Development of Modified Phenylalanine Ammonia-lyase for the Treatment of Phenylketonuria

Woomi Kɪм*

Department of Pharmacology, Kosin University College of Medicine, Busan 602-703, Republic of Korea

Abstract − Phenylketonuria (PKU) is an inherited metabolic disorder caused by mutations in the phenylalanine catabolic enzyme, phenylalanine hydroxylase (PAH). The use of phenylalanine ammonia-lase (PAL) by oral and parenteral routes as a therapeutic drug for PKU has been severely limited due to inactivation by intestinal proteolysis and immune reactions. PEGylation was applied to PAL to reduce the degrees of antigenicity and proteolytic inactivation. Kinetic experiments with native PAL and pegylated PALs were performed, and pH stability, temperature stability, and protease susceptibility were evaluated. Enzyme linked immunosorbent assay (ELISA) was carried out to measure the immune complex between pegylated PALs and antiserum that had been extracted from a PAL-immunized mouse. Pegylated PAL, especially branched pegylated PAL (10 kDa, 1:32), was more active for phenylalanine and more stable in pancreatic proteases than native PAL. Native PAL was optimal at pH 8.5, corresponding to the average pH range of the small intestine; the same finding was noted for pegylated PALs. All linear and branched pegylated PALs had low reactivity with mouse antiserum, especially the 1:16 formulation with linear 5-kDa PEG and the 1:32 formulation with branched 10-kDa PEG. Therefore, we suggest the 1:32 formulation with branched 10-kDa PEG as the most promising formulation for enzyme replacement therapy.

Keywords: Phenylketonuria (PKU), Phenylalanine ammonia-lyase (PAL)

INTRODUCTION

Phenylketonuria (PKU) is an inherited metabolic disorder caused by mutations in the phenylalanine catabolic enzyme, phenylalanine hydroxylase (PAH) (Scriver and Kaufman, 2001). A few studies have focused on the application of phenylalanine ammonia-lyase (PAL) for enzyme replacement therapy for patients with PKU (Hoskins and Gary, 1982). In 1980, Hoskins and colleagues reported their initial trials of the use of PAL from *Rhodotorula glutinis* for PKU enzyme substitution applications (Hoskins *et al.,* 1980). However, native PAL is very susceptible to protease inactivation (Gilbert and Jack, 1981; Gilbert and Tully, 1985) and the sensitivity of PAL to protease inactivation or low activity in gastric conditions limits further development of native PAL as a clinical therapeutic (Gilbert and Jack, 1981). Sarkissian *et al.* considered the parenteral application of PAL for reducing the plasma con-centration of phenylalanine (Sarkissian *et al.,* 1999). They reported that intraperitoneal injections of unmodified PAL lowered blood L-phenylalanine levels in the PKU model, but repeated injections led to immunogenic reductions in efficacy due to elicitation of an immune response.

Various formulations have been tested to prevent protease inactivation or to suppress the immunogenicity of PAL (Ambus *et al.,* 1983; Bourget and Chang, 1984; Bourget and Chang, 1989; Chang *et al.,* 1995). Microencapsulated PAL in artificial cells administered orally to rats with PKU was more effective than a phenylalanine-free diet (Bourget and Chang, 1989). However, the restriction was that microencapsulated PAL demonstrated activity that was only 20% of native PAL enzyme activity (Bourget and Chang, 1986). This would be limited to patients with mild PKU, and thus diet control should be recommended as therapy (Fritz *et al.,* 1976). Therefore, the most important condition during-modification was maintaining enzyme activity in addition to protection from pancreatic proteolysis and/or immune reaction to effectively function in reducing phenylalanine levels.

---

*Corresponding author
Tel: +82-51-990-6437   Fax: +82-51-990-3084
E-mail: kwm@ns.kosinmed.or.kr

Under these circumstances, one approach to overcome the reduction in the activity of PAL might be the use of PEGylation derivatization, since the covalent coupling of activated PEG molecules to the protein (PEGylation) has been shown to allow retention of bioactivity, and even increases circulation half-times (Delgado *et al.,* 1992; Mehvar, 2000; Veronese *et al.,* 2002; Greenwald *et al.,* 2003; Harris and Chess, 2003). A growing number of pegylated enzymes are being approved by the FDA (Harris and Chess, 2003). Thus, PEGylation can also be applied to PAL. In this study, we generated PAL: PEG-conjugates to investigate whether pegylated PAL can maintain its enzymatic activity and protect itself from degradation by pancreatic proteases and/or immune reactions.

