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Short communication

Pre-column derivatisation method for the measurement of glycosylated hydroxylysines of collagenous proteins

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Abstract

Measurement of the glycosylated hydroxylysines galactosyl- and glucosylgalactosylhydroxylysine (GH and GGH) in combination with other amino acids has been based on ion-exchange chromatography followed by reaction with ninhydrin. Here, a rapid and sensitive high-performance liquid chromatographic method with fluorimetric detection has been developed and employed to determine the glycosylated hydroxylysine residues in alkaline collagen hydrolysates. After hydrolysis, amino acids were derivatised with 9-fluorenylmethyl chloroformate and separated on a Micropak ODS-80TM reversed-phase column (150×4.6 mm). With a multistep gradient system all amino acids were separated in less than 30 min, including the collagen-specific hydroxylysine, hydroxyproline and the glycosylated hydroxylysines. The method was used to evaluate the glycosylation levels of human articular cartilage derived from femoral head, femoral condyle, tibial plateau and ankle. GGH was highest in cartilage from femoral head and ankle; GH showed no differences between the different sources of cartilage.

Keywords: Derivatisation, LC; Hydroxylysines; Collagen; Galactosylhydroxylysine; Glucosylgalactosylhydroxylysine

1. Introduction

Collagen, an important structural protein in connective tissues, undergoes considerable post-translational modifications. The major modification of the triple helical domain involves enzymatic hydroxylation of prolyl and lysyl residues and the covalent attachment of carbohydrates on hydroxylysyl (Hyl) residues. These events occur intracellularly during the period that the nascent α-chain is in a non-helical conformation; folding of the α-chains into a triple helix prevents further hydroxylation and hydroxylysyl glycosylation [1,2].

The biological role of the two Hyl glycosides, the monosaccharide galactosylhydroxylysine (GH) and the disaccharide glucosylgalactosylhydroxylysine (GGH), has not been clearly established. A decreased fibril formation rate was found for glycosylated collagen [3–5]. Furthermore, the carbohydrates, which are oriented parallel to the backbone of the collagen molecule [3], disturb the complementary positioning of collagen molecules, resulting in thinner fibrils [3,6–8]. Furthermore, the spatial effect of the glycosides may be responsible for a decreased

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packing density of glycosylated collagen molecules in fibrils [9]. In addition, glycosylation plays a role in the resistance of collagen towards digestion by mammalian collagenase [3,10]. It has been postulated that excessive glycosylation of hydroxylysine correlate with the severity of *osteogenesis imperfecta* [11,12] and *chondrodysplasias* [13].

GH and GGH have been found in all animal phyla, including the most primitive metazoan, the sponge [14,15]. In mammals, the extent of glycosylation of a single collagen type is known to be tissue-specific [8]. In addition, species-specific and age-related differences have been reported within the same tissue [6]. The microheterogeneity of collagen is further enhanced by varying the ratio of GH to GGH [16]. So far, the measurement of glycosylated Hyl in alkaline hydrolysates of connective tissues or purified collagens has been based on the time-consuming ion-exchange chromatographic separation with post-column colorimetric detection by reaction with ninhydrin. Here we describe elution conditions with reversed-phase high-performance liquid chromatography (RP-HPLC) using an improved pre-column derivatization method with 9-fluorenylmethyl chloroformate (FMOC) [17] for rapid and sensitive determination of glycosylated Hyl in alkaline hydrolysates of collagens and connective tissues.

2. Experimental

2.1. Reagents and cartilage specimens

Acetone, tetramethylammonium chloride, boric acid and sodium hydroxide were purchased from Merck (Darmstadt, Germany). HPLC-grade methanol and acetonitrile was obtained from Rathburn (Walkerburn, UK). Sodium azide (Baker grade) and citric acid were from Baker (Deventer, Netherlands); FMOC was supplied by Fluka (Buchs, Switzerland). Homoarginine and amino acid standard for collagen hydrolysates (A-9531) was obtained from Sigma (St. Louis, MO, USA). This standard contains the amino acids normally found in acidic protein hydrolysates as well as hydroxyproline (Hyp) and Hyl. Acid soluble human placental collagen types III, IV and V were purchased from Biofine (Leiden, Netherlands); bovine type II collagen was purified from nasal cartilage according to Miller et al. [18]. Full depth healthy cartilage specimens were obtained post-mortem from hip, knee and ankle of a 50 year old woman.

