Journal of proteome research

Article

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Stable Isotope-Labeled Collagen: A Novel and Versatile Tool for Quantitative Collagen Analyses Using Mass Spectrometry

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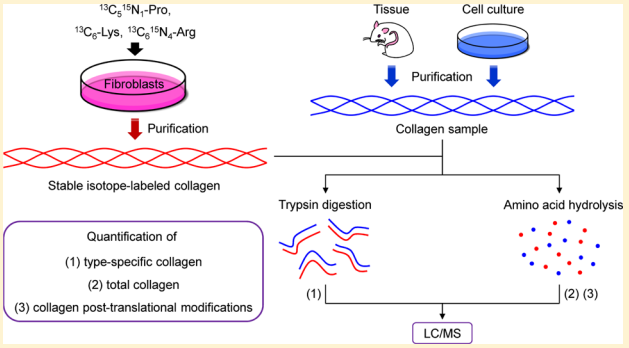
Supporting Information

ABSTRACT: Collagens are the most abundant proteins in animals and are involved in many physiological/pathological events. Although various methods have been used to quantify collagen and its post-translational modifications (PTMs) over the years, it is still difficult to accurately quantify type-specific collagen and minor collagen PTMs. We report a novel quantitative method targeting collagen using stable isotope-labeled collagen named "SI-collagen", which was labeled with isotopically heavy lysine, arginine, and proline in fibroblasts culture. We prepared highly labeled and purified SI-collagen for use as an internal standard in mass spectrometric analysis, particularly for a new approach using amino acid hydrolysis. Our method enabled accurate collagen analyses, including quantification of (1) type-specific collagen (types I and III in this paper), (2) total collagen, and (3) collagen PTMs by LC−MS with high sensitivity. SI-collagen is also applicable to other diverse analyses of collagen and can be a powerful tool for various studies, such as detailed investigation of collagen-related disorders.



KEYWORDS: *collagen, stable isotope labeling, post-translational modification, mass spectrometry, quantification*

INTRODUCTION

Collagens that consist of X-Y-Gly triplets are the major proteins of extracellular matrix in connective tissues. The tissues comprise various types of collagen (I−XXVIII), such as types I and III in skin and types II, IX, and XI in cartilage. Almost all Pro at the Y position is hydroxylated to 4-hydroxyproline (4-Hyp), and 3-hydroxyproline (3-Hyp) infrequently presents at the X position. Modification of Lys to hydroxylysine (Hyl) is observed at the Y position, and additional O-glycosylation steps sometimes occur to generate galactosyl-hydroxylysine (GHL) and glucosyl-galactosyl-hydroxylysine (GGHL). Qualitative and quantitative abnormalities of collagen are related to many disorders. For example, collagen is well-known to increase in fibrosis, and concomitantly, the type III/type I collagen ratio decreases relative to the normal state in lung and liver fibrosis.[1−3] In addition, decreases in Hyl content in both type I and type III collagens from fibrotic lung have been reported.[1] In osteogenesis imperfecta (OI) characterized by brittleness of the bone, decreases in collagen content and increases in Hyl glycosides occur in connective tissues.[4] Monitoring of these qualitative and quantitative changes in collagen and its post-translational modifications (PTMs) may provide insight into collagen-related disorders. In addition, a robust assay system of these collagen parameters has the potential to be used for screening and evaluation of drugs for the collagen-related disorders using experimental animals or cultured cells.

Various quantitative methods of collagen and its PTMs have been developed.[5−7] In general, total collagen has been quantified using 4-Hyp following acid hydrolysis, and analysis of collagen PTMs has been performed using acid and alkaline hydrolysis. Additionally, collagen type-specific detection and quantification have been performed by using antibodies. However, in some cases, it is difficult to quantify type-specific collagen and minor collagen PTMs because of insufficient analytical accuracy and sensitivity. For example, because collagens consist of X-Y-Gly triplets, cross-reaction of antibodies between structurally related collagen types is a concern, particularly cross-reaction between type I and type III collagens.[8] Similarly, 3-Hyp content is extremely low (approximately one residue per 1000 amino acid residues in type I collagen); therefore, accurate quantification of 3-Hyp with discrimination from 4-Hyp concomitantly with separation from other amino acids has been challenging. We believe that such difficulties in collagen analyses have been one of the major bottlenecks to further understanding of the biological significance of qualitative and quantitative changes in collagen under various conditions.

