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Oxidation of Crocein Orange G by Lignin Peroxidase Isoenzymes
Kinetics and Effect of H_2O_2

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ABSTRACT

The ligninolytic enzyme system of *Phanerochaete chrysosporium* is able to decolorize several recalcitrant dyes. Three lignin peroxidase isoenzymes, LiP 3.85, LiP 4.15, and LiP 4.65, were purified by preparative isoelectric focusing from the carbon-limited culture medium of *P. chrysosporium.* Based on amino terminal sequences, the purified isoenzymes correspond to the isoenzymes H8, H6, and H2, respectively, from the *N*-limited culture. The purified isoenzymes were used for decolorization of an azo dye, Crocein Orange G (COG). According to the kinetic data obtained, the oxidation of COG by lignin peroxidase appeared to follow Michaelis-Menten kinetics. Kinetic parameters for each isoenzyme were determined. The inactivating effect of ascending H_2O_2 concentrations on COG oxidation is shown to be exponential within the used concentration range. The best degree of decolorization of 100 μ*M* COG was obtained when the H_2O_2 concentration was 150 μ*M*. This was also the lowest H_2O_2 concentration for maximal decolorization of 100 μ*M* COG, regardless of the amount of lignin peroxidase used in the reaction.

Index Entries: Azo dye; decolorization; lignin peroxidase; *Phanerochaete chrysosporium.*

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INTRODUCTION

A wide variety of dyes are used by industry and released into the environment in industrial effluents. These dyes have to be highly stable in everyday use and resistant to microbial degradation. Azo dyes are the largest class of dyes used in industry *(1)*, and they appear to be resistant to aerobic degradation by bacteria *(2,3)*. However, a few azo dyes have been demonstrated to be degradable by some aerobic and anaerobic bacteria *(4)*. Under anaerobic conditions, the azo linkage can be reduced to generate aromatic amines that are colorless, but which can be toxic and potentially carcinogenic *(5,6)*. In mammals, this process is performed by anaerobic bacteria of intestinal microflora *(4,7)*. Also, cytochrome P-450 *(8)* and a flavin-dependent cytosolic reductase *(9)* are able to reduce azo dyes to aryl amines.

The lignin-degrading white rot fungus, *Phanerochaete chrysosporium*, is able to degrade a wide range of aromatic pollutants *(10–14)*, including azo dyes *(15–17)*. In each case, evidence for the lignin-degradative system of *P. chrysosporium* being involved in the degradation of these pollutants has been presented.

The oxidation of azo dyes by peroxidases of *P. chrysosporium* has been studied previously, using either the whole culture of the fungus *(15,16,18)* or purified peroxidases *(17,19–21)*. In the studies in which purified peroxidases were used, the reaction conditions have been constant. An exception has been the authors' previous work *(17)*, in which the reactions were done at different pHs. For this article, the authors have studied the H_2O_2-dependent oxidation of an azo dye, Crocein Orange G (COG), by three major lignin peroxidase (LiP) isoenzymes and, as a result, kinetic parameters for the oxidation of COG are presented for each isoenzyme. Also, the effects of H_2O_2 and pH on oxidation were further studied, and the conditions for the maximal decolorization of COG were determined.

MATERIALS AND METHODS

Chemicals

The azo dye, 6-hydroxy-5-(phenylazo)-2-naphthalenesulfonic acid monosodium salt (COG) (Fig. 1), was purchased from Aldrich Chemie GmbH, Steinheim, Germany. Dye content of the product was 70%. The remaining 30% of the product was UV-inactive material, such as inorganic salts (C. Behringer, Aldrich Chemie, personal communications). Hydrogen peroxide solution was prepared daily by diluting the stock solution with purified water, and the concentration was determined at 240 nm, using the extinction coefficient of $39.4/(M \times \text{cm})$ *(22)*. All other chemicals were of analytical grade, and used as such.

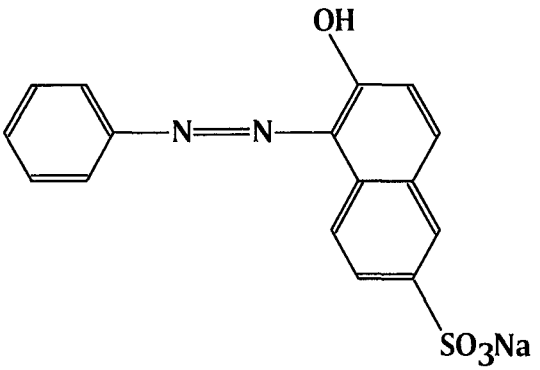

Fig. 1. Chemical structure of Crocein Orange G.

