INACTIVATION OF PEROXIDASE AND LIPOXYGENASE IN GREEN BEANS, PEAS AND CARROTS BY A COMBINATION OF HIGH HYDROSTATIC PRESSURE AND MILD HEAT TREATMENT

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

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ABSTRACT

INACTIVATION OF PEROXIDASE AND LIPOXYGENASE IN GREEN BEANS, PEAS AND CARROTS BY A COMBINATION OF HIGH HYDROSTATIC PRESSURE AND MILD HEAT TREATMENT.

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In this study, the efficiency of high-pressure treatment (HHP, 250 - 450 MPa) with the combination of heat treatment (20 - 70^{o}C) on peroxidase (POD) and lipoxygenase (LOX) inactivation in green beans, green peas and carrots was investigated for blanching purposes. Two steps treatments were also performed by pressurization at 250 MPa at 20°C for 15- 60 min and then by water blanching at 40-70°C.

For green beans, 25 % residual activity was obtained by water blanching at 50°C for 15 min after the pressurization at 250 MPa for 60 min. The enzyme inactivation in green peas was 78 % with water blanching at 50^{o}C for 30 min after holding at 250 MPa for 60 min. When the carrots were water blanched at 50°C for 30

min after HHP treatment at 250 MPa for 15min, 13 % residual activity was obtained. During the experiments, the stability gain or the activation of latent form of the enzymes were observed prior to inactivation.

For carrots, LOX activity could not be measured. For green beans, 22 % LOX inactivation was obtained by holding at 250 MPa for 15 min and then by water blanching at 40°C for 5 min. For green peas, the multiple treatment of 250 MPa for 30 min and water blanching at 50°C for 30 min provided 70% inactivation.

To obtain the enzyme inactivation higher than 90 % for blanching purposes, the pressure applied must be increased.

Key words: high hydrostatic pressure, green bean, green pea, carrot, blanching, peroxidase, lipoxygenase

ÖZ

YÜKSEK HİDROSTATİK BASINÇ VE DÜŞÜK ISIL İŞLEM KOMBİNASYONU İLE YEŞİL FASÜLYE, BEZELYE VE HAVUÇTA PEROKSİDAZ VE LİPOKSİGENAZIN İNAKTİVASYONU

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Bu çalışmada, Yüksek hidrostatik basınç (YHB, 250-450 MPa) ve düşük ısıl işlem (20 - 70^{o}C) kombinasyonunun yeşil fasülye, bezelye ve havuçta bulunan peroksidaz (POD) ve lipoksigenaz'ın (LOX) inaktivasyonu üzerindeki verimliliği, farklı uygulama süreleri için araştırılmıştır. YHB ve su ile haşlamanın iki basamaklı uygulaması da yapılmıştır. Two steps treatments were also performed by pressurization at 250 MPa at 20°C for 15- 60 min and then by water blanching at 40-70°C. Bu uygulamalar için, sebze numuneleri 20°C'de 15-60 dakika boyunca 250 MPa basınca tabi tutulmuş ve daha sonra 40-70°C arasında su ile haşlanmıştır.

Yeşil fasülyeler için, 20°C'de 60 dakika boyunca 250 MPa basınca tabi tutulduktan sonra 15 dakika boyunca 50°C'de haşlama sonucunda enzim aktivasyonu yaklaşık olarak 25%'e düşmüştür. Oda sıcaklığında 250 MPa (60 dakika) basınca tabi tutulduktan sonra 50°C'de 30 dakika boyunca su ile haşlanmış bezelyelerde 78%

enzim inaktivasyonu görülmüştür. 20°C'de 15 dakika boyunca 250 MPa basınca tabi tutulduktan sonra 50°C'de 30 dakika boyunca haşlanan havuçlarda 13% enzim aktivasyonu elde edilmiştir. Deneyler süresince, inaktivasyondan önce, sebze tipi ve basınç-sıcaklık kombinasyonuna bağlı olarak, kararlılık kazancı veya enzimlerin gizli formlarının aktivasyonu gözlemlenmiştir.

Havuçlarda LOX aktivitesi ölçülememiştir. Yeşil fasülyeler için, 15 dakika boyunca 250 MPa basınç ve daha sonra 40°C'de 5 dakika boyunca haşlama sonucunda 22% LOX inaktivasyonu elde edilmiştir Bezelyeler için, 30 dakika boyunca 250 MPa basınç ve 50°C'de 30 dakika haşlama uygulaması 70% inaktivasyon yaratmıştır.

Daha etkili inaktivasyon elde etmek için uygulanan basıncın arttırılması gerekmektedir.

Anahtar kelimeler: yüksek hidrostatik basınç, yeşil fasülye, bezelye, havuç, haşlama, peroksidaz, lipoksigenaz

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INTRODUCTION

Enzymes in plant tissues can have undesirable or desirable effects on the quality of fruits and vegetables such as the post-harvest senescence, oxidation of phenolic substances, starch-sugar conversion, post-harvest demethylation of pectic substances leading to softening of plant tissues during ripening. Table 1 shows the enzymatic activities responsible for the quality deterioration of fruits and vegetables. The major factors useful in controlling enzyme activities are temperature, water activity, pH and the use of chemicals during processing.

Table 1. Enzymes responsible for quality deterioration in fruit and vegetables

Enzyme	Quality deterioration
lipoxygenase, lipase, protease	off-flavor
pectic enzymes, cellulose	textural change
polyphenol oxidase, peroxidase, lipoxygenase, chlorophyllase	color change
ascorbic acid oxidase, thiaminase	nutritional loss

1.1 Blanching

Blanching is the pre-processing thermal treatment commonly applied to fruits and vegetables before freezing, drying and canning for different purposes as shown in Table 2 (Garrote et al. 2004). The most important reason for blanching of

vegetables is to inactivate enzymes that can affect the quality. Blanching sets the color and shortens the drying time by affecting the tissue walls so moisture can be removed more rapidly. Blanching before freezing also reduces cooking time of finished products.

Table 2. Purposes of blanching

Purpose	Freezing	Drying	Canning
Enzyme inactivation	+	+	+
Microbial population reduction	+	+	
Tissue gas removal	+	+	+
Product shrinkage/wilting	+	+	+
Increase the peeling efficiency	+	+	+
Cleaning	+	+	+
Color stabilization	+	+	+
To aid in vacuum formation			+

Blanching can be carried out by different methods. Commercial blanching is usually done in hot water or in steam. In water blanching, the product is submerged in water at 85-100°C for approximately 1-10 min. Steam blanching is carried out in continuous installations with conveyer belts made from metallic sieves. Water blanching takes less time than steam blanching but usually results in a greater loss of nutrients (mineral salts, vitamins, sugars, etc.). Cooling of vegetables after blanching is performed in order to avoid excessive softening of the tissues by cooling in cold water or by using pre-cooled air. For drying, the vegetables are conveyed directly from blanching equipment to drying installations without cooling.

Blanching of vegetables prior to freezing has several advantages, but also a number of disadvantages. The advantages include stabilization of texture, flavor and nutritional quality, destruction of microorganism and wilting of leafy vegetables, which assists in packing. However, since blanching is a heat treatment, changes associated with mild thermal processing can be expected. These include loss of

turgor in cells, due to thermal destruction of membrane integrity, and partial degradation of cell wall polymers. A further effect of thermal processing is the degradation of chromophores such as chlorophyll, resulting in color change. Pigment degradation will continue to take place through frozen storage. Blanching can lead to thermally induced degradation of nutrients such as to whether or not the unblanched product has adequate stability to retain structural integrity and quality attributes upon freezing and during frozen storage, and a decision must be reached as to the extent of blanching needed to ensure optimum product quality (Bahçeci et al. 2004).

Process optimization involves measuring the rate of enzyme destruction, so the blanch time is just long enough to destroy the indicator enzyme (Cano, 1997; Bahçeci et al. 2004). Many processors utilize a heat treatment sufficient to inactivate peroxidase, one of the more stable enzymes present, and not incidentally, one of the enzymes whose activity is relatively easy to measure. Some researches suggested that targeting peroxidase leads to a more severe heat treatment than is required for many vegetables and that the enzymes responsible for quality loss, which have been identified, have a lower stability than peroxidase (POD) (Bahçeci et al. 2004). Therefore, the complete activity loss of POD is not required at the end of the blanching. Generally, a residual activity of PODs of about 3-10 % after blanching was recommended. Recently, use of lipoxygenase (LOX) as an indicator of proper blanching has been recommended as more significant in determining storage stability in frozen vegetables (Sheu & Chen, 1991; Barrett & Theeralkulkait, 1995; Bahçeci et al. 2004).

POD and LOX inactivation and ascorbic acid retention were studied during water and microwave blanching of peas, green beans and carrots by Güneş and Bayındırlı (1993). LOX and POD were used as blanching indicator enzymes during the experiments. Ascorbic acid retention during blanching was higher in vegetables when LOX was selected as the indicator enzymes for the blanching treatments. Also ascorbic acid retention was found to be higher in microwave compared to water blanched vegetables.

Kidmose and Martens (1999) studied the effect of microwave, steam (3 min at 90°C) and water blanching (4 min at 90°C) on the texture and nutritional quality of carrots. Microwave heating causes some dehydration and also that the rapid removal of water may increase the mechanical strength of the tissue possibly by increasing the crystallinity of cellulose and hemicellulose in the cell wall. Microwave blanching did not result in improved texture compared to the conventional blanching methods.

An extensive study on the effects of blanching (water and steam blanching) and long-term freezing storage (-20°C for 18 months) on more than 20 commonly vegetables was performed by Puupponen-Pimia et al. (2003). Significant losses of antioxidant activity (20–30%) and total phenolics were detected in most of the vegetables after blanching. Freezer storage slightly decreased phenolic content and antioxidant activity. Dietary fiber components of peas were either not affected by blanching or increased slightly. Soluble fiber almost doubled, while insoluble and total dietary fiber increased by about 20% for the Snake variety. Double amount of soluble fiber was also measured in the Avola variety after blanching. Some losses of minerals by leaching were observed during water blanching. Vitamin C losses were 20–30% in pea samples in which the original vitamin content was high (>30mg /100 g). However, when the original vitamin content was lower (<20mg /100 g), no losses were detected during processing. Folic acid of vegetables were very sensitive to thermal treatment, with more than half of the vitamin being lost after blanching, but was stable during storage at 4^{o}C. Losses were between 12 and 35% for peas. Carotenoids, sterols pentosans, pectins and fructo-oligosaccharides were not significantly affected by blanching or storage. In the same study, soluble, insoluble and total dietary fiber components increased in carrots during processing. This can be explained by the better washing-out of small molecules and the increase of fiber mass fraction in water-cooled samples when compared with air-cooled samples. Vitamin C contents of carrots decreased by around 30% during blanching and no significant further losses were detected during storage. When comparing water- and air-cooled samples, higher losses were found in water-cooled samples. Carotene contents increased during blanching then decreased slightly during storage. It was

also observed that when carrot cubes and slices (harvest year 1997) were stored frozen for 1 year, carotene contents fell dramatically. However, such a decrease was not found in the next harvest year (1998), when carotene contents in the raw material were already much lower. The amount of total phenolics and radical scavenging activity were not affected by blanching and storage.

As a conclusion, during blanching of vegetables, the heat treatment applied depends on the specificity of vegetables, the objectives that are followed and the subsequent processing/ preservation methods. Because various types of vegetables differ in size, shape, heat conductivity, the natural levels of their enzymes, enzymatic distribution within the tissue, inactivation kinetic parameters and relative proportions of heat-labile and heat-resistant fractions, blanching treatments have to be established on an experimental basis according to the literature.

