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Multicomponent analysis by off-line combination of synchronous fluorescence spectroscopy[1] and capillary electrophoresis of collagen glycation adducts[2]

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Abstract

Capillary electrophoresis separation and synchronous fluorescence spectral detection was used off-line to reveal the nature of fluorescent adducts formed in vivo in the collagen molecule and their distribution in the molecule. It was shown that by using the $\Delta\lambda$ in the area of the Stokes shift for the analyzed entities (~10 nm for pentosidine, 4,5(E)-epoxy-2(E)-heptenal and 4,5(E)-epoxy-2(E)-decenal lysine adducts) a distinct profile of spectral bands can be obtained allowing for differentiation of the several entities involved. In combination with capillary electrophoretic separation of the CNBr peptides the location of individual adducts was possible: while pentosidine (and, perhaps, pentosidine related compounds K_1–K_4) is found in the large α_1(I)CB_6 and α_2(I)$CB_{3,5}$ peptides along with a complete set of the other fluorescent adducts, low-molecular-mass peptides originating from the terminal region of the molecule are devoid of any fluorescence. All other parts of the molecule possess synchronous fluorescence profiles corresponding to the intact molecule except that they are devoid of pentosidine. The results indicate random distribution of fluorescent adducts in the collagen molecule and, in a broader context, indicate the usefulness of multicomponent analysis by means of combining synchronous luminescence spectra and capillary electrophoresis.

Keywords: Fluorescence detection; Detection, electrophoresis; Collagen; Peptides; Proteins

1. Introduction

While in young animals skin or tendon collagen is rather readily solubilized by acid buffers, with age it becomes less soluble even under aggressive conditions such as pepsin or cyanogen bromide digestion [1–4]. Progressive insolubility is caused by time dependent formation of covalent cross-links between collagen molecules in fibrils. The formation of the best characterized cross-links such as pyridinoline or hydroxylysinonorleucine is enzymatically regulated; these cross-links are situated in the telopeptide region of the molecule and molecules joined with such cross-links can be solubilized by pepsin which splits the short telopeptide extensions, or by cyanogen bromide which cleaves the main polypeptide chains of fibre forming collagens.

In addition to these ''physiological'' cross-links, evidence has accumulated during the past decade [5] that glycation or reactions with reactive products of lipid metabolism [6] is the basis for the formation of additional cross-links, the formation of which is not

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[1]This technique is also referred to as synchronous acquisition/scanning mode.

[2]These results were partly presented in The First International Cyber Congress on Analytical BioSciences, Aug 21–Oct 20, 1997.

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subject to any enzymatic regulation and reflects strictly the chemical reactivity of the functional groups involved, namely the free ε-amino groups of lysine and the aldehydic moiety of the reactive metabolites. In this process the aldehyde (open chain) glucose or the aldehydic products of lipid metabolism (stemming from unsaturated fatty acids) condense spontaneously with the free amino groups of proteins (both ε-amino group of lysine and the free N-terminal amino group of the protein) (for review see Ref. [7]). Though in this communication we limit ourselves to the study of collagen as a model protein, it has to be emphasized that the nonenzymatic modifications can be found in a number of other proteins; the first to be mentioned is, perhaps, haemoglobin (as a matter of fact glycated haemoglobin is routinely assayed in diabetic patients), but lens proteins, serum albumin, lipoproteins and others were shown in the literature to undergo this category of modifications as well.

Glycated collagens (both in vivo and in vitro) exhibit decreased solubility, increased fluorescence and changes in mechanical properties consistent with aging [8–11]. Increased fluorescence of the modified proteins is the most marked feature of the nonenzymatically modified collagens. While in vitro glucose–collagen adduct formation is rather nonspecific, we were able to document that in vivo only specific domains of the collagen molecule are predisposed for this reaction [12].

It has been mentioned already that collagen fluorescence can be used to reveal the extent of post-translational nonenzymatic modification (glycation) of the protein. Routinely, the formation of fluorescent products (advanced glycation end products) is monitored at 370/440 nm; when recording the emission spectrum a very broad fluorescence band is observed. On the other hand this broad peak can be easily distinguished from the so far only well characterized fluorescent product, pentosidine with the fluorescence parameters 330/385 nm. The situation is further complicated by the fact that pyridinoline, a ''physiological'' cross-link stemming from a condensation reaction between δ-semialdehyde of α-aminoadipic acid (arising from enzymatically regulated lysine oxidation) and an unmodified lysine residue is also a fluorescent entity. The other complication is based on the fact that the broad luminescence peak observed at 370/440 nm is unlikely to represent a single chemical compound. As shown in Table 1 there is a number of products that may be expected to participate in the formation of the broad emission peak, arising from both glycation reactions and reactions with products of lipid metabolism, all of which are unlikely to be distinguished by simple measuring of fluorescence spectra, though reports using measurement at different wavelengths can be traced in the literature [13]. Even the well distinguished peak of pentosidine is likely to be composed from more than a single compound (pentosidine isomers, see Ref. [14]). A further complication stems from the fact that not only the aldehydic form of glucose can participate in the fluorescent product formation but also glucuronamides can be involved. Finally the already formed glucose adducts can be further modified by the reaction of another glucose molecule to add to the complexity of arising fluorescent products.

