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Effect of various inhibitors on enzymatic browning, antioxidant activity and total phenol content of fresh lettuce (*Lactuca sativa*)

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Abstract

In this study, polyphenol oxidase (PPO) was isolated from fresh lettuce. Its optimum temperature and pH were found to be 40 °C and 7.0, respectively. Lettuce PPO was shown to use catechin, catechol, chlorogenic acid, caffeic acid and gallic acid as substrates. Among the substrates used, the greatest substrate specificity was observed with chlorogenic acid. Lettuce PPO was sensitive to some inhibitors. Ascorbic acid, cysteine, oxalic acid and citric acid were tested as potential inhibitors of lettuce PPO. Cysteine was the most effective inhibitor. Total phenol and total antioxidant activity contents were also determined in the presence of these inhibitors at room and refrigerator temperatures. Ascorbic acid and cysteine increased the total antioxidant activity of lettuce while citric and oxalic acids had no effect on the total antioxidant activity. Lettuce phenolics were protected from oxidation by ascorbic acid and cysteine.
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Keywords: Lettuce (*L. sativa*); Polyphenol oxidase; Inhibitors; Antioxidant activity

1. Introduction

Minimally processed lettuce has become popular because of the increased consumption of fast food and prepared salads. Lettuce is highly susceptible to enzymatic browning, thereby causing economic loss to the producer. The control of browning from harvest to consumer is critical for minimising product loss (Martin-Diana et al., 2005). Enzymatic browning generally results in loss of nutritional, functional and organoleptic qualities, such as darkening, softening and off-flavour development. Degree of browning depends on phenol content and polyphenol oxidase (PPO) activity (Zawistowsky, Biliaderis, & Eskin, 1991).

PPO is a copper-containing oxidoreductase, which catalyses two reactions: the *o*-hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones. The main step in enzymatic browning is the oxidation of phenol compounds by PPO in the presence of oxygen (Kavrayan & Aydemir, 2001). These quinones then undergo subsequent reactions to form dark-coloured compounds. (Eidhin, Murphy, & O'beirne, 2005).

The increased use of minimally processed lettuce and increased restriction on chemical treatment to prevent browning has created the need to understand the browning reactions, and to find simple, natural treatments to control it (Martin-Diana et al., 2005). Among the compounds that have been shown to inhibit browning are sulphites, ascorbic acid and its derivatives, oxalic acid, citric acid, and thiol compounds such as cysteine.

Significant levels of antioxidants and phenolic components have been detected in lettuce (Heimler, Isolani, Vignolini, Tombelli, & Romani, 2007). The concentrations of flavonoids and phenolic acids in lettuce are sensitive to environmental conditions (Liu et al., 2007). For increasing the shelf-life of food products and preventing loss of sensory and nutritional quality, the addition of antioxidants has become increasingly popular (Ponce, Del Valle, & Rural, 2004). Some browning inhibitors may also act as antioxidant compounds.

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In this study, PPO was partially purified from lettuce. The effect of different substrates and inhibitors on PPO activity, total antioxidant activity and total phenol content were investigated.

2. Materials and methods

2.1. Materials

Lettuce was obtained from a local market in Ankara, Turkey. PVPP, oxalic acid, ascorbic acid, Folin-Ciocalteu reagent and cysteine were purchased from Merck (Darmstadt, Germany), and citric acid from Carlo Erba (Milan, Italy). Caffeic acid, chlorogenic acid and gallic acid were purchased from Acros (Loughborough, UK). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), ABTS (2,2-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt), catechol and potassium persulfate were obtained from Sigma-Aldrich (St. Louis, Mo).

2.2. PPO extraction and purification procedure

Lettuce (30 g) was homogenised in 90 ml of distilled water with PVPP (3%). The slurry was centrifuged at 15,000*g* for 15 min. The supernatant was concentrated by ultrafiltration using an Amicon PM 30 membrane, having a molecular weight cut off of 30 kDa. The concentrated enzyme was used as partially purified crude enzyme extract.

