Oxidation of catechol, (+)-catechin, and caffeic acid by the laccase from *Trametes versicolor* and tyrosinase from *Agaricus bisporus**

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Abstract: Laccase and tyrosinase are copper-containing phenol oxidases, which are known to have multiple roles in nature, such as melanin pigment formation in fungi, microorganisms and animal tissues, transport, uptake and storage of metal ions and enzymatic browning of fruits, and vegetables. In this study, a comparative analysis of the oxidation and polymerization of catechol, (+)-catechin and caffeic acid was analyzed in the presence of laccase from *Trametes versicolor* and tyrosinase from *Agaricus bisporus*. Products were first analyzed by HPLC and they were further characterized by LC-ESI/MS. A dimer, a trimer and an oligomer of catechol were observed by laccase but tyrosinase resulted in just a dimer formation. Catechin products from laccase included type-A and type-B dimers, as well as a trimer and a tetramer. Tyrosinase yielded only a type-B dimer from (+)-catechin as well as a trimer and a tetramer. Caffeic acid was oxidized only by laccase, yielding different types of dimers and tetramers. Specificity of laccase and tyrosinase-catalyzed oxidation products of the three ortho-diphenolic compounds, catechol, (+)-catechin and caffeic acid were quite different. This might occur because of the difference in the electron transfer system and the radicals produced during the oxidation of phenolic compounds, which were then coupled to give more stable dimer, trimer, tetramers and oligomers.

Keywords: Caffeic acid, catechin, catechol, oxidation, phenol exidase

Katekol, (+)-katekin ve kafeik asidin *Trametes versicolor* lakkazı ve *Agaricus bisporus* tirozinazı varlığında oksidasyonu

Öz: Sakkaz ve tirosinazlar doğada küflerde, mikroorganizmalarda ve hayvan dokularında melanin pigment oluşumu, metal iyonlarının taşınması, alınması ve depo edinmesinde ve meyve ve sebzelerin enzimatik kararmasında rol oynayan bakır içerikli fenol oksidazlardır. Bu çalışmada katekol, (+)-katekin ve kafeik asidin *Trametes versicolor lakkazı* ve *Agaricus bisporus* tirozinazı varlığında oksidasyonu ve polimerizasyonunun karşılaştırmalı analizi yapılmıştır. Ürünler öncelikle HPLC daha sonra LC-ESI/MS ile analiz edilmiştir. Lakkaz varlığında katekolun oksidasyonu sonucu dimer, trimer ve oligomer, katekinin oksidasyonu sonucu ise tip-A ve tip-B dimerler ile trimer ve tetramer oluştururken tirosinaz varlığında katekolün sadece dimer oluşturduğu, katekinin ise tip-B dimer ile trimer ve tetramer oluşturduğu gözlemlenmiştir. Kafeik asit sadece lakkaz tarafından okside edilmiş ve farklı tiplerde dimer ve tetramer ürünleri vermiştir. Lakkaz ve tirosinaz ile katalizlenen katekol, (+)-katekin ve kafeik asit orto-difenolik bileşiklerinin oksidasyon ürünleri oldukça farklılık göstermiştir. Sonuçlarda gözlemlenen değişiklik, elektron sistemlerindeki farklılık ve fenolik bileşiklerin oksidasyonu sırasında oluşan radikallerin birden fazla dimer, trimer, tetramer ve oligomer verecek şekilde bir araya gelmesinden kaynaklamış olabilir.

Anahtar kelimeler: Fenol oksidaz, katekin, katekol, kafeik asit, oksidasyon

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Introduction

Phenol oxidases (POs) are copper-containing enzymes capable of catalyzing the oxidation of various phenolic compounds in the presence of molecular oxygen. Phenol oxidases are classified into three groups: laccases (E.C. 1.10.3.2), catechol oxidases (E.C. 1.10.3.1) and tyrosinases (E.C. 1.14.18.1) (29). Laccases and tyrosinases are in different classes, depending on their optical and electron paramagnetic resonance (EPR) spectroscopic features (16, 39).

Laccase is one of the few lignin-degrading enzyme (37) and first described by Yoshida in 1883 (40). It has the ability to catalyze the single-electron oxidation of polyphenols, methoxy-substituted phenols, diamines, aromatic amines, ascorbate and thiols (34, 40). Laccase contains 4 copper atoms and oxidizes wide range of organic and inorganic substrates with a single electron removal mechanism and it does not exhibit monophenol hydroxylase activity (35). In oxidation reaction catalyzed by laccase, high reactive radicals are produced and further these can lead to polymerization, hydration, and disproportionation (40).

Tyrosinase is bifunctional, type-3 copper-protein as it has both monophenol hydroxylase (cresolase activity) and diphenol oxidation activity (catecholase activity) (24) and oxidizes its substrates by removing a pair of electrons from the substrate undergone a polymerization (3, 4).

Catechol oxidase is a dicopper protein (12) and it is related to both tyrosinase and hemocyanin (38). It mainly has diphenolase activity (20) and oxidizes o-diphenols such as catechols, but also shows monophenolase activity (13).

Phenolic compounds are plant secondary metabolites (33) and they are known both by their antioxidant capacities and also as pro-oxidant (21). They play an important role in multiple biological effects. They have anti-oxidant activity by acting as scavengers of oxygen radicals (7) and reactive nitrogen species (32). They are inhibitors of the N-nitrosation reactions (33) and show anti-carcinogenic, anti-viral and anti-inflammatory activity (6, 22, 27, 30). On the other hand, phenols are very abundant environmental pollutant (10). They are often produced and enter the environment as wastes from several types of industrial and agricultural activities (17, 18) and have ability to undergo autoxidation reactions and quinine formations (33), which are dangerous to living organisms because of their toxicity (18).

Phenolic compounds are different in size, ranging from simple phenols to polyphenols (28). The simplest phenolic compound is catechol. Catechol contains two hydroxyl groups in ortho-positions, which are very reactive and quickly undergone an autoxidation reactions (36). Polyphenols can be classified, according to their structures, into three groups: phenolic acids, flavonoids, and tannins (9). Caffeic acid is the main representative of hydroxycinnamic acid. It is found in all plants and important intermediate in the biosynthesis of lignin (5). Catechin is the main representative of flavan 3-ols. It can be found in peach (8), vinegar (15) and many other foods and responsible for the enzymatic browning (31).

Oxidoreductive enzymes such as laccases and tyrosinases are able to transform phenols through oxidative coupling reactions with production of polymeric products (10). Formation of oligomers or polymers could make phenolic compounds more stable. This type of polymerization could also decrease the pro-oxidant/toxic effect of phenolics (21). The present study is the first report on the comparison of the phenol oxidation products catalyzed by two typical phenol oxidases, namely laccase from *Trametes versicolor* and tyrosinase from *Agaricus bisporus*. In this respect, 3 phenolic compounds were selected among the simple phenols and different classes of plant phenolics and were oxidized by laccase and tyrosinase under selected conditions. These enzymes have industrial significance such as in biotechnology, food processing, medicine, and the textile and pulp and paper industry, especially

due to their ability to polymerize compounds (1, 14, 19, 25, 26). Due to the complexity of the oxidation reactions and the importance of phenol oxidases in the industry, it was of interest to compare the oxidation products of these three different o-diphenolic compounds in the presence of laccase and tyrosinase, under similar experimental conditions. The oxidation products were analyzed by HPLC, LC-ESI/MS, and UV–vis spectroscopy.

