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Strategies to maximize the encapsulation efficiency of phenylalanine ammonia lyase in microcapsules

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Abstract

The activity of phenylalanine ammonia lyase (PAL) encapsulated in cellulose nitrate microcapsules is only 23% of the activity of PAL in Tris buffer. This lower activity is partially due to its incomplete encapsulation. The objective of the study was to maximize the encapsulation efficiency (EE) of PAL by optimizing different formulation parameters. PAL was purified using size exclusion chromatography and then radioiodinated using the Iodo-gen reaction. Use of ^{125}I-PAL showed that PAL had an EE of 45% in cellulose nitrate microcapsules. Formulation parameters including concentration of polymer in solution, stirring speed and ratio of aqueous phase volume to organic phase volume were individually optimized to maximize the EE of PAL. The reformulated microcapsules showed an EE of PAL of 80%. The dramatic increase in EE was reflected in a marked (119%) increase in the activity of encapsulated PAL compared to its activity in the original microcapsules. Eighty two percent of the encapsulated PAL was physically present in the aqueous core while 18% was entrapped in the microcapsule membrane. Distribution of PAL activity in the microcapsule was in concordance with its physical distribution.

Keywords: Microcapsules; Phenylalanine ammonia lyase; Phenylketonuria; Encapsulation efficiency; Catalytic activity; Protein formulation

1. Introduction

Phenylketonuria (PKU) is an inherent disorder of metabolism, which if left untreated could result in severe mental retardation. PKU is a result of a deficiency or defect in the phenyalanine hydroxylating (PAH) system that metabolizes phenylalanine to tyrosine. The resulting high concentrations of phenylalanine (Phe) in blood results in mental retardation, microcephaly, spasticity and tremors. A novel therapy for the treatment of PKU has been proposed by Bourget and Chang (1984) that involves encapsulation and subsequent oral administration of phenylalanine ammonia lyase (PAL). PAL is an enzyme that is obtained from plants and it catalyzes the conversion of Phe to a non-toxic product namely, transcinnamic acid (TCA). Phe is an essential amino acid derived from diet. The hope of this novel therapy is that in the gastrointestinal tract Phe will diffuse into the microcapsules and will be metabolized by PAL before it can be absorbed into the bloodstream. The consequent decrease in Phe absorption will be beneficial to PKU patients.

Our earlier studies have shown that the enzymatic activity of encapsulated PAL was 23% of the activity of PAL in solution (Habibi-Moini and D'mello, 2001). This decrease in activity of encapsulated PAL was determined to be due to incomplete encapsulation of enzyme and losses during manufacture of the microcapsules. The low activity of PAL in the microcapsules makes this therapy economically unfeasible. The overall objective of our research is to improve the activity of the encapsulated PAL. The specific aim of the current study is to increase the encapsulation efficiency (EE) of the PAL in the microcapsules by optimizing formulation variables.

PAL was microencapsulated by the interfacial coacervation method using cellulose nitrate as the microcapsule membrane. Briefly, microcapsules were made by dispersing an aqueous solution of PAL in 10% hemoglobin solution with water saturated ether with the help of stirring. Then, cellulose nitrate in a mixture of ether:ethanol (82.5:17.5, v/v) was added to the dispersion. Cellulose nitrate is very hydrophobic and it therefore precipitates around the aqueous phase droplets to form the microcapsules. The microcapsules were hardened by *n*-butyl

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benzoate, washed with Tween 20 and finally suspended in Tris buffer. In our previous studies ^{14}C hemoglobin was used to measure the EE of PAL (Habibi-Moini and D'mello, 2001). It was assumed that since hemoglobin and PAL form a homogenous solution in the aqueous phase, the EE of hemoglobin can serve as a surrogate marker of the EE of PAL. The EE of PAL could not be measured directly as radioactive PAL was not available. In the current studies, we purified commercially available PAL, radioiodinated the purified PAL and used it to directly determine the EE of PAL in the cellulose nitrate microcapsules. We then compared the EE of hemoglobin as measured by ^{14}C hemoglobin with the EE of PAL measured by ^{125}I PAL. These comparisons were used to test the validity of our previous assumptions. Finally, ^{125}I PAL was used to determine the effect of alteration of select formulation variables on the EE of PAL. The three formulation parameters selected were stirring speed, concentration of cellulose nitrate, and ratio of organic phase volume to aqueous phase volume. A review of the literature showed that these formulation variables can affect the EE of substrates in microcapsules.

2. Materials and methods

2.1. Materials

Phenlyalanine ammonia lyase, grade 1, from Rhodotorula glutinis, triglyceride reagent A, transcinnamic acid, bovine serum albumin, Tween 20, hemoglobin and ^{14}C methylated hemoglobin were obtained from Sigma Aldrich (St. Louis, MO). 12% Tris–HCl gel, Tris/glycine/SDS buffer, Lamelli buffer, Coomassie Blue R 250 dye powder and 2-mercaptoethanol were obtained from Bio-Rad (Hercules, CA), and Universol cocktail was obtained from MP-Biomedicals (Irvine, CA). Colloidion containing 5% (w/v) cellulose nitrate was obtained from Spectrum Chemicals (New Brunswick, NJ). Cellulose nitrate is soluble in ether, methanol, acetone, and glacial acetic acid and its oral LD 50 in rats and mice is greater than 5000 mg/kg.

