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Inhibition of loquat enzymatic browning by sulfhydryl compounds

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Abstract

The effectiveness of a series of sulfhydryl compounds in inhibiting polyphenoloxidase (PPO) activity in a model system (chlorogenic acid solution) and in loquat juices was evaluated. Application of different concentrations of sulfhydryl compounds to chlorogenic acid solution and fresh loquat juice showed that L-cysteine appeared to be an effective browning inhibitor. The required concentration of L-cysteine for 90% browning inhibition depended on loquat cultivars, and ranged from 0.6 mM for "Nagasaki" to 2.0 mM for "Yukawa" or "Toi". The difference may be due to the concentration of endogenous phenolic compounds inherent in each cultivar. The results from oxidized product analysis by loquat PPO implied that the mechanism of browning inhibition by thiols involved the formation of a thiol-conjugated reaction product.

Keywords: Loquat; Phenolics; Polyphenoloxidase; Browning; Sulfhydryl compounds; L-Cysteine

1. Introduction

The browning reaction in fruits and vegetables, resulting from mechanical or physiological injury during post-harvest storage or processing, is a widespread phenomenon. It is generally considered to be an undesirable reaction because of the unattractive appearance and concomitant development of an off-flavour (Friedman, 1996). Browning is caused mainly by enzymatic oxidation of endogenous phenols into quinones, which then polymerize into brown products. Thus, polyphenolic compounds and polyphenol oxidase (PPO) are directly responsible for the enzymatic browning.

Loquat (*Eriobotrya japonica* Lindl.) is widely cultivated in the subtropical regions of China, Japan, India, Israel and the Mediterranean area (Shaw, 1980). Loquat fruit is consumed mainly as fresh fruit. Processing of loquat fruit is limited by its rapid browning after it is peeled or crushed. Loquats have a relatively high concentration of polyphenols, of which the main phenolic compounds in ripe fruit are chlorogenic acid, neochlorogenic acid, hydroxybenzoic acid and 5-feruoylquinic acid (Ding, Chachin, Ueda, Imahori, & Kurooka, 1999). PPO enzyme, extracted from loquat fruit has been shown to have very high activity towards chlorogenic acid. The optimum pH and temperature for loquat PPO activity were pH 4.5 and 30 °C, respectively (Ding, Chachin, Ueda, & Imahori, 1998a).

Techniques have been developed to prevent PPO activity, which requires a unique approach, depending on the characteristics of the plant tissues and the PPO. Among the compounds that have been shown to inhibit the PPO activity are sulfites, ascorbic acid and its derivatives, and thiol compounds such as cysteine (Dudley & Hotchkiss, 1989; Langdon, 1987; Santerre, Cash, & VanNorman, 1988). Sulfur dioxide (SO_2) is one of the most effective inhibitors and has been used for many years (Mayer, Heral, & Shain, 1964). However, its use is being discontinued because of a number of reports that some humans, especially asthmatics, may be sensitive to sulfite (FDA, 1990; Sapers, 1993). Therefore, an alternative to SO_2 is obviously needed. Most of the alternatives in the food industry are formulations of ascorbic and citric acids (Hsu, Sheih, Bill, & While, 1988). However, these preparations are less effective than SO_2, since ascorbic acid is quickly consumed in the process of reducing quinones formed by the reaction of PPO with the substrate. Recent studies have shown that sulfhydryl (SH or thiol) compounds are good inhibitors of the enzyme PPO (Ding et al., 1998a; Friedman & Bautista, 1995). It was therefore of interest to compare

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the relative effectiveness of structurally different thiols in inhibiting the oxidase in loquat juices as well as in pure enzyme solution.

This study was conducted to evaluate the effectiveness of a series of sulfhydryl compounds in inhibiting polyphenoloxidase (PPO) activity in a model system (chlorogenic acid solution) and in loquat juices.

2. Materials and methods

2.1. Materials

The fruits used for various cultivars were obtained from Nagasaki Fruit Tree Experiment Station, Japan. The samples were harvested at ripe stage (suitable for fresh consumption), brought to the laboratory immediately, and then peeled and analyzed.

