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Preparation and application of cross-linked aggregates of chloroperoxidase with enhanced hydrogen peroxide tolerance

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ABSTRACT

A cross-linked enzyme aggregate (CLEA®) of chloroperoxidase (CPO) was created that exhibited greatly improved stability in the presence of hydrogen peroxide concentrations as high as 1.2 M. The CPO-CLEA was generated by oxidizing the protein with sodium periodate and precipitating and cross-linking with ammonium sulfate and sodium borohydride. CLEA® production parameters, including the concentrations of these three reagents, were optimized to maximize the activity of the biocatalyst in oxidizing 7-azaindole to 7-azaoxindole. Additionally, the in situ production of the CLEA® was demonstrated, resulting in a process for converting >90% of 5 g/l 7-azaindole in <1 h while requiring neither gradual peroxide addition nor immobilized enzyme isolation.

1. Introduction

Chloroperoxidase is an exceptionally versatile biocatalyst, catalyzing the sulfoxidation, epoxidation, dismutation, halogenation and oxidation of a broad spectrum of compounds [1–3]. This diversity, coupled with the generally high regio- and enantio-selectivity of the enzyme, have made chloroperoxidase a potentially very attractive tool for the asymmetric organic synthesis of industrially relevant products and intermediates. The primary obstacle to the widespread application of the biocatalyst is the relatively rapid inactivation that it undergoes in response to commonly used pH levels, temperatures and organic solvent and oxidant concentrations. While pH, temperature and solvent parameters can be readily adjusted to increase the stability of the enzyme, the high degradation rate of the enzyme heme in the presence of even small 30 μM concentrations of hydrogen peroxide [4] poses a greater challenge.

To alleviate the toxic effect that peroxide has on chloroperoxidase, many methods have been explored as means for maintaining a low level of oxidant in the reaction system. These include the continuous [5] or sensor-controlled [6] addition of hydrogen peroxide, and the in situ formation of peroxide through enzyme-catalyzed glucose oxidation [7], autoxidation of a sacrificial reductant [8] or electrochemical generation [9]. In addition to requiring additional instrumentation and raw materials, these techniques also have potential disadvantages associated with volume expansions, byproduct formation and locally high peroxide concentrations.

As an alternative to the modification of the reaction system, the biocatalyst itself may also be modified to enhance its stability. To this end, the additive use of polyethylene glycol has been shown to improve the thermostability of chloroperoxidase [10,11]. Immobilization of the enzyme on silica has been demonstrated as increasing its solvent [12,13], temperature [14] and pH [15] tolerance. Similar benefits were seen in modifying the chemical makeup of the biocatalyst through alkylation, amidation and cross-linking reactions [16]. Also, genetic manipulation of the enzyme through random mutagenesis [17] and directed evolution [18] has resulted in variants with greater activity at high solvent concentration. Although these improvements certainly provide significant benefits, only one has been described as also increasing the absolute stability of the biocatalyst in the presence of increasing peroxide concentrations [15].

Another recently developed biocatalyst modification technique involves the formation of cross-linked enzyme aggregates (CLEAs) [19] that in many cases have been shown to possess superior properties to those of the native enzymes [20]. In this process, protein molecules are precipitated and chemically bonded to one another through, for example, the addition of glutaraldehyde. The use of glutaraldehyde to cross-link solubilized chloroperoxidase [16] or to create cross-linked crystals [21] has been successfully demonstrated in previous works, however in these cases either the majority of the enzyme remained in a water-soluble form or enzyme crystallization was required prior to cross-linking. In this work, we present the first production of chloroperoxidase CLEAs

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(CPO-CLEAs), and show that they possess very high tolerance of large peroxide concentrations. In thus eliminating the need for a gradual introduction of oxidant to chloroperoxidase-catalyzed reactions, their operational simplicity, as well as industrial potential, are significantly increased. Additionally, in describing what we believe to be the first example of in situ CLEA® formation, we have removed all steps associated with the isolation of the new catalyst form, further streamlining the process.