1.2 Peroxidase

PODs (donor: hydrogen peroxide oxidoreductase) are a group of enzymes that catalyze oxidation-reduction reactions. They are given the official EC number 1.11.1.X where X is the digit that represents the type of POD. POD catalyzes reactions in which H_2O_2 is an electron acceptor and a compound AH_2 as a donor of hydrogen atom:

$$H_2O_2 + AH_2 \rightarrow H_2O + A$$

The action of POD is primarily control the level of peroxides, which are generated in almost every reaction in living cells. It constitutes a major activity for the plants to avoid the detrimental effect of free radicals. Beside this one, this enzyme system has gained much attention recently due to its role in modulating the mechanical properties of cell walls during extension. POD can generate phenolic cross-links connecting biopolymer chains (Renier and Macheix, 1996; Sanchez et al. 1996; Schopher, 1996).

PODs can be found in all parts and organelles of the cell, varying in amount and activity with physiological state (Gaspar et al. 1986). PODs found in plants are iron-containing enzymes. There are many different PODs, and each enzyme differs in structure at the active site by only a small degree. The iron is bound on four sides (horseradish POD: arginine, phenylalanine, and two histidine) by nitrogen, making up the heme center (Prater, 2002). In vegetables, PODs are located in soluble form in the cell cytoplasm and in insoluble form as ionically bound and as covalently bound to the cell wall. The bound form can be converted into soluble form by a temperature dependent conversion reaction. All of the POD fractions are present in plant tissues as a combination of various isoenzymes with different thermal stability. The differences in heat resistance of the isoenzymes vary considerably with the vegetable source and origin (Güneş and Bayındırlı, 1993; Tijskens et al. 1997; Garrote et al. 2004).

Tijskens et al. (1997) measured POD activity in peaches, carrots and potatoes after blanching treatments at different constant temperatures. The bound form can be converted into the soluble form by a temperature-dependent conversion reaction. According to their results related to the water blanching of carrots, the inactivation started at temperatures higher than 50°C. Short time inactivation can be obtained in a temperature range of 70-95°C. Güneş and Bayındırlı (1993) showed that the short-time inactivation of heat resistant POD fraction in green beans, green peas and carrots could be obtained at temperatures higher than 70°C.

1.3 Lipoxygenase

Lipoxygenase (linoleate: oxygen oxidoreductase E.C. 1.13.1.X) catalyzes the oxidation of methylene-interrupted unsaturated fatty acids and their esters such as linoleic and linolenic acids. The reaction of oxygen with unsaturated lipids (RH)

involves initiation, propagation, and termination processes. The resulting unstable lipid free radicals (R•) react with oxygen to form peroxy radicals (ROO•). In this propagation process, ROO• react with more RH to form lipid hydroperoxides (ROOH):

$RH \rightarrow R^{\bullet} + H^{\bullet}$

$R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$

$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet}$

Decomposition of lipid hydroperoxides has biological effects and causes flavor deterioration in foods. This decomposition proceeds by cleavage of RO-OH to form alkoxy radicals LO•. These radicals undergo carbon-carbon cleavage to form breakdown products including aldehyde, ketones, alcohols, hydrocarbons, esters, furans, and lactones. LOX catalyzes the addition of oxygen to the reaction to form hydroperoxides. The complete lipid oxidation is composed of four parts (a) the activation of enzyme, (b) pathway with O_2, (c) pathway without O_2, and (d) the nonenzymatic pathway. The reactions are similar to those occurring during autoxidation, but LOX can act much more rapidly than autoxidation and is more specific in terms of end products.

LOX is very important to food scientists for a number of reasons. LOX can affect color, flavor (off-flavors in frozen vegetables), and nutritive properties such as the destruction of vitamin A, loss of essential polyunsaturated fatty acids (linoleic acid), and interaction of enzymatic product with some essential amino acids that lower the quality of protein (Malekian et al. 2000). Lipase and LOX enzymes generally are activated when tissue is disrupted or injured. Sequential enzyme action on lipids starts with the release of fatty acids (lipolytic enzymes). Among the free fatty acids the polyunsaturated are oxidized to fatty acid hydroperoxides by LOX.

LOX, found throughout the plant and animal kingdoms, also have key roles in some metabolic pathways, including the synthesis of prostaglandins and jasmonates. LOX is an ideal biocatalyst using just atmospheric oxygen for the conversion of polyunsaturated fatty acids into many potentially useful and valuable chemicals, which may include leukotrienes and lipoxins. It is possible that the plant enzymes

will have other uses as biocatalysts for production of aroma compounds when available in larger quantities and lower price.

Kinetics of thermal inactivation of pea seed LOX and the effect of additives on their thermostability was investigated (Busto et al. 1999). It was suggested that LOX in crude enzyme extracts was stabilized at 50^{o}C due to protection by some constituents, possibly including starch and proteins. Separate tests at 70^{o}C in the presence of additives (polyols, detergents and small ions) showed that sucrose was the most effective stabilizer and increased the stability of pea LOX by 400-600%.

Williams et al. (1986) and Gunes and Bayındırlı (1993) reported that LOX was the primary cause of development of off-flavors in English green peas and green beans. Pea and green bean LOX were more heat sensitive than POD. Therefore, a less severe heat treatment was required to inactivate LOX in green peas and green beans. According to the study of Güneş and Bayındırlı (1993), LOX activity in carrots was not detected. This was due to the very low enzyme activity in carrots or the presence of strong antioxidants such as carotenoids. This result was also supported by the study of Moral et al. (2002). Thermal stability of LOX and POD in fresh asparagus tips extracts and partially purified asparagus LOX and POD were compared (Ganthavorn et al. 1991). Their results showed differences, since heating at 50, 60 and 70°C resulted in higher percentages of residual LOX activity than POD activity. Heating asparagus tips for 10 min at 60°C, resulted in retention of 46 and 71% for POD and LOX activities, respectively. Inactivation of LOX followed first order kinetics while inactivation of POD followed a biphasic curve.

Gokmen et al (2002) reported that crude LOX extract from green pea was found to be very stable at 60 °C, only 30% of the original activity was inactivated after heating the crude LOX extract at 60 °C for 30 min whilst about 80% of the original activity was inactivated after heating it at 65 °C for 30 min. LOX was almost completely inactivated after 3.0 min at 70 °C and 1.5 min at 75 °C.

In the study of Bahçeci et al. (2004), POD and LOX inactivation in green beans were considered as the indicator of blanching adequacy. At 60°C, 90% of LOX activity was lost within the first 10 min, but the residual activity remained relatively stable during further 20 min of blanching. A blanching treatment at 70°C for 2 min was found sufficient to inactivate 90% of LOX activity. POD was determined to be more resistant to heat treatment than LOX in green beans. 90% of inactivation of green bean POD could be achieved by a blanching treatment at 90 °C for 3 min.

1.4 High Pressure Processing

Thermal treatment is predominantly used in the food industry for product safety. Heat treatment may cause undesirable protein denaturation, non-enzymatic browning and loss of vitamins and also color and flavor compounds. New technologies such as microwave, pulsed electric field, ultrasound, high hydrostatic pressure (HHP) are being evaluated as an alternative to heat treatments for food processing (Knorr, 1999; Bozoglu, 2001; Lado and Yousef, 2002; Knorr, 2003; Food and Drug Administration, http://vm.cfsan.fda.gov/ ~comm/ift-hpp.html).

Firstly, HHP is being evaluated as an alternative to heat treatments for preserving foods to destroy microorganisms without preservatives (Cheftel, 1992; Farr, 1990; Patterson et al. 1995; Knorr, 1999; Alpas et al. 1999; Smelt, 1998; San Martin et al. 2002; Tewari: http://www.avure.com/science-hpp-review.htm; Food and Drug Administration, http://vm.cfsan.fda.gov/~comm/ift-hpp.html). Vegetative cells are inactivated at about 300 MPa at ambient temperature, while spore inactivation requires much higher pressures (600 MPa or more) in combination with a temperature rise to 60–70°C. Certain enzymes are inactivated at 300 MPa, while others are very difficult to inactivate at all within the pressure range. Moisture content is extremely important in this context, little effect being noticeable below 40% moisture content (Ludikhuyze et al. 2002).

HHP also named as high pressure processing (HPP), or ultra high pressure (UHP) processing, subjects liquid and solid foods, with or without packaging, to pressures between 100 and 800 MPa. High pressure processing involves subjecting food material to a high hyrostatic pressure and held isobarically for a specified period of time before release (Tedford et al, 1999). In operation, after all air has been removed, a pressure-transmitting medium (water) is pumped from a reservoir into the pressure vessel. This is termed 'indirect compression' and requires static pressure seals. Another method, termed 'direct compression' uses a piston to compress the vessel, but this requires dynamic pressure seals between the piston and internal vessel surface, which are subject to wear and are not used in commercial applications. Batch systems for food processing are available of up to 500 L in volume and for pressures up to 800 MPa (Ludikhuyze et al. 2002).

Two principles underlie the effect of HHP. Firstly, the principle of Le Chatelier, according to which any phenomenon (phase transition, chemical reaction, change in molecular configuration) accompanied by a decrease in volume can be enhanced by pressure. Secondly, pressure is instantaneously and uniformly transmitted independent of size and geometry of the food i.e. the food will be compressed by a uniform pressure from every direction and then return to its original shape when the pressure has been released. This is known as isostatic pressure (Knorr, 1993; Tewari, 1999; Alpas et al, 1999).

For economic reasons, there is a general tendency to apply pressures lower than 600 MPa. The ACB company (USA) has developed a semi-continuous horizontal pressure vessel with a double set of pistons for loading and unloading in a straight line. Semi-continuous processing of fruit juices at 4000–6000 L/h, using pressures of 400–500 MPa for 1–5 min at ambient temperature, is used by a company in Japan. Overall estimates by several equipment manufacturers point towards investment costs for a commercial system in the range 0.5–2 million Euro and production costs at 400 MPa of 0.1–0.2 Euro/ kg of processed product. A high pressure plant for fruit juice pasteurisation is about 20 times the cost of an equivalent heat exchanger system (Manvell, 1996). Actual costs will depend on chamber

capacity and time–pressure–temperature combinations in processing. Investment cost will be about 75% of total production costs.

The work of compression during HHP treatment can increase the temperature of foods through adiabatic heating approximately 3 °C per 100 MPa, depending on the composition of the food. For example, if the food contains a significant amount of fat, such as butter or cream, the temperature rise can be larger. Foods cool down to their original temperature on decompression if no heat is lost to or gained from the walls of the pressure vessel during the hold time at pressure. Meyer (2000) has proposed that the heat of compression can be used in a high-temperature, short-time combined thermal and pressure treatment, since the temperature of a product can be raised from 100 to 120 °C by a quick compression to 700 MPa and brought back to 100 °C by a quick decompression.

Pressures used in the HHP of foods appear to have little effect on covalent bonds, therefore this method does not destroy vitamins and the product retains its natural flavor and color. Due to this advantage of the method, HHP treated food products (e.g. juices, dairy product, meat, fish, fruits and vegetables) have been delivered to the Japanese, US and European market (Knorr, 2002). Also substantial accomplishments have been obtained in the field of gelatinization of polysaccharides as well as in the pressure mediated modification of real food systems especially fruits and vegetables, meat, fish and milk and dairy products. Pressure stabilization of enzymes (e.g. amylase) at high temperatures has also been observed which offers the potential for the improvement of various food processes (e.g. conversion of polymers). Pressure induced phase transitions such as pressure shift freezing or pressure assisted thawing offer many possible applications including reduced structural damage of frozen products, or bacterial starter cultures, increased thawing rates as compared to conventional thawing of foods.

Knorr (2002) reported that the future opportunities of HHP include the optimization of pressure assisted blanching, the development of pressure supported freezing and thawing processes and the utilization of pressure homogenization.

Further, combination processes (e.g. with heat) or the use of high pressure as pretreatment for permeabilization of biological membranes to aid subsequent mass transfer reactions should be developed. Beside these, the studies related to the optimum design of the package and other suitable packaging materials are important research areas in the future.

1.5 Effect of high pressure processing on enzymes

Effect of HHP on enzymes has been divided in two classes: Relatively low pressures of 100–200 MPa have been shown to activate monomeric enzymes, whereas higher pressures generally cause enzyme inactivation.

