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Effect of glutathione and Maillard reaction products prepared from glucose or fructose with glutathione on polyphenoloxidase from apple—II. Kinetic study and mechanism of inhibition

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Abstract

Maillard reaction products (MRPs) prepared from aqueous equimolar glucose or fructose with glutathione (GSH) model solutions (0.25 M), when heated at 90 °C for 15–39 h, were previously recognized as strong apple PPO inhibitors. This paper reports the inhibition mode of the purified *o*-diphenoloxidase activity from apple, using 4-methylcatechol as the substrate. Assuming a reversible inhibition, Lineweaver-Burk plotting showed that both MRPs were mixed-type inhibitors, glucose/GSH being the most efficient model system, with K_i values ranging between 11.6 and 1.1 μl MRPs, according to the heating treatment and the sugar tested. Enzyme inactivation took place during pre-incubation (0–180 min) of PPO with various MRPs at 0 °C, and was only partly reversed by exhaustive dialysis or gel filtration. Activity initially lost was also partly restored when cupric ions ($CuSO_4$) were added to the reaction medium, suggesting a chelating effect of copper ion at the active site of PPO, as already observed with cysteine-derived MRPs. MRPs derived from the tripeptide GSH were more potent inhibitors of apple PPO than those prepared from cysteine.

Keywords: Maillard reaction products; Glutathione; Apple PPO; Inhibition kinetic; Copper

1. Introduction

Polyphenoloxidase or PPO (EC 1.10.3.1) is a copper-containing oxidoreductase which is mainly responsible for browning of fruit and vegetables during post-harvest handling and processing. Research into antibrowning agents is still encouraged in order to replace sulfites which are, up to now, the most powerful and cheapest chemicals but are responsible for adverse effects in some asthmatics. In this regard, thiol derivatives have been reported to act as direct inactivation agents of PPO from various vegetal sources (Espin & Wichers, 2001; Motohashi, Nishikawa, & Mori, 1991; Richard-Forget, Cerny, Fayad, Saunier, & Varoquaux, 1998; Roux, Billaud, Maraschin, Brun-Mérimée, & Nicolas, 2003) and some MRPs have also been tested for their ability to reduce enzymatic browning (Nicoli, Elizalde, Pitotti, & Lerici, 1991; Pitotti, Nicoli, Sensidoni, & Lerici, 1990; Tan & Harris, 1995). Although undesirable effects may occasionally result from the Maillard reaction, it has very interesting potential because it involves natural precursors and is often used to generate desirable flavour as well as colour components, and yields a number of products exhibiting antioxidant properties towards compounds very susceptible to oxidation by oxygen in air, more particularly in lipid systems (Lingnert & Eriksson, 1980a, b). Moreover, some MRPs of model systems exhibit a metal binding ability (Bersuder, Hole, & Smith, 2001; Gomyo & Horikoshi, 1976; Rendleman, 1987; Wijewickreme & Kitts, 1998; Wijewickreme, Kitts, & Durance, 1997) and owing to the desmutagenic action and the scavenging activity (against reactive oxygen species) of other model MRPs, these effects are the subject of considerable research from the viewpoint of preservation of foodstuffs (Chuyen, 1998; Manzocco, Calligaris, Mastrocola, Nicoli, & Lerici, 2001; Morales & Babbel, 2002; Namiki, 1988).

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In a preceding work, we have shown that MRPs synthesized by reacting equimolar solutions of GSH with either an aldose sugar, D-glucose, or a ketose sugar, D-fructose (each 0.25 M), under various combinations of temperature and time of heat treatment were very potent inhibitors of enzymatic browning as well as of enzyme activity (Billaud, Brun-Mérimée, Louarme, & Nicolas, in press). Still, the mechanism of enzyme inhibition remains to be elucidated and compared with that obtained from cysteine-derived MRP, which is the purpose of the present study. Moreover, results will show whether they are also potential metal cation chelators and could bind copper at the active site of the enzyme.

