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Original Paper

Posttranslational modifications of collagen studied by off-line coupling of HPLC and CE

A typical example of non-enzymatic change of collagen is glycation (the Maillard reaction, formation of advanced glycation end products) resulting from the reaction of sugars with the ε-amino group of lysine. Posttranslational non-enzymatic modifications of collagen by sugars were studied. Collagenous tissues were incubated as a test protein separately with both glucose and ribose. The collagen mixture was digested by bacterial collagenase and separated by reversed-phase HPLC (in a Jupiter Proteo 90 A column). The eluate from this HPLC separation was collected as seven fractions and consecutively analysed by CE in a bare fused silica capillary (57/50 cm × 75 mm id) using 100 mM sodium 1-heptanesulfonate in 100 mM phosphate buffer, pH 2.5 (NaH_2PO_4 adjusted to pH by phosphoric acid). The chromatographic and electromigration behaviour of individual peptides varied considerably. This off-line HPLC-CE coupling made it possible to discover minor changes in the structure of collagen caused by posttranslational modifications. A new HPLC-CE technique for peptide analysis was developed, and applied to the identification of posttranslational modifications in slowly metabolised test proteins.

Keywords: Capillary electrophoresis / Collagen / HPLC / Posttranslational modification / Proteomics

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1 Introduction

The depository effects of collagen (a well-known long-lived protein) are very difficult to assess. Collagen accumulates reactive metabolites through reactions that are not regulated by enzymes. A typical example of these non-enzymatic changes is glycation (the Maillard reaction, formation of advanced glycation end products) resulting from the reaction of the oxo-group of sugars with the free amino group of the protein (typically the ε-amino group of lysine). Collagens represent a large family of closely related proteins (27 members identified so far). Most of these proteins are ubiquitous in vertebrate tissues and the proportions of the different types vary over a broad range. The set of all collagen proteins can be roughly divided into two subfamilies: Those which form large structures like tendons, skin, cartilages, *etc.*, are typical in having most of the molecule built from the typical collagenous sequence (Gly-Pro-X tripeptides); those which are present at considerably lower concentrations possess large non-collagenous terminal domains. The major supporting collagens are the fibrous types (I, II, III) in which the monomeric molecules polymerise to form striated fibres that are stabilised by intermolecular cross-links, thereby preventing the long rod-like collagen molecules from sliding past each other under stress. Type IV forms a non-fibrous structure as a support for a more flexible basement membrane.

Collagen is relatively resistant to enzymatic degradation, except for some type-specific collagenases. The direct detection of posttranslational non-enzymatic modifications is practically impossible in a large protein molecule (M_r about 110 000 of one α-chain, i.e. 1/3 of a collagen triple helix). This leads to a very complex peptide mixture (theoretically about 172 peptides could arise from the naturally occurring mixture of collagen type I and III). One of the ways of identifying such alterations can be a deep enzymatic fragmentation by collagenase, resulting in a set of small peptides (typically tripeptides). The changes in these peptides caused by modification can be visualised in the chromatographic and electrophoretic profile (peptide mapping). In protein chemistry, peptide mapping, employing both chromatographic and electromigratory methods, is a widely used approach [1]. These changes of peptides are associated with inter- and intramolecular cross-linking and side-chain modifications.

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Cross-linking involves two different mechanisms: a precise enzymatic process during development and maturation, and a subsequent non-enzymatic adventitious reaction with reactive compounds (*e.g.* sugars) during ageing. The mechanism of enzyme-derived cross-linking in which the N- and C-terminal lysines are oxidatively deaminated by lysyl oxidase to form lysyl aldehyde is now well established [2]. The non-enzymatic modification by sugars is known as glycation and plays a central role in the pathogenesis of ageing [2]. The glycation of proteins, including collagen, is initiated by the reversible Schiff base condensation of the oxo group of the sugar (*e.g.* glucose) with amino groups, such as lysine side-chains, followed by a largely irreversible rearrangement that yields ketoamine Amadori products [3]. There are a number of situations in which the proteins present in tissues undergo minor non-enzymatic modifications, which are extremely difficult to assess.

