Post-catch Textural Changes and Softening of the Flesh of Seafoods

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Cover Motif

The front cover depicts line drawings of the molecular structure of some of the compounds found in the extracellular matrix of muscle. Taken from the cover of the Program of the International Meeting on the Biology and Pathology of the Extracellular Matrix, Lorne, November 1991.

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(II) NON-TECHNICAL SUMMARY

Background

This project was aimed at gaining a greater understanding of the complex processes that cause the softening and gaping in fish flesh. Fish flesh is divided into a series of muscle blocks by sheets of connective tissue. When fish is cooked, this connective tissue is gelatinised and the flesh separates into flakes which are blocks of cooked muscle fibres. The connective tissue is mostly comprised of collagen and fine collagen fibres form a net around each individual muscle fibre anchoring it at both ends.

Previous work had indicated that the major site of degradation in the flesh occurred at the junctions between the muscle fibres and the connective tissue and in the fine collagen fibres that enclose the muscle fibres. The softening of the structure and breakdown of the collagen is assumed to be due to enzymes present in the tissue. Collagen is normally regarded as a very stable protein so the presence of such powerful enzymes would be unusual. If such enzymes could be extracted and purified they would have considerable use in the food industry because they would be active at low temperatures and could be used to tenderise meats without encouraging the growth of harmful bacteria.

It is now known that there are several different genetic types of collagen, each with different structures, composition and properties and each with different functions in the living tissue. The major structural collagen type found in the skin and connective tissues of fish and other animals is type I. This type had been identified in the skin of blue grenadier and the first step in this project was to use these techniques on muscle tissue.

Collagen extraction

The techniques to isolate type I collagen from fish flesh were more complicated than those appropriate for fish skin because of the greater need to clean up extraneous material, but successful methods for its isolation were developed. The properties of type I collagen from the skin and muscle of the blue grenadier were compared with type I collagen from other species of fish.

During the investigation it became apparent that another newly described form of collagen, type V, was a critical component of fish connective tissue. This collagen is only present in low proportions, but is very important in maintaining flesh integrity. It occurs in admixture with type I and may even form the core within strands of type I fibres. This makes it difficult to extract and isolate in its intact form. It also seems to be much more labile than type I. Emphasis thus changed from seeking enzymes active against type I collagen to isolating and purifying type V collagen. Only when purified type V was obtained could the enzymes responsible for its degradation be detected.

Type V collagen was isolated from bream and from rainbow trout but isolation from the blue grenadier proved far more difficult than was anticipated. The extraction of flesh from air freighted, frozen fish or from fish immediately after catch (i.e. on the boat) has not helped to improve the negligible yield of type V collagen from the muscle of the blue grenadier. We are still unsure whether type V collagen in the muscle of the blue grenadier is absent, degraded or simply difficult to extract.

We have collaborated with Professor Kenji Sato of Kyoto Prefectural University in Japan who is the world expert on isolation and characterisation of fish collagens, particularly type V collagen. After extensive experimentation, his group too has been unable to isolate intact type V collagen from samples and extracts of blue grenadier.

Enzyme activity

Studies on the flesh extracts have found some enzyme activity against an artificial substrate, but no activity (using a different system) against type I collagen isolated from blue grenadier. It is now considered that these results are, however, unlikely to relate to type V collagen degrading activity.

Collagen films and solutions

Tissues such as skin are discarded during fish processing but they are rich in collagen which may have uses as a food ingredient and as 'natural' films or wraps for other seafood products. Neutral collagen solutions can be used to coat fillets, filling gaps and imperfections prior to them being smoked. The use is limited by the fact that the collagen does not form a good pellicle and does not absorb the smoke well enough. Collagen films can be used as wraps or as layers but they do not rehydrate or adhere to other product components. Very thin films may perform much better.

Implications for other projects

The basic expertise, information and understanding of fish collagens obtained in this project has been used in other projects which otherwise could not have been tackled so readily:

1. NSC 92/125.02 Value-added beche-de-mer;
2. NSC 92/125.06 Development of a process to remove skin from small fish;
3. NSC 92//125.11 Development of a process to manufacture powdered shark cartilage;
4. NSC 92/125.18 The manufacture of powdered fish collagen for use as a finings agent by the brewing industry.

Conclusions

The conclusions from our collaborative investigations with Professor Sato are that the type V collagen is of prime importance in maintaining muscle structure, that it can degrade rapidly in chilled storage and that this degradation is most probably due to specific cleavage of the telopeptides on the molecules or to rupture of intermolecular crosslinks on the type V collagen.

This project has advanced the understanding of post-mortem softening and gaping in chilled fish by ascertaining that future work needs to be focussed not on type I collagen, but on type V collagen and other lesser components of the extracellular matrix. To study this, it would be necessary to develop other techniques of isolating type V collagen in a form with its telopeptides and crosslinks intact.

(III) BACKGROUND

From original application (1989)

This project represents a new initiative in understanding textural change in raw fish flesh. Much previous work on texture has been concerned with the muscle (myofibrillar) proteins while the collagen of the connective tissue, which envelopes each muscle fibre and separates each muscle block, has been regarded as stable in chill storage. Earlier work on fish collagen assumed only one collagen type (Kimura, 1985). It is now known that in mammals there are at least fifteen different types, each with specialised properties (Martin *et al.*, 1985). Some types occur only in specific locations, while others (such as type I) are widespread, particularly in skin and muscle (Sikorski *et al.*, 1984). Only types I, II and III have so far been reported in fish (Kimura, 1985) and even these have some different properties to their homologues in mammalian flesh. It is highly likely that type IV occurs in fish muscle basement membranes (Kefalides, 1973).

Recent research (Bremner and Hallett, 1985, 1986; Hallett and Bremner, 1988) indicated that degradative changes to the fine structure of the junctions (myotendinous junctions) between the collagen fibres of the connective tissue sheets and the ends of the muscle fibres themselves may be responsible for the loosening of the integrity of the whole three-dimensional structure and softening of the flesh. The destruction of the fine collagen fibres and membranes appears to be due to attack by an enzyme (or enzymes). Details of the fine structure of the myotendinous junctions are still unclear but there is a network of very fine connecting fibrils which proceed from the muscle cell membrane through the basement membrane to the collagen fibres. These collagen fibres protrude into gaps and clefts in the muscle fibre ends and, along with the fine connecting fibrils, are responsible for stabilising the structure (Hallett and Bremner, 1988) and for transmitting the forces of muscular contraction which power the swimming movements. When these fine interlocking connections are degraded, the whole structure is loosened even though the major structural components remain intact. The nature of the fine connecting fibrils and the collagen fibres that hold the flesh together is unknown. The nature and location of the degradative enzymes and whether anything can be done about them is also unknown.

This project is concerned with how the whole structure of muscle cell and connective tissue fits together to form a coherent flesh and how this structure changes after death. The collagen content of fish muscle is of considerable influence on the texture of the raw flesh (Sato *et al.*, 1986).

The unique feature of the enzymes is that, even at chill temperatures, they are very active against the fine collagen fibres as well as against the basement membrane (containing type IV and V collagen). As stated, collagen is normally regarded as a fairly stable molecule resistant to enzymic attack. An analogous situation occurs with invasive tumours where the cancer cells produce collagenases which break down adjoining cell membranes. This process, however, occurs at normal mammalian body temperatures, not at 0°C! Thus, the particular nature of the fish enzymes is of great interest and potential value.

There has been considerable previous research on enzymes in the digestive organs in fish (Haard, 1986), proteases and cathepsins in the flesh (Reddi *et al.*, 1972) and on the application of these enzymes to food processing (Haard, 1986), but not on the presence of collagenases in fish.

Subsequent developments

Around the time that this project commenced, Japanese workers reported the presence of another collagen, type V, in fish muscle. Sato *et al.* (1988) reported the presence of type V in the white muscle of carp in a higher proportion than it occurs in mammalian muscle. Electrophoresis of the fractions after treatment in reducing conditions with 2-mercaptoethanol did not change the band pattern indicating the absence of reducible thiol bonds and hence the absence of type III collagen. Further studies have shown the presence of type V collagen in lizard fish, Japanese eel, sturgeon, spotted shark and lamprey indicating widespread occurrence of type V in both elasmobranches and teleosts (Sato *et al.*, 1989). The relative concentration of type V to type I collagen was higher in the endomysial than in the myocommatal fraction in carp and spotted mackerel. Both the type I and the type V were less soluble in the endomysial than in the myocommatal fraction. Three distinct chains of type V were identified from two molecular forms stated as $[\alpha 1(V)]_2\alpha 2(V)$ and $\alpha 1(V)\alpha 2(V)\alpha 3(V)$, with a higher proportion of the latter occurring in the endomysium in comparison to the myocommata.

This recent evidence indicates that not only can there be differences between major tissue groups (e.g. muscle, skin or swimbladder), but that differences in the chain structure of collagen can occur within different domains in the one tissue. These differences in chain composition are likely to result in slightly different properties and stabilities in the collagen.

In fish muscle, type V collagen probably plays a role similar to that of type III in mammalian muscle in that it forms copolymers with type I and acts to control fibril diameter (Keene *et al.*, 1987; Birk *et al.*, 1990, Adachi and Hayashi, 1986). Collagen fibrils of different diameters are to be found in the myomata near muscle fibre ends and in the invaginations of the myotendinous junction.

As the Japanese workers made further progress, it became apparent that type V collagen was an important component in maintaining structural integrity. Using a compression test as a means of applying a force to muscle segments, Ando *et al.* (1991) demonstrated by light microscopy and

Scanning Electron Microscopy (SEM) that a gradual disintegration of the extracellular matrix occurred during chilled storage of rainbow trout (*Oncorhyncus mykiss*).

(IV) NEED

From original application (1989)

There is a need to enhance the value of seafoods for both the domestic and export markets. In addition to the strict handling and processing techniques, a basic knowledge of the biochemical and structural processes which cause many species of fish to deteriorate after catching is essential. Softening and gaping of the flesh and fluid (drip) loss from the tissue are important fishery problems worldwide. The blue grenadier (*Macruronus novaezelandiae*) is a prime Australian example. The yield and profit from soft fish is generally lower because of processing problems (e.g. loss of drip fluids, trimming losses) and restricted product range.

The products from fish farming and the trap and line-caught fish destined for the sushi and sashimi markets or for haute cuisine do not suffer from such extreme changes as in blue grenadier but because of their value there is still the need to understand the factors that alter the texture and make them less prized.

Work on basic problems such as these is of wide benefit to the industry, since the problem ranges over all sectors and over all seafoods, including molluscs, crustaceans and fish. Since the work will be done at first on blue grenadier, the likely beneficiary will be the trawlfish sector. No attempt is made in this document to put a value on this fast growing sector.

(V) OBJECTIVES

1. To examine in detail the mechanisms of post-mortem softening of fish flesh due to overall changes in its three-dimensional structure.

2. To isolate and characterise the collagenolytic enzymes responsible which actively degrade the connective tissue (collagen).

3. To locate the enzymes in the tissue.

4. To explore potential uses for the enzymes in seafood and food processing.

This work is designed to give an understanding of the underlying mechanisms controlling textural changes in seafoods and the structural changes that lead to decreased yields through drip loss. The trawlfish, blue grenadier, will be used as example.
A further objective is to provide recommended handling procedures, if appropriate.

Progress

This project application, written in 1987 and approved in 1988, was converted to the standard FRDC format on 10 February 1993. The milestones have been expressed directly consistent with the original application. The thrust of the experimental part of the project became centred around the development and refinement of methods for the extraction and identification of type V collagen, and the preparation of small amounts of type V collagen. This has proved more difficult than was apparent at the start of the project. As a task, it was deceptively elusive since success always seemed imminent.

Objective 1, a major objective, was met in full and a review of the mechanisms involved was published (Bremner, 1992).

Objectives 3 and 4 were contingent on achieving objective 2 and this was thwarted by the unforeseen difficulties encountered in extracting and purifying sufficient type V collagen to provide a suitable test system to show the presence of these enzymes. Objectives 2, 3 and 4, cannot be fulfilled until advances occur in collagen isolation and purification.

(VI) METHODS

Extraction and separation of collagen types

Detailed flow charts of the methods for extracting separating and refining the collagen types and their fractions is given as an Appendices 1, 2, 3, 4 and 5.

Extraction of bulk amounts of collagen

Ten kg of blue grenadier skin was obtained from Petuna Seafoods of Tasmania. Four separate extractions of collagen were made to obtain 11.25g of freeze dried collagen. Discolouration, a problem with the salt precipitated extracts, was removed with activated carbon or diatomaceous earth columns. This caused a considerable reduction in yield.

(VII) RESULTS

Experimental approach

The approach to this work was to:

1. apply techniques developed to isolate collagen from fish skin to muscle tissue.
2. confirm presence of type I collagen in muscle tissue.
3. develop methods to isolate type V collagen from fish muscle of species in which it was known to occur.
4. apply methods to seek type V collagen in blue grenadier flesh.
5. refine methods to produce type V collagen intact with telopeptides simultaneously to above.
6. seek enzyme and collagenase activity in extracts of fish flesh.
7. develop methods to distinguish collagenase activity from proteinase activity.
8. apply these methods to extracted fish collagens to seek activity.

In addition to this, uses for extracted collagen were sought.

Histological exploration of collagen types

A parallel approach to identifying collagen types and their location in the muscle was taken using histological staining. A special staining procedure has been reported to be capable of differentiating between collagen types I, II and III in organs of fish (Junqueira *et al.*, 1978). Trials were initiated to apply the stain to tissue sections, both fresh and aged, to look for any changes in appearance. This was to be followed by checking whether the stain could be applied to individual collagen bands on electrophoretic gels. If the different collagen fractions stained differently and, if the stain for type V were distinctive, then this would provide a rapid means of assessing the presence of type V in tissue.

The site under investigation was where the muscle fibres attach to the connective tissue of the myocommata, mainly comprised of collagen. The selective stain for collagen, Sirius Red F3BA, distinguishes between different collagen types, which become birefringent in polarized light. Proteoglycans present on the collagen fibres may also stain and these can be removed by digestion with papain to enhance the birefringency. This stain is better than a general connective tissue stain such as Mallory's Trichrome.

Both formalin and Bouin's fixatives have been used in conjunction with this stain and these fixatives were evaluated using mullet tissue which had been mistreated and which exhibited some gaping. Tissue fixed by both methods stained well. The collagen fibres themselves showed some distortion when fixed in formalin. This may have been due to a change in osmotic levels when soaked in formalin. A saline formalin was prepared for further trials.

The mullet exhibited gaping when samples were taken probably dué to rough handling after filleting. The collagen layer was intact and it was difficult to remove the proteoglycan with a papain digestion. The collagen sheath surrounding several fibres was visible after papain digestion. There was, however, major proteolysis of the muscle fibres leaving holes where damaged tissue was washed away.

Samples of blue grenadier from Tasmania for histological and enzymic evaluation were not available due to bad weather and gear failure and this work had to be postponed several times.

Some sections from wax embedded blue grenadier tissue retained from earlier work (in 1988) were cut and stained with Sirius Red F3BA. The samples were taken from a fresh pre-rigor fish and from one fish stored on ice for 12 days. The collagen stained well and, in tissue fixed immediately after catch, the fine collagen fibres at the end of the muscle can be seen stained pink/red (Figure 1). Under polarised light these appear as fine gold fibres (Figure 2). A similar situation was observed with sections of muscle from fish which had been stored in ice for 12 days after catch (Figures 3 and 4). No obvious differences due to degradation during storage were noted.

Collagen fibres exhibiting different colours gold or purple were evident in different sites in the tissue sections when examined under polarised light. It is not clear whether this was due to differences in section thickness, differences in the type of collagen in the fibres or to the proteoglycans associated with them.

When the sections were digested in papain for 2 or 24 hours to remove proteoglycans, differences were apparent. A 2 hr digestion with papain did little to remove the proteoglycans on the fresh sample. It took a 24 hr incubation in papain (37°C) to remove the bulk of the proteoglycans and collagen. The fish stored on ice for 12 days was more susceptible to papain digestion with a 2 hr exposure (Figures 5 and 6) looking similar to the fresh tissue after a 24 hr incubation. This is indicative of the general loosening and degradation of the extracellular matrix that occurs during post mortem chilled storage.

The treatment with papain did not otherwise make interpretation of the staining patterns any clearer. A source of a specific stain for proteoglycans, Cupromeronic blue (Scott, 1991) was sought but it was no longer available. The method for its synthesis was obtained but the technique was considered to be beyond the resources of the project.

Sirius Red F3BA is clearly a good stain for general examination of tissue since examination of sections under polarised light can aid in interpretation of structural relationships. However, further work is necessary before it can be used to locate type V collagen in fish tissue. It would be important to know whether the stain had affinity for the intact collagen molecule or whether it was substantive to the telopeptide regions of the molecule. Recent work indicates that the telopeptides of type V collagen are quite labile. The treatment of sections with papain would be likely to result in the destruction of at least type V collagen. Thus, both proteoglycan and collagen may be degraded in this step and the results be uninterpretable, particularly since the specific stain for proteoglycan is unobtainable. This work was halted until such time as pure preparations from fish of collagen types I and V, both intact and without telopeptides, were available to examine specific staining characteristics.

Uses for Extracted Collagen

Collagen was extracted in experimental quantities from the skins of several commercial species to explore its use as a potential ingredient in fish processing. One potential use is as a binder or film on smoked fish fillets to mask imperfections and physical defects on the surface due to gaping or poor handling. Application of a solution of collagen to the surface of a fillet, either by dipping or brushing it on, results in a surface film which tends to cover imperfections when the fillet is dried and smoked.

Films of fish collagen that are almost clear and colourless can be cast by drying from solution. The use of these sheets in fabricated seafoods to separate layers and mimic the structure of fish flesh was explored. The films were also tried as wraps for fillets and for surimi products.

Smoking experiments

Collagen from coral trout dissolved in acetic acid was applied to fillets of blue grenadier which were then subjected to either hot or cold smoking. A reasonable pellicle did form on the cold smoked fillets. However, the residual acetic acid served to soften the connective tissue in the fillets resulting in gaping flesh. Hot smoked fillets almost fell apart.

This meant that collagen could not be used in the acetic acid solution used to extract it and that further steps to prepare it had to be taken. Collagen which had been precipitated by salt was prepared and freeze dried. This material was then resuspended as a dip solution for the fillets. This resulted in good coverage and gaps and imperfections on the surface were filled. However, the collagen did not take up the smoke well resulting in white patches on the finished product (Figure 7). The addition of glucose to the dip to improve browning was tried. Although a 1.5 hr drying stage before smoking the fillets was used, the resulting fillets were still too moist, were quite soft and no pellicle was visible at the end of the process.

A variety of collagen concentrations were tried but there was little difference between fillets and gaping was still evident. It was concluded that the difficulties in preparation of collagen free from acid and the fact that it did not take up smoke meant that this approach was not worth pursuing.

Several films of collagen were made using acetic acid extracted collagen from tropical species of fish, coral trout and maori cod. The collagen solutions were dried using de-humidified air. 1.5L of solution was placed in a 27.5 x 37.5cm plastic lined tray in the de-humidifier over two nights (day time temperatures were above 30°C which exceeds the denaturation temperature for collagen).

