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Effect of glutathione and Maillard reaction products prepared from glucose or fructose with glutathione on polyphenoloxidase from apple—I: Enzymatic browning and enzyme activity inhibition

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Abstract

The effect of unheated and heated glutathione (GSH) and Maillard reaction products (MRPs), derived from equimolar mixtures (0.25 M) glucose or fructose with GSH, on purified apple polyphenoloxidase (PPO) activity was investigated by polarography and spectrophotometry, using 4-methylcatechol as the main phenolic substrate. When assayed alone, glutathione interacted with enzyme-generated *o*-quinones, giving rise to colourless conjugates, as demonstrated by high pressure liquid chromatography (HPLC). By polarography, increasing concentrations (0–300 mM) of GSH, resulted in a high inhibitory effect on PPO activity, mostly due to a drop of pH of the reaction solutions to acidic values. Upon heating GSH at 90 °C, thermal degradation product formation was responsible for a partial PPO inhibition. GSH-derived MRPs highly inhibited PPO activity, inhibition efficiency increasing with heating time (2–39 h) and temperature (80–110 °C). Compared with MRPs prepared from hexose with cysteine, those from GSH exhibited a more potent inhibitory effect, due to the presence of an additional carboxylic function on the tripeptide. Therefore, these MRPs could be considered as potential natural inhibitors for use with minimally processed fruits.

Keywords: Maillard reaction products; Glutathione; PPO; Enzymatic browning; Enzyme inhibition

1. Introduction

Polyphenoloxidase or PPO (EC 1.10.3.1) catalyzes the oxidation of *o*-phenolic substrates to *o*-quinones, which are subsequently polymerized to dark-coloured pigments. This metalloenzyme, widely distributed in plants, is considered to be the main contributor to browning, discolouration and darkening in fruits and vegetables (Mayer & Harel, 1979, 1991; Vamos-Vigyazo, 1981; Zawistowski, Biliaderis, & Eskin, 1991). Among the numerous compounds capable of reducing enzymatic browning and/or oxidoreductase activity, the use of natural inhibitors of PPO is still stimulated by the need to replace sulphiting agents in order to prevent or minimize the loss of fresh or processed foodstuffs (Nicolas, Richard-Forget, Goupy, Amiot, & Aubert, 1994; Vamos-Vigyazo, 1995). In this area, non-enzymatic browning reactions of the Maillard system, between carbonyl and amino compounds, taking place during storage and food processing, at relatively mild heating temperatures (70–110 °C), are very important in the food industry. In particular, the Maillard reaction affects sensory characteristics as with sulfur-containing components, which generate meat-like aromas through their reaction with reducing sugars. In addition, compounds or classes of compounds with antioxidative properties may be formed, depending on which amino acids and which sugars participate in the reaction as well as on the reaction conditions. In fact, Maillard reaction products (MRPs) are known for their ability to retard lipid oxidation in vitro as well as in vivo (Chuyen, 1998; Lingnert & Eriksson, 1980a,b, 1981) and to inhibit certain oxidoreductases such as PPO (Nicoli, Elizalde, Pitotti, & Lerici, 1991; Pitotti, Nicoli, Sensidoni, & Lerici, 1990; Tan & Harris, 1995), peroxidase (Nicoli et al., 1991; Pitotti, Elizalde, & Anese, 1995), superoxide dismutase (Ukeda, Hasegawa, Ishi, &

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Sawamura, 1997), xenobiotic enzyme systems (Kitts, Wu, & Powrie, 1993) and hydrolase activity (Hirano, Miura, & Gomyo, 1996; Öste, Dahlqvist, Sjöström, Noren, & Miller, 1986; Öste, Miller, Sjöström, & Noren, 1987; Pitotti, Dal Bo, & Stecchini, 1994; Schumacher & Kroh, 1996). Although the antioxidative mechanism of MRPs is still unclear, it is thought that the reductone structures, the electron donor property, the chelating properties of melanoidins against transition metals, as well as the scavenging activity of Amadori compounds and melanoidins against reactive oxygen species, are important factors promoting inhibition of lipid and phenolic oxidation reactions.

Since the complexity in chemical composition of foods renders study of the different chemical properties of browning reactions difficult, simple model systems, based on combinations of sugars and amino acids, have frequently been used. We have demonstrated in two preceding papers that MRP model mixtures from glucose or fructose with cysteine, heated at 90 °C for periods of time ranging between 2 and 39 h were potent inhibitors of enzymatic browning as well as apple polyphenoloxidase activity (Billaud, Roux, Brun-Mérimée, Maraschin, & Nicolas, 2003). These effects were believed to partly arise from a chelating action of copper ions at the active site of the oxidoreductase (Roux, Billaud, Maraschin, Brun-Mérimée, & Nicolas, 2003). Moreover, glucose was shown to be the most active sugar in producing inhibitory derivatives in the browning reaction with L-cysteine. To add further support to the possible involvement of Maillard reaction products derived from a thiol precursor, we have extended the study to the inhibitory potency of various amounts of MRPs derived from hexose/glutathione, heated at various temperatures (80–110 °C) for different times (2–39 h), on enzymatic browning and apple PPO activity in order to optimize organoleptic properties of fruit and vegetables.

2. Materials and methods

2.1. Materials

Apples from the variety Red Delicious were picked at commercial maturity and used as PPO source. The enzyme was purified from the cortex according to the method of Janovitz-Klapp, Richard, and Nicolas (1989) in three steps, i.e. extraction, fractional precipitation by ammonium sulfate (164 g l^{-1}, 30% saturation) and hydrophobic chromatography with phenyl-Sepharose CL4B (Pharmacia, Uppsala, Sweden). Active fractions were pooled, stored at +4 °C and constituted the enzymatic extract used for subsequent experiments.

