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PROTEOLYSIS OF SKELETAL MUSCLE IN YELLOWFIN TUNA (*THUNNUS ALBACARES*): EVIDENCE OF CALPAIN ACTIVATION

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Abstract—1. White muscle of yellowfin tuna is subject to a form of deterioration known as "burnt tuna".

2. TEM and SDS–PAGE were used to quantify cellular differences in deteriorated white muscle of yellowfin tuna.

3. Electron micrographs showed a significant loss of Z-disc integrity and an increase in intracellular edema in burnt tuna.

4. Electrophoresis established that a specific doublet of proteins, 42 kD and 46 kD was lost.

5. Proteolysis of isolated myofibrils incubated in calpain (EC 3.4.22.17) was greatest at pH 7.5 and was selective for intermediate molecular weight proteins.

6. This evidence suggests that burnt tuna is a specific and limited proteolysis of myofibrillar structural proteins characteristic of calpain proteolysis.

INTRODUCTION

"Burnt tuna" is the term used by fishermen and commercial buyers to describe white muscle of yellowfin tuna (*Thunnus albacares*) that appears pale, grainy, exudative and unacceptable for the sashimi market (i.e. raw consumption). Since this muscle deterioration first appears *post mortem* and progresses for hours afterwards it is not considered a muscle disease but instead a breakdown of metabolic or proteolytic regulation associated with death. Burnt tuna appears in approximately 25% of the tuna caught by commercial handliners in Hawaii (Bourke, 1985) and elsewhere (Gibson, 1981) producing real economic losses to the sashimi fishery. Besides the clear monetary motive for identifying the cause of burnt tuna, it affords an opportunity to examine the control of muscle proteolysis.

Sport-caught fish and those landed by the night handline fishery put up a fierce struggle during capture. Since burnt tuna occurs more often in fish captured by these procedures, struggling has been presumed to contribute to its cause. Tuna white muscle has extraordinarily high lactate dehydrogenase activity (LDH) and produces some of the highest muscle lactate concentrations found in nature (Guppy and Hochachka, 1978). Therefore, high lactic acid concentrations and low muscle pH were once thought to be responsible for burnt tuna (Konagaya and Konagaya, 1978). However, failure to find a close correlation between pH and burnt tuna (Cramer *et al.*, 1981) or products of acid-mediated proteolysis (Hochachka and Brill, 1987) led to the formulation of new theories regarding the etiology of this muscle degeneration. Hochachka and Brill (1987) and later Watson *et al.* (1988) proposed an alternate hypothesis, that burnt tuna was caused by a calcium activated neutral proteinase (calpain) which cleaved structural proteins within muscle fibers rather than the contractile proteins.

While these theories were consistent with what was known about burnt tuna, they were untested. The purpose of this investigation was to evaluate the hypothesis that burnt tuna was the result of calpain proteolysis. This was done by quantifying the microanatomical and biochemical differences between burnt and unburnt muscle in whole *post mortem* tuna to determine their consistency with calpain proteolysis. Also, yellowfin tuna held in captivity were used to directly assess the effects of exhaustive exercise and calpain treatment on tuna muscle. In brief, this study was designed to answer several questions: (1) How does burnt tuna differ from other tuna microanatomically and biochemically? (2) Can burnt tuna be caused by severe exercise alone? and (3) Is it merely an accelerated *post mortem* generalized proteolysis or is it a specific proteolysis consistent with what is known of calpain?

MATERIALS AND METHODS

Sample collection

For analysis of ultrastructure of normal muscle, small (~2 kg) wild yellowfin tuna were caught at sea, stunned by a blow to the head and muscle samples taken by needle biopsy.

Changes in muscle structure caused by severe exercise alone were examined using captive yellowfin tuna held at the Kewalo Research Facility (Honolulu Laboratory, National Marine Fisheries Service, Honolulu, U.S.A.). Individual fish were dip netted from their holding tanks, biopsied and quickly moved to a doughnut shaped tank. The fish were

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then chased to exhaustion, sacrificed by a blow to the head, and the muscle biopsied immediately.

Sampling of yellowfin tuna at the Honolulu Fish Auction is complicated by uncontrollable variables such as sex of the fish, fight time, vigorousness of the capture struggle, time of year, and the nutritional state of the animal at the time of hooking, as well as a variety of *post mortem* treatments administered before the fish reaches the auction. Therefore, all samples taken from a commercial fish auction were from partially burnt fish so each animal acted as its own control for these variables.

