Developmental Stage-dependent Regulation of Prolyl 3-Hydroxylation in Tendon Type I Collagen*

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Background: The physiological role of 3-hydroxyproline (3-Hyp) rarely found in collagen is unclear. Recent studies have suggested a function of prolyl 3-hydroxylation in fibril assembly and its relationships with certain disorders, including recessive osteogenesis imperfecta and high myopia. However, no direct evidence for the physiological and pathological roles of 3-Hyp has been presented. In this study, we first estimated the overall alterations in prolyl hydroxylation in collagens purified from skin, bone, and tail tendon of 0.5–18-month-old rats by LC-MS analysis with stable isotope-labeled collagen, which was recently developed as an internal standard for highly accurate collagen analyses. 3-Hyp was found to significantly increase in tendon collagen until 3 months after birth and then remain constant, whereas increased prolyl 3-hydroxylation was not observed in skin and bone collagen. Site-specific analysis further revealed that 3-Hyp was increased in tendon type I collagen in a specific sequence region, including a previously known modification site at Pro707 and newly identified sites at Pro716 and Pro719, at the early ages. The site-specific alterations in prolyl 3-hydroxylation with aging were also observed in bovine Achilles tendon. We postulate that significant increases in 3-Hyp at the consecutive modification sites are correlated with tissue development in tendon. The present findings suggest that prolyl 3-hydroxylation incrementally regulates collagen fibril diameter in tendon.

Type I collagen is the major component of connective tissues, such as skin, bone, and tendon. Collagens consist of repeating Gly-X-Y triplets and have characteristic post-translational modifications (PTMs), including 3-hydroxyproline (3-Hyp) and 4-hydroxyproline (4-Hyp). Almost all Pro residues lying in the Y position are hydroxylated to 4-Hyp (~100 residues/1000 amino acid residues in type I collagen), which stabilizes the collagen triple helix (1). In contrast, Pro residues at the X position are rarely hydroxylated to 3-Hyp, which was identified in collagen more than 50 years ago (2). Although 3-Hyp was reported to slightly increase the stability of the triple-helical structure of collagen (3), the biological function of this minor modification is not well understood (4). The 3-Hyp levels in type I collagen vary according to tissue type, e.g. 0.5 residues in skin, 0.7 residues in bone, and 2.4 residues in tail tendon per 1000 amino acid residues in our previous study on Sprague-Dawley rats at 5 weeks of age (5).

Originally, α1(I) Pro986 was identified as the primary 3-Hyp site, and is usually almost fully hydroxylated (6). Prolyl 3-hydroxylase (P3H) 1 is responsible for the reaction by forming a ternary complex with cartilage-associated protein and cyclophilin B (7, 8). Mutations in the complex components were recently reported to cause severe forms of recessive osteogenesis imperfecta (9–11), thus indicating the importance of prolyl 3-hydroxylation and attracting increased attention to the modification. MS has enabled high-sensitive and site-specific analysis of collagen PTMs, including 3-Hyp (12–16). Using LC-MS, Eyre and colleagues (12, 17, 18) identified novel 3-Hyp sites in type I collagen, including α1(I) Pro707, α2(I) Pro707, and a C-terminal (GPP)n motif, which are mainly modified by P3H2 (19). Detailed analyses using LC-MS have also provided clues about the function of 3-Hyp. For example, P3H2 mutations were reported to be associated with non-syndromic severe high myopia in humans (20, 21). Site-specific LC-MS analysis further revealed that prolyl 3-hydroxylation was decreased at specific sites in collagens from eye tissues of P3H2-null mice, suggesting that the under 3-hydroxylation causes high myopia (19). In another P3H2 KO mouse model, a lack of 3-Hyp in type IV collagen resulted in embryonic lethality, because 3-Hyp in type IV collagen showed a crucial role in preventing maternal platelet aggregation (22).

