Research Article

Inhibition of polyphenoloxidase activity by mixtures of heated cysteine derivatives with carbonyl compounds

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It had previously been shown that soluble Maillard reaction products (MRP) made from thiol compounds and glucose or fructose contained powerful inhibitors of various fruit and vegetable polyphenoloxidase (PPO) activity. In MRP from cysteine and glucose, the amount of hydroxymethylfurfural (HMF) formed increased with the increase in glucose concentration (0 – 1 M), particularly under acidic (pH 2) conditions. Using model mixtures containing a preheated cysteine-derived compound and a carbonyl component, especially HMF, furfural and benzaldehyde, we showed that the neoformed compounds produced exhibited a stronger inhibitory potency toward PPO activity of eggplant, apple, and mushroom than former MRP. Optimal reaction conditions for the formation of inhibitory compounds when HMF reacted with preheated cysteine were investigated. It was found that a reactants molar ratio of 1:1 and a reaction time exceeding 1 h were the most efficient reaction conditions to generate inhibitory compounds. The stability of the newly formed products, evaluated during storage, showed that their inhibitory potency was globally kept at 4, 21, and 37°C for 72 h but was unstable when stored at −20°C and lost when exposed to UV radiations for 24 h.

Keywords: Cysteine, 5-HMF / Inhibition / Neoformed compounds / Polyphenoloxidase

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1 Introduction

Oxidation of phenolic substrates, catalyzed by polyphenoloxidase (PPO) action in the presence of molecular oxygen, so called enzymatic browning, occurs once vegetal or animal tissues have been damaged by slicing, cutting, or pulping. It leads to the formation of brown pigments and altering both the organoleptic and biochemical characteristics of the products. The rate of enzymatic browning is mainly governed by the active PPO content and the phenolic profile of the tissues. Sulfiting agents are the most widely used compounds to control the deteriorative effects of browning. However, they are subject to regulatory restrictions owing to their adverse effects on health. There is therefore a considerable research on proposing appropriate sulfite substitutes for use in raw and processed foodstuffs [1]. Among the numerous antioxidant compounds tested to avoid or delay the enzymatic browning process, natural sources of inhibitory products such as Maillard reaction products (MRP) generated from glucose or fructose with glycine [2, 3] and other amino acids [4, 5] or caramelization products [6] have been proven capable of inhibiting PPO activity. MRP from glucose or fructose with thiol reactants (cysteine or glutathione) have been shown to highly inhibit apple and eggplant PPO activity, especially when prepared under acidic conditions [7 – 11]. Maillard reaction is a complex set of reactions between amino and aldehyde or ketone compounds to produce a wide range of intermediate products, for which a large number of antioxidant properties have been described so far. They are to a large extent dependent on their capability of free radical scavenging [12], che-

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Abbreviations: HMF, 5-hydroxymethylfurfural; **MRP,** Maillard reaction products; **PPO,** polyphenoloxidase

lating di- and trivalent metals [13 – 15] and/or they present a reducing activity [16]. Antioxidant MRP properties have been reported to be strongly affected by the physico-chemical environment of the model systems and the processing conditions [17]. This point raises the question of knowing which compounds possessing inhibitory potency on PPO activity are produced during the heating of thiol compounds with carbohydrates and the reaction conditions most strongly affecting the induction of inhibitory compounds. It is well established that during heating of hexoses and pentoses under acidic conditions, accumulation of furanic compounds such as 5-HMF and 2-furaldehyde, two key intermediates from both caramelization and the Maillard reaction is favored through enolisation, dehydration, and cyclization reactions of the reducing sugar or the Amadori product [18 – 22].

The present work was undertaken to compare the inhibitory potency toward PPO activity of apple, eggplant, and/or mushroom of cysteine-derived MRP and the model mixture consisting of heat-treated cysteine and HMF. The processing conditions (heating time, temperature, and concentration ratio of the reactants) and stability of inhibitory compounds under various storage conditions (time, temperature, and pH of the mixture) were also studied, using the model mixture containing hydroxymethylfurfural (HMF) and heated cysteine (115°C for 200 min).

Data are also presented on the inhibitory effect of various binary model systems prepared by reacting a carbonyl compound (aliphatic or cyclic) with a cysteine-related compound.

2 Materials and methods

2.1 Materials

Apple (*Var.* Red Delicious) and purple eggplant (*Solanum melongena*) fruits were purchased at maturity from the local market and used as a source of PPO (EC 1.10.3.1). The enzyme was partially purified from the cortex (apple) [23] and the flesh part (eggplant) [11] of the vegetal. Mushroom tyrosinase (EC 1.14.18.1, 600 U/mg) was purchased from Sigma – Aldrich Chemical (St. Quentin Fallavier, France) and diluted (1 mg/mL) in sodium phosphate buffer (0.05 M, pH 6.5) and stored at +4°C.