MATERIALS AND METHODS

PEGylation

PEG: PAL conjugates were produced by coupling either linear 5-20 kDa methoxy-PEG-SPA or 10-40 kDa branched methoxy-(PEG)$_2$-NHS (Nektar Therapeutics) to PAL (Hershfield *et al.,* 1991). The concentration of PAL in the reaction mixture was 1 mg/ml in 50 mM potassium phosphate buffer, pH 8.5. Pegylated PAL was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% Bis-Tris gels in 3-(*N*-morpholino) propane sulfonic acid (MOPS) buffer.

PAL activity assay

The activity of PAL/ -pegylated PAL using L-phenylalanine as a substrate was assayed at room temperature by measuring the production of trans-cinnamic acid, which was monitored by the increase in absorbance at 290 nm (Hoskins, 1968). The reaction mixture contained 22.5 mM phenylalanine in 100 mM Tris-HCl buffer, pH 8.5. The molar extinction coefficient of trans-cinnamic acid at 290 nm is 10.238 L$\cdot$ M^{-1} $\cdot$ cm^{-1}.

Kinetic analysis

Kinetic experiments were performed on a Cary UV spectrophotometer (Cary 50). All experiments were conducted at room temperature. For standard measurements, the final enzyme concentration was 0.0035 mg/ml, but for kinetic studies the enzyme concentration in the assay was adjusted so that the slope at 290 nm per minute was in the range of 0.005 to 0.02. The velocity of the reaction (*v*) is the number of substrate molecules converted to trans-cinnamic acid per minute.

Temperature and pH stabilities of PAL

Native PAL and pegylated PAL (1 mg/ml) were stored at 4°C and $-$80°C in closed bottles and the residual enzyme activity was measured for 6 months. The PAL or pegylated PAL was incubated in 100 mM Tris-Cl buffer of pH 7.5 or pH 8.5, at 37°C for 0 to $-$200 hours. After incubation, substrate solutions containing 22.5 mM L-phenylalanine in 100 mM Tris-Cl buffer were added and incubated at 37°C for the assay. The production of trans-cinnamic acid was monitored at 290 nm.

Protease susceptibility

To evaluate the effects of pancreatic proteases on PAL and pegylated PAL activities, the enzymes (0.9 mg/ml) in 100 mM Tris-Cl buffer at pH 8.5 were incubated with the protease mixture at 37°C for 1 hour. The pancreatic protease mixture contained trypsin (20 μg/ml), chymotrypsin (30 μg/ml), carboxypeptdase A (20 μg/ml), and carboxypeptidase B (30 μg/ml), elastase (10 μg/ml). Samples of 50 μl were collected every 10 minutes and for the residual PAL activity. At the same time intervals, samples of 100 μl were collected and subjected to SDS-PAGE.

ELISA

The ELISA plate (Nunc Maxisorp) was coated with purified native PAL or pegylated PAL (2-8 μg/ml in Tris-Cl buffer, pH 8.5) for 2 hours at 37°C. Antisera for positive control were obtained from wild PAL-treated mice and were diluted 0-5,000 fold. Negative control serum was prepared by sampling from a buffer-treated mouse. HRP-Goat anti-mouse IgG (1:2,500, 50 μl) was used as the secondary antibody. Absorbance was measured at 450 nm, and antibody (Ab) titer expressed as OD/μl, which was calculated as A (450 nm) $\times$ dilution factor/100 μl (Bos *et al.,* 1981).

Statistical analysis

The data were analyzed using the unpaired or paired Student's *t*-test, and values were considered significantly different when the *p* value was < 0.05. One-way analyses of variances were tested, and non-significant differences between the groups, which presented as same letters, were followed by Tukey's multiple comparison test. The results are expressed as mean ± SEM.

