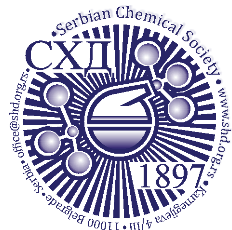

Journal of the Serbian Chemical Society
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J. Serb. Chem. Soc. 89 (2) 151–164 (2024)
JSCS–5712

Original scientific paper
Published 2 March 2024

Oxidation of 1,5-benzodiazepine oximes catalysed by peroxidases

LIDIJA KOSYCHOVA*, LINA REKOVIC, IRINA BRATKOVSKAJA, INGRIDA RADVEIKIENE and REGINA VIDŽIŪNAITĖ**

Institute of Biochemistry, Life Sciences Center, Vilnius University, Saulėtekio al. 7, Vilnius 10223, Lithuania

(Received 20 May, revised 12 September, accepted 18 September 2023)

Abstract: Oxidation of 1,3,4,5-tetrahydro-2*H*-1,5-benzodiazepine oximes catalysed by horseradish peroxidase (HRP) and recombinant *Coprinus cinereus* peroxidase (rCiP) was studied spectrophotometrically. The reaction rate dependences on the substrate and hydrogen peroxide concentrations were investigated; the values of apparent K_M and V_{max}, catalytic, oxidation and reduction constants (k_{cat}, k_{ox} and k_{red}, respectively) were calculated. The reactivity constants for the reactions catalysed by rCiP were higher than those for the HRP. Since oximes can have different structures depending on pH, the influence of pH on the rate of oxidation of compounds was studied. The dependences of the oxidation rate of the investigated oximes on the pH of the buffer solution were determined, and the p*K*a values of the amino acids of peroxidases responsible for the rate of catalysis were obtained. The HRP activity dependence on pH has a classical bell-shaped character, while rCiP dependence has a complex character.

Keywords: HRP; rCiP; 1,3,4,5-tetrahydro-2*H*-1,5-benzodiazepines.

INTRODUCTION

Nitrogen-containing heterocyclic compounds are widely distributed in nature. Both exocyclic and endocyclic organic nitrogen of heterocycles, as molecular centers, can participate in various metabolic transformations, one of which is biological oxidation. Biological nitrogen oxidation as a metabolic pathway was identified by Keese in 1959.[1] N-hydroxy compounds are biologically active secondary metabolites that can be further oxidised *in vivo* and are often sources of NO. The importance of such compounds and the mechanism of their effect on living organisms became particularly evident at the end of the last century, when R. F. Furchgott, L. J. Ignarro and F. Murad received the Nobel Prize in 1998. It should be noted that the synthesis of the first cyclic hydroxamic acid 2-hydroxy-

*,** Corresponding authors. E-mail: (*)lidija.kosychova@bchi.vu.lt, (**)regina.vidziunaite@bchi.vu.lt
https://doi.org/10.2298/JSC230520068K

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-2-pyridone was published by Shaw in 1951, and later in 1956 Clauson-Kaas synthesised derivatives of this compound that had antimicrobial properties.[2,3] The N-hydroxy group is the pharmacophore required for the biological activity of the entire drug molecule. This group significantly changes the properties of the molecule – solubility, acid–base properties and binding to the target protein. Such compounds are characterized by a wide spectrum of biological activity.[4,5]

Due to the widespread use of these compounds in medicine, the chemistry of these materials has expanded over the past few decades, producing a series of structurally novel compounds with a wide range of reactivity and stability.

Among these compounds are the pharmacologically active drugs benzodiazepines (BZDs) and their derivatives.[6] They are widely used as anti-inflammatory, analgesic, hypnotic, sedative and antidepressant agents.[6,7] Benzodiazepines bind to γ-aminobutyric acid ($GABA_A$) receptors, enhance the inhibitory effect of the neurotransmitter $GABA_A$ by removing benzodiazepine metabolites. In the liver benzodiazepines are metabolized by the action of cytochrome P450 enzymes and excreted in the urine.[8]

The 1,5-BZDs have received a lot of attention in medical research, as fewer side effects of new derivatives with enhanced pharmacological activity have been reported. These derivatives are a poorly studied class of compounds.[9] Data on their oxidation are inconsistent in the literature. Therefore, intensive transformation studies of such compounds are currently underway. One of the strategies for improving the properties of ketone drugs is the development of prodrugs by synthesising oxime precursors.[10,11] However, NO binding in the molecule to be used as a drug is known to be a risk factor, as most drugs containing amino and/or nitro groups generate reactive N–OH metabolites.[12] The high reactivity of N–OH metabolites towards nucleophiles is further enhanced by interaction with inorganic sulphate to form the o-sulphate esters, which are further ionized to form electrophilic reactive nitrene ions that covalently bind to various cellular components. The primary N-hydroxy metabolites of drugs with increased electronic density on the nitrogen atom are chemically unstable and can be further converted to secondary metabolites – nitrons, nitroses and nitro compounds.[12] Therefore, the characterization of 1,5-benzodiazepines with an iminoxy group and studies of their oxidative conversion are currently very relevant, since the resulting products can be toxic and the intensive use of these compounds can pose a danger to the environment.[13,14] Data from studies on the biodegradation of this type of compound using microorganisms and/or their enzymes have emerged recently.[15–18] Whereas, due to their chemical structure these N-hydroxyimines will have a spatially stable oxamide moiety that is likely to facilitate interaction with the enzymes. Benzodiazepine-containing drugs or other similar compounds can be oxidised not only by cytochrome P450 but also by human peroxidases (myeloperoxidases, eosinophil peroxidases, catalases). Peroxidases can

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be found in all life domains.[19] Evolutionarily related heme-containing peroxidases are found in bacteria and organelles of prokaryotic origin (class I), fungi (class II) and plants (class III).[20] In the view of the wide applications of peroxidases in the key areas of biotechnology and bioremediation of contaminating environmental pollutants and industry, they are considered as one of the important industrial enzymes.

