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Activity and Stability of Chloroperoxidase in the Presence of Small Quantities of Polysaccharides: A Catalytically Favorable Conformation Was Induced

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Abstract Chloroperoxidase (CPO) is thought to be the most versatile heme-containing enzyme with enormous applications in organic synthesis, biotransformation, pharmaceutical production, and detoxification of environmental pollutants. Any improvement in the stability of this enzyme will greatly enhance its application in the mentioned areas. In the present study, the effects of three polysaccharides (soluble starch, β-cyclodextrin, and dextrin) on the stability of CPO at elevated temperatures (20, 30, 35, 40, and 50 °C) or in aqueous–organic solvents media (methanol, dioxane, DMSO, and DMF) were investigated. An improved catalytic performance of CPO was observed in the presence of a small amount of the three polysaccharides, where dextrin provided the most effective promotion. The changes of enzyme structure and microenvironment around heme in the presence of additives were studied by fluorescence, circular dichroism, and UV–vis spectra analyses, as

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well as kinetic parameters measurement. A catalytically favorable structure of CPO was induced, including the strengthening of the α-helix structure and more exposure of heme for easy access of the substrate, resulting in an increase of catalytic turnover frequency (k_{cat}) and the improvement of affinity and selectivity of CPO to substrate. The results revealed that the introduction of trace soluble starch, β-cyclodextrin, and dextrin (<10 μmol/L) in reaction media was an effective strategy for the enhancement of the thermodynamic and the operational stability of the enzyme, which are promising in view of the industrial applications of this versatile biological catalyst.

Keywords Chloroperoxidase · Activity · Polysaccharide · Spectroscopy analysis · Kinetic parameters

Introduction

Chloroperoxidase (CPO) is a versatile biocatalyst with extensive catalytic properties, such as halogenase-, peroxidase-, catalase, and cytochrome P450-like activities. It catalyzes the sulfoxidation, epoxidation, dismutation, halogenation, and oxidation of a broad spectrum of compounds [9, 10, 20, 22, 45]. This diversity, coupled with the generally high regio- and enantioselectivity of the enzyme, has made chloroperoxidase a promising catalyst for the asymmetric organic synthesis of industrially relevant products and intermediates. However, the potential industrial application of this enzyme would be limited by the relatively rapid inactivation it undergoes in response to commonly used temperatures, organic solvents, and oxidant concentrations.

Several strategies for improving the catalytic performance and operational stability of CPO were utilized; for example, the use of polyethylene glycol could improve the thermostability of CPO [48, 60]. The immobilization of CPO on silica gel [18, 34, 43, 51], talc [2], mesoporous materials [1, 6, 23–25, 30, 50, 52], and magnetic particles [5, 21, 56] was demonstrated to increase its tolerance to organic solvent, elevated temperature, and low pH. Genetic manipulation of CPO through random mutagenesis [54] and directed evolution [28, 44, 58, 59] could result in variants with enhanced specific activity.

Among these techniques, the introduction of efficient stabilizing additives is the simplest method for improving the catalytic properties and thermal stability of CPO. In this work, we present the investigation of the effect of three polysaccharides (soluble starch, β-cyclodextrin, and dextrin) on CPO activity and stability. The performance of CPO in the presence of polysaccharides was evaluated according to its activity measurement using the chlorination of monochlorodimedon (MCD) to dichlorodimedon (DCD) as a well-functioning model reaction. Though the stabilizing effect of monosaccharides and disaccharides on enzyme has been extensively discussed [7, 8, 12, 16, 19, 29, 32, 33, 38, 40, 42], such as trehalose, sorbitol, mannitol, sucrose, glucose, erythritol, xylitol, and lactitol, polysaccharides were rarely reported [11, 13]. Our results indicated that all of them display positive effects. These findings are promising in view of the potential industrial applications of this versatile biological catalyst due to the low cost, operational simplicity, no toxic effect on product, as well as the environment-friendly nature of these additives. The possible stabilizing effect of polysaccharides on CPO was discussed through a kinetic assay and UV–vis, circular dichroism (CD), and fluorescence spectra analyses.

Materials and Methods

Enzyme and Chemicals

Chloroperoxidase was isolated from the growth medium of *Caldariomyces fumago* according to the method established by Morris and Hager [35] with minor modifications, using acetone rather than ethanol in the solvent fractionation step. The enzyme solution was concentrated to 6.5 mg/mL CPO with R_z=1.18 (R_z=purity standard=A_{398}/A_{280}=1.44 for pure enzyme) and an activity of 7,670 U/mL based on the standard MCD assay [17].

MCD was obtained from Fluka. All other chemicals were purchased from Xi'an Chemical Co. Ltd. They are of analytical grade unless otherwise indicated.