Materials and Methods

Catechol, caffeic acid and (+)-catechin were purchased from Sigma-Aldrich (Taufkirchen, Germany). Acetonitrile and methanol were purchased from Merck (Darmstadt, Germany); other chemicals were obtained from various commercial suppliers. All the solvents used for chromatography were of HPLC grade. Commercial *Trametes versicolor* laccase (23.1 units mg-1 solid) and *Agaricus bisporus* tyrosinase (4276 units mg-1 solid) were purchased from Sigma-Aldrich (Taufkirchen, Germany).

Phenol oxidation: The reaction mixtures for the enzymatic oxidation of the 3 phenolic compounds by 10 µg laccase (23.1 U mg-1) and 10 µg tyrosinase (4.2 U µg-1) consisted of 10 mM phenolic compound dissolved in 2 mL absolute methanol, 2 mL 100 mM potassium citrate buffer (pH 4.8 for laccase and pH 6.5 for tyrosinase). Mixtures were incubated at 25°C for 1 hour.

HPLC and LC-ESI/MS conditions: A Shimadzu SIL-20AHT (Kyoto, Japan) instrument equipped with variable wavelength absorbance detector (UV-vis detector) set a 254 nm and reversed phase C18 column (Inertsil ODS-3, 4.6×250 mm i.d., GL Sciences Inc., Torrance, CA, USA) held at a constant temperature at 40 ºC was used for separations. The eluent consisted of 0.04% acetic acid in water (solvent system A) and acetonitrile: methanol: distilled water (52:37:11 v/v) (solvent system B) was pumped at 1.0 mL min-1 with the following linear gradient mode: 100% solvent A applied for 2 min, followed by rapid increase by 12% in B for 5 min, solvent B was held for 2 min at 12%, increased to 18% over 17 min, increased to 35% over 20 min, and finally held at 50% for 2 min. An Agilent 1100 series LC-MSD instrument with a Waters Spherisorb S5ODS2 (25cm X 4.6 mm i.d.) column (adjust to 25 oC) was used for LC-ESI/MS analysis. Gradient mode was applied with two solvent systems which are: 0.05% acetic acid and acetonitrile: methanol: distilled water (52:37:11 v/v). Negative electrospray mode was applied. The mass spectrometer was operated to scan 100-1000 m/z and the optimized electrospray parameters were used as: drying gas flow: 0.6 mL/min, nebulizer pressure: 40 psig, drying gas temperature: 350 °C, capillary voltage: 3 kV and fragmentor: 70 eV.

Results and Discussion

Oxidation of catechol: Laccase-catalyzed oxidation of catechol yielded three major products, namely PLcat-1 with 21.9 min, PLcat-2 with 23.1 min and PLcat-3 with 24.7 min of retention times (RT) (Figure 1a). PLcat-1 gave molecular ion with m/z ratio of 216.9, and is possibly a dimer of ortho-quinone, which is derived from the oxidation of catechol (Figure 1b). PLcat-2 with m/z ratio of 324.7, is likely to be a trimer (Figure 1c) and PLcat-3, with m/z ratio of 977.8 appears to be an oligomer of catechol (Figure. 1d). Tyrosinase resulted in a single major product from catechol, namely PTcat-1 with 16.1 min of RT (Figure 2a) which is likely to be a catechol dimer (m/z 219.1) (Figure. 2b). The m/z ratios of PLcat-1 (214.9) and PTcat-1 (219) indicated dimer formation. In the literature, C-C and C-O-C coupled dimer formations have been observed in the enzyme-catalyzed oxidations of catechol (2, 11).

Oxidation of catechin: Four major products PLcath-1, PLcath-2, PLcath-3 and PLcath-4 were detected as a result of laccase-catalyzed oxidation of catechin (RT 14.8 min, 21.8 min

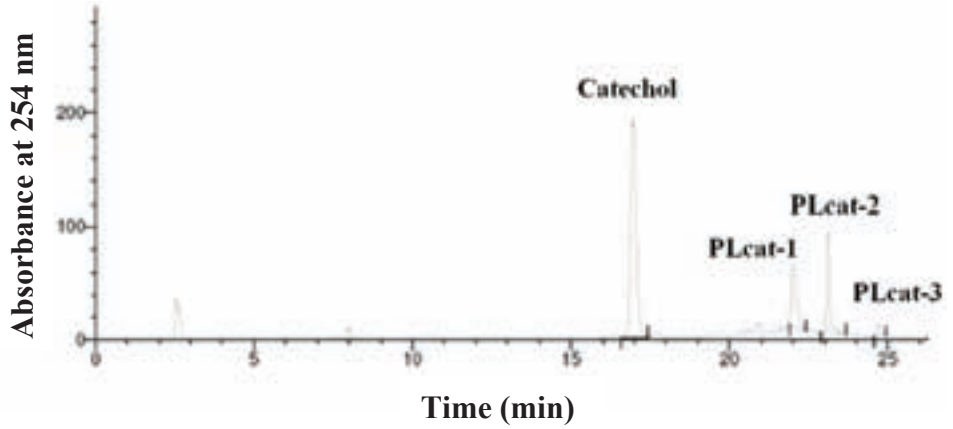

Figure 1a. HPLC chromatogram of 10 mM catechol oxidized by laccase (23.1 U mg^{-1}) for 1 h at 25 º C

Şekil 1a. *Lakkaz (23.1 U mg^{-1}) ile 1 sa. 25 º C'de 10 mM katekol oksidasyonunun HPLC kromatogramı*

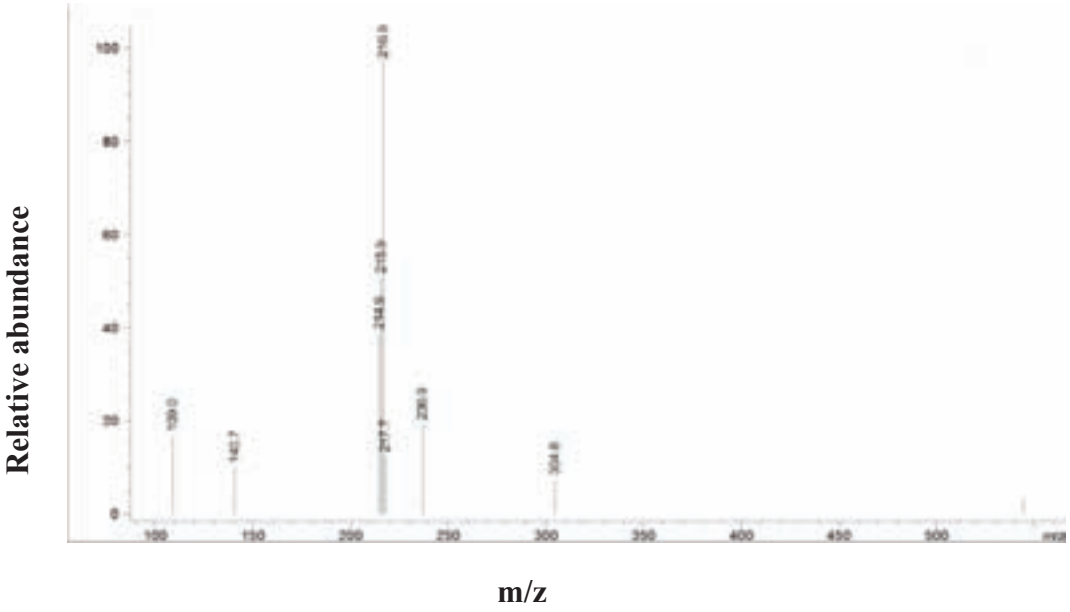

Figure 1b. Mass spectrum of the catechol dimer (PLcat-1, m/z=216.9)

Şekil 1b. *Katekol dimerinin kütle spektrumu (PLcat-1, m/z=216.9)*

22.9 min, and 24.4 respectively) (Figure 3a). As the retention time for (+)-catechin was 16.8 min, PLcath-1 was eluted earlier than catechin, suggesting, it is more hydrophilic in nature than catechin. We suggest that PLcath-1 with an m/z ratio of 576.6 (Figure 3b) is a hydrophilic type B dimer, PLcath-2 (m/z 574.6) is a hydrophobic type A dimer, which most likely condenses with catechin to form the reduced trimer with m/z 865 (Figure 3c) and Pcath-3 with m/z value of 574.6 is possibly the dimer of catechin, condensed to form the reduced tetramer (m/z 1150.3) (Figure 3d). PLcath-4 could not be detected during LC-ESI/MS most probably because of the larger mass to charge ratio of the product.

