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Purification and Characterization of Polyphenol Oxidase, Peroxidase and Lipoxygenase from Freshly Cut Lettuce (*L. sativa*)

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Summary

Enzymatic reactions taking place in minimally processed vegetables are considered as a major problem, because they adversely affect sensorial and nutritional quality. Polyphenol oxidase (PPO), peroxidase (POD) and lipoxygenase (LOX) from lettuce were purified on a column packed with positively charged diethylaminoethyl (DEAE) cellulose by applying pH gradient elution from pH=4.0 to 9.0. The main purified fractions (PPO1 and PPO4, POD1 and POD2, LOX1 and LOX2) were characterized for enzyme concentration--reaction rate relationship, thermal stability, pH activity and kinetic parameters. Kinetic properties of each isoform were considerably different. Cysteine was found as the most effective inhibitor of both fractions of PPO. Kinetic parameters of lettuce POD were presented using guaiacol at various H_2O_2 concentrations. β-carotene directly influences lettuce LOX in the reaction medium available for the catalytic conversion of linoleic acid into hydroperoxides. Ascorbic and oxalic acids appear as effective PPO inhibitors, protecting phenolic compounds against oxidation in lettuce. Understanding the characteristics of deteriorative enzymes becomes important to maintain suitable conditions for fresh-like quality of lettuce. The results can be useful to keep the nutritional quality of minimally processed lettuce during shelf-life.

Key words: lettuce, polyphenol oxidase, peroxidase, lipoxygenase, browning, inhibitors

Introduction

Minimally processed fruits and vegetables have gained increasing popularity, as they meet the consumers' demand for fresh ready-to-use products (*1*). Among them, lettuce has become popular because of the increased consumption of fast food and ready made salads. However, shredding or cutting of lettuce provokes wound-induced physiological and biological reactions (*2*). Therefore, considering the importance of lettuce, it has become important to understand the factors affecting its quality (*3*). Besides microbial contamination, deteriorative enzymes such as lipoxygenase (LOX), peroxidase (POD) and polyphenoloxidase (PPO), which are active in minimally processed lettuce, are considered as the factors responsible for the deterioration during shelf-life. Since endogenous oxidation enzymes may affect the organoleptic properties such as colour, taste and aroma of lettuce, their characterization is important for keeping the high quality of the product (*4*).

The shelf-life of minimally processed lettuce is limited by the enzymatic browning reaction (*5*). Browning, which is mediated by endogenous PPO and POD activities, is a major problem in the food industry and is believed to be one of the main causes of quality loss during postharvest handling and processing (*6*).

PPO, also known as tyrosinase (monophenol, *o*-diphenol:oxygen oxidoreductase; EC 1.14.18.1), is a copper--containing enzyme that catalyzes two different reactions using molecular oxygen: the hydroxylation of mono-

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phenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity). In living tissues, the phenolic substrate and the enzyme are separated within the cell, but upon extraction or other cell-damaging treatments, the enzyme and substrate may come into contact, leading to browning and thus altering not only the structural and functional properties of the protein, but also its nutritive value (*7*). POD (donor: H_2O_2 oxidoreductase; EC 1.11.1.7), is a glycoprotein whose primary function is to oxidize phenolic compounds at the expense of H_2O_2. POD is a widely distributed plant enzyme responsible not only for browning but also for discolouration, off-flavours and nutritional damage (*8*). Quality deterioration, such as off-flavours, off-odours, and off-colours, in unblanched vegetables has been ascribed to the oxidative deterioration of unsaturated lipids by LOX action (*9*). LOX (linoleate:oxygen oxidoreductase; EC 1.13.11.12) catalyses the bioxygenation of polyunsaturated fatty acids containing a *cis*,*cis*-1,4--pentadiene unit to form conjugated hydroperoxydienoic acids (*10*). It has been associated with quality deterioration because of its involvement in off-flavour and odour production, loss of pigments such as carotenes and chlorophylls, and destruction of essential fatty acids (*11*).

The reduction of deterioration reactions is one of the main objectives of the food industry. During the processing of lettuce, LOX, POD and PPO enzymes can cause deterioration in the product, thus changing its characteristics (*12*). Isolation and characterization of the enzyme forms is important to understand more fully the role of LOX, POD and PPO in lettuce.

There have been studies of PPO purification and prevention of browning in lettuce. However, no published data could be found in literature relating to isoenzymes of PPO, POD and LOX enzymes, their characteristics and inhibition mechanism in lettuce. Therefore, the purpose of the present study is to develop a rapid and accurate procedure for purification of three important enzymes involved in enzymatic deterioration reactions from fresh cut lettuce, their partial characterization and to elucidate the mechanism of their inhibition by chosen chemical compounds.

Material and Methods

Chemicals and reagents

Oxalic acid, citric acid, ascorbic acid, cysteine, hydrogen peroxide and guaiacol were purchased from Merck (Darmstadt, Germany). Catechol, polyvinilpolypyrrolidone (PVPP), diethylaminoethyl (DEAE) cellulose, β-caroten, chlorogenic acid, caffeic acid, ferulic acid, *p*-coumaric acid, gallic acid, protocatechuic acid and phloridzin were purchased from Sigma-Aldrich (Steinheim, Germany).

Purification and assays of PPO, POD and LOX from lettuce

Lettuce was obtained from the local market. A mass of 30 g of lettuce was homogenized in 90 mL of distilled water with PVPP (3 %, by mass per volume). The slurry was centrifuged at 15 000×*g* for 15 min. The supernatant was used as the crude enzyme extract.

