JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Use of Experimental Design Methodology To Prepare Maillard Reaction Products from Glucose and Cysteine Inhibitors of Polyphenol Oxidase from Eggplant (*Solanum melongena*)

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Polyphenol oxidase (PPO) from eggplant was extracted and partially purified by a two-step fractionation−precipitation using ammonium sulfate and phenylsepharose hydrophobic interaction chromatography. The eggplant PPO extract was characterized concerning its kinetic properties. Optimal conditions to obtain Maillard reaction products (MRPs) with a maximal inhibitory potency (IP) toward PPO activity were determined using the surface response methodology and a four-factor and five-level experimental design. The MRPs were prepared from cysteine (0.25 M) and glucose (0−1 M), at several initial pH values (2−6) and at differing heating times (3−19 h) and temperatures (95−115 °C). The maximal IP was obtained after heating a model system of glucose/cysteine (1/0.25 M) at pH 2 for 3 h 20 min at 115 °C. The soluble part of this MRP, called MRP_{IPmax}, was a noncompetitive inhibitor toward eggplant PPO. The IP of MRP_{IPmax} on PPO activity was very potent as compared to that displayed by benzoic, *p*-coumaric, and *t*-cinnamic acids, as well as sorbic acid and 4-hexylresorcinol. The activity of preincubated PPO at 0 °C with MRP_{IPmax} was only slightly restored after dialysis or gel filtration.

KEYWORDS: Maillard reaction products; cysteine; eggplant; polyphenol oxidase; enzymatic browning

INTRODUCTION

To promote fruit and vegetable consumption, food manufacturers are taking up the challenge of proposing them in ready-to-eat packages. Therefore, they have to cope with enzymatic browning due to endogenous phenolic compound oxidation catalyzed by polyphenol oxidase (PPO, EC 1.10.3.1) in the presence of oxygen. In addition to spoiling the products by shortening the shelf life of fresh-cut fruits and vegetables (*1*), enzymatic browning also results in a loss of their nutritional and visual qualities (*2*). Eggplant is rich in phenolic compounds (*3*) showing antimutagenic activities (*4*) and high oxygen radical scavenging capacity. It is also particularly sensitive to this browning especially after being cut up into slices for industrial ready-to-cook ratatouille meals.

Current means of controlling enzymatic browning in processed fruits and vegetables are based on the use of various physical or chemical methods (*5*). Although enzymatic browning is successfully controlled by sulfites and analogues, the Food and Drug Administration has banned its use in several fresh fruits and vegetables (*6*) due to their harmful effects on health (*7*). Thus, finding alternative solutions to prevent enzymatic browning is a challenge for food scientists in their effort to develop natural and nontoxic antioxidants.

Maillard reaction products (MRPs) exhibit free radical scavenging actions (*8*, *9*), metal ion chelating properties, and chain breaking and oxygen scavenging (*10*−*12*) and reducing activities (*13*, *14*). Among these antioxidant properties, specific MRPs also prove to be capable of preventing enzymatic browning (*15*, *16*). The Maillard reaction is a complex set of reactions between amino and carbonyl compounds producing a wide range of intermediate products and finally brown pigments (melanoidins) (*17*). The nonenzymatic browning is influenced by many factors, including reactant concentration, temperature, time, initial pH, and water activity as well as the nature and the ratio of the reactants (*18*, *19*). It has recently been shown that MRPs from glucose and sulfhydryl amino compounds (L-cysteine or glutathione) have an effective inhibitory effect on apple PPO (*15*). The purpose of the present work was to investigate the effects of time, heating temperature, pH, and glucose concentration and their interactions on the inhibition properties of MRPs on eggplant PPO. In this respect, a four-factor and five-level central composite experimental design was used to determine experimental conditions of MRP preparation and obtain potent inhibitors toward a partially purified PPO from eggplant. In addition, some kinetic properties of this PPO have been studied as well as the effect of classical chemical inhibitors.

MATERIALS AND METHODS

Materials. Purple eggplant fruits (*Solanum melongena*) were purchased at maturity from the local market and used as the PPO source. All chemicals used were the purest available. The PPO substrates [4-methylcatechol (4-MC), (+)-catechin, (−)-epicatechin, pyrocatechol,

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10.1021/jf060228r CCC: $33.50
Published on Web 06/03/2006