2.2. Quantification of GH and GGH

The GH and GGH were prepared from commercially available natural sea sponges as described previously [19]. The concentration GH and GGH was determined by quantitatively converting them to Hyl by heating at 110°C for 4 h in 6 *M* HCl. The amount of Hyl was measured after derivatisation with FMOC and subsequent separation with RP-HPLC, using the Hyl in the Sigma amino acid standard as the reference [17].

2.3. Alkaline hydrolysis

About 20 mg cartilage (dry weight) or 10 mg collagen was hydrolysed in sealed 7 ml PTFE containers (Tuf-Tainers; Pierce, Rockford, IL, USA) with 800 μl 2 *M* NaOH at 110°C for 20–24 h. After neutralising the hydrolysate with 800 μl 2 *M* HCl the mixture was diluted 100 times with 0.1 *M* borate pH 11.4 (0.1 *M* boric acid adjusted to pH 11.4 with 5 *M* NaOH) containing 6 μ*M* homoarginine; 200-μl aliquots were used for derivatisation. As it was found that the presence of proteoglycans does not affect the analysis no attempt was made to remove the proteoglycans before hydrolysis.

2.4. Derivatisation

Samples (200 μl) were mixed with 200 μl acetone containing 6 m*M* FMOC and allowed to stand at room temperature for 40 min. Termination of the reaction and removal of excess reagent and acetone was achieved by extraction with 600 μl pentane [17]. After two additional extractions, 400 μl 25% (v/v) acetonitrile in 0.25 *M* boric acid was added. A 50-μl aliquot of the derivatisation mixture was injected into the HPLC system.

The routinely used amino acid standard was prepared by adding 120 μl of the Sigma stock solution to 100 ml 0.1 *M* borate pH 11.4 containing 6 μ*M* homoarginine (internal standard), 2.44 μ*M*

GGH and 2.73 μ*M* GH. With the derivatisation protocol described above, 50 pmol of most of the amino acids were applied on the column (exceptions: Hyp and Pro 250 pmol, homoarginine 100 pmol, GGH 41 pmol, GH 46 pmol, Cys 25 pmol).

Hyl, GH and GGH are expressed as moles per mole collagen, assuming 300 hydroxyproline residues per collagen molecule.

2.5. Chromatography

RP-HPLC of derivatised amino acids was performed on a Micropak ODS-80TM column (150× 4.6 mm I.D.; Varian, Sunnyvale, CA, USA) thermostatted at 40°C as described elsewhere [17]. For elution a modified gradient was used (Table 1); the flow-rate was maintained at 1.4 ml/min throughout the analysis. Eluent A was 20 m*M* citric acid containing 5 m*M* tetramethylammonium chloride and 0.01% (w/v) sodium azide, adjusted to pH 2.85 with 20 m*M* sodium acetate containing 5 m*M* tetramethylammonium chloride and 0.01% (w/v) sodium azide. Solvent B was 80% (v/v) of 20 m*M* sodium acetate solution containing 5 m*M* tetramethylammonium chloride and 0.01% (w/v) sodium azide (adjusted to pH 4.5 with concentrated phosphoric acid) plus 20% (v/v) methanol. Solvent C was acetonitrile. Fluorescence was monitored at 254/630 nm. The emission wavelength of 630 nm was selected instead of the widely used 310–350 nm since it results in a three-fold improvement of sensitivity [17].

