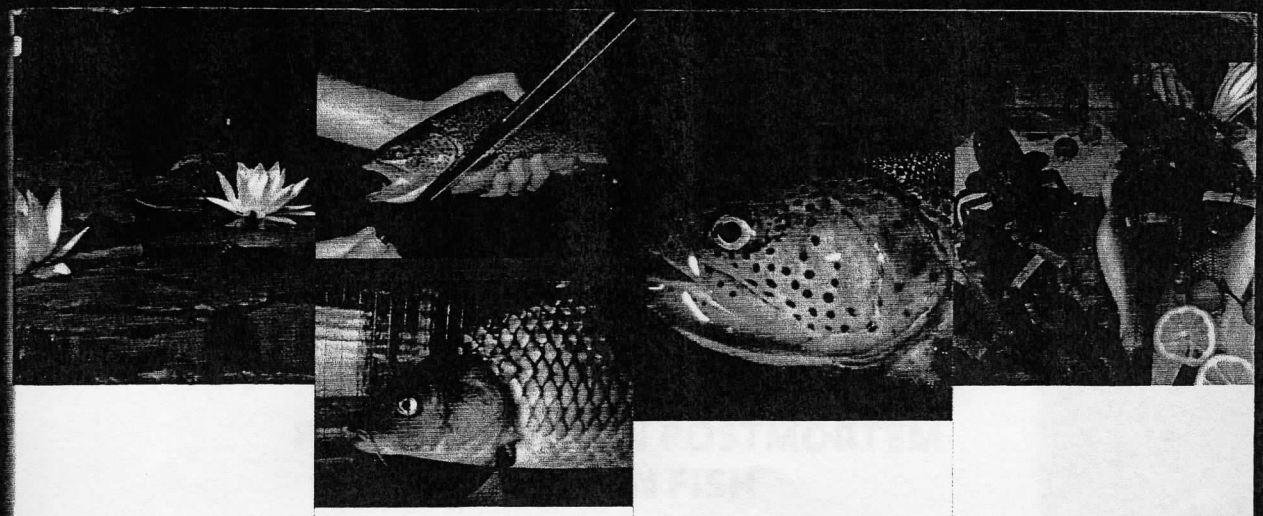

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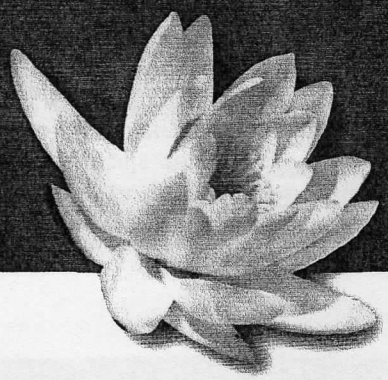

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RECENT VIEWS ON POSTMORTEM AGING IN FISH

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NOVIJI POGLEDI NA POSTMORTALNE PROMENE MIŠIĆA RIBE

Prošireni abstrakt

Odmah nakon prekida cirkulisanja krvi, pa zbog toga i nedostatka izvora kiseonika, glikogen podlaže anaerobnom razlaganju i preko niza fosforilovanih derivata glukoze prelazi u mlečnu kiselinu, koja se nagomilava u mišićima, što rezultira smanjenjem pH vrednosti sa vrednosti blizu 7,4 na šest, ponekada i nižu. Mišićni osmotski pritisak raste u roku od sat vremena nakon smrti, sadržaj ATP (adenozin trifosfat) opada a lipidi oksidišu. Trimetilaminoksid (TMAO) prelazi u trimetilamin (TMA), usled dejstva endogenih enzima, a kasnije bakterijskih - kada počne mikrobiološka aktivnost. Količina azot-oksida i reaktivnih metabolita kiseonika se takođe povećava. Mitohondrije i sarkoplazmatski retikulum prestaju sa radom tokom pada pH vrednosti i promena osmotskog pritiska, što rezultira oslobađanjem kalcijumovih jona u citosol, gde njihova koncentracija može da dostigne i 0.2 mM slobodnog kalcijuma.

Proteoliza komponenata citoskeleta rezultira degradacijom miofilamenata. Kod ribe, u zavisnosti od vrste, ona može da obuhvati degradaciju titina, nebulina, distrofina, oslobađanje α-aktinina, proteolizu miozina i delokalizaciju tropomiozina. Sarkoplazmatski 16 kDa protein (označen kao nukleozid difosfat kinaza) takođe podleže proteolizi tokom skladištenja. Tropomiozin se oslobađa iz miofibrila, a sadržaj ektrahovanog tropomiozina raste između 0 i 48 sati u prisustvu 5 mM EGTA. U ekstrakciji sa 5 mM Ca^{2+}, tropomiozin je pronađen u manjoj količini nakon 48 sati.

