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Enzymatic Reactions in Supercritical Gases

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ABSTRACT

The enzyme polyphenol oxidase has been found to be catalytically active in supercritical carbon dioxide and fluoroform: it readily oxidizes *p*-cresol and *p*-chlorophenol to their corresponding *o*-benzoquinones.

Index Entries: Enzymatic reactions, in supercritical gases; reactions, enzymatic, in supercritical gases; supercritical gases, enzymatic reactions in; gases, enzymatic reactions in supercritical; polyphenol oxidase, reactions in supercritical gases; carbon dioxide, reactions of polyphenol oxidase in supercritical; fluoroform, reactions of polyphenol oxidase in supercritical; *p*-cresol, oxidations by polyphenol oxidase in supercritical gases; *p*-chlorophenol, oxidations by polyphenol oxidase in supercritical gases.

INTRODUCTION

Supercritical gases whose pressure-dependent dissolving properties were first described over 100 years ago (*1*) are receiving resurgent attention in the development of new separation processes (*2*). They offer the chemical process industries the potential to achieve lower energy separations (*3*) and are being evaluated by the food industry as replacements for

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traditional hydrocarbon and chlorinated solvents (*4*). More recently, application to the fractionation of refractory polymers and purification of reactive monomers has been described (*5*).

Other advantageous properties of supercritical gases include a zero surface tension. Thus they can be effective in depositing materials in microporous solids (*6*). Since pressure reduction through a valve, for example, can occur within microseconds or less, nucleation rates in gaseous solutions can far surpass those achievable in traditional crystallization or precipitation methods, and extremely small particles of solids can be produced (*6a*). Finally, supercritical gases have two other properties that are important: they have higher diffusivities than conventional solvents and they are fully miscible with other gases, such as oxygen, so that virtually any desired concentration can be achieved to carry out homogenous or heterogeneous chemical reactions. It is the last property, miscibility with oxygen, that is exploited in the enzyme-catalyzed oxidation of phenols described in this paper.

It is becoming increasingly clear that many, perhaps most enzymes can function as catalysts in nearly anhydrous organic solvents (*7*). Supercritical gases constitute the "next frontier" as the medium for enzymatic reactions that may have a considerable promise in biotechnological applications. Therefore, we have undertaken a study to determine whether enzymes can work in supercritical gases. (When this work was completed and the manuscript was in preparation, a paper by Randolph et al. (*8*) appeared that described alkaline phosphatase-catalyzed hydrolysis of *p*-nitrophenyl phosphate in supercritical carbon dioxide.) Recently, Kazandjian and Klibanov (*9*) have found that mushroom polyphenol oxidase can regioselectively oxidize phenols in organic solvents. In the present study, we have established that this enzymatic conversion (Fig. 1) can also be carried out in supercritical gases.

EXPERIMENAL

Materials

Mushroom polyphenol oxidase (listed as tyrosinase) (EC 1.14.18.1) was purchased from Sigma Chemical Co. as a solid with a specific activity of 2430 units/mg (1 unit is defined as the enzyme activity resulting in an

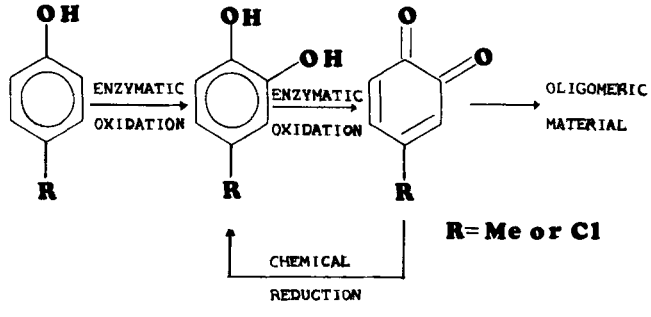

Fig. 1. Diagram of the oxidation of phenols by polyphenol oxidase.

increase in absorbance at 280 nm of 0.001 units/min at pH 6.5 and 25°C in a 3 mL reaction volume containing L-tyrosine).

