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Characterization of Collagen from the Swimbladder of Catfish (*Tachysurus maculatus*)

C. ROSE[1], A.B. MANDAL[2] and K. T. JOSEPH[1]

[1]Division of Biological Sciences and
[2]Division of Chemical Sciences
Central Leather Research Institute
Chennai 600 020, India

Abstract

Collagen was isolated from swimbladder tissue of *Tachysurus maculatus* by acid extraction. Electrophoresis on SDS-PAGE showed that the α_1 and α_2 chains are present in the molar ratio of 2:1. Chromatography on CM-cellulose, in addition to confirming the chain composition, showed the presence of reduced amounts of the crosslinked dimers β_{11} and β_{12} after the collagen was denatured. Thus, the native collagen molecule in this study has the formula, $(\alpha_1)_2\ \alpha_2$ and corresponds to a Type I collagen. Molecular sieve chromatography gave an elution profile similar to rat tail tendon (RTT) collagen. Amino acid composition showed a higher content of Met and lower content of OH-Pro than mammalian collagen. The shrinkage temperature (T_s) of the swimbladder tissue was 54.5°C. The single peak obtained around T_s by hydrothermal isometric tension (HIT) measurements indicated the presence of only heat-labile cross-links.

Introduction

Fish collagen showed comparatively less Pro and OH-Pro than mammalian collagen (Piez and Gross 1960), which was held responsible for the reduced shrinkage temperature (Doty and Nishihara 1958). The cross-linking of individual chains within the molecule was also reported to determine the stability of collagen. Miller *et al.* (1967) showed that the cross-linked components like β_{11} and β_{12} were absent when the Type I collagen of tissue from a lathyritic animal was chromatographed on CM-cellulose. Additionally, Naito *et al.* (1994) confirmed by NMR that the interchain Gly-NH...O=C-X_{aa} or Pro hydrogen-bond is believed to be essential for stabilizing the coiled-coil triple helix conformation. These stabilizing forces are affected by physical (Fujimori 1966; Bailey *et al.* 1974) chemical or thermal treatments (Burges and Hynes 1959; Steven and Tristram 1962; Rose *et al.* 1988; Dombi *et al.* 1993; Rose and Mandal 1996), and such forces, including the intermolecular cross-links, determine the solubility of collagen (Tanzer *et al.* 1973). Recently (Dombi *et al.* 1993), the total insoluble collagen in rat skin has been correlated with the extent of collagen cross-links and in turn with the tensile strength of the tissue.

According to preliminary studies carried out in this laboratory, the swimbladder of catfish (*Tachysurus maculatus*) was mainly collagen and was highly soluble in an acetic acid solution. In India, enormous quantities of

catfish (*Tachysurus species*) are available from both inland and marine waters. The swimbladder, which constitutes about 3-5% of the total body weight of the fish, is presently collected during processing and exported to other Asian countries for use in soup, etc.

Although reports are available on the amino acid composition, stability (Piez and Gross 1960) and the type of cross-links (Bailey 1970) in fish collagen from several fish species, information on collagen of warm-water catfish seems to be limited. The present study is directed towards determining the properties of swimbladder collagen.

Materials and Methods

Swimbladders

Marine catfish (*Tachysurus maculatus*) from the Bay of Bengal (Marina Beach, Madras) were dissected and their swimbladders removed, cleaned and washed thoroughly with cold distilled water. The fish were caught during the fall season.

Extraction and Purification of Collagen

The cleaned swimbladders were cut into small pieces (about 5 x 5 mm) and the collagen extracted according to the method of Piez *et al.* (1963) using 0.5 M acetic acid at 4°C for 48 h. After extraction, it was clarified by centrifugation at 15,000 rpm for 20 min (IEC refrigerated centrifuge, Bombay, India) and the supernatent collected.

The soluble collagen was salted out using NaCl (5% w/v) and the precipitate was collected by centrifugation. The precipitate was redissolved in 0.5 M acetic acid, and these steps were repeated once. The collagen in 0.5 M acetic acid was exhaustively dialyzed against 0.02 M Na_2HPO_4 in a pre-washed dialysis tubing until precipitation was complete. After centrifugation, collagen was dissolved in 0.5 M acetic acid, and dialyzed against 0.05 M acetic acid before being lyophilized. Rat tail tendon of albino rats (King Institute, Madras, India) were separated and the collagen was extracted using 0.5 M acetic acid as done for fish collagen. The lyophilized collagen sample was used for comparision studies with fish collagen.