Enzyme extract (25 μl) 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 absorbance at 420 nm, using UV–visible spectrophotometry (Shimadzu 2101 PC, Tokyo, Japan) with a 1 cm pathlength cuvette. PPO activity was defined as increase in absorbance per minute in 1 ml of reaction mixture (ΔA_{420}/min/ml).

2.3. Enzyme activity

Enzyme activity, as a function of pH, was determined with 1 mM catechol in 0.067 M phosphate buffer, ranging from pH 4.0 to 9.0. PPO activity as a function of temperature was determined at various temperatures from 10 to 70 °C.

2.4. Substrate specificity and enzyme kinetics

Michaelis-Menten constant (K_m) and maximum reaction velocity (v_m) were determined for five substrates (catechol, catechin, chlorogenic acid, caffeic acid and gallic acid). Data were plotted as $1/v$ and $1/S$ concentration according to the method of Lineweaver and Burk (1934).

2.5. Inhibitor effects

The effects of several inhibitors (ascorbic acid, cysteine, citric acid and oxalic acid) on lettuce PPO activity were studied. PPO activities were measured at two inhibitor concentrations. Values $1/v$ and $1/S$ were plotted in Lineweaver Burke graphs. Finally K_i values were obtained from graphs.

2.6. Determination of antioxidant activity

Total antioxidant activity values of fresh-cut lettuce treated with various PPO inhibitors (ascorbic acid, cysteine, citric acid and oxalic acid) were determined as described elsewhere (Re, Pellegrini, Proteggente, Pannala, & Yang, 1999). ABTS radical cation ($ABTS^{++}$) was obtained by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark for 12–16 h before use. The radical was stable in this form for more than two days when stored in dark at room temperature.

Lettuce (3 g) was homogenised in 9 ml of distilled water, ascorbic acid (0.5%), cysteine (0.05%), citric acid (0.05%), and oxalic acid (0.05%) solutions, respectively. Aliquots were taken from the slurry at 0, 0.25, 0.5, 1, 2, 4 and 6 h. The slurry was centrifuged at 15,000*g* for 15 min. The supernatant was used for antioxidant activity measurement. The ABTS solution was diluted with distilled water to an absorbance of 0.70 ± 0.02 at 734 nm and equilibrated at 30 °C. After addition of 2.95 ml diluted ABTS solution to 5 μl antioxidant compounds or Trolox standards in ethanol, the absorbance was measured at 30 °C after mixing for 6 min. Solvent blanks were run in each assay. The percentage inhibition of absorbance at 734 nm was calculated and plotted as a function of concentration of antioxidants and Trolox for the standard reference data. Total antioxidant activity was expressed as mM Trolox equivalent antioxidant activity/g lettuce (mM TEAC/g lettuce).

2.7. Total phenol content

Total amount of phenols was determined by using Folin-Ciocalteu's phenol reagent and spectrophotometric determination. Lettuce (3 g) was homogenised in 9 ml of distilled water, ascorbic acid (0.5%), cysteine (0.05%), citric acid (0.05%), and oxalic acid (0.05%) solutions, respectively. Samples were taken from the slurry at 0, 0.25, 0.5, 1, 2, 4 and 6 h. The slurry was centrifuged at 15,000*g* for 15 min. One hundred microlitres of clear supernatant was mixed with 4 ml saturated sodium carbonate (75 g/l). After 5 ml of 0.2 N Folin-Ciocalteu reagent was added, the reaction mixture was stored at 50 °C for 30 min before measuring the absorbance at 765 nm. Total phenol content was expressed as mg gallic acid equivalents/g lettuce (mg GAE/g lettuce).

