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The chiral proteomic analysis applied to aging collagens by LC-MS: Amino acid racemization, post-translational modifications, and sequence degradations during the aging process

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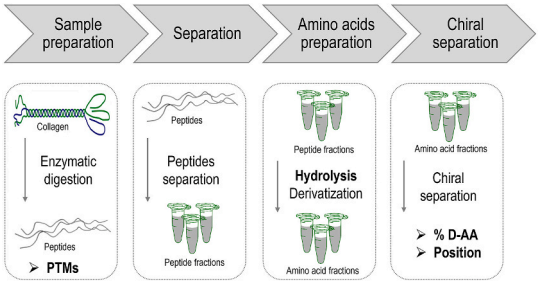
HIGHLIGHTS

- DCl/D_2O protein hydrolysis limit the amino acid racemization.
- % D-amino acids in collagen is progressive according to age.
- A fifth of the collagen sequences is lost during aging.
- Hydrophilic PTMs decrease and hydrophobic PTMs increase during aging.
- Determination of the exact positions of D-amino acids and PTMs.

GRAPHICAL ABSTRACT



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ABSTRACT

Collagen is the most abundant protein in the animal and human bodies, and it is not exempt from this aging phenomenon. Some age-related changes may appear on collagen sequences, such as increased surface hydrophobicity, the appearance of post-translational modifications, and amino acids racemization. This study has shown that the protein hydrolysis under deuterium conditions is privileged to limit the natural racemization during the hydrolysis. Indeed, under the deuterium condition, the homochirality of recent collagens is preserved whose amino acids are found in their L-form. However, in aging collagen, a natural amino acid racemization was observed. These results confirmed that the % D-amino acids are progressive according to age. The collagen sequence is degraded over time, and a fifth of the sequence information is lost during aging. Post-translational modifications (PTMs) in aging collagens can be a hypothesis to explain the modification of the hydrophobicity of the protein with the decrease of hydrophilic groups and the increase of hydrophobic groups. Finally, the exact positions of D-amino acids and PTMs have been correlated and elucidated.

1. Introduction

Aging is a natural and uncontrolled phenomenon that the animal body, including human, undergoes. *In vivo*, molecules are not exempt from this aging process. Indeed, some age-related protein changes may appear like a loss of proteolytic capacity [1–3], increased surface

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hydrophobicity [1], appearance of post-translational modifications such as oxidation [1,4,5], phosphorylation [4], methylation [4,6], deamination [5,7], and acylation (particularly acetylation [4], carbonylation [5], carboxymethylation [5]) [1], and racemization of amino acids [8–13].

Recent animal and human proteins are found in their L-amino acid forms. However, during the natural aging process, an amino acid racemization can take place in proteins *via* amino acids racemases (enzymatic process) or/and *via* a succinimidyl intermediate (non-enzymatic process) [14]. This amino acid racemization affects the three-dimensional protein conformation and can induce some aggregation [15–18], disorder [16], malfunctions [16], misfolding [16], and toxicity [16]. In addition, the amount of D-amino acids in proteins is progressive according to age. In 1975, D-Asp enrichment in human tooth enamel proteins *via* natural racemization was measured as a content of approximately 0,1% per year [19]. Furthermore, some D-amino acids were related to aging human proteins and can be linked to aging diseases. It is the case of D-Asp in elastin linked to arteriosclerosis [20,21], D-Asp in β-amyloid linked to Alzheimer's disease [22], D-Asp, D-Asn, D-Ser, and D-Thr in α-crystallin linked to cataract [9]. The complete list and the exact position of these D-amino acids in till now described protein sequences are recently summarized [14].

D-amino acids are also found in aging and/or ancient proteins and can be used for the age-estimation. Indeed, different studies used the quantification of D-Asp from bone [23], lens [24], and tooth [25,26] for age-estimation, using a HCl/H_2O protein hydrolysis. However, in a hydrogenated environment, a natural racemization of L-amino acids to its enantiomer can take place. Different kinetics were observed according to the nature of amino acids [27,28]. To prevent this amino acids racemization during the acidic hydrolysis, a DCl/D_2O condition is privileged [14,24,29,30]. Indeed, in a deuterium environment, the hydrogen on the alpha carbon was exchanged with a deuterium atom and decreases considerably the racemization. Yasunaga et al. compared the racemization of D-Asp for age-estimation under HCl/H_2O and DCl/D_2O hydrolysis conditions. Errors in age estimations were decreased by at least one-half compared with that of the HCl/H_2O hydrolysis method [24].

Recently, chiral chromatographic and electrophoretic separation methods for the enantioseparation of (un-)derivatized D- and L-amino acids were described [14]. Mass spectrometry optimization can also enhance the discrimination of derivatized D- and L-amino acids, specifically using ion-mobility mass spectrometry [31,32].

Collagen is the most abundant protein in animal bodies, including humans, and is the major structural component of the extracellular matrix present in tissues and organs [33,34]. To date, 28 different types of collagens were described [33]. However, the most common of them is collagen type I which is found in 90% [34]. Its three-dimensional structure is a triple-helix form made up of polypeptide chains α 1 and 2 (COL1A1 and COL1A2). The appearance of D-amino acids in the collagen protein reverses the rotation of the triple helix and decreases the helix-helix interactions until the overall destabilization of the three-dimensional structure [35,36]. However, D-amino acids can also be involved in the cross-linking of assembly molecular material. Indeed, in aging collagen, the presence of D-Ala and D-Glu destabilized the structure while D-Lys stabilized it [2].

For this work, the amino acids racemization in collagen samples (type I alpha 1 and 2) from different organisms (bovine and rat) and at different ages (recent, 4-months and 4-years) were studied. Artificial aging was applied to standard bovine collagens to mimic natural aging. A comparison of HCl/H_2O and DCl/D_2O effects on collagen hydrolysis was studied. The amount of D-amino acids in bovine collagen after artificial aging was compared to that obtained in rat collagen all along the natural aging at the same age. After enzymatic treatment, arising peptides at different ages were compared. Common peptides between recent and aging collagen were identified as peptides from enzymatic digestions, and non-common peptides come from aging degradations. The evolution of post-translational modifications on the collagen sequences was also studied according to age. Finally, the exact position of D-amino acids and amino acids modified by post-translational modifications were elucidated.

