Kinetics of enzymatic modification of quercetin with cysteine by horseradish peroxidase

Sasa R Savic and Zivomir B Petronijevic*
Faculty of Technology, University of Nis, Bulevar Oslabodjenja 124, 16000 Leskovac, Serbia

Received 14 September 2013; revised 03 April 2013

The kinetic mechanism of enzymatic modification of flavonol quercetin with L-cysteine by horseradish peroxidase (HRP) was studied. Reaction of modification of quercetin was followed by recording spectral changes over time at 380 nm. All reactions were performed in 100 mM phosphate buffer pH, 6.0 at 20ºC. Kinetic parameters were determined from graphics of linear Michaelis-Menten equation. The values obtained at specified intervals were:

\[ V_{\text{max}} = 0.17 \div 0.91 \text{ \Delta A}_{380}/\text{min}, \]
\[ K_m = 0.023 \div 0.5 \text{ mM}, \]
\[ k_{\text{cat}} = 0.21 \div 1.14 \text{ \Delta A}_{380}/\text{min} \cdot \text{mM}^{-1} \]
\[ V_{\text{max}}/K_m = 0.83 \div 26.55 \text{ \Delta A}_{380}/\text{min} \cdot \text{mM}^{-1}. \]

It was found that all investigated reactions of the modification of quercetin with L-cysteine by HRP followed an ordered mechanism. We propose that HRP initially reacts with \( \text{H}_2\text{O}_2 \) than with quercetin and finally with L-cysteine, leading to the introduction of L-cysteine in the structure of quercetin.

Keywords: Horseradish peroxidase, Quercetin, Cysteine, Kinetic mechanism, Spectrophotometry

Peroxidases are enzymes which are widely distributed in eukaryotes and prokaryotes, and have a pivotal role in biology. Most of peroxidases are hemoproteins containing ferriprotoporphyrin IX as the prosthetic group. The family of peroxidases includes plant ascorbate peroxidases, yeast cytochrome \( c \) peroxidase, mammalian haloperoxidases (myeloperoxidase and lactoperoxidase), fungal and other plant peroxidases.

Horseradish peroxidase (HRP, EC 1.11.1.7) is a well-known and highly investigated member of the peroxidase family that catalyzes the oxidation of flavonoids and phenolic substrates to the free phenoxyl or semiquinone radicals. Previous research has shown that the oxidation process of phenolic compounds can be considered as a modified type of ping-pong kinetics, which can be represented by the following reaction scheme:

HRP + \( \text{H}_2\text{O}_2 \) \( \rightarrow \) HRP-I + \( \text{H}_2\text{O} \) \( \quad ... (1) \)

HRP-I + \( \text{AH}_2 \) \( \rightarrow \) HRP-II + \( \cdot \text{AH} + \text{H}_2\text{O} \) \( \quad ... (2) \)

HRP-II + \( \text{AH}_2 \) \( \rightarrow \) HRP + \( \cdot \text{AH} + \text{H}_2\text{O} \) \( \quad ... (3) \)

where HRP-I is an active intermediate, HRP-II is the reduced active form of HRP, \( \cdot \text{AH} \) is free radical and \( \text{AH}_2 \) is an aromatic compound.

HRP provides wide substrate specificity to the donor of hydrogen, but recently attention has focused on polyphenols with complex structures, such as flavonoids. Flavonoids are a large group of polyphenols which are widely distributed in plants, vegetables, fruit juices and a variety of beverages (tea, coffee, wines and fruit drinks). Experimental studies have demonstrated that they possess numerous biological and pharmacological effects, including antioxidant, anti-mutagenic, anti-carcinogenic, anti-ulcer, probiotic, anti-microbial, anti-allergic, antiviral and antiinflammatory properties.