2.2. Removal of glycerol from commercial PAL solution

PAL is commercially available in a 60% (v/v) glycerol solution. As previously described, glycerol was removed by dialyzing the PAL solution using Centricon concentrator tubes (Millipore Corporation, Billerica, MA) with a molecular weight cut off of 100 kDa (Habibi-Moini and D'mello, 2001). Glycerol-free PAL was diluted with pH 8.5 Tris buffer and aliquots stored at −80 °C. All subsequent experiments were performed with glycerol-free PAL.

2.3. Size exclusion chromatography (SEC) of PAL

Size exclusion chromatography was performed to determine the purity of PAL. Sixty microliters of a 0.5 mg/mL of glycerol-free PAL solution in Tris buffer pH 8.5 was injected onto a 7.8 mm × 300 mm TSK G3000 SWXL size exclusion column (TosoHaas, Montgomeryville, PA). Protein was eluted with a 50 mM, 0.15 M ionic strength, pH 7.2 phosphate buffer at flow rate of 0.5 mL/min and the eluent was monitored at a wavelength of 210 nm. Data were collected and analyzed using ChromPerfect Software 5.5 (Justice Innovations Inc., Denville, NJ).

2.4. Purification of PAL

PAL required for radioiodination was purified with SEC chromatography using conditions described in the previous section. Sixty microliters of a 0.5 mg/mL solution of glycerol-free PAL in Tris buffer pH 8.5 was loaded onto the column. The fraction corresponding to the largest peak was collected in a 1.5 mL centrifuge tube and denoted as Fraction 1. The fraction corresponding to the next two peaks was also collected and denoted as Fraction 2. The procedure was repeated twice to obtain adequate amounts of the purified PAL.

The volume of the fractions collected was large and they were subsequently concentrated in a 0.5 mL Centricon tube using a 10 kDa molecular weight cut off membrane (Millipore Corporation, Billerica, MA). Each fraction was loaded into a Centricon tube and centrifuged at 10 000 × g for 45 min at 4 °C until approximately 60 μL of the retentate remained. The protein concentration of the retentate was measured using the Bradford's method (Bradford, 1976).

2.5. Evaluation of PAL purity

Purity of PAL was evaluated by subjecting protein from concentrated fractions 1 and 2 in the SEC to SDS-PAGE analysis. SDS-PAGE was carried out with a Bio-Rad mini protean 3 gel assembly using 12% Tris–HCl gels from Bio-Rad laboratories (Hercules, CA). Aliquots of sample were mixed with Lamelli sample buffer containing 2-mercaptoethanol and were heated in a boiling water bath for 3 min. Samples containing 2–10 μg of protein were loaded onto the gel in a volume of 40 μL. The gel was run for 45 min at a constant 150 V at room temperature with Tris/glycine/SDS buffer. Gels were stained with Coomassie blue staining solution for 2 h. After destaining the gels overnight, images of the gels were obtained on a gel documentation system (UVP, Upland, CA). Integrated optical density measurements were used to compare the intensity of the protein bands.

2.6. Radioiodination of PAL

Radioiodination was conducted using the Iodo-gen method (Salacinski et al., 1979) and was performed according to the indirect protocol by Pierce Biotechnology, Inc. (Rockford, IL). Briefly, the precoated Iodo-gen (1,3,4,6-tetrachloro-3 alpha,6 alpha-diphenylglycouril) tube was wetted with 1 mL of 125 mM High Tris iodination buffer containing 0.15 M NaCl at pH 7.3. The buffer was decanted out and 2.33 mCi of Na ^{125}I was added into the tube. The Na ^{125}I was reacted with the Iodo-gen for 7 min to activate the iodide. Activated iodide solution was added into 3 μg of the purified PAL sample in the microcentrifuge tube. PAL was reacted with the iodide solution for 7 min at room temperature and mixed gently by flicking the tube every 30 s. Unreacted sodium iodide was removed using an ion exchange column.

2.7. Preparation of microcapsules

Microcapsules were made by the process of interfacial coacervation as described previously (Bourget and Chang, 1985; Habibi-Moini and D'mello, 2001). Briefly, 500 μL of aqueous phase containing glycerol-free PAL in 10% hemoglobin solution was stirred at speed of 250 rpm with 5 mL of water saturated ether. Next, 5 mL of 4% cellulose nitrate dissolved in a mixture of ether:ethanol (82.5:17.5, v/v) was added and stirred for 60 s. The microcapsules were formed due to precipitation of cellulose nitrate around the aqueous droplets. After 45 min the microcapsules settled down and the top organic phase was collected and denoted as organic washing. Next, 6 mL of *n*-butyl benzoate was added to harden the microcapsules. After 30 min the top organic solvent was collected and combined with the previous organic washing. The microcapsules were washed with Tween 20 solution. The wash solution was separated from the microcapsules by centrifugation at 150 × *g* for 2 min, collected and denoted as the aqueous washing. Finally, the washed microcapsules were suspended in 3 mL of pH 8.5 Tris buffer.

