Isolation and Characterization of Flavonol Converting Enzymes from *Mentha piperita* Plants and from *Mentha arvensis* Cell Suspension Cultures

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Peroxidases from several plants, including horseradish peroxidase, were capable of converting flavonols to the corresponding 2,3-dihydroxyflavanones in presence of H_2O_2. Contrastingly, protein extracts from *Mentha piperita* plants and *Mentha arvensis* cell suspension cultures performed the same enzymatic step in absence of H_2O_2, but only with quercetin, not with kaempferol. H_2O_2-independent, quercetin converting enzymes were isolated and purified from these extracts, and they could be classified in two groups according to the extent of stimulation of the enzyme reaction by H_2O_2. Enzymes from group I were stimulated by exogenous H_2O_2, and they resembled horseradish peroxidase in several aspects. They possessed IAA oxidase activity, but quercetin was the preferred substrate. Enzymes from group II from the plants appeared to be a distinctly different set of enzymes. They were not stimulated by H_2O_2, but required molecular oxygen and converted only 3,3′,4′-trihydroxyflavones under aerobic conditions. Also, they showed no Soret-bands and possessed no IAA oxidase activity. These proteins appear to be a new class of enzymes participating in the first step of flavonol degradation in plants.

Introduction

Peroxidases participate in the degradation of flavonoids. It has been shown with plant suspension cultures that these enzymes oxidized flavonols, flavanones, chalcones, aurones and liberated the B rings as the corresponding benzoic acids [1–4]. Flavonols were converted in a first enzymatic step (Fig. 1) in the presence of H_2O_2 to the equivalent 2,3-dihydroxyflavanones by peroxidases from various plant tissues [4–5]. With the enzyme preparations from most tissues the presence of H_2O_2 was a stringent requirement, and quercetin and kaempferol were converted at similar rates [4–6]. Protein preparations from *Mentha piperita* plants, however, were capable of converting quercetin, but not kaempferol, without addition of H_2O_2 [7]. This report describes the isolation and characterization of flavonol-converting enzymes from *Mentha* plants and *Mentha* cell suspension cultures. The properties of these enzymes were compared with those of horseradish peroxidase in order to compare H_2O_2-independent and H_2O_2-dependent conversion of flavonols and the possible indoleacetic acid oxidase function of both enzymes.

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Abbreviations: EDTA, ethylendiaminetetraacetic acid; DIECA, sodium diethyldithiocarbaminate; IAA, indoleacetic acid.

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Materials and Methods

Plant material

Mentha piperita plants were grown in the Botanic Garden of the University. Aerial parts were harvested in July/August, and they were used immediately for the preparation of acetone dry powder.

Cell cultures

Cultures were started from seeds of *Mentha arvensis*, and callus material was kept on a medium as previously described [8], but without cholin. Cell suspension cultures were kept in 200 ml-Erlenmeyer flasks on a gyrotory shaker (120 rpm) in the dark.

Chemicals

Kaempferol and quercetin were purchased from Roth, Karlsruhe. The flavonol methylethers were obtained from Dr. Wollenweber, Darmstadt. All enzymes and biochemicals were purchased from Merck, Darmstadt; Boehringer, Mannheim; and

Serva, Heidelberg. Dowex resins were obtained from Serva, Heidelberg, while DEAE-cellulose and reagents for column chromatography came from BioRad, Richmond. CM-Sephadex and Sephadex were handled according to the manufacturer's advice.

Extraction of enzymes

Acetone powder was prepared with 80% aqueous acetone at $-18\,^{\circ}C$ from frozen plants or cell culture material which had been thoroughly crushed in the presence of liquid nitrogen. The powder was stored over P_2O_5 where it kept its activity for several months.

Acetone powder (25 g) was extracted by stirring for 30 min with 1250 ml of 0.25 M potassium phosphate buffer (pH 7.5) in the presence of 3 g Polyclar AT and 0.1 M ascorbate. The mixture was centrifuged at $27\,000 \times g$ for 20 min, and the supernatant fluid was filtered through glass wool. The eluate was brought to 80% saturation with solid ammonium sulfate and stirred for 1 h. Precipitated protein was collected by centrifugation as above and dissolved in 30 ml extraction buffer. This solution was filtered through a Sephadex G-25 (coarse) column, previously equilibrated with 0.1 M ascorbate and 0.1 M potassium phosphate (pH 7.5). The proteins were dialyzed overnight against two batches of 5 l of 5 mM Tris/HCl-buffer (pH 7.5) and finally concentrated by ultrafiltration in an Amicon cell with a PM 10 filter. A series of experiments had shown that this procedure was optimal with respect to yield of enzyme activity and efficient removal of the large amounts of phenolic compounds present in the *Mentha* plants.

