0273-2289/92/32(1/3)—0111$03.20

Kinetics and Activity Distribution of Urease Coencapsulated with Hemoglobin Within Polyamide Membranes

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Received July 15, 1991; Accepted November 4, 1991

ABSTRACT

A 91.5% mass yield of urease and hemoglobin (Hb), co-encapsulated within polyamide membranes, was determined spectrophotometrically. The specific activity yield of microencapsulation was 84%, twofold higher than values previously reported, as a result of optimization of encapsulation conditions. The kinetic parameters and pH activity profiles of intracapsular urease were determined to be similar to those corresponding to the free enzyme. Similar activities were also observed for intact and microcapsule homogenate, indicating minimal mass transfer and diffusional limitation. The active configuration of the enzyme appears to remain intact upon microencapsulation. The application of a kinetic model for encapsulated urease further indicated that the kinetics were reaction-controlled with minimal mass transfer restrictions.

Index Entries: Urease; hemoglobin; microencapsulation; polyamide membrane; observed and specific yield.

INTRODUCTION

A number of techniques have been developed for enzyme immobilization, including covalent binding, adsorption, matrix entrapment, and microencapsulation (*1–3*). Microencapsulated enzymes are separated

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from the external solution by means of an ultrathin, semipermeable polymeric membrane that restricts access of large-mol-wt components such as antibodies and proteins but prevents loss of the immobilized biocatalyst. Low-mol-wt materials such as substrate molecules are permitted to diffuse through the membrane to the site of catalytic activity, along with concomitant diffusion of product to the bulk solution.

Microcapsules or artificial cells have potential for use in medicine as artificial liver (*4–6*) and kidney (*7–8*), in enzyme therapy (*9–11*), and as blood substitutes (*12–14*), as well as in biotechnology (*15*) to immobilize enzymes (*16*) and biological cells (*17,18*).

Although interfacial polymerization has been applied to enzyme immobilization, it is often thought to result in considerable loss of enzyme activity. Chang (1971) reported activity yields of 30% after encapsulating urease (*19*). The addition of Hb as a protective agent against enzyme denaturation in the aqueous phase improved the long-term stability of urease and other enzymes but did not increase the activity yield. Microencapsulation of carbonic anhydrase by interfacial polymerization resulted in 10% retention of enzyme activity (*20*). The activity yield of encapsulated asparaginase reported by Mori et al. was only 37% (*21,22*). Wood and Whateley (1982) encapsulated α-chymotrypsin and histidase and obtained activity yields of 15 and 30%, respectively (*23*). Ostergaard and Martiny (1973) reported a relative activity yield of 20% with microencapsulated β-galactosidase (*24*). On the other hand, Sundaram (1973) reported 90% activity yield of urease after encapsulation but obtained only 30% urease activity in the presence of hemoglobin (*25*). Monshipouri and Neufeld (1991) demonstrated an 83% yield of encapsulation of urease within nylon membranes (*26*). High activity yields of entrapped enzyme relative to the mass of entrapped enzyme (92.5%) were reported, suggesting minimal effects of mass transfer limitation.

Despite the high cost, factors leading to low enzyme activities following microencapsulation have rarely been investigated. Low activity yields may be the result of loss of enzyme during preparation, extremes of pH during interfacial polymerization reactions, enzyme denaturation because of solvent exposure, and membrane diffusional restrictions. Enzyme loss and denaturation, or the amount of enzyme incorporated into the membrane during formulation, have not been reported in most of the studies noted above since the mass yield of microencapsulation was not determined. Consequently, it is often difficult to separate the effects of mass transfer and diffusion on the rates of reaction from that of enzyme loss and denaturation. Therefore, the kinetic parameters of encapsulated enzyme are often ambiguous.

In this study, the mass yield of hemoglobin–urease co-encapsulation was examined by spectrophotometry. The specific activity yields and hence the efficiency of the microencapsulation procedure were determined. The kinetic parameters of microencapsulated urease were mea-

sured, and the effect of diffusion limitation on the observed rate of urea hydrolysis was evaluated. The conclusions based on the experimental results were confirmed using a model developed by Sundaram (1973) (*25*).