For the bioanalysis of peptides and proteins in biological samples, one-dimensional separation methods often do not have sufficient selectivity. In these cases, multidimensional methods may offer a higher selectivity [4–10]. LC-CE coupling instead of an LC-LC combination is generally more orthogonal. Also, the high separation speed of CE is advantageous, especially in the development of compatible multidimensional systems. In addition, CE has a larger separation efficiency. However, especially when trace level concentrations have to be determined, the analyte concentration is often insufficient to be detected. The on-line coupling of LC and CE is complicated by two additional problems: the large difference between the peak volume of LC and the injection volume of CE and the way the electrode is used at the coupling end of the capillary when the voltage is applied.

The transfer solvent from the first dimension should preferably allow the peptides or proteins to stack in the CE capillary. Consequently, the ionic strength of the LC effluent has to be as low as possible. Most LC-CE systems are compatible, because they use a slowly eluting LC system and fast eluting CE [11, 12].

The differences in peak volumes of LC (mL range) and the injection volume of CE (nL range) are large in LC-CE coupling. However, the disadvantage of using nano LC in the first dimension is the relatively small amount of sample that can be injected. Consequently, using nano LC, the sensitivity of the total system will not always be improved. The stainless steel tubing of the LC dimension in most cases serves as the inlet electrode for the CE. In this case, the inlet electrode of the CE is grounded, while the outlet electrode has a positive or negative potential [13–20].

A combination of HPLC and CE is not often used, there are only a few papers dealing with separation of complex peptide mixtures or peptide maps [21, 22]. In our study we utilised deep cleavage of the parent collagen molecules by *Clostridium histolyticum* collagenase. The peptide maps obtained by HPLC and CE were too complicated, therefore an off-line coupling of these techniques was developed. The peptide mixture was divided into five fractions by HPLC. These fractions were further characterised by CE [23]. In the paper presented here, we tried to improve this method (mainly the HPLC step) and validate it for the analysis of collagen changes caused by posttranslational modifications by sugars.

2 Material and Methods

2.1 Chemicals

All chemicals used were either of analytical grade or the highest available purity. Calcium chloride and phosphate were obtained from Lachema (Brno, Czech Republic), collagen type I (bovine Achilles tendon, purity 99%) and collagenase (E.C. 3.4.24.3., 0.8 U/mg, from *Clostridium histolyticum)*, glucose, ribose, and sodium 1-heptanesulfonate were obtained from Sigma (St. Louis, MO, USA), trifluoroacetic acid and acetonitrile (HPLC gradient grade) were purchased from Merck (Darmstadt, Germany). All other chemicals were obtained from Sigma (St. Louis, MO, USA). All solutions were prepared in Milli-Q Water (Millipore, Bedford, MA, USA).

2.2 Instruments

The HPLC apparatus used was a HP 1100 LC system (Hewlett-Packard, Palo Alto, CA, USA) consisting of a degasser, a binary pump, an autosampler, a thermostated column compartment, and a diode array detector.

CE was performed on a P/ACE 5000 instrument (Beckman Instruments, Fullerton, CA, USA).

2.3 Sample preparation

2.3.1 Preparation of posttranslationally modified collagen

Collagen was diluted in 0.2 M phosphate buffer pH 7.4 at a concentration of 10 mg/5 mL and incubated at 37°C for 48 h with one of the following oxo-compounds: 180 mg/10 mL glucose or 150 mg/10 mL ribose. Collagen incubated under the same conditions in the buffer alone served as control.

2.3.2 Tissues and animals used

Two types of collagen samples were used: a) commercially obtained collagen; b) rat skin (six-month old male Wistar strain rats). Every group contained six samples: (1) control collagen (group Cc); (2) glucose collagen (Gc) – samples of commercially obtained collagen incubated with glucose; (3) ribose collagen (Rc) – samples of com-

mercially obtained collagen incubated with ribose; (4) control skin (group Cs); (5) glucose skin (Gs) – samples of rat skin incubated with glucose; and (6) ribose skin (Rs) – samples of rat skin incubated with ribose.

2.3.3 Collagen digest

To obtain peptide maps, collagen was digested by bacterial collagenase (collagen/collagenase ratio, 50:1, m/m). The samples were incubated at 37°C for 48 h in a collagenase-activating buffer (200 mmol/L NH_4HCO_3, 1 mmol/L $CaCl_2$, pH = 7.8). Blank samples were prepared by incubation of the enzyme solution alone under identical conditions. After the incubation was complete, the vials were centrifuged for 5 min at $2000 \times g$ and the supernatants removed to other vials and stored at −18°C.