Table 1. Independent Variables and Their Coded and Actual Values Used in the Experimental Design

			coded levels				
independent variable	units	symbols	−2	−1	0	1	2
heating time	h	X_1	3	7	11	15	19
temperature	°C	X_2	95	100	105	110	115
glucose concentration	M	X_3	0.2	0.4	0.6	0.8	1
initial pH		X_4	2	3	4	5	6

pyrogallol, tyrosine, *p*-cresol, *p*-phenylenediamine, and chlorogenic, caffeic, hydrocaffeic, dihydroxyphenylacetic, and protocatechuic acids] and the inhibitors (4-hexylresorcinol and benzoic, *t*-cinnamic, *p*-coumaric, and sorbic acids), L-cysteine (free base), ascorbic acid, polyvinylpolypyrrolidone (PVPP), D-glucose, and 5,5′-dithio-bis(2-nitrobenzoic) acid (DTNB) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Other chemicals were purchased from VWR International−Merck-Eurolab (Fontenay s/bois, France). The water used was purified using Analyst HP purification Purite Select limited (Oxon, England).

Preparation of MRPs Using the Experimental Design. MRPs were obtained by heating aqueous solutions (initial pH ranging from 2 to 6) of cysteine (0.25 M) and glucose (0−1 M) in an air convection oven (Memmert ULE 400) for 3−19 h at temperatures ranging from 95 to 115 °C. Once the initial pH of the model systems had been adjusted using either phosphoric acid (1 M) or sodium hydroxide (1 M), aliquots of solutions were placed in Pyrex vials sealed with silicone−Teflon septa and metallic caps. After they were heated, the vials were cooled in ice and the soluble part of the mixtures was used to determine both their remaining thiol content and their inhibitory properties. The inhibitory potency (IP) on eggplant PPO activity using 10 μL of 10-fold dilutions of MRPs (equivalent to 1 μL) was calculated according to the following formula:

$$\mathrm{IP} = \frac{100}{\text{residual activity}} - 1$$

A four-factor fractional factorial design (central composite face design) with five central points and six star points was developed according to the principles of the response surface methodology (*20*). Thirty experimental runs were carried out to evaluate both first- and second-order effects of the experimental factors (X_1, time of heating; X_2, temperature of heating; X_3, initial glucose concentration; and X_4, initial pH level) on the dependent variables (experimental responses, IP and residual thiol concentrations). The initial experimental conditions used in the preparation of model MRP mixtures are given in **Table 1**. An assay without glucose was added, the reduced centered coordinates of which were $X_1 = X_2 = 0$, $X_3 = -3$, and $X_4 = 0$. The effects of unexplained variability in the observed responses due to extraneous factors were minimized by randomizing the order of the experiments.

The response surface regression procedure and data analysis were performed using Statgraphic Plus 5.1 software (Statistical Graphics Corp.). Within the limits of the experimental domain, the generalized second-order polynomial model used in the response surface analysis was as follows:

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum \sum_{i<j=1}^{4} \beta_{ii} X_i X_j \qquad (1)$$

where Y is the estimated response; β_0, β_i, β_{ij}, and β_{ii} are the regression coefficients for intercept, linear, interactive, and quadratic effects, respectively; and X_i and X_j are the independent variables.

Results reported in this part are the average of at least three measurements, and the average of data was considered. The significance level for the process variables considered was $P \leq 0.05$.

Total Free Thiol Amount Determination. The amount of total free thiol compounds was determined in alkaline medium (*21*) with an excess of DTNB by adding 0.2 mL of diluted sample (1/100 or 1/200) to 0.5 mL of 10 mM DTNB solution—prepared in 0.1 M phosphate buffer (pH 7.5)—and 4.5 mL of Tris-HCl buffer solution (0.2 M, pH 8.2). After 15 min, the absorbance at 412 nm was measured. The thiol content was calculated with the molar extinction coefficient ($\epsilon = 13500$ M^{-1} cm^{-1}) previously determined on cysteine.

Enzyme Activity and Protein Determination. The PPO activity was measured by polarography at 30 °C in a 1.5 mL reaction cell containing 4-MC (20 mM in air-saturated Mac-Ilvaine buffer, pH 5.0) as the substrate. The reaction rate was calculated from the initial slope of the progress curve giving oxygen uptake vs time using a Clark oxygen microprobe. The activity was expressed as nmoles of oxygen consumed per second (nkat) under the assay conditions. For inhibition studies with cysteine or MRPs, aliquots (10−100 μL) of either diluted or nondiluted solutions were added to the substrate solution just before the enzyme extract. Except for the preincubation experiments, all determinations were performed at least in triplicate and averages of data were considered. The protein content was determined according to the dye-binding method of Bradford (*22*) with bovine serum albumin as the standard.