Cheftel (1992), Mozhaev et al. (1996), Knorr (1999), Lullien-Pellerin and Balny (2002), Hendrickx et al. (1998), Lullien-Pellerin and Balny (2002) and Tewari et al. (http://www.avure.com/ science-hpp-review.htm) reviewed the effect of high pressure on proteins/enzymes. The primary structure of proteins (amino acids in a polypeptide chain joined by covalent bonding) will remain intact during pressure treatment since covalent bonds are almost unaffected by high pressure. Secondary structure (coiling of peptide chains joined with hydrogen bonding) changes occur at very high pressures (300-700 MPa), which might be explained by the rupture of hydrogen bonds (which are enhanced at low pressures). Significant tertiary structure (arrangement of chains into globular shape by long distance non covalent bonding) changes are observed beyond 200 MPa. The quaternary structure (various compact structures or sub-units joined by non-covalent bonding), mainly maintained by hydrophobic interactions, is the most sensitive structure to pressure. Moderate pressures (<150 MPa) were found to favor the dissociation of oligomeric proteins, a phenomenon always accompanied with negative and sometimes large volume changes. Dissociation can be followed by subunit aggregation or by precipitation. Generally, oligomer dissociation occurs at pressures (<150–200 MPa) lower than those at which unfolding of monomers is observed. Pressure above 150–200 MPa induces unfolding of proteins and reassociation of subunits from dissociated

oligomers. Pressure-dissociated subunits undergo time-dependent conformational changes.

The denaturation of proteins by high pressure is not identical to the temperature-induced process. (Mozhaev et al. 1996). The exact unfolding mechanism by which the pressure-induced protein is denatuted is not yet fully understood altough it has been suggested that pressure causes changes in the structure of the protein molecules due to the cleavage of weak hydrogen bonds and van der Waals forces while covalent bonds are unaffected (Mozhaev et al. 1996). This ensures the retention of essential vitamins and nutrients and thereby improving the quality of product (Tedford, 1999)

The effect of HHP on the rate and degree of irreversible enzyme denaturation depends on the composition and structure of enzyme, pH, pressure, temperature and type and concentration of solutes in the liquid that the enzyme presents (Cano et al. 1997; Castellari et al. 1997; Gomes and Ledward, 1996; Gomes, et al. 1997; Goodner et al. 1998; Nienaber and Shellhammer, 2001; Ogawa et al. 1990; Quaglia et al. 1996; Tedjo et al. 2000; Weemaes et al. 1998; Ludikhuyze etal. 2003). Seyderhelm et al. (1996) ranked the enzymes according to the their pressure induced inactivation in the following order (from low to high): LOX, lactoperoxidase, pectinesterase, lipase, phosphatase catalase, polyphenol oxidase, POD by considering distinct conditions within a pressure range of 0.1-900 MPa, a temperatures range of 25 to 60°C and pH from 3 to 7 with treatment times of 2 to 45 min in model buffer solutions.

In addition to conformational changes, enzyme activation can rise from pressure-induced decompartmentalization. In intact tissues, enzymes and substrate are often separated by compartmentalization, which can be destroyed upon application of pressure. Pressure-induced membrane damage cause enzyme-substrate contact. The enzymatic reaction resulting from this contact can, in turn, be accelerated or decelerated by pressure. Concerning pressure inactivation, there seems to be a minimum pressure below, which no or little enzyme inactivation occur. For some enzymes, there seems to be a maximum pressure above which further pressure

increase does not result in additional inactivation. This has been attributed to an enzyme portion that is irreversibly converted to the inactive form, while a fraction is converted to a very pressure-resistant form. Upon pressure release, the pressure-resistant fraction reverts to the equilibrium state, while the irreversibly inactivated enzyme remains unchanged.

Ludikhuyze *et al.* (1997) determined kinetic parameters of pressure-temperature inactivation of α-amylase under dynamic conditions of pressure cycling. They reported that multiple pressure treatments had a more pronounced effect on the inactivation of α-amylase as compared to a single-cycle process.

With respect to enzymes related to quality of fruit and vegetable products, for which HHP treatment is believed to offer great potential in the area of preservation and processing and therefore the recent research efforts have mainly been focused on enzyme inactivation.

1.6 Effect of high pressure processing on quality related enzymes in fruits and vegetables

Some key enzymes in fruit and vegetable processing include polyphenoloxidase, which is responsible for enzymatic browning, LOX which induces changes in flavour, colour and nutritional value, pectinmethylesterase which is responsible for cloud destabilization and consistency changes and POD which gives rise to unfavourable flavours. Because of their importance in food industry, thermal inactivations of these enzymes have been studied extensively. All throughout the last decade, the studies on pressure inactivation of enzymes have consistently been increasing.

The inactivation of avocado polyphenol oxidase (pH 7) was investigated in different pressure (0.1-900 MPa) and temperature (25-80ºC) combinations. Low-pressure applications protect this enzyme from thermal inactivation (Weemaes *et al.* 1998 a and b). For many sources (e.g. apple, pear, potato, strawberry), the activation

of PPO was observed at pressures below those needed for inactivation, which could be ascribed to conformational changes or to conversion of a latent enzyme form to an active form by release from the membrane. In tissues, apparent activation of PPO may take place as a result of membrane alterations and decompartmentation of the enzyme and its substrate (Asaka *et al.* 1994; Gomes and Ledward, 1996; Jolibert *et al.* 1995). Gomes and Ledward (1996) investigated the effect of pressure treatment (100-800 MPa for 1-20 min at room temperature) on polyphenol oxidases from mushrooms, potatoes, and apples. For potatoes, enzyme activity was consistently lost with increasing pressure, but after 10 min at 800 MPa, 40% of enzyme activity remained. For mushrooms, enzyme activity was increased after treatment at 400 MPa for 10 min and 60% of enzyme activity remained after processing at 800 MPa for 10 min. They concluded that enzyme sources/type affected the efficiency of HHP on their inactivation. Jolibert *et al.* (1995) studied the effects of HHP on polyphenol oxidase in fruits (plums, apricots, strawberries, and apples). They suggested that a total inactivation of fruit enzyme activity may be achieved at 600 MPa and the inactivation dependent on the substrate, fruit species, and pH, since they found that HHP increased apple-browning, but reduced the enzyme activity in plums at the same HHP treatment conditions. Castellari *et al.* (1997) studied the effect of HHP on polyphenol oxidase activity of grape must and reported that limited polyphenol oxidase inhibition was obtained between 300 and 600 MPa. They suggested that very high pressures (> 900 MPa) along with mild thermal treatment (40-50°C) should be used to totally inactivate polyphenol oxidase activity. Knorr (1993) reported that pressure treatment at 100–400 MPa could cause a decrease in polyphenol oxidase activity in potatoes. Pressure treatment at 500MPa has been suggested for PPO inactivation in banana.

Cano *et al.* (1997) studied the pressure and temperature effects on inactivation of POD, polyphenol oxidase, and pectin esterase enzymes in strawberry puree and orange juice. They subjected the samples to a pressure in the range of 50-400 MPa at 20-60°C. They found that significant inactivation of strawberry polyphenol oxidase activity (60%) occurred up to 250 MPa and but POD activity was reduced by 25%. In orange juice, POD and pectin esterase activities were reduced by

50% at 35°C. In strawberry puree, POD was increasingly inactivated up to 300MPa for treatments at 20°C for 15 min. Above 300 MPa, POD activity was slightly increased. Above 45°C, a decrease in activity was found for all pressures (50-400 MPa). At room temperature, the activity of orange POD decreased continuously up to 400MPa (processing time 15 min). The highest inactivation rate (50%) was found at 32°C. High-pressure treatments at 32-60°C adversely increased POD activity in orange juice.

Goodner *et al.* (1998) studied the inactivation of pectin esterase in orange and grapefruit juices by high pressure. They reported that treatment at 600 MPa caused substantial inactivation of the heat labile form of pectin esterase, whereas the heat stable form was not affected. They also reported that grapefruit pectin esterase was more sensitive to pressure than orange pectin esterase. Almost 90% inactivation of pectin methylesterase in orange juice was reported by Irwe and Qisson (1994) after pressure treatment at 400 MPa with 1–2 min holding time.

Rovere *et al.* (1996) studied the inactivation of POD, polyphenol oxidase and pectin esterase by combining pressures up to 100 MPa with temperatures of 20-88°C. They concluded that all of the enzymes could be inactivated using 100 MPa at 50-60°C.

HHP inactivation of commercial amylase in apple juice was evaluated under various conditions (100– 400 MPa, 0–60 min and 6–40 °C) by Riahi and Ramaswamy (2004). Inactivation of amylase was more pronounced at higher-pressure levels, higher temperature, longer treatment times and lower pH of juice. The pressure effect on amylase was assumed to follow a dual inactivation behavior, with a pressure pulse effect followed by a first order rate inactivation during the pressure hold. The concept of instantaneous pressure inactivation due to the application of a pressure pulse has been also reported for the other enzymes by Basak & Ramaswamy (1996).

Ibarz *et al.* (1996) investigated inhibition of polyphenol oxidase activity in apple slices during HHP. They immersed apple slices in water or ascorbic acid, citric acid, or 4-hexylresorcinol (4-HR) solutions as anti-browning agents at 50 ppm, prior to subjecting them to high pressures in the range of 138 - 690 MPa for 5 min. They did not find any polyphenol oxidase inactivation at 138 MPa (except in slices immersed in 4-HR), and suggested that to inhibit polyphenol oxidase activity, pressures > 276 MPa should be used or prior treatment with 4-HR should be done.

Quaglia et al. (1996) studied the effect of HHP treatment on green peas at different operation conditions (magnitude of pressure from 400 to 900 MPa and treatment times of 5 and 10 min) combination with a mild heat treatment at 60 °C for blanching purposes. The residual POD activity was 12% at 900 MPa for 5 min. For green beans, a treatment of 900MPa for 10 min at room temperature was needed to cause an 88% reduction of POD activity. A combination with temperature treatments enhanced the inactivating effect at 600MPa.

The combined effects of pressure and temperature on the activity of POD in carrot extract were investigated in the pressure range of 0.1–600 MPa and the temperature range of 25–45 °C. At lower pressures (<396 MPa), carrot POD stability increased compared to unpressurized samples. At low temperatures (25 and 35°C), residual enzyme activity did not show significant decreases for pressures up to 600 MPa. With treatment at 600 MPa and 25 °C for 15 min, 55.3% POD inactivation was achieved, whereas 91.2% inactivation was observed at 600 MPa and 45°C. Holding the sample at 45°C for 15 min without pressure application reduced POD activity by 29.4%. When combined HHP and temperature treatment was compared with thermal treatment at ambient pressure (0.1 MPa), it was seen that carrot POD stability increases compared to unpressurized samples up to 396 MPa, which proved the antagonistic effects of pressure and temperature at low pressures. An antagonistic effect of pressure and temperature on avocado polyphenoloxidase at pressure below 250 MPa and 62.5°C has already been demonstrated (Tedjo et al. 2000). Regeneration of carrot POD was studied by treating the crude extract at 506 MPa for 15 min at temperatures of 25, 35, 40 and 45 °C and storing the samples at 4 °C. The

regeneration of POD activity with the combined HHP and temperature treatments followed first order kinetics at 25, 35 and 40°C. With treatment at 506 MPa and 45 °C, no regeneration was observed indicating that in these conditions, carrot POD was irreversibly inactivated. When the temperature of the pressure application was increased, the regeneration of the carrot POD was decreased, and to obtain irreversible inactivation of POD, temperatures equal to or higher than 45°C were necessary at 506 MPa.

The effect of HHP treatment on the activities of LOX and POD were studied by Tedjo et al. (2000). HHP treatment (240 MPa, 55°C, 15 min) of LOX and POD in 30% sucrose solution without buffer led to approximately 80 and 50% residual activity, respectively. At low pressures up to approximately 200 MPa (55°C, 15 min) an increase in LOX activity (100-120%) was observed. Total inactivation at the given temperature and time was obtained at 570 MPa. POD activity was reduced to 50% at 240 MPa, (55°C, 15 min, in 30% sucrose solution). The sugar solution provides a protective effect on the enzymes.

