Free Rad. Res., Vol. 25, No. 1, pp. 5–12
Reprints available directly from the publisher
Photocopying permitted by license only

Horseradish Peroxidase-Catalyzed Conjugation of Eugenol with Basic Amino Acids

MARISA H.G. MEDEIROS*, PAOLO DI MASCIO, ADRIANA P. PINTO, REINALDO R. VARGAS, and ETELVINO J.H. BECHARA

Instituto de Química da Universidade de São Paulo, CP 26.077, 05599–970 São Paulo, Brazil

Accepted by Professor H. Sies

(Received August 29th, 1995; in revised form, November 21st, 1995)

L-Lysine is shown to yield an adduct with the quinone methide intermediate formed during the horseradish peroxidase (HRP)-catalyzed aerobic oxidation of eugenol (4-allyl-2-methoxyphenol). Adduct formation is evidenced by (i) lysine quenching of the characteristic quinone methide absorption band measured at 350 nm; arginine and γ-aminobutyric acid, but not alanine or propionic acid showed similar behaviour (ii) lysine-promoted a 400 mV decrease of the eugenol oxidation voltammetric wave (1.00 V), concomitantly with an increase in current intensity and (iii) reverse phase HPLC isolation of the lysine eugenol adduct, followed by GC-MS analysis. The MS spectrum is consistent with a 2:1 lysine:eugenol adduct (MW = 455). If operative *in vivo*, binding of lysine to eugenol might lead to protein inactivation and possibly be involved in eugenol toxicity.

Key words: Eugenol, horseradish peroxidase, amino acids, eugenol-amino acid conjugation

INTRODUCTION

Eugenol (4-allyl-2-methoxyphenol) is the main essential oil found in clover and in several other plants. Given its antiseptic and analgesic properties, eugenol is commonly used in dental materials.[1,2] Eugenol has also been employed to treat gastrointestinal problems and chronic diarrhea.[3,4] However, several toxic side effects of eugenol have been described on human oral mucosal fibroblasts, at concentrations higher than 3 mmol/l.[5] Toxicological properties of eugenol are well documented such as depression of the central nervous system,[6] cell respiration inhibition[7] and polymorphonuclear cell migration inhibition.[8]

In an attempt to explain the molecular basis of eugenol toxicity, Thompson *et al.*[9] provided spectrophotometric evidence suggesting the involvement of a very reactive quinone methide intermediate in the aerobic oxidation of eugenol catalyzed by horseradish peroxidase (HRP, EC 1.11.1.7). This intermediate was found to be intercepted by glutathione and to covalently bind to heat-inactivated rat liver S-9 protein. The final products from eugenol oxidation were observed to be highly cytotoxic to isolated rat hepatocytes.

*To whom correspondence should be addressed: Departamento de Bioquímica, Instituto de Química da Universidade de São Paulo, CP 26.077, CEP 05599–970 São Paulo, Brazil. FAX: + + 55-0118155579, Phone: + + 55-0118187953, e-mail: mhgdmede@quim.iq.usp.br

Using GC-MS, HPLC and cyclic voltammetry techniques, we here provide evidence for the formation of an adduct between eugenol and lysine during the HRP-catalyzed reaction. Adduct formation might be involved in the mechanism responsible for extensive covalent binding of eugenol to proteins and play an important role on the cytotoxic properties of eugenol.

MATERIALS AND METHODS

Materials

All reagents were analytical grade chemicals. Horseradish peroxidase, eugenol, L-arginine and L-lysine monohydrocloride were obtained from Sigma and other chemicals from Merck. The concentration of horseradish peroxidase was determined spectrophotometrically ($\varepsilon_{403} = 1.1 \times 10^5\ M^{-1}\ cm^{-1}$),[10] and that of H_2O_2, according to Cotton and Dunford.[11]

Unless otherwise stated the spectrophotometric studies were performed on a Zeiss DMR-10 or on a Beckman DU-70 spectrophotometer. The HPLC apparatus consisted of Waters 600E Multisolvent Delivery System, Waters Model U6 K Universal Injector, Waters 745B Data Module, Waters 484 Tunable Absorbance Detector and a Merck-Hitachi system Model L-3000 with Multi Chanel Photodetector.