Chloroperoxidase Activity Assay

CPO catalytic activity was determined by the MCD chlorination assay. It was used as a model reaction because an elegant study has been carried out for this reaction system in the absence of polysaccharide [15, 31], and therefore we can focus on how polysaccharide influences the activity of CPO. The activity measurement was based on the loss of absorbance at 278 nm accompanying the conversion of MCD to DCD. The assay mixture contained 25 μM MCD, 2 mM KCl, 200 μM H_2O_2, and 5 μM CPO in 0.1 M phosphate buffer (pH 2.75) in the presence or absence of additives in a total volume of 1 mL. The CPO activity was evaluated by the specific initial reaction rate, ν (moles of MCD consumed per unit of time), which is calculated from the slope of changes (recorded continuously) in absorbance versus time. All sets of experiments were reproduced three times under identical operating conditions in order to ensure the data accuracy, the discrepancy of which was below 5%.

Stability Tests

CPO and corresponding polysaccharides were pre-incubated for 60 min at elevated temperatures (20, 30, 35, 40, and 50 °C) in a closed thermostatic water bath. Then, the thermal stability was determined as a function of additives concentration or of the reaction time in the presence of additives at the indicated concentration and expressed by relative chlorination activity (the ration of activity in the presence of additives to that in pure buffer after being pre-incubated at the same temperature).

In aqueous–organic solvents media, the activity was determined before a pre-incubation of CPO with polysaccharides at 25 °C. The samples contained different amounts of the organic solvent and a fixed concentration of the additive, or contained a fixed concentration of the organic solvent and various amounts of additives. The aliquots were withdrawn at appropriate time points for activity measurement.

UV–Vis Spectra Analysis

The spectra of 5 μM CPO in pure 0.1 M phosphate buffer (pH 5.0) was recorded from 300 to 900 nm by a UV-1700 spectrophotometer (Shimadzu) in a 1-mL quartz cuvette at 25 °C. A pre-incubation for 60 min was needed in the presence of additives.

Circular Dichroism Measurement

The CD spectra of 2 μM CPO in pure 0.1 M phosphate buffer (pH 5.0) were recorded from 190 to 700 nm by a CD spectrometer (Applied Photophysics Ltd.) in a 200-μL quartz cuvette at 25 °C. A pre-incubation for 60 min was needed in the presence of additives.

Fluorescence Spectra Analysis

The fluorescent spectra of 2 μM CPO in pure 0.1 M phosphate buffer (pH 5.0) was recorded by a PE LS55 Fluorescence spectrophotometer (PerkinElmer) in a 100-μL quartz cuvette at 25 °C. The samples were excited at 280 nm and the fluorescence spectra recorded from 320 to 360 nm. A pre-incubation for 60 min was needed in the presence of additives.

Kinetic Parameter Measurements

The kinetic assay for the chlorination of MCD by CPO was carried out over the substrate (Cl^-) concentration range of 0.1–50 mM. Besides Cl^-, the assay mixture contained 5 μM CPO, 25 μM MCD, and 200 μM H_2O_2 in 0.1 M phosphate buffer (pH 2.75) in the absence or presence of additives at their optimum concentration. The reaction follows Michaelis–Menten kinetic characteristics. Kinetic parameters K_m and V_{max} values were obtained by a linear regression analysis of the double-reciprocal Lineweaver–Burk plots. The turnover number, k_{cat}, was calculated according to the following equation:

$$V_{max} = k_{cat}[E_0]$$

where E_0 was the initial concentration of CPO.

Results and Discussion

Dependence of Enzyme Activity on the Concentration of Polysaccharides

An enhancement of CPO activity was achieved in the presence of polysaccharides, and this enhancement depended greatly on the additive concentration. As shown in Fig. 1, a bell-shaped behavior was observed. The highest relative chlorination activity of CPO (ratio of activity in the presence and absence of additives) was enhanced 1.32 times at a starch concentration of 5 μmol/L, 1.35 times at a β-cyclodextrin concentration of 8.5 μmol/L, and 1.58 times at a dextrin concentration of 14 μg/mL. Dextrin showed the most efficient effect.

Effect of Polysaccharides on CPO Tolerance to Hydrogen Peroxide

MCD chlorination catalyzed by CPO with different concentrations of the oxidant (H_2O_2) in the presence of polysaccharide at its optimum concentration (as indicated above) was investigated. The results of are summarized in Fig. 2. Unfortunately, the tolerance of CPO to H_2O_2 remained 200 μmol/L in the presence of additives, just as in the pure buffer.

The poor stability of CPO to H_2O_2 has been well elucidated [14, 41, 47], which is primarily due to its sensitivity to H_2O_2 in the presence of a reducing substrate. CPO is firstly oxidized by H_2O_2 into an oxyferryl porphyrin π-radical cation intermediate (compound I). In the next step, the substrate is oxidized by a one-electron transfer process and compound II is formed. The main problem causing inactivation is in the next step in