Yellowfish tuna judged to be burnt by the wholesale buyers at the United Fishing Agency Honolulu Fish Auction (Honolulu, U.S.A.) were sampled for electron microscopy and SDS–PAGE. The judgment of the buyers was based on a visual analysis of flesh quality from quartered fish. Three paired biopsies were taken from areas judged to be completely burnt and adjacent areas that were designated as totally unburnt (saleable for sashimi). Tissue was immediately placed in buffered fixative for microscopy. Similarly paired biopsies from ten yellowfin tuna were collected for SDS–PAGE. 400 mg samples were sealed in polyethylene tubes, immediately placed on dry ice, and stored in a −90°C freezer until electrophoresis.

Post mortem age is also unknowable in auction sampled yellowfin tuna. To record *post mortem* changes in vigorously exercised, large (>40 kg) fish, needle biopsies were taken from sport-caught yellowfin tuna at the Kona Hawaii Billfish and the Hawaiian International Billfish Tournaments. Samples were obtained as soon as the fish were brought to the dock in Kailua-Kona (Hawaii) and at intervals until the time of sale. The time of death of tournament-caught fish is recorded, and all biopsies were taken at known *post mortem* intervals which could be compared to samples from auction sampled fish.

Electron microscopy

Muscle was placed in either 0.15 M phosphate buffered fixative, 0.15 M sodium cacodylate buffered fixative or 0.2 M sodium cacodylate buffered fixative, each containing 2.5% glutaraldehyde. Samples were secondarily fixed in 2% osmium tetroxide plus 0.08% potassium ferrocynide. After secondary fixation, samples were dehydrated and embedded in LX112 resin. Thin sections were cut with a diamond knife, mounted on 200 mesh unsupported copper grids, and stained with saturated uranyl acetate in water followed by Reynolds' lead citrate (Reynolds, 1963). Sections were viewed and photographed using a Philips 300 electron microscope set at 60 kilovolts.

Analysis of ultrastructural quality

At least four blocks were prepared from each biopsy and two blocks per biopsy were thin-sectioned longitudinally. From each of these blocks, 25 thin sections were cut and mounted on to 5 copper grids. One of these grids was chosen at random to be viewed under the electron microscope. The first grid square encountered that contained tissue was photographed in its entirety at a magnification of 3200×. Sixteen to 30 negatives were made from each grid square (depending upon the amount of overlap), developed and printed at a standard size of 8 × 10. Montages were then created.

A test grid of 88 squares was overlaid on the montage. Ten squares were chosen at random, and the Z-discs within that square were categorized: (1) = Z-disc completely intact, (2) = Z-disc with one hole only, (3) = Z-disc with multiple holes, (4) = Z-disc position identified only by I bands, (5) = No Z-disc position identifiable.

The test grid was moved to an adjacent area until the entire montage was covered. Z-discs from the montage were then summed by category.

Numbers of Z-discs from burnt tuna micrographs were pooled by condition category for all three fish. The same was done for unburnt samples. Since categories 1–3 represented existing Z-discs these data were pooled by cell and this total used for F-tests, paired t-tests and analysis of variance (ANOVA) to determine differences in the number of Z-discs/cell between burnt and unburnt tuna.

The volume of intracellular edema, as a percentage of cell volume, was measured using point analysis and the same 88-square test grid. Edema was calculated as a percentage of total cell volume. Mean percentage of edema for each cell was analyzed by F-test. The limit for statistical significance was $P < 0.05$.

Sodium dodecyl-sulfate–PAGE

Samples were prepared from 100 mg of either burnt or unburnt muscle in 4 ml of homogenizing buffer (in mM: 100 KCl, 1 EDTA, 50 imidizole, 5 mercaptoethanol, pH 7.5). Tissues were homogenized by four 10 sec bursts at 70% speed in a Tissumizer tissue grider and then centrifuged at 800 *g* for 15 min. Aliquots of the supernatant and homogenate were prepared for electrophoresis as per Laemmli (1970). Samples were placed on a 5% stacking gel over a 10% separating gel, electrophoresed and stained overnight with 0.1% Coomassie Blue R250 (Sigma, St Louis, MO) made up in 10% acetic acid and 50% methanol. Destaining was done with 7% acetic acid. Molecular weight of proteins was estimated by relative mobility using known molecular weight compounds electrophoresed simultaneously as standards. Gels were analyzed by densitometry to determine the quantity of individual muscle proteins remaining in burnt and unburnt tuna.