2. Materials and methods

2.1. Materials

PPO from apple (var. Red Delicious) was prepared as previously described (Billaud, Roux, Brun-Mérimée, Maraschin, & Nicolas, 2003). All chemicals used were the purest available. Glutathione (GSH) reduced form, D-glucose, D-fructose and 4-methylcatechol (4-MC) were obtained from Sigma-Aldrich Chemical Co (St Louis, MO, USA). Chemicals used in the preparation of buffers, purification of apple PPO and copper sulfate pentahydrate were purchased from Merck-eurolab, Div. Prolabo (Fontenay S/Bois, France).

2.2. Preparation of Maillard reaction products (MRP) model systems

Glucose, fructose and GSH (each 0.25 M) were used to prepare different model aqueous solutions: GSH (thermal degradation products) and equimolar glucose/GSH or fructose/GSH (MRPs). Aliquots (1.5 ml) of these model solutions were placed without control of pH in Pyrex vials (2 ml) sealed and heated at 90 °C in an air convection oven (Memmert, ULE 400) for predetermined periods of time varying between 1 and 39 h. Afterwards, the vials were removed, immediately cooled in ice and directly used to determine their inhibition potency on PPO activity.

2.3. Enzyme determination

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 MRPs, 4-MC was varied from 1 to 20 mM in the control. Each inhibitor was added at three concentrations before incorporating in the enzyme extract. All determinations were conducted in duplicate and the averages reported. Kinetic parameters K_m and V_m were determined by using non-linear regression data analysis software developed for IBM PC (Leatherbarrow, 1987).

2.4. Pre-incubation of apple PPO with MRPs

To look into the mechanism of inhibition, the effect of the enzyme pre-incubation with the inhibitor, prior to the addition of the phenolic substrate, was also tested. Pre-incubation mixtures consisted of 0.9 ml of the buffered solution of purified PPO and 0.1 ml of different MRP model systems. The mixture was left at 0 °C for 0–180 min. At regular intervals of time, 100 μl aliquots, corresponding to 8 nkat incubated enzyme, were withdrawn and added to 1.4 ml of 20 mM 4-MC and the reaction monitored for oxygen uptake.

3. Results and discussion

3.1. Study of the inhibition kinetics of MRPs on PPO activity

The effect of concentration of MRPs (0–100 μl) on PPO was first determined, using model solutions heated at 90 °C for various heating times (6, 8, 15 and 39 h). It was seen that the longer the heating time, the stronger the inhibitory effect at a given concentration of MRPs present in the reaction vessel, as shown in Fig. 1 for 15 and 39 h. In the same way, the extent of inhibition increased, with increasing amounts of MRPs heated at a predetermined temperature, until it reached a plateau close to 60 and 6 μl respectively for 15 and 39 h of heating. Moreover, the inhibitory effectiveness of compounds formed from GSH with glucose (G-GSH) was higher than when MRPs were produced with fructose (F-GSH), as recently observed with L-cysteine-derived Maillard products (Roux et al., 2003).

These data imply that oxygen consumption velocity could not be driven to zero, in contrast with either pure competitive, non competitive or uncompetitive inhibitors. They also indicate that the nature of the thiol compound plays an important role in eliciting PPO inhibitory activity as, according to initial concentrations of reactants adopted in the model mixtures (1 M/1 M versus 0.25 M/0.25 M with L-cysteine and GSH-derived MRPs), GSH remains the most powerful agent to exert a high inhibitory potency in the MRPs.

The effect of equimolar sugar-GSH mixtures heated at 90 °C for 4–39 h on enzyme activity, assayed by polarography, was next studied by considering 4-MC as

