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Aspirin in retrieving the inactivated catalase to active form: Displacement of one inhibitor with a protective agent

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Abstract

Aspirin as a potential drug is able to bind to different targets and also could affect on the binding process of other ligands. In the present work, aspirin was considered as a protective agent to retrieve the inactivated catalase by farnesiferol C (FC) through displacement manner. The catalytic assessment revealed that aspirin is able to remarkably retrieve the activity of FC-catalase from 4.2±0.2% to 98±0.1% compare to the control sample. Furthermore, displacement study and CD spectroscopy indicated that aspirin could reduce the stability of FC-catalase complex. Based on the obtained data, it is shown that the binding of aspirin to catalase led to decrease the affinity of catalase to the inhibitor. The releasing analysis of FC from the complex showed that the dissociation constant (K_d) of FC-catalase was increased, considerably from 8.9±0.2 μM to 256±01 μM in the presence of aspirin at 298 K. Also, molecular simulation proved the instability of FC-catalase following the binding of aspirin to the complex.

Key words: Aspirin, catalase, inhibitor, molecular simulation

1. Introduction

Aspirin (Acetylsalicylic acid, $C_9H_80_4$) is a well-known drug that is used commonly for treatment of pains, aches and fever. It is reported that aspirin has anti-inflammatory properties and can be considered as a blood thinner. It was shown that this drug is able to interact with various proteins such as transferrin, albumin and immunoglobulin based on the acetylation process [1, 2]. Aspirin could acetylate the serine residues in different targets such as cyclooxygenases, which is related to the reduction of prostaglandin synthesis [3-5]. Furthermore, it is revealed that aspirin has capability to affect the binding process of other drugs or compounds on targets. Previous reports indicated that aspirin is a protective agent for catalytic function of catalase [6]. Also, it is referred that this drug is able to prevent the non- enzymatic glycation of catalase [6, 7].

Catalase (EC 1.11.1.6) as a main H_2O_2 scavenger, neutralizes hydrogen peroxide into the molecules of water and oxygen. This enzyme is an essential antioxidant enzyme that controls the level of oxidative stress in human body [8, 9]. It is previously verified that oxidative stress is a main cause of cellular damage in different chronic diseases such as cancers and diabetics [10-12]. Hydrogen peroxide as main reactive oxygen species has crucial roles in the regulation of various biological processes (as a signaling factor) [4, 13, 14]. It was shown that the concentration of hydrogen peroxide is essential to initiate some specific biological responses. For example, the higher concentrations (20-fold) of hydrogen peroxide is required to induce apoptotic process in mammalian cells [15-17]. Also, higher concentrations of hydrogen peroxide are associated with different macromolecule damages such as destruction of DNA or proteins [18]. These evidences show the importance of catalase in regulation of the level of H_2O_2 in cells by sensing different regulators.

Bovine liver catalase (BLC), as a model for assessment of human catalase, has been considered in many researches. Based on BLAST research, it is confirmed that human catalase (PDB code 1F4J) and bovine liver catalase (PDB code 1TGU) have approximately similar structure (91% homological identity and 100% similarity in the catalytic sites) [19]. In the present study, aspirin as a protective agent for catalytic function of catalase was used to retrieve the inactivated catalase by farnesiferol C (FC), as previously reported [20].

This study was aimed to evaluate the retrieving effects of aspirin on the inactivated catalase to active form by different experimental and simulation methods. Further, the replacement mechanism of the inhibitor and aspirin as a protective agent was recognized in the present work.

2. Materials and Methods

Bovine liver catalase (~250 kDa) and dialysis membrane with molecular weight cut off at 3500 Da were provided from Sigma Aldrich, USA. Sodium phosphate salts (Na_2HPO_4/ NaH_2PO_4), aspirin, H_2O_2 (30%) and Amicon® Filter (0.1-1.5 µm pore size) were purchased from Merck Company, Germany. Farnesiferol C (FC) was purified from *Ferula szowitsiana* as reported previously [20]. UV–visible spectrophotometer (T-60, PG Instruments LTD., Leicestershire, UK) was used to determine the concentration of H_2O_2 at 240 nm by using extinction coefficient (ε_{240}, 43.6 $M^{-1}cm^{-1}$) [21]. FC-catalase concentration was calculated spectrophotometrically by using ε_{405}, of 3.24×10^5 $M^{-1}\,S^{-1}$ for catalase tetramer [22].

2.1 Catalase assay

The catalase activity was assessed by the decomposition of hydrogen peroxide (H_2O_2) via spectroscopic method at 240 nm (A_{240}) based on Beers and Sizer method [23]. The initial

catalase velocity (V_i) was obtained from the reduction slope of H_2O_2 to recognize the activity of catalase. All assays were performed in 50 mM sodium phosphate buffer, pH 7.0, in the considered time period (60 s) at 298 K. The optimal concentration of the substrate (H_2O_2) was 30 mM for all assays [24].

2.2 The reversibility rate of FC-catalase complex

First of all, in order to identify the effect of aspirin on the catalytic function of inactivated catalase by farnesiferol C (FC), a fixed concentration (5 nM) of the enzyme was incubated in the presence different concentrations of FC (0-10 μM) for 5 minutes in 50 mM sodium phosphate buffer, pH 7.0, at 298 K. Then, maximum concentration of FC (10 μM) that is required to completely inactivate the catalytic function of catalase was chosen for further studies. The provided samples were filtered with Amicon® Filter (0.1-1.5 μm pore size) to separate the created FC-catalase complexes from the free concentration of FC in the solution. Thereafter, the purified FC-catalase solution was incubated in the dark and shaking condition (20 rpm) for 30 minutes at 298 K, in order to assess the reversibility or separation of FC from the enzyme. To analyze the effects of aspirin on the reversibility rate of FC from the complex, different concentrations of aspirin (0-20 μM) were added to the FC-catalase solution and then incubated under the same dark and shaking condition (20 rpm) for 30 minutes. Following that, the catalytic function of the FC-catalase solutions was monitored each 5 minutes, orderly after addition of different concentrations of aspirin (0-20 μM). Also, the effect of aspirin on the activity of catalase was measured as a control sample.