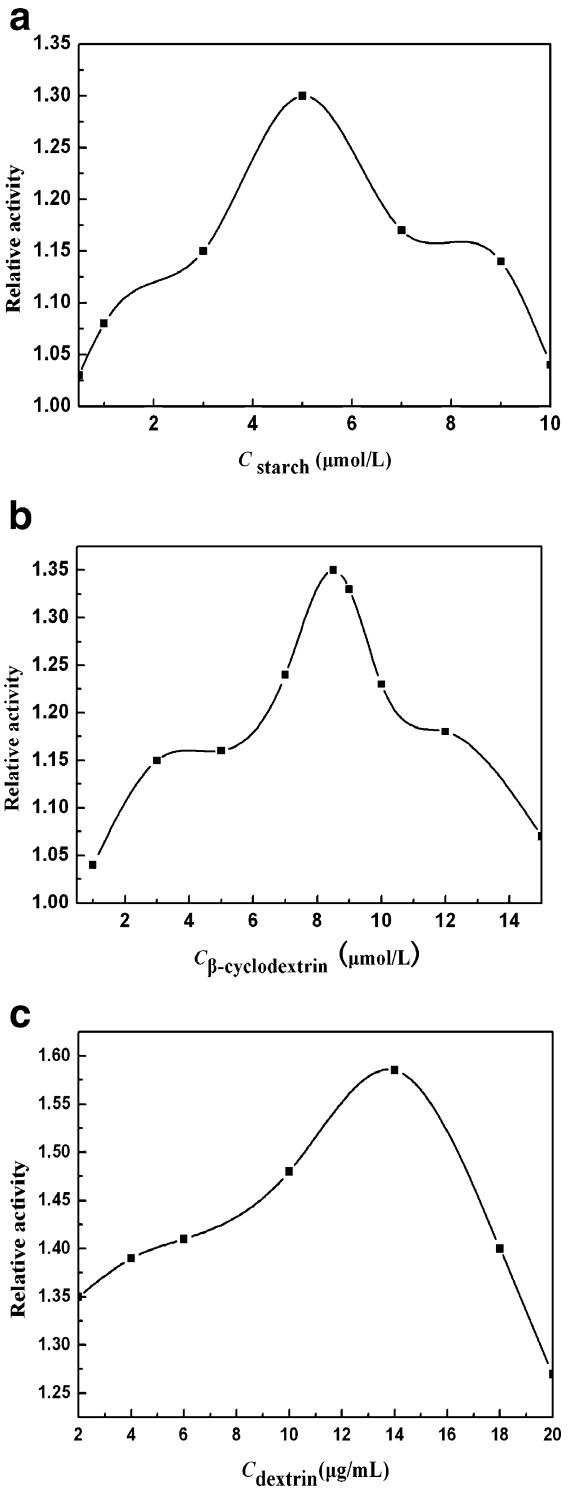


Fig. 1 Effect of additive concentration on the chlorination activity of CPO in 0.1 M phosphate buffer at 25 °C (pH 2.75), expressed as relative activity (ratio of CPO activity in the presence of additive to that in pure buffer). **a** Soluble starch. **b** β-cyclodextrin. **c** Dextrin

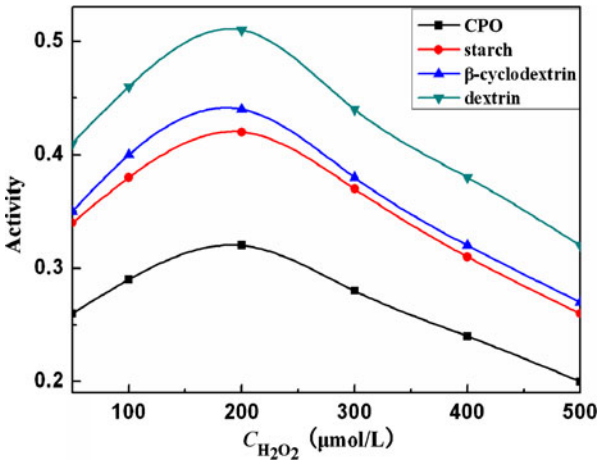


Fig. 2 Hydrogen peroxide tolerance of CPO in the presence of additives at their optimum concentration (5 μmol/L soluble starch, 8.5 μmol/L β-cyclodextrin, and 14 μg/mL dextrin, respectively) or in pure buffer

which the substrate competes with H_2O_2 for compound II. When compound II reacts with H_2O_2, this would lead to the conversion of the prosthetic group into the highly reactive peroxy-iron(III) porphyrin species (compound III). Compound III could decompose in at least three different ways. One is that the peroxyl radical oxidizes the porphyrin ring, resulting in heme degradation through the formation of an open-chain tetrapyrrole structure and the consequent release of free iron ions into the solution [53]. The second possibility was that compound III returned to its native form after the oxidation of the surrounding protein molecule so that an oxidized amino acid side chain is formed. The third possibility was the release of peroxyl radical, which can also attack other proteins and produce oxidative inactivation. Our results supported the first possible way of compound III decomposition. The crystal structures of CPO [49] showed that the heme edge was not accessible directly, and there was a small opening above the heme which could allow access of substrate to the active center. But the size and structure of the substrates would be restricted by this channel. The impossible access of polysaccharide to the CPO active site through the substrate channel due to steric hindrance made it impossible for polysaccharide to hold down the oxidation of the porphyrin ring. Moreover, if the second and third ways were true, the oxidation damage of the surrounding protein molecule should be inhibited by the additives because of the protection of polysaccharides forming a glass state around the enzyme molecule surfaces [26]; however, this result was not presented in our results. The conclusion that the main molecular event during the peroxide-mediated inactivation of chloroperoxidase was heme destruction has recently been testified [4].