MATERIALS AND METHODS

Reagents

Bovine serum Hb and Jack Bean urease type VI were purchased from Sigma Chemical Co. (St. Louis, MO). A stock organic solvent consisting of chloroform and cyclohexane (1:4 v/v) was prepared. The emulsifer Span 85 (Atkemix, Montreal, Que) was dissolved (0.4% v/v) in a solvent solution of chloroform and cyclohexane (1:4 v/v). Trephthaloyl chloride was dissolved in organic solvent solution (0.3% w/v) for 60 min at 4°C and filtered. The dispersing solution consisted of Tween 20 in distilled water (50% v/v) adjusted to pH 7.0 with borax buffer. Hexamethylene diamine (Aldrich, Milwaukee, WI) was dissolved in 0.45*M* sodium bicarbonate buffer to a concentration of 0.4*M*, and the pH was adjusted to 9.0 with HCl. All solutions were freshly prepared and cooled to 4°C prior to microcapsule preparation.

Preparation of Nylon Microcapsules

Urease was dissolved in 1 mL of a 10% (w/v) Hb solution and mixed with an equal vol of hexamethylene diamine solution. The mixture was emulsified for 1 min using a magnetic stir bar, aided by the addition of 15 mL span solution. The trephthaloyl chloride solution (15 mL) was subsequently added to initiate the interfacial polymerization reaction. The reaction was terminated after 3 min by addition of 20 mL solvent solution. The microcapsules were allowed to settle, the supernatant discarded, and 30 mL of 50% tween solution added to disperse the microcapsules. The resulting suspension was mixed and gradually diluted to 150 mL with distilled water. The microcapsules were then separated from the supernatant by centrifugation at 3000 rpm for 3 min and washed twice with distilled water. Microcapsules were suspended in 4°C distilled water, and the experiments were performed within 3 h of preparation.

Activity Assay

Activity measurements were performed in a pH-stat titrator (Radiometer, Copenhagen). Urease activity was measured in 40 mL of 0.33*M* urea solution at 25±1°C. Urea was converted to ammonia according to:

$$CO(NH_2)_2 + H_2O \xrightarrow{\text{urease}} CO_2 + 2NH_3$$

Catalysis was determined at a set-point pH by the rate of 0.1M HCl addition to neutralize the ammonia (*27,28*). Ammonia was determined by phenol-hypochlorite reaction (*29*). One International Unit (IU) of urease activity liberates 1 μmol NH_3/min at pH 7.0 and 25(±1)°C.

Total activity (IU) was obtained by assaying the activity of the total microcapsule suspension following microencapsulation. Observed activity (IU/mg) was calculated by dividing the total activity by the initial mass of urease (mg) prior to encapsulation. Specific activity (IU/mg) was determined by dividing total activity by the mass of intracapsular urease determined spectrophotometrically. The observed activity yield (%) was the ratio of observed activity to the activity of free enzyme in solution. Specific activity yield (%), or the effectiveness factor, was defined as the ratio of specific activity of intracapsular enzyme to the activity of free urease in solution.

The activity of membrane-bound urease was determined by homogenizing a fixed volume of a microcapsule suspension in a tissue grinder (Fisher Scientific, Montreal, Que) and centrifuging the membrane debris. The supernatant was discarded, and the membrane fraction washed and recentrifuged to separate the membrane fraction from the freely soluble enzyme. The membrane fraction was assayed for urease activity.

Hemoglobin Concentration Measurement

Since Hb is a combination of ferro (Fe^{2+}) and ferri (Fe^{3+}) derivatives with different spectral absorptivities, the solution was reduced with $Na_2S_2O_4$ prior to absorbance measurement. The extinction coefficient of ferrohemoglobin at 555 nm and at pH 7.0 was $1.03 \times 10^4\ M^{-1}cm^{-1}$ (*30*).

A fixed vol of microcapsule suspension was homogenized and centrifuged at 4000 rpm for 7 min to separate membrane debris. Membrane-bound Hb was determined by difference spectra at 555 nm between the absorbance of intact urease–Hb microcapsules and that of the homogenate supernatant representing freely soluble Hb.

