Amino Acids (2005) 29: 283–287
DOI 10.1007/s00726-005-0218-5

Amino Acids
Printed in Austria

Phenylalanine ammonia-lyase modified with polyethylene glycol: Potential therapeutic agent for phenylketonuria

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Received April 1, 2005
Accepted May 4, 2005
Published online June 28, 2005;

Summary. Phenylketonuria (PKU) is an autosomal recessive genetic disease caused by the defects in the phenylalanine hydroxylase (*PAH*) gene. Individuals homozygous for defective *PAH* alleles show elevated levels of systemic phenylalanine and should be under strict dietary control to reduce the risk of neuronal damage associated with high levels of plasma phenylalanine. Researchers predict that plant phenylalanine ammonia-lyase (PAL), which converts phenylalanine to nontoxic *t*-cinnamic acid, will be an effective therapeutic enzyme for the treatment of PKU. The problems of this potential enzyme therapy have been the low stability in the circulation and the antigenicity of the plant enzyme. Recombinant PAL originated from parsley (*Petroselinum crispum*) chemically conjugated with activated PEG_2 [2,4-bis(*O*-methoxypolyethyleneglycol)-6-chloro-*s*-triazine] showed greatly enhanced stability in the circulation and was effective in reducing the plasma concentration of phenylalanine in the circulation of mice. PEG-PAL conjugate will be an effective therapeutic enzyme for the treatment of PKU.

Keywords: Phenylketonuria – Phenylalanine ammonia-lyase – Phenylalanine – Polyethylene glycol – Enzyme replacement therapy

Introduction

Phenylketonuria (PKU) is an autosomal recessive disorder of phenylalanine metabolism (Folling, 1994). PKU is characterized by abnormally high levels of phenylalanine in the circulation, and the absence or markedly reduced enzymic activities of phenylalanine hydroxylase (PAH) in the liver (Folling, 1994; Jervis, 1960). PAH converts phenylalanine to tyrosine and is responsible for the first step of the phenylalanine metabolism. Genetic defects of PAH cause the systemic accumulation of phenylalanine followed by its abnormal metabolism, which leads to the excretion of phenylpyruvic acid in the urine (Boscott et al., 1953). A high concentration of phenylalanine in the circulation is thought to be toxic to the central nervous system due to its inhibitory effect on catecholamine synthesis (McKean, 1972).

At present, a low-phenylalanine diet is the only effective remedy to prevent mental retardation, epilepsy and growth defects associated with PKU (Bickel et al., 1953; Woolf and Vulliamy, 1951). The dietary control, at high costs to affected children and to their families, needs to be continued for life because its interruption may lead to irreversible neurological disorders (Stark, 1998). Nevertheless, one study showed that the plasma phenylalanine levels of most PKU patients in late adolescence were above the recommended levels due to patients' difficulty in accepting the dietary control (Walter and White, 2004), so, an alternative therapy that ensures high quality of life of patients is needed.

Years ago, researchers proposed that the plant enzyme phenylalanine ammonia-lyase (PAL) might be a therapeutic agent for PKU (Ambrus et al., 1978). PAL catalyzes the conversion of phenylalanine to *t*-cinnamic acid, a harmless metabolite that can be rapidly excreted in urine as hippurate (Hoskins et al., 1984). As PAL does not require a coenzyme or cofactor, recombinant PAL will be a suitable agent to reduce the plasma phenylalanine to harmless levels. However, to develop an enzyme therapy using PAL, three major problems need to be resolved. First, large amounts of purified PAL with high specific activity need to be available. Second, as a plant enzyme, PAL needs to be tolerated by the immune system of PKU patients. Otherwise, the repeated administration of PAL

will induce antibody production against PAL, leading to the rapid removal of PAL activity and to harmful allergic reactions. Finally, PAL needs to be stable in the circulation to ensure the therapeutic effects over a long period.

Recently, a large amount of active recombinant parsley (*Petroselinum crispum*) PAL was prepared by using a designed gene and an expression system using molecular chaperone in *Escherichia coli* (Baedeker and Schulz, 1999). Therefore, in the present study, we applied a chemical modification technique (Inada et al., 1989) to render PAL non-immunogenic and stable in the circulation.

Materials and methods

Phenylalanine ammonia-lyase (PAL)

The nucleotide sequence of the *PAL* gene originating from parsley (*Petroselinum crispum*) was designed for translation in *Escherichia coli* by assembly polymerase chain reaction, and the recombinant PAL was expressed in *Escherichia coli* with a molecular chaperone as described (Baedeker and Schulz, 1999). PAL with specific activity of 20.2 units/mg was used for chemical modification.