Because of its ubiquitous nature, quercetin (3,3´,4´,5,7-pentahydroxyflavone, Fig. 1) is the most studied of the dietary flavonoids and one of the most abundant plant-derived polyphenols widely consumed with a human diet. Quercetin containing the 3´,4´-dihydroxy structure in

![Fig. 1—Quercetin structure and division of band I and band II related to UV–VIS absorption bands]
the B ring (catechol B ring) is known to possess a high antioxidant ability by scavenging free radicals. It is a strong antioxidant against lipid peroxidation in phospholipid bilayers and in human low-density lipoprotein13,21,27,28.

As quercetin has a free hydroxyl group at the 3-position, it can also act as an inhibitor of tyrosinase29,30. Thus, quercetin is simultaneously a substrate and an inhibitor of the tyrosinase, depending upon the manner in which it binds to the copper atoms of the active site. Previous studies have shown that oxidation of quercetin by oxidative enzymes, such as polyphenol oxidase and peroxidase give three major oxidation products, which could react with various nucleophilic agents building adducts23,29.

In this study, we have investigated the kinetic mechanism of enzymatic modification of quercetin with L-cysteine by HRP and determined of the values of kinetic parameters $K_m$, $V_{max}$ and $k_{cat}$ for the substrates. Study of the kinetic mechanism of enzymatic modification of quercetin can provide useful information to the field of chemistry and biochemistry of food and drugs that contain flavonoids.

Materials and Methods

Reagents

Horseradish peroxidase (298 U/mg; using pyrogallol), quercetin, dimethyl sulfoxide (DMSO) and L-cysteine were purchased from Sigma (Germany). Stock solutions of quercetin (10 mM) and L-cysteine (20 mM) were prepared in DMSO and aqua destilata, respectively.

Enzyme

A 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 using a $\varepsilon_{403} = 102.0 \text{mM}^{-1}\text{cm}^{-1}$.

Spectrophotometric assays

Kinetic assays were carried out by measuring the appearance of the product in the reaction medium on VARIAN Cary-100 Spectrophotometer controlled 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. First, the reaction mixture was scanned at wavelengths of 200-500 nm and then only at a wavelength of 380 nm at every 10 s for a period of 120 s. All measurements were performed at 20°C. The blank contained 100 mM phosphate buffer, pH 6.0.

Enzymatic reaction was performed in a manner that phosphate buffer, pH 6.0 and water were always the first added in a tube. Then was added 10 mM solution of quercetin, but not much earlier before the start of the reaction, due to sensitivity to light. Thereafter, 20 mM solution of L-cysteine was added, followed by 2 µM solution of HRP and finally the reaction started by adding 10 mM solution of H$_2$O$_2$ with efficient mixing on the vortex. The concentrations of the substances in the reaction mixture are given in the figures legends.

The determination of the proportion of enzymatic reaction in overall reaction was done by comparing the reaction rate in the presence and absence of the enzyme, respectively, and the kinetic mechanism was determined by the method of Haldane and Dalziel31.

Results and Discussion

Based on previous study, it is known that the solution of quercetin in phosphate buffer (Fig. 2) shows two distinct absorption bands in the UV-VIS spectrum — the first one originates from the absorption of the A ring and is located at about 255 nm and the second one is at about 372 nm, originating from the absorption of rings B and C (Fig. 1)15.

Modification of quercetin with L-cysteine by HRP

It is known that the oxidation of quercetin primarily produces o-quinones, which react with different nucleofiles, resulting into enzymatic modification of quercetin27,29. Figure 3 shows the change in absorption spectrum of the reaction mixture during the reaction of modification of quercetin with L-cysteine.
The presence and position of two absorption bands at 256 and 372 nm indicated that they originated from quercetin, which was expected and was consistent with previously published results. During the reaction, there was a proportional decrease in intensity of absorption lines that originated from quercetin, and also resulted in the formation of two new absorption maximum at wavelengths of 290 and 332 nm. At the same time, there was appearance of new absorption maximums between two isosbestic points (281 and 340 nm), confirming the expectations the quercetin as a substrate for HRP. The isosbestic points indicated that the product (o-quinone) was stable in the absence of nucleophile (L-cysteine).