3. Result and discussion

3.1. Characterisation of partially purified lettuce PPO activity

Optimum pH for partially purified lettuce PPO was found to be 7.0, using catechol as substrate (Fig. 1). The

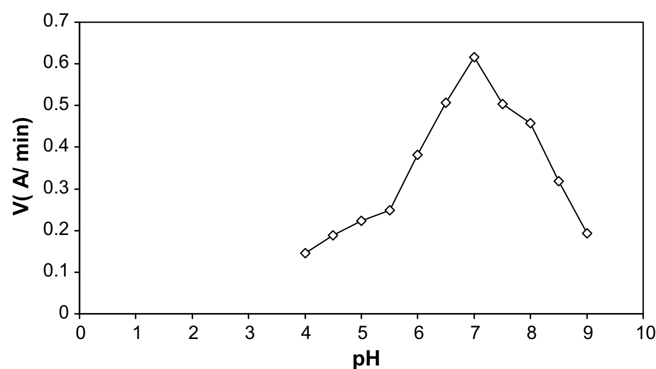

Fig. 1. Optimum pH for lettuce PPO.

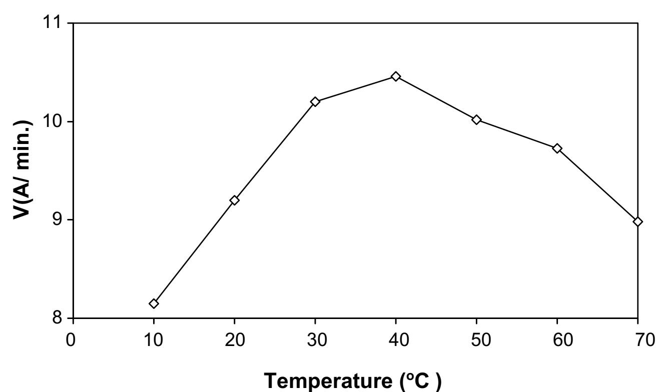

Fig. 2. Optimum temperature for lettuce PPO.

enzyme was still active at extreme pH values. Relative activities were 24% and 31% at pH 4.0 and pH 9.0, respectively. The optimum temperature for lettuce PPO was 40 °C (Fig. 2).

Many phenolic compounds are both good browning substrates and good antioxidants. Their role as substrates for oxidative browning is important for foods (Robards, Prenzler, Tucker, Swatsitang, & Slover, 1999).

v_m/K_m ratio was taken as the criterion to evaluate substrate specificity (Palmer, 1995). Although catechin seemed to have the highest specificity to PPO, when considering the ratio of v_m/K_m, chlorogenic acid was the most efficient substrate (Table 1). Melo, Shimizu, and Mazzafera (2006) also found that chlorogenic acid is the best substrate for coffee leaves PPO. Lettuce PPO was shown to have no specific activity toward gallic acid, but gallic acid itself was found to inhibit PPO activity. Maximum substrate specificity of PPO was found for chlorogenic acid, then catechol, catechin, caffeic acid and gallic acid, respectively.

3.2. Effect of inhibitors

The effects of four inhibitors, namely ascorbic acid (10–20 μM), cysteine (5–10 μM), oxalic acid (0.1–0.14 mM) and citric acid (0.1–0.14 mM) on crude lettuce PPO activity are shown in Table 2. The results show that the most effective inhibitor of lettuce PPO is L-cysteine.

Table 1
K_m and v_m values of lettuce PPO for different substrates

	K_m (μM)	v_m (min^{-1})	v/K_m
Chlorogenic acid	2.18	31.3	14.3
Catechol	15.0	75.2	5.02
Catechin	22.9	115	5.01
Caffeic acid	2.15	2.15	1.0
Gallic acid	0	0	0

Table 2
Inhibition modes of lettuce PPO

	I (M)	K_i (μM)	Type of inhibition
Ascorbic acid	1×10^{-5} 2×10^{-5}	12.11	Competitive
Cysteine	5×10^{-6} 10×10^{-6}	5.06	Competitive
Oxalic acid	1×10^{-4} 1.4×10^{-4}	10.0	Non-competitive
Citric acid	1×10^{-4} 1.4×10^{-4}	10.0	Non-competitive

Since enzymatic browning is in most cases a quality indicator, considerable effort has been devoted to its control. Heat treatments, such as blanching, are sometimes not desirable; a wide range of chemical compounds has been proposed to inhibit PPO (Richard-Forget, Goupy, & Nicolas, 1992).

Inhibition types of the inhibitors of lettuce PPO are as follows: ascorbic acid, competitive; cysteine, competitive; citric acid, non-competitive; oxalic acid, non-competitive.

