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Application of an enzyme chip to the microquantification of L-phenylalanine

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Abstract

We describe here a new microquantification method of L-phenylalanine concentration in an extract from a dried blood spot by using the diaphorase–resazurin system. To miniaturize the fluorometric enzymatic microplate assay for the diagnosis of phenylketonuria, an enzyme chip immobilized with His-tag fused phenylalanine dehydrogenase (PheDH) was developed. His-tag fused PheDH was immobilized on the surface of nickel-coated slide glass. A microarray sheet (8 × 30 well) was fabricated with poly(dimethylsiloxane) (PDMS) using the photolithographic technique. An enzyme reaction chamber in a double-layered structure was constructed with different types of microarray PDMS sheets on the surface of Ni-coated slide glass immobilized with His-tagged PheDH. To evaluate the affinity toward the Ni-chelating ligand, eight kinds of His-tagged PheDH variants were constructed and expressed. $(His)_6$- and $(His)_9$-PheDH variants at the N terminus showed high adsorption ratio to Ni-chelating ligand. The V_{max} and k_{cat} values of the $(His)_6$-PheDH variant at the N terminus for L-phenylalanine were higher than those of the $(His)_9$-PheDH variant, and the $(His)_6$-PheDH variant was found to be most suitable for immobilization onto nickel-coated slide glass. Fluorescence formed by resazurin-coupled enzymatic reaction (in a 0.2-μl reaction mixture) on the enzyme chip exhibited good linearity and a correlation coefficient up to 12.8 mg/dl of the L-phenylalanine-containing sample extracted from a dried blood spot on filter paper.

Keywords: Phenylalanine dehydrogenase; *Bacillus badius*; Phenylketonuria; Enzyme chip; Polydimethylsiloxane

Phenylketonuria (PKU)[1] is a group of inborn metabolism errors in which the conversion of L-phenylalanine (L-Phe) to L-tyrosine is impaired. This disease is due to an autosomal recessive inheritance that codes for a type of phenylalanine hydroxylase with reduced enzymatic activity, resulting in high levels of L-Phe in inborn fluid and phenylpyruvate in urine. Early quantitative determination of plasma L-Phe is essential for the diagnosis of PKU and control of dietary therapy of PKU patients.

Neonatal mass-screening for PKU has been established in most countries including Japan. The Guthrie method [1], utilizing a bacterial inhibition assay, is the universal diagnostic procedure; however, it requires empirical observation and a long incubation time, and it is sometimes difficult to record accurate diagnostic results. Fluorometric [2], spectrophotometric [3], enzymatic [4], and chromatographic [5,6] methods have been described for newborn screening for such as PKU, maple syrup urine disease (MSUD), homocystinuria (HCU), and galactosemia (GAL). In 1984, NAD^+-dependent phenylalanine dehydrogenase (PheDH) activity was detected in *Brevibacterium* sp. by Hummel et al. [7]. PheDHs derived from *Sporosarcina ureae* R04 and *Bacillus sphaericus* R79a were first purified, crystallized,and characterized by Asano et al. [8,9] in 1987. They also found PheDH which is highly specific toward L-Phe in *Bacillus badius* IAM 11059 [10] and *Microbacterium*

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[1] *Abbreviations used:* IMAC, immobilized metal affinity chromatography; PheDH, phenylalanine dehydrogenase; PKU, phenylketonuria; MSUD, maple syrup urine disease; HCU, homocystinuria; GAL, galactosemia; PDMS, poly(dimethylsiloxane); AB-NTA, *N*-(5-amino-1-carboxypentyl)iminodiacetic acid; IPTG, isopropyl-β-D-thiogalactoside.

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sp. DM 86-1 [11]. These gene codings for PheDH from *S. ureae*, *B. sphaericus* [12], and *B. badius* [13] were also cloned and applied to enzymatic amino acid synthesis [14–16]. The discovery of these enzymes impacted the diagnosis of PKU, because it became possible to develop a colorimetric enzyme microassay of L-Phe in blood [17–20]. In Japan and several western countries, the fluorometric microplate assay with PheDH, diaphorase, and resazurin has been developed and is extensively used for the practical screening of PKU during the past decade [18].