RESULTS

Activity of native PAL and pegylated PAL

The reaction velocities (*v*) of native PAL and pegylated PAL were dose-dependent at phenylalanine concentration ranges from 0.02 to 11.25 mM. The V$_{max}$ values of native
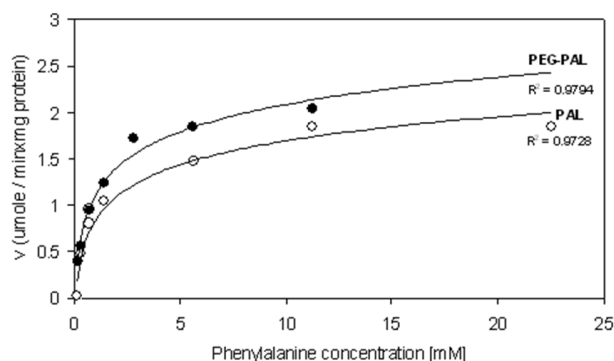
Fig. 1. Activity of native PAL and pegylated PAL on phenyla-lanine. The activity of native PAL and branched pegylated PAL (10 kDa, 1:32) on phenylalanine (0-25 mM) was assayed by measuring the production of trans-cinnamic acid, which was monitored as the increase in the absorbance at 290 nm.

Table I. The specific activity of native PAL and pegylated PAL

Enzyme	Specific activity (μmole/ min · mg protein)
Native PAL	1.77 ± 0.03
Pegylated PAL	
Linear PEG, 5 kDa (1:8)	1.79 ± 0.09
Linear PEG, 20 kDa (1:2)	1.84 ± 0.05
Branched PEG, 10 kDa (1:28)	1.89 ± 0.18
Branched PEG, 10 kDa (1:32)	2.03 ± 0.06

Data are expressed as mean ± SEM (n=5, $p < 0.05$).

PAL and pegylated PAL were 1.77 and 2.03 μmole/ min · mg protein, respectively. The K_m of native PAL was 4.77×10^{-4} M and that of pegylated PAL was 3.61×10^{-4} M (Fig. 1). The specific activity of all pegylated PALs was higher than that of native PAL (Table I). Branch pegylated PAL (10 kDa, 1:32) had the strongest activity for phenylalanine.

Temperature and pH stabilities

Native PAL (1 mg/ml), stored at -80°C for 20 weeks, possessed 87% of its original activity. In contrast to native PAL, all linear pegylated PALs showed no loss of activity. Two different molar ratios of branched pegylated PAL pos-sessed 85% (10 kDa, 1:28) and 97 percent (10 kDa, 1:32) of the original activity. Although PAL had an optimum pH at 8.5, when it was incubated for 150 hours at 37°C, only 61.56% of the original activity remained. Native PAL at pH 7.5 was more stable than native PAL at pH 8.5 after 150 hours of incubation at 37°C (Fig. 2). Pegylated PAL was al-so optimal at pH 8.5 and pegylated PAL had similar stabil-ity to native PAL at pH 7.5. Below pH 5.5, native PAL and pegylated PAL possessed 33% and 27% of their activity, respectively, after 1 week.
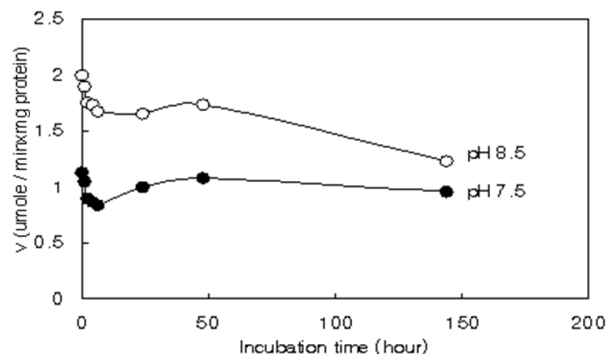

Fig. 2. The stability of native PAL at pH 7.5 or 8.5. Native PAL was incubated in 100 mM Tris-Cl buffer of pH 7.5 or 8.5 at 37°C. After incubation, a substrate solution containing 22.5 mM L-phenylalanine in 100 mM Tris-Cl buffer was added and incubated at 37°C for the assay. The production of trans-cinnamic acid was monitored at 290 nm.