CM-cellulose chromatography

Denatured collagen was fractionated on CM-52 (Whatman, Maidstone, Kent, England) at 45°C according to Piez et al (1963). Prior to this, the column was equilibrated with a 0.02 M sodium acetate buffer containg 1 M urea (Sigma, St. Louis, MO, USA), pH 4.8. Lyophilized collagen was dissolved at 5 $mg.ml^{-1}$ in the same buffer warmed to 45°C for 30 min and then applied to a water jacketed column (1.6 x 10 cm I.D.). After initially washing the column with buffer, the bound collagen chains were eluted using a linear gradient from 0 to 0.1 M NaCl over a total volume of 800 ml. The flow rate

was 120 ml·h^{-1}. The effluent was monitored with a UV monitor (LKB 2238 UVICORD SII, Uppsala, Sweden) at 230 nm. Absorbance of the collected fractions was measured at 230 nm using a 1 cm cuvette (Spectronic 21, Bausch & Lomb, Rochester, New York, U.S.A).

SDS-PAGE

Lyophilized catfish collagen, after denaturation, was separated on 5% polyacrylamide gels using a vertical slab gel electrophoresis system (Hoefer Scientific Instruments, San Francisco, California, USA) in Tris-glycine buffer, pH 8.3 containing 0.2% (w/v) SDS according to the method of Laemmli (1970). The chemicals used were of electrophoresis grade from Sigma. After completion of the electrophoretic run, the gel was removed from the plates and immersed for about 1 h at room temperature in a staining solution consisting of 50% (v/v) methanol, 10% (v/v) acetic acid, 40% (v/v) water and 0.25% Coomassie Brilliant Blue, with moderate shaking. Destaining was carried out overnight in a solution of methanol (10% v/v) - acetic acid (7% v/v) - water (83% v/v). The gel was then photographed.

The gel was also scanned starting from the sample application end using a GS 300 Transmittance Scanning Densitometer (Hoefer Scientific Instruments, San Francisco, California, USA) at a speed of 13 cm.minute^{-1} and the % transmission was recorded simultaneously in a chart recorder.

Molecular sieve chromatograph

The catfish swimbladder collagen was fractionated on Ultrogel ACA-22 (LKB, Uppsala, Sweden) gel filtration medium, (2% polyacrylamide and 2% agarose) as described by Kimura *et al.* (1981). The sample was dissolved in 2 ml of 0.05 M Tris-HCl, pH 7.5, containing 4 M urea. The mixture after denaturation at 45°C for 30 min was applied to a column (1.6 x 90 cm ID) and eluted at 5.6 ml.h^{-1} with the same buffer. Fractions of 2.8 ml were collected and the absorbance determined at 230 nm. Appropriate fractions were pooled and dialysed against distilled water.

Amino acid analysis

Amino acid analysis was performed on an automatic amino acid analyzer (LKB, B alpha Model 4151, Uppsala, Sweden) after the hydrolysis of fish collagen in 6 N HCl at 110°C for about 20 h in a sealed tube and preparation of sample according to Miller *et al.* (1969). However, the OH-Pro content was determined by the method of Neuman and Logan (1950).

Determination of carbohydrate

Collagen was hydrolyzed with 0.5 N sulphuric acid at 100°C in a sealed tube for 4 h. Total carbohydrate content of the suitably diluted hydrolysate was determined by the phenol - sulphuric acid method (Dubois *et al.* 1956).

Measurement of hydrothermal isometric tension (HIT)

Two fish weighing 1.5 kg (about 30 cm long) and 4.5 kg (about 90 cm long), respectively, were cut open immediately after landing and their bladders removed. It was then carefully freed of other adhering tissues and stored at -20°C until use. The bladder samples from each of the above said fish (small and big) were taken for HIT measurements using an Instron tensile testing machine (Model 1112 Instron Ltd, Bucks, HP, UK.) A specimen of bladder 4mm width and 10mm long was punched out using a dye, paralleled to the backbone.The specimen of tissue was clamped between two holders then immersed in distilled water bath. Minimum tension was appplied and the sample was allowed to relax. When the stress relaxation became steady, the temperature was raised from 25° to 100°C, at $3°C \cdot min^{-1}$ or until the tissue broke.