All chemicals used were the purest available. L-Cysteine (Cyst, free base), reduced glutathione (GSH), D-glucose, D-fructose, sodium fluoride, 4-methylcatechol (4-MC), (+) catechin and chlorogenic acid were obtained from Sigma-Aldrich Chemical Co (St Louis MO, USA). Chemicals used in the preparation of buffers, purification of apple PPO and acetic acid, were purchased from Merck-eurolab, Div. Prolabo (Fontenay S/Bois, France). Acetonitrile was supplied from Acros organics (New Jersey, USA). Water used was purified by Analyst HP purification, Purite Select limited (Oxon, England).

2.2. Preparation of Maillard reaction products (MRPs)

Glucose or fructose (0.25 or 1 M), L-Cyst (1 M) and GSH (0.25 M) were used to prepare thermal degradation products of GSH, and different model aqueous solutions of MRPs, composed of glucose or fructose with either Cyst (1 M/1 M) or GSH (0.25 M/0.25 M), using the procedure already described by Billaud et al. (2003). Aliquots of the soluble part of the heated mixtures were directly used to determine losses in apple PPO activity and spectrophotometric readings. The remaining part was stored at −20 °C for GSH loss determinations.

2.3. Colour intensity and pH measurements

UV-visible spectra and absorbance measurements of MRP aqueous solutions at 340–360 nm were recorded with a Hewlett-Packard (Bracknell, UK) model 8453A spectrophotometer equipped with a photodiode-array detector using a 10 mm cell. When necessary, appropriate dilutions were made in order to achieve an absorbance <1.8 in the 340–360 nm region. Absorbance index was defined as : absorbance at 340, 350 or 360 nm × dilution factor.

An Orion model 410A pH meter and a Radiometer analytical XC111 thin-probe combination electrode were used for pH measurements.

2.4. Enzyme determination

2.4.1. Spectrophotometric measurements

The rate of 4-MC oxidation by the purified apple PPO was determined at 25 °C in a 10 mm path cell on a Hewlett Packard 8453A photodiode array spectrophotometer. Typically, assay mixtures (3 ml total volume) contained 20 mM 4-MC dissolved in 0.1 M air-saturated citrate-phosphate (McIlvaine's) buffer at pH 4.5. The reaction was initiated by adding aliquots of enzymatic extract (max. volume of 0.05 ml corresponding to approximately 7–10 nkat) and various additives as specified below in the assay medium. The oxidation of phenolic substrate was followed by monitoring the increase in absorbance at 400 nm resulting from the oxidation of 4-MC in the presence of oxygen (Mason &

Peterson, 1965). Activity was determined by measuring the maximal slope from the linear part of the curve and defined as absorbance unit A.U s^{-1} for a total volume of 3 ml. Unless otherwise indicated, PPO was added last.

Enzyme-activity data are averages of duplicate measurements. Variation between duplicates was less than 5%.

2.4.2. Polarographic measurements

PPO activity was determined polarographically at 30 °C in a 1.5 ml reaction cell containing 20 mM 4-MC in 0.1 M air-saturated McIlvaine's buffer (pH 4.5) as the substrate. The reaction rate was calculated from the initial slope of the progress curve giving oxygen uptake versus time using a Clark oxygen micro-probe. Activity was expressed as nmoles of oxygen consumed per second (nanokatals) under the assay conditions. For inhibition studies by GSH or MRPs, aliquots (1–100 μl) were added to the phenolic solution before the enzyme extract. All determinations were performed in duplicate.

2.5. Analytical HPLC analyses with diode-array detection (DAD)

2.5.1. Reaction and oxidation products analysis during enzymatic catalysis of 4-MC

The HPLC method used to analyze for reaction and oxidation products during the course of 4-MC (2 mM) oxidation by apple PPO in the absence or in the presence of GSH (2 mM) was the procedure previously described by Billaud et al. (2003), using a Waters (Milford, MA, USA) liquid chromatograph equipped with a Model 600 pump system controller, a Model 996 photodiode array detector with Millenium 32 chromatography manager software, a rotary valve (Rheodyne model 8125) fitted with a 20 μl sample loop and a Nucleosil 5 C18 (150 × 4.6 mm i.d) column from Macherey-Nagel (Duren, Germany), protected with a guard column of the same material (8 × 4 mm i.d). The detection was monitored at 280 nm and UV–visible spectra were recorded from 210 to 450 nm. Vanillic acid was used as the internal standard.

2.5.2. Heated thiols and MRP solutions analysis

During the heating, at 90 °C, of thiol model solutions (1 M cysteine or 0.25 M GSH) for various periods of time, remaining thiol concentration was monitored by HPLC, using the same column as described above. Elution was isocratic using 5% acetonitrile in an *o*-phosphoric acid aqueous solution (pH 4.2), with a 0.8 ml min^{-1} flow rate. A calibration curve was obtained by injecting 20 μl each of 7 standards containing from 2 to 50 mM cysteine or GSH, using the DAD detector set at 230 nm. Solvents and thiol solutions were filtered through a 0.2 μm nylon membrane filter before use. Under these chromatographic conditions, retention times of cysteine and GSH were, on average, 2.5 and 3.5 min, respectively.

After heating of model MRP solutions (fructose/GSH or glucose/GSH), the total reaction products were analyzed by HPLC-DAD, using a 5 μm YMC ODS AQ column (250 × 4.6 mm i.d., AIT Chromato, France), protected with a guard column of the same material (8 × 4 mm i.d.). The elution solvent was water/acetonitrile (95/5). Samples were 5 fold diluted, filtered through a 0.2 μm nylon membrane and run isocratically at a flow of 0.8 ml min^{-1}. The detection was monitored at 280 nm and UV–visible spectra were recorded from 260 to 500 nm.

