STRUCTURE/FUNCTION RELATIONSHIP OF CHANNEL CATFISH (*Ictalurus punctatus*) MUSCLE PROTEINS SUBJECTED TO pH-SHIFT PROCESSING

By

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To my Lord and Savior Jesus Christ, my wife Alyson and the past three generations of my family, thank you for paving the way making this milestone possible and welcoming me to your ranks

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Novel processing utilizing pH-shift technology applied to muscle-based systems modifies or increases raw product utilization. The use of pH-shift processing on muscle products and by-products allows for the separation of muscle proteins from lipids, collagen, skin, bones and other undesirable components. Undesirable materials are not easily separated from whole muscle or other comminuted muscle products such as surimi. During pH-shift processing, animal and fish muscle including channel catfish muscle is modified by the reduction of lipid and heme content, altering protein composition and thermal gelation properties. Reduction of lipid and heme content increases whiteness and oxidative stability of protein isolates. Changes in protein composition are associated with the removal of sarcoplasmic proteins including heme proteins, some endogenous enzymes, collagen and other protein components. Modification of thermal gel properties induced variable gel strengths. It was hypothesized that pH-shift processing will structurally modify channel catfish muscle proteins resulting in functional properties leading to the expansion, utilization and production of muscle based products. The model system in this study was developed to provide the basis for implementation of economical and environmental utilization of current seafood by-products by pH-shift processing.

The results show that pH-shift processing modifies muscle proteins structurally and functionally. pH-shift processing reduced the relative content of alpha helix to beta structure of muscle proteins after isoelectric precipitation indicating a molten globular state. Muscle proteins in a molten globular state showed reduced myosin ATPase activity, altered protein surfaces, modified thermal sensitivity, increased susceptibility to enzymatic crosslinking and modified solubility. These changes lead to changes in the physical properties of pH-shift processed muscle proteins. Alkali processed catfish showed increased gel rigidity, gel strength and gel flexibility compared to acid processed catfish which exhibited inconsistent functional performance, increasing and decreasing in gel rigidity, gel strength and gel flexibility.

These results show that pH-shift processing of channel catfish muscle provides highly functional isolates with a broad range of applications by inducing structural modification of muscle proteins. This innovative process yields muscle proteins with good color, high oxidative stability and modified functionality, which can find many applications in formulated comminuted muscle food products. Furthermore, this technology can be an important contribution to better utilization of seafood and land animal by-products.

CHAPTER 1
INTRODUCTION

Background

Presently the world demand for seafood products exceeds the sustainable limits of the available fisheries (Hultin and Kelleher 2000). The supply traditionally has been based on wild caught products; however, the demand for high quality seafood products has not been fulfilled. The investigation into economically viable alternative production methods and utilization of currently harvested products is of interest to fill the pressing needs of the consuming public. The advent of commercially viable aquaculture in the past 50 years has helped to alleviate some of the stress on wild stocks (Harvey 2002). The supply of aquacultured seafood products has come under fire for reducing the price of high value wild species such as salmon, shrimp and grouper (Eaglea and others 2004). Aquaculture also has been criticized for unmanaged waste production, increase in fish disease and damage to the aquifer (Barton 1997). Thus the need for value-added products is of great interest to commercial producers as the profit margins are getting smaller and the cost of production is rising (Harvey 2002). The utilization of value addition with modern processing and preservation has allowed for increased commercial production of high value products from lower value fish species and by-products, but many useable protein sources are still diverted into animal feed or waste (Kristinsson and Rasco 2002).

One of the products commercially produced as a value addition product is surimi (Park 2005). Surimi is not a new product. Surimi originated centuries ago as a high value product in Japan (Park 2005). A variety of structured products can be formed from surimi, including the traditional kamaboko (Park 2005). Kamaboko was traditionally made by first producing surimi by mincing freshly ground fish meat, washing it with pure water or water with low concentrations of salt, dewatering the washed tissue, forming a sausage, and cooking the sausage

to form kamaboko. The value of quality surimi and kamaboko was high due to the requirement to use fresh fish and the very short shelf-life of either the raw or cooked material (Park 2005). Commercialization of surimi production and subsequent products made it necessary to extend their shelf-life. Freezing surimi was one of the prerequisites to its success as a global commodity, but surimi is particularly sensitive to freeze thaw denaturation (Park 2005). Cryoprotection increased protein stability to freeze thaw treatments by utilizing small carbohydrates which stabilized the muscle proteins during freeze thaw processes. This has been attributed to paving the way for the commercialization of surimi (Park 2005). The current surimi production uses lower value white fish, primarily Alaskan Pollack and Pacific Whiting as the base material. The use of Alaskan Pollack and Pacific Whiting for the production of surimi allows for commercial processors to fill the commodities market with two products, surimi and frozen fillet blocks (Park 2005).

Surimi processing does have drawbacks such as still mainly requiring fresh fish (Park 2005). This has led to the utilization of large trawlers which catch, process and freeze surimi either on the water or on shore (Park 2005). Concurrently on these trawlers the alternative product, frozen fish fillet blocks and mince also are produced (Park 2005). Even though surimi can allow for increased utilization and profit margins from these species, there are many other commercially harvested species which are not used for surimi and go to animal feed or other lower value products (Hultin and Kelleher 2000). Traditional commercial surimi processing cannot easily handle complex materials, such as whole fish, species rich in dark muscle, high in fat or processing byproducts (Hultin and Kelleher 2000). Not only is it difficult to recover proteins effectively from complex materials, but these materials lead to major color and oxidation problems resulting in surimi with poor functionality (Hultin and Kelleher 2000). The

by-products from fish processing which contain the most muscle tissue are the frame and the trimmings. The frame is the backbone and ribs left after the fillet is removed. The trimmings are the parts of the fillet which are removed to increase the quality and acceptability of a fillet; a useable trimming from catfish is the nugget or belly flap. Large amounts of unprocessed by-products such as frames and trimmings are not used for human consumption but rather end up in animal feed, bone meal or fish meal if used at all (Hultin and others 2000). Unutilized by-products end up as waste material and add disposal costs and increase the biological effluent from the processing plant (Hultin and others 2000). Usable meat left on the frame and other processing by-products can be used for human consumption leaving enough remaining material to satisfy the animal feed market (Hultin and Kelleher 1999). Therefore a major need exists to develop and use alternative techniques to provide economical advantages and recover these proteins for high value products to minimize the impact on wild stocks and also satisfy the large demand for seafood based protein.

pH-Shift Processing

A recently developed technology in fish and meat processing allows for the potential use of discarded raw materials for human consumption. The primary drawback to using these materials is the high level of pro-oxidants in particular heme proteins, collagen, phospholipids and triacylglycerols all of which contribute to reduced consumer acceptability (Kristinsson and Hultin 2003b). Removal of these components resulting in a relatively pure protein product made possible by acid or alkali solubilization of muscle proteins with recovery at or near the average isoelectric point of a muscle homogenate (Hultin and Kelleher 1999; Hultin and others 2000; Kristinsson and others 2005b). Isoelectric recovery is done after unwanted components are removed by either filtration or centrifugation at low or high pH (Hultin and Kelleher 1999; Hultin and others 2000; Kristinsson and Hultin 2004b; Kristinsson and others 2005b). This “pH-

shift process" allows for the recovery of proteins with high functionality at a relatively low cost to the producer (Hultin and Kelleher 1999; Hultin and others 2000; Kristinsson 2002; Kristinsson and Ingadottir 2006; Kristinsson and others 2005b). pH-shift processing relies on electrostatic repulsion between muscle proteins to impart solubility (Kristinsson and Hultin 2003b). This is possible after the muscle has been thoroughly homogenized and diluted with water. Dilution allows for the dispersion of proteins and reduction of viscosity to a sufficient degree facilitating solubilization by allowing for the necessary molecular spacing to occur. HCl and NaOH are used to adjust the pH to extreme low or high pH, however the effect of other acids and bases are under investigation (Raghavan and Kristinsson 2007b). After the appropriate pH has been reached, i.e. the pH necessary to achieve solubility, the insoluble material is removed (Hultin and Kelleher 1999; Hultin and others 2000). If centrifugation is used, the insoluble material will produce a three phase separation; a top layer and bottom sediment, with the soluble proteins comprising the middle phase (Kristinsson and others 2005b). The top fat layer is comprised primarily of triacylglycerides and some emulsified proteins (Kristinsson and others 2005b). The sediment layer consists of phospholipids, collagen, bones, skin, scales and some poorly soluble proteins, including a small percentage of the actomyosin complex, actin and myosin among other proteins also found in the soluble fraction (Kristinsson and others 2005b). After separation, the middle soluble layer is collected and adjusted to the average pI (~5.3-5.5) of the muscle protein slurry (Hultin and Kelleher 1999; Hultin and others 2000). The proteins are recovered by dewatering using centrifugation, filtration or screening (Hultin and Kelleher 1999; Hultin and others 2000). This gives a partly dewatered protein isolate of 70-80% moisture. The proteins retained, for the most part, are the myofibrillar proteins, although some of the sarcoplasmic proteins are retained as well, increasing the recovery over conventional washing processes to

recover muscle proteins (e.g. surimi processing) (Hultin and Kelleher 1999; Hultin and others 2000). This is particularly the case for the acid-aided process where significant amounts of heme proteins (denatured and oxidized) are retained whereas in the alkali-aided process very low levels of heme proteins are retained and they remain in their native and reduced state (Kristinsson and Hultin 2004a). The sarcoplasmic proteins include not only the heme proteins but also endogenous enzymes and other small proteins which can have negative effects on the quality of the final protein product (Kristinsson and others 2005a). The removal of the sarcoplasmic proteins, especially the heme proteins increases the whiteness of the proteins isolate, leads to less oxidation and improves gel strength (Kristinsson and others 2005a).

Research to date has shown that during pH-shift processing, protein structure is disrupted and may be partially responsible for the effects on the functional properties of the pH-shift process isolate as compared to conventional methods (e.g. surimi processing) (Choi and Park 2002; Kristinsson and Hultin 2003b; Shann-Tzong and others 1998; Yongsawatdigul and Park 2004). Conformational changes occurring during the pH-shift isolation process are linked to differences observed between acid and alkali solubilization on the physical nature of the isolate (Kristinsson and Hultin 2003a). When utilizing the acid solubilization process higher puncture force and lower deformation values are observed than with conventional surimi. However isolate gels from the alkali process show both higher puncture and deformation values than isolate gels from the acid process and gels from conventional surimi processing (Choi and Park 2002; Perez-Mateos and others 2004; Undeland and others 2002). Kristinsson and Demir demonstrated that among four species, catfish, mackerel, mullet and croaker, alkali solubilization produced higher gel storage modulus (i.e. gel rigidity/firmness) after cooling than either surimi or acid processing (Kristinsson and Demir 2003). In all three species except croaker, acid

processing produced a much lower storage modulus than the other processes (Kristinsson and Demir 2003).

The acid and alkali-aided technology is expected to provide the seafood industry a new powerful tool in the production of high quality muscle protein isolates from inexpensive sources (Kristinsson and others 2005b). The effect isolation conditions have on muscle proteins is still under investigation and is relatively unknown. There are changes noted in the function, structure and conformation of single proteins (e.g. myosin) subjected to the process as well as whole protein isolates from various species compared to proteins subjected to conventional processes (e.g. surimi processing) or no processing (Hultin and Kelleher 2000; Kristinsson and Demir 2003; Kristinsson and Hultin 2003a; Undeland and others 2002). The pH-shift process has been successful on many aquatic species as well as turkey and beef (Kristinsson and Hultin 2003b; Kristinsson and Ingadottir 2006; Kristinsson and others 2005b; Liang and Hultin 2003; Mireles Dewitt and others 2002; Undeland and others 2002; Yongsawatdigul and Park 2004). The proteins extracted and concentrated during pH-shift processing, when compared to similar processes such as surimi, show altered physical properties (Kristinsson and others 2005b). Further understanding on changes that occur to muscle proteins during the pH-shift processing and the mechanism responsible may lead to tailor-made protein isolates with the functionality desired for specific products, additives or utilizations.

Protein Gelation

The primary functionality of interest for surimi and protein isolates is gelation. Gelation is the interaction of molecules, primarily polymers giving rise to a three dimensional network entrapping solvent within the network (Stone and Stanley 1992). Water is typically the solvent in food polymers (Stone and Stanley 1992). Protein gelation can and does occur via different mechanisms, leading to differences in texture and quality (Stone and Stanley 1992). During

protein gelation, especially fish protein gelation, there are three phases of the protein paste/gel formation. Phase one involves the uncooked raw product or the sol which has little to no detectable tensile strength. The next phase is variable depending on the treatment. "Suwari" is the process when the sol is set at medium to high temperatures and this is also the point at which protein cross-linking enzymes (e.g. transglutaminase) may be added to increase the setting effect at the elevated temperature. "Suwari" setting temperatures are highly species dependent, but usually do not exceed 50°C. Then the final phase of fish protein gelation is kamaboko the final cooked and cooled gel product (Stone and Stanley 1992).

The three main types of protein gels are reversible gels, chemically/enzymatically set gels and thermally set gels (Lanier 2000). The latter two are irreversible (Lanier 2000). Reversible gels are unique since they are initiated by heating during which the solution becomes highly fluid and upon cooling the gel is formed (Lanier 2000). Collagen forms these type of gels due to its unique structure imparted by the high percentage of proline and hydroxyproline (Kołodziejska and others 2004). Collagen structure is comprised of a super structure forming a triple helix with three collagen strands interacting to make large long strands (Ockerman and Hansen 2000). The collagen complex loses most of its structure during the heating process as its non-covalent interactions are broken leading to an unfolded scrambled chain, then upon cooling the regions rich in proline and hydroxyproline reform hydrophobic patches and associate, next hydrogen bonds form and, thus a, cold set gel is formed (Oakenfull and others 1997). This gel is reversible, and melts on heating, due to hydrophobic interactions and hydrogen bonding providing the basis for the three dimensional structure (Oakenfull and others 1997).

Enzymatically and chemically set gels are formed with either enzymes or chelating agents respectively. The enzymes and chelating agents will not, for the most part, independently form

a gel but are used in setting or curing prior to heating the protein paste or solution to complete gel formation. Transglutaminase (TGase) is one of the most common enzymes used to increase the strength of protein gels (Walsh and others 2003). TGase is a calcium dependent enzyme which mediates an acyl transfer between glutamine and lysine bound in a peptide/protein forming an ε-(γ-glutamyl) lysine cross-link. The covalent cross-linking of these amino acids gives increased strength the network (Lee and others 1997; Walsh and others 2003).

The final and most common method of protein gel formation is thermally setting the gel. Thermally setting a gel is done primarily by heating a protein solution. The heating process unfolds the proteins which allows for interaction of hydrophobic groups and disulfide interchanges. Upon cooling the protein solution, the hydrophobic interactions are further set and reformation of hydrogen bonds in a different order from the native system leading to the three dimensional network of the newly created the gel structure (Oakenfull and others 1997).

Many different factors affect protein-protein interaction during the gel forming process. Muscle proteins specifically include ionic strength, ionic type, pH, protein hydrophobicity, reactive groups on proteins (e.g. SH groups), protein source and type, post-mortem age, chemical additives and thermal treatment regiment (Oakenfull and others 1997).

The need to solubilize muscle proteins with salt is a believed prerequisite of well dispersed gel network formation (Feng and Hultin 2001). Solubility was attributed to the increased binding and interaction of protein systems with water leading to increased protein-protein interaction, water binding and reduced cook loss (Feng and Hultin 2001). High ionic strength is not necessary and low ionic strength, depending on pH leads to favorable conditions improving gel formation (Chang and others 2001). If the pH of a low ionic strength muscle protein system is slightly alkaline (e.g. pH 7.2-7.6), enough electrostatic repulsion will form between the

proteins to produce a well dispersed, ordered protein network (Chang and others 2001) This network will form a strong gel on heating and cooling, capable of binding high levels of water due to the gel pressure formed because of strong repulsive forces between proteins making up the gel network (Kristinsson and Hultin 2003c).

The pH of a sol paste is very important to form a good gel (Feng and Hultin 2001). Characteristics of a good gel include incorporating water into the gelled network and retaining the water after setting (Kristinsson and Hultin 2003c). The effect of pH on proteins is very specific due to the ionic nature of proteins (Feng and Hultin 2001). The interaction of proteins with both acid and base can result in different structural modifications and intermolecular interactions greatly affecting the taste, texture, tensile strength and water-holding capacity of raw and cooked gels (Kristinsson and Hultin 2003a, 2003b, 2003c). A change in pH leads to a change in surface amino acid ionization of the protein (Feng and Hultin 2001). A good muscle protein paste and gel has a proper balance between electrostatic repulsion and attraction (Feng and Hultin 2001). If the pH of the system is reduced close to the isoelectric point (pI) of the proteins, they will aggregate in the raw system and associate so closely that they have no water holding capacity and will form a brittle, dry gel (Lanier and Lee 1992). As mentioned above, at a higher pH there is enough electrostatic repulsion that the proteins will still associate via other means and produce open areas which are filled by water, giving rise to the texture and mouth feel desired in a good muscle protein gel (Feng and Hultin 2001). If the pH is changed too far above or below the pI, the total surface charge increases repulsion between proteins and results in the behavior of muscle proteins to the point that the interactions necessary to form a gel are not possible even on cooking (Kristinsson 2003; Vega-Warner and Smith 2001).

Proteins have hydrophobic amino acid residues, normally associated in groups to produce hydrophobic patches, which gives rise to a certain hydrophobicity of a protein molecule which may vary depending on solution conditions (Lanier 2000). Hydrophobic residues play a very important role in the development of all levels of protein structure. Interactions of hydrophobic patches (and their exposure) during heating (and cooling) are one of the main driving forces of protein gelation as they provide much of the initial interaction and association of proteins (Lanier 2000). Hydrophobic interactions are attributed to the increase in gel strength during heating (Lanier 2000). Hydrophobicity and hydrophobic interaction of proteins are mediated by a) the structure of the protein and b) by the external factors of the matrix and physical treatment (Lanier 2000). These factors include temperature, pH, salts, lipid content, lipid oxidation products, free fatty acids and many others (Lanier 2000).

Protein source has a great influence on the gelation characteristics of the system by dictating the types of proteins which are present to participate in gelation (Lanier 2000). Protein source is not limited to species differences, be it between fish species or between land animal and fish species (Lan and others 1995a; Lanier 2000; Luo and others 2001b). Protein source also pertains to the location within the animal, the difference between dark and light muscle or between muscle types (Vega-Warner and Smith 2001). The presence of specific proteins and concentrations of specific proteins can vary greatly between muscle types (Nowsad and others 2000). Differences observed between proteins, isolated under the same conditions, have been seen from various physical and chemical analyses done on the proteins, including gel strength, solubility, temperature stability, hydrophobicity, and other chemical indicators (Oakenfull and others 1997).

The postmortem age of the raw material from which proteins are recovered from is highly important (Lanier 2000). Some animal species have a shelf life of 2-3 weeks, e.g. beef and pork, and thus will still form an acceptable gel based product after that time, whereas some fish species have a very short shelf life and their proteins can be negatively affected very quickly (Lan and others 1995b). This is because of endogenous enzymes and the rigor state of the muscle (Lanier 2000). Processing fish muscle pre- vs. post-rigor can have an effect on the recovery and state of the isolated proteins (Lanier 2000).

Additives such as sugars, polyols and polysaccharides are commonly added to minimize muscle protein denaturation and retain functionality during freezing and thawing. These additives allow for long term frozen storage and make large scale processing of muscle protein ingredients, such as surimi possible. The addition of these compounds affects the gelation characteristics of the muscle proteins (Lanier and Lee 1992).

Thermal input has a profound affect on the gelation and gel strength of muscle proteins (Riemann and others 2004a). The effect of thermal input in a gelation system can be equated to the D value during canning, i.e. there is a relationship between time and temperature and the gel quality (Riemann and others 2004a). During heat induced gelation, it has been demonstrated that the longer the time taken to reach the end point temperature the greater the gel strength (Riemann and others 2004a). This is assumed to be due to the increased energy added to the system during slow heating in contrast to rapid heating. Studies where gels have been heated rapidly but held at the end point temperatures to give equivalent energy input as the slow heating rate have also shown to give similar increases in gel strength as slow heating, supporting the energy input theory (Riemann and others 2004a).

Rheology

Gelation and gel formation can be studied and described using rheology (Steefe 1992). Rheology is the study of the flow properties of a material (Owusu-Apenten 2005). Rheological tests apply a rotational or tangential force to a material and the response of the material to this force and its mode of application provide information which relates to its characteristics of flow (Owusu-Apenten 2005). The three main parameters which are controlled in the determination of flow are force of application, distance of head movement or degree of oscillation and the frequency of application (Steefe 1992). Macro-rheology is the study of tensile properties prior, during and after breaking and/or reforming of the material's three dimensional structure (Steefe 1992). Micro-rheology studies the same tensile properties as macro-rheology, however the breakage/resolution of the three dimensional structure may not be involved (Steefe 1992). There is a linear viscoelastic range of most if not all gels (Owusu-Apenten 2005). This region is the area where there is deformation of the three dimensional network, however the bond interactions are not broken (Owusu-Apenten 2005).

Macro-rheology is utilized as either an end-point determination or stop-point determination of gel properties (Owusu-Apenten 2005). As macro-rheology is destructive to the material, except in reformation or continuous flow studies, it renders the material unusable for future tests (Owusu-Apenten 2005). The other method of macro-rheology, which is continuous testing, is flow properties which may or may not render the sample unusable for future studies (Owusu-Apenten 2005). Common rheology parameters followed during testing are break point or shear strength, initial viscosity, which is the force required to begin flow, and continuous viscosity which his the force required to maintain flow (Steefe 1992). Two characteristics associated with continuous flow tests are shear thinning, where viscosity is reduced, and shear thickening, where viscosity is increased (Owusu-Apenten 2005). These tests provide very valuable information

and are the primary methods of quality control (QC) for many food products, especially in starches (Ta 2001). Among products evaluated using this method are pork, surimi, food starches and many others (Steefe 1992).

Micro-rheology determines different aspects of structure and change in structure than macro-rheology (Owusu-Apenten 2005). The changes monitored utilizing micro-rheology occur mostly during the formation of a gel (Steefe 1992). Micro-rheology is less characterized on a broad scale for QC utilization. Micro-rheology is very sensitive in its measurements and values; thus a greater degree of variation may be seen between samples and instruments (Ta 2001). Micro-rheology, when dealing with food polymers, remains the predominant method of choice in basic research of materials (Steefe 1992). Micro-rheology is, however, also used in QC for other polymer research pertaining to fluids, adhesives and rubbers (Ta 2001). The crossover for food polymer analysis on a basic level to QC requires not a greater understanding of micro-rheology but a standardization and data bank development of micro rheological data for specific materials and the correlation of those materials to currently held standard values for the other tests used in QC labs (Ta 2001).

The basis for all rheological testing is on the deformation of a material when under stress, ie., the stress strain relationship. The differences between micro-rheology and macro-rheology are the use of shear stress and shear strain or the force and distance a material can withstand at failure or when the structure breaks. Macro-rheology utilizes the shear stress and shear strain where as micro-rheology utilizes stress and strain parameters which may not shear the material. To do this in micro-rheology, oscillatory stress is used in place of continuous stress. The parameters which are then calculated in oscillatory stress testing are different that those observed in continuous stress tests. The total response of the material to oscillatory stress is made up of

the two components of the stress applied the in-phase stress, that is the stress applied moving away from the zero point and the out-of-phase stress which is the removal of the stress from the material or the probe moving back to the zero position. The in-phase stress is referred to as the storage modulus (G') and can be viewed as the resistance of the material to movement or the solid fraction of a material. The out-of-phase stress is referred to as the loss modulus (G") and is considered the liquid phase of the material. The G' represents the material as it is resistant to motion, this resistance to an applied force, when a small enough stress and strain are used, which does not break, shear, the three dimensional structure of the material is testing the intramolecular strength of the material to tangential compression. As the probe returns to the zero position the G" is testing for the compliance of the material to a non-fracture stress. This is the ability of the material, though neither the stress nor strain is large enough to induce flow or breakage of the structure, to move into space created by the positive stress applied by the probe and thus the resistance of the material to the negative stress. The effect of the sinusoidal movement of the head, G' and G", the resistance of the material to the movement of the head both forward and back alter sin wave during the motion of the probe. The alteration of the sin wave is represented at the tan delta. Tan delta ranged from 0-1 (G"/G'), or is the tangent of the degrees of change of the sin wave during the motion of the probe, 0 being a pure solid and 1 being a pure liquid or 0 difference being there is no modification of the sin wave G' and G" are equal and 1 being there is no G" pressure of the material or no space filling occurring. This relationship and the analysis of tan delta in rheological studies allows for the determination of the phase change of a material as it transitions from a more liquid like material to a more solid like material or vice versa. This transition period of a material from a more liquid like to a more solid like material is also determined by the change of G' and G" or the transition from greater out-of-phase resistance

(G") to greater in-phase resistance (G'). As the G' increases and the G" decreases or vice versa, if these two values cross during a treatment, this point is known as the gelation point, or where the material is equally solid and liquid. The transition of the material at this point is either going from a more solid to a more liquid material (melting) or a more liquid to a more solid material (gelling) (Oakenfull and others 1997; Owusu-Apenten 2005; Steefe 1992; Stone and Stanley 1992; Ta 2001).

The micro-rheology as discussed above provides valuable information about what is occurring during a treatment study. After the tests have been done to determine what is going on in a system it is then necessary to test how strong the gel is. This is done using shear stress and shear strain, causing the failure of the material and thus determining how strong it is after or at some point during a treatment. This is done in one of two ways 1) torsional stress or 2) tangential stress. That is either twisting or puncturing a material. The shear values of each of these types of testing, both shear stress and shear strain, are dependent on the type of force applied to a material. The torsional resistance of a material may not be the same as the tangential resistance of a material as the structure of some materials are very directional sensitive. In food systems the tangential and torsion resistance of a material has been shown to provide a good indication of the mouth feel and texture. Both of these tests are also used as quality determinants in muscle products, especially comminuted muscle products. The use of shear stress and shear strain have lead to the development of texture profile analysis of foods and is a primary component used to determine the grade and thus value of comminuted muscle products, especially surimi (Oakenfull and others 1997; Owusu-Apenten 2005; Park 2005; Steefe 1992; Stone and Stanley 1992; Ta 2001).

Key Muscle Proteins of Interest for the pH-shift Process

Myosin is the primary muscle protein comprising ~50% of the myofibrillar fraction of muscle (Kristinsson and Hultin 2003a). Myosin is composed of a long helical rod, where most of its secondary structure comes from, and a large bulky head group where most of its tertiary structure is found. This structure of myosin allows it to be a good gel former (Kristinsson and Hultin 2003a). The head is globular in nature with hydrophobic sites on the exterior of the protein designed to interact with actin during muscle contraction (Ruppel and Spudich 1996). These hydrophobic sites are blocked by meromyosins and troponin, primarily (Berne and others 2004). In a living system the release of calcium moves troponin and in the presence of ATP the light chains are moved by a conformational shift in myosin, which looks like the myosin head sitting up and making the exposed hydrophobic site on the S-1 fraction of the myosin head available to actin (Berne and others 2004). The structural and functional design of the proteins as they are in the living system still holds the predominant role in the mechanism of gelation. In a non-living system when muscle is processed and cooked to form a gel, the sequence of events is quite different.

The role of myosin in muscle gel formation is a cascade of events, the sequence of which is vaguely proposed to be myosin-myosin head interaction at hydrophobic sites causing close association between myosin molecules at lower temperatures as heat denaturation is taking place (Hettiarachchy 1994). The heat denaturation moves and or removes both tropomyosin and meromyosin from the hydrophobic patches on the myosin head allowing for this interaction (Hettiarachchy 1994). After this association, disulfide interchanges, mediated by increasing temperatures, are proposed to take place to some extent in the lower S-2 fragment of the myosin head (Stone and Stanley 1992). These disulfide interchanges hold the myosin-myosin linkage, created by the hydrophobic attraction, increasing the overall tensile strength of the system (Stone

and Stanley 1992). During the heating process, the myosin tail is partially uncoiled exposing the buried hydrophobic patches in the tail portion as the myosin tail has on its surface predominantly hydrophilic residues (Stone and Stanley 1992). The heat denaturation of the myosin tail causes a scrambling effect in the myosin system allowing for the now exposed hydrophobic patches of the myosin tail to interact and form new associations with other uncoiled myosin tails (Stone and Stanley 1992). This rearrangement of the myosin tail along with the polymerization of the myosin head is the strengthening effect of myosin gelation (Stone and Stanley 1992). This is because of the increased disorder in the gel system which is a consequence of the disruption of the natural organized tight packing of myosin, and other proteins, in the myofibrillar unit (Hettiarachchy 1994).

Protein solubility based on pH and ionic strength is attributed to the effect of ionization of the surface of the protein. Myosin has more than half of its amino acids as hydrophilic residues and 80% of these are exposed to the solvent (Lanier and Lee 1992). The increasing concentration of either positive or negative ions, depending on the pH and/or ionic strength of the solvent, water in this case, proceed to interact on the surface of the protein, forming a charged shell around the protein (Lanier 2000). This shell shields the ionic bridges between the proteins, allowing for separation of the proteins and eventually solubilizing proteins. The ion shell around the protein also increases the association of water around the protein surface, resulting in soluble proteins (Lanier 2000). Once the salt bridges are weakened/broken by ion shielding, increased water association is possible well beyond the hydration level of the ion alone. This increased amount of water associated with the protein increases its total level of hydration and, thus, that level of hydration is perceived as solubility of the protein (Stefansson and Hultin 1994). This mechanism of solubility is more evident in the myofibrillar fraction of

muscle than in the sarcoplasmic fraction, as sarcoplasmic proteins are fairly soluble under most conditions (Kristinsson and others 2005b). It is this aspect of sarcoplasmic proteins, mainly hemoglobin and myoglobin, that allows for their separation (remaining soluble in the aqueous phase) from the protein isolate during isoelectric precipitation where primarily myofibrillar proteins aggregate (Hultin and Kelleher 1999; Hultin and others 2000). This removes off color associated with pelagic surimi and increases the stability of the protein isolate as both myoglobin and hemoglobin are active in lipid and protein oxidation (Hultin and Kelleher 2000).

Objectives

The overall objective of this project was to investigate how different acid (pH 2.0, 2.5 and 3.0) and alkali (pH 10.5, 11.0, 11.5) treatments followed by isoelectric precipitation influenced the structure, conformation and functionality of catfish muscle proteins. The ultimate goal of this work was to attempt to build an understanding how specific pH unfolding and refolding regimes affect the proteins and connect structural/conformational changes to functional changes. We hypothesized that different pH treatments lead to differences in structure and conformation which are responsible for changes in functionality (gelation). Basic work in this area is expected to further our knowledge of muscle protein gelation and provide insight for production of muscle proteins with specialized functional characteristics.

The specific objectives of this project were:

1) Investigate the effect of different acid- and alkali-aided unfolding followed by isoelectric precipitation on the gel forming properties protein isolates during thermal treatment.
2) Investigate the effect of different acid- and alkali-aided unfolding followed by isoelectric precipitation on the strength, elasticity and quality of thermally set protein isolate gels.

3) Investigate the effect of different acid- and alkali-aided unfolding followed by isoelectric precipitation on the chemical properties of protein isolates (surface hydrophobicity, ATPase activity, sulfhydryl groups and protein solubility).
4) Investigate the effect of different acid- and alkali-aided unfolding followed by isoelectric precipitation on the conformation, structure and structural stability of protein isolates.
5) Investigate the effect of heating rate on the gel formation, strength, elasticity and quality of protein isolates made from acid- and alkali-aided unfolding followed by isoelectric precipitation.

Experimental Design

The study of pH-shift processing in catfish muscle was conducted on catfish fillets. The use of catfish fillets as the raw material was chosen to provide a stable, high quality raw material. Catfish was chosen as the raw material due to its importance in the southeastern United States and increasing consumption within this region and through out the entire United States. The experiments used to study pH-shift processing, outlined in figures 1-1 through 1-4 and described in the next chapter, were conducted due to their common use in meat and meat products. Each method used to investigate the properties of pH-shift processed catfish muscle was replicated on independent batches of catfish fillets. This was determined acceptable by power analysis (α=0.05, β=0.80). The chemical analyzes used were specifically chosen for their previously established correlation to the physical/textural properties of muscle products. The determination of the physical properties of pH-shift processed catfish muscle was the initial focus, as previous research had indicated that pH-shift processing will modify physical properties. The reason for the modification of the physical properties (figure 1-2) would be further understood through the investigation of the chemical properties (figure 1-3) and structural properties (1-4) of muscle proteins. The combination of these three properties will provide a deeper look into how different the physical/textural properties in meat are formed.

