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Oxidative Activation of the Propolis Hapten Isoprenyl Caffeate

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The reactions of isoprenyl caffeate, the strongest allergen known to be present in propolis, with plausible functional groups of proteins were studied. The thiols glutathione and cysteine, which add to isoprenyl caffeate after having been oxidised to the corresponding quinone, served as model substances. The structure of the thiol adducts was determined by spectroscopic methods, addition being found to occur at C-2. The results support the hypothesis that the catecholic propolis hapten is a pro-hapten that forms a complete antigen after oxidation to caffeate quinone and addition to nucleophilic groups of proteins. ***Key words: thiol; glutathione; cysteine; quinone; allergic; contact dermatitis; 3-methylbut-2-enyl caffeate; pro-hapten.***

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Propolis, or bee glue, a product produced by bees, has a long tradition in many parts of the world of being used as a constituent in folk medicinal remedies (1). Although allergic contact dermatitis to propolis is reported occasionally in beekeepers, its major occurrence is in persons who make use of natural-product remedies or of "biocosmetics", many of which are derived from beeswax or contain it. When propolis is found in such products its presence is not always declared. At the same time, not all products derived from beeswax contain propolis.

Propolis is a complex mixture of many compounds, and gas chromatography-mass spectrometry (GC-MS) analysis has led to the identification of more than 150 of its constituents (2). Cases of allergic dermatitis to propolis have been described in recent papers (3, 4). Hausen et al. reported several esters of caffeic acid found in propolis to be strong sensitisers (5). The presence of the catecholic moiety of these esters was found to increase the sensitising capacity in comparison to esters of isoferulic acid, which also are present in propolis (Fig. 1). The strongest sensitiser that has been detected in propolis is isoprenyl caffeate **1** (3-methylbut-2-enyl caffeate = 1,1-dimethylallyl caffeate) (3, 4). In this paper a new method for the analysis of isoprenyl caffeate **1** is introduced, a method based on high performance liquid chromatography (HPLC).

The mechanism for the formation of a complete antigen from isoprenyl caffeate is unknown. Isoprenyl caffeate **1**, a catechol derivative having an α,β-unsaturated ester group in the side-chain, contains two functionalities frequently found in haptens (6). Either of these functional groups can be the site of a protein-coupling to the isoprenyl caffeate through the addition of a thiol group to the α,β-unsaturated ester, in analogy to the reaction of glutathione with other α,β-unsaturated esters (7), or to the caffeate quinone obtained after oxidation of the catechol function. The reactions of isoprenyl caffeate **1** with nucleophilic protein model compounds such as glutathione and cysteine were studied both before and after enzymatic oxidation of the catecholic structure to the corresponding quinone. Only the oxidised isoprenyl caffeate showed measurable reactivity. The adducts that were formed were isolated and their structures determined by nuclear magnetic resonance (NMR) and mass spectrometry.

MATERIALS AND METHODS

Chemicals

Methanol, tetrahydrofuran (THF) and acetone HPLC-grade (Lab-scan), phosphoric acid (Merck) and ethanol 99.5% UV-grade (Kemetyl, Sweden) were used as received. Cysteine was obtained from Janssen Chimica. Tyrosinase from mushroom (>2000 units/mg solid) and glutathione were obtained from Sigma Chemical. The water used was deionised on an Elgastat® spectrum water purification system. Isoprenyl caffeate was prepared synthetically according to a procedure developed by Wasser, Frosch and Benezra (8) and was then compared with synthetic material kindly supplied by Prof. P. Frosch. The synthesis involved a Wittig reaction of 3,4-dihydroxybenzaldehyde with triphenylisoprenyl phosphonium bromide, and the structure was confirmed by NMR and MS. Spectroscopic data on isoprenyl caffeate has been presented in a recent paper (9).

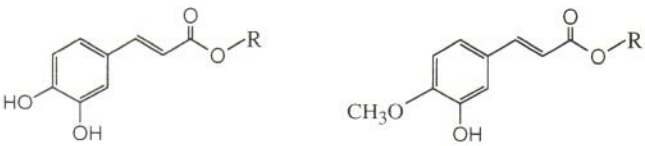
Chromatography

HPLC analysis was performed using a Waters 600 pump, a Nucleosil® 100–5C_{18} column (200×4.6 mm i.d.) and an LDC Spectromonitor D UV-detector set at 330 nm. Injections were made using a Rheodyne 7161 sampling valve injector provided with a 20 μl loop. The mobile phase consisted of aqueous phosphoric acid (25 mM, pH 3.50, 40%) and methanol (60%). The mobile phase flow rate at ambient temperature was 1.0 ml/min. Prior to injection into the HPLC system, samples were filtered through a syringe filter provided with a PTFE membrane (0.5 μm pore size).

