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Evaluation of possible reasons for the low phenylalanine ammonia lyase activity in cellulose nitrate membrane microcapsules

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Abstract

Microencapsulated phenylalanine ammonia lyase (PAL) exhibits a marked reduction in activity compared to the activity of the free enzyme in pH 8.5 Tris buffer. The purpose of this investigation was to evaluate the contribution of incomplete entrapment, the internal environment of cellulose nitrate membrane microcapsules, the diffusional barrier of the membrane and the microcapsulation process to the low activity of encapsulated PAL. A solution of PAL and 10% w/v hemoglobin was incorporated into cellulose nitrate membrane microcapsules. Hemoglobin incorporation was used as a surrogate marker of PAL entrapment. Using ^{14}C hemoglobin, the encapsulation efficiency was determined to be 70% and suggested that incomplete entrapment might partially account for the low activity of encapsulated PAL. The effect of the internal environment of the microcapsule (10% hemoglobin solution) on PAL activity was evaluated by comparing enzyme activity in 10% w/v hemoglobin solution and pH 8.5 Tris buffer. Similar K_M and V_{max} values of PAL in the two media indicated that the internal environment of the microcapsule did not contribute to the reduction in activity of the encapsulated enzyme. The contribution of a membrane diffusional barrier was determined by breaking the putative barrier and measuring PAL activity in intact and broken microcapsules. Similar activity of PAL in these two conditions is evidence for the lack of a diffusional barrier. The effect of the microencapsulation process on PAL activity was evaluated by comparing K_M and V_{max} of free and encapsulated PAL. Similar K_M values in these two media suggested that the process did not affect the conformation of PAL. However, encapsulated PAL had a 50% lower V_{max} value compared to free PAL, which showed that the microencapsulation process deactivated a substantial proportion of the enzyme. © 2001 Published by Elsevier Science B.V.

Keywords: Microencapsulation; Coacervation; Cellulose nitrate membrane microcapsules; Phenylalanine ammonia lyase; Hemoglobin; Entrapment efficiency; Diffusional barrier

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1. Introduction

Phenylketonuria (PKU) is caused by a genetic defect in the phenylalanine hydroxylating system in the liver, which results in the accumulation of phenylalanine in the body and leads to serious neuropsychological outcomes in patients. The current treatment strategy for PKU is a low phenylalanine diet. However, the available diets are expensive and barely palatable, which in turn reduces their acceptance by patients. Also, diet therapy requires frequent biochemical monitoring to ensure a normal phenylalanine balance in the body (Smith, 1993).

Currently, a novel method for the treatment of PKU is being developed, which involves microencapsulation of the enzyme responsible for metabolizing phenylalanine. The microencapsulated enzyme is administered orally along with diet. Phenylalanine is an essential amino acid, which is obtained only from diet. It is hoped that in the gastrointestinal tract, the phenylalanine molecules will diffuse into the microcapsules before they can be absorbed and the microencapsulated enzyme will metabolize them into less harmful products. The resultant decrease in the absorption of phenylalanine would be of benefit to phenylketonurics. Phenylalanine hydroxylase, which is the physiologically relevant enzyme in the body, requires another enzyme (dihydropteridine reductase) as well as two coenzymes (tetrahydrobiopterin and NADH) for its activity. Therefore, microencapsulation of this multi-species complex might be a difficult problem. On the other hand, phenylalanine ammonia lyase (PAL) is an enzyme that converts phenylalanine to a nontoxic product, namely trans cinnamic acid. Also, PAL does not need any other enzymes or coenzymes for its activity. Therefore, it might be easier to microencapsulate PAL and to evaluate the efficiency of this oral therapy in normalizing phenylalanine levels in the body.

Some investigators have microencapsulated PAL, administered it orally and demonstrated its effectiveness in lowering plasma phenylalanine levels in animal models of PKU (Chang et al., 1995). PAL was dissolved in 10% w/v hemoglobin solution and microencapsulated by the interfacial coacervation method using cellulose nitrate as the microcapsule membrane. It is believed that hemoglobin preserves the physical stability of the enzyme during microcapsule manufacture and also enhances structural integrity of the microcapsules (Chang et al., 1966; Mori et al., 1972; Chang, 1987). However, despite the presence of hemoglobin, microencapsulated PAL is only 20% as active as the free enzyme in buffer solution (Bourget and Chang, 1984). The reasons for the lower activity of microencapsulated PAL have not been examined. Microencapsulated PAL has lower activity probably because of its incomplete entrapment into microcapsules during its manufacture, due to an unfavorable environment within the microcapsules, the presence of a diffusional barrier to the passage of substrate/product molecules, inactivation of the enzyme during the process of microencapsulation or most likely due to a combination of these factors. Our objective was to evaluate the contribution of each of these factors to the lowering of activity of encapsulated PAL.