Several strategies can be applied to analyze such a complex mixture. The first obvious approach is to distinguish between hydrolytically stable and unstable adducts; alternatively the armamentarium of chromatographic and/or electromigration methods can be made use of (for review see Ref. [15]).

Table 1
Spectral parameters of fluorescent products in collagen and tyrosine

	λ_{exc} (nm)	λ_{em} (nm)
Pentosidine	335	385
	(328)	(378)
K_1–K_4 adducts (pentosidine related)	340	390
Malondialdehyde adduct	385	457
Glucosone intermediates	373	448
PEN K_2 (LM1) adduct[a]	360	440
EH[b] lysine adduct	350	390
	380	430
ED[b] lysine adduct	350	410
	400	430
Glucuronic acid adducts	350	410
Pyridinoline	295	400
	(270)	(389)
Tyrosine	280	330

[a] PEN K_2 (LM1) adduct is a trivial name for a so far poorly characterized adduct.

[b] ED and EH stand for 4,5(E)-epoxy-2(E)-decenal and 4,5(E)-epoxy-2(E)-heptenal, respectively.

However, none of the separation technologies applied so far offered satisfactory results. Moreover direct on-line measurement (HPLC or capillary electrophoresis hyphenation) of fluorescence spectra is precluded by low detector sensitivities of the commercially available devices.

On the other hand it is absolutely clear that further investigation of the fluorescent adducts requires new dimensions to be included. In this work, an attempt is made to evaluate the presence of different fluorescent entities by applying the so-called synchronous excitation technique (synchronous scanning mode). The idea of synchronous excitation luminescence was first suggested by Lloyd [16] and further theoretically developed by Vo Dinh [17]. Although forensic researchers have often employed this technique in an empirical manner [18,19], the effective use of this technique has been limited so far. Apparently the lack of specific information and methodology made it difficult for analysts to exploit fully the possibilities offered by this approach. Practical applications have been limited so far only to providing fingerprints of complex samples such as crude oils of various origins [20]. The aims of this paper are to develop a simple method offering trace analysis of nonenzymatically generated fluorescent entities and to investigate how the technique of synchronous excitation can be applied to obtain specific information of both analytical and biological interest.

2. Principle and theory

A luminescent compound excited a fixed wavelength λ' yields a distribution pattern $E_M(\lambda)$ referred to as an emission spectrum. The luminescence signal at emission wavelength λ designed as I_λ depends on the value E_M and is proportional to the spectral radiance of luminescence $R_{\lambda'}$ emitted by the compound excited at λ':

$$I(\lambda) = kR_{\lambda'}E_M(\lambda) \quad (1)$$

where k is a constant.

Considering the validity of the Lambert law for dilute solutions, $R_{\lambda'}$ can be expressed as

$$R_{\lambda'} = k'Y_L(\lambda')I_0(\lambda')\varepsilon(\lambda')cd \quad (2)$$

where Y_L is the luminescence quantum yield, I_0 the exciting light intensity, ε the molar extinction coefficient, c the concentration of the analyte, d the thickness of the sample and k' an experimentally determined constant.

The product $Y_L I_0 \varepsilon$ depends exclusively on λ' and is proportional to the excitation spectrum $E_X(\lambda')$ (which is determined by scanning the excitation wavelength):

$$E_X(\lambda') = k''Y_L(\lambda')I_0(\lambda')\varepsilon(\lambda') \quad (3)$$

where k'' is a constant.

The synchronous luminescence intensity (I_s) can be expressed by combining Eqs. (1)–(3) as a functions of λ and λ':

$$I_s(\lambda', \lambda) = KcdE_X(\lambda')E_M(\lambda) \quad (4)$$

with $K = kk'k''^{-1}$.

With the synchronous technique

$$\lambda - \lambda' = \Delta\lambda, \text{ i.e., } \lambda = \lambda' + \Delta\lambda \quad (5)$$

Consequently we can write:

$$I_s = KcdE_X(\lambda - \Delta\lambda)E_M(\lambda) \quad (6)$$

which is the fundamental equation of synchronous spectrometry.

This equation brings about not only an improvement in sensitivity (by involving two functions instead of only one in the conventional luminescence method), but mainly adds to selectivity by introducing the parameter $\Delta\lambda$ which can be experimentally selected.