A recent study implied developmental regulation of prolyl 3-hydroxylation (23). The C-terminal (GPP)n motif in type I collagen had a high 3-Hyp content in adult human tendon compared with that in fetal human tendon, although such age-dependent alterations were not observed for α1(I) Pro986 and α2(I) Pro707, which were completely hydroxylated in fetal tendon. We considered that variations in 3-Hyp levels with aging could be important clues for elucidating the function of 3-Hyp. Age-related alterations in collagen PTMs, such as hydroxylation of Pro and Lys (24) and glycosylation of hydroxylysine (Hyl) to galactosyl-hydroxylysine (GHL) and subsequent gluco-
syl-galactosyl-hydroxylysine (GGHL) (25), have been examined. However, no studies have thoroughly estimated the effects of age on prolyl 3-hydroxylations, because of analytical difficulties for such a minor modification. Recently, we developed stable isotope-labeled collagen (SI-collagen), in which Pro, Lys, Arg, and collagen PTMs are all substituted with stable isotopically heavy ones, to enable highly accurate collagen analyses through its use as an internal standard in LC-MS (5, 26).

Here, we first estimated the 3-Hyp contents in collagens from skin, bone, and tail tendon of Sprague-Dawley rats over a wide age distribution using the new method. Rapid and significant increases in 3-Hyp were observed for tendon collagen until 3 months after birth, whereas such marked variations were not observed for skin and bone collagens. In further experiments analyzing tryptic peptides by LC-MS, we identified novel 3-Hyp sites at Pro\(^{716}\) and Pro\(^{719}\) adjacent to Pro\(^{707}\) in tail tendon type I collagen, and site-specific analysis revealed that 3-Hyp increased at the consecutive modification sites only in tendon at the early ages.

**Experimental Procedures**

**Ethics Statement**—All animal studies were approved by the Experimental Ethical Committee of Nippi Research Institute of Biomatrix.

**Extraction and Purification of Tissue Collagens**—Skin, bone, and tail tendon were dissected from male Sprague-Dawley rats at 0.5, 1, 2, 3, 6, 12, and 18 months of age (Charles River Laboratories Japan). Fetal bovine Achilles tendon was obtained from Japan Bio Serum, and adult bovine Achilles tendon was obtained from Shibaura Zoki. The femurs were demineralized in 0.5 M acetic acid) to exclude type V collagen, and the precipitates was analyzed by acid hydrolysis (6N HCl, 110 °C for 20 h in the gas phase under N\(_2\)) using SI-collagen as an internal standard (5). The acid hydrolysate was subjected to LC-MS analysis using a hybrid triple quadrupole/linear ion trap 3200 QTRAP mass spectrometer (AB Sciex) coupled to an Agilent 1200 Series HPLC system (Agilent Technologies). Sample separation was performed using a ZIC-HILIC column (3.5 μm particle size, length × inner diameter 150 mm × 2.1 mm; Merck Millipore), and the peak areas of the eluted amino acids were quantified in multiple reaction monitoring mode with Analyst software 1.6.2 (AB Sciex). The relative value of the 3-Hyp content was calculated according to the following formula: (3-Hyp/[\(^{13}\)C\(_5\)\(^{15}\)N\(_3\)]-3-Hyp)/(Arg/[\(^{13}\)C\(_5\)\(^{15}\)N\(_3\)]Arg). Pro, 4-Hyp, Lys, total Hyl (Hyl + GGHL + GGHL), Hyl, GHL, and GGHL were also analyzed by acid hydrolysis and alkaline hydrolysis (2 n NaOH, 110 °C for 20 h in the gas phase under N\(_2\)) with SI-collagen in a similar manner. The absolute 3-Hyp content expressed as residues/1000 total residues was calculated using the predetermined 3-Hyp content in SI-collagen (5).

For site-specific analysis of 3-Hyp, collagen samples were digested with sequencing grade-modified trypsin (Promega) at 37 °C for 16 h following heat denaturation at 60 °C for 30 min. The tryptic peptides were analyzed by LC-MS using an Ascentis Express C18 HPLC column (5 μm particle size, length × inner diameter 150 mm × 2.1 mm; Supelco) at a flow rate of 500 μl/min with a binary gradient as follows: 98% solvent A (0.1% formic acid) for 2.5 min, linear gradient of 2–50% solvent B (100% acetonitrile) for 12.5 min, 90% solvent B for 2.5 min, and 98% solvent A for 2.5 min. Peptide identification was performed by searching the acquired MS/MS spectra against the UniProtKB/Swiss-Prot database (release 2011_08, July 2011) with ProteinPilot software 4.0 (AB Sciex). The relative abundance of prolyl 3-hydroxylation at each modification site was semiquantitatively estimated by the relative peak area ratio of extracted ion chromatograms for each 3-Hyp variant of the peptides containing the sites.

