Umbelliferone as a Small Molecular Peroxidase Mimic towards Sensitive Detection of H₂O₂ and Glucose

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In this work, umbelliferone, a kind of coumarin derivative, was proved to exhibit peroxidase-like activity that could catalyze the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) in the presence of hydrogen peroxide to generate a blue-colored oxide (oxTMB). The catalytic mechanism is similar to that of native enzymes (*e.g.* horseradish peroxidase, HRP) and nanozymes, which follow the Michaelis-Menten kinetics behavior. Meanwhile, the 7-hydroxyl group of umbelliferone plays a significant role in the peroxidase-like activity. Compared with enzymes and nanozymes, this small molecular mimic enzyme possesses the advantages of low cost, simple molecular structures, small molecular weight and high stability against harsh conditions. Based on the favorable peroxidase mimetic activity of umbelliferone, a convenient, practical and sensitive H₂O₂ and glucose detection method was successfully established. This work not only opens some new inspirations into seeking for novel molecular enzyme mimetics with excellent catalytic activities, but also provides promising assays for clinical diagnosis.

Keywords Umbelliferone, 3,3',5,5'-tetramethylbenzidine, peroxidase mimic, glucose, chromogenic reaction

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Introduction

Natural enzymes are biological catalysts; although a few are RNA molecules, most enzymes are generally proteins that increase the rate of a chemical reaction by lowering their activation energy and make their conversion of substrate to product occur many millions of times faster.¹ Due to their special selectivity, high catalytic efficiency occurs under eco-friendly reaction conditions;² natural enzymes have recently been applied to the chemical industry, biotechnology, environmental analysis,³ especially to biosensing and immunoassays.⁴ Although natural enzymes are highly active and specific during their catalytic behavior, their expensive cost, difficult purification process, low operation stability and rigorous storage conditions hamper their extensive applications. Hence, increasing attention has been paid by researchers to discovering and constructing enzyme mimics with similar functions to that of natural enzymes.⁵ Since Yan's group reported on the intrinsic peroxidase-like activity of ferromagnetic nanoparticles in 2007,⁶ a series of nanomaterial-based artificial enzymes, including metal nanoparticles,⁷⁻⁹ metal oxides and their combined nanostructures,¹⁰⁻¹² and carbon-based materials,¹³ have also been affirmed to have peroxidase-like activities during the past few decades. They occupied excellent superiorities over traditional natural enzymes, such as easy preparation and storage, design flexibility and good stability under diversified conditions. Unfortunately, dissatisfied activity differences from batch to batch, easy aggregation with high concentrations have

seriously restricted their comprehensive utilization.¹⁴

Therefore, that is of vital importance to find substitutes with remarkable catalytic activity similar to that of natural enzymes and nanozymes. Our group had found that fluorescein exhibits noteworthy peroxidase-like activity.¹⁵ This opens small molecules as peroxidase mimics, and provides some new inspirations into seeking for novel molecular enzyme mimics with excellent catalytic activities. In addition, we also investigated the peroxidase-like activity of other xanthene dyes with similar structure to fluorescein, such as rhodamine B, rhodamine 6G, and found that their xanthene rings without any hydroxyl groups did not possess peroxidase-like activity.¹⁵ Based on this result, we may infer the hydroxyl groups could play a crucial role in the catalytic systems. Therefore, we speculated that other substances with hydroxyl groups and similar molecular structure to fluorescein perhaps have the same activity. Herein, we found another kind of small molecules, umbelliferone, a kind of coumarin derivative that possess intrinsic peroxidase-like activity in the catalytic oxidation of peroxidase substrate 3,3',5,5'-tetramethylbenzidine (TMB) in the presence of H₂O₂ (illustrated in Scheme 1).

Umbelliferone is also known as 7-hydroxycoumarin, displayed in Scheme 1, which is derived from the plant family Umbelliferae¹⁶ and is present in many plants, such as carrot, coriander and garden angelica.¹⁷ This compound is also commercially available in large quantities.¹⁸ Meanwhile, it possesses a wide range of bioactivities such as anti-inflammatory, antitumor promoting and antiproliferative.^{19,20} Further, this compound is also the main metabolite of coumarin with strong fluorescence, and has served as a core to prepare a range of coumarin derivatives for extensive applications.²¹ However, to the best of our knowledge, its catalytic activity has not been

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Scheme 1 Schematic illustration of a peroxidase-like catalytic reaction of umbelliferone for colorimetric detection of H₂O₂ and glucose by coupling with glucose oxidase (GOx).

reported so far. Herein, we demonstrated umbelliferone to have good peroxidase-like catalytic performance. Compared with natural enzymes and some artificial nanozymes,²² umbelliferone is a particularly efficient catalyst owing to its high abundance, low cost, remarkable thermal stability and good tolerance of high H₂O₂ concentrations and strong acid or alkali environments. At the same time, umbelliferone, with a simple and similar molecular structure, low molecular weight and fast reaction time, is better than fluorescein. Hence, umbelliferone could have great potential as an ideal candidate for biomimetic catalysis.

