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Combining [$^{13}C_6$]-phenylisothiocyanate and the Edman degradation reaction: a possible breakthrough for absolute quantitative proteomics together with protein identification

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This manuscript describes the results of a preliminary experiment performed as 'proof of concept' of a novel approach to absolute quantitation of proteins without the use of standard proteins. Absolute quantitation remains a challenging issue in the proteomics field. Therefore, we propose a combination of [$^{13}C_6$]-phenylisothiocyanate (PITC) and the Edman degradation reaction as a possible breakthrough. [$^{13}C_6$]-PITC was synthesized from [$^{13}C_6$]-aniline with *O,O'*-di-2-pyridyl thiocarbonate to prepare [$^{13}C_6$]-phenylthiohydantoin (PTH)-amino acids as internal standards. Upon the Edman degradation reaction, it has been confirmed that a model protein, bovine serum albumin (BSA), releases the *N*-terminal amino acid quantitatively as PTH-Asp. The standard curve of PTH-Asp against [$^{13}C_6$]-PTH-Asp showed good linearity ($r^2 = 0.9977$). BSA could be quantified as PTH-Asp using the standard curve. In addition, the residual des-Asp^1-BSA provided sufficient information for further protein identification. Copyright © 2009 John Wiley & Sons, Ltd.

It has been more than a decade since the word 'proteome' was introduced.[1] The mass spectrometry (MS)-based protein identification methodology named 'proteomics' immediately spread with the high expectation[2] that it could express phenotypes under various physiological states as the next era of genomics.[3] However, researchers have noticed that quantitation based on this methodology was not as simple as hoped. For MS-based analyses, the stable isotope dilution technique is the most powerful technique for quantitation.[4] Therefore, several stable isotope labeling methods have been applied for quantitative proteomics. Oda *et al*. introduced the technique for the first quantitative proteomics.[5] They used ^{15}N-labeled media for the cell cultures and achieved accurate quantitation of protein expression and site-specific phosphorylation. This concept was further improved by Mann and colleagues in a method named SILAC (Stable Isotope Labeling with Amino acids in Cell culture).[6] They used [D_3]-leucine instead of ^{15}N-labeled media to facilitate further applications for mammalian systems and tandem mass spectrometry (MS/MS)-based protein identifications. These methodologies enabled the introduction of stable isotopes for the entire proteome; however, it is limited to cell experiments. On the other hand, Fenselau and colleagues have reported the use of tryptic digestion in ^{18}O-water as a method to introduce ^{18}O into whole digested peptides.[7] In a different approach to introducing stable isotopes, several stable isotope labeled tags have been reported. Gygi *et al*. have reported quantitative proteomics attaching a deuterated biotin tag to cysteines in the technique named Isotope-Coded Affinity Tags (ICAT).[8] This methodology has been further improved as *cleavable* ICAT, which has a cleavable site on the reagent to overcome the low recovery from the avidin column and is labeled with ^{13}C instead of deuterium to reduce the isotope effect on chromatography.[9] In addition, resins attached with deuterated tags have been designed by Zhang *et al*. to facilitate the clean-up step.[10] Isobaric Tag for Relative and Absolute Quantitation (iTRAQ^{TM}) was developed next.[11] The iTRAQ^{TM} reagents are a set of isobaric reagents that have four different mass combinations to enable simultaneous identification and quantitation of up to four different experiments. Because the reagents can react through the lysine residue and the *N*-terminal amino group, more peptides can be labeled than with ICAT. All of the above methodologies have made major contributions to the proteomics field; however, absolute quantitation remains a challenging issue.[12]

Absolute quantitation can be attained if limited numbers of proteins are targeted, such as amyloid betas in cerebrospinal fluid,[13] membrane transporter proteins,[14] and phosphorylation on the kinase activation loop of cellular focal adhesion kinase.[15] These approaches are possible when sufficient amounts of standards and stable isotope labeled standards (or tryptic peptides) are available to prepare standard solutions. However, it is virtually impossible to obtain large enough amounts of standards and the corresponding stable isotope labeled internal standards (ISs) for the entire

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proteome. Therefore, a novel approach is needed to attain absolute quantitation in global proteomics.

