EFFECT OF ULTRASOUND AND TEMPERATURE ON TOMATO PEROXIDASE

M.Sc. Thesis
in
Food Engineering
University of Gaziantep

Supervisor
Assist. Prof. Dr. Çiğdem SOYSAL

By
Songül ŞAHİN ERCAN
JUNE 2009

T.C.
UNIVERSITY OF GAZİANTEP
GRADUATE SCHOOL OF
NATURAL & APPLIED SCIENCES
(Food Engineering Department)

Name of the thesis: Effect of ultrasound and temperature on tomato peroxidase
Name of the student: Songül ŞAHİN ERCAN
Exam date: 22 June 2009

Approval of the Graduate School of Natural and Applied Sciences

Prof. Dr. Ramazan KOÇ
Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

Prof. Dr. Ahmet KAYA
Head of Department

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

Assist. Prof. Dr. Çiğdem SOYSAL
Supervisor

Examining Committee Members

Prof. Dr. Zerrin SÖYLEMEZ (Chairman) ______________________

Prof. Dr. Sevim KAYA ______________________

Assoc.Prof. Dr. Hüseyin BOZKURT ______________________

Assist. Prof. Dr. Sema KOÇ KAYHAN ______________________

Assist. Prof. Dr. Çiğdem SOYSAL ______________________

ABSTRACT

EFFECT OF ULTRASOUND AND TEMPERATURE ON TOMATO PEROXIDASE

ŞAHİN ERCAN, Songül
M.Sc. in Food Engineering
Supervisor: Assist. Prof. Dr. Çiğdem SOYSAL
June 2009, 74 Pages

Activity of tomato peroxidase (POD) was determined by using guaiacol and o-dianisidine as hydrogen donors. Activity of tomato POD for inactivation experiments was determined by using o-dianisidine as hydrogen donor because low K_m (Michaelis-Menten constant) value was found by using o-dianisidine. Thermal inactivation of tomato POD was performed at temperatures of 63, 64, 65, 66 and 67°C. 50% reduction in POD activity was observed at 63, 64, 65, 66 and 67°C for 15.5, 10.5, 6.5, 5 and 3 minutes, respectively. The effect of ultrasound on tomato POD inactivation was performed at 15, 25, 40, 50 and 75% ultrasonic powers. It was observed that as the ultrasonic power increased, inactivation rate increased. 100% POD inactivation was observed at 50% and 75% powers for 150 and 90 seconds, respectively. Regeneration of tomato POD activity was investigated for the samples exposed to ultrasound at different ultrasonic powers (15, 25, 40, 50 and 75%) and stored at 4°C for one week period. It was observed that regeneration increased as the ultrasonic power increased. There was no regeneration in the samples which 100 % enzyme inactivation obtained by ultrasound. Vitamin C and total bacteria of tomato extract after heat and ultrasonic treatment were used for quality indicator. When approximately 50% POD was inactivated, vitamin C of tomato extract was decreased 27% by heat treatment and no reduction by ultrasonic treatment was observed. Higher amount of total bacteria reduction was observed by ultrasonic treatment.

Key Words: Tomato, peroxidase, kinetics, heat, ultrasound, vitamin C.

ÖZ

ULTRASON VE SICAKLIĞIN DOMATES PEROKSİDAZI ÜZERİNE ETKİLERİ

ŞAHİN ERCAN, Songül
Yüksek Lisans Tezi, Gıda Mühendisliği Bölümü
Tez Yöneticisi: Yrd.Doç. Dr. Çiğdem SOYSAL
Haziran 2009, 74 Sayfa

Domates peroksidazının aktivitesi hidrojen verici olan guaykol ve o-dianisidin kullanılarak belirlendi. Düşük Km (Michaelis-Menten sabiti) değeri o-dianisidin kullanılarak bulunduğu için etkisizleştirme deneylerinde domates peroksidazının aktivitesi o-dianisidin kullanılarak belirlendi. Domates peroksidazının ısıl işlem ile etkisizleştirilmesinde 63, 64, 65, 66 ve 67°C'de çalışıldı. Peroksidaz aktivitesindeki %50 düşüş 63, 64, 65, 66 ve 67°C'de sırasıyla 15,5, 10,5, 6,5, 5 ve 3 dakika olarak belirlendi. Ultrasonun domates peroksidazı üzerine etkisi %15, 25, 40, 50 ve 75 ultrasonik güçlerde çalışıldı. Ultrasonik güç arttıkça etkisizleştirme hızının arttığı belirlendi. Peroksidazın %100 etkisizleştirilmesi %50 ve %75 ultrasonik güçde sırasıyla 150 ve 90 saniye olarak belirlendi. Domates peroksidaz aktivitesinin geri dönüşümünü incelemek için numuneler farklı ultrasonik güçlere (%15, 25, 40, 50 ve 75) maruz bırakılarak 4°C'de bir hafta boyunca saklandı. Ultrasonik güç arttıkça geri dönüşümün hızlandığı belirlendi. Enzimin %100 etkisizleştirildiği numunelerde geri dönüşümün olmadığı gözlendi. Isı ve ultrason işleminden sonra domates özütündeki vitamin C ve toplam bakteri kalite göstergesi olarak kullanıldı. Peroksidazın yaklaşık olarak %50 etkisizleştirildiği noktada, ısıl işlem ile domates özütünde %27 vitamin C kaybı belirlenirken, ultrasonik işlem sonucu vitamin C miktarında hiçbir düşüş gözlenmedi. Ultrasonik işlemle toplam bakteri miktarında daha fazla bir düşüş belirlendi.

Anahtar Kelimeler: Domates, peroksidaz, kinetik, ısı, ultrason, vitamin C.

ACKNOWLEDGEMENTS

I wish to express my special deepest gratitude to my supervisor Assist. Prof. Dr. Çiğdem SOYSAL for her excellent guidance, patience, suggestions and continuous encouragement throughout the preparation of this thesis.

I would like to thank to Department of Food Engineering.

I wish to thank my beloved parents for always believing me and supporting me throughout my education.

Special thanks go to my husband for his understanding and constant encouragement through this study.

CONTENTS

Page

ABSTRACT. iii
ÖZET iv
ACKNOWLEDGEMENTS v
CONTENTS vi
LIST OF TABLES ix
LIST OF FIGURES x
LIST OF ABBREVIATIONS xiii
CHAPTER 1: INTRODUCTION . ..……... 1
1.1. Origin of enzyme 2
1.2. Physiological role of POD 3
1.3. Physical properties 4
1.3.1. Isoelectric point (pI) 4
1.3.2. Moleculer weight 4
1.3.3. Isoenzymes 5
1.3.4. Nonprotein moeity 5
1.4. Catalytic properties …….…... 6
1.4.1. Substrate specificity 7
1.4.2. Optimum temperture 8
1.4.3. Optimum pH 8
1.4.4. Inhibitors and activators 10
1.4.5. Michaelis-Menten constant (Km) 11
1.5. Inactivation of POD. 12
1.5.1. Inactivation kinetics 12

1.5.2. Thermal inactivation. 13
1.5.3. Inactivation by ultrasound 15
1.5.3.1. Ultrasonic science 15
1.5.3.2. Mechanisms and effect. 17

1.5.3.3. Application of sonication 18
1.5.3.4. Application of ultrasound on enzyme inactivation 19
1.5.4. Effect of POD in food processing. 22
1.5.5. Effect of heat and ultrasound on vitamin C and microorganisms. . . . 23
1.5.6. Tomato. 25
1.6. Aim of the thesis 27

CHAPTER II: MATERIALS AND METHODS. 28
2.1. Materials 28
2.2. Methods 28
2.2.1. Composition of tomato 28
2.2.1.1. Total moisture 28
2.2.1.2. Total sugar 28
2.2.1.3. Ash content 29
2.2.1.4. Protein content 29
2.2.1.5. Vitamin C content . . .. .29
2.2.2. Extraction of tomato POD. 30
2.2.3. POD assay and kinetics 30
2.2.4. POD assay and kinetics by guaiacol 30
2.2.5. POD assay and kinetics by o-dianisidine 31
2.2.6. Inactivation of tomato POD 31
2.2.6.1. Thermal treatment 31
2.2.6.2. Ultrasound treatment 31
2.2.7. Regeneration of tomato POD 32
2.2.8. Microbial analysis. 32
2.2.9. Statistical analysis 32

CHAPTER III: RESULT AND DISCUSSION 34
3.1. Composition of tomato 34
3.1.1. Moisture content. 34
3.1.2. Total sugar content. 34
3.1.3. Ash content. 35
3.1.4. Protein content. 35
3.1.5. Vitamin C content. 35
3.2. Activity of tomato POD. 35
3.2.1. Activity by guaiacol 35
3.2.2. Activity by o-dianisidine 35
3.3. Kinetics of tomato POD. 37
3.3.1. Kinetics by guiacol 39
3.3.2. Kinetics by o-dianisidine 40
3.4. Comparison of kinetic results 41
3.5. Inactivation of tomato POD. 42
3.5.1. Thermal inactivation 42
3.5.2. Ultrasound treatment 47
3.5.3. Regeneration of tomato POD 50
3.5.4. Effects of inactivation methods on vitamin C and microorganisms 55

CHAPTER IV: CONCLUSION 59
REFERENCES 61

LIST OF TABLES

Page

Table 1.1. Optimum pH values of POD from various sources 9

Table 1.2. Thermal inactivation kinetic models of POD for some vegetables.. 13

Table 3.1. Results of ANOVA of thermal inactivation of tomato POD 43

Table 3.2. Reaction rate constants (k) and decimal reduction times (D) of tomato POD by thermal inactivation 46

Table 3.3. Results of ANOVA of ultrasonic inactivation of tomato POD 49

Table 3.4. Reaction rate constants (k) of tomato POD by ultrasonic inactivation. 49

Table 3.5. Model constants obtained at 40% ultrasonic power for the regeneration of tomato POD 54

Table 3.6. Model constants obtained at 50% ultrasonic power for the regeneration of tomato POD. 54

Table 3.7. Model constants obtained at 75% ultrasonic power for the regeneration of tomato POD. 54

Table 3.8. Effects of inactivation methods on vitamin C of tomato extract. 55

Table 3.9. Aerobic plate count of tomato extract treated with heat and ultrasound. 56

Table 3.10. Mold-yeast count of tomato extract treated with heat and ultrasound 57

LIST OF FIGURES

Page

Figure 1.1. Motions of bubbles during cavitation 18

Figure 3.1. Calibration curve for sugar determination 34

Figure 3.2. Change of POD activity with guaiacol concentration with 98.7 mM H_2O_2, Extract: 0.572 mg protein/mL 36

Figure 3.3. Effect of pH on POD actvitiy with guaiacol, buffer: 0.01 M, H_2O_2: 98.7 mM, Guaiacol: 0.1% (v/v), Extract: 0.572 mg protein/mL, T= 25°C 36

Figure 3.4. Change of POD activity with o-dianisidine concentration with 32.6 mM H_2O_2, Extract: 0.572 mg protein/mL. 37

Figure 3.5. Effect of pH on POD activitiy with o-dianisidine, buffer: 0.01 M, H_2O_2: 32.6 mM, o-dianisidine: 0.05% (w/v), Extract: 0.572 mg protein/mL, T= 25°C 38

Figure 3.6. Change of POD activity with tomato extract by o-dianisidine at 460 nm (▼), by guaiacol at 470 nm (●). 38

Figure 3.7. Initial rate versus substrate concentration by guaiacol, Extract: 0.572 mg protein/mL. 39

Figure 3.8. Lineweaver-Burk plot of peroxidase action on hydrogen peroxide in the presence of guaiacol 40

Figure 3.9. Initial rate versus substrate concentration by o- dianisidine, Extract: 0.572 mg protein/mL 41

Figure 3.10. Lineweaver-Burk plot of peroxidase action on hydrogen peroxide in the presence of o-dianisidine 42

Figure 3.11. Thermal inactivation of tomato POD at 63 (●), 64 (○), 65 (▼), 66 (∇), 67°C (■) 43

Figure 3.12. Natural logarithm of residual tomato POD activity versus heating time at 63 (▲),64 (■), 65 (●), 66 (▼), 67 (♦) °C 44

Figure 3.13. Arrhenius plot of the inactivation rates of tomato POD. 45

Figure 3.14. Temperature of the extract after sonication at 75% (■), 50% (∇), 40% (▼), 25% (○) and 15% (●) ultrasonic powers. 47

Figure 3.15. Ultrasonic inactivation of tomato POD at 15% (●), 25% (○), 40% (▼), 50% (∇), 75% (■) powers. 48

Figure 3.16. Natural logarithm of residual tomato POD activity versus heating time at ultrasonic powers of 15% (●), 25% (■), 40% (▲), 50% (▼), 75% (♦) 49

Figure 3.17. Regenerated POD activity versus storage time when samples were treated at 15% ultrasonic power for 20 (●), 40 (■), 60 (▲), 90 (◘), 120(▼), 150 (♦) seconds. 51

Figure 3.18. Regenerated POD activity versus storage time when samples were treated at 25% ultrasonic power for 20 (●), 40 (■), 60 (▲), 90 (♦), 120(◘), 150 (▼) seconds 51

Figure 3.19. Regenerated POD activity versus storage time when samples were treated at 40% ultrasonic power for 20 (●), 40 (■), 60 (▲), 90 (◘), 120(▼), 150 (♦) seconds 52

Figure 3.20. Regenerated POD activity versus storage time when samples were treated at 50% ultrasonic power for 20 (♦), 40 (◘), 60 (▼), 90 (■), 120(●), 150 (▲) seconds 52

Figure 3.21. Regenerated POD activity versus storage time when samples were treated at 75% ultrasonic power for 20 (●), 40 (■), 60 (▲), 90 (▼) seconds 53

LIST OF ABBREVIATIONS

AA	Ascorbic acid
ABTS	2,2-Azino-bis-3-ethylbenzothiazol-6-sulfonic acid
ACC	1-Aminocyclopropane-1-carboxylic acid
AOAC	Association of Offical Analytical Chemists
ANOVA	Analysis of variance
D	Decimal reduction time (min)
DCP	2,6-dichlorophenolindophenol
E_a	Activation energy (kj/mol)
FAD	Flavin adenine dinucleotide
H_2O_2	Hydrogen peroxide
HTST	High temperature and short time treatments
IAA	Indoleacetic acid
k	Reaction rate constant (min^{-1})
kDa	Kilodalton
kHz	KiloHertz
Km	Michaelis-Menten constant
KP	Potassium phosphate
m/o's	Microorganisms
MS	Manosonication
MTS	Manothermosonication
NADPH	Nicotinamide adenine dinucleotide phosphate
PCA	Plate count agar
PDA	Potato dextrose agar
pI	Isoelectric point
POD	Peroxidase
US	Ultrasonication
TS	Thermosonication

CHAPTER I

1. INTRODUCTION

In 1855, Schoenbein observed that extracts from some mushrooms and animal tissues caused the development of a blue color in guaiacol solutions in the presence of air or with dilute solutions of hydrogen peroxide. This activity was due to the presence of peroxidase (Whitaker, 1993).

POD (donor: H_2O_2 oxido-reductase; EC 1.11.1.7) constitute a group of glycoproteins the main function of which is the oxidation of different substrates at the expense of H_2O_2. PODs are ubiquitous, iron-containing enzymes, which oxidize phenolic compounds and related substances, using activated oxygen released from H_2O_2 or organic peroxidase (Robinson, 1991).

To prevent unwanted changes during storage, fruit and vegetable products are generally subjected to some type of treatment during processing in order to inactivate these enzymes. A heat treatment, such as blanching, pasteurization or commercial sterilization is most commonly used (Anthon et al, 2002). Heat, compared to other food preservation methods, has the important advantage of ensuring food safety and long preservation due to its destructive effect on enzymes and microorganisms.

POD is considered to have an empirical relationship to off- flavours and off-colours in raw and unblanched frozen vegetables (Lopez et al, 1994). Therefore, the inactivation of this enzyme increases the shelf life of vegetables during frozen storage (Williams et al, 1986; Barret and Theerakulkait, 1995). POD activity of plant foods is widely used as an index of blanching because of its high termostability. It is considered that any heat treatment sufficient to destroy peroxidase activity will also destroy most other enzyme systems (Serrano-Martinez et al, 2008). However, the non-specific effect of heat can cause reductions in nutritive and sensorial quality of many attempts have been made to design alternative procedures for food preservation and sanitation (Barbosa-Canovas et al, 1998). Some of the common non-thermal

alternatives to thermal processing of foods include pulse- electric field inactivation, high pressure, microfiltration and ultrasonication. In recent years, the food industry has discovered that ultrasonic waves have a wide variety of applications in the processing and evaluation of products. From grading beef to sterilization, ultrasound has a number of applications in an increasing number of areas in the food industry. In combination with heat, it can accelerate the rate of sterilization of foods, thus lessening both the duration and intensity of thermal treatment and the resultant damage (Piyasena et al, 2003). The advantages of ultrasound over heat pasteurization include: the minimizing of flavor loss, especially in sweet juices; greater homogeneity; and significant energy savings (Earnshaw et al, 1995).

1.1. Origin of Enzyme

POD extensively distributed in higher plants (e.g., horseradish, turnip, fig sap), animals (e.g., tryptophan pyrrolase, iodine peroxidase of thyroid) and microorganisms [e.g., cytochrome *c* peroxidase of yeast (Cruz and Fatibello-Filho, 1998)].

Plant PODs can be subdivided into three subgroups (acidic, neutral and cationic) according to their isoelectrophoretic mobilities. Based on differences in primary structure, the plant peroxidase superfamily can be divided into three classes: I, II and III. Class I peroxidases include intracellular enzymes in plants, bacteria and yeast. Class II peroxidases are extracellular peroxidases of fungi. Class III comprises classical plant secreted peroxidases. Class III peroxidases enzymes have approximately 300 amino acids (Clemente, 2002).

According to current classification, the plant peroxidase superfamily includes prokaryotic enzymes (bacterial catalase-peroxidases, yeast cytochrome *c* peroxidase, plant ascorbate peroxidases), fungal enzymes (lignin and manganese peroxidases and planttype enzymes) as well as those from plants.

They are present in plant cells in soluble, ionically or covalently bound to cell wall components forms. These enzymes are widely distributed in higher plants where they are involved in various processes e.g. cell elongation by cell wall-bound peroxidase, lignification process and plant defense mechanisms (Belcarz et al, 2008).

POD has been reported as found in many plants those of including strawberry (Civello et al, 1995), spring cabbage (Belcarz et al, 2008), orange (Clemente, 2002), kiwifruit (Fang et al, 2008), mint leaves (Shalini et al, 2008), red pepper (Serrano-Martinez et al, 2008), horseradish (Veitch, 2004; Sariri et al, 2006), cocoa beans (Sakharov and Ardila, 1999), Brussel sprout (Forsyth and Robinson, 1998), pumpkin (Gonçalves et al, 2007), carrots (Soysal and Söylemez, 2005), melon (Rodriguez Lopez et al, 2000), maize root (Maksimoviç et al, 2008), butternut squash (Agüero et al, 2008), tomato (Savic et al, 2008) and sunflower roots (Jouili et al, 2008).

1.2. Physiological Role of POD

The enzyme is involved in many plant functions such as hormone regulation, defence mechanisms, indoleacetic acid degradation during maturation and senescence of fruits and vegetables and lignin biosynthesis. Because of its multiple functions, the enzyme is commonly found as several isoenzymes in plants. In the presence of peroxide, POD produces phytotoxic free radicals which react with a wide range of organic compounds (ascorbic acid, carotenoids and fatty acids), leading to losses in the colour, flavour and nutritional value of raw and processed foods (Serrano-Martinez et al, 2008).

POD participates in the late stages of the lignin-forming process and in the protection of tissues damaged by, or infected with, pathogenic microorganisms. The capacity *in vitro* of the enzyme to convert 1-aminocyclopropane-l-carboxylic acid (ACC) into ethylene has already been established. Moreover, it can also degrade chlorophyll *in vitro* in the presence of phenols and participate in the oxidation of indoleacetic acid (IAA) proposed a model in which the capacity to oxide ACC and IAA was assigned to basic PODs, while acid peroxidases would participate in the lignification process (Civello et al, 1995).

POD is the most widely used enzyme as a biochemical indicator of disease, cellular injury, trauma damage, infection, etc, in plants. POD activity appears to increase during ripening and senescence in fruits, e.g. mango, grape, apple, pear and banana. It plays regulatory role in plant systems such as ethylene biogenesis, membrane integrity, respiration control, and oxidative metabolism of auxin and cytokinin (Ory and Angelo, 1977).

1.3. Physical Properties

1.3.1. Isoelectric Point (pI)

POD found in higher plants include basic, neutral, or acidic pI, and a single vegetable may contain several isoenzymes having wide range of pI values. Acidic PODs have been found in turnip roots, pI 3 (Duarte-Vazquez et al, 2000); pepper fruits, pI 3.8 (Pomar et al, 1997); the soluble fraction of potato tuber sprouts, pI 3 (Boucoiran et al, 2000) and the salt extract of tomato, pI 3.5 (Marangoni et al, 1989). Basic PODs have been found in turnip roots, pI 8.5 (Duarte-Vazquez et al, 2000), and the soluble fraction of potato tuber sprouts, pI 10 (Boucoiran et al, 2000). The pI of neutral turnip POD has been found to be 7.2 (Duarte-Vazquez et al, 2001).

POD from ripening banana fruit is composed of at least twelve isoenzymes which have isoelectric points ranging from approximately 3.3 up to 9.5 (Ory and Angelo, 1977). Major three isoenzymes of horseradish POD, have pI of 6.1, 6.9 and 8.9 (Wong, 1995). The pI values of the purified isoperoxidase from orange were 4.5, 5.2, and 9.0 (Clemente, 2002). Two POD isoenzymes were detected in strawberry fruit; they were of the basic type isoelectric points 9.5-10.0 (Civello et al, 1995). Isoperoxidase from scented-geranium callus have isoelectric points 9.1, 9.0, 8.6 (Lee et al, 2001). Isoelectric points of four isoperoxidase of Brussel sprout are 3.5, 4.9, 9.5, 8.8 (Forsyth and Robinson 1998). The pI of broccoli was approximately 4, 5 and 8 for acidic, neutral and basic PODs, respectively (Thongsook and Barrett, 2005a), while that of marula fruit was 5.7 (Mdluli, 2005).