3. Results and discussion

3.1. Effect of unheated and heated thiol solutions on enzymatic browning and PPO activity

When apple PPO was incubated in the presence of 4-MC (20 mM), a yellow colouration developed which rapidly turned to brown, together with oxygen uptake. When GSH (0–1 mM) was initially present in the reaction medium, there was only a slight decrease in oxygen consumption (< 5%) but colour development, measured by the absorbance at 400 nm, steadily decreased and disappeared with 1 mM of the reductant (Fig. 1). Thus,

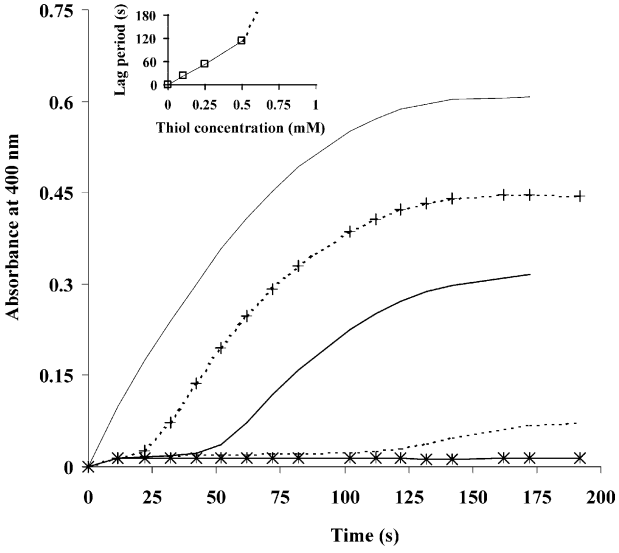

Fig. 1. Spectrophotometric determination of 4-MC oxidation by apple PPO recorded at 400 nm using GSH as inhibitor at different concentrations : 0 mM (—), 0.1 mM (+ - - +), 0.25 mM (—), 0.5 mM (- -) and 1 mM (×–×). The 3 ml reaction mixture contained 0.1 M McIlvaine's buffer (pH 4.5), 20 mM 4-MC, 10 nkat PPO and the indicated concentrations of GSH, at 25 °C. Inset : Effect of GSH concentration on the length of the lag period. The lag time was estimated from the interception of the extrapolated linear portion of the progress curve with the baseline.

the presence of GSH did not affect the phenol oxidation but enzymatic browning inhibition was due to the reaction of the reducing agent with enzyme-generated products (*o*-quinones) yielding adducts with lower extinction coefficients at 400 nm than those of coloured products formed in the absence of GSH. In this way, it provoked an initial lag period (Fig. 1, inset) and a concomitant decrease in the maximal rate of primary oxidation product formation following this lag period. In the range tested, the higher concentration of GSH resulted in a lag period >3 min. These data confirm those obtained with an excess of thiol compounds in the reaction medium, namely cysteine (Dudley & Hotchkiss, 1989; Janovitz-Klapp, Richard, Goupy, & Nicolas, 1990a; Richard-Forget, Goupy, Nicolas, Lacombe, & Pavia, 1991; Richard-Forget, Goupy, & Nicolas, 1992; Tan & Harris, 1995; Robert, Richard-Forget, Rouch, Pabion, & Cadet, 1996; Billaud et al., 2003), the dipeptide cysteine-glutamic acid (Richard-Forget, Cerny, Fayad, Saunier, & Varoquaux, 1998), GSH (Golan-Goldhirsh & Whitaker, 1984; Kahn, 1985), captopril (Espin & Wichers, 2001) and other bioactive sulfur compounds (Motohashi, Nishikawa, & Mori, 1991) with PPO from various origins. In order to confirm that the interaction of the sulphydryl compound, with *o*-quinone, led to an adduct formation with generation of a colourless conjugate, PPO oxidation of 4-MC in the presence of GSH was also monitored by CLHP at 280 nm (Fig. 2). In the absence of GSH in the reaction mixture, accumulation of *o*-quinones was confirmed by the increase in absorbance at 400 nm (Fig. 2A) and the 4-MC primary oxidation products formation (peak 2). The addition of 2 mM GSH after 200 s of 4-MC oxidation catalyzed by PPO resulted in the disappearance of absorbance at 400 nm (Fig. 2B), typical CLHP chromatograms obtained attesting to the disappearance of the quinone peak (peak 2) and the presence of an addition compound (peak 3), with no regeneration of the parent phenol (peak 1). The structures of the adduct compounds with caffeoyltartaric acid or 4-MC were previously identified as 2-*S*-glutathionylcaffeoyltartaric acid and 5-*S*-glutathione-3,4-dihydroxytoluene, respectively, by Cheynier, Trousdale, Singleton, Salgues, and Wilde (1986) and Richard-Forget et al. (1991), using the tripeptide GSH as the reductant. Spectra of the parent phenol and the glutathionyl adduct product, obtained from the chromatograms using a diode array detector, are shown in Fig. 2C. According to Sanada, Nakashima, Suzue, and Kawada (1976) and Richard-Forget et al. (1991, 1998), the high absorbance around 255 nm gives evidence of an attack of the thiol group of GSH occurring at the 5 position of the ring of the *o*-quinone.

Increasing concentrations of GSH (1-300 mM) in the reaction medium resulted in a gradual loss of enzyme activity, when assayed by polarography (Table 1). Comparison of the inhibitory potencies of thiol compounds, i.e. cysteine (Cyst) and GSH, revealed that the latter exhibited a much higher effect on 4-MC oxidation catalyzed by PPO, whatever the concentration of the reductant added in the reaction mixture. Concomitantly, increasing concentrations of GSH added to the substrate solution were accompanied by a drop in the pH of the solutions to acidic values. Compared to the Cyst structure (1 COOH, 1 SH, 1 NH_2 free functions), GSH possesses an additional COOH function which is responsible for lowering the pH of the subsbtrate solution, in a concentration-dependent way. Thus, it appears that the resulting acidification highly contributes to the inhibitory effect of solutions containing GSH, keeping PPO away from its optimum pH of activity. As a matter of fact, when the effect of GSH was measured for a constant pH value and using equivalent concentrations of thiol, its inhibitory potency remained close to that of Cyst (24–25%), with 300 mM Cyst and GSH, respectively.

