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Improved Substrate Specificity of Phenylalanine Dehydrogenase for L-Phenylalanine Sensor

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L-Phenylalanine (L-Phe), which is one of the essential amino acids, is important for the human body because it is used in the biosynthesis of neurotransmitters. Phenylalanine dehydrogenase (PheDH) has been studied as a material for the L-Phe sensor. In particular, the accurate and simple determination of L-Phe concentrations in human samples is needed for the diagnosis and treatment of patients with phenylketonuria. Thermostable PheDH from the bacterium *Thermoactinomyces intermedius* is suitable as a sensor material; however, it is reactive to not only L-Phe but also L-tyrosine (L-Tyr). Thus, we attempted to improve its substrate specificity for the accurate measurement of L-Phe. In this study, we developed the PheDH mutant with improved substrate specificity by amino acid substitution. The PheDH mutant, in which the 290th asparagine residue was substituted with aspartic acid (N290D), is considered to form a new interaction in the substrate binding site and has a markedly reduced reactivity with L-Tyr compared with the wild-type PheDH; the relative activities (L-Tyr/L-Phe) of the wild-type PheDH and the PheDH N290D mutant were 64.0 and 1.5%, respectively. An enzymatic assay using this mutant was established to quantify the L-Phe concentration in human plasma. The relative errors ranged from −10.3 to 7.4% when compared with the quantitative value determined by high-performance liquid chromatography/electrospray ionization mass spectrometry. Furthermore, the immobilized mutant showed a concentration-dependent reactivity with L-Phe and no reactivity with L-Tyr, which indicate its potential use as a sensor material.

1. Introduction

Amino acids are essential components in the body, and information on the concentrations of amino acids in the body can be an important indicator of health. For example, the molar ratio of free branched-chain amino acids (valine, leucine, and isoleucine) to aromatic amino acids (phenylalanine and tyrosine, Fischer's ratio) or tyrosine (branched chain amino acid and tyrosine ratio) in plasma is used as an indicator of liver function.[1–3] The quantifications of leucine and

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methionine in the blood are diagnostic methods for inborn errors of amino acid metabolism, such as maple syrup urine disease and homocystinuria, respectively.[4–6] Thus, an accurate and simple method of quantifying amino acids is important for disease diagnosis.

L-Phenylalanine (L-Phe) is one of the essential amino acids contained in breast milk and various foods. It is important because it is converted to tyrosine in the human body and used in the biosynthesis of neurotransmitters.[7] However, patients with phenylketonuria (PKU), one of the inborn errors of metabolism, have elevated L-Phe levels in biological fluids due to reduced phenylalanine hydroxylase activity, which is known to cause growth failure and irreversible neurological damage if untreated.[7,8] Therefore, the accurate and simple quantification of L-Phe is very important in neonatal mass screening and point-of-care testing during treatment monitoring.

Phenylalanine dehydrogenase (PheDH, EC 1.4.1.20) has been studied as a material for PKU sensors.[7,8] This enzyme catalyzes the oxidation of L-Phe using the oxidized form of *β*-nicotinamide adenine dinucleotide (NAD^+) as a cofactor. Since NAD^+ is converted to the reduced form (NADH) during enzyme reaction, the concentration of L-Phe can be determined by an enzymatic method using a water-soluble tetrazolium salt and an electron mediator.[9] For the commercial use of enzymes, enzyme stability is one of the important factors to consider when developing an enzymatic analysis method.[10] Therefore, we focused on thermostable PheDH from the bacterium *Thermoactinomyces intermedius* as a material for sensors.[11,12] However, by confirming the substrate specificity, we showed that PheDH was also active against L-tyrosine (L-Tyr), which is structurally similar to L-Phe.[13,14] In previous studies, PheDH was investigated to improve its substrate specificity by amino acid substitutions, but these mutants had low activity for L-Phe or still had activity for L-Tyr.[13,14] Therefore, we considered that thermostable PheDH would be more suitable as a sensor material for accurate L-Phe analysis if its substrate specificity is significantly improved while maintaining its activity.

