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Studies to reveal the nature of interactions between catalase and curcumin using computational methods and optical techniques

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Abstract

Curcumin is an important antioxidant compound, and is widely reported as an effective component for reducing complications of many diseases. However, the detailed mechanisms of its activity remain poorly understood. We found that curcumin can significantly increase catalase activity of BLC (bovine liver catalase). The mechanism of curcumin action was investigated using a computational method. We suggested that curcumin may activate BLC by modifying the bottleneck of its narrow channel. The molecular dynamic simulation data showed that placing curcumin on the structure of enzyme can increase the size of the bottleneck in the narrow channel of BLC, and readily allow the access of substrate to the active site. Because of the increase of the distance between amino acids of the bottleneck in the presence of curcumin, the entrance space of substrate increased from 250 Å³ to 440 Å³.

In addition, the increase in emission of intrinsic fluorescence of BLC in presence of curcumin demonstrated changes in tertiary structure of catalase, and possibility of less quenching. We also used circular dichroism (CD) spectropolarimetry to determine how curcumin may alter the enzyme secondary structure. Catalase spectra in the presence of various concentrations of curcumin showed an increase in the amount of α -helix content.

Keywords

Catalase activity; Main channel; Curcumin

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1. Introduction

Increased levels of reactive oxygen species (ROS), as a result of diminished capacity of intracellular anti-oxidant defense systems, contribute to pathogenesis of many diseases including cancer, diabetes, and cardiovascular diseases. ROS are chemically reactive molecules which are mainly byproducts of normal cell metabolism. The mass generation of ROS may lead to cell damage and likely affect proteins, lipids, and nucleic acids. As an important oxidant agent, hydrogen peroxide is one of ROS species whose increased production can damage the β -cells in the pancreatic Langerhans islands [1–3]. Hydrogen peroxide has also been reported as an inhibitor of insulin signaling [4]. Catalase is one of the antioxidant enzymes playing a predominant role in elimination of ROS, particularly hydrogen peroxide, by converting it into a less reactive gaseous oxygen and water molecules [5]. A catalase deficiency may increase the likelihood of developing type-2 diabetes mellitus. Catalase activity in blood of patients with diabetes was significantly lower than healthy human subjects [6,7], and similarly in patients with schizophrenia and atherosclerosis [8]. Acatalasemia (<10% of normal activity) and hypocatalasemia (~50% of normal activity) are two categories of genetic deficiencies of erythrocyte catalase [9].

Many studies have investigated different components and various factors that affect catalase activity, and subsequently result in improvement of the enzymatic efficiency through modifications of some amino acid residues [10–13]. Based on the X-ray crystal structure of bovine liver catalase (BLC), there is a main channel in the protein structure that is responsible for connecting the deeply buried heme with the enzyme surface. The steric hindrance and the hydrophobic nature of this channel restrict substrate access [14]. The length of the main channel, from the protein surface to the heme, is 22–55 Å [15]. The first 20 Å of the main channel, starting from the surface, is not as significant as the final 15 Å, since this region of the protein does not restrict the accessibility of substrate to the heme cavity.

The value of 0–15 Å above the heme, starting from conserved Asp¹²⁷, is a narrow channel and plays a significant role in the catalytic function of catalase [16]. Chelikani et al. suggested that the three-dimensional organization and shape of the main channel regulate the efficiency of catalase activity [15]. Fourteen amino acid residues of the narrow channel are as follow: Val⁷³, His⁷⁴, Val¹¹⁵, Asp¹²⁷, Pro¹²⁸, Asn¹⁴⁷, Phe¹⁵², Phe¹⁵³, Phe¹⁶⁰, Phe¹⁶³, Ile¹⁶⁴, Gln¹⁶⁷, Trp¹⁸⁵, and Leu¹⁹⁸. The conserved amino acid residues of the narrow channel can significantly affect enzyme activity [17]. Zamocky et al. demonstrated that the side chains of these residues are important in construction of the channel's bottleneck. They showed substitution of Val¹¹¹, 8 Å away from the heme group, with a smaller amino acid residue such as Ala increased the access of large substrates and enhanced enzyme peroxidatic activity [18]. Chelikani et al. pointed out the importance of a potential field between Asp¹⁸¹ with a negative charge, and heme with a positive charge. They reported that the mutation of Asp¹⁸¹, at a distance of 12 Å from the heme, to Ala, Asn, Gln, Ile, or Ser reduced enzymatic activity [19]. Also, it is well accepted that the amino acid side chains forming the narrow channel play a significant role in substrate access and catalase reaction. It was reported that catalase from *Exiguobacterium oxidotolerans* T-2-2T (EKTA catalase) can decompose hydrogen peroxide more rapidly than BLC or *Micrococcus luteus* catalase