Preparation of PEG-PAL

Activated PEG_2 [2,4-bis(*O*-methoxypolyethylene glycol)-6-chloro-*s*-triazine] (Ono et al., 1991) with a molecular weight of 10,000 was provided by Seikagaku Kogyo Co. (Tokyo, Japan). PEG-PAL was prepared as follows. To 2.0 ml of PAL (5.0 mg/ml) dissolved in 0.4 M borate buffer (pH 10.0) was added a given amount of activated PEG_2 (0, 100, 200 mg). The mixture (2.0 ml) was stirred at 37°C for 1 hr to complete the reaction. The protein concentration was determined by the Biuret method (Layne, 1957). The degree of modification was determined by measuring the amount of free amino groups in PEG-PAL with trinitrobenzene sulfonate (Habeeb, 1966). The immunoreactivity of PEG-PAL and native PAL was evaluated by measuring the binding ability towards anti-PAL antibodies by the competitive enzyme-linked immunosorbent assay (ELISA) (Nakamura, 1986).

Assay for enzyme activity

PAL activity was determined by monitoring the production of *t*-cinnamic acid at 290 nm. The reaction mixture contained sodium borate (33 mM, pH 8.7), L-phenylalanine and enzyme solution in a total volume 3.0 ml, and it was incubated at 30°C (Havir, 1981). The amount of *t*-cinnamic acid formed was calculated from the increase in absorbance using a molar extinction coefficient for *t*-cinnamic acid of 10^4 L cm^{-1} $mole^{-1}$ (Zucker, 1969). One unit of activity is defined as the amount of enzyme required for the formation of 1 μmol of product in 1 min under the assay condition.

Mice

BALB/c mice (female, 2 months old) were obtained from Sankyo Laboratory Service (Tokyo, Japan) and were maintained in the animal facility in Toin University of Yokohama. Mice were sacrificed using CO_2.

Pharmaco-kinetic studies

Native PAL and PEG-PAL were compared for their clearance rates as well as for their capacities to reduce phenylalanine concentrtion in the circulation of mice. For the clearance rates, 3.0 units of native PAL or PEG-PAL were injected intravenously. For the capacities to reduce plasma phenylalanine levels, 15 units of native PAL or PEG-PAL were injected intraperitoneally. At a given time, blood was taken from the orbital venous plexus using capillary tube and the PAL activity in the serum was determined. To determine the phenylalanine concentration in the blood, an aliquot of serum was deproteinated with 5-sulfosalicylic acid and then reacted with N-hydroxysuccinimidyl-6-aminquinolinyl carbamate, and the amount of labeled phenylalanine was measured with high performance liquid chromatography using an octadecyl silica gel column with the AccQ Tag™ amino acid analysis system (Waters Corporation, Milford, MA).

Results

Modification of phenylalanine ammonia-lyase (PAL) from parsley with polyethylene glycol (PEG)

Table 1 summarizes the chemical modification of PAL with activated PEG_2 [2,4-bis(*O*-methoxypolyethyleneglycol)-6-chloro-*s*-triazine]. The degree of modification of amino groups in PAL was enhanced by increasing the amount of activated PEG_2 added. The immunoreactivities of PAL conjugated with activated PEG_2 (PEG-PAL) decreased as the degree of modification increased. PEG-PAL, in which 23% of the total 41 amino groups in the PAL molecule were modified, lost its immunoreactivity toward the antibody nearly completely. Nevertheless, this

Table 1. Chemical modification of phenylalanine ammonia-lyase (PAL) with polyethylene glycol (PEG)

Molar ratio[a] ($PEG_2/-NH_2$)	Degree of modification[b] (%)	Enzymic activity[c] (%)	Immunoreactivity[d] (%)
0	0	100	100
6.3	2	58	31
12.5	23	15	0.8

Recombinant phenylalanine ammonia-lyase (PAL) from parsley (*Petroselinum crispum*) was conjugated with activated PEG_2 (MW: 10000) at different molar ratios in the reaction mixture as described in *Materials and methods*.

[a] Molar ratio of activated PEG_2 to ε-amino groups of a PAL molecule.

[b] Percentages of amino groups reacted with activated PEG_2. A PAL molecule has a total of 41 amino groups.

[c] Native PAL has a specific activity of 20.2 μmol/min mg of protein.

[d] Reactivity of PEG-PAL conjugates towards rabbit anti-PAL antibody as determined by competitive enzyme-linked immunosorbent assay.

preparation retained 15% of the enzymic activity. The following experiments were conducted using this preparation of PEG-PAL.