Chemicals used for the preparation of MRP and mixtures of heated cysteine derivatives with carbonyl compounds (collated in Table 2) as well as 4-methylcatechol (4-MC) were obtained from Sigma – Aldrich Chemical (St. Louis, MO). Chemicals used in the preparation of buffers and the purification of apple and eggplant PPO were purchased from VWR International, Merck-eurolab (Fontenay S/Bois, France). ACN was of HPLC grade (Acros Organics, Noisy-Le-Grand, France). The water was purified using Analyst HP purification Purite Select limited (Oxon, England).

2.2 Model systems preparation

2.2.1 MRP preparation

Glucose (0 – 1 M) and L-cysteine (0.25 M) aqueous solutions were used to prepare model MRP. Aliquots (1.5 mL) of these solutions were placed either without pH control or after adjusting the pH to 2 with concentrated H_3PO_4 in Pyrex vials (2 mL) sealed with silicone-Teflon septa and metallic caps (Interchim, Montluçon, France). Samples were heated at 115°C for 200 min, in an air convection oven (Memmert, ULE 400). These heating conditions led to MRP presenting maximal inhibitory potency toward PPO activity from eggplant [11]. After the vials were cooled in ice, aliquots of the soluble part of MRP were used to determine both their inhibitory effect on PPO activity and their 5-HMF content.

2.2.2 Neoformed compounds mixtures preparation

In a first series of experiments, a cysteine aqueous solution (10 mM) was first heated at 115°C for 200 min. The water-cooled solution was mixed with a carbonyl compound (4 mM). After 180 min reaction at 0°C, aliquots (10 µL) of the reaction mixture were taken for inhibition studies on PPO from apple.

In another series of experiments, an aqueous cysteine-derivative solution (10 mM) was preheated at 115°C for 200 min as described above. The water-cooled solution was mixed with 5-HMF (4 mM) and after 180 min reaction at 0°C aliquots (10 µL) of the reaction mixture were taken for inhibition studies on PPO activity from apple.

2.3 Determination of pH and HMF amounts

An Orion model 410A pH meter and an analytical XC111 Radiometer with thin-probe combination electrodes were used for pH measurements. The amount of 5-HMF present in MRP model systems was determined by HPLC with a Waters system (Milford, MA, USA) equipped with a Model 600 pump system controller, a Model 996 DAD and a YMC-ODS AQ (250 × 4.6 mm) column from A.I.T (France). Samples were filtered through 0.20 µm nylon filters and injected into the HPLC. HMF was separated using water/ACN 95 : 5 v/v as mobile phase, at a flow of 0.8 mL/min and detected at 285 nm. Quantification was performed by calibration with an external standard.

2.4 Enzyme determination

PPO activity was determined polarographically at 30°C in a 1.5 mL reaction cell containing 2 (mushroom PPO) or 20 (apple and eggplant PPO) mM 4-MC in 0.1 M air-saturated McIlvaine's buffer pH 4.5 (apple and mushroom PPO) or 5.0 (eggplant) as the substrate. The reaction rate was calculated from the initial slope of the progress curve giving oxy-

gen uptake *versus* time using a Clark oxygen microprobe. Activity was expressed as nmoles of oxygen consumed *per* second (nanokatals) under the assay conditions. For inhibition studies, MRP (1–10 μL) were added to the reaction medium before the enzyme extract.

2.5 Experimental design

The effects of initial concentrations (0.8–9.2 mM) of both HMF and heated cysteine (115°C for 200 min) on the production of mixture inhibitors of PPO activity were optimized according to a rotatable two-variable five-level central experimental design (Table 1). Five replications of the central point were determined to calculate the experimental error. The 13 trials were analyzed according to linear and quadratic models. Three measurements of the residual PPO activity from eggplant with 10 μL of the mixture taken after 60 min of contact at 0°C were determined. Within the limits of the experimental domain, a second-order polynomial model was fitted to the dependent variable. Analysis of variance and *t*-test of the coefficients of the model were used to check the reliability of the polynomial and the significance of the parameters. The level of statistical significance for the process variables was defined at $p \leq 0.01$.

2.6 Statistical analysis

Analyses performed in this work were carried out in triplicate and the averages of data were considered. The error bars represent SD.

