DEGRADATION OF 3-HYDROXYFLAVONE BY HORSE RADISH PEROXIDASE

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Summary: A hydroxylic group in position 3 and a double bond between positions 2 and 3 is the minimum requirement for flavones to be substrates for horse radish peroxidase (EC 1.11.1.7). 3-Hydroxyflavone, fulfilling these requirements, yields on enzymatic cleavage salicylic, phenylglyoxylic and probably benzoic acid.

Flavones have been recognized as a new class of substrates for peroxidases (1) and the degradation of flavones in plants is mediated by these enzymes (2). The flavones used as substrates in these studies had a rather complex substitution pattern, e.g. kaempferol having OH-groups at positions 3, 4', 5 and 7. Therefore, the minimum requirement of hydroxylic substituents in a flavone or flavanone for being a substrate of horse radish peroxidase was investigated. Using the simplest substrate found in these studies, 3-hydroxyflavone, we looked for its degradation products in order to understand the prevailing scission scheme.

MATERIALS AND METHODS

For all experiments horse radish peroxidase grade II (50 IU/mg, RZ 0.6, Boehringer-Mannheim) was used. In

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spectral runs, solutions of the substrates in diethylene glycol (approx. 1.5 mM) were diluted with 9 volumes 10 mM Na-pyrophosphate buffer (pH 8). 2 ml of the solution obtained were mixed in the cuvette with 0.2 ml 2.4 mM H_2O_2 and 50 µl enzyme solution (1 mg/ml). The reference cuvette contained all ingredients with the exception of the organic substrate. Spectra were run before and after adding H_2O_2 or enzyme. A preparative run with 3-hydroxyflavone was performed as described in reference 1. 3-Hydroxyflavanone was prepared according to reference 3.

RESULTS AND DISCUSSION

With flavone, flavanone and 3-hydroxyflavanone (isomeric mixture) no spectral changes were observed after the addition of H_2O_2 and peroxidase, while in the case of 3-hydroxyflavone, H_2O_2 and a small amount of peroxidase produced a gradual decrease of all maxima (fig. 1). Following the addition of more enzyme, a spectrum with maxima at approx. 310 nm and 250 nm developed. As the optical densities decreased at all wavelengths, no isobestic points could be observed.

The chromatographic separation of the acidic scission products from 3-hydroxyflavone, shown in fig. 2, permitted to identify salicylic acid both by its violet coloration with $FeCl_3$-spary and by UV and mass spectra (138 = M^+, 120 = $M^+ - H_2O$, 92 = $M^+ - H_2O - CO$) of the eluted material. The presence of phenylglyoxylic acid could be recognized, both by the UV spectrum of the eluted corresponding spot

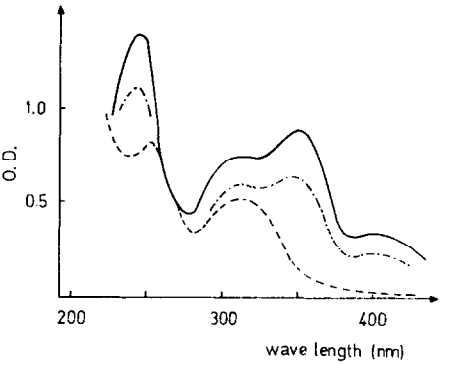

Fig. 1. Degradation of 3-hydroxyflavone by peroxidase and 0,21 mM H_2O_2.
(——) = only H_2O_2, (---) = with 220 mIU/ml enzyme after 5 min., (-.-) = with 2.2 mIU/ml enzyme after 80 min..

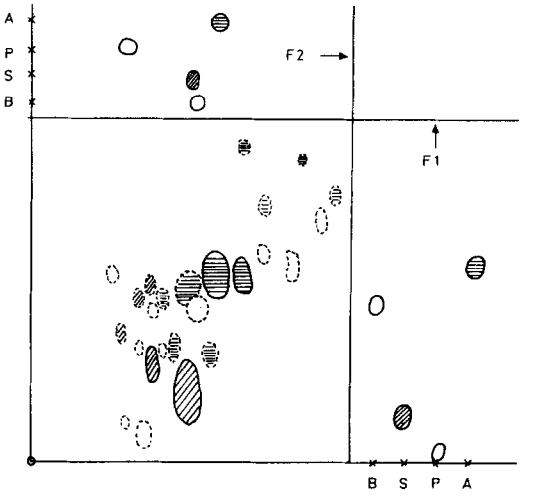

Fig. 2. Chromatogram of scission products from 3-hydroxyflavone and peroxidase/H_2O_2 (silica gel F_{254} Merck, activated. F1 = front solvent benzene-acetone-water-methanol 16/28/1/1, F2 = front solvent toluene-ethyl formate-formic acid 5/4/1. Detection by UV and $FeCl_3$-spray. B = benzoic acid, S = salicylic acid. P = phenylglyoxylic acid, A = 3-hydroxyflavone. ● = fluorescence at 366nm, ◍ = coloration with $FeCl_3$, ○ = absorption at 254 nm, ◌ = faint spots).