2. Materials and methods

2.1. Chemicals

Acetic acid (AcOH, ≥99,8%), acetic acid-d4 (DOAc, ≥99,9 atom % D), acetonitrile (ACN, ≥99,9%), ammonium hydrogen carbonate (NH_4HCO_3, ≥99%), chloroform (99,8%), α-chymotrypsin from bovine pancrease, type II (≥40 units/mg protein), collagen from bovine achilles tendon, type I (bovine collagens), deuterium chloride solution (DCl, ≥99,9 atom %D), deuterium oxide (D_2O, 99.9 atom %D), dimethyl sulfoxide (DMSO, ≥99,9%), ethyl acetate (EtOAc, ≥99,7%), formic acid (FA, ≥98%), heptafluorobutyric acid (HFBA, ≥99,5%), methanol (MeOH), Nα-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (FDAA, Marfey's reagent, ≥99%), pepsin from porcine stomach mucosa (≥2500 units/mg protein (E1%/280)), phosphate buffer solution (1 M, pH 7,4), proteinase K from *Tritirachium album* (≥30 units/mg protein), sodium bicarbonate ($NaHCO_3$, ≥99,5%), tris(hydroxymethyl)aminomethane (Tris, ≥99,8%), trypsin from porcine pancreas (13,000–20,000 BAEE units/mg protein) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetone (≥99,5%) and sodium chloride (NaCl) were from Penta (Chrudim, Czech Republic). Ultrapure water was obtained using a Milli-Q system from Millipore (Bedford, MA, USA)

Hereditary hypertriglyceridemic (HTG) rats were originally selected from Wistar rats as previously described [37]. HTG rats aged 4-months (bred in the Institute of Physiology, Academy of Sciences of the Czech Republic, Prague) were studied. Age-matched normotensive Wistar-derived Lewis rats were used as controls. Rats were housed under controlled conditions (temperature 23 ± 1 °C, 12 h: 12 h light-dark cycle). They were fed a standard rat chow (Velaz, ST-l) containing 0.4% sodium chloride. Water and food were available *ad libitum*.

Amino acid: D-α-alanine (D-Ala, ≥98%), D-arginine (D-Arg, ≥98%), L-arginine (L-Arg, ≥98%), D-aspartic acid (D-Asp, 99%), D-cysteine hydrochloride monohydrate (D-Cys, ≥98%), L-cysteine (L-Cys, 97%), D-glutamic acid (D-Glu, ≥99%), glycine (Gly, ≥99%), D-histidine monohydrochloride monohydrate (D-His, ≥98%), DL-5-hydroxylysine hydrochloride (DL-Hyl, ≥98%), *trans*-4-hydroxy-D-proline (D-Hyp, 97%), DL-isoleucine (DL-Ile, 99%), D-leucine (D-Leu, 99%), D-lysine (D-Lys, ≥98%), DL-methionine sulfoxide (DL-Met sulfoxide, ≥98.5%), L-methionine sulfoxide (L-Met sulfoxide, ≥98%), D-phenylalanine (D-Phe, ≥98%), D-proline (D-Pro, ≥99%), L-proline (L-Pro, ≥99%), DL-serine (DL-Ser, ≥98%), D-serine (D-Ser, ≥98%), D-threonine (D-Thr, ≥98%), L-threonine (L-Thr, ≥98%), D-tryptophan (D-Trp, ≥98%), L-tryptophan (L-Trp, ≥98%), D-tyrosine (D-Tyr, 99%), L-tyrosine (L-Tyr, ≥98%), and D-valine (D-Val, ≥98%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). DL-α-alanine (DL-Ala), L-glutamic acid (L-Glu), DL-leucine (DL-Leu), DL-methionine (DL-Met), and DL-β-phenyl-α-alanine (DL-Phe) were obtained from Reachem (Mississauga, Canada). L-aspartic acid (L-Asp, 98%), L-histidine (L-His, 98%), L-methionine (L-Met, 98%) and L-valine (L-Val, 98%) were obtained from Roana (Budapest, Hungary). L-isoleucine (L-Ile, *allo*-isoleucine free), L-lysine monohydrochloride (L-Lys) were obtained from Calbiochem (San Diego, USA). L-hydroxyproline NH·CH2·CH(OH)·CH2 (L-Hyp, ≥98,5%) was obtained from BDH (BDH Chemicals Ltd., Poole, UK).

2.2. Sample preparations

2.2.1. Standard and proteinogenic amino acids preparation

Standard amino acids preparation method: standard amino acids were dissolved in D_2O at 50 mM.

Amino acids derivatization method: To standard and proteinogenic amino acid solutions, 100 μL of $NaHCO_3$ (1 M) in D_2O was added, and

200 μL of 38.7 mM Nα-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (FDAA) in acetone. Solutions were vortexed and incubated at 40 °C for 60 min. Reactions were quenched by the addition of 50 μL of DCl (6 M) in D_2O. Compounds of interest are extracted by ethyl acetate (0.5 mL). A saturated sodium chloride solution (300 μL) was added in the aqueous phase to increase the difference in density between the aqueous and organic phases and facilitate the extraction. Undesired hydrogen fluoride formed during the derivatization reaction stays in the aqueous phase and is not injected to limit the HPLC and LC column degradations. From extracted organic phases, 20 μL of these solutions in 20 μL of DMSO were analyzed by HPLC at 340 nm or LC-MS.

2.2.2. Recent and aging collagens preparation

2.2.2.1. Artificial aging of bovine collagens. Bovine collagen samples (n = 6) in phosphate buffer (100 mM, pH 7,4) at 2 mg mL^{-1} in glass tubes, were placed outside under natural meteorological conditions for 4-months and 4-years.

2.2.2.2. Extraction of natural aging HTG rat collagens. Tendons from the tails of HTG rats (n = 2) were scraped with a scalpel, then rinsed three times with 1 M NaCl solution, each for 24h at 4 °C. Extracted collagens were rinsed with ultrapure water and lyophilized.

2.2.2.3. Enzymatic digestion. Recent and aging bovine and rat collagens type I alpha 1 and 2 were digested successively by pepsin (0.1 mg mL^{-1} in 3% AcOH, 50/1 sample/enzyme, w/w) and trypsin (0.02 mg mL^{-1} in 50 mM NH_4HCO_3, 50/1 sample/enzyme, w/w). Collagen residues were lyophilized after both enzymatic treatments. Bovine and rat 4-months aging collagens produce an insoluble material after these treatments while recent and 4-years aging collagens do not. These insoluble parts were then digested by proteinase K (0.05 mg mL^{-1} in 10 mM Tris, 50/1 sample/enzyme, w/w), and resulting insoluble parts were digested by chymotrypsin (0.04 mg mL^{-1} in 50 mM NH_4HCO_3, 50/1 sample/enzyme, w/w). The totality of bovine and rat 4-months aging collagens was soluble after chymotrypsin enzymatic treatment. Each enzymatic digestion was performed overnight at 37 °C in the darkroom.