Table II. Protease susceptibilities of PAL and pegylated PALs

Enzyme	Residual activity (%)[a]	T[b,c]
Native PAL	0	a
Pegylated PAL		
Linear PEG, 5 kDa (1:8) + linear PEG, 20 kDa (1:2)	15.62 ± 5.08	b
Linear PEG, 20 kDa (1:2)	16.19 ± 1.41	b
Branched PEG, 40 kDa (1:2)	51.22 ± 4.71	c
Branched PEG, 10 kDa (1:28)	43.75 ± 3.10	c
Branched PEG, 10 kDa (1:32)	75.71 ± 4.85	d

[a]The residual activity of native PAL and pegylated PAL was calculated by measuring the remaining activity after incubation with the pancreatic protease mixture for 10 minutes at 37°C. Data are expressed as mean ± SEM (n=5, [b]$p < 0.01$). Statistical signi-ficances were tested by one-way analysis of variances among groups. [c]The same letters indicate a non-significant difference between groups on the basis of Tukey's multiple comparison test.

Protease susceptibility

After incubation with the pancreatic proteases mixture for 10 minutes at 37°C, native PAL showed no activity (Table II). In contrast to native PAL, 5 pegylated PALs maintained their activity, with the amount of residual activ-ity (%) varying from 19.14 to 73.37 (Table II). Branched pe-gylated PAL (10 kDa, 1:32) was the most effective in main-taining its activity in the pancreatic protease mixture (Table II). The experiments with SDS-PAGE showed different pat-terns of bands (Fig. 3). Compared to the bands of native PAL and pegylated PAL with Tris-Cl buffer, the bands of native PAL and pegylated PALs with pancreatic proteases were more and moved lower from the original bands (Fig. 3). In addition, more bands with smaller molecular weights appeared after 30 minutes of incubation (Fig. 3).
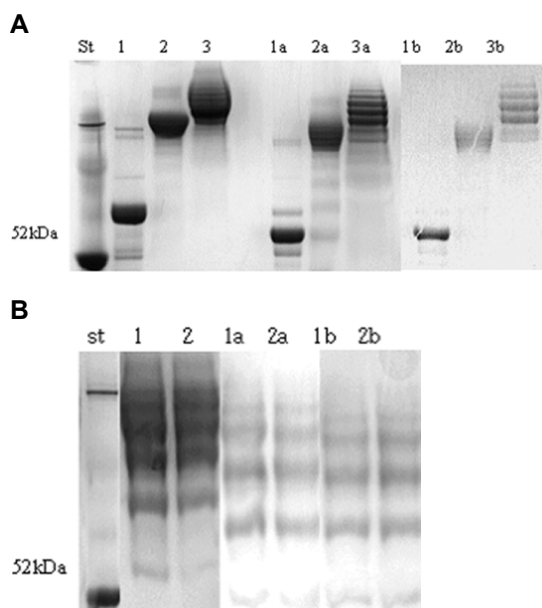
A

B

Fig. 3. Digestions of linear and branched pegylated PALs by pancreatic proteases on SDS-PAGE. (A) Native PAL and linear pegylated PALs (0.9 mg/ml) in 100 mM Tris-Cl buffer at pH 8.5 were incubated with reaction buffer (1, 2, 3) or the protease mixture (1a, 2a, 3a, 1b, 2b, 3b) at 37°C for 0 to $-$30 minutes. After 10 (1a, 2a, 3a) or 30 (1b, 2b, 3b) minutes of incubations, 100 μl of each sample was analyzed by SDS-PAGE on 4-10% Bis-Tris gels. St: High-molecular-weight, standard marker, 1: native PAL, 2: 5-kDa linear pegylated PAL (1:32), 3: 20-kDa linear pegylated PAL (1:8). (B) Branched pegylated PALs (0.9 mg/ml) in 100 mM Tris-Cl buffer at pH 8.5 was incubated with reaction buffer (1, 2) or the protease mixture (1a, 2a, 1b, 2b) at 37°C for 0 to $-$30 minutes. After 10 (1a, 2a) or 30 (1b, 2b) minutes of incubations, 100 μl of each sample was analyzed by SDS-PAGE on 4-10% Bis-Tris gels. St: High-molecular-weight, standard marker, 1: 10-kDa branched pegylated PAL (1:28), 2: 10-kDa branched pegylated PAL (1:32).

