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Mini Review

Protein thaw loss in meat systems: Biochemical influence towards meat authentication of fresh versus thawed

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Abstract

Meat quality is always subject to consumer scrutiny when purchasing from retail markets on mislabeling as fresh meat. Repeated cycles of 'freeze-thaw' degrade the quality of meat. Existing studies have primarily embarked on physical, chemical and biochemical changes induced by variable storage conditions. The authentication of fresh versus thawed meat quality can be further explored with the data involving a series of biochemical pathways that were largely well-studied in living muscle tissues. However, these pathways are less predictable in post-slaughter condition where muscle turns to meat. In addition, there is far less known about how various management or environmental stimuli impact these pathways, either by substrate load or altered cellular environment during storage. Though the rate of post-slaughter metabolism is quite important in driving meat quality development, it is also fairly well established. Alternatively, the biochemical mechanisms responsible for the cessation of postmortem metabolism, or protracted carbohydrate metabolism are particularly puzzling. Likewise, there is little information about the relationship between volatility profiles of biomolecules with regards to functional groups, enzymatic activity, protein solubility and protein surface properties in meat during storage. The studies of these changes could be used to distinguish between fresh and thawed meat.

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Introduction

Various physiological and biochemical changes influence the quality of meat. As a major component of muscle tissue, proteins play a major role in muscle foods. Understanding the mechanism of protein changes induced during technological processing, and the rationale behind the variable outcome of processing will greatly aid the improvement of processing technologies. These mechanisms are further aggravated by changes in temperatures (Pearson and Young, 2012). The quality of frozen meat is related to freezing and thawing processes. Freezing rate and the small ice crystals formation in freezing are critical for minimizing tissue damage and drip loss during thawing. During thawing process, meats are subject to damage by chemical and physical changes and microbial attacks (Kalichevsky *et al*., 1995). For assurance of meat quality, quick thawing at low temperature avoiding notable rise in temperature and increased dehydration of meat is desirable. Various aspects of effect of freeze-thaw process on meat quality have been reviewed (Kalichevsky *et al*., 1995; Bendixen, 2005; Ballin and Lametsch, 2008; Sehar *et al*., 2013).

A perception of the changes that freezing and thawing bring about in different meat types and cuts is essential to the meat industry, as their main objective is to produce better-quality products with high resale values that are both attractive and pleasant to the consumer. Despite a countless of advances, questions remain regarding the mechanisms controlling or impacting post-slaughter metabolism and how physiological and tissue based homeostatic set points are maintained or breeched by various management practices ultimately leading altered meat quality. Various biochemical mechanisms responsible for cessation of post-slaughter metabolism have been studied by researchers. These comprise the relationship between biomolecules (NAD, lactic acid), functional group, enzymatic activity, protein solubility, and protein surface properties (hydrophilic/hydrophobic). All these cause changes in physical properties of proteins, including fragmentation, aggregation, loss of solubility and functionality and decreased susceptibility to proteolysis in meat during storage (Estevez, 2011). Studies of these factors can

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enable us to distinguish between fresh and thawed meat. Precise mechanisms by which fresh and thawed meat can be destroyed due to the influence of these undesirable biochemical dis-harmonization changes are not fully clear. The identification of routes of mechanisms involved in determination of the quality of meat is essential to establish the potential implication of such fluctuations on particular (species) meat quality.

This review focuses on the data and numbers pertaining to biochemical changes in protein as an expression of the non-homeostatic biochemical changes to meat proteins at cellular level. It would be a good resource that unravels and peels the vast existing current knowledge on this topic and aims to elucidate what future challenges could be hold. We have covered only meat quality trait focusing on the linkage between functional groups, protein surface properties, enzymatic activity and protein solubility that were thoroughly studied and how each factor contributes to meat authenticity.

