ORIGINAL ARTICLE

Laccase-catalyzed carbon–nitrogen bond formation: coupling and derivatization of unprotected L-phenylalanine with different *para*-hydroquinones

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Abstract Unprotected L-phenylalanine was derivatized by an innovative enzymatic method by means of laccases from *Pycnoporus cinnabarinus* and *Myceliophthora thermophila*. During the incubation of L-phenylalanine with *para*-hydroquinones using laccase as biocatalyst, one or two main products were formed. Dependent on the substitution grade of the hydroquinones mono- and diaminated products were detected. Differences of the used laccases are discussed. The described reactions are of interest for the derivatization of amino acids and a synthesis of pharmacological-active amino acid structures in the field of white biotechnology.

Keywords Laccase · Cross-coupling · Phenylalanine · Derivatization · Unprotected amino acid · Quinone

Introduction

Enzyme catalyzed reactions are currently of considerable interest for new biotechnological purposes. A great advantage of these reactions is the utilization of mild and environmental-friendly conditions, for example none or less toxic educts, ambient temperature and atmospheric pressure. Thus, also substances which are sensitive towards chemical synthesis procedures, e.g. amino acids can be derivatized. The so far known chemical derivatization methods of amino acids are complicate and mostly connected with a use of dangerous compounds, for example the usage of sodium periodate (Lai et al. 1996). The laccase-catalyzed reaction is an environmental-friendly method for the derivatization of unprotected amino acids.

Laccases [E.C. 1.10.3.2] are polyphenoloxidases, which oxidize aromatic phenols to reactive radicals, which can undergo non-enzymatic coupling reactions (Davin and Lewis 2005; Burton 2003; Claus 2003; Gianfreda et al. 1999; Thurston 1994; Bollag 1992). Therefore, interest in the potential of these enzymes in biotransformations used in organic synthesis has increased (Mikolasch et al. 2006; Niedermeyer et al. 2005; Schauer et al. 2001) especially to show great promise for the derivatization of amino acids (Manda et al. 2006).

To further determine the potential of laccases for amino acid derivatization, laccases of *Pycnoporus cinnabarinus* and *Myceliophthora thermophila* were used for reactions of L-phenylalanine with different *para*-hydroquinones. In this study, we show that L-phenylalanine can be derivatized by different substituted *para*-hydroquinones under laccase-catalyzed conditions. Depending on the substituents mono- or diaminated products were formed.

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Material and methods

Chemicals

L-phenylalanine was purchased from Serva Feinbiochemica GmbH & Co. (Heidelberg, Germany). 1,4-Hydroquinone

and methyl-1,4-hydroquinone were products of Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and 2,3-dimethyl-1,4-hydroquinone was obtained from Arcos Organics (New Jersey, USA). Trimethyl-1,4-hydroquinone was purchased from Alfa Aesar GmbH & Co. KG (Karlsruhe, Germany).

Enzymes

The laccase was obtained from *Pycnoporus cinnabarinus* SBUG-M 1044. The white rot fungus was isolated from an oak tree in northern Germany and is deposited at the strain collection of the Department of Biology of the University of Greifswald (SBUG).

Cultivation of *Pycnoporus cinnabarinus* SBUG-M 1044 and crude preparation of laccase as we reported previously (Jonas et al. 1998).

Myceliophthora thermophila laccase (expressed in genetically modified *Aspergillus* sp.) was obtained from Novozymes (Bagsværd, Denmark).

Measurement of laccase activity

The activity of laccase was determined spectrophotometrically at 420 nm using ABTS (2,2′-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt) as substrate (Bourbonnais and Paice 1990) and the method described by Jonas et al. (1998).

Experimental procedures

For analytical experiments the educts L-phenylalanine (1 or 100 mM) and the respective 1,4-hydroquinone (1 mM) were incubated with laccase (activity 487 nmol ml^{-1} min^{-1}). For the reaction with *Pycnoporus cinnabarinus* laccase, 20 mM sodium acetate buffer (SAB) pH 5 was used and the reaction with the laccase of *Myceliophthora thermophila* proceeded in citrate phosphate buffer (CPB, 18 mM citrate, 165 mM phosphate) pH 7. The reaction mixture was incubated in 5-ml-brown-glass-bottles with 2 ml sample volume at room temperature with agitation at 200 rpm.