2.8. Measurement of EE of microcapsules with ^{14}C hemoglobin and ^{125}I PAL

An aliquot of glycerol-free PAL solution, 0.2 μCi of ^{14}C hemoglobin or 2–3 μCi of ^{125}I PAL and 14% (w/v) hemoglobin solution were mixed together such that the concentration of hemoglobin in the aqueous phase was 10% (w/v). 0.5 mL of this aqueous phase was used to prepare the microcapsules as described in Section 2.7. The organic and aqueous washings were collected, weighed, their density measured and used to compute their volume. The suspended microcapsules in pH 8.5 Tris buffer were weighed and homogenized with a hand held tissue grinder. The density of the homogenate was measured and used to compute the total volume of the homogenate. The radioactivity in the aqueous phase (prior to encapsulation) and in the microcapsule homogenate were measured after treatment of the samples with 30% (v/v) hydrogen peroxide to bleach the color of hemoglobin. The chemiluminescence in the samples was eliminated by leaving them overnight in the dark prior to counting. The EE of hemoglobin or PAL in the microcapsules was computed as shown in Eq. (1).

$$\%\ \text{EE of hemoglobin or PAL} = \frac{\text{DPM in the total volume of microcapsules homogenate}}{\text{DPM in the 0.5 mL of aqueous phase prior to encapsulation}} \quad (1)$$

Similarly, the radioactivity in the aqueous and organic washings were also measured and used to compute the mass balance of ^{14}C hemoglobin and ^{125}I PAL.

2.9. Optimization of various formulation variables to improve the encapsulation efficiency of PAL

We determined the effects of varying the levels of three individual formulation parameters on the EE of PAL. The three parameters were stirring speed, concentration of the polymer solution and ratio of the organic phase volume to aqueous phase volume. Microcapsules were prepared by the general procedure as described in Section 2.7. Table 1 shows the formulation parameter values for each set of experiments. The microcapsules prepared according to the method described previously are referred to as the original microcapsules. The EE of PAL in each microcapsule formulation was measured with ^{125}I PAL as described in Section 2.8.

2.10. Distribution of PAL within the cellulose nitrate membrane microcapsules

Distribution of PAL within the microcapsules was studied using ^{125}I PAL. After measuring the EE of PAL in the microcapsule homogenate, 0.1 mL aliquots of the remaining homogenate was taken in four different 1.5 mL polypropylene centrifuge tubes. The samples were centrifuged at 7900 × *g* for 10 min and the supernatant was collected. The sediment was washed with 50 μL of Tris buffer and the washing pooled with the supernatant from the previous centrifugation. The radioactivity in the supernatant and the sediment was measured after treatment of the samples with 30% (v/v) hydrogen peroxide to bleach the color of the hemoglobin. The chemiluminescence in the samples was eliminated by leaving them overnight in the dark prior to counting. The amount of the PAL in the supernatant and sed-

Table 1
Conditions for evaluating effects of various formulation variables on EE of PAL in the microcapsules

Formulation	Concentration of cellulose nitrate in ether:ethanol solution (%)	Volume of aqueous phase (mL)	Volume of water saturated ether (mL)	Stirring speed (rpm)	Volume of cellulose nitrate in ether:ethanol solution (mL)	Phase volume ratio[a]
Original	4.0	0.5	5	250	5	20:1
F1	4.0	0.5	5	155	5	20:1
F2	4.5	0.5	5	250	5	20:1
F3	5.0	0.5	5	250	5	20:1
F4	4	0.5	6.5	250	6.5	26:1
F5	4	0.5	8	250	8	32:1
F6	4	0.5	11	250	11	44:1
F7	4	0.5	13	250	13	52:1

[a] Phase volume ratio is the ratio of organic phase volume: aqueous phase volume. Organic phase volume is the sum of volume of water saturated ether and volume of cellulose nitrate in ether:ethanol solution.

iment was reported as percent of total as shown in Eqs. (2) and (3).

$$\%\ \text{PAL in the supernatent} = \frac{\text{DPM in the supernatent}}{\text{DPM in the supernatant} + \text{DPM in the sediment}} \quad (2)$$

$$\%\ \text{PAL in the sediment} = \frac{\text{DPM in the sediment}}{\text{DPM in the supernatant} + \text{DPM in the sediment}} \quad (3)$$

2.11. Measurement of PAL activity within the cellulose nitrate membrane microcapsules

Microcapsules were prepared using a 25 μL aliquot of glycerol-free PAL solution as described earlier. Microcapsules were suspended in Tris buffer and the volume made up to 3mL. 200 μL of the microcapsule suspension was added into 1.8 mL of 4.44 mM Phe solution. The final concentration of Phe in 2 mL of the reaction mixture was 4 mM. The reaction mixture was incubated for 7 min at 37 °C. The reaction was stopped by the addition of trichloroacetic acid solution (10%, v/v). The product formed during the enzymatic reaction, namely transcinnamic acid, was measured by high pressure liquid chromatography as described previously (Habibi-Moini and D'mello, 2001). The maximal activity which is the rate of product formation in the linear phase was computed as μM/min and expressed as a percent of its control.