For ion exchange chromatography, a column packed with DEAE-cellulose (10×2.5 cm) was used. Packed column was washed and equilibrated with phosphate buffer (pH=4) before use. Prior to elution, 5 mL of crude enzyme extract were loaded onto the column. The column was eluted with a linear discontinuous gradient of 0.01 M sodium phosphate buffers from pH=4.0 to 9.0, each step increased by 0.25 at room temperature. The eluate fractions were collected as 5-mL aliquots and assayed for their PPO, POD and LOX activities. Aliquots having PPO, POD or LOX activity were assigned as the corresponding isoenzymes and used for further characterization. Protein concentration of each fraction was also determined by using dye-binding method (*13*).

Polyphenoloxidase assay

PPO activity was measured using the method described by Altunkaya and Gökmen (*14*) with minor modifications. A volume of 25 μL of enzyme extract was added to 0.3 mL of 1 mM catechol solution in 0.067 M phosphate buffer to initiate the reaction (final volume was 2.5 mL). Initial rate of quinone formation was monitored as an increase in the absorbance at 420 nm using UV-VIS spectrophotometer (Shimadzu UV-2101 PC, Shimadzu Corp, Kyoto, Japan) with a 1-centimeter path length cuvette.

Peroxidase assay

POD activity was measured spectrometrically using the method described by Gökmen *et al*. (*15*). The mixture containing equal concentrations of guaiacol and H_2O_2 (240 mM each) was used as substrate. The substrate solution (2.9 mL) was transferred into a cuvette in 0.067 M phosphate buffer and the reaction was started by adding 0.1 mL of partially purified enzyme extract. Initial rate of brown colour formation was monitored as an increase in the absorbance at 420 nm using UV-VIS spectrophotometer with a 1-centimeter path length cuvette.

Lipoxygenase assay

A modified spectrometric method described by Gökmen *et al.* (*16*) was used. The substrate solution was prepared by mixing 157.2 mL of pure linoleic acid, 157.2 mL of Tween 20 and 10 mL of deionized water. The solution was clarified by adding 1 mL of 1 M NaOH and diluting to 200 mL with 0.067 M sodium phosphate buffer at pH=6.0; giving a final concentration of linoleic acid of 2.5 mmol/L. 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 partially purified 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 M 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 M NaOH both stopped the enzymatic reaction and ensured the optical clarity by formation of NaCl of unreacted linoleic acid prior to the absorbance reading. The formation of hydroperoxides was monitored spectrophotometrically (Shimadzu UV-2101 PC) with a 1-centimeter path length cuvette) as an increase of absorbance at 234 nm due to the presence of a conjugated hydroperoxydiene moiety.

Characterization of purified polyphenoloxidase, peroxidase, and lipoxygenase

A total of four PPO (PPO1, PPO2, PPO3 and PPO4), one POD and two LOX (LOX1 and LOX2) isoenzymes were obtained by using ion-exchange column chromatography with pH gradient. Isoenzymes having the maximum activities (PPO1, PPO4, POD1, POD2, LOX1 and LOX2) were characterized in terms of pH and temperature optima, kinetic parameters, substrate specificity (for PPO), and the effects of different inhibitors (for PPO and LOX). The pH activity profiles were determined in 0.067 M phosphate buffers at different pH values ranging from pH=4.0 to 9.0. At optimum pH for each enzyme, activities were also determined as a function of temperature ranging from 10 to 70 °C. Substrate specificities and kinetic parameters of PPO were determined for five substrates including catechol, catechin, chlorogenic acid, caffeic acid and gallic acid by means of Michaelis-Menten plots.

Effects of inhibitors on polyphenoloxidase and lipoxygenase activities

The inhibitory effects of different compounds on PPO and LOX were tested on isoforms (PPO1, PPO4, LOX1 and LOX2) which were separated by using ion-exchange column chromatography with pH gradient (Fig. 1). Ascorbic acid, cysteine, citric acid and oxalic acid were tested as the potential inhibitors of PPO, while only β-carotene was used as the inhibitor of LOX. The stock solutions of ascorbic acid, cysteine, citric acid and oxalic acid were prepared in water at a concentration of 1.0 mM. The stock solution of β-carotene was prepared by dissolving 1 mg of β-carotene in 1 mL of dichloromethane containing 40 μL of Tween 80 (*17*). Dichloromethane was evaporated to dryness. The residue was redissolved in 7.36 mL of 0.67 mM EDTA solution in water to set the final concentration of β-carotene solution to 0.25 mM. The solutions were prepared daily and kept at 4 °C prior to use. The inhibitory effects of the above mentioned compounds were determined. Catechol (1 mM) and constant volume of enzyme (25 μL) in 0.067 M phosphate buffer (pH=7.0) were run in the presence and absence of constant inhibitor concentrations (final volume was 2.5 mL) in order to monitor the inhibition mechanism of PPO inhibitors, whereas 12 mM linoleic acid and constant volume of enzyme (50 mL) in 0.067 M phosphate buffer (pH=6.0) were applied in the presence and absence of constant β-carotene concentrations to observe the effect of β-carotene on LOX. Inhibition constants and the type of inhibitions were determined by means of Lineweaver-Burk plots (*18*). Modified spectrophotometric method described by Gökmen *et al.* (*15*) was used.