All cyclic voltammetric measurements were performed with a home-built combined potentiostat wave form generator and sweep generator using a Houston Model RE 0074 PAR x-y recorder for the registration of the voltammograms.

Gas chromatography combined with mass spectrometry (GC-MS) were taken using a Varian Model 3400 with a DB5 column (25 m, 0.2 id) with helium as a carrier gas, combined with a ITD 800 Finnigan-Mat mass selective detector. The ionization was provided by electron impact (energy 70 eV). The column temperature was maintained at 100°C for 1 min, raised to 250°C with a 10°C min^{-1} rate, and maintained at this temperature.

Conjugation of eugenol with lysine and arginine

Twenty µl of 50 mM eugenol in acetonitrile were mixed with lysine or arginine in 0.10 M phosphate buffer pH 7.4 (1.0 ml final volume) at 25 °C. The reaction was initiated by simultaneous addition of 30 nM HRP and 7.5 µM H_2O_2.

Eugenol treatment with alanine, propionic acid and γ-aminobutyric acid

Alanine, propionic acid and γ-aminobutyric acid (50 mM) were also incubated with the eugenol/HRP/H_2O_2 system under the same conditions as for analysis of lysine.

Chromatographic analysis of eugenol conjugates

Formation of eugenol-lysine conjugates in the reaction mixtures was monitored at 260 nm by reverse phase HPLC. The reactions were stopped by addition of 0.2 ml 50% trichloroacetic acid into the reaction mixture, followed by centrifugation and injection onto a 3.9 mm × 15 cm Waters Nova-Pak C-18 reverse phase column to remove HRP from the sample. Product elution was carried out through an isocratic 60% methanol mobile phase at a flow rate of 0.20 ml/min.

RESULTS

Spectrophotometric studies

Addition of micromolar amounts of HRP to eugenol in H_2O_2-containing buffered solution gave a product with an absorption peak at 350 nm (Figure 1). This product has previously been attributed to a quinone methide derivative[9]; eugenol has a maximum absorption at 280 nm. Pre-addition of 10 mM lysine to the HRP and eugenol-containing standard mixture resulted in

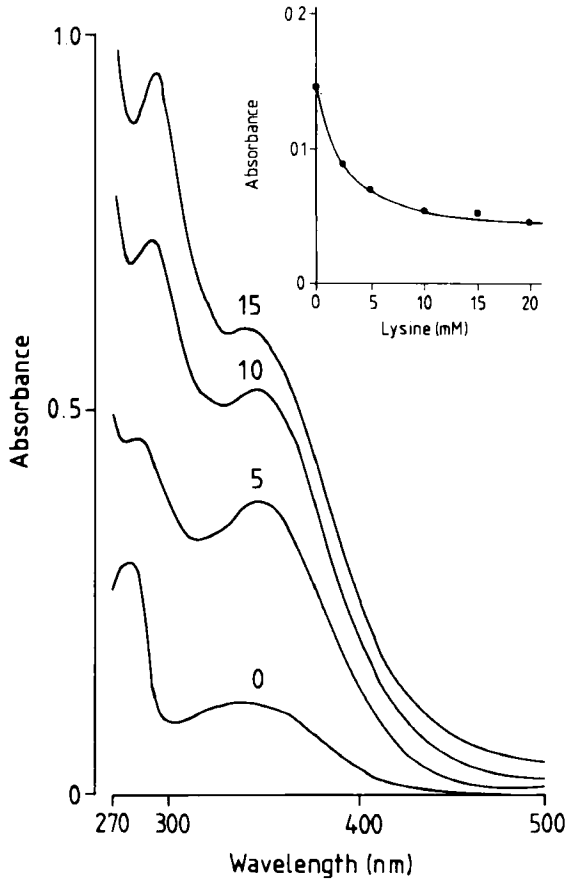


FIGURE 1 Spectral changes of the eugenol/H_2O_2/HRP system with the time. Conditions: eugenol (1.0 mM); H_2O_2 (7.5 μM); HRP (30 nM) in 0.10 M phosphate buffer pH 7.4 at 25 °C. Traces represent scans at 5 min intervals after initiating the reaction. Inset, concentration effect of lysine on the characteristic absorbance of the quinone methide from eugenol/H_2O_2/HRP at 5 min reaction time, followed at 346 nm, on a Beckman DU 70 spectrophotometer.

