Muscle to meat conversion in common carp (cyprinus carpio): new insights involving apoptosis

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MUSCLE TO MEAT CONVERSION IN COMMON CARP (Cyprinus carpio): NEW INSIGHTS INVOLVING APOPTOSIS

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Abstract – Tenderization of fish muscle results in its deterioration. Different proteolytic systems exist within the muscular cell. In this decade we have integrated a new process capable to better explain the meat tenderization process namely apoptosis. This process is orchestrated by the family of cysteine aspartate-specific proteases named caspases, which are probably the first proteolytic system to be implicated in *postmortem* proteolysis and meat tenderization. Our study contribute to understanding proteolysis integrating the apoptosis process in the biochemistry of fish muscle. We have choosen three hallmarks of apoptosis: phosphatidylserine (PS) externalization observed by using a FITC-annexin V conjugate as a specific probe, cytochrme c liberation from mithochondria and actin degradation by western blot using a polyclonal anti-actin and anticytochrome c (Cyt c) antibody. Results demonstrate that proteolysis begin just after fishing and confirmed the degradation of actin probably by caspase 3 and release of cytochrome c by mitochondria immediately after death. These results must be completed in future works to elucidate the mechanisms of action of these proteolytic systems.
Key Words – fish proteolysis, apoptosis, caspases

I. INTRODUCTION

Proteolysis plays a critical role in postmortem aging of muscle resulting in tenderization, a clear understanding of the mechanisms responsible remains elusive. In contrast to mammalian muscles, for which tenderness is beneficial to meat quality, tenderization of fish muscle results in its deterioration and loss of quality [1-2-3-4].
The meat tenderising process is unanimously recognized to be enzymatic in nature [6]. In fish muscle, different proteolytic systems exist within the muscular cell: a multicatalytic complex or proteasome, a lysosomal system including aspartic and cysteine acidic cathepsins, the cytosolic calcium dependent calpains, as well as cytoplasmic aminopeptidases, alkaline proteases, and connective tissue hydrolytic enzymes such as elastase and collagenase [2-5].
Ouali [6] proposed to reconsider the knowledge accumulated on meat ageing by integrating a new possible way to explain meat tenderisation namely program cell death or apoptosis. This process is orchestrated by a family of cysteine aspartate-specific proteases called caspases [7]. In postmortem muscle, caspases are probably the first proteolytic system to be implicated in *postmortem* proteolysis and meat tenderization [6-7]. Some recent studies have proved apoptosis onset in post mortem skeletal muscle cells by observing a rapid phosphatidylserine (PS) externalization, cell shrinkage [8], and a progressive degradation of actin by caspases [9-10]. Evidence has shown that the first step of the conversion of muscle to meat is the triggering of programmed cell death or apoptosis. However, there were quite a few studies that correlate ~~the~~ apoptosis with meat quality, thus being the actual processes of meat quality development far to be understood [10]. This study was designed to improve our understanding on the proteolysis integrating the apoptosis process in fish muscle biochemistry. Three different hallmarks of apoptosis have been selected and monitored during the first few days after animal bleeding. These are phosphatidylserine (PS) externalization, cytochrme c liberation from mithochondria and actin degradation, a protein proposed to be a good marker of apoptosis development.

II. MATERIALS AND METHODS

Animals and muscle sampling. Ten common carp *(Cyprinus carpio* L.) ,15-20 cm body long were captured from Beni Haroune dam and

used in the present work. Animals were killed by decapitation, and *longissimus dorsi* were excised at 0, 0.25, 0.5, 1, 2, 3, 4, 5, 6, 10 and 24 h postmortem. Muscles were maintained on the carcass. Carcasses were stored on ice at 0 C for 2 days.

pH measurement. Longissimus Muscle samples (0.5 g) were homogenized in 9 volumes of cold 5 mM iodoacetate, and the pH determined on the suspension using a WTW pH meter (Amilabo, Chassieu, France) equipped with a combined glass electrode.

