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Enhancing the peroxidase-like activity of ficin via heme binding and colorimetric detection for uric acid

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ABSTRACT

Ficin, a classical sulfhydryl protease, was found to possess intrinsic peroxidase-like activity. In this paper, we have put forward a novel strategy to improving the peroxidase-like activity of ficin through binding heme. Heme-ficin complexes were successfully obtained by simple one-step syntheticism. The results demonstrated that the catalytic activity and efficiency of heme-ficin complexes were about 1.7 times and 3 times higher than those of native ficin, respectively. Taking advantages of the high peroxidase-like activity, the heme-ficin complexes were used for colorimetric determination of uric acid with a low detection limit of 0.25 μM. Based on the excellent selectivity and sensitivity, we detected the concentration of uric acid in human serum successfully. On the basis of these findings, the heme-ficin complexes are promising for wide applications in various fields. Thus we not only optimized the peroxidase-like activity of the ficin, but also established a new strategy for development of artificial enzyme mimics by mimicking the architecture of the active site in horseradish peroxidase.

1. Introduction

Artificial enzyme mimics have drawn considerable attentions in recent years, which are regarded as the alternatives for natural enzymes because of their excellent comprehensive properties. So far, more and more nanoparticle-based artificial enzyme mimics, such as metal-based nanoparticles [1–3], graphene-based materials [4,5], carbon nanodots [6–8], and metal-organic frameworks [9–11], have been designed and constructed. Unfortunately, some of these non-biological enzyme mimics, which often need complicated preparation procedures and modification process to avoid aggregation, have low biological compatibility and poor reproducibility in activity. Horseradish peroxidase (HRP) as a kind of nature biological enzyme, have been narrowed their industrial applications primarily for relatively high costs of preparation, complicated purification and meticulous storage environment [12,13]. Nevertheless, the intrinsic overwhelming superiorities of nature enzymes that we still can't ignore, such as high-catalytic activity, good biological compatibility and low toxicity [14–17]. Development of artificial enzyme mimics based on the nature enzymes is highly desirable.

Ficin was found to possess intrinsic peroxidase-like activity as classical cysteine protease that isolated from the latex of fig trees, which was firstly reported by our group in 2017 [17]. There is no denying that the catalytic efficiency of ficin is lower than HRP. However, compared with HRP, ficin could be used more extensively in analytic applications due to their outstanding robustness against harsh pH and temperature, higher affinity to H_2O_2 [17]. It could be mentioned that ficin is a simple enzyme while HRP is a conjugated enzyme containing the ferroprotoporphyrin cofactor in which iron plays a crucial role in the catalytic process [14,18–20]. In nature, the enzyme has evolved activity sites where the distribution of basic functional groups follows the precise spatial structure to activate the catalytic center effectively. Heme, a cofactor in HRP, is activated by H_2O_2 via the collaboration among a proximal His ligand, a distal His, and a distal Arg residue to form a high oxidation state intermediate compound I that accepts the electrons from the reducing substrate, which leading to a variety of basic biological functions [14].

A great number of efforts had been expended on incorporating heme into protein-like scaffolds in the looking for a similar active site promoting the creation of compound I [21–23]. Heme could bind His residues in amyloid-β to form a peroxidase mimic which plays an important role in the treatment of Alzheimer's disease(AD) [24–27], suggesting that heme also could bind His residues in ficin which contains about 200 amino acids to form complexes. In this paper, we have put forward a novel strategy to incorporate heme into ficin to obtain peroxidase-like complexes, in which ficin could offer a native protein microenvironment more than simple amino acid residue. To verify our hypothesis, we studied the UV–vis spectra, fluorescence spectroscopy and circular dichroism of the native ficin, heme and heme-incubated

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ficin to indicate the formation of heme-ficin complexes. Then we investigated the catalysis performance of heme-ficin complexes compared with native ficin and heme. And our results indicate heme-ficin complexes have enhanced peroxidase activity effectively.

Uric acid (UA), an end product of purine metabolism, is an important biomarker of hyperuricemia in urine and serum can lead to several pathological symptoms such as gout, arthritis, neurological, renal, cardiovascular and kidney related disease. Hence, precise detection of UA concentration in urine and serum samples is a key of the early stage diagnosis and warning of these conditions. Colorimetric and fluorometric methods have always attracted considerable interest owing to its simple, cost-effective and routine analysis [28–32]. Based on the excellent peroxidase-like activity of heme-ficin complexes, we have applied the complexes to the colorimetric detection of uric acid with uricase. This system exhibited excellent selectivity and sensitivity for uric acid determination. And we detected the concentration of uric acid in human serum successfully, suggesting its great potential for biocatalysis and bioassays in the future.

2. Experiment section

2.1. Reagents

Premium grade ficin (F4165, powder, molecular weight 23.8 kDa) were purchased from Sigma-Aldrich (America). Hemin (98%, which is referred to as heme in this paper) was obtained from Adamas (Shanghai, China). Uric acid and uricase were purchased from Yuanye Bio-Technology (Shanghai, China). NaCl, KCl, glucose, sucrose, urea, tryptophan, L-cysteine, ascorbic acid (AA) were purchased from Aladdin (Shanghai, China). Hydrogen peroxide (H_2O_2, 30%) was obtained from Chongqing Chuandong Chemical Co., Ltd. (Chongqing, China). The phosphate saline buffer (PBS) containing Na_2HPO_4 and NaH_2PO_4 was prepared and the pH was adjusted with H_3PO_4 or NaOH. All the commercial available regents were used without further purification. All solutions were prepared using ultrapure water (18.2 MΩ cm^{-1}) from a Milli-Q automatic ultrapure water system.