LC−MS has been used in many protein studies, and recently, in collagen studies with increasing frequency.[9−14] Using LC−MS, type-specific quantification of collagen (types I−V) has been performed following trypsin digestion.[10] In addition, amino acids, including minor collagen PTMs, are comprehensively detected by multiple reaction monitoring (MRM)

Received: March 4, 2014
Published: June 23, 2014

   dx.doi.org/10.1021/pr500213a | J. Proteome Res. 2014, 13, 3671−3678

analysis with high selectivity and sensitivity, and 3-Hyp and 4-Hyp can be analyzed separately on hydrophilic interaction liquid chromatography (HILIC) columns without derivatization (Figure S1 in the Supporting Information).[9] However, retention time shifts, which are particularly significant with HILIC columns, and ion suppression effects sometimes critically impair quantitative accuracy in LC−MS analysis. In 2002, the stable isotope labeling by amino acids in cell culture (SILAC) method was established by the Mann group to achieve accurate proteomic quantification in MS analysis.[15] In this technique, two cell populations are combined after metabolic labeling of proteins with either "light" or stable isotope-labeled "heavy" amino acids (typically Arg and Lys in combination with trypsin digestion). Every peptide pair is distinctively quantified by MS analysis, leading to accurate protein quantification with elimination of any errors. This metabolic labeling technique is also applicable to tissue samples, for example, by spiking in SILAC-labeled cells as internal standards.[16,17] In addition, "absolute SILAC" was developed for absolute quantification of selected single proteins by using purified SILAC-labeled recombinant proteins, which were produced in a cell-free system or in an *Escherichia coli* strain, as internal standards.[18] The method enabled accurate and robust quantification of targeted proteins. However, highly efficient purification of the recombinant proteins is challenging in some cases, and PTM analysis is not available because the recombinant proteins are not post-translationally modified.

In the present study, we produced stable isotope-labeled collagen named "SI-collagen", which was labeled with $^{13}C_5{}^{15}N_1$-Pro, $^{13}C_6$-Lys, and $^{13}C_6{}^{15}N_4$-Arg in the culture of human embryonic lung (HEL) fibroblasts. The metabolic labeling resulted in isotopically heavy Pro, 3-Hyp, 4-Hyp, Lys, Hyl, GHL, GGHL, and Arg in the collagen. SI-collagen was purified from the culture medium and mixed into each collagen sample before sample preparation procedures as an internal standard. Our method using SI-collagen enabled accurate quantification of (1) type-specific collagen (types I and III in this paper) designated as [Col_{type}], (2) total collagen designated as [Col_{total}], and (3) collagen PTMs designated as [Col_{PTMs}] after trypsin digestion or amino acid hydrolysis. We first maximized the labeling efficiency and purity of SI-collagen because unlabeled analytes from SI-collagen could potentially hamper accurate quantification, particularly in the new approach using amino acid hydrolysis. Collagens from skin, bone, and tail tendon of rats were analyzed using SI-collagen to demonstrate the major advantages of the method in collagen analyses.

EXPERIMENTAL SECTION

Materials and Reagents

DMEM, pepsin−agarose, pepsin, and *trans*-3-hydroxy-L-proline were purchased from Sigma-Aldrich (St. Louis, MO). SILAC DMEM medium, dialyzed FBS, $^{13}C_6$-Lys, and $^{13}C_6{}^{15}N_4$-Arg were purchased from Thermo Scientific (Hudson, NH). PicoTag Sample Tubes and Oasis MCX were purchased from Waters (Milford, MA). Penicillin−streptomycin was purchased from MP Biomedicals (Santa Ana, CA), FBS was purchased from Intergen (Purchase, NY), $^{13}C_5{}^{15}N_1$-Pro was purchased from Cambridge Isotope Laboratories (Andover, MA), L-ascorbic acid phosphate magnesium salt *n*-hydrate was purchased from Wako Chemicals (Osaka, Japan), and sequencing-grade-modified trypsin was purchased from Promega (Madison, WI). Standards of type I and type III collagens were purified from human placenta by pepsin digestion and differential salt precipitation,[19] and standards of GHL and GGHL were purified from natural sponge.[6]

Cell Culture

HEL fibroblasts were maintained in DMEM containing 0.5% penicillin−streptomycin and 10% FBS. Cells were incubated at 37 °C in a humidified 5% CO_2 incubator.

Preparation of SI-Collagen

SILAC DMEM medium, which lacks Lys, Arg, Pro, and Gln, was supplemented with 0.5% dialyzed FBS, 100 mg/L $^{13}C_6$-Lys, 100 mg/L $^{13}C_6{}^{15}N_4$-Arg, 200 mg/L $^{13}C_5{}^{15}N_1$-Pro, and 200 μM L-ascorbic acid phosphate. HEL cells were cultured in the labeling medium with collection and changing of the medium performed every 3 days. The collected culture medium was digested by pepsin−agarose (0.1 mg/mL in 0.1 N HCl) with gentle mixing at 4 °C for 16 h. After removal of the immobilized pepsin by centrifugation, salt precipitation was performed with 1 M NaCl for 3 h on ice. Collagen was precipitated by centrifugation at 16 000*g* for 10 min at 4 °C and extensively washed with 1 M NaCl in 0.1 N HCl and 95% ethanol. The purified SI-collagen was dissolved in 5 mM acetic acid.

