https://communications.science.ankara.edu.tr

Commun.Fac.Sci.Univ.Ank.Ser. C Biology
Volume 30, Number 2, Pages 134-147 (2021)
ISSN 1303-6025 E-ISSN 2651-3749
DOI: 10.53447/communc.934706

Received by the Editors: May 08, 2021; Accepted: August 02, 2021

IN SILICO PROOFS FOR PHLORIDZIN, NARINGENIN, AND CINNAMIC ACID AS ALPHA-AMYLASE ACTIVATORS, WHICH IS IMPORTANT IN INDUSTRIAL MICROBIOLOGY OR BIOCHEMICAL ENGINEERING

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ABSTRACT. Enzymes are commonly defined as biological catalysts, regulating particular biochemical reactions. α-Amylase (EC 3.2.1.1) is one of the industrially important enzymes, which are extensively used in starch hydrolyzing processes, such as brewing, fermentation, detergent production, food processing, etc. This enzyme breaks down α-1,4 glycosidic bonds in amylose or amylopectin. The end products from amylose are maltotriose and maltose. Maltose, glucose, and limit dextrin are formed from amylopectin. There are many studies in the literature regarding the α-amylase inhibitors, which have the potentials of being used in diabetes and obesity. However, there is a very limited number of studies in the literature about the activation of this enzyme, which could be harmful to such diseases. This study aims to support the activation activity of phloridzin, naringenin, and cinnamic acid for α-amylase, which was previously proved experimentally, with some *in silico* tests.

1. INTRODUCTION

Enzymes are commonly defined as biological catalysts, regulating particular biochemical reactions. α-Amylase (EC 3.2.1.1) is one of the industrially important enzymes, which are extensively used in starch hydrolyzing processes, such as brewing, fermentation, detergent production, food processing, etc. [1,2]. This enzyme breaks down α-1,4 glycosidic bonds in amylose or amylopectin chains [3]. α-Amylase cleaves long polysaccharide chains. The end products from amylose are maltotriose and maltose. Maltose, glucose, and limit dextrin are formed from amylopectin. [2].

Keyword and phrases. Alpha amylase, activator, phloridzin, naringenin, cinnamic acid, *in silico*

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Several industrially important crops, such as potato, maize, rice, and wheat, contain starch as a storage polysaccharide. The industries based on a large scale of starch processing increased in the last century. As a reason for this, the importance of these crops increased too.

α-Amylase has several applications in the industry. And the bread industry is one of them. In the bread industry, right after the dough was prepared, a fermentation process starts. In this process, the yeasts convert sugar to alcohol and CO_2. This process helps the dough to rise. In the process of preparing the dough, adding α-amylase will hydrolyze the starch present in the wheat flour. This hydrolyzation directly affects the fermentation process. It leads to an improvement in the taste, the volume of bread, the texture of crumbs, toasting qualities, and the crust color [4,5].

α-Amylase is widely used in the production of glucose and fructose syrups, specifically at the liquefaction step to partially hydrolyze starch into short-chain dextrins, so that the viscosity of the starch suspension is reduced [5,6].

Besides, α-amylase is also used in the paper industry. During the paper production process, starch is used to coat paper for increasing strength and smoothness, and to ease writing and erasability. However, the viscosity of the natural starch suspension is considerably high and α-amylase is used to decrease the viscosity of the starch solution to make it suitable for a continuous paper production process [6,7].

Enzymes have been commonly using in detergents for laundry and dishwashing for a long time. Because they can considerably improve the potential of the detergent to remove the stain. The aim of using α-amylase in detergents is to digest food particles containing starch to form smaller oligosaccharides soluble in water [8].

Starch obtained from potatoes or corn is frequently used in ethyl alcohol production and using α-amylase converts starch into fermentable sugars. Adding this enzyme increases the rate of conversion for microorganisms producing their starch degrading enzymes and it is necessary for microorganisms, which are not able to produce any starch decomposing enzyme by themselves, to utilize this carbon source for alcohol production [9]. Since ethyl alcohol is the most commonly used liquid biofuel, α-amylase also has great importance in producing fuel alcohol [10].

There are many studies in the literature regarding the α-amylase inhibitors because they have the potentials of being used in diabetes and obesity. However, there is a very limited number of studies in the literature about the activation of this enzyme,

which could be harmful in diseases such as diabetes and obesity [11]. One of these studies shows that phloridzin, naringenin, and cinnamic acid, which are plant secondary metabolites, are acting as α-amylase activators [12].