In the present work, the mechanism, optimal reaction conditions, catalytic kinetics, stability and robustness under harsh conditions of the peroxidase-like activity of umbelliferone were considered. Furthermore, we investigated the effect of the 7-hydroxyl group on its molecular structure to the peroxidase-like activity.²³ Subsequently, in view of the excellent peroxidase-like activity of umbelliferone, we successfully established a convenient, brief and sensitive H₂O₂ and glucose detection system. This system will provide some novel ideas concerning practical chemical analysis.

Experimental

Reagents and chemicals

Umbelliferone (Umb, GC grade, ≥98%), coumarin (Cou, GC grade, 99.0%) and esculetin (Esc, HPLC grade, 98.0%) were purchased from TCI Chemical Industry (Tokyo, Japan). 4,7-Dihydroxycoumarin (4,7-DHC, 97.0%), glucose oxidase (GOx, EC1.1.3.4.47, 200 U/mg) and 3,3',5,5'-tetramethylbenzidine (TMB) (99%) were purchased from Sigma-Aldrich. 5,7-Dihydroxycoumarin (5,7-DHC, 98.0%) was purchased from Ark Pharm, Inc. (Chicago, USA). H₂O₂ (30%) and glucose were purchased from Chongqing Chuandong Chemical Co. Ltd. (Chongqing, China). Superoxide dismutase (SOD, 2500 U/mg) was purchased from Sangon Biotech (Shanghai) Co., Ltd. Acetate (HPLC grade, ≥99.9%) was obtained from Shanghai Mackin biochemical Co. Ltd. (Shanghai, China). Sodium acetate anhydrous (99.99%, metals basis) and sodium azide (99.5%) were obtained from Aladdin (Shanghai, China). All other reagents were of analytical grade, and used without further purification. All aqueous solutions were prepared using Milli-Q water (Millipore, USA) throughout the study.

Fig. 1 Absorption spectra of different catalytic reaction systems: (a) TMB + H₂O₂ (black line), (b) TMB + Umb (green line), (c) Umb + H₂O₂ (blue line), (d) TMB + H₂O₂ + Umb (red line).

Results and Discussion

Peroxidase mimetic activity of umbelliferone

To examine the peroxidase-like activity of umbelliferone, the catalytic oxidation of the peroxidase substrate TMB in the absence or presence of H₂O₂ was implemented, and the absorption spectra were monitored. The characteristic blue-colored products (oxTMB) had typical absorption peaks at 652 nm stemming from the distinctive blue charge-transfer complex between di-amine and di-imine.²⁴ It can be easily observed from Fig. 1 and Fig. S1, that the TMB + H₂O₂ system revealed a light-blue color with a low absorption peak at 652 nm (curve a, Fig. 1); the TMB + Umb or Umb + H₂O₂ system was almost colorless and the absorbance at 652 nm was near zero (curves b and c, Fig. 1), while the TMB + H₂O₂ + Umb system showed a dark-blue color with an obvious absorption peak (curve d, Fig. 1). The chromogenic reaction catalyzed by Umb showed an observably higher reaction velocity than the reaction catalyzed only by H₂O₂ (Fig. S2). This indicated the catalytic oxidation of TMB derived from the contribution of Umb. Moreover, it was found that the catalytic reaction system is dependent on the concentrations of umbelliferone. As shown in Fig. S3, with the increase of the concentrations of umbelliferone, the absorbance value at 652 nm was gradually raised. The results both further testified that Umb acts as a peroxidase

mimic toward TMB. To further characterize the peroxidase-like activity of Umb, we repeated the experiments using other peroxidase substrates in place of TMB, including *o*-phenylenediamine (OPD) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). As shown in Fig. S4, that Umb not only catalyzes the oxidation of TMB producing a blue color, but also OPD to given an orange color and ABTS to given a green color. These results indicate that Umb has same peroxidase-like activity if other colorimetric substrates, such as OPD and ABTS, are used.

