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Stabilization of phenylalanine ammonia lyase against organic solvent mediated deactivation

R.M. Shah, Anil P. D'mello *

University of the Sciences in Philadelphia, Department of Pharmaceutical Sciences, 600 South 43rd Street, Philadelphia, PA 19104, United States

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Abstract

A potential novel therapy for phenylketonuria involves oral administration of microencapsulated phenylalanine ammonia lyase (PAL), an enzyme that converts phenylalanine to transcinnamic acid. A drawback of this potential therapy is that encapsulated PAL retains only 23% of its activity. Microcapsules are prepared by emulsifying PAL in 10% hemoglobin solution with water-saturated ether (WSE) and subsequent addition of cellulose nitrate dissolved in ether:ethanol (E:E) mixture. The objective of this paper was to determine the contribution of emulsification to the overall loss in activity of encapsulated PAL, and to devise strategies to protect PAL against such loss in activity. Emulsification was simulated by stirring the aqueous phase containing PAL with the organic phase. The mixture was then centrifuged, and the protein content and catalytic activity of PAL in the aqueous phase was measured. Emulsification of PAL solution with WSE caused no loss in activity but resulted in a loss in protein content of aqueous phase. Size exclusion chromatography and gel electrophoresis studies showed that the loss was primarily due to the specific loss of impurities in the PAL sample. Emulsification of PAL solution with E:E resulted in a 50% decrease in its activity. Among the additives, hydroxypropyl-γ-cyclodextrin and hydroxy propyl-β-cyclodextrin protected PAL against emulsion mediated loss in activity.

Keywords: Phenylalanine ammonia lyase; Emulsion; Organic solvents; Cyclodextrins; Catalytic activity; Microcapsule

1. Introduction

Phenylketonuria (PKU) is caused by genetic defect in the phenylalanine hydroxylating system; a system that normally converts phenylalanine (Phe) to tyrosine. The resultant build up of Phe in blood leads to serious neuropsychological outcomes in patients. Phe is an essential amino acid obtained from diet. The only therapy currently available for PKU patients is a diet that is low in phenylalanine. The diet is low in natural high quality protein and is supplemented by a semi synthetic formula consisting of essential nutrients. The major drawbacks of diet therapy are its high cost and poor patient compliance due to the diet's poor palatability. The diet regimen is easier to maintain in very young patients but gets increasingly difficult to adhere to as the patient gets older. Previous guidelines required the diet to be maintained until the patients were 8–12 years old. However, increasing cases of mental degeneration with older patients who have been weaned off the diet led to the institution of new guidelines which require the diet to be continued for life. In pregnant PKU patients the diet needs to be followed rigorously as poor dietary compliance results in microcephaly, intrauterine growth retardation, congenital heart disease and mental retardation in the fetus (Hanley, 2004).

Due to difficulties associated with diet therapy, there is a need for newer therapies for PKU patients. Recently gene therapy has been attempted using viral as well as non-viral vectors. So far this therapy has been unsuccessful either due to an inability to deliver the gene or due to the inability to express it for a sustained period (Ding et al., 2004; Kim et al., 2004). Another therapeutic approach in PKU involves parental administration of phenylalanine hydroxylase (PAH), the physiological enzyme involved in the metabolism of Phe in the body. However injection of recombinant PAH results in toxic shock. Attempts have been made to pegylate PAH and increase the residence time of injected PAH in circulation and reduce its toxic effect (Gamez et al., 2004). The therapy, while promising, has not been tested in vivo.

* Corresponding author. Tel.: +1 215 596 8941; fax: +1 215 895 1161.
E-mail address: a.dmello@usip.edu (A.P. D'mello).

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A novel therapy has been proposed for PKU which involves the oral administration of Phenylalanine ammonia lyase (PAL). PAL is an enzyme that is present in plants, does not require any cofactors for its activity, and catalyzes the conversion of Phe to a non-toxic product namely transcinnamic acid (TCA). TCA is further metabolized to hippuric acid which is excreted in the urine. Since Phe is an essential amino acid derived from food, the hope of this novel therapy is that orally administered PAL will metabolize Phe in the gastrointestinal tract before it is absorbed into the bloodstream. The consequent decrease in Phe absorption will be beneficial to PKU patients. Preliminary studies in PKU patients have shown that orally administered PAL is effective in reducing blood levels of Phe (Hoskins et al., 1980). In these studies, PAL was administered in large doses in enteric coated, hard gelatin capsules. Oral PAL therapy was able to reduce the Phe levels in blood by about 25% although there was large intersubject variability. A large dose of PAL was administered to probably account for the expected proteolytic degradation of the enzyme in the stomach. Unfortunately the high doses of PAL required and the associated costs have made the therapy economically unfeasible.