2. Experimental

2.1. Enzymes and chemicals

Chloroperoxidase from *Caldariomyces fumago* was obtained in the form of a 25 kU/ml solution from Codexis (Redwood City, CA). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Chloroperoxidase activity assay

A pH 3.5 solution of 500 μM 2-chloro-5,5-dimethyl-1,3-cyclohexamide (monochlorodimedone), 20 mM potassium chloride, 2 mM hydrogen peroxide and 50 mM citrate was prepared. To 190 μl of this mixture was added 1–10 μl of a chloroperoxidase sample solution with an expected activity concentration of approximately 5 U/ml. The rate of change in absorbance of the resulting reaction as measured at 278 nm is directly proportional to the actual enzyme activity present in the chloroperoxidase sample.

2.3. CPO-CLEA production with glutaraldehyde cross-linking

A mixture of 90 μl acetonitrile, 7 μl chloroperoxidase solution and 3 μl 5 M glutaraldehyde was prepared and aged for 1 h at room temperature with occasional gentle stirring, causing the formation of an enzyme aggregate. The mixture was then diluted with the addition of 900 μl of 100 mM pH 4 citrate buffer, vortexed for 10 s and microfuged for 2 min. The observed dissolving of the enzyme aggregate upon vortexing and microfuging was indicative of incomplete cross-linking of the protein.

2.4. CPO-CLEA production with periodate oxidation

A mixture of 40 μl each of chloroperoxidase solution and 33 mM sodium periodate was aged for 1 h at 4 °C. The temperature was then increased to room temperature and 720 μl of a pH 8.1 saturated ammonium sulfate solution was added. After an additional hour with mixing, 40 μl of 33 mM sodium borohydride was added and the mixture was further stirred for 1 h prior to microfuging for 1 min. The supernatant was removed and the resulting CPO-CLEA pellet was stored at 4 °C.

2.5. Oxidation of 7-azaindole with in situ generated CPO-CLEA

A mixture of 1.7 ml each of chloroperoxidase solution and 33 mM sodium periodate was aged for 1 h at 4 °C. The temperature was then increased to room temperature and 30.6 ml of a pH 8.1 saturated ammonium sulfate solution was added. After an additional hour with mixing, 1.7 ml of 33 mM sodium borohydride was added and the mixture was further stirred for 1 h. Starting material was then added in the form of a solution of 1 g 7-azaindole in a mixture of 41.7 ml *tert*-butanol and 125 ml of 0.1 M pH 4.5 acetate buffer. The reaction was begun with the addition of 21.7 ml of 11.7 M hydrogen peroxide. A conversion of >90% 7-azaindole to 7-azaoxindole as assayed by HPLC and NMR was achieved in 50 min.

3. Results and discussion

3.1. Chloroperoxidase cross-linking with glutaraldehyde

Development of a CPO-CLEA production protocol was begun by following published generalized procedures for precipitant selection and glutaraldehyde concentration optimization [22]. In a screen of eight precipitants – ethanol, isopropanol, *tert*-butanol, acetone, acetonitrile, dimethoxyethane, saturated ammonium sulfate and polyethylene glycol PEG200 – all but ethanol and dimethoxyethane were found to give solid enzyme aggregates that retained >90% activity upon resolubilization through dilution of the precipitant. However, when these aggregates were treated with glutaraldehyde at concentrations as high as 1 M prior to this dilution and a centrifugation, no significant solids could be isolated. Also, the majority of the initial enzyme activity could be accounted for in the resulting supernatants. Together these findings suggest that minimal cross-linking of the aggregates is taking place.

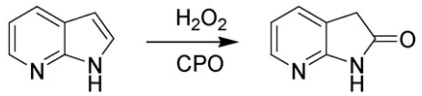
The formation of CLEA® enzyme preparations through this method is expected to occur through the covalent bonding of the amine functionality of lysine residues of the protein with the aldehyde functionality of glutaraldehyde. The number and frequency of lysine constituents of the chloroperoxidase amino acid sequence are listed in Table 1. In comparing these with analogous values in the table for other enzymes that have been previously used to generate CLEA® biocatalysts, it can be seen that chloroperoxidase contains less lysine. This is true of both the percentage of the sequence that is lysine, and to an even larger extent the absolute number of lysine residues. It is therefore reasonable to assume that there are fewer opportunities for the chloroperoxidase protein to react with the glutaraldehyde cross-linker to fix the enzyme molecules into a water-insoluble aggregate configuration.