Microorganism and Culture Conditions

P. chrysosporium VKM-F-1767 (ATCC 24725) was provided by Jakob Reiser (Institut für Biotechnologie, Zurich, Switzerland), and was maintained on 2% malt agar slants.

P. chrysosporium was grown immobilized at 30°C in the carbon-limited liquid culture medium described by Kirk et al. *(23)*, with some modifications *(24)*. The carrier for immobilization *(25)*, nylon web (Bear-Tex, Visella Oy, Valkeakoski, Finland), was washed 3× with boiling water, and sterilized in a fermentor (Chemap AG, Volketswil, Switzerland) in 13 L of the growth medium. Glucose, vitamins, and minerals were sterilized separately then added in the medium. When the temperature of the medium had fallen to 30°C, the medium was inoculated with 5×10^8 spores.

During the growth, glucose concentration was monitored using glucose kit from Sigma, St. Louis, MO. After the glucose concentration of the medium had decreased from 2.0 to 0.2 g/L, LiP production was activated by adding veratryl alcohol to the final concentration of 2.5 m*M* *(25)*. At the same time, 430 mL of minerals *(24)* and Tween-80 (to the final concentration of 0.05%) were added, and the aeration was switched from air to pure oxygen. LiP activity was measured during the growth by monitoring the oxidation of veratryl alcohol at 310 nm *(26)*. The culture fluid (crude lignin peroxidase) was harvested when the maximal LiP activity was obtained.

Purification of Lignin Peroxidase

Crude lignin peroxidase was separated from the mycelia by filtration through Whatman 3MM chromatography paper (Whatman, Clifton, NJ) and microporous membrane (Pellicon, Millipore, Bedford, MA). After filtration, extracellular fluid was concentrated from 12 L to 3 mL by consecutive concentration steps. The first step involved tangential-flow

ultrafiltration (Pellicon) using Millipore type PTGC polyethersulfone filter with a molecular exclusion limit of 10 kDa. Following the first ultrafiltration, the fluid was concentrated using Amicon stirred-cell concentrators (Amicon, Beverly, MA) having a molecular mass cut-off of 10 kDa. The fluid was further concentrated using Macrosep™ (Filtron Technology, Northborough, MA) and Ultrafree-CL (Millipore) centrifugal concentrators having a molecular mass cut-off of 30 and 10 kDa, respectively.

Individual isoenzymes were isolated by preparative isoelectric focusing (IEF), as previously described *(27)*. Pharmalytes 2.5–5 and 4–6.5 (Pharmacia, Uppsala, Sweden) were used for preparation of IEF gel. The gel was prerun in a Multiphor II (Pharmacia) at 15°C for 1 h with 400 V constant voltage. After that, 1 mL crude LiP was loaded on the gel, and the gel was run further for 8 h with 400 V. At the end of the run, the voltage was raised to 1000 V for 1 h to sharpen the separated isoenzyme bands. After focusing, gel slices containing the protein bands were cut out and the protein was eluted from the gel with water. Eluates were freed from ampholytes by dialyzing against 2 m*M* Na-tartrate, pH 4.5, and concentrated using Ultrafree-CL concentrators. Isoelectric points of the proteins were determined as previously described *(27)*.

Analytical IEF and SDS-PAGE *(28)* were performed with the Pharmacia PhastSystem, using the precast PhastGel Dry IEF and PhastGel Gradient 10–15 gels. pH gradient for IEF was made by mixing three parts of Pharmalyte 2.5–5 and one part of Pharmalyte 4–6.5 carrier ampholytes. Rehydration of dry IEF gel and separation methods for IEF and SDS-PAGE were performed according to the manufacture's instructions. After electrophoresis, the gels were stained with Coomassie brilliant blue.

Enzyme and Protein Assays

Lignin peroxidase activity was determined by the method of Tien and Kirk *(26)*. One unit (U) of LiP activity was defined as 1 μmol of veratryl aldehyde produced in 1 min. Protein concentrations were estimated by the method of Bradford *(29)*, using BSA as a standard. Concentrations of the purified isoenzymes were also determined at 408 nm, using the extinction coefficient of 169/(m*M* × cm) *(30)*. For calculations, *M*, of 40,000 was used for LiP.