A traditional and well-known method for amino acid sequencing in a peptide, reported in 1950, is the Edman degradation reaction; it was used almost exclusively for protein identification before MS-based proteomics appeared.[16] The degradation reaction consists of three steps: (i) Edman reagent (phenylisothiocyanate, PITC, Fig. 1(a)) is allowed to react with the *N*-terminal α-amino group of the peptide; (ii) the resulting phenylthiocarbamoyl derivative (PTC-peptide, Fig. 1(b)) is cleaved at the first amide bond to produce the anilinothiazolinone derivative (ATZ-amino acid, Fig. 1(c)) and des-1 peptide (Fig. 1(d)); and (iii) the relatively labile ATZ-amino acid is converted into the more stable phenylthiohydantoin derivative (PTH-amino acid, Fig. 1(e)). The PTH-amino acids can be identified by their retention times using high-performance liquid chromatography–ultraviolet detection (HPLC-UV), and the remaining des-1 peptide can be returned to the first step to identify the next amino acid. Automated,[17] miniaturized,[18] and fluorescence[19] methods have also been used to improve the methodology. In addition, the Edman degradation reaction from gel samples has been reported recently.[20]

There have also been some attempts reported to use a combination of Edman reagent and MS as proteomics tools. Chait *et al.* have reported a novel protein ladder sequencing based on partial Edman degradation.[21] The methodology has been used for combinatorial chemistry because of its high throughput and good cost performance.[22] Aebersold *et al.* have reported several Edman-type reagents to enhance the ionization of peptides.[23] Recently, Marekov and Steinert have reported that charge derivatization using 4-sulfophenylisothiocyanate (SPITC) had high ability to enhance peptide sequencing by post-source decay matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS.[24] Guillaume *et al.* have reported that the combination of [$^{13}C_6$]-SPITC and SPITC can be further applied, not only to protein identification, but also to quantitation.[25] Therefore, a combination of Edman reagent and MS has the potential ability in the proteomics field for both sequencing and quantitation. However, no one has tried it for absolute quantitation. We assumed that proteins could be quantified as PTH-amino acids released from the proteins if the following degradation is quantitative for proteins.

There are three major potential advantages of the original Edman degradation reaction for absolute quantitative proteomics (Fig. 2): (i) all PTH-amino acids are commercially available as standards; (ii) the reaction is well studied and automation can be used; and (iii) des-1 protein can be used for further protein identification after protease digestion. Recently, Brune *et al.* have reported the great possibility of quantitation of Edman degradation with detailed data as a study by the Edman Sequencing Research Group.[26] Therefore, we believe that the Edman degradation reaction as used in combination with stable isotope labeled PTH derivatives of selected amino acids could formulate the basis of a possible breakthrough for quantitative proteomics and concomitant protein identification.

To prove the concept, we have demonstrated here the quantitation of bovine serum albumin (BSA) without a protein standard by analyzing the PTH-Asp released from the *N*-terminus. The standard curve was prepared using commercially available PTH-Asp together with [$^{13}C_6$]-PTH-Asp as the IS prepared from novel [$^{13}C_6$]-PITC (Fig. 3). The protein identification from des-Asp^1-BSA was also demonstrated by the peptide mass fingerprinting (PMF) method.

EXPERIMENTAL

Chemicals and materials

Aniline, ethylmercaptan, triethylamine, arginine (Arg), tryptophan (Trp), $CDCl_3$ (containing 0.03% tetramethylsilane (TMS)), ethyl acetate, *n*-hexane, ammonium bicarbonate, iodoacetamide (IAA), dithiothreitol (DTT), Na_2EDTA, and HCl were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Phenylisothiocyanate (PITC), *O*,*O*′-di-2-pyridyl thiocarbonate, and 2,5-dihydroxybenzoic acid (DHB) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). N_2 gas and [$^{13}C_6$]-aniline were purchased from Taiyo Nippon Sanso Co. (Tokyo, Japan). PTH-Asp, PTH-*nor*-Val, PTH-amino acids mixture standard, trifluoroacetic acid