U mišićnim ćelijama postoje različiti proteolitički sistemi: multikatalitički kompleks, lizozomalni sistem koji uključuje aspartamske i cisteinske kisele katepsine, citosolni Ca-zavisni kalpaini i citoplazmatične aminopeptidaze, alkalne proteaze, kao i hidrolitični enzimi vezivnog tkiva, elastaze i kolagenaze.

Multikatalitički kompleks ili *proteazom* učestvuje u razgradnji hormona, antigena, transkripcionih faktora i ubikvitin konjugovanih i oksidovanih proteina. Za aktivnost 26S proteazoma protreban je ATP i hidrolizovani ubikvitin konjugovani proteini. Proteazom 20S, koji predstavlja deo 26S proteazoma, postoji u latentnoj formi koja može da se aktivira različitim jedinjenjima.

Katepsini su kisele proteaze, najčešće lokalizovane u lizozomima. Stoga su uglavnom neaktivni u tkivu živih organizama, ali bivaju oslobođeni usled povreda ili smrzavanja i odmrzavanja u mišićima postmortem. Katepsini mogu biti specifični po svom aktivnom mestu (aspartam, cistein ili serin proteaza), kao i po svojoj supstrat specifičnosti ili osetljivosti na određene inhibitore. U lizozomima se može naći najmanje 13 različitih katepsina. Pored njih katepsini B, D, L su već izolovani iz ćelija ribe. Katepsini B, D, L i H su najzastupljenije grupe katepsina u lizozomima mišićnih ćelija ribe.

Kalpaini su cistein proteaze, aktivne pri neutralnoj pH vrednosti i zavisne od kalcijuma. Ubikvitarni kalpain se sastoji od kalpaina I ili mikro(μ)-kalpaina, koji zahteva mikromolarne koncentacije kalcijuma za punu aktivnost (10 do 50 μM) i kalpaina II ili mili(m)-kalpaina, koji zahteva milimolarne koncentracije kalcijuma (300 μM do 1 mM). Kalpainski heterodimeri se razdvajaju u prisustvu kalcijuma. Mala subjedinica (30 kDa) je ista kod svih kalpaina, dok je velika subjedinica (80 kDa) specifična za određeni tip kalpaina i odgovorna za katalitičku aktivnost. Aktivnost kalpaina, aktivnih pri intracelularnoj fiziološkoj pH vrednosti, je u velikoj meri regulisana *in vivo*. Regulacija aktivnosti kalpaina je kompleksna i nije poznata u potpunosti. Četiri osobine kalpaina su kalcijum zavisne: vezivanje za celularne komponente, kao što su membrane, vezivanje za kalpastatin, proteolitička aktivnost i autoliza.

Metaloproteaze matriksa (MMP-Matrix metalloproteases) predstavljaju brojnu familiju strukturno sličnih endopeptidaza, odgovornih za katabolizam vezivnog tkiva. One su sposobne da razgrade različite tipove kolagena i proteine citoskeleta povezujući sarkolemu za ekstracelularni matriks. Oni su cink i kalcijum zavisni enzimi, klasifikovani u 4 podfamilije: kolagenaze, želatinaze, stromelizin i membranski tip MMP-a.

Postmortalne promene mesa ribe vezane su za metaboličke promene (prekid nukletida, hidroliza i oksidacija masti, pad pH, rast osmotskog pritiska, povećanje koncentracije Ca^{2+}, povećanje azot oksida i slobodnih radikala), za promene strukture miofibrila (oslobađanje i proteolizu α-aktinina, degradaciju titina, proteolizu nebulina i miozina, delokalizaciju tropomiozina, proteolizu troponina T, pristustvo 30kDa fragmenta, degradaciju aktina, dezmina i distrofina). U ovim promenama učestvuje brojni faktori,a promene neminovno dovode do gubitka svežine ribe.

***Ključne reči**: riba, postmortalne promene, strukturne promene, svežina*

INTRODUCTION

Quality attributes of fish flesh, including food safety, organoleptic features, nutritive value quality and aptitude to industrial processing, influence on consumption and acceptability of fish as food. Fish sensorial changes and texture properties are closely linked to freshness. Along with

ante mortem muscle biochemistry, postmortem biochemical processes are directly linked to final quality attributes. The understanding of postmortem mechanisms is a prerequisite for an accurate control of commercialized fish quality by the identification of objective markers or indicators as measure of freshness.

