ACTION OF HORSE RADISH PEROXIDASE UPON SOME FLAVONES

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1. Introduction

Flavonoids are easily degraded by microbial enzymes [1, 2]. There are even some reports available, in which the action of isolated fungal enzymes upon flavonoids is described: a laccase (EC 1.10.3.1) from *Polyporus versicolor* is able to attack 3-substituted flavonols and flavanones [3] and another dioxygenase from *Aspergillus flavus* degrades 3-hydroxyflavones to carbon monoxide and hydroxyphenylcarbonic acids [4].

There has been evidence, that in plants too metabolizm of flavonoids takes place, rutine giving for example protocatechuic acid and phloroglucinol-carboxylic acid [5], whereas with an extract from *Cicer arietinum L.* a formal hydratation of the $C_2 - C_3$ double bond of 3-hydroxyflavones has been reported [6]. This reaction occurred only in the presence of both oxygen and mercaptoethanol.

In no case have isolated plant enzymes been used for the conversion of flavonoids.

We want to report a reaction of a single enzyme, horse radish peroxidase (EC 1.11.1.7), with some flavonoids, which may be of some interest for studies of flavonoid metabolism in plants.

2. Materials and methods

Most experiments have been performed with horse radish peroxidase from Calbiochem, grade B. In some instances electrophoretically pure peroxidase from Worthington has been used. Morin p.a. (Riedel de Haën) has been recrystallized twice from aqueous methanol, rutin puriss. (Roth), quercetin puriss. (Fluka), kaempferol puriss. (Roth) and hesperidin krist. (Merck) were used as such. For the isolation of morin scission products 2 l of its 0.17 mM solution in 75 mM pyrosphosphate buffer of pH 8 were mixed with 0.1 mg peroxidase (Calbiochem) in 1 ml buffer and 2 ml of a 0.22 M H_2O_2 solution. The additions of enzyme solution were repeated at intervals of 20 min until no further change of absorption at 405 nm was noticed. The same was done with further additions of H_2O_2. The resulting solution was acidified with HCl to pH 2, extracted with ethyl acetate and the content of the extract was isolated and chromatographed as usual.

Enzyme assays have been performed using guaiacol as substrate [7].

3. Results and discussion

On mixing solutions of kaempferol (3,4′, 5,7-tetrahydroxyflavone), morine (2′, 3, 4′, 5, 7-pentahydroxyflavone), quercetin (3, 3′, 4′, 5, 7-pentahydroxyflavone) or rutin (3-[6-0-(α-L-rhamnopyranosyl)-β-D-glucopyranosyloxy]-3′, 4′, 5, 7-tetrahydroxyflavone) in ethanol with solutions of horse radish peroxidase and H_2O_2 in buffer very distinct changes of the absorption spectra of these solutions, are observed, which do not occur, when enzyme is omitted from the mixture (fig. 1a–d).

With hesperidin (a 3′, 5-dihydroxy-4′-methoxy-7-[6-0-(α-L-rhamnopyranosyl)-β-D-glucopyranosyloxy]-flavanone) almost no spectral changes result (fig. 1e).

These few observations allow only a limited speculation on the minimum structural requirement for the action of peroxidase on flavones: the 3-hydroxyflavone structure seems necessary, but it cannot be said, whether the 3 OH-group may be glycosylated, since rutin possesses two adjacent OH-groups in the B ring, which may fulfill per se the structural requirement for a peroxidase substrate [8].