In the microencapsulation process, PAL and hemoglobin are mixed to form a solution which is then microencapsulated. Direct spectrophotometric determination of microencapsulated PAL is not possible due to the high concentration of hemoglobin. Therefore, we decided to measure the extent of hemoglobin entrapment and use it as a surrogate marker of the entrapment efficiency of PAL. Hemoglobin entrapment was measured by using ^{14}C hemoglobin in the preparation of microcapsules and measurement of the entrapped radioactivity.

The activity of free PAL is determined in Tris buffer. However, the internal environment of the microcapsules consists of a 10% w/v hemoglobin solution. Although hemoglobin could preserve the physical stability of PAL, it may have a detrimental effect on the activity of the enzyme. We determined the contribution of the internal environment of the microcapsules to the lower activity of microencapsulated PAL by measuring and comparing the maximum velocity (V_{max}) and Michaelis constant (K_M) of PAL in Tris buffer and 10% hemoglobin solution.

The cellulose nitrate membrane of the microcapsules may exert a diffusional barrier for the transport of substrate and product molecules, which in turn could lower apparent enzyme activity. We determined the possible existence of such a diffusional barrier by two approaches. First, we measured PAL activity in an in-vitro incubate at different stirring rates. Direct dependence of PAL activity on stirring rate would suggest that phenylalanine diffusion through the stagnant layer around the microcapsules was a rate-limiting step. In our second approach, we removed the putative barrier by breaking the microcapsule membrane and compared PAL activity in intact and broken microcapsules. We hypothesized that the presence of a diffusional barrier would result in lower PAL activity in the intact microcapsules compared to the broken microcapsules.

In the process of microcapsule preparation, PAL comes into contact with organic solvents and chemicals. Such contact could alter the conformation and/or denature PAL and decrease its activity. We determined possible losses of PAL activity during microcapsule manufacture by measuring K_M and V_{max} of encapsulated PAL and comparing them to similar parameters determined for the free enzyme in buffer solution. In order to have an accurate comparison, we first determined pH and stirring rates of the reaction medium under which maximum activity of free and encapsulated enzyme is obtained. K_M and V_{max} of free and microencapsulated PAL were then measured under these optimized conditions.

2. Materials and methods

2.1. Materials

Centricon concentrator tubes were purchased from Amicon (Beverly, MA). Coomassie plus protein assay reagent and bovine serum albumin standard were obtained from Pierce (Rockford, IL). Collodion and *n*-butyl benzoate was purchased from Spectrum (New Brunswick, NJ). Ether and HPLC grade methanol were obtained from Fisher (Pittsburgh, PA). Phenylalanine ammonia lyase (from *Rhodotorula glutinis*, Grade I) and all other materials and reagents were purchased from Sigma (St. Louis, MO).

2.2. Extraction of glycerol from enzyme solution

PAL is commercially available in a 60% v/v glycerol solution. Results of earlier studies suggest that the cellulose nitrate membrane of microcapsules is unstable in the presence of glycerol (Bourget and Chang, 1984). Therefore, glycerol in the enzyme solution was removed prior to microencapsulation by dialysis using Centricon concentrator tubes (molecular weight cut-off of 100 000 kDa). PAL solutions were taken into Centricon tubes and centrifuged three times for 30 min on each occasion at 4°C and $1000 \times g$. The retentate containing the glycerol free enzyme was recovered and used for further studies. In order to confirm complete removal, glycerol in the filtrate of dialysis tubes was quantitated using an enzymatic assay with a colorimetric end point at a wavelength of 540 nm, by the method of McGowan et al. (1983) using the Sigma triglyceride diagnostic kit.

2.3. Preparation of cellulose nitrate membrane microcapsules

Cellulose nitrate membrane microcapsules were prepared by using the method of Chang et al. (Chang, 1987). The entire process was conducted at 4°C. Hemoglobin solution was prepared by stirring lyophilized bovine hemoglobin in pH 8.5 Tris buffer for 2 h at 4°C. The solution was filtered through a Whatman No. 42 filter paper and its concentration was determined spectrophotometrically by the method of Drabkin et al. (Drabkin and Austin, 1935) using the Sigma total hemoglobin reagents. Next, PAL was dissolved in the hemoglobin solution and the final concentration of hemoglobin was adjusted to 10% w/v. The aqueous mixture was emulsified in ether to yield a water in oil emulsion, followed by coacervation of cellulose nitrate on the surface of aqueous droplets. After aspirating off the organic solvents, *n*-butyl benzoate was added to stabilize the microcapsule membrane. After 30 min, butyl benzoate was aspirated off and the microcapsules were

washed three times in Tween-20 solution to remove any remaining organic solvents. Finally, the microcapsules were suspended in pH 8.5 Tris buffer. In all experiments, microcapsules were freshly prepared on the day of the experiment.