Stabilized Effect of Polysaccharides on CPO Activity at Elevated Temperatures

Moderately high temperatures are often needed in industrial application in order to accelerate the reaction rate, increase solubility, reduce solution viscosity, and avoid microbiological pollution. However, thermo-inactivation is often a dilemma in enzyme industrial applications [3, 37, 46]. How to improve the thermostability of enzymes is a very important aim, but also a challenge.

The effect of polysaccharides on the thermal stability of CPO was investigated as a function of additive concentration at temperatures of 20, 30, 40, and 50 °C, respectively. Compared with the slightly enhanced activity in room temperature, the protective effect of

polysaccharides on CPO activity was more attractive at higher temperatures, shown in Fig. 3a–c in the presence of starch, β-cyclodextrin, and dextrin, respectively. CPO chlorination activity was 1.7, 1.43, and 2.29 times at 35 °C in the presence of starch, β-cyclodextrin, and dextrin. Even at 40 °C, the activity was still higher than that at 20 °C. However, a promotion on enzymatic inactivation at 50 °C was observed when additive concentration was low. The remaining activity of CPO via incubation time at 50 °C in the presence of polysaccharides at their optimum concentration or in pure buffer was presented in Fig. 4.

The effects of polysaccharides on CPO stability may be due to the following three aspects: (1) the hydroxyl group in polysaccharides would replace water to bind with amide located at the molecular surface at multipoints [11]. This binding resulted in a more rigid structure of the protein through non-covalent multipoint cross-links, reducing the protein chain mobility of CPO and so increasing its optimum temperature and its resistance to thermo-deactivation. In the present study, the results indicated that dextrin, with lower molecular weight and more open-chain structure compared with the other two additives, has a stronger binding ability and, therefore, has a more efficient stabilization effect on CPO thermostability. (2) In a number of cases, irreversible thermo-inactivation of enzymes has been attributed to various polymolecular processes, including aggregation [36]. It is reported that apoenzyme (control) is much more sensitive toward aggregation as compared with holoenzyme [32]. This may be due to the formation of disulfide bonds (S–S), which has been suggested to cause aggregation. It is possible that the lowering of aggregation in the presence of sugars is, at least partly, due to an increase in the cohesive force of the medium and the lowering of protein diffusion. (3) Polysaccharides would further form a glass state around the enzyme molecules [26] (Scheme 1) to prevent the enzyme from losing activity caused by exposure to high temperatures.

Effects of Polysaccharides on CPO Stability Against Organic Solvents

CPO-catalyzed reactions are in an aqueous solution [48, 55]. But the yield of products is usually not satisfactory due to the low solubility of organic substrates in aqueous solution, which often limits CPO for broader applications. Therefore, the introduction of an organic solvent into the enzymatic reaction media as a co-solvent is regarded as a good strategy to make the substrate-to-enzyme contact more efficient. However, according to Khmelnitsky et al. [27], organic solvent molecules tend to displace water from the hydration shell of the proteins, which would result in an inactivation of the enzyme. In a previous study, we demonstrated [57] that the CPO-catalyzed epoxidation of 3-chloropropene was inhibited when organic solvents were used as a co-solvent, such as DMSO, DMF, CH_3OH, CH_3CN, and CH_3OCH_3.

The activity of CPO in organic–water binary solvent was investigated as a function of the organic solvent (methanol, dioxane, DMSO, and DMF) content in the presence of polysaccharides at their optimum concentration at 25 °C (expressed as residual activity), shown as Fig. 5. Moreover, the CPO stability via incubation time in the presence of polysaccharides in media containing 25% (*v*/*v*) methanol, dioxane, DMSO, and DMF was investigated and presented in Fig. 6 with water–DMSO as an example.

The extent of stabilization of polysaccharide on the enzyme depended on its structure. The function of polysaccharide was postulated mainly as: (1) polysaccharides interacted with water molecules to be hydrated. This process would decrease the water activity and modify the physicochemical properties of the media, which was favorable for the stability of enzymes. (2) These highly polar polyhydroxy compounds around the enzyme surface