Blue grenadier and threadfin salmon fillets were wrapped in the films after brining and drying. The collagen sheet on half the batch was wet with water before smoking to soften the sheet so that it would mould to the fillet. After smoking, the fillets wrapped in collagen had less gaping than untreated fillets but had not taken up colour from the smoke which did not seem to be trapped on the collagen film (Figure 8). The collagen film also had not shrunk onto the fillets and was easily removed. Those fillets in which the film had first been wetted had an improved appearance.

To determine whether heating would assist shrinkage of the collagen sheet the fillets were the cooked in the smoker for 20 min at consecutive temperatures, 35, 45 and 55°C. There was little effect on the collagen films.

More smoking experiments were undertaken using:

(a) salt precipitated collagen; and
(b) collagen which was dialysed and lyophilised (with 0.4% glucose)

Blue grenadier and threadfin fillets were brined (5 min, 0.7% NaCl), dried (2 hr, 15°C), left as controls or soaked for 10 min in 1% collagen, smoked for 4 hr and chilled overnight at 5°C.

The results using the collagen which had been precipitated to treat the fillets were that a film of sticky white collagen filled in the gaping, but had little visual appeal as no smoke colour was

taken up. The lyophilised collagen treated fillets had little gaping and better colour but there was still some white patches from excess collagen. Overall, there was still not enough colour and all fillets were too moist.

To form a pellicle and dry the fillets better, they were stored overnight at 5°C after brining (5 min in 0.137% NaCl). A 1% precipitated collagen solution was brushed onto one of the fillets (No.1) and stored overnight, although to reduce excess storage time this fillet had no brining. The next day some of the brined fillets were brushed with collagen and dried for 4 hrs (No.2) and 0 hr (No.3). These fillets and a control with no collagen were cold smoked for 6 hrs and kept chilled overnight. All the fillets had better colour than previous attempts. Treatment No.1 produced a very moist fillet with no visible membrane, it held together in the middle with no gaping but not on the edges. Treatment No.2 resulted in a much drier fillet, the collagen filled in the gaping, the edges held together, but the collagen did not take up the smoke colour. Treatment No.3 gave a drier fillet but tearing at the edges was evident and the collagen in the gaping did not colour. The method in which the fillets were dried for four hours was preferable but the overall appearance of all the smoked fillets was poor when compared to commercial products. High ambient temperatures made it difficult to maintain stable conditions in the smoker.

The overall conclusion was that while collagen, applied either as a dip or as a film, could help keep fillets intact and smooth out surface imperfections, it did not allow the formation of the desirable shiny surface pellicle and did not absorb smoke. The result was an uneven appearance in colour, the exact opposite of what the application was designed to achieve.

Collagen films combined with surimi

Collagen from orange roughy skins and from maori cod was extracted and dialysed to make collagen films in the dehumidifier. The orange roughy collagen was difficult to desiccate, possibly due the residual presence of fat even though large amounts were removed during preparation.

The maori cod collagen films were combined with surimi to imitate a sushi roll or layered reformed fish fillet where there is an alternation between connective tissue (collagen) and the muscle protein (surimi). Surimi dilutions of 1:1.5, 1:2 and 1:3 were combined with the collagen films and baked or steamed. The higher the dilution of surimi, the more fragile was the product. Steam was also more destructive to the product. The combination of surimi at the lowest dilution with a collagen film gave the best results. The major difficulty was the reluctance of the reformed collagen sheet to rehydrate, soften and blend in with the surimi (Figure 9). There was also insufficient cohesion between the materials. Collagen films prepared from tropical species were quite heat stable once formed, as noted in the smoking experiments.

This work has indicated that collagen films may be useful as wraps in special applications, however, it is obvious that specialised equipment would be required to produce much thinner films than can be obtained by evaporation.

The search for Collagenase Enzymes

Rapid screening for collagenase

A rapid screening method for collagenase activity was developed in which a film of collagen is applied to a microscope slide and drops of test solution are placed on this film to test action against it. The slides were dipped in hot agar, set overnight and dipped in a 2% collagen solution, drained and stored in a humid container. For use, a 3 µl droplet was placed on surface with collagen and the slide incubated at 37°C for a minimum of 30 minutes. After incubation, the slides were washed with distilled water and stained with Coomassie Blue. Clear areas at the application site indicate collagenase activity.

Testing the system

The system was checked by treating the slides with various test solutions, solvent blanks, enzyme solutions and solutions with known activity against type I collagen. The solvents were 0.05M Tris buffer containing 0.005M $CaCl_2$ (pH7.5) and either 0.006g/ml Trypsin 242 units activity, 0.005g/ml Pepsin 40 units activity or bacterial collagenase of high (309 units) or low (19 units) activity. The pepsin showed some activity against the collagen but left sufficient present to retain the stain. The bacterial collagenase cleared the area but at lower concentrations of collagenase, some staining was apparent. The behaviour of pepsin could be viewed as a false positive as it could easily be misread as collagenase activity being present if found independently on a slide.

New slides were prepared with (a) 2% fish collagen dissolved in water, (b) 2% fish collagen dissolved in 10% acetic acid or (c) 1% bovine tendon collagen dissolved in 10% acetic acid. The same solutions were tested on these slides and incubated for 45 min at 37°C. Slides coated with solution a lost collagen at the collagenase site while pepsin did not totally remove collagen but resulted in clearing around the edges of the droplet. The buffer behaved similarly. This behaviour was similar for the acetic acid dissolved fish collagen. The bovine collagen did not stick as readily to the slide as the fish collagen. It was also more difficult to dissolve in 10% acetic acid. Pepsin led to clearing at the edge with staining in the centre, while the collagenase cleared completely.

Testing with marine collagenase

Crab hepatopancreas is known to contain collagenase, so the system was used to test this marine source. Slides were treated with crab extracts the presence and absence of calcium, an ion essential for collagenase activity. The extracts were (a) a raw sample, (b) a centrifuged and dialysed extract, (c) a fraction precipitated by salting out at a strength of 1M NaCl using a bulk DEAE separation, and (d) a fraction from a DEAE Sepharose liquid chromatography column. Although the bovine collagen slide was again faint after staining, there was clearing present on all slides and sites. It is unlikely that any false positives were present and no apparent difference was observed when $CaCl_2$ was present. Samples (c) and (d) caused some swelling to the agar which may help with removal of collagen during the washing and staining. Thus, the test system was positive for marine as well as bacterial collagenase.

Fresh crabs were caught in the Brisbane River (26/8/1992) to provide new hepatopancreatic extracts. The tissue was homogenised with 0.1M Tris-HCl buffer (pH 7.5), dialysed and run on a DEAE Sepharose ion exchange column where 10mL fractions were collected. Activity was characterised as being similar to separations achieved during earlier work. Four collagen slides were treated with raw extract and various fractions. A large number of fractions caused clearing even outside the region of the peak known to have collagenase activity. This would suggest that other types of enzyme could give a positive result limiting the use of this method for screening purposes.

Testing on blue grenadier extracts

Freshly caught unfrozen whole blue grenadier were obtained from Mr John Sealey of Gail Jeanette Fisheries, Portland, Victoria. The fish had been kept on ice only for 24 hours after capture and were still in rigor. Four fish had a fillet removed and the rest of the fish frozen. Fish 1 exhibited tearing or gaping at the top of the midsection. Fish 2 was still in rigor and some gaping occurred near the head section. Fish 3 was damaged mid dorsal below the fin with some bruising. This fish exhibited some signs of coming out of rigor. Fish 4 was also in this state although the fillet was in good condition. About 60g of muscle tissue was kept for enzyme analysis while the remainder was stored for collagen analysis. The method of extraction of collagenase from rat uterus described by Weeks *et al.* (1976) was applied to the fish samples. As mammalian collagenase is usually associated with collagen, a heating step is employed to release it from tissue homogenates. The samples were homogenised with 0.25% Triton X-100 and 0.01M $CaCl_2$ and centrifuged at 2°C and 6000xg for 20 min. The supernatant for rat tissue is discarded but for the fish it was retained. The pellet is normally resuspended to the original volume in 0.05M Tris, buffer (pH 7.4) containing 0.1 M $CaCl_2$, but for compatibility with the FALGPA method, Tricine buffer was used. This was placed in metal centrifuge tubes and heated in a 60°C water bath for 4 min with manual agitation. The samples were chilled, centrifuged and the supernatants dialysed for 4 hr in 10mM Tricine buffer (pH 7.4) containing 2mM $CaCl_2$ and 0.08M NaCl and spun again to remove sediment.

The samples were applied to collagen coated slides. Incubation (30 min) suggested that some activity may be present. After 1 hr, all dialysed samples and two of the supernatant samples exhibited no clearing while the other supernatant samples had rings around stained centres. The 1.5 hr incubation showed little clearing. All the above samples were tested for protease activity using azocasein but no activity was detected. Thus, the test results on blue grenadier extracts were ambivalent and the method was not persevered with. The other assay technique using FALGPA activity was pursued.

Detection of collagenase activity using the artificial substrate FALGPA

Methods for determination of collagenase activity of tissue extracts are almost as varied as the collagenase sources that have been examined. The Sigma Chemical Company describes collagenase activity for their products as collagen digestion units per mg solid and FALGPA hydroloysis units per mg solid. The product definition for the latter is the peptide compound N-(3-[2-furyl]acryloyl)-leu-gly-pro-ala which is described as a substrate specific for bacterial

collagenase and not hydrolysed by the well known proteases such as trypsin, thermolysin or elastase (VanWart and Steinbrink, 1981).

The reaction conditions for this method are a 0.05mM concentration of substrate in a buffer of 50mM Tricine buffer (pH 7.5) containing 0.4M NaCl and 10mM $CaCl_2$. The initial velocity for the first 10% hydrolysis resulting in a decrease of absorbance at 324nm is the main parameter for measurement..

Test solutions

The test solutions used on slides were also were also examined for FALGPA activity. A 25 ml collagenase addition to 700ml FALGPA resulted in a drop in absorbance of 0.019 per minute for the first 99 seconds. Pepsin, trypsin and crab hepatopancreatic extract (prepared as part of a separate research project) resulted in low absorbance changes (0.003 - 0.004). The latter solution was applied as a model for testing raw extracts from seafood as collagenase activity has already been found in the crab digestive system.

Tests on crab extracts

The fractions of hepatopancreas extract taken from crab caught in the Brisbane River were tested for FALGPA activity. Fifty samples were tested but little activity could be detected. The raw extract showed considerable activity, although the high turbidity present made repeat runs variable. Calcium was added to the test solutions with many repeat readings but there was no change in behaviour in the fractions.

Thus, no collagenolytic activity (as determined by activity against FALGPA) could be found in the fractions, even though it was apparently present in the raw extract and activity was found on the slide screening test.

Tests on blue grenadier extracts

No FALGPA activity was found in the dialysed samples but minor activity was evident in the first supernatant for all the fish extracts. After the sensitivity of the test was improved by increasing the substrate concentration from 0.05mM to 0.125mM, definite, although low activity was discernible. The amount of background of the absorbance plot also increased, but did not interfere with the overall drop in absorbance. From this experiment, it was decided to use 0.125mM FALGPA for screening then dilute to 0.05mM for use against the standard curve of collagenase activity for more accurate measurement. This method did not seem very sensitive as a narrow range of concentration is needed to get accurate responses.

Further tests on blue grenadier extracts

One of the whole frozen blue grenadier after 1 month storage was defrosted overnight. The fish was filleted and two muscle samples taken and processed by the above method. The supernatant was pooled while the extract from the heated pellet was kept separate. FALGPA activity was tested for all the samples but the results were difficult to interpret. It became apparent that a

variety of volumes need to be tested to reach a concentration which will give a confident indication of activity.

After 2 months of frozen storage, the frozen fish halves were defrosted overnight, fillets were removed and 25 g taken for extraction for enzyme analysis as previously. Again the FALGPA activity was found only in the first supernatant in similar amounts to that of the fish when fresh. The heat stability of the active samples was also tested. Aliquots of the first supernatant were subjected to the same heating conditions as the pellets, then chilled and tested for FALGPA activity. The remaining unheated supernatants were pooled, dialysed in buffer for 4 hr and 25mL loaded onto a DEAE Sepharose column. The Tricine buffer (225mL) was followed by a 600mL gradient from 0-1M NaCl using a flow rate of 50mL/hr and fraction size of 10mL. FALGPA activity was determined for each fraction. Activity was detected in tubes 14 and 15, but, by referring to the absorbance at 280nm profile, there were two peaks present here.

The fraction size (10 to 2.5mL), flow rate (50 to 30mL/hr) and gradient starting time and volume were changed to improve the separation. Several more runs of blue grenadier muscle extract (each time a new fillet was defrosted) were carried out to improve separation. The amount of Triton used during homogenisation was reduced to increase activity levels. The salt content of the buffer (which eluted the protein peaks from the column) was removed from the column buffer. When the parameters were then considered sufficient to give good separation, a final run using a Tricine buffer was made. A total gradient volume of 350mL was started when fraction 36 came off the column. This resulted in good peak definition and separation although the peak containing the FALGPA activity came off the column at a early stage of the salt gradient, between 0.1M and 0.25M. Figure 10 shows the 280nm absorbance, conductivity and FALGPA activity for each fraction. Using the Folin assay, the column load was 350mg tyrosine equivalents per mL of sample. The active fractions were pooled and frozen.

Effect of specific inhibitors

Specific inhibitors are used to characterise the activity and nature of enzymes and to block extraneous activity. In these trials, general inhibitors of proteases were included. These inhibitors should not affect the collagenases which are metalloproteinases.

To investigate the effect of inhibitors, a 0.1M Tris-HCl buffer (pH 7.5) containing 0.02% NaN_3 and 0.1M $CaCl_2$ was substituted for the Tricine buffer. The reaction rates using Tris buffer are similar to Tricine up to pH 7.5 but are inhibited markedly above this pH. The fish muscle was homogenised directly and divided into equal portions and extracted using the same method as previously except that the buffer was substituted for Triton. Inhibitors (sodium azide, PMSF, NEM, Pepstatin A and Leupeptin) were added to one homogenate buffer. This extract was quite cloudy after centrifugation.

To improve retention time, the salt gradient contained 150mL buffer and 150mL 0.5M NaCl buffer. The absorbance profiles of the muscle extract with no inhibitors at a wavelength of 280nm can be seen in Figure 11. Because of the higher protein load, separation of individual peaks was not as effective as previous runs. The absorbance profile of the muscle extract with inhibitors present can be seen in Figure 12. The various fractions containing different protein

peaks were pooled, dialysed with distilled H_2O overnight and subsamples freeze dried. FALGPA activity was indeterminate for both sets of pools.

The FALGPA activity was examined for the pooled protein peaks from the inhibitor free run and individual fractions from the inhibitor treated fish muscle extract. Peak 3 from the uninhibited extract had a high level of activity. When some EDTA was added, this activity dropped and when EDTA was in excess, no activity was observed. It was difficult to confirm activity in any of the inhibitor treated extract fractions.

A fish which had been frozen overnight was extracted and run on the column with a calcium free Tris buffer. A similar pattern to the previous inhibitor treated run was obtained. No FALGPA activity was detected until calcium was added.

Hydroxyproline assays as a measure of collagenase activity

Hydroxyproline is an amino acid unique to collagen and estimates of the amounts released on incubation of collagen with enzyme solutions are a measure of their activity. The method of hydroxyproline determination by Woessner (1961) was used to detect any possible collagenase action against the collagen used.

Fresh blue grenadier was obtained from Petuna Seafoods of Tasmania to repeat the previous experiment. The extract profiles can be seen in Figure 9 (no inhibitors) and Figure 10 (when inhibitors were present). Pool 7 for both runs was obtained when the 1M NaCl buffer eluted from the column. 0.5mL samples of fresh extract and from each peak were incubated at 4°C with 0.5mL coral trout collagen (0.142g/25mL, A230nm=2.6). Calcium was added to the collagen solution used with the inhibited extract and pooled peaks as this was absent from the original buffer. A series of controls contained the above mixtures plus 0.5mL 30% TCA. The tubes were placed on a stirrer and mixed for 96 hr (no inhibitors) or 72 hr (inhibitors present). The reaction in the various tubes at the end of incubation was tested without the addition of TCA. A day later, trichloracetic acid was added to stop the reaction.

Hydroxyproline estimations were done on the samples with no inhibitors present (Table 1) and when they were included (Table 2). The pooled peak numbers for each chromatographic separation are not necessarily of the same protein peak only its elution position from the column.

The levels of hydroxyproline released from collagen by the extracts were greater than from the collagen or extract peak alone. This indicates some general collagenolytic activity on a low scale. However, the extracts treated with TCA, which should have acted as a comparison, also showed similar or higher levels of hydroxyproline. The addition of TCA to the solutions after a further 24 hr incubation also resulted in some samples with higher levels of hydroxyproline. This suggests that TCA has some ability to release hydroxyproline from the collagen used in this experiment. This occurred also for the samples which included inhibitors.

TABLE 1: Free hydroxyproline content of incubated tubes - (no inhibitors)

Contents of tube	Conc. (μg/mL)	Total (μg)	Effect of TCA at 120hr (μg/mL)
Collagen	1.8	0.9	0
Extract	17.6	8.8	
Extract+collagen	11.2	11.2	7.6
Extract+collagen+TCA	7.9	11.8	
Peak 1	17.3	8.6	
Peak 1+collagen	14.9	14.9	12.6
Peak 1+collagen+TCA	10.0	15.0	
Peak 2	19.4	9.7	
Peak 2+collagen	11.2	11.2	8.9
Peak 2+collagen+TCA	8.7	13.0	
Peak 3	12.1	6.0	
Peak 3+collagen	8.7	8.7	12.1
Peak 3+collagen+TCA	9.6	14.3	
Peak 4	8.5	4.2	
Peak 4+collagen	6.8	6.8	10.4
Peak 4+collagen+TCA	8.1	12.2	
Peak 5	1.4	0.7	
Peak 5+collagen	0.2	0.2	0
Peak 5+collagen+TCA	0.2	0.3	
Peak 6	1.8	0.9	
Peak 6+collagen	0.3	0.3	0
Peak 6+collagen+TCA	0.1	0.2	
Peak 7	3.4	1.7	
Peak 7+collagen	1.7	1.7	0.9
Peak 7+collagen+TCA	1.3	2.0	

TABLE 2: Free hydroxyproline content of incubated tubes (inhibitors present)

Contents of tube	Conc. (μg/mL)	Total (μg)	Effect of TCA at 96hr (μg/mL)
Collagen	6.2	2.5	12.5
Extract	4.0	1.6	
Extract+collagen	6.4	5.1	7.9
Extract+collagen+TCA	6.4	7.7	
Peak 1	1.1	0.4	
Peak 1+collagen	4.0	3.2	6.7
Peak 1+collagen+TCA	5.2	6.2	
Peak 2	2.5	1.0	
Peak 2+collagen	4.9	3.9	8.1
Peak 2+collagen+TCA	6.2	7.4	
Peak 3	0.5	0.2	
Peak 3+collagen	4.3	3.4	6.9
Peak 3+collagen+TCA	5.4	6.4	
Peak 4	5.7	2.3	
Peak 4+collagen	5.7	4.5	6.6
Peak 4+collagen+TCA	5.3	6.3	
Peak 5	4.0	1.6	
Peak 5+collagen	5.5	4.4	8.0
Peak 5+collagen+TCA	5.5	6.5	
Peak 6	2.0	0.8	
Peak 6+collagen	5.3	4.2	6.8
Peak 6+collagen+TCA	5.5	6.6	
Peak 7	2.9	1.1	
Peak 7+collagen	4.9	3.9	8.0
Peak 7+collagen+TCA	6.2	7.4	

The levels of hydroxyproline obtained in those tubes to which inhibitors were added were generally lower than in the corresponding tubes where no inhibitors were present. This indicates that the collagenolytic activity present in the extract fractions is probably due to general proteases rather than specific collagenases.