**N-terminal Amino Acid Sequence Analysis**—A tryptic peptide containing the novel 3-Hyp sites was purified using an Ascentis Express F5 HPLC column (2.7 μm particle size, length × inner diameter 150 mm × 2.1 mm; Supelco) with MS monitoring. N-terminal amino acid sequence analysis of the collected fraction was performed by a Procise 492 protein sequencer (Applied Biosystems) in pulsed-liquid mode.

**Results**

3-Hyp Increases in Tendon Collagen at Young Ages—The age-related alterations in prolyl hydroxylation in skin, bone, and tail tendon collagens from Sprague-Dawley rats (0.5–18 months of age) were estimated by LC-MS analysis following acid hydrolysis, using SI-collagen as an internal standard (Fig. 1). The Pro and 4-Hyp contents were revealed to remain definitely constant over the entire period in all three tissues. In contrast, the 3-Hyp content notably varied with age in tendon collagen (Fig. 1C). The 3-Hyp level increased by 3-fold (from ~0.8 to 2.4 residues/1000 total residues) from 0.5 to 3 months of age, and subsequently remained high until 18 months of age. The absolute 3-Hyp contents are summarized in Table 2. In contrast to tendon collagen, 3-Hyp slightly decreased in skin collagen until 3 months of age (from ~0.6 to 0.5 residues/1000 total residues).

The total 3-Hyp content in the collagen samples was analyzed by acid hydrolysis (6 N HCl, 110 °C for 20 h in
The 3-Hyp level in bone collagen was almost unchanged with age (\(\sim\)0.7 residues/1000 total residues) (Fig. 1B). Although we focused on prolyl 3-hydroxylation in the present study, Lys hydroxylation/glycosylation also showed tissue-specific alterations with aging (Fig. 1). Novel 3-Hyp Sites in Type I Collagen Specific for Tendon—The dramatic and tendon-specific increases in the overall extent of prolyl 3-hydroxylation led us to predict that there were some unidentified 3-Hyp sites in tendon. We thoroughly searched for novel 3-Hyp sites in tryptic digests of collagen from tail tendon of 18-month-old rats by LC-MS. The MS/MS-based peptide identification raised two prospective 3-Hyp sites at Pro716 and Pro719 in type I collagen. Tryptic peptides containing the known 3-Hyp sites at Pro707 (VGP707OGPSGNAPGPGOPVGK; O indicates 4-Hyp) and a2(I) Pro707 (TGP707OGPSGTGPOGPOGAAGK) were found to have two further hydroxylation forms (Fig. 2). Pro716 and Pro719 lying in the Gly-Pro-Hyp motif sequence were hydroxylated in both a2(I) (Fig. 2A) and a2(I) (Fig. 2B), judging from the +16 Da mass shift in the MS/MS spectra.

The presence of 3-Hyp at the newly identified sites was verified by N-terminal amino acid sequence analysis of a purified double-substituted (2 \(\times\) 3-Hyp) peptide containing a2(I) Pro707, Pro716, and Pro719 (Fig. 3). Three split 3-Hyp peaks were observed at cycle 3 corresponding to the known 3-Hyp site at a2(I) Pro707. Three 3-Hyp peaks were similarly observed at cycle 12 corresponding to a2(I) Pro716. Furthermore, one of the three 3-Hyp peaks was obviously detected at cycle 15 corresponding to a2(I) Pro719, although it was near the detection limit. We were unable to purify a sufficient amount of the 2 \(\times\) 3-Hyp peptide containing a1(I) Pro707, Pro716, and Pro719 for N-terminal sequence analysis because of its low abundance. However, from the results for a2(I), it was conceivable that the +16 Da mass shifts at a1(I) Pro716 and Pro719 (Fig. 2A) were also attributed to prolyl 3-hydroxylation.

