Biochemical Characterizations of Phenylalanine Ammonia-Lyase and its Mutants to Develop an Enzymatic Therapy for Phenylketonuria

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Enzyme substitution with recombinant phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) is currently being explored for treatment of phenylketonuria (PKU), an autosomal recessive genetic disorder with mutations of the gene encoding phenylalanine-4-hydroxylase (EC 1.14.16.1). However, oral administration of PAL is limited because of proteolytic digestion in the gastrointestinal tract. The aim of this study was to determine the biochemical properties of PAL and delineate the susceptibility of wild-type PAL to pancreatic proteolysis by exploring several mutants, and to develop therapeutic drugs with PAL for PKU. The specific activity of PAL was assayed and its optimal pH, temperature stability, and intestinal protease susceptibility were investigated. Its $V_{max}$ values for phenylalanine and tyrosine were 1.77 and 0.47 μmol/min/mg protein, respectively, and its $K_m$ values were $4.77 \times 10^3$ and $4.37 \times 10^3$ M, respectively. PAL showed an optimal pH at 8.5, corresponding to the average pH range of the small intestine. It showed no loss of activity at 80°C for 5 months and possessed 93.4% of its activity under 4°C for 4 wks. PAL was susceptible to chymotrypsin digestion and, to a lesser extent, to trypsin, elastase, carboxypeptidase A, and B. The trypsin and chymotrypsin cleaving sites were mutated to investigate protection from pancreatic digestion and the specific activities of these mutants were evaluated. The six mutants displayed low specific activities compared to the wild-type, suggesting that the primary trypsin and chymotrypsin cleaving sites may be essential for catalytic reaction. The PAL mutants could therefore be applied as a pretreatment modality without susceptibility to proteolytic attack, however, additional modification for enhancing enzymatic activity is needed to reduce the Phe levels effectively.

Key words: Phenylketonuria, phenylalanine ammonia-lyase, chymotrypsin, trypsin, proteolysis

Introduction
Phenylketonuria (PKU) is an inborn error of amino acid metabolism resulting from impaired activity of hepatic phenylalanine hydroxylase (PAH), the enzyme responsible for phenylalanine metabolism [8,19]. Patients with PAH mutations, leading to PKU and hyperphenylalaninemia, display impaired neurophysiological functioning and reduced cognitive development [20,22]. The current treatment regime for PKU involves adherence to a life-long restricted diet low in protein and especially the amino acid phenylalanine. This dietary therapy is difficult to maintain and does not always eliminate the damaging neurological effects that can be caused by elevated phenylalanine levels [7,14,16,24]. Therefore, a novel therapeutic modality would assist the current dietary therapy and prevent the neurological damages seen in individuals with PKU, particularly for those patients with the severest forms. Enzyme substitution therapy with recombinant phenylalanine ammonia-lyase (PAL) is currently being explored. PAL is indicated for irreversible elimination of L-phenylalanine from the diet of new borns with PKU [11,13,17]. PAL requires no cofactors for degrading Phe, and trans-cinnamate (a PAL product) has shown very low toxicity and no embryotoxic effects in experimental animals [13]. Trans-cinnamic acid is converted to benzoic acid in the liver, which is then excreted via the urine mainly as hippurate [11,13]. Although PAL potentially has various therapeutic applications, its use may be limited by the paucity of pharmacological knowledge regarding this enzyme. For example, oral administration of PAL may be limited by proteolytic digestion in the gastrointestinal tract. Therefore, characterization of the its pH, temperature stability, substrate affinity, and intestinal protease susceptibility is required.

Materials and Methods
PAL activity
The activity of PAL from Rhodospirillum toruloheides was

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assayed by using a Cary UV spectrophotometer (Cary 50) in kinetics mode. The activity of PAL using L-phenylalanine as substrate was assayed at room temperature by measuring the production of trans-cinnamate in terms of increase in the absorbance at 290 nm. The molar extinction coefficient of cinnamic acid at 290 nm is 10.2381 L/M/cm. The reaction mixture contained 22.5 mM phenylalanine in 100 mM Tris-HCl buffer (pH 8.5). The activity of one unit of PAL was defined as the amount of enzyme producing 1 mole of trans-cinnamic acid per minute at room temperature (mol/min/mg protein). To measure the PAL activity for tyrosine, 0.176-1.406 mM of L-tyrosine in Tris-HCl buffer (pH 8.5) was used. The absorbance of trans-coumaric acid was monitored at 315 nm. The molar extinction coefficient of coumaric acid at 315 nm is 10.001 L/M/cm. For standard measurements the final enzyme concentration was 0.0035 mg/ml, but for the kinetic studies, the enzyme concentration in the assay was adjusted so that the slope at 290 nm / min was in the range of 0.005- 0.02.