Commercial soybean LOX in 0.2 M citrate-phosphate were subjected to pressures of 0.1, 200, 400, and 600 MPa for 20 min. The enzyme was stable at atmospheric pressure (0.1 MPa) over a wide pH range (5-9). In citrate-phosphate buffer, after treatment at 200 MPa with increasing pressure, the pH stability range becomes narrower (7-8) (Tangwongchai et al. 2000).

As to pressure inactivation, the studies have been performed for tomato, soybean, green bean and pea LOX. Pressures in a range between 400 and 600 MPa have been reported for inactivation (Heinisch *et al.* 1995; Ludikhuyze *et al.* 1998; Tangwonchai *et al.* 2000; Indrawati, 2000). Several combinations of high pressure (up to 750MPa) and temperature (0-75°C) for a period of 5 min have been used for the inactivation of soybean LOX in Tris buffer, pH 8.3 (Heinissh et al. 1995). Pressure resistance of the enzyme increased with increasing enzyme concentration and decreased with decreasing pH in the range 9±5.4. Furthermore, the pressure stability was much higher in Tris buffer than in McIllvaine buffer. LOX inactivation

in green beans due to high-pressure treatment were studied in the pressure-temperature ranges of 0.1 up to 650 MPa and -10 up to 70°C by Indrawati et al. (2000a). At ambient pressure, in whole green beans LOX was less thermostable than in the green bean juice at temperatures below 68°C whereas the stability ranking was reverse at temperatures above 68°C. The pressure needed to obtain the same rate of LOX inactivation at a given temperature was lower in whole green beans than in the juice. Application of high-pressure treatment at low/subzero temperature resulted in an antagonistic effect on LOX inactivation for both systems, whereas no such effect was found above room temperature. In the selected pressure range, inactivation was similar at 0 or -10°C and less rapid than at 50°C. High pressure blanching would therefore be possible, preferably at higher temperatures. For green bean and peas it was noted that pressure stability of LOX decreased with increasing system complexity, i.e. inactivation occurred faster *in* situ (in the intact vegetable) as compared to a crude extract (Indrawati, 2000b). It has also been reported that green bean POD showed 75% residual activity after high-pressure application at 500 MPa for 60 s at ambient temperature (Krebbers et al. 2002).

Kim et al. (2002) investigated the effects of the combined treatment of high pressure and heat on carrot juice. The optimum process condition was estimated at 395 to 445 MPa, 70°C, for 8 to 11 min. The predicted inactivation values of pectin methylesterase, polyphenoloxidase and LOX were 90, 96 and 98%, respectively. A combined treatment of high-pressure carbon dioxide and HHP was investigated as a non-thermal processing technique to enhance the safety and shelf life of carrot juice by Park et al. (2002). A combined treatment of 4.90 MPa-high-pressure carbon dioxide and 600 MPa-HHP effectively inactivated enzymes. The residual activities of polyphenoloxidase, LOX, and pectinmethylesterase were less than 11.3%, 8.8%, and 35.1%, respectively. Enzyme activities showed a strong correlation with pH, which was dependent on the pressure of carbon dioxide.

Shook et al. (2001) determined HPP's ability to inactivate LOX, pectinesterase, and polygalacturonase in diced tomatoes. Processing conditions used were 400, 600, and 800 MPa for 1, 3, and 5 min at 25 and 45°C. The magnitude of

applied pressure had a significant effect on inactivating LOX and polygalacturonase, with complete loss of activity occurring at 800 MPa. At 400 MPa, LOX inactivation was greater at 25℃ that at 45℃. Pectinesterase was very resistant to pressure treatment.

1.7 Effect of high pressure processing on the quality of fruits and vegetables

In general, significant effects of pressures up to 350 MPa applied to plant systems were not observed on overall texture and structure (Knorr, 1995). But several studies revealed that pressure treatment of fruit and vegetables can cause both firming and softening (Basak and Ramaswamy, 1996) and the effects also dependent on the pressurization time. The softening curves revealed that texture changes caused by pressure occurred in two phases: a sudden loss as a result of the pulse action of pressure followed by further gradual loss during pressure holding phase. At low pressure (100 MPa), instantaneous texture change was caused by compression of cellular structures without disruption, while at higher pressure (>200 MPa) severe texture loss occurs owing to rupture of cellular membranes. In many cases, pressure-treated vegetables do not soften during subsequent cooking, which is attributed to the action of pectin methylesterase that is only partially inactivated by pressure. Simultaneous disruption of cell structures allows interaction of the enzyme with the pectic substance. Hence, the deesterfied cell wall pectin can crosslink with divalent ions, leading to increased compactness of cellular structure. Knorr (1999) reported that heat and mass transfer in plant tissues can be influenced by HHP, resulting in improved release of metabolites, increased drying rates of vegetables, or reduced oil uptake of french fries. HHP also can cause structural changes in structurally fragile foods such as strawberries. Cell deformation and cell membrane damage can result in softening and cell serum loss. Usually these changes are undesirable, but these food products can be used in the manufacture of fruit jellies and jams, fruit juices and salad dressing after processing.

The influence of high pressure on functional properties of a choice of fruits and vegetables (carrots, tomatoes and broccoli) was evaluated by Butz et al. (2002 and 2003) . The treatments were at pressures around 600 MPa and also in combination with elevated temperatures and in comparison with thermal treatment were investigated. In most cases high pressure induced changes in the structure of the products which resulted in altered properties such as higher glucose retardation index and water retention or reduced extractability.

Bignon (1996) observed that vitamin A, C, B and E content of fruit and vegetable products is not significantly affected by pressure treatment if compared with thermal treatment. Beside these, in the case of strawberries, the decrease in vitamin C content during storage after pressure treatment (400– 600MPa/15–30min) was found to be much lower compared to the fresh products (Sancho et al. 1999). The pressure–temperature stability of ascorbic acid in buffer, orange juice and tomato juice was performed by Van den Broeck *et al.* (1998). They found only significant degradation of ascorbic acid when pressure of about 850 MPa was combined with temperatures between 60 and 80°C, and more in tomato and orange juice than in buffer.

The effects of HHP on naturally present microbial flora, texture, color and ascorbic acid content of whole green beans were evaluated and compared to conventional blanching techniques (4 min at 90°C) by Krebbers et al. (2002). HHP treatment (500 MPa, ambient temperature) showed a large retention of firmness and ascorbic acid for green beans compared to conventional preservation methods. Quaglia et al. (1996) studied the effect of HHP treatment on green peas at different operation conditions (400-900 MPa for treatment times of 5-10 min) combination with a mild heat treatment at 60 °C for blanching purposes. While treatment times did not affect the parameters measured, increase in pressure resulted in a higher level of POD inactivation and retention of ascorbic acid. Pressurized samples did not show differences in texture, although a significant reduction in firmness was observed with respect to fresh peas. Among the conditions tested, 900 MPa for 5 min gave the best

results with respect to fresh peas (residual POD activity and ascorbic acid content 12 % and 82%, respectively).

The effect of high-pressure treatment (200-600 MPa for 20 min) on the texture of cherry tomatoes and on the key softening enzymes (pectinmethylesterase and polygalacturonase) was investigated by Tangwongchai et al. (2000). When subjected to high-pressure treatment whole cherry tomatoes showed increasing textural damage with increasing pressures up to 400 MPa. However, treatment at pressures 500-600 MPa led to less apparent damage than treatment at 300 and 400 MPa. It was concluded that the textural changes in tomato induced by pressure involve at least two related phenomena. Initially, damage is caused by the greater compressibilty of the gaseous phase (air) compared to liquid-solid components, giving rise to a compact structure which, on pressure release, is damaged as the air rapidly expands, leading to increases in membrane permeability. The damage also enables enzymatic action to increase, causing further cell damage and softening. The major enzyme involved in the further softening is polygalacturonase, which is inactivated at 500 MPa and above, and not pectinmethylesterase, which in the whole fruit, is barotolerant. In the study of Shook et al. (2001) the processing conditions used were 400-800 MPa for 1-5 min at 25 and 45°C. Percent soluble solids, pH, titratable acidity, and Hunter color values did not differ significantly among the high-pressure-processed diced tomatoes as compared to the control. Apparent protein content decreased in the pressure-processed samples, due possibly to protein denaturation, loss of solubility, and/or a decrease in dye binding sites to assay protein content. For many fruit and vegetable products high pressure treatment was noted largely to preserve fresh colour (Ludikhuyze et al. 2002).

1.8 Aim of the study

These results demonstrate that HHP treatment is a promising technology for use as an alternative to thermal treatment to inactivate the enzymes. But the combination of HHP treatment with one or more additional factors such as low pH,

use of natural compounds and heat treatment are more reliable to reduce enzyme activity. HHP are also under consideration to reduce energy, processing time, wastewater and nutrient loss as alternatives to conventional water and steam blanching. Some of the represented studies showed that enzyme inactivation in fruit and vegetable juices or extracts cannot represent the inactivation of the same enzyme in whole plant structure.

Carrot, green beans and peas are important economic products in Turkey. In this study, the efficiency of HHP treatment with the combination of low heat treatment on POD and LOX inactivation was determined in green beans, peas and carrots for blanching purposes.

CHAPTER 2

MATERIALS AND METHODS

2.1 Vegetables

Green beans, peas and carrots were purchased from a local market in Ankara. The vegetables rinsed in tap water and prepared for treatments in the following manner: the peas were removed from their pods. The carrots and green beans were 5-8 cm in length. The carrots were cut into two pieces along the length and cylindrically shaped.

2.2 High Pressure Treatment

HHP treatments were performed in a designed and constructed lab-scale unit with 10 ml pressure vessel (maximum T: 50^{o}C). Time to reach the desired pressure and also for decomposition was approximately 5-10 seconds for the designed system (Figure 2.1). The main parts of the HHP equipment are a cylindrically designed pressure vessel, a pressure pump, temperature control device and other system controls.

Temperature inside the vessel is controlled at the intervals of 10-90°C by the temperature control device. The liquid inside the vessel is warmed prior to pressurization to the desired temperature by an electrical heating system around the chamber. The rate of pressure increase is about 140 MPa/min, and pressure comedown time is less than 2 min. An automatic device sets the pressure level and the temperature of pressurization.

Fresh vegetables were packed in polyethylene film (carrots and green beans were cut in 7 cm length and 1 cm radius, 9 beans were squeezed into film) and placed inside the cylindrical vessel of HHP equipment and then the chamber was closed. Before the pressurization, the samples were kept in the chamber for 1-2 minutes for the temperature equilibration.

Figure 2.1 HHP Equipment

The vegetable samples were pressurized at 250-450 MPa and 20-50°C for 15 - 60 minutes. After pressurization the samples were immediately removed and put into an ice bath. Multiple treatments of HHP and water blanching were also performed. After the vegetable samples were pressurized at 250 MPa at 20°C for 15- 60 minutes, they were water blanched at 40-70^{o}C for 5 - 30 min. All the experiments and measurements were replicated twice.

2.3 Thermal Treatment (Water Blanching)

A constant temperature water bath with shaker was used as the water blanching equipment. Green beans, carrots and peas were blanched in water at 40-70^{o}C for 5, 15, 30, 45 and 60 minutes. All the experiments and measurements were replicated twice.

The treatments applied to the selected vegetables were given in Table 3 and Table 4.

