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Study of lipoxygenase and peroxidase as blanching indicator enzymes in peas: change of enzyme activity, ascorbic acid and chlorophylls during frozen storage

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Abstract

In this study, the effects of different blanching conditions on residual lipoxygenase (LOX) and peroxidase (POD) activities and quality changes in peas during frozen storage were studied. Peas were analysed for LOX and POD activities, ascorbic acid and chlorophylls *a* and *b* contents at 1, 2, 3, 6, 9 and 12 months of frozen storage. No regeneration of LOX and POD activities was determined in frozen-stored peas at −18 °C. The degradations of ascorbic acid and chlorophylls followed first-order kinetics. The half-lives of ascorbic acid, chlorophylls *a* and *b* derivatives in unblanched peas were found to be 3.30, 14.01 and 36.76 months during storage, respectively. Blanching at both 70 °C for 4.0 min and 80 °C for 2.0 min increased the half-life of ascorbic acid while it decreased those of chlorophylls *a* and *b*. Overall results suggested a blanching time of 2.0 min at 80 °C to inactivate 90% of initial POD activity, so, to retain quality parameters such as ascorbic acid and chlorophyll pigments in a storage period of 12 months at −18 °C.

Keywords: Lipoxygenase; Peroxidase; Ascorbic acid; Chlorophylls; Blanching indicator; Frozen storage

1. Introduction

Freezing is one of the most important methods for retaining quality during long-term storage and it is often considered to be the simplest and natural way to preserve vegetables (Cano, 1996). However, most raw vegetables can be stored only a short time even at sub-zero temperatures. This is because of the changes in texture, color, flavor and nutritional quality that occur as a result of the action of several enzymes that are still active after harvest (Williams, Lim, Chen, Pangborn, & Whitaker, 1986). For this reason, blanching to inactivate enzyme is a critical step prior to freezing (Barrett, Garcia, Russell, Ramirez, & Shirazi, 2000). Even though the primary aim of blanching is the inactivation of enzymes that caused undesirable changes during the processing and subsequent storage of the products, it has also a number of other advantages including color stability, improvement in texture and decrease in microbial population (Murcia, Lopez-Ayerra, & Garcia-Carmona, 1999). However, the severity of the blanching process should be limited in order to maintain color, texture, flavor and nutritional quality.

To determine the adequacy of the blanching process, many enzymes have been suggested as indicators of sufficient heat treatment. Among various enzymes, POD has been the most popular indicator enzyme in the blanching process (Hemeda & Klein, 1991). POD appears to be one of the most heat stable enzymes present in vegetables and it has been generally accepted that if POD is destroyed then it is quite unlikely that other enzymes will have survived (Halpin & Lee, 1987). However, heating vegetables to a temperature high

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enough to inactivate POD is generally more than enough to destroy the undesirable enzymes, since POD activity has not been shown to be directly responsible for quality deterioration during frozen storage of vegetables. The use of LOX as indicator of proper blanching has been recommended as more significant in determining storage stability in frozen vegetables (Williams et al., 1986; Sheu & Chen, 1991; Barrett & Theerakulkait, 1995; Nagy-Gasztonyi, Kardos-Neumann, & Biacs, 2000). LOX has been associated with quality deterioration because of its involvement in off-flavor and odor production, loss of pigments such as carotenes and chlorophylls, and destruction of essential fatty acids (King & Klein, 1987).

In this study, a re-evaluation of the use of LOX and POD as indicator enzyme for optimizing the blanching conditions of peas was attempted in terms of the stabilities of some quality attributes such as ascorbic acid and chlorophylls during frozen storage.

2. Materials and methods

2.1. Enzyme assays

Preparation of crude enzyme extract. Enzyme extraction was carried out by homogenizing 10 g of pea with 50 ml of distilled water at 4 °C in a Virtis homogenizer for 2 min at medium speed. The slurry was filtered through three layers of cheesecloth and centrifuged at $25000 \times g$ for 20 min at 4 °C. The supernatant was used as the crude enzyme extract. The protein determinations were carried out using the dye-binding method of Bradford (1976). A standard curve was constructed using bovine serum albumin in the concentration range of 50–1200 μg/ml in which a linear response ($r = 0.996$) was observed.