Using morin as substrate we determined the H_2O_2-

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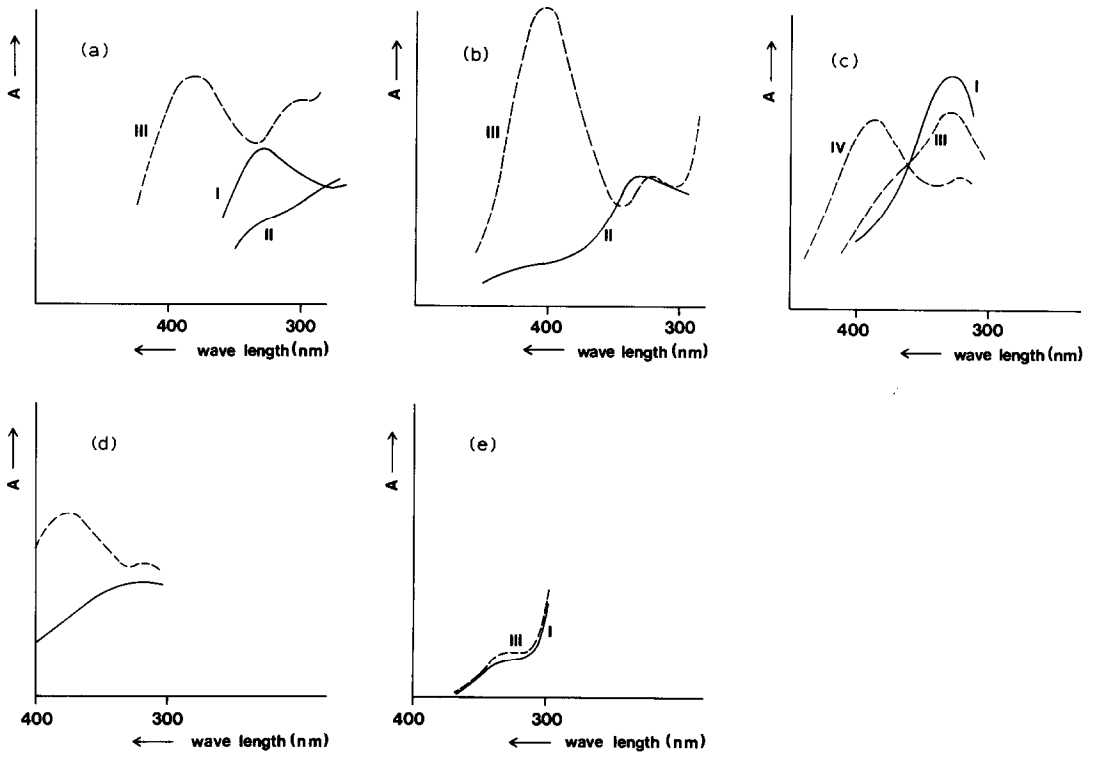

Fig. 1. a–e: Spectral changes of flavone and flavanone solutions upon action of horse radish peroxidase and H_2O_2. a = kaempferol (45 μM, 0.18 mM H_2O_2, 10 mM pyrophosphate pH 8, 25°C, I = in presence of 1.2 IU enzyme/ml after 90 sec, II = same as in I but after 30 min, III = without enzyme); b = morin (90 μM, 0.25 mM H_2O_2, 10 mM pyrophosphate pH 9, 40°C, II = in presence of 0.16 IU enzyme/ml after 30 min, III = without enzyme); c = quercetin (48 μM, other conditions as in a, I = in presence of 1.2 IU enzyme/ml after ca. 90 sec, III = without enzyme after 90 sec, IV = without enzyme after 90 min); d = rutin (42 μM, other conditions as in a, I = in presence of 1.2 enzyme/ml after 90 min, III = without enzyme); e = hesperidin (53 μM, other conditions as in a, I = in presence of 1.2 IU enzyme/ml after 90 min, III = without enzyme). Kaempferol, quercetin and rutin were added as ethanolic solutions, hesperidin as solution in dimethylsulfoxide immediately before running the spectra.

requirement (fig. 2), which was identical to the H_2O_2-requirement with guaiacol as substrate. The pH-optimum for the morin oxidation is near 8 (fig. 3). Furthermore, we observed, an inhibition of the reaction at high substrate concentrations. In order to get some insight into the reaction sequence we tried to isolate products from the reaction mixtures with morin. The resulting thin layer chromatogram is shown in fig. 4: we are almost certain, that 2.4-dihydroxybenzoic acid is one of the many products present, both by identical chromatographic behaviour with authentic material (the spot giving a blue fluorescence) and by the mass spectrum of the material eluted from the chromatogram (*m/e* = 44, 81, 82, 110).