The control consisted of activity in an equivalent amount of glycerol-free PAL solution in pH 8.5 Tris buffer.

2.12. Determination of distribution of PAL activity within the microcapsules

Two milliliters of the microcapsule suspension was homogenized in a hand held tissue grinder. A three hundred microliter aliquot of the homogenate was taken in a 1.5 mL polypropylene centrifuge tube. The samples were centrifuged at 7900 × *g* for 10 min and the supernatant collected. The sediment was washed with 50 μL of Tris buffer and the washing pooled with the previously collected supernatant. The volume of the supernatant was made up to 500 μL and the volume of the sediment was made to 250 μL with pH 8.5 Tris buffer. Two hundred microliters of the homogenized microcapsules, 200 μL of the sediment suspension and 200 μL of the supernatant solution were separately added to 1.8 mL aliquots of 4.44 mM Phe solution. The final concentration of the Phe in 2 mL of the reaction mixture was 4 mM. The reaction mixture was incubated for 7 min at 37 °C. The reaction was stopped by the addition of trichloroacetic acid solution (10%, v/v). The product formed during the enzymatic reaction, namely transcinnamic acid, was measured by high pressure liquid chromatography as described previously (Habibi-Moini and D'mello, 2001).

The maximal activity in the microsomal homogenate which is the rate of product formation in the linear phase was computed as μM/min and expressed as a percent of its control. The control consisted of activity in an equivalent amount of glycerol-free PAL solution in pH 8.5 Tris buffer.

The activity of the PAL in the supernatant and sediment was reported as percent of total activity as shown in Eqs. (4) and (5).

$$\%\ \text{activity of PAL in the supernatant} = \frac{\text{PAL activity in the supernatant}}{\text{PAL activity in the supernatant} + \text{PAL activity in the sediment}} \quad (4)$$

$$\%\ \text{activity of PAL in the sediment} = \frac{\text{PAL activity in the sediment}}{\text{PAL activity in the supernatant} + \text{PAL activity in the sediment}} \quad (5)$$

2.13. Statistics

Results are presented as mean ± S.D. Wherever appropriate differences between groups were evaluated with a Student *t*-test (two groups) or a one-way ANOVA (more than two groups) at an alpha level of 0.05.

3. Results

3.1. SEC and SDS-PAGE of glycerol-free PAL solution

As shown in Fig. 1, glycerol-free PAL solution in Tris buffer showed three major peaks in SEC. We suspected that the peak at 16.7 min represented PAL while the remaining peaks in the chromatogram were the impurities. This result suggested the presence of impurities in the PAL sample.

PAL is a 320 kDa homotetramer containing four 80 kDa monomers. As shown in Fig. 2, SDS-PAGE of glycerol-free PAL solution (lane 3) resulted in a major band at the 80 kDa region representing the monomer and number of other bands below 80 kDa. These results confirmed the presence of impurities in this PAL sample.

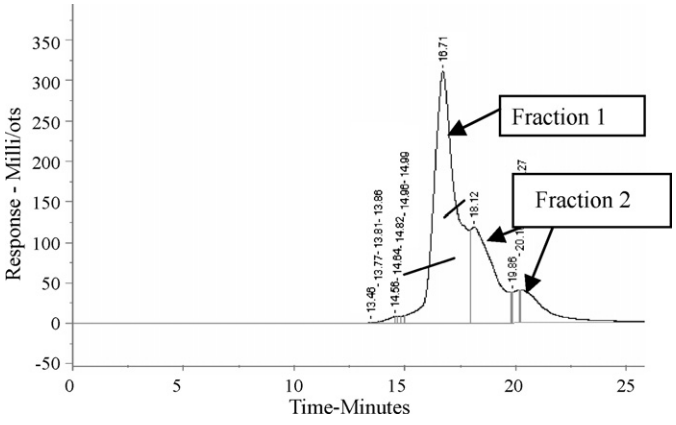

Fig. 1. Size exclusion chromatography of glycerol-free PAL solution. The peak at 16.7 min was collected as Fraction 1. Peaks at 18.12 and 20.2 min were collected as Fraction 2. The black dashes on the peak with a retention time of 16.7 min represent the region of collection for Fraction 1.

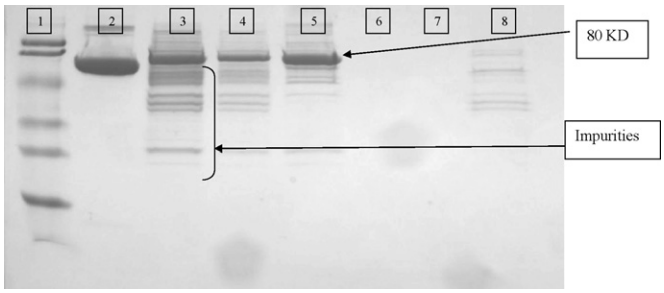

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of PAL. Lane 1 contains the SDS-PAGE markers, lane 2 contains 10 μg of albumin (66,000 Da), lane 3 contains 10 μg of protein from a glycerol-free PAL solution, lane 4 contains 5 μg of protein from a glycerol-free PAL solution, lane 5 contains 5 μg of protein from concentrated Fraction1, lanes 6 and 7 are empty, lane 8 contains 2 μg of protein from concentrated Fraction 2. The SDS-PAGE markers include from top to bottom phosphorylase B (111,000 Da), bovine serum albumin (93,000 Da), ovalbumin (53,500 Da), carbonic anhydrase (36,100 Da), soybean trypsin inhibitor (29,500 Da), and lysozyme (21,300 Da).

