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RESEARCH PAPER

A new approach for quantitative analysis of L-phenylalanine using a novel semi-sandwich immunometric assay

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Abstract Here, we describe a novel method for L-phenylalanine analysis using a sandwich-type immunometric assay approach for use as a new method for amino acid analysis. To overcome difficulties of the preparation of high-affinity and selectivity monoclonal antibodies against L-phenylalanine and the inability to use sandwich-type immunometric assays due to their small molecular weight, three procedures were examined. First, amino groups of L-phenylalanine were modified by "*N*-Fmoc-L-cysteine" (FC) residues and the derivative (FC-Phe) was used as a hapten. Immunization of mice with bovine serum albumin/FC-Phe conjugate successfully yielded specific monoclonal anti-FC-Phe antibodies. Second, a new derivatization reagent, "biotin linker conjugate of FC-Phe *N*-succinimidyl ester" (FC(Biotin)-NHS), was synthesized to convert L-phenylalanine to FC-(Biotin)-Phe as a hapten structure. The biotin moiety linked to the thiol group of cysteine formed a second binding site for streptavidin/horseradish peroxidase (HRP) conjugates for optical detection. Third, a new semi-sandwich-type immunometric assay was established using pre-derivatized L-phenylalanine, the monoclonal anti-FC-Phe antibody, and streptavidin/HRP conjugate (without second antibody). Using the new "semi-sandwich" immunometric assay system, a detection limit of 35 nM (60 amol per analysis) and a detection range of 0.1–20 μM were attained using a standard L-phenylalanine solution. Rat plasma samples were analyzed to test reliability. Intra-day assay precision was within 6 % of the coefficient of variation; inter-day variation was 0.1 %. The recovery rates were from 92.4 to 123.7 %. This is the first report of the quantitative determination of L-phenylalanine using a reliable semi-sandwich immunometric assay approach and will be applicable to the quantitative determination of other amino acids.

Keywords Semi-sandwich immunometric assay · Amino acids · Phenylalanine · Derivatization · Pre-derivatization

Introduction

Amino acids have important roles in protein synthesis, metabolic pathways, and taste stimuli. Therefore, they have been studied in various research fields. A well-known amino acid target in clinical examination is L-phenylalanine for phenylketonuria (PKU) newborn blood spot screening [1, 2]. The concentration ratio of branched chain amino acids to L-tyrosine (BCAA/Tyr) for the BCAA-to-Tyr ratio (BTR) tests is analyzed to evaluate the severity of hepatic failure [3]. In addition, analysis of plasma free amino acids is used as a favorable biomarker for understanding the disease mechanisms and diagnosis, either during early disease or to determine future risk. This is because they circulate abundantly between multiple organ systems and are influenced by metabolic variations in specific damaged organs [4–19]. To accelerate amino acid research and clinical applications in the future, methods of amino acid quantification should be easy to use, have low cost and high reliability, and be able to analyze multiple samples.

Quantification of amino acids has been widely performed using chromatographic separation techniques coupled with

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various detectors [20–25]. These conventional or cutting-edge instrumental analyses used for laboratory analysis are robust and reliable and provide precise and accurate data. However, they require skillful operators and expensive instruments. In addition, they are time-consuming to use for multiple assays. Recently, a direct infusion method with ultrahigh-resolution mass spectrometry has been used for metabolic research and diagnosis testing fields [26, 27]. Although the performance of the high-throughput screening is powerful, it requires a high-grade mass spectrometry instrument. In addition, the influence of ion suppression and overlapping signals of identical compositional formula compounds on quantitative analysis is unavoidable. Enzymatic amino acid determination is an alternative inexpensive method with higher accuracy; additionally, it is simple to operate and can perform multiplex sample analysis. Analytical kits using enzymatic reaction mechanisms such as the BTR test [3], L-glutamate determination [28], and PKU screening are available. However, one drawback of the enzymatic approach is an incomplete set of enzymes for all proteinogenic amino acid analysis. And few of the amino acid metabolic enzymes have the acceptable characteristics of enzymatic activity, stability, and cross-reactivity for quantitative amino acid analysis.

In contrast, enzyme-linked immunosorbent assays (ELISAs) [29], based on antigen–antibody reactions, have been widely used for the detection of protein biomarkers and small compounds such as environmental pollutants. The first advantage of ELISAs is the remarkable property of monoclonal antibodies to bind target molecules with high affinity and specificity. In addition, antibodies are highly stable, which is desirable for commercialization. Furthermore, ELISAs have high-throughput ability for sample processing using commercially available 96-well plates and automated instruments. Considering these advantages, we developed a new immunometric assay for L-phenylalanine analysis as a model study.

First, the preparation of a monoclonal antibody that recognized L-phenylalanine with high affinity and selectivity was required. In general, it is difficult to obtain antibodies against small endogenous compounds from immunized animals due to their low immunogenicity. To increase immunogenicity in mice, Fmoc-L-cysteine (FC)-based modification of L-phenylalanine was adopted and we successfully obtained monoclonal anti-FC-Phe antibodies. Next, a novel "semi-sandwich-type" [30, 31] immunometric assay system was established using the derivatization reagent FC(Biotin)-*N*-hydroxysuccinimide (NHS; Fig. 1). The novel approach reported here provides a higher sensitivity and broader working range for L-phenylalanine detection without the need for two or more specific antibodies. Analysis of rat plasma samples was performed to assess its reliability. This is the first report of the quantitative determination of L-phenylalanine in biological samples using an immunometric assay approach, and this may be useful for future quantitative analysis of amino acids.

