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Analytica Chimica Acta 352 (1997) 257–270

ANALYTICA CHIMICA ACTA

Reversed-phase chromatography of pentosidine-containing CNBr peptides from collagen

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Abstract

Reversed-phase chromatography with a C_4 macroreticular sorbent was elaborated and optimized for the separation of collagen type I and III CNBr peptides. In its final stage the method allows separation of α_1(I)CB_6 and α_2(I)$CB_{3,5}$ peptides which are the only ones in which pentosidine fluorescence was detected. In both these fractions non-pentosidine fluorescence was observed as well; however, it exhibited a shorter emission maximum (425 nm) as compared to the emission maximum for advanced glycation products (excitation at 370 nm, emission 440 nm). Five fractions were accumulated and evaluated for the presence of fluorescent glycation products. Fraction 1 and 4 were completely devoid of any fluorophores. Fraction 2, however, exhibited a spectrum typical for advanced collagen glycation products (370/440 nm), though it did not contain any distinct pentosidine fluorescence peak. The only fractions which exhibited typical pentosidine fluorescence were fractions 3 and 5 (see Fig. 1 for fraction identification). Fluorescence of the peak of ~60 000 rel. mol. mass (fraction 5) was attributed to collagen type I fragment (α_2(I)$CB_{3,5}$) present in this fraction as no fluorescence was observed with the contaminating α_1(III)CB_9 fragment. © 1997 Elsevier Science B.V.

Keywords: RP-chromatography; CNBr-peptides; Collagen; Pentosidine; Pentosidine unrelated glycation products

1. Introduction

Considerable evidence has accumulated over the past years regarding non-enzymatic post-translational modification of proteins [1–3]. Though the mechanism of chemical reactions involved is far from being completely elucidated, it is generally accepted that at some stage (or some of the arising products) may act as cross-linking agents introducing protein (typically collagen) polymerization. However, it is also felt that the main proportion of the modification products are non-crosslink entities. Some (but for not all) modifications result in fluorescent products. Of these pentosidine (ex/em 335/385 nm) has been fully characterized [4]. It has been also proven, that most collagen preparations exhibit a 370/440 nm fluorescence which probably stems from more than one chemical entity. On the other hand, Monnier et al. [3] speculate that the major cross-link is a non-fluorescent non-ultraviolet active cross-link between two lysine residues, which includes a fragmentation product of glucose linked in a non-reducible bond labile to both strong acids and base. An indication about fluorescent elements arising from the lipoperoxidation pathway was reported in the literature [5].

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PII S0003-2670(97)00121-9

From the analytical point of view there are several points to be emphasized before undertaking any study on the biological role of fluorescent adducts no matter whether they originate by the glycation or lipoperoxidation pathway. At the very end of these post-translational modification insoluble proteins arise; for assaying the fluorescent products the proteins must be brought into solution. Unfortunately, it has been proven that with the exception of pentosidine most of these adducts are unstable both under acid and alkaline hydrolysis; consequently some other solubilization technique has to be applied or the adducts have to be stabilized before protein hydrolysis. To our knowledge no positive results regarding adducts stabilization before hydrolysis have been reported so far. Secondly, studies on model compounds in vitro have demonstrated that the excitation/emission maxima of the arising adducts do not differ considerably, all compounds studied have the excitation maximum between the range 335 and 370 nm and the emission maximum between 385 and 440 nm which makes multicomponent analysis by e.g. synchronous spectrofluorometry difficult [6].

As we have shown in our previous reports [7–9] based on the earlier investigation of Fietzek and Kühn [10], van der Rest et al. [11,12] and Miller et al. [13,14], a productive and reliable method of bringing tissue collagen (particularly types I, III and V) into solution is based on CNBr cleavage of this protein. The small number of methionine residues in the molecule results in a relatively limited number of cleavage products. Their distribution is unique for each type of collagen which provides a useful means for collagen type identification and structural analysis [7]. This strategy has been applied many times in the past for the identification of pathological collagens.

The techniques used for separation of the arising mixture of collagen CNBr peptides were both electromigration and chromatographic. Ion exchange chromatography, reversed phase chromatography, gel electrophoresis and more recently also capillary electrophoresis have been used (for a brief survey of analytical strategies applied see [8]). While ion exchange separations yielded incomplete separations of complex CNBr mixtures and as a matter of facts were applicable to purified constituting α-chains of fibre forming collagens only, gel electrophoresis offered better resolution but at the expense of less reliable quantitation. At the present stage of knowledge there are only two strategies that can be successfully used for the separation of complex CNBr mixtures. Either some type of reversed-phase chromatography originally introduced by van der Rest et al. [11] or capillary electrophoresis in acidic buffers as described in our previous communications [7–9].

The profiles obtained both by reversed phase HPLC and capillary electrophoresis were remarkably similar; this has been explained by assuming a similar separation mechanism of both techniques in the particular case of fibre forming collagens. These proteins display a high internal homogeneity; consequently the bigger the CNBr released fragment, the larger its hydrophobic domain will be. Assuming further the hydrophobic interaction either with the reversed-phase packing (in case of HPLC) or bare silica capillary wall (in case of capillary electrophoresis) the larger CNBr peptides released from fibre forming collagens will come later in front of the detector's window. As a matter of fact most of the released CNBr peptides exhibit a linear relationship between retention (migration) time and their molecular mass [8,9].

In this report we have applied the macroporous C_4 reversed phase packing for the separation of CNBr peptides of rat tail tendon collagen and attempted to evaluate the individual fractions for the content of fluorescent adducts, with the hope to reveal either fluorescence rich fragments (and possibly localizing them in the molecule) and/or to reveal cross-linked peptides in the mixture particularly if their rel. mol. mass will be higher than of the regularly occurring peptides of unmodified collagens.

The reason for using C_4 macroporous reticular sorbent for reversed-phase separation of CNBr released peptides was that from all the techniques used in our laboratory (C_{18} macroporous packing RPLC and capillary electrophoresis) this one had the highest selectivity. Application of the CZE techniques was precluded by the fact that none of our CZE devices was equipped with a fluorescence detector.

2. Experimental

2.1. Preparation of CNBr peptides

CNBr peptide mixtures were prepared either from commercial collagen preparations or from tissue sam-

ples. Collagen type I CNBr peptides were obtained from collagen purchased from Sigma (St. Louis, MO, USA), type III collagen was prepared in the laboratory from rat tail tendons of Wistar male rats aged 12 and 18 months following limited pepsin digestion and selective salt precipitation according to established procedures [15]. These samples were treated with CNBr without chain separation in 70% formic acid as described by Scott and Veis [16]. This procedure yielded a some proportion of uncleaved peptides which emerged closely to the joint peak of α_1(I)CB_7 and α_1(I)CB_8. In order to maximize the CNBr cleavage in some experiments the reaction time was increased up to 24 h, however, the cleavage was never complete (see [11]).

Peptide preparation from tissue slices followed generally the same procedure: the tissues were incubated in 0.2 M ammonium bicarbonate pH 7.0 containing 25% β-mercaptoethanol to reduce oxidized methionyl residues and thereby to enhance CNBr cleavage [17]. The resulting peptide mixture was lyophilized. Lyophilized CNBr peptide preparations from tissue samples were redissolved in Milli-Q water, centrifuged and lyophilized again; before analysis they were preferably in 2% aqueous heptafluorobutyric acid. Using of formate buffer (20 mM), 1% aqueous acetic acid or just Milli-Q water offered identical results in the chromatographic separations step.