Optimization of reaction conditions

Similar to HRP and nanozymes,²⁵ the peroxidase-like activity of Umb was dependent on the pH, temperature, H₂O₂ concentrations and reaction time. To confirm this, the reaction conditions of Umb and HRP were conducted varying pH value from 1.0 to 7.0, the temperature from 15 to 60°C, the H₂O₂ concentrations from 0 to 6.0 mM and the reaction time from 15 to 180 min. As displayed in Fig. S5a, the absorbance increased with the pH and reached a maximum at pH 3.0, but then dropped down. It is worth noting that the molecular structure of umbelliferone constantly changed under different pH conditions, similar to hymecromone.²⁶ As shown in Fig. S6, in the strong acid environment, the carbonyl group of umbelliferone was protonated²⁴ (inset in type I), which increased the degree of conjugation of benzopyrone and contributed to the peroxidase-like activity of umbelliferone. Meanwhile, over a range of pH 3–6, the carbonyl group of umbelliferone was gradually deprotonated, which exists as a neutral molecule²⁷ (inset in type II), the peroxidase activity was gradually reduced; when the pH was continually raised, the hydroxyl group of umbelliferone was ionized to become an anion and the pyran ring was open, which decreased the degree of conjugation and rigidity of umbelliferone²⁷ (inset in type III, IV). This could explain that during the ionization and hydrolyzation process, the peroxidase-like activity of umbelliferone is gradually changed and the maximum catalytic activity is obtained at pH 3.0.

Similarly, as shown in Fig. S5, the catalytic activity of umbelliferone with the change of the other reaction conditions constantly fluctuate and the maximum activity was obtained under the optimal reaction conditions: temperature 50°C (Fig. S5b), H₂O₂ concentrations 4.0 mM (Fig. S5c) and reaction time 90 min (Fig. S5d). The optimal condition was similar to that of natural enzyme (*e.g.* HRP). What is more, compared to HRP, umbelliferone exhibited remarkable tolerance when it was situated in high H₂O₂ concentrations, which could establish extensive applications under various conditions.

The robustness of umbelliferone under harsh environment

The influences on the peroxidase mimetic catalytic activity of umbelliferone while being placed in different temperatures and pH environments for some time were explored. It was expected to be more stable than natural enzymes (*e.g.* HRP). First, we incubated Umb and HRP within a range of pH values (pH 1–12) or different temperatures in a range of 4–90°C for 2 h, and then the activities were measured under the optimal conditions, as described in supplementary information (SI). It can be observed from Fig. S7a that the relative activity was almost retained at more than 65% over the range of pH from 1 to 12. Especially, with a change of the pH, the catalytic activity of umbelliferone was gradually reduced until equilibrium occurred. Meanwhile, as displayed in Fig. S7b, the umbelliferone kept almost 90% of the peroxidase-like activity over a wide range of temperatures from 4 to 90°C. However, the results showed that the activity of HRP was largely inhibited after incubation at lower or higher

pH or temperatures over 40°C. These directly demonstrated the robust stability of umbelliferone as a small molecular enzyme mimic, compared to natural enzyme.

Reactive species analysis

To confirm the generation of reactive species during the catalytic process, reactive species trapping experiments were carried out.²⁸ As displayed in Fig. S8, •OH radical scavengers, such as ascorbic acid (AA), thiourea (TU), caused a dramatic decrease in the absorbance at 652 nm. However, a small change in the absorption occurred after adding ¹O₂ radical scavengers, such as sodium azide (NaN₃) and O₂^{•−} radical scavengers, superoxide dismutase (SOD). The results pointed out that the generation of •OH radicals played a critical role in oxidizing TMB to produce a blue color.

In addition, in order to further investigate the mechanism of the peroxidase-like activity of umbelliferone, Electron Spin Resonance (ESR) was used to demonstrate the production of free radicals (•OH) during the catalytic reaction procedure using DMPO as a spin trap.²⁹ As observed from Fig. S9, weak characteristic peaks can be observed in the DMPO + H₂O₂ system. In contrast, a stronger characteristic peak with an intensity ratio of 1:2:2:1 can be found in the DMPO + H₂O₂ + Umb system, indicating the production of the •OH radical which is similar to the behavior of peroxidase.³⁰ Moreover, with an increase in the concentrations of umbelliferone, the ESR signal intensity increased. This result provides direct evidence that umbelliferone possesses peroxidase mimetic activity.

