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Improvement of Chloroperoxidase Catalytic Activities by Chitosan and Thioglycolic Acid

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Abstract The chlorination and oxidation activity of chloroperoxidase (CPO) was enhanced 32 and 20% by chitosan, and oxidation activity 24% by thioglycolic acid, respectively. The pH optimum of chlorination reaction shifted 3 units to a less acidic environment by chitosan (from 2.75 to 5.75). The kinetic parameters indicated both the affinity and specificity of CPO to substrate were improved.

Keywords Chloroperoxidase · Activity · Chitosan · Thioglycolic acid · Kinetic parameters

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1 Introduction

Heme enzyme chloroperoxidase (CPO, EC 1.11.1.10), a monomeric glycoprotein isolated from *Caldariomyces fumago* [1], is thought to be the most versatile and unusual enzyme for synthetic applications in the peroxidases superfamily [2–5]. And how to improve the catalytic activities of this promising enzyme attracts a lot of interests now. For example, Site-directed mutagenesis was employed to replace the proximal heme thiolate ligand (C29) and glutamic acid (E183) with histidine residue [6, 7]. E183 and C29 have long been thought to play an essential role in its chlorination and epoxidation activities. However, the analysis of the produced mutants showed that the chlorination, peroxidation, epoxidation, and catalase activities of these recombinant enzymes was not enhanced obviously, and even reduced compared with wild type CPO, except that the epoxidation activity of E183H is enhanced by over 2.5-fold.

Besides the mutagenesis technique, a lot of efforts have been made to enhance the stability and activity of CPO [8–11]. Among these approaches, additive is a simple way that can improve the activity of enzyme. Moreover, there have been many reported additives. For example, the presence of PEG as additive in solution improved catalytic performance of many enzymes, such as glucose-6-phosphate dehydrogenase, and R-chymotrypsin [12, 13]. In addition, it had been reported that chitosan induced a significant increase of the activities of polyphenoloxidase (PPO) and peroxidase (POD) [14]. Thioglycolic acid showed a positive effect on catalase activity [15].

However, little work could be found for the effect of additives on catalytic properties and activity of CPO. In this study, Polyhydroxy compounds, compounds

containing sulfur and nitrogen atom ligand, and some surfactants were investigated in order to find some efficient additives.

2 Experimental

2.1 Chemicals and Instruments

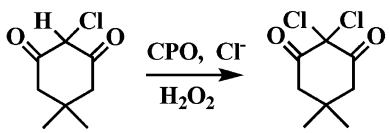
Chloroperoxidase was isolated from the growth medium of *C.fumago* according to the method established by Morris and Hager [1] with minor modifications, using acetone rather than ethanol in the solvent fractionation step. The enzyme had a specific activity of 7,400 U/mL based on the standard MCD assay ($Rz = 1.41$).

Chitosan (90% deacetylated) was purchased from Yuanju Bioengineering Company (Shanghai, China), and it was dissolved in 10% citrate solution at room temperature. Monochlorodimedon (MCD) was obtained from Fluka. Citrate, acetic acid, sodium citrate, amino acids, thioglycolic acid and hydrogen peroxide (30% in aqueous solution) were obtained from Xi'an Chemical Co. Ltd. All chemicals are of analytical grade unless otherwise indicated.

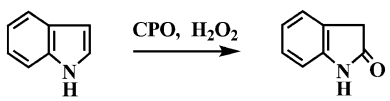
Enzyme assay was performed on a Shimadzu UV-1700 UV–visible spectrophotometer.