2.2. *Chromatographic conditions for separation of pentosidine containing peptides*

HPLC was carried out with a Waters automated gradient controller (Millipore, Milford, MA, USA) with Waters Model 510 pumps. The steel column packed with macroporous C_4 resin Supelcosil LC-304 (250 × 4.6 mm i.d., 5 μm, 300 Å pores; Supelco, Bellefonte, PA, USA) with 2 cm guard column packed with the same material was mounted in the instrument. The sample (CNBr peptide mixture 30 mg) was dissolved in 0.5 ml 2% heptafluorobutyric acid (HFBA) and 20 μl was injected into the column. Elution was done by a linear gradient H_2O (A) 50% acetonitrile (B) (both containing 0.1% HFBA) from 30% B to 100% B at 60 min followed by 10 min isocratic washing with 100% B. The flow rate was 1.0 ml/min and the column temperature was held at 60°C. The eluate was monitored by absorbance at 210 nm using a Waters 490E detector. Peak areas were integrated using software Apex v3.1 (DataApex, Prague, Czech Republic).

2.3. *Preparative chromatography of marker peptides and proof of their identity*

The liquid chromatography equipment used for the isolation of marker peptides was composed of two 6000A pumps, a model 660 gradient programmer and model U6K injector, all from Waters (Millipore, MA, USA). Two types of columns were used:

1. For the isolation of α_1(I)CB_2, α_1(III)CB_2 and α_1(V)CB_1 peptides the initial peptide mixtures obtained by cleaving individual collagen types were chromatographed on a cation exchange column (Mono S HR 5/5, Pharmacia, Uppsala, Sweden) in 20 mM (Na^+) sodium formate (pH 3.8 starting buffer) at a flow-rate 1.0 ml/min (column 35 × 1.5 cm i.d.). For this chromatographic procedure peptides were dissolved in starting buffer and 0.5 ml aliquots were injected. The column was run with the starting buffer for 10 min followed by a linear gradient 0.4 M NaCl in the starting buffer to create the salt gradient (total running time was 40 min). The effluent was monitored at 200 nm; the peaks of interest were collected in 10 ml vials. The whole procedure followed that described by Miller et al. [14].
2. Isolation of the α_2(I)CB_4 peptide was done by reversed-phase chromatography as described by van der Rest et al. [11] and in our previous communication [7]. The column used was Vydac TP 201 column (The Separations Group, Hesperia, CA) (250 × 4.6 mm i.d., 10 μm particle size, 30 nm pore size). Detection was done at 200 nm and the fraction eluting at 40–42 min was collected. Elution was done with a linear gradient 12.8–44.8 (v/v) acetonitrile in water; starting and limiting eluent were 10 mM with respect to heptafluorobutyric acid (HFBA). The flow-rate applied was 1.0 ml/min. This procedure was also used for the separation of other peptides after collagen type I collagen needed for peak identification in the capillary electrophoresis runs.

Table 1
A survey of chromatographic procedures used for the separation of pentosidine containing peptides and peptides used as markers for the C_4 macroreticular resin separation of the whole mixture

Sample	Purpose	Column (chromatographic system)	Mobile phase
(1) Complex mixture of CNBr peptides derived from collagen type I and III	Separation of pentosidine containing peptides	C_4 column Supelco LC-304 reversed-phase sorbent (250×4.6 mm i.d., 5 μm, 300 Å pores)	Elution with a linear water–acetonitrile gradient (both containing 0.1% heptafluorobutyric acid)
(2) Isolation of α_1(I)CB_2, α_1(III)CB_2 and α_1(V)CB_1 [a] peptides	Preparation of marker peptides	Cation exchange column, Mono S HR 5/5 Pharmacia	20 mM sodium formate pH 3.8 (10 min) followed by a linear gradient to 0.4 M NaCl in the starting buffer
(3) Isolation of α_2(I)CB_4	Preparation of marker peptide	Vydac TP 201, 10 μm particle size, 300 Å pore size	Linear gradient 12.8–44.8 v/v acetonitrile in water, all buffers 10 mM with respect to heptafluorobutyric acid
(4) Isolation of α_2(I)$CB_{3,5}$	Identical with item 1, i.e. C_4 column Supelco LC 304 reversed-phase sorbent (250×4.6 mm i.d., 5 μm, 300 Å pores). For separation α_2(I)$CB_{3,5}$ peptide separation the peptide was accumulated from 10 subsequent runs (Fraction 5 shown in Fig. 1).		

[a] The α_1(V)CB_1 was present and prepared from young tendons only.

The overall strategy of separation of pentosidine containing peptides and peptides used as markers is shown in Table 1.

2.4. Measurement of fluorescence spectra

Fluorescence spectra were measured with LS 50B Luminescence Spectrometer (Perkin–Elmer, Beaconsfield, UK). On-line luminescence detection was precluded by the low sensitivity of our fluorescence detector.

2.5. CNBr peptides extraction from polyacrylamide gel

CNBr peptides were extracted from polyacrylamide gel using two Amicon products (Amicon, a Grace Co., Beverly, MA, USA; Protein peptide recovery from polyacrylamide gel with Micropure™ inserts and Microcon™ microconcentrators. Ultrafiltration protocol, Pub. 311). By this way the α_1(I)CB_6 peptide was obtained and identified in the chromatographic profiles (data not shown).

2.6. Pentosidine preparation

The pentosidine was prepared by modified procedure of Dyer et al. [18]. Briefly, an aqueous solution (1000 ml, 100 mM N^{α}-acetylarginine, lysine and glucose in 200 mM phosphate buffer, pH 9.0) was heated at 65°C for one day. The solution was lyophilized and the product was extracted with 200 ml of methanol. Methanol was removed by rotary evaporation, the residue dissolved in 150 ml H_2O (with 0.1% HFBA) and 1 ml aliquots were applied to a Sep-Pak C18 catridge (Waters), washed with H_2O (with 0.1% HFBA), crude product was eluted by 15% acetonitrile (with 0.1% HFBA). Eluates were pooled and concentrated to dryness and then dissolved in 1% trifluoracetic acid (TFA). Pentosidine was in two subsequent separation steps purified by HPLC on Supelcosil LC-318 column (250 × 4.6 mm i.d., 5 mm, 300 Å pores). In the first step separation was done in a system with 0.1% TFA and in the second step TFA was replaced by 0.1% HFBA. After de-acetylation by heating in 6 M HCl for 1 h at 110°C, pentosidine was finally purified by the system with 0.1% HFBA. Identification of pentosidine was confirmed by measuring of its fluorescence spectra.