N-terminal sequencing was carried out at the Turku Centre for Biotechnology protein-sequencing facility with an Applied Biosystems (Foster City, CA) model 477A protein sequenator.

Kinetic Assays and Dye Decolorization

For each isoenzyme, kinetic studies were conducted at five different H_2O_2 concentrations (88–878 μ*M*). In addition to H_2O_2, reaction mixtures

contained a constant concentration of LiP and varying concentrations of COG in 50 m*M* sodium tartrate (pH 3.5) in a total volume of 2 mL. Reactions were run at room temperature and initiated by addition of enzyme. The oxidation of COG was monitored as a decrease of absorbance at 482 nm, and quantitated using an extinction coefficient of 26,000/ ($M \times$ cm), determined with standard solutions of COG.

Similarly, for each isoenzyme, total number of turnovers was calculated at five different H_2O_2 concentrations. Reaction mixtures contained LiP in the concentration of 50 n*M* and five different COG concentrations. After the reactions were complete, a decrease of absorbance at 482 nm was measured, using similar set of reactions without H_2O_2 as standards. Amounts of oxidized COG were calculated from the absorbance differences, using the extinction coefficient of COG. The resulting concentrations were divided by those of the enzyme.

Unless otherwise stated in the text, the dye decolorization reaction mixtures consisted of 100 m*M* COG, 0.5 μ*M* LiP, and 50–400 μ*M* H_2O_2 in 50 m*M* sodium tartrate, in a total volume of 1 mL. The reaction mixtures were permitted to stabilize 1 min before the reactions were initiated by the addition of H_2O_2. Decolorization of COG was monitored at 482 nm for 10 min at four different pHs (2.5, 3.0, 3.5, and 4.5) in the case of every isolated isoenzyme.

As a control, COG was incubated with 400 μ*M* H_2O_2 without the enzyme. No changes in absorbance or shift in the absorbance maxima were observed in the control.

All spectrophotometrical measurements were done as duplicates using Biochrom 4060 UV-Visible Spectrophotometer (Pharmacia) with version 2.0 Applications Software.

RESULTS AND DISCUSSION

Lignin Peroxidase Isoenzymes

LiP isoenzymes from the carbon-limited culture medium of *P. chrysosporium* were purified by preparative IEF. Prior to IEF, the culture medium was concentrated to a total protein concentration of 35 mg/mL. The purity of the isolated isoenzymes was analyzed by analytical IEF and SDS-PAGE, and the preparations were seen to be pure by these criteria (data not shown). LiP 3.85, LiP 4.15, and LiP 4.65 (named by their p*I*s) were the three isoenzymes of the purified lignin peroxidases used for decolorizing COG, and their specific activities were 27.5, 30.4, and 41.8 U/mg, respectively. Based on amino terminal sequences, the isoenzymes LiP 3.85, LiP 4.15, and LiP 4.65 correspond to the isoenzymes H8, H6, and H2, respectively, from the *N*-limited culture *(31,32)* (Table 1).

Steady-State Kinetics

The dependence of the initial rates of COG disappearance on COG concentrations were determined at five constant H_2O_2 concentrations. The concentration of LiP isoenzymes was kept low (25–50 n*M*) and the assay period was short enough (5 s) to ensure that only a small fraction of COG (less than 5%) was consumed.

The initial rate (v) of COG oxidation by LiP 4.65, at a H_2O_2 concentration of 615 μ*M*, is presented in Fig. 2. The initial rate rose with increasing COG concentrations. The data was fitted to the Michaelis-Menten equation

$$v = V_{max}/(1 + K_M/[COG])$$

The apparent maximum rate (V_{max}) and apparent Michaelis constant (K_M) were estimated by nonlinear regression. On the basis of Fig. 2, it is clear that kinetic data fitted well with the Michaelis-Menten equation, indicating that the oxidation of COG by LiP and H_2O_2 followed Michaelis-Menten kinetics.

The values of the kinetic parameters at five different H_2O_2 concentrations are shown in Table 2. The V_{max} values were practically the same in each case, and the values of K_M seemed to increase with the H_2O_2 concentration. This suggests that H_2O_2 and COG compete for LiP, obviously for the compound II (Fig. 3), during the oxidation, as observed previously in the oxidation of phenols *(33)*.