2.2 Enzyme Activity Assay

Enzyme activity was based on chlorination assay of a model substrate, monochlorodimedon (MCD) (Scheme 1) and oxidation assay of a substrate, indole (Scheme 2). CPO chlorination activity measurement was determined by following the decrease of absorbance of the substrate MCD at 278 nm, and indole oxidation activity measurement was tested according to the decrease of absorbance of the substrate indole at 270 nm. All measurements were carried out in a 3-mL quartz at room temperature. The chlorination activity is tested in pure phosphate buffer (0.1 M, pH = 2.75) containing 2.5 mM MCD, 20 mM KCl, and 0.1 M H_2O_2 or in the presence of additives, while oxidation activity was tested in 0.05 M pure citrate buffer (pH = 5) with 200 μM indole, 300 μM H_2O_2 or in the presence of additives. The enzymatic reaction was started by addition of 5 μL of CPO buffered stock solution (37 U/mL).



Scheme 1 Chlorination of monochlorodimedon (*MCD*) to dichlorodimedon (*DCD*) catalyzed by chloroperoxidase



Scheme 2 Oxidation of indole to oxindole catalyzed by chloroperoxidase

CPO activity, evaluated by the specific initial reaction rate r (moles of substrstes consumed per unit of time), was calculated from the slope of changes in absorbance versus time. All sets of experiments were reproduced several times under identical operating conditions in order to increase the accuracy of the findings, and each data point of a set of results was obtained at least three times and the discrepancy was below 5%.

2.3 Determination of Kinetic Parameters

The kinetic parameters, K_m and V_{max} values of MCD chlorination and indole oxidation were determined by measuring initial rates of the reactions with hydrogen peroxide as substrate. Michaelis–Menten kinetic characteristics were found both for the chlorination and oxidation reactions. K_m and V_{max} values were calculated from Lineweaver–Burk plots by linear regression analysis.

$$v^{-1} = \left\{\frac{K_m}{V_{max}}\right\}[S]^{-1} + V_{max}^{-1} \quad (1)$$

where $[S]$ was the concentration of substrate, v and V_{max} represented the initial and maximum rate of reaction, respectively.

3 Results

3.1 Effect of Additives on CPO Chlorination Activity

The effect of some additives including polyhydroxy compounds (chitosan, β-cyclodextrin), compounds containing sulfur and nitrogen atom ligand (thioglycolic acid, mercaptopropanoic acid, sodium thiosulfate, DL-threonine, DL-methionine, arginine, tryptophan, cysteine, histidine, glycine, phenylalanine, hydroxylamine hydrochloride, hydrazine sulfate, sodium p-toluenesulfonate) were examined in a 3-mL quartz at room temperature by a Shimadzu UV-1700 UV–visible spectrophotometer.

Among the above additives, only chitosan and thioglycolic acid have positive effect. As shown in Figs. 1 and 2, CPO chlorination activity was enhanced to a maximum 32% compared to that in pure buffer at chitosan concentration of 0.0225 mg/mL, and about 20% at thioglycolic acid concentration of 5 μM.

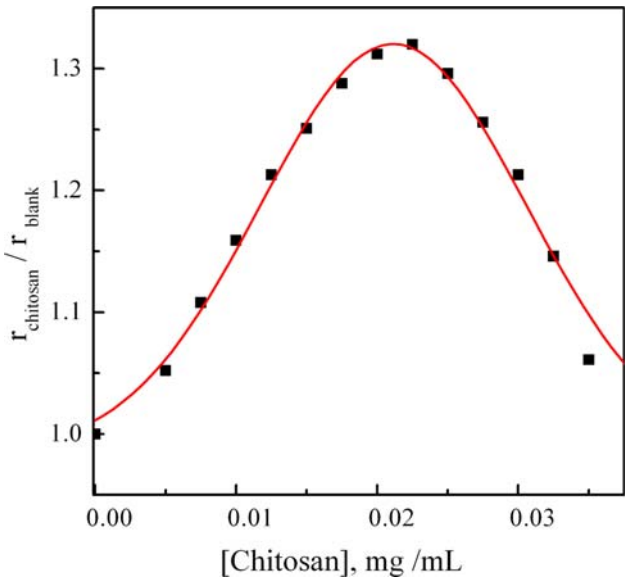


Fig. 1 Effect of chitosan concentration on the chlorination activity of chloroperoxidase in 0.1 M phosphate buffer, pH = 2.75 at room temperature