The effects of several inhibitors (ascorbic acid, cysteine, citric acid and oxalic acid) on lettuce PPO activity and β-carotene on lettuce LOX activity were studied. PPO and LOX activities were measured at two inhibitory concentrations.

Effects of polyphenoloxidase inhibitors in lettuce

In order to determine any protective action of enzyme inhibitors against the oxidation of phenolic compounds, the slurry was prepared by homogenizing 3 g of lettuce with 9 mL of water (control), ascorbic acid (0.5 %), citric acid (0.5 %), oxalic acid (0.5 %) and cysteine (0.05 %). After 1 h, the slurry was centrifuged at 15 000×*g* for 15 min. The supernatant was used for HPLC analysis. Chromatographic analyses were performed on an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) consisting of a photodiode array detector, quaternary pump, autosampler and column oven. Phenolic compounds were separated on a Waters Atlantis C18 (Waters Corporation, Milford, MA, USA) column (250×4.6 mm, 5 μm) using a linear gradient elution program with a mobile phase containing solvent A (*V*(formic acid)/*V*(H_2O)=1:99) and solvent B (methanol) at a flow rate of 0.8 mL/min. The solvent gradient was programmed as follows: linear gradient elution from 10 % B to 60 % B, 0–15 min; isocratic elution of 60 % B, 15–20 min; linear gradient elution from 60 % B to 10 % B, 20–25 min; isocratic elution of 10 % B, 25–30 min. The chromatograms were recorded at 280 nm by monitoring the spec-

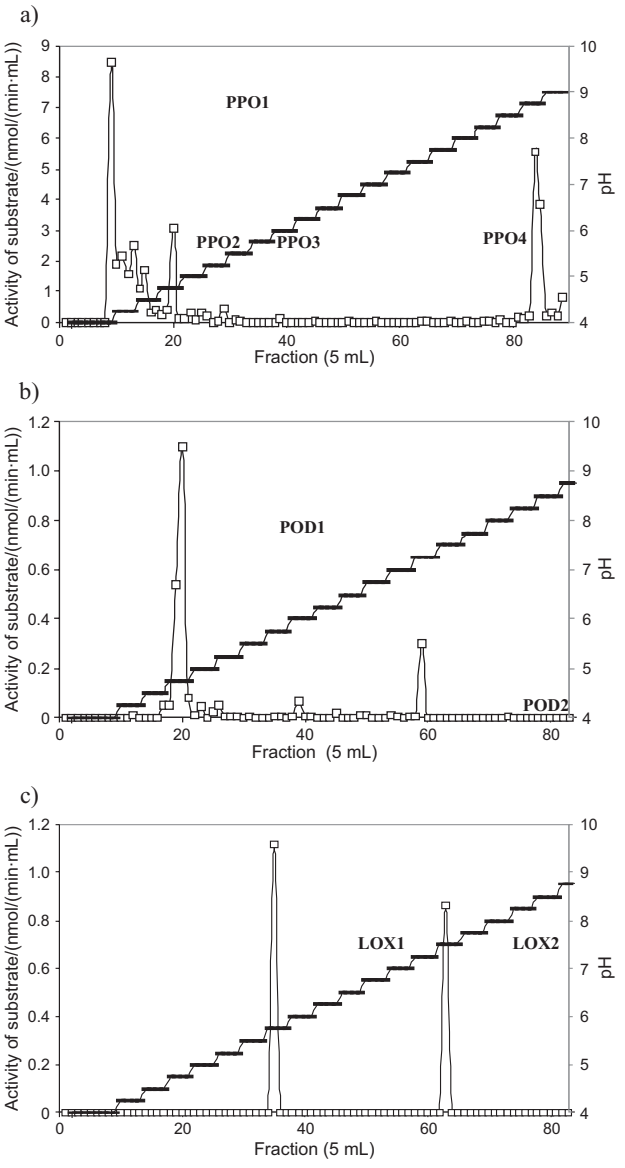

Fig. 1. The activities of: a) PPO, b) POD and c) LOX fractions obtained using DEAE-cellulose column chromatography

tra within a wavelength range of 190–400 nm. Identification of phenolic acids was accomplished by comparing the retention times and absorption spectra of peaks in the samples to those of standard compounds. The quantification of phenolic compounds was based on calibration curves built for each of the compounds identified in the samples.

Results and Discussion

Purification of enzymes

In this study, simple purification protocols are proposed for separation. The stability of each enzyme varied in response to factors such as storage time, temperature, pH, ions present in buffer solutions and the presence of protective agents and detergents. In this way, an important problem to be solved before the development of a purification procedure from a plant homogenate is to remove or inactivate plant cell secondary metabolites which hinder the recovery of the enzymes and strongly lower the yield. Tissue homogenization during the isolation of PPO and POD enzymes, whose intermediates (quinones) may also form covalent linkages that may not be reversible, initiates browning reactions (*19*). Thus, the undesirable effects of degradation of polyphenolic compounds were prevented by the addition of PVPP during the homogenization of lettuce tissue to obtain the crude enzyme extract.

DEAE ion-exchange chromatography was selected to remove the contaminating proteins and to purify different oxidative enzymes in lettuce. Purification of enzymes was carried out by contacting an impure liquid enzyme preparation containing enzyme and soluble impurities (hinder the recovery of the enzymes and strongly lower the yield) with DEAE in a column. Thus, the soluble impurities are preferentially adsorbed by DEAE and the adsorbed enzyme is displaced from the DEAE to produce a purified liquid enzyme preparation containing higher enzyme activity than before purification (*20,21*).

