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KINETIC MECHANISM OF ENZYMATIC MODIFICATION OF RUTIN WITH 3-METHYL-2-BENZOTHIAZolinONE HYDRAZONE BY HORSERADISH PEROXIDASE

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The kinetic mechanism of enzymatic modification of rutin with 3-methyl-2-benzothiazolinone hydrazone by horseradish peroxidase was studied. The reaction of the rutin modification was followed by recording spectral changes over the time at 510 nm. All reactions were performed in 100 mM phosphate buffer pH 6.0 at 20 °C. Kinetic parameters were determined from the graphics of linear Michaelis-Menten equation. It was found that all the investigated reactions of the rutin modification with 3-methyl-2-benzothiazolinone hydrazone by HRP take place in an ordered mechanism. We propose that peroxidase initially reacts with hydrogen peroxide, then with 3-methyl-2-benzothiazolinone hydrazone and finally with rutin which further leads to the formation of azo-dye.

Keywords: horseradish peroxidase; rutin; 3-methyl-2-benzothiazolinone hydrazone; kinetic mechanism

Introduction

Peroxidases are enzymes which are widely distributed in nature and have a pivotal role in biology [1]. They have the ability to oxidize phenolic substrates in the presence of H₂O₂, and can produce the reactive oxygen species (ROS) such as superoxide anion (O₂⁻) or hydroxyl radical (OH⁻) through the hydroxyl cycle [2,3]. Horseradish peroxidase (HRP, EC 1.11.1.7) is one of the heme peroxidases and a highly investigated member of the peroxidase family that catalyzes the oxidation of flavonoids and phenolic substrates to the free phenoxyl or semiquinone radicals [1,2,4-9]. The previous research has shown that the oxidation process of phenolic compounds can be considered as a modified type of ping-pong kinetics [10,11], which can be represented by the following reaction scheme:

HRP + H₂O₂ → HRP-I + H₂O...............................(1)
HRP-I + AH₂ → HRP-II + ∙AH + H₂O..................(2)
HRP-II + AH₂ → HRP + ∙AH + H₂O....................(3)

where HRP-I is an active intermediate, HRP-II is a reduced active form of HRP; ∙AH is a free radical and AH₂ is an aromatic compound.

Flavonoids are aromatic compounds with complex structures and they have great abundance in the human diet. Flavonoids are widely distributed in plants, vegetables, fruit juices, and a variety of beverages (tea, coffee, wines, and fruit drinks) [6,12,10-18]. In previous experimental studies its demonstrated that they possess numerous biological and pharmacological effects including antioxidant, antimitagenic, anticarcinogenic, antiulcer, probiotic, antimicrobial, antiinflammatory properties [3,10-12,14,16-19]. Rutin (quercetin-3-rhamnosyl glucoside) is a low molecular natural flavone derivative (Figure 1), which is considered as one of the most promising quercetin derivatives from the biochemical and pharmacological point of view [20,21].

Figure 1. The chemical structure of rutin (a) and MBTH (b).

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It is widely distributed in vegetables and fruits, but recent studies have shown that buckwheat is a major dietary source of rutin [22,23]. Rutin has a significant anti-inflammatory, antitumour and antioxidant activity which makes it a popular ingredient of numerous multivitamin preparations and herbal remedies [23,24]. These properties help in preventing diseases and have a significant stability effect of the genetic material.

On the other hand 3-methylbenzothiazolin-2-one hydrazone (MBTH) is a compound which was introduced as a reagent in analytical chemistry for the detection and determination of aldehydes, indoles, aromatic amines, iminohetero-aromatic compounds, arylalkylamines, carbazoles, phenols, etc. [25,26]. In 1970s the first studies about the horseradish peroxidase-catalyzed oxidative coupling reactions appeared which demonstrated the potential of a chromogenic system based on the oxidative coupling of MBTH and aromatic amines by peroxidase [27]. About ten years later, the investigators reported a sensitive and versatile chromogenic assay for peroxidase and peroxidase-coupled reactions based on the oxidative coupling of MBTH and 3-(dimethylamino) benzoic acid. After that, Setti et al. [26] showed that HRP catalyzes an oxidative coupling reaction between MBTH and o-, m-, and p-methoxyphenols. The obtained results showed that reactions follow a modified type of ping-pong kinetics [26].

Realizing the increasing importance of rutin in human nutrition and health in a proper way, it is of a great interest to study the rutin oxidation and its possible modifications, such as the nature of the modified products. The objectives of this study were to investigate the possibility of the reaction of enzymatic modification of rutin with MBTH by HRP and to evaluate the kinetic mechanism, as well as to determine the values of kinetic parameters $K_m$, $V_{max}$ and $k_{cat}$ for substrates. Furthermore, we have evaluated the possibility of the formation of azo-dye by oxidative coupling of MBTH and rutin as the polyphenol compound.