Steady-state kinetic study

We performed a kinetic analysis to measure the steady-state kinetic parameters of the chromogenic reaction catalyzed by umbelliferone. Typical Michealis-Menten curves were obtained by drawing the initial reaction velocities against substrate concentrations. The corresponding concentrations of the oxidized peroxidase substrate was obtained by the Lambert-Beer law using the value $\epsilon_{\text{oxTMB}} = 39000 \text{ M}^{-1} \text{ cm}^{-1}$ at 652 nm based on the absorbance data and the Michaelis-Menten constant was calculated from the Lineweaver-Burk double-reciprocal plot,^{31,32} based on

$$1/v = K_m/V_{\text{max}} \cdot (1/[S] + 1/K_m). \quad (1)$$

As displayed in Fig. 2 (a, b), the reaction rate mounted gradually with increasing TMB or H₂O₂ concentrations until achieving the reaction equilibrium. The steady-state kinetic parameters are summarized in Table 1. The results indicated the catalytic reaction behavior of umbelliferone is similar to that of HRP which followed the Michaelis-Menten kinetics behaviors. The K_m value is represented by the degree of affinity between the enzymes and the substrate: a lower K_m indicates a higher affinity. As can be seen in Table 1, compared with other artificial enzymes, the umbelliferone has a smaller K_m value for H₂O₂, which was almost 3-times lower than HRP,¹⁵ indicating a relatively higher affinity to H₂O₂. The K_m value of Umb with TMB was higher than that of HRP.¹⁵ Figure 2 (c, d) showed double reciprocal plots at different H₂O₂ and TMB concentrations, respectively. The lines in each plot are almost parallel, which is the characteristic of a typical ping-pong mechanism,³³ as was observed for HRP.³⁴ This means that the umbelliferone binds and reacts with one substrate (either TMB or H₂O₂), and then releases a product before reacting with another substrate.

Fig. 2 Steady-state kinetic assay and catalytic mechanism of Umb (a – d).

Table 1 Comparison of the apparent Michaelis-Menten constant (K_m) and maximum reaction rate (V_{max}) between umbelliferone and HRP

Catalyst	Substrate	K_m/mM	$V_{max}/10^{-8} \text{ M s}^{-1}$	K_{cat}/s^{-1}
Umb	TMB	1.58 ± 0.132	0.335 ± 0.016	0.112×10^{-5}
	H_2O_2	0.184 ± 0.01	0.077 ± 0.001	0.026×10^{-5}
HRP ¹⁵	TMB	0.16 ± 0.025	4.27 ± 0.456	2.08×10^2
	H_2O_2	0.52 ± 0.011	1.82 ± 0.015	8.02×10^3

The peroxidase mimetic catalytic activity of different umbelliferone analogs

In this work, we demonstrated that Umb facilitated $\cdot\text{OH}$ radical production by catalyzing the decomposition of H_2O_2 to oxide TMB, which is similar to the behavior of fluorescein with intrinsic peroxidase-like activity.¹⁵ This result may derive from the existence of hydroxyl groups on the Umb.¹⁵ We suspect that the 7-hydroxyl group of Umb could contributed to catalyze the oxidation of TMB and the peroxidase-like activity perhaps enhance with an increase of the number of hydroxyl groups of Umb. Thus, the peroxidase activities of four umbelliferone analogs were investigated. The differences among umbelliferone analogs are mainly the numbers and location of the hydroxyl groups on the benzopyrone (Fig. S10). As exhibited in Fig. S11a, the results indicated that the Cou (the 7-hydroxyl group of umbelliferone was replaced by hydrogen), has a lower catalytic activity than that of umbelliferone. This conformed preliminarily to our surmise that the existence of the hydroxyl group could play a crucial role in the peroxidase-like activity. Nevertheless, contrary to our guess, along with the increase in the number of hydroxyl groups and the change of the location of the hydroxyl groups on the benzopyrone, the umbelliferone

analogs (Esc, 4, 7-DHC and 5, 7-DHC) showed a weak peroxidase-like activity, even less than that of the blank system (without any catalysts). The reasons maybe that the umbelliferone analogs besides coumarin with the numbers of hydroxyl groups increased and the location of the hydroxyl groups changed, acting as antioxidants, and possessed free-radical scavenging properties³⁵ that captured the $\cdot\text{OH}$ radical of the reaction system and induced a disappearance of the characteristic absorption peak of oxTMB at 652 nm.

To further investigated this idea, first we carried out the chromogenic reaction of TMB in the assistance of Umb under the optimal conditions; we added different umbelliferone analogs (Esc, 4, 7-DHC and 5, 7-DHC) with different concentrations and incubated for 30 min; finally, we recorded the change of the spectra over 500 – 800 nm. As shown in Fig. S11 (b, c and d), with the increase of the concentrations of Esc, 4, 7-DHC and 5, 7-DHC, the absorbance at 652 nm was dramatically decreased. The results manifested that they could act as antioxidants to lead in the fading of oxTMB. Therefore, it demonstrated that with an increase of the numbers of hydroxyl groups, the peroxidase-like activity of umbelliferone analogs is not as good as expected.