Materials and methods

Apparatus

Immunometric assays were performed using a microplate reader, SpectraMax M2e (Molecular Devices, Sunnyvale, CA), and an automatic microplate washer, MW-96CR (Biotec Co., Ltd, Tokyo, Japan). A CLASS-VP series high-performance liquid chromatography (HPLC) system equipped with a UV detector (Shimadzu Corporation, Kyoto, Japan) was used for the analysis and fractionation of synthesized materials. For confirmation of reactants, mass spectra were identified using a liquid chromatography–mass spectrometry (LC/MS) system. Agilent 1100 series (Agilent Technologies, Inc., CA) was used for separation and API365 LC/MS/MS system (Applied Biosystems, Foster City, CA) was used for detection in positive scan mode. Each bovine serum albumin (BSA)/hapten conjugate solution was analyzed with AXIMA® series matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS; Shimadzu Corporation) to confirm maleimide–sulfhydryl coupling between the hapten and the carrier protein. A Cadenza C18 column (4.6×250-mm ID; Imtakt Corporation, Kyoto, Japan) was used at 40 °C. The flow rate was maintained at 1 mL/min throughout the analysis. Solution A consisted of water/acetonitrile (95:5) containing 0.1 % formic acid and solution B consisted of water/acetonitrile (10:90) containing 0.1 % formic acid and were used for mobile phases. Appropriate gradient conditions for each compound were used for chromatic analysis and purification.

Materials

N,*N*-bis-Fmoc-L-cystine $(Fmoc\text{-}Cys)_2$ and L-cystinyl-bis-L-phenylalanine $(Cys\text{-}Phe)_2$ were purchased from Bachem. Fmoc-OSu (*N*-(9-fluorenylmethoxycarbonyloxy)succinimide) and dithiothreitol (DTT) were purchased from Wako Pure Chemical Industries, Ltd. *N*,*N*-dicyclohexylcarbodiimide (DCC) and NHS were obtained from Tokyo Chemical Industry Co., Ltd. AccQ Fluor reagent (*N*-hydroxysuccimidyl-6-aminoquinolinylcarbonate) kit was acquired from Waters Corporation. *p*-*N*,*N*,*N*-trimethylammonioanilyl *N*′-hydroxysuccinimidyl carbamate iodide (TAHS) was chemically synthesized as previously reported [32]. All antibodies and the peroxidase-conjugated streptavidin used in ELISA assays were purchased from Jackson ImmunoResearch (West Grove, PA). *o*-Phenylenediamine (OPD) and 30 % hydrogen peroxide solution were purchased from Kanto

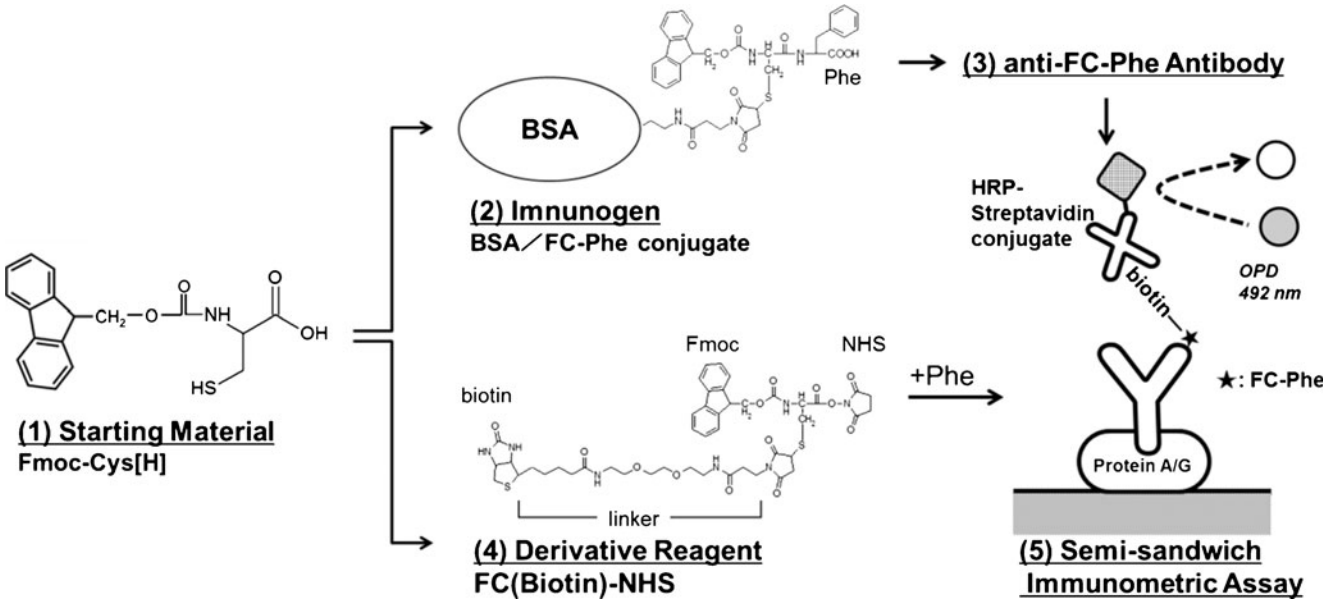

Fig. 1 Principle of the novel semi-sandwich immunometric assay. (*1*) Basic structure (Fmoc-Cys[H]). (*2*) Immunogen (BSA/FC-Phe conjugate). (*3*) Immunization of mice with the immunogen and preparation of monoclonal antibodies. (*4*) Derivatization reagent (FC(Biotin)-NHS). (*5*) Detection at 492 nm. *BSA* bovine serum albumin, *HRP* horseradish peroxidase, *OPD* *o*-phenylenediamine, *Phe* phenylalanine, *NHS* *N*-hydroxysuccinimide, *FC* *N*-Fmoc-L-cysteine

Chemical Co., Inc. A blocking reagent, Block Ace, was purchased from Bio-Rad Laboratories, Inc. All standard amino acids were obtained from Sigma-Aldrich, Japan.

Preparations of four haptens

Fmoc-Cys[H]-Phe (FC-Phe) was synthesized as follows: 5 mL of 2 mM L-cystinyl-bis L-phenylalanine in water/acetonitrile (50 %, *v*/*v*) and 0.5 mL of 250 mM Fmoc-OSu in acetonitrile were mixed and heated at 55 °C for 30 min. After cooling to room temperature, 1 mL of 3 M aqueous DTT was added to the reaction mixture and heated again at 55 °C for 120 min. The resulting mixture was fractionated using reversed-phase HPLC and lyophilized to give FC-Phe as a white powder (yield, 58 %). A portion of the lyophilized powder was redissolved and analyzed using a mass spectrometer. The value of the observed protonated ion, $[M+H]^+$ at *m*/*z*491.4, was identical to the theoretical value.