In the PPO assays, when ascorbic acid was used as an inhibitor, changes in absorbance could not be detected at the beginning of the reaction, and a lag period was observed. Similar results were found by Dincer, Çolak, Aydin, Kadioglu, and Güner (2002). After the lag period, when nearly all ascorbic acid is converted to dehydroascorbic acid, the amount of *o*-quinones formed by action of PPO increases. The quinones then polymerise and or combine together with amino compounds to form high molecular weight brown pigments (Duangmal & Owusu Apenten, 1999). Kavrayan and Aydemir (2001) and Aydemir (2004) have showed that ascorbic acid is competitive to peppermint PPO and artichoke PPO, as found in this study.

Cysteine is more effective inhibitor for PPO (Eidhin et al., 2005). The action of cysteine is complex. During enzymatic oxidation, it traps the *o*-quinone by forming cysteinyl adducts. Cysteine–quinone adducts have been proved to be competitive inhibitors of PPO. With a sufficient amount of cysteine, the phenol is fully degraded in cysteinyl adducts without colour formation (Richard-Forget et al., 1992).

Oxalic acid is a natural component of a large number of plants, such as asparagus, broccoli, Brussels sprout, carrot, garlic, lettuce, onion, parsley, pea, potato, radish, spinach, tomato and turnip, but it is not an approved food additive (Yoruk, Yoruk, Balaban, & Marshall, 2004). Inhibition of PPO by oxalic acid was shown, due to its binding with the active sites of copper, to form an inactive complex (Prenen, Boer, & Mees, 1984). The extent of inhibition was influenced not only by oxalic acid concentration, but also by pH. Oxalic acid diminished the catechol-quinone formation and no quinone bleaching was observed. Different sources of PPO exhibit different types of inhibition mechanism. Son, Moon, and Lee (2001) found that oxalic acid shows competitive inhibition on mushroom PPO. Aydemir (2004) found that oxalic acid showed non-competitive inhibition to artichoke PPO, and competitive inhibition to celery root PPO (Aydemir & Akkanlı, 2006).

Citric acid has been reported extensively for its inhibitory activity on PPO and its antibrowning activity in minimally processed fruits and vegetables (Ahvenaien, 1996). Citric acid exerts a double inhibitory effect, by lowering the pH (below that necessary for optimal PPO activity)