Further experiments along similar lines were inconclusive and this approach was halted.

Collagen isolation and characterization

Collagen isolated from the skin of fish is predominantly (almost 100%) composed of type I collagen. By comparison, collagen isolated from the muscle of fish is mainly (>95%) type I with a smaller contribution from type V collagen. The isolation and characterisation of type I collagen from the skin and muscle of the Blue Grenadier is reasonably straightforward and can now be done routinely. Type I collagen from the skin and muscle of the Blue Grenadier has been compared with type I collagen from other species of fish.

The technique to extract type V collagen from the muscle of fish (particularly Blue Grenadier) has been arrived at following considerable experimentation and discussion with fellow researchers. To date, only tiny amounts of type V collagen have been isolated, leaving concerns that either the extraction technique (which takes 3-4 weeks to complete) is not working, or there is little or no type V collagen present in the flesh of the Blue Grenadier. The absence of type V collagen from Blue Grenadier fillets would be quite unusual, but would be an explanation for the unfortunate gaping problem observed in Blue Grenadier fillets with storage. Without a reliable source of type V collagen from the Blue Grenadier, it is not possible to study the potential enzymatic degradation of the collagen. The first step in the understanding of the gaping problem in the Blue Grenadier is to isolate and characterise Blue Grenadier type V collagen. This has proved far more difficult than was first expected. A summary of the experiments undertaken to isolate the type V collagen from Blue Grenadier flesh are presented below.

Initial studies involved optimizing the procedures necessary for the preparation of type I collagen from the skin of fish. Appendices 1 and 2 outline in some detail the methods necessary for the isolation of acid-soluble and pepsin-soluble collagen from the skin of fish, respectively. Acid-soluble collagen is predominantly intact collagen chains (i.e. with the N- and C-terminal telopeptides still attached). The incorporation of pepsin into the extraction procedure results in scission of the telopeptides from the collagen and an increase in the solubility of the collagenous fraction. Collagen chains in the tissue matrix can become intertwined with other collagen molecules and matrix components. It is often necessary to use chemical or enzymatic methods to assist in the separation of these components. The major collagenous component of fish skin is type I collagen. To minimize the potential for endogenous collagenolytic degradation, all extractions were performed at cold-room temperature (4°C). The first step in the collagen extraction methods is solubilization of all non-collagenous proteins with caustic soda (0.1 N NaOH). Collagen is not solubilized in caustic soda, but is readily soluble in acetic acid. Most other proteins are not soluble in acetic acid. This selective solubility of collagen is the first method used for the extraction of collagen from fish tissues.

Beyond this primary extraction, more selective fractionations of the collagenous components can be undertaken. Selective salt-precipitation is used to isolate all or part of the collagen components. Type I collagen can be extracted by precipitation from an acetic acid solution with 2.0 M NaCl. Experience has shown that it is necessary to allow solutions being salt-precipitated at least 16 hours to "mature". If this is not done, the yields of collagen are significantly reduced. It appears that it is necessary to have a "nucleus" around which the collagen can bind. The centrifugation conditions (13000 g/20 min/4°C) were derived from the literature and various discussions.

A third level of fractionation can be achieved using various concentrations of ammonium sulfate dissolved in acetic acid. This mode of protein fractionation permits the separation of one protein (or collagen type) from another. The fractionation can be further enhanced by the use of chromatographic procedures (e.g. gel permeation, ion exchange or high performance liquid chromatography).

The preparation of acid-soluble type I collagen from fish skin required the removal of any non-collagenous protein with caustic soda, the solubilization of any collagen in acetic acid, and the sequential precipitation of the type I collagen with 2.0 M NaCl and 11.5% ammonium sulfate. This procedure is outlined in more detail in Appendix 1.

The acid-soluble type I collagen from fish skin is readily soluble in acetic acid. The collagen which can not be solubilised in acetic acid requires further treatment with pepsin. The pepsin excises the ends of each collagen chain, allowing the insoluble collagenous material to become more soluble. The technique for isolating pepsin-soluble type I collagen from fish skin (Appendix 2) is essentially the same as outlined in Appendix 1, except that the proteins are treated with pepsin early in the preparation.

The preparation of type V collagen from fish muscle was considerably more complicated than that required for type I collagen. This was in large part because the proportion of type V collagen was more than 100 times less than type I collagen. The precautions taken in the preparation of type I collagen must be carefully adhered when type V collagen is being prepared. One step which required particular care was "maturation" of the various solutions prior to salt-precipitation. After much trial-and-error, it was found that the degassing of all solutions prior to centrifugation was essential, particularly for the preparation of type V collagen. For the far more abundant type I collagen, degassing was not as necessary. Presumably, there was enough type I collagen to produce a "nucleus" for collagen precipitation. The precipitation of the small amount of type V collagen in solution has been suggested to be adversely affected by the presence of air bubbles. Fish type V acid-soluble collagen is not able to be fractionated in acetic acid with NaCl. Type I acid-soluble collagen and types I and V pepsin-soluble collagens can be fully fractionated with NaCl in acid solutions. Type V acid-soluble collagen can be precipitated with 2.0 M NaCl, but cannot be further fractionated.
Type V acid-soluble collagen from fish muscle can be prepared according to Appendix 3. In brief, white muscle is extracted in caustic soda, solubilized in acetic acid and precipitated with NaCl. Type V acid-soluble collagen can then be separated by column chromatography. Pepsin-soluble type V collagen required treatment with pepsin, and could be fractionated

with salt and ammonium sulfate (Appendix 4). Final purification ultimately required fractionation by column chromatography (high performance liquid chromatography, HPLC).

Initial extractions of Blue Grenadier flesh (free from bone, skin and fat) were performed on samples of frozen material air-freighted from Tasmania. No type V collagen was detected at the completion of the extraction procedure. It was not observed as a contaminant in any of the other fractions obtained.

Enzyme activity tests (zymography and an artificial substrate assay) were performed using fish type I collagen as substrate. The results were difficult to interpret suggesting the need to use fish type V collagen as the substrate.

Further extractions of Blue Grenadier flesh investigated the influence of a large fat layer which remained after several extraction steps and may contribute to the lack of type V collagen in the extractions.

A field trip to Tasmania was undertaken in April 1993 to collect and process Blue Grenadier flesh immediately after catch. Samples were homogenised immediately after catch into 0.1 N NaOH to minimise the possibility of degradation of type V collagen by endogenous enzymes. The flesh was stored in plastic bottles at 4°C until it could be concentrated (by centrifugation) and transported to Brisbane.

These samples of Blue Grenadier flesh extracts were processed. The fat layer was again observed. To discount the possibility that the type V collagen was being lost in this lipid layer, it was separated and dissolved in solvents (chloroform/methanol). No protein was extracted from this material. Again, no type V collagen was able to be isolated.

In June 1993, Dr Davis undertook a study tour to France and Japan. Discussions were held with IFREMER in Nantes, France, a group with extensive experience with fish type I collagen. They could make no suggestions as to why type V collagen could not be extracted from the fish muscle.

Discussions were also held with Institute for the Biochemistry and Chemistry of Proteins (IBCP) in Lyon, France, a group very experienced in the isolation and characterisation of type V collagen from many species (particularly mammalian). There were no apparent problems with the procedure which we were using to extract the type V collagen from fish muscle which would compromise the yield of type V collagen.

Discussions were also held with Professor Sato in Kyoto, Japan, a world expert in the isolation and characterization of type V collagen from fish. Professor Sato suggested preparing type V collagen from a readily available aquacultured species of fish to test the method, and that I be very careful to degas the material before centrifugation.

The effect of degassing on the final extraction of type V collagen was thoroughly investigated, but no improvement in yield was observed with degassing.

Additionally, aquacultured salmon flesh (no bone, skin and fat) was similarly extracted. The technique is straightforward, but tedious, and a small amount of type V collagen was extracted from the salmon. There were problems with oil in the flesh which complicated the extraction procedure.

Further samples of Blue Grenadier flesh from a sample of frozen material air-freighted from Tasmania, were used to compare homogenisation and dicing procedures prior to extraction of the flesh in the alkaline solution (0.1 N NaOH). Dicing was less harsh, requiring more time for extraction in NaOH, but did not improve the yield of type V collagen. Subsequent preparations were diced finely and degassed thoroughly before centrifugation.

Blue Grenadier flesh (again free from bone, skin and fat) was extracted from frozen material air-freighted from New Zealand. One of these samples was from fish which exhibited bruising at the filleting step. Another sample was described as "pink" and was appreciably tougher to fillet. These samples were analysed along with control fish, to determine whether there were differences in type V collagen extractability.

In an attempt to rule out extraction error, we extensively extracted flesh in an alkaline solution (0.1 M sodium hydroxide) to remove non-collagenous proteins, dissolved in acetic acid, enzyme (pepsin) digested and concentrated (freeze-dried).

Extracts containing acid-soluble and pepsin-soluble type V collagen isolated from the New Zealand Blue Grenadier muscle (prepared according to Appendices 3 and 4) were freeze-dried prior to the chromatographic steps. Pepsin-soluble material was able to be easily resolubilized in acetic acid, but SDS-Page analysis indicated that extensive degradation of the sample had occurred (Figure 13a). No intact type V collagen was able to be visualized. The freeze-dried acid-soluble material was far more difficult to redissolve, but was not as severely degraded (Figure 13b). Chromatographic purification could continue with the acid-soluble, but not the pepsin-soluble collagen. The degradation of the pepsin-soluble fraction may have occurred during sampling or processing. The chromatographic procedures resulted in the separation of type V collagen from type I collagen and a number of other co-extracted molecules. The initial chromatographic separation of acid-soluble collagen (using a DEAE-cellulose column) was able to separate type I collagen from type V collagen (Figure 14). Type I collagen did not bind to the column and eluted directly. Bound material could be eluted with a buffer containing a higher concentration of salt. The type V collagen was further purified by absorption and selective elution (with a salt gradient) from an SP-Fractogel column. Type V collagen could be observed in this high molecular weight fraction following limited pepsin digestion and SDS - PAGE Analysis (Figure 15). When a sample of this high molecular weight material was applied to a Reversed Phase HPLC column and desalted, two distinct peaks (fractions A and B) were unexpectantly observed (Figure 16). An amino acid analysis (Table 3) of these fractions resulted in a composition which was intermediate between types I and V collagen. The most appropriate interpretation of these results is that the high molecular mass component which was subjected to HPLC consisted of cross-linked type I and V collagens. The distinct peaks on HPLC separation are most likely due to different proportions of the two collagen types.

Table 3: Amino acid composition of Fractions A and B from hoki ASC with hoki type I and eel type V collagen subunit chains

(residues/1 000 residues)

	Hoki Collagens					Eel type V collagen			
	Fr. A	Fr. B	α1(I)	α2(I)	α3(I)	α1(V)	α2(V)	α3(V)	α4(V)
Asp	56.9	18.8	47	52	49	40.8	35.2	42.9	36.1
Gly	85.7	77.8	82	65	88	101.0	94.8	99.3	84.0
Hyp	72.0	70.1	69	60	60	98.5	100.4	82.4	97.0
Ser	60.4	62.1	46	53	19	39.0	45.0	38.9	39.1
Gly	329.0	331.9	345	347	347	325.5	338.5	336.0	353.8
His	7.9	6.7	3	11	12	7.9	8.2	7.0	2.8
Arg	52.4	52.6	48	53	42	46.3	56.5	45.0	54.3
Thr	25.4	26.4	26	26	24	27.4	33.9	21.3	23.4
Ala	102.3	112.0	135	121	138	42.4	53.7	50.8	86.2
Pro	95.0	97.0	96	99	94	147.9	123.3	137.7	132.2
Tyr	2.8	3.5	1	5	1	3.5	1.2	3.0	1.1
Val	18.7	19.8	17	23	17	15.1	23.3	16.7	11.0
Met	14.5	16.6	15	13	10	6.7	8.7	7.9	5.9
Ile	10.3	9.8	8	11	10	11.2	9.3	16.0	9.2
Leu	18.1	19.0	14	23	14	32.8	28.7	36.7	22.6
Hyl	10.5	6.6	4	7	4	28.7	14.4	32.6	16.1
Phe	14.1	15.0	15	9	15	11.2	9.9	11.4	12.6
Lys	21.5	24.0	29	22	26	14.1	14.8	14.8	12.4

JAM Ramshaw *et al.*, 1988, Arch. Biochem. Biophys., 267, 497-502.

K Sato *et al.*, 1994, J. Agric. Food Chem., 42, 675-678.

If this phenomenon can be explained by the covalent cross-linking of types I and V collagen at the telopeptides, it would be the first demonstration of cross-linking of heterotype collagens in fish tissues. The quantities of material isolated from these preparations was very small (milligrams) and was almost completely used in the characterization outlined above.

The isolation of fish muscle collagen (which was not type I collagen) was not pure enough (i.e. apparently, mixtures of types I and V collagen) and not in sufficient quantity (i.e. most was used for characterization) for studies of specific type V collagenolysis to be undertaken. Studies of the susceptibility of fish type I collagen to enzymatic degradation "in vitro" showed little evidence that degradation of type I collagen contributed to the gaping of Blue Grenadier flesh. Type V collagen isolated from Blue Grenadier muscle may have been considerably more susceptible to enzymatic digestion than type I collagen and the type V collagens isolated from the muscle of other fish species. This may explain the difficulties experienced in the early extraction trials and in the pepsin-treated type V collagen fraction.

Conclusion

No specific collagenase activity was detected with any degree of certainty by any of the methods. This is consistent with the interpretation that the enzymes responsible for post-mortem textural changes may act on the telopeptide regions of the molecules rather than on the triple helical regions of the collagen. If this is the case, then the enzyme(s) may be from of a variety of enzyme types. This work has shown the need to isolate and purify type V collagen with the telopeptides intact, as well as the type V triple helical regions.

(VIII) BENEFITS

The benefits of the project are in the understanding gained about the structure and the biochemistry of softening of the flesh as outlined in the attached paper. There are, at the moment, no direct quantifiable benefits.

(IX) INTELLECTUAL PROPERTY

No intellectual property arises directly from this research.

(X) FURTHER DEVELOPMENTS

The importance of the development of methods to identify, isolate and locate type V collagen should not be underestimated. Apart from the value of this information in understanding the softening and gaping process, recent information indicates that there may be other biological uses of type V collagen if it can be extracted insufficient quantity and purity from fish wastes. The type V collagen binds to bioactive materials such as heparin and insulin and it inhibits the growth of vein endothelial cells. It is possible that the type V

collagen can be used as an anti-angiogenic compound to restrict the proliferation of arteries to cancerous tissues and as a carrier for delivery of drugs with specific bioaffinity.

(XI) STAFF

HA Bremner	Senior Principal Scientist	IFIQ	20%
CKC Davis	Chemist	IFIQ	100%
SL Slattery	Chemist	IFIQ	20%

(XII) FINAL COSTS

A Statement of expenditure has been previously forwarded to FRDC.

(XIII) DISTRIBUTION LIST

ASIC	AUSEAS
AFMA	TDPIF
CSIRO Fisheries	U of Q, Gatton
NFITC	UTAS Launceston
IFIQ	QDPI

APPENDIX 1: Method for the preparation of fish skin acid-soluble type I collagen

Diced skin (muscle-free) was stirred overnight in 10 volumes (w/v) of cold caustic soda (0.1 N NaOH) to dissolve any non-collagen protein.

The precipitate, following centrifugation (13000 g/20 min/4°C), was resuspended in 20 volumes (v/v) of cold water and stirred overnight.

The precipitate was extracted with 10 volumes (v/v) of acetic acid (0.5 M) pH 2.5 for 2 days at 4°C.

Acid-soluble collagen remained in the supernatant following centrifugation and could be concentrated by salt-precipitation (2.0 M NaCl) at 4°C overnight.

The precipitate (salt-precipitated acid-soluble collagen) could be resuspended in 50 mM Tris buffer (pH 7.5) containing 2.4 M NaCl by stirring overnight at 4°C.

The precipitate at this step could be solubilized again in acetic acid, precipitated with 11.5% (w/v) ammonium sulfate, and resolubilized (by dialysis) in acetic acid.

This final solution was the acid-soluble type I collagen from the skin of fish and could be freeze-dried to obtain a powdered product.

APPENDIX 2: Method for the preparation of fish skin pepsin-soluble type I collagen

Diced skin (muscle-free) was stirred overnight in 10 volumes (w/v) of cold caustic soda (0.1 N NaOH) to dissolve any non-collagen protein.

The precipitate, following centrifugation (13000 g/20 min/4°C), was resuspended in 20 volumes (v/v) of cold water and stirred overnight.

The precipitate was extracted with 10 volumes (v/v) of acetic acid (0.5 M) pH 2.5 for 2 days at 4°C.

Acid-soluble collagen remained in the supernatant and further purified as is outlined in Appendix 1. The acid-insoluble collagen (in the precipitate) could be partially solubilized with pepsin. The precipitate was resuspended in 10 volumes (v/v) of cold acetic acid containing porcine pepsin (25 mg/100 mL) and stirred at 4°C for 2-3 days. Maximal solubilization was achieved if the insoluble precipitate was re-extracted with pepsin 3 times.

The pooled supernatants (containing pepsin-solubilized collagen) were concentrated by salt-precipitation (2.0 M NaCl) at 4°C overnight.

The precipitate (salt-precipitated pepsin-soluble collagen) could be resuspended in 50 mM Tris buffer (pH 7.5) containing 2.4 M NaCl by stirring overnight at 4°C.

The precipitate at this step could be solubilized again in acetic acid, precipitated with 11.5% (w/v) ammonium sulfate, and re-dissolved (by dialysis) in acetic acid.

This final solution was the pepsin-soluble type I collagen from the skin of fish and could be freeze-dried to obtain a powdered product.

APPENDIX 3: Method for the preparation of fish muscle acid-soluble type V collagen

Fish flesh (white muscle only) was homogenized twice in 10 volumes (w/v) of cold caustic soda (0.1 N NaOH) to dissolve any non-collagen protein.