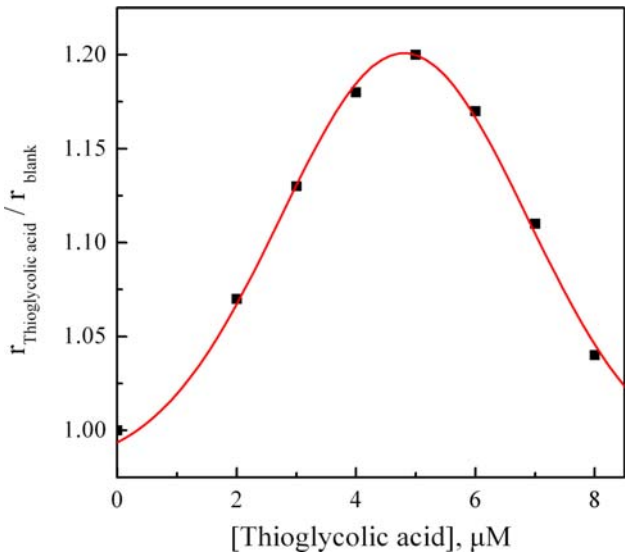


Fig. 2 Effect of thioglycolic acid concentration on the chlorination activity of chloroperoxidase in 0.1 M phosphate buffer, pH = 2.75 at room temperature

3.2 Effect of pH on CPO Chlorination Activity

Curve 3a in Fig. 3 shows that chlorination activity of CPO changed in a pH-dependent manner. The CPO-catalyzed MCD decay decreased with an increase of pH. When chitosan was introduced, it was supposed that the enzymatic activity would change as the dot line as shown in curve 3b in Fig. 3. However, to our surprise, curve 3c in Fig. 3 reveals an obvious difference with our thoughts. The activity began

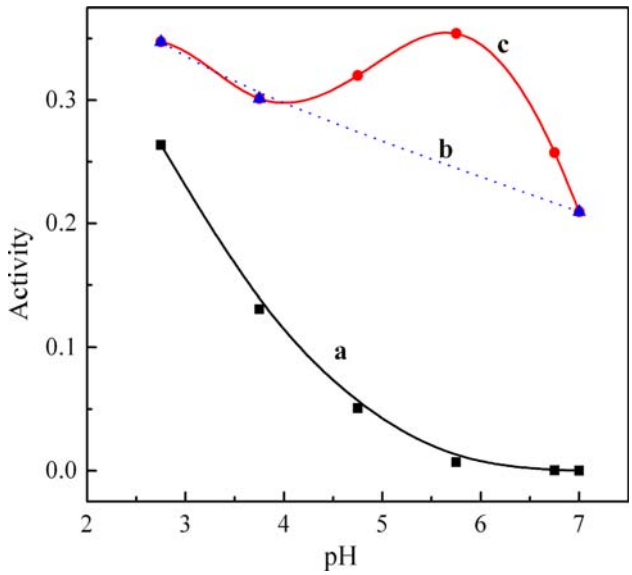


Fig. 3 Effect of pH on the chlorination activity of chloroperoxidase at room temperature

to increase slightly at pH = 4.75 and appeared a maximum at pH = 5.75. As we know, the suitable pH of the chlorination reaction in which the substrates are peroxides and chloride anion, are usually below 4 [16], and the pH optimum of this reaction is very acidic (2.75). However, in this work, the suitable pH extends over a broad range (below 7) in the presence of chitosan, moreover, the optimal pH of the CPO chlorination reaction shifted 3 pH Units to a less acidic environment (from 2.75 to 5.75) by chitosan, which is beneficial to the stability of CPO.