Glass beads (100–230 mesh) were obtained from Potters Industry (NJ) and washed with 5% nitric acid prior to use. All chemicals and solvents were purchased from commercial suppliers and were of analytic grade or the highest purity available. Gases containing 2% oxygen were supplied by Matheson.

Methods

Bioreactor

Polyphenol oxidase (100 mg) was dissolved in 6 mL of 50 m*M* phosphate buffer (pH 8) and then 10 g of glass beads (100–230 mesh) were added. The slurry was thoroughly mixed, spread on a watch glass, and left to dry at room temperature with occasional mixing until visibly dry.

The reaction was conducted in a 30 × 1.5 cm stainless-steel tube (Autoclave Engineers). Glass wool that had been moistened with water was introduced at the base of the reactor and a further piece of glass wool was added. The substrate (1.85 mmol) was then introduced, followed by another layer of glass wool. The immobilized enzyme (3.5 g) was hydrated with 0.35 mL of 50 m*M* phosphate buffer (pH 8), mixed with glass balls (3 mm in diameter) and also placed in the reaction vessel. A further glass wool plug was added prior to sealing the vessel and connecting to the high pressure rig (shown diagramatically in Fig. 2). The reaction vessel was heated to the required temperature either by a glascol tape or by immersion in a thermostatically controlled water bath. The temperature used was 36°C for carbon dioxide and 34°C for fluoroform (±2°C).

Static Operation

Two gas-tight valves were introduced at either end of the reaction vessel (A and B in Fig. 2) so that it could be removed from the high pressure rig to allow agitation. Gas was introduced into the reactor and compressed to 5000 psi (34 MPa). (When fluoroform was used, it and oxygen were mixed in the appropriate proportions and then compressed to 5000

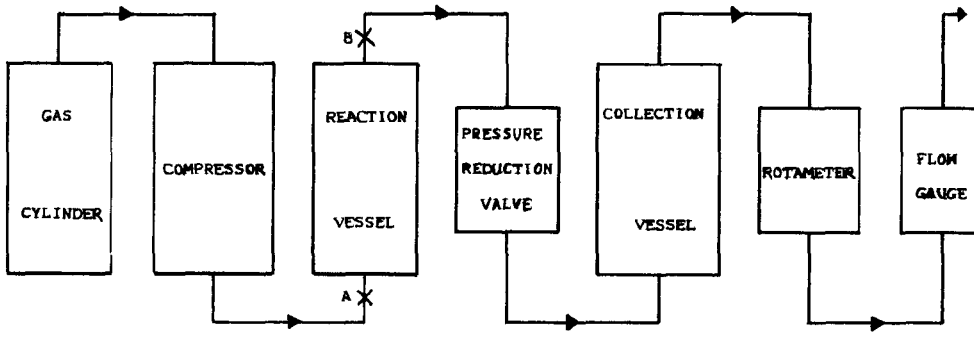

Fig. 2. Schematic representation of reaction equipment.

psi). After agitation for 40 min. the reaction vessel was reconnected to the rig and flushed out with 150 L (STP) of supercritical gas. After passing through a pressure reduction valve, the material was collected in a U tube and dissolved in chloroform. The solution was shaken with 10 mL of 0.27*M* aqueous ascorbic acid solution to reduce the *o*-benzoquinone formed. The two layers were separated and analyzed by HPLC and GLC.

Flow Operation

When the reaction was performed under flow conditions, an identical system was used except that the valves (A and B) were omitted. The gas was pumped into the vessel raising the pressure to 5000 psi. The pressure reduction valve was opened to give the required flow rate, determined on the rotameter (Fisher-Porter, Series 10A 35) or the dry test meter (Singer, DTM-200), while maintaining the required pressure during the flow of 200 L (STP). In the case of fluoroform, 90 L (STP) were passed through, and then the reactor was swept out with 150 L (STP) of carbon dioxide. The effluent was collected in a U tube, reduced, and analyzed as described below. The enzyme was removed from the vessel and washed with chloroform to remove any oligomeric material insoluble in the supercritical gas.