An interesting extension of oral PAL therapy has now been proposed and it attempts to protect PAL from proteolytic damage by encapsulating it in cellulose nitrate polymer microcapsules (Bourget and Chang, 1985). These microcapsules can be given orally and the cellulose nitrate membrane which is not permeable to large molecular weight proteases should protect the enzyme from degradation in the gastrointestinal tract. However, a review of the literature and studies in our laboratory has shown that encapsulated PAL retains only about 23% of its original activity (Bourget and Chang, 1984; Habibi-Moini and D'mello, 2001). Studies designed to identify the exact steps in the encapsulation process where loss of activity occurs have revealed that 30% of the activity is lost due to incomplete encapsulation of PAL into the microcapsules, while the manufacturing process accounts for the remaining 47% loss in activity (Habibi-Moini and D'mello, 2001). The overall objective of our research is to improve the activity of encapsulated PAL.

The commonly used procedure to encapsulate proteins involves initial emulsification of an aqueous solution of the protein with organic solvents. A review of the literature suggests that emulsification of proteins with organic solvents in the manufacture of microcapsules is the major reason for loss in protein activity (Morlock et al., 1996; Sah, 1999a). We make microcapsules by emulsifying an aqueous solution of PAL in 10% hemoglobin solution with water-saturated ether. Cellulose nitrate dissolved in a mixture of ether:ethanol is then added to the primary emulsion and the microcapsules are formed by coacervation. Our first objective is to determine the contribution of the emulsification steps to the loss in activity of encapsulated PAL. The emulsification step is simulated by adding the organic solvents to the aqueous phase in the absence of the polymer. Then the emulsion is broken by centrifugation and the aqueous phase is evaluated for loss in protein content and activity of PAL. In the literature various additives have been evaluated for their ability to stabilize proteins during emulsification with organic solvents (Meinel et al., 2001; Morlock et al., 1996; Sah, 1999b). Therefore, the second objective of the paper is to evaluate select additives for their ability to stabilize PAL against emulsification mediated loss in activity.

2. Materials and methods

2.1. Materials

Collodion, hemoglobin, phenylalanine ammonia lyase (from Rhodotorula glutinis, grade I), Tween 20®, L-phenylalanine, bovine serum albumin (BSA), hydroxypropyl-γ-cyclodextrin (average degree of substitution of 0.52 hydroxypropyl groups per glucose unit), hydroxypropyl-β-cyclodextrin (average degree of substitution of 0.67 hydroxypropyl groups per glucose unit), 2-mercaptoethanol and ^{14}C methylated hemoglobin, were obtained from Sigma–Aldrich (St. Louis, MO). Lamelli buffer, SDS low molecular weight markers, 12.5% Tris–HCl ready gels and Bio-Rad protein reagent were obtained from Bio-Rad Laboratories (Hercules, CA).

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. Preparation of the microcapsules

Microcapsules were made by the process of interfacial coacervation as described previously (Habibi-Moini and D'mello, 2001) Briefly PAL in 10% hemoglobin was stirred with water-saturated ether for 60 s to form a w/o emulsion. Next, cellulose nitrate dissolved in a mixture of ether:ethanol [82.5:17.5, v/v] was added to the emulsion and the mixture was allowed to stand for 45 min. Microcapsules are formed due to precipitation of cellulose nitrate around the aqueous droplets. The coat was hardened by soaking them in *n*-butyl benzoate. Finally, the solvent was removed and microcapsules were suspended in Tris buffer (pH 8.5). The entire procedure was conducted at 4 °C.

2.4. Emulsification experiments

2.4.1. Emulsification of the aqueous phase with water-saturated ether

The experiment simulated the first step in the manufacture of microcapsules, and enabled us to determine possible losses of PAL activity in this step. Five hundred micro liters of aqueous phase which consisted of either PAL in 10% hemoglobin or PAL in Tris buffer (pH 8.5) was stirred with 5 mL of water-saturated ether for 2 h at 4 °C in a glass beaker. The mixture was then transferred to a glass centrifuge tube and centrifuged at 150 × *g* for 2 min. The top organic phase was aspirated off, and

the bottom aqueous phase was collected and its volume measured. Unemulsified aqueous phase consisted of PAL either in 10% hemoglobin or in Tris buffer (pH 8.5), which was not subject to emulsification. Aliquots of emulsified and unemulsified aqueous phase were used to determine the maximal activity of PAL and protein content.

2.4.2. Emulsification of the aqueous phase with water-saturated ether plus ether:ethanol [82.5:17.5, v/v] mixture

The experiment simulated the first two steps in the manufacture of microcapsules and enabled us to estimate possible losses in PAL activity in these steps. Five hundred micro liters of aqueous phase which consisted of either PAL in 10% hemoglobin or PAL in Tris buffer (pH 8.5) was stirred with 5 mL of water-saturated ether in a glass beaker. After 60 s, 5 mL of mixture of ether:ethanol [82.5:17.5, v/v] was added and the mixture was stirred for 2 h at 4 °C. The mixture was then transferred to a glass centrifuge tube and centrifuged at 150 × *g* for 2 min. The top organic phase was aspirated off, while the bottom aqueous phase was collected and its volume measured. Unemulsified aqueous phase consisted of PAL either in 10% hemoglobin or in Tris buffer (pH 8.5) which was not subject to emulsification. Aliquots of emulsified and unemulsified aqueous phase were used to determine the maximal activity of PAL and protein content.