3.3 Effect of Additives on CPO Oxidation Activity

The indole oxidation catalyzed by CPO was investigated both in pure citrate buffer (pH = 5) and in the presence of all the above additives. Only thioglycolic acid can enhance the enzymatic activity to 24% at concentration of 0.5 μM (Fig. 4).

3.4 Kinetic Analysis of Decomposition of Hydrogen Peroxide Catalyzed by CPO

The enzyme "CPO" can catalyzes the decomposition of hydrogen peroxide both in the presence and absence of halogen anion [17]. The complex formation of CPO with hydrogen peroxide was related to the mechanism of the chlorination and oxidation reactions. The kinetic analysis was carried out by adding hydrogen peroxide and chloride salt in relation to the chlorination activity, and adding hydrogen peroxide only to oxidation activity. The initial reaction rates of the catalytic decomposition of the substrate H_2O_2 were measured at different substrate

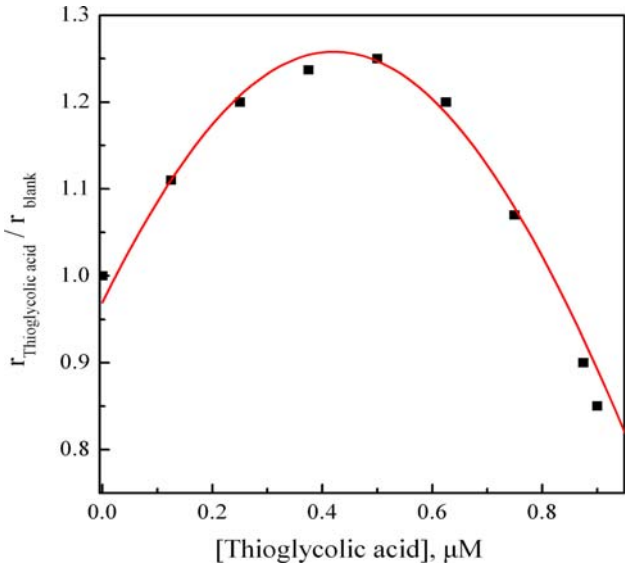


Fig. 4 Effect of thioglycolic acid concentration on the oxidation activity of chloroperoxidase in 0.05 M citrate buffer, pH = 5 at room temperature

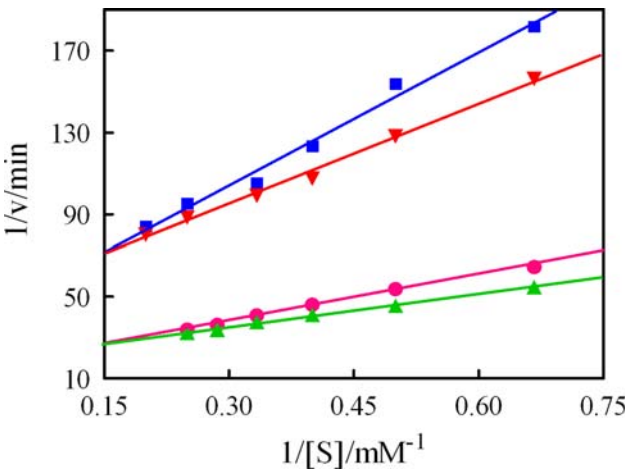


Fig. 5 Lineweaver–Burk plot for the decomposition of hydrogen peroxide (0.05 M) catalyzed by CPO at room temperature: 0.1 M phosphate buffer, pH = 2.75 (■) pure buffer, (▼) thioglycolic acid (5 μM) and 0.05 M citrate buffer, pH = 5 (●) pure buffer, (▲) thioglycolic acid (5 μM)

concentrations in the presence of thioglycolic acid. The Lineweaver–Burk plot of 1/*V* versus 1/*S* was shown in Fig. 5. The kinetic parameters of enzymatic chlorination and oxidation reactions were presented in Table 1.

