange peels contain considerable PME activity (300-350 mL NaOH/min/100 g) that is quite stable during season for at least 5 months. The enzyme was ionically bound to cell walls and can not be extracted by homogenization with water. However, the addition of suitable amounts of NaCl (10 g /100 g extraction mixture) to pellet, obtained by homogenization of peels several times with water, and 30 min mixing (at 200 rpm) may be effectively used to extract the enzyme. The PME in orange peels contains almost the same amount of heat stable and heat labile fractions and the enzyme can not be activated by mild heating. A slight activation (almost 20 %) may be achieved by adding 1 mM CaCl<sub>2</sub> to enzyme extracts. However, at higher concentrations the addition of CaCl<sub>2</sub> was inhibitory. The PME activity in extracts, stabilized by use of 0.1 % Na-benzoate and 0.1 % K-sorbate, is stable almost 5 months at + 4 °C (maintains > 90 % of its activity). Thus, the commercial preparations of enzyme may be obtained in liquid form. The extracted PME was successfully used to prepare edible films from citrus pectin.

For the extraction of PPO, on the other hand, mushroom stems were first processed to acetone powder. The acetone powders were then extracted with Na-phosphate buffer and partially purified with ammonium sulfate (90 % saturation) or acetone precipitation (2-fold). Following dialysis, the recoveries and purification folds obtained from the partial purification of monophenolase activity of PPO from the same acetone powder were 74-86 % and 3.4-4.3 and 55-67 % and 5.4-6.2 for ammonium sulfate and acetone precipitations, respectively. Thus, it appears that the ammonium sulfate precipitation gives a higher yield but lower purity. The monophenolase activity of partially purified PPO is heat labile and showed inactivation above 45 °C. The enzyme exhibited a pH optimum between pH 6.0 and 8.0. The pH stability of enzyme was maximal at pH 7.0 and 8.0. However, at pH 4.0 the enzyme lost most of its activity after 24 h incubation. The optimum temperature of enzyme was found as 40 °C. The monophenolase activity of PPO enzyme showed no stability in acetone powders at + 4 °C. However, it showed good stability at -18 °C for two months with retention of

60-70 % of its activity. The PPO partially purified with ammonium sulfate precipitation and dialysis, and lyophilized by using dextran or saccharose as supporting materials also retained its monophenolase and diphenolase activities for three months at -18 °C. The effect of lyophilization with dextran on temperature stability of enzyme was insignificant. However, lyophilization with dextran reduced the pH stability of monophenolase activity at 4.0 moderately. In addition to its monophenolase activity on tyrosine and diphenolase activity on L-DOPA, PPO lyophilized with dextran can also use phloridzin as substrate. Thus, it appears that the enzyme may be used in different food applications including the production of antioxidants and colorants, modification of proteins, fermentation of cocoa and black tea, etc.

## ÖZ

Bu çalışmada pektin metilesteraz (PME) ve polifenol oksidaz (PPO) enzimlerinin sırasıyla portakal kabukları ve mantar saplarından eldesi için pratik ve etkili olabilecek ekstraksiyon ve kısmi saflaştırma prosedürleri geliştirilmiştir. Ayrıca enzimlerin bazı karakteristikleri de incelenmiş ve sıvı veya liyofilize haldeki stabil preparatları da hazırlanmıştır. Çalışmada Valencia portakal kabuklarında kayda değer miktarda PME aktivitesi tesbit edilmiş (300-350 mL NaOH/dak/100gr) ve bu aktivitenin sezon boyunca en az 5 ay stabilitesini koruduğu belirlenmiştir. Portakal kabuklarında bulunan enzim, hücre duvarına iyonik olarak bağlı olup su ile ekstrakte edilememekte, ancak buna karşın kabukların su ile birkaç kez homojenizasyonu ve filtrasyonu ile elde edilen kitleden uygun oranda NaCl ilavesi (10 gr/100 gr ekstraksiyon karışımı) ve karıştırma ile (200 rpm'de 30 dak) etkili bir şekilde ekstrakte edilebilmektedir. Portakal kabuklarındaki PME aktivitesinin yaklaşık yarısı ısıya dirençli, yarısı da ısıya duyarlı enzim fraksiyonlarından oluşmakta olup, ısının enzim üzerinde aktive edici bir özelliği belirlenmemiştir. Enzim ekstraktına 1 mM CaCl<sub>2</sub> ilavesi ile zayıf bir aktivasyon (yaklaşık % 20) sağlanabilmekte, ancak buna karşın yüksek konsantrasyonlardaki CaCl<sub>2</sub> ilavesi inhibe edici özellik göstermektedir. % 0.1 Na-benzoat ve % 0.1 K-sorbat ile stabilize edilmiş ekstraktlardaki PME aktivitesi + 4 °C'de yaklaşık 5 ay stabildir (% 90'ın üzerinde aktivitesini korumaktadır). Buna göre portakal kabuğundan elde edilen PME'nin ticari preparatlarının sıvı formda hazırlanmasında herhangi bir sakınca bulunmamaktadır. Bu çalışmada hazırlanmış olan PME preparatı narenciye pektininden yenilebilir film üretiminde başarılı bir şekilde kullanılmıştır.

Diğer yandan, PPO'nun ekstraksiyonu için öncelikle mantar sapları aseton tozuna işlenmiştir. Aseton tozları daha sonra Na-fosfat tamponu ile ekstrakte edilmiş ve % 90 amonyum sülfat çöktürmesi ya da 2-kat aseton çöktürmesi ve bunu takip eden diyaliz işlemi ile kısmi olarak saflaştırılmıştır. Aynı aseton tozundaki PPO'nun monofenolaz aktivitesinin, amonyum sülfat veya asetonla çöktürme yoluyla kısmi saflaştırılması ile elde edilen geri kazanım ve saflık katsayıları sırasıyla % 74-86 ve 3.4-4.3 ve % 55-67 ve 5.4-6.2'dir. Buna göre amonyum sülfat çöktürmesinin daha yüksek verime karşın daha düşük saflık sağladığı görülmektedir. Kısmi olarak saflaştırılmış PPO enziminin monofenolaz aktivitesinin ısıl direnci düşük olup inaktivasyonu 45 °C'ın üzerinde başlamaktadır. Enzim, pH 6.0 ve 8.0 arasında optimum aktivite göstermiş olup pH 7.0 ve 8.0'deki stabilitesi maksimumdur. Ancak buna karşın enzim, pH 4.0'te 24

saat inkübasyon sonucunda aktivitesinin büyük kısmını kaybetmiştir. Enzimin optimum sıcaklığı 40 °C olarak belirlenmiştir. PPO enziminin monofenolaz aktivitesi, aseton tozu halinde + 4 °C'de stabilite göstermemiş, ancak buna karşın - 18 °C'de iki ay süreyle % 60-70 oranında aktivitesini korumuştur. Ayrıca amonyum sülfat ve diyaliz ile kısmi saflaştırılmış ve destek maddesi olarak dekstran ve sakkaroz eklenerek liyofilize edilmiş PPO, -18 °C'de 3 ay süresince monofenolaz ve difenolaz aktivitesini korumuştur. Dekstranla liyofilizasyonun enzimin ısı stabilitesi üzerinde herhangi bir etkisi belirlenmemiştir. Ancak buna karşın dekstranla liyofilizasyon, pH 4.0'deki monofenolaz aktivitesinin stabilitesini kısmen azaltmıştır. Tirozin üzerindeki monofenolaz aktivitesi ve L-DOPA üzerindeki difenolaz aktivitesinin yanısıra dekstranla liyofilize edilmiş PPO, floridzin'i de substrat olarak kullanabilmektedir. Bu sonuç enzimin antioksidan ve renk maddelerinin üretimi, proteinlerin modifikasyonu, kakao ve siyah çayın fermantasyonu gibi farklı gıda uygulamalarında kullanılabileceğini göstermektedir.

## TABLE OF CONTENTS

LIST OF FIGURES .....	xii
LIST OF TABLES .....	xiv
CHAPTER 1. INTRODUCTION.....	1
CHAPTER 2. ENZYME PRODUCTION FOR INDUSTRIAL APPLICATIONS	3
2.1. Extraction of Enzymes .....	3
2.1.1. Cell disruption methods.....	5
2.1.1.1. Mechanical methods.....	5
2.1.1.2. Nonmechanical methods .....	5
2.2. Clarification of Enzyme Extracts .....	6
2.2.1. Centrifugation.....	6
2.2.2. Filtration .....	6
2.2.3. Flocculation and flotation.....	7
2.3. Concentration of Enzymes .....	7
2.3.1. Addition of a dry matrix polymer.....	7
2.3.2. Freeze-drying (Lyophilization) .....	7
2.3.3. Ultrafiltration.....	8
2.3.4. Precipitation .....	9
2.3.4.1. Precipitation by increasing the ionic strength (salting out) .....	9
2.3.4.2. Precipitation by decreasing the ionic strength (salting in) .....	10
2.3.4.3. Precipitation by organic solvents.....	10
2.3.4.4. Precipitation by alteration of pH .....	11
2.3.4.5. Precipitation by organic polymers.....	11
2.3.4.6. Precipitation by denaturation.....	11
2.3.5. Aqueous two-phase partitioning.....	12
2.3.6. Removal of salts and exchange of buffers.....	12
2.3.6.1. Dialysis .....	12
2.3.6.2. Diafiltration .....	13



2.3.6.3. Gel filtration .....	13
2.4. Purification .....	13
2.5. Product Formulation.....	14
CHAPTER 3. PECTIN METHYLESTERASE .....	16
3.1. Pectin methylesterase and Other Pectinases.....	16
3.2. Sources of PME.....	16
3.3. The Effects of PME on Food Quality.....	17
3.4. Industrial Applications of PME.....	19
3.4.1. Clarification of fruit juices .....	19
3.4.2. Firming of fruits and vegetables before processing .....	21
3.4.3. Modification of pectin .....	21
3.4.4. Production of low sugar jams, jellies, and compotes .....	22
3.4.5. Other applications.....	22
CHAPTER 4. POLYPHENOL OXIDASES.....	24
4.1. Polyphenol oxidases .....	24
4.2. Substrates of PPO .....	26
4.3. Sources and Some Characteristics of PPO .....	27
4.4. The Effects of PPO on Food Quality.....	29
4.5. Industrial Applications of PPO.....	29
4.5.1. Enzymatic cross-linking of proteins or polysaccharides.....	30
4.5.2. Production of flavonoid-derived colorants and antioxidants.....	30
4.5.3. The removal of haze forming polyphenols from beverages.....	31
4.5.4. Oxygen scavenging and removal of undesirable phenolics from food ...	32
4.5.5. Removal of undesirable phenolics from wastewaters .....	32
4.5.6. Analytical and clinical applications of PPO.....	33
4.5.6.1. Production of biosensors .....	33
4.5.6.2. Clinical applications .....	33
CHAPTER 5. MATERIALS AND METHODS .....	35
5.1. Materials.....	35
5.2. Methods.....	35
5.2.1. Methods related to PME enzyme .....	35
5.2.1.1. PME extraction.....	35

5.2.1.2. Determination of PME activity .....	37
5.2.1.3. Effect of mild heating on PME activity.....	37
5.2.1.4. Effect of CaCl <sub>2</sub> on PME activity .....	38
5.2.1.5. Preparation of a commercial PME preparation and test of its stability .....	38
5.2.1.6. Test of obtained PME in the preparation of edible pectin films.....	38
5.2.2. Methods related to PPO enzyme .....	39
5.2.2.1. Acetone powder preparation.....	39
5.2.2.2. PPO extraction .....	39
5.2.2.3. Ammonium sulphate precipitation .....	39
5.2.2.4. Acetone precipitation.....	39
5.2.2.5. Determination of PPO activity .....	40
5.2.2.6. Characterization studies.....	40
5.2.2.7. Storage stability of PPO in acetone powders .....	41
5.2.2.8. Preparation of commercial PPO preparations and test of their storage stabilities .....	41
5.2.2.9. The effect of lyophilization with dextran on temperature and pH stability of PPO.....	41
5.2.3. Determination of protein content.....	42
 CHAPTER 6. RESULTS AND DISCUSSION .....	 43
6.1. The Results Obtained for PME Enzyme .....	43
6.1.1. Change of PME activity in orange peels during season.....	43
6.1.2. Effect of different extraction procedures on PME activity .....	44
6.1.3. Effect of NaCl concentration on PME activity extracted from orange peels.....	45
6.1.4. Effect of extraction period on PME activity extracted from orange peels.....	46
6.1.5. Effect of mild heating on PME activity.....	47
6.1.6. Effect of CaCl <sub>2</sub> on PME activity.....	48
6.1.7. Stability of the prepared PME during storage .....	49
6.1.8. Test of obtained PME in the preparation of edible pectin films .....	50
6.2. The Results Obtained for PPO Enzyme .....	52

6.2.1. Monophenolase and diphenolase activities of PPO.....	52
6.2.2. Distribution of PPO in mushrooms .....	53
6.2.3. Partial purification of PPO .....	54
6.2.4. Characterization of monophenolase activity .....	56
6.2.5. Stability of monophenolase activity in acetone powders .....	60
6.2.6. Stability of the prepared PPO during storage .....	61
6.2.7. The effect of lyophilization with dextran on temperature and pH stability of PPO .....	63
6.2.8. The ability of the prepared PPO to oxidize phloridzin.....	64
 CHAPTER 7. CONCLUSIONS.....	 66
 REFERENCES.....	 68

## LIST OF FIGURES

Figure 2.1.	The important steps of enzyme production .....	4
Figure 2.2.	Phase diagram for water .....	8
Figure 2.3.	Typical profile for ammonium sulphate precipitation .....	10
Figure 3.1.	Deesterification of pectin by PME .....	16
Figure 4.1.	The reactions of polyphenol oxidases .....	25
Figure 4.2.	The nonenzymatic reactions during formation of dark colored melanins .....	25
Figure 4.3.	Some of the good substrates of PPO in plants .....	26
Figure 5.1.	Standart curves for protein determination .....	42
Figure 6.1.	Change of PME activity in orange peels during season .....	43
Figure 6.2.	Effect of NaCl concentration on PME activity extracted from peels .....	46
Figure 6.3.	Effect of extraction period on PME activity extracted from peels ...	46
Figure 6.4.	Effect of heating for 30 min at different temperatures on PME activity .....	47
Figure 6.5.	Effect of heating at 50 °C on PME activity .....	48
Figure 6.6.	Effect of different CaCl <sub>2</sub> concentrations on PME activity .....	49
Figure 6.7.	Stability of the prepared PME during storage at + 4 °C in liquid form .....	50
Figure 6.8.	The effect of prepared orange peel PME on film forming ability of pectin .....	51
Figure 6.9.	Monophenolase activity of PPO from mushroom stems .....	52
Figure 6.10.	Diphenolase activity of PPO from mushroom stems .....	53
Figure 6.11.	Temperature profiles of monophenolase activity of PPO partially purified with different procedures from different acetone powders.	57
Figure 6.12.	The effect of pH on monophenolase activity of PPO partially purified with different procedures from different acetone powders.	58
Figure 6.13.	pH stabilities of monophenolase activity of PPO partially purified with different procedures from different acetone powders .....	59
Figure 6.14.	Optimum temperature for monophenolase activity of PPO .....	60

Figure 6.15. The stability of monophenolase activity of PPO in acetone Powders .....	60
Figure 6.16. The stability of monophenolase activity of PPO in different lyophilized forms stored at -18 °C.....	62
Figure 6.17. The stability of diphenolase activity of PPO in different lyophilized forms stored at -18 °C.....	62
Figure 6.18. The effect of lyophilization with dextran on heat stability of monophenolase activity of PPO .....	63
Figure 6.19. The effect of lyophilization with dextran on pH stability of monophenolase activity of PPO .....	64
Figure 6.20. The phloridzin oxidation of PPO lyophilized with dextran.....	65

## LIST OF TABLES

Table 2.1. Different methods for cell disruption .....	5
Table 2.2. The main chromatographic techniques and their separation principles .....	14
Table 3.1. Some properties of PME's from different fruits and vegetables .....	18
Table 4.1. Some properties of PPOs from different fruits and vegetables .....	28
Table 6.1. The PME activities obtained by applying different extraction procedures.....	44
Table 6.2. Distribution of PPO monophenolase and diphenolase activities in mushrooms.....	54
Table 6.3. Results of partial purification with ammonium sulfate and acetone precipitation of PPO monophenolase activity from mushroom stems .....	55

## CHAPTER 1

### INTRODUCTION

Recently, extensive studies have been conducted related to the use of plant enzymes in food industry. For example, Ridgway et al (1997) extracted and used apple polyphenol oxidase (PPO) successfully for biosynthesis of antioxidant compounds 3-hydroxyphloridzin and 3-hydroxyphloretin from phloridzin and yellow/orange colored dimerized oxidation products of phloridzin that may be used as food colorants. Ridgway and Tucker (1999) also developed a procedure for the partial purification of commercially suitable PPO from apple leaf. However, since PPO in apples can not oxidize the p-diphenols (laccase activity) and lacks monophenolase activity it may only be used for some specific applications.

The monophenolase activity of PPO is essential for many different applications such as the production of plant pigments such as red-violet betalains and gold colored aurones (Strack and Schliemann 2001) and biosynthesis of antioxidant compounds such as hydroxytyrosol (Espin et al. 2001). Thus, with its hydroxylation and high oxidation capacity PPO in mushrooms is quite suitable for many food applications including fermentation of cocoa and tea (Ridgway et al. 1997, Selamat et al. 2002), removal of undesirable odors caused by volatile sulfur compounds (Negishi et al. 2001, Negishi et al. 2002) and enzymatic cross-linking of proteins (Thalman and Lötzbeyer, 2002). The ability of mushroom PPO to oxidize sinapic acid was also reported by Choi and Sapers (1994). Thus, as done by Lacki and Duvnjak (1998) with *Trametes versicolor* PPO, removal of sinapic acid and increase of the nutritional values of canola meal and canola protein concentrates may be conducted by mushroom PPO. One of the most interesting features of mushroom PPO is its ability to inhibit the attachment of some bacteria. For example, Cowan et al (2000) demonstrated that by oxidizing the critical tyrosine residues of glucan binding lectin and glucosyltransferases, PPO prevents the attachment of *Streptococcus sobrinus*, bacteria responsible from the formation of oral cavities, to glucans deposited on the tooth surface. Kolganova et al (2002) also showed that the PPO reduces the adhesion of some viruses and pathogenic bacteria to buccal epithelial cells, while unaffected the attachment of probiotic bacteria. These findings are quite interesting and may open the way of using PPO in foods such as gums and confectionaries to increase the tooth health of people. Out of food industry,

mushroom PPO may also be used to remove undesirable phenols from wastewaters (Ikehata and Nicell 2000) and produce biosensors for the detection and quantification of phenolic compounds (Rubianes and Rivas, 2000, Climent et al. 2001). Several clinical applications such as using PPO as a catalyst to produce L-DOPA, a drug for the treatment of Parkinson's disease (Sharma et al. 2003), a marker of vitiligo, an autoimmune disease and a tumor suppressing and prodrug therapy agent (Seo et al. 2003) also attracts considerable interest.

Another enzyme, attracting great attention in food processing is pectin methylesterase (PME). The fungal PME is now extensively used in beverage industry for fruit juice extraction and clarification (Alkorta et al. 1998, Bhat, 2000, Demir et al. 2001, Cemeroğlu and Karadeniz 2001, Kashyap et al. 2001, Sarioğlu et al. 2001). The use of enzyme in the modification of pectin, oil extraction, firming of fruit and vegetables and peeling of fruits becomes also very popular (Ralet et al. 2001, Schmelter et al. 2002, Degraeve et al. 2003, Suutarinen et al. 2000, Suutarinen et al. 2002, Pszczola, 2001, Kashyap et al. 2001, Vierhuis et al. 2003, Pretel et al. 1997, Janser, 1996). Currently, the plant PME is not used in food industry. However, there are some successful experimental studies related to the use of commercial citrus PME for the firming of fruits and vegetables and modification of pectin (Suutarinen et al. 2000, Massiot et al. 1997).

In the production of commercial enzymes, microorganisms are the primary sources. However, 15 % of the enzyme production is still provided by extracting from animal or plant sources. In this study, the main objectives were; (1) to develop some simple and effective extraction and/or partial purification procedures for PPO and PME enzymes from agro-industrial wastes and (2) to process the extracted enzymes to commercially suitable preparations. The waste materials used in the study were mushroom stems and orange peels for the PPO and PME enzyme, respectively. These, materials are known as rich sources of the indicated enzymes (Moore and Flurkey 1989, Ratcliffe et al. 1994, Seo et al. 2003, Cemeroğlu et al. 2001, Nielsen and Christensen 2002, Johansson et al. 2002, Cameron et al. 1994, Cameron et al. 1998). The enzymes obtained from edible plant materials mostly need no toxicity tests for food applications. Also, because of the non-complex nature of the plant extracts these enzymes may easily be used as crude or partially purified preparations. Moreover, the use of waste materials in enzyme extraction may provide an extra income to factories and reduces the costs of waste material treatments.