2.7. Glycation of collagen

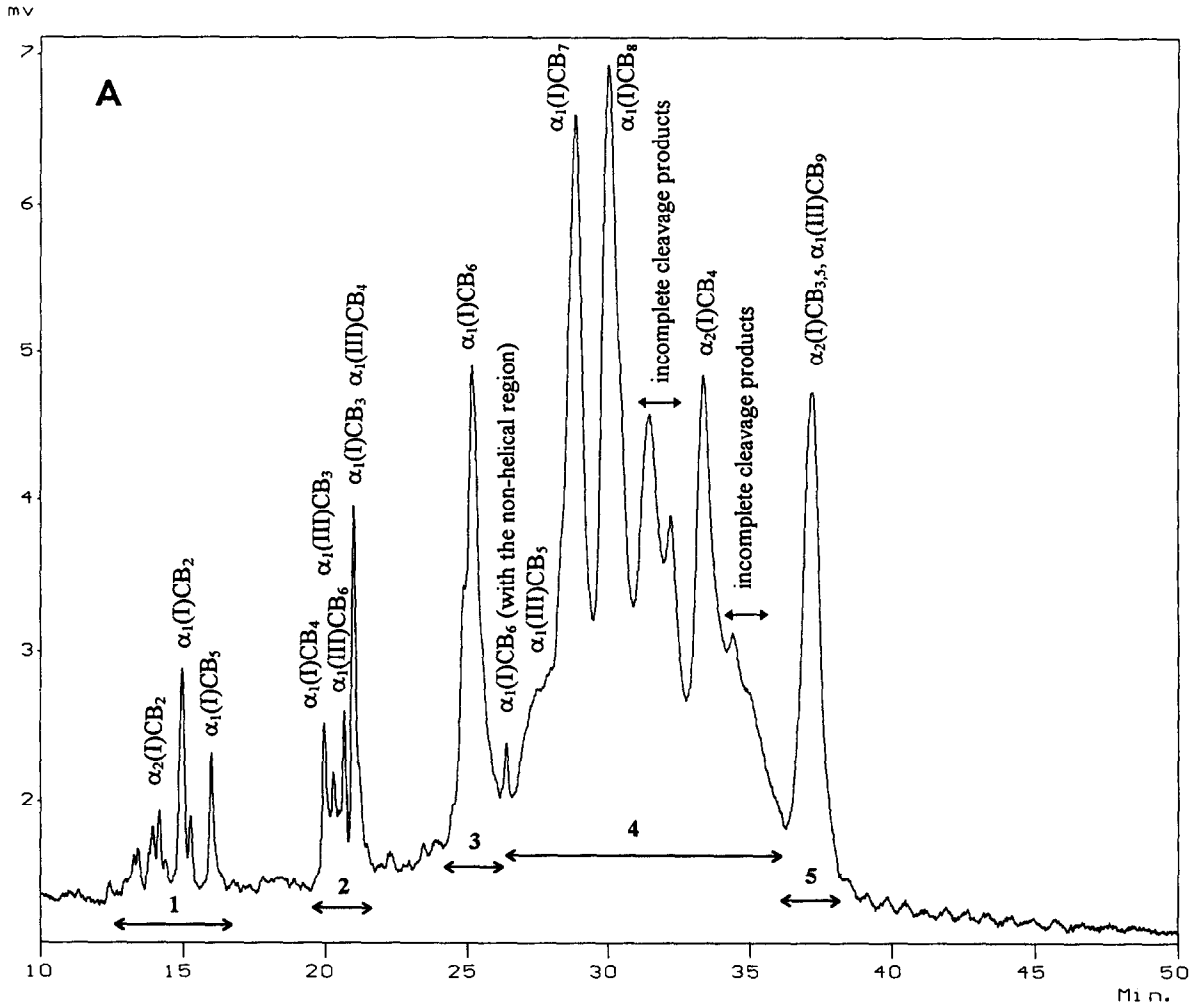
Glycation of collagen was performed at 100 mM phosphate buffer pH 7.4 at 37°C, collagen at concentration 0.5 mg/ml was glycated by glucose (1 mg/ml) for 10 days.

2.8. Chemicals

All chemicals used were either of the analytical grade or highest available purity. Formic acid (88%) was obtained from Lachema (Brno, Czech Republic), collagen type I, pepsin (activity 1 500–3 500 units per mg protein), 2-mercaptoethanol, CNBr, lysine, N^{α}-acetylarginine, glucose, guanidine hydrochloride and ammonium bicarbonate were products of Sigma. Sodium formate was purchased from Baker (Phillipsburg, NJ, USA). Phosphate buffer pH 2.5 was either purchased from Bio Rad or prepared from sodium monophosphate (Lachema) with pH adjusted by phosphoric acid (Lachema). All solutions were prepared in Milli-Q water (Millipore, Bedford, MA, USA).

3. Results

Separation of CNBr peptides in the reversed phase mode on the macroreticular resin yielded slightly better resolution than the separation on the C_{18} sorbent as reported in our previous communication [7]. Besides CNBr peptides from collagen type I α_1 and α_2 chains we were able to identify several peptides which belonged to the type III collagen. Collagen preparations were obtained from animals aged 12 and 18 months (tail tendons) as specified in Section 2. Typical runs are shown in Fig. 1. No pentosidine related fluorescence was observed in fraction 4 containing incompletely cleaved collagen fragments. On the other hand in all collagen preparations the propor-



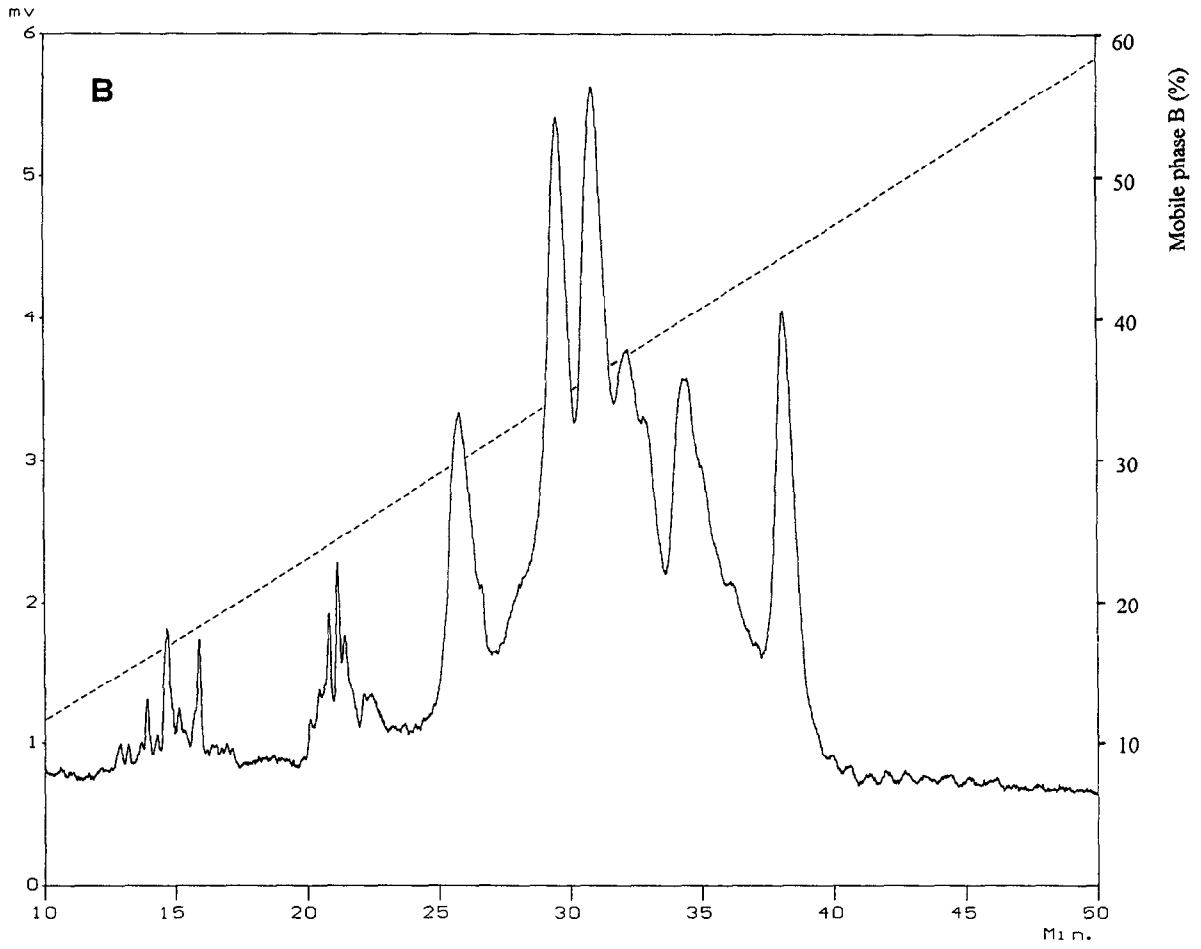


Fig. 1. Reversed-phase profiles of CNBr peptides of 12 and 18 months old rats. Identification of peaks is shown in the Figure, bars indicate fractions collected for further fluorimetric assay as described in Section 2. (A) 12 months old animals, (B) 18 months old animals. 20 μl sample (30 mg of the CNBr peptide mixture reconstituted in 0.5 ml of 2% aqueous heptafluorobutyric acid) was loaded on the column.

tion of the incompletely cleaved CNBr fragments was similar, through no clearcut separation of these fragments was possible under the conditions used.