Table 3. The treatment conditions applied to green beans, green peas and carrots for POD activity

	Water Blanching				HHP treatment																Two Step Treatment 250MPa +20 °C											
	40	50	60	70	300MPa				350MPa				400MPa				450MPa				15 min				30 min				60 min			
					20	30	40	50	20	30	40	50	20	30	40	50	20	30	40	50	40	50	60	70	40	50	60	70	40	50	60	70
Green Beans	+	+	+	+	+	+	+	+	+	+	+	+													+	+	+	+	+	+	+	+
Green Peas	+	+	+	+	+	+	+			+															+	+	+	+	+	+	+	+
Carrots	+	+	+	+	+	+			+				+				+				+	+	+		+	+	+	+				

Table 4. The treatment conditions applied to green beans and green peas for LOX activity

	Water Blanching				HHP treatment																Two Step Treatment 250MPa +20 °C											
	40	50	60	70	300MPa				350MPa				400MPa				450MPa				15 min				30 min				60 min			
					20	30	40	50	20	30	40	50	20	30	40	50	20	30	40	50	40	50	60	70	40	50	60	70	40	50	60	70
Green Beans	+	+	+	+																	+	+	+		+	+	+					
Green Peas	+																				+	+			+	+						

2.4 Enzyme Assays

2.4.1 Preparation of Crude Enzyme Extracts

Preliminary experiments were performed to determine the ratio between sample weight and extracting buffer solution volume (g sample: ml buffer) for reproducibility and linearity between enzyme activities. To obtain vegetable extracts, samples were cut to smaller size. Samples were then mixed with the cold (4°C) sodium phosphate buffer (PH = 6.5) in different proportions. The proportions were 30:70 w/v for green beans and 30:140 w/v for green peas and 30:40 w/v for carrots, respectively. Each sample-buffer mixture was homogenized in by a hand blender for 2 min. The suspensions were filtered through cheesecloth to remove solid particles. These methods used for the preparation of the enzyme extracts were used for both untreated and HHP and /or heat treated vegetables to measure POD and LOX activities.

2.4.2 Peroxidase Assay

POD substrate solution was prepared daily by mixing 0.5 ml guaiacol (99.5 %, Sigma), 0.5 ml hydrogen peroxide (30%, Pancreac), and 99 ml sodium phosphate buffer (PH 6.5). POD assay was conducted by mixing 0.1 ml enzyme extract with 3.5 ml substrate solution. POD activity was measured at 25ºC as the initial increase in absorbance at 470 nm (UV-Vis spectrophotometer 1202, Shimadzu) by reporting the data in 10 sec intervals for 4 min. The blank was prepared with 0.1 ml water and 3.5 ml substrate solution (Gunes and Bayındırlı, 1993). From the plot of absorbance vs time, the initial rate of reaction was determined by considering the initial slope of the line. One unit of activity was defined as an initial absorbance change of 0.001 / min under the assay conditions. Per cent residual POD activity was calculated as 100 times of the enzyme activity in treated samples per the enzyme activity in untreated control sample.

2.4.3 Lipoxygenase Assay

LOX was assayed using the method of Bahçeci and others (2004) with some modifications. The LOX substrate solution was prepared by mixing 160 µl linoleic acid (Sigma), 160 µl Tween 20, 10 ml water and clarified by adding 1 ml sodium hydroxide (1 N). The stock was diluted with sodium phosphate buffer (PH = 6) to obtain 200 ml substrate solution volume. The solution was mixed with a vortex for 30 min to allow solubilization of oxygen within the substrate solution. For the LOX assay, 29 ml substrate solution was put in 30^{o}C water bath and 2 ml of crude enzyme extract was added to the solution. LOX assay was conducted by mixing 750 µl of this mixture with 3 ml sodium hydroxide (0.1 N) in a glass tube in certain time intervals. LOX activity was measured as the change in absorbance at 234 nm for 3 min. The blank was prepared with 750 µl of substrate solution and 3 ml sodium hydroxide (0.1 N). Enzymatic activity measurements were done by using spectrophotometer (UV-Vis spectrophotometer 1202, Shimadzu). From the plot of absorbance vs time, the initial rate of reaction was determined by considering the initial slope of the line. One unit of activity was defined as an initial absorbance change of 0.001 / min under the assay conditions. Per cent residual LOX activity was calculated as 100 times of the enzyme activity in treated samples per the enzyme activity in untreated control sample.

CHAPTER 3

RESULTS AND DISCUSSION

In vegetable processing, blanching to inactivate enzymes is important to prevent quality changes during storage of green beans, peas and carrots. Since its wide distribution in vegetable tissue, resistance to heat and ease of measurement in processing, the inactivation of POD during heat treatment is one of the most investigated topics in relation to blanching. Thermal inactivation of POD in vegetables is generally biphasic due to the presence of heat-labile and heat resistant isoenzyme fractions. Thermal denaturation of POD can be due to the dissociation or modification of the prosthetic group and conformational change in the enzyme. No general rule has yet been established as to what extent the presence of residual enzyme activity is suitable from the aspect of quality preservation of processed vegetables during storage. In most cases the process variables and controls have been obtained by investigation of the effect of temperature and exposure time on enzyme inactivation with regard given to vegetable quality. For frozen storage, Böttcher (1975) recommended blanching until 2-6.3 % and 0.7-3.2% POD activity left by depending on variety for peas and green beans, respectively. Generally, 90% or higher reductions of POD activity are required in vegetables to obtain the optimum vegetable quality during frozen storage.

3.1 Peroxidase Inactivation

3.1.1 Peroxidase Inactivation in Green Beans

Figure 3.1 shows the residual enzyme activities during water blanching of green beans at different temperatures. The non-linearity of the curve on log scale (not shown in the text) indicated the presence of POD fractions with different thermostabilities. POD activity was reduced to 13 % residual activity only at 70^{o}C in 15 min that is a long time for blanching purposes and also the reduced activity was not sufficient for blanching purposes. Reduced blanching time is important for the industry by decreasing energy cost and also would result in more desirable product quality. It was reported that POD in green beans is completely inactivated by 2 min blanching at 93.3^{o}C (Barret & Threekulkait, 1995). In the study of Bahçeci et al. (2004), the retention of POD was approximately 80 % at 70°C for 20 min blanching and 90 % of POD inactivation could be achieved by a blanching treatment at 90°C for 3 min. Güneş and Bayındırlı (1993) reported approximately 30% residual activity in green beans after blanching at 96°C for 10 min while microwave blanching (2450 MHz, 650 W) provided 5% retention of the enzyme after 3 min treatment. So in order to analyze a blanching process, the variety of the vegetable, the proportion of resistant and labile isoenzymes, the end point that the analytical method to be used may detect, are very important beside of the heat transfer conditions.

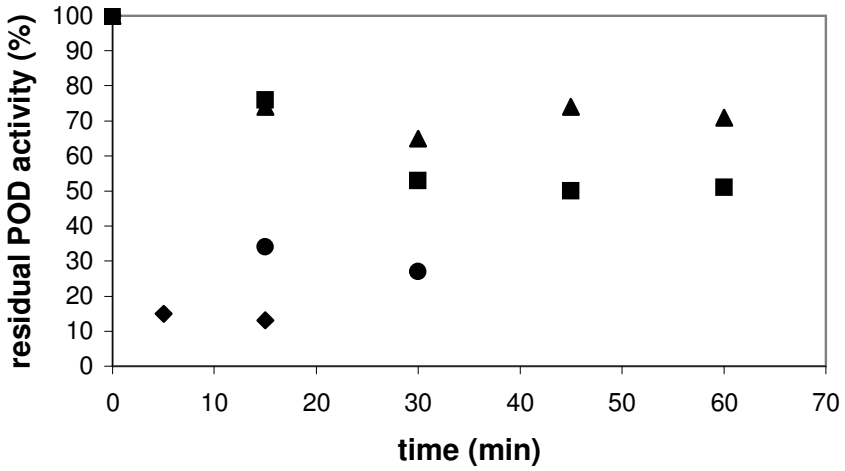
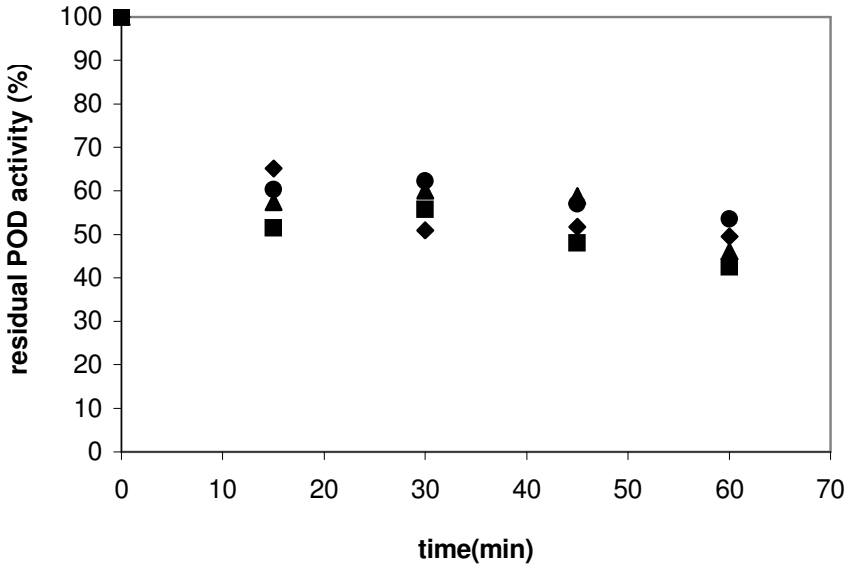


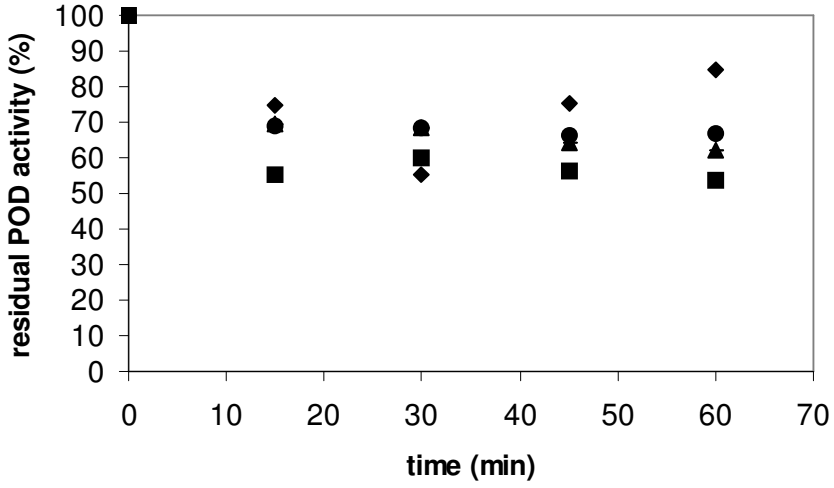
Figure 3.1 Residual POD activities in green beans after water blanching at 40°C (▲), 50°C (■), 60°C (●), 70°C (♦)

HHP treatment can be used due to some advantages over thermal treatment such as minimal heat damage problems, retention of freshness and color and also reduced waste problem and energy usage. The combinations of heat and HHP treatments were considered for the inactivation of POD in selected vegetables for blanching purposes. Figure 3.2.a and b show the effect of HHP treatment on POD activity in green beans under 300 MPa and 350 MPa at different temperatures. Treatment at 300 and 350 MPa and 20-50°C could be used for blanching purposes due to the remaining POD activity that is higher than 10%. The pressure effect follows a dual inactivation behavior due to the presence of isoenzymes or an initial pressure pulse effect followed by further gradual loss during pressure holding phase. Knorr (1999) reported that the pressure resistance of enzymes was not related to thermal resistance. Riahi et al. (2004) were studied the pulse effect on α- amylase activity in apple juice. At 20° C, the pulse inactivation is about 30.5% at 100 MPa and after 60 min holding time, the inactivation increases to 73.2%. On the other hand, at 400 MPa, the pulse pressure inactivation is 93.1%, and following a holding time of 60 min it increases to 98.1%. At 30 °C, a single pressure pulse at 400 MPa resulted in more that 98.3% inactivation of amylase in apple juice at pH 3.0. Thus, the pressure effect on amylase was assumed to follow

a dual inactivation behavior with a pressure pulse effect followed by a first order rate inactivation during the pressure hold. The concept of instantaneous pressure inactivation due to the application of a pressure pulse has been also reported for pectin methyl esterase in orange juice by Basak & Ramaswamy (1996).