Horseradish peroxidase (HRP) is well known for its high catalytic activity and broad specificity for electron donors.[21] A fungal peroxidase from *Coprinus cinereus* (CiP; identical to *Arthromyces ramosus* peroxidase), has attained much attention because of its high specific activity, and broad substrate specificity similar to that of the HRP.[22] Notably, CiP consists of a single species of enzyme, whereas HRP consists of at least 12 isozymes possessing different catalytic properties. The mutants of recombinant CiP (rCiP) demonstrate an elevated stability compared with CiP and/or HRP.[23] For these reasons rCiP was selected for our study and HRP was used for comparison as in investigation of other scientists.[24]

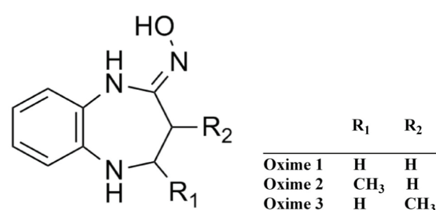
The aim of this work was to investigate and compare the oxidation of 1,5-benzodiazepine oximes catalysed by these peroxidases, to determine the main kinetic parameters, oxidation and reduction constants and the influence of pH on these transformations, since such studies are relevant, but no similar studies were found in the literature.

EXPERIMENTAL

Enzymes and chemicals

Peroxidase (EC 1.11.1.7) from horseradish (HRP, type 1) was obtained from "Reanal" (Hungary). Recombinant peroxidase from the basidiomycete *Coprinus cinereus* (rCiP) was received from "Novozymes A/S" (Denmark).

The 1,3,4,5-tetrahydro-2*H*-1,5-benzodiazepine oximes **1**–**3** (Fig. 1) synthesis was performed as described.[25]



	R_1	R_2
Oxime 1	H	H
Oxime 2	CH_3	H
Oxime 3	H	CH_3

Fig. 1. Chemical structures of 1,3,4,5-tetrahydro-2*H*-1,5-benzodiazepine oximes **1**–**3**.

96 % Ethanol was received from "Sigma Chemical Co". Acetic acid, sodium acetate, boric acid, potassium dihydrophosphate and potassium hydroxide were of analytical grade and received from "Sigma". Britton–Robinson buffer solution was obtained by titrating mixture of 20 mM H_3BO_3, 20 mM KH_2PO_4, 20 mM CH_3COOH with 0.3 M NaOH until necessary pH.

The hydrogen peroxide (30 %) was from "Roth" (Germany). Buffer solutions, solutions of enzymes and hydrogen peroxide were prepared in double distilled water. The concentrations of enzymes and hydrogen peroxide were determined spectrophotometrically.[26-28]

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Samples of oximes **1**–**3** were weighed and dissolved in ethanol. The final concentration of ethanol in the solutions for kinetic measurements was 2 vol. %. pH of buffer solutions was determined by using universal pH-meter WTW GmbH (Germany).

Kinetic measurements and calculations

Spectrophotometric measurements were performed using computer-controlled "Nicolet evolution 300" spectrophotometer (Thermo electron Corporation, USA) in Britton–Robinson buffer solution, pH 7.0, at 298 K. Absorbance spectra changes were registered in the interval from 210 to 650 nm. The kinetics of substrate and product concentrations were calculated by using extinction coefficients at 225 and 550 nm, respectively. The extinction coefficients were determined experimentally, and the values are 9.6, 11.0 and 10.5 $mM^{-1} cm^{-1}$ at 225 nm and 3.1, 2.5 and 3.2 $mM^{-1} cm^{-1}$ at 550 nm, for oximes **1**, **2** and **3**, respectively.

The rate of substrate oxidation (V) was calculated by substrate concentration kinetic curve fitting. In the case of exponential function (decrease or increase): $V = kc_0$; k is the first order reaction constant and c_0 is the initial concentration of a substrate. For the linear dependence the initial rate was calculated as a slope. To analyze the rate dependence on the substrate concentration and determine the apparent kinetic parameters V_{max} and K_M of the reactions, the Michaelis–Menten equation was used. Catalytic constants were calculated as $k_{cat} = V_{max}/[E]$, whereas bimolecular enzyme and substrate reactivity constants (k_{ox} and k_{red}) were calculated as $k_{ox/red} = k_{cat}/K_M$.

The influence of buffer solution pH on the oxidation rate of oximes **1**–**3** catalysed by HRP or rCiP was investigated using spectrophotometric method by following spectral changes at 225 and 550 nm. The dependence of the reaction rate on buffer solution pH was studied using Britton–Robinson buffer solution in pH interval 3.5–12.0, at 298 K.

For data fittings the programs GraFit (Erithacus Software Ltd.), SigmaPlot 12.0 and MathCad 2001 (MathSoft, Inc.) were used.

RESULTS AND DISCUSSION

*Determination of kinetic parameters of peroxidase-catalysed oxidation of oximes **1–3***

The 1,5-benzodiazepine oximes (oximes **1**–**3**) were enzymatically oxidised by recombinant *Coprinus cinereus* (rCiP) and horseradish (HRP) peroxidases. The synthesised oximes **1**–**3** had similar absorbance spectra in the UV region with maxima at 225, 255 and 290 nm (Fig. 2). During the oxidation process the decrease of absorbance was observed at 225 and 255 nm while moderate changes were observed at 290 nm. At the same time, the new absorbance maxima appeared at 360 and 550 nm. This is supposedly related to the formation of an intermediate which is further converted to the final product. This assumption was supported by the initial increase and further decrease of absorbance at 360 and 550 nm during the oxidation of the studied oximes.