Preparation of isolated myofibrils

Myofibrils were prepared from captive yellowfin tuna according to the method of Etlinger and Fischman (1973) except for the elimination of 0.02% deoxycholate in one wash and the use of three washes instead of ten. The purity of the prepared myofibril fraction was verified by TEM and light microscopy. Preparations that contained only myofibrils (i.e. no whole cells or membrane fragments) were placed in a solution of 100 mM KCl, 0.25 mM thioglycollic acid and 50% glycerol (Reddy *et al.*, 1983) stored in a −20°C freezer.

Incubation of myofibrils

Calpain was purchased from Sigma and kept at −20°C until it was used. It was then thawed and diluted 1 unit/ml, with enzyme buffer (in mM: 20 Tris, and 1 EDTA, pH 7.5). All proteinase used was from the same lot number. The ratio of calpain/myofibrils was 1:100 w/w.

Myofibrils were incubated (at 25°C) at three pHs (5.5, 6.8 and 7.5) using a buffer modified from Reddy *et al.* (1983). The only changes were an increase in $CaCl_2$ from 1 mM to 5 mM and an increase in myofibrillar concentration from 1 mg/ml to 5 mg/ml. The reaction volume was 1 ml. Reactions were stopped at 10 min intervals by the addition of 125 µl of 10% TCA. After a 10 min centrifugation at 14,000 *g* the supernatant was withdrawn and the absorbance measured at 280 nm in a Beckman Model 35 Spectrophotometer to determine the quantity of acid-soluble peptides released from the myofibrils. Absorbance readings from five to seven myofibril incubations at each pH were averaged and regression lines calculated. Pellets were prepared for SDS–PAGE.

RESULTS

Electron microscopy

Auction sampled yellowfin tuna. Morphological preservation in 0.2 M (i.e. 400 mOsm) sodium cacodylate buffer was slightly better than with the other concentrations of fixatives. There was less swelling of the sarcoplasmic reticulum (SR) and

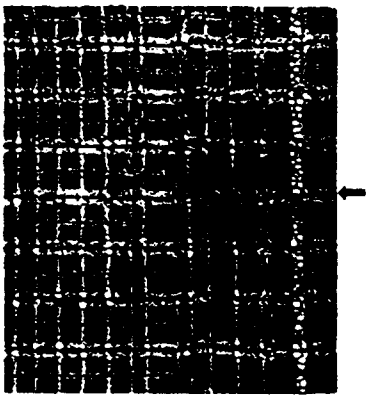

Fig. 1. White muscle from a small (<2 kg), wild yellowfin tuna biopsied immediately *post mortem*. The animal was captured by hook and line and quickly brought to the boat with a minimal amount of struggling. Note the integrity of the Z-discs (arrow indicated Z-disc) and the absence of intracellular edema. Mag. 8300×.

slightly less disruption of the mitochondria in the 400 mOsm buffer with no deterioration in the quality of myofibrillar fixation.

An electron micrograph of white muscle from wild yellowfin tuna is shown in Fig. 1. The salient features of this normal muscle are the integrity of the Z-disc and the absence of intermyofibrillar space. In contrast, burnt tissue sampled at the Honolulu Fish Auction (Fig. 2) exhibits Z-disc disintegration or even absence, and relatively large spaces between myofibrils, indicative of edema. The unburnt cells from the same fish (Fig. 3) more closely resemble myofibrils from freshly caught, wild tuna. Note that the Z-discs are present, but that a few holes indicate some limited proteolysis. Some intracellular edema also is evident, but it is much less than in burnt muscle. Samples taken from a yellowfin tuna judged to be completely

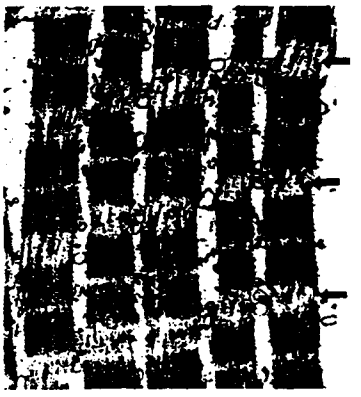

Fig. 2. White muscle sampled from a yellowfin tuna at the Honolulu Fish Auction. The sample was taken from an area of muscle judged to be severely burnt. Note the Z-discs (arrows indicate Z-disc position) show significant deterioration. 17,000×.