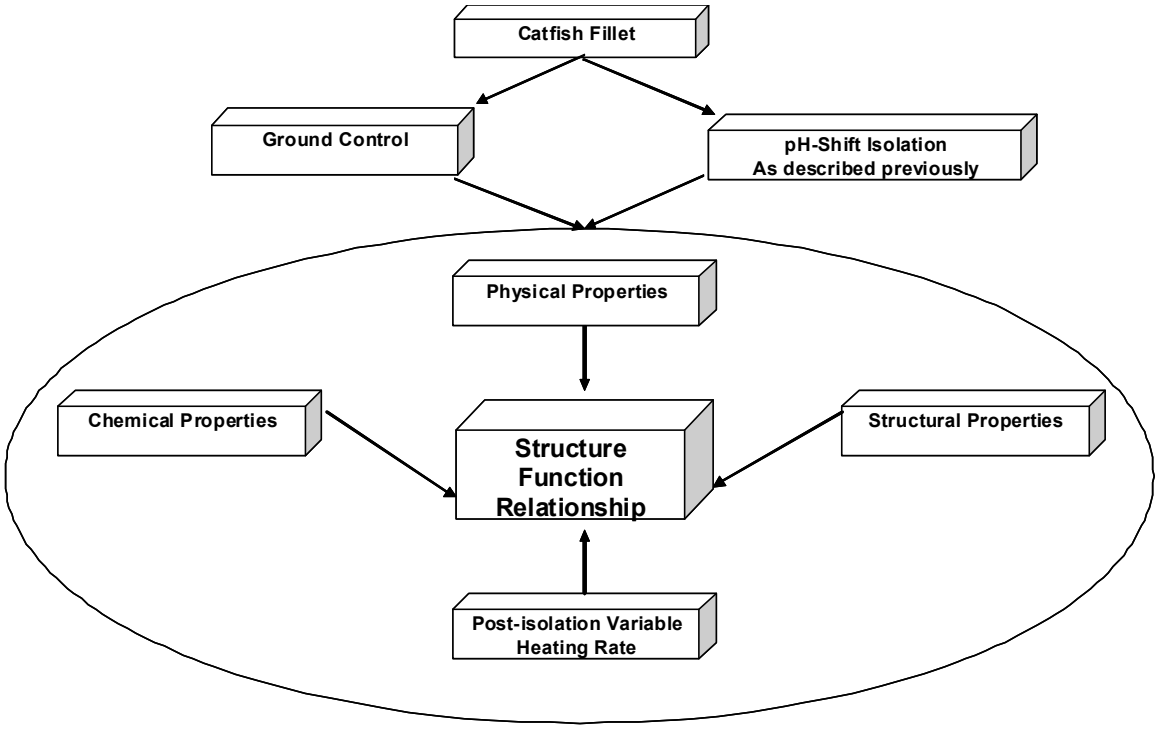

Figure 1-1: Figure summarizing the overall experimental design of the structure/function relationship of channel catfish (*Ictalurus punctatus*) muscle proteins subjected to pH-shift processing.

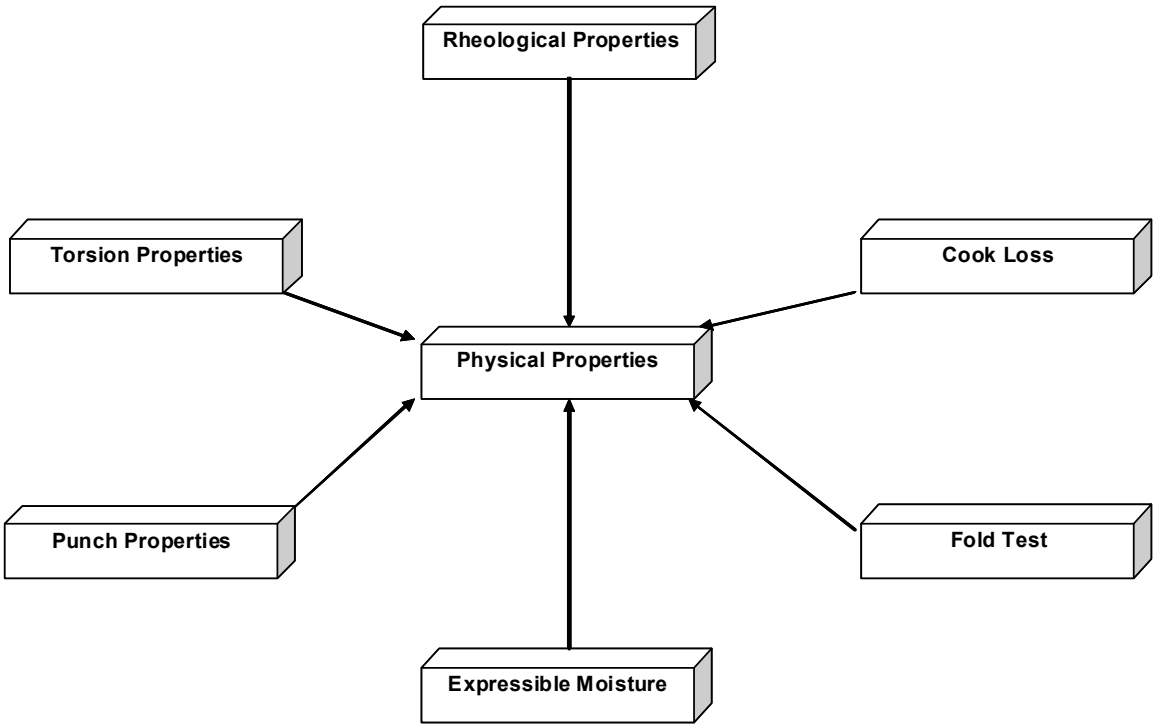

Figure 1-2: Experimental design of the investigations into the physical properties of channel catfish (Ictalurus punctatus) muscle proteins subjected to pH-shift processing.

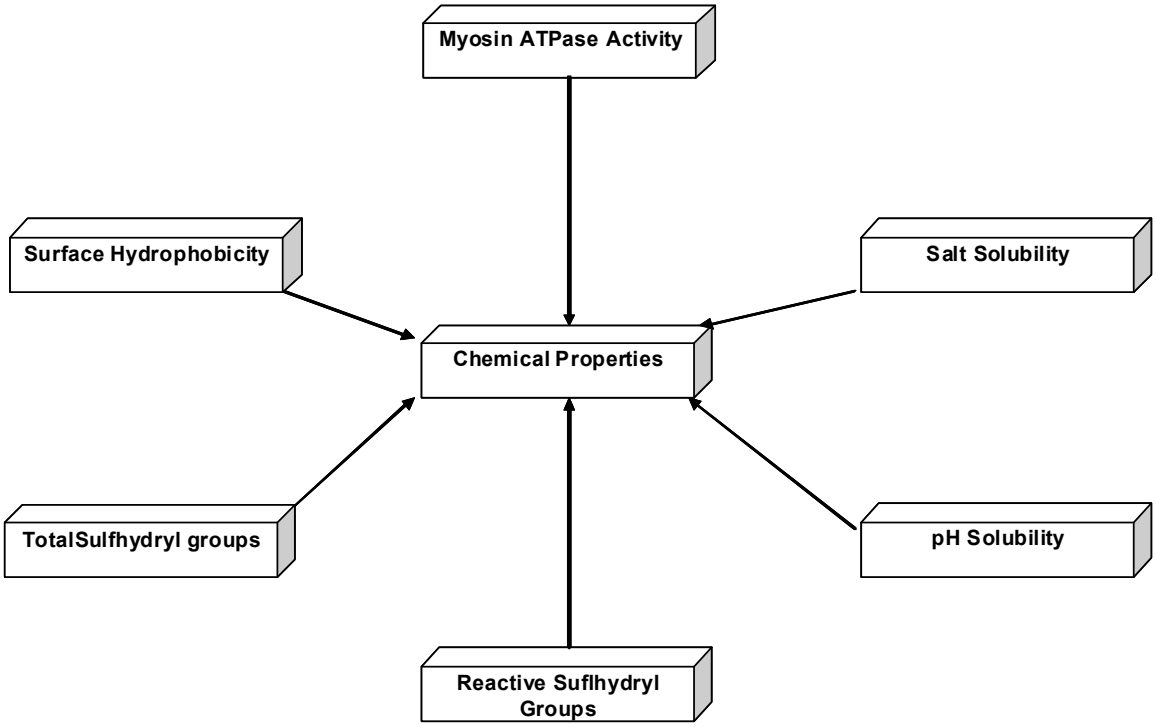

Figure 1-3: Experimental design of the investigation into the chemical properties of channel catfish (*Ictalurus punctatus*) muscle proteins subjected to pH-shift processing.

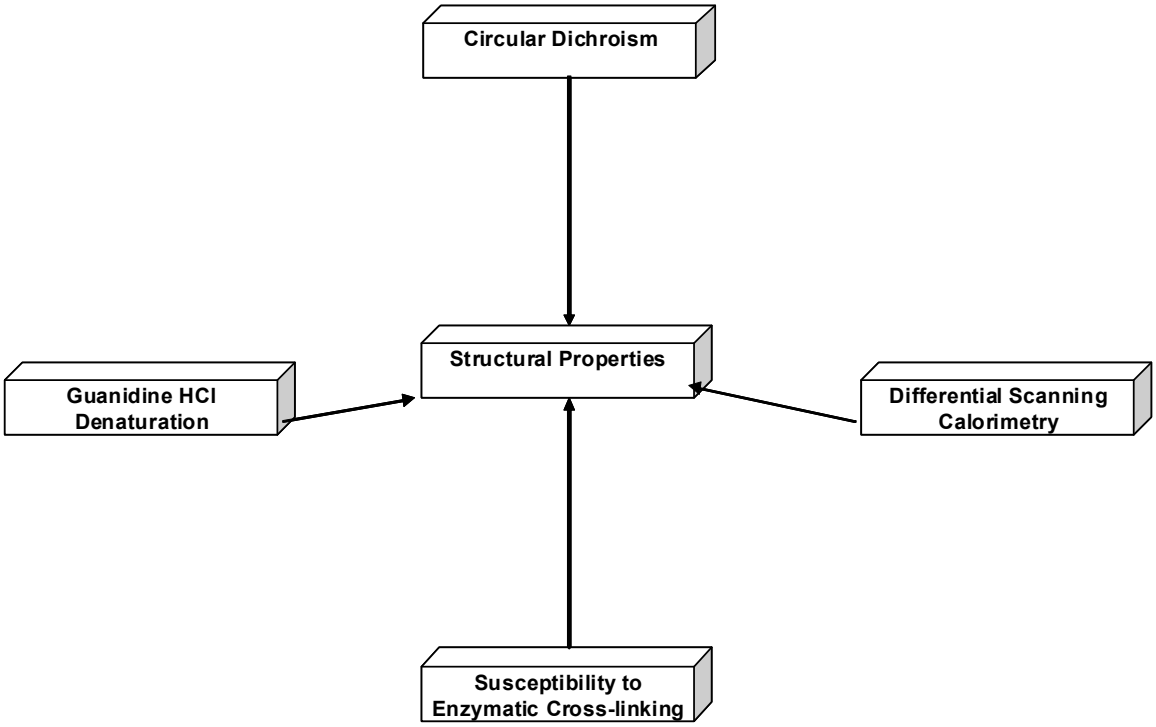

Figure 1-4: Experimental design of the investigation into the structural properties of channel catfish (*Ictalurus punctatus*) muscle proteins subjected to pH-shift processing.

CHAPTER 2
METHODS

Raw Material

The raw material used in these studies was fresh catfish fillets obtained 1-3 days post harvest from a local supplier. Catfish fillets were only purchased which were determined to be within 3 days of packaging. The catfish fillets were purchased and immediately transported on ice to the laboratory and processed the same day.

Preparation of Protein Isolates

Protein isolates were prepared according to figure 2-1. Fresh fillets were initially ground in an Oster heavy duty food grinder (Niles, Ill., U.S.A.) for the preliminary disruption and collection of the muscle tissue. Following grinding, the comminuted meat was diluted 1:2 (w/v) with deionized (DI) water and homogenized in a Waring blender for two bursts of 30 seconds. Following homogenization, the resulting muscle tissue slurry was further diluted to give a final dilution ratio of 1:6 (w/v) muscle to DI water. This slurry was manually stirred with a plastic spatula to achieve good homogeneity. The pH of the slurry was adjusted according to the methods described below, using either 2N NaOH or 2N HCl as needed for the pH desired, with continuous manual mixing. Upon reaching the desired pH, insoluble material was removed by centrifugation (Sorvall RC-5B centrifuge with a GS-3 rotor, Kendro Laboratory Products, Newtown, Conn., U.S.A.) at 10,000 x g for 20 minutes at 5^{o}C. Following centrifugation, the soluble middle layer was collected through a kitchen strainer with a mesh size of approximatly 0.25 mm to minimize contamination with other separated materials. The soluble material was readjusted to pH 5.5 as described above. After readjustment the solution was centrifuged to remove excess water and remaining soluble proteins at 10,000 x g for 20 minutes. The

precipitated protein was collected by decanting the supernatant containing the unprecipitated proteins and removing it with a steel spatula. All of the precipitate from each solubilization pH was combined from the centrifuge bottles into one protein isolate. This dewatered protein isolate was further dewatered by placing the combined precipitate into cheesecloth and hand squeezing until the moisture content was below 80%. Moisture content was determined using a Cenco infrared moisture analyzer (CSC Scientific, Fairfax, Va., U.S.A.). Upon completion of manual dewatering the protein isolation was complete. Preliminary unpublished investigation of protein isolates in this laboratory found the shelf life of catfish protein isolates to be 5-7 days on ice. All protein isolates were stored on ice at the precipitation pH and used within 5 days.

Protein Concentration

Protein concentration in the isolates and the subsequent solutions was determined using the Biuret method, as described by Torten and Whitaker (1964), with of 10% w/v deoxycholic acid in water added at 10% v/v of the protein-Biuret reagent to minimize turbidity from any remaining lipids in the samples. Protein concentration was measured based on a standard curve based on BSA.

Protein Surface Hydrophobicity

Protein surface hydrophobicity was conducted according to Liang and Kristinsson (2005). To measure surface hydrophobicity the isolate was diluted to give a stock solution of 10 mg/ml in a 20 mM tris-HCl buffer, 0.6 M NaCl, pH 7.2. The stock solution was serially diluted to obtain a concentration curve. Increasing volume (100 µl, 200 µl, 300 µl, 400 µl and 500 µl) of the stock solution were added to Tris-HCl buffer to a final volume of 4.0 ml. Then, 10 µl of 6-propionyl-2-(dimethylamino) naphthalene (PRODAN) (11.35 µg/ml in methanol) was added and the samples mixed for ~15 seconds. After mixing the samples were then incubated for 15 min in the dark. All sample preparations and incubations were performed on ice in disposable test tubes.

After incubation, the sample was transferred to a fluorescence cuvette and the fluorescence emission intensity scanned between 380-560 nm with excitation at 365 nm in a Perkin Elmer LS 45 Luminescence Spectrophotometer (Norwalk, CT). As the isolate is a collection of proteins, the fluorescence peak of the samples is a relatively flat and broad peak between 430-460 nm. The maximal fluorescence of the protein isolate was taken and used as the wavelength for analysis. The surface hydrophobicity was calculated as the slope of the net fluorescence versus protein concentration (mg/ml) of the samples.

Circular Dichroism (CD)

CD was done according to Kristinsson and Hultin (2003a). The isolate was diluted and homogenized for 1 minute on ice at speed 2 in a Bio-homogenizer (M133/1281-0, Bio Spec Products Inc., Bartlesville, OK) in 20 mM Tris-HCl buffer, pH 7.2 with 600 mM NaCl to a concentration of 10 mg/ml. This protein stock solution of 10 mg/ml was prepared and diluted to 2 mg/ml 1 hr before analysis and held on ice. For analysis of secondary structure the sample was scanned from 260-200 nm in a 0.1 cm quartz cuvette, 0.2 nm resolution scanned at 50 nm/min on a Jasco J-500C circular dichroism spectropolarimeter (Jasco Inc, Easton, MD) at room temperature. The time duration of the scan was no more than 10 minutes. Differences in total alpha helix beta structure and random coil structures present were determined from DICHROWEB using the K2D analysis program (Lobley et. al., 2002).

Isolate Susceptibility to Unfolding in Guanidine Hydrochloride (Gu-HCl)

Catfish protein isolate and the control, untreated ground catfish muscle, were treated with Gu-HCl over the range of 0-6M in 0.5 M increments. For assessment of denaturation, 222 nm was used as an indicator wavelength of alpha helical content, scanning from 220-225 nm in a 0.10 cm quartz cuvette, 0.2 nm resolution scanned at 50nm/min on a Jasco J-500C circular dichroism spectropolarimeter (Jasco Inc, Easton, MD) at room temperature. The protein

concentration was 1 mg/ml. Samples were allowed to incubate for 5 minutes on ice prior to reading.

Differential Scanning Calorimetry (DSC)

DSC was conducted according to Fukushima and others (2003) on a MicroCal DSC (MicroCal, LLC, North Hampton, MA). Isolate were diluted in sample buffer (20 mM tris-HCl, pH 7.2 with 600 mM NaCl) to a protein concentration of 10 mg/ml and homogenized for 1 minute on ice at speed 2 in a Bio-homogenizer (M133/1281-0, Bio Spec Products Inc., Bartlesville, OK). The sample was degassed under vacuum for 5 min at 5^{o}C. After degassing, 0.6 ml was loaded into the sample cell, the reference cell contained sample buffer. The sample was then linearly heated at 1^{o}C/min from 5^{o}C-80^{o}C. Analysis of the data was conducted on the software provided by the manufacturer, Origin Pro 7.5, for determination of both exothermic and endothermic events.

Reactive Sulfhydryl Groups

Reactive sulfhydryl groups were determined according to Kim and others (2003). The isolate was diluted and homogenized for 1 minute on ice at speed 2 in a Bio-homogenizer (M133/1281-0, Bio Spec Products Inc., Bartlesville, OK) in 20 mM Tris-HCl buffer, pH 7.2 with 600 mM NaCl to 250 µg/ml. After dilution, 80 µl of 10 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) was added to the sample. This mixture was then incubated for 1 hr on ice. After incubation the sample was read at 420 nm using an Agilent 8453 diode array UV-visible spectroscopy system (Agilent Technologies Deutschland GmbH, Waldbronn, Germany). Micromolar determination of SH groups per gram in the sample were done using the following equation:

$$SH/g = [absorbance \times dilution\ factor \times 100]/[13600(mol/cm) \times sampleconcentration(mg/ml)]$$

Total Sulfhydryl Groups

Total sulfhydryl groups were determined according to Kim and others (2003) . The isolate was diluted and homogenized for 1 minute on ice at speed 2 in a Bio-homogenizer (M133/1281-0, Bio Spec Products Inc., Bartlesville, OK) in 20 mM Tris-HCl buffer, pH 7.2 with 600 mM NaCl to 250 µg/ml. After dilution, 0.5 ml of diluted isolate was mixed with 2.5 ml urea buffer. The urea buffer contained 8 M urea, 0.2 M Tris-HCl, 2% SDS, 10 mM EDTA and was adjusted to pH 8.5. After mixing with urea, 50 µl of 10 mM DTNB was added to the sample. The mixture was incubated in a water bath for 15 minutes at 40°C. After incubation the sample was read at 420 nm using an Agilent 8453 diode array UV-visible spectroscopy system (Agilent Technologies Deutschland GmbH, Waldbronn, Germany). Total sulfhydryl groups were calculated using the following equation.

$$SH/g = [absorbance \times dilution\ factor \times 100]/[13600(mol/cm) \times sampleconcentration(mg/ml)]$$

Myosin ATPase Activity Assay

Myosin ATPase activity was determined by the method of Perry (1955). The buffers to initiate the enzymatic hydrolysis of ATP by myosin were prepared prior to sample preparation. The reaction buffers used in this assay were 0.2 M glycine-NaOH buffer (pH 9.0), 0.1 M calcium chloride, 0.05 M ATP, sodium salt (pH 6.8) and 15% trichloroacetic acid (TCA). Liberation of inorganic phosphate was monitored in this reaction as the determinant of enzymatic function. For the estimation of inorganic phosphate the following buffers were used: 12% TCA, 10% (w/v) ammonium molybdate stock solution in 10N sulfuric acid. This ammonium molybdate stock was used in the preparation of the ferrous sulfate – ammonium molybdate reagent which was made fresh the day of analysis. For analysis of myosin ATPase activity the isolate was diluted to 1 mg/ml in Tris-HCl buffer containing 600 mM NaCl pH 7.2 and homogenized

thoroughly. This was kept on ice until needed. In separate tubes for each reaction, prior to isolate addition, the reaction buffer was prepared by adding 1.3 ml glycine-NaOH buffer, 0.2 ml calcium chloride, 0.3 ml ATP. This mixture was then incubated at 25^{o}C for 5 min to allow the temperature to equilibrate. After temperature equilibration, 0.2 ml of the individual isolate solution was added to each reaction tube with proper mixing and allowed to incubate at 25^{o}C for 5 min. After 5 min, 1 ml of 15% TCA was added to each reaction tube to stop the reaction. This was centrifuged for 10 minutes at 25,000 x g to precipitate the proteins from solution and leaving liberated phosphate from ATP in solution. Next 0.5 ml of supernatant was added to 3.2 ml of 12 % TCA with good mixing and left to stand at 25^{o}C for 10 minutes. This solution (3.0 ml) of this solution was removed and mixed with 2 ml of the ferrous sulfate – ammonium molybdate reagent and incubated at room temperature for 1 min. After 1 min of incubation the solution was transferred to a cuvette and read at 363 nm using an Agilent 8453 diode array UV-visible spectroscopy system (Agilent Technologies Deutschland GmbH, Waldbronn, Germany). The color of this reaction intensifies with time so only one reaction was done at a time to standardize the incubation time of all samples to 1 min. The results of myosin ATPase activity were assayed as relative results to the control. The control absorbance was set to 100% and the percent activity of the isolates was calculated from the absorbance of the samples relative to the control.

$$activity = pH\,treated\,sample / control\,sample$$

Susceptibility of Proteins to Transglutaminase-Induced Cross Linking

The susceptibility of pH-shift processed catfish muscle to protein cross linking was assayed using the commercial transglutaminase (TGase) Activia TI from Ajinomoto LLC (Ajinomoto Food Ingredients LLC Chicago Il). 0.2% TGase (w/w) was added to isolate diluted to 10% solids in 20mM Tris-HCl buffer, 600mM NaCl. TGase activity was assayed by using an

AR2000 advanced research rheometer (TA Instrument, New Castle, DE) with a head with a flat cross-hatched polyacrylic surface and thermally controlled plate. The gap was 1000 microns and the head lowered onto the sample using the controlled speed function provided by the software. The samples were tested in oscillatory mode under controlled frequency at 0.1 Hz, and strain controlled at 0.01. TGase activity was monitored by the increase in G' over 1 hour at 30^{o}C. Activity was compared against samples with no TGase added. G' was plotted against time and the slope of the line from linear regression was used to calculate the TGase activity. The activity coefficient was calculated as the ratio of treated to untreated samples.

Oscillatory Rheology

For the gelation tests, the isolates were rehydrated to 10% protein and pH adjusted to 7.2 with 20 mM Tris-HCl and HCl/NaOH. For the added NaCl samples, NaCl was added at 2% w/w. The sample was homogenized at speed 1 using an Ultra-Turrax T18 homogenizer. The protein paste was then transferred to a peltier plate at 5°C attached to an AR2000 advanced research rheometer (TA Instrument, New Castle, DE) and a head with a flat cross-hatched polyacrylic surface. The gap was 1000 microns and the head lowered onto the sample using the controlled speed function provided by the software. The isolates were subjected to heating (5-80°C) and cooling (80-5°C), with testing conducted in oscillation mode. The storage modulus (G'), loss modulus (G") and tan delta (δ) was followed as a function of heat and time. All experiments were done under a controlled strain of 0.01 and controlled frequency of 0.1 Hz.

Torsion Gel Testing

Protein paste was prepared as above, except at 20% protein concentration. Proteins were stuffed in cylindrical tubes 10 cm long by 1.8 cm in diameter and cooked at 80°C for 20 min in a water-bath. The interior wall of the tube was coated with a lecithin based spray. The cooked samples were then removed from the casing after cooling for 30 min on ice, held overnight on

ice, and subsequently cut into 2.54 cm long pieces. These pieces were glued to plastic disks (Gel Consultants Inc., Raleigh, N.C.) using an instant adhesive, Krazy glue (Toagosel Co., LTD. Nishi-shinnbashi Minato-Ku, Tokyo). Dumbbell shaped samples were then milled from each sample to 1 cm diameter in a machine mill (Electro Sales Co., Somerville, Mass., U.S.A.), wrapped in plastic wrap to prevent moisture loss, and brought to room temperature prior to torsion testing. For testing, 4 gel specimens were vertically mounted and sheared to the point of fracture at 2.5 rpm in a modified Brookfield viscometer (Gel Consultants Inc., Raleigh, N.C). Stress (kPa) and strain (dimensionless) at fracture were calculated with the manufacturer's software for each sample, corresponding to the strength and deformation of the gels, respectively (Hamann and others 1990).

Punch Test

Punch test samples were prepared by adjusting protein isolates to 20% solids according to the moisture content and stuffed into plastic tubing, 1.25 inches in diameter. These samples were cooked for 20 min at 80°C, and then cooled rapidly in ice water. Samples were air freighted the same day they were cooked to the laboratory of Dr. Herbert O. Hultin in Gloucester MA (University of Massachusetts Marine Station) where punch testing was conducted The samples were cut to 1.0 inch length and 1.25 inch diameter. The sample was placed on the machine table and the head adjusted to just above the sample surface. A 5 mm head was used at a speed of 1.0 cm/min with force and distance at failure reported. Punch testing was conducted on a Rheo Tex Model AP-83 (Sun Science Co., Seattle, Wa).

Fold Test

A gel fold test was conducted according to Nowsad and others (2000). The test was conducted by slicing 2-mm thick sample disc from the cylindrical gels, mentioned above, and folding them into halves and quarters. The scales are: 5= no crack when folded into a quarter,

4=no crack when folded into half but crack when folded into quarter, 3=crack when folded into half, 2=broke and split into halves when folded into half, 1=broke and split prior to being folded into half.

Gel Water Holding Capacity

Gel water holding capacity was conducted according to Nowsad and others (2000). Expressible moisture of the gels was determined by compressing a 1.0 g spherical gel slice between 4 double layers of filter paper at a pressure of 20 Kg/cm^2 for 1 min and calculated from weight before and after the compression. Expressible moisture was determined as the percentage of original weight after compression.

Cook Loss

Cook loss of gels was determined as the weight of the protein paste before heat treatment compared to the weight of the gels after cooking. Gels were prepared and heated as described above (torsion gel testing). After cooking the gel was removed from the tube, lightly dried and reweighed. The weight of the tube was subtracted from the total initial weight and then compared to the final weight of the cooked gel. Percent cook loss was determined by:

$$Cook\,Loss = \{1 - [Gel\ Weight/(Initial\ Weight - Tube\ Weigh)]\} \times 100$$

Cook loss is in percent, gel weight is the weight of the cooked protein paste after being removed from the tube, initial weight is the capped tube plus the raw gel stuffed inside, tube weight is the weight of the tube after the gel has been cooked and removed from the tube.

Heating Rate Gelation Studies

Heating rate gelation studies were conducted at North Carolina State University in the laboratory of Dr. Tyre Lanier, Raleigh, NC. Torsion tubes, 10 cm in length and 1.8 cm in diameter were filled with protein paste and subsequently heated from 10^{o}C to a 70^{o}C internal endpoint, either rapidly by a cylindrical microwave (Riemann and others 2004b), at 20 or

98°C/min to test rapid heating effects, slowly at 1°C/min by immersion in a programmable water bath or placed directly in an 70°C water bath for 15 minutes. Temperatures were measured by fiber optic probe (Riemann and others 2004b). Upon reaching 70°C, rapidly heated samples were held for 0 or 20 min prior to rapid cooling by immersion in ice water for sufficient internal cooling to <10°C for ~30 min. Samples were held static in a cylindrical microwave applicator (length 16 cm, radius 12.5 cm) and heated using power settings calculated previously. After reaching a final temperature of 70°C, this temperature was maintained in gels during the subsequent holding period by utilizing feedback software (Riemann and others 2004b).

Rheological changes (storage modulus, G') of pastes/gels were non-destructively and continuously measured as pastes were heated, held and cooled using a 40 mm, 4 degrees slope cone and plate attachment of a constant stress, small strain rheometer (Stresstech, Rheologica instruments AB, Lund, Sweden). Oscillation parameters were those used in Riemann and others (2004). Heating conditions were at either 20°C/min, the most rapid heating rate possible for this apparatus, 0.5°C/min, 1°C/min, 2°C/min or 5°C/min to an endpoint temperature of 70°C followed by holding for 0 or 20 min prior to cooling at 5°C/min to 10°C.

Protein Solubility as a Function of pH

The isolate was diluted to 10 mg/ml in DI water with thorough homogenization using a tissue homognizer (Ultra-Turrax T18, IKA Works Inc. Wilmington, NC). After dilution in water, 2 ml diluted protein was added to 2 ml buffer with good mixing. The pH range was from 1.5-12 in 0.5 increments. For pH 1.5-6.5, 2 mM citric acid buffer was used and for pH 7.0-12, 2 mM sodium phosphate buffer was used. The buffers were adjusted to the desired pH prior to protein addition. After protein addition, the mixture was allowed to incubate on ice for 10 minutes. After incubation the protein solution was centrifuged at 3,000 x g for 10 minutes. After centrifugation, the supernatant was sampled for protein concentration using the biuret

method (Torten and Whitaker 1964). Protein concentration was calculated from a standard curve based on bovine serum albumin (BSA). Percent solubility was then calculated as a percentage of an uncentrifuged control by the following equation:

$$[protein(mg/ml)\sup ernant]/[protein(mg/ml)total]$$

Protein Solubility as a Function of Salt Concentration

The isolate was diluted to 5 mg/ml in 20 mM Tris-HCl at pH 7.2 without added NaCl with thorough homogenization using an Ultra-Turrax T18. After dilution in buffer, 4 ml was taken and the appropriate amount of NaCl added to each tube to achieve 0 mM, 150 mM, 300 mM 450 mM or 600 mM concentrations. After the appropriate NaCl concentration was achieved, the samples were centrifuged at 3000 x g for 10 minutes at 5°C. After centrifugation, the supernant was collected in duplicate and soluble protein was determined according to the biuret method (Torten and Whitaker 1964). The soluble protein was compared to an uncentrifuged control. Percent soluble protein was calculated as

$$(protein(mg/ml)\sup ernant)/(protein(mg/ml)total)$$

Statistical Analysis

Experimental design, as shown in figures 1-1 – 1-4, was conducted, in duplicate, on replicate isolations as discussed above. A replicated (N=2) was determined as acceptable due to achieving an acceptable power (β=0.80, α=0.05).

One-way independent measures analyses of variance were used to examine the effects of all methods used except for the analysis of pH solubility and salt solubility which were tested with 2-way ANOVA analysis. The Kruskal-Wallis ANOVA testing by ranks was used on data which did not pass normality. Post hoc analysis was conducted only in the presence of significant population differences. ANOVA statistical comparisons were conducted with

SigmaStat, (Systat Software Inc. San Jose Ca) with a significance level of $p<0.05$. After SigmaStat completed the ANOVA analysis, the post hoc analysis recommended by SigmaStat was used. In most cases this was Tukey's test. Dunn's test was used for all punch test analysis; force, distance and jelly strength. The Holm-Sikak method was used with the torsion stress, expressible moisture and ATPase activity. The two-way ANOVA analysis used the Holm-Sidak pair wise comparisons. The two-way ANOVA tables and the post hoc tests are reported in appendix A. The heating rate study was analyzed with Graph Pad QuickCalcs online calculator software (Graph Pad Software Inc. San Diego Ca). Analysis used an unpaired t-test with a manual bonferoni correction, overall significance of $p<0.05$ with the individual significant p-values of $p<0.004$.

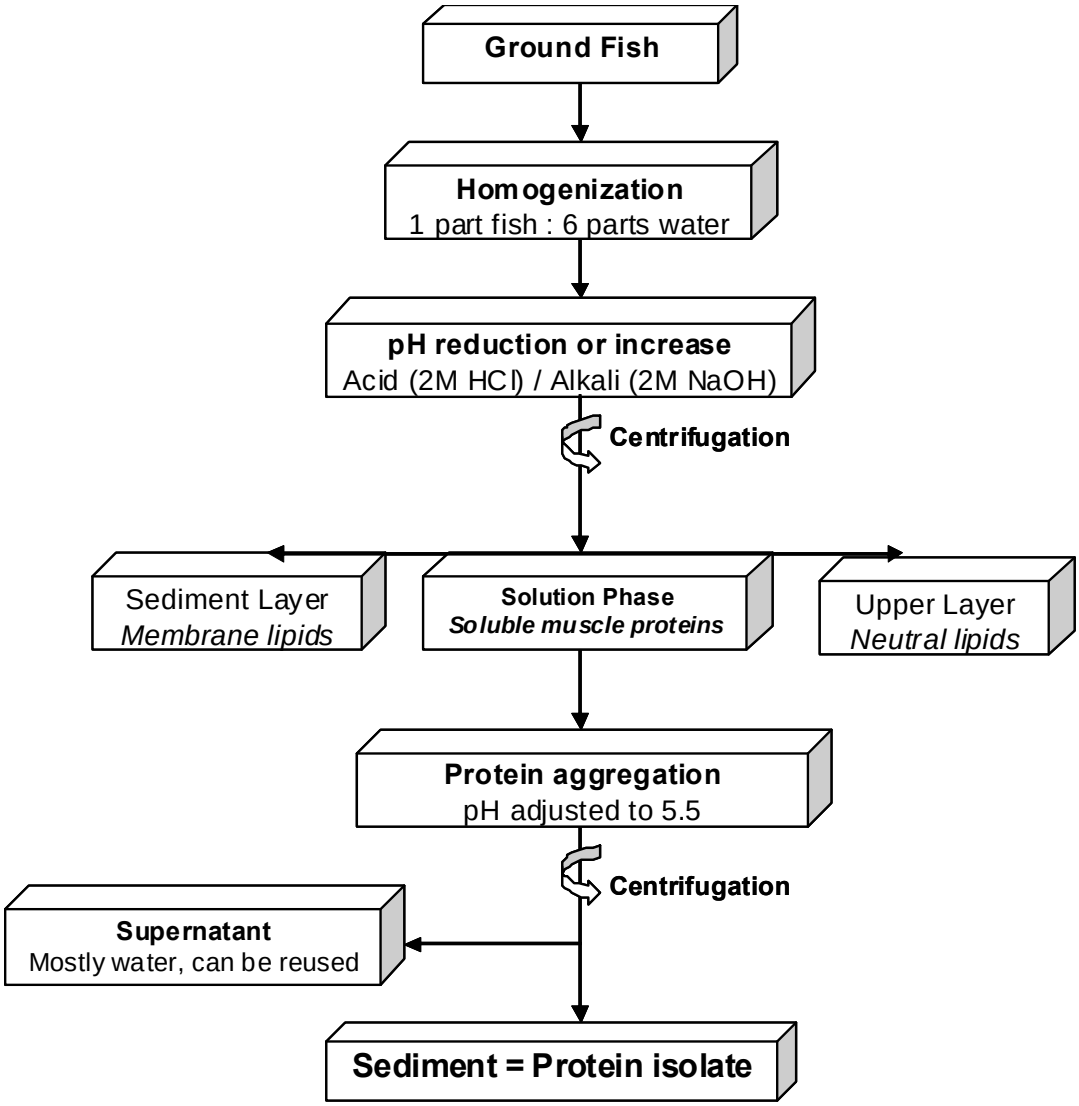

Figure 2-1: The process used in acid and alkali pH shift processing.