Extraction and Purification of Collagen from Rat Tissues

Collagens were purified from skin, bone, and tail tendon, as described previously.[20] In brief, all tissues were dissected from male Sprague−Dawley (SD) rats at 5 weeks of age (Japan SLC, Shizuoka, Japan). The center of the femur was powdered under liquid nitrogen after demineralization by 0.5 M EDTA (pH 7.8) for 3 days at 4 °C, which was sufficient time for the bone obtained from young rat. Tissues were treated with 5 mg/mL pepsin in 0.5 M acetic acid at 4 °C for 1 day (skin and tail tendon) or 1 week (bone) to extract collagen. The solubilized collagens were purified by salt precipitation with the same procedure as for SI-collagen. The collagens were further purified by isoelectric precipitation (pH 8.0 adjusted by Tris-HCl) at 4 °C for 16 h; after centrifugation, the precipitated collagens were dissolved in 5 mM acetic acid.

Amino Acid Hydrolysis

After drying up collagen sample solution in PicoTag sample tubes with a centrifugal evaporator CVE-3100 (EYELA, Tokyo, Japan), acid hydrolysis was performed with 6 N HCl/1% phenol at 110 °C for 20 h in the gas phase under N_2. The acid hydrolysate was dissolved in 0.1% acetic acid/5 mM ammonium acetate in 50% acetonitrile for LC−MS analysis or dissolved in 20 mM HCl for analysis using an amino acid analyzer. Alkaline hydrolysis was performed with 2 N NaOH in PicoTag sample tubes at 110 °C for 20 h under N_2.[21] The alkaline hydrolysate was neutralized with 30% acetic acid and cleaned on a mixed-mode cation-exchange sorbent (Oasis MCX). The eluate was dried up and dissolved in 0.1% acetic acid/5 mM ammonium acetate in 50% acetonitrile for LC−MS analysis.

Absolute total collagen concentration was calculated from the total amount of amino acids. SI-collagen, rat collagens, and human type I and type III collagens were subjected to acid hydrolysis and subsequently analyzed by an L-8900 amino acid analyzer (Hitachi, Tokyo, Japan) using citrate buffer and a sodium chloride gradient. Amino acid in the eluate was monitored by postcolumn reaction with ninhydrin. The amino acid composition of SI-collagen was determined by acid and

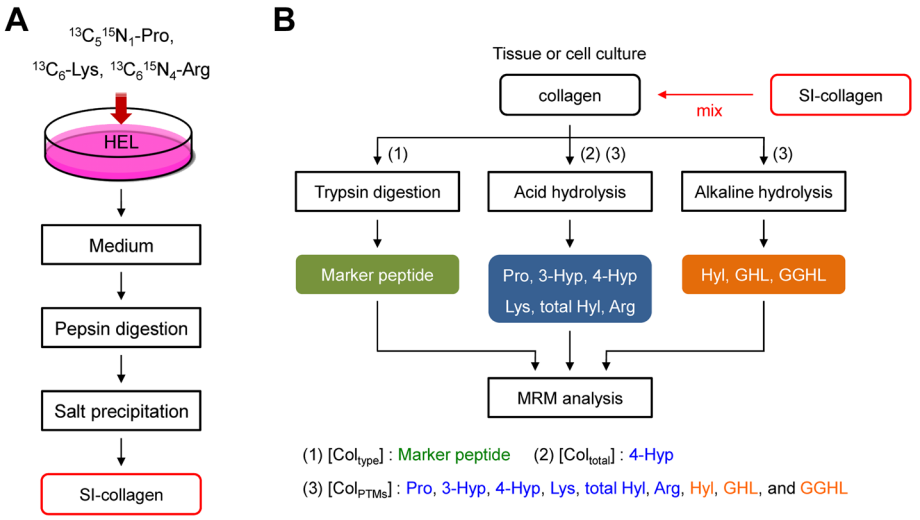


Figure 1. Schematic flowchart of quantitative collagen analyses using SI-collagen. (A) Stable isotope labeling of collagen was performed in a medium containing 0.5% FBS and ascorbic acid supplemented with $^{13}C_5{}^{15}N_1$-Pro, $^{13}C_6$-Lys, and $^{13}C_6{}^{15}N_4$-Arg in the culture of HEL cells. Immobilized pepsin and salt precipitation were used to purify SI-collagen from the culture medium. (B) SI-collagen is first mixed into each collagen sample derived from tissue or cell culture. Next, trypsin digestion is performed for (1) quantification of type-specific collagen designated as [Col$_{type}$], acid hydrolysis is performed for (2) quantification of total collagen designated as [Col$_{total}$], and acid and alkaline hydrolysis are performed for (3) quantification of collagen PTMs designated as [Col$_{PTMs}$]. Finally, the resulting amino acids and peptides are measured by MRM analysis. The stable isotope-labeled amino acids and peptides from SI-collagen are used as internal standards.

alkaline hydrolysis and expressed in residues/1000 total amino acid residues. The 3-Hyp and GHL/GGHL contents in SI-collagen were measured by MRM analysis, as described later using standard of those amino acids as internal standards, and other amino acids were measured by the amino acid analyzer.