the variable substrate, in order to get information about the kinetic behaviour of inhibitory properties of these MRP model systems. As illustrated by the Lineweaver-Burk double-reciprocal plots (Fig. 2), in the absence (control) or in the presence of variable amounts (1.0–5.0 μl) of MRPs heated for 15 h, curves intersected on the 1/[S] axis (Fig. 2A) and below the 1/[S] axis, at the left of the ordinate axis (Fig. 2B) with model systems prepared from fructose and glucose, respectively. According to this pattern and assuming a reversible inhibition, the former MRPs were non-competitive inhibitors with 4-MC since they did not affect the apparent K_m and decreased the apparent V_m. For their part, MRPs containing glucose appeared as a mixed-type inhibitor, with a decrease in apparent V_m and, conversely, an increase in apparent K_m. Curves associated with F-GSH mixtures heated at 90 °C for 4 and 8 h always displayed a pattern of non-competitive inhibition. After 39 h of heating, they showed a mixed-type inhibition, similar to the one observed for the G-GSH mixtures heated for 4, 8, 15 and 39 h (not shown). According to Segel (1975) and from rapid equilibrium assumptions, results demonstrated that MRPs exhibited a hyperbolic mixed inhibition. Corresponding equilibria and velocity equations for this type of inhibition were comparable to those recently reported when L-cysteine was the amino acid source (Roux et al., 2003). The inhibition constant values (apparent K_i, α and β), calculated from the secondary plots of $[(1/V_m \text{ app})-(1/V_m)]$ versus 1/[I] and $[(V_m \text{ app}/K_m \text{ app})-(V_m/K_m)]$ versus 1/[I], I representing the amount of inhibitor tested (μl) are given in Table 1. They were determined using non-linear regression by minimizing the sum of squares of the differences between experimental and theoretical values. It should be taken into consideration that the equilibrium constant for the inhibitor binding (K_i) parameter is expressed in μl for the analysis and comparison of both MRP model mixtures. As a result, their overall inhibitory potencies could be analyzed as reactants in a complex food matrix, as is often the case.

In summary, results obtained by the current kinetic study are consistent with those of the previous report when L-cysteine was the thiol compound tested, with K_i values which decreased with the duration of heating until 15 h at 90 °C. Likewise, when α values were close

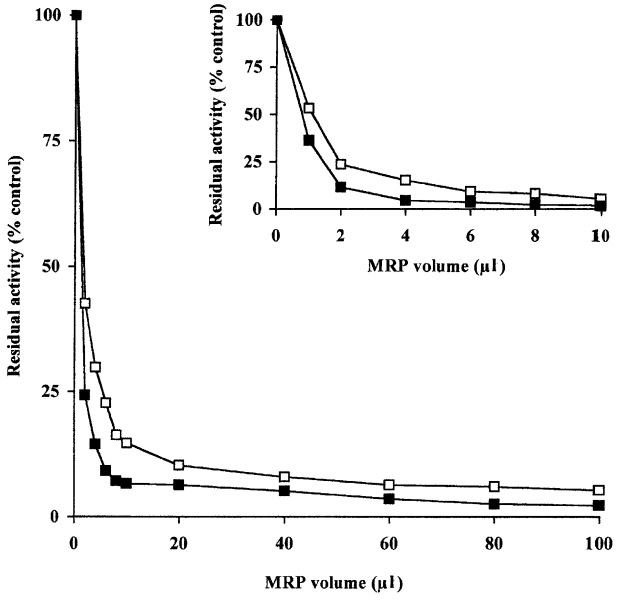

Fig. 1. Inhibitory effect of different amounts (0–100 μl) of MRPs derived from fructose/GSH (□—□) and glucose/GSH (■—■) aqueous model systems during heat treatments at 90 °C for 15 or 39 h (inset), on residual apple PPO activity. Activity was measured by polarography at 30 °C, using 4-MC (20 mM) as the substrate, 8 nkat PPO and different volumes of MRP solutions. It was expressed as a percentage maximum activity measured without MRPs in the reaction medium.