Postmortem proteolysis, actin degradation and cytochrome C liberation. Proteolysis, time course degradation of actin and cytochrome c (cyt c) liberation were investigated using fish Longissimus muscle from the same animals. After animal bleeding, muscle was maintained on the carcass, and samples were collected at each sampling times (0.25, 0.5, 1, 2, 3, 4 , 5, 10 and 24 h postmortem) for pH measurement, actin proteolysis and cytochrome C liberation. For extraction of myofibrillar proteins we refer to [11]. After that, Samples were subjected to SDS–PAGE (12%) according to Laemmli [12]. Actin degradation and cytochrome c liberation were therefore followed by western blot using a polyclonal anti-actin and anticytochrome c antibody, as previously described [13].

Phosphatidylserine (PS) externalization. We have used a FITC-annexin V conjugate as a specific probe to bind PS. To attest the extracellular location of PS, we referred to laminin, a major component of the basal lamina. In living cells, the primary function of the laminin protein family is the anchorage of the plasma membrane to the extracellular matrix. Hence, these proteins are located in the vicinity of the external leaflet of the plasma membrane [14] and constitute an interesting internal control to verify that the PS labeling is extracellular and not intracellular. Phosphatidylserine externalization was investigated using strips from *longissimus dorsi* muscles excised 0 h and 6 h postmortem. (For experimental details see [8].

III. RESULTS AND DISCUSSION

PS externalization Regarding postmortem samples, as depicted in **Fig.1 A-B-AB** (0h postmortem sample) and **Fig.1 C-D-CD** (6h). Immunostaining of fish muscle fibers prepared 0 h **(Fig.1 B)** or 6h **(Fig.1 D)** postmortem with a laminin rabbit polyclonal antibody revealed with a cyanin 3-conjugated goat anti-rabbit IgG led to specific fluorescent red . Merging images corresponding to laminin and PS labeling led to a yellow color, a result indicative of a co-localization of laminin and PS-bound annexin V. Such color change was observed both in 0 h **(Fig.1 AB)** and 6 h **(Fig.1 CD)** postmortem samples. The co-localization of both antigens confirms that annexin V is bound to extracellular PS and not to intracellular acidic phospholipids. It is noteworthy that the yellow color is more intense in the 6h sample, suggesting an increase in the number of externalized PS between 0 and 6 h postmortem.

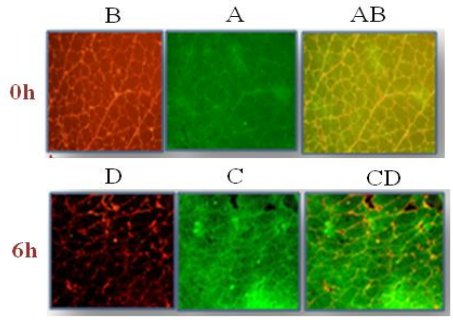

Fig.1- Samples from fish longissimus muscle excised 0 h postmortem. (0h) and 6 h postmortem (6 h). Samples were primarily labeled with a FITC annexinV conjugate at 0 h (A) and 6h (C) postmortem. Note the greater fluorescent labeling of the 6h sample when compared to 0 h.The same samples were then labeled with a rabbit specific laminin antibody revealed with a cyanine 3-goat anti-rabbit IgG conjugate at 0 h (B) and 6 h postmortem (D). Note the lower fluorescent labeling of the 6 h sample when compared to 0 h probably indicative of the degradation of the laminin. Merge images from 0-h samples (AB) and 6h samples (CD).