As shown in Fig. 2, the MS/MS fragmented \(y_{10}\) ion included Pro716 and Pro719, but not Pro707. In the MS/MS spectrum of the 3 \(\times\) 3-Hyp peptide containing a2(I) Pro707, Pro716, and Pro719 (lower spectrum in Fig. 2B), a single +32 Da mass variant (2 \(\times\) 3-Hyp) was observed for the \(y_{10}\) ion (m/z 912.44), indicating the presence of 3-Hyp at both Pro716 and Pro719. On the other hand, in the case of the 2 \(\times\) 3-Hyp peptide (upper spectrum in Fig. 2B), only a +16 Da mass variant (1 \(\times\) 3-Hyp) was observed for the \(y_{10}\) ion (m/z 896.45), indicating only one 3-Hyp at either Pro716 or Pro719. There was no double-substituted peptide without 3-Hyp at Pro707, meaning that prolyl 3-hydroxylation first occurred at Pro707 in the consecutive 3-Hyp sites in a2(I). Furthermore, Pro716 appeared to be secondarily hydrox-
ylated as seen in the example of the $y_9$ ion, which included Pro$^{719}$, in the $2 \times 3$-Hyp peptide (upper spectrum in Fig. 2B). The $0 \times 3$-Hyp fragment ($y_9$ ion; m/z 783.40) was dominantly detected compared with the $1 \times 3$-Hyp fragment ($y_9$ ion; m/z 799.39). These MS/MS fragmentation patterns were similarly observed for $\alpha1$ (I) (Fig. 2A). Taken together, $\alpha1/\alpha2$ (I) Pro$^{716}$ and Pro$^{719}$ are secondary 3-Hyp sites adjacent to Pro$^{707}$ in tendon type I collagen. Prolyl 3-hydroxylation at the sites was also observed in collagen from Achilles and patellar tendons (data not shown).

**Prolyl 3-Hydroxylation at Specific Sites Increases in Tendon Type I Collagen at Young Ages**—The tryptic peptides containing the previously known and newly identified 3-Hyp sites in type I collagen are summarized in Table 1. Fig. 4 shows the extracted ion chromatograms of the 3-Hyp-containing peptides from tail tendon type I collagen derived from young rats (0.5 months)
and adult rats (18 months). Although α1(I) Pro986 was almost completely hydroxylated in both young and adult rats (Fig. 4A), varied prolyl 3-hydroxylation was observed for α1(I) Pro707,716,719 (0–3 3-Hyp; 3 3-Hyp peptide was under the detection limit) and α2(I) Pro707,716,719 (0–3 3-Hyp) (Fig. 4, B and C). It was evident that the prolyl 3-hydroxylation level at α1/α2(I) Pro707,716,719 was significantly higher in adult rats compared with young rats. Prolyl 3-hydroxylation at the newly identified modification sites (Pro716 and Pro719) was not detected for skin and bone type I collagens (Fig. 5).

We further estimated the influence of aging on prolyl 3-hydroxylation at each modification site in tendon type I collagen by analyzing tail tendons from 0.5–18-month-old rats (Fig. 6C).

The relative abundance of 3-Hyp at 0.5 months of age was 42.0% for 1 3-Hyp, 0% for 2 3-Hyp, and 3.4% for 3 3-Hyp. The actual 3-Hyp level in the 3 3-Hyp peptide was probably close to 0% in the 0.5-month-old tendon, because the peptide peak overlapped with the nonspecific peak indicated in Fig. 4C. Higher resolution of LC separation or MS detection is required to accurately analyze the minor hydroxylation form. Similar to α1(I) Pro707,716,719, the 3-Hyp level increased until 3 months of age (68.3% for 1 3-Hyp, 16.9% for 2 3-Hyp, and 8.2% for 3 3-Hyp). The absolute 3-Hyp content at each modification site is summarized in Table 2. Unlike tendon, the peptides containing 2 × or 3 3-Hyp, which represent prolyl 3-hydroxylation at α1/α2(I) Pro716 and Pro719, were not detected for type I collagens from skin and bone (Fig. 2).
A and B). Consistent with the amino acid analysis shown in Fig. 1, A and B, the 3-Hyp levels at both α1(I) Pro986 and α1/α2(I) Pro707 were slightly decreased in skin (Fig. 6A) and nearly unchanged in bone (Fig. 6B) with age.