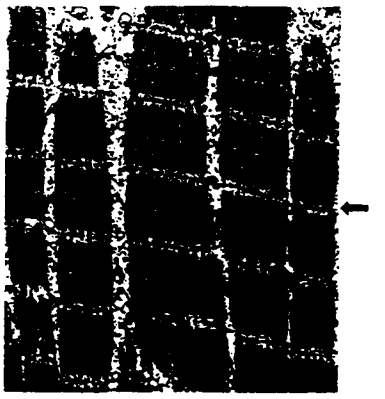

Fig. 3. White muscle from the same fish as in Fig. 2. This sample was taken from an area of muscle judged to be unburnt. The Z-discs have some holes (arrow indicated Z-disc position), but are largely intact. 17,000×.

unburnt show the same degree of Z-disc integrity as unburnt tuna (Fig. 4).

Significantly more Z-discs were present in unburnt cells (*F*-test). Z-discs/cell in unburnt and burnt cells from the same fish also were significantly different (paired *t*-test). A significant difference in the number of Z-discs/cell existed between burnt and unburnt cells for all three fish (ANOVA). The ANOVA used to test the hypothesis that the reduction in Z-disc number seen in burnt tuna is uniform across fish showed no significant differences between burnt cells in any of the fish sampled. Since burnt cells from different fish were more similar than burnt and unburnt cells from the same fish, the data from all three fish were pooled. Figure 5 shows the percentage of Z-discs/condition category. Muscle tissue classified as burnt clearly has significantly more disorganized Z-discs (categories 3–5) than tissue classified as unburnt. Mean percentage of edema was also significantly greater in burnt than in unburnt yellowfin tuna cells (*F*-test). In summary, burnt yellowfin tuna tissue exhibited more edema and fewer Z-discs per cell than unburnt tissue.

Fig. 4. White muscle from a yellowfin tuna judged to be 100% unburnt. 17,000×.

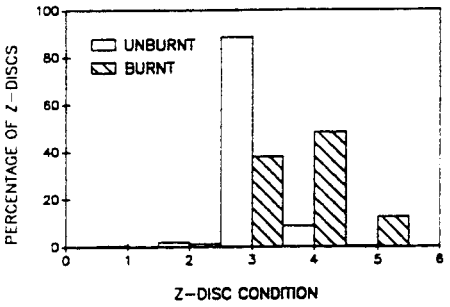

Fig. 5. Frequency histogram of Z-disc condition in burnt and unburnt muscles from fish sampled at the Honolulu Fish Auction.

Exercised yellowfin tuna. Of the three captive fish exercised to exhaustion and sacrificed only one had any myofibrillar disorganization and it was unrelated to that seen in burnt tuna. The muscle fibrils were torn mid-A band (Fig. 6). Some mechanical stress of swimming appears to have caused this rending of the myofibrils while leaving the Z-discs undisturbed. These results show that burnt tuna is not the direct result of mechanical disruption due to severe exercise. While exercise can cause major myofibrillar changes in muscle, it alone does not cause disruption that resembles burnt tuna.

Post mortem *deterioration.* All 8 *post mortem* samples taken from tournament-caught yellowfin tunas showed muscular changes similar to those in auction sampled unburnt tuna. A typical muscle fiber (Fig. 7), taken 16 hr *post mortem* had only minimal Z-disc damage. Despite intense exercise white muscle from these yellowfin tunas remained unburnt long after death, that is, it was saleable as sashimi and retained the Z-disc in muscle ultrastructure.

Is burnt tuna the unavoidable result of *post mortem* deterioration? In other words, given sufficient time i.e. more than 16 hr, will all muscle eventually become burnt? A yellowfin tuna sampled 21 days *post mortem* had no more Z-disc damage (Fig. 8) than muscle 16 hr old (Fig. 7) despite advanced *post mortem* age and decayed appearance of the flesh. Since burnt tuna is not inevitable, it is probably the result of a specific proteolytic cascade rather than an accumulated general auto-digestion.

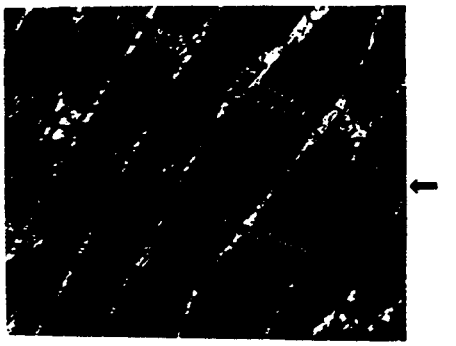

Fig. 7. White muscle sample taken from a yellowfin tuna caught using sportfishing gear during the Hawaiian International Billfish Tournament. Note that even after severe exercise during capture, the muscle showed little Z-disc damage 16 hr *post mortem* (arrow indicates Z-disc position). 17,000 ×.