In this report, amino acid substitution was used to achieve low substrate specificity with L-Tyr based on a model structure constructed by a computational method. The engineered PheDH mutant showed very low substrate specificity with L-Tyr. This result demonstrates the possibility of developing a colorimetric method to accurately analyze the L-Phe concentrations in human plasma samples. Furthermore, the PheDH mutant immobilized on a plate also maintained its substrate specificity and showed concentration-dependent activity against L-Phe without reacting with L-Tyr, indicating its potential for use as a sensor material.

2. Materials and Methods

2.1 Structure model building of PheDH

The complex structure model of PheDH from *T. intermedius*, NADH, and L-Phe was constructed by homology modeling as a template for the X-ray crystal structure of PheDH from *Rhodococcus* sp. (PDB ID: 1C1D) using BIOVIA Discovery Studio software (San Diego, CA, USA). PheDH from *Rhodococcus* sp., which is the only PheDH with reported X-ray crystal structures,[15,16] has the highest homology to PheDH from *T. intermedius* among the known

complex structures containing a substrate, with 33% identity and 49% similarity. BIOVIA Discovery Studio was also used for estimating the minimum distances and interactions between any atom pair of amino acids and L-Phe. The mutants were designed by observing the substrate binding site and using an in-house mutant design program.

2.2 Cloning and mutagenesis of PheDH

The amino acid sequence of wild-type PheDH from *T. intermedius* (UniProt ID: P22823) was reverse-translated to DNA using GENEius software (Eurofins Genomics K.K., Tokyo, Japan). For cloning into the pET-24a(+) vector (Merck, Darmstadt, Germany) at *Nde*I and *Bam*HI sites and adding the His-tag to the N-terminus of this construct, the sequences 5'-catatgcatcaccatcaccaccac-3' and 5'-taatgaggatcc-3' were added to the 5' and 3' termini of the PheDH sequence, respectively. The construct was chemically synthesized (Eurofins Genomics K.K., Tokyo, Japan). The PheDH mutant, in which the 290th asparagine residue (Asn290) was substituted with aspartic acid (N290D), was prepared by amino acid substitution using KAPA HiFi HS ReadyMix (Nippon Genetics, Tokyo, Japan). *Escherichia coli* XL10-Gold cells were transformed with plasmids for plasmid purification.

2.3 Expression and purification of PheDH for mutant screening

E. coli BL21 (DE3) cells were transformed with plasmids and colonies were inoculated into lysogeny broth medium containing 25 μg/mL kanamycin at 37 ℃. His-tag fusion proteins were expressed by induction using 0.5 mM isopropyl-β-D-thiogalactopyranoside when the optical density at 600 nm reached 0.8 to 1.0. The culture temperature during induction was maintained at 30 ℃. The cells were incubated overnight and harvested by centrifugation. The cells were washed using 0.9% (w/v) NaCl, the NaCl solution was removed by centrifugation, and the cells were resuspended in lysis buffer [50 mM Tris-HCl (pH 9.0), 1000 mM NaCl, and 50 mM imidazole] and disrupted for 20 min at 180 W with an ultrasonic disintegrator (ISONATOR 201M, Kubota, Tokyo, Japan). The extract was centrifuged for 10 min at 15000 × *g*. The supernatant was loaded onto an open column packed with 5 mL of Ni Sepharose 6 Fast Flow resin (Cytiva, Tokyo, Japan), and the resin was washed with lysis buffer to elute unbound proteins. The bound proteins were eluted with 50 mM Tris-HCl (pH 9.0), 1000 mM NaCl, and 500 mM imidazole. The eluted protein solution was loaded onto a HiPrep 26/10 desalting column (Cytiva) equilibrated with 100 mM Tris-HCl (pH 8.0) and 0.1 mM ethylenediaminetetraacetic acid (EDTA) using an ÄKTA Explorer 10S system (Cytiva). Target proteins were stored at 4 ℃ until use.