Protease susceptibility of PAL

Intestinal proteases (Sigma), such as trypsin, chymotrypsin, elastase, carboxypeptidase A, and B were used for evaluating time-dependent proteolysis. Wild-type PAL and different concentrations of proteases were incubated at room temperature and 37°C for 2-90 min. After incubation, the residual activity was assayed and SDS-PAGE was performed for detecting the digested fragments of native PAL after proteolysis.

SDS-PAGE

Gel electrophoresis in 4-12% NuPAGE Bis-Tris gel with 1× 3-(N-morpholino) propane sulfonic acid (MOPS) -SDS buffer (pH 7.7) was performed. Approximately 17 μg of PAL was incubated with each protease in 60 μl of reaction mixture at 37°C for 10 and 30 min, followed by addition of 11 μl of 4× LDS sample buffer and 0.5 M DTT. The gels were run for 1 h at 195 V and then stained with Coomassie G-250.

Mutation of the trypsin and chymotrypsin cleavage sites

Site-directed mutagenesis was performed using the Quik Change kit (Stratagene, U.S.). Trypsin and chymotrypsin recognition sites of PAL were mutated. Mutations in the trypsin site were Arg123His, Arg123Ala and Arg123Gln, and in the chymotrypsin site were Tyr110His, Tyr110Ala and Tyr110Leu.

Results

Biochemical characteristics of PAL

The V_max values of PAL for phenylalanine and tyrosine were 1.77 and 0.47 μmol/min/mg protein, respectively, and the K_m values were 4.77×10^{-4} and 4.37×10^{-4} M, respectively (Fig. 1). The enzymatic activities were assayed at various pH levels at room temperature. Wild-type PAL showed an optimal pH at 8.5. It showed a broadening of the optimal pH 9.5 and still had 33% of its pH 8.5 activity at pH 10.5 (Fig. 2). PAL was stored at 4°C and -80°C in closed bottles, and the residual enzymatic activity was measured for 4 wks. PAL stored at -80°C for 4 weeks did not show any change in activity and that stored at 4°C for 4 wks, still possessed 93.4% of its original activity. No loss of activity was detectable in the stock solutions (7 mg/ml) stored at -80°C for 5 months. The PAL activity was stable for 1 hr in Tris-HCl buffer (pH 8.5) at room temperature.

![Fig. 1. Lineweaver-Burk plots of PAL activities for phenylalanine (a) and tyrosine (b).](image-url)
Evaluation of PAL susceptibility against pancreatic proteases

To determine the effects of proteases on PAL activity, the enzyme in reaction buffer was incubated with various concentrations of trypsin, chymotrypsin, elastase, carboxypeptidase A, and B for 10 min and 30 min (Fig. 3, Table 1). Wild-type PAL showed no activity when incubated with chymotrypsin (30 μg/ml) for 10 min (Table 1). PAL was susceptible to chymotrypsin digestion and a lesser extent to trypsin, elastase, carboxypeptidase A, and B. In the case of trypsin digestion, wild-type PAL was not completely digested at low protease concentration (Fig. 3). In contrast, digestion with chymotrypsin was completed within 10 min (Fig. 3).

Trypsin and chymotrypsin cleaving sites mutants

Mutants of the trypsin and chymotrypsin primary recognition sites were created by site-directed mutagenesis: R123H, R123A, and R123Q for the trypsin site, and Y110H, Y110A, and Y110L for the chymotrypsin site. All the mutants were expressed at levels similar to the wild-type protein and did not show any sign of aggregation. Testing for protease susceptibility indicated that the point mutations provided protection to the three R123 mutants (the intact protein size were retained even on trypsin exposure) and three Y110 mutants (low activity negated any observations of about residual activity, but SDS-PAGE analysis indicated retention of intact protein size upon chymotrypsin incubation). All the mutants had decreased activity relative to wild-type PAL (Table 2), however, reduced proteolytic susceptibility was evident in numerous samples (as determined by SDS-PAGE analysis of PAL mutant protease digestions).