The precipitate, following centrifugation (13000 g/20 min/4°C), was extracted twice in 10 volumes (v/v) of acetic acid (0.5 M, pH 2.5) for 2 days at 4°C.

Acid-soluble collagen remained in the supernatant following centrifugation and could be concentrated by salt-precipitation (2.0 M NaCl) at 4°C overnight.

The precipitate (salt-precipitated acid-soluble collagen) could be resuspended in 50 mM Tris buffer (pH 8.6) containing 50 mM NaCl and 5.0 M urea.

The material which remained in the supernatant was applied to a DEAE-cellulose column. Type I collagen did not bind to the column (i.e. eluted directly). High molecular weight bound material (type V collagen) was eluted using a 0.5 M NaCl step gradient.

The eluate was dialysed into 20 mM acetate buffer (pH 4.8) containing 5 M urea and applied to an SP-Fractogel column. In the presence of low salt, all proteins, became bound to this column. Type V collagen was eluted using a linear salt gradient (from 0 to 0.7 M NaCl).

This final solution was the acid-soluble type V collagen from the flesh of fish and could be dialysed against acetic acid and freeze-dried to obtain a powdered product.

This material was applied to a reverse-phase HPLC and 2 peaks eluted. These peaks were analysed for amino acid composition.

APPENDIX 4: Method for the preparation of fish muscle pepsin-soluble type V collagen

Fish flesh (white muscle only) was homogenized twice in 10 volumes (w/v) of cold caustic soda (0.1 N NaOH) to dissolve any non-collagen protein.

The precipitate, following centrifugation (13000 g/20 min/4°C), was extracted twice in 10 volumes (v/v) of acetic acid (0.5 M, pH 2.5) for 2 days at 4°C.

Acid-soluble collagen remained in the supernatant and was further purified as is outlined in Appendix 3. The acid-insoluble collagen (in the precipitate) could be partially solubilized with pepsin. The precipitate was resuspended in 10 volumes (v/v) of cold acetic acid containing porcine pepsin (25 mg/100 mL) and stirred at 4°C for 2-3 days. Maximal solubilization was achieved if the insoluble precipitate was re-extracted with pepsin 3 times.

The pooled supernatants (containing pepsin-solubilized collagen) were concentrated by salt-precipitation (2.0 M NaCl) at 4°C overnight.

The precipitate was resuspended twice in 50 mM Tris buffer (pH 7.5) containing 4.4 M NaCl by stirring overnight at 4°C.

The precipitate (containing all collagenous material) was resuspended three times in 50 mM Tris buffer (pH 7.5) containing 2.4 M NaCl by stirring overnight at 4°C.

Soluble collagenous material was precipitated by increasing the salt concentration to 4.4 M NaCl again.

The precipitate at this step could be solubilized again in acetic acid, precipitated with 11.5% (w/v) ammonium sulfate.

The supernatant from the previous step was further fractionated with ammonium sulfate to a final concentration of 20% (w/v).

The precipitate obtained here (i.e. the 11.5-20% (w/v) ammonium sulfate cut) was resuspended twice in 50 mM Tris buffer (pH 8.2) containing 50 mM NaCl and 2.0 M urea.

The desalted eluate was to a SP-Fractogel column. In the presence of low salt, all proteins are bound by this column. Any collagen species present are eluted using a linear salt gradient (from 0 to 0.7 M NaCl).

The various peaks could be dialysed against acetic acid and freeze-dried to obtain a powdered product.

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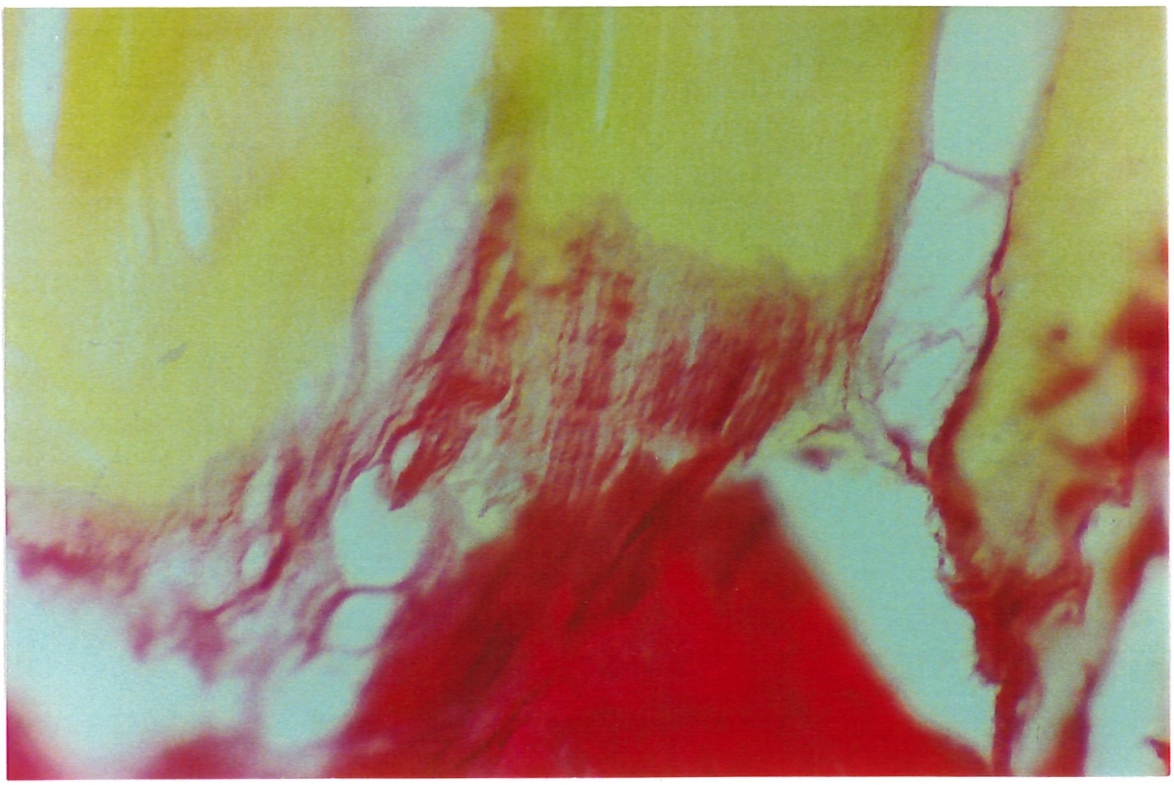

Figure 1 Muscle tissue from blue grenadier fixed directly after catching. The section is stained with Sirius Red and photographed under normal light. The muscle fibres appear yellow and the collagen fibres are red. Fine collagen fibres proceed from the mass of the collagenous connective tissue around the edge and base of the fibres. Magnification X 100.

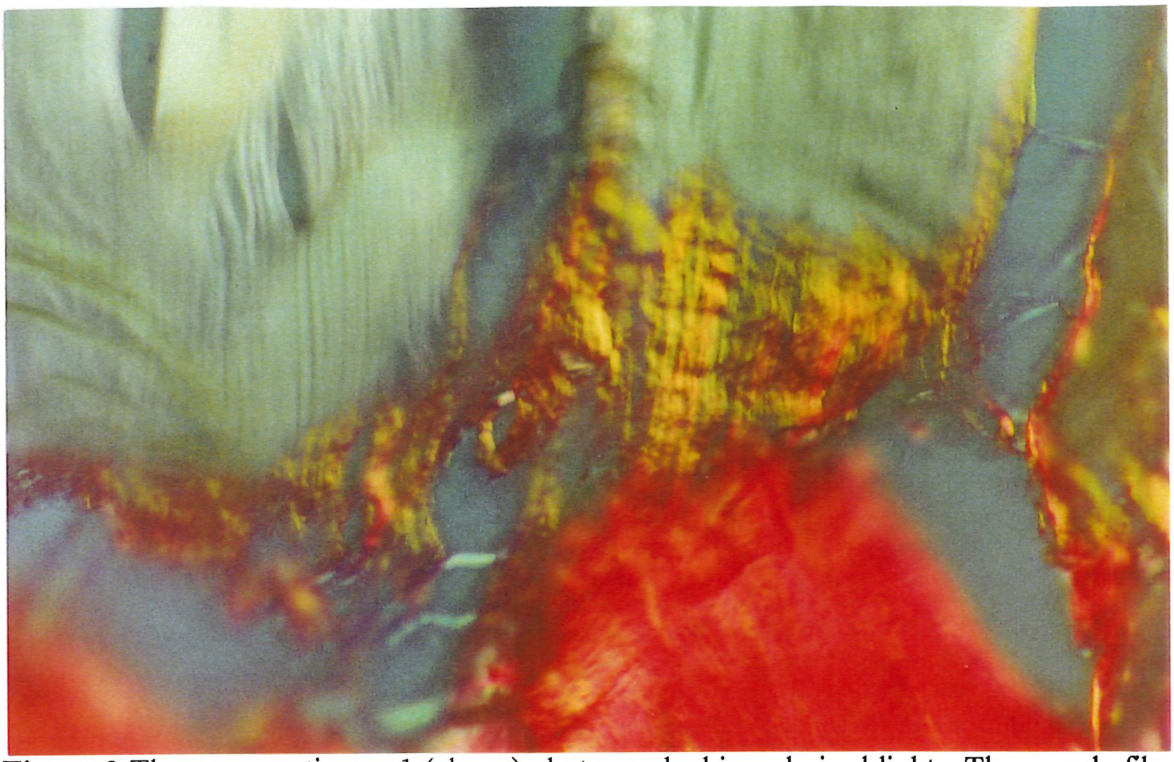

Figure 2 The same section as 1 (above) photographed in polarised light. The muscle fibres appear grey/green and the collagen mass as red with the finer collagen fibres being gold and purple.

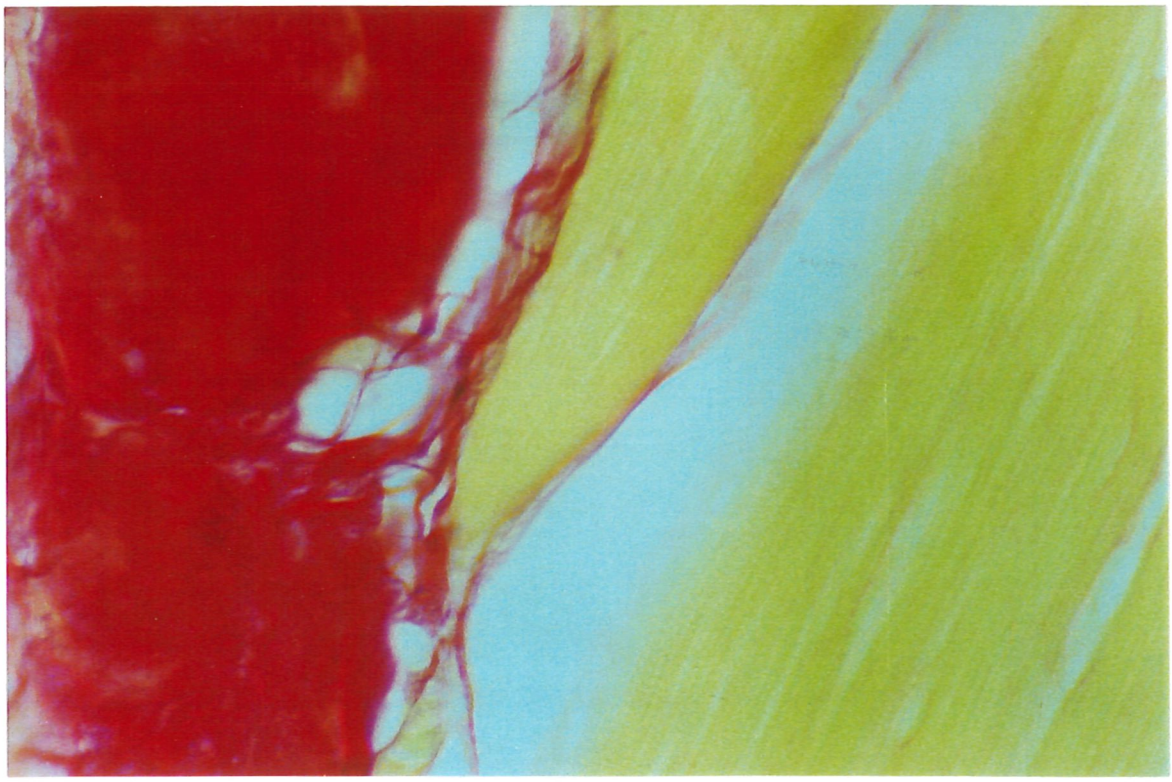

Figure 3 Muscle tissue from blue grenadier stored twelve days in ice. Section photographed in normal light. Fine collagen fibres (red) still connect the major muscle fibres (yellow) to the myocommatal connective tissue

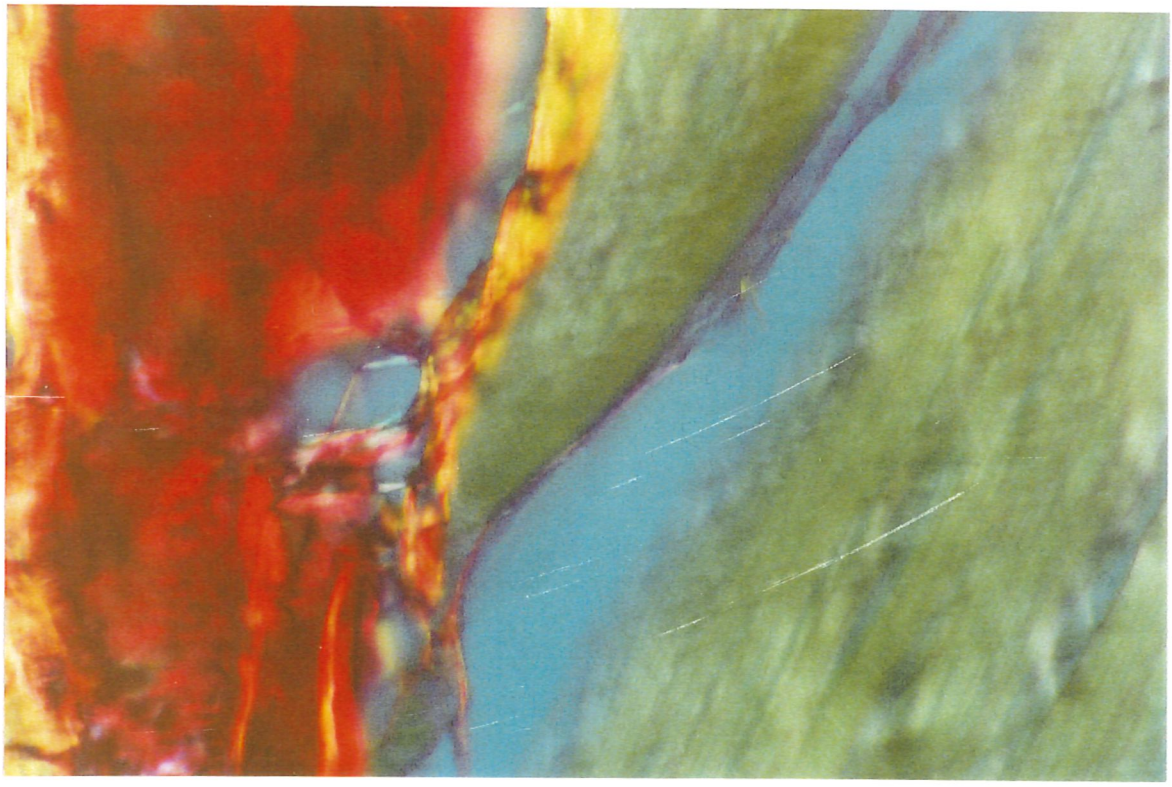

Figure 4 The same section as in 3 (above) photographed in polarised light.

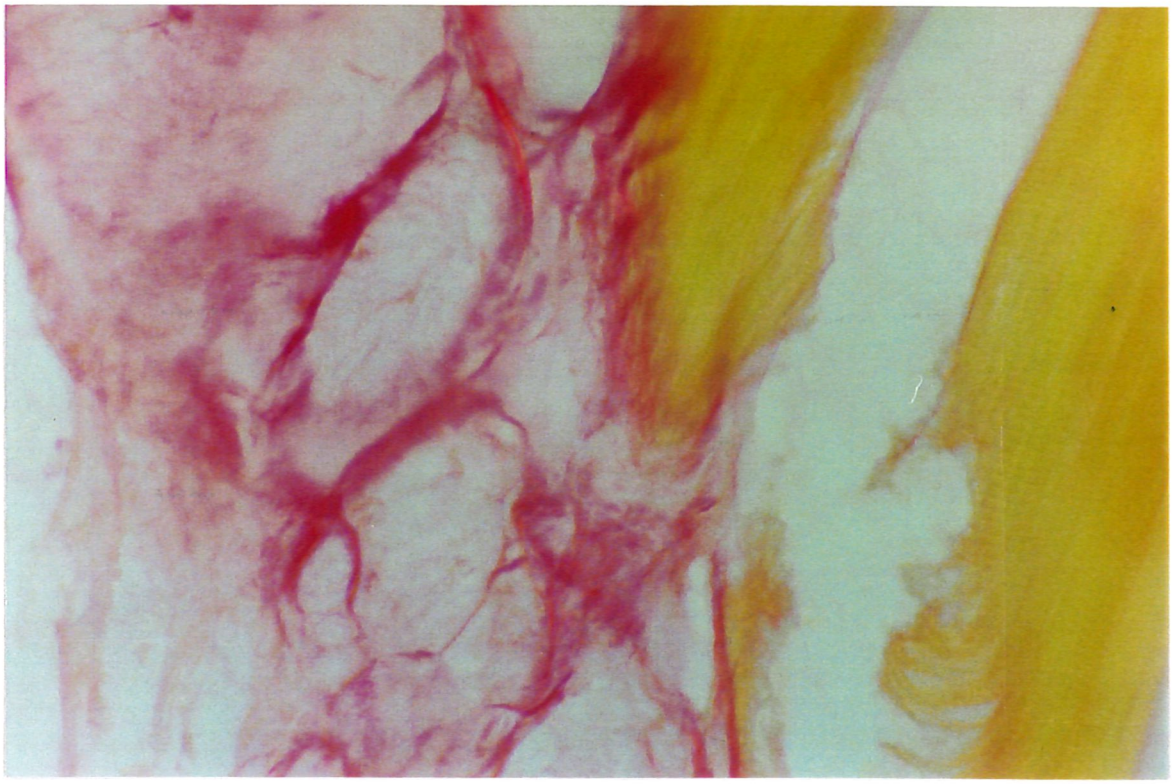

Figure 5 Muscle from a blue grenadier stored twelve days in ice. The section has been digested for two hours with papain then photographed in normal light. Considerable digestion of the connective tissue has occurred.