TAHS-Cys[H]-Phe (TC-Phe) was prepared as follows: 1 mL of 20 mM L-cystinyl-bis L-phenylalanine in 10 % hydrochloric acid solution, 3 mL of 200 mM sodium borate buffer, and 1 mL of 200 mM TAHS in acetonitrile were mixed and heated at 55 °C for 20 min. After cooling to room temperature, 0.5 mL of 3 M aqueous DTT was added to the reaction mixture and maintained at 40 °C for 60 min. After cooling to room temperature, 2.25 mL of 0.2 % acetic acid was added. The resulting mixture was fractionated using reversed-phase HPLC and lyophilized to give TC-Phe as a white powder (yield, 49 %). $[M+H]^+$ at *m*/*z*445.5 (LC/MS) was identical to the theoretical value.

AQC-Cys [H]-Phe (AC-Phe) (AQC; 6-aminoquinolyl-*N*-hydroxysuccinimidyI carbamate) was synthesized in the same manner as TC-Phe using 1 mL of 100 mM of AQC reagent in acetonitrile. The value of the observed protonated ion, $[M+H]^+$ at *m*/*z*439.5 (LC/MS), was identical to the theoretical value.

L-Cysteinyl-L-phenylalanine (Cys[H]-Phe) was prepared by the reduction of L-cystinyl-bis L-phenylalanine as follows: 5 mL of 2 mM L-cysteinyl-y-phenylalanine in water/acetonitrile (50 %, *v*/*v*) and 1 mL of 3 M DTT in water were mixed and heated at 55 °C for 60 min. After cooling to room temperature, the resulting mixture was fractionated using reversed-phase HPLC and lyophilized to give Cys[H]-Phe as a white powder (yield, 79 %). $[M+H]^+$ at *m*/*z*269.3 (LC/MS) was identical to the theoretical value.

Preparation of the derivatization reagent, Fmoc-Cys[Biotin]-NHS

Fmoc-Cys[Biotin]-NHS (FC(Biotin)-NHS) was prepared as follows: 10 mL of 2 mM *N*,*N*-bis-Fmoc-L-cystine in 50 mM sodium phosphate buffer (pH 6.0) and 1 mL of 3 M DTT in water were mixed and heated at 55 °C for 30 min for conversion to the reduced form. After cooling to room temperature, the resulting mixture was purified using reversed-phase HPLC and lyophilized to give Fmoc-L-cysteine (yield, 77 %). Redissolved Fmoc-L-cysteine in 5.83 mL of sodium phosphate buffer (pH 6.0) was added to 2.38 mL of 40 mM maleimide–PEG2–biotin (Thermo Scientific) in 50 mM sodium phosphate buffer (pH 7.3)

containing 0.1 % EDTA and incubated at room temperature for 2 h. The resulting mixture was fractionated using reversed-phase HPLC and lyophilized to give Fmoc-Cys[Biotin] (FC(Biotin); yield, 90 %). The $[M+H]^+$ value by LC/MS analysis at *m/z*869 was identical to the theoretical value. Then, 0.869 mg (1 μmol) of FC(Biotin), 12 μL of 0.1 M DCC in acetonitrile, and 12 μL of 0.1 M NHS in acetonitrile were added to 56 μL of acetonitrile. The solution was mixed and incubated at room temperature for 120 min, at which point the FC(Biotin) peaks (two diastereomers at the maleimide–sulfhydryl binding position) disappeared and the expected FC(Biotin)-NHS peak intensity reached a maximum. The production of derivatization reagent, Fmoc-Cys[Biotin]-NHS (FC(Biotin)-NHS, was confirmed by LC/MS ($[M+H]^+$ at *m/z*1,017 as FC(Biotin)-Phe after reaction with L-phenylalanine).

Derivatization condition of L-phenylalanine and other amino acids by FC(Biotin)-NHS

Two microliters of FC(Biotin)-NHS solution, 4 μL of aqueous 0.1 M L-phenylalanine solution, and 4 μL of 0.1 M sodium borate buffer (pH 9.0) were added to 10 μL of acetonitrile. The mixture was incubated at room temperature for 15 min, followed by quenching with 20 μL of 0.2 % aqueous acetic acid. The resulting mixture containing FC(Biotin)-Phe was stored at 4 °C until use. The same derivatization procedure was used for other individual products (the proteinogenic amino acids tested and L-DOPA) or the mixture of amino acids used for cross-reaction monitoring. Production of the objective reactant was confirmed by monitoring the molecular ion weight using LC/MS.

Some purified FC(Biotin)–amino acids (AA=Phe, Tyr, Gly, Ala, Val, Ile, Leu) were prepared by further isolation using HPLC to assess the hapten/antibody binding without the effect of the remaining reagents. The concentration of each resulting FC(Biotin)-AA was quantified by HPLC using UV absorbance of Fmoc residue at 262 nm.

Preparation of BSA/hapten conjugates as immunogens

Each hapten (FC-Phe, TCs-Phe, AC-Phe, or Cys-Phe) was conjugated to BSA using Imject® Maleimide Activated BSA (Thermo Fisher Scientific, Rockford, IL). The maleimide-activated BSA solution (2 mg/mL) in 50 mM phosphate buffer (pH 7.3) containing 0.1 M EDTA was mixed with each hapten solution (0.5 mg/200 μL) in dimethyl sulfoxide and maintained at room temperature for 2 h. The resulting BSA/hapten conjugate solutions were subjected to ultrafiltration using Amicon® Ultra-4 centrifugal filter (MWCO=10 kDa) and then washed seven times with 50 mM phosphate buffer (pH 7.3) to remove excess unreacted hapten and EDTA. The conjugates were quantified using the Bradford method (reading at 595 nm) and finally adjusted to a concentration of 0.5 mg/mL. The number of haptens binding to BSA was analyzed by MALDI-TOF-MS.