2.4 Enzymatic assay

PheDH activity and substrate specificity were measured by a colorimetric assay. The reaction mixture contained 100 mM Gly-KCl-KOH (pH 10.0), 1 mM NAD^+, 10 μg/mL PheDH solution, and 1 mM substrate solution in a total volume of 200 μL. L-Phe, L-Tyr, D-Phe, L-tryptophan,

L-histidine, L-methionine, L-ethionine, L-valine, L-leucine, L-isoleucine, L-*allo*-isoleucine, L-alanine, L-2-aminobutyrate, L-norvaline, and L-norleucine were used as substrates. The absorbance values at 340 and 800 nm were measured at 37 ℃ for 5 min using a microplate reader (Molecular Devices, San Jose, CA USA). The absorbance value change was determined as the absorbance value at 340 nm minus the absorbance value at 800 nm.

2.5 Human plasma analysis

Three samples of human plasma were purchased from Kohjin Bio (Saitama, Japan) and used in this study (Table 1). Frozen plasma was thawed and centrifuged to remove precipitates immediately before use. The reaction mixture contained 100 mM Tris-HCl (pH 8.0), 1 mM NAD^+, 1 mM 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8; Dojindo Laboratories, Kumamoto, Japan), 10 U/mL diaphorase (TOYOBO, Osaka, Japan), 1 mM EDTA (pH 8.0), 0.1% (w/v) sodium oxamate, 100 mM NaCl, 0.1% (v/v) polyoxyethylene sorbitan monolaurate (Tween20), the PheDH N290D mutant solution, and the substrate solution in a total volume of 300 μL. The PheDH N290D mutant amounts of 200, 300, 400, and 500 μg/mL were investigated at the L-Phe substrate concentration of 400 μM, and finally, 500 μg/mL was adopted. L-Phe solutions of 0, 25, 50, 100, 200, and 400 μM concentrations were used for the preparation of a standard curve. The substrate solutions (L-Phe or human plasma) were mixed with a reaction mixture at a 50-fold dilution. The reaction was initiated by adding the enzyme to the reaction. The absorbance values at 450 and 800 nm were measured at 37 ℃ for 5 min using a microplate reader to reach the endpoint of the reaction. The absorbance value change was determined as the absorbance value at 450 nm minus the absorbance value at 800 nm. The detection limit of the analysis procedure was calculated as an average value plus 3.3 times the standard deviations of the reaction mixture with 0 μM L-Phe. To evaluate the performance of the enzymatic assay, instrumental and enzymatic analysis values were compared. Instrumental analysis was performed using high-performance liquid chromatography/electrospray ionization mass spectrometry (HPLC/ESI–MS, UF-Amino Station; Shimadzu Corporation, Kyoto Japan).[17]

2.6 Enzyme immobilization in microplate

Three hundred microliters of the PheDH N290D mutant at 50 μg/mL was added per well of Clear Flat-Bottom Immuno Nonsterile 96-Well Plates #439454 (Thermo Fisher Scientific, MA, USA). The temperature during overnight immobilization was kept at 4 ℃. The solution of the PheDH N290D mutant was removed from each well, these wells were then washed with 350 μL

Table 1
Plasma samples used in this study.

Plasma	Donor(s)	Amino acid concentration (μM)	
		L-Phe	L-Tyr
A	Multiple donors (20 individuals)	60.5	67.9
B	Single donor	70.9	67.7
C	Single donor	38.9	46.5

of wash buffer [50 mM sodium phosphate buffer (pH 8.0), 200 mM NaCl, and 0.05% (v/v) Tween20], and the wash buffer was removed. Reaction mixtures contained 100 mM Tris-HCl (pH 8.0), 1 mM NAD^+, 1 mM WST-8, 10 U/mL diaphorase, 0.1% (v/v) Tween20, 100 mM NaCl, and substrate solution in a total volume of 300 µL. L-Phe or L-Tyr was used as a substrate. The substrate concentration range was 0–200 µM, and the substrate solutions were mixed with the reaction mixture at a 10-fold dilution. Absorbance value changes at 450 and 800 nm were measured at 37 °C for 30 min using the microplate reader. The absorbance value change was determined as the absorbance value at 450 nm minus the absorbance value at 800 nm.