(MLC). This finding has been attributed to the larger bottleneck of the EKTA narrow channel [17].

A significant antioxidant effect has been reported for curcumin (diferuloylmethane), the most effective component of turmeric plant. This important natural compound is also used as a flavor in Indian cuisine [20–23], and it is responsible for the yellowish color of curry. Many experimental studies show that curcumin is used as a remedy in human illnesses including cancer, diabetes, Alzheimer, hepatic disorders, rheumatism, anorexia and Parkinson [24–27]. It is suggested that curcumin has a significant ability to inhibit ROS production, which is caused by high glucose levels in erythrocyte of diabetic patients [28]. Curcumin can also bind specifically to α -synuclein oligomers and reduce its toxicity in Parkinson's disease [29]. The molecular mechanism by which curcumin prevents or attenuates complication of various diseases is not yet known. Thus, studying the effect of curcumin, as an important antioxidant, on the structural and functional properties of proteins and enzymes that are involved in diseases has not only theoretical significance but also clinical applications. In the current study, we demonstrated that interaction of curcumin with catalase resulted in activation of the enzyme. We have studied the mechanism of this activation of BLC by curcumin employing various computational and experimental techniques. We demonstrated that this interaction has important impact on the conformation, and possibly accessibility of the enzyme active site.

2. Materials and methods

2.1. Material

Curcumin and bovine liver catalase (BLC) were purchased from Sigma–Aldrich. The concentration of stock solution of catalase was measured by its optical density at 405 nm, using $3.24 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ for the molar extinction coefficient [30] and 250,000 Da for the molecular mass of BLC. Curcumin stock solution (9 mM) was prepared in methanol. This stock solution was further diluted with twice distilled water and working solution was prepared freshly before using. The used concentration of methanol did not affect the structure and activity of catalase because it was diluted about 1000 times. All the tests were carried out at room temperature and at least 3 times.

2.2. Methods

2.2.1. Enzyme assay—Catalase activity was determined by measuring the rate of hydrogen peroxide decomposition. The samples containing catalase (30 nM) with different concentrations of curcumin were prepared in phosphate buffer (pH 7.4) 1 h before testing. The reaction was recorded immediately after mixing 10 μL , per samples, with 990 μL hydrogen peroxide (12 mM). The reduction of absorbance at 240 nm resulted from H_2O_2 elimination was followed. One unit of activity was defined as the amount of enzyme that decomposes 1 μM hydrogen peroxide in 1 min. The concentration of stock solution of catalase was determined by measuring its absorbance at 405 nm, using $3.24 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ for the molar extinction coefficient [30] and 250,000 Da for the molecular mass of BLC. The concentration of hydrogen peroxide was obtained by UV–vis spectrophotometry (Varian

UV–vis spectrophotometer, model Carry 100 Bio) at 240 nm using $43.6 \text{ M}^{-1} \text{ cm}^{-1}$ as an extinction coefficient (Scheme 1).

