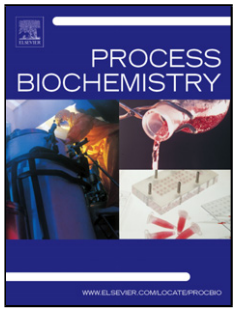

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Chemical modification of chloroperoxidase for enhanced stability and activity

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ABSTRACT

Chloroperoxidase from *Caldariomyces fumago* (CPO, EC 1.11.1.10) is one of the most interesting enzymes from the group of heme peroxidases and has been extensively applied in synthetic processes. Nevertheless, the practical application of CPO is limited due to its very low operational stability, especially in presence of peroxidative compounds. For this reason, effect of chemical modifications of CPO in the stability of the enzyme was studied. Side-chain selective modifications of amino groups of Lys residues, and carboxyl groups of Asp and Glu residues, as well as crosslinking and periodate oxidation of sugar moiety were carried out. The stability of modified CPOs was evaluated at elevated pH and temperature, and in presence of *tert*-butyl hydroperoxide. Effect of modification of CPO on the performance of the reaction of Cbz-ethanolamine oxidation was studied as well. Those modifications that involved carboxyl groups via carbodiimide coupled method and the periodate oxidation of the sugar moiety produced better catalysts than native CPO in terms of stability and activity at elevated pH values and temperatures.

KEYWORDS

Chemical modifications; Chloroperoxidase from *Caldariomyces fumago*; Cbz-ethanolamine oxidation; Cbz-glycinal synthesis; peroxide dependent inactivation.

1. INTRODUCTION

A chemical modification of enzymes is a useful and inexpensive method for the improvement of their efficiency as biocatalysts. Chemical modifications of enzymes allow the introduction of a wide variety of functional groups. The alterations of the properties of the enzyme are the result of the conformational changes which occur due to the modifications of its primary structure [1-5].

A number of chemical methods has been reported whereby the stability, selectivity or activity of the enzymes can be changed [6-10]. Thus, chemical modification of the protein as a tool for searching improved enzymes represents an alternative to site-directed mutagenesis and directed evolution [1]. The main disadvantage of these methods is the lack of control with respect to the extent and regiochemistry of the reaction [2]. Some of the commonly used methods of chemical modifications are side-chain selective modifications, crosslinking and modification of the sugar moiety of the protein.

Side-chain selective modifications involve the use of reagents that react, under certain specified conditions, with a single or a limited number of side-chain groups in a rather predictable way [11]. Many of these modifications involve the ε-amino groups of Lys and carboxyl groups of Asp and Glu, since these amino acids are usually abundantly found on the protein surface. For the selective acylation of ε-amino groups of Lys, a variety of acidic or dicarboxylic anhydrides (e.g. maleic or phthalic) has been used [11-12]. Modifications of carboxyl groups of the protein (Glu and Asp) commonly involve the activation of the carboxyl group by a water-soluble carbodiimide and the subsequent reaction of the activated carboxyl group with a nucleophile [13-14].

The technique of chemical crosslinking of proteins has been used widely for the enhancement of their stability by increasing the rigidity of the active structure of the enzyme by inter- or intramolecular crosslinking and it can be achieved by the use of a variety of bi- or poly-functional crosslinkers [15]. Dimethyl suberimidate (DMS), is a commonly used crosslinker that contains imidoester group at each end of an 8-atom spacer arm and represents a highly specific reagent for amino groups in proteins [16].

The chemical modification of sugar chains of the protein may alter the catalyst stability, since these chains provide important physical properties of the glycoproteins (conformational stability, charge and waterbinding capacity), but are not directly involved in catalysis [17]. Some authors pointed out cross-linking as a result of the formation of Schiff base bonds between the protein amino groups and sugar aldehyde groups formed after the periodate oxidation of the protein, though these linkages are usually present in a very low amount [18-19].

Depending on the nature and the amount of the reagent, the enzyme and the conditions used for modification, the complete modification of all such side chains is not always achieved. Selection of the conditions and the amount of the reagent used is limited by the stability of the enzyme at these conditions, and therefore cannot be always optimized for a complete modification [3, 14]. The loss of enzymatic activity after such modification can be caused by the procedure used for modification (pH, temperature, presence of reagents etc.), by alteration of the active conformation of the enzyme or can be the result of the modification of essential residues in the active site of the enzyme [5].

Chloroperoxidase (CPO; EC 1.11.1.10) from the fungus *Caldariomyces fumago* is one of the most promising enzymes from the group of heme peroxidases, expressing peroxidase, halogenase, catalase and oxygen insertion activities [20]. CPO was recently

found to catalyze selective oxidation of amino alcohols to amino aldehydes by using *tert*-butyl hydroperoxide as an oxidant. This biotransformation has a great importance in organic chemistry [21-22], as produced amino aldehydes are widely applied for the synthesis of important pharmaceuticals and natural products [23-27] and as transition state analogue inhibitors for various proteases [28-29].

Nevertheless, the main shortcoming of CPO and all heme peroxidases is their low operational stability caused by rapid irreversible inactivation in the presence of peroxides [30]. The improvement of the operational stability of CPO has been achieved so far via immobilization [22, 31-32]; addition of stabilizing agents [33-34] or antioxidants [35]; continuous, step-wise or controlled feed-on-demand addition of the peroxide [21, 36-38]; and *in situ* generation of hydrogen peroxide catalyzed by glucose oxidase [39-40].

In this work, the effects of chemical modifications of CPO by maleic anhydride (MA), phthalic anhydride (PA), carbodiimide (EDAC) in the presence of ethanolamine (EA) or ethylenediamine (EDA), dimethyl suberimidate (DMS) and sodium periodate on the thermostability, pH stability, and tolerance to *tert*-butyl hydroperoxide as well as on the reaction performance for the oxidation of Cbz-ethanolamine to Cbz-glycinal were investigated.

2. MATERIALS AND METHODS

2.1. Materials

CPO from *C. fumago* was obtained from Chirazyme Labs (Greenville, NC, USA) as a solution of partially purified enzyme (11.6 mg protein/mL). Monochlorodimedone (1,1-dimethyl-4-chloro-3,5-cyclohexanedione), Cbz-ethanolamine (benzyl-N-(2-hydroxyethyl)-carbamate, 98%), Cbz-glycine, *tert*-butyl hydroperoxide (70% (w/w) in

water), dimethyl suberimidate, ethanolamine, ethylenediamine, sodium periodate and N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) were purchased from Sigma Aldrich (St. Luis, MO, USA). Phthalic anhydride was purchased from Kemika (Zagreb, Croatia). Maleic anhydride was purchased from Serva Electrophoresis GmbH (Heidelberg, Germany). Cbz-glycinal (benzyl (2-oxoethyl)-carbamate) was purchased from Sunshine Chemlab, Inc (Downingtown, PA, USA). All the other reagents and solvents were obtained from various commercial suppliers and were of the highest available purity and of analytical grade.

2.2. Chloroperoxidase activity assay

The optical assay for the measurement of CPO chlorination activity is based on the decrease in absorbance for a solution of monochlorodimedone, MCD (ε_{278}=12200 $M^{-1}\cdot cm^{-1}$) as it is converted to dichlorodimedone, DCD (ε_{278}=120 $M^{-1}\cdot cm^{-1}$), by CPO according to the method described by Morris and Hager [41]. The assay mixture contained 100 mM potassium phosphate buffer pH 2.75, 20 mM KCl, 2 mM hydrogen peroxide and 0.16 mM MCD. The absorbance was measured at 25ºC at a wavelength of 278 nm. One unit of CPO is defined as the amount of enzyme required to catalyze the conversion of 1 μmol of MCD to DCD per minute using 100 mM potassium phosphate buffer pH 2.75 at 25ºC. Based on this assay, specific activity of purified CPO was 1400 U/mg.