2.3.4 Preparation of internal standards for CE

A 0.1-mg portion of diglycine (Gly-Gly) was dissolved in 100 μL of water (1 mg/mL). During the experiments, 10 μL of diglycine was added to the first fraction of the sample, 6 μL of diglycine was added to the second fraction, 23 μL of diglycine to the third fraction, 6 μL of diglycine to the fourth fraction, 11 μL of diglycine to the fifth fraction, 10 μL of diglycine to the sixth fraction, and 15 μL of diglycine to the seventh fraction.

2.4 Separation conditions

2.4.1 Reversed phase HPLC

Chromatographic separation was carried out on a Jupiter Proteo 90 A, 250 × 2 mm (from Phenomenex, Torrance, CA, USA), using a Synergi MAX-RP, 4 × 2.0 mm id as precolumn.

Separation was achieved with a linear gradient between mobile phase A (water–trifluoroacetic acid, 100:0.1, v/v) and B (acetonitrile–trifluoroacetic acid, 100:0.085, v/v). The concentration of B was raised linearly from 0% B to 30% B over the first 30 min and from 30% B to 100% B over the next 20 min. The column was then washed with 100% B for the next 5.5 minutes. The flow rate was 0.25 mL/min, the column temperature was held at 25°C, and UV detection was performed at 214 nm.

Seven fractions were collected (the first: from 0 to 3.9 min retention time, the second: from 3.9 to 6.5 min, the third: from 6.5 to 14.4 min, the fourth: from 14.4 to 16.6 min, the fifth: from 16.6 to 21.2 min, the sixth: from 21.2 to 25.1 min, and the seventh: from 25.1 to 32.1 min). The third, sixth, and seventh fractions were evaporated to half of their original volume, the other fractions were analysed without evaporation.

2.4.2 Capillary electrophoresis

Separations were run at 10 kV in a bare fused silica capillary (57 cm, 50 cm to the detector, 75 μm id) at 20°C. A pH 2.5, 100 mM sodium 1-heptanesulfonate in 100 mM phosphate buffer (NaH_2PO_4 adjusted to pH by phosphoric acid) was used as the background electrolyte. UV absorbance at 214 nm was used for detection.

Injection was performed hydrodynamically by overpressure (3.4 kPa, 10 s). Before analysis, the capillary was conditioned with the run buffer (4 min). The capillary was flushed stepwise with the run buffer (1 min), water (1 min), 1 mol/L NaOH (3 min), water (1 min), 3 mol/L HCl (1 min), and water (1 min) every day.

2.5 Analysis of electrophoretic profiles

The peptide content of the individual electrophoretic profiles was measured by valley-to-valley integrations, and the relative amount of peptides was calculated taking the area of the internal standard as 100%. Each measurement was carried out in at least four parallel determinations and the mean values were calculated. The peak area difference (if any) was calculated using a conventional *t*-test (significant change ($p < 0.05$) compared to the controls). The relative standard deviation of the correlated migration times was lower than 5%.

3 Results

In our previous work [23] we demonstrated that peptide maps obtained by HPLC and CE alone were too complicated. Therefore an off-line coupling of these techniques was developed. The peptide mixture was divided into five fractions by HPLC. These fractions were further characterised by CE [23]. In the paper presented here, we tried to improve on this method (mainly the HPLC step) and applied it to the study of the posttranslational modification of collagen by sugars.

The first separation of peptides arising from collagenase digest was carried out using RP-HPLC. RP-HPLC is capable of partially separating 46 peptide peaks from rat skin (collagen type I and III) (see Fig. 1).

Those profiles were hardly distinguishable. For this reason we separated these mixtures by CE. Unfortunately we obtained similar results – a hardly recognisable mixture of peaks (data not shown).