2.4. Determination of the entrapment efficiency of hemoglobin

2.4.1. Determination of entrapped ^{14}C hemoglobin

Microcapsules were prepared without PAL but in the presence of a 10% hemoglobin solution containing 0.7 μCi ^{14}C hemoglobin. After preparation, the microcapsules were homogenized by a hand-held tissue grinder. The homogenate was incubated with an equal volume of a 30% v/v hydrogen peroxide solution for 1 h at ambient temperature, to bleach the hemoglobin color and thereby minimize its quenching effect. The samples were then counted to determine the extent of hemoglobin entrapment. On each day, the experiment was conducted in triplicate and the experiment was repeated on 3 separate days.

2.4.2. Mass balance of hemoglobin during the process of microencapsulation and distribution of hemoglobin within the microcapsules

Microcapsules were prepared without PAL, but in the presence of a 10% hemoglobin solution containing 0.7 μCi ^{14}C hemoglobin. During the preparation of microcapsules, radioactivity in aliquots of aspirated organic solutions and aqueous washings were measured in order to determine the contribution of different steps in the process to the loss of hemoglobin. Radioactivity in the solution in which the microcapsules were stored was also measured to evaluate the extent of possible leakage of hemoglobin from the microcapsules. Finally, the microcapsules were homogenized with a hand-held tissue grinder and radioactivity in aliquots of the homogenates was measured to determine the extent of entrapped hemoglobin.

The remaining homogenate was centrifuged at $8800 \times g$ and radioactivity of the supernatant and the sediment was measured to determine the relative amounts of free hemoglobin inside microcapsules (supernatant) and the hemoglobin bound to the microcapsule membrane (sediment). Prior to counting, all samples were bleached with a 30% hydrogen peroxide solution. On each day, the experiment was conducted in triplicate and the experiment was repeated on 3 separate days.

2.5. Role of the internal environment of the microcapsules — comparative V_{max} and K_M of PAL in Tris buffer and 10% w/v hemoglobin solution

Phenylalanine, ranging in concentrations from 50 to 1500 μM, was incubated with PAL in either pH 8.5 Tris buffer or 10% w/v hemoglobin solution at 37°C under constant stirring. The total volume of the reaction was 0.4 ml. The enzymatic reaction was stopped after 7 min with trichloroacetic acid (10% w/v) solution. Preliminary studies had indicated that product formation was linear for at least 7 min. Concentration of the product, trans cinnamic acid, was measured by HPLC and velocity was measured as the rate of increase of product concentration. Velocity (V) versus $V/[S]$ data were fitted to the Eadie–Hofstee equation listed below and estimates of K_{M} and V_{max} were obtained.

$$V = V_{\mathrm{max}} - K_{\mathrm{M}}.V/[S]$$

where $[S]$ is phenylalanine concentration.

V_{max} values were normalized based on PAL concentrations. Concentration of PAL was determined spectrophotometrically according to the method of Bradford (1976) using the Coomassie plus protein assay reagent and bovine serum albumin as the standard. On each day, the experiment was conducted in triplicate and the experiment was repeated on 3 separate days.

2.6. Determination of the existence of a diffusional barrier

2.6.1. Effect of stirring rate on the activity of microencapsulated PAL

Phenylalanine solution (12.8 mM) was incubated with microencapsulated PAL at 37°C. The incubate was stirred at rates ranging from a setting of two to ten (100–1000 rpm) in the Pierce Reacti-Therm III magnetic stirrer.

2.6.2. PAL activity in pH 8.5 Tris buffer within intact microcapsules and in homogenized microcapsules

Phenylalanine solution (12.8 mM) was incubated with either free PAL in pH 8.5 Tris buffer or with PAL in intact or homogenized microcapsules at 37°C in pH 8.5 Tris buffer. All incubates were stirred at a stirrer setting of six.

2.6.3. Evaluation of leakage of PAL from the microcapsules

After preparation, microcapsules were suspended in pH 8.5 Tris buffer. One hour later, aliquots of the Tris buffer solution were taken and incubated with 12.8 mM phenylalanine solution at 37°C. The incubate was stirred at a stirrer setting of six. This experiment was performed to evaluate the possible leakage of PAL from the microcapsules into the Tris buffer solution.

In Section 2.6.1, Section 2.6.2 and Section 2.6.3, the total volume of the reaction mixture was 2.0 ml. The enzymatic reaction was stopped after 7 min with trichloroacetic acid (10% w/v) and concentration of the product, trans cinnamic acid, was measured using an HPLC method. In cases where intact microcapsules were incubated with phenylalanine solution, the microcapsules were homogenized in a hand-held tissue grinder prior to determination of trans cinnamic acid concentration.

2.7. Role of the microencapsulation process

2.7.1. Measurement of K_M and V_{max} of PAL in pH 8.5 Tris buffer and in intact microcapsules

Phenylalanine, ranging in concentrations from 50 to 1500 μM, was incubated with either free PAL in pH 8.5 Tris buffer or with microencapsulated PAL. Incubations were conducted at 37°C under constant stirring and the total volume of the reaction mixture was 2.0 ml. The enzymatic reaction was stopped after 7 min with trichloroacetic acid (10% w/v) solution and concentration of the product, trans cinnamic acid, was measured by HPLC. In cases of microencapsulated PAL, the microcapsules were homogenized in a hand-held tissue grinder prior to determination of trans cinnamic acid concentration. K_M and V_{max} values of PAL were determined using the Eadie–Hofstee equation. V_{max} values were normalized to PAL concentration. PAL concentrations were determined according to the method of Bradford using the Coomassie plus protein assay reagent and bovine serum albumin as the standard (Bradford, 1976). On each day, the experiment was conducted in triplicate with freshly prepared microcapsules and the experiment was repeated on 3 separate days.