The synchronous signal has (as demonstrated by Vo Dinh [17]) a capability of simplifying the spectra; this unique feature is the consequence of the restrictive character of the two practically mirror symmetric functions $E_M(\lambda)$ and $E_X(\lambda')$; $E_M(\lambda)$ is limited on the short wavelength side while $E_X(\lambda')$ is limited on the long wavelength side of the spectrum. Consequently the synchronous signal which represents the product of $E_M(\lambda)$ and $E_X(\lambda')$ must necessarily have a limited spectral band width (for completeness it is necessary to remember that there is always a small wavelength difference of about 3 nm between the peaks of the 0–0 band in the excitation and emission spectra called the Stokes shift).

Diffuse fluorescence peaks are the main drawback

of measuring fluorescence spectra. The reason is twofold: first, the emission peaks are intrinsically broad, second in complex mixtures (as, e.g., represented by fluorescent glycation products) severe overlapping of individual peaks occurs. The advantage of synchronous technique is that it is capable of minimizing the effects of these two sources of diffuseness. If either one of the two functions, $E_M(\lambda)$ or $E_X(\lambda')$ has a resolved structure in a given spectral range, a narrow peak is obtained, increasing thereby the chance of obtaining spectra with a resolved structure.

Another advantage of the synchronous technique is simplification of the complex quasilinear spectra. Namely a signal is observed only when $\Delta\lambda$ matches of the interval between one absorption and one emission band. If a selected $\Delta\lambda$ interval matches one unique pair of absorption and emission bands, the synchronous spectrum shows a single peak only.

In this way in multicomponent mixtures the spectra of individual components will be simplified and interference resulting from spectral superposition will be considerably reduced. More deep analysis shows that characteristic intense peaks are in the synchronous technique increased more strongly and the interfering effect of weak bands is reduced even in situations where by coincidence two (or several bands) in the emission and excitation spectra show identical $\Delta\lambda$ intervals.

From what has been said the magnitude of $\Delta\lambda$ is critical. In general the simplicity of the spectrum increases as $\Delta\lambda$ is decreased.

3. Materials and methods

3.1. Capillary electrophoresis

All separations were done on a SpectraPhoresis 500 TSP instrument, obtained from Watrex (Prague, Czech Republic) using PC 1000 software version 2.6. The capillary used was 70 cm long (63 cm to the detector)×75 μm I.D., uncoated, run at 15 kV if not specified otherwise (yielding 30–70 μA current depending on temperature). Separations were done with 20–100 m*M* phosphate buffers either purchased from Bio-Rad (Richmond, CA, USA, catalogue No. 148-5010 with polymeric modifier) or laboratory prepared. Concerning the separation of marker peptides only small differences were seen between the two types of electrolytes used. Runs at 25°C and 50°C were performed to reveal the influence of denaturation of the large peptides present in the mixture and buffer concentration (20–100 m*M* buffers were used as specified in the text). Depending on the protein content in the sample 1–8 s hydrodynamic sample application (vacuum 10.3 kPa) was used. UV absorbance was detected at 200 nm.

3.2. Preparation of CNBr peptides

CNBr peptides were separated from collagen type I (Sigma, St. Louis, MO, USA). These samples were treated with CNBr without chain separation in 70% formic acid as described by Scott and Veis [21]. This procedure yielded some proportion of uncleaved peptides which emerged closely to the joint peak of α_1(I)CB_7 and α_1(I)CB_8; therefore in some experiments the reaction time was increased up to 24 h.

Peptide preparation directly from tissue slices followed generally the same procedure following incubation of the tissues in 0.2 *M* ammonium hydrogencarbonate pH 7.0 containing 25% β-mercaptoethanol to reduce oxidized methionyl residues and thereby to enhance CNBr cleavage [22]. Lyophilized CNBr peptide preparations from tissue samples were redissolved in Milli-Q water, centrifuged and lyophilized again, before analysis they were reconstituted either in formate buffer, 1% acetic acid or water at a concentration 400 μg/ml.

3.3. Isolation of marker peptides and proof of their identity

Isolation for marker peptides was done as described previously [23] using HPLC and 12.8–44.8% acetonitrile gradient (all sorbents made 10 m*M* with respect to trifluorobutyric acid) with Vydac TP 201 column (Separation Group, Hesperia, CA, USA; 250×4.6 mm, 10 μm peptide size, 300 Å pore size). Flow-rate applied was 1 ml/min.

3.4. Fluorescent spectra measurement

The respective samples (peptide fractions) were collected after designated periods of time in the

cathodic vials respecting the time interval needed for transporting the analytes from the detector window to the cathodic compartment; usually in five fractions as described in Section 4. Material from 20 runs was collected; UV absorbance at 200 nm was determined and the solution was adjusted to 10 arbitrary units (a.u.) in a 1 cm cuvette (this was done to obtain comparable peptide concentrations in different samples). Fluorescence spectra were obtained on a LS-50B luminescence spectrometer (Perkin-Elmer, Beaconsfield, UK). Fluorescence emission was observed at right angles to the incident radiation which impinged upon samples maintained at 25°C in quartz cells of 1 cm path. A slide width of 5 nm was used.