Skeleton of meat: biomolecules

Many changes take place during meat thaw particularly in the perspective of protein components within cellular environment as well as in meat integrity as a whole. Carbonylation is one of the most remarkable chemical modifications in oxidized proteins. In practice, protein carbonyls are quantified by the dinitrophenylhydrazine (DNPH) method to assess P-OX in meat systems. The formation of protein carbonyls from particular amino acid side chains leads to impair the conformation of proteins and thus ultimately denaturation and loss of functionality (Estevez, 2011). Some of the arbitrary corresponding events documented in the past decades would be such as scenario whereby decline in oxygen demand during maturation appeared to be related to corresponding decrease in NAD concentration. Concentration of NAD, oxygen uptake and metmyoglobin reducing activity (MRA) in both beef and lamb muscles are decreased with time as the meat ages or is frozen. The metmyoglobin begins to accumulate on the surface of the meat at a rapid rate. The NADH and MRA are lost during thawing because of exudate leaching. This leads to increase in oxidation and loss of color (Atkinson and Follett, 1973; Abdallah *et al.*, 1999).

Study done by Flores *et al*. (2000) showed that peptide fractions isolated from pork meat can be used as potential markers of meat quality. Peptide fractions were used to distinguish exudative from non-exudative meats as well as to differentiate dark, firm and dry meat from the rest. Volatile basic nitrogen (VBN) index is used for evaluating food decay from degradation of proteins and non-protein nitrogenous. It has been observed that when volatile basic nitrogen level increases, it indicates the food is stared to decay. If meat is stored at 4°C between 4th to 8th day, VBN is increased dramatically (Hsieh *et al*., 2010; Umuhumuza and Sun, 2010).

Lipid oxidation generally increases with repeated freeze thaw cycles (Yarnpakdee *et al*., 2012). Qi *et al.* (2012) have found that total and myofibrillar protein solubility and intramuscular free fatty acids concentration were decreased after one cycle of freeze and thaw but then increased gradually with further cycles. Loss of redness caused by myoglobin (Castro-Giráldez *et al.*, 2011) with drip during thawing and formation of metmyoglobin was documented as well (Benjakul and Bauer, 2001). Increase in yellowness due to lipid oxidation and browning reaction was also observed (Thanonkaew *et al*., 2006). Freeze-thaw cycles also cause disintegration of muscle structure in resulting re-distribution of pro-oxidants; e.g. non-heme iron and lipid oxidation acceleration (Qi *et al*., 2012). Aliani *et al.* (2013) have studied the post-slaughter changes in ATP metabolites, reducing and phosphorylated sugars in chicken meat. They have found that glucose-6-phosphate concentrations were inversely related to the pHu (ultimate pH) of the breast meat of chicken throughout chilled storage. An ATP, ADP, AMP were rapidly broken down, concentration of IMP rose rapidly and remained high (Aliani *et al*., 2013). Concentration of inosine, ribose and hypoxanthine increased gradually post-slaughter as studied by others (Hernández-Cázares *et al*., 2000).

Decrease efficiency of sarcoplasmic reticulum Ca^{2+} ATPase was observed because of intracellular Ca^{2+} overload (Chen *et al*., 2011). Mitochondria accumulate substantial amounts of Ca^{2+} and sustained increases in matrix Ca^{2+} contribute to permeability transition whereby the inner membrane becomes permeable to solutes. It also triggers mitochondria swelling and collapse of mitochondrial energy. Mitochondrial ATP synthase run in reverse, hydrolyzing glycolytically-produced ATP in order to maintain a membrane potential. An ATP hydrolysis by the F1F0 ATPase would directly influence cellular ATP consumption, promote glycolysis and pH decline post-slaughter (England *et al*., 2013). Glucose released from muscle glycogen is dependent on a muscle-specific glycogen phosphorylase that is activated by Ca^{2+}, epinephrine, and AMP. When glycogen is not a limiting factor, pHu could be determined by AMP-dependent activity because the disappearance of AMP induces an inhibition of biochemical reactions in postmortem muscle (Rammouz *et al*., 2004).