2.3 Inhibitor- aspirin displacement study

In order to assess the effect of aspirin on the separation of FC from the complex, 10 μM of free catalase was incubated in the presence of a fix concentration of FC (100 μM) in 50 mM sodium phosphate buffer, pH 7.0, at 298 K. After giving the appropriate incubation time (20 minutes) for maximum binding of FC to the enzyme, the mixture sample was filtered to ensure about the separation of free concentration of FC from the mixture. Also, the concentration of unbound FC to the enzyme was measured via UV-spectroscopy method based on Beer-Lambert law. Then, 2 ml of the supplied FC-catalase/catalase solution was inserted into each of 6 provided test tubes. These samples were then put in an equilibrium dialysis system for 30 minutes and the releasing rate of FC was monitored every 5 minutes by a UV-visible spectrophotometer. To find out the effect of aspirin on the releasing rate of FC from the complex, the fresh FC-catalase/catalase solution was provided by the same method. Subsequently, various concentrations of aspirin were added into 5 out of 6 test tubes. The sixth test tube was considered as a blank sample that contained only FC-catalase/catalase solution. The final ratio between FC-catalase/catalase and aspirin was 1:1, 1: 2, 1: 3, 1: 4 and 1:5. Then, the samples were put in the equilibrium dialysis system for 30 minutes and the free concentration of FC was measured by a UV-visible spectrometer.

2.4 Circular dichroism (CD) measurements

In order to identify the effect of aspirin on the secondary structure of FC-catalase complex (3 μM), the CD spectra of the complex were recorded by a J-810 spectropolarimeter (Jasco Co., Tokyo, Japan) at the far-UV regions from 200 to 260 nm [25]. To this purpose, 4 μM of free catalase was incubated with a fix concentration of FC (40 μM) in 50 mM sodium phosphate buffer, pH 7.0, at 298 K for 10 minutes. Then, the mixed solution was filtered for elimination of

the free FC from the solution. Following that, different concentrations of aspirin (10, 20 and 30 μM) were added to the FC- catalase solution, gradually and then incubated for 10 minutes again. Also, the CD spectra of the free enzyme and FC-catalase were monitored as control samples. CDNN program was implemented to estimate the percentage of the secondary structure elements [26].

2.5 Molecular simulation

In order to simulate the interaction process of aspirin with the complex of FC-catalase, the x-ray structure of bovine liver catalase with PDB code: 1TGU was obtained as a model from the RCSB website (http://www.rcsb.org). The 3D molecular structure of FC and aspirin were drawn and optimized by Gaussian 09 package. Furthermore, the complex of FC-catalase was created by Autodock tools as previously reported. The FC-catalase complex was considered as a target for further investigations. Then, the docking simulation of aspirin and FC-catalase complex was done by AutoDock v4.2 software. The docking parameters of this study were completely same according to our previous studies [20]. Lamarckian genetic algorithm was implemented to run docking simulation. Thereafter, the obtained .dlg file was used to create aspirin-FC-catalase complex for the next molecular dynamic simulation (MD). The MD simulation was done by Amber 12 program as described previously. Chimera 1.10.1 program was used for visualization of the simulation results [27].

2.6 Statistical analyses

Data were collected from three independent experiments (at least), and analyzed with one-way ANOVA by SPSS 16 software (SPSS INC, Chicago, IL, USA). Also, error bars were revealed by standard deviation (means ± SD (n=3)).

3. Results and Discussion

3.1 Catalytic function of the inhibited catalase in the presence of aspirin

As previously reported, FC is a well inhibitor for catalase that is able to significantly decrease the activity of catalase via mixed manner, while aspirin did not have any significant effect on the catalytic function of catalase. To investigate the potential reversibility effects of aspirin on the inactivated catalase by FC, catalase (5 nM) was incubated in the presence of different concentrations of FC (0-10 μM) for 5 minutes to find the appropriate concentration of FC which approximately inactivate catalase (5 nM). In the presence of FC, as an inhibitor in catalase solution, only free catalase is catalytically active; however, because catalase is an oligomeric enzyme, there is basically more than one "on" and "off" state in this enzyme. Catalase with a tetrameric structure is able to be in one of five various catalytic states based on the number of FC molecules that are bound to the enzyme. Therefore, the saturation of all catalytic active sites of catalase with FC is associated with the completely inactivation of the enzyme. Thus, higher concentration of FC (10 μM) was used to approximately inactivate all catalytic sites of catalase (Fig. 1). In the next step, the stability of the complexes was measured by maintaining the complex solution under the dark and shaking condition (20 rpm) for 30 minutes. Then, the activity of catalase was measured, orderly every 5 minutes to check the reversibility of the catalytic function. As shown in Fig. 2, the activity of the inhibited catalase

was not reversed considerably within the desired time frame, which indicates that FC-catalase is a stable complex. However, by addition of different concentrations of aspirin (0-20 μM) to the FC- catalase solution in the same condition, the catalytic function of catalase retrieved approximately 95%, compared with the activity of free catalase (100%) (Fig. 2). These findings revealed that aspirin is able to accelerate the separation process of FC from the complex, which leads to the activation of catalase.