CHAPTER 3
CHANNEL CATFISH (*Ictalurus punctatus*) MUSCLE PROTEIN ISOLATE PERFORMANCE PROCESSED UNDER DIFFERING ACID-AIDED AND ALKALI-AIDED pH VALUES.

Introduction

The need for an increase in the production of seafood products has become a clear concern with international seafood shortages projected to be seen as early as 2015 (FAO Subcommittee on Aquaculture, Beijing China 2002). One of the methods which may alleviate some of the stress currently on the seafood marked is the utilization of byproducts. Byproducts are considered what is left over from the whole fish after the primary products have been removed. In catfish these are the fillet and the belly flaps ("nugget"). These byproducts provide an inexpensive raw material which increases the total utilization, profitability and management of the fisheries resources.

The production of catfish in the United States has been steadily increasing for the last 35 years (Harvey 2002). In the filleting process the utilization based on live weight is at best 45%, comprising fillets and nuggets. The byproducts are the frame and cutoffs, these portions of the fillet/nugget are not acceptable to the consumer. This is a substantial amount of usable material that, with the appropriate processing, may be used for the production of value-added protein isolates. A relatively new process developed by Hultin and coworkers (Hultin and Kelleher 1999) utilizes the solubilization properties of specific muscle tissues, primarily myofibrillar proteins, at extreme low or high pH. This property of myofibril proteins allows for the removal of membranes and neutral lipids, connective tissue, bones, scales and other non-solublized components.

The effect of pH-shift processing on muscle proteins has been studied by several workers. Currently it has been demonstrated that pH-shift processing can modify the physical properties of the resulting protein isolate. This has been demonstrated in species ranging from cold water

fish to land animals. The modification of physical properties of pH-shift produced isolates has resulted in a wide range of effects. Acid (pH 2.5) treatment of myofibrillar proteins showed a decrease in the storage modulus of cod muscle proteins where as alkali (pH 11.0) treatment has shown an increase in the storage modulus when compared to a surimi control (Kristinsson and Hultin 2003b). An increase in storage modulus refers to a stiffer gel and might indicate that a treatment may lead to stronger and better gels. In Atlantic croaker, however, both acid (pH 2.5) and alkali (pH 11.0) processing showed an increase in the storage modulus as compared to a surimi control (Kristinsson and Liang 2006). Yongsawatdigul and Park (2004) made rockfish protein isolates using both acid and alkali pH-shift processing and compared their gelation properties with washed muscle and whole muscle homogenates. Compared to muscle and washed muscle, isolates from alkali-aided processing had an increase in gel elasticity and force needed to penetrate the gel while isolates from acid-aided processing showed a decrease in both (Yongsawatdigul and Park 2004). It is unknown what the specific effects of solubilizing muscle proteins at different high or low pHs will have on the functional properties of recovered muscle proteins.

Muscle gelation is a multi step process with the final result being a three dimensional network. This network may be studied utilizing different methods which provide information on the various mechanisms involved in its formation and its properties. The modifications, treatments and additives of a formulated muscle product are a directly influence of the final gel network formed. The changes to the three dimensional gel structure by utilizing the acid or alkali solubilizing process have shown changes in gel strength which can be determined using the Hamann torsion test and the punch test. Small strain oscillatory testing on isolates made with the pH shift process has shown increases in G' both after heating (~80°C) and upon cooling

(~5°C) (Park 2005). However, decreases have also been shown with acid processed protein isolates. Inconsistent results from acid pH processing raise the question of a species/pH relationship.

Acid and alkali solubilization may be done over a range of pH. The muscle proteins of interest, mainly actin and myosin, are solubilized under acidic conditions between 2.0-3.0 and 10.5-11.5 under basic conditions. This can yield proteins of varying levels of unfolding. It is hypothesized that utilizing different solubilizing conditions within this pH range will produce differences in gel forming properties and also differences in the physical properties in the final gel.

Methods

Raw Material

The raw material used in these studies was fresh catfish fillets obtained 1-3 days post harvest from a local supplier. Catfish fillets were only purchased which were determined to be within 3 days of packaging. The catfish fillets were purchased and immediately transported on ice to the laboratory and processed the same day.

Preparation of Protein Isolates

Protein isolates were prepared according to figure 2-1. Fresh fillets were initially ground in an Oster heavy duty food grinder (Niles, Ill., U.S.A.) for the preliminary disruption and collection of the muscle tissue. Following grinding, the comminuted meat was diluted 1:2 (w/v) with deionized (DI) water and homogenized in a Waring blender for two bursts of 30 seconds. Following homogenization, the resulting muscle tissue slurry was further diluted to give a final dilution ratio of 1:6 (w/v) muscle to DI water. This slurry was manually stirred with a plastic spatula to achieve good homogeneity. The pH of the slurry was adjusted according to the methods described below, using either 2N NaOH or 2N HCl as needed for the pH desired, with

continuous manual mixing. Upon reaching the desired pH, insoluble material was removed by centrifugation (Sorvall RC-5B centrifuge with a GS-3 rotor, Kendro Laboratory Products, Newtown, Conn., U.S.A.) at 10,000 x g for 20 minutes at 5^{o}C. Following centrifugation, the soluble middle layer was collected through a kitchen strainer with a mesh size of approximately 0.25 mm to minimize contamination with other separated materials. The soluble material was readjusted to pH 5.5 as described above. After readjustment the solution was centrifuged to remove excess water and remaining soluble proteins at 10,000 x g for 20 minutes. The precipitated protein was collected by decanting the supernatant containing the unprecipitated proteins and removing it with a steel spatula. All of the precipitate from each solubilization pH was combined from the centrifuge bottles into one protein isolate. This dewatered protein isolate was further dewatered by placing the combined precipitate into cheesecloth and hand squeezing until the moisture content was below 80%. Moisture content was determined using a Cenco infrared moisture analyzer (CSC Scientific, Fairfax, Va., U.S.A.). Upon completion of manual dewatering the protein isolation was complete. Preliminary unpublished investigation of protein isolates in this laboratory found the shelf life of catfish protein isolates to be 5-7 days on ice. All protein isolates were stored on ice at the precipitation pH and used within 5 days.

Protein Concentration

Protein concentration in the isolates and the subsequent solutions was determined using the Biuret method, as described by Torten and Whitaker (1964), with of 10% w/v deoxycholic acid in water added at 10% v/v of the protein-Biuret reagent to minimize turbidity from any remaining lipids in the samples. Protein concentration was measured based on a standard curve based on BSA.

Oscillatory Rheology

For the gelation tests, the isolates were rehydrated to 10% protein and pH adjusted to 7.2 with 20 mM Tris-HCl and HCl/NaOH. For the added NaCl samples, NaCl was added at 2% w/w. The sample was homogenized at speed 1 using an Ultra-Turrax T18 homogenizer. The protein paste was then transferred to a peltier plate at 5°C attached to an AR2000 advanced research rheometer (TA Instrument, New Castle, DE) and a head with a flat cross-hatched polyacrylic surface. The gap was 1000 microns and the head lowered onto the sample using the controlled speed function provided by the software. The isolates were subjected to heating (5-80°C) and cooling (80-5°C), with testing conducted in oscillation mode. The storage modulus (G'), loss modulus (G") and tan delta (δ) was followed as a function of heat and time. All experiments were done under a controlled strain of 0.01 and controlled frequency of 0.1 Hz.

Torsion Gel Testing

Protein paste was prepared as above, except at 20% protein concentration. Proteins were stuffed in cylindrical tubes 10 cm long by 1.8 cm in diameter and cooked at 80°C for 20 min in a water-bath. The interior wall of the tube was coated with a lecithin based spray. The cooked samples were then removed from the casing after cooling for 30 min on ice, held overnight on ice, and subsequently cut into 2.54 cm long pieces. These pieces were glued to plastic disks (Gel Consultants Inc., Raleigh, N.C.) using an instant adhesive, Krazy glue (Toagosel Co., LTD. Nishi-shinnbashi Minato-Ku, Tokyo). Dumbbell shaped samples were then milled from each sample to 1 cm diameter in a machine mill (Electro Sales Co., Somerville, Mass., U.S.A.), wrapped in plastic wrap to prevent moisture loss, and brought to room temperature prior to torsion testing. For testing, 4 gel specimens were vertically mounted and sheared to the point of fracture at 2.5 rpm in a modified Brookfield viscometer (Gel Consultants Inc., Raleigh, N.C). Stress (kPa) and strain (dimensionless) at fracture were calculated with the manufacturer's

software for each sample, corresponding to the strength and deformation of the gels, respectively (Hamann and others 1990).

Punch Test

Punch test samples were prepared by adjusting protein isolates to 20% solids according to the moisture content and stuffed into plastic tubing, 1.25 inches in diameter. These samples were cooked for 20 min at 80°C, and then cooled rapidly in ice water. Samples were air freighted the same day they were cooked to the laboratory of Dr. Herbert O. Hultin in Gloucester MA (University of Massachusetts Marine Station) where punch testing was conducted The samples were cut to 1.0 inch length and 1.25 inch diameter. The sample was placed on the machine table and the head adjusted to just above the sample surface. A 5 mm head was used at a speed of 1.0 cm/min with force and distance at failure reported. Punch testing was conducted on a Rheo Tex Model AP-83 (Sun Science Co., Seattle, Wa).

Fold Test

A gel fold test was conducted according to Nowsad and others (2000). The test was conducted by slicing 2-mm thick sample disc from the cylindrical gels, mentioned above, and folding them into halves and quarters. The scales are: 5= no crack when folded into a quarter, 4=no crack when folded into half but crack when folded into quarter, 3=crack when folded into half, 2=broke and split into halves when folded into half, 1=broke and split prior to being folded into half.

Gel Water Holding Capacity

Gel water holding capacity was conducted according to Nowsad and others (2000). Expressible moisture of the gels was determined by compressing a 1.0 g spherical gel slice between 4 double layers of filter paper at a pressure of 20 Kg/cm^2 for 1 min and calculated from

weight before and after the compression. Expressible moisture was determined as the percentage of original weight after compression.

Cook Loss

Cook loss of gels was determined as the weight of the protein paste before heat treatment compared to the weight of the gels after cooking. Gels were prepared and heated as described above (torsion gel testing). After cooking the gel was removed from the tube, lightly dried and reweighed. The weight of the tube was subtracted from the total initial weight and then compared to the final weight of the cooked gel. Percent cook loss was determined by:

$$Cook\,Loss = \{1 - [Gel\ Weight / (Initial\ Weight - Tube\ Weigh)]\} \times 100$$

Cook loss is in percent, gel weight is the weight of the cooked protein paste after being removed from the tube, initial weight is the capped tube plus the raw gel stuffed inside, tube weight is the weight of the tube after the gel has been cooked and removed from the tube.

Statistical Analysis

Experimental design, as shown in figures 1-1 – 1-4, was conducted, in duplicate, on replicate isolations as discussed above. A replicated (N=2) was determined as acceptable due to achieving an acceptable power (β=0.80, α=0.05).

One-way independent measures analyses of variance were used to examine the effects of all methods used. The Kruskal-Wallis ANOVA testing by ranks was used on data which did not pass normality. Post hoc analysis was conducted only in the presence of significant population differences. ANOVA statistical comparisons were conducted with SigmaStat, (Systat Software Inc. San Jose Ca) with a significance level of $p<0.05$. After SigmaStat completed the ANOVA analysis, the post hoc analysis recommended by SigmaStat was used. In most cases this was

Tukey's test. Dunn's test was used for all punch test analysis; force, distance and jelly strength. The Holm-Sikak method was used with the torsion stress and expressible moisture.

Results

Rheological Changes in Protein Isolates During Thermal Gelation

The storage modulus (G') measures the resistance of a material to positive movement of a probe. Positive movement is the duration of movement during the oscillation of the head which is moving away from the zero point. G' is used to determine the solid fraction of a material during testing. In this study G' was followed with temperature to assess the change in the solid-like characteristics of protein isolates. G' showed a decrease for all isolates without added NaCl from 5°C until 45°C, and after 45°C, G' began to increase (figure 3-1, 3-4, 3-7, 3-10, 3-13, 3-16). For isolates made under acidic conditions, G' increased until 60°C for pH 2.0 samples without added NaCl and pH 2.5 samples with and without NaCl and then began to decline until the final temperature of 80^{o}C was reached (figures 3-1, 3-4, 3-25). The other isolates produced under acidic conditions, pH 2.0 with added NaCl and pH 3.0, exhibited a decrease in G´ until ~72°C. Acid isolates with added NaCl showed an initial decrease in G' which ended at ~30°C. Further decrease in G' was then observed at about 70-72°C. Isolates produced under basic conditions with and without added NaCl did not show a second decrease in G' after the initial decrease ending at 45°C.

The G' of gel pastes with no salt added prior to heat treatment (figure 3-43) were not significantly different ($p>0.05$) within the acid treated samples. The alkali processed gel paste treatment pH 11.5 was significantly higher ($p<0.05$) in G' than pH treatment 10.5, but there were no other significant differences between the individual alkali treated pH treatments. Between individual acid and alkali pH treatments, pH 2.5 was significantly lower ($p<0.05$) in G' than all alkali pH treatments and pH treatment 2.0 was significantly ($p<0.05$) lower in G' than pH

treatment 11.5. The control sample was significantly lower ($p<0.05$) in G' than all alkali pH treatments but was not significantly different ($p>0.05$) from any of the acid treatments. The G' of the gel paste with 2% added salt (figure 3-46) was not significantly different ($p>0.05$) within the acid treated samples. The alkali treatment pH 11.5 was significantly ($p<0.05$) higher in G' than pH treatments 2.5, 3.0 and 10.5. The control paste was significantly ($p<0.05$) higher in G' than pH treatments 2.5, 3.0 and 10.5. No other significant differences ($p>0.05$) were present between gel pastes.

Samples treated at low pH had G' values significantly lower ($p<0.05$) than samples treated at high pH after heating at 80°C and after cooling to 5°C (Figure 3-45). This difference was found for treatment conditions containing NaCl and also for those without added NaCl. The differences between low and high pH treatment were not the same in the presence or absence of added salt. Without the addition of salt pH treatments 2.0 and 2.5 were significantly different ($p<0.05$) from pH treatment 11.5 with pH treatment 2.5 also being significantly different ($p<0.05$) from pH treatment 11.0 when heated to 80^{o}C and when cooled to 5^{o}C. With the addition of salt pH treatments 3.0 and 2.5 were significantly different ($p<0.05$) from pH treatment 11.5 after cooling to 5^{o}C however after heating to 80^{o}C and before cooling pH treatment 3.0 was significantly different ($p<0.05$) from pH treatments 11.0 and 11.5.

The rheology studies also provided information about the loss modulus (G") of the protein isolates as well as the storage modulus. The loss modulus is considered the viscous or liquid like fraction of a viscoelastic material. The G" of catfish protein isolates in the absence of salt are shown in figures 3-2 (pH 2.0), 3-5 (pH 2.5), 3-8 (pH 3.0), 3-11 (pH 10.5), 3-14 (pH 11.0) 3-17 (11.5) and 3-20 (whole muscle). The G" of catfish protein isolates treated in the presence of salt are shown in figures 3-23 (pH 2.0), 3-26 (pH 2.5), 3-29 (pH 3.0), 3-32 (pH 10.5), 3-35 (pH 11.0),

3-38 (pH 11.5) and 3-41 (whole muscle). The changes in G" during both the heating and cooling phase in acid treated samples without added salt showed a decline in G" during heating with the minimum at the end of the heating phase, 80^{o}C. During the cooling phase the G" increased, after decreasing during heating. The G" after cooling was slightly higher than the starting G" but did not show the increases seen in G'. Alkali treated samples in the absence of added salt showed a decrease in G" during heating but the decrease during heating in all alkali samples was characterized by a sharp dip beginning below 40^{o}C and ending just below 60^{o}C. The increase in G" after the minimum point of the dip did not continue to increase but began to decrease again. Untreated muscle showed a similar trend to alkali treated samples but showed a more gradual decline before increasing. After the increase in G" during heating of the untreated muscle, G" began to decline at 70^{o}C whereas the alkali treated samples after a minor increase, began to decrease at 60^{o}C for pH treatments 11.0 and 11.5. Samples from the pH 10.5 treatment however showed a similar trend to the untreated muscle at higher temperatures, beginning to decrease in G" at 70^{o}C. In the presence of added salt, pH treated samples showed a G" trend similar to that of G' though the value was lower.

Tan delta is the relationship of G"/G' which provides an analysis of the transition of a material from a more viscous material to a more elastic material. A perfectly elastic material is represented as tan delta equal to 0, or stress and strain are perfectly in phase and a perfectly viscous material is represented as tan delta equal to 90 or stress and strain are perfectly out of phase (Park 2005). Tan delta was analyzed in all samples by merging and smoothing replicate rheograms. The tan delta results are shown in figures 3-3 (pH 2.0), 3-6 (pH 2.5), 3-9 (pH 3.0), 3-12 (pH 10.5), 3-15 (pH 11.0), 3-18 (pH 11.5), 3-21 (whole muscle) for samples treated without salt. In the absence of salt the tan delta transition showed more of a peak increase in tan delta

prior to the start of the decline in tan delta. The decline in tan delta began at 37°C in samples subjected to pH-shift processing. The whole muscle samples did not show a peak increase but a gradual increase in tan delta leading to a gradual decline in the absence of added salt. Samples treated with either acid or alkali processing in the presence of added salt is shown in figures 3-24 (pH 2.0), 3-27 (pH 2.5), 3-30 (pH 3.0), 3-33 (pH 10.5), 3-36 (pH 11.0), 3-39 (pH 11.5) and 3-42 (whole muscle). Samples treated with either acid or alkali processing showed a gradual decline in slope beginning between 19-22°C during heating and did not increase after this decline began. The tan delta for the control however increased to between 28°C and 42°C with the peak ranging over 5°C from 42-47°C with no change in tan delta then began the decrease, forming a bell curve. After the decline began at 47°C no increase in tan delta was observed.

Gel Quality of Isolates as Assessed by Torsion Testing

The results of torsion testing are shown in figures 3-49, 3-50. Alkali aided isolate gels 11.0 and 11.5 had significantly higher ($p < 0.05$) stress and strain values than acid aided isolate gels or control. Acid and alkali treated isolate gels were significantly higher in stress ($p < 0.05$) but not significantly different ($p > 0.05$) in strain from the control. Alkali aided isolate gels showed significant differences ($p < 0.05$) within the high pH group, with pH 11 samples showing significantly higher stress differences ($p < 0.05$) from pH 10.5 and 11.5, with no significant differences ($p > 0.05$) between isolates made at pH 10.5 or 11.5. There were no significant differences ($p > 0.05$) in strain within the alkali aided isolate. Significant differences ($p < 0.05$) were observed between all pH treatments compared to the control for stress but no differences were observed in strain values.

Gel Quality of Isolates as Assessed by Punch Testing

Acid and alkali aided isolate gels 2.5, 10.5, 11.0 and 11.5 had a significantly higher ($p < 0.05$) break force than control samples shown in figures 3-51. The distance required to break all

of the gels did not show significant differences ($p>0.05$) except pH 3.0 was significantly lower ($p<0.05$) than pH 10.5, and the control. The control had a significantly lower ($p<0.05$) force than pH treated samples 2.0, 10.5, 11.0 and 11.5. The control was significantly higher ($p< 0.05$) in the distance needed to break the gel than gels made with isolate from pH 3.0 treatment. No other significant differences ($p>0.05$) were observed between the control and pH treated samples.

Gel Quality of Isolates as Assessed by Expressible Moisture

The results from expressible moisture testing of the samples are shown in figure 3-54. The results of the study show gels made with acid-aided isolates have higher expressible moisture than gels made with alkali-aided isolates. Significant differences ($p<0.05$) were observed between gels made with alkali treatments 11.0 and 11.5 when compared to the acid treatment at pH 2.0 and the control. Other individual pH treatments from acid- or alkali-aided isolates did not show any significant differences ($p>0.05$).

Gel Quality of Isolates as Assessed Fold Test

The fold test conducted in this study is shown in figure 3-55. These results did not show any significant differences ($p>0.05$) between gels from the pH-shift process and the control gels. All gels made with isolates from the alkali-aided process treatment and the control gels scored a 5, which is the highest score. The gel made with the isolate from the acid-aided process ranged from 2.5-4, but were not significantly different from the control and the alkali-aided gels.

Gel Quality of Isolates as Assessed by Cook Loss

The cook loss results of this study are shown in figure 3-56. The results of this study did not show any significant differences ($p>0.05$) between gels made with isolates from the acid-aided process as compared to gels made with isolates from the alkali-aided process. Individual pH treatments within samples treated at low pH or at high pH did not show any significant differences ($p>0.05$). Individual pH treatments or grouped pH treatments did not show any

significant differences ($p>0.05$) as compared to the whole muscle control except pH treatment 11.0 was significantly ($p<0.05$) lower than the control.

Discussion

The G' or rigidity modulus is the resistance to gel structural movement of the network which is being formed. G' is derived in oscillatory testing as the fraction of the sigmoid movement of the head which is positive or negative displacement from 0 as compared to the fluid fraction of oscillatory measurement which is the return to 0 from positive or negative movement of the head. As the myofibrillar proteins set into a gel structure the resistance to movement of the curing system is increased by molecular interactions. The rheological testing of isolate measured non-fracture parameters using micro-strain oscillatory testing. The resistance to movement, or decreasing fluidity of the gel network is shown as increased G'. The rheological findings of this study show that pH treatments used to produce isolate significantly influence the final rigidity of the thermally set gel.

The protein paste prior to heat treatment, in both the presence and absence of salt, showed the same trend as final gel rigidity of the thermally set and cooled gels (figures 3-43 and 3-45 (no salt, respectively) and 3-46 and 3-48 (added salt, respectively)) except for pH 3.0 treatment. The pH 3.0 treatment was equal to pH 2.5 treatment prior to heating, but after heating decreased in G' relative to the other samples. The trends of the gel paste prior to heat treatment may provide an indication of the relationship of the final gel rigidity of the thermally treated muscle paste with the pH shift processed catfish muscle used in this study; however, the relative increase [(final G'/ initial G')*100] in gel rigidity from the raw paste to the final cooked gel ranged from 200-2000%. Due to the consistent trend of the raw paste with the final gel rigidity, standardizing the start point of rheological testing (initial G'- final G') did not affect the final results of the testing even with the out of trend raw paste seen in pH treatment 3.0.

The acid treated samples had a higher susceptibility to movement whereas alkali treated samples had a decreased susceptibility or increased structural rigidity (G') when compared to untreated muscle. These results are inconsistent with results shown for Atlantic croaker which showed an increase in gel rigidity over muscle without pH-shift processing, for both acid (pH 2.5) and alkali (pH 11.0) treatments (Kristinsson and Liang 2006). Isolates produced at pH 2.0 with added salt increased above that of the untreated catfish muscle. It can be concluded from the differentiation of gel rigidity as indicated by G' that pH processing at different low or high pH values allows for the modulation of the gel strength. This modulation of gel strength can now be tailored as the final product requires.

The rheological results of acid aided isolate made from the lowest pH treatment (highest degree of ionization at the low pH values tested) show gel rigidity increased above that of the untreated control in the presence of added salt (figure 3-48). When the treatment pH used was to achieve solubility and separation was less extreme (pH 2.5 and 3), the rigidity of the gel was equal to or less than the control. These results are similar to those shown by Raghavan and Kristinsson (2007a), where catfish myosin increased in gel rigidity as pH of acid solubilization decreased. Previous studies on acid solubilization of muscle proteins from sources other than catfish have shown acid solubilization increasing gel strength above that of untreated controls (Kristinsson and Liang 2006; Mireles Dewitt and others 2007). However, the solubilization pH used in these experiments was not as low as the most extreme pH used here. The increase in gel rigidity above the control in only the most extreme pH treated sample indicates that a high net positive charge which influences the level of unfolding is necessary to produce increased gel rigidity above that of an untreated control is species specific. Studies have demonstrated that extreme pH may lead to a partially refolded state, often called a molten globule. This modified

structure may be predisposed to promote more protein-protein interactions which can promote the formation of gels and produced stronger gels via more extensive protein-protein interactions during thermal processing. Rheological studies on isolated muscle proteins, specifically catfish myosin, have shown that acid treatment resulted in a gel which increased in G' above untreated control (Raghavan and Kristinsson 2007a). Increasing gel rigidity of catfish myosin with less extreme ionization/unfolding than that used here, indicates the contribution of other myofibril proteins co-precipitated with myosin in the whole protein system (Kristinsson and others 2005b) after pH adjustment will greatly influence the final rigidity an isolated muscle system is able to achieve. Thus, when using a whole muscle system it is important to optimize the pH used. The higher low pH values used to make acid aided catfish protein isolates (in the presence of salt) resulted in lower G', These results indicate that treatment with less [H+] subjected to the muscle proteins produces isolates which do not promote the stronger interactions occurring with the isolates made with the treatments with the highest level of [H+].

No significant differences ($p>0.05$) were observed between alkali treated gels in the presence or absence of added NaCl. Previous studies investigating multiple pH treatments on tilapia white muscle reported an improvement of gel properties with alkali treatment but decreased gelling ability of acid treated proteins (Ingadottir 2004). The increase in gel rigidity with alkali treated samples is consistent with previously published data on pH-shift processed muscle at alkali pH. The lack of differences in alkali treated catfish samples indicates that the molecular transitions induced by alkali pH treatment produces protein structures (in the presence of 2% NaCl) with similar interaction potentials at all alkali pH values tested,, thus resulting in unfolded structures which have similar gel forming abilities. As muscle gels are formed in the presence of salt, it may be concluded that alkali solubilized isolate produces a gel network with

the highest resistance to compressibility of the samples tested and all alkali treatments tested results in gel networks with equivalent non-fracture compressibility.

During the heating phase, all isolates and the control in the absence of added salt demonstrated a similar decline in G' until about 45^{o}C, which indicates that the molecular interactions induced by the thermal input during this range were similar for all isolates under the conditions tested. In the absence of salt, and at the pH tested, there is a significant repulsion between the muscle proteins at low temperatures, which may be modified as the system is heated, leading to a more fluid system, hence lower G' as seen here in this study. Above approximately 45^{o}C, all isolates and control samples with no added salt demonstrated an increase in G', indicating the development of thermally induced protein-protein interactions. While alkali-aided isolates made from pH 11 and 11.5 treatments (figures 3-13 and 3-16) showed a gradual increase in G' until 80°C (maximum point of heating), the acid-aided isolates (figures 3-1, 3-4 and 3-7) peaked at about 60°C (pH 2.0 and 2.5) and about 70°C (pH 3.0), demonstrating they had different and weaker interactions than the alkali-aided isolates. The isolate made with pH 10.5 treatment (figure 3-10) had a similar gelation behavior as the acid-aided isolates, peaking at about 70°C, suggesting it had different protein-protein interactions than the other alkali-aided treatments.

Gelation behavior of isolates in the presence of salt was different than in the absence of salt. This is not unexpected as the presence of salt can cause partial solubilization of the proteins and can also induce protein unfolding. Prior to heating the G' of the samples with salt was lower than the sampled without added salt. This is most likely due to the fact that salt would screen the electrostatic repulsive charges between then proteins, and thus lead to a more fluid system, hence lower G'. Small changes were seen in G' during heating, until between 35-40°C for the acid-

aided gels (figures 3-22, 3-25 and 3-28) and about 40-45°C for the alkali-aided gels (figure 3-31, 3-34 and 3-37). Unfolding is a prerequisite to thermal gelation of muscle proteins, thus suggesting that proteins in the acid-aided isolates were more susceptible to heat and presumably thermally unfolded at a lower temperature than the proteins in the alkali-aided process. Both the acid- and alkali-aided isolates had a distinctly different gelation behavior on heating in the presence of salt compared to the control (figure 3-40), which demonstrated an expected dip in G' between 35 and 50°C. This clearly shows that the pH-shift process leads to changes with the proteins in the isolate which in turn have different gel forming properties.

All isolates, and control, regardless of the presence or absence of salt demonstrated a significant increase in G' during the cooling phase. The final G' was in all cases substantially higher than the initial G', but the difference between the two varied greatly between treatments. The difference in initial and final G' can be an indicator of the ability of a sample to produce a strong gel. The larger the final G', the more rigid and strong the gel could be. The increase in initial and final G' for acid-aided gels in the absence of salt ranged from 1.8-3.3 fold, while it was 5.9-6.4 fold for the alkali-aided gels. Although the control increased its G' 5.5 fold, the final G' was lower than the G' of alkali-aided gels. In the presence of salt the increase in G' was 3.6-13 fold for the acid-aided gels but 14-25 fold for the alkali-aided gels. The control had an 8 fold increase in G'. The much larger increase in G' during the heating phase of gelation exhibited by alkali processed muscle proteins, clearly indicates that high pH processing results in protein conformations and supramolecular structures which promote stronger protein-protein interactions during heating and particularly cooling than acid-aided gels and untreated minced catfish muscle. The larger increase in G' on cooling for the alkali-aided gels compared to the acid-aided gels may suggest they lead to increased hydrogen bonding on cooling (Lanier 2000).

Muscle protein gels are characterized from a quality standpoint by color, gel strength and flexibility. Gel strength is evaluated as resistance to structural breakage or the stress coefficient. The gel flexibility is the structural elasticity or the strain coefficient. The stress and strain at fracture of muscle gel may be analyzed in two ways; 1) the rotational stress and strain and 2) the tangential stress and strain. Torsion testing analyzes the rotational stress and strain by measuring the force and distance required to twist a cylindrical gel segment until it breaks in half. Tangential fracture measurement is done using the punch test. The punch test lowers a probe into a flat cross-section of the gel and measures the force and distance required to puncture the gel. These two tests are frequently used to determine physical qualities attributed to the quality of muscle protein products.

Torsion testing is the study of the rotational fracture properties of muscle proteins gels and was used in this study to compare pH treated and untreated samples using a method widely accepted for surimi testing. Four primary texture profile properties which have been attributed to surimi gels by torsion testing (Park 2005), those are mushy, brittle, rubbery, and tough (Park 2005). Mushy is described by having low stress and low strain, with brittle having high stress and low strain. The other two are high strain descriptors with rubbery having low stress and tough having high stress. However, the results with catfish protein isolate, as seen in figure 3-57, do not fall within the parameters of a typical texture profile graph, as the gel stress is too high except for the control which is considered brittle. As seen in figure 3-57 all treatments resulted in product that had brittle properties (Park 2005).

The differentiation between acid and alkali treated samples in the way they respond to rotational fracture indicates alkali treatment forms both a stronger and more elastic gel. The individual pH treatments did show differences with pH 2.5 having the lowest stress and pH 11.0

the highest. The desired textures of a restructured muscle product are important in the processing chosen for the production of that product. Kim and others (1996) showed conventional processing of catfish by-products resulted in a surimi product with acceptable textural properties for the production of shellfish analogs with the addition of cryoprotectants and starch. The torsion results of pH-shift processing of catfish muscle when compared to the results of Kim and others (1996) showed that protein gels (with no added cryoprotectants) made from alkali processing produced a stronger gel than either acid or no processing. The increased strength of pH-shift processed gels are however categorized as brittle according to the torsion texture profile (Park 2005). Acid processing of catfish, however, has led to mixed results. The acid results reported here for catfish are similar to Mireles Dewitt and others (2007) who showed that acid treatment resulted in an improvement in gel strength as compared to a ground muscle control.

The pH-shift process exhibited higher shear stress and lower shear strain than the processing and formulation additives used in the products tested to develop the torsion texture profile (Park 2005). The pH-shift processing of catfish muscle proteins compared to croaker, whiting, rockfish and cod since alkali treatment increased gel strength and elasticity of all species compared to acid treatment and no treatment. These differences show that the pH used to produce isolate do in fact provide protein isolates with different rotational physical resistance.

