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Note

Sensitive, enzyme-catalyzed, chromogenic reagent for hydrogen peroxide and other peroxygen compounds on thin-layer chromatographic plates

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Several chromogenic spray reagents have been described for the detection of hydrogen peroxide and other peroxides on thin-layer chromatographic (TLC) plates. Easily oxidizable inorganic ions or organic chromogens are commonly used. Aromatic diamines or substituted aromatic amines are oxidized to colored quinoneamines, but heating or catalysis by metal ions or enzymes is required. The sensitivity of the detection system is a function of the extinction coefficient of the colored product or complex, and, in practical use, the rate at which the chromophore develops.

In this paper we describe the application of the peroxidase catalyzed oxidation of the aromatic amine, leuco crystal violet (LCV), to the detection of hydrogen peroxide. Enzyme catalysis and the extremely high molar absorptivity of crystal violet afford this detection system improved sensitivity relative to existing methods.

EXPERIMENTAL

Materials

All chemicals were obtained commercially and were used as received unless otherwise noted. Leuco crystal violet (ICN Pharmaceuticals) was purified by dissolving 3 g in 200 ml of benzene and extracting with four 250-ml portions of 5% aqueous sodium hydroxide. Evaporation of the organic layer yielded a dark brown solid which was crystallized three times from benzene and three times from 95% ethanol, m.p. 174–176°C (Lit.[1] 173–175°C). Horseradish peroxidase (320 purpurogallin units/mg) was obtained from Sigma.

Solution preparation

All aqueous solutions were prepared with demineralized water. LCV stock solution consisted of 50 mg of purified material in 100 ml of 0.5% aqueous hydrochloric acid. Spray reagents were prepared immediately prior to use and stored at 5°C during experiments. Reagent 1 consisted of 3 ml of LCV stock solution dissolved in 8

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ml of pH 4.5 one molar acetate buffer; reagent 2 was an aqueous peroxidase solution (0.25 mg/ml).

Thin-layer chromatography

TLC was carried out on 0.25-mm silica gel 60 F_{254} plates (E. Merck) or on 0.5-mm layers prepared in our laboratory. Silica gel G (25 g) was suspended in 80 ml of 95% ethanol, spread to a thickness of 0.5 mm on 20 × 20 cm glass plates with a thin-layer spreader (Desaga-Brinkmann Model 611), air dried for 0.5 h, activated for 1 h at 100°C, and stored in a dessicator. Aliquots of 5 μl were applied to the adsorbent and the carrier solvent was evaporated. Plates were developed in various solvent systems (see Results and discussion) in chromatography tanks fitted with filter paper liners.

Chromogenic sprays

After evaporation of the mobile phase, 20 × 20 cm chromatoplates were sprayed with reagent 1 for 5 sec and with reagent 2 for approximately 2 sec; a 10-min room temperature incubation followed. The spray reagent should be used only in a well-ventilated fume hood.

The response of hydrogen peroxide to the LCV–peroxidase reagent was compared to 5% aqueous potassium iodide and 0.1% N,N-dimethyl-*p*-phenylenediamine (DMPD) reagents. Hydrogen peroxide solutions (5 μl) ranging from 30 μM to 1.0 mM were spotted in triplicate on 0.5-mm TLC plates, subjected to the chromogenic treatments, and their responses noted. DMPD treated plates were heated for 10 min at 100°C to allow color development.

RESULTS AND DISCUSSION

Sensitivity of detection reagents

Hydrogen peroxide was visualized on TLC plates using each chromogenic spray reagent (Table I); the limit of detection for hydrogen peroxide was reduced from approximately 90 ng with DMPD to approximately 1.5 ng using the peroxidase-catalyzed LCV spray. With the LCV reagent hydrogen peroxide and other peroxygen compounds were detected as intense violet spots on a light field. The background in the case of DMPD, however, was pink, increasing the detection limit slightly. The background response observed may be due to autoxidation of the chromogen during heat treatment. Iodine and crystal violet spots faded after several hours, but spots formed on DMPD treated plates were persistent.

Peroxidases, such as hemin of blood, have been used to catalyze the oxidation of 2,7-diaminofluorene to a blue quinoneimine; the reaction has been used as a test for organic peroxides in fats and oils[2]. Mottola *et al.*[1] utilized the peroxidase-catalyzed oxidation of the leuco base of crystal violet as a spectrophotometric assay for oxidants in solution. A single hydride transfer from the leuco base extends π electron delocalization from 7 to 22 atomic centers (Fig. 1) producing an intensely colored quinoid compound (ε = 75,000 $l \cdot mol^{-1} \cdot cm^{-1}$ at 596 nm). The chromophore formed by DMPD, in contrast, exhibits an extinction coefficient of only 9400 at 558 nm (ref. 3) accounting, in part, for the increased sensitivity of the LCV detection reagent.