The increases in prolyl 3-hydroxylation at α1/α2 (I) Pro707, Pro716, and Pro719 during young ages were similarly observed in collagen from Achilles tendon and patellar tendon (data not shown). In addition, the site-specific altera-

FIGURE 4. Extracted ion chromatograms of tryptic peptides containing 3-Hyp sites of tail tendon type I collagen. Tail tendon type I collagens from 0.5- and 18-month-old Sprague-Dawley rats were analyzed by LC-MS following trypsin digestion. Monoisotopic extracted ion chromatograms of peptides containing 0 × 3-Hyp (blue) and 1 × 3-Hyp (red) were extracted for (A) α1(I) DGLNGLOGPIGP986OGPR, and those containing 0 × 3-Hyp (blue), 1 × 3-Hyp (red), 2 × 3-Hyp (green), and 3 × 3-Hyp (pink) were extracted for (B) α1(I) VGP707OGPSGNAGP716OGP719OGPVGK and (C) α2(I) TGP707OGPSGITGP716OGP719OGAAGK. Detailed information for these 3-Hyp-containing peptides is summarized in Table 1. The 3 × 3-Hyp peptide in B was under the detection limit. Asterisks indicate nonspecific peaks or other collagen-derived peptide peaks whose m/z overlapped with the monoisotopic m/z ranges of the 3-Hyp-containing peptides.

6, A and B). Consistent with the amino acid analysis shown in Fig. 1, A and B, the 3-Hyp levels at both α1(I) Pro986 and α1/α2(I) Pro707 were slightly decreased in skin (Fig. 6A) and nearly unchanged in bone (Fig. 6B) with age.
tions in the 3-Hyp level were also observed in bovine tendon (Fig. 7). The total 3-Hyp content in adult Achilles tendon collagen was ~2.5 times higher than that in fetal Achilles tendon collagen (Fig. 7A). Furthermore, similar to rat collagen, prolyl 3-hydroxylation increased with aging at \( \alpha 1/\alpha 2(I) \) Pro707,716,719 in bovine Achilles tendon (Fig. 7, C and D), whereas the 3-Hyp level at \( \alpha 1(I) \) Pro986 slightly decreased during the same period (Fig. 7B).

Contamination with other tissue collagens, such as those from skin having low 3-Hyp contents, critically influences

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**FIGURE 5.** Extracted ion chromatograms of tryptic peptides containing 3-Hyp sites of type I collagens from skin and bone of 18-month-old rats. Monoisotopic extracted ion chromatograms of peptides containing 0 × 3-Hyp (blue) and 1 × 3-Hyp (red) were extracted for (A) \( \alpha 1(I) \) DGLNLOGPIGP986OGPR. In a similar manner, peptides containing 0 × 3-Hyp (blue), 1 × 3-Hyp (red), 2 × 3-Hyp (green), and 3 × 3-Hyp (pink) were extracted for (B) \( \alpha 1(I) \) VGP707OGPSGNAGP716OGP719OGPVGK and (C) \( \alpha 2(I) \) TGP707OGPSG716OGP719OGAAGK. Asterisks indicate nonspecific peaks or other collagen-derived peptide peaks, whose m/z overlapped with the monoisotopic m/z ranges of the 3-Hyp-containing peptides.
the quantitative accuracy of the estimation of 3-Hyp contents in tendon samples. To ensure the purity of the tissue collagen samples in the present study, we monitored a tryptic peptide from type I collagen /H92512 chain in which Pro891 is potentially hydroxylated to 4-Hyp. We found tissue-specific and stationary hydroxylation rates at /H92512(I) Pro891 that were ~100% in skin, 60–80% in bone, and 20–40% in tendon (Fig. 8). The tendon samples from rats at 0.5–18 months of age were confirmed to be pure tendon collagen using the tissue marker peptide (Fig. 8D).

**Discussion**

In this study, our highly accurate analytical approaches using LC-MS demonstrated that 3-Hyp is significantly increased in type I collagen from rat tail tendon between 0.5 and 3 months after birth at consecutive modification sites, including Pro707, Pro716, and Pro719. Except for these increases at young ages in tendon, no other notable increases in 3-Hyp were observed for collagen from skin, bone, and tendons. The maintained level of 3-Hyp after the drastic increases in tendon indicates that the
alterations are correlated with tissue development in tendon, rather than with aging. 3-Hyp was suggested to be involved in collagen fibril assembly (12, 18, 29). Previous studies indicated that the collagen fibril diameter in rat tail tendon increases rapidly until 3–4 months after birth (30–32). These observations correspond well to the specific increases in 3-Hyp during 0.5–3 months of age obtained in the present study. Although KO of P3H1, which is responsible for prolyl 3-hydroxylation at /H9251/1(I)/Pro986, was reported to result in a slight increase in collagen fibril diameter in newborn tendon (15), we suggest that at least 3-Hyp at /H9251/1/2(I)/Pro707, Pro716, and Pro719 contributes to the incremental regulation of fibril diameter. The 3-Hyp content in tendon is known to be higher than those in skin and bone (5, 33); however, the 3-Hyp level did not differ much among these tissues at 0.5 months of age (Fig. 6). Subsequently, until 3 months of age, 3-Hyp was significantly increased in tendon, slightly decreased in skin, and constant in bone. The unique tissue-specific properties of collagen fibrils may partly arise through the alterations in prolyl 3-hydroxylation at the early stage of development. The bovine data comparing fetal Achilles tendon and adult Achilles tendon (Fig. 7) suggest that the developmental alterations in prolyl 3-hydroxylation are common to other animals, probably including humans.