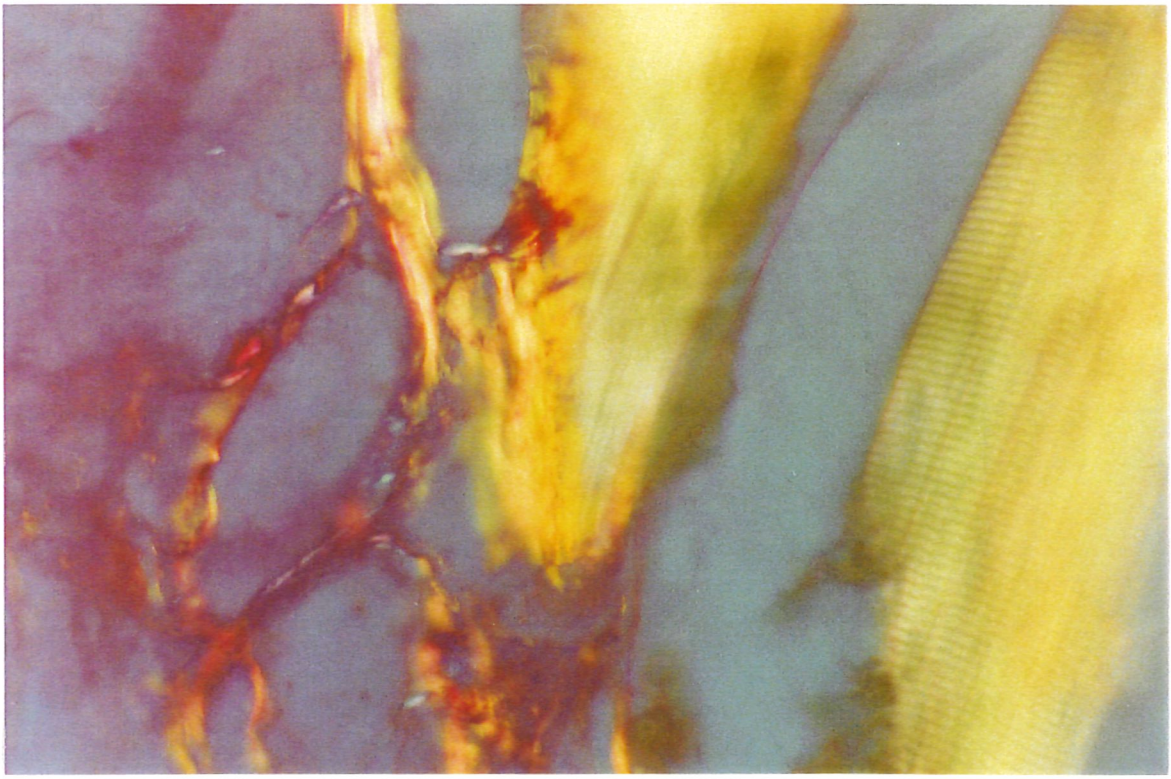

Figure 6 The same section as in 5 (above) photographed in polarised light.

Figure 7: Smoked sides of Blue Grenadier. Note the gaping in the lower fillet which is untreated. The upper fillet has been coated with fish collagen which has filled the gaps, but has not taken up smoke, leaving white patches.

Figure 8: Smoked sides of Blue Grenadier. The lower untreated side exhibits a considerable degree of gaping. The upper side has been wrapped in a collagen film which holds the flesh together, but does not take up the smoke as well.

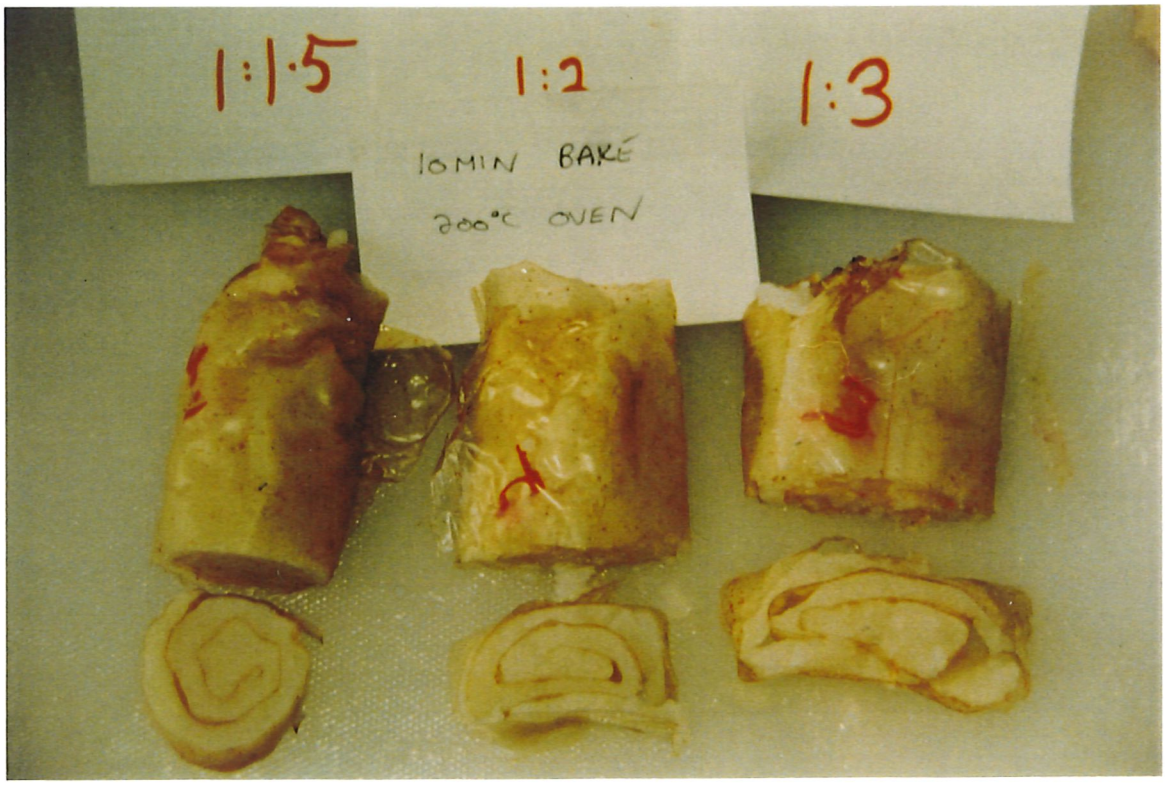

Figure 9: Fish surimi diluted with water at ratios of 1:1.5, 1:2 and 1:3, wrapped in collagen film, then cooked. The surimi does not adhere well to the collagen which does not rehydrate easily.

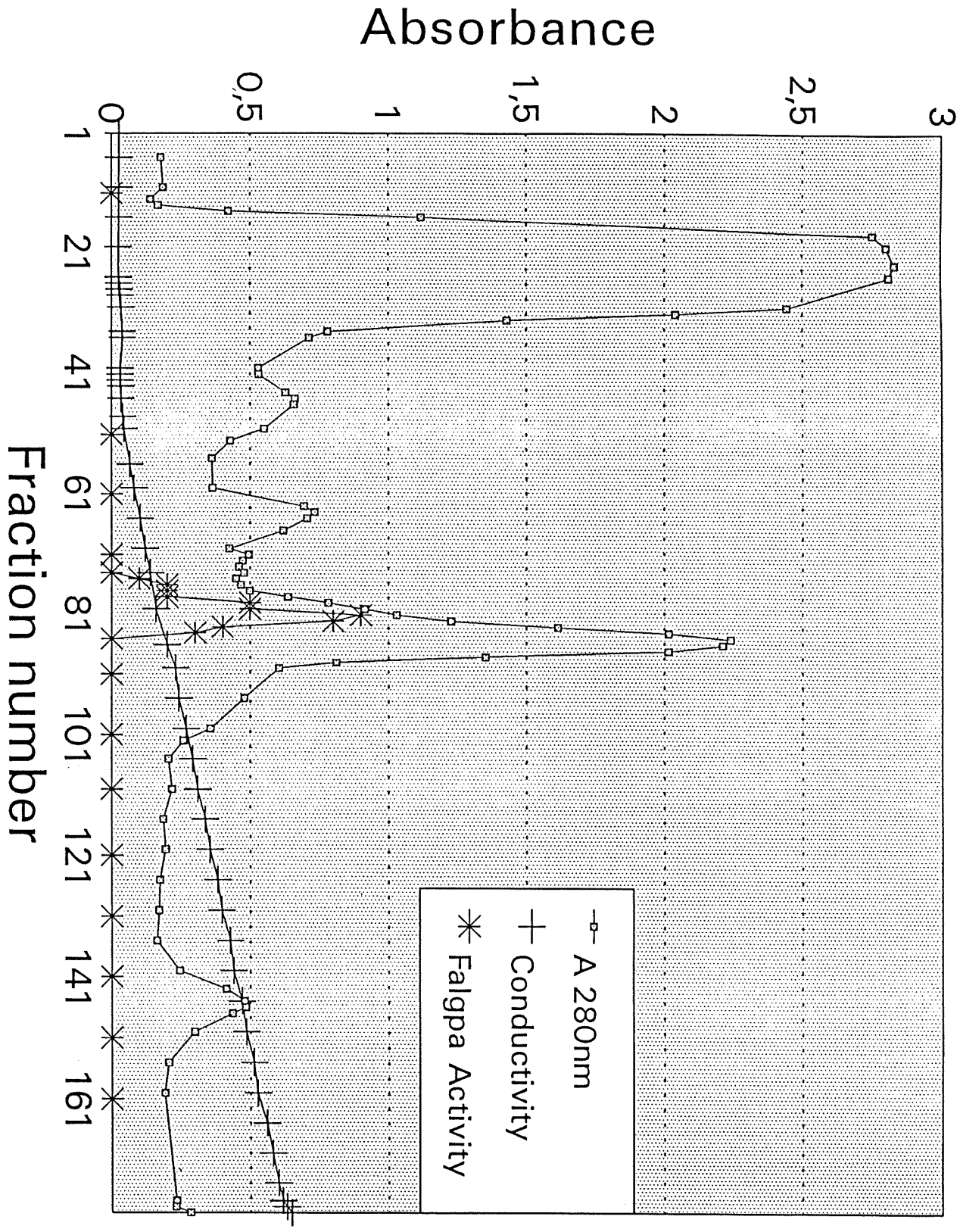

Figure 10: Fractionation of a Blue Grenadier muscle extract using DEAE-cellulose chromatography.

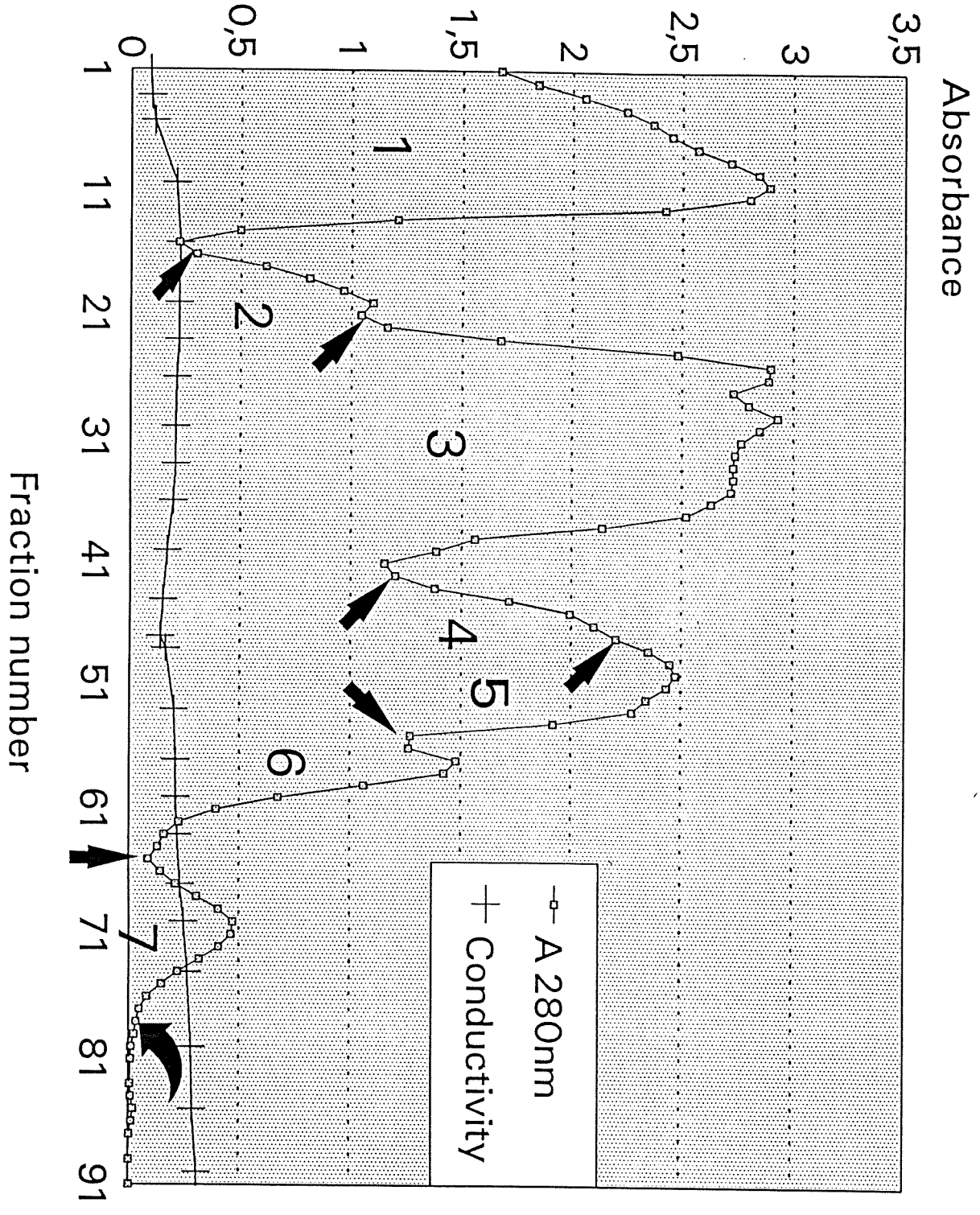

Figure 11: The effect of the absence of inhibitors on the proteolytic activity of DEAE-cellulose fractionated Blue Grenadier muscle extract.

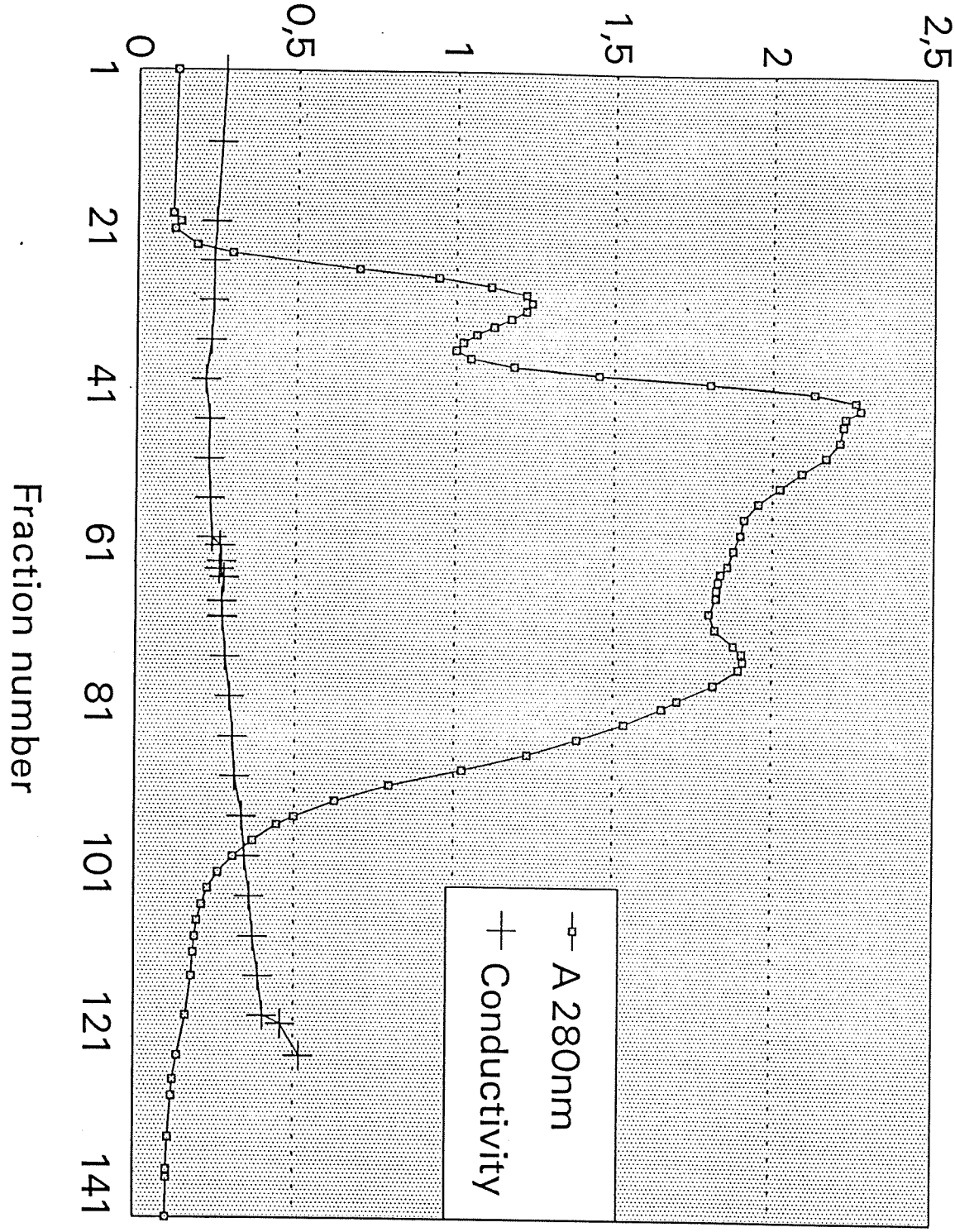

Figure 12: The effect of the presence of inhibitors on the proteolytic activity of DEAE-cellulose fractionated Blue Grenadier muscle extract.