Quantification of Total Collagen Using SI-Collagen

Rat collagen samples were mixed with SI-collagen at a collagen concentration ratio of roughly 5:1 and subjected to acid hydrolysis, followed by MRM analysis of 4-Hyp. Analysis was performed on a hybrid triple quadrupole/linear ion trap 3200 QTRAP mass spectrometer (AB Sciex, Foster City, CA) equipped with an electrospray ionization source. The instrument was coupled to an Agilent 1200 Series HPLC system (Agilent Technologies, Palo Alto, CA). The acid hydrolysate was loaded onto a ZIC-HILIC column (3.5 μm particle size, L × I.D. 150 mm × 2.1 mm; Merck Millipore, Billerica, MA) at a flow rate of 200 μL/min and separated by a binary gradient as follows: 90% solvent B (100% acetonitrile) for 5 min, linear gradient of 10−95% solvent A (0.1% acetic acid/5 mM ammonium acetate) for 15 min, 95% solvent A for 5 min, and 90% solvent B for 5 min. Analyst 1.5.2 (AB Sciex) was used to perform data acquisition and analysis. The light and heavy MRM transitions of 4-Hyp are shown in Table S1 in the Supporting Information. The total collagen concentration was determined by the peak area ratio of 4-Hyp to $^{13}C_5{}^{15}N_1$-4-Hyp.

Quantification of Type-Specific Collagen Using SI-Collagen

Rat collagen samples were mixed with SI-collagen at a collagen concentration ratio of roughly 5:1. The samples were denatured at 60 °C for 30 min and digested by trypsin (1:100 enzyme/substrate ratio) in 100 mM Tris-HCl/1 mM $CaCl_2$ (pH 7.6) at 37 °C for 16 h. The tryptic peptide solutions were acidified with formic acid and loaded onto an Ascentis Express C18 HPLC column (5 μm particle size, L × I.D. 150 mm × 2.1 mm; Supelco, Bellefonte, PA) at a flow rate of 500 μL/min. The separation conditions by a binary gradient were as follows: 98% solvent A (0.1% formic acid) for 2 min, linear gradient of 2−60% solvent B (100% acetonitrile) for 4 min, 90% solvent B for 2 min, and 98% solvent A for 2 min. The quantification of type I and type III collagens was performed by calculating the peak area ratio of the corresponding light and heavy marker peptides in MRM analysis using the Scheduled MRM algorithm. The MRM transitions of marker peptides of type I and type III collagens are shown in Table S2 in the Supporting Information. The concentrations of type I and type III collagens in SI-collagen were determined in advance using human type I and type III collagens as internal standards of known concentrations, respectively.

Quantification of Collagen PTMs Using SI-Collagen

Rat collagen samples were mixed with SI-collagen at a collagen concentration ratio of roughly 5:1. The samples were then subjected to acid and alkaline hydrolysis, and the resulting amino acids were measured by MRM analysis using the ZIC-HILIC column with the same analytical conditions for 4-Hyp in quantification of total collagen. The light and heavy MRM transitions of amino acids and collagen PTMs are shown in Table S1 in the Supporting Information. The relative values of Pro, Lys, and their PTMs were calculated according to the following formula: (light/heavy)/(light Arg/heavy Arg).

RESULTS

Preparation of Highly Labeled and Purified SI-Collagen

We optimized several conditions for incorporating stable isotope-labeled amino acids into collagen and also optimized the purification procedure. The preparation steps of SI-collagen are illustrated in Figure 1A. HEL cells, which secrete type I collagen with a trace amount of type III collagen into the culture medium in the presence of ascorbic acid,[22] were metabolically labeled with $^{13}C_5{}^{15}N_1$-Pro, $^{13}C_6$-Lys, and $^{13}C_6{}^{15}N_4$-Arg. The pepsin-resistant characteristic of collagen conferred by its triple helical structure allows a simple and efficient purification process with pepsin digestion of the

dx.doi.org/10.1021/pr500213a | J. Proteome Res. 2014, 13, 3671−3678

culture medium, followed by salt precipitation. To avoid even trace contamination of other proteins, we reduced the serum concentration in the culture from 10 to 0.5% and used immobilized pepsin, which is easily removed by centrifugation after the reaction. By use of these procedures, SI-collagen composed of type I and type III collagens was highly purified from the culture medium (Figure S2 in the Supporting Information).

In mammalian cells, Pro is metabolically converted from either Arg or Glu (Figure S3 in the Supporting Information),[23] which potentially decreases the labeling efficiency of Pro and Hyp in collagen. The Arg to Pro conversion has been reported to be prevented by the addition of 200 mg/L Pro in SILAC labeling,[24,25] and blocking of the Glu to Pro conversion by the addition of Pro has also been observed.[26] To assess the effect of Pro addition for labeling of 4-Hyp in SI-collagen, we examined varied amounts of $^{13}C_5{}^{15}N_1$-Pro (50, 200, and 350 mg/L) to minimize the conversions from Arg and Glu (Figure 2).

