COMMERCIAL LACCASE OXIDATION OF PHENOLIC COMPOUNDS

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Lacazele sunt enzime aparţinând clasei oxidazelor, ce conţin în centrul catalitic patru atomi de cupru. Aceştia transferă electroni de la substratul ce se oxidează la cofactorul oxigen, acesta din urmă transformându-se în apă. Compuşii fenolici sunt cei mai potriviţi ca substrat pentru acest tip de enzime. Lucrarea prezintă rezultatele obţinute la oxidarea următorilor compuşi: 1,2-dixidroxibenzen (catecol),(2R,3S)-2-(3,4-dihidroxifenil)-3,4-dihidro-2H-croman-3,5,7-triol (catehină) si 3,4-dihidroxi-fenilalanina (DOPA) cu o lacază comercială, utilizând spectroscopia de masă pentru evidenţierea structurii produşilor obţinuţi.

Laccases are enzymes belonging to the class of oxidases, containing four copper atoms into the catalytic centre. These metal atoms make the electron transfer between the substrate and the cofactor, namely oxygen, turning the later into water. The most suitable substrates for laccases are polyhydroxy-phenols. The oxidation of 1,2-dihydroxybenzene (catechol), (2R,3S)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol (catechin) and 3,4-dihydroxyphenylalanine (DOPA) by a commercial laccase was performed and the products structures were proposed based on MS analysis.

Keywords: commercial laccase, catechol, catechin, DOPA, oxidation, MS spectra

1. Introduction

Polyphenols are phytochemicals, including a large variety of structures from small molecules, improperly called like this, up to polymer structures. Polyphenols are important components of food possessing anti-inflammatory, antioxidative, chemopreventive and neuroprotective activities [1-5].

The estimated intake from vegetables and fruits of the dietary polyphenols is approximately 1 g/day [6].

After absorption, the polyphenols are subject to three type of reaction: methylation, sulfonation and glucuridonation. These polyphenolic compounds are synthesized by higher plants such as teas, vegetables, fruits, tobaccos, etc [7]. A

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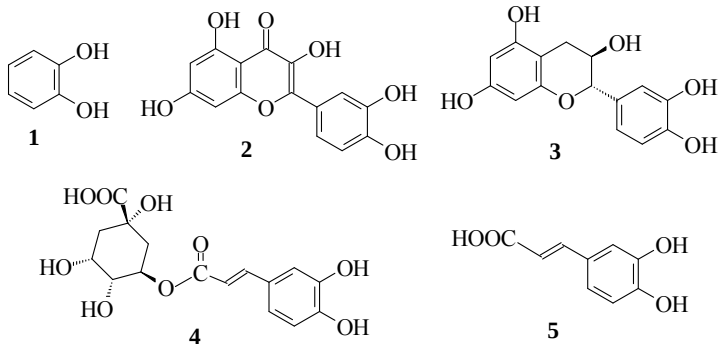
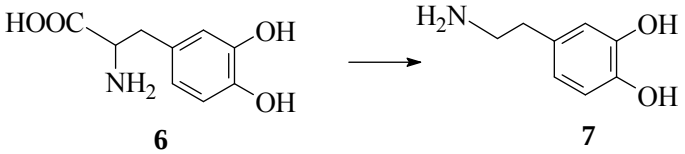
catechol (**1**) moiety, characteristic also to polyphenols like: quercitin (**2**), catechin (**3**), chlorogenic acid (**4**) and caffeic acid (**5**) are transformed mainly by a methylation reaction [8] but metabolization by oxidation also occurs.



Another important molecule with a catechol moiety is 3,4-dihydroxy-phenylalanine (DOPA, **6**), the precursor of the neurotransmitter dopamine (**7**).



The food technologists have been able to demonstrate the nutritional benefits of these phenolics, taking in account their antioxidant capacity or free radical scavenging, acting as food preservative, antimicrobial, antimutagenic, with a number of therapeutic and pharmaceutical properties [9].

During the oxidative metabolization of these compounds, they give rise to toxic molecules like the superoxide anion ($^{\cdot}O_2^-$) which may produce DNA damages [10, 11]. Simultaneously, processing in air presence the food containing catechol and other phenols, dark polymers are obtained as oxidation products, fact leading to the decline of food quality.