Microcapsule Volume Concentration

Volumetric and cumulative size distributions were determined by laser beam scattering with a 2602-LC particle analyzer (Malvern Instruments, Southborough, MA) according to the log normal distribution model (*31*). The mean diameter and the arithmetic SD [$\sigma_a = 0.5(d_{84} - d_{16})$] were calculated from the cumulative distribution curve. Volume concentration is the vol of the incident beam containing solids and can be calculated from Lamberts-Beer law.

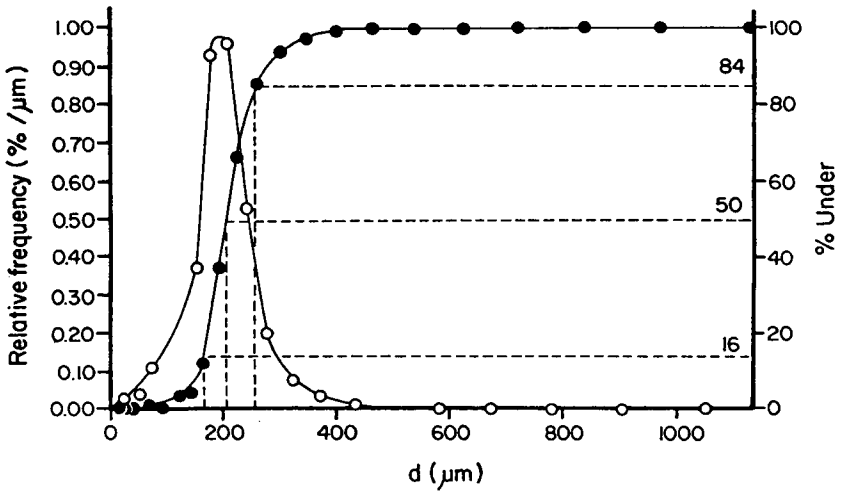

Fig. 1. The volumetric and cumulative size distribution of urease-Hb microcapsules. —○— = Volumetric distribution, and —●— = Cumulative distribution.

RESULTS

Volumetric and cumulative size distributions of urease-Hb microcapsules with polyamide membranes, are illustrated in Fig. 1 The size distribution curve shows symmetry around a mean value of 185 μM. The actual mean, corresponding to 50% cumulative size distribution, is 205 μm. This is caused by a small number of large microcapsules in the 450–1050 μm diameter range. The arithmetic SD for this particular preparation was found to be 57.5 μm, and the vol concentration was 0.067%.

Microencapsulated urease activity was assayed by means of a pH-stat autotitrator at a pH set-point of 7.0. Activity was dependent on reactor agitation, as seen in Fig. 2. The observed activity increased linearly with mixing rate to 1220 rpm, at which point no further increase in activity was observed. At mixing rates higher than 1700 rpm, breakage of microcapsules was observed. For subsequent experiments, a mixing rate of 1700 rpm was selected for reaction rate measurements.

Figure 3 shows the absorbance spectra of urease-Hb microcapsules in the visible region. Neither urease nor the nylon membrane absorbed at 555 nm, hence the absorbance was the result of reduced Hb. Based on the absorbance reading, the concentration of encapsulated Hb was determined to be 45.8 mg/mL, representing a mass yield of 91.5%. The percentage of Hb incorporated into the membrane during polymerization accounted for 35.8% of total microencapsulated Hb. Since urease and Hb were combined

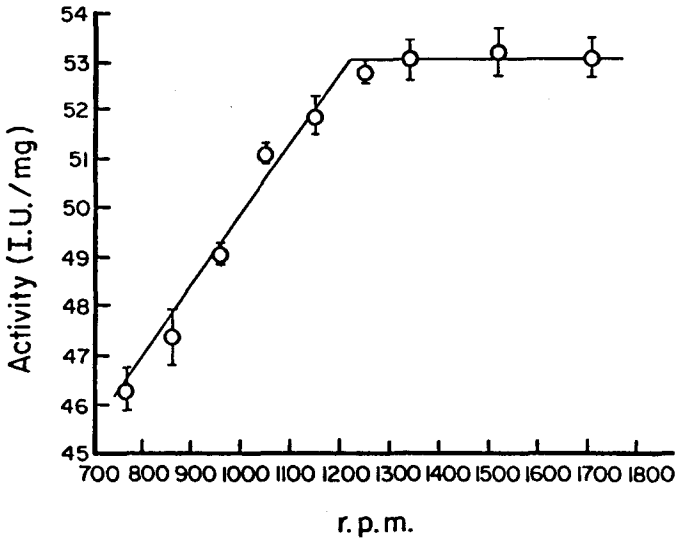

Fig. 2. The dependence of microencapsulated urease activity on reactor agitation rate at pH 7.0 and 25°C.