(a)



(b)

Figure 3.2. Residual POD activities in green beans treated at 300 MPa (a) 350 MPa (b) and 20°C (▲), 30°C (■), 40°C (●), 50°C (♦)

Increasing the pressure from 300 MPa to 350 MPa did not provide an increase in the inactivation rate. Interestingly, an increase in the activity for POD from 55 to 84 %was observed at 350 MPa and 50°C after an exposure time of 30 min up to 60 min. This may arise from changes due to the release of membrane-bound enzymes and may relate to the pressure-activated latent form. According to the explanation of Balny et al.(1997), the effects of pressure and temperature on biochemical systems are antagonistic: increased temperature leads to disorder, increased pressure to order. The pressure at which proteins denaturate can be lower at low temperature compared to that at higher temperature and generally the denaturation temperature of proteins increases with increasing pressure up to 100 MPa, after that, if pressure is increased more, the temperature of denaturation decreases. Mozhaev et al., (1996) showed that the protection effect of high hydrostatic pressure becomes more pronounced at higher temperatures. Although, the complete inactivation of chymotrypsin in reaction mixture at 55°C occurs instantaneously at atmospheric pressure, under a pressure of 360 MPa at 50°C, the same enzyme shows activity which is more than 30 times higher than the activity at 20°C and 1 atm. A possible explanation for stabilization was given as counteracting effects of pressure and temperature on the formation or disruption of intramolecular interactions and opposing effects on interactions of the enzyme with water molecules.

Most effective results were obtained by HHP treatment (250 MPa, 20°C, 30 and 60 min) and then water blanching (40 and 50°C) as shown in Figure 3.3. The residual enzyme activity was higher than 70% after water blanching at 40 and 50°C for 15 min (Figure 3.1), but in two steps treatment, a significant decrease was observed in POD activity within the first 15 min. Increasing the pressurization time from 15 min to 30 min was not effective to reduce more POD activity. Approximately 25 % residual activity was obtained with the combined effect of 250 MPa/20°C (60 min) and 15 min of water blanching at 50^{o}C. Figure 3.4 shows two steps treatments with higher water blanching temperatures (60 and

70 °C). By the two steps treatments of 250 MPa (20°C/60min) and water blanching at 60°C and 70°C for 15 min, 71 % and 88% inactivation of POD were achieved in green beans, respectively. POD activity was reduced to 13 % residual activity by water blanching at 70°C in 15 min without HHP treatment (Figure 3.1). It was reported that whole green bean POD showed 75% residual activity after high-pressure application at 500 MPa for 60 s at ambient temperature (Krebbers et al., 2002). In the same study, the researchers were also applied pulsed HHP treatment that the samples were preheated at 75°C for 2min, HHP treated by holding 80 s at 1000 MPa with a second pressure pulse of 1000 MPa after 30 s at 0.1 MPa. The pulsed treatment provided more than 99% reduction in activity.

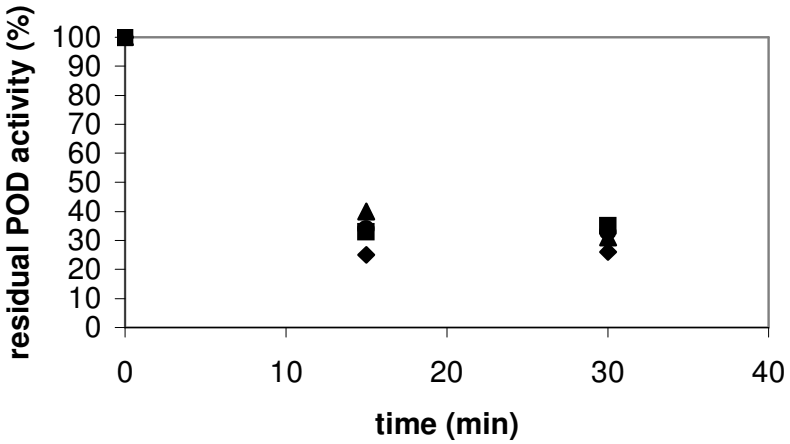


Figure 3.3. Residual POD activities in green beans: water blanching at 40°C after holding at 250 MPa &20°C for 30 min (▲), water blanching at 40°C after holding at 250 MPa &20°C for 60 min (■), water blanching at 50°C after holding at 250 MPa &20°C for 30 min (●), water blanching at 50°C after holding at 250 MPa &20°C for 60 min (♦)

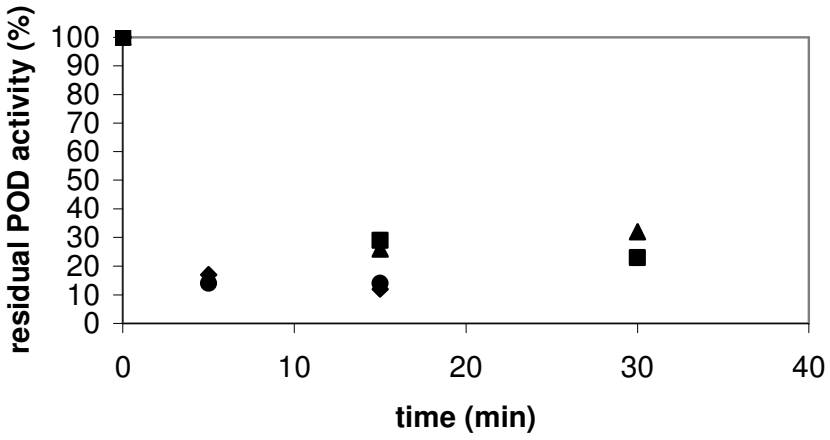


Figure 3.4. Residual POD activities in green beans: water blanching at 60°C after holding at 250 MPa &20°C for 30 min (▲), water blanching at 60°C after holding at 250 MPa &20°C for 60 min (■), water blanching at 70°C after holding at 250 MPa &20°C for 30 min (●), water blanching at 70°C after holding at 250 MPa &20°C for 60 min (●)

3.1.2 Peroxidase Inactivation in Green Peas

If the inactivation of POD was selected as the indicator, the water blanching at 40, 50, 60 and 70°C cannot be used as blanching purposes for green peas as shown in Figure 3.5. Generally, the retention of the activity was higher than 60 % at a blanching time of 30 min for the selected temperatures. Water blanching at 60°C provided an increase POD activity (123 % at 15 min). In the study of Güneş and Bayındırlı (1993), 90% reduction in POD activity in green peas was obtained after water blanching of 12 min at 96°C. According to the same study, a treatment condition of 70°C and 10 min provided approximately 60 % reduction in the activity. Williams et al. (1986) reported that about 30 % of POD activity is lost within 6 min, 50 % within 40 min and the remaining 50 % activity was stable for 70 min in peas homogenate at 60°C. The enzyme was significantly stable in the whole pea than the pea homogenate. At 70°C, 50% of the activity

was lost in 5 min with about 40% of the activity being relatively stable in whole peas.

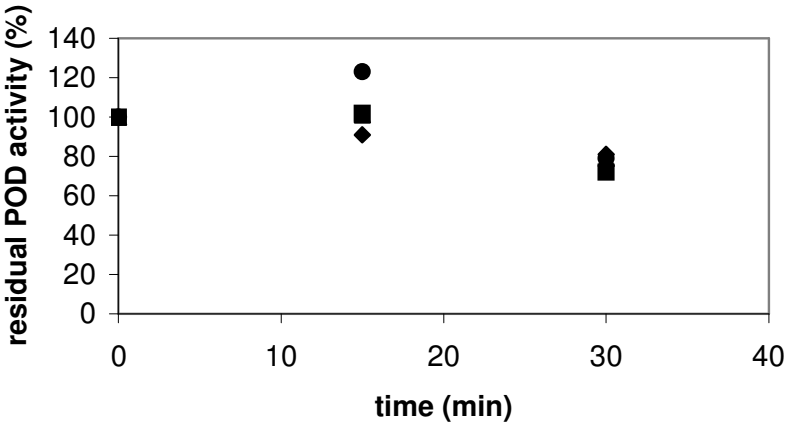


Figure 3.5. Residual POD activities in green peas after water blanching at 40°C (▲), 50°C (■), 60°C (●), 70°C (♦)

Figure 3.6 shows the effect of HHP treatment at 300 MPa and 20, 30 and 40°C on POD activity of green peas at different temperatures. An effective inactivation was not obtained at 300 MPa with the selected temperatures and at the treatment at 30°C, an increase in the activity was observed (100-106% in 45 min). Increasing the pressure from 300 to 350 MPa caused only activation in 60min (100-118 %) and Quaglia et al. (1996) studied the effect of HHP treatment on green peas at different operation conditions (magnitude of pressure from 400 to 900 MPa at 40 and 60°C and treatment times of 5 and 10 min). In general, an increase in pressure resulted in a reduction of enzyme activity and a nonuniform behavior was detected for the residual activity with respect to time in their study. The residual POD activity was 12% at 900 MPa for 10 min. Combination with thermal treatment provided an enhancement on the pressure effect at 600 MPa, but an enhancement was not detected for 700 MPa. This behaviour was explained by different response of isoenzymes to heat and pressure.

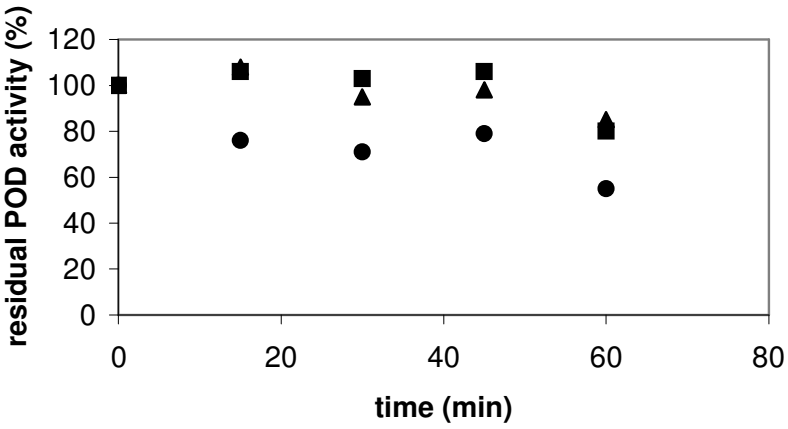


Figure 3.6. Residual POD activities in green peas treated at 300 MPa and 20°C (▲), 30°C (■), 40°C (●)

Water blanching after HHP treatment was also applied for peas as shown in Figure 3.7. A 50% inactivation of POD was achieved with water blanching at 40°C for 30 min after a treatment at 250 MPa (60min) at ambient temperature. Compared to pressure treatment at 300 MPa, the multiple treatments caused more inactivation. Increasing the temperature from 40 to 50°C provided higher retention of enzyme activity. The enzyme inactivation was 78 % with water blanching at 50°C for 30 min after a treatment at 250 MPa (60min) at ambient temperature.

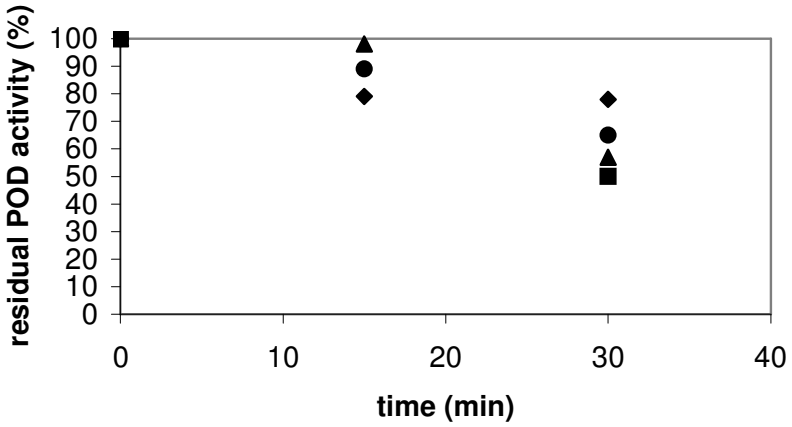


Figure 3.7. Residual POD activities in green peas: water blanching at 40°C after holding at 250 MPa &20°C for 30 min (▲), water blanching at 40°C after holding at 250 MPa &20°C for 60 min (■), water blanching at 50°C after holding at 250 MPa &20°C for 30 min (●), water blanching at 50°C after holding at 250 MPa &20°C for 60 min (♦)

3.1.3 Peroxidase Inactivation in Carrots

Water blanching was more effective on POD inactivation in carrots as shown in Figure 3.8. 9% residual activity remained in carrot after heat treatment at 60°C for 30 min. The results obtained in this study were similar to the results obtained by Tijskens et al., (1997) where POD activity in carrots was measured by using o-dianisidine and H_2O_2 as substrate after blanching treatments at different temperatures. According to their results related to the water blanching of carrots, short time inactivation can be obtained in a temperature range of 70-95°C and two isoenzymes exist, one bound and one soluble. The bound form can be converted into the soluble form by a temperature-dependent conversion reaction. Also, POD in carrot extract was inactivated by approximately 29 % after 15 min of heating at 45°C and complete inactivation was obtained after 10 min heating at 75°C (Soysal et al., 2004).