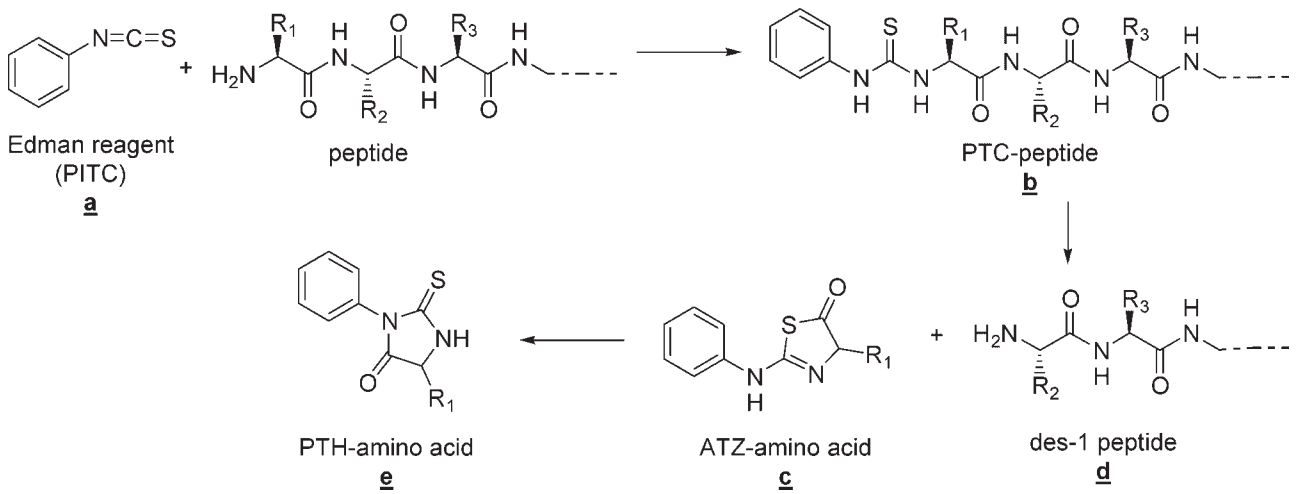

Figure 1. Edman degradation reaction. (a) Edman reagent (phenylisothiocyanate, PITC), (b) phenylthiocarbamoyl (PTC)-peptide, (c) anilinothiazolinone (ATZ)-amino acid, (d) des-1-peptide, (e) phenylthiohydantoin (PTH)-amino acid.

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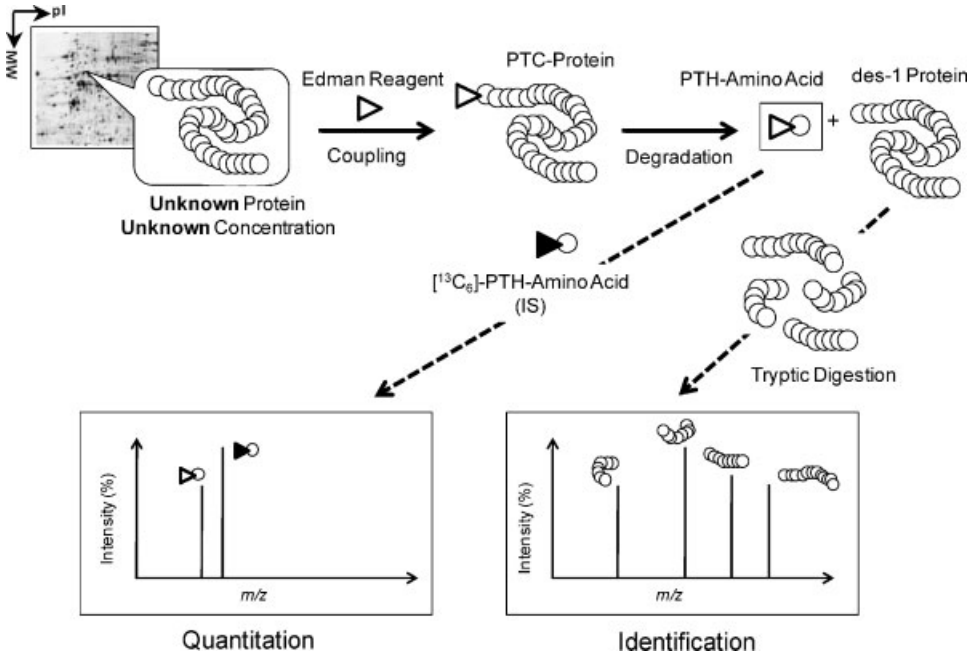

Figure 2. Concept of quantitative proteomics together with protein identification.

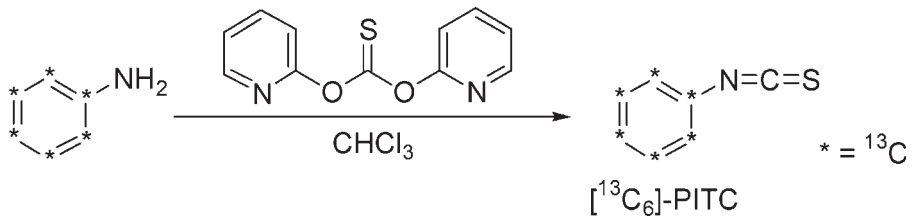

Figure 3. Structure and synthesis of [$^{13}C_6$]-Edman reagent.