2.2.2.4. Hydrolysis. Proteins and peptides hydrolysis method: intact proteins and each peptide fraction were hydrolyzed in individual glass tubes placed into 10-mL Pierce Reacti-vials (Kimble Chase, Mexico) that had been purged with nitrogen thrice. After 18 h at 130 °C in 400 μL 1:1 DCl/(2H4)acetic acid (DCl/DOAc) on the bottom of the vial, 200 μL of D_2O was added to the proteinogenic amino acid solution obtained in each tube. Then, these proteinogenic amino acids were derivatized following the amino acids derivatization method described in section 2.2.1.

2.3. HPLC parameters

2.3.1. HPLC for amino acids analysis

Analyses were performed in a HPLC system from Agilent (Agilent Technologies, Santa Clara, USA). Mobile phase A constituted to 0.1% of FA in ultrapure water (v/v) and mobile phase B constituted to 0.1% of FA in ACN (v/v). The chromatographic column was the Phenomenex Aeris 3.6 μm peptide XB-C18, 250 × 2.1 mm (Phenomenex Inc., Torrance, USA). The mobile phase operated in gradient mode, starting at 2% mobile phase B and increasing for 30 min to reach 8% B, 110 min to reach 40%, and then increasing to 100% B at 125 min, remaining for 20 min, and returning to 2% B for 10 min maintaining constant until the end of the analysis. The flow rate was set at 0.250 mL min^{-1}, the column was held at ambient temperature (25 °C), the injection volume was 5 μL, and UV detection was performed at 340 nm.

The Agilent HPLC system was connected to a maXis quadrupole time-of-flight (Q-TOF) mass spectrometer with ultrahigh resolution (Bruker Daltonics, Bremen, Germany).

2.3.2. HPLC for peptides analysis

Analyses were performed on an HPLC system from Agilent (Agilent Technologies, Santa Clara, USA) consisting of a degasser, binary pump, autosampler, thermostatted column compartment and multi-wavelength detector. Mobile phase A constituted to 0.1% of HFBA in ultrapure water (v/v) and mobile phase B constituted to 0.1% of HFBA in ACN (v/v). Elution was achieved using a linear gradient when separation was started by running the system isocratically for 2 min with 2% of mobile phase B, followed by a gradient elution to 35% B at 40. min, Next gradient was 10 min–100% B. Finally, the column was eluted with 100% B for 10 min. Equilibration before the next run was achieved by washing with buffer A for 10 min. The chromatographic column was the Jupiter 4 μm Proteo 90 Å column (250 × 2 mm ID, Phenomenex Inc., Torrance, USA). The flow-rate was 0.250 mL min^{-1}, the column temperature was held at 25 °C and UV absorbance detection was done at 214 nm, injection volume was 20 μL.

Bovine and rat collagen peptides obtained after enzymatic treatments were separated and collected in several fractions, *e.g.* 31 fractions for collagen sample treated by pepsin and trypsin, 41 fractions after proteinase K treatment, and 37 fractions after chymotrypsin treatment. Chromatograms with fraction delimitations are presented in supplementary materials (Figs. S1, S2, and S3).

2.4. NanoLC-MS parameters

The nanoHPLC apparatus used for protein digest analysis was a Proxeon Easy-nLC (Proxeon, Odense, Denmark). It was coupled to an ultrahigh resolution MaXis Q-TOF (quadrupole – time of flight) mass spectrometer (Bruker Daltonics, Bremen, Germany) by nano-electrosprayer. The nLC-MS/MS instruments were controlled with the software packages HyStar 3.2 and micrOTOF-control 3.0. The data were collected and manipulated with the software packages ProteinScape 3.0 and DataAnalysis 4.0 (Bruker Daltonics).

Five microliters of the peptide mixture were injected into an NS-AC-12dp3-C18 Biosphere C18 column (particle size: 3 μm, pore size: 12 nm, length: 200 mm, inner diameter: 75 μm) with an NS-MP-10 Biosphere C18 precolumn (particle size: 5 μm, pore size: 12 nm, length: 20 mm, inner diameter: 100 μm), both manufactured by NanoSeparations (Nieuwkoop, Holland).

The separation of peptides was achieved via a linear gradient between mobile phase A (ultrapure water) and B (ACN), both containing 0.1% FA (v/v). Separation was started by running the system with 5% mobile phase B, followed by a gradient elution to 7% B at 5 min, and 30% B at 180 min. The next step was a gradient elution to 50% B in 10 min and then a gradient to 100% B in 10 min. Finally, the column was eluted with 100% B for 20 min. Equilibration between the runs was achieved by washing the column with 5% mobile phase B for 10 min. The flow rate was 0.200 μL min^{-1} and the column was held at ambient temperature (25 °C).

2.5. MS parameters

Online nano-electrospray ionization (easy nano-ESI) was used in positive mode. The ESI voltage was set to +4.5 kV, scan time: 3 Hz. Operating conditions: drying gas (N_2): 4 L min^{-1}; drying gas temperature: 180 °C; nebulizer pressure: 100 kPa. Experiments were performed by scanning from 50 to 2200 *m/z*. The reference ion used (internal mass lock, chip cube high mass reference (HP-1221), Agilent Technologies, Waldbronn, Germany) was a monocharged ion of $C_{24}H_{19}F_{36}N_3O_6P_3$ (*m/z* 1221.9906). Mass spectra corresponding to each signal from the total ion current chromatogram were averaged, enabling an accurate molecular mass determination. All HPLC-MS and nanoLC-MS analyses were done in duplicate.

