Substrate specificity of catechol oxidase from *Lycopus europaeus* and characterization of the bioproducts of enzymic caffeic acid oxidation

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Abstract The substrate specificity of catechol oxidase from *Lycopus europaeus* towards phenols is examined. The enzyme catalyzes the oxidation of *o*-diphenols to *o*-quinones without hydroxylating monophenols, the additional activity of tyrosinase. Substrates containing a -COOH group are inhibitors for catechol oxidase. The products of enzymic oxidation of caffeic acid were analyzed and isolated by HPLC with diode array detection. The neolignans of the 2,3-dihydro-1,4-benzodioxin type (3, 6–8), 6,7-dihydroxy-1-(3,4-dihydroxyphenyl)-2,3-dicarboxy-1,2-dihydronaphthaline (1) 6,7-dihydroxy-1-(3,4-dihydroxyphenyl)-3-carboxynaphthaline (5) and 2,6-bis-(3′,4′-dihydroxyphenyl)-1-carboxy-3-oxacyclo-(3,0)-pentan-2-on-1-ene (4) were formed. A reaction mechanism for the formation of (1, 4 and 5) is discussed.

Key words: Type 3 copper center; Caffeic acid; Enzyme kinetic; Polyphenol oxidase; Lignan

1. Introduction

The oxidation reaction associated with the darkening of damaged tissue in fresh fruits, vegetables and in senescent leaves is catalyzed by polyphenol oxidases (PPOs) [1]. PPOs are also called *o*/*p*-diphenoloxidases or phenolases. They are of widespread appearance among plants and exert their activity on a large number of substrates. PPOs can be further divided into tyrosinases, laccases, or catechol oxidases. All these enzymes reduce molecular oxygen to water during the catalysis of *o*-diphenols to *o*-quinones, but only tyrosinase (EC 1.14.18.1 monophenol monooxygenase) can also catalyze the hydroxylation of monophenols in the ortho position. Laccase (EC 1.10.3.2) is the key enzyme in the biosynthesis of lignin. It oxidizes *o*-diphenols as well as *p*-diphenols and is quite distinct from catechol oxidase (EC 1.10.3.1), which is only active on *o*-diphenols. There are reports in the literature describing the substrate specificity of polyphenol oxidases and tyrosinases. For polyphenol oxidases, other authors have investigated various substrates and have studied the effect of substitution in the ring of phenolic substrates on the velocity of the enzymic reaction ([2] and references therein, [3]). Less detailed information is known about the substrate specificity of catechol oxidases.

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Dedicated to Prof. Dr. Dr. H. Witzel on the occasion of his 75th birthday, deceased 1 September 1996.

Two types of phenolic browning reactions are known: (1) enzymic oxidation when a polyphenol oxidase is present [4,5]; (2) substantial non-enzymic autooxidation can take place [6]. Browning occurs in the early stages of oxidation and correlates with the oxidative disappearance of caffeic acid. The early browning suggests that its formation is not dependent on the formation of large molecules with extended conjugation but rather the formation of small products, i.e. dimers and trimers, in which enough conjugation is present to extend into the visible range [7]. The different steps leading to the quinones and condensation products are not understood yet and only a few hypotheses have been proposed concerning the mechanism of their formation [8,9]. The oxidation products of caffeic acid obtained by non-enzymic oxidation have been described and characterized [6,10].

Spectroscopic studies on the type 3 copper enzymes catechol oxidase [11,12] and tyrosinase ([13] and references therein) revealed the surrounding of the two copper ions in the oxy, deoxy and met form. A X-ray structure analysis is available for the met, deoxy and the phenylthiourea inhibited form of a catechol oxidase from *Ipomoea batatas* [14]. The excellent matching of the EPR, UV/Vis and resonance Raman data for all type 3 copper proteins indicate a near identity of copper coordination and oxygen binding in this family of type 3 copper proteins [13] although tyrosinase catalyzes two different reaction types whereas catechol oxidase oxidizes exclusively catechols to quinones.

To understand the reaction of catechol oxidase and to point out differences to tyrosinase, the specificity of catechol oxidase towards different substrates is studied. In the present paper catechol oxidase is tested towards 16 phenolic substrates. Knowledge about the substrate specification of catechol oxidases is further needed to understand the browning process and how to control it. For a better understanding of the catalytic mechanism of this enzyme we isolated and identified a new reaction product of the enzymic caffeic acid oxidation. Dimerization and cyclization leads to a new caffeic acid lactone derivative. A mechanism for the product formation is proposed.