(TFA), ammonium acetate, chloroethyl carbonate, phosphoryl chloride, formic acid, Wakogel® FC-40 silica gel (for flash chromatography, 20–40 μm), and tris(hydroxymethyl)aminomethane (Tris) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). HPLC grade acetonitrile and ethanol were obtained from Kanto Chemical Co. Inc. (Tokyo, Japan). Purified water was purchased from Daiwa-Yakuhin Co. Ltd. (Sendai, Japan) and further filtered using a CPW-100 Ultrapure water system (Advantec Toyo Kaisha, Ltd., Tokyo, Japan). Bovine serum albumin (BSA), α-cyano-4-hydroxycinnamic acid (CHCA), ACTH (18–39), and bovine insulin oxidized β-chain were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Angiotensin (Ang) I was purchased from American Peptide Company, Inc. (Sunnyvale, CA, USA). Human Ang II was obtained from Calbiochem/EMD Chemicals, Inc. (San Diego, CA, USA). Ang IV was purchased from the Peptide Institute, Inc. (Osaka, Japan). Oasis® HLB cartridges (1 cc, 10 mg) were obtained from Waters Co. (Milford, MA, USA). Slide-A-lyzer® Mini-Dialysis units (3500 molecular weight cutoff (MWCO)) were purchased from Thermo Fisher Scientific Inc. (Rockford, IL, USA). Sequencing-grade trypsin and guanidine-HCl were purchased from Promega Co. (Madison, WI, USA).

HPLC analyses of PTH-amino acids

Chromatography was carried out using a Nanospace SI-1 semimicrocolumn HPLC system (Shiseido Co. Ltd., Tokyo, Japan) that consisted of two model 2001 semimicropumps, a model 2002 UV-Vis detector, and a model 7125 six-port syringe loading sample injector valve (Rheodyne Co., Cotati, CA, USA). A Wakosil II 3C18 RS column (150 × 2.0 mm i.d., 3 μm, 300 Å; Wako Pure Chemicals Co., Osaka, Japan) and a YMC-Pack ODS-AM column (150 × 6.0 mm i.d., 5 μm, 120 Å, YMC Co. Ltd., Kyoto, Japan) were used for systems 1 and 2, respectively. Solvent A was 20 mM ammonium acetate buffer (pH 4.88)/acetonitrile (95:5, v/v), and solvent B was 20 mM ammonium acetate buffer (pH 4.88)/acetonitrile (5:95, v/v). The linear gradient for system 1 was as follows: 10% B at 0 min, 50% B at 10 min, 50% B at 20 min, 95% B at 21 min, 95% B at 30 min, 10% B at 31 min, and 10% B at 45 min with a flow rate of 0.2 mL/min. The linear gradient for system 2 was as follows: 10% B at 0 min, 50% B at 10 min, 95% B at 11 min, 95% B at 25 min, 10% B at 26 min, and 10% B at 40 min with a flow rate of 2.0 mL/min. All separations were performed at ambient temperature. HPLC-UV was performed at 269 nm and the data were processed using Chromato-PRO (Runtime Instrument Co., Sagamihara, Japan).

MALDI-TOF MS analyses of PTH-amino acids and tryptic peptides

MALDI-TOF MS experiments were carried out using a Voyager-DE STR MALDI-TOF mass spectrometer (Applied Biosystems, Framingham, MA, USA) located in the Biomedical Research Core, School of Medicine, Tohoku University.

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The mass spectrometer was equipped with a nitrogen laser (337 nm, 3 ns pulse width, 20 Hz repetition rate) and had a flight path of 200 cm. All spectra presented were acquired in positive-ion mode in reflection mode with the following parameters. For PTH-amino acids: accelerating voltage, 10 kV; grid voltage, 70%; extraction delay time, 100 ns; low mass gate, 100 Da; shots in the spectrum, 100–150. For tryptic peptides: accelerating voltage, 20 kV; grid voltage, 64%; extraction delay time, 100 ns; low mass gate, off; shots in the spectrum, 100. TOF MS experiments were performed in the range of m/z 100–1500 for PTH-amino acids and 500–5000 for tryptic peptides, respectively. All acquired data were processed using Data Explorer version 4.0.0.0.

Instrumental analyses

^{1}H-NMR

NMR spectra were recorded at 25°C at 500 MHz using an ECP-500 (JEOL Ltd., Tokyo, Japan) equipped with a 3 mm indirect detection gradient probe (Wilmad Glass Co. Inc., Buena, NJ, USA). [$^{13}C_6$]-PITC (ca. 1 mg) was dissolved in 150 μL of $CDCl_3$ (containing 0.03% TMS). Data processing was conducted directly on the NMR spectrometer. Chemical shifts are reported on the δ scale (ppm) by assigning the TMS peak.

High-resolution FABMS

High-resolution mass spectra were recorded using a JMS-700 double-focusing magnetic sector mass spectrometer (JEOL Ltd., Tokyo, Japan).

IR

IR spectra were determined at ambient temperature using an FTIR-400 (JASCO Co., Tokyo, Japan). [$^{13}C_6$]-PITC was diluted with $CHCl_3$. An aliquot of the solution was dropped on a KBr disk and analyzed after drying.