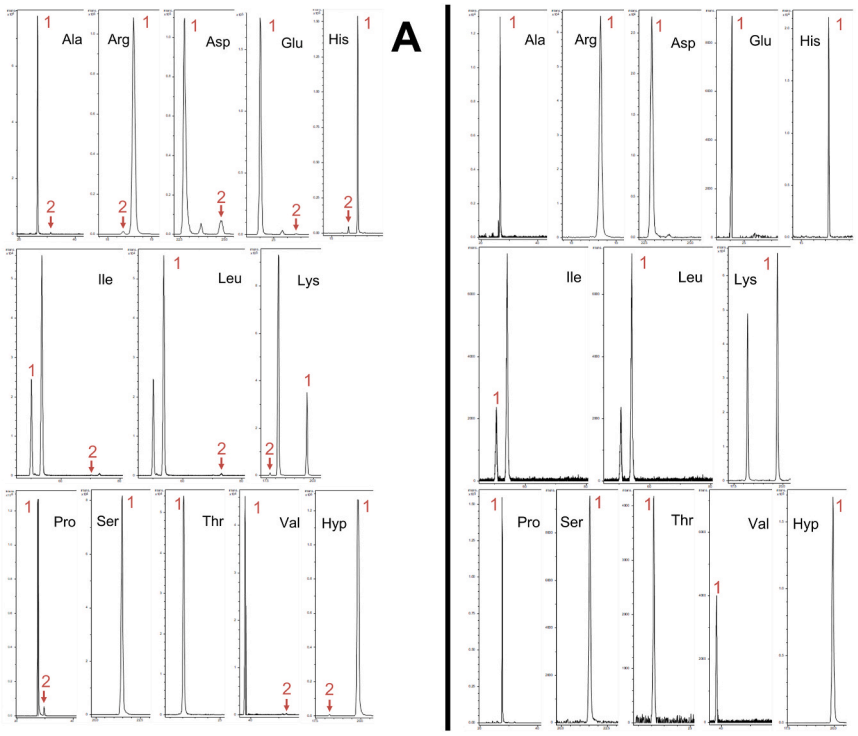

Fig. 1. Effect of hydrolysis conditions on proteinogenic amino acid racemization from standard bovine collagens. Hydrolysis conditions: HCl/H_2O (A) and DCl/D_2O (B). Peak 1 identifies amino acids in their L-form and Peak 2 in their D-form. Exact masses of derivatized amino acids: 342,104420 (Ala), 427,168420 (Arg), 386,094250 (Asp), 400,109900 (Glu), 408,126220 (His), 384,151370 (Ile/Leu), 651,211740 (Lys), 368,120070 (Pro), 358,099340 (Ser), 372,114990 (Thr), 370,135720 (Val) and 384,114990 (Hyp).

2.6. Peptide identifications

Peptide data were processed using ProteinScape software (Bruker Daltonics, Bremen, Germany). Collagen type I alpha 1 and 2 chains were identified by correlating tandem mass spectra to the NCBI, IPI, and SwissProt databases, using the MASCOT search engine (http://www.matrixscience.com). The taxonomy was limited to *Bos taurus* and *Rattus norvegius* due to the provenance of the samples. SemiTrypsin was chosen as the enzyme parameter. Three missed cleavages were allowed, and an initial peptide mass tolerance of ±15.0 ppm was used for MS and ±0.03 Da for MS/MS analysis. Arginine, asparagine, and glutamine were assumed to be deamidated, methionine to be dioxidized, lysine, methionine, and proline to be oxidized, lysine, serine and threonine to be formylated, lysine to be acetylated, carbamylated, carboxylated and carboxymethylated, arginine, aspartic acid, cysteine, histidine, lysine, serine, threonine, and tyrosine were allowed to be phosphorylated. All these possible modifications were set to be variable. Monoisotopic peptide charge was set at 1+, 2+, and 3+. The Peptide Decoy option was selected during the data search process to remove false-positive results. Only significant hits (accepted as identified on ProteinScape) were selected.

3. Results and discussion

3.1. Effect of hydrolysis conditions

Fig. 1 shows the amino acid racemization of recent bovine collagens under HCl/H_2O (Fig. 1A) and DCl/D_2O (Fig. 1B) hydrolysis conditions. Amino acid racemization takes place only under HCl/H_2O hydrolysis conditions for all amino acids except Ser and Thr which were not racemized. Methionine, phenylalanine, tryptophan, and tyrosine were not detected. For this work, a DCl/D_2O hydrolysis is privileged to limit the racemization during the hydrolysis and not distort the results when determining the % D-amino acids.

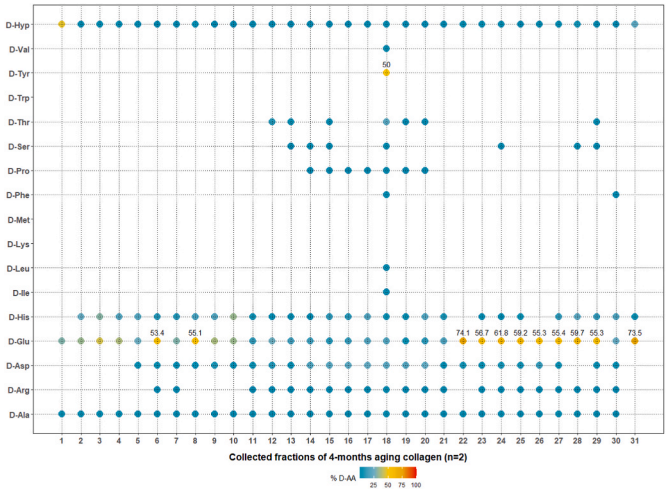

Fig. 2. Representation of % D-amino acids in 4-months aging bovine collagen fractions (n = 2) after pepsin and trypsin enzymatic treatment. Percentages of D-amino acids superior to 50% are indicated on the graph. All percentages of D-amino acids are summarized in Table S1. The total % RSD of %D-amino acids was 4.37%.

3.2. D-amino acids in aging collagens

This chiral analysis method, described in section 2.3.1 and newly developed for this work using standard amino acids, was then used to confirm the presence of D-amino acids in bovine collagen after *in vitro* artificial aging, at different ages. In addition, this analytical method was also used for the detection of D-amino acids in rat collagen with *in vivo* natural aging. Preliminary study was performed to confirm the presence of amino acid only in their L-form in recent bovine collagen (Table S5). During the aging process, the artificial amino acid racemization, applied to bovine aging collagens, mimes the natural amino acid racemization. Most of the different amino acids were racemized over time. It is the case

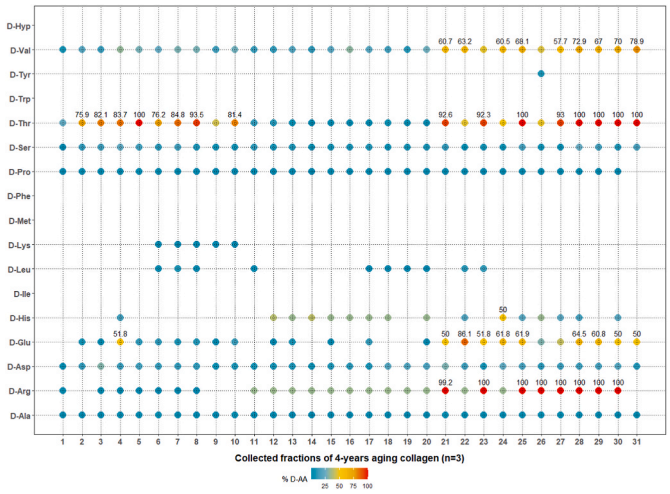

Fig. 3. Representation of % D-amino acids in 4-years aging bovine collagen fractions (n = 3) after pepsin and trypsin enzymatic treatment. Percentages of D-amino acids superior to 50% are indicated on the graph. All percentages of D-amino acids are summarized in Table S2. The total % RSD of %D-amino acids was 10.97%.

of D-Arg, D-Asp, D-Leu, D-Pro, D-Ser, D-Thr, and D-Val most frequently racemized in 4-years than in 4-months collagen samples. In addition, the percentage of Arg, His, Thr, and Val in their D-form was higher in the older sample (Figs. 2 and 3, Figs. S4 and S5, Tables S1–S5).