Chromatography on DEAE-cellulose

Protein from up to 100 g acetone powder and freed from phenols as described above was subjected to column chromatography on a DEAE-cellulose column (1.8×47 cm) equilibrated with 5 mM Tris/HCl-buffer (pH 7.5). Non-binding protein was eluted with the same buffer at a rate of 15 ml/h. Fractions of 5 ml were collected, and bound protein was eluted with a linear KCl-gradient (0 – 0.4 M). After measurement of enzyme activity, protein was recovered by lyophilisation. It was dissolved in 5 ml Tris/HCl (pH 7.5) and dialyzed against the same buffer overnight. In other experiments smaller columns were used, and 0 – 0.3 M KCl-gradients were applied. Enzymes were stored in 5 mM Tris/HCl (pH 7.5) at $-20\,^{\circ}C$ for at least 2 months without loss of activity.

Enzyme assays

Standard incubations with quercetin or other flavonols contained in a final volume of 2 ml: 0.1 M citrate-phosphate buffer (pH 5.5), enzyme, and 50 μM of the flavonol. The flavonols were first dissolved in 50 μl of ethylene glycolmonomethylether. For peroxidatic reactions the incubations were supplemented with 2.5 mM H_2O_2. The assays were carried out at $25\,^{\circ}C$, and they were started by addition of enzyme. The reaction was followed by recording the decrease in flavonol absorption at 370 nm in a Zeiss PM 4 spectrophotometer, and the rate of flavonol conversion was calculated from a standard curve. Absorption spectra of incubations were routinely measured between 200 – 400 nm with a Leitz Unicam SP 8000 spectrophotometer.

This assay was also used to determine the influence of inhibitors on the conversion of quercetin: EDTA ($10^{-5} - 10^{-2}$ M), KCN ($10^{-6} - 10^{-4}$ M), NaN_3 ($10^{-6} - 10^{-3}$ M), DIECA ($5 \times 10^{-8} - 10^{-5}$ M), and ascorbate ($10^{-6} - 10^{-3}$ M). Inhibitors were preincubated with enzyme for 5 min prior to the start of reactions. The influence of superoxide dismutases and catalase was measured at pH 5.5 and 7.5. Between 50 and 1000 units were applied per incubation. IAA-Oxidase activity was determined in a 2 ml-assay which contained 50 mM potassium phosphate (pH 5.9), 0.1 mM $MnCl_2$, 0.05 mM 2,4-dichlorophenol, 0.13 mM IAA, and enzyme. Incubations were performed at $30\,^{\circ}C$, and formation of 3-methylene oxindole was recorded between 230 – 430 nm. Reactions containing simultaneously IAA and quercetin were followed by difference spectroscopy with two reference cuvettes which contained either IAA or quercetin. Spectra were recorded every 5 min using the reference cuvettes alternatively.

Determination of protein

Protein was measured according to Lowry *et al.* [9]. Eluates from DEAE-columns were assayed by measuring absorption at 280 nm.

Anaerobic incubations and Warburg-manometry

The standard assay for quercetin conversion was increased to 4 ml. Anaerobic incubations were carried out in Thunberg cuvettes, and air was removed by flushing with oxygen-freed nitrogen for 20 min. For Warburg-manometry the concentration of quercetin was increased to 0.45 or 0.53 mM. Oxygen consumption was followed for about 100 min at 25 °C.

Isolation of quercetin conversion product

The 2,3,5,7,3′,4′-hexahydroxyflavanone was isolated and characterized as described [5].

Molecular weight determination

The molecular weight of enzymes was estimated by Sephadex G-100 gel permeation chromatography or by sucrose gradient centrifugation. The following standard proteins were used to calibrate the Sephadex columns: Aldolase (M_r 147 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 45 000), chymotrypsinogen A (M_r 25 700), and cytochrome C (M_r 12 300). Sucrose gradient centrifugation was performed in an IEC/B 60 ultracentrifuge with the rotor 488. Aldolase (0.1 mg) as reference protein and enzymes from *Mentha* (0.1 – 0.5 mg) were centrifuged for 22 h at 270 000 × *g*. The results were evaluated according to Martin and Ames [10], and aldolase activity was determined with a published procedure [11].

Analytical gel electrophoresis

A previously published method [12] was applied. The quercetin-converting enzymes were extracted from the gel with 0.1 M citrate-phosphate-buffer (pH 5.5). Peroxidase activity was stained by incubating the gels for 30 min at 35 °C in a solution of 1 M Tris/HCl-buffer (pH 8.0), 1 mM benzidine and 10 mM H_2O_2. Protein was stained with Coomassie Brilliant Blue [13] and glycoproteins with the Schiff reagent [14].

Results

Enzymes from plants

Crude protein preparations from *Mentha piperita* converted the flavonol quercetin (λ_{max} 255, 375 nm) to a compound (λ_{max} 292 nm) which was shown by its UV-data and by chromatographic comparison to be identical with the previously characterized 2,3,5,7,3′,4′-hexahydroxyflavanone [5] (Fig. 1). Kaempferol was converted to its corresponding 2,3-dihydroxyflavanone by these preparations only after the addition of H_2O_2.