## CHAPTER 2

### ENZYME PRODUCTION FOR INDUSTRIAL APPLICATIONS

#### 2.1. Extraction of Enzymes

In the production of commercial enzymes, microorganisms are the primary sources. Currently, 50 % of the commercial enzymes are obtained from fungi and yeast and 35 % are obtained from bacteria. The remaining enzyme production, on the other hand, is conducted by extraction from plant or animal sources (Rolle, 1998). A good example for commercial animal origin enzymes is rennet, whereas papain may be an example for the plant origin enzymes (Gölker, 1990).

Following the production of enzymes by microbial fermentation or providing suitable plant or animal sources the first step to obtain industrial enzymes is extraction. In this process, the enzymes are extracted from a source or from a fermentation media and then in the second step the extracts are purified by further processing. According to the mode of application, the degree of purity may range from raw enzymes (sometimes relatively crude preparations in the form of plant extracts, chopped fruits, leaves and pounded grains) to highly purified forms. The overall steps for the preparation of enzymes from different sources were given in Figure 2.1.

For the extraction of an enzyme from a plant material (root, stem, grain, nuclear sap, etc.), the material is first ground or minced with different crushers or grinders. At this step, processes such as peeling, removal of seeds, etc. may also be applied. After that, desired enzymes can be extracted with water and/or suitable buffer solutions. On the other hand, when animal organs are used in the extraction, they must be transported and stored at low temperatures for short times to retain the enzymatic activity. Most animal proteins are present in specific muscles or organs surrounded with a fatty layer that often interacts with subsequent purification steps. Thus, before freezing operation fats and connective tissues should be removed. The enzyme containing frozen organ or tissue can be cut, minced or homogenized with a blender, grinder or mill (for hard tissues) for producing a cell paste. Then, the enzyme is extracted with an appropriate buffer solution.

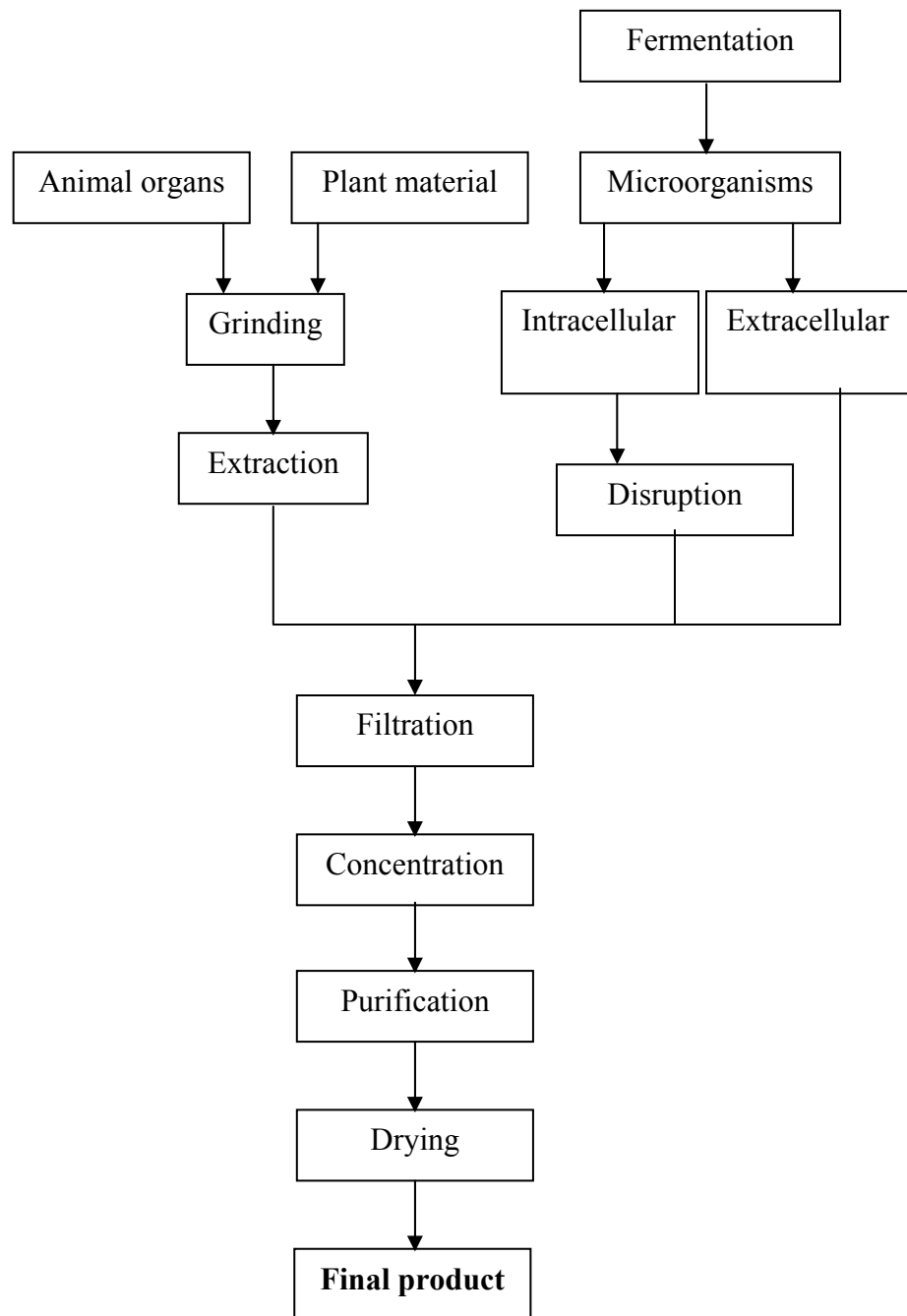


Figure 2.1. The important steps of enzyme production (Gölker, 1990)

For the production of microbial enzymes, cultivation and then fermentation are applied after selection of a suitable microorganism. When enzyme is extracellular, there is no other process required at this step. However, if the enzyme is intracellular, for releasing the enzyme, different cell disruption techniques are applied (Gölker, 1990; Temiz, 1998).

### 2.1.1. Cell disruption methods

There are different methods for cell disruption based on the cell type, and the nature of the intracellular product. However, only a few of these methods are used in large scale production. These methods can be classified as mechanical and non-mechanical methods (Table 2.1).

#### 2.1.1.1. Mechanical methods

Among the mechanical methods, high pressure homogenization is the most common one working well on laboratory scale. The principle of this method is based on the subjection of the cell suspension to high pressure by extruding through a valve to atmospheric pressure and disruption of the cells by shearing forces and simultaneous decompression.

On large scale processes, disruption with ball mills is used frequently. This method is based on breaking of cells with balls generally having a diameter of 0.2 to 1 mm. Ultrasonic vibrators are also sometimes used for the disruption of cell wall and membrane of bacterial cells (Gölker, 1990, Shuler and Kargi 2002).

Table 2.1. Different methods for cell disruption (Gölker, 1990).

<b>Mechanical methods</b>	<b>Nonmechanical methods</b>
High pressure homogenization	Drying (freeze-drying, organic solvents)
Grinding (ball mill)	Lysis (Physical: freezing, osmotic shock; Chemical:
Ultrasound	detergents, antibiotics; Enzymatic: lysozyme)

#### 2.1.1.2. Nonmechanical methods

Drying of microorganisms and preparation of acetone powders are applied to alter the cell wall and to permit subsequent extraction of the cell contents. To lyse the cell walls, different methods are applied by physical, chemical and enzymatic means. With osmotic shock or freezing, the cell wall and membrane can be disturbed to release the enzyme easily into the extraction media. Different detergents can also be used to

dissociate proteins and lipoproteins from the cell membranes. Another alternative is the application of lytic enzymes such as lysozyme. However, this treatment is too expensive (Gölker, 1990, Salusbury, 1995, Shuler and Kargi 2002).

## **2.2. Clarification of Enzyme Extracts**

During purification of enzymes, clarification is applied to remove particulate material (e.g. cells, organelles, debris or precipitated macromolecules) from the surrounding liquid (e.g. fermentation medium or buffer). The size, shape, and resistance to shear of the bulk should be taken into account for the selection of the most suitable clarification technique. Because of the small size of bacterial cells and slight difference between the density of fermentation medium and these cells, clarification is very difficult in microbial enzyme production. However, large cells such as yeast cells can be collected by decantation. In contrast, animal or plant residues can easily be separated with centrifugation or filtration (Gölker, 1990, Whittington, 1995).

### **2.2.1. Centrifugation**

The principle of this method is based on the application of radial acceleration to a particle suspension by rotational motion. As a result, particles denser than the bulk fluid move outwards and separated. Generally, centrifugation is applied to separate particles that size is between 100 and 0.1  $\mu\text{m}$ . There are different types of centrifuges such as tube centrifuges and continuous flow centrifuges used at the laboratory scale and plant scale, respectively.

### **2.2.2. Filtration**

The filtration is defined as the separation of solid particles from liquid or gaseous streams. This is the most cost-effective method for the separation of large particles and cells from fermentation broth. There are different types of filters such as drum filter, rotary vacuum filter, ultrafilter, microfilter, and plate and frame filter. Continuous rotary vacuum filters are the most widely used types in the fermentation industry.

### **2.2.3. Flocculation and flotation**

The principle of this method is based on the agglomeration of coagulated dispersed colloids in the medium with the addition of flocculating agents such as polyelectrolytes or  $\text{CaCl}_2$ . The agglomerates formed can be removed by filtration or centrifugation. However, when there are no stable agglomerates formed, particles can be removed by flotation. In this method, the particles adsorbed onto gas bubbles and accumulated at the top of the extract (Gölker, 1990).

## **2.3. Concentration of Enzymes**

After clarification, the enzyme concentration of the obtained extract is often very low. Thus, a concentration step is generally required in order to aid subsequent purification steps. The major concentration procedures that do not inactivate enzymes are; (1) addition of a dry matrix polymer; (2) freeze-drying (lyophilization); (3) ultrafiltration; (4) precipitation, (5) aqueous two-phase partitioning, and (6) removal of salts and exchange of buffers

### **2.3.1. Addition of a dry matrix polymer**

The principle of this method is based on the addition of a dry inert matrix into the protein solution. The pores of these matrixes are quite small. Thus, this allows the absorption of water and small molecules but not the proteins. To separate matrix, the extract was then centrifuged, filtered or settled by the gravity (Harris, 1995).

### **2.3.2. Freeze-drying (Lyophilization)**

The principle of this method is based on the removal of water from a frozen solution by sublimation under vacuum. The phase diagram showing the sublimation of water was given in Figure 2.2.

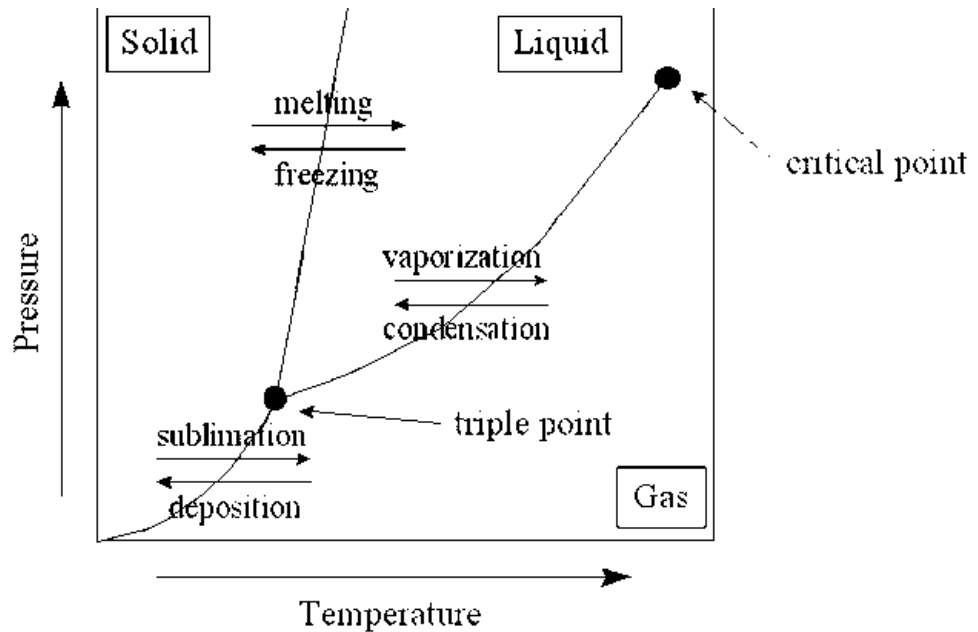


Figure 2.2. Phase diagram for water

Freeze-drying can be applied both for concentration and preservation of proteins by preparing their dehydrated form as dry powder. There are different freeze-dryers, which may differ in their specifications according to the model and manufacturer. However, in general, the process occurs first by the removal of bulk water from the frozen protein solution by sublimation under vacuum (primary drying) and then the removal of remaining bound water from the protein by controlled heating (secondary drying). As a result, the protein was prepared as a dry powder with a moisture level lower than 1 %. Freeze-drying is a time consuming process and require high capital and running costs. However, it is very suitable for high value biomolecules like enzymes (Fagain, 1996, Shuler and Kargi 2002).

### 2.3.3. Ultrafiltration

The principle of this method is based on the transport of a protein solution through a filter medium or membrane in the ultrafiltrate and retention of the solute behind the membrane in the retentate. The driving force for the separation is the pressure difference across the membrane. The diameters of the membrane pores range between 1 and 20 nm that may separate proteins in the range of 5.000-500.000 Daltons.

### **2.3.4. Precipitation**

Enzymes are very complex protein molecules having both hydrophobic and hydrophilic groups. The solubility of a protein molecule in an aqueous solvent is determined by the distribution of these groups onto its surface. Protein precipitates may be formed due to aggregation of protein molecules by adjusting the system temperature, pH, ionic strength and dielectric constant. Currently, precipitation is usually used as a crude separation and partial purification step. Also, it can be used for concentrating proteins prior to analysis or a subsequent purification step. After the precipitation, the precipitate is collected by filtration or centrifugation, dissolved in a suitable buffer or water, desalted if necessary and used in the subsequent purification steps (Harris, 1995).

#### **2.3.4.1. Precipitation by increasing the ionic strength (salting out)**

This method is related with the hydrophobic nature of the protein surface. The solubility of proteins in an aqueous solvent is determined by distribution of hydrophobic and hydrophilic groups on their surfaces. In proteins, most of the hydrophobic groups exist in the interior parts whereas some exist at the surface, often in patches. In solution, the surface of proteins is surrounded by a water jacket that prevents the protein-protein interactions by shielding the hydrophobic areas. After the addition of high concentrations of salts to a protein solution, the added ions interacted with water strongly, remove water surrounding the protein and expose hydrophobic patches. In such a case, the contact of different proteins' hydrophobic groups causes aggregation. The aggregates formed are collected by centrifugation and redissolved in fresh buffer or water. In practice, this is the most commonly used method for fractionation of proteins.

The optimum salt concentration required to obtain the desired precipitation changes according to the properties of protein, type of salt used, and the method of contact. Experimentally, the optimum salt concentration is determined by finding the concentration of total protein and concentration and/or activity of target protein at different salt concentrations. Figure 2.3 shows the typical profile of precipitation with ammonium sulphate, the most commonly used salt for protein precipitation. The ammonium sulphate precipitation may be applied simply by bringing the salt concentration to optimum precipitation conditions of the target protein. Also, before the precipitation of the target protein, the solution may first be brought to a percent

saturation that precipitates only the proteins other than the target protein. In such a two step precipitation process, a higher purity is obtained for the target protein.

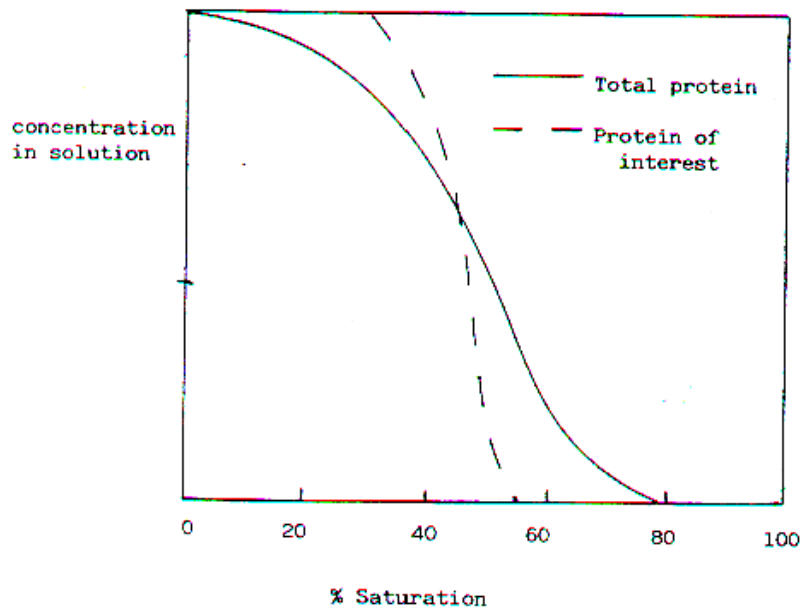


Figure 2.3. Typical profile for ammonium sulphate precipitation

#### 2.3.4.2. Precipitation by decreasing the ionic strength (salting in)

The principle of this method is based on the reduction of ionic strength in the medium. In this case, ionic interactions between protein molecules increase leading to aggregation and precipitation. However, since low ionic strengths can only be achieved by addition of water, the application of this method decreases the concentration. This form of precipitation may occur at later stages of purification (when removing salts by gel filtration, dialysis, or diafiltration) (Harris, 2001).

#### 2.3.4.3. Precipitation by organic solvents

This type of precipitation is based on decreasing the solubility of proteins, by reducing the dielectric constant (solvating power) of the protein solution. A reduction in dielectric constant causes the formation of stronger electrostatic forces between the protein molecules and enhances protein-protein interactions that resulted with agglomeration and subsequent precipitation.



Acetone and ethanol are the most commonly used solvents. Generally, an equal volume of acetone but four volumes of ethanol is used for precipitation. Thus, because of the little amounts needed for the precipitation acetone is more preferable. Optimum organic solvent concentration can be determined experimentally with a similar method like in salting-out. Precipitation by organic solvents should be applied at or below 0 °C. At higher temperatures, the protein may change its conformation, enabling the solvent to access the protein interior and disturb the hydrophobic interactions at these locations. After collecting the pellet by centrifugation, the remaining solvent can be removed by dialysis, gel-filtration or evaporation at reduced pressure.

#### **2.3.4.4. Precipitation by alteration of pH**

This method is based on adjusting the pH of the extract around the isoelectric point (pI) of the target protein. At this pH, the negative charges of one protein molecule attract the positive charges of the other. Thus, as occurs in the precipitation by solvents, electrostatic attraction causes the aggregation. The protein aggregates may be collected by centrifugation and redissolved in suitable buffers or water.

#### **2.3.4.5. Precipitation by organic polymers**

The mechanism of this method is similar to the method of precipitation with organic solvents but this one requires lower concentrations. The most widely used organic polymer for this kind of precipitation is polyethylene glycol (PEG).

#### **2.3.4.6. Precipitation by denaturation**

Precipitation by this method can be applied if the contaminating proteins, but not the target protein, are denatured by changes in temperature and pH, or addition of organic solvents. During denaturation, the tertiary structure of proteins is disrupted and the random coil structures formed cause aggregation. Aggregate formation is highly influenced by the pH and ionic strength. Close to the pI of the protein and at lower ionic strength the aggregation accelerates considerably.

### **2.3.5. Aqueous two-phase partitioning**

The principle of this method is based on the extraction of soluble proteins between two aqueous phases containing incompatible polymers, or a polymer and a high ionic strength salt. Due to the incompatibility, when the polymers are mixed, large aggregates formed tend to separate due to steric exclusion. Most soluble and particulate matter partition to the lower, more polar phase, whilst proteins partition to the upper, less polar phase. After that the separation of phases can be performed by decantation or centrifugation.

Separation of proteins among each other can be achieved by manipulating the partition coefficient by altering the average molecular weight of polymers, the type of ions in the system, ionic strength, or presence of hydrophobic groups. In this method, by application of sequential partitioning steps or alternatively by mixing of polymers to yield more than two phases, a higher degree of purification can be achieved (Andrews and Asenjo 1995, Shuler and Kargi 2002).

### **2.3.6. Removal of salts and exchange of buffers**

During purification of proteins, it is necessary to change the buffer of extract or to remove salts from the extract. For this purpose, different methods can be applied such as dialysis, diafiltration, and gel filtration.

#### **2.3.6.1. Dialysis**

Dialysis is a membrane separation technique. In this method, the protein solution is placed in a special semi-permeable membrane bag usually made from cellulose and the bag is placed in a selected solvent. Small molecules can pass freely across the membrane until their concentration is same on the both sides of the membrane whilst large molecules are retained. The driving force for diffusion of the salts across the membrane is the relative concentration of the salts in the two solutions. The solvent, which is normally water or buffer, is replaced with a fresh one at regular intervals. During dialysis, the medium should be stirred continuously.