Although α-amylase inhibitors are proposed to have great importance in the literature, it is clear that the activation of α-amylase could potentially lead to improvements in several industrial processes.

This study aims to support the activation activity of phloridzin, naringenin, and cinnamic acid for α-amylase, which was previously proved experimentally, with some *in silico* tests.

2. MATERIALS AND METHODS

2.1 Target enzyme preparation

The X-ray crystal structure of human salivary amylase (PDB ID: 1SMD) [13] was downloaded from the Protein Data Bank (http://www.rcsb.org/structure/1SMD). In the structure of amylase, a Cl^- ion and a Ca^{2+} ion were present. The Cl^- ion was interacting with Arg195, Asn298, and Arg337. The Ca^{2+} ion was interacting with Asnl00, Arg158, Asp167, and His201. Therefore, before performing molecular docking, Cl^- ion, Ca^{2+} ion, and water molecules in the structure of the enzyme were deleted from the X-ray crystal structure of the enzyme by Discovery Studio Visualizer v.20.1.0.19295 [14]. The 3D structure of the enzyme was further modified by adding charges and H atoms by AutoDock 4.2 [15].

2.2 Compound preparation

The 3D structures of phloridzin, naringenin, and cinnamic acid were downloaded from PubChem (National Institute of Health). Their structures were protonated at pH 7.4, Gasteiger charges, and 3D coordinates were assigned by Open Babel v.3.1.1 [16,17].

2.3 Prediction and analysis of active sites

The pockets and their amino acid sequences were determined by CASTp v.3.0 [18]. The potential pockets were also visualized by UCSF Chimera v.1.14 software [19]. Potential binding pockets, which are suitable for the interaction between the enzyme and the activators, obtained from CASTp were used in molecular docking analysis.

2.4 Virtual screening by molecular docking

To obtain possible docking conformations and orientations of phloridzin, naringenin, and cinnamic acid, when binding to α-amylase, virtual screening by molecular docking was performed by AutoDock 4.2 [15]. The best binding location and position of the compounds were determined by their binding affinities.

In this process, firstly a virtual screening for compounds was performed by rigid molecular docking with a grid box covering the possible pocket. The central coordinates grid box was set to 8.349, 58.709, and 19.099 with a grid point spacing of 0.450 Å. In performing molecular docking, phloridzin, naringenin, and cinnamic acid kept flexible, while α-amylase was rigid. As a final point, the lowest binding energies of all possible conformations and orientations were chosen for further analysis.

In addition to AutoDock 4.2, the interaction scores of phloridzin, naringenin, and cinnamic acid were determined by iGEMDOCK v.2.1 (Graphical Drug Design System for Docking, Screening, and Post-analysis) [20].

2.5 Visualization

The 2D interactions of the compounds with α-amylase were analyzed by using LigPlot+ v.2.2 software [21]. This software gives better visualization to understand the nature of interactions between the compound and enzyme in the docking, indicating the hydrogen bonds, hydrophobic bonds with the length of bonds.

3. RESULTS

3.1 Target enzyme and compound preparation

Target enzyme and compounds, which were prepared according to the methods given in the previous section, are given in Figure 1.