Table 1
Chromatographic gradient conditions for HPLC analysis of FMOC derivatised amino acids including glycosylated hydroxlysines

Time (min)	Eluent A (%)	Eluent B (%)	Eluent C (%)
0	75	–	25
11.5	60	–	40
13	60	–	40
13.1	–	64	36
21.5	–	65	35
25	–	50	50
30	–	25	75
32	–	25	75
38	75	–	25

2.6. Data analysis

Data, based on the peak area, are presented as mean±standard deviation. Quantification of GH and GGH was based on the total area of the doublet. Statistical differences between groups were evaluated using Student's *t*-test (unpaired, two-sided), $P<0.05$ was considered to represent significant differences between groups.

3. Results and discussion

Several papers address the determination of collagen specific amino acids in acid hydrolysates by means of pre-column derivatisation (e.g., [20–22]), so far no attempt has been made to do the same for hydroxylysine glycosides in alkaline hydrolysates. Despite the fact that pre-column derivatisation has been employed to quantify GH and GGH in urine as a tool for monitoring bone collagen breakdown [23–29], no protocol has been described for the analysis of GH and GGH in combination with the collagen specific amino acids Hyl and Hyp or any other amino acid. Fig. 1 shows a typical chromatographic analysis of FMOC-derivatised standard containing the com-

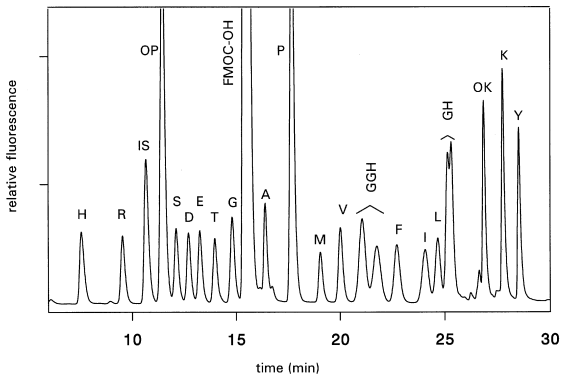

Fig. 1. Elution profile of an amino acid standard derivatized with FMOC. Each amino acid represents 50 pmol, except Hyp and Pro (250 pmol), homoarginine (100 pmol), GH (46 pmol), GGH (41 pmol) and Cys (25 pmol). The peaks are labelled by one-letter codes for the usual amino acids; FMOC-OH, hydrolysis product of FMOC with water; GGH, glucosylgalactosylhydroxylysine; GH, galactosylhydroxylysine; I.S., internal standard (homoarginine); OK, hydroxylysine; OP, hydroxyproline.

mon amino acids as well as Hyp, Hyl, GH and GGH. Homoarginine, used as internal standard, eluted at the beginning of the chromatogram just in front of Hyp. All amino acids (including Ile and Leu) are adequately separated, thus allowing accurate determination of their absolute amounts. The detection limit (signal-to-noise ratio=3) for GH and GGH was 200 fmol and 500 fmol, respectively. For both glycosides, a linear response was obtained from 1 to 600 pmol. Relative standard deviations at an amount of about 50 pmol ranged from 1.6 to 7.4% for both glycosides (mean, 3.8).

With the exception of GH and GGH, all amino acids resulted in single peaks. The doublets seen by GH and GGH result from two diastereoisomers [19,24,28,30]. These diastereoisomers are not both found in vivo; they result from the alkaline hydrolysis. For example, in non-hydrolyzed urine only one diastereoisomer is found, whereas two diastereoisomers are present in alkaline hydrolysates of the same urine [25,28].

GH and GGH are not commercially available. They can be synthesized [31] or purified from alkaline hydrolysates of e.g., anterior lens capsules, basement membranes and sponges [19,30,32–34]. Despite the lack of a standard, concentrations of purified GH and GGH can easily be determined by converting them to Hyl by hydrolysis in 6 *M* HCl for 4 h at 110°C. The concentration can subsequently be calculated on the basis of a primary Hyl standard. By doing so, it was established that FMOC derivatives of GH and GGH do not show the same fluorescence response. The molar response relative to the internal standard, homoarginine, was 2.41±0.034, 2.74±0.045, 1.99±0.057 and 2.13±0.064 for GGH, GH, Hyl and Lys, respectively. The response ratio of about two in comparison to homoarginine implies that GH and GGH, like Hyl and Lys, are disubstituted FMOC derivatives.