3.2 Displacement study

To understand the effect of aspirin on the releasing process of FC from the complex, the FC-catalase/catalase solution was assessed in the presence of different concentrations of aspirin according to the equilibrium dialysis method. It was shown that without addition of aspirin, the free concentration of FC was negligible in the mix solution, confirming the results of catalytic assessment (Table 1). However, as listed in Table 2, the releasing of FC from the FC-catalase/catalase (10 μM) was significantly increased from 3.7±0.01% to 73±0.03% with raising the concentration of aspirin, while the concentration of free aspirin (50 μM) was remarkably reduced from 100±0.01% to 36.0±0.03%. It is suggested that the increment of the concentration of aspirin is able to reduce the stability of FC-catalase complex and even decrease the affinity of the enzyme to FC. Furthermore, the dissociation constant (K_d) of FC was analyzed in the absence or presence of aspirin through equilibrium dialysis method by using Scatchard plot. Considering to the interaction of FC with catalase, the dissociation constant can be written as follows:

$$\text{Catalase} + \text{FC} \rightleftharpoons \text{FC-catalase}$$

$$K_d = \frac{[E][L]}{EL} = \frac{([E_t]-[EL])[L]}{[EL]}$$

where [E], [L] and [EL] are the concentrations of the free enzyme, free FC and FC-catalase/catalase, respectively, and $[E]_t$ is the total catalase concentration.

Equilibrium dialysis method is a cost-efficient technique for assessment of the reversibility of inhibitor/enzyme binding process. The K_d values of FC are calculated to be 256±01 μM and 8.9±0.2 μM in the presence or absence of aspirin, respectively (Fig. 3 and 4). The smaller value of the dissociation constant refers to the more tightly bound of FC, or the higher affinity between catalase and FC. As shown in Figs. 3 and 4, the dissociation constant of FC was increased approximately 28 times in the presence of aspirin compared with aspirin free condition. These results revealed that the separation rate of FC from the complex was increased remarkably by raising the additional concentrations of aspirin to the mixture. It means that aspirin is able to enhance the reversibility rate of FC by raising the instability of FC-catalase complexes.

3.4 CD spectra

To recognize the effect of aspirin on the secondary structure of FC-catalase, the CD spectra of FC-catalase solution (3 μM) was monitored at far-UV regions (200- 260 nm) in 50 mM phosphate buffer, pH 7.0, at 298 K as a control sample. Then, the FC-catalase solution was incubated with different concentrations of aspirin (10, 20 and 30 μM) for 10 minutes. It is reported that FC is able to significantly change the secondary structure of catalase, especially α-helix and β-sheet elements [20]. It is shown that by raising the concentration of aspirin (10, 20 and 30 μM) to the FC-catalase mixture, the secondary structural changes of FC-catalase was limited (Table 3) (Fig.5). These results revealed that aspirin could return the conformational changes of the complex to the close of the free catalase.

3.5 Molecular docking simulation

Based on our previous report, to simulate the effect of aspirin on the stability of FC-catalase complex, MD simulation was done by Amber 12 suits [20]. The results revealed that after binding of aspirin to FC-catalase complex, the stability of the complex was considerably reduced, which provides an appropriate condition for releasing of FC from the complex (Figs. 6 and 7). It was also shown that by binding of aspirin to the complex, the stability of FC-catalase was reduced, that is associated with breaking the complex down. As previously reported, FC has interaction with the following residues: Met 338, Glu 343, Asn 337 and Phe 339, in a cavity between β-sheet and wrapping domains through hydrophobic forces [20]. MD simulation results revealed that after binding aspirin into the FC-catalase complex (a cavity between helical domain and β-barrel), the distance between the aromatic ring of FC and Phe 339 increased significantly in the FC-catalase complex (Fig.8). The aromatic ring of FC is one of the main hydrophobic center in this molecule that contains different pharmacological and biological properties [28]. Phe 339 is also one of the important residues in the interaction between FC and catalase. This residue plays a main role in the formation of hydrophobic network in the binding process [20]. Therefore, by increasing the distance between the aromatic ring of FC and Phe 339, the hydrophobic network between FC and catalase was broken down. Consequently, FC molecule would be released by binding of aspirin to the complex. These results verify our experimental data.

4. Conclusion

Many studies show that the activity of catalase is essential for controlling the level of oxidative stress in different diseases such as diabetic [29-31]. Low catalase activity is shown in

different chronic diseases such as diabetes mellitus, hypertension and vitiligo [32]. Acatalasemia (<10% of normal catalase activity) as the inherited catalase deficiency is a good example to understand the importance of catalase fully activity in the cells [33]. Despite different problems of acatalasemia for the patients with oral gangrene, altered lipid and homocysteine metabolism, it is strongly associated with the increment of diabetes mellitus risk [33]. Considering the association of catalase inactivation with different diseases, identification of different catalase protective molecules is an essential subject to preserve catalase activity in the cells. Catalase as a potential target in liver can be affected by the binding of various exogenous compounds such as FC. Coumarins, especially FC have significant hepatotoxic effects [34]. As previously reported, FC could remarkably inhibit the catalytic function of catalase (<10% of normal activity) though the changing of conformation of the heme site [20]. Therefore, protection of this enzyme from the toxic compounds such as FC is very important issue in the clinical studies. In the present work, the potential of aspirin in retrieving the inactivated catalase to active form was confirmed. It is shown that aspirin could significantly retrieve the catalytic function of FC-catalase complex compared to the control samples. Furthermore, it is verified that the dissociation constants of FC-catalase were increased by binding of aspirin to the complex. CD spectra indicated that the stability of FC-catalase complex was reduced by binding of aspirin. These results were also supported by the results of molecular simulation study.