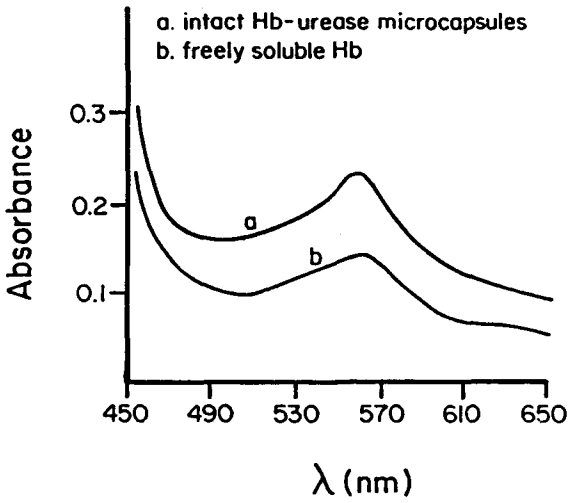

Fig. 3. Absorbance spectra of reduced Hb in urease-Hb microcapsules (**A**) and of the freely soluble form (**B**) in 0.01*M* phosphate buffer at 25°C.

prior to microencapsulation, the mass yield of intracapsular urease was assumed to be 91.5%. The mass and activity distribution of urease co-encapsulated with Hb is presented in Table 1. The specific activity of intracapsular urease was found to be 60.5 IU/mg, and the specific activity yield of microencapsulation was 84%. An activity assay on the membrane fraction of homogenized urease-Hb microcapsules indicated that urease activity was not associated with the membrane. Similarities between the activities of intact (60.5 IU/mg) and homogenized (61.7 IU/mg) microcapsules implied minimal effect of membrane diffusional limitation on kinetics.

Table 1
Summary of HB-Urease Activity Assays[a]

Hb/Urease	Mass	Activity
Freely soluble urease in Hb solution	10.0 mg	72.0 IU/mg
Microencapsulated urease	9.2 mg	60.5 IU/mg
Yield of encapsulation	91.5%	—
Specific activity yield	—	84.0%
Encapsulated soluble fraction[b]	—	61.7 (100%)
Membrane-bound fraction[b]	—	0.0
Activity of combined released fractions	—	61.7 (100%)

[a] Assay conditions were 0.33 *M* urea, pH 7.0, 25°C; and Hb concentration in the aqueous phase prior to microencapsulation was 50 mg/mL.
[b] Activity of released soluble fraction and released membrane-bound fraction upon rupture of microcapsules; % activity in comparison to combined released fractions.

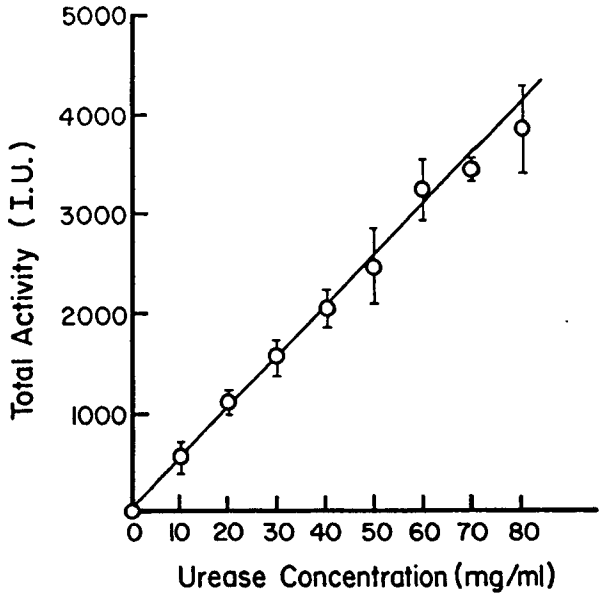

Fig. 4. Activity of urease coencapsulated with Hb under varying initial urease concentrations.