3. Results and Discussion

3.1 PheDH N290D mutant with high substrate specificity

Wild-type PheDH from *T. intermedius* was highly reactive not only with L-Phe but also with L-Tyr, so we attempted to improve its substrate specificity for use as a sensor material for the accurate measurement of L-Phe in human plasma. A mutation in the substrate binding site of the enzyme has been reported to improve its substrate specificity.[18] Therefore, we focused on the amino acid residues around the substrate binding site of PheDH using the structure model of PheDH to improve the substrate specificity of the wild-type PheDH by amino acid substitution. The structure model of PheDH from *T. intermedius* was prepared on the basis of the X-ray structure of PheDH from *Rhodococcus* sp., which has the highest homology among the known complex structures containing a substrate [Fig. 1(a)].

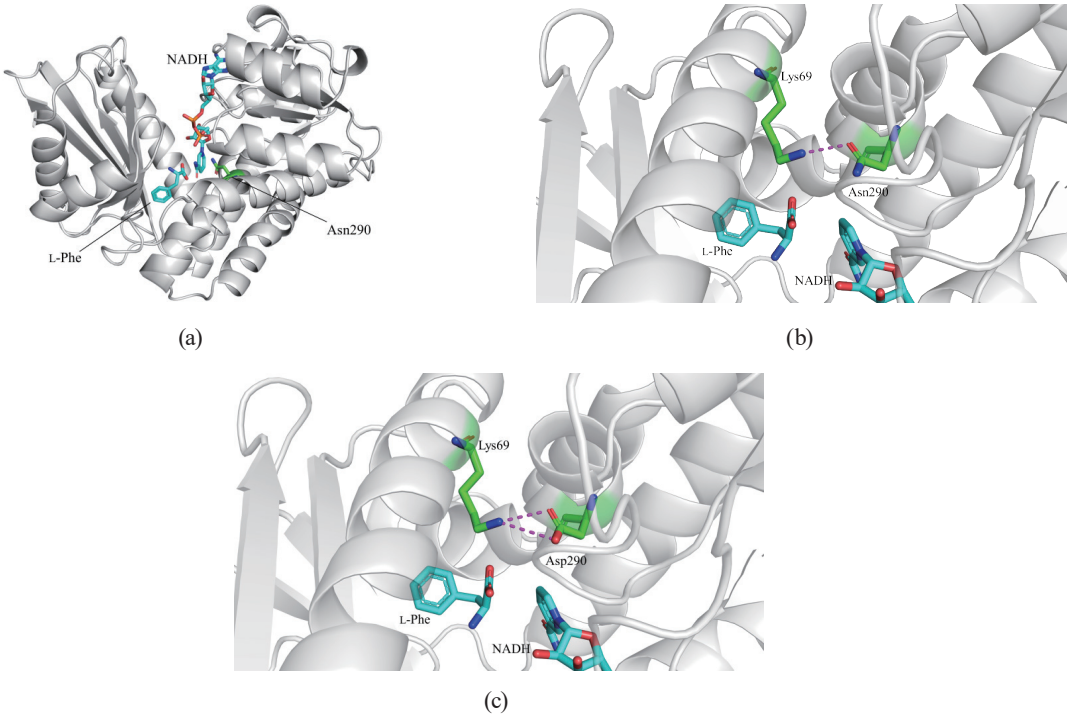

Fig. 1. (Color online) Structure model of PheDH. (a) Overall model structures of PheDH. (b) Close-up view of wild-type PheDH substrate binding site. The dotted magenta line shows a hydrogen bond between Asn290 and Lys69. (c) Close-up view of PheDH N290D mutant substrate binding site. The dotted magenta lines show the salt bridges between the 290th aspartic acid residue (Asp290) and Lys69.

