Possible involvement of aminotelopeptide in self-assembly and thermal stability of collagen I as revealed by its removal with proteases

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Running title: Characterization of protease-treated type I collagen

1 The abbreviations used are: CD, circular dicroism; ASC, acetic acid-soluble collagen; CBB, Coomassie Brilliant Blue; MMP, matrix metalloprotease; MT-MMP, membrane type-MMP; kDa, kilodalton; PBS, phosphate-buffered saline.
SUMMARY

The functions of aminotelopeptide and N-terminal crosslinking of collagen I were examined. Acetic acid-soluble collagen I (ASC) was purified from neonatal bovine skin and treated with three kinds of proteases. The amino acid sequencing analysis of the N-terminus showed that ASC contained a full-length aminotelopeptide. Pepsin and papain cleaved the aminotelopeptide of the α1 chain at the same site and the aminotelopeptide of the α2 chain at different sites. Proctase-treated ASC lost the whole aminotelopeptide and the N-terminal sequence began from the tenth residue inside the triple helical region. The rates of fibril formation of pepsin-treated ASC and proctase-treated ASC were the same and were slower than that of ASC. The denaturation temperatures, monitored by CD ellipticity at 221nm, of ASC, pepsin-treated or papain-treated collagens were the same at 41.8˚C. Proctase-treated ASC showed a lower denaturation temperature of 39.9˚C. We also observed the morphology of the collagen fibrils under an electron microscope. The ASC fibrils were straight and thin while the fibrils of pepsin-treated ASC were slightly twisted, and the fibrils from papain- and proctase-treated ASC were highly twisted and thick. When the collagen gel strength was examined by a modified method of viscosity-measurement, ASC was the strongest, followed by pepsin-treated ASC, and papain- and proctase-treated ASCs were the weakest. These results suggest that the aminotelopeptide plays important roles in fibril formation and thermal stability. In addition, the functions of intermolecular crosslinking in aminotelopeptides may contribute to the formation of fibrils in the correct staggered pattern and to strengthening the collagen gel.
INTRODUCTION

The collagens of types I, II, III and V/XI are grouped as fibrillar collagens, and collagens I, II, III and V are able to form fibrils in exactly a 67-nm staggered manner in vitro (1). The existence of collagen amino- and carboxytelopeptides in the collagen molecules accelerates the assembly of collagen fibrils (2) and are necessary for contraction of the collagen gel lattice by dermal cells (3). Collagens solubilized in acetic acid solution began to assemble fibrils on warming and neutralization of pH. The speed of fibril formation, the fibril thickness and fibril length were easily affected by pH, concentration of NaCl, and temperature (4, 5). The fibril diameter of type I collagen is reduced by the addition of type V collagen (6) or the existence of age-related crosslinking in the triple helical region (7). The fibril formation of acid-soluble collagen (ASC) is faster than that of pepsin-treated ASC, and pepsin-treated ASC fibril is released from the fibrils to the solution again on incubation at low temperature (8). In fact, the pepsin-treated ASC have short telopeptides, because the molecular weight of pepsin-treated ASC is smaller than that of intact ASC by SDS-PAGE analysis (2, 3, 9). The telopeptide may affect the lag time and speed of fibril formation, but it is not known which portion of the telopeptide is important because the exact cleavage sites by pepsin have not been determined.

Several crosslinkings in the telopeptide region such as hydroxylsinonorleucine (10) and age-dependent mature crosslinking such as histidinohydroxylsinonorleucine (HHL) (11, 12) or pentosidine (9, 13) were previously reported. The types I, II, and III collagen molecules contain four major sites involved in crosslinking: allysine or hydroxyallysine at 9 (K-9) in the aminotelopeptide, hydroxylysine at 103 (K-103), hydroxylysine at 946 (K-946) in the triple helical region, and allysine or hydroxyallysine in the carboxytelopeptide (K-1046) of the α1 chain in type I collagen. Intermolecular crosslinks between allysine at K9 and at K946 and between at K1046 and at K103 stabilize a 4-D staggered arrangement between molecules that is important for fibril structure formation (summarized by Piez, 14). The type I collagen contains three allysines in the aminotelopeptides, and the structure of crosslinking on three α chains of purified collagen was suggested (15). Recently, Prockop and Fertala illustrated that the self-assembly of collagen began
from the carboxy-terminal portion, because synthetic peptides corresponding to the
carboxytelopeptide region in the α1 and α2 chains could inhibit fibril formation, but synthetic
peptides corresponding to the aminotelopeptide region only partially inhibited it (16). This is an
important illustration in which the first step of collagen fibril formation is the interaction of the
telopeptide region and the triple helical domain of collagen.