2.4.3. Evaluation of the ability of different excipients to protect PAL during emulsification

Different excipients were added to the aqueous phase in order to evaluate their ability to protect the activity of PAL upon emulsification with ether:ethanol. Individual excipients were added to a solution of PAL in Tris buffer (pH 8.5) to obtain the following final concentration of excipients in the aqueous phase: 25% polyethylene glycol 400 (PEG 400), 10% bovine serum albumin (BSA), 1% Tween 20®, 25% hydroxypropyl-γ-cyclodextrin (HP-γ-CD), 5% hydroxypropyl-β-cyclodextrin (HP-β-CD). Five hundred micro liters of excipient containing aqueous phase was stirred with 5 mL of water-saturated ether for 5 s. To this primary emulsion 5 mL of ether:ethanol [82.5:17.5, v/v] mixture was added and stirred for 2 h at 4 °C in a glass beaker. The mixture was then transferred to a glass centrifuge tube and centrifuged at 150 × *g* for 2 min. The top organic phase was aspirated off, while the bottom aqueous phase was collected and its volume measured. Unemulsified aqueous phase consisted of a solution of PAL along with the individual excipient that was not subject to emulsification. Aliquots of emulsified and unemulsified aqueous phase were used to determine the maximal activity of PAL and protein content.

2.5. Measurement of maximal activity (V_{max}) of PAL

Previous studies in our laboratory using Phe as substrate had indicated that the Michaelis–Menton constant (km) for PAL was 451 μM. The maximal activity of PAL in aliquots of emulsified and unemulsified aqueous phase was determined by incubating 384 μL of Phe solution with 16 μL of the aqueous phase at 37 °C. The final concentration of Phe in the reaction mixture was 4 mM. The reaction was stopped after 7 min 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 and expressed as μM/min/μg of protein.

2.6. Evaluation of possible migration of protein into the organic phase during emulsification

To determine if proteins migrated into the organic phase during emulsification, PAL in Tris buffer (pH 8.5) was emulsified with water-saturated ether for 2 h at 4 °C in a glass beaker. The mixture was then transferred to a glass centrifuge tube and centrifuged at 150 × *g* for 2 min. The top organic phase was collected and evaporated to dryness under nitrogen. The dried organic phase was reconstituted with Tris buffer (pH 8.5) and its protein content measured using a modified Bicinchoninic assay (BCA assay).

2.7. Evaluation of adsorption of protein on the glass surface

One possible area where protein could be lost during the emulsification procedure was to adsorption on the glassware. To evaluate protein adsorption on the glass during emulsification, PAL in Tris buffer was stirred with water-saturated ether in an unsilanized glass beaker for 2 h at 4 °C. Next, the mixture was transferred to an unsilanized glass centrifuge tube and centrifuged at 150 × *g* for 2 min. The top organic phase was aspirated off and the aqueous phase collected. The protein content of the aqueous phase was measured by the Bradford assay and expressed as the percent of its control. The control consisted of a PAL solution stored in a polypropylene tube for 2 h.

The presence of organic solvent during emulsification could modify the adsorption of the protein on the glass. Therefore we also investigated protein adsorption on glassware without the presence of organic solvent by stirring a solution of PAL in Tris buffer in an unsilanized glass beaker for 2 h at 4 °C. The solution was then transferred to an unsilanized glass centrifuge tube and centrifuged at 150 × *g* for 2 min. The protein content of the solution was measured by the Bradford's protein assay and was compared to the protein content of a control PAL solution kept in a polypropylene tube.

In an indirect method to verify that adsorption onto glassware is a source of protein loss during emulsification, we attempted to decrease protein adsorption onto glassware during emulsification. We then computed the expected resultant increase in protein content of the emulsified aqueous phase. Silanization is known to decrease protein adsorption onto glassware (Wu and Chen, 1989). Therefore, we repeated all the aforementioned experiments with emulsified and unemulsified aqueous phases using silanized glassware. Glassware was silanized by soaking in a 5% solution of dimethyldichlorosilane in toluene for 30 min. Glassware was then triple rinsed with toluene followed

by methanol and dried overnight. The control consisted of PAL solution stored in a polypropylene tube. Amount of protein in the aqueous phase of the experimental sample was computed and expressed as a percent of its control.

2.8. Determination of protein amount in the aqueous phase

Protein amounts were measured by one of the following methods depending on the specific objectives of individual experiments. The Bradford assay (Bradford, 1976) was conducted using a kit from Bio-Rad Laboratories (Hercules, CA); the Bicinchoninic assay (BCA assay) (Brown et al., 1989) using a QP-BCA kit from Sigma–Aldrich (St. Louis, MO). Protein amount was also estimated by measurement of aqueous phase absorbance at 280 nm.

In some experiments, we suspected that the emulsification procedure resulted in partial precipitation of PAL in the aqueous phase. In order to determine the amount of precipitated PAL we first needed to resolubilize it. A modified BCA assay using a kit from Sigma–Aldrich (St. Louis, MO) was employed in such cases. Briefly, all proteins in the post-emulsified aqueous phase were precipitated with 0.15% sodium deoxycholate and 6.1N (100%, w/v) trichloroacetic acid. The samples were then centrifuged at 8800 × *g* for 15 min. The supernatant was decanted and the pellet was resolubilized with a 5% (w/v) solution of sodium dodecyl sulfate in 0.1N NaOH. The resolubilized solution was incubated for 1 h at 60 °C with the BCA reagent and its absorbance was measured at 562 nm.