Analytical Methods

Analysis of the aqueous solution containing the catechol produced by reduction of the *o*-benzoquinone was performed by HPLC. The separation was carried out on a Partisil reverse-phase ODS 3 column (Whatman) using acetonitrile (32.5%) and phosphate buffer (16 m*M*, pH 3) with a flow rate of 2 mL/min. Detection was by UV at 280 nm, and peak areas were determined by integration (Hewlett Packard 3390A).

The chloroform solution was dried ($MgSO_4$), filtered, and analyzed for the remaining substrate by GLC. The analysis was conducted at 105°C on a 50-cm packed column (10% silicone UCW-982 on 80–100 mesh Chromosorb W AW-DMCS B79) with N_2 as the carrier gas (flow rate, 30 mL/min) and flame ionization detection (detector and injector temperatures set at 250°C).

The amount of polymeric material was determined by evaporation of the chloroform solution under vacuum and weighing, an allowance being made for residual substrate.

Molecular Weight Distribution of Polymeric Material

The molecular weight distribution of the polymeric material produced in the reaction was determined by gel permeation chromatography using a lipophilic Sephadex resin (LH-20 and LH-60). A column was prepared with either LH-20 or LH-60 (depending on requirements) that had been preswollen by heating at 90°C for 20 min in dioxane. The reaction mixture was dissolved in dioxane, a 0.1 mL portion was placed on

the top of the column, and 1 mL fractions of the eluant were collected. The fractions were then analyzed by UV at 270 nm to determine where the material had eluted. The columns were precalibrated using samples of defined molecular weight (polystyrene, MW 321,000, 2000, 1000, α-tocopherol, phenanthrenequinone, and *p*-cresol).

Enzyme Activity

The immobilized enzyme (500 mg) was placed in 5 mL of a 50 m*M* solution of *p*-cresol in chloroform, followed by addition of 0.05 mL of 50 m*M* phosphate buffer (pH 8). The suspension was shaken and 0.05 mL aliquots removed at predetermined intervals. The chloroform solution was added to 0.2 mL of 25 m*M* ascorbic acid in methanol to reduce the benzoquinone. The levels of *p*-cresol and 4-methylcatechol were quantified by HPLC. The amount of substrate loss/product accumulation was plotted against time and the enzyme activity was taken as the initial slope of either of these curves.

RESULTS AND DISCUSSION

When polyphenol oxidase and *p*-cresol were contacted in either supercritical fluoroform or carbon dioxide containing oxygen, the effluent from the reaction vessel was orange/yellow in color (a similar color was seen when the reaction was carried out in chloroform). In control experiments, using either the immobilized enzyme in the absence of substrate or substrate in the absence of enzyme, no color was seen in the effluent from the reactor. This suggested that some enzyme-catalyzed reaction had occurred.

The use of both static and flow systems was examined for the enzymatic oxidation of *p*-cresol. It was found that although similar substrate conversions (70–80%) could be achieved using either method, the flow system allowed the isolation of more catecholic material (4.8 vs 2.4%) after chemical reduction, and therefore it was used in all further investigations.

When the oxidation of *p*-cresol was conducted in fluoroform/oxygen, at 5000 psi with a flow rate of 1 L (STP)/min, about 70% of the substrate was oxidized by the enzyme. (This is based on a 20% physical loss of material, which was the amount lost in control experiments in the absence of enzyme.) The majority of the enzymatically formed *o*-benzoquinone polymerized before it was swept out of the reaction vessel; however, some was found in the effluent. (This was determined as the catechol, after reduction with ascorbic acid, and was equivalent to 4.8% of the substrate added.) The polymeric material accounted for 70% of the substrate added and its nature is discussed later.