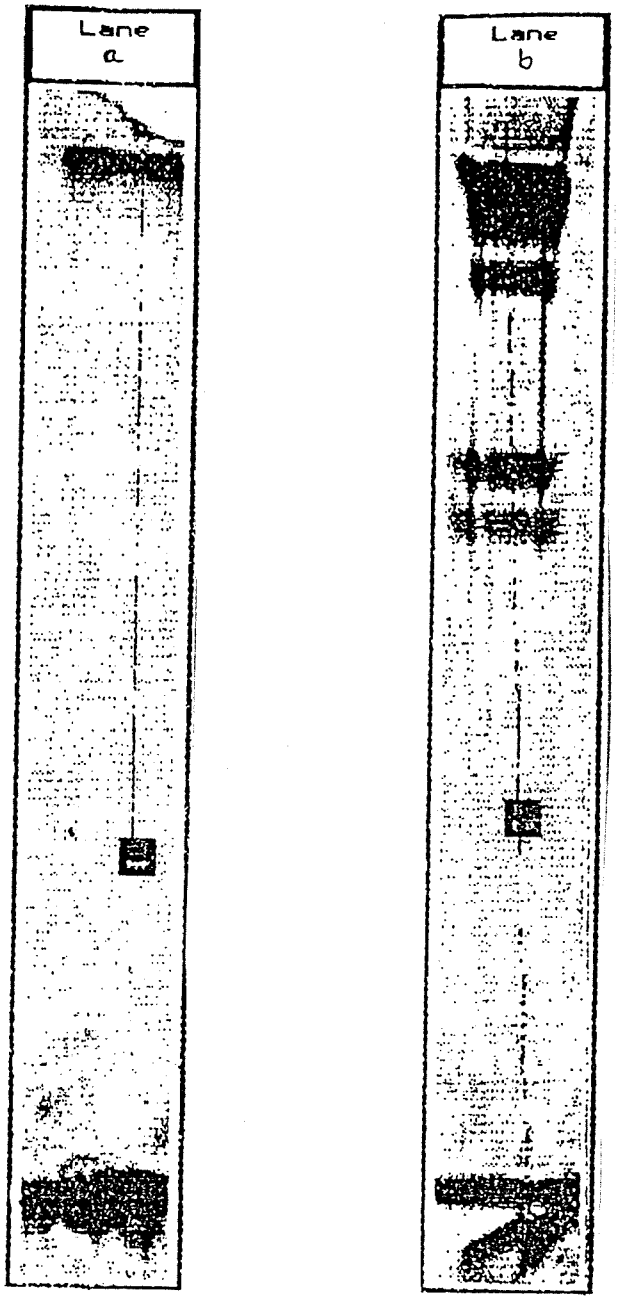

Figure 13: Effect of pepsin digestion on extration of type V collagen from Blue Grenadier muscle a) extraction in acid alone, b) extraction in acid containing pepsin.

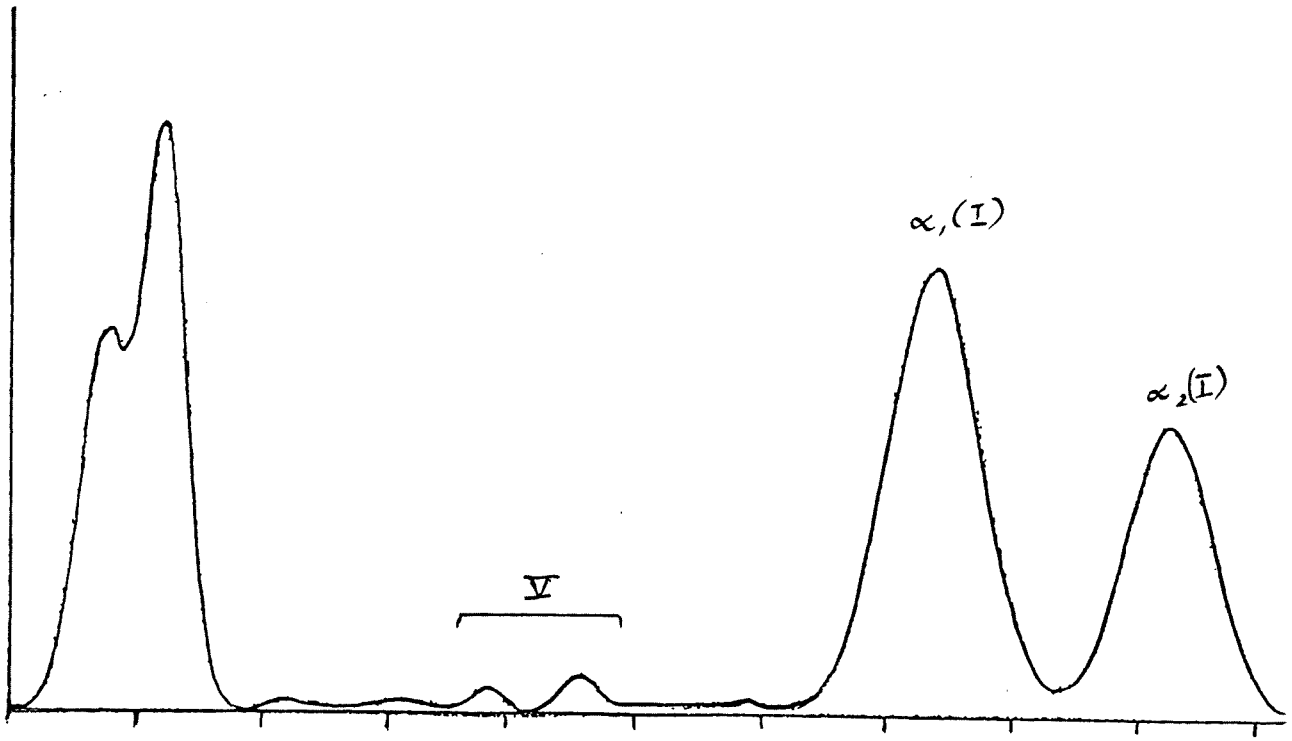

Figure 14: Separation of type I and Type V collagen using DEAE - cellulose chromatography.

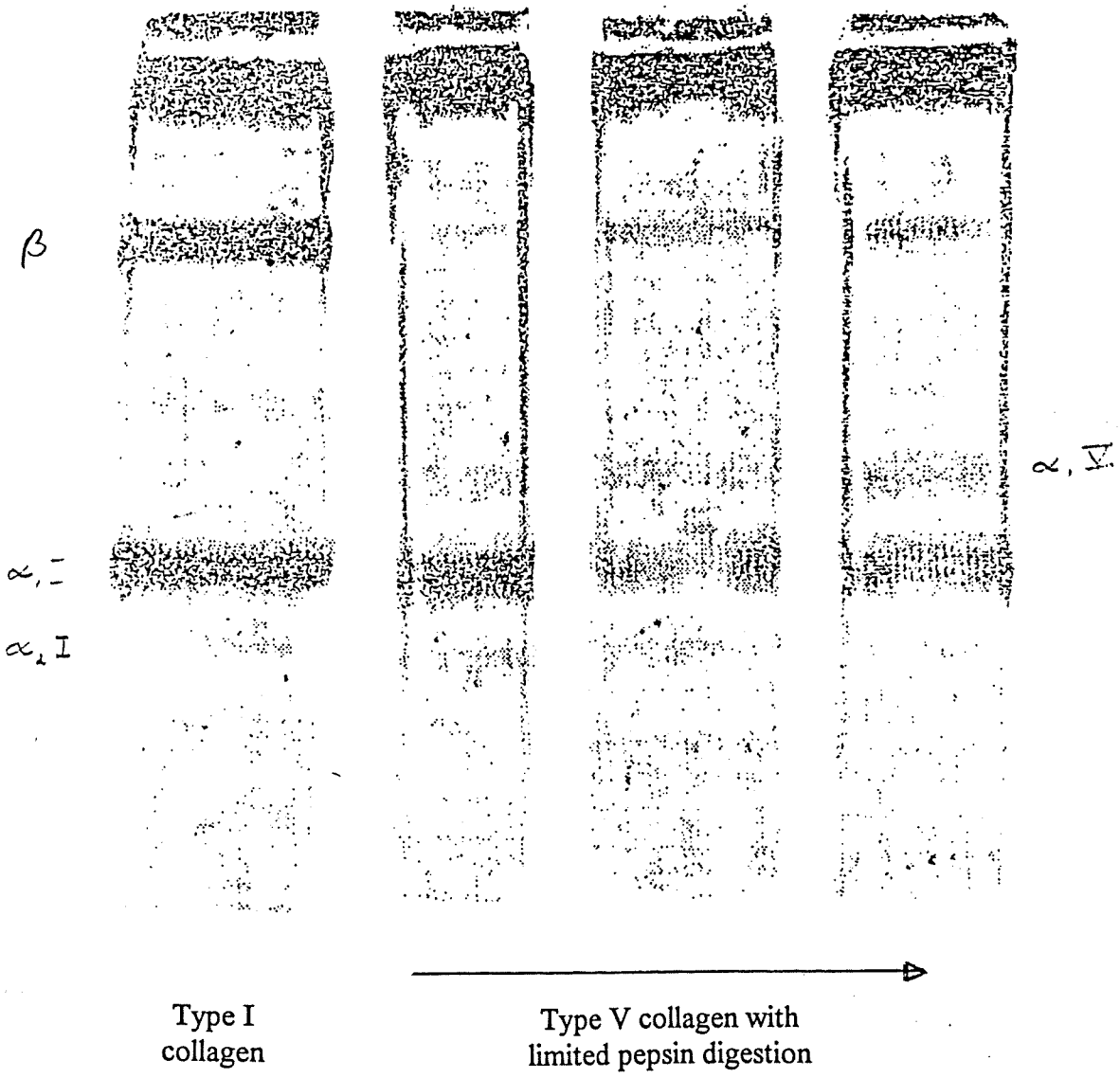

Figure 15: Effects of limited pepsin digestion on release of type V collagen from high molecular weight fraction.

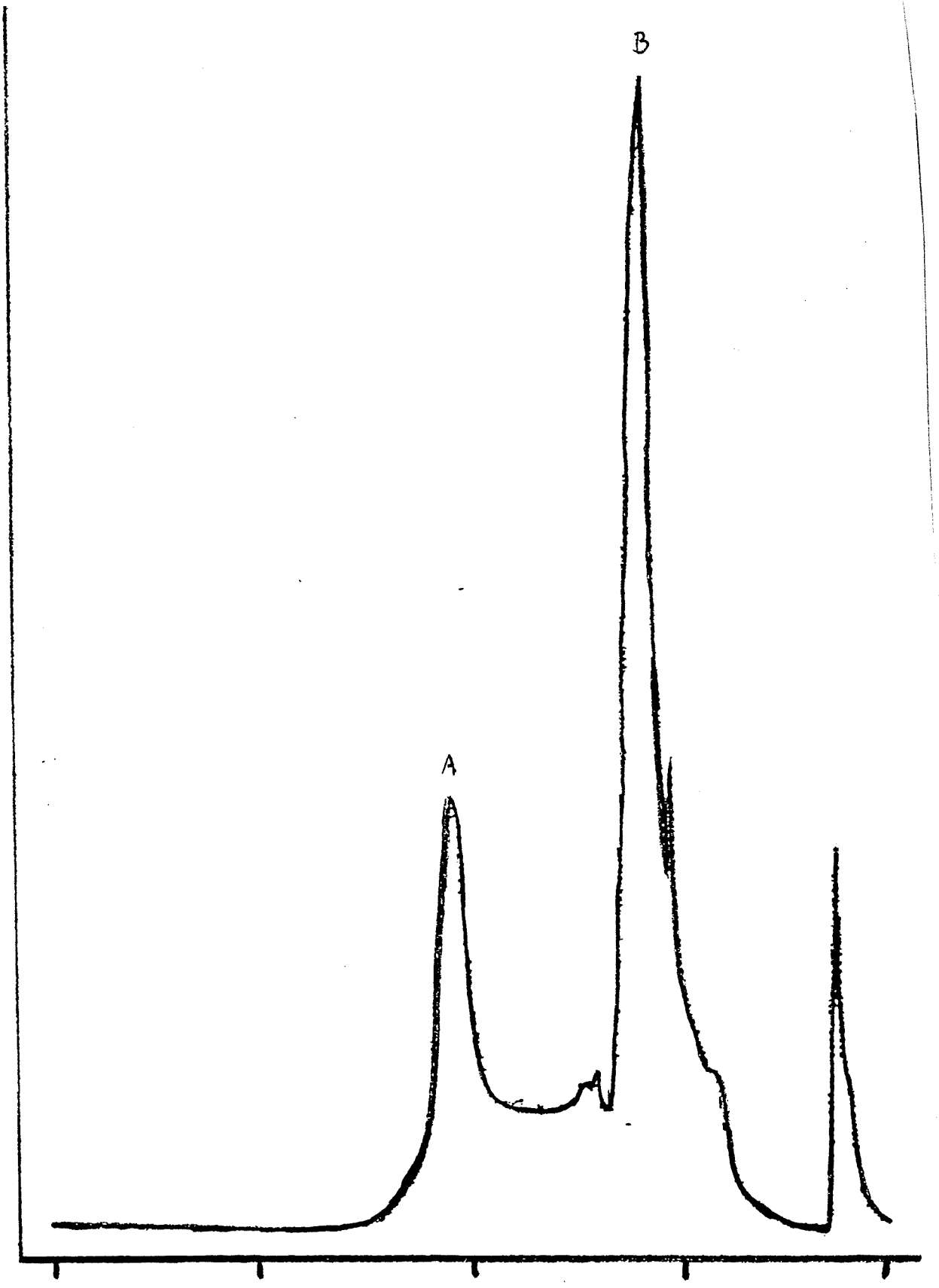

Figure 16: Reverse Phase HPLC chromatogram of Blue Grenadier type V collagen.

H.H. Huss et al., (eds.) Quality Assurance in the Fish Industry

Fish flesh structure and the role of collagen - its post-mortem aspects and implications for fish processing

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Abstract

Structural links between the muscle cells and the connective tissues of fish provide the necessary integrity for the flesh to withstand the effects of post-harvest handling, processing and storage. Therefore, a fundamental knowledge of the components of the delicate and complex structure of fish flesh is essential in order to understand the changes that occur post-harvest. This understanding can then lead to techniques and processes to minimise change, or, to use it to advantage.

Minor components which form the interfaces and adhesive links are as important in this regard as are the major components. Within the muscle cell, elements of the cytoskeleton serve to link the major proteins, actin and myosin, into an ordered structure. External to the cell, collagen is the major connective tissue in the structure and it is dominant in determining the textural attributes of the raw flesh. The links between the muscle cells and the external connective tissue occur mainly at the myotendinous junction where the forces of muscular contraction are transmitted from within each cell to the connective tissue of the myocommata.

Post-mortem changes occur within the muscle cell in the elements of the cytoskeleton, in interactions between the proteins in the cell and externally in the links between the cell and its envelope and in the myotendinous junction. The relative importance of these phenomena varies with species and circumstance, but each affects the characteristics of the flesh during processing.

This review concentrates on the external structures and on changes occurring at the myotendinous junction and in the fine collagen fibrils of the endomysium.

INTRODUCTION

Sikorski *et al.* 1984 [1] have reviewed collagen in fish, Howgate [2] has summarised work on fish muscle structure up until 1980 and the continued output of Love and his colleagues in a series of investigations spanning many years, most recently reviewed in 1988 [3], provide the background for this paper and reference is mostly restricted to recent work. In order to discuss the nature of post-mortem change and the mechanisms and

possible agents causing these changes, it is necessary to first describe the flesh structure including the myotendinous junction and the muscle cell constituents, the basement membrane and the extracellular matrix.

FISH FLESH STRUCTURE

The flesh of teleost fish is constructed of adjacent muscle blocks, called myotomes, separated from each other by sheets of collagenous tissue called myocommata [4]. Within each myotome, the muscle fibres (myomeres, sarcomeres or myofibrils in the literature on mammalian muscle) run approximately parallel to each other but at varying angles to the myocommatal sheet to accomodate the juxtapositional rhythmical contractions that occur during swimming so that all the fibres in the myomere contract to a similar extent when the fish bends. This results in maximum power output at a given rate of contraction [5]. The myocommata are connected internally to the skin and to the skeletal system and are also linked to the membrane dividing the fish into epaxial and hypaxial planes and to the median vertical septum.

The junctions between the myomeres and the myocommata in fish are equivalent to the myotendinous junction in mammalian muscle. In fish, this junction is formed by fine collagenous processses which have their origin in the myocomma and which then proceed as sheaths to surround each muscle fibre [6,7].

GENERAL ASPECTS OF THE MYOTENDINOUS JUNCTION

Early work with the electron microscope established that the muscle fibre terminated in finger-like projections which were bounded by the cell membrane and that the actin filaments terminated inside the cell and that collagen fibres did not penetrate the cell membrane and were never found within the muscle cell [8,9]. A complicated series of folds and invaginations at the myotendinous junction increase the area of contact by a factor of about 20 to 30 times for fast twitch muscles [10] and 50 times for tonic cells [11]. This reduces the load on the cell membrane by an order of magnitude [12]. The geometry of attachment of the actin filaments to the sarcolemma ensures that the forces are transmitted in shear rather than in tensile mode [13].

THREE STRUCTURAL DOMAINS

Muscle cells are bounded by a continuous cell membrane called the sarcolemma. External to this is the basement membrane and the endomysial layer of fine collagen fibres. The myotendinous junction can be considered as three major structural domains which are in close proximity: (i) the internal elements of the muscle cell and the sub-sarcolemmal surface; (ii) the membranes and fine processes at the junction; and (iii) the external connective tissue stroma which eventually attaches to the tendon.

The internal structure

Most of the detailed work on muscle components has been done on mammalian muscle and a recent comprehensive review of muscle structure and biochemistry is given by Pearson and Young [14]. The presence of many of the minor proteins of the mammalian cytoskeletal system has not yet been established for fish muscle.

Muscle fibres are comprised of bundles of fibrils (myofibrils) arranged longitudinally within the muscle cell and organised within a cytoskeletal framework of desmin-containing intermediate filaments [15]. The basic repeat unit of the myofibril is the sarcomere (often termed myomere in fish) and each sarcomere is bounded at each end of its long axis by the electron-dense structure known as a Z disc (or Z band, or Z line) (see Figure 2,3 later). Actin filaments extend from one Z disc to the next and, in cross-section, are arranged in an hexagonal array parallel to and around the myosin rods. The myosin rods themselves do not attach to the Z disc but occur in the mid-portion of the sarcomere. The rod-shaped protein tropomyosin occupies the grooves of the actin helix providing a structure for the globular protein troponin to attach to at regular intervals. Titin (originally named connectin, when first isolated in a slightly impure form [16]), together with nebulin, forms 'gap filaments' which join the thick myosin filaments from their ends to the Z disc and which thus stabilize the myosin in the centre of the sarcomere [17].

The protein components of the Z disc have not been fully resolved, but alpha-actinin, a protein with actin-bundling properties, comprises about 50%. Actin filaments from adjacent sarcomeres overlap into the structure. Other proteins that have been suggested as components are Z-protein, amorphin, Eu-actinin, Z-nin, filamin, zeugmatin and another 220 Kdalton protein [18]. Zeugmatin is present in the early formation of Z disc structures grown in cell culture, before alpha-actinin is observed, and hence probably plays some organisational role in development and organisation [19]. At the edge of each fibre, elements of the cytoskeleton link the filaments from the terminal Z disc to an electron-dense meshwork at the sub-sarcolemmal surface. The terminal Z discs near the sarcolemma have been found to contain much less alpha-actinin and it is assumed that this indicates that thin filaments are not bundled by alpha-actinin near the sarcolemma [20] and that Z discs in different locations are not homogeneous in composition. Talin, a 225 Kdalton protein has also been located at myotendinous junctions and is a component of the digit-like processes that extend into the tendons there and may be involved in force transmission [21].

The cytoskeletal proteins, the skelemins, are located at the periphery of the M discs while vinculin is organised along the sarcolemma in an array of rib-like bands termed costameres [22]. Transverse sarcomeric filamentous systems organised at the Z and M band levels link these structures to the sarcolemma [23].