LOX assay. A modified spectrophotometric method described by us elsewhere was used (Gökmen, Bahçeci, & Acar, 2002a). The substrate solution was prepared by mixing 157.2 μl of pure linoleic acid, 157.2 μl of Tween-20 and 10 ml of deionized water. The solution was clarified by adding 1 ml of 1 N NaOH and diluting to 200 ml with M/15 sodium phosphate buffer, pH 6.0; giving a 2.5 mmol/l final concentration of linoleic acid. The substrate solution (29 ml) was transferred into a flask placed in a temperature controlled water-bath set at 30 °C. The substrate solution was aerated by a gentle stream of air for 2 min and the reaction was started by adding 1 ml of crude enzyme extract into the flask. The aliquots of 1 ml from the reaction medium were transferred into glass tubes containing 4 ml of 0.1 N NaOH solutions at time intervals of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 5.0 min. The use of 0.1 N NaOH both stopped the enzymatic reaction and ensured the optical clarity by formation of Na-salt of unreacted linoleic acid prior to absorbance reading. The formation of hydroperoxides was monitored spectrophotometrically (Shimadzu model 2101PC scanning spectrophotometer with 1 cm path length) as the increase of absorbance at 234 nm due to the presence of a conjugated hydroperoxydiene moiety. The blank solution was prepared by mixing 1 ml of substrate solution with 4 ml of 0.1 N NaOH solutions. One unit of LOX activity was defined as an increase in absorbance of 0.001 at 234 nm per minute per mg of protein under assay conditions.

POD assay. POD activity was measured spectrophotometrically using a mixture of equal volumes of guaiacol (0.5 ml/100 ml) and H_2O_2 (0.5 ml/100 ml) solutions as substrate based on the method of Sheu and Chen (1991). The substrate solution (2.9 ml) was transferred into a cuvette and the reaction was started by adding 0.1 ml of crude enzyme extract. One unit of POD activity was defined as an increase in absorbance of 0.001 at 420 nm per minute per mg of protein under assay conditions.

2.2. Blanching and frozen storage

An ordinary bench top constant temperature water-bath (50 l) was used as the blanching equipment. Peas obtained from a local market in Ankara were podded, washed and drained prior to blanching. Approximately 0.5 kg of pea was blanched in water at a temperature range of 60–90 °C for different times as indicated in Fig. 1 to determine the thermal inactivation rates of LOX and POD. Each measurement was repeated three times. Then, two blanching conditions of 70 °C for 4 min and at 80 °C for 2 min were chosen for 90% or greater inactivation of LOX and POD enzymes, respectively. After blanching, samples were plunged into an ice water-bath, drained, wrapped in polyethylene bags and stored at −18 °C up to 12 months. Measurements of LOX and POD activities, ascorbic acid and chlorophylls in peas were performed at 0, 1, 2, 3, 6, 9, 12 months during storage period.

2.3. HPLC analyses of ascorbic and dehydroascorbic acid in Pea

10 g of peas and 50 ml of metaphosphoric acid solution (5 g/100 ml) were homogenized using a Virtis homogenizer at medium speed for 2 min. The homogenized sample was filtered through a filter paper (Schleicher & Schuell no. 589) and then 0.45 μm disposable membrane filter (Millipore, regenerated cellulose acetate). The clarified sample was divided into two parts, and one part was analysed for AA content of samples. Dithiothreitol was added into the other part at a ratio of 1 mg/ml and it was kept in the dark for 90 min to convert any DHAA to AA. After complete