Results and Discussion

Characterization by SDS-PAGE

The nature of the collagen fractions was evaluated by SDS-PAGE. As shown in Fig. 1, the presence of two α chain components, designated α_1 and α_2, and of two ß chains, $ß_{11}$ and $ß_{12}$, was evident. The electrophoretic pattern of these chains was comparable to that of rat tail tendon (RTT) collagen, which has been reported as Type I (Piez *et al.* 1963). Octopus skin collagen, which has been characterized as a Type I-like collagen (Kimura *et al.* 1981), also showed a similar pattern. Recently, Type I collagen have also been identified from other fish and reported (Kimura *et al.* 1988; Matsui *et al* 1989). The α_1 chain of fish collagen was more mobile than the RTT α_1 chain. The α_1 and α_2 chains of acid extracted from catfish swimbladder collagen have a densitometric ratio of approximately 2:1 (seen in Fig. 2), suggesting that the collagen is of Type I nature.

Characterization by ion-exchange chromatography

Catfish swimbladder collagen, after denaturation, was chromatographed on CM-cellulose. A chromatogram (Fig. 3) shows the presence of both the α_1 and α_2 chains. The shoulder following the α_1 peak was found to contain $ß_{11}$. The other ß chain, $ß_{12}$, eluted in the small peak preceeding the a peak. Identification of both a and ß peaks was based on their elution pattern as reported by Piez *et al.* (1963) for RTT Type I collagen and Kimura *et al.* (1981) for octopus collagen. The chromatographic and electrophoretic properties of all components are very similar to those of vertebrate Type I collagens as reported by Piez *et al.* (1963) and Furthmayr and Timpl (1971).

Molecular weight determination

The elution profile of denatured catfish swimbladder collagen on molecular sieve chromatography is shown in Fig. 4. The column was initially calibrated

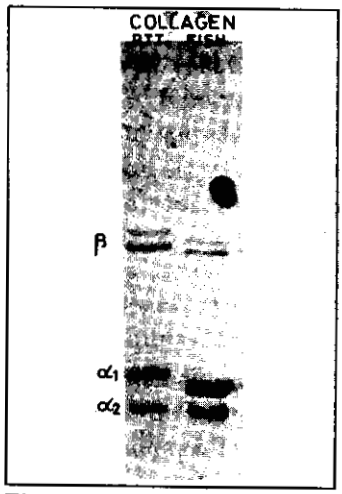

Fig. 1. SDS-PAGE of catfish collagen on 5% gel.
(a) Rat tail tendon Type I collagen
(b) Catfish swimbladder collagen

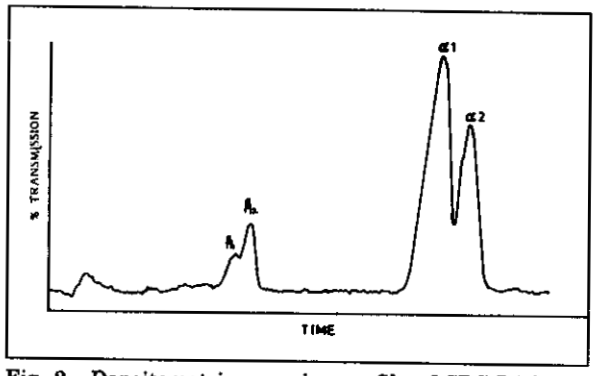

Fig. 2. Densitometric scanning profile of SDS-PAGE of catfish swimbladder collagen.

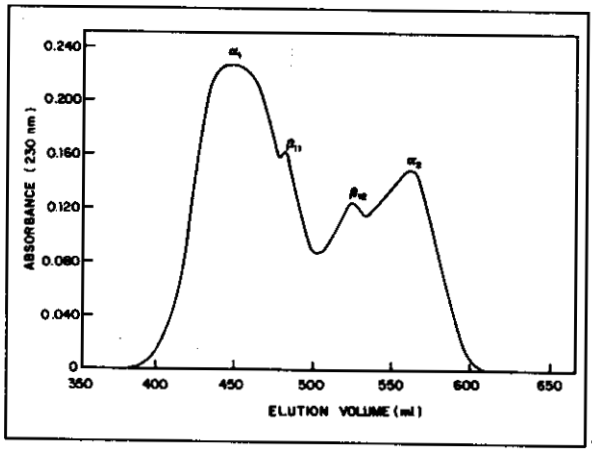

Fig. 3. CM-Cellulose chromatography of catfish swimbladder collagen.