Two branched pegylated PALs retained their activity in the pancreatic proteases mixture longer than native PAL (Fig. 4). After 1 minute of incubation in the pancreatic protease mixture, the residual activity of native PAL decreased to less than 30%. On the other hand, branched pegylated PAL (10 kDa, 1:32) kept its activity rate higher than 50% even after 30 minutes of incubation with the pancreatic protease mixture (Fig. 4).

ELISA

This graph showed a linear dose-dependent line for positive control. The absorbance increased proportionally to the dilution factors of positive control. For negative control, the absorbance showed a steady and flat line (Fig. 5).

As shown in Fig. 6 and Fig. 7, all linear and branched pegylated PAL showed strong protections against immune
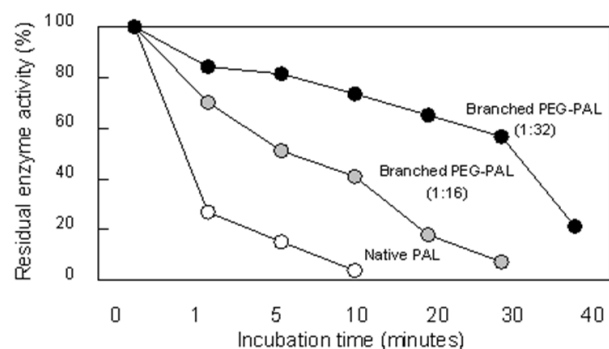

Fig. 4. Inactivation of branched pegylated PAL by pancreatic proteases. The residual enzyme activity of native PAL and branched pegylated PAL was calculated by measuring the remaining activity after incubation with the pancreatic protease mixture for 0-40 minutes at 37°C. Branched PEG (10 kDa) was used for PEGylation of native PAL.
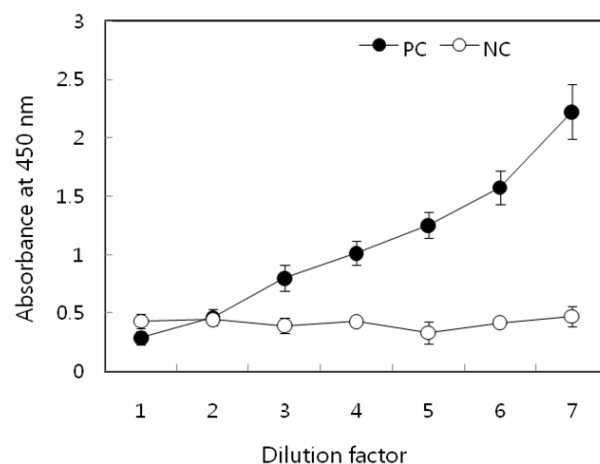

Fig. 5. Dilution linearity for ELISA. PC: positive control (antisera from native PAL-treated-mice), NC: negative control (antisera from buffer-treated-mice). Antisera were diluted to the concentrations of 0-1/5,000. Dilution factors were represented as 1 (0), 2 (1:160,000), 3 (1:80,000), 4 (1:40,000), 5 (1:20,000), 6 (1:10,000), and 7 (1:5,000). Data are expressed as mean $\pm$ SEM ($p < 0.05$).

reactions compared to native PAL. All linear pegylated PAL showed little reaction to the antibody (Fig. 6).

All branched pegylated PALs showed little reaction with the antibody, especially branched pegylated PAL (10 kDa, 1:32) (Fig. 7).