3 Results and discussion

3.1 Comparison of the inhibitory potency of MRP and neoformed compounds toward PPO activity

It was recently shown that MRP prepared under acidic conditions (pH 2) with glucose (1 M) and cysteine (0.25 M) aqueous solutions heated at 115°C for 200 min led to maximal inhibition of partially purified PPO from eggplant [11]. The inhibitory potency of these MRP was also tested toward PPO activity from apple (Fig. 1). It appeared that the higher the glucose concentration, the higher the inhibitory potency. When the pH value of the glucose–cysteine solutions was adjusted to 2 before heating, MRP exhibited a more potent effect at any glucose concentration, confirming the importance of a low initial pH value to generate inhibiting compounds. The amount of HMF formed during the heating treatment was also determined in MRP. Above 0.4 M in glucose, the higher the glucose concentration in the model systems, the higher the HMF production, all the more clearly at pH 2. These results confirm the hypothesis that HMF production is favored under acidic conditions [21].

Table 1. Independent variables and their coded and actual values

		Coded levels				
Independent variable	Symbol	−1.41	−1	0	1	1.41
Cysteine conc. (mM)	A	0.8	2	5	8	9.2
HMF conc. (mM)	B					

When glucose and cysteine solutions were heated separately before mixing, the resulting model mixture was more efficient than MRP prepared under the same reaction conditions (not shown). This result suggests that HMF produced from heated glucose solutions reacted with compound(s) derived from heated cysteine, generating reaction products which were very active toward PPO activity from apple.

In these experiments, the control solutions (unheated glucose–cysteine mixtures and HMF solution) were devoid of inhibiting efficiency on PPO activity.

Therefore, to evaluate the role of both HMF and compounds thermally derived from thiol reactant in the inhibiting effects observed, two sets of experiments were carried out.

3.2 Nature of the carbonyl compound

In a first series of experiments (Table 2A), the reaction between various aqueous solutions of carbonyl compounds (4 mM) and heated cysteine (10 mM) was allowed to proceed at 0°C for 180 min.

As seen in Fig. 2 with some model mixtures, the inhibiting efficiency of model mixtures was found to affect the time-course of the reaction of PPO by a rapid decrease in the initial rate. For the most potent mixtures, this inhibitory effect appeared to be quite stable after at least 60 min of reaction at 0°C. It was first verified that under the assay conditions neither the thermal degradation products from cysteine nor carbonyl compounds tested alone were *per se* active on PPO activity. These results contrast with those of [24–27] for which some pure aromatic aldehydes such as cuminaldehyde, isopropylsalicylaldehyde or HMF were found to present inhibitory effects on mushroom tyrosinase, when monophenolase and *o*-diphenolase activities of the enzyme were determined by spectrophotometry.

As seen in Table 2A, mixtures containing an aliphatic carbonyl or dicarbonyl molecule (acetaldehyde, formaldehyde, glutaraldehyde, butanedione) were totally devoid of efficacy toward PPO activity inhibition or showed only a slight inhibitory effect (glyoxal).

The same trend was observed with cyclic carbonyl compounds consisting of a pentane (cyclopentanone), a hexane (cyclohexane carboxaldehyde), or a furan (2-furanone) ring, indicating that the absence or the presence of a single

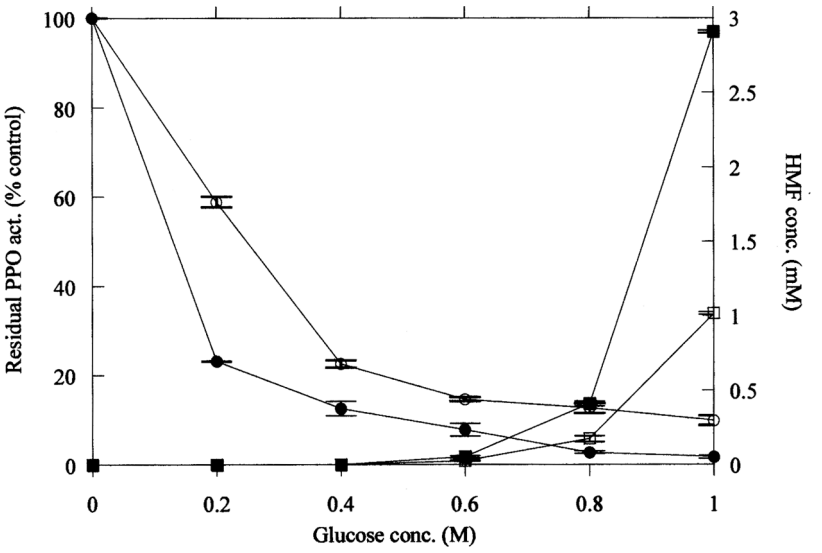

Figure 1. Effect of glucose concentration (0–1 M) of model systems MRP prepared with cysteine (0.25 M) and glucose aqueous solutions heated at 115°C for 200 min on PPO activity from apple and on HMF content (mM). The inhibitory potency of MRP was determined with samples prepared without pH control (○) or with pH adjusted to 2 (●) before heating. HMF concentration was determined in MRP prepared without pH control (□) or with pH adjusted to 2 (■) before heating. Activity was measured by polarography at 30°C, using 4-MC (20 mM) as the substrate (pH 4.5), 9 nkat PPO and 1 μL MRP. It was expressed as% maximum activity measured without MRP in the reaction medium.

