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Regioselective hydroxylation of diverse flavonoids by an aromatic peroxygenase

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ABSTRACT

Aromatic peroxygenases are extracellular fungal biocatalysts that selectively oxidize a variety of organic compounds. We found that the peroxygenase of the fungus *Agrocybe aegerita* (*Aae*APO) catalyzes the H_2O_2-dependent hydroxylation of diverse flavonoids. The reactions proceeded rapidly and regioselectively yielding preferentially monohydroxylated products, e.g., from flavanone, apigenin, luteolin, flavone as well as daidzein, quercetin, kaempferol, and genistein. In addition to hydroxylation, *O*-demethylation of fully methoxylated tangeretin was catalyzed by *Aae*APO. The enzyme was merely lacking activity on the quercetin glycoside rutin, maybe due to sterical hindrance by the bulky sugar substituents. Mechanistic studies indicated the presence of epoxide intermediates during hydroxylation and incorporation of H_2O_2-derived oxygen into the reaction products. Our results raise the possibility that fungal peroxygenases may be useful for versatile, cost-effective, and scalable syntheses of flavonoid metabolites.

1. Introduction

Flavonoids are the most incident antioxidants in higher plants with a large structural diversity.[1] The precondition to their radical scavenging effect is the number and location of phenolic groups.[2] For example the *ortho*-dihydroxy (catechol) substitution raise the radical scavenging activity.[3] However, the synthesis of these complex compounds is complicated given that the selective transfer of oxygen atoms to non- or little activated carbons is still a challenging reaction in chemical synthesis.[4] Therefore, multi-step syntheses are predominantly used in industrial processes of chemical hydroxylation.[5,6] Though progress has been reported in using hydrogen peroxide and metal catalyst for the oxidation of benzene and toluene derivatives, the number of direct hydroxylations as well as their selectivity is still limited.[7,8] More recently, a paper reported an efficient one-step oxidative modification of hydroxylated flavonoids with 2-iodoxybenzoic acid (IBX) in order to obtain catecholic flavonoids but not C-6 hydroxylation was observed.[9] Another approach would be to use biocatalysts, such as cytochrome P450 monooxygenases (P450s) for highly selective one-step reactions under environmentally sound conditions.[10] Nowadays this type of biotransformation is rarely used in chemical industry and restricted to whole cell processes, since P450s are poorly stable, catalytically slow, and require expensive cofactors as well as associated proteins.[11] Another approach, the use of laboratory-evolved and engineered P450s for the H_2O_2-dependent monooxygenation via the so-called peroxide 'shunt' pathway, has been demonstrated but needs further optimization.[12] Thus, biotransformations based on the activity of stable extracellular oxidoreductases would offer an elegant alternative. Possible candidates are found within the fungal proteomes including oxidases, peroxidases, as well as aromatic peroxygenases (APOs, EC 1.11.2.1).[13] Enzymes of the latter group have been found in agaric basidiomycetes, and act as functional hybrids of heme thiolate peroxidases and P450s.[14] The best-characterized fungal aromatic peroxygenase, from *Agrocybe aegerita* (*Aae*APO), is involved in the H_2O_2-dependent hydroxylation/epoxidation of aromatic rings and benzylic compounds,[14–20] phenol oxidation,[15,17] sulfoxidation of tricyclic heterocycles,[16] N-oxidation of pyridine derivatives,[21] and cleavage of diverse ethers.[22] Here we demonstrate the catalytic potential of APOs for the H_2O_2-dependent regioselective hydroxylation of diverse flavonoids by the use of *Aae*APO.

2. Results

2.1. Hydroxylation of flavonoids

In qualitative experiments done with continuous H_2O_2 supply, we found that *Aae*APO monooxygenated diverse flavonoids including flavones, flavanones, flavonols, isoflavons, and anthocyans (Table 1). The products were ring-hydroxylated compounds, which

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were identified by HPLC/MS based on authentic standards or via NMR. Notably, quercetin (**2**), daidzein (**4**), apigenin (**5**), kaempferol (**6**), and luteolin (**8**) yielded only one monohydroxylated flavonoid (MHF), whereas genistein (**1**) gave two MHFs. The flavonoids naringenin (**3**), flavone (**9**, and its derivatives 7- and 5-hydroxy flavone), and flavanone (**7**, and its derivatives 7-, 2′- and 3′-hydroxyflavanone) yielded diverse reaction products including MHFs, dihydroxylated products, and other derivatives. The flavonoid diglycoside rutin (**10**) was not oxidized by *Aae*APO. Some of the MHFs formed during the *Aae*APO-catalyzed oxidation were further characterized.