N-(2-mercaptopropiony)glycine (MPG) and L-cysteine methyl ester (L-CME) were obtained from Sigma Chemical Co. Mercaptopyridine (MP), L-cysteine, *N*-acetylcysteine (*N*-AC), glutathione (reduced, GTT) and chlorogenic acid were obtained from Wako Pure Chemical Industries (Osaka, Japan).

2.2. PPO extraction and purification procedures

PPO was extracted from fresh Mogi loquats harvested at the ripe commercial stage from trees grown on the farm of the College of Agriculture, Osaka Prefecture University (Osaka, Japan). Peeled loquat pulp was homogenized with 1.5-fold its weight of 0.1 M sodium phosphate buffer (pH 7.2), 30 mM sodium ascorbate, and 10% Polyclar SB-100. The crude juice was squeezed through four layers of cheesecloth and the juice, containing the enzyme, was centrifuged for 30 min at 10 000× ***g***. The loquat PPO was purified 422-fold from the pulp of fruit in five steps: fractional precipitation by extractions, ammonium sulfate, Toyopearl HW-55 chromatography, DEAE-Toyopearl and FPLC (Mono Q column) chromatographies, as described previously (Ding et al., 1998a).

2.3. Determination of inhibition of pure loquat PPO by thiols

Chlorogenic acid (1.0 mM of final concentration) was mixed with different concentrations of the various inhibitors (SH) prior to addition of pure loquat PPO (approximately 400 unit/ml). All of the enzymatic reactions were carried out with chlorogenic acid in a reaction cell at pH 4.5 and 30 °C. The increase in absorbance at 400 nm was measured. An increase in absorbance of 0.01 per min at 30 °C was defined as 1 unit of PPO activity. The results were expressed as percentage of the activity of non-inhibitor.

2.4. Analyses of oxidized products and chlorogenic acid

After incubation for 0, 1.0 and 24 h at 30 °C, the reaction solution was directly used as a sample for HPLC analysis. All samples were analyzed immediately by using an HPLC which was equipped with a Hitachi L-6200 intelligent pump, a Hitachi D-2000 Chromato-integrator and two detectors. A photodiode array UV-vis detector (Shimadzu, SPD-M6A) was used for the characterization of each peak at 280 and 325 nm, and a Hitachi L-4200 UV-vis detector for quantification at 280 nm. After injecting a known amount of extract into a GL Sciences Inertsil ODS-2 (4×250 mm) column, solvent A, acetic acid/water (5:95, v/v), and solvent B, acetic acid/acetonitrile/water (5:80:15, v/v) were used to elute the column. During analysis the solvent gradient was programmed from 0 to 50% B in A for 50 min, at a flow rate of 1.0 ml/min at room temperature (23 °C). Peaks were detected simultaneously at 280, 325 and 400 nm after injection of 20 μl of the sample. All assays were determined in triplicate. The chlorogenic acid, which remained after incubation, was expressed as a percentage of the initial content.

2.5. Measurement of browning in loquat juice

Pulp (200g) was homogenized for 1 min with an equal weight of chilled distilled water and filtered at 4 °C. An aliquot of diluted loquat juice was incubated at 30 °C for different times with various concentrations of the inhibitors, as indicated in the results and, for the control, no inhibitor was added. Then an equal volume of 4% metaphosphoric acid solution was added to stop the oxidizing reaction and the solution centrifuged at 10 000×***g*** for 20 min. The absorbance of the supernatant