Three major products PTcath-1, PTcath-2 and PTcath-3 were detected by the tyrosinase catalyzed oxidation of (+)-catechin (RT, 14.8 min, 21.8 min and 22.9 min respectively) as shown in Figure 4a. According to m/z ratios on LC-ESI/MS, PTcath-1 is a hydrophilic, type B

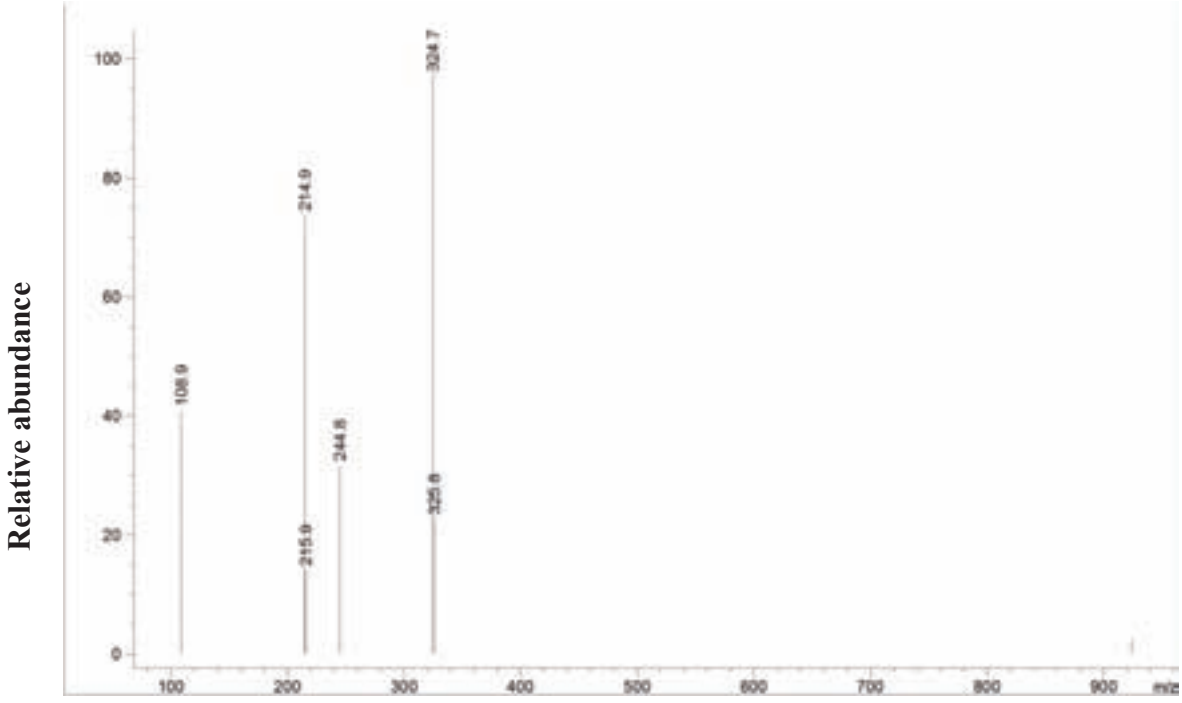

Figure 1c. Mass spectrum of the catechol trimer (PLcat-2, m/z=324.7)
Şekil 1c. *Katekol trimerinin kütle spektrumu (PLcat-2, m/z=324.7)*

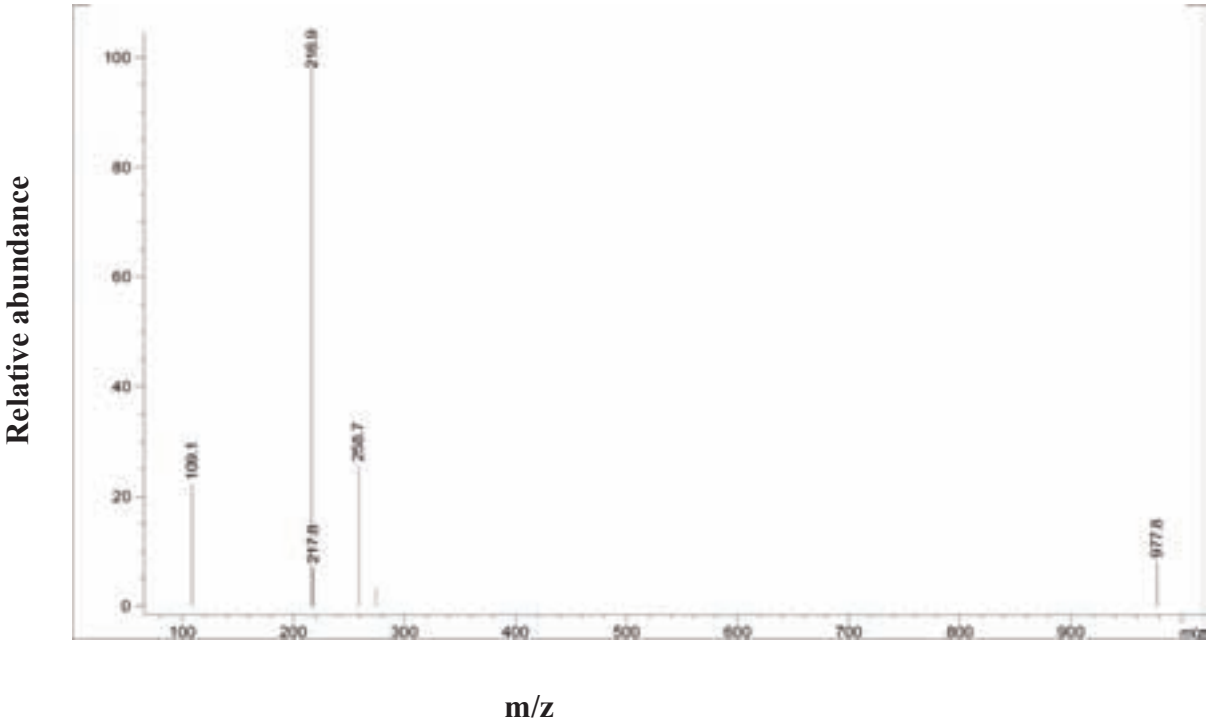

Figure 1d. Mass spectrum of the catechol oligomer (PLcat-3, m/z=977.8)
Şekil 1d. *Katekol oligomerinin kütle spekturumu (PLcat-3, m/z=977.8)*