Table 1
Mono-hydroxylated products identified by mass spectrometry in the course of flavonoid oxidation by *Aae*APO. The *m*/*z* value for the major observed diagnostic ion as well as the yield for selected products is given. TC—total conversion

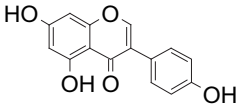
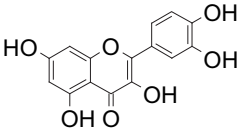
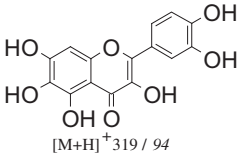
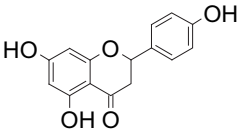
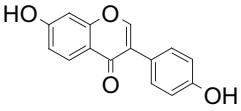
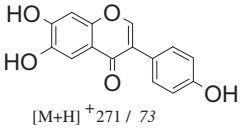
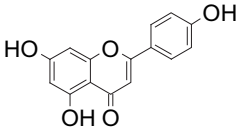
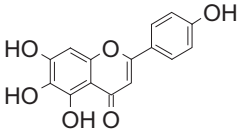
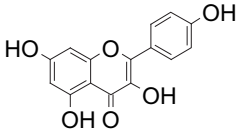
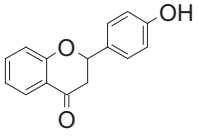
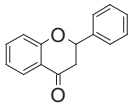
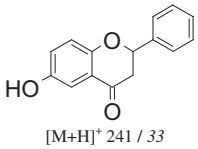
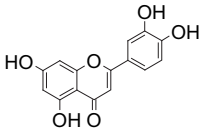
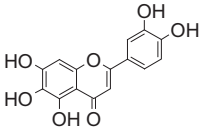
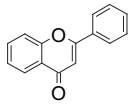
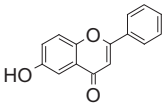
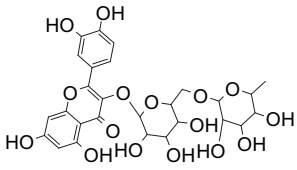
	Substrate	TC (%)	Product(s) *mz*/yield (%)
1		99	$[M+H]^+$ 287[b] $[M+H]^+$ 287[b]
2		94	 $[M+H]^+$319 / *94*
3		77[a]	$[M+H]^+$ 289[b] $[M+H]^+$ 289[b]
4		73	 $[M+H]^+$271 / *73*
5		51	 $[M+H]^+$287 / *42*
6		47	$[M+H]^+$ 303[b]
			$[M+H]^+$ 241 / *4*
7		43	
			$[M+H]^+$ 241 / *33*

Table 1 (*continued*)

	Substrate	TC (%)	Product(s) *mz*/yield (%)
8		33	 $[M+H]^+$ 303
9		13	 $[M+H]^+$ 239 / *5*, $[M+H]^+$ 239[b], $[M+H]^+$ 239[b]
10			No product formation

[a] An unidentified reaction product appeared.
[b] A dihydroxylated derivative was detected.

For example, 4′-hydroxyflavanone and 6-hydroxyflavanone were released during the *Aae*APO-catalyzed oxidation of flavanone (**7**). Moreover, products of the *Aae*APO-catalyzed oxidation of apigenin (**5**) and luteolin (**8**) showed molecular ions at *m*/*z* 287 $[M+H]^+$ and *m*/*z* 303 $[M+H]^+$ indicating the incorporation of an hydroxyl group. The respective ^1H and ^{13}C NMR spectra in both cases, confirmed a new hydroxyl group at the C6-position with chemical shifts of 129.19 ppm (instead of 98.90 ppm) for 6-hydroxyapigenin and 129.19 ppm (instead of 98.88 ppm) for 6-hydroxyluteolin (Fig. 1).

