PHYSICOCHEMICAL PROPERTIES OF COLLAGEN ISOLATED FROM CALF LIMED SPLITS

by

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ABSTRACT

Samples of collagen extracted from calf limed splits by three different methods (acetic acid, pepsin, and alkali treatments) were characterized with respect to their thermal stability, subunit composition, and morphology of fibril network. Differential scanning calorimetry showed that the denaturation temperature of pepsin-treated collagen was very similar to that of commercial type I collagen but much higher than those of acetic acid-treated collagen and alkali-treated collagen. Analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Western blotting revealed that the subunit composition in collagen solution samples appeared to be major type I collagen and minor type III collagen. Scanning electron microscopy showed that acetic acid-treated collagen had a porous fibrillar network structure, while pepsin-treated collagen and alkali-treated collagen had membranous networks resembling that of commercial type I collagen.

INTRODUCTION

In recent years, collagen has been widely used in various products and fields, including foods,[1] cosmetics,[2] medical material and cell culture technology.[3,4,5] Much interest has recently been shown in collagen as a potential biomaterial by researchers in the field of tissue engineering. As a protein, collagen has a specific amino acid sequence, size and structure that give it its basic qualities as a biomaterial suitable for medical products. The attractiveness of collagen as a biomaterial is also largely due to the fact that it is a natural material of low immunogenicity and is therefore recognized by the body as a normal constituent rather than foreign matter.[6] Collagen is different from gelatin. Gelatin has been denatured but collagen still holds its native form. Collagen type I molecules are characterized by a triple helix formation. The triplex consists of two $\alpha 1$ chains and an $\alpha 2$ chain of over 1000 residues in length. Each collagen chain adopts a left-handed helical conformation, and the three strands intertwine with a right-handed superhelical twist. Collagen is generally produced for industrial use from animal pelts. The usual procedure for obtaining collagen from pelts is illustrated in Figure 1.[7]

As shown in Figure 1, there are three methods by which collagen can be obtained from skins: (i) extraction with acid, (ii) digestion with pepsin, and (iii) treatment with alkali. In this study, we examined the chemical and biological properties of collagen obtained by using these methods from calf limed splits, which are by-products of the leather manufacturing process. The purpose of our study was to establish another method to obtain collagen for use as a biomaterial from limed splits instead of unlimed pelts.

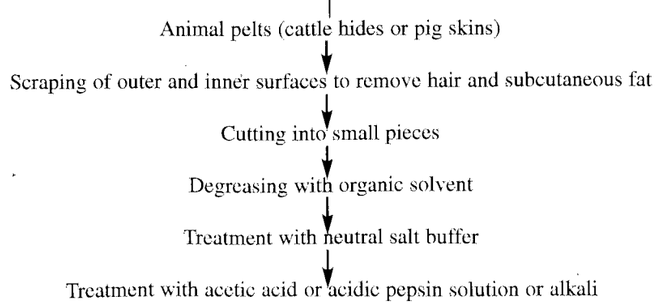

Figure 1.- Procedure for obtaining collagen from animal pelts.

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EXPERIMENTAL

Materials

Pepsin (1:10,000) was produced by Nacalai Tesque Co., Ltd. (Kyoto, Japan). Sodium lauryl sulfate, coomassie brilliant blue R-250, and acrylamide were also obtained from Nacalai. Tris and N,N′-methylenebisacrylamide were supplied by Life Technologies, Inc. (U.S.A.). TEMED was produced by Cibcobrl (Grand Island, N.Y., U.S.A.). Ammonium persulfate was provided by Bio-Rad Co. (U.S.A.). Commercial acid soluble bovine type I collagen (0.5%, pH=3.0) was made from fresh bovine skin by KOKEN Co., Inc. (Tokyo, Japan). Type III collagen was extracted from fresh amnia of cattle embryos by pepsin method in our laboratory. Anti-collagen type III antiserum was prepared from bovine and provided by LB-1387, LSL (Tokyo, Japan).