Sodium dodecyl sufate–PAGE

Auction sampled yellowfin tuna. SDS–PAGE analysis showed no discernible difference in the amount of alpha-actinin in burnt and unburnt tissue. This was unexpected considering the Z-disc disintegration seen in the electron micrographs. However, as shown in Fig. 9, a doublet of proteins (42 and 46 kD) is missing in burnt tuna, both from supernatant and homogenate preparations.

Ten paired biopsies were taken from auction sampled yellowfin tuna white muscle and the tissue supernatant electrophoresed on PAGE gels. Analysis by densitometry showed that the 42,000 and 46,000 Da proteins were both significantly diminished

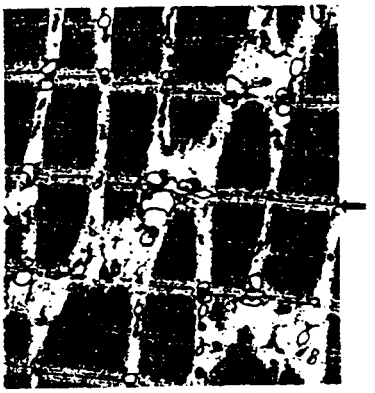

Fig. 6. Myofibrils taken immediately *post mortem* from a yellowfin tuna exercised to exhaustion. Note that myofilaments are torn at mid-A band but that the Z-discs are intact (arrow indicates Z-disc position). 17,000 ×.

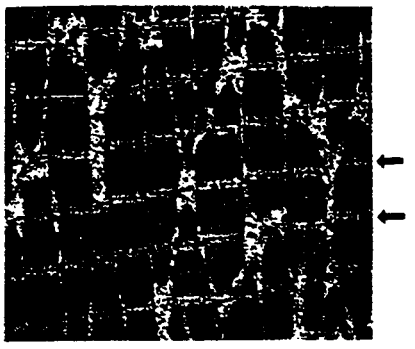

Fig. 8. Yellowfin tuna sampled at the Honolulu Fish Auction >21 days *post mortem.* Note that the muscle still had intact Z-discs despite the extreme decay (arrows indicates Z-disc positions). 16,000 ×.

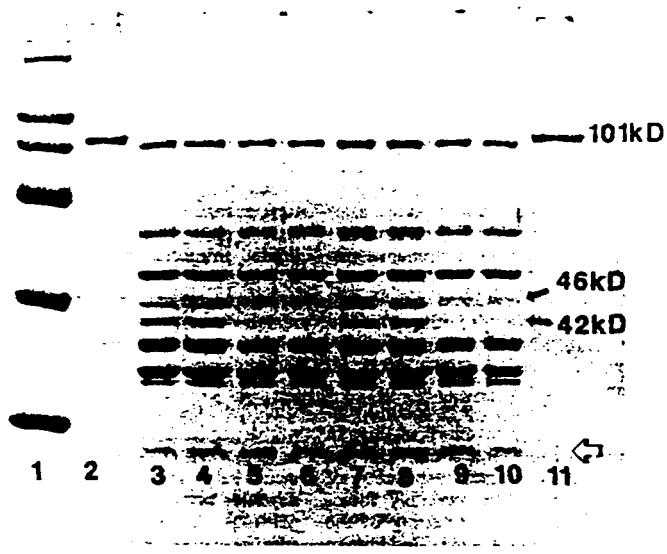

Fig. 9. Polyacrylamide gel of paired burnt and unburnt samples from two yellowfin tuna. Lane 1: High molecular weight standard, lane 2: alpha-actinin standard lanes 3, 4, 7, 8: supernatant of unburnt muscle, lanes 5, 6, 9, 10: supernatant of burnt muscle, lane 11: alpha-actinin standard. Note the missing doublet of proteins (46 and 42 kD) in burnt muscle samples.

in burnt tuna in comparison with unburnt tuna ($P < 0.05$).

Myofibril incubation with calpain. Myofibrils incubated at pH 7.5 with purified calpain demonstrated a faster rate of proteolysis than those incubated at more acidic pHs (Fig. 10). Electrophoresis of myofibril pellets remaining after incubation showed a preferential proteolysis of low molecular weight proteins (Fig. 11). However, the 46 kD and 42 kD proteins missing in burnt tuna were not completely hydrolysed during the calpain incubation.