Muscle metabolism postmortem

Immediately after the cessation of the circulation of the blood and, consequently of oxygen supply, the stored carbohydrate glycogen is anaerobically degraded and lactic acid accumulates in muscle (Watabe et al.,1989), resulting in a pH drop from a value close to 7,4 to 6 in fish, sometimes below (Church, 1998). Muscle osmotic pressure increases within hours postmortem, ATP (adenosine triphosphate) decreases and lipids are oxides. Trimethylamine oxide (TMAO) is changed to trimethylamine (TMA) by endogenous enzymes and later by bacteria when microbial activity begins. Nitric oxide and reactive species of oxygen also increase. Mitochondria and sarcoplasmic reticulum deterioration due to pH fall and osmotic pressure changes results in the release of calcium ions in the cytosol where concentration can reach 0, 2 mM free calcium.

The onset and extent of rigor mortis are biochemically characterized by a total exhaustion of energy-rich compounds. ATP depletion initiates the rigor mortis process and at less than 2 μM ATP, actin and myosin form inextensible actomyosin, which causes stiffness of the whole body in rigor mortis. The rigor generally begins one to six hours after death in fish (Watanabe i Turner, 1993), in particular, it is maximal one day and a half after death for sea bass muscle stored at 0°C. This condition usually lasts for a day and then the resolution of rigor mortis makes the muscle less rigid and no longer elastic. The rate in onset and resolution of rigor mortis varies from species to species and is affected by temperature, handling, and stress before slaughter, size and physical condition of the fish, biological status of fish and temperature during storage before rigor onset. Also exists individual variations. Rigor is very dependent on stress before slaughter: control salmon shows maximum rigor at about 8 hours postmortem, but highly stressed fish can enter rigor as soon as 30 minutes postmortem (Skjervold et al., 2001).

Sometime after death, an opposing process called tenderization begins within hours postmortem and continues during postmortem storage. Several studies showed that tenderization begins in the early stage of postmortem storage of fish and mammals (Koohmaraie, 1996). The key structures which are degraded are the cytoskeletal links to sarcomeres and to the plasma membrane (Taylor et al., 2002). Tenderization rate and extent vary depending on species and other factors.

Proteolysis of cytoskeletal components results in myofilament degradation. In fish, depending on species, this may include degradation of titin, nebulin, dystrophin, α-actinin release, myosin proteolysis and tropomyosin delocalization (Ofsad et al., 1996, Papa et al., 1997). The costameres which link sarcomeres to the sarcolemma are also degraded within 24h postmortem, Most of the changes are common among different fish species but they may occur at different rates. In particular, sea bass muscle changes include the detachment of sarcolemma, the degradation of titin and nebulin as well as the release and proteolysis of α-actinin from the Z line and the degradation of dystrophin. Desmin remained unchanged after a 4 days cold storage in sea bass muscle. A sarcoplasmic 16 kDa protein (identified as nucleoside diphosphate kinase)

was also shown to undergo proteolysis during storage (Verrez-Bagnis et al., 2001). Tropomyosin was released from the myofibrils; the content of extracted tropomyosin increased with time between 0 and 48 h in the presence of 5 mM EGTA. In extracts with 5 mM Ca^{2+}, tropomyosin was observed in lower quantities after 48 h.

Connective tissue collagen is degraded in fish after death as shown by scanning electron microscopic analysis of muscle as a progressive breakdown of the collagen junctions between the myocommata and the muscle fibers during storage in ice. The structural change in collagen fibrillar network in fish correlates with the postmortem

tenderization. Collagen fibrils in the pericellular connective tissue are disorganized and degraded and space between fibers increases. The decrease of type V collagen content has a correlation with the postmortem softening of fish meat during chilled storage (Shigemura et al., 2003).

ROLE OF PROTEASIS IN POSTMORTEM AUTOLYSIS OF FISH MUSCLE

Deterioration of fish flesh results from the complex combination of physical, chemical, biochemical and microbial processes. However, the first changes occurring in postmortem fish muscle are due to endogenous enzymes promoting proteolysis of muscle proteins and connective tissue as well as fat hydrolysis. Indeed, the muscle is not significantly contaminated by bacteria at this stage.

Proteases in fish muscle

Different proteolytic system exist within the muscular cell: a muticatalytic 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.

The multicatalytic complex is involved in the degradation of hormones, antigens, transcription factors and ubiquitin-conjugated or oxidized proteins. The 26S proteasome requires ATP for activity and hydrolyses ubiquitin-conjugated proteins. The 20S proteasome, which in fact is also a part of 26S proteasome, exists as a latent form possibly activated by different compounds (Aoki et al., 2000).

Cathepsins are acid proteases usually located in organelles called lysosomes and thus are for the most part inactive in living tissue, but become released at sites of injury or upon freezing and thawing of postmortem muscle. Cathepsin can be distinguished by their active site (aspartic, cysteine, serine proteases) as well as by their substrate specificity and inhibitor sensitivity. Lysosomes are known to harbor at least 13 cathepsins.

