Studies on Collagenase of Fish—I.
Existence of Collagenolytic Enzyme in Pyloric Caeca of Seriola quinqueradiata

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A collagenolytic activity capable of degrading native collagen under non-denaturing conditions was detected in the pyloric caeca of yellow-tail, Seriola quinqueradiata. The pyloric caecum enzyme was capable of reducing the specific viscosity of carp skin collagen at pH 7.5 and 20°C. Crude enzyme extracts from the pyloric caeca, which is a digestive organ, exhibited significant neutral protease activity. The effects of α-chymotrypsin and trypsin on the viscosity of collagen were rather small. However, the pyloric caecum enzyme produced marked viscosity reduction in collagen preparations. This indicates that a collagenolytic enzyme, distinct from α-chymotrypsin and trypsin, may exist in the pyloric caecum extracts.

The enzyme preparation was also capable of degrading native, reconstituted, carp skin collagen fibrils at pH 7.5 and 25°C, and insoluble bovine tendon collagen at pH 7.5 and 30°C. α-Chymotrypsin and trypsin, each at a concentration equal to the amount of total protein in the enzyme preparation, had practically no effect on the release of bound hydroxyproline from collagen fibrils. The results corroborate the existence of a collagenolytic enzyme in the pyloric caeca of yellow-tail.

The first animal collagenolytic enzyme capable of degrading native collagen at neutral pH was demonstrated in a culture of tadpole tissue by Gross and Lapierre1). Subsequently, similar collagenases have been isolated and characterized from tadpole tail fin2–3), rat uterus4) and skin5), epithelium and mesenchyme of healing rabbit wound6), normal human skin7), and rheumatoid synovial tissue8–9). Generally, these enzymes can be obtained only by tissue culture techniques; the two exceptions being human granulocyte collagenase10) and crustacean hepatopancreas collagenase11) which have been extracted directly from cells and tissue. All these enzymes are able to attack native collagen in a highly specific manner at neutral or slightly alkaline pH.

Many fishes, especially carnivorous ones, feed on animal tissues containing collagen as a constituent protein. Since certain proteases such as trypsin, chymotrypsin, and pepsin are unable to attack the native collagen helix under non-denaturing conditions, it seems reasonable to assume that a specific enzyme capable of degrading native collagen may be present in the digestive organs of these fishes. A true collagenase functioning as a digestive enzyme has only been demonstrated in the hepatopancreas of a crustacean, Uca pugilator, by Eisen and Jeffrey12). The presence of collagenase has not been reported...
hitherto in fish.

This paper reports the existence of a collagenolytic enzyme in the pyloric caeca of yellow-tail, *Seriola quinqueradiata*.

**Materials and Methods**

**Reagents.** Trypsin (2× crystallized, from bovine pancreas), α-chymotrypsin (3× crystallized, from bovine pancreas) and bovine Achilles tendon collagen were purchased from Sigma Chemical Company, U.S.A., and casein (acc. to Hammarsten) from E. Merk, Germany. Other reagents used were of analytical grade.

**Preparation of enzyme from the pyloric caeca of yellow-tail.** Acetone powder was prepared from the pyloric caeca of fresh yellow-tail (about 1 kg in body weight) and stored in a refrigerator. The acetone powder stocked was homogenized with cold 0.05 M Tris-HCl buffer, pH 7.5, containing 0.005 M CaCl₂. The homogenate was centrifuged at 10,000× g for 30 min. The supernatant was filtered through cotton to remove the lipid material present at the surface and dialyzed against the same buffer overnight in a cold room. This was then assayed for collagenolytic and caseinolytic activities. In the case of assay for collagenolytic activity by viscometric method, the enzyme was prepared as follows. The pyloric caeca were homogenized with 6 volumes of cold 0.05 M Tris-HCl buffer, pH 7.5, containing 0.005 M CaCl₂ and 0.4 M NaCl, and centrifuged at 10,000× g for 30 min. The supernatant was filtered through cotton to remove the lipid material present at the surface and assayed for collagenolytic activity by viscometric method.

**Isolation of acid-extracted carp skin collagen.** The skin from carp, *Cyprinus carpio*, (about 300 g in body weight) was used as the source of collagen. All operations were performed at about 5°C. The skin was cut into small pieces and washed with a large volume of water. The washed skin pieces were initially extracted three times in 1.0 M NaCl to remove neutral salt extractable fraction. The residue was then extracted with 100 volumes of 0.1 M acetic acid for 24 hr with stirring. The extract was clarified by centrifugation at 10,000× g for 30 min and the collagen was precipitated by dialysis against 0.01 M disodium phosphate for 48 hr. The resultant collagen precipitate was separated by centrifugation and redissolved in 0.1 M acetic acid with gentle stirring. After centrifugation at 18,000× g for 30 min, the collagen solution was again dialyzed against several changes of 0.01 M disodium phosphate for 48 hr. The collagen precipitate was collected by centrifugation and stored at −20°C until used.