On the other hand, analytical methods using high-performance liquid chromatography (HPLC) [21] or tandem mass spectrometry (MS/MS) [22,23] have also been developed for the screening of inborn metabolism errors such as PKU, MSUD, and HCU. The enzymatic analytical method is still convenient and economical to use rather than HPLC and MS/MS analytical methods, because a fairly large amount of an organic solvent has been used as the mobile phase in the latter methods and the MS/MS method presents one or more of the following drawbacks: securement of a number of neonates to warrant the expensive cost of installation, retainment of high vacuum, laborious derivatization to ionize the ampholytic substances, and difficulty in discriminating structural and optical isomers.

Protein microarrays have been expanded with rapid advances in the "-omics" studies, antibody technology, high-throughput screening, etc. The major advantages of protein microarray technologies include a highly parallel and miniaturized solid-phase assay, high-throughput approaches, and low consumption of reagents and samples. In recent years, several types of microarray chips have been developed, and a DNA microarray scanner was used for the detection of fluorescence Cy-dyes [24–26].

The current screening method for PKU by using PheDH has been put into practice for more than a decade. On the other hand, the microarray chip has been widely used in gene diagnosis and the "-omics" studies. It has been desired to miniaturize the fluorometric enzymatic assay of PKU for integration of the newborn screening methods. It will be possible to expand this technology to the diagnosis for many diseases in the future. To address this requirement, we developed an enzyme chip method which is a miniaturized method of the fluorometric enzyme microplate assay for PKU screening. We successfully immobilized His-tag fused PheDH on an enzyme chip and used it for the quantitative determination of L-Phe in dried blood spots.

Materials and methods

Reagents and chemicals

Dried blood spots and the standard PKU microplate assay kit (PKU-R) were obtained from Sapporo IDL (Sapporo, Japan). *N*-(5-Amino-1-carboxypentyl)iminodiacetic acid (AB-NTA) was obtained from Dojindo Laboratories Co. (Kumamoto, Japan). High-density amino group DNA microarray slide glass was purchased from Matsunami Glass Ind., Ltd. (Osaka, Japan). OmniCoat, NANO XP SU-8 50 and SU-8 developer were purchased from Micro-Chem Corp. (Newton, MA, USA). SYLGARD 184 base and SYLGARD 184 curing agent were purchased from DowCorning Corp. (Midland, MI, USA). β-NAD^+ and diaphorase were purchased from Oriental Yeast Co. (Osaka, Japan), Chelating-Sepharose Fast Flow was from Amersham Bioscience (Uppsala, Sweden), and the Protein Assay Kit was from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). *Bam*HI, *Nde*I, and *Xho*I were purchased from Takara Shuzo (Shiga, Japan), Toyobo (Osaka, Japan), and New England Biolabs (UK) Ltd. (England, UK), respectively. *Escherichia coli* JM109 from Novagen (Madison, WI, USA) and *E. coli* BL21(DE3) and pET21(+) from Invitrogen (Carlsbad, CA, USA) were used as a host and an expression vector. All other chemicals were of analytical grade.

PheDH and His-tagged PheDH

PheDH from *B. badius* IAM 11059 was purified and the *pdh* gene was cloned as described previously [10,13]. The *pdh* gene from *B. badius* was amplified by polymerase chain reaction (PCR) from plasmid pBB1 [13] using oligonucleotides to introduce various numbers of consecutive histidine residues followed by a *Nde*I site at the N terminus and *Xho*I site at the C terminus. The following oligonucleotides were used as primer sets: fBBnh3, 5′-gctcatatgcatcatcatgcgatgagcttagtagaaaaaaca-3′; fBBnh6, 5′-gctcatatgcatcatcatcatcatcatgcgatgagcttagtagaaaaaaca-3′; fBBnh9, 5′-gctcatatgcatcatcatcatcatcatcatcatgcgatgagcttagtagaaaaaaca-3′; fBBnh12, 5′-gctcatatgcatcatcatcatcatcatcatcatcatcatcatcatgcgatgagctagtagaaaaaaca-3′; and rBBnh2, 5′-taatctcgaggattagttgcgaatatccca-3′. After cleavage with respective restriction enzymes, the PCR products were introduced into pRSET-B vector DNA which had been digested with the same restriction endonucleases. Likewise, the *pdh* gene was also amplified by PCR using oligonucleotides to introduce a *Bam*HI site at the N terminus and *Xho*I site followed by various numbers of histidines at the C terminus. The following oligonucleotides were used as primer sets: rBBch3, 5′-cgtaatctcgagtcagtggtggtggttgcgaatatcccattt-3′; rBBch6, 5′-cgtaatctcgagtcagtggtggtggtggtggttgcgaatatcccattt-3′; rBBch9, 5′-cgtaatctcgagtcagtggtggtggtggtggtggtggtggttgcgaatatcccattt-3′; rBBch12, 5′-cgtaatctcgagtcagtggtggtggtggtggtggtggtggtggtggtggttgcgaat atcccattt-3′; and fBBnch1, 5′-aaggatccgatgagcttagtagaaaaaaca-3′. The primers were designed to contain *Bam*HI and *Xho*I sites (underlined). After cleavage with respective restriction enzymes, the PCR products were inserted into pET21(+) vector DNA. After each plasmid, pBBNH3, pBBNH6, pBBNH9, pBBNH12, pBBCH3, pBBCH6, pBBCH9, and pBBCH12, was transformed into *E. coli* JM109 and the constructs were verified by sequencing, these plasmids were further transformed into *E. coli* BL21(DE3) for the expression of the enzyme. *E. coli* BL21 (DE3) harboring the above-mentioned plasmids were grown at 37 °C for 10 h with Luria–Bertani (LB)