1.3.2. Molecular Weight

The molecular weights of POD from various sources have been reported between 26–70 kDa. Some of the molecular masses reported are 42 kDa for peanut POD (Chibbar and Huystee, 1984), 56 kDa for strawberry POD (Civello et al, 1995), 26–48 kDa for brussel sprout POD (Forsyth and Robinson, 1998), 40-46 kDa for horseradish POD (Paul and Stigbrand, 1970), 34 kDa for green asparagus POD (Wang and Luh, 1983), 48 kDa for oil palm leaf and rice PODs (Ito et al, 1991; Deepa and Arumughan, 2002) and 43 kDa for tomato POD (Jen et al, 1980). The differences observed are attributed to post translational modifications of the polypeptide chain including the number and composition of glycan chains present in plant PODs (Van Huystee et al, 1992; Duarte-Vazquez et al, 2001).

1.3.3. Isoenzymes

The occurence of multiple forms of peroxidase was first found in horseradish roots. This tissue contains several major isoenzyme forms, which are similar in molecular weight and amino acid composition (Ory and Angelo, 1977). It was found that for vegetables and fruits the various kinds of isoperoxidases including the anionic and cationic types with low and high pI values respectively can be extracted as both soluble and ionically bound forms (Forsyth and Robinson, 1998).

PODs exist in numerous isoenzymatic forms and are separated into anionic (acidic) and cationic (basic) types according to their isoelectric points. Anionic PODs are involved in the processes of lignification and suberinization. Nevertheless, cationic PODs are also involved in the biosynthesis of lignin and suberin (Hatzilazarou et al, 2006).

There are at least eight distinguishable isoperoxidases in Korean radish, and six of them were purified to near homogeneity. The physicochemical, catalytic and immunological properties of Korean radish peroxidases showed that different isoperoxidases might have different specificities toward physiological substrates in vivo (Lee et al, 2001).

It has been reported that POD isoenzymes from various sources have different molecular weights ranging from 30-60 kDa, e.g. eleven isoenzymes of orange POD have molecular weights between 22-44 kDa (Clemente, 1998), the neutral and basic broccoli PODs had molecular masses of 43 kDa, and the acidic peroxidase had a molecular mass of 48 kDa (Thongsook and Barrett, 2005a) and two POD isoenzymes were detected in strawberry fruit; they were of the basic type and had molecular masses of 58.1 and 65.5 kDa (Civello et al, 1995).

1.3.4. Nonprotein Moeity

PODs are heme-containing enzymes that reduce H_2O_2 to oxidize a wide variety of organic and inorganic compounds. PODs have been classified as iron containing peroxisases and flavoprotein peroxidases. The iron containing peroxidases are further subgrouped into ferriprotoporphyrin peroxidases and verdoperoxidases.

The ferriprotoporphyrin group include peroxidases from higher plants (horseradish, Japanese radish, turnip, fig sap), animals (tryptophan pryrolase, iodine peroxidase of thyroid), and microorganisms (cyctochrome c peroxidase of yeast). These peroxidase all contain ferriprotoporphyrin III as a prosthetic group which can be removed from the protein moeity on treatment with acidic acetone. The enzymes are brown in color when highly purified due to the ferriprotoporphyrin III cofactor (Whitaker, 1993).

The verdoperoxidases are found in myelocytes (myeloperoxidase), milk (lactoperoxidase), and in other tisses. The prosthetic group of these enzymes is an iron porphyrin nucleus but is not ferriprotoporphyrin III. Highly purified myeloperoxidase and lactoperoxidase are green because of their absorbance maxima in the region 570 to 690 nm (as well as near 403 nm).

Flavoprotein peroxidases have been purified from several streptococci, including Streptococcus faecalis, and from several animal tissues. The prosthetic group of these peroxidases is FAD (Whitaker, 1993).

1.4. Catalytic Properties

There are four types of catalytic activity reported for PODs. These are the peroxidatic, oxidatic, catalytic and hydroxylation reactions. POD catalyzes oxidatic reactions (in which oxygen is the electron acceptor) as well as the classic peroxidatic reactions (in which hydrogen peroxide is the electron acceptor) (Kay et al,1967; Swedın and Theorell, 1940). The peroxidatic reaction, more generally thought to be of most physiological significance, has been studied more extensively than other three reactions.

Peroxidatic reactions occur in the presence of a wide variety of hydrogen donors, including p-cresol, guaiacol, resorcinol, benzidine and o-dianisidine. Certain PODs appear to have a greater affinity for specific hydrogen donors such as NADH, glutathione or cytochrome C. One approach to elucidate hydrogen donors of physiological importance is the use of affinity chromotography. The preferred oxidant for peroxidatic reaction is hydrogen peroxide although other PODs are effective substrates. In postharvest tissues the peroxidatic reaction of most obvious importance at this time is lignification which can profoundly influence the

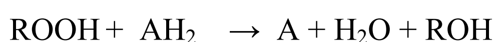
toughening of vegetables such as beans and asparagus. It has also been suggested that the peroxidatic reaction functions to protect the cellular milieu from PODs which may cause an imbalance in redox potential and damage membranes, enzymes, etc.

In the absence of hydrogen donor, POD can convert hydrogen peroxide to water and oxygen although this reaction is some 1000 times slower than the peroxidatic and oxidatic reactions. Finally in the presence of certain hydrogen donors, such as dihydroxyfumaric acid, and molecular oxygen, peroxidase can catalyze hydroxylation of a variety of aromatic compounds notably tyrosine, phenylalanine, p-cresol and benzoic acid. The metabolism of phenolic substances is a particular importance to the quality of postharvest fruits and vegetables in that they may act as effectors of hormone metabolism, intermediates in lignin biosynthesis and may result in discoloration resulting from their enzymic or non-enzymic oxidation.

The oxidatic reaction requires the presence of molecular oxygen and a suitable hydrogen donor. Examples of so-called redogenic hydrogen donors are indole - 3-acetic acid (IAA), oxalacetic acid, ascorbic acid and hydroquinone. The IAA oxidase function of peroxidase appears to be extremely important in postharvest fruit and vegetables. There is also recent evidence that cytokinins are oxidized by POD. It may also that the oxidatic function may contribute to deteriorative reactions such as membrane lipid oxidation and oxidation of essential thiol groups. There is considerable evidence that the enzyme which catalyzes the oxidatic degradation of the plant growth hormone, indole-3- acetic acid, is a peroxidase (Ray, 1958).

1.4.1. Substrate Specificity

PODs are oxidizing enzymes that catalyze the oxidation of many substances. The oxidation reaction proceeds with the aid of hydrogen peroxide that is ultimately reduced to water (Sariri et al, 2006). This enzyme catalyses the following reaction:



$$ROOH + AH_2 \rightarrow A + H_2O + ROH$$

where A is a hydrogen donor (Göğüş and Fadıloğlu, 2006). Some well known peroxidase substrates (hydrogen donors) namely, catechin, vanillin, *o*-dianisidine

(Lee et al, 2001; Leon et al, 2002; Clemente, 2002; Soysal and Söylemez, 2005; Doğan et al, 2007;) , o-phenylenediamine (Lee et al, 2001; Sakharov et al, 2002; Leon et al, 2002; Doğan et al, 2007), diaminobenzidine and tropolone (Ratcliffe et al, 1994), 2,2-azino-bis-3-ethylbenzothiazol-6-sulfonic acid (ABTS) (Rodrigo et al, 1996; Rani and Abraham, 2006; Serrano-Martinez et al, 2008), guaiacol (Thongsook and Barrett, 2005a; Ghamsari et al, 2007; Jouili et al, 2008; Rudra et al, 2008), catechol (Sakharov and Ardila, 1999; Troıanı et al, 2003), and pyrogallol (Soysal and Söylemez, 2005).

The substrates of POD can be conventionally divided into two groups by the rate of their oxidation. The group of slowly oxidized substrates includes electron donors. In particular, it includes substances with high functional activity in plants such as NADH, indole-3-acetic acid (IAA), and ascorbic acid (AA). POD catalyzes oxidation of indole-3-acetic acid both with and without hydrogen peroxide (Rogozhin and Rogozhin, 2004).

On the other hand PODs can be categorized into two different groups. PODs which oxidize guaiacol (o-methoxyphenol), as a commonly used reducing substrate *in vitro,* are referred to as guaiacol peroxidases. They are located in cytosol, vacuole, cell wall, apoplast and extracellular medium, but not in organelles and are assumed to be involved in a range of processes related to plant growth and development. Ascorbate peroxidases belong to another group of plant peroxidases that show preference for ascorbic acid as reducing substrate (Ghamsari et al, 2007).

1.4.2. Optimum Temperature

Different enzyme sources show different optimum temperature values. Optimum temperature of POD from various sources change in the range of 25-40°C. Vanilla POD showed its maximum activity at 16°C (Marquez et al, 2008), grape POD at 40°C (Sciancalepore et al, 1985), strawberry POD at 30°C (Civello et al, 1995) and sunflower POD at 40°C (Jouili et al, 2008).

1.4.3. Optimum pH

It is well known that the optimal conditions for catalysis by different PODs are not identical. The various pH dependence of POD reactions have been reported for

different substrates (Sakharov et al, 2002). It was reported that, when guaiacol was used as H donor, broccoli POD showed maximum activity at pH 4-5 for the acidic POD and pH 6 for both the neutral and basic PODs. (Thongsook and Barrett, 2005a). On the other hand, optimum pH values of peroxidases obtained from various sources using different substrates show differences (Table 1.1)

Table 1.1. Optimum pH values of POD from various sources

Source	Optimum pH					
	ABTS	**o-Phenylenediamine**	**o-dianisidine**	**Catechol**	**Guaiacol**	**Ref.**
Corn step water	3.4	5.2	5.2	-	3.7-5.2	Gray and Montgomery, (2003)
Sweet potato	4.5	5.0	4.5	5.5	3.5-5.5	Leon et al, (2002)
Royal palm tree	3.0	5.0	5.2	5.0	5.5	Sakharov, (2001)
Oil palm tree	3.0	5.5	5.5	4.5	5.5	Sakharov et al, (2002)
Salvia A_1	4.5	2.5	5.0	7.0	6.0	Doğan, (2007)
Salvia A_2	4.0	2.5	6.0	6.0	7.0	Doğan, (2007)
Salvia A_3	3.5	2.5	6.0	7.0	7.0	Doğan, (2007)

PODs purified from various sources have been reported to have their pH optima mostly in the region of 4.5-6.5. The optimum pH for acidic turnip peroxidases was reported to be between 5 and 5.5 with ABTS as H donor (Duarte-Vazquez et al, 2000). The POD isoenzyme from tomato juice was reported to have an optimum pH of 5.5 with guaiacol as H donor (Jen et al, 1980). The optimum pH for strawberry POD was found to be at pH 6 (Civello et al, 1995), and for potato sprouts and tubers

was 4-4.5, respectively (Boucoiran et al, 2000). Sakharov (2001) has reported that the heme-peroxidase from palm tree leaves is stable over a broad pH-range, maximum stability being found at pH 7.0.

It was found that the pH curve of Tartary buckwheat bran POD, exhibited a broader stable range at pH 4.5 to 9.0 with guaiacal as substrate, its optimum pH at nearly 6.5 and it was stable against acidic and alkaline conditions (Zhang et al, 1999). Vanilla POD had maximum activity at a pH of approximately 3.8 (Marquez et al, 2008). The optimum pH for palm leaf POD is 5.0 (Deepa and Arumughan, 2002), for marula fruit is 4.0 (Mdluli, 2005), and for peanut POD it is 3.6 (Ravindra and Van-Huystee, 1984). The POD isoenzymes from broccoli were reported to have an optimum pH of 6.0 for neutral peroxidase and 4.0 for acidic peroxidase (Thongsook and Barrett, 2005a).

1.4.4. Inhibitors and Activators

Inhibitors can diversely affect the rate of the reactions catalyzed by POD. The cyanide, fluoride, and azide ions inhibit the catalytic activity of POD by formation of stable complexes at the sixth coordination position of the heme iron, preventing the reaction with H_2O_2. A series of organic compounds inhibit peroxidase by reacting with its intermediates. Some of these compounds, such as ascorbic acid, NADH, sugars, and thiourea, belong to slowly oxidized POD substrates that inhibit the oxidation of o-dianisidine by tightly binding in the active site of the enzyme (Vallee and Ulmer, 1972).

Some substances inactivate POD by chemically reacting with it. For example, irreversible inactivating action of phenylhydrazine results from the modification of functional groups of POD (presumably, a tryptophan residue) by freeradical products of its noncatalytic and catalytic oxidation that are formed upon incubation with POD. This leads to a change in the conformation of the enzyme active site, a partial exhibition of the heme into solution, and its subsequent destruction. Thiourea is not only a competitive reversible inhibitor of the POD oxidation of o-dianisidine but can also modify the enzyme to cause its inactivation (Rogozhin et al, 2000).

Inhibitory effect of some metal ions on POD activity was also reported. POD isolated from Eupatorium odoratum, was found to be stable to metal ions like Hg^{2+}, K^{+}, Ca^{2+} where as slight decrease in enzyme activity was observed by Mg^{2+}, Zn^{2+} and Mn^{2+} (Rani and Abraham, 2006).

It was found that some metal ions tested had a low inhibitory effect on vanilla bean POD (Mg^{2+}, Cu^{2+} and Ca^{2+}) while Fe^{2+} and Hg^{2+} had important peroxidase inhibitory effect (Marquez et al, 2008).

Little is known about the effect of metal ions on plant POD activity (Marquez et al, 2008). It was reported that sodium was found to increase the activity of POD isolated from Eupatorium odoratum in presence of Cl^{-} (Rani and Abraham, 2006). Guaiacol or o-dianisidine oxidation rates were increased by $CaCl_2$ (Vianello et al, 1997). Activation of POD by glucose and sucrose was observed (Nicoli et al, 1991).

1.4.5. Michaelis-Menten constant (Km)

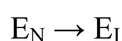
POD from different sources have different kinetics. Also, each POD isoenzyme show different reaction rate with different hydrogen donor. Green pea has three isoenzymes. Guaiacol and pyrogallol were used as hydrogen donor and hydrogen peroxide used as substrate for each isoenzyme. K_m values for guaiacol as hydrogen donor was found as 10.2, 10.8 and 10.2 mM and for hydrogen peroxide it was found as 2.6, 7.2 and 4.3 mM for each isoenzyme. K_m values for pyrogallol as hydrogen donor was found as 4.2, 6.2 and 2.0 mM and for hydrogen peroxide peroxide it was found as 2.6, 2.8 and 3.0 mM for each isoenzyme (Halpin et al, 1989).

Apparent K_m values of carrot POD was reported as 1.4 mM for guaiacol, 7.7x 10^{-3} mM for o-dianisidine and 0.34 mM for pyrogallol (Soysal and Söylemez, 2005). For Vanilla bean peroxidase Km value was found as 3.8 mM for guaiacol (Marquez et al, 2008). Km value of palm leaf peroxidase was found as 3.96 mM (Deepa and Arumughan, 2002), spinach peroxidase as 1.66 mM (Chen and Asada, 1989) and pea peroxidase as 0.04 mM (Mittler and Zilinskas, 1991).

1.5. Inactivation of POD

1.5.1. Inactivation kinetics

Enzyme inactivation often follows first order kinetics; by the definition that a single molecule undergoes a conformational change (Kuldiloke, 2002). If first order kinetics can be assumed, the process is



$$E_N \rightarrow E_I$$

where E_N is the native or active enzyme, E_I is the inactive enzyme, and k is the specific rate constant for the inactivation process. Mathematically the decrease in active enzyme becomes

$$-d[E_N]/dt = k[E_N] \quad \text{or} \quad -dA/dt = kA$$

where *A* is enzyme activity, then integration gives

$$\ln([E_N]/[E_{Ni}]) = -kt \quad \text{or} \quad \ln(A/A_i) = -kt$$

where E_{Ni} is the initial native, active enzyme and A_i is initial enzyme activity.

Thermal inactivation can also be described as using k value, where k represents the inactivation rate constant (min^{-1}) and the temperature inactivation can be expressed according to Arrhenius equation.

$$\ln A = -kt$$

$$\ln(k) = \ln(k_{ref}) - E_a/R\ (1/T - 1/T_{ref})$$

where k_{ref} is the inactivation rate constant at reference temperature (min^{-1}), T_{ref} is absolute reference temperature, E_a is activation energy (J/mol), R is universal gas constant (8.314 Jmol/K).

The relationship between decimal reduction time and the inactivation rate constant is given by the equation:

$$D = 2.303/k$$

1.5.2. Thermal inactivation

POD is considered to be most heat stable enzyme in vegetables and is therefore used as indicator of blanching efficiency. Thermal inactivation of POD depends on the nature, thickness, and geometry of the vegetables, and the applied time-temperature combination. Heating time can be significantly reduced if an individual quick blanching process is followed. Kinetic inactivation behaviour of some vegetables were explained by first order reaction kinetics (Table 1.2). The first order reaction kinetics can adequately describe POD inactivation (Sun, 2005).

Table1.2. Thermal inactivation kinetic models of POD for some vegetables

Source	Temperature range (°C)	Kinetic model	References
Potato	60–85	First order	Anthon and Barrett, (2002)
Tomato	66–72	First order	Anthon et al, (2002)
Green beans	70–95	First order	Bifani et al, (2002)
Carrot	60–85	First order	Anthon and Barrett, (2002)
	35–75	Biphasic first order	Soysal and Soylemez, (2005)
Broccoli	70–95	Biphasic first order	Morales-Blancas et al, (2002)
Asparagus	70–95	Biphasic first order	Morales-Blancas et al, (2002)
Watercress	40–92.5	Biphasic first order	Cruz et al, (2006)

The first-order kinetic model is based on the assumption that the disruption of a single bond or structure is sufficient to inactivate the enzyme. The following processes have been found to be involved in thermal denaturation of peroxidase: (i) disassociation of the prosthetic group from the haloenzyme, (ii) a conformation

change in the apoenzyme, and (iii) modification or degradation of the prosthetic group. The first order kinetic model is based on the assumption of that the disruption of a single bond or structure is sufficient to inactivate the enzyme (Shalini et al, 2008). Biphasic first order model was based on the presence of two isoenzyme groups with distinct thermal stabilities, heat labile fraction that inactivates rapidly and a heat resistant fraction which can not be inactivated in shorter times (Weng et al, 1991). Lencki et al, (1992) inferred that when an apparent first-order behavior is observed during inactivation of the enzyme, the rate at which one of the phenomena happens determines the rate of inactivation and, when the rates of several of the phenomena involved are similar, non-linearity in the inactivation process results. The concept that thermal inactivation curves are the cumulative form of the chronological distribution of degradation events has been substantiated by several researchers. These, in turn, represent the spectrum of resistances to thermal inactivation within the enzymes. Regardless of the specific biophysical mechanisms that actually cause inactivation of enzymes, thermal residual activity curve indicates the total destructive effect of heat on the affected enzyme (Shalini et al, 2008).

Blanching is the common term used to describe heat inactivation of enzymes naturally present in the vegetables and always involves short time heating for periods of 2-3 min at temperatures between 70°C and 100°C. However, for such short time periods the relationship between blanching time at selected temperatures and residual POD activity shows non-linear kinetics (Robinson et al, 1989; Khan and Robinson, 1993).

Forsyth et al (1999) were reported that for many of the short time-heat treatments, non-linear curves consisting of approximately three phases: first an initial steep nearly straight line for a relatively short period, an intermediate curved portion and finally a shallow almost straight line for the third phase. Several mechanisms have been proposed to explain the observed deviation from linear first order kinetics. Frequently the presence of a mixture of thermostable and heat-labile isoperoxidases has been suggested to account for the non-linear heat inactivation plots observed in crude samples. In many instances it is highly probable that mixtures of heat-resistant and heat-labile isoenzymes were present and responsible for the deviation from first order kinetics as described typically by Ling and Lund (1978).

It has been suggested that the heat-inactivation process may involve the formation of thermostable aggregates (Lopez et al, 1994) or the process may follow series type inactivation kinetics with the formation of partially inactivated intermediates (Henley and Sadana, 1985). As an enzyme is a complex molecule with a three dimensional structure, heat-inactivation may well not follow first order kinet ics. For peroxidases, which also contain a haem substituent, it seems even more likely that different types of intrinsic conformational and chemical changes will continually take place during the early stages of heat inactivation. Tamura and Morita (1975) first suggested the loss of haem to form an apo-peroxidase. It has shown that haem fragments were formed during inactivation of a commercial preparation of horseradish POD and that non-linear kinetics of heat inactivation of horseradish peroxidase may be due to recovery and regeneration of peroxidase activity at acidic pH values (Adams, 1997a).

1.5.3.Inactivation by Ultrasonic

1.5.3.1. Ultrasonic science

Systematic studies of physical, chemical, and biological effects produced by ultrasound began in early 1900s. In 1917, Lord Raleigh developed a mathematical model for cavitation bubble collapse while investigating the problem of high-speed propeller erosion (Alliger,1975). Also in 1917, Langevin discovered that sound rays killed fish while studying sonar for antisubmarine warfare. The late 1920s was also an important time in ultrasound research with potential application in the food industry, with a number of important ultrasound effects identified (Nyborg, 2000; Nyborg, 2001).

Sound is nothing more than waves of compression and expansion passing through gases, liquids or solids. Human can sense these waves directly through our ears if they have frequencies from about Hertz to 16 kHz (the Hertz unit is cycles of compression or expansion per second; kiloHertz, abbreviated kHz, is thousands of cycles per second). These frequencies are similar to low frequency radio waves, but sound is intrinsically different from radio or other electromagnetic radiation. For example, electromagnetic radiation (radio waves, infrared, visible light, ultraviolet, x-rays, gamma rays) can pass through a vacuum without difficulty; on the other

hand, sound cannot because the compression and expansion waves of sound must be contained in some form of matter (Suslick, 1994).

Ultrasound is a form of energy generated by sound (really pressure) waves of frequencies that are too high to be detected by human ear, i.e. above 16 kHz (Jayasooriya et al, 2004). Ultrasound when propagated through a biological structure, induces compressions and depressions of the medium particles and a high amount of energy can be imparted. In dependence of the frequency used and the sound wave amplitude applied a number of physical, chemical and biochemical effects can be observed which enables a variety of applications (Got et al, 1999; Knorr et al, 2004).