Edman degradation reaction for BSA

Each BSA solution (20, 50, 100, 200, 500, 1000, 2000, 5000, 10000, 20000, and 50000 pmol/10 μL in 0.1 M HCl) was transferred to a microcentrifuge tube and evaporated under a N_2 stream using a Jet Air vaporizer (Ishii Shoten Co., Tokyo, Japan). Each residue was allowed to react with 10 μL of PITC solution (PITC/triethylamine/water/ethanol 1:1:1:7, v/v/v/v) at 55°C for 15 min on a heating block (Yamato Scientific Co., Ltd., Tokyo, Japan). The reaction mixture was evaporated under a N_2 stream and the residue was then washed with *n*-hexane/ethyl acetate (15:1, v/v, 200 μL), ethyl acetate (200 μL), and acetone (200 μL × 3). Ethylmercaptan in TFA (0.1%, v/v, 20 μL) was added to the tube at 55°C for 15 min on a heating block. After evaporation under a N_2 stream, the residue was redissolved in 25% (v/v) aqueous TFA (20 μL) and heated at 65°C for 30 min. At that stage, an aliquot of this solution can be used for further protein identification if required. PTH-*nor*-Val (1 nmol/100 μL of 50% aqueous ethanol, v/v) was added to each tube as an IS. An aliquot (5 μL) of the final solution was analyzed using HPLC system 1. The peak area ratio (PTH-Asp/PTH-*nor*-Val) was then plotted against BSA concentration. The calculations were performed using Microsoft Office Excel 97–2003 with the following criteria: curve, linear; origin, ignored. The above experiment was performed in triplicate ($n = 3$).

Synthesis of [$^{13}C_6$]-PITC

The isothiocyanate group was constructed by the method described by Bures *et al.*[27] with minor modifications. [$^{13}C_6$]-Aniline (250 mg, 2.5 mmol) in $CHCl_3$ (2 mL) was allowed to react with *O*,*O*′-di-2-pyridyl thiocarbonate (1.25 g, 5 mmol) at room temperature for 3 h then at 50°C for 1 h. After the reaction, the solvent was evaporated and the residue was applied to a flash silica gel chromatography column with *n*-hexane to remove excess reagent. The eluate was then evaporated to give [$^{13}C_6$]-PITC as a colorless oil (392 mg, quant.). High-resolution FABMS calculated for $^{13}C_6^{12}C_1H_5N_1S_1$: theoretical, m/z 141.0344; found, m/z 141.0324 ($M^{+\cdot}$). ^{1}H-NMR (500 MHz, $CDCl_3$, δ): 7.05–7.25 and 7.35–7.55 (doublet of multiplets, $J(^{13}C-H) = 167$ Hz, H_{Ar}, 5H). IR (KBr, cm^{-1}): 2078 (vs, NCS).

Synthesis of [$^{13}C_6$]-PTH-Asp

Ang II (7 mM in 0.1 M HCl, 10 μL × 10) solvent was evaporated under a N_2 stream. The residue was allowed to react with 10 μL of [$^{13}C_6$]-PITC solution ([$^{13}C_6$]-PITC/triethylamine/water/ethanol 1:1:1:7, v/v/v/v) at 55°C for 15 min on a heating block. The reaction mixture was dried under a N_2 stream and the residue was then washed with *n*-hexane/ethyl acetate (15:1, v/v, 200 μL) and ethyl acetate (200 μL). Ethylmercaptan in TFA (0.1%, v/v, 20 μL) was added to the tube at 55°C for 15 min on a heating block. After evaporating the solvent under a N_2 stream, the residue was redissolved in water (20 μL) at 55°C for 5 min and 50% (v/v) aqueous TFA (20 μL) at 55°C for 60 min. The reaction mixture was dried under a N_2 stream and the residue was then dissolved in 50% (v/v) ethanol (50 μL). The solution from each tube was then applied to HPLC system 2, the fraction between t_R 4.3 and 5.3 min was collected, dried under a N_2 stream, and redissolved in 50% (v/v) aqueous ethanol (7 mL) as [$^{13}C_6$]-PTH-Asp stock solution (100 μM). MALDI-TOF MS (DHB): m/z 257.07 (MH^+).