Activity of microcapsule suspension (IU) vs free urease concentration in the aqueous phase (mg/mL) prior to microencapsulation is plotted in Fig. 4. Total activity increased linearly with initial urease concentration over the range considered. In Table 2, the variations of specific activity yield (%) with urease concentration in the aqueous phase prior to microencapsulation are presented. The specific activity yields, or effectiveness factors, ranged from 72 to 84%.

Table 2
Variations of Specific Activity Yields
With Enzyme Concentration
Within Urease-Hb Microcapsules

Urease (mg/mL)	Specific activity yield (%)
10	83.8±1.8
20	83.5±0.7
30	79.6±2.4
40	76.5±2.3
50	75.4±5.0
60	82.5±3.5
70	75.4±1.0
80	72.1±5.4

Note: the mass yield of microencapsulation was 91.5%. SD were based on three replicate measurements.

Table 3
Comparison Between Specific Activities
of Intact and Homogenized Urease Microcapsules
at Different pH Values[a]

	Specific Activity (IU/mg)	
pH	Intact	Homogenized
3	21.7±7.5	21.4± 8.0
4	46.7±4.0	45.0± 3.5
5	64.2±6.5	70.0±11
6	74.0±8.0	78.9± 2.0
7	59.1±4.0	67.5± 2.0
8	43.3±7.0	43.5± 2.0

[a]0.33*M* urea buffer; 25(±1)°C; and [urease]=30 mg/mL.

The specific activities of intact Hb microcapsules (60 mg/mL) are compared with the activity of the homogenate over a range of pH values (Table 3). There was little difference between the activities of encapsulated enzyme and the microcapsule homogenate, suggesting that the membrane did not interfere with kinetics.

The dependence of urease activity on pH is plotted in Fig. 5. The pH optimum of free urease was 6.0, coinciding with the pH optimum of intracapsular urease shown in Fig. 6. Thus, no shift in pH optimum was observed for the encapsulated urease. The rate of urea hydrolysis at different substrate concentrations is shown in Table 4. Comparison between the

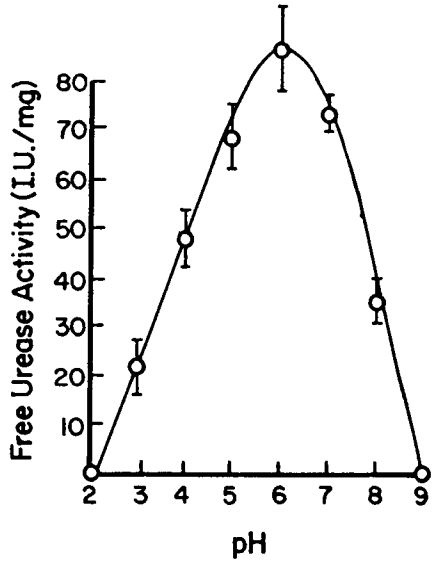

Fig. 5. The pH activity profile of free urease at 25°C.

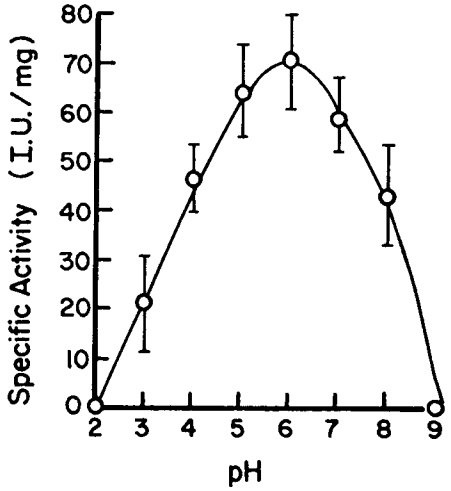

Fig. 6. The pH activity profile of urease coencapsulated with Hb at 25°C.