Using FMOC as the pre-column derivatisation agent, Miller et al. [18] reported the elution of glycosylated Hyl under chromatographic conditions similar to those described in this paper. However, the two peaks designated as GH and GGH [18] are actually the diastereoisomers of GGH. With their gradient, we found that the diastereoisomers of GH coelute as a single peak with Leu (data not shown). The diastereoisomers of GH can be separated from Leu by adjusting the slope of the gradient (Table 1, Fig. 1). The identity of the GH and GGH peaks was corroborated by mild acid hydrolysis in 0.2 *M* hydrochloric acid at 110°C for 2 h in glass vials fitted with PTFE-lined screw caps [30]. In agreement with literature data [30], GGH was converted to a compound that co-eluted with GH, whereas the conversion product of GH co-eluted with Hyl (data not shown). To further substantiate our findings, alkaline hydrolysates of different collagen types were subjected to amino acid analysis. The GGH/Hyl ratio increased in the order type III<type II<type V<type IV collagen (Fig. 2, Table 2). This is in agreement with glycosylation values found by post-column derivatisation [35]. Furthermore, substantial amounts of GH were only observed in type II collagen (Fig. 2, Table 2).

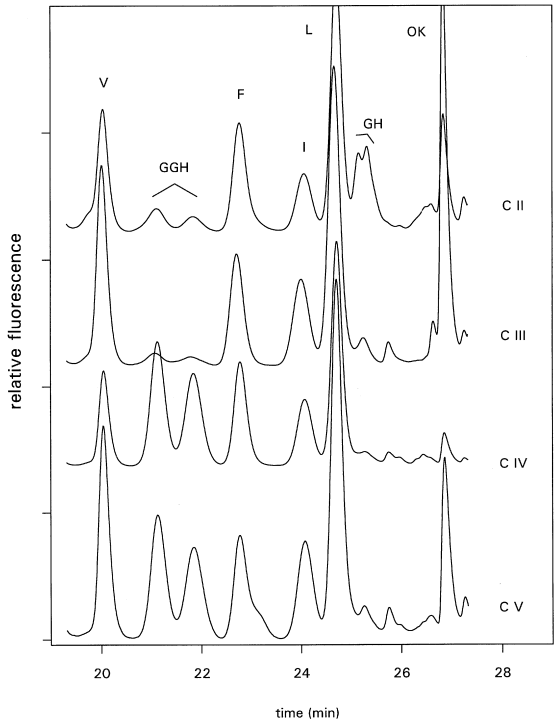

Fig. 2. Reversed-phase chromatography of FMOC derivatized amino acids from alkaline hydrolysed collagens. Only the relevant section of the elution profile, containing Hyl and the glycosylated hydroxylysines, is shown. Collagen type II (C II) was from bovine nasal cartilage, whereas the collagens type III (C III), IV (C IV) and V (C V) were obtained from human placenta. For abbreviations see Fig. 1.

Table 2
Glycosylation ratios of different collagen types

Collagen sample	% of hydroxylysine glycosylated	Ratio GGH/Hyl	Ratio GGH/GH
Bovine type II (n=2)	51.6±2.1	0.33±0.01	0.46±0.08
Human type III (n=3)	6.8±0.2	0.073±0.002	–
Human type IV (n=3)	82.3±3.1	4.78±1.08	–
Human type V (n=4)	56.0±3.2	1.28±0.16	–