Most interesting amino acid fractions of bovine collagens were selected and compared to the same amino acid fractions of rat collagens at the same age. The selection of fractions was made based on the high percentage of amino acid racemization, and the diversity of the nature of amino acids racemized. The comparison of the percentage of D-amino acids is shown in Figs. S6 and S7 and Tables S2 and S6. Into selected fractions of soluble part of collagen after pepsin and trypsin enzymatic treatment, some D-amino acids, *e.g.* D-Arg, D-Ile, D-Leu, D-Phe, D-Pro, and D-Tyr, were present in bovine collagen after artificial aging and not in rat collagen with natural aging. Other amino acids were less frequently present in rat collagen with natural aging, such as D-Asp, D-His, D-Ser, and D-Hyp. Conversely, D-Thr and D-Val were more frequently present in rat collagen with natural *in vivo* aging. Concerning the % D-amino acids, D-Glu, D-His, D-Thr, and D-Val were most racemized in their D-form in rat collagen with natural aging. The same trend was observed in selected fractions of the insoluble part of bovine and rat collagens after pepsin, trypsin, and proteinase K enzymatic treatment (Fig. 4 and Tables S3 and S7). Some D-amino acids, *e.g.* D-Arg, D-Lys, D-Phe, and D-Pro, were present in bovine collagen and not in rat collagen. D-Asp, D-His, D-Ser, and D-Hyp were less frequently present in rats. Conversely, D-Thr was more frequently present in rat collagen. Concerning the % D-amino acids, D-Glu and D-Thr were most racemized in their D-form in rat collagen with the natural aging, and D-Val was most racemized in bovine collagen after the artificial aging.

3.3. Peptide analysis

Peptides in each fraction from recent and aging collagens were analyzed by nanoLC-MS (sections 2.4 and 2.5) and identified (section 2.6)

3.3.1. Position of D-amino acids

The identification of peptides in each collected fraction (for the method see section 2.6) allowed us to determine their position on the bovine and rat collagen sequences (type I alpha 1 and 2). Regrouping the racemization rate of each amino acid in each fraction (section 3.2) and the peptide identification in these same fractions, the exact position of amino acids totally racemized in their D-form have been determined on the bovine and rat collagen sequences (Figs. 5 and 6, S8 and S9, Table 1 and S8).

In bovine collagens, 21 specific sites of Thr were identified as totally racemized in the D-form for 4-months aging sample and 18 specific sites for 4-years aging sample in the COL1A1 collagen sequence. Their exact positions have shown that 70% of them were common at both ages. For COL1A2, 14 and 15 Thr specific sites were found in the 4-months and 4-years aging samples respectively, and 45% were common at both ages. These specific sites of D-Thr totally racemized represent 48% (4-months) and 41% (4-years) of total Thr on the bovine COL1A1 sequence. For bovine COL1A2, these D-Thr specific sites represent 32% (4-months) and 34% (4-years) of total Thr on the protein sequence. D-Val was exclusively totally racemized in 4-months aging collagens and represent 19% of total Val on bovine COL1A1 and 4% on bovine COL1A2 sequences. D-Arg was entirely racemized in 4-years aging collagens and represented 75% of total Arg on bovine COL1A1 and 63% on bovine COL1A2 sequences.

For amino acids totally racemized in fractions of 4-month aging rat collagens selected according to most promising bovine fractions at the same age, 12 specific sites for D-Glu (17%) and 6 for D-Val (14%) were identified in COL1A1. In COL1A2, 10 specific sites for D-Glu (16%) and 6 for D-Val (12%) were identified.

3.3.2. Peptides from enzymatic digestion and aging degradation

After successively enzymatic digestions by pepsin and trypsin, all

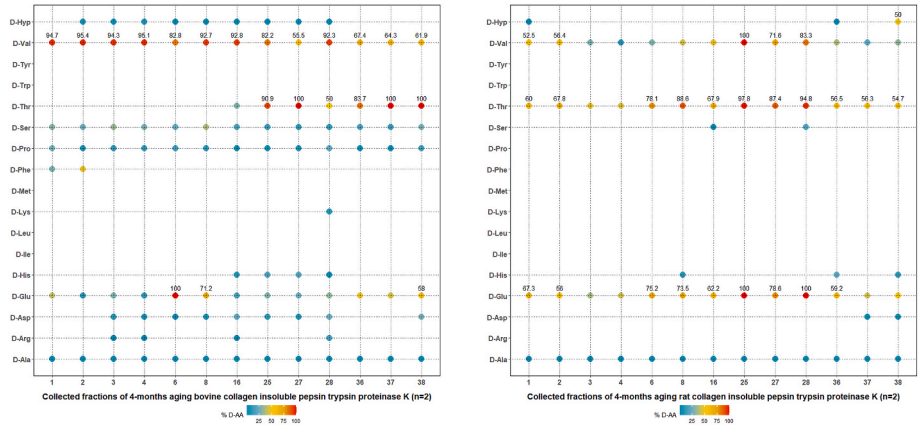

Fig. 4. Representation of % D-amino acids in selected fractions of bovine and rat aging collagen after pepsin, trypsin, and proteinase K enzymatic treatment. Percentages of D-amino acids superior to 50% are indicated on the graph. All percentages of D-amino acids are summarized in Tables S3 and S7. The total % RSD of %D-amino acids from rat collagen was 7.07%.