Thawing increases molecular mobility resulting in the relaxation of meat components frozen in a rigid state. Such relaxation may sometimes allow the reabsorption of the fluids (soluble proteins, vitamins and salts) by the meat, but with a risk of meat discoloration and lipid oxidation if the thawing time is too long (Liu and Chen, 2001; Medina *et al.*, 2009). Although, meat color is defined by myoglobin oxygenation and the oxidative status of the heme iron, the concentration of residual oxygen and the radical formation is major cause of color deterioration in stored meat.

Major form of programmed cell death (PCD) is apoptosis, regulated by central nervous system or by the target cell itself. Recently, the role of apoptosis in meat tenderization and various biomarkers (proteins and enzymes) involved in meat tenderness have been reviewed (Ouali *et al.*, 2013). The conversion of muscle into meat occurs in pre-rigor step, rigor step and tenderization step. Apoptosis induces a series of biochemical and structural changes in dying cells. Cell contents totally disappear due to unfavorable environmental modifications like pH, ionic strength, low energy availability etc. The respiratory chain loses its capacity to oxidize molecular oxygen; the mitochondrial external membrane becomes permeable to all protein compounds localized in the inter-membrane space, including cytochrome C, a central caspase 9 activator. Calcium from endoplasmic reticulum is transferred to the mitochondria that become overloaded with calcium causing irreversible alteration of their internal membrane. Molecular oxygen, not oxidized by the respiratory chain, forms free oxygen radicals which oxidize all cellular compounds. Apoptosis starts within few minutes after death, first oxygen radicals are generated by mitochondria that initiate the autocatalytic process. This autocatalysis continues during the whole storage period even at low temperature including freezing (Ouali *et al*., 2006).

Factors that manipulate reaction of biochemical mechanisms

Functional groups

The current poultry meat grading system is based on aesthetics and does not categories high or low quality meat in terms of functional properties (Barbut, 1997). Meat exudates have been correlated with total protein content. Removing meat exudates and avoiding freeze can slow down the quality deterioration of meat during cold storage. Exudates are formed in higher amount at -20°C than cold storage at 1°C. This leads to increase in drip loses and Warner-Bratzler shears force along with a decrease in lightness and redness (Atkinson and Follett, 1973). Fractions of bound water present in muscle cells are very resistant to freezing, but other fractions are easily exuded from muscle cells by freezing and thawing. Freezing induces distortion of tissue structure and mechanical damage due to the formation of ice crystals. However, no effect of freeze-thaw on protein concentration has been reported (Kim *et al*., 2013). Changes in level of nitrogen in meat homology tend to convey a message. As reported in literature, extractability of nitrogen decreases quickly after death of animals. When meat chilled at 4°C for a week, was stored at 20°C, amount of extractable nitrogen showed a higher value than that from the meat immediately after death (Hashimoto *et al*. 1959).

Heme pigments in poultry muscle include myoglobin, hemoglobin and cytochrome C. Increase in darker color of meat is related with Increase in myoglobin content, higher pH, cytochrome C. Generally, pH of meat and color are correlated; higher pH is observed with dark color broiler chicken whereas low pH is observed in light colored chicken breast fillets. Muscle with higher pH (~6.6) contains more cytochrome C than muscle with lower pH (6.0 to 6.1). Chilling methods (ice slush against air chilled) may influence cytochrome C content of chicken meat but no explanation is available (Boulianne and King, 1998; Holownia *et al*., 2003). Oxidation-reduction potential (ORP) determines the inter-conversion of hemochrome (Fe^{2+})/hemichrome (Fe^{3+}) iron of globin. Concentration of ferric ion affects the production of carbonyls in HRGS-oxidized muscle proteins leading to denaturation (Park *et al*., 2007).

