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Organic Synthesis Using Enzymes in Two-Phase Aqueous Ternary Polymer Systems

Sir:

The practicality of enzymes as catalysts in organic synthesis often depends on the efficiency with which they can be recovered from product mixtures and reused.[1,2] Two general approaches are presently available to the design of synthetic reactors based on enzymes: either the enzymes may be immobilized on (in) an insoluble support, or they may be used in solution and reisolated by ultrafiltration, adsorption, precipitation, or other methods.[2] When applicable, immobilization is usually the preferable approach: immobilized enzymes often enjoy protection against deactivation by adventitious proteases and are not exposed to the potentially deactivating conditions encountered during isolation from solution. Immobilized enzymes are, however, not applicable in reactions involving insoluble substrates, or in sequences requiring the enzymes to associate with or dissociate from other insoluble proteins or macromolecules during reaction.[3] Further, partial or complete deactivation often accompanies the immobilization of sensitive enzymes.

We wish to describe a new strategy for utilization of enzymes as catalysts in organic synthesis based on their partition in aqueous two-phase ternary polymer systems. Many aqueous polymer solutions show low mutual solubility, and two-phase ternary polymer systems have been extensively utilized for biochemical separations.[4,5] The partition of material between the two phases depends on the composition, pH, and ionic strength of the system,[4] and is also affected by the presence of polyelectrolytes[6] or specific ligands covalently bound to one of the polymers.[7] Characteristically, the partition coefficients, K,[8] of various proteins between the phases of the system formed from dextran and poly(ethylene glycol) (PEG) in water[9] are in the range $K = 0.1$ to 10.[4] Low molecular weight substances such as inorganic salts, amino acids, sugars, and nucleotides partition almost equally between the two phases (i.e., $K = 1$). We take advantage of the difference in K between substrate, product, and enzymatic catalyst to construct a two-phase biosynthetic reactor (TPBR). An efficient TPBR should consist of two, immiscible, aqueous polymer phases in which the enzyme is partitioned predominantly into one phase. If the partition coefficient for enzyme is far from unity, and that for substrates and products is close to unity, it is possible to separate the enzymatic catalyst from products efficiently by extraction. To assess the influence of the magnitude of K of an enzyme in a two-phase system on the operation of a TPBR, it is useful to analyze a simple model. We assume that the initial quantity of the enzyme added to the TPBR is A_0, the volume of the upper (enzyme poor) phase is V_1 and its enzyme concentration is C_1, and the volume of the lower (enzyme rich) phase is V_2 and its enzyme concentration is C_2. The loss of enzyme from the lower phase in each stage of separation is described by eq 3. For

$$K = C_1/C_2 \tag{1}$$

$$A_0 = A_1 + A_2 = C_1V_1 + C_2V_2 \tag{2}$$

$$A_1 = A_0(1 + V_2(KV_1)^{-1})^{-1} \tag{3}$$

an enzyme with $K = 0.001$ in a TPBR with $V_1/V_2 = 100$, the loss of enzyme from the lower phase to the upper in one stage would be 9.1%. Since for most enzymes values of K <0.001 or >1000 are unlikely,[3] countercurrent operation with multiple partition of the product mixture is necessary to minimize loss of the enzyme from a TPBR.

The operation of a single stage of this type of reactor has been demonstrated using a model system based on glucose 6-phosphate dehydrogenase (G-6-PDH, D-glucose 6-phosphate:$NADP^+$-oxidoreductase, EC 1.1.1.49) isolated from Torula yeast.[10] The partition coefficient of G-6-PDH in the commonly used dextran-PEG two-phase system is $K = 0.20$ (Table I); this value indicates that the protein partitions to the extent of 83% in the lower (dextran rich) phase at a phase volume ratio, $V_1/V_2 = 1$. Because this partition ratio

Table I. Partition Coefficients, K, for G-6-PDH, Its Conjugate with Modified Polyethylene Glycol, Substrate, Cofactor, and Product in Dextran-PEG and Ficoll-UCON Two-Phase Systems