Each fibril is bathed in cytoplasm and is partly enshrouded by the membranous sarcoplasmic reticulum containing the sarcoplasm. This sarcoplasmic reticulum swells out to form the terminal cisternae which in apposition with those of the adjacent sarcomere and the intermediate element, the T tubule, make up the triad structure. In fish, the triads are situated external to the Z disc but in mammals the triad occurs in the midportion of the sarcomere near the junction of the A and I bands.

Near the end of a muscle fibre, the sarcomeres anastomose around the invaginations of the sarcolemma. Actin fibres reach from the last complete Z disc to the internal surface

of the sarcolemma where they attach in an electron dense sub-sarcolemmal layer. This layer is comprised of both globular densities and fine linear elements which run approximately parallel to the major actin fibres [24] and is involved in anchoring the actin to the interior of the sarcolemma, referred to as the internal lamina [25]. The proteins talin and vinculin are found at this location and it is considered that these may be the force-transmitting and attaching proteins [21]. Longitudinal muscle growth occurs at the fibre ends [26] and glycogen granules, mitochondria, polysomes and ribosomes occur with notable frequency near the fibril ends [27].

The basement membrane

The basement membrane, sometimes called the basal lamina, occurs adjacent to the sarcolemma as part of the integral structure marking the boundaries of cells, although it may not follow exactly all the convolutions of the sarcolemma. The basement membrane serves a variety of functions such as molecular ultrafiltration and tissue organization and mediation of interactions between specific cell layers and their underlying stroma. The outer area of the basement membrane, the *lamina densa*, is an electron-dense area that is comprised mainly of collagen, mostly type IV. The *lamina lucida (rara)* lies between the *lamina densa* and the sarcolemma and is more transparent to electrons. In the *lamina densa*, the type IV collagen molecules form unique end-to-end associations to construct a network of molecules which comprise the structural framework of the membrane [28]. Several other components, in particular laminin and heparin sulphate proteoglycan, form parts of the overall structure. The large molecular weight glycoprotein laminin has been localised to the basement membranes of skeletal muscle and is distributed throughout the *lamina densa* and the *lamina lucida* [29,30]. The proteoglycans are comprised of a central protein core with covalently bound glycosaminoglycan side chains which affect permeability and cell attachment [30].

It was proposed that collagen fibres from the connective tissue were attached to the basement membrane. The analogy of a rope untangling at its end to provide the fibres which were woven into the carpet of the basal lamina was employed to convey the concept of how force may be transmitted from the muscle to the tendon [31]. Tropocollagen molecules were proposed as the links between the sarcolemma and the basal lamina.

Low [32,33] described a set of fibrous structures with a diameter in the range of 4 to 12 nm. These structures, which were finer than the collagen fibrils, appeared to link the collagen fibrils. He termed them 'microfibrils', although this term has other connotations in collagen chemistry. 'Microfibrils' were also reported in muscle-tendon transitions in the papillary muscle of the heart, muscle from the tip of the tongue, the diaphragm and the gastrocnemius of the guinea pig [34] and it was proposed that these elements actually passed through the basal membrane and fused with the outer electron-dense layer of the sarcolemmal membrane. Similar structures were demonstrated [35] in the myotendinous junctions of muscles of the hagfish (*Myxine glutinosa* Linnaeus) where spine-like structures about 6 nm in diameter at 15-25 nm intervals extended from the external leaflet of the plasma membrane to the internal surface of the *lamina densa*. Further work [36] showed similar 'intermediary' structures in the myotendinous junctions of the lamprey [37]. When detergent and EGTA solvents were used to disrupt the sarcolemma, the small filamentous structures that cross the *lamina lucida* remained intact and tension could still be transmitted

across the myotendon, indicating that these filaments were attached to elements of the contractile structure not just to the sarcolemma alone [38]. From this and other evidence, three important structural facets were proposed: one that binds actin near the sarcolemma to transmit the contraction; another that crosses the hydrophobic portion of the membrane; and a third that transmits tension from the membrane to the *lamina densa* [38]. Further work indicated that the filaments of the *lamina lucida* are composed of two subdomains: one closely associated with the sarcolemma the other with the *lamina densa*. The connection between the layers is ionic, not covalent [38]. There does not appear to have been any further advances in ascertaining the identity and structure of these filaments.

Nakao [36] first observed that the basement membrane, which otherwise is continuous, is absent in the terminal ends of the invaginations in the finger-like projections of the ends of the muscle cells in the lamprey and the tadpole. This absence is unusual since no breaks occur in the sarcolemma.

The extracellular matrix

The major structural feature of the extracellular matrix is the collagenous network that surrounds each cell and which forms the tendons and ligaments that attach to the skeletal system. In the endomysium, the fibres are fine and tend to form a lace network around the muscle fibre [39]. Branched reticular fibres, now considered to be type III collagen, form rows arranged obliquely or perpendicularly to the long axis of the muscle [40]. Larger diameter collagen fibres of the perimysium surround the muscle fibre bundles and these merge into the major sheets of collagen of the epimysium which cover individual muscles to become tendons.

Fibroblasts, with their flattened dendritic processes, are often present at boundaries between adjacent layers of collagen fibres. These fibroblasts, derived from mesenchymal cells, produce most of the collagen adjacent to the muscle cell ends. Adipose cells, mast cells and macrophages containing lysosomes are regularly noted in the looser connective tissues. Eosinophilic leukocytes and other plasma cells occur nearby. Elastin fibres, which play a role in the providing some of the elastic properties of the tissue are also present.

In muscle tissue, the large collagen fibres are almost invariably type I collagen. In mammalian muscle, smaller proportions of type III collagen occur, while types IV and V are associated with the basement membrane. The collagen fibres embed in a matrix of proteoglycans. They are often crimped and run in layers in different directions to provide a strong flexible structure. The fibres have a range of diameters according to their position and function (from 30 nm to over 100 nm).

Early studies [31] indicated that finer connections appeared to join the collagen fibres. Some external tissues contain 'anchoring fibrils' with the recently discovered type VII collagen [41,42] as the primary structural agent. This collagen forms an extended network of these fibrils between anchoring plaques in the *lamina lucida* of many epithelial tissues [43]. Collagen type VII has not been found in muscle or other internal organs.

THE MYOTENDINOUS JUNCTION IN FISH

Schwarzacher [44] compared muscle fibre-tendon junctions in the seahorse with those from the cat, rat, mouse and frog. There were considerable similarities and all the junctions revealed folds and finger-like projections at the ends enveloped by the basement membrane [45]. The myotendon junction of the flowing muscle of the spinal cord of the pipefish was similar to the appearance of that in the tadpole tail. In the primitive hagfish (*M. glutinosa* L.), spine-like projections and thread-like cones between the *lamina densa* and the external surface of the sarcolemma were found [35]. They were considered to be the same feature, namely ring-like structures that, according to the angle of section, could appear either as spines or threads. Fibres from the I band (actin fibres) attaching to the sub-sarcolemmal surface in electron-dense areas were observed and were considered to be analogous to Z discs [35]. The sequence of events in the longitudinal growth of myofibrils of the skeletal muscle of *Macropodus opercularis* is that high ribosomal concentrations occur in the short terminal myofibril region along which actin filaments are formed [27]. As the terminal portion enlarges, myosin filaments are evident and A and I bands appear, followed by the Z disc which forms in close contact with the sarcolemma and gradually detaches from it. In the lamprey, the sarcolemma at the terminal end of the invagination frequently showed specific coupling with the cisterns of the sarcoplasmic reticulum. The basal lamina was partially or completely deficient in this area [36]. Although this allowed for the possibility of collagen fibres to be in direct contact with the sarcolemma, no definite relationship between them was found.

Recent studies

The three-dimensional structure of the junction and the effects of post-mortem storage have recently been reported [46-48]. In the blue grenadier (*Macruronus novaezelandiae* Hector), a network of fine collagen fibres emerges from the myocomma to envelope the muscle fibre along its length (Figure 1) [cf. 49,50]. The muscle fibres fit into socket-like indentations in the myocomma which are revealed when the muscle fibres are removed [48].

The microstructure at the myotendinous junction at the base of a fibre reveals folds and invaginations in the fibre ends filled with fine collagen fibres (Figure 2,3) which may protrude up to 10 μm into the fibre end. The sarcolemma forms a continuous boundary to the cell (Figures 2,3,4), but the basement membrane lying outside this appears to be discontinuous near the ends of the invaginations which often occur in close apposition to a vesicle of the sarcoplasmic reticulum [48]. Fine connections of an unknown nature exist between the collagen fibres, between these fibres and the basement membrane, and between the basement membrane and the sarcolemma (Figure 5,6). Within the muscle cell, the regular banding pattern typical of skeletal muscle is evident (Figure 2,3,4) and fine filaments, resembling actin, proceed from the what appears to be the last Z disc to the inner surface of the sarcolemma, where they appear to be attached by some electron-dense material (Figure 4). In all respects, these studies confirm the nature of the myotendinous junction as discussed above. The structure at the periphery of the fibre is also typical [48].

It has recently been shown in guppy muscle (*Lebistes reticulates*) that the structure of what appears to be the terminal Z disc is not a Z disc but some as yet uncharacterized

electron-dense band. The actin bundling protein alpha-actinin was absent and actin filaments were observed to pass through the band without any alteration in orientation as indicated by decoration with heavy meromyosin [51].

FISH COLLAGENS

The collagens of fish are, in general, much more easily solubilised than those of mammals. The major collagen present is type I [1,52]; type II has been found in the cartilage, skin and notochord of lampreys [53-55]; type V has been reported in carp and spotted mackerel [57] and in lamprey along with type IX [56]. Evidence for the fibre-forming type III collagen common in mammals has not been found in any investigation to date. Type IV has not been reported either, probably because it has not been specifically sought. Nonetheless, fish have quite clearly delineated basement membranes and it is reasonable to assume that type IV collagen is present. In the intramuscular tissue of teleost fish only types I and V have been demonstrated to be present.

Piez [57] was the first to demonstrate the presence of three different α-chains in the skin of cod and more recently they were also reported in rockfish [58]. Kimura [52] and Kimura and Ohno [59] found the $\alpha 1\alpha 2\alpha 3$ heterotrimer of type I collagen in the skin of carp and alaska pollock, whereas the swimbladders of these species contained the dimer $\alpha 1_2\alpha 2$. This indicated tissue-specific localisation of the two molecular forms. The three different α-chains in the type I collagen of the skin of fish from ten different orders was reported, but the occurence was not consistent within an order. The widespread occurrence of three different α-chains led the authors [60] to suggest that the gene for the $\alpha 3$ chain may be universally present in teleosts, having arisen about the time of the adaptive variation of the bony fish, but that it may be quiescent or only poorly expressed in some species. In contrast, the collagens of the lower vertebrate species, such as lamprey and shark, do not exhibit the $\alpha 3$ chain at all. The occurrence of the $\alpha 1\alpha 2\alpha 3$ trimer in the type I collagen from the skin of the blue grenadier was also reported [61]. Within the muscles of fish, studies of the type I collagen of the myocommata indicate that eel and mackerel have the $\alpha 1\alpha 2\alpha 3$ heterotrimer, that saury contains only $\alpha 1$ and $\alpha 2$ chains and that carp and chum salmon seem to possess two different heterotrimers, with $\alpha 1_2\alpha 2$ as a major component and $\alpha 1\alpha 2\alpha 3$ as a minor component [62].

The presence of type V collagen in the white muscle of carp in a higher proportion than it occurs in mammalian muscle has recently been reported [56]. Electrophoresis of the fractions, after treatment in reducing conditions with 2-mercaptoethanol, did not change the band pattern, indicating the absence of reducible thiol bonds and hence the absence of type III collagen. Further studies showed the presence of type V in lizard fish, japanese eel, sturgeon, spotted shark and lamprey, suggesting widespread occurrence of type V in both elasmobranchs and teleosts [63]. The relative concentration of type V collagen to type I was higher in the endomysial fraction than in the myocommatal fraction from carp and spotted mackerel. Both the type I and the type V were less soluble in the endomysial fraction than in the myocommatal fraction. Three distinct chains of type V were reported to occur in the molecular forms $\alpha 1_2\alpha 2$ and $\alpha 1\alpha 2\alpha 3$. A higher proportion of the heterotrimer was found in the endomysium in comparison to the myocommata. This recent

evidence indicates that, not only can there be differences between major tissue groups (e.g. muscle, skin or swimbladder), but that differences in the chain structure can occur within different domains in the one tissue. These differences in chain composition are likely to result in slightly different properties and stabilities in the collagen.

In fish muscle, type V collagen probably plays a role similar to that of type III in mammalian muscle in that it forms copolymers with type I and acts to control fibril diameter [64-66]. Collagen fibrils of different diameters are to be found in the myocommata near muscle fibre ends and in the invaginations of the myotendinous junction. Borresen [67] developed a method for preparing the muscle cell envelope from cod (*Gadus morhua*). These envelopes were tubular structures with an outer three-dimensional network of fibres (30-60 nm in diameter) and an inner membrane about 2000 nm thick. Further work using this method showed that the cell membrane was composed of three layers [49]. The innermost layer, the sarcoplasmic membrane, was 8-16 nm thick. The middle layer, the basement membrane, was 50-70 nm thick and the outer layer, which was mostly collagen, was approximately 600-1100 nm thick. This collagen was shown to be type I, possessing a chain structure $\alpha 1_2 \alpha 2$ containing the acid- and heat-stable cross-link hydroxy-lysino-5-keto-norleucine.

Collagen content and texture

The content of collagen in fish muscle varies considerably from species to species and is found in increasing proportion in the tail region. In the main edible portion, concentrations of 0.3% to 3% are common [1,52,68], but even within species there is seasonal variation as the body reserves are depleted during spawning and migration. Since muscle is not conserved there is greater reliance on the connective tissues to hold the fish together.

Figure 1. Pre-rigor blue grenadier muscle. Fine collagen fibres (**C**) connect the muscle fibres (**F**) to the myocomma (**M**). Bar 100 μm.

Figure 2. Muscle fibre end from a pre-rigor fish showing collagen-filled (**C**) grooves and folds bounded by the basement membrane. Bar 5 μm.

Figure 3. Pre-rigor fish at a muscle fibre end with an elongated collagenous invagination penetrating into the myofibrils. Bar 5 μm.

Figure 4. Muscle fibre base showing fine filamentous fibres (**arrow**) extending from the terminal segment to the inner surface of the sarcolemma (**S**). Bar 2 μm.

Figure 5. Cross-section of a groove at the muscle fibre end showing well-defined sarcolemma (**S**) and basement membrane (**arrow**). Bar 500 nm.

Figure 6. Detail of Figure 5 showing fine connections (**arrow**) between collagen fibres, in cross-section, and basement membrane. Other connections across the *lamina lucida* between the basement membrane and the sarcolemma and between collagen fibres are evident. Bar 100 nm.

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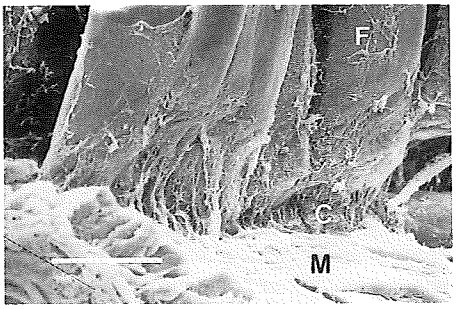

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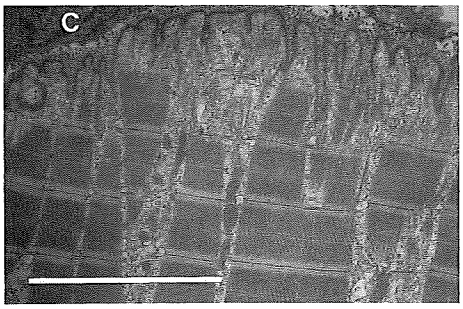

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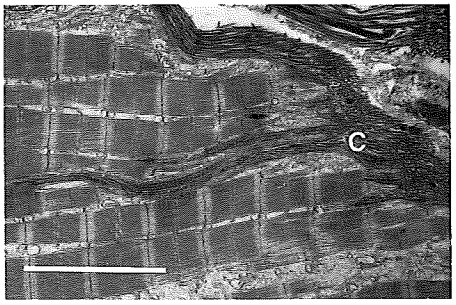

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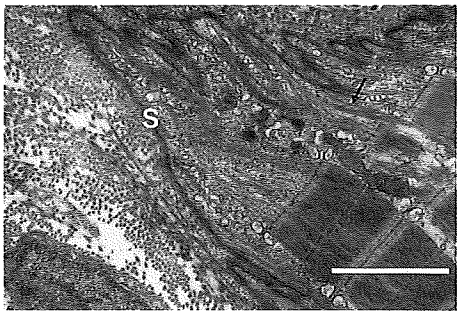

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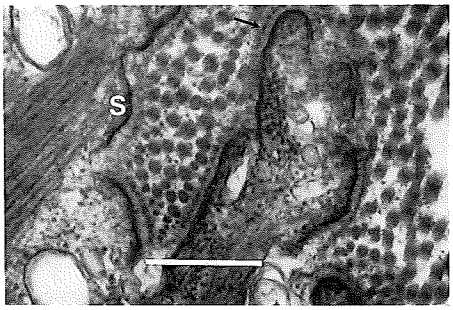

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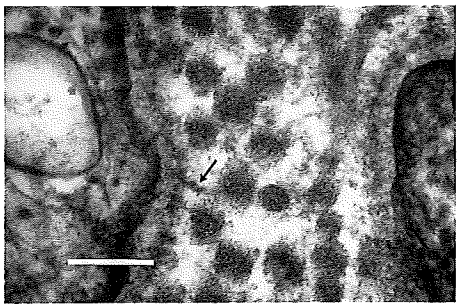

Figure 6.

Collagen content should affect the textural properties of the muscle but this relationship is not straightforward because of the seasonal turnover and other species effects. Sato *et al.* [68] investigated the collagen content and the texture of twenty two species of fish and concluded that there was a broad relationship between collagen content, swimming motion and the raw texture of the flesh prepared for sashimi. A high collagen content (near 2%) meant that the species were too tough for sashimi. Hatae *et al.* [69] also reported a significant correlation between collagen and raw texture. In cooked fish, the reverse is true and the collagen softens and the myofibrillar component toughens to become the dominant component [70]. Indeed, a model has been proposed to explain textural differences between species on the basis of fibre diameter and the amount of coagulated sarcoplasmic material that sticks the fibres together and impedes them sliding across on another when chewed [71]. Other workers have found no relationship between collagen and the raw texture in a limited number of species [72].