Figure 4 shows the change of absorbance during the time in the reaction medium in the presence and in absence of HRP. As for the kinetic characterization of the reaction of modification of quercetin with L-cysteine, the results indicated that the reaction was mostly enzymatic (about 90%) and non-enzymatic part comprised only about 10%.

Kinetic mechanism of quercetin enzymatic modification with L-cysteine by HRP

The dependence of the quercetin modification rate on quercetin concentration is shown on Fig. 5, in which the enzyme shows Michaelis-Menten type kinetics. Michaelis-Menten type kinetics was also obtained in all other investigated cases.

The kinetic parameters of reaction modification of quercetin with L-cysteine by HRP are presented in Table 1. The present values of $K_m$ referred to the substrate, which was variable at constant concentrations of two other substrates. Looking at the obtained values of the kinetic parameters $V_{max}$ and $K_m$ (Table 1), it could be concluded that in all cases with an increasing of concentration of one of the substrate led to increasing of $V_{max}$. Thus, it can be concluded that during the reaction the substrate inhibition was not there.

Lineweaver-Burk plots for the kinetic data of quercetin modification are shown in Fig. 6 and it could be concluded that the speed of reaction modification of quercetin increased with increase...
in the concentration of H$_2$O$_2$. The location and layout lines on the Lineweaver-Burk plots indicated that this enzymatic modification of quercetin followed ordered or random mechanism. Further, a drawing of a graph of the $K_m$ for quercetin as the function of H$_2$O$_2$ concentration (Fig. 7A) also suggested that the process of modification of quercetin with L-cysteine followed the ordered mechanism.

Figure 7 shows that the process of quercetin modification with L-cysteine followed an ordered mechanism in all cases. According to our knowledge, this is the first report of the kinetic mechanism of quercetin enzymatic modification with L-cysteine by HRP. Based on this results, it was estimated that peroxidase initially reacted with H$_2$O$_2$ than with quercetin and finally with L-cysteine which further led to the introduction of L-cysteine in the structure of quercetin.