In the food industry, a division into two distinct categories of ultrasound applications is done. For the classification of ultrasound applications the energy amount of the generated sound field, characterised by sound power (W), sound intensity (W/m^2) or sound energy density (Ws/m^3), is the most important criterion. Low energy (low power, low-intensity) ultrasound applications involve the use of frequencies higher than 100 kHz at intensities below 1 W/cm^2. Low intensity ultrasound uses a so small power level that the ultrasonic waves cause no physical or chemical alterations in the properties of the material through which the wave passes, that is it is generally nondestructive. They are successfully used for noninvasive monitoring of food processes. The most widespread application of low intensity ultrasound in the food industry is as an analytical technique for providing information about the physicochemical properties of foods, such as composition, structure and physical state (McClements, 1995; Fellows, 2000; Jayasooriya et al, 2004; Knorr et al, 2004).

The other group is high energy (high power, high-intensity) ultrasound which uses intensities higher than 1 W/cm^2 (typically in the range 10-1000 W/cm^{-2}) at frequencies between 18 and 100 kHz. Physical, mechanical or chemical effects of ultrasonic waves at this range are capable of altering material properties (e.g. physical disruption, acceleration of certain chemical reactions). High-intensity ultrasound has been used for many years to generate emulsions, disrupt cells and disperse aggregated materials. More recently various areas have been identified with greater potential for future development, e.g. modification and control of crystallization processes, degassing of liquid foods, enzymes inactivation, enhanced

drying and filtration and the induction of oxidation reactions. The beneficial use of the sound energy is realized through the various effects the ultrasound generates upon the medium where it transmits (Dolatowski et al, 2007).

Ultrasound has advantages over other traditional analytical techniques because measurements are rapid, non-destructive, precise, fully automated and might be performed either in a laboratory or on line. One of the most widespread and most promising ultrasonic applications is the utilization of ultrasound for composition measurement (Simal et al, 2003).

1.5.3.2. Mechanisms and effect

As ultrasound passes through a liquid, the expansion cycles exert negative pressure on the liquid, pulling the molecules away from one another. If the ultrasound is sufficiently intense, the expansion cycle can create cavities in the liquid. Once formed, small gas bubbles irradiated with ultrasound will absorb energy from the sound waves and grow. Cavity growth depends on the intensity of the sound. At high intensities, a small cavity may grow rapidly through inertial effects.

At lower acoustic intensities cavity growth can also occur by a slower process called rectified diffusion (Figure 1.1). Under these conditions a cavity will oscillate in size over many expansion and compression cycles. The growing cavity can eventually reach a critical size where it can efficiently absorb energy from the ultrasonic irradiation. Called the resonant size, this critical size depends on the liquid and the frequency of sound; at 20 kHz, for example, it is roughly 170 micrometers. At this point the cavity can grow rapidly during a single cycle of sound. Once the cavity has overgrown, either at high or low sonic intensities, it can no longer absorb energy as efficiently. Without the energy input the cavity can no longer sustain itself. The surrounding liquid rushes in, and the cavity implodes. It is the implosion of the cavity that creates an unusual environment for chemical reactions (Suslick, 1994).

The ability of ultrasound to cause cavitation depends on ultrasound characteristics (e.g. frequency, intensity), product properties (e.g. viscosity, surface tension) and ambient conditions (e.g. temperature, pressure). The ultrasound intensity required to cause cavitation increases markedly above about 100 kHz (Dolatowski et al, 2007).

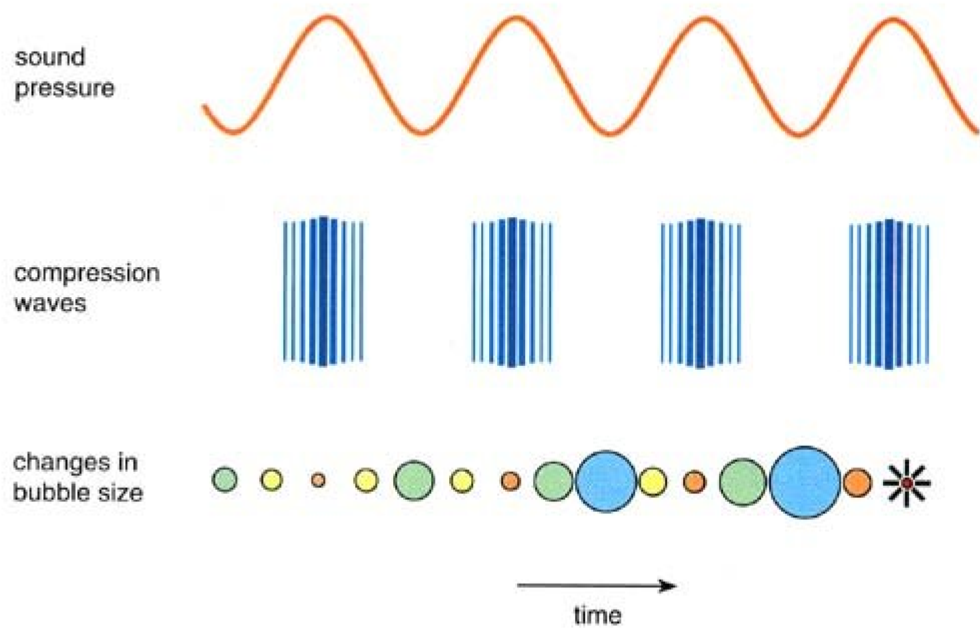


Figure 1.1. Motions of bubbles during cavitation

The inactivation effect of ultrasound is attributable mainly to a phenomenon called cavitation. Cavitation refers to the formation, growth, and implosion of tiny gas bubbles or cavities in a liquid when ultrasound travels through it. Extreme physical phenomena (1000 K and 500MPa) at micro-scale take place when the bubbles collapse, and these phenomena are considered to be the cause of enzyme inactivation. The stable cavitating bubbles interacting with the acoustic field generate strong micro-streaming and high shear, which also contribute to the observed enzyme inactivation (Raviyan et al, 2005).

Compression of a gas generates heat. This localized temperature increase by ultrasound treatment is difficult to measure and an overall increase of temperature is observed during treatment, which depends on the nature of the medium and the total energy input of ultrasound treatment (Susclik, 1994).

1.5.3.3 Application types of sonication

Ultrasonication (US) is operated at low temperature. Therefore, a product with heat sensible components can be treated. However, the treatment time is actually long during the inactivation of enzymes and/or microorganisms, which may cause high

energy requirement. Normally, this treatment will need to be combined techniques to optimize the process.

Thermosonication (TS) is a combined method of ultrasound and heat. The product is subjected to ultrasound and moderate heat simultaneously. This method produce a greater effect on enzyme inactivation than heat alone. Heat combined with ultrasound reduces process temperatures and processing times, for pasteurization or sterilization processes that achieve the same lethality values as with conventional processes (Mason et al, 1996; Villamiel et al, 1999).

Manosonication (MS) is a combined method in which ultrasound and pressure are applied. MS provides to inactivate enzymes and/or microorganisms by combining ultrasound with moderate pressure 100-300 kPa at low temperatures.

Manothermosonication (MTS) is a combined method in which heat and ultrasound under pressure are applied. The ultrasound generates the cavitation or bubble implosion in the media. This implosion can cause inactivation of enzyme and destruction of microorganisms. The simultaneous pressure treatment maximizes the intensity of the explosion, which increase the level of inactivation. MTS treatments allow to inactivate several enzymes at lower temperatures and/or in a shorter time than thermal treatments at the same temperatures (Vercet et al, 2001).

1.5.3.4. Application of ultrasound on enzyme inactivation

Enzyme inactivation is a requisite for stabilisation of some food materials. Although it can be easily achieved by heat treatment, there are some cases where the high heat resistance of some enzymes makes heat treatments not a solution of the problem but a problem by itself, because heat can negatively modify some food properties such as flavour, colour or nutritional value. This is the driving force for the increased interest in alternative methods of enzyme inactivation. One such alternative method is ultrasound, i.e. sonic waves above 20 kHz (Vercet et al, 2000).

Enzyme inactivation by ultrasound depends on the conditions of the treatment as well as on the nature of the enzyme. MTS treatment has an increased effectiveness of enzyme inactivation compared with ultrasound alone (Earnshaw et al, 1995). The

effect of MTS on lipoxygenase, peroxidase, and polyphenoloxidase, as well as heat-resistant lipase from Pseudomonas fluorescens, within the temperature range of 110-140°C MTS inactivated the enzyme more effectively than the heat treatment alone (Vercet et al, 1997). MTS at 650 kPa and 140°C reduce the level of protease activity to 6% and lipase to 7% when compared to activity levels obtained when employing thermal treatment alone. However, the effective improvement achieved using this combined treatment decreased as the treatment temperature increased. MTS could be useful to inactivate those enzymes within food materials that do not require such high temperatures for preservation (Zeuthen and Sorensen, 2003).

Ultrasound increases the effectiveness of heat inactivation of enzymes and has been demonstrated in enzymes derived from plants soybean lipoxygenase (Lopez et al, 1994), horseradish peroxidase (Lopez et al, 1994; Lopez and Burgos, 1995; De Gennaro et al, 1999), watercress peroxidase (Cruz et al, 2006), mushroom polyphenol oxidase (Lopez et al, 1994), orange pectinmethylesterase (Vercet et al, 1999), tomato pectinmethylesterase, and polygalacturonase (Vercet et al, 2002b), animal tissues (porcine heart malate dehyrogenase) (Özbek and Ülgen, 2000), microorganisms (Pseudomonas fluorescens lipase and protease) (Vercet et al, 2001), alcohol dehydrogenase and glucose-6-phosphate dehyrogenase from baker´s yeast (Özbek and Ülgen, 2000), β-galactosidase from Escherichia coli (Özbek and Ülgen, 2000), and milk (bovine lactoperoxidase and alkaline phosphatase (Ertugay et al, 2003), alkaline phosphatase, γ-glutamyltranspeptidase and lactoperoxidase (Villamiel and Jong, 2000).

The ultrasound stability of individual proteins varies between the enzymes (Lopez et al, 1994; Özbek and Ülgen, 2000; Vercet et al, 2001; Vercet et al, 2002b) and also depends on ultrasound treatment conditions (Raviyan et al, 2005), the composition of treatment medium, treatment pH, and whether they are bound (e.g., membrane-bound proteins) or free (e.g., cytoplasmic proteins). Enzyme inactivation generally increases with increasing ultrasound power, ultrasound freqency, exposure time, amplitude level, cavitation intensity, processing temperature and processing pressure, but decreases as the volume being treated increases (Özbek and Ülgen, 2000; Vercet et al, 2001; Vercet et al, 2002a; Raviyan et al, 2005).

A combined heat and ultrasound treatment can produce a markedly greater effect on enzyme inactivation than heat alone. Orange pectinmethylesterase in orange juice was inactivated relatively slow by heat alone (72°C, D value of 500 min) while the combined heat and ultrasound treatment (72°C, 20 kHz, 117 µm, 350 kPa) gave a much lower D value (1.2 min) (Vercet et al, 1999). Ultrasound was also effective at a temperature (38°C) where thermal inactivation is nonsignificant, giving a pectinmethylesterase in orange juice, D value of 11 minute. The rate of inactivation of tomato pectinmethylesterase was also greatly increased by a combination heat and ultrasound, with increasing cavitation intensity dramatically increasing the rate of inactivation (Raviyan et al, 2005). Smilarly for Pseudomonas fluorescens heat resistant lipase, a combined ultrasound and thermal treatment reduced lipase D values by 25 to 86 and protese D values by 15 to 67 compared to the heat only treatment at the same temperature (Vercet et al, 2002b) .

In 1994 a research group headed by Burgos initiated the study of the application of MTS to model enzymes releavent to the food industry (peroxidase, lipoxygenase and polyphenoloxidase) in model buffer systems. MTS treatments proved to be much more efficient than heat treatment for inactivating these enzymes, especially those which are more thermally labile (lipoxygenase and polyphenoloxidase) (Sun, 2005).

Pectic enzymes of tomatoes, pectic methylestearase and the two endopolygalacturonase isozymes are also inactivated by MTS treatments with much higher efficiency, both in model systems (Lopez et al, 1998) and in tomato juice (Vercet et al, 2002a). General trends arose from all these enzyme inactivation studies; thermobile enzymes are more sensitive to ultrasound than those which are heat resistant (Sun, 2005).

The use of ultrasound at ambient pressure has also been successfully used to inactivate food relevant enzymes. Peroxidase was inactivated by combinations of heat and ultrasound at neutral (Gennaro et al, 1999) or low pH (Yoon-Ku et al, 2000) and lipoxygenase has been shown to be inactivated at low sonication intensities (Thakur and Nelson, 1997).

1.5.4. Effect of POD in food processing

POD is a ubiquitous enzyme in plant cells. It is related to food quality in processing, and can contribute to adverse changes in the flavor and color of both raw fruits and processed products. Miesle et al (1991) pointed out that POD promotes lipid oxidation with consequent off-flavor formation. In fact, phenolic oxidation mediated by POD is believed to be associated with deterioration in flavor, color, texture and nutritional qualities of processed foods (Fang et al, 2008).

PODs have a broad substrate specificity towards different H-donors, including phenols, aromatic amines (Burnette, 1977), anthocyanins, lignin and vitamin C. The enzyme's involvement in such a range of reactions has led to POD being implicated in several development and postharvest changes in plant tissues (Haard, 1977); but this has also made its physiological role in living plants and its deleterious action on food quality difficult to assign precisely. A relationship with off-flavour in peas was claimed by Wagenknecht and Lee (1958) and Pinsent (1962). Wagenknecht and Lee (1958) also found that off-flavour developed when preparations of horseradish PODs were added to pea slurries. PODs have also been implicated in loss of colour and changes in texture, e.g. toughening of asparagus tips due to lignification (Haard, 1977). Bruemmer et al (1976) found a negative correlation between POD activity and flavour scores for orange juices.

POD is the most thermally stable enzyme in vegetable systems and for this reason, it is usually used as an index of blanching effectiveness. Heating for complete inactivation of POD is more than adequate to destroy the enzymes responsible for quality loss and often leads to overblanching. Therefore, the quality of the blanched products may be better if there is some activity of POD left at the end of the blanching (Agüero et al, 2008).

It catalyses a reaction in which hydrogen peroxide acts as the acceptor and another compound acts as the donor of hydrogen atoms. It is involved in enzymatic browning since diphenols may function as reducing substrates in this reaction. The involvement of POD in browning is limited by the availability of electron acceptor compounds such us superoxide radicals, hydrogen peroxide and lipid peroxides (Serrano-Martinez et al, 2008).

This enzyme is of great interest in food technology because of its influence on the quality of raw and processed fruits and vegetables**.** In the presence of peroxide, POD produces phytotoxic free radicals which react with a wide range of organic compounds (ascorbic acid, carotenoids and fatty acids), leading to losses in the colour, flavour and nutritional value of raw and processed foods (Serrano-Martinez et al, 2008).

It has generally been observed that POD is quite stable to adverse conditions encountered during food processing such as elevated temperatures, freezing, ionizing radiation, dehydration and even when inactivated by such treatments is capable of re-naturation. Regenaration of POD activity can posses a serious limitations to a food processing operation. For example, fruit and vegetables subjected to high temperature and short time treatments (HTST) are particularly prone to POD regeneration and associated quality change during storage. Regeneration of POD occurs within hours or days following thermal processing and may occur even after several months in frozen fruits and vegetables. The resistance of POD to thermal inactivation, together with ubiquity and direct involment in deterioration of food quality, has lead to the wide use of POD activity as an index of processing efficiency by blanching and other heat treatments (Ory and Angelo, 1977).

1.5.5. Effect of heat and ultrasound on vitamin C and microorganisms

In the food industry, eliminating harmful microorganisms and inactivating enzymes are important for food quality and also for public health. That is the reason why heat treatment is the most utilized method for stabilizing foods because of its capacity to destroy microorganisms and also inactivating enzymes. Heat can alter the organoleptic properties of foods and diminish the contents or bioavailability of some nutrients (Ulusoy et al, 2007).

In recent years, the food industry has discovered that ultrasonic waves have a wide variety of applications in the processing and evaluation of products. From grading beef to sterilization, ultrasound has a number of applications in an increasing number of areas in the food industry. In combination with heat, it can accelerate the rate of sterilization of foods, thus lessening both the duration and intensity of thermal treatment and the resultant damage. (Piyasena et al, 2003).

In modern food technology, the trend is to maximize the nutrients retention in both processing and storage. It is generally observed that, if ascorbic acid is well retained, the other nutrients are also well retained. Hence, ascorbic acid is usually considered as an index of nutrient quality during processing and storage of foods (Nicoleti et al, 2004).

Vitamin C is instable thermic and therefore in fruit and vegetables it provides an indication of the loss of other vitamins and acts as a valid criterion for other sensorial or nutritional components, such as natural pigments and aromatic substances. Its concentration decreases during storage, depending on storage conditions, such as temperature, oxygen content and lights (Matei et al, 2009).

The rate of vitamin C destruction is increased by the action of metals, especially copper and iron, and enzymes. Availability of oxygen, prolonged heating in the presence of oxygen and exposure to light are all harmful factors to vitamin C content of foods (Deman, 1990). When oxygen is present, the contribution of the anaerobic degradation to the total vitamin C loss is small or not noticeable, compared to the aerobic degradation which has a much higher degradation rate (Villota and Hawkes, 1992). Some studies have been reported on the degradation of vitamin C in thermally processed fruits and vegetables like grapefruit juice (Saguy et al, 1978), orange juice (Johnson et al, 1995), lime, lemon, grapefruit and tangerine (Alvarado and Viteri, 1989), cupuaçu nectar (Vieira et al, 2000) tomatoes (Dewanto et al, 2002), green asparagus (Esteve et al, 1999), amla (Nisha et al, 2004), drumstick (Bineesh et al, 2005) and strawberry products (Castro et al, 2004).

Investigation of ultrasound as a potential microbial inactivation method began in the 1960s, after it was discovered that the sound waves used in anti-submarine warfare killed fish (Earnshaw et al, 1995). The mechanism of microbial killing is mainly due to thinning of cell membranes and production of heating (Butz and Tauscher, 2002; Fellows, 2000). During the sonication process, waves are created when a sonic wave meets a liquid medium, thereby creating regions of alternating compression and expansion (Sala et al, 1995). These regions of pressure change cause cavitation to occur, and gas bubbles are formed in the medium. These bubbles have a larger surface area during the expansion cycle, which increases the diffusion of gas, causing

the bubble to expand. A point is reached where the ultrasonic energy provided is not sufficient to retain the vapour phase in the bubble; therefore, rapid condensation occurs. The condensed molecules collide violently, creating shock waves. These shock waves create regions of very high temperature and pressure. The pressure changes resulting from these implosions are the main bactericidal effect in ultrasound. The hot zones can kill some bacteria, but they are very localized and do not affect a large enough area.

1.5.6. Tomato

The tomato is a member of the Solanaceae family. Botanically, it is a berry fruit, but it is cultivated and used as a vegetable. It is a tender, warmseason perennial. Nutritionally, the tomato is a good source of vitamin A and C. Composition data varies due to the wide range of species, stage of ripeness, year of growth, climatic conditions, light, temperature, soil, fertilization, irrigation, and other conditions of cultivation, and handling and storage (Yılmaz, 2001).

Tomato is one of the main vegetables consumed fresh in the world. However, tomato fruit is still mainly used as raw material for industrial processing. For example, in the USA, more than 80 % of the tomato annual consumption is consumed in the form of processed products (juices, purees or pastes). For these reasons, the optimum blanching conditions should have specific inactivation of the deleterious enzymes and minimization of quality losses (Wang et al, 2008).

Wild tomato plants are still found from Ecuatorto Chile, as well as on the Galapacos Island, although only two have edible fruit: *Lycopersicon esculentum* (the commen tomato in wide used today) and *Lycopersicon pimpielliform* (sometimes cultivated under the name currant tomato). A small fruited type *Lycopersicon esculentum* var. *cerasiforme*, cultivated under the name cherry tomato, is widely distributed as a wield plant in the tropics and subtropics (Jones, 2007).

Types of tomatoes based on plant characteristics :

(a) Midget, patio, or dwarf tomato varieties have very compact vines best grown in hanging baskets or other containers. The tomatoes produced may be, but are not

necessarily, the cherry type (1 inch diameter or less). Some produce larger fruit. These plants are usually short-lived, producing their crop quickly and for a short period.

(b) Compact or determinate tomato plants refers to the plant habit of growing to a certain size, setting fruit, and then declining. Most of the early ripening tomato varieties are determinate.

(c) Indeterminate tomato plants are the opposite of the determinate types. The vines continue to grow until frost or disease kills them. These are the standard, all-summer tomatoes that most people like to grow. They require support of some kind for best results, since otherwise the fruit would be in contact with the soil, thus susceptible to rot.

Types of tomatoes based on fruit characteristics

(a) Cherry tomatoes have small, cherry-sized (or a little larger) fruits often used in salads. Plants of cherry tomatoes range from dwarf (Tiny Tim) to sevenfooters (Sweet 100). One standard cherry tomato plant is usually sufficient for a family, since they generally produce abundantly.

(b) Beefsteak type tomatoes are large-fruited types, producing a tomato slice that easily covers a sandwich, the whole fruit weighing as much as two pounds or more. These are usually late to ripen, so plant some standard-sized or early tomatoes for longest harvest.

(c) Paste tomatoes have pear-shaped fruits with very meaty interiors and few seeds. They are less juicy than standard tomatoes and are without a sizeable central core. Paste tomatoes are a favorite for canning since they don’t have to be cut up and since they are so meaty.

(d) Color of tomatoes include orange, yellow, pink, or striped, and often the only way to get a specific one is by growing your own. Most are heritage varieties obtained through seed-saver groups. Tests have shown that there is no relationship between color and acidity of tomatoes.

(e) Winter storage tomatoes are a relatively new item for gardeners. The plants are set out later in the season than most tomatoes and fruit are harvested partially ripe. If properly stored, they will stay fresh for twelve weeks or more. While the flavor does not equal that of summer vine-ripened tomatoes, many people prefer them to grocery store tomatoes in winter (Relf et al, 2004).

1.6. Aim of the thesis

POD is one of the most heat stable and widely distributed enzymes in the plant kingdom. Active PODs are known to alter food flavor, color, texture and nutritional qualities of raw and processed foods (Svensson et al, 1977).

Application of heat treatment is the most utilized method for stabilising foods, because of its capacity to destroy microorganisms and inactivate enzymes. However, since heat can impair, as well, many organoleptic properties of foods and reduce the contents or bioavailability of some nutrients (Lopez et al, 1994). This is the driving force for the increased interest in alternative methods of enzyme inactivation. One such alternative method is ultrasound, i.e. sonic waves above 20 kHz (Suslick, 1990). The advantages of ultrasound over heat treatment include: Minimization of flavor loss, greater homogenity and significant energy savings (Earnshaw et al, 1995).

