ORIGINAL ARTICLE

Quercetin Fatty Acid Esters: from Synthesis to the Mushroom Tyrosinase Inhibition

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KEYWORDS
Mushroom tyrosinase; Quercetin; PUFA; Inhibition; Instability

ABSTRACT: New complexes of quercetin esterification with alpha linolenic acid (ALA) and linoleic acid (LA) were applied as inhibitor of tyrosinase as the main melanogenesis enzyme. The most abundant flavonoid compound, quercetin was considered as the base of esterification with poly unsaturated fatty acids (PUFA). The new derivatives including quercetin-ALA (complex I) and quercetin- LA (complex II) were designed and their impacts on mushroom tyrosinase (MT) were assessed by experimental and theoretical studies. The new complexes I and II were induced competitive inhibition on tyrosinase enzyme with Ki of 0.59 and 0.40 mM, respectively. The molecular analysis of docking revealed that the complex II has a better ability to interact with enzyme than the complex I and the nature of interactions was obeyed from hydrophobic manner. So, the esterification of quercetin by above mentioned fatty acids achieved strength inhibitors against tyrosinase and because of their abundant in natural sources and importance in lifestyle, it is proposed to utilize them in medicine, cosmetics, agriculture and food industries. Their other biological properties need more investigations.

INTRODUCTION

Tyrosinase [EC 1.14.18.1] as a hetero-tetramer (H₂L₂) enzyme with molecular weight of 120 kDa and copper containing active site catalysis the first enzymatic reactions of melanogenesis and plays a crucial role in the biosynthesis of melanin pigments from phenolic compounds [1]. Tyrosinase activity and inhibition is a very important aspect in the quality of fruits, vegetables and beverages, and unwanted browns through processing, storage and crop cultivation in food industry [2, 3].

Mushroom tyrosinase from Agaricus bisporus conducted as the subject of many researches particularly designing of new inhibitors for effectual control of these enzymes from kinetics and thermodynamic point of view [4].

So far many tyrosinase inhibitors from the chemical and biological recourses investigated in previous studies. The natural flavonoids, including 5, 7-dihydroxyflavone (chrysin), extracted from plants, honey, and propolis has been inhibiting the enzymatic oxidation of DOPA (3, 4-Dihydroxyphenylacetic acid) [5-7].

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Flavonoids have a lot of scientific attractiveness due to research and evidence in the field of health, they use as antioxidant, anti-inflammatory and antithrombotic, improve lipid profiles, decrease blood pressure and improve endothelial function and prevent cardiovascular disease (CVD) [8-10]. From public health outlook, the use of the most important sources of flavonoids, such as fruits, vegetables and juices among adolescents offers a preventive potential for some diseases at a young age [11]. Linoleic acid (LA), α-linolenic acid (ALA), and their metabolites have been applied as therapeutic agents in patients suffering from variety of skin disorders forms [12,13]. The fatty acids physiological functions in skin have been investigated by analyzing the syndromes that related to essential fatty acid deficiency followed by loss of epidermal barrier function. The administrations of LA to animals with necessary fatty acid-deficiency not only amend the barrier function of their epidermis but also decrease the redness and scaling of the diseased skin [14].

Tyrosinase as a key molecule of adenylyl signaling pathway have been proposed as a potential target of new agents for skin depigmentation and treatment of hyperpigmentation disorder [15]. Thus, pursuing our previous studies on flavonoids [6], aromatic [16] and aliphatic [17] acids on mushroom tyrosinase inhibition, the aim of this comprehensive study was focused on new design complexes of quercetin with ALA and LA on activity, structure and stability of mushroom tyrosinase to understand the mechanisms through which the conjugations of two important natural compounds flavonoids and polyunsaturated fatty acids acts in controlling tyrosinase activity in agriculture, Food stuffs and medicine.

**MATERIALS AND METHODS**

**Materials**

Mushroom Tyrosinase (MT) (specific activity 3600 units/mg; Sigma-Aldrich co), quercetin, polyunsaturated fatty acids, α-Linolenic Acid (ALA), Linoleic Acid (LA), L-DOPA, isopropanol, and 1-Anilino-8-Naphthalene Sulfonat (ANS) were purchased from Sigma- Aldrich Co. Isopropanol was applied to make all stock solutions of substrates and inhibitors in this research. Taking Fisher method, Biological complexes I and II were synthesized by esterification of quercetin a member of flavonoid family with ALA and LA which are PUFAs, respectively (Figure 1).

**Esterification analysis**

Esterification process has done with the Fischer method. Fischer esterification or Fischer–Speier esterification is a distinct type of esterification in which acid catalyst, a carboxylic acid and an alcohol or phenol groups will be refluxed [18,19]. The mechanism for an acid Fischer esterification in this paper, the ester bond shaped amongst the ortho quercetin situation and each one of the polyunsaturated fatty acids (α-Linolenic Acid and Linoleic Acid) that is highlighted in Figure 1A-B.