Calibration curve using PTH-Asp and [$^{13}C_6$]-PTH-Asp for BSA quantitation

The calibration curve was prepared with PTH-Asp standard and [$^{13}C_6$]-PTH-Asp. PTH-Asp solution (10, 20, 50, 100, 200, 500, or 1000 pmol/10 μL of 50% aqueous ethanol, v/v) and [$^{13}C_6$]-PTH-Asp solution (1000 pmol/10 μL of 50% aqueous ethanol, v/v) were mixed. BSA (1 nmol/10 μL in 0.1 M HCl) was allowed to react with Edman reagent as described in the HPLC-UV experiment and [$^{13}C_6$]-PTH-Asp (1000 pmol/10 μL of 50% aqueous ethanol, v/v) was added as an IS. For MALDI-TOF MS analyses, aliquots (0.5 μL) were loaded on the MALDI sample plate followed by 0.5 μL of 100 mM DHB in a mixture of water/acetonitrile/TFA (50:50:0.1, v/v/v) containing internal calibrants (Arg, 10 pmol, MH^+ 175.1195; Trp, 10 pmol, MH^+ 205.0977; and Ang IV 1 pmol, MH^+ 775.4143) and allowed to dry at room temperature. After the MALDI-TOF MS analyses, the peak area ratios of PTH-Asp/[$^{13}C_6$]-PTH-Asp (monoisotopic MH^+; intensity @ 251/intensity @ 257) were plotted against PTH-Asp concentrations. The calculations were performed using Microsoft

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Office Excel 97–2003 with the following criteria: curve, linear; origin, ignored. The above experiment was performed in triplicate ($n = 3$).

Tryptic digestion for des-Asp^1-BSA

After Edman degradation, the solution containing des-Asp^1-BSA coexisting with PTH-Asp and [$^{13}C_6$]-PTH-Asp in 12.5% (v/v) aqueous TFA (40 μL) was evaporated and redissolved in reduction and carboxymethylation (RCM) buffer (1.65 M Tris-HCl, 7.3 M guanidine-HCl, 0.03 M EDTA, pH 8.3, 50 μL). DTT was added to produce a final concentration of 10 mM and incubated at 60°C for 1 h to reduce the disulfide bonds. The reduced cysteines were then alkylated upon the addition of IAA at a final concentration of 55 mM and incubated at room temperature in the dark for 45 min. The reduced and alkylated proteins were dialyzed with Slide-A-lyzer® Mini Dialysis units (3500 MWCO) against water (1 L, 1 h × 3). After evaporating the solution under a N_2 stream, the residue was redissolved in 100 mM ammonium bicarbonate (40 μL) and digested using sequencing-grade trypsin (enzyme/protein 1:165, w/w) with overnight incubation at 37°C. The pH of the digested protein sample was then lowered to 3 with 1% (v/v) aqueous TFA solution. The sample was desalted using an Oasis® HLB cartridge by washing with water (1 mL) and eluted with 75% (v/v) aqueous acetonitrile (1 mL). After evaporating the eluate under a N_2 stream, the residue was redissolved in the mixture of water/acetonitrile/TFA (50:50:0.1, v/v/v, 20 μL). The aliquots (0.5 μL) were loaded on the MALDI sample plate followed by addition of 0.5 μL of matrix solution (saturated CHCA in water/acetonitrile/TFA 50:50:0.1, v/v/v) and allowed to dry at room temperature. The calibrant solution (1 pmol Ang II, MH^+ 1046.5418; 1 pmol Ang I, MH^+ 1296.6853; 1 pmol ACTH (18–39), MH^+ 2465.1989; and 10 pmol bovine insulin oxidized β-chain, MH^+ 3494.6513) in saturated CHCA solution (water/acetonitrile/TFA 50:50:0.1, v/v/v) was used for the external calibration. Protein identification was carried out using a PMF tool, MS-Fit in ProteinProspector Version 5.2.1 Basic, developed in the UCSF Mass Spectrometry Facility,[28] with the following criteria: database, SwissProt. 2008.12.16; digest used, trypsin; maximum number of missed cleavages, 2; constant modifications, carbamidomethyl (C), minimum matches, 4; sort type, score sort; considered modifications, oxidation of M; min precursor ion matches, 1; MOWSE On, 1; MOWSE P factor, 0.4; masses, monoisotopic; mass tolerance, 0.2 Da. Peaks for which the signal/noise ratio was greater than 14.0 were used for PMF.

RESULTS AND DISCUSSION

Quantitation of the Edman degradation reaction

Quantitation of the Edman degradation reaction has mainly been studied for peptides. Recently, Brune *et al*. have reported the important possibility of quantitative Edman degradation with detailed data as a study by the Edman Sequencing Research Group.[26] They used three peptides, KAQYARSVLLEKDAEPDILELATGYR (peptide B), RQAKVLLYSGR (peptide C), and RQAK(Ac)VLLYSGR (peptide C*), and have shown favorable data. The data encouraged us to investigate the yield of PTH-amino acids from larger proteins to see if this reaction can be used for the key reaction in our strategy. Therefore, the degradation yield of BSA (67 kDa) was examined to optimize the reaction conditions. In our conditions, the released PTH-Asp and des-1 protein were not separated from each other to avoid reducing the recovery and further protein digestion in the same tube. The response of released PTH-Asp from BSA showed a good linearity ($y = 0.0006x - 0.0559$, $r^2 = 0.9998$) throughout the range of 20–50000 pmol/tube (Fig. 4).