### **2.3.6.2. Diafiltration**

In diafiltration, ultrafiltration membranes may be used to separate small molecules like salts, sugars, or alcohols from the protein solutions. The driving force for the filtration is pressure and the protein of interest is retained on the filter. The process, applied by the addition of water or buffer to the protein solution, is continued until the ionic strength of the filtrate reaches to that of the added water or buffer.

### **2.3.6.3. Gel filtration**

This method is a molecular sieving process that can be used to fractionate molecules according to size. The sieving medium is a gel, which has pores of a fixed diameter, smaller than the protein molecules. Thus, when protein solutions are applied to a column, filled with this gel, small molecular weight substances such as salts are retained by the gel particles while protein molecules are excluded. The elution of column with a buffer or water is enough to elude and collect the salt free proteins from the column.

## **2.4. Purification**

Partial purification is sufficient for many industrial applications. However, for analytical purposes and medical use enzymes must be highly purified. The most widely used technique for this purpose is chromatography. The general principle of this method is based on differential separation of sample components between a mobile phase (passing fluid mixture) and a stationary phase (a bed of adsorbent material). The four mainly used chromatographic techniques are ion exchange, affinity, gel filtration and hydrophobic interaction chromatography. The type of the chromatographic technique to be used depends on the properties of the protein and the aim(s) of the purification process (Table 2.2).

Hydrophobic chromatography is applied when the aqueous phase is at high ionic strength. Gel-filtration chromatography can be used for purification, buffer exchange, desalting or molecular weight determination.

Table 2.2. The main chromatographic techniques and their separation principles

<b>Chromatographic technique</b>	<b>Properties of protein affecting separation</b>
Ion-exchange	Charge
Affinity	Specific binding
Gel-filtration	Molecular size
Hydrophobic interaction	Exposed hydrophobic groups at the surface

For a complete purification, multiple types of chromatographic techniques are applied following to each other. Among these techniques ion-exchange and affinity chromatography are the most widely applied methods for purification of proteins from bioprocesses.

## **2.5. Product Formulation**

The storage time of enzymes may vary from a few days to more than one years depending on the nature of enzyme and storage conditions. Different factors like proteolysis, aggregation, and certain chemical reactions cause loss or deterioration of enzymes' biological activities. Considering their stability during transport and storage, the produced enzymes are formulated in different ways.

In liquid enzyme formulations, to prevent the microbial contamination, different antimicrobials can be added to enzyme extract. Alternatively, to avoid the addition of antimicrobials, enzyme solutions can be filtered through a 0.22  $\mu\text{m}$  filter that excludes all bacteria.

To stabilize the activity of enzymes, the addition of low molecular weight substances like glycerol or sucrose can be used frequently. Salts like ammonium sulphate can also stabilize proteins in solution by forcing the protein molecules to adopt a tightly packed, compact structure by salting out hydrophobic residues.

In some cases, enzymes only can be stored by storage in refrigerator at 4-6  $^{\circ}\text{C}$  in 50 % glycerol or as slurries in approximately 3 M ammonium sulphate. However, the proteins that loose their activity at refrigerator temperatures require lower temperatures such as  $-18$  or  $-20$   $^{\circ}\text{C}$ .

Application of drying is another alternative for stable storage of enzymes. The drying conditions of the enzyme are selected according to the heat sensitivity, physical

properties and desired final moisture content of the product. For example, freeze-drying can be used for drying of heat sensitive enzymes. Other drying alternatives for heat stable enzymes are vacuum tray dryers, rotary vacuum dryers, spray dryers, and pneumatic conveyor driers (Fagain, 1996, Gölker, 1990, Shuler and Kargi 2002 ).

Another application for enzyme stabilization is the immobilization of enzyme on a solid carrier. Sharma et al (2003) studied the storage stability of immobilized tyrosinase by two different immobilization methods and found that in any case the storage stability of immobilized enzyme derivatives is higher than the storage stability of soluble enzymes.

## CHAPTER 3

### PECTIN METHYLESTERASE

#### 3.1. Pectin methylesterase and Other Pectinases

Pectin methylesterase (PME, E.C. 3.1.1.11) is a member of pectinases, a group of enzymes that use pectic substances as substrate. According to their action on pectic substances, pectinases are classified as deesterifying and depolymerizing enzymes (Alkorta et al. 1998, Cemeroglu et al. 2001). PME catalyzes the deesterification of pectin and produces liberated carboxyl groups and methanol as product (Figure 3.1). Other members of pectinases such as polygalacturonase (PG) and pectate lyase (PL) are depolymerization enzymes acting on different forms of pectin.

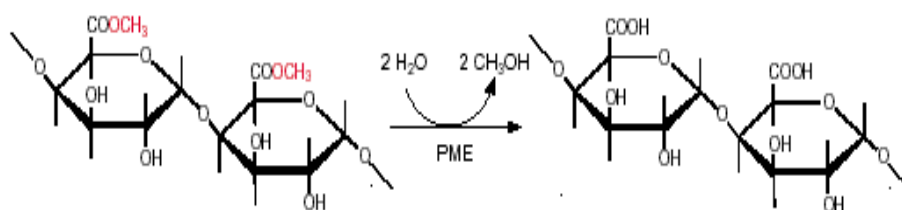


Figure 3.1. Deesterification of pectin by PME (Micheli,2001)

Pectin, is the principal polysaccharide of the middle lamella and it forms approximately 30 % of polysaccharides constituting the cell wall in higher plants. Pectins contain a backbone of smooth homogalacturonan regions, (1→4) linked  $\alpha$ -D-galacturonic acid units that may be methyl esterified at C 6 position, and hairy rhamnogalacturonic regions that interrupt the galacturonic acid residues with (1→2) linked  $\alpha$ -L-rhamnopyranosyl residues carrying neutral sugar side chains (Ralet et al. 2001, Johansson et al. 2002, Wicker et al. 2003, Giovane et al., 2004).

#### 3.2. Sources of PME

PME exists in all higher plants, but it is particularly abundant in citrus fruits (Johansson et al. 2002). The enzyme is also produced by some bacteria and fungi that are pathogenic to plants (Johansson et al. 2002, Giovane et al. 2004). In plants, by

working in coordination with other pectinases such as PL and PG, PME prepares and modifies the pectin. Also, it was reported that the enzyme plays some important roles in cellular adhesion, stem elongation, development, dormancy breakage, fruit ripening, seed and pollen germination, pH regulation, and defense mechanisms of plants against pathogens (Pimenta-Braz et al. 1998, Micheli, 2001, Johansson et al. 2002).

PME is a cell wall bound enzyme that two or more isoforms were detected in higher plants differing in molecular weight, biochemical activity and physical and chemical characteristics (Cameron et al. 1998, Corredig et al. 2000). The action pattern of the PME on pectic substances and the kinetic properties of the enzyme are highly affected from the source. For example, plant PMEs and bacterial PMEs from *Erwinia chrysanthemi* generally remove methyl ester groups of pectin linearly (single chain mechanism). In this action pattern, the binding of enzyme is followed by conversion of all subsequent substrate sites on the polymer chain. In contrast, the fungal PMEs (from *Aspergillus niger*) act on pectin with the multiple chain mechanism. In this type of action pattern, for each enzyme-substrate complex formation the enzyme catalysis the limited number of substrate sites randomly. It was reported that the random deesterification is conducted by acidic PMEs whereas linear deesterification is conducted by alkaline PMEs (Denes et al., 2000). Recently, it has also been reported that the mode of deesterification depends also on the initial degree of methylesterification of pectins and pH of medium (Micheli, 2001).

### **3.3. The Effects of PME on Food Quality**

As indicated above, pectin acts as a cement material among the plant cells and plays an important role for the texture of fruits and vegetables. In unripe fruits, the pectin is insoluble and bound to cellulose microfibrils in the cell walls. During ripening, processing and storage, naturally occurring PME catalyzes the specific demethylation of pectin at C6 position of galacturonic acid units. The demethylized pectin is a good substrate for PG, and other depolymerization enzymes that degrade pectin and cause the loosening of cell walls. This kind of softening, mediated by PME, is a great problem in fruit and vegetable processing industry (particularly in freezing and canning industry). Thus, PME from different fruits and vegetables such as sweet cherry (Alonso et al. 1996); carrot (Ly-Nguyen et al. 2002); acerola fruit (Aparecida de Assis et al. 2002);

Table 3.1. Some properties of PME<sub>s</sub> from different fruits and vegetables

Source	Optimum pH	Optimum temperature (°C)	Temperature stability <sup>a</sup>	References
Sweet cherry (Isoforms, I <sub>1</sub> to I <sub>4</sub> )	I <sub>1</sub> : 7.0 I <sub>2</sub> : - I <sub>3</sub> : < 5.2 I <sub>4</sub> : 5.8	-	I <sub>1</sub> and I <sub>3</sub> are thermolabile I <sub>2</sub> and I <sub>4</sub> are thermostable 100 % (1 min , 60-70 °C)	Alonso et al (1996)
Carrot	7.3-7.4	48.5	50 % (10 min, 55 °C) 5 % (10 min, 60 °C)	Ly-Nguyen et al (2002)
Acerola fruit	9.0	-	125.5 % (90 min, 98 °C)	Aparecida de Assis et al (2002)
Strawberry	6.9-7.0	59-60	-	Ly-Nguyen et al (2002)
Mandarin	9.0	-	20 % (1 min, 70 °C)	Rillo et al (1992)
Navelina Orange	7.0	50	-	Christensen et al (1998)
Valencia orange peel	9.0 (6.0 at 1.2 % NaCl)	-	-	Savary et al (2002)
Commercial orange PME	9.0 (6.0 at 1.2 % NaCl)	55-60	50 % (5 min, 55 °C) inactivated at 65 °C	Savary et al (2002)
Green bean pod isoforms (P1 and P2)	P1: 6.5-9 P2: 7-9	-	P1: inactivated (4 min, 80 °C) P2: inactivated (8 min, 60 °C)	Laats et al (1997)
Apple	6.5-7.5	-	100 % (1 min, 40 °C) inactivated (1 min, 90 °C)	Castaldo et al (1989)

<sup>a</sup> % retained activity after the indicated heating



strawberry (Ly-Nguyen et al. 2002); mandarin (Rillo et al. 1992); Navelina Orange (Christensen et al. 1998); Valencia orange peel (Savary et al. 2002); green beans (Laats et al. 1997); apple (Castaldo et al. 1989) has been extracted, purified and characterized (Table 3.1).

Because of its close relation with the cloud loss of fruit juices, the enzyme PME is also attracts a great attention in fruit juice industry. Particularly in citrus juices, that cloud stability is an important quality criteria, the inactivation of enzyme by heat treatment is essential (Giovane et al. 2004). If the enzyme can not be inactivated immediately after fruit juice extraction, it demethylates the pectin rapidly. The low methoxy pectin cross-links readily with divalent ions such as  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  and forms some insoluble pectates. This induces the loss of cloud stability by precipitating pulp-particles and separating fruit juice serum. Such products are not attractive for consumers. Also, during concentration of such fruit juices, the gelation occurred causes problems during reconstitution, pumping and blending operations (Cameron et al. 1998).

The PME in citrus juices is more heat stable than the microorganisms in the juice. Thus, the heat treatment applied to these fruit juices (90 °C for 0.5-1 min) for pasteurization is more severe than that desired for microbial inactivation (Cemeroğlu and Karadeniz, 2001).

### **3.4. Industrial Applications of PME**

Pectinases have been utilized in the commercial sector for wine and fruit juice industry since 1930. Commercial pectinase preparations, account for about 25 % of the global food enzymes, are produced generally from fungal sources (from *Aspergillus niger*) and used in many applications in food industry without purification. According to the specific objective(s) of the processes, the enzyme PME is used alone or in combination with other enzymes, mostly with other pectinases. The processes that PME involved are introduced below.

#### **3.4.1. Clarification of fruit juices**

In fruit juices, pectic substances are the main substances responsible for the cloudiness. Thus, in the production of clear fruit juices the pectin in juices should be

degraded by the application of pectinases. The degradation of pectin reduces also the viscosity of fruit juices and eases their filtration and concentration. To degrade pectin, PME is used in combination with other pectinases such as PG and PL. Following the degradation of pectin, the viscosity of fruit juice drops and suspended cloud particles lost their stability and precipitated.

In the clarification of fruit juices, the PME enzyme may also be used alone. However, such a treatment needs the addition of  $\text{CaCl}_2$  to fruit juice. This removes pectin in fruit juice as insoluble calcium pectates and causes the clarification (Massiot et al. 1997, Alkorta et al. 1998, Wicker et al. 2002).

In fruit juice industry, pectinases may also be used to increase the yield of fruit juice extraction. The treatment of mash with pectinases causes the degradation of cell wall and middle lamella of plant cells. This releases the fruit juice and increases yield. Another application of pectinases in fruit juice industry involves liquefaction of fruit mashes. In such an application, the pectinases are combined with cellulases and hemicellulases for the complete disruption of the cell walls. This method can alternatively be used for the production of fruit juices from tropical fruits (e.g. banana) that can not be processed with classical methods (Cemeroğlu and Karadeniz, 2001; Demir et al. 2001). In all these enzymatic treatments, degradation of pectin facilitates pressing and increase juice yield. Also, the aromatic quality of fruit juices increases and amount of waste material reduces (Sarioğlu et al. 2001).

In industry, the use of pectic enzymes in fruit juice processing has traditionally been conducted in batch reactors using soluble enzymes. This operation causes the loss of enzyme. Also, the presence of enzyme in the final product and alteration of organoleptic properties are inevitable (Demir et al. 2001). Thus, different studies have been conducted for the immobilization of pectinases. For example, Demir et al. (2001) studied the use of immobilized commercial pectinase named 'Pectinex Ultra SP-L' for mash treatment of carrot puree. This treatment maintains almost 93 % of the enzyme after the fifth treatment. However, in practice the application of pectinases are still conducted by the classical methods.

Pectinases are also used in wine-making industry. The utilization of these enzymes influences the methanol concentration in musts and wines. However, their use increases the volume of free-run juice (by breakdown of polysaccharides and solubilization of middle lamella), extraction yield of polyphenols, and color and

aromatic compounds (Pimenta-Braz et al. 1998, Rogerson et al. 2000, Kashyap et al. 2001).

#### **3.4.2. Firming of fruits and vegetables before processing**

Texture, an important quality attribute of fresh and processed fruits and vegetables, is closely related with the structural integrity of the primary cell walls and middle lamella, which are mainly composed of pectic substances. In industry, most of the processes such as blanching, freezing, dehydration, pasteurization and sterilization applied to preserve fruits and vegetables cause irreversible physical damages on cellular tissues. However, the negative effects of processing on texture can be overcome by applying different processes, which involve the use of PME enzyme. For example, the application of PME and  $\text{CaCl}_2$  by vacuum infusion is now used for firming fruits and vegetables (Degreave et al. 2003). The mechanism of firming in this method is based on the demethylation of naturally occurring pectin in plant tissues by the action of PME and the chelation of the added or natural calcium with the free carboxyl groups generated in pectin molecules. Such a chelation causes the formation of networks among pectin molecules (the egg box model), stabilizes pectin and increases the firmness of plant tissues. Some of the successful applications of enzymatic firming by use of PME involves, firming of strawberries before jam making and freezing (Suutarinen et al. 2000, Suutarinen et al. 2002) and firming of apples, strawberries, and raspberries before pasteurization (Degreave et al. 2003).

Besides commercial PMEs, the in situ PME in the product may also be used for firming of fruits and vegetables. In this method, the in situ PME should be activated by low temperature blanching. The free carboxyl groups produced then were cross-linked with divalent ions in the medium or  $\text{CaCl}_2$  added to form a stable network. Firming by this method is suitable to apply for whole or sliced potatoes to be processed to French fries (Yemenicioğlu, 2002).

#### **3.4.3. Modification of pectin**

Pectin produced from apple and orange peels has widespread application in food industry as gelling, thickening, and stabilizing agents. The degree of esterification of

pectin molecule greatly influences its functional properties. The degree of esterification (DE) of pectin may range between 0 and 100 % and on the basis of DE, pectins are divided into two groups; high methoxylated (HM) pectins with a DE higher than 50 %, and low methoxylated (LM) pectins with a DE lower than 50 %. Pectin can be modified by PME to obtain the required DE value (Morris et al. 2000, Schmelter et al. 2002). Pectins with low DE are particularly useful to obtain gels without using sugar and acid.

#### **3.4.4. Production of low sugar jams, jellies, and compotes**

Traditionally, HM pectin is used for the preparation of jams and jellies. The gelling mechanism of HM pectin is based on hydrophobic interactions and dehydration at low pH (< 4.0). In such gels, the presence of high concentrations of sugar (> 60 %) is essential for gelling. In contrast, LM pectin forms gels by ionic interactions in which calcium or other divalent cations interact with free carboxylic acid of two adjacent chains, and give rise to cross-linking of these chains. In such a gelling mechanism, the sugar concentration is not very important. Thus, LM pectins are suitable for the production of low sugar (diabetic) jams and jellies (Cemeroğlu and Acar, 1986).

#### **3.4.5. Other applications**

Pectinases, including PME, can also be used in some different industrial applications. One of these applications is oil extraction. Oils from coconut germ, sunflower seed, palm kernel, rape seed are industrially extracted with organic solvents. The most commonly used solvent is hexane, which is a potential carcinogen. Thus, alternatively, cell wall degrading enzymes, PME and other pectinases are used in combination to extract oil in different crops by liquefying the structural components of their cell walls. Pectinase preparations (such as Olivex) are also used in olive oil industry to increase the oil extraction output and to improve certain olive oil quality indicators (Kashyap et al. 2001, Vierhuis et al. 2003).

Another application of combinational use of PME, other pectinases and cellulases is the peeling of fruits. Peeling of fruits has traditionally been applied by hand or treatment with steam, boiling water, acid or alkali. But these methods sometimes cause poor product quality (losses of fruit juice and/or disintegrations at the fruit

surface) in delicate fruits. Also, in chemical methods, the disposal of the used peeling solution is a great problem. Therefore, application of pectinases and cellulases by vacuum infusion can be used as an alternative method for peeling of delicate fruits. For example, Pretel et al (1997) applied a commercial preparate (Rohament PC) containing pectinases and cellulases to remove peels and skins of oranges and to obtain whole fruit segments.

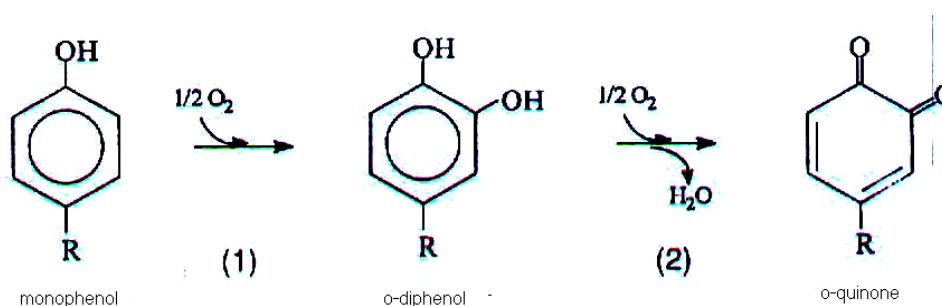
## CHAPTER 4

### POLYPHENOL OXIDASES

#### 4.1. Polyphenol oxidases

Polyphenol oxidases (PPOs) are copper containing oxidoreductases that catalyze the hydroxylation and oxidation of phenolic compounds in the presence of molecular oxygen. According to the type of their substrates PPOs can be divided into three groups namely tyrosinase, catechol oxidase and laccase. Tyrosinase (EC. 1.14.18.1) catalyzes the hydroxylation of monophenols to o-diphenols (monophenolase or cresolase activity) and the oxidation of o-diphenols to o-quinones (diphenolase or catecholase activity). Catechol oxidase (EC. 1.10.3.1) catalyzes only the oxidation of o-diphenols whereas laccase (EC. 1.10.3.2) catalyzes the oxidation of o-diphenols as well as p-diphenols to the corresponding quinones. Both laccase and catechol oxidase cannot catalyze the hydroxylation reactions (Figure 4.1).