<table>
<thead>
<tr>
<th>Pancreatic protease</th>
<th>Concentration (μg/ml)</th>
<th>Residual activity of PAL (%)*</th>
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</thead>
<tbody>
<tr>
<td>No protease</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Trypsin</td>
<td>10</td>
<td>90.5</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>81.1</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>72.2</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>36.5</td>
</tr>
<tr>
<td>Elastase</td>
<td>10</td>
<td>93.3</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>87.3</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>77.1</td>
</tr>
<tr>
<td>Carboxypeptidase A</td>
<td>10</td>
<td>95.5</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>92.0</td>
</tr>
<tr>
<td>Carboxypeptidase B</td>
<td>10</td>
<td>93.2</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>88.6</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>78.4</td>
</tr>
</tbody>
</table>

*The residual activity of PAL was calculated by measuring the remaining activity of PAL after 10 min incubation with each protease.

Discussion

Numerous proteins are used therapeutically to alleviate metabolic deficiencies caused by genetic disorders [18]. The
Table 2. Specific activities of the various PAL mutants

<table>
<thead>
<tr>
<th>PAL / mutant</th>
<th>Specific activity (μmol/mg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAL</td>
<td>1.32</td>
</tr>
<tr>
<td>R123A</td>
<td>0.11</td>
</tr>
<tr>
<td>R123H</td>
<td>0.074</td>
</tr>
<tr>
<td>R123Q</td>
<td>0.033</td>
</tr>
<tr>
<td>Y110H</td>
<td>0.084</td>
</tr>
<tr>
<td>Y110A</td>
<td>0</td>
</tr>
<tr>
<td>Y110L</td>
<td>0</td>
</tr>
</tbody>
</table>

efficacy of protein therapeutics can be enhanced by biochemical and enzymatic characterization, leading to improved stability, activity, and/or altered activity [5]. PAL (EC 4.3.1.5) is a non-mammalian enzyme widely distributed in plants, some fungi, and can also be recombinantly produced in Escherichia coli [1,5,9,15,21]. Numerous studies have focused on the application of PAL to enzyme substitution treatment for PKU [10-12,17,21-23]. In 1999, Sarkissian and colleagues reported their initial studies on the use of PAL from Rhodosporidium toruloides for PKU enzymatic substitution [23]. PAL from Rhodosporidium toruloides requires full characterization to elucidate the optimal conditions for achieving the best catalytic performance in enzymatic replacement therapy. Although this treatment has promising effects, the PAL activities for both Phe and Tyr compared with those of PAH may be offsets. PAL catalyzes the deamination of both Phe and Tyr to form trans-cinnamic acid and trans-coumaric acid, respectively. Abell et al. have reported that the Vₘₚ values of PAL from Rhodotorula glutinis for phenylalanine and tyrosine are 1.47 and 0.7 μmol/min/mg protein, respectively [1]. In the current study, PAL was obtained from Rhodosporidium toruloides and its Vₘₚ values for phenylalanine and tyrosine were 1.77 and 0.47 μmol/min/mg protein, respectively. Although the Vₘₚ of PAL from Rhodosporidium toruloides for Phe is higher than that from Rhodosporidium glutinis, the Vₘₚ of PAL from Rhodosporidium toruloides for Tyr is lower than that from Rhodosporidium glutinis. These results indicated that PAL from Rhodosporidium toruloides has greater affinity for Phe than from of Rhodosporidium glutinis, and it may be better to use PAL from Rhodosporidium toruloides for enzymatic replacement therapy. The Vₘₚ value for Phe was significantly higher than that for Tyr, indicating that the affinity for Phe is substantially stronger than that for Tyr, and therefore, the catalytic effect exerted on Tyr is negligible. PAL therapy lowers the Tyr concentration in PKU patients to a small extent, and the role of Tyr in the pathogenesis and therapeutics of PKU is a matter of continued debate. However, Tyr should still be supplemented along with PAL therapy in PKU patients.

With the ultimate aim of developing an optimized form of PAL for clinical application, it is important to understand the baseline activity and stability of PAL under both working and storage conditions. Purified PAL from Rhodotorula toruloides at a concentration of 7 mg/ml showed no loss of activity at -80°C for 5 months and still possessed 93.4% of its activity under 4°C for 4 wks. PAL was very stable under wide temperature ranges, whereas recombinant PAH loses its activity rapidly upon production and purification in E.coli [7,19]. The optimal pH of PAL (pH 8.5) corresponds to the average pH range of the small intestine. The optimal pH of PAL from Rhodotorula glutinis and Rhodotorula rubra were 8.75 and 8.0, respectively [1,3]. At physiological pH (7.5), the activity of PAL is about 51% of the maximum. For investigating oral administration of PAL, both enzymatic activity and its stability should be evaluated in gastrointestinal fluid. PAL from Rhodosporidium toruloides reportedly has no activity at pH 2.2 and a half-life of 3.5 min in duodenal juice [11].