3.2. Chloroperoxidase cross-linking following oxidation

An alternative methodology for enzyme aggregate cross-linking was then used in which the enzyme is first treated with an oxidant to generate aldehyde groups on the protein. The enzyme is then

Table 1
Lysine and serine contents of enzyme proteins used in CLEA® preparations

Enzyme	NCBI sequence ID	Lysine residues (number)	Lysine residues (% of total)	Serine residues (number)	Serine residues (% of total)
M. esculenta hydroxynitrile lyase [23]	P52705	20	7.8	10	3.9
E. coli penicillin acylase [19]	AAA2434	44	5.2	44	5.2
P. fluorescens nitrilase [24]	AAW79573	12	3.4	17	4.9
A. niger phytase [22]	AAU93518	16	3.4	53	11.3
C. antarctica lipase B [22]	CAA83122	11	3.2	32	9.4
R. erythropolis alcohol dehydrogenase [22]	AAN73270	11	3.2	16	4.6
A. niger glucose oxidase [22]	ABG66642	16	2.8	35	6.0
C. fumago chloroperoxidase	CAA77015	4	2.3	12	6.9



Scheme 1. Oxidation of 7-azaindole to 7-azaoxindole with chloroperoxidase.

precipitated with ammonium sulfate and a reductant is added to cause the aldehydes of the enzyme to bond with the amines of the precipitant [25]. In this new protocol, the sites of cross-linking on the protein surface are alcohol-containing serine amino acids that have been oxidized to yield aldehydes. Because chloroperoxidase contains three times as many serine constituents as it does of lysine, as is shown in Table 1, this approach had the potential to remedy the incomplete cross-linking observed earlier.

In addition, chloroperoxidase has been shown to be a heavily glycosylated protein with 25–30% of the molecule comprised of carbohydrate [26], and an oxidant is at least as likely to create new aldehyde moieties through the cleavage of these glycols as it is through reactions with the serine residues. These new aldehydes are then free to also form imines with the amine precipitant, or to create acetals with alcohols of the glycoprotein, polymerizing carbohydrates associated with the same or different enzyme molecules [27]. In either case, the end result is again the desired cross-linking of the enzyme aggregate.

As with the glutaraldehyde cross-linking procedure, when the enzyme was aged sequentially with sodium periodate, ammonium sulfate and sodium borohydride a solid aggregate resulted. However, in this case the aggregate remained a solid after being placed in an aqueous environment. To test this CLEA® enzyme preparation for activity, the solid was isolated and used to catalyze the oxidation of 7-azaindole shown in Scheme 1 [28]. Indole oxidations constitute a very interesting class of reactions catalyzed by chloroperoxidase, as there are relatively few alternate routes leading to oxindole, in particular with the extremely high specificity exhibited by the biocatalyst [29]. Additionally, derivatives of 7-azaoxindole are notable for their anti-inflammatory and analgesic properties as well as their anti-cancer potential through inhibition of protein kinase [30,31].

Results of 7-azaindole oxidations using both the CPO-CLEA and the original enzyme solution are given in Table 2. Hydrogen peroxide in the amount of 0.15 mol eq. relative to that of the 7-azaindole was directly added to each reaction in the form of a single dose, and conversion to the 7-azaoxindole product was assayed by HPLC. In these and other oxidation reactions described in this work, no other products were detected by either HPLC or ^{13}C NMR. Catalysis with the liquid chloroperoxidase yielded nearly the theoretical maximum production within the first 30 min of the reaction. In contrast, the CPO-CLEA produced approximately 1/3 as much azaindole in the same amount of time. By allowing this reaction to continue overnight, though, the final conversion nearly matched that of the liquid chloroperoxidase case.