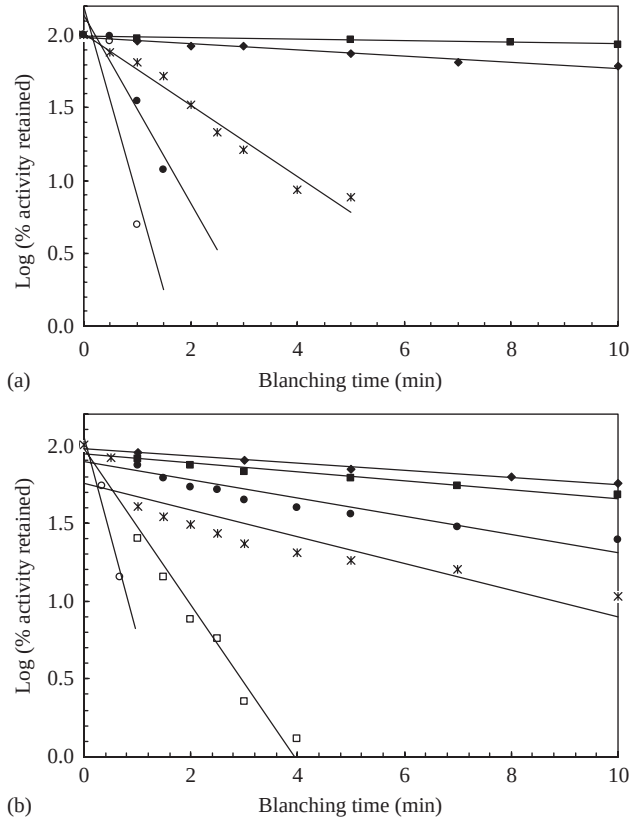

Fig. 1. Thermal inactivation curves of pea enzymes at various temperatures (a) LOX (—■—, 60 °C; —◆—, 65 °C; —*—, 70 °C; —●—, 75 °C; —○—, 80 °C). (b) POD (—◆—, 60 °C; —■—, 65 °C; —●—, 70 °C; —*—, 75 °C; —□—, 80 °C; —○—, 90 °C).

conversion of DHAA was achieved, the sample was analysed for its total AA content by HPLC. DHAA content of pea was calculated by subtraction of initial AA content from total AA after conversion. HPLC analyses were performed using the conditions described by us elsewhere (Gökmen, Kahraman, Demir, & Acar, 2000).

2.4. HPLC analyses of chlorophylls in pea

Ten grams of sample was weighed into a homogenizer cup that contained 0.1 g $CaCO_3$ and 1 ml of butylated hydroxy toluene solution (1 g/100 ml) to stabilize pigments against tissue acids and oxidation, respectively. Fifty milliliter of methanol was added and the mixture was simultaneously homogenized and extracted in a Virtis homogenizer at medium speed for 2 min. After tissue was thoroughly disintegrated, the extract was filtered through a filter paper (Schleicher & Schuell no. 589) into a 100 ml volumetric flask. The residue and filter paper were returned to the homogenizer cup with additional 50 ml portion of methanol, and the extraction was repeated. The final residue was washed with methanol to remove the traces of pigments. The filtrates were combined and completed to volume with methanol. The extraction and filtration steps were carried under low light intensity and at low temperature (4 °C), respectively, to avoid pigment degradations. The extract was filtered through a 0.45 μm membrane filter (Millipore, regenerated cellulose acetate). HPLC analyses were performed using the conditions described by us elsewhere (Gökmen, Bahçeci, & Acar 2002b).

3. Results and discussion

3.1. Heat stabilities of LOX and POD

Fig. 1 shows the residual activities of LOX and POD in peas as a function of blanching time at various temperatures during water blanching. Thermal inactivation of both LOX and POD enzymes followed first-order kinetics. Some researchers observed biphasic curves during thermal inactivation studies of LOX and POD in vegetables (Wang & Luh, 1983; Ganthavorn, Nagel, & Powers, 1991; Güneş & Bayındırlı, 1993; Yemenicioğlu, Özkan, Velioğlu, & Cemeroğlu, 1998; Morales-Blancas, Chandia, & Cisneros-Zevallos, 2002). Biphasic model proposed by Ling and Lund (1978) assumes the presence of an enzyme system formed by two groups differing in their thermal stability, a heat-labile fraction and a heat-resistant fraction. The data that show an initial steep straight line, an intermediate curved portion and a final straight line with a shallow slope by semi log scale usually applies this approximation. However, our experimental data fitted well to first-order monophasic kinetics at all temperatures studied with regression coefficients (r) ranging from 0.88 to 0.99. POD was found to be more heat stable than LOX in peas during water blanching with an exception occurred at 60 °C. D values of POD and LOX of peas were 4.06 and 17.16 min at 70 °C, and were 0.59 and 1.99 min at 80 °C, respectively (Table 1).