2.2. Preparation of heme-ficin complexes

A stock solution of heme (40 mM) was prepared in 100 mM NaOH solution and stored at 4 °C in the dark, then was diluted to 2.0 mM in 50 mM PBS (pH = 7.0) before use. The stock solution of ficin (2.0 mg/mL) was freshly prepared in 50 mM PBS (pH = 7.0). Then incubating 1 equiv of both heme and ficin solutions for 8 h at 4 °C in the dark. The molar ratio of heme to ficin was maintained at 25:1.

2.3. Peroxidase-like activity

The peroxidase-like activity of heme-ficin complexes was measured using TMB as the chromogenic substrate in the presence of H_2O_2 following the increase in absorbance at 652 nm. In brief, 200 μL heme-ficin complexes (0.50 μg/mL, which is the concentration of ficin presented in the complexes), 200 μL TMB (8.0 mM) and 200 μL H_2O_2 (8.0 mM) were orderly added into 200 μL PBS (pH = 5.0) and the final volume of the mixture was adjusted to 2.0 mL with ultrapure water. The mixed solution was then incubated in 30 °C water bath. After incubation for 210 min, the UV–vis absorption spectra of the solution were measured on a UV-2450 UV–vis spectrophotometer (Shimadzu, Japan).

2.4. Electron spin resonance (ESR)

Twenty μL ficin or heme-ficin complexes was added into 0.20 M PBS buffer (pH = 5.0), 20 μL of 15% H_2O_2, and 50 μL 100 mM 5,5-dimethyl-1-pyrolin-N-oxide (DMPO) and proper amount of ultra-pure water into a plastic tube. The prepared sample solution was transferred to a quartz capillary tube and placed in the ESR cavity. DMPO was used to form the DMPO/•OH spin adduct. The ESR spectra were obtained on a Bruker ESR 300 E with a microwave bridge (receiver gain, 1×10^5; modulation amplitude, 2 Gauss; microwave power, 10 mW; modulation frequency, 100 kHz).

2.5. Kinetic assays

Steady-state kinetic assays were carried out by monitoring the absorbance change at 652 nm at different reaction time. Experiments were carried out at 30 °C in 1.0 mL reaction buffer solution (20 mM PBS, pH = 5.0) contains 0.10 μg/mL heme-ficin complexes as catalyst in the presence of H_2O_2 and TMB by varying concentrations of TMB at a fixed concentration of H_2O_2 or vice versa. The Michaelis-Menten constant was calculated based on the Lineweaver-Burk plot: $1/v = (K_m/V_{max}) \cdot (1/[S] + 1/K_m)$, where v is the initial velocity, V_{max} is the maximal reaction velocity, and [S] is the concentration of substrate and K_m is the Michaelis constant. The Michaelis constant is equivalent to the substrate concentration at which the rate of conversion is half of V_{max}. K_m indicates the affinity of the enzyme to the substrate: a lower K_m value means a higher affinity.

2.6. Detection of hydrogen peroxide and uric acid

H_2O_2 detection was carried out as follows: a) 1200 μL ultrapure water, 200 μL 0.20 M PBS (pH = 5.0), 200 μL of the 1.0 μg/mL heme-ficin complexes, 200 μL of 0.80 mM TMB solution, and 200 μL of H_2O_2 with different concentrations were added into a 2.0 mL centrifuge tube and mixed together; b) the mixed solution was incubated for 210 min at 30 °C and then for standard curve measurement.

Uric acid detection was performed as follows: a) 10 μL of 3.0 mg/mL uricase and 200 μL of uric acid of different concentrations in 0.02 M PBS (pH = 8.5) were incubated at 37 °C for 15 min; b) 200 μL of 1.0 μg/mL heme-ficin complexes, 200 μL of 0.80 mM TMB and 1390 μL of 0.20 M PBS (pH = 5.0) were added to the above uric acid reaction solution; c) the mixed solution was incubated at 30 °C for 210 min and then for standard curve measurement.

2.7. Uric acid Determination in Serum Samples

Serum samples were obtained from two healthy volunteers. The collected samples were first treated by ultrafiltration with 5000 Da at 1000 rpm for 30 min. Then, the filtrates were diluted 2 times using ultrapure water. The subsequent operations for uric acid detection in serum samples were the same as described above except the replacement of uric acid with serum samples. And the volunteers' consent and approval from the Institutional Research Ethics Committee of Southwest University hospital were obtained for research purposes.

3. Results and discussion

3.1. Formation of heme-ficin complexes

It has been well recognized that heme is an indispensable factor for some metabolism of organisms including oxygen transportation, catalysis and electron transfer [33]. Free heme, possessed low peroxidase-like activity, were available for binding His residues in amyloid-β (Aβ), which may hold a key towards development of the cure of AD [25]. Meanwhile, the catalytic rate of heme-Aβ complex was ~ 3 times faster than free heme [25]. In this study, we prepared heme-ficin complexes by mixing heme and ficin together, as shown in Scheme 1a. The main fabrication conditions we have optimized, which could be mainly influenced by pH and the molar ratio of heme to ficin that were investigated by measuring the corresponding peroxidase-like activities. As shown in Fig. S1a, at weakly acidic and neutral pH, we can obtain complexes with higher catalytic activity, and the optimal pH for fabricating complex was 7.0. The molar ratio of heme to ficin varied from 1