

## FLUORESCENCE OF COLLAGEN – PROPERTIES OF TYROSINE RESIDUES AND ANOTHER FLUORESCENT ELEMENT IN CALF SKIN COLLAGEN

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### 1. Introduction

Recent studies have revealed two types of ultra-violet fluorescence in collagen [1–3]. The first type with fluorescence parameters of 275 nm/315 nm is due to tyrosine residues; the nature of the compound responsible for the “long-wave” UV fluorescence (345 nm/440 nm) remains obscure [1, 2]. The relationship between collagen cross-linking and long-wave UV fluorescence have been considered by LaBella and Thornhill [1]. These authors showed the accumulation with age both of long-wave fluorescence and cross-linking density. The only direct evidence associating cross-linking and such fluorescence in collagen is available from the irradiation studies of Fujimori [2]. Hoermann and Balekjian [3] studying the luminescence properties of individual  $\alpha$ -chains were able to show that collagen appears to constitute a special class of proteins with regard to its luminescence properties. According to their experiments, the element responsible for emission at 395 nm is not associated with the cross-link formation. Furthermore, dityrosine, which is one of the fluorescent cross-link sites in resilin and elastin, is absent in collagen [4] or present in trace amounts only [5]. Because the fluorescent element (or elements) is apparently important both in collagen structure and physiology, an attempt was made to localize these elements within the structure

and to get a somewhat deeper insight into the tyrosine fluorescence in collagen.

### 2. Material and methods

Calf skin acid soluble collagen prepared by the procedure described by Rubin et al. [6] was used within this study. Three different preparations were compared in order to eliminate artifacts from the preparative procedure and in two cases the resulting acid-soluble collagen was further purified by the procedure described by Jackson and Cleary [7] to eliminate fluorescence quenching which might be due to the sorption of smaller peptides as indicated by Francois et al. [8]. Pepsin and pronase treatment was done according to Drake et al. [9] and a Farrand spectrofluorimeter was used for measuring both excitation and luminescence spectra. All runs were done at room temperature. Except when stated otherwise all measurements were done in 0.05% acetic acid and the concentration of collagen was estimated according to the hydroxyproline content of the sample. Generally samples containing 10 to 60 micrograms of hydroxyproline per ml were suitable for fluorescence measurements. For quantitation all concentrations were adjusted to 21.5 micrograms of hydroxyproline per ml of the sample. A Technicon analyser was used for aminoacids analysis.

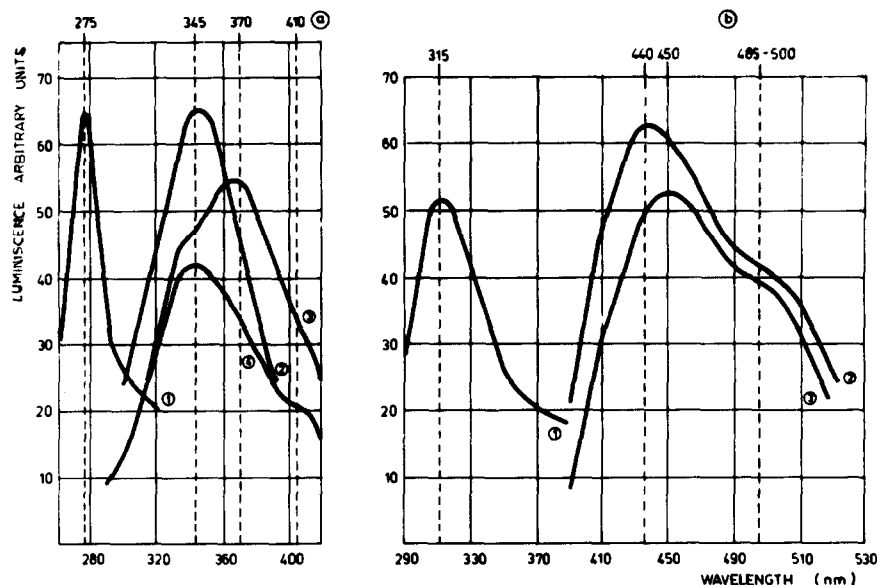


Fig. 1. Fluorescence action (a) and emission (b) spectra of acid soluble collagen (0.06% solution of ASC preparation in 0.05% acetic acid). (1) Tyrosine fluorescence. (2) Near UV fluorescence. (3) The same preparation alkalinized to pH 12. (4) Action spectra at the absorbancy wave-length 485 nm.