Preparation of limed splits

Calf skins were collected from a farm and washed thoroughly with water for one day. They were then limed with 1.5% Na_2S and 5% lime powder, calculated on the basis of skin weight, as the usual method used in leather manufacturing to remove hair, at 25°C for 18 hours. The limed skins were then fleshed and split.

Preparation of solubilized collagen

The splits were neutralized with 1.5% HCl, based on the weight of limed splits. The pH of splits was reduced from 12 to 5-6. After washing with water, the splits were cut into pieces and pulverized with a mill (FRITSCH puluterisette 14).

- Acid-solubilized collagen[8]: twenty grams of split pieces was homogenized and stirred in 1000 ml of 0.5 M acetic acid at 4°C for 48 hours.
- Pepsin-digested collagen[9]: twenty grams of split pieces was digested in 1000 ml of 0.5 M acetic acid solution containing 0.4 g pepsin at 4°C for 24 hours.
- Alkali-treated collagen[10]: twenty grams of split pieces was treated in 1000 ml of 30 g NaOH and 19 ml monomethylamine solution at 20°C for 5 days.

The soluble collagen solutions thus obtained were all clarified as follows. The resulting viscous solution was centrifuged at 10,000 rpm for 15 min to remove insoluble substances and salted out at 3 M NaCl, and the solution was then centrifuged at 12,000 rpm for 30 min. The precipitate was dissolved in 0.5 M acetic acid. The solution was then salted out at 0.7 M NaCl and centrifuged at 11,000 rpm for 30 min again, and the precipitate was dissolved in 0.5 M acetic acid again. There was no dialization treatment to these collagen solutions.

Determination of the concentration of collagen in solution

Collagen concentration was determined indirectly from the hydroxyproline concentration, which was analyzed by the method of Bergman and Loxley.[11] The collagen sample was hydrolyzed in 6 M HCl at 110 °C for 24 hours, and after HCl had been evaporated, the hydrolysate was dissolved in acetate/citrate buffer (pH 6.8) for the assay. An absorbance of 1.40 corresponded to 40 mg/ml of hydroxyproline at 558 nm, and the collagen content was calculated by multiplying the hydroxyproline content by 7.52.

Determination of denaturation temperature

A 20-µl portion of the collagen solution was sealed in an aluminium cell. The thermal denaturation curve of collagen solution was obtained by using a DSC apparatus (Shimadzu Differential Scanning Calorimeter DT-40) under the following conditions: heating rate, 5 °C/min; sensitivity, 3.0 mJ/s.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 5% gels containing 3.6 M urea. Collagen samples were heated at 100°C for three minutes in 0.5M Tris-HCl buffer (pH 6.8) containing 3.6 M urea, 1% SDS, 10% glycerol and 0.01% bromophenol blue. Gels were stained for 30 minutes with 0.25% comassie brilliant blue R-250 solution and destained with 7.5% acetic acid/5% methanol solution. The percent distribution of collagen among the different bands as absorption intensity was determined by scanning the gel using a dual wavelength flying spot scanner (Shimadzu CS-9000).

Identification by Western blotting analysis

The separated bands of collagen samples on SDS-PAGE were electroblotted onto a nitrocellulose membrane by Trans-Blot (SD, Bio-Rad). Before the collagen type III antibody reacted randomly with the bands on the membrane, a blocking solution (3% casein, 1% BSA and 0.05% NaN_3 in Tween 20-PBS solution) was used to prevent nonspecific reactions. The transferred proteins were visualized immunochemically with DAB after reaction with peroxydase-conjugated goat anti-rabbit IgG.