Asn290 of PheDH is located around the substrate binding site, the distance between Asn290 and L-Phe is 3.9 Å, and Asn290 interacts with the 69th lysine residue (Lys69) through hydrogen bonding, which is important for substrate binding [Fig. 1(b)].[19] We considered that increasing the interaction with Lys69 would increase the rigidity and reduce the flexibility of the substrate binding site. As a result, the substrate specificity would be improved because its alteration strongly suppresses the entry of L-Tyr, which is slightly larger than L-Phe, to the substrate binding site. The PheDH N290D mutant was designed on the basis of this approach. The substituted aspartic acid residue of the PheDH N290D mutant interacts with Lys69 by bonding through two salt bridges [Fig. 1(c)], and thus, the structure of the substrate binding site may be considered rigid. The evaluation of substrate specificity by the enzymatic assay showed that the N290D mutation markedly reduced the reactivity of the PheDH N290D with L-Tyr to much less than that of the wild-type PheDH (Table 2). The relative activities, i.e., ratios of L-Tyr to L-Phe, were 64.0 and 1.5% for the wild-type PheDH and PheDH N290D mutant, respectively. Relative activities for the following substrates were also confirmed as in previous studies using the wild-type PheDH:[13,14] D-Phe, L-tryptophan, L-histidine, L-methionine, L-ethionine, L-valine, L-leucine, L-isoleucine, L-*allo*-isoleucine, L-alanine, L-2-aminobutyrate, L-norvaline, and L-norleucine. Although there were slight activities for L-leucine (0.5%) and L-norleucine (1.5%), the other substrates were inert. The wild-type PheDH substrate specificities for L-leucine and L-norleucine were 3.9 and 6.3%, respectively.[13] Therefore, the substrate specificity of the PheDH N290D mutant was improved compared with that of the wild-type PheDH. On the other hand, the activity of the PheDH N290D mutant toward L-Phe was reduced to 53.8% of that of the wild-type PheDH. The increased interaction with Lys69 was considered to decrease the activity of the PheDH N290D with L-Phe because it would be difficult for various substrates to enter the substrate binding site. However, compared with L-Phe, it is more difficult for the larger L-Tyr to enter the substrate binding site, and thus, the substrate specificity of PheDH was considerably improved.

3.2 L-Phe quantification in human plasma using PheDH N290D mutant

The PheDH N290D mutant, which has a high substrate specificity and half the activity of the wild-type PheDH, was evaluated by a colorimetric assay to verify whether L-Phe in human plasma can be quantified (Fig. 2). In enzymatic assays, it is important to select the optimum enzyme concentration and reaction time from the viewpoint of analytical performance. The absorbance values became maximum in 5 min when the PheDH N290D mutant amount was 400 or 500 μg/mL (Fig. 3). In this study, 500 μg/mL of the PheDH N290D mutant was used per

Table 2
Relative activity of PheDH (n = 6, mean). The relative activities of each PheDH with L-Phe as the substrate were taken as 100%.

Substrate	Relative activity (%)	
	Wild type	N290D
L-Phe	100	100
L-Tyr	64.0	1.5

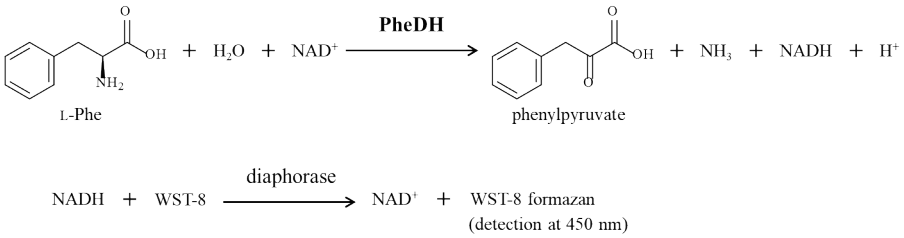

Fig. 2. Scheme for colorimetric L-Phe quantification.