In the previous communication we developed an off-line coupling method of CE and HPLC [23]. This method was based on the fractionation of an HPLC run into five fractions and analysis of these fractions by CE. In this paper we used much better HPLC separation (based on separation with a Jupiter Proteo column instead of a Zorbax Eclipse XDB C18 column) and divided the HPLC run into seven fractions (in contrast to the previous method [23]

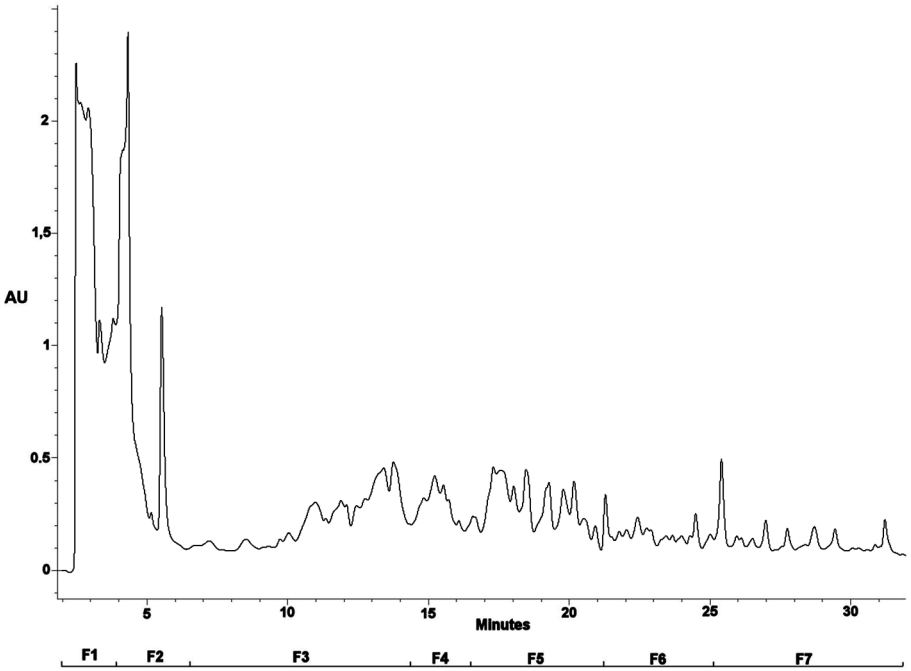

Figure 1. Chromatographic profile of bacterial collagenase digest of rat skin collagen (controls); fraction collection in the preparative run is indicated below (F1–F7). For details see Section 2. *x* axis – migration time; *y* axis – absorbance at 214 nm.

where we divided it only into five fractions). These fractions (1–7) were collected from each category of samples for further (off-line) separation by capillary electrophoresis (see Fig. 1). An internal standard (peptide Gly-Gly) was added to every fraction for better recognition, comparison of the runs, and calculation of the relative areas of the peaks. Application of an internal standard improved the method in comparison to previous experiments [23].

This off-line combination enabled the total recognition of over 160 peptide peaks (as compared to the theoretical 172 peptides in the rat collagen mixture of collagen types I and III) in the electropherograms of all seven HPLC fractions (see Fig. 2). The previous method of off-line coupling of HPLC and CE was able to detect up to 150 peaks [23]. This improvement was mainly due to the use of an HPLC column with a greater ability to separate collagenous peptides.

We applied this method for the study of the posttranslational modifications of collagen by sugars. The collagen peptide profiles of the controls and treated samples (glucose and ribose incubated *in vitro*) are considerably different for both collagen categories (commercially obtained collagen and the rat skin samples). These changes are particularly apparent in detail in the electropherograms. These modifications reflect the reaction of free amino groups of the protein with the oxo-groups of sugars (ribose and glucose). The analyses of all collagens/tissues were performed at least four times (on different samples) and the significance of all changes was statistically verified (Student's test, significance $p < 0.05$). The main