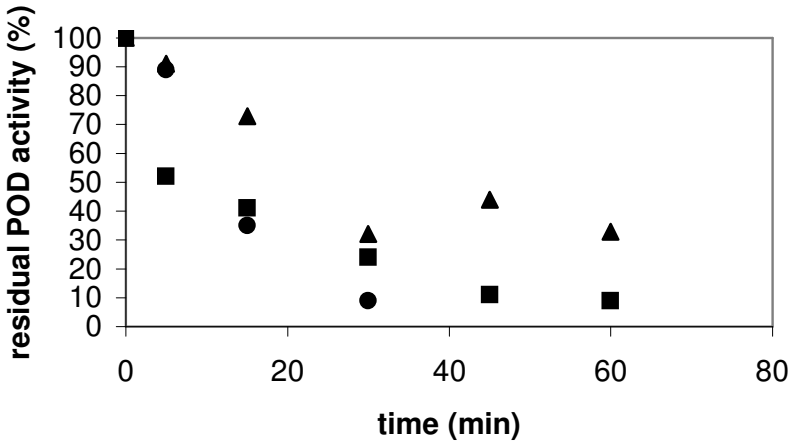


Figure 3.8. Residual POD activities in carrots after water blanching at 40°C (▲), 50°C (■), 60°C (●)

The effect of HHP treatment at different pressures at 20°C on POD activity in carrots was presented in Figure 3.9. Carrot POD was decreased to 16 % residual activity within the first 30 min at treatment conditions of 350 MPa and 20^{o}C and then it decreased to 9% in 60 min. However, a gradual decrease in the enzyme activity could be seen in the first 15 min at 300 and 450 MPa, the effectiveness of 400- 450 MPa was lower than that of 350 MPa. According to the study of Soysal et al. (2004), at 25 and 35°C, POD activity in carrot extract did not show significant decreases for pressures up to 600 MPa. With treatment at 600 MPa and 25°C for 15 min, 55 % POD inactivation was achieved, whereas 91 % inactivation was obtained at 600 MPa and 45°C. They also reported that carrot extract POD stability increases compared to unpressurized samples up to 396 MPa.

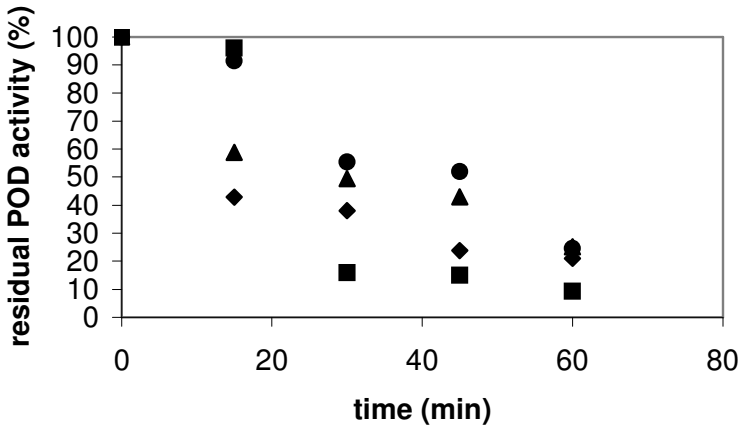


Figure 3.9. Inactivation of POD in carrots treated at 20°C and 300 MPa (▲), 350 MPa (■), 400 MPa (●), 450 MPa (♦)

Water blanching after HHP treatment was also effective for the inactivation of POD in carrots as shown in Figure 3.10. When the carrots were water blanched at 50°C for 30 min after HHP treatment at 250 MPa, 20°C for 15min, 13 % residual activity was obtained. But treating carrots for 60 min at treatment conditions of 350 MPa and 20^{o}C provided 9 % residual POD activity.

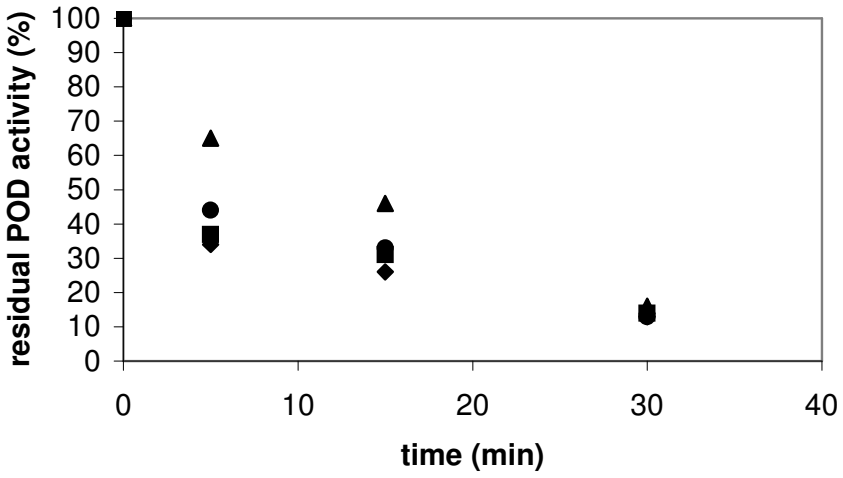


Figure 3.10. Residual POD activities in carrots: water blanching at 40°C after holding at 250 MPa &20°C for 15 min (▲), water blanching at 40°C after holding at 250 MPa &20°C for 30 min (■), water blanching at 50°C after holding at 250 MPa &20°C for 15 min (●), water blanching at 50°C after holding at 250 MPa &20°C for 30 min (♦)

Although the inactivation of POD higher than 90% can not be obtained at the selected pressure and temperature ranges for green beans and green peas, water blanching at lower temperatures after HHP treatment 250 MPa provided better results if compared with only HHP treatments at lower temperatures. Researchers have recently suggested that the indicator should be the enzyme directly involved with major deteriorative changes in vegetables during storage. In vegetables, the most important quality attribute during storage is off-flavor development and color loss that is mainly catalyzed by LOX (Barret & Theeralkukait, 1995). A less severe heat treatment is required for the inactivation of LOX if compared with POD in green beans and peas (Gunes & Bayındırlı, 1993). As the indicator enzyme, the use of less heat resistant LOX provides both saving in energy and in product quality during blanching.

3.2 Lipoxygenase Inactivation

For carrots, LOX activity could not be detected with the assay used in this study. This may be due to very low LOX activity in carrots or the presence of strong antioxidants such as carotenoids. Blancas et al. (2002) also could not detected LOX activity for carrots. The same result was obtained in the study of Gunes and Bayındırlı (1993) that they used a different assay (KI-starch method) for the measurement of LOX activity. In the literature, there are only a few articles related to LOX activity in carrots. Park et al. (2002) assayed LOX activity in carrot juice by using linoleic acid as the substrate and they studied the effect of HHP on the quality of carrot juices. According to their results, to lower the residual activity to less than 30%, 600 MPa of HHP applications at 25°C for 10 min was required. The same researcher group in a different study (Kim et al., 2001) investigated the combination of heat and HHP treatment for carrot juice quality and reported that heat treatment at 105°C for 30 s inactivated 54 % of LOX and pressure treatment at 300 MPa and 25°C for 10 min inactivated 83 % of LOX. Increasing the pressure caused the enzyme activation. The combined treatment (300-500 MPa and 50-70°C for 10 min) was effective to inactivate LOX that the enzyme showed less than 10 % residual activity at all combined treatment conditions according to their studies.

3.2.1 Lipoxygenase Inactivation in Green Beans

In order to compare heat resistance of POD and LOX, LOX activities were measured in green beans during water blanching at different temperatures (Figure 3.11). Although the enzyme inactivation was observed at 50, 60 and 70°C, a blanching time of 5 min provided 136 and 105 % activation of LOX at 40 and 50^{o}C, respectively. While 16% residual LOX activity was reached in 15 min at 60^{o}C, the same result was achieved in 6 min at 70^{o}C. A treatment condition at 60°C for 45 min reduced the activity to 6.5 %. In the study of Bahçeci et al.

(2004), it was reported that 90 % of LOX activity was lost within the first 10 min, but the residual activity remained stable during further 20 min of blanching at 60°C and a blanching treatment at 70°C for 2 min was found as sufficient for 90 % inactivation. If the effect of the time and temperature combinations of this study is compared with the results of Bahçeci et al. (2004), the inactivation was slightly lower than the inactivation reported in their study. Güneş and Bayındırlı (1993) obtained approximately 30 % remaining activity after water blanching at 70 °C for 6 min. If the LOX inactivation is compared with the POD inactivation (Figure 3.1), LOX in green beans is more heat sensitive at 60 and 70°C.

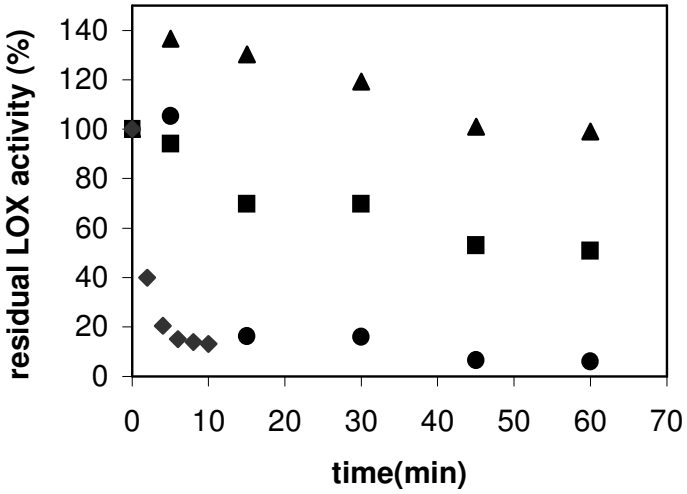


Figure 3.11. Residual LOX activities in green beans after blanching at 40°C (▲), 50°C (■), 60°C (●), 70°C (♦)

Figure 3.12 shows the effect of water blanching after pressurization at 250 MPa and 20°C on LOX activity in green beans. HHP treatment 250 MPa and then water blanching at 40°C was not effective on LOX inactivation. 22 % LOX inactivation was obtained by holding at 250 MPa for 15 min and then by water blanching at 40°C for 5 min and no more inactivation was determined in further times up to 60 min. At the same water blanching temperature, holding 30 min instead of 15 min at 250 MPa provided an increase in the stability of the enzyme.

Although, only water blanching at 50°C for 30 min caused 30 % inactivation, 58 % inactivation was obtained by water blanching at 50°C for 30 min after pressurization at 250 MPa for 30 min. Treatment at 250 MPa for 15 min instead of 30 min caused an activity increase (154 %) in 5 min water blanching at 50°C and 35 % inactivation was obtained at a treatment of 30 min. After pressurization at 250 MPa and 20°C for 15 or 30 min, the water blanching at 60°C for 30 min provided 2-3 % residual LOX activity. Indrawati et al. (2000a) studied the inactivation of LOX in crude green bean extract at 200 to 700 MPa and -10 to 60 °C and observed that an increase in pressure at constant temperature enhanced the LOX inactivation rate, whereas at constant pressure, maximal stability was observed around 20°C. The same research group (Indrawati et al., 2000b) also reported that the intact green bean LOX was less thermostable than in the juice at temperatures below 68 °C whereas the stability ranking was reverse at temperatures above 68 °C at ambient pressure conditions. The pressure needed to obtain the same rate of LOX inactivation at a given temperature was lower in beans than in the juice.