Peroxidative activity of CPO was assayed by the guaiacol method by measuring the increase in absorbance (A_{436}) as it was converted to tetraguaiacol ($\varepsilon_{436\ nm}$=25.5 $M^{-1}cm^{-1}$) in a 3 mL assay mixture containing 18 mM guaiacol, 0.3 mM hydrogen peroxide and 100 mM acetate buffer pH 5.0, at 25ºC. The reaction was initiated upon the addition of 0.1 mL of the enzyme and monitored by the increase in absorbance at 436 nm. One unit

of peroxidative activity is defined as the amount of the enzyme required to catalyze the conversion of 1 μmol of guaiacol per minute at a temperature of 25ºC.

The change in the absorbance in both activity assays was measured in UV-Visible Spectrophotometer Cary 50 (Varian, Palo Alto, CA, USA) using quartz cuvettes.

2.3. Chemical modifications of CPO

2.3.1. Modification of amino groups of CPO

Mixtures containing 1.66 mg/mL of CPO and 2 mg/mL of maleic or phthalic anhydride in 100 mM sodium acetate buffer at pH 5.0 in the final volume of 0.5 mL were incubated at 4ºC. In case of modification with phthalic anhydride, 0.02 mL of DMF were added (corresponding to a concentration of 4% (v/v)) in order to dissolve it. After 1 h of incubation the mixtures were desalted using Sephadex G-25 column and stored at 4ºC.

2.3.2. Modification of carboxyl groups of CPO

Modification of carboxyl groups of CPO was carried out by following this procedure: mixtures containing 1 mg/mL of CPO, 5 mM of EDAC and 25 mM of ethanolamine (EA) or ethanoldiamine (EDA) in 100 mM MES buffer at pH 5.0 were incubated during 1.5 h at room temperature. Then 100 μL of 0.5 M sodium acetate buffer pH 5.0 was added in order to stop further reaction. Finally, the mixtures were desalted using Sephadex G-25 column and stored at 4ºC.

2.3.3. Crosslinking of CPO with DMS

A mixture containing 1.66 mg/mL of CPO and 4 mg/mL of DMS in 100 mM sodium acetate buffer at pH 5.0 in the final volume of 0.5 mL was incubated at 4ºC for 1 h and finally desalted using Sephadex G-25 column and stored at 4ºC.

2.3.4. Periodate oxidation of CPO

For the modification with sodium periodate, the mixture containing 1.66 mg/mL of CPO and 25 mM $NaIO_4$ in 50 mM acetate buffer at pH 5.0 was incubated in a final volume of 0.5 mL for 1 h at 4ºC in dark. Then 10 µL of 5 M ethylene glycol was added in order to stop the further oxidation. Finally, the mixture was dialyzed in 20 mM acetate buffer pH 5.0 and stored at 4ºC.

2.4. Determination of the degree of modified amino groups

The degree of modification of amino groups was determined as a percentage of those amino groups in native enzyme, and it was measured spectophotometrically at 420 nm with 2,4,6-trinitrobenzenesulphonic acid (TNBS) [42]. 0.225 mL of 0.2 M borate buffer, pH 9.2, and 0.125 mL of TNBS (1.18 mg/mL) were added to 0.05 mL of the sample containing native or modified CPO in the concentration of 0.74 mg/mL. The reaction was stopped after 30 min by adding 0.125 mL of 2 M NaH_2PO_4 and 18 mM Na_2SO_3, and the absorbance (A_{420nm}) was measured using a Philips UV-VIS-NIR PU 8630 spectrophotometer. The percentage of modified amino groups was calculated as a percentage of A_{420nm} obtained for the modified CPO respect to that value for native CPO. The experiments were performed in duplicate.

2.5. Isoelectric focusing and zymogram

Isoelectric focusing was performed using Multiphor II electrophoresis system (Pharmacia-LKB Biotechnology) according to manufacturer's instruction. Focusing was carried out on 7.5% acrylamide gel with ampholytes in a pH range 3.5–5.2 (Bio-Rad, USA), at 7 W constant powers for 1.5 h at 10ºC. Low pI kit (GE Healthcare) was used as isoelectric point (pI) markers. After the run, native and modified chloroperoxidases were detected using in-gel peroxidation activity staining (zymogram detection) using a solution that contained 8.5 mM guaiacol and 0.95 mM hydrogen peroxide in 100 mM

sodium acetate buffer pH 5.0. CPO activity appeared as orange bands on clear background.

Figure 4. was prepared using PyMOL (http://pymol.org). The structure coordinates with accession code 1cpo has been downloaded from the Protein Data Bank.

2.6. Stability of chemically modified CPOs

Effect of pH on CPO stability was studied at pH 7.0. The reaction medium (50 mM potassium phosphate pH 7.0) containing the modified or native enzyme was incubated at room temperature (25ºC). Effect of temperature on CPO stability was studied at 50ºC by incubating native or modified CPO in 50 mM sodium acetate buffer pH 5.0. Effect of the presence of *t*-BuOOH was studied by incubating native or modified CPOs in 50 mM acetate buffer pH 5.0 containing 3 mM *t*-BuOOH at room temperature (25ºC).

Added CPO peroxidation activity was 1 U/mL, in all experiments. After different times of incubation aliquots of enzyme solution were withdrawn and residual peroxidation activity was measured.

The inactivation kinetics was studied by plotting time course of inactivation on semi-log plot, and calculating half life of the enzyme from the slope of linear regression. It was observed that the deactivation follows first order kinetics; hence from the regression of the type $f=a\cdot\exp(-b\cdot t)$, the half life was calculated as $t_{1/2}=\ln 2/b$.

2.7. Oxidation of Cbz-ethanolamine to Cbz-glycinal catalyzed by native and modified CPOs

Cbz-ethanolamine was dissolved at concentration of 20 mM in 100 mM acetate buffer at pH 5.0 or 100 mM phosphate buffer at pH 7.0 in a final volume of 1 mL. Then 500 U/mL (chlorination activity) of native or modified CPO was added to the reaction medium. The reaction was started by adding *tert*-butyl hydroperoxide continuously, by manual addition of one pulse (10.7 μL) per hour during 8 h of reaction. After this time

the addition was stopped and restarted after 24 h in case that CPO activity was already detected. Concentration of peroxide stock solution was within a range of 0.28-1.12 M in order to reach peroxide addition rate of 3-12 mM/h. The reactions were performed at room temperature (25ºC) or at 50ºC using mild agitation. Reaction samples were withdrawn periodically and analyzed by liquid chromatography in order to quantify Cbz-ethanolamine and Cbz-glycinal concentrations and to determine the activity of CPO. Each measurement was carried out in duplicate.

Pseudo half life of CPO was determined experimentally and represents the time at which CPO activity decays to the half of the activity initially added. Conversion and yields of the products were defined respectively as the percentage of the consumed Cbz-ethanolamine or produced products, related to initially added concentration of Cbz-ethanolamine. These values were additionally corrected by the dilution factor which was calculated for each set of measurements taking into account the volume of the peroxide added in pulses and the volume of the reaction medium withdrawn for the analysis. Initial reaction rate represents the rate of the Cbz-ethanolamine consumption during the period of linear change in concentration.