DISCUSSION

On a cellular level, burnt yellowfin tuna was found to be significantly different from unburnt tuna in a predictable way. The anatomical differences in Z-disc integrity are consistent and quantifiable. Burnt and unburnt tuna are also distinct from one another regarding the amount of 42 and 46 kD protein they contain. Given the unique characteristics of burnt tuna a specific proteolytic pathway is implicated in its formation.

Electron micrographs of white muscle from sport caught yellowfin tuna sampled *post mortem* showed Z-disc damage and *post mortem* edema undistinguishable from that seen in unburnt tissue of partially burnt fish at the Honolulu Fish Auction. Clearly, unburnt tissue from partially burnt fish is not anatomically exceptional, it is simply undergoing changes common to *post mortem* yellowfin tuna

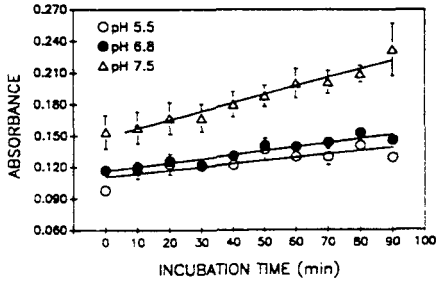

Fig. 10. Quantity of acid-soluble peptides (absorbance measured at 280 nm) released from isolated myofibrils of yellowfin tuna muscle following incubation with calpain. Error bars are SEM. Lines shown are based on linear regression.

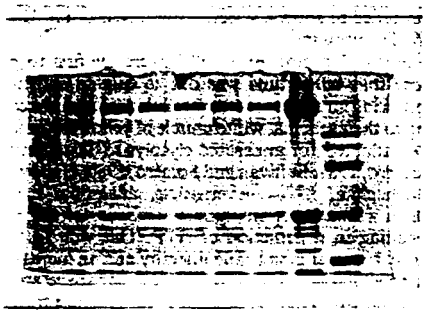

Fig. 11. Polyacrylamide gel of myofibrils incubated with calpain at pH 7.5. Lane 1: actin standard, lanes 2–8: myofibrils incubated for 90, 80, 70, 50, 30, 10 and 0 min, lane 9: high molecular weight standard.

muscle. However, burnt tuna is more than simply accelerated *post mortem* deterioration as suggested by Davie and Sparksman (1986) and Hochachka and Brill (1987). If that were the case we would expect to see all tuna muscle eventually look burnt. However, electron micrographs from muscle sampled 21 days *post mortem* demonstrated that this is not always true.

It was important to establish whether or not burnt tuna was caused by the physical stress of exercise on the myofibrils since all tuna struggle during capture. Myofibrils of captive yellowfin tuna biopsied after strenuous exercise have the same tears mid-A band as occurs in human myofibrils following strenuous sustained exercise (O'Reilly *et al.*, 1987; Warhol *et al.*, 1985), but do not have the frayed Z-discs of burnt tuna. Since cutting in oblique instead of longitudinal section could produce the same impression of mid-A band fractures, we sectioned multiple blocks in multiple orientations and submitted the micrographs for independent evaluation (Evans, pers. comm.) who concurred that we were seeing myofibril tearing. In tuna, exhaustive exercise causes three primary changes in muscle fibers: intracellular edema, a spreading of the myofibrils, with the Z-discs generally intact although often out of register, and tearing of the sarcomere, generally in mid-A-band. In human muscle this is caused by mechanical stress on the myofibril rather than by proteolysis. While exercised muscle shows interesting ultrastructural changes, it does not resemble the specific Z-disc dissolution of burnt tuna myofibrils.

Electron micrographs show that Z-disc disintegration, coupled with intracellular edema are ultrastructural benchmarks of tissue designated as burnt. Since these characteristics are well accepted results of calpain activity (Reddy *et al.*, 1983; Ishiura, 1981), we conclude that the protease calpain is more active in "burnt" muscle than in normal *post mortem* tissue.

Attempts to identify the 46 and 42 kDa proteins using Western blot were unsuccessful, perhaps due to the incompatibility of mammalian antibodies and fish proteins. However, desmin and vimentin, located at the Z-disc have degradation products in this molecular weight range (Lazarides, 1982) which would be partly degraded in normal tissue and completely degraded at higher calpain activation rates. Incubation of isolated myofibrils in calpain produced the expected increase in proteolysis at physiological pH, but failed to show a selective hydrolysis of the 42 and 46 kDa proteins.