2.2.2. Computational methods—Avogadro program was used to draw the structure of curcumin [31]. Gaussian 09 (Rev. D.01) was used for the optimization of curcumin structure [32]. The B3LYP method using the 6–31G(d) basis set was applied in the mentioned step, and the minimum nature of the structure was confirmed by the absence of the imaginary frequency [32]. AutoDock Vina was used to perform the docking studies and the result of this step was analyzed using AutoDock Tools 4 [33,34]. The point numbers equal to 100 in the directions X and Y, and 110 in the direction Z were used to prepare the grid box to cover the whole protein structure. Iterated Local Search global optimizer implanted in the AutoDock Vina was used in docking study [35]. MD simulations were carried out using Amber12 suite of program [36]. The general Amber force field (GAFF) parameters were applied for the curcumin using antechamber program of the Amber Tools 12 [37,38]. The RESP charges were calculated for the heme moiety at the B3LYP/6–31 g (d) level of theory. The complex of the final result of docking studies was used as the starting point of the MD simulation. The system was solvated using TIP3P water model [39]. At first 2000 steps of minimizations were performed to remove the close contacts. The first 1000 steps carried out using steepest descent algorithm and the second 1000 steps using conjugate gradient algorithm. The Langevin thermostat was used to adjust the temperature to 300 K with gradual increase during 25,000 steps, and then kept at this temperature for 75,000 steps [40]. Two successive runs were carried out, including 100,000 steps for each one to obtain the constant density and equilibrate the system. Finally, a 10 ns simulation was carried out.

The 1TGU.pdb was used to obtain the structure of the protein. All the water molecules and ligand, except the heme moieties, were removed from the pdb file. Because the attachment positions for the curcumin on catalase have not been previously determined, the docking studies were carried out to determine its most favorable positions. Four successive docking studies were carried out to determine the position of four curcumin molecules. In each docking the previously docked curcumins were regarded in the structure as well. The most stable structure based on the docking binding energies was selected to perform MD simulation studies to evaluate the effect of the presence of the curcumin on the structure of the protein. The MD simulations were carried out with the details mentioned in the computational method section.

2.2.3. Intrinsic fluorescence spectroscopy—The effect of curcumin on tertiary structure of catalase was determined by recording emission spectra of BLC in absence and presence of curcumin using a spectrofluorometer (Cary Eclipse; Varian). Catalase solution in 50 mM phosphate buffer (pH 7.4) was prepared with curcumin. The mixture was incubated at room temperature for 1 h. We used the incubated catalase in buffer as control. The data was collected in the wavelength range of 300–500 nm with an excitation wavelength of 280 nm and the slit widths of 10 nm.

2.2.4. Circular dichroism (CD) spectropolarimetry—Catalase (0.2 mg/mL) was incubated with different concentrations of curcumin in phosphate buffer (pH 7.4) for 1 h. Far-UV CD spectra of catalase in absence and presence of curcumin were acquired using a

circular dichroism spectropolarimeter model 215 (Aviv Instruments Inc. Lakewood, NJ, USA) in the wavelength range of 190–280 with a spectral resolution of 1 nm, scan speed of 20 nm/min and a band width of 1 nm. Also, quartz cells with 0.1 cm path length were used, and all measurements were done at 25°C. Phosphate buffer spectrum was subtracted from all CD spectra. Processed data were used to analyze the secondary structural elements of the native and the enzyme in presence of curcumin using a CDNN program version 2.¹

3. Results and discussion

3.1. The effect of curcumin on catalase activity

The effect of different concentrations of curcumin on catalase activity was evaluated. Fig. 1 shows that measuring the rate of hydrogen peroxide decomposition in the presence of different concentrations of curcumin reveals that curcumin has significant effect on catalase activity. By increasing concentration of curcumin, the catalase activity was increased. The catalytic activity reached a maximum of more than two-fold when curcumin concentration was increased to 500 nM, and then remained almost constant in the presence of higher concentrations of curcumin.

Although, curcumin undergoes degradation in phosphate buffer [41], it has been reported that some proteins can have important role in stabilizing curcumin in phosphate buffer as a result of binding of curcumin to proteins[42,43]. So we tested curcumin stability in the presence of catalase by UV–vis spectrophotometry. Absorption spectral features of curcumin indicated that catalase can stabilize and suppress degradation of curcumin with a yield of approximately 90% after 30 min (Fig. 2).

It is important to note, curcumin is stabilized due to binding with catalase, then curcumin does not hydrolyses. So there are not degradation products of curcumin and cannot interfere in this process.

3.2. The effect of curcumin on the size of the bottleneck in the main channel of BLC and its reactivity

The data on curcumin, as an activator of catalase, are novel but the question is how curcumin accelerates catalase activity? Does the increase in enzyme activity come from the specific effect of this ligand on the enzyme active site? Could curcumin alter the physicochemical properties of the non-catalytic part of the enzyme? In order to answer these questions and confirm our observations, we followed both experimental and computational approaches. At first, we looked at the specific effect of curcumin on the heme. Upon addition of curcumin to the enzyme solution, no significant changes were observed in the absorption spectra of BLC at 409 nm which corresponds to characteristic absorption of the porphyrin – soret band (results not shown).