In all protein assays bovine serum albumin was used as the standard.

2.9. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out with a Bio-Rad mini protean 3 gel assembly using12% Tris–HCl gels from Bio-Rad Laboratories (Hercules, CA). Aliquots of emulsified and unemulsified aqueous phase were mixed with Lamelli sample buffer containing 2-mercaptoethanol and were heated in a boiling water bath for 3 min. Samples containing 7 μg of protein were loaded onto the gel in a volume of 40 μL. The gels were run for 45 min at a constant 150 V at room temperature. 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 protein bands in emulsified and unemulsified samples.

2.10. Size exclusion chromatography (SEC)

Loss of protein in the aqueous phase due to emulsification was evaluated using SEC. Equal aliquots of protein from emulsified and unemulsified aqueous phase were 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 was collected and analyzed using ChromPerfect Software (Justice Innovations Inc., Denville, NJ).

2.11. Statistics

Results are presented as mean ± S.D. Wherever appropriate differences between groups were evaluated with a Student's *t*-test (two groups) or a one-way ANOVA (more than two groups) at an alpha level of 0.05. Data that failed the normality or equal variance test were analyzed by the non-parametric Mann–Whitney rank sum test at an alpha level of 0.05.

3. Results

3.1. Emulsification of the aqueous phase with water-saturated ether

3.1.1. Emulsification of PAL in 10% hemoglobin solution with water-saturated ether

As shown in Table 1, emulsification of a 0.5 mL PAL in a 10% hemoglobin solution with water-saturated ether did not alter the volume of the aqueous phase. The activity of PAL in the unemulsified aqueous phase (PAL in a 10% hemoglobin solution) was 310 ± 26 μM/min (mean ± S.D.). The activity of PAL in the aqueous phase after emulsification with water-saturated ether was 289 ± 15 μM/min, and was similar to its activity in the unemulsified aqueous phase ($P > 0.05$, *t*-test). In both cases activity of PAL is expressed per total volume of the aqueous phase. We could not express activity per μg of PAL because

Table 1
Volume, protein content and activity of PAL in the aqueous phase upon emulsification with water-saturated ether

Parameter	Aqueous phase			
	PAL in 10% hemoglobin		PAL in Tris buffer	
	Unemulsified	Emulsified	Unemulsified	Emulsified
Volume of aqueous phase (μL)	500 ± 0[a]	498 ± 8[N.S.]	500 ± 0	521 ± 13[*]
Activity in aqueous phase (μM/min)	310 ± 26	289 ± 15[N.S.]	253 ± 39	231 ± 31[N.S.]
Protein content of the aqueous phase (μg)	N/D	N/D	61.8 ± 4.0	36.2 ± 2.5[*]
Activity (μM/min/μg of protein)	N/D	N/D	4.1 ± 0.5	6.5 ± 1.2[*]

N.S., not significantly different from the unemulsified sample; N/D, not determined. See text for additional details.
[a] Mean ± S.D.; $n = 3$–7.
[*] Significantly different from the unemulsified sample.

it was not possible to specifically measure aqueous phase PAL content in the presence of very large amounts of hemoglobin.

3.1.2. Emulsification of PAL in Tris buffer with water-saturated ether

As shown in Table 1, emulsification of PAL in Tris buffer (pH 8.5) with water-saturated ether produced a small, but statistically significant, increase in the volume of the aqueous phase. The activity of PAL expressed per total volume of the aqueous phase was also not affected by emulsification with water-saturated ether. Surprisingly, there was a 40% decrease in the protein content (Bradford's assay) of the aqueous phase upon emulsification with water-saturated ether. Consequently, PAL activity expressed per μg of protein increased after emulsification.

To confirm this unusual finding of decrease in aqueous phase protein levels but lack of change in PAL activity, we re-computed the protein content of the aqueous phase by measuring its absorbance at 280 nm. Aromatic amino acids like tryptophan, tyrosine, histidine, and phenylalanine absorb maximally at 280 nm and thus proteins can be quantitated at this wavelength. The absorbance of the emulsified aqueous phase was 42 ± 7% less than that of the unemulsified aqueous phase. The magnitude of decrease in absorbance was similar to percent decrease in protein content observed with the Bradford's method and confirmed the emulsification mediated loss in protein content.

3.2. SDS-PAGE of emulsified aqueous phase

PAL is a 320,000 Da homotetramer containing four 80 kDa monomers. As shown in Fig. 1, SDS-PAGE of unemulsified PAL solution resulted in a major band at the 80 kDa region representing the monomer and a number of other bands below 80 kDa, suggesting the presence of impurities in the PAL sample.