Analytical HPLC

For routine analysis, the reaction mixtures were analyzed using an HPLC system LC-10AT VP (Shimadzu, Germany) consisting of a FCV-10AL VP pump, SPD-M10A VP diode array detector, and a SCL-10A VP control unit controlled by Class-VP version 6.12 SP5. The separation of the substances was achieved on an endcapped, 5-µm, LiChroCART® 125-4 RP18 column (Merck, Darmstadt, Germany) at a flow rate of 1 ml/min. A solvent system consisting of methanol (eluent A) and 0.1% phosphoric acid (eluent B), starting from an initial ratio of 10% A and 90% B and reaching 100% methanol within 14 min, was used.

Isolation of transformation products

At first, a liquid–liquid-extraction with ethylacetate was performed to remove undesired byproducts.

Then a RP18 silicagel column (polypropylene 6 ml, 2000 mg adsorbent material, Merck, Darmstadt, Germany) was charged with 1 ml of the aqueous phase from the liquid–liquid-extraction. The amino acid L-phenylalanine was eluted with 30 ml citrate phosphate buffer, 1 ml 5%, 2 ml 10% methanol in water and 1 ml 100% methanol. The cross-coupling products were eluted with additional 1 ml 100% methanol. For mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy the isolated products (**3c** and **4a**) were dried by lyophilization.

Characterization

The products were characterized by liquid chromatography/mass spectrometry (LC/MS). The standard atmospheric pressure ionization (API) mass spectrometry experiments were performed using an Agilent Series 1100 HPLC system and an Agilent 1946C quadrupole mass spectrometer (Waldbronn, Germany). The MS was used with both, atmospheric pressure chemical ionization (APCI) and electrospray ionization (API-ES) sources. HPLC separation was performed on a LiChroCART® 125-4, LiChrosphere® 100 RP-18e column (Merck, Darmstadt, Germany) at 25°C at a flow rate of 1 mL/min within a 14-min gradient from 10 to 100% methanol in 0.1% aqueous formic acid. APCI/API-ES MS conditions (positive and negative ion mode) were as follows: nebulizer and drying gas, nitrogen; nebulizer pressure, 30 psig; drying gas flow, 10 L/min; vaporizer temperature (for APCI), 350°C; drying gas temperature, 250°C; capillary voltage, 4 kV; corona current (for APCI), 4 µA.

The LC high-resolution mass spectrometry experiments were performed on an Agilent Series 1200 HPLC system and an Agilent 1969A time-of-flight mass spectrometer (Waldbronn, Germany). The TOF-MS conditions (neg. and pos. ion mode) with a dual sprayer API-ES source were as follows: nebulizer and drying gas, nitrogen; nebulizer pressure, 40 psig; drying gas flow, 10 L/min; drying gas temperature, 350°C; capillary voltage, 4 kV; fragmentor voltage, 175 V; skimmer voltage, 60 V; octopole voltage, 250 V; mass reference (*m/z*), 121.05087 and 922.00979 in pos. ion mode, and 112.98558 and 1033.98810 in neg. ion mode. HPLC separation was performed on a Zorbax Eclipse XDB-C8, 4.6 × 50 mm, 3.5 µm, column (Agilent, Germany) at 25°C at a flow rate of 0.5 mL/min within a 14-min gradient from 10 to 100% methanol in 0.1% aqueous formic acid.

The nuclear magnetic resonance (NMR) spectra for product **3c** and **4a** (^{1}H-, ^{13}C-NMR, DEPT) were recorded on different Bruker Avance 600 instruments (Karlsruhe, Germany) at 600 MHz in MeOH-d$_4$. Tetramethylsilane was used as internal standard.

2-(3,6-Dioxocyclohexa-1,4-dienylamino)-3-phenylpropionic acid (3a)

Synthesis and isolation as described above. **3a** was only present in a mixture with **4a**, due to the fast reaction of **3a** to **4a**. Yield for **3a** and **4a** 66.8%. R$_f$ (HPLC) 10.52 min, UV/VIS λ_{max} 211, 260, 477 nm.