Scanning electron microscopy

The morphological characteristics of collagen solutions produced by the different methods were studied by scanning electron microscopy. Collagen solution was dropped on the surface of a circular piece of glass of about 10 mm in diam-

eter and prefixed in 2.5% glutaraldehyde in a 0.2 M phosphate buffer solution (pH=7.4) for more than 2 days at 4°C. The specimens on the pieces of glass were immersed in a 1% aqueous solution of tannic acid for 2 hours and rinsed in distilled water for several hours, and then fixed in a 1% aqueous solution of OsO_4 for 2 hours. The specimens were dehydrated in a series of ethanol concentrations, put into t-butyl alcohol and dried in a freeze dryer (EYELA FD-1). The dried specimens were mounted on metal stubs with double-face adhesive tape and then coated with gold using a sputter coater (Osmium Plasma Coater). The samples were then introduced into the specimen chamber of a scanning electron microscope (JEOL JSM-6301F) with an accelerating voltage of 5 kV, and the surface morphology was examined.

Results And Discussions

Extractability of collagen from limed split

We compared the results obtained by using several methods for solubilizing collagen from calf limed splits. The results are shown in Table I.

TABLE I
Extractability of Collagen from Calf Limed Splits by Different Methods

Extraction Method	Extraction Time, days	Extracted Collagen fromLimed Splits, %
Acetic Acid	2	54
Pepsin	1	86
Alkali	5	88

About half of the collagen could be extracted from a calf limed split by acetic acid treatment, while most of the collagen was solubilized by both pepsin treatment and alkali treatment. However, since the time required for pepsin treatment (one day) was much less than that required for alkali treatment (five days), pepsin treatment is a better method than either alkali treatment or acetic acid treatment for producing a large amount of collagen solution from limed splits.

Thermal stability of collagen

The thermal denaturation curves of different collagen solutions are shown in Figure 2.

The peak values of denaturation temperature (Td) obtained from DSC curves are summarized in Table II. The table shows that the thermal stability of collagen solution increases in the following order: alkali-treated collagen, acetic acid-treated collagen, pepsin-treated collagen and commercial type I collagen.

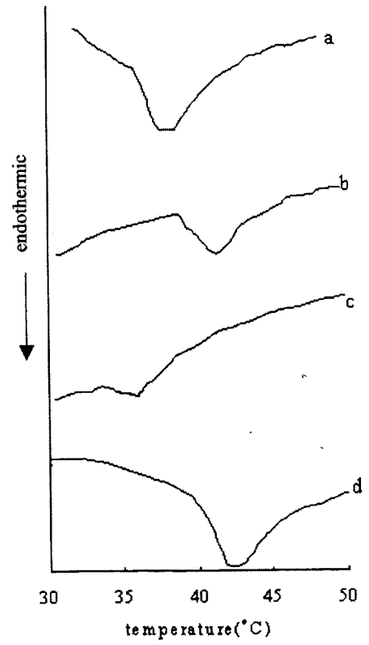

Figure 2. - DSC curves of (a) acetic acid-treated collagen, (b) pepsin-treated collagen, (c) alkali-treated collagen and (d) commercial type I collagen.

TABLE II
Td Values of Collagen Extracted from Limed Splits

Extraction Method	Denaturation Temperature Td, °C
Acetic acid-treated collagen	38
Pepsin-treated collagen	41.5
Alkali-treated collagen	36
Commercial type I collagen	42

As shown in Figure 2 and Table II, pepsin-treated collagen has a Td of 41.5 °C and commercial type I collagen has a Td of 42°C, indicating that pepsin-treated collagen and commercial type I collagen have almost the same degrees of resistance to heat denaturation. However, Td of acetic acid-treated collagen and that of alkali-treated collagen were 38°C and 36°C, respectively, about 4°C and 6°C lower, respectively, than that of commercial type I collagen.

