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Cross-linking of collagen CNBr peptides by ozone or UV light

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Insoluble collagen from rat tail tendon was digested with cyanogen bromide. The resultant peptides were dissolved in 0.1% SDS solution and separated by gel filtration and gel electrophoresis. Cross-linking occurred in CNBr-cleaved peptides when they were exposed to ozone or biologically effective UV (300 nm) radiation. The enhancement of a blue fluorescence at 430 nm (excited at 350 nm) was found to be associated with oxidized, cross-linked peptides. Polymeric peptides, formed in collagen with aging, also exhibited enhanced blue fluorescence.

Collagen; Crosslinking; Peptide; Fluorescence; Ozone; Ultraviolet irradiation

1. INTRODUCTION

The involvement of oxidation in aging of collagen has long been suspected [1,2]. Moreover, the oxidation of collagen has also been implicated in environmental (pollutants, ozone, solar UV radiation) and pathological (oxygen radicals in inflammation, connective tissue disorders) damage. These processes, however, have many unclarified aspects. Recent studies revealed the cross-linking/polymerization of collagen by hydroxyl radical (·OH) [3] and ozone (O_3) [4] as well as the degradation by O_3, ·OH and superoxide anion ($O_2^{\cdot -}$) [4-6]. Our previous study on 3-hydroxypyridinium (pyridinoline)-free type I collagen showed the presence of a novel 360-370 nm fluorescence which was found to be susceptible to both O_3 and UV (300 nm) radiation [7]. In order to elucidate the structural and fluorescence changes in collagen, occurring under oxidative stress, we here investigated the oxidation and photo-oxidation of collagen peptides by O_3 and UV radiation, respectively.

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2. MATERIALS AND METHODS

Insoluble collagen was obtained from dissected and homogenized rat (about 2 years old) tail tendon, after washing with 0.15 M NaCl/5 mM phosphate, 1 N NaCl/50 mM Tris and 6 M guanidine/50 mM Tris (all pH 7.5) and repeated extraction with 0.5 M acetic acid. Lyophilized collagen (60 mg) was digested with CNBr (360 mg) in 70% formic acid (6 ml) at 30°C for 6 h. The digests were lyophilized and dissolved in 0.1 or 1% SDS, 0.1 M borate buffer (pH 8.2). The solubilized peptides were applied to a Sephacryl S-200 column (1.3 × 200 cm) and eluted with 0.1% SDS buffer. The peptides were also subjected to SDS-polyacrylamide gel electrophoresis using a 7.5% gel. After staining with Coomassie blue and destaining, the gels were scanned with a Joyce Loebl densitometer. O_3 (0.12 μmol/min) was produced by an O_3 generator (model SOG-2, Ultraviolet Products) and passed through the solutions in a conical vessel. UV (300 nm) radiation (1.8×10^{15} quanta/s) was produced by a 150 W xenon lamp connected to a Bausch and Lomb high-intensity monochromator (bandwidth ±10 nm). Absorbance and fluorescence intensities were measured using a Shimadzu spectrophotometer and a Perkin-Elmer fluorescence spectrophotometer, respectively.

3. RESULTS

The solubility of CNBr peptides larger than α1CB6 was found to be dependent upon the concentration of SDS. Fig.1A (curve 1) shows a gel-filtration profile of a CNBr digest of insoluble collagen, dissolved in 1% SDS buffer (12 mg/3 ml). Polymeric peptides (1st peak), migrating at the

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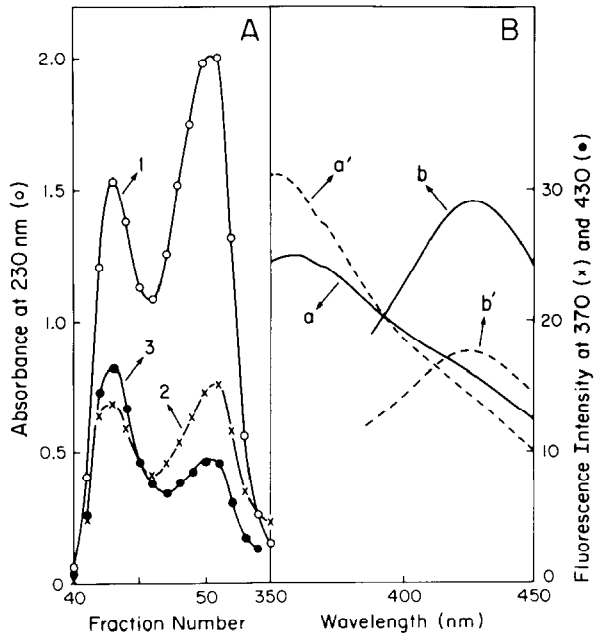