Materials and methods

Chemicals
Rutin, dimethyl sulfoxide (DMSO) and MBTH were purchased from Sigma (Germany). A stock solution of rutin (10 mM) and MBTH (2 mM) was prepared in DMSO and aqua destilata, respectively.

Horseradish peroxidase (298 U/mg) was purchased from Sigma (Germany). The 2 μM stock solution of HRP was prepared by dissolving the 0.34 mg of the solid HRP in 10 ml of cold 50 mM phosphate buffer pH 6.0. The enzyme concentration was calculated by using $\varepsilon_{280} = 102.0 \text{ mM}^{-1} \text{ cm}^{-1}$.

Spectrophotometric assays
Kinetic assays were carried out by measuring the appearance of the product in the reaction medium on VARIAN Cary-100 Spectrophotometer and controlled by using a VARIAN Cary-100 UV-Winlab software in quartz cuvettes dimensions 1×1×4.5 cm. The total amount of the reaction mixture was 5 ml. Firstly, the reaction mixture was scanned at wavelengths of 800-200 nm, and then only at the wavelength of 510 nm, every 5 s for a period of 60 s. All measurements were performed at the temperature of 20 °C. The blank contained 100 mM phosphate buffer pH 6.0.

The enzymatic reaction was performed in a manner that phosphate buffer (pH 6.0) and water were always first measured in a tube. Then, the solution of rutin was added but not too long before the start of the reaction itself due to the sensitivity to light. Afterwards the solution of MBTH was added, then the solution of HRP and finally the reaction began by adding the solution of hydrogen peroxide with efficient mixing on the vortex. The concentrations of the substances in the reaction mixture are given in Results and discussion, below the figures.

The determination of the enzymatic proportion in the overall reaction was done by measuring the rate of the reaction in the presence and absence of HRP. The kinetic mechanism of the reaction was determined by the method of Haldane and Dalziel [28].

Results and discussion

Enzymatic modification of rutin with MBTH by HRP
Generally, the absorption spectrum of flavonoids consists of two absorption bands in the range of 240-400 nm. Band I, originating from the absorption of the B-ring is covering the range of 300-380 nm (with $A_{max}$ position around 350-370 nm), while band II originating from the absorption of the A-C benzoyl system is covering the range of 240-280 nm (with $A_{max}$ position around 260-270 nm); a week band with the absorption maximum around 300 nm was also detected, and it has been attributed to the C-ring only [29].

The changes in the absorption spectrum of the reaction mixture during the reaction modification of rutin with MBTH by HRP are shown in Figure 2. The presence and position of two absorption bands at 260 and 370 nm indicate that they originate from rutin (Figure 2, increment (a)), which was expected and is consistent with previously published results [30]. In addition, there is the third absorption maximum on 300 nm which originates from MBTH (Figure 2, increment (a)). During the reaction there is a proportional decrease in the intensity absorption lines that originate from rutin (260 and 370 nm) and MBTH (300 nm), and also results in the formation of new absorption maximum at the wavelength of 510 nm. These changes in the absorption spectrum indicate that during the reaction the formation of azo-dye (with $A_{max}$ 510 nm) by the oxidative coupling of MBTH and rutin as a polyphenol compound occurs, which was expected if we take into account the results obtained by Setti et al. [26].

As for the kinetic characterization of the reaction modification of rutin with MBTH by HRP, Michaelis-Menten type kinetics was obtained in all the investigated cases
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(Fig. 2 - increment (b)). Figure 2 (increment (c)) shows the change of the absorbance during the time in the reaction medium in the presence and absence of HRP. The obtained results indicate that the reaction modification of rutin with MBTH by HRP is enzymatic. These experimental results are expected and they are compatible with the previous results obtained by Setti et al. [26].

Table 1. The values of the kinetic parameters obtained in reaction modification of rutin with MBTH by HRP

<table>
<thead>
<tr>
<th>Case</th>
<th>Rutin, mM</th>
<th>MBTH, mM</th>
<th>H₂O₂, mM</th>
<th>Keq, mM</th>
<th>V_max, ΔA₅₁₀ min⁻¹</th>
<th>K_cat, ΔA₅₁₀ min⁻¹ mM⁻¹</th>
<th>V_max/K_cat, ΔA₅₁₀ min⁻¹ mM⁻²</th>
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<td>Variable</td>
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<td>0.051</td>
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<td>0.068</td>
<td>0.085</td>
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<tr>
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</table>