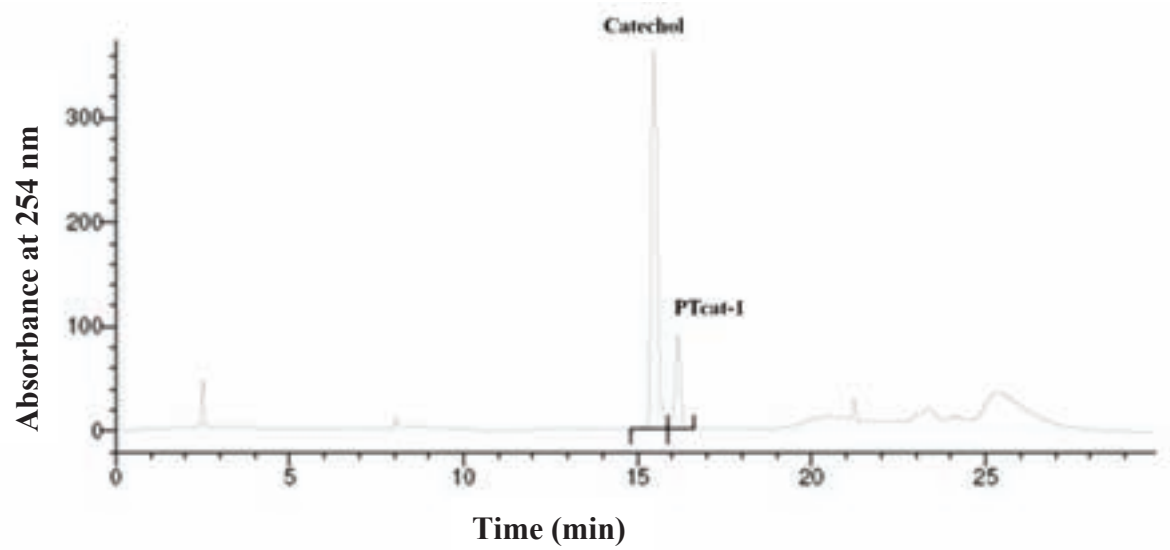

Figure 2a. HPLC chromatogram of 10 mM catechol oxidized by tyrosinase (4.2 U μg-1) for 1 h at 25 ° C

Şekil 2a. *Tirosinaz (4.2 U μg-1) ile 1 sa. 25 ° C'de 10 mM katekol oksidasyonunun HPLC kromatogramı*

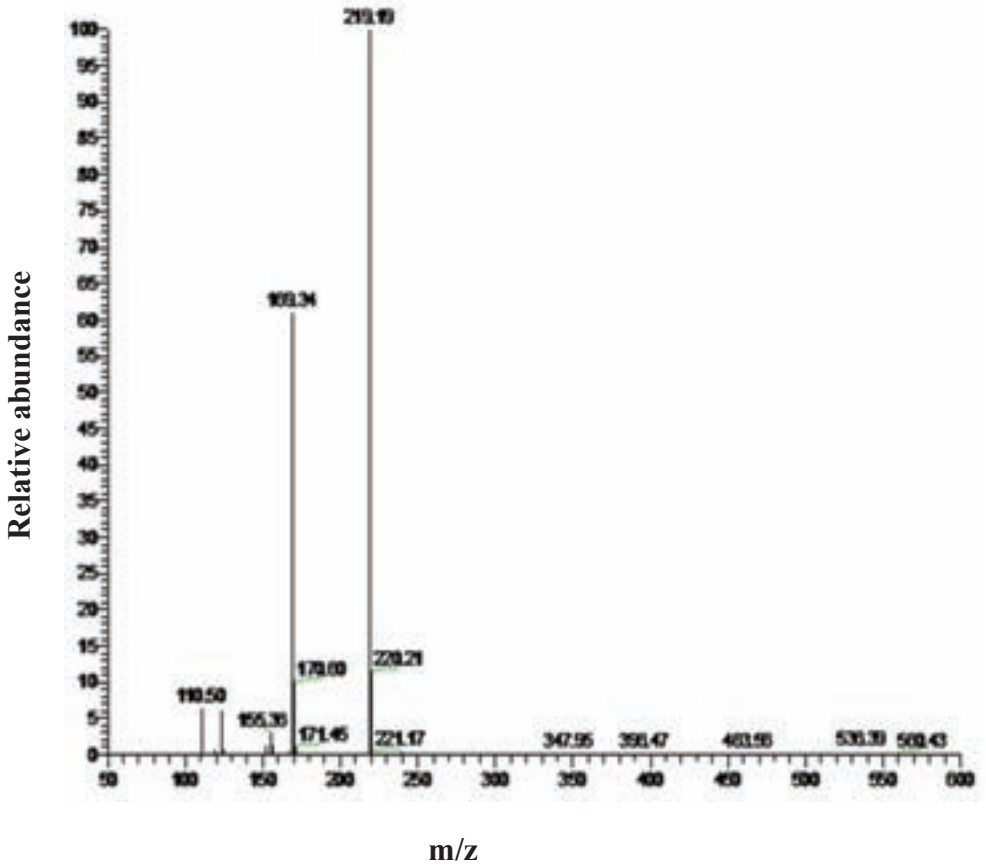

Figure 2b. Mass spectrum of the catechol dimer (PLcat-1, m/z=219.1)

Şekil 2b. *Katekol dimerinin kütle spekturumu (PLcat-1, m/z=219.1)*

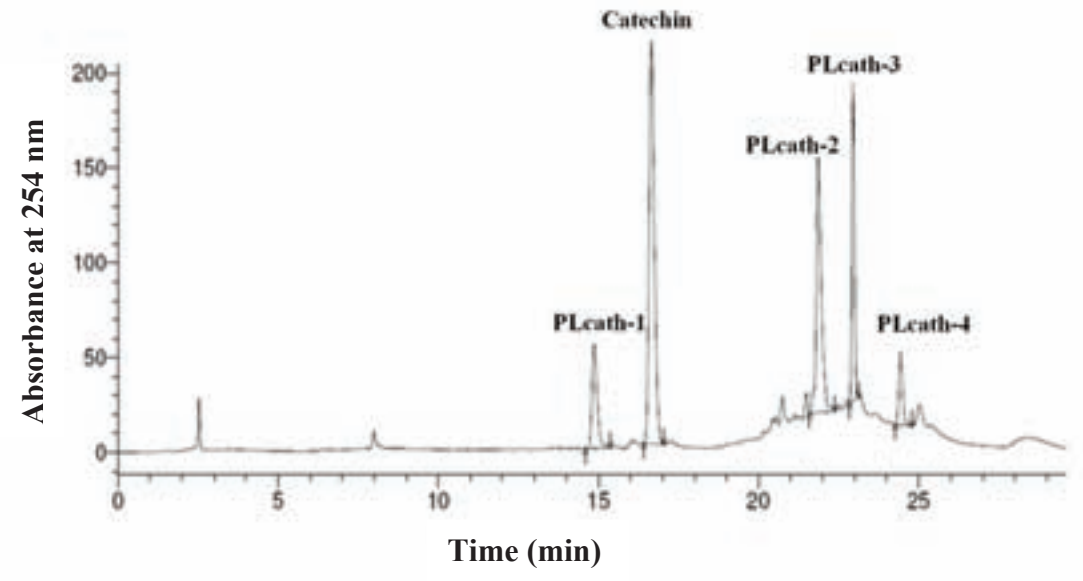

Figure 3a. HPLC chromatogram of 10 mM catechol oxidized by tyrosinase (4.2 U μg^{-1}) for 1 h at 25 ° C

Şekil 3a. *Lakkaz (23.1 U mg^{-1}) ile 1 sa. 25 ° C'de 10 mM katekin oksidasyonunun HPLC kromatogramı*