The method was applied to articular cartilage obtained post-mortem from different joints of a 50 year old woman. Interestingly, the different joints showed different lysylhydroxylation and glycosylation levels (Fig. 3). Total levels of Hyl glycosides (GH and GGH) were highest in cartilage from femoral head and ankle; significantly lower levels were observed in femoral condyle and tibial plateau (Fig. 3A). The same was found for the total amount of hydroxylysines (i.e., the sum of free and glycosylated hydroxylysines) (Fig. 3B). The glycoside specific for type II collagen (GH), showed no differences between the different sources of cartilage (Fig. 3A). The observed differences in total glycoside levels are therefore entirely due to differences in GGH levels (Fig. 3A). This is also reflected in the ratio GGH/GH: the femoral head shows the highest ratio whereas in the femoral condyle the lowest ratio is observed (Fig. 3D). Interestingly, the fraction of Hyl that has been glycosylated was identical in all cartilages studied (Fig. 3C), suggesting that differences in the amounts of glycosides result from differences in lysyl hydroxylation levels. Although the cartilaginous collagen network consists of a polymeric framework of collagen type II, IX and XI, the main constituent in adult human articular cartilage is collagen type II, accounting for at least 95% of the total dry weight of the network [36]. Therefore, our data can be considered to reflect predominantly differences in glycosylation/lysyl hydroxylation of collagen type II. Although the extent of glycosylation of a single collagen type is known to be tissue-specific, species-specific and age-specific [6,8], we are not aware of any reports that show differences within the same tissue type. Inasmuch hydroxylation of Lys and its subsequent glycosyla-

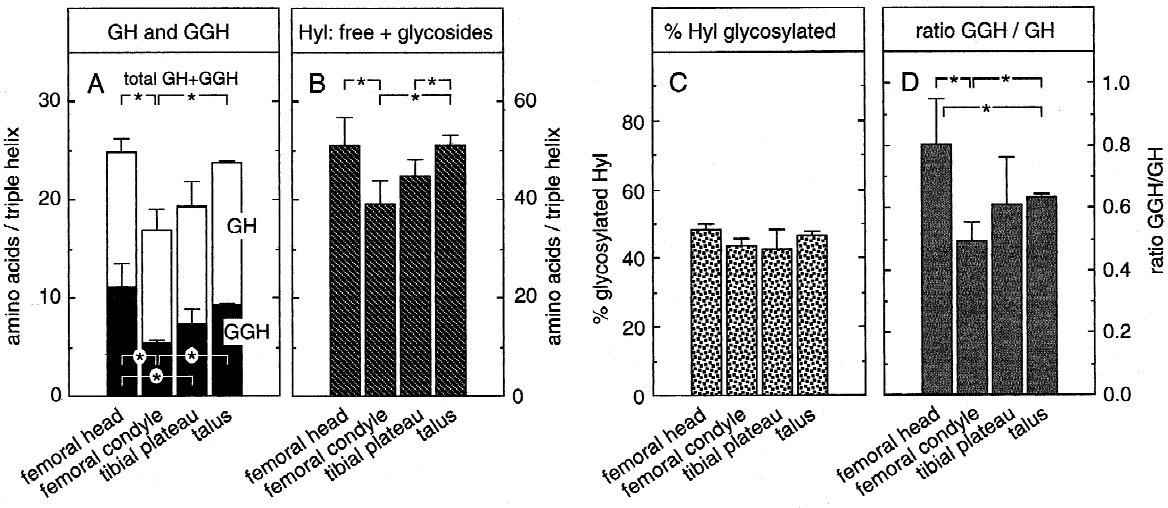

Fig. 3. Glycosylated hydroxylysine and lysylhydroxylation levels in human articular cartilage obtained from hip (femoral head), knee (femoral condyle and tibial plateau) and ankle (talus) of a single donor. Amino acids are expressed as moles per mole collagen, assuming 300 hydroxyproline residues per collagen molecule. Statistical differences between groups are marked with asterisks. (A) GH (white bar) and GGH (black bar) residues/triple helix; (B) total amount of Hyl (=free Hyl and glycosylated Hyl)/triple helix; (C) and (D) % Hyl glycosylated and ratio GGH/GH, respectively.

tion are entirely intracellular processes [1,2], the observed variations reflect phenotypic differences of chondrocytes in the different joints. This phenomenon is presently investigated in more detail.

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