2.8. Cbz-ethanolamine, Cbz-glycinal and Cbz-glycine quantification

Concentrations of Cbz-ethanolamine, Cbz-glycinal and Cbz-glycine were estimated by analytical HPLC Dionex UltiMate 3000 with UltiMate 3000 Variable Wavelength Detection (Sunnyvale, USA) employing a reversed-phase column X Bridge C18, 5 μm, 4.6x250 mm from Waters (Wexford, Ireland). The solvent system consisted of solvent A, composed of 0.1% v/v trifluoroacetic acid (TFA) in H_2O, and solvent B, composed of 0.095% v/v TFA in H_2O/CH_3CN 1:4. The solvents were eluted at a flow rate of 1 mL/min using a gradient from 20% B to 36% B over 24 min and peaks were detected at 200 nm and 30ºC. Quantitative analysis of products was performed from peak areas by

the external standard method by means of a prior calibration with samples of known concentration.

3. RESULTS AND DISCUSSION

3. 1. Chemical modifications of CPO

CPO has 321 amino acids with predominantly acidic residues and a pI in a range of 3.2-4.0. It is rich in aspartic acid, glutamic acid, serine and proline; these four amino acids constitute about 45% of the total amino acid content. Besides, CPO is a glycoprotein with a carbohydrate content of 25-30% [41]. The conditions for chemical modifications of CPO were selected in order to assure the sufficient amount of modifying agent while minimizing the inactivation of enzyme by the process of modification.

Chemical modifications of CPO were carried out via six different methods: modifications of Lys residues with maleic and phthalic anhydride, modifications of carboxyl groups with EDAC and EA or EDA, crosslinking with DMS and periodate oxidation (Figure 1). Even though, in general, chemical modifications of Lys residues with maleic and phthalic anhydride and crosskinking with DMS optimally take place at alkaline conditions (pH 8-9), CPO had to be modified by this methods at acidic conditions, in order to avoid the fast irreversible inactivation which occurs on high pH values [22]. The retained activities of modified CPOs for chlorination of MCD and peroxidation of guaiacol were measured and compared to those of native enzyme (Figure 2). The loss of both chlorinating and peroxidative activity was observed for all types of modification, within the range of 39.5 and 56.8% for the chlorinating activity, and 16.4 and 44.5% for peroxidative activity. In all cases, higher losses were observed for chlorinating than for peroxidative activity. This could be due to the fact that CPO

possesses at least two different binding sites for the substrates for peroxidation reactions, occurring via different mechanisms [43].

The degree of the modification was determined by the indirect measurement of the remained amino groups by the reaction with TNBS, for CPO modified with MA, PA, DMS and periodate (Table 1). The percentage of modified amino groups had similar values in all cases, possibly due to the accessibility of the Lys residues for the chemical modifications and it was more than 70%, even for the periodate oxidation of CPO. The possible explanation in the latter case is that formed aldehyde groups on the sugar moiety of CPO react with amino groups forming Schiff base bonds.

In order to check out whether the modifications caused the change in the overall charge of the enzyme molecule, the analysis by isoelectic focusing was performed for native and modified CPOs for the pH range of 3.5-5.2 (Figure 3). As it can be seen, no change in pI value was observed after the modifications of CPO with maleic and phthalic anhydrides, DMS and $NaIO_4$, while for both methods corresponding to the modification of carboxyl groups, a slight increase of pI was detected. Modifications of the Lys residues of the enzyme deplete the positive charge in the enzyme molecule. Hence, it could have been expected that the enzyme modified in that way will have lower pI value than the native one. However, since in the molecule of CPO only five Lys residues are present (Figure 4), which based on the results obtained by TNBS assay means that four lysines were modified, it is reasonable that no visible change in pI value occurred. On the contrary, the modifications of carboxyl groups eliminate the negative charge, and thus, the increase of the pI of CPO after such a modification could have been expected, since CPO contains a great number of superficial Asp and Glu residues [41].

3.2. Oxidation of Cbz-ethanolamine to Cbz-glycinal catalyzed by chemically modified CPOs

Modified CPOs were tested as biocatalysts for the oxidation of Cbz-ethanolamine to Cbz-glycinal and were compared to the native enzyme. The reactions of oxidation of Cbz-ethanolamine were performed by using CPO chlorinating activity of 500 U/mL and by adding *tert*-butyl hydroperoxide in pulses at the rate of 3 mM/h, based on the previously reported results [21]. When applied in the reaction, native and all modified CPOs lost their activity after only 24 h of reaction. Even so, yields of Cbz-glycinal and conversions of Cbz-ethanolamine were slightly improved for all modified CPOs compared to the native one, except for CPO modified with phthalic anhydride (Table 2). Similar to the reaction catalyzed by native CPO, in all the experiments, between 8.5 and 13% of Cbz-glycine as the product of further oxidation of the amino aldehyde by CPO.

The improvement of the reaction performance could be either due to the improvement of the stability of CPO in the presence of peroxide or due to the improved affinity for the substrate. Therefore the stability of all modified CPOs was examined in the presence of 3 mM *tert*-butyl hydroperoxide. As it was expected, in absence of substrate the inactivation of the enzyme was fast in all cases. The half lives of modified CPOs were slightly lower compared to the native enzyme (Figure 5) indicating that none of the modifications made the heme group less prone to destruction by peroxides. Hence, production improvement when using modified enzymes was possibly due to improved affinity of CPO for Cbz-ethanolamine.

3.3. Effect of pH on the stability of modified CPOs and the reaction of oxidation of Cbz-ethanolamine

CPO has a very narrow interval of pH in which activity is preserved [22]. Native CPO and all modified CPOs were almost completely stable at pH 5.0 and room temperature during 3 days of incubation (data not shown). Stability of modified CPOs was then studied at pH 7.0 at room temperature in absence of substrates. CPO-MA, CPO-PA and CPO-DMS resulted less stable at pH 7.0 than the native CPO, while all other modified CPOs showed improved stability on this pH value (Figure 6). The most drastic improvement was observed for CPO-EDAC-EA and periodate oxidized CPO, with half lives 2.1 and 1.5-fold improved, respectively.

Expecting the improvement in the reaction performance at pH 7.0 by using the most stable modified preparations at this pH value (CPO-EDAC-EA, CPO-EDAC-EDA and CPO-$NaIO_4$), the reaction of oxidation of Cbz-ethanolamine was performed and their efficiency as catalysts at these conditions was compared to the one of native CPO.

All three modified preparations tested resulted to be better catalysts at pH 7.0 than the native CPO. In all cases, slightly higher yields of Cbz-glycinal were produced at higher reaction rates than in the reaction catalyzed by native CPO (Experiments 1-4 in Tables 3 and 4). The yields of Cbz-glycinal were improved from 9.8% (value obtained with native CPO) to more than 11% when modified CPOs were used, with almost no Cbz-glycine produced. The productivity improvement, even being low, could be due to the slightly improved stability of the enzyme and the reaction rate when using modified CPOs at these conditions or improved substrate affinity. Although half life times were higher than that of native enzyme, they were low for all of the CPOs, indicating the rapid inactivation of the enzyme. Relatively low yields were obtained in all cases, resulting in around 90% of activity loss after only 2 hours of reaction.

