THE IMMOBILIZATION OF UREASE USING LIQUID-SURFACTANT MEMBRANES

Sheldon W. May and Norman N. Li
Corporate Research Laboratories
Esso Research and Engineering Company
Linden, N.J. 07036

Received May 2, 1972

SUMMARY

Hydrocarbon-based liquid-surfactant membranes have been used to immobilize urease, and the immobilized enzyme has been shown to retain catalytic activity. Furthermore, no significant leakage of the enzyme into the external aqueous solution was detected. The apparent Km value determined for immobilized jack bean urease is approximately 50 times as large as the value which we have obtained for soluble jack bean urease under similar conditions.

Recently, we have been investigating the use of semi-permeable liquid-surfactant membranes as a method for the immobilization of enzymes. This approach offers an alternative to the more usual methods of enzyme immobilization, such as entrapment inside a gel matrix or covalent attachment to an inert support (1,2), and is conceptually similar to the encapsulation method of Chang, which employs solid polymer membranes (3,4,5). Moreover, microcapsules containing enzymes or other proteins are of special interest, since they may be viewed as models for the study of processes which occur in living cells.

The liquid-surfactant membranes which we use are films composed of surfactants, additives and solvent. Such membranes have been employed very successfully in separation processes, and a great deal of information regarding their chemical and physical properties is available (6-10). In our experiments an emulsion composed of individual droplets of the aqueous enzyme solution surrounded by the hydrocarbon based liquid membrane is prepared, and

the enzymatic reaction of interest is carried out by dispersing these droplets in an aqueous solution of the appropriate substrate. Among the advantages of this method are the ease of preparation of the desired emulsion and the possibility of recovering the immobilized enzyme by simple mechanical disruption of the liquid membrane. In this communication we report the results of our studies on urease immobilized using this liquid membrane technique

EXPERIMENTAL

Materials

Crude, crystalline urease was purchased from Worthington Biochemical Corporation (Lot URC-1AA, 510U/mg), Cleland's reagent (dithiothreitol, A grade) from Calbiochem, and urea from Fisher.

Preparation of Liquid Membrane Emulsions

The emulsion was prepared by adding an aqueous urease solution dropwise to the membrane-forming solution with vigorous agitation (350 rpm) (7,8). The final weight ratio of the former to the latter phase was 0.82. The enzyme solution was composed of 0.064% urease in distilled water and the membrane-forming solution was composed of 2% Span-80, 3% ENJ-3029 and 95% S100N. Span-80 is sorbitan monooleate manufactured by Atlas Chemical Industries. ENJ-3029 is a high molecular weight amine with an average molecular weight of 2000 manufactured by ENJAY Additives Laboratories, and S100N is a high molecular weight isoparaffin having an average molecular weight of 386.5, a cloud point of 93°F, a pour point of 90°F, and a gravity of 0.836 gram per cc between 18° and 140°F.

pH Stat Measurements

In these experiments, the enzymatic hydrolysis of urea was monitored using Radiometer type SBR2c/SBU1/TTA3 titration equipment, TTT11b titrator, and pH meter 26. In all cases, the titrant was N/10 HCl (Fisher certified). In a typical experiment with immobilized urease, 30 ml of a solution of the desired amount of urea in 0.1 M NaCl were placed in the titration vessel. To this were added 1 ml of a 0.025 M solution of phos-

phate buffer, pH 7.0, and 0.1μ moles of Cleland's reagent, and the pH of the resulting mixture was checked. Finally, 2 ml of the enzyme-containing emulsion were added to the rapidly stirring titration vessel, and the reaction was monitored at pH 6.7 ± 0.05 by recording the rate of addition of N/10 HCl.

The emulsion used in these experiments has a tendency to adhere to the electrodes of the titrator, and thus to interfere with the response of the instrument. To avoid this problem, we took care to add the emulsion quickly to the rapidly stirring reaction vessel, and to direct the stream of emulsion toward the mechanical stirrer and away from the electrodes during the addition. In this manner, adhesion to the electrodes was minimized, since the emulsion was immediately dispersed into the aqueous solution. In addition, the electrodes were scrupulously washed and cleaned after each experiment, and their response toward a series of standard buffers was checked periodically.