To investigate further the effect of H_2O_2 on LiP during COG oxidation, the authors determined the total number of turnovers in the reaction of LiP with COG at five different H_2O_2 concentrations. The total number of turnovers decreased with ascending H_2O_2 concentrations, as shown in the Fig. 4. The result suggests that the inactivating effect of H_2O_2 is exponential within the used concentration range. The effect of ascending COG concentrations on the total number of turnovers was opposite to that of H_2O_2, and is linear within the used concentration range (data not shown).

Combined Effects of H_2O_2 Concentration and pH

The decolorization of 100 μ*M* COG by the three isoenzymes at different H_2O_2 concentrations at four different pHs was also studied. As shown above, during the oxidation of COG, high H_2O_2 concentrations inactivate LiP. Previously, it had been shown that during the oxidation LiP inactivates at low pH *(34)*.

Generally, the best degree of decolorization of 100 μ*M* COG was obtained when the concentration of H_2O_2 was 150 μ*M* (Fig. 5); but at pH 4.5, the optimal H_2O_2 concentration was 100 μ*M*. In H_2O_2 concentration

Table 1
Amino Terminal Sequences of Major LiP Isoenzymes from *P. chrysosporium* Grown in *C*-Limited Culture

Protein	*N*-terminal amino acid sequence																			
LiP 4.65[a]	V	A	C	P	D	G	V	H	T	A	S	N	A	A	C	C	A	W	F	P
H2[b]	V	A	C	P	D	G	V	H	T	A										
H2[c]	V	A	X	P	?	G	V	H	T	A	S	N	A	A	X	X	A			
LiP 4.15[a]		A	T	C	A	N	G	K	T	V	G	D	A	S	C	C	A	W		
H6[b]		A	T	C	A	N	G	K	T	V	G									
LiP 3.85[a]		A	T	C	S	N	G	K	T	V	G	D	A	S	C	C	A			
H8[b]		A	T	C	S	N	G	K	T	V	G									

[a] This work;
[b] From ref. *31*.
[c] From ref. *32*.

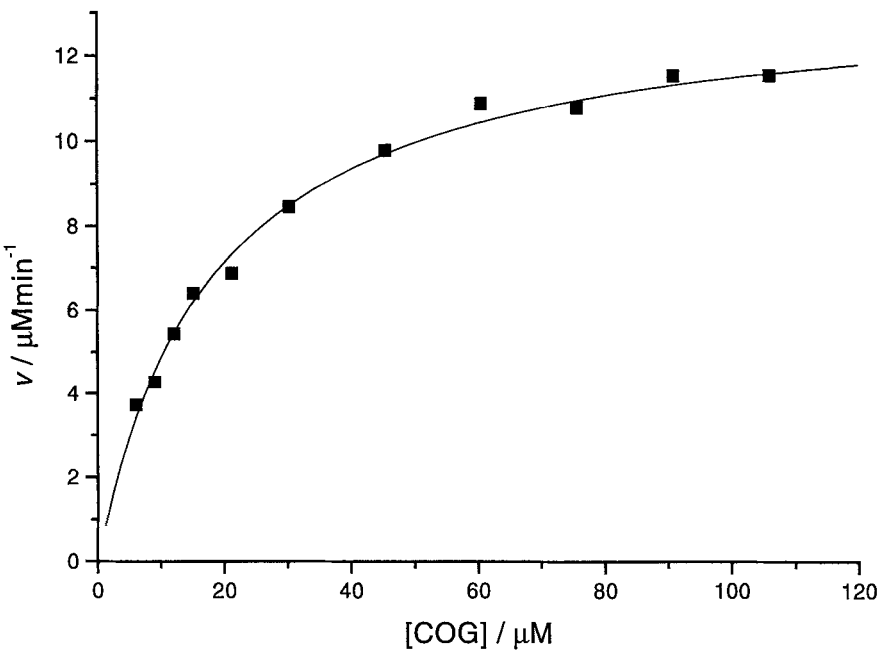

Fig. 2. Steady-state initial rates (■) of oxidation of COG by LiP 4.65 at H_2O_2 concentration of 615 μ*M*. Theoretical curve was obtained by fitting the data to the Michaelis-Menten equation.