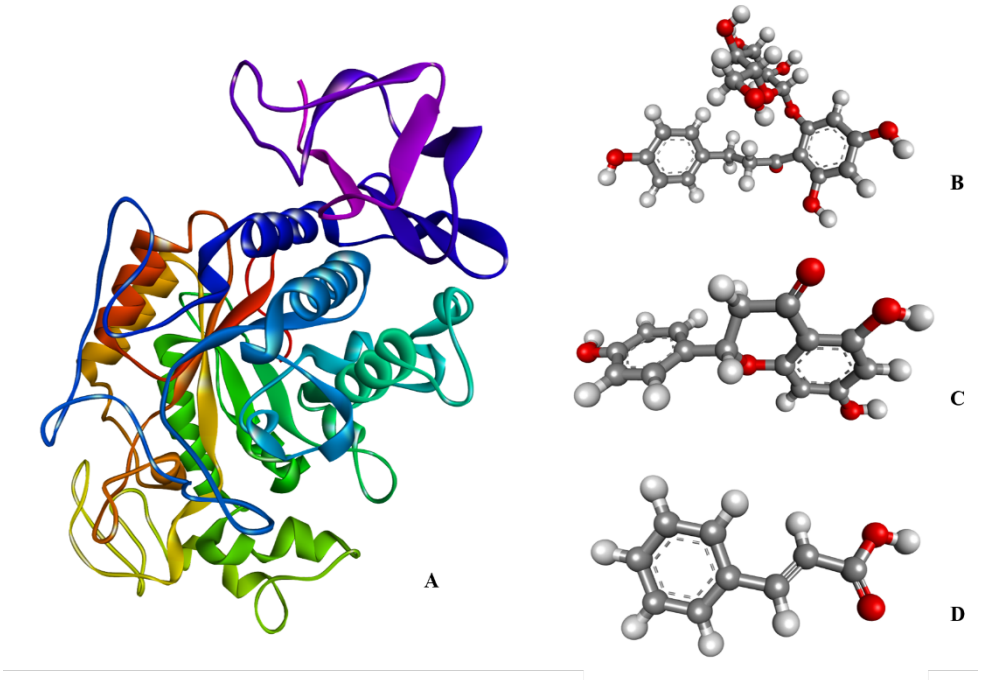

FIGURE 1. 3D Structure of a. α-amylase, b. phloridzin, c. naringenin, d. cinnamic acid.

3.2 Prediction and analysis of active sites

The pockets, where phloridzin, naringenin, and cinnamic acid can bind were predicted by CASTp v.3.0 [18]. According to the results, 86 possible pockets are present in α-amylase. In these pockets, solvent-accessible (SA) areas are observed to be ranging between 159.662 and 0.000 $Å^2$ and solvent-accessible (SA) volumes between 177.930 and 0.000 $Å^3$.

However, seven of them can be accepted as major pockets having volume (SA) higher than 10.000 $Å^3$. The locations of these seven major pockets are shown in Figure 2a and the data regarding these pockets are given in Table 1.

The pockets, where the substrate binds, were visualized by UCSF Chimera v.1.14 software [19] (Figure 2b).

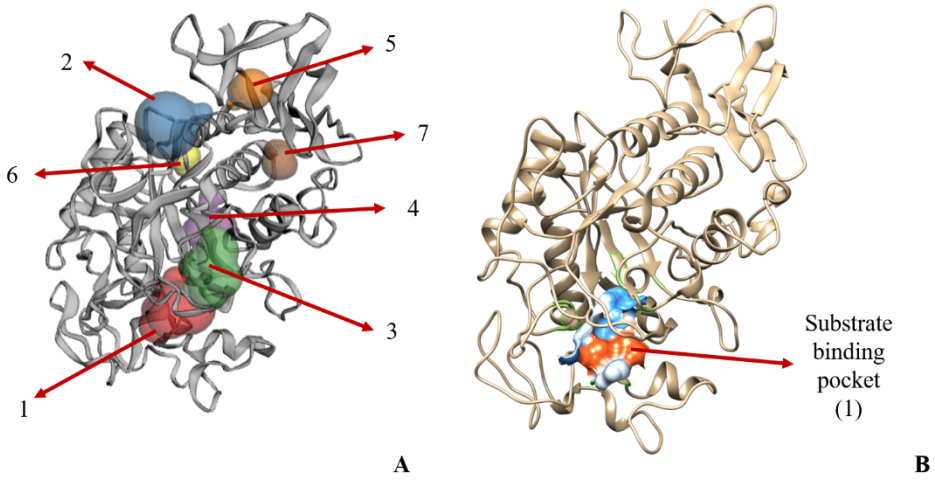

FIGURE 2. a. Major pockets of α-amylase (Numbers are showing pocket IDs), b. Substrate binding pocket.

TABLE 1. Data about major pockets.

ID	MS Volume	SA Volume	Pocket MS Area	Pocket SA Area	# openings	Mouth MS Area	Mouth SA Area
1	457.9	177.9	241.0	159.7	1	130.3	61.2
2	226.9	97.4	111.6	80.0	1	138.7	68.1
3	343.4	93.9	234.8	136.8	1	110.2	43.7
4	220.1	43.2	177.4	89.9	3	112.9	33.1
5	106.2	18.7	91.1	38.5	1	50.2	15.2
6	72.6	12.8	61.7	25.9	1	54.1	14.3
7	81.0	11.2	75.9	26.8	1	32.0	7.9

MS volume - pocket volume based on the molecular surface
SA volume - pocket volume based on the solvent-accessible surface
pocket MS area - pocket molecular surface area
pocket SA area - pocket solvent-accessible surface area
openings - number of mouths, or openings to the external molecular surface
mouth MS area - total area of mouth opening(s) based on the molecular surface
mouth SA area - total area of mouth opening(s) based on the solvent-accessible surface

3.3 Molecular docking analysis

In molecular docking analysis, phloridzin, naringenin, and cinnamic acid were docked in pockets of α-amylase to predict the best possible binding pose of these

compounds with higher binding scoring. The 3D and 2D interactions between phloridzin and α-amylase is given in Figure 3a and 3b respectively.