Similar results were obtained by Janovitz-Klapp, Richard, Goupy, and Nicolas (1990a,b) when testing the slight effectiveness of cysteine as an inhibitor of apple PPO activity. Conversely, Golan-Goldhirsh, and Whitaker (1984) showed that, with mushroom tyrosinase, 0.25 mM reduced GSH decreased the initial rate of oxygen uptake by about 35% compared to the control. At higher concentrations (up to 0.8 mM) of the thiol compound, no additional effect was seen. Therefore, it can be deduced that PPO enzymes, according to the vegetal source, have differential sensitivities to thiol treatment. Based on our kinetic and spectral data, we concluded that thiol compounds did not inhibit apple PPO per se but they gave an apparent inhibition of

Table 1
Inhibitory effect of thiol (cysteine or glutathione) concentration on apple PPO activity in relation to changes in pH of solutions

Thiol conc. (mM)	Initial pH		PPO activity			True Inhibitory effect of GSH (%)[c] (5)–(4)
	+ Cyst (1)	+ GSH (2)	+ Cyst[b] (3)	GSH[a] (4)	+ GSH[b] (5)	
0	4.7	4.5	0	0	0	0
1	4.7	4.3	5.5	3.9	6.7	2.8
10	4.7	4.0	14.1	14.7	30	15.3
25	4.7	3.7	21.4	26.9	43	16.1
100	4.7	3.2	22.5	60.8	79.1	18.3
200	4.6	3.1	23.2	63.2	86.8	23.6
300	4.6	3.0	24.8	64.7	88.9	24.2

Activity was measured by polarography at 30 °C, using 10 nkat PPO and thiol compounds at the indicated concentrations.

[a] Inhibitory effect is expressed as % of inhibition due to the nominal pH (col. 2) compared to the activity measured at pH 4.5, using McIlvaine's buffer solutions.

[b] Inhibitory effect is expressed in % inhibition compared to the activity measured in the absence of thiol.

[c] The true inhibitory effect of GSH was obtained by the difference in inhibitory effects due to GSH addition (col. 5) minus that due to the decrease of pH (col. 4).