Table 2
Initial test of CPO-CLEA activity through oxidation of 7-azaindole

Enzyme	Time (h)	Reaction yield (%)
CPO solution	0.5	13
	20	14
CPO-CLEA	0.5	4
	20	12

Conditions: CPO-CLEA was prepared with 1 μl CPO, 3 μl 0.1 M $NaIO_4$, 140 μl sat. NH_4SO_4 and 12 μl 0.1 M $NaBH_4$. Oxidations contained 20 mg 7-azaindole, 200 μl *tert*-butanol, 120 μl 0.5 M pH 4.5 OHAc, 600 μl H_2O, 12 μl 12 M H_2O_2 and either CPO-CLEA or 1 μl CPO.

From these findings it can be seen that the CPO-CLEA does indeed retain catalytic activity through its enzyme immobilization process. Calculating the absolute activity recovery through this immobilization was beyond the scope of this experiment, but the data collected indicate that there is a markedly lower activity in the isolated CLEA® form than in the original liquid enzyme preparation, which could be caused by several potential factors. Any one of the chemical modification steps of oxidation, cross-linking or reduction may have interfered with the amino acids residing in the catalytic site of the enzyme, degrading its effectiveness. There may be significant losses associated with the difficulty in isolating the very small mass of solid produced in the CLEA® production. Additionally, the centrifugation associated with this isolation may compress the protein aggregate, decreasing the accessibility of interior enzyme components to the reaction substrates [32]. Nonetheless, this initial test did confirm the ability of the CPO-CLEA to catalyze an important oxidation, and to retain activity for longer than 30 min in the presence of a high hydrogen peroxide concentration.

3.3. CPO-CLEA tolerance of hydrogen peroxide

To further test this hydrogen peroxide tolerance a series of reactions were carried out involving 7-azaindole oxidation by either the CPO-CLEA or the chloroperoxidase solution at several different peroxide concentrations. The results of this experiment are summarized in Fig. 1 In agreement with earlier observations, when 25 mM (0.1 mol eq. relative to azaindole) of peroxide is added, the maximum attainable conversion of 10% is seen for both biocatalysts. As the peroxide concentration increases, though, there is no corresponding improvement in product yield when using the chloroperoxidase solution. This indicates that any increase in either the reaction kinetics or the maximum attainable yield is offset by the toxic effect of the higher peroxide levels on the biocatalyst. As the amount of peroxide added to the reaction exceeds 200 mM, this toxic effect becomes enough to prevent nearly any conversion from occurring.

For reactions catalyzed by CPO-CLEA, the peroxide tolerance profile shown in Fig. 1 is significantly different. Here, as the initial hydrogen peroxide concentration is raised from 25 to 125 mM, the yield of 7-azaoxindole is also raised from approximately 10–32%, or 73% of the theoretical maximum based on the limiting peroxide. At this optimal concentration the positive role of peroxide as an oxygen source for the reaction is balanced by its negative role as an enzyme deactivator. For peroxide levels greater than 125 mM, a drop in product yield is seen analogous to the one observed with

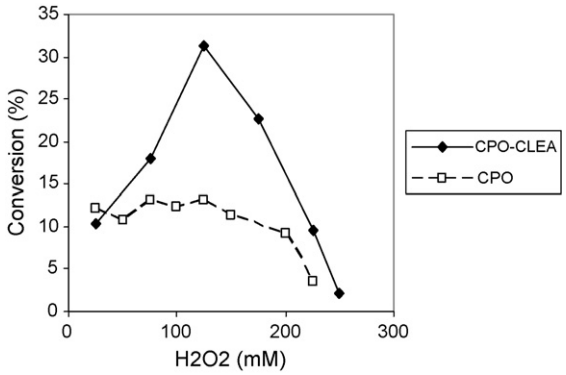


Fig. 1. Effect of hydrogen peroxide on 7-azaindole oxidation by CPO-CLEA. Conditions: CPO-CLEA was prepared with 5 μl CPO, 15 μl 0.1 M $NaIO_4$, 300 μl sat. NH_4SO_4 and 60 μl 0.1 M $NaBH_4$. Oxidations contained 28 mg 7-azaindole, 230 μl *tert*-butanol, 140 μl 0.5 M pH 4.5 OHAc, 560 μl H_2O, the amount of H_2O_2 shown and either CPO-CLEA or 5 μl CPO.

the liquid chloroperoxidase solution. Despite this drop, for all cases above 25 mM hydrogen peroxide, the CPO-CLEA was found to produce more oxindole than the non-cross-linked enzyme did.

