Involvement of Type V Collagen in Softening of Fish Muscle during Short-Term Chilled Storage

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Type V collagen became solubilized in softened sardine muscle after 1 day of chilled storage with the concomitant weakening of pericellular connective tissue induced by disintegration of thin collagen fibrils. However, no significant changes were observed in the structure of interstitial connective tissue or biochemical properties of type I collagen. Z disk in myofibrils showed structural changes, but no significant loss of longitudinal continuity of myofibrils was observed even at the deteriorated Z disk from the muscle destroyed by compression test. On the other hand, tiger puffer muscle did not show significant softening during the storage, with no significant changes in structure of connective tissues and biochemical properties of collagens. These facts suggest that degradation of type V collagen causes disintegration of the thin collagen fibrils in pericellular connective tissue, weakening pericellular connective tissue and resulting in postharvest softening.

Keywords: *Collagen; postharvest storage; fish; connective tissue; muscle; collagen V*

INTRODUCTION

Firmness of fish flesh decreases rapidly during postharvest storage with few exceptions (Hatae et al., 1985; Toyohara and Shimizu, 1988; Ando et al., 1991a−c, 1992, 1993, 1995; Sato et al., 1991). Ando and his coworkers have demonstrated by using a compression technique, which simulated the biting of fish flesh, and a light microscope that the softening is caused by weakening of pericellular connective tissue in muscle (Ando et al., 1991c, 1993). It has been speculated that weakening of the Z disk in myofibrils may contribute to postharvest softening of fish flesh (Tokiwa and Matsumiya, 1969; Seki and Watanabe, 1982; Tachibana et al., 1988, 1993; Seki and Tsuchiya, 1991; Tsuchiya et al., 1992). In these studies, weakening of the Z disk was estimated after extensive destruction of muscle. Therefore, the contribution of weakening of the Z disk to the softening of fish flesh needs to be tested by using techniques that can simulate the biting action of teeth, such as a compression test.

Collagen, the fibrillar component of connective tissue, forms a family designated by type and a Roman numeral. In fish intramuscular connective tissue, type I and V collagens and their submolecular species were isolated (Kimura and Ohno, 1987; Sato et al., 1991, 1994a,b). Sato et al. (1991, 1994a) demonstrated that type V collagen was solubilized specifically in softened rainbow trout (*Oncorhychus mykiss*) muscle. On the basis of these findings, we proposed a hypothesis that the type-specific changes of collagens play a significant role in the weakening of pericellular connective tissue and resultant postharvest softening of fish flesh (Sato et al., 1991, 1994a; Ando et al., 1992, 1993, 1995). Postmortem changes of intramuscular type V collagen in other fish species have not been examined until now. In the present study, sardine and tiger puffer muscles, which show extensive and no significant softening, respectively, were examined to study postmortem structural changes in myofibrils and intramuscular connective tissue along with biochemical changes in fibril-forming collagens (types I and V).

MATERIALS AND METHODS

Materials. Live sardine *Sardinops melanosticta* (body weight; 20−50 g) and tiger puffer *Fugu rubripes* (700−900 g) were obtained commercially and filleted. The fillets were used for experiments immediately or after 1 day of storage at 5 °C in a polyethylene bag.

Evaluation of Firmness. Firmness of fish flesh was evaluated by puncture test as described previously (Ando et al., 1991c). Briefly, a muscle slice of 10 mm thickness was excised perpendicular to the orientation of muscle fibers. A cylindrical plunger (3 mm diameter) was pierced into the slice parallel to the orientation of muscle fibers at a speed of 60 mm/min by using a Rheometer (Yamaden Model 3305, Tokyo, Japan). The maximum force to penetrate muscle was regarded as the breaking strength (*g*). Results were expressed as an average of 5−10 determinations and the standard deviation (SD). In addition, softening of fish muscle by storage was also evaluated organoleptically by paired preferential test [refer to, for example, Meilgaard et al. (1991)].

Compression Test. To detect tissues weakened by chilled storage, a compression test was performed according to the method of Ando et al. (1991c). The muscle cube (10 × 10 × 10 mm) was excised from fresh and stored fillets and subjected to compression parallel to the orientation of muscle fibers at 100 g/cm^2 with a cylindrical plunger (40 mm diameter). The structures of intramuscular connective tissues and myofibrils in the compressed muscle were observed with light and transmission electron microscopes.

