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Multicopper Oxidase-Catalyzed Biotransformation of Dihydroquercetin[1]

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Abstract—Multicopper oxidases such as bilirubin oxidase (BOD) from *Myrothecium verrucaria* and laccase (LC) from the basidial fungus *Trametes hirsuta* have been used as catalysts in dihydroquercetin (DHQ) oxidative polymerization. The conditions selected enabled good yields of DHQ oligomers, which were then analyzed using UV-vis, FTIR, ^{1}H and ^{13}C NMR spectroscopy. DHQ oligomers synthesized using both enzymes showed higher thermostability as compared with the monomer. Depending on the oxidase, the products of DHQ polymerization differed in physicochemical properties, and as shown by NMR studies, had different structures.

Keywords: biocatalysis, bilirubinoxidase, fungal laccase, dihydroquercetin, enzymatic polymerization, NMR investigation

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Biocatalysis, which is an environmentally friendly method, is increasingly becoming the alternative of chemical synthesis due to the mild conditions of the reaction. Oxidoreductases are an important tool in the oxidation of various natural compounds in order to produce novel pharmaceuticals. Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) and bilirubin oxidase (bilirubin: oxygen oxidareductase, EC 1.3.3.5) belong to multicopper oxidases and catalyze one-electron oxidation of inorganic and organic compounds by molecular oxygen, with the concomitant reduction of oxygen to water [1, 2]. Aromatic compounds are oxidized in the presence of these enzymes by a radical mechanism with the subsequent coupling of intermediates, which finally results in the formation of oligomers and polymers [3–8]. Enzyme-catalyzed polymerization of natural physiologically active compounds, including various flavonoids, may enable the production of new derivatives for application as pharmaceuticals. As was reported in [9–12], high molecular weight natural polyphenols exhibit enhanced physiological properties as compared with their monomers.

Dihydroquercetin belongs to natural flavonoids (Fig. 1, *1*) and is close to guercetin (*2*) and rutin (*3*). As stated in [13] DHQ is not genotoxic. DHQ has a wide range of pharmaceutical effects, in particular antioxidant, angioprotective, hepatoprotective, antitumoral and other ones [14–17]. Furthermore, DHQ also shows regulatory properties and can control DNA reparation, apoptosis, and mitochondrial biogenesis, as well as regulate the activity of certain enzymes [14, 18–21]. It is known that many flavonoids, including DHQ, decompose rather quickly in vivo, while their relatively high molecular weight derivatives have a longer lifespan [22].

The above data show promise for the synthesis of novel DHQ derivatives, which prompted us to investigate the potential of multicopper oxidases, namely a fungal laccase and bilirubin oxidase, for DHQ oxidative polymerization, as well as the structure and physicochemical properties of the products obtained.

EXPERIMENTAL SECTION

Chemical agents. KH_2PO_4, NaOH, citric acid, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), *N*-methylpyrrolidone, tetrahydrofuran (THF), 1,1'-di-phenyl-2-picrylhydrazyl radical (DPPH$^{\cdot}$), and dimethylsulfoxide (DMSO) from Marbiopharm (Russia) were used without further purification. All the solutions were prepared using deionized water obtained in a Milli Q system (Milli-

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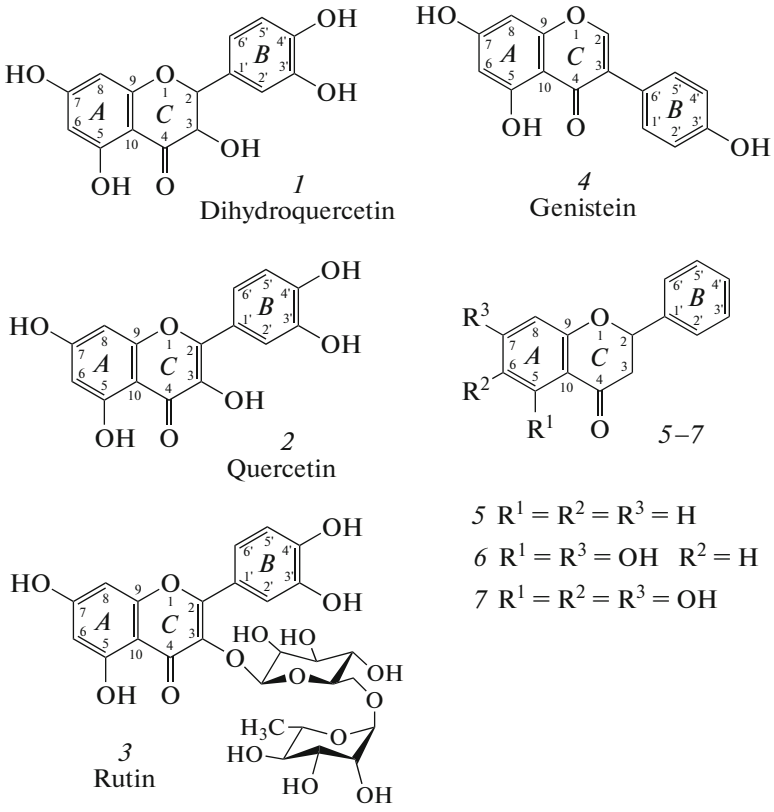