Results presented in Fig. 2 show changes of optical density, when oxime **1** is oxidised by rCiP. Analogous changes in optical density and oxidation reaction rate were observed in all studied cases and are dependent on the structure of oximes **1**–**3**, the concentrations of enzymes HRP and rCiP, hydrogen peroxide and oximes, as well as buffer pH solution.

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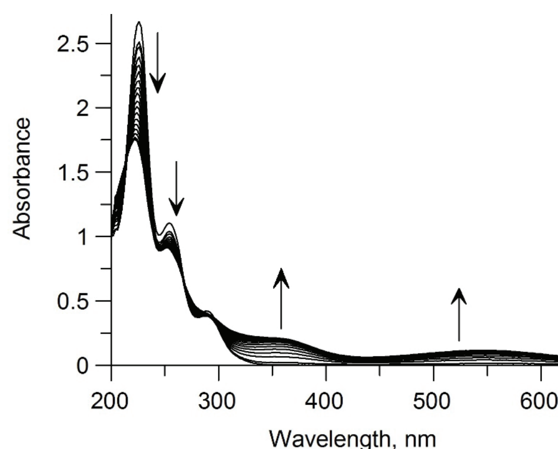

Fig. 2. The change in the absorbance spectra of oxime **1** during the oxidation catalysed by rCiP peroxidase. Conditions: 100 μM H_2O_2, rCIP 1.0 nM, 100 μM oxime **1**. 0.06 M Britton–Robinson buffer solution, pH 7.0, 298 K.

*The dependence of the oxidation rate on the concentration of oximes **1–3** and on hydrogen peroxide concentration*

The HRP- and rCiP-catalysed oxidation of oximes **1–3** in the presence of H_2O_2 was performed at pH 7.0. The substrate consumption was followed at 225 nm, and the formation of an intermediate product at 550 nm. The rate of oximes oxidation was directly proportional to rCiP or HRP concentrations.

The determined dependences of the oxidation rate on the concentrations of oximes **1**–**3** are shown on Fig. 2. The oxidation rate was determined by fitting the kinetic curves using an exponential decay function and it was found that the dependence of the oxidation rate of oximes **1**–**3** on the concentration of these substrates is hyperbolic and corresponds to the Michaelis–Menten equation. The apparent values of the parameters K_M and V_{max} were determined, and also the catalytic and oxidation constants were calculated and are presented in Table I. Although the parameters obtained during the formation of the primary product are similar to those of the disappearance of the oxime, evaluating and comparing the obtained kinetic parameters is not completely correct, because during the formation of the primary product its transformation into the final product also takes place.

TABLE I. Kinetic parameters of oximes **1**–**3** oxidation at 225 nm

Oxime	HRP			rCIP		
	K_M / μM	k_{cat} / s^{-1}	k_{ox} / μM^{-1} s^{-1}	K_M / μM	k_{cat} / s^{-1}	k_{ox} / μM^{-1} s^{-1}
1	200.0±20.0	250.0±25.0	1.2±0.2	160.0±40.0	3500.0±900.0	10.0±1.0
2	250.0±25.0	26.0±8.0	0.14±0.03	120.0±25.0	230.0±25.0	1.9±0.2
3	50.0±10.0	150.0± 20.0	3.1±0.2	80.0±20.0	640.0±100.0	8.0±1.4

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As can be seen from the data in Table I, peroxidases exhibit different affinity and reactivity to the oximes **1**–**3**. The lowest K_M values were obtained for oxime **3** during both the HRP-catalysed and rCiP-catalysed oxidation, 50 and 80 μM, respectively.

The highest oxidation (k_{ox}) and catalytic (k_{cat}) constants were obtained during the oxidation of oxime **1**, whereas they were slightly lower for oxime **3.** The oxidation constants of oxime **2** by both HRP and rCiP were significantly lower. In addition, the values of the constants k_{cat} and k_{ox} obtained with rCiP are significantly higher than those obtained with HRP, which allows us to conclude that rCiP catalyses oxidation processes more efficiently. These results are in good agreement with the literature data on the oxidative capacity of heme-containing peroxidases. It is known that rCiP has better oxidizing power than HRP[29] and the data obtained confirm this.

The investigation of the oximes oxidation rate on the concentration of H_2O_2 showed that the concentrations of H_2O_2 higher than 100 μM were inhibiting the reaction rate, thus they were not used for the calculation of the kinetic constants. The apparent values of the K_M and V_{max} were determined from obtained dependences and the catalytic and reduction constants were calculated and are presented in Table II.

TABLE II. Kinetic parameters of H_2O_2 reduction at 225 nm

Oxime	HRP			rCIP		
	K_M / μM	k_{cat} / s^{-1}	k_{red} / μM^{-1} s^{-1}	K_M / μM	k_{cat} / s^{-1}	k_{red} / μM^{-1} s^{-1}
1	15.0±4.0	110.0±10.0	7.0 ±2.0	38.0±16.0	460.0±80.0	12.1±3.3
2	8.5±0.8	12.5 ±2.0	1.5±0.3	37.0±21.0	220.0±50.0	6.1±2.3
3	25.0±7.0	130.0±15.0	5.2±1.0	51.0±17.0	450.0±60.0	8.8±1.7

As can be seen from the data in Table II, the reactivity constants for the reactions catalysed by rCiP were higher than those for the HRPs in all cases analysed. This indicates that rCiP oxidises 1,5-benzodiazepine oximes significantly more efficiently than HRP.

The pH dependence of oximes 1–3 oxidation rates

The changes of absorption spectra during the oxidation of oxime **1** catalysed by rCiP at different buffer solution pH are presented in Fig. 3. Similar spectral changes were observed during oxidations of oximes **2** and **3** catalysed by both peroxidases used in this work.