3.4. CLEA® production process optimization

To increase the amount of initial chloroperoxidase activity that was retained in the CLEA® formulation, the concentrations of sodium periodate oxidant, ammonium sulfate precipitant and sodium borohydride reductant were next optimized. By testing multiple batches of CPO-CLEA, each created with protocols using different amounts of these three reagents, their individual and combined effects were determined.

The concentration of saturated ammonium sulfate used to precipitate the enzyme and supply the cross-linking amine were found to not have an effect when present at concentrations of >70% (v/v) in the precipitation step. Below this level, there was a decrease in recovered catalytic activity. The concentration of sodium periodate used to oxidize the enzyme protein was also not found to have a significant impact on the resulting CLEA® activity. In contrast, there was a strong correlation between the amount of sodium borohydride used in the reduction step and the chloroperoxidase activity retained through the immobilization process, with higher concentrations resulting in a greater activity loss. Representative results are presented in Fig. 2, which plots a fitted surface based on observed oxidation results for CPO-CLEAs created with different ammonium sulfate and sodium borohydride amounts. It can be seen from the surface plot that when large amounts of sodium borohydride are added to the CLEA® formation reaction, the enzyme activity will be mostly lost unless the borohydride is diluted through a coinciding increase in the amount of ammonium sulfate solution present. As a result of these and other findings, the periodate and borohydride used to create the CPO-CLEAs were each reduced from 12 to 1.3 μmol per KU of chloroperoxidase, and the ammonium sulfate solution volume was set at 90% (v/v).

The influence of the length of time of the oxidation, precipitation and reduction steps was also examined, and it was found that if these step lengths were set at 15 or 30 min, little to no solid CLEA® could be isolated. Alternatively, with steps of 90 min, solid CLEA® was produced, but its activity in oxidation reactions was less than that of CLEA® generated with the optimal 60 min step durations.

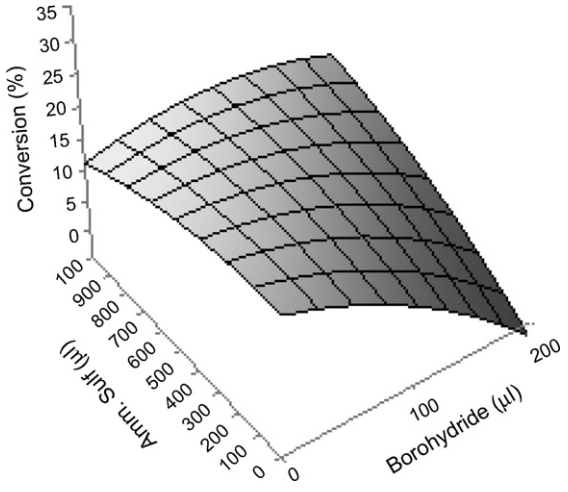


Fig. 2. Effects of precipitant and reductant concentrations on CPO-CLEA. Conditions: CPO-CLEA was prepared with 5 μl CPO, 5–45 μl 0.1 M $NaIO_4$ and sat. NH_4SO_4 and 0.1 M $NaBH_4$ in the amounts shown. Oxidations contained 15 mg 7-azaindole, 180 μl *tert*-butanol, 110 μl 0.5 M pH 4.5 OHAc, 440 μl H_2O, 8.4 μl H_2O_2 and CPO-CLEA.