medium [27] supplemented with 50 μg/ml ampicillin, and the expression protein was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactoside (IPTG). After 4 h of induction at 30 °C, the cells were harvested by centrifugation, washed twice with saline, and stored at −30 °C until use.

Purification of His-tagged PheDH

The washed cell paste was suspended with a 5× volume of 20 mM Tris–HCl buffer, pH 8.0, containing 0.5 M NaCl and 2 mM 2-mercaptoethanol. The cells were disrupted by ultrasonication for 20 min with Kubota Ultrasonicator Insonator Model 210 apparatus (19 kHz, Kubota, Tokyo, Japan). To remove the cell debris, the sonicated suspension was centrifuged at 28,000*g* for 20 min at 4 °C, and the cell-free extract was obtained. The cell-free extract was applied to Ni^{2+}-saturated chelating-Sepharose Fast Flow column (1.5 by 10 cm; Amersham Bioscience) equilibrated with 20 mM Tris–HCl buffer, pH 8.0, containing 0.5 M NaCl and 2 mM 2-mercaptoethanol. After washing with the same buffer containing 25 mM imidazole, the enzyme was eluted with 20 mM Tris–HCl buffer, pH 8.0, containing 0.5 M NaCl, 2 mM 2-mercaptoethanol, and 500 mM imidazole. Active fractions were combined and concentrated with ultrafiltration using Centriprep YM-10 (Millipore Corp., Bedford, MA, USA). The concentrate was dialyzed against 20 mM Tris–HCl buffer, pH 8.0, containing 0.1 mM EDTA and 5 mM 2-mercaptoethanol. The dialysate was loaded onto a Pre-packed High-Q column (5 ml, Bio-Rad Laboratories, Inc.) equilibrated with 20 mM Tris–HCl buffer, pH 8.0, containing 0.1 mM EDTA and 5 mM 2-mercaptoethanol. After washing with the same buffer, the enzyme was eluted with a linear gradient from 0 to 0.75 M of NaCl. The active fractions were combined and concentrated with ultrafiltration using Centriprep YM-10. The concentrate was injected into a Superdex 200 HR 10/30 column (Amersham Bioscience) with an ÄKTA FPLC system (Amersham Bioscience) equilibrated with 20 mM Tris–HCl buffer, pH 8.0, containing 0.1 mM EDTA, 5 mM 2-mercaptoethanol, and 0.2 M NaCl. The enzyme was eluted with the same buffer, and the active fractions were concentrated and dialyzed against 20 mM Tris–HCl buffer, pH 8.0, containing 0.1 mM EDTA and 5 mM 2-mercaptoethanol. The final preparation was diluted to 50% with glycerol and stored at −30 °C until use.

Enzyme assay and protein determination

The standard assay of PheDH activity was performed at 25 °C by measuring the reduction of β-NAD^+ at 340 nm in a poly(methylmethacrylate) (PMMA) cuvette placed in the beam of a 1-cm light path. The reaction mixture contained 100 μmol of glycine–KCl–KOH buffer, pH 10.4, 2.5 μmol of β-NAD^+, 10 μmol of L-Phe, and the enzyme solution in a total volume of 1.0 ml. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of NADH in the oxidative deamination. Protein concentration was determined with a Protein Assay Kit (Bio-Rad Laboratories, Inc.) by the method of Bradford [28] with bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA) as a standard, and the eluate fractions were measured with absorbance at 280 nm.