Table 4
Rate of Urea Hydrolysis With Soluble
and Microencapsulated Urease at Varying Urea Concentrations

	Soluble	Microencapsulated	
Urea (m*M*)	Activity (IU/mg)	Observed activity (IU/mg)	Specific activity (IU/mg)
100	63.3±1.0	54.2±4.0	59.2±7.6
40	57.1±5.5	48.6±4.0	53.2±7.3
10	38.5±3.0	34.5±2.5	37.7±4.8
8	34.7±3.5	27.0±1.0	29.5±2.7
4	23.3±5.0	19.2±1.5	20.9±2.8

Table 5
Kinetic Parameters of Freely Soluble and Microencapsulated Urease

Enzyme form	K_M (m*M*)	V_{max} (IU/mg)	K_{cat} (min^{-1})
Freely soluble	7.6	68.0	4.1×10^4
Encapsulated (observed)	8.4	59.2	3.1×10^4
Encapsulated (specific)	8.4	61.7	3.7×10^4

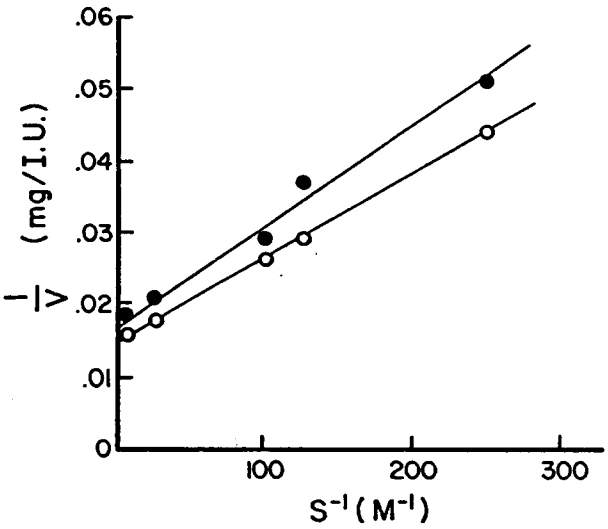

Fig. 7. Lineweaver-Burk plot of free and microencapsulated urease at pH 7.0 and 25°C. ● = Encapsulated urease, ○ = Free urease.

free and encapsulated forms of the enzyme was conducted with equal concentrations of the enzyme. Microencapsulated urease appeared to follow Michaelis-Menton kinetics, similar to that of the free form.

The kinetic parameters for urea hydrolysis reaction with freely soluble and microencapsulated forms of urease at different urea concentrations are presented in Table 5. Lineweaver-Burk plots of free and microencapsulated urease (Fig. 7) were used to determine the kinetic parameters (K_M and V_{max}) of the respective enzyme forms. The Michaelis constant (K_M) of free urease (7.6 mM) was similar to that of the microencapsulated urease (8.4 mM), within experimental error. K_{cat} values of free and encapsulated forms (using specific reaction rates) were $4.1\times10^4\ min^{-1}$ and $3.7\times10^4\ min^{-1}$, respectively. In Fig. 8, a plot of V_c/V vs S'/K_M for predicted values of ϕ are given according to a model that considers the effects of mass transfer and

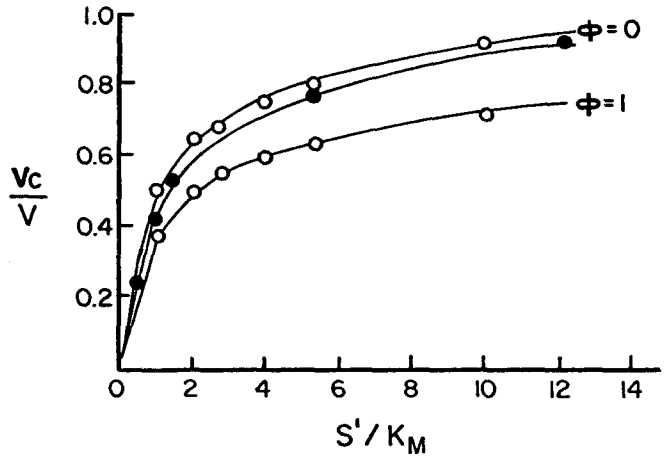

Fig. 8. Plot of V_c/V vs S'/K_M. The parameter ϕ refers to the ratio of reaction to mass transfer rate. ○ = Predicted values, ● = Experimental values.

diffusion on the kinetics of microencapsulated urease (*25*). The parameter ϕ is often referred to as *Thiele modulus,* which is the ratio of reaction to mass transfer rate. Application of the data obtained in this study to the above model results in a plot with a Thiele modulus approaching zero. It appears that reaction rates catalyzed by intracapsular urease are considerably less than mass transfer rates, indicating reaction control of the process.