The decrease of absorbance was observed at 225 and 255 nm while two new absorbance maxima at 360 and 550 nm appeared during the oxidation of oxime **1** catalysed by rCiP at pH 7.0 (Fig. 3d). Similar changes were registered at pH 9.0 (Fig. 3e). Conversely, the expected maxima in the long wave region (550 nm) were diminished at pH 10.0 (Fig. 3f). In the acidic media at pH 3.5–4.5 and 5.5

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both of the absorbance maxima at 360 and 550 nm were absent (Fig. 3a and b) or insignificant (Fig. 3c), instead noticeable increase of the absorbance was observed at 240–260 nm and 280–340 nm regions.

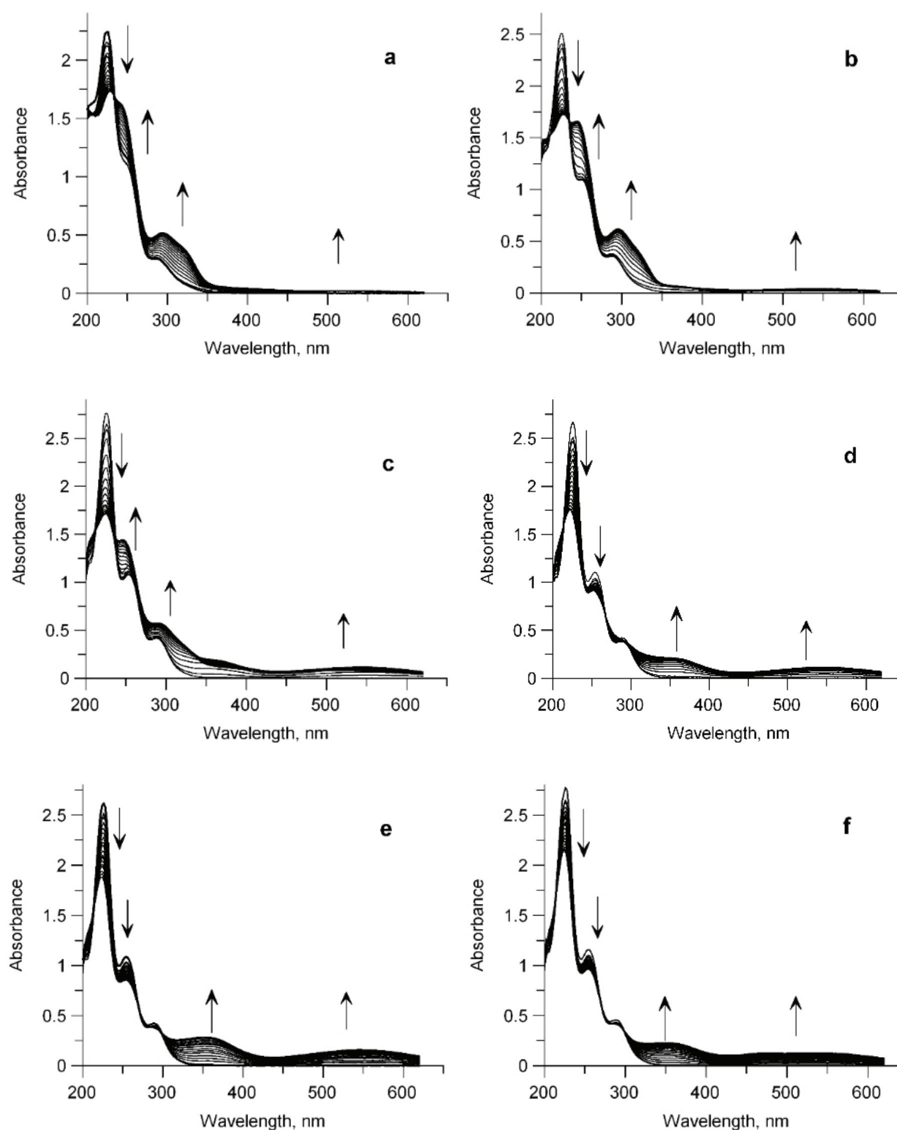

Fig. 3. The changes in the absorbance spectra during the oxidation of oxime **1** catalysed by rCiP at different buffer solution pH. Experimental conditions: oxime **1** – 100 μM; H_2O_2 – 100 μM; pH – 3.5 (a), 4.5 (b), 5.5 (c), 7.0 (d), 9.0 (e) and 10.0 (f); 0.06 M Britton–Robinson buffer solution, 298 K.

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Based on the data from the literature[30] and the obtained results, the spectral changes can be explained by the formation of the initial reactive product during the enzymatic oxidation of oximes **1**–**3**. Presumably radical species which absorb UV light in the long wave region transform further into the final product during secondary non-enzymatic conversion step. This is in agreement with Fukunishi *et al*., who showed that the iminoxical radicals capable of recombining through O–N, O–C, and N–N bonds to form the corresponding dimers are generated by oxidation of substituted benzaldehyde oximes with H_2O_2 and HRP.[31] Moreover, Aveline *et al.* showed that *N*-hydroxy-2(1*H*)-pyridone (N-HP) was capable of forming poorly reactive radicals.[30]

Our obtained results coincide with the data presented in work by Aveline *et al*. These results also confirm the presumption that initial product, which formed during peroxidase-catalysed oxidation of oximes **1**–**3**, can be radical.[30] If the radicals of investigated **1**–**3** oximes are protonized, positive charge from oxygen can be delocalized to the tertiary carbon. Since the reactivity of the formed radical is low, its disappearance is predetermined by radical recombination reactions. In acidic region, at pH 4.5–5.5, pronounced maxima at 360 and 550 nm were not observed, but intensive absorbance increases at 240–260 nm and 280–340 nm was registered. Probably an intermediate compound was formed during the oxidation reaction, which was further converted to the final product. These compounds are believed to be unstable in acidic buffer solutions because no distinct absorption maxima at 360 and 550 nm were observed.