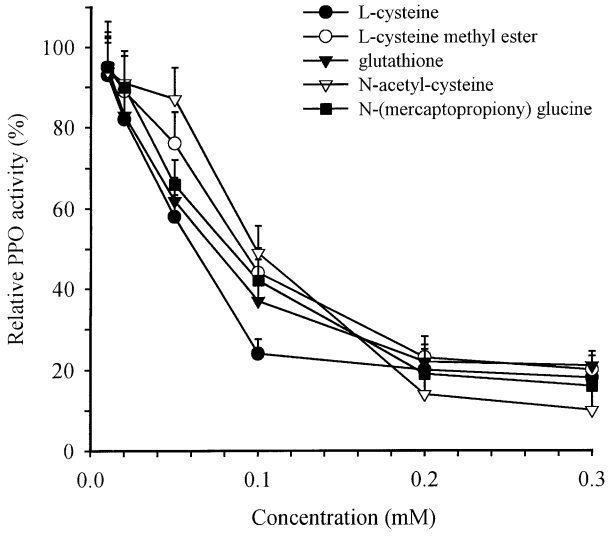

Fig. 1. Changes in the activity of polyphenol oxidase (PPO) purified from loquat (cv. Mogi) fruit in solutions containing various SH-compounds. Reactions were performed at pH 4.5 and 30 °C.

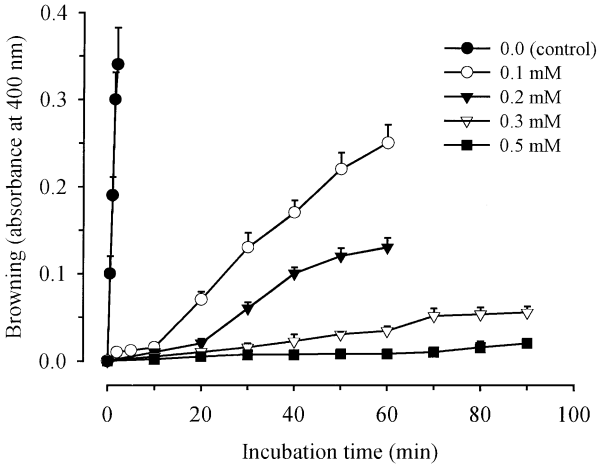

Fig. 2. Progress curve for browning of chlorogenic acid oxidized by loquat PPO in various concentrations of cysteine.

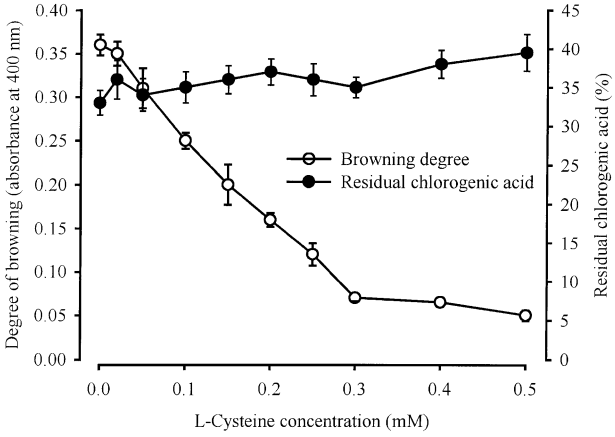

Fig. 3. Changes in the browning degree and residual chlorogenic acid after 1 h of incubation with loquat PPO in various concentrations of L-cysteine.

was determined with a spectrophotometer set at 400 nm to evaluate the degree of browning. For the initial value of absorbance of loquat juice, 2 mM sodium fluoride and 15 mM ascorbic acid was added to inhibit the browning reaction.

3. Results and discussion

3.1. Prevention of enzymatic browning of chlorogenic acid caused by loquat PPO

Chlorogenic acid is the predominant phenolic compound in all cultivars of loquat (Ding, Chachin, Ueda, & Sato, 1998b) and mainly contributes to the browning of loquat juice. Our studies previously revealed that chlorogenic acid had a high affinity towards loquat PPO (Ding et al., 1998a). Therefore, we used the chlorogenic acid solution as a model system to investigate the inhibitory effects of enzymatic browning.