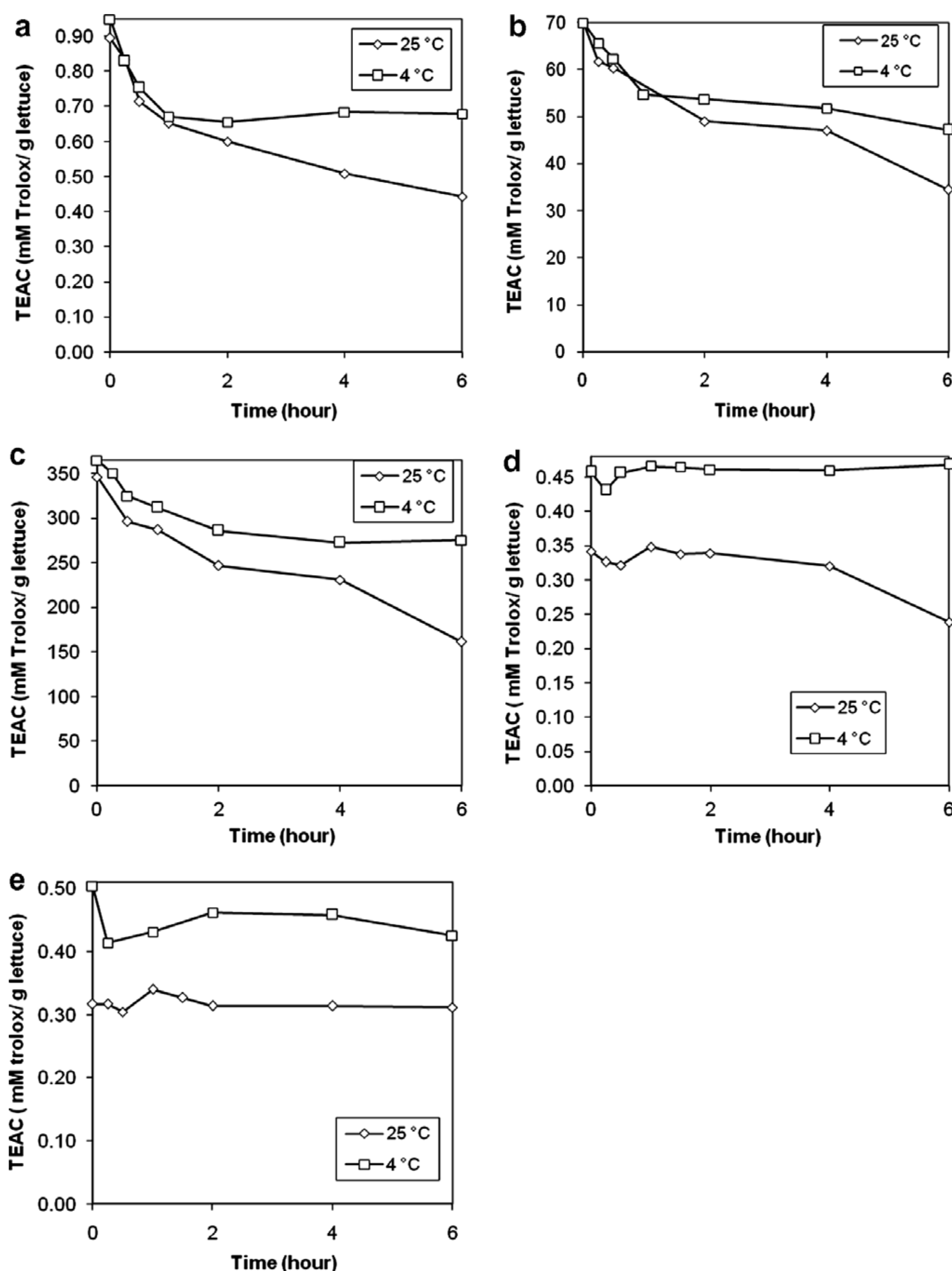

Fig. 3. Changes in total antioxidant activity of lettuce: (a) untreated control, (b) 0.5% ascorbic acid, (c) 0.05% cysteine, (d) 0.05% citric acid and (e) 0.05% oxalic acid, at 4 and 25 °C.

and by chelating copper (Ibrahim, Osman, Saari, & Abdul-Rahman, 2004). Citric acid was found to be a non-competitive inhibitor to lettuce PPO.

3.3. Effect of inhibitors on total antioxidant activity

In this study, total antioxidant activity of lettuce was monitored at 4 and 25 °C for 6 h, in the presence of ascorbic acid, cysteine, oxalic acid and citric acid. Fig. 3 illustrates the decline of total antioxidant activities under different conditions.

The antioxidant activity of lettuce is derived from phenolic compounds. Flavonoids are phenol derivatives and found in substantial amounts in lettuce. Their potential antioxidant activity has been investigated several times. Various natural and synthetic flavonoids are found to be efficient radical scavengers, especially superoxide radicals. Superoxide radical is slowly but efficiently inactivated by phenol derivatives because of the high redox potential of phenols. It means that the reduction potential of the flavonoid radicals is lower than that of alkylperoxyl and superoxide radicals. So, flavonoids may inactivate these