loss of the absorbance peak (330–360 nm) relative to the control, suggesting adduct formation in analogy to that observed with glutathione.[9] The absorbance decrease due to lysine addition occurred in a concentration-dependent manner (Figure 1, inset). In order to ascertain whether the α- or the ε-amino group is involved in the reaction with eugenol, the effect of various amino compounds on the rate of quinone methide formation was measured. Under the same experimental conditions, described in legend to Figure 1, the decrease of the quinone methide absorbance at 350 nm was negligible upon addition of 50 mM alanine (5%), but significant with 50 mM lysine (37%), 50 mM arginine (53%), or 50 mM γ-aminobutyric acid (32%). Propionic acid (50 mM) had no effect on the quinone methide absorbance, indicating that the carboxylic group is not involved in adduct formation. Since neither alanine nor propionic acid decreased the absorbance levels at 350 nm, it is conceivable that the ε-amino group but not the α-amino group of lysine is responsible for the reaction with eugenol/HRP.

Voltammetric studies

Evidence for eugenol-amino acid adduct formation was also obtained by cyclic voltammetry, with the phenoxyl radical being generated electrochemically rather than enzymatically. Figure 2A (curve a) shows the cyclic voltammogram of eugenol (17 mM) in acetonitrile-water (1:1 v/v) and its first oxidation peak potential at 1.00 V (*vs* Ag/Ag^+). When 30 μl of 0.30 M lysine was added, the oxidation potential dropped to 0.73 V (curve b). Upon addition of another 30 μl lysine aliquot, the oxidation potential became less positive (0.60 V) and the current intensity increased (curve c). The oxidation potential remained constant when more lysine was added to the solution. This same behavior was observed for arginine, in which case the oxidation potential dropped to 0.55 V (Figure 2B). An interesting aspect was observed when successive adduct voltammetric cycles were recorded: the oxidation potential increased concomitantly with a high decrease of the current intensity (Figure 2C). This is expected from electrode inactivation due to adduct polymerization. As a control, upon addition of NaOH to obtain the phenolate form of eugenol, the cyclic voltammetric waves were completely different from those traced with both arginine and lysine (data not shown).

When 3 mM reduced glutathione was added to 13 mM eugenol, slight changes towards higher (1.10 V) voltammetric field values were observed; no current intensification, however, was observed (data not shown). Accordingly, conjugation of