In fresh condition, there are more reactive sulphydryl groups than in frozen condition. Freezing induces more protein oxidation (Chan *et al.*, 2011). Protein carbonyl was measured to evaluate the degree of protein oxidation (Xia *et al*., 2009). Biogenic amines (BA) can be used as marker for freshness meat. Various biogenic amines of importance are serotonin, tyramine, tryptamine, putrescine, cadaverine, spermidine, spermine etc. These BA are formed because of enzymatic action on various amino acids. Increased level of BA indicates the meat spoilage by microorganism during storage. Biogenic amines can cause potential health risk, especially when coupled with additional factors, such as monoamine oxidase inhibitors, drug, alcohols and gastrointestinal diseases. Vinci and Antonelli (2002) have studied the red and white chicken meat and level of various BA (chicken and beef). Moreover, Balamatsia *et al.* (2006) have evaluated the formation of various BA in

breast chicken meat during storage under aerobic and modified atmospheric packaging (MAP) conditions at 4°C. They have found that the levels of putrecine, cadaverine and tyramine are increased during storage and these may be used as quality index. Silva and Glória (2002) have also studied the changes in levels BA in chicken (breast and thigh) and chicken-based meat products after slaughter and during storage at 4±1°C. They concluded that quality index, based on the ratio of spermidine and spermine (Spd/Spm), can be used as quality index for proper storage of chicken meat. Hajduk (1999) had studied the effect of method of preservation (freeze-drying and freezing at -30°C) on DNA, ADA (adenosine deaminase) and PNP (purine nucleoside phosphorylase) activity. He found that there is no effect on DNA content however, activity of ADA and PNP had been decreased. Moreover, freezing is a better method than freeze-drying.

Protein surface properties

Protein conformation is affected by temperature, pH, storage conditions, repeated cycles of freeze-thaw etc. Significant decrease in sarcoplasmic protein surface hydrophobicity was reported in frozen samples when compared to fresh samples. Myofibrillar protein surface hydrophobicity was significantly higher in high pH meat compared to that of low and normal pH meat (Lee *et al.*, 2010). However, it has been revealed that there are no statistical differences in myofibrillar protein surface hydrophobicity between low and normal pH meat, indicating a similar pattern of protein folding (Hopkins *et al.,* 2000). Moreover, Chan *et al*. (2011) have reported an increase in surface hydrophobicity of chicken natural actomyosin (NAM) during freezing and frozen storage. This increase may be due to mild denaturation of muscle proteins without aggregation, causing protein unfolding during freezing. Acidification of muscle decreases protein charges and increases their hydrophobicity leading to reduced water retention. Very poor correlation had been reported between the increase in extracellular space and muscle pH (Hollung *et al.,* 2007).

Enzymatic activity

Generally, meat will undergo autolytic changes that include proteolysis and lipolysis. There is hardly any report of lipolytic activity of microorganisms leading to spoilage of meat. This is primarily because proteolytic activity is much faster than the lipolytic activities at cold temperature (Sarika *et al*., 2011). In a study, it was concluded that aerobic energy metabolism increased after slaughter with an increase in the enzymes involved in both the glycolysis and TCA cycle. It is because of an increase in the level of a wide range of metabolic enzymes and stress proteins after slaughter. Activities of many glycolytic enzymes such as enolase, aldehyde dehydrogenase, phosphoglycerate kinase as well as other enzymes involved in oxidative metabolism such as ATP-specific succinylCoA synthetase and isocitrate dehydrogenase are increased. Glycerol-3-phosphate dehydrogenase 1 (GPD1), ADP-ribosylhydrolase like 1, biliverdin reductase B, and cytochrome bc1 complex, are not directly involved in glycolytic pathway but take part in production of NAD+, which may enter the glycolytic and TCA pathway to drive the synthesis of ATP. This leads to increase in energy metabolism in post-slaughter via glycolytic pathway in first four hours after slaughter (Hollung *et al*., 2007).