From the results of the stability at this pH value, especially for CPO-EDAC-EA where a drastic improvement of the stability was observed, much better reaction performance could have been expected. This is probably due to the fact that the very rapid inactivation observed in all cases was partly caused by the high pH value, but more significantly by the accumulation of the peroxide in the reaction medium, which, as it was already shown, has slightly higher effect on the modified CPOs than on the native one. CPO requires one mol of peroxide to oxidize one mol of Cbz-ethanolamine to Cbz-glycinal [21]. The half life of enzyme can be significantly prolonged if the peroxide addition rate is adjusted to the reaction rate. However, in all cases, the rate of peroxide addition was higher than the reaction rate.

3.4. Effect of the temperature on the stability and reaction of oxidation of Cbz-ethanolamine catalyzed by modified CPOs

CPO resulted to be very stable at room temperature, but the increase of the temperature might be desired in order to accelerate the reaction, and therefore the consumption of the peroxide. The lower accumulation of the peroxide will hence, have a positive effect on the stability of CPO. Therefore, the thermostability of native and modified CPOs was studied at 50ºC and pH 5.0. At these conditions, all the modified CPOs resulted to be more stable than the native CPO, those modified with carbodiimide coupled method being the most stable ones, with 1.8-fold improved half lives for the modification with EDA and 4.8-fold for modification with EA, compared to the soluble CPO (Figure 7).

The reactions of oxidation of Cbz-ethanolamine were then carried out at the same temperature by using those modified preparations that resulted more stable at this temperature (CPO-EDAC-EA, CPO-EDAC-EDA and CPO-$NaIO_4$). Use of high temperatures may be beneficial due to the generally higher reaction rates, which in case

of the reaction catalyzed by CPO means also faster consumption of *t*-BuOOH, i.e. less accumulation.

t-BuOOH was added at the rate of 3 mM/h in order to assure a low concentration in the reaction medium, and therefore preserve the activity of the enzyme. At these conditions native CPO lost more than 70% of the initial activity after only one hour of reaction, while after the same reaction time the modified ones lost within 54-60% of activity; consequently, higher yields of Cbz-glycinal were produced (Experiments 5-8 in Tables 3 and 4). The biggest improvement in the conversion and product yield was accomplished when CPO-$NaIO_4$ was used as biocatalyst, reaching a final yield of Cbz-glycinal of 21.3%, while the most stable one resulted to be CPO-EDAC-EA.

The values of initial reaction rates obtained for the modified CPOs were 3.4 mM/h, 4.5 mM/h and 5.2 mM/h for CPO-EDAC-EA, CPO-EDAC-EDA and CPO-NaIO4, respectively. All these values exceeded the rate of peroxide addition, which was in this case possible, since due to very high reaction and enzyme inactivation rates, it was measured only in first half an hour of the reaction, while the addition of t-BuOOH was performed by means of one pulse each hour. Therefore, the concentration of peroxide was limiting the overall reaction rate.

In order to avoid the limitation by peroxide concentration, the reaction of oxidation of Cbz-ethanolamine with native CPO was performed at 50ºC with peroxide addition rates of 3, 6, 9 and 12 mM/h, reaching final yields of Cbz-glycinal of 13.9%, 14.3%, 15.6% and 16.1%, respectively. As the highest yield of Cbz-glycinal was reached when peroxide was added at the rate of 12 mM/h, the reactions catalyzed by modified CPOs were performed by adding the peroxide at this rate.

Even though almost complete inactivation of CPO was observed after only 1 h of reaction, the Cbz-glycinal yields were further improved. In fact, almost the final

conversion achieved occurred after only 30 minutes of reaction, the time in which more than 20% of the yields of the product were reached when using modified CPOs, and around 15% when using the native one (Figure 8).

In these conditions reaction was not limited by peroxide concentration, but the presence of peroxide caused more rapid CPO inactivation. Even so, final reaction yields and productivities were improved compared to those obtained when the peroxide was added at the rate of 3 mM/h (Experiments 9-12 in Tables 3 and 4).

Modified CPOs resulted better catalysts than the native one in all the aspects: the conversions of Cbz-ethanolamine were improved from 18.5% when native CPO was used to more than 26% for the reactions catalyzed by modified CPOs; yields of Cbz-glycinal were 16.1% and 21.5-22.1% for native and modified CPOs respectively; the values of half lives were higher for modified compared to the native CPO; and finally, around 1.3-fold improvement in reaction rates was achieved for all the modified CPOs tested.

Even though chemical modifications enabled the enhancement of the stability and activity of CPO, the problem of its operational instability in batch reactor, was not entirely resolved. There are numerous perspectives for further improvement of the performance of chemically modified CPOs. Operational stability of enzyme depends on its design and on the reactor fashion [44]. The use of continuous reactor could be highly beneficial for reaching higher productivities, and besides, the peroxide accumulation would be avoided. Immobilization of chemically modified CPOs could result in further improvement of stability and allow easier use in continuous reactors or reuse. The chemical modification of enzymes may improve their immobilization by reinforcing adsorption or by improving multipoint covalent attachment. Besides, improvement of

enzyme stability accomplished by chemical modification can facilitate the selection of the immobilization conditions [45-46].

4. CONCLUSIONS

In this work, different types of chemical modifications were studied in order to improve the properties of CPO as catalyst. While those modifications that involved the amino groups of the enzyme, both by selective reaction with dicarboxylic anhydrides (maleic and phthalic) or by crosslinker DMS had less or no effect on the improvement of the properties of CPO, those which modified carboxyl groups via EDAC coupling and the periodate oxidation of the sugar moiety produced better catalysts than native CPO. These modified CPOs showed to be more stable on high pH values and temperatures, and consequently better overall reaction performances for the oxidation of Cbz-ethanolamine to Cbz-glycinal were accomplished in these conditions. Improvement of the stability in both cases could be due to the crosslinking, in the first case by using EDAC as a zero-length crosslinker, and in the second one by the formation of Schiff base bonds between the aldehyde groups formed on the sugar moiety and the amino groups of the protein.

Design of CPO with wider range of conditions in which it is active is of great importance for its further application, since it may improve or facilitate its immobilization. Besides, Cbz-glycinal, as well as other amino aldehydes, is widely used as starting compounds for the synthesis of important pharmaceuticals and natural products. For this purpose, there is an increasing trend for application of enzymes such as CPO in multistep one-pot reactions where improved stability of the enzyme is beneficial for better compatibility of enzymes.

5. ACKNOWLEDGEMENTS

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Captions to Figures

Figure 1. Reaction scheme for chemical modification of CPO by using maleic (A) and phthalic anhydride (B), EDAC and nucleophile ethanolamine (C) and ethylenediamine (D), sodium periodate (E) and DMS (F). R represents either another molecule of CPO or functional group within the same molecule.

Figure 2. Effect of modifications of CPO on the chlorinating (black bars) and peroxidative (grey bars) activity. Each value represents the mean of two measurements. The error bars represent the standard deviation of the measurements.

Figure 3. Isoelectric focusing of native (1) and modified CPOs: CPO-MA (2), CPO-PA (3), CPO-DMS (4), CPO-$NaIO_4$ (5), CPO-EDAC-EA (6) and CPO-EDAC-EDA (7) for the pH range of 3.5-5.2; Zymogram determination of active enzyme was performed with guaiacol assay.

Figure 4. Structure of the CPO. The heme in the center is colored red, polypeptide chain green, while five lysine residues are outlined in blue. Lys177 and Lys211 are parts of helixes and Lys112, Lys115 and Lys145 are parts of loops. Glycocomponent of protein is presented with residues of arabinose, mannose, xylopyranose and N-acetyl-D-glucosamine visible in crystal structure.