2. Materials and methods

2.1. Materials

Substrates: tyrosine (Sigma), 2-methoxyphenol (Sigma), 4-hydroxy-3-methoxy-cinnamic acid (Roth), catechol (Sigma), hydroquinone (Sigma), 3,4-dihydroxybenzoic acid, caffeic acid (Fluka), dihydrocaffeic acid (Roth), chlorogenic acid (Roth), verbascosid (Roth), 3,4,5-trihydroxybenzoic acid (Roth), luteolin-7-glycoside (Roth), quercetin (Roth), rosmarinic acid (Roth), myricetin (Roth), rutin (Roth), and

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ellagic acid (Roth) were purchased and used as received. All other chemicals were of analytical grade. The substrates were dissolved just before the assay. Ultrapure water was used throughout this research.

2.2. Enzyme source

Catechol oxidase from *Lycopus europaeus* was purified by a procedure of Rompel et al. [12]. The protein concentration was determined both by the method of Bradford [15], with BSA as a standard, and by measuring the absorbance at 280 nm. The molecular mass of the *Lycopus* enzyme was determined to be 39.8 kDa [12] using MALDI-MS and based on this value the enzyme concentration was calculated.

2.3. Spectrophotometric assays

Kinetic assays were performed by measuring the appearance of the products in the reaction medium using a Shimadzu 2100 UV/Vis spectrophotometer. The temperature was controlled at 20°C by using a circulating water bath with a heater/cooler and checked using a thermometer with a precision of ±0.1°C. The reference cuvette contained all the compounds except the substrate in a final volume of 1 ml.

2.4. Diphenolase activity

The activity of the enzyme during the purification was monitored by spectrophotometric measurements of the oxidation products. Catechol oxidase activity was measured towards 3 mM catechol. The reaction was performed at 20°C in 1 ml of reaction mixture containing 0.1 M sodium phosphate buffer pH 6.5, 3 mM substrate and suitable amounts of enzyme. One unit of enzyme activity is defined as the amount of enzyme which increased the absorbance by 1/min during of the oxidation of 3 mM catechol at 20°C [12].

2.5. Kinetic data analysis

For each compound the spectrum of the product was recorded and maximum absorbances were taken from these spectra. The reaction rates were estimated by drawing tangents to the slopes of the time dependent recordings of the absorbance. The graphical method of Lineweaver and Burk was used to determine K_m and V_{max} values [16].

2.6. Preparation of oxidation products

Caffeic acid (180 mg) was added to 200 ml H_2O and the pH adjusted to 6.0 with 2 N NaOH to dissolve the phenol and to work in a range where the enzyme is close to its optimum pH. To start the reaction, 800 U of catechol oxidase from *Lycopus europaeus* were added. Oxygen was bubbled through while the mixture was stirred on a magnetic stirrer. After 15 min the reaction was stopped with 130 mg KCN. The solution was frozen at −70°C and lyophilized. The lyophilized product was stored at −20°C. 50 mg of the oxidation product was re-dissolved in 0.5 ml H_2O/0.025% trifluoroacetic acid (TFA) and the solution was filtered through a 0.45 μm filter.

2.7. Preparative separation

For analytical HPLC separation a Merck/Hitachi 655 system was used (consisting of a Merck/Hitachi 655 processor A, a Merck/Hitachi 655 variable wavelength UV monitor, a Merck/Hitachi 655 A-11 liquid chromatograph, a Merck/Hitachi 655 A-71 proportioning valve). The injection volume was 100 μl. For separation, mobile phases consisting of acetonitrile containing 0.025% TFA as mobile phase A and H_2O containing 0.025% TFA as mobile phase B were used. A Knauer rp-18, 10 μm (40×1 cm) column was used. Starting with 78% mobile phase B, a linear gradient was run to 68% B in 40 min with a flow rate of 6 ml/min. The column was washed and equilibrated with 78% B for 15 min before the next sample was injected. Fractions obtained were then re-chromatographed on an analytical HPLC system to verify the purity of each fraction. All fractions were refrigerated and kept in the dark. The matching fractions were pooled and concentrated under reduced pressure by a rotary evaporator. Care was taken not to exceed 35°C.

2.8. Mass spectrometry

DCI (direct chemical ionization mass) spectra were collected on a Finnigan MAT 8230. Inlet was a heated wire and NH_3 was used as reactant gas. Mass spectra were recorded at the Institute of Organic Chemistry, University of Münster.

2.9. NMR

^{1}H NMR spectra were recorded at a Bruker WH 300 using deuterated solvents.

^{1}H NMR spectra were recorded at the Institute of Organic Chemistry, University of Münster.

3. Results

3.1. Substrate specificity

The activity of catechol oxidase from *Lycopus europaeus* has been tested on some naturally occurring phenols and their kinetic data are shown in Table 1. The structures of the substrates are given in Fig. 1.