Skin collagens of fetal and newborn bovines were easily solubilized using acetic acid, because
the collagens containing N-terminal crosslinkings were acid-soluble and contained a negligible
amount of mature crosslinking in the triple helical region (9). Thus, the type I collagen isolated from
juvenile skin tissue is a good material for investigating telopeptide crosslinking. In the present
experiment, the N-terminal cleavage sites by three kinds of proteases were determined by protein
sequencing. The results showed that the length of the aminotelopeptides and the number of
crosslinkings present were different depending on the proteases used. We compared the natures of
these collagens from fibril formation, thermal stability, morphological changes in the fibril and fibril
strength, and discussed possible functions of the aminotelopeptide in those processes.
EXPERIMENTAL PROCEDURES

Reagents--- Pepsin and papain were purchased from Wako Pure Industries, Inc. (Osaka, Japan). Mixture of Proctase A (EC 3.4.23.18) and proctase B (EC 3.4.23.19), the aspartic protease extracted from Aspergillus niger var. macrosporus was purchased from Meiji Seika (Tokyo, Japan). Other chemicals were products of Wako Pure Chemical Industries, Inc. (Osaka, Japan).

Preparation of acid-soluble type I collagen from bovine skin--- Bovine skins were obtained from several farms in the eastern areas of Hokkaido, Japan. The ages of the bovines used were fetus and newborn. The hair and lipids were carefully removed from 100 g of bovine skin, and the skin was washed overnight at 4°C with a washing buffer containing 10 mM disodium phosphate, pH 7.6, 1 mM N-ethylenediaminetetraacetic acid (EDTA), 0.1 mM N-ethylmaleimide, and 10 μM phenylmethylsulfonyl fluoride (PMSF). The washed skin was minced, homogenized in fresh washing buffer, and centrifuged at 10,000 x g for 30 min at 4°C. The pellet was washed twice with the washing buffer, suspended in 10 volumes of diethyl ether-ethanol (mixed at 1:1) to remove the lipids, and filtered using a filter paper. This washing was repeated at least 3 times until the supernatant became colorless. The final pellets were air-dried and stored at -20°C.

Collagen was extracted from the dried skin by gently shaking it at 4°C for 3 days in 50 mM acetic acid (pH 4.2) to obtain the acid-soluble collagen (ASC). After extraction, the suspension was centrifuged at 10,000 x g for 30 min at 4°C. To the supernatant, an equal volume of 4 M NaCl in 50 mM acetic acid was added, and the mixture was incubated overnight at 4°C and centrifuged. The pellet was resuspended in 10 volumes of 50 mM acetic acid and incubated overnight. After removal of the insoluble materials by centrifugation at 10,000 x g for 30 min at 4°C, a 1/5 volume of 5 M NaCl was added to the supernatant. After centrifugation, the pellet containing type I collagen was resuspended in 10 volumes of 50 mM Tris-HCl, pH 7.5, containing 0.45 M NaCl, incubated overnight, and centrifuged. The supernatant was dialyzed against 50 mM Tris-HCl, pH 7.5, containing 1.2 M NaCl, and centrifuged again to remove type III collagen as an insoluble material. To the supernatant (type I collagen-rich fraction), 1 N HCl was added to adjust the pH to 3.5, and...
centrifuged. The pellet was resuspended in 50 mM acetic acid. The final collagen preparation was subjected to SDS-PAGE on 5% polyacrylamide gel containing 3.6 M urea to check the purity of the type I collagen fraction. Type I collagen fraction was dialyzed against 5 mM acetic acid, filtered through a 0.8 μm pore filter, and stored at 4°C. The collagen concentrations were determined by BCA protein assay reagent (Pierce, Rockford, IL, USA) using 1 mg/ml collagen solution as a standard.