### 3. Results

In luminescence spectra two main maxima, namely 275 nm/315 nm (tyrosine) and 345 nm/440 nm were observed. In addition a broad plateau was observed on the emission curve between 485 nm and 500 nm, and an additional peak was identified in the excitation spectra at 410 nm (fig. 1). No differences were observed when acid-soluble collagen prepared according to Rubin et al. [6] and a purified preparation [7] were used, implying that adsorbed small molecular weight peptides, if present at all, do not influence the fluorescence properties of the structure. No fluorescence contribution of phenylalanine residues was found.

As far the intensity of the tyrosine fluorescence is concerned, no changes were recorded in native, heat denaturated and LiCl denaturated collagen. However, a significant decrease in fluorescence intensity was detected in urea-denaturated samples (table 1). If the tyrosine-derived fluorescence (emission at 315 nm) is related to the intensity of fluorescence of free tyrosine solution of equal concentration at pH 7, the relative tyrosine fluorescence

in collagen ( $R_{\text{tyr}}$ ) is 0.1; if the same preparation is treated with pepsin and about 1% of all aminoacids are split off, the  $R_{\text{tyr}}$  increases threefold, if the same sample is treated with pronase,  $R_{\text{tyr}}$  equals 0.5.

If pronase and pepsin treated collagen preparations are dissolved in 0.05% acetic acid containing 4 M LiCl, no difference in  $R_{\text{tyr}}$  was detected. Similarly in heated solutions the intensity of tyrosine fluorescence was changed.

In long-wave fluorescence (345 nm/440 nm) pepsin treatment results in an increase of fluorescence intensity of the sample by approximately one third, however, pronase treatment removes the long-wave fluorescence almost completely (fig. 2). In the dialysable fraction in pronase treated collagen, a new fluorescence 380 nm/440 nm was observed, while nothing but tyrosine fluorescence was detected in peptides split off by pepsin.

Acrylamide gel electrophoresis verified that after pronase treatment no further breakdown of the molecule occurred (only  $\alpha$ -fraction was detected). In agreement with previously reported data [9] 1% of aminoacids was split by pepsin and 5% by pronase; aminoacid analyses verified that out of the total 12

Table 1

Intensity of tyrosine fluorescence (275 nm/315 nm) of various collagen preparation. Hydroxyproline concentration of all samples 21.5 micrograms per 1 ml (collagen concentration  $1.10^{-5}$  M); spectra (except denaturated samples) run in 0.05% acetic acid. Molecular weight of collagen assumed  $3.10^5$ ; collagen-hydroxy proline ratio = 14. Fluorescence intensity of milimolar tyrosine solution 19.5 fluorescence units. Fluorescence expressed in arbitrary units.

Collagen sample*	Fluorescence intensity of a $10^{-5}$ M solution of collagen	No. of tyrosine residues per $3.10^5$ g of collagen preparation	Contribution of fluorescence intensity of one tyrosine residue in collagen ( $I_{coll}$ )	$R_{tyr} = \frac{I_{coll}}{I_{tyr}}$ **
Acid soluble collagen I	25.8	12	2.15	0.11
preparation (ASC) II	25.8	12	2.15	0.11
III	24.6	12	2.05	0.10
Pepsin treated ASC	51.5	8	6.45	0.33
Pronase treated ASC	49.8	5	9.95	0.51
ASC after 10 min heating to 60°C	28.1	12	2.34	0.12
ASC in 4 M LiCl	23.4	12	1.95	0.10
ASC in 6M urea	4.68	12	0.39	0.02
Pepsin treated ASC heated to 60°C 10 min	58.4	8	7.30	0.36
Pronase treated ASC heated to 60°C 10 min	52.8	5	10.55	0.54
Pepsin treated ASC in 4 M LiCl	59.3	8	7.42	0.38
Pronase treated ASC in 4 M LiCl	55.7	5	11.15	0.57
Pepsin treated ASC in 6 M urea	2.97	8	0.37	0.02
Pronase treated ASC in 6 M urea	2.95	5	0.59	0.03
ASC alcohol reprecipitated	25.8	12	2.15	0.11

\* If not stated otherwise all data referred to ASC preparation No. 1.

\*\*  $I$  designates fluorescence intensity of collagen sample and tyrosine solution of equal concentration. Concentration of collagen solution is considered in terms of moles of tyrosine per litre.

residues of tyrosine per one molecule of collagen, four are split by pepsin and additional three by pronase.