Table 2
Kinetic Parameters for COG Oxidation by LiP Isoenzymes at Five Different H_2O_2 Concentrations

	LiP 4.65					LiP 4.15					LiP 3.85				
$[H_2O_2]$ (μM)	88	263	439	615	878	88	263	439	615	878	88	263	439	615	878
V_{max} (μM/min)	12.9	13.8	14.8	13.6	13.0	15.6	18.0	14.3	14.2	12.8	15.1	15.5	14.8	14.8	15.0
K_M (μM)	10.2	14.4	18.4	18.0	19.7	11.5	16.1	15.9	18.0	17.4	11.4	13.0	12.3	12.7	18.1
k_{cat}(1/s)	8.6	9.2	9.9	9.0	8.6	8.3	9.6	7.6	7.6	6.9	6.7	6.9	6.5	6.5	6.6
k_{cat}/K_M ($1/\mu M \times 1/s$)	0.85	0.64	0.54	0.50	0.44	0.72	0.60	0.48	0.42	0.40	0.59	0.53	0.53	0.51	0.37

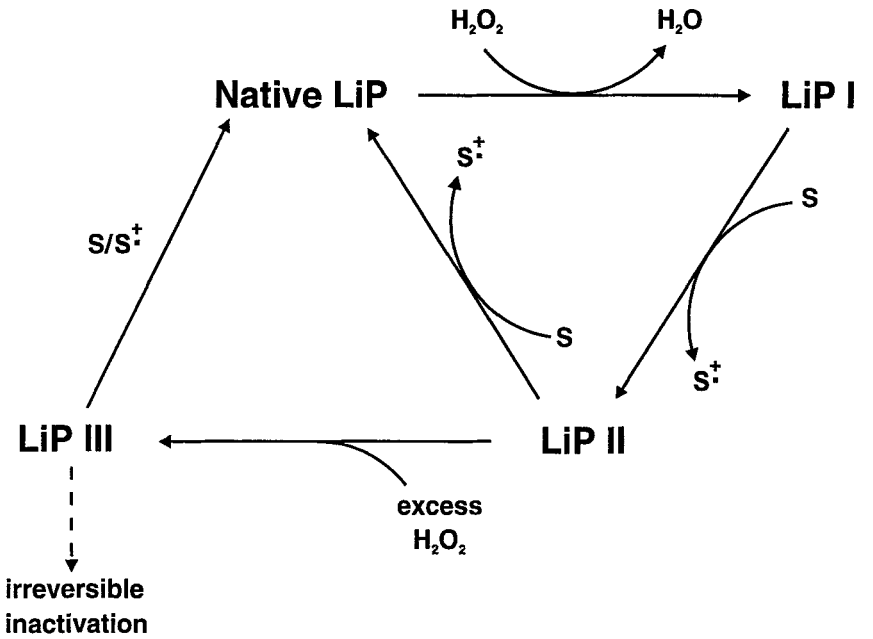

Fig. 3. Schematic representation of the four oxidation states of LiP and their relationships. The catalytic cycle of the enzyme consisted of native LiP, LiP I, and LiP II. LiP III is converted to native enzyme by the substrate (S) *(38)*, or by the cation radical of the substrate ($S^{\cdot+}$) *(39)*.

more than 300× the LiP concentration, the decolorization ability of the isoenzymes went down with increasing H_2O_2 concentration (Fig. 5), and this effect grew when the pH of the reaction was raised. The optimal range of the H_2O_2 concentration for decolorization became narrower when the pH of the reaction was raised (Fig. 5). This may be caused by a lower rate of the decolorization reaction at a higher pH because the optimum pH for most LiP isoenzymes is 2.3, and for LiP 4.15, it is 3.2 *(27)*. The lowered reaction rate gives a better opportunity for H_2O_2 to react with compound II.

The inactivation of LiP at low pH was seen only when the concentrations of H_2O_2 were below the optimum. In those cases, the best decolorization of the dye for each isoenzyme was obtained at the highest pH. In contrast to that, in optimal H_2O_2 concentration or above it, the best decolorization was gained at the lowest pH, because high concentrations of H_2O_2 inactivate the enzyme. These results clearly show that, at H_2O_2 concentrations higher than optimal, the inactivating effect of H_2O_2 exceeded that of pH. The only exception was LiP 4.15 at pH 2.5, at which the enzyme had only little decolorization activity. At the H_2O_2 concentrations lower than optimal, the inactivating effect of pH was the most significant.