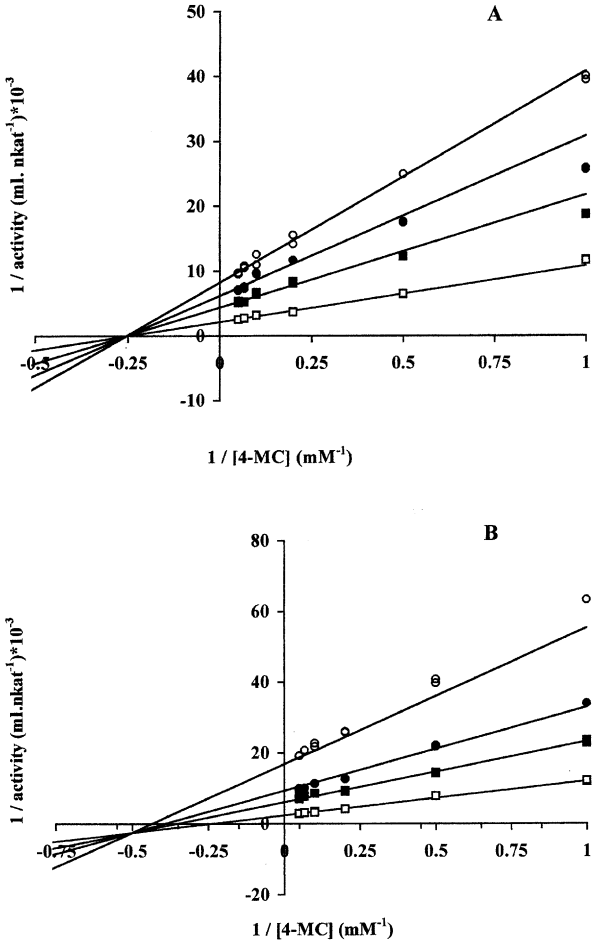

Fig. 2. Inhibitory effects of various amounts of MRPs derived from fructose/GSH (A) and glucose/GSH (B) aqueous model systems heated at 90 °C for 15 h on apple PPO (pH 4.5) at 30 °C with 4-MC (1–20 mM) as the substrate. Reciprocal plots of MRP inhibition on PPO activity was determined for 0 (□), 1.5 (■), 3.0 (•) and 5.0 (○) μl MRPs (A) and for 0 (□), 1.0 (■), 2.0 (•) and 5.0 (○) μl MRPs (B). Theoretical lines were determined by applying the velocity equation for a hyperbolic mixed-type inhibition, using kinetic constant values given in Table 1. PPO activity was followed by polarography using 10 nkat PPO.

Table 1
Evolution of the inhibition constant values (K_i, α and β) of MRPs (0.25 M/0.25 M aqueous solutions) derived from glucose/glutathione (G-GSH) and fructose/glutathione (F-GSH) mixtures heated at 90 °C for various lengths of time, acting on PPO from apple.

Time of heating (h)	K_i (μl)		α	β
	G-GSH	G-CYST[a]	G-GSH	
4	11.6	5.6	0.2	0
8	1.5	0.6	1.0	0
15	1.1	1.9	0.5	0.05
39	3.2	1.7	0.3	0.01
	F-GSH	F-CYST[a]	F-GSH	
4	6.3	22	1.1	0
8	4.6	2.2	1.0	0
15	1.2	2.6	0.9	0.1
39	1.8	3.4	0.4	0

The K_m value, using 4-MC (1–20 mM) as the substrate, was 7 mM and V_m value was on average 860 nkat ml^{-1}. These values were calculated from the assays performed by polarography in air-saturated McIlvaine's buffer at 30 °C and at pH 4.5, shown in Fig. 2.

[a] Values obtained by Roux et al. (2003) with MRPs (1 M/1 M) aqueous mixtures derived from glucose-cysteine (G-CYST) and fructose-cysteine (F-CYST), heated under the same conditions. For the sake of comparison and due to the differences in the initial concentrations for cysteine in sugar and thiol, the K_i values must be multiplied by 4 for comparison with those obtained with GSH in this study.

to 1 and β values adjacent to zero, a pure non-competitive inhibition was obtained, while when $\alpha = 1$ and $0 < \beta < 1$, a partial non-competitive inhibition was observed. After correction of initial concentrations of thiol and sugar (multiplied by 4), the K_i values obtained with cysteine by Roux et al. (2003) are much greater than those obtained with GSH under the same heating conditions. This illustrates that the inhibitory potency is higher with GSH-derived MRPs than those derived from cysteine.