3.5. Isolation of fluorescent marker compounds

Pentosidine was prepared according to Mikšík et al. [24], 4,5(E)-epoxy-2(E)-decenal (ED) and 4,5(E)-epoxy-2(E)-heptenal (EH) adducts were laboratory prepared according to the procedure described by Zamora and Hidalgo [6].

For marker studies pentosidine was used as such; the ED and EH lysine products were allowed to react with lysine in the following way (for detailed procedure see Zamora and Hidalgo [6]). A solution of either ED or EH containing 0.12 mmol in 1.0 ml was dissolved in the respective amount of 0.3 *M* sodium lysine monohydrochloride (47.6 mg per ml) and stirred overnight at room temperature. Identity of the ED and EH was based on the comparison of ^{1}H NMR spectra obtained with literary data [6].

Malondialdehyde adduct was obtained by incubating pentalysine (Sigma, Prague, Czech Republic, catalogue No. L 9151) with 0.12 mmol solution of malondialdehyde (Lachema, Brno, Czech Republic) in 0.3 *M* sodium phosphate buffer at room temperature overnight upon sieving.

3.6. Chemicals

All chemicals used were either of analytical grade or highest available purity. Formic acid (88%) was obtained from Lachema, collagen type I, pepsin (activity 1500–3500 U/mg protein), 2-mercaptoethanol, CNBr, guanidine hydrochloride and ammonium hydrogencarbonate 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).

4. Results

Luminescence spectrum of collagen preparations usually reveals two broad bands at namely at 340/385 (Fig. 1) and 370/440 nm. The former corresponds to pentosidine and pentosidine related products (K_1–K_4), the latter represents a complex band corresponding to several chemical entities (for fluorescent compounds in collagen see Table 1). The complexity of the band emitting at ~440 nm can be partly resolved by running the spectra at different

Fig. 1. Luminescence spectrum of rat skin collagen obtained at 340 nm excitation.

excitation wavelengths, namely at 335 nm for pyridinoline, 350 nm for lipid derived lysine adducts and at 370–375 nm for what is tentatively called advanced glycation end products (Fig. 2a–c). With tendon collagen usually better spectra are obtained compared to skin preparations. Accepting the idea of slow posttranslational modification of proteins it is not surprising that with collagen preparations obtained from old rats the spectral bands are less distinct, a phenomenon that can be ascribed to both a higher concentration of the different adducts in the collagen structure and/or to the fact that more fluorescent entities are involved. In any case it is clear that simple measurement of luminescence spectra is not a method with sufficient resolving power to distinguish between the different adducts involved. Consequently two ways to get some idea about the complexity of the arising luminescence products are at hand. First to use synchronous fluorescence spectra and, second, to combine synchronous fluorescence detection with some separation technique (typically capillary zone electrophoresis). These two tasks had to be solved separately as on-line synchronous luminescence detection is not possible owing to the lack of adequate equipment.

While the conditions for the very separation both by capillary electrophoresis and reversed-phase chromatography have been reported in our previous communications [23] there is no reference to conditions of the synchronous luminescence detection: basically either the $\Delta\lambda$ used should match the interval between one absorption and one emission band [20] or should exploit the Stokes shift $\delta\lambda_S$ (which for a given environment is a constant). Fig. 3 reveals synchronous fluorescence spectra at different $\Delta\lambda$ for a commercial bovine Achilles tendon preparation. While with, e.g., polyaromatic hydrocarbons it is known that in most cases the Stokes shift is about 3 nm, with the fluorescent entities this value has not been reported in the literature so far. From Fig. 3 it further follows that using the interval matching the absorption and emission bands does not improve the situation too much; the reason apparently is that the differences in spectral parameters ($\Delta\lambda$) of individual presumptive chemical species are very close, most of them being in the range of ~50 nm. On the other hand, however, the Stokes shift value for our pentosidine preparation was shown to be less than 10 nm and a similar value was obtained for the EH and ED adducts. By comparing the synchronous fluorescence spectrum with synchronous spectra of pure compounds available to us it was possible to ascribe at least some bands in the luminescence profile to individual compounds. There is a distinct band corresponding to pentosidine, which, however, comprises possibly also the so called pentosidine related products (K_1–K_4, reported luminescence parameters 340/390 nm), EH and ED lysine revealed a single band in the range of 430 nm; by incubating a collagen preparation with malondialdehyde an increase of the peak in the 457 nm region was observed; this peak was tentatively ascribed to the malondialdehyde adduct.