Two features that should be emphasized when evaluating the resulting collagen CNBr peptide profile:

1. Among the collagen samples analyzed we were unable to detect a peptide exhibiting a molecular mass larger than 63 000 representing the joint peak of α_2(I)$CB_{3,5}$ (rel. mol. mass 60 800) and α_1(III)CB_9 (62 300).
2. When comparing collagen preparations obtained from 12 and 18 months old animals a number of small peaks decreased or even vanished in the latter collagen preparations. These peaks cannot be attributed to any of the known CNBr fragments and seem to be impurities stemming from other connective tissue proteins which survived the extraction procedure.

It is worth noting that no substantial difference between the two groups of animals (and as a matter of fact also no difference when compared to commercial calf skin collagen type I preparation) was seen in the proportion of peaks that correspond to incompletely cleaved products. Hence, it can be concluded that collagen preparations from old and adult animals (at

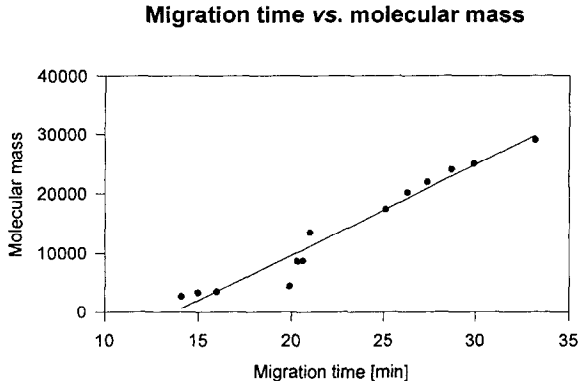


Fig. 2. Migration time vs. molecular mass plot of CNBr marker peptides; $y=-20822.5+1522.3x$ ($R=0.978$, $P<0.0001$).

least as far as rat tendon and calf skin collagen is concerned) are equally accessible to CNBr solubilization from the tissue. A more detailed study on changes in CNBr profiles and fluorescent moieties with age and different physiological conditions will be published elsewhere.

When plotting rel. mol. mass vs. retention time the dependence shown in Fig. 2 was observed. This indicates that the mechanism determining separation on the C_4 resin is the same as that with C_{18} resin and capillary electrophoresis in bare capillaries as reported earlier [9].

Regarding the luminescent elements in collagen it was proven that a particular collagen preparation no matter whether solubilized by pepsin, collagenase or CNBr treatment exhibits the same proportion of products with the 335/380–395 nm and 370/420–440 nm luminescence parameters corresponding to pentosidine (335/380–395 nm) and advanced glycation products (370/420–440 nm). Typical luminescence spectra for CNBr solubilized collagen obtained from rat tail tendons of 12 months old animals are shown in Fig. 3. Identity of the pentosidine peak in these spectra was ascertained by spiking with pentosidine standard. The other peak was ascribed to non-pentosidine glycation related luminescence. This conclusion is based on the results shown in Fig. 4. A clear increase of luminescence between 400 and 420 nm (aside to an increase of the pentosidine peak) can be observed which is in the literature ascribed to non-pentosidine glycation products [1–3].

Direct evaluation of individual peaks by HPLC online fluorescence measurement was precluded by the fact that at the expected fluorescence parameters no adequate response of the detector was obtained and the system was clearly below its detection limit.

Overloading of the column was possible but only at the expense of an intolerable loss of selectivity. Therefore, we have decided to ascertain the presence or absence of luminescent elements in individual fractions off-line after having collected a number of fractions. The fraction collection scheme is shown in Fig. 1(A). It turned out that fractions No 1 and 4 were completely devoid of any fluorescence attributable either to pentosidine or to other glycation products (data not shown). Fraction 4 possessed a broad emission peak both at 335 and 370 luminescence excitation wavelengths (Fig. 5). It clearly showed the absence of a distinct pentosidine peak; however, other luminescent entities were apparently present. In fraction 3 (corresponding to the α_1(I)CB_6 peptide) distinct luminescence maxima were seen both at 335 and 375 luminescence excitation (Fig. 6). Fraction 5 revealed a spectrum typical for pentosidine (see Fig. 7) and some luminescence at 370 nm excitation indicating the presence of both pentosidine and other glycation products as well. This fraction contained peptides α_2(I)$CB_{3,5}$ (rel. mol. mass 60 800) and α_1(III)CB_9 (rel. mol. mass 62 300).

However, further purification of this peak by ion exchange chromatography revealed that all the luminophores present in peptide were stemming from collagen type I only.

4. Discussion

A plethora of candidate products that possibly could cause collagen fluorescence has been described in the literature (for a brief survey see Table 2). Of these, however, only pentosidine and advanced glycation end products have been shown to be present in collagen structures while the evidence for the presence of the others is only indirect. To obtain a direct proof would require a smart combination of physiological animal models with adequate separation technologies. In the present communication we have focused on finding two of the luminescent elements, namely pentosidine and non-pentosidine glycation products in the