A standard solution of isoprenyl caffeate **1** was prepared by dissolving isoprenyl caffeate (10 mg) in methanol (10 ml). The methanol solution was diluted with a KH_2PO_4-buffer solution (0.5 M, pH 6.5) to the desired concentration.

Spectroscopic analysis

^{1}H NMR spectra were recorded using Varian XL300 and Bruker ARX500 spectrometers, the chemical shifts being reported in parts per million (ppm) relative to CHD_2OD (3.31 ppm) and HDO (4.80 ppm), the coupling constants (J) being given in Hz. The atom numbers are given in Fig. 2. Mass spectra (FAB ionisation, positive ions) were recorded with a Jeol SX102 spectrometer. UV-spectra were obtained using a Perkin-Elmer 550 S UV-Vis spectrophotometer.



Ester of caffeic acid Ester of isoferulic acid

Fig. 1. The structures of esters of caffeic acid and esters of isoferulic acid.

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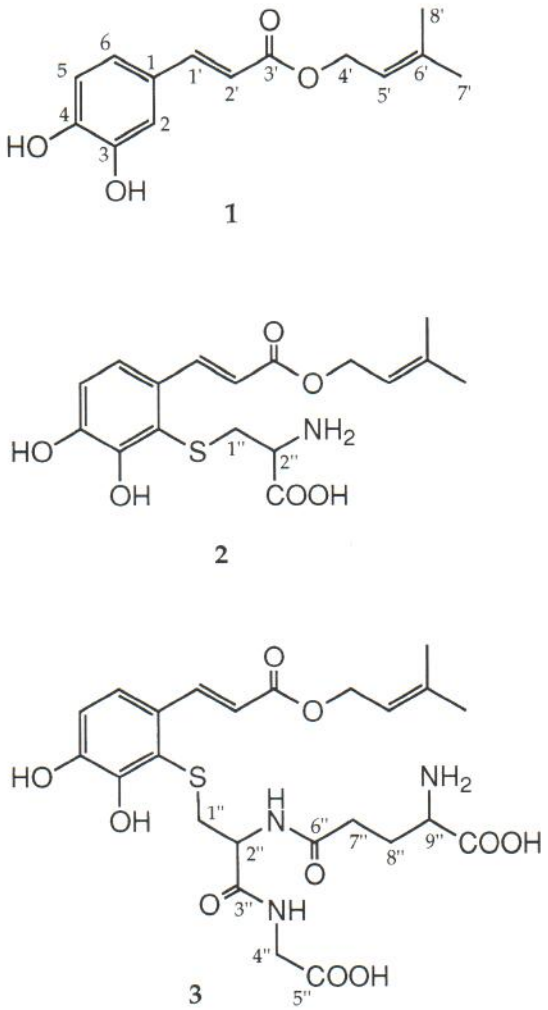

Fig. 2. The structures of isoprenyl caffeate **1**, and of its adducts with cysteine **2**, and with glutathione **3**.