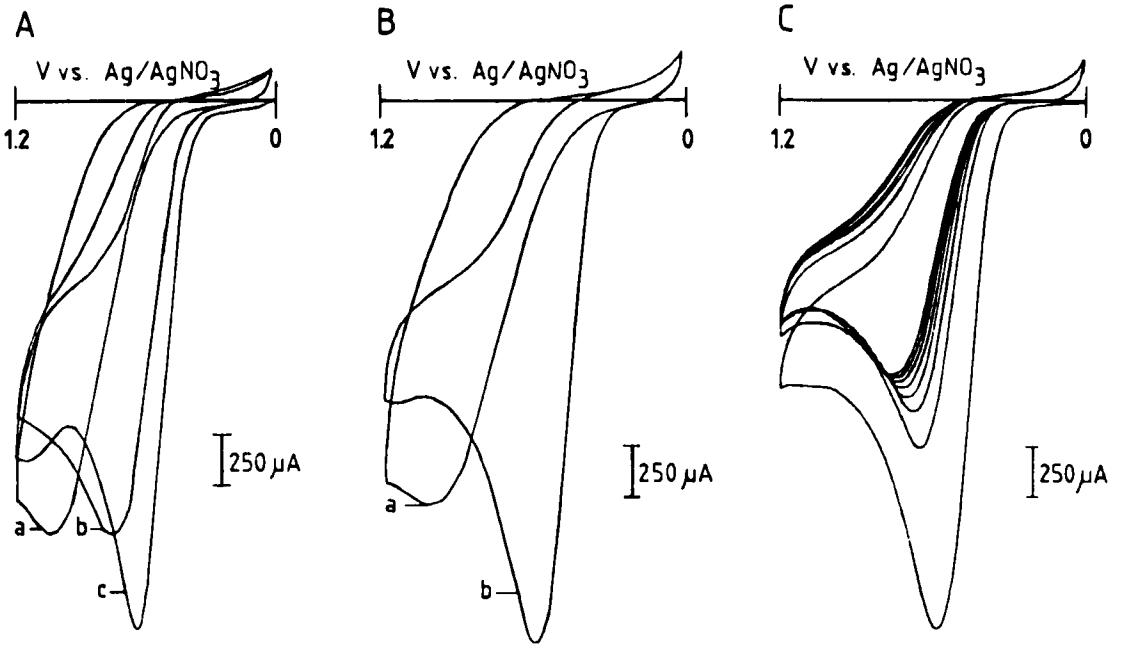


FIGURE 2 Cyclic voltammograms of eugenol in the absence and presence of lysine and arginine in acetonitrile:water (1:1 v/v). A: (a) eugenol (17.0 mM), (b) plus 1.5 mM lysine, (c) plus 4.5 mM lysine. B: (a) eugenol (13.0 mM), (b) plus 3.0 mM arginine. C: eugenol (13.0 mM) in the presence of arginine (3.0 mM) at consecutive cycles.

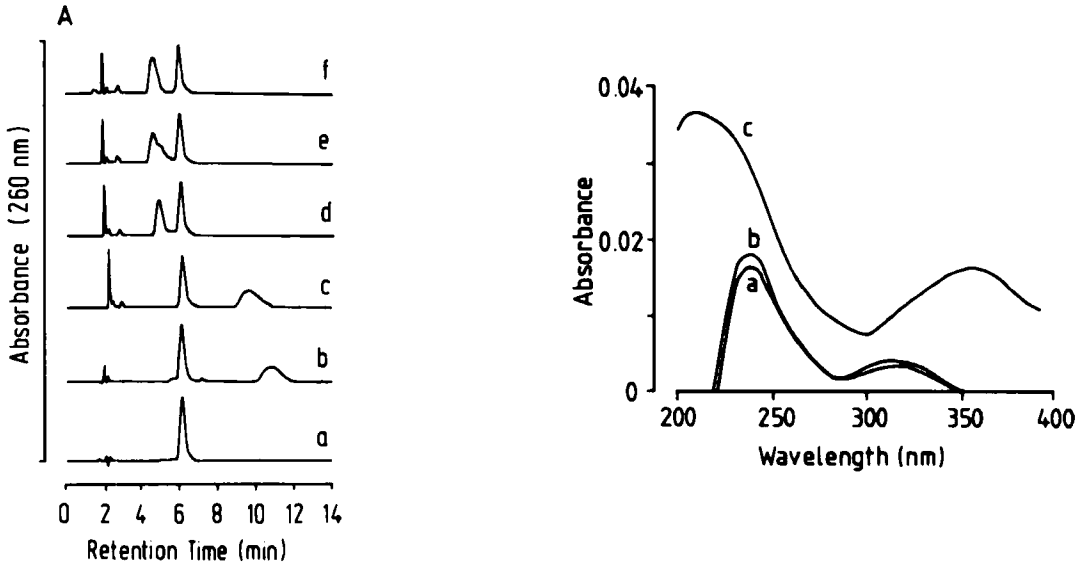
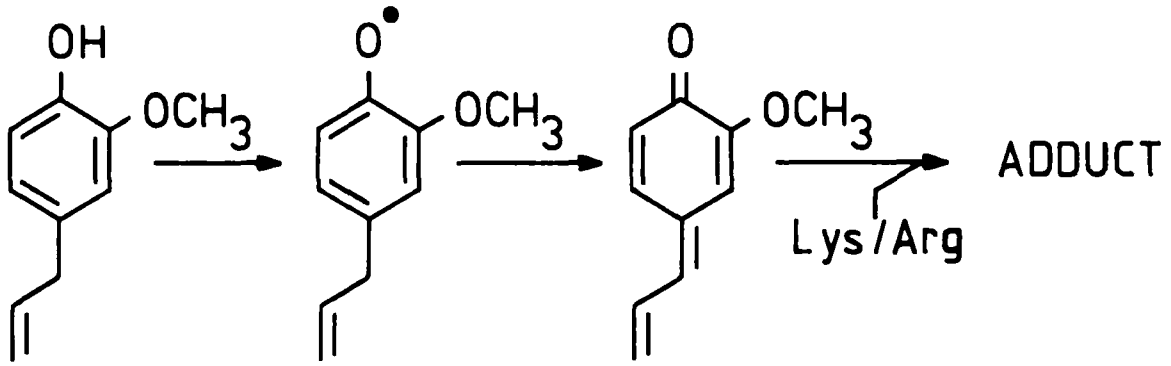