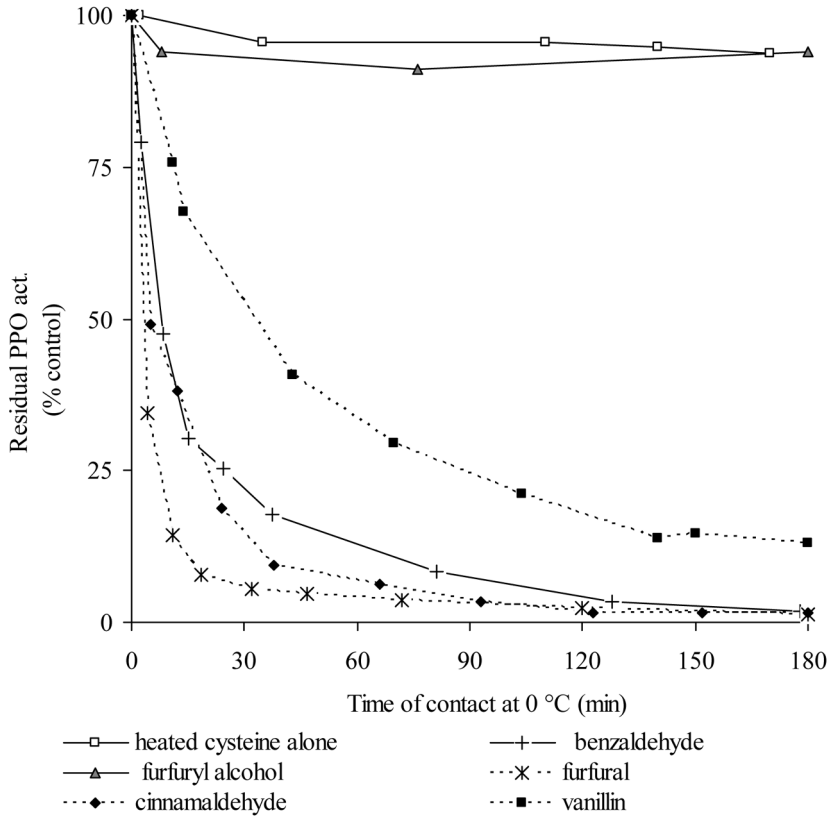

Figure 2. Effect of the time of contact on the inhibitory effect of mixtures prepared with preheated (115°C, 200 min) cysteine (10 mM) and a carbonyl compound (4 mM) on PPO activity from apple. The time of contact between the two reactants ranged from 3 to 180 min at 0°C. PPO activity was measured as in Fig. 1 with 10 μL model mixture added to the reaction medium before the PPO extract.

double bond on the ring was not favorable to the formation of inhibitory compounds.

With regards to benzaldehyde derivatives, the lengthening of the lateral chain (benzeneacetaldehyde) drastically reduced the inhibitory ability of benzaldehyde. Conversely, the presence of a double bond on this lateral chain (cinnamaldehyde) rendered the compound as reactive as benzaldehyde after it had been mixed with heated cysteine. The introduction of either a hydroxyl group at the *ortho* (salicylaldehyde) or *para* (*p*-hydroxybenzaldehyde) or a methoxy group at the *meta* (*m*-anisaldehyde) or *para* (*p*-anisaldehyde) position of the benzene ring did not significantly or only slightly lowered the inhibitory potency of the reaction mixture. The simultaneous presence of a hydroxyl and either a methoxy (vanillin) or an ethoxy (ethylvanillin) groups respectively on the 4th and 3rd positions of the benzaldehyde induced a more significant decrease in the efficacy of the compounds (4.6 and 6.5 times lower), compared

to benzaldehyde, attesting to the effect of an electron-withdrawing group to the benzene structure for the formation of active compounds. Likewise, when an electron-donating radical such as an isopropyl group was present at the *para* position (cuminaldehyde) of the aldehyde group, the resulting inhibitory effect was 7.7 times lower than with benzaldehyde.

When furan compounds such as furfural and its hydroxymethyl derivative HMF, were used in place of benzaldehyde for reactions, neoformed compounds turned out to be highly active and as efficient as benzaldehyde with less than 2 and 4% of the original PPO activity remaining, respectively. Conversely, oxidation (2-furoic acid), reduction (furfuryl alcohol), or methylation (furyl methylketone) of the carbonyl group was accompanied by a great loss in inhibitory potency with, on average, a loss of 6.5% of the initial PPO activity.