Use of POD as blanching indicator for peas required more blanching time than that of LOX. Although our results obtained for peas agree very well with the results of many other researchers (Williams et al., 1986; Halpin & Lee, 1987; Güneş & Bayındırlı, 1993), heat stability of LOX was reported to be higher than that of POD in asparagus (Ganthavorn et al., 1991) and in fresh pinto beans (Yemenicioğlu, et al., 1998). In these studies, LOX activities were assayed as the increase of absorbance at 234 nm in the cuvette containing enzyme extract and linoleic acid substrate solution buffered to pH of 6.0–6.5. However, the low solubility of linoleic acid is a common problem as pointed out by some researchers during spectrophotometric assay of LOX (Suurmeijer, Perez-Gilabert, van der Hijden, Veldink, & Wliegenthart, 1998; Parraud, Kermasha, & Bisakowski, 1999). The reaction medium losses its optical clarity which results in an increase of absorbance at 234 nm to

Table 1
Thermal inactivation rate constants (k) and decimal inactivation times (D[a]) of pea LOX and POD enzymes at various temperatures

Temperature (°C)		k (min^{-1})	D (min)	r	N
60	LOX	0.0122	188.77	0.974	5
	POD	0.0534	43.13	0.982	6
65	LOX	0.0725	31.76	0.989	7
	POD	0.0665	34.63	0.957	7
70	LOX	0.5667	4.06	0.991	9
	POD	0.1342	17.16	0.954	11
75	LOX	1.4865	1.55	0.944	4
	POD	0.1969	11.69	0.885	11
80	LOX	3.8980	0.59	0.881	3
	POD	1.1563	1.99	0.986	8
90	POD	2.9252	0.79	0.979	3

[a] $D = \frac{2.303}{k}$.

be measured as a "pseudo" LOX activity even if no enzyme extract is present in the reaction medium. Therefore, the modified spectrophotometric method described by us elsewhere was used to measure LOX activity in peas more accurately in this study (Gökmen et al., 2002a).

3.2. Storage tests

Enzyme activity. Conventionally the vegetables are blanched to the point of destruction of POD activity and it is generally accepted that if POD is destroyed then it is quite likely that other enzymes will not survive. In this study, two separate water-blanching operations performed at 70 °C for 4.0 min and 80 °C for 2.0 min were applied for 90% inactivation of initial LOX and POD activities, respectively. Blanched and unblanched peas were then subjected to a storage test at −18 °C up to 12 months to re-evaluate the use of POD and LOX as indicator enzyme for optimizing the blanching conditions of peas.

The regeneration of POD activity after heat treatment was reported by some researchers in model conditions (Adams, Harvey, & Dempsey, 1996; Rodrigo, Rodrigo, Alvarruiz, & Frigola, 1997), but not encountered during frozen storage of vegetables (Sheu & Chen, 1991; Barrett & Theerakulkait, 1995). In this study, no regeneration of LOX and POD activity was determined in any blanched samples during storage at −18 °C (Fig. 2). LOX in unblanched peas decreased to 80% of its initial activity and remained relatively stable later on during storage while POD activity continued to decrease through storage period. These results showed blanching conditions of 70 °C for 4.0 min and 80 °C for 2.0 min were sufficient to inactivate LOX and POD, respectively. However, the residual POD activity in peas was ca. 25%

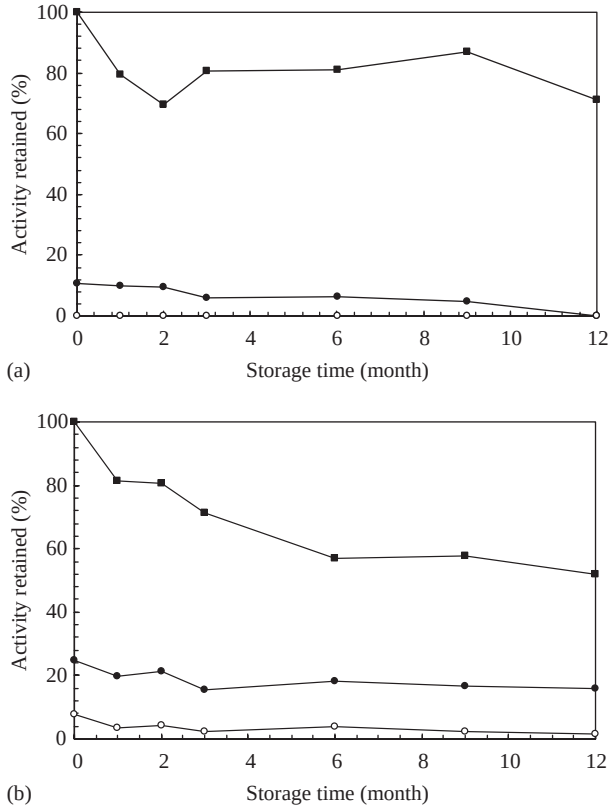

Fig. 2. Changes of enzyme activities in pea during frozen storage at −18 °C. (a) LOX (—■—, unblanched; —●—, 70 °C × 4.0 min; —○—, 80 °C × 2.0 min). (b) POD (—■—, unblanched; —●—, 70 °C × 4.0 min; —○—, 80 °C × 2.0 min).

of its initial activity after blanching at 70 °C for 4.0 min, and remained relatively stable during frozen storage.