3.2. Inhibitory effect of MRPs during pre-incubation experiments with purified PPO extract

Inactivation studies are usually carried out under two sets of assay conditions. In a first series of experiments, the reaction was started by adding the enzyme as the last component in the reaction medium, allowing us to check the continuous inhibitory effect of MRPs on PPO activity. In a second series of experiments, to check the possible direct inactivation of the enzyme by inhibitors, PPO was pre-incubated with MRPs obtained after heating mixtures at 90 °C for 4, 8, 15 or 39 h. The reaction was started by adding the phenolic substrate (20 mM) as the last component (pH 4.5). The rate of inactivation of apple PPO was determined by plotting the percent of remaining activity versus the pre-incubation time (Figs. 3 and 4). In the standard assay, in which water replaced MRPs, no significant loss in activity was recorded during the 180 min of pre-incubation (not shown). On addition (to both series) of MRPs, significant changes in oxygen uptake

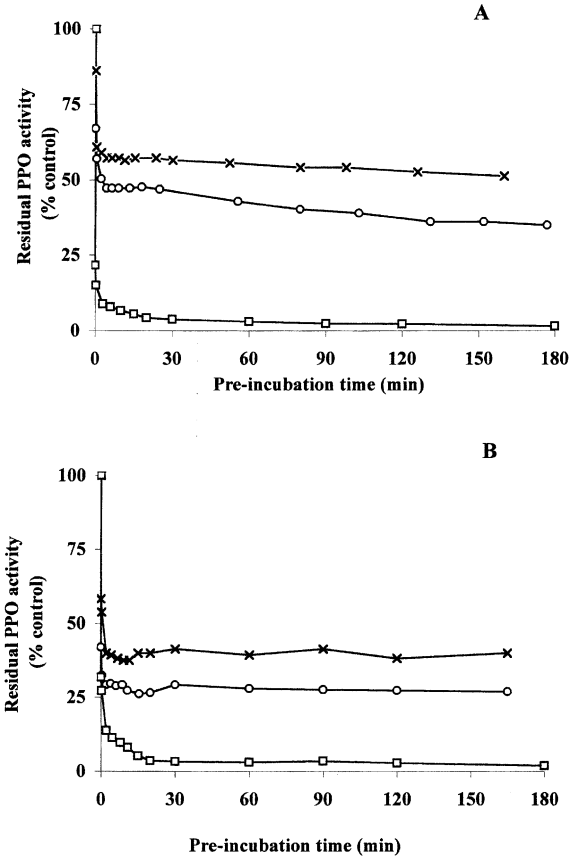

Fig. 3. Effect of MRPs prepared from fructose/GSH (A) and glucose/GSH (B) aqueous model systems (each 0.25 M), all heated at 90 °C for 4 h (×—×), 8 h (○–○) or 39 h (□—□) on the rate of apple PPO inactivation during a pre-incubation time varying from 0 to 180 min. In the assay medium, PPO (900 μl) was pre-incubated with MRPs (100 μl) at 0 °C. After pre-incubation for the appropriate time, PPO activity was measured.

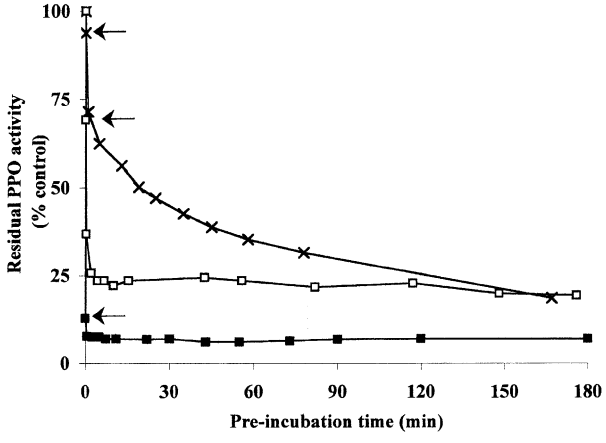

Fig. 4. Effect of 0.25 M GSH (×—×) and 0.25 M/0.25 M MRPs prepared from fructose/GSH (□—□) or glucose/GSH (■—■) aqueous model systems, all heated at 90 °C for 15 h, on the rate of apple PPO inactivation during a pre-incubation time varying from 0 to 180 min. Conditions of pre-incubation and activity measurements are the same as described in Fig. 3. Arrows indicate the t_0 pre-incubation time for each assay medium.