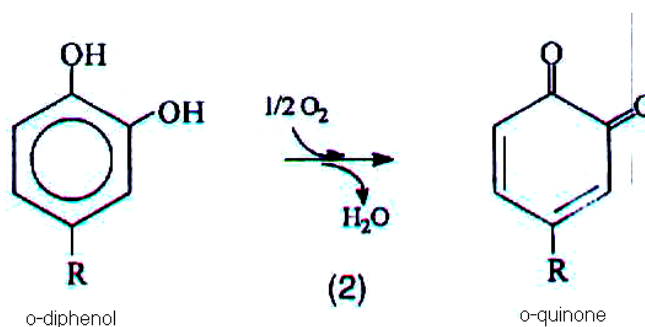
A



(1) Cresolase activity

(2) Catecholase activity

B



C

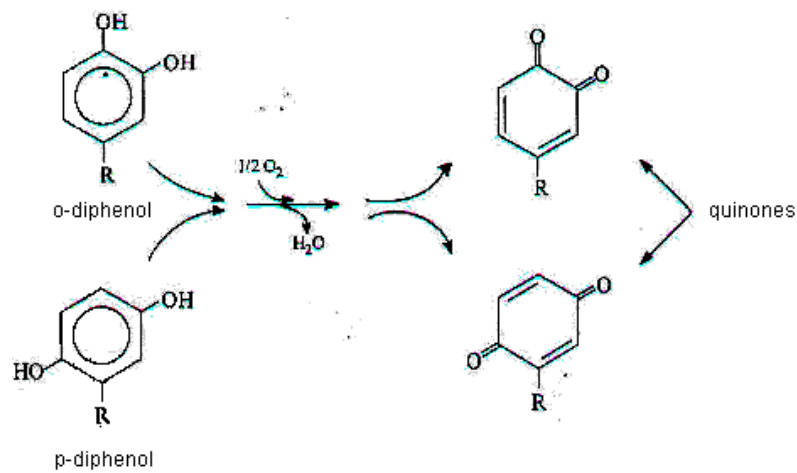


Figure 4.1. The reactions of polyphenol oxidases (Cemeroğlu et al. 2001)

(A: Reaction mechanism of tyrosinase; B: Reaction mechanism of catechol oxidase; C: Reaction mechanism of laccase)

The o-quinones formed by PPOs are very reactive and turn spontaneously to tri hydroxy benzenes (THB). The THBs then interact with o-quinones in the medium to form hydroquinones that further polymerize to complex and dark colored pigments referred melanins (Figure 4.2). This reaction is called the enzymatic browning and it is undesirable during processing of fruits and vegetables.

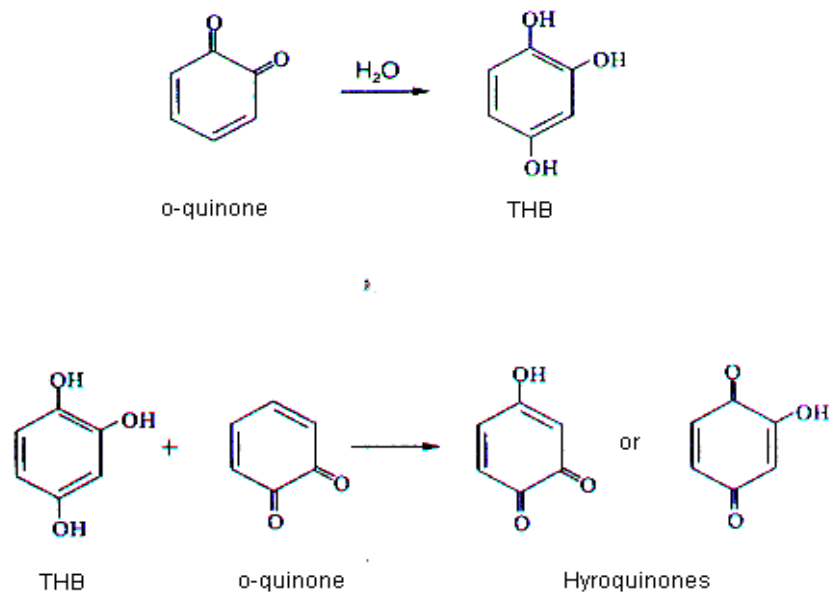
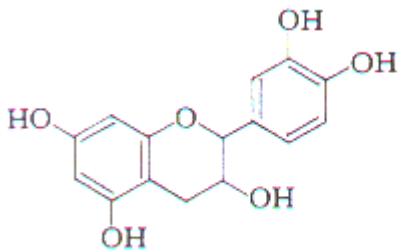


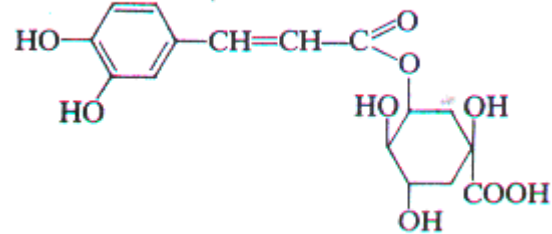
Figure 4.2. The nonenzymatic reactions during formation of dark colored melanins (Cemeroğlu et al. 2001)

## 4.2. Substrates of PPO

Phenolic substances, the natural substrates of PPO, occur in many fruits and vegetables to provide color pigments and characteristic flavor. Although, fruits and vegetables contain a wide variety of phenolic compounds only a few of them are good substrates of PPOs. The most important natural substrates of PPOs in fruits and vegetables are catechins, cinnamic acid esters (especially chlorogenic acid), 3-4-dihydroxy phenylalanine (DOPA) and tyrosine (Vamos Vigyazo, 1981) (Figure 4.3.).



Catechin



Chlorogenic acid



Tyrosine



L-DOPA

Figure 4.3. Some of the good substrates of PPO in plants



The substrate specificity of PPO and the available phenolic substrates in different fruits and vegetables vary greatly. For example, in mushrooms and potatoes the precursor of enzymatic browning is tyrosine. However, in peaches and pears the primary phenolic compounds causing enzymatic browning are tannins and chlorogenic acid, respectively. The substrate specificity of PPO may also be different in various sections of the plant. For example, in apple peel and flesh the precursors of enzymatic browning are chlorogenic acid and catechins, respectively (Davidek et al. 1990).

### **4.3. Sources and Some Characteristics of PPO**

PPOs are widely distributed in nature. These enzymes are abundant in higher plants but found also in animals and some microorganisms, especially in fungi. The properties of PPO from different sources vary considerably. For example, it was reported that the substrate specificity of plant PPO is broader than that for the PPO in animals (Burton, 1994).

In plants, PPOs are located mainly in thylakoid membrane of chloroplasts and mitochondria. The enzymes exist also in the vesicles or other bodies in nongreen plastid types and cytoplasm (Nicolas et al., 1994). In higher plants and fungi there are different isoforms of tyrosinases such as immature, mature latent and active forms. For example, in mushrooms almost ~ 98-99 % of the PPO is present in its latent form (Vamos-Vigyazo, 1981, Nicolas et al. 1994, Minussi et al. 2002, Seo et al. 2003).

In living organisms, PPO has many different roles. For example, in insects they are responsible for the melanization and sclerotization of the exoskeleton. In crustaceans such as octopus, they form the black colorant sepia. In animals and human, the enzyme is responsible for hair and skin coloration whereas in plants it has vital roles in the defense mechanisms against insects and microbial pathogens (Strack and Schliemann 2001).

The activity and biochemical characteristics of PPO change according to the source of enzyme (Table 4.1). However, in most cases optimum pH of enzyme varies between 5.0 and 7.0. The enzyme mostly shows low pH stability between 3.0 and 4.0. Also, the PPO enzymes do not belong to an “extremely heat-stable enzyme” group and short exposures to temperatures between 70 ° and 90 °C are sufficient to inactivate them (Vamos-Vigyazo, 1981).

Table 4.1. Some properties of PPOs from different fruits and vegetables

Source	Optimum pH	Optimum temperature (°C)	Temperature stability <sup>a</sup>	Reference
<b>Banana peel</b>	6.5 (Dopamine)	30 (Dopamine)	90 % (30 min, 60 °C) inactivated at 80 °C	Yang et al (2001)
<b>Banana pulp</b>	6.5 (Dopamine)	30 (Dopamine)	80 % (10 min, 70 °C)	Yang et al (2000)
<b>Chinese cabbage</b>	5 (Catechol)	50 (Catechol)	unstable > 50 °C	Nagai and Suzuki (2001)
<b>Cabbage</b>	7.6 (Phloroglucinol)	40 (Phloroglucinol)	40 % (10 min, 100 °C)	Fujita et al (1995)
<b>Malatya apricot</b>	8.5 (Catechol)	-	50 % (47 min, 60 °C) 50 % (16 min, 80 °C)	Arslan et al (1998)
<b>Grape</b>	5 (Catechol)	-	inactivated at 70 °C	Garcia and Buzaleh (1994)
<b>Amasya apple</b>	7.0 (Catechol) 8.6 (pyrogallol) 6.6 (L-DOPA)	15 (Catechol) 50 (L-DOPA) 70 (Pyrogallol)	-	Oktay et al (1995)
<b>Artichoke</b>	5-7 (Catechol) 8 (L-DOPA) 6.5 (Pyrogallol)	25 (Catechol)	50 % (6 min, 60 °C) 50 % (4 min, 70 °C)	Aydemir (2004)
<b>Iceberg lettuce</b>	4.4 and >7.5 (-SDS) >5.0 (+SDS)	-	inactivated at 90 °C	Chazarra et al (1996)
<b>Coffee</b>	6.0-7.0	25-30	-	Mazzafera and Robinson (2000)

<sup>a</sup> % retained activity after the indicated heating

#### **4.4. The Effects of PPO on Food Quality**

The enzymatic browning reaction is a widespread phenomenon, mostly undesirable in food technology due to the formation of unpleasant discoloration and development of some off-flavors. Thus, numerous studies have been conducted related to the inhibition and inactivation of PPO by chemicals and thermal processes, respectively. Most of these studies are related with the light colored plant products such as apple (Oktay et al. 1995, Yemenicioğlu et al. 1997), potato (Coetzer et al. 2001, Yemenicioğlu, 2002), and mushroom (Kermasha et al. 1993, Friedman and Bautista 1995, Gomes and Ledward 1996, Jimenez and Garcia-Carmona 1997, Rodziguez-Lopez et al, 1999, Weemaes et al. 1997, Beaulieu et al. 1999, Devece et al. 1999, Fan and Flurkey 2004).

A novel and promising approach for the control of PPO activity in fruits and vegetables without the use of chemicals is the use of molecular biology. By employing an antisense RNA technique Bachem et al (1994) blocked PPO gene expression in potatoes and obtained very low PPO activity-containing cultivars. Coetzer et al (2001) also reduced the PPO activity in potatoes by using sense or antisense RNA techniques and successfully controlled their enzymatic browning. One of the concerns related to these methods is that PPO in plants is involved in disease and pest resistance (Li and Steffens 2002). The inactivation of PPO enzymes in plants may cause a susceptibility increase to diseases and may encourage a wider pesticide use. In fact, hypersusceptibility to pathogens was reported in tomatoes with reduced PPO activity induced by the use of an antisense technique (Thomas-Berberan and Espin 2001). Thus, alternative strategies should be developed to increase the application potential of molecular techniques.

#### **4.5. Industrial Applications of PPO**

In industry, the commercial PPOs used are laccase obtained from *Trametes versicolor*, a white-rot fungus, by fermentation and tyrosinase extracted from mushrooms (*Agaricus bisporus*). Recently, extensive studies have been conducted related to the use of PPO in food industry and in other branches of industry. Enzymatic cross-linking of proteins and polysacharrides, production of color compounds and antioxidants, clarification of beverages, production of biosensors are some of the

example uses of the enzyme. These uses of PPO in industry are discussed with further details below.

#### **4.5.1. Enzymatic cross-linking of proteins or polysaccharides**

There is an increasing interest in food industry for finding new functional ingredients. For example, the modification of proteins to improve or change their functions has become very popular. One of the potential methods for the modification of proteins is cross-linking. This application may improve the functions of proteins related to gel formation, foaming, emulsification, etc.

The modification of proteins by PPO is done by mixing enzyme, target protein(s) and suitable phenolic compounds. The oxidation of phenolic compounds by PPO produces o-quinones that may undergo a spontaneous Michael-like addition with amino-sulphydryl or pyrrolidine side chain of proteins. Thus, with this reaction the proteins are cross-linked by the oxidized phenolic compounds (protein-oxidized phenolic compound-protein). In some proteins, the protein-protein cross-linking with PPO may also occur without using any phenolic compounds. In such proteins, the tyrosine groups in protein oxidized by PPO provide the desired cross-linking. Thalmann and Lötzbeyer (2002), by use of mushroom tyrosinase, studied the cross-linking of lysozyme and whey proteins such as  $\alpha$ -lactalbumin,  $\beta$ -lactoglobulin in the presence or absence of a low molecular weight phenolic compound, caffeic acid. In the presence of phenolic compound, these workers reported the successful modification of all indicated proteins with cross-linking. However, in the absence of phenolic compound only  $\alpha$ -lactalbumin showed cross-linking.

In the presence of suitable phenolic compounds, the cross-linking of some polysaccharides is also possible (polysaccharide-oxidized phenolic compound-polysaccharide). For example, Micard and Thibault (1999) reported the use of laccase and ferulic acid for cross-linking of sugar beet pectin. The gel obtained from this modified pectin is thermo-irreversible and maintains its structure during heating operation (Minussi et al. 2002).

#### **4.5.2. Production of flavonoid-derived colorants and antioxidants**

Some plant secondary metabolites, including the flavonoids, show antioxidant activity. Also, there are some natural plant metabolites with stable and attractive color.

These natural compounds may be used in place of synthetic antioxidants and colorants. One of these plant based flavonoids is phloridzin which found in apples. The bioconversion of phloridzin to a number of derivatives provides some novel compounds with higher antioxidant activities. Ridgway et al (1997) extracted and used apple PPO successfully for biosynthesis of antioxidant compounds 3-hydroxyphloridzin and 3-hydroxyphloretin from phloridzin and yellow/orange colored dimerized oxidation products of phloridzin that may be used as food colorants. Ridgway and Tucker (1999) also developed a procedure for the partial purification of commercially suitable PPO from apple leaf. However, since PPO in apples can not oxidize the p-diphenols (laccase activity) and lacks monophenolase activity it may only be used for some specific applications. By use of tyrosinases it is also possible to obtain some plant pigments such as red-violet betalains and gold colored aurones (Strack and Schliemann 2001) and biosynthesis of antioxidant compounds such as hydroxytyrosol (Espin et al. 2001).

#### **4.5.3. The removal of haze forming polyphenols from beverages**

The most frequently encountered problem in wine, beer, and fruit juices is the haze formation, resulted from protein-polyphenol interaction. Thus, different methods have been used in order to remove the haze forming phenolic compounds from the beverages. For example, the haze forming polyphenols may effectively be removed from the beverages by use of polyvinylpolypyrrolidone (PVPP) columns. However, because of its health problems on workers and low biodegradability in the wastewaters it is difficult to handle PVPP in factories (Minussi et al. 2002).

The use of in situ PPO or commercial PPO (Laccase) to selectively oxidize haze-forming polyphenols is an alternative to the application of PVPP columns. It was demonstrated that the phenolic compounds oxidized by the PPO and polymerized may be flocculated and removed from the beverages (Minussi et al. 2002). The removal of oxygen during enzymatic oxidation enhances the storage stability of beers (Minussi et al. 2002). In contrast, the oxidative changes caused by the enzyme affect the aromatic quality of apple juices adversely (Cemeroğlu and Karadeniz 2001).

#### **4.5.4. Oxygen scavenging and removal of undesirable phenolics from food**

Because of their high oil content, foods such as salad dressings and mayonnaise are susceptible to oxidation. The oxidation of fatty acids in these foods by the dissolved oxygen causes the formation of undesirable volatile compounds. Thus, the removal of dissolved oxygen in these foods by PPO improves their quality (Minussi et al. 2002).

In cocoa beans, the enzymatic oxidation of phenolic compounds responsible from astringency and bitterness is an important PPO application. Selamat et al (2002) studied the enrichment of fermented cocoa beans with mushroom PPO showed that the concentration of undesirable phenolic compounds in cocoa beans may be reduced effectively to acceptable levels. Also, laccase from *Coriolus versicolor* studied for the treatment of cocoa bean removed the phenolic compounds responsible from bitterness and other unpleasant tastes successfully (Minussi et al. 2002).

The undesirable phenolic compounds exist also in canola meal, a by product obtained after oil extraction from canola seeds. Although, it is a good source of protein with a favorable amino acid composition, its high phenolics content prevents the use of canola meal as human food or animal feed. Thus, there are some studies to remove undesirable phenolic compounds, especially sinapic acid, from canola meal by *Trametes versicolor* PPO (Lacki and Dunjvak 1998). The ability of mushroom PPO to oxidize sinapic acid in canola meal was also reported by Choi and Sapers (1994). Thus, PPO from different sources may be suitable to treat the canola meal.

#### **4.5.5. Removal of undesirable phenolics from wastewaters**

Aromatic compounds including phenolics and aromatic amines present in wastewaters of chemical, textile, wood preservation and food industries are very harmful for the environment. Thus, there are some studies to oxidize and remove these undesirable phenolic compounds from wastewaters by peroxidases (Minussi et al. 2002, Duran and Esposito 2000, Ikehata and Nicell 2000). As alternative to peroxidases, the immobilization of tyrosinases and/or laccases on different supports and treatment of wastewaters is also studied by different workers. For example, *Coriolopsis gallica* (white-rot fungus) laccase was studied to degrade tannins in waste waters from beer factories and the effectiveness of *Pleurotus ostreatus* laccase was also tested for the treatment of wastewater from olive oil production (olive mill wastewater). This

treatment reduced the phenolic content of olive mill wastewater almost 90 %. However, no toxicity reduction was observed in the treated wastes (Minussi et al. 2002).

#### **4.5.6. Analytical and clinical applications of PPO**

##### **4.5.6.1. Production of biosensors**

Biosensors are specific devices that detect, transmit, and record information regarding a physiological or biochemical change in a medium. In practice, it is a probe integrating a biological component with an electric transducer thereby converting a biochemical signal into a quantifiable electrical response. Due to their biological specificity, enzymes are widely used as biological sensing elements (Minussi et al. 2002).

It was reported that a number of biosensors have been developed containing laccase for immunoassays and determination of phenolic compounds and tyrosinase for the detection and quantification of phenolic compounds (Minussi et al. 2002, Rubianes and Rivas 2000, Climent et al. 2001). For example, tyrosinase and catechol oxidase containing biosensors were also developed for detection of phenolic compounds in olive oil, green tea, grape and olive extracts and beer. A PPO biosensor for detection of pesticides in spiked apples and a PPO-alkaline phosphatase combinational biosensor for detection of phosphatase in drinking water were also developed (Mello and Kubota 2002).

##### **4.5.6.2. Clinical applications**

One of the most interesting features of mushroom tyrosinase is its ability to inhibit the attachment of some bacteria. For example, Cowan et al (2000) demonstrated that by oxidizing the critical tyrosine residues of glucan binding lectin and glucosyltransferases, tyrosinase prevents the attachment of *Streptococcus sobrinus*, a bacteria responsible from the formation of oral cavities, to glucans deposited on the tooth surface. Kolganova et al (2002) also showed that the tyrosinase reduces the adhesion of some viruses and pathogenic bacteria to buccal epithelial cells, while unaffected the attachment of probiotic bacteria. These findings are quite interesting and

may open the way of using mushroom PPO in foods such as gums and confectionaries to increase the tooth health of people.

Several other clinical applications such as using tyrosinase as a catalyst to produce L-DOPA, a drug for the treatment of Parkinson`s disease (Sharma et al. 2003), a marker of vitiligo, an autoimmune disease and a tumor suppressing and prodrug therapy agent (Seo et al. 2003) also attracts considerable interest.



## CHAPTER 5

### MATERIALS AND METHODS

#### 5.1. Materials

Oranges (cultivar Valencia from Antalya, Turkey) were purchased from a local market in İzmir. In the laboratory, the juices of oranges were extracted with a home type manual extractor and the peels obtained were frozen and kept at  $-18^{\circ}\text{C}$  until used in the experiments. The commercially cultivated mushrooms (*Agaricus bisporus*) were purchased from a local market in İzmir, Turkey. In the laboratory, the stems of mushrooms, cut approximately 1 cm below their caps, were collected and processed to acetone powder immediately. The citrus pectin (Galacturonic acid content 79 %, methoxy content 8 %), dialysis tubing (prepared as described in the product information), insoluble PVPP (polyvinylpolypyrrolidone), and dextran were purchased from Sigma Chem. Co. (St Louis, Mo., USA). Ammonium sulphate (for biochemistry), and L-tyrosine (for biochemistry) were purchased from Merck (Darmstadt, Germany). 3-(3,4 Dihydroxyphenyl)-L-alanine and phloridzin dihydrate were purchased from Fluka (Steinheim, Germany). Saccharose was purchased from Pancreac (Barcelona, Spain) and sodium benzoate and potassium sorbate were purchased from AppliChem (Darmstadt, Germany). All the other chemicals were reagent grade.