Extraction and Partial Purification of Eggplant PPO. All steps were carried out at 4 °C. After they were peeled, the eggplants (100 g) were quickly cut into small pieces and homogenized three times for 30 s, using an Ultra-Turrax T 25 (IKA Labortechnik) with 250 mL of 0.1 M phosphate buffer (pH 7.0) containing ascorbic acid (10 mM), cysteine (2.5 mM), and PVPP (12.5% w/w). After centrifugation at 37800*g* for 30 min (Beckman, model J2-21), the supernatant was filtered to obtain the crude extract. The enzyme solution was fractionated with solid ammonium sulfate (20−70% saturation). The precipitated PPO was collected by centrifugation, and the pellet was dissolved in 80 mL of 0.1 M phosphate buffer (pH 7.0) containing 0.6 M in ammonium sulfate (buffer A). This last fraction, called P_{20-70}, was then dialyzed (Spectra/Por 4: 12000−14000 Da, Fischer Bioblock, Illkirch, France) overnight at 4 °C against buffer A to give the P_{20-70D} fraction. This fraction was applied on to a Phenyl-Sepharose CL-4B column (2 cm × 20 cm, Pharmacia, Uppsala, Swedden) preequilibrated with buffer A at a flow rate of 100 mL h^{-1}. The column was then washed with two column volumes of buffer A. The PPO was eluted by two column volumes of water/ethylene glycol (EG) (95/5 v/v), and the gel was regenerated by washing with water/EG (50/50 v/v). Fractions of 6 mL were collected and assayed for PPO activity and absorbance at 280 nm.

Kinetic Properties of Eggplant PPO. The enzyme activity toward 4-MC (20 mM) and chlorogenic acid (5 mM) was determined between pH 3 and pH 8 using MacIlvaine (pH 3−6) and 0.1 M phosphate buffer (pH 6−8).

The Michaelis constants (K_m) and maximum velocities (V_m) toward several phenolic compounds as well as the apparent inhibition constants (K_{iapp}) of several carboxylic acids, sorbic acid, and 4-hexylresorcinol were determined at pH 5.0 using GraFit 4.0 Data Analysis software. Each inhibitor was added before the enzyme extract.

Preincubation of Eggplant PPO with MRP_{IPmax}. The effect of the enzyme preincubation with the inhibitor, prior to the addition of the phenolic substrate, was tested according to Brun-Mérimée et al. (*23*). Preincubation mixtures (90/10 v/v) containing eggplant PPO with either water (control), MRP_{IPmax}, cysteine (0.25 M), or heated cysteine (0.25 M at pH 2 and 115 °C for 3 h 20 min) were left at 0 °C for 0−180 min. At regular intervals of time, aliquots (20 μL) were withdrawn and added to 1.4 mL of 20 mM 4-MC and the reaction was monitored for oxygen uptake. Ten nanomoles of oxygen consumed per second was systematically added in the control assays.

RESULTS AND DISCUSSION

Enzyme Extraction and Partial Purification. The effects of several additives in the medium on the PPO activity extraction were studied. Eggplant shows a high level of phenolic compounds that can interfere with the extraction of the enzyme. By selectively adsorbing some phenols, the PVPP prevents their complexation with proteins (*24*) and their oxidation to quinones. The latter can covalently link to proteins and irreversibly inhibit enzymatic activity (*25*). According to preliminary experiments,

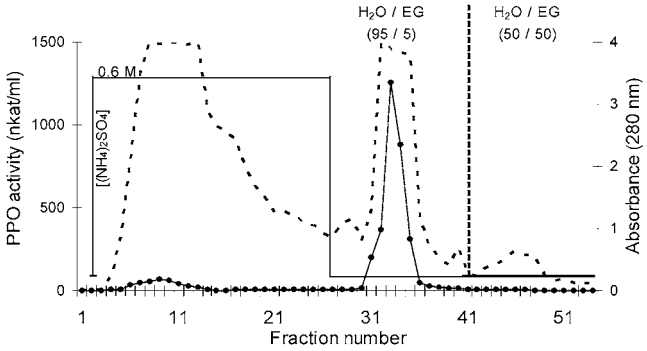

Figure 1. Elution profiles of enzyme activity (●) and absorbance at 280 nm (- - -) of eggplant PPO by HIC on Phenyl Sepharose CL4B. PPO activity was measured by polarography using air-saturated 4-MC (30 °C, 20 mM, and pH 5.0) as the substrate.