Fig. 1. Structures of certain flavonoids.

pore, USA). Dehydroquercetin (≥96%) was purchased from BioChemMak-ST (Russia).

Enzymes. The laccase was isolated from a culture liquid of the basidial fungus *Trametes hirsuta* as described in [23]. The enzyme homogeneity was checked by SDS electrophoresis. Its specific activity was 158 U/mg protein. The amount of enzyme catalyzing 1 μmol ABTS for 1 min was taken as an activity unit. Bilirubin oxidase from *Myrothecium verrucaria* "Amano 3" (Amano Enzyme Inc., Japan) was additionally purified by ion-exchange chromatography using a Toyopearl DEAE-650M (Toson Biosience, Japan). The specific activity of the resultant enzyme preparation was 110 U/mg protein, ABTS being used as substrate.

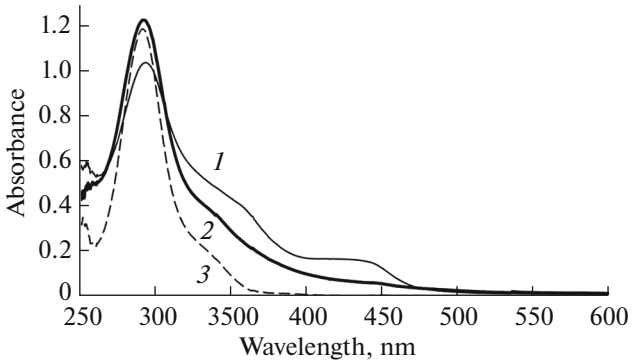

Fig. 2. UV-vis spectra of DHQ monomer (*1*), LC-oligoDHQ (*2*) and BOD-oligoDHQ (*3*) in DMSO solutions.

Enzyme-catalyzed DHQ polymerization. DHQ oligomers were synthesized in buffer solutions containing 3% v/v ethanol at room temperature under aerobic conditions. BOD-mediated DHQ polymerization was carried out in 0.1 M K-phosphate buffer, pH 7.0, and LC-mediated DHQ polymerization, in 0.1 M citrate-phosphate buffer, pH 4.5. In a typical experiment, 10 mg of DHQ was dissolved in 0.3 mL of ethanol, and then 10 mL of the corresponding buffer was added to the solution. The final DHQ concentration in the reaction mixture was 3.2 mmol. DHQ polymerization initiated by adding the enzyme was performed in the air at room temperature (21–22°C) with constant stirring for 24 h until insoluble products were formed. The specific activity of the two enzymes in the reaction mixture was 0.4 U/mL. The DHQ oligomers formed were separated by centrifugation, washed many times with 3% v/v ethanol, dried and used in experiments.

Characterization of DHQ oligomers. The average molecular weight and polydispersity index of the DHQ oligomers produced in the enzyme-catalyzed reaction were determined by gel exclusion chromatog-

raphy using a RI detector. The molecular weight of LC-oligoDHQ was determined on a PL-Gel C column ("Phenomenex") using THF as a mobile phase, and the molecular weight of BOD-oligoDHQ, on a PL Gel MIXED-C column ("Agilent") using *N*-methylpyrrolidon as a mobile phase. The columns were calibrated by polysterene standards.

UV-vis spectra were recorded using a Shimadzu UV1240 mini spectrophotometer (Japan). FTIR spectra were obtained in KBr pellets using a Frontier FT-IR/FIR spectrometer (PerkinElmer Inc.). Thermogravimetric analysis of the samples was carried out in N_2 using a NETZSCH STA 409 PC/PG apparatus with a heating rate of 10°C/min.