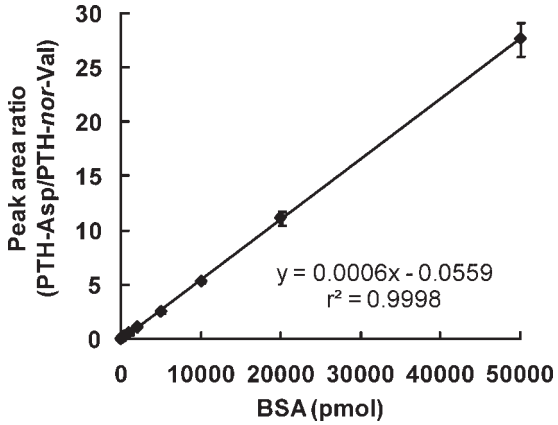

Figure 4. Production of PTH-Asp released from BSA by the Edman degradation reaction.

Synthesis of [$^{13}C_6$]-Edman reagent

An attempt to introduce ^{13}C on PITC has already been reported by Ares *et al*.[29] However, only one ^{13}C was introduced on the isothiocyanate group (Phe−N=^{13}C=S) for the purpose of protein probing by ^{13}C-NMR. For our purpose, a 1 Da shift is not enough as an IS, so we have prepared [$^{13}C_6$]-PITC labeled on the phenyl group because [$^{13}C_6$]-aniline is commercially available. As the preliminary experiment, three different methods were tried to synthesize PITC from [^{12}C]-aniline. The reaction between aniline and carbon disulfide followed by E1 elimination using chloroethyl carbonate[30] and phosphoryl chloride[29] gave 40% and 90% yields, respectively. The reaction with *O*,*O*′-di-2-pyridyl thiocarbonate[27] gave 90% yield. Therefore, a one-step reaction with *O*,*O*′-di-2-pyridyl thiocarbonate was chosen for [$^{13}C_6$]-PITC synthesis (Fig. 3). The yield from [$^{13}C_6$]-aniline was quantitative and the product was sufficiently isotopically pure (>99.9%) for further preparations of [$^{13}C_6$]-PTH-amino acids as ISs.

Synthesis of [$^{13}C_6$]-PTH-amino acid as IS

All [$^{13}C_6$]-PTH-amino acids (ISs) for 20 amino acids can be prepared simply using the reaction of [$^{13}C_6$]-PITC and individual amino acid standards (or an amino acid standard mixture). In this experiment, only [$^{13}C_6$]-PTH-Asp was synthesized for the further model experiments with BSA. The yields of PTH-amino acids from individual amino acids were lower than that from the peptide in our preliminary experiments (~50%). Therefore, [$^{13}C_6$]-PTH-Asp was prepared from Ang II. In the future, reaction of PITC and carboxyamidated-amino acids ($NH_2-CHR-CONH_2$) will be

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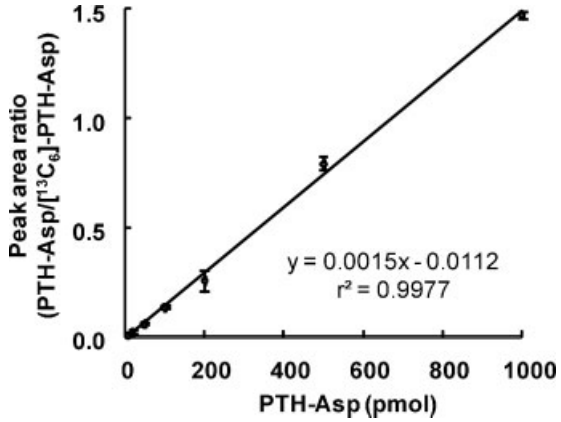

Figure 5. Calibration curve for PTH-Asp against [$^{13}C_6$]-PTH-Asp.

evaluated for efficient preparation of [$^{13}C_6$]-PTH-amino acids as a general method. The [$^{13}C_6$]-PTH-Asp prepared here was sufficiently isotopically pure (>99.9%) to be used as an IS, and was applied for further experiments.

Calibration curve of PTH-Asp over [$^{13}C_6$]-PTH-Asp

The commercial availability of individual and mixtures of 20 PTH-amino acids is one of the biggest advantages of this strategy. Therefore, calibration curves can be prepared for the entire proteome without standard proteins using the corresponding [$^{13}C_6$]-PTH amino acids as ISs. A calibration curve of PTH-Asp against [$^{13}C_6$]-PTH-Asp was prepared in the range from 10 pmol to 1 nmol for the next model experiment using BSA. The samples were analyzed using MALDI-TOF MS. The peak area ratios (PTH-Asp/[$^{13}C_6$]-PTH-Asp) were plotted against PTH-Asp concentrations. The calibration curve showed good linearity over the entire range ($y = 0.0015x - 0.0112$, $r^2 = 0.9977$) (Fig. 5). The correlation coefficient (r) was slightly lower than that from the HPLC-UV experiment because of the higher background in the low mass range and the lower dynamic range based on the use of MALDI-TOF MS.