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and by its mass spectrum (m/e = 150, 105, 77, 51). Furthermore, the occurrence of benzoic acid among the degradation products is made highly probable, both by

chromatographic comparison with a reference sample and by the characteristic multiple UV maxima or shoulders of the eluted material at 276, 270, 263, 258 and 252 nm (fig. 3). A mass spectrometric confirmation, however, could

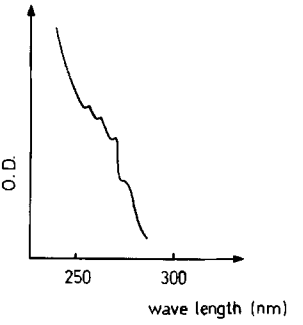
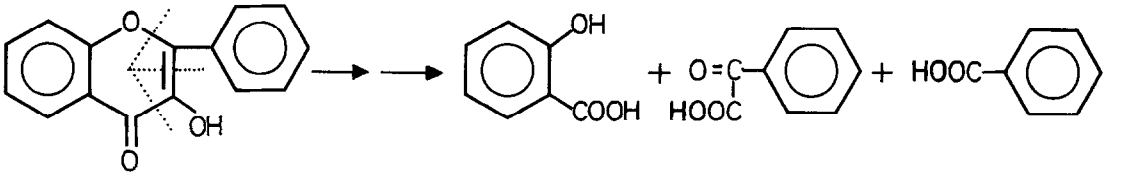


Fig. 3. UV-spectrum of the spot "benzoic acid" in methanolic solution.

not be achieved. In some runs a spot with bluish-white fluorescence was observed close to the spot of undegraded 3-hydroxyflavone. This fluorescent substance was slightly more lipophilic in solvent 2 than 3-hydroxyflavone: possibly this spot represents 2.3-dihydroxyflavanone formed in analogy to the reported hydratation of kaempferol by cell cultures of Cicer arietinum L. (4).

These results point to the following scission scheme of 3-hydroxyflavone



This formation of benzoic acid from ring B, is analogous

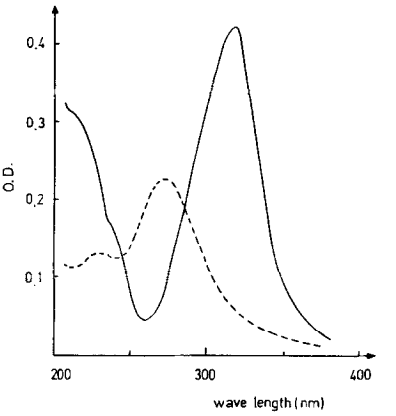


Fig. 4. Degradation of phloroglucine acetophenone by peroxidase (1.4 IU/ml) and 0.23 mM H_2O_2. (——) = spectrum in presence of H_2O_2, (---) = spectrum after 30 min. standing with enzyme.

to the reaction with morine (1). For the first time a molecule with intact ring A, salicylic acid, was observed. This conservation of ring A does not correspond in all respects to the current view about the enzymatic degradation of flavonoids (5) and differs from our own statements using morine (2'.3.4'.5.7-pentahydroxyflavone) as substrate (1). On the other hand, it has been reported that phloroglucine and its carboxylic acid are not degraded by action of cell suspension cultures (5). We therefore looked for the ability of peroxidase to degrade other phloroglucine derivatives and noticed, indeed, a very distinct reaction with phloroglucine acetophenone (fig. 4): the degradability of phloroglucine compounds thus depends strongly on their substituents.

Therefore, it appears that the conservation of ring A with 3-hydroxyflavone as substrate was possible only because of the absence of other hydroxylic groups in ring A. The

easy degradation of 3-hydroxyflavone by peroxidase-H_2O_2 in our experiments is, however, in contrast to the observation of 3.4'.7-trihydroxyflavone as a product in the conversion of 2'.4.4'-trihydroxychalcone by peroxidase-H_2O_2 by other authors (6).

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