^{1}H and ^{13}C NMR spectra of DHQ and oligoDHQ were recorded for the solutions in DMSO-D_6 using a Bruker AVANCE 600 spectrometer with an operating frequency of 600.03 MHz for ^{1}H nuclei.

Antioxidant activity was measured spectrophotometrically at 515 nm using DPPH$^\cdot$ in methanol [24].

RESULTS AND DISCUSSION

In our preliminary experiments, we obtained pH dependences of LC and BOD activity in DHQ monomer oxidation. LC shows the optimal activity at pH 4.0–4.5, while BOD is most active within neutral pH values. It is noteworthy that no insoluble DHQ oligomers are formed in LC-catalyzed synthesis at pH > 6.7. The maximum yield of insoluble products was ~32% at pH 4.5. In contrast, in the BOD-catalyzed reaction, the maximum yield of insoluble DHQ oligomers (~36%) was observed at pH 7.0 and no insoluble DHQ oligomers appeared at pH 4.5.

The physicochemical properties of DHQ oligomers synthesized at pH 4.5 using the fungal laccase (LC-oligoDHQ) and those obtained at pH 7.0 in BOD-catalyzed DHQ oxidation (BOD-oligoDHQ) were compared. The DHQ oligomers showed different solubility in organic solvents. Thus both LC-oligoDHQ and BOD-oligoDHQ were soluble in DMSO and methanol, but only LC-oligoDHQ dissolved entirely in THF.

Size exclusion chromatographic studies showed that LC-oligoDHQ had the number average molecular weight (Mn) of 1000 g/mol and the polydispersity index (PDI) of 1.4, while BOD-oligoDHQ had Mn = 2800 g/mol and PDI = 8.6.

As compared with DHQ monomer spectrum, UV-vis spectra of oligoDHQ (Fig. 2) synthesized using both enzymes had a long wavelength tail indicating the presence of extended conjugation [25]. In contrast to LC-oligoDHQ spectrum, the spectrum of BOD-oligoDHQ showed a distinct absorbance band at 420–450 nm.

The thermogravimetric analysis (TGA) showed that the stability of DHQ and oligoDHQ was approximately equal up to 240–250°C. At higher temperatures all samples studied suddenly lost weight, the thermal stability of oligoDHQ being higher than that of DHQ, i.e. oligoDHQ lost 20% of the weight at 385°C, while the same weight loss of DHQ was observed at 306°C.

The antioxidant activity of DHQ oligomers and DHQ monomer was defined as the antioxidant concentration which is required to decrease the initial DPPH$^\cdot$ concentration by 50%. First, we measured the DPPH$^\cdot$ reduction rate for different antioxidant concentration. The absorbance reached steady state values ~25 min after adding each oxidant. The antioxidant activity was 4 μg/mL for BOD-oligoDHQ, 10 μg/mL for LC-oligoDHQ, and 7 μg/mL for DHQ monomer. Thus, products with a higher antioxidant activity are formed in BOD-catalyzed polymerization as compared to that of the monomer.

The structure of enzyme-synthesized oligoDHQ was studied by FTIR and NMR spectroscopic methods. The FTIR spectra of BOD-oligoDHQ and LC-oligoDHQ differed in the peak intensity and shape in the ranges of 1400–1550, 1350–1400 and 960–1000 cm^{-1}. The two absorption spectra resemble the DHQ monomer vibration spectrum, but their peaks are wider and more smoothed. The broad peak with the maximum at 3400 cm^{-1} corresponds to the vibration of the O–H linkage of phenolic and hydroxyl groups [26], the peak at 1640 cm^{-1} can be attributed to the carbonyl groups [6, 10], the peak at 1150 cm^{-1} is due to the vibration of Caryl–O linkage [27], and the low peaks in the range 780–810 cm^{-1} can result from the vibrations of C=C and C–H linkages of the aromatic ring [6]. It is noteworthy that the FTIR spectra of oligo-DHQ had no absorption bands at 870, 1200, 1240 cm^{-1}, which correspond to cooperative vibrations of the C_{aryl}–O–C_{aryl} linkages. However, these data on the DHQ oligomer structure can hardly be regarded as reliable. Therefore, we used ^{1}H and ^{13}C NMR spectroscopy to study the structure of the enzyme-synthesized DHQ oligomers in detail.