Preparation of Sample for Microscopic Observations. Muscle cubes were fixed in Bouin fixative and embedded in

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S0021-8561(96)00661-9 CCC: $14.00

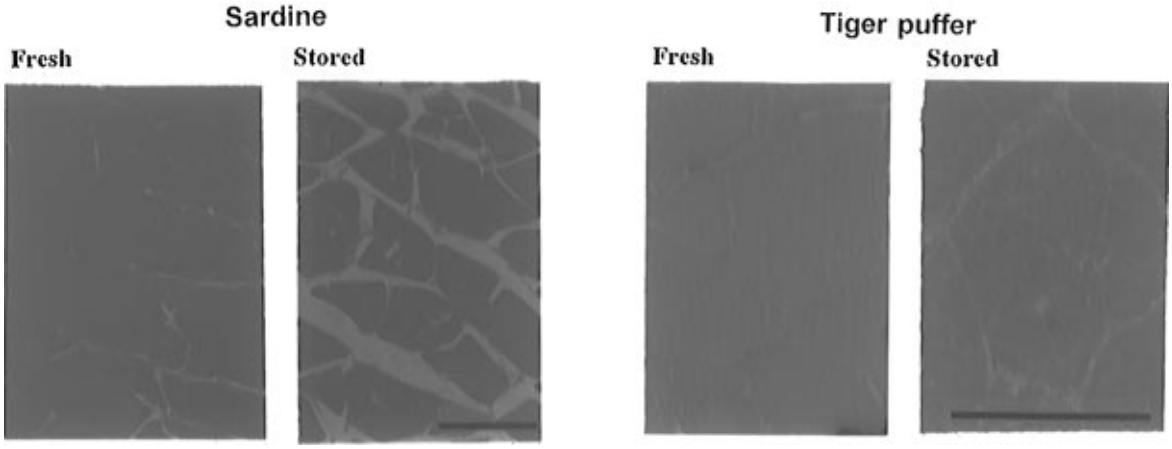

Figure 1. Transverse sections of the compressed sardine and tiger puffer muscles. The pericellular connective tissue in the softened sardine muscle was broken by the compression. Magnifications: 200× for sardine, 400× for tiger puffer. Bars represent 100 μm. (Figure is reproduced here at 65% of original.)

paraffin. Thin sections of 7 μm were prepared using a Microtome (PR-50, Yamato Kohki, Urawa, Japan) and stained with hematoxyline and eosin for light microscopic (Nikon FX-PH-21, Tokyo, Japan) observation.

For a transmission electron microscope (Hitachi H-800, Hitachi, Japan), muscle cubes were fixed in Karnovsky fixative and 1% osmic acid and then dehydrated in ethanol series. The fixed sample was embedded in Epok 812 (Nissin EM, Tokyo, Japan). An ultrathin section was prepared using an Ultramicrotome (DuPont MT-6000, Wilmington, DE) and stained with uranyl acetate and lead citrate.

Determination of Type I and V Collagens. Intramuscular connective tissues were prepared according to the method of Sato et al. (1987) employing the preliminary extraction with 0.1 M NaOH at 5 °C. The preliminary extraction excludes the effect of endogenous proteases on collagen during preparation and does not denature or solubilize collagen (Sato et al., 1987). Collagens in the intramuscular connective tissues of sardine and tiger puffer were successively solubilized by extraction with 0.5 M acetic acid and limited pepsin digestion, and the remainder was solubilized by heating in 0.1% sodium dodecyl sulfate (SDS) as described previously (Sato et al., 1989). The contents of type I and V collagens in the resultant acid-soluble, pepsin-solubilized, and insoluble collagen preparations were calculated on the basis of the hydroxyproline content of the hydrolysates and the relative staining intensity of type V collagen α1(V) and α3(V) bands to the total collagenous bands on SDS−PAGE gels as described previously (Sato et al., 1989).

SDS−PAGE was performed according to the method of Laemmli (1970) using 7.5% gel. The gels were stained with Coomassie Brilliant Blue R-250 and scanned and processed with a Chromatoscaner CS-930 (Shimadzu, Kyoto, Japan) or a Masterscan (CSPI, Billerica, MA).

Hydrolysis of collagen preparation and determination of hydroxyproline were performed as described previously (Sato et al., 1995).

RESULTS

Postharvest Softening. As shown in Table 1, the breaking strength of sardine muscle decreased significantly after 1 day of chilled storage, indicating that postharvest softening of sardine muscle occurred. The postharvest softening of sardine flesh was also confirmed organoleptically ($n = 11$, $P < 0.01$). On the other hand, no significant decrease in breaking strength was observed with the stored tiger puffer flesh. These data agreed with previous observations (Ando et al., 1991a).