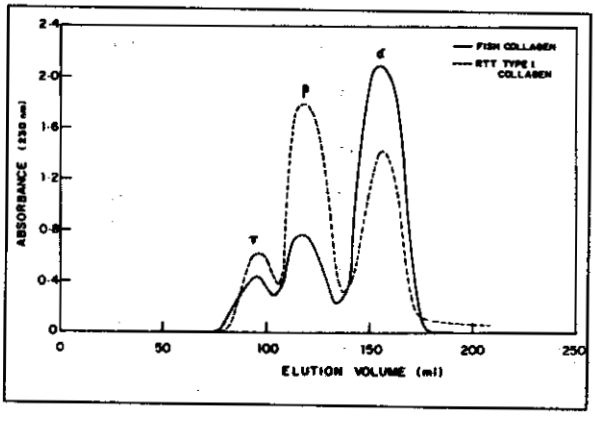

Fig. 4. Molecular sieve chromatography of catfish swimbladder collagen.

with denatured RTT Type I collagen. The α-components as well as the dimeric (ß) and trimeric (γ) components eluted in the corresponding regions of RTT collagen suggesting that the α chain of catfish collagen has a molecular weight of approximately 100,000 daltons similar to that of RTT. Similar results have been reported for the collagen of carp swimbladder (Gallop 1955a, b). Another

observation in this study was the significant reduction in the areas under the cross-linked components (ß and γ) and a corresponding increase in the peak area of monomeric component (a), when compared with that of RTT collagen (Fig. 4), which has been used as the standard Type I collagen as it has been well characterized (Piez *et al.* 1963). The reduced amount of cross-linked components was an indirect index of intramolecular urea susceptible bonds (Whitney and Tonford 1962).

Amino acid analysis

Table 1 shows the amino acid composition of catfish swimbladder collagen. The amino acid composition of dogfish shark skin (Lewis and Peiz 1964) and calf skin collagens are also presented in Table 1 for reasons of comparison. The total content of Pro and OH-Pro obtained for catfish swimbladder is similar to that reported for carp swimbladder (197/1000 residues) by Doty and Nishihara (1958) and for pike skin (199/1000 residues) by Gustavson (1956), whereas for cod (Lewis and Piez 1964) and halibut (Gustavson 1956) collagens, these values were 160/1000 residues and 171/1000 residues, respectively. A high amino acid content of 232/1000 residues has been reported for calf skin collagen. These investigators and Harrington and Von Hippel (1961) have also suggested that the variation in the amino acid content is reflected by a change in the shrinkage temperature. Comparing these values, the catfish collagen shows higher amino acid content than the cold water fish and lower level than the calf skin collagen.

TABLE 1. Amino acid composition of collagen (residues per 1000 residue).

Amino acids	Catfish bladder	Dogfish shark[1]	Calf skin[2]
Alanine	121.0	110.0	112.0
Glycine	339.0	339.0	320.0
Valine	11.4	27.8	20.0
Leucine	19.4	25.5	25.0
Isoleucine	9.5	16.7	11.0
Proline 108.0	99.0	138.0	
Phenylalanine	17.5	12.4	13.0
Tyrosine	10.0	2.7	2.6
Serine	24.1	59.0	36.0
Threonine	22.9	24.0	18.0
Methionine	15.0	15.7	4.3
Arginine 43.2	53.0	50.0	
Histidine	7.3	11.7	5.0
Lysine 31.0	25.7	27.0	
Aspartic Acid 35.4	44.0	45.0	
Glutamic acid	84.1	69.0	72.0
Hydroxyproline	90.0	60.0	94.0
Hydroxylysine	11.3	6.3	7.4
Total	1000.1	1001.5	1000.3

[1]Lewis, M.S and K.A. Piez (1964).
[2]Doty, P and T. Nishihara (1958).