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Tables

Table 1 The displacement rate of FC in the FC-catalase/catalase (10 μM) solution during 30 minute (The obtained data are expressed as mean ±SD of replicates, Ob. No. = Observation number, conc. = Concentration).

Ob. No.	Time (minutes)	Free conc. of FC ($\times 10^{-6}$ M)	% Displacement of FC
1	5	1.4±0.01	1.4±0.01
2	10	1.8±0.04	1.8±0.03
3	15	2.6±0.01	2.6±0.01
4	20	3.8±0.015	3.8±0.05
5	25	4.9±0.02	4.9±0.02
6	30	5.6±0.03	5.6±0.03

Table 2. Effect of aspirin on the separation rate of FC from the FC-catalase complex after 30 minutes incubation (The obtained data are expressed as mean ±SD of replicates, Ob. No. = Observation number, conc. = Concentration).

Ob. No.	Added aspirin conc. ($\times 10^{-6}$ M)	[aspirin] / [FC-catalase/catalase]	Free conc. of FC ($\times 10^{-6}$ M)	% Displacement of FC
1	0	0	3.7±0.01	3.7±0.01
2	10	1	28±0.04	28±0.03
3	20	2	46±0.01	46±0.01
4	30	3	58±0.015	58±0.05
5	40	4	64±0.02	64±0.02
6	50	5	73±0.03	73±0.03

Table 3. The percentage of secondary structural elements in FC-catalase in the absence and presence of aspirin at 298 K.

Samples	α-helix (%)	β-sheet (%)	β-turns (%)	random coil (%)
Catalase (3 μM)	27.1	21.5	17.9	33.7
FC-catalase (3 μM)	32.3	17.6	19.1	31.0
FC-catalase + aspirin (10 μM)	31.4	18.2	19.1	31.3
FC-catalase + aspirin (20 μM)	30.7	18.5	18.5	32.3
FC-catalase + aspirin (30 μM)	29.3	19.4	18.6	32.9

Figure captions

Figure 1. The relative activity of catalase in the presence of different concentrations of FC (0-10 μM) in 50 mM sodium phosphate, pH 7.0, at 298K.

Figure 2. Effects different concentrations of aspirin (0-20 μM) on the catalytic function of the inactivated catalase by FC during 30 minutes in 50 mM sodium phosphate, pH 7.0, at 298 K.

Figure 3. Scatchard plot of the releasing rate of FC from the complex in the absence of aspirin over 30 minutes.

Figure 4. Scatchard plot of the releasing rate of FC from the complex in the presence of different concentrations of aspirin after 30 minutes incubation.

Figure 5. CD spectra of FC-catalase (4 μM) by a gradual addition of aspirin (a-d: 0, 10, 20 and 30 μM) after 10 minutes incubation in 50 mM sodium phosphate, pH 7.0, at 298 K.

Figure 6. The binding site of aspirin to FC-catalase.

Figure 7. The structural stability of FC-catalase during MD simulation in presence of aspirin.

Figure 8. 3D representation of the changes in FC-catalase before and after binding of aspirin. The distance between the aromatic ring of FC and Phe 339 during the MD simulation.