FIGURE 3 A, HPLC separation of the products from the eugenol/H_2O_2/HRP reaction mixture. Conditions as described in Fig. 1. Trace *a*, control (eugenol); *b*, *c*, *d*, *e* and *f* are traces for eugenol/H_2O_2/HRP system taken at 0, 15, 30, 60, 90 min, respectively. The reaction is started by addition of HRP(zero time). B, absorption spectra of the reaction product at *a*, 0; *b*, 15; *c*, 60 min reaction times, corresponding to the peaks with retention times at ca *a*, 11; *b*, 9; *c*, 5 min.



SCHEME 1 Lysine and arginine-eugenol adduct formation from the HRP-catalyzed aerobic oxidation of eugenol.

eugenol with glutathione, in the presence of HRP/H_2O_2, has also been reported.[9]

HPLC analysis and GC-MS adduct identification

Evidence for eugenol-amine adduct formation was also obtained by HPLC analysis. Aliquots of the reaction mixtures were analysed by reverse phase HPLC using isocratic mobile phase of 60% methanol as described in Materials and Methods.

Figure 3A depicts the HPLC chromatograms of the eugenol/H_2O_2/HRP system (control) at various reaction times. Upon HRP addition, the eugenol peak (*ca.* 6 min retention time) and two small peaks (*ca.* 2 and *ca.* 11 min retention time) were observed. The 11 min retained product collapses after 30 min reaction, with concomitant formation of another compound observed at *ca.* 5 min. The absorption spectrum of the eluted unstable product measured at 0, 15 and 60 min, showed a hypochromic shift from 320 to 350 nm (Figure 3B).

Figure 4A shows that in the presence of lysine, other products are formed. Immediately upon addition of HRP, a main peak with retention time of 3.2 min is observed. This product disappears with the time giving rise to another product with retention time of *ca.* 4.2 min. After 30 min reaction, only a stable product with 4.0 min retention time is formed. The corresponding peak does not change in the following 8 h of reaction. The absorption spectrum of the final product, after HPLC separation, shows maximum absorbance at 315 nm (Figure 4B).

When arginine is added in the beginning of the reaction, a stable HPLC peak with 3.6 min retention time is observed (Figure 5), having the same absorption spectrum (not shown) as that of the eugenol/lysine/HRP system.

In an attempt to identify the structure of the eugenol-lysine adduct, the reaction mixture was applied onto a SEP-PAK C18 cartridge to remove protein and eluted with 60% methanol. The eluates were colleted, evaporated to dryness and resuspended in CCl_4. The fraction with absorbance similar to that detected by HPLC attributable to the eugenol-lysine adduct (315 nm), was submitted to GC-MS analysis as described in Materials and Method. The analysis showed that this fraction contained a mixture of six products, of which only one gave a fragment attributable to the lysine residues (27 min GC retention time). The mass spectrum of this produce gave a base peak at m/z^+ 456 which can be assigned to the molecular peak consistent with a 1:2 eugenol:lysine adduct for $C_{22}N_4O_6H_{40}$; (M-CH_3), (M-H_3CO), (M-CH_2 = CH), (M-Lys) were observed at m/z^+ 441, 426, 429, 311, respectively.