Oxidation of isoprenyl caffeate 1 by tyrosinase in the presence of cysteine

To a solution of **1** (10.7 mg, 43.1 μmol) in methanol (1.0 ml) and 0.5 M KH_2PO_4-buffer (pH 6.5, 1.5 ml), mushroom tyrosinase (4.6 mg in 2.0 ml buffer) was added. Cysteine (25.0 mg, 206.3 μmol) was added to the solution dry. After 20 min the activity of the tyrosinase was stopped by adding HCl (6 M, 10 drops) to obtain pH = 3 in the reaction mixture. The mixture was cooled and then centrifuged (15 min, 5000 rpm). The pellet was dissolved in methanol (1.8 ml) and purified by column chromatography (Lobar®, LiChroprep® RP-18, 40–63 μm; water/acetonitrile/acetic acid, 75:25:1), yielding 1.1 mg (3.0 μmol, 7%) of 2-S-cysteinyl-isoprenyl caffeate **2** as a white solid. MS [m/z (% rel. int.)]: 390 M + Na^+ (35), 368 M + H^+ (10), 299 (20), 146 (75), 113 (100). UV (methanol/water) I_{max} 328, 250, 198 nm. ^1H NMR (500 MHz, CD_3OD): 8.45, d, $J_{1'-2'} = 15.9$, 1'-H; 7.17 and 6.83, d and d, $J_{5-6} = 8.4$, 5-H and 6-H; 6.32, d, $J_{1'-2'} = 15.9$, 2'-H; 5.41, tm, $J_{4'-5'} = 7.2$, 5'-H; 4.69, d, $J_{4'-5'} = 7.2$, 4'-H_2; 3.38, dd, $J_{1''a-2''} = 4.3$, $J_{1''b-2''} = 9.8$, 2''-H; 3.24, dd, $J_{1''a-1''b} = 14.1$, $J_{1''a-2} = 4.3$, 1''-Ha; 2.92, dd, $J_{1''a-1''b} = 14.1$, $J_{1''b-2''} = 9.8$, 1''-Hb; 1.78 and 1.77, s and s, 7'-H_3 and 8'-H_3.

Oxidation of isoprenyl caffeate 1 by tyrosinase in the presence of glutathione

To a solution of **1** (10.3 mg, 41.5 μmol) in methanol (0.5 ml) and 0.5 M KH_2PO_4-buffer (pH 6.5, 10.0 ml), mushroom tyrosinase (3.7 mg in 1.0 ml buffer) was added. Glutathione (63.0 mg, 205.0 μmol) in 1.0 ml buffer was added. After 5 min the activity of the tyrosinase was stopped by the addition of HCl (6 M, 40 drops) to obtain pH = 3 in the reaction mixture. The mixture was then purified by column chromatography (Lobar®, LiChroprep® RP-18, 40–63 μm; water/acetonitrile/acetic acid, 75:25:1), yielding 2.0 mg (3.6 μmol, 8.7%) 2-S-glutathionyl-isoprenyl caffeate **3** as a white solid. MS [m/z (% rel. int.)]: 554 M + H^+ (3), 277 (22), 240 (55). UV (Water) I_{max} 272, 326 nm. ^1H NMR (500 MHz, D_2O): 8.22, d, $J_{1'-2'} = 15.9$, 1'-H; 7.27 and 6.96, d and d, $J_{5-6} = 8$, 5-H and 6-H; 6.36, d, $J_{1'-2'} = 15.9$, 2'-H; 5.45, m, 5'-H; 4.72, d, $J_{4'-5'} = 7$, 4'-H_2; 4.23, dd, $J_{1''a-2''} = 4$, $J_{1''b-2''} = 8$, 2''-H; 3.68, t, $J_{8''-9''} = 7$, 9''-H; 3.59, s, 4''-H_2; 3.31, dd, $J_{1''a-1''b} = 15$, $J_{1''a-2''} = 4$, 1''-Ha; 3.08, dd, $J_{1''a-1''b} = 15$, $J_{1''b-2''} = 8$, 1''-Hb; 2.41, m, 7''-H_2; 2.04, m, 8''-H_2; 1.78 and 1.76, s and s, 7'-H_3 and 8'-H_3.

RESULTS

The addition of cysteine and glutathione to oxidised isoprenyl caffeate, resulting in the formation of the adducts **2** and **3**, respectively, was confirmed by MS and the structures of the adducts were determined by NMR. In both adducts two aromatic protons in ortho-position to one another (coupling constants $J_{5-6} = 8.4$ Hz and $J_{5-6} = 8.5$ Hz, respectively) could be detected. Thus the thiols add predominantly to the C-2 position in the aromatic ring.

The analysis of isoprenyl caffeate **1** and the adducts **2** and **3** that were formed was performed using the same chromatographic conditions (see Material and Methods). Isoprenyl caffeate showed a retention time of 12 min (k' = 4.2). The capacity factor was k' = 2.5 for compound **2** and k' = 1.9 for compound **3**, the same chromatographic system being used in both cases.