Determination of L-Phe concentration

L-Phe concentration was standardized with the dried blood spots containing fixed amount of L-Phe as an authorized method used in Japanese local institutes of health. A 3-mm (diameter) disk was punched from a dried blood spot on filter paper and put into each well of a 96-well flat-bottomed black microplate. To fix the erythrocyte in the filter paper, 10 μl of fixing solution (acetone:ethanol:ultra pure water = 7:7:2 (v/v/v)) was added to the filter paper and the microplate was left for 1 h at 37 °C in an oven. To extract L-Phe and other soluble components, 100 μl of extraction buffer (50 mM Tris–HCl buffer, pH 8.9, containing 40 μM resazurin) was added to each well and the microplate was left for 1 h at room temperature with the top of the plate sealed to prevent vaporization. Each extract (75 μl) was transferred into a microtube, and 15 μl of 25 mM β-NAD^+ and 10 μl of 0.2 mg/ml diaphorase from *Clostridium kluyveri* (EC 1.6.99.2, Oriental Yeast Co., Osaka, Japan) dissolved in 0.1 M potassium phosphate buffer, pH 7.5, were added. The mixture (0.2 μl) was poured into the enzyme reaction chamber constructed with PDMS sheet on an Ni–NTA-modified slide glass and sealed with a cover glass. The enzyme reaction was performed at 25 °C for 1 h, followed by fluorometric scanning at 532 nm of excitation wavelength and 585 nm of emission wavelength using Hitachi Software CRBIO II-e (Hitachi Software Engineering Co., Ltd., Tokyo, Japan). The fluorescent intensity was analyzed with DNAsis array software (Hitachi Software).

Preparation of PDMS microarray

The mold for the preparation of the PDMS microwell array sheet was manufactured by the photolithographic patterning technique of thick film photoresist on a slide glass (76 × 26 mm). OmniCoat adhesive agent (MicroChem) was spun on top of a slide glass, which had been washed with acetone and cleaned by ultrasonication at 2500 rpm for 15 s. NANO XP SU-8 50 resist (MicroChem) was coated onto the adhesive-agent-treated slide glass by spinning at 2500 rpm for 15 s. The coated slide glass was baked at 100 °C for 30 min and cooled to room temperature. The coating procedure was repeated three times, and about 0.3 mm thickness of resist coating was obtained. The mold patterning photo-mask prepared by computer illustration software (Adobe Illustrator, Adobe Corp., San Jose, CA, USA) was layered onto the resist-coating substrate and exposed at near-UV (at 350–400 nm) for 90 s. The exposed slide glass was baked at 100 °C for 10 min and then dipped into SU-8 developer (MicroChem) to remove the raw resist.

The obtained circular-type SU-8 mold was arrayed with 0.6-mm-tall column on the surface of the slide glass. The grid-type SU-8 mold (1.6 mm of square grid) was also prepared as described above.

Two types of PDMS microarray sheets were prepared as follows: SYLGARD 184 base (DowCorning) and SYLGARD 184 curing agent (DowCorning) were mixed with a ratio of 10 to 1, and the mixture was degassed for 20 min with an aspirator. It was dropped onto the circular-type SU-8 mold (8 × 30 wells), layered onto slide glass on a silicon sheet (0.5 mm thickness), clamped, and heated at 60 °C for 50 min in an oven. The treated PDMS sheet was detached from the SU-8 mold slide glass plate in ethanol and then immersed in 3 N HCl to improve the hydrophobicity of the surface. Likewise, the grid-type PDMS sheet (8 × 30 wells) was also obtained as described above. The PDMS sheets were placed on top of each other, adhered by compression without glue, and used as the enzyme reaction chamber sheet.

Preparation of Ni–NTA slide glass and enzyme immobilization

To prepare Ni–NTA slide glass for immobilization of His-tagged PheDH, the DNA microarray slide glass induced with high-density amino groups (Matsunami Glass, Osaka, Japan) was preserved in 12.5% (v/v) glutaraldehyde to activate the amino groups on the surface of the slide glass at room temperature for 1 h with gentle shaking (Figs. 1A and B). After the slide glass was soaked and stirred at room temperature for 1 h in 2 mM AB-NTA solution, the excess AB-NTA was washed with ultrapure water. To protect the excess amino groups from other chemical reactions, the slide glass was soaked in 50 mM L-lysine solution for 1 h. After being washed with ultrapure water, the AB-NTA-coated slide glass was placed for 1 h in 1% (w/v) $NiSO_4 \cdot 6H_2O$ and used as an Ni–NTA-modified slide glass for the immobilization of His-tag fused PheDH variant (Fig. 1C).