Protease treatment of type I collagen --- Purified acid-soluble type I collagen (ASC) (2 mg/ml) was incubated at 4 °C overnight in 0.5 M acetic acid containing 0.2 mg/ml pepsin, or 0.1 M tartaric acid-0.1 M sodium tartarate (pH 2.8) containing 0.2 mg/ml proctase. In the case of papain-treatment, ASC (2 mg/ml) was treated with 0.2 mg/ml papain at 20°C for 3 days in 50 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl. To terminate the papain reaction, 10 mM iodoacetate was added to the reaction mixture, which was then incubated for 1 h, and the pH was adjusted to 3.5 by addition of HCl. Protease-treated collagens were precipitated with 1 M NaCl at 4°C overnight and centrifuged at 10,000 x g for 20 min. Pellets were washed once with 0.5 M acetic acid containing 1 M NaCl and dialyzed to 5 mM acetic acid. Collagens dissolved in 5 mM acetic acid were filtered through a 0.8 μm pore filter and stored at 4°C.

Determination of amino acid sequences --- Type I collagens (10 μg) were subjected to SDS-PAGE containing 3.6 M urea and 5% acrylamide gel, then transferred to a PVDF membrane by a semi-dry cassette using thick filter paper filled with 6-amino-n-capronic acid buffer for 3 h at 1 mA/cm². After transfer, the PVDF membrane was stained with 0.1% Coomassie Brilliant Blue (CBB) containing 50% methanol and 10% acetic acid and destained with 50% methanol. The visible bands were cut, and their amino acid sequences were determined using amino acid sequencer (Applied Biosystems, Procise 491).
Fibril formation--- Fibril formation was performed as described before (5). Briefly, the collagen solution in 5 mM acetic acid was neutralized in a disposal polystyrene cuvette (Kartell) by addition of phosphate-buffered saline (x3 concentrated) with gentle shaking on ice. The final concentration of collagen was adjusted to 0.25 mg/ml. The neutralized collagen solution was incubated at 37˚C, and the turbidity at 530 nm was measured every 2 min. To examine the fibril stability at low temperature, the formed fibrils were incubated on ice for 5 min, and the turbidity at 530 nm was measured. Usually, the process of collagen fibril formation comprises three phases. These are the lag phase, the growth phase and the plateau. To define the lag time, we used the extrapolated line of the slope at half-maximal turbidity crossing the abscissa on the time scale.

Circular dichroism spectra measurement--- ASC or protease-treated ASCs (0.05 mg/ml) in 5 mM acetic acid were placed in a spectropolarimeter Jasco-600 (Japan Spectroscopic), and their circular dichroism (CD) ellipticity at 221 nm was monitored. The denaturaton temperature ($T_m$) was determined by monitoring the ellipticity of the collagen solution in a cell of which the temperature was increased from 20˚C to 50˚C at a constant rate (0.25˚C/min). The triple helix content of non-denatured collagen was adjusted to be 100%, and the value of the heat-denatured collagen to be 0 %. $T_m$ was defined as the temperature that gave the midpoint of ellipticities between 20˚C and 50˚C.
Measurement of relative collagen gel strength--- The mechanical strength of the collagen gel was measured by a modified method of viscosity determination (17). Fibril gels were formed from the collagen solutions at various concentrations by incubating in phosphate-buffered saline containing phenol red and NaHCO₃, pH 8.0, at 37°C for 1 h. A lead ball (1.65-1.70mm in diameter and 29.1±2.6 mg in weight) was laid carefully on the gel and incubated at 37°C for 10 min before being photographed.