### Table 1—The obtained values of kinetic parameters in reaction of modification of quercetin with L-cysteine by HRP

<table>
<thead>
<tr>
<th>Case</th>
<th>Quercetin (mM)</th>
<th>L-Cysteine (mM)</th>
<th>H$_2$O$_2$ (mM)</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ ($\Delta A_{380}$ min$^{-1}$)</th>
<th>$k_{cat}$ ($\Delta A_{380}$ min$^{-1}$ nM$^{-1}$)</th>
<th>$V_{max}/K_m$ ($\Delta A_{380}$ min$^{-1}$ mM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.04</td>
<td>0.50 ± 0.1</td>
<td>0.42 ± 0.01</td>
<td>0.52 ± 0.01</td>
<td>0.83 ± 0.055</td>
<td>2.15 ± 0.03</td>
<td>4.44 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>0.06</td>
<td>0.38 ± 0.04</td>
<td>0.48 ± 0.02</td>
<td>0.60 ± 0.02</td>
<td>1.25 ± 0.03</td>
<td>2.89 ± 0.08</td>
<td>6.04 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>0.08</td>
<td>0.25 ± 0.11</td>
<td>0.59 ± 0.02</td>
<td>0.74 ± 0.02</td>
<td>2.35 ± 0.065</td>
<td>4.44 ± 0.02</td>
<td>9.18 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>0.21 ± 0.13</td>
<td>0.63 ± 0.03</td>
<td>0.78 ± 0.03</td>
<td>2.98 ± 0.08</td>
<td>5.10 ± 0.015</td>
<td>12.63 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>0.12</td>
<td>0.15 ± 0.02</td>
<td>0.67 ± 0.02</td>
<td>0.83 ± 0.02</td>
<td>4.44 ± 0.02</td>
<td>6.04 ± 0.02</td>
<td>14.12 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>0.16</td>
<td>0.12 ± 0.02</td>
<td>0.72 ± 0.02</td>
<td>0.91 ± 0.02</td>
<td>6.04 ± 0.02</td>
<td>10.33 ± 0.02</td>
<td>20.66 ± 0.02</td>
</tr>
<tr>
<td>B</td>
<td>0.05</td>
<td>0.09 ± 0.02</td>
<td>0.17 ± 0.01</td>
<td>0.21 ± 0.01</td>
<td>1.75 ± 0.015</td>
<td>2.15 ± 0.03</td>
<td>4.44 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>0.08 ± 0.02</td>
<td>0.20 ± 0.01</td>
<td>0.26 ± 0.01</td>
<td>2.10 ± 0.015</td>
<td>2.89 ± 0.08</td>
<td>6.04 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>0.07 ± 0.01</td>
<td>0.22 ± 0.02</td>
<td>0.28 ± 0.02</td>
<td>3.13 ± 0.015</td>
<td>4.44 ± 0.02</td>
<td>9.18 ± 0.02</td>
</tr>
<tr>
<td>C</td>
<td>0.04</td>
<td>0.13 ± 0.05</td>
<td>0.39 ± 0.03</td>
<td>0.49 ± 0.03</td>
<td>3.13 ± 0.04</td>
<td>6.04 ± 0.02</td>
<td>12.63 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>0.08</td>
<td>0.12 ± 0.02</td>
<td>0.41 ± 0.02</td>
<td>0.52 ± 0.02</td>
<td>3.47 ± 0.02</td>
<td>7.10 ± 0.015</td>
<td>14.20 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>0.10 ± 0.04</td>
<td>0.43 ± 0.01</td>
<td>0.54 ± 0.01</td>
<td>4.18 ± 0.025</td>
<td>10.33 ± 0.02</td>
<td>20.66 ± 0.02</td>
</tr>
<tr>
<td>D</td>
<td>0.10</td>
<td>0.28 ± 0.12</td>
<td>0.79 ± 0.02</td>
<td>0.98 ± 0.02</td>
<td>2.81 ± 0.07</td>
<td>4.44 ± 0.02</td>
<td>9.18 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>0.26 ± 0.14</td>
<td>0.81 ± 0.03</td>
<td>1.02 ± 0.03</td>
<td>3.13 ± 0.085</td>
<td>6.04 ± 0.02</td>
<td>12.63 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>0.40</td>
<td>0.21 ± 0.07</td>
<td>0.83 ± 0.05</td>
<td>1.04 ± 0.05</td>
<td>3.91 ± 0.06</td>
<td>7.10 ± 0.015</td>
<td>14.20 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>0.80</td>
<td>0.19 ± 0.04</td>
<td>0.91 ± 0.04</td>
<td>1.14 ± 0.04</td>
<td>4.73 ± 0.04</td>
<td>10.33 ± 0.02</td>
<td>20.66 ± 0.02</td>
</tr>
<tr>
<td>E</td>
<td>0.08</td>
<td>0.10 ± 0.04</td>
<td>0.71 ± 0.01</td>
<td>0.89 ± 0.01</td>
<td>1.43 ± 0.09</td>
<td>2.15 ± 0.03</td>
<td>4.44 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>0.08 ± 0.03</td>