3.2. Purification of PAL and evaluation of PAL purity

As described in Section 2 glycerol-free PAL solution was purified by collecting two fractions corresponding to peaks in the SEC chromatograms. The fractions were concentrated and PAL purity was evaluated by subjecting the retentates of the two fractions to SDS-PAGE. As shown in Fig. 2, lane 3 which contained 10 μg of protein loaded from the commercial glycerol-free PAL solution exhibited an intensity of the 80 kDa band of 612 integrated optical density (IOD) units. Lane 5 which contained 5 μg of protein loaded from the retentate of concentrated Fraction 1 exhibited an intensity of the 80 kDa band of 689 IOD units. This result showed that there was approximately a 2-fold refinement of PAL in the retentate of Fraction 1 compared to the commercial PAL solution. Lane 8 which contained 2 μg of protein loaded from the retentate of Fraction 2 did not show any band at the 80 kDa position but showed a large number of bands in the region representing the impurities. This result suggested that the retentate of Fraction 2 represented the impurities in the PAL solution.

3.3. Radioiodination of PAL

The total yield of radioiodinated PAL in aqueous phase was 13.6% of the added sodium iodide. About 317 μCi of ^{125}I PAL was obtained and was stored at −80 °C.

3.4. Comparison of the EE of hemoglobin using ^{14}C hemoglobin and the EE of PAL using ^{125}I PAL

We measured the EE of hemoglobin using ^{14}C hemoglobin and compared it to the EE of PAL measured with ^{125}I PAL. Table 2 shows mass balance of ^{125}I PAL and ^{14}C hemoglobin during the manufacture of the microcapsules. A major portion of both ^{125}I PAL and ^{14}C hemoglobin (≈50%) was lost in the aqueous washings. A much smaller amount of both isotopes (≈3–5%) was lost in the organic washing. The EE of PAL in the microcapsule was 45.1%, which was not significantly different from the 42.8% EE of hemoglobin in the microcapsules. This result validated the assumption in the previous study that the EE of hemoglobin in the microcapsules is a good surrogate marker of the EE of PAL (Habibi-Moini and D'mello, 2001).

Table 2
Mass balance of ^{14}C hemoglobin and ^{125}I PAL during preparation of the original cellulose nitrate microcapsules

Fraction	% of ^{14}C hemoglobin[a]	% of ^{125}I PAL
Organic washing	5.6 ± 0.9[b]	3.3 ± 1.5
Aqueous washing	50.1 ± 3.8	49.0 ± 5.7
Inside microcapsules	42.8 ± 1.5	45.1 ± 2.9[N.S.]
Total	98.5 ± 2.3	97.4 ± 3.8

N.S.: not significantly different from the ^{14}C hemoglobin sample ($p > 0.05$, t-test).
[a] % radioactivity obtained in various fractions compared to the initial amount of radioactivity added.
[b] Mean ± S.D. ($n = 5$).

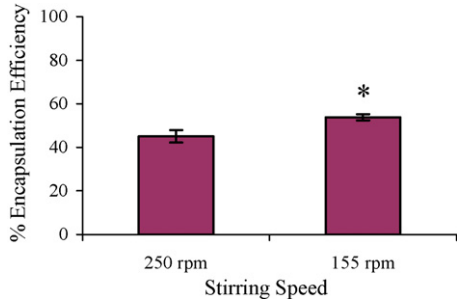

Fig. 3. Effect of decrease in the stirring speed on the EE of PAL as determined by ^{125}I PAL. Sample size = 3–5. *Significantly different from the sample stirred at 250 rpm ($p < 0.05$, t-test).

3.5. Effect of alteration of select formulation variables on the encapsulation efficiency of PAL measured using ^{125}I PAL

As shown in Fig. 3, reducing the speed of stirring from 250 to 155 rpm increased the EE of PAL from 45.1 ± 2.9% (mean ± S.D.) to 53.8 ± 1.4%. As shown in Fig. 4, increasing

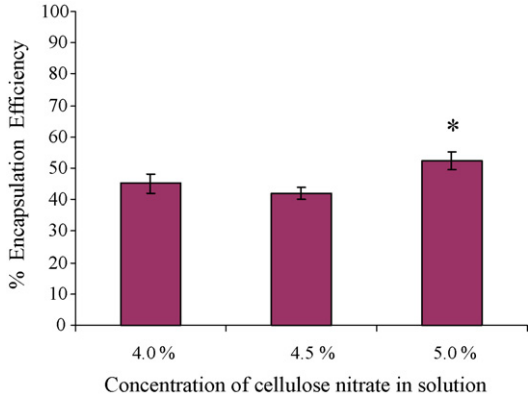

Fig. 4. Effect of increasing concentration of cellulose nitrate on EE of PAL as determined by ^{125}I PAL. Sample size = 3–5. *Significantly different from the 4% and 4.5% sample ($p < 0.05$, one-way ANOVA).