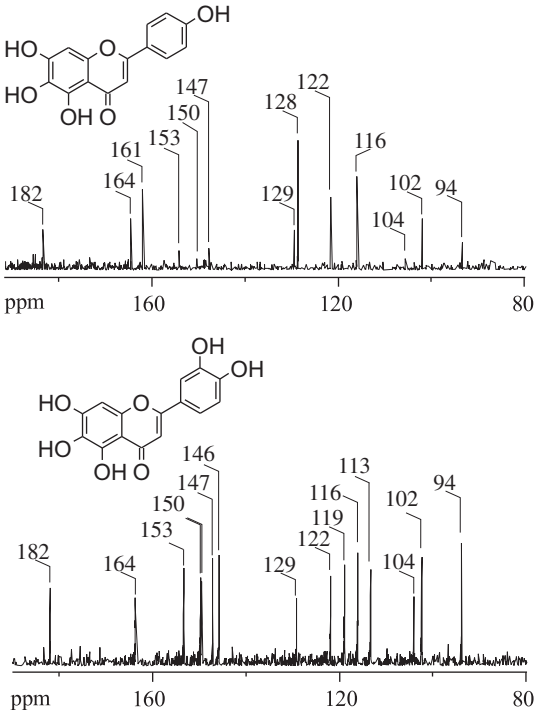


Fig. 1. ^{13}C NMR spectra of products obtained from the reaction of *Aae*APO with apigenin and luteolin. Upper: 6-hydroxyapigenin, Lower: 6-hydroxyluteolin.

2.2. Source of the oxygen introduced during hydroxylation of flavonoids

The reaction mechanism of flavonoid hydroxylation was investigated to identify the source of incorporated oxygen. HPLC/MS analysis showed that the peroxygenase-catalyzed hydroxylation of daidzein (**4**) in the presence of 90 atom % $H_2{}^{18}O_2$ resulted in 90% ^{18}O incorporation into the hydroxyl group of the resulting 6-hydroxydaidzein, as evidenced by the shift of the principal molecular ion from *m*/*z* 271 to *m*/*z* 273 (Fig. 2).

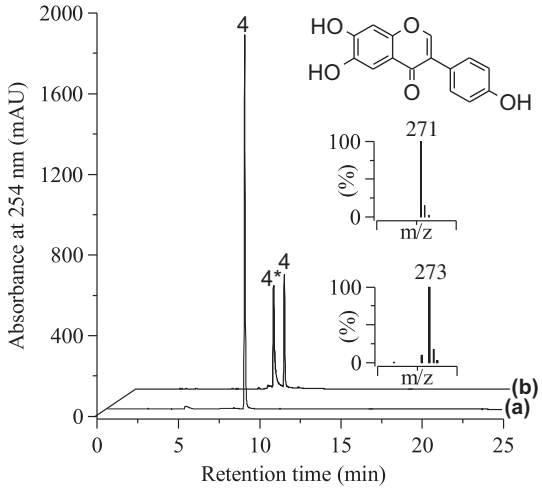


Fig. 2. HPLC elution profiles of a reaction mixture obtained after the *Aae*APO-catalyzed oxidation of daidzein (**4**; b) and control without enzyme (a). Insets show the molecular ions of 6-hydroxydaidzein (**4***) obtained from the oxidation of daidzein by *Aae*APO in the presence of natural abundance H_2O_2 (top) and $H_2{}^{18}O_2$ (bottom). The latter proves the incorporation of peroxide-oxygen into the phenolic group.

2.3. Epoxide formation

The possible formation of epoxide intermediates was studied for the reaction of *Aae*APO with flavone (**9**; Fig. 3) and in fact, the formation of a corresponding epoxide-like reaction product (proposed to be 5,6-dihydro-5,6-epoxyflavone or 6,7-dihydro-6,7-epoxyflavone) (**9′**) with a molecular mass of 238 and a characteristic UV-absorption spectrum (maxima 267 nm and 294 nm) was observed. This intermediate spontaneously hydrolyzed under acidic conditions to form 6-hydroxyflavone (**9***) with the same molecular mass but a different UV spectrum (maxima at 269 nm and 305 nm).