MS (LC/MS): m/z (relative intensity): API-ES, pos. ion mode 272 ([M + H]$^+$, 65), 226 ([M − CO$_2$H–H]$^+$, 100).

HRMS for **3a**: [M + H]$^+$ (C$_{15}$H$_{14}$NO$_4$): calcd.: 272.0917; found: 272.0911 (−2.4 ppm).

2-[4-(1-Carboxy-2-phenylethylamino)-3,6-dioxocyclohexa-1,4-dienylamino]-3-phenylpropionic acid (4a)

Synthesis and isolation as described above. Red brown solid. Yield 71.3%. R$_f$ (HPLC) 12.95 min, UV/VIS λ_{max} 205, 342, 460 nm; 600 MHz ^{1}H-NMR (MeOH-d$_4$), see Table 1.

MS (LC/MS): m/z (relative intensity): API-ES, pos. ion mode 1325 ([3 M + Na]$^+$, 28), 891 ([2 M + Na]$^+$, 42), 457 ([M + Na]$^+$, 68), 435 ([M + H]$^+$, 100); APCI, pos. ion mode 435 ([M + H]$^+$, 15), 391 ([M − COO + H]$^+$, 100).

Table 1 ^{1}H-NMR data of the transformation product (**4a**) resulted from the reaction of 1,4-hydroquinone (**1a**) and L-phenylalanine (**2**) with laccase (MeOH-d$_4$ as solvent)

C	^{13}C	^{1}H	^{1}H–^{13}C correlations
2′, 6′	130.6	7.17, d, 4H, (7.4)	C3, C1′, C2′, C6′
3′, 5′	129.5	7.23, dd, 4H, (7.0, 7.8)	C1′, C3′, C5′
4′	127.8	7.15, m, 2H	C2′, C6′
2″, 5″	93.7	5.11, s, 2H	C1″, C3″, C4″, C6″
2	60.1	4.05, dd, 2H, (4.6, 6.8)	C1, C3, C1′, C1″, C4″
3	39.0	3.25, dd, 2H, (4.6, −13.7)	C1, C2, C1′, C2′, C6′
3	39.0	3.05, dd, 2H, (6.8, −13.7)	C1, C2, C1′, C2′, C6′
3″, 6″	179.1		
1	176.1		
1″, 4″	152.2		
1′	138.7		

^{1} H, J (in Hz) values in parentheses. Chemical shifts are expressed in δ (ppm) calibrated on the resonances of TMS

HRMS for **4a**: [M + H]$^+$ (C$_{24}$H$_{23}$N$_2$O$_6$): calcd.: 435.1551; found: 435.1549 (−0.4 ppm).

[M − H]$^-$ (C$_{24}$H$_{21}$N$_2$O$_6$): calcd.: 433.1405; found: 433.1411 (+1.3 ppm).

2-(5-Methyl-3,6-dioxocyclohexa-1,4-dienylamino)-3-phenylpropionic acid (3b)

R$_f$ (HPLC) 11.75 min, UV/VIS λ_{max} 208, 275, 477 nm.

MS (LC/MS): m/z (relative intensity): API-ES, pos. ion mode 286 ([M + H]$^+$, 100), 240 ([M − CO$_2$H–H]$^+$, 83).

HRMS for **3b**: [M + H]$^+$ (C$_{16}$H$_{16}$NO$_4$): calcd.: 286.1074; found: 286.1081 (+2.7 ppm).

2-[4-(1-Carboxy-2-phenylethylamino)-5-methyl-3,6-dioxocyclohexa-1,4-dienylamino]-3-phenylpropionic acid (4b)

R$_f$ (HPLC) 13.57 min, UV/VIS λ_{max} 346, 515 nm.

MS (LC/MS): m/z (relative intensity): API-ES, pos. ion mode 919 ([2 M + Na]$^+$, 68), 471 ([M + Na]$^+$, 29), 449 ([M + H]$^+$, 100), 403 ([M− CO$_2$H–H]$^+$, 76).

HRMS for **3b**: [M + H]$^+$ (C$_{24}$H$_{23}$N$_2$O$_6$): calcd.: 449.1707; found: 449.1705 (−0.4 ppm).