Postharvest Structural Changes. As shown in Figure 1, detachment of muscle fibers by compression occurred in the softened sardine muscle, whereas muscle fibers in the fresh sardine muscle were almost intact after the compression, indicating that sardine pericellular connective tissue, which integrated muscle fibers, was weakened by the storage. On the other hand, no significant weakening of pericellular connective tissue was observed in tiger puffer muscle after storage.

Table 1. Postharvest Change in Breaking Strength (*g*) of Sardine and Tiger Puffer Muscles

	sardine[a]	tiger puffer
fresh	127 ± 19	916 ± 197
stored	34 ± 2*	788 ± 170

[a] Asterisk indicates significant difference ($P < 0.01$).

Fine structures of pericellular connective tissue and myocommata, an interstitial connective tissue of fish flesh (Love and Robertson, 1968), were examined by transmission electron microscope. In sardine, most of collagen fibrils in the pericellular connective tissue disappeared after storage, whereas collagen fibrils in the myocommata remained almost intact after storage (Figure 2). However, disappearance of collagen fibrils did not result from compression (data not shown). Therefore, it is proposed that disintegration of the collagen fibrils occurred during storage, resulting in weakening of the pericellular connective tissue. It should be noted that the diameter of collagen fibrils in the pericellular connective tissue (20−30 nm) is smaller than those in myocommta (30−40 nm). On the other hand, no significant structural changes in collagen fibrils in intramuscular connective tissue after storage were detected in tiger puffer (Figure 3).

The fine structure of myofibrils in the compressed muscles is shown in Figure 4. In the fresh muscle, Z disks and M lines, which are located in the center of actin and myosin filaments, respectively, can be identified. However, after storage, the structure of sardine Z disks and M lines became obscure and indistinguishable. No significant loss of longitudinal continuity of myofibrils was observed even with the deteriorated disks after compression.

Postharvest Biochemical Changes of Collagens. Postharvest changes in the intramuscular collagens are summarized in Table 2. In the softened sardine muscle, solubilization of type V collagen occurred, while no significant changes were observed in content and solubility of type I collagen. Type V collagen content was reduced to half by storage. Collagens in tissue are insolubilized by covalent cross-links. Cross-links are formed preferentially between triple-helical and globu-

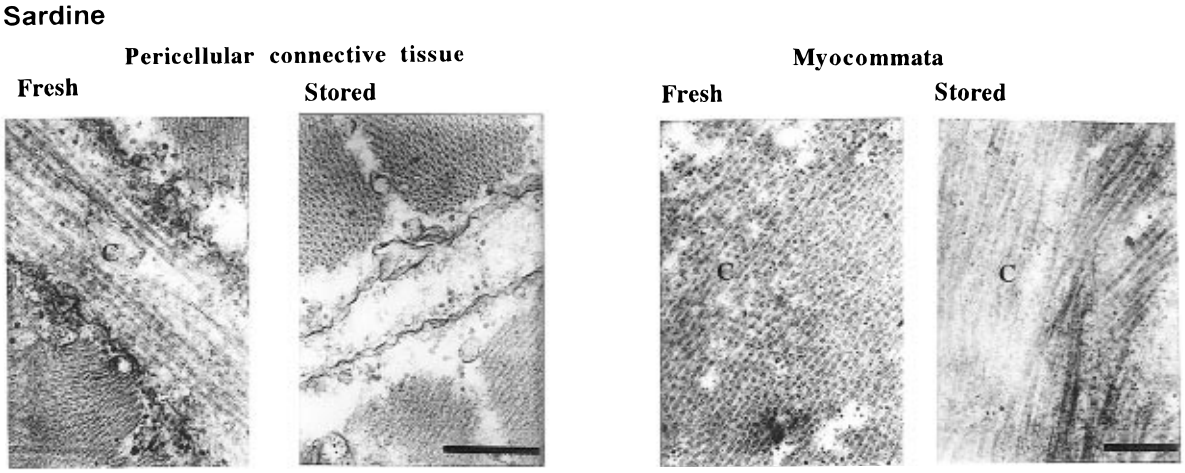

Figure 2. Transmission electron micrographs of pericellular connective tissue and myocommata in the fresh and stored sardine muscle. C, collagen fibrils. Magnifications: 40000× for pericellular connective tissue, 30000× for myocommata. Bars represent 500 nm. (Figure is reproduced here at 65% of original.)