In order to determine the effect of curcumin as a ligand on the structural pocket of enzyme, docking studies and MD simulations were carried out with the use of the 1TGU pdb file as receptor. The most stable structure based on the docking binding energies was selected to

¹<http://bioinformatik.biochemtech.uni-halle.de/cdnn>.

perform MD simulation studies. Curcumin was placed on the structural pocket of enzyme and it was stabilized with hydrogen binding to the enzyme's amino acids. Interaction of one curcumin with enzyme in the last step of MD is shown in Fig. 3. Fig. 3A shows putative binding site of curcumin on BLC. In this position, hydrogen bonds were found to occur between hydrogen of hydroxyl group of curcumin and residues Gly120 and His254 of chain C of the enzyme (Fig. 3B). Gly120 is a part of turn from domain C, while His 254 belonging to α -helix of domain C. That is worthwhile to report the aromatic rings of curcumin are located between His 254 of chain C and Arg 126, Phe 199 and His 254 of chain B. According to the Wang's results [44], this location could be interfering with π - π interaction and aromatic ring stacking of these amino acids. This might be an initial factor for conformational changes of entire domain and occurred at the interval between the aforementioned amino acids from chains C and B.

It was important for us to know whether the placement of curcumin on the structure of BLC causes specific changes and enzyme activation. We focused on a specific part of the enzyme, the structure of the enzyme which is responsible for connecting the deeply buried heme with the surface, named the main channel. The substrate access to the active site of this enzyme is not feasible except through this channel. 15 Å above the heme starting from conserved Asp 127 is a narrow channel that plays a significant role in the catalytic function of catalase. This narrow channel strongly limits the access of substrate to the enzyme active site. Since four residues including Trp185, Asp127, Gln167 and Leu198 are located at the neck of the narrow channel 15 Å from the iron, the distances between Gln167–Leu198 and Asp127–Trp185 are very important [17]. Thus, we determined the distance between these amino acids before and after the interaction with curcumin.

Curcumin located on the structure of the enzyme caused maximum distance between Gln167–Leu198 and Asp127–Trp185. As it shown in Fig. 4, it is clear that the distance between the mentioned amino acid residues were increased during the MD simulation in the presence of curcumin. The changes in position of amino acids are shown in Fig. 5. Because of the increase of the distance between amino acids of the bottleneck, the entrance space of substrate increased from 250 Å³ to 440 Å³. The changes of the shape and volume of the neck of narrow channel was measured using KVfinder [46]. The RMSD of the backbone of the protein during the MD simulation is presented in Fig. 6, showing the stability of the protein structure during MD simulation.

3.3. Tertiary structure of BLC upon interaction with curcumin

Intrinsic (Trp) fluorescence spectrum can give information about the changes in protein three dimensional structure. Fluorescence spectra of BLC with different concentrations of curcumin were recorded. Fig. 7 shows the quantum yields for BLC increase when they interact with curcumin. Changes in emission spectrum of Trp may be caused by protein conformational transitions. Trp fluorescence can be quenched by neighboring protonated acidic groups such as Asp or Glu as quenching groups. Steric effects and distance between the side chain of Trp and quenching groups are important in fluorescence quantum yield. The increase in emission of intrinsic fluorescence of BLC in presence of curcumin supports the possibility of less quenching, which may be due to increased distance between Trp and

quenching groups. Control experiments were done only with curcumin and buffer, which did not show any significant fluorescence emission so the results can be state as a curcumin effect on the tertiary structure of enzyme.