[M + Na]$^+$ (C$_{24}$H$_{22}$N$_2$O$_6$Na): calcd.: 471.1527; found: 471.1524 (−0.7 ppm).

2-(4,5-Dimethyl-3,6-dioxocyclohexa-1,4-dienylamino)-3-phenylpropionic acid (3c)

Synthesis and isolation as described above. Dark red to purple solid. Yield 60.5%. R$_f$ (HPLC) 12.95 min, UV/VIS λ_{max} 204, 289, 477 nm. ^{1}H-NMR δ 7.20 (m, ^{3}J = 6.7 Hz, 4H, H-2′, H-3′, H-5′, H-6′), 7.15 (t, ^{3}J = 6.7 Hz, 1H, H-4′), 5.26 (s, 1H, H-2″), 3.97 (dd, ^{3}J = 4.6 Hz, ^{3}J = 7.7 Hz, 1H, H-2), 3.24 (dd, ^{3}J = 4.6 Hz, ^{2}J = −13.8 Hz, 1H, H-3), 3.04 (dd, ^{3}J = 7.7 Hz, ^{2}J = −13.8 Hz, 1H, H-3), 1.96 (d (s), ^{5}J = 1.0 Hz, 3H, aromatic methyl group), 1.95 (d (s), ^{5}J = 1.0 Hz, 3H, aromatic methyl group).

^{13}C-NMR δ 183.46 (C-3″); 180.60 (C-6″); 172.89 (C-1); 143.79; 141.18; 135.19; 133.91; 126.63; 126.19; 125.54; 124.58; 123.79; 94.05 (C-2″); 56.22 (C-2); 35.18 (C-3); 8.91 (aromatic methyl group); 7.99 (aromatic methyl group).

MS (LC/MS): m/z (relative intensity): APCI, pos. ion mode 300 ([M + H]$^+$, 26), 256 ([M − CO$_2$ + H]$^+$, 100); API-ES, neg. ion mode 298 ([M − H]$^-$, 30), 254 ([M − COO-H]$^-$, 100).

HRMS for **3c**: [M + H]$^+$ (C$_{17}$H$_{18}$NO$_4$): calcd.: 300.1230; found: 300.1228 (−0.6 ppm).

[M + Na]$^+$ (C$_{17}$H$_{17}$NO$_4$Na): calcd.: 322.1050; found: 322.1051 (+0.6 ppm).

3-Phenyl-2-(2,4,5-trimethyl-3,6-dioxocyclohexa-1,4-dienylamino)propionic acid (**3d**)

R_f (HPLC) 13.53 min, UV/VIS λ_{max} 203, 287, 494 nm.

MS (LC/MS): m/z (relative intensity): API-ES, pos. ion mode 336 ($[M + Na]^+$, 12), 256 ($[M + H]^+$, 63); 270 ($[M - COO\text{-}H]^-$, 100).

HRMS for **3c**: $[M + Na]^+$ ($C_{18}H_{19}NO_4Na$): calcd.: 336.1206; found: 336.1199 (-2.2 ppm).

Results

General observations

The heteromolecular transformation of 1,4-hydroquinone and methyl-1,4-hydroquinone with L-phenylalanine (educts **1a**, **1b** and **2**, Fig. 1) in the presence of laccase from *Pycnoporus cinnabarinus* and *Myceliophthora thermophila* resulted in the formation of two main products for each reaction **3a**, **3b** and **4a**, **4b** (Fig. 1). Whereas in the reaction of 2,3-dimethyl-1,4-hydroquinone (**1c**) and L-phenylalanine only one main coupling product (**3c**) was formed. Due to their characteristic UV/VIS-spectra, formation of products and decrease of substrates could easily be detected by HPLC using a diode array detector (200–595 nm).

In all performed heteromolecular transformations, the product yield was low with an educt concentration of 1:1 mM, and in the reaction mixture of trimethyl-1,4-hydroquinone (**1d**) no products were detected. Through a rise of amino acid concentration from 1:1 to 1:100 mM the reaction rate and the product yield could be increased. The highest product yield was achieved with the laccase of *M. thermophila* at a pH of 7 and a 1:100 mM concentration of the educts.