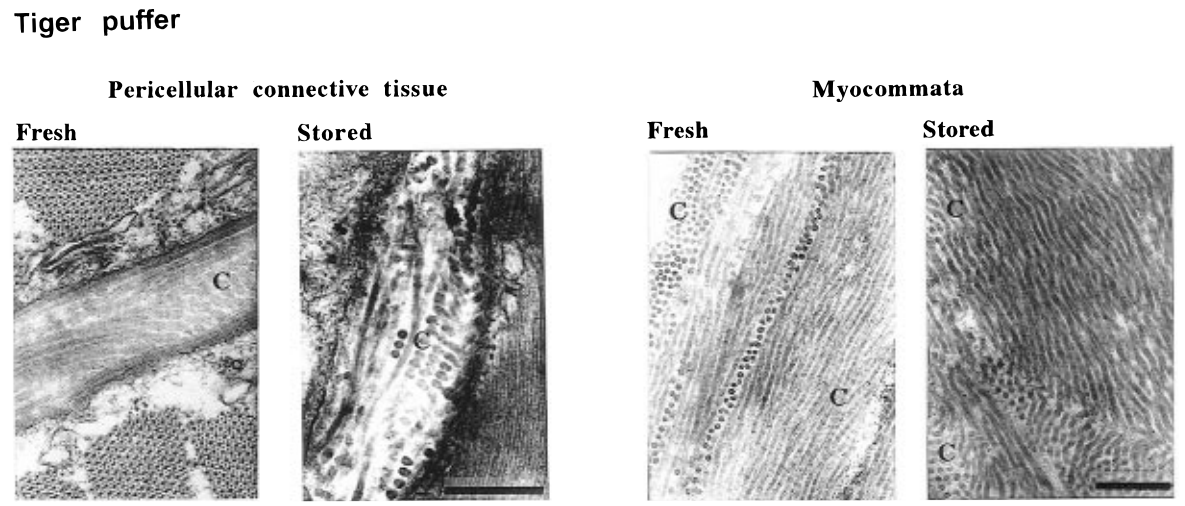

Figure 3. Transmission electron micrographs of tiger puffer intramuscular connective tissues. Magnifications: 40000× for pericellular connective tissue, 30000× for myocommata. Bars represent 500 nm. (Figure is reproduced here at 65% of original.)

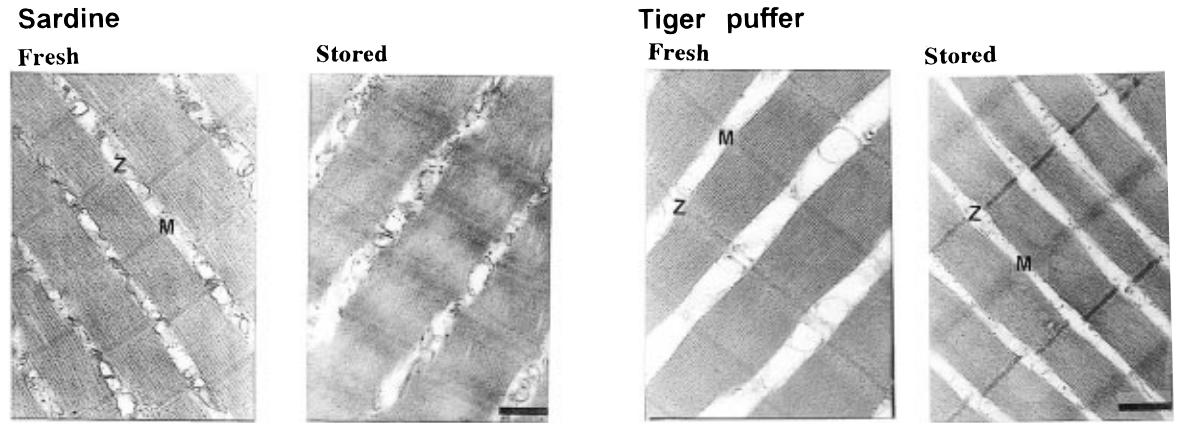

Figure 4. Transmission electron micrographs of longitudinal sections of myofibrils. Z, Z disk; M, M disk. Magnification: 20000×. Bars represent 500 nm. (Figure is reproduced here at 65% of original.)