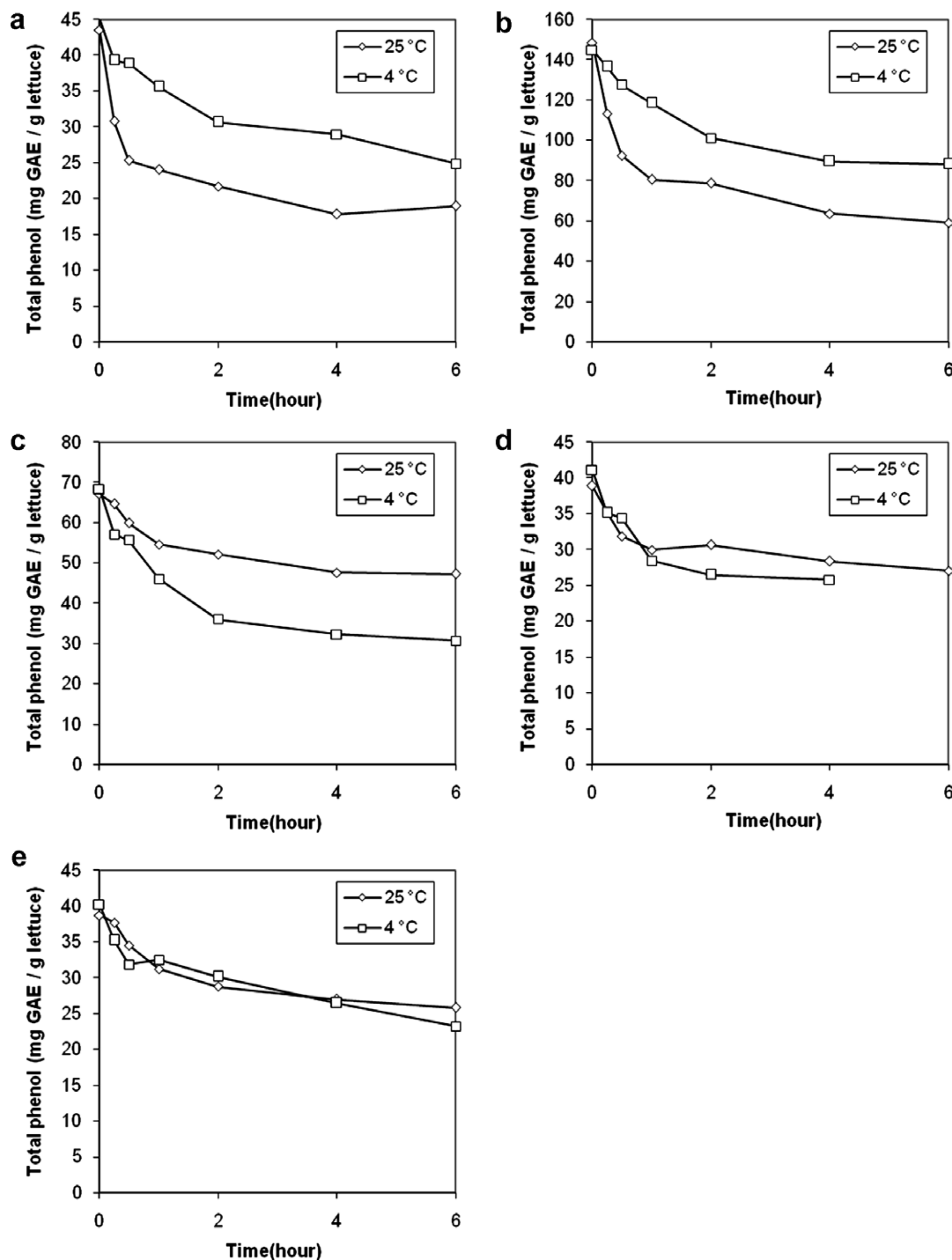

Fig. 4. Changes in total phenolic content of lettuce: (a) untreated control, (b) 0.5% ascorbic acid, (c) 0.05% cysteine, (d) 0.05% citric acid and (e) 0.05% oxalic acid at 4 and 25 °C.

damaging species and prevent the deleterious consequences of their reactions (Jovanovic, Steenken, Tosic, Marjanovic, & Simic, 1994).

Decrease in antioxidant capacity of lettuce was observed at the two temperatures studied. Yamaguchi et al. (2003) reported similar results. They showed a decrease in the radical-scavenging activity of lettuce, broccoli and burdock within 15 min. Total antioxidant activity decreased faster at 25 °C than at 4 °C. Cold conditions could protect phenolic loss and also antioxidant activity loss. However, in the presence of ascorbic acid and cysteine, total antioxidant activity increased, as they are major antioxidants.