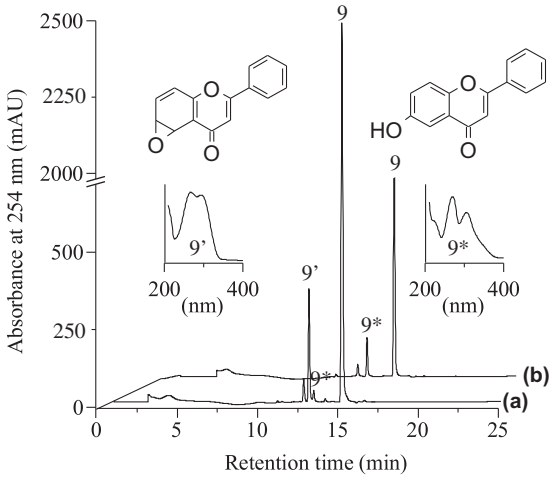


Fig. 3. HPLC elution profiles of reaction mixtures obtained from the *Aae*APO-catalyzed oxidation of flavone (**9**). (a): original reaction mixture at pH 9; (b): same reaction mixture at pH 2 after acidification with TCA. The insets show the UV spectrum of the suggested flavone oxide (**9′**) and 6-hydroxyflavone (**9***).

2.4. Kinetics

Since apigenin (**5**) was well oxidizable by *Aae*APO and gave a single reaction product, we used a calibrated HPLC method to monitor the production of 6-hydroxyapigenin at pH 7.0, assuming steady-state conditions. The results gave a k_{cat} of 26 s^{-1} and a K_m of 290 μM (catalytic efficiency; $k_{cat}/K_m = 8.97\times10^4$ s^{-1} M^{-1}).

2.5. *O*-Demethylation of tangeretin

Beside ring-hydroxylation, *O*-demethylation of tangeretin (5,6,7,8,4′-pentamethoxyflavone, Fig. 4) was observed, and although in this case no authentic standard was available, the *m*/*z* value and the UV data of the reaction product strongly indicated the formation of 4′-hydroxy-5,6,7,8-tetramethoxy-flavone (*m*/*z* 359 $[M+H]^+$, UV-absorption maxima at 270 nm and 325 nm, Fig. 4). Formaldehyde, the second reaction product of this ether cleavage, was identified as its 2,4-dinitrophenylhydrazone derivative (*m*/*z* 209 $[M-H]^-$).

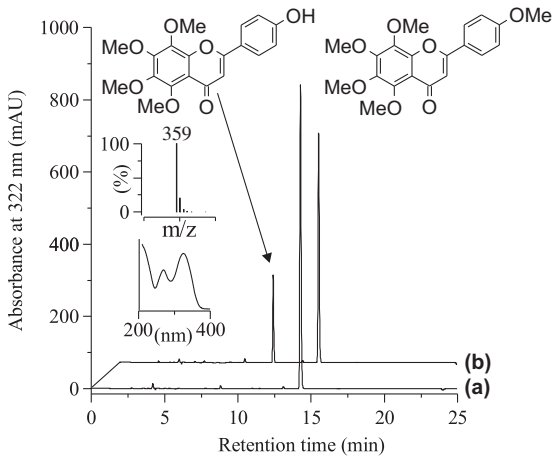


Fig. 4. HPLC elution profiles of a reaction mixture obtained from the *Aae*APO-catalyzed demethylation of tangeretin (b) and a respective control without enzyme (a). The insets show the mass (ESI-MS) and the UV spectra of the proposed metabolite 4′-hydroxy-5,6,7,8-tetramethoxyflavone.