#### 5.2. Methods

##### 5.2.1. Methods related to PME enzyme

###### 5.2.1.1. PME extraction

For the extraction of PME enzyme five different extraction procedures were tested;

**Extraction procedure # 1:** Homogenization of orange peels (30 g) with cold distilled water (90 mL) / Filtration from cheese-cloth (4 layers) to collect the enzyme extract (the pellet was discarded) / Centrifugation of enzyme extract (at 3500 g for 15 min at  $4^{\circ}\text{C}$ )

**Extraction procedure # 2:** Homogenization of orange peels (30 g) with 1 M NaCl solution (90 mL) / Filtration from cheese-cloth (4 layers) to collect the enzyme extract (the pellet was discarded) / Centrifugation of enzyme extract (at 3500 g for 15 min at 4 °C)

**Extraction procedure # 3:** Homogenization of orange peels (30-50 g) with cold distilled water (150 mL) / Filtration from cheese-cloth (2 layers) to collect the pellet (the water extract was discarded) / The pellet was once more homogenized with cold water (150 mL) / Filtration from cheese-cloth (2 layers) to collect the pellet (the water extract was discarded) / The pellet was mixed with 6 or 10 g NaCl and the final weight of mixture was made up to 100 g with distilled water / The mixture was mixed for 30 or 45 min for the extraction of enzyme / Filtration from cheese-cloth (4 layers) to collect the enzyme extract (the pellet was discarded) / Centrifugation of enzyme extract (at 3500 g for 15 min at 4 °C).

**Extraction procedure # 4:** Homogenization of orange peels (30 g) with cold distilled water (150 mL) / Filtration from cheese-cloth (2 layers) to collect the pellet (the water extract was discarded) / The pellet was once more homogenized with cold distilled water (150 mL) / Filtration from cheese-cloth (2 layers) to collect the pellet (the water extract was discarded) / The pellet was mixed with 6 g NaCl and the final weight of mixture was made up to 100 g with distilled water / 2 g PVPP was added to medium and the mixture was mixed for 45 min for the extraction of enzyme / Filtration from cheese-cloth (4 layers) to collect the enzyme extract (the pellet was discarded) / Centrifugation of enzyme extract (at 3500 g for 15 min at 4 °C)

**Extraction procedure # 5:** Homogenization of orange peels (30 g) with cold acetone at -18 °C (200 mL) / Filtration from a Buncher funnel, containing Whatman No:1, under vacuum to collect the pellet (the acetone extract was discarded) / The pellet was once more homogenized with cold distilled water (150 mL) / Filtration from cheese-cloth (2 layers) to collect the pellet (the water extract was discarded) / Pellet was mixed with 6 g NaCl and the final weight was made up to 100 g with distilled water / The mixture was mixed for 45 min for the extraction of enzyme / Filtration from cheese-cloth (4 layers) to collect the enzyme extract (the pellet was discarded) / Centrifugation of enzyme extract (at 3500 g for 15 min at 4 °C).

### **5.2.1.2. Determination of PME activity**

For the determination of PME enzyme activity titrimetric and spectrophotometric methods were used. In the titrimetric assays, the method given in Yemenicioğlu (2002) was used with slight modifications. The reaction mixture contained 1 or 3 mL of enzyme extract and 20 mL of 0.5 % pectin solution prepared in 0.1 M NaCl. The pH of the reaction mixture was brought to 7.5 with 0.1 N NaOH and kept constant for 5 or 10 min by titrating with 0.01 N NaOH. The titrations were performed in a double walled magnetically stirred cell connected to a circulating water bath working at 30 °C. The enzyme activity was expressed as percent initial activity or amount of 0.01 N NaOH spend in titrations per minute per mL of enzyme extract (mL 0.01 N NaOH/min/mL). All activity measurements were performed for three times and averages were calculated.

In spectrophotometric assays the method given in Hagerman and Austin (1986) was used with slight modifications. The reaction mixture was formed by mixing 2.3 mL of 0.3 % pectin solution prepared in 0.1 M NaCl, 0.5 mL of 0.01 % (w/v) bromothymol blue prepared in 0.003 M sodium phosphate buffer at pH 7.5 and 0.1 mL crude enzyme. The decrease in absorbance at 620 nm was monitored by using a Shimadzu (Model 2450) spectrophotometer, equipped with a constant temperature cell holder working at 30 °C. The enzyme activity was determined from the slope of the initial linear portion of abs versus time curve and expressed as Unit. One Unit was defined as that amount of enzyme that caused 0.001 changes in absorbance in 1 min.

### **5.2.1.3. Effect of mild heating on PME activity**

To determine the effects of mild heating on PME, pieces of orange peels (obtained from 16 peel halves from different oranges) were put into sacks made from cheese-cloth and incubated between 30 ° and 55 °C for 30 min in a circulating water bath. At the end of the incubation period the PME was extracted from the peels by the extraction procedure # 3 (by using 10 g NaCl and 30 min mixing for enzyme extraction) and tested for enzyme activity by the titrimetric method.

#### **5.2.1.4. Effect of CaCl<sub>2</sub> on PME activity**

To determine the effect of CaCl<sub>2</sub> on PME activity, the activity of enzyme was determined in the presence of 0.75-50 mM CaCl<sub>2</sub> (The final concentrations in reaction mixture). The enzyme extract used in these experiments was obtained with the extraction procedure # 3 (by using 10 g NaCl and 30 min mixing for enzyme extraction). Enzyme activities were determined by the spectrophotometric method by including 0.1 mL CaCl<sub>2</sub> (at varying concentrations) to the reaction mixture.

#### **5.2.1.5. Preparation of a commercial PME preparation and test of its stability**

In order to obtain a commercial PME enzyme preparation, the PME was extracted by extraction procedure # 3 (by using 10 g NaCl and 30 min mixing for enzyme extraction). To clarify the preparation, the enzyme extract was incubated for 1 week and the precipitate formed was collected by applying centrifugation at 3500 g for 15 min at 4 °C. To prevent the microbiological spoilage, 0.1 % K-sorbate and 0.1 % Na-benzoate were then added to the clear enzyme extract that was at pH 3.8. The storage stability of the enzyme was determined by monitoring enzyme activity at 4 °C with or without the presence of 1 mM CaCl<sub>2</sub>. The activity of enzyme was tested by the titrimetric method.

#### **5.2.1.6. Test of obtained PME in the preparation of edible pectin films**

For the preparation of pectin films, the following steps were followed; (1) 18 mL 2 % pectin solution was demethylized by 1 mL PME preparation containing 1.0 mL 0.01 N NaOH/min/mL enzyme activity. The demethylation was conducted at room temperature until 2.1 mL, 1 M NaOH was spent in the titration; (2) 10 g demethylized PME solution was pipetted onto a 9.8 cm diameter glass petri dish and left 1 day at room temperature to dry; (3) The cross-linking of film was conducted by adding 5 mL of 1 M CaCl<sub>2</sub> onto the dried pectin film and by applying further drying for 5-6 h at room temperature. At the end of the drying period, the film was peeled from the petri dish and wetted to check whether insoluble Ca-pectate structure is formed by the action of PME.

## **5.2.2. Methods related to PPO enzyme**

### **5.2.2.1. Acetone powder preparation**

To obtain the acetone powder, 100 g mushroom stems were homogenized in a Waring blender for 3 min with 200 mL cold acetone at -18 °C and 2 g insoluble PVPP. The slurry obtained was filtered under vacuum from a Buncher funnel containing a Whatman No:1 filter paper and the solid residue remained on the filter paper was collected. The homogenization with 200 mL cold acetone and filtration were then repeated for two more times for the collected residue without using PVPP and the light brown colored powder, left overnight to evaporate the acetone, was stored at – 18 °C until used for enzyme extraction.

### **5.2.2.2. PPO extraction**

The enzyme extraction from acetone powder was conducted by mixing 3 g acetone powder, 3 g insoluble PVPP, 50 mL 0.05 M, pH 7 Na-phosphate buffer and stirring for 30 min at + 4 °C with a magnetic stirrer. The extract was then filtered from a four layers of cheese-cloth and clarified by centrifuging 15 min at 10000 x g and + 4 °C.

### **5.2.2.3. Ammonium sulfate precipitation**

For ammonium sulphate precipitation, solid  $(\text{NH}_4)_2\text{SO}_4$  was slowly added to crude enzyme extracts up to 90 % saturation. The mixture was stirred slowly at + 4 °C for 2 h and the precipitate was collected by applying 45 min centrifugation at 15000 x g and + 4 °C. The precipitate collected was then dissolved in 20 mL of 0.05 M Na-phosphate buffer at pH 7 and dialyzed 24 h at + 4 °C against 2000 mL distilled water by three changes.

### **5.2.2.4. Acetone precipitation**

When PPO was partially purified with acetone, two volume of cold acetone at -18 °C was added to one volume of crude enzyme extract. After 10 min stirring at + 4 °C, the precipitate formed was collected by 45 min centrifugation at 15000 x g and 0

°C, and dissolved in 15 mL of 0.05 M Na-phosphate buffer at pH 7. The enzyme was then dialyzed for 24 h at + 4 °C against 2000 mL distilled water by three changes.

#### **5.2.2.5. Determination of PPO activity**

The PPO activities were determined at + 30 °C by using a Shimadzu (Model 2450) spectrophotometer equipped with a constant temperature cell holder. The monophenolase activity of PPO was determined by forming the following reaction mixture; 0.5 mL enzyme extract, 1.5 mL, 0.05 M Na-phosphate buffer at pH 7 and 1 mL, 0.25 or 2 mM tyrosine. The increase in absorbance was recorded at 280 nm and enzyme activity was determined from the initial linear portion of absorbance vs. time curve coming after the initial lag period. The catechol oxidase activity of PPO, on the other hand, was determined by mixing 0.1 mL enzyme extract, 0.1 mL 10 mM L-DOPA and 2.8 mL, 0.05 M Na-phosphate buffer at pH 7. The increase in absorbance was determined at 475 nm, and enzyme activity was calculated from the slope of the initial linear portions of absorbance vs. time curve. The activities of enzymes were expressed as percent initial activity or Unit, which gives the amount of enzyme that causes 0.001 absorbance change in one minute.

#### **5.2.2.6. Characterization studies**

The temperature profiles of PPO were determined by 30 min incubation of enzyme extracts in TIT (Thermal Inactivation Time) tubes (i.d., 9 mm; wall thickness, 1 mm) between 35 ° and 60 °C.

The optimum temperature was determined by measuring enzyme activities between 25 and 40 °C.

The optimum pH was determined by changing the standard reaction mixture to 0.2 mL enzyme extract, 2 mL, 0.1 M acetate (at pH 4 and 5), Na-phosphate (at pH 6.0, 6.6 and 7.0) or Tris-HCl (at pH 8.0) buffers and 0.8 mL, 2 mM tyrosine.

The pH stabilities were determined by mixing 0.2 (or 0.5) mL enzyme extract and 0.4 (or 1) mL of 0.1 M buffer (suitable buffer as given in optimum pH

determination) at pH 4.0, 5.0, 6.0, 6.6, 7.0 or 8.0. The enzyme-buffer mixtures formed were incubated at 4 °C for 24 h and their remaining activities were determined at pH 7.0 by using the following reaction mixture; 0.5 mL enzyme-buffer mixture, 1.5 mL, 0.5 M Na-phosphate buffer at pH 7.0 and 1 mL, 2 mM tyrosine prepared in 0.5 M Na-phosphate buffer at pH 7.0.

#### **5.2.2.7. Storage stability of PPO in acetone powders**

In order to investigate the storage stability of PPO in acetone powders, 0.5 g acetone powder was distributed to different test tubes. Some of these tubes were incubated in a refrigerator at almost + 4 °C, whereas the others were incubated in a deep-freezer working at -18 °C. The monophenolase activity of samples was determined by using 0.25 mM tyrosine in the reaction mixture.

#### **5.2.2.8. Preparation of commercial PPO preparations and test of their storage stabilities**

To obtain commercial PPO preparations ammonium sulphate precipitated and partially purified enzymes were lyophilized by using 1 g dextran or saccharose as supporting material. In lyophilization, a Labconco (FreeZone®, 6 liter) freeze dryer working at almost - 47 °C collector temperature and 50-100 x 10<sup>-3</sup> mBar vacuum was used. After lyophilization the samples were stored in a deep-freezer at -18 °C and their monophenolase and diphenolase activities were determined at different time intervals.

#### **5.2.2.9. The effect of lyophilization with dextran on temperature and pH stability of PPO**

The effects of lyophilization on temperature stability of PPO lyophilized with dextran were determined by 30 min incubation of the enzyme solution (prepared by dissolving 100 mg lyophilized enzyme in 5 mL 0.05 M Na-phosphate buffer at pH 7) at 50° or 60 °C.

The effect of lyophilization with dextran on pH stability was determined by treating the enzyme solution as described in section 5.2.2.6. But the tests were conducted only at pH 4.0 and 6.0 and the incubation period was extended to 48 h. In all

stability tests, unlyophilized enzyme extracts containing 0 % and 1 % dextran were used as control.

### 5.2.3. Determination of protein content

The protein content of PME and PPO extracts was determined by the method of Lowry by using bovine serum albumin as a standard (Figure 5.1). The average of five values was used to calculate the protein content.

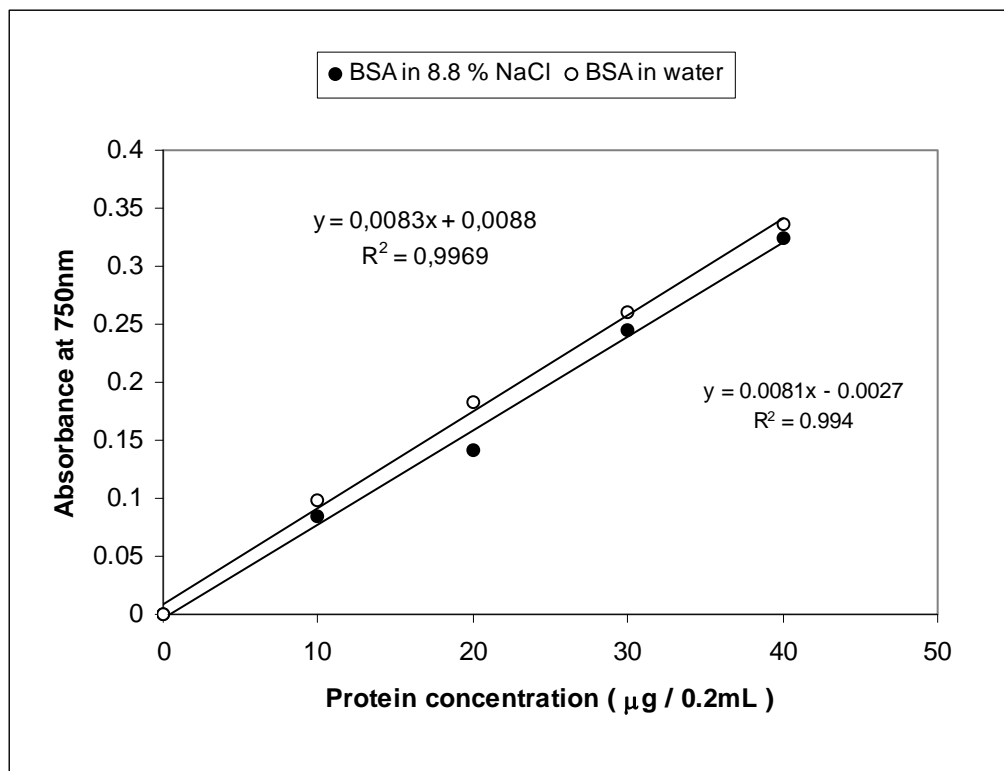


Figure 5.1. Standart curves for protein determination



## CHAPTER 6

### RESULTS AND DISCUSSION

#### 6.1. The Results Obtained for PME Enzyme

##### 6.1.1. Change of PME activity in orange peels during season

To determine whether orange peels are suitable for PME extraction or not, the activity of enzyme was monitored during season. As seen in Figure 6.1, at the beginning of the season a significant PME activity exists in orange peels. A slight reduction occurred in PME activity at the third month of storage. However, during 5 months period the enzyme maintained most of its activity. These results clearly showed the suitability of using orange peels as source of PME enzyme.

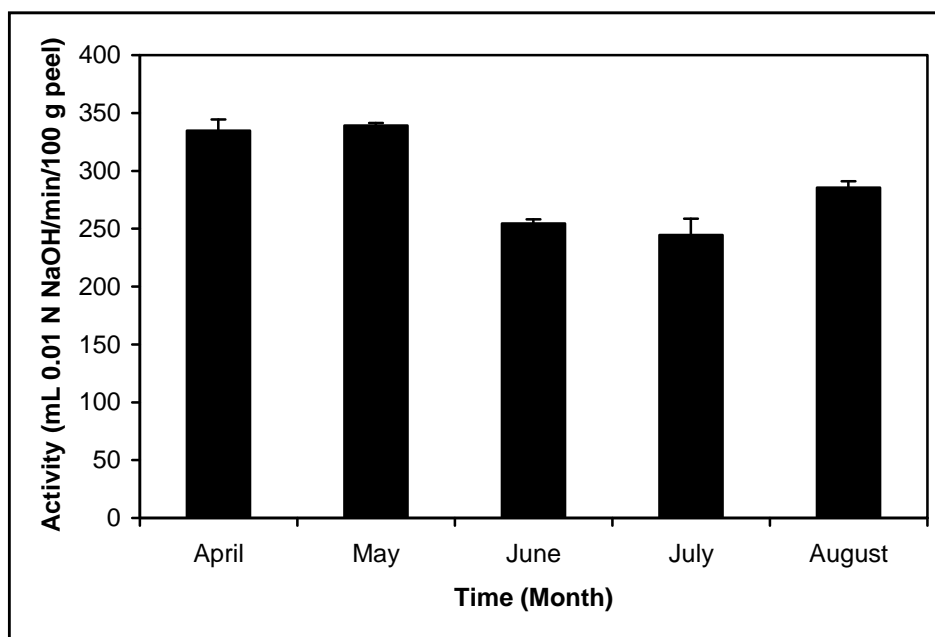


Figure 6.1. Change of PME activity in orange peels during season (The enzyme was extracted with 1 M NaCl from the pellet obtained by homogenization of orange peels with water)

### 6.1.2. Effect of different extraction procedures on PME activity

After determining the suitable source for the extraction of PME, the effectiveness of different extraction procedures were tested. The very low PME activity in extract obtained from the filtration and centrifugation of peel homogenate prepared with cold distilled water (Extraction procedure # 1) clearly showed that the PME in orange peels is ionically or covalently bound to cell walls (Table 6.1). This result is in line with Wicker et al (2002) who reported that the PME is a cell wall bound enzyme that may only be extracted at high salt concentrations. Thus, to extract the ionically bound enzyme, homogenization of peels was also conducted with 1 M NaCl solution (Extraction procedure # 2). However, this caused the formation of a very thick gel in the extraction medium.

Table 6.1. The PME activities obtained by applying different extraction procedures

Extraction procedure	Activity (mL 0.01 N NaOH/min/mL)	Total activity <sup>a</sup> (mL 0.01 N NaOH/min)
<b>Extraction procedure # 1</b> (filtration and centrifugation of peel homogenate prepared by cold distilled water)	0.05	6.5
<b>Extraction procedure # 2</b> (filtration and centrifugation of peel homogenate prepared by 1 M NaCl solution)	Gel formation	Gel formation
<b>Extraction procedure # 3</b> (filtration and centrifugation of NaCl containing extract of pellet obtained after 2 times homogenization of orange peels with cold distilled water)	1.39	92.1
<b>Extraction procedure # 4</b> (filtration and centrifugation of NaCl and PVPP containing extract of pellet obtained after 2 times homogenization of orange peels with cold distilled water)	1.45	94.3
<b>Extraction procedure # 5</b> (filtration and centrifugation of NaCl containing extract of pellet obtained first by homogenization of orange peels with cold acetone and then homogenization with cold distilled water)	1.40	98.3

<sup>a</sup> 30 g peels were used in all extractions

It appears that the homogenization in presence of NaCl extracted both pectic compounds and PME. With such a contact, the demethylation of pectin and gelling occurred very rapidly. Thus, to remove the soluble pectic substances, the peels were homogenized 2 times with cold water before extraction with NaCl solution (Extraction procedure # 3). This treatment eliminated the gel formation completely and enabled the extraction of PME from the washed pellet effectively.

To minimize the potential tanning effect of phenolic compounds on PME and to prevent the loss of enzyme activity by this undesirable reaction, the addition of PVPP during extraction of pellet was also tested (Extraction procedure # 4). However, this did not affect the PME activity extracted from the peels considerably. The homogenization of peels with acetone, to remove phenolic compounds more effectively, was also conducted before extraction of pellet. However, this treatment did not also yield a considerably higher PME activity. Thus, it seems that the tanning effect does not cause any activity loss during PME extraction.