<td>0.59 ± 0.01</td>
<td>0.74 ± 0.01</td>
<td>7.11 ± 0.02</td>
<td>2.89 ± 0.08</td>
<td>6.04 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>0.40</td>
<td>0.04 ± 0.02</td>
<td>0.36 ± 0.04</td>
<td>0.45 ± 0.04</td>
<td>10.00 ± 0.03</td>
<td>4.44 ± 0.02</td>
<td>9.18 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>0.80</td>
<td>0.03 ± 0.01</td>
<td>0.38 ± 0.02</td>
<td>0.48 ± 0.02</td>
<td>14.07 ± 0.015</td>
<td>7.10 ± 0.015</td>
<td>14.20 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>1.20</td>
<td>0.03 ± 0.01</td>
<td>0.77 ± 0.03</td>
<td>0.96 ± 0.03</td>
<td>26.55 ± 0.03</td>
<td>10.33 ± 0.02</td>
<td>20.66 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>1.60</td>
<td>0.02 ± 0.01</td>
<td>0.45 ± 0.02</td>
<td>0.56 ± 0.02</td>
<td>19.57 ± 0.02</td>
<td>14.20 ± 0.02</td>
<td>28.40 ± 0.02</td>
</tr>
<tr>
<td>F</td>
<td>0.08</td>
<td>0.14 ± 0.07</td>
<td>0.63 ± 0.01</td>
<td>0.78 ± 0.01</td>
<td>5.68 ± 0.01</td>
<td>7.10 ± 0.015</td>
<td>14.20 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>0.06</td>
<td>0.11 ± 0.04</td>
<td>0.56 ± 0.04</td>
<td>0.70 ± 0.04</td>
<td>5.09 ± 0.04</td>
<td>7.10 ± 0.015</td>
<td>14.20 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>0.08</td>
<td>0.08 ± 0.02</td>
<td>0.50 ± 0.03</td>
<td>0.63 ± 0.03</td>
<td>6.02 ± 0.03</td>
<td>7.10 ± 0.015</td>
<td>14.20 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>0.05 ± 0.02</td>
<td>0.44 ± 0.05</td>
<td>0.55 ± 0.05</td>
<td>8.98 ± 0.05</td>
<td>7.10 ± 0.015</td>
<td>14.20 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>0.12</td>
<td>0.05 ± 0.02</td>
<td>0.53 ± 0.02</td>
<td>0.66 ± 0.02</td>
<td>11.28 ± 0.02</td>
<td>7.10 ± 0.015</td>
<td>14.20 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>0.14</td>
<td>0.03 ± 0.02</td>
<td>0.48 ± 0.02</td>
<td>0.60 ± 0.02</td>
<td>14.12 ± 0.02</td>
<td>10.33 ± 0.02</td>
<td>20.66 ± 0.02</td>
</tr>
</tbody>
</table>
Fig. 6—Lineweaver-Burk plots for the reaction modification of quercetin with L-cysteine by HRP at 20°C [The reaction medium contained 40 ÷ 120 µM quercetin, 0.8 mM L-cysteine, 50 mM sodium phosphate buffer (pH 6.0), 0.8 nM H2O2 and 0.04 ÷ 0.16 mM H2O2]

Fig. 7—Dependance of Michaelis constants in relation to the concentration of substrate [For more explanation of Figure A, B, C, D, E and F see Table 1]

quercetin. This conclusion was in accordance with the published results by Fenoll et al.29, when tyrosinase was present in reaction. This can be written as follows32:

\[
E \rightarrow EA \rightarrow EAB \rightarrow EABC \rightarrow E, P, Q, R \quad \ldots (4)
\]

where A is H2O2, B is quercetin, C is L-cysteine 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:

\[
\frac{V_{\text{max}}}{v_o} = \left( 1 + \frac{K_C}{C} + \frac{K_B K_C}{BC} + \frac{K_A K_B K_C}{ABC} \right). \quad \ldots (5)
\]

In summary, reaction of the modification of quercetin with L-cysteine by HRP was mostly enzymatic with the non-enzymatic reaction making 10% of the total reaction. Further, all the reactions of quercetin modification with L-cysteine followed an ordered kinetic mechanism.

Acknowledgements

This work was supported by the Ministry of Education and Science of the Republic of Serbia under Project No. TR-34012 and OI-172044.

References

10 Procházková D, Boušová I & Wilhelmová N (2011) Fitoterapia 82, 513–523