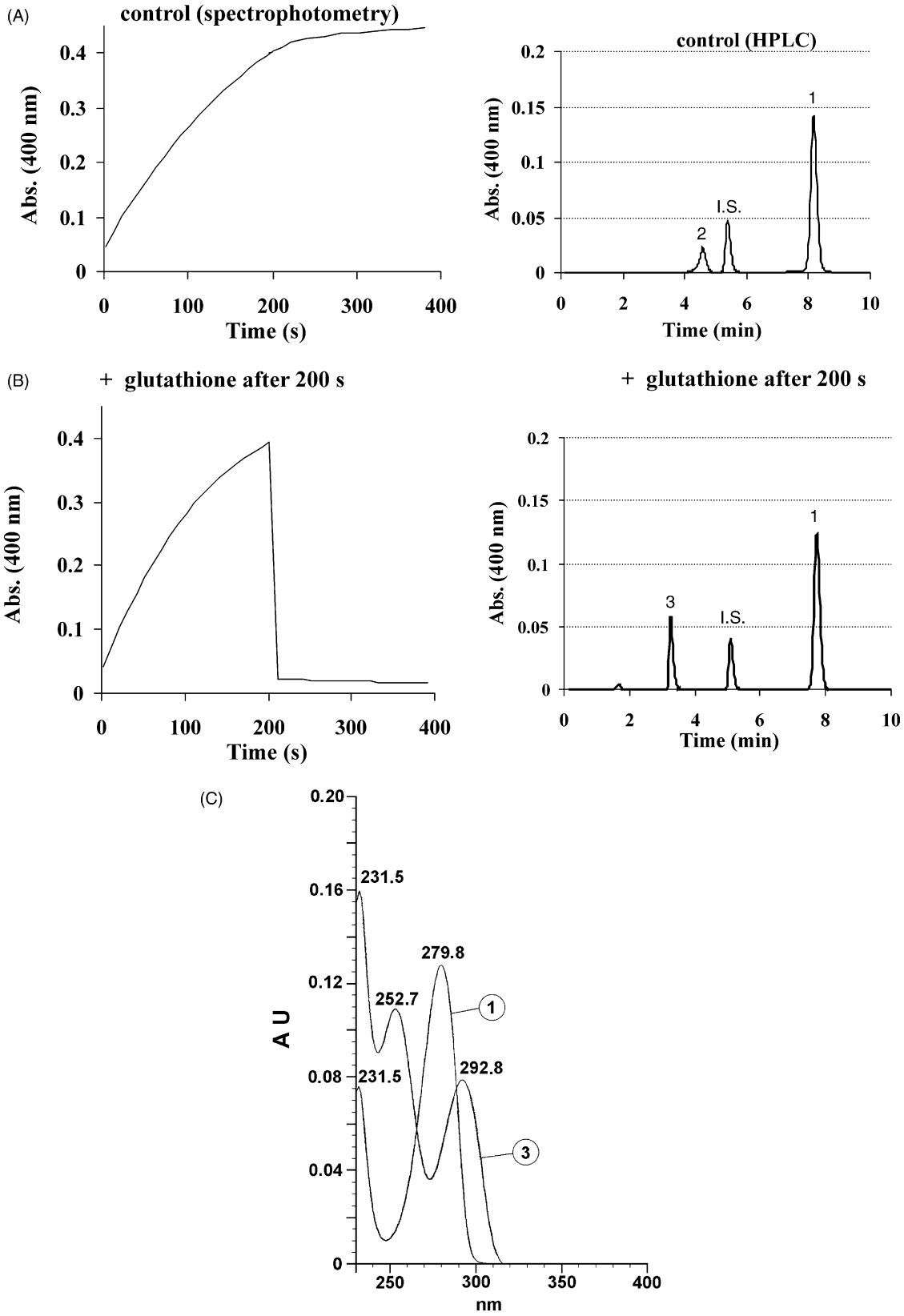

Fig. 2. Spectrophotometric determination (left panels) of *o*-quinone formation and HPLC chromatogram of reaction products (right panels) of 4-MC (20 mM or 2 mM) oxidation, in the absence (A) or in the presence of GSH (2 mM, B) added after 200 s of oxidation by 9 nkat (spectrophotometry) or 24 nkat (HPLC) of apple PPO in 3 ml of McIlvaine's buffer at pH 4.5 and at 25 °C. 1, 4-MC; 2, *o*-quinone of 4-MC; 3, 5-*S*-glutathionyl 4-MC; I.S., vanillic acid (internal standard). Overlay UV spectra (C) of 4-MC and glutathionyl adduct product was obtained by diode array detection. The displayed spectra were taken at HPLC peak apices 1 and 3, respectively.

activity due to their ability to conjugate with primary oxidation products formed in the reaction.

Effects of thiol compounds on potential inactivation of protein enzyme were further tested by incubating PPO at 0 °C with either Cyst or GSH (5 or 10 mM, respectively), in the absence of phenolic substrate. Remaining activity was assayed spectrophotometrically and polarographically on aliquots withdrawn during the incubation (5–180 min). Similar effects to that presented above were obtained, namely, a lag time before any change in absorbance at 400 nm was measurable and a decrease in the rate of product formation, whereas the decrease in oxygen uptake remained slight (results not shown). Thus, at these concentrations, cysteine and GSH were not effective in inactivating apple PPO during 3 h incubation.

In another experiment, the formation of inhibitory compounds on heating aqueous solutions of Cyst (1 M) and GSH (0.25 M) at 90 °C for various lengths of time (0–48 h) was investigated. When assayed spectrophotometrically, it emerged that, after heating GSH solutions at 90 °C for 6 h and at GSH concentrations equivalent to unheated solutions, a lag period was again observed before recording a change in absorbance at 400 nm, with a similar effect upon the maximum rate of reaction. When assayed polarographically and in the course of heating (4–48 h), the inhibitory potency of GSH on oxygen uptake gradually increased until it resched almost 50% after 48 h of heating (Table 2). This increase in inhibitory effect, which progressed with the time of heating, seemed to coincide with thermal degradation product formation from GSH. Compared with the thiol amino acid previously studied (Billaud et al., 2003), the tripeptide was more rapidly converted to compounds responsible for the inhibition observed. In this way, GSH was more heat-labile than Cyst, as unreacted GSH was reduced to nearly 70 and 30% of its original value after 8 and 48 h of heating, respectively, compared to 84 and 60% residual Cyst after heating 1 M solutions in identical conditions (results not shown).

3.2. Effect of MRPs derived from glucose or fructose/GSH solutions on enzymatic browning and PPO activity

3.2.1. Heat treatment effect on the spectrophotometric and chromatographic characteristics of ose/GSH model mixtures

According to the nature of the reactants involved, the Maillard reaction development is generally measured by the absorbance increase, either at 294 (early MRPs), 320–350 (soluble pre-melanoidins) or 420-450 (advanced MRPs) nm, corresponding to colour intensity of the reaction medium, as well as the formation of specific compounds, some of which possessed antioxidant properties (Eiserich, Macku, & Shibamoto, 1992; Kirigaya, Kato, & Fujimaki, 1968; Wijewickreme, Kitts, & Durance, 1997).

The pigment formation from MRP soluble materials was evaluated from their visual colour (Table 3). Thus, when D-glucose or D-fructose were heated with GSH in aqueous solutions, the formation of a yellow colour could be observed during heating for 6 h, which turned, during prolonged heating, strong orange, but never developed a dark brown colour as previously observed during similar heating conditions with hexose/Cyst systems. Inasmuch as concentration of reactants was 4-fold higher in these latter model mixtures, this is not surprising. Browning products derived with glucose increased more rapidly than with fructose, revealing that the aldohexose was a more reactive precursor of Maillard products than the ketohexose. Absorbance spectra in the region 250–450 nm for the Maillard products derived from D-glucose (0.25 M/0.25 M), heated at 90 °C for times ranging from 4 to 39 h, (Fig. 3A), showed two absorbance maxima at 285 and 350–360 nm. Resulting absorbance spectra of MRPs prepared

Table 2
Effect of time (0–48 h) of heating at 90 °C of aqueous GSH solutions (0.25 M) on pH, residual GSH and inhibitory effect on PPO activity

Time of heating at 90 °C (h)	pH of heated GSH solutions	GSH remaining (% control)[a]	Inhibitory effect[b]
0	4.25	100	100
4	4.12	93.5	95.3
6	4.12	85.1	84.9
8	4.14	66.6	76.6
15	4.16	53.6	71.1
20	4.16	47.0	68.5
24	4.15	37.5	66.4
39	4.15	35.9	58.3
48	4.14	32.6	52.7

Activity was measured by polarography at 30 °C, using 4-MC (20 mM) as the substrate at pH 4.5, with 10 nkat PPO and 100 µl heated GSH solution.

[a] HPLC quantification of GSH.

[b] Inhibitory effect is expressed as % of inhibition compared to the activity measured with unheated GSH in the reaction medium.