The effects of pancreatic proteases such as trypsin, chymotrypsin, elastase, carboxypeptidase A, and B on PAL activity allowed evaluation of the susceptibility in the duodenum. Inactivation of PAL in pancreatic proteases was due to the enzymatic susceptibility to chymotrypsin rather than to trypsin, elastase, carboxypeptidase A, and B. To preserve the PAL activity in intestinal fluids, PAL must be protected from chymotrypsin trypsin digestion, therefore, pretreatment is necessary mainly against chymotrypsin and trypsin. The primary trypsin site of PAL was identified as Arg123, and the primary chymotrypsin sensitive site was identified as Tyr110 [2,6]. Stabilization of the protein by removal of these sites could be used for oral PKU therapy. These sites were mutated and six mutants were created (R123H, R123A, R123Q, Y110H, Y110A, Y110L). All six mutants displayed low specific activity. The primary trypsin and chymotrypsin sites might be essential for the catalytic reaction. Accordingly, additional modifications for enhancing the enzymatic activity are needed to reduce the Phe levels effectively. Therefore, further processing is necessary for clinical application of PAL.

References

1. Abell, C. W. and R. S. Shen. 1987. Phenylalanine ammoo-
초록: 페닐케톤노증의 효소처리 개발을 위한 phenylalanine ammonia-lyase 및 유전자 변이형의 생화학적 특성

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페닐케톤노증은 상염색체 열성으로 진단되며, phenylalanine-4-hydroxylase (PAH, EC 1.14.16.1)의 돌연변이에 의해 효소 활성화를 초래하는 질환이다. 최근 유전자 재조합된 phenylalanine ammonia-lyase (PAL)의 이용 효소 대체요법이 보고된 바 있다. 이 효소를 경구용 약제로 개발하기 위하여 효소활성을 나타내기 위한 촉매 조건들을 알아야 하며, 의장관내 소화효소에 의해 분해되지 않는 구조적 안정성을 유지하여야 한다. 따라서 본 연구에서는 PAL의 생화학적 특성을 규명하고, 이를 바탕으로 의장관내 소화효소로부터 저항할 수 있는 변이형들을 만들고자 하였으며, 이러한 구조적 변화를 통하여 효소의 특이 활성도가 유지될 수 있는지를 보고자 하였다. PAL의 특이 활성도를 측정하였고, 효소 활성을 나타내기 위한 최적 pH, 온도 변화에 따른 효소 활성도, 단백분해효소에 의한 활성도 변화를 측정하였다. PAL의 Vmax은 폐널알란닌과 토로신에 대하여 각각 1.77, 0.47 μmol/ mg x protein으로 나타났으며, Km은 폐널알란닌에 대하여 4.77×10⁻⁴ M, 토로신에 대하여 4.37×10⁻⁴ M로 나타났다. 또한 pH 8.5에서 가장 높은 활성을 나타내었는데, 이는 소장의 평균 pH와 유사하다. PAL의 효소 활성은 -80°C에서 5개월 동안 유지되었으며, 4°C에서 1주일 동안 93.4%의 활성을 유지하였다. PAL은 킴트립신에 의해 쉽게 분해되었으며, 이를 약한 절도 트립신, elastase, carboxypeptidase A, B에 의해 분해되었다. 혈장 소화효소에 대한 저항성을 증가시키기 위하여 트립신, 킴트립신 절단부위 아미노산을 변이시키 유전자 변이형을 만들었고, 효소 활성도를 측정하였다. 6개의 유전자 변이형은 모두 저하된 효소 활성도를 나타내었는데, Y110H는 0.084, Y110A와 Y110L은 0, R123A는 0.11, R123H는 0.074, R123Q는 0.033으로 나타났다. 이러한 결과는 트립신 및 킴트립신 절단부위 아미노산이 PAL의 효소 활성에 필수적인 역할을 하고 있음을 나타낸다. PAL 변이형은 단백분해작용으로부터 보호할 수 있는 전처치 방법이지만, 페닐알란닌을 효과적으로 저하시키기 위해서 효소활성을 유지할 수 있는 다음 단계의 처치가 필요하다.