Substance	Partition coefficient, K^a	
	Dextran-PEG[b]	Ficoll-UCON[c]
G-6-PDH (Torula yeast)	0.20	0.0080
G-6-PDH (bakers yeast)	0.073	0.0083
G-6-PD-PEG conjugate	27.0	1.0
Glucose 6-phosphate	0.90	0.68
6-Phosphogluconate	0.99	0.81
$NADP^+$	0.78	0.44
NADPH	0.84	0.73

[a] Determined at 25°. [b] Two-phase system of the total composition: dextran 7.0% (w/w), poly(ethylene glycol) 4.0% (w/w), and triethanolamine buffer (20 mM, pH 7.0) 89% (w/w). [c] Two-phase system with total composition: Ficoll 10.0% (w/w), UCON 50 HB 5100 10.0% (w/w), and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (100 mM, 50 mM potassium chloride, 10 mM magnesium chloride, 2 mM EDTA, 4 mM β-mercaptoethanol, 1 mg/ml bovine serum albumin (Sigma, fraction V, pH 7.5) 80.0% (w/w).

would be unsatisfactory for efficient "immobilization" of the enzyme in a TPBR without several countercurrent extraction stages, other two-phase systems were explored which were expected to minimize or maximize K. Systems incorporating ionic polymers were avoided, on the (untested) belief that they might interact with the enzyme, substrate, or product in undesirable ways. The most useful system seemed to be that which involved neutral polymers having the largest possible difference in hydrophilicity; we employed Ficoll (a synthetic polymer of sucrose) and UCON 50 HB 5100 (a copolymer of ethylene glycol and propylene glycol). The partition coefficient for G-6-PDH in this system was 0.008.[11]

A representative experimental procedure for the conversion of glucose 6-phosphate to 6-phosphogluconate illustrates the synthetic application of this two-phase system. Reaction was carried out in a 150-ml polyethylene centrifuge tube, equipped with a Teflon-coated magnetic stirring bar and a pH electrode. The UCON phase (90.0 ml) was added into the tube, and, with gentle stirring, 0.855 g (1.00 mmol) of $NADP^+$ and 0.300 g (1.05 mmol) of glucose 6-phosphate were dissolved in this solution. The solution was adjusted to pH 7.51 by adding a few drops of 50% aqueous sodium hydroxide. G-6-PDH (220 U.) was dissolved in 10.0 ml of the Ficoll phase, and added to the well-stirred UCON phase. The pH of the heterogeneous reaction mixture decreased sharply during the first 5 min, and became constant at pH 7.25 after 30 min at 25°. The two liquid phases were separated by centrifugation. Appropriate assays indicated the presence of 203 U. (92%) of G-6-PDH in the Ficoll phase; the experimental recovery of the enzyme was in excellent agreement with value of 93% calculated using the experimental partition coefficients. The yields of 6-phosphogluconate[10] and NADPH spectrophotometrically determined in the UCON phase were 81 and 84%, respectively; both yields are consistent with the partition coefficient and phase volume ratio used. Further separation and purification of the products by DEAE cellulose anion exchange chromatography yielded the pure ammonium salts of 6-phosphogluconate and NADPH in 57 and 59% isolated yields, respectively.