Table 3
Evolution of the visual colour and absorbance values at 340, 350 or 360 nm of MRP (0.25 M/0.25 M) aqueous solutions heated at 90 °C for various lengths of time

Heating time (h)	Glucose/GSH			Fructose/GSH		
	Absorbance[a]		Visual colour	Absorbance[a]		Visual colour
	350 nm	360 nm		340 nm	350 nm	
2	0.17	0.17	Colourless	0.45	0.39	Colourless
4	0.70	0.75	Very light yellow	1.50	1.20	Very light yellow
6	2.00	2.05	Light yellow	3.05	2.45	Light yellow
8	4.60	4.70	Yellow	4.80	3.80	Yellow
15	10.05	10.20	High yellow	8.90	7.15	High yellow
20	15.3	15.65	Light orange	14.10	11.70	Light orange
24	26.6	27.15	Orange	18.40	15.90	High orange
39	42.8	43.90	High orange	22.85	19.50	High orange

[a] Absorbance index is : absorbance × dilution factor.

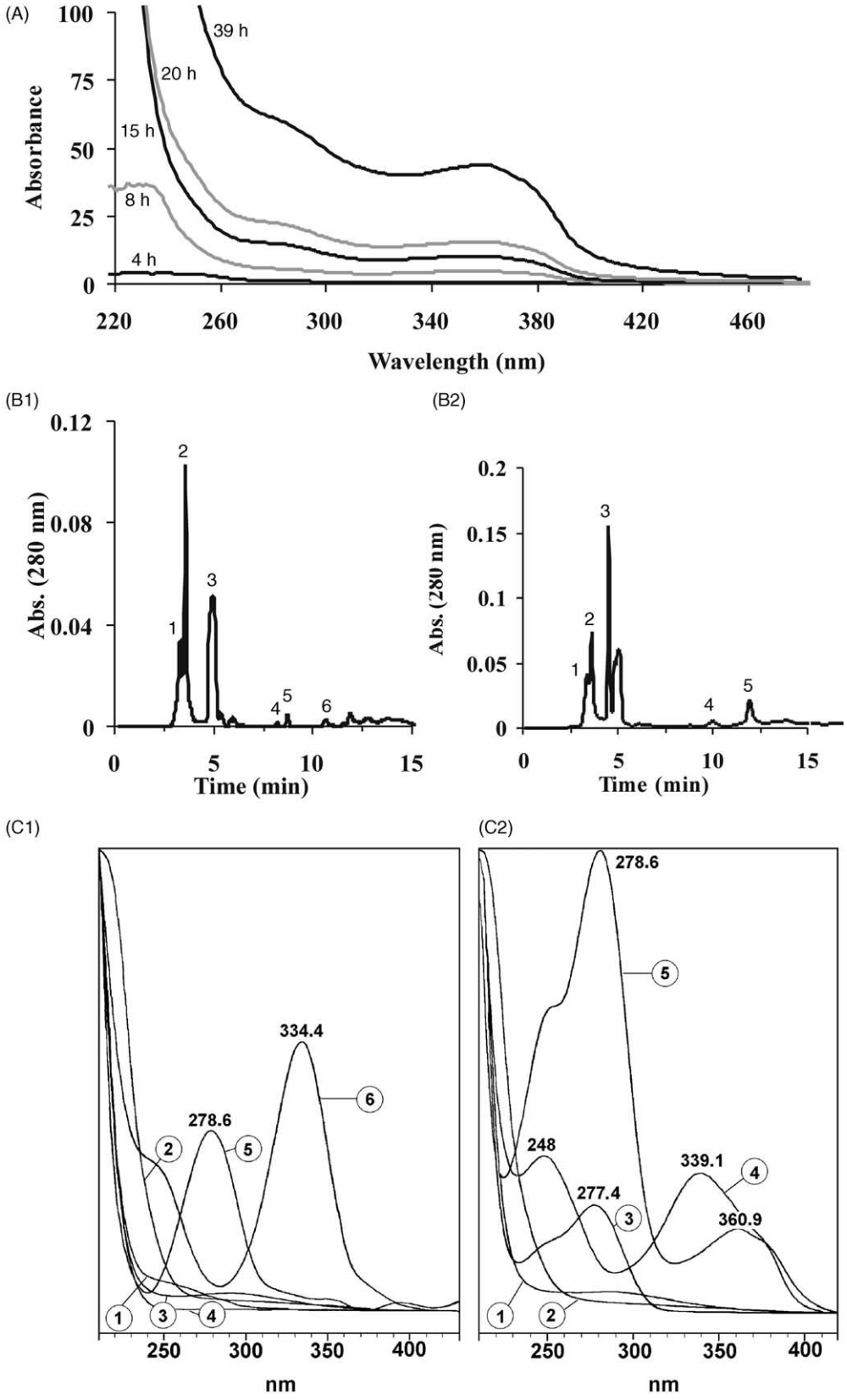

Fig. 3. Evolution of UV–visible spectra of MRPs derived from glucose/GSH (0.25 M/0.25 M) solutions heated at 90 °C for 0–39 h (A) and HPLC elution profiles recorded at 280 nm of fructose/GSH (B1) and glucose/GSH (B2) solutions heated at 90 °C for 15 h. The UV–visible spectra of the major formed compounds were obtained from the chromatograms B1 and B2 (respectively C1 and C2) using a diode array detector. Wavelengths of absorption maxima of the spectra are indicated.

with D-fructose were quite similar, with two absorbance maxima at 282 and 340–350 nm (not shown). These results suggested that, actually, the development of the Maillard reaction during heat tratment of model mixtures corresponded to intermediate MRPs and was better evaluated by recording absorbance at 340–360 nm than at 294 or 420 nm, as usually determined with heterocyclic components and brown pigments from heated amino acid-glucose systems, respectively (Friedman & Molnar-Perl, 1990; Wijewickreme et al., 1997).

Preliminary HPLC study was next used to compare chromatographic profiles of the reaction products after heating reactants at 90 °C for 15 h (Fig. 3B1 and B2), monitored at 280 nm. Both chromatograms showed partly resolved peaks at the beginning of the run, between 3 and 6 min and some discrete peaks between 8 and 15 min. The chromatograms resemble each other in the overall peak profile up to 15 min, although the absorption intensity levels of most of them differ greatly, except for a distinct peak at 4 min (peak 3 in Fig. 3B2) that did not appear with fructose-derived MRPs. Part of the products formed eluted early (peaks 1–3) on the column and should therefore be more polar and/or smaller molecules than those eluting after 8 min (peaks 4–6).