In the next step of our experiments we attempted to run the synchronous luminescence spectra of individual sections of the collagen molecule; fragmentation was done by CNBr cleavage and no difference was seen in the profile of the spectral bands of the whole molecule and CNBr peptide mixture (data not shown).

The whole profile was divided into five sections as shown in Fig. 4, the peptide mixture corresponding to a particular section was accumulated in the cathodic vial and after the adjustment to equal concentration the synchronous luminescence spectra were run from each fraction. In concert with our previous communication it was demonstrated that the pentosidine fluorescence was present in the α_1(I)CB_6 and α_2(I)$CB_{3,5}$ peptides only. The set of peptides emerging at the beginning of the electropherogram [α_1(I)CB_2, α_1(I)CB_4, α_2(I)CB_1 and α_1(I)CB_5] was practically devoid of any fluorescence. The second set of peptides [α_1(I)CB_5, α_2(I)CB_2, α_1(I)CB_3] comprised all peaks seen in the total profile except the peak of pentosidine. The synchronous luminescence profile of the α_1(I)CB_6 peptide (Fig. 5) was practically undistinguishable from the profile of the whole molecule, perhaps with some preponderance for pentosidine (a larger peak). The same was observed with the α_2(I)$CB_{3,5}$ peak. The remaining peaks possess all the luminescence bands except the band of pentosidine.