Characterization of SDS-PAGE electrophoresis of collagen

Figure 3 shows the electrophoresis patterns of the commercial collagen and collagen samples isolated from limed splits on sodium dodecyl sulfate-polyacrylamide gel. All of these collagen samples displayed two α bands (α1 and α2), one or two β bands (β11 and β12) and one or two γ bands. The molecular weights of α1 and α2 were both about

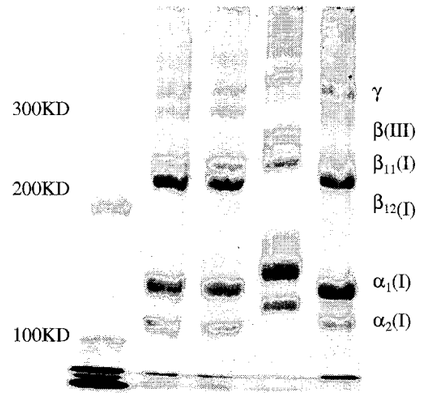

Figure 3.- SDS-PAGE electrophoresis of a) SDS-PAGE molecular weight standards, b) acetic acid-treated collagen, c) pepsin-treated collagen, d) alkali-treated collagen and e) commercial type I collagen.

100,000, and the molecular weights of β and γ were about two-times and three-times greater than that of α, respectively. The α, β and γ chains of acetic acid-treated collagen and pepsin-treated collagen showed the same migration patterns as that of commercial type I collagen. However, the migration of α, β and γ chains of alkali-treated collagen was significantly slower than that of commercial type I collagen, and moreover, alkali-treated collagen had three β bands, respectively β(III), β_{11}(I), β_{12}(I). The result showed that type III collagen existed in alkali-treated collagen solution.

In order to determine the relative ratios of subunits in collagen solutions, the percent distributions of collagen in the different SDS-PAGE bands were also calculated. One-dimensional densitograms of SDS-PAGE electrophoresis for these collagen samples are shown in Figure 4. The densitogrames show the percentage distributions of α, β and γ bands.

The weight ratios of the subunit chains for various kinds of collagen were calculated from the area percentages of peaks illustrated in Figure 4, and the results are shown in Table III.

The weight ratio of β to α for pepsin-treated collagen was 0.53, but the ratio of β to α for acetic acid-treated collagen was about two-times larger than that for pepsin-treated col

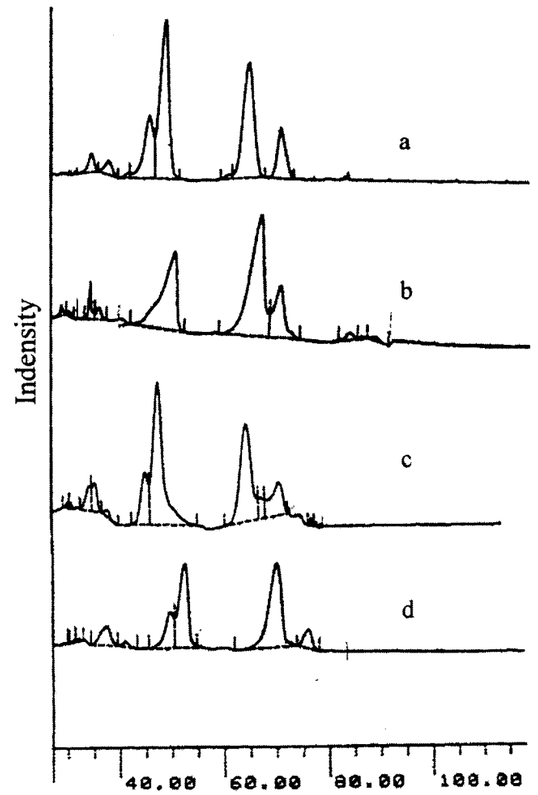

Figure 4.- One-dimensional densitograms of SDS-PAGE electrophoresis for acetic acid-treated collagen (a), pepsin-treated collagen (b), alkali-treated collagen (c) and commercial type I collagen (d).

lagen. The ratio of β to α for alkali-treated collagen was 1.38, which was also much higher than that for pepsin-treated collagen. The weight ratios of α_1 to α_2, β to α and γ to α for pepsin-treated collagen were similar to those ratios for commercial type I collagen.