Figure 5. Half lives of native and modified CPO in the presence of 3 mM *tert*-butyl hydroperoxide. The stability experiments were performed in 100 mM sodium acetate buffer pH 5.0 at room temperature.

Figure 6. Half lives of native and modified CPOs in 50 mM sodium phosphate buffer pH 7.0 at room temperature.

Figure 7. Half lives of native and modified CPOs in 100 mM sodium acetate buffer pH 5.0 at 50ºC.

Figure 8. Time-course of Cbz-glycinal production catalyzed by native CPO (●), CPO-EDAC-EA (□), CPO-EDAC-EDA (Δ) and CPO-$NaIO_4$ (○) using *tert*-butyl hydroperoxide as oxidant at the addition rate of 12 mM/h. 500 U/mL of CPO were added to the reaction medium that contained 20 mM of Cbz-ethanolamine in 100 mM sodium acetate buffer pH 5.0 at 50ºC. The end of reaction was considered when no CPO activity was detected. Each value represents the mean of two measurements. The error bars represent the standard deviation of the measurements.

Highlights

- Chloroperoxidase from *Caldariomyces fumago* was chemically modified by 6 methods.
- By using modified CPOs higher yields of Cbz-glycinal were achieved.
- CPOs modified by carbodiimide and periodate were more stable at higher pH values.
- CPOs modified by carbodiimide and periodate had improved thermostability.
- Higher Cbz-glycinal yields at pH 7.0 and 50ºC were reached by using modified CPOs.

Table 1. Percentage of modified amino groups determined by the reaction with TNBS. Each value represents the mean of two measurements. The errors represent the standard deviation of the measurements.

Type of modification	% of modified amino groups
CPO-MA	77.5 ± 1.8
CPO-PA	73.5 ± 1.6
CPO-DMS	77.5 ± 1.6
CPO-NaIO4	76.1 ± 0.0

Table 2. Oxidation of Cbz-ethanolamine to Cbz-glycinal catalyzed by native and modified CPOs using *tert*-butyl hydroperoxide as oxidant at the addition rate of 3 mM/h. 500 U/ml of CPO measured by MCD assay were added to the reaction medium that contained 20 mM of Cbz-ethanolamine in 100 mM sodium acetate buffer pH 5.0 at room temperature. The end of reaction was considered when no CPO activity was detected, which was after 24 h in all the experiments.

	Conversion (%)	Yield of Cbz-glycinal (%)	Yield of Cbz-glycine (%)
Native CPO	56.6	45.8	10.8
CPO-MA	59.3	46.4	12.9
CPO-PA	52.4	43.9	8.5
CPO-EDAC-EA	58.7	47.5	11.1
CPO-EDAC-EDA	59.1	47.7	11.3
CPO-DMS	60.2	48.9	11.3
CPO-$NaIO_4$	60.7	47.5	13.2

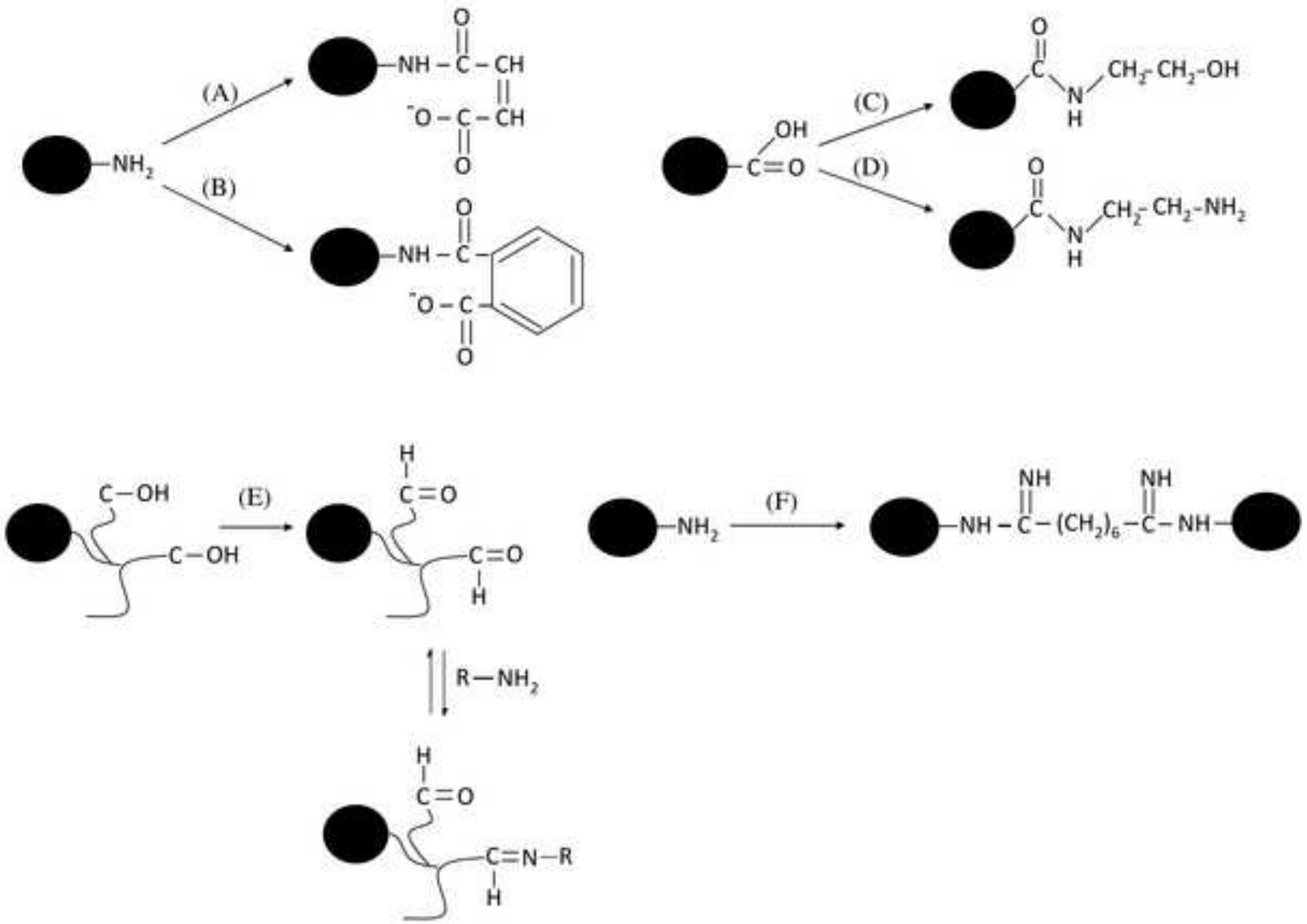
Table 3. Conditions for Cbz-ethanolamine oxidation catalyzed by native and modified CPOs.

Experiment	Biocatalyst	*t*-BuOOH addition rate (mM/h)	pH	T (ºC)
1	native CPO	3	7.0	25
2	CPO-EDAC-EA	3	7.0	25
3	CPO-EDAC-EDA	3	7.0	25
4	CPO-$NaIO_4$	3	7.0	25
5	native CPO	3	5.0	50
6	CPO-EDAC-EA	3	5.0	50
7	CPO-EDAC-EDA	3	5.0	50
8	CPO-$NaIO_4$	3	5.0	50
9	native CPO	12	5.0	50
10	CPO-EDAC-EA	12	5.0	50
11	CPO-EDAC-EDA	12	5.0	50
12	CPO-$NaIO_4$	12	5.0	50

Table 4. Results for Cbz-ethanolamine oxidation catalyzed by 500 U/ml of native or modified CPOs. Conditions of each experiment are summarized in Table 3.