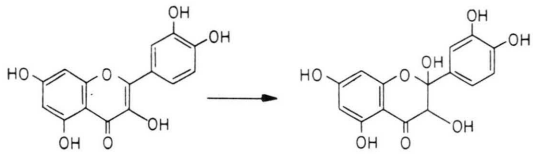


Fig. 1. Conversion of quercetin to 2,3,5,7,3′,4′-hexahydroxyflavanone. Peroxidases from most plants show a strict requirement of H_2O_2 for this reaction. Enzymes from *Mentha* need molecular oxygen, but not H_2O_2 for the same reaction.

Aerial parts of *Mentha piperita* are exceptionally rich in polyphenol material so that special precautions for extractions of active enzymes had to be taken [15]. Despite of these precautions yield and stability of the enzymatic activity were greatly affected by darkening of extracts in the early steps of purification. It was therefore necessary to prepare acetone dry powder with acetone/water mixtures to increase removal of phenolics [16], and to extract protein from the powder in the presence of ascorbate and Polyclar AT. Further removal of phenols from protein was achieved by precipitation with ammonium sulfate and by Sephadex G-25 filtration. The last two steps had to be carried out also in the presence of ascorbate.

After removal of ascorbate by dialysis, the quercetin-converting enzyme activity was separated by chromatography on DEAE-cellulose into 3 active fractions. Each fraction was rechromatographed on DEAE-cellulose, resulting in a further separation of enzyme activities. Altogether 5 active fractions were obtained (Table I) which converted quercetin in the absence of H_2O_2. Based on the rate of stimulation of quercetin-conversion by H_2O_2, two groups of enzymes were distinguished (P I, P II).

The purity of these protein fractions was tested by alcaline disc gel electrophoresis under nondenaturing conditions (Fig. 2). Fractions PIa and PIb contained peroxidase activity, but only in case of PIa peroxidase activity coincided with activity for quercetin conversion without H_2O_2. PIIa to PIIc showed several protein bands, but activity for the H_2O_2-independent conversion of quercetin could well be assigned to one individual band in each case. Peroxidase activity could not be detected in PII-frac-

Table I. Protein fractions obtained from *Mentha piperita* plants after double chromatography on DEAE-cellulose, and some properties of the fractions with respect to conversion of quercetin (see Fig. 1). The last column shows the molecular weight of the enzymes as determined by gel permeation chromatography (left set of data) or by sucrose gradient centrifugation (right set of data).

Enzyme fraction	Elution with KCl [mM]	Conversion of quercetin without H_2O_2 [mkat/kg]	Conversion of quercetin with H_2O_2 [mkat/kg]	K_m [10^{-5} M]	V_{max} [mkat/kg]	Molecular weight [daltons $\times 10^{-3}$]
PIa	0	30	214	12.5	100	68 , 70
PIb	30 – 40	25	92	3.3	70	– * , 62
PIIa	60 – 70	139	199	7.1	220	68 , 57
PIIb	120 – 140	89	110	5.6	10 **	68 , 68
PIIc	150 – 180	92	100	6.3	170	68 , 62

* Not determined.

** This protein fraction had been subjected to an additional purification step by chromatography on Sephadex G-100, resulting in a great loss enzyme activity (see text).

tions, thus giving additional support for distinguishing two groups of enzymes (see Table I). In contrast to the peroxidases in PIa and PIb none of the bands found to be active in quercetin conversion without H_2O_2 could be stained with the Schiff reagent. Fraction PIb appeared to be a mixture of PIa and PIIa.

Further attempts to purify enzyme fractions failed because chromatography on hydroxy apatite led to inactive preparations, and the enzymes were not bound by CM-Sephadex. Chromatography on Sephadex G-100 or G-200 resulted in substantial losses in enzyme activity of up to 70%. Sephadex G-100 chromatography was therefore only occasionally used to obtain material for determination of molecular weights and other enzyme properties. Most studies were conducted with the fractions described in Table I.

Properties of enzymes from plants

Molecular weights were determined both by gel permeation chromatography on Sephadex G-100 and by linear sucrose gradient centrifugation. The data are also given in Table I. The pH-optima for the H_2O_2-independent conversion of quercetin were determined over the range of pH 3 to pH 10. Crude