As the coated enzyme concentrations of native PAL and branched pegylated PAL increased higher from 2 μg to 8 μg, the immune reaction between the native PAL and the antibody as positive control increased. As for the two branched pegylated PALs, their reactions were not enhanced despite the increase in enzyme concentration
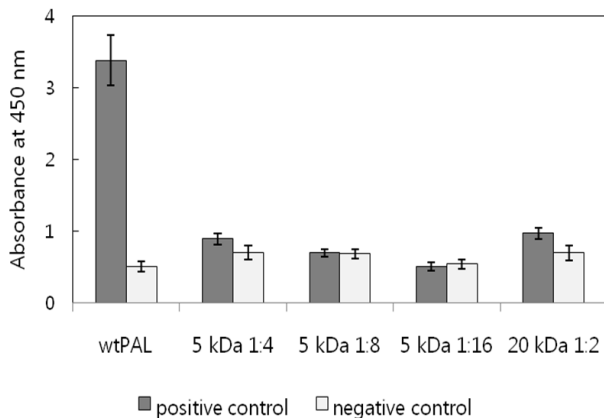
Fig. 6. ELISA of linear pegylated PAL. The ELISA plate was coated with 8 ug/ml of wild PAL or linear pegylated PAL in Tris-Cl buffer, pH 8.5, for 2 hours at 37°C. Antisera for positive control were obtained from a native PAL-treated-mice and diluted 5,000-fold. Negative control serum was prepared by sampling from buffer-treated-mice. Data are expressed as mean ± SEM ($p < 0.005$).
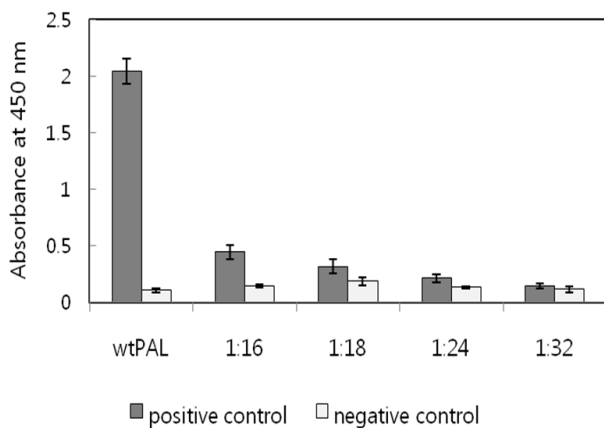

Fig. 7. ELISA of branched pegylated PAL. The ELISA plate was coated with 4 mg/ml of wild PAL or 10 kDa of branched pegylated PAL in Tris-Cl buffer, pH 8.5, for 2 hours at 37°C. Antisera for positive control were obtained from native PAL-treated-mice and diluted 5,000-fold. Negative control serum was prepared by sampling from buffer-treated-mice. Data are expressed as mean ± SEM ($p < 0.001$).

(Fig. 8).

DISCUSSION

The use of PAL as a therapeutic drug for PKU via the oral and parenteral routes has been severely limited due to inactivation by intestinal proteolysis and immune reactions. In this paper, PEGylation was applied to PAL to reduce the degree of immune reaction and proteolytic inactivation
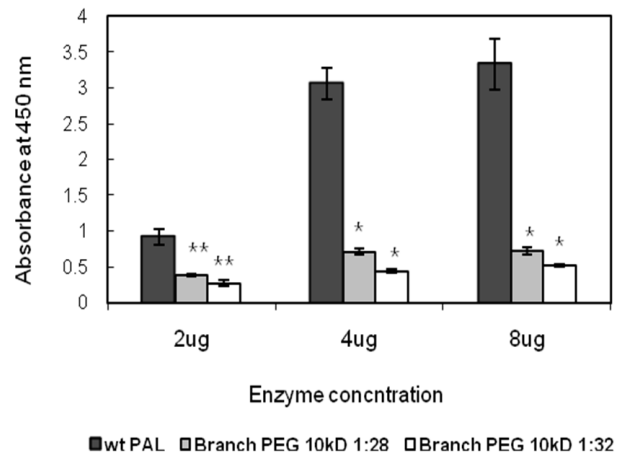

Fig. 8. ELISAs for different concentrations of PAL and branched pegylated PAL. The ELISA plate was coated with 2-8 μg/ml of wild PAL or branched pegylated PAL in Tris-Cl buffer, pH 8.5, for 2 hours at 37°C. Antisera were obtained from wild PAL-treated-mice and diluted 5,000-fold. Data are expressed as mean ± SEM (*$p < 0.001$, **$p < 0.01$).

while maintaining PAL activity. Because all the lysine residues (29) of PAL are on the surface of the molecule (Monfardini *et al.,* 1995; Veronese *et al.,* 1996; Veronese, 2001), it was presumably available for PEGylation and easy to conduct experiments with a variety of molar ratios during PEGylation. All the pegylated forms of PAL were retained to some extent and possessed increased catalytic activity compared to native PAL (Table 1). Especially, branched pegylated PAL (10 kDa, 1:32) showed an increased V_{max} value and a decreased K_m value (Fig. 1). We also tested resistance against pancreatic proteases by comparing the activities of linear and branched pegylated PAL to that of native PAL. All pegylated PALs were more stable in the pancreatic protease mixture than native PAL (Table 2). Branched pegylated PAL (10 kDa, 1:32) showed the strongest protective effect against proteolysis (Table 2), although the effect was not retained for more than 50 minutes (Fig. 4). From these results, PEGylation did not consistently protect PAL from intestinal proteolysis, and therefore, more advanced modification of PAL should be developed.