Developmental regulation of prolyl 3-hydroxylation in the C-terminal (GPP)n motif of tendon type I collagen was mentioned in a recent paper comparing young and adult tendons (23). We were not able to detect tryptic peptides containing the C-terminal (GPP)n motif, because pepsin-extracted collagen shows heterogeneous C-terminal cleavage sites that may prevent reliable analysis of 3-hydroxylation at these sites (23). Other collagen extraction methods using acid, heating, or cyanogen bromide are required to analyze the C-terminal modification sites. However, the modification level at the C-terminal sites can be roughly estimated by subtracting the sum of the site-specific 3-Hyp contents (Fig. 6) from the total 3-Hyp content (Fig. 1). For example, in the 3-month-old tendon, the total 3-Hyp content and sum of the site-specific 3-Hyp contents were 2.4 and 1.4 residues/1000 total residues, respectively (Table 2). The 3-Hyp content in the C-terminal (GPP)n motif is thus approximated to 1.0 residue/1000 total residues, provided there are no more unidentified 3-Hyp sites. In contrast, the total 3-Hyp content and sum of the site-specific 3-Hyp contents were ~2.4 and 1.4 residues/1000 total residues, respectively (Table 2). The 3-Hyp content in the C-terminal (GPP)n motif during the developmental stage in tendon.

α1(I) Pro986 is hydroxylated by P3H1 (8), whereas α1(I)/α2(I) Pro707 and the C-terminal (GPP)n motif are mainly hydroxylated by P3H2 (19). In the present study, the order of prolyl 3-hydroxylation at the consecutive 3-Hyp sites appeared to be Pro707 > Pro716 > Pro719 based on the MS/MS fragmentation
patterns, as mentioned under “Results” (upper spectra in Fig. 2, A and B). The definitive order of prolyl 3-hydroxylation suggests that Pro716 and Pro719 are modified by P3H2 after the hydroxylation of Pro707. In addition, the 3-Hyp level at the highly hydroxylated Pro986 in tendon decreased slightly with age, especially until 3 months of age, in contrast to the consecutive modification sites at which the 3-Hyp level increased significantly during the same period. We suspect that opposite outcomes arise through the difference in the responsible P3H enzyme. This hypothesis can be confirmed by analyzing the P3H2-null tendon.

In this study, we focused on prolyl 3-hydroxylation of collagen. However, amino acid analysis showed significant alterations in glycosylation of Hyl in skin, bone, and tail tendons with different profiles (Fig. 1), which would partly arise through cross-link formation (34, 35). Hyl glycosides (GHL and GGHL) are minor modifications similar to 3-Hyp, and are thus difficult to analyze accurately. However, we previously developed a specific purification method for GHL/GGHL-containing peptides to allow highly sensitive analysis of these minor glycosylations (13, 36). Although age-related analysis of GHL/GGHL has been already conducted at the amino acid level (25), site-specific analysis using this method may lead to further novel findings.

We have observed that the 3-Hyp level in collagen shows individual variability and is relatively easy to alter in response to physiological and pathological conditions compared with 4-Hyp (data not shown). Prolyl 3-hydroxylation might be responsible for temporal regulation of the mechanical/structural properties of connective tissues, and the 3-Hyp level could be an indicator of the qualitative status of tissue collagens. Although the biological significance of the increases in 3-Hyp in tendon remains to be clarified, the tissue-, developmental stage-, and modification site-specific alterations imply its key role in the tissue. Further studies are warranted for this minor, but important, modification.

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