To immobilize His-tagged PheDH variant onto the surface of slide glass, an Ni–NTA-modified slide glass was soaked in 10 ml of enzyme solution (0.2 U/ml dissolved in 15 ml of 20 mM Tris–HCl buffer, pH 8.0, containing 0.5 M NaCl and 2 mM 2-mercaptoethanol) and gently stirred for 1 h at 4 °C. To remove unimmobilized enzyme, the slide glass was washed twice with the same buffer without the enzyme at 4 °C, dried, and stored at 4 °C until use (Fig. 1D).

Immediately prior to use, the improved circular-well-arraying PDMS sheet (Fig. 2A) was adhered to the grid-well-arraying PDMS sheet (Fig. 2B), and then the constructed PDMS sheet in a double-layered structure was placed directly on the surface of the Ni–NTA-modified slide glass which had been immobilized with His-tagged PheDH variant (Fig. 2).

Results

Expression and adsorption of His-tag fused PheDH

To evaluate the suitability of the enzyme activity and immobilization toward the Ni–NTA ligand on the surface

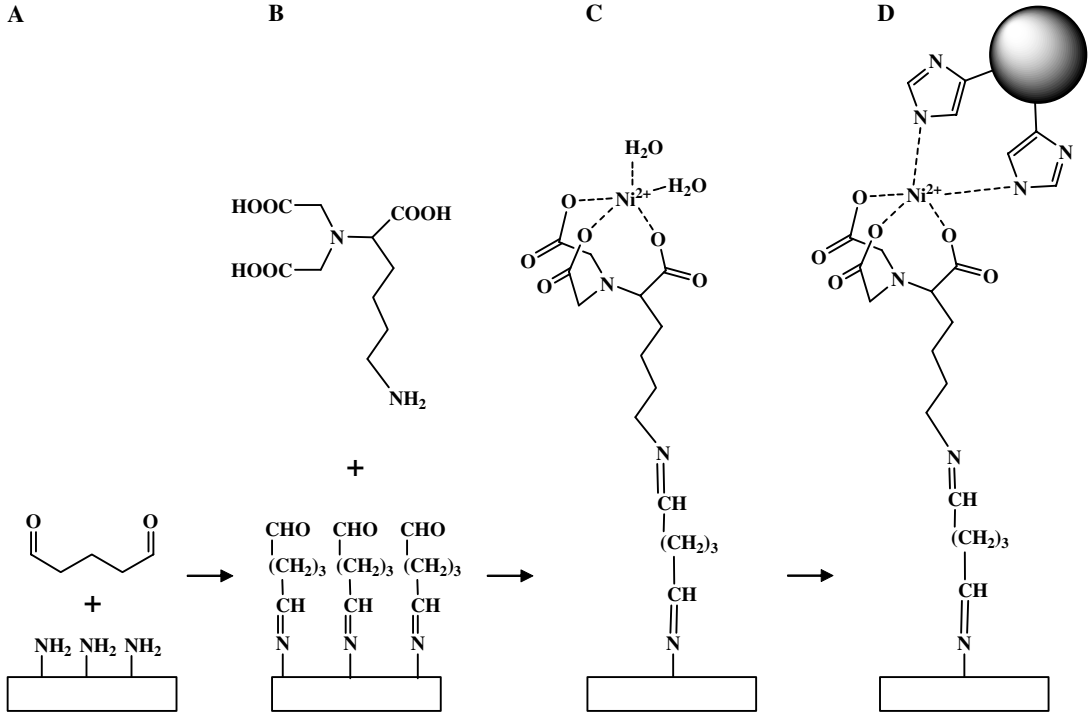

Fig. 1. Strategy for the preparation of an Ni–NTA-modified slide glass immobilized with His-tagged PheDH (A) Amino-group-induced slide glass was activated with glutaraldehyde. (B) *N*-(5-Amino-1-carboxypentyl)iminodiacetic acid was bonded chemically to the activated slide glass. (C) Nickel ion was linked with AB-NTA slide glass. (D) His-tag fused PheDH was immobilized on the Ni–NTA-modified slide glass.