![Figure 1](image-url)
Experimental studies

Catecholase reactions of MT

Catecholase activity of the enzyme was carried out after MT oxidation reaction of L-DOPA to DOPA-quinone at 475 nm using UV-2100 spectrophotometer (Rayleigh). These reactions were performed with 40 units of MT and in a variety of concentrations of L-DOPA (0.1, 0.5, 1, 1.5, 2 and 2.5 mM) as substrates, whether the inhibitors I and II exist or not (0, 0.01, 0.02 and 0.04 mM).

Enzyme-substrate reaction was done in 25°C, pH 6.8 and 120 sec with the incubation time of 3 min for inhibitors and all the experiments were replicated three times, for at least [20]. The enzyme kinetic parameters including $K_m$, $V_{max}$ and $K_i$ were obtained through Michaelis-Menten and Lineweaver-Burk equations, as the complexes I and II are available.

Regular Secondary structure analysis by CD

The interactions of CD spectra of MT with inhibitors were obtained in a quartz cell which is 0.1 cm long and with the volume of 0.3 ml, using Aviv, Model 215, and U.S.A. spectropolarimeter at the incubation time of 3 min.

Various concentrations of complexes I and II (20, 40 and 60 µM) and 40mM of enzyme were applied for evaluation of regular secondary structures. The recorded CD spectra depicted at far-UV region wavelength (190-260 nm) were considered to obtaining of α-helix, β-sheet, turn and random coil contents of the enzyme by CD deconvolution software [21].

Theoretical analysis

The mushroom tyrosinase crystal structure with 3.25Å was taken from Protein Data Bank (PDB ID: 5M6B). Loosed remainders of crystallographic structure file included of residues 462-469, that were restored applying a software called MODELLER by multi-templates approximate [22]. Then, using the Auto Dock Tool software [23], they were added to tyrosinase, after the designation of the polar hydrogen atoms gasteiger charges [24]. Molecular dynamics simulation with force field CHARMM27 was used to improve the side chain and protein equilibrium along with the correct configuration of atoms [25].

Processing of molecular docking of the ligands to tyrosinase was carried out by Auto Dock Vina software [26]. All the calculations of docking were done, taking the optimization algorithm of repetitive local search due attention to the flexibility of ligand and protein. A grid box of 20x20x14 Å³ points were determined and put in the enzyme active site district. The grid distance was set at 1000Å³ and other parameters were ignored. Next docking, the best conformation with the lowest binding energy was picked as the outcome. Hydrophobic and hydrogen interactions of the mushroom tyrosinase-ligand complex and the length of the hydrogen bonds were analyzed via Lig-Plot software [27].

RESULTS

Complexes I and II esterification and their competitive inhibition on enzyme diphenolase activity

As referred in our previous study FTIR data displayed the shifting of C=O absorption bond from 1709 cm$^{-1}$ to 1739 cm$^{-1}$. These results showed that which functional group of –COOH were converted into –COOC- and the formation of complex I and II was confirmed [19, 26].

After analyzing of Lineweaver-Burk double reciprocal plots and their secondary curves the competitive mode of inhibition was obtained after incubation of enzyme with complex I and II in diphenolase activity using L-DOPA as substrate. Maximum velocity ($V_{max}$) and Michaelis–Menten constant ($K_m$) of the enzyme were calculated 0.0189 mM/min and 1.873 mM, respectively, from the Y and X intercepts of first the line in Figure 2A-B.
Figure 2. Lineweaver-Burk plots of MT diphenolase activity with L-DOPA as substrate in different concentrations of [A] Complex I: 0 μM (♦), 10 μM (■), 20 μM (▲), 30 μM (×); [B] Complex II: 0 μM (♦), 10 μM (■), 20 μM (▲), 30 μM (×), 40 (Ж). Inset: Slopes against inhibitor concentration in secondary plots to obtaining the inhibition constant ($K_i$) from the abscissa-intercepts.
Circular dichroism study of the enzyme secondary structure

As depicted in Figures 3A-B the addition of the two complexes led to significant changes in the secondary structure of MT. The alterations of the enzyme secondary structure contents in the far-UV-CD spectra obey from a concentration dependent manner.

Figure 3. MT Far UV-CD spectra in the presence of different concentrations (0, 20, 40 and 60 µM) of: [A]: Complex I and [B]: Complex II.
As illustrated in Table 1[28, 6]. The amounts of alpha-helix and beta-sheets of sole MT were obtained 63.1 and 9.1%, respectively. The interactions of enzyme with two complexes resulted in decrease in the sum α-helix and β-sheet structures with the coincidence of increasing in the random coil values.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Inhibitor Concentration [µM]</th>
<th>α-Helix [%]</th>
<th>β-Sheet [%]</th>
<th>β-turn [%]</th>
<th>Random.Coil [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT</td>
<td>0</td>
<td>63.1</td>
<td>9.1</td>
<td>15.9</td>
<td>11.9</td>
</tr>
<tr>
<td>Complex I</td>
<td>20</td>
<td>44.6</td>
<td>18.3</td>
<td>18.1</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>42.7</td>
<td>17.6</td>
<td>19.5</td>
<td>21.4</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>39.5</td>
<td>20.9</td>
<td>19.6</td>
<td>20.7</td>
</tr>
<tr>
<td>Complex II</td>
<td>20</td>
<td>52.8</td>
<td>12.4</td>
<td>17.8</td>
<td>16.2</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>45.8</td>
<td>14.2</td>
<td>18.9</td>
<td>21.2</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>38.2</td>
<td>16.6</td>
<td>20.8</td>
<td>24.5</td>
</tr>
</tbody>
</table>