Immunization protocol and evaluation of antisera

Ten micrograms of each BSA/hapten conjugate prepared was emulsified in Freund's complete adjuvant (Difco, Detroit, MI) and subcutaneously injected into the back of 4-week-old BALB/c mice (three females; Charles River Japan Inc., Kanagawa, Japan). The second and third immunizations were conducted 2 and 4 weeks after the first immunization under the same conditions, except for using Freund's incomplete adjuvant (Difco). One week after the third immunization, antibody titers in murine antisera were tested. Briefly, haptens were immobilized on a 96-well microtiter Reacti-Bind Maleimide Activated Plate (Thermo Fisher Scientific) by maleimide–sulfhydryl group coupling. Blood samples from immunized mice were centrifuged (3,000 rpm, at 4 °C, 15 min) and the obtained sera were serially diluted with 0.1 % gelatin-containing PBS (10^5-fold). After the addition of the diluted serums, the wells were incubated at room temperature for 1 h and washed with PBS containing 0.05 % Tween-20 (PBST) three times. After 1-h incubation with peroxidase conjugate affinity-purified goat anti-mouse IgG Fc-specific antibody (20 ng/100 μL per well, Jackson ImmunoResearch), the wells were washed with PBST and reacted with OPD (100 μL, 0.04 %, in 20 mM sodium citrate buffer, pH 5.0)/hydrogen peroxide (100 μL, 0.018 %). After adding 100 μL of 1 M aqueous sulfuric acid solution, absorbance was measured at 492 nm. The mouse with the highest antisera titer was administered a final (fourth) immunization and subjected to splenectomy.

Cloning of hybridoma and preparation of monoclonal anti-FC(Biotin)-Phe antibodies

The production of monoclonal antibodies was conducted based on a procedure by Kohler and Milstein [33]. Spleen cells from a mouse with the highest antibody titers for each immunogen were fused with P3-Ag-X3 myeloma cells using PEG1500 to assist fusion. Eight 96-well microtiter plates, which were used for the antibody titer tests, were also used for primary and secondary screening. Wells with an absorbance above 3.0 were considered positive.

In the cloning step, a limiting dilution method was adopted. Selected parent cell cultures from the secondary screening were subjected to cloning steps. The cell cultures were serially diluted by factors of 10, 100, and 1,000 with medium containing 10 % (*v*/*v*) BM-condemned H1 (Roche Diagnostics, Tokyo, Japan) and placed in wells to incubate. Detection of binding to immobilized haptens on 96-well

microtiter plates was performed as above, and clones with an absorbance above 3.0 were subjected to re-cloning for single clone selection. For large-scale preparation of purified monoclonal antibodies, ascites were prepared after intraperitoneal administration of hybridoma strains to BALB/c mice. The obtained ascites were purified with a Protein G column (GE Healthcare, Japan) to isolate monoclonal antibodies and cryopreserved at −20 °C until required.

New semi-sandwich immunometric assay procedure for standard L-phenylalanine analysis

A new semi-sandwich immunometric assay for L-phenylalanine analysis was established according to the following procedure. First, monoclonal antibodies that specifically recognized FC-Phe were immobilized on 96-well microtiter Reacti-Bind Protein A/G-coated plates (Thermo Fisher Scientific). Purified monoclonal antibody (100 μL of 0.1 μg/mL in PBS, pH 7.3) was dispensed to the 96-well plates and incubated at room temperature for 2 h. Then, the plates were washed three times with PBST. In the second step, the purified derivatized L-phenylalanine was analyzed. Purified FC(Biotin)-Phe was serially diluted with PBS containing 0.1 % gelatin solution (GPB) with/without 10 % acetonitrile and 100 μL of the solution was added to wells and incubated at room temperature for 60 min. After washing with PBST, 100 μL of 1 μg/mL streptavidin/HRP conjugate solution (Jackson ImmunoResearch) in 0.1 % gelatin containing PBS(GBP)/Applie block (95:5, Seikagaku Biobusiness Co., Ltd., Tokyo, Japan) was added to the wells, mixed, incubated at room temperature for 60 min, and then washed three times with PBST. Detection was performed using the reaction of OPD (100 μL, 0.04 %, in 25 mM sodium citrate buffer, pH 5.0)/hydrogen peroxide (6 μL, 0.018 % in water). After adding 100 μL of 1 M aqueous sulfuric acid solution, absorbance was measured at 492 nm and a response curve was obtained.

Cross-reactivity tests for the selection of monoclonal anti-FC-Phe antibodies

Selection of the best-performing monoclonal anti-FC-Phe antibodies regarding cross-reactivity was performed using purified FC(Biotin)-AA (Phe, Tyr, Gly, Ala, Val, Leu, and Ile). Assays were conducted as for the semi-sandwich immunoassay described above.

Selectivity test for characterization of FC-Phe#07MAb

Selectivity of FC-Phe#07MAb was examined using the pre-derivatized reaction solution of the individual or mixture of amino acids. The reaction solution was diluted 3,000-fold prior to the immunoassay. Samples were assayed using the new semi-sandwich immunoassay procedure using GPB with and without 10 % acetonitrile as a hapten/antibody reaction buffer.

Quantitative determination of L-phenylalanine in rat plasma

The new immunoassay approach was applied to biological samples using FC-Phe#07 monoclonal antibodies (MAb). Rat plasma samples (female Sprague–Dawley, 20 weeks old) were deproteinized with 50 % acetonitrile, followed by vortexing and 5 min centrifugation at 15,000 rpm. Four microliters of deproteinized plasma or standard L-phenylalanine solution, 4 μL of 0.1 M sodium borate buffer (pH 9.0), and 1 μL of the derivatization reagent, FC(Biotin)-NHS, were added to 11 μL of acetonitrile and incubated at room temperature for 15 min. After adding 20 μL of 0.2 % acetic acid, the reaction mixtures were diluted 3,000-fold with GPB plus 10 % acetonitrile and 5 % blocking reagent for the semi-sandwich immunometric assay, in accordance with the regulations set by the Animal Investigations Committee of the Ajinomoto Corporation.