2.7.2. pH — activity profile for free and microencapsulated PAL

Phenylalanine solution (12.8 mM) was incubated with either free or microencapsulated PAL at 37°C at pH values ranging from 2 to 11.5. Discrete pH values were achieved by using the following buffers: 0.1 M citrate buffer (pH 2–6), 0.1 M Tris buffer (pH 7.5–8.5) and 0.1 M glycine buffer (pH 9.5–11.5). The incubate was stirred at a stirrer setting of six.

2.7.3. Determination of the relative activity of free and membrane bound PAL within microcapsules

Cellulose nitrate microcapsules containing PAL were homogenized with a hand-held tissue grinder. A portion of the homogenate was centrifuged at $8800 \times g$. Aliquots of the supernatant, the resuspended sediment and the original homogenate were incubated with phenylalanine solution (12.8 mM) at 37°C in pH 8.5 Tris buffer at stirrer setting of six.

In Section 2.7.2 and Section 2.7.3, the total volume of the reaction mixture was 2.0 ml. The enzymatic reaction was stopped after 7 min with trichloroacetic acid (10% w/v). Concentration of the product, trans cinnamic acid, was measured with an HPLC method.

2.8. Chromatography

Trans cinnamic acid was determined using the HPLC method of Ruhul Amin et al. (1995) with the following modifications. A reversed phase column RP-18 (150 × 3.9 mm ID, 4 μM particle size) was used, along with a mobile phase of 50 mM phosphate buffer (pH 6.5): methanol (92:8, v/v). The flow rate was 1.0 ml/min and the column

temperature was set at 40°C. The eluent was monitored by a UV detector at 270 nm.

2.9. Statistical analysis

Differences between groups were evaluated either with a Student's t-test (two groups) or a one-way ANOVA (more than two groups). All tests were conducted at a significance level of 0.05.

3. Results

3.1. Extraction of glycerol from enzyme solution

The results of glycerol assay on the dialysate indicated that 103% ± 2.5 (mean ± S.D., $n = 4$) of the glycerol in the original enzyme solution was extracted into the dialysate. This suggests that dialysis using Centricon concentrator tubes was capable of removing all the glycerol from the enzyme solution.

3.2. Determination of the entrapment efficiency of hemoglobin

3.2.1. Determination of entrapped ^{14}C hemoglobin
Measurement of ^{14}C radioactivity within microcapsules indicated that 71.2% ± 0.5 (mean ± S.D., $n = 9$) of hemoglobin was encapsulated within the microcapsules. This suggests the coacervation process was unable to encapsulate ≈30% of the hemoglobin.

3.2.2. Mass balance of hemoglobin during the process of microencapsulation and distribution of hemoglobin within microcapsules
A mass balance study was performed in order to identify and measure the contribution of steps in the microencapsulation process where unencapsulated hemoglobin was lost. The results in Table 1 show that unencapsulated hemoglobin was primarily found in the organic solvent fraction and the aqueous washings. Radioactivity in the supernatant solution in which the microcapsules were finally stored was small and suggested that leakage of hemoglobin through the microcapsule membrane upon storage was minimal. The mass balance

Table 1
Mass balance of ^{14}C hemoglobin during the preparation of microcapsules

Fraction	% Radioactivity[a] (mean ± S.D.)[b]
Organic mediums	7.1 ± 2.2
Aqueous washings	15.6 ± 4.8
Supernatant of the microcapsules	0.08 ± 0.02
Microcapsules	69.7 ± 3.0
Total[c]	92.4 ± 4.0

[a] All percent data are expressed as a percent of initial radioactivity.
[b] $N = 9$.
[c] Sum of percent radioactivity in the different fractions.

studies showed that ≈70% of hemoglobin was encapsulated and confirmed the results of our previous experiments. Evaluation of the fate of entrapped hemoglobin showed that 82% ± 2 (mean ± S.D.; $n = 9$) of the encapsulated hemoglobin was bound to the cellulose nitrate membrane, while only 9.9% ± 0.4 of the encapsulated hemoglobin was free.