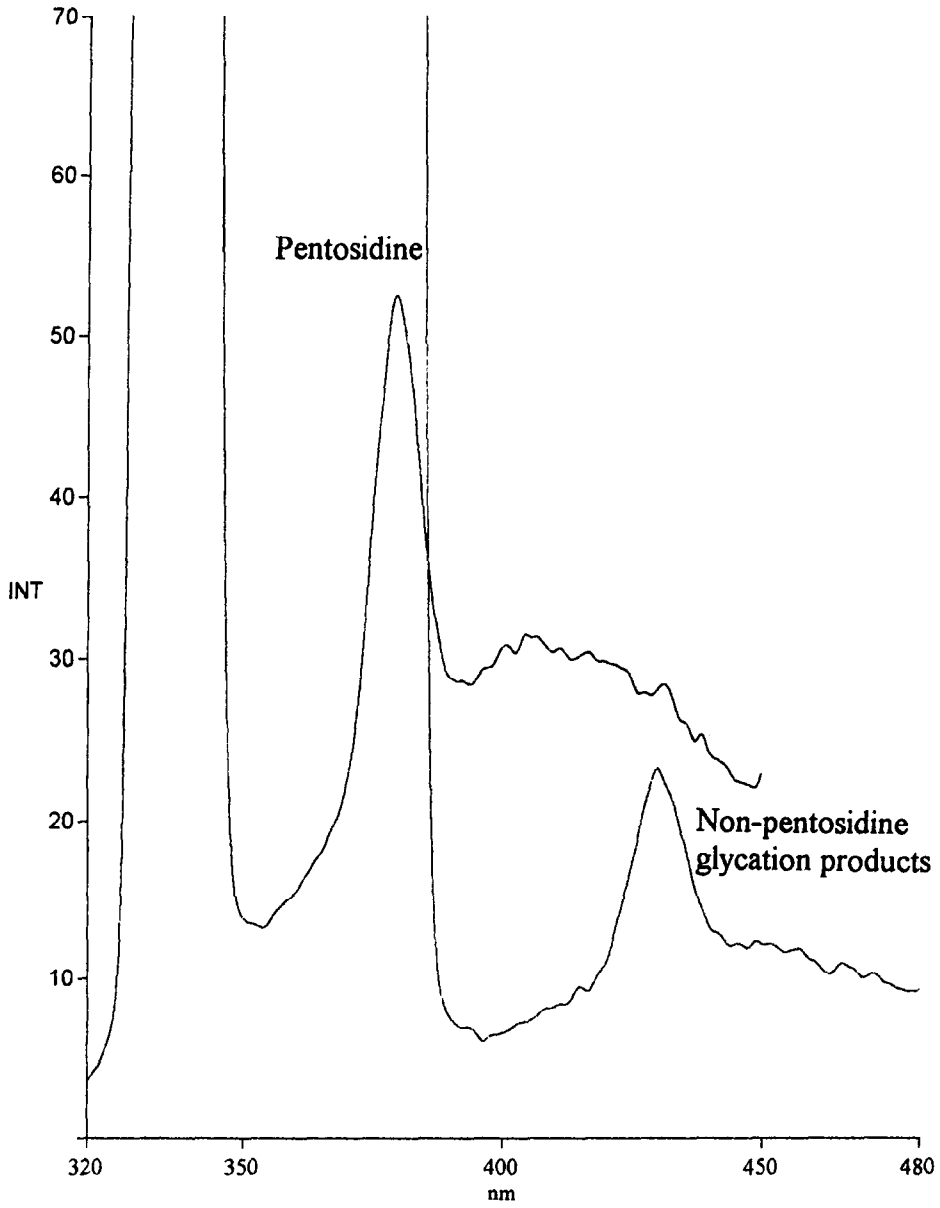


Fig. 3. Typical luminescence emission spectrum of CNBr solubilized collagen from 12 months old rat tendons. Upper curve excitation for pentosidine at 335 nm; lower curve excitation for non-pentosidine glycation products (other than pentosidine) at 370 nm. Crude mixture, without separation.

collagen structures of rat tail tendons. It was demonstrated that CNBr solubilization of the collagenous component is an adequate procedure not affecting the luminescence parameters and their intensity. This is an important point because it has to be remembered that the CNBr cleavage is carried out in highly concentrated formic acid (70%). It has been demonstrated by Tanaka et al. [27] already in 1988 that the recovery of collagen from rat tail tendon by CNBr cleavage is nearly 100% (99.6±2.3% as determinated by standard hydroxyproline assay with the collagen sample obtained from rats aged 12 months). In the same paper

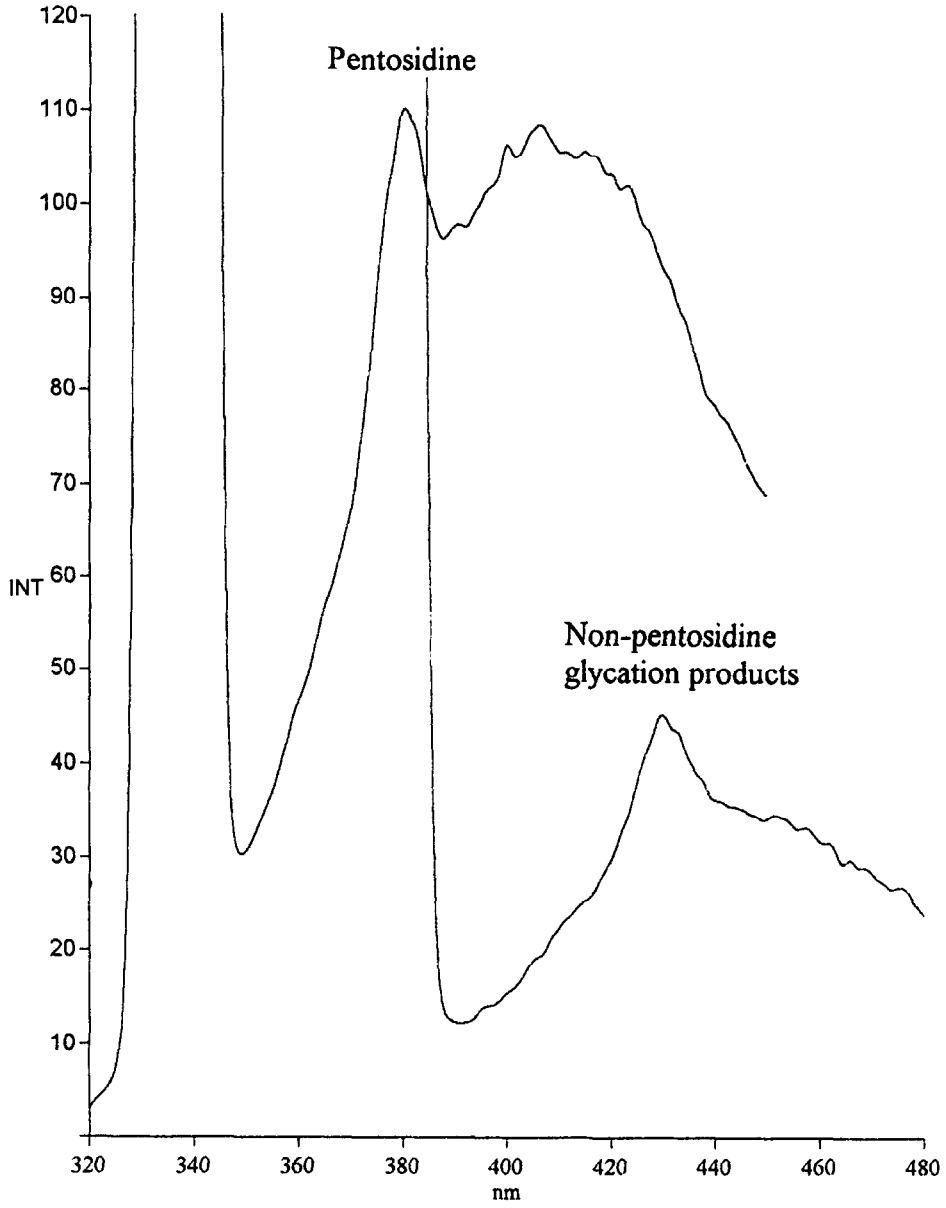


Fig. 4. Typical luminescence emission spectrum of CNBr solubilized in vitro glycated collagen (for details see Section 2). Upper curve excitation at 335 nm for pentosidine, lower curve excitation at 370 nm for glycation products other than pentosidine. Note about two-fold increase in luminescence intensity when compared with spectra shown in Fig. 3. Crude mixture, without separation.

by Tanaka et al. [27] it has been documented that in vitro incubation of rat tail tendons with ribose results in binding of this aldehydic sugar to all major CNBr peptides of collagen with some preference to α_2(I)$CB_{3,5}$ and the triple helical region of α_1(I)CB_6. The authors of this paper were precluded to express more quantitative data as their results were based on the evaluation of polyacrylamide gel separation of CNBr peptides labeled with C^{14} ribose. It was also shown that glucose is capable of similar reactions, however, at a much lesser rate and to a much lower extent.