Plasma half-lives of native PAL and PEG-PAL

Native PAL or PEG-PAL was injected intravenously into BALB/c mice. Time courses of the clearance of native PAL and PEG-PAL are shown in Fig. 1. The half-lives of native PAL and PEG-PAL were 2 and 32 hrs, respectively, showing the markedly enhanced stability of PEG-PAL in the circulation. Seven days after the first injection, these mice were tested for the second injections. Native PAL was cleared more rapidly (half life <0.5 hr) at day 7 than at day 0. After the second injection, PEG-PAL showed a similar time-course of the clearance as occurred after the first injection (Fig. 1).

Effect of native PAL and PEG-PAL on the plasma phenylalanine levels

To compare the enzymatic effects on the plasma phenylalanine levels, native PAL or PEG-PAL was administered intraperitoneally into BALB/c mice. Figure 2A compares the changes of PAL activities in the plasma of mice administered either native PAL or PEG-PAL. In mice treated with native PAL, PAL activity appeared to reach a transient peak at 6 hr after the administration, and it became undetectable at 12 hr. In mice administered with PEG-PAL, although the activity appeared with a slower kinetics, the PEG-PAL remained stable in the circulation. A half-maximum level of PAL activity was observed even at 48 hr. Figure 2B shows the time courses of the plasma phenylalanine concentrations in mice treated with native PAL or PEG-PAL. In mice treated with native PAL, the plasma phenylalanine concentration became undetectable soon after the administration. Subsequently, nearly normal levels of plasma phenylalanine levels were recovered within 2 days. In contrast, in mice treated with PEG-PAL, plasma phenylalanine concentration decreased more slowly than in mice injected with native PAL, and reached 10% of the initial level 24 hrs after injection. Subsequently, low levels of plasma phenylalanine concentrations (<25%) were maintained even 72 hrs after injection.

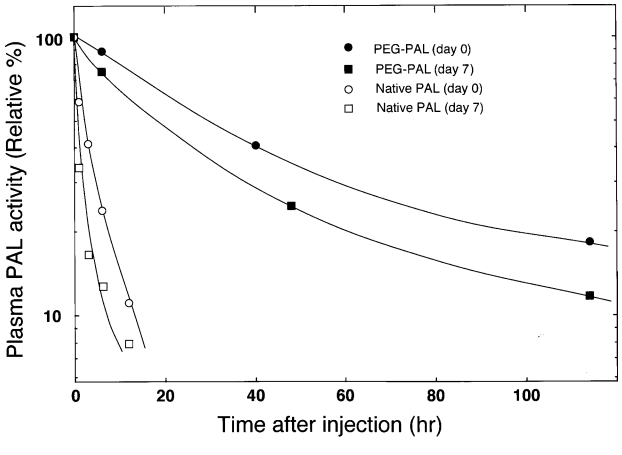

Fig. 1. Clearance rates of native phenylalanine ammonia-lyase (native PAL) and polyethylene glycol-modified PAL (PEG-PAL) in the circulation of BALB/c mice. Three units of native PAL or PEG-PAL were injected intravenously at days 0 and 7. Time courses of the serum PAL activities after the first (day 0) and the second injections (day 7) are shown