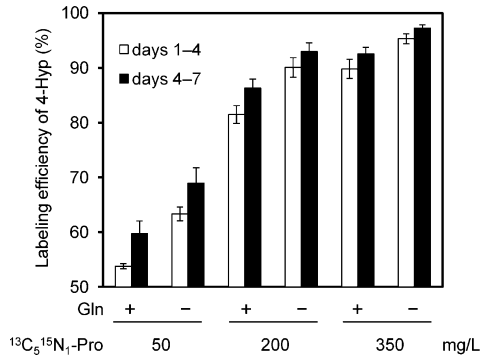


Figure 2. Efficiency of stable isotope labeling of 4-Hyp. HEL cells were cultured with 50, 200, and 350 mg/L $^{13}C_5{}^{15}N_1$-Pro in the presence or absence of 300 mg/L Gln. The labeled collagens purified from the culture media of days 1−4 and days 4−7 were subjected to acid hydrolysis; subsequently, 4-Hyp and $^{13}C_5{}^{15}N_1$-4-Hyp were measured by MRM analysis. The labeling efficiency of 4-Hyp was estimated as peak areas of the heavy 4-Hyp relative to the sum of the peak areas of the light and heavy 4-Hyp. The data represent the mean ± SD (n = 3).

Concomitantly, Gln was depleted to minimize the Glu to Pro conversion. On days 4−7 on which the labeling efficiency reached the maximum (data not shown), the $^{13}C_5{}^{15}N_1$-Pro addition directly affected the labeling efficiency in a dose-dependent manner at a range of 50−350 mg/L. In addition, the removal of Gln from the culture medium increased the labeling efficiency regardless of the $^{13}C_5{}^{15}N_1$-Pro concentrations. Similar results were observed for Pro labeling (Figure S4 in the Supporting Information). These results indicate that both the addition of Pro and depletion of Gln efficiently prevent undesirable conversion to Pro, which leads to predominant $^{13}C_5{}^{15}N_1$-Pro incorporation into the collagen. In the following experiment, we adopted the culture conditions of 200 mg/L $^{13}C_5{}^{15}N_1$-Pro without Gln, in which high labeling efficiency of 4-Hyp and Pro was observed at levels nearly comparable to those of 350 mg/L $^{13}C_5{}^{15}N_1$-Pro. The amount of Pro and Gln in the culture medium had little effect on collagen yield under the conditions (Figure S5 in the Supporting Information).

By use of the culture conditions with the addition of 100 mg/L $^{13}C_6$-Lys and 100 mg/L $^{13}C_6{}^{15}N_4$-Arg coupled to the purification process, highly efficient labeling of SI-collagen was achieved for each labeled amino acid ranging from 94.8% for Lys to 98.1% for Arg on days 4−7 (Table 1 and Figure S1 in the Supporting Information). The amino acid composition of SI-collagen was almost identical to that of human type I collagen (Table 1).[1,27] The total collagen yield calculated by summing the amino acid concentrations of SI-collagen was 23.6 ± 2.7 μg in the 3 day culture per 100 mm dish. The respective collagen yields of types I and III were estimated to be 18.3 ± 1.6 and 1.7 ± 0.4 μg, which were measured by MRM analysis of tryptic peptides, as described later with purified human type I and type III collagens used as internal standards. These parameters allowed absolute quantification in collagen analyses, including [Col_{type}], [Col_{total}], and [Col_{PTMs}] quantifications, described in the next section. SI-collagen from a 3 day culture in a 100 mm dish can be used to analyze >1000 samples by conventional LC−MS in [Col_{total}] quantification (>25 samples in [Col_{type}] and [Col_{PTMs}] quantifications); therefore, the cost for analyzing one sample in [Col_{total}] quantification is <$0.05 for the labeling reagents (<$2.00 in [$Col_{type}$] and [$Col_{PTMs}$] quantifications). In addition, SI-collagen can be continuously prepared by collecting and changing the labeling medium.

Strategy of Quantitative Collagen Analyses Using SI-Collagen

The workflow of collagen analyses using SI-collagen is shown in Figure 1B. SI-collagen is first mixed into each collagen sample from tissue or cell culture as an internal standard. The sample is then subjected to (1) trypsin digestion for [Col_{type}] quantification that is type-specific analysis of type I and type III collagens, (2) acid hydrolysis for [Col_{total}] quantification using 4-Hyp, and (3) acid and alkaline hydrolysis for [Col_{PTMs}]

Table 1. Efficiency of Stable Isotope Labeling and Amino Acid Composition of SI-Collagen[a]

			residues/1000 total residues[b]	
	labeling efficiency (%)[c]	residues/1000 total residues[d]	human type I collagen	human type III collagen
Pro	97.6 ± 0.2	104.8	120.3	107.0
3-Hyp	97.3 ± 0.1	1.8	1.0	0
4-Hyp	97.6 ± 0.2	100.8	103.0	125.0
Lys	94.8 ± 0.4	23.8	23.3	30.0
Hyl	96.6 ± 0.3	9.7	10.0	5.0
GHL	96.7 ± 0.7	0.6	1.0	
GGHL	96.5 ± 0.3	1.4	1.3	
Arg	98.1 ± 0.3	47.9	50.0	46.0