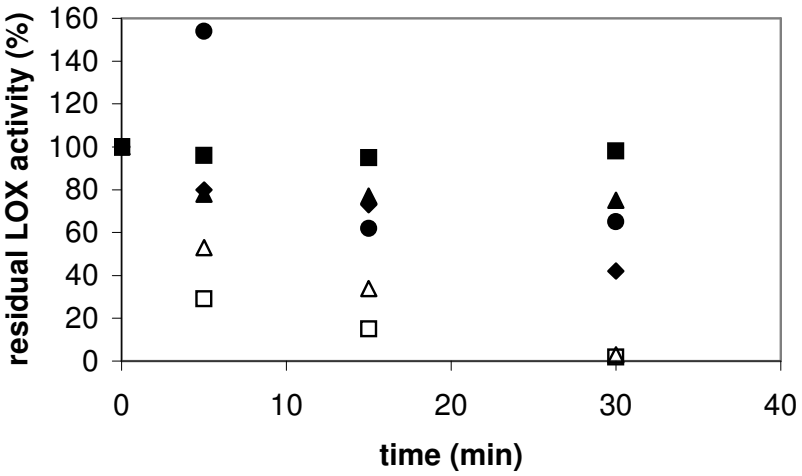


Figure 3.12. Residual LOX activities in green beans: water blanching at 40°C after holding at 250 MPa &20°C for 15 min (▲), water blanching at 40°C after holding at 250 MPa &20°C for 30 min (■), water blanching at 50°C after holding at 250 MPa &20°C for 15 min (●), water blanching at 50°C after holding at 250 MPa &20°C for 30 min (♦), water blanching at 60°C after holding at 250 MPa &20°C for 15 min (Δ), water blanching at 60°C after holding at 250 MPa &20°C for 30 min (□)

3.2.1 Lipoxygenase Inactivation in Green Peas

Figure 3.13 shows the effect of the similar treatments on LOX activity in green peas. Water blanching at 40°C for 60 min causes 40% inactivation. After HHP processing at 250 MPa and 20°C for 30 min, water blanching at 40°C for 5 min provided approximately the same result for green pea LOX. While, the multiple treatment of 250 MPa for 30 min and water blanching at 50°C for 30 min provided 70% inactivation, the multiple treatment of 250 MPa for 15 min instead of 30 min with the same water blanching condition caused only 17 % inactivation. This shows the stability gain during pressurization. According to the study of Indrawati et al. (2001), pressure levels below 200 MPa retarded thermal inactivation of LOX in green peas at temperatures above 60°C. Above 60°C,

enzyme activation under pressure occurred prior to inactivation. Shook et al. (2001) showed that LOX inactivation in tomatoes at 400 MPa was greater at 25°C than at 45°C.

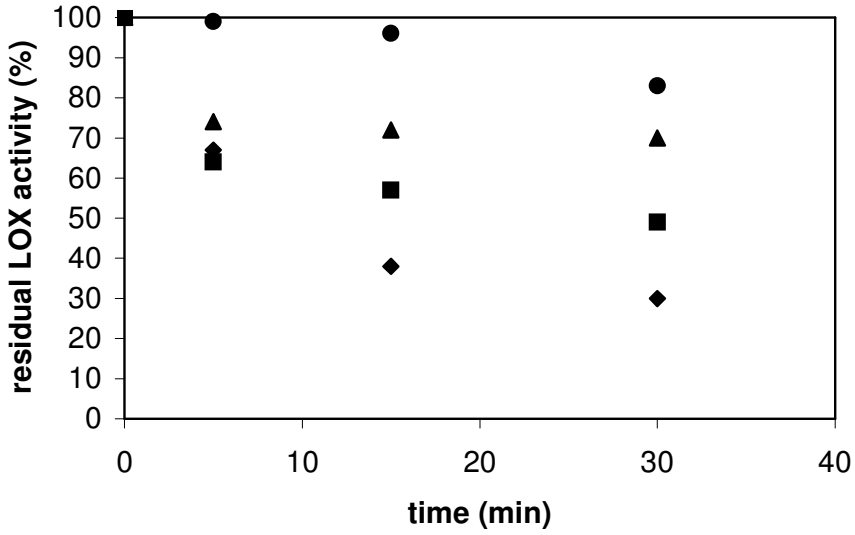


Figure 3.13 Residual LOX activities in green peas: water blanching at 40°C after holding at 250 MPa &20°C for 15 min (▲), water blanching at 40°C after holding at 250 MPa &20°C for 30 min (■), water blanching at 50°C after holding at 250 MPa &20°C for 15 min (●), water blanching at 50°C after holding at 250 MPa &20°C for 30 min (♦)

It is clear that the resistances against thermal/pressure inactivation of POD and LOX are higher in the selected ranges of pressure and temperature. The resistances of POD and LOX of green beans and green peas are not too different from each other. To obtain more effective inactivation, there is a requirement of increasing the pressure applied.

CHAPTER 4

CONCLUSIONS AND RECOMENDATIONS

Blanching of vegetables prior to freezing has several advantages, but also a number of disadvantages. The advantages include stabilization of texture, flavor and nutritional quality, inactivation of enzymes and microorganism and wilting of leafy vegetables, which assists in packing. However, due to blanching is a heat treatment, changes associated with mild thermal processing can be expected.

The inactivation of the most heat resistant POD is used to show the adequacy of the blanching of vegetables. Another important enzyme found in vegetables is LOX that affects the quality of vegetables during storage.

For thermal treatment without pressure application, temperatures above 70°C can be used for POD inactivation in the selected vegetables for blanching purposes. By combination with HHP treatment, the temperature of thermal treatment can be reduced. Pressure effect on POD follows a dual inactivation behavior, with a pressure pulse effect followed by inactivation.

After HHP processing at 250 MPa and 20°C for 15-30 min, water blanching at lower temperatures (40-50°C) provided significant reductions in POD and LOX activities of the selected vegetables. It is clear that the resistances against thermal/pressure inactivation of POD and LOX are higher in the selected ranges of pressure and temperature. The enzymes from different origins display different pressure stabilities, but the resistances of POD and LOX of green beans and also green peas are not too different from each other. Higher pressures than the pressures used in this study may be combined with mild heat (<50°C) to obtain more sufficient enzyme inactivation in the selected vegetables in shorter process times. Economic

feasibility by HHP processing requires that the treatment conditions must be adjusted to achieve the lowest pressure/temperature/ shortest time combinations needed to inactivate the enzymes of concern from the vegetables to be treated. Ideally, pressures should be no greater than about 600 MPa to reduce cost.

Further research may be carried to understand the effect of HHP on different vegetables and enzymes. Also further studies could be done to investigate the effects of HHP on the shelf life, physical and chemical properties of vegetables with the optimization of the process.

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APPENDIX A

Experimental Data

Table A.1 Effect of water blanching on POD activity in green beans

Residual POD activity (%) P (MPa)		
T (°C)	t (min)	0.1 MPa
40	0	100
	15	74
	30	65
	45	74
	60	71
50	0	100
	15	76
	30	53
	45	50
	60	51
60	0	100
	15	34
	30	27
70	0	100
	5	15
	15	13

Table A.2 Effect of HHP treatment on POD activity in green beans

Residual POD activity (%) P (MPa)			
T (°C)	t (min)	300 MPa	350 MPa
20	0	100	100
	15	57	69
	30	60	68
	45	58	64
	60	46	62
30	0	100	100
	15	51	59
	30	55	58
	45	47	56
	60	42	53
40	0	100	100
	15	60	69
	30	62	68
	45	56	66
	60	53	66
50	0	100	100
	15	65	74
	30	50	55
	45	51	75
	60	49	84

Table A.3 Effect of two step treatment on POD activity in green beans

Residual POD activity (%) P (MPa)			
T (°C)	t (min)	250 MPa+20°C	
		30 min	60 min
40	0	100	100
	15	40	33
	30	31	35
	45		
	60		
50	0	100	100
	15	34	25
	30	33	26
	45		
	60		

Table A.4 Effect of two step treatment on POD activity in green beans

Residual POD activity (%) P (MPa)			
T (°C)	t (min)	250 MPa+20°C	
		30 min	60 min
60	0	100	100
	15	26	29
	30	32	23
70	0	100	100
	5	14	17
	15	14	12

Table A.5 Effect of water blanching on POD activity in green peas

Residual POD activity (%) P (MPa)		
T (°C)	t (min)	0.1 MPa
40	0	100
	15	101
	30	79
	45	
	60	
50	0	100
	15	102
	30	72
60	0	100
	15	123
	30	79
70	0	100
	5	91
	15	81

Table A.6 Effect of HHP treatment on POD activity in green peas

Residual POD activity (%) P (MPa)			
T (°C)	t (min)	300 MPa	350 MPa
20	0	100	
	15	108	
	30	95	
	45	98	
	60	85	
30	0	100	100
	15	106	95
	30	103	85
	45	106	118
	60	80	108
40	0	100	
	15	76	
	30	71	
	45	79	
	60	55	

Table A.7 Effect of two-step treatment on POD activity in green peas

Residual POD activity (%) P (MPa)			
T (°C)	t (min)	250 MPa+20°C	
		30 min	60 min
40	0	100	100
	15	98	103
	30	57	50
	45		
	60		
50	0	100	100
	15	89	79
	30	65	78

Table A.8 Effect of water blanching on POD activity in carrots

Residual POD activity (%) P (MPa)		
T (°C)	t (min)	0.1 MPa
40	0	100
	5	91
	15	73
	30	32
	45	44
	60	33
50	0	100
	5	52
	15	41
	30	24
	45	11
	60	9
60	0	100
	5	89
	15	35
	30	9

Table A.9 Effect of HHP treatment on POD activity in carrots

		Residual POD activity (%) P (MPa)			
T (°C)	t (min)	300 MPa	350 MPa	400 MPa	450 MPa
20	0	100	100	100	100
	15	58	96	91	42
	30	49	16	48	38
	45	42	14	42	38
	60	25	9	24	28

Table A.10 Effect of two step treatment on POD activity in carrots

Residual POD activity (%) P (MPa)			
T (°C)	t (min)	250 MPa+20°C	
		15 min	30 min
40	0	100	100
	5	65	37
	15	46	31
	30	16	14
	45		
	60		
50	0	100	100
	5	44	34
	15	33	26
	30	13	13
	45		
	60		

Table A.11 Effect of HHP treatment on LOX activity in green beans

Residual LOX activity (%) P (MPa)		
T (°C)	t (min)	0.1 MPa
40	0	100
	5	136
	15	130
	30	119
	45	101
	60	99
50	0	100
	5	94
	15	69
	30	70
	45	52
	60	51
60	0	100
	5	105
	15	16
	30	15
	45	6
	60	6
70	0	100
	2	40
	5	20
	6	15
	8	14
	10	13

Table A.12 Effect of two step treatment on LOX activity in green beans

Residual LOX activity (%) P (MPa)			
T (°C)	t (min)	250 MPa+20°C	
		15 min	30 min
40	0	100	100
	5	78	96
	15	77	95
	30	75	98
	45		
	60		
50	0	100	100
	5	154	80
	15	62	73
	30	65	42
	45		
	60		
60	0	100	100
	5	53	29
	15	34	15
	30	3	2
	45		
	60		

Table A.13 Effect of two step treatment on LOX activity in green peas

Residual POD activity (%) P (MPa)			
T (°C)	t (min)	250 MPa+20°C	
		15 min	30 min
40	0	100	100
	5	74	64
	15	72	57
	30	70	49
	45		
	60		
50	0	100	100
	5	99	67
	15	96	38
	30	83	30