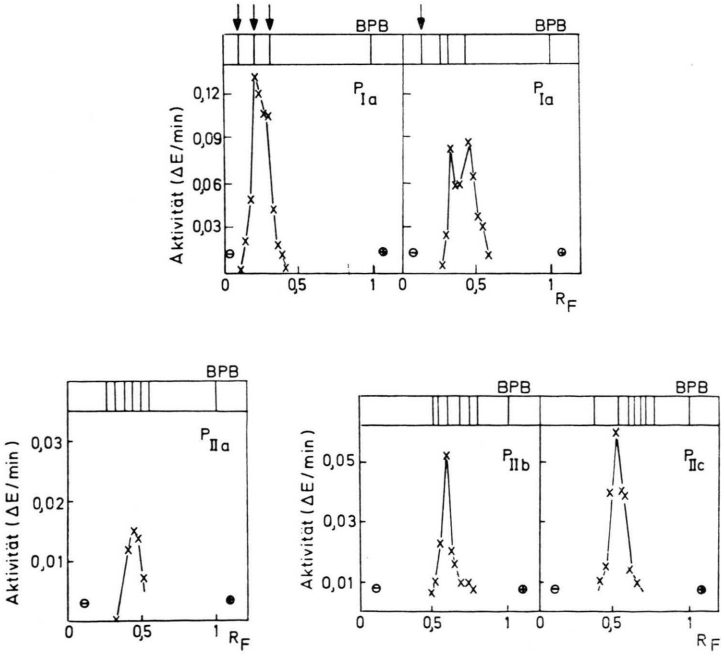


Fig. 2. Alcaline disc gel electrophoresis of protein fractions PIa to PIIc. The upper part of each graph shows the number and relative migration of protein bands, arrows indicate the positions of peroxidase activity as stained with benzidine/H_2O_2 (see Materials and Methods). The lower part of each graph depicts position and activity of enzymes converting quercetin in absence of H_2O_2. BPB: Bromophenol blue.

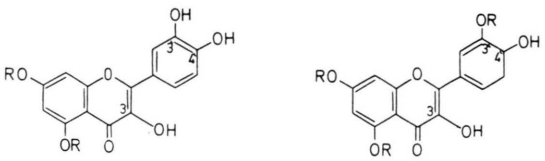
protein preparations as well as fractions PIa to PIIc all showed optimal activity around pH 5.5. The K_m-values for quercetin as obtained from Lineweaver-Burk plots are also given in Table I. They were measured at substrate concentrations from 1.5×10^{-5} to 10^{-4} M. These limits were determined either by the sensitivity of the photometric test at lower concentrations or by the solubility of quercetin and severe substrate inhibition at higher concentrations.

Experiments under anaerobic conditions with the fractions PIa and PIIb showed that molecular oxygen was necessary for the conversion of quercetin with both groups of enzymes. However, addition of H_2O_2 to anaerobic incubations restored enzyme activity completely, even with PIIb, which was not stimulated by H_2O_2 under aerobic conditions (see Table I). During anaerobic conditions no bathochromic shift in quercetin absorption was observed, suggesting that no flavonol-heavy metal interaction occurred [17, 18]. Quantitative determinations of oxygen consumption during quercetin conversion by enzyme PIIb were carried out by Warburg-manometry. In five parallel experiments the reaction was carried to almost completion which resulted in an oxygen consumption of 0.46 ± 0.053 µmol O_2 per µmol quercetin.

Due to thier ferriprotoporphyrin IX prosthetic group peroxidases strongly absorb at 400 – 410 nm [19, 20, 21]. Such measurements of Soret-bands with enzymes PIa to PIIc and horseradish peroxidase are shown in Table II. Despite of some protein impurities (see Fig. 2) PIa and PIb showed well distinguished peaks at 403 nm. The much higher values for the ratio of heme to protein absorption of the PI enzymes in comparison to those of the PII group suggest that PIa and PIb contained peroxidases similar to horseradish peroxidase with respect to the prosthetic group.

Substitution patterns of flavonol substrates

The results presented so far indicate that enzymes from group PII were partly different from typical peroxidases. This difference was further supported by studies on the conversion of quercetin- and kaempferol-methylethers by PIa, PIIa, PIIb, and horseradish peroxidase. All possible combinations of monomethylethers to tetra- or penta-methylethers, respectively, were investigated. With reference to Fig. 3 the results may be summarized as follows:



A B

Fig. 3. Pattern of free hydroxyl groups required for H_2O_2-independent (A) and peroxidatic (B) conversion of flavanols by enzymes from *Mentha* and by horseradish peroxidase.

1. Enzymes from group PII converted all substrates with free hydroxyl groups in positions 3′, 4′ and 3 (Fig. 3 A). The reactions required oxygen, but not H_2O_2. Due to the strict dependence on free vicinal hydroxyl groups in ring B, these enzymes did not attack kaempferol or its derivatives.

2. Enzymes from group PI converted all flavonols with free hydroxyl groups in positions 4′ and 3 (Fig. 3 B), but only in presence of H_2O_2. Under aerobic conditions in the absence of H_2O_2 a low rate of conversion was observed if flavonols contained an additional free hydroxyl group in position 3′ (quercetin or its derivatives). Kaempferol was therefore not converted by this group of enzymes in the absence of H_2O_2.

3. The reaction of horseradish peroxidase with all flavonols was only possible in presence of H_2O_2, and free hydroxyl groups in positions 4′ and 3 were necessary (Fig. 3 B).

Inhibitor studies

Various studies with inhibitors aiming at determination of heavy metal content of the investigated enzymes are also summarized in Table II. Values for inhibition with KCN and NaN_3 were for all enzymes in the same order of magnitude, indicating that iron ions might be involved in the enzymatic reaction.