SDS-PAGE of an equal amount of protein from the emulsified aqueous phase showed that the intensity of 80 kDa band was 19% greater than that in the unemulsified aqueous phase [emulsified = 404 ± 73 (mean ± S.D.; $n = 9$) integrated optical density (IOD) units; unemulsified = 328 ± 60 IOD units; $P < 0.05$, t-test]. Additionally, in the emulsified aqueous phase sample the intensities of bands representing the impurities were less than or missing when compared to similar bands in the SDS-PAGE profile of the unemulsified aqueous phase. These results suggest that an aliquot of protein in the emulsified aqueous phase contains a larger percent of PAL compared to an equal aliquot of protein in the unemulsified aqueous phase.

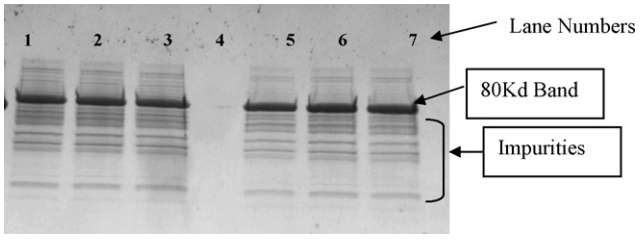

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel analyses of unemulsified and emulsified aqueous phase. All lanes were loaded with 7 μg of protein. Lanes 1–3 represent the unemulsified aqueous phase, lane 4 is empty, and lanes 5–7 represent the emulsified aqueous phase.

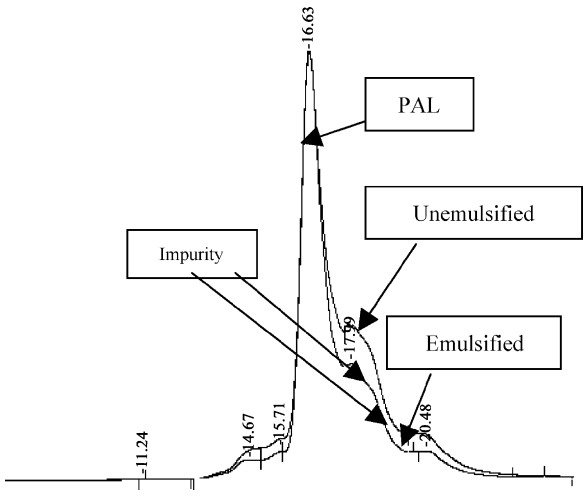

Fig. 2. Size exclusion chromatography of an equal amount of protein from samples of unemulsified and emulsified aqueous phases.

3.3. SEC of PAL upon emulsification with water-saturated ether

Protein from the unemulsified and emulsified aqueous phases (14 μg) was loaded on the SEC column. As shown in Fig. 2, there are three major peaks in the chromatogram. The largest peak in both chromatograms represents PAL and the two peaks following it represent impurities in the PAL sample. The area of the PAL peak in the emulsified aqueous phase was 23% larger than that in the unemulsified aqueous phase. Also, the combined area of peaks representing the impurities was about 24% smaller in the emulsified aqueous phase than in the unemulsified aqueous phase. These results also suggest that an aliquot of protein in the emulsified aqueous phase contains a larger percent of PAL compared to an equal aliquot of protein in the unemulsified aqueous phase.

3.4. Evaluation of different factors that could contribute to loss in protein during the emulsification procedure

3.4.1. Migration of protein into organic phase

After emulsification of the aqueous phase (PAL in Tris buffer) with water-saturated ether, the organic phase was collected and evaporated to dryness. The residue was reconstituted with Tris buffer and its protein content measured using a modified BCA assay. The assay did not reveal presence of protein in the reconstituted sample. This result suggests lack of significant migration of protein into the organic phase after emulsification with water-saturated ether.

3.4.2. Emulsification mediated precipitation of protein in the aqueous phase

Protein content of the post-emulsified aqueous phase was measured by BCA assay and modified BCA assay. In the modified BCA assay all protein in the aqueous phase is precipitated

and then resolubilized by sodium dodecyl sulfate. Therefore, the modified BCA measures both soluble and precipitated protein while the BCA assay measures only the soluble protein. The protein content of the post-emulsified aqueous phase was 60 ± 4% of its unemulsified control determined by BCA assay and 62 ± 4% of its control as determined by the modified BCA assay. The similarity of the protein content measured by the two assays suggests an absence of emulsification induced precipitation of the protein in the aqueous phase.

3.4.3. Protein adsorption on to glassware

Results in Sections 3.4.1 and 3.4.2 show that protein did not migrate into the organic phase nor did it precipitate in the aqueous phase. It is therefore conceivable that protein was adsorbed on to the glassware used in the emulsification procedure. To evaluate this possibility, the aqueous phase was emulsified with water-saturated ether in unsilanized glassware. The amount of protein recovered in the post-emulsified aqueous phase was 50 ± 9% (Bradford assay) of its unemulsified control stored in a polypropylene tube. An aliquot of the unemulsified aqueous phase was also kept in contact with the unsilanized glassware for 2 h and the amount of protein recovered was 68 ± 4% of its unemulsified control stored in the polypropylene tube. These results suggest that proteins in the aqueous phase can adsorb onto glass, and emulsification and the consequent contact with organic solvents increases protein adsorption onto glass.