The aim of this thesis was to inactivate tomato POD with heat and ultrasound treatments and to determine kinetic properties of tomato POD by using guiacol and o-dianisidine as hydrogen donors. Effects of ultrasound and heat treatments on microorganisms and vitamin C were studied.

CHAPTER II
MATERIALS AND METHODS

2.1. Materials

Ripe, fresh tomatoes were supplied from commercial markets. o-dianisidine, guaiacol and hydrogen peroxide were obtained from Sigma Chemical Company, (USA). The stock solution of o-dianisidine was prepared freshly in 100% methanol and solutions of other reagents, guaiacol and hydrogen peroxide were prepared using distilled water. The common reagents used were all reagent grade (Merck or Riedel de Haen).

2.2. Methods
2.2.1. Composition of tomato
2.2.1.1. Total moisture
Moisture content of tomato was determined by both oven and infrared dryer. It was determined according to AACC Approved Methods, 1995. For oven method, tomato was heated at 105 °C for 3.5 hours and for infrared dryer at 105 °C for 30 minute.

2.2.1.2. Total sugar
Sugar content was determined by using phenol-sulfuric acid method (Dubois et al, 1956). 10 gram of tomato was hydrolysed in 450 ml of 6 N HCl solution at 98°C for 8 hours. Then, it was filtered by using Whatman 40 filter paper. Obtained hydrolysate was used for determination of the total sugar content of tomato. 2 mg/mL glucose was used as the stock solution and dilutions in the range of 10 to 50 mg/mL were prepared and used as the standard. Then, 0.1 mL of 80% (w/v) phenol was added to each of samples. After the addition of 5 mL of 96% sulfuric acid rapidly, it was waited for 10 minute. After waiting, tubes were shaked and placed in water bath (25-30°C) for 20 min. The absorbance of obtained orange-yellow color of Total sugar content of tomato was determined as glucose by using calibration curve.

2.2.1.3. Ash content

Ash content of tomato was determined in Nüve MF 120 type oven (according to AACC Approved Methods, 1995). Defined amounts of blended tomato was ignited at 550°C for 4 hours. Ash content was calculated from the remaining sample.

2.2.1.4. Protein content

Protein content of tomato was determined by Kjeldahl method (AOAC, 1990) with digestion, distillation and titration steps. For digestion: 10 gr potassium sulfate, one spatule of copper sulfate and boiling chips were added to digestion tube that contain 10 gr of blended tomato sample and heated until digestion was completed. Clear light green color indicated the end of the digestion. For distillation: 50 mL water, 50 mL of 50% sodium hydroxide and steam was used in instrument and distillate was obtained into erlenmayer flask which has 25 mL of 4% boric acid. For titration: methyl red was dropped as an indicator and the samples were titrated by 0.1 N hydrochloric acid. Obtained volume from titration was used for the determination of amount of $N\text{-}NH_4$ and protein content was obteined by multiplying this value by 6.25.

2.2.1.5. Vitamin C content

Indephenol titration method was used for the determination of vitamin C content of tomato extract (Egan et al, 1981). In this method ascorbic acid was determined by titration with the dye 2,6-dichlorophenolindophenol (DCP) which is reduced by the ascorbic acid to a colourless form. Reagents:

Acid solution: 2 gr of metaphosphoric acid (HPO_3) was dissolved with distilled water at 60 °C . Next, it was cooled to room temperature and 8 mL glacial acetic acid was added and volume was completed to 100 mL with distilled water. The soluion was kept in refrigerator and used within 7-10 days.

Dye solution: 250 mg 2,6-dichlorophenolindophenol sodium salt was dissolved in water at 60°C and made up to 500 mL. The solution was coolled to room temperature then stored at refrigerator. Dye solution must be protected from air and light and filtered before use.

Standard ascorbic acid solution: 100 mg ascorbic acid was dissolved in 10 mL acid solution and then volume of the solution was completed to 100 mL with distilled water. Solution was prepared daily.

For the standardization of dye solution; 10 mL ascorbic acid solution was mixed with 10 mL acid solution and titrated with dye solution until pink colour was observed for at least 5 second. Then dye titer was calculated in terms of mg ascorbic acid per mL dye solution.

For determination of vitamin C in tomato extract; 10 mL tomato extract was mixed with 10 mL acid solution and titrated with dye solution. Obtained titer volume and dye titer were used for the calculation of ascorbic acid content in tomato.

2.2.2. Extraction of tomato POD

Tomatoes were washed and cut into small pieces and blended in Waring blender at high speed for 30 sec with 20% (w/v) water addition at 4°C . The slurry was filtered through two layers of cheese cloth and the filtrate was centrifuged for 10 min at 11000 rpm in an Eppendorf model 5810 R centrifuge at 15°C (Hemeda and Klein, 1990). The supernatant was kept frozen approximately at -20°C and used as the enzyme source.

2.2.3. POD assay and kinetics

POD activity of tomato was measured using guaiacol and o-dianisidine as hydrogen donors. All analysis were done at least in dublicate. The changes in absorbance were read by using PerkinElmer Lambda 25 UV-VIS spectrophotometer at 25°C. The wavelengths of 470 nm for guaiacol and 460 nm for o-dianisidine were used for activity measurements. Kinetic parameters such as K_m and V_{max} were determined using Lineweaver-Burk plot.

2.2.4. POD assay and kinetics by guaiacol

POD activity was determined spectrophotometrically as the change in absorbance at 470 nm. The reaction mixture contained 1.6 mL of 0.01 M KP buffer (pH 6.5) containing 1 mL 1.0% (v/v) guaiacol; 0.3 mL of 0.1% (v/v) H_2O_2 and 0.1 mL

enzyme extract (Cruz et al, 2006; Sakharov and Ardila, 1999). For the determination of kinetic parameters, H_2O_2 concentrations from 0.0987 mM to 9.78 mM were used at 75.4 mM guaiacol concentrations.

2.2.5. POD assay and kinetics by o- dianisidine

POD activity was determined spectrophotometrically at 460 nm as described by Robinson et al, (1989). The reaction mixture consisted of 2.7 mL of 0.01 M acetate buffer (pH 5.0); 0.1 mL of 0.1% (v/v) H_2O_2, 0.1 mL of 0.05% (w/v) o- dianisidine in methanol and 0.1 mL enzyme extract (Shalini et al, 2008). For the determination of kinetic parameters, H_2O_2 concentrations from 0.0653 mM to 1.956 mM were used at 6.8 x 10^{-5} mM guaiacol concentrations.

2.2.6. Inactivation of tomato POD

2.2.6.1. Thermal treatment

Thermal treatment of tomato POD was performed at varying temperatures in the range 63, 64, 65, 66 and 67°C with exposure times 2, 4, 6, 8, 10, 12, 15, 20 and 25 min. Crude extract (1.5 mL) in test tubes were heated in the water bath at defined temperatures and samples were removed at different time intervals and rapidly cooled in ice-bath to stop thermal inactivation instantaneously. Enzyme activity was determined with o-dianisidine as hydrogen donor.

2.2.6.2. Ultrasound treatment

The ultrasonication experiments were carried out at 23 kHz on a Soniprep 150 Ultrasonic Disintegrator equipped with a horn of 9.5 mm diameter. The tip of the horn was immersed about 1 cm into 10 ml solution to be processed. The solution was processed with the sonication horn for 20-150 s and amplitude level changed between 3-15 μm. Throughout this study these levels were mentioned as ultrasonic power. Amplitude level of 3 μm corresponded to 15% ultrasonic power, and amplitude levels of 5, 8, 10 and 15 μm corresponded to 25, 40, 50 and 75% ultrasonic powers, respectively. The temperature inside the solutions was checked before and after the process. Samples were removed at different time intervals and rapidly cooled in ice-bath. Enzyme activity was determined with o-dianisidine as hydrogen donor.

2.2.7. Regeneration of tomato POD

Tomato extract was treated with ultrasound and regeneration of POD activity was determined. Enzyme extract was processed with the sonication horn amplitude level between 3-15 μm for 20-150 seconds and then enzyme activity was determined with o-dianisidine as hydrogen donor. Samples were stored at 4°C and residual enzyme activity was measured daily. Peroxidase activity was expressed as percent of residual activity.

2.2.8. Microbial analysis

Total bacteria and mold-yeast counts were made for ultrasound treated, heat treated and untreated tomato extract. Aerobic plate count (PCA) for total bacteria and potato dextrose agar (PDA) for mold yeast count was used for analysis (Jay, 1986).

A 0.2 ml of sample was transferred into corresponding labelled plate and spread plated over the agar surface. Inoculated PCA plates were incubated at 37°C at 24 hours. Inoculated PDA plates were incubated at 25°C for 2 to 5 days. After incubation, number of colony forming units were counted and expressed as number of microorganisms per gram of tomato extract.

2.2.9. Statistical analysis

SPSS 8.0 software for Window was used to perform statistical anaysis. Experimental results were subjected to two way analysis of variance (ANOVA). A level of significance of $P<0.05$ is used throughout the analysis. Duncan's multiple range test method was used for determination of the effects of inactivation parameters. Equations used for modelling:

- Hyperbolic equation of (2.1) was used for Michaelis-Menten kinetics by guaiacol and o-dianisidine where

$$y = ax/(b+x) \qquad (2.1)$$

$a = V_{max}$ (abs/min) and $b = K_m$ (M), y = enzyme activity (abs/min), x = substrate concentration (M).

- Exponential equation of (2.2) was used for regenerated peroxidase activity versus storage time when samples were treated at 40%, 50% and 75% ultrasonic powers where

$$y = a(1-e^{-bx})+c \qquad (2.2)$$

a is the amplitude of the exponential, b is the rate constant (min^{-1}), c is the intercept (activity at time=0), a+c is the maximum of the exponential (regeneratable A_{max}).

- Linear regression eqution of (2.3) was used for linerization of many graphes.

$$y=ax+b \qquad (2.3)$$

Such as calibration curve for sugar determination, change of POD activity with tomato extract by o-dianisidine and guaiacol, Lineweaver-Burk plot of POD action on hydrogen peroxide in the presence of guaiacol and o-dianisidine, the plot of natural logarithm of residual activity versus heating time, Arrhenius plot of the inactivation rates of tomato POD, temperature of the extract after sonication, the plot of natural logarithm of ultrasonic inactivation of tomato POD, regenerated POD activity versus storage time when samples were treated at 15% and 25% ultrasonic powers.

CHAPTER III
RESULT AND DISCUSSION

3.1. Composition of tomato

3.1.1. Moisture content

Moisture content was found to be 93.88% and 93.32% by infrared dryer and oven method, respectively. It was reported that moisture content of tomato change between 93-94% (Suarez et al, 2007) and 94.7% by Elliott et al (1981).

3.1.2. Total sugar content

Sugar content of tomato was determined by using phenol-sulfuric acid method. Total sugar content of the tomato was found as 5.06% by using calibration curve shown in Figure 3.1. Jones (2007) reported the similar value of 3.9-4.3%. Glucose was used as standard sugar that suitable to correlate the obtained values with literature values.

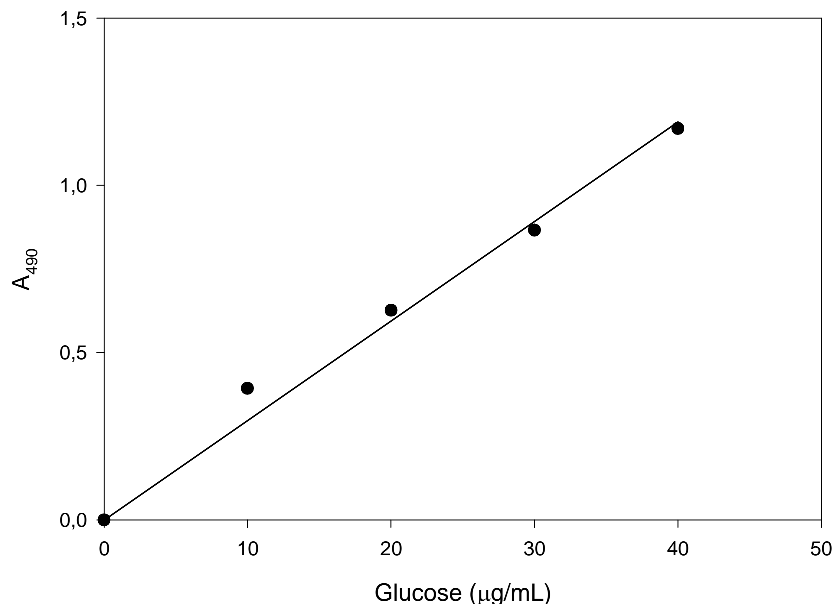


Figure 3.1. Calibration curve for sugar determination

3.1.3. Ash content

Ash content was found to be 0.55%. It was reported that ash content of tomato change between 0.50-0.75% (Suarez et al, 2007).

3.1.4. Protein content

Protein content of tomato was found as 0.47%. This value was lower than the values reported as 0.73-0.83% by Suarez et al, (2007) and as 1.1% by Jones (2007).

3.1.5. Vitamin C content

Vitamin C content of tomato was found as 20.8 mg per 100 g tomato by indephenol titration method. This value was lower than those reported as 23.4 mg /100 g tomato by Jones, (2007) and 25 mg /100 g tomato by Grolier et al (1998).

3.2. Activity of tomato POD

Peroxidase activity of tomato extract was determined by using guaiacol and o-dianisidine as hydrogen donors at temperature of 25°C.

3.2.1. Activity by guaiacol

POD activity of tomato extract was determined by using guaiacol (2-methoxyphenol) as hydrogen donor. The increase in absorbance as a result of colored products of oxidized guaiacol was measured at 470 nm. Highest activity was observed at 0.1% (v/v) guaiacol and 0.1% (v/v) hydrogen peroxide (Figure 3.2). The effect of pH on the activity of enzyme was studied with 0.01 M potassium phosphate buffer (pH of 6.0, 6.5 and 7.0) and 0.01 M acetate buffer (pH of 4.5, 5.0 and 5.5). Peroxidase exhibited its maximum activity at pH 6.5 (Figure 3.3).

3.2.2. Activity by o-dianisidine

POD activity of tomato extract was determined by using o-dianisidine (3,3'-dimethoxybenzidine) as hydrogen donor. Hydrogen peroxide reacts with o-dianisidine in the presence of peroxidase to form a colored product. The increase in absorbance as a result of colored products of oxidized o- dianisidine was measured at 460 nm. Highest activity was observed at 0.05% (w/v) o-dianisidine and 0.1% (v/v) hydrogen peroxide (Figure 3.4). The effect of pH on the activity of enzyme was studied with 0.01 M potassium phosphate buffer (pH of 6.0, 6.5 and 7.0) and 0.01 M

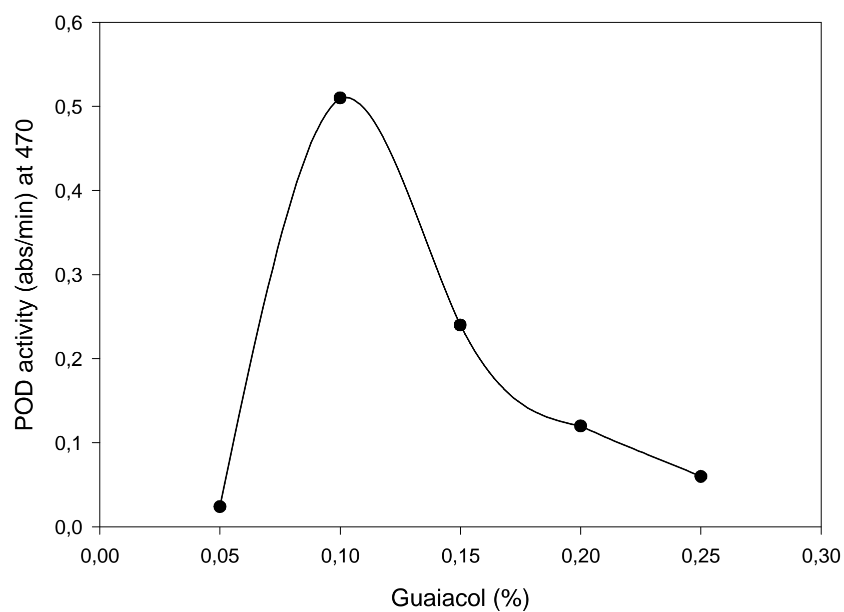


Figure 3.2. Change of POD activity with guaiacol concentration with 0.978 mM H_2O_2, Extract: 0.572 mg protein/mL

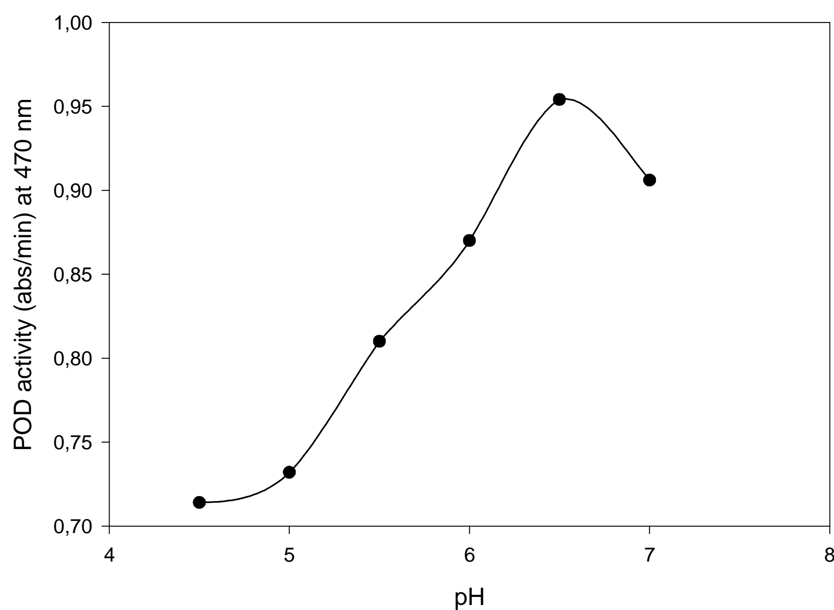


Figure 3.3. Effect of pH on POD activitiy with guaiacol, buffer: 0.01 M, H_2O_2: 0.978 mM, Guaiacol: 0.1% (v/v), Extract: 0.572 mg protein/mL, T= 25°C

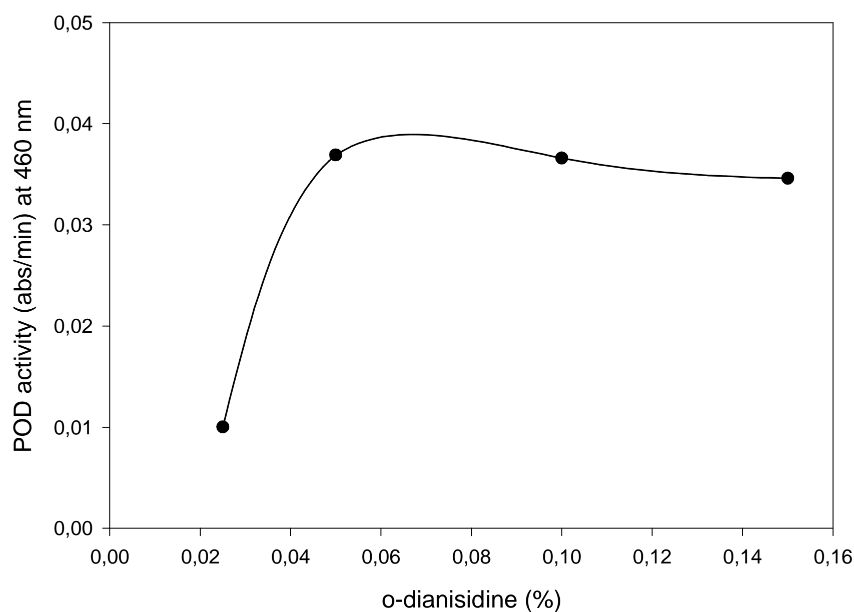


Figure 3.4. Change of POD activity with o-dianisidine concentration with 0.326 mM H_2O_2, Extract: 0.572 mg protein/mL

acetate buffer (pH of 4.5, 5.0 and 5.5) at fixed substrate (0.326 mM) and o-dianisidine ($6.8x10^{-5}$ mM) concentrations. Maximum enzyme activity was observed at pH 5.0 (Figure 3.5). Enzyme activity versus enzyme concentration at 0.1% (v/v) hydrogen peroxide concentration showed linear relationship with both guaiacol and o-dianisidine (Figure 3.6.).

3.3. Kinetics of tomato POD

Kinetics of tomato POD was determined by using guaiacol and o-dianisidine as hydrogen donors. By guaiacol, peroxidase activity was determined by KP buffer (pH 6.5) at 470 nm and by o-dianisidine it was determined by acetate buffer (pH 5) at 460 nm.