Electron microscopy--- The collagen fibrils were pre-fixed with 3.7% paraformaldehyde containing 0.02% picric acid for 30 min at 37°C to avoid dissolution of the fibril at low temperature and were fixed with 2.5% glutaraldehyde containing 0.2% tannic acid for 30 min. The fibrils were then stained with 0.5% uranyl acetate (pH 4.4) and phosphotungstic acid (pH 1.8) for observation by transmission electron microscopy (6). For the observation by scanning electron microscopy, the collagen fibrils were fixed with modified Zamboni's solution containing 0.2% picric acid and 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 30 min. They were then cut with a razor blade to reveal the internal structure of the gels. The specimens were fixed again for 30 min with 1% osmium tetroxide in Zamboni's solution. They were dehydrated with ethanol and dried in a critical point dryer (Hitachi HCP-2, Tokyo, Japan). The dried specimens were placed on specimen stages using a piece of double-sided adhesive tape and coated with platinum in a sputter-coater (Model 108 auto, Cressington Co., England). All of the specimens were observed under a scanning electron microscope (Hitachi S-4500, Tokyo, Japan) or a transmission electron microscope (JEM1200 EX/JEOL, Japan).
RESULTS

SDS-PAGE and N-terminal sequence analysis of protease-treated collagens --- Removal of the aminotelopeptides by pepsin was often used for solubilization of collagen with a high extraction rate. The cleavage sites in collagens by pepsin and proctase were expected to be different because the solubilization rate by proctase was higher than that by pepsin (9). To compare the size of these collagen preparations, the collagens were subjected to SDS-PAGE on 5% gel to determine their relative molecular weights. Their apparent molecular weights on SDS-PAGE were as follows (Fig. 1A); the 140 kDa $\alpha_1$ chain and 125 kDa $\alpha_2$ chain in the acid soluble collagen (ASC), the 137 kDa $\alpha_1$ chain and 125 kDa $\alpha_2$ chain in pepsin- and papain-treated ASC, the 135 kDa $\alpha_1$ chain and 120 kDa $\alpha_2$ chain in proctase-treated ASC. These molecular weights were calculated using a protein marker kit (Bio-Rad) as a standard. A small amount of type III collagen was detected in protease-treated collagens, such as 137 kDa for pepsin and 135 kDa for proctase. These differences in molecular weights showed that the collagens were cleaved at different sites in the aminotelopeptide or carboxytelopeptide depending on the proteases used. In addition to the $\alpha$ chains, two $\beta$ chains, $\beta_{11}$ and $\beta_{12}$, were detected in ASC and pepsin-treated ASC. The ratios of $\alpha$ chains and $\beta$ chain were compared densitometrically as the bands stained with CBB. In ASC, the densities of the $\beta_{11}$, $\beta_{12}$, $\alpha_1$, and $\alpha_2$ chains were 6.6%, 24.9%, 45.5%, and 22.9%, respectively. In pepsin-treated ASC, the densities of the $\beta_{11}$, $\beta_{12}$, $\alpha_1$, and $\alpha_2$ chains were 0.2%, 25.8%, 48.3%, and 25.6%, respectively. In papain-treated ASC, the densities of the $\beta_{11}$, $\beta_{12}$, $\alpha_1$, and $\alpha_2$ chains were 1.8%, 10.1%, 57.9%, and 30.2%, respectively. In proctase-treated ASC, the densities of the $\beta_{11}$, $\beta_{12}$, $\alpha_1$, and $\alpha_2$ chains were 0%, 5.2%, 58.0%, and 36.7%, respectively. Thus, the amount of $\beta_{12}$ and $\beta_{11}$ was 4:1 and $\alpha_1$ and $\alpha_2$ was 2:1 in the ASC, but the amount of $\beta_{11}$ decreased in pepsin-treated ASC ($\beta_{12}:\beta_{11}=26:1$). In papain- and proctase-treated ASCs, only small amounts of $\beta$ chains were detected. These results mean that the proteases removed the crosslink-containing aminotelopeptide, and one $\beta$ chain was converted to two $\alpha$ chains.