### **6.1.3. Effect of NaCl concentration on PME activity extracted from orange peels**

To determine the optimum extraction conditions for the PME, by using Extraction procedure # 3, the extraction of pellet was conducted at different NaCl concentrations. As seen in Figure 6.2 when 2-10 g NaCl presents in 100 g extraction mixture, the activity of PME extracted increased by increasing the NaCl concentration. The activity extracted was maximal when 10 g NaCl presents in 100 g extraction mixture. However, it declined when NaCl concentration was further increased to 12 g / 100 g. Between 2-4 g / 100 g NaCl concentration the increase of NaCl concentration increased the specific activity of enzyme considerably. At 6 and 8 g / 100 g NaCl concentrations, on the other hand, the specific activity of enzyme reduced slightly and significantly, respectively. This was apparently related with the increased extraction of proteins (other than the enzyme) at this NaCl concentration. Further increase of NaCl concentration to 10 g / 100 g once more reduced the solubility of the proteins other than the enzyme and this again increased the specific activity of the PME.

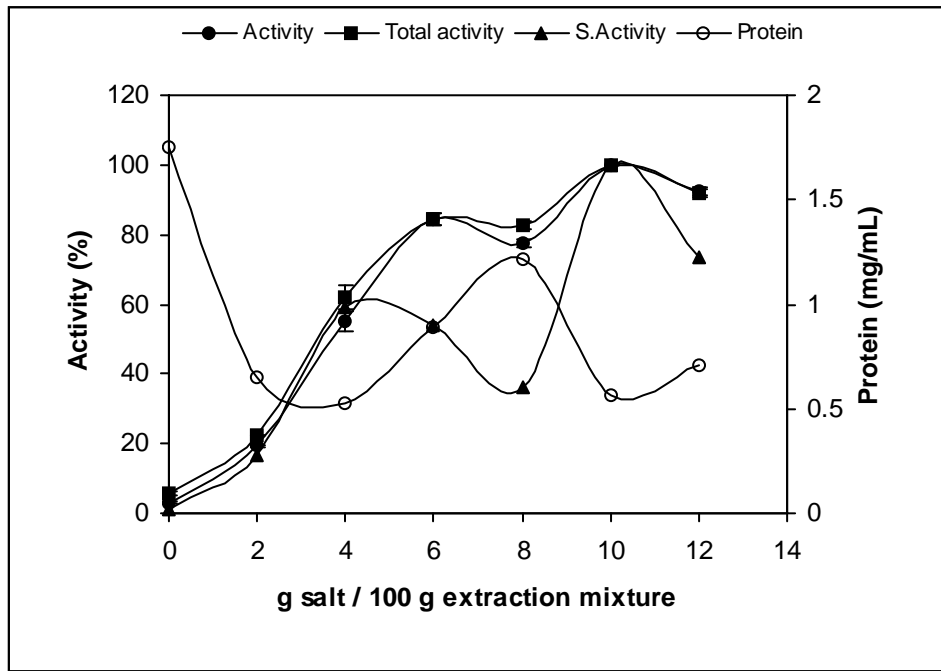


Figure 6.2. Effect of NaCl concentration on PME activity extracted from peels

#### 6.1.4. Effect of extraction period on PME activity extracted from orange peels

For extraction procedure # 3, the effect of extraction period of pellet at constant stirring rate (at 200 rpm) on PME activity was also investigated (Figure 6.3).

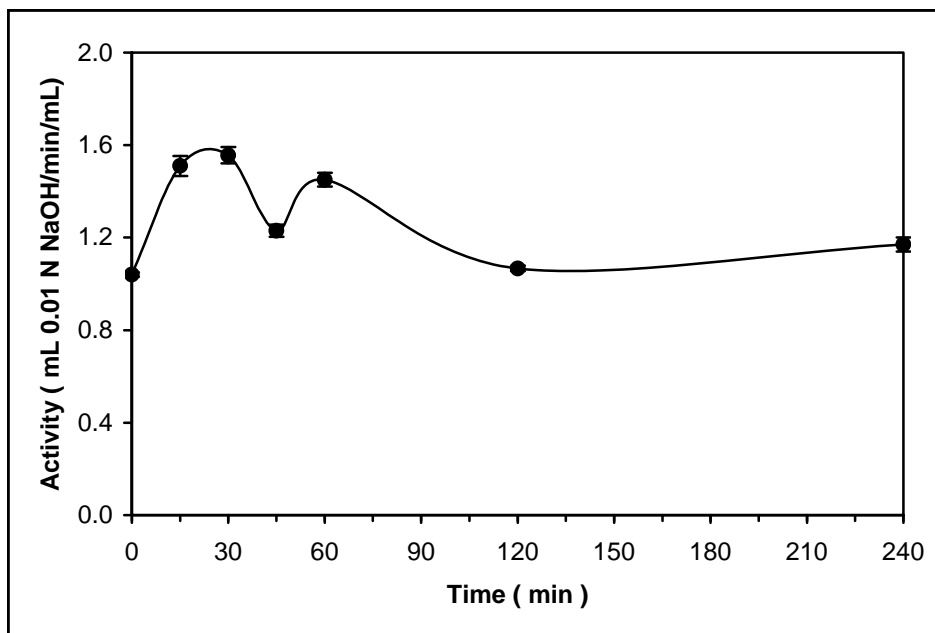


Figure 6.3. Effect of extraction period on PME activity extracted from peels

The high initial PME activity at 0 time indicated that most of the enzyme activity released from the cell walls without stirring. Stirring for 15 or 30 min increased the activity of PME in extracts almost 50 %. However, further stirring reduced the activity of enzyme to initial value gradually. The reduction of enzyme activity may be related with the solubilization of some enzyme inhibitors from the pellet. Thus, to obtain the maximum recovery, it is essential to apply the proper extraction period.

### 6.1.5. Effect of mild heating on PME activity

In literature, it was reported that the application of mild heating might cause the activation of PME enzyme (Yemenicioğlu, 2002, Degreave et al. 2003). It was thought that the activation is resulted from the contact of enzyme with intracellular electrolytes after the loss of cellular membrane integrity. Thus, for the activation of PME enzyme the orange peels were heated over 30-55 °C for 30 min before they were used in the extraction. The results given in Figure 6.4 indicate that the orange peel PME does not activated under the studied heating conditions. However, the peak point observed at 50 °C may be an indication of activation that may occur more intensively at shorter or longer heating times. Thus, the heating study was once more repeated specifically at this temperature.

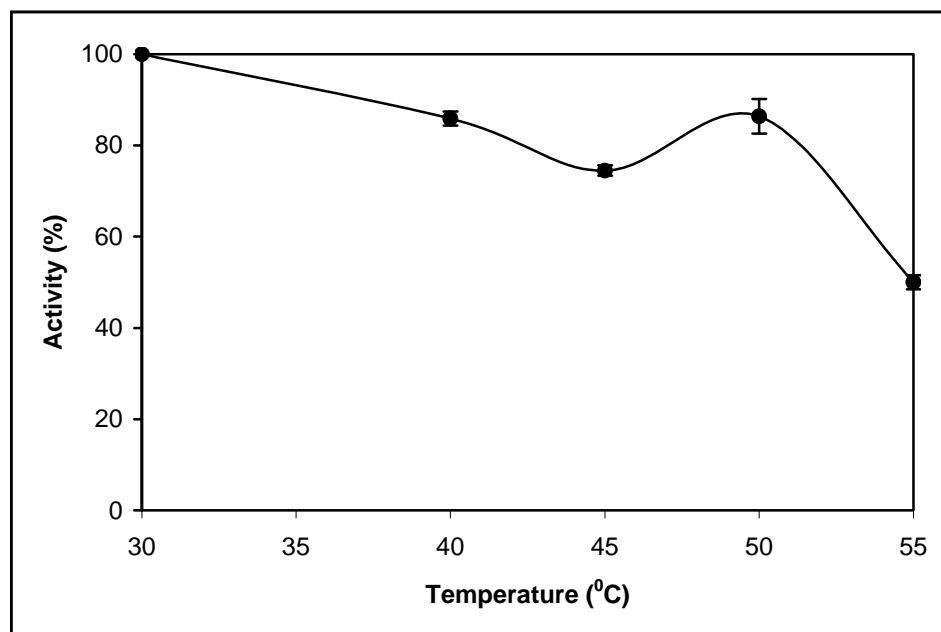


Figure 6.4. Effect of heating for 30 min at different temperatures on PME activity

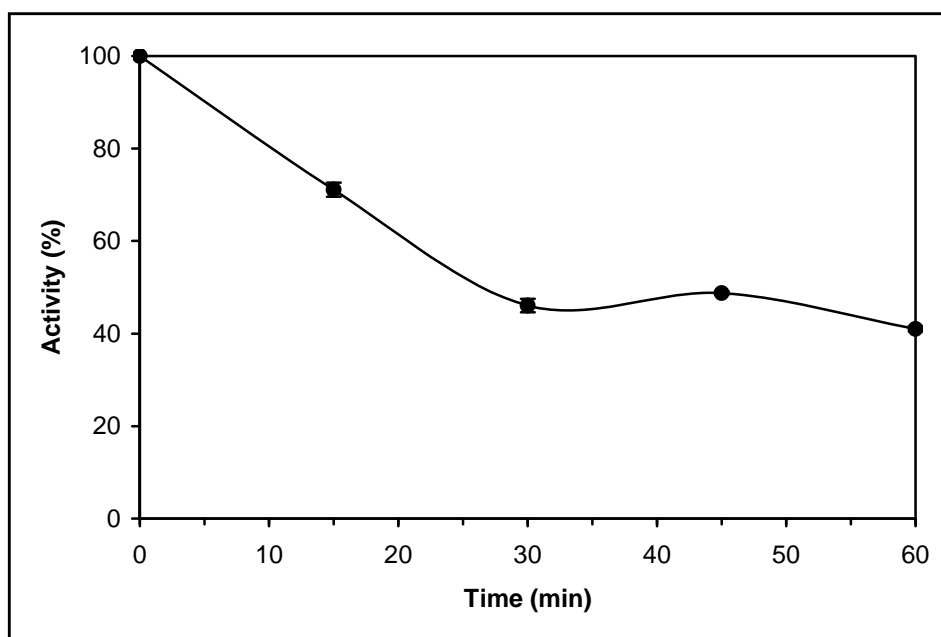


Figure 6.5. Effect of heating at 50 °C on PME activity

However, as seen in Figure 6.5, 15-60 min heating at this temperature did not cause the activation of enzyme. In fact, because of the heat labile portion of PME, the heating study at 50 °C yielded a heat inactivation curve. The biphasic inactivation curve enabled us to calculate the percentages of heat stable and heat labile fractions of the enzyme as described by Yamamoto et al (1962) by extending the heat stable portion of heat inactivation curve and finding its intercept on the y-axis. The percentage of the heat labile and heat stable enzyme fractions of PME, calculated by this method, were almost same (50 % for each fraction).

#### 6.1.6. Effect of CaCl<sub>2</sub> on PME activity

It is well known that the cations such as Ca<sup>++</sup> ions may also activate the PME enzyme. It was reported that the activation of PME by cations is related with the competitive displacement of PME bound to blocks of carboxylic groups on pectin. As a result of this competition, PME becomes free for further catalyses of pectin (Alonso et al. 1996, Leiting and Wicker 1997, Sun and Wicker 1999).

The results of our activity measurements conducted between 0.75 and 50 mM CaCl<sub>2</sub> concentrations were given at Figure 6.6. As seen in this figure, a slight activation of PME (almost 20 %) occurred only at 1 mM CaCl<sub>2</sub> concentration.

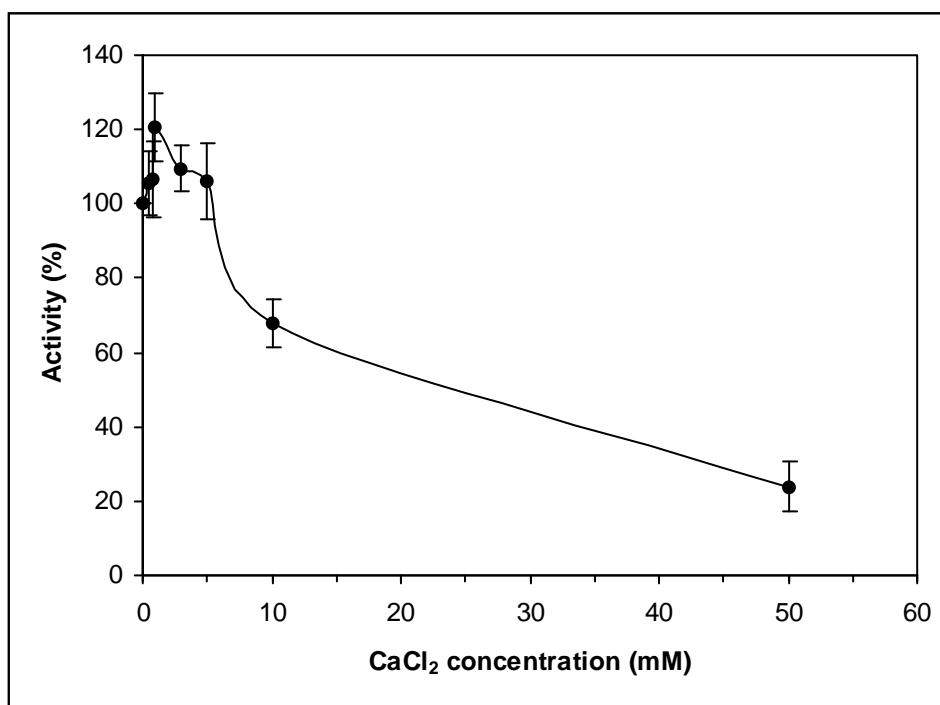


Figure 6.6. Effect of different CaCl<sub>2</sub> concentrations on PME activity

At other concentrations between 0 and 5 mM the activation was inconsiderable. At  $\geq 10$  mM concentration, on the other hand, the effect of CaCl<sub>2</sub> was inhibitory. Nari et al (1991) attributed the inhibitory effect of high cation concentrations to the competition between PME and cations for free carboxylic acid groups on pectin. Thus, during storage or commercial applications of orange peel PME, the concentrations of Ca<sup>++</sup> should be chosen carefully.

#### 6.1.7. Stability of the prepared PME during storage

In industry most of the fungal PME preparations are supplied in liquid form. Thus, to determine whether plant PME preparations may be stored in liquid form, the stability of PME activity was investigated during cold storage (+ 4 °C) of enzyme extract containing 0.1 % K-sorbate and 0.1 % Na-benzoate as preservatives. The enzyme extract, cold stored at +4 °C, showed good stability for 5 months with retention of 90-95 % of its activity (Figure 6.7). Thus, it appears that the plant PME preparations may also be supplied in liquid form.

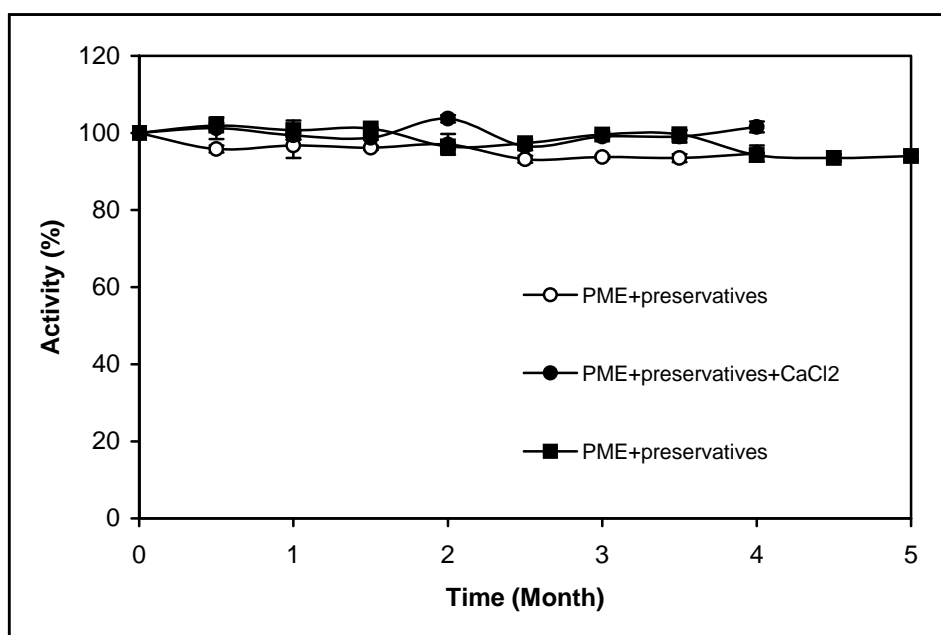


Figure 6.7. Stability of the prepared PME during storage at + 4 °C in liquid form

In this study, no significant activatory effect of  $\text{Ca}^{++}$  ions was observed on PME enzyme. However, to determine the effect of  $\text{Ca}^{++}$  ions at long periods of time, the stability test was also conducted in the presence of  $\text{Ca}^{++}$  ions. However, in the presence of preservatives, even the slight activatory effect of 1 mM  $\text{CaCl}_2$  was not observed. Also, the presence of  $\text{Ca}^{++}$  ions did not affect the activity of prepared PME enzyme during storage.

#### 6.1.8. Test of obtained PME in the preparation of edible pectin films

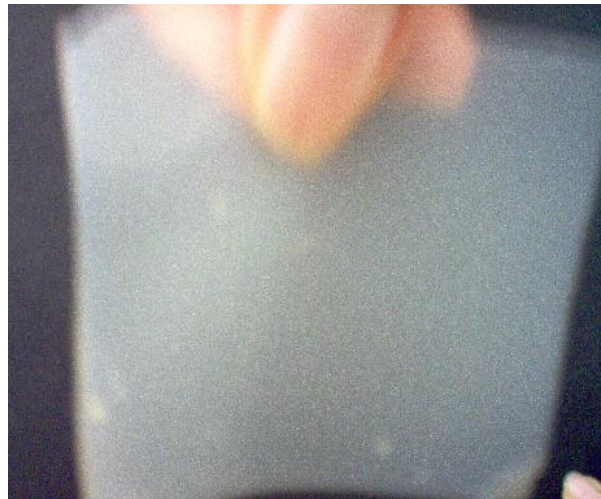
In this study, the suitability of the obtained PME for the preparation of edible pectin films was also tested. The effect of PME on film formation was determined by casting and drying the PME treated and untreated pectin solutions into petri dishes. Before cross-linking with  $\text{CaCl}_2$ , the PME treated dried pectin is cloudy whereas untreated dried pectin is transparent. Following the cross-linking the PME treated pectin forms cloudy, films that may easily be peeled from the petri dishes when wetted (Figure 6.8).

The untreated pectin, on the other hand, did not form films after cross-linking and solubilized when wetted with water. This result indicates the suitability of using PME from orange peels in the pectin film coating of foods. However, further studies are



needed to optimize the film forming conditions and to characterize the obtained film properties.

**A**



**B**



Figure 6.8. The effect of prepared orange peel PME on film forming ability of pectin  
A: The film of PME treated pectin; B: Control (PME untreated pectin)

## 6.2. The Results Obtained for PPO Enzyme

### 6.2.1. Monophenolase and diphenolase activities of PPO

In this study, the monophenolase and diphenolase activities of mushroom PPO were determined by using tyrosine and L-DOPA as substrates, respectively. The progress curves for monophenolase activity contain a very long lag phase that is typical for this kind of activity (Vamos-Vigyazo, 1981) (Figure 6.9). Thus, the slopes of the linear portions of progress curves coming after the lag phases were used for the determination of enzyme activity. On the other hand, because diphenolase activity of PPO does not contain any lag phases, the slopes of the initial linear portions of progress curves were used for the calculation of enzyme activity (Figure 6.10).