[a]Data represent the mean ± SD or mean (n = 3). [b]Reference data from a previous study.[27] [c]Labeling efficiency of each amino acid was estimated by MRM analysis of acid- and alkaline-hydrolyzed SI-collagen (days 4−7). [d]Content of the labeled amino acids in SI-collagen was determined using an amino acid analyzer and LC−MS and is expressed in residues/1000 total residues.

dx.doi.org/10.1021/pr500213a | J. Proteome Res. 2014, 13, 3671−3678

quantification. Subsequently, amino acids in the hydrolysates or peptides in the digests are measured by MRM analysis with an HILIC column or a C18 column, respectively. The three types of concentration values of collagen samples are calculated from the peak area ratio of the amino acids or peptides relative to the isotopically heavy ones from SI-collagen. In a previous study reporting "absolute SILAC", which uses isotopically heavy recombinant proteins as internal standards and thus is comparable to [Col_{type}] quantification, the linearity of the protein quantification was excellent over a broad range of heavy to light protein ratio (1:100 to 100:1).[18] In addition, we confirmed that the accuracy and linearity of quantification of 4-Hyp used for [Col_{total}] and [Col_{PTMs}] quantifications were also excellent ranging from 1:300 to 30:1 of SI-collagen to collagen sample ratio with and without ovalbumin spiked before acid hydrolysis as background (Figure S6 in the Supporting Information). In the following experiments, SI-collagen was mixed into collagen samples at a collagen concentration ratio of roughly 1:5, which was determined with balance of detection sensitivity and cost.

Hyl and GHL/GGHL are analyzed following alkaline hydrolysis because carbohydrate moieties of GHL/GGHL are labile to acid hydrolysis.[28] Other amino acids, including 3-Hyp, 4-Hyp, and total Hyl (Hyl + GHL + GGHL), are analyzed following acid hydrolysis. Arg is used for normalization of the collagen amount in [Col_{PTMs}] quantification. Because Arg is not post-translationally modified and its amino acid composition stays constant in collagen, the relative amounts of each amino acid are calculated according to the following formula: (light/heavy)/(light Arg/heavy Arg). Selection of marker peptides of the collagens is important for ensuring the accuracy of [Col_{type}] quantification. We selected peptides having more than two labeled sites, which dramatically decreases unlabeled peptide identical to the light peptide in SI-collagen. In addition, tryptic peptides cleaved at Lys lying in the Y position were excluded because the site is potentially modified to Hyl and GHL/GGHL. We also gave priority to peptides having high sequence homology among species as marker peptides to apply the method for various species, including human, bovine, rat, and mouse. In this paper, we used six marker peptides for quantification of type I and type III collagens (GVQGPOGPAGPR and GVVGLOGQR for type I collagen alpha 1 chain, EGPVGLOGIDGR and GPSGPQGIR for type I collagen alpha 2 chain, and GROGLOGAAGAR and GLAGPOGMOGPR for type III collagen alpha 1 chain; O indicates 4-Hyp, and stable isotopically labeled sites are underlined). Other species' collagens can be quantified by reselecting the marker peptides as needed, and collagen types other than types I and III can also be analyzed by our method; for example, stable isotope-labeled cartilage collagens, including types II, IX, and XI, will be able to be prepared in the culture of chondrocytes.[29]

Quantitative Analyses of Collagens from Skin, Bone, and Tail Tendon Using SI-Collagen

To evaluate the quantitative performance of collagen analyses using SI-collagen, we used collagens purified from skin, bone, and tail tendon of SD rats by pepsin digestion, salt precipitation, and isoelectric precipitation. First, [Col_{total}] and [Col_{type}] quantifications of the samples were performed by acid hydrolysis and trypsin digestion, respectively. SI-collagen was mixed into 20 μg of the collagen samples in which the collagen concentrations were predetermined from the total amino acid concentration after acid hydrolysis. As shown in Figure 3, the

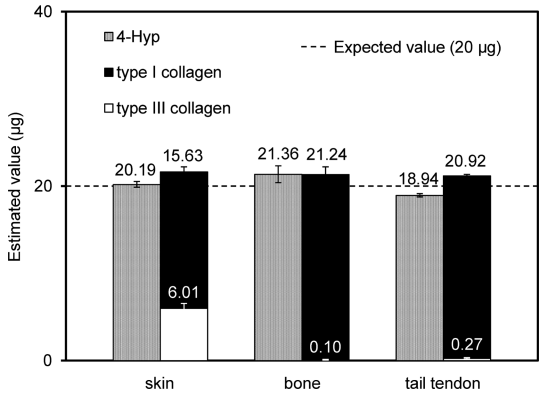


Figure 3. Quantification of total and type-specific collagen using SI-collagen. Twenty micrograms of collagen samples, which were purified from skin, bone, and tail tendon of SD rats at 5 weeks of age, was mixed with SI-collagen. One-tenth of each sample was subjected to acid hydrolysis, and the remaining ones were subjected to trypsin digestion. The [Col_{total}] concentrations of the samples were calculated from the relative peak areas of light 4-Hyp compared with those of heavy 4-Hyp in MRM analysis. Similarly, [Col_{type}] concentrations of type I and type III collagens were calculated from the relative peak areas of light tryptic peptides compared with those of heavy ones. The data represent the mean ± SD (n = 3).