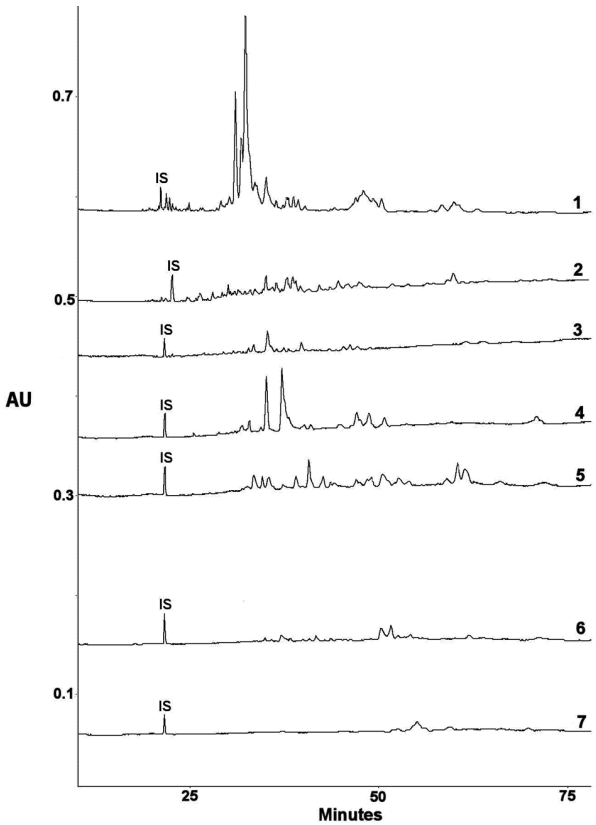

Figure 2. CE profiles of seven collected HPLC fractions of bacterial collagenase digest (control skin samples). The numbers of the individual runs represent the respective fractions obtained by RP-HPLC. (IS indicates the internal standard.) *x* axis – migration time; *y* axis – absorbance at 214 nm.

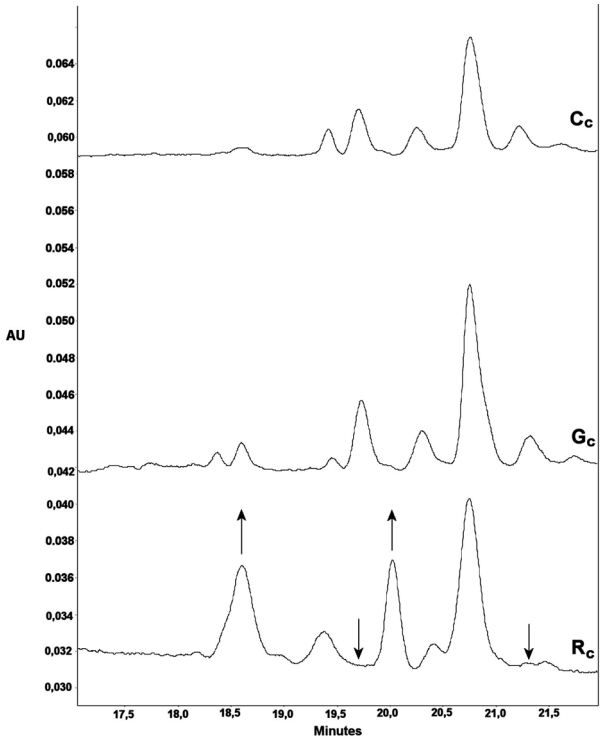

Figure 3. Enlarged sections of the electrophoretic profiles of the peptides from the first HPLC fraction of the digested control commercial collagen (Cc), and of the digest of this collagen treated before digestion by glucose (Gc) or ribose (Rc). *x* axis – migration time; *y* axis – absorbance at 214 nm.

changes were mostly present in the first and second RP-HPLC fractions of the commercially obtained collagen samples (see Fig. 3 and Fig. 4) and in the fourth RP-HPLC fraction of the rat skin samples (see Fig. 5). Most of the changes in the electropherograms were found in the commercially obtained collagen samples incubated with ribose (6 changes) (Fig. 3 and Fig. 4), and three changes in the skin samples (Fig. 5). Comparison of the control and glucose in the commercially obtained collagen samples did not reveal any significant changes. This documents the different specificity and reactivity of the two sugars. In most cases the changes were smaller (non-significant) in glucose-treated samples, but significant in ribose-treated ones – which is in agreement with the higher reactivity of ribose (Fig. 4).