Results and discussion

Strategy for new semi-sandwich-type immunometric assay for L-phenylalanine analysis

To develop a novel semi-sandwich-type immunometric assay, there were two problems to be solved: (1) it is difficult to obtain anti-L-phenylalanine MAbs from immunized animals and (2) reliable sandwich-type immunometric assays (two-site immunoassay) cannot be applied to L-phenylalanine assays because of their small size. The main reason we wanted to purify MAbs is that they are more homogenous and have a higher specificity than polyclonal antibodies. These features can lead to higher reproducibility between experiments, especially when used for sandwich immunometric assays. In general, carrier protein/hapten conjugates are usually used as immunogens to stimulate an animal immune system because small antigens have low immunogenicity. Since L-phenylalanine is small and an endogenous compound, further methods were required to obtain L-phenylalanine-specific MAbs. Figure 1 (upper panel) shows the strategy for the acquisition of MAbs for L-phenylalanine assays. An amino group was modified by FC residues to enhance immunogenicity prior to BSA conjugation.

Competitive immunoassay approaches [34–36] have been exclusively used for small compound analysis. Non-competitive [37, 38] immunometric assays, especially sandwich-type immunoassays, allow reliable experimentation compared with competitive immunoassays in terms of higher precision and sensitivity, shorter reaction time, and

broader working range [31]. Since the sandwich-type immunoassay requires two or more distinct antibodies, the monoclonal anti-FC-Phe antibody obtained in this study was not applicable to common sandwich-type immunoassays. Figure 1 (lower panel) illustrates the pre-derivatization method for L-phenylalanine adopted using the newly synthesized derivatization reagent, FC (Biotin)-NHS. This reagent was designed to provide L-phenylalanine with an epitope (FC-Phe) for antibody recognition and a second binding site for optimal detection. By coupling these ideas, we developed a novel semi-sandwich immunometric assay approach using a single monoclonal antibody, which could be used for reliable immunometric assays for L-phenylalanine measurement.

Optimization of hapten design

To obtain MAbs for L-phenylalanine analysis, haptens were designed based on the amino group modification of L-phenylalanine by X-L-cysteinyl structures (XC). Group X was expected to provide an exogenous and complex structure to enhance immune responses and higher affinity by expanding the contact area between the hapten and antibody. A cysteine residue was induced as a juncture to the X moiety, L-phenylalanine and carrier protein.

The four haptens of X-L-cysteinyl-L-phenylalanine, where X was Fmoc (F), AQC (A), and TAHS (T), were synthesized from L-cystinyl-bis-L-phenylalanine. The dipeptide of L-cysteinyl-bis-L-phenylalanine (C-Phe) was also prepared as a control to assess the contribution of the X moiety. The immunogens (BSA/hapten conjugates) were prepared using maleimide–sulfhydryl coupling between maleimide-activated BSA and hydrogen sulfide on the cysteine side chain. The resulting conjugates of BSA/FC-Phe, BSA/AC-Phe, BSA/TC-Phe, and BSA/C-Phe were confirmed with MALDI-TOF-MS, and the number of conjugated haptens was estimated to be 10, 8, 9, and 19, respectively. Antibodies against FC-Phe, AC-Phe, TC-Phe, and C-Phe were raised in three mice immunized with each immunogen.

Figure 2 shows the antibody titers in antisera from immunized mice at day 28. Immunoassays were performed using target hapten-immobilized 96-well microtiter plates, prepared by coupling maleimide-activated plates and cysteine sulfhydryl groups from each hapten. The antibody titers from BSA/XC-Phe-immunized mice showed a tendency to be higher than from BSA/C-Phe-treated mice; the highest antibody titers were obtained from BSA/FC-Phe-immunized mice. In addition, Table 1 shows the number of hybridoma cell strains that survived the monoclonization procedure. The results also suggested that the Fmoc-bearing immunogen had the highest efficiency for obtaining hybridoma cells. For the C-Phe-containing immunogen, all clones failed at the cloning steps. This suggested that the introduction of a unique structure (X) effectively enhanced the immune responses. Considering the potency of hybridoma preparation and the availability of the materials for hapten synthesis, FC-Phe was finally chosen as the hapten for our approach. For large-scale preparation of monoclonal anti-FC-Phe antibodies, ascites were collected after intraperitoneal administration of ten hybridoma cell strains to BALB/c mice.

Design and synthesis of the pre-derivatization reagent, FC(Biotin)-NHS, for a novel semi-sandwich immunometric assay

The derivatization reagent, FC(Biotin)-NHS, was designed for the semi-sandwich immunoassay for L-phenylalanine quantitative determination. The reagent has three branches, which are linked to Fmoc, a biotin linker and an NHS ester from the L-cysteine residue. Fmoc was important for yielding an effective epitope for anti-FC-Phe antibodies. The biotin linker provided a second binding site for streptavidin/HRP conjugate for optical detection. The role of the NHS ester was to react with the amino groups from amino acids. The derivatization reagent was synthesized via three steps from bis-Fmoc-L-cystine. To optimize the condensation reaction of the reagent with amino acids, the buffer pH, reaction time, and reaction temperature were investigated by monitoring the product peak with HPLC (UV, 265 nm) and were determined as pH 9.0 (sodium borate buffer), 15 min, and room temperature, respectively. Finally, the derivatization reagent was provided as a reaction mixture of FC(Biotin) with NHS at the time of use because of its higher reactivity.