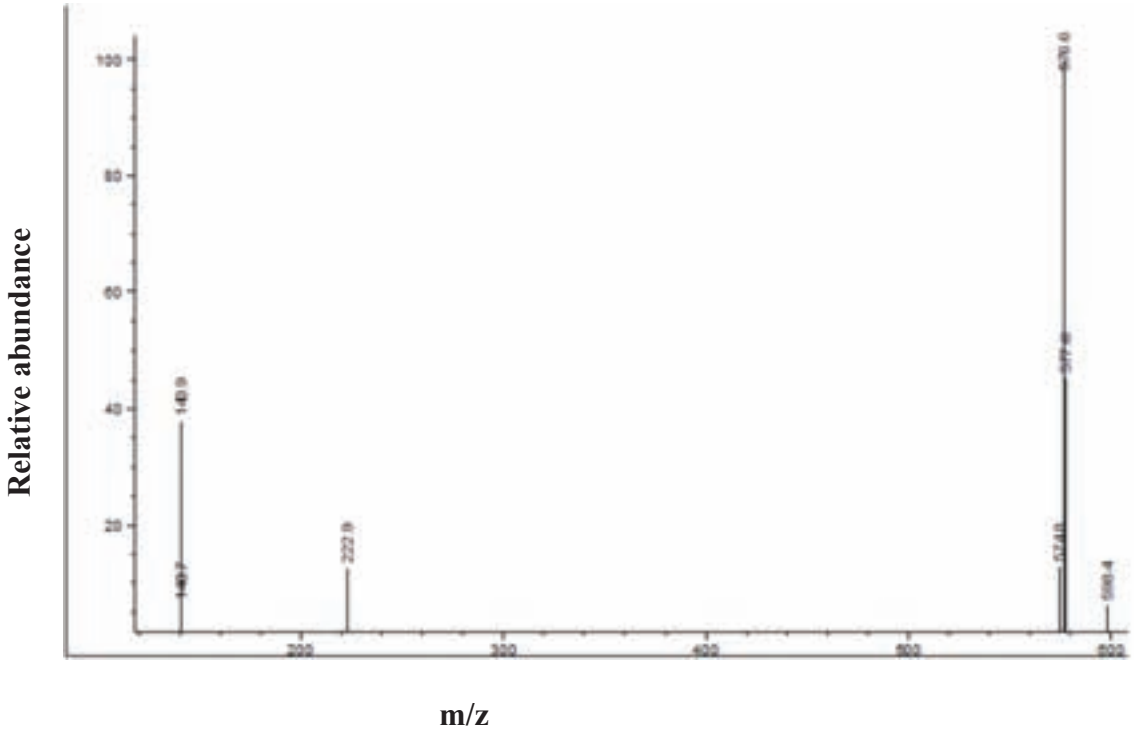

Figure 3b. Mass spectrum of the hydrophilic dimer (PLcath-1, m/z=576.6)

Şekil 3b. *Hidrofilik dimerin kütle spektrumu (PLcath-1, m/z=576.6)*

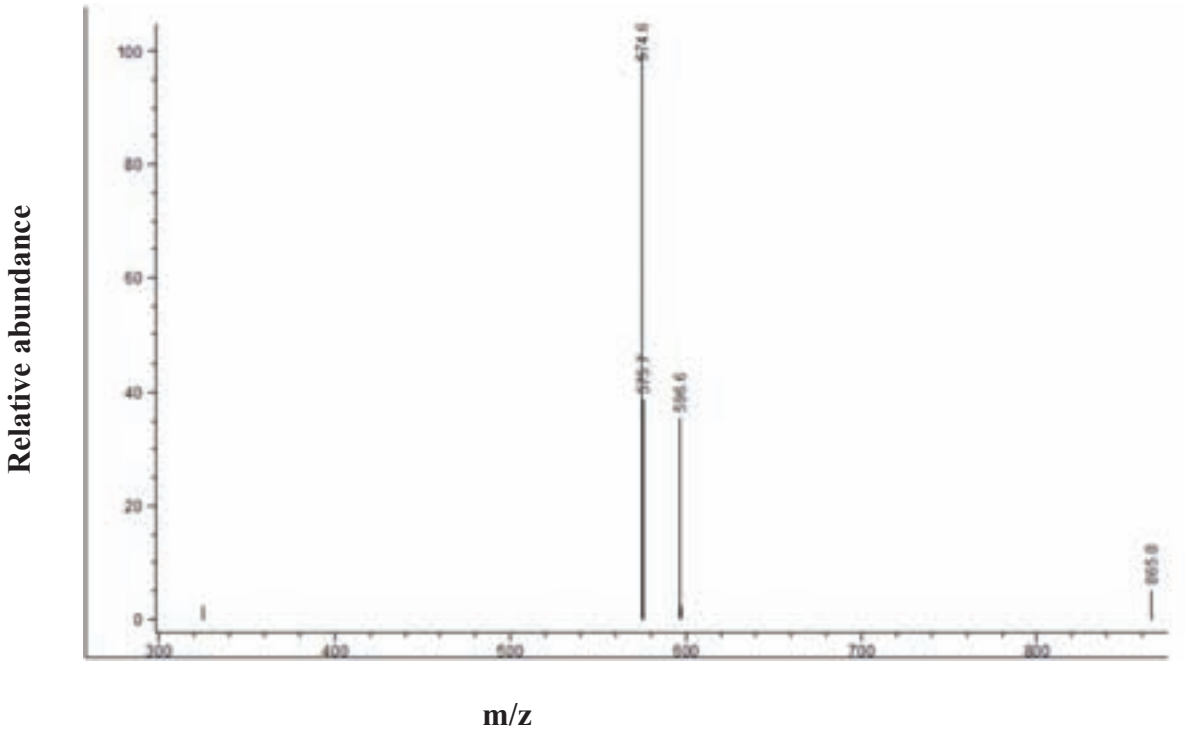

Figure 3c. Mass spectrum of the hydrophobic dimer (PLcath-2, m/z= 574.6)
Şekil 3c. *Hidrofobik dimerin kütle spekturumu (PLcath-2, m/z= 574.6)*

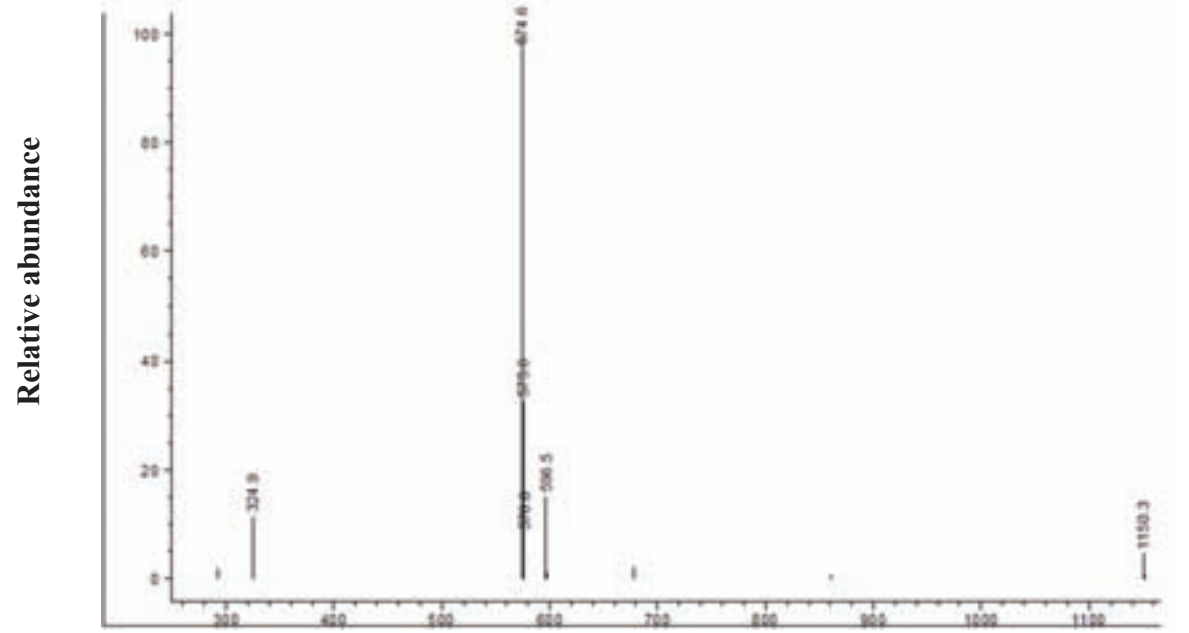

Figure 3d. Mass spectrum of the reduced tetramer (PLcath-3, m/z=1150.3)
Şekil 3d. *İndirgenmiş tetramerin kütle spekturumu (PLcath-3, m/z=1150.3)*