Using *M. thermophila* laccase (1:100 mM, **1a**:**2**) the concentration of **3a** (first product) was approx. 13-fold higher after 20 min and those of **4a** (second product) approx. 3-fold higher after 24 h than in the reaction with *P. cinnabarinus* laccase (1:100 mM) (Fig. 2). By means of the laccase of *M. thermophila* the concentration of product **3c** was 27-fold higher in the 1:100 mM attempt (2,3-dimethyl-1,4-hydroquinone:L-phenylalanine) after 24 h compared with the 1:1 mM reaction.

In the reaction mixture of **1d** and **2** (1:100 mM) only traces of one coupling product (**3d**) were detected, due to the characteristic UV/VIS-spectra and MS measurements (for data see "Characterization" section).

In all laccase-catalyzed reactions of **1a-d**, respectively, one product was detected, which was identified through the retention time (HPLC) and UV/VIS-spectrum as the corresponding quinonoid form of the substrate (data not shown), as described for other *para*-hydroquinones (Mikolasch et al. 2006; Manda et al. 2005; Niedermeyer et al. 2005). In all cross-coupling reactions, the respective hydroquinone was converted within 20 min completely into the corresponding quinone.

The concentration of these quinones decreased during the incubation of the reaction mixture, under simultaneous formation of heteromolecular coupling products. This means that the reactions proceeded in two steps. First the quinones are formed, which then react with the amino acid resulting in the development of a monoaminated heteromolecular coupling product, as recently described (Mikolasch et al. 2006; Manda et al. 2005; Niedermeyer et al. 2005). Further reactions with the amino partner to diaminated products are dependent on the substitution grade of the *p*-hydroquinone. Whereas **3a** reacted with **2** forming **4a**, an diaminated product, for **3c** no further reaction with **2** could be detected.
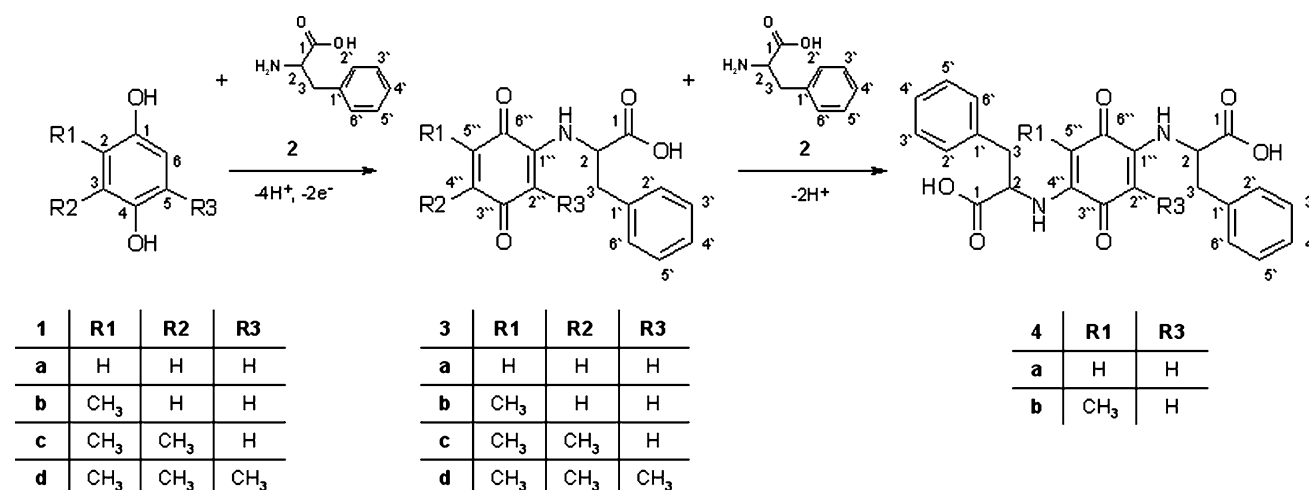