Quantitation and qualification of BSA

The method was applied to BSA to prove our proposed concept. The PTH-Asp released from the *N*-terminus of BSA (1.0 nmol) was clearly found together with [$^{13}C_6$]-PTH-Asp (IS, 1.0 nmol) (Fig. 6). The peak area ratio (PTH-Asp/[$^{13}C_6$]-PTH-Asp = 1.01) was ideal for the quantitation. This suggests that the Edman degradation is the key reaction to attain absolute quantitative proteomics without protein standards. Then, the protein digestion procedure was optimized for further protein identification. To reduce disulfide bonds, RCM buffer was required to denature the aggregated des-Asp^1-BSA resulting from the Edman degradation conditions. After the following *S*-alkylation with IAA, des-Asp^1-BSA was successfully digested using trypsin (Fig. 7). Using the PMF method, des-Asp^1-BSA has been assigned to BSA as the top score (6.97×10^4) with 25.0% amino acid sequence coverage (152/607 amino acids). This suggests that des-1 proteins can provide sufficient information to identify the intact protein by the PMF method, even if the *N*-terminal amino acids are missing. MS/MS-based techniques can therefore give better identification.

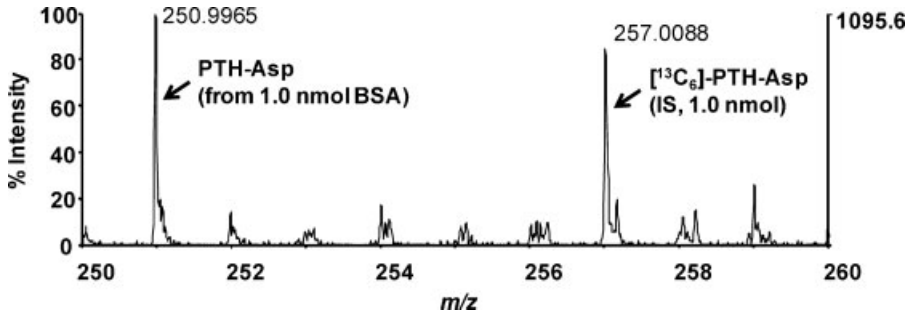

Figure 6. MALDI-TOF MS spectrum of PTH-Asp released from BSA by the Edman degradation reaction.

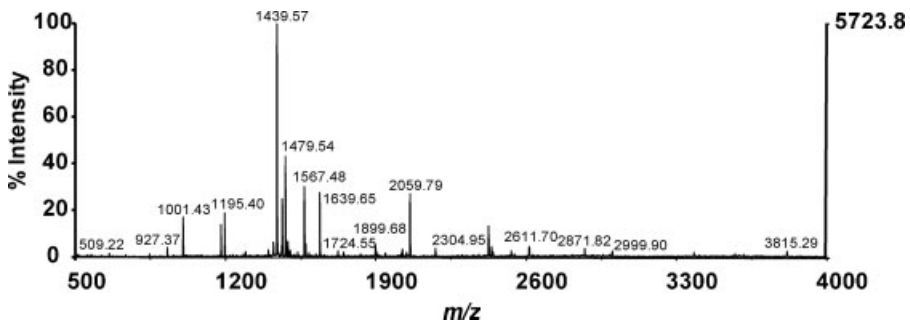

Figure 7. MALDI-TOF MS spectrum of des-Asp^1-BSA after tryptic digestion.

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CONCLUSIONS

Here, we have proved the concept of absolute quantitative proteomics using the combination of [$^{13}C_6$]-PITC as ISs for products of the Edman degradation reaction. Also, we have shown that analysis of tryptic fragments of the residual des-1 protein can provide sufficient information for protein identification. Because the concept worked, we are now focusing on ways to solve the potential problems for practical use. For instance, a spot from a 2-D gel normally contains multiple proteins, and some of them could have the same *N*-terminal amino acid. For this problem, we are trying to use not only PTH-amino acids from the first cycle, but also those from the second cycle together for the quantitation. In addition, use of an LC/MS system can solve the problems of coexisting proteins that have the same mass *N*-terminal amino acids, such as Leu vs. Ile and Gln vs. Lys, chromatographically. This should further increase the sensitivity because the current sensitivity with MALDI-TOF MS is limited by the high background from the matrix in the low molecular weight region for PTH-amino acids (*m*/*z* 193–322). Besides those steps, enzymatic or chemical digestions prior to the Edman reaction will be tried for *N*-protected and *N*-truncated proteins.

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