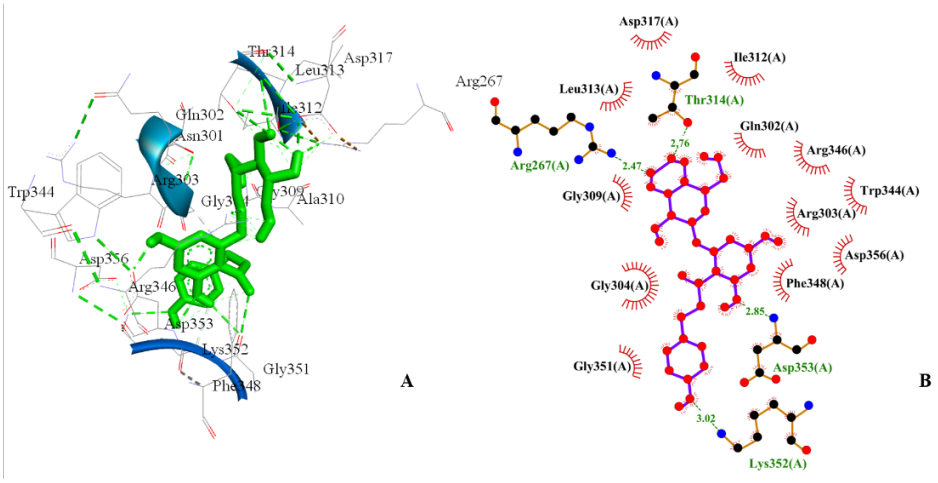

FIGURE 3. The interaction between phloridzin and α-amylase a. 3D, b. 2D.

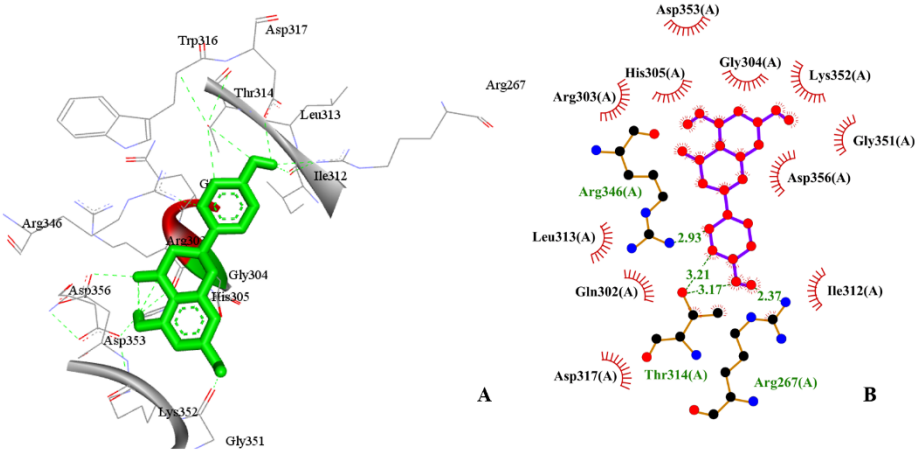

FIGURE 4. The interaction between naringenin and α-amylase a. 3D, b. 2D.