Spectrophotometric Methods

To 60 ml of an 0.45 M urea solution containing 0.1 M NaCl were added 0.2μ moles of Cleland's reagent and 0.1 ml of a 1% ethanolic phenolpthalein solution. The absorbance of the resulting solution at 550 nm was recorded on an ACTA V spectrophotometer. Ten ml of the enzyme-containing emulsion were then added to the stirring solution to initiate the reaction. After the desired time interval, a sample was withdrawn and centrifuged for 3 minutes at 3000 rpm. The lower, aqueous layer was separated and its absorbance at 550 nm was recorded.

RESULTS AND DISCUSSION

Our initial experiments with urease immobilized via the liquid membrane technique were aimed at (1) determining whether or not the immobilized enzyme retained substantial catalytic activity toward urea, and (2) testing for leakage of the enzyme into the continuous aqueous phase. Figure 1 presents the results of a typical experiment performed using the pH-stat

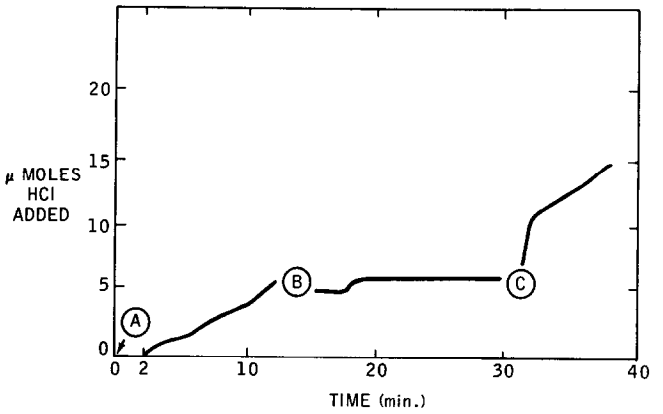

Figure 1. Hydrolysis of Urea by Immobilized Urease, Followed by pH Stat Method. At point A, 2ml of the enzyme-containing emulsion was added to a solution prepared as outlined in the Experimental Section, with $[urea]_o$ = 0.43 M. At point B, recording and stirring were interrupted, the reaction solution was removed, and the aqueous and emulsion layers were allowed to separate. About 80% of the aqueous layer was transferred to a clean titration vessel, and after adjusting its pH to 6.7, recording was resumed. At point C, recording was interrupted, the emulsion and residual aqueous layer were readded, and recording was then resumed.

method. These results clearly indicate that the immobilized urease retains substantial enzymatic activity, and that no detectable leakage of enzyme into the continuous aqueous phase occurs during the reaction. In control experiments, where the enzyme was contained in the continuous aqueous phase rather than in the hydrocarbon phase, removal of the emulsion did not terminate the reaction.

In order to eliminate the possibility that the liquid membrane emulsion was responsible for artifacts in the response of the pH-stat electrodes (see Experimental Section), the enzymatic hydrolysis of urea was monitored spectrophotometrically using phenolpthalein as an indicator. The results of these experiments confirm those obtained in the pH-stat experiments. The absorbances at 550 nm of the aqueous layers of samples withdrawn from the reaction mixture after 500, 2000 and 2800 seconds were 0.036, 0.178 and 0.383, respectively. Moreover, the absorbance of each of these samples was stable for several minutes, after which it slowly decreased, due, evidently, to the

uptake of carbon dioxide (cf. similar observations in reference 11). Since the addition of 1 unit of soluble urease to these aqueous samples caused an immediate increase in absorbance, at an initial rate of 0.04 per min, it is apparent that no detectable leakage of the immobilized urease into the aqueous layer has occurred. Thus, the results of both the pH-stat and spectrophotometric experiments clearly demonstrate that our liquid membrane technique is a viable method for the immobilization of enzymes.