Supercritical carbon dioxide was used to study the effect of higher flow rates on the reaction. With these higher flow rates the level of conversion was lower (typically 15–20%) because of a shorter residence time

of the substrate in the reactor. However, this change did not significantly affect the polymerization reaction, and most of the *o*-benzoquinone formed still polymerized. (The amounts of material found were between 15 and 20% of the added substrate.) After reduction of the effluent from the reactor, small quantities of catechol were detected showing that some benzoquinone was flushed out of the reactor before it polymerized (typically 0.4–0.6% of added substrate).

The polymerization of the *o*-benzoquinone is probably promoted by its low solubility in the supercritical phase, which limits diffusion away from the aqueous layer around the enzyme in which it polymerizes. Kazandjian and Klibanov (*9*) also observed a similar rapid polymerization when they conducted the oxidation in hexane rather than chloroform. It is known that solvent properties of hydrocarbons and supercritical carbon dioxide are similar (*10*).

Oligomeric material in the chloroform solution accounted for most of the consumed substrate, thus demonstrating that the enzymatically formed *o*-benzoquinone polymerized. Using gel permeation chromatography, the polymeric materials from a number of reactions, conducted under different conditions, were examined. All samples had similar molecular weight distributions (Fig. 3). The molecular weights ranged from about 200 to 6500 with a maximum occurring at about 300, indicating that dimers and trimers were the major species present. A sample obtained by the reaction of polyphenol oxidase with *p*-cresol, conducted in hexane as a solvent, showed a similar molecular weight distribution.

When another substrate, *p*-chlorophenol, was oxidized by polyphenol oxidase under the same conditions (except twice as much enzyme was used), there was a 27% loss in substrate. Again, the majority, of the *o*-benzoquinone formed polymerized (22.5% based on initial substrate) and only a small amount of catecholic material (0.15%) was detected after reduction. In this case the physical loss of material was smaller (5%), which was probably caused by the slightly higher boiling point of the phenol.

It is important to note that exposure of the enzyme to supercritical carbon dioxide or fluoroform containing oxygen, but no phenolic substrate, caused little or no inactivation. However, the enzyme was inactivated during the oxidation process. With *p*-chlorophenol, 64% inactivation was observed at 23% conversion. With *p*-cresol, 90% conversion gave 90% inactivation and 30% conversion gave 36% inactivation.

This work has demonstrated that an enzyme can function in supecritical gases. The amount of catecholic product isolated was low, but this can be readily explained in terms of the particular system employed: the unstable *o*-benzoquinone polymerizing rapidly in the reaction medium. The use of supecritical gases as a medium to conduct enzyme-catalyzed reactions constitutes a new and interesting research area. In particular, the use of the medium as a reactant in an enzyme reaction could offer a fruitful direction of research. We are at present