4 Discussion

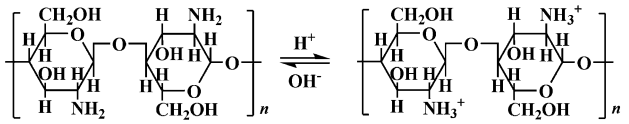
4.1 Chitosan

Chitosan is a natural nontoxic biopolymer, this natural polymer has excellent properties such as biocompatibility, biodegradability, and commercial availability at relatively low cost. In the molecule chains of chitosan, there are high contents of free amine (–NH_2) and hydroxyl (–OH) functional groups which can participate in chemical reactions. In our present work, the results suggest that chitosan could effectively activize chloroperoxidase. Firstly, the –NH_2 in chitosan molecule chains was protonated in acidic system, as described by Scheme 3. The protonated amino groups (–NH_3^+) provided chitosan with adsorption properties, and could attract and enrich Cl^- (substrate) in the vicinity of CPO active site. The close proximity of the chloride to the heme enabled it more easily to act as a donor for Compound I (an oxyferryl porphyrin π-cation radical intermediate $[(Fe^{IV} = O)\bullet]^+$, Scheme 4). Secondly, protonated chitosan as an electron acceptor, could accept the electrons which were given by the penta-coordinate ferric resting state Fe^{III} and the porphyrin. This would make it easier for the formation of Compound I. Thirdly, chitosan has capability of changing the polar property of water molecule by forming H-bonding. As a result, it made the content of helix structure in enzyme protein molecule added and molecule chains of enzyme stretched. These changes were favorable to stabilize the conformation of CPO molecule.

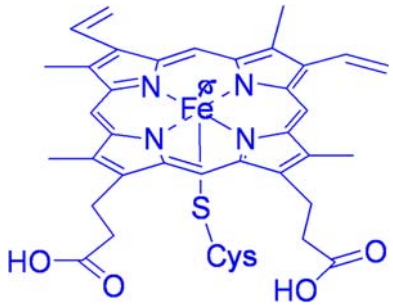
The effect of pH on CPO chlorination activity was interesting. A model for the halogenation reactions has been proposed in which halide ions compete with a carboxyl group for a location near the heme group [16]. At low pH, protonation of the carboxy1 group permits halide ion to supply the negative charge preferred at this location.

Table 1 Kinetic parameters of CPO (for hydrogen peroxide substrate) in the presence of thioglycolic acid ($SHCH_2COOH$) at room temperature (0.1 M phosphate buffer, pH = 2.75; 0.05 M citrate buffer, pH = 5)

Enzyme	V_{max} (min^{-1})	K_m (10^{-3} mol L^{-1})	$k_{cat} \times 10^6$ (min^{-1})	k_{cat}/K_m (μmol L^{-1} min^{-1})
CPO + PBS	0.069	5.34	6.88	1.29
CPO + PBS + $SHCH_2COOH$	0.053	2.89	5.32	1.84
CPO + Citrate buffer	0.024	4.93	2.38	0.48
CPO + Citrate buffer + $SHCH_2COOH$	0.020	3.19	2.05	0.64



Scheme 3 Forms of the chitosan in aqueous solution



Scheme 4 Ironic porphyrin structure of CPO active center

The close proximity of the halide to the heme enables it to act as a donor for Compound I. The low pH requirement for the binding of halide is responsible for the low pH optimum of the halogenation reactions. The protonated chitosan would release H^+ to the solution as shown in Scheme 3 at a higher pH. It was clearly useful to protonate the carboxyl group near the heme group of CPO. This is favorable for the location of chloride ion and acting as a donor for compound I, and promoting the generation of DCD from the substrate MCD.

