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Use of Dinitrofluorobenzene to Modify Enzymes for Enhanced Solubilization and Activity in Organic Solvents

Scientific Note

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INTRODUCTION

It is now known that many enzymes can function as biocatalysts in, or in contact with, various organic solvents (*1–5*). As a result, such enzymes can now be considered for many different applications not previously thought to be possible in organic solvents. Approaches taken with this type of biocatalytic system have included the use of immobilized enzymes (*6*), enzymes particulates (*7*), enzymes in micelles (*8*), or enzymes in an aqueous phase that is in contact with the organic phase (*6*). These are effective ways of using biocatalysts for some applications providing the reaction is carried out in the liquid phase. However, if the enzyme must interact with a solid substrate, such as coal in the organic phase, it is desirable for the enzyme to be present in the organic media in a soluble form, since direct molecular interactions are a requirement. Some enzymes have minimal solubility in polar organics, especially those that contain water (*9*). However, there is only a limited solubility of enzymes in nonhydrous, polar organic solvents, since proteinaceous molecules are primarily hydrophilic. It may be possible to modify enzymes chemically to increase hydrophobicity and, thus, increase organic solubilization while maintaining catalytic activity. Several methods have been studied to

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modify enzymes, including the attachment of polyethylene glycol and palmitoyl chloride, both of which contribute to increases in organic solubilization (*10–13*). Another approach being investigated uses 2,4 dinitrofluorobenzene (DNFB) to attach dinitrophenyl (DNP) groups to enzymes (*14*). It has now been shown that such modified enzymes are much more hydrophobic with increased solubilization in a variety of organic solvents. Reducing enzymes that have been modified with DNFB have been used to increase the liquefaction of coal particles significantly in organic solvents, such as benzene and pyridine.

CHEMICAL MODIFICATION OF ENZYMES

Proteins have a large number of different types of chemically active groups, resulting from branching amino acids and terminal groups, that can be utilized as reaction sites for the chemical modification of enzymes (*15*). Sanger found that DNFB could interact with free amino groups in proteins resulting in the addition of numerous DNP groups (*16,17*). It has now been shown that the resulting DNP-protein is much more hydrophobic and, thus, should be much more soluble in nonpolar organic solvents.

This type of reagent was originally used to determine the total number of free amino groups, so reaction conditions were chosen to carry out the complete interaction. Unfortunately, excessive dinitrophenylization also adversely affects enzyme activity, since interaction can also occur within the active biocatalytic site. Thus, it will be important to determine conditions in which appreciable numbers of DNP groups can be attached to the protein molecule without significant effect on the biocatalytic activity. This can be done either by using relatively mild conditions for the DNFB reaction with incomplete interaction or by protecting the active sites by substrate adsorption prior to the DNFB reaction. These two techniques are being investigated, and preliminary results are reported here.

MATERIALS AND METHODS

Materials

DNFB and cytochrome c were obtained from Sigma Chemical Co. The primary reducing enzyme, hydrogenase, used in this study was isolated from *Proteus vulgaris* using an extraction and purification method previously described (*13,18*). Organic solvents and other chemical reagents used were obtained from Fisher Scientific Co. or EM Science Co.

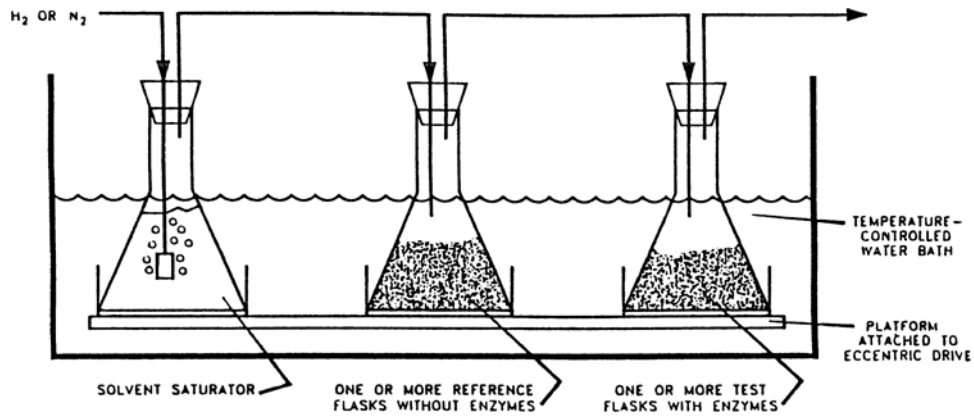

Fig. 1. Experimental setup for determining coal liquefaction/solubilization in shake flasks. Experimental system for coal conversion tests.

Illinois #6 bituminous coal samples were obtained from the Pennsylvania State University Coal Sample Bank. The coal was size-reduced by ball-milling, and the $-270+325$ mesh fraction was used.