It was important to test the stability of the activity under the physiologic condition to use PAL for enzyme replacement therapy. PAL was optimal at pH 8.5, corresponding to the average pH range of the small intestine, and the activity of pegylated PAL was similar to that of native PAL at pH 8.5. This indicates that PAL has an advantage when it is administered through an oral route and so does pegylated PAL. However, the activity of native and pegylated PAL dropped below pH 5.5. The activity of pegylated PAL may not be stable against gastric acidity of pH 2.2. Hoskins

suggested an encapsulated PAL for bypassing the stomach, although it showed a lower activity than native PAL (Hoskins *et al.,* 1980). Unfortunately, the reduced activity of the encapsulated PAL was not enough to control the plasma phenylalanine level in PKU. Encapsulation needed additional processes to enhance the activity of PAL for oral administration. Thus, PEGylation with encapsulation may be recommended, since the activity of PAL could be enhanced by pegylation and gastric degradation could be protected by encapsulation.

On the other hand, we had to overcome some problems related to PAL's immunogenicity for parenteral administration (Fritz *et al.,* 1976). For the quantitation of the immune complex between pegylated PAL and antibody, ELISA was carried out. As shown in Figs. 6 and 7, all linear and branched pegylated PALs had low reactivity with the mouse antiserum, especially the 1:16 formulation with linear 5-kDa PEG and the 1:32 formulation with branched 10-kDa PEG (Fig. 8). The data indicated that all PEGs shielded the PAL's surface to abrogate antibody binding to PAL, and, therefore, PEGylation of PAL almost completely attenuated immunoreactivity. Branched PEG, which is characterized by two linear PEG chains with one reactive group, has recently been developed (Monfardini *et al.,* 1995; Veronese *et al.,* 1996; Veronese, 2001). The bulky branched structure of PEG probably may not allow the pancreatic protease to reach the protein surface, which is why it can protect partially itself against the pancreatic protease's attack partially (Table 2). Of all branched pegylated PAL in our experiments, 10 kDa of branched pegylated PAL (1:32) was the most effective in protecting itself from immunogenicity, although the protection against pancreatic proteolysis was not perfect. Therefore, we suggest that the 1:32 formulation with branched 10-kDa pegylated PAL is the most promising formulation for enzyme replacement therapy.

In conclusion, pegylated PAL might be a useful pretreatment modality for increasing or maintaining PAL's catalytic activity while effectively protecting itself from degradation by immune reactions. Pegylated PALs, especially branched PEG-PAL (10 kDa, 1:32), should be further evaluated both *in vitro* and *in vivo*, in order to assess its potential use in the treatment of human PKU patients. PEGylation may provide a new prospect for both oral and parenteral PAL therapies for patients with PKU.

ACKNOWLEDGMENTS

REFERENCES

Ambrus, C. M. *et al.* (1983). In vivo safety of hollow fiber enzyme-reactors with immobilized phenylalanine ammonia-lyase in a large animal model for phenylketonuria. *J. Pharmacol. Exp. Ther.* **224**, 598-602.

Bos, E. S., van der Doelen, A. A., van Rooy, N. and Schuurs, A. H. (1981). 3,3',5,5' -Tetramethylbenzidine as an Ames test negative chromogen for horse-radish peroxidase in enzyme-immunoassay. *J. Immunoassay.* **2**, 187-204.

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

Bourget, L. and Chang, T. M. (1986). Phenylalanine ammonia-lyase immobilized in microcapsules for the depletion of phenylalanine in plasma in phenylketonuric rat model. *Biochim. Biophys. Acta.* **883**, 432-438.

Bourget, L. and Chang, T. M. (1989). Effects of oral administration of artificial cells immobilized phenylalanine ammonia-lyase on intestinal amino acids of phenylketonuric rats. *Biomater. Artif. Cells Artif. Organs.* **17**, 161-181.