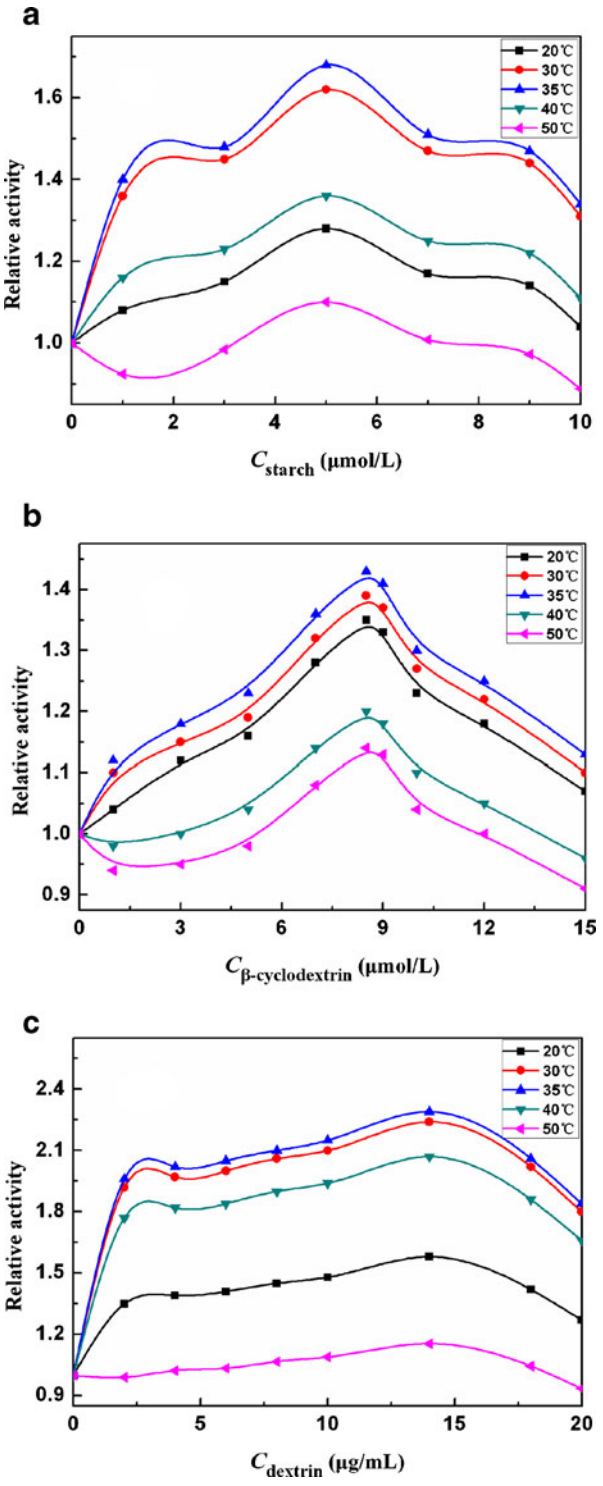


Fig. 3 Dependence of the relative activity of CPO (ratio of CPO activity in the presence of additive to that in pure buffer at the same temperature) on additive concentration at elevated temperatures. **a** Soluble starch. **b** β-cyclodextrin. **c** Dextrin

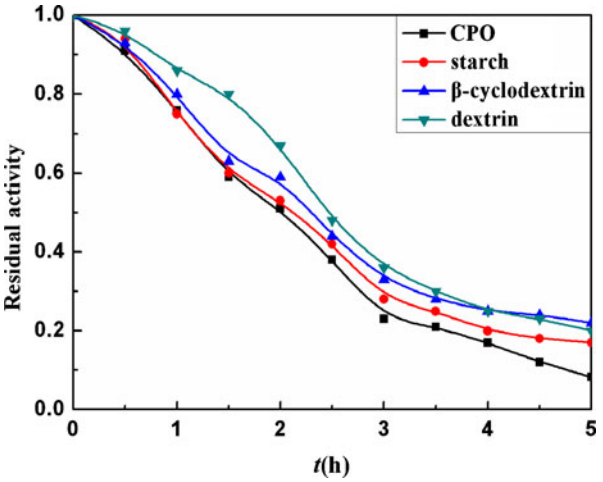


Fig. 4 Remaining activity of CPO via incubation time at 50 °C in the presence of additives at their optimum concentration (5 μmol/L soluble starch, 8.5 μmol/L β-cyclodextrin, and 14 μg/mL dextrin, respectively) or in pure buffer

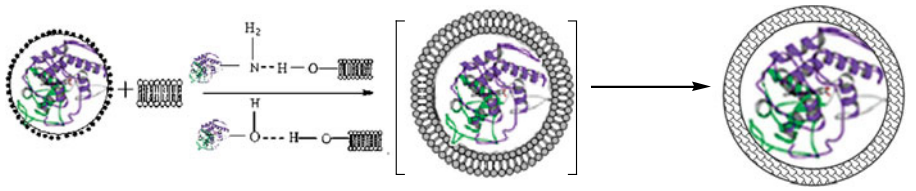
probably protected the native structure of CPO in organic media. (3) Polysaccharide could increase the hydrophobicity of the enzyme's surface, preventing hydrophilic CPO from inactivation due to exposure to hydrophobic organic solvents.

Structure Changes of CPO in the Presence of Polysaccharides

Fluorescence Spectroscopy Analysis

The CPO samples were excited at 280 nm, and the fluorescence emission showed a maximum around 340 nm caused mainly by five Trp residues of CPO [39].