The comparative analysis of ^{1}H and ^{13}C NMR spectra of DHQ monomer and oligomers (Figs. 3 and 4, respectively) was performed in presumption that the DHQ carbon skeleton is rather stable and remains unchanged in course of enzyme-catalyzed polymerization.

The reference of DHQ proton signals is shown in Fig. 3a. In order to reveal hydroxyl protons in ^{1}H NMR spectra, the solutions of oligoDHQ in DMSO-D_6 were diluted 15 times with solution of D_2O (10 μL) in DMSO-D_6 (630 μL). As a result, the intensity of signals of hydroxyl protons decreases markedly due to the substitution of protons with deuterium atoms (Figs. 3c, 3e), which allowed us to recognize the peaks of the OH-groups. A comparison of areas at 8.3–13.5 ppm enables us to conclude that all the phe-

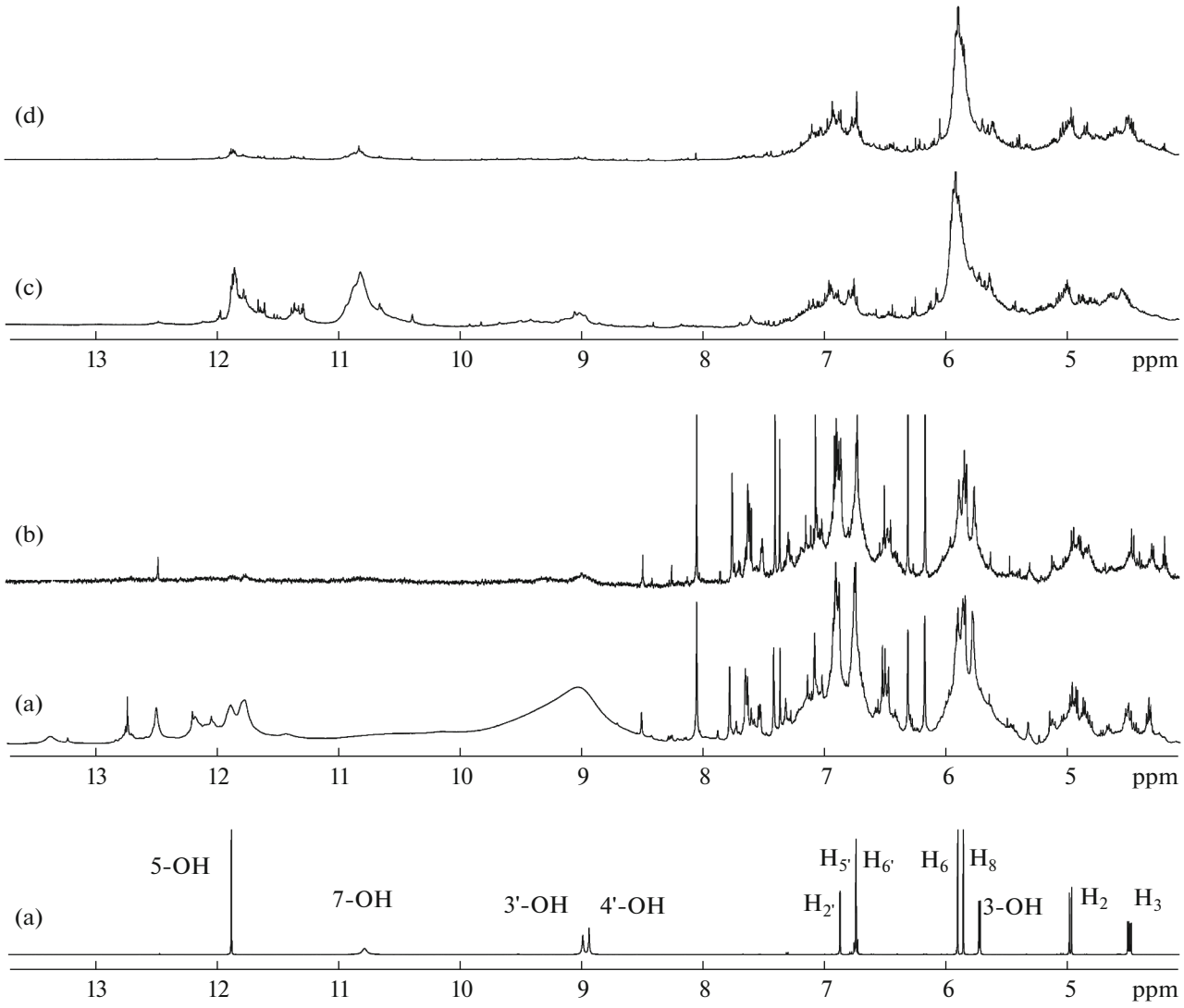

Fig. 3. ^{1}H NMR spectra: (a) DHQ, (b) BOD-oligoDHQ, (c) diluted solution of BOD-oligoDHQ with addition of D_2O (10 μL), (d) LC oligoDHQ and (e) diluted solution of LC-oligoDHQ with addition of D_2O (10 μL).

nolic hydroxyls are conserved in the samples of BOD-oligoDHQ (Figs. 3b, 3c) and LC-oligoDHQ (Figs. 3d, 3e). The hydroxyl protons 7-OH, 3'-OH and 4'-OH are significantly broadened due to fast exchange processes, while the protons of 5-OH groups in both DHQ monomer and oligomers participate in the formation of a very stable hydrogen bond with the