Chang, T. M., Bourget, L. and Lister, C. (1995). A new theory of enterorecirculation of amino acids and its use for depleting unwanted amino acids using oral enzyme-artificial cells, as in removing phenylalanine in phenylketonuria. *Artif. Cells Blood Substit. Immobil. Biotechnol.* **23**, 1-21.

Delgado, C., Francis, G. E. and Fisher, D. (1992). The uses and properties of PEG-linked proteins. *Crit. Rev. Ther. Drug Carrier Syst.* **9**, 249-304.

Fritz, R. R., Hodgins, D. S. and Abell, C. W. (1976). Phenylalanine ammonia-lyase. Induction and purification from yeast and clearance in mammals. *J. Biol. Chem.* **251**, 4646-4650.

Gilbert, H. J. and Jack, G. W. (1981). The effect of proteinases on phenylalanine ammonia-lyase from the yeast *Rhodotorula glutinis*. *Biochem. J.* **199**, 715-723.

Gilbert, H. J. and Tully, M. (1985). Protection of phenylalanine ammonia-lyase from proteolytic attack. *Biochem. Biophys. Res. Commun.* **131**, 557-563.

Greenwald, R. B., Choe, Y. H,, McGuire, J. and Conover, C. D. (2003). Effective drug delivery by PEGylated drug conjugates. *Adv. Drug Deliv. Rev.* **55**, 217-250.

Harris, J. M. and Chess, R. B. (2003). Effect of pegylation on pharmaceuticals. *Nat. Rev. Drug Discovery.* **2**, 214-221.

Hershfield, M. S., Chaffee, S., Koro-Johnson, L., Mary, A., Smith, A. A. and Short, S. A. (1991). Use of site-directed mutagenesis to enhance the epitope-shielding effect of covalent modification of proteins with polyethylene glycol. *Proc. Natl. Acad. Sci. USA.* **88**, 7185-7189.

Hoskins, D. S. (1968) The presence of a carbonyl group at the active site of L-phenylalanine ammonia-lyase. *Biochem. Biophys. Res. Commun.* **32**, 246-253.

Hoskins, J. A. and Gray, J. (1982). Phenylalanine ammonia-lyase in the management of phenylketonuria: the relationship between ingested cinnamate and urinary hippurate in

humans. *Res. Commun. Chem. Pathol. Pharmacol.* **35**, 275-282.

Hoskins, J. A., Jack, G., Wade, H. E., Peiris, R. J., Wright, E. C., Starr, D. J. and Stern, J. (1980). Enzymatic control of phenylalanine intake in phenylketonuria. *Lancet.* **23**, 392-394.

Mehvar, R. (2000). Modulation of the pharmacokinetics and pharmacodynamics of proteins by polyethylene glycol conjugation. *J. Pharm. Sci.* **3**, 125-136.

Monfardini, C., Schiavon, O., Caliceti, P., Morpurgo, M., Harris, J. M. and Veronese, F. M. (1995). A branched monomethoxypoly(ethylene glycol) for protein modification. *Bioconjug. Chem.* **6**, 62-69.

Sarkissian, C. N., Shao, Z., Blain, F., Peevers, R., Su, H., Heft, R., Chang, T. M. and Scriver, C. R. (1999). A different approach to treatment of phenylketonuria: phenylalanine degradation with recombinant phenylalanine ammonia lyase. *Proc. Natl. Acad. Sci. USA.* **96**, 2339-2344.

Scriver, C. R. and Kaufman, S. (2001). *The metabolic and molecular bases of inherited disease.* pp. 1667-1724. McGraw-Hill Press, New York.

Veronese, F. M. (2001). Peptide and protein PEGylation: a review of problems and solutions. *Biomaterials.* **22**, 405-417.

Veronese, F. M., Caliceti, P., Schiavon, O. and Sergi, M. (2002). Polyethylene glycol-superoxide dismutase, a conjugate in search of exploitation. *Adv. Drug Deliv. Rev.* **54**, 587-606.

Veronese, F. M., Monfardini, C., Caliceti, P., Schiavon, O., Scrawen, M. D. and Beer D. (1996). Improvement of pharmacokinetic, immunological and stability properties of asparaginase by conjugation to linear and branched monomethoxypoly (ethylene glycol). *J. Control Release.* **40**, 199-209.