1	R1	R2	R3
a	H	H	H
b	CH₃	H	H
c	CH₃	CH₃	H
d	CH₃	CH₃	CH₃

3	R1	R2	R3
a	H	H	H
b	CH₃	H	H
c	CH₃	CH₃	H
d	CH₃	CH₃	CH₃

4	R1	R3
a	H	H
b	CH₃	H

Fig. 1 1,4-hydroquinone, methyl-1,4-hydroquinone, 2,3-dimethyl-1,4-hydroquinone and trimethyl-1,4-hydroquinone (substrates **1a–d**), respectively with L-phenylalanine (educt **2**) and the products (**3a–d**), (**4a, b**) as results of laccase-catalyzed reaction
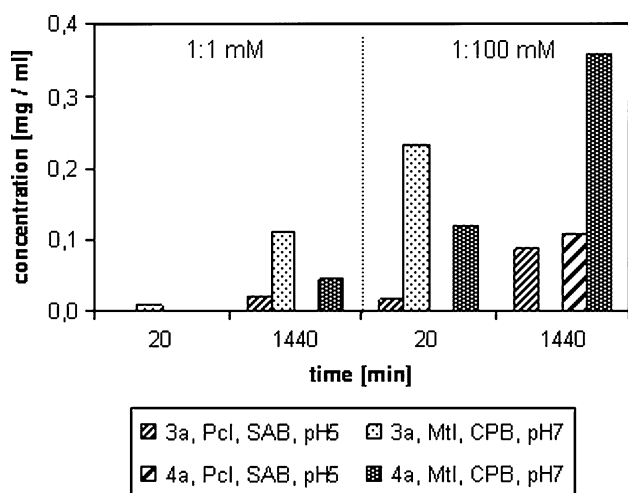
Due to the highest yield of the main coupling products **3a**, **3c** and **4a**, the reaction with *Myceliophthora thermophila* laccase, 1:100 mM (1,4-hydroquinone/2,3-dimethyl-1,4-hydroquinone:L-phenylalanine) was chosen for the characterization of the products.

Detailed structural characterization of three products

By means of HPLC-MS the reaction product **3a** was identified as quinonoid structure, arising from the oxidation of 1,4-hydroquinone and further coupling with one molecule L-phenylalanine.

In accordance with the first heteromolecular coupling product, the second coupling product **4a** was identified as quinonoid structure. But while **3a** was formed of one molecule benzoquinone and one molecule L-phenylalanine, product **4a** was constituted of one molecule benzoquinone and two molecules of the amino acid L-phenylalanine. The structure was revealed by MS measurements and NMR analysis. In the API-ES positive ion mode the typical fragment ion of the decarboxylated form of the product was found. The analysis of product **3a** by high-resolution mass spectrometry (HRMS) in positive ion mode showed the expected ions $[M + H]^+$ at $m/z = 272.0911$. This measured value, with an error of -2.4 ppm, was in adequate conformance with the calculated values for the formula $C_{15}H_{14}NO_4$. The HRMS of product **4a** in the positive ion mode showed a signal at $m/z = 435.1549$ for the quasi-molecular ion $[M + H]^+$. This value confirmed the calculated mass of the quasi-molecular ion $[M + H]^+$ with a deviation of -0.4 ppm. Additionally, an intensive signal was detected in negative ion mode at $m/z = 433.1411$ for the $[M - H]^-$ ion, in good accordance with the supposed value $m/z = 433.1405$, too.

^{1}H-NMR spectral data of **4a** showed characteristic signals for both educts (Table 1). Multiplicity of the H5″ and H2″ protons indicated substituents at the C1″ and the C4″ position. In the ^{13}C-NMR spectrum of **4a,** the signal for C3″ and C6″ in the range of 180 ppm indicated a quinonoid character of the product. The HMBC spectrum of **4a** (Table 1) showed correlations between the protons H5″/H2″ and the quinone carbonylcarbons confirming the oxidation of the *p*-hydroquinone to a quinone as described previously (Mikolasch et al. 2006; Manda et al. 2005; Niedermeyer et al. 2005; Tatsumi et al. 1994a, b). All facts together proved **4a** to be aminated at the C1″ and the C4″ position of the quinone ring.