4.2 Thioglycolic Acid

Thioglycolic acid could enhance the catalase activity because it interacted with tryptophan residue of catalase molecule, and make the electrons transfer to ferric resting state Fe^{III} of ironic porphyrin, which caused the environmental architecture of active center in catalase changed and facilitated to generate compound I [15]. In this work, as we expected, thioglycolic acid has a same positive effect on CPO chlorination and oxidation activities. Thioglycolic acid improved the charge density of ferric ion of CPO active center and made it easier to transfer electrons to antibonding orbital of oxygen atom. Moreover, it can assist cleavage of an O–O bond in H_2O_2 molecule to produce Compound I. However, the mercaptopropanoic acid can not activate CPO activity. Perhaps the longer carbon chain would weaken the electronic effect.

The kinetic parameters (Table 1) of CPO in the presence of thioglycolic acid show that the value of K_m decreased when 5 μM thioglycolic acid was added, which demonstrates CPO has a higher affinity to the substrate H_2O_2. The higher ratio of k_{cat}/K_m increase indicated CPO has a better specificity to the substrate due to the changes of environment around ironic porphyrin. Hence, from the view of dynamics, it could draw a conclusion that thioglycolic acid was able to improve catalytic activity of CPO.

5 Conclusion

In summary, chitosan and thioglycolic acid are found to be efficient additive in the CPO-catalyzed reactions. The chlorination activity was enhanced 32% at chitosan concentration of 0.0225 mg/mL, and 20% at thioglycolic acid concentration of 5 μM compared with that in pure phosphate buffer. The oxidation activity increased 24% at thioglycolic acid concentration of 0.5 μM. Moreover, the suitable pH extends over a broad range (below 7) in the presence of chitosan, moreover, the optimum pH is 5.75 of the CPO chlorination reaction, which shifted 3 pH Units to a less acidic environment (from 2.75 to 5.75) by chitosan and is beneficial to the stability of CPO. The kinetic parameters indicated that both the affinity and specificity of CPO to substrate were improved in the presence of chitosan and thioglycolic acid. The reasons of activation of CPO catalytic activities by chitosan and thioglycolic acid are different.

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References

1. Morris DR, Hager LP (1966) J Biol Chem 241:1763
2. Shaw PD, Hager LP (1961) J Biol Chem 236:1626
3. Adam W, Lazarus M, Saha-Möller CR, Weichold O, Hoch U, Häring D, Schreier P (1999) J Adv Biochem Eng Biotechnol 63:73
4. Dembitsky VM (2003) Tetrahedron 59:4701
5. Kiljunen E, Kanerva LT (2000) J Mol Catal B Enzym 9:163
6. Yi XW, Conesa A, Punt PJ, Hager LP (2003) J Biol Chem 278:13855
7. Rai GP, Sakai S, Flórez AM, Mogollon L, Hager LP (2001) Adv Synth Catal 343:6
8. Franssen MCR, Weijnen JGJ, Vincken JP, Laane C, Vander PHC (1998) Biocatalysis 1:205
9. Tzialla AA, Kalogeris E, Gournis D, Sanakis Y, Stamatis H (2008) J Mol Catal B Enzym 51:24
10. Zhi LF, Jiang YC, Wang YS, Hu MC, Li SN, Ma YJ (2007) Biotechnol Prog 23:729
11. Spreti N, Germani R, Incani A, Savelli G (2004) Biotechnol Prog 20:96
12. Narayanan R, Zhu GY, Wang P (2007) J Biotechnol 128:86
13. Simon LM, Kotorman M, Garab G, Laczko I (2002) Biochem Biophys Res Commun 293:416
14. Liu J, Tian SP, Meng XH, Xu Y (2007) Postharvest Biol Tec 44:300
15. Liu JZ, Yang HY, Zhao JL, Weng LP, Ji LN (1998) Acta Scientiarum Naturalium Universitatis Sunyatseni 37:125
16. Morris DR, Hager LP (1970) J Biol Chem 245:3135
17. Morris DR, Hager LP (1970) J Biol Chem 245:3129