Fig. 1 illustrates the concentration dependence of the inhibition of loquat PPO by five thiol chemicals. PPO activity was assayed spectrophotometrically at 400 nm at 30 °C with 1.0 mM chlorogenic acid in pH 4.5 McIlvaine buffer. The results showed that increasing the concentration of thiol compounds improved the inhibitory effects on enzymic browning (Fig. 1). Once the activity had decreased to 20%, the inactivation occurred at a slower rate. The inhibitory effects of thiol compounds on pure PPO at 0.1 mM were as follows: L-cysteine > glutathione > *N*-(mercaptopropiony) glucine > L-cysteine methyl ester > *N*-acetyl-cysteine (Fig. 1).

Fig. 2 shows the time course of the oxidation of chlorogenic acid catalyzed by pure loquat PPO in various concentrations of L-cysteine. An initial delay (lag period) in the browning product and accumulation curves was observed in the presence of the inhibitor. As the cysteine concentration increased, the lag period lengthened and the extent of browning decreased. The inhibitory effects of browning improved as the amount of cysteine increased. When 0.3 mM of cysteine was added to the model solution, enzymatic browning was completely inhibited.

3.2. Mechanism of browning inhibition by L-cysteine

The browning reaction compounds and residual chlorogenic acid were studied in order to establish whether L-cysteine reacts with quinone and/or directly inhibits the enzyme. Fig. 3 shows the degree of browning and the residual chlorogenic acid after 1 h of incubating with PPO and various concentrations of cysteine. The spectrophotometrically assessed browning decreased with increasing L-cysteine concentration. However, residual chlorogenic acid content was relatively stable, regardless of the concentration of L-cysteine (Fig. 3). When the concentration of cysteine was increased to more than 0.3 mM, the chlorogenate solution did not turn brown, but the residual chlorogenic acid was only 39%. This implied that 61% of chlorogenic acid was oxidized after 1 h of incubation. The results indicated that, under these conditions, cysteine did not influence the activity of PPO, although it inhibited browning colour formation.

Chromatographic analysis of solutions, in which chlorogenic acid was oxidized in the presence of 0.3 mM cysteine (Fig. 4A), showed that the disappearance of chlorogenic acid was accompanied by the formation of a new compound (peak 1, retention time, 15.89 min). The spectrum of this new compound exhibited a maximum absorbance wavelength at 327 nm in the same region as that of chlorogenic acid, but the shoulder at 300 nm disappeared. On the other hand, many oxidized products were observed when chlorogenic acid was oxidized by loquat PPO in the absence of cysteine (Fig. 4B). Those results suggest that the newly formed compound

