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

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Abstract – Phenylketonuria (PKU) is an inherited metabolic disorder caused by mutations in the phenylalanine catabolic enzyme, phenylalanine hydroxylase (PAH). The use of phenylalanine ammonia-lyase (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) (Scrivan 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 concentration 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.

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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; Mehrvar, 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 native PAL and 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 L-phenylalanine in 100 mM Tris-Cl buffer, pH 8.5. The molar extinction coefficient of trans-cinnamic acid at 290 nm is 10.238 L·M$^{-1}$·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), carboxypeptidase 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) × 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
Table I. The specific activity of native PAL and pegylated PAL

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

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 $4.77 \times 10^{-4}$ M and that of pegylated PAL was $3.61 \times 10^{-4}$ 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 possessed 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 also optimal at pH 8.5 and pegylated PAL had similar stability 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.

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 activity (%) varying from 19.14 to 73.37 (Table II). Branched pegylated PAL (10 kDa, 1:32) was the most effective in maintaining its activity in the pancreatic protease mixture (Table II). The experiments with SDS-PAGE showed different patterns 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).
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 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 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 μg/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. 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).

**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 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 Vmax value and a decreased Km 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 end, may provide a new prospect for both oral and parenteral PAL therapies for patients with PKU.

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.

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REFERENCES