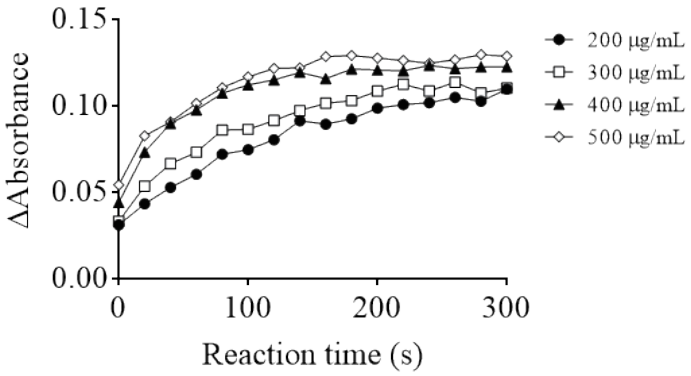

Fig. 3. Confirmation of optimal PheDH N290D mutant amounts in the assay. The vertical axis shows the change in absorbance value (the absorbance value at 450 mm minus the absorbance value at 800 nm) determined by the enzymatic method using the PheDH N290D mutant. Filled circles, open squares, filled triangles, and open diamonds indicate the PheDH N290D mutant amounts in each assay of 200, 300, 400, and 500 μg/mL, respectively.

measurement, and the reaction time was set to 5 min to maintain the robustness of this analysis method. L-Phe solutions of 0, 25, 50, 100, 200, and 400 μM concentrations were added to the reaction mixtures. The absorbance values were monitored for 5 min to obtain the L-Phe standard curve. The correlation coefficient was 0.998 (Fig. 4), the coefficient of variation (CV) was less than 18.2% (n = 3), and the limit detection was 1.1 μM from the standard deviations of the blank sample to which no L-Phe was added. The concentration of L-Phe in a healthy person's plasma ranges from 43 to 73 μM.[20] Patients with PKU are found to have L-Phe blood levels of 120 to 360 μM.[21] The standard curve for the PheDH N290D mutant covered both concentration ranges of L-Phe, which indicates the potential for use in point-of-care testing during the treatment monitoring of PKU patients. The guideline on ligand binding assay, a method for detecting an analyte using binding reagents such as enzymes against a substrate, indicates a CV standard of within 20%.[22] This assay using the PheDH N290D mutant was confirmed to satisfy this criterion. The concentration-dependent reaction was confirmed with this mutant, so it was used to quantify the L-Phe in human plasma.

Three human plasma samples were added to reaction mixtures to measure L-Phe concentrations without deproteination and derivatization pretreatments for evaluating the simplicity and accuracy of this method using the PheDH N290D mutant. The relative errors of the enzymatic method ranged from −10.3 to 7.4% compared with HPLC/ESI–MS (Table 3). The

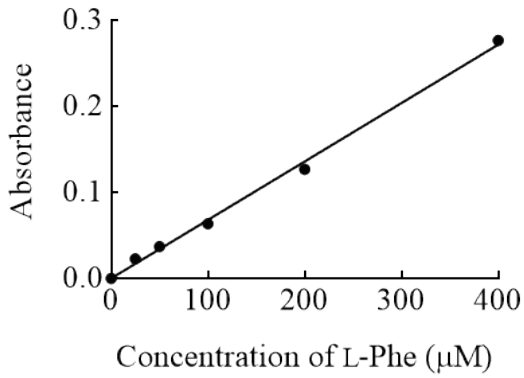

Fig. 4. L-Phe standard curve for PheDH N290D mutant. The vertical axis shows the absorbance value change at 450 nm minus 800 nm per 5 min determined by the enzymatic method using the PheDH N290D mutant (n = 3, mean). The linear regression equation was $y = 0.0006789x + 0.0002148$, and the correlation coefficient was 0.998.

Table 3
Quantification of L-Phe in human plasma by PheDH N290D enzymatic method.