For each of the substrates the wavelength at which the corresponding oxidation product exhibited maximum absorption is determined (see also Table 1). For compounds without a pronounced maximum in their absorption spectra, the 420 nm wavelength was chosen for spectrophotometric determinations. The values for K_m and k_{cat} were obtained by evaluation of Lineweaver-Burk [16] plots of kinetic measurements.

The affinity of catechol oxidase from *Lycopus europaeus* to

Table 1
Substrate specificity of catechol oxidase from *Lycopus europaeus*

Substrate	ε-maximum (nm)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
Monophenols				
Tyrosine			No interaction	
2-Methoxyphenol			No interaction	
4-Hydroxy-3-methoxy-cinnamic acid			No interaction	
Diphenols				
Catechol	420	5.0	160	32
Hydroquinone			No interaction	
3,4-Dihydroxybenzoic acid			Inhibition	
Caffeic acid	420	5.0	106	21.2
Dihydrocaffeic acid	420	4.3	113	26.3
Chlorogenic acid	420	3.5	156	44.6
Verbascosid	460	2.0	150	75
Triphenols and polyphenols				
3,4,5-Trihydroxybenzoic acid			Inhibition	
Luteolin-7-glycoside	490	8.0	22.7	2.8
Quercetin	470	0.8	193	241.3
Rosmarinic acid	330	2.0	109	54.5
Myricetin	470	1.0	213	213
Rutin			No interaction	