Studies with EDTA, however, showed that metal ions of *Mentha* enzymes, if participating in the enzyme reaction, were nevertheless not readily accessible. While activity of horseradish peroxidase was inhibited to 50% with 3.4 µM EDTA, PII enzymes required approximately 10 mM EDTA for the same degree of inhibition in the conversion of quercetin. The copper chelating agent DIECA which has been shown to inhibit Cu-containing enzymes very strongly [17, 22, 23] (100% inhibition of quercetinase at 0.5 µM [17]), was also tested (Table II).

Enzyme fraction	$A_{403\,nm}$	$A_{280\,nm}$	A_{403}/A_{280}	Inhibitor concentration [μM] for 50% inhibition by		
				KCN	NaN_3	DIECA
PIa	0.20	0.93	0.22	14.5	450	4.5
PIb	0.12	0.87	0.14	4.0	−*	−*
PIIa	0.05	0.99	0.05	0.2	250	0.23
PIIb	0.06	1.04	0.06	1.0	400	5.0
PIIc	0.06	0.90	0.07	1.8	−*	−*
HRP	0.59	0.93	0.63	1.0	560	10**

Table II. Soret-bands and inhibitor studies with the enzyme fractions from *Mentha piperita* and with horseradish peroxidase (HRP). The inhibitors were tested in the standard assay for quercetin conversion in absence of H_2O_2.

* Not determined.
** This was highest concentration tested. It resulted in 30% inhibition of enzyme activity.

Except for PIIa the results do not show any specific inhibition by this reagent.

The O_2-consuming conversion of quercetin by enzymes PIa to PIIc was in each case strongly inhibited by reducing agents such as NADH, NADPH, 2-mercaptoethanol, and ascorbic acid. The latter compound yielded total inhibition at 50 μM. In contrast to this value, the H_2O_2-dependent conversion of quercetin by horseradish peroxidase was only weakly inhibited (5%) at this ascorbate concentration. The *Mentha* enzymes readily oxidized ascorbate when incubated together with quercetin, but conversion of quercetin was only observed after complete oxidation of ascorbate. Previous reports have shown that various H_2O_2-consuming enzyme reactions can be inhibited very efficiently by catalase [24, 25], such as the reaction shown in Fig. 1 when catalyzed by horseradish peroxidase [6]. The conversion of quercetin by enzymes PIa, PIIa and PIIb in the absence of H_2O_2 was not effected by incubations with even high concentrations (50 μg/ml) of commercial catalase. H_2O_2 can thus be eliminated as a free molecular intermediate in the oxygen-dependent conversion of quercetin by *Mentha* enzymes.

Similarly, attempts to inhibit quercetin conversion by *Mentha* enzymes PIa and PIIa – PIIc with superoxide dismutases failed. Incubations with even high amounts (500 units/ml) of an Fe-containing superoxide dismutase from *Spirulina* or a Cu/Zn-containing superoxide dismutase from spinach did not slow down the quercetin conversion, indicating that free superoxide anion may not be involved in this reaction.

Equivalent studies to document inhibition by mannitol [26] did not effect the quercetin reaction catalyzed by enzymes PIa, PIIa and PIIb, so that free hydroxyl radicals are also not likely to be involved.

Studies with indoleacetic acid

Peroxidases from numerous plants are known to exhibit IAA oxidase activity [27, 28] which depends on O_2 and can be measured in presence of Mn^{2+}-ions and 2,4-dichlorophenol. Quercetin has repeatedly been assumed to inhibit this oxidase activity [29 – 31].

Table III presents the results of studies with horseradish peroxidase and enzymes PIa to PIIc where IAA and quercetin as well as mixtures of both compounds were assayed for O_2-dependent conversion of quercetin under conditions of IAA oxidase activity. These results were obtained by difference spectroscopy measuring IAA at 279 nm and quercetin at 375 nm.

IAA oxidase activity of horseradish peroxidase did not convert quercetin according to Fig. 1 unless H_2O_2 was added, but the flavonol completely inhibited IAA oxidation in the oxidase reaction. In contrast to enzymes of group PII the enzymes PIa and PIb possessed IAA oxidase activity leading to

Table III. Quercetin, IAA, or mixtures of both compounds as substrates for the enzyme fractions from *Mentha piperita* and for horseradish peroxidase (HRP). The assays were carried out under conditions of IAA oxidase activity (see Materials and Methods) and in absence of H_2O_2, if not stated otherwise.

Enzyme fraction	IAA	Quercetin	IAA plus quercetin	
PIa	+	+	−	+
PIb	+	+	−	+
PIIa	−	+	−	+
PIIb	−	+	−	+
PIIc	−	+	−	+
HRP	+	−	−	−
HRP with H_2O_2	−	+	−	+

−, No measurable enzyme activity within 2 h.
+, At least 30% conversion within 30 min.

3-methyleneoxindole. This latter reaction was again inhibited by a preferential conversion of quercetin when IAA/flavonol mixtures were assayed. IAA oxidation even appeared permanently inactivated by quercetin or its degradation products. Therefore, enzymes from group PII differ both from horseradish peroxidase by their ability to convert quercetin with O_2 only and from PI enzymes by their lack of IAA oxidase activity.