TABLE I

RESPONSES OF HYDROGEN PEROXIDE DETECTED WITH LCV–PEROXIDASE, POTASSIUM IODIDE, OR DMPD

+ = Positive response relative to background; ± = approximate detection limit; NR = no reaction observed.

Hydrogen peroxide (ng)	*LCV–peroxidase*	*Potassium iodide*	*DMPD*
$6.0 \cdot 10^3$	+	+	+
$1.5 \cdot 10^3$	+	+	+
$3.6 \cdot 10^2$	+	±	±
90	+	NR	±
25	+	NR	NR
6.0	+	NR	NR
1.5	+	NR	NR

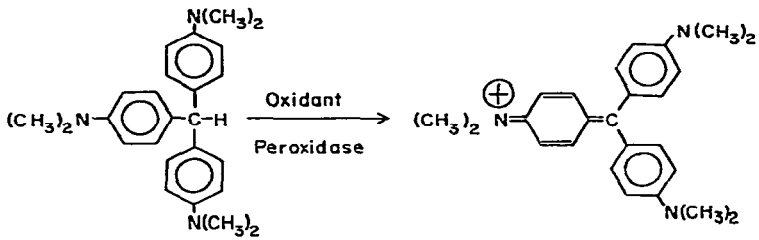

Fig. 1. Peroxidase catalyzed oxidation of the leuco base of crystal violet.

Other peroxygen compounds can be detected colorimetrically using the peroxidase-catalyzed assay (Fig. 2). The response of the system was greatest to stoichiometric amounts of hydrogen peroxide relative to *m*-chloroperoxybenzoic acid (MCPBA) or cumene hydroperoxide. Although we have not tested the sensitivity of the chromogenic spray reagent toward these organic peroxides on TLC plates, the

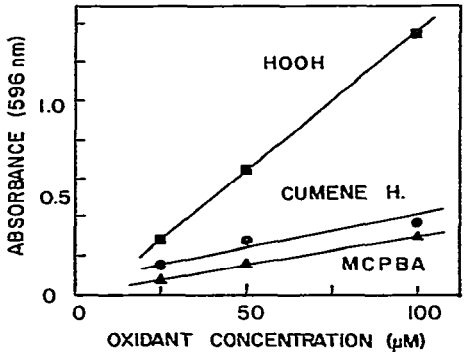

Fig. 2. Standard response curves for hydrogen peroxide, MCPBA, and cumene hydroperoxide generated with the spectrophotometric assay of Mottola *et al.*[1] Peroxide and MCPBA were incubated 10 min; cumene hydroperoxide required 1 h.

TABLE II

CHROMATOGRAPHIC MOBILITY OF HYDROGEN PEROXIDE

Solvent system compositions represent volume ratios. Adsorbents: A = 0.5 mm silica gel G; B = 0.25 mm silica gel 60.

Solvent system	*Adsorbent*	R_F *value*
n-Butanol–acetic acid–water (4:1:1)	A	0.54
n-Butanol–diethyl ether–water (10:10:1)[4]	A	0.62
Ethyl acetate–benzene–acetic acid (90:60:1)	A	0.27
Toluene–methanol (7:3)[4]	A	0.23
Acetic acid–chloroform–methanol (10:10:1)	B	0.45
Chloroform–acetic acid (16:1)	B	0.01
Chloroform–methanol–acetic acid (15:4:1)	B	0.12
Methanol–acetic acid (20:1)	B	0.56

system would conceivably function as it does in solution; the detection limit for MCPBA on a chromatographic plate is estimated to be 50 ng.

Successful use of the reagent requires purification of the commercially available LCV, a black substance. Extraction with aqueous base and several recrystallizations from 95% ethanol yielded a white powder with a high, narrow melting point range.

Solvent systems

The mobility of hydrogen peroxide varied widely with the composition of the mobile phase (Table II). Spots tended to tail when large amounts of peroxide were chromatographed and R_F values were somewhat larger (up to 5%). Tailing was eliminated, however, by use of the more polar, acidic solvents.

CONCLUSION

Trace amounts of hydrogen peroxide and other peroxygen species can be detected and differentiated using TLC and the chromogenic spray based on the peroxidase catalyzed oxidation of LCV. The technique represents a significant improvement in sensitivity over existing chromogenic reagents.

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