3.3. Role of the internal environment of the microcapsules — comparative V_{max} and K_M of PAL in Tris buffer and 10% hemoglobin solution

Fig. 1 shows a representative Eadie–Hofstee plot of PAL in pH 8.5 Tris buffer and 10%

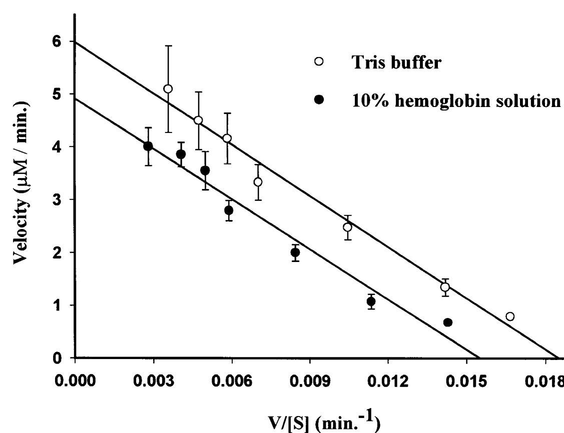

Fig. 1. Eadie–Hofstee plot for PAL in pH 8.5 Tris buffer and 10% hemoglobin solution. Each point represents the mean ± S.D. of three measurements.

Table 2
K_M and V_{max} of PAL in pH 8.5 Tris buffer and 10% hemoglobin solution

Parameter (units)	pH 8.5 Tris buffer	10% Hemoglobin solution	*P* value
K_M (μM)	319 ± 23[a]	324 ± 29	NS[b]
V_{max} (μM/min.mg protein)	1.39 ± 0.08	1.30 ± 0.06	NS

[a] Mean ± S.D., $N = 9$.
[b] Not significant; Student's *t*-test.

hemoglobin solution, while Table 2 shows comparative kinetic analyses of the data. The K_M and V_{max} values of PAL in these two media were not statistically different.

3.4. Determination of the existence of a diffusional barrier

3.4.1. Effect of stirring rate on the activity of microencapsulated PAL

As shown in Fig. 2, activity of encapsulated PAL was similar at different stirring rates (one-way ANOVA, $P = 0.24$). In subsequent experiments, PAL activity was measured at stirrer setting of six.

3.4.2. PAL activity in pH 8.5 Tris buffer, within intact microcapsules and in homogenized microcapsules

As shown in Fig. 3, the activity of PAL in intact microcapsules is ≈ 23% of activity of free enzyme in pH 8.5 Tris buffer. This confirms literature reports, which show that microencapsulation produces a marked reduction in PAL activity (Bourget and Chang, 1984). Interestingly, the activity of PAL in intact and homogenized microcapsules is similar. This observation suggests that the cellulose nitrate membrane does not exert a diffusional barrier to the transport of phenylalanine molecules into the microcapsules.

The aforementioned conclusion assumes that homogenization of microcapsules did not affect the activity of the encapsulated PAL. In order to confirm this assumption, we examined the effect of homogenization on PAL activity. We dissolved PAL in pH 8.5 Tris buffer, homogenized the solution in a hand-held tissue grinder and determined PAL activity before and after the homogenization process. As shown in Fig. 4, homogenization decreased enzyme activity by ≈ 65%. However, within microcapsules PAL is dissolved in 10% hemoglobin solution that could possibly modulate the detrimental effect of homogenization on PAL activity. In order to evaluate the possible protective effect of hemoglobin during the homogenization process, we dissolved the enzyme in 10% hemoglobin solution and then homogenized it. As shown in Fig. 4, in the presence of 10% hemoglobin, homogenization did not affect PAL activity. But in our studies designed to measure the activity of encapsulated PAL, microcapsules were reconstituted in a volume of 3 ml and then homogenized. The final hemoglobin concentration in the microcapsule homogenate is 1.2% and it was unclear if this lower concentration of hemoglobin was also protective Therefore, we dissolved PAL in 1.2% hemoglobin solution,

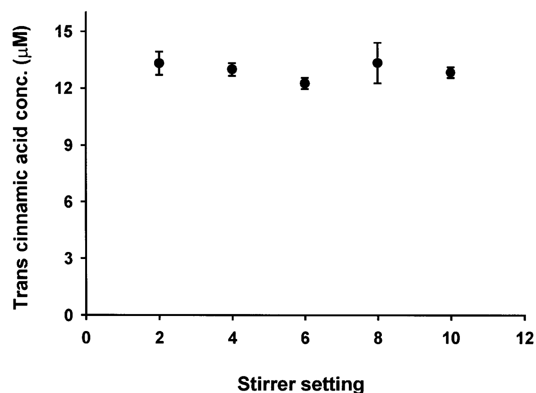

Fig. 2. Effect of stirring rate on the activity of microencapsulated PAL. Each point represents the mean ± S.D. of three measurements.