To determine if silanization was effective in decreasing protein adsorption onto glass, we repeated the aforementioned experiment in silanized glassware. The amount of protein recovered in the unemulsified aqueous phase sample was 87 ± 2% of its control stored in polypropylene tube (Bradford's method). The minimal loss of protein in this sample, and the significant increase in recovery (19%) compared to the recovery from corresponding unsilanized glassware indicates that silanization can reduce protein adsorption onto glassware ($P < 0.05$, one-way ANOVA). Next, we determined if silanization could also reduce protein adsorption onto glassware during the emulsification procedure. The amount of protein recovered in the post-emulsified aqueous phase in silanized glassware was 62 ± 5% of its control stored in polypropylene tube. The smaller (12%) but statistically significant increase ($P < 0.05$, one-way ANOVA) in the amount of protein recovered from post-emulsified aqueous phase in silanized glassware compared to the corresponding recovery from unsilanized glassware suggest that silanization was also able to reduce protein adsorption from PAL solution during emulsification. The results also support the suggestion that adsorption onto glassware is an important reason for the loss of protein during emulsification.

3.5. Emulsification of the aqueous phase with water-saturated ether and ether:ethanol mixture

3.5.1. Emulsification of PAL in 10% hemoglobin solution with water-saturated ether and ether:ethanol [82.5:17.5, v/v] mixture

Emulsification of PAL in 10% hemoglobin with water-saturated ether and ether:ethanol caused extensive precipitation of the hemoglobin immediately upon addition of the ether:ethanol mixture. At the end of the emulsification process, no discernible aqueous phase was observed. We weighed the total amount of precipitate and measured the PAL activity in a weighed aliquot of the precipitate. As shown in Table 2, PAL activity in the total amount of the precipitate was 50% less than the activity in the unemulsified aqueous phase.

3.5.2. Emulsification of PAL in Tris buffer (pH 8.5) with water-saturated ether and ether:ethanol [82.5:17.5, v/v] mixture

As shown in Table 2, emulsification of PAL in Tris buffer (pH 8.5) with water-saturated ether plus ether:ethanol resulted in a 20% decrease in the volume of the aqueous phase. The activity of PAL in the total aqueous phase was 24% lower than aqueous phase activity in the unemulsified sample while aqueous phase protein levels decreased by 40% after emulsification. The activity of PAL expressed per μg of protein was not altered after emulsification suggesting that the loss of PAL activity was approximately commensurate with the loss of PAL protein.

3.5.3. Emulsification of PAL with water-saturated ether and ether:ethanol [82.5:17.5, v/v] in presence of various additives

As shown in Table 3, emulsification of aqueous phase with ether:ethanol in the absence of any additive results in a 20% decrease in the volume of aqueous phase compared to the

Table 2
Volume, protein content and activity of PAL in the aqueous phase upon emulsification with water-saturated ether and ether:ethanol [82.5:17.5, v/v]

Parameter	Aqueous phase			
	PAL in 10% hemoglobin		PAL in Tris buffer	
	Unemulsified	Emulsified	Unemulsified	Emulsified
Volume/amount of aqueous phase (μL/μg)	500 ± 0[a]	505 ± 58 μg[b]	500 ± 0	402 ± 38[*]
Activity in aqueous phase (μM/min)	242 ± 25	120 ± 12[*]	259 ± 27	198 ± 45[*]
Protein content of the aqueous phase (μg)	N/D	N/D	59.8 ± 5.7	35.8 ± 5.8[*]
Activity (μM/min/μg of protein)	N/D	N/D	4.4 ± 0.8	5.5 ± 1.2[N.S.]

N.S., not significantly different from the unemulsified sample; N/D, not determined. See text for additional details.

[a] Mean ± S.D.; $n = 4$–5.

[b] Upon emulsification with ether:ethanol mixture proteins instantly and completely, precipitated and no discernible aqueous phase was observed. See text for details.

[*] Significantly different from the unemulsified sample.

Table 3
Volume, protein content and activity of PAL in aqueous phase containing various additives upon emulsification with water-saturated ether and ether:ethanol [82.5:17.5, v/v]

Additive	Volume of aqueous phase (μL)		PAL activity in aqueous phase (μM/min)		Protein content of the aqueous phase (μg)	
	Control	Sample	Control	Sample	Control	Sample
No additive	500 ± 0[a]	402 ± 38*	259 ± 27	198 ± 45*	59.8 ± 5.7	35.8 ± 5.8*
25% PEG 400	500 ± 0	336 ± 31*	264 ± 29	141 ± 5*	55.3 ± 0.9	37.5 ± 2.7*
1% Tween 20	500 ± 0	359 ± 19*	347 ± 18	277 ± 22*	N/D[b]	N/D
10% BSA	500 ± 0	370 ± 33*	407 ± 21	313 ± 8*	N/D	N/D
2% HP-γ-CD	500 ± 0	467 ± 16*	223 ± 15	227 ± 11[N.S.]	67 ± 1.2	46.7 ± 12.6*
5% HP-β-CD	500 ± 0	440 ± 9[N.S.]	295 ± 32	268 ± 31[N.S.]	70.3 ± 2.9	61.4 ± 8.8[N.S.]