DISCUSSION

The aerobic oxidation of eugenol catalyzed by HRP was previously shown to proceed *via* an

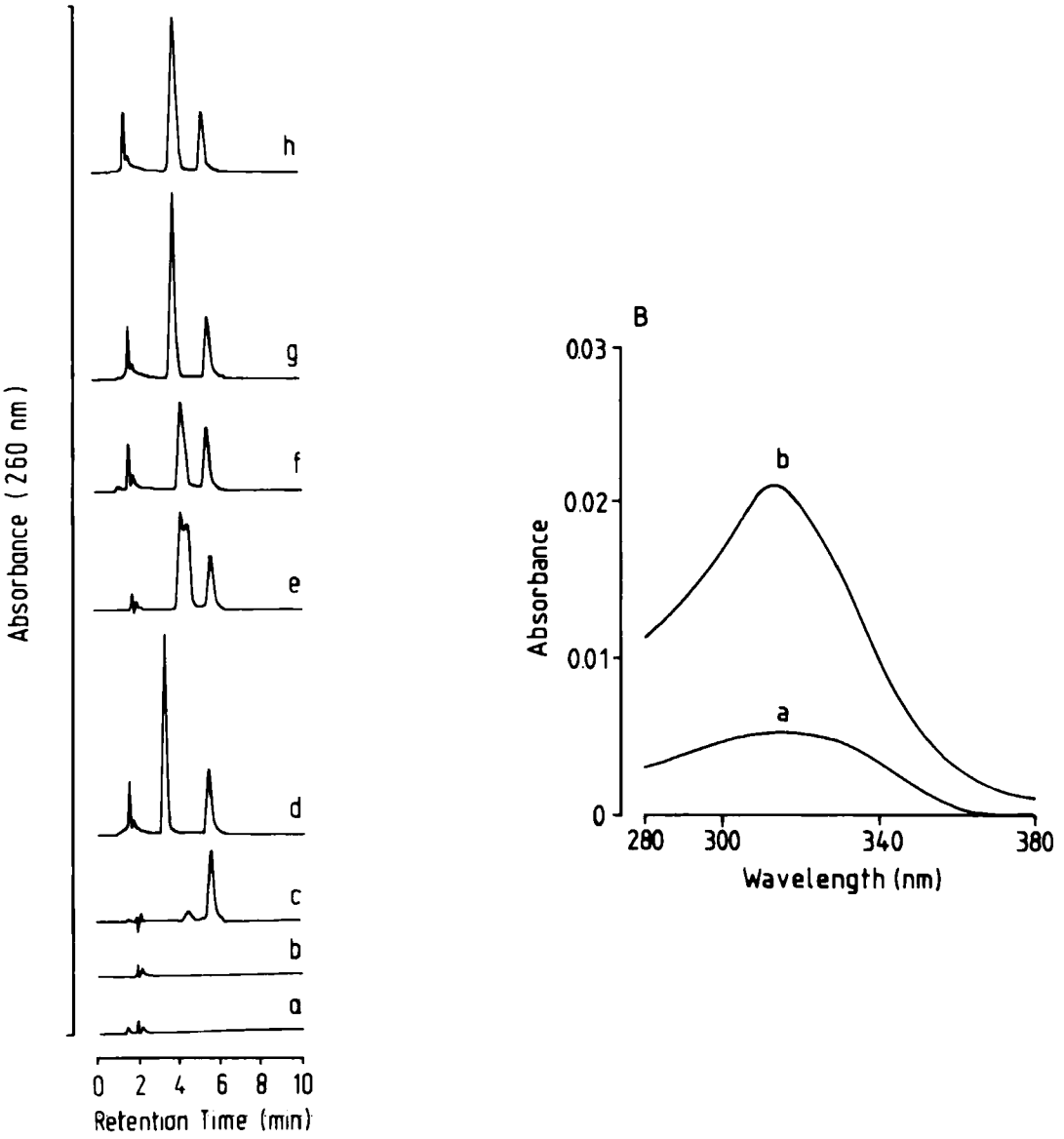


FIGURE 4 A, HPLC separation of the products from the eugenol/H_2O_2/HRP system in the presence of lysine (50 mM). Conditions as described in Fig. 1. Trace *a*, control (lysine); *b*, control (lysine/H_2O_2/HRP); *c*, control (eugenol); *d*, *e*, *f*, *g* and *h* reaction mixture at 0, 10, 15, 30, 60 min respectively. The reaction is started by addition of HRP. B, absorption spectra of the reaction products at *a*, 0 and *b*, 60 min retention times, corresponding to the peaks with retention times at 3.5 and 4.0 min, respectively.