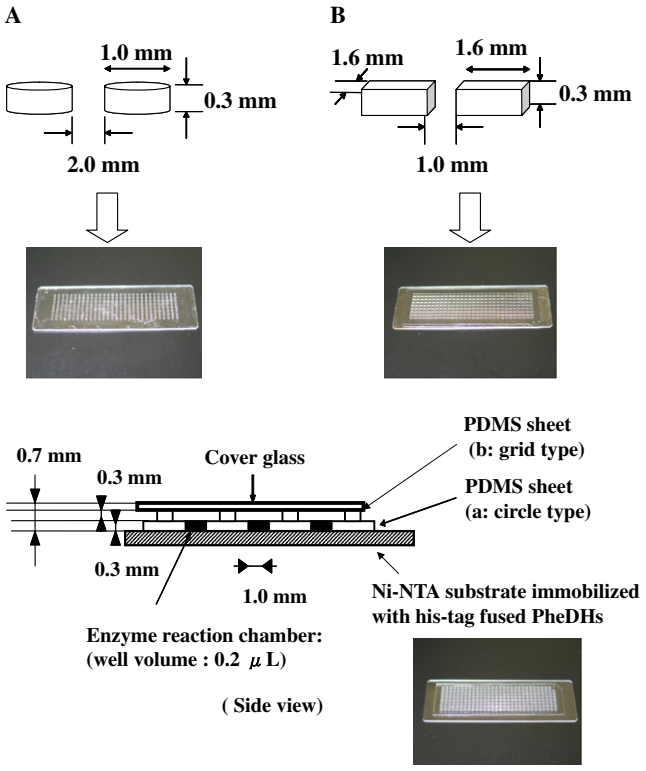

Fig. 2. Schematic general view of the enzyme chip and the SU-8 photoresist molds. The enzyme chip was constructed from three parts. (A) The mold for the circular-type PDMS sheet was used as the enzyme reaction chamber. (B) The mold for the grid-type PDMS sheet was used to prevent vaporization and cross contamination in each well. The general view shows the side view, and His-tagged PheDH variant was immobilized on the surface of the Ni–NTA-modified slide glass.

of slide glass, eight kinds of His-tag fusion PheDHs, derived from *B. badius* IAM 11059 and conjugated with $(His)_3$-, $(His)_6$-, $(His)_9$-, and $(His)_{12}$-peptides at the N and C termini, were constructed and expressed in *E. coli* BL21 (DE3) harboring pBBNH3, pBBNH6, pBBNH9, pBBNH12, pBBCH3, pBBCH6, pBBCH9, and pBBCH12, respectively. *E. coli* BL21 (DE3) harboring these plasmids were cultivated at 37 °C for 10 h in LB medium, pH 7.5, supplemented with 50 μg/ml ampicillin. The enzyme was induced by the addition of 0.5 mM IPTG and further cultivated at 30 °C for 4 h. The specific activities of His-tagged PheDH variants were assayed as shown in Table 1. The specific activities of His-tagged PheDH variants at the N terminus were higher than those of at the C terminus. Although the highest specific activity was obtained with $(His)_{12}$-PheDH at the N terminus, $(His)_{12}$-PheDH at the C terminus exhibited only slight specific activity.

Table 1
Effect of the number of consecutive histidine residues on specific activity in a cell-free extract of expressed proteins

	Specific activity (U/mg)			
	3 × His	6 × His	9 × His	12 × His
N terminus	0.50	0.17	0.15	0.77
C terminus	0.12	0.24	0.14	0.10

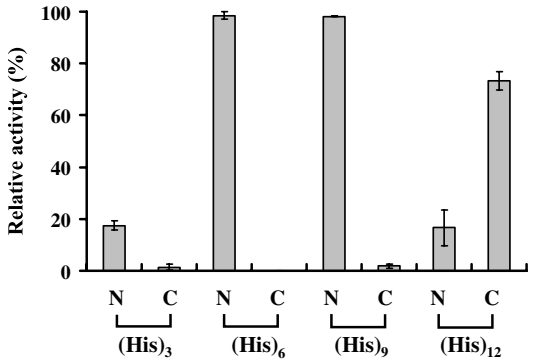

Fig. 3. Adsorption ability of His-tagged PheDH variants to Ni-chelating resin. Various numbers of consecutive histidine residues were conjugated at the N terminus of PheDH. Recovery relative activity was defined as the rate of total activity of the eluent fraction to the applied total activity.