Enzyme isolation by any of the precipitation methods is normally followed by chromatographic separation. Denaturation or loss in activity of enzymes during extraction could occur. Therefore, adsorption to ion exchanger could be appropriate and can achieve adequate concentration of diluted protein solutions (*8*).

Lettuce PPO, POD and LOX enzymes in the crude extract were purified on an ion-exchange column packed with DEAE-cellulose applying a pH gradient to further understand the characteristics of these enzymes. Partial purification of each enzyme was demonstrated in Table 1.

The elution profile of PPO, POD and LOX isoenzymes on DEAE-cellulose packed column is shown in Fig 1. PPO was found as the most active oxidation enzyme in lettuce followed by POD and LOX. A total of four PPO isoenzymes was assigned as the most active forms in lettuce without considering the fractions having only traces of PPO activity. The peaks that were assigned as PPO1, PPO2, PPO3 and PPO4 isoforms eluted at pH values of pH=4.25, 4.5, 4.75 and 8.75, respectively. PPO1 and PPO4 fractions were characterized without considering minor fractions. There were two isoforms of POD and two isoenzymes of LOX found in lettuce. The peak assigned as POD eluted at pH=4.75 and 7.0, while the peaks assigned as LOX1 and LOX2 eluted at pH=5.75 and 7.50, respectively.

Table 1. Partial purification of PPO, POD and LOX enzymes

Purification steps	*V*/mL	Total activity/U	*m*(total protein)/mg	Specific activity/(U/mg)	Recovery/%	Purity/fold
PPO						
crude extract		2.830	478.8	0.006	100	
DEAE-cellulose chromatography	30					
PPO1	5	0.067	7.9	8.48	2.4	1413
PPO2	10	0.219	93.0	2.35	7.8	392
PPO3	5	0.057	18.0	3.16	2.0	527
PPO4	10	0.083	18.0	4.60	3.0	767
POD						
crude extract		0.580	478.8	0.0012	100	
DEAE-cellulose chromatography	30					
POD1	5	0.052	18.0	2.90	9	2417
POD2	10	0.077	29.0	3.75	7	987
LOX						
crude extract		1.19	478.8	0.003		
DEAE-cellulose chromatography	30					
LOX1	5	0.24	217.0	1.10	20	367
LOX2	10	0.17	194.0	0.86	14	292

PPO, POD and LOX do not exist as single enzymes in fruits and vegetables; like many other plant enzymes, they are present in a number of enzymatic forms which can be detected by different methods (*12*). However, the physiological significance of the presence of multiple isoenzymes is unknown so far (*8*). The existence of multiple forms of PPO, POD and LOX is also well documented for some fruits and vegetables (*8–10,13*). In the case of PPO, proteolytic processing may play a role in this heterogeneity, as well as participating in the activation of the latent forms of the enzyme. As regards POD, in most plant materials both basic and acidic POD isoenzymes have been found in the cell wall, whereas only the basic ones are located in vacuoles. On account of this, POD is thought to be involved in the oxidation of both cell wall and vacuolar phenols, as part of a series of metabolic reactions associated with cell wall rigidification and phenolic turnover and degradation (*8*). LOX enzymes are predominantly located in the cytoplasm, but they are also associated with vacuoles, mitochondria, chloroplasts, microsomal membranes, plasmalemma and lipids. The presence of two LOX isoenzymes in cucumber, tomato and apple has been reported earlier (*10,11*). In this paper, simultaneous purification and characterization of isoenzymes of lettuce PPO, POD and LOX are described for the first time. The isoenzymes differ mainly in the broadness of their pH activity range, optimum temperature and in the product they form during oxidation.

pH and temperature optima of purified enzymes

pH is a determining factor in the expression of enzyme activity as it alters the ionization states of amino acid chains or the ionization of the substrate (*19*). For the determination of pH optima of lettuce PPO, POD and LOX enzymes, the chromatographic peaks exhibiting corresponding activities were analyzed separately. Interestingly, isoforms of PPO and LOX from lettuce had similar pH activity pattern. An optimum pH=7.0 was observed for lettuce PPO1, PPO4 and POD1 (the activity of POD2 was too low for characterization). LOX1 and LOX2 isoforms were found to have optimum pH=6.0 and 7.0, respectively. The observed pH optima of purified isoforms of lettuce PPO were similar to those reported previously for crude PPO extract (*14*). The pH optimum of POD from lettuce has been reported to be between pH=6.0 and 8.5 (*22*). This relatively broad range is probably due to the presence of isoenzymes having different pH optima. The purified LOX isoenzymes were found to be more stable under acidic than under neutral conditions. Soybean LOX has been reported to have similar pH activity pattern (*23*).

The purified lettuce PPO1 and PPO4 isoenzymes had an optimum activity at 40 and 30 °C, respectively. There was a gradual decrease in the activity of PPO isoenzymes at temperatures exceeding 40 °C due to denaturation. The optimum temperature of crude lettuce PPO has been reported previously as 40 °C (*13*). The purified lettuce POD1 had an optimum temperature of 30 °C. The same temperature optimum has been reported for strawberry POD (*22*). The two isoforms of LOX had a temperature optimum of 40 and 30 °C, respectively. Banana leaf LOX has also showed optimum activity at 40 °C (*23*).