5. Discussion

Evaluating of synchronous luminescence spectra

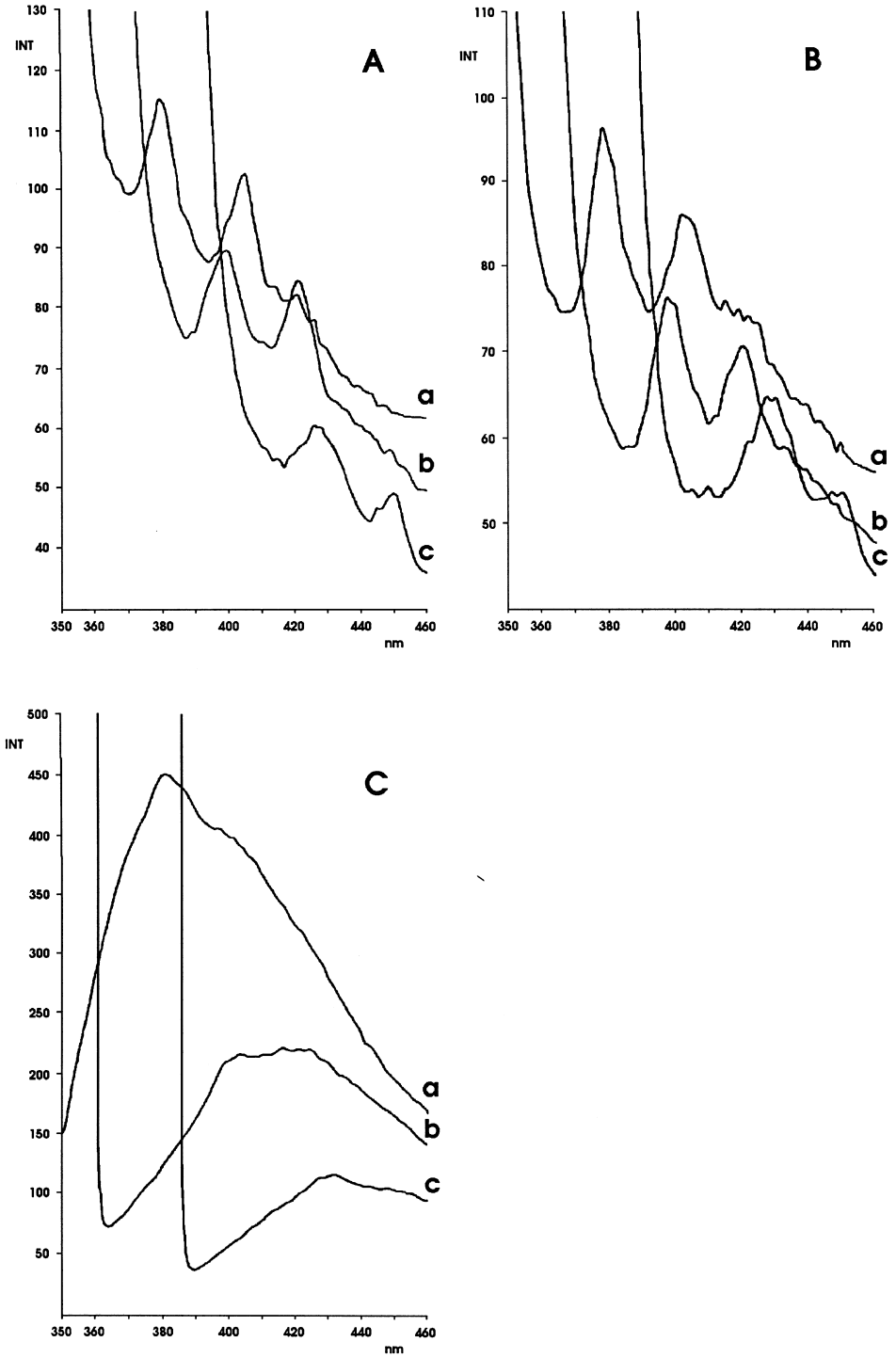

Fig. 2. Luminescence spectra of rat tail tendon collagen run at difference excitation wavelengths (a, 335 nm; b, 350 nm; c, 375 nm; A, 6; B, 12 and C, 18 months old rats).

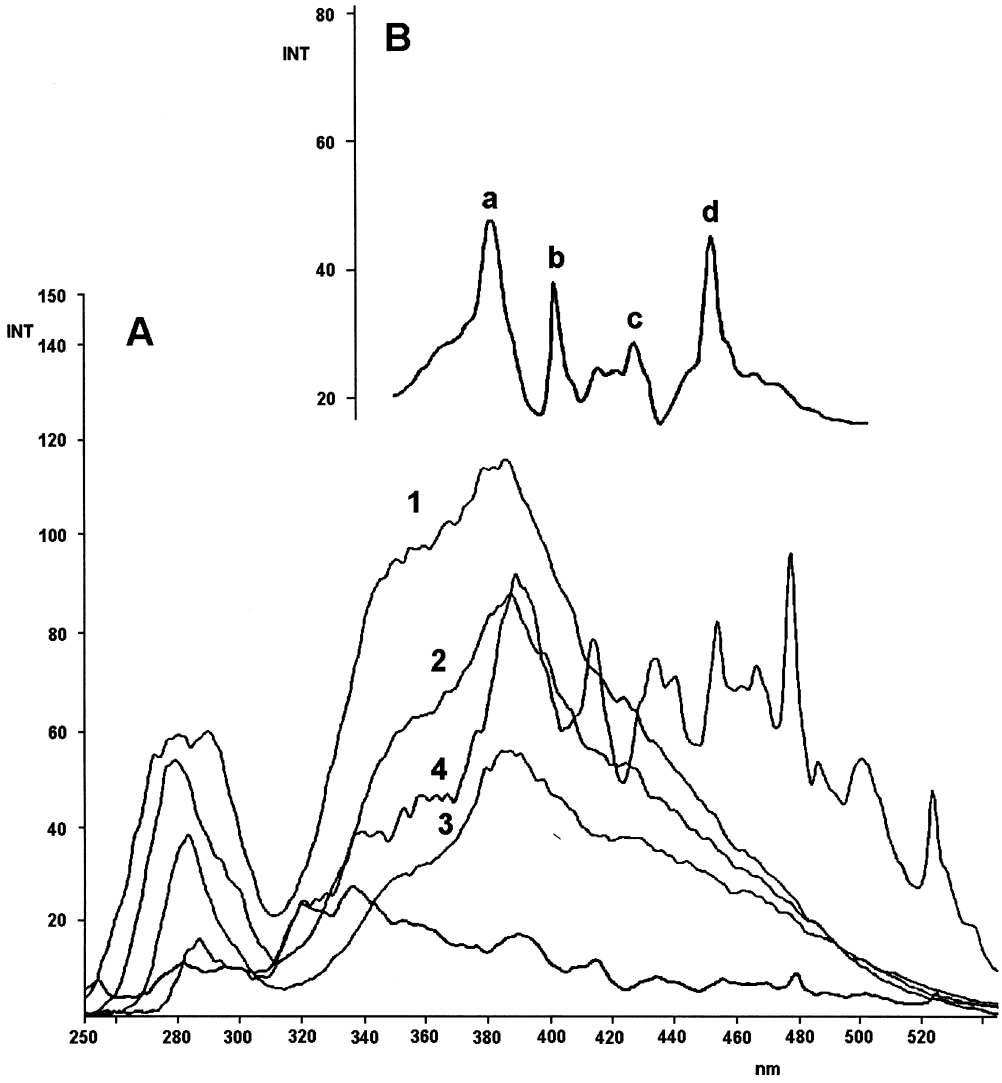

Fig. 3. Synchronous fluorescence spectra of commercial type I collagen preparation at different $\Delta\lambda$ (1, 40 nm; 2, 30 nm; 3, 20 nm and 4, 10 nm) (A) and comparison with a model mixture of standards (a, pentosidine; b, pyridinoline; c, 4,5(E)-epoxy-2(E)-decenal and 4,5(E)-epoxy-2(E)-heptenal adducts; d, malondialdehyde adduct) (B) (identical *x*-axis).

of collagen fractions accumulated in a capillary electrophoresis step revealed useful data for obtaining an idea about which types of adducts are present in which part of the molecule. From the separation point of view it appears that combination of synchronous fluorescence profiles with either capillary electrophoresis (and/or liquid column chromatography) appears a powerful tool for analysing complex mixtures of fluorescent compounds.