The results obtained during the oxidation of oximes at neutral pH and basic buffer solutions (pH 7.0–9.0) also agree with the data presented in the literature.[30] In the beginning there was an increase in absorption during the oxidation of the oxime and a further decrease at 360 and 550 nm. It has also been observed that the compounds formed in alkaline solutions are converted to the final product more slowly than in acidic solutions. The intermediate compound probably is more stable and less reactive in alkaline solutions. It should be mentioned that the absorbance maximum at 550 nm was absent while a new one at 460 nm was registered in extremely basic medium (pH 11.0). It is possible that the studied oximes **1**–**3** could have been deoximated into their carbonyl form.[4,32] Similar spectral changes were also registered during the oxidation of oximes **2** and **3** catalysed by both peroxidases.

The rate of oxidation of oximes **1**–**3**, as well as intermediate compounds formed during the enzymatic reaction and their further transformation into the final product depended on the pH of the buffer solution (Fig. 4). As can be seen from results presented in Fig. 4 a and b, the pH dependence of HRP activity upon oxidation of oximes **1**–**3** was classically bell-shaped. For oximes **1** and **3**, the maximum HRP activity was observed in the pH range from 5.5 to 8.0, while the pH optimum for oxime **2** was shifted to a more acidic range from 4.5 to 7.0. A

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similar trend was observed for the rate of formation of intermediate product at 550 nm at the same pH of the buffer solution (Fig. 4 b).

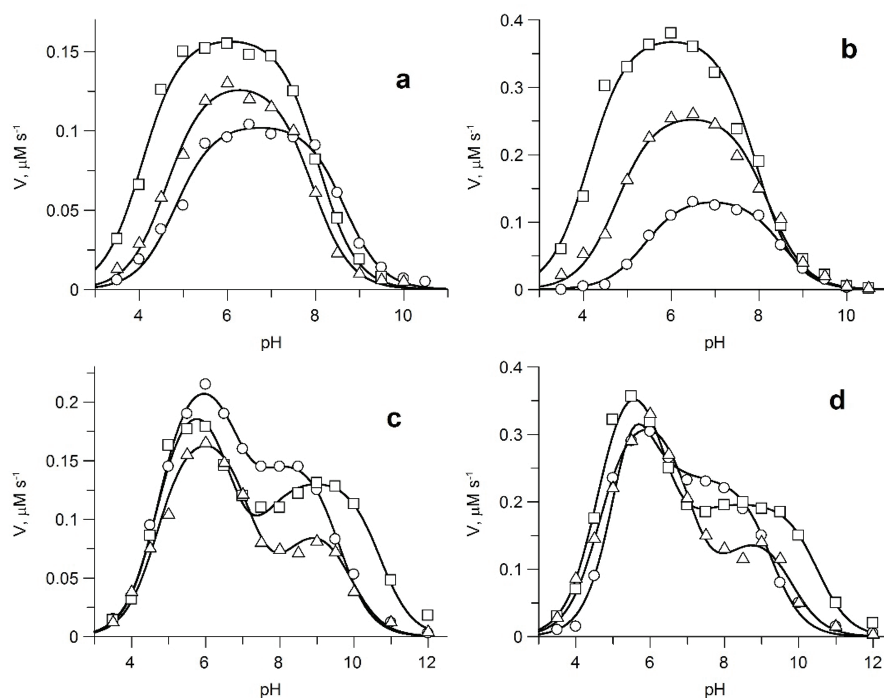

Fig. 4. The pH effect on the oxidation rates of oximes **1**–**3** catalysed by HRP and rCiP. The disappearance of substrate was followed at 225 nm (a, c); the formation of initial product was followed at 550 nm (b, d). Conditions: oximes – **1** (○), **2** (□) and **3** (△) – 100–110 μM; H_2O_2 – 100–110 μM; 0.06 M Britton–Robinson buffer solution, 298 K.

Determined reaction rate dependences on the buffer solution pH were complex when reaction was catalysed by rCiP Fig. 4 c and d. The dependences of oxidation rates of oximes **1**–**3** on the buffer solution pH, characterized by disappearance (225 nm) and appearance of intermediate product (550 nm), showed that rCiP is able to oxidise oximes **1**–**3** in a broader pH range from 3.5 to 11.0 with the highest rate observed in pH 5.5–6.5 interval. In highly acidic or basic buffer solutions the reaction rate decreased, and the pH curve shape differed from the classic bell form. To calculate the p*K*a values, the resulting curves were decomposed into individual peaks (deconvolution) and the resulting peaks were smoothed to a bell shape using a theoretical model. The determined pKa values of rCiP- and HRP-catalysed oxidation of oximes **1**–**3** are presented in Table III.

Peroxidases share a common catalytic mechanism for the degradation of H_2O_2.[33] The peroxidase reaction is a two-electron oxidation–reduction with three distinct steps.[34] The peroxidase catalytic cycle is described by the equations else-

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where.[22] During this reaction, radicals are formed, which then disproportionate or initiate various non-enzymatic reactions, including degradation or polymerization processes.