The spectrofluorometric titration results in Fig. 7 showed a minor increase of fluorescence emission with the increase of additive concentration up to their optimum concentration. No concomitant red shift, caused by an increase in the polarity of the environment, was observed. Most fluophor in CPO situated deeply inside the protein molecule, surrounded by a hydrophobic environment, so no red shift of the emission peak



A: native CPO with a hydration layer

B: polysaccharide

C: CPO with a protective cover formed by polysaccharide

D: the protective cover turned into glass state at high temperature

Scheme 1 Proposed stabilized effect of polysaccharides

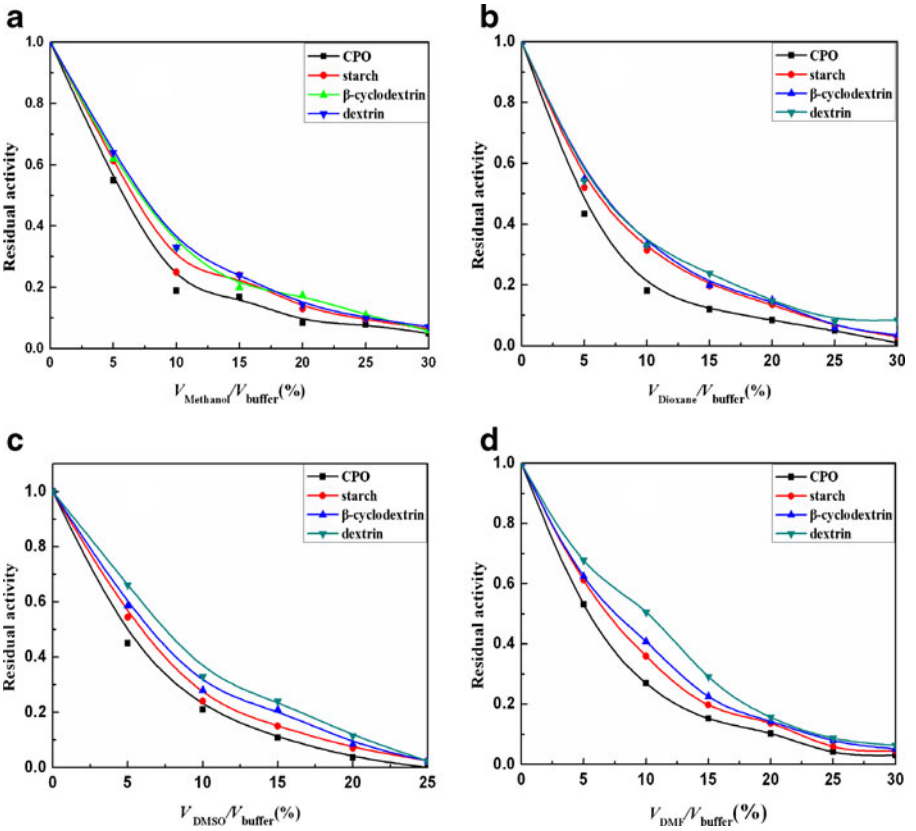


Fig. 5 Residual activity of chloroperoxidase via organic solvent percentage in aqueous–organic solvent media in the presence of additives at their optimum concentration (5 μmol/L soluble starch, 8.5 μmol/L β-cyclodextrin, and 14 μg/mL dextrin, respectively) or in pure buffer. **a** Methanol. **b** Dioxane. **c** DMSO. **d** DMF

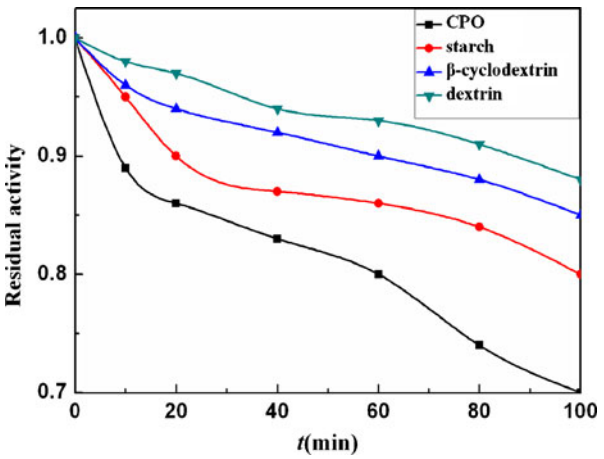


Fig. 6 Residual activity via incubating time in aqueous–organic solvent media in the presence of additives at their optimum concentration (5 μmol/L soluble starch, 8.5 μmol/L β-cyclodextrin, and 14 μg/mL dextrin, respectively) or in pure buffer. The percentage of organic solvent in aqueous–organic solvent media was 25% (*v*/*v*)