carbonyl oxygen of ring C (Fig. 1), which is confirmed by the presence of relatively narrow and clearly distinguished peaks at 11.5–13.5 ppm (Fig. 3b) and at 11.3–12.3 ppm (Fig. 3d). It should be noted that the signal of the hydroxyl group at C3 is lacking in the spectra of oligoDHQ, which enable us to assume that LC- and BOD-catalyzed DHQ polymerization is starting with oxidation of the hydroxyl group at C3.

Broadened signals with a high integral intensity are observed in the area 5.5–8.0 ppm of the proton spectra of both oligoDHQ, which can be assigned to the corresponding signals of DHQ monomer aromatic protons H2', H5', H6', H6 and H8. However, in the LC-oligoDHQ spectrum (Figs. 3d, 3e) the signals of the phenyl protons H2', H5' and H6' show a markedly lower intensity as compared with the intensity of the protons H6 and H8. The ^{1}H NMR spectrum of BOD-oligoDHQ (Fig. 3b) exhibits peaks at 6.0–6.6 ppm and 7.0–8.1 ppm, which are lacking in the spectrum of the DHQ monomer. These signals can most likely be assigned to the phenyl protons corresponding to the protons of related flavonoids 2–7 (Fig. 1) with a double bond C2=C3. In addition, the spectra of DHQ oligomers show broad signals in the area of aliphatic protons H2 and H3 (4.5–5.8 ppm), but their total integrated intensity is noticeably lower as compared with the intensity of the phenyl protons.