DISCUSSION

Volumetric size distribution of the microcapsules was determined by particle analyzer using a laser diffraction technique. Mean diameters and SD obtained from volumetric size distributions have led to a better estimate of the specific surface area of microcapsules than that obtained from numeric size data (*32*). Thus, volumetric size distributions are particularly beneficial in cases where the kinetics of intracapsular enzyme is mass transfer or surface area controlled. In this study, the size distribution was used to determine the volume concentration of the suspension.

The mass yield of urease-Hb encapsulation was determined using spectrophotometry. The reduction of Hb (Fe^{+3} to Fe^{+2}) using sodium dithionite permitted determination of protein concentration at 555 nm through the heme (Fe) groups. Urease or the encapsulating membrane material did not absorb at this wavelength. Based on absorbance measurements of reduced Hb, it was determined that 91.5% of the original Hb and urease were encapsulated. The urease mass yield determined directly on nylon microcapsules containing pure urease was 83% (*26*), similar to the mass yield reported here; thus, protein loss occurred during the formulation.

The specific activity yields with varying initial enzyme concentrations were in the range of 72–85%, which are at least twofold higher than previously reported values (*7,22,25*). The balance of the enzyme appears to be denatured during the encapsulation procedures according to the mass and activity balance. In a previous study (*26*), pure microencapsulated urease showed higher specific activity (92.5%) than that of urease–Hb microcapsules (72–85%) in this study. It appears that the protective effect of Hb against interfacial denaturation of the urease is minimal. The discrepancies between the results of this experiment and those reported previously in the literature may be explained in terms of mass yield of microencapsulation. In this study, specific activity yield or effectiveness factor of intracapsular urease was determined and used to estimate the efficiency of microencapsulation. In most previous studies, the mass yield of encapsulation had not been determined because it was assumed that the observed activity yield was the same as the intracapsular enzyme effectiveness factor. This assumption in cases where the mass yield of encapsulation is low, e.g., 10%, could result in serious error in determining the activity yield (*20*).

Buffer effects may also lead to underestimation of the absolute value of reaction rates. Although this error may not be of significance for effectiveness factor determination, it may manifest itself when determining the percentage intracapsular enzyme, which is cross-linked to the membrane. For example, the assay of the membrane fraction of homogenized urease microcapsules performed in phosphate buffer indicated no enzyme activity associated to the membrane (*7*). Assays at a fixed pH without buffer demonstrated that 6% of intracapsular enzyme was cross-linked to the membrane (*26*). This discrepancy may be explained in terms of the inhibitory effect of the phosphate buffer on the urease active site (*33*).

The activity assay conducted on the membrane fraction of homogenized urease–Hb microcapsules indicated that there was no activity associated with the membrane. However, spectrophotometric results suggest that 35.8% of the Hb may be membrane-bound. The most likely amino acids taking part in membrane formation are lysine, arginine, and histidine because of the presence of amino (NH_4^+) groups. The wt fraction of these residues in Hb is 22.5% (*34*). The corresponding value for urease based on amino acid analysis was 19.2% (*35*). This similarity between Hb and urease suggests that both proteins should be incorporated to the same extent within the forming nylon membrane. It was shown that 6% of encapsulated urease is incorporated into the nylon membrane (*26*), if it is assumed that a similar amount of Hb is incorporated into the nylon membrane, the balance (29.2%) may be an adsorbed fraction. Horbett et al. (1977, 1978) demonstrated that Hb has been shown to have a high affinity for diverse polymeric surfaces (*36,37*).

The pH activity profiles of some immobilized enzymes are known to shift to higher or lower pH values as a result of charge interactions of the

support with the reactants or products of enzyme reaction (*23,38*). The pH of the enzyme microenvironment may be different from that of the bulk solution. Although the pH activity profile of urease has previously been reported, the reaction rate was found to be a function of reaction conditions and the type of isozyme used (*39*). For example, a pH optimum of 6.65 was obtained for urease type IV - Sigma (*40*) and an optimum of 7.0 at 0.2*M* urea determined for urease obtained from Worthington (*41*). In this study, the pH optimum was found to be 6.0 for free urease, and shifts in the pH activity profile of encapsulated urease–Hb were not observed. These results suggest that the microenvironment of urease in the microcapsules is similar to that of the bulk solution. There is no net charge on the polyamine membrane within a broad range of pH, so that charge interactions between the support and reaction products are not expected.