The cleavage sites by proteases in ASC were determined by amino acid sequencing of their N-terminal portions (Fig. 2A). Amino acid sequencing of ASC gave only a small amount of the I-S-
V-P sequence in the α1 chain showing that the N-terminus of the telopeptides may be blocked. Pepsin-treated ASC gave the sequence of I-S-V-P- in the α1 chain and D-A-K-G-G-G-P in the α2 chain. The recovery of the lysine residue at the third position of the α2 chain was small compared with that in other cycles. This may indicate that the lysine residue at this position underwent hydroxylation, but we could not identify the derivative as a hydroxylysine residue. Interestingly, the sequence of the β12 chain in pepsin-treated ASC started from D followed by E/A, and stopped before K. These results suggest that the third K in the β12, thus in the α2, chain of pepsin-treated ASC is involved in crosslinking. Similarly, the β11 chain in pepsin-treated ASC started from D, followed by E, and stopped before K. Papain-treated ASC had a telopeptide starting from I-S-V-P in the α1 chain and from G-G-G-P or a small amount of L-M-G-P- in the α2 chain. The amino acid sequences of proctase-solubilized collagens started from G-L-P- in the α1 chain and from G-P-P- in the α2 chain which was inside the triple helical region. A scheme of a collagen heterotrimer consisting of β12 and α1 chains, but not β11 and α2 chains, was shown (Fig. 2B).

*Fibril formation and fibril stability at low temperature*--- The fibril formation of ASC and ASCs treated with pepsin or proctase was examined (Fig. 3). In the case of ASC, the turbidity began to increase 2 min after warming and reached a plateau after 8 min. The lag times for pepsin- and proctase-treated ASC was longer than that of ASC (4 min) and reached a plateau after 12 min. The time course of fibril formation of papain-treated ASC was similar to that of pepsin-treated ASC (data not shown). The turbidity of the ASC fibrils at the plateau was much lower than that of pepsin-treated ASC fibrils. When the collagen fibrils formed were cooled again on ice, the fibrils of protease-treated collagens melted and became clear solution, while the intact ASC fibrils were stable even under cold conditions (Fig. 3, 4°C), as described before (8).

*Thermal stability of collagens*--- The thermal stability of ASC, pepsin-, papain- or proctase-treated ASC was examined by CD spectroscopy. In Fig. 4, we assumed the value of ellipticity at 20°C to be 100% helicity and the value at 50°C to be 0% helicity of the collagen molecules. The denaturation
curve of ASC, pepsin-treated, and papain-treated collagen preparations showed the same denaturation temperature ($T_m=41.8 ^\circ C$) (Fig. 4, A-C). On the other hand, the proctase-treated ASC showed a monophasic pattern in the denaturation curve, and the denaturation temperature was lower than that of the other collagens ($T_m=39.9 ^\circ C$) (Fig. 4, D).

**Electron micrograph of collagen fibrils**--- It was reported that ASC and pepsin-treated ASC assemble to form fibrils *in vitro* (2, 8, 9, 15). We examined the structure of reconstituted fibrils from papain- and proctase-treated ASC by scanning electron microscopy (Fig. 5, A-H) or transmission electron microscopy (Fig. 5, I-L). At low magnification, the scanning electron micrograph showed that the fibrils of ASC constituted a mesh-like structure, while the fibrils of pepsin-, papain- and proctase-treated ASCs had a smaller meshwork and a sponge-like structure (Fig. 5, A-D). The individual fibrils of ASC were straight (Fig. 5, E), the fibrils of papain- and proctase-treated ASCs were twisted and thicker than that of ASC (Fig. 5, G and H), and the fibrils of pepsin-treated ASC showed loosely twisted fibrils (Fig. 5, F). The transmission micrograph showed that all 4 kinds of collagen preparations assembled into a 67-nm staggered pattern as indicated by the arrowheads (Fig. 5, I-L). The 67-nm staggered patterns were clearly observed in the case of ASC or pepsin-treated ASC, but less clear in papain- and proctase-treated ASCs.