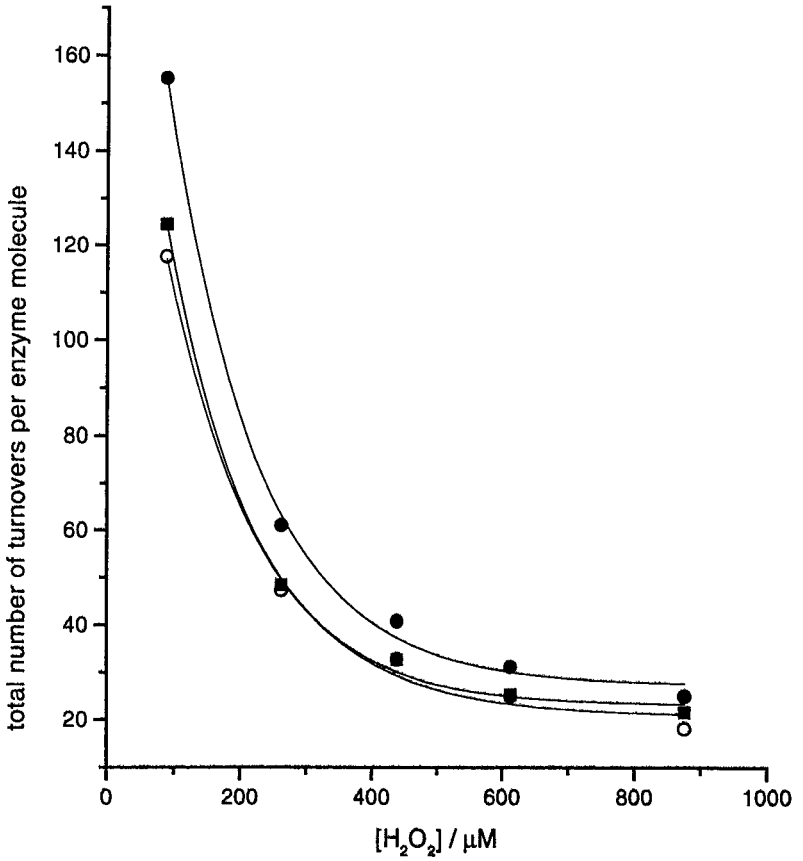

Fig. 4. Effect of H_2O_2 concentration on the total number of turnovers of LiP isoenzymes during COG oxidation at pH 3.5. After the reactions were complete, amounts of oxidized COG were calculated and the resulting concentrations were divided by those of the enzyme. The concentrations of LiP and COG in the reactions were 50 n*M* and 17.4 µ*M*, respectively. ●, LiP 4.65; ○, LiP 4.15; ■, LiP 3.85.

A short preincubation of LiP at low pH (pH 2.5) did not affect the decolorization ability of the enzyme. The decolorization reactions described in Materials and Methods were also done by adding H_2O_2 and the buffer at the same time. The incubation of LiP at low pH was thus omitted, but that had no effect on results (data not shown). As mentioned, LiP 4.15 was an exception. When the decolorization reaction at pH 2.5 was initiated by adding the buffer and H_2O_2 simultaneously, the decolorization of COG by LiP 4.15 was considerably better, compared with the reaction initiated by adding H_2O_2 only (Fig. 6). LiP 4.15 has been previously reported to be more sensitive to low pH than are the other LiP isoenzymes (*27,35*). The reason for this is unknown at present.