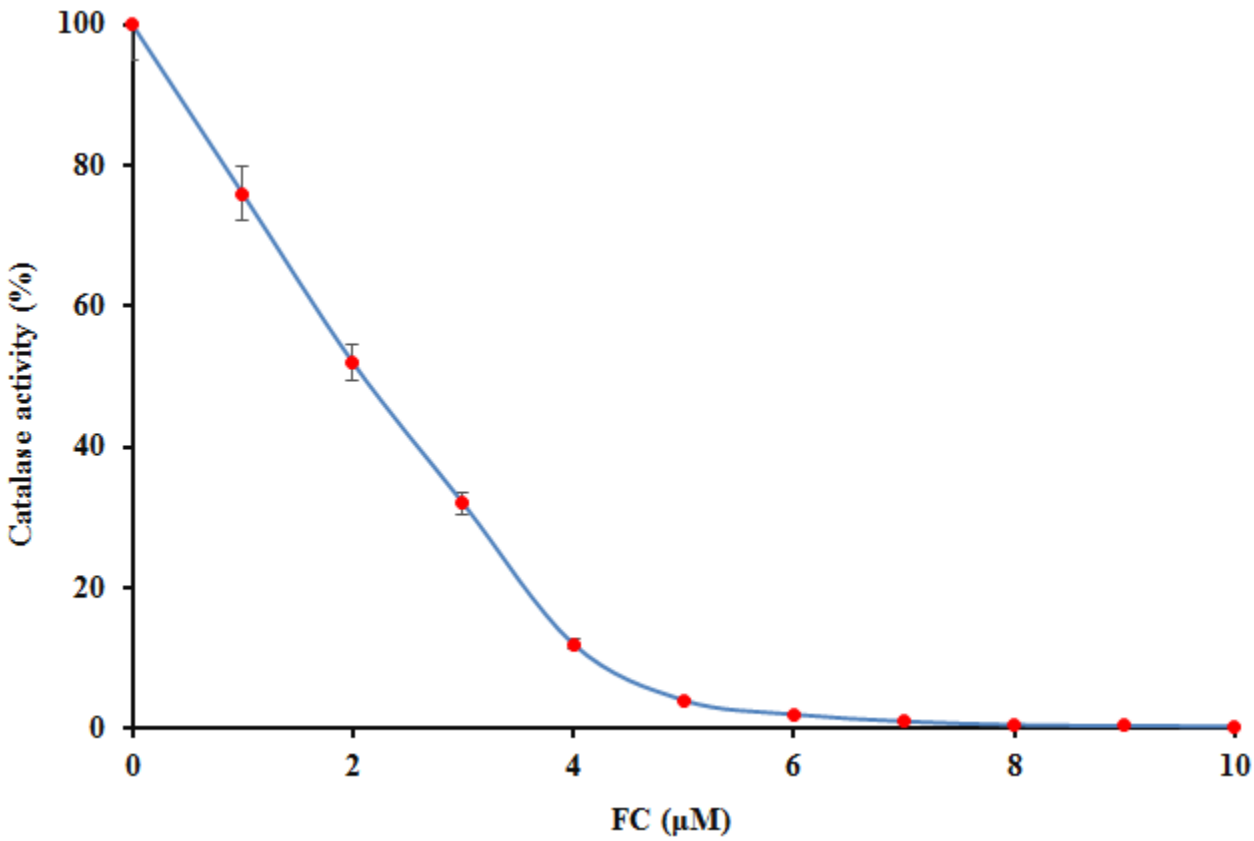


Figure 1

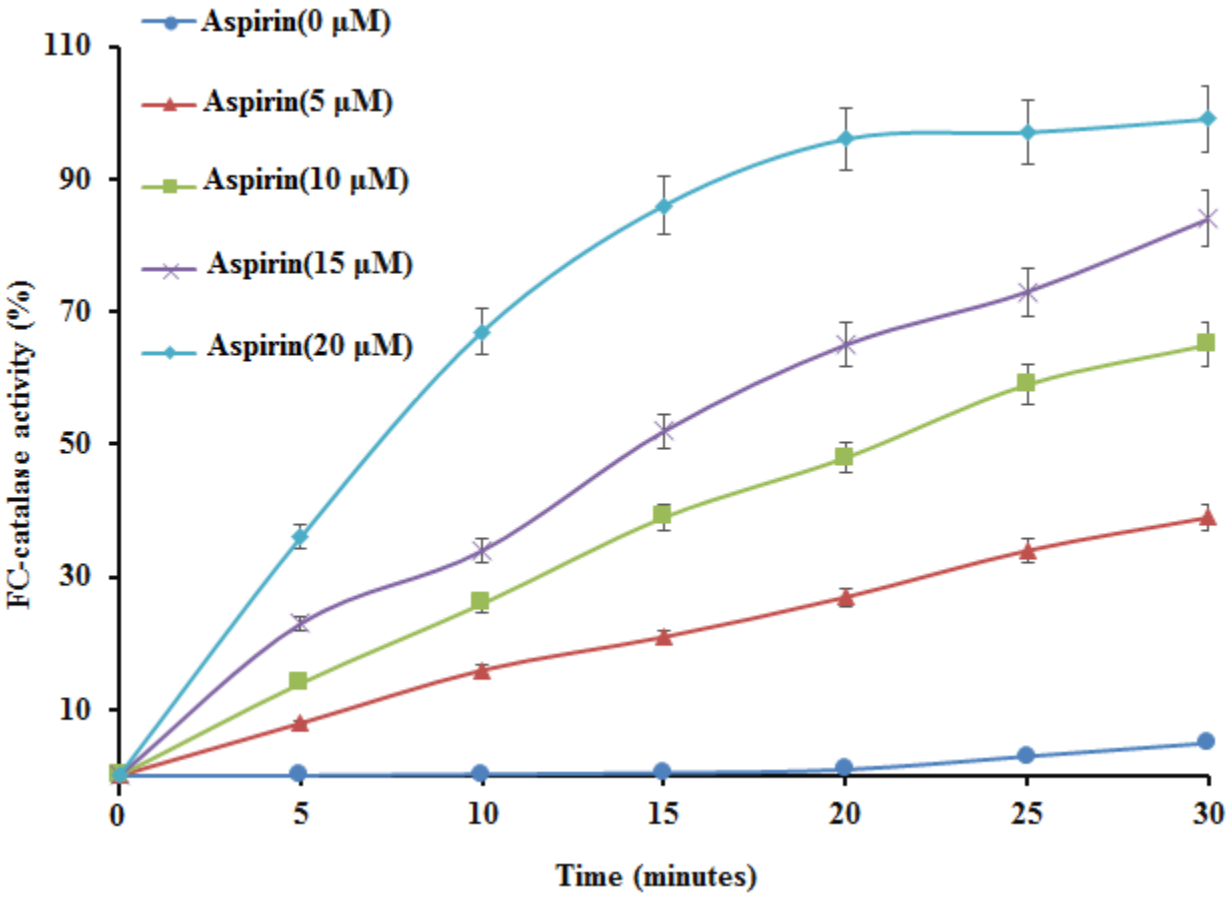
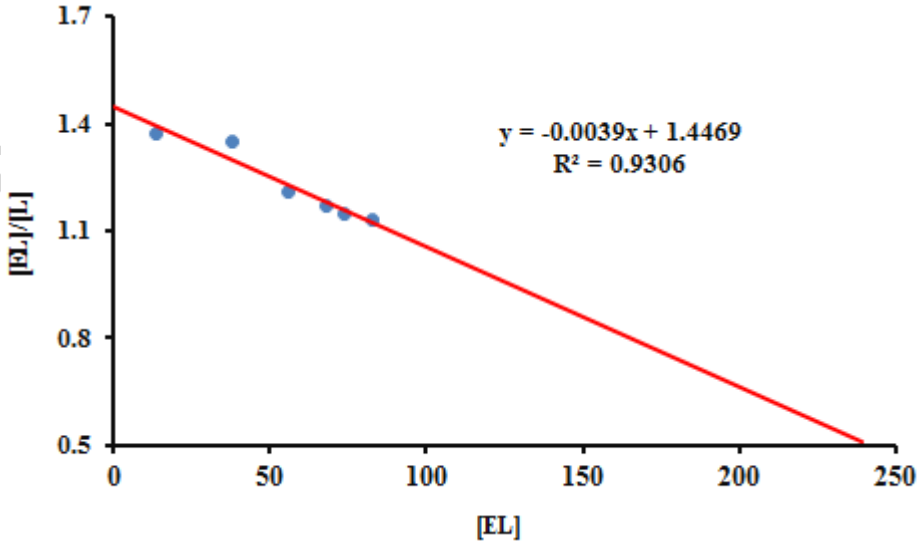