were recorded, attesting that PPO was inhibited per se. With increase in heating time, compounds present in the model systems became more active. With MRPs prepared from F-GSH (Fig. 3A), after heating model mixtures for 4 and 8 h, less than 55 and 39% of the initial activity was left, respectively, after 3 h of incubation. This trend was the same with MRPs derived from G-GSH (Fig. 3B), although to a greater extent, namely 38 and 28%. When heating time was prolonged to 39 h for both MRPs, a similar level of inactivation (<3% of the control activity left) was obtained. Furthermore, maximal inhibition was reached after only 4 (4 and 8 h-treated MRP) min and 20 (39 h-treated MRP) min of direct contact with PPO.

A more detailed pre-incubation study with extracts of GSH alone or contained in MRPs, all heated at 90 °C for 15 h was carried out (Fig. 4). The results showed that the direct addition of 100 µl extract to 900 µl PPO caused the inhibition of enzyme by nearly 6.5, 31 and 87%, respectively for GSH, F-GSH and G-GSH, as indicated by the arrows. The inhibitory activity, provoked by MRPs, was further increased by carrying on the incubation time until 4.5 (F-GSH) and 5 (G-GSH) min; afterwards it stabilized.

From this study, it also appears that, concomitantly with the loss of GSH during heating, thermally-formed degradation products, originating from the thiol compound, were responsible for a gradual decrease in PPO activity. Nevertheless, an inhibitory potency, comparable to that supplied by fructose-derived MRPs, was only reached after an incubation period longer than 150 min. As previously suggested with L-cysteine-derived MRPs, aqueous model systems could contain, in addition to intermediate or advanced Maillard products, some thermal degradation components originating from the thiol compound which would join in the global inhibitory effect recorded on PPO activity.

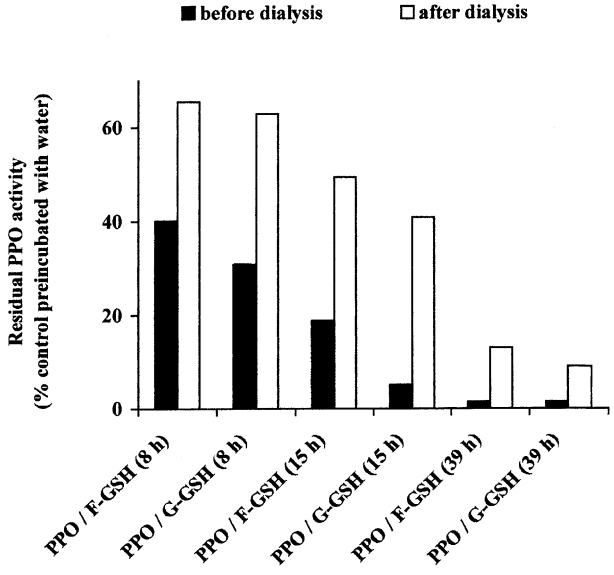

Fig. 5. Effect of dialysis performed at +4 °C on the restoration level of apple PPO activity following a 3 h pre-incubation time of PPO (900 µl) with MRPs derived from fructose/GSH (F-GSH) and glucose/GSH (G-GSH) aqueous model systems heated at 90 °C for 8, 15 or 39 h. PPO activity was measured by polarography at 30 °C and at pH 4.5, using 4-MC (20 mM) as the substrate and 8 nkat dialyzed PPO extract and expressed as a percentage maximum enzymatic activity of the control assay medium in which water (100 µl) replaced MRPs.