**The strength of collagen fibrils (gels)**--- Type I collagen fibrils strengthen many tissues such as skin, bone, cartilage, and tendon. We compared the fibril strength between ASC and protease-treated collagens. A lead ball (1.7 mm in diameter) was laid on the collagen fibril at various concentration and whether the fibril sustained the lead ball was observed. The lead balls were supported on ASC at higher concentrations than 0.125 mg/ml, and on pepsin-treated ASC at higher concentrations than 0.25 mg/ml (Fig. 6, A and B). In contrast, papain-treated ASC or proctase-treated ASC could not support the lead balls even when the concentration was increased to 1.3 mg/ml (Fig. 6, C and D). Thus, the ASC fibrils were the strongest, followed by pepsin-treated ASC fibrils, and papain- or proctase-treated ASC fibrils were the weakest against mechanical pressure.
DISCUSSION

Solubilization of collagen from tissue is an old but still challenging issue. For a long time, acid treatment or enzyme digestion was the major method for the extraction of collagen in native form. Recently, we found that proctase treatment was an efficient method for collagen extraction from aged bovine skin (9). Although characterization of these collagens extracted by different ways was not sufficient, most researchers did not seem to be very careful in the preparation method when they used collagen as the adhesion molecule for many cells. In this report, we identified the cleavage sites in type I collagen using several proteases and examined several properties of the derived collagens.

The inability of N-terminal sequencing of acid-soluble collagen (ASC) may mean that ASC contains the full-length aminotelopeptide. Cutting the sites by pepsin in the aminotelopeptide occurred between G-12 and I-13 of the α1 chain and at F-2 and D-3 of the α2 chain. This cutting position explains the decrease in the β11 component in the pepsin-treated ASC (Fig. 1). When K-9 in the aminotelopeptide was intramolecularly crosslinked, the cutting site in the α1 chain was shifted between Y-6 and D-7 (Fig 2A and B). Pepsin left the crosslinking between α1 and α2 undigested (Fig. 1, lane pepsin, β12). It is formed between allysine in the α1 chain (K-9) and lysine in the α2 chain (K-5). There is a gap of three residues between the α1 chain and the α2 chain before the triple helix. The conformation of the α1 chain aminotelopeptide is thought to have an unusual hairpin-like structure that is essential for both assembly into well-ordered fibrils and correction of crosslinking (10). The hairpin conformation in the α1 chain may explain how this gap is compensated. On the other hand, papain removed the first 12 residues from the α1 chain and 5 residues from the α2 chain and, thus, no crosslinking remained in the residual aminotelopeptides. A small amount of the α2 chain cleaved between G-13 and L-14 was detected, which is the same cleavage site by MMP-13 (Sato and Hattori, unpublished result) or MT-MMP (18). Proctase, that was the most effective enzyme to solubilize the collagen in our experiments, cleaved ASC between R-25 and G-26 at the beginning of the triple helical region in the α1 and α2 chains resulting in the blunt N-terminal ends. Unfortunately, because we could not identify the cutting site(s) in the C-
terminal region of protease-treated ASCs, the function of carboxytelopeptide and carboxyterminal crosslinking could not be discussed here.