Running the spectra at $\Delta\lambda$ matching the interval between one absorption and one emission band did not offer the expected result. However, using $\Delta\lambda$ in the range of the Stokes shift of pentosidine, EH and ED lysine adducts, yielded a set of fluorescence bands out of which four bands could be tentatively ascribed to pentosidine (and pentosidine related products), EH and ED lysine and malondialdehyde adducts. From the spectra presented it also emerges

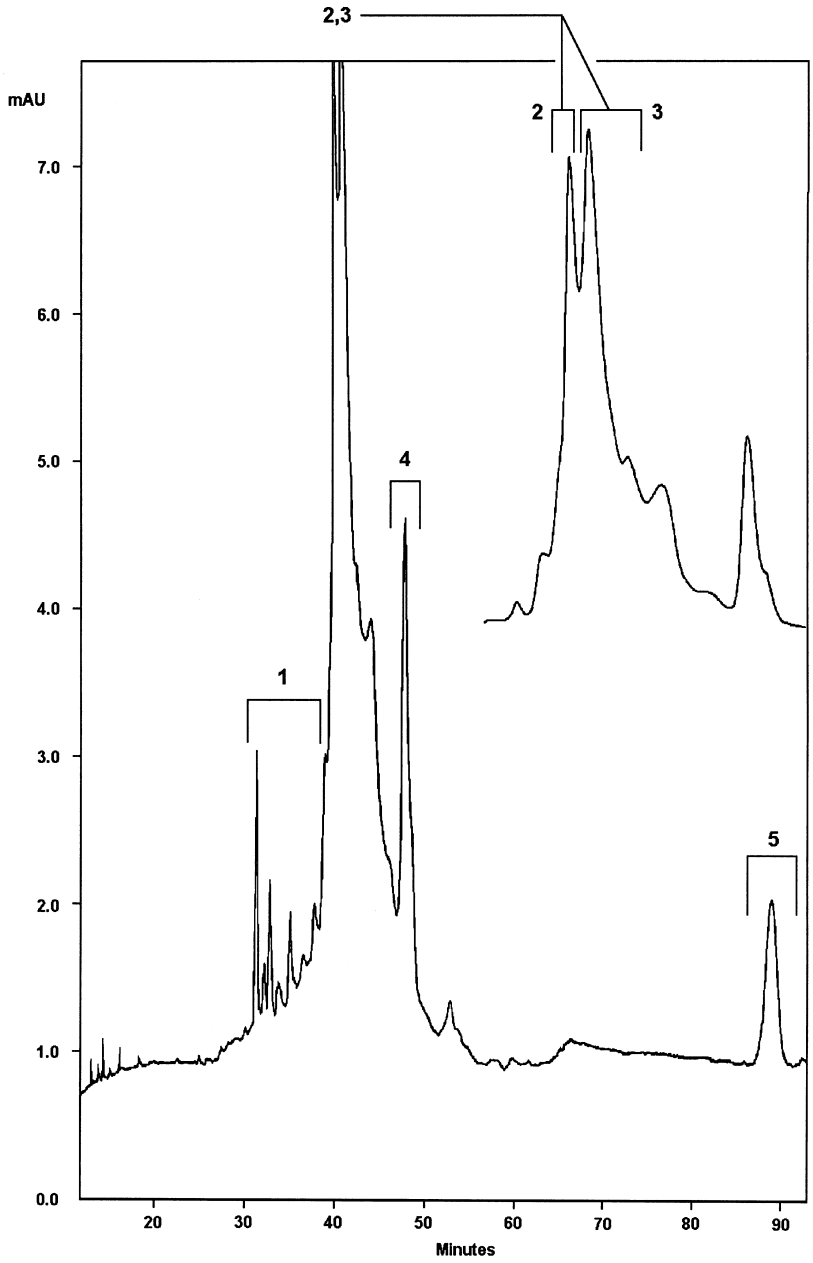

Fig. 4. Capillary zone electrophoresis of rat skin collagen peptides; animals aged 18 months; horizontal bars indicate fractions collected and accumulated. For synchronous fluorescence spectra of fractions containing or not containing pentosidine see Fig. 5. Peptides in individual fractions: (1) α_1(I)CB$_2$, α_1(I)CB$_4$, α_2(I)CB$_1$, α_1(III)CB$_2$, α_1(I)CB$_5$, α_2(I)CB$_2$, α_1(I)CB$_3$; (2) α_1(I)CB$_6$; (3) α_1(I)CB$_7$, α_1(I)CB$_8$, incomplete cleavage products; (4) α_2(I)CB$_4$; (5) α_2(I)CB$_{3,5}$, [α_1(III)CB$_9$]$_3$. Inset: Enlarged section of the electropherogram around the α_1(I)CB$_6$ peptide. Bars indicate timing of fraction collection. The insert shows the enlarged section of the electropherogram between 37 and 47 min.