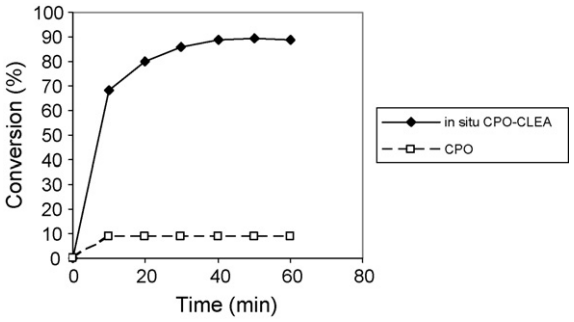


Fig. 3. Oxidation of 7-azaindole to 7-azaoxindole with in situ CPO-CLEA and single direct addition of H_2O_2: CPO-CLEA was prepared with 1.7 ml CPO, 1.7 ml 33 mM $NaIO_4$, 30.6 ml sat. NH_4SO_4 and 1.7 ml 33 mM $NaBH_4$. Oxidations contained 1 g 7-azaindole, 42 ml *tert*-butanol, 125 ml 0.1 M pH 4.5 OHAc, 22 ml H_2O_2 and in situ CPO-CLEA or 1.7 ml CPO.

3.5. In situ CPO-CLEA production

As the concentrations of the reagents associated with the CLEA® formation are much lower than those of the substrates of the 7-azaoxindole formation, it was theorized that they would have very little impact on this oxidation were they to remain in the reaction system. If this is the case, then it would be possible to add the oxidation reaction components directly to the mixture associated with CLEA® production, removing the need for all manipulations associated with isolation of the CPO-CLEA. This would in turn provide a much more straightforward process as well alleviate potential sources for activity loss during the isolation, such as aggregate compression due to centrifugation and challenges associated with recovering a small, fine solid mass.

As a test, two batches of CPO-CLEA were generated with the optimized procedure described above, but while one was washed and isolated prior to use in an oxidation reaction the other was allowed to remain in its mixture of periodate, ammonium sulfate and borohydride. When 0.5 mol eq of hydrogen peroxide relative to 7-azaindole was added to reactions catalyzed by these two CLEAs, the in situ variety converted 30% of the starting material in the time required for the isolated version to convert 10%.

Remarkably, in subsequent experiments it was found that not only did this in situ CLEA® have a higher initial activity than its isolated counterpart, but it also had an even greater tolerance for very high hydrogen peroxide levels. With this increased resistance it became possible to directly add peroxide to reactions at concentrations in excess of 1 M, enabling >90% conversion of 7-azaoxindole without the need for any gradual addition method. This improved process was tested at the 1-g scale using both in situ CPO-CLEA® and liquid chloroperoxidase solution, and reaction yields for both cases are shown in Fig. 3. Less than 1 h of oxidation reaction time was needed for the in situ CLEA® to convert 90% of the 7-azaindole substrate. Under identical reaction conditions, the liquid chloroperoxidase solution was only capable of converting approximately 10% of the 7-azaindole substrate in the first few minutes of the reaction before being completely inactivated.

4. Conclusions

The instability of chloroperoxidase in the presence of even very low concentrations of hydrogen peroxide has been a significant barrier to its practical application. In this work, a novel cross-linked aggregate of the enzyme has been shown to have greatly enhanced tolerance of this oxidant. It should be noted that the azaindole oxidation processes described here exhibit chloroperoxidase total turnover numbers (ttn) on the order of 10^6, and are thus not yet

as enzyme-efficient as those described elsewhere in the literature with chloroperoxidase ttn values approaching 10^7 [6]. However, application of the novel immobilized form of the enzyme eliminates the need for these more elaborate reaction systems designed so as to maintain as low a level of peroxide as possible. Also, in a further simplification of the processes of creating and using the new biocatalyst, the CLEA® was generated in situ with subsequent enzyme-catalyzed oxidations carried out immediately afterwards in the same vessel. A potential area for future work involves further improving the activity retention of the enzyme in the immobilization process by, for example, exploring the use of alternate cross-linkers such as diamines of varying size [25]. Additionally, the application of in situ CLEA® technology described here to other biocatalysts and reactions should be explored.

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