TABLE III. The p*K*a values determined from the pH dependences of oximes **1**–**3** oxidation rate for HRP and rCiP catalysed reactions

Oxime	HRP		rCIP			
	p*K*a1	p*K*a2	p*K*a1	p*K*a2	p*K*a3	p*K*a4
1	4.8±0.04	8.6±0.04	4.7±0.02	7.0±0.04	7.4±0.03	9.7±0.02
2	4.0±0.10	8.0±0.10	4.7±0.02	6.6±0.02	7.3±0.02	10.7±0.02
3	4.6±0.03	8.4±0.03	4.7±0.03	7.2±0.06	8.2± 0.01	9.9±0.01

It is known that the rate of formation of an intermediate compound (radical) is independent of pH in the range of 6–10. However, in an acidic medium, the formation of an intermediate compound is determined by another titratable group with pKa 5.0 located in the catalytic center of the enzyme. Abelskov and Smulevich with co-autors concluded that this titratable group is the proximal His183, but not as expected the distal His55, and the latter is responsible for conversion of the Fe–OOH complex to intermediate compound (radical).[22,35]

In this work, the oxidation of oximes **1**–**3** with rCiP yielded pKa 4.7 and with HRP yielded pKa 4.0–4.8 for the first titratable group. It is as the protonated substrate, since the analysed substrates may have a different form in over the entire pH range (Fig. 5).

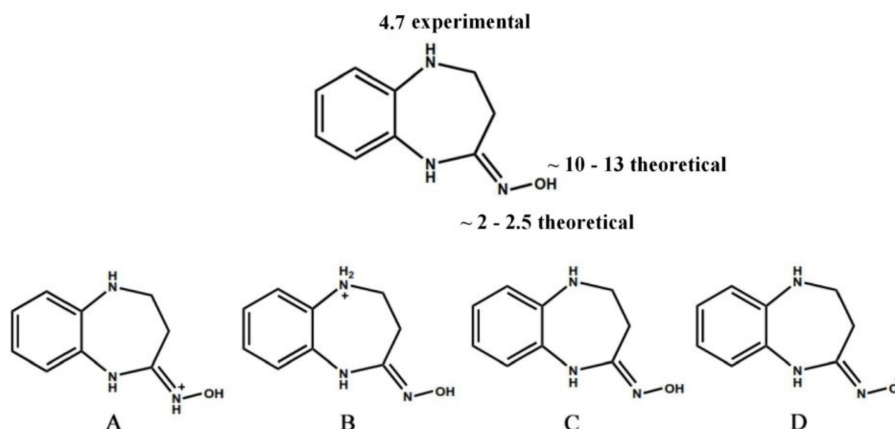

Fig. 5. Structures of oxime **1** that can be formed at different pH of the buffer solution.

Thus, oxime **1** has four potential ionization forms. In a strongly acidic medium, the nitrogen protonation of the $=NH^{+}$–OH group (form A) takes place. When the medium is acidified, one of the nitrogen atoms is protonated (form B), for which the pKa value is experimentally determined in this work to be 4.7. In

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neutral media, oxime **1** exists in the C form. According to the literature, the anion in aqueous solutions has the pKa 10–13, so in a strongly alkaline medium the oxime =N–OH group is deprotonated, *i.e.*, the =N–O$^-$ anion (D form) is formed.[4,36] But the strongly acidic (pH < 3) and alkaline (pH > 11) regions are outside the limits of the studied pH dependences and therefore may not be analysed.

The obtained p*K*a 4.7 of the first titratable group in the reaction with rCiP was very close to the values determined for oximes **1–3** by spectroscopic methods, 4.56, 4.60 and 4.51 (fluorescence data), respectively.[25] Therefore, it cannot be unambiguously determined whether this titratable group belongs to the enzyme or to the substrate, since the enzyme has distal His55 (p*K*a = 5.1) or proximal His183 (p*K*a= 5.0).[22] However, it turned out that the p*K*a of the first titratable group is 4.6–4.8 in both HRP- and rCiP-catalysed oxidation. The fact that the pKa of this group is independent of the enzyme used is a possible proof that the first titratable group belongs to the substrate. pKa values are very sensitive to the environment, and the catalytic centers of HRP and rCiP have their own differences, although they consist of the same amino acids. The second titratable group was found to have p*K*a 8.6 (1), 8.0 (2) and 8.4 (3), Table III. It is well known from the literature that the properties of HRP are unambiguously related to the distal histidine with p*K*a of 8.6, 8.8, which is capable of forming a hydrogen bond with ferrile oxygen [Fe(IV)=O] in compound II.[37,38] The obtained p*K*a values of the second titratable group were slightly different depending on the compound being oxidised. Therefore, it can be assumed that the substrate could influence the distribution of electron density in the His+–H–O–Fe group of atoms. The third titratable group (for rCiP) obtained in this work has p*K*a 9.7 and p*K*a 10.5–10.7 for the oxidation of oxime **2** with hydrogen peroxide and rCiP. It is known that the nitrogen of distal arginine in the catalytic center of HRP, HRP C, CiP and CCP heme peroxidases in the case of cpd I and II is capable of forming hydrogen bond with oxygen of Fe(IV)=O group of enzyme. In CiP peroxidase distal Arg has p*K*a ≈ 10.[22] In order to complete the peroxidase catalytic cycle the hydroxyl or one water molecule must dissociate and the ferric oxygen must take up one or two protons. In the case when Arg is deprotonated, the proton transfer becomes impossible and the rate decreases.

It should be mentioned that the deprotonation pKa of the =N–OH group of oximes **1**–**3** was not determined experimentally. If the obtained p*K*a 9.7 and p*K*a 10.5–10.7 are the conversion of the substrate to the anionic form, the rate may decrease due to the fact that the anionic form is not a peroxidase substrate. This is shown in the literature in studies with phenolic substrates (*p*-methoxyphenol) and HRP C.[22]

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CONCLUSIONS

Spectrophotometric investigations of oxidation of oximes **1**–**3** catalysed by horseradish peroxidase (HRP) and fungal peroxidase (rCiP) showed how the rate of oxidation depends on the concentration of oximes and hydrogen peroxide, the nature of the enzymes, the structure of the oximes as well as from the pH of the buffer solution.