Different treatments for avoiding such transformations have been suggested, among these a free [12, 13] or immobilized [14, 15] laccase treatments have been also proposed.

A study of three representative phenolic compounds: catechol (1,2-dihydroxybenze, **1**), catechin [(2R,3S)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol, **3**] and DOPA (3,4-dihydroxy-phenylalanine, **6**) transformations by a commercial laccase treatment was performed and the product structures are proposed based on experimental MS data analysis.

2. Experimental

Materials

All the substrates used were products supplied by Sigma, having the following purity: catechol (**1**) 99%, catechin (**3**) 98% and DOPA (**6**) of analytical purity. The other reagents were purchased from Sigma being of analytical grade.

Laccase

The experimental enzyme was Laccase Roglyr Lite 1540, a commercial product supplied by Hungarian Industry Products KFT and produced by Rotta Manheim. The protein content of the commercial product was resolved using Bradford method based on the measurement of the absorbance of the complex with Coomassie Brilliant Blue G250 at 595 nm. As standard bovine serum albumin was employed [16]. A content of 25.7±1.2 mg protein was evidenced per gram commercial product, the major component of the commercial product being adipic acid, added for stabilizing the enzyme.

Laccase activity

The activity of the commercial enzyme was measured using as substrate a solution of 60mM 2,2′-azinobis-(3-etylbenzothiazoline-6-sulfonate) (ABTS) in 100 mM acetate buffer. The absorbance corresponding to the radical-cation resulting from ABTS at 420 nm (ε_{420}=3.6·10^{4}M^{-1}cm^{-1}) was monitored [17]. Enzyme-specific activity was expressed as units per protein content (U g−1). One unit of laccase activity (U) represents the amount of enzyme required for oxidizing 1 μmol of ABTS per minute.

Catechol, catechin and DOPA oxidation with the commercial laccase

Samples of 100-150 mg of substrates have been dissolved into 2.5 mL acetate buffer, of pH 4. Solutions of 1g commercial laccase in 12 mL of the same buffer have been prepared. Each substrate solution was treated with 1.5 mL of the enzyme solution with stirring, at room temperature, for 24 h. After the water removal the samples have been analyzed by mass spectrometry.

Mass spectra analysis

ESI-mass spectra were carried out on a MSQ+ Thermo Fisher Scientific LC-MS spectrometer, in isocratic mobile phase (100% CH_3CN).

The experimental conditions are as follows:

- Negative ion mode;
- Single quadrupole;
- Probe temperature 500°C;
- Needle voltage 2 kV;
- Cone 40 V;

Samples of 5 mg from the analyzed compounds were dissolved in 1mL acetonitrile and were introduced into the mass spectrometer at a flow rate of 0.5 mL/min through a Surveyor LC Pump Plus (a dual-piston, quaternary, low-

pressure mixing pump with a built-in vacuum degasser and pulse dampener). The analysis was performed in the range of 0-1500 m/z. From all the spectra the peak characteristic to adipic acid anion (m/z=145) had been taken out.

3. Results and discussion

Laccases (EC 1.10.3.2) are enzymes belonging to the first class of enzymes, namely *oxidoreductases*. These enzymes are produced by bacteria, fungi and plants [18] and may be obtained in large quantities favoring large scale applications [19, 20]. Laccases take electrons and protons from different substrates and transfer it *via* four copper atoms to oxygen, the cofactor, which is turned into water according the relation (1) [21].

$$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O \qquad (1)$$

The electrons are taken by one electron steps, the substrates giving free radicals as intermediates. The free radicals are stabilized by reactions like: hydration, elimination, addition and polymerization [22].

The three studied substrates: **1**, **3** and **6** have been treated with catalytic amount of a solution of commercial laccase in acetate buffer (pH=4)

The protein content of the enzyme was 25.7±1.2 mg protein/g commercial product. As previously [23] shown, the specific activity of the enzyme measured (toward ABTS as substrate) is 1301.2 Ug^{-1} at pH of 3.8 and it is decreasing at higher pH values. Thus, the phenolic compound oxidation was performed at a pH close to optimum pH value, insuring a high activity for the enzyme. The analysis of the mass spectra of the products resulted from the three studied compounds revealed a number of transformations presented below.

For catechol (**1**) the main product is a compound with m/z =197 (see Figure 1).