Punch testing, which is a study of tangential fracture properties of muscle protein gels, was also used in this study to compare pH treatments and untreated samples. This is the other fracture testing method widely used in the surimi industry. The parameters recorded are the resistance and distance traveled at breakage. Breakage is when the structure of the gel gives way and can no longer hold its cohesive form. The results of this study indicate that pH treatment

directly affects the punch test scores as shown in figures 3-51, 3-52, and 3-53. Other studies have shown surimi from carp form a gel with lower break force and distance than acid or alkali processing (Luo and others 2001a). The effect of changing the punch test scores is that the surimi quality grade may be based on the jelly score (Park 2005) obtained from punch test values, the jelly score is the multiplication of force times distance. The jelly score for pH-shift processed catfish muscle is shown in figure 3-53. The problems with the jelly score are many (Park 2005), but as the jelly score is the product of gel force and gel deformation the quality of the protein isolate is not reported accurately. The results of this study show that pH-shift processing does not change the gel structure as assessed by the punch test as dramatically as seen in other testing methods, specifically torsion testing.

The fold test is a semi-quantitative test used to determine the flexibility of cooked muscle protein gels rapidly. Based on the 1-5 or grading scale the fold test allows for a five point scoring of the gels. The fold test allows for the determination of gels which are considered to be too brittle. However the fold test does not account for gels which may be too elastic. The fold test is one of the primary methods used in grading surimi. The lack of significant difference ($p>0.05$) between all pH treated samples and the control indicates that catfish muscle proteins with or without pH shift processing will form acceptable gels under these test conditions. The alkali results are consistent with pH-shift processing of tilapia (Ingadottir 2004). However, acid processed tilapia also resulted in fold test scores of 5 which the catfish isolate gels from the acid-aided process did not. The lack of significant difference ($p>0.05$) between the acid treatment and the alkali treatment or the control, but with inconsistent results in acid processed catfish muscle as compared to the consistent results of acid processed tilapia muscle, indicate that the acid processing of catfish muscle may lead to a more complex material than the same process did

with tilapia. The production of surimi from catfish, however, showed acceptable results with the addition of cryoprotectants and starch (Kim and others 1996). This indicates that based on the fold test, pH-shift processing of catfish muscle, though inconsistent, under the acidic conditions tested here, provides a gel structure acceptable for the production of a surimi product without the addition of cryoprotectants or other additives.

The press test investigates water holding capacity of the gel matrix formed after cooking. The water holding capacity of muscle gels is directly related to the firmness of the gel network (Kim and others 1996). The results of the study show that alkali treated isolate, pH 11.0 and 11.5, had significantly higher water holding capacity than acid treated isolate, pH 2.0, and the control. Water holding capacity of pH-shift processed catfish muscle when compared to catfish surimi indicates that pH-shift processing resulted in decreased water holding capacity compared to washed catfish surimi with added cryoprotectants which had an expressible moisture of less than 2 percent (Kim and others 1996). The results of Kim and others (1996) indicate that catfish mince with or without cryoprotection produced a weak gel with puncture forces less than 500g. These results are inconsistent with the results found in this study showing that even though there is a reduction in water holding capacity, the preparation of thermally set gels without cryoprotection does result in strong gel formation. The stability of pH-shift processed catfish muscle on frozen storage and the need for cryoprotection has yet to be determined.

Cook loss determines the amount of weight a product may lose during thermal processing. This weight loss is very important as increased cook loss reduces the final retail weight of the finished product and is a direct economic loss for the processor. Cook loss is a form of water holding capacity of muscle gels as the minced muscle product transitions from a protein paste to a thermally set network. The lack of significant difference ($p>0.05$) between the pH treated

samples and the control except for pH treatment 11.0 indicates that the water holding capacity of pH processed isolate during thermal transition is not affected by all pH treatments except pH 11.0. The low cook loss of all samples in the current study (less than 3%) indicated that the isolates have a better water holding capacity than that reported for acid processed catfish surimi with added cryoprotectants and phosphates, which are known to aid in water retention (Mireles Dewitt and others 2007). This means that pH-shift processing of catfish muscle may provide a protein isolate with comparable or improved properties of phosphates in muscle systems but without the addition of phosphates.

Conclusions

The results of the gel properties of pH-shift processed catfish muscle indicate that pH processing results in improved gel strength over conventional processing according to the catfish results published by Kim and others (1996). These results also indicate that the range of textural possibilities has not been fully explored. The brittle gels formed, based on the textural schematic of Park (2000), indicate that optimization of the gel properties of pH-shift processed catfish muscle is crucial before commercial processing is possible. Increasing the shear strain above 2.25 and decreasing the shear stress below 70 kPa is just the first step in developing a pH-shift processed catfish muscle into a useable primary product. The most desirable method for achieving the reduction in shear stress and the increase in shear strain is the addition of water to the gel paste. The pH-shift processed isolates were tested at 80% moisture compared to 78% moisture used by Kim and others (1996). This suggests that pH-shift processed isolates may require a lower protein concentration than conventionally processed catfish muscle. Additional benefits to the processor and the consumer if pH-shift processing is used include taking advantage of the different solubilization pH values. pH-shift processing allows the processor to have greater control over the desired texture for specific applications as seen within the textural

ranges published here. This data also suggests that individual isolates may be combined to provide an even broader range of textures within a single comminuted product if the appropriate processing conditions are used.

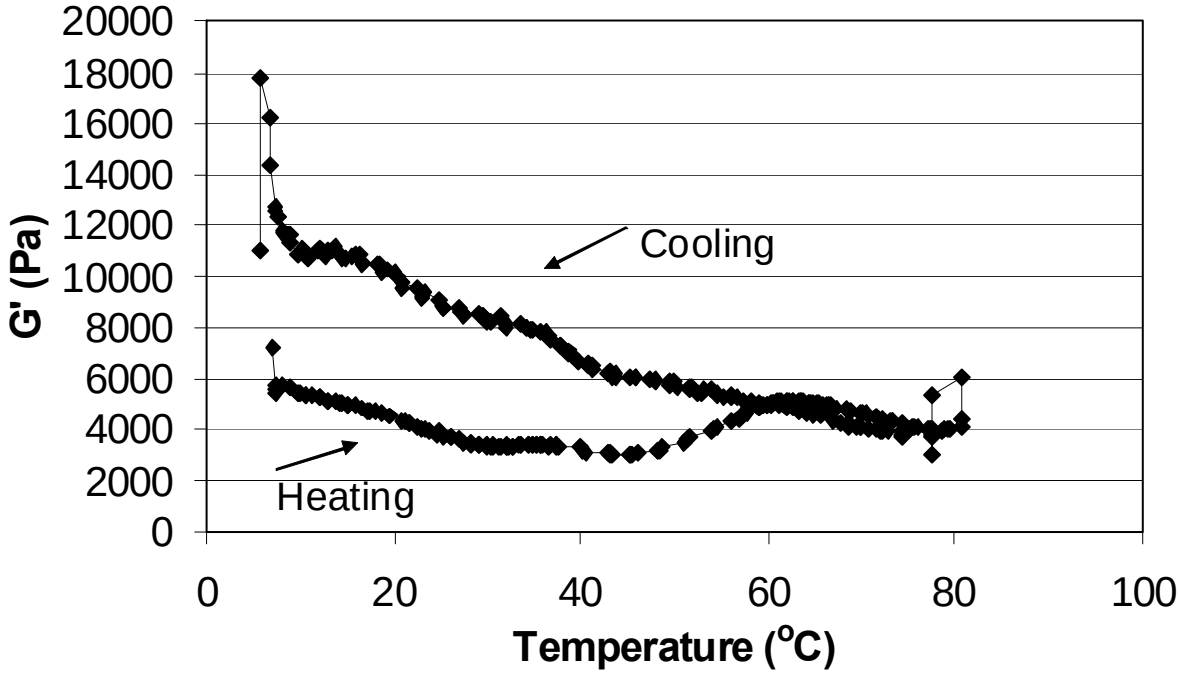

Figure 3-1: The storage modulus (G') of catfish protein isolates produced at pH 2.0 during heating and cooling as tested by small strain rheology. The gel was developed at 10% solids, 2°C/min in the absence of added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

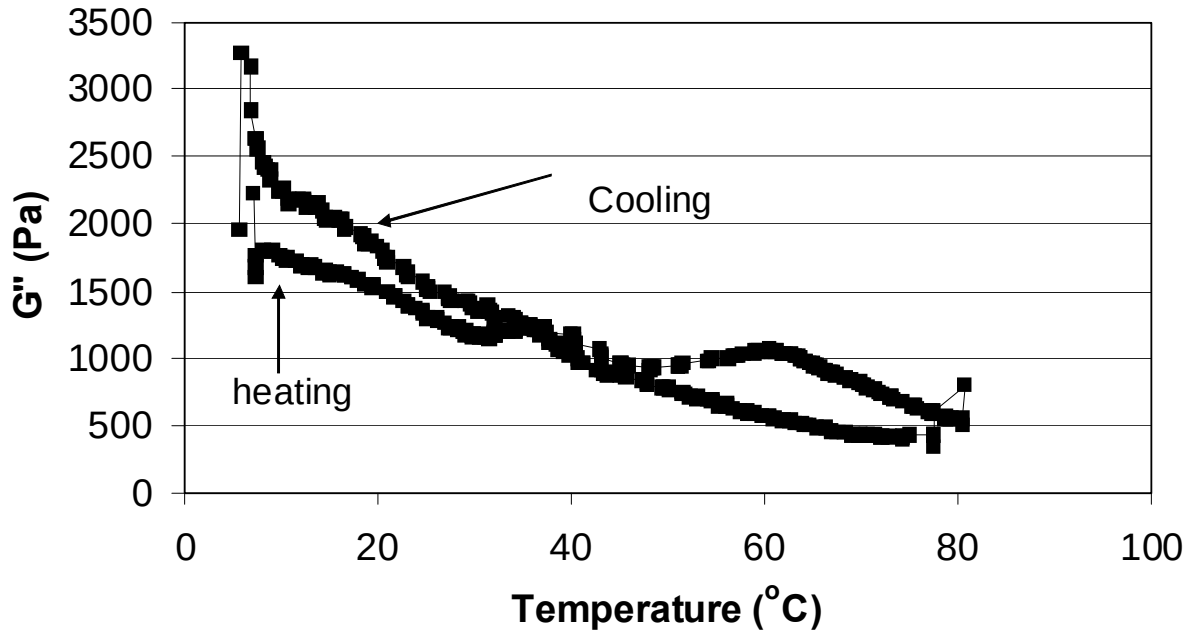

Figure 3-2: The loss modulus (G") of catfish protein isolates produced at pH 2.0 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min in the absence of added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

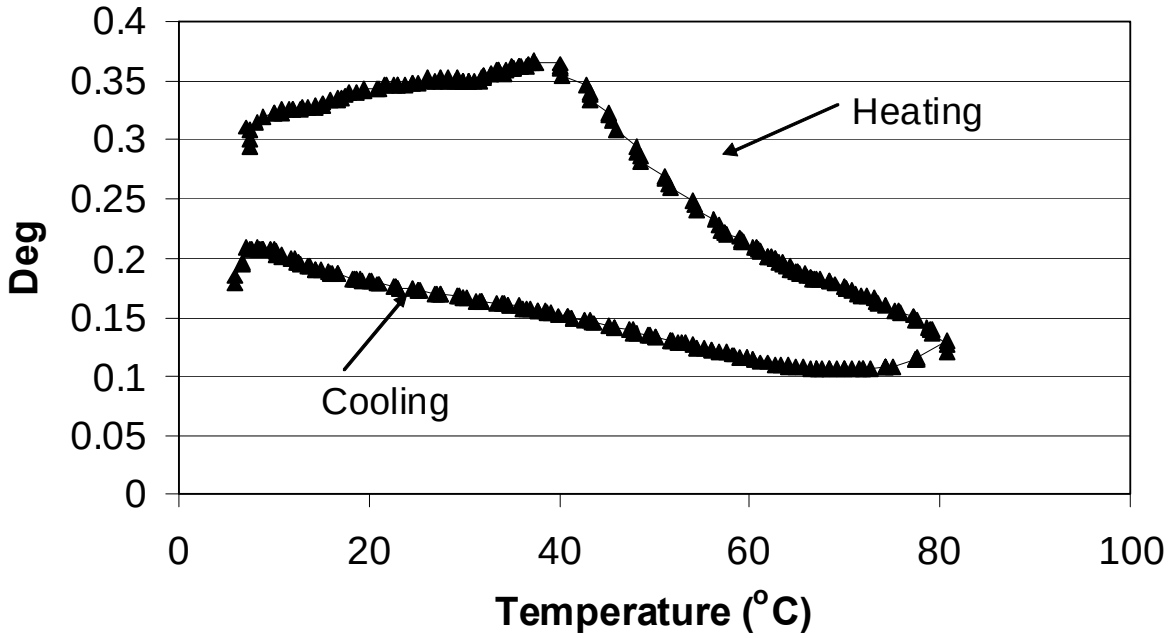

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Figure 3-3: The tan delta (G"/G') of catfish protein isolates produced at pH 2.0 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min in the absence of added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

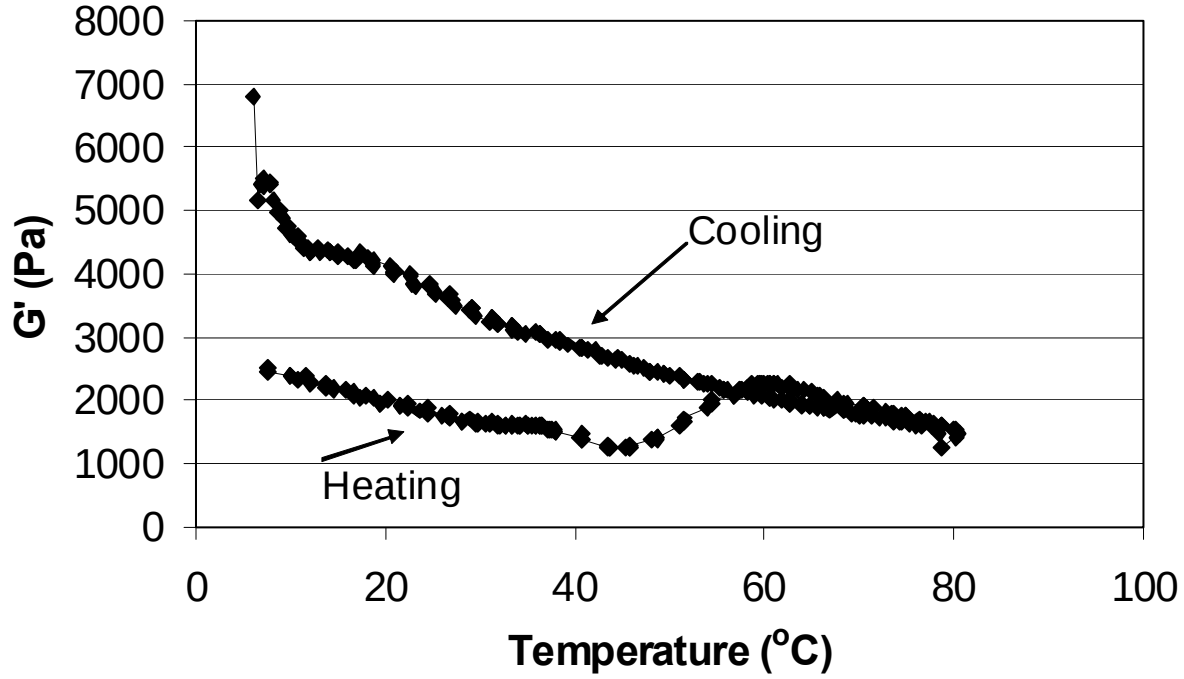

Figure 3-4: The storage modulus (G') of catfish protein isolates produced at pH 2.5 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min in the absence of added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

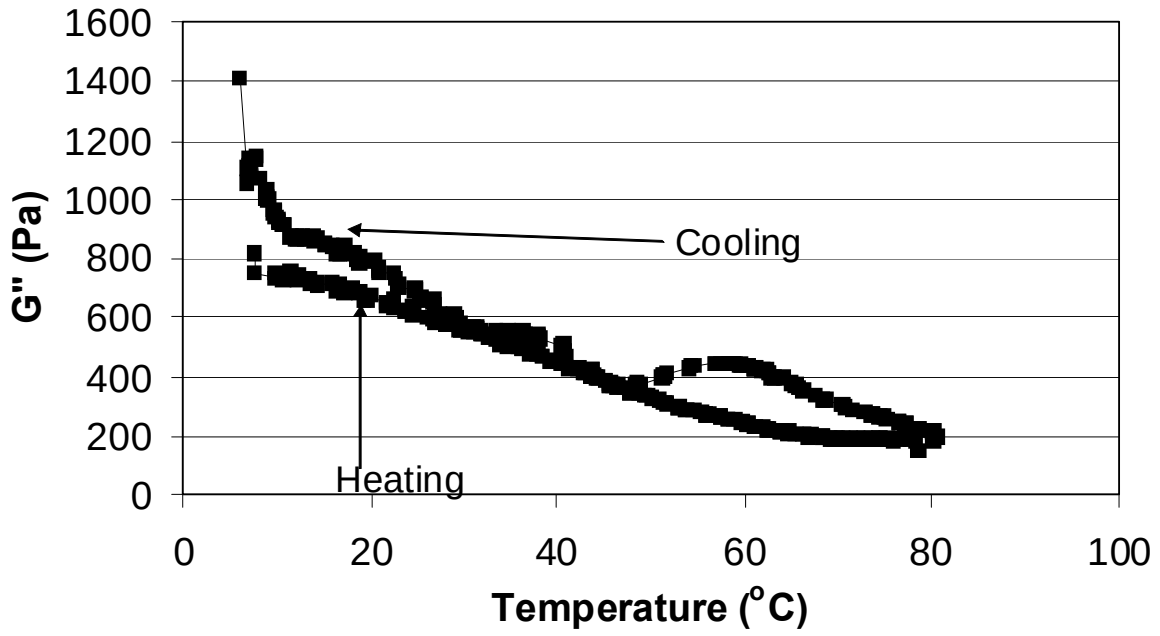

Figure 3-5: The loss modulus (G") of catfish protein isolates produced at pH 2.5 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min in the absence of added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

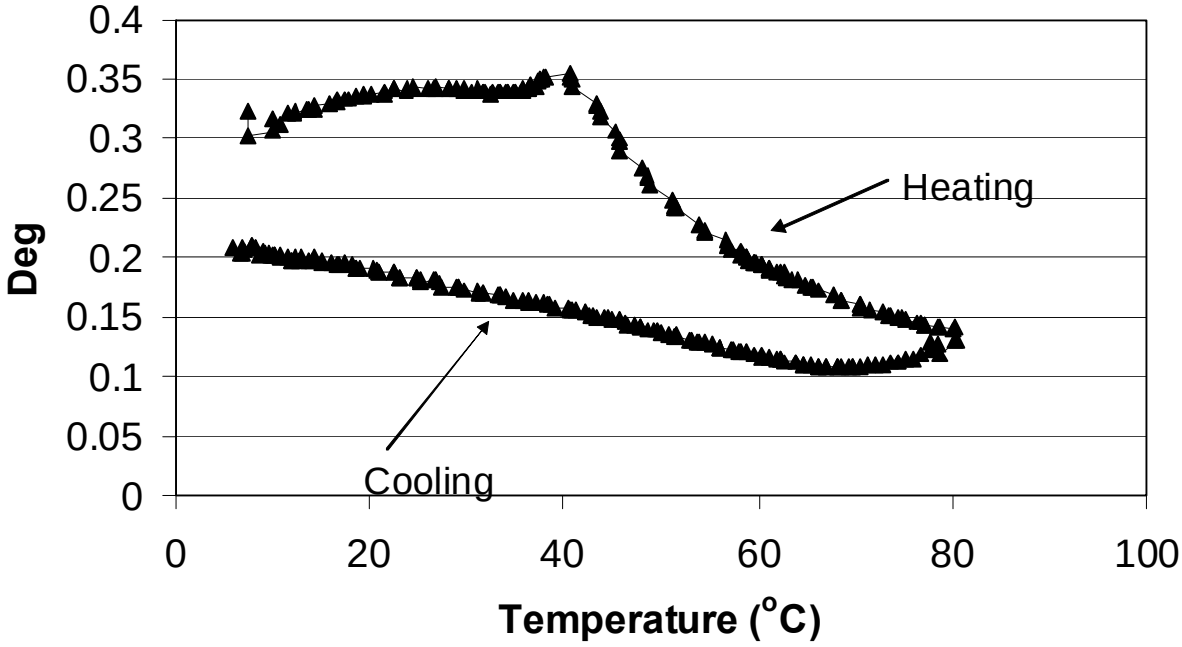

Figure 3-6: The tan delta (G"/G') of catfish protein isolates produced at pH 2.5 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min in the absence of added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

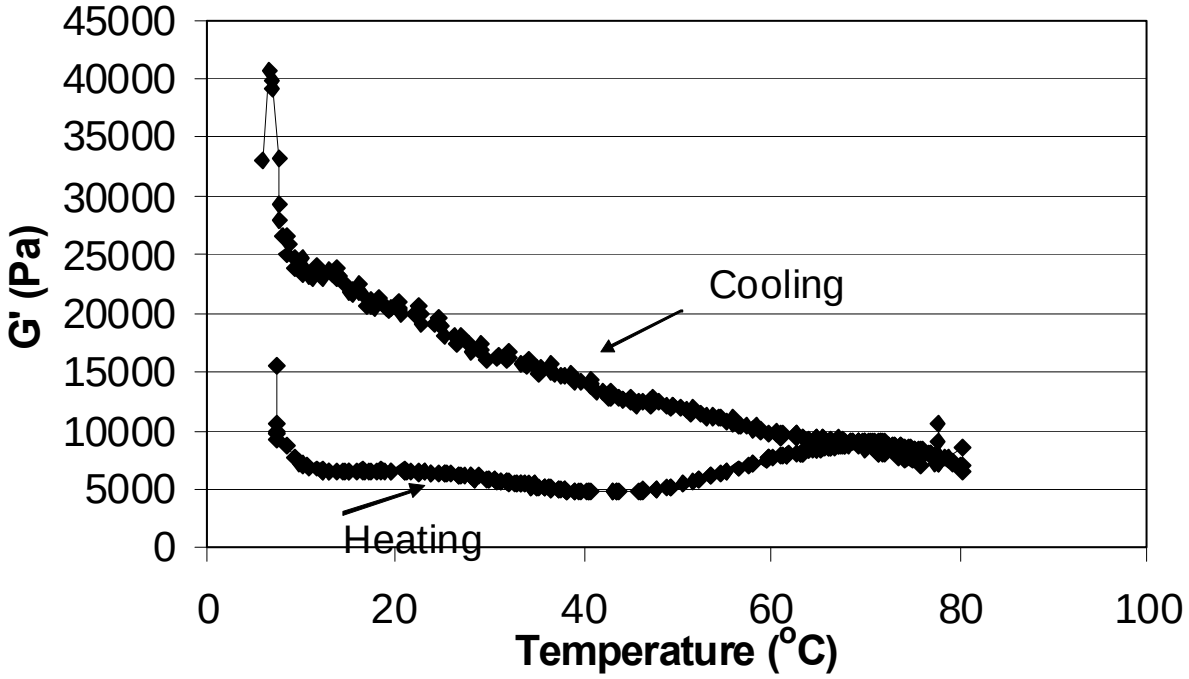

Figure 3-7: The storage modulus (G') of catfish protein isolates produced at pH 3.0 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min in the absence of added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

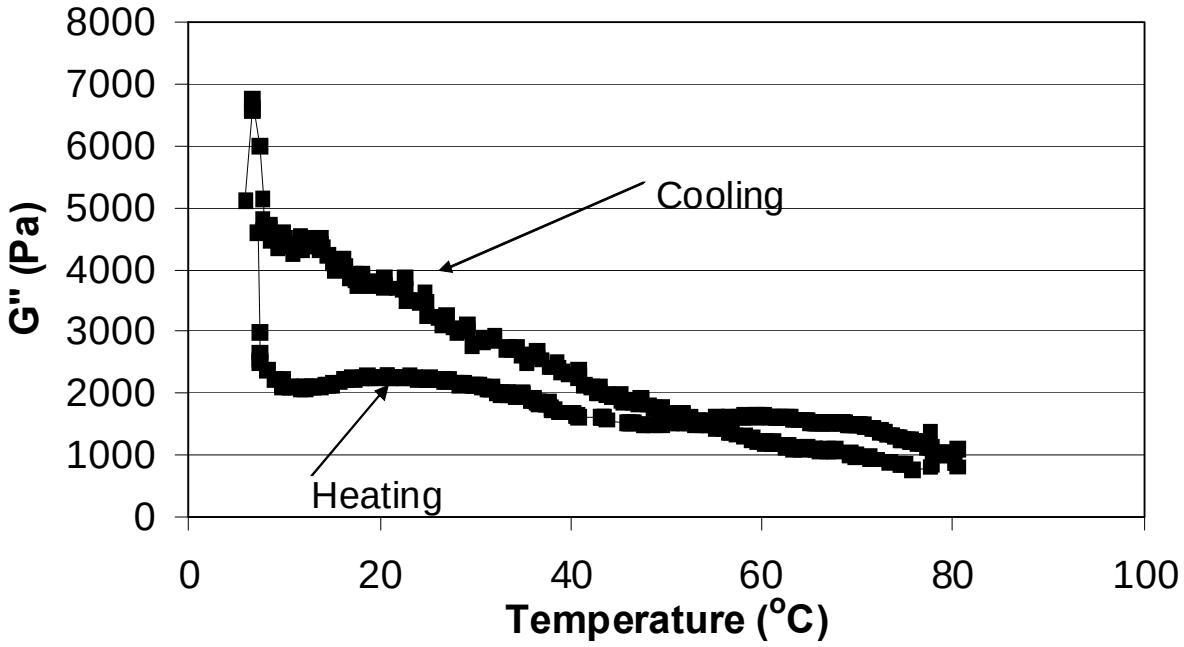

Figure 3-8: The loss modulus (G") of catfish protein isolates produced at pH 3.0 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min in the absence of added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

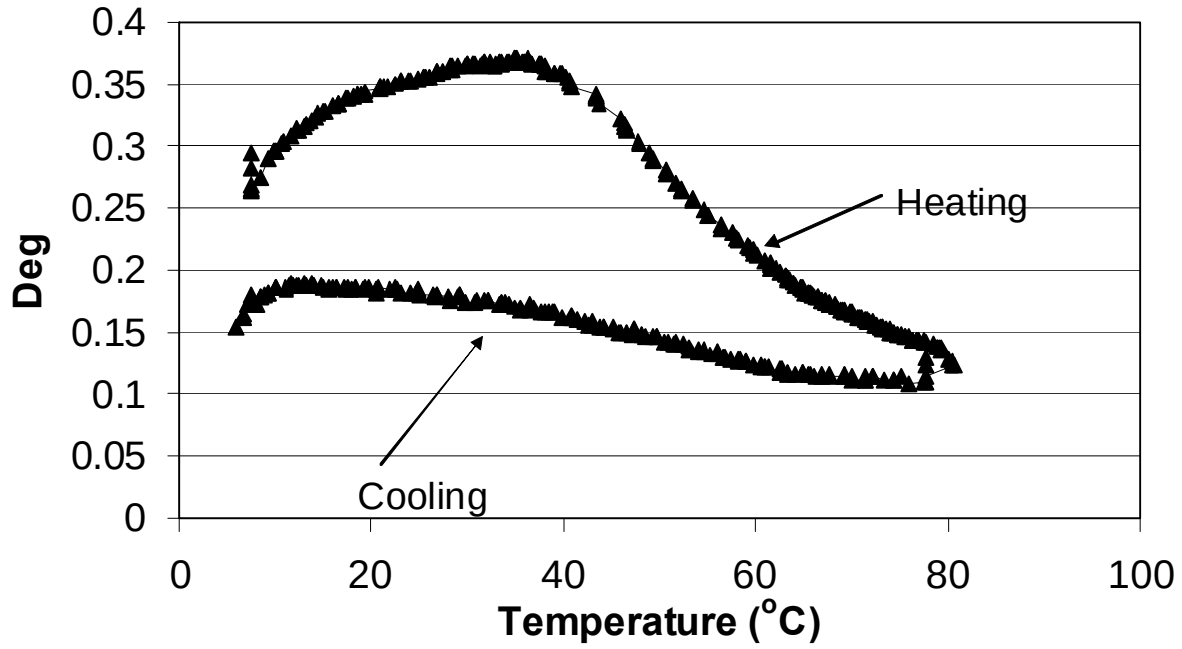

Figure 3-9: The tan delta (G"/G') of catfish protein isolates produced at pH 3.0 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min in the absence of added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

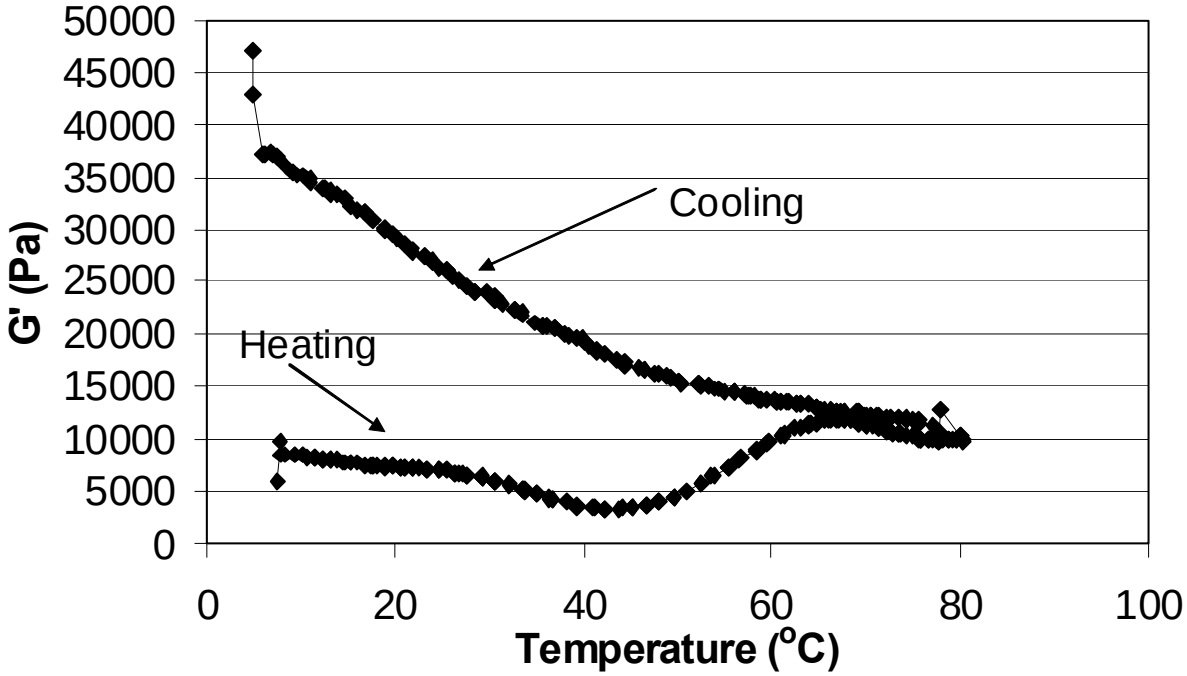

Figure 3-10: The storage modulus (G') of catfish protein isolates produced at pH 10.5 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min in the absence of added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

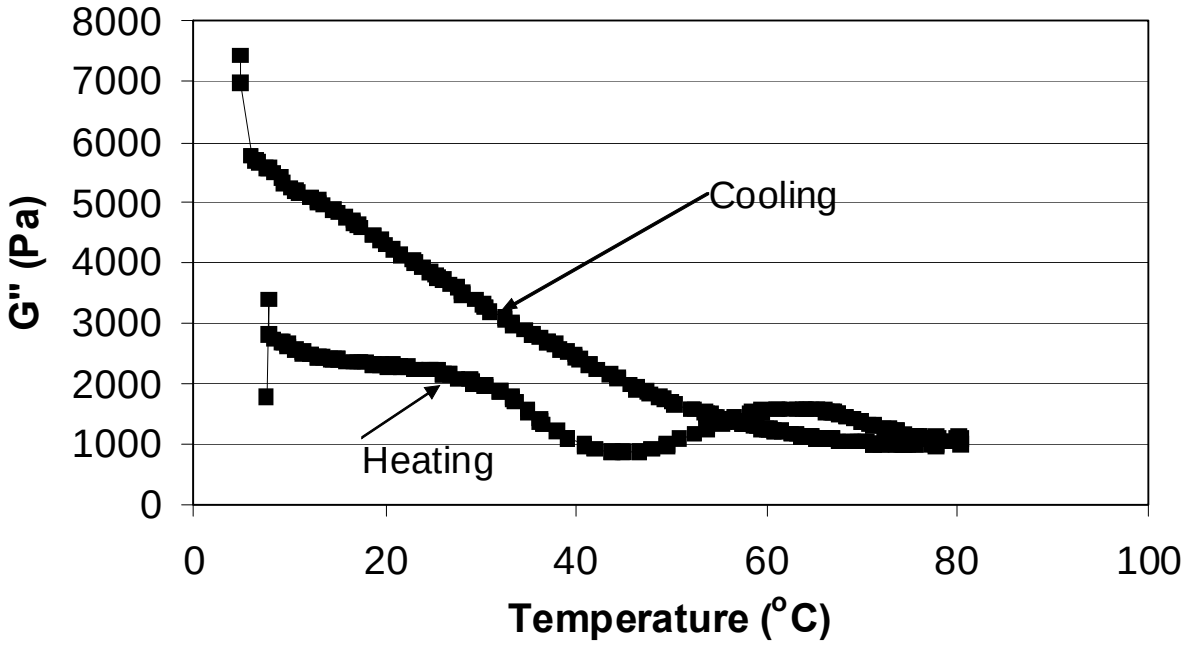

Figure 3-11: The loss modulus (G") of catfish protein isolates produced at pH 10.5 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min in the absence of added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