3. Discussion

*Aae*APO selectively hydroxylated a variety of flavonoids in the presence of hydrogen peroxide, predominantly at the C6-position. We could show by ^{18}O-labeling studies that the *Aae*APO-catalyzed hydroxylation of flavonoids is a true peroxygenation, i.e., the transferred oxygen comes from the cosubstrate, H_2O_2. The results further indicate that the ring-hydroxylation of flavonoids proceeds via epoxide intermediates (Fig. 5). This picture is consistent with previous results, which proved the initial formation of an epoxide in the course of naphthalene oxidation by *Aae*APO yielding, after hydrolysis, 1-naphthol and 2-naphthol as major and minor products, respectively.[18]

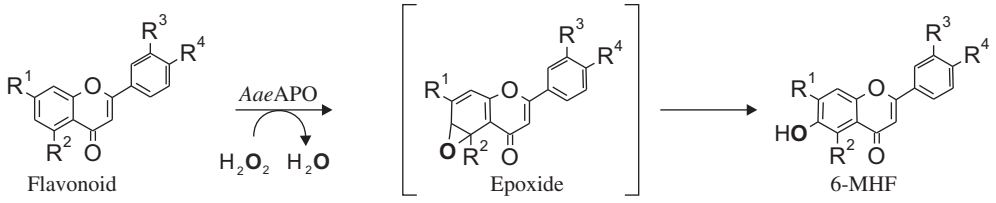


Fig. 5. Proposed reaction mechanism of flavonoid hydroxylation by *Aae*APO.

The kinetic values observed during apigenin hydroxylation established that the apparent catalytic efficiency (k_{cat}/K_m=8.97×10^4 s^{-1} M^{-1}) ranges between the data observed for the *Aae*APO-catalyzed N-oxidation[21] (3.04×10^3 s^{-1} M^{-1}) on the one hand and aromatic ring-hydroxylation[23] (5.17×10^5 s^{-1} M^{-1}) as well as *O*-dealkylation[22] (5.03×10^5 s^{-1} M^{-1}) on the other hand. It is interesting to compare the above values with those obtained for functionally similar enzymes. Some P450s that hydroxylate flavonoids, such as the CYP105D from *Streptomycetes avermitilis* bind them more strongly with K_m values around 20 μM, but have much lower k_{cat} values in the vicinity of 0.25 s^{-1} or less (k_{cat}/K_m=1.25×10^4 s^{-1} M^{-1}).[24,25] That means, the catalytic efficiency of apigenin (**5**) hydroxylation by *Aae*APO is about seven-times more higher than that of P450s.

Besides ring-hydroxylation, selective *O*-demethylation of 5,6,7,8,4′-pentamethoxyflavone was observed. The demethylation at C4′-position is very probable due to the steric hindrance of other positions.[22] Moreover the UV-absorption spectrum of the product is very similar to the UV-absorption spectrum reported for 4′-hydroxy-5,6,7,8-tetramethoxyflavone.[26] The *O*-dealkylation activity of *Aae*APO has been demonstrated for diverse ethers and may be useful for the selective removal of protecting methyl groups frequently used in chemical synthesis including flavonoids.[22]

The chemical synthesis of oxyfunctionalized flavonoids usually needs several steps; thus for 6-hydroxyapigenin synthesis, at least seven steps are required.[27–31] We demonstrated that *AaeAPO* selectively hydroxylated flavonoids in C6-position. Despite our moderate yields, our method is an efficient alternative procedure to the use of 2-iodoxybenzoic acid because of the distinction in the position of hydroxylation.[9]

An alternative pathway is the one-step biotransformation of apigenin by P450s using either whole cells or the isolated biocatalyst. However, these approaches entail some difficulties: (a) the heterologous expressed flavonoid 6-hydroxylase from soy bean was shown to convert many flavonoids, such as flavone to 6-hydroxyflavone but failed to oxidize isoflavones,[32] (b) whole cell transformation of the glucuronide baicalin by *Coryneum betulinum* as well as hydroxylation of apigenin by rat liver microsomes containing CYP1A and CYP3A yielded the desired 6-hydroxyapigenin but also gave further reaction products, such as 4′-hydroxyapigenin, products of glycoside hydrolysis, luteolin, and isoscutellarein,[25,26,33] and (c) the reaction products 6-hydroxyflavanone and 4′-hydroxyflavanone can be produced by whole cell transformation of flavanone in *Aspergillus niger* and *Penicillium chermesinum* but the formation of diverse byproducts was reported here as well.[34]