estimated values of [Col_{total}] quantified using the 4-Hyp/$^{13}C_5{}^{15}N_1$-4-Hyp ratio were fairly close to the expected values ±10% for all samples, which demonstrated the high accuracy and precision of the quantification using SI-collagen. Similarly, the estimated values of [Col_{type}] also closely agreed with the expected values as the sum of type I and type III collagens. In addition, trace type III collagens in bone (0.5% of the total) and tail tendon (1.3% of the total) could be quantified. Although type III collagens in bone and tendon have been difficult to detect because of their low occupancy in the tissues,[8,30] even by LC−MS,[10] SI-collagen with the optimized MRM analysis enabled detection and precise quantification of those trace collagens.

Next, [Col_{PTMs}] quantification was performed following amino acid hydrolysis with SI-collagen. The alkaline hydrolysates desalted on a cation exchange column and the acid hydrolysates were subjected to MRM analysis. The results of relative [Col_{PTMs}] quantification of rat collagens from skin, bone, and tail tendon are shown in Figure 4. Dynamic variations of collagen PTMs among the tissues were observed. For example, significantly higher content of 3-Hyp was observed in tail tendon (462.3% to skin), which has been recently located at C-terminus of type I collagen by LC−MS,[31] while the amounts of Pro and 4-Hyp were unchanged among the tissues. Furthermore, significant decreases in GHL and GGHL in tail tendon (30.3% and 17.4% to skin, respectively) were observed with increasing Hyl (159.7% to skin). These results were highly reproducible and consistent with those of previous reports.[31−33] The use of amino acid composition values of SI-collagen enabled calculation of the absolute [Col_{PTMs}] values as residues/total 1000 residues (Table S3 in the Supporting Information).

DISCUSSION

Qualitative and quantitative changes in collagen, including variations in relative distribution of different collagen types in tissues and variations in collagen PTMs, have been reported in many types of disorders, such as fibrosis,[1−3] cancer,[34,35]

dx.doi.org/10.1021/pr500213a | J. Proteome Res. 2014, 13, 3671−3678

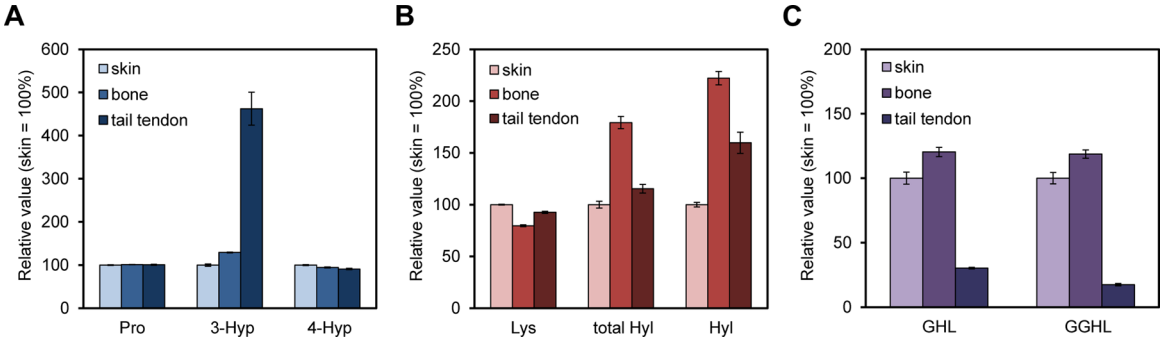


Figure 4. Quantification of collagen PTMs using SI-collagen. Collagen samples, which were purified from skin, bone and tail tendon of SD rats at 5 weeks of age, were mixed with SI-collagen. The samples were subjected to acid and alkaline hydrolysis, and the resulting amino acids were analyzed by MRM analysis. Data are expressed as relative values, with skin being 100% in (A) Pro, 3-Hyp, and 4-Hyp; (B) Lys, total Hyl, and Hyl; and (C) GHL and GGHL, calculated according to the following formula: (light/heavy)/(light Arg/heavy Arg). The data represent the mean ± SD (n = 3).

diabetes,[36,37] OI,[4] and osteoporosis.[38] However, accurate quantification of subtle changes in collagen has been challenging with traditional methods, and the functional and pathological significance of these changes remain unclear. In this study, we developed a novel method using SI-collagen for accurate collagen analyses, including quantification of type-specific collagen ($[Col_{type}]$), total collagen ($[Col_{total}]$), and collagen PTMs ($[Col_{PTMs}]$). We used stable isotope labeling in cell cultures with heavy Pro, Lys, and Arg to produce SI-collagen capable of being used as an internal standard for mass spectrometric analysis. Optimized culture conditions coupled to a purification process were used to achieve highly efficient labeling of SI-collagen (>94% in all the labeled amino acids), which led to accurate quantitative collagen analyses. Our method can detect even subtle changes in collagen and provide insights into various types of studies, including the previously investigated ones.