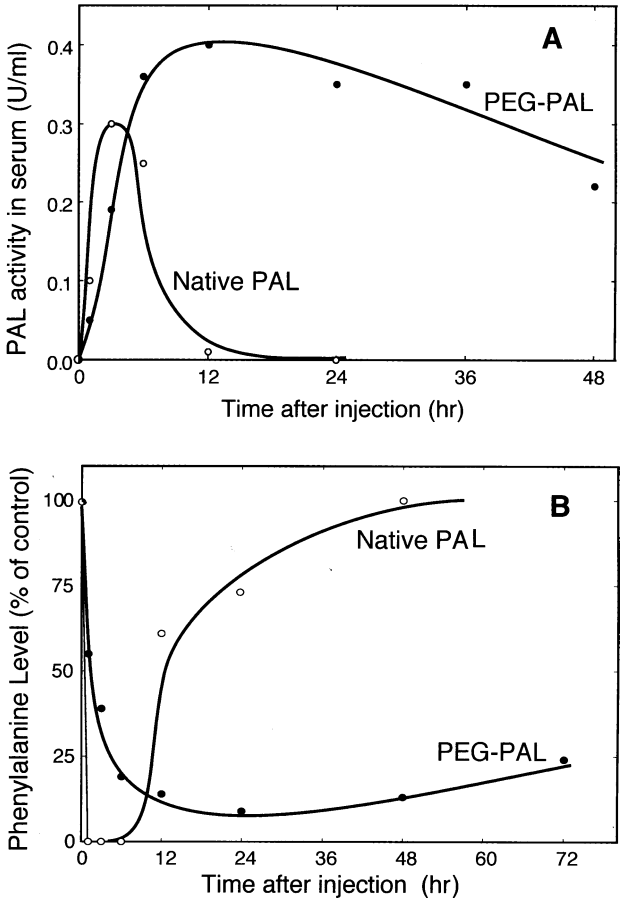

Fig. 2. Effects of native PAL and PEG-PAL on the plasma phenylalanine levels. BALB/c mice received 15 units of either native PAL or PEG-PAL. (**A**) Time courses of phenylalanine ammonia-lyase (PAL) activity in sera of mice after intraperitoneal injection of native PAL (open circles) or polyethylene glycol-modified PAL (PEG-PAL) (closed circles). (**B**) Time courses of the serum phenylalanine concentrations in mice after intraperitoneal injection of native phenylalanine ammonia-lyase (native PAL) (open circles) or polyethylene glycol-modified PAL (PEG-PAL) (closed circles)

Discussion

PEG is a linear, non-toxic and non-immunogenic synthetic polymer. It is well established that covalent attachment of PEG enhances the plasma half-lives of foreign proteins. When L-asparaginase with anti-tumor activity was conjugated with PEG, patients with acute lymphoblastic leukemia were successfully treated with PEG-asparaginase (Yoshimoto et al., 1986). Similarly, severe combined immunodeficiency in patients with genetic defects of the adenosine deaminase (ADA) gene was ameliorated by treatment with ADA conjugated with PEG (Davis et al., 1981; Hershfield, 1995).

In the present study, we tested the possibility that PEG-PAL may be used as a therapeutic agent for PKU. The plasma half-life of PEG-PAL was 16 times as long as that of native PAL in mice when injected intravenously (Fig. 1). Effects of native PAL and PEG-PAL on plasma phenylalanine levels were compared in mice injected intraperitoneally with these enzymes. In these mice, time courses of the change of the serum enzymic activity reflected two processes, transfer of these enzymes from the peritoneum to the circulation, and their subsequent clearance from the circulation (Fig. 2A). Native PAL appeared rapidly in the circulation. However, its effect on plasma phenylalanine level was only transient due to its rapid clearance. PEG-PAL appeared slowly in the circulation presumably due to its increased molecular weight. Nevertheless, the effect of PEG-PAL on the plasma phenylalanine level was much more intense than native PAL due to its higher stability in the circulation. In mice received PEG-PAL, low levels of plasma phenylalanine ($<0.4\,mg/dl$) were observed for more than 72 hrs (Fig. 2B). This observation is encouraging, since the recommended level of plasma phenylalanine is between 2 and $4\,mg/dl$ in infants with PKU under dietary control.

To our knowledge, this is the first demonstration that plasma phenylalanine levels can be successfully modulated by the administration of an exogenous enzyme. In 1979, Wieder et al. first reported the conjugation of PAL from yeast with PEG and proposed its potential to be used as an anti-tumor enzyme. Recently, Gamez et al. (2004) reported the conjugation of recombinant phenylalanine hydroxylase (PAH) with PEG and suggested that PEG-PAH may be used in enzyme replacement therapy of PKU. However, modulation of the plasma phenylalanine level was demonstrated by none of these previous conjugates.

As PAL is a plant enzyme, the problem associated with the therapy using PEG-PAL may be the immunogenicity of PAL. In repeated injection of native PAL, half life in the circulation was markedly reduced (Fig. 1) and anti-PAL antibody was detected in the sera of mice (data not shown). In the case of repeatedly injection with PEG-PAL, the half-life was not shortened markedly at day 7 (Fig. 1). It was likely that reduced immunoreactivity of PEG-PAL towards antibody contributed to the stability of PEG-PAL in the repeated injection. It is well established that PEG-protein antigen conjugates do not elicit immune response towards the protein antigen, but rather induce immune tolerance specific to the protein antigen (Lee and Sehon, 1977; Kawamura et al., 1985; Saito et al., 2000). We demonstrated that PEG-protein antigen conjugate induces the clonal deletion in the thymus of antigen-specific Th cells that help the antibody production by antigen-specific B cells (Saito et al., 2000). Therefore, PEG-PAL may be optimized for the immune tolerance induction by either chemical and/or genetic modification of PAL, and will be administered safely to PKU patients without the risk of harmful allergic reactions. PEG-PAL may also be administered orally, which is preferable to intravenous injection for PKU patients. Further studies are ongoing in our laboratory to explore these possibilities.

Acknowledgement

We would like to thank Bärbel Dirr (Institut für Organische Chemie und Biochemie, Albert-Ludwigs-Universität Freiburg) for technical help. This work was supported by the Science Research Promotion Fund from the Promotion and Mutual Aid Corporation for Private Schools of Japan.

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