Shake-Flask Tests

Most of the tests were made in shake flasks with mixing induced by an eccentric drive operating at 100 rpm. The tests were made in 25- or 50-mL glass flasks in a temperature-controlled bath. All tests with DNFB were made at $25 \pm 1°C$, and tests for the conversion of coal to liquids were made at 30°C. In the latter type of tests, 10 mL of liquid were used with 0.05 to 0.01 g of coal. Generally, a series of tests were made simultaneously using a single gas stream that progressed through the head space in each flask in the entire sample chain (Fig. 1). This gas was presaturated with the solvent mixture being used.

At the completion of each test with coal, the solid residue was removed by successive centrifugation and washing. The first wash solution was the same solvent as that used in the tests, and this was followed by acetone and finally water. The supernatants were removed by siphoning, and spectral measurements were made on the liquid.

Methods for Enzyme Modification

DNFB was used as the primary reactant to add DNP groups to enzyme molecules. The experimental method is still being optimized, but it is based on the method originally developed by Sanger (*14,16,17*). Because DNFB has minimal solubility in water, ethyl alcohol or another appropriate organic solvent must be added to the reaction mixture to increase the solubility. Typically, 28–65 vol% of alcohol was used with 0.1–3

vol% of the DNFB. The enzyme was added to a concentration of about 10 mg/mL, and the reaction solution was buffered to a pH of 8.5 with a 7-m*M* phosphate buffer and/or $NaHCO_3$. All tests were made at ambient temperature (25 ± 1°C) while being exposed to air for 0.5–2 h.

At the end of the reaction, the pH was reduced to 7.0 by the addition of 1*M* HCl, and the resulting product mixture was dialyzed overnight against 50 times the volume of the same ethanol–water–buffer mixture also at a pH of 7, but without DNFB. A cellulose membrane with a mol wt cutoff of 12,000–14,000 daltons was used (Spectra/Por dialysis membrane from Spectrum Medical Industries, Inc.). After dialysis, the modified enzymes were converted to the reduced state by the addition of 5 mg/mL sodium dithionite, and then lyophilized and stored under N_2 until used.

When the higher concentrations of DNFB and/or extended contact time were used, a portion of the modified enzyme actually precipitated and was recovered as a yellow solid that had very low water solubility. At milder conditions (0.1 vol% DNFB and 0.5 h of contact time), the modified enzyme was still soluble in the alcohol–water solution; but even after dialysis, the solution had a yellow color indicative of DNP addition.

Measurement of Enzyme Activity

Activity of the hydrogenase in either aqueous or aqueous-alcohol solutions was measured by the technique developed by Ballantine and Boxer (*19*). This is a spectrophotometric technique in which H_2-dependent reduction of benzyl viologen is determined at 600 nm. Although cytochrome c has little actual enzyme activity, it is important to be able to measure the amount of the oxidizable protein present, since it an act as an effective reducing agent. Total oxidizable cytochrome c was determined spectrophotometrically at 550 nm after first reducing with sodium dithionite using a method similar to that presented by Errede et al. (*20*).

RESULTS AND DISCUSSION

DNP groups can be added to reducing enzymes to increase their solubility in organic solvents. Enzyme activity can also be affected at high levels of DNP; however, modified enzymes clearly maintain activity in organic solvents as indicated by the enhanced liquefaction/solubilization of coal. Both pyridine and benzene have been shown to be useful solvents for this application.

Chemical Modification of Enzymes

Enzymes can be chemically modified with DNFB resulting in a higher solubilization in organic solvents (Table 1). The solubility of the DNP-enzymes increases as the hydrophobicity of the solvents decreases. In

Table 1
Solubility of Enzymes in Different Organic Solvents
After Modification with DNFB[a]

Solvent	Hydrophobicity log P[b]	Average solubility, mg/mL	
		DNP-hydrogenase	DNP-cytochrome c
Dioxane	−1.1	15.5	~20
Pyridine	0.7	9.4	~20
Benzene	2.0	2.0	2.8
Toluene	2.5	0.9	0.9

[a]Approximately 0.2 g of freeze-dried DNP-enzyme formed with about 0.2% DNFB in the alcohol-water solution at 25°C for 1 h was contacted with 10 mL of dry solvent in a shake flask under nitrogen at 30°C for 1 h. The solid residue was removed by centrifugation, and a portion of the supernatant was dried to determine the amount of solubilization.
[b]Log P is defined as the logarithm of the partition coefficient in a standard octanol-water, two-phase system (*12*).

this case, hydrophobicity is defined as log P, where P is the partition coefficient in a standard octanol–water, two-phase system (*21*). A maximum value of about 20 mg/mL of the modified proteins was observed in the most polar solvent. Small quantities of the unmodified enzymes are also dissolved in some organic solvents varying from undetectable in benzene and toluene (<0.1 mg/mL) to barely detectable in pyridine and dioxane (<0.5 mg/mL). In general, these unmodified enzymes were deactivated in the solvent of lowest hydrophobicity.