Kinetic characterization of purified enzymes

Polyphenoloxidase

Number of phenolic compounds have been shown to act as the substrates for PPO in the literature (*24*). In this study, dihydroxy- and trihydroxyphenols, namely catechol, chlorogenic acid, caffeic acid, catechin and gallic acid were used to test substrate specificity of lettuce PPO. The apparent kinetic parameters of the most active isoforms of lettuce PPO (PPO1 and PPO4) are given in Table 2. The lettuce PPO utilized *o*-diphenols as the substrate, while no activity was detected for trihydroxyphenols such as gallic acid using v_{max}/K_m as the criterion for catalytic efficiency (*25*). The substrate specificity of two fractions was quite different in terms of v_{max}/K_m values. The order of affinity of substrates for lettuce PPO1 was as follows: chlorogenic acid>caffeic acid>catechin>catechol>gallic acid, while that of substrate for lettuce PPO4 isoforms was: catechol>catechin>caffeic acid>chlorogenic acid. Lettuce PPO1 and PPO4 isoforms were shown to have no specific activity towards gallic acid, but gallic acid itself was found to inhibit PPO activity. Chlorogenic acid has been reported previously as the best substrate for PPO from coffee leaves, whereas finding of catechol as the best substrate for lettuce PPO4 is similar to that found for numerous plant PPOs (*8,23*).

Peroxidase

Kinetic parameters for POD1 were determined using guaiacol as the reducing substrate. The activity of POD1 showed Michaelis-Menten relationship at various H_2O_2 concentrations. The K_m and v_{max} values of lettuce POD were found to be 0.33 mM and 0.24 μmol/min, respectively. The significance of low K_m value for H_2O_2 reflects a high number of H_2 or hydrophobic interactions between the substrate and the heme group at the enzyme active site (*22*).

Table 2. Kinetic parameters of lettuce PPO1 and PPO4 isoenzymes for different substrates

	K_m/mM		v_{max}/min^{-1}		v_{max}/K_m	
	PPO1	PPO4	PPO1	PPO4	PPO1	PPO4
chlorogenic acid	2.18	0.22	31.25	0.01	14.33	0.05
catechol	15.00	0.14	75.18	0.08	5.02	0.57
catechin	22.90	0.09	114.94	0.01	5.01	0.13
caffeic acid	2.15	0.19	2.15	0.08	1.00	0.41
gallic acid	0.00	0.00	0.00	0.00	0.00	0.00

Lipoxygenase

Variation in linoleic acid concentration between 0–12 mM in the absence of any inhibitor resulted in Michaelis-Menten curve with K_m=0.33 mM and v_{max}=0.24 μmol/min for LOX1 and K_m=0.98 mM and v_{max}=0.24 μmol/min for LOX2 at pH=6.0.

Effect of inhibitors on lettuce polyphenoloxidase and lipoxygenase

Polyphenoloxidase activity

The effects of four inhibitors, namely ascorbic acid (0.012–0.040 mM), cysteine (0.001–0.003 mM), oxalic acid (0.04–0.80 mM) and citric acid (0.04–0.80 mM) on lettuce PPO1 and PPO4 activities were investigated in this study. The mode of inhibition and the values of inhibition constants (K_i) are given in Table 3. Among others, L-cysteine was found as the most effective inhibitor of lettuce PPO1 and PPO4 isoforms, followed by ascorbic acid, oxalic acid and citric acid. For both fractions, the type of inhibition was competitive for cysteine and ascorbic acid, and non-competitive for citric and oxalic acids.

Previous studies have shown that enzymatic browning of plants catalyzed by PPO may be delayed or eliminated by removing the reactants such as oxygen and phenolic compounds, or by using PPO inhibitors (*19*). Inhibition of PPO by cysteine is attributed to the stable colourless products formed by reaction with *o*-quinones (*26*). Ascorbic acid acts more as an antioxidant than as an enzyme inhibitor because it reduces the initial *o*-quinone formed by the enzyme to the original diphenol before it undergoes secondary reaction which leads to browning. Inhibition of PPO by oxalic and citric acids has been attributed to their binding with active site copper to form an inactive complex. The extent of inhibition is not influenced only by oxalic or citric acid concentration, but also by pH (*27*).

Lipoxygenase activity

The effect of β-carotene (0.110–0.992 mM) on the inhibition of lettuce LOX1 and LOX2 activity was also determined. β-carotene was found a very effective inhibitor of lettuce LOX isoenzymes, with inhibition constants (K_i) of 0.804 mM for LOX1 and 0.290 mM for LOX2. The inhibition of both LOX isoforms by β-carotene was non-competitive.

Carotenoids are widespread in plants, where they function as auxiliary light-harvesting pigments and quenchers of harmful reactive species like chlorophyll and singlet oxygen (*17*). The most characteristic feature of the carotenoid structure is the long system of alternating double and single bonds that form the central part of the molecule. This constitutes a conjugated system in which the π-electrons are effectively delocalized over the entire length of the polyene chain. This feature is responsible for the molecular shape, chemical reactivity and light-absorbing properties, and hence colours of carotenoids (*16*). Carotenoids may function as antioxidants by preventing or delaying oxidation of lipids with regard to their structural properties (*9*). Inhibition of LOX activity by some chemicals such as dihydrolipoic acid (*24*) and tetrapetalone (*25*) has also been reported. Recently, during co-oxidation of β-carotene by LOX-mediated hydroperoxidation reactions, inhibition of LOX activity by β-carotene was reported (*17*).