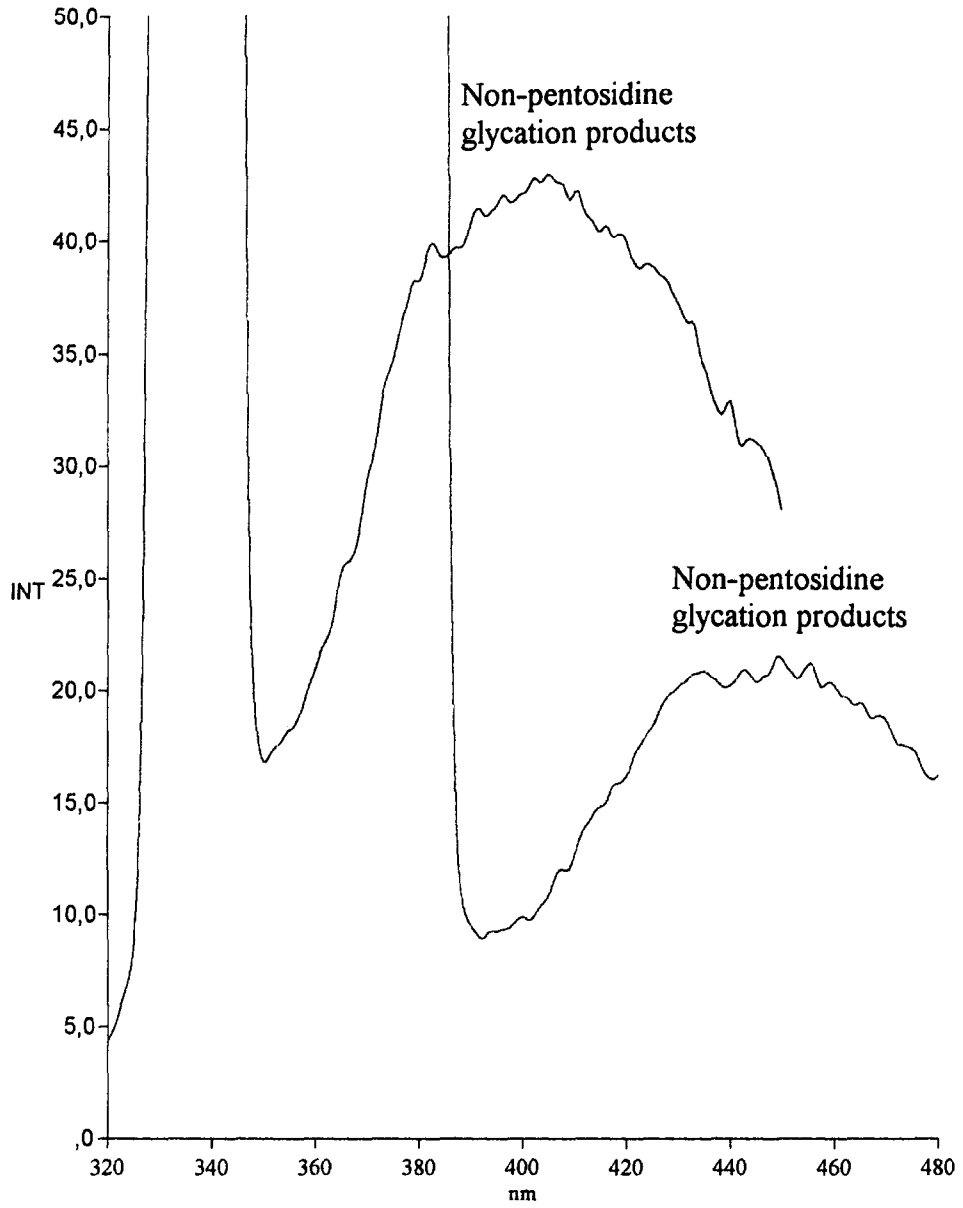


Fig. 5. Luminescence profile of fraction No 2 (for fraction identification see Fig. 1, fraction emerging around 20 min) containing α_1(I)CB_4, α_1(III)CB_3, α_1(III)CB_6, α_1(I)CB_3 and α_1(III)CB_4 peptides. Upper curve excitation at 335 nm, lower curve excitation at 370 nm. Note the shift of the emission spectrum excited at 370 nm towards the red region of the spectrum. The 370/440 nm maximum has been repeatedly reported as typical for advanced glycosylation products [22,23].

One has to keep in mind that the in vitro interactions between aldehydic sugars and collagen polypetide chains are run under much harsher conditions compared to the in vivo conditions. However, in vivo there is much more time available for the glycation reaction to proceed. It is therefore not surprising that the in vivo glycation does not affect all the CNBr peptides equally; those which seemed most prone to the glycation reaction (α_1(I)CB_6, α_2(I)$CB_{3,5}$), indeed, seem to undergo the glycation reaction in vivo. In the α_1(I)CB_6