lar (referred to telopepptide) regions. The triple-helical region resists most proteolytic enzymes except for collagenase. On the other hand, telopeptides are susceptible to other proteases such as pepsin [refer to recent reviews by Miller (1984) and van der Rest and Garrone (1991)]. The solubilization of sardine type V colagen, therefore, could be attributed to cleavage of triple helix by collagenase, cleavage of telopeptides by other protease, and/or cleavage of cross-links. As shown in Figure 5, collagenous fragments, which had greater mobility than monomeric subunits of collagens (α chains), were not detected in all collagen preparations, which indicates no significant cleavage of triple-helical domains of collagens by collagenase. Approximately half of type V collagen became soluble to cold 0.1 N NaOH. Together with these facts, it is proposed that specific cleavage of telopeptides and/or cross-links of type V collagen occurred during storage of sardine muscle. On

Table 2. Postharvest Changes in Collagen Preparations by 1-Day-Chilled Storage of Sardine and Tiger Puffer

	collagen content (mg/100 g of muscle)		solubility[a] of collagen (%)					
			type I			type V		
	type I	type V	ASC	PSC	ISC	ASC	PSC	ISC
sardine								
fresh	266 ± 9	26.5 ± 4.6	36.0 ± 6.4	34.8 ± 5.2	29.3 ± 1.3	5.6 ± 0.7	61.9 ± 1.3	32.5 ± 0.6
stored	210 ± 19	11.6 ± 1.2*	31.6 ± 4.5	42.0 ± 6.1	26.3 ± 1.9	32.1 ± 4.1*	41.3 ± 0.2*	26.5 ± 4.0*
tiger puffer								
fresh	948 ± 9	6.9 ± 2.1	20.5 ± 3.6	75.8 ± 3.3	3.7 ± 1.1	33.2 ± 3.1	61.1 ± 8.3	5.7 ± 5.8
stored	905 ± 10	8.4 ± 4.3	18.3 ± 4.6	79.6 ± 5.4	2.1 ± 0.9	24.3 ± 3.2	72.8 ± 5.2	3.0 ± 1.5

[a] ASC, acid-soluble; PSC, pepsin-solubilized; ISC, insoluble collagens. Asterisk indicates significant difference.

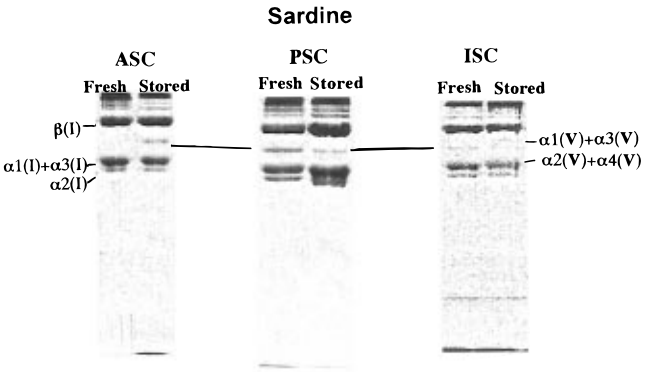

Figure 5. SDS−PAGE patterns of collagen preparations from the fresh and stored sardine muscles. ASC, acid-soluble collagen preparation; PSC, pepsin-solubilized collagen preparation; ISC, insoluble collagen preparation. ASC was digested with pepsin before electrophoresis to detect type V collagen subunits. Subunit composition of fish collagens is as follows: type I, α1(I)α2(I)α3(I), and $[\alpha1(I)]_2\alpha2(1)$ (Kimura and Ohno, 1987); type V, α1(V)α3(V)α4(V), and $[\alpha1(V)]_2\alpha2(V)$ (Sato et al., 1994b). α1(I), α3(I), α2(V), and α4(V) migrate to the same position. β(I) represents cross-linked dimers of type I α chains. The high molecular aggregates with lower mobility than β chains have been believed to be cross-linked polymers of type I collagen subunits.

the other hand, tiger puffer collagens (types I and V) showed no significant changes in the SDS−PAGE patterns (data not shown).