Experiment	Conversion (%)	Cbz-glycinal yield (%)	Yield of Cbz-glycine (%)	Half life, $t_{1/2}$ (h)	Initial reaction rate (mM /h)
1	11.9	9.8	0.8	0.47	1.8
2	12.0	11.5	0.7	0.61	2.3
3	12.3	11.3	1.0	0.56	2.2
4	11.3	11.0	0.2	0.55	2.0
5	15.6	13.9	1.6	0.51	1.8
6	19.8	18.2	2.9	0.90	3.4
7	21.4	18.3	3.1	0.76	4.5
8	25.8	21.3	4.5	0.80	5.2
9	18.5	16.1	2.4	0.22	6.7
10	26.7	21.6	5.1	0.29	9.9
11	26.2	21.5	4.7	0.30	9.2
12	26.1	22.1	4.0	0.29	8.9

Figure(s)



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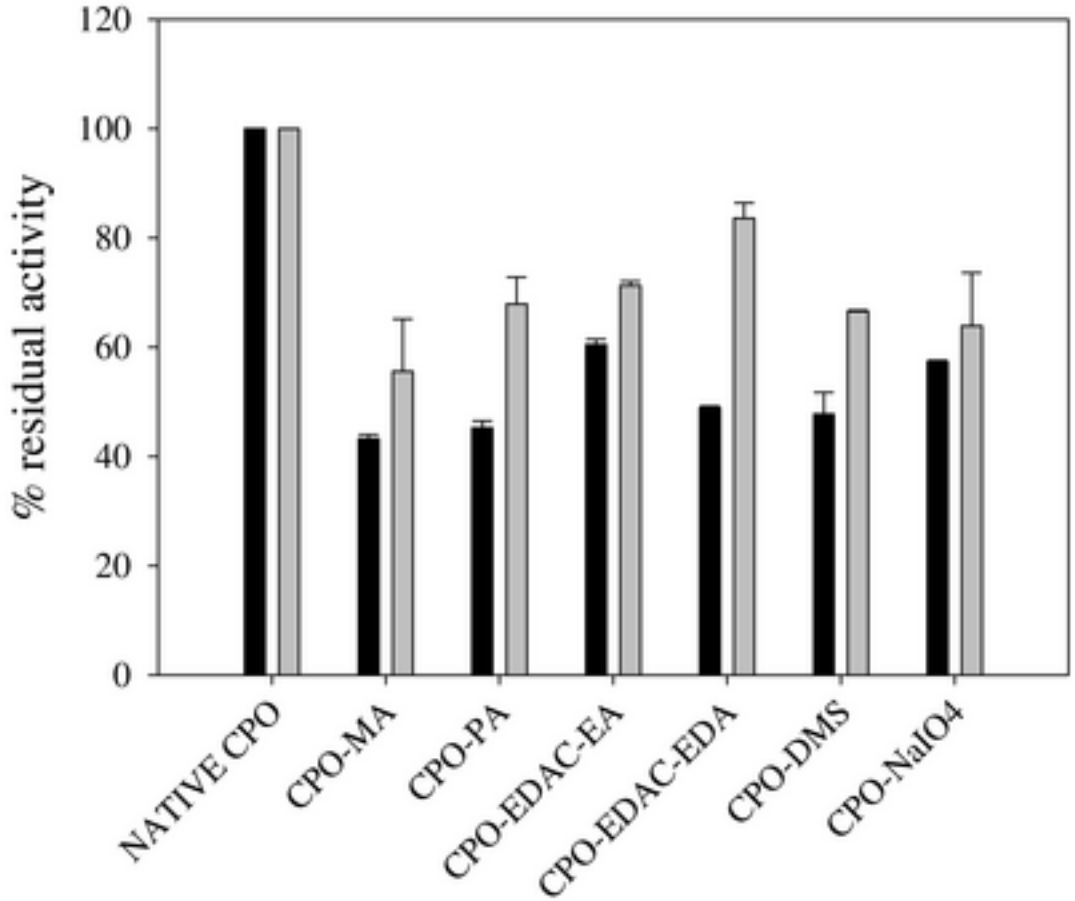
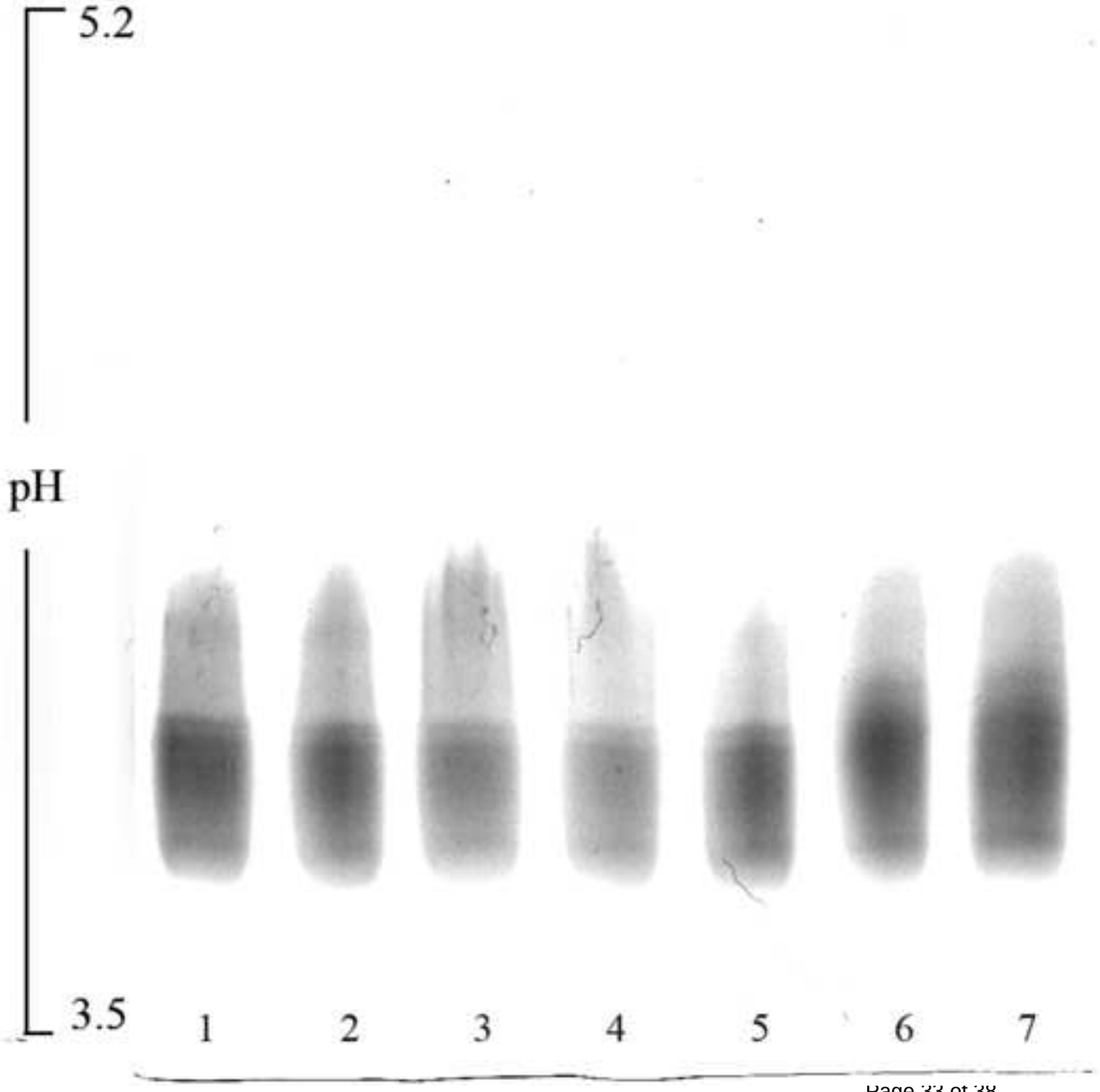