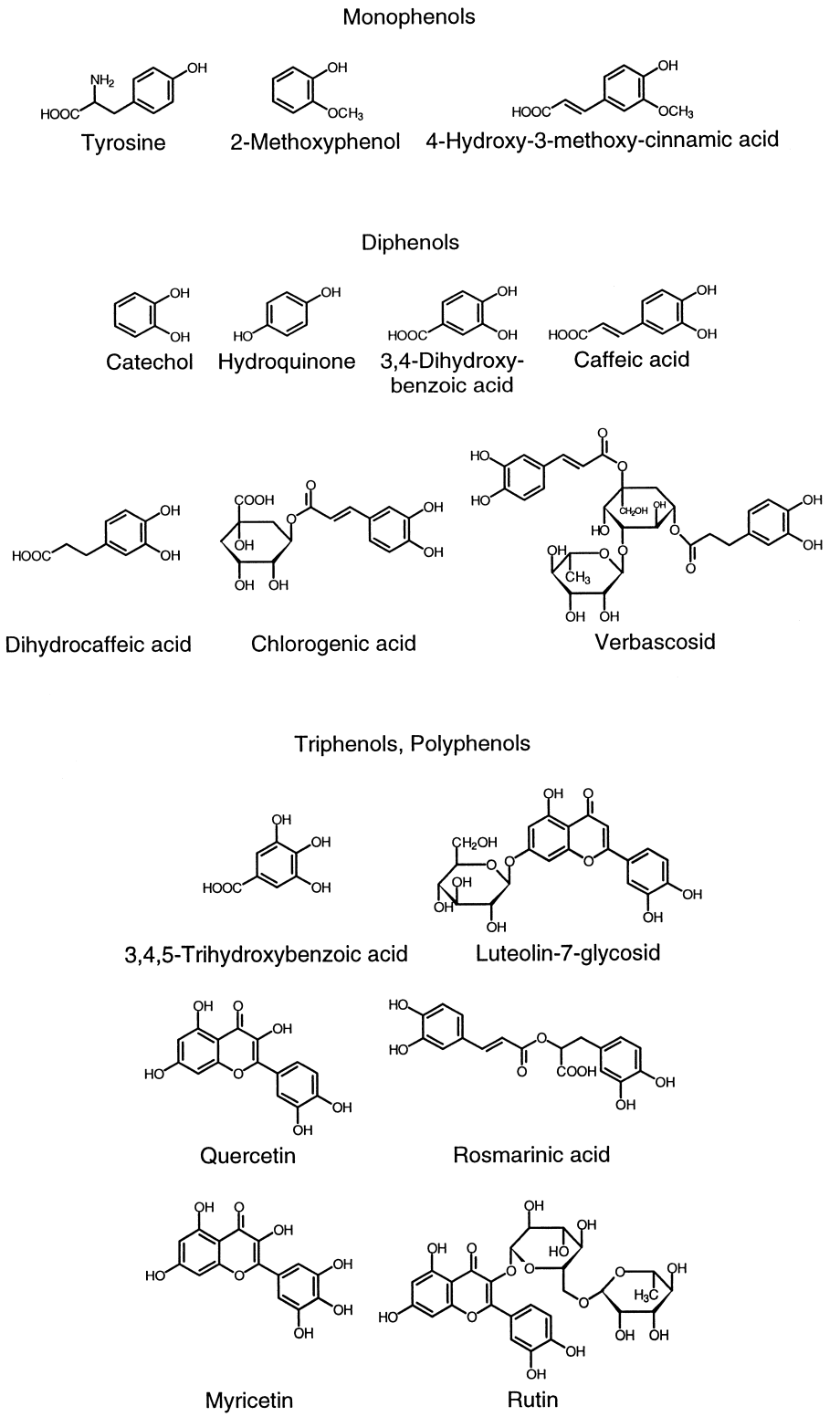

Fig. 1. Structures of the substrates tested for catechol oxidase reactivity.

the phenolic substrates is relatively low. Among the phenols there are substrates and inhibitors, and in some cases no reaction at all was observed with the enzyme. In common with polyphenol oxidases isolated from a variety of higher plant tissues (e.g. tobacco [17], banana [18], sweet potato [11,19], and peach [20]), the *Lycopus* enzyme lacks monophenolase activity and is consequently not a tyrosinase. In particular, this catechol oxidase is unable to convert *p*-diphenols to the corresponding quinone, thus, hydroquinone shows no interaction with catechol oxidase.

Catechol oxidase oxidizes *o*-diphenols as catechol and the natural substrate caffeic acid. Methylation of one of the two

hydroxy groups also leads to no interaction of the substrate with the enzyme. This is demonstrated in the case of catechol (interaction) and *o*-methoxyphenol (no interaction) as well as caffeic acid (interaction) and 4-hydroxy-3-methoxy-cinnamic acid (no interaction).

Catechol oxidase oxidizes a wide range of *o*-diphenols. With some substrates classical Michaelian kinetics were observed, while some others exhibited the phenomenon of inhibition by access substrate. The results are described, below, in groups according to the form of the lines in the Lineweaver-Burk plots.

Lineweaver-Burk plots showed linear lines for the substrates catechol, verbascosid, luteolin-7-glucoside, quercetin, and myricetin. Catechol oxidase appears to possess an extended substrate binding site, since the enzyme-substrate affinity is relatively insensitive to the substrate's bulkiness (compare for example catechol and verbascosid).

Caffeic acid, dihydrocaffeic acid, chlorogenic acid and rosmarinic acid show the phenomenon of inhibition by access substrate. It is remarkable that the phenomenon of inhibition by access substrate occurs with substrates exhibiting a -COOH group in their structure (see compounds caffeic acid, dihydrocaffeic acid, chlorogenic acid and rosmarinic acid, see Fig. 1).

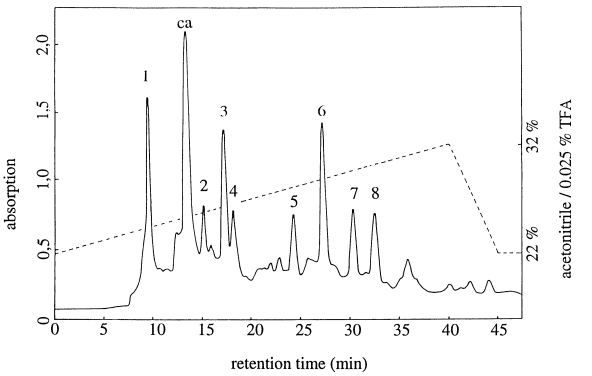

Fig. 2. Mixture of the reaction products (**1**–**8**) after 15 min reaction of 1 mmol caffeic acid (ca) with 800 U catechol oxidase, stopped by addition of KCN and separated on a reversed phase C18 HPLC column.

Obviously a -COOH group can inhibit the activity of catechol oxidase from *Lycopus*. As expected, the compounds 3,4-dihydroxybenzoic acid and 3,4,5-trihydroxybenzoic acid are inhibitors for catechol oxidase. Both compounds contain -COOH residues directly on the phenolic ring.