Piez and Gross (1960) suggested that the few hydroxyl groups of OH-Pro are compensated for by an equal increase in Ser and Thr to maintain a nearly constant number of hydroxyl groups, which maintains the structural conformation of the collagen molecule. The Thr and Ser content of catfish collagen is comparable to that in carp (Doty and Nishihara 1958; Piez *et al.* 1963) and the total number (137) of hydroxyl groups is also comparable to carp swimbladder. A significantly higher content of met residues was observed compared to mammalian collagen. A decreased level of hydrophobic amino acids (Val, Leu and Isl) was also observed, a factor which may render the collagen molecules more susceptible to urea denaturation. It is generally accepted that urea acts on hydrophobic links in addition to hydrogen bonds (Whitney and Tanford 1962). A decreased amount of Arg is associated with an increase in the other basic amino acids, namely, Lys, OH-Lys and His. The proportion of Gly and Ala are close to the generalization (Eastoe and Leach 1958) of one Gly for every three residues and one Ala for every nine residues. These values are similar to those for sturgeon swimbladder (Eastoe 1957). The catfish collagen was considered fairly pure based on its carbohydrate content (0.45%). According to Kubota and Kimura (1975), collagen with 0.3-0.7% hexose was considered to be pure. The increased Tyr content (10/1000 residues) might have come from the non-helical ends as the collagen was extracted non-enzymatically.

Collagen cross-linking and aging in catfish swimbladder

The hydrothermal isometric tension (HIT) curves obtained for catfish swimbladder showed both similarities and differences when compared with the HIT results obtained for skins of various fishes (Cohen-Solal *et al.* 1981) and rat, with increasing age from birth to 18 months (Allain *et al.* 1978). The bladder tissue of smaller fish (weighing 1.5 Kg) displayed only one maximum, i.e., a small peak (Fig. 5a) at the shrinkage temperature (55°C). But the skins of carp, catfish, sole and pike displayed two maxima, one at 51-65°C and one towards 100°C, which represent the presence of heat-labile and heat-stable bonds, respectively (Cohen-Solal *et al.* 1981). However, the skins of older fish - carp, plaice, dogfish (>2 years) studied by Cohen-Solal *et al.* (1981) had a single peak with tension dropping almost to zero well below 100°C.

For the bladder tissue of bigger catfish (weighing 4.5 Kg), no clear peak was observed and the tension started to drop close to the shrinkage point and reached almost zero by 68°C (Fig. 5b). From these studies, it can be suggested that the collagen of catfish bladder is much less cross-linked when compared to the skin collagen of other fishes (Cohen-Solal *et al.* 1981) and rat skin collagen (Allain *et al.* 1978). Also, the failure to exhibit a clear peak by this tissue (Fig. 5b) may be due to the presence of relatively less amount of reducible cross-links compared to the bladder tissue of smaller fish (Fig. 5a). This is dependent on age, tissue and, in particular, species (Robins *et al.* 1973; Allain *et al.* 1978; Cohen-Solal *et al.* 1981).

The Schiff base (aldimine bond) is the most common cross-link found in collagen. The proportion of these reducible cross-links increases rapidly during growth in mammals. As the growth rate slows down, these bonds decrease

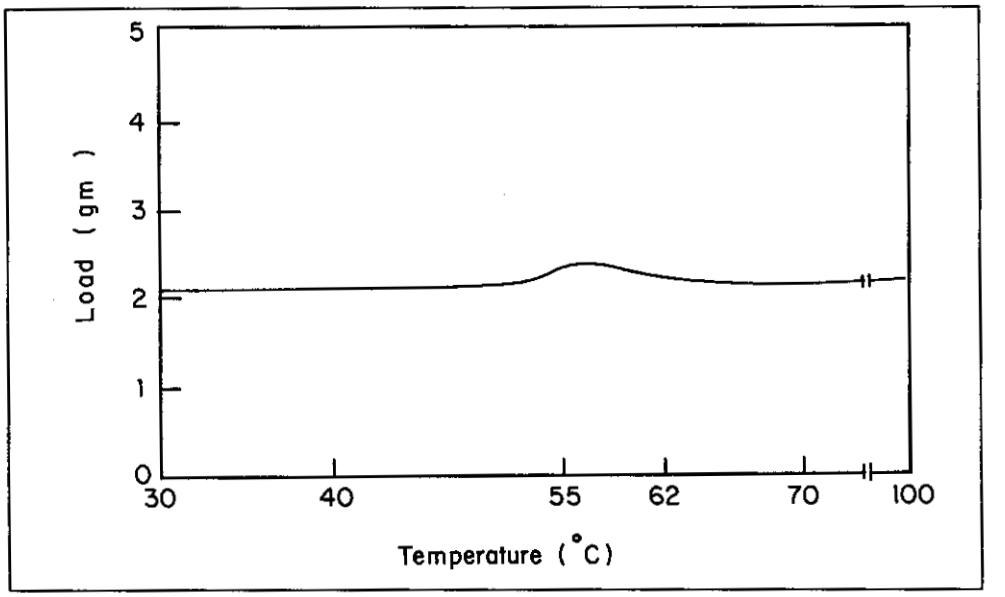

Fig. 5(a) Hydrothermal isometric tension of swimbladder tissue of smaller catfish (weight 1.5 kg).