Next, we examined the activity of *in vitro* fibril formation. Pepsin- or proctase-treated collagens could form the fibrils like ASC. Even after removing all of the aminotelopeptides, as in the case of proctase digestion, collagen retained the fibril forming activity with the D banding pattern (Figs. 3 and 5). However, the time courses of fibril formation were different. Pepsin- or proctase-treated ASC had a longer lag time than ASC in fibril formation, and their final turbidity was higher than that of ASC fibrils. This suggests that the removed N-terminal 6 residues (pQ-L-S-Y-G-Y) or 12 residues including K-9 (pQ-L-S-Y-G-Y-D-E-K-S-T-G) in the $\alpha_1$ chain, and/or 2 residues (pQ-F) in the $\alpha_2$ chain, may contribute to accelerate fibril assembly and shorten the lag time. In contrast, intramolecular crosslinking between $\alpha_1$ and $\alpha_2$, which was still preserved in pepsin-treated ASC, did not contribute to shortening the lag time. The differences in the final turbidity between the different collagen preparations were explained as follows. When we examined the morphology of the collagen fibril by electron microscopy, the diameter of the fibril from ASC was thinner than that from pepsin- or proctase-treated ASC (Fig. 5). If the concentration of collagen is kept the same, the turbidity of a thin fibril may be lower than that of a thick fibril. In the previous report, we found that the turbidity of reconstituted fibrils of type I collagen from aged (8 years old) bovine skin was 8 times lower than that of collagen from young bovine skin (9). In that case, the fibril diameter of the collagen from 8-year bovine skin was thinner (50 nm) than that of the collagen from fetal bovine skin (80-150 nm) (7).

Next we examined the contribution of aminotelopeptide to the thermal stability of the collagen triple helix using CD analysis. By the proctase digestion, the denaturation temperature of collagen remarkably decreased but the papain and pepsin digestions did not affect the thermal stability of collagen (Fig. 4). These results suggest that the first three G-X-Y residues in the triplets are important for stabilizing collagen triple helical conformation, but the aminotelopeptide did not contribute to the thermal stability.
The fine structure of collagen fibrils was examined by electron microscopy. Fibrils from papain- or proctase-treated ASC were twisted with each other, while that from ASC showed a straighter morphology (Fig. 5). Thus, the aminotelopeptide may also be involved in the spiral structure of the collagen fibril. The D period pattern of the fibril from papain- and proctase-treated ASCs was not clear compared with those from pepsin-treated ASC and ASC. These results indicate that the function of crosslinking in the aminotelopeptide is to assemble the fibrils into a correctly staggered pattern as suggested by Nagan and Kagan (10). Twisted fibrils were also observed in alkali-treated collagen which lost the telopeptides (5). The papain-treated ASC fibrils were flat and more twisted than the proctase-treated ASC fibrils. The reason is not clear at this moment.

The role of intermolecular crosslinking in gel strength was also examined. The gel strength of ASC was the strongest, pepsin-treated ASC was weaker than ASC, and the collagens lacking aminotelopeptides were weakest against mechanical pressure (Fig. 6). It was reported that the ASC molecules were connected to each other by intermolecular crosslinking during fibril formation, and this is why the fibril from ASC was stable under cold conditions after being formed (Fig. 3, ref. 5, 8, 19). However, the intermolecular crosslinking in the pepsin-treated ASC fibril was generated very slowly and required several hours (8). The number of crosslinkings generated via lysine-aldehyde correlates with gel strength (14). When the aldehyde was reduced by sodium borohydride, the formed fibril was released again at 4°C (19) like the pepsin-treated ASC (Fig. 3). It is then suggested that the ASC fibril contains the highest level of intermolecular crosslinking, next is the pepsin-treated ASC, and a negligible level exists in papain- or proctase-treated ASC with no crosslinking site in the aminotelopeptides.

These differences in the features of collagens obtained by different methods suggest the roles of the aminotelopeptide in the crosslinking, thermal stability, fibril structure and gel strength. These differences may affect the cell behavior in the collagen. For example, we recently found that collagen gel contraction by human foreskin fibroblasts was very weak in the collagen fibril from papain- or proctase-treated collagen (data not shown). These results suggest that gel strength
obtained by crosslinking(s) is crucial for collagen gel contraction. This experiment also suggests the importance of crosslinking between aminotelopeptides to strengthen bone, skin, or blood vessels.

Finally, we mention the function of cleaved collagen fragments in vivo. Recently, it was reported that several collagenases, MT1-MMP and MT3-MMP (18), can cleave the α2 chain of type I collagen between G-11 and L-12 (see Fig. 2B), and also MMP-13 could do so at exactly the same position by papain digestion (Sato and Hattori, unpublished results). In humans, MMP-13 is expressed in skin fibroblasts cultured in the collagen gel (20) and in chronic skin inflammation (21), or chondrocytes in osteoarthritis (23). A further study of papain-treated ASC will provide us with information about the functions of collagen in degrading tissue and applications to therapy.