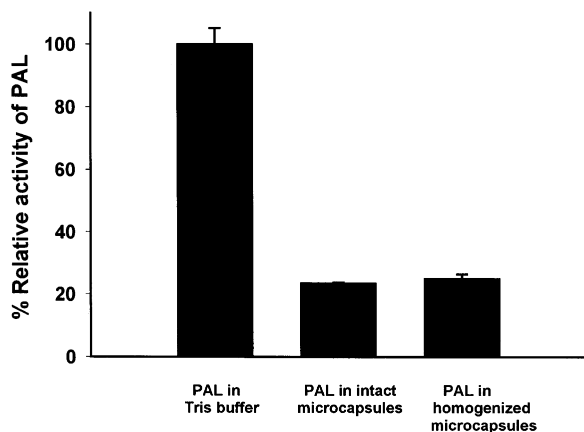

Fig. 3. Relative activity of PAL in pH 8.5 Tris buffer and within intact or homogenized microcapsules. Each value represents the mean ± S.D. of triplicate measurements. Enzyme activities in intact and homogenized microcapsules are expressed as a percent of PAL activity in Tris buffer. The activity of PAL in Tris buffer is 1.28 μM/(min.mg protein).

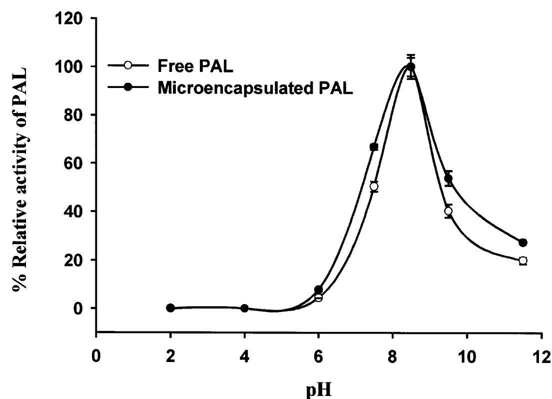

Fig. 5. pH-activity profile for free and microencapsulated PAL. Each point represents the mean ± S.D. of three measurements. Free and microencapsulated enzyme activities at different pH values are expressed as a percent of the respective activities at a pH of 8.5. The actual activity of free PAL at a pH of 8.5 is 1.49 μM/(min.mg protein) while the activity of microencapsulated PAL at a pH of 8.5 is 0.34 μM/(min.mg protein).

homogenized it and determined the enzyme activity before and after homogenization. As shown in Fig. 4, a lower concentration of hemoglobin was also effective in protecting PAL activity against the detrimental effects of homogenization.

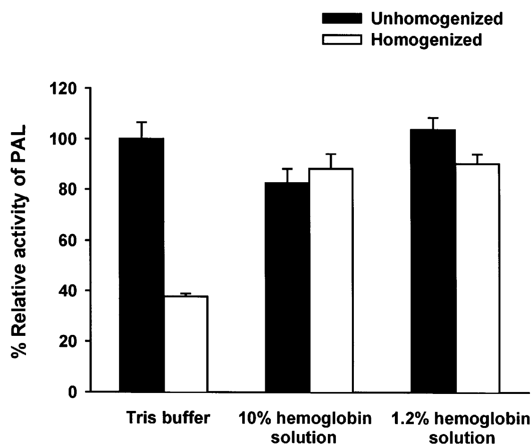

Fig. 4. Effect of homogenization on the activity of PAL under different conditions. Each value represents the mean ± S.D. of triplicate measurements. Enzyme activities in the different conditions are expressed as a percent of the activity of unhomogenized PAL in Tris buffer. The activity of unhomogenized PAL in Tris buffer is 1.53 μM/(min.mg protein).

3.4.3. Evaluation of the leakage of PAL from the microcapsules

One hour after microcapsule preparation (1 h), an aliquot of the supernatant solution in which the microcapsules are stored was incubated with phenylalanine. No activity of PAL was detected in this solution. This suggests that there is no leakage of PAL through the cellulose nitrate membrane of microcapsules for at least 1 h after their preparation.

3.5. Role of the microencapsulation process

3.5.1. pH-activity profile for free and microencapsulated PAL

Fig. 5 shows the pH-activity profiles of free and encapsulated PAL. At pH values of 7.5, 9.5 and 11.5, the encapsulated enzyme exhibits slightly higher relative activity than the free enzyme. However, both free and encapsulated enzymes exhibit peak activity at a pH of 8.5. In our subsequent experiments, PAL activity was measured at a pH of 8.5.

3.5.2. Measurement of K_M and V_{max} of PAL in pH 8.5 Tris buffer and in intact microcapsules

In order to evaluate in greater detail the effect of microencapsulation process on PAL activity,

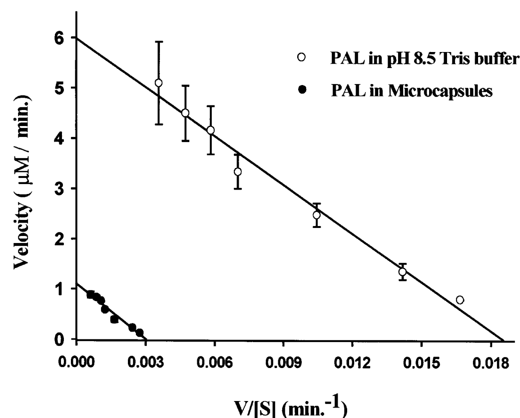

Fig. 6. Eadie–Hofstee plot for PAL in pH 8.5 Tris buffer and within intact microcapsules. Each point represents the mean ± S.D. of three measurements. At some points, the error bar is within the symbol.

kinetic parameters of PAL were measured in Tris buffer and intact microcapsules. Fig. 6 shows the Eadie–Hofstee plot of the kinetic data, while Table 3 shows enzyme kinetic parameters of PAL under the two conditions. The K_M values of free and microencapsulated PAL were not statistically different which suggests that the microencapsulation process did not affect the conformation of PAL. However, the V_{max} of encapsulated PAL was only 21% that of the free enzyme which suggests that the process decreased the amount of functional enzyme.