It was clear from these results that β-carotene completely inhibited LOX at sufficient concentration. It has been proposed that β-carotene breaks the chain reaction at the beginning of linoleic acid hydroperoxidation catalyzed by LOX by means of its strong radical scavenging activity and by keeping LOX in the inactive form (*27*). β-carotene directly influences the amount of enzyme in the reaction medium available for the catalytic conversion of linoleic acid into corresponding hydroperoxides. Thus, increasing the concentration of β-carotene in the reaction mixture results in a decrease in the rate of conjugated diene formation (*16*).

Effect of poliphenoloxidase inhibitors on phenolic content of lettuce

Effects of PPO inhibitors on the content of naturally occurring phenolic compounds in lettuce were determined by HPLC analysis. The aim was to investigate how various inhibitors influence polyphenol profile of lettuce during enzymatic oxidation processes.

Relative changes in the concentration of individual phenolic compounds in lettuce as influenced by the addition of ascorbic acid, citric acid, oxalic acid and cysteine were compared. Protocatechuic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid and phloridzin were identified by matching the retention time of the compounds determined in the lettuce extract to those of pure standards. The peaks identified in lettuce extract were further confirmed by comparing their UV spectra to those of pure standards.

The interaction of lettuce phenols with ascorbic acid, cysteine, citric acid or oxalic acid during oxidation has been investigated. Data presented in this paper show that the quantity of all identified phenolic compounds in lettuce decreased with the passage of time. Percentage of decrease of phenolic compounds in the presence of inhibitors was as follows: ascorbic acid>oxalic acid>citric acid>cysteine. While the differences among samples with applied ascorbic acid, citric acid, oxalic acid and cysteine were found to be significant, control sample and that with cysteine were not ($p<0.01$). Ascorbic, citric and oxalic acids could be recommended to prevent PPO-mediated enzymatic browning and loss of polyphenols against oxidation. Synergistic effect of these inhibitors and phenolic compounds in slowing down browning was also observed.

As a result of enzymatic oxidation, approx. 90 % decrease in the concentration of phenolic compounds was observed in control within 1 h (Fig. 2). Phenolic compounds and PPO originally exist separately in different organelles in plant cells. When the plant is damaged, phenolic compounds and PPO come into contact and in their reaction, parts of phenolic compounds are isomerized (*25*). A sharp decrease in the concentration of phenolic compounds was also observed in control sample.

The addition of anti-browning agents is one of the typical methods used to inhibit the browning reactions. Treatment with ascorbic acid was found the most effective way in delaying the oxidation of phenolic compounds. Ascorbic acid acts as a protector of pigments preserving

Table 3. Modes of inhibition of lettuce PPO1 and PPO4 isoenzymes for different inhibitors

	c/mM		K_i/mM		Type of inhibition	
	PPO1	PPO4	PPO1	PPO4	PPO1	PPO4
ascorbic acid	$1.2\cdot10^{-2}$	$2.8\cdot10^{-2}$	$9.2\cdot10^{-2}$	$7.5\cdot10^{-2}$	competitive	competitive
	$1.6\cdot10^{-2}$	$4.0\cdot10^{-2}$				
L–cysteine	$1.1\cdot10^{-3}$	$2.0\cdot10^{-3}$	$3.0\cdot10^{-3}$	$2.0\cdot10^{-3}$	competitive	competitive
	$2.4\cdot10^{-3}$	$3.6\cdot10^{-3}$				
oxalic acid	$4.0\cdot10^{-2}$	$1.5\cdot10^{-2}$	$1.5\cdot10^{-1}$	$4.7\cdot10^{-1}$	non-competitive	non-competitive
	$7.8\cdot10^{-2}$	$2.5\cdot10^{-2}$				
citric acid	$4.1\cdot10^{-2}$	$1.5\cdot10^{-2}$	$1.1\cdot10^{-1}$	$4.17\cdot10^{-1}$	non-competitive	non-competitive
	$8.0\cdot10^{-2}$	$2.5\cdot10^{-2}$				