Based on the dependences of the reaction rates on the concentrations of oximes **1**–**3** and hydrogen peroxide, the apparent K_M and V_{max} values were determined, and the catalytic, oxidation and reduction constants were calculated. Determined reactivity constants indicate that rCiP oxidises 1,5-benzodiazepine oximes significantly more efficiently than HRP. The highest catalytic, oxidative and reduction constants were obtained in the oxidation of oxime **1**, and the lowest in the oxidation of oxime **2**. It was established that hydrogen peroxide concentrations higher than 100 μM inhibit reactions catalysed by peroxidases. Apparently, the reactivity of oximes depends on their structure, the position of the $–CH_3$ group in the oxime molecule, and interaction with the enzyme.

The dependences of the oxidation rates of oximes and the resulting intermediate products on the pH of the buffer solution, as well as the p*K*a values of the amino acids of the enzymes involved in catalysis, were obtained. It was determined that if the dependence of HRP activity on pH is classically bell-shaped, then the dependence of rCiP is complex. rCiP oxidises 1,5-benzodiazepine oximes over a wider pH range than HRP. Such pH dependencies are likely determined by the ionization forms of the oximes at various pHs, their p*K*a values, and the properties of the enzyme.

The results show that for practical applications in the oxidation of benzodiazepine oximes, rCiP is preferable to HRP because it oxidises more efficiently, works in a wider pH range, and can be synthesised cheaper, in large quantities, and with suitable activity.

ИЗВОД

ОКСИДАЦИЈА ОКСИМА 1,5-БЕНЗОДИАЗЕПИНА КАТАЛИЗОВАНА ПЕРОКСИДАЗАМА

LIDIJA KOSYCHOVA, LINA REKOVIC, IRINA BRATKOVSKAJA, INGRIDA RADVEIKIENE
и REGINA VIDŽIŪNAITĖ

Institute of Biochemistry, Life Sciences Center, Vilnius University, Saulėtekio al. 7, Vilnius 10223, Lithuania

Спектрофотометријски је испитивана оксидација оксима 1,3,4,5-тетрахидро-2*H*--1,5-бензодиазепина катализована пероксидазом из рена (HRP) и рекомбинантном пероксидазом гљиве *Coprinus cinereus* (rCiP). Анализиран је утицај концентрације супстрата и водоник пероксида на брзину реакције. Израчунате су вредности K_M и V_{max}, као и константе каталитизе, оксидације и редукције (k_{cat}, k_{ox} и k_{red}). Константе реактивности за реакцију катализовану ензимом rCiP су биле веће него са HRP. Пошто се структура оксима мења у зависности од pH, даље је испитиван утицај pH на брзину оксидације испитиваних једињења. Утврђена је зависност брзине оксидације оксима од pH пуфера и

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добијене pK_a вредности амино киселина пероксидаза које су одговорне за брзину катализе. Зависност активности HRP од pH прати класични облик звона, док је зависност rCiP комплексниja.

(Примљено 20. јуна, ревидирано 12. септембра, прихваћено 18. септембра 2023)

REFERENCES

1. W. B. Jakoby, *Metabolic Basis of Detoxication: Metabolism of Functional Groups*, Elsevier Science, Amsterdam, 1982 (ISBN: 9780323137997)
2. E. N. Shaw, *2-hydroxy-pyridine*-N-*oxide and process for preparing same*, 1951 (http://www.google.com/patents/US2540218) Accessed May 8, 2023
3. N. K. F. W. Clauson-Kaas, N. Elming, J. Tormod Nielsen, *Derivatives of N-hydroxy pyridines and process of production*, 1956 (http://www.google.ch/patents/US2748142) Accessed May 8, 2023.
4. S. B. King, *C-Nitroso Compounds, Oximes,* N-*Hydroxyguanidines and* N-*Hydroxyureas*, John Wiley & Sons, Ltd., New York, 2005 (https://doi.org/10.1002/3527603751.CH7)
5. R. Rani, C. Granchi, *Eur. J. Med. Chem.* **97** (2015) 505 (https://doi.org/10.1016/J.EJMECH.2014.11.031)
6. P. Aastha, K. Navneet, A. Anshu, S. Pratima, K. Dharma, *Res. J. Chem. Sci.* **3** (2013) 90 (http://www.isca.me/rjcs/Archives/v3/i7/14.ISCA-RJCS-2013-057.php)
7. P. Jara-Ulloa, S. Catalán-Caro, C. A. Escobar, *J. Chil. Chem. Soc.* **59** (2014) 2520 (https://doi.org/10.4067/S0717-97072014000200027)
8. M. Whirl-Carrillo, R. Huddart, L. Gong, K. Sangkuhl, C. F. Thorn, R. Whaley, T. E. Klein, *Clin. Pharmacol. Ther.* **110** (2021) 563 (https://doi.org/10.1002/CPT.2350)
9. O. Mazimba, T. C. Molefe, *Int. J. Chem. Stud.* **3** (2015) 46 (https://www.chemijournal.com/archives/2015/vol3issue3/PartA/3-2-9.1.pdf)
10. L. Kosychova, R. Vidziunaite, G. Mikulskiene, I. Bratkovskaja, R. Janciene, *Arkivoc* **2015** (2015) 71 (https://doi.org/10.3998/ARK.5550190.P008.772)
11. S. Kumar, H. Kavitha, S. Arulmurugan, B. Venkatraman, *Mini. Rev. Org. Chem.* **9** (2012) 285 (https://doi.org/10.2174/1570193X11209030285)
12. K. D. Krewulak, H. J. Vogel, *Biochim. Biophys. Acta – Biomembr.* **1778** (2008) 1781 (https://doi.org/10.1016/J.BBAMEM.2007.07.026)
13. Y. Zhang, S. U. Geißen, C. Gal, *Chemosphere* **73** (2008) 1151 (https://doi.org/10.1016/j.chemosphere.2008.07.086)
14. T. Kosjek, S. Perko, M. Zupanc, M. Zanoški Hren, T. Landeka Dragičević, D. Žigon, B. Kompare, E. Heath, *Water Res.* **46** (2012) 355 (https://doi.org/10.1016/j.watres.2011.10.056)
15. T. Hata, H. Shintate, S. Kawai, H. Okamura, T. Nishida, *J. Hazard. Mater.* **181** (2010) 1175 (https://doi.org/10.1016/J.JHAZMAT.2010.05.103)
16. E. Marco-Urrea, M. Pérez-Trujillo, C. Cruz-Morató, G. Caminal, T. Vicent, *Chemosphere* **78** (2010) 474 (https://doi.org/10.1016/J.CHEMOSPHERE.2009.10.009)
17. A. Jelic, C. Cruz-Morató, E. Marco-Urrea, M. Sarrà, S. Perez, T. Vicent, M. Petrović, D. Barcelo, *Water Res.* **46** (2012) 955 (https://doi.org/10.1016/J.WATRES.2011.11.063)
18. S. Ostadhadi-Dehkordi, M. Tabatabaei-Sameni, H. Forootanfar, S. Kolahdouz, M. Ghazi--Khansari, M. A. Faramarzi, *Bioresour. Technol.* **125** (2012) 344 (https://doi.org/10.1016/J.BIORTECH.2012.09.039)