Hochachka and Brill (1987) were the first to propose that burnt tuna was due to calpain activation following an increase in intracellular calcium. According to their schema, white muscle of yellowfin tuna are well adapted for anaerobic glycolysis (Castellini and Somero, 1981; Sullivan and Somero, 1980; Guppy and Hochachka, 1978) nevertheless, anaerobic metabolism will cease some minutes or hours after death, resulting in a depletion of ATP. This will, in turn, cause a loss of membrane integrity and an increase in intracellular calcium from extracellular space as well as from SR. High intracellular Ca^{2+} concentrations will activate calpain and the result should be burnt tuna. Yet under the worst of circumstances, only about 25% of handline caught tuna are burnt. Watson *et al.* (1988) proposed that high levels of circulating catecholamine could account for this difference. Besides their role in cell calcium regulation, catecholamines are also accelerators of calpain proteolysis (Toyo-oka, 1982). While this remains an interesting possibility, the transient nature of catecholamines make it untestable in auction sampled animals. However, there are other conditions which would allow escape from what appears to be an inevitable proteolytic cascade.

Our current hypothesis is as follows. If yellowfin tuna engage in an aerobic capture struggle by using red muscle or are in a poor nutritional state, i.e. have limited intracellular glycogen, they will have little opportunity to accumulate lactate and the accompanying protons before landing. If intracellular pH remains high *post mortem*, and within the range where calpain is most active, proteolysis of intermediate molecular weight proteins is probable. This is consistent with Yu and Lee (1986) and Zeece *et al.* (1986) who found that *post mortem* substrate proteolysis was pH dependent, with muscle incubated at high pH having a specific removal of the Z-disc. Muscle incubated at low pH had well preserved Z-discs but degraded myosin and M-lines, whereas intermediate pH (5.8–6.3) resulted in little proteolysis of any substrate. In other words, yellowfin tuna that become burnt have limited intracellular glyocgen which allows a minimal drop of pH and optimal conditions for calpain activity while at the same time inhibiting lysosomal proteases.

Some field evidence indirectly support this hypothesis. More female yellowfin tuna caught near the Hawaiian Islands become burnt during spawning season, when the high metabolic cost of gonadal tissue synthesis could lead to depletion of intracellular glycogen stores (Nakamura, 1987). Although muscle glycogen content of large yellowfin tuna caught near the Hawaiian Islands has never been measured, skipjack tuna (*Katsuwonus pelamis*) caught in the same area appear to be malnourished. High concentrations of muscle anserine and carnosine are indicative of starving skipjack tuna, and higher anserine levels have been found in skipjack tuna caught near the Hawaiian Islands than in fish caught elsewhere (Abe *et al.*, 1985, 1986). Moreover, anserine and carnosine have recently been found to act directly as potentiators of calpain activity (Johnson, 1990). Now that "burnt" tissue can be identified in small quantities of muscle, these possible mechanisms can be investigated.

In conclusion, we have demonstrated that burnt tuna is anatomically and biochemically different from unburnt tuna in a specific and quantifiable way. The degradation of Z-discs and the loss of the 42 and 46 kDa proteins is not caused by exercise alone, nor is it an accelerated generalized *post mortem* proteolysis. While the unique proteolytic pathway leading to burnt tuna bears many of the earmarks of calpain activation, we were unable to establish by incubation that calpain is responsible. It is possible that the same pre-death conditions which increase calpain activity also stimulate another cytoplasmic proteinase which operates independently or synergistically with calpain. Details of the exact proteolytic sequence still need to be described and could provide valuable insights into proteolytic regulation during ischemia.

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REFERENCES

Abe H., Brill R. W. and Hochachka P. W. (1986) Metabolism of L-histidine, carnosine and anserine in skipjack tuna. *Physiol. Zool.* **59**, 439–450.

Abe H., Dobson G. P., Hoeger U. and Parkhouse W. S. (1985) Role of histidine-related compounds on intracellular buffering in fish skeletal muscle. *Am. J. Physiol.* **249**, R449–R454.

Bourke R. (1985) Hilo Ika-shibi fishery 1984 survey: Problems of a maturing fishery and a potential solution to the burnt tuna problem. Abstract, 37th Tuna Conference. National Marine Fishery Service.

Castellini M. A. and Somero G. N. (1981) Buffering capacity of vertebrate muscle: correlations with potentials for anaerobic function. *J. Comp. Physiol.* **143**, 191–198.