Enzymes from cell suspension cultures

The exceptionally high polyphenol content of *Mentha piperita* plants which severely hampered isolation of enzymes suggested studies with cell suspension cultures. It was hoped that growth of cultures in the dark would result in a much lower polyphenol content [32].

Cultures were started from *Mentha arvensis* seeds because *Mentha piperita* is an infertile hybrid and aseptic tissues suitable for tissue cultures could not be obtained. Light-coloured, well growing calli [8] were used to establish cell suspensions. They excreted considerable amounts of H_2O_2-requiring peroxidase activity into the growth medium. Exocellular enzyme activity for quercetin conversion without H_2O_2 was not detected. Cell extracts, however, possessed both enzyme activities at varying levels depending on the growth phase of the culture (not shown).

Although the suspension cultures were kept for more than 6 months in the dark with repeated subculturing, they still contained high amounts of polyphenols. The isolation of enzymatically active protein preparations was therefore carried out under the same precautions as described above for the plant material. The colourless protein preparations thus obtained and subjected to DEAE-column chromatography were assayed for H_2O_2-independent quercetin conversion. Four out of the 5 enzyme activities so obtained were further studied. Based on stimulation of quercetin conversion by H_2O_2 (compare Table I) 2 groups of enzymes could be distinguished (Table IV).

As measured by UV-absorption, CI but not the CII enzymes seemed to possess a ferriprotoporphyrin moiety (Table IV), while molecular weights were all in the range of 57 000 daltons. Alcaline disc gel electrophoretic studies revealed 4 protein bands each in both CI and CIIa. In case of CI all of these bands showed peroxidase activity, but with CIIa only one peroxidase band could be detected. When stained with the benzidine/H_2O_2-reagent, CIIb and CIIc were completely free from peroxidatic activity though several protein bands were discernable. With respect to substrate specificity (Fig. 3), inhibition by various compounds (Table II), IAA oxidase activity and its inhibition by the preferred substrate quercetin (Table III) CI appeared very similar to PIa and CIIa – CIIc similar to PIIa – PIIc.

Discussion

Flavonols have been recognized as a new class of substrates for peroxidases [4, 5, 33]. Due to the large molar extinction coefficient of flavonols the H_2O_2-dependent conversion of these compounds according to Fig. 1 may be recommended as a very sensitive test for these enzymes. Heme-containing peroxidases such as horseradish peroxidase will convert all 4′,3-dihydroxyflavones (Fig. 3 B) in this reaction. In fact, it was possible to use this reaction in a coupled enzyme test to search for flavonol 3-

Table IV. Protein fractions obtained from *Mentha arvensis* cell suspension cultures after chromatography on DEAE-cellulose, and some properties of the fractions with respect to the conversion of quercetin. The enzyme from the cell cultures are referred to as C in analogy to the enzymes P from the plants.

Enzyme fraction	Elution with KCl [mM]	Conversion of quercetin		$A_{403\,nm}$	$A_{280\,nm}$	A_{403}/A_{280}	Preferred substrate *	
		without H_2O_2 [mkat/kg]	with H_2O_2 [mkat/kg]				IAA	quercetin
CI	0	3.8	2980	0.20	0.53	0.38	–	+
CIIa	65	32	94	n. d.**	n. d.	n. d.	–	+
CIIb	110	94	86	0.05	0.95	0.05	–	+
CIIc	150	23	31	0.05	0.92	0.06	–	+

* The assays contained IAA and quercetin simultaneously, and they were carried out under the same conditions as described in Table III for the plant enzymes.

** n. d., Not determined.

glycoside specific β-glycosidases in plants [34] because flavonol 3-glycosides are not converted by these peroxidases.

In contrast to the typical, widely distributed peroxidases such flavonol-converting enzymes as isolated from a *Mentha* species seem to be a group of enzymes not previously described. The O_2-requiring flavonol converting enzymes from *Mentha piperita* plants and *Mentha arvensis* cell cultures occurred as a set of several isozymes which were so similar in their properties and identical in number that there was no significant difference between the plant and the cell culture enzymes. Also, all *Mentha* enzymes were rather similar with respect to pH-optimum, molecular weight, and oxygen requirement. The purification of the enzymes could not be carried to homogeneity, but they were sufficiently enriched and separated to clearly determine the main enzymatic characteristics. Though the *Mentha* enzymes (PII and CII) and true peroxidases (other plant peroxidases [4, 35] and peroxidases excreted by cell cultures into the medium [6, 35]) catalyzed the same basic reaction (Fig. 1), they differed in various aspects (see Tables I – III). Thus, the PII enzymes showed no reaction towards well established peroxidase substrates such as benzidine or guaiacol, used oxygen only in combination with 3,3′,4′-trihydroxyflavones, were no glycoproteins, showed no Soret-bands, and lacked IAA oxidase activity. With the exception of the lacking IAA oxidase activity the PII enzymes were partly comparable to apoperoxidases, as obtained by removal of the heme prosthetic group [36]. It should be mentioned that there was also a difference between enzymes PI/CI and horseradish peroxidase in so far, as the latter enzyme was strictly dependent on H_2O_2 for the conversion of all flavonols according to Fig. 1.