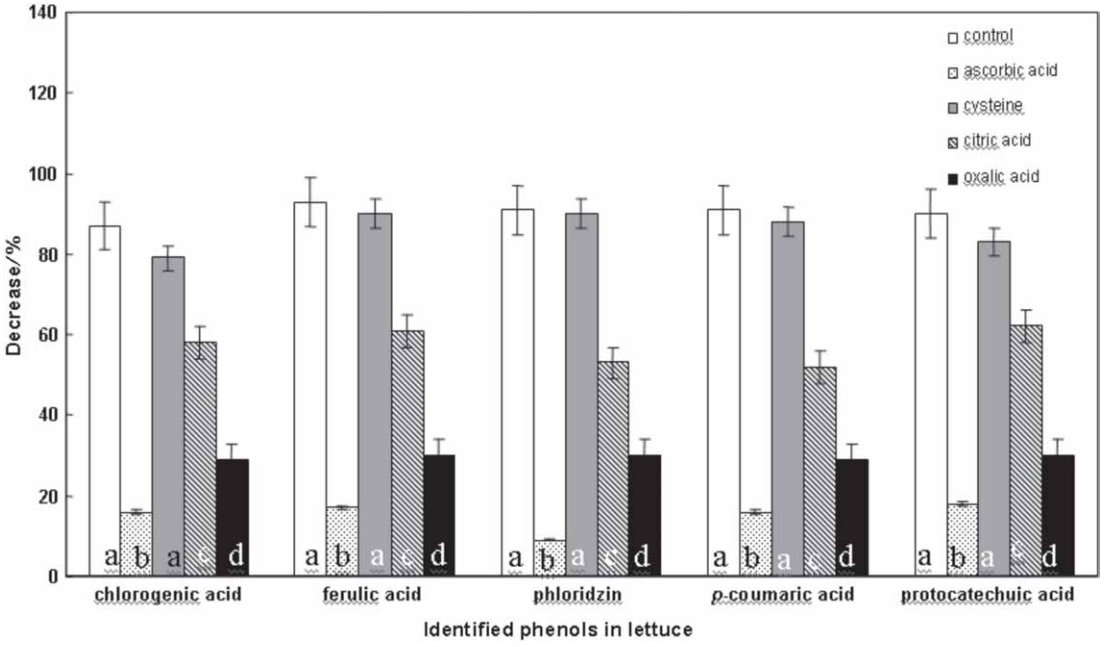

Fig. 2. Decrease of individual phenolic compounds identified in lettuce after 1 h at 25 °C in the presence of various PPO inhibitors ($p<0.05$); letters represent significant differences between the effects of applied inhibitors

them from chemical and biochemical oxidation by competing with amides and amine-carbonyl interactions that result in browning. An alternative mode of action may be suppression of free radical formation involved in browning reaction. There is also evidence that ascorbic acid has polyphenol-protective and -enhancing activities, probably due to the reduction of oxidized phenols and regeneration of polyphenols. A cooperative action between ascorbic acid and polyphenols could be beneficial in enhancing the ability of the latter to rescue cells from damage induced by an oxidative stress (*28*).

Although cysteine allowed permanent protection against enzymatic browning, phenolic compounds could not be protected against oxidation using cysteine. With an excess amount of cysteine (cysteine to phenol ratio above 1), the phenol is fully degraded into cysteinylquinil adducts (CQAC) without colour formation. When cysteine/phenol ratio is below 1, *o*-quinones are formed in excess and are able to react with the CQAC with regeneration of phenols and formation of highly coloured pigments. Therefore, provided this critical level in cysteine concentration is exceeded, thiols could in theory allow permanent protection against enzymatic browning (*26*). Loss of phenolic compounds is undesirable from a nutritional point of view.

Oxalic acid was also found very effective in delaying the oxidation of phenolic compounds in lettuce. Citric acid was less effective than oxalic acid in the prevention of oxidation during storage. Oxalic acid seems to inhibit PPO by chelating copper from the active site of the enzyme since oxalic acid has high affinity to form metal complexes with copper ion. Citric acid lowers the pH and also chelates the copper at the active site of the PPO. Its inhibitory effect could be related to the phenolase copper-chelating power. Especially at pH values below 4, the looser binding of copper at the active enzyme site causes the PPO activity to decrease further, permitting the citric acid to remove the copper (*29*).

Conclusion

This paper has analyzed the characteristics of PPO, LOX and POD of lettuce, which are responsible for the

browning and bleaching phenomenon. Browning and its possible results were strongly emphasised because most of the enzymatic activity comes from PPO and POD.

Minimally processed lettuce offers consumers highly nutritious, convenient and healty food while still maintaining the desired freshness. Throughout the preparation and storage period of ready-to-eat lettuce, oxidative enzymes (PPO and POD) and their respective substrates can lead to enzymatic browning, which decreases both nutritional and visual quality of the product. Enzymatic browning in foods is reasonably well understood, but prevention of browning is not the only factor to be sure about quality. Besides anti-browning property of inhibitors, protection of nutritional properties should be taken into consideration to maintain the fresh-like quality. Among PPO inhibitors tested in this study, ascorbic acid and oxalic acid have a great capability for the prevention of the degradation of phenolic compounds in freshly cut lettuce. Hence, it appears that they have strong potential for practical applications. Quality deterioration, such as off-flavours, off-odours, and off-colours, caused by LOX action during processing could be prevented by β-carotene. Understanding the characteristics of enzymes that cause deterioration and their inhibition mechanism becomes important to maintain suitable conditions for fresh-like quality during shelf-life. As a consequence, formulations including additives have to be optimized to succeed in the control of enzymatic deteriorations and loss of nutritional quality.