120
100
80
60
40
20
0
% residual activity
NATIVE CPO
CPO-MA
CPO-PA
CPO-EDAC-EA
CPO-EDAC-EDA
CPO-DMS
CPO-NaIO4

Figure(s)



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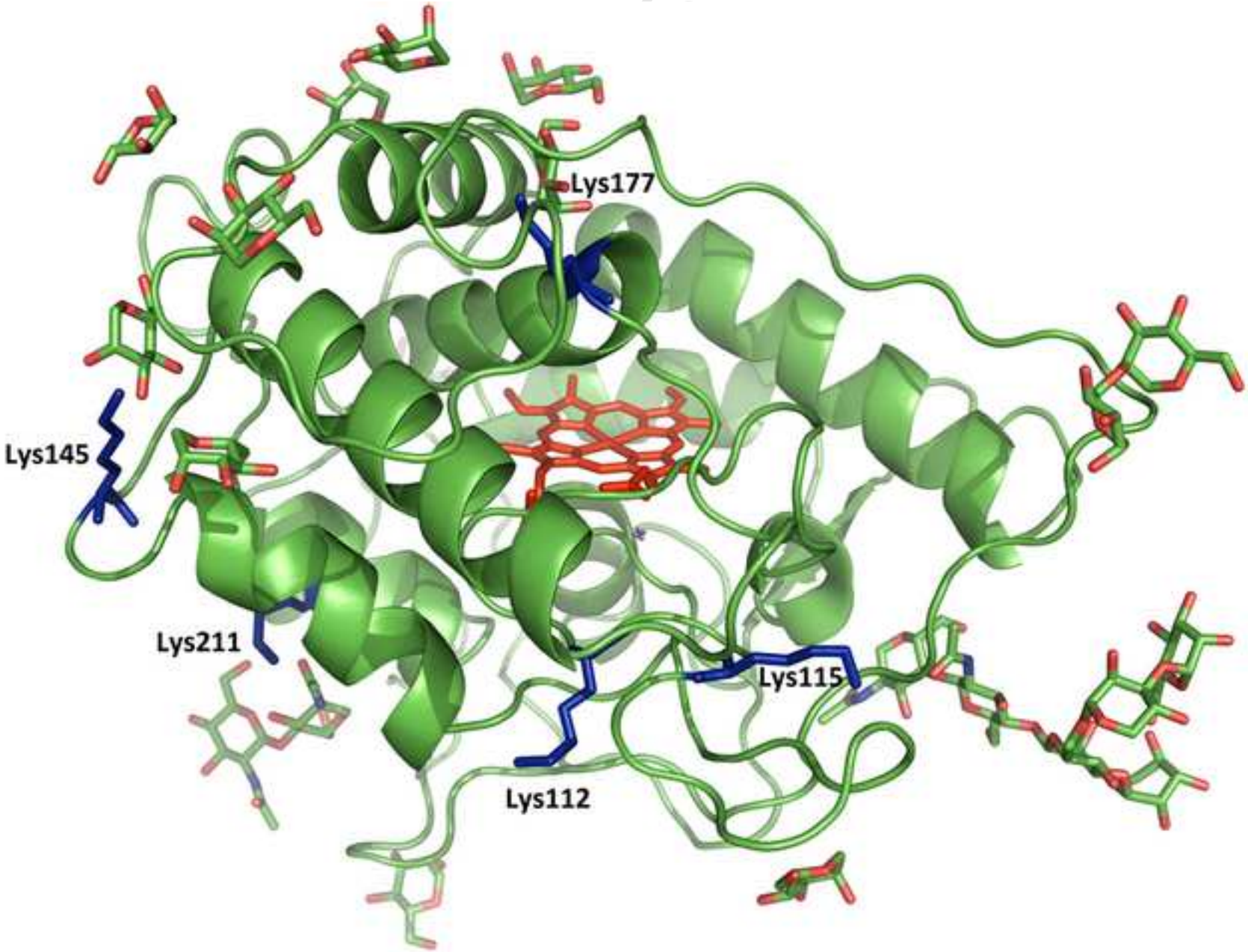