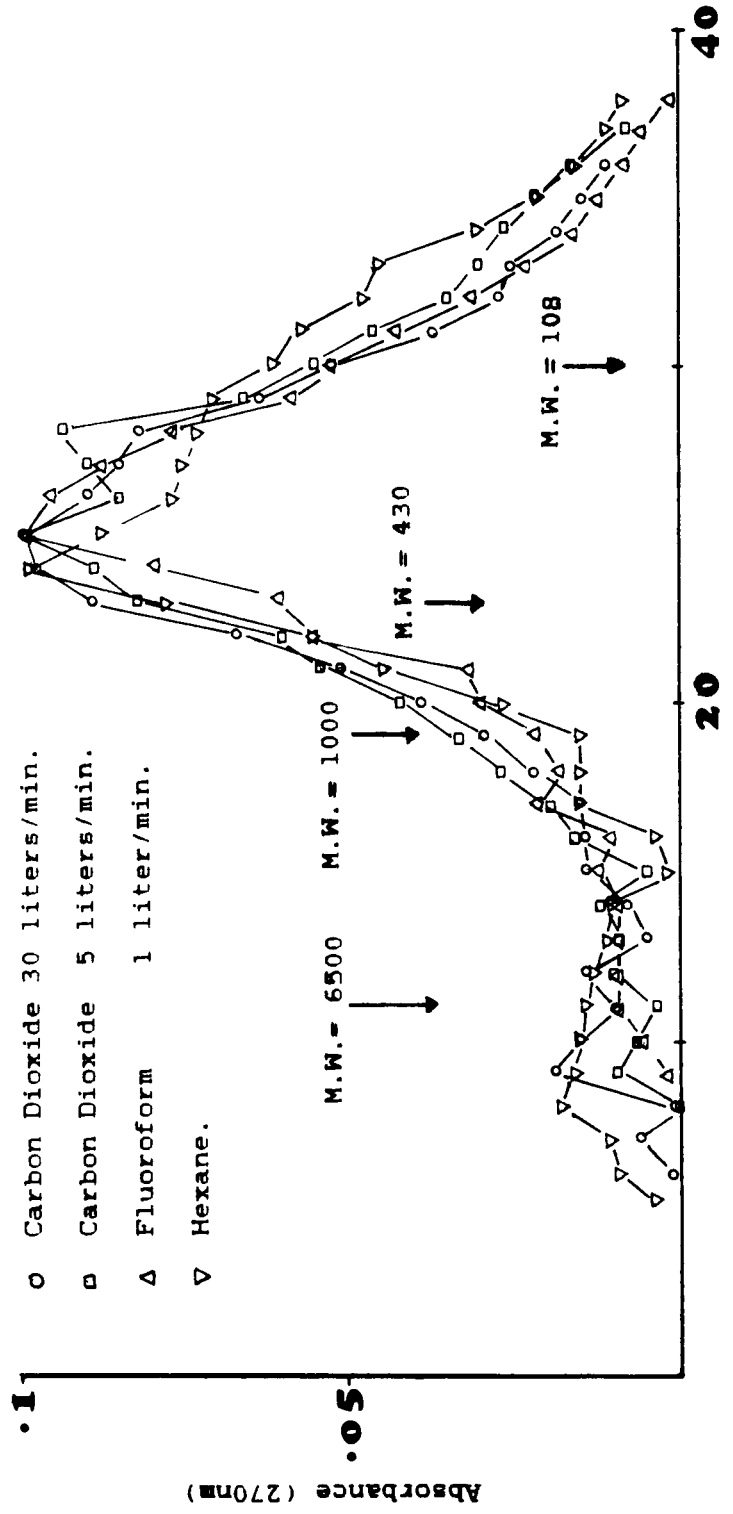

Fig. 3. Elution pattern of polymeric material from a Sephadex LH-60 column.

investigating this possibility by using a decarboxylase enzyme in the presence of supercritical carbon dioxide. The objective is to reverse the normal decarboxylation reaction under the high carbon dioxide pressure. If that proves successful, it would open whole new avenues of research.

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REFERENCES

1. Hannay, J. B., and Hogarth, J. (1879), *Proc. Royal Soc. (London)* **29,** 324.
2. Basta, N., and McQueen, S. (1985), *Chem. Eng.* **92**(3), 14.
3. de Filippi, R. P., and Vivian, J. A. (1982), US Patent No. 4,349,415.
4. Friedrich, J. P., List, G. R., and Leakin, A. J. (1982), *J. Amer. Oil Chem. Soc.* **59,** 288.
5. Krukonis, V. J. (1985), *Polymer News* **11,** 7.
6. Worthy, W. (1981), *C & E News* **59**(31), 16.
6a. McHugh, M. A., and Krukonis, V. J. (1986), in *Supercritical Fluid Extraction, Performance, and Practice,* Krukonis, V. J., ed., Butterworth, Stoneham, MA, chapter 12.
7. Zaks, A., and Klibanov, A. M. (1985), *Proc. Natl. Acad. Sci. USA* **82,** 3192.
8. Randolph, T. W., Blanch, H. W., Praunsnitz, J. M., and Wilke, C. R. (1985), *Biotech. Lett.* **7,** 325.
9. Kazandjian, R. Z., and Klibanov, A. M. (1985), *J. Amer. Chem. Soc.* **107, 448.**
10. Hyatt, J. A. (1984), *J. Org. Chem.* **49,** 5097.