Numerous investigators reported strong inhibition of the peroxidase-catalyzed IAA oxidase activity by various phenolic compounds including quercetin [31, 37]. IAA oxidase activity and its inhibition by quercetin appears to be a general property of all heme-containing peroxidases [27, 28, 38], such as again found with horseradish peroxidase (Table II). In contrast to data by Sano [31], obtained with a peroxidase from *Pisum sativum*, our studies with IAA/quercetin mixtures and horseradish peroxidase showed no indication of any quercetin destruction without H_2O_2 under conditions of IAA oxidase activity. The postulated alteration of quercetin as described by Sano [31] which led first to a short-lived purple compound (λ_{max} 530 nm) and then within ten seconds to a stable compound (λ_{max} 490 nm), may possibly be explained by a transformation of the heme group of the peroxidase [39] upon addition of quercetin, but not likely by a destruction or chemical change of the flavonol. Such changes should be detectable between 250 and 370 nm, however, they were not found in our studies nor were they demonstrated by Sano [31].

As expected [40], peroxidases will convert flavonols in flavonol/IAA mixtures when incubated in the presence of H_2O_2 (see Table III). In contrast to true peroxidases and enzymes from group PI, the PII enzymes possessed no IAA oxidase activity. It is suggested that this lack of enzymatic activity may be related to the finding that they possessed no Soret-bands [40]. This seems to be supported by our data (Tables II, III) with enzymes from group PI which are supposed to contain heme groups and which, when measured separately, converted both, IAA or quercetin, under conditions of the IAA oxidase assay. In IAA/quercetin mixtures the flavonol constituted the preferred substrate leaving IAA essentially unchanged as long as phenolic material was present. The observations with horseradish peroxidase, PI enzymes and IAA/flavonol mixtures seem to suggest that both, quercetin and/or the corresponding 2,3-dihydroxyflavanone inhibited IAA oxidation by scavenging the oxidizing equivalents at active intermediates.

Future experiments on flavonol conversion will especially have to investigate further the stoichiometric aspects of the involvement of H_2O_2 and O_2, respectively, with regard to the reaction shown in Fig. 1. This reaction represents the addition of the elements of water to a double bond. When catalyzed by a H_2O_2-requiring peroxidase one can visualize a catalase type of activity of a peroxidase [40] to be involved according to the equation:

Kaempferol + H_2O_2 → 2,3,5,7,4′-pentahydroxyflavanone + 0.5 O_2.

Such a reaction would be very drastically inhibited by the addition of catalase, as indeed shown to be the case with various flavonols and horseradish peroxidase [4]. In case of enzymes PIIa – PIIc and CIIa – CIIc from *Mentha*, oxygen was required for the conversion of quercetin. The measured stoichiometry of 0.5 mol O_2/mol quercetin would place this reaction in the role of a mono-

oxygenase. Since no cofactor or reducing agent were, however, added to the reaction system it could be formally represented by a backward peroxidase reaction such as:

Quercetin + 0.5 O_2 + 2 H_2O → 2,3,5,7,3′,4′-hexahydroxylflavanone + H_2O_2.

Such H_2O_2-generating reactions of plant peroxiase-oxidase enzymes are known [41], and they show similar properties with respect to inhibitory compounds as those tested in our experiments. The *Mentha* enzymes, however, also differ from those enzymes [41] because they seem to lack the porphyrin moiety.

The O_2-dependent conversion of flavonols by the *Mentha* enzymes was restricted to compounds with two adjacent hydroxyl groups in ring B (see Fig. 3 A), and these groups may fulfill the structural requirements for an oxygen-dependent oxidase reaction [40]. It is therefore also possible that a transient oxidation of these hydroxyl groups by oxygen occurs with the intermediate formation of H_2O_2. The Fe-ions of the *Mentha* enzymes which are implied by the strong inhibition exercised by KCN form suitable constituents of the enzymes for such a reaction. The possible intermediate formation of H_2O_2 and its participation in the enzyme reaction may be supported by the observation that under anaerobic conditions the PII enzymes converted quercetin after addition of H_2O_2. This interpretation is not necessarily contradicted by the finding that catalase did not inhibit the conversion of flavonols, if the H_2O_2 or its active equivalent is thought to be tightly bound to the enzyme. The intermediate formation with the *Mentha* enzymes of oxidizing equivalents is indicated by the strong inhibition found with various reducing agents such as NADH or ascorbate. The same type of argument may, however, also be used with respect to the negative results obtained with superoxide dismutase or hydroxyl-radical trapping reagents.

Both, the H_2O_2-dependent peroxidase reaction of 4′-hydroxyflavonols and the O_2-requiring oxidase reaction of quercetin will also have to be analyzed for the origin of the oxygen atom incorporated into the flavonol molecule. The rather great lability of 2,3-dihydroxyflavanones [5], however, will severely hamper any studies with ^{18}O. In order to characterize the chemical nature of the oxygen species involved in the enzyme reaction with the PII group further studies are in progress.