3.3.1. Kinetics by guiacol

Kinetics of tomato POD was studied at 75.4 mM guaiacol and 0.0987 mM to 9.78 mM hydrogen peroxide concentration at fixed enyzme concentration. Data obtained from experiments was used for nonlinear equation of (y = ax / (b+x)) by using Sigma

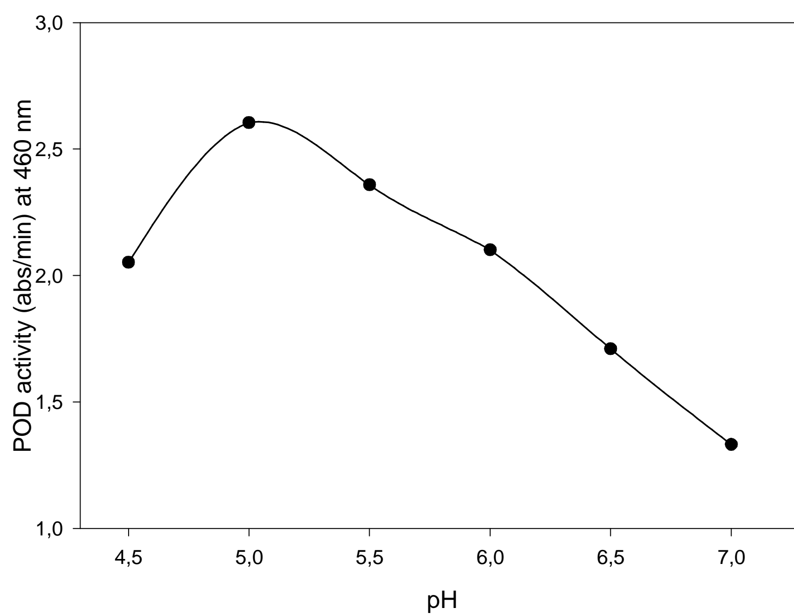


Figure 3.5. Effect of pH on POD activitiy with o-dianisidine, buffer: 0.01 M, H_2O_2: 0.326 mM, o-dianisidine: 0.05% (w/v), Extract: 0.572 mg protein/mL, T= 25°C

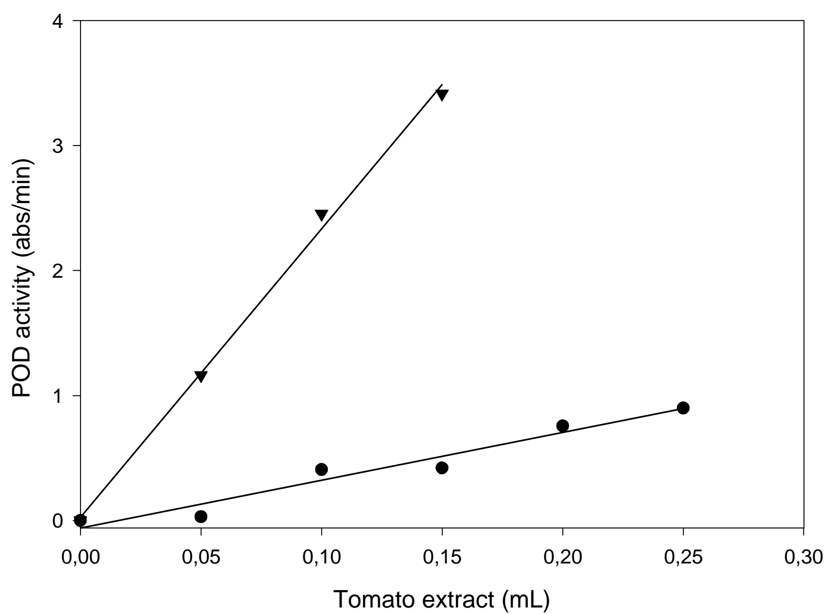


Figure 3.6. Change of POD activity with tomato extract by o-dianisidine at 460 nm (▼), by guaiacol at 470 nm (●)

Plot 2000 (Jandel Sientific, San Francisco, USA), where a = V_{max} (abs/min) and b = K_m (M). Then predicted data and equation's constants were calculated. Experimental data and predicted data from the model were compared with correlation coefficients (r^2), that was found as 0.97. Figure 3.7 shows that enzyme follows Michaelis Menten Kinetics in the substrate concentration ranges of 0.0987 mM to 9.78 mM.

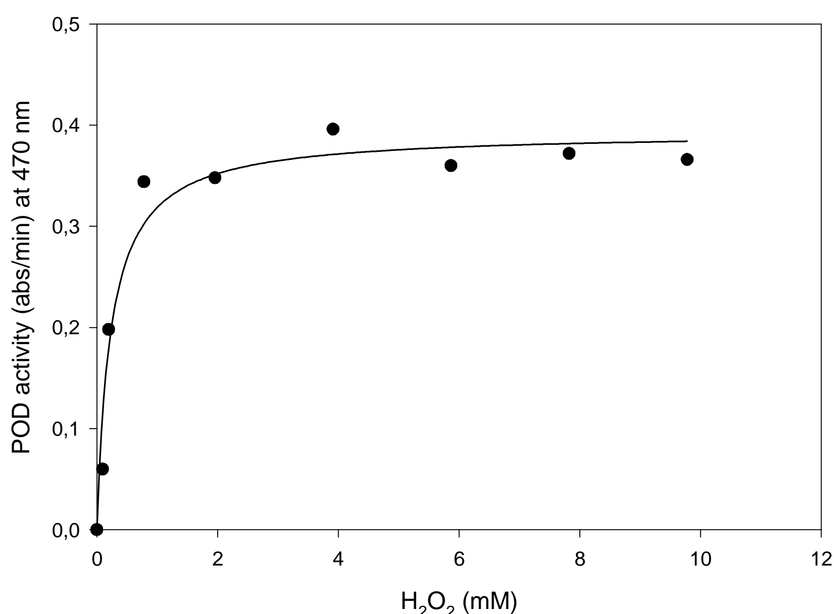


Figure 3.7. Initial rate versus substrate concentration by guaiacol, Extract: 0.572 mg protein/mL

Equation of y = ax / (b+x) was obtained by Michaelis-Menten kinetics. Constants of equation (a, b) were calculated, where a= $3.9x10^{-1}$ and b= $2.34x10^{-1}$. The Lineweaver-Burk plot was obtained as linear and linear correlation coefficient (r^2) was found as 0.90 in Figure 3.8 and V_{max} and K_m values were found as 0.52 abs/min and 0.66 mM, respectively. K_m value for hydrogen peroxide by using guaiacol as hydrogen donor was higher than the value of 4 mM for tomato peroxide (Jen et al, 1980) and 7.2 mM for green pea (Halpin et al, 1989).

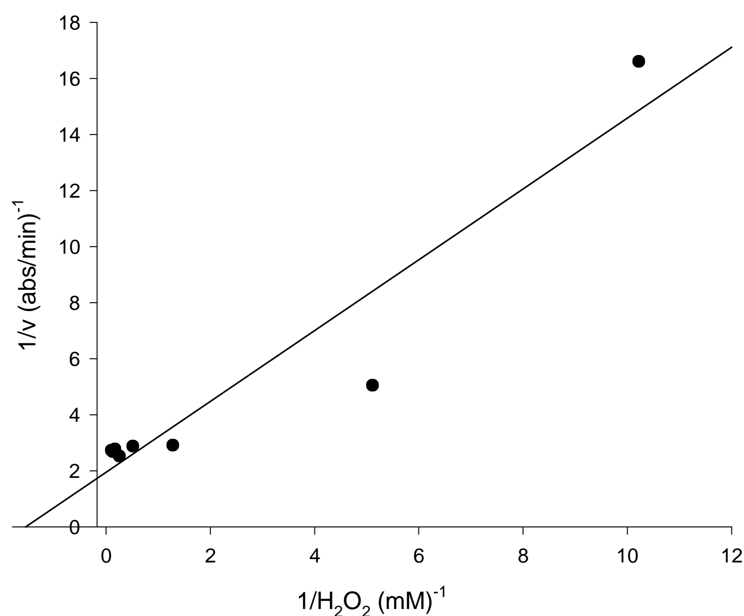


Figure 3.8. Lineweaver-Burk plot of POD action on hydrogen peroxide in the presence of guaiacol

3.3.2. Kinetics by o-dianisidine

Kinetics of tomato POD was studied at 6.8 x 10^{-5} mM o-dianisidine in methanol and 0.0653 mM to 1.956 mM hydrogen peroxide concentration at fixed enzyme concentration. Data obtained from experiments was used for nonlinear equation of (y = ax/(b+x)) where a = V_{max} (abs/min) and b = K_m (M). Then predicted data and equation′s constants were calculated. Experimental data and predicted data from the model were compared with correlation coefficient (r^2) that was found as 0.91. Figure 3.9 shows that enzyme follows Michaelis Menten Kinetics in the substrate concentration ranges of 0.0653 mM to 1.956 mM.

Equation of y = ax / (b+x) was obtained by Michaelis-Menten kinetics. Constants of equation (a, b) were calculated, where a= $9.705x10^{-1}$ and b= $6.685x10^{-2}$. The Lineweaver-Burk plot was obtained as linear (Figure 3.10) and linear correlation coefficient (r^2) was found as 0.91. V_{max} and K_m values were found as 1.14 abs/min and 0.16 mM, respectively. K_m value for hydrogen peroxide by using o-dianisidine as hydrogen donor was found as $7.7x10^{-3}$ mM for carrot peroxidase (Soysal and Söylemez, 2005).

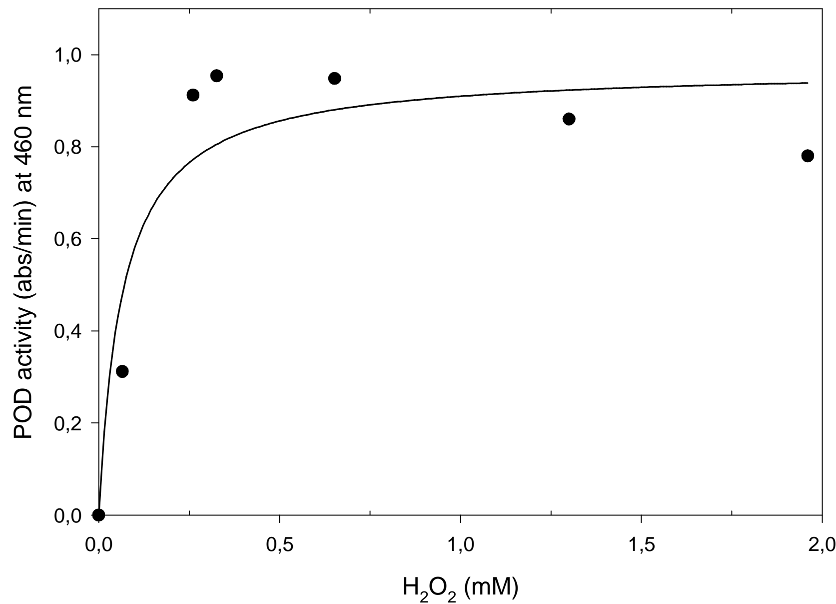


Figure 3.9. Initial rate versus substrate concentration by o- dianisidine, Extract: 0.572 mg protein/mL

3.4. Comparison of the kinetic results

Enzyme activity is generally defined as the amount of enzyme that caused an absorbance change of 0.001 per min under experimental conditions (Doğan et al, 2007). V_{max} is the maximum rate of reaction which occurs when the enzyme is completely saturated with substrate, K_m is a Michaelis-Menten constant and is an essential parameter for characterization of a certain enzyme-substrate couple. A low value of K_m indicates a high affinity to the enzyme for its substrate.

V_{max}/K_m ratio is called catalytic power and is a good parameter for finding the most effective substrate (Doğan et al, 2002). For guaiacol V_{max}/K_m ratio was found as 0.78, for o-dianisidine it was found as 7.13. The K_m values for guaiacol and o-dianisidine were obtained as 0.66 mM and 0.16 mM. The lowest K_m and the highest V_{max}/K_m ratio were obtained with o-dianisidine. Lower K_m value indicates higher tendency of enzyme towards hydrogen peroxide. As a result, when o-dianisidine was used as hydrogen donor, tomato POD showed higher tendency towards hydrogen peroxide. Also, when o-dianisidine was used as hydrogen donor reaction rate was higher than guaiacol. Because V_{max} of o-dianisidine was 2.2 times higher than that of

guaiacol with same enzyme concentration. So, o-dianisidine was used as hydrogen donor for the further inactivation experiments.

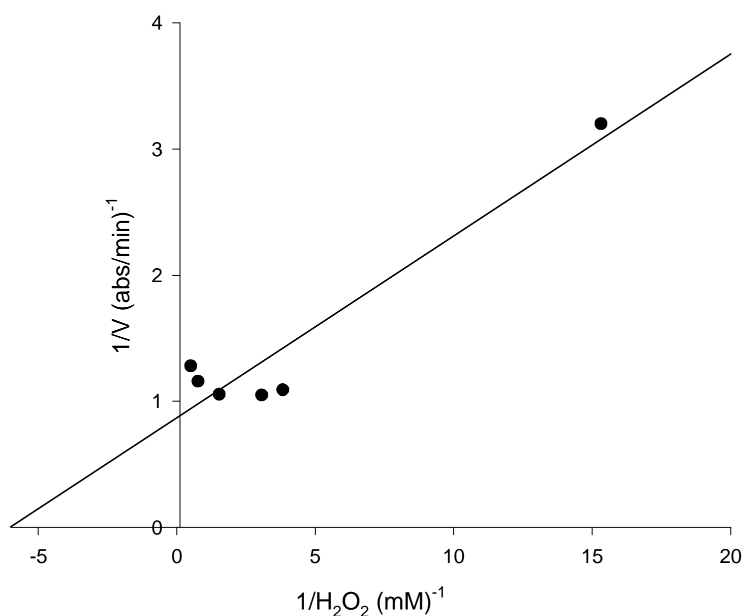


Figure 3.10. Lineweaver-Burk plot of peroxidase action on hydrogen peroxide in the presence of o-dianisidine

3.5. Inactivation of tomato POD

Inactivation of tomato peroxidase was studied by thermal and ultrasonic treatments.

3.5.1. Thermal inactivation

Thermal inactivation of tomato POD was performed at temperatures of 63, 64, 65, 66 and 67°C. For the statistical analysis; two way ANOVA was applied to the experimental data. There was a significant interaction ($P<0.05$) among time and temperature. They had a significant effect ($P<0.05$) on the inactivation of tomato POD (Table 3.1). Residual activity was used for the determination of effect of heat treatment on tomato POD. The residual peroxidase activities against time for different processing temperatures are presented in Figure 3.11.

Table 3.1. Results of ANOVA of thermal inactivation of tomato POD

Source	Sum of squares	df	Mean Squares	F	Significance
Time	18.071	9	2.008	506.426	0.000
Temperature	4.526	6	0.754	190.245	0.000
Time-Temp	1.280	54	$2.370x10^{-2}$	5.977	0.000
Error	0.278	70	$3.965x10^{-3}$		

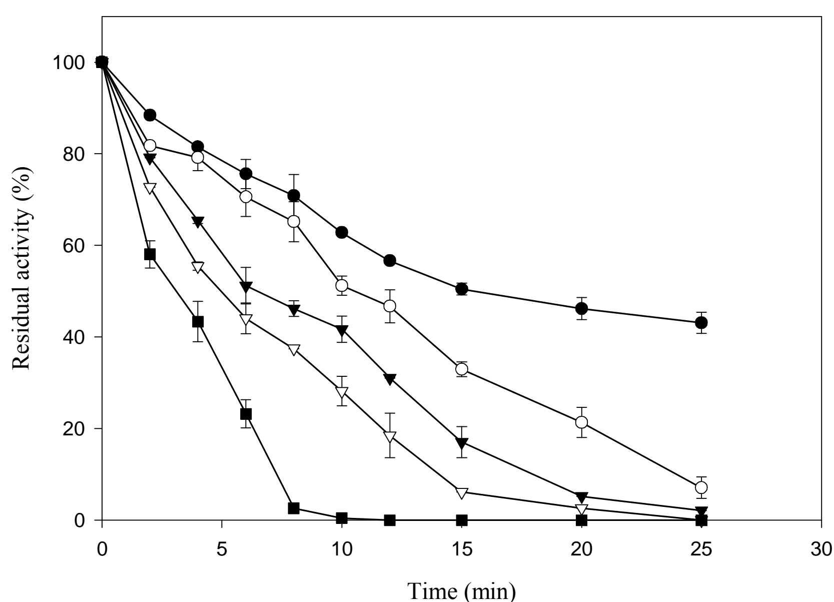


Figure 3.11. Thermal inactivation of tomato POD at 63 (●), 64 (○), 65 (▼),66 (∇), 67°C (■)

From the graph, it could be seen that the rate of POD inactivation depended on temperature and increased with increasing temperature (P<0.05). 50% reduction in POD activity was observed for 15.5, 10.5, 6.5, 5 and 3 min of heating at 63, 64, 65, 66 and 67°C, respectively. Sciancalepore et al (1985) reported that 50% reduction in grape POD activity for 2 minutes of heating at 65°C and also in another study it was found that for Borbon grape cultivar, POD activity decreased about 58% at 60°C for 6 minutes of heating (Troıanı et al, 2003). Cano et al (1990) treated whole peeled

bananas in boiling water for 11 minutes and observed 96-100% reduction in the activity of POD enzyme.

Thermal inactivation of POD in tomato showed first order kinetics. The plot of natural logarithm of residual activity versus heating time could be fitted to linear regression model (Figure 3.12). The results of Anthon et al (2002) were in good agreement with this study. They were reported a simple first order inactivation for POD from tomato juice. Peroxidase inactivation in different vegetables, such as broccoli, green asparagus, carrots and watercress, has been reported to follow a biphasic first-order model (Cruz et al, 2006; Morales-Blancas et al, 2002). For carrots, potatoes, tomato and green beans, a first-order model was used to describe the enzyme inactivation (Anthon and Barrett 2002; Anthon et al, 2002; Bifani et al, 2002).

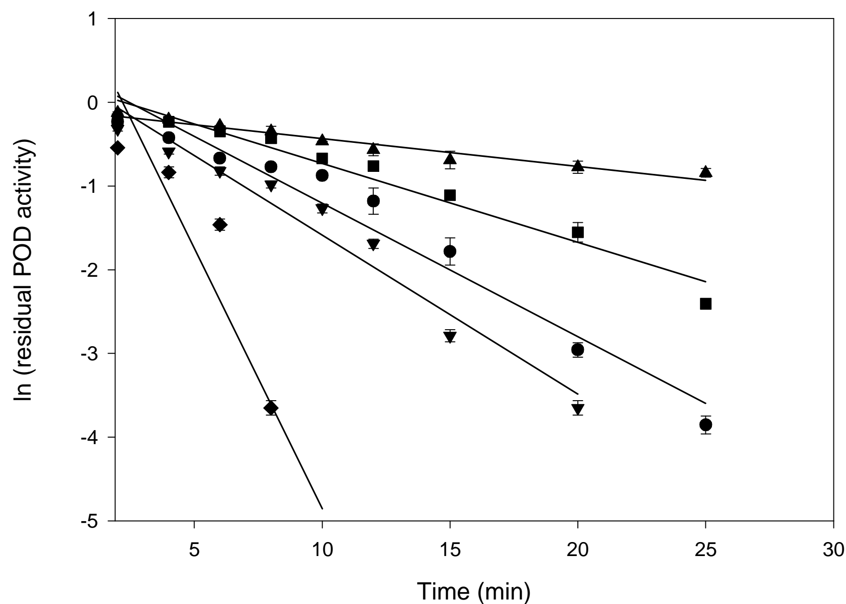


Figure 3.12. Natural logarithm of residual tomato POD activity versus heating time at 63 (▲),64 (■), 65 (●), 66 (▼), 67 (♦) °C

Simple first-order kinetics are not usually obtained for the inactivation of POD in crude plant homogenates because most plants contain multiple isoforms of peroxidase. Tomato mesocarp has been shown to contain only one isoform of POD

(Marangoni et al, 1989; Andrews et al, 2000). In most cases, even purified single isoforms of POD does not show simple, linear first-order kinetics. In this regard, the peroxidase from tomatoes appears to be unusual (Anthon et al, 2002). But, for carrot, potatoe, tomato and green bean, a first-order model was used to describe the enzyme inactivation (Anthon and Barrett, 2002; Anthon et al, 2002; Bifani et al, 2002).

From the slopes of lines, inactivation rate constant, k, were calculated by linear regression and calculated according to the equation (Anthon and Barrett, 2002):

$\ln A/A_0 = -\ kt$

where A_0 is the initial enzyme activity and A is the activity after heating at time t. The obtained k values were used to plot of lnk versus 1/T (Arrhenius plot) (Figure 3.13.), showing a simple linear fit (r^2=0.95).

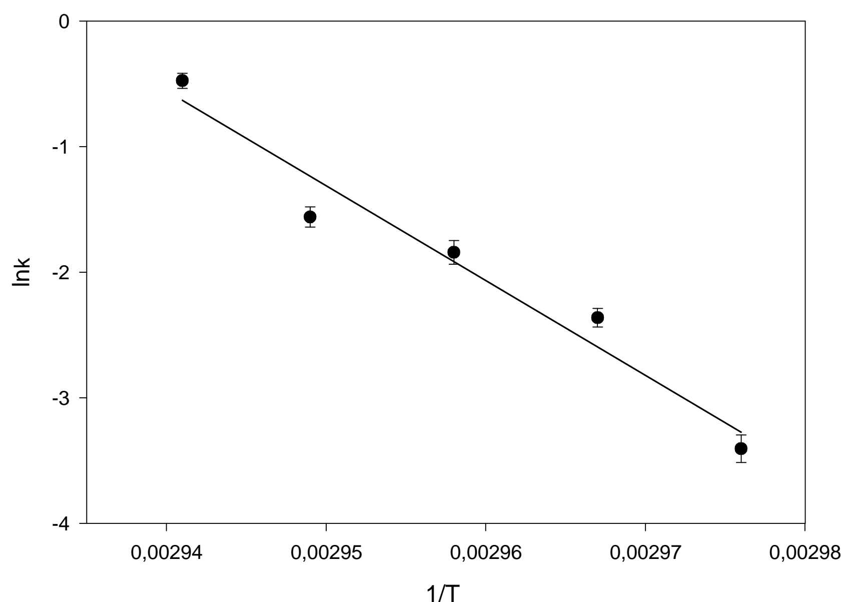


Figure 3.13. Arrhenius plot of the inactivation rates of tomato POD

E_a value is an important parameter for the inactivation of enzymes. E_a was calculated from the slope of Arrhenius plot. It was found as $14.9x10^4$ J/mol. Similar results were reported for the activation energies for red pepper peroxidase $15.1x10^4$ J/mol

(Serrano-Martinez et al, 2008), for butternut squash POD 14.9x10^4 J/mol (Agüero et al, 2008) and for asparagus POD 14x10^4 J/mol (Ganthavorn et al, 1991). However, higher E_a values were obtained for tomato POD enzymes. Anthon et al (2002) found the activation energies as 54.6x 10^4 J/mol and 55.7x10^4 J/mol. A higher E_a value (32.2x 10^4 J/mol) was also reported for POD from soybeans (Mceldoon and Dordick, 1996). Anthon et al (2002) was reported that E_a for the inactivation of POD enzyme are generally in the range of 80 to 200 kJ/ mol.

In some cases inactivation is given as a D value (decimal reduction time), the time required to reduce the enzyme activity to 10% of its original value (Liu et al, 2008). The D value is directly related to the inactivation rate constant k by :

$$D = 2.303/k$$

D values for POD was also determined (Table 3.2.). From the table it can be seen that as temperature increases, k values increases and time required to reduce the enzyme activity to 10% of its original value decreases ($P<0.05$).