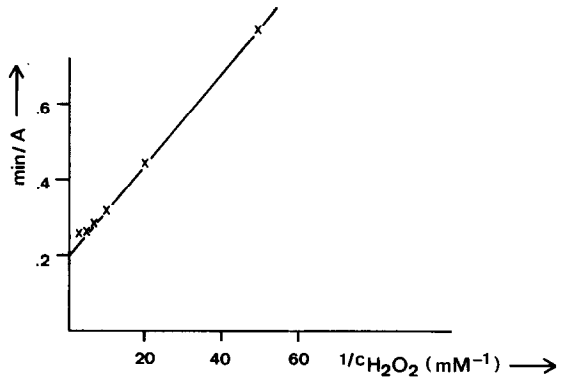

Fig. 2. H_2O_2-requirement for the oxidation of morin by horse radish peroxidase (19 mIU/ml, 105 μM morin, 10 mM pyrosphate pH 7.5, 25°C, wavelength 405 nm).

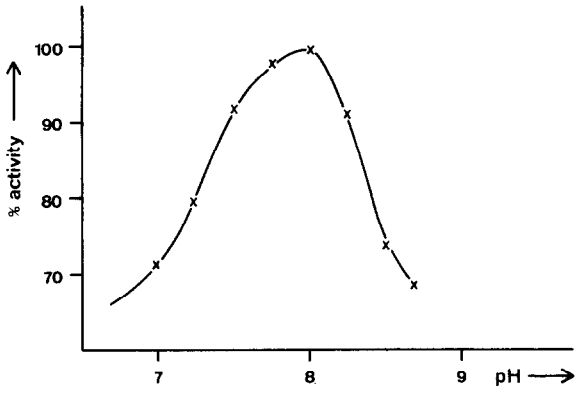

Fig. 3. pH-optimum of morin degradation by horse radish peroxidase (19 mIU/ml, RZ 3.1 Worthington, 80 μM morin, 0.14 mM H_2O_2, 10 mM pyrophosphate + 10 mM phosphate, 25°C, wavelength 405 nm).

The presence of phloroglucinolcarboxylic acid and phloroglucinolglyoxylic acid in the scission products cannot be excluded.

With kaempferol, there is clear evidence of a biphasic, consecutive reaction: immediately after mixing the solutions one observes a new maximum at 330 nm, which disappears after another 30 min incubation.

Our results point to a pathway of flavonoid degradation in plants, in which oxidative enzymes such as peroxidase are involved, and which seems to be different from reported degradations [6].

In the meantime a report has been published by W. Barz and his group referring to peroxidases as responsible for the degradation of some flavonols, flavanones, aurones and chalcones in cell cultures of various plants (Z. Physiol. Chem. (1973), 354, 1170, 1203).

Acknowledgement

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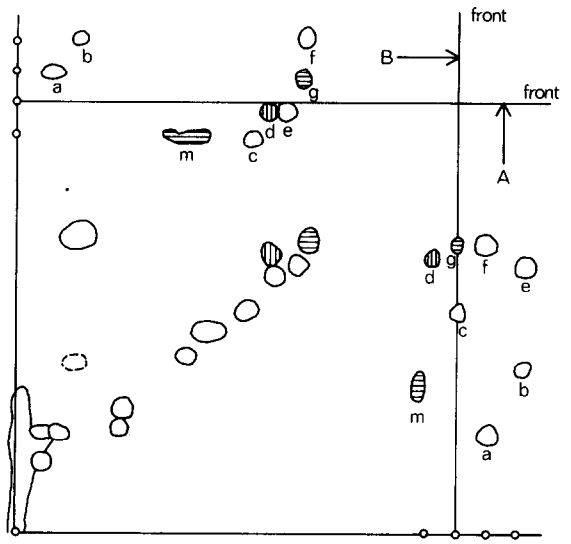

Fig. 4. Chromatogram of morin scission products by peroxidase/H_2O_2 (silica gel F_{254} Merck) activated. direction A: CH_2Cl_2–ethyl acetate–formic acid = 5:4:1. direction B; benzene–acetone–methanol–water = 80:140:5:5. Detection by UV and spraying with Echtblausalz®-solution. m = morin, a = phloroglucinolglyoxylic acid, b = phloroglucinolcarboxylic acid, c = phloroglucine, d = 2,4-dihydroxybenzoic acid, e = phloroglucinolacetophenone, f = 2,4-dihydroxyacetophenone, g = 2,4,4′-trihydroxychalcone, ◐ = blue fluorescence, ⊖ = yellow fluorescence).

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