3.5.3. Determination of the relative activity of free and membrane bound PAL within microcapsules

Microcapsules were homogenized and centrifuged. The activity of PAL in the supernatant was designated as activity of free enzyme, while the activity of PAL in the sediment was considered to be the activity of membrane bound enzyme. Free PAL activity accounted for 63.9% ± 2.4 (mean ± S.D., $n = 3$) of the total activity of encapsulated enzyme while membrane bound PAL activity accounted for 39.5% ± 1.7 ($n = 3$) of the total activity.

4. Discussion

Microencapsulated PAL is a promising therapy for PKU though its clinical use may be limited because of poor activity of the encapsulated enzyme. The reasons for the low activity of intracapsular enzyme are still unclear. It is possible that during the process of manufacture, incomplete entrapment of PAL within microcapsules is partially responsible for the reduced activity of encapsulated enzyme. Surprisingly, to the best of our knowledge, there is little developed methodology to measure the entrapment yield of enzymes into microcapsules.

In our studies, the easiest way to measure entrapment efficiency of PAL into the microcapsules was to break them and measure the amount of PAL released. However, the very high concentration of co-entrapped hemoglobin interfered with the spectrophotometric determination of PAL and precluded the use of this method. Measurement of the activity of entrapped PAL as a marker of its entrapment efficiency would not be accurate, since all of the entrapped enzyme may not necessarily be active. One method to accurately measure en-

Table 3
K_M and V_{max} of PAL in pH 8.5 Tris buffer and within intact microcapsules

Parameter (units)	pH 8.5 Tris buffer	Intact microcapsules	P value
K_M (μM)	319 ± 23[a]	333 ± 25	NS[b]
V_{max} (μM/min.mg protein)	1.39 ± 0.08	0.29 ± 0.03[c]	<0.05

[a] Mean ± S.D., $N = 9$.
[b] Not significant; Student's t-test.
[c] Assumes complete entrapment of PAL.

trapment efficiency of PAL is to use labeled enzyme. However, radiolabeled PAL is not commercially available and we do not have the facilities to label it. Hemoglobin, a co-entrapped solute that is necessary for microcapsule manufacture is commercially available as ^{14}C hemoglobin. Therefore, we encapsulated a solution of ^{14}C hemoglobin, measured its encapsulation yield and used it as a marker of the entrapment efficiency of PAL. In a multicomponent solution, the use of encapsulation efficiency of one component to reflect the entrapment efficiency of another component seems a reasonable premise and has been previously used (Monshipouri and Neufeld, 1992). Quantitation of ^{14}C hemoglobin in homogenized microcapsules indicated that 70% of the originally added hemoglobin was entrapped within the microcapsules. Mass balance studies confirmed our 70% estimate of hemoglobin entrapment efficiency and showed that $\approx 23\%$ of the hemoglobin is lost in the organic media and aqueous washings. The hemoglobin present in these fractions probably represents unencapsulated hemoglobin and hemoglobin that leaked from partially formed microcapsules. We had determined that hemoglobin does not leak from microcapsules after final manufacture. Assuming that a similar fraction of PAL is encapsulated, our data suggest that incomplete entrapment partially accounts for the lower activity of microencapsulated PAL. Other investigators have used radiolabeled albumin and fibrinogen and reported an encapsulation yield of $\approx 65\%$ for these proteins in polyamide membrane microcapsules (Wood and Whateley, 1982). Similar to our results, these authors indicated that most of the unencapsulated protein was lost in the organic media and aqueous washings.

Microencapsulated PAL reportedly looses 80% of its activity (Bourget and Chang, 1984). We have shown that incomplete entrapment accounts for $\approx 30\%$ of this loss. In an attempt to identify other factors that produce the unexpectedly low activity of encapsulated enzyme, we examined the effect of the internal environment of the microcapsules on the activity of PAL. Similar K_M and V_{max} values of PAL in Tris buffer and 10% hemoglobin solution suggested that the internal environment of the microcapsules was not responsible for the lower activity of encapsulated PAL.