Fig.1. Separation of polymeric from non-polymeric 1% SDS-soluble peptides (A) and their fluorescence spectra (B). (A) Gel-filtration pattern. Curves: 1, 230 nm absorbance; 2, 370 nm fluorescence; 3, 430 nm fluorescence. (B) Fluorescence spectra of fractions 43 (a,b) and 51 (a',b').

void volume, were separated from non-polymeric peptides (2nd unresolved peak). Gel-electrophoresis experiments indicate that non-polymeric peptides are mainly a mixture of α1CB7, 8/α2CB3, 4, 5 (24–31 kDa). These peptides exhibited two kinds of fluorescence bands: one at 360–370 nm (excited at 300 nm) and the other at 430 nm (excited at 350 nm). It was found that the 430 nm blue fluorescence of polymeric peptides was stronger than that of non-polymeric peptides, whose fluorescence of 370 nm was markedly higher than that of 430 nm (fig.1A, curves 2,3). These changes are also shown in the fluorescence spectra of polymeric peptides in fraction 43 (fig.1B, spectra a,b), compared with those of non-polymeric peptides in fraction 51 (fig.1B, spectra a',b'). Spectra a and a' were excited at 300 nm, with b and b' being excited at 350 nm.

CNBr peptides (24 mg/3 ml) were also soluble in 0.1% SDS buffer when heated to 60°C. Upon cooling to room temperature, however, insoluble gel-like material was gradually formed. After being allowed to stand overnight, 0.1% SDS-soluble peptides were separated by centrifugation from 0.1% SDS-insoluble peptides. The gel-filtration pattern of the former (fig.2A, curve 1) was different from that of the latter dissolved in 1% SDS buffer (fig.2A, curve 4). Fig.2A (curve 1) shows the presence of polymeric peptides (1st peak) and the smaller peptide α1CB6 (20 kDa) (4th peak). Limited amounts of other larger peptides appeared as two resolved peaks (2nd and 3rd peaks). In contrast, 0.1% SDS-insoluble peptides contained an unresolved mixture of large peptides (2nd peak) with some polymeric peptides (1st peak), but not α1CB6 (fig.2A, curve 4).

When 0.1% SDS-soluble peptides were exposed to O_3, the 230 nm absorbance of polymeric peptides (1st peak) was not greatly reduced, whereas the other peaks were either markedly reduced or appreciably shifted (fig.2B, curve 1). A most remarkable change was an increase in the 430 nm blue fluorescence in polymeric peptides (cf. fig.2B, curve 3, with fig.2A, curve 3). This increase is attributed to the polymerization (cross-linking) of non-polymeric peptides by O_3 oxidation. Polymerized species also migrated at the void volume. The small decrease in 230 nm absorbance at the 1st peak indicates simultaneous degradation of some original polymeric peptides by O_3. The depolymerization of polymeric collagen aggregates by O_3 was also shown in our previous studies [7]. It should be noted that O_3 treatment of a CNBr digest in alkaline solution (1 N NaOH) completely fragmented the whole peptides.

Fig.3A shows a densitometer trace from an electrophoretic gel of 0.1% SDS-soluble peptides, exhibiting the presence of (a) α1CB6 doublet with and without non-helical peptides, (b) α1CB8, (c) a mixture of other large peptides (α1CB7, α2CB3,4,5) and (d,e) high-molecular-mass peptides of 60–80 kDa. Upon exposure to O_3, α1CB6 (a) disappeared and other peptides (b,c)/polymeric species (d,e) were also reduced (fig.3B). With these changes, large polymers with greater molecular masses than collagen γ-component (300 kDa) were formed, most of which could not enter the gel. Neither polymerization nor the associated enhancement of the 430 nm fluorescence was observed in 1% SDS-solubilized intact peptides as well as 0.1% SDS-insoluble peptides, when treated with O_3.