This preliminary study points out the importance of the presence of both an unsaturated ring and a free carbonyl group for inhibitory potency to be exhibited. It could be assumed that electronic delocalization is involved in the chemical reaction with compounds thermally generated from cysteine. Results also suggest that the positions and polarities of the groups on the rings play an important role in their reactivity and in the formation of active compounds.

3.3 Nature of the cysteine-derived compound

HMF was selected as the carbonyl reactant. Various cysteine-derived components (10 mM) were heated at 115°C for 200 min before they reacted with HMF (4 mM) for 5 to 180 min at 0°C (Table 2B). Mixtures from the heating of cysteine and cysteine methylester were the most efficient once they had reacted with HMF. The earliest such reaction was after 60 min of contact. *N*-acetylcysteine and homocysteine also reduced PPO activity, although to a lesser extent. Conversely, mixtures containing HMF and a heated cysteine derivative in which the thiol group was methylated (methionine, methylcysteine) or oxidized (cysteic acid) generated compounds eliciting minimal effect or were almost totally devoid of inhibitory potency. The same was true when the carboxyl group (cysteamine) or the amino group (3-mercaptopropionic and thiolactic acids) was lacking. According to these data, heating conditions seemed sufficient to allow hydrolysis of the acetyl (*N*-acetyl-cysteine) and ester (cysteine methylester) functions and to generate reactive compounds with HMF.

Unheated ammonium sulfide was also included in this study because it corresponded to a soluble form of hydrogen sulfide which could be generated during the heating of cysteine solutions [28]. Although not an inhibitor *per se*, once mixed with HMF, this compound gave rise to potent inhibitory compounds toward PPO activity.

At this concentration level, unheated cysteine and sulfite derivatives were devoid of inhibitory potency when tested alone or after reacting with HMF for 180 min. The absence of inhibition was also noticed when these compounds were heated alone. With the exception of homocysteine, cysteine-derived compounds displayed a similar level of inhibitory potency toward PPO activity from apple as did MRP prepared with glucose and cysteine-related reactants [10]. It seems that the initial simultaneous presence in the

Table 2. Inhibitory potency of the different compounds used in this study, determined on PPO activity from apple after 180 min of contact between the reactants at 0°C. PPO activity was measured as described in Fig. 1, except that 10 μL mixtures were added to the substrate solution before the PPO extract. (**A**) Each carbonyl reactant (4 mM) was mixed with preheated (115°C, 200 min) cysteine (10 mM); (**B**) Each cysteine-derived reactant (10 mM) was preheated (115°C, 200 min) before mixing with 5-HMF (4 mM)

Nature of the reactant	Residual PPO activity (%)
(**A**) Carbonyl reactant	
Salicylaldehyde	$1.33 \pm 0.16^{a)}$
Furfural	$1.74 \pm 1.6^{a)}$
Cinnamaldehyde	$1.76 \pm 0.22^{a)}$
Benzaldehyde	$2.03 \pm 1.4^{a)}$
5-HMF	$3.52 \pm 2.5^{a,b)}$
p-Anisaldehyde	$3.70 \pm 0.47^{a,b)}$
m-Anisaldehyde	$4.80 \pm 0.30^{a,b)}$
p-Hydroxybenzaldehyde	$7.53 \pm 0.31^{b,c)}$
Ethylvanillin	$9.41 \pm 2.3^{c,d)}$
Vanillin	$13.2 \pm 1.9^{d,e)}$
Cuminaldehyde	$15.6 \pm 0.60^{e)}$
2-Furyl methylketone	$93.7 \pm 1.9^{f)}$
Furfuryl alcohol	$94.8 \pm 1.9^{g)}$
Glyoxal	$94.8 \pm 1.9^{g)}$
Benzeneacetaldehyde	$95.0 \pm 2.1^{g)}$
2-Furoic acid	$95.7 \pm 3.0^{g)}$
Cyclopentanone	$95.0 \pm 2.1^{g)}$
2-Furanone	$95.1 \pm 1.4^{g)}$
Cyclohexane carboxaldehyde	$96.5 \pm 3.4^{g,h)}$
Acetaldehyde, formaldehyde	$100^{h)}$
2,3-butanedione	$100^{h)}$
Glutaraldehyde	$100^{h)}$
Heated cysteine (alone)	$93.7 \pm 1.9^{g)}$
(**B**) Cysteine-derived reactant	
L-Cysteine methylester	$1.41 \pm 0.07^{a)}$
Cysteine	$1.73 \pm 0.14^{a)}$
Ammonium sulfide*	$2.76 \pm 0.55^{a)}$
N-acetylcysteine	$18.2 \pm 0.61^{b)}$
Homocysteine	$30.5 \pm 0.92^{c)}$
Cysteamine	$84.0 \pm 2.37^{d)}$
Methylcysteine	$88.6 \pm 2.02^{e)}$
Cysteic acid	$92.2 \pm 2.19^{f)}$
Methionine	$97.5 \pm 1.22^{g)}$
Thiolactic acic	$97.2 \pm 0.56^{g)}$
3-Mercaptopropionic acid	$98.1 \pm 1.12^{g)}$

Values sharing different letters (a – h) showed significant differences for residual PPO activity (Duncan's test, $p \leq 0.01$).