The exposure of enzymes to solvents and various reagents is often associated with the loss of enzyme activity. Therefore, the possibility of urease structural and thereby activity change during microencapsulation should be considered. The close similarity observed between the K_M values of free (7.6 m*M*) and intracapsular urease (8.4 m*M*) suggests that urease did not undergo a conformational change, which would alter its activity following microencapsulation.

The similarity between the kinetic parameters of free and intracapsular urease also suggests that the nylon membrane did not create a significant mass transfer/diffusional barrier to the reactants and products. At high rates of mixing, external mass transfer limitations were minimized. Hence, the only differences between the reaction rates of microencapsulated urease vs the free form will be the result of internal mass transfer and diffusion limitations. A 2.5-fold decrease in the mean diameter of the microcapsule preparation did not result in any effects on the observed reaction rates (unpublished data); thus, the internal mass transfer effects on the observed rates appear to be insignificant. Finally, the close similarity between the activities of intact and homogenized urease–Hb microcapsules suggests that urea and ammonia diffusion rates through the membrane occur at rates higher than kinetic rates.

Sundaram (1973) developed a model that predicts the reaction rate per unit vol of total suspension as follows (*25*):

$$V_c / V = (\phi - 1 + K_M / S')\,(-1 + \sqrt{1 + 4\,K_M / S' / (\phi - 1 + K_M / S')^2})\,/$$

$$2\,K_M / S' + (\phi - 1 + K_M / S')\,(-1 + \sqrt{1 + 4\,K_M / S' / (\phi - 1 + K_M / S')^2})$$

where Φ is given by $\Phi = V \cdot r/3DS'$.

V_c is the reaction rate per capsule volume (IU/mg); V is the reaction rate per unit of suspension volume (IU/mg); S' is the bulk substrate concentration (*M*); Φ is the ratio of reaction to mass transfer rate; r is the mean radius of the microcapsules; and D is the diffusivity of the substrate in solution. A plot of V_c/V vs S'/K_M for the data obtained in this study resulted

in a curve with the Thiele modulus ϕ approaching zero. It appears that the reaction rate is slower than the mass transfer rate, resulting in kinetic rates similar to those of the free soluble enzyme.

The similarity between the kinetic parameters and the pH activity profiles of free and intracapsular urease do not agree with the results obtained by Sundaram (1973), who showed a shift in the pH activity profile as well as an increase in K_M upon encapsulation (*25*). This was explained by assuming a diffusion barrier as a result of the presence of the nylon membrane. The difference between the results of the two studies may be explained in terms of the preparation procedures. The procedure of Miyawaki et al. (1979) was followed in this study as conditions optimal for encapsulation (*42*). This procedure differed from that of Sundaram in terms of the aqueous pH, time of polymerization, and the type of acid dichloride used. Sundaram's membrane may have a decreased porosity relative to that used in this study, resulting in significant diffusion limitations.

In summary, urease microencapsulation in the presence of Hb demonstrated high yields of mass and activity. The activity yields were at least twofold higher than previously reported values. This is in accordance with the yields obtained previously on pure urease microencapsulation. The pH activity profiles of urease co-encapsulated with Hb was the same as the free enzyme, which suggested that the microenvironment of microencapsulated urease may be the same as in the free solution. This was further supported by the close similarity between the K_M values of free and microencapsulated urease. These findings suggest that the role of diffusion limitation caused by the membrane on the overall kinetics of microencapsulated urease may be minimal, and enzyme conformational change during microencapsulation is unlikely.

ACKNOWLEDGMENTS

The authors wish to thank the Natural Sciences and Engineering Research Council of Canada for providing financial assistance for the project. Partial funding was also received from the Ministere de l'Education du Québec through a research grant to the Artificial Cells and Organs Research Centre of McGill University.

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