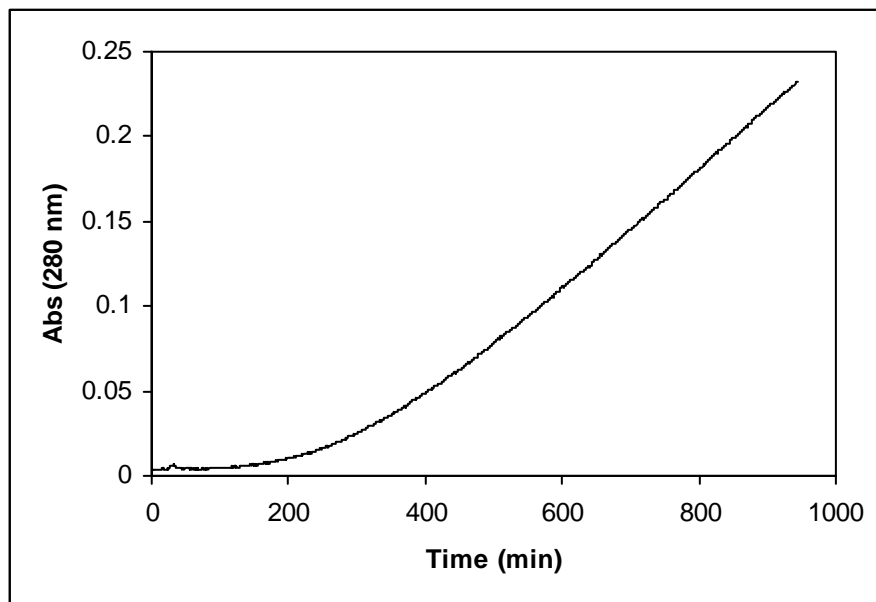


Figure 6.9. Monophenolase activity of PPO from mushroom stems

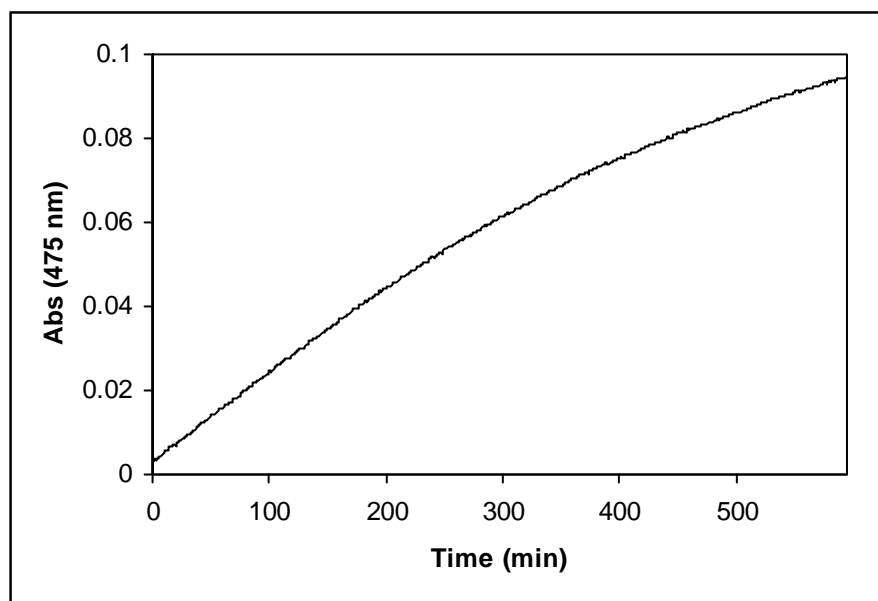


Figure 6.10. Diphenolase activity of PPO from mushroom stems

In plants, the monophenolase activity of PPO is significantly lower than its diphenolase activity. Also, it was reported that the monophenolase activity of PPO is less stable and may be lost significantly during the purification of the enzyme (Vamos-Vigyazo, 1981). The monophenolase activity of PPO is essential for many of its food applications, including the production of antioxidants, modification of proteins, inhibition of microbial attachment, etc. Thus, in this study the purification, characterization and stability tests were based primarily on the monophenolase activity of PPO enzyme.

### 6.2.2. Distribution of PPO in mushrooms

To determine the distribution of PPO in mushrooms, monophenolase and diphenolase activities of enzyme were determined in acetone powder (AP) extracts obtained separately from mushroom caps and stems (Table 6.2). The results of this study showed that the mushroom caps contain almost 3-4 and 2-3 fold higher monophenolase and diphenolase activity than mushroom stems, respectively. However, because of the higher protein content in AP extracts of mushroom caps, the specific activities of enzymes in different sections were not that much different. The activity measurements conducted in acetone powders showed the considerable differences in the PPO activities of different parties of mushroom stems. However, the diphenolase/monophenolase activity ratios of these mushrooms were almost same and

indicated that the monophenolase activity occupies a greater portion of PPO activity in mushroom caps. These results are in line with those of Devecce et al (1999) who compared the PPO activities in stem and caps of mushrooms and found 1.4 fold higher PPO activity in mushroom caps (cap flesh + cap skin). Moore and Flurkey (1989) also found the total monophenolase activity in mushroom cap skin and cap flesh 3 fold higher than that in mushroom stems.

Table 6.2. Distribution of PPO monophenolase and diphenolase activities in mushrooms

Parameter	Monophenolase		Diphenolase		Ratio <sup>a</sup>	
	Activity		Activity			
	Stem	Cap	Stem	Cap	Stem	Cap
<b>Acetone powder-1</b>						
Activity (U/mL)	45	143	255	475	5.7	3.3
S. activity (U/mg)	95	129	538	429		
<b>Acetone powder-2</b>						
Activity (U/mL)	97	409	514	1679	5.3	4.1
S. activity (U/mg)	141	329	750	1350		

<sup>a</sup>The ratio of diphenolase activity to monophenolase activity

### 6.2.3. Partial purification of PPO

In this study, the partial purification of PPO was conducted mainly by 90 % ammonium sulphate precipitation and a following dialysis. As seen in Table 6.3, for ammonium sulphate precipitation, the recovery and purification fold of monophenolase activity in AP-3 varied between 38-44 % and 1.2-2.6, respectively. These recoveries and purities were almost two fold lower than those obtained for the partial purification of monophenolase activity from AP-4 and AP-5 by using the same procedure (74-86 % and 3.4-4.3, respectively). This difference in the indicated parameters may be related with the higher initial activity of monophenolase in crude extracts of AP-3. The activity of PPO in mushrooms may be affected by the presence of its in situ activators such as benzyl alcohol and ethylene. In mushrooms, the PPO activators may also be some proteases such as serine proteases (Espin et al. 1999).

Table 6.3. Results of partial purification with ammonium sulfate and acetone precipitation of PPO monophenolase activity from mushroom stems

Type of extract	Volume (mL)	Activity (U/mL)	Protein (mg/mL)	Specific activity (U/mg)	Recovery (%)	Purity (fold)	Ratio <sup>a</sup>
<b>Acetone powder-3</b>							
Crude extract-3.1	28	134	0.62	216	100	1	-
Crude extract-3.2	26.5	98 (665) <sup>b</sup>	0.45	218 (1500)	100	1	6.8
<b>0-90% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitation and 24 h dialysis of crude extract-3.1</b>							
	16	90	0.16	563	38	2.6	-
<b>0-90% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitation and 24 h dialysis of crude extract-3.2</b>							
	18.5	62 (537)	0.24	258 (2238)	44 (56)	1.2 (1.5)	8.7
<b>Acetone powder-4</b>							
Crude extract-4.1	21	51 (310)	0.53	96 (585)	100	1	6.1
Crude extract-4.2	27	51 (292)	0.44	116 (664)	100	1	5.7
Crude extract-4.3	33	40 (198)	0.47	85 (421)	100	1	5.0
Crude extract-4.4	34	42 (232)	0.46	91 (504)	100	1	5.5
<b>0-90% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitation and 24 h dialysis of crude extract-4.1</b>							
	16	49 (255)	0.12	408 (2125)	74 (63)	4.3 (3.6)	5.2
<b>0-90% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitation and 24 h dialysis of crude extract-4.2</b>							
	21.5	55 (260)	0.14	393 (1857)	86 (71)	3.4 (2.8)	4.7
<b>Acetone precipitation (2-fold) and 24 h dialysis of crude extract-4.3</b>							
	14	63 (170)	0.12	525 (1417)	67 (36)	6.2 (3.4)	2.7
<b>Acetone precipitation (2-fold) and 24 h dialysis of crude extract-4.4</b>							
	18	44 (138)	0.09	489 (1533)	55 (32)	5.4 (3.0)	3.1

cont. on next page

**Table 6.3. (cont.)**

<b>Acetone powder-5</b>							
<b>Crude extract-5.1</b>	67	82	0.56	146	100	1	4.8
		(395)		(705)			
<b>0-90% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitation and 24 h dialysis of crude extract-5.1</b>							
	50	89	0.17	524	81	3.6	4.4
		(387)		(2276)	(73)	(3.2)	

<sup>a</sup>The ratio of diphenolase activity to monophenolase activity; <sup>b</sup>The values given in parenthesis were for diphenolase activities

Thus, it is likely that the PPO in AP-3 is an activated enzyme form that showed less stability during purification. Also, it is possible that the activated enzyme transformed to its native state with the removal of potential enzyme activators by the partial purification. After ammonium sulfate precipitation and dialysis, the diphenolase/monophenolase activity ratio of PPO in AP-3 reached the highest ratio obtained in this study. This result clearly indicated the different nature of PPO in this acetone powder.

The effects of 2 fold acetone precipitation and 90 % ammonium sulfate precipitation on purification parameters were compared in AP-4 extracts. The results showed that the recovery of monophenolase and diphenolase activities was considerably lower in acetone precipitation. Particularly, the greater loss of diphenolase activity by acetone precipitation reduced the diphenolase/monophenolase ratios of PPO almost 1.5-1.7 fold, compared to those ratios for the PPO purified by ammonium sulfate precipitation. Acetone precipitation did not affect purification fold of diphenolase activity but purification fold of monophenolase activity increased almost 1.3-1.8 times.

#### **6.2.4. Characterization of monophenolase activity**

To determine the possible variations in the characteristics of PPO from mushroom stems, thermal stability, pH optimum and stability, and optimum temperature of monophenolase activity were determined for enzymes obtained from different APs with different partial purification procedures.

As seen in Figure 6.11, monophenolase activity of PPO is not very heat stable. In 35-45 °C range, the enzyme did not show a considerable inactivation after 30 min heating. The inactivation of enzyme starts at > 45 °C and accelerates by further increase

of heating temperature. However, the enzyme maintains  $\geq 20\%$  of its activity after 30 min at  $60\text{ }^{\circ}\text{C}$ .

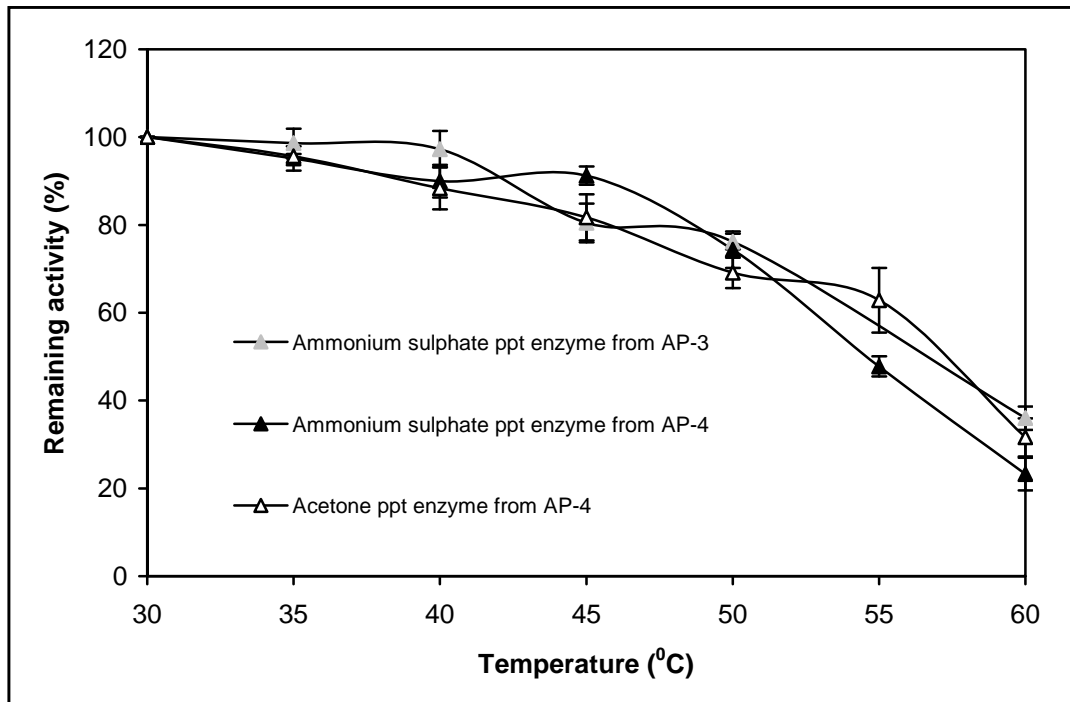


Figure 6.11. Temperature profiles of monophenolase activity of PPO partially purified with different procedures from different acetone powders

In literature, there are very limited reports related to the heat stability of monophenolase activity in mushrooms. Ikehata and Nicell (2000), investigating the potential uses of PPO in industry, reported that the commercial PPO from mushrooms lost 90 % of its monophenolase activity at  $50\text{ }^{\circ}\text{C}$  for 38 min (at pH 7.0). Weemaes et al (1997) also reported the times needed for the 90 % inactivation of diphenolase activity of mushrooms at  $60\text{ }^{\circ}\text{C}$  as 2.2-4.7 min (at pH 6.5). Thus, it is clear that the monophenolase activity obtained from mushroom stems is much more heat stable than these enzymes. On the other hand, the thermal stability of enzyme is not different in APs obtained from different parties of mushroom stems. Partial purification with acetone precipitation or ammonium sulfate precipitation did not also affect the thermal stability of the enzyme.

Monophenolase activity of PPO from mushroom stems has optimum pH in the range of 6-8, but the enzyme did not show its activity below pH 6.0 (Figure 6.12). Also, as observed for the temperature stabilities, the pH profiles of enzyme did not change

considerably by the variation of AP and purification procedure. The pH profiles obtained in this study are quite similar with that reported by Ikehata and Nicell (2000) for the monophenolase activity of commercial mushroom PPO. The only major difference is that at pH 5.0, commercial mushroom PPO showed almost 35 % of its monophenolase activity observed at optimum pH.

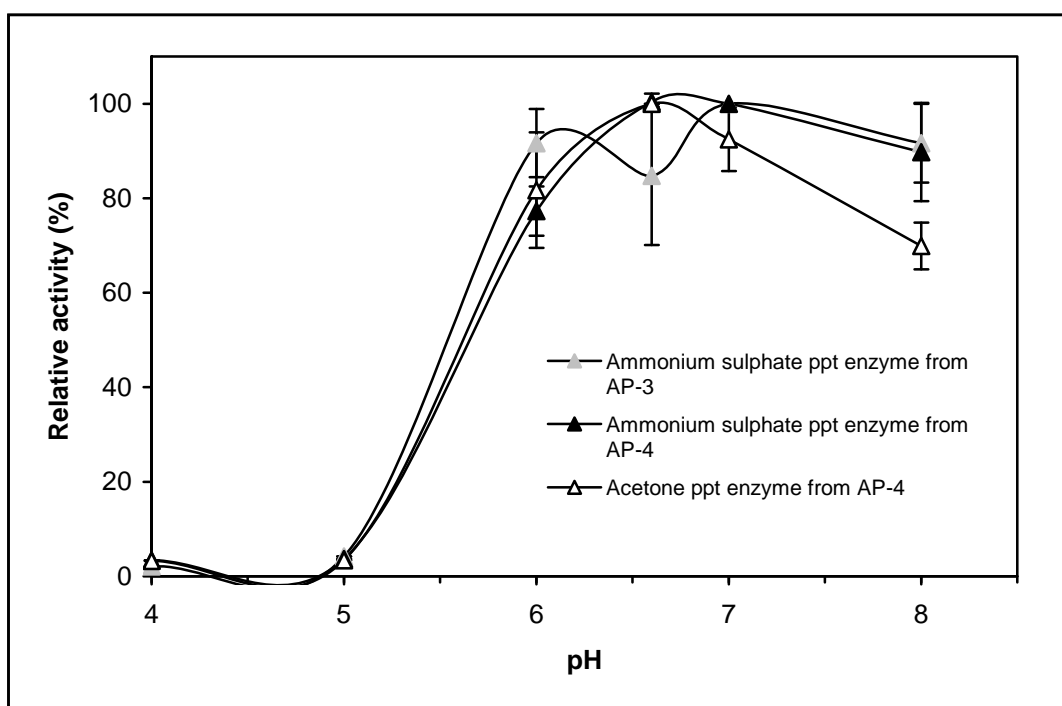


Figure 6.12. The effect of pH on monophenolase activity of PPO partially purified with different procedures from different acetone powders

The enzyme showed its maximum pH stability at pH 7.0 and 8.0 (Figure 6.13). At these pH values, there is no considerable activity loss for 24 h incubation period. In 5.0-6.6 pH range, the enzymes extracted from AP-4 showed moderate stability and maintained  $\geq 60$  % of their initial monophenolase activity. However, in the same pH range, the enzyme extracted from AP-3 lost most of its activity at pH 6.0. This finding supports our hypothesis that the tyrosinase in this AP may be different from the others and helps explaining the low recovery of monophenolase activity from this AP during partial purification. In this study, after ammonium sulfate precipitation, the enzyme was dissolved in pH 7.0 buffer and then dialyzed against distilled water. Following dialysis, the pH of enzyme dropped to the pH range of 6.5-7.0. Thus, it seems that the loss of enzyme activity occurred during 24 h dialysis of the enzyme extract. On the other hand,



all the enzyme extracts obtained in this study, lost most of their monophenolase activity in 24 h at pH 4.0. Our results of pH stability test are quite different from those of monophenolase activity of commercial mushroom PPO studied by Ikehata and Nicell (2000). These researchers reported 100 % and 65 % loss of monophenolase activity in 8 h and 16 h at pH 5.0 and pH 8.0, respectively. Thus, it is clear that the monophenolase activity of tyrosinase used in this study have greater pH stability.

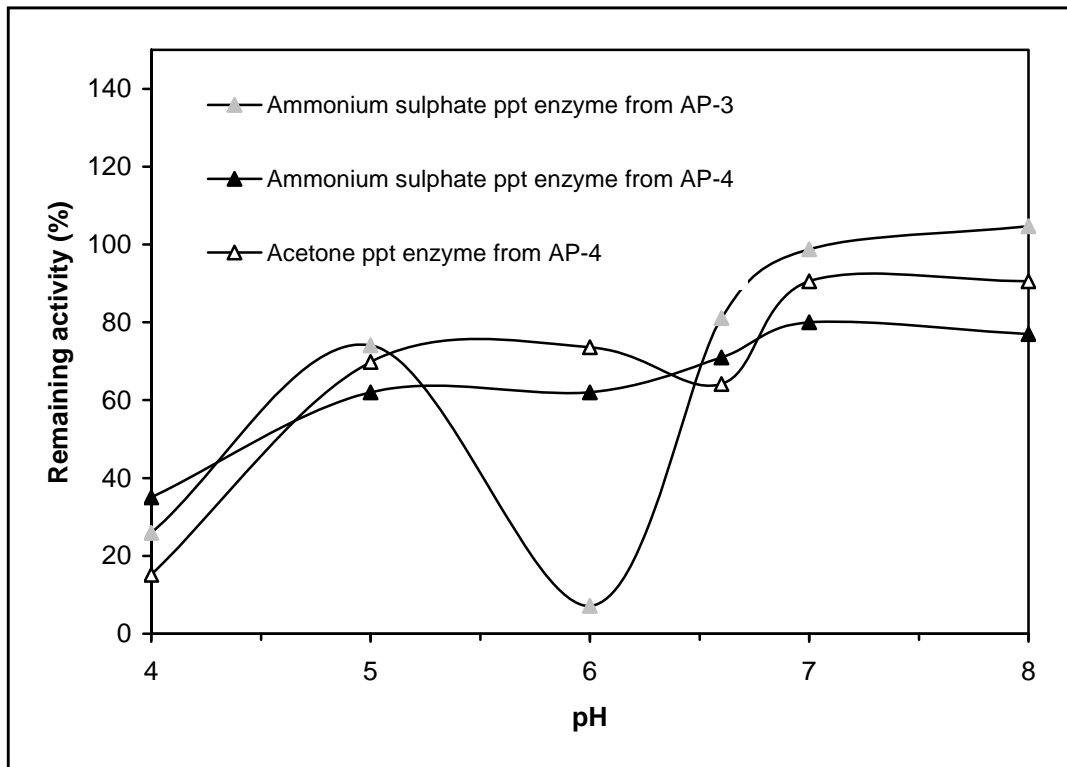


Figure 6.13. pH stabilities of monophenolase activity of PPO partially purified with different procedures from different acetone powders (for 24 h incubation at different pHs)

To determine the optimum temperature of monophenolase activity, measurements were conducted between 25-40 °C by using the ammonium sulfate precipitated enzyme (Figure 6.14). In this temperature range, the enzyme activity increases by increasing the measurement temperature. At 40 °C the enzyme showed almost two fold greater activity compared to its activity at 25 °C.