**Preparation of collagen substrate.** The acid-extracted carp skin collagen stocked at −20°C was redissolved in 0.1 M acetic acid with stirring to a final concentration of about 0.2%, then dialyzed against several changes of phosphate buffer (pH 7.6, I=0.4) for 3 days and against a large volume of 0.4 M NaCl for 24 hr, followed by centrifugation
at 29,000×g for 1 hr.

Assays for collagenolytic activity.

1) Viscometric method. Changes of specific viscosity were measured in Ostwald viscometer with water flow times at 20°C ranging from 46 to 56 sec. At zero time, 1.0 ml of enzyme solution and 2.0 ml of 0.05M Tris-HCl buffer, pH 7.5, containing 1.0 M NaCl were added to 2.0 ml of 0.15% collagen in 0.4 M NaCl. After thorough mixing, the reaction mixture was transferred to a viscometer and changes of viscosity were measured periodically at 20°C.

2) Release of soluble fragments from native, reconstituted, acid-extracted carp skin collagen fibrils. The assay method used was a modification of the one employed by NAGAI et al. The modification was introduced in order to use non-labelled collagen instead of 14C-labelled one. Reconstituted collagen fibrils were prepared by incubating 1.0 ml of 0.18% carp skin collagen in test tubes at 25°C for 1 hr. The resulting collagen gel was disrupted with micro-spatula. One ml of enzyme solution to be assayed was added to gel. The reaction mixture was incubated for a given time at 25°C and filtered through glass fibre paper (Whatman GF/C) under reduced pressure. The residual gel on fibre paper was washed with 0.05 M Tris-HCl buffer, pH 7.5, and hydrolyzed in sealed tube with 6 N HCl for 3.5 hr at 130°C for the determination of hydroxyproline.

3) Release of soluble fragments from bovine Achilles tendon collagen. The tendon collagen was cut into small pieces. 15 mg of these collagen pieces was put into a test tube, 1.0 ml of 0.05 M Tris-HCl buffer, pH 7.5, added to it and then allowed to stand overnight in a refrigerator. To this collagen suspension was added 1.0 ml of enzyme solution to be assayed. The reaction mixture was incubated for 8 hr at 30°C and centrifuged at 10,000×g for 10 min. An aliquot of the supernatant was hydrolyzed in sealed tube with 6 N HCl for 3.5 hr at 130°C for the hydroxyproline determination.

Other assays. Caseinolytic activity was determined by the method of YOSHIDA and NODA. Briefly, the reaction mixture contained 0.2 ml of enzyme solution and 4.0 ml of 0.6% casein in 0.04 M Tris-HCl buffer, pH 7.5. After incubation at 30°C, the reaction was terminated by adding 2.0 ml of 10% TCA. The mixture was filtered through Toyo filter paper No. 5C and optical density of the filtrate was measured at 275 mμ. Protein was determined by the method of LOWRY et al. and micro-Kjeldahl method. Hydroxyproline was measured by the method of WOESSNER.

Results

Viscosity reducing activity of pyloric caecum enzyme. The enzyme preparation from the pyloric caeca of yellow-tail acts on carp skin collagen in solution. At pH 7.5 and 20°C it is capable of producing 75% reduction in specific viscosity after 60 min (Fig. 1). No change in control viscosity was observed during the experimental period. Higher
concentration of enzyme caused a greater loss of the specific viscosity (Fig. 2). The presence of non-collagenolytic proteases has been known in pyloric caecum of various fishes. Therefore, crude enzyme preparation used in Fig. 1 was assayed for caseinolytic activity at pH 7.5 and 30°C. In 2.37 mg of crude enzyme protein was contained the activity equivalent to 0.22 mg of bovine $\alpha$-chymotrypsin or 0.25 mg of bovine trypsin.

![Fig. 1](image1.png)  
**Fig. 1.** Effect of pyloric caecum enzyme on viscosity of carp skin collagen at 20°C and pH 7.5. Enzyme protein in reaction mixture was 2.73 mg. $\alpha$-Chymotrypsin and trypsin controls contained 0.5 mg enzyme. Initial specific viscosity was 2.15.

![Fig. 2](image2.png)  
**Fig. 2.** Relationship between loss of specific viscosity of carp skin collagen and protein concentration of pyloric caecum enzyme. Reaction mixture was incubated at 20°C and pH 7.5 for 30 and 60 minutes. Control specific viscosity was 2.15.

Since considerable caseinolytic activity was detected, bovine $\alpha$-chymotrypsin and trypsin were examined with respect to their viscosity reducing abilities so as to assess the extent of loss of viscosity by caseinolytic activity in the enzyme preparation. $\alpha$-Chymotrypsin and trypsin, each at a concentration of 0.5 mg in the reaction mixture, reduced the specific viscosity to about 27% and 17%, respectively, of the control value within 60 min. Loss of the viscosity by $\alpha$-chymotrypsin was greater than that by trypsin.