Ascorbic and dehydroascorbic acid. In vegetables, AA is one of the most readily destroyed nutrients because it is readily oxidized, pH and light sensitive and affected by naturally occurring enzyme ascorbic acid oxidase. It oxidizes to DHAA and irreversibly hydrolyses into diketogluconic acid, which has no vitamin activity (Gökmen et al., 2000). The average AA and DHAA contents of unblanched fresh peas were found to be 26.12 mg/100 g and 2.15 mg/100 g, respectively. AA and DHAA contents of peas decreased to 25.85 mg/100 g and 1.98 mg/100 g after blanching at 70 °C for 4.0 min, and to 25.69 mg/100 g and 1.67 mg/100 g after blanching at 80 °C for 2.0 min, respectively.

The loss of AA during frozen storage followed first-order kinetics. The kinetic parameters (first-order rate constants, half-lives) and regression coefficients were given in Table 2. The AA contents of unblanched peas decreased significantly during storage (Fig. 3). There were significant differences between AA contents of unblanched and blanched pea samples after 12 months of frozen storage. The losses of AA were found to be 90.62%, 44.95% and 35.27% after 12 months in unblanched, 70 °C × 4.0 min blanched and 80 °C × 2.0 min

Table 2
The first order degradation rate constants (k) and half-lives ($t_{1/2}$) of AA, Chl-*a* and Chl-*b* in peas during frozen storage at −18 °C

	Rate constant (k), 1/month	Half-life[a] ($t_{1/2}$), month	r
AA			
Unblanched	0.210	3.30	0.979
70 °C × 4 min	0.054	12.76	0.916
80 °C × 2 min	0.037	19.00	0.847
Chl-*a*			
Unblanched	0.049	14.01	0.924
70 °C × 4 min	0.083	8.35	0.980
80 °C × 2 min	0.061	11.42	0.978
Chl-*b*			
Unblanched	0.019	36.76	0.979
70 °C × 4 min	0.057	12.14	0.991
80 °C × 2 min	0.040	11.42	0.996

[a] $t_{1/2} = \frac{0.693}{k}$.

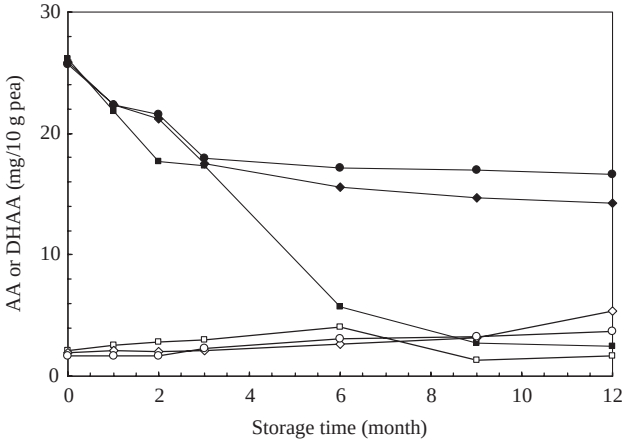

Fig. 3. Changes of AA and DHAA contents of pea during frozen storage at −18 °C. (—■—, Unblanched, AA; —♦—, 70 °C × 4.0 min, AA; —●—, 80 °C × 2.0 min, AA; —□—, Unblanched, DHAA; —◇—, 70 °C × 4.0 min, DHAA; —○—, 80 °C × 2.0 min, DHAA).

blanched peas, respectively. Half-life of AA degradation increased from 3.30 months to 12.76 months by blanching at 70 °C for 4.0 min, and to 19.00 months by blanching at 80°C for 2.0 min (Table 2). The initial AA to DHAA ratio of 12.13 in unblanched fresh peas changed to 1.48, 2.66 and 4.52 in unblanched, blanched at 70 °C for 4.0 min and blanched at 80 °C for 2.0 min samples, respectively. This is very important from the viewpoint of keeping vitamin C in its relatively more stable form of AA in frozen vegetables. It has also been reported by other researchers that vitamin C content of blanched vegetables decreased slowly during storage (Halpin & Lee, 1987; Sheu & Chen, 1991).