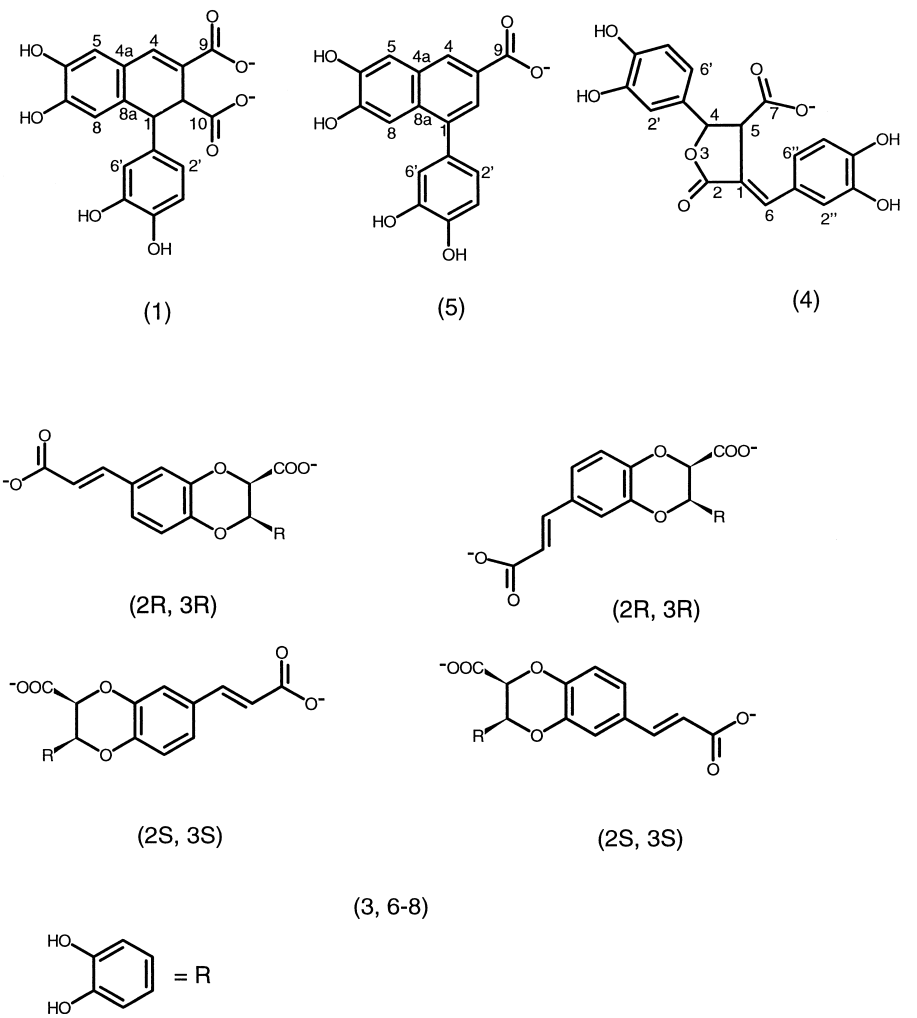

Fig. 3. Structure of 6,7-dihydroxy-1-(3,4-dihydroxyphenyl)-2,3-dicarboxy-1,2-dihydronaphthaline (**1**), 6,7-dihydroxy-1-(3,4-dihydroxyphenyl)-3-carboxynaphthalin (**5**) and 2,6-bis-(3′,4′-dihydroxyphenyl)-1-carboxy-3-oxacyclo-(3,0)-pentan-2-on-1-ene (**4**) and **3**, **6**–**8** which are the caffeicins (A–D) according to [6].

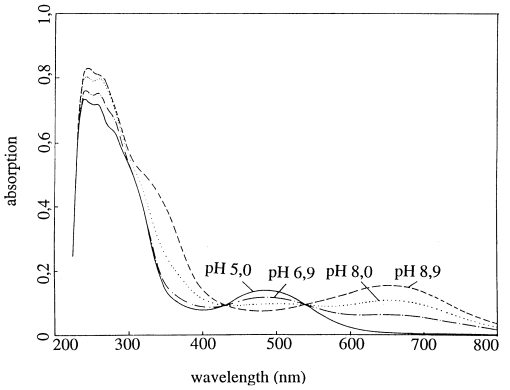

Fig. 4. UV/Vis spectrum of **2**.

More information about the substrate specificity can be obtained by comparing the kinetic data values summarized in Table 1. The compounds myricetin and quercetin show the greatest turnover and the highest enzyme-substrate affinity. The low K_m values for myricetin and quercetin compared to the values of other substrates point towards a higher affinity of this substrate to the enzyme. The k_{cat} value is only doubled whereas the k_{cat}/K_m value is 4–5 times the value compared to other substrates. In myricetin and quercetin, the position three in the chromane ring is occupied by a hydroxy group. Luteoline-7-glucoside and rutin exhibit a very similar structure due to their basic frame work but both show only a low affinity or no interaction. This great difference in reactivity is due to the fact that in rutin and luteoline-7-glucoside the position three in the chromane ring is not occupied by a free hydroxy group that influences the stability of the resulting quinone. The hydroxy group can stabilize the produced quinone by mesomeric effects, which cause a decrease of activation energy for the formation of the enzyme substrate complex.