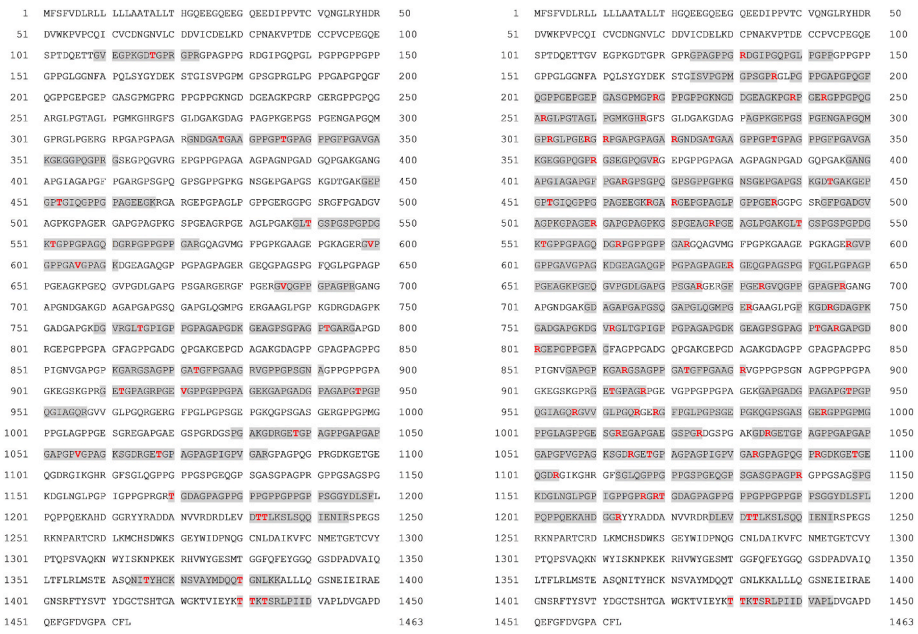

Fig. 5. Exact position of D-amino acids totally racemized in their D-form on the bovine collagen COL1A1 sequence at 4-months (left) and 4-years (right).

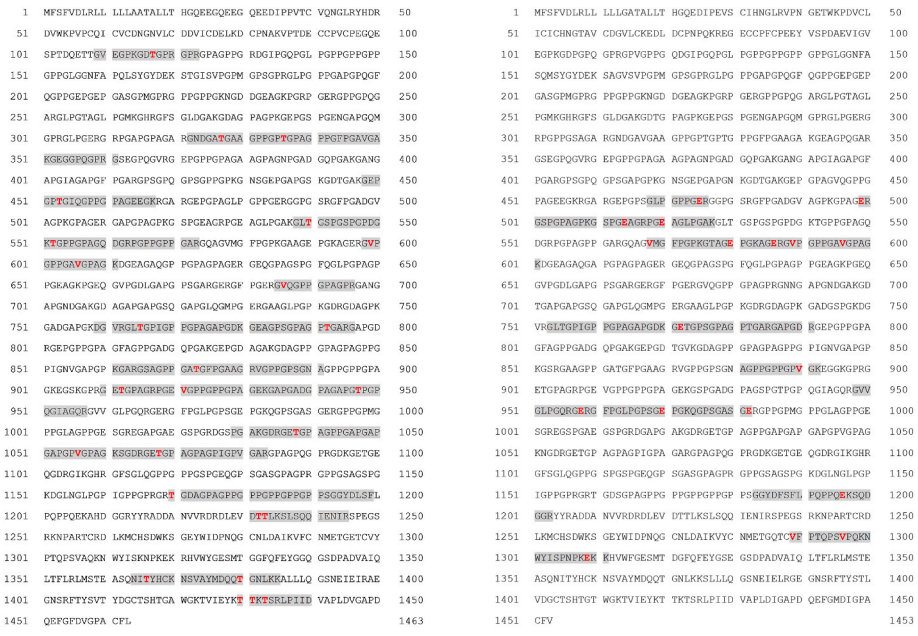

Fig. 6. Exact position of D-amino acids totally racemized in their D-form on the bovine collagen COL1A1 sequence at 4-months (left) and on the rat collagen COL1A1 sequence at 4-months (right).

peptides and residues from recent and aging bovine collagens (COL1A1 and COL1A2) were soluble and identified by nanoLC-MS (section 2.4). Peptides from the insoluble part of collagen protein after pepsin and trypsin treatment at 4-months of aging were not added in the study. Arising peptides in aging soluble collagen samples common to arising peptides in recent collagen were identified as peptides from enzymatic digestions. Other peptides were identified as peptides from aging degradation (Fig. 7). For aging bovine collagens type I alpha 1 and 2 (COL1A1 and COL1A2), the number of peptides from aging degradation is higher than arising peptides from enzymatic digestions. Indeed at 4-months, 46% (COL1A1) and 48% (COL1A2) of peptides come from aging degradations for both collagen types, and 55% (COL1A1) and 46% (COL1A2) at 4-years. The total of arising peptides from enzymatic digestion and aging degradation did not reach 100% for both collagen chains and at both ages. Approximately, a fifth of the sequence information was lost during aging.

3.3.3. *Post-translational modifications during the aging process*

Hydrophilic (*e.g.* oxidation, dioxidation, deamidation, phospho, sulfo) and hydrophobic (*e.g.* acetyl, carbamyl, carboxy, carboxymethyl, carboxyethyl, formyl, methyl) post-translational modifications were studied on the bovine and rat collagen sequences (COL1A1 and COL1A2). Differences according to the organism, age, and artificial/natural aging were observed. The number of modifications and the corresponding percentage according to the total number of possible modified sites are summarized in Table 2 and S9-11 for each sample at different ages.

Based on previous results (see section 3.3.2), about a fifth of bovine

Table 1
Exact position of D-amino acids totally racemized in their D-form on the bovine collagen sequences at different ages.

		COL1A1	COL1A1			COL1A2	COL1A2
AA	Position	4-months	4-years	AA	Position	4-months	4-years
R	131		✓	R	31		✓
	186		✓		38		✓
	219		✓		41		✓
	239		✓		130		✓
	243		✓		154		✓
	252		✓		163		✓
	267		✓		178		✓
	303		✓		232		✓
	309		✓		280		✓
	311		✓		325		✓
	321		✓		340		✓
	360		✓		379		✓
	369		✓		397		✓
	414		✓		411		✓
	468		✓		421		✓
	471		✓		430		✓
	486		✓		438		✓
	510		✓		448		✓
	527		✓		474		✓
	563		✓		484		✓
	573		✓		571		✓
	597		✓		586		✓
	630		✓		607		✓
	675		✓		643		✓
	684		✓		661		✓
	696		✓		669		✓
	732		✓		673		✓
	744		✓		691		✓
	762		✓		706		✓
	795		✓		739		✓
	801		✓		775		✓
	864		✓		792		✓
	881		✓		828		✓
	917		✓		877		✓
	957		✓		904		✓
	966		✓		924		✓
	969		✓		946		✓
	993		✓		976		✓
	1013		✓		994		✓
	1025		✓		1003		✓
	1035		✓		1015		✓
	1065		✓		1051		✓
	1083		✓		1078		✓
	1092		✓		1116		✓
	1104		✓		1203		✓
	1140		✓		1216		✓
	1167		✓				
	1169		✓				
	1213		✓				
	1435		✓				
T	117	✓		T	29		✓
	327	✓	✓		168	✓	✓
	336	✓	✓		207		✓
	444		✓		211		✓
	453	✓	✓		337	✓	✓
	540	✓	✓		382	✓	✓
	552	✓	✓		424	✓	
	765	✓			561	✓	
	792	✓	✓		601	✓	✓
	873	✓	✓		658	✓	
	912	✓	✓		741	✓	✓
	947	✓	✓		754	✓	
	1038	✓			784	✓	✓
	1068	✓	✓		793	✓	
	1097		✓		832	✓	✓
	1170	✓	✓		852	✓	✓
	1232	✓	✓		858	✓	✓
	1233	✓	✓		1319		✓
	1366	✓			1325		✓
	1380	✓			1331		✓
	1430	✓	✓				
	1431	✓	✓				