3.3. Effect of MRP removal from the inactivated apple PPO extract

With the aim of determining whether the enzyme activity could be recovered after removal of MRP model mixtures from the PPO solution, various pre-incubation conditions were selected, for which PPO activity had been partially or almost completely inhibited. The control samples and MRP-treated PPO preparations were then subjected to either 24 h dialysis against two changes of phosphate buffer (pH 6.5) or gel filtration chromatography on Sephadex G-25M (PD10 mini-columns, Pharmacia). With these two similar approaches, the restoration of enzymatic activity was attributed to removal of molecules with MM <12 kDa from the pre-incubation medium. Results were expressed as residual PPO activity, in % control pre-incubated with water (Fig. 5). It appears that PPO activity was only partly restored after being initially repressed, whatever the MRP model system selected. Remaining PPO activities, left after 3 h of incubation in the presence of MRPs were, as already noted, higher with those prepared from F-GSH than those coming from G-GSH, averaging 30–40 (8 h heating), 5–19 (15 h heating) and 1.5 (39 h heating) % of control activity. Following exhaustive dialysis, there was from 7.5 to 36% increase in activity, the regained activity being less when model systems were heated for 39 h at 90 °C.

These data indirectly suggested that, in agreement with our interpretation concerning MRPs prepared from L-cysteine, only part of MRPs did not bind irreversibly to the enzyme according to a chelating effect of these compounds or possibly that the metalloenzyme-chelator complex was not totally dissociable.

3.4. Effect of Cu^{2+} ions, added as $CuSO_4$, on restoration of PPO activity

Since apple PPO contains copper as a functional group (Zawistowski, Biliaderis, & Eskin, 1991) and inactivation by MRPs could be related to interaction with the metal cation (Nicoli et al., 1991; Roux et al., 2003; Tan & Harris, 1995), a last experiment was carried out to assess the possible reversion of enzymatic activity inhibition by MRPs after addition of free Cu^{2+} ions to the reaction medium, as previously shown with PPO from various sources (Kahn & Andrawis, 1985; Satô, 1980; Son, Moon, & Lee, 2000; Watanabe, Hiraoka,

Masada, & Satô, 1991). Thus, 10 μl $CuSO_4$ solutions (0–3 mM final concentration) were added before addition of 10 μl MRP to the phenolic substrate (1.4 ml). 9 nkat of PPO were introduced last before recording O_2 consumption. As seen in Fig. 6, when residual PPO activity was plotted versus $CuSO_4$ concentration, the extent of reactivation of the enzyme was dependent on Cu^{2+} concentration, in a non-linear fashion, i.e. most of the activity being recovered at ca. 1.5 mM copper sulfate, when model systems were heated for 2–24 h. It was also observed, from the control curves, that added copper sulfate alone did not inhibit *o*-diphenolase activity of PPO while, in the absence of Cu^{2+} ions, supply of the unheated model system hexose/GSH provoked a recovery of 86–88% of the original activity, attesting to a slight inhibitory effect of thiol and/or sugar reactant on PPO. In this last case, no restoration of activity was shown when $CuSO_4$ was varied from 0.075 to 3 mM. These experiments showed that a brief contact between MRPs and copper sulfate, prior to the addition of the enzymatic extract, reduced the inhibitory effect of MRPs on apple PPO. Moreover, at higher concentrations of copper sulfate, it appeared that, globally, the higher the heating treatment of model mixtures, the lower was the maximal reversal of the inhibitory effect of 10 μl MRPs.