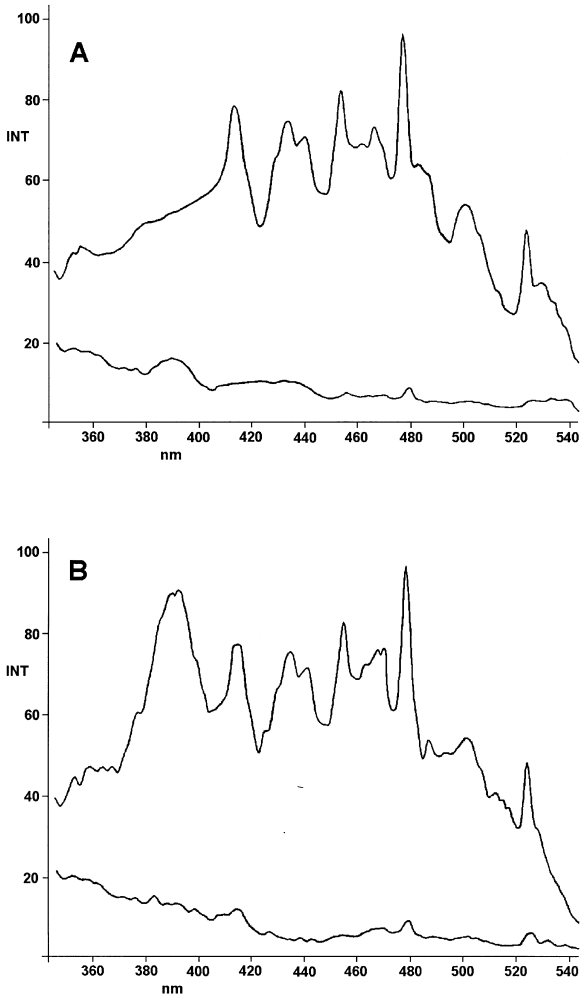

Fig. 5. Typical synchronous scans ($\Delta\lambda$ 10 nm) of fractions obtained by capillary electrophoresis. (A) Fraction No 3 (Fig. 4) containing α_1(I)CB_7 and α_1(I)CB_8 peptides along with incomplete cleavage products; (B) Fraction No 2 (Fig. 4) containing α_1(I)CB_6 peptide. Synchronous spectra of peptide α_2(I)$CB_{3,5}$ indicated the presence of pentosidine in a similar way as in scan B; Fraction 1 and 4 were analogous to scan seen in plate A. Lower line in each plate refers to blank.

that in the collagen preparations there were also entities exhibiting fluorescence bands at higher wavelengths than the malondialdehyde–lysine adduct.

Another important fact observed was that CNBr released peptides and the parent molecule gave (at the corresponding concentrations) a virtually identical set of fluorescence bands. This allowed us to solubilize tissue collagen by the CNBr technique from tissues; were it not so the fluorescence spectra would have to be limited to the hydrolytically stable adducts only.

In concert with our previous report [12] we have found that the small molecular mass peptides are devoid of any fluorescence adducts; pentosidine occurs in the α_1(I)CB_4 and α_2(I)$CB_{3,5}$ peptide only; all the other peptides exhibited similar synchronous fluorescence profiles which suggests a rather random distribution of the arising adducts along the molecule. A similar conclusion was drawn by Tanaka et al. [25] from their studies of collagen glycation in vitro based, however, on a completely different analytical approach. It was emphasized that in particular the adducts which are of cross-linking nature can be found at several places in the collagen molecule mediating both inter- and intramolecular cross-linking of collagen polypeptide chains.

6. Conclusions

An off-line combination of synchronous spectrofluorometry with capillary electrophoresis has been used for separating sections of the collagen molecule and revealing the presence of a number of fluorescent adducts, pentosidine and other fluorophores (related to age pigments). Collagen fragmentations were done by CNBr cleavage and it was demonstrated that while pentosidine can be found in the C-terminal region only, all the other fluorescent compounds are randomly distributed along the polypeptide chain. It was also demonstrated that the combination of synchronous spectrometry with a separation technique (capillary electrophoresis) represents a powerful tool for analyzing complex mixtures of luminescent entities and, if bound to the polypeptide chain, also for locating the luminescent adducts to a specified section of the collagen molecule.

Acknowledgements

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