one-electron transfer pathway, yielding a phenoxyl radical,[9] which is polymerized to a complex insoluble material. This is also suggested here by the inactivation of the electrode during cyclic voltammetry of either lysine or arginine and eugenol containing mixtures (Figure 2). In addition to polymerization, the phenoxyl radical can undergo further oxidation to a quinone methide, indicated by a characteristic absorption band at 350 mm.[9] Myeloperoxidase, eosinophil peroxidase, prostaglandin H synthase and rat intestinal peroxidase also give this H_2O_2-dependent reaction.[11] In the presence of reduced glutathione, an eugenol-glutathione conjugate is formed;

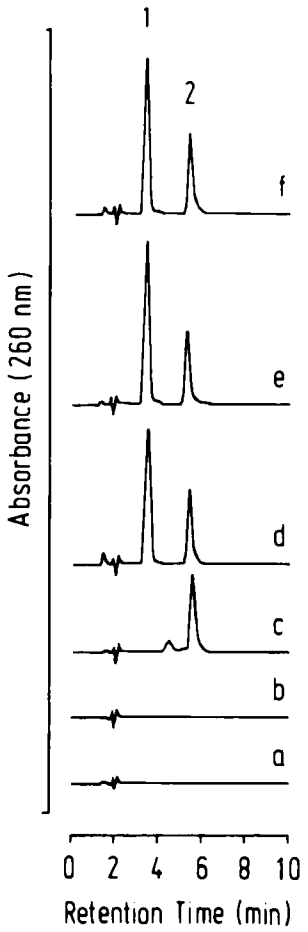


FIGURE 5 A, HPLC separation of the products from the eugenol/H_2O_2/HRP system in the presence of arginine (50 mM). Conditions as described in Fig. 1. Trace *a*, control (arginine); *b*, control (arginine H_2O_2/HRP); *c*, control (eugenol); *d*, *e* and *f* are due to eugenol/H_2O_2/HRP/arginine at 10, 60 min, respectively. The reaction is started by addition of HRP (time 0).

ascorbate and glutathione can also reduce the eugenol radical back to the parent compound.[9]

We have now reexamined the HRP-catalyzed oxidation of eugenol in the presence of amino nucleophiles such as lysine and arginine with the aim to explain possible conjugation between proteins and eugenol through amino acid adduct formation with the eugenol radical or with the quinone methide. Our results indicate that indeed these amino acids react with eugenol as judged from the loss of the quinone methide absorption at 350 nm and also from the concentration-dependence on lysine (Figure 1, inset).

Using the cyclic voltammetry technique, we also demonstrated formation of a compound with lower oxidation potential than that of eugenol from HRP/H_2O_2-treated eugenol-aminoacid mixtures (Figure 2). Pre-association of the amino acid with eugenol is suggested by the gradual shift in the oxidation potential from 1.00 V to 0.60 V (lysine) or 0.55 V (arginine) before reaching the constant value, even when more amino acid is added. The voltammetric runs pointed to a titration of the electrochemically generated eugenol derivative by the amino acid.

The lysine-eugenol adduct together with by-products, were further detected by HPLC analysis (Figure 3). Arginine and γ-aminobutyric acid, but not alanine, also lead to eugenol-adduct formation attesting to non-participation of the α-aminogroup of the amino acids in the reaction with the quinone methide intermediate as proposed by Thompson et al[9] when studying the eugenol glutathione cycle (Figure 5 and Results). The chemical identification of all products obtained from lysine and eugenol is quite complex because coupling of free radicals is expected to occur during the reaction, as demonstrated by Sarkanen and Wallis[12] using isoeugenol as substrate. The GC-MS analysis of the products obtained from lysine and eugenol reaction indicates formation of a 1:2 eugenol-lysine adduct. The accompanying products detected by GC-MS analysis are expected from eugenol radical polymerization as already mentioned by Thompson et al.[9]

From the biological point of view, formation of quinone methide may be responsible for covalent binding of eugenol to proteins[13] and hence their inactivation. It was suggested that eugenol can potentially interfere with the vital polymorphonuclear leukocyte functions. In this work we show that binding of eugenol to proteins may occur by conjugation of eugenol oxidation products with lysine and arginine. If operative *in vivo*, these processes could be implicated in the toxicological reactions involving eugenol.