Table 3.2. Reaction rate constants (k) and decimal reduction times (D) of tomato POD by thermal inactivation

Temperature (°C)	kx10^3(min^{-1})*	D (min)*
63	33.2 ± 0.11^a	69.4 ± 4.7^a
64	94.2 ± 0.07^{ab}	24.4 ± 2.12^b
65	158.6 ± 0.09^{ab}	14.5 ± 1.54^b
66	210.5 ± 0.08^b	10.9 ± 2.6^b
67	621 ± 0.06^c	3.7 ± 0.22^b

*Means with same letter do not show significant differences at P = 0.05.

When the temperature was increased from 63 to 67°C, time needed to achive 90% reduction in POD activity was reduced from 69.4 to 3.7 min. Increasing the temperature increased the inactivation rate, which has been demonstrated in many tomato enzyme thermal inactivation tests (Crelier et al, 2001; Lopez et al, 1997). Gennaro et al, (1999) was reported the D of horseradish POD of 56 min at 80°C,

Kuldiloke (2002) was reported the D values of tomato POD range from 37 min at 40°C to 8 min at 70°C.

3.5.2. Ultrasound treatment

Ultrasonic inactivation of tomato POD was performed by sonication at 15, 25, 40, 50 and 75% ultrasonic power for 20-150 s. At 75% ultrasonic power sonication was stopped at 90 second. Because, when the sample was exposed to sonication at that power for 90 second, temperature of the extract was increased to 71°C that no POD activity was observed and inactivation effect of ultrasound could not be detected. Figure 3.14. shows the temperature change of the extract after the sonication. It was observed that temperature of the extract was increased with ultrasonic power and time.

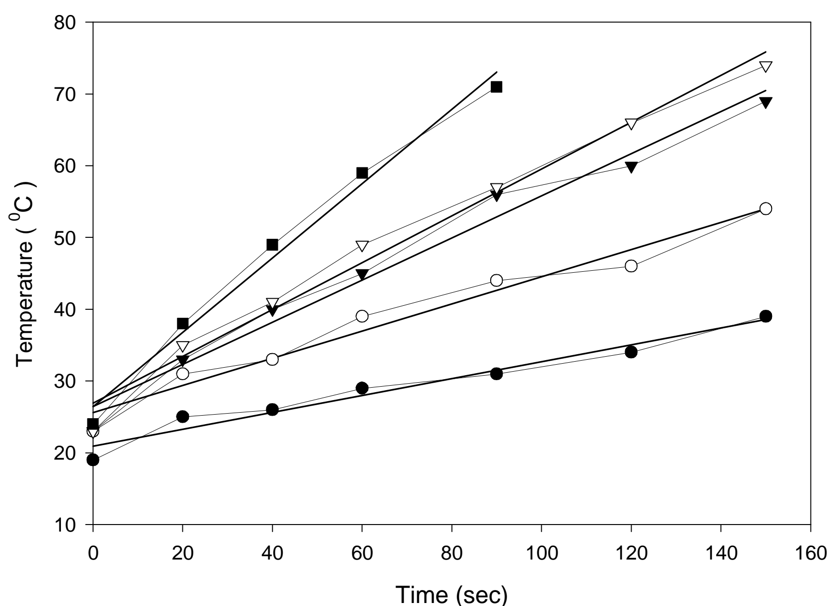


Figure 3.14. Temperature of the extract after sonication at 75% (■), 50% (∇), 40% (▼), 25% (○) and 15% (●) ultrasonic powers

Effect of ultrasound on tomato POD activity was shown in Figure 3.15. From the inactivation plot it can be easily seen that there was a sharp decrease in enzyme activity for the first 20 seconds at all powers. As the ultrasonic power increased, inactivation rate increased (Figure 3.15). Time and ultrasonic power had a significant effects on ($P<0.05$) the inactivation of tomato POD. Also, there was a significant

interaction between time and ultrasonic power (Table 3.3). As a result the residual activities of tomato POD decreased significantly ($P<0.05$) with rise of ultrasonic power and time. When ultrasound was applied for 150 seconds at ultrasonic powers of 15, 25 and 40%, inactivation of tomato POD were 35.84%, 36.07% and 83.79%, respectively. On the other hand, 100% POD inactivation was observed at 50% power for 150 seconds and same reduction was observed at 75% power for 90 seconds of ultrasonication.

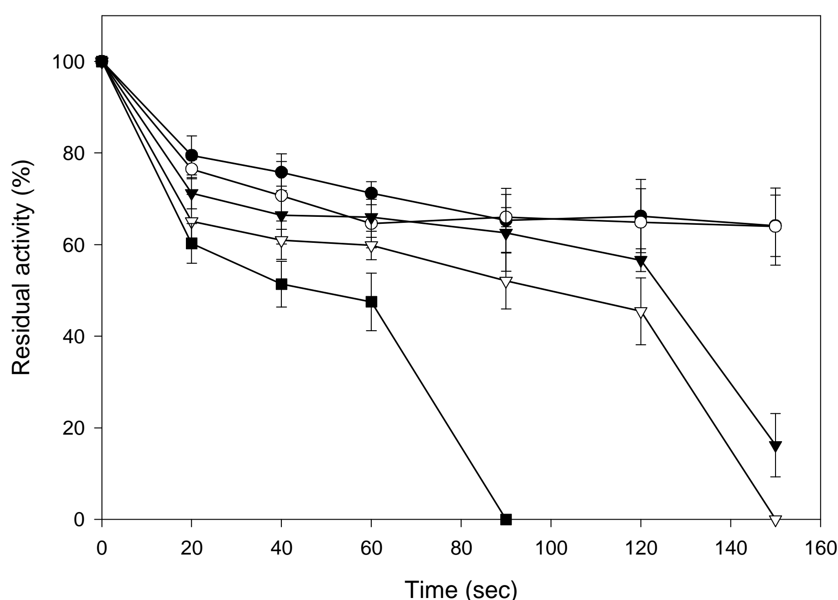


Figure 3.15. Ultrasonic inactivation of tomato POD at 15% (●), 25% (○), 40% (▼), 50% (∇), 75% (■) powers

The semi-log plots of the residual activity versus heating time were linear at all ultrasonic powers studied, consistent with inactivation occurring by a simple first-order process (Fig. 3.16).

The plots of ln residual activity versus ultrasonication time could be fitted to linear regression model. Raviyan et al (2005) and Gennaro et al (1999) reported that in all experiments of pectinmethylesterase and POD inactivation exhibited first order kinetics by ultrasonication. From the slopes of these lines k values were calculated (Table 3.4). As the ultrasonic power increased, inactivation rate constans were increased ($P<0.05$). This result is in agreement with the results of Tiwari et al (2009).

Table 3.3. Results of ANOVA of ultrasonic inactivation of tomato POD

Source	Sum of Squares	df	Mean Squares	F	Significance
Power	2.246	4	0.561	165.341	0.000
Time	5.127	6	0.854	251.622	0.000
Power-Time	1.681	24	$7x10^{-2}$	20.622	0.000
Error	0.119	35	$3.395x10^{-3}$		

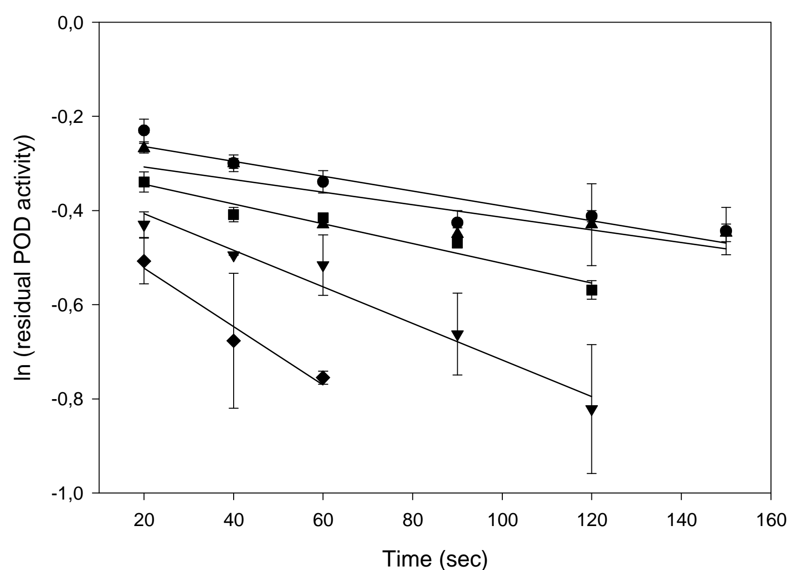


Figure 3.16. Natural logarithm of residual tomato POD activity versus heating time at ultrasonic powers of 15% (●), 25% (■), 40% (▲), 50% (▼), 75% (♦)

Table 3.4. Reaction rate constants (k) of tomato POD by ultrasonic inactivation

Ultrasonic power (%)	$kx10^3(min^{-1})^*$
5	1.54 ± 0.042[a]
25	1.58 ± 0.021[b]
40	2.20 ± 0.016[ab]
50	3.88 ± 0.012[b]
75	6.18 ± 0.025[c]

*Means with same letter do not show significant differences at P = 0.05.

3.5.3. Regeneration of tomato POD

Heat treatment is commonly used to inactivate an active enzyme. However, it is well-known that POD can recover its activity after heat treatment with time (Thongsook et al, 2005b). POD can regain enzymic activity, which is believed to be responsible for further loss of quality by the development of off-flavor in vegetables (Lu and Whitaker, 1974). Although the reactivation of POD has been commonly observed in heat-treated plant-based foods, little work has been attempted to investigate the detailed mechanisms involved in the heat inactivation and reactivation of this enzyme. Knowledge of the mechanism of POD reactivation in vegetables important to the food industry may be used to prevent enzyme reactivation, which would help prolong the shelf life of processed vegetable products (Thongsook et al, 2005b).

Regeneration of POD following heat inactivation has been reported for some vegetable and fruit extracts, e.g., kohlrabi (Vamos-Vigyazo, 1981), Brussels sprouts and cabbage (Mclellan and Robinson, 1981), apples (Moulding et al, 1987), pears (Moulding et al, 1989), mango (Khan and Robinson, 1993) and orange (Lu and Whitaker, 1974).

POD activity versus ultrasonication time at 15% and 25% ultrasonic powers could be fitted to linear models with high correlation coefficients (0.90 to 0.97) as shown in Figure 3.17. and 3.18. Reactivation increased as the ultrasonication power increased (Figure 3.17 and 3.18). For 15% power and 150 sec ultrasonication, residual activity increased 22%, at 25% power and same ultrasonication exposure time residual activity increased 36%.

Regeneration of tomato POD treated at 40%, 50% and 75% power was not linear. POD activity versus ultrasonication time at 40%, 50% and 75% powers could be fitted to exponential equation;

$$y = a(1-e^{-bx})+c$$

where; a is the amplitude of the exponential, b is the rate constant (min^{-1}), c is the intercept (activity at time=0), a+c is the maximum of the exponential (regeneratable A_{max}) (Figure 3.19, 3.20 and 3.21).

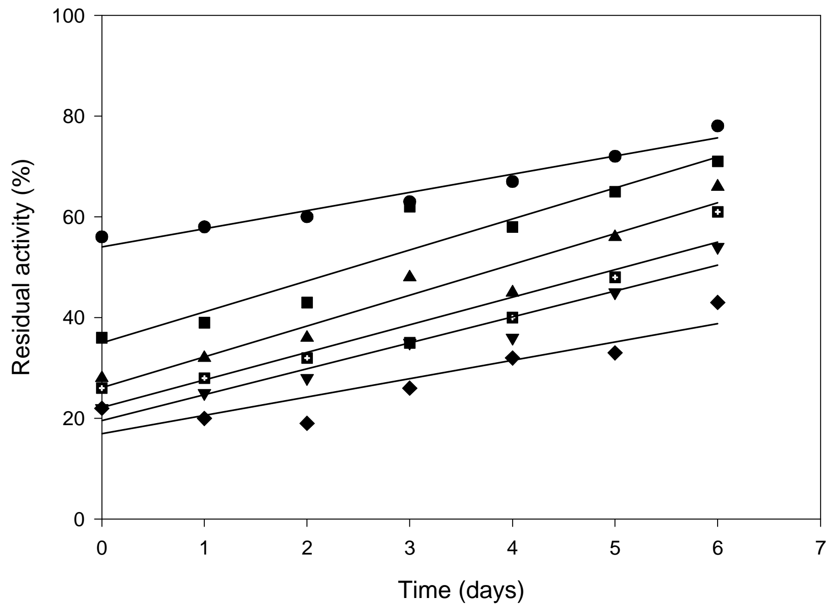


Figure 3.17. Regenerated POD activity versus storage time when samples were treated at 15% ultrasonic power for 20 (●), 40 (■), 60 (▲), 90 (◘), 120(▼), 150 (♦) seconds

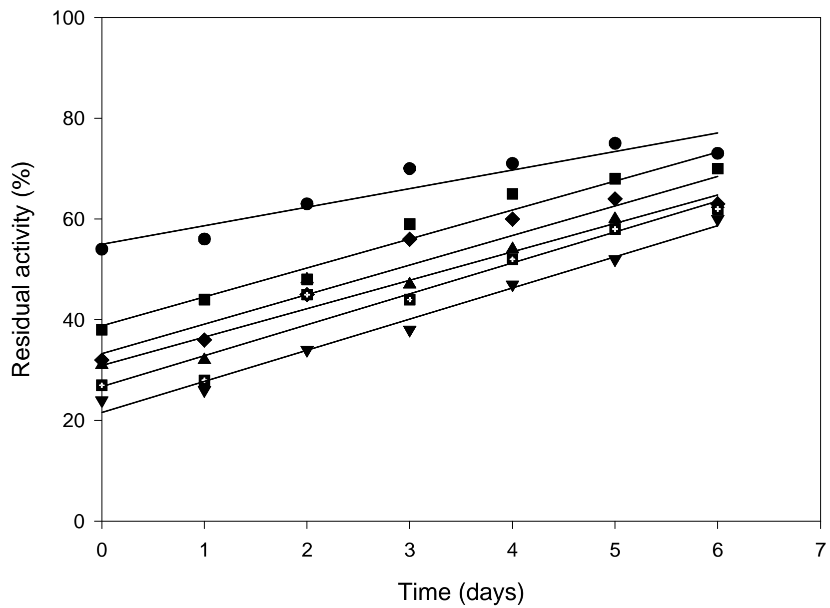


Figure 3.18. Regenerated POD activity versus storage time when samples were treated at 25% ultrasonic power for 20 (●), 40 (■), 60 (▲), 90 (♦), 120(◘), 150 (▼) seconds

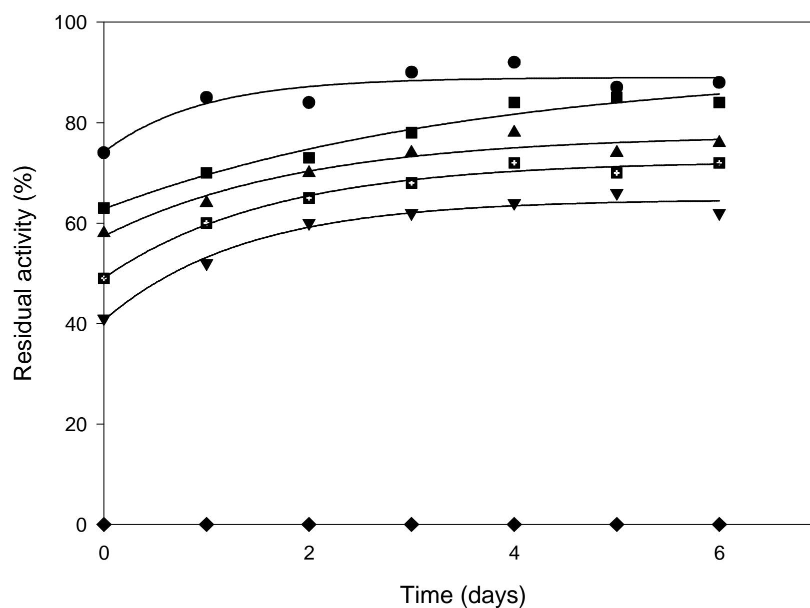


Figure 3.19. Regenerated POD activity versus storage time when samples were treated at 40% ultrasonic power for 20 (●), 40 (■), 60 (▲), 90 (◘), 120(▼), 150 (♦) seconds

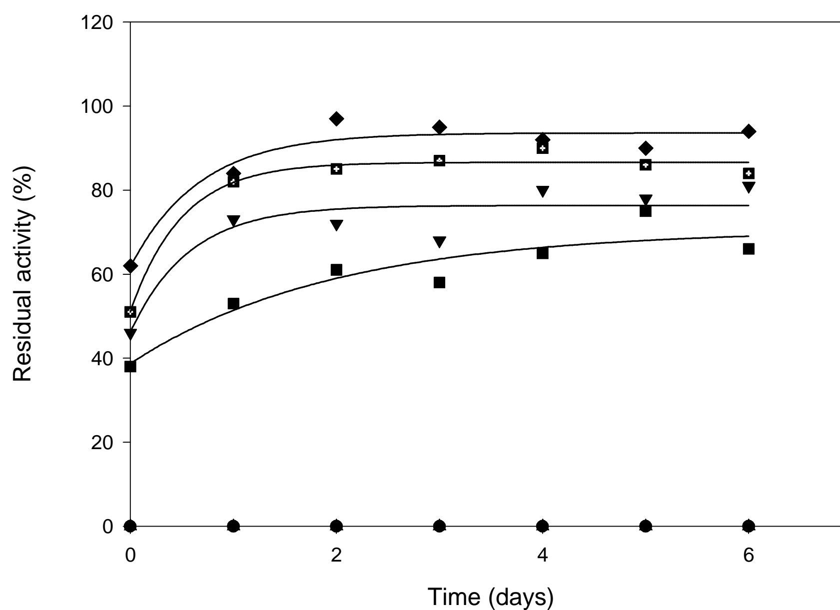


Figure 3.20. Regenerated POD activity versus storage time when samples were treated at 50% ultrasonic power for 20 (♦), 40 (◘), 60 (▼), 90 (■), 120(●), 150 (▲) seconds

Similar regeneration kinetics were reported for thermal inactivation of asparagus POD by Rodrigo et al (1996) and for thermal and high hydrostatic pressure inactivation of carrot POD (Soysal et al, 2004).

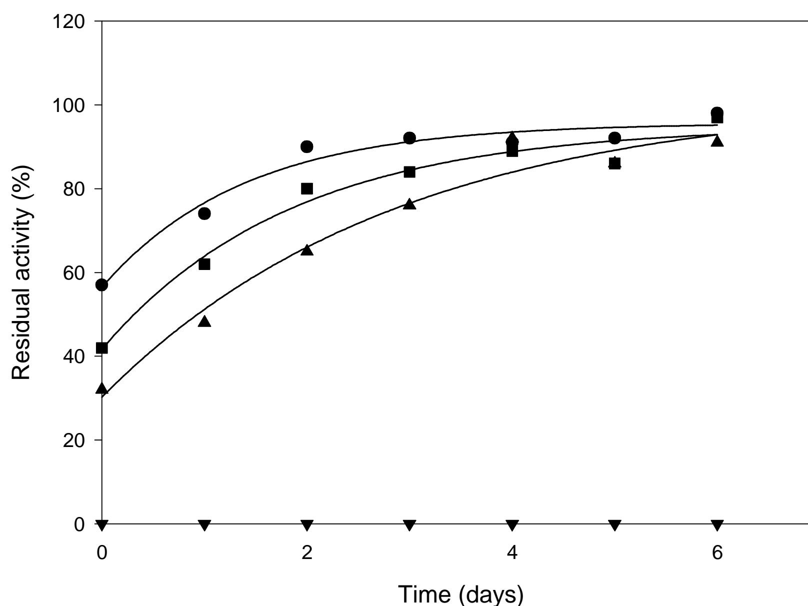


Figure 3.21. Regenerated POD activity versus storage time when samples were treated at 75% ultrasonic power for 20 (●), 40 (■), 60 (▲), 90 (▼) seconds

At 40% power for 120 sec no activity was observed as a result no reactivity was observed. Same result was observed at 50% power for 90 sec and at 75% power for 60 sec. At these powers reactivation increased as the ultrasonication power increased (Figure 3.19, 3.20 and 3.21). 23% POD regeneration was observed for enzyme treated at 40% ultrasonic power for 60 sec. At the same conditions 28% and 59% tomato POD regeneration was observed at 50% and 75% ultrasonic powers, respectively. Table 3.5, 3.6 and 3.7 shows the model constants obtained at 40%, 50% and 75% powers for the regeneration of tomato POD.

After certain heating conditions, inactivated PODs such as horseradish, kohlrabi, tobacco, orange or pears regain their enzymic activity (regeneration) during storage in an extract (Burnette, 1977; Moulding et al, 1989; Vamos-Vigyazo, 1981; Mclellan and Robinson, 1984) while no POD reactivation was observed in palm tree, potato, cauliflower, turnip, mango or grape (Robinson et al, 1989; Khan and Robinson, 1993; Vamos-Vigyazo, 1981; Rodriguez et al, 2002) due to the modifications such

as digestion, aggregation, loss of prostetic group, etc (Schmid et al, 1993). The variability in regeneration, depending on the extent of haem polymerization, has been proposed as the major cause of the non-linear first-order kinetics of inactivation; further support for this proposal was provided by the observation that simple first-order inactivation kinetics was followed when no regeneration occurred (Adams, 1997b). The study of POD inactivation kinetics could be very useful to optimize blanching parameters utilized to avoid POD regeneration, which causes off-flavour and off-colour formation (Sergio et al, 2007).

Table 3.5. Model constants obtained at 40% ultrasonic power for the regeneration of tomato POD

Time (sec)	a	b	c	a+c	r^2
20	14.82	1.05	74.15	88.97	0.86
40	28.47	0.27	62.78	91.25	0.97
60	20.19	0.51	57.38	77.57	0.95
90	23.29	0.61	49.07	72.36	0.99
120	24.04	0.73	40.68	64.72	0.98

Table 3.6. Model constants obtained at 50% ultrasonic power for the regeneration of tomato POD

Time (sec)	a	b	c	a+c	r^2
20	31.86	1.50	61.72	93.58	0.95
40	35.57	2.0	51.01	86.58	0.98
60	30.17	1.76	46.17	76.34	0.86
90	31.79	0.51	38.73	70.52	0.89

Table 3.7. Model constants obtained at 75% ultrasonic power for the regeneration of tomato POD

Time (sec)	a	b	c	a+c	r^2
20	39.19	0.72	56.48	95.67	0.97
40	53.30	0.54	41.69	94.99	0.98
60	71.59	0.35	30.25	101.8	0.98

3.5.4. Effects of inactivation methods on vitamin C and microorganisms

Effect of inactivation methods on vitamin C of tomato extract was determined because vitamin C is the least stable of all vitamins and is easily destroyed during processing and storage. The effects of inactivation methods on vitamin C of tomato extract are shown in Table 3.8. A significant decrease (P<0.05) in ascorbic acid content of tomato extract (mg/100g sample) was observed as a function of temperature and treatment time (Table 3.8).