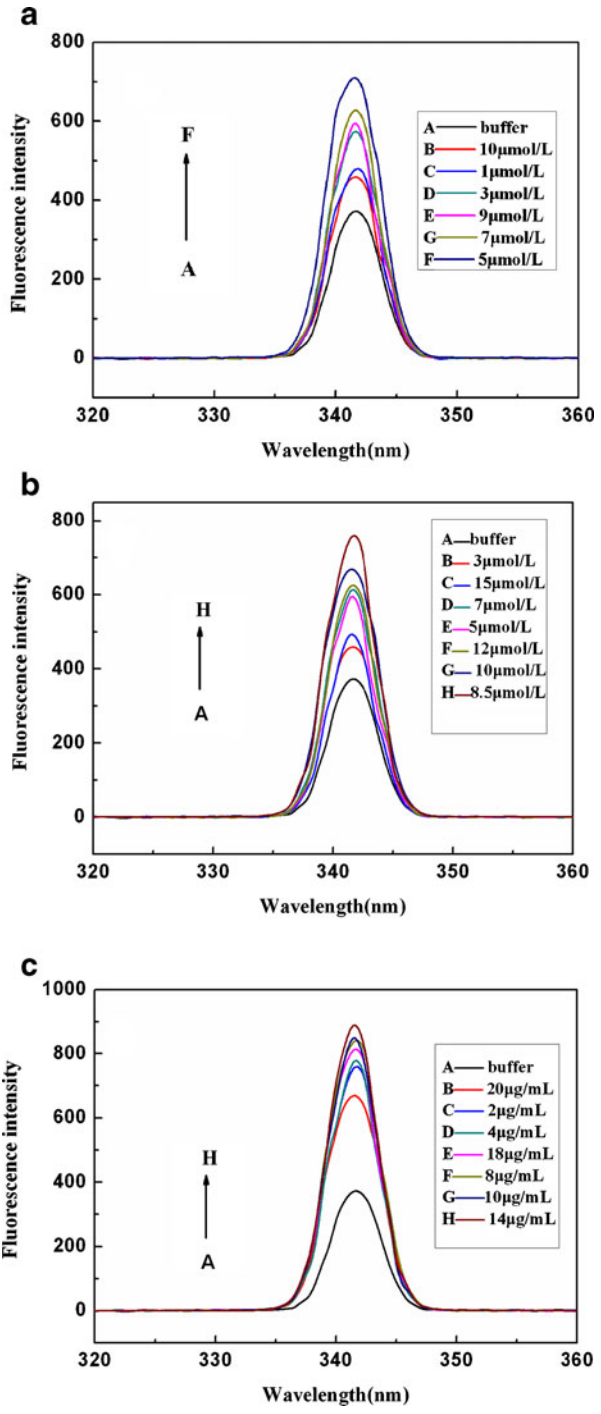


Fig. 7 Fluorescence spectra of CPO in the presence of soluble starch, β-cyclodextrin, and dextrin at different concentrations or in pure buffer (0.1 M phosphate, pH 5.0) at 25 °C. **a** Soluble starch, **b** β-cyclodextrin. **c** Dextrin

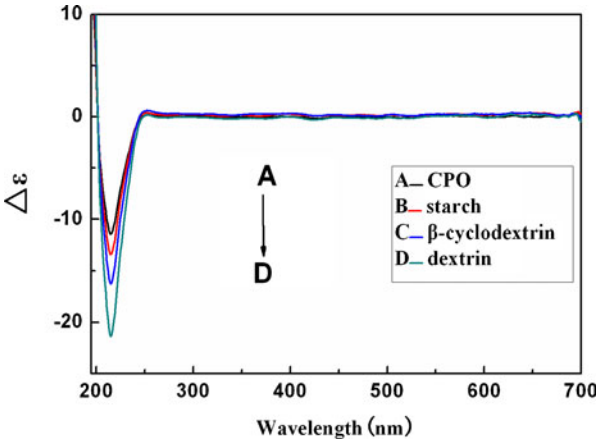


Fig. 8 CD spectrum of CPO in the presence of additives at their optimum concentration (5 μmol/L soluble starch, 8.5 μmol/L β-cyclodextrin, and 14 μg/mL dextrin, respectively) or in pure buffer (0.1 M phosphate, pH 5.0) at 25 °C

indicating no uncoiling of the α-helix occurred in the presence of polysaccharide. The increase of fluorescence yield could be attributed to the energy transfer from Tyr to Trp, resulting in a fluorescence quenching of Tyr while increasing Trp fluorescence. This implied that the α-helix secondary structure of CPO was strengthened, so the distance between Tyr and Trp decreased. This was also confirmed by CD analysis.

Circular Dichroism Spectroscopy Analysis

CD spectroscopic data were used for the detection of the changes in the heme protein structure [19], as follows: (1) far-UV (200–250 nm) CD: changes in the secondary structure of the protein; (2) near-UV (250–320 nm) CD: changes in the tertiary structure of the protein; (3) CD at 320–450 nm: the structural changes of the protein surrounding the heme.

As shown in Fig. 8, the far-UV CD spectroscopy of CPO had a large negative band around 208 nm, which indicated that CPO was a typical α-helix protein. This negative absorption became stronger when polysaccharide was added. This increase was due to the strengthening of the α-helix structure of CPO (shown in Table 1), which was consistent with the result from the fluorescence assay. Both the changes of tertiary structure (250–350 nm) and the structural changes surrounding the heme were very tiny in the CD spectroscopy.