Table 2. Purification of Eggplant PPO

purification step	volume (mL)	activity (μkat)	protein (mg)	specific activity (μkat mg^{-1})	yield (%)	purification (fold)
crude extract	275	91.3	192	0.48	100	
S_{20}	275	74.5	168	0.44	81.6	0.9
P_{20-70D}	68	69.1	99	0.70	75.7	1.5
most active fraction (F33)	6	20.2	18	1.13	22.1	2.4
pooled active fractions	50	40.3	88	0.46	44.1	1.0

the PVPP concentration was fixed to 12.5% to provide optimal stability to the crude extract. In the same way, ascorbic acid (10 mM) was used to reduce quinones and avoid their polymerization responsible for the enzymatic inactivation (*26*). Cysteine (2.5 mM) was also added to the extraction medium to form colorless cysteine−quinone adducts (*27*).

Adding Triton X-100 to the medium extract did not increase the extraction yield of eggplant PPO, meaning that the enzyme was readily soluble in the extraction solution. The effect of ammonium sulfate saturation concentrations on eggplant PPO solubility was studied in a range of 10−80% saturation (data not shown). Up to 20% saturation (S_{20}), more than 90% of PPO activity remained in the supernatant. Thereafter, 90% of the initial PPO activity precipitated with 70% saturation in ammonium sulfate. Therefore, a two-step fractionation (20−70%) with ammonium sulfate made it possible to eliminate close to 40% of the total protein while saving close to 80% of the PPO activity. The pellet was dissolved in buffer A and then dialyzed against this buffer.

Ion exchange chromatography on DEAE cellulose has previously been associated with gel filtration on Sephadex G-100 to purify eggplant PPO (*28*, *29*), but hydrophobic interaction chromatography (HIC) has never been used. **Figure 1** shows the elution pattern of the enzyme activity and the absorbance at 280 nm on the Phenyl-Sepharose CL4B gel. The bulk of PPO activity was eluted with water/ethylene glycol (95/5 v/v). The active fractions (33 and 34) were pooled and used for further experiments. They remained stable at −20 °C for more than 9 months. As compared with PPO from other fruits or vegetables, PPO from eggplant seems to be particularly hydrophobic, similar to the PPO from endive core (*30*).

The results of the purification process (**Table 2**) showed that up to the dialysis step, the yield remained higher than 75% and then fell to 44% for the pooled active fractions after the HIC step. Further experiments carried out with less hydrophobic supports such as TSK Toyopearl-butyl 650 M (Supelco, Tosoh) and Pentyl-Agarose (Sigma) did not result in better separation.

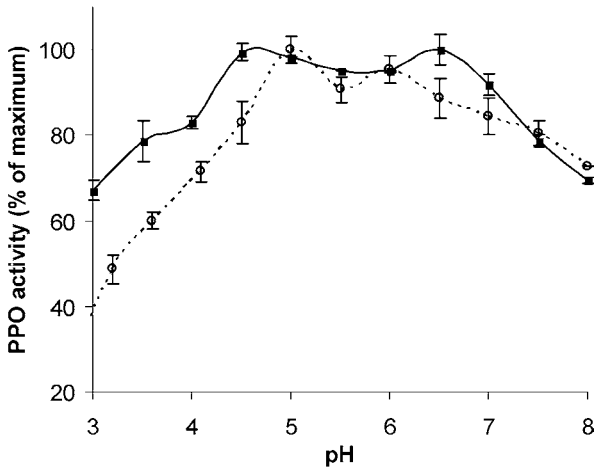

Figure 2. Effect of pH on PPO activity. PPO activity was measured by polarography at 30 °C using air-saturated 4-MC (20 mM, ■) or chlorogenic acid (5 mM, ○) as the substrates in the presence of 10 nkat PPO. PPO activity was expressed as a percentage of the maximum activity measured for both substrates.

Isoelectrofocalization was conducted on Phastsystem apparatus (Pharmacia) with Phastgels (IEF 3-10, Amersham Biosciences). Specific revelation (4-MC) and the Coomassie dye-binding method showed at least two isoforms (pH 5.7−6.0 and pH 6.8−7.1) in the HIC extract (not shown). Although not very efficient, this process made it possible to eliminate nonprotein material and provided a partially purified but highly stable PPO that expresses the global enzyme activity located in eggplant.

Enzyme Characterization. The PPO extracted from eggplant showed a broad range of maximum activity between pH 4.5 and pH 6.5 using 4-MC (20 mM) as the substrate and between pH 4.8 and pH 6 using chlorogenic acid (5 mM) as the substrate. According to **Figure 2**, the PPO activity was 70 (4-MC) and 40% (chlorogenic acid) of its optima at pH 3 whereas it was 60% of its optima at pH 8. These results are in partial agreement with those of Fujita and Tono (*29*) who found a maximum activity at pH 4.0 using a spectrophotometric method at 420 nm with chlorogenic acid (1 mM). Conversely, Shatta and El-Shamei (*31*) found a maximal eggplant PPO activity toward catechol at pH 7.0 in phosphate buffer solution. Moreover, Pérez-Gilabert and García-Carmona (*26*) showed that the eggplant catecholase activity at pH 4 and pH 9 was close to 70% of its maximum value found at pH 5.5 with *tert*-butylcatechol as the substrate. In addition, Dogan et al. (*32*) indicated that the pH for maximum eggplant PPO activity is dependent on the cultivars.