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[1] W. Barz and W. Hösel, The Flavonoids (T. J. Mabry, J. B. Harborne, and H. Mabry, eds.), Chapter 17, Chapman and Hall, Ltd., London 1975.
[2] W. Barz, Physiol. Végét. **15,** 261–277 (1977).
[3] W. Barz, Ber. dtsch. Bot. Gesellsch. **88,** 71–81 (1975).
[4] W. Hösel, G. Frey, and W. Barz, Phytochem. **14,** 417–422 (1975).
[5] W. Hösel and W. Barz, Biochim. Biophys. Acta **261,** 294–303 (1972).
[6] E. Muhle, W. Hösel, and W. Barz, Phytochemistry **15,** 1669–1672 (1976).
[7] W. Hösel, Dissertation, Universität Freiburg/Brsg. 1972.
[8] M.-L. Lin and E. J. Staba, Lloydia **24,** 139–145 (1961).
[9] H. O. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, J. Biol. Chem. **193,** 265–275 (1951).
[10] R. G. Martin and B. N. Ames, J. Biol. Chem. **236,** 1372–1379 (1961).
[11] H. U. Bergmeyer and E. Berut, Methoden der enzymatischen Analyse (H. U. Bergmeyer, ed.), 2. edit., **Vol. 1,** p. 1062, Verlag Chemie, Weinheim 1970.
[12] H. R. Maurer, Disc Electrophoresis, W. de Gruyter, Berlin, New York 1971.
[13] A. Chrambach, R. A. Reisfeld, M. Wyckoff, and J. Zaccari, Anal. Biochem. **20,** 150–154 (1967).
[14] R. M. Zacharius, T. E. Zell, J. H. Morrison, and J. J. Woodlock, Anal. Biochem. **30,** 148–152 (1969).
[15] W. D. Loomis and J. Battaile, Phytochemistry **5,** 423–438 (1966).
[16] G. W. Sanderson and G. R. Roberts, Biochem. J. **93,** 419–423 (1964).
[17] T. Oka, F. J. Simpson, and H. G. Krishnamurty, Can. J. Microbiol. **18,** 493–508 (1972).
[18] J. Jurd, Chemistry of Flavonoid Compounds (T. A. Geissmann, ed.), p. 107, Pergamon Press (1962).
[19] L. M. Shannon, E. Kay, and J. Y. Lew, J. Biol. Chem. **241,** 2166–2172 (1966).
[20] H. Theorell, Enzymologia **10,** 250–258 (1942).
[21] T. Yonetani, Adv. Enzymol. **33,** 309–335 (1970).
[22] T. Oka and F. J. Simpson, Biochem. Biophys. Res. Commun. **43,** 1–5 (1971).
[23] W. S. Pierpoint, Biochem. J. **98,** 567–580 (1966).
[24] V. S. Kalyanaraman, S. A. Kumar, and S. Mahadevan, Biochem. J. **149,** 577–584 (1975).
[25] R. H. Kenten, Biochem. J. **55,** 350–360 (1953).
[26] E. W. Kellog and I. Fridovich, J. Biol. Chem. **250,** 8812–8817 (1975).
[27] J. P. Gove and M. C. Hoyle, Plant Physiol. **56,** 684–687 (1975).
[28] B. Darbyshire, Physiol. Plant. **29,** 293–297 (1973).
[29] H. Shinshi and M. Noguchi, Phytochemistry **14,** 1255–1258 (1975).

[30] J. W. Pickering, B. L. Powell, S. H. Wender, and E. C. Smith, Phytochemistry **12,** 2639–2643 (1973).
[31] H. Sano, Biochim. Biophys. Acta **227,** 565–575 (1971).
[32] E. Teuscher, Pharmazie **28,** 6–18 (1973).
[33] W. Schreiber, Biochem. Biophys. Res. Commun. **63,** 509–514 (1975).
[34] E. Surholt and W. Hösel, Phytochemistry **17,** 873–877 (1978).
[35] J. Berlin and W. Barz, Z. Naturforsch. **30 c,** 650–658 (1975).
[36] B. Z. Siegel and A. W. Galston, Science **157,** 1557–1559 (1967).
[37] J. W. McClure, The Flavonioids (T. J. Mabry, J. B. Harborne, and H. Mabry, eds.), Chapter 18, Chapman and Hall, Ltd., London 1975.
[38] M. C. Hoyle, Plant Physiol. **60,** 787–793 (1977).
[39] H. Yamazaki and I. Yamazaki, Arch. Biochem. Biophys. **154,** 147–159 (1973).
[40] I. Yamazaki, Molecular Mechanisms of Oxygen Activation (O. Hayaishi, ed.), Chapter 13, Academic Press, New York, London 1974.
[41] V. S. Kalyanaraman, S. Mahadevan, and S. A. Kumar, Biochem. J. **149,** 565–576 (1975).