The amount of DNFB interaction affects the solubilization of the enzyme with a larger amount of dinitrophenylization, resulting in a larger amount of solubilization in the organic solvent. As shown in Fig. 2, after a 0.5-h reaction time, there was a corresponding increase in solubilization of the modified enzyme as the concentration of DNFB in the reaction solution was increased from 0 to 0.67 vol%. However, there was also a corresponding decrease in the enzyme activity, apparently owing to the addition of DNP in the region of the active sites. The enzyme activity of the more highly dinitrophenylized enzyme was only 40% of the original value.

This enzyme deactivation could be at least partially blocked by the addition of a substrate, such as benzyl viologen, to the enzyme mixture prior to the addition of the DNFB. In preliminary tests of this concept, when 0.17% DNFB was used to react with hydrogenase for 30 min, the resulting enzyme activity was only 86.5% of the starting material. However, the addition of 20 m*M* benzyl viologen to the reaction solution resulted in biocatalytic activity of modified hydrogenase very close to the original value. Although the effect of substrate inclusion was relatively small, this may indicate that incorporation of the substrate in the chemical modification mixture protects some of the active sites from interaction with the phenylizing reagent. Early experimental results on this approach

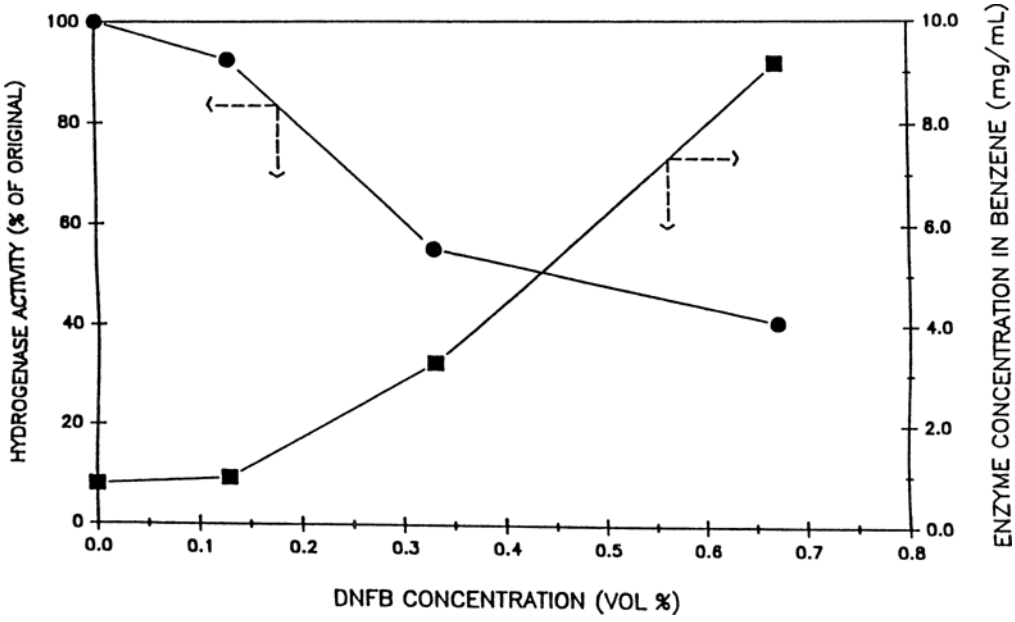

Fig. 2. The effects of DNFB concentration on the solubilization of the modified enzyme in benezene and on enzyme activity.

have been somewhat variable, so additional research will be required to understand and use this technique fully.

Coal Conversion

In previous work, it was shown that the maximum enzyme enhancement of coal conversion to liquids occurred when both the modified hydrogenase and modified cytochrome c were present in the organic solvent (*14,22*). This was presumably because of the reducing properties of the cytochrome c that could be utilized by the biocatalyst, hydrogenase. That enzyme also could use the molecular hydrogen that was present in all tests. The measurement of the increase in light absorbency of the interacting liquid at the wavelength of maximum change was used as a qualitative indication of coal conversion during the course of the 24-h test and the loss of weight of coal conversion during the course of the 24-h test when compared to the starting material represented the overall coal conversion.