Preliminary test of a novel semi-sandwich immunometric assay

In the first step for the establishment of a novel semi-sandwich immunometric assay, feasibility was assessed using purified monoclonal anti-FC-Phe antibodies from ascites. The purified analyte was used to exclude the influence of the remaining derivatization reagents on antibody recognition. The obtained monoclonal anti-FC-Phe antibodies (FC-Phe#01–#10MAb) were immobilized onto a protein A/G-coated 96-well plate. After incubation of serially diluted FC(Biotin)-Phe standard solutions and washing, it was incubated with streptavidin/HRP conjugate solution. Optical detection of the captured antigens was carried out using a reaction system consisting of HRP/H_2O_2/OPD (at 492 nm). Typical dose–response curves with a sigmoidal shape were obtained for five monoclonal antibodies (#02, #03, #07, #09, and #10). Other monoclonal antibodies showed little response against FC(Biotin)-Phe; therefore, they were considered to have insufficient binding affinities to haptens. Thus, the principle of our semi-sandwich immunometric

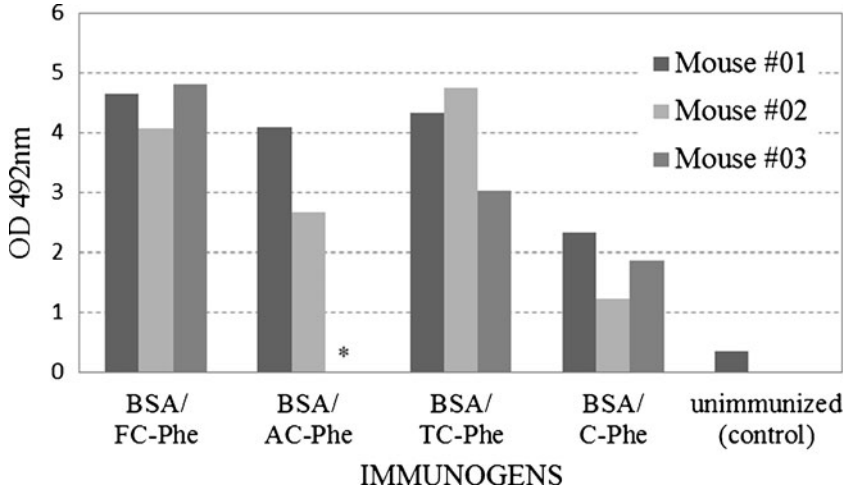

Fig. 2 Comparison of antibody titers at day 28 in mice immunized with immunogens (BSA/FC-Phe, BSA/AC-Phe, BSA/TC-Phe, and BSA/C-Phe). The presence of antibodies was analyzed with ELISA using each hapten immobilized on a 96-well microtiter plate after diluting the sera 10^5-fold. For unimmunized mice, an FC-Phe-immobilized plate was used. *BSA/AC-Phe-immunized mouse #03 died during the experiment. *OD* optical density, *BSA* bovine serum albumin, *Phe* phenylalanine

assay was confirmed using FC-Phe(#02, #03, #07, #09, and #10)MAb and a purified hapten of FC(Biotin)-Phe.

Selection of the best-performing monoclonal anti-FC-Phe antibody

The monoclonal anti-FC-Phe antibody with the best performance was selected from among the five clone (FC-Phe(#02, #03, #07, #09, and #10)MAbs according to its affinity and specificity (Fig. 3). To assess its specificity to L-phenylalanine, a primary cross-reactivity test was performed by comparing the dose–response curves of the purified FC(Biotin)-Phe and other derivatives, FC(Biotin)-(Tyr, Leu, Ile, Val, Ala, Gly, and OH). FC(Biotin)-(Tyr, Leu, Ile, and Val) were chosen to access the impact of hydrophobicity on its selectivity and FC(Biotin)-(Ala and Gly) were used to verify recognition of the phenylalanine side chain by comparing it with samples with no (Gly) or small (Ala) side chains. FC(Biotin)-OH corresponded to the structure of the hydrolyzed derivatization reagent. As a result, FC-Phe(#02, #03, #09)MAbs were excluded as they demonstrated low sensitivity and selectivity. FC-Phe#10MAb demonstrated a relatively higher selectivity, but could not distinguish between the derivatives of phenylalanine and luecine. Consequently, FC-Phe#07MAb was selected for the semi-sandwich immunoassay, although it demonstrated slight cross-reactivity to FC(Biotin)-Leu (11.2 %) and Tyr (12.6 %).

Optimization of a new semi-sandwich immunometric assay and selectivity test for FC-Phe#07MAb

We optimized the immunoassay conditions using FC-Phe#07MAb. To assess the potency under actual usage conditions, a mixture of amino acids were subjected to analysis through the pre-derivatization step. Cross-reactivity was examined by comparing three dose–response curves to (A) derivatized L-phenylalanine alone and (B) a mixture of the derivatized L-phenylalanine and 19 other amino acids (Fig. 4a). The difference of the responses between curves (A) and (B) maybe caused by the sum of cross-reactivity observed in Fig. 4, and the optimization of the reaction conditions is essential for accurate quantitative determination.

To decrease the cross-reactivity of FC-Phe#07MAb for other amino acids, a solution at the antigen–antibody incubation step was investigated. Figure 4b shows the result of the addition of 10 % acetonitrile to the buffer. The difference between the dose–response curves of the derivatized L-phenylalanine alone (C) and a mixture of the derivatized L-phenylalanine and 19 other amino acids (D) was significantly minimized, while the sensitivity of L-phenylalanine was almost unaffected. This result suggests that the L-phenylalanine structure in the hapten was specifically recognized by FC-Phe#07MAb under these conditions. The effect of acetonitrile content was decided at 10 % since a 5 % content did not sufficiently improve sensitivity and above 10 % caused a

Table 1 Preparation of monoclonal anti-derivatized L-phenylalanine antibodies for various hapten structures

Step	Procedure	Survived number	Hapten structures			
			FC-Phe	AC-Phe	TC-Phe	C-Phe
Step 1	Primary screening	Positive wells	31	75	165	9
Step 2	Secondary screening		14	12	26	Not measured
Step 3	Cloning	Cell lines	11	11	16	3
Step 4	Re-cloning		10	9	12	0
Step 5	Final positive confirmed		10	5	6	0