Chlorogenic acid, the main hydroxycinnamic derivative present in eggplant (*3*), was the most efficient substrate at pH 5.0 (**Table 3**). This result is consistent with other works on eggplant PPO (*29*). Caffeic acid presented a good efficiency due to its strong affinity toward the enzyme (K_m = 0.80 mM). According to our results, PPO from eggplant shows a strong stereospecificity toward the two flavan-3-ols diastereoisomers. The affinity of eggplant PPO is six times higher for (−)-epicatechin than for (+)-catechin. Moreover, no detectable activity was observed with monophenols such as tyrosine and *p*-cresol or with pyrogallol and *p*-phenylenediamine. These results are in agreement with those of Dogan et al. (*32*) who indicated that eggplant PPO is a diphenol oxidase.

The benzoic, *t*-cinnamic, *p*-coumaric, and sorbic acids as well as the 4-hexylresorcinol were competitive inhibitors of eggplant PPO (**Table 4**). According to the inhibition constants (K_{iapp}) at

Table 3. Substrate Specificity of Eggplant PPO at pH 5.0

substrate	K_m (mM)	V_m (% 4-MC)	V_m/K_m
4-MC	5.5	100	18
chlorogenic acid	1.2	82	68
dihydroxyphenylacetic acid	18	70	3.9
pyrocatechol	19	46	2.4
hydrocaffeic acid	3.7	41	11
(–)-epicatechin	0.70	34	49
(+)-catechin	4.3	26	6.0
caffeic acid	0.80	23	29
pyrogallol	ND[a]		
protocatechuic acid	ND		
tyrosine	ND		
p-cresol	ND		
p-phenylenediamine	ND		

[a] ND, not detectable.

Table 4. Inhibition of Eggplant PPO Activity by Various Compounds Using 4-MC (1.25–20 mM) as the Substrate

compound	pH	K_{iapp} (mM)
benzoic acid	4	0.94
benzoic acid	5	0.83
t-cinnamic acid	5	0.11
p-coumaric acid	5	0.26
sorbic acid	5	6.7
4-hexylresorcinol	5	1.5

pH 5, the *t*-cinnamic and *p*-coumaric acids are the most efficient inhibitors. These results are in agreement with those found with the apple PPO (*33*). Some differences, however, appear between the two enzymes. For instance, for eggplant PPO, the inhibition properties of benzoic acid ($pK_a = 4.18$) were almost unaffected by a decrease in pH in the reaction medium (pH 4), whereas for apple PPO the same drop in pH value resulted in a large decrease in the K_{iapp} value. This phenomenon means that for apple PPO, the benzoic acid is a much better inhibitor than the benzoate anion whereas for eggplant PPO, benzoic acid and benzoate anion are almost equivalent competitive inhibitors.

Concerning 4-hexylresorcinol, which has been patented for inhibiting the enzymatic browning of shrimp (*34*), it appeared less efficient with eggplant PPO at pH 5 ($K_{iapp} = 1.5$ mM) than with endive PPO ($K_{iapp,pH4} = 0.56$ mM) or mushroom tyrosinase ($K_{iapp} = 0.004$ mM) at pH 4.5 (*35*). Pérez-Gilabert and García-Carmona (*26*) using *tert*-butylcatechol at pH 5.5 as the substrate reported a K_{iapp} of 0.50 μM toward PPO catecholase activity determined spectrophotometrically at 400 nm.

Study of the MRP_{IPmax}. The experimental design was set up to study and model the effect of initial pH, glucose concentration, heating temperature, and time on the formation of inhibitory MRP derived from glucose and cysteine (0.25 M) as well as on the residual thiol levels. The values of the regression coefficients obtained according to (eq 1) were expressed for actual variables (**Table 5**). Residual errors vs predicted values were randomly distributed for both variables.

The Ellman reagent was used to determine the total soluble free thiol compound amount during the Maillard reaction. For this response, the linear effects of the glucose concentration, the heating temperature, and the time were significant as well as the quadratic effect of the initial pH. All of these factors had negative effects on the residual thiol concentration. According to this experimental design, the minimum level of residual thiol was obtained for a low glucose concentration (0.17 M), a high initial pH level (pH 6.0), and extensive heating conditions (115 °C for 19 h). These results were consistent with prior studies on the effects of time, temperature, glucose concentration, and initial pH on the intensity of the Maillard reaction (*13*, *19*).