3.2. Bioproducts of enzymic caffeic acid oxidation

Caffeic acid oxidation was followed spectrophotometrically at pH 6.0. A chromatogram of the oxidation products after 15 min of oxidation at pH = 6.0 is shown in Fig. 2. The HPLC peaks were numbered according to their retention time. A chromatogram of non-enzymic caffeic acid oxidation has been reported and discussed before [6,10].

The products were chromatographically separated to obtain structural and physical information. After separation by HPLC, diode array detection gave UV/Vis spectra from 200 to 370 nm.

Product **1** has been identified as 6,7-dihydroxy-1-(3,4-dihydroxyphenyl)-2,3-dicarboxy-1,2-dihydronaphthaline. The structure is shown in Fig. 3. The DCI method showed a molecular mass of 358 g/mol. The UV/Vis spectrum of **1** exhibits an absorption maximum at 240 nm and three less intense broad absorption features at 290, 305 and 322 nm. ^{1}H NMR signals are as follows: 3.8 (d, J = 4.0 Hz, H-2), 4.33 (d, J = 4.0 Hz, H-1), 6.35 (dd, J = 2.0: 8,4 Hz, H-6′), 6.58 (s, H-8), 6.6 (d, J = 2.0 Hz, H-2′), 6.65 (d, J = 8.4 Hz, H-5′), 6.88 (s, H-5), 7.6 (s, H-4). **1** was first identified and described by Agata et al. [21] as a tetrameric derivative of caffeic acid from *Rabdosia japonica*. Furthermore, **1** was identified as a product of non-enzymic caffeic acid oxidation [6,10].

Product **5** could be identified as 6,7-dihydroxy-1-(3,4-dihydroxyphenyl)-3-carboxynaphthaline. The structure is shown in Fig. 3. **5** can be obtained by decarboxylation of **1**. **5** exhibits a relative molecular mass of 312 g/mol. The UV/Vis spectrum shows an intense absorption maximum at 250 nm and a broad shoulder between 290 and 330 nm. ^{1}H NMR data are as follows: 6.8 (dd, J = 2.2: 8.2 Hz, H-6′), 6.95 (d, J = 2.2 Hz, H-2′), 6.98 (d, J = 8.2 Hz, H-5′), 7.32 (s, H-8), 7.41 (s, H-5), 7.72 (d, J = 1.7 Hz, H-4), 8.34 (d, J = 1.7 Hz, H-2). **5** still shows a low affinity to catechol oxidase, because of the diphenolic groups. **5** is a product of non-enzymic caffeic acid oxidation [10] and the chemical synthesis of **5** has also been described [22].

Product **2** could only be obtained in small amounts and the product is not stable. Furthermore it is the only oxidation product that contains absorption bands in the visible range. Fig. 4 shows the UV/Vis spectrum obtained at different pH values. Below pH 7.5, **2** shows a red color, otherwise a blue color. The appearance of two isosbestic points at 435 and 542 nm is a direct evidence that two different compounds are present depending on the pH value. The appearance of a red product has also been observed by Sugumaran et al. [23] and Kumada et al. [24]. Kumada et al. investigated the tyrosinase catalyzed reaction of caffeic acid whereas Sugumaran et al. investigated the reaction of tyrosinase with 1,2 dehydro-*N*-acetyldopamine. They considered this red product as a quinone methide or a semiquinone radical.

Product **4** could be identified as 2,6-bis-(3′,4′-dihydroxyphenyl)-1-carboxy-3-oxacyclo-(3,0)-pentan-2-on-1-ene. The structure is shown in Fig. 3. **4** exhibits a relative molecular mass of 358 g/mol. The UV/Vis spectrum of **4** shows an intense absorption maximum at 250 nm and a broad shoulder between 290 and 330 nm. ^{1}H NMR signals are as follows: 4.02 (d, J = 3.1 Hz, H-5), 5.62 (d, J = 3.1 Hz, H-4), 6.80 (d, J = 8.3 Hz, H-5′), 6.89 (d, H-2′), 6.91 (dd, J = 8.3: 1.9 Hz, H-6′), 6.95 (d, J = 8.5 Hz, H-5″), 7.09 (dd, J = 8.5 Hz, H-6′), 7.14 (d, H-2″), 7.59 (s, H-6). Due to peak overlapping it was not possible to determine the coupling constants for the aromatic protons H-2′, H-6″ and H-2″. Kumada et al. [24] isolated a dihydrodicaffeic acid dilactone using a tyrosinase from mushroom (*Inonotus* species K-1410). The formation of a dihydrodicaffeic acid lactone during the enzymic oxidation of caffeic acid has never been described before.