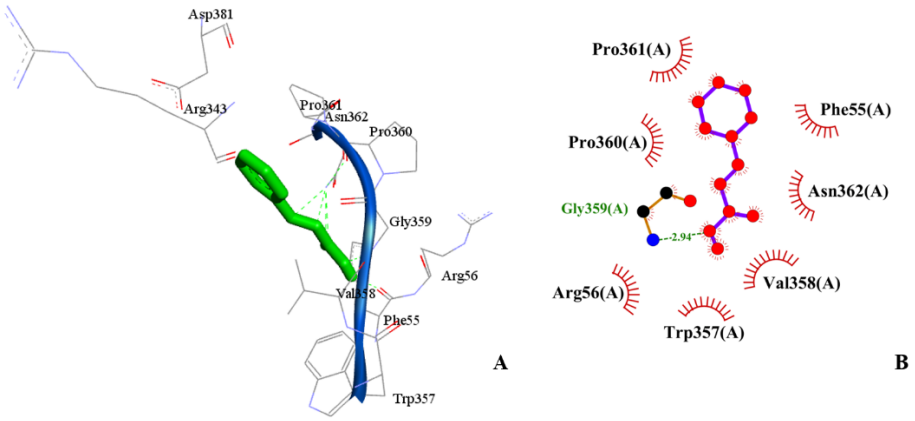

FIGURE 5. The interaction between cinnamic acid and α-amylase a. 3D, b. 2D.

The interactions between naringenin and cinnamic acid with α-amylase are given in Figure 4 and Figure 5 respectively.

The best docking possibilities binding to the major pockets are calculated and ten lowest binding energies for the compounds are given in Table 2.

The molecular docking results are mainly based on the binding energies of activators to α-amylase for all possible interactions. The lowest binding energy of phloridzin was found as -10.90 kcal/mol. This value was -9.61 kcal/mol for naringenin and -6.26 kcal/mol for cinnamic acid. Phloridzin showed slightly less binding energy, which means better binding affinity than naringenin and cinnamic acid (Table 2).

The post-analysis was performed for phloridzin, naringenin, and cinnamic acid by iGEMDOCK. Tested compounds were ranked by using both scores based on energies and pharmacological interactions. If a negative value for binding energy was observed, it means this interaction will be spontaneous. In addition, if this negative value is higher, the chance of being accepted as a drug candidate will be higher too [22]. The lowest binding energies in an enzyme-compound interaction mean the compound is fitting to the target enzyme.

TABLE 2. Ten lowest binding energies of phloridzin, naringenin, and cinnamic acid (kcal/mol) to the major pockets according to AutoDock.

	Phloridzin		Naringenin		Cinnamic Acid	
	Location	Binding Energy	Location	Binding Energy	Location	Binding Energy
1	10.832816 49.702500 34.319368	10.90	10.014391 50.022522 33.974087	9.61	-6.508833 51.789833 32.205000	6.26
2	-8.484868 49.323789 1.942500	10.78	10.109304 49.859130 34.176913	9.46	8.428250 48.213917 34.076167	6.17
3	10.948658 49.839184 35.124105	10.22	10.049913 49.873478 34.189913	9.45	8.504333 48.417750 34.221750	6.15
4	13.770711 70.880921 8.066816	9.43	10.026652 49.791565 34.320826	9.42	8.331667 48.276250 34.041750	6.12
5	12.006921 51.628395 35.093316	9.29	8.375673 58.626996 19.156482	9.41	8.542833 48.553250 34.300250	6.12
6	5.442263 44.231526 21.051895	8.78	10.034609 49.802565 34.336565	9.40	8.365744 58.647360 19.114551	6.10
7	-14.476605 51.435658 20.360789	8.72	10.019870 49.771478 34.356087	9.39	18.432917 68.508750 12.722417	5.95
8	15.198237 67.743842 8.683421	8.67	10.279739 49.822043 33.458652	9.27	18.444417 68.570167 12.626167	5.93
9	11.109500 54.599237 -2.566579	8.65	10.555043 49.672130 33.804870	9.04	3.142667 44.169167 20.271250	5.64
10	9.845132 44.627816 19.466105	8.51	3.795130 43.357913 21.198739	9.02	4.586167 45.664917 20.692333	5.52

Among the screened compounds, phloridzin (-103.02 kcal/mol) has the lowest binding energy and cinnamic acid (-58.29 kcal/mol) has the highest binding energy (Table 3).

TABLE 3. Binding energies of phloridzin, naringenin, and cinnamic acid according to AutoDock and iGEMDOCK.