Cramer J. L., Nakamura R. M., Dizon A. E. and Ikehara W. N. (1981) Burnt tuna: Conditions leading to rapid deterioration in the quality of raw tuna. *Mar. Fish. Rev.* **43**, 12–16.

Davie P. S. and Sparksman R. I. (1986) Burnt tuna: An ultrastructural study of postmortem changes in muscle of yellowfin tuna (*Thunnus albacares*) caught by rod and reel and southern bluefin tuna (*Thunnus maccoyii*) caught on handline or longline. *J. Food Sci.* **51**, 1122–1128, 1168.

Etlinger J. D. and Fischman D. A. (1973) M and Z band components and the assembly of myofibrils. *Cold Spring Harbor Symp. Quant. Biol.* **37**, 511–521.

Evans, W. J. Dept. of Pathology, Brigham and Women's Hospital, Boston, MA, U.S.A. (personal communication).

Gibson D. J. M. (1981) *A Handbook on Processing Southern Bluefin Tuna for the Fresh Chilled Sashimi Market in Japan*. NZ Fishing Industry Board, Wellington, New Zealand.

Guppy M. and Hochachka P. W. (1978) Controlling the highest lactate dehydrogenase activity known in nature. *Am. J. Physiol.* **234**, 136–140.

Hochachka P. W. and Brill R. W. (1987) Autocatalytic pathways to cell death: a new analysis of the tuna burn problem. *Fish Physiol. Biochem.* **4**, 81–87.

Ishiura S. (1981) Minireview: Calcium-dependent proteolysis in living cells. *Life Sci.* **29**, 1979–1987.

Johnson P. (1990) Calpains (intracellular calcium-activated cysteine proteinases): structure–activity relationships and involvement in normal and abnormal cellular metabolism. *Int. J. Biochem.* **22**, 811–822.

Konagaya S and Konagaya T. (1978) Denaturation at moderate temperatures of myofibrillar protein of red-meat fish: A possible cause of yake-niku. *Bull. Tokai Fish. Res. Lab.* **96**, 67–74.

Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680.

Lazarides E. (1982) Biochemical and immunocytological characterization of intermediate filaments in muscle cells. In: *Methods in Cell Biology*, **25**, 333–353. Academic Press, New York.

Nakamura R. (1987) Dept. of Animal Science, Univ. of Hawaii, Honolulu, HI, U.S.A. (personal communication).

O'Reilly K. P., Warhol M. J., Fielding R. A., Frontera W. R., Meredith C. N. and Evans W. J. (1987) Eccentric induced muscle damage impairs muscle glycogen repletion. *J. Appl. Physiol.* **63**, 252–256.

Reddy M. K., Rabinowitz M. and Zak R. (1983) Stringent requirement for Ca^{++} in the removal of Z-lines and alpha-actinin from isolated myofibrils by Ca^{++}-activated neutral proteinase. *Biochem. J.* **209**, 635–641.

Reynolds E. S. (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.* **17**, 208.

Sakamoto S. and Seki N. (1985) Limited hydrolysis of carp myosin by calpains. *Bull. Jpn Soc. Sci. Fish.* **51**, 1551–1557.

Sullivan K. M. and Somero G. N. (1980) Enzyme activities of fish skeletal muscle and brain as influenced by depth fo occurrence and habits of feeding and locomotion. *Mar. Biol.* **60**, 91–98.

Toyo-oka T. (1982) Phosphorylation with cyclic adenosine 3′:5′ monophosphate-dependent protein kinase renders bovine cardiac troponin sensitive to the degradation by calcium-activated neutral protease. *Biochem. Biophys. Res. Comm.* **7**, 44–50.

Warhol M. J., Siegel A. J., Evans W. J. and Silverman L. M. (1985) Skeletal muscle injury and repair in marathon runners after competition. *Am. J. Pathol.* **118**, 331–339.

Watson C., Bourke R. E. and Brill R. W. (1988) A comprehensive theory on the etiology of burnt tuna. *Fishery Bull.* **86**.

Yu L. P. and Lee Y. B. (1986) Effects of postmortem pH and temperature on bovine muscle structure and meat tenderness. *J. Food Sci.* **51**, 774–780.

Zeece M. G., Robson R. M., Lusby M. L. and Parrish F. C. Jr (1986) Effect of calcium activated protease (CAF) on bovine mofibrils under different conditions of pH and temperature. *J. Food Sci.* **51**, 797–803.