UV-visible spectra, corresponding to peaks 1–6 (Fig. 3C1) and 1–5 (Fig. 3C2), were registered on a diode array detector. Most of the compounds showed a maximum absorbance at 210–230 nm, close to 280 nm and around 330–370 nm. Although the spectra of major products did not correspond to known products (in view of possible coelutions), those from peaks 1–3, with retention times ranging from 3.2 to 5.1 min and a maximum absorbance near 220 nm suggest a thiol structure, similar to thermal degradation compounds of GSH chromatographed under the same conditions (not shown). Thus, although not identified, they are probably related to sugar- and thiol-derived components, as well as unaltered parent reactants. In the same way, the spectrum of the peak denoted 5 in Fig. 3C2, exhibited a spectrum similar to that of 5-hydroxymethyl-2-furfuraldehyde, a well-known thermal degradation compound of glucose (Baltes, Kunert-Kirchoff, & Reese, 1989; Monti, Bailey, & Ames, 1998). It is noteworthy that, to our knowledge, investigations on the structure of compounds isolated from sugar-GSH heated systems are very limited and usually refer to the volatile fraction determination (Zhang & Ho, 1991; Tai & Ho, 1998).

Overall, this preliminary chromatographic and spectrophotometric analysis, from both MRP systems, can give an insight into differences and similarities of the total reaction products of these model systems, in which inhibitory potency seems to be dependent on the reactivity of hexoses and, consequently, on the reaction products formed. However, the origin, composition and structure of components or classes of components present in these soluble extracts and responsible for an potential inhibitory effect on PPO activity are not resolved.

3.2.2. *Effect of heat treatment time on the inhibitory effect towards PPO activity caused by sugar/GSH model mixtures*

In any MRP system tested, although free GSH level progressively decreased during heat treatment, owing to its consumption in the Maillard reaction, the concentration of unreacted GSH remained in excess in the reaction medium. Accordingly, inhibition studies were only followed by means of measurements of oxygen uptake.

The influence of GSH concentration was first explored to define PPO inhibition conditions. It emerged that, at the concentration of 0.25 M, inhibition exerted by the glucose/GSH system was of the same order of magnitude as that of glucose/Cyst at the concentration 1 M. Therefore, an equimolar (0.25 M) hexose/GSH mixture was adopted as standard for inhibition studies. In these experiments, the addition of MRPs (0–40 μl) to the reaction mixture did not cause changes in the pH of the model solutions.

As expected, inhibitory potency of both MRPs increased parallel to increasing the time of heating reactants at 90 °C from 2 to 39 h (Fig. 4). A highly significant reduction in PPO activity was already noticed after 2 h of heating, more particularly when glucose was heated with an equimolar amount of GSH. After heating for 39 h, 93 and more than 97% PPO activity was inhibited upon addition of 25 μl of glucose- and fructose-derived model systems, respectively. We had previously

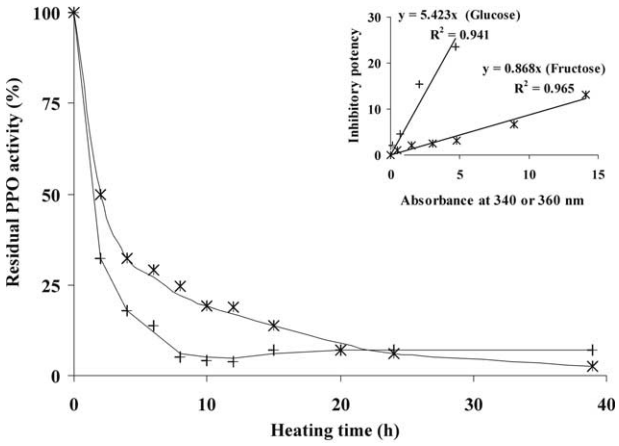

Fig. 4. Effect of MRPs derived from fructose/GSH (×–×) and glucose/GSH (+–+) aqueous model systems during heat treatment at 90 °C for different times (0–39 h) on apple PPO activity. PPO activity was measured by polarography at 30 °C, using 4-MC (20 mM) as the substrate (pH 4.5), 10 nkat PPO and 25 μl MRP solutions. It is expressed as % maximum activity measured without MRP in the reaction mixture. Inset : Relation between absorbance monitored at 340 (fructose/GSH, ×–×) or 360 (glucose/GSH, +–+) nm of aqueous model solutions heated at 90 °C for different times (0–20 h) and their inhibitory potencies (see text) on apple PPO activity.

observed a similar pattern with MRPs containing 1 M Cyst as the amino group. To investigate whether the antioxidant activity of MRPs did correlate with absorbance values taken at 340–360 nm (Table 3), used as indicator of heating intensity, inhibitory potency was plotted versus absorbance (Fig. 4, inset). For convenience, inhibitory potency was calculated from the expression $[(V_0/V_{MRP})-1]$, where V_0 = control activity and V_{MRP} = activity in the presence of MRPs. As a result, a linear positive relation between the increase in inhibitory potency of MRPs and the increase in absorbance recorded at 340 (fructose/GSH) or 360 (glucose/GSH) nm was obtained, the glucose/GSH system being the most reactive one. However, beyond 8 (glucose/GSH) and 20 (fructose/GSH) h of heat treatments, highly coloured compounds were formed but they were partly devoid of inhibitory potency. This may be related to a partial destruction of active compounds which could result from polymerization and/or insolubilisation reactions affecting these Maillard products. Thus, for these MRPs, the inhibitory effect may correlate with thermal product formation in the range 340–360 nm during short times of heating at 90 °C, which indicates that the method previously used to predict the inhibitory capability in MRPs, prepared with Cyst and heated at 90 °C (Billaud et al., 2003), is still suitable for use with GSH and allows the prediction of the inhibitory level of glucose or fructose/GSH model mixture by simple absorbance measurements at these selected wavelengths.