The non-oxidised isoprenyl caffeate did not react with cysteine or glutathione at a measurable rate. The enzymatic oxidation of isoprenyl caffeate **1** by tyrosinase proceeded smoothly, the quinone formed reacting with the cysteine and the glutathione. The adducts 2-S-cysteinyl-isoprenyl caffeate **2** and 2-S-glutathionyl-isoprenyl caffeate **3** were the main products, as shown by analysis with HPLC. The isolated yields were low, due to the fact that the amounts recovered during the purification steps were low, and to the fact that unreacted isoprenyl caffeate remained when the reactions were stopped. It was necessary to stop the reaction at the point chosen, since the thiol adducts formed would otherwise have been oxidised further to the corresponding quinones.

For the standard solution of isoprenyl caffeate, the chromatogram showed two peaks that were in equilibrium with each other. For the water-based solutions that we studied the two peaks were well separated, the first peak having an area of about 4% of the main peak. For the solutions in organic solvents such as methanol, in contrast, the first peak was about half the intensity of the main peak. An equilibrium of this sort may be indicative of some type of stereoisomerism, one that reaches a steady-state after several hours. The chemical basis of the equilibrium found here is currently under investigation.

DISCUSSION

Delayed allergic contact dermatitis is usually caused by organic haptens of low molecular weight. The haptens couple to a macromolecule, presumably a protein, to yield a complete antigen, which is then processed in the Langerhans' cells before being presented to the T-lymphocytes. The molecular structure of several thousand different haptens has been determined. Also,

the cellular morphologic changes following allergic reactions are well known. However, the interaction of haptens with a macromolecule prior to the processing to the resulting antigen in the Langerhans' cells is not well characterised chemically. Some haptens, such as acrylate and epoxy derivatives, contain electrophilic functions which react with nucleophilic sites in proteins, whereas other classes of haptens, e.g. the catechols, are devoid of reactive functionalities. However, in many instances such functionalities can be activated metabolically. It appears in the present case that the effects of the allergenic catechols may be caused by the enzymatic conversion of these to reactive ortho-quinones. This has been proposed earlier and has been found to be supported by changes in the UV-spectra of oxidised poison ivy catechols observed after the addition of albumin and chemical nucleophilic reagents (10). Schmidt et al. have proposed that a radical mechanism is responsible for the activation of phenols and that this mechanism may also apply to the catechols (11).

In the present study the chemical reactivity of isoprenyl caffeate, the strongest hapten known to be found in propolis, was examined. No reaction was registered when glutathione was added to the non-oxidised isoprenyl caffeate. The catecholic structure of isoprenyl caffeate was found to be easily oxidised, a reaction that was catalysed under mild conditions by mushroom tyrosinase. Tyrosinase is also an epidermal enzyme present in the melanocytes. Mushroom tyrosinase is known to be less specific than human tyrosinase (12, 13). Glutathione is the major intracellular low-molecular thiol. Its addition to electrophilic organic compounds is catalysed by glutathione-S-transferase. However, its addition to strongly electrophilic compounds such as ortho-quinones has been found to occur rapidly, even without enzymatic catalysis (14). The reactivity of the ortho-quinone of isoprenyl caffeate formed through oxidation by tyrosinase was studied here in the presence of a thiol, either glutathione or cysteine, which served as models of nucleophilic proteins. Both of the thiols added rapidly to the quinone, yielding 2-S-cysteinyl-isoprenyl caffeate **2** and 2-S-glutathionyl-isoprenyl caffeate **3**, respectively, as products. In earlier studies we have noted that glutathione adds predominantly in the 5-position to such ortho-quinones as dopaquinone and p-tert-butylquinone (14, 15). The unsaturated conjugated ester in the side-chain gives the isoprenyl caffeate quinone an electron distribution that differs from that of a non-conjugated ortho-quinone. This may facilitate a nucleophilic attack in the 2-position.

The products isolated in this study indicate isoprenyl caffeate to be a pro-hapten that can be oxidised enzymatically in the cells of the skin to a reactive intermediate, one which reacts with nucleophiles by addition, mainly to the 2-position. It appears reasonable to assume that the nucleophile may be either cysteine, glutathione or a thiol group of a protein. Since the thioether bond formed is a strong covalent bond, the protein-isoprenyl caffeate adduct can presumably be formed outside the Langerhans' cells and, after being phagocytosed, be processed in the Langerhans' cells to the allergenic antigen presented to the T-lymphocytes.

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