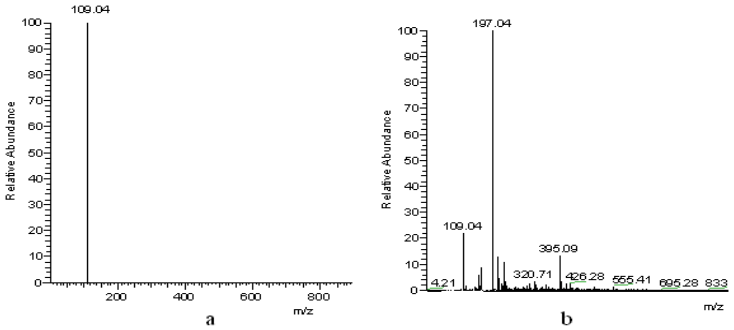
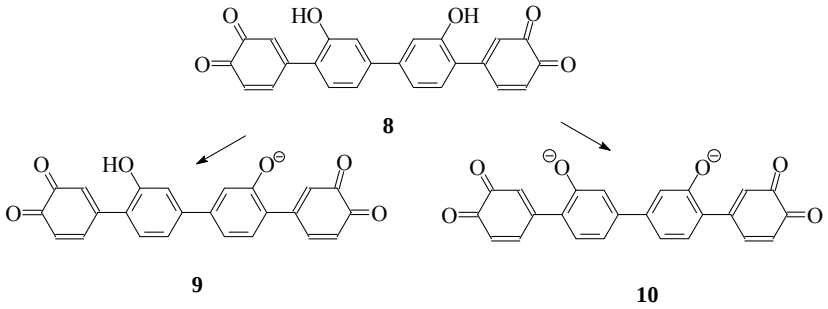


Fig.1. Mass spectra of catechol (a) and its laccase oxidation products (b)

This main product seems to be a poly-condensation tetramer with most probably the structure **8** giving rise to a monoanion **9** (m/z=395), as well as a dianion **10** (m/z =197):



8

9 **10**

Similar products have been observed for compounds with catechol moiety [24]. Higher molecular structures, reported as oxidation products when using a pure laccase [25], have not been observed in the case of the commercial enzyme oxidation. The mild conditions attributed to the oxidation with the commercial laccase Roglyr 1540 are illustrated also by the presence of some unchanged **1** (m/z =109).

For catechin (**3**) the MS experimental data are shown in Figure 2:

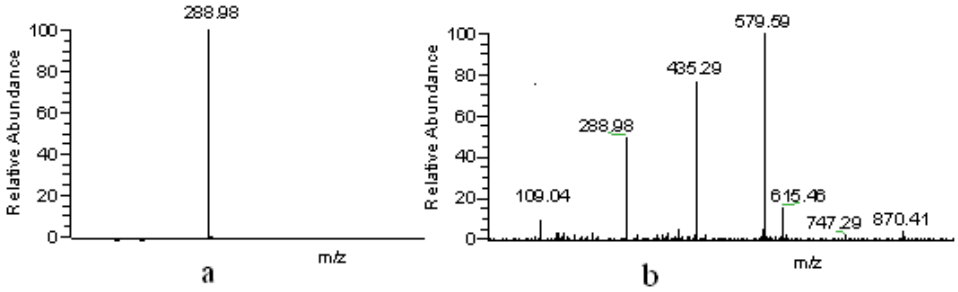
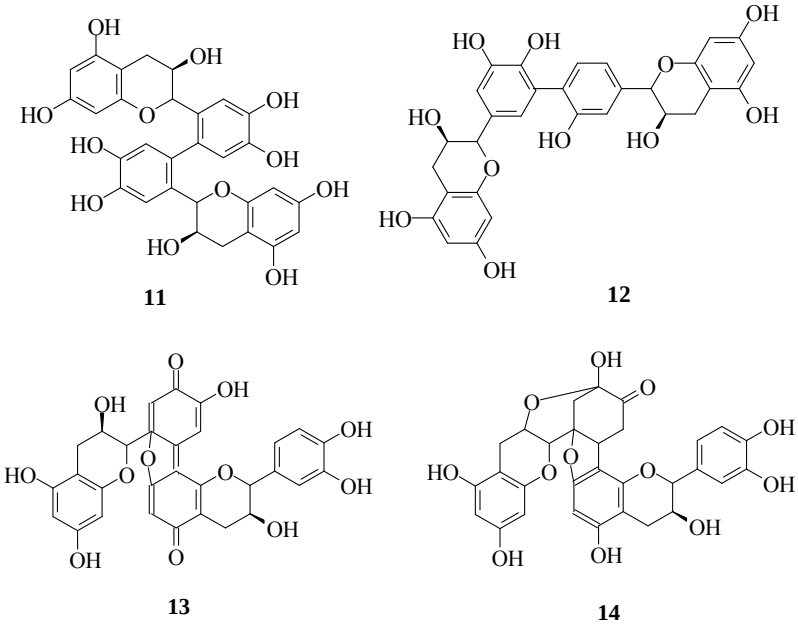