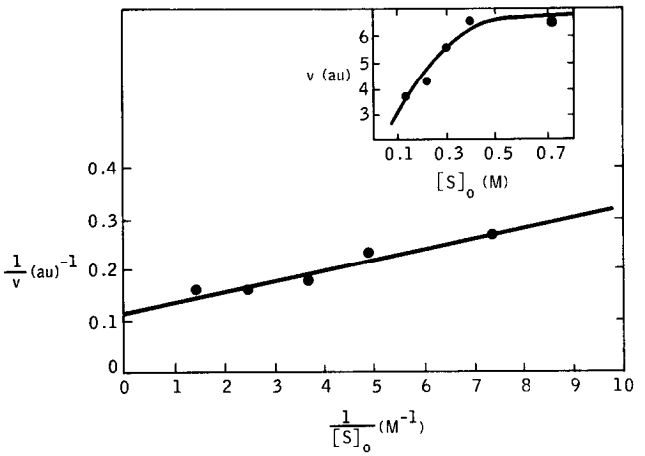

Figure 2. Saturation Curve and Lineweaver-Burk Plot for the Hydrolysis of Urea by Immobilized Urease, pH 6.7 and 25°. The reaction was followed using the pH-stat method, and initial rates are expressed in arbitrary units. The plot gives K_m (app) = (slope) (1/intercept) = 0.18 M.

Figure 2 presents a Lineweaver-Burk plot and a saturation curve for the immobilized-urease-catalyzed hydrolysis of urea at pH 6.7 and 25°. The reaction was followed using the pH-stat method, and initial rates were taken immediately following the short lag period (about 2 min.) caused by the presence of a small amount of phosphate buffer in the reaction vessel (see Experimental Section). The value of Km (app) obtained from Figure 2 is 0.18 M, which is approximately 50 times as large as the value of 3.4×10^{-3} M which we obtained for soluble jack bean urease under similar conditions (cf. ref. 12). Thus it would appear that some diffusion control is exerted on the

reaction by the presence of the liquid membrane (13,14)[1]. However, we recognize that there are complications in the interpretation of the plots in Figure 2, since a gradient of substrate concentrations within the microcapsule would cause the reaction to change from first toward zero order, and the kinetic equations which would apply are therefore not necessarily of simple form (13,14,15)[2]. In addition, it is possible that the diffusion of hydrolysis products to the external solution is relatively slow, and thus, the measured rate of reaction may not actually reflect the true turnover rate of the enzymatic reaction. Moreover, such restricted diffusion would cause the local pH in the vicinity of the immobilized enzyme to be more alkaline than that of the external solution, and the activity of the immobilized urease would thereby be affected. Finally, it should be noted that in the absence of information on the concentration of active enzyme after immobilization, the extent of denaturation caused by the preparation of the emulsion cannot be accurately assessed.

ACKNOWLEDGMENT

The authors acknowledge the excellent technical assistance of Mr. A. Felix and Mr. T. Hucal.

REFERENCES

1. G. Kay, Process Biochemistry, 3, 36 (August, 1968).
2. L. Goldstein and E. Katchalski, Fresenius' Z. Anal. Chem. 243, 375 (1968).
3. T. M. S. Chang, Ph.D. thesis, McGill University, Montreal, Canada, 1965.
4. T. M. S. Chang, Science 146, 524 (1964).
5. T. M. S. Chang, F. C. MacIntosh, and S. G. Mason, Canad. J. Physiol. Pharmacol 44, 115 (1966).
6. N. N. Li, U.S. Pat. 3,410,794 (Nov. 12, 1968).
7. N. N. Li, J. Am. Inst. Chem. Eng. 17, 459 (1971).

(1) Actually, the data presented here do not distinguish between physical adsorption of the enzyme onto the hydrocarbon membrane and encapsulation.
(2) A critical question in this regard is whether or not the internal solution of a small microcapsule of the order of 1-10μ in diameter can be considered to be homogeneous.

8. N. N. Li, Ind. Eng. Chem. Process Design Develop. 10, 215 (1971).
9. N. N. Li and W. J. Asher, "Blood Oxygenation by Liquid Membrane Permeation," presented at 162nd Am. Chem. Soc. Meeting in Washington on September 13, 1971.
10. U.S. Patent pending.
11. R. L. Blakeley, J. A. Hinds, H. E. Kunze, E. C. Webb and B. Zerner, Biochemistry 8, 1991 (1969).
12. F. J. Reithel in The Enzymes IV, Edited by P. D. Boyer, Academic Press, New York, p. 1 (1971).
13. P. R. Rony, Biotechnology and Bioengineering 13, 431 (1971).
14. R. Goldman, O. Kedem, and E. Katchalski, Biochemistry 10, 165 (1971).
15. V. Kasche, H. Lundquist, R. Bergman and R. Axen, Biochem, Biophys. Res. Commun. 45, 615 (1971).