Another series of experiments was also carried out to determine efficiency of inhibition for MRPs heated for 2 h at various temperatures. This showed that the extent of inhibition increased with treatment temperature in the range 80–110 °C (Table 4), MRPs prepared from GSH exhibiting a much stronger effect than MRPs from Cyst, whatever the hexose tested. Once again, glucose led to production of MRPs which inhibited the enzyme more effectively than fructose.

3.2.3. Influence of the nature of the phenolic substrate on the level of PPO inhibition by MRPs

Chlorogenic acid and (+) catechin, which are the major phenolic compounds found in fruit and vegetables, are often used as substrates for the inhibition studies of PPO (Kermasha, Goetghebeur, Monfette, Metche, & Rovel, 1993; Oszmianski & Lee, 1990; Richard-Forget et al., 1992). Therefore, in our study, the inhibitory effect of MRPs was studied with chlorogenic acid, a hydroxycinnamic derivative, and (+) catechin, a flavan 3-ol compound, and compared with the *o*-diphenolic 4-MC (Table 5). The inhibition, exerted by MRPs on the rate of oxidation of these phenolic substrates by apple PPO, indicated that both MRPs inhibited enzyme per se. Whatever the substrate, the higher the concentration in the reaction medium, the lower the residual PPO activity, MRPs prepared from thiol with glucose containing globally more potent inhibitors than those derived from fructose. The same tendency was shown with thermally treated reaction mixtures of hexose with GSH, compared with Cyst. Apart from the fructose/Cyst model system, residual PPO activity recorded was lower when (+) catechin was the phenolic substrate.

It is largely accepted that polyphenol antioxidant ability varies greatly, depending on chemical structure

Table 4
Comparison of the inhibitory effect of MRPs prepared from fructose or glucose with Cyst (1 M/1 M) or GSH (0.25 M/0.25 M) aqueous solutions heated at rising temperatures (80–110 °C) for 2 h on apple PPO activity

Temperature (°C)	Residual PPO activity (%)[a]			
	Fructose-GSH	Fructose-Cyst	Glucose-GSH	Glucose-Cyst
80	70.3	95.6	72.1	94.9
90	42.8	87.6	38.4	83.5
95	27.4	50.6	13.3	33.3
100	9.2	34.8	3.6	11.6
105	7.5	16.9	1.3	5.6
110	2.4	6.7	0[b]	0[b]

Activity was measured by polarography at 30 °C, using 4-MC (20 mM) as the substrate, at pH 4.5, with 7 nkat PPO and 40 μl MRP (1.5 ml total volume).

[a] Expressed in % maximum PPO activity measured without MRPs in the reaction medium.

[b] No PPO activity was detected.

Table 5
Effect of selected phenolic substrates[a] on the rate of apple PPO inhibition by MRPs derived from fructose or glucose with glutathione (0.25 M/0.25 M) and fructose or glucose with cysteine (1 M/1 M) aqueous model solutions heated at 90 °C for 15 h

MRP volume (μl)	Residual PPO activity (% control)		
	4-MC	Chlorogenic acid	(+) catechin
Fructose/GSH			
2	40.0	39.0	53.8
25	3.3	4.5	11.1
Fructose/Cyst			
2	62.7	68.2	64.8
25	25.5	21.6	19.0
Glucose/GSH			
2	14.1	14.4	23.7
25	4.5	3.5	3.6
Glucose/Cyst			
2	38.0	43.5	40.2
25	4.9	3.3	5.5

Activity was measured by polarography at 30 °C and at pH 4.5, using 8 nkat PPO and 2 or 25 μl MRP. It was expressed as percent of the control without MRP.

[a] Final phenolic substrate concentrations in the reaction mixture were: 4-MC, 20 mM; chlorogenic acid, 10 mM; (+) catechin, 10 mM (according to the K_m values taken from Janovitz-Klapp et al., 1989).

and concentration (Lee & Lee, 1997; Natella, Nardini, Di Felice, & Scaccini, 1999; Nicoli, Calligaris, & Manzocco, 2000; Scott, Butler, Halliwell, & Aruoma, 1993). It was previously demonstrated, using (+) catechin-PPO or -tyrosinase in a model system, that the parent flavanol or flavanol-enzyme reaction products exhibited a high level of antioxidant activity, owing to their ability to donate a hydrogen atom and their free radical scavenging activity (Cheigh, Um, & Lee, 1995). In this study, it is therefore possible that they partially protect PPO by interacting with MRPs during the catalysis reaction.

4. Conclusions

Under the tested experimental conditions, and the initial concentration of reactants, soluble material derived from heated glucose or fructose/GSH mixtures displayed a higher inhibitory potency on apple PPO activity than that prepared from Cyst.

It is well known that the Maillard reaction is a cascade of consecutive and parallel reactions steps influenced by various critical process parameters. Among them, temperature and duration of heating are believed to play a crucial role. Reactivities of sugar and amino groups are also highly influenced by the pH, Aw, the nature and the concentration of the precursors, and the quantitative ratio of reducing sugar to amino compound. Consequently, a thorough study is still needed to better understand the effects of such variables on the course of the Maillard reaction in order to be able to apply this knowledge, subsequently, to the prediction and control of inhibitory compound generation in model systems containing thiol compounds. A comparison of the effects of MRPs prepared from the cysteine-containing tripeptide (γGlu-Cys-Gly) with those directly derived from cysteine would clarify any structure/activity relationships. We are currently working on this.

Moreover, investigation of the mechanism of apple PPO inhibition by these model MRPs is essential and will be discussed in the next paper.

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