Control – unemulsified aqueous phase; Sample – post-emulsified aqueous phase. N.S., not significantly different from the control.
[a] Mean ± S.D.; $n = 3$–5.
[b] N/D, not determined.
* Significantly different from the control.

unemulsified control. The presence of PEG 400, Tween 20®, and BSA also resulted in marked decrease (26–33%) in the volume of the post-emulsified aqueous phase while inclusion of HP-γ-CD and HP-β-CD produced much smaller losses in the volume of the post-emulsified aqueous phase.

The loss in PAL activity of the emulsified aqueous phase in the absence of any additive was 24% compared to its unemulsified control. PEG 400 exacerbates the emulsification mediated loss in PAL activity (47% loss in activity) while Tween 20® and BSA do not confer any protection as evidenced by the 20–23% loss in activity in the presence of these agents. Interestingly, HP-β-CD and HP-γ-CD completely protected PAL against emulsification mediated losses in activity.

In comparison with an unemulsified control, emulsification without an additive results in a 40% loss in protein content of the aqueous phase. Emulsification in the presence of HP-γ-CD and PEG 400 also resulted in marked (30%) loss of protein content in the post-emulsified aqueous phase. Interestingly, inclusion of HP-β-CD prevented any emulsification mediated loss in the protein content in aqueous phase. We were unable to measure protein content of aqueous phases containing Tween 20® and BSA due to their interference in the protein assay.

4. Discussion

Our previous studies have shown that PAL encapsulated in cellulose nitrate membrane microcapsules looses 77% of it activity (Habibi-Moini and D'mello, 2001). Further investigation has revealed that 30% of the activity is lost due to incomplete encapsulation while the remainder is lost due to the manufacturing procedure of the microcapsules. Results of our present studies suggest that within the manufacturing process, the initial emulsification step is partially responsible for loss in PAL activity. We also evaluated a series of agents for their ability to minimize the emulsification mediated loss of PAL activity. These studies identified HP-γ-CD and HP-β-CD as particularly effective in protecting PAL activity from organic solvent mediated losses.

The primary emulsification step is the first step in the preparation of microcapsules. It leads to the formation of an aqueous solvent–organic solvent interface. Proteins are surface active and tend to migrate towards the solvent–solvent interface formed during emulsification. In order to lower the interfacial tension, the protein becomes unfolded resulting in its hydrophobic core lying towards the organic solvent. The unfolding of protein results in aggregation and finally a loss in its enzymatic activity (Perez and Griebenow, 2001). Simulation of the emulsification step in our studies showed that water-saturated ether either in the presence or absence of 10% hemoglobin did not cause any loss in PAL activity. These results suggest that PAL is resistant to the deactivating effects of ether and that hemoglobin is not required to protect the activity of PAL. Other investigators have shown that the enzyme deactivating effects of ethers are directly dependent on their hydrophobicity and on the exposure time of the enzyme to the ethers (Hickel et al., 2001). Diethyl ether used in our studies has a log P of 0.8 and it was in contact with the PAL solution for 2 h. Its lower hydrophobicity compared to higher order ethers like dibutyl ether (log P of 3.21) and shorter exposure times in the experiment probably accounts for its lack of effect on the activity of PAL. Also, most studies use sonication in the production of the primary emulsion while our procedure uses relatively gentle stirring. The gentle stirring creates a relatively smaller interfacial area (compared to sonification) which limits the exposure of PAL to diethyl ether and accounts for the absence of deactivation of the enzyme.

While emulsification of PAL with ether did not produce loss in activity, there was a surprising loss in the protein amount of the aqueous phase (Table 1). This result suggested that a portion of protein in the aqueous phase is not contributing to activity and selective loss of this protein fraction during emulsification results in decrease in protein content without a loss of overall activity. The commercial PAL solution obtained from Sigma and used in our studies contained impurities or possible degradation products of PAL (Khot, 2005). Analysis of post-emulsified aqueous phase with SDS-PAGE showed a selective decrease in amount of non-PAL protein and a consequent refinement of PAL. SEC data confirmed these results which show that emulsification with water-saturated ether selectively removed a portion of the non-active impurities resulting in a decrease in protein levels without a change in PAL activity. Repeating these experiments with pure PAL will help reconfirm that emulsification with water-saturated ether does not affect PAL protein amount

or PAL activity. Other investigators have reported similar refinement of protein content upon treatment with ether (Curzon and Vallet, 1960). Efforts to identify the location of the lost protein showed that this protein did not migrate into the organic phase nor did it precipitate in the aqueous phase. Experiments with silanized glassware revealed that emulsification with organic solvent facilitated selective adsorption of protein impurities or degradation products on the glassware. Such selective adsorption of less stable structural variants of proteins has previously been demonstrated in the literature (Lee et al., 2004).

Addition of ether:ethanol represents the second step in the manufacture of microcapsules. Unlike ether alone, emulsification with ether:ethanol mixture resulted in a marked loss in activity of PAL. Experiments with a PAL solution in the absence of hemoglobin showed that hemoglobin exacerbated the loss in the activity of PAL. The presence of ethanol during emulsification caused total precipitation of the hemoglobin from the aqueous phase. There is evidence in the literature for this ability of ethanol to specifically precipitate hemoglobin (Frantzen et al., 1997). The marked reduction of PAL activity in this system is probably due to the inability of PAL to work efficiently while being entrapped in the precipitated hemoglobin.