Damage to cell organelles due to storage condition causes release of its content. The lysosomes release various digestive enzymes like lipase carbohydrases, proteases, nucleases and phosphatases. Amount of these enzymes is increased in meat press juice after freeze-thawing due to damage of cell compartments. To discriminate between fresh and thawed meat, numerous enzymatic methods have taken advantage of this enzymatic release. Proteases are released from lysosomes during freezing and it is possible that proteolytic degradation of individual enzymes could cause a decrease in their activities in the press juice. This will complicate the discrimination between fresh and long term frozen meat when the juice is analyzed.

The HADH (β-hydroxyacyl-CoA-dehydrogenase) enzymatic method has been a choice for discrimination between fresh and thawed meat as long as the whole meat sample has been frozen below -12°C. The HADH method is not applicable to ground meat, because grinding releases HADH from the mitochondria, as is the case in frozen (-12°C) thawed meat (Ballin and Lametsch, 2008). This spectrophotometric method measures the conversion rate of NADH to NAD+ by monitoring the decrease in absorption at 340 nm. Ellerbroek *et al*., 1995 have observed that level of N-acetyl-β-glucosaminidase is significant between fresh and frozen meat in pig and beef (Ellerbroek *et al.*, 1995). Other enzymes have been also investigated in post-mortem muscle. They consist of dipeptidyl peptidases (DPP), aminopeptidases and calpain system. Enzymes-citrate synthase and lactate dehydrogenase were reported as indicative of the aerobic and anaerobic capacity. Muscle glycolytic potential is used to predict meat quality (Remignon *et al*., 2006). Endopeptidases affect the texture of

meat; tenderization is affected by post mortem pH because of calpain and lysosomal cathepsin activities. Exopeptidase act over polypeptides and proteins generating smaller peptides and free amino acids that are responsible of flavor changes. Dipeptidyl peptidase (DPP) generates dipeptides from the amino terminus of polypeptides and proteins. Aminopeptides are the enzymes involved in the last step of the proteolytic chain. In fact, these enzymes contribute to taste development during meat ageing and meat products. Differences in enzyme activities among classes, mainly in exoproteases, could be used as indicators or markers for post mortem quality in a broad time range (2 to 24 h) (Toldrá and Flores, 2000).

An ATPase activity is regulated by the presence of calcium ions in mammalian myosin. Chan *et al.* (2011) have studied the activity of Ca^{2+}-ATPase in low, normal, and high pH meat as a function of frozen storage and concluded that there was no significant difference in Ca^{2+}-ATPase activity among these meat samples in fresh condition. However, a significant reduction was observed in Ca^{2+}-ATPase activity in the frozen samples as compared to fresh samples. Decrease in Ca^{2+}-ATPase activity due to change in the conformation of enzyme or aggregation of myosin molecules. Decrease in Ca^{2+}ATPase activity may be associated with oxidation of sulphydryl groups on the myosin globular head. Freezing and frozen storage cause a marked decrease in Ca^{2+} ATPase activity and increase in Mg^{2+}-EGTA-ATPase activity. This leads to translation into denaturation of myosin and the troponin-tropomyosin complex (Leygonie *et al*., 2012).

Phospholipase A2 (PLA2) and or protease enzyme activities and proteoglycans) may contribute to reduce meat quality characteristics of low pH Turkey meat. Biochemical properties of protein of low, normal and high pH meat tend to become similar after freezing (Chan *et al.*, 2011). Activities of glycolytic enzymes decreased when muscle pH value was high at 24 hour just before freezing. Remaining glycogen in muscles was degraded and rigor could occur during thawing, pH decline and sarcomere shortening within the first 10 freeze-thaw cycles. Melting and reformation of ice crystals may destroy lysosomes and induce the release of lysosomal enzymes. These enzymes partially participate in the degradation of myofibrillar proteins (Qi *et al.*, 2012).