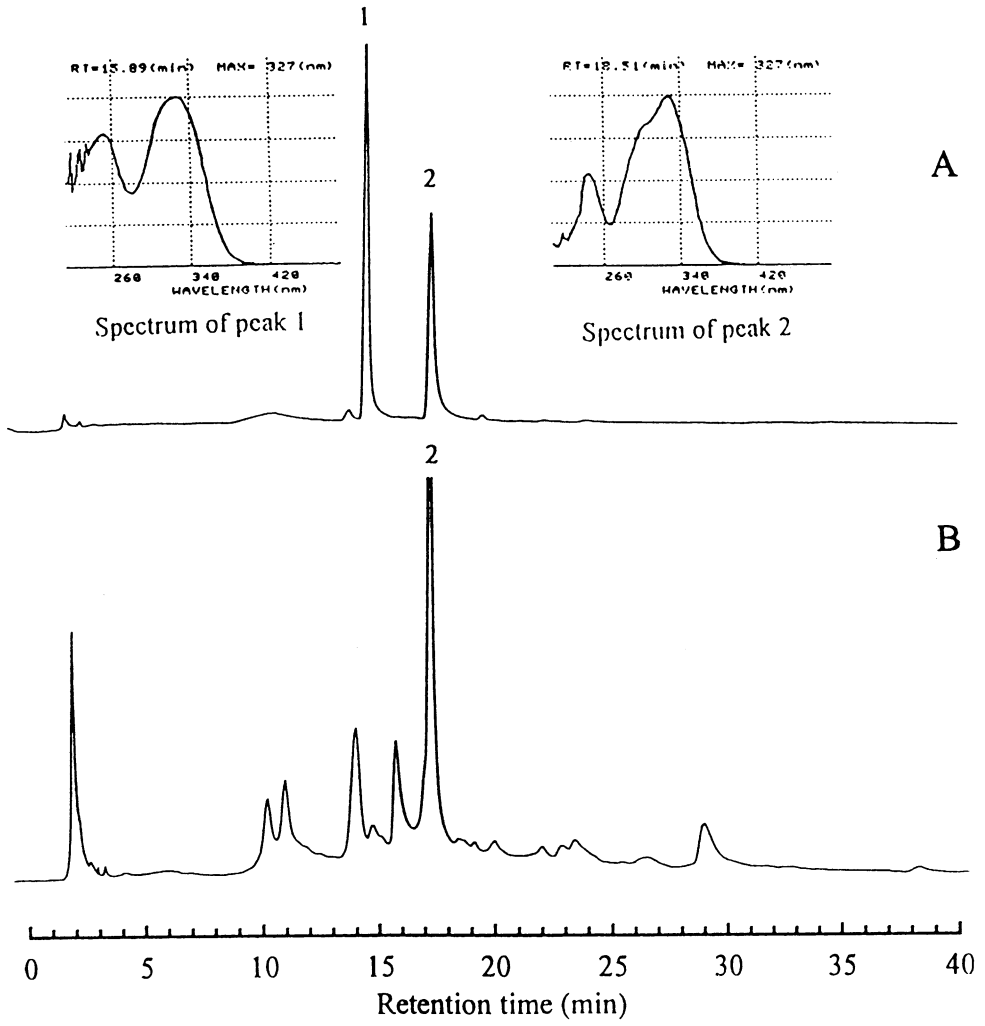

Fig. 4. HPLC chromatograms of oxidized products of chlorogenic acid by loquat PPO in (A) the presence of 0.3 mM cysteine and (B) in the absence of cysteine. Peak 1, newly formed compound; peak 2, chlorogenic acid. Spectra of peak 1 and 2 were in water–acetonitrile solution (adjusted to pH 2.6 with acetic acid).

is probably a colourless complex between quinone and cysteine, since this new compound has the same maximum absorbance wavelength as that of chlorogenic acid. Thus, the PPO activity cannot be determined by spectrophotometer in the presence of cysteine due to its product having the same maximum absorbance at 325 nm as chlorogenic acid. Likewise, the PPO activity seemed to be inhibited in the presence of cysteine when it was measured at 400 nm because the oxidized products did not turn brown. Therefore, changes in the activity of PPO in solutions containing various SH-compounds (Fig. 1) were strictly due to the browning inhibition by various SH-compounds.

The mechanism of browning inhibition by L-cysteine has been controversial for a long time. There are two main opinions which exist: (1) inhibition results from the formation of a conjugate between quinone and cysteine (Richard-Forget, Rouet-Mayer, Goupy, Friend, & Nicolas, 1992); and (2) cysteine may directly inhibit the enzyme by combining irreversibly with copper at the active site (Valero, Varon, & Garcia-Carmona, 1991). The latter opinion holds true to the fact that cysteine affects the PPO directly, because SH groups of cysteine have a strong affinity for copper and displaces histidine residues liganded to the copper of the active site of PPO and/or completely remove the copper from the enzyme (Lerch, 1987; Martinez, Solano, Penafiel,

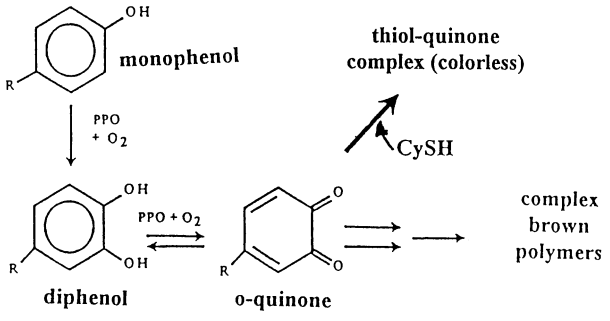

Fig. 5. Possible effect of L-cysteine on the enzymatic oxidation of chlorogenic acid. CySH, L-cysteine.