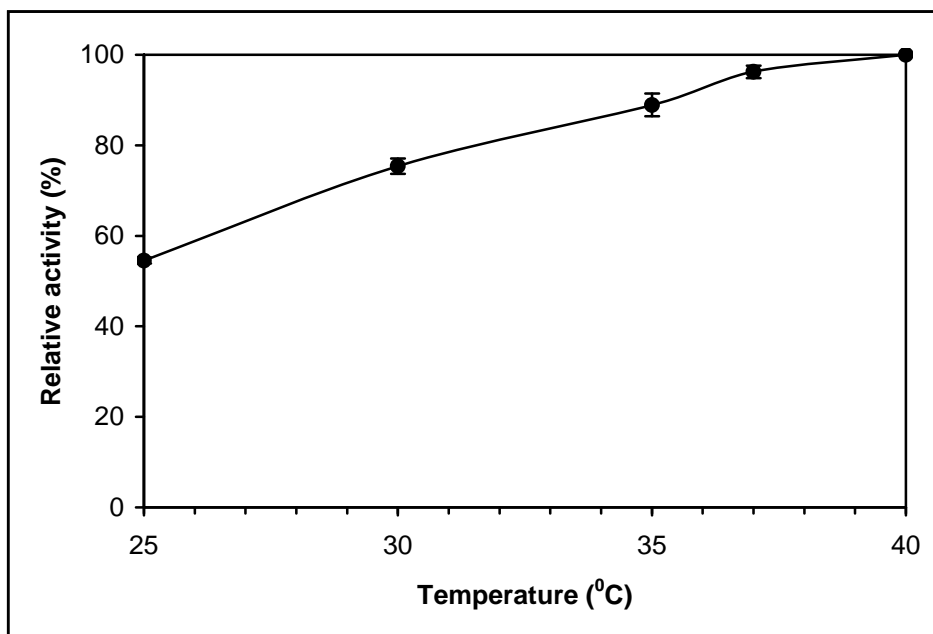


Figure 6.14. Optimum temperature for monophenolase activity of PPO

### 6.2.5. Stability of monophenolase activity in acetone powders

In the production of PPO from mushroom stems, the acetone powders may be used as source of PPO for enzyme extraction. Thus, the stability of monophenolase activity was investigated during cold (+ 4 °C) and frozen storage (-18 °C) of acetone powders (Figure 6.15).

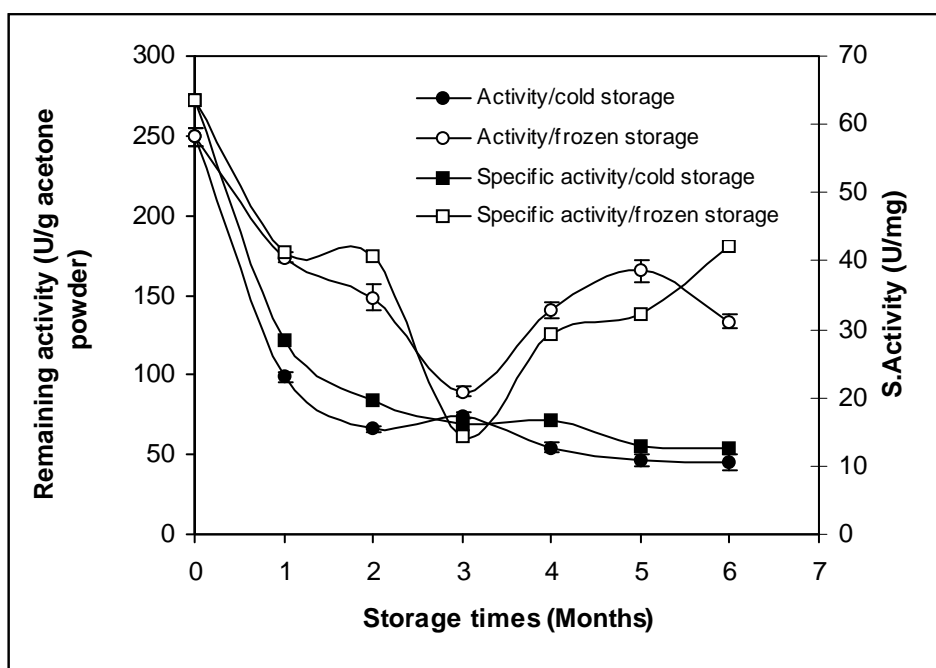


Figure 6.15. The stability of monophenolase activity of PPO in acetone powders

During storage at different temperatures, the activities and specific activities of enzyme changed with quite similar patterns. The enzyme has no stability in cold stored acetone powder. However, acetone powder stored at -18 °C showed good stability for two months with retention of almost 60-70 % of its monophenolase activity. Further storage of acetone powder at -18 °C caused first a reduction and then an increase in the activity of enzyme. It is hard to explain this fluctuation in enzyme activity. However, it may be related with the reversible modifications in enzyme conformation that occurred during storage.

#### **6.2.6. Stability of the prepared PPO during storage**

The stability of monophenolase and diphenolase activity of partially purified and lyophilized PPO was investigated during frozen storage at -18 °C. As seen in Figures 6.16 and 6.17, lyophilization with saccharose or dextran did not cause a significant difference in the monophenolase and diphenolase activity of PPO. However, the specific activities of enzyme lyophilized with dextran are higher than those for the enzyme lyophilized with saccharose. Because of the reduced protein content during storage, the specific monophenolase and diphenolase activities of enzymes showed a significant increase at the second month of storage. Such a reduction in protein content may be observed because of the modification of the proteins. For example, the increased molecular interactions between proteins and carbohydrates may have caused the masking of proteins, particularly other than the enzyme.

At the third month of the storage, a sharp drop was observed in the specific monophenolase and diphenolase activity of PPO lyophilized with dextran. This clearly shows the reversible nature of interactions during storage of PPO enzyme. On the other hand, the increase of the specific activities of PPO lyophilized with saccharose continues also at the third month. At the end of three months the loss of enzyme activities was negligible. Thus, these results clearly prove the suitability of using dextran and saccharose as supporting material for the lyophilization of PPO enzyme.

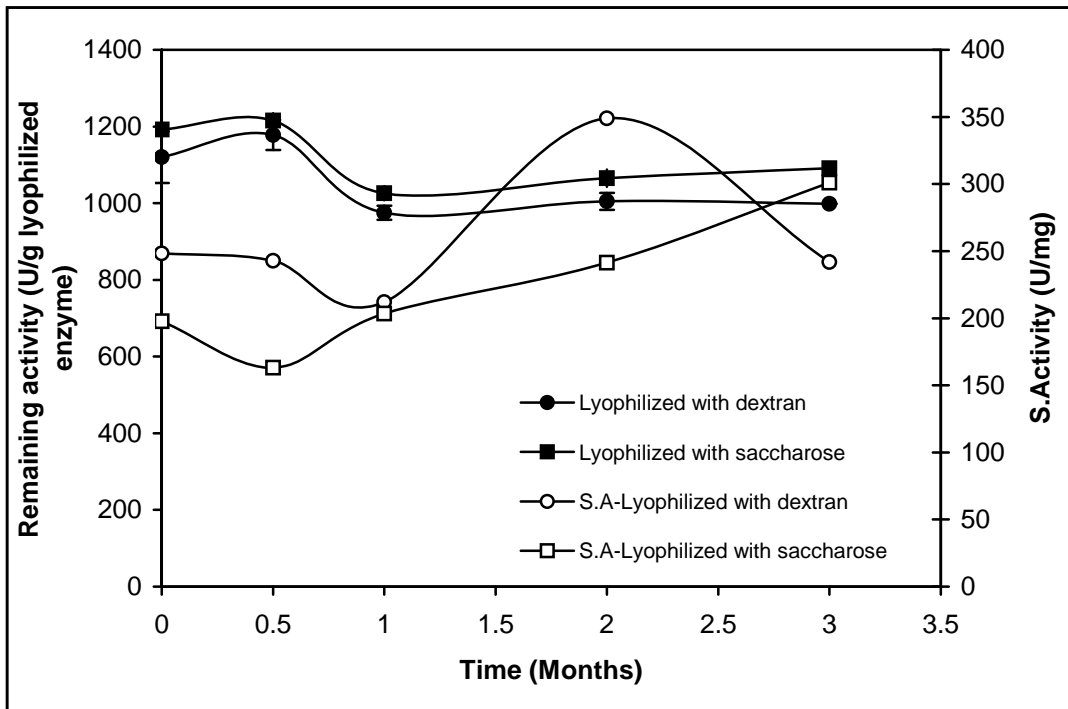


Figure 6.16. The stability of monophenolase activity of PPO in different lyophilized forms stored at  $-18\text{ }^{\circ}\text{C}$  (S.A.: specific activity)

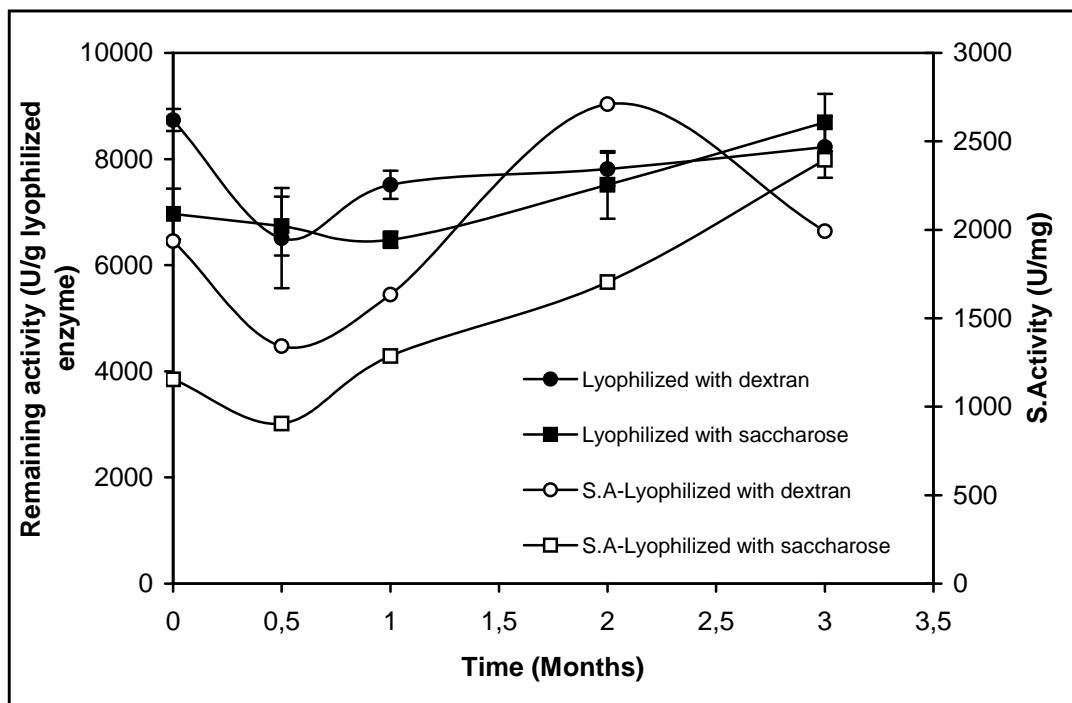


Figure 6.17. The stability of diphenolase activity of PPO in different lyophilized forms stored at  $-18\text{ }^{\circ}\text{C}$  (S.A.:specific activity)

### 6.2.7. The effect of lyophilization with dextran on temperature and pH stability of PPO

The lyophilization itself and/or the supporting carbohydrates may affect the characteristics of enzyme. In the literature, it was reported that lyophilization with polysaccharides such as dextran may cause the stabilization of enzymes (de la Casa et al. 2002). It was thought that the stabilization occurs because of the binding of polysaccharides to proteins and trapping of the surrounding water molecules to create a protective shield to the hydrophobic regions of enzyme (Longo and Combes 1999). However, the main draw back of this strategy was that a hydrophilic molecule may also facilitate the contact of enzyme surface with damaging interactions occurred in aqueous medium (Longo and Combes 1999). Thus, enzyme-polysaccharide interaction may result either with stabilization or destabilization. Figure 6.18 and 6.19 shows the effect of lyophilization with dextran on the thermal and pH stability of monophenolase activity, respectively.

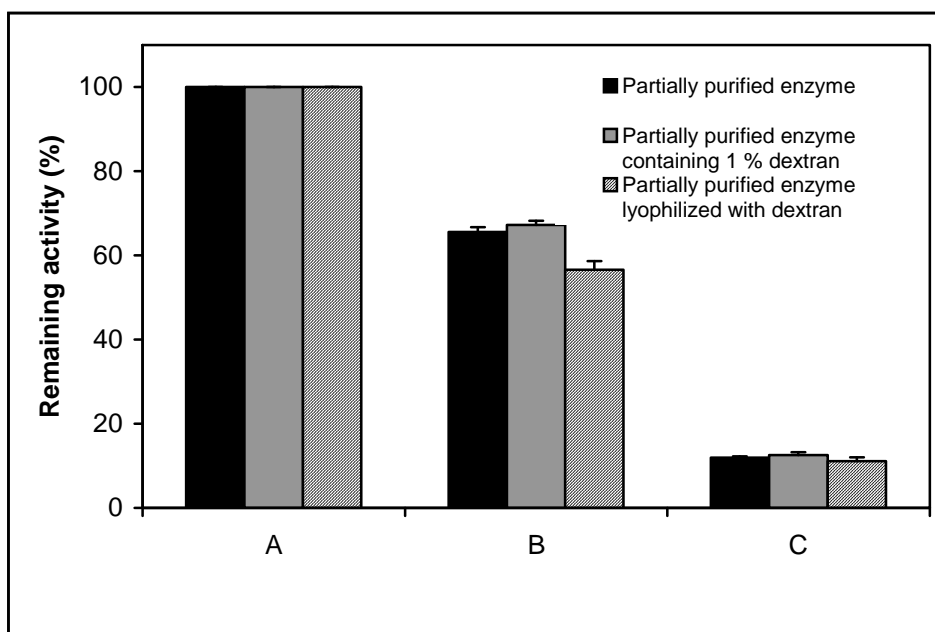


Figure 6.18. The effect of lyophilization with dextran on heat stability of monophenolase activity of PPO (A: activity of unheated enzyme (control), B: activity of enzyme heated at 50 °C for 30 min, C: activity of enzyme heated at 60 °C for 30 min)

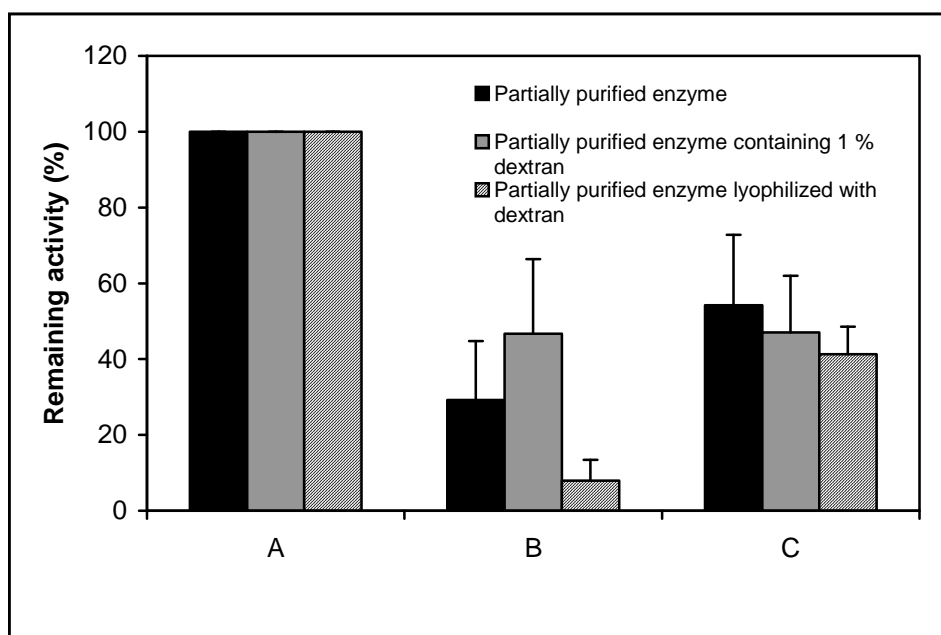


Figure 6.19. The effect of lyophilization with dextran on pH stability of monophenolase activity of PPO (A: initial enzyme activity at pH 7 (control), B: remaining enzyme activity at pH 7.0 after 48 h incubation at pH 4.0, C: remaining enzyme activity at pH 7.0 after 48 h incubation at pH 6.0)

The results of this study clearly showed that the lyophilization with dextran or the presence of dextran did not affect the thermal stability of monophenolase activity. On the other hand, lyophilization with dextran reduced the pH stability of the monophenolase activity at pH 4.0. The presence of 1 % dextran in enzyme extract did not cause the same destabilization. In fact, a slight stabilizing effect was observed for dextran in this sample at pH 4.0. Thus, it is likely that the reduced pH stability of enzyme may be related with the lyophilization process.

#### 6.2.8. The ability of the prepared PPO to oxidize phloridzin

In this study, the ability of PPO lyophilized with dextran to oxidize phloridzin was also tested spectrophotometrically at 420 nm. As seen in Figure 6.20, the lag phase of the enzyme for phloridzin oxidation is very long. However, the PPO prepared is capable to catalyse the oxidation of phloridzin. The calculated activity of the PPO lyophilized with dextran is 1440 U/g for this substrate. The oxidation of phloridzin by

the enzyme indicated that the prepared PPO is suitable for the production of yellow/orange colored oxidation products of phloridzin that are used as food colorants.

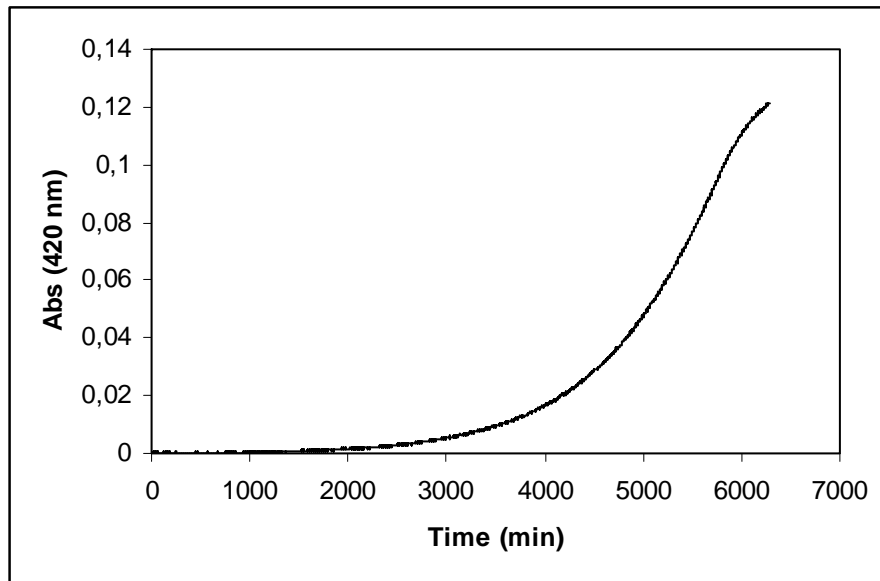


Figure 6.20. The phloridzin oxidation of PPO lyophilized with dextran (The reaction mixture was formed by mixing 0.5 mL, 2.2 mM phloridzin + 2 mL 0.05 M acetic acid-sodium acetate buffer at pH 5.0 + 0.5 mL enzyme)

## CHAPTER 7

### CONCLUSIONS

#### **The results obtained for PME enzyme**

Valencia orange peels contain considerable amount of PME activity that is quite stable during season (300-350 mL NaOH/min/100 g peel).

Before the extraction of PME, to prevent the gelling of extract, the soluble pectin in orange peels should be removed by homogenization with water several times.

The PME is ionically bound to cell walls. Thus, the pellet obtained from orange peels by washing with water should be extracted in the presence of suitable amounts of NaCl (6-10 g NaCl/100 g extraction mixture).

Mild heating and CaCl<sub>2</sub> did not activate the PME significantly.

The liquid extracts of PME stabilized by 0.1 % Na-benzoate and 0.1 % K-sorbate are quite stable for 5 months at + 4 °C with retention of almost 95 % of their activity. Thus, the commercial preparations of PME from orange peels can be stored in liquid form.

The PME preparation obtained can successfully be used to prepare edible pectin films from citrus pectin.

#### **The results obtained for PPO enzyme**

Acetone powders can be used to obtain buffer extracts of PPO from mushroom stems. The powders maintain most of their enzyme activity at -18 °C for two months.

The crude buffer extracts obtained did not show browning and can be further purified by ammonium sulfate or acetone precipitation and a following dialysis. The recoveries and purification folds obtained from the partial purification of monophenolase activity of PPO from the same acetone powder were 74-86 % and 3.4-4.3 and 55-67 % and 5.4-6.2 for ammonium sulfate and acetone precipitations, respectively. Thus, it appears that the ammonium sulfate precipitation gives a higher yield but lower purity.

The characteristics of PPO such as thermal stability, optimum pH and pH stability are almost same in acetone powders from different parties of mushroom stems.



Also, the indicated characteristics of the enzyme are not affected from the partial purification procedure.

The thermal stability and pH stability of monophenolase activity of PPO from mushroom stems are significantly greater than those reported for the monophenolase activity of commercial PPO from whole mushrooms.

The monophenolase and diphenolase activities of PPO lyophilized by using dextran or saccharose as supporting materials are quite stable at -18 °C for minimum three months. Thus, these preparations of PPO may be used as a source for the commercial applications of the enzyme.

The lyophilization of enzyme with dextran did not affect the thermal stability of the PPO enzyme. However, the stability of the enzyme at pH 4.0 reduced moderately.

The PPO preparation obtained contains monophenolase and diphenolase activities. Also, the prepared enzyme can use phloridzin as a substrate. These results indicate that the PPO prepared may be suitable for many different food applications including protein modification, production of antioxidants and colorants, cocoa and tea fermentations etc.

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