Western blotting analysis

Since the SDS-PAGE pattern of type III collagen was

TABLE III
Weight Ratios of Subunit Components for Various Collagen Solutions

Collagen Sample	Ratio of subunit component		
	α_1/α_2	β/α	γ/α
Acetic acid-treated collagen	3.05	1.26	0.15
Pepsin-treated collagen	2.97	0.53	0.18
Alkali-treated collagen	4.01	1.38	0.13
Commercial type I collagen	2.79	0.80	0.19

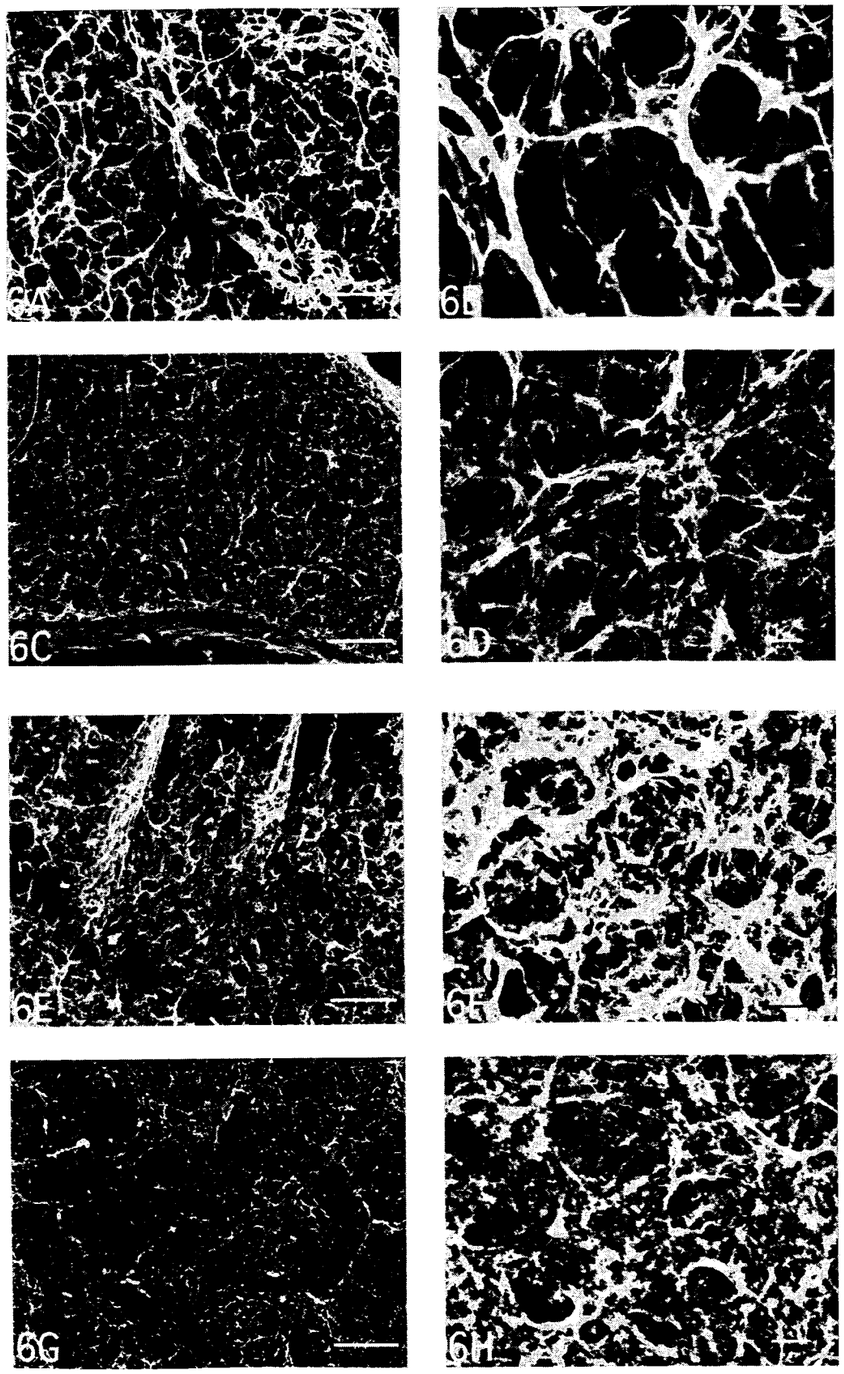

Figure 6.- Scanning electron microscopy of acetic acid-treated collagen (Fig. 6A and 6B), pepsin-treated collagen (Fig. 6C and 6D), alkali-treated collagen (Fig. 6E and 6F) and commercial type I collagen (Fig. 6G and 6H), at magnification of 10000x (Fig. 6A, 6C, 6E and 6G, bars: 10 μm) and 5000´ (Fig. 6B, 6D, 6F and 6H, bars: 1 μm).

almost the same as that of type I collagen, the collagen samples were electroblotted from SDS-PAGE gel onto a nitrocellulose membrane and then reacted with an anti-type III collagen antibody. The bands of Western blotting analysis are shown in Figure 5.

The results of Western blotting analysis showed that there was a small amount of type III collagen in the different collagen samples derived from limed splits. However, there was no α band, and only β and γ bands were found in type III collagen of different collagen samples. Moreover, the amounts of type III collagen in acetic acid-treated collagen and pepsin-treated collagen solutions were less than that in alkali-treated collagen solution.

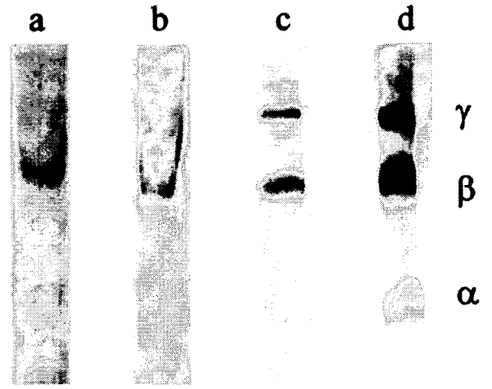

Figure 5.- Immunoblotting analysis of type III collagen in a) acetic acid-treated collagen (60 μl), b) pepsin-treated collagen (60 μl), c) alkali-treated collagen (30 μl) and d) commercial type I collagen (30 μl).

Observation by scanning electron microscopy

Observation of the appearance and network structure of the collagen samples isolated from limed splits was carried out by scanning electron microscopy (SEM), and SEM images are shown in Figure 6. SEM images of the collagen sample extracted by acetic acid (Figure 6A and 6B) showed that the fibrous texture had a fibril network with a porous structure. As shown in Figure 6C, 6D, 6E and 6F, pepsin-extracted collagen and alkali-treated collagen appeared to have fibrous networks with membranous structures, features similar to those of the fibrous structure of commercial type I collagen (Figure 6G and 6H).

CONCLUSIONS

The collagen solutions obtained from calf limed splits, which were characterized with DSC, SDS-PAGE and SEM, were found to have physicochemical properties similar to those of commercial type I collagen obtained from cattle pelts. The features of collagen solution extracted by the pepsin method, a method that not only enables more collagen to be extracted but also takes less time than the acetic acid method and alkali method, were almost the same as those of commercial type I collagen. However, in order to reuse collagen isolated from limed splits as a biomaterial, the cell biochemical properties of collagen, such as collagen fibril formation and activity for cell adhesion, must be elucidated. Studies aimed at elucidation of the cell biochemical properties of collagen isolated from limed splits are in progress in our laboratory and the results will be present in a future paper.

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