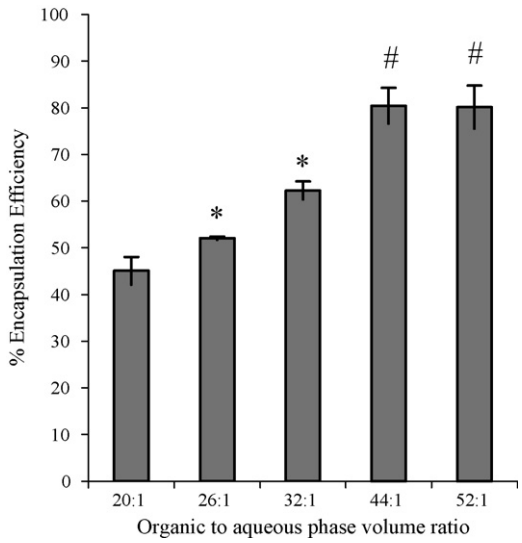

Fig. 5. Effect of increase in the organic to aqueous phase volume ratio on EE of PAL as determined by ^{125}I PAL. Sample size = 2–5. *Significantly different from all other samples ($p < 0.05$, one-way ANOVA). #Significantly different from the 20:1, 26:1 and 32:1 samples ($p < 0.05$, one-way ANOVA).

the concentration of cellulose nitrate from 4% to 4.5% did not affect the EE of PAL. However, further increase in the cellulose nitrate concentration to 5% significantly increased the EE of PAL from 45.1 ± 2.9% to 52.4 ± 2.8%. The ratio of organic phase volume to aqueous phase volume in the original procedure was 20:1. This ratio was increased by keeping the volume of the aqueous phase constant while increasing the volumes of the water saturated ether and polymer solution equally (see Table 1). As shown in Fig. 5, increasing the organic to aqueous phase volume ratio increased the EE of PAL. Increasing the phase volume ratio from 20:1 to 44:1 increased the EE of PAL from 45.1 ± 2.9% to 80.4 ± 3.8%. Further increase in the phase volume ratio did not result in any additional increase in the EE of PAL.

The microcapsules manufactured using a stirring speed of 250 rpm, cellulose nitrate concentration of 4% and organic to aqueous phase volume ratio of 44:1 and with an EE of 80% were designated as the reformulated microcapsules.

3.6. Distribution of ^{125}I PAL within the original and reformulated microcapsules

A sample of the homogenized microcapsules was centrifuged and its supernatant and sediment were collected. The radioactivity in the supernatant represented the percent of PAL in the aqueous core of the microcapsules and the radioactivity in the sediment represented the percent of PAL bound to the membrane of the microcapsules. In the original microcapsules, 82.1 ± 1.5% (mean ± S.D., $n = 5$) of PAL was in the aqueous core of the microcapsules while 17.9 ± 1.5% was bound to the membrane of the microcapsules. In the reformulated microcapsules, 81.5 ± 2.8% ($n = 3$) of PAL was in the aqueous core of the microcapsules while 18.5 ± 2.8% was bound to the membrane of the microcapsules.

3.7. Activity of PAL in the original and reformulated microcapsules

The activity of encapsulated PAL in the original microcapsules was 32.9 ± 0.6% (mean ± S.D., $n = 4$) of the activity of PAL in a solution of pH 8.5 Tris buffer. Assuming an encapsulation efficiency of 45% in these microcapsules, it suggests that approximately 73% of the encapsulated enzyme was active. The activity of the encapsulated PAL in the reformulated microcapsules was 72.2 ± 0.5% ($n = 3$) of the activity of PAL in a solution of Tris buffer. Assuming an encapsulation efficiency of 80% in these microcapsules, it suggests that approximately 90% of the encapsulated enzyme was active.

3.8. Distribution of PAL activity in the reformulated microcapsules

An aliquot of the homogenized reformulated microcapsules was centrifuged and the supernatant and sediment were collected and analyzed for the activity of PAL. The activity in the supernatant represented the activity of the PAL in the aqueous core of the microcapsules while the activity in the sediment represented the activity of PAL bound to microcapsule membrane. The activity of PAL in the total microcapsule homogenate was 86.0 ± 7.8% of the activity of unencapsulated PAL in solution in pH 8.5 Tris buffer. The activity of PAL free in the aqueous core of the microcapsules was 85.7 ± 3.3% of the total activity in the homogenate of the microcapsules while the activity of PAL bound to the membrane of the microcapsules was 14.3 ± 3.3% of the total activity in the homogenate of the microcapsules.

4. Discussion

In the present studies we purified glycerol-free PAL solution, radioiodinated the purified PAL and used the ^{125}I PAL to measure the EE of PAL within the microcapsules. We then compared the EE of hemoglobin to the EE of PAL in the microcapsules and demonstrated them to be similar. Finally, select formulation variables were optimized to increase the EE of PAL. Compared to the original microcapsules, the reformulated microcapsules exhibited markedly increased EE of PAL, and showed a correspondingly higher activity of encapsulated PAL.