* Ammonium sulfide was not preheated before reacting with HMF.

Table 3. Comparative inhibitory effect of MRP derived from glucose–cysteine (1 M/0.25 M) [**A**] and preheated cysteine-HMF (5 mM each) [**B**] aqueous model systems on PPO activity from apple, eggplant, and mushroom

Model system	Residual PPO activity (% control)		
	Apple PPO	Eggplant PPO	Mushroom PPO
Cysteine-glucose derived MRP [**A**]	2.1 ± 0.4	1.6 ± 0.3	6.5 ± 1.4
Heated cysteine-HMF mixture [**B**]	7.0 ± 0.8	6.3 ± 0.6	44.0 ± 3.1

PPO activity was followed by polarography using 9 nkat PPO and 1 [**A**] or 5 [**B**] μL inhibitory model system (cysteine equivalent concentration of 176 or 17.6 μM respectively in the reaction media). Heat-treatment of glucose–cysteine [**A**] solution (initial pH = 2) or cysteine alone [**B**] was performed at 115°C for 200 min.

molecule of SH, NH_2, and COOH groups, either in a free state, or released in the course of heating, is necessary for the formation of active precursors during heating. Nevertheless, the carboxyl function proved unnecessary when using unheated ammonium sulfide.

Unreacted cysteine or related compound containing a free SH group were not involved in the formation of inhibitory compounds with HMF, as judged from the lack of inhibitory property of furfurylmercaptan, a thiol-furan conjugate tested under the same assay conditions (not shown). According to [29], this odorant compound is formed during the reaction of HMF with either H_2S or sulfur-containing amino acids, the amount increasing significantly under acidic conditions.

Although the nature of the chemical reaction between carbonyl compounds and some compounds derived from heat-treated cysteine is unknown, it is plausible that resulting products, owing to their strong inactivating effect on PPO activity, would react with nucleophilic groups such as sulfhydryl, amino or hydroxyl groups of the enzyme active site, bringing about conformational change. They could also potentially chelate the copper ion at the active site of the PPO, a copper-metalloenzyme that would explain the strong inhibition noticed.

Thus, in the presence of heated cysteine, benzaldehyde, salicylaldehyde, cinnamaldehyde, furfural, and HMF were among the most prone to generate highly inhibitory compounds. To make comparison between the inhibitory effect of cysteine-derived MRP and these model mixtures easier and because HMF was present in MRP samples, particular emphasis was placed on the heated cysteine–HMF mixture. When residual activity of PPO from various sources was recorded after direct addition of either MRP or the model mixture (Table 3), the latter was found to be more efficient than MRP in its inhibitory potency, owing to the initial cysteine concentrations tested in the reaction medium, which is ten times lower than in MRP. As a general rule, PPO from apple and eggplant were 3–7 times more sensitive to the inhibitory compounds than PPO from mushroom when the *o*-diphenolase activity was followed using 4-MC as a substrate.

3.4 Effect of the concentration of reactants and their ratio on inhibition of PPO activity

According to the experimental design, the best fit equation relative to the main, interactive and quadratic effects of cysteine and HMF concentrations on residual PPO activity from eggplant was as follows:

$$Y = 82.9 - 6.13*[A] - 7.85*[B] + 0.167*[A]^2 - 0.026*[A*B] + 0.29*[B]^2 \quad (1)$$

The R-squared statistic indicates that the model as fitted explains 88.9% of the variability in the residual PPO activity. According to the analysis of variance, two effects (linear effects of cysteine and HMF concentration) were statistically significant at the 99.0% confidence level (not shown). What is more, as the values of the estimated effects of the cysteine and HMF concentration were close (–25.3 and –28.3 ± 2.2, respectively), equimolar mixtures were advisable. Also, under our experimental conditions and considering the fact that no factor had any significant quadratic effect, we can say that the higher the concentration of the mixture, the higher the inhibitory potency.

3.5 Effect of heat treatment of cysteine solution on the formation of inhibitory compounds *versus* PPO activity

Cysteine solutions (10 mM) were heated at various temperatures (90–120°C) and for different lengths of time (0–360 min). The resulting solutions were mixed with HMF (5 mM final concentration) and kept at 0°C for 60 min, after which the inhibitory effect of the mixtures was assessed. As can be seen in Fig. 3, enzyme activity decreased with the increase in temperature and heating time. A maximum inhibition of the enzyme was recorded, after which it remained almost constant with prolonged durations of heating. The experimental curves were modeled according to a first-order model

$$A = A_f + (A_0 - A_f)\ \exp^{-kt} \quad (2)$$

where A represents the remaining PPO activity (% control) at the time t, A_0 and A_f are the initial and final activity after heating respectively, and k is the first-order kinetic rate constant.