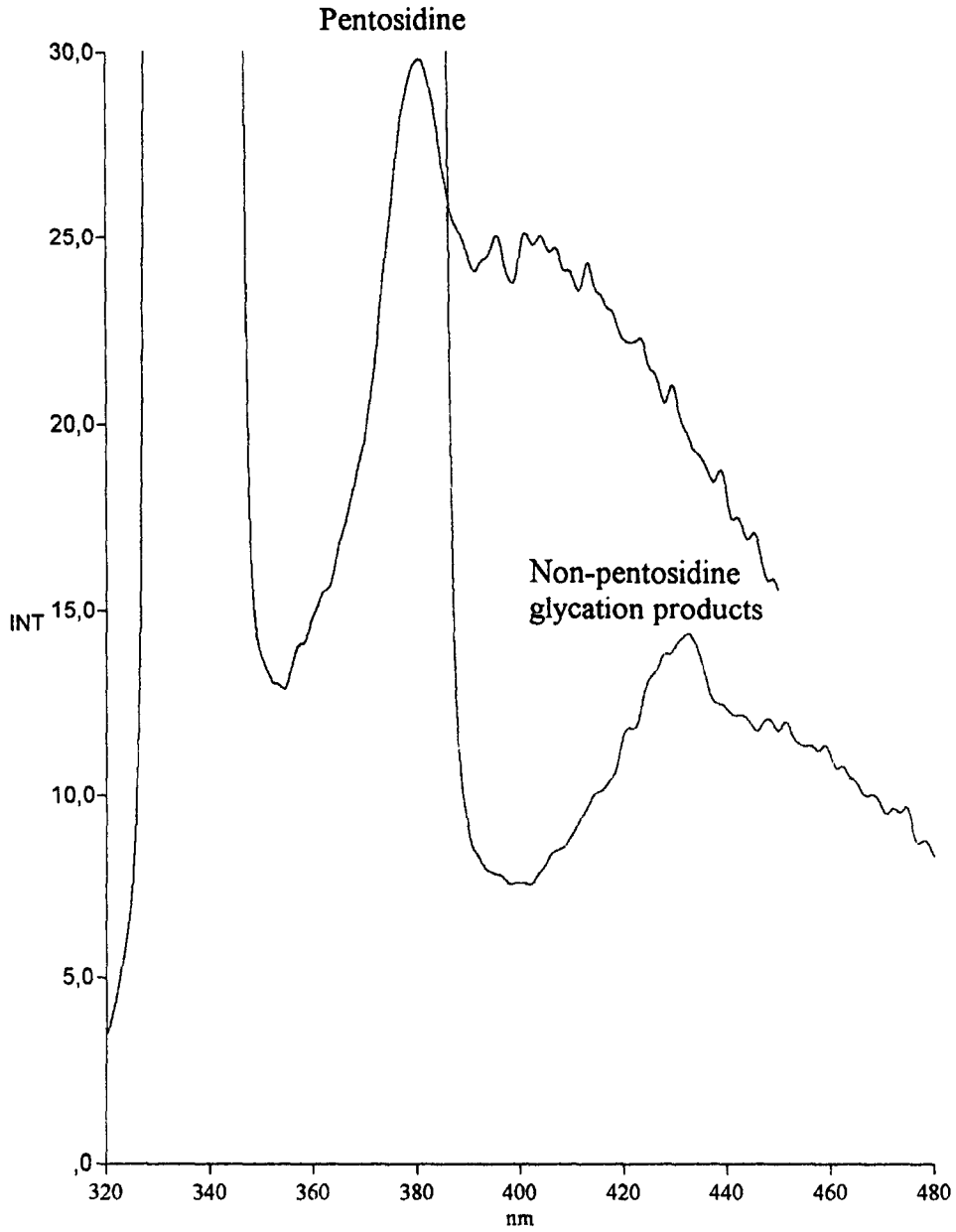


Fig. 6. Typical luminescence profile of fraction No 3 containing α_1(I)CB_6 peptide only (for fraction identification see Fig. 1, this fraction emerges around 25 min retention time from the macroreticular C_4 resin). A distinct peak of pentosidine is seen at the excitation wavelength 335 nm (upper curve) while at 370 nm the presence of other glycation products can be visualized (lower curve).

and α_2(I)$CB_{3,5}$ peptides both types of glycation products, i.e. pentosidine and non-pentosidine glycation products were found, there were no fluorophores present in peptides constituting fractions 1 and 4, i.e. in peptides α_2(I)CB_2, α_1(I)CB_2, α_1(I)CB_5, α_1(I)CB_7, α_1(I)CB_8 and the incomplete cleavage products eluting in fraction 4 (numbering of fractions refers to Fig. 1).

In the paper of Tanaka et al. [27] it is further shown that by glycation in vitro β_{11}, β_{12} and β_{22} components

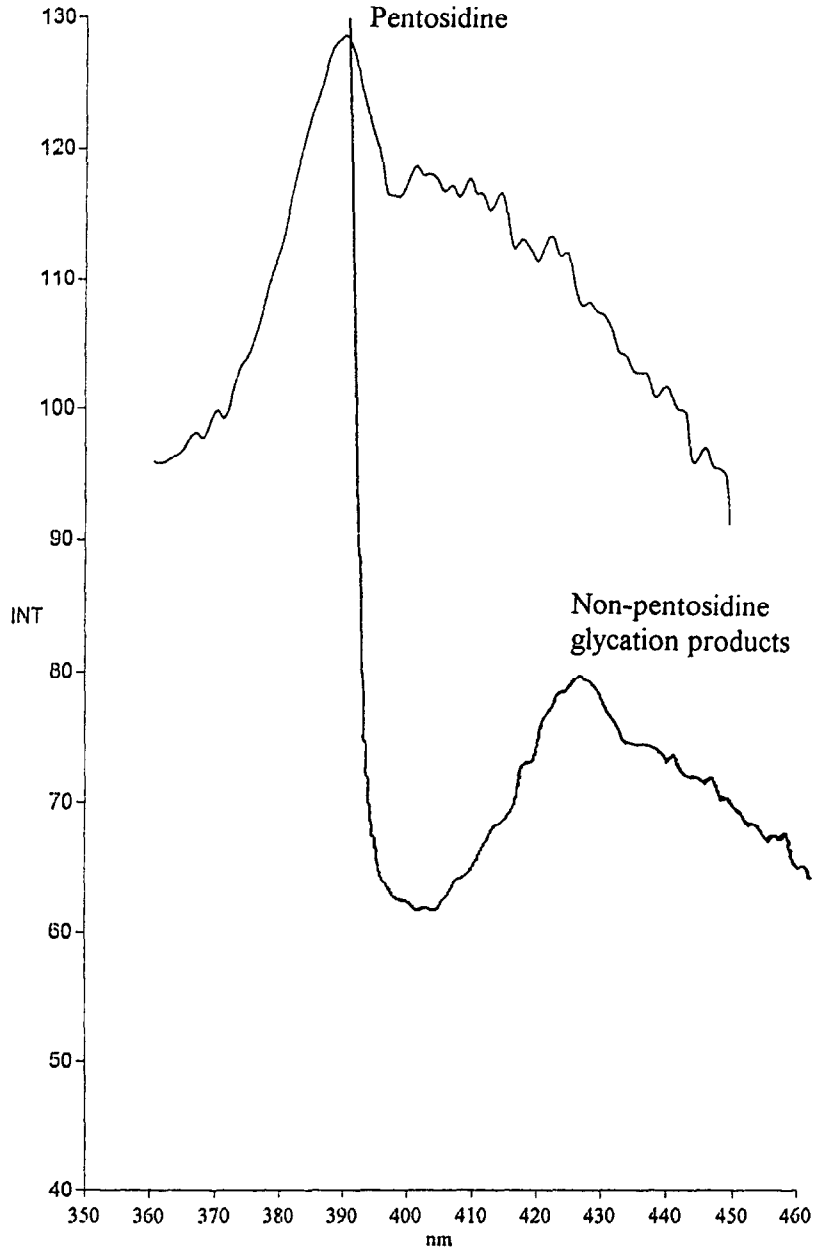


Fig. 7. Typical luminescence profile of fraction No 5 containing the α_1(I)$CB_{3,5}$ peptide (for fraction identification see Fig. 1, this fraction emerges between 35–40 min retention time from the macroreticular C_4 resin). Upper curve – excitation for the presence of pentosidine (335 nm), lower curve – excitation for non-pentosidine glycation products (370 nm).

of collagen type I are formed. On the other hand we were unable to prove systematic occurrence of CNBr peptides with a higher molecular mass than about 60 000 (we have seen it in two cases only, data not shown). Because, pentosidine which is typically a cross-linking aminoacid should involve two polypeptide chains, our results strongly suggest that in vivo glycation occurs preferably at two places in the mole-

Table 2
Luminescence parameters of different products arising by post-translational modifications of amino groups

Products	Excitation (nm)	Emission (nm)	Reference
Products arising from interaction with reducing sugars			
Pentosidine	335 (328)	385 (378)	[19,20]
Glycation related products	335 (328)	385 (378)	[21,20]
Advanced glycation end products	370	440	[22,23]
Fluorophore LM-1	360	460	[24]
3-Deoxyglucosone (glucose)/butylamine adduct FL-C	370	455	[25]
Lipid peroxidation related products			
Malondialdehyde/collagen adduct	390	460	[5]
Hydroxynonenal/collagen adduct	356	460	[5]
4,5(*E*)-epoxy-2(*E*)-decenal/butylamine adduct	350	430–440	[26]
4,5(*E*)-epoxy-2(*E*)-decenal/lysine adduct	350	~395	[26]
4,5(*E*)-epoxy-2(*E*)-heptenal/butylamine adduct	360	~450	[26]
4,5(*E*)-epoxy-2(*E*)-heptenal/lysine adduct	350	430–450	[26]
13-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid/butylamine adduct	350	440	[26]
13-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid/lysine adduct	350	~450	[26]

cule, involving the α_1(I)CB_6 and probably α_2(I)$CB_{3,5}$ peptide. For sure the fluorescent compounds seem to have nothing in common with the CNBr resistant portion of the collagen molecule. On the other hand, however, the well documented polymerization of the parent collagen α-chains is likely to proceed through one big peptide (like those specified above) with another small peptide attached. In a preliminary quantitation (by peak area) of the CNBr peptides released, it was indeed observed that in preparations obtained from 18 months old animals the peptide α_2(I)CB_2 (rel. mol. mass 2700) vanished nearly completely and at least one other (α_1(I)CB_4, rel. mol. mass 4400) was considerably decreased. On the other hand the area percentage of α_1(I)CB_6 (rel. mol. mass 17400) increased from 13.92% to 17.09% between the preparations obtained from 12 and 18 months old animals. Similarly the peak of α_2(I)$CB_{3,5}$ showed a 2.71% increase. Further investigation is, however, needed to obtain a complete picture of collagen cross-linking through glycation products under different physiological conditions and with advancing age.