Figure 2



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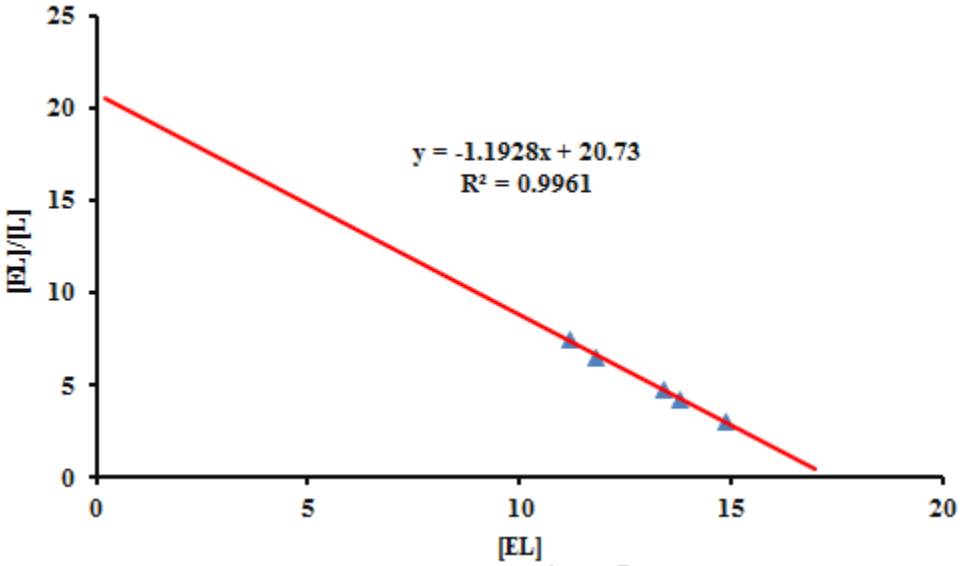