The heteromolecular product **3c** has a monoaminated quinonoid structure, as shown with MS and NMR measurements. In the API-ES (neg. ion mode) and APCI (pos. ion mode) measurements the anticipated quasi-molecular ions $[M - H]^-$ at $m/z = 298$ and $[M + H]^+$ at $m/z = 300$ were detected. The investigations concerning product **3c** by high-resolution mass spectrometry in positive ion mode showed signals for the $[M + H]^+$ ion at $m/z = 300.1228$ and for the $[M + Na]^+$ ion at $m/z = 322.1051$ which were in good accordance with the theoretical values of $m/z = 300.1230$ (-0.6 ppm) and $m/z = 322.1050$ ($+0.6$ ppm), respectively.

The final confirmation of the structure was accomplished with different NMR measurements in deuterated methanol-d_4 (^{1}H-, ^{13}C-NMR, DEPT). In evaluation of the ^{1}H- and ^{13}C-NMR-spectra it could be stated that both the number and the multiplicity of resonance signals within the aromatic range changed in comparison with **1c**. In the ^{1}H-NMR-spectrum of **3c** appears a signal at 5.26 ppm, which could be assigned to the H6″. This could be attributed to the coupling of one molecule **2** at C1. The aromatic signals of **2** are high-field-shifted in the coupling product. Additionally all signals show more varied couplings due to the substitution at the amino group caused by steric effects of **1c**. In the ^{13}C-NMR-spectrum, two signals in the range of 180 ppm indicated a quinonoid character of the product. Including the results of DEPT the structure of **3c** could be confirmed, as 2-(4,5-dimethyl-3,6-dioxocyclohexa-1,4-dienylamino)-3-phenylpropionic acid (Fig. 1).

Discussion

Structural characterization of three reaction products showed that in the case of laccase-catalyzed reaction, derivatization of the amino acid L-phenylalanine with *para*-dihydroxylated compounds depends on the number and position of substituents of the substrates.

The *p*-hydroquinone-substrates can be divided into two groups. There are on the one hand alkylated *p*-hydroquinones as used in this study and on the other hand

2,5-dihydroxybenzoic acid derivatives as used by Mikolasch et al. (2006), Manda et al. (2005) and Niedermeyer et al. (2005).

We pointed out that enzymatic derivatization of amino acids is possible with alkylated *p*-hydroquinones, namely 1,4-hydroquinone as the basic structure, methyl-1,4-hydroquinone, 2,3-dimethyl-1,4-hydroquinone and trimethyl-1,4-hydroquinone, while Manda et al. (2006) derivatized L-tryptophane with 2,5-dihydroxy-*N*-(2-hydroxyethyl)-benzamide only. 2,5-Dihydroxy-*N*-(2-hydroxyethyl)-benzamide is a substance of the 2,5-dihydroxybenzoic acid derivative group.

As mentioned above the laccase catalyzes a radical formation. The radicals can be transformed into quinones, and then a nucleophilic attack of the amino acid is possible. The nuclear amination resulting in the formation of a C–N-coupled heteromolecular product, consisting of the respective quinone and the amino acid L-phenylalanine, as described for other aminations (Mikolasch et al. 2006; Manda et al. 2005; Niedermeyer et al. 2005; Michalek and Szarkowska 1959).

In the reaction of hydroquinones with amines, two aminations dependent on the substitution grade are conceivable. First one amine is coupled to the hydroquinone, confirmed by the structural characterization of the products **3a** and **3c**. A further amine molecule can then react with the *para*-position to the first amine group, if there is no other substituent. That was confirmed by the structural analysis of **4a** (Fig. 1). The same reaction mechanism could be observed with **1b** and **2**. Nevertheless, the *ortho*-position for the diamination would be possible, but it is not very likely due to the steric covering of the *ortho*-position of the first amino substituent. The preferential substitution is in *para*-position (Yamaoka and Nagakura 1971), as described likewise for other diaminations (Niedermeyer et al. 2005; Berger 1988; Finley 1988) (Fig. 3).

Remarkable is the fact that small amounts of **3d** were formed from **1d** under the used reaction conditions (1:100 mM, **1d**:**2**), whereas Niedermeyer et al. (2005) did not detect any products in the reactions of trimethyl-1,4-hydroquinone and aromatic amines with a concentration of 1:5 mM. Our results suggest that changes in molarity can initiate the formation of products.