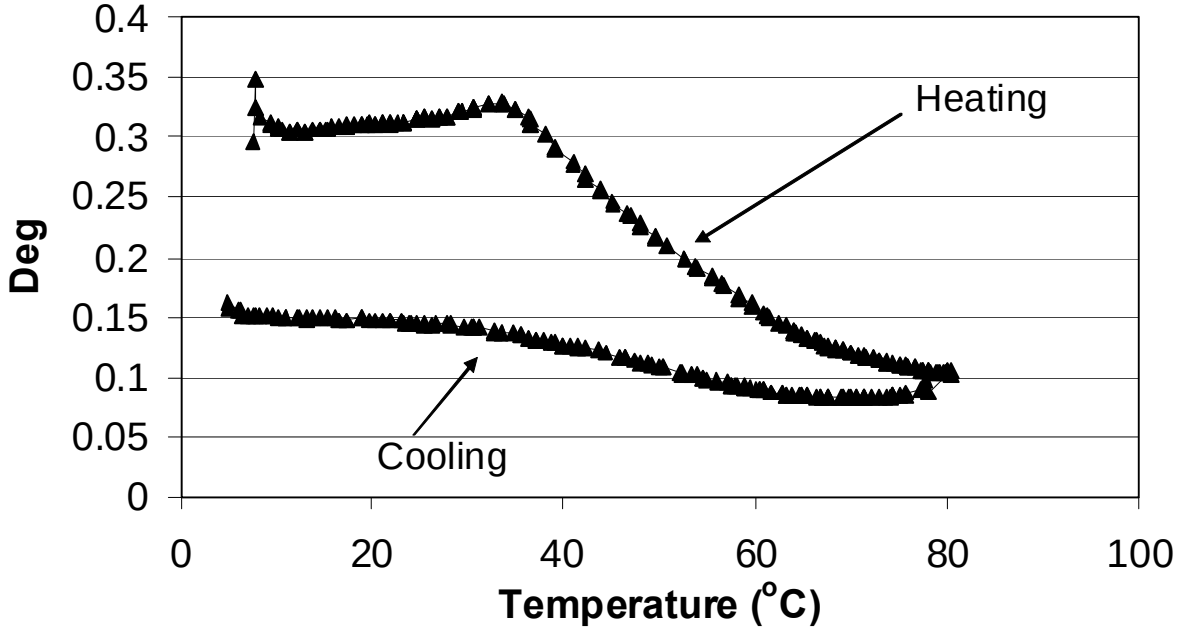

Figure 3-12: The tan delta (G"/G') of catfish protein isolates produced at pH 10.5 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min in the absence of added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

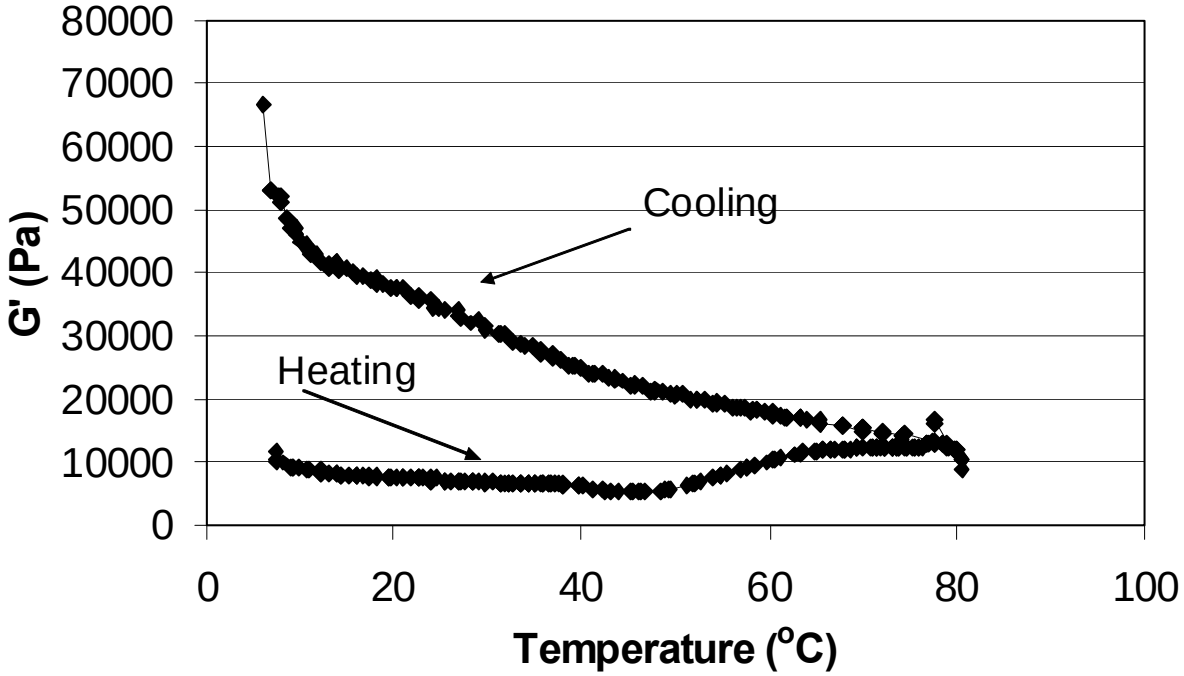

Figure 3-13: The storage modulus (G') of catfish protein isolates produced at pH 11.0 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min in the absence of added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

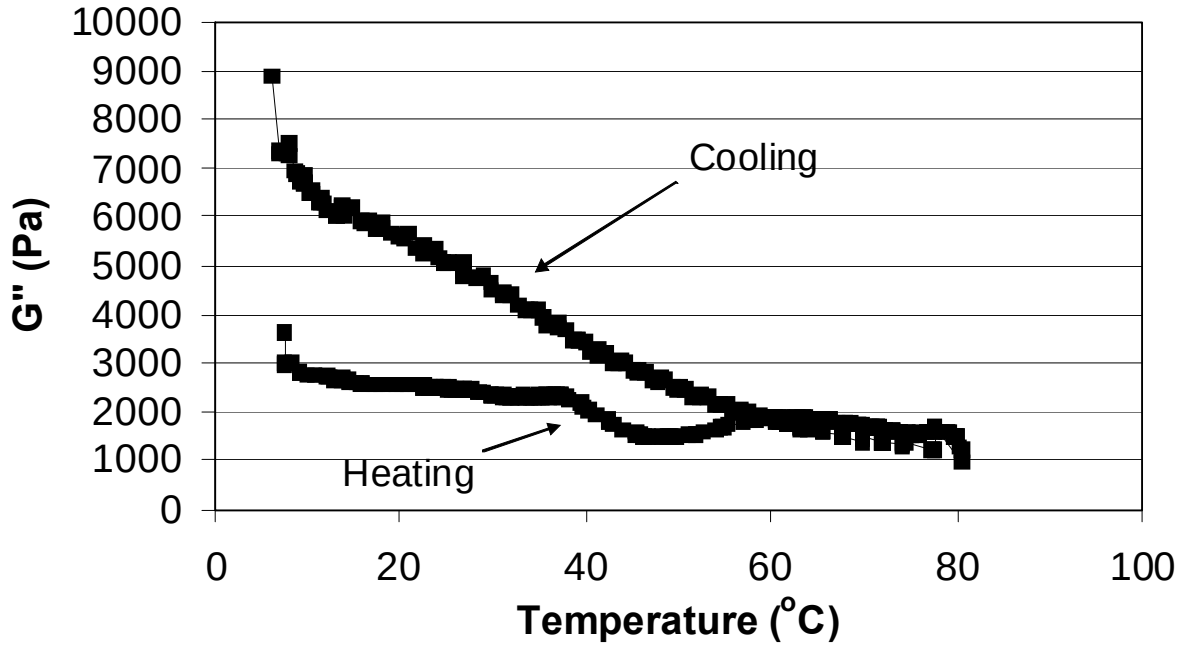

Figure 3-14: The loss modulus (G") of catfish protein isolates produced at pH 11.0 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min in the absence of added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

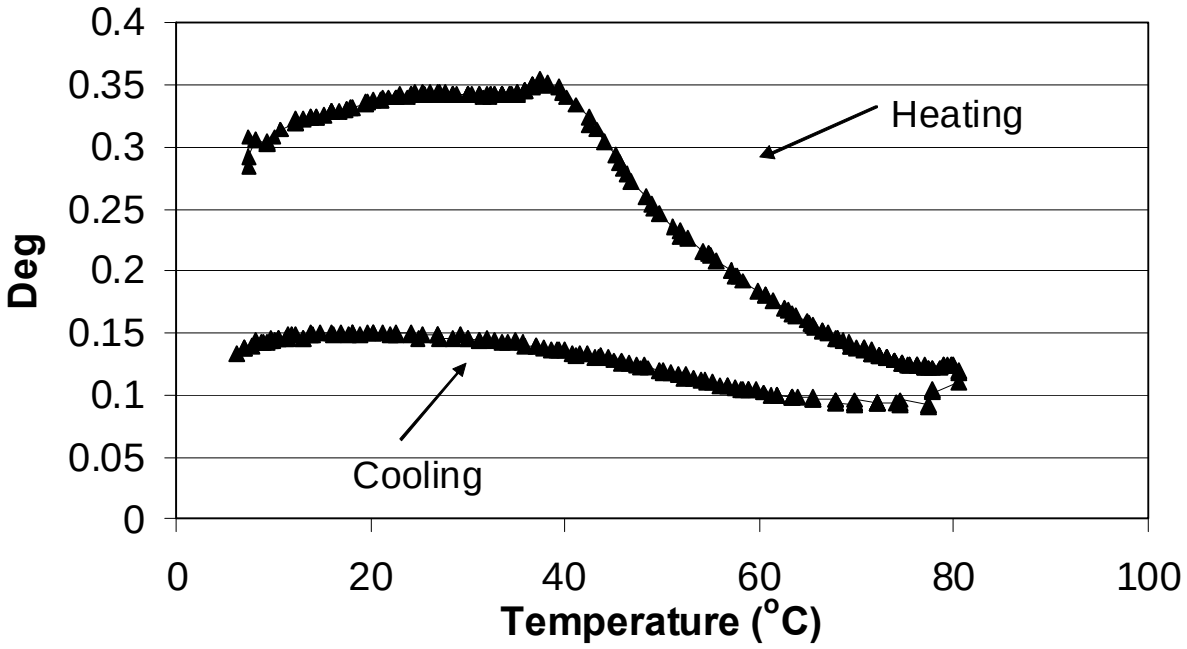

Figure 3-15: The tan delta (G"/G') of catfish protein isolates produced at pH 11.0 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min in the absence of added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

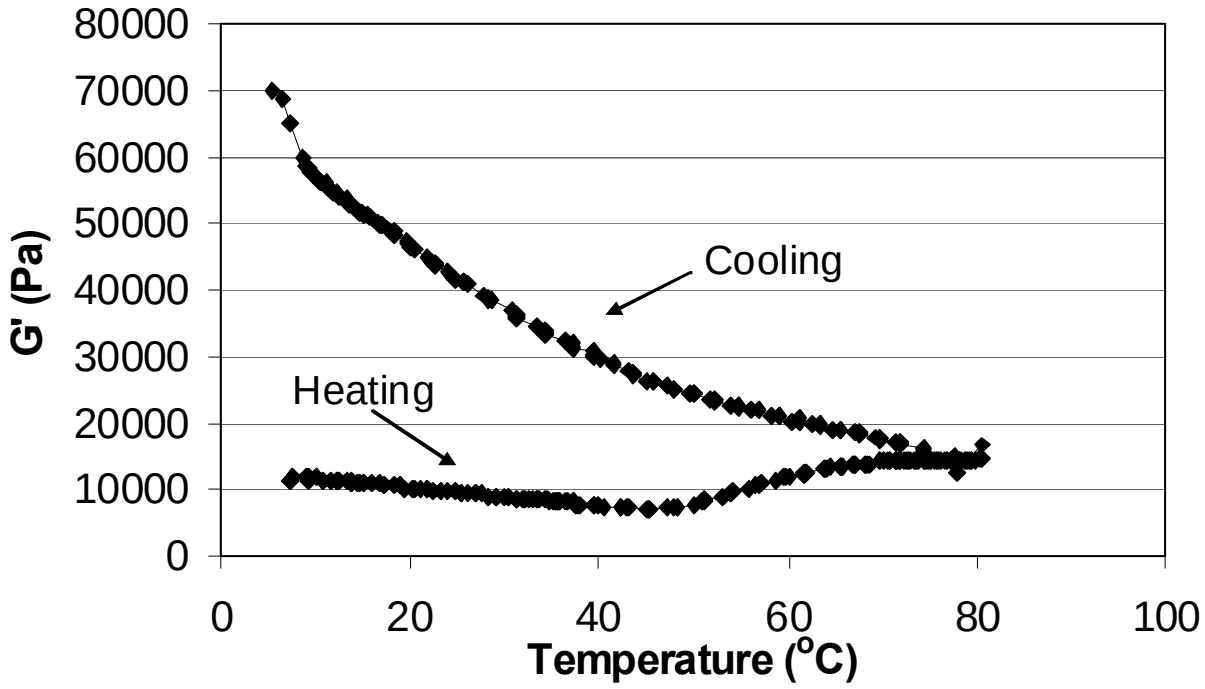

Figure 3-16: The storage modulus (G') of catfish protein isolates produced at pH 11.5 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min in the absence of added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

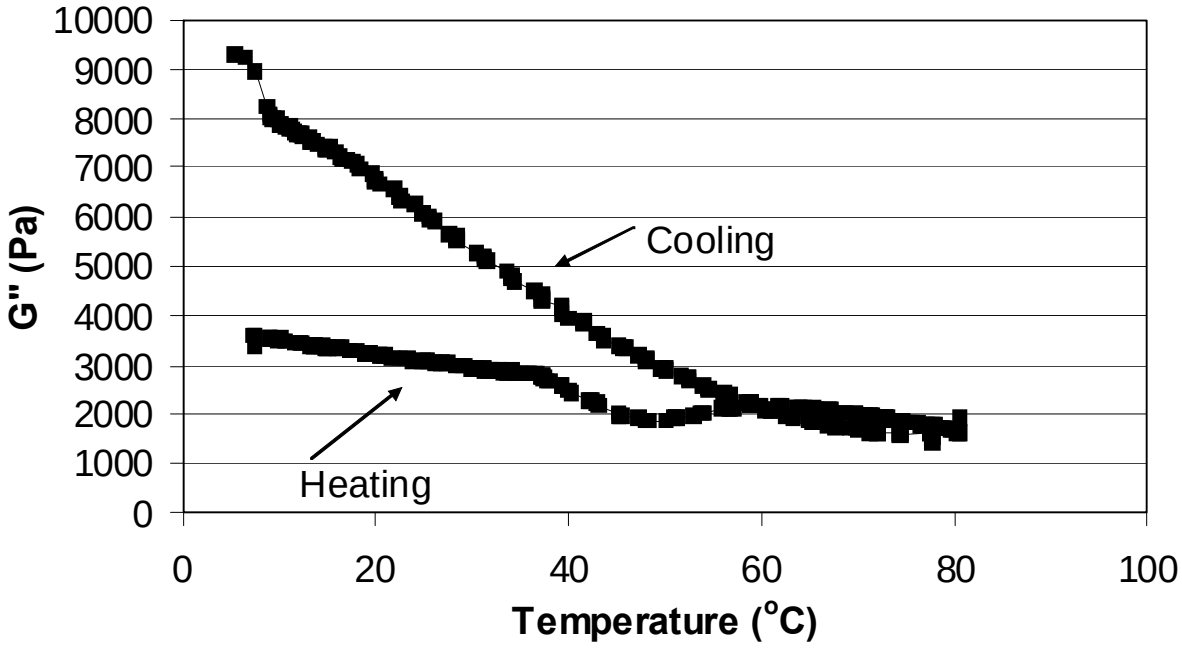

Figure 3-17: The loss modulus (G") of catfish protein isolates produced at pH 11.5 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min in the absence of added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

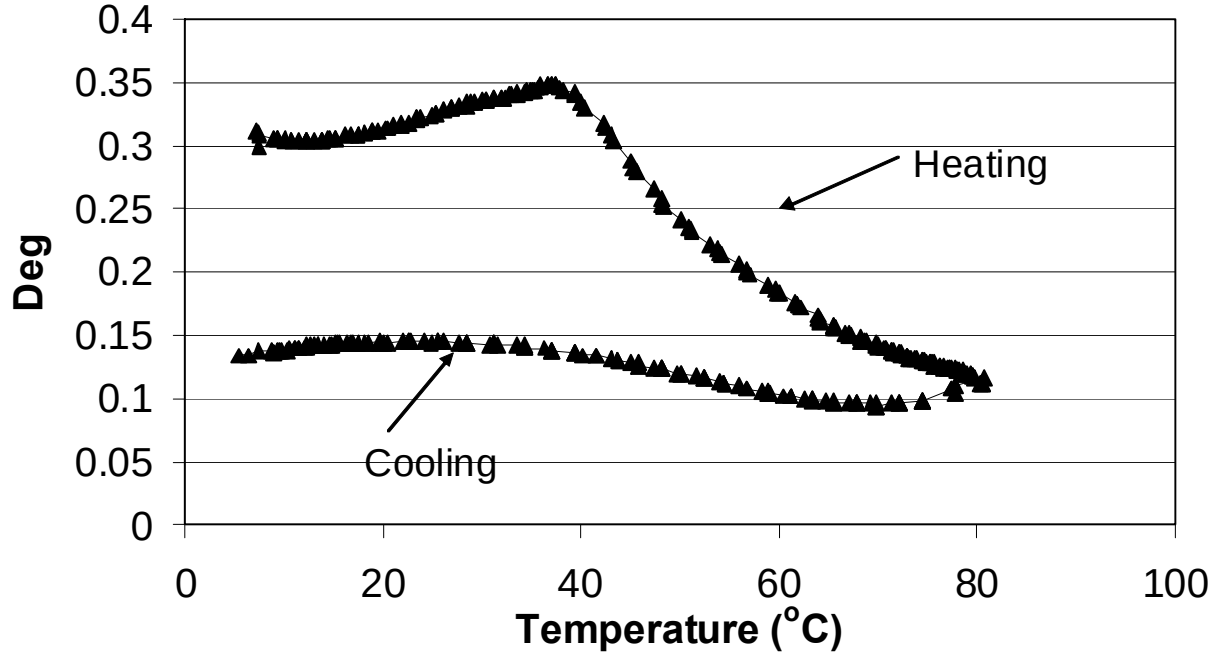

Figure 3-18: The tan delta (G"/G') of catfish protein isolates produced at pH 11.5 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min in the absence of added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

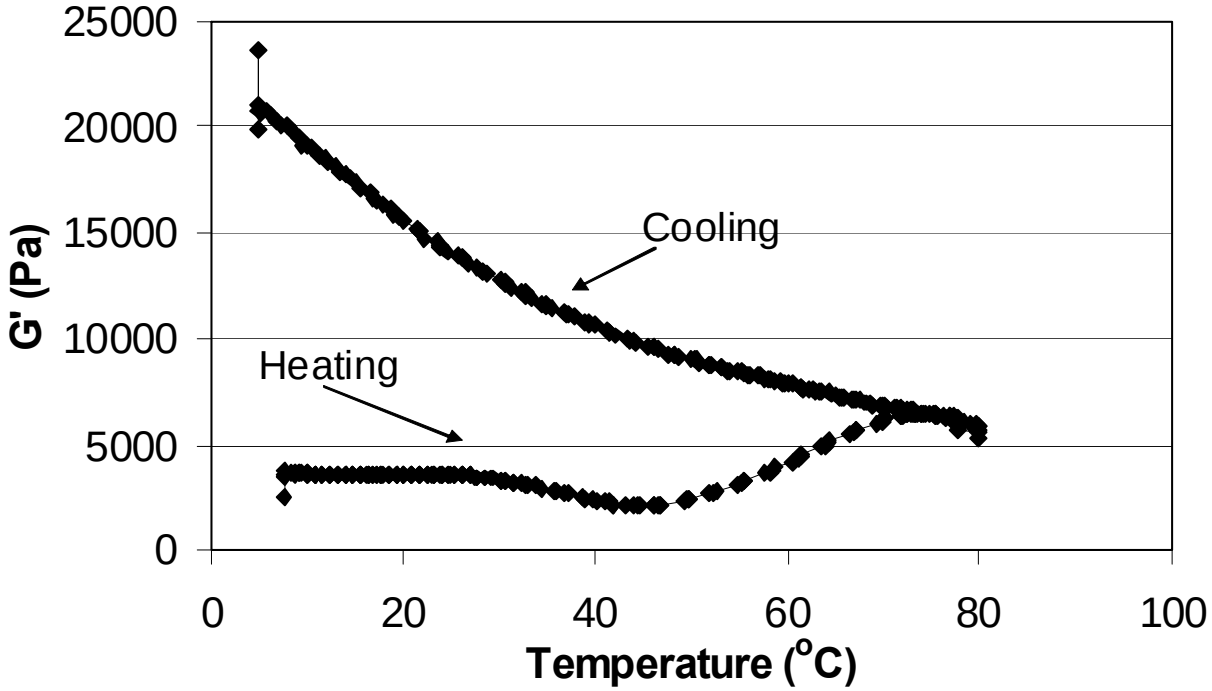

Figure 3-19: The storage modulus (G') of untreated catfish muscle during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min in the absence of added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

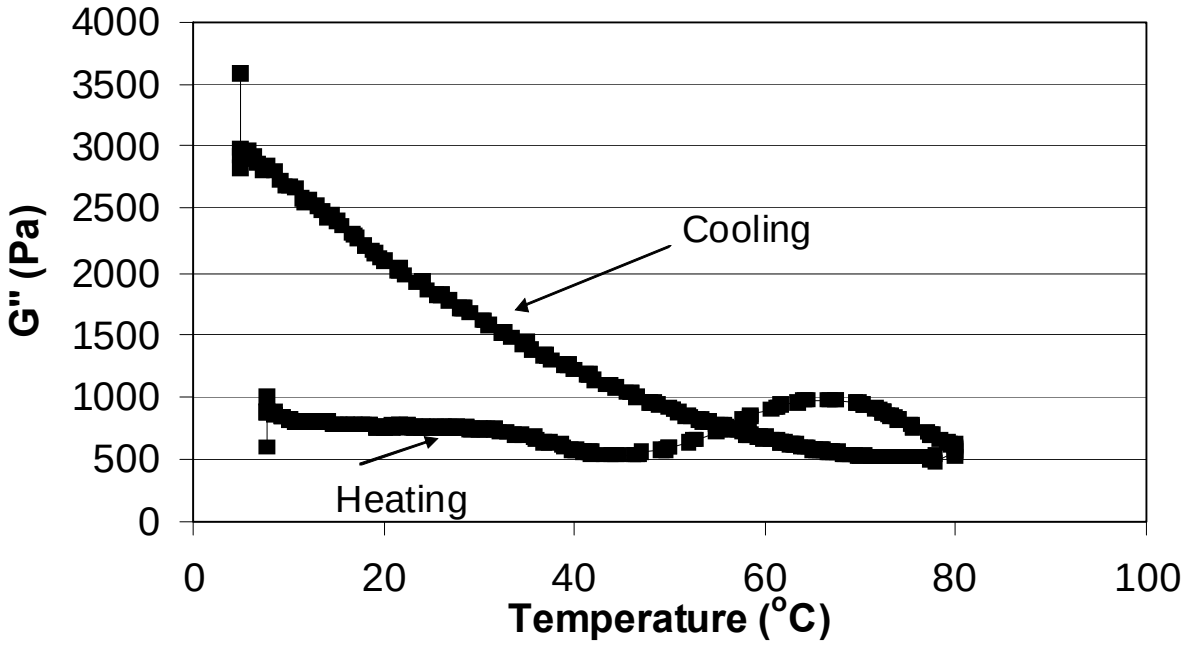

Figure 3-20: The loss modulus (G") of untreated catfish muscle during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min in the absence of added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

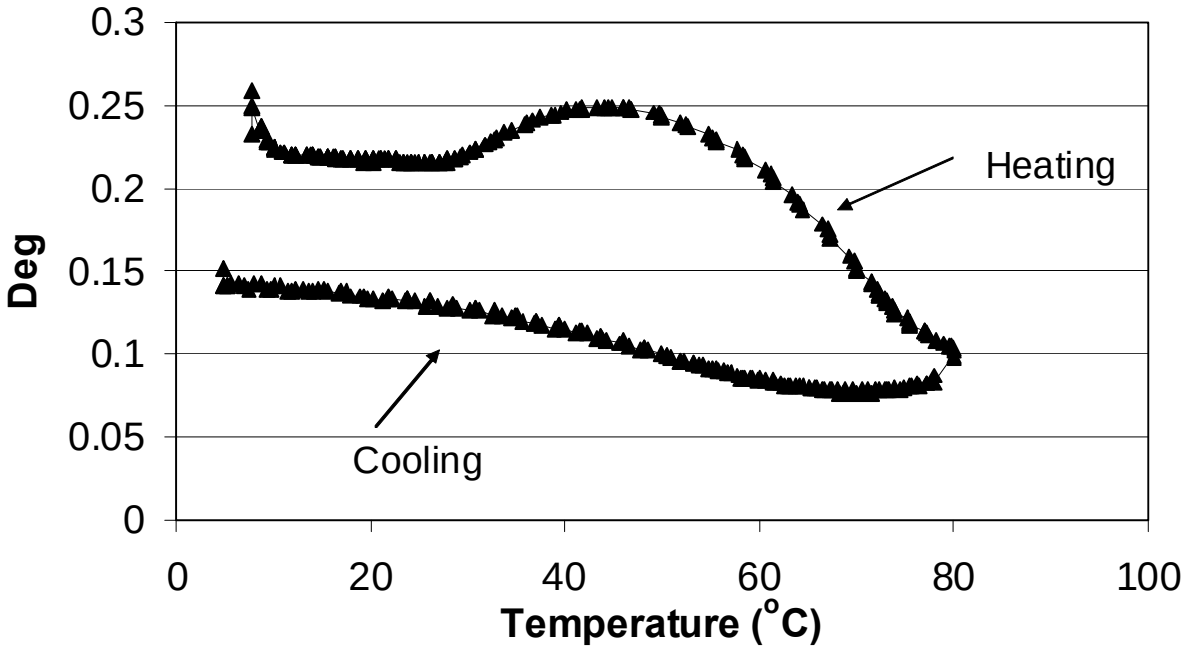

Figure 3-21: The tan delta (G"/G') of untreated catfish muscle during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min in the absence of added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

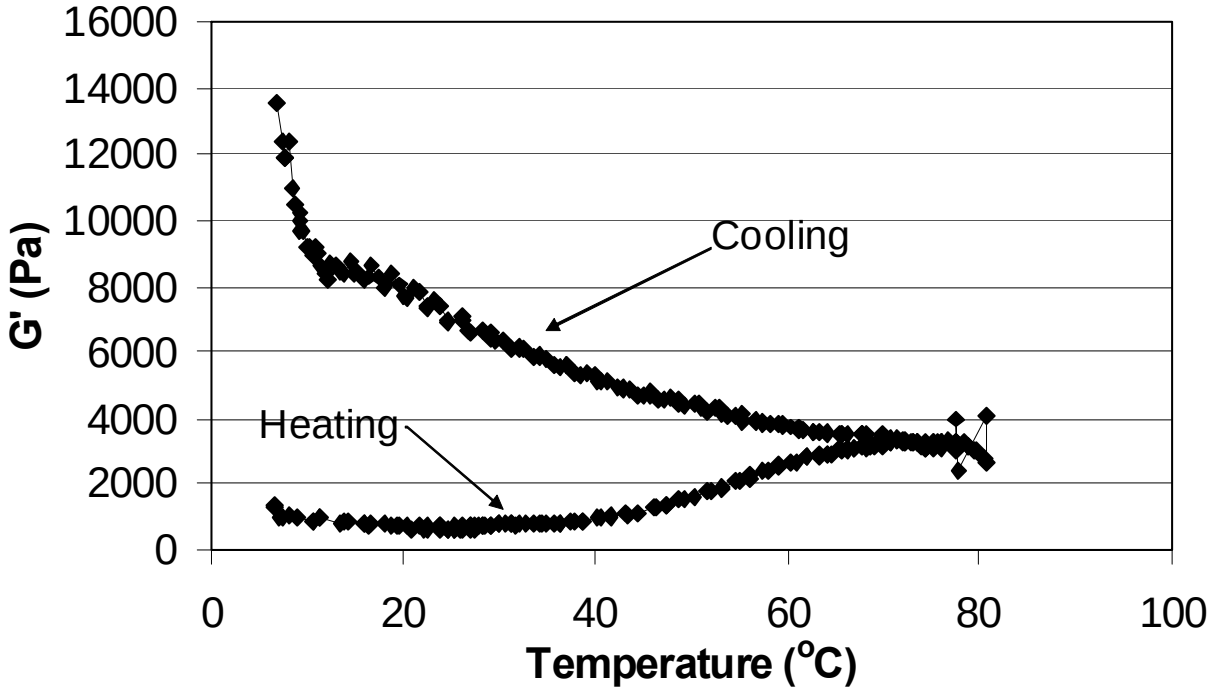

Figure 3-22: The storage modulus (G') of catfish protein isolates produced at pH 2.0 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min with 2% added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

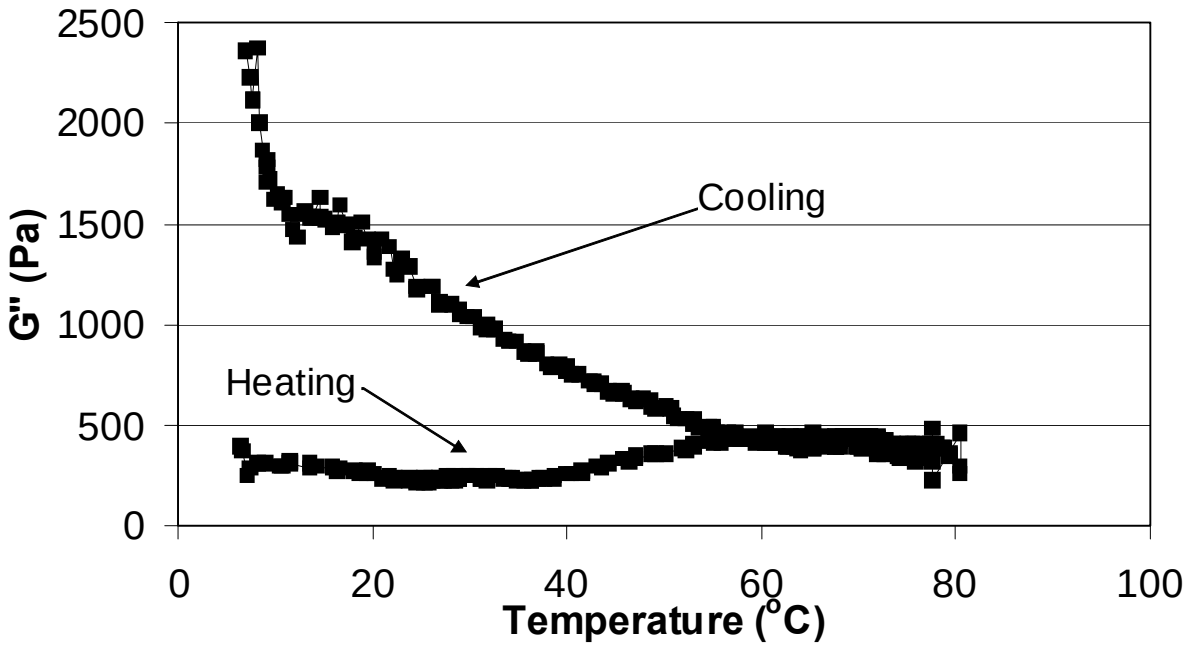

Figure 3-23: The loss modulus (G") of catfish protein isolates produced at pH 2.0 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min with 2% added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