Emulsification of PAL with ether:ethanol mixture in the absence of hemoglobin also caused a significant loss of activity. Water molecules play an important part in keeping the protein in its native conformation. The layer of water surrounding the protein forms hydrogen bonds with hydrophilic regions in the protein and keeps the protein hydrated and its structure rigid (Stevenson, 2000). Organic solvents that are miscible with water can strip water molecules surrounding the protein and can denature the protein. It has also been shown that as the concentration of organic solvent in the aqueous phase increases there is an increase in denaturation of protein (Halling, 1994; Khmelnitsky et al., 1991). Polar solvents like ethanol are highly miscible with water and have a tendency to strip water surrounding the protein. This could lead to loss in folded structure of the protein and probably accounts for the loss in the activity of PAL upon emulsification with ether:ethanol. Simon and associates have reported similar decreases in activities of trypsin and chymotrypsin in ethanolic solution and these decreases were accompanied by less compact structures of the protein (Simon et al., 2001). This reasoning could also explain why in our previous experiments emulsification with ether did not deactivate PAL. The solubility of ether in water is only about 6.9%. It is possible that at this concentration ether was unable to strip the critical number of water molecules required to alter the folded structure of the protein and was therefore not able to deactivate PAL.

Our final studies evaluated a series of additives for their ability to protect PAL from emulsification mediated loss in activity. The specific additives used in our studies were chosen because of the proven ability to protect a variety of proteins from losses against organic solvent mediated deactivation. Results of our studies showed that PEG, Tween 20® and BSA were unable to protect the activity of PAL. On the other hand, HP-γ-CD and HP-β-CD were successful in completely protecting the activity of PAL during emulsification and HP-β-CD was even able to prevent loss of aqueous phase protein content. Cyclodextrins are non-reducing oligosaccharides prepared by enzymatic degradation of starch and are chemically substituted to produce modification like HP-γ-CD and HP-β-CD. Cyclodextrins have a circular structure with an internal cavity that is hydrophobic while groups outside the cavity are hydrophilic (Albers and Muller, 1995). It has been shown that upon addition of cyclodextrins to a protein solution, the aromatic amino acids on the surface of the protein are covered by the hydrophobic cavity of the cyclodextrins. Many molecules of cyclodextrin can interact physically with one molecule of protein and the magnitude of this interaction is dependent on the number of hydrophobic amino acids on the surface of the protein (Brewster et al., 1991). The net result of the interaction is an increase in the surface hydrophilicity of the protein which in turn increases its resistance to deactivation by water-ethanolic solutions (Vinogradov et al., 2001). Interactions between HP-γ-CD, HP-β-CD and PAL could explain the observed increase in stability of PAL in ethanolic solutions. A review of the literature indicates that cyclodextrins can also decrease loss in protein activity at solvent–solvent interfaces by reducing organic solvent mediated aggregation of the protein (Morlock et al., 1996; Sah, 1999b). It is conceivable that such a protective mechanism might also be operational in our system.

When PAL was emulsified with ether:ethanol mixture we observed a decrease in the volume of the aqueous phase. The ether used in this mixture was not saturated with water. Since water is partially miscible in ether and the presence of a large excess of ether compared to the volume of the aqueous phase resulted in possible migration of a portion of the aqueous phase and its solubilization in the ether:ethanol phase. The ether used in the mixture was not saturated with water prior to use because during the preparation of microcapsules the cellulose nitrate dissolved in the ether:ethanol mixture precipitates in the presence of minute traces of dissolved water. In contrast, when the aqueous phase was emulsified with ether alone, the ether was saturated with water prior to use which probably accounts for the lack of emulsion mediated loss of aqueous phase volume. It is also important to note that in the aqueous phase/ether/ethanol system, the ethanol is miscible with the other two components. Therefore, ethanol could change the miscibility of the other two components in each other which could also account for the loss in aqueous phase volume. Interestingly, the two cyclodextrins markedly reduced the loss in aqueous phase volume upon emulsification with the ether:ethanol mixture. Cyclodextrins are known to confer osmotic pressure to the aqueous phase (Zannou et al., 2001) which could have prevented the migration of water into the organic phase. Bovine serum albumin at the concentration used would also be expected to produce high osmotic pressure in solution and prevent the loss of aqueous phase volume. Surprisingly, it was unable to do so, and the reasons are presently unclear.

In conclusion we demonstrate that emulsification of PAL with ether:ethanol is the main reason for the loss in its activity, and the presence of hemoglobin in the aqueous phase exacerbated this loss. Addition of 2% HP-γ-CD and 5% HP-β-CD to the aqueous phase completely prevented emulsion mediated loss in PAL activity. In the next series of experiments we will attempt

to prepare microcapsules containing PAL along with HP-γ-CD or HP-β-CD, and determine the activity of encapsulated PAL in these reformulated microcapsules.

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