Phe phenylalanine

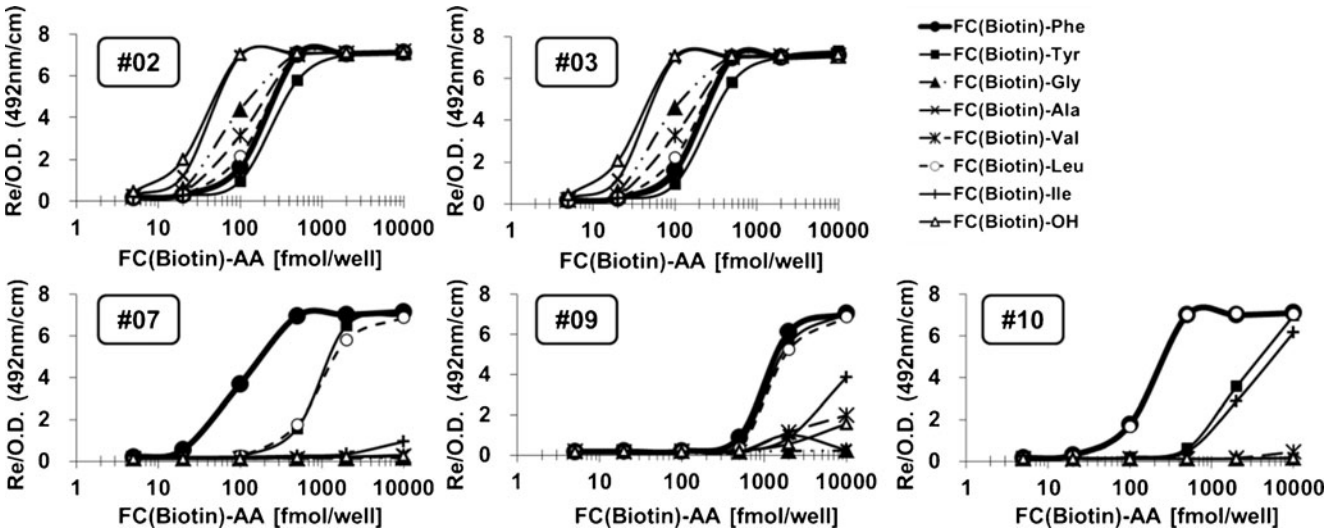

Fig. 3 Dose–response curves for eight FC-AA standard solutions using monoclonal anti-FC-Phe antibodies. *Horizontal axis*, Absolute quantity of FC(Biotin)-AA] (in femtomoles per well: AA=Phe, Tyr, Gly, Ala, Val, Ile, Leu, OH). *Vertical axis*, Relative optical density (*OD*) at 492 nm. The plots were obtained from the average values of duplicate assay results

decrease of responses. Greater than 10 % addition of the organic solvent might interfere with the hydrophobic interactions between FC-Phe#07MAb and FC(Biotin)-Phe or denaturize the antibody structure. Thus, it was considered that 10 % acetonitrile addition improved the recognition mode of the antibody to the hapten or increased the effective washing of nonspecifically absorbing other derivatized amino acids in the well. Methanol addition was not effective in selectivity improvement (data not shown). Changing the reaction times (30–120 min) or pH conditions did not affect selectivity, but reduced the intensity.

After optimizing the semi-sandwich immunometric assay conditions, the standard calibration curve was determined to various L-phenylalanine concentrations including the pre-derivatization step (Fig. 5). It covered a working range of 0.1–20 μmol/L, and the limit of detection (LOD) was calculated as 35 nmol/L. Because the original L-phenylalanine solution was diluted 60,000-fold throughout the assay protocol, the LOD value corresponded to an absolute amount of 60 amol per well (100 μL). It is surprising that an immunoassay for a small substance has such a good performance.

A previous study attempted to develop new sandwich immunometric assays applicable to small compounds [31] using "anti-metatype antibodies" as second antibodies, which recognized a particular antigen–antibody complex but did not bind to the antibody or the antigen alone. In the case of dioxin analysis, a LOD of 1 fmol was attained [39]. Another study used antibodies that could bind to an immobilized cyclodextrin–hapten complex [40]. However, the production of those antibodies from immunized animals was considered too difficult. Kobayashi et al. [30] tried to isolate evolved single-chain Fv fragments from a phage display that recognized vitamins/β-cyclodextrin complex and improved the sensitivity to a nanomole range of detection. The "open-sandwich immunoassay" [41] was based on the

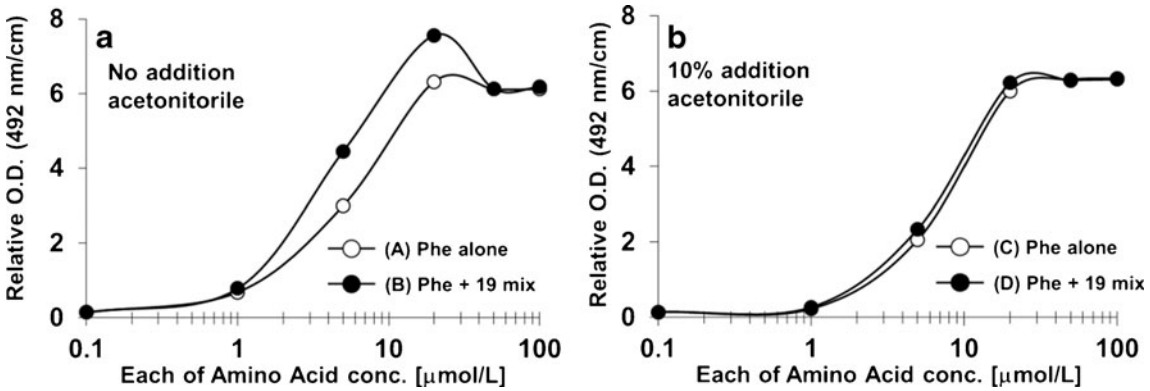

Fig. 4 Cross-reactivity test of FC-Phe#07MAb. Response of the semi-sandwich immunometric assay using FC-Phe#07MAb. Both (**a**) and (**b**) demonstrate the L-phenylalanine selectivity of the FC-Phe#07MAb antibody under different reaction buffer conditions (with and without the addition of 10 % acetonitrile). *OD* optical density, *Phe* phenylalanine. The plots were obtained from the average values of duplicate assay results