Next, we explored the possibility that the stagnant diffusion layer around the microcapsules and the cellulose nitrate membrane of microcapsules exerted a diffusional barrier to the passage of substrate/product and thereby reduced the apparent activity of PAL. We found enzyme activity to be independent of stirring rate. This suggested that the stagnant diffusion layer is not rate limiting with respect to the diffusion of phenylalanine molecules into the microcapsules. Other investigators reported that within polyamide membrane microcapsules, an increase in stirring rate was associated with a proportional increase in the activity of urease; suggestive evidence for a diffusional barrier (Monshipouri and Neufeld, 1992). We also destroyed the putative membrane barrier by homogenizing the microcapsules and observed that PAL activity in intact and homogenized microcapsules was similar. This result suggests that the cellulose nitrate membrane of microcapsules did not interfere with the diffusion of phenylalanine molecules.

We evaluated the effect of microcapsule manufacturing process on the activity of PAL by measuring and comparing K_M and V_{max} values of the free and microencapsulated enzyme. For meaningful comparisons, these parameters had to be measured under conditions in which the free and encapsulated PAL exhibited maximum activity. Free PAL is reported to have maximum activity at a pH of 8.5 (Abell and Shen, 1987), while the activity of the encapsulated enzyme is not well established. In our studies, both free and microencapsulated PAL exhibited maximum activity at a pH of 8.5 indicating the microcapsulation process did not alter the pH of maximal activity. Bourget and Chang also reported that in cellulose nitrate membrane microcapsules, free and microencapsulated PAL exhibited maximal activity at a pH of 8.5 ± 1. They reported markedly higher relative activity of microencapsulated PAL compared to the free enzyme at low and high pHs and attributed that to the high concentration and buffering capacity of coencapsulated hemoglobin (Bourget and Chang, 1985).

In our next experiments, we determined possible leakage of PAL from the microcapsules. One hour after preparation of the microcapsules, we

observed no leakage of PAL through the cellulose nitrate membrane. Khanna and Chang (1990) microencapsulated histidase within cellulose nitrate membrane microcapsules and also reported no leakage of the enzyme through the membrane. Other investigators who encapsulated asparaginase and histidase inside polyamide membrane microcapsules showed that this membrane is also impervious to the passage of the encapsulated enzymes (Mori et al., 1972; Wood and Whateley, 1982).

We compared PAL activity in pH 8.5 Tris buffer and within intact microcapsules and showed that the encapsulated enzyme had an activity which was only ≈23% of the activity of free enzyme in buffer solution. In these experiments, the free and encapsulated PAL was incubated at a phenylalanine concentration (12.8 mM) that is much greater than the K_M (0.32 mM) of the enzyme. Consequently, the measured activity of the enzyme reflects the V_{max} of PAL. These experiments did not evaluate the effect of microencapsulation on K_M of the enzyme. In order to evaluate the effect of the microencapsulation process on both the V_{max} and K_M of PAL activity, kinetic analysis of free and encapsulated PAL was conducted at varying phenylalanine concentrations. As observed before, the V_{max} of encapsulated PAL was ≈21% that of the free enzyme. The decreased V_{max} suggests that during the microencapsulation process, PAL was extensively denatured and deactivated. However, the unchanged K_M of PAL in the two states indicates that the small fraction of encapsulated enzyme that did not denature was conformationally intact and functional. Similar to our findings, Kondo and Muramatsu (1976) showed that microcapsulated arginase lost 88% of its activity due to denaturation of the enzyme after contact with organic solvents during the microcapsule manufacturing process. Other investigators who encapsulated histidase, catalase and lactase within cellulose nitrate membrane microcapsules also reported that microencapsulation did not affect K_M values, but markedly decreased V_{max} of the encapsulated enzymes (Poznansky and Chang, 1974; Khanna and Chang, 1990; Wang and Shao, 1993). These authors suggested that the low V_{max} values were the result of diffusional barrier exerted by the cellulose nitrate membrane to the permeation of substrate molecules. In the present study, we have ruled out the presence of a diffusional barrier as a cause for the low V_{max} of encapsulated PAL.

We measured distribution of PAL activity within the microcapsules and observed that both free and membrane bound PAL are enzymatically active. Free PAL accounted for ≈64% of the total activity, while the remaining activity was associated with the enzyme bound to the cellulose nitrate membrane. A more informative method to evaluate the percent of free and membrane bound enzyme activity within microcapsules is to express them as specific activity, i.e. activity per milligram of protein. However, since we could not directly measure the amount of PAL at each location, we could not compute specific activity. Future studies will attempt to radiolabel PAL and use it to directly compute encapsulation efficiency and distribution of specific activity of the enzyme within the microcapsule.

In summary, we have shown that upon encapsulation, PAL looses 80% of its activity. We have identified steps where loss of activity occurs. The internal environment of the microcapsules does not contribute to the loss of activity. The cellulose nitrate membrane does not offer a diffusional barrier to the passage of substrate molecules. About 30% loss of activity occurs due to incomplete entrapment of PAL into the microcapsules. The remaining 50% loss of activity occurs due to denaturation of PAL during microcapsule manufacture. Future studies will try to elucidate the mechanisms involved in these losses and devise strategies to minimize them.

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