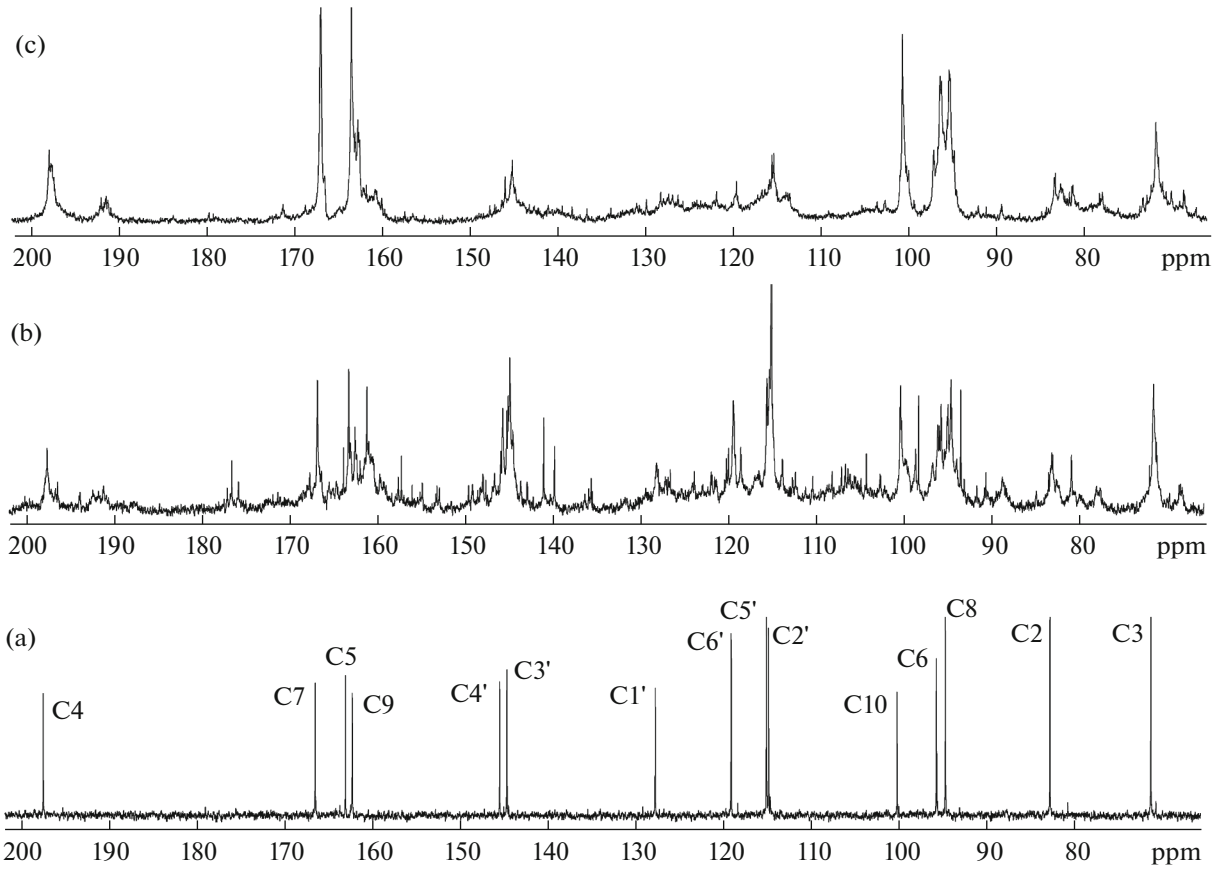

Fig. 4. ^{13}C-{^{1}H} NMR spectra: DHQ (a), BOD-oligoDHQ (b), and LC-oligoDHQ (c).

Thus, the comparative analysis of the proton spectra of DHQ monomers and oligomers allowed us to conclude that DHQ polymerization mediated by the two oxidases starts with the oxidation of the hydroxyl group in position 3, the most visible changes occurring in positions 2 and 3 of ring C. The alkoxy radical formed at the first stage attacks another DHQ molecule via radical or electrophilic substitution of a sufficient aromatic hydrogens in any possible positions in both rings A and B in BOD-catalyzed polymerization and via substitution predominantly of aromatic hydrogens in ring B in LC-catalyzed polymerization.

A comparison of ^{13}C NMR spectra of DHQ monomer (Fig. 4a) with those of BOD-oligoDHQ (Fig. 4b) and LC-oligoDHQ (Fig. 4c) revealed that all the peaks in the spectrum of the carbon atoms in DHQ monomer were also present in the spectra of DHQ oligomers. For example, the broadened peaks arising from the carbonyl carbon of oligoDHQ at 197–198 ppm correspond to the position of the carbonyl group in the monomer (197.7 ppm). However, of the most interest are the peaks in the spectra of DHQ oligomers which are lacking in the spectrum of DHQ monomer. In particular, it is a group of peaks arising from quaternary carbons at 191–193 ppm, which are especially conspicuous in the spectrum of LC-oligoDHQ. Analysis of the literature [28–31] enables us to assume that these peaks ought to be assigned to C4 carbonyl group, and the highfield shift may be attributed to the gamma-effect arising due to the replacement of the hydroxyl in position 3 with an alkoxy group. ^{13}C NMR spectrum of BOD-oligoDHQ (Fig. 4b) revealed peaks of quaternary carbons at 176.0–177.5 ppm, which may also be attributed to C4 carbonyl group. The change in the chemical shift is caused by the formation of double bonds C2=C3, as in the case of quercetin (*2*), rutine (*3*), flavone (*5*) and its derivatives (*6*)–(*7*). The formation of the double bond in BOD-oligoDHQ is borne out by the presence of signals of quaternary carbons at 135.5–136.0 ppm (C3 atom in quercetin (*2*) and rutin (*3*)) and at 146.7–162.0 ppm (C2 atom in flavonoides (*2*)–(*7*)) [28–31].