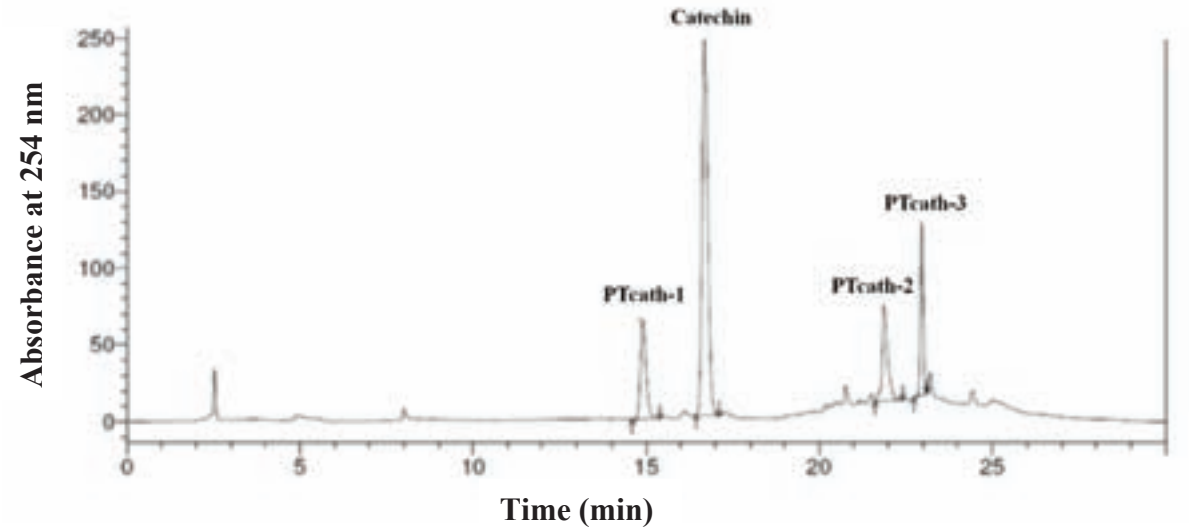

Figure 4a. HPLC chromatogram of 10 mM catechin oxidized by tyrosinase (4.2 U µg-1) for 1 h at 25°C

Şekil 4a. *Tirosinaz (4.2 U µg-1) ile 1 sa. 25°C'de 10 mM katekin oksidasyonunun HPLC kromatogramı*

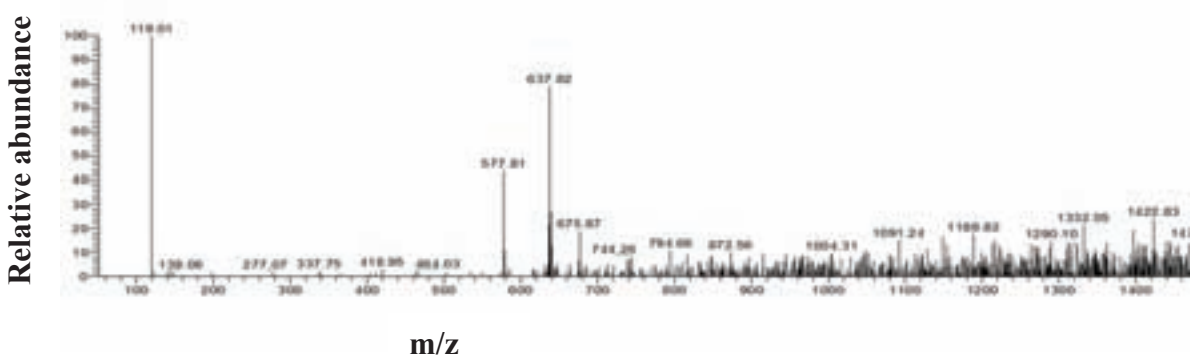

Figure 4b. Mass spectrum of PTcath-1 (m/z=577.8)

Şekil 4b. *PTcath-1 (m/z=577.8) kütle spekturumu*

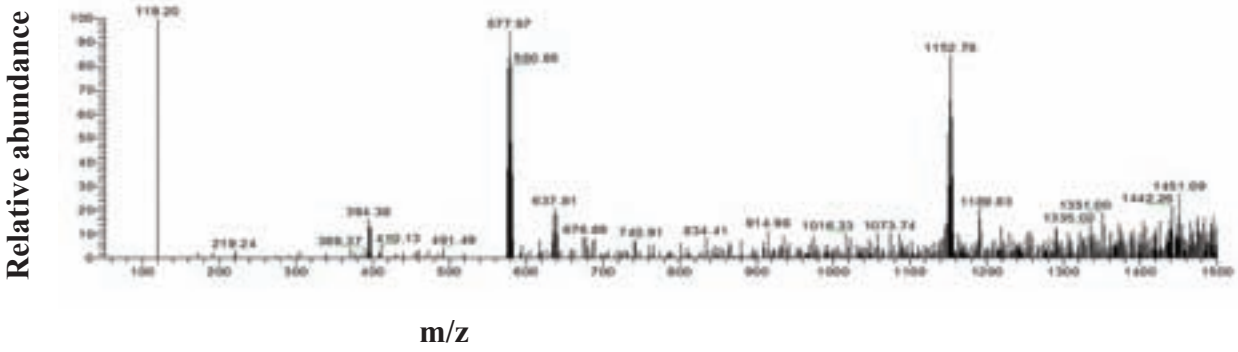

Figure 4c. Mass spectrum of PTcath-2 (m/z=1152.7)

Şekil 4c. *PTcath-2 (m/z=1152.7) kütle spekturumu*

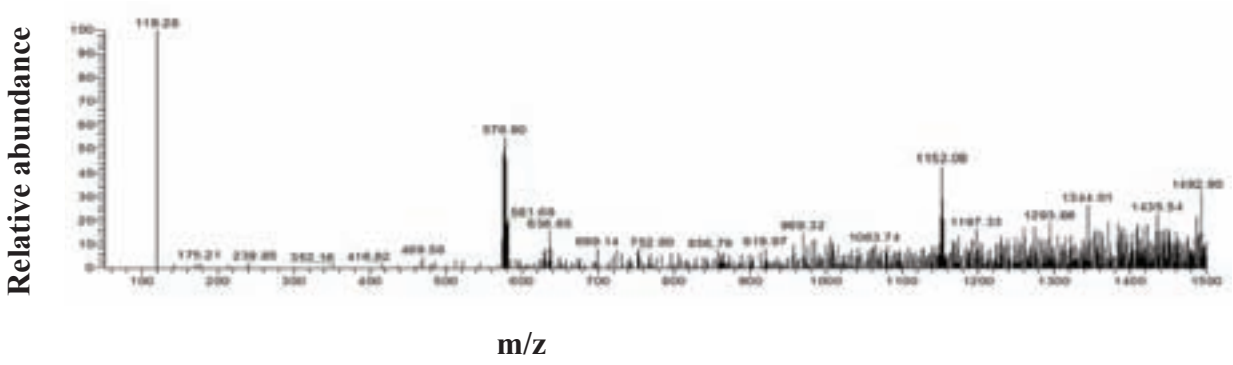

Figure 4d. Mass spectrum of PTcath-3 (fragments, m/z=1152 and m/z =576.9)
Şekil 4d. *PTcath-3 (fragmanlar, m/z=1152 and m/z =576.9) kütle spekturumu*

dimer (m/z 577.8), PTcath-2 could be a tetramer (m/z 1152.7) which likely condenses from the hydrophobic, type A dimer and PTcath-3 is most likely a polymer of catechin (m/z 1435.5) and give fragments with m/z 1152 and 576.9 (Figure 4b, 4c, 4d respectively).