One change was observed by comparing the controls and glucose-treated rat skin samples – both electrophoretic profiles (control and glucose treated) were very similar (Fig. 5). Three significant changes were found in ribose-treated rat skin samples (Fig. 5). This partially reveals the chemism of the posttranslational modifications of proteins by sugars, where the sugar with the more available oxo-group is more reactive [24]. For this reason ribose interacts with the protein at a higher rate and the

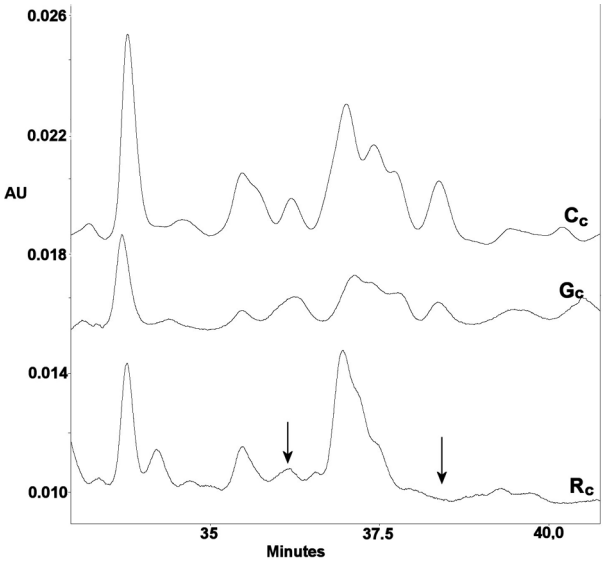

Figure 4. Enlarged sections of the electrophoretic profiles of the peptides from the second HPLC fraction of the digested control commercial collagen (Cc), and of the digest of this collagen treated before digestion by glucose (Gc) or ribose (Rc). *x* axis – migration time; *y* axis – absorbance at 214 nm.

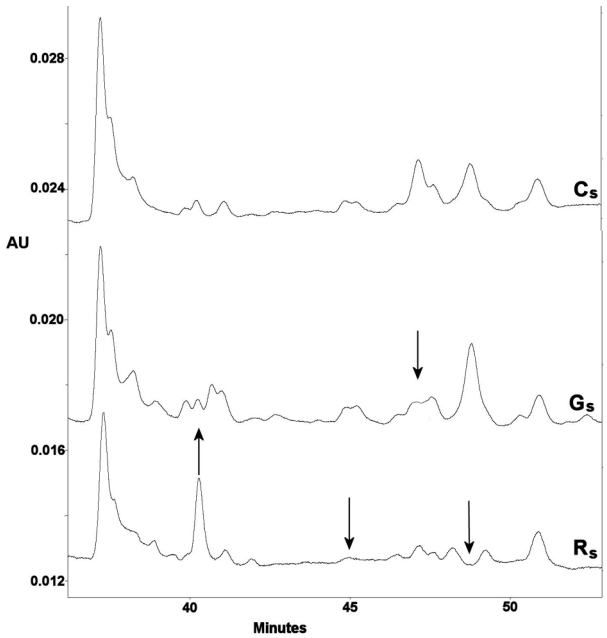

Figure 5. Enlarged sections of the electrophoretic profiles of the peptides from the fourth HPLC fraction of the digested control rat skin collagen (Cs), and of the digest of this collagen treated before digestion by glucose (Gs) or ribose (Rs). *x* axis – migration time; *y* axis – absorbance at 214 nm.

amount of products formed is significantly higher in both types of samples – the commercially obtained collagen and the rat skin samples. With glucose, the extent of posttranslational modifications is lower and the inter-

mediates of these modifications are more likely to form than their advanced products. This could be the reason why in one case we investigated changes in glucose and not in ribose (Fig. 5). Similar changes in the extent of posttranslational modification were also observed in albumin, for example [25].

4 Discussion

Peptide mapping after specific enzymatic cleavage followed by the off-line coupling of two separation methods was found to be a reliable method for assaying the non-enzymatic posttranslational modifications of collagen. By exploiting the complementary nature of HPLC and CE separations of deeply degraded collagenous structures (using *Clostridium histolyticum* collagenase for protein fragmentation), it is possible to detect several minor, but significant, differences in a very complex set of peptides. These differences stem from the non-enzymatic reaction of free amino groups of the protein with glucose and ribose. The high reactivity of ribose with collagen compared to the reactivity of glucose was confirmed. This approach appears to be a handy tool for revealing differences in complex peptide profiles.

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