Proteolysis. Proteolysis of cytoskeletal components results in myofilament degradation [15-16]. In fish, depending on species, this may include degradation of titin, nebulin, dystrophin [17-18-19], α-actinin release [20-21], myosin proteolysis, and tropomyosin delocalization [18]. Most of the changes are common among different fish species but they may occur at different rates. Different proteolytic systems are involved for this proteolysis. We have: a multicatalytic complex or proteasome, a lysosomal system including aspartic and cysteine acidic cathepsins, the cytosolic calcium dependent calpains and, more recently, caspases. In postmortem muscle, caspases are probably the first proteolytic system to be

implicated in *postmortem* proteolysis and meat tenderization [6-7]. In our study we have detected three areas (I, II, III) of proteins degradation **(Fig.2).** In addition, by means of western blotting using a rabbit polyclonal anti-actin antibody we have confirmed the degradation of actin, probably by the action of caspase 3 by **(Fig.3).**

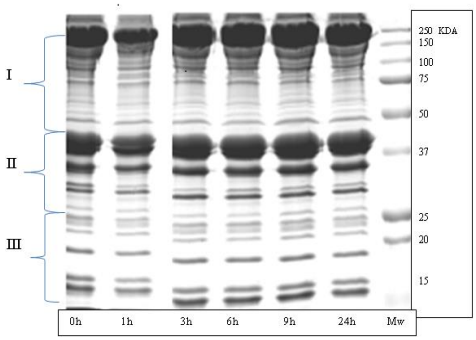

Fig2. 12% SDS-PAGE of the protein degradation profile of fish muscle (supernant). There are three areas: I, II and III indicating differents molecular mass values. I: 175- 47 KDa, II: 47- 30 KDa, III: 30- 14 KDa. This profile demonstrate that proteolysis begin early at 0h post mortem and We got the same protein fragments with all times post mortem.

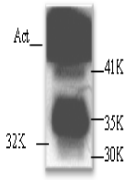

Fig.3: Actin fragments identified in the soluble fraction of fish L. muscle having molecular mass values around 41, 35, 32 and 30 kDa, respectively

Actin degradation and liberation of cytochrome c. Degradation of actin and liberation of cyt c were investigated by SDS–PAGE at different postmortem sampling times. In the soluble fraction (supernatant), western blot carried out with a polyclonal anti-actin antibody revealed a series of fragments with Mr ranging between 41 and 30 KDa **(Fig.3)**. The amount of the 32-kDa actin fragment increased sharply just after death and reached its maximum level about 4–5 h postmortem. Interestingly, this maximum coincides with the pH plateau observed between 4 and 5 h postmortem. The amount of 32 KDa decreased thereafter to reach a minimum level about 24 h postmortem suggesting that this fragment is further degraded to smaller ones not detectable by western blotting in the soluble fraction **(Fig.4)** As assessed by western blot and densitometry on the release of cyt c into the cytosol starts immediately after death and reach its maximum concentration about 10 h postmortem at a pH value of 6.8 **(Fig.4)**. These results match with the study of Becila [8].

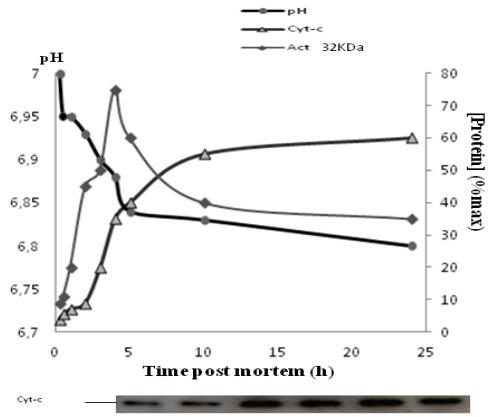

Fig.4: Postmortem changes in muscle pH, soluble 32 kDa actin fragment levels and in the extent of Cytochrome c release in *Longissimus* fish muscle maintained on the carcass which was stored at 0°C for 24 h.

IV. CONCLUSION

Proteolysis and improper storage of whole fish will cause undesirable changes in the texture of many fishes. In this report we demonstrate that proteolysis begin early and just after fishing. Several enzymatic mechanisms generate proteolysis and the recent one is provided by apoptotic caspases. The mastery of their mode of action just after fishing will help to ensure the freshness of the fish. Further works are needed to elucidate the mechanisms of action of these systems.

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