Fig. 2. Mass spectra of catechin (**a**) and its laccase oxidation products (**b**)

Traces of dihydroxybenzene (m/z=109) due to a molecular fragmentation of catechin (**3**) are observed. A quantity of **3** (anion m/z=289) remained in the reaction mixture due to the mild oxidation conditions. A dimer (m/z =579) is also formed by the association between catechin anion and catechin (**3**). The peak at m/z = 435 corresponds most probably to the degradation (possible loss of a dihydroxy-benzene moiety, water, etc.) of one of the previously described

dimmers **11** -**14**, or other complex structure that may be obtained through oxidation reactions [24, 25].



11 **12**

13 **14**

Products with higher molecular mass are only in traces like in the oxidation of compound **3**.

DOPA oxidation in the similar conditions gave mainly degradation products (see Figure 3).

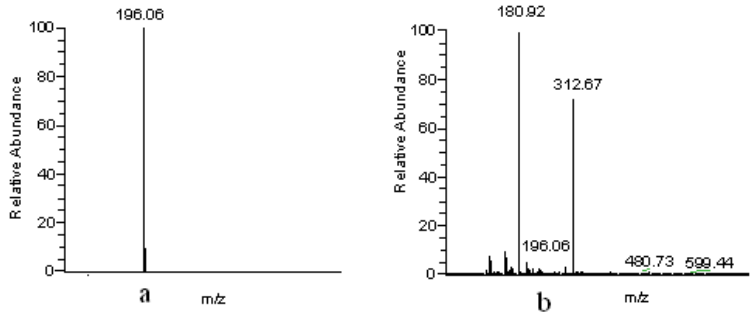
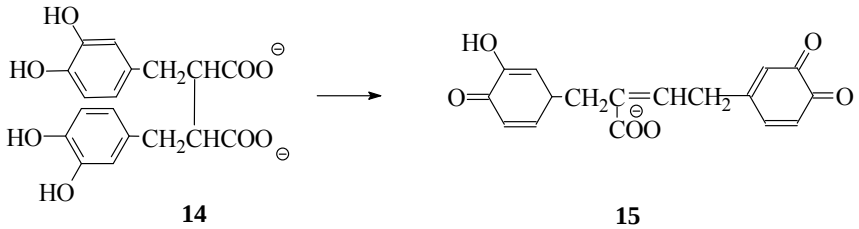


Fig. 3. Mass spectra of DOPA (**a**) and its laccase oxidation products (**b**)

According to the MS data the DOPA was transformed by a mild oxidation reaction into dimmers. The most appropriate products seemed to be the dianion **14**

(m/z=180) obtained by oxidative elimination of the NH_2 group and the anion **15** (m/z=312) resulted after further oxidation and decarboxilation of **14**.



14 **15**

No polymer was observed also in DOPA oxidation with the commercial laccase, in catalytic amount.

Comparable results (formation of dimmers and not polymers) have been previously evidenced during the treatment of apple juice with an immobilized laccase [15].

4. Concluding remarks

The MS experimental data have definitely shown that the commercial laccase has a mild oxidative effect on the studied phenolic compounds. Thus, the insoluble polymers, evidenced after pure laccase treatment, previously described, are not obtained in this case. Such results recommend the commercial enzyme to be used for the stabilization of food containing phenolic compounds, due to the formation of soluble products. The polymer formation which generates solid deposits is avoided by such treatment resulting in the preservation of food product quality.

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