We evaluated the adsorption ability of His-tagged PheDH variants toward the Ni–NTA ligand on the surface of slide glass using immobilized metal affinity chromatography (IMAC) (Fig. 3). $(His)_6$- and $(His)_9$-PheDH variants at the N terminus showed high adsorption ability (>98%) to Ni-chelating Sepharose Fast Flow resin. $(His)_3$- and $(His)_{12}$-PheDH variants at the N terminus had high specific activity in the cell-free extracts, while the binding abilities of these variants were significantly lower than those of $(His)_6$- and $(His)_9$-PheDH variants at the N terminus. On the other hand, $(His)_{12}$-PheDH variant at the C terminus was also adsorbed to Ni-Chelating resin (73%). However, V_{max} value of $(His)_{12}$-PheDH variant at the C terminus (11.2 ± 0.11 U/mg) was markedly lower than those of $(His)_6$- (85.6 ± 1.8 U/mg) and $(His)_9$- (56.2 ± 2.6 U/mg) PheDH variants at the N terminus. From these results, it was revealed that the site-specific conjugation of His-tag at the N terminus of PheDH with six consecutive histidine residues was most suitable for the immobilization toward Ni–NTA-modified slide glass.

Detection and quantification of L-Phe in a dried blood spot

Linear calibration curve was obtained for L-Phe in the range of 0 to 12.8 mg/dl with the dried blood spot filter papers (Fig. 4). Each plot was displayed as an average value of triplicate fluorescence (mean ± SD, respectively). CVs ranged from 0.3 to 14.2% in the concentration range from 0.4 to 12.8 mg/dl in the dried blood spots. Precision of the assay was calculated by replicate analysis of the same spot sample. The fluorescence intensities in arbitrary units with the reagent blank subtracted were proportional to the concentration of L-Phe in an enzyme chip immobilized with $(His)_6$-PheDH variant at the N terminus. The linearity of the chip was confirmed up to 12.8 mg/dl for L-Phe and had good correlation, $r^2 = 0.992$. The concentrations of the control, which had been estimated to be 2.0 ± 0.4 and 3.9 ± 0.8 mg/dl by HPLC, calculated from the calibration curve were 1.7 ± 0.072 (CV 4.2%) and 2.8 ± 0.25 (CV 9.2%) mg/dl, respectively.

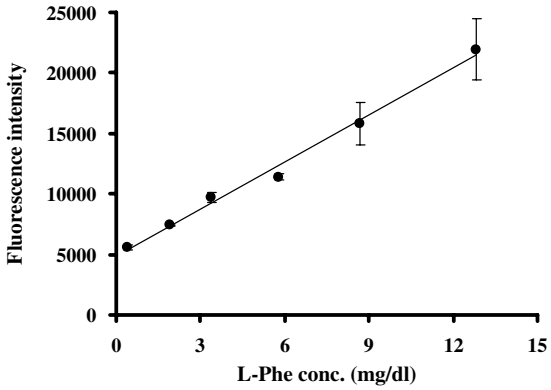

Fig. 4. Calibration curve of L-Phe extracted from dried blood spot filter paper. The extracts, β-NAD^+, resazurin, and diaphorase mixtures (0.2 μl) were dispensed into each well. The enzyme chip immobilized with $(His)_6$-PheDH variant at the N terminus was incubated in an oven at 25 °C for 60 min. After incubation, the formed fluorescence was scanned with a DNA microarray scanner and visualized with imaging analysis software. L-Phe concentrations are plotted as mean ± SD ($n = 3$).