		Phloridzin	Naringenin	Cinnamic Acid
AutoDock	Estimated Free Energy of Binding (kcal/mol)*	-10.90	-9.61	-6.26
	Final Intermolecular Energy (kcal/mol)	-15.07	-10.80	-7.15
	vdW + Hbond + desolv Energy (kcal/mol)	-15.07	-10.80	-7.15
	Electrostatic Energy (kcal/mol)	0.00	0.00	0.00
	Final Total Internal Energy (kcal/mol)	-4.88	-1.05	-0.25
	Torsional Free Energy (kcal/mol)	+4.18	+1.19	+0.89
	Unbound System's Energy (kcal/mol)	-4.88	-1.05	-0.25
	Estimated Inhibition Constant (nM)	10.31	90.90	25.90
iGEMDOCK	Total Energy (kcal/mol)	-103.02	-96.14	-58.29
	vdW (kcal/mol)	-78.78	-75.43	-54.79
	H-bond (kcal/mol)	-24.24	-20.71	-3.5
	Electrostatic Energy (kcal/mol)	0.00	0.00	0.00

* Estimated Free Energy of Binding = Final Intermolecular Energy + Final Total Internal Energy + Torsional Free Energy-Unbound System's Energy
- nM: nanomolar, μM: micromolar
- vdW: Van der Waals, H-bond: Hydrogen Bond

The lowest binding energy of phloridzin means phloridzin can bind easier to the enzyme than cinnamic acid. This can also be explained by comparing Figures 3 and 5. Figure 5 shows that cinnamic acid binds to the location (-6.508833, 51.789833, 32.205000) by only one hydrogen bond with **Gly 359**, but phloridzin binds to the location (10.832816, 49.702500, 34.319368) by four hydrogen bonds with **Arg 267**, **Thr314**, **Lys352** and **Asp353**. Table 3 also shows that the hydrogen bonding energy for cinnamic acid was -3.5 kcal/mol, where this value for phloridzin was -24.24 kcal/mol. In addition, the energy for Van der Waals interactions was lower in the binding of phloridzin to the enzyme. These data clearly present that phloridzin can bind to the enzyme easier than cinnamic acid.

4. DISCUSSION

In this current study the activation activities of phloridzin, naringenin, and cinnamic acid for α-amylase, which were previously proved experimentally, with some *in silico* tests.

According to the current literature, there are very few studies regarding the effect of phloridzin, naringenin, and cinnamic acid on α-amylase.

Menshaz and Altuner [12] previously proved that phloridzin, naringenin, and cinnamic acid are acting as activators for α-amylase.

In addition, Yusoff et al. [23] observed that phloridzin significantly reduced glucose absorption rate in rats.

Previous studies also proved that naringenin increases the α-amylase activity [24]. On contrary, some studies proposed naringenin as an α-amylase inhibitor [25,26]. As can be seen, sometimes consistent and sometimes inconsistent results were found in the literature. There are too many parameters in enzyme activity studies that can affect these inconsistencies. A detailed analysis should be done to understand where these inconsistencies arise.

Whether these compounds inhibit or activate α-amylase, *in silico* proofs in this recent study clearly show that they can bind to the enzyme to the locations rather than the substrate binding active site.

Figure 3 shows the interaction between phloridzin and α-amylase. The amino acid residues taking the role in this interaction are **Arg267**, Gln302, Arg303, Gly304, Gly309, Ile312, Leu313, **The314**, Leu317, Trp344, Arg346, Phe348, Gly351, **Lys352**, **Asp353**, and Asp356, where the amino acid residues written in bold are involving in active interaction with phloridzin.

Figure 4 shows the interaction between naringenin and α-amylase. The amino acid residues taking the role in this interaction are Phe55, **Arg56**, Trp357, Val358, Gly359, Pro360, Pro361, and Asn362, where the amino acid residue written in bold is involving in active interaction with naringenin.

Figure 5 shows the interaction between cinnamic acid and α-amylase. The amino acid residues taking the role in this interaction are **Arg267**, Gly302, Arg303, Gly304, His305, Ile312, Ile313, **The314**, Asp317, **Arg346**, Gly351, Lys352, Asp353, and Asp356, where the amino acid residues written in bold are involving in active interaction with naringenin.

Qian et al. [27] proposed that His101, His201, His299, and His305 are some of the important amino acid residues forming hydrogen bonds with inhibitors. The analysis showed that cinnamic acid interacts with His305 amino acid residue. This probably

causes a change in the active site of the enzyme. Thus, the substrate can bind effectively.

The results showed that phloridzin and cinnamic acid can bind to similar locations, where both of the compounds interact with **Arg267** and **The314** amino acid residues. The interaction location of naringenin is very close both to phloridzin and cinnamic acid, but not with the same amino acid residues.

As a result, it can be proposed that although all compounds activate the enzyme, the mechanisms of action of activation are different from each other.

Declaration of Competing Interests: The author declares no conflict of interest.

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