Table 3.8. Effects of inactivation methods on vitamin C of tomato extract

Inactivation method	Inactivation parameters	Peroxidase inactivation (%)	Vitamin C* (mg/100g sample)	Reduction of vitamin C (%)
No treatment		0	20.8[a]	-
Heat treatment	63°C, 10 min	29	16.8 ± 0.42[b]	19.3
	64°C, 10 min	37.4	16.2 ± 0.28[c]	22.2
	65°C, 10 min	47.3	15.2 ± 0.14[d]	27
	66°C , 10 min	61.3	14 ± 0.28[e]	32.7
	66°C, 25 min	100	12.5 ± 0.14[f]	40
	67°C, 10 min	100	12.7 ± 0.14[f]	39
Ultrasonic treatment	15%, 90 sec	34.7	20.8 ± 1.13[a]	0
	25%, 90 sec	34	20.8 ± 1.13[a]	0
	40%, 90 sec	37	20.8 ± 1.13[a]	0
	50%, 90 sec	47	20.8 ± 1.13[a]	0
	75%, 90 sec	100	18.3 ± 0.56[c]	12

*Means with same letter do not show significant differences at P = 0.05.

On the other hand there was no significant difference (P>0.05) in ascorbic acid content of tomato extract after ultrasonic treatment. For heat treatment at 67ºC for 10 min and at 66ºC for 25 min 100% peroxidase inactivation was observed and vitamin C reduction at these treatment conditions were 39% and 40%. For ultrasonic treatment, 12% reduction was observed in vitamin C after 90 sec at 75% power ultrasonicatin that was the point of 100% peroxidase inactivation was achieved. Results shows that thermally processed tomato extract had a significantly (P<0.05) lower concentration of vitamin C than sonicated. These results were also in

agreement with those reported for orange juice by Tiwari et al (2008). They found that sonication improved retention of vitamin C in orange juice compared to thermal processing. Also, Cruz et al (2008) compared ascorbic acid content of watercress after heat and ultrasonic treatment and found that ascorbic acid retention after sonication was at higher levels.

Effect of inactivation methods on aerobic plate count and mold yeast count contents were shown in Table 3.9 and 3.10.

Table 3.9. Aerobic plate count of tomato extract treated with heat and ultrasound

Inactivation method	Inactivation parameters	Peroxidase inactivation (%)	Number of m/o's /ml sample	Reduction of m/o's (%)
No treatment		0	120	-
Heat treatment	63°C, 10 min	29	50	58.3
	64°C, 10 min	37.4	35	71
	65°C, 10 min	47.3	25	79.2
	66°C , 10 min	61.3	0	100
	66°C, 25 min	100	0	100
	67°C, 10 min	100	0	100
Ultrasonic treatment	15%, 90 sec	34.7	50	58.3
	25%, 90 sec	34	50	58.3
	40%, 90 sec	37	25	79.2
	50%, 90 sec	47	0	100
	75%, 90 sec	100	0	100

For the aerobic plate count 100% reduction of m/o's was obseved at the point of 100% peroxidase inactivation (at 66°C for 25 min and 67°C for 10 min). Also, same reduction of m/o's was observed after 90 sec at 75% power ultrasonication. On the other hand, 47.3% peroxidase inactivation was observed after 10 min at 65°C, at that point microbial content reduction was 79.2%. Same peroxidase inactivation (47%) was observed with ultrasonic treatment (for 90 sec at 50% power), but at that

treatment 100% m/o's reduction was observed. Similar results were also observed for the mold-yeast count of tomato extract. The higher inactivation rate at greater amplitudes was observed and it could be due to an increase in the number of bubbles undergoing cavitation per unit of time (Suslick, 1990) or to an increase in the volume of liquid in which cavitation is liable to occur (Suslick, 1988).

Table 3.10. Mold-yeast count of tomato extract treated with heat and ultrasound

Inactivation method	Inactivation parameters	Peroxidase inactivation (%)	Number of m/o's /ml sample	Reduction of m/o's (%)
No treatment		0	65	-
Heat treatment	63°C, 10 min	29	0	100
	64°C, 10 min	37.4	0	100
	65°C, 10 min	47.3	0	100
	66°C , 10 min	61.3	0	100
	66°C, 25 min	100	0	100
	67°C, 10 min	100	0	100
Ultrasonic treatment	15%, 90 sec	34.7	50	23
	25%, 90 sec	34	50	23
	40%, 90 sec	37	25	61.5
	50%, 90 sec	47	0	100
	75%, 90 sec	100	0	100

The effectiveness of an ultrasound treatment is dependent on the type of bacteria being treated (Sala et al, 1995). Microorganisms (especially spores) are relatively resistant to the effects, thus extended periods of ultrasonication would be required to render a product safe. If ultrasound were to be used in any practical application, it would most likely have to be used in conjunction with pressure treatment (manosonication), heat treatment (thermosonication) or both (manothermosonication) (Piyasena et al, 2003). Other factors that are known to affect the effectiveness of microbial inactivation are amplitude of the ultrasonic waves, exposure/contact time,

volume of food being processed, the composition of the food and the treatment temperature (USDA, 2000).

CHAPTER IV

CONCLUSION

Highest activity for guaiacol was observed at 0.1% (v/v) guaiacol and 0.1% (v/v) hydrogen peroxide and highest activity for o-dianisidine was observed at 0.05% (w/v) o-dianisidine and 0.1% (v/v) hydrogen peroxide concentrations.

According to kinetic results, V_{max} and K_m values for guaiacol were found as 0.52 abs/min and 0.66 mM, for o-dianisidine 1.14 abs/min and 0.16 mM, respectively. Lower Km value indicates higher tendency of enzyme towards hydrogen peroxide. So, o-dianisidine was used as hydrogen donor for the further inactivation experiments.

Thermal inactivation of POD in tomato showed first order kinetics. Ea was calculated from the slope of Arrhenius plot and found as $14.9x10^4$ J/mol. 50% reduction in POD activity was observed for 15.5, 10.5, 6.5, 5 and 3 min of heating at 63, 64, 65, 66 and 67°C, respectively.

Ultrasonic inactivation of tomato POD showed first order kinetics. When ultrasonic power increased, first order inactivation rate constans were increased.

It was observed that at 15 and 25% ultrasonic powers, residual enzyme activity for regeneration of tomato POD increased linearly, whereas at powers of 40, 50 and 75 % residual enzyme activity increased nonlinearly. There was no regeneration in the samples which 100 % enzyme inactivation obtained by ultrasound.

A significant decrease ($P<0.05$) in ascorbic acid of tomato extract (mg/100g sample) was observed as a function of temperature and treatment time with heat treatment. No significant difference ($P>0.05$) in ascorbic acid of tomato extract was observed after ultrasonic treatment. When approximately 50% POD was inactivated,

vitamin C content was decreased 27% by heat treatment and no reduction by ultrasonic treatment was observed.

When approximately 50% POD was inactivated, aerobic plate count reduction was 79.2% by heat treatment and 100% by ultrasonic treatment. For mold-yeast count, at all heat treatment conditions 100% reduction was observed but for ultrasonic treatment reduction range was between 23-100% for all treatment condition.

REFERENCES

AACC (1995). *Approved Methods of the American Association of Cereal Chemists*. 9th edn., 45-48. Minnesota, USA, The American Association of Cereal Chemists.

Adams, J.B. (1997a). Electrospray mass spectrometric study of haem changes during peroxidase denaturation. *Food Chemistry*, **58**, 173- 175.

Adams, J.B. (1997b). Regeneration and the kinetics of peroxidase inactivation. *Food Chemistry,* **60,** 201–206.

Agüero, M.V., Ansorena, M.R., Roura, S.I., del Vale, C.E. (2008). Thermal inactivation of peroxidase duringblanching of butternut squash. *Lebensmittel-Wissenschaft und-Technologie*, **41** , 401–407.

Alliger, H. (1975). Ultrasonic distruption. *American laboratory*, **10**, 75-85.

Alvarado, J.D., Viteri, N.P. (1989). Effect of temperature on the aerobic degradation of vitamin C in citric fruit juices. *Archives Latinoamerican Nutrition*, **39**, 601−612.

Andrews, J., Malone, M., Thompson, D.S., Ho, L.C., Burton, K.S. (2000). Peroxidase isozyme patterns in the skin of maturing tomato fruit. *Plant Cell Environment*, **23**, 415-422.

Anthon, G.E., Barrett, D.M. (2002). Kinetic parameters for the thermal inactivation of quality-related enzymes in carrots and potatoes. *Journal of Agricultural and Food Chemistry*, **50**, 4119–4125.

Anthon, G.E., Sekine, Y., Watanabe, N., Barrette, D.M. (2002). Thermal inactivation of pectin methylesterase, polygalacturonase, and peroxidase in tomato juice. *Agricultural and Food Chemistry*, **50**, 6153-6159.

AOAC (1990). Offical Methods of Analysis of Association of Offical Analytical Chemists. Edited by Kenneth Helrich, Fifteenth ed., Arlington, Virginia, USA, pp. 74.

Barbosa-Canovas, G.V., Pothakamury, U.R., Palou, E., Swanson, B.G. (1998). *Nonthermal preservation of foods*, Macel Dekker, Inc. New York, pp. 354.

Barret, D.M., Theerakulkait, C. (1995). Quality indicators in blanched, frozen, stored vegetables. *Food Technology*, **49**, 62–65.

Belcarz, A., Ginalska, G., Kowalewska, B., Kulesza, P. (2008). Spring cabbage peroxidases – Potential tool in biocatalysis and bioelectrocatalysis. *Phytochemistry,* **69**, 627–636.

Bifani, V., Inostroza, J., Cabezas, M. J., Ihl, M. (2002). Determinacion de parametros cine´ticos de peroxidasa y clorofila a en judias verdes (Phaseolus vulgaris cv. Win)

estabilidad del producto congelado. *Revista de Quimica Teorica y Aplicada Febrero LIX* , **467,** 57–64.

Bineesh, N.P., Singhal, R.S., Pandit, A.B. (2005). A study on degradation kinetics of ascorbic acid in drumstick (Moringa olifera) leaves during cooking. *Journal of Science of Food and Agriculture,* **85**, 1953−1958.

Boucoiran, C.F.S. Kijne, J.W. Recourt, K. (2000). Isolation and partial characterization of thermostable isoperoxidases from potato (*Solanum tuberosum* L.) tuber sprouts. *Journal of Agricultural and Food Chemistry*, **48**, 701-707.

Bruemmer, J.H., Roe, B., Bowen, E.R. (1976). Peroxidase reactions and orange juice quality. *Journal of Food Science,* **41**, 186-189.

Burnette, F.S. (1977). Peroxidase and its relationship to food flavor and quality: A review. *Journal of Food Science,* **42**, 1–6.

Butz, P., Tauscher, B. (2002). Emerging technologies: chemical aspects. *Food Research International,* **35** , 279– 284.

Cano, P., Marin, M.A., Fuster, C. (1990). Effects of some thermal treatments on polyphenoloxidase and peroxidase activities of banana (*Musa cavendishii,* var. *enana*). *Journal of Science of Food and Agriculture***, 51**, 223-231.

Castro, I., Teixeira, J.A., Salengke, S., Sastry, S.K., Vicente, A.A. (2004). Ohmic heating of strawberry products: electrical conductivity measurements and ascorbic acid degradation kinetics. *Innovative Food Science and Emerging Technologies,* ***5****,* 27−36.

Chen, G., Asada, K. (1989). Ascorbate peroxidase in tea leaves: Occurrence of two isozymes and the differences in their enzymatic and molecular properties. *Plant Cells Physiology,* **30**, 1987–1998.

Chibbar, R.N., Van Huystee, R.B. (1984). Characterization of peroxidase in plant cells. *Plant Physiolology*, **75**, 956-958.

Civello, P.M., Martinez, G.A., Chaves, A.R., Anon, M.C. (1995). Peroxidase from Strawberry Fruit *(Fragaria ananassa* Duch.): Partial Purification and Determination of Some Properties. *Journal of Agricultural and Food Chemistry*, **43**, 2596-2601.

Clemente, E. (1998). Purification and thermostability of isoperoxidase from orange. *Phytochemistry,* **49**, 29-36.

Clemente, E. (2002). Peroxidase from oranges (*Citrus sinenses L.*) Osbeck. *European Food Research and Technology,* **215**, 164–168

Crelier, S., Robert, M.C., Claude, J., Juillerat, M.A. (2001). Tomato (Lycopersicon esculentum) pectinmethylesterase and polygalacturonase behaviors regarding heat- and pressure-induced inactivation. *Journal of Agricultural and Food Chemistry,* **49**, 5566–5575.

Cruz, V.I., Fatibello-Filho, O. (1998). Flow injection spectrophotometric determination of hydrogen peroxide using a crude extract of zucchini (*Cucurbita pepo*) as a source of peroxide. *The Analyst*, **123**, 1809-1812.

Cruz, R.M.S., Vieira, M.C., Silva, C.L.M. (2006). Effect of heat and thermosonication treatments on peroxidase inactivation kinetics in watercress (Nasturtium officinale). *Journal of Food Engineering*, **7**, 8–15.

Cruz, R.M.S., Vieria, C.M., Silva, C.L.M. (2008). Effect of heat and thermosonication treatments on watercress (Nasturtium officinale) vitamin C degredation kinetics. *Innovative Food Science and Emerging Technologies,* **9**, 483-488.

De Gennaro, L., Cavella, S., Romano, R., Masi, P. (1999). The use of ultrasound in food technology I: inactivation of peroxidase by thermosonication. *Journal of Food Engineering*, **39**, 401–407.

Deepa, S., Arumughan, C. (2002). Purification and characterization of soluble peroxidase from oil palm (Elaeis guinensis Jacq.) leaf. *Phytochemistry*, **61**, 503–511.

Deman, J. (1990). *Principles of food chemistry* (2nd ed.). New York: Van Nostrand Reinhold, pp. 350−351

Dewanto, V., Wu, X., Adom, K. K., Liu, R. H. (2002). Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. *Journal of Agricultural and Food Chemistry*, **50**, 3010−3014.

Doğan, M., Arslan, O., Doğan, S. (2002). Substrate specificity, heat inactivation and inhibition of polyphenol oxidase from different aubergine cultivars. *International Journal of Food Science and Technology,* **37***,* 415–423.

Doğan, S., Turan, P., Doğan, M., Arslan, O., Aklan, M. (2007). Variations of peroxidase activity among Salvia species. *Journal of Food Engineering*, **79**, 375–382.

Dolatowski, J.Z., Stadnik, J., Stasiak, D. (2007). Application of ultrasound in food technology. *Acta Scientiarum Polonorum Technologia Alimentaria,* **6**, 89-99.

Duarte-Vazquez, M.A., Garcia-Almendarez, B.E., Regalado, C., Whitaker, J.R. (2000). Purification and partial characterization of three turnip (*Brassica napus* L. V*ar. esculenta DC*) peroxidases. *Journal of Agricultural and Food Chemistry*, **48**, 1574-1579.

Duarte-Vazquez, M. A., Garcia-Almendarez, B. E., Regalado, C., Whitaker, J. R. Purification and properties of a neutral peroxidase isozyme from turnip (*Brassica napus* L. V*ar. Purple top white globe*) roots (2001). *Journal of Agricultural and Food Chemistry*, **49**, 4450- 4456.

Dubois, M. Gilles, K.A. Hamilton, J.K. Rebers, P.A. and Smith, F. (1956). Colorimetric method for determination of sugars and releated substances. *Analytical Chemistry,* **28**, 350-356.

Earnshaw, R.G., Appleyard, J., Hurst, R.M. (1995). Understanding physical inactivation processes: combined preservation opportunities using heat, ultrasound and pressure. *International Journal of Food Microbiology,* **28** , 197– 219.

Egan, H. , Kirk, R.S. and Sawyer, R. (1981). *Pearson's chemical analysis of foods*. (8th ed.). Churchii Livingstone, London, pp. 196-197.

Ellıott, J., Mulvıhıll, E., Duncan, C., Forsythe, R., Krıtchevsky, D. (1981). Effects of Tomato Pomace and Mixed-Vegetable Pomace on Serum and Liver Cholesterol in Rats. *The Journal of Nutrition*, **111**, 2203-2211.

Ertugay, M.F., Yüksel, Y., Sengul, M. (2003). The effect of ultrasound on lactoperıxidase and alkaline phosphatase enzymes from milk. *Milchwissenschaft*, **58**, 593-595.

Esteve, M. J., Farre, R., Frigola, A., Garcia-Cantabella, J. M. (1999). Determination of ascorbic and dehydroascorbic acids in blood plasma and serum by liquid chromatography. *Journal of Chromatography B: Biomedical Sciences and Applications*, **688**, 345−349.

Fang, L. , Jiang, B. , Zhang, T. (2008). Effect of combined high pressure and thermal treatment on kiwifruit peroxidase. *Food Chemistry*, **109**, 802–807.

Fellows, P. (2000). *Food Processing Technology: Principles and Practice*. (2nd ed) CRC Press, New York. pp. 367-371.

Forsyth, J.L., Robinson, D.S. (1998). Purification of Brussels sprout isoperoxidases. *Food Chemistry*, **63**, 221-234.

Forsyth, J.L., Owusu Apenten, R.K., Robinson, D.S. (1999). The thermostability of purified isoperoxidases from Brassica oleracea var. gemmifera. *Food Chemistry,* **65** 99-109.

Ganthavorn, C., Nagel, C.W., Powers, J.R. **(**1991). Thermal inactivation of asparagus lipoxygenase and peroxidase. *Journal of Food Science*, ***56***, 47.

Gennaro, L.D., Cavella, S., Romano, R., Masi, P. (1999). The use ultrasound in food technology I: inactivation of peroxidase by thermosonication. *Journal of Food Engineering,* **39**, 401-407.

Ghamsari, L., Keyhani, E., Golkhoo, S. (2007). Kinetic properties of guaiacol peroxidase activity in Crocus sativus L. Corm during rooting. *Iranian Biomedical Journal* , **11,** 137-146.

Gonçalves, E.M., Pinheiro, J., Abreu, M., Brandao, T.R.S., Silva, C.L.M. (2007). Modelling the kinetics of peroxidase inactivation, colour and texture changes of pumpkin (Cucurbita maxima L.) during blanching. *Journal of Food Engineering*, **81**, 693–701.

Got, F., Culioli, J., Berge, P., Vignon, X., Astruc, T., Quideau, J.M., Lethiecq, M. (1999). Effects of high-intensity high frequency ultrasound on ageing rate, ultrastructure and some physicochemicalproperties of beef. *Meat Science*, **51**, 35-42.

Göğüş, F., Fadıloğlu, S. (2006). *Food chemistry.* Nobel Yayın Dağıtım. Ankara, pp. 260.

Gray, J.S.S., Montgomery, R. (2003). Purification and characterization of a peroxidase from corn steep water. *Journal of Agricultural and Food Chemistry,* **51**, 1592–1601.

Grolier, P. , Bartholin, G., Broers, L., Caris-Veyrat, C., Dadomo, M., Di Lucca, G., Dumas, Y., Meddens, F., Sandei, L., Schuch, W. (1998). Composition of tomatoes and tomato products in antioxidants. Tomato and health seminar, Pamplona, pp.19.

Haard, N.F. (1977). Physiological roles of peroxidase in postharvest fruits and vegetables. *ACS Symposium Series*, **47**, 143-171.

Halpin, B.E., Pressey, R., J. And Mondy, N. (1989). Purification and characterization of peroxidase isoenzymes from gren peas (Pisum Sativum). *Journal of Food Science*, **54**, 644-649.

Hatzilazarou, S.P. , Syros, T.D., Yupsanis, T.A., Bosabalidis, A. M., Economou, A. S. (2006). Peroxidases, lignin and anatomy during in vitro and ex vitro rooting of gardenia (Gardenia jasminoides Ellis) microshoots. *Journal of Plant Physiology*, **163**, 827-836.

Hemeda, H.M. and Klein, B.P. (1990). Effects of naturally occuring antioxidants on peroxidase activity of vegetable extracts. *Journal of Food Science*, **55**, 184-187.

Henley, J.P., Sadana, A. (1985). Categorisation of enzyme deactivations using a series-type mechanism. *Enzyme Microbial Technolology*,**7**, 50-60.

Ito, H., Hiraoka, N., Ohbayashi, A., Ohashi, Y. (1991). Purification and characterization of rice peroxidases. *Agricultural and Biological Chemistry*, ***55***, 2445-2454.

Jay, J.M. (1986). *Modern food microbiology.* New York: Van Nostrand Reinhold Company Inc., pp. 275-277.

Jayasooriya, S.D., Bhandari, B.R., Torley, P., Darcy, B.R. (2004). Effect of high power ultrasound waves on properties of meat: a review. *International Journal of Food Properties,* **2**, 301-319.

Jen, J.J., Seo, A., Flurkey, W.H. (1980). Tomato peroxidases purification via hydrophobic chromatography. *Journal of Food Science,* **45**, 60-63.

Johnson, J.R., Braddock, R.J., Chen, C.S. (1995). Kinetics of ascorbic acid loss and nonenzymatic browning in orange juice serum: Experimental rate constants. *Journal of Food Science*, **60**, 502−505.

Jones, B.J. (2007). Tomato Plant Culture: In the Field, Greenhouse, and Home Garden (2nd ed.). *Plant characteristics and physiology*. Keillor, G. (Ed.) CRC pres., pp.120-127.

Jouili, H., Bouazizi, H., Rossignol, M., Borderies, G., Jamet, E., El Ferjani, E. (2008). Partial purification and characterization of a copper-induced anionic peroxidase of sunflower roots. *Plant Physiology and Biochemistry*, **44**, 1-8.

Kay, E., Shannon, L.M., Lew, J.Y. (1967). Peroxidase Isozymes from Horseradish Roots. *The Journal of Biological Chemistry*, **242**, 2470-2473.

Khan, A.A., Robinson, D.S. (1993). The thermalstability of purified mango isoperoxidases. *Food Chemistry*, **47**, 53-59.