It is well established, that in mammalian tissues, collagen cross-linking increases with the age of the animal and that the tissue increases in toughness. Because of the seasonal changes occurring in most fish species, this relationship is less clear. It is not just the amount or proportion of collagen present but the degree to which it is crosslinked that affects texture. Montero and Borderias [73] measured collagen content, the proportions of α, β and γ chains and the shear force in the muscle of trout (*Salmo irideus* Gibb) from four different size (age) groups. Although there was a higher proportion of connective tissue in the oldest fish, it had slightly higher acid solubility and fewer cross-links and did not give higher shear strength values. It is generally regarded that there are higher levels of connective tissue near the tail region [6] and this was confirmed recently for trout and hake, with higher levels of connective tissue in the ventral than the dorsal portions for the trout [74]. Shear strength values were highest nearer the tail as was a higher proportion of insoluble collagen. The amino acid composition, chain structure and type of collagen from trout and hake were also characterised in samples from the skin, myotomes, fasciae and myocommata. The collagen from all sites in both species was mainly type I and type III was not detected. The amino acid compositions differed from mammalian sources and the fish skin collagen was less cross-linked than the collagen from the fish muscle.

POST-MORTEM CHANGES

During post-harvest storage, textural changes occur in many fish species long before they are spoilt. The result is that the flesh softens and gapes, trimming losses occur, products have a poorer appearance and are downgraded, and, in extreme cases, mechanical processing becomes impractical since the fillets fall apart in the skinning operation. Softening and gaping is a common problem in the merluccid hakes [75-78] and in the related species blue grenadier (hoki, *M. novaezelandiae*) [79]. Farmed species also have this problem [80]. It is clear that there are differences between related species and that circumstances affect whether gaping occurs. Whole cod stored in ice showed no gaping whatsoever, even after 6 weeks storage when the fish were thoroughly spoilt [81]. This lack of change was further borne out with measurement of the forces need to pull samples

apart at the myocommata. No change in cohesiveness was found for cod stored in ice for up to 26 days. Whole muscles loaded to failure fail at or near the myotendinous junction in the region between the cell membrane and the *lamina densa* of the basement membrane [82].

Post-mortem change attributed to changes in the collagen.

Unfortunately, there are few published investigations into the nature of the specific changes that occur. Using goldfish (*Carassius auratus*) as a model species, changes in the structure could be observed in post-rigor fish held in ice for 3-4 days, in comparison to pre-rigor fish [83]. Shrinkage and distortion of the myofibres, an increase in the extracellular space, loss of configuration of the endomysium and breakdown of the connective tissue occurred. Further changes to these structures occurred with subsequent storage. Similar structural changes in the flesh of the major carp (*Labeo calbasu*) during chilled storage were also observed histologically [84]. Disorganisation of the structure was evident when the samples were examined after 7 days. By 14 days, fissures appeared in the cells and partial disappearance of the connective tissue structure was apparent.

Texture, determined as shear force using the Kramer shear press, was related to an increase in heat-soluble collagen in rockfish stored in ice [85]. A significant decrease in the solubility of collagen from trout muscle occurs during rigor, followed by an increase in solubility during storage post-rigor [86]. The proportions of heat- and acid-soluble collagen increased during and after rigor while the levels of insoluble collagen and shear strength progressively decreased. Proteolytic activity increased post-rigor.

Changes in the myotendinous junction observed with SEM and TEM

The structure of the myotendinous junction of the blue grenadier did not change during rigor mortis, but after 8 days storage in ice the basement membrane and the collagen fibrils in the tubular invagination were degraded [48]. Progressive deterioration of the fine collagen results in detachment of the muscle fibre from the myocomma (Figure 7) and notable deterioration within the muscle fibre end leading to the production of vesicles (Figure 8). This is in accord with the results shown by SEM where the fine collagen fibres of the cell envelope are degraded (Figure 9) and muscle fibres are shown detached from the myocomma (Figure 10).

The progressive deterioration and disruption of the structure is consistent with it being attacked by enzymes. Collagen is normally considered to be quite a stable protein, yet these micrographs indicate digestion within a few days at 0°C. This implies that either these collagens are very susceptible to attack, or that the enzymes present are very active. Even if the extent of disruption seen in Figures 7 & 8 is partly an artefact of preparation, the structure must have been severely weakened to allow this to occur [46-48].

Use of antibodies to identify collagen fibre types and to detect changes

An immunogold method [29, Bremner unpublished] using an antibody to purified fish type I collagen [61] and protein A-gold complex was used to label the myocommatal collagen (Figures 11,12). The individual collagen fibres were not defined well enough to be able to state unequivocally whether, or not, they were labelled with the gold. Collagen *in situ* can be difficult to stain and, in these preparations, the normal post-fixation with

osmium could not be included since this would have destroyed the antigenic sites. Had the technique proved adequate, then antibodies to other collagen types would have been used to localize them in the tissue and identify the fine fibrils in the invaginations. These fibrils may be copolymers of more than one type with the minor component buried in the interior as occurs in other tissues [64-66] making them inaccessible for reaction with antibody unless suitably treated.

Although rockfish soften in storage and their collagen becomes more heat-soluble, no collagen breakdown products were detected by an antibody to bovine type I collagen when extracts were run on electrophoretic gels [87]. This antibody reacted with intact collagen, with cyanogen bromide peptides and with peptides produced by collagenase treatment of purified rockfish collagen. If the collagen was degraded, either the fragments were degraded so rapidly once they were released from the fibrils that they were lost in the extraction procedure, or they were of such nature that they did not react with the antibody. If the detected increase in heat-soluble collagen was due to another collagen type present in minor proportion, such as type V, then reaction of the fragments with the antibody could not be expected.

Proteases and collagenases

Collagen is degraded by a number of enzymes acting in concert, with the initial attack on undenatured molecules performed by a collagenase acting at a specific site, followed by other enzymes acting on the fragments [88]. Historically, the mammalian collagenases are defined by their ability to cleave the triple helical region of the native collagen molecule at a specific locus. The discovery of a broader range of collagens with globular

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Figure 7. Muscle fibre base has separated from the myocommata, the invaginations are empty and amorphous material occurs near the collagen fibres in blue grenadier stored 8 days in ice. Bar 1 μm.

Figure 8. Vesicles have formed in the ends of the myofibrils. The sarcolemma is mostly intact (**arrows**) but only degraded material remains in the invaginations. Bar 1 μm.

Figure 9. Disruption of fine collagen fibres (**C**) of muscle cell envelope, allowing muscle fibres (**F**) to part from myocomma (**M**) in spotted trevally stored 4 days in ice. Bar 100 μm.

Figure 10. Muscle fibre (**F**) completely detached from myocomma in blue grenadier stored 11 days in ice. Bar 100 μm.

Figure 11. Gold particles (15 nm diam.) label myocomma (**M**) of pre-rigor blue grenadier, with only a few random labels in the muscle fibre (**F**). Bar 1 μm.

Figure 12. Gold particles label myocommatal collagen fibres of rock cod fixed within 30 seconds of death. Bar 1 μm.

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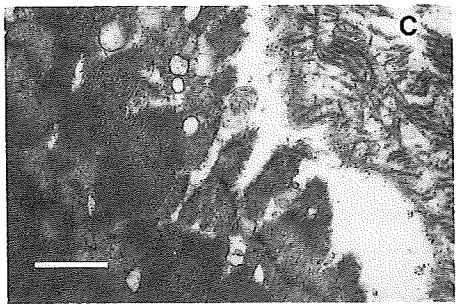

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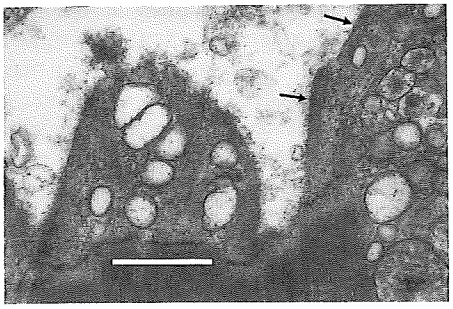

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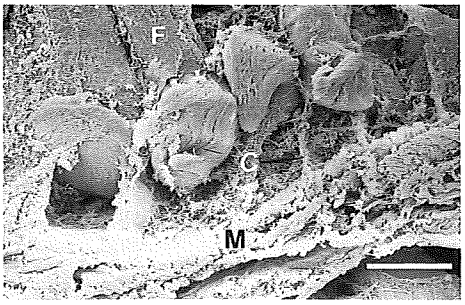

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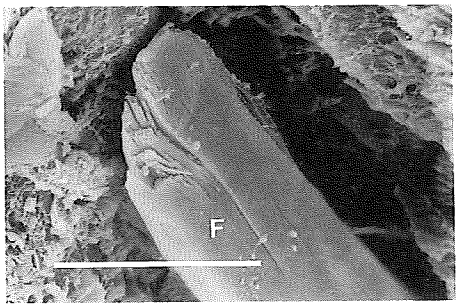

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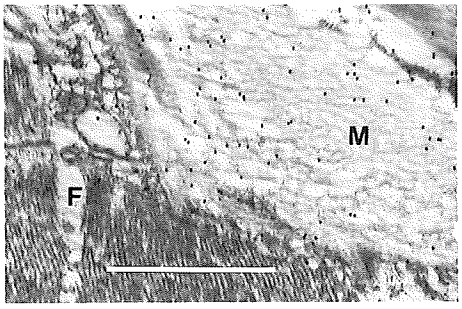

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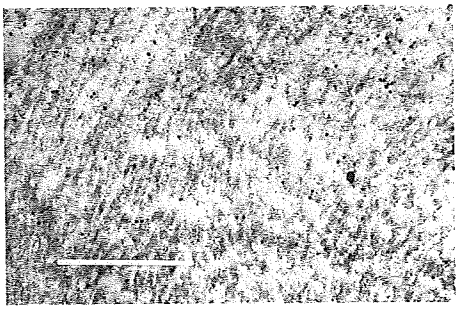

Figure 12.

domains and discontinuities in the helix has required that this simple definition be modified. Furthermore, serine proteases and a heterogeneous group of proteases can degrade some collagen types. Two main groups of collagenases have emerged - the 'classical' collagenases which degrade types I, II and III collagens and type-specific interstitial and basement membrane collagenases which attack types IV and V [89]. Collagenases are mostly metallo-endoproteases having a pH optimum in the range 7 to 8 which are activated and stabilised by calcium ions. Consequently, they are inhibited by metal chelators such as EDTA. They have been isolated from a wide variety of tissues, but not as yet from fish muscle tissue although collagenase from fish caeca and pancreas has been known for some years [90].

The collagenases that degrade types I, II and III collagen do not affect types IV and V collagen. Specific collagenases which degrade type IV have been isolated from tumours, but it can be degraded by other metalloproteinases including gelatinase, proteoglycanase, serine proteases, neutrophil elastase and mast cell chymase. Type V is attacked by metalloproteinases which are also gelatinases of a molecular weight greater than the classical collagenases [89,91].

Type V collagen isolated from bovine bone is susceptible to attack by trypsin at 35°C [92] at a site which may represent the natural target domain for cleavage *in vivo* and which is at the opposite end of the molecule to the site at which type I is attacked by collagenase. This indicates that, *in vivo*, separate enzyme systems are necessary for types I and V to be copolymers in the same fibril.

After synthesis, collagenases are secreted into the extracellular matrix and it is obvious there must be specific inhibitory mechanisms to prevent spontaneous tissue destruction. The collagenases, present in the tissue in latent form as zymogens, are activated by a wide variety of proteolytic enzymes such as trypsin, plasma kallikrein, cathepsin B, plasmin [93], but once activated they must be controlled. In human tissue, the main control mechanism seems to be through small cationic glycoproteins known as Tissue Inhibitors of Metallo Proteinases, or TIMP. They are ubiquitous and their relatively low molecular weight, 28.5 Kdalton, allows them ready access within the extracellular matrix where they play a major role in collagenase inhibition [89]. Other control mechanisms such as the α_2-macroglobulin molecule in plasma are too large to penetrate the tissue.

The basement membrane degrading enzyme, collagenase IV/V -gelatinase, is more easily released by kallikrein and becomes active in the extracellular space before other lysosomal proteinases are released [94]. Tissue kallikrein, a serine protease (specifically an arginyl esteropeptidase), also activates type I collagenase and is a likely candidate to perform these functions *in vivo*. It may also be an activating factor in post-mortem tissue. There are about twenty kallikreins which seem to be ubiquitous. So far they have only been reported in the skeletal muscle of the rat. The lysosomes in fish muscle are commonly associated with the connective tissue [94] so that cathepsin B, which could also activate this collagenase, would be released by post-mortem disruption of the lysosomes. The concentration of calcium ions would not be a factor limiting collagenase activity due to leakage from the sarcoplasmic reticulum. Indeed, it has been shown that the introduction of Ca^{++} into fibroblasts promotes a cascade of proteolytic events culminating in activation of collagenase [93].

The question is whether these mechanisms are present in fish tissue and whether they

are active at post-mortem pH and at chill temperatures. The type IV/V basement membrane collagenase isolated from human leukocytes has a pH optimum between 7 and 8, with about 50% of this optimum activity at pH 6 [96]. No information relating to kallikrein in fish has been found but it is likely to be present and, since it is active at physiological pH, it is likely to retain activity at post-mortem pH. However, it too is subject to the effect of inhibitors in the serum, similar to trypsin inhibitors. Cathepsin B may not be very active at post-rigor pH.

Freezing damage

Although a comprehensive coverage of frozen storage changes is outside the scope of this paper, it is pertinent to note here some of the recent observations that changes in the collagen contribute to changes in texture which occur in frozen stored fish.

Both trout (*S. irideus*) and hake (*Merluccius merluccius* Linnaeus) collagens exhibited a decrease in the proportion of α-chains and a concomitant increase in γ- chains during frozen storage at -18°C after only 25 days, with further changes occurring progressively. The amount of insoluble collagen in the hake samples also increased with the period of storage [97]. This increase in collagen insolubility in hake flesh and decrease in the percentage of heat-soluble collagen with period of frozen storage was confirmed in further studies and it was suggested that aggregation of hake collagen was due to reaction with formaldehyde produced by breakdown of trimethylamine oxide [74,98]. This is also consistent with the other reports [2,100] that suggested that the remnants of the sarcoplasmic reticulum could serve as a glue to cement the fibres to produce a tougher product, the opposite of the situation that occurs in chilled storage.

Walton and Gill [50] suggest that the collagen layer of the endomysium may be the cementing agent responsible for cell strength. They found that the level of salt soluble collagen of Atlantic cod (*G. morhua*) decreased with frozen storage and high molecular weight complexes of both myocommatal and endomysial collagens were formed. They also suggested the possibility of complexes between sarcoplasmic proteins and the endomysial collagen. If such complexes were not denatured during cooking they would increase the toughness of the flesh in a manner consistent with the model proposed by Hatae *et al.* [71].

Changes in the muscle

It has long been known that fish flesh has greater catheptic activity than mammalian muscle [101] and that it possesses higher concentrations of enzymes responsible for proteolysis and amino acid metabolism [102]. Neutral proteases are found in many species [103]. Recent work, stimulated by the need to understand the modori phenomenon during the setting of surimi gels, has uncovered a number of proteases, mostly serine proteases, bound to both the sarcoplasmic and myofibrillar fractions of the muscle in a variety of species [104-107]. These enzymes are active during the heating step in forming fish gels and it is not clear what role they may play in the live fish, but it seems unlikely that they are active in the same way in chill stored fish. Cathepsin L from lysosomes has been implicated in the extensive muscle softening observed in chum salmon [108] and has been shown to increase in activity in the muscle of ayu (sweet fish, *Plecoglossus altivelis*) as the fish approaches maturity. Lysosomes have been located within fish muscle cells mostly

concentrated at the periphery [95]. These lysosomes break down post-rigor. Other cathepsins (such as cathepsins A and B) require a pH lower than occurs in post-mortem fish muscle. Even the pepstatin-sensitive cathepsins (mainly cathepsin D) are not active at pH 6.5 [109]. However, in fish such as tuna, where the pH is often lower than this, it is likely to be one of the factors responsible for the honeycomb problem in the flesh that is sometimes encountered [110]. White croaker (*Micropogon opercularis*) contains an endogenous serine proteinase which degrades the cytoskeletal network [111,112], which initiates protein turnover *in vivo* and which can completely disrupt the myofibrils (at 37°C) and degrade the major proteins [113]. When croaker were stored at 0°C for 7 days, after dipping in azide to prevent bacterial growth, only minimal changes were found in the major proteins of the myofibril. There was considerable breakdown of nebulin, a major cytoskeletal protein of the trabecular network [111]. Desmin, troponin and Z lines were shown to be stable under these conditions, whereas these entities are known to be degraded in post-mortem storage of beef muscle along with titin and alpha-actinin [114]. The connectin fraction of carp muscle also exhibits change when extracted from fish that have been stored chilled for 7 days at 25°C [115].

There are two Ca^{++}-activated neutral proteinases that are considered to be responsible for post-mortem softening of sheep muscle [116,117]. Calpain I requires only 0.1 mM Ca^{++} for activation while Calpain II is active at higher concentrations of calcium. Both enzymes are subject to inhibition by calpstatin. Calpain II has been found in both carp (*Cyprinus carpio* [118] and tilapia (*Tilapia nilotica* x *Tilapia aurea*) [119]. Calpstatin and a trypsin inhibitor is also present in carp [120].

OVERALL MECHANISMS

Hatae *et al.* [121] examined electrophoretic patterns of sarcoplasmic alkali-soluble and stroma proteins of five species of fish stored for 14 days at 4°C as well as physically measuring a range of textural properties using a General Foods type Texturometer. They concluded that post-mortem softening of the flesh was "more affected by the changes of the muscle structure than by the changes of the component proteins". Similarly, Toyohara and Shimizu [122] stated that "the weakening of muscle may be explained not as a proteolytic breakdown of myofibrils, but as a decomposition of the muscle structure". These observations, in conjunction with those presented here, lead to the conclusion that integrity is due to minor components which link the major components together and that proportionately small changes in these minor components can have disproportionately large effects on the structure, and hence the texture, of the flesh.

There are three possible explanatory mechanisms for post mortem softening:-

(a) some major components within either the myofibrils or in the extracellular connective tissue degrade, or
(b) links, bonds and connections that organise and stabilize the structure between the muscle components degrade, or
(c) both of these mechanisms occur.

Hypothesis (b) has considerable attraction as an explanation of the changes occurring early in the storage period before they are sufficiently gross to be detected by such means as alteration in an electrophoretic pattern.

CONCLUSION

The structure of fish flesh is complicated and intricate and the interrelationships between all the components is, as yet, obscure. Topics for further research include:-

- Establishing the type and location of minor collagens in fish muscle
- Establishing the nature of the fine connections in the myotendinous junction
- Determining the presence, location and activity of kallikrein
- Extracting and characterizing collagenases and proteases that act on the extracellular matrix
- Further identification of enzymes that degrade the cytoskeleton and their occurrence in commercial species
- Examining the use of suitable inhibitors to both extra and intracellular enzymes
- Exploring further uses of these enzymes in food processing.

The initial softening in texture that occurs in many species is due to changes in the cytoskeleton and in the collagen produced by enzymes acting on structural links and bonds. Internally, serine proteases and cathepsin L are the most likely agents, while the extracellular matrix collagenases active against collagen types I, IV and V are implicated as initiators of breakdown.

There is a need for considerably more work to elucidate the components of fish muscle and the processes of deterioration. Only then can specific methods to minimise these effects be placed on a rational basis.

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