Figure 4

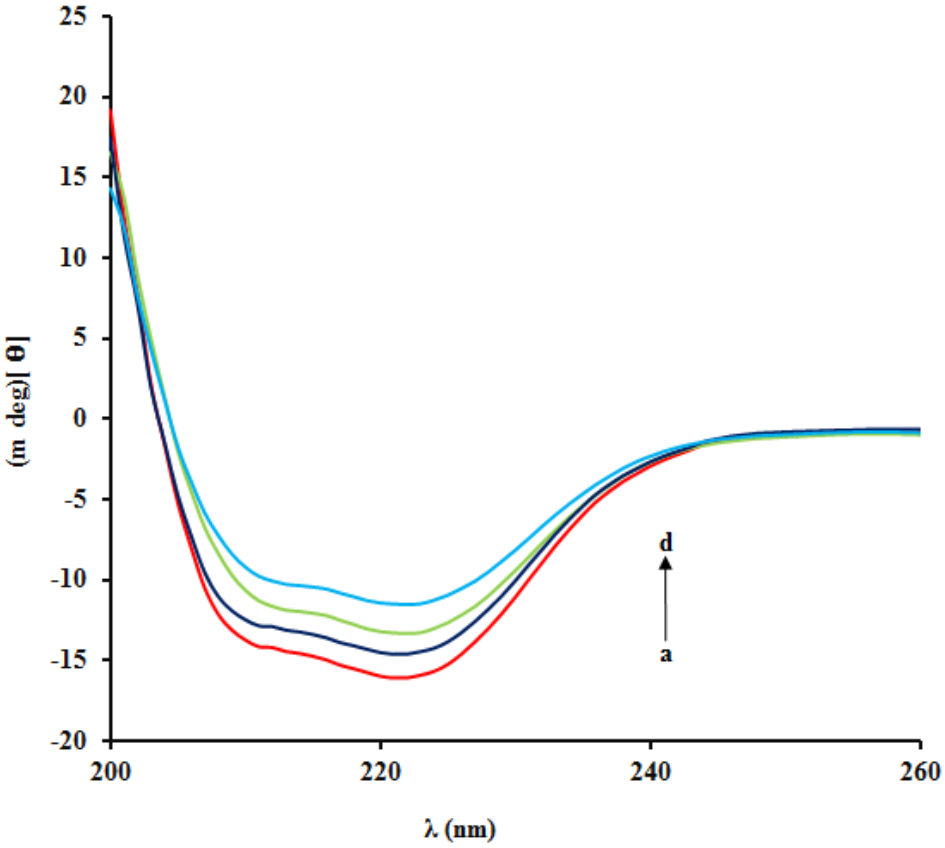
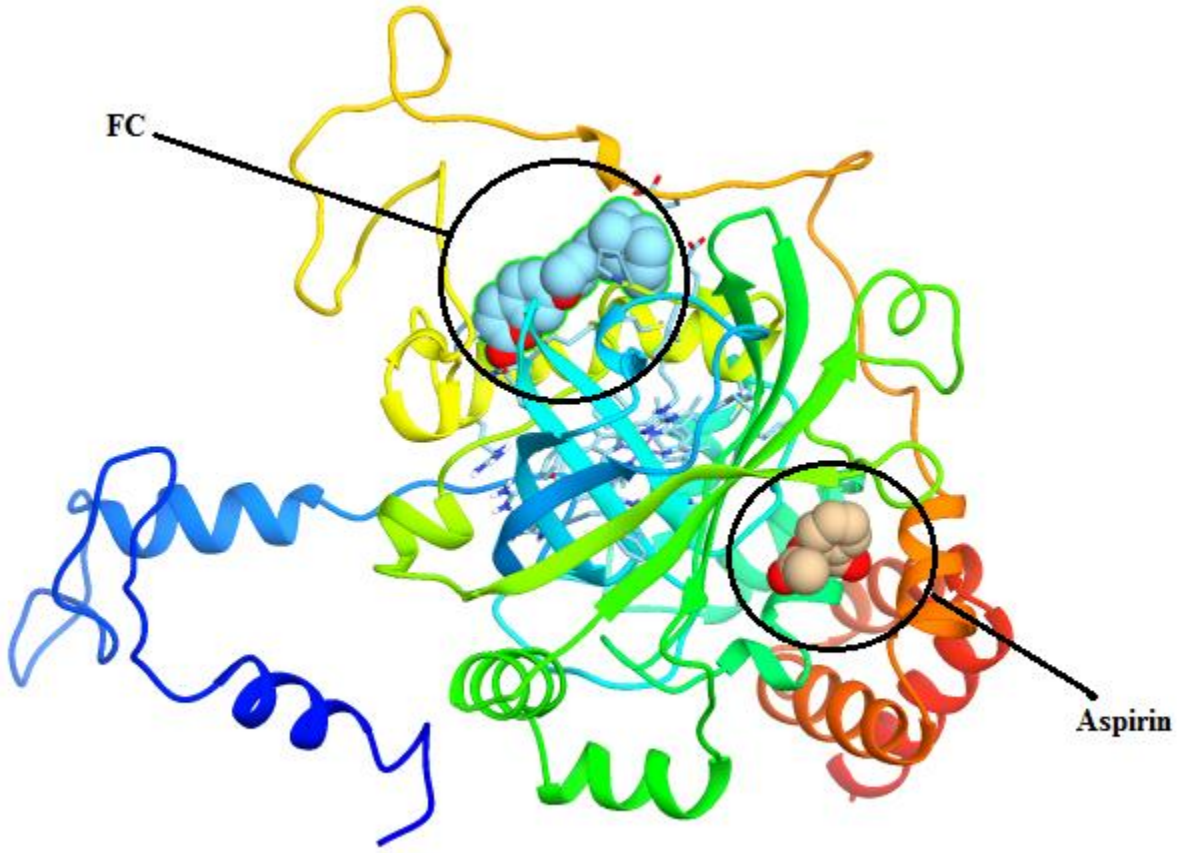


Figure 5



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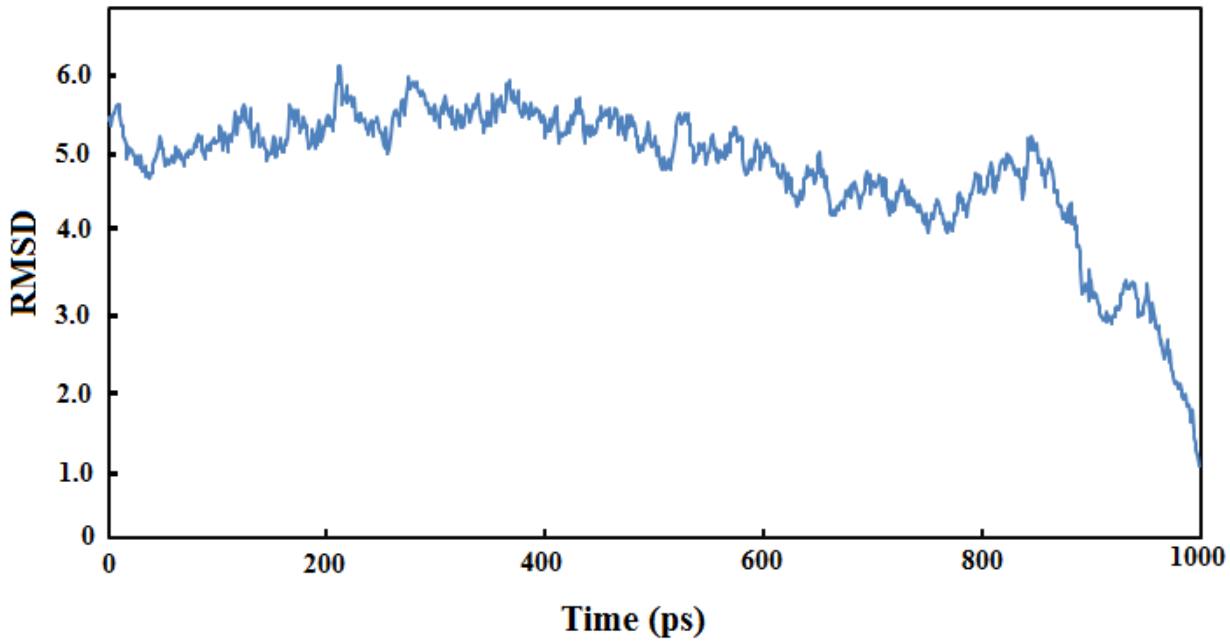