**Degradation of carp skin collagen fibrils by pyloric caecum enzyme.** Pyloric caecum enzyme is also active on native, reconstituted, carp skin collagen fibrils at pH 7.5 and 25°C. Activity was almost linear with the time of incubation when determined on the basis of release of soluble bound hydroxyproline from fibrillar collagen (Fig. 3A). The relationship between activity and enzyme protein concentration was not so ideal, possibly because of the presence of non-collagenolytic proteases in the crude preparation (Fig. 3B). $\alpha$-Chymotrypsin and trypsin, each at a concentration nearly equal to the amount of total protein in an aliquot of crude preparation, released only small amount of soluble fragments from collagen fibrils as compared with pyloric caecum enzyme. These results indicate that a specific enzyme, other than $\alpha$-chymotrypsin or trypsin, capable of degrading native collagen is present in the pyloric caeca of yellow-tail.

**Degradation of bovine Achilles tendon collagen by pyloric caecum enzyme.** When
the crude enzyme preparation was incubated with 15 mg of bovine Achilles tendon collagen for 8 hr at pH 7.5 and 30°C, about 20% of the total hydroxyproline present in the substrate was released, whereas no hydroxyproline was released in the absence of enzyme (Fig. 4). This indicates that pyloric caecum enzyme is also capable of degrading native insoluble collagen under conditions that do not denature collagen.

**Discussion**

Some properties of carp skin collagen used in this study are summarized in Table 1. These data are consistent with the analyses of carp collagen by PIEZ and GROSS for hydroxyproline content, GALLOP for specific optical rotation, and BOEDTKER and DOTY for denaturation temperature. These results indicate that carp skin collagen is not denatured in its preparing process and can be used as substrate for assay of collagenolytic activity. However, the collagen preparation is supposed to contain some impurity since it was not clarified by high-speed centrifugation.

The enzyme preparation from the pyloric caeca of yellow-tail is capable of reducing the viscosity of carp skin collagen at pH 7.5 and 20°C. Since the pyloric caeca of the fish is the major organ involved in the digestion of ingested protein, crude enzyme preparation contains a high level of caseinolytic activity. To know how this activity might affect on native collagen is important in order to distinguish this action from that of collagenolytic activity. The effect of trypsin on collagen viscosity at 20°C was rather small. This viscosity reduction might possibly be due to the presence of impurity in
collagen, since it has been demonstrated that trypsin is relatively ineffective in reducing the viscosity of native collagen solution, as also the content of cross-linked components in native collagen\(^{18,19}\). \(\alpha\)-Chymotrypsin was more effective in the reduction of collagen viscosity than trypsin. It has been demonstrated that \(\alpha\)-chymotrypsin is capable of reducing the viscosity of collagen but is unable to produce cleavages in the native helix of collagen\(^{18,19}\). The fact that the crude extract from the pyloric caeca of yellow-tail produces a considerable decrease in the viscosity of collagen as compared with trypsin and \(\alpha\)-chymotrypsin strongly suggests that a collagenolytic enzyme capable of degrading native collagen is present in the extract.

In addition to the above-mentioned data, crude pyloric caecum preparation is capable of degrading extensively native, reconstituted, carp skin collagen fibrils at 25°C and insoluble bovine Achilles tendon collagen at 30°C. \(\alpha\)-Chymotrypsin and trypsin, however, are practically unable to degrade carp skin collagen fibrils. These results corroborate the existence of a collagenolytic enzyme in the pyloric caeca of the fish.

In contrast to most of the known human and animal collagenases, pyloric caecum collagenolytic enzyme is directly extractable from fresh tissues and appears to play a significant role in the digestion of collagen present in the ingested animal tissues. In this respect it resembles the collagenase from the hepatopancreas of the crab\(^{11}\).

Further studies are required to resolve whether the action of pyloric caecum enzyme is similar to that of other animal collagenases.

### Table 1. Some properties of carp collagen.

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<th>Skin collagen</th>
<th>Scale gelatin</th>
<th>Swim bladder collagen</th>
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<tr>
<td>Hydroxyproline (%)</td>
<td>9.9</td>
<td>9.9*</td>
<td>9.7*</td>
</tr>
<tr>
<td>([\alpha]_D)</td>
<td>(-378^\circ)</td>
<td>(-350^\circ)*</td>
<td>(-350^\circ)**</td>
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<td>Denaturation temperature (°C)</td>
<td>29</td>
<td>29</td>
<td>29***</td>
</tr>
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* Piez and Gross\(^{15}\)
** Gallop\(^{16}\)
*** Boedtker and Doty\(^{17}\)

References