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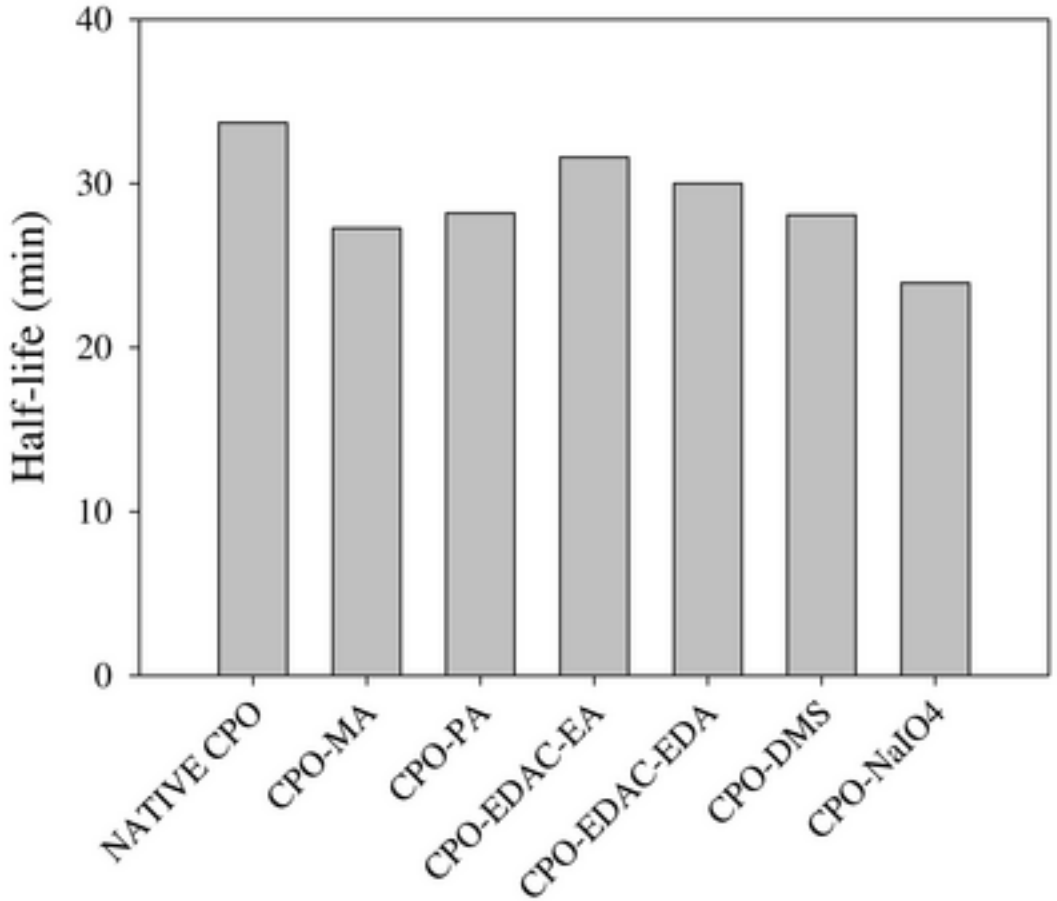
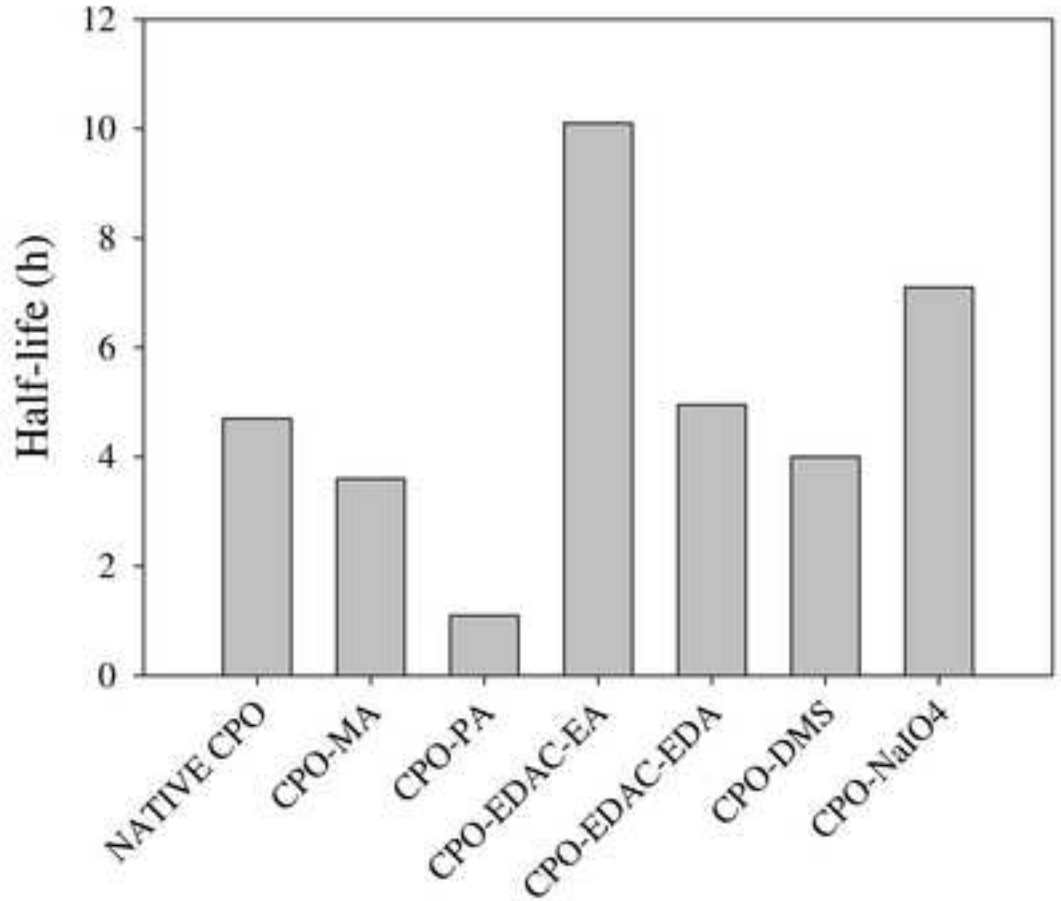
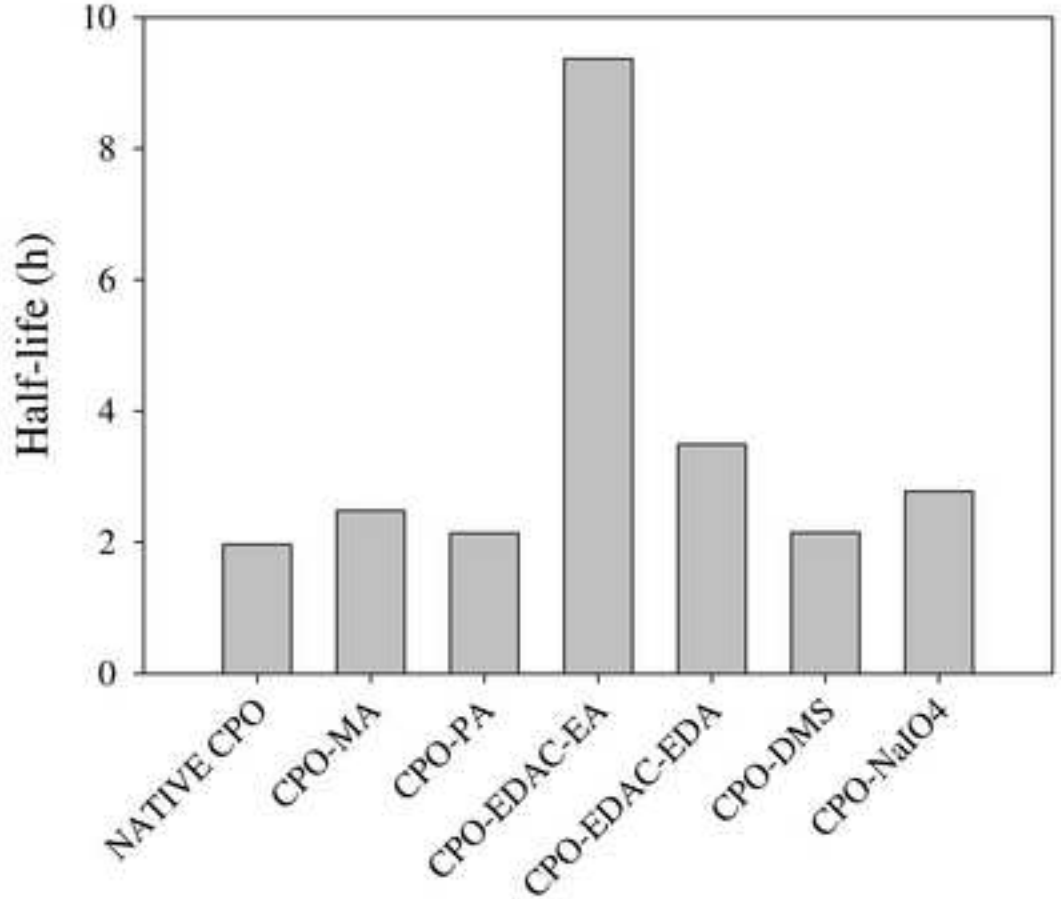

Half-life (min)
40
30
20
10
0
NATIVE CPO
CPO-MA
CPO-PA
CPO-EDAC-EA
CPO-EDAC-EDA
CPO-DMS
CPO-NaIO4

Figure(s)


Half-life (h)
0
2
4
6
8
10
12
NATIVE CPO
CPO-MA
CPO-PA
CPO-EDAC-EA
CPO-EDAC-EDA
CPO-DMS
CPO-NaIO4

Figure(s)


Half-life (h)
10
8
6
4
2
0
NATIVE CPO
CPO-MA
CPO-PA
CPO-EDAC-EA
CPO-EDAC-EDA
CPO-DMS
CPO-NaIO4

Figure(s)