The first-order kinetics gave good correlation coefficients (Table 4). The k values for the formation of inhibitory precursors from cysteine and A_f were calculated from nonlinear regression by minimizing the sum of squares of the

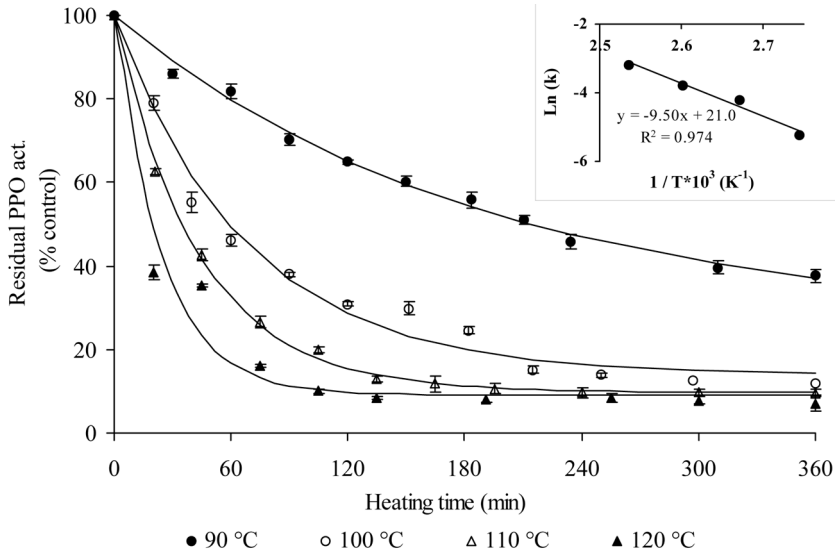

Figure 3. Changes in the inhibitory potency of cysteine solutions *versus* heating time (0–360 min) at different temperatures (90–120°C). Inset: determination of activation energy (E_a) value for the production of precursors of inhibitory compounds derived from heated cysteine, according to Eq. (3). Mixtures consisting of heated cysteine-HMF (5 mM each) were tested on PPO activity from apple after 60 min of contact at 0°C. Activity was measured by polarography in the presence of a 2 μL model mixture.

Table 4. Results from fitting the reaction rate data to the proposed first-order kinetic model (Eq. 2)

Temperature of heat treatment (°C)	k ($\times 10^{-3}$/min)[a]	R^2	A_f(%)
90	5.32	0.974	26.2
100	14.8	0.907	13.9
110	23.1	0.985	9.8
120	41.3	0.857	9.2

a) Values are obtained from Fig. 3.

differences between experimental and theoretical values. As expected, k values increased on increase in the temperature.

The temperature sensitivity was described by an Arrhenius-type dependence for the model system studied according to the equation:

$$k = \frac{-E_a}{RT} \quad (3)$$

where E_a is the activation energy (kJ/mol), R the universal gas constant (8.314 J/molK), and T the absolute temperature (K).

E_a for the production of active precursors from heated cysteine was determined to be 79 kJ/mole with the Arrhenius plot (Fig. 3, inset). This value was 2.4-fold lower than that obtained for the production of inhibitory MRP from glucose–cysteine model systems (E_a = 191 kJ/mole) and acting on PPO activity from apple [30]. This observation indicates that cysteine heated in solution is less temperature sensitive compared to MRP in the formation of inhibitory compounds.

We can thus consider that heating a cysteine solution at 115°C for 200 min gives rise to maximal precursors to inhibitory compounds. These heating conditions of the cysteine solution were used to study the stability of compounds formed after reaction with HMF.

3.6 Stability of the inhibitory neoformed compounds towards temperature and pH

The stability of the inhibitory effect in the preheated cysteine-HMF equimolar (5 mM) model mixture was checked during 3 days of storage at four different temperatures (37, 21, 4, or −20°C, respectively). Some fractions were also maintained at 21°C under UV radiations. Samples were taken at 1, 24, and 72 h and analyzed for their inhibitory potency *versus* PPO activity from eggplant (Fig. 4). According to the analysis of variance for the residual PPO activity, both the storage time and the type of treatment had statistically significant effects on the response at the 99.0% confidence level. Using a Duncan's multiple comparison procedure to discriminate among the means, it appeared that there was a 1% risk of calling one or more pair significantly different when their actual difference equal to 0.