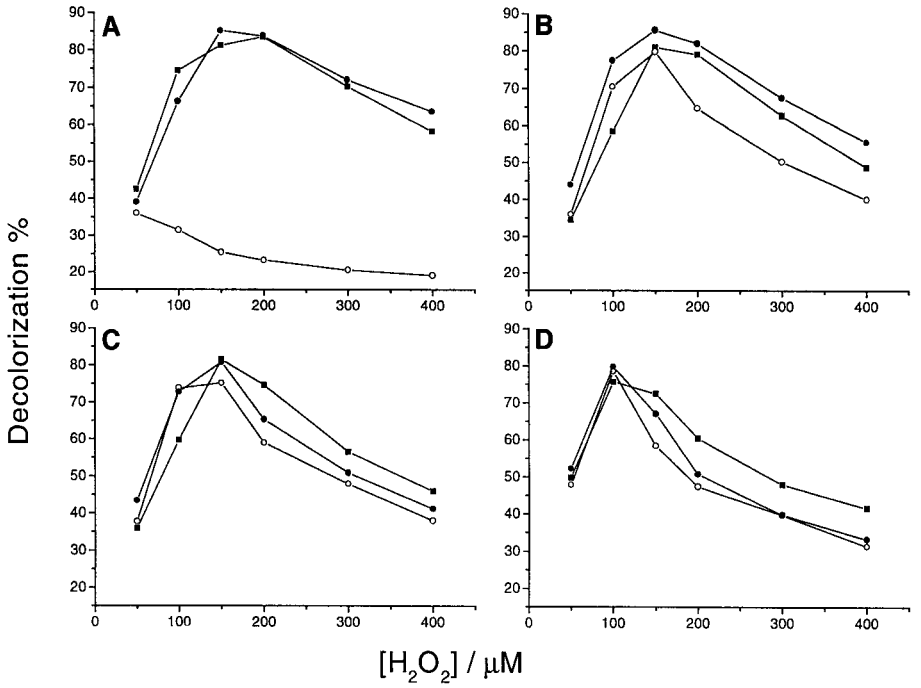

Fig. 5. Dye decolorization by three LiP isoenzymes isolated from *P. chrysosporium*. Reaction mixtures consisted of 100 μ*M* COG, 0.5 μ*M* LiP, and 50–400 μ*M* H_2O_2 in 50 m*M* Na-tartrate. pHs of the reactions were 2.5, 3.0, 3.5, and 4.5 (panels A, B, C, and D, respectively). ●, LiP 4.65; ○, LiP 4.15; ■, LiP 3.85.

Effect of LiP–H_2O_2 Ratio

It is important to optimize the concentration of H_2O_2, as well as the ratio of LiP to H_2O_2 used in the reaction, to achieve the maximal decolorization. Because high H_2O_2 concentrations inactivated LiP, the lowest H_2O_2 concentration needed to decolorize 100 μ*M* COG was determined. For this, the reactions were carried out keeping the LiP–H_2O_2 ratio at 1:300 (the ratio that gave the best decolorization results in previous reactions), but the absolute amounts of these substances were varied (Fig. 7A), and a similar set of reactions was also performed with the LiP–H_2O_2 ratio at 1:150 (Fig. 7B). These two sets of reactions demonstrated that the lowest H_2O_2 concentration required for complete decolorization of 100 μ*M* COG was 150 μ*M*, regardless of the amount of the LiP isoenzymes. This concentration was equal to the H_2O_2 concentration that gave the highest rate of decolorization with 100 μ*M* COG (Fig. 5). These reactions also showed that the degree of decolorization did not rise when the amount of LiP was raised, suggesting that the conversions were complete. On the contrary,