DISCUSSION

For decades, weakening of Z disk and resultant fragmentation of myofibrils have been speculated to play significant roles in the postharvest softening of fish flesh (Tokiwa and Matsumiya, 1969, Seki and Watanabe, 1982; Tachibana et al., 1988, 1993; Seki and Tsuchiya, 1991; Tsuchiya et al., 1992). In these studies, weakening of Z disk and resultant fragmentation of myofibrils were evaluated by determining myofibril length remaining after vigorous stirring in low ionic strength buffer using a Waring blender, which destroyed muscle structure extensively. Recently, Tachibana et al. (1993) demonstrated that the structure of Z disk in red seabream *Pagrus major* muscle lost its intact structure after 1 day of iced storage. A similar phenomenon was observed in the Z disk of softened sardine muscle (see Figure 4). However, no significant cleavage occurred at the deteriorated Z disk after compression, which simulated the biting of fish flesh, while the pericellular connective tissue was disintegrated (Figures 1 and 4). We, therefore, believe that the destruction of Z disk induced by vigorous treatment using a Waring blender should not be linked directly to the softening of fish flesh. In addition, it has been demonstrated that the softening occurs frequently during pre- or in-rigor state of fish with few exceptions (Ando et al., 1991a). If fish flesh were softened by the weakening of the Z disk and resultant fragmentation of myofibrils, such muscle could not contract and transfer stress to tendon and myocommata to maintain rigor mortis. Together with these facts, it is unlikely that the structural and mechanical changes of Z disk play a significant role in the rapid softening of fish flesh by chilled storage at early storage. We however, do not deny the possibility that weakening of the Z disk might be involved in other kinds of textural changes in different storage conditions.

In addition to weakening of the Z disk, degradation of connectin (alternatively referred to as titin), a myofibrillar protein, has been assumed to contribute to the tenderization of fish flesh (Kumano and Seki, 1993). Electrophoretic studies have demonstrated that the α-form of connectin decreases during storage with increase in the β-form. The conversion to β form occurs in accordance with the softening of carp and rainbow trout muscles (Kumano and Seki, 1993). In our preliminary experiment, α-connectin, however, decreased more in tiger puffer muscle than in sardine muscle during 1 day of chilled storage (Kubota et al., 1996). In addition, degradation of connectin has not been accompanied by any structural changes that could be linked to postharvest softening. Therefore, it is unlikely that postmorten change in connectin plays a significant role in rapid softening of raw fish meat.

We propose that rapid softening of fish flesh during short-term chilled storage is caused by degradation of type V collagen and resultant weakening of intramuscular pericellular connective tissue on the basis of the following facts. First, type V collagen was solubilized specifically in the softened muscle by chilled storage. Second, disintegration of thin collagen fibrils and weakening of the intramuscular pericellular connective tissue occurred specifically in the softened muscle. Third, diameters of the collagen fibrils in the pericellular connective tissue of sardine were significantly smaller than those in myocommata. A similar observation has been reported in hoki muscle (Hallett and Bremner, 1988). It has been demonstrated that the diameter of collagen fibril decreases with increase of type V collagen in fibril (Adachi and Hayashi, 1986; Birk, et al., 1990). It can be postulated that the thin collagen fibrils in the pericellular connective tissue consist of a larger portion of type V collagen than the thick fibrils in myocommata. Therefore, the solubilization of type V collagen during chilled storage can be linked to disintegration of the thin collagen fibrils and weakening of the pericellular connective tissue and resultant softening of muscle.

In the present paper, we demonstrate that degradation of type V collagen rather than degradation of myofibril proteins can be linked to postharvest softening of fish flesh. However, direct evidence for postharvest change in telopeptides and cross-links of type V collagen cannot be obtained by the present approach, because

the present collagen preparations were digested by pepsin to detect type V collagen bands by SDS−PAGE, resulting in removal of telopeptides. The breakthrough might be achieved by preparation of intact type V collagen with telopeptides, which has not been isolated by the conventional salt fractionation. It would also be useful to prepare antibodies against type V collagen to identify microlocalization of type V collagen in tissues, because telopeptides have stronger antigenecity than the triple-helix region (Schmitt et al., 1964; Ponz et al., 1970).

In tiger puffer muscle, the collagen fibrils in the pericellular connective tissue remained almost intact after storage. It should be noted that the diameter of collagen fibrils in tiger puffer pericellular connective tissue was the same as those in myocommata of both fishes. It could therefore be assumed that type I collagen might be present in excess of type V collagen in tiger puffer pericellular connective tissue, and type V collagen might be masked with type I collagen, protecting it from proteolytic attack. The lower ratio of type V collagen to type I collagen in tiger puffer muscle than in sardine, carp, and mackerel *Pneumatophorus japonicus* (Sato et al., 1991) supports this hypothesis. There is, however, another possibility that the localization and quantity of the factor(s) which degrade type V collagen might be different between tiger puffer and other fishes. To solve these problems, further studies on preparation of intact fish type V collagen and its specific antibody are now in progress.

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Received for review September 9, 1996. Accepted November 12, 1996.[⊗] This work was supported by a grant from the Ministry of Education, Science and Culture of Japan (1995).

JF9606619

[⊗] Abstract published in *Advance ACS Abstracts,* January 15, 1997.