Inhibiting properties of the mixtures were statistically stable at 21 and 37°C for at least 24 h. At 4°C, mixtures remained stable for at least 72 h even though the increase of the inhibitory potency between 1 and 24 h was statistically significant. When frozen (−20°C) or submitted to UV radiations at 21°C, there was a significant loss of inhibitory potency in the course of the storage time.

Hence, a contact between the reactants for 60 min at –20°C did not seem sufficient to allow the formation and stabilization of inhibitory potency (Fig. 3), the reaction being stopped or deviated. It can also be hypothesized that low energy bonds (hydrophobic) are involved in the formation of inhibitory compounds and very low temperatures are not recommended for maintaining the efficacy of the

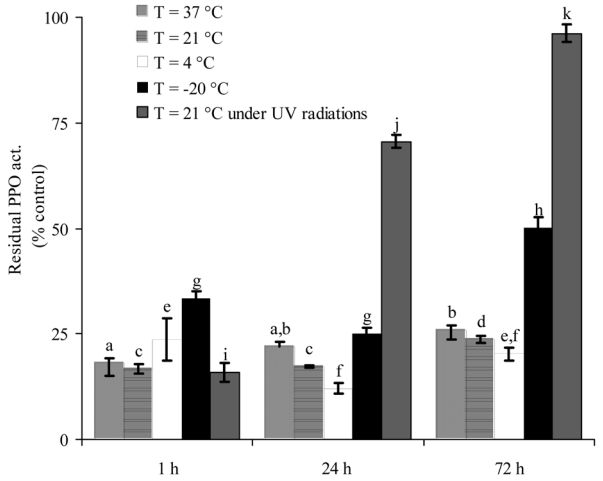

Figure 4. Effect of storage time and temperature on inhibitory compounds stability in preheated (115°C, 200 min) cysteine-HMF (5 mM each) mixtures. Model mixtures were tested on PPO activity from apple after 1–72 h of contact at different temperatures. Activity was measured by polarography in the presence of 5 μL model mixture. Values sharing different letters showed significant differences for the same temperature in the course of time (Duncan's test, $p \leq 0.01$).

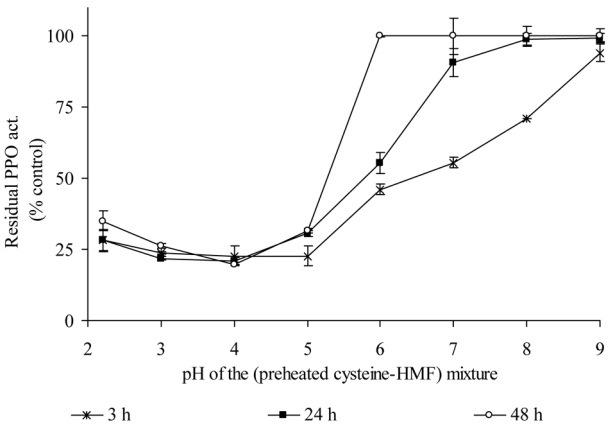

Figure 5. Effect of pH and storage time (3–48 h) on the stability of inhibitory potency of model mixtures. Model mixtures (preheated cysteine-HMF, 5 mM each) were tested on PPO activity from apple after 60 min of contact at 0°C. Activity was measured by polarography in the presence of 5 μL model mixture.

mixture. From a technological point of view, it should be possible to use such a mixture based on the quite good stability of compounds stored at 4–21°C.

The effect of pH on the stability of the active compounds formed after reacting preheated cysteine with HMF showed that they were pH-sensitive (Fig. 5). The lower the pH, the greater the formation and stability of inhibitory compounds. Conversely, near neutral or alkaline conditions (pH 6–9), there was minimal production of such compounds. They probably degraded since they repressed PPO activity to a maximum of about 54 and 45% inhibition after 3 and 24 h storage for the mixtures prepared at pH 6. The inhibitory potency was completely lost after 48 h storage at room temperature.

In accordance with [31], the reaction of an aldehyde compound (furfural) with an amino group increases with pH, as the concentration of unprotonated amine increases. As a result, the reaction will probably generate compounds with no inhibitory properties toward PPO activity.

Taken as a whole, these data underline that temperature and pH of the reaction medium are found to markedly influence the composition and stability of aqueous mixtures containing cysteine and carbonyl compounds. Consequently, storage at room temperature under acidic conditions is best for maintaining the inhibitory properties of the preheated cysteine-HMF mixture.

4 Concluding remarks

The present study showed that some simple model mixtures produced from heat-treated cysteine and carbonyl compounds of variable structure (*e.g.*, benzaldehyde, HMF) contain neoformed compounds displaying PPO inhibiting properties more potent than that of cysteine-derived MRP. The improvement of the reaction conditions between heated cysteine and HMF was the prerequisite research prior to the fractionation–purification as well as elucidations of the mechanism and the components involved. These topics are presently under study.

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