Broad peaks in the spectra of both DHQ oligomers correspond to the signals of quaternary and CH carbons in rings A and B of monomeric DHQ. At the same time, the peaks of C5, C6, C7, C8 and C10 in ring A of LC-oligoDHQ (Fig. 4c) remain virtually in the same position as in the monomer, while the signals due to all the six carbons in ring B are noticeably broader and have a markedly lower intensity as com-

pared with the carbons in ring A. Also numerous narrow peaks of quaternary carbons are observed at 123–147 ppm. This type of spectrum most likely indicates that LC-mediated DHQ polymerization proceeds via substitution of hydrogen atoms for an alkoxy radical in all three possible positions of ring B (H2', H5', H6').

One further comment should be made about ^{13}C NMR spectrum of BOD-oligoDHQ (Fig. 4b). This spectrum has some peculiarities, namely, four groups of signals attributed to quaternary carbon atoms at 121.9–128.4 ppm, i.e. in the region of monomer C1' signal at 128 ppm. This may indicate that in ring C the position of C1` signal is noticeably affected by changes in the neighboring carbons, which lead to the formation of a double bond between C2 and C3, as in the case of quercetin (2) and rutin (3) [29, 30]. Thus, unlike LC mediated DHQ polymerization, BOD-mediated DHQ polymerization is accompanied by the formation of the C2=C3 double bond, which may be considered as evidence that stabilization of the intermediate alkoxy radicals proceeds via hydrogen abstraction at C2.

The signals of quaternary carbons C5, C7, C9, C10 and CH carbons (C6, C8) in ^{13}C NMR spectrum of BOD-oligoDHQ have the same positions as in the spectrum of DHQ monomer, but they are significantly broadened (Fig. 4b). Numerous narrow peaks of quaternary carbons observed at 145.0 and 164.0 ppm correspond to C5 and C7. The spectrum also has intensive signals arising from CH groups at 90.7, 93.6, 98.4 and 98.7 ppm. These chemical shifts are in good agreement with the data for quercetin (*2*), rutin (*3*) and substituted flavones (*6* and *7*) [28–31]. The signals of quaternary carbon atoms within the region of 104.4–112.4 ppm are observed. Their peaks are in reasonable coincidence with the shifts of C10 for quercetin (*2*), rutin (*3*), genistein (*4*) and substituted flavones (*6*)–(*7*) [28–31]. The peak of the CH carbon at 107.2 ppm may correspond to C3 of flavones (*5*)–(*7*), which is associated with the formation of the C2=C3 double bond.

Broad intensive signals are observed in both BOD-oligoDHQ and LC- oligoDHQ spectra in the region characteristic of aliphatic C2 and C3 of the parent monomer (83.0 and 71.5 ppm, respectively).

Thus, the results of the comparative analysis of ^{1}H and ^{13}C NMR spectra of DHQ monomer, BOD-oligoDHQ and LC-oligoDHQ, as well as some literature data on related compounds [28–31] allow us to make some assumptions. BOD- and LC-catalyzed DHQ oxidation starts with hydrogen abstraction from the aliphatic hydroxyl group at C3 to produce an active radical. In LC-catalyzed DHQ polymerization, the alkoxy radical then predominantly replaces hydrogen atoms in positions 2', 5', 6' in the ring B of another monomer molecule. In BOD-catalyzed DHQ polymerization, the radical can be attached to phenyl carbons in positions 6, 8 of ring A and in positions 2', 5', 6' of ring B. Also the removal of H2 and H3 followed by the formation of a double bond between C2 and C3 with or without preservation of the bond C3–O may occur. So, BOD-oligoDHQ seem to have a rather irregular structure and contain all possible fragments in different combination. Comparison of the results of the enzyme-catalyzed DHQ polymerization enables us to assume that LC-catalyzed reaction produces short 2–4 unit oligomers while BOD-catalyzed oligomers are of different length and have a more complex structure.

CONCLUSIONS

In this work the enzymatic synthesis of biologically active compound dihydroquercetin (DHQ) using laccase from *Trametes hirsuta* and bilirubin oxidase from *Myrothecium verrucaria* was described. It has been shown that the oligomers formed at optimal pH values differ not only in the average molecular weight and polydispersity index but also in structure. Both DHQ oligomers are more thermostable than the monomer. Besides, BOD-catalyzed oligomers have a higher antioxidant activity as compared to the monomer. Comparison of ^{1}H and ^{13}C NMR spectra of DHQ oligomers, monomer and related flavonoids suggests that the products of the enzyme-catalyzed DHQ polymerization have an irregular structure.

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