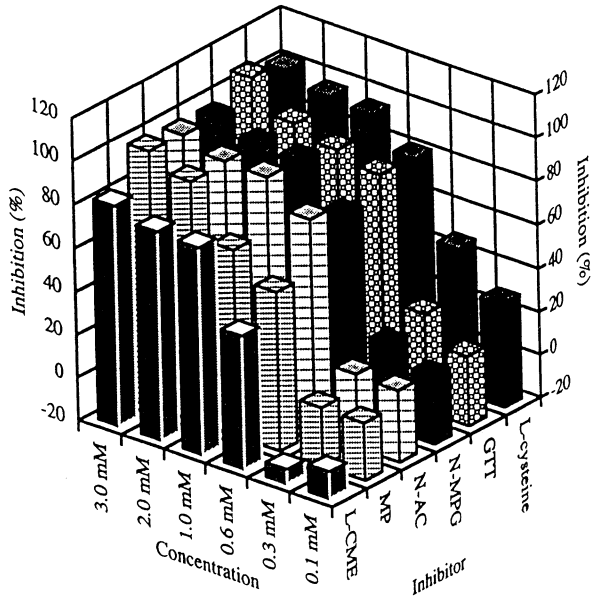

Fig. 6. Inhibition (%) of browning in the fresh loquat (cv. Mogi) juice incubated with various inhibitors for 1 h at 30 °C.

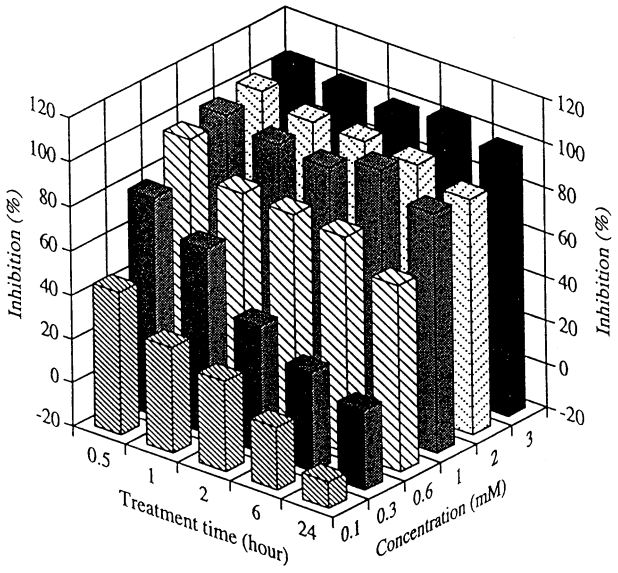

Fig. 7. Prevention of browning in fresh loquat (cv. Mogi) juice incubated at 30 °C and different time with various concentrations of L-cysteine.

Galindo, Iborra, & Lozano, 1986). The resulting structural modifications of the active site inactivate the enzyme. However, the results from our experiment suggested that the major effect was caused by the formation of thiol-conjugated reaction products (Fig. 5) rather than direct enzymatic inhibition of protein. These results also implied that the browning inhibition by cysteine, to reduce quinone back to chlorogenic acid, did not exist.

3.3. Prevention of enzymatic browning by thiols in loquat juice

It was important to examine whether the in vitro effectiveness of the above SH compounds on loquat pure PPO would also hold true in vivo in the case of crushed loquat juice. The following equation was used to calculate the percent inhibition:

$$\text{Inhibition of browning (\%)} = (A_{400}\ \text{control} - A_{400}\ \text{treated}) \times 100/A_{400}\ \text{control}.$$

where A_{400} treated is the difference between the measured A_{400} value (SH inhibitor added) at time t and the corresponding value at zero time. A_{400} control was the difference between the measured A_{400} value (no inhibitor added) after incubation for time t and the corresponding value at zero time. Zero time was defined as about 1–2 min after the oxidation process.