Tests on bituminous coal at 30°C in shake flasks indicated that there was a significant enhancement of coal conversion when the modified mixed enzymes were present in benzene (Fig. 3). Liquefaction/solubilization of the coal over the 24-h period was 3.4% on an ash-free and water-free basis in benzene alone, whereas it was 19.7% when the modified

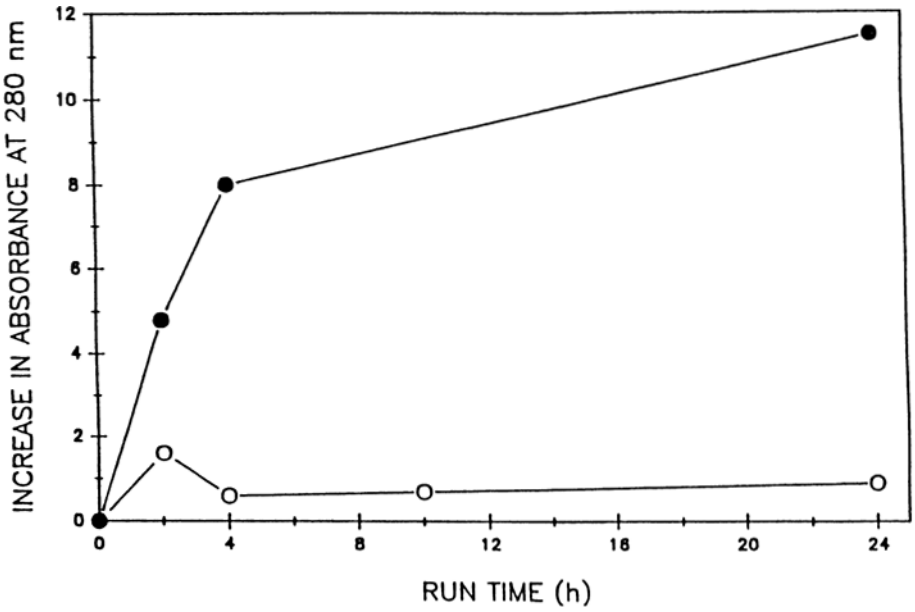

Fig. 3. Enhancement of the conversion of bituminous coal in benzene by a mixture of 0.6 mg/mL DNP-hydrogenase and 0.3 mg/mL cytochrome c at 30°C in a shake flask under H_2.

mixed enzymes were also present. There was also an indication that some of the modified proteins precipitated during the tests, thus contributing to additional solid residue that would erroneously reduce the apparent coal conversion. Based on the change in absorbance of the reaction mixture, it appears that most of the coal conversion occurred during the first 4 h of the test. These results are even more remarkable when it is recognized that the coal conversion occurred at very mild operating conditions.

The modified enzymes also increased coal liquefaction/solubilization in pyridine (Fig. 4), although the maximum amount of coal conversion was $< 10\%$. This indicates that the more polar solvents may not be as effective for this type of enzyme interaction.

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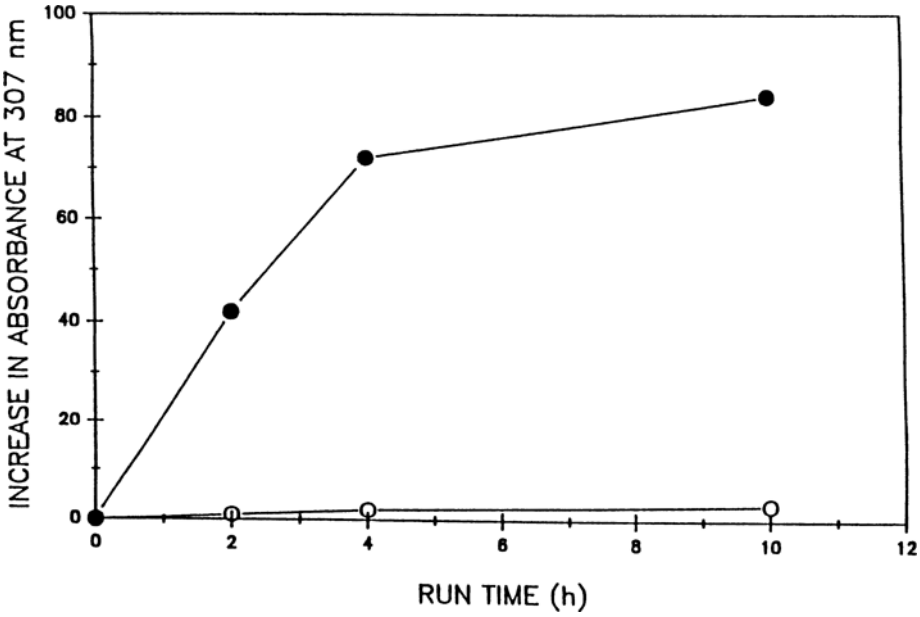

Fig. 4. Enzyme-enhanced bioconverion of bituminous coal in pyridine by a mixture of 1 mg/mL of DNP-hydrogenase and 4 mg/mL of DNP-cytochrome c at 30°C under an H_2 atmosphere.

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