Chlorophylls and color. Similar to AA, the losses of Chl-*a* and Chl-*b* during storage followed first-order kinetics. The kinetic parameters (first-order rate constants, half-lives) and regression coefficients were given in Table 2. Chl-*a* and Chl-*b* contents of fresh unblanched peas were found to be 164.77 and 75.83 mg/kg, respectively. The first-order degradation rate constants of Chl-*a* and Chl-*b* were 0.0495 and 0.0189/month, respectively. Several other studies have also reported that Chl-*a* derivative degraded faster than Chl-*b* (Buckle & Edwards, 1970; Schwartz & von Elbe, 1983). The Chl-*a* and Chl-*b* contents of peas decreased to 94.15 and 59.55 mg/kg after 12 months of storage.

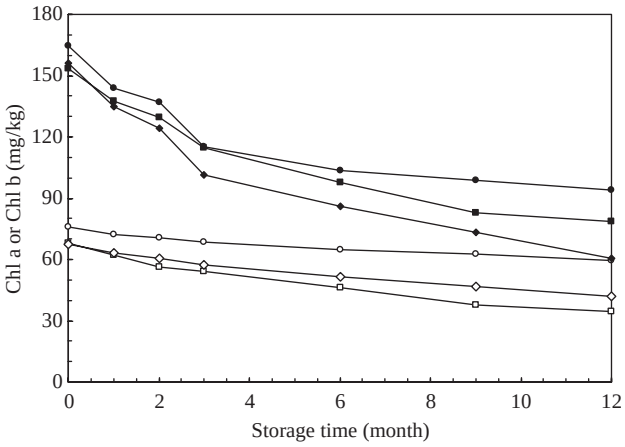

Fig. 4. Changes of Chl-*a* and Chl-*b* contents of pea during frozen storage at −18 °C (—●—, Unblanched, AA; —♦—, 70 °C × 4.0 min, AA; —■—, 80 °C × 2.0 min, AA; —○—, Unblanched, DHAA; —◇—, 70 °C × 4.0 min, DHAA; —□— 80 °C × 2.0 min, DHAA).

Chl-*a* and Chl-*b* were converted into their corresponding epimers at ratios of 2.17–2.80% and 5.84–6.72% following blanching. There was no change in color since epimers have the same color with their parent chlorophylls. However, blanching prior to frozen storage adversely affected the stability of Chl-*a* and Chl-*b* in peas. The respective half-lifes of Chl-*a* and Chl-*b* decreased from 14.01 months and 36.76 months to 8.35 months and 12.14 months by blanching at 70 °C for 4.0 min and to 11.42 months and 17.50 months by blanching at 80 °C for 2.0 min (Table 2).

The loss of natural color during storage of frozen green vegetables has attracted much attention. Campbell (1937) showed that color deterioration in frozen peas stored above –18 °C was due to conversion of Chl-*a* and Chl-*b* to the corresponding pheophytins. In acid solutions, the magnesium in chlorophylls *a* and *b* is replaced by hydrogen to give the corresponding pheophytins. In this study, 8.35–8.62 mg/kg of pheophytin-*a* was found in blanched peas only after 12 months of storage (Fig. 4).

Hunter L, a and b values of pea samples were recorded three times before and after frozen storage. The L value of 67.56 decreased to 64.34 in unblanched and to 55.64 and 57.26 in 70 °C × 4.0 min blanched and 80 °C × 2.0 blanched peas after 12 months storage

period. The Hunter stimulus value (−a/b) which related to green color of peas as suggested by Francis and Clydesdale (1975) was 0.52 in unblanched fresh peas and slightly decreased to 0.48–0.49 in blanched samples after 12 months of storage.

4. Conclusion

This study revealed that there is a direct correspondence between the water blanching conditions and the quality of pea during frozen storage. POD was found more heat stable than LOX in peas, and blanching to the point of POD inactivation instead of LOX appeared to be necessary to retain quality in terms of vitamin C and chlorophyll derived pigments during frozen storage. Blanching conditions suggested from our study was 2.0 min at 80 °C to inactivate 90% of initial POD activity in peas.

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