A direct method to measure the EE of PAL is to use radioactive PAL. However, this is not commercially available. Using SEC and SDS-PAGE we confirmed that the PAL purchased from Sigma is impure. Radioiodinating it would simultaneously radioiodinate the impurities which would result in erroneous estimates of the EE of PAL. Therefore, we purified the commercial PAL sample before using it to measure the EE of PAL. PAL was purified by separating PAL from its impurities on a size exclusion column, and collecting and concentrating the peak corresponding to pure PAL. Purity of PAL was then verified using SDS-PAGE. The results showed that there was a 2-fold refinement of the commercially purchased PAL sample. The purified PAL was subject to radioiodination.

Previous studies in our laboratory showed that PAL encapsulated in cellulose nitrate microcapsules lost 77% of its

activity primarily due to losses in the manufacturing procedure and due to incomplete encapsulation of PAL in the microcapsules (Habibi-Moini and D'mello, 2001). In those studies it was assumed that during microcapsule manufacture since hemoglobin and PAL form a homogenous aqueous phase, the EE of hemoglobin represented the EE of PAL. Therefore, the EE of ^{14}C hemoglobin was used as a surrogate marker of the EE of PAL (Habibi-Moini and D'mello, 2001). The EE of proteins in microcapsules is partially dependent on the specific properties of the proteins (Blanco and Alonso, 1998). Hemoglobin and PAL have different charge, molecular weight, surface activity and could interact differently with cellulose nitrate which could cause them to be encapsulated with different efficiency. It is therefore conceivable that ^{14}C hemoglobin may not be good marker for the EE of PAL in the microcapsules, and our previous assumption may not be entirely valid. In the present studies we had relatively pure ^{125}I PAL and were able to test our previous assumption by measuring and comparing the EE of both co-encapsulated proteins. Our results show that the EE of hemoglobin and PAL are similar which confirms that the EE of hemoglobin is a good marker of the EE of PAL. To the best of our knowledge, these results are the first experimental evidence to demonstrate that when an aqueous solution of two proteins is encapsulated the EE of one protein is a good surrogate marker of the EE of the other co-encapsulated protein. These results also demonstrated that the EE of PAL was 45% and incomplete encapsulation was a major reason for the poor activity of encapsulated PAL.

Next, various formulation variables were optimized to maximize the EE of PAL in the microcapsules. In these studies the EE of PAL was measured using ^{125}I PAL. A decrease in the stirring speed from 250 to 155 rpm during emulsification increased the EE of PAL. Decrease in stirring speed increases the size of the aqueous phase droplets in the w/o emulsion and consequently reduces the surface area of the dispersed aqueous phase. Therefore, there is a larger amount of polymer per unit surface area of the aqueous droplets which probably accounts for the increase in the EE of PAL. The literature confirms that the stirring speed used in the manufacture of the primary emulsion can affect the EE of substrates in microcapsules (Chen and Lu, 1999; Park et al., 2006). Increasing the concentration of cellulose nitrate increased the EE of PAL in the microcapsules probably due to an increase in the amount of polymer per unit surface area of the aqueous droplets. Similar results have been observed in the manufacture of PLGA microsphere (Park et al., 2006; Torres et al., 1996). In our studies increasing the concentration of the cellulose nitrate, increased the viscosity of the polymer solution. We were unable to increase the concentration of the cellulose nitrate solution to greater than 5% as the consistency of the polymer solution became gel like and was unsuitable for use in the preparation of microcapsules.

Increasing the organic to aqueous phase volume ratio from 20:1 to 44:1 dramatically increased the EE of PAL in the microcapsules from 45% to 80%. The phase volume ratio was increased by keeping the volume of the aqueous phase constant and increasing the volume of the water saturated ether and ether:ethanolic solution of cellulose nitrate. Increasing the volume of the organic phase increased the amount of polymer in the formulation which probably resulted in encapsulation of the enzyme with greater efficiency. Also, the larger amount of polymer probably resulted in the precipitation of a greater amount of polymer around the aqueous droplets and formation of a thicker coat. During our studies we did note that microcapsules with high EE were difficult to break which suggested a thicker or harder polymer coat. In confirmation of our findings, Herrmann and Bodmeier (1995) showed a decrease in encapsulation of pseudoephedrine HCl content with decrease in organic to aqueous phase volume ratio during preparation of poly(methacrylate) microsphere. Freytag et al. (2000) also observed a decrease in EE of an oligonucleotide in poly(lactide) microparticle with a decrease in the ratio of organic phase volume to aqueous phase volume.

Next, we reformulated the microcapsules using a stirring speed of 250 rpm, a 4% cellulose nitrate solution and an organic to aqueous phase volume ratio 44:1. There was a 119% increase in the maximal activity of PAL in the reformulated microcapsules compared to the activity in the original microcapsules. In both original and reformulated microcapsules when the activity of PAL was expressed as a percent of the encapsulated enzyme, it became evident that the increase in activity in the reformulated microcapsules was primarily due to their markedly higher encapsulation efficiency. These results suggest that poor encapsulation was the primary reason for the low activity of encapsulated PAL. The data also show that 73% and 90% of the encapsulated enzyme was active in the original and reformulated microcapsules, respectively. This result suggests that PAL is minimally deactivated during the manufacturing process and that reformulation was able to further reduce the losses of PAL activity during the manufacturing procedure.