The UV-vis spectra and LC-ESI/MS data suggest that first, catechin is oxidized into the semiquinone and the o-quinone then, o-quinone is reduced to the hydrophilic dimer (type B dimer) (31), which corresponds to PLcath-1 and PTcath-1. Thus both enzymes yield the type B dimer. Type B dimer is then enzymatically oxidized into the hydrophobic type-A dimer, which corresponds to PLcath-2. Hydrophobic type dimer structure could not be determined during the oxidation of catechin by tyrosinase. Once formed, the hydrophobic dimer was further enzymatically oxidized into an o-quinone which, most likely, condensed with catechin to form the reduced trimer (31), corresponding to PLcath-3, and then to the tetramer, corresponding to PLcath-4.

Oxidation of caffeic acid: Laccase catalyzed oxidation of caffeic acid yielded three major peaks. These were PLcaf-1 with 20.8, PLcaf-2 with 21.6 and PLcaf-3 with 23.4 min RT (Figure 5a). LC-ESI/MS analysis indicated that, PLcaf-1 has m/z ratio of 714.5, [M2-H]- likely to be a tetramer of caffeic acid. PLcaf-1 yielded fragments with m/z ratios of 356.8, dimer of caffeic acid, and fragments with m/z ratio of 269 [M-2CO2-H]- and 158.9 (Figure 5b) probably corresponding to the degradation products of the tetramer or dimer that losses of CO2 and H+. PLcaf-2 is a dimer of caffeic acid with m/z ratio 312.7 [M-CO2-H]- and gave one fragment with m/z ratio 178.9 (Figure 5c). PLcaf-3 is a tetramer of caffeic acid with m/z 736.4 [M2+Na-H]- and gave the base peak has m/z ratio of 312.8 [M-CO2-H]- and two important fragments, one with m/z 268.9 [M-2CO2 -H]- and the other with 178.8 (Figure 5d).

Four different types of dimer structures were defined for caffeic acid in the literature (33). The caffeicin-like dimer formation can explain the fragments catalyzed by laccase and obtained during mass spectroscopy. In this type of mechanism, one or two hyroquinone hydroxyl groups and the side chain double bond involves during the polymerization (33). It is hypothesized that a proton transfer occurs between the remaining COOH group and the carbanion in the decarboxylated anion (m/z 313) (33). Lighter fragments (m/z 179) occur because of further CO2 loss which is combined with a RDA rearrangement (33). This type of dimer formation was also detected by the CATPO-catalyzed oxidation of caffeic acid (23) and could explain the formation of PLcaf-2 and PLcaf-3.

PLcaf-1 yielded fragments with m/z ratios of 356, dimer of caffeic acid, and fragments with m/z ratio of 269 [M-2 CO2-H]- and 159. The formation of peaks at m/z 159 can be explained by the formation of a C-C type coupling of caffeic acid, through hydrogen transfers and double-bond displacements, as described in Pati (2006). Furthermore, among six of the

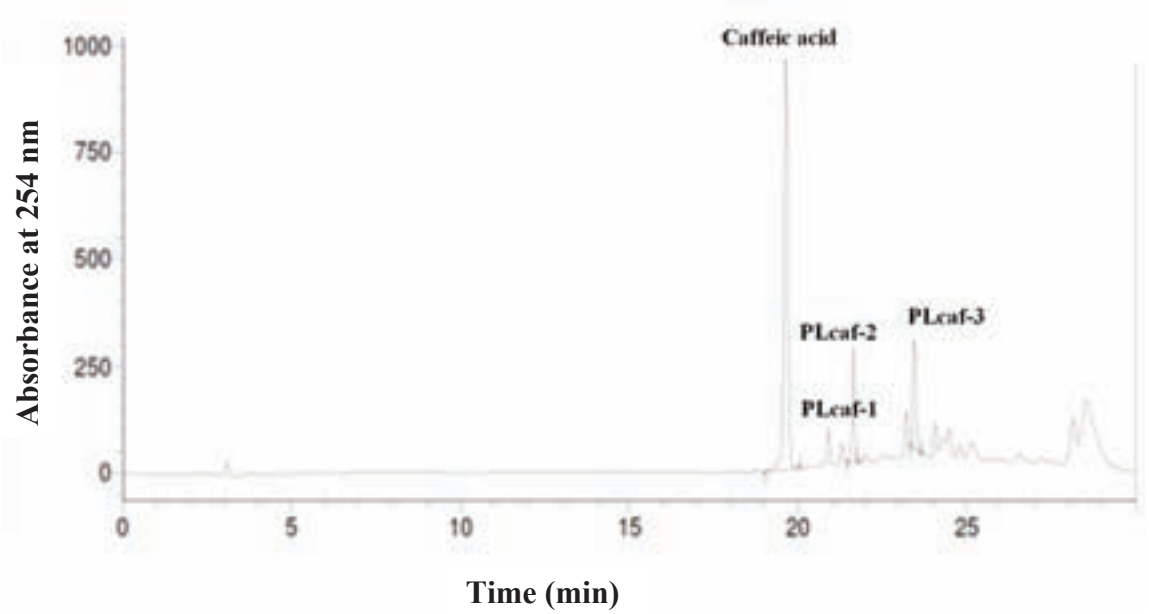

Figure 5a. HPLC chromatogram of 10 mM caffeic acid oxidized by laccase (23.1 U mg-1) for 1 h at 25°C

Şekil 5a. *Lakkaz (23.1 U mg-1) ile 1 sa. 25°C'de 10 mM kaffeik asit oksidasyonunun HPLC kromatogramı*

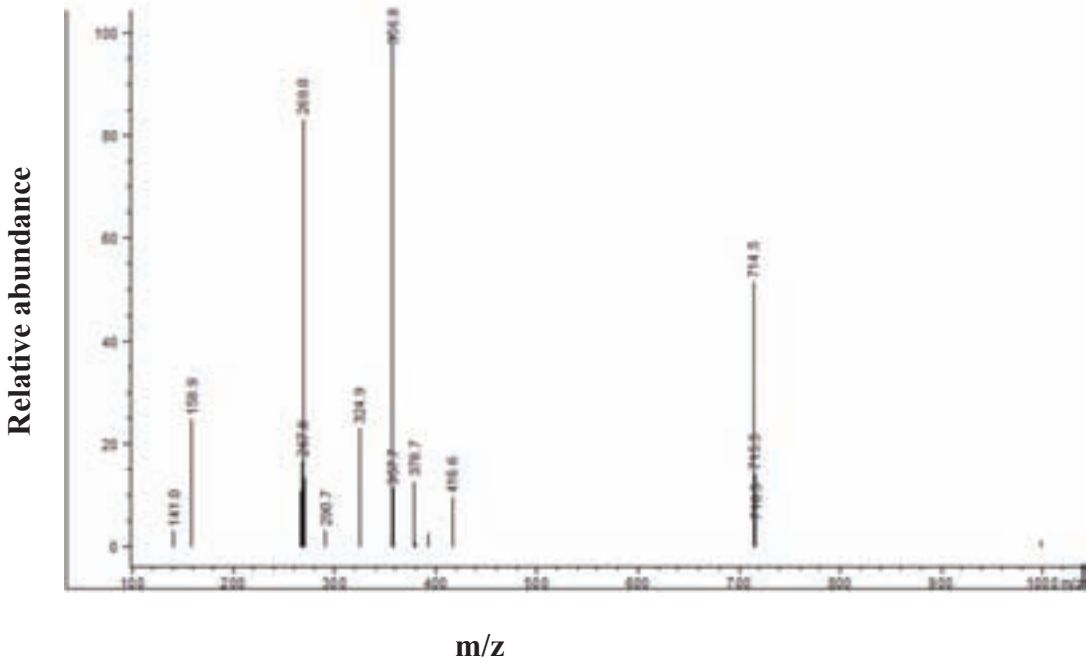

Figure 5b. Mass spectrum of PLcaf-1, (the main peak, m/z=714.5- tetramer of caffeic acid, the base peak, m/z= 356.8-dimer of caffeic acid, the fragments; m/z= 269, 158.9)