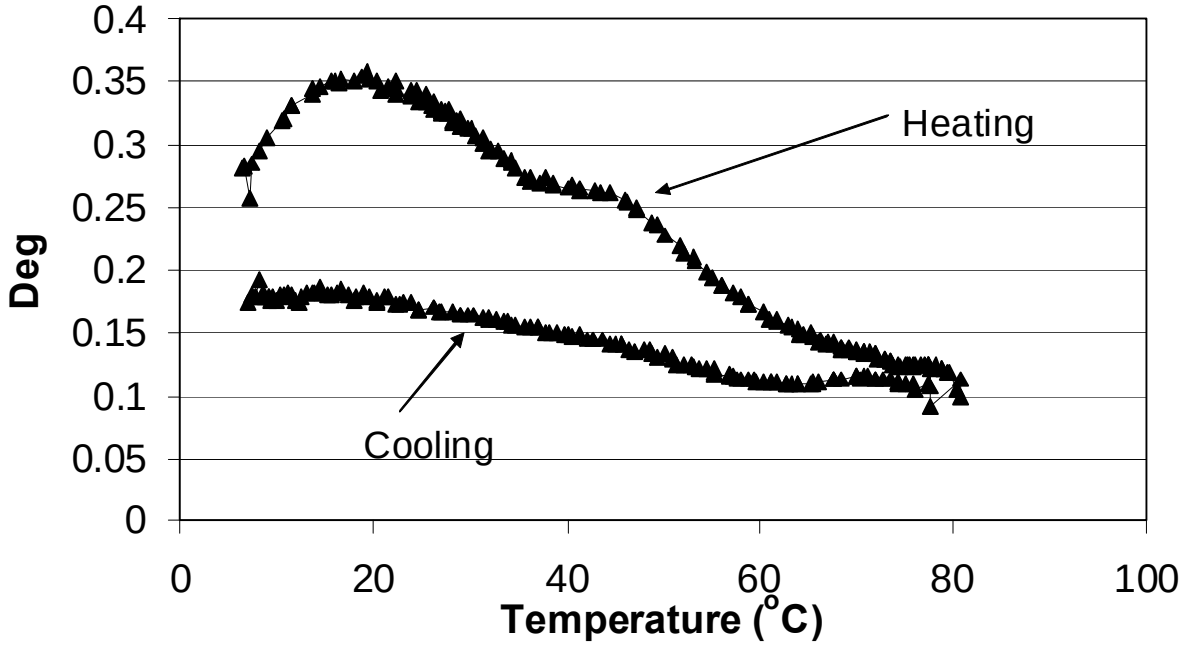

Figure 3-24: The tan delta (G"/G') of catfish protein isolates produced at pH 2.0 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min with 2% added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

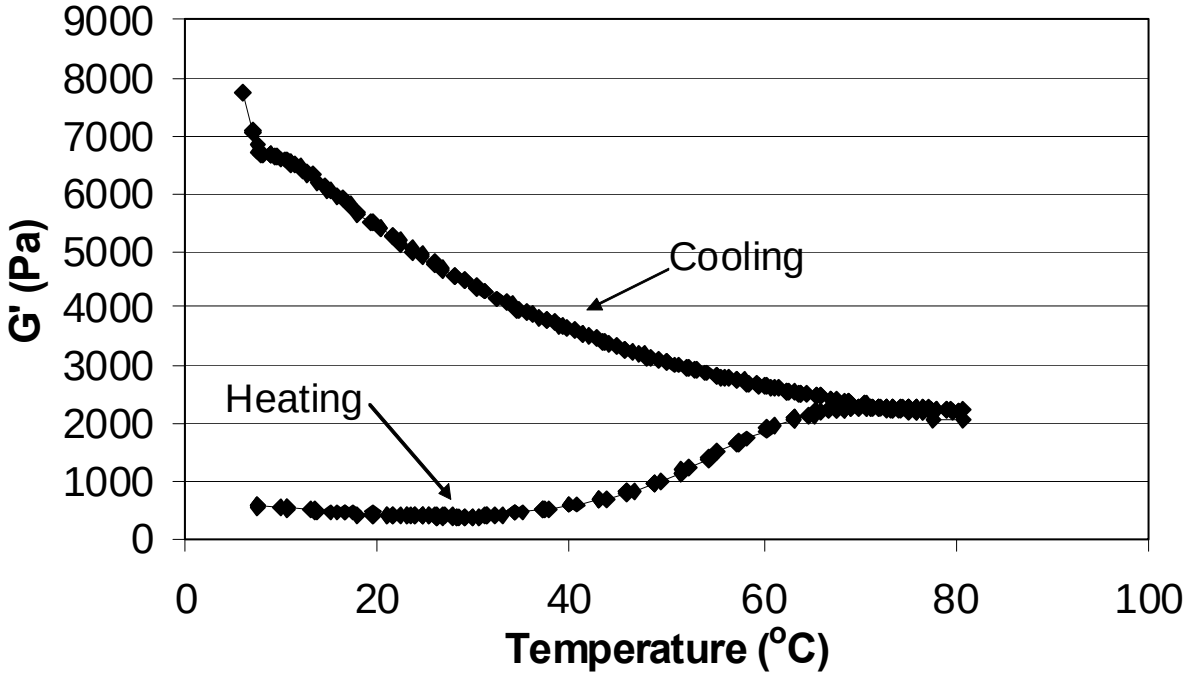

Figure 3-25: The storage modulus (G') of catfish protein isolates produced at pH 2.5 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min with 2% added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

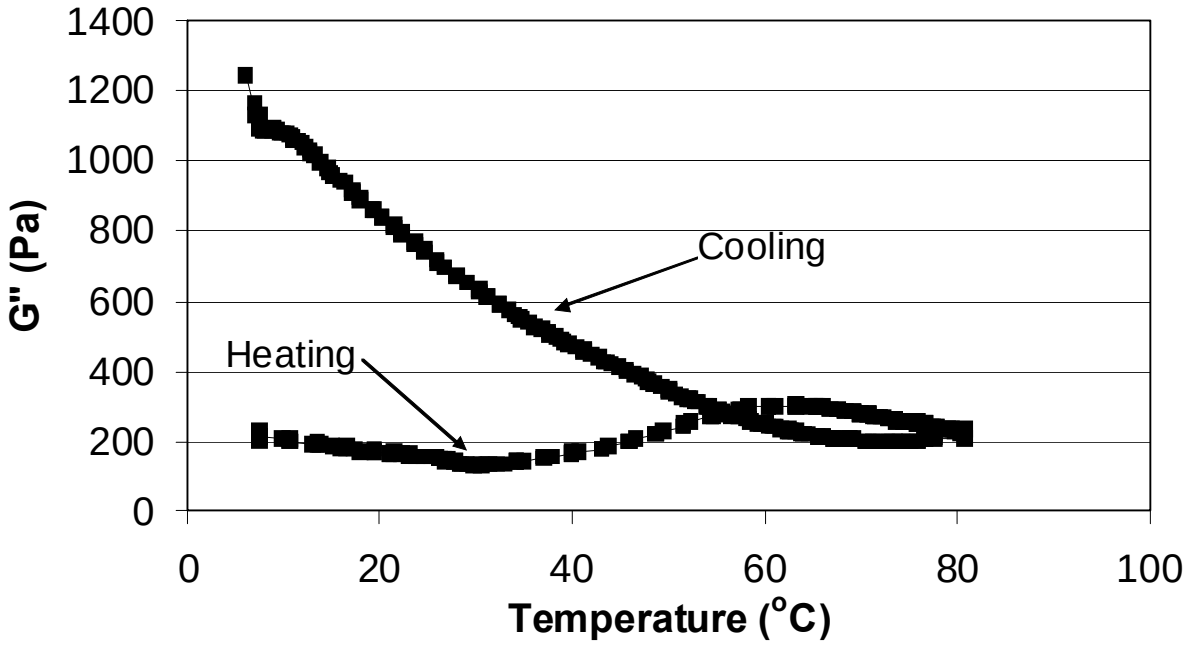

Figure 3-26: The loss modulus (G") of catfish protein isolates produced at pH 2.5 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min with 2% added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

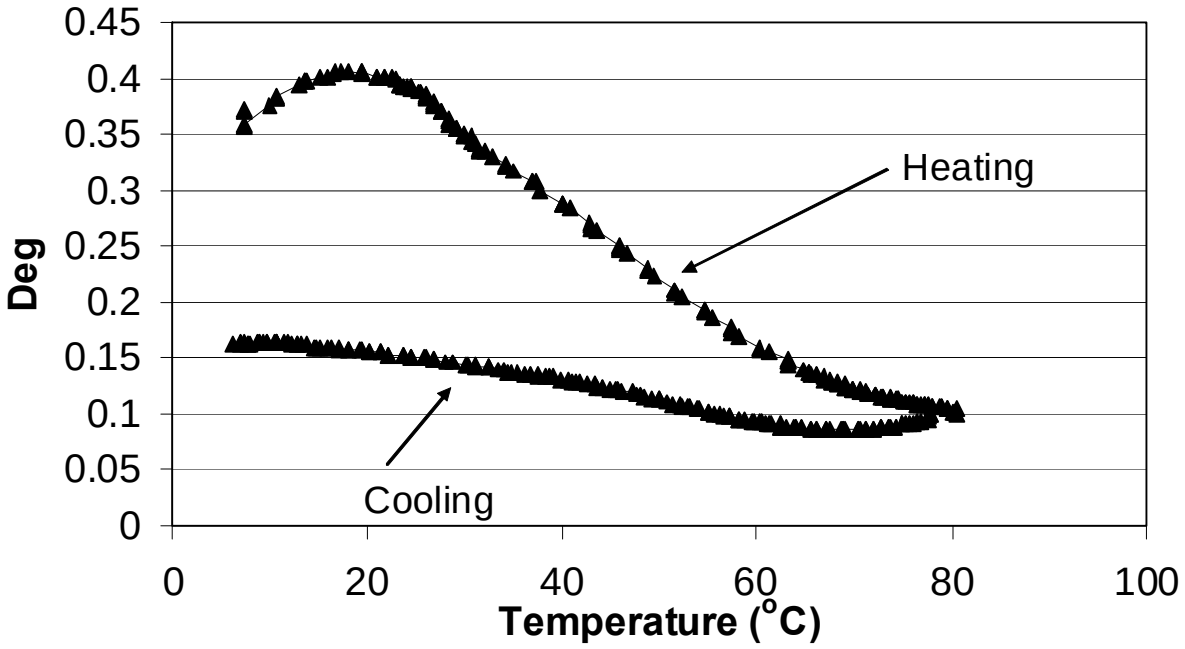

Figure 3-27: The tan delta (G"/G') of catfish protein isolates produced at pH 2.5 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min with 2% added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

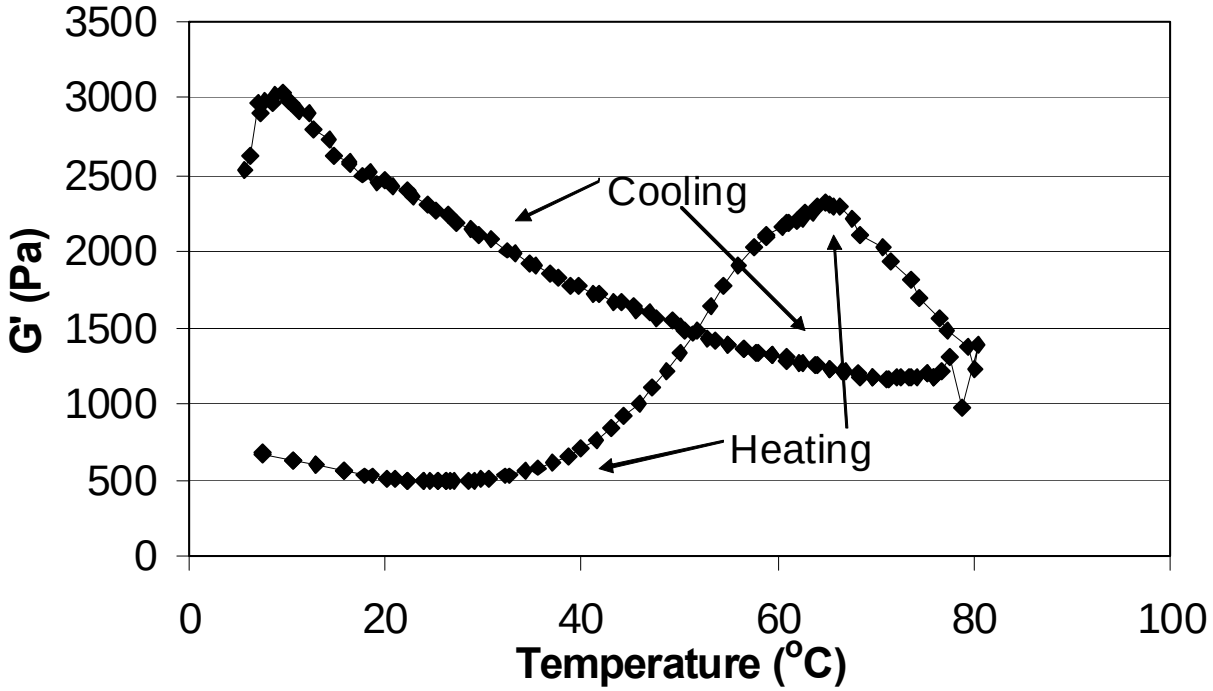

Figure 3-28: The storage modulus (G') of catfish protein isolates produced at pH 3.0 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min with 2% added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

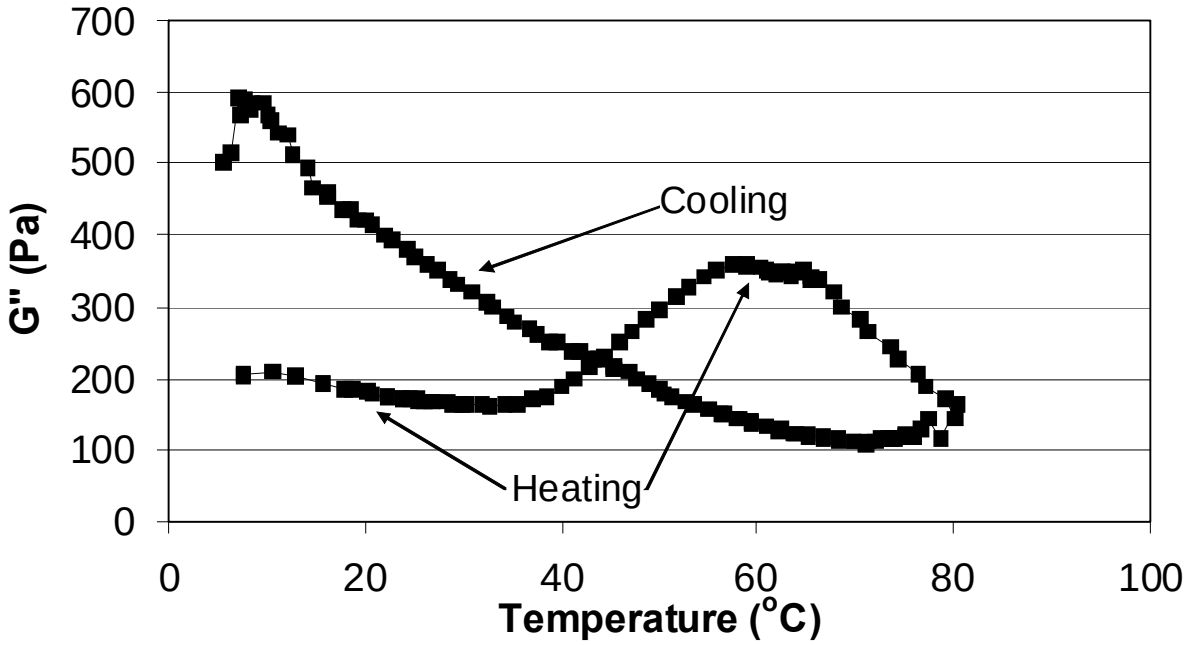

Figure 3-29: The loss modulus (G") of catfish protein isolates produced at pH 3.0 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min with 2% added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

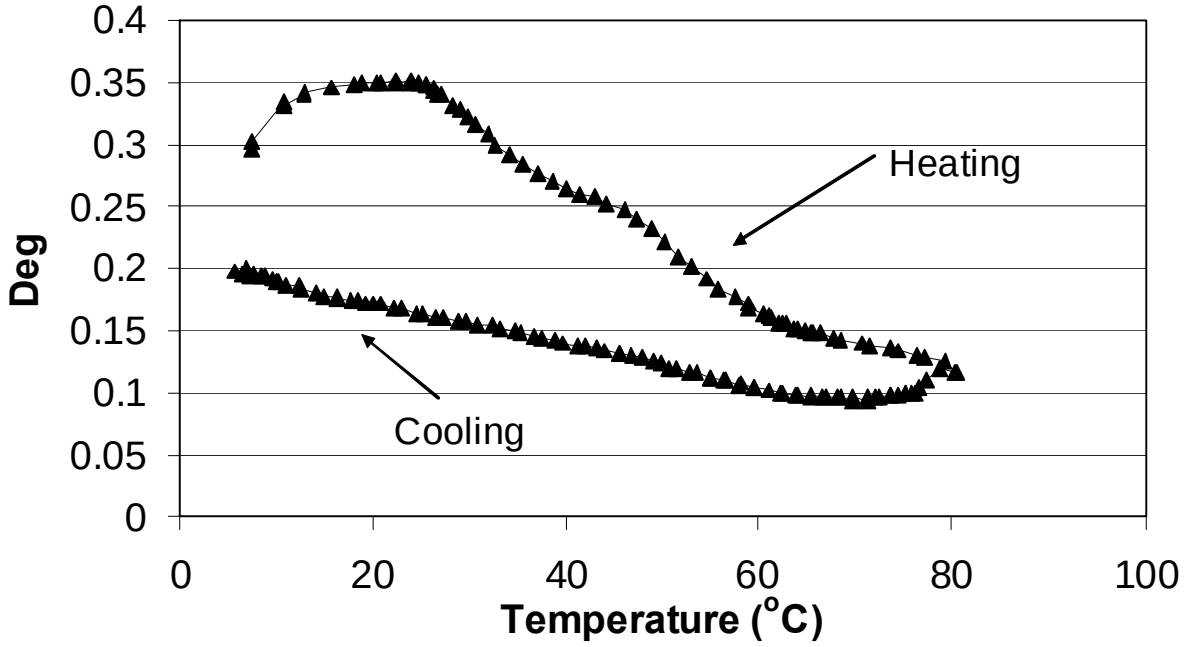

Figure 3-30: The tan delta (G"/G') of catfish protein isolates produced at pH 3.0 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min with 2% added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

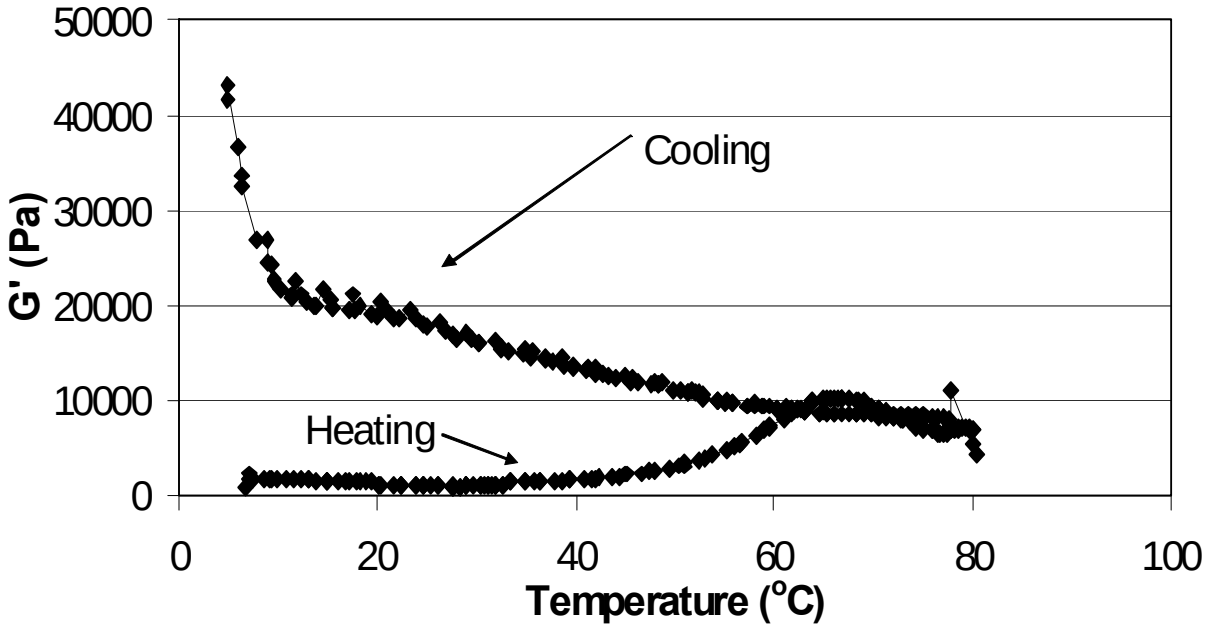

Figure 3-31: The storage modulus (G') of catfish protein isolates produced at pH 10.5 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min with 2% added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

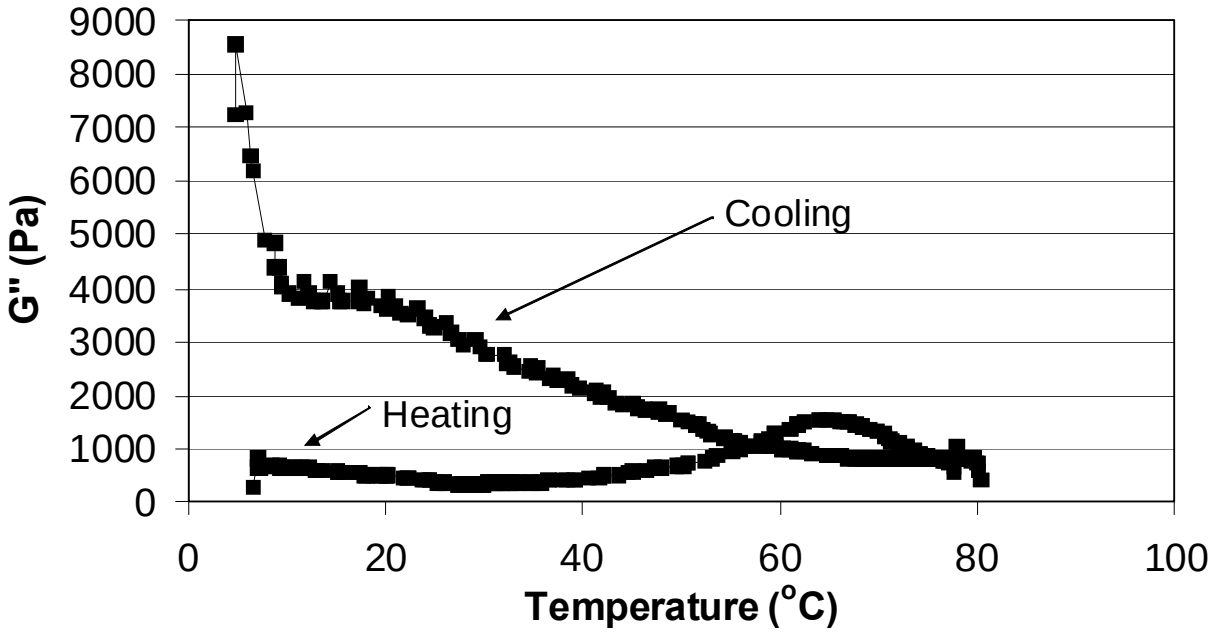

Figure 3-32: The loss modulus (G") of catfish protein isolates produced at pH 10.5 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min with 2% added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

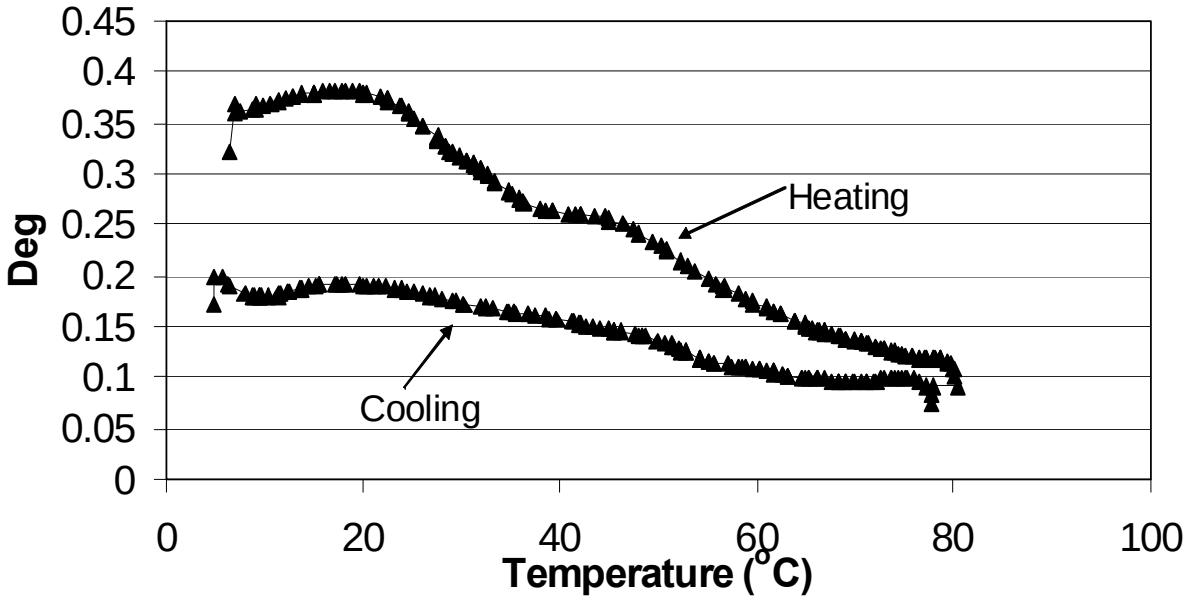

Figure 3-33: The tan delta (G"/G') of catfish protein isolates produced at pH 10.5 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min with 2% added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

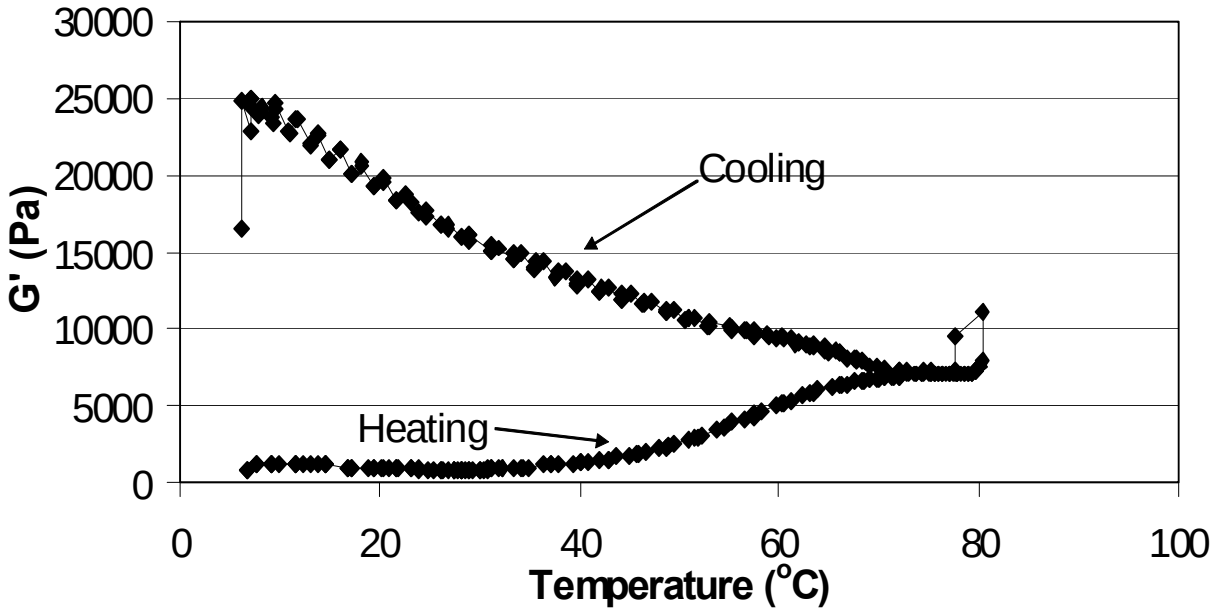

Figure 3-.34: The storage modulus (G') of catfish protein isolates produced at pH 11.0 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min with 2% added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

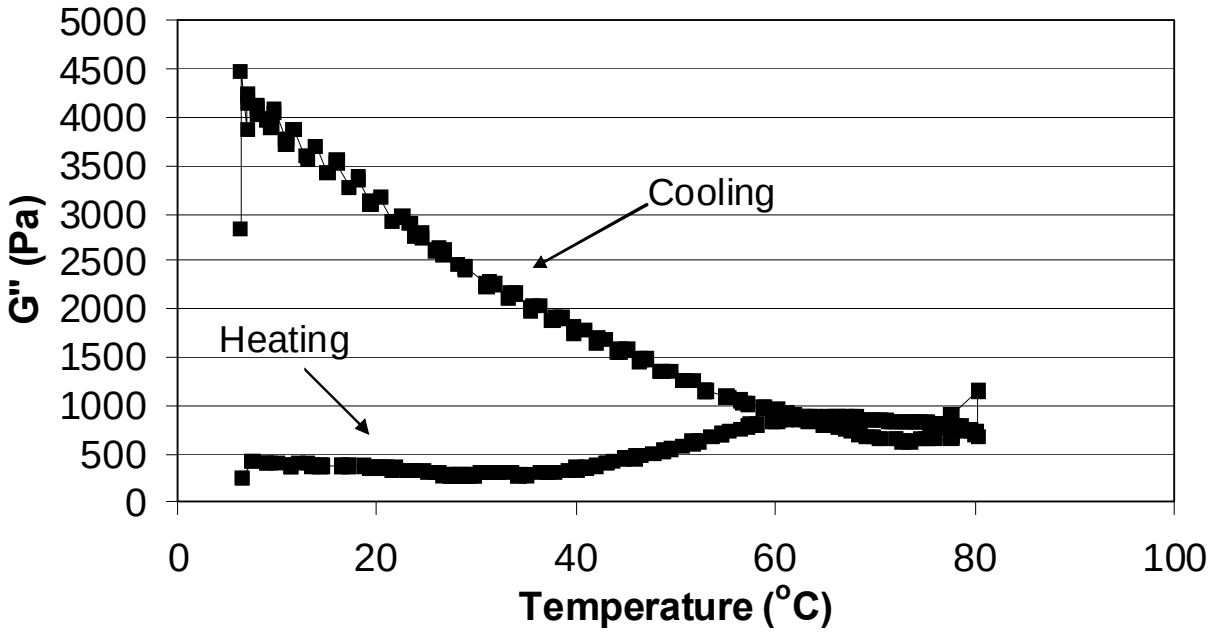

Figure 3-35: The loss modulus (G") of catfish protein isolates produced at pH 11.0 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min with 2% added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

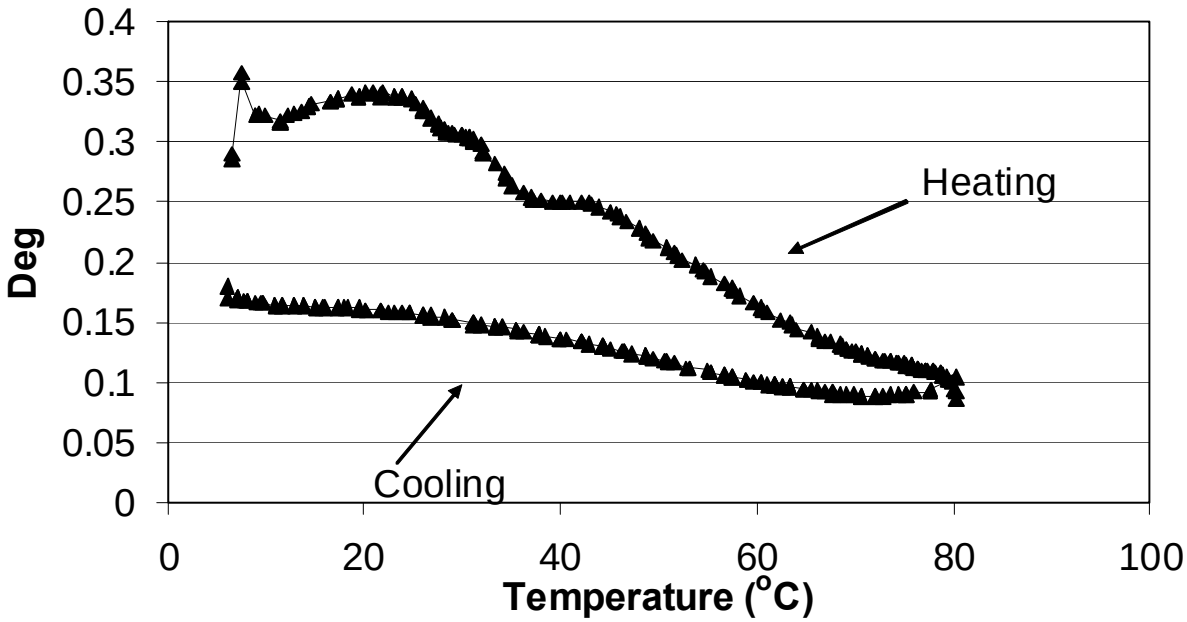

Figure 3-36: The tan delta (G"/G') of catfish protein isolates produced at pH 11.0 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min with 2% added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