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19. H. Kellner, P. Luis, M. J. Pecyna, F. Barbi, D. Kapturska, D. Kruğer, D. R. Zak, R. Marmeisse, M. Vandenbol, M. Hofrichter, *PLoS One* **9** (2014) e95557 (https://doi.org/10.1371/JOURNAL.PONE.0095557)
20. K. G. Welinder, J. M. Mauro, & L. Norskov-Lauritsen, *Biochem. Soc. Trans.* **20** (1992) 337 (https://doi.org/https://doi.org/10.1042/bst0200337)
21. S.-J. Kim, J.-A. Lee, Y.-H. Kim, & B.-K. Song, *J. Microbiol. Biotechnol.* **19** (2009) 966 (https://doi.org/10.4014/jmb.0901.018)
22. A. K. Abelskov, A. T. Smith, C. B. Rasmussen, H. B. Dunford, K. G. Welinder, *Biochemistry* **36** (1997) 9453 (https://doi.org/10.1021/BI970387R)
23. Y. Yao, L. Huang, Y. Xu, Q. X. Li, *J. Agric. Food Chem.* **70** (2022) 646 (https://doi.org/10.1021/acs.jafc.1c06261)
24. R. Ivanec-Goranina, J. Kulys, *Cent. Eur. J. Biol.* **3** (2008) 224 (https://doi.org/10.2478/S11535-008-0021-X)
25. L. Rekovic, L. Kosychova, I. Bratkovskaja, R. Vidziunaite, *J. Serb. Chem. Soc* **83** (2018) 343 (https://doi.org/10.2298/JSC180226090R)
26. L. X. Shannon, E. Kay, J. Y. Lew, *J. Biol. Chem.* **241** (1966) 2166 (https://doi.org/https://doi.org/10.1016/S0021-9258(18)96680-9)
27. Z. S. Farhangrazi, I. Yamazaki, L. S. Powers, B. R. Copeland, T. Nakayama, T. Amachi, *Biochemistry* **33** (1994) 5647 (https://doi.org/https://doi.org/10.1021/bi00184a038)
28. D. P. Nelson, L. A. Kiesow, *Anal. Biochem.* **49** (1972) 474 (https://doi.org/https://doi.org/10.1016/0003-2697(72)90451-4)
29. E. Torres, M. Ayala, *Biocatalysis based on heme peroxidases: Peroxidases as potential industrial biocatalysts*, Springer, Berlin, 2010 (https://doi.org/https://doi.org/10.1007/978-3-642-12627-7_3)
30. B. M. Aveline, I. E. Kochevar, R. W. Redmond, *J. Am. Chem. Soc.* **118** (1996) 10124 (https://doi.org/https://doi.org/10.1021/ja961989l)
31. K. Fukunishi, K. Kitada, I. Naito, *Synthesis (Stuttgart)* **3** (1991) 237 (https://doi.org/https://doi.org/10.1055/s-1991-26433)
32. S. Sahu, S. Sahu, S. Patel, S. Dash, B. K. Mishra, *Indian J. Chem.* **47** (2008) 259. (https://nopr.niscpr.res.in/handle/123456789/1410)
33. M. Nissum, A. Feis, G. Smulevich, *Biospectroscopy* **4** (1998) 355 (https://doi.org/https://doi.org/10.1002/(SICI)1520-6343(1998)4:6<355::AID-BSPY1>3.0.CO;2-I)
34. P. Tn, T. L. Poulos, J. Kraut, *J. Biol. Chem.* **255** (1980) 8199 (https://doi.org/https://doi.org/10.1016/S0021-9258(19)70630-9)
35. G. Smulevich, A. Feis, C. Focardi, J. Tams, K. G. Welinder, *Biochemistry* **33** (1994) 15425 (https://doi.org/https://doi.org/10.1021/BI00255A024)
36. S. Ramalingam, M. Karabacak, S. Periandy, N. Puviarasan, D. Tanuja, *Spectrochim. Acta, A* **96** (2012) 207 (https://doi.org/10.1016/j.saa.2012.03.090)
37. S. Hashimoto, Y. Tatsuno, T. Kitagawa, *Proc. Natl. Acad. Sci. U. S. A.* **83** (1986) 2417 (https://doi.org/https://doi.org/10.1073/PNAS.83.8.2417)
38. I. Yamazaki, M. Tamura, & R. Nakajima, *Mol. Cell. Biochem.* **40** (1981) 143 (https://doi.org/https://doi.org/10.1007/BF00224608).

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