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REFERENCES


FIGURE LEGENDS

Fig. 1. **SDS-PAGE analysis of isolated collagens.**
Acid-soluble collagen (ASC), pepsin-treated ASC (pepsin), papain-treated ASC (papain) and proctase-treated ASC (proctase) (3 μg/lane) were heat-denatured under reduced condition, and subjected to SDS-PAGE on 5% acrylamide gel containing 3.6 M urea to separate type I and type III collagens, and stained with Coomassie Brilliant Blue. The molecular mass standards are indicated in the left margin. Arrowheads in the right margin indicate the collagen bands.

Fig. 2. **N-terminal sequences of isolated type I collagens.**
Purified acid-soluble type I collagen (ASC), pepsin-treated ASC (pepsin), papain-treated ASC (papain), and proctase-treated ASC (proctase) (10 μg/lane) were heat-denatured under the reduced condition and subjected to SDS-PAGE on 5% acrylamide gel containing 3.6 M urea. Collagens were transferred to PVDF membrane using 6-amino-n-capronic acid buffer, and the membrane was stained with CBB. The collagen bands of α1(I), α2(I), β12(I), β11(I) were cut out and were analyzed by a 491 Procise Protein Sequencer (Applied Biosystems). In A, the amino acid sequences identified in each band are shown. n.t., not tested; n.d., not detected. In B, cleavage sites in each chain are schematically shown, and possible intramolecular and intermolecular crosslinking sites between lysine residues are combined by the bold line. pQ, pyroglutamic acid; O, hydroxyproline.

Fig. 3. **Fibril formation of type I collagen is delayed by pepsin or proctase treatment.**
Type I collagen preparations, ASC (○), pepsin-treated ASC (□), and proctase-treated ASC (△), were suspended in phosphate-buffered saline and incubated at 37°C. Fibril formation of collagen molecules was monitored by measuring the turbidity at 530 nm. After 30 min when the fibril formation reached a plateau, the solution was incubated on ice for 5 min, and the turbidity was measured again at 530 nm.
Fig. 4. **Thermal stability of collagens.**
The CD ellipticity at 221 nm of collagen solutions was monitored on increasing the temperature from 20˚C to 50˚C at a constant rate (0.25˚C/min).  A, ASC; B, pepsin-treated ASC; C, papain-treated ASC; and D, proctase-treated ASC.  Half maximal temperatures are indicated by arrows and $T_m$ values in the margin.

Fig. 5. **Electron micrographs of type I collagens.**
Acid-soluble type I collagen (ASC) (A, E, I), pepsin-treated ASC (B, F, J), papain-treated ASC (C, G, K), and proctase-treated ASC (D, H, L) were allowed to form fibrils and were observed by electron microscopy.  Low-power scanning electron micrographs are shown in A-D (bar, 20 µm), and magnified scanning electron micrographs are shown in E-H (bar, 1 µm).  Transmission electron micrographs are shown in I-L (bar, 100 nm).  The 67-nm staggered arrangements of collagen molecules are indicated by arrowheads.

Fig. 6. **Effect of pepsin-, papain- or proctase-treatment of ASC on relative fibril strength.**
The collagen fibril strength was measured by a modified method of viscosity measurement.  A, ASC; B, pepsin-treated ASC; C, papain-treated ASC; D, proctase-treated ASC.  The concentration of collagen in each tube is indicated as mg/ml.  The three separate experiments were done, and the same results were obtained.
### A

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<th>β12</th>
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* n.t.: not tested  
** n.d.: not detected  

### B

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[Image of test tubes with varying concentrations]
Possible involvement of aminotelopeptide in self-assembly and thermal stability of collagen I as revealed by its removal with protease
Kaori Sato, Tetsuya Ebihara, Eijiro Adachi, Seiichi Kawashima, Shunji Hattori and Shinkichi Irie

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