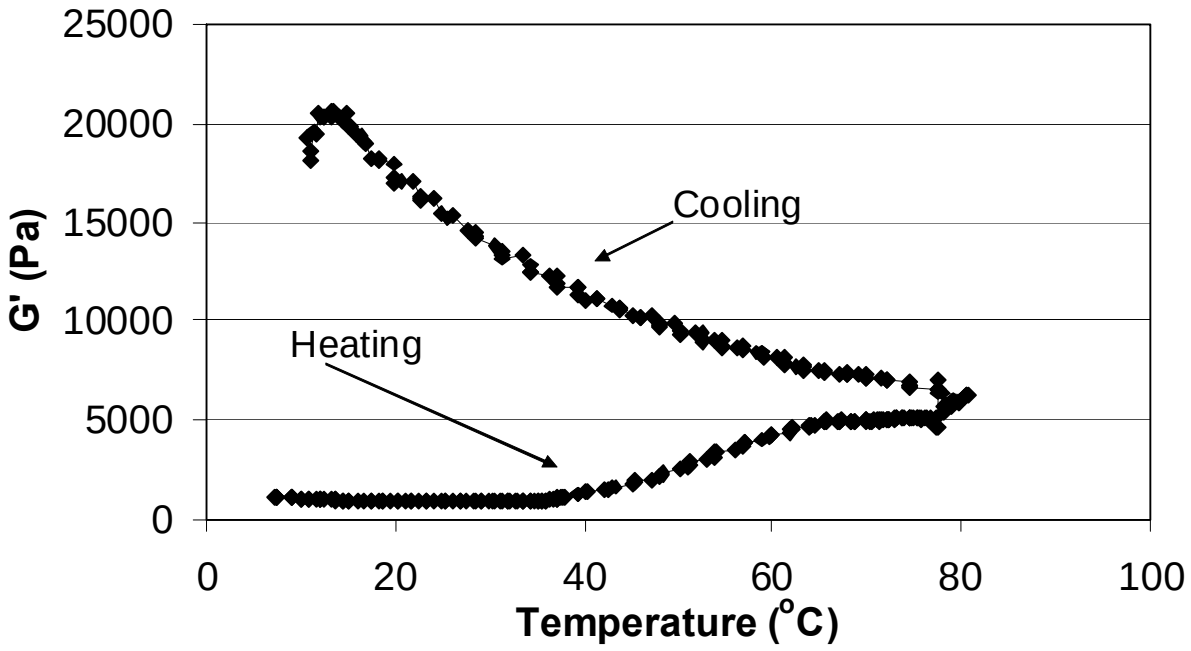

Figure 3-37: The storage modulus (G') of catfish protein isolates produced at pH 11.5 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min with 2% added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

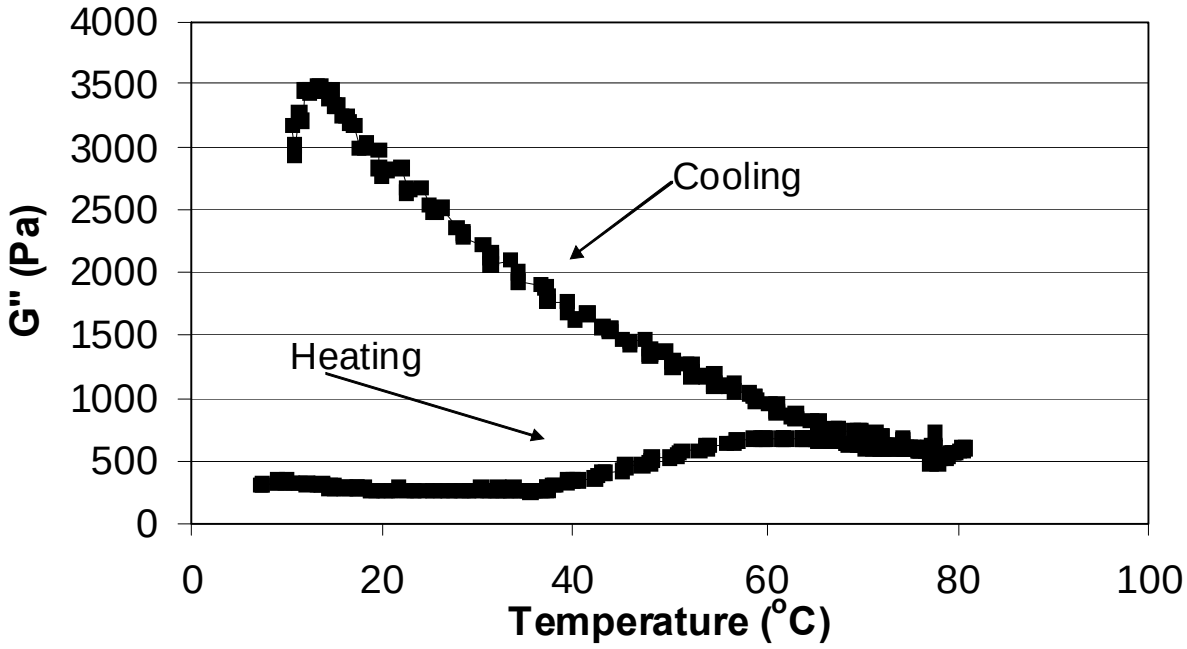

Figure 3-38: The storage modulus (G") of catfish protein isolates produced at pH 11.5 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min with 2% added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

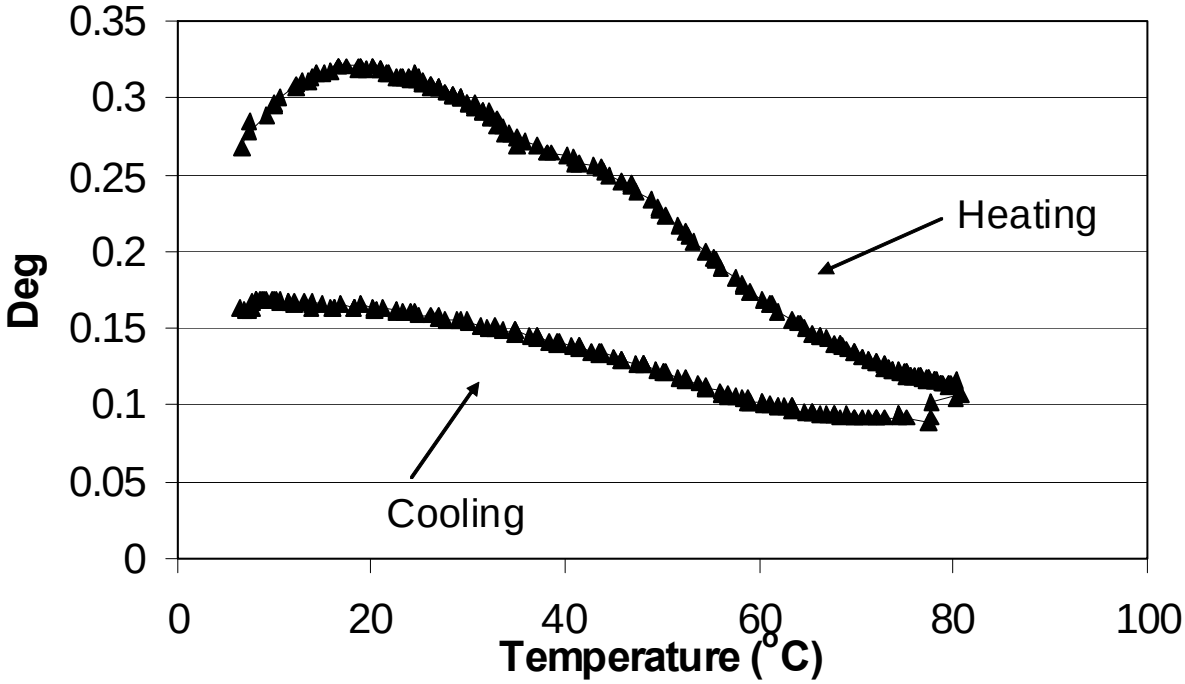

Figure 3-39: The tan delta (G"/G') of catfish protein isolates produced at pH 11.5 during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min with 2% added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

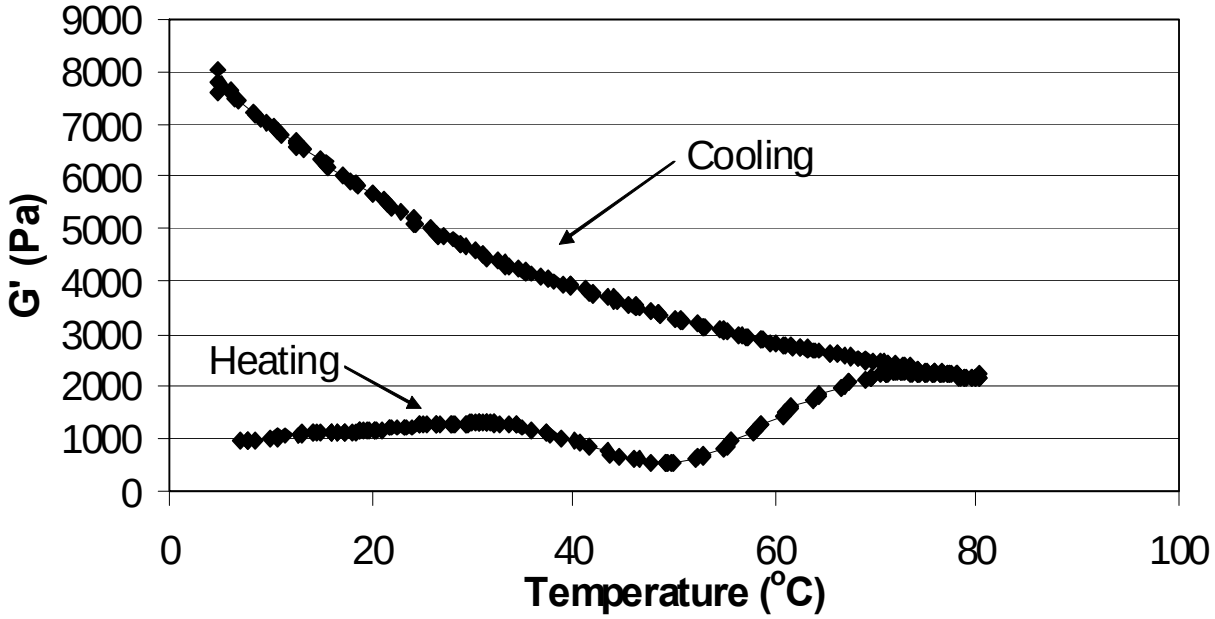

Figure 3-40: The storage modulus (G') of untreated catfish muscle during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min with 2% added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

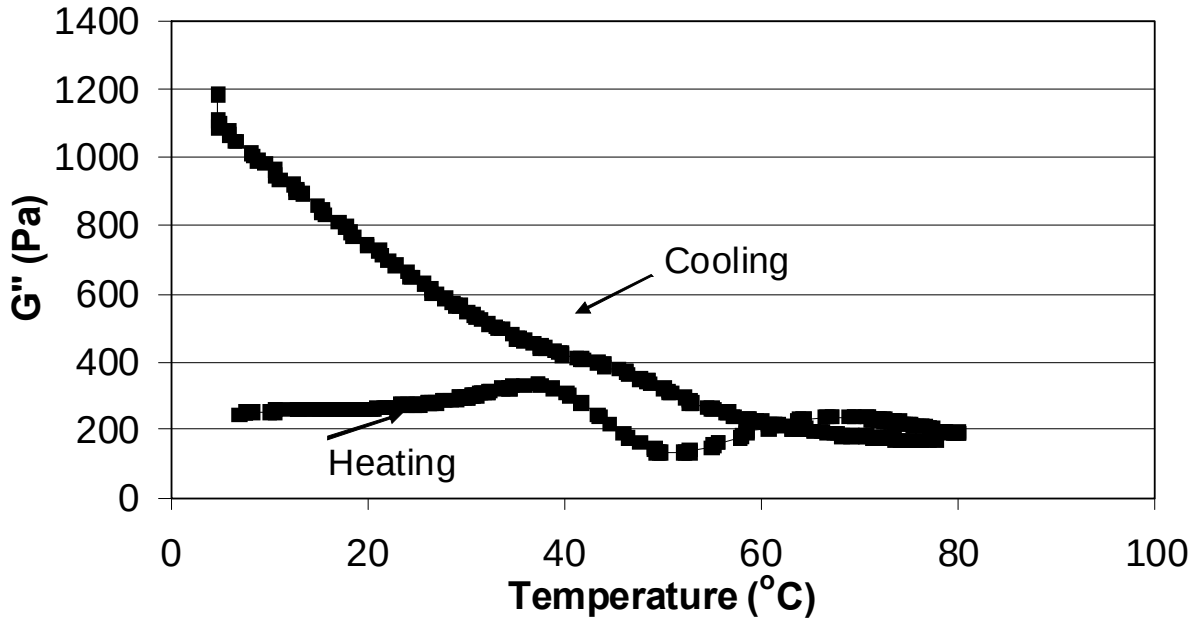

Figure 3-41: The loss modulus (G") of untreated catfish muscle during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min with 2% added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

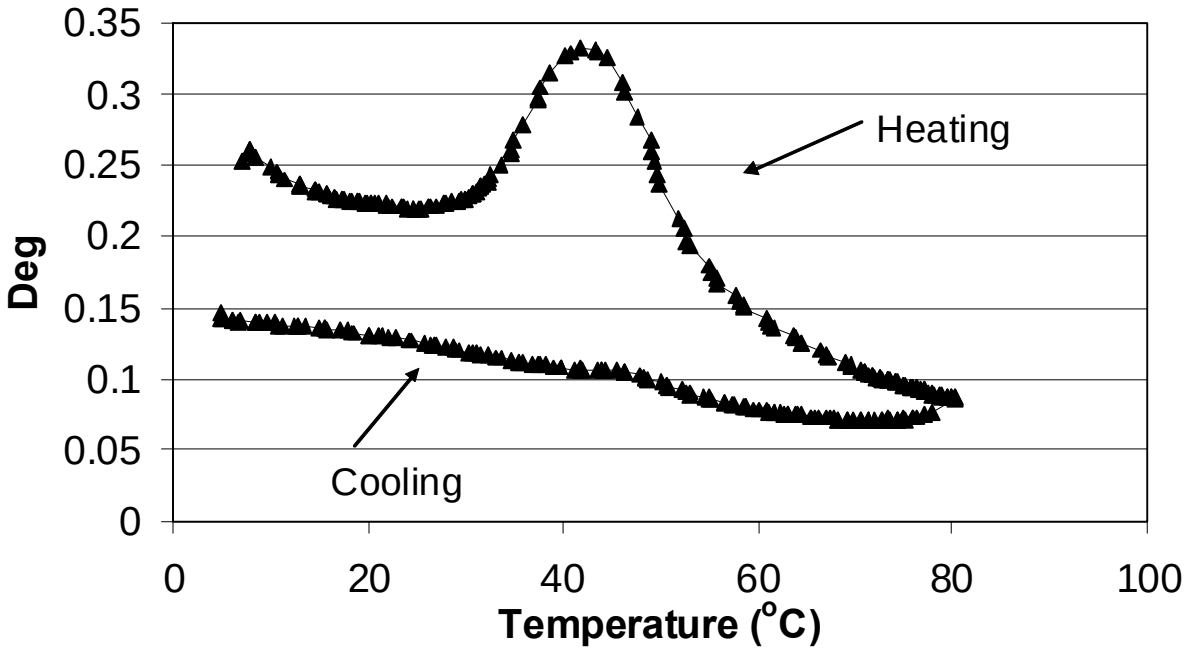

Figure 3-42: The tan delta (G"/G') of untreated catfish muscle during heating and cooling as tested by small strain rheology. The gel was developed at 2°C/min with 2% added NaCl. Testing was conducted with controlled strain, 0.01 at a frequency of 0.1 Hz. Plate on plate geometry was used with a 40 mm cross-hatched acrylic head set to a gap of 1000 μm. The development of thermal gelation during heating and cooling shows the physical transitions of proteins subjected to pH-shift processing.

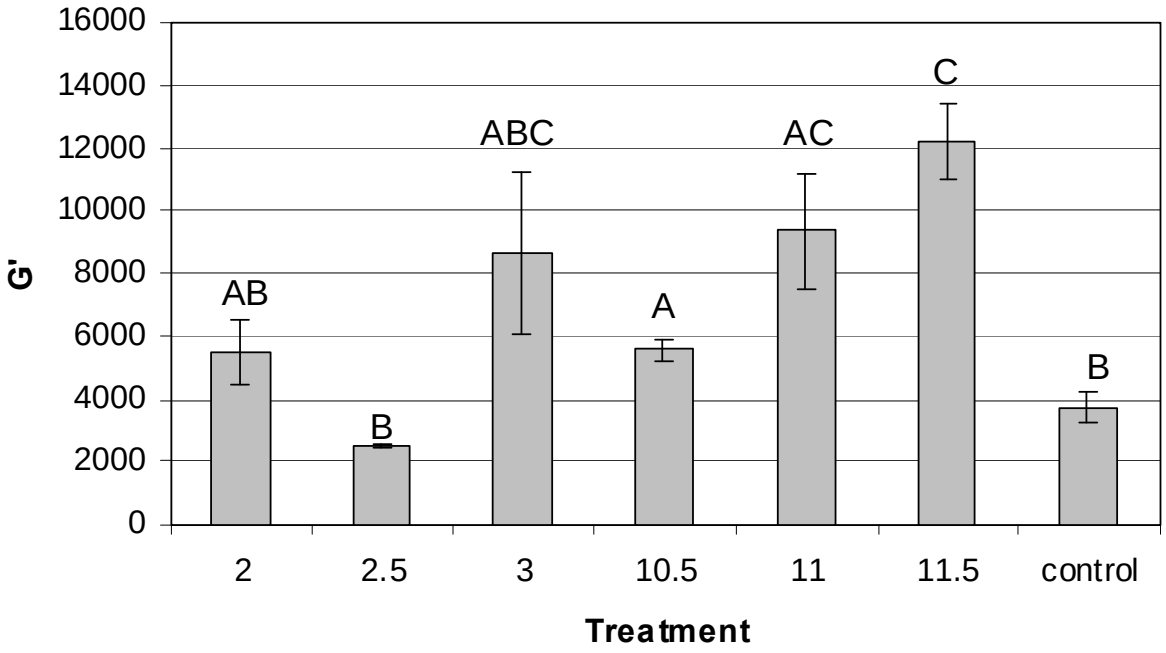

Figure 3-43: The storage modulus (G') of catfish protein isolates compared to unprocessed muscle (control) prior to heating, in the absence of added NaCl. The significant differences present between samples ($p<0.05$) are represented by the letters above the column. Similar letters represent no significant difference ($p>0.05$) between treatments.

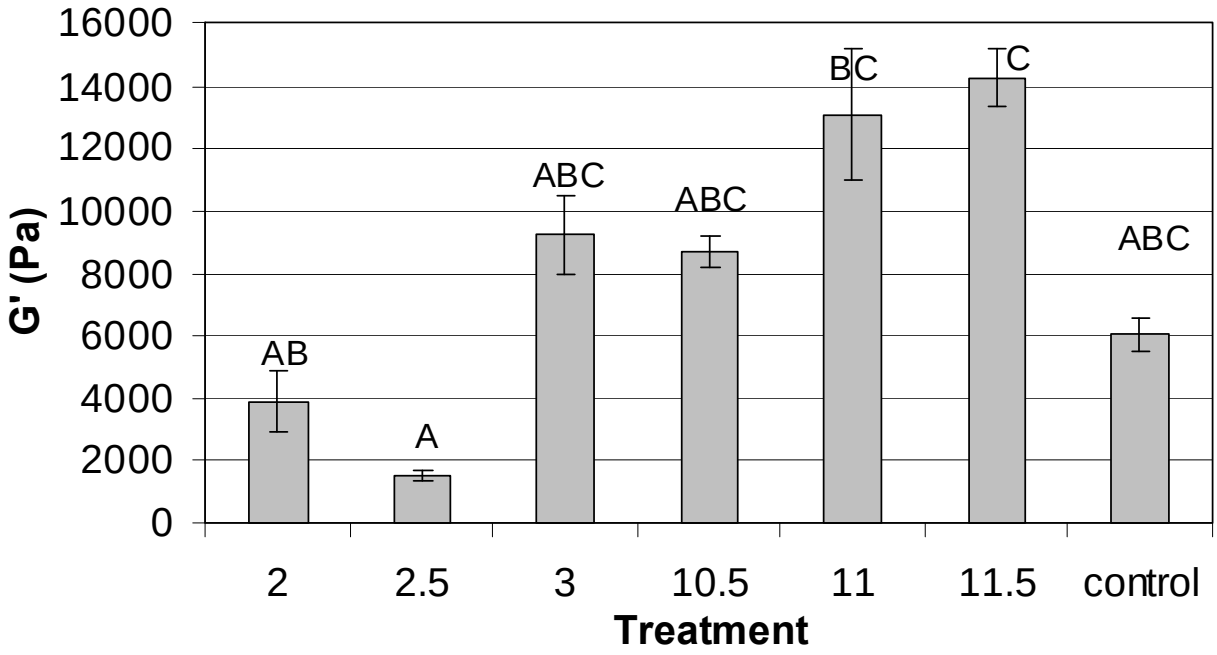

Figure 3-44: The storage modulus (G') of catfish protein isolates compared to unprocessed muscle (control) after heating to 80°C, in the absence of added NaCl. The significant differences present between samples ($p<0.05$) are represented by the letters above the column. Similar letters represent no significant difference ($p>0.05$) between treatments.

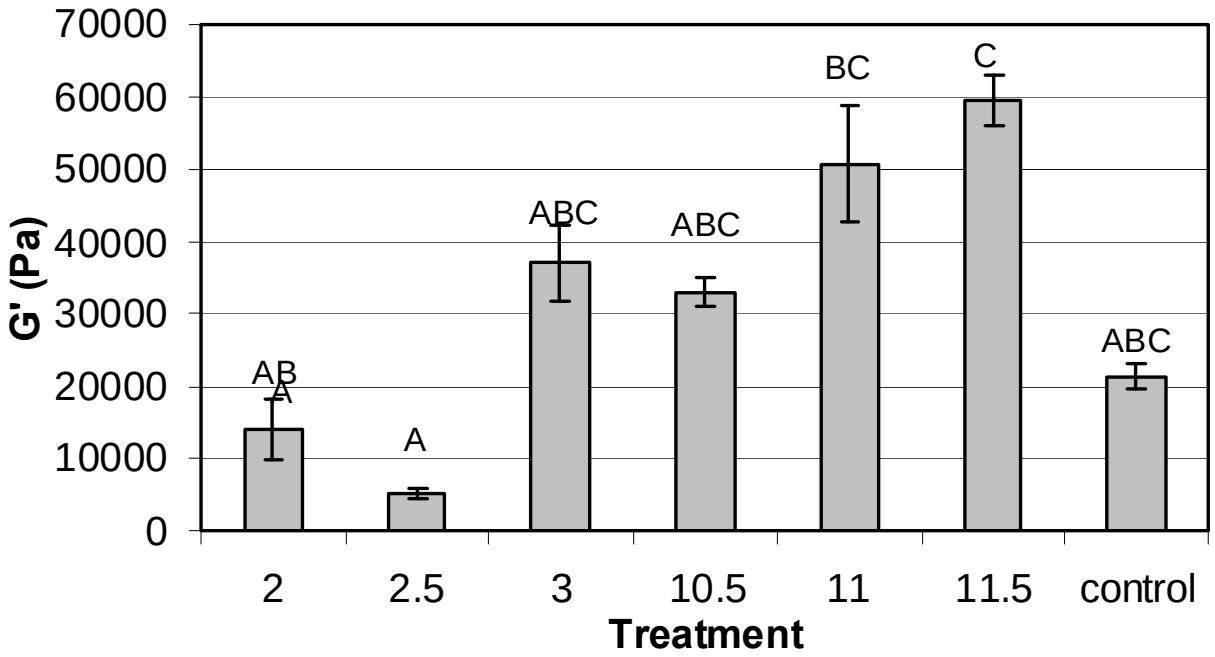

Figure 3-45: The storage modulus (G') of catfish protein isolates compared to unprocessed muscle (control) after cooling to 5°C, in the absence of added NaCl. The significant differences present between samples ($p<0.05$) are represented by the letters above the column. Similar letters represent no significant difference ($p>0.05$) between treatments.

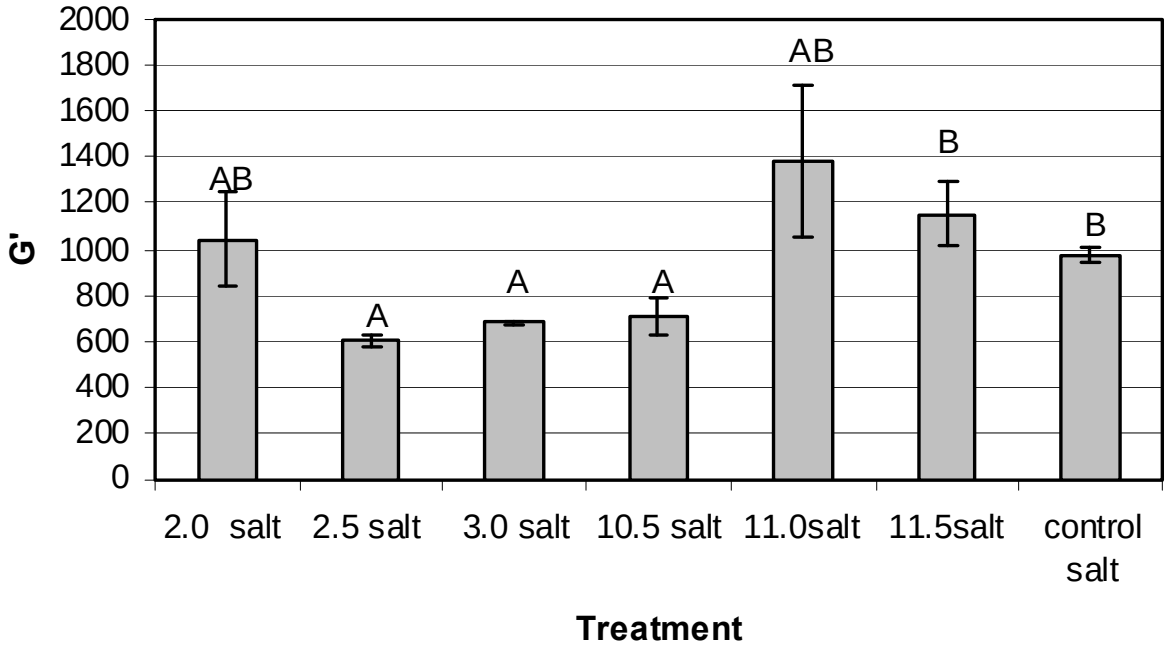

Figure 3-46: The storage modulus (G') of catfish protein isolates compared to unprocessed muscle (control) prior to heating, in the presence of 2% added NaCl. The significant differences present between samples ($p<0.05$) are represented by the letters above the column. Similar letters represent no significant difference ($p>0.05$) between treatments.

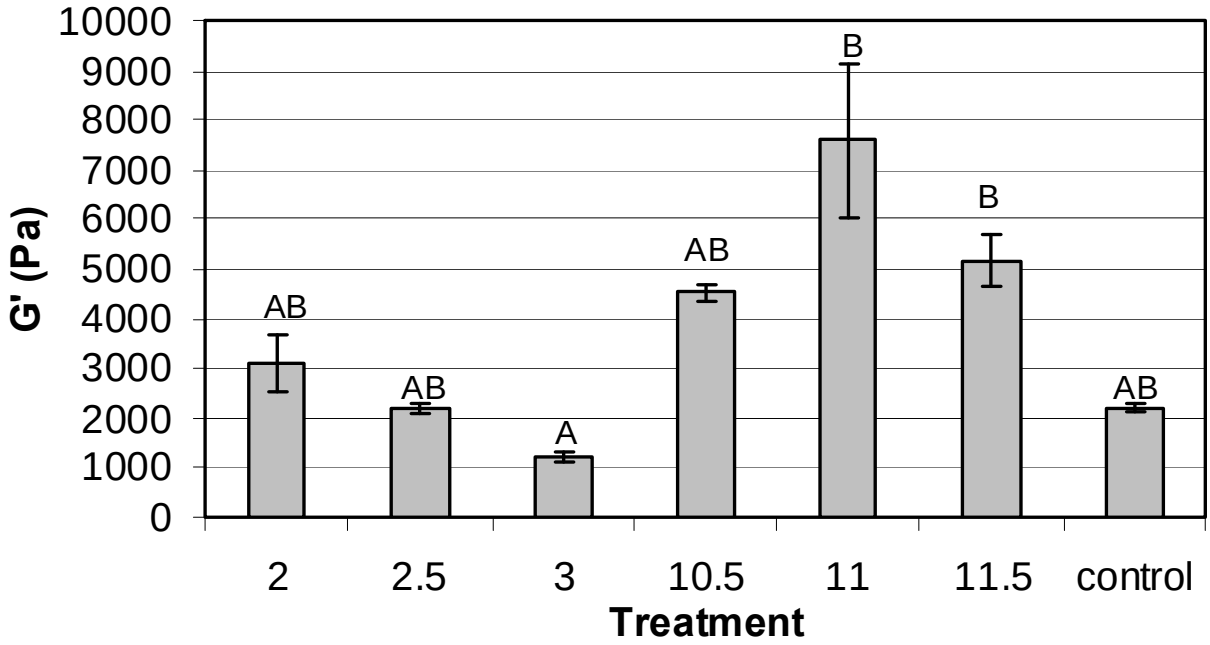

Figure 3-47: The storage modulus (G') of catfish protein isolates compared to unprocessed muscle (control) after heating to 80°C, in the presence of 2% added NaCl. The significant differences present between samples ($p<0.05$) are represented by the letters above the column. Similar letters represent no significant difference ($p>0.05$) between treatments.

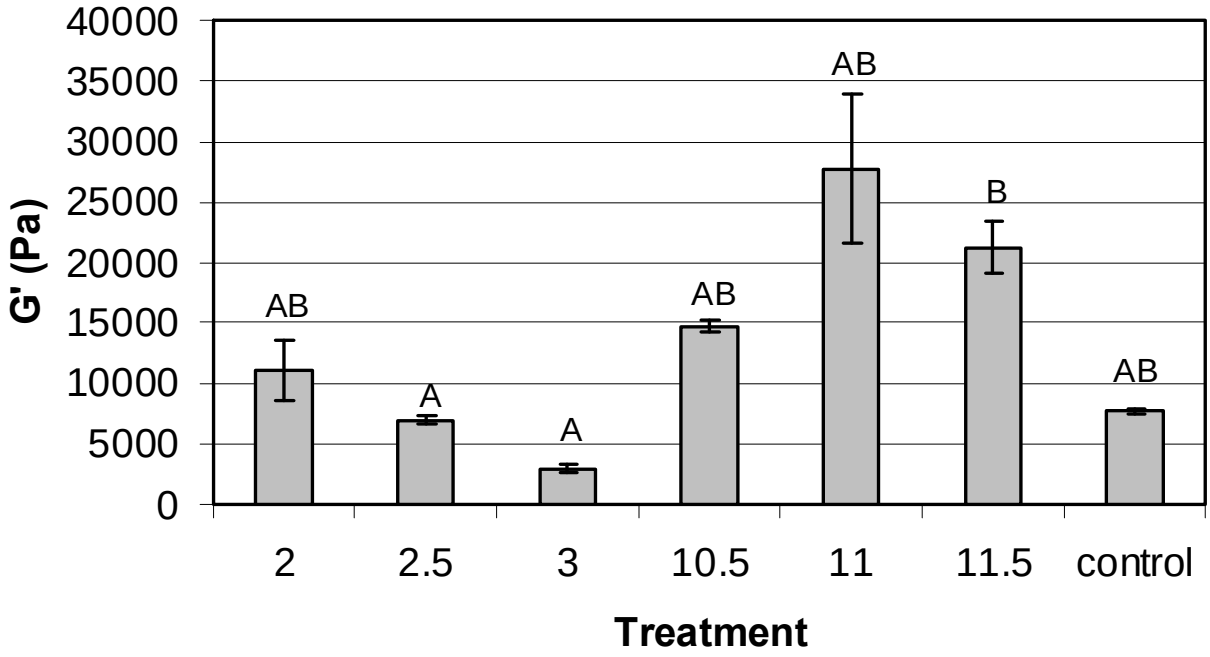

Figure 3-48: The storage modulus (G') of catfish protein isolates compared to unprocessed muscle (control) after cooling to 5°C, in the presence of 2% added NaCl. The significant differences present between samples ($p<0.05$) are represented by the letters above the column. Similar letters represent no significant difference ($p>0.05$) between treatments.

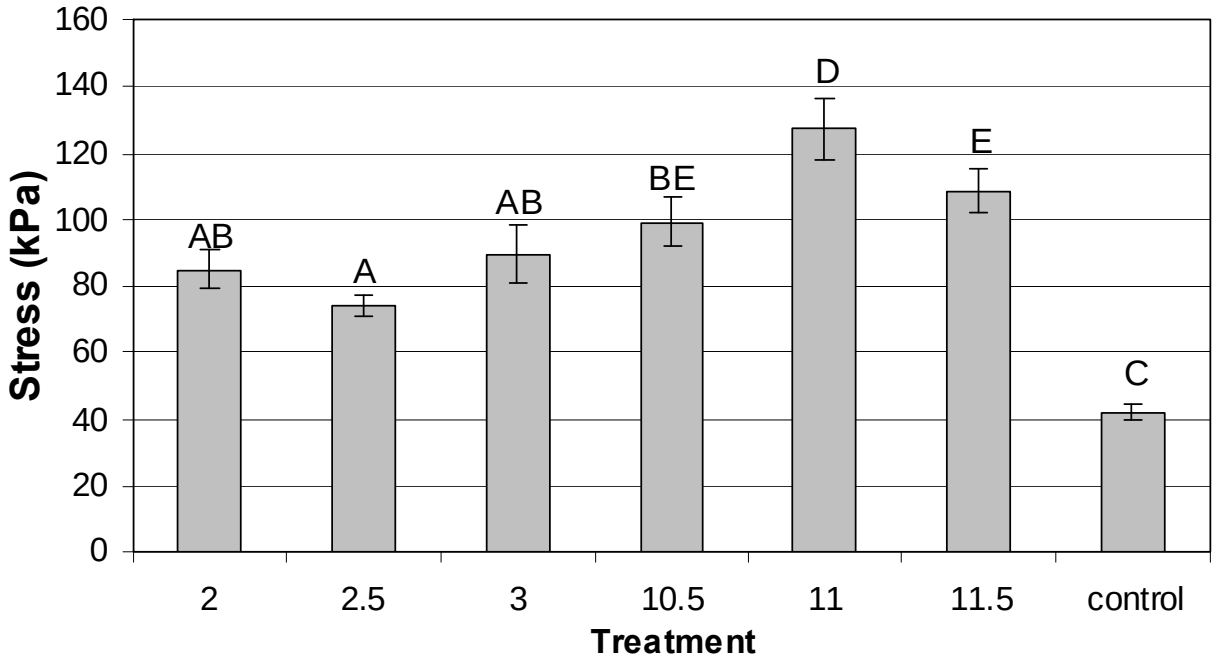

Figure 3-49: The torsion shear stress of catfish protein isolates and unprocessed muscle (control) heat set gels as tested by torsion shear. The gel was developed with static heating (80^{o}C for 20 min) and 48 hrs cold setting time, with 2% added NaCl. The significant differences present between samples ($p<0.05$) are represented by the letters above the column. Similar letters represent no significant difference ($p>0.05$) between treatments.