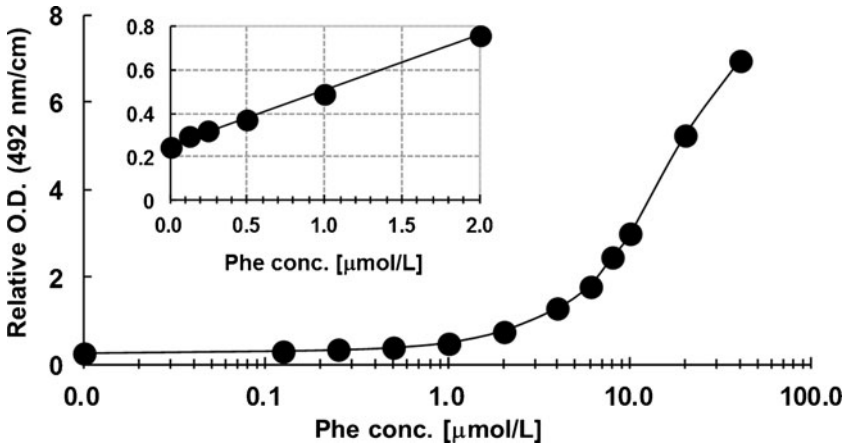

Fig. 5 Standard curve obtained with L-phenylalanine in semi-sandwich immunometric assay using FC-Phe#07MAb throughout all procedures, including pre-derivatization. *OD* optical density, *Phe* phenylalanine. Standard deviations to the plots were within 0.04 (*N*=4)

principle of stabilization of the antibody-variable region Fv upon binding the antigen. The VH fragment, which played the role of a second binding site, was bound to an immobilized VL fragment in the presence of antigen. Although they could be applied to various smaller targets, they required molecular biology and protein engineering techniques. Our novel semi-sandwich immunometric assay had the advantage of not requiring the production of two or more distinct antibodies or the use of special techniques. The biotin moiety of FC(Biotin)-Phe formed a second binding site for the streptavidin/HRP conjugate for optical detection. Regarding the distance of two-site binding (antigen–antibody binding and avidin–biotin binding), the model study indicated that at least a 11.5-Å distance was required for a successful sandwich assay [42]. In our case, the estimated distance between biotin and cysteine was 29.1 Å and is considered sufficient for simultaneous binding by FC-Phe#07MAb and the streptavidin/HRP conjugate.

Assessment of a novel semi-sandwich immunometric assay by rat plasma analysis

To assess the reliability of the proposed semi-sandwich immunoassay coupled with the pre-derivatization method, L-phenylalanine concentration was determined in rat plasma samples. In the first step, deproteinization of the sample was investigated. Although conventional immunoassays usually do not require pretreatment due to the specificity of antibodies, the efficiency of derivatization can be affected by unfavorable reactions with the amino groups in proteins. Deproteinization was successfully achieved by the addition of acetonitrile (final concentration, 50 %). Filtration using membrane filters (MWCO=5,000) was not successful, probably because of the insufficient removal of small proteins. The supernatants from deproteinized samples were derivatized according to the protocol described in this study. After dilution of the reaction mixtures 3,000-fold with buffer, 100 μL of the solutions was analyzed. Consequently, L-phenylalanine concentration in the rat plasma sample was determined to be 78.3 μM using the external standard method. Intra-day precision, expressed as percent coefficient of variation (CV) of repeatability, was within 6 % (*N*=3) for days 1 and 2. The variation between days 1 and 2 was 0.8 μM (0.1 %). The result of recovery rate testing by standard addition at four concentrations (50, 100, 200, and 400 μM) was 92.4–123.7 %, indicating that detection was not affected by matrix components in the plasma samples (Table 2). This result was consistent with that from LC/MS analysis [22] (76.6 μM). Taken together, our novel semi-sandwich immunometric assay was successfully applied to the measurement of L-phenylalanine from rat plasma samples with good performance.

Table 2 L-Phenylalanine determination by novel semi-sandwich immunoassay using FC-Phe#07MAb

Addition of standard L-Phe(μM)	Day 1 (*N*=3)				Day 2 (*N*=3)		
	Mean (μM)	SD (μM)	CV (%)	Recovery (%)	Mean (μM)	SD (μM)	CV (%)
0	78.3	4.7	6.0	–	79.8	4.1	5.2
+50 μM	124.5	–	–	92.4	–	–	–
+100 μM	190.8	–	–	112.5	–	–	–
+200 μM	325.7	–	–	123.7	–	–	–
+400 μM	530.2	–	–	113.0	–	–	–

N number, *SD* standard deviation, *CV* coefficient of variation, *Phe* phenylalanine

Conclusions

A novel semi-sandwich immunometric assay was successfully developed for the quantitative analysis of L-phenylalanine. This approach included three processes. The first was the preparation of a monoclonal anti-FC-Phe antibody (FC-Phe#07MAb) from immunized mice. Next was the design of the derivatization reagent, FC(Biotin)-NHS, and a pre-derivatization step that converted L-phenylalanine to an epitope structure for antibody binding. The reagent also provided a second binding site that permitted a sandwich-like immunoassay by streptavidin/HRP conjugate binding. The last step was the optimization of the assay conditions. The novel approach enabled L-phenylalanine to be analyzed similar to that of a reliable sandwich assay. The limit of detection and working range obtained were 35 nmol/L (60 amol) and 0.1–20 μmol/L, respectively. From the rat plasma analysis, acceptable intra-day precision was obtained with <6 % CV. Day 1 and 2 difference was 0.1 %. The recovery rates were 92.4–123.7 %. To our knowledge, this is the first report of the quantitative determination of L-phenylalanine using a sandwich-like immunometric assay. We hope that this method will be applicable for the analysis of other amino acids.

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