Protein solubility

Protein solubility is a good indicator of protein denaturation. Loss of protein solubility has been shown to accompany loss of quality during frozen storage. During frozen storage, loss of sulfhydryl group content of muscle tissue and formation of phenol-reagent positive materials occurred more rapidly in low pH samples than in high pH samples; leading to loss of protein solubility (Khan and Nakamura, 1972). Changes in protein composition after slaughter between the soluble and insoluble protein fractions had been reported. Connection between the stability of myofibrillar proteins and solubility of easily soluble proteins, such as metabolic enzymes and cellular defense or stress proteins was reported. The occurrence of these easily soluble proteins in insoluble protein fraction could be due to precipitation or aggregation, thereby going from soluble to an insoluble state. Mechanism involved could be isoelectric precipitation caused by pH decline and modification of proteins (Hollung *et al*., 2007). Greater solubility was observed in muscle tissue frozen at -10°C. This occurred in both raw and cooked muscle tissue. The total solubility content of raw muscle was greater when frozen at -10°C (Huber and Stadelman, 1970).

Barbut (1997) had shown that lower protein solubility of pale soft exudate broiler/Turkey breast meat as compared to normal meat. Likewise, It had been revealed that normal pH meat had the same extent of protein denaturation as low pH meat as shown by protein solubility. Freezing causes significant reduction (33%) in total protein solubility. This reduction is related to instability of proteins due to aggregation behavior during freezing process. They have also found a reduction in solubility chicken actomyosin after frozen storage. This is because of association or dissociation of actomyosin leading to formation of aggregates (Chan *et al*., 2011). However, sarcoplasmic protein solubility is increased in the frozen samples (90 mg/g) compared to fresh samples (80 mg/g). After frozen storage, sarcoplasmic proteins became more folded preventing exposure of hydrophobic groups on the protein surface as shown by decrease in protein surface hydrophobicity; thus, provides Interaction of proteins with the surrounding water. Owen (1996) has concluded that fewer the number of surface hydrophobic patches, the greater the solubility observed. A study on the effect of freeze-thaw process on eating and technological quality attributes of ovine longissimus dorsi muscle was carried out. They have found that solubility of myofibrillar and total proteins decreased after 1 cycle of freeze and thaw but increased greatly during further cycles. Solubility of myofibrillar proteins could be related to the proteolytic degradation of myfibrillar and total proteins during thaw. Previous studies have shown that the solubility of myofibrillar proteins

decreases due to oxidative deterioration of muscle protein during freezing and thawing (Qi *et al*., 2012). A good indicator of protein denaturation is related to its hydrophobicity/hydrophilicity balance. Though protein solubility has an impact on freeze-thaw meat but in overwhelming majority of studies correlations mainly done on enzymatic activity, thus now indeed a correlation between multiple contributing factors discussed above should be given equal importance to fully establish the value of the biochemical changes upon freeze-thaw condition on meat.

Conclusion

Pertaining to conversion of muscle to meat, questions are yet to be addressed. Mechanism responsible for the cessation of anaerobic glycolysis postmortem has eluded researchers for the past 60 years, but the pH-mediated inactivation of glycolytic enzymes or a loss of adenosine nucleotides is likely. As technological and applications of proteomics has been more widely used, the term itself is currently expanding from its classic status as biochemistry at an unprecedented high throughput scale to much broader definition of panoramic protein characterization. This includes global characterization of functionality, modification states and isoform patterns of proteins. The challenge of defining biological system is far beyond the capabilities of any single functional molecular tool like proteomics; hence data from many levels of biology and many technologies, including those of pre and post-genomic tools must be integrated, in order to understand complex biological systems, and their influence on animal production and meat quality. This truly requires interdisciplinary collaborations between a broad range of sciences, including those of physiology, genetics, cell biology, computer sciences, as well as from the animal production and from food industry (Bendixen, 2005).

Globally, companies rely on air chilling systems for poultry that is to be sold as a "fresh", chilled product and speculated that the market will not accept a full return to immersion techniques (James *et al.*, 2006). Various reported data could eventually summarize that rapid thaw in water bath seems to be an eye opener to food safety guidelines without deleterious effects on meat quality (Eastridge and Bowker, 2011) whereas slow freezing rates and longer frozen storage duration reduce meat quality (Muela *et al*., 2010).

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