(*continued on next page*)

Table 1 (*continued*)

		COL1A1	COL1A1			COL1A2	COL1A2
	1433	✓	✓				
V	599	✓		V	156	✓	
	606	✓			157	✓	
	686	✓					
	921	✓					
	1056	✓					

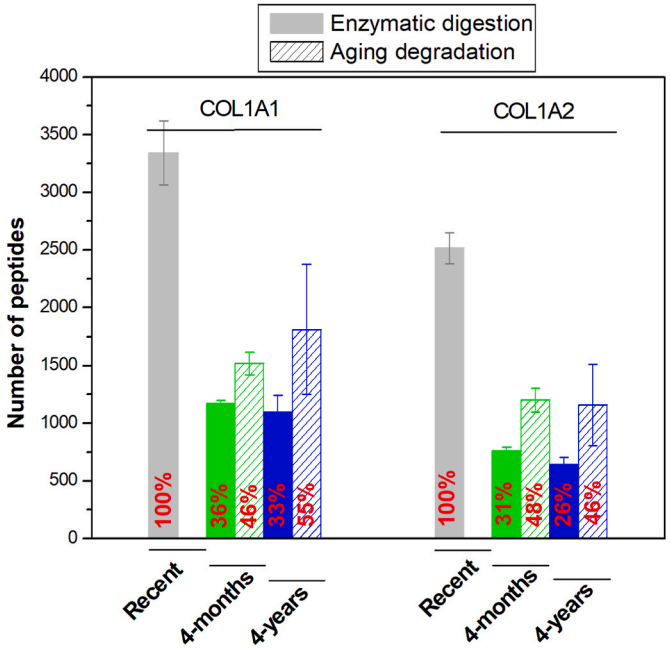

Fig. 7. Number of arising peptides from enzymatic digestion and aging degradation in recent, 4-month, 4-year bovine artificial aging collagens.

sequence information was lost during aging. The post-translational modifications studies show that the percentage of hydrophilic modifications (deamidation, phospho and sulfo) in COL1A1 decreases considerably. Exception for oxidation modifications on lysines, methionines, and prolines (exact positions in Figs. S10–S12, Tables S9 and S10), which increase in 4-months samples, before decreasing in older samples in correlation with the degradation and the loss of sequence information. On the other hand, the polypeptide chain alpha 2 seems more subject to hydrophobic (acetyl, carbamyl, carboxymethyl, formyl, and methyl) modifications than the COL1A1, during the aging process. Exception for oxidation and phospho modifications which increase (Table 2). In sum, the decrease of hydrophilic groups and the increase of hydrophobic groups during aging may be a hypothesis to explain the evolution of the insolubility of collagen along life, to add to the protein degradations. The comparison between artificial (bovine) and natural (rat) aging on post-translational modifications on collagen sequences is summarized in Table 3. The percentages of modifications were slightly higher under artificial aging.

3.3.4. *Comparison of exact positions of D-amino acids and posttranslational modifications*

Both amino acid racemization and post-translational modifications can appear, simultaneously or not, during aging. Fig. 8 and S14-18 regroup the exact position of D-amino acids totally racemized and the exact position of post-translational modifications.

Table 2
Total number and percentage of post-translational modifications on bovine collagen COL1A1 and COL1A2 sequences at different artificial aging.

COL1A1							
Modifications	Amino acids	Recent (n = 3)	% recovery	4-months (n = 2)	% recovery	4-years (n = 3)	% recovery
Oxidation	KPM	79	22,8%	98	28,2%	65	18,7%
Dioxidation	M	0	0%	0	0%	0	0%
Deamidation	NQR	5	3,4%	4	2,7%	3	2,0%
Phospho	CDKHRSTY	27	8,0%	20	5,9%	9	2,7%
Acetyl	K	0	0%	1	1,8%	0	0%
Carbamyl	K	0	0%	0	0%	0	0%
Carboxy	K	0	0%	0	0%	0	0%
Carboxymethyl	K	0	0%	0	0%	0	0%
Carboxyethyl	K	0	0%	0	0%	0	0%
Formyl	KST	9	5,3%	12	7,1%	9	5,3%
Methyl	DE	4	3,0%	3	2,2%	2	1,5%
Sulfo	STY	4	3,2%	4	3,2%	1	0,8%
COL1A2							
Modifications	Amino acids	Recent (n = 3)	% recovery	4-months (n = 2)	% recovery	4-years (n = 3)	% recovery
Oxidation	KPM	40	13,8%	56	19,4%	40	13,8%
Dioxidation	M	0	0%	0	0%	0	0%
Deamidation	NQR	4	2,6%	3	1,9%	4	2,6%
Phospho	CDKHRSTY	9	2,9%	16	5,2%	17	5,6%
Acetyl	K	0	0%	0	0%	1	2,0%
Carbamyl	K	0	0%	1	2,0%	0	0%
Carboxy	K	0	0%	0	0%	0	0%
Carboxymethyl	K	0	0%	0	0%	1	2,0%
Carboxyethyl	K	0	0%	0	0%	0	0%
Formyl	KST	4	2,5%	6	3,8%	7	4,5%
Methyl	DE	2	1,9%	3	2,9%	3	2,9%
Sulfo	STY	1	0,8%	1	0,8%	0	0%

Table 3
Number and percentage of post-translational modifications on bovine and rat collagens COL1A1 and COL1A2 sequences at 4-months after pepsin and trypsin enzymatic treatment.