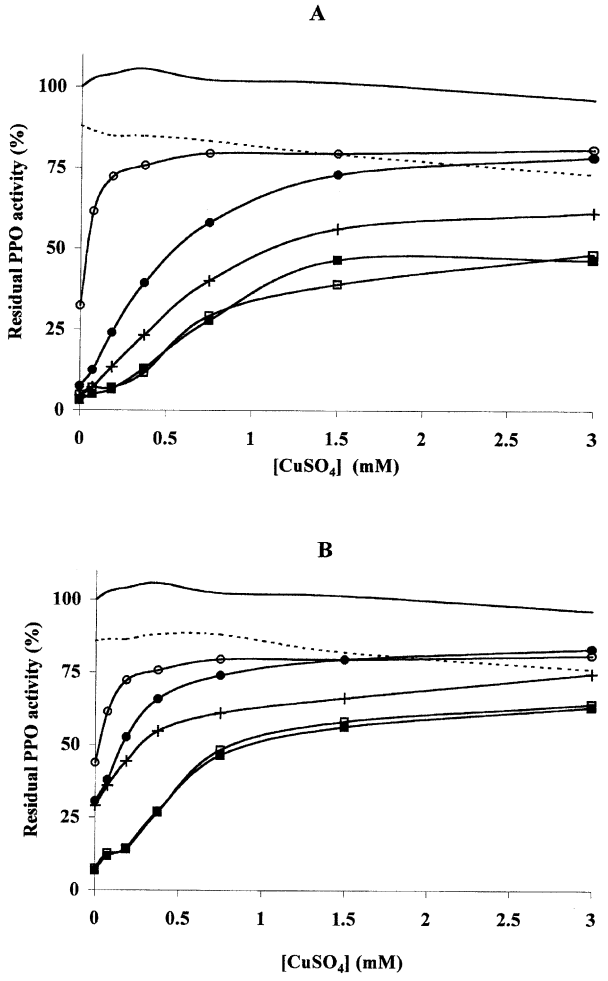

Fig. 6. Relationship between the copper concentration (added as 0–3 mM $CuSO_4$, final concentration) and restoration of the inactivated apple PPO following addition of MRPs derived from glucose/GSH (A) and fructose/GSH (B) aqueous model systems heated at 90 °C for 2 (○—○), 6 (●—●), 8 (+—+), 19 (■—■) or 24 (□—□) h in the reaction medium. PPO activity was measured by polarography at 30 °C and at pH 4.5, using 4-MC (20 mM) as the substrate, 10 μl $CuSO_4$, 10 μl MRPs and 9 nkat PPO (1.5 ml total volume) and expressed in % maximum enzymatic activity of the control assay medium without MRPs. Control curves showing the direct effect of increasing concentrations of $CuSO_4$, either with 10 μl water (—) or 10 μl unheated sugar with GSH (each 0.25 M, - - - -), on PPO activity.

Taken as a whole, these results can be explained, at least partly, by a chelating effect on the copper present in PPO by some MRPs generated in our model systems. A reasonable assumption is that the Cu^{2+}-binding ability of MRPs can affect the availability of the metal cation during the enzymatic catalysis of 4-MC. Accordingly, depending on its concentration in the reaction medium, copper sulfate could compete with copper, from the active site of the oxidoreductase, for active components present in these MRPs.

The effect of various MRP fractions (reductones, premelanoidins and/or melanoidins) on the interaction with polyvalent divalent cations has already been the subject of a number of works (Asakura, Nakamura, Inoue, Murata, & Homma, 1990; Friedman, 1996; Homma, Aida, & Fujimaki, 1986; Moon, Murata, & Homma, 1994; O'Brien & Morrissey, 1997; Wijewickreme, Kitts, & Durance, 1997). Nevertheless, the nature of the putative binding sites on model MRPs is still unknown. Most likely, they are free carbonyl groups of compounds formed which possess acidic properties and are probably associated with thiol amino acid moieties of MRP. Indeed, Rendleman and Inglett (1990) have reported that Cu^{2+} ions have the ability to form strong complexes with various model melanoidins from glucose-glycine mixtures, two H^+ ions being generally released from each Cu^{2+} ion that became bound. This would explain, at least partly, slight differences established in the inhibitory potency of MRPs derived from hexose with either L-cysteine or GSH on PPO activity, the tripeptide containing, in addition to the functional sulfhydryl group, an additional carboxyl group in its structure.

4. Conclusions

In this study, we have shown that MRPs containing GSH (and cysteine as well) behave like mixed-type inhibitors whose action is partly reversible and leads to a strong inactivation of apple PPO, possibly by chelating the copper ion at the active site of the enzyme.

Further investigations of the structures of inhibitory products and the mechanisms of formation by the Maillard reactions are still required. In this way, by optimizing processing conditions, it may be possible to enhance the formation of antioxidant MRPs, thus

maintaining the overall inhibitory potency against oxidoreductase activity.

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