Table 5. Regression Coefficients with Actual Values According to Eq 1 for the Response Model of the Residual Thiol Concentration and the IP[a]

factors	[thiol compounds] (mM)	IP
β_0	–3.44	–239
β_1 (time in h)	**0.00705**	14.9
β_2 (temperature in °C)	**0.086**	**3.03**
β_3 ([glucose] in M)	**–1.35**	**–208**
β_4 (initial pH)	0.0438	**–15.2**
β_{11} (time^2)	–0.000145	–0.0675
β_{12} (time × temperature)	–0.000156	**–0.125**
β_{13} (time × [glucose])	0.00234	–0.633
β_{14} (time × initial pH)	0.000781	0.0170
β_{22} (temperature^2)	–0.000493	–0.00908
β_{23} (temperature × [glucose])	**–0.0188**	**2.44**
β_{24} (temperature × initial pH)	0.000625	–0.130
β_{33} ([glucose]^2)	–0.133	20.9
β_{34} ([glucose] × initial pH)	0.0406	–9.87
β_{44} (pH^2)	**–0.0173**	0.238

[a] In bold are regression coefficients for factors with significant effects at $P \leq 0.05$.

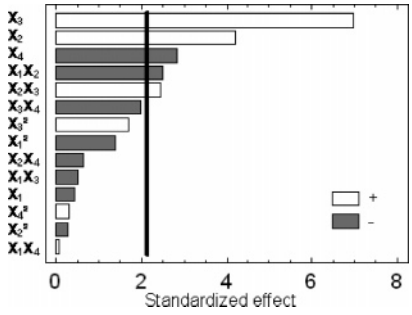

Figure 3. Pareto chart for IP representing the standardized effects of the experimental factors. X_1, time of heating; X_2, temperature of heating; X_3, initial glucose concentration; and X_4, initial pH level. Positive effects are in white, and the line indicates the confidence level of 95% (i.e., $P = 0.05$). Factors with standardized effect values to the right of this line are statistically significant.

According to the standardized Pareto chart for IP resulting from the variance analysis (**Figure 3**), the linear effects of the glucose concentration (X_3), temperature (X_2), and initial pH (X_4) and the interactions between the time and the temperature of heating (X_1 X_2) and the temperature and the glucose concentration (X_2 X_3) were significant at the statistical level of 5%. The model fitted with the observations with a R^2 of 86% and led to an optimized MRP when a model system of glucose/cysteine (1/0.25 M) at pH 2 was for 3 h 20 min at 115 °C. This MRP called MRP_{IPmax} was at the limit of the experimental domain but had been fixed according to experimental constraints. Moreover, no experimental parameters had a significant quadratic effect (X_i^2), explaining the maximal conditions obtained by the model. It should also be noted that heating time had no significant linear (X_1) or quadratic effect (X_1^2), indicating that, in our study, the temperature was the main parameter to take into account in the heating process of the Maillard reaction. The IP value expected by the model for this MRP_{IPmax} was 61.0. Even if not within the initial experimental domain, this predicted value was experimentally verified. Under these conditions, we obtained an IP of 61.9 ± 1.7 for MRP_{IPmax} (four replicates).

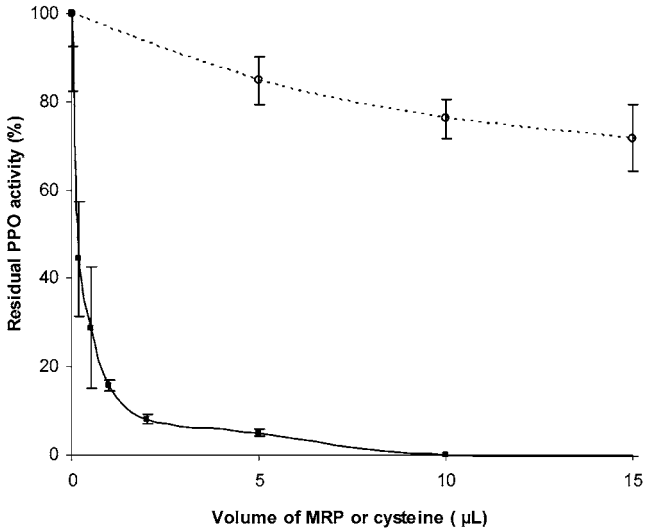

Figure 4. Dose response curves for the inhibitory effect of heat at 115 °C for 3 h 20 min cysteine (0.25 M) (○) or glucose/cysteine (1/0.25 M), pH 2 (■), on eggplant PPO activity. PPO activity was followed by polarography using 10 nkat PPO (see the conditions in **Figure 1**) and expressed as a percentage of maximum activity measured without inhibitors in the reaction medium.