Altogether the amination of unsubstituted and monomethylated 1,4-hydroquinone leads to two coupling products, heteromolecular di- and trimer. In contrast to this reaction pattern, the heteromolecular dimer is the final product in the transformation reactions of 2,3-dimethyl-1,4-hydroquinone and trimethyl-1,4-hydroquinone respectively with L-phenylalanine, developed after the first nucleophilic addition. Due to the methyl groups at C2 and C3 at the aromatic ring of **1c** and additionally at C5 of **1d** a second amination is hindered. A diamination of substituted
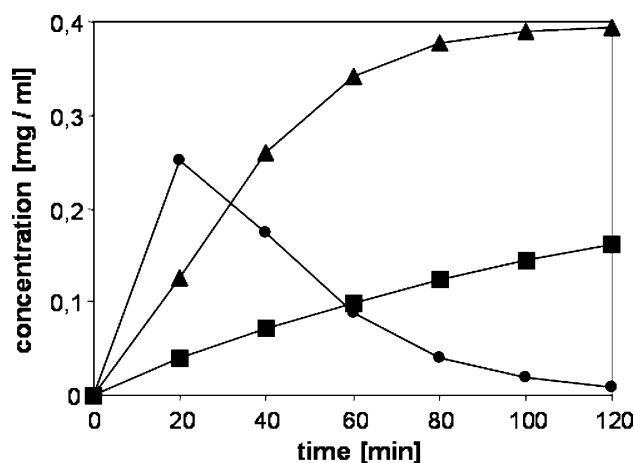

Fig. 3 Kinetics of product formations (**3a** *filled circle*; **3c** *filled square*, **4a** *filled triangle*) during laccase-catalyzed reaction of 1,4-hydroquinone (educt **1a**) and 2,3-dimethyl-1,4-hydroquinone (substrate **1c**), respectively, with L-phenylalanine (educt **2**) (1:100 mM)

1,4-benzoquinones is however not impossible in principle. With hydroxy, alkoxy or halogen substituents the reaction happens usually under substitution of the functional group and also alkyl groups can be replaced in some cases by amino groups (Ulrich and Richter 1977). Chakraborty et al. (1998) determined in the reaction of 2,3-dimethyl-1,4-hydroquinone and octadecylamine, a substitution of a methyl group, which was in *para*-position to the first bound octadecylamine.

Here it is to be pointed out that these reactions were chemically catalyzed, without participation of laccase. Due to the mild reaction conditions in laccase-catalyzed synthesis, compared to chemical methods, no diamination was determined with laccase.

The reactions of **1a** and **1c,** respectively, with **2** showed many parallels.

Concerning product concentration the heteromolecular transformation reactions were affected by laccase and pH-value of the used buffer solution respectively. Moreover, the product yields were depended on the quantity of the amino acid.

The highest product yield was reached with the laccase of *M. thermophila* and an excess of amino acid. This corresponds with the experiments of Niedermeyer et al. 2005. There it was stated that with *Trametes spec.* laccase (pH-optima 5) the product formation happens slower and with lower yield than with *M. thermophila* laccase (pH-optima 7). Furthermore, increasing the ratio of the amino partner in comparison with the alkylated hydroquinone of 5:1 led to faster turnover rates and higher product yields (Niedermeyer et al. 2005).

Nevertheless, an influence of organic acids of the buffer cannot be excluded, since these can already cause an oxidation of the substrate (Jonas 1997).

In summary, the experiments confirmed, that a derivatization of unprotected amino acids, like L-phenylalanine catalyzed by laccase is possible. The presented method is a new one pot synthesis for the production of molecules with probably new properties. So these derivatized amino acids could be the basis of new pharmaceuticals. Especially in the connection with quinones this could be promising. 1,4-Quinones are components of important bioactive compounds, for example antibiotics or chemotherapeutics (Pachatouridis et al. 2002; Mizushina et al. 2000; Fleck et al. 1978; Falci et al. 1977).

Our described synthesis method is suitable to render the properties of the amino acids and/or of the used hydroquinone. The biological characteristics of the new compounds will be prospectively tested in future.

The application possibilities of the new enzymatically synthesized compounds is manifold and the laccase could be specifically chosen for the respective utilization, for example temperature-resistance or pH-stability. In the future the applicability of laccase will be optimized and enhanced to improve the utilization of the coupling products.

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