When a CNBr digest in 0.1% SDS buffer (16

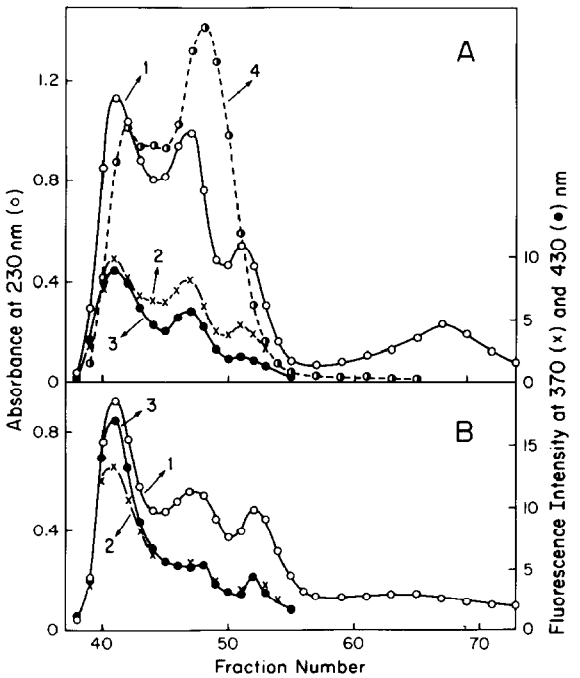

Fig.2. Gel-filtration profiles of 0.1% SDS-soluble (curves 1–3) and -insoluble (curve 4) peptides before (A) and after (B) O_3 treatment (1 h). Curves: 1,4 230 nm absorbance; 2, 370 nm fluorescence; 3, 430 nm fluorescence.

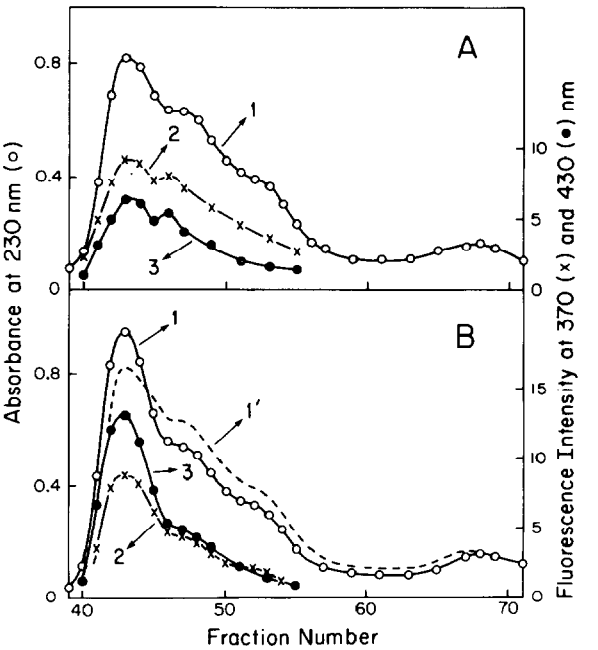

Fig.4. Gel-filtration profiles of 0.1% SDS-soluble peptides before (A) and after (B) UV (300 nm) irradiation (5 h). Curve 1′ (B) is the same as curve 1 (A). Curves: 1,1′, 230 nm absorbance; 2, 370 nm fluorescence; 3, 430 nm fluorescence.

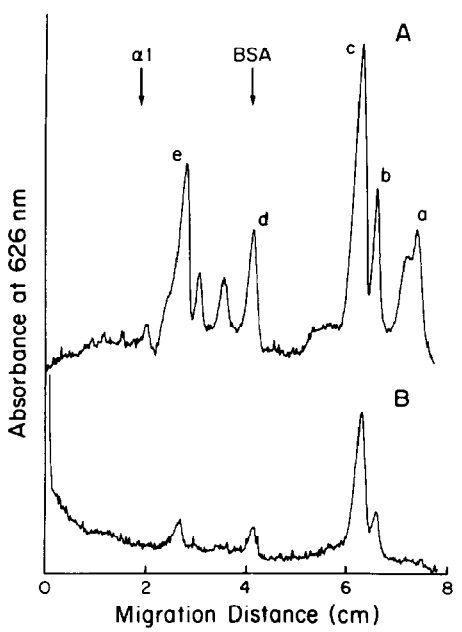

Fig.3. Densitometer traces from electrophoresis gels of 0.1% SDS-soluble peptides before (A) and after (B) O_3 exposure (1 h). Arrows indicate positions of type I collagen α_1 chain (95 kDa) and bovine serum albumin (BSA) (68 kDa).