We noted some apparent size limitations on flavonoid substrates for the *Aae*APO. Although the enzyme hydroxylated quercetin to a monooxygenated product, it failed to hydroxylate the quercetin glycoside rutin (quercetin 3-*O*-glucorhamnoside). This result suggests that the active site of the *Aae*APO cannot accommodate larger flavonoid glycosides with more than one sugar residue, since the apigenin 7-*O*-glucoside was slowly converted (data not shown). Recent studies have been consistent with this picture, showing that the size of the substrate has an influence on the oxygen rebound at the active site.[22]

4. Conclusion

The aromatic peroxygenase of *A. aegerita* monooxygenated a variety of flavonoids. According to the molecular structure of identified hydroxylated metabolites, the enzyme regioselectively hydroxylates the C6-position of flavonoids. We could show by ^{18}O-labeling studies that the *Aae*APO-catalyzed hydroxylation of flavonoids is a true peroxygenase reaction that proceeds via initially formed epoxide intermediates. These results raise the possibility that fungal peroxygenases may be useful for versatile, cost-effective, and scalable syntheses of hydroxylated flavonoids.

5. Experimental

5.1. Reactants

Flavonoids were purchased from Extrasynthese (Genay, France) except tangeretin and flavone, which were obtained from TCI (Zwijndrecht, Belgium) and Alfa Aesar (Karlsruhe, Germany), respectively. Organic solvents and H_2O_2 were purchased from Merck (Darmstadt, Germany) and from J. T. Baker (Mallinckrodt Baker B.V., AA Deventer, Holland). $H_2{}^{18}O_2$ (90 atom %, 2% wt/vol), was obtained from Icon Isotopes (New York, USA). All other chemicals were purchased from Sigma–Aldrich (Schnelldorf, Germany).

Extracellular *Aae*APO (isoform II, 44 kDa) was produced with the mushroom *A. aegerita* and purified as described previously.[14] The enzyme preparation was homogeneous by SDS polyacrylamide gel electrophoresis and exhibited an A_{418}/A_{280} ratio of 2.2. The specific activity of the peroxygenase was 62 U mg^{-1}, where 1 U represents the oxidation of 10^{-3} mmol of 3,4-dimethoxybenzyl alcohol to 3,4-dimethoxybenzaldehyde in 1 min at 23 °C.[14]

5.2. Incubation conditions

Typical reaction mixtures (0.2–1.5 mL) contained purified peroxygenase (1–2 U mL^{-1}), potassium phosphate buffer (50 mM, pH 7.0 resp. pH 9.0), 20% (vol/vol) DMF, and the flavonoid substrate (1–10 mM). Ascorbic acid (4 mM) was used to inhibit further oxidation of the phenolic products that were released.[15,17] The reactions were started by the addition of H_2O_2 (0.1–2.2 mM) via single injection or continuous syringe pump supply at room temperature under vigorous stirring. The reaction mixtures were analyzed after 8 h of incubation.

5.3. Product identification

Reaction products were analyzed by HPLC using an Agilent HP 1200 liquid chromatograph (Agilent, Waldbronn, Germany) equipped with diode array (DAD) and mass detectors (LC/MSD VL), or a ProStar 210–510 liquid chromatograph (VARIAN, Walnut Creek, USA) equipped with a diode array detector (DAD). Both systems were used along with Synergi 4u Fusion RP 18 80A reversed phase columns (4.6 mm diameter by 250 mm length, 4 μm particle size, Phenomenex Ltd., Aschaffenburg, Germany), which was eluted at 1 mL min^{-1} and 40 °C with aqueous 0.01% vol/vol ammonium formate (pH 3.5)/acetonitrile (95:5), followed by a 25-min stepwise acetonitrile gradient (5 min to 50%, 15 min to 90%, 2 min to 5%, 3 min at 5% acetonitrile). Products were identified relative to authentic standards, based on their retention times, UV-absorption spectra, and $[M+H]^+$ or $[M-H]^-$ ions.