The similarities of these UV/Vis spectra of caffeic acid and spectra of products **3**, **6**, **7**, and **8** suggest similar chromophores and indicate that these products are structural analogues possibility modified caffeic acid oligomers. Since the spectra do not exhibit a 40–80 nm bathochromic shift, they can not be quinoid but possibly phenolic products. Some side chain conjugation still exists in the oxidized products to give the 290 and 326 nm peak although the 326 nm absorbance is decreased indicating the involvement and loss of half of the side chain conjugation during oxidation. Furthermore an increase in absorption can be seen at 200 nm. ^{1}H NMR investigations confirmed the UV/Vis data. Products **3**, **6**–**8** are structural analogues (isomers). Cilliers and Singleton [6] have observed four products after non-enzymic oxidation of caffeic acid. They exhibit the same UV/Vis and ^{1}H NMR spectra as we obtained for products **3**, **6**–**8**. Cilliers and Singleton [6] called these compound caffeicins A–D. Caffeicins are deduced to be neolignans of the 2,3-dihydro-1,4-benzodioxin type. The structure of the caffeicins is shown in Fig. 3 and a

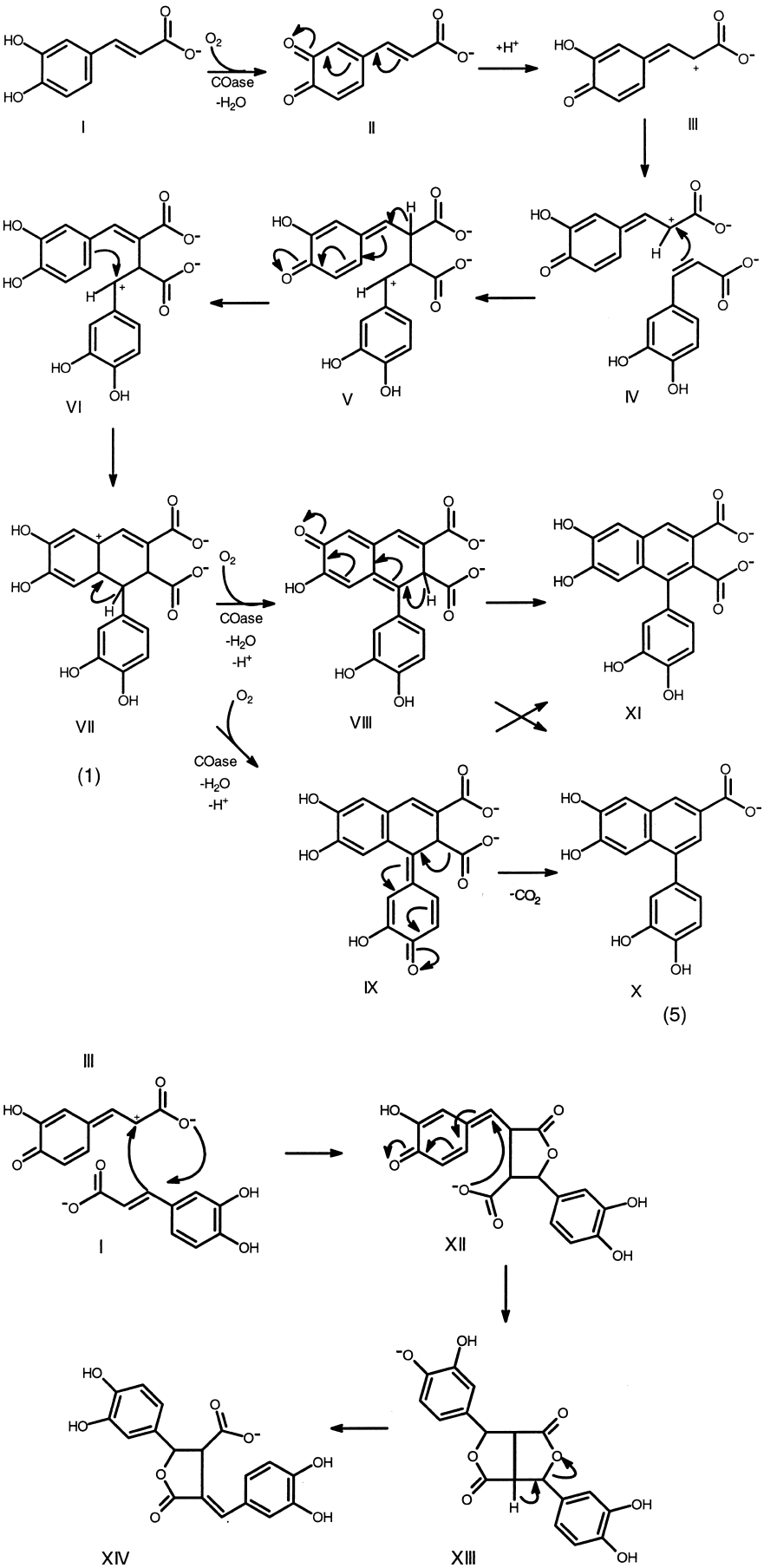

Fig. 5. Reaction mechanism for the formation of **1**, **4** and **5**. COase: catechol oxidase.

postulated mechanism for the formation of these products is published in [6].