Figure 2. UV-VIS spectrum of the reaction medium. The assay medium (5 mL) contained 80 μM rutin, 40 μM MBTH, 16 nM HRP and 50 mM sodium phosphate buffer (pH 6.0) at 20 °C. The reaction was started by the addition of hydrogen peroxide (0.1 mM). Scans were recorded every minute during 10 min. The increments show: (a) The absorption spectrum of rutin and MBTH. The concentration of rutin and MBTH in 100 mM phosphate buffer were 80 and 40 μM, respectively. (b) The dependence rate of the rutin modification with MBTH by HRP towards its concentration. The reaction medium contained 80 μM rutin, 16 nM HRP, 50 mM sodium phosphate buffer (pH 6.0), 0.1 mM hydrogen peroxide and variable concentrations of MBTH. (c) Spectrophotometric recordings of the rutin modification in the presence and absence of HRP were done at 510 nm. The reaction medium contained 80 μM rutin, 40 μM MBTH, 16 nM HRP, 50 mM sodium phosphate buffer (pH 6.0) and 0.1 mM H₂O₂.
In Table 1 the kinetic parameters of rutin modification with MBTH by HRP are presented. The presented values of $K_m$ refer to substrate which was variable, at constant concentration of other substrates. By looking at the obtained values of the kinetic parameters $V_{max}$ and $K_m$ (Table 1), it can be concluded that in all cases the increase of the concentration of one of the substrates leads to the increase of $V_{max}$ of the rutin enzymatic modification. According to this, it can be concluded that in this relationship for the measured concentration in the reaction within the reaction time, the substrate inhibition was not present.

Lineweaver-Burk plots for the kinetic data of the rutin modification with MBTH by HRP are shown in Figure 3. By looking at Figure 3 the following can be concluded: firstly, the increase of the concentration of hydrogen peroxide increases the speed of the reaction modification of rutin; secondly, the location and layout lines on the Lineweaver-Burk plots indicates the ordered or random mechanism of this enzymatic modification of rutin.

![Figure 3. Lineweaver-Burk plots for the reaction modification of rutin with MBTH by HRP at 20 °C and pH 6.0. The reaction medium contained 40 μM MBTH, 16 nM HRP, 50 mM sodium phosphate buffer (pH 6.0) and variable concentrations of rutin and H$_2$O$_2$.](image)

Then, a drawing of the graph of the $K_m$ for rutin in the function of the hydrogen peroxide concentration (Figure 4 A) shows that the process of the rutin modification with MBTH by HRP is performed by an ordered mechanism.

Figure 4 shows that the process of the rutin modification with MBTH by HRP takes place in an ordered mechanism in all cases. According to our knowledge this is the first report of the kinetic mechanism of enzymatic modification of rutin with MBTH by HRP. Based on the these results, it is estimated that peroxidase initially reacts with hydrogen peroxide, then with MBTH and finally with rutin which further leads to the formation of azo-dye. This conclusion is similar to the one which was found by Setti, in the reaction between $o$-, $m$-, and $p$-methoxyphenols and MBTH in the presence of horseradish peroxidase and H$_2$O$_2$ as an oxidative agent, as well as to the investigation in the reaction between $o$-, $m$-, and $p$-methoxyphenols and MBTH in the presence of laccase [26,27].

![Figure 4. The dependance of Michaelis constants in relation to the concentration of substrate. For more explanation of Figure A, B and C see Table 1.](image)
where: \( A \) is hydrogen peroxide, \( B \) is MBTH, \( C \) rutin and \( P \), \( Q \) and \( R \) are the products.

If we assume that \( V_{\text{max}} = k_{\text{cat}}[E]_0 \), then the velocity equation for the above mechanisms is as follows:

\[
V_{\text{max}} = \frac{K_C}{1 + \frac{K_B}{BC} + \frac{K_A}{ABC}} \quad \text{(5)}
\]

In this equation \( A \), \( B \) and \( C \) are the concentrations of substrates (hydrogen peroxide, MBTH and rutin, respectively), whereby \( K_A \), \( K_B \) and \( K_C \) are Michaelis-Menten constants for hydrogen peroxide, MBTH and rutin.

Conclusions

To sum up, in the reaction of the enzymatic modification of rutin with MBTH by HRP non-enzymatic reaction is not present, it is just enzymatic. All the reactions of the rutin modification with MBTH by HRP take place in an ordered kinetic mechanism. We propose that peroxidase initially reacts with hydrogen peroxide, then with MBTH, and finally with the rutin which further leads to the formation of azo-dye.

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References


Izvod

**KINETIČKI MEHANIZAM ENZIMSKE MODIFIKACIJE RUTINA SA 3-METIL-2-BENZOTIAZOLINON HIDRAZONOM POMOĆU PEROKSIDAZE IZ RENA**

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**Ključne reči:** peroksidaza iz rena; rutin; 3-metil-2-benzotiazolinon hidrazon; kinetički mehanizam