Calpains are cysteine proteases active at neutral pH and are dependent upon calcium. Ubiquitous calpain include calpain I or micro (μ)-calpain which requires micromolar calcium concentration for full activity (10 to 50 μM) and calpain II or milli (m)-calpain requiring millimolar calcium concentrations (300 μM to 1 mM). Calpains are heterodimers dissociating in presence of calcium. The small subunit (30 kDa) is common to ubiquitous calpains and the large subunit (80 kDa) is specific to the calpain type and is responsible for catalytic activity. Calpains, active at intracellular physiological pH, are highly regulated in vivo. The regulation of calpain activity is complex and not fully understood. The regulation system is based on calcium binding, subunit association, interaction with calpastatin (the endogenous inhibitor specific to calpains) and cellular membranes as well as limited autolysis in the presence of calcium allowing proteolytic activity, but increasing instability. Calcium is binding in the calpain molecule resulting in a conformational switch allowing the alignment of the catalytic amino acid trio Cys, His, Asn. Four properties of calpains are calcium dependent: binding to cellular components such as membranes, calpastatin binding, proteolytic activity and autolysis (Hosfield et al., 1999).

Matrix metalloproteases (MMP) represent a large family of structurally related endopeptidases responsible for connective tissue catabolism. MMPs are able to degrade different types of collagen and cytoskeletal proteins connecting the sarcolemma to the

extracellular matrix. They are zinc and calcium-dependent enzymes, classified into four subfamilies: collagenase, gelatinase, stromelysin and membrane type MMP.

Reports on other proteolytic activities such as serine proteases, neutral proteases or alkaline proteases from fish muscle are fragmented.

Contribution of proteases to postmortem autolytic changes

Until now an understanding of the complex mechanisms responsible for postmortem fish muscle changes hasn't reached general agreement. For fishes, of the different intracellular proteolytic systems identified so far, two major pathways were generally distinguished for muscle proteins degradation during postmortem storage: the cathepsins and the calpains. A rapid decrease in pH after death could indicate that lysosomal acidic proteinases may be active if they are liberated from lysosomes to reach the substrate. Cathepsins D and L are believed to play a major role in the autolytic degradation due to their wide pH range of activity while other cathepsins are active at pH values too low to be of physiological significance. The high cathepsin content in spawning fish and rapid degradation of postmortem muscle indicates a possible role also existing in normal fish. Also supporting a role for cathepsin is the degradation of connective tissue proteins within days in fish, connective tissue proteins are not susceptible to most proteases but are cathepsin substrates.

The second protease system which can degrade connective tissue is the metalloproteases. These proteases are constitutively inactive in mammals and require signal transduction pathway activation. There is report of their activity in some species of fish, but no studies of their role in texture.

A number of *in vitro* studies have clearly demonstrated the susceptibility of numerous myofibrillar proteins to proteolysis by calpains and lysosomal proteinases. In sea bass muscle, calpain was able to release α-actinin and tropomyosin from myofibrils in vitro. Calpains and cathepsins degrade myosin heavy chain, α-actinin and desmin while actin and tropomyosin appear to be sensitive to cathepsins B, D, L. Troponin T was degraded by cathepsins B and L. Minor changes of some other myofibrillar of cytosolic proteins were also observed (creatine kinase and other identified proteins).

Changes in collagen have been attributed to collagenases; they have also been related to the process responsible for gaping phenomenon in which the muscle fibers are gradually disconnected from the myocommata during chilled storage due to collagen fibers breakdown. It has been pointed out at role of matrix metalloproteases in the disintegration of the intramuscular connective tissue that induces the postmortem tenderization of fish muscle. It has been observed both quantitative and qualitative differences in collagenolytic activities in the muscle of different fish species (Kubota et al., 2003, Delbarre-Ladrat et al., 2004). None of these systems alone can explain all the changes observed postmortem, Synergy between proteases and other environment factors exists. During rigor mortis, since osmotic pressure is modified, ionic strength increases and may become high enough to weaken the myofibrillar structure, making it more susceptible to proteolysis.

CONCLUSION

Postmortem changes in fish are related to metabolic changes (nucleotides breakdown, lipid hydrolysis and oxidation, decrease of pH, increase in osmotic pressure, Ca^{2+} increase, increase in nitric oxide and free radicals) and structural changes (myofibrillar

structure: α-actinin realease, α-actinin proteolyses, titin degradation, nebulin proteolysis, myosin proteolysis, tropomyosin delocalization, tropomyosin proteolysis, troponin T proteolysis, 30 kDa fragment appearance, actin degradation; desmin degradation, dystrophin degradation).

In these changes is involved big number of factors (nucleotide-degrading enzymes, phospholipases, oxidation status in cell, glycogen degradation and lactic acid accumulation, protein deterioration, increase in osmotic pressure and pH fall, calpains, cathepsins, calcium) and these changes will inevitably lead to loss of fish freshness.

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