Acknowledgments

This work was supported by the Conselho Nacional para o Desenvolvimento Científico e Tecnológico (CNPq), the Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) and the Interamerican Development Bank-University of São Paulo Program (BID-USP).

References

1. D.M. Dickey, A.H. El-Kafrawy and D.F. Mitchell (1974) Clinical and microscopic pulp response to a composite restorative material. *Journal of the American Dental Association*, **88,** 108–113.
2. G.C. Paffenbarger and N.W. Rupp (1979) In Kirk-Othmer Encyclopedia of Chemical Technology, Vol. 7, 3rd Ed., edited by M. Grayson (New York: John Wiley & Sons), pp. 463–464, 498–499.
3. A.A. Fernandes, J.W. Alencar, F.J. Abreu Matos, A.A. Craverio, C.H.S. Andrade, M.C. Fonteles, G.S.B. Viana, L.R. Capelo and F.F. Matos (1978) Canelas silvestres nordestinas: aspectos botânicos, químicos e farmacológicos. *Ciência e Cultura*, **32S,** 26–35.
4. I.A. Tavares, S. Jacobs, F. Capasso, N. Mascolo, G. Autor, V. Romano and G. Di Carlo (1988) The biological activity of eugenol, a major constituent of nutmeg (*Myristitca fragrans*): studies on prostaglandins, the intestine and other tissues. *Phytotherapy Research*, **2,** 124–130.
5. J.H. Jeng, L.J. Hahn, F.J. Lu, Y.J. Wang and M.Y.P. Kuo (1994) Eugenol triggers different pathobiological effects on human oral mucosal fibroblasts. *Journal of Dental Research*, **73,** 1050–1055.
6. A.B. Sell and E.A. Carlini (1976) Anesthetic action of methyleugenol and other eugenol derivatives. *Pharmacology*, **14,** 367–377.
7. W.R. Hume (1984) An analysis of the release and the diffusion through dentin of eugenol from zinc oxide-eugenol mixtures. *Journal of Dental Research*, **62,** 1013–1015.
8. P.G. Fotos, C.J. Woolverton, K. Van Dyke and R.L. Powell (1987) Effects of eugenol on polymorphonuclear cell migration and chemiluminescence. *Journal of Dental Research*, **66,** 774–777.
9. D. Thomspon, K. Norbeck, L. Olsson, D. Constantin-Teodosiu, J. Van der Zee and P. Moldéus (1989) Peroxidase-catalyzed oxidation of eugenol: formation of a cytotoxic metabolite. *Journal of Biological Chemistry*, **264,** 1016–1021.
10. K. Yokota and I. Yamazaki (1977) Analysis and computer simulation of aerobic oxidation of reduced nicotinamide adenine dinucleotide catalyzed by horseradish peroxidase. *Biochemistry*, **16,** 1913–1920.
11. M.L. Cotton and H.B. Dunford (1973) Studies on horseradish peroxidase XI: On the nature of compounds I and II as determined from kinetics of the oxidation of ferrocyanide. *Canadian Journal of Chemistry*, **51,** 582–587.
12. K. Sarkanen and A.F. Wallis (1973) Oxidative dimerizations of (E)- and (Z)-isoeugenol (2-methoxy-4-propenylphenol) and (E)- and (Z)-2,6-dimethoxy-4-propenylphenol. *Journal of the Chemical Society Perkin I*, 1869–1878.
13. D. Thomspon, D. Constantin-Teodosiu, K. Norbeck, B. Svensson and P. Moldéus (1989) Metabolic activation of eugenol by myeloperoxidase in polymorphonuclear leucocytes. *Chemical Research in Toxicology*, **2,** 186–192.