Using ^{125}I PAL as a marker, we determined the physical distribution of PAL within the microcapsules. About, 80% of the encapsulated PAL was located in the aqueous core of the microcapsules and only 20% of it was bound to the cellulose nitrate membrane. Previous studies have determined the distribution of the co-encapsulated hemoglobin within cellulose nitrate microcapsules. Those results demonstrate that, in contrast to PAL, 82% of the co-encapsulated hemoglobin was bound to cellulose nitrate membrane and only 10% was located in the aqueous core of the microcapsules (Habibi-Moini and D'mello, 2001). These data suggest that though the EE of PAL and hemoglobin are similar, they are differentially distributed within the cellulose nitrate microcapsules. In our studies a small amount of PAL (about 0.13% of the total protein in aqueous phase) is co-encapsulated with an enormous amount of hemoglobin. It is possible that due to this high concentration of hemoglobin, a major portion of hemoglobin lies at the interface and gets entrapped into the cellulose nitrate membrane during the formation of the microcapsules. Hemoglobin at the interface probably minimizes PAL presence at the interface and confines most of it in the core of the aqueous phase. The isoelectric point of hemoglobin at 7.2 while that of PAL is 5.2 and therefore both proteins are negatively charged in the pH 8.5 Tris buffer aqueous phase (Habibi-Moini, 2000). The cellulose nitrate membrane possesses carboxyl groups which gives it a negative charge. It is also possible that while both proteins are repelled by the

membrane, a major portion of hemoglobin gets entrapped in the membrane owing to its much higher concentration.

Finally, we determined the activity of encapsulated PAL within the reformulated microcapsules. Distribution of PAL activity was similar to the physical distribution of PAL in the microcapsules. The major portion of PAL activity was present in the aqueous core while a small fraction of PAL activity was present in the membrane. The presence of catalytic activity in PAL trapped in the membrane was a surprising finding. It is unlikely that this activity was due to loosely adsorbed PAL from the aqueous core since the membranes were thoroughly washed prior to determination of catalytic activity.

Our data suggest that PAL encapsulated in the reformulated microcapsules can be an economically feasible therapy for PKU. Our future work will determine the stability of the reformulated microcapsules in simulated gastrointestinal fluids and their efficacy will be tested in a mouse model of PKU.

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References

Blanco, D., Alonso, M.J., 1998. Protein encapsulation and release from poly(lactide-co-glycolide) microspheres: effect of the protein and polymer properties and of the co-encapsulation of surfactants. Eur. J. Pharm. Biopharm. 45, 285–294.

Bourget, L., Chang, T.M., 1984. Artificial cell-microencapsulated phenylalanine ammonia-lyase. Appl. Biochem. Biotechnol. 10, 57–59.

Bourget, L., Chang, T.M., 1985. Phenylalanine ammonia-lyase immobilized in semipermeable microcapsules for enzyme replacement in phenylketonuria. FEBS Lett. 180, 5–8.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Chen, W., Lu, D.R., 1999. Carboplatin-loaded PLGA microspheres for intracerebral injection: formulation and characterization. J. Microencapsul. 16, 551–563.

Freytag, T., Dashevsky, A., Tillman, L., Hardee, G.E., Bodmeier, R., 2000. Improvement of the encapsulation efficiency of oligonucleotide-containing biodegradable microspheres. J. Control. Release 69, 197–207.

Habibi-Moini, S., 2000. Evaluation of phenylalanine ammonia lyase activity within cellulose nitrate membrane microcapsules. Ph.D. University of the Sciences in Philadelphia, Philadelphia.

Habibi-Moini, S., D'mello, A.P., 2001. Evaluation of possible reasons for the low phenylalanine ammonia lyase activity in cellulose nitrate membrane microcapsules. Int. J. Pharm. 215, 185–196.

Herrmann, J., Bodmeier, R., 1995. Somatostatin containing biodegradable microspheres prepared by a modified solvent evaporation method based on W/O/W-multiple emulsions. Int. J. Pharm. 126, 129–138.

Park, J.H., Ye, M., Yeo, Y., Lee, W.K., Paul, C., Park, K., 2006. Reservoir-type microcapsules prepared by the solvent exchange method: effect of formulation parameters on microencapsulation of lysozyme. Mol. Pharm. 3, 135–143.

Salacinski, P., Hope, J., McLean, C., Clement-Jones, V., Sykes, J., Price, J., Lowry, P.J., 1979. A new simple method which allows theoretical incorporation of radio-iodine into proteins and peptides without damage [proceedings]. J. Endocrinol. 81, 131P.

Torres, A.I., Boisdron-Celle, M., Benoit, J.P., 1996. Formulation of BCNU-loaded microspheres: influence of drug stability and solubility on the design of the microencapsulation procedure. J. Microencapsul. 13, 41–51.