This example indicates the feasibility of carrying out enzymatically catalyzed organic synthesis in a two-phase aqueous ternary polymer system, and demonstrates the ease of recovery of enzyme from product in such a system. Although the partition coefficient of native G-6-PDH from Torula yeast was satisfactory for direct use in the Ficoll-UCON system (Table I), the partition coefficients of other enzymes of interest in synthesis are not, and it is not necessarily the case that manipulation of polymer compositions will generate ternary two-phase systems in which some arbitary enzyme will show both satisfactory partition coefficients, good activity, and long operating lifetime. We have briefly explored the practicality of modifying the enzyme instead of the ternary polymer system to influence its partitioning characteristics. α,ω-Di-*p*-nitrobenzoxypoly(ethylene-glycol) (**1**) was synthesized by the reaction of the potassium salt of PEG with *p*-nitrobenzyl bromide in DMSO. Compound **1** was reduced with alkaline aqueous hydrosulfite to α,ω-di-*p*-aminobenzoxypoly(ethylene glycol), to which G-6-PDH was coupled by treatment with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride in a phosphate buffer at pH 4.80. The crude G-6-PDH–PEG conjugate (22% yield, calculated on the basis of enzymatic activity), had K = 3.0 in the dextran–PEG two-phase system, and was assumed to be a mixture of conjugates with different K values. It was purified by successive partition between PEG and dextran phases until a constant K = 27 was reached (9.5% yield, based on original activity). Thus, conjugation of G-6-PDH with a functionalized polyethylene glycol results in a 135-fold increase in partition coefficient (Table I), with the G-6-PDH–PEG conjugate partitioning, as expected, predominantly into the more hydrophobic PEG phase in a PEG–dextran two-phase system. This result emphasizes the potential flexibility of the TPBR; it is possible to manipulate the partition coefficients of enzymes between the two phases by choice of polymers, by changing ionic strength and composition and pH, and by chemical modification of the enzymes; control of the partition coefficients of starting materials, products, and cofactors should also be possible. Judicious adjustment of all of these partition coefficients should make it possible to separate enzymatic activity from products efficiently in many fully developed systems.

A number of questions must be explored before a large-scale TPBR based on cell-free enzymatic catalysis can be constructed routinely. First, how do the polymers that compose the two phases influence substrate, product, and cofactor binding and substrate turnover at the enzyme active site? Second, what are the characteristics of diffusion of substrates and products within a single phase and between two phases of the ternary polymer system, and how does this diffusion influence the productivity of a TPBR? Third, what are the most efficient methods of dispersing the phases in one another, and of separating them after reaction? Fourth, how are enzymatic and cofactor lifetimes in these systems maximized? Fifth, what are the most efficient methods for separating polymers from products? Work on these problems is in progress. Although these questions cannot presently be answered, it is clear that two-phase ternary aqueous polymer systems already provide a useful basis for utilizing enzymes in bench-scale organic synthesis, and offer a potentially practical approach to large-scale synthesis with enzymes whose constitution or mechanism of action preclude conventional immobilization methods.

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(8) K = (protein concentration in the upper phase)/(protein concentration in the lower phase).

(9) Dextran (M_w = 500 000)–poly(ethylene glycol) (M_w = 6000–7500) two-phase system of the total composition: dextran 7% (w/w), poly(ethylene glycol) 5% (w/w), water 88% (w/w).[4] This two-phase system is one of those most commonly used.

(10) Throughout this work a commercially available enzyme (Sigma, Type XII, 320 I.U.) was used without further purification; G-6-PDH and 6-phosphogluconate were assayed spectrofluorimetrically following the procedure reported by O. L. Lowry and J. W. Passonneau in "A Flexible System of Enzymatic Analysis", Academic Press, New York and London, 1972, pp 68 and 205; dextran (Sigma, M_w = 500 000), PEG (Polysciences, M_w = 7500), Ficoll (Sigma, M_w = 400 000), UCON 50 HB 5100 (Union Carbide, M_w = 5100) were used as purchased.

(11) The stability of the native enzyme in the Ficoll phase was good: its half-life was approximately 6 days at 25°. The catalytic activity of the enzyme in this medium was about 70% of its activity in an analogous solution prepared by omitting the organic polymer. In general, enzymes tolerate high concentrations of materials related to these polymers without loss of activity: J. J. O'Malley and R. W. Ulmer, *Biotech. Bioeng.*, **15,** 917 (1973); C. Toniolo, G. M. Bonora, and A. Fontana, *Int. J. Peptide Protein Res.*, **6,** 283 (1974); S. L. Bradbury and W. B. Jakoby, *Proc. Nat. Acad. Sci. U.S.A.*, **69,** 2373 (1972).

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