#### 4. Discussion

Data reported above allow several conclusions about the properties and localization of fluorescent

elements in collagen. The relative tyrosine fluorescence  $R_{tyr}$  is rather low (0.1) in native preparation ranging generally in the magnitude usual for denaturated (randomly coiled) proteins. This implies that a very intensive quenching must occur in the molecule. However, if the terminal regions are split off, the fluorescence intensity increases threefold in the case of pepsin and fivefold in the case of pronase, suggesting that fluorescence quenching occurs in the

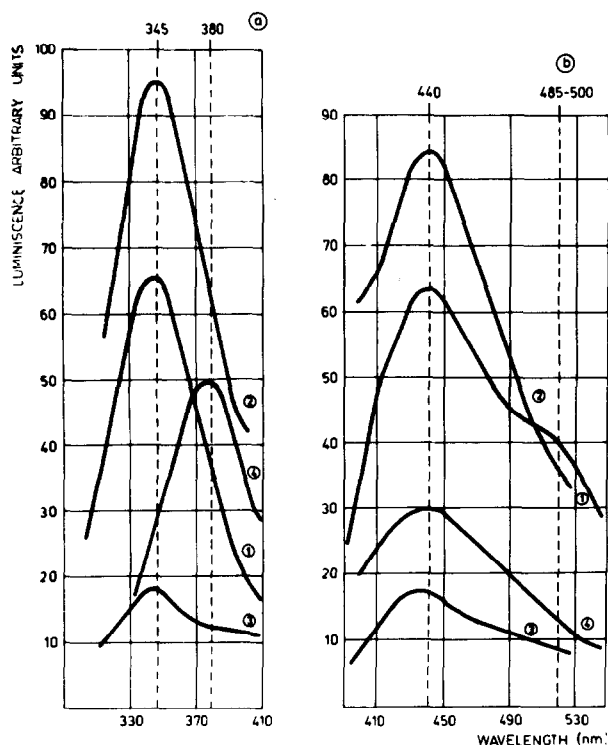


Fig. 2. Near UV fluorescence of collagen preparations treated with enzymes. (1) Acid soluble collagen (0.06% solution of ASC preparation in 0.05% acetic acid). (2) The same preparation treated with pepsin. (3) The same preparation treated with pronase. (4) Fluorescence of dialysable peptides after pronase treatment. Acid soluble collagen used is preparation I (see table 1).

terminal regions of the molecule. Further purification by Jackson's procedure [7] did not alter the overall picture. Presumably adsorbed peptides do not play a role in fluorescence quenching.

There are two other reasons which must be taken into consideration. Fluorescence quenching in the terminal region may be due to a high structural freedom in this region which allows translational and vibrational degradation of energy. It is also possible that the sequence rich in aspartic and glutamic acid results in fluorescence quenching as described recently by Cowgill [10]. In this latter case, a sequence where tyrosine and glutamic acid are intimately related should be isolated from the dialysable fraction of pronase and/or pepsin digest of collagen. Indeed in our previous paper [11] the

presence of a tetrapeptide glu.gly.gly.tyr was reported in the peripheral region of the molecule (present in the pronase digest).

The fact that denaturation (urea excepted) does not influence the fluorescence characteristics of the preparation indicates that tyrosine residues are not accessible to the surrounding solvent. The neighbourhood of tyrosine residues appears to be not substantially attacked by heating in acid solution (pH 4.8) for ten minutes at 60°C nor by exposure to 4 M LiCl. On the other hand, urea denaturation results in a marked decrease in fluorescence. This means that the vicinity of tyrosine residues was involved in the reaction. Thus one can conclude that tyrosines are mainly located in hydrophobic regions not accessible to the surrounding solvent; unmodified tyrosine residues are apparently not involved in interactions of two  $\alpha$ -chains in the molecule, and thus they do not contribute to the overall stability of the triple helix.

The ease of energy transfer along the body of collagen molecule appears to be due to the unique aminoacid sequence rather than to the secondary structure. It is not yet clear whether there could be any energy transfer between the other fluorescent element and tyrosine residues; it can be stated, however, that the 345 nm/440 nm fluorescence is located in the peripheral region of the molecule and more specifically its location is presumably sub-terminal as it is split by pepsin but it is liberated by the action of pronase. Should the terminal localization be taken into consideration then one has to assume the polytopic action of pronase in the  $\alpha$ -chain.

The position of the excitation maxima of the element responsible for the long-wave UV fluorescence is relatively unstable. After alkali and after enzymic splitting the values 370 nm and 380 nm were obtained.

## References

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