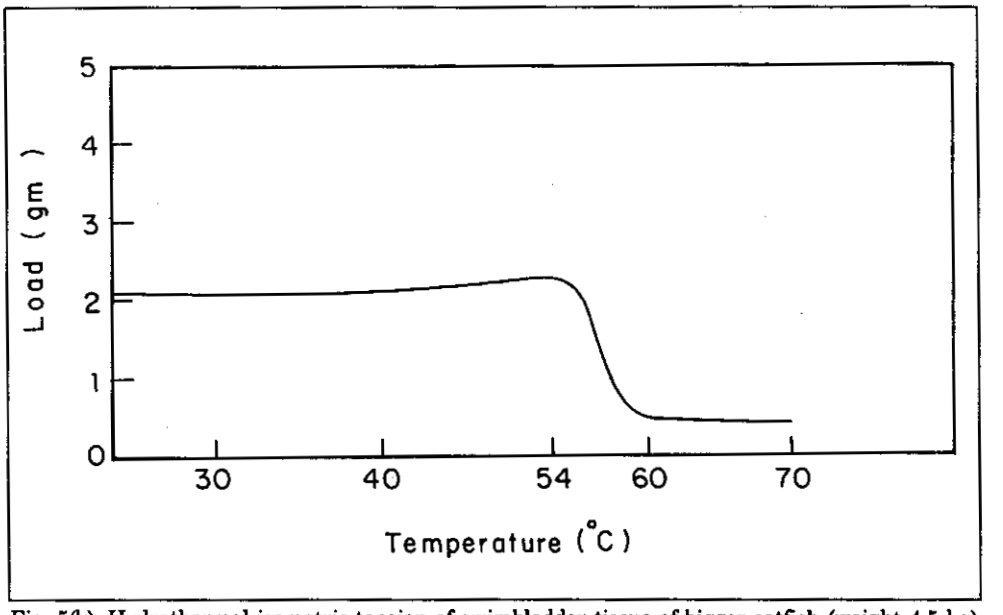

Fig. 5(b). Hydrothermal isometric tension of swimbladder tissue of bigger catfish (weight 4.5 kg).

until maturity and then remain at a low value or disappear alogeth er (Robins *et al.* 1973). The results for catfish bladders were in good agreement with the above studies, i.e. the proportion of reducible cross-links was higher for smaller (younger) fish compared to the bigger (older) one. The peak obtained for the smaller fish may be due to the destruction of small amounts of heat labile reducible cross-links as reported by Allain *et al.* (1978). The second maximum, which is attributed to the presence of heat-stable reducible cross-links (Cohen-Solal *et al.* 1981) was not obtained. Since no second peak was obtained, the heat-stable bonds are apparently not present in the catfish bladders studied.

The absence of cross-links, therefore, leads to the high solubility (>90%) of catfish swimbladder in acetic acid solution. These observations are in good

agreement with earlier reports on other species (Cohen-Solal *et al.* 1981; Robins *et al.* 1973; Dombi *et al.* 1993).

Conclusion

Catfish are an abundantly available species in Indian waters. Their swimbladder is a rich source of Type I collagen. It is highly soluble in dilute acid solution and therefore result in the high yield of collagen, probably due to the absence of heat-stable cross-links. Collagen, in general, is widely used in the field of food, cosmetics, medical and so on. Various physical forms of collagen and their clinical applications have been reviewed by Chvapil (1977). Therefore, catfish collagen as such, or, if necessary, in its suitably modified form, could be used in these fields. For instance, the required degree of stability which is desirable for medical application has been achieved by introducing crosslinks (Chvapil *et al.* 1973) by treating collagen with tanning agents or gamma irradiation. The telopeptide region attached to the collagen extracted by acid solubilisation could be removed by enzyme treatment (Schmitt *et al.* 1964) to obtain a relatively less immunogenic collagen. Apart from medical use, collagen, in its soluble form, is widely used in cosmetic formulations and beer clarification. Moreover, the adequate availability of the starting material would mean adequate inexpensiveness of the end product for widespread utilization.

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