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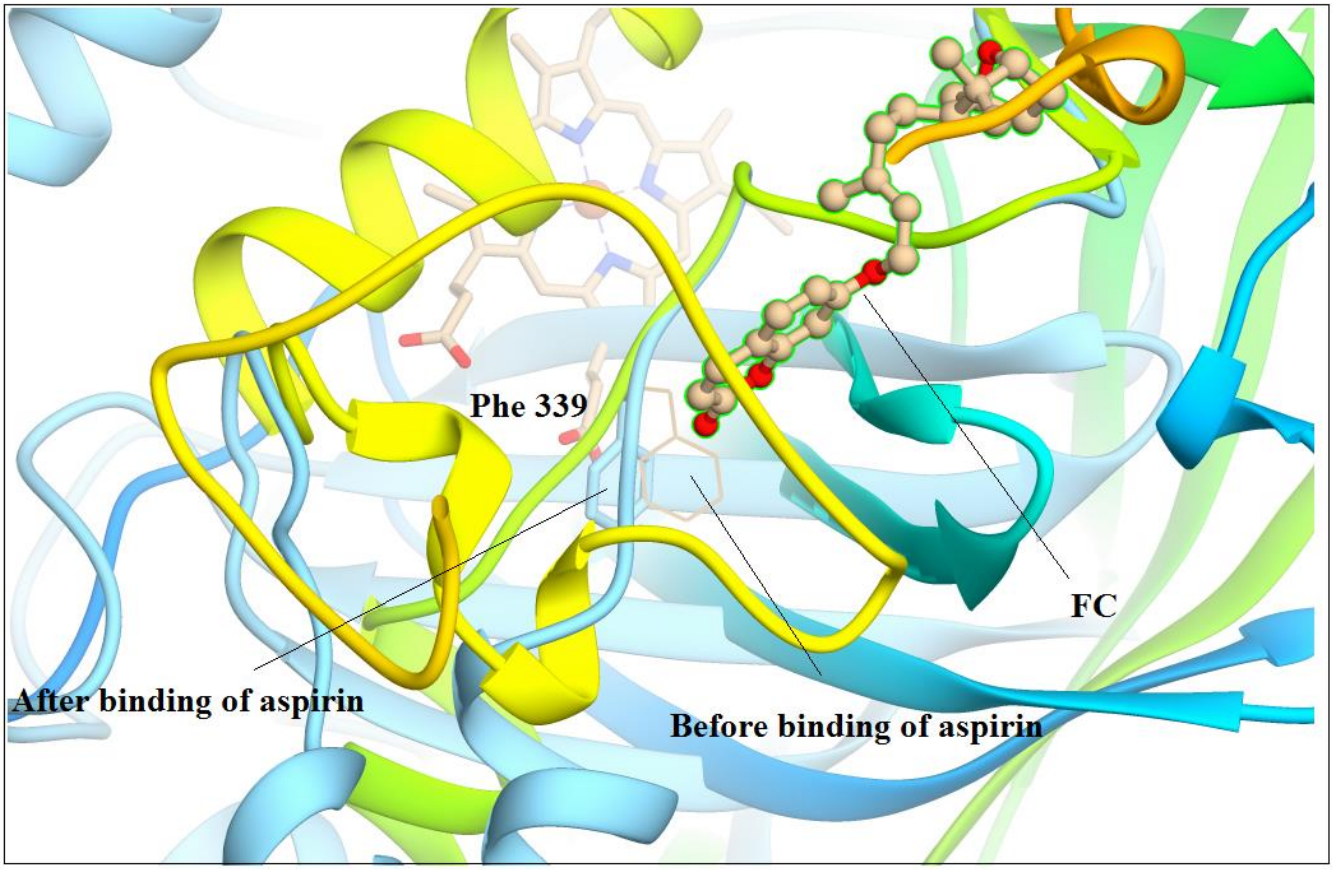

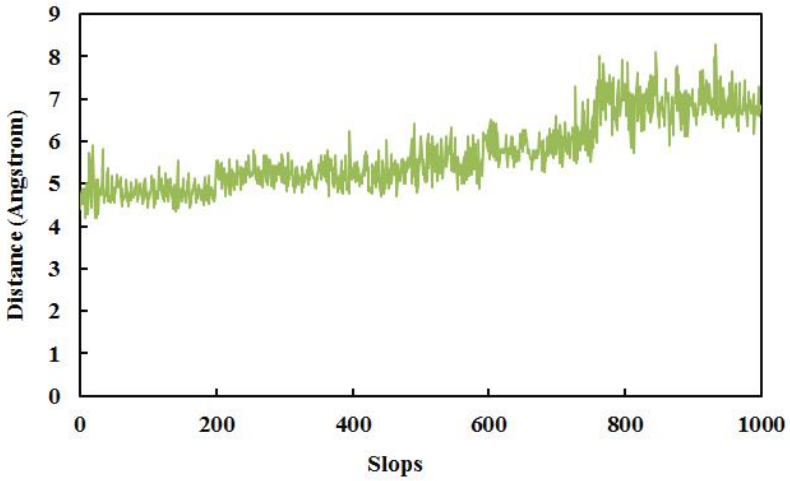

Figure 8

Highlights

- Aspirin was able to remarkably retrieve the FC-induced inactive catalase to active form
- Aspirin could reduce the stability of FC-catalase complex
- The binding of aspirin to catalase led to decrease the affinity of catalase to the inhibitor
- The dissociation constant of FC-catalase was significantly increased in the presence of aspirin