Colorimetric detection of H_2O_2 and glucose

As demonstrated above, concerning the peroxidase mimetic properties of umbelliferone being dependent on the H_2O_2 concentrations, a colorimetric detection method of H_2O_2 was successfully established. Figure S12 (a, b) shows the absorption spectra of chromogenic systems in the presence of H_2O_2 with different concentrations. The absorbance at 652 nm was proportional to the H_2O_2 concentrations from 1.0 to 100.0 μM , accompanying the progression of the blue color (inset in Fig. S12b); and the linear regression equation was $A_{652\text{nm}} = 0.00232C + 0.01197$ ($R^2 = 0.9966$) with a detection limit of

Fig. 3 (a) Typical absorption spectra obtained for the detection of glucose using umbelliferone as a peroxidase mimic under the optimized conditions. (b) Linear calibration plot of glucose detection using umbelliferone as an artificial enzyme.

Table 2 Comparison between the values obtained by our proposed method and hospital for the determination of glucose in serum samples

Sample	Proposed method/mM	RSD, % (<i>n</i> = 3)	Hospital result/mM
1	4.52 ± 0.05	1.4	4.64
2	4.65 ± 0.08	2.4	4.71

0.5 μM ($\text{LOD} = 3\sigma/\kappa$ method, in which σ means the standard deviation of blank experiment ($n = 11$), and κ means the slope of calibration curve), which is comparable to other peroxidase mimic-based methods reported (as shown in Table S1). The good sensitivity to H_2O_2 -based umbelliferone as a peroxidase mimic was a significant prerequisite for H_2O_2 -mediated glucose detection by coupling with glucose oxidase (GOx). The glucose could be catalyzed to generate gluconic acid and H_2O_2 by GOx in the presence of molecular oxygen,³⁶ which is a crucial indicator concerning the diagnosis of diabetes mellitus in clinical medicine. Furthermore, when combined with GOx, a quantitatively colorimetric detection method for glucose can also be established. As shown in Fig. 3, the absorbance at 652 nm was proportional to the glucose concentrations from 5.0 to 100.0 μM , and the linear regression equation is $A_{652\text{nm}} = 1.8727C + 0.0152$ ($R^2 = 0.9975$), with a detection limit as low as 2.5 μM and the color variation can be observed by naked eyes. The detection limit is comparable to the previous reports of state-of-the-art glucose sensors (as shown in Table S2). To evaluate the selectivity of this proposed system, we utilized fructose, lactose, sucrose and maltose as negative controls. The results (Fig. S13) demonstrated that these glucose analogues were negligible compared with that of glucose, even at concentrations of 5-fold higher (except for the maltose with 2-fold higher) than that of 0.1 mM glucose. This indicates that this proposed method had good selectivity for glucose, which could be attributed to the high affinity of GOx for glucose.³⁷

In order to further demonstrate the practical application of the constructed colorimetric approach for glucose in real samples, two serum samples taken from two healthy subjects in our laboratory workers were used for glucose detection; and the volunteers' consent and approval from the Institutional Research

Ethics Committee of Southwest University hospital were obtained for research purposes. As shown in Table 2, the results obtained by our colorimetric method agree satisfactorily with those obtained in the hospital. The biggest relative standard deviation (RSD) is 2.4% for the three independent measurements, demonstrating good reproducibility of the method. The above results imply that the proposed system can be applicable for reliable determinations of glucose in human blood samples.

Conclusions

In summary, we have testified that umbelliferone, a kind of coumarin derivative, could act as a novel type of peroxidase mimic to catalyze the oxidation of the peroxidase substrate TMB in the presence of H_2O_2 to generate a blue product. The catalytic activity of umbelliferone was dependent on the pH, reaction temperature, concentration of H_2O_2 and incubation time. On this basis, a convenient, brief and sensitive H_2O_2 and glucose detection method was successfully established. H_2O_2 can be detected with a linear range from 1.0 to 100.0 μM and a detection limit of 0.5 μM . Coupled with glucose oxidase, a linear range from 5.0 to 100.0 μM and a detection limit of 2.5 μM was obtained for glucose detection. What is more, the demonstrated colorimetric method was applied to glucose determination in human serum, and the values of the glucose levels agree very well with that obtained in hospitals, exhibiting good accuracy and reproducibility. This work not only provides some new insights into seeking for novel molecular enzyme mimetics with excellent catalytic activities, but also expands the application of molecular enzyme mimetics as a substitute of peroxidase-like catalysts.

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Supporting Information

This material is available free of charge on the Web at <http://www.jsac.or.jp/analsci/>.

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