	Plasma		
	A	B	C
L-Phe concentration by HPLC/ESI–MS (μM)	60.5	70.9	38.9
L-Phe concentration by enzymatic method (μM)	65.0	67.0	34.9
Relative error (%)	7.4	−5.5	−10.3

guideline on ligand binding assay indicates a relative error standard within ±20%.[22] Therefore, these results indicated that the enzymatic method using the PheDH N290D mutant can correctly determine the L-Phe concentration in non-pretreated human plasma even though L-Tyr is included in the plasma at a concentration similar to that of L-Phe.[20] The decreased activity of the PheDH N290D mutant compared with that of the wild-type PheDH had no effect on the L-Phe analysis because the analysis was finished in just 5 min. Additionally, the advantage of this enzymatic method over HPLC/ESI-MS is that no pretreatment such as the deproteination and derivatization of samples before analysis is required for accurate L-Phe quantification.

3.3 Application of PheDH N290D for immobilized-enzyme-based L-Phe sensor

The PheDH N290D mutant could accurately quantitate L-Phe in human plasma. We thus confirmed its applicability as a material for enzyme-based sensors. If this enzyme is not deactivated during immobilization, it can be used as a sensor material. The sensor for L-Phe was prepared by immobilizing the PheDH N290D mutant on a 96-well plate for enzyme-linked immunosorbent assay. L-Phe at concentrations between 0 and 200 μM as the substrate and the reaction mixtures were added to each well of the enzyme-immobilized sensor. Linear standard curves were obtained with absorbance values at 30 min, and the correlation coefficient was calculated to be 0.972 (Fig. 5). Similarly, the response of the enzyme-immobilized sensor with L-Tyr at concentrations of 0–200 μM was monitored, but no response was obtained. It was

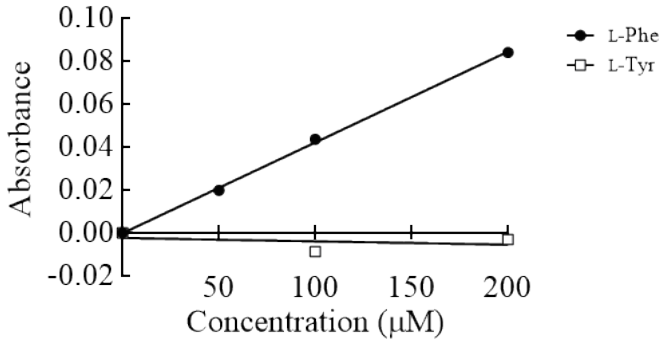

Fig. 5. Standard curve for immobilized PheDH N290D mutant. Filled circles and open squares are the measured values at each concentration of L-Phe and L-Tyr, respectively. The vertical axis represents each absorbance value at 450 nm per 30 min (n = 1). The absorbance values were corrected by subtracting the blank value. The correlation coefficient was 0.972.

considered that it could quantify the concentrations of L-Phe in plasma without the effect of L-Tyr even if the PheDH N290D mutant was immobilized because the concentration of L-Tyr in human plasma is 42–81 μM.[20]

4. Conclusions

In this study, we focused on thermostable PheDH from *T. intermedius* as a material for L-Phe sensors. Although the high thermostability of this enzyme makes it suitable as a sensor material, this enzyme was active not only with L-Phe but also with L-Tyr, so we attempted to improve its substrate specificity for the accurate measurement of L-Phe. The structure model of PheDH was prepared to develop a PheDH mutant with high substrate specificity by amino acid substitution. As a result, we successfully engineered the PheDH N290D mutant. This enzyme showed suppressed reaction with L-Tyr, and it could quantify L-Phe in human plasma accurately and simply without the pretreatment of samples. Furthermore, the PheDH N290D mutant was immobilized on a plate to evaluate whether it can be used as a sensor material. The immobilized enzyme also reacted with L-Phe in a concentration-dependent manner, the correlation coefficient was 0.972, and it did not react with L-Tyr. These results indicate that the PheDH N290D mutant has potential as a sensor material with L-Phe specificity.

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