Knorr D., Zenker M., Heinz V., Lee D.U. (2004). Applications and potential of ultrasonics in food processing. *Trends in Food Science and Technology,* **15**, 261-266.

Kuldiloke, J. (2002). Effect of ultrasound, temperature and pressure treatments on enzyme activity and quality indicators of fruit and vegetable juices. Ph. D. Thesis. Der Technischen Univesitat Berlin, pp.64.

Lee, S.H. , Kim, E.S., Lee, M.Y. (2001). Purification and characterization of a cationic isoperoxidase from scented-geranium. *Phytochemistry*, **58**, 859–864.

Lencki, W.R., Arul, J., Neufeld, R.J. (1992). Effect of subunit dissociation, denaturation, aggregation, coagulation and decomposition on enzyme inactivation kinetics. II. Biphasic and grace period behavior. *Biotechnology and Bioengineering*, **40**, 1427–1434.

Leon, J.C., Alpeeva, I.S., Chubar, T.A., Galaev, I. Yu., Csoregi, E., Sakharov, I. Yu. (2002). Purification and substrate specificity of peroxidase from sweet potato tubers. *Plant Science*, **163,** 1011–1019.

Ling, A.C., Lund, D.B. (1978). Determining kinetic parameters for thermal inactivation of heat-resistant and heat-labile isozymes from thermal destruction curves. *Journal of Food Science*, **43**, 1307-1310.

Liu, X., Gao, Y., Peng, X., Yang, B., Xu, H., and Zhao, J. (2008). Inactivation of peroxidase and polyphenol oxidase in red beet (*Beta vulgaris L.*) extract with high

pressure carbon dioxide. *Innovative Food Science and Emerging Technology*, **9**, 24-31.

Lopez, P., Sala, F.J., de la Fuente, J.L., Condon, S. , Raso, J., Burgos, J. (1994). Inactivation of peroxidase, lipoxygenase and polyphenol oxidase by manothermosonication. *Journal of Agricultural and Food Chemistry,* **42**, 252–256.

Lopez, P., Burgos, J. (1995). Peroxidase stability and reactivation after heat treatment and monothermosonication. *Journal of Food Science,* **60**, 451-455, 482.

Lopez, P., Sanchez, A.C., Vercet, A., Burgos, J. (1997). Thermal resistance of tomato polygalacturonase and pectinmethylesterase at physiological pH. *Zeitschrift fur Lebensmittel-Untersuchung und -Forschung,* **204**, 146–150.

Lopez, P., Vercet, A., Burgos, J. (1998). Inactivation of tomato pectic enzymes by manothermosonication. *Zeitschrift für Lebensmittel-Untersuchung und Forschung A,* **207**, 249-25.

Lu, A.T. and Whitaker, J.R. (1974). Some factors affecting rates of heat inactivation and reactivation of horseradish peroxidase. *Journal of Food Science*, **39**, 1173-1178.

Maksimoviç, J.D., Maksimoviç, V., Zivanovic, B., Sukalovic, V.H.T., Vuletic, M. (2008). Peroxidase activity and phenolic compounds content in maize root and leaf apoplast, and their association with growth. *Plant science,* in press. doi: 10.1016/j.plantsci.2008.06.015.

Marangoni, A. G., Brown, E. D., Stanley, D. W., Yada, R. Y. (1989). Tomato peroxidase: rapid purification and partial characterization. *Journal of Food Science*, ***54***, 1269-1271.

Marquez, O., Waliszewski, K.N., Oliart, R.M., Pardio, V.T. (2008). Purification and characterization of cell wall-bound peroxidase from vanilla bean. *Lebensmittel-Wissenschaft und-Technologie*, **41**, 1372–1379.

Mason T.J. , Paniwnyk L., Lorimer J.P. (1996). The uses of ultrasound in food technology. *Ultrasonics Sonochemistry*, **3**, 253-260.

Mateı, N., Soceanu, A., Dobrınas, S.,Magearu, V. (2009). Kinetic study of ascorbic acid degradation from grapes. *Ovidius University Annals of Chemistry*, **20**, 132-136.

McClements, D.J. (1995). Advances in the application of ultrasound in food analysis and processing. *Trends in Food Science and Technology*, **6** , 293–299.

Mceldoon, J.P., Dordick, J.S. (1996). Unusual thermal stability of soybean peroxidase. *Biotechnology Progress*, **12**, 555-558.

Mclellan KM, Robinson, D.S. (1981). *Food Chemistry*, **7**, 257–266.
McLellan, K.M., Robinson, D.S. (1984). Heat stability of peroxidase from orange. *Food Chemistry*, **13,**139–147.

Mdluli, K.M. (2005). Partial purification and characterization of polyphenoloxidase and peroxidase from marula fruit (Scleorocarya birrea subsp. Caffra). *Food Chemistry*, **92**, 311–323.

Miesle, T.J., Proctor, A., Lagrimini, L.M. (1991). Peroxidase activity, isoenzymes, and tissue localization in developing high-bush blueberry fruits. *Journal of the American Society for Horticultural Science,* **116***,* 827–830.

Mittler, R., Zilinskas, B. (1991). Purification and characterization of pea cytosolic ascorbate peroxidase. *Plant Physiology*, **97**, 962–968.

Morales-Blancas, E.F., Chandia, V.E., Cisneros-Zevallos, L. (2002). Thermal inactivation kinetics of peroxidase and lipoxygenase from broccoli, green asparagus and carrots. *Journal of Food Science*, **67**, 146–154.

Moulding, P.H., Grant, H.F., McLellan, K.M., Robinson, D.S. (1987). *International Journal of Food Science and Technology,* **22**, 391–397.

Moulding P.H., Goodfellow J., Mclellan K.M., Robinson D.S. (1989). *International Journal of Food Science and Technology,* **24**, 269–275.

Nicoleti, J.F., Silveira-Junior, V., Telis-Romero, J., Telis, V.R.N. (2004). Ascorbic acid degradation during convective drying of persimmons with fixed temperature inside the fruit. *Proceedings of the 14th International Drying Symposium*, Sao Paulo, Brazil, vol. C, pp. 1836-1843.

Nicoli, M.C., Elizalde, B.E., Pitotti, Lerici, C.R. (1991). Effects of sugars and maillard reactions products on polyphenol oxidase and peroxidase activity in food. *Journal of Food Biochemistry*, **15**, 169-184.

Nisha, P., Singhal, R.S., Pandit, A.B. (2004). A study on degradation kinetics of ascorbic acid in amla (Phyllanthus emblica L.) during cooking. *International Journal of Food Sciences and Nutrition,* **55**, 415−422.

Nyborg, W.L. (2000). Biological effects of ultrasound: development of safety guidelines. Part I: personal histories. *Ulrasound in Medicine and Biology*, **26**, 911-964.

Nyborg, W.L. (2001). Biological effects of ultrasound: development of safety guidelines. Part II: general review. *Ulrasound in Medicine and Biology*, **27**, 301-333.

Ory, R.L., St. Angelo, A.J. (1977). *Enzyme in Food and Beverage Processing.* American Chemical Socity. Washington, D.C. USA. pp. 143-171.

Özbek, B., Ülgen, K.O. (2000). The stability of enzymes after sonication. *Process Biochemistry*, **35**, 1037-1043.

Paul, K.G., Stigbrand, T. (1970). Four isoperoxidases from horseradish root. *Acta Chemica Scandinavica,* **24**, 3607-3617.

Pinsent, B.R.W. (1962). Peroxidase regeneration and its effect on quality in frozen peas and thawed peas. *Journal of Food Science*, **27**, 120-126.

Piyasena, P., Mohareb, E., Mckellar, R.C. (2003). Inactivation of microbes using ultrasound: a review. *International Journal of Food Microbiology*, **87**, 207-216.

Pomar, F., Bernal, M. A., Diaz, J., Merino, F. (1997). Purification, characterization and kinetic properties of pepper fruits acidic peroxidase. *Phytochemistry*, **46**, 1313-1317.

Rani, N.D., Abraham, T.E. (2006). Kinetic study of a purified anionic peroxidase isolated from Eupatorium odoratum and its novel application as time temperature indicator for food materials. *Journal of Food Engineering*, **77**, 594–600.

Ratcliffe, B., Flurkey, W.H., Kuglin, J., Dawley, R. (1994). Tyrosine, laccase and peroxidase in mushrooms (agaricus, crimini, oyster and shiitake). *Journal of Food Science*, **59**, 824-827.

Ravindra, N., Van-Huystee, R. (1984). Characterization of peroxidase in plant cells. *Plant Physiology*, **75**, 956–958.

Raviyan, P., Zhang, Z., Feng, H. (2005). Ultrasonication for tomato pectinmethylesterase inactivation: effect of cavitation intensity and temperature on inactivation. *Journal of Food Engineering*, **70**, 189–196

Ray, P.M. (1958). Destruction of auxin. *Annual Review Plant Physiolology,* **9**, 81-118.

Relf, D., McDanial, A., Morse, R. (2004). Tomatoes. *Environmental Horticulture*, 426-418.

Robinson, D.S., Bretherick, M.R., Donnelly, J.K. (1989). The heat stability and isoenzyme composition of peroxidases in Ohane grapes *International Journal of Food Science and Technology,* **24**, 613-618.

Robinson, D.S. (1991). Peroxidases and Catalases in foods. Elsevier Science Publishers, UK, pp. 1–47.

Rodrigo, C., Rodrigo, M., Alvarruiz, A., Frigola, A. (1996). Thermal inactivation at high temperatures and regeneration of green asparagus peroxidase. *Journal of Food Protection,* **59,** 1065-1071.

Rodriguez Lopez, J. N., Espin, J.C., Amor, F. Del, Tudela, J., Martinez, V., Cerda, A., Garcia-Canovas, F. (2000). Purification and kinetic characterization of an anionic peroxidase from melon (*Cucumis melo* L.) cultivated under different salinity conditions. *Journal of Agricultural and Food Chemistry*, ***48***, 1537.

Rodriguez, A.L., Pina, D.G., Yelamos, B., Castillo Leon, J.J., Zadan, G.G., Villar, E., Gavilanes, F., Roig, M.G., Sakharov, I.Y., Shnyrov, V.L. (2002). Thermal

stability of peroxidase from the african oil palm tree *Elaeis guineensis*, *Europe Journal Biochemistry,* **269,** 2584–2590.

Rogozhin, V.V., Kutuzova, G.D., Ugarova, N.N. (2000). Inhibition of Horseradish Peroxidase by N-Ethylamide of o-Sulfobenzoylacetic Acid. *Russian Journal of Bioorganic Chemistry,* **26**, 138-141.

Rogozhin, V.V., Rogozhin,T.V. (2004). The Role of Indole-3-Acetic Acid in Peroxidase Oxidation of Ascorbic Acid Catalyzed by Horseradish Peroxidase. *Biology Bulletin*, **31**, 342–345.

Rudra, S.G., Shivhare, U.S., Basu, S., Sarkar, B.C. (2008). Thermal Inactivation Kinetics of Peroxidase in Coriander Leaves. *Food Bioprocess Technology,* **1**, 187–195.

Saguy, I., Kopelman, I. J., Mizrahi, S. (1978). Simulation of ascorbic acid stability during heat processing and concentration of grapefruit juice. *Journal of Food Process Engineering*, **2**, 213−225.

Sakharov, I.Y., Ardila, G.B. (1999). Variations of peroxidase activity in cocoa (Theobroma cacao L.) beans during their ripening, fermentation and drying. *Food Chemistry*, **65**, 51-54.

Sakharov, I.Y. (2001). Unusual stability of the hemeperoxidase from palm tree leaves Elaeis guineensis. *Journal of Inorganic Biochemistry,* **86**, 415.

Sakharov, I.Y., Vesga Blanco,M.K., Sakharov, I.V. (2002). Substrate Specificity of African Oil Palm Tree Peroxidase. *Biochemistry (Moscow)*, **67**, 1043-1047.

Sala, F.J., Burgos, J., Condon, S., Lopez, P., Raso, J. (1995). *Effect of heat and ultrasound on microorganisms and enzymes.* In: Gould, G.W. (Ed.), New Methods of Food Preservation. Blackie Academic and Professional, London, pp. 176–204.

Sariri, R., Jafarian, V., Sajedi, R.H., Khajeh, K. (2006). Inhibition of horseradish peroxidase by thiol type inhibitors: Mercaptoethanol and mercaptoacetic acid. *Journal of Molecular Liquids* , **128**, 175–177.

Savic, S., Stikic, R., Radovic, B. V., Bogicevic, B., Jovanovic, Z., Sukalovic, V.H.-T. (2008). Comparative effects of regulated deficit irrigation (RDI) and partial root-zone drying (PRD) on growth and cell wall peroxidase activity in tomato fruits. *Scientia Horticulturae*, **117**, 15–20.

Schmid, F.X., Mayr, L.M., Mucke, M., Schonbrunner, E.R. (1993). Prolyl isomerases: Role in protein folding. *Advance in Protein Chemistry,* **44***,* 25–66.

Sciancalepore, V., Longone, V., Alviti, F.S. (1985). Partial purification and some properties of peroxidase from malvasi grapes. *American Society for Enology and Viticulture,* **36**, 105-110.

Serrano-Martinez, A., Fortea, M.I., del Amor, F.M., Nunez-Delicado, E. (2008). Kinetic characterisation and thermal inactivation study of partially purified red pepper (*Capsicum annuum L.*) peroxidase. *Journal of Food Chemistry*, **107**,193-199.

Sergio, L., Pieralice, D., Venere, D.D., Cardinali, A. (2007). Thermostability of soluble and bound peroxidases from Artichoke and a mathematical model of its inactivation kinetics. *Food Technology Biotechnology*, **45**, 367-373.

Shalini, G.R., Shivhare, U.S., Basu, S. (2008). Thermal inactivation kinetics of peroxidase in mint leaves. *Journal of Food Engineering*, **85**, 147–153.

Simal, S., Benedito, J., Clemente, G., Femenia, A., Rossello, C. (2003). Ultrasonic determination of the composition of a meat – based product. *Journal of Food Engineering*, **58**, 253-257.

Soysal, Ç., Söylemez, Z., Bozoğlu, F. (2004). Effect of high hydrostatic pressure and temperature on carrot peroxidase inactivation . *Journal European* **Food Research** *and* **Technology**, **218,** 152–156.

Soysal, Ç., Söylemez, Z. (2005). Kinetics and inactivation of carrot peroxidase by heat treatment. *Journal of Food Engineering*, **68,** 349–356.

Suarez, M.H., Rodriguez, E.M.R., Romeo, C.D. (2007). Chemical composition of tomato (Lycopersicon esculentum) from Tenerife, the Canary Islands. *Food Chemistry*, **106**, 1046–1056.

Sun, Da-Wen. (Eds). (2005). *Emerging Technologies for food processing*. Elsevier Academic Press, USA, pp. 323-345.

Suslick, K.S. (Eds.). (1988). *Homogeneous sonochemistry. In Ultrasound. Its chemical, physical, and biological effects.* VCH Publishers, Inc., New York, pp. 123-163.

Suslick, K.S. (1990) . Sonochemistry. *American Association for the Advancement of Science,* **247,** 1439-1445.

Suslick, K.S. (1994). *The chemistry of ultrasound*. From *The Yearbook of Science and the Future* ; Encyclopaedia Britannica, Chicago, pp. 138-155.

Svensson, S., Hoyem, T., Kvale, O. (1977). (Eds.). *Inactivation of enzymes during thermal processing in physical, chemical and biological changes in foods caused by thermal processing.* London, Applied Science Publishers, pp.168.

Swedın, B., Theorell, H. (1940). Dioximaleic acid oxidase action of peroxidase. *Nature*, **145**, 71.

Tamura, Y., Morita, Y. (1975). Thermal denaturation and regeneration of Japanese-radish peroxidase. *Journal of Biochemistry*, **78**, 561-567.

Thakur, B.R., Nelson, PE. (1997). Inactivation of lipoxygenase in whole soy floor suspension by ultrasonic cavitation. *Die Nahrung*, **41**, 299-301

Thongsook, T., Barrett, D.M. (2005a). Purification and Partial Characterization of Broccoli (Brassica oleracea Var. Italica) Peroxidases. *Journal of Agricultural and Food Chemistry*, **53**, 3206-3214.

Thongsook, T., Barrett, D.M. (2005b). Heat Inactivation and Reactivation of Broccoli Peroxidases. *Journal of Agricultural and Food Chemistry*, **53**, 3215-3222.

Tiwari, B.K., Donnell, C.P.O., Muthukumarappan, K., Cullen, P.J. (2008). Ascorbic acid degredation kinetics of sonicated orange juice during storage and comparison with thermally pasteurized juice. *Food Science and Technology*, in press. doi: 10.1016/j.lwt.2008.10.009.

Tiwari, B.K., Muthukumarappan, K., O'Donnell, C.P., Cullen, P.J. (2009). Inactivation kinetics of pectin methylesterase and cloud retention in sonicated orange juice. *Innovative Food Science and Emerging Technologies,* **10**, 166-171.

Troıanı, E.P., Tropıanı, C.T., Clemente, E. (2003). Peroxidase and polyphenol oxidase in grape. *Cience agrotecnologia,* **27**, 635-642.

Ulusoy, H.B., Colak, H., Hampikyan, H. (2007). The use of ultrasonic waves in food technology. *Research Journal of Biological Science*, **2**, 491-497.

USDA, 2000. U.S. Food and Drug Administration Report. Kinetics of Microbial Inactivation for Alternative Food Processing Technologies: Ultrasound. Published June 2, 2000. Available at http://www.vm.cfsan.fda.gov/~comm/ift-us.html.

Vallee, B., Ulmer, D. (1972). Biological effects of mercury, cadmium and lead. *Annual Review in Biochemistry*, **49**, 91–128.

Vamos-Vigyazo, L. (1981) Polyphenol oxidase and peroxidase in fruits and vegetables. *CRC Crit Rev Food Science Nutrition*, **15**, 49–127.

Van Huystee, R.B., Xo, Y.J., Odonnell, J.P. (1992).Variation in soret band absorption of peroxidase due to calcium. *Plant Physiology and Biochemistry*, **30**, 293-297.

Veitch, N.C. (2004). Horseradish peroxidase: a modern view of a classic enzyme. *Phytochemistry*, **65**, 249–259.

Vercet, A., Lopez, P., Burgos, J. (1997). Inactivation of heat resistant lipase and protease from Pseudomonas fluorescens by manothermosonication. *Dairy Science*, **80**, 29-36.

Vercet, A., Lopez, P., Burgos, J. (1999). Inactivation of heat resistant pectinmethylesterase from orange by manothermosonication. *Journal of Agricultural and Food Chemistry*, **47**, 432-437.

Vercet, A., Burgos, J., Crelier , S., Lopez-Buesa, P. (2000). Inactivation of proteases and lipases by ultrasound. *Innovative Food Science and Emerging Technologies*, **2**,139-150.

Vercet, A., Burgos, J., Crelier, S., Lopez-Buesa, P. (2001). Inactivation of protease and lipase by ultrasound. *Innovation Food Science and Technologies*, **2**, 139-150.

Vercet, A., Sanchez, C., Burgos, J., Montanes, L., Lopez-Buesa, P. (2002a).The effects of manothermosonication on tomato pectic enzymes and tomato paste rheological properties. *Journal of Food Engineering*, **53**, 273-278.

Vercet, A., Burgos, J., Lopez-Buesa, P. (2002b). Manathermosonication of heat-resistant lipase and protease from *Pseudomonas fluorescence*: effect of pH and sonication parameters. *Journal of Dairy Research*, **69**, 243-254.

Vianello A, Zancani M, Nagy G, Macri F. (1997). Guaiacol peroxidase associated to soybean root plasma membranes oxidizes ascorbate. *Journal of Plant Physiolology*, **150**, 573–577

Vieira, M.C., Teixeira, A.A., Silva, C.L.M. (2000). Mathematical modeling of the thermal degradation kinetics of vitamin C in cupuaçu (Theobroma grandiflorum) nectar. *Journal of Food Engineering*, **43**, 1−7.

Villota, R., Hawkes, J.G. (1992). Handbook of Food Engineering, In D. R. Heldman & D. B. Lund (Eds.), *Reaction kinetics in food systems*, New York: Marcel Dekker, pp. 39−144.

Villamiel, M., van Hamersveld, E.H., de Jong, P. (1999). Review: effect of ultrasound processing on the quality of dairy products. *Milchwissenschafts*, **54**, 69–73.

Villamiel, M., de Jong, P. (2000). Influence of high intensity ultrasound and heat treatment in continuous flow on fat, proteins, and native enzymes of milk. *Journal of Agricultural and Food Chemistry*, **48**, 472-478.

Wagenknecht, A.C., Lee, F.A. (1958). Enzyme action and off-flavour in frozen peas. *Food Research,* **23**, 25-31.

Wang, Z., Luh, B.S. (1983). Characterization of soluble and bound peroxidases in green asparagus. *Journal of Food Science,* **48**, 1412.

Wang, M., Sun, J., Feng, W., Cao, J., Jiang, W. (2008). Identification of a ripening-related lipoxygenase in tomato fruit as blanching indicator enzyme. *Process Biochemistry*, **43**, 932–936

Weng, Z., Hendrickx, M., Maesmans, G., Gebruers, K., Tobback, P. (1991). Thermostability of soluble and immobilized horseradish peroxidase. *Journal of Food Science,* **56**, 574-578.

Whitaker, J.R. (1993). *Principles of Enzymology for the Food Science*.(2nd ed). CRC Pres. University of California, Davis, USA, pp. 230-290.

Williams, D.C., Lim, M.H., Chen, A.O., Pangborn, R.M., Whitaker, J.R. (1986). Blanching of vegetables for freezing which indicator enzyme to choose? *Food Technology*, **40**, 130–139.

Wong W.S.D. (1995). Food Enzymes Structure and Mechanism.Chapman and Hall, pp.321.

Yılmaz, E. (2001). The Chemistry of Fresh Tomato Flavor. *Turkish Journal of Agriculture Forestry*, **25**, 149-155.

Yoon-Ku, J., Park, S.O., Bong-Soo, N. (2000). Inactivation of peroxidase by hurdle technology. *Food Science and Biotechnology*, **9**, 124-129.

Zeuthen, P., Sorensen, L.B. (2003). *Food Preservation Techniques*. CRC Pres. Washington, pp. 303-337.

Zhang, Z., Wang, Z., Lın, R. (1999). Purification and some properties of a peroxidase from Tartary buckwheat bran. *Fagopyrum*, **16**, 57-60.