Discussion

To immobilize the Ni–NTA-modified slide glass, we constructed variants of PheDH from *B. badius* [10] fused with various lengths of His-tag peptides. PheDH catalyzes the reversible NAD^+-linked oxidation–reduction reaction and is highly specific toward L-Phe as a substrate for the oxidative deamination reaction. Wild PheDH from *B. badius* has been used for the fluorometric enzyme assay of PKU screening in Japan, and the gene of this enzyme was cloned by Yamada et al. [13]. We reported that the thermostabilization of PheDH from *B. badius* was increased by chemical modification with β-cyclodextrin derivatives [29]. In this study, we examined the effect of His-tag conjugation toward PheDH on specific activity and site-specific immobilization. Although $(His)_3$- and $(His)_{12}$-PheDH variants at the N terminus exhibited higher specific activity toward L-Phe than those of $(His)_6$- and $(His)_9$-PheDH variants, excellent affinity toward Ni-chelating resin was obtained with $(His)_6$- and $(His)_9$-PheDH variants at the N terminus. On the other hand, all of the His-tagged PheDH variants at the C terminus showed similar specific activity and could not be adsorbed to Ni-chelating resin except for $(His)_{12}$-PheDH variant. The kinetic parameters of $(His)_6$-PheDH variant at the N terminus showed excellent values compared with those of $(His)_9$-PheDH variant at the N terminus and $(His)_{12}$-PheDH variant at the C terminus. PheDH from *B. badius* has an octameric structure [10], and it is thought that this phenomenon was caused by steric hindrance influenced by the His-tag length and fused position to the protein. Since site-specific His-tag fusion and IMAC have been widely used [24,30–32], we examined various conditions for immobilization of the target enzyme in this study and found that the $(His)_6$-PheDH variant at the N terminus was most suitable.

The micro enzyme reaction chamber in a double-layered structure was prepared with PDMS and adhered to the surface of an Ni–NTA-modified slide glass immobilized with His-tagged PheDH variants at the N terminus. When an untreated PDMS sheet was used as the enzyme reaction chamber, no fluorescence was observed in microwells which had been unable to achieve contact between the reaction mixture and the surface of the enzyme-immobilized Ni–NTA slide. It was thought that this phenomenon was caused by the water-repellent property of PDMS. Recently, it has been reported that the surface of hydrophobic elastomer could be modified using a variety of plasma created from gases and microwave-oven generated plasma [33,34]. In this experiment, when the PDMS sheet was pretreated with 3 N HCl, the reaction mixture could contact the surface of the Ni–NTA-modified slide glass immobilized with His-tagged PheDH well, and fluorescence was clearly detected.

The microquantification of L-Phe extracted from dried blood spots was successful even in only 0.2 μl of enzyme reaction volume. So far, a 96-well microplate has been used for the quantification of L-Phe in dried blood spots or plasma specimens by colorimetric or fluorometric methods [17–20]. In these methods, an enzyme reaction mixture (100–200 μl) has usually been required, whereas microquantification by an enzyme chip in our study was realized with only 0.2 μl. Furthermore, the enzyme chip could be used without an enzyme mixture because PheDH was already immobilized on the chip. In this study, we demonstrated experimentally for the first time that the enzyme chip can be applied to the diagnosis of PKU because it showed the same accuracy and reliability as with the current PKU diagnosis kit. Thus, the enzyme chip prepared in this study has significant advantages over the microplate assay method.

In 2001, a proteome chip for the screening of biochemical activities was reported by Zhu et al. [24]. They described the cloning, expression, purification, and arraying of 5800 yeast proteins. These cloned proteins were expressed as glutathione *S*-transferase–polyhistidine (GST-His × 6) tag fusions at their N terminus and site-specifically immobilized on a nickel-coated slide glass. On the other hand, Lesaicherre et al. [25] demonstrated that an intein-mediated expression system is effective for the site-specific immobilization of expressed proteins onto avidin-functionalized slide glass. We also succeeded in the site-specific immobilization of His-tagged PheDH at the N terminus onto the surface of an Ni–NTA-modified slide glass. While Cy-dye-labeled proteins and green fluorescence protein are used to detect fluorescence in their methods, resolfin fluorescence was used in the enzyme chip method established in this study. Thus far, the fluorescence Cye-dye method has been widely used for various DNA and protein microarrays [24–26]. This is the first report in which the resolfin fluorescence method was applied to the protein microarray system.

In this work, we showed that the diaphorase–resazurin system can be applied to DNA microarray scanner system. Although diaphorase could still be coimmobilized with the immobilized His-tag fusion PheDH, we demonstrated here

that the His-tag-fused PheDH-immobilizing enzyme chip is applicable for the diagnosis of PKU. Moreover, the enzyme chip fabricated in this experiment could be used for other assays when immobilized with other NAD^+-dependent oxidoreductases. In the future, we hope that the method using this chip could be used in the preliminary neonatal mass-screening for PKU, MSUD, and GAL.

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