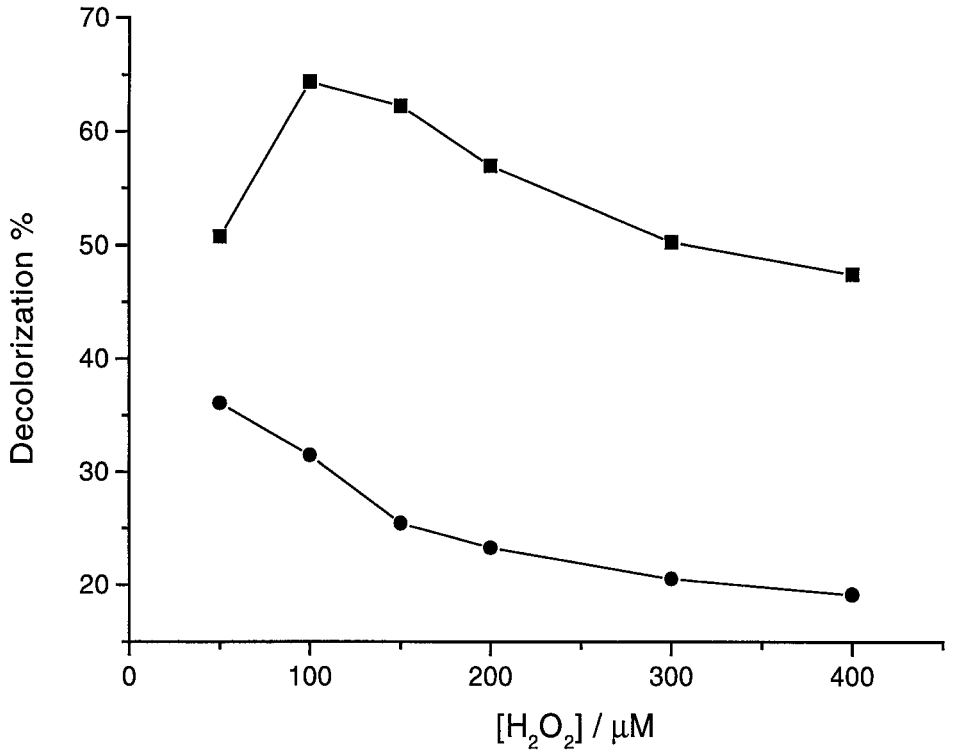

Fig. 6. Effect of low pH on decolorization ability of LiP 4.15. Symbol ● represents the reaction in which LiP 4.15 was incubated at pH 2.5, 1 min before the reaction was initiated. Symbol ■ represents the reaction that was initiated by simultaneously adding buffer and H_2O_2. The preincubation of LiP 4.15 at low pH was thus omitted.

the decolorization level even seemed to go down slightly when the amount of LiP and H_2O_2 was raised (Fig. 7).

CONCLUSIONS

LiP isoenzymes have the potential to be used in biodegradation reactions of aromatic pollutants. When LiPs are used to oxidize different substrates, it is important to optimize reaction conditions to avoid inactivating conditions, in order to gain the maximal oxidation. If the reaction contains too little H_2O_2, it will be used up before the substrate is fully oxidized. On the other hand, excess H_2O_2 converts the active enzyme to the compound III, which reacts with excess H_2O_2, resulting in irreversible inactivation of the enzyme *(36,37)*, if the substrate cannot effectively revert the compound III to the native enzyme. A proper concentration of H_2O_2 in a reaction depends on a concentration of the substrate. Also, the LiP–H_2O_2 ratio in the reaction affects the result of the reaction, and, because of that, the con-

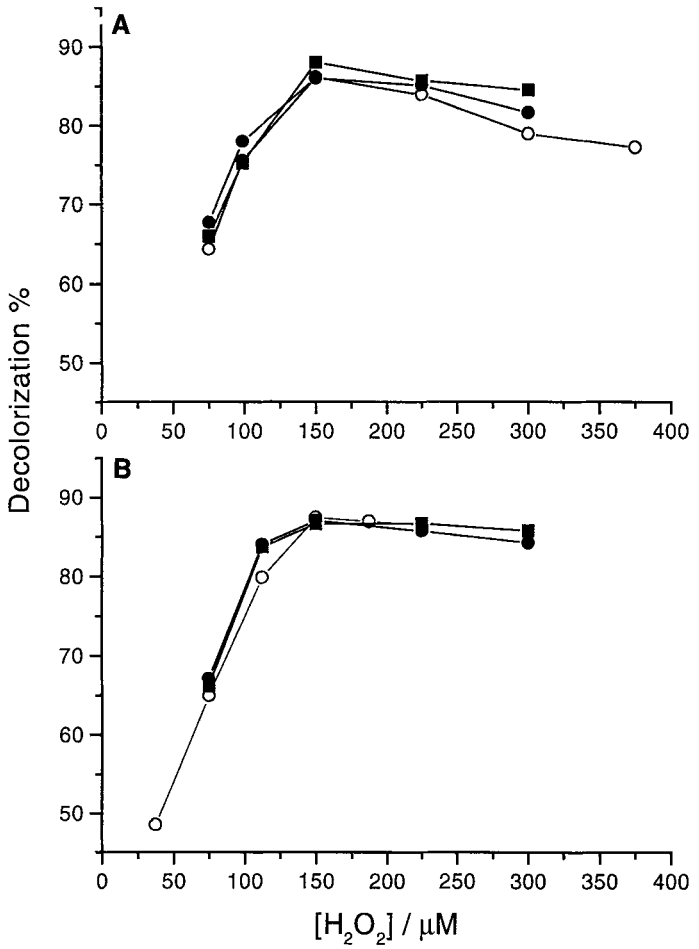

Fig. 7. Effect of H_2O_2 and LiP concentrations on COG decolorization. Reaction mixtures consisted of 100 μ*M* COG, 0.25–2 μ*M* LiP, and 37.5–375 μ*M* H_2O_2 in 50 m*M* Na-tartrate at pH 3.5. Two sets of the reaction were made: One set consisted of the reactions in which LiP–H_2O_2 ratio was kept at 1:300 **(A)** and the other set consisted of the reactions in which LiP–H_2O_2 ratio was kept at 1:150 **(B)**; ●, LiP 4.65; ○, LiP 4.15; ■, LiP 3.85.

centration of LiP for the reaction should be chosen on the basis of the concentration of H_2O_2 in the reaction.

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