Various volumes (0.2−15 μL equivalent to 0.035−2.61 mM thiol reactant) of MRP_{IPmax} were added to the reaction medium containing eggplant PPO (10 nkat) and 4-MC (20 mM, pH 5.0). The residual PPO activity (**Figure 4**) was almost undetectable when 10 μL of MRP_{IPmax} was added in the reaction medium. In comparison, the addition of 15 μL of heated cysteine (0.25 M, pH 2) equivalent to 2.61 mM of thiol reactant led nearly to 30% inhibition. These results proved that the strong IP of the MRP was only partially due to the heated cysteine products alone. Under the assay conditions, unheated cysteine (0.25 M; 0−15 μL) had no effect on PPO activity.

To characterize the inhibition due to MRP_{IPmax}, the influence of 4-MC concentration (1.25−20 mM) on the inhibition level was investigated at pH 5.0 with 3 volumes of highly diluted ($1.0-1.5 \times 10^3$ folds) MRP_{IPmax} equivalent to 0.010, 0.013, and 0.020 μL of initial MRP_{IPmax}. Assuming reversibility for the PPO−MRP_{IPmax} complex, a noncompetitive inhibition can be deduced from the Lineweaver−Burk double-reciprocal plots (**Figure 5**). This type of inhibition was observed by Brun-Mérimée et al. (*22*) for MRP derived from an equimolar (0.25 M) mixture of fructose/glutathione heated at 90 °C for 15 h with apple PPO. The apparent inhibition constant for MRP_{IPmax} was found at 0.013 μL (equivalent to 2.26 μM of cysteine before heating). This value, very low in comparison to *t*-cinnamic acid ($K_{iapp} = 100$ μM), the most powerful competitive inhibitor for eggplant tested in the same conditions in this study, highlighted the very strong inhibitory effect of MRP toward eggplant PPO activity.

To assess the possible inactivation of eggplant PPO by MRP, experiments were carried out with PPO incubated with MRP_{IPmax} and compared to the incubation experiments with water, unheated cysteine (0.25 M, pH 2), or cysteine (0.25 M, pH 2) heated at 115 °C for 3 h 20 min. The inactivation rate of eggplant PPO was determined by plotting the residual PPO activity vs the incubation time (**Figure 6**). When incubated for 3 h with water, almost no loss in PPO activity was observed with water while a loss lower than 30% was recorded with unheated cysteine. In the same conditions, the addition of heated cysteine resulted in rapid (less than 2 min) loss of the activity (80%) and after 3 h only 7% of the original activity remained

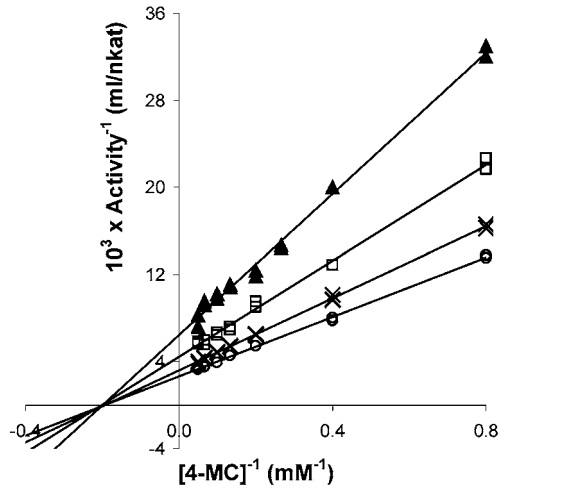

Figure 5. Inhibition of eggplant PPO activity with an equivalent of 0.010 (×), 0.013 (□), and 0.020 μL (▲) obtained after dilution of MRP_{IPmax}. The control was done without MRP_{IPmax} (○) (see the conditions in **Figure 1** with various 4-MC concentrations).