**Molecular Docking**

After docking, Quercetin with ALA and Quercetin with LA was examined using Lig-Plot software. As illustrated in Table 2 [29] the best possible condition of interaction that achieved in Quercetin with n-6(complex II). The affinity of the Quercetin with ALA complex and Quercetin with LA complex was -4.5, -6.5 kcal/mol, respectively (Figure 4).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Complex I</th>
<th>Complex II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Afinity Energy[Kcal/mol]</td>
<td>-4.5</td>
<td>-6.5</td>
</tr>
<tr>
<td>Bond Lenght[A°]</td>
<td>2.92</td>
<td>3.08</td>
</tr>
</tbody>
</table>

**Table 2. Calculated molecular docking parameters**

<table>
<thead>
<tr>
<th>Residues</th>
<th>Complex I</th>
<th>Complex II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ser282, Pro284, Pro277, Gly281, Val283, Phe264, His263, His61, His259, His 244, Asn260, His85, Glu322 and Asn81</td>
<td>Glu322, Val283, Pro281, Ser228, Pro277, Arg268, Gly281, Phe264, His263, His85, His259 and Asn260</td>
</tr>
</tbody>
</table>

**Figure 4.** Best docked conformations of complex I-MT (A) and Complex II-MT (B).
DISCUSSION

Esterification of quercetin with ALA and LA produced the two new complexes I and II. They induced enzyme instability through the reduction of regular secondary structures and competitive inhibition of MT with Ki 0.59 and 0.40 mM, respectively. Increasing of quercetin lipophilicity as one of the main goals of modifications resulted in more biochemical and pharmacological efficacy and higher permeability through cell membranes [30]. Esterification of flavonoids with respective fatty acids may represent a solution to increase the stability of flavonoids in lipophilic media [31]. Because of unwanted auto-oxidation of fatty acids; their conjugation with natural compounds such as flavonoids could be blocking this unfavorable process [32]. However, designing new tyrosinase inhibitors from these natural sources is an important issue in cosmetic and medicinal products to preventing from hyperpigmentation [33].

From the previous studies, the quercetin, LA and ALA alone showed inhibitory effect on MT with Ki 0.74, 0.53 and 0.34mM respectively [6, 34]. The kinetic assay of MT catecholase reaction with new design complexes I and II in Figure 2A-B showed a competition mode of inhibition with Ki 0.59 and 0.40 mM. So, the complex II induced more impact than complex I in enzyme inhibition.

Comparison of the molecular structures of complex I, II with quercetin and ALA, AL with that of L-DOPA reveals that two inhibitors designed is structurally bulkier than both quercetin and ALA, AL and has extra steric hindrance for binding of substrate to the enzyme active site. The esterification of quercetin with PUFAs produced a giant molecule that interacts with the hydrophobic pocket on enzyme active site. These data are in agreement with the crystallography structure analysis of MT proposed a bi copper active site that surrounded with four- helix bundles making a tunnel -like structure for substrate accessibility [30, 35]. Interactions of substrates and inhibitors with MT active site are related to the their coordinations with the cluster of coppers and the hydrophobic pocket that serounded the active site. Also the docking style of these compounds to the MT active site are not expected to be too different excepted the type of substrate interance to the active site pocket. Thus, the enzyme conformational change is related to the substrate interaction with the pocket and its coordination to the copper in active site [17].

In line with above explanations of enzyme-inhibitor interactions and competitive mode of inhibition, the theoretical analysis of the complexes with enzyme active site revealed that they could impact with enzyme by hydrophobic interactions with the binding affinity of -4.5 and -6.5 for I and II complexes, respectively. Thus, the complex II show the higher impact than the other one. The change of CD spectra in Figure 3 and their analysis in Table 1 emphasizes to the significant reduction in the regular structure of α- helix and β-sheet collectively and increasing of the random coil structure and enzyme instability.

CONCLUSIONS

Thus, according to experimental and theoretical data, the complex of the quercetin and LA has more impact on mushroom tyrosinase in compare with esterification of quercetin with ALA. As natural new design compounds, they proposed as a good candidate for unfavorable activities of tyrosinase enzyme in medicine. These natural flavonoids-fatty acids derivatives potentially proposed for utilizing as skin-whitening agents in cosmetic formulations.

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REFERENCES