3.4. Secondary structure of BLC upon interaction with curcumin

The effect of curcumin on the secondary structure of catalase was obtained by far-UV CD spectrum. The chemical modification of carboxylic group of aspartic and glutamic acid causes significant changes in the secondary structure of the protein. Changes in the secondary folding level of BLC in the absence and presence of curcumin (0–1000 nM) are shown in Fig. 8. All changes in CD results are in comparison with the control sample (without any curcumin) so the results can be stated as a curcumin effect on the second structure of enzyme. Negative extremes in 209 nm, which corresponds to π - π * transition of α -helix and 225 nm which is assigned to π - π * transition, for both the α -helix and random coil are seen in all spectra [47]. The contents of secondary structure of each spectrum were measured using the CDNN Program Version 2. Table 1 shows that the catalase spectra in the presence of various concentrations of curcumin suggested an increase in the α -helix content which was accompanied with a reduction in the random coil. This structural alteration may suggest stabilization of the enzyme

4. Conclusion

Previous studies showed that curcumin can affect as an allosteric effector in sirtuin activation [48] but there isn't any study in activation of catalase by curcumin. Evidences in human clinical trials indicated that curcumin is safe and there is no dose-limiting toxicity. Thus, the superiority of this study compared to previous studies in the activation of catalase, which all have used industrial chemical components [49,50], is the use of curcumin as a safe natural component.

In this paper we discuss a possible mechanism by which curcumin activates BLC. Our study reinforced the hypothesis that curcumin can increase enzyme activity by affecting arrangements of amino acid residues in structural pocket of enzyme. Increase of distance between the residues of the bottleneck of narrow channel, which determines the amount of substrate entering the active site, facilitated the substrate access to the enzyme active site. This finding could be regarded as one of the possible reasons for the observed increased activity.

There are important aspects in this achievement. Catalase activation with a natural antioxidant component, which doesn't have toxicity, can provide useful results in the health field. Previous studies have shown that catalase activity of animals with tumor is markedly reduced [51–54]. Moreover this enzyme can effectively remove residual hydrogen peroxide in various industries such as dairy, textile, and electronics. Catalase activation provides appropriate way to reduce enzyme consumption and cause economic efficiency.

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Fig. 1.
Relative activity of BLC vs. various curcumin concentrations, using H₂O₂ as a substrate.

Fig. 3. Interaction of one curcumin with amino acids of pocket of BLC in the latest step of MD. (A) Putative binding site of curcumin (shown in stick) on BLC (shown with ribbons). (B) curcumin interactions with residues forming the putative binding site in the latest step of MD drawn by LigPlot software [45].

Fig. 4.
Distance between (A) Gln167–Leu198 and (B) Asp127–Trp185 during the MD simulation.

Fig. 5. 3D representation of the changes in the neck of the narrow channel of Catalase before (A) and after (B) treatment with curcumin. Amino acids of narrow channel entrance and heme group in active site are showed in red line representation. Yellow arrow pointing to the entrance of the active site. As it can be seen from this figure, neck of the channel is more capacious and its volume changed from 240 Å³ to 440 Å³ after treatment with curcumin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 6.
Stability of the protein structure during MD simulation.

Fig. 7. Intrinsic fluorescence spectra of the native and modified forms of BLC. (A) Native BLC; (B) BLC +Cur (50 nM); (C) BLC +Cur (100 nM); (D) BLC +Cur (300 nM); (E) BLC +Cur (500 nM); (F) BLC +Cur (1000 nM). Experimental conditions: fluorescence measurements were carried out with 0.2 mg/mL of BLC in 50 mM phosphate buffer (pH 7.0) at 25 °C. The excitation wavelength was 295 nm using a slit width of 10 nm.

Fig. 8.

The far-UV circular dichroism spectra of BLC in absence and presence of curcumin of BLC. (A) Native BLC; (B) BLC +Cur (100 nM); (C) BLC +Cur (200 nM); (D) BLC +Cur (300 nM); (E) BLC +Cur (500 nM); (F) BLC +Cur (1000 nM). Experimental conditions: the spectra were taken of proteins dissolved at a concentration of 0.2 mg/mL in 50 mM phosphate buffer at pH 7.0.

Scheme 1.
Curcumin formula.

Table 1

The percentage of the secondary structure obtained by CDNN Program Version 2.

[Curcumin] (nM)	Secondary structure (%)		
	α -Helix	β -Sheet	Random coil
0	28.3	17.4	37.6
100	28.2	17.4	37.6
200	28.5	17.3	37.5
300	32.3	16.7	34.5
500	33.4	16.5	33.6
1000	35.6	16.2	32.1