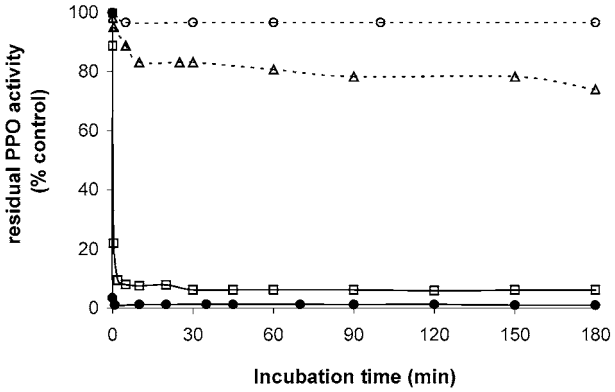

Figure 6. Effect of unheated cysteine (0.25 M, △), heated cysteine (115 °C for 3 h 20 min, 0.25 M, □), and MRP_{IPmax} (●) on PPO activity (90/10 v/v) in the course of incubation time (0–180 min) at 0 °C. The control (○) was incubated with water (see the conditions in **Figure 1**).

whereas with MRP_{IPmax}, the loss was immediate and reached 97%. After 3 h of incubation time, the residual activity with MRP_{IPmax} was below 1%.

In an attempt to release the enzyme from the inhibitory compounds and to determine whether the inhibition of PPO was reversible, the incubation systems with heated cysteine on one hand and with MRP_{IPmax} on the other hand were dialyzed against water for 24 h at 0 °C. **Figure 7** shows that after dialysis, the PPO was only partially recovered. The phenomenon was more pronounced with the heated cysteine/PPO incubate (30% of recovery) than with the MRP_{IPmax}/PPO incubate (only 5% of recovery). A similar result was obtained when a gel filtration chromatography on Sephadex G-25M (PD10 minicolumns, Pharmacia) was applied to the two incubates (not shown). It thus appears that the inhibition of eggplant PPO by heated cysteine is partially reversible and is partially due to small molecules, which can be eliminated by dialysis (or gel filtration). In comparison, inhibition of eggplant PPO by MRP_{IPmax} was nearly irreversible, suggesting that the MRP (or the products issued from the reaction of heated cysteine and caramelization of glucose) may irreversibly denature the major part of the enzyme or chelate the copper present in the enzyme to form a complex, which cannot be totally dissociated by dialysis (or gel filtration).

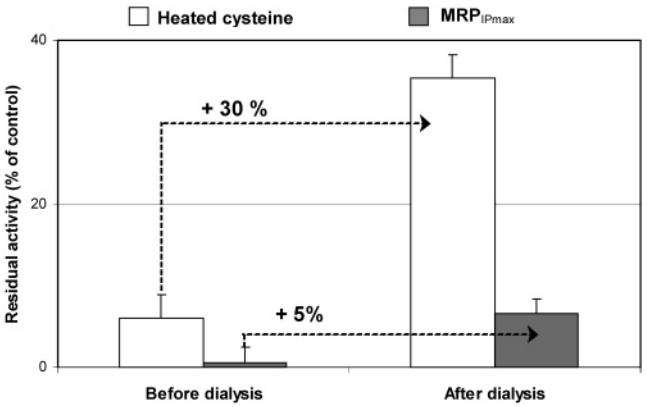

Figure 7. PPO activity recovery after dialysis of incubation media containing heated cysteine (115 °C for 3 h 20 min, 0.25 M) and MRP_{IPmax} in white and gray, respectively. The incubation media consisted of PPO and inhibitors (90/10 v/v) maintained at 0 °C for 180 min (see the conditions in **Figure 1**).

As compared to other chemical inhibitors (4-hexylresorcinol and aromatic carboxylic compounds), MRP_{IPmax} appears to be much more efficient as an inhibitor of the eggplant PPO activity. The apparent inhibition constant of these compounds thus indicates that the addition of less than 2.5 μM (equivalent cysteine before heating) is sufficient to inhibit the eggplant PPO activity by 50%. In addition, the inhibition appears almost irreversible. The strong potency of these MRPs to inhibit the browning of fresh cut apple, mushroom, and eggplant was shown even at low concentrations without effects on the color of the products (*36*). These results are of great importance for manufacturers considering shelf life product constraints linked to fresh fruits and vegetable marketing. Further experiments at a larger scale could show whether the replacement of sulfite by MRPs in food processes would prevent the enzymatic browning leading to the development of ready-to-eat or ready-to-cook fruits and vegetables. Nevertheless, even if used as aromas or naturally neoformed in heated foodstuffs, MRPs are suspected of negative effects on health, suggesting that toxicological analysis of these compounds should be carefully carried out prior to their utilization in food industries. The next challenge of this research will be the fractionation and the identification of the compounds involved in these antioxidant properties in order to characterize their toxicological behaviors and anti-browning effects while widening their potential interest by testing them on other oxidation phenomena.

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Received for review January 25, 2006. Revised manuscript received May 4, 2006. Accepted May 8, 2006.

JF060228R