Ascorbic acid suppressed free radicals by the formation of ascorbyl radicals (Yamaguchi, Yoshimura, Nakazawa, & Ariga, 1999). The ene-diol structure plays an effective role in scavenging free radicals (Darkwa, Mundoma, & Simoti, 1998). It has a synergistic effect with phenols. Miller and Rice-Evans (1997) studied orange, apple and blackcurrant drinks and concluded that phenolic constituents of beverages can retard the oxidative decomposition of vitamin C. In addition, under mild oxidation conditions, after 24 h, a significant decline in total antioxidant activity was demonstrated in this experiment. Among the phenols, quercetin had the greatest effect on ascorbic acid.

Cysteine is a free radical scavenger because of the presence of a thiol group. It has three reactive centres: the carboxylic acid, the amino group and the sulphur centre. The only reactivity of physiological significance was at the sulphur centre. Reactions of cysteine with radicals show that the thiol group is capable of mopping up free radicals by forming radicals especially with reactive oxygen species (Darkwa et al., 1998). Bassil, Makris, and Kefalas (1999) showed that antioxidant activity was increased due to a positive conjugation effect exerted by sulphur, which may weaken the O–H bonds of the phenolics. A possibility synergistic effect of cysteine and plant polyphenols, such as protocatechuic acid, and its analogues was also demonstrated by Saito and Kawabata (2004).

Citric and oxalic acids have no significance effect on the antioxidant activities of lettuce. These acids reduced pH sharply, so antioxidant activity may decrease slightly as a result of pH drop. Low pH may also decrease the activity of PPO, so that polyphenols cannot be oxidised. Ascorbic acid also reduced pH but is different from the other acids, as it has a reductant feature. Ascorbic acid is a common component of vegetables and has strong radical-scavenging activity. After vegetables are cut and exposed to oxygen, it is oxidised to dehydroascorbic acid and has no radical-scavenging activity (Yamaguchi et al., 2003).

3.4. Total phenol content

Changes in total phenolic content with time in the control and 0.5% ascorbic acid, 0.05% cysteine, 0.05% citric acid and 0.05% oxalic acid at 4 and 25 °C were investigated. Total phenol content showed a decreasing trend with time. Phenolic content decreased much more slowly at 4 °C than at 25 °C, because low temperatures slow down plant metabolic processes, such as respiration, ethylene production and enzyme activity. Optimum storage temperature can vary from species to species and cultivar to cultivar. The most frequently used temperature is 4 °C, considered the optimal for many leafy vegetables. A low temperature must be maintained during the entire postharvest period to guarantee quality (Ferrante & Maggiore, 2007) (see Fig. 4).

Yamaguchi et al. (2003) studied changes in total phenol content in heated and unheated lettuce after 15 min. While phenolic content remained constant in heated lettuce, a drastic decrease was observed in unheated samples. Some phenolic compounds are known to be substrates of PPO. The decrease of total phenol content is probably due to the oxidation by PPO. It was also reported that quercetin was oxidised directly by PPO.

In the presence of ascorbic acid and cysteine, total phenol content increased. This may be due to synergism of the compounds with phenols (as mentioned above). Oxalic and citric acids decreased phenol content very slightly. This result may depend on pH. These acids may prevent the loss of phenolic compounds during this time interval.

The decrease in antioxidant activity paralleled the decrease in total phenol content. The results also showed that there was a good correlation between antioxidant activity and total phenol content of the control, and in the presence of ascorbic acid and cysteine ($r > 0.90$ for each). No correlation was found in the presence of citric and oxalic acids.

In conclusion, PPO and polyphenols are found in different organelles of plant cells.When tissue is damaged, they meet and react with each other. As a result the antioxidant capacity and total phenol contents decrease. These findings show that inhibitors can be used to prevent loss of antioxidant activity and antioxidants, such as polyphenols. Ascorbic acid, cysteine, citric acid and oxalic acids prevent browning of lettuce. In addition, ascorbic acid and cysteine increase the total antioxidant activity of lettuce.

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