Şekil 5b. *PLcaf-1, (ana pik, m/z=714.5-kaffeik asitin tetrameri, temel pik, m/z= 356.8-kaffeik asit dimeri, fragmanlar; m/z= 269, 158.9) kütle spekturumu*

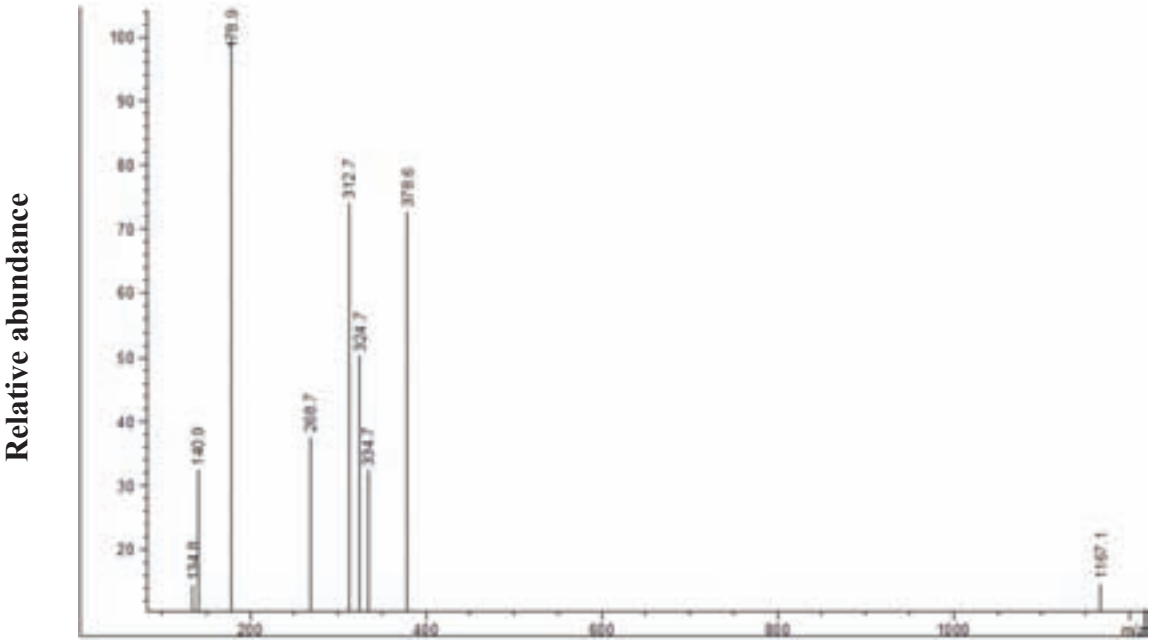

Figure 5c. Mass spectrum of PLcaf-2 (m/z=312.7, fragment, m/z=178.9)
Şekil 5c. *(m/z=312.7, fragman, m/z=178.9) kütle spekturumu*

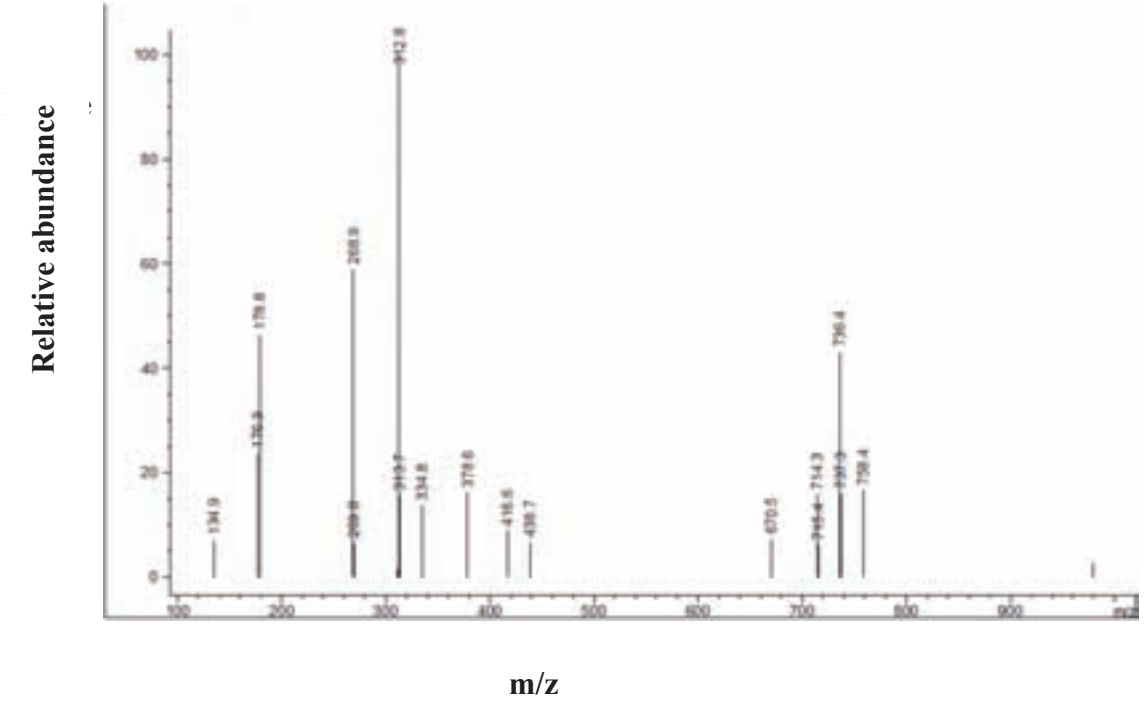

Figure 5c. Mass spectrum of PLcaf-3 (the main peak, m/z =736.4 the base peak, m/z=312.8, and fragments, m/z=268.9 and 178.8)
Şekil 5c. *PLcaf-3 (ana pik, m/z =736.4 temel pik, m/z=312.8, ve fragmanlar, m/z=268.9 and 178.8) kütle spekturumu*

possible dimeric structures, potentially generated after C-C coupling, C2-C2, C2-C5, and C2-C6 type ones seems less likely due to the steric hindrance (33). On the other hands, tyrosinase catalyzed caffeic acid oxidation was ended with no oxidation products.

Conclusion

Based on the overall results, substrate specificity and laccase and tyrosinase catalyzed oxidation products of the three ortho-diphenolic compounds, catechol, (+)-catechin and caffeic acid are quite different. This may occur because of the difference in the electron transfer system and the radicals produced during the oxidation of phenolic compounds which are then coupled to give more stable dimer, trimer, tetramers and oligomers. However, these remain to be determined. Also, these different types of oxidation products will be assayed for their antioxidant and anticancer capacities. Further structure determination of the oxidation products is in progress.

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