mg/2 ml) was allowed to stand for 1 week at room temperature after heating to 60°C, 0.1% SDS-soluble peptides gave rise to a less discrete elution pattern as shown in fig.4A (curve 1) or fig.4B (curve 1′). Upon irradiation with UV (300 nm) light, the 230 nm absorbance of polymeric peptides (fractions 42–44) increased at the expense of other non-polymeric peptides (fig.4B, curves 1,1′). In contrast to O_3 oxidation, UV (300 nm) radiation was not effective at degrading polymeric peptides originally present. The 430 nm blue fluorescence of UV-irradiated polymeric peptides also increased (curves 3, fig.4A,B) apparently at the expense of the 370 nm fluorescence (curves 2, fig.4A,B). Gel-electrophoresis experiments revealed that UV-polymerized products were rich in intermediate polymers of 100–150 kDa.

4. DISCUSSION

The present results provide novel information concerning the vulnerability of collagen peptides to ozone and UV attack. The significance of this

work can be seen in two ways. On the one hand, these environmental modes of chemical attack are important physiologically; on the other, oxidation by O_3 and UV modifies the structural and fluorescence properties of collagen components.

Collagens have long been known to exhibit fluorescence. Since the finding of blue fluorescence in uterine collagen [8], age-related blue 420–460 nm fluorescence has been reported in collagens from various tissues [9–14]. Another 395 nm fluorescence, due to pyridinoline, is present in hard tissue collagens (achilles tendon, cartilage, bone, dentin), but not in soft tissue collagens (skin, cornea, tail tendon) [15,16]. This study shows the presence of both 430 nm blue fluorescence and the novel 360–370 nm fluorescence in CNBr peptides from pyridinoline-free rat tail tendon collagen. Furthermore, enhancement of the 430 nm blue fluorescence at the expense of that of 360–370 nm is found to be associated with the oxidation and cross-linking of some CNBr peptides. Similar changes were also observed in calf cornea collagen. The 370 nm fluorescence of free fluorophores, released from collagen by acid hydrolysis, was also found to change gradually to blue fluorescence through auto-oxidation as well as O_3 or UV (300 nm) light (unpublished).

The selective solubility of CNBr peptides in 0.1% SDS solution permitted observation of the cross-linking of certain peptides by oxidation. With the concentration of CNBr digests (8 mg/ml), α1CB6 is completely soluble, but only limited amounts of polymeric and larger peptides are soluble, in 0.1% SDS buffer. As observed in 1% SDS extracts or 0.1% SDS-insoluble peptides, the high concentration of large peptides apparently prevents their cross-linking. The reason for this inhibition is not clear.

In bovine tendon collagen, polymeric CNBr peptides, designated poly-α1CB6, have been shown to accumulate upon aging with the decrease in α1CB6 but other peptides are also involved [17]. The material was polydisperse with a molecular mass range of 50–350 kDa [18]. Our studies imply that age-related cross-linking occurs as the result of oxidation. UV (300 nm) radiation may cause photosensitized oxidation via the formation of singlet oxygen by energy transfer from the triplet state of UV (300 nm)-absorbing photosensitizers (370 nm fluorophores) to oxygen [19]. Further studies in progress on the effect of chemically produced singlet oxygen support this view.

Partial degradation of CNBr peptides in buffer solution (pH 8.2) and their complete fragmentation in alkaline solution by O_3 are consistent with the effect of ·OH on these peptides. ·OH is the main product formed in the decomposition of O_3 in water. O_3 decomposition is catalyzed by OH^- and therefore is pH-dependent [20]. In alkaline solution (at pH >10), O_3 is rapidly converted to ·OH. In neutral solution, O_3 reacts with organic substances essentially as O_3 itself. It has recently been shown that radiolytically generated ·OH fragments proteins in the presence of oxygen by oxidizing and cleaving proline residues [21,22]. Collagen containing a large number of proline residues can be expected to be degraded by ·OH [4].

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