Reiser et al. [28] investigated preferential sites of glycation in α_1(I)CB_3 and α_2CB_{3-5} peptides. In case of the α_1(I)CB_3 peptide Lys-434 was prefered (from the five lysine residues present) and in the case of α_2CB_{3-5} peptide three residues (namely Lys-453, Lys-479 and Lys-924) from a total of 18 lysine residues and one hydroxylysine residue contained more than 80% of the glucose adducts in the peptide. These preferential glycation sites were highly conserved with aging. In our results some fluorophores were observable in fraction 2 containing the α_1(I)CB_4, α_1(III)CB_2, α_1(III)CB_6, α_1(I)CB_3 and α_1(III)CB_4 peptides. On the contrary to the non-pentosidine fluorescence present in peptides α_1(I)CB_6 and α_1(I)$CB_{3,5}$ the fluorescence exhibited 420 nm emission when excited at 375 nm which is slightly shorter than the parameters of fluorescence of advanced glycation products (375/440 nm).

From the analytical point of view we present here a simple procedure that allows to assign pentosidine and non-pentosidine glycation products to particular regions of collagen α-chains.

5. Conclusion

In this paper we present a reversed phase chromatographic procedure, that exploits the properties of a C_4 modified macroreticular sorbent for the separation of CNBr peptides released from tissue collagen. Individual peptides were tentatively identified by co-chromatography of the respective isolates the nature of which was further confirmed by routine polyacrylamide gel electrophoresis. Because, on-line detection of the fluorescent elements was beyond the possibilities of the fluorescence detector used (and to our best

knowledge no on-line fluorescence detector allowing the detection and scanning of the minute amounts of fluorescent entities in collagen is currently available on the market), we have applied an off-line approach; we succeeded in optimizing the separation conditions in such a way that we were able to accumulate two peptides, α_1(I)CB$_6$ and α_2(I)CB$_{3,5}$ which contained both the pentosidine and non-pentosidine fluorescence. However, the non-pentosidine fluorescence exhibited a maximum at 420 nm which is less than the usually reported 340 nm. On the other hand a complex peak (fraction No 2 in our procedure) exhibited the presence of a fluorophore the parameters of which are in full agreement with the so called advanced glycation end products (375/440 nm). It was confirmed that in reversed phase separations, the elution of individual CNBr peptides occurs in a sequence following the increasing hydrophobicity of the solutes and, owing to the large internal homogenity of collagen constitution α-chains, also following the increasing molecular mass of these peptides. No peptides larger than 63 000 rel. mol. mass units were observed in our preparations indicating that the pentosidine crosslinking reaction binds always a large and a small CNBr peptide of the constituting collagen α-chains.

Acknowledgements

This work was supported by the Grant Agency of the Czech Republic, Grant No. 303/94/1725.

References

[1] K.M. Reiser, Proc. Soc. Exp. Biol. Med. 196 (1991) 17.
[2] V.M. Monnier, J. Gerontol. 45 (1990) 105B.
[3] V.M. Monnier, M. Glomb, A. Elgawish, D.R. Sell, Diabetes 45 (1996) S67.
[4] D.R. Sell, R.H. Nagaraj, S.K. Grandhee, P. Odetti, A. Lapolla, J. Fogarty, V.M. Monnier, Diabetes/Metabolism Rev. 7 (1991) 239.
[5] P. Odetti, M.A. Pronzato, G. Noberasco, L. Cosso, N. Traverso, D. Cottalasso, M.M. Marinari, Lab. Invest. 70 (1994) 61.
[6] T. Vo-Dinh, Anal. Chem. 50 (1978) 396.
[7] J. Novotná, Z. Deyl, I. Mikšík, J. Chromatogr. B 681 (1996) 77.
[8] Z. Deyl, J. Novotná, I. Mikšík, D. Jelínková, M. Uhrová, M. Suchánek, J. Chromatogr. B 689 (1997) 481.
[9] I. Mikšík, J. Novotná, M. Uhrová, D. Jelínková, Z. Deyl, J. Chromatogr. A 772 (1997) 213.
[10] P.P. Fietzek, K. Kühn, Int. Rev. Connect. Tissue Res. 7 (1976) 1.
[11] M. van der Rest, H.P.J. Bennet, S. Solomon, F.H. Glorieux, Biochem. J. 191 (1980) 253.
[12] M. van der Rest, P.P. Fietzek, Eur. J. Biochem 125 (1982) 491.
[13] E.J. Miller, R.K. Rhodes, D.K. Furuto, Collagen Rel. Res. 3 (1983) 79.
[14] E.J. Miller, D.K. Furuto, A.J. Narkates, Anal. Biochem 196 (1991) 54.
[15] J.P. Borel, A. Randoux, in L. Robert, M. Moczar, E. Moczar (Eds.), Methods in Connective Tissue Research, Frontiers of Matrix Biology, vol. 10, Karger, Basel, 1985, p. 1.
[16] P.G. Scott, A. veis, Connect. Tiss. Res. 4 (1976) 107.
[17] E.J. Miller, E.H. Epstein, K.A. Piez, Biochem. Biophys. Res. Commun. 42 (1971) 1024.
[18] D.G. Dyer, J.A. Blackledge, S.R. Thorpe, J.W. Baynes, J. Biol. Chem. 266 (1991) 11654.
[19] D.R. Sell, V.M. Monnier, J. Biol. Chem. 264 (1989) 21597.
[20] D.G. Dyer, J.A. Dunn, S.R. Thorpe, K.E. Bailie, T.J. Lyons, D.R. McCance, J.W. Baynes, J. Clin. Invest. 91 (1993) 2463.
[21] R.H. Nagaraj, M. Prabhakaram, B.J. Ortwerth, V.M. Monnier, Diabetes 43 (1994) 580.
[22] I. Mikšík, Z. Deyl, J. Gerontol. 46 (1991) B111.
[23] J.T. Wu, M.-C. Tu, P. Zhung, J. Clin. Lab. Anal. 10 (1996) 21.
[24] D.R. Sell, V.M. Monnier, Connect. Tiss. Res. 19 (1989) 77.
[25] F. Hayase, H. Hinuma, M. Asano, H. Kato, S. Arai, Biosci. Biotech. Biochem. 58 (1994) 1936.
[26] R. Zamora, F.J. Hidalgo, Biochim. Biophys. Acta 1258 (1995) 319.
[27] S. Tanaka, G. Avigad, E.F. Eikenberry, B. Brodsky, J. Biol. Chem. 263 (1988) 17650.
[28] K.M. Reiser, M.A. Amigable, J.A. Last, J. Biol. Chem. 267 (1992) 24207.