The reaction products 6-hydroxyapigenin and 6-hydroxyluteolin were analyzed via ^1H and ^3C NMR (Bruker, 400 MHz, Bruker BioSpin GmbH, Rheinstetten, Germany). For these measurements, the substances were dissolved in deuterated dimethyl sulfoxide. Quantitative amounts (5 mg) of 6-hydroxyapigenin (yield 17.5%) and 6-hydroxyluteolin (yield 16.5%) were obtained from semi-continuous enzymatic conversions.

For apigenin: the complete reaction mixture (65 mL) contained the peroxygenase (1.2 U mL^{-1}), potassium phosphate buffer (38.3 mM, pH 7.0), DMF (16% vol/vol), apigenin (1.5 mM), ascorbic acid (12.3 mM), and H_2O_2 (6.4 mM). H_2O_2 was continuously added using a syringe pump (for 67 h). Apigenin and ascorbic acid (one fourth of the total amount) were added at 0 h, 16 h, 24 h, and 43 h.

For luteolin: the complete reaction mixture (124 mL) contained the peroxygenase (1.3 U mL^{-1}), potassium phosphate buffer (40.5 mM, pH 7.0), DMF (16% vol/vol), luteolin (0.8 mM), ascorbic acid (6.5 mM), and H_2O_2 (4.4 mM). H_2O_2 was continuously added using a syringe pump (for 43 h). Luteolin and ascorbic acid were added (one third of the total amount) at 0 h, 9 h, and 19 h.

The samples were then concentrated and purified on a solid phase extraction system (for apigenin: Mega BE C18, 6 mL, 1 g, VARIAN, Walnut Creek, USA; for luteolin: Strata X 33u Polymeric RP, 12 mL, 500 mg, Phenomenex, Aschaffenburg, Germany) and fractionation using preparative reversed phase HPLC on a Gemini NX12C18 column (110 Å, 21.2 mm×150 mm, 10 μm particle size; AXIA, Phenomenex, Aschaffenburg, Germany).

The reaction product formaldehyde was determined as its 2,4-dinitrophenylhydrazone adduct by HPLC[22] using the same HPLC apparatus as above but on a Luna C18 column (4.6 mm×150 mm, 5 μm particle size, Phenomenex Ltd., Aschaffenburg, Germany), which was eluted at 0.35 mL min^{-1} and 40 °C with aqueous 0.01% vol/vol ammonium formate (pH 3.5)/acetonitrile, 70:30 for 5 min, followed by a 25-min linear gradient to 100% acetonitrile. The formaldehyde-dinitrophenylhydrazone was identified relative to an authentic standard, based on its retention time (12.4 min), UV-absorption spectrum (223, 253, 355 nm), and $[M-H]^-$ ion (m/z 209).

The assumed epoxide product (5,6-dihydro-5,6-epoxyflavone or 6,7-dihydro-6,7-epoxyflavone) was analyzed by reversed phase HPLC using the above apparatus but with a Gemini 3u C6—Phenyl 110 Å column (2 mm diameter by 150 mm length, 3 μm particle size, Phenomenex Ltd., Aschaffenburg, Germany). A mixture of 5 mM ammonium formate buffer (pH 8.0) and acetonitrile was used as solvent at constant 40 °C at a flow rate of 0.2 mL min^{-1} under the gradient conditions described for the Synergi 4u Fusion column.

5.4. Enzyme kinetics

The kinetics of apigenin hydroxylation were analyzed in stirred reaction mixtures (0.20 mL, 23 °C) that contained 10^{-4} mM of *Aae*APO, potassium phosphate buffer (100 mM, pH 7.0), 4 mM of ascorbic acid, 4×10^{-2} mL DMF and 5×10^{-3}–5×10^{-1} mM of the flavonoid. The reactions were initiated with 1.8–2.2 mM H_2O_2 and stopped by addition of 2×10^{-2} mL of 50% trichloroacetic acid after 20 s, at which time less than 5% of the apigenin had been consumed. The resulting 6-hydroxyapigenin was quantified by HPLC as described above, and the K_m and k_{cat} values for apigenin were calculated by nonlinear regression using the Michelis–Menten model in the Sigma Plot 11.0 Enzyme Kinetics 1.3 program as apparent constants.[35]

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References and notes

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