We established a new approach using amino acid hydrolysis in combination with an stable isotope labeling technique for quantitative collagen analyses, including analysis of overall changes in PTMs. Recently, we developed a novel method using hydrazide chemistry for specific purification of GHL/GGHL-containing peptides to identify the GHL/GGHL sites presenting as minor modifications.[39] In addition, we combined SILAC with the hydrazide capture method to quantitatively analyze overglycosylated collagen produced by skin fibroblasts from OI patients and demonstrated that the collagen overglycosylation in OI proceeds only at specific sites.[40] These methods allowed highly sensitive identification and quantification of the glycosylation sites in collagen. However, although peptide-based analysis is suitable for site-specific quantification of PTMs, simple and straightforward analysis of overall PTM changes is useful in some cases, such as when a robust assay system is required to screen numerous samples. Amino acid analysis using SI-collagen allows comprehensive quantification of collagen PTMs, including minor modifications, with high accuracy and sensitivity. This is the first study in which amino acid analysis has been used in a SILAC-based approach except for assessment of labeling efficiency and amino acid conversion as total protein mixture in SILAC experiments.[41]

In one important aspect, both SI-collagen and collagen samples must be highly purified from cell culture or tissue for amino acid analysis. After acid and alkaline hydrolysis, amino acids from collagen samples and those from contaminated proteins cannot be discriminated. In addition, because Arg is used for normalization of the collagen amount in $[Col_{PTMs}]$ quantification, Arg from contaminated proteins critically impairs the quantitative accuracy of the analysis. These were the major reasons why highly pure SI-collagen was required, although the noncollagenous protein impurities have little effect on $[Col_{type}]$ and $[Col_{total}]$ quantifications. The purification procedure for SI-collagen was optimized in this study. However, for collagen samples, the extraction and purification conditions are different in each case. We have to highly purify the collagen and consider the purity when preparing the collagen samples from tissues for $[Col_{PTMs}]$ quantification. Similarly, we need to be considerably attentive to the collagen type composition of the collagen samples in $[Col_{PTMs}]$ quantification because content of collagen PTMs significantly differs between the collagen types, except for 4-Hyp. For example, relative to type I collagen, type V collagen has approximately three times higher content of 3-Hyp and ten times higher content of Hyl glycosides.[27] Thus, even trace difference of the collagen type composition between the collagen samples may influence the results and impair the quantitative accuracy. We should compare basically the same types of collagen samples in the case of $[Col_{PTMs}]$ quantification, although all skin, bone, and tail tendon collagens, which were compared in this study, are primarily composed of type I collagen. In addition, complete extraction of collagen from tissues is difficult because of its insoluble nature. Therefore, analysis of all collagen present in tissues remains a challenge, although collagen from culture medium can be analyzed absolutely by mixing SI-collagen into the medium before the purification process, including pepsin digestion and salt precipitation.

SI-collagen can be used for many applications other than the three types of analyses described in this paper. For example, cellular internalization efficiency of collagen is able to be analyzed using SI-collagen instead of radio isotope-labeled collagen or fluorescent-labeled collagen (Figure S7 in the Supporting Information).[42] Increases in the internalization efficiency by the addition of a lysosomal cysteine protease inhibitor and predenaturation of the added SI-collagen were observed in agreement with the results of a previous report.[42] In addition, the acid hydrolysate of SI-collagen can be utilized as internal standards of collagen PTMs, which are not commercially available (Figure S8 in the Supporting Information), such as for quantification of 4-Hyp in blood and urine. In conclusion, SI-collagen allows simple, sensitive,

dx.doi.org/10.1021/pr500213a | J. Proteome Res. 2014, 13, 3671−3678

and accurate quantification in various collagen analyses and will be a powerful tool for collagen research.

■ ASSOCIATED CONTENT

Supporting Information

MRM chromatograms of Pro, Lys, Arg, and collagen PTMs from SI-collagen. SDS-PAGE analysis of SI-collagen. Proline metabolism. Efficiency of stable isotope labeling of Pro. Collagen yield with varying amounts of Gln and Pro in the culture. Linear dynamic range of amino acid analysis using SI-collagen. Collagen internalization assay using SI-collagen. Correction of ion suppression effect using acid hydrolysate of SI-collagen. Light and heavy MRM channels of Pro, Lys, Arg, and collagen PTMs. Light and heavy MRM channels of marker peptides of type I and type III collagens. Absolute quantification of collagen PTMs using SI-collagen. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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