4. Discussion

Catechol oxidase from *Lycopus europaeus* catalyses exclusively *o*-diphenols. The K_m values of the different substrates are in general higher than the values reported for fungi [25–27] and bacteria [28] polyphenol oxidases indicating that this plant enzyme is not so specific as the one from fungi and bacteria. The enzyme is insensitive to the substrate's bulkiness. The crystal structure of a catechol oxidase from *Ipomoea batatas* [14] showed that the catalytic pocket will not be able to take up larger substrates as a whole.

It was found that carboxy groups inhibit catechol oxidase activity. This finding is in line with investigations of Balasingham et al. [29] and Kuttner et al. [30], who pointed out that a PPO from mushrooms (*Psalliota campestris*) is inhibited by benzoic acid and its derivatives and that a tyrosinase from mushroom (*Amanita muscaria*) is inhibited by benzoic acid at pH 5 [31]. The effect of inhibition might be due to the following binding modes: (a) a -COOH group coordinates the two copper atoms 2.9 Å apart [32]; (b) the -COOH group binds at a positively charged amino acid residue near the catalytic pocket. Consequently the active site is blocked for binding of substrate.

Enzymic oxidation of caffeic acid by catechol oxidase leads in the first step to the corresponding quinone. The formation of the quinone has been observed before for the oxidation of caffeic acid by other polyphenol oxidases [33–36] and the oxidation with $KMnO_4$ (10–20%) [10,37]. The comparison of enzymic caffeic acid oxidation with the oxidation by $KMnO_4$ (20%) showed that both oxidations partially led to the same products.

We clearly showed the structure of products **1**, **4** and **5**. In the following, we postulate a mechanism for their formation. The first step is the oxidation of caffeic acid (I) in the presence of oxygen and catechol oxidase from *Lycopus europaeus* is the formation of the quinone (II). It is well known that II rearranges to the quinone methide (III) [38]. It follows a nucleophilic addition of a caffeic acid molecule (IV), which leads to the formation of a σ-bonding to the α-C atom (V). The β-C atom takes over the positive charge. The methide tautomerizes to the aromatic system (VI), which forms a six-membered ring by electrophilic substitution (VII). The product is 6,7-dihydroxy-1-(3,4-dihydroxyphenyl)-2,3-dicarboxy-1,2-dihydronaphthaline (**1**), which is difficult to obtain by enzymic oxidation because it undergoes further oxidation. The oxidation of VII leads to the quinones and the methides (VIII and IX). Both can eliminate CO_2 in position 2, producing X which was identified as 6,7-dihydroxy-1-(3,4-dihydroxyphenyl)-3-carboxynaphthaline (**5**). At basic pH it should be possible to obtain 6,7-dihydroxy-1-(3,4-dihydroxyphenyl)-2,3-dicarboxynaphthaline (XI). Since we worked at pH 6, we were not able to isolate this product. The mechanism for the formation of **4** is shown in the lower part of Fig. 5. Again we postulate the formation of **4** via the quinone methide.

5. Conclusions

In summary, catechol oxidase from *Lycopus europaeus* catalyzes the oxidation of catechols to quinones. The enzyme shows no tyrosinase activity and catalyzes the oxidation of a variety of *o*-diphenols. A -COOH group can inhibit the activity of catechol oxidase. The products of enzymic oxidation of caffeic acid were analyzed and isolated by HPLC. The neolignans of the 2,3-dihydro-1,4-benzodioxin type (**3**, **6**–**8**) as well as 6,7-dihydroxy-1-(3,4-dihydroxyphenyl)-2,3-dicarboxy-1,2-dihydronaphthaline (**1**) 6,7-dihydroxy-1-(3,4-dihydroxyphenyl)-3-carboxynaphthaline (**5**) and 2,6-bis-(3′,4′-dihydroxyphenyl)-1-carboxy-3-oxacyclo-(3,0)-pentan-2-on-1-ene (**4**) could be identified. This is the first description of the formation of a dihydrodicaffeic acid lactone during the enzymic oxidation of caffeic acid. A mechanism for the formation of **1**, **4** and **5** was discussed.

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