References

1. R. Ibrahim, A. Osman, N. Saari, R.A. Rahman, Effects of anti-browning treatments on the storage quality of minimally processed shredded cabbage, *J. Food Agric. Environ. 2* (2004) 54–58.
2. E. Cantos, J.C. Espin, F.A. Tomas-Barberan, Effect of wounding on phenolic enzymes in six minimally processed lettuce cultivars upon storage, *J. Agric. Food Chem. 49* (2001) 322–330.
3. M. Ihl, L. Aravena, E. Scheuermann, E. Uquiche, V. Bifani, Effect of immersion solutions on shelf-life of minimally processed lettuce, *Lebensm. Wiss. Technol. 36* (2003) 591–599.
4. U. Schweiggert, A. Schieber, R. Carle, Inactivation of peroxidase, polyphenoloxidase, and lipoxygenase in paprika and chili powder after immediate thermal treatment of the plant material, *Innov. Food Sci. Technol. 4* (2005) 403–411.
5. K. Duangmal, R.K. Owusu Apenten, A comparative study of polyphenoloxidases from taro (*Colocasia esculenta*) and potato (*Solanum tuberosum* var. *Romano*), *Food Chem. 4* (1999) 351–359.
6. P.M.A. Toivonen, D.A. Brummell, Biochemical bases of appearance and texture changes in fresh-cut fruit and vegetables, *Postharvest Biol. Technol. 48* (2008) 1–14.
7. T. Aydemir, Partial purification and characterization of polyphenol oxidase from artichoke (*Cynara scolymus* L.) heads, *Food Chem. 87* (2004) 59–67.
8. K.M. Mdluli, Partial purification and characterisation of polyphenol oxidase and peroxidase from marula fruit (*Sclerocarya birrea* subsp. Caffra), *Food Chem. 92* (2005) 311–323.
9. S. Yoon, B.P. Klein, Some properties of lipoxygenase isoenzymes, *J. Agric. Food Chem. 2* (1979) 955–962.
10. S. Aziz, Z. Wu, D.S. Robinson, Potato lipoxygenase catalysed co-oxidation of β-carotene, *Food Chem. 64* (1999) 227–230.
11. D.L. King, B.P. Klein, Effect of flavonoids and related compounds on soybean lipoxygenase-1 activity, *J. Food Sci. 52* (1987) 220–221.
12. C. Mantovani, E. Clemente, Peroxidase and polyphenoloxidase activity in tomato *in natura* and tomato purée, *Acta Sci. Technol. 32* (2010) 91–97.
13. M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem. 72* (1976) 248–254.
14. A. Altunkaya, V. Gökmen, Effect of various inhibitors on enzymatic browning, antioxidant activity and total phenol content of fresh lettuce (*Lactuca sativa*), *Food Chem. 107* (2008) 1173–1179.
15. V. Gökmen, K.S. Bahçeci, A. Serpen, J. Acar, Study of lipoxygenase and peroxidase as blanching indicator enzymes in peas: Change of enzyme activity, ascorbic acid and chlorophylls during frozen storage, *LWT - Food Sci. Technol. 38* (2005) 903–908.
16. V. Gökmen, S. Bahçeci, J. Acar, Characterization of crude lipoxygenase extract from green pea using a modified spectrophotometric method, *Eur. Food Res. Technol. 215* (2002) 42–45.
17. A. Serpen, V. Gökmen, Effect of β-carotene on soybean lipoxygenase activity: Kinetic studies, *Eur. Food Res. Technol. 224* (2007) 743–748.
18. H. Lineweaver, D. Burk, The determination of enzyme dissociation constants, *J. Am. Chem. Soc. 56* (1934) 658–666.
19. U. Gawlik-Dziki, U. Złotek, M. Świeca, Characterization of polyphenol oxidase from butter lettuce (*Lactuca sativa* var. *capitata* L.), *Food Chem. 107* (2008) 129–135.
20. R.K. Scopes: *Protein Purification: Principles and Practice*, Springer-Verlag, Berlin, Germany (1987).
21. C.T. Mant, R.S. Hodges: *High Peformance Liquid Chromatography of Peptides and Proteins*: *Seperation, Analysis and Conformation*, CRC Press, Boca Raton, FL, USA (1991).
22. G.H. Onsa, N. bin Saari, J. Selamat, J. Bakar, Purification of characterization membrane-bound peroxidases from *Metroxylon sagu*, *Food Chem. 85* (2006) 365–376.
23. J.M. Kuo, A. Hwang, D.B. Yeh, M.H. Pan, M.L. Tsai, B.S. Pano, Lipoxygenase from banana leaf: Purification and characterization of an enzyme that catalyzes linoleic acid oxygenation at the 9- position, *J. Agric. Food Chem. 54* (2006) 3151–3156.
24. S. Dogan, M. Dogan, Determination of kinetic properties of polyphenol oxidase from *Thymus* (*Thymus longicaulis* subsp. *chaubardii* var. *chaubardii*), *Food Chem. 88* (2004) 69–77.
25. T. Palmer: Kinetics of Single Substrate Enzyme Catalyzed Reactions. In: *Understanding Enzymes*, Prentice Hall, Upper Saddle River, NY, USA (1995) pp. 107–127.
26. F.C. Richard, P.M. Goupy, J.J. Nicolas, J.M. Lacombe, A.A. Pavia, Cysteine as an inhibitor of enzymatic browning. 1. Isolation and characterization of addition compounds formed during oxidation of phenolics by apple polyphenol oxidase, *J. Agric. Food Chem. 39* (1991) 841–847.
27. G. Rapeanu, A. Loey, C. Smout, M. Hendrickx, Biochemical characterization and process stability of polyphenoloxidase extracted from Victoria grape (*Vitis vinifera* ssp. *sativa*), *Food Chem. 94* (2006) 253–261.
28. G.A. González-Aguilar, S. Ruiz-Cruz, H. Soto-Valdez, F. Vázguez-Ortiz, R. Pacheco-Aguilar, C.Y. Wang, Biochemical changes of fresh-cut pineapple slices treated with antibrowning agents, *Int. J. Food Sci. Technol. 40* (2005) 377–383.
29. J.A. Prenen, P. Boer, E.J.D Mees, Absorption kinetics of oxalate from oxalate-rich food in man, *Am. J. Clin. Nutr. 40* (1984) 1007–1010.