COL1A1					
Modifications	Amino acids	4-months bovine (n = 2)	% recovery	4-months rat (n = 2)	% recovery
Oxidation	KPM	89	25,5%	57	16,4%
Dioxidation	M	0	0%	0	0%
Deamidation	NQR	3	2%	2	1,4%
Phospho	CDKHRSTY	18	5,6%	4	1,2%
Acetyl	K	1	1,8%	0	0%
Carbamyl	K	0	0%	0	0%
Carboxy	K	0	0%	0	0%
Carboxymethyl	K	0	0%	0	0%
Carboxyethyl	K	0	0%	0	0%
Formyl	KST	8	5,0%	3	1,8%
Methyl	DE	3	2,1%	1	0,7%
Sulfo	STY	2	1,7%	0	0%
COL1A2					
Modifications	Amino acids	4-months bovine (n = 2)	% recovery	4-months rat (n = 2)	% recovery
Oxidation	KPM	39	13,2%	31	10,7%
Dioxidation	M	0	0%	0	0%
Deamidation	NQR	2	1,3%	2	1,3%
Phospho	CDKHRSTY	12	4,0%	5	1,6%
Acetyl	K	0	0%	0	0%
Carbamyl	K	0	0%	0	0%
Carboxy	K	0	0%	0	0%
Carboxymethyl	K	0	0%	0	0%
Carboxyethyl	K	0	0%	0	0%
Formyl	KST	4	2,7%	4	2,5%
Methyl	DE	3	2,8%	1	1,0%
Sulfo	STY	1	0,9%	1	0,8%

4. Conclusion

Artificial aging was successfully applied to standard collagen to mimic the natural aging in biological samples. Several collagen samples from different organisms (bovine and rat), were studied at different ages (recent, 4-months, 4-years). DCl/D_2O hydrolysis was privileged to HCl/H_2O to limit the natural racemization of amino acids during the protein hydrolysis. This new chiral analysis method allowed us to determine the percentage of D-amino acids in collagen according to age. Results presented show a % D-amino acids – age correlation. Peptide analysis shows that the amount of peptides from aging degradation is higher than arising peptides from enzymatic digestions. The collagen protein sequence is increasingly degraded over time. The post-translational modifications study showed a decrease of hydrophilic groups and an increase of hydrophobic groups during aging. These sequence modifications may be a hypothesis to explain the evolution of the insolubility of collagen throughout life. The percentage of post-translational modifications was not significantly different between artificial and natural aging in both organisms. The combination of these results allowed us to determine the exact positions of D-amino acids and PTMs.

CRediT authorship contribution statement

Marine Morvan: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Ivan Mikšík:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

```
   1  MFSFVDLRLL LLLAATALLT HGQEEGQEEG QEEDIPPVTC VQNGLRYHDR   50
  51  DVWKPVPCQI CVCDNGNVLC DDVICDELKD CPNAKVPTDE CCPVCPEGQE  100
 101  SPTDQETTGV EGPKGDTGPR GPRGPAGPPG RDGIPGQPGL PGPPGPPGPP  150
 151  GPPGLGGNFA PQLSYGYDEK STGISVPGPM GPSGPRGLPG PPGAPGPQGF  200
 201  QGPPGEPGEP GASGPMGPRG PPGPPGKNGD DGEAGKPGRP GERGPPGPQG  250
 251  ARGLPGTAGL PGMKGHRGFS GLDGAKGDAG PAGPKGEPGS PGENGAPGQM  300
 301  GPRGLPGERG RPGAPGPAGA RGNDGATGAA GPPGPTGPAG PPGFPGAVGA  350
 351  KGEGGPQGPR GSEGPQGVRG EPGPPGPAGA AGPAGNPGAD GQPGAKGANG  400
 401  APGIAGAPGF PGARGPSGPQ GPSGPPGPKG NSGEPGAPGS KGDTGAKGEP  450
 451  GPTGIQGPPG PAGEEGKRGA RGEPGPAGLP GPPGERGGPG SRGFPGADGV  500
 501  AGPKGPAGER GAPGPAGPKG SPGEAGRPGE AGLPGAKGLT GSPGSPGPDG  550
 551  KTGPPGPAGQ DGRPGPPGPP GARGQAGVMG FPGPKGAAGE PGKAGERGVP  600
 601  GPPGAVGPAG KDGEAGAQGP PGPAGPAGER GEQGPAGSPG FQGLPGPAGP  650
 651  PGEAGKPGEQ GVPGDLGAPG PSGARGERGF PGERGVQGPP GPAGPRGANG  700
 701  APGNDGAKGD AGAPGAPGSQ GAPGLQGMPG ERGAAGLPGP KGDRGDAGPK  750
 751  GADGAPGKDG VRGLTGPIGP PGPAGAPGDK GEAGPSGPAG PTGARGAPGD  800
 801  RGEPGPPGPA GFAGPPGADG QPGAKGEPGD AGAKGDAGPP GPAGPAGPPG  850
 851  PIGNVGAPGP KGARGSAGPP GATGFPGAAG RVGPPGPSGN AGPPGPPGPA  900
 901  GKEGSKGPRG ETGPAGRPGE VGPPGPPGPA GEKGAPGADG PAGAPGTPGP  950
 951  QGIAGQRGVV GLPGQRGERG FPGLPGPSGE PGKQGPSGAS GERGPPGPMG 1000
1001  PPGLAGPPGE SGREGAPGAE GSPGRDGSPG AKGDRGETGP AGPPGAPGAP 1050
1051  GAPGPVGPAG KSGDRGETGP AGPAGPIGPV GARGPAGPQG PRGDKGETGE 1100
1101  QGDRGIKGHR GFSGLQGPPG PPGSPGEQGP SGASGPAGPR GPPGSAGSPG 1150
1151  KDGLNGLPGP IGPPGPRGRT GDAGPAGPPG PPGPPGPPGP PSGGYDLSFL 1200
1201  PQPPQEKAHD GGRYYRADDA NVVRDRDLEV DTTLKSLSQQ IENIRSPEGS 1250
1251  RKNPARTCRD LKMCHSDWKS GEYWIDPNQG CNLDAIKVFC NMETGETCVY 1300
1301  PTQPSVAQKN WYISKNPKEK RHVWYGESMT GGFQFEYGGQ GSDPADVAIQ 1350
1351  LTFLRLMSTE ASQNITYHCK NSVAYMDQQT GNLKKALLLQ GSNEIEIRAE 1400
1401  GNSRFTYSVT YDGCTSHTGA WGKTVIEYKT TKTSRLPIID VAPLDVGAPD 1450
1451  QEFGFDVGPA CFL                                         1463
```

Fig. 8. 4-years bovine collagen COL1A1 sequence modifications: post-translational modifications (red), amino acids in their D-form totally racemized (blue), and both modifications on the same amino acid (green). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2023.341260.

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