UV–Vis Spectroscopy Analysis

Native CPO has a maximum absorption at 398 nm, named Soret band, which is the characteristic π–π^* electron transition of the porphyrin ring (Fig. 9). The absorption of the Soret band was increased compared with that in pure buffer with no concomitant shift,

Table 1 Content (α-helix, β-Turn and Rndm.Coil) of CPO by CD spectra analysis using Deconvolution software in the presence of additives or in pure buffer (0.1 M phosphate buffer, pH 5.0 at 25 °C)

	CPO (%)	CPO+starch (%)	CPO+cyclodextrin (%)	CPO+dextrin (%)
α-Helix	90.3	92.5	95.4	98.1
β-Turn	5.5	4.8	3.4	1.5
Rndm.Coil	4.2	2.7	1.2	0.4

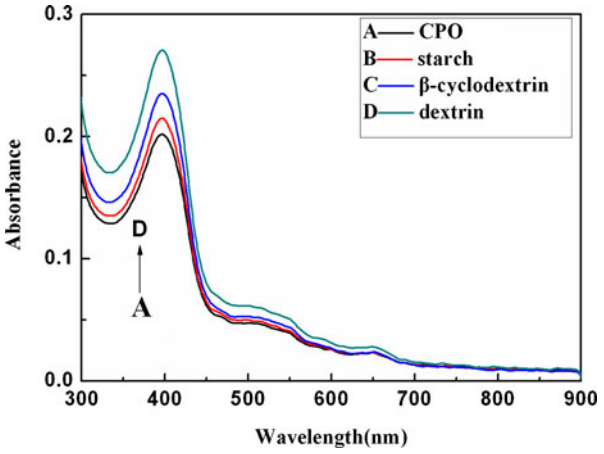


Fig. 9 UV–vis spectra of CPO in the presence of additives at their optimum concentration (5 μmol/L soluble starch, 8.5 μmol/L β-cyclodextrin, and 14 μg/mL dextrin, respectively) or in pure buffer (0.1 M phosphate, pH 5.0) at 25 °C

which indicated that the additives did not bind at heme or at other positions inside the active center, so we conclude that presumably, this increase was caused by the change of microenvironment around heme; that is, heme became more exposed for easier access in the presence of polysaccharides, so it was easier for CPO to bind with substrates in such an environment.

Dextrin showed the most favorable data, which indicated that dextrin was the most efficient additive. This conclusion was consistent with the one drawn from the activity measurements,

Kinetic Parameters Analysis

The kinetic parameters of MCD chlorination were measured and listed in Table 2, including apparent Michaelis constant ($K_{m,\ app}$, this enzymatic reaction had two substrates and the Michaelis constant measured under the particular conditions of a defined concentration of the invariant substrate), catalytic turnover frequency (k_{cat}), and second-order rate constants (k_{cat}/K_m). Compared with that in aqueous buffer solutions, $K_{m,\ app}$ decreased while k_{cat} and k_{cat}/K_m increased in the presence of polysaccharide. The enhanced k_{cat} corresponded to the improved catalytic performance of CPO. The reduced $K_{m,\ app}$ showed that the affinity between the enzyme and substrate increased, while the augment of k_{cat}/K_m indicated that the specificity of CPO to the substrate was improved in these media containing polysaccharide. The changes of kinetic parameters were different, and dextrin had the best performance.

Table 2 Kinetic parameters of CPO in the presence of additives or in pure buffer (0.1 M phosphate buffer, pH 2.75 at 25 °C)

Enzyme	K_m (mol L^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (mol^{-1} L s^{-1})
CPO	1.87×10^{-2}	7.07×10^{3}	3.79×10^{5}
CPO+starch	1.42×10^{-2}	1.03×10^{4}	7.24×10^{5}
CPO+β-cyclodextrin	1.13×10^{-2}	1.15×10^{4}	1.02×10^{6}
CPO+dextrin	6.02×10^{-3}	1.20×10^{4}	1.98×10^{6}

Substrate concentration range (Cl^-) was 10–200 mM

Meanwhile, it has been reported that the final halogen ion transfer occurs outside the CPO active site [31], which is a non-enzymatic process. So here, the effects of polysaccharides may be related to the two cases: (1) the improvement of conditions of the non-enzymatic chlorination by HOCl and (2) the rate of HOCl formation by the enzyme. We probed this problem by mixing HOCl and MCD in the absence and presence of the different polysaccharides. No obvious change of reaction rate was found, which indicated that the effect of additives should be related to the enhancement of HOCl formation by CPO due to the improved affinity and specificity of CPO to Cl^- in the presence of polysaccharides. This is consistent with the above observation of kinetic parameters analysis.

Conclusions

On the whole, the presented study showed that the catalytic performance of CPO, including its activity and stability against elevated temperatures or organic solvents, was efficiently improved by the introduction of polysaccharides (soluble starch, β-cyclodextrin, and dextrin) as additives, in which dextrin had the best performance. The promotion depended on the additives' concentrations.

The UV–vis, CD, and fluorescence spectroscopy of CPO in the presence of polysaccharides indicated that the α-helix secondary structure of CPO was strengthened and that heme became more exposed for easier access of substrates to the active site in CPO, which corresponded to the enhanced catalytic turnover number (k_{cat}).

The kinetic parameters showed that both the affinity and specificity of CPO to the substrates were improved in the presence of all the three additives.

These three additives are conveniently commercially available with low cost, so this strategy for the promotion of enzyme performance is promising in view of the industrial applications of this versatile biological catalyst.

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