Application of different concentrations of sulfhydryl compounds to fresh juice of loquat showed that, when the concentrations were increased up to 1.0 mM, all of the SH-compounds prevented browning effectively (Fig. 6). The concentration of inhibitors required to

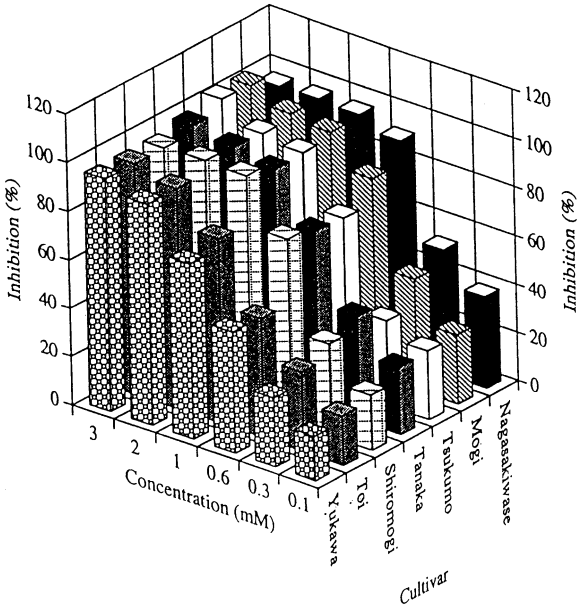

Fig. 8. Changes of browning inhibition (%) of loquat juice from different cultivars incubated with various concentration of L-cysteine for 1 h at 30 °C.

inhibit browning in loquat juice is much greater than that for pure PPO. Among the six thiol compounds, L-cysteine appeared to be the most effective inhibitor followed by *N*-acetyl-cysteine, *N*-(2-mercaptopropiony)glycine and reduced glutathione.

On the basis of the cited observations, the effectiveness of L-cysteine in inhibiting browning of loquat juice was investigated more extensively. L-Cysteine prevented browning significantly at the first 30 min of incubation. However, the effectiveness of browning inhibition decreased with extended incubation time (Fig. 7).

To evaluate the effectiveness of browning inhibitor on different cultivars, seven cultivars grown in Japan with different browning potential were used. The data in Fig. 8 shows that the required concentration of L-cysteine for 90% inhibition ranged widely from 0.6 mM for "Nagasaki" to 2.0 mM for "Yukawa" and "Toi". The rest of the cultivars required concentrations from 0.6 to 1.0 mM. The difference may be due to the concentration of endogenous phenolic compounds inherent in each cultivar (Ding et al., 1998b).

The results presented earlier demonstrated that L-cysteine is the most effective inhibitor of browning in pure chlorogenic acid solution as well as in loquat juice. *N*-acetyl-cysteine, *N*-(2-mercaptopropiony)glycine and reduced glutathione have similar effects on the browning inhibition of loquat juice. The browning of the chlorogenic acid solution was oxidized by pure loquat PPO and the browning of loquat juice can be considered to result from endogenous PPO that oxidized endogenous phenolics. The effective concentrations of SH-containing compounds in the inhibition of browning of loquat juice varied, depending on the cultivars used to make the juices, the storage time of juice and the type of SH compounds. Whether any of the compounds evaluated in this study are usable in foods awaits further study. One of the most potent compounds, *N*-(2-mercaptopropionyl)glycine, is not a naturally-occurring amino acid of peptides and therefore is not usable in foods. L-Cysteine is very effective in inhibiting browning, but Molnar-Perl and Friedman (1990) reported that the addition of 1.7 mM of L-cysteine may produce undesirable odours. *N*-acetyl-cysteine and reduced glutathione had the same relative effectiveness on browning at the same concentration as cysteine and no off-flavours. These compounds have also been shown to reduce browning of apple and banana slices (Buta, Moline, Spaulding, & Wang, 1999; Moline, Buta, & Newman, 1999) and have potential to be used commercially to substitute sulfite as anti-browning agents.

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