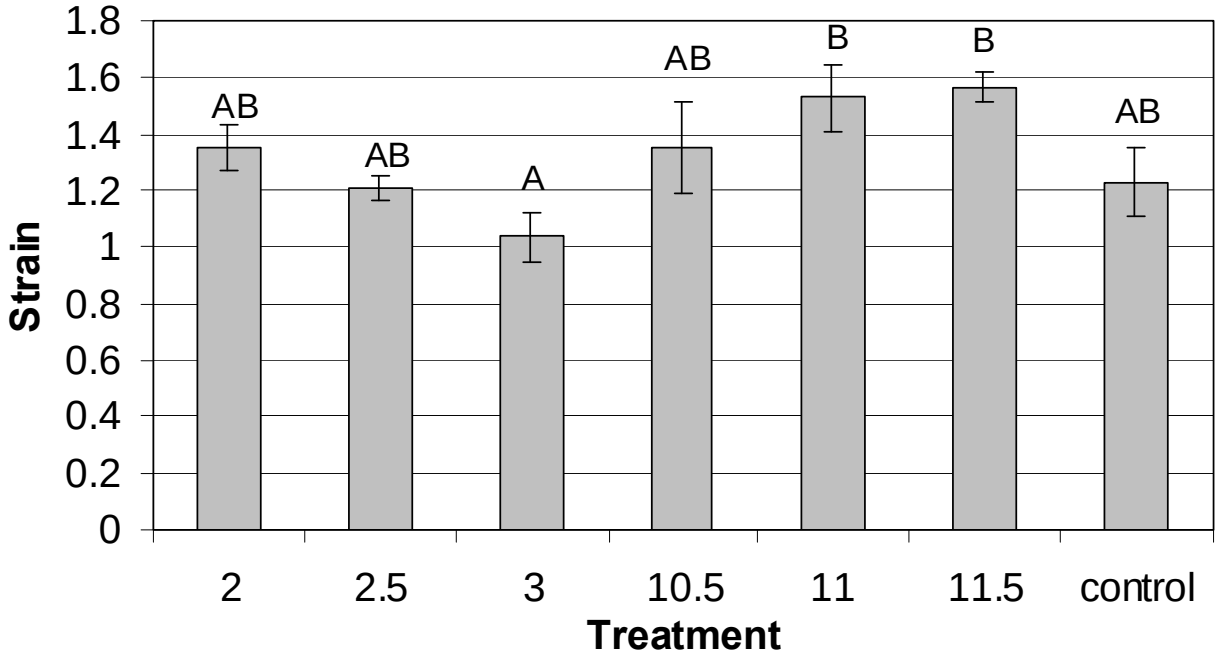

Figure 3-50: The torsion shear strain of pH-shift processed catfish protein isolates and unprocessed muscle (control) heat set gels as tested by torsion shear. The gel was developed with static heating (80^{o}C for 20 min) and 48 hrs cold setting time, with 2% added NaCl. The significant differences present between samples ($p<0.05$) are represented by the letters above the column. Similar letters represent no significant difference ($p>0.05$) between treatments.

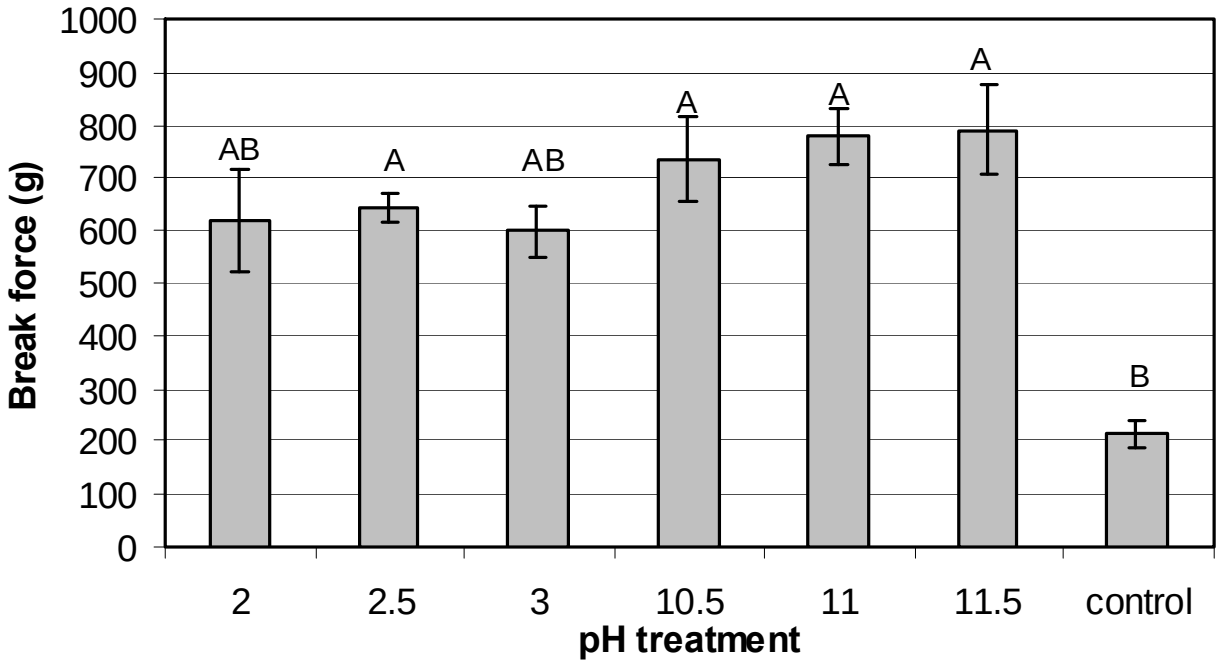

Figure 3-51: The tangential shear stress of pH-shift processed catfish protein isolates and unprocessed muscle (control) heat set gels as tested by punch testing. The gel was developed with static heating (80^{o}C for 20 min) and 48 hrs cold setting time, with 2% added NaCl. The significant differences present between samples ($p<0.05$) are represented by the letters above the column. Similar letters represent no significant difference ($p>0.05$) between treatments.

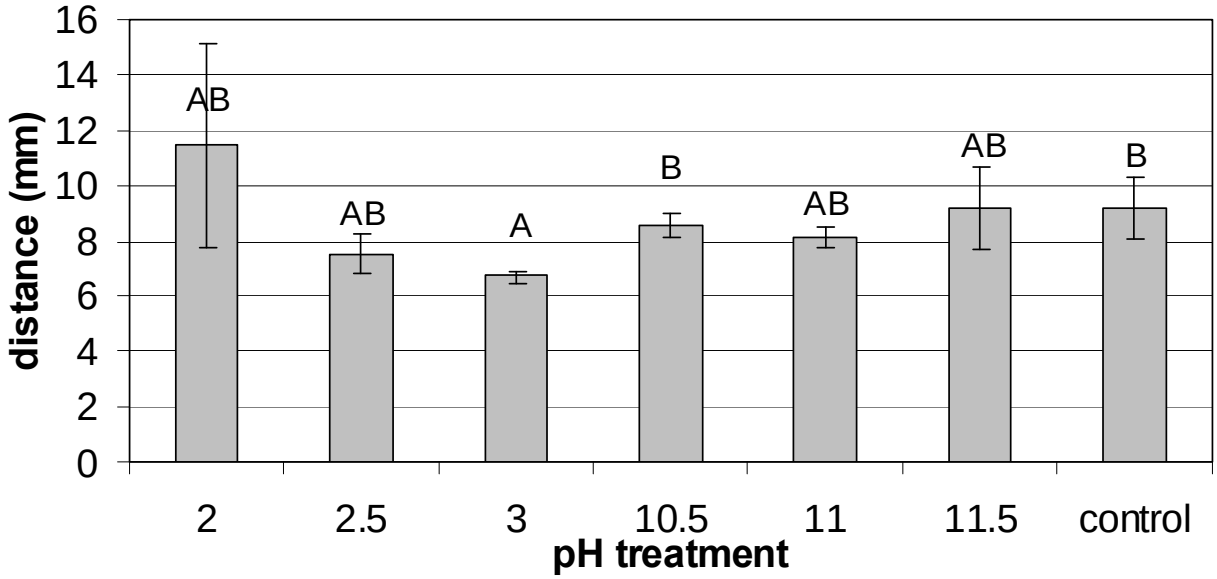

Figure 3-52: The tangential shear strain of pH-shift processed catfish protein isolates and unprocessed muscle (control) heat set gels as tested by punch testing. The gel was developed with static heating (80^{o}C for 20 min) and 48 hrs cold setting time, with 2% added NaCl. The significant differences present between samples ($p<0.05$) are represented by the letters above the column. Similar letters represent no significant difference ($p>0.05$) between treatments.

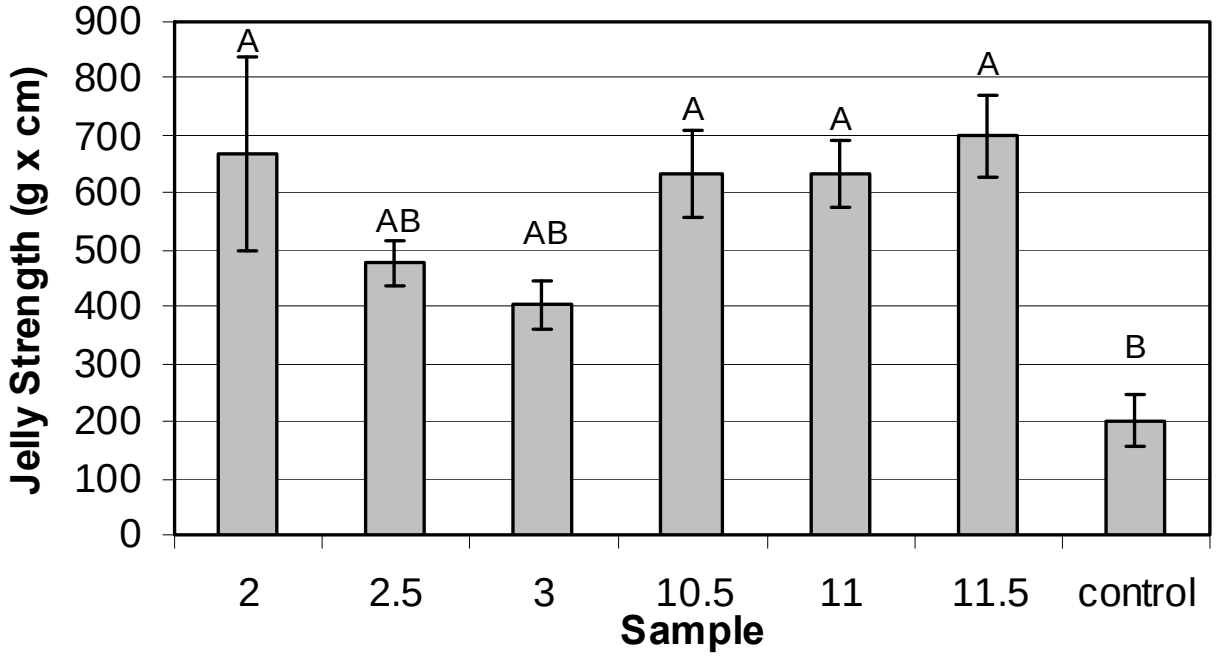

Figure 3-53: The jelly strength of pH-shift processed catfish protein isolates and unprocessed muscle (control) heat set gels. Jelly strength was calculated from the punch test values and is tangential shear stress*shear strain.. The gel (with 2% added NaCl) was developed with static heating (80^{o}C for 20 min) followed by 48 hrs cold setting time. The significant differences present between samples ($p<0.05$) are represented by the letters above the column. Similar letters represent no significant difference ($p>0.05$) between treatments.

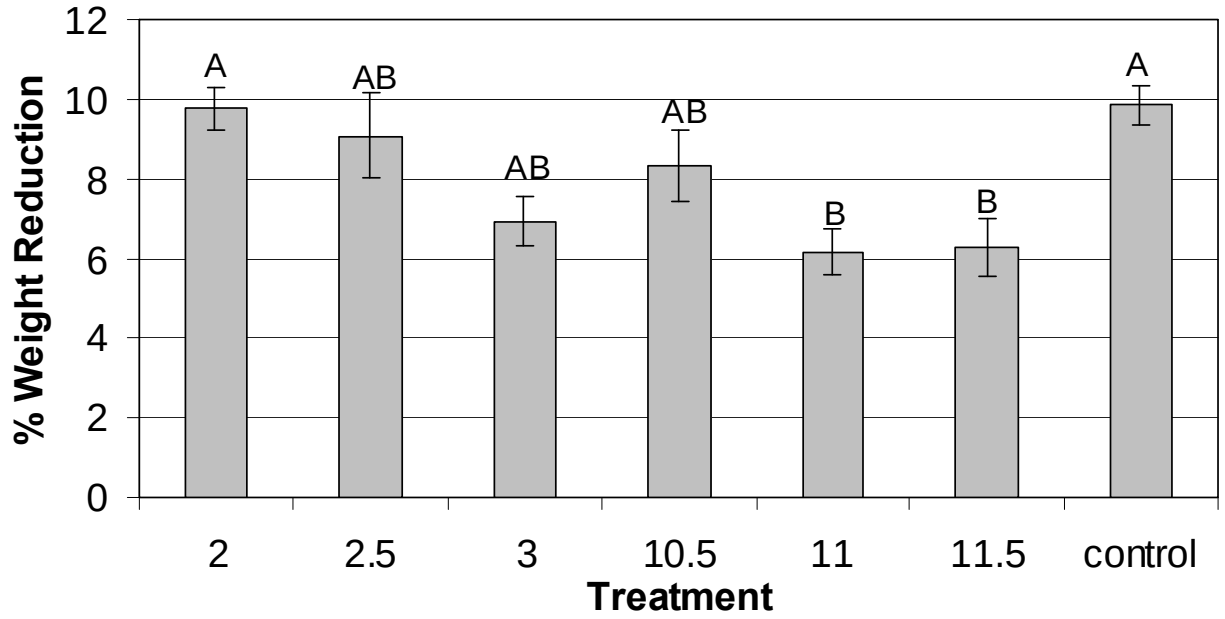

Figure 3-54: The expressible moisture of pH-shift processed catfish protein isolates and unprocessed muscle (control) heat set gels as tested by placing a 3 Kg weight on a gel slice, 1.8 cm in diameter and 3 mm in thickness. The weight loss is assumed to be water leakage from the gel matrix and is a determinant of water holding capacity of the gel matrix. The gel was developed with static heating (80^{o}C for 20 min) and 48 hrs cold setting time, with 2% added NaCl. The significant differences present between samples ($p<0.05$) are represented by the letters above the column. Similar letters represent no significant difference ($p>0.05$) between treatments.

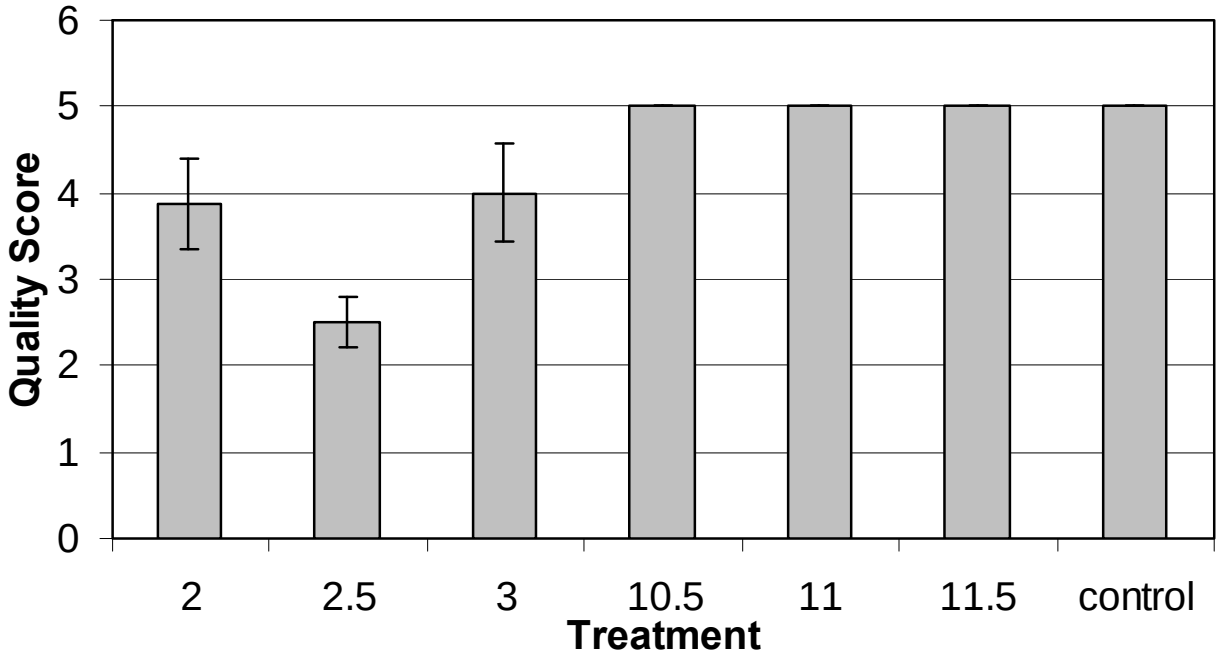

Figure 3-55: The shear stress of pH-shift processed catfish protein isolates and unprocessed muscle (control) heat set gels as tested by folding a 3 mm gel slice, 1.8 cm in diameter, in half then quarters. The gel score is assessed as 5= no crack when folded into a quarter, 4=no crack when folded into half but crack when folded into quarter, 3= no crack when folded in half but split when folded into quarter, 2=crack when folded into half, 1=broke and split into halves. The gel was developed with static heating (80^{o}C for 20 min) 48 hrs cold setting time, with 2% added NaCl. No significant differences were present between any of the samples tested.

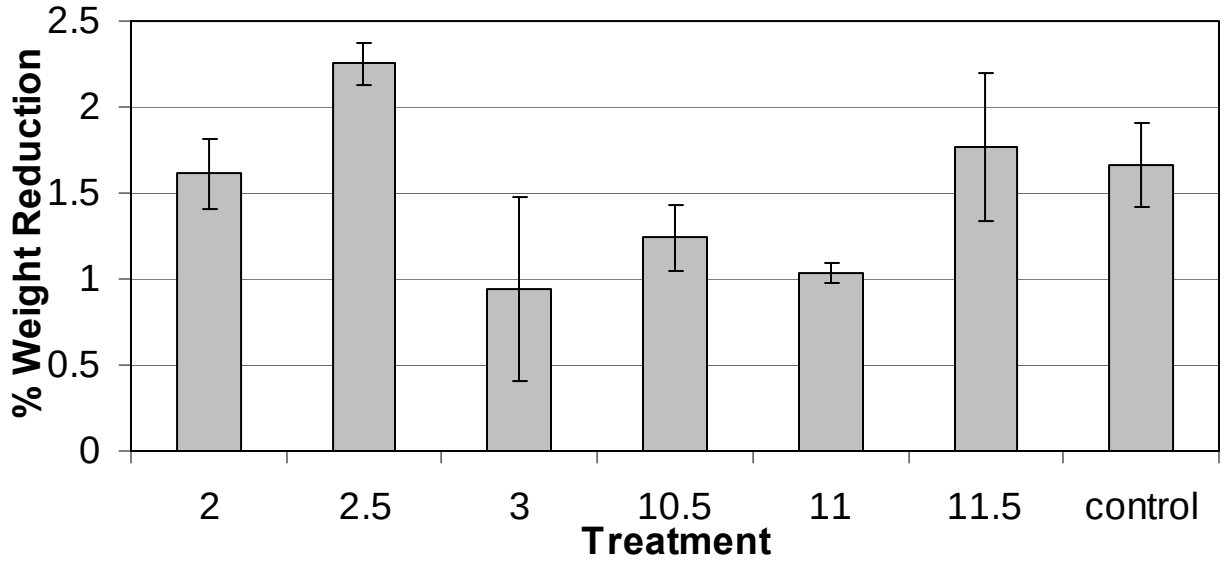

Figure 3-56: The weight loss of pH-shift processed catfish protein isolates and unprocessed muscle (control) heat set gels with 2% added NaCl developed with static heating (80^{o}C for 20 min), 48 hrs cold setting (4°C), as compared to the weight before cooking and setting. No significant differences were present between samples ($p>0.05$).

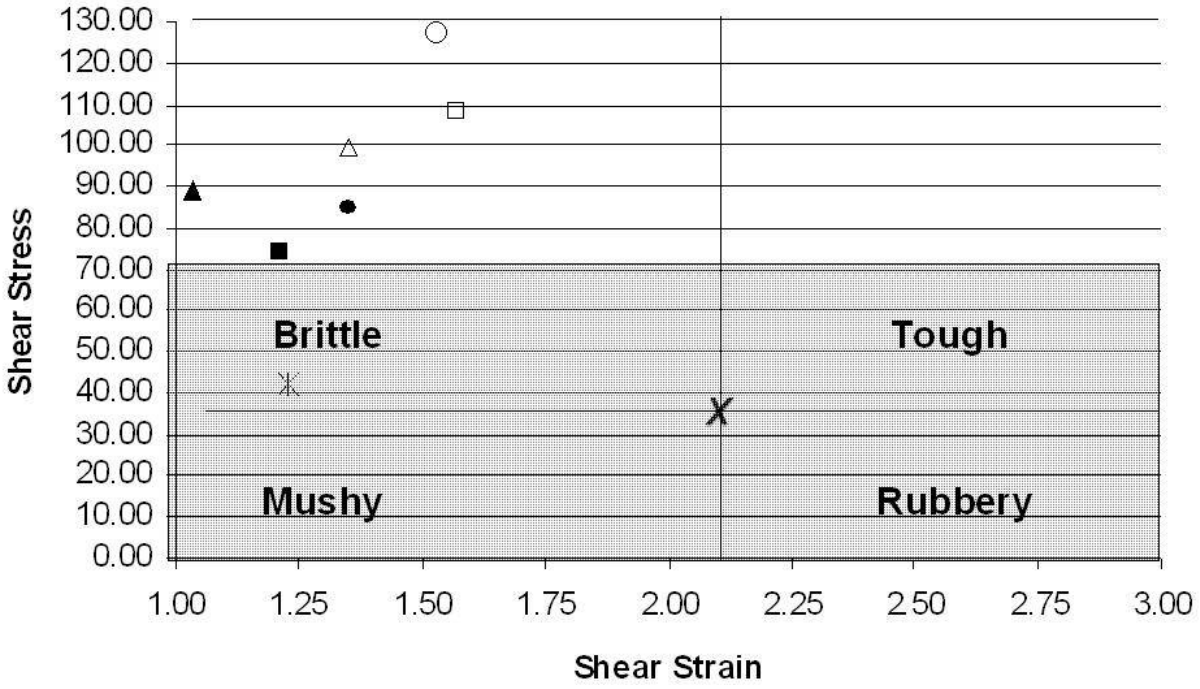

Figure 3-57: Torsion shear strain vs. shear stress of pH-shift processed catfish muscle and an untreated control. The texture profile associated with torsion testing is according to Park (2000). The X in the middle represents the crossing points of the different textures. The shaded box is the extent which the texture profile was determined. The points from pH-shift processing outside of the shaded box (all points except the untreated control) are above the maximum stress value listed in the texture profile. The pH-shift samples are pH 2.0(●), pH 2.5 (■), pH3.0 (▲), pH 10.5 (Δ), pH 11.0 (○), pH 11.5 (□), untreated control (Ж).

CHAPTER 4
CHEMICAL PROPERTIES OF ACID AIDED AND ALKALI AIDED PROTEIN ISOLATES FROM CATFISH (*Ictalurus punctatus*)

Introduction

Channel catfish (*Ictalurus punctatus*) is one of the fastest growing aquatic products in the United States. The production and utilization of aquacultured catfish in the United States has increased over the past 20 years and recently stabilized to a production level of 600,000 pounds from 1999-2001 (Harvey 2002). The primary product of catfish is the fillet, with the secondary product being the belly flaps, or the "nugget". The quality and potential utilization of catfish for other purposes is under on-going investigation with promising results (Kim and others 1996; Kristinsson and others 2005b; Suvanich and others 2000). The over-harvesting of all aquatic resources was recently highlighted by Worm and others (2006) with the projected depletion of aquatic resources by 2048. This becomes more of an issue since approximately 40% of the live weight of many harvested species is discarded. Methods are currently being investigated which will put more of the total seafood catch into the human consumable market and potentially diminish or greatly relieve some of the current strain on the fisheries. The present seafood catch totals approximately 100 million tons (Kristinsson and others 2005b) and can be far better utilized if new economic technologies are developed. To achieve optimally utilize material currently being discarded or diverted into other products not intended for human use, it is of the utmost importance to understand the chemistry of these material so novel processes can be developed.

The chemical properties of muscle proteins in meat and meat products are known to affect quality, application and formulation ranging from whole meat to comminuted products (Lanier 2000). The chemistry of muscle proteins directed for human consumption is investigated to better understand the effect of inherent chemical properties on the functional properties and

quality of proteins. The main functional properties of importance to proteins are their gelation properties which dictate the difference textural properties you can achieve with muscle protein products, and also their water holding capacity. Myosin, actin and the complex actomyosin are known to be the primary contributors of textural properties of muscle gels (Park 2005). The chemical properties of proteins in muscle vary widely, but are known to affect the utilization of muscle proteins for food applications, including protein gels. The chemical properties of proteins include but are not limited to myosin ATPase activity, reactive and total sulfhydryl groups and surface hydrophobicity (Lanier 2000).

Myosin ATPase activity is used as an indicator of structural changes in myosin and/or actomyosin integrity during post-harvest handling and processing (Park 2005). These structural changes have been found to correlate to changes in the gelling properties of muscle proteins. Yongswawatdigul and Park (2002) state that the gelling differences are due to the oxidation of -SH groups at the active site on the head portion of the myosin heavy chain.

Modification of -SH groups may be monitored by measurement of reactive and total sulfhydryl groups (Liang and Kristinsson 2005). Protein sulfhydryl groups in gelled systems are believed to provide structural stability to the gel network by undergoing disulfide interchanges and -SH oxidation which contributes to disulfide bridges formed during gelation (Visschers and De Jongh 2005). The presence and availability of -SH groups to participate in forming disulfide bridging increases protein-protein interactions and thus the integrity of the gel network (Visschers and De Jongh 2005).

Surface hydrophobicity of proteins in gel networks is known to impart structural integrity to the gel (Lanier 2000). As the gel is formed, the hydrophobic patches of proteins exposed to the solvent interact to bury these exposed hydrophobic patches through protein-protein

interactions. Protein surface hydrophobicity and physical properties (i.e. with torsion testing) have been found to correlate (Lanier 2000). Hydrophobic interactions are believed to occur after disulfide interchanges as mentioned above during the heat induced gel formation of muscle proteins (Smyth and others 1998). The hydrophobic interactions which are partly directed by disulfide links bring proteins into the preliminary three-dimensional network allowing for hydrogen bonding to occur during the cooling phase of heat induced gels (Smyth and others 1998). Modification of sulfhydryl and/or hydrophobic properties of muscle proteins would be one way of directing protein gelation as well as investigating the chemical mechanisms responsible for differences observed in the gel networks formed when implementing new technologies of muscle protein recovery (Lanier 2000).

Acid-aided and alkali-aided processing is a new technology aimed at utilizing underutilized products and byproducts of muscle-based systems (Hultin and Kelleher 2000). Acid aided and alkali aided processing utilizes the solubility properties of muscle proteins, specifically the myofibrillar proteins. Solubility is imparted to myofibrillar proteins by the increase or decrease in pH to a point where the proteins are highly ionized resulting in their solubility. Solubility of myofibril proteins is important because it allows for the separation and removal of unwanted materials such as collagen and fat. These unwanted materials are not easily separated or removed in other recovery processes, such as surimi. Acid-aided and alkali-aided processing of channel catfish has been shown to provide an improved protein isolate by reducing the lipid and heme content. The reduction of lipid and heme content increases whiteness and increases the oxidative stability of the isolate (Kristinsson and others 2005b). Acid aided and alkali aided processing has been shown to alter the structural states of muscle proteins in cod and catfish myosin (Davenport and Kristinsson. 2003; Kristinsson and Hultin 2003a; Raghavan and

Kristinsson 2007a, 2007b) and whiting and rockfish protein isolate made from whole ground muscle (Choi and Park 2002; Yongsawatdigul and Park 2004). However, the structural stability of catfish muscle proteins as a whole when subjected to the acid aided or alkali aided processing has not yet been investigated. We hypothesize that acid-aided and alkali-aided processing will structurally modify channel catfish muscle proteins. The goal of this study is to characterize the effect of acid and alkali processing on some key structural properties of catfish muscle protein.

Methods

Raw Material

The raw material used in these studies was fresh catfish fillets obtained 1-3 days post harvest from a local supplier. Catfish fillets were only purchased which were determined to be within 3 days of packaging. The catfish fillets were purchased and immediately transported on ice to the laboratory and processed the same day.

Preparation of Protein Isolates

Protein isolates were prepared according to figure 2-1. Fresh fillets were initially ground in an Oster heavy duty food grinder (Niles, Ill., U.S.A.) for the preliminary disruption and collection of the muscle tissue. Following grinding, the comminuted meat was diluted 1:2 (w/v) with deionized (DI) water and homogenized in a Waring blender for two bursts of 30 seconds. Following homogenization, the resulting muscle tissue slurry was further diluted to give a final dilution ratio of 1:6 (w/v) muscle to DI water. This slurry was manually stirred with a plastic spatula to achieve good homogeneity. The pH of the slurry was adjusted according to the methods described below, using either 2N NaOH or 2N HCl as needed for the pH desired, with continuous manual mixing. Upon reaching the desired pH, insoluble material was removed by centrifugation (Sorvall RC-5B centrifuge with a GS-3 rotor, Kendro Laboratory Products, Newtown, Conn., U.S.A.) at 10,000 x g for 20 minutes at 5^{o}C. Following centrifugation, the

soluble middle layer was collected through a kitchen strainer with a mesh size of approximatly 0.25 mm to minimize contamination with other separated materials. The soluble material was readjusted to pH 5.5 as described above. After readjustment the solution was centrifuged to remove excess water and remaining soluble proteins at 10,000 x g for 20 minutes. The precipitated protein was collected by decanting the supernatant containing the unprecipitated proteins and removing it with a steel spatula. All of the precipitate from each solubilization pH was combined from the centrifuge bottles into one protein isolate. This dewatered protein isolate was further dewatered by placing the combined precipitate into cheesecloth and hand squeezing until the moisture content was below 80%. Moisture content was determined using a Cenco infrared moisture analyzer (CSC Scientific, Fairfax, Va., U.S.A.). Upon completion of manual dewatering the protein isolation was complete. Preliminary unpublished investigation of protein isolates in this laboratory found the shelf life of catfish protein isolates to be 5-7 days on ice. All protein isolates were stored on ice at the precipitation pH and used within 5 days.

Protein Concentration

Protein concentration in the isolates and the subsequent solutions was determined using the Biuret method, as described by Torten and Whitaker (1964), with of 10% w/v deoxycholic acid in water added at 10% v/v of the protein-Biuret reagent to minimize turbidity from any remaining lipids in the samples. Protein concentration was measured based on a standard curve based on BSA.

Protein Surface Hydrophobicity

Protein surface hydrophobicity was conducted according to Liang and Kristinsson (2005). To measure surface hydrophobicity the isolate was diluted to give a stock solution of 10 mg/ml in a 20 mM tris-HCl buffer, 0.6 M NaCl, pH 7.2. The stock solution was serially diluted to obtain a concentration curve. Increasing volume (100 µl, 200 µl, 300 µl, 400 µl and 500 µl) of

the stock solution were added to Tris-HCl buffer to a final volume of 4.0 ml. Then, 10 µl of 6-propionyl-2-(dimethylamino) naphthalene (PRODAN) (11.35 µg/ml in methanol) was added and the samples mixed for ~15 seconds. After mixing the samples were then incubated for 15 min in the dark. All sample preparations and incubations were performed on ice in disposable test tubes. After incubation, the sample was transferred to a fluorescence cuvette and the fluorescence emission intensity scanned between 380-560 nm with excitation at 365 nm in a Perkin Elmer LS 45 Luminescence Spectrophotometer (Norwalk, CT). As the isolate is a collection of proteins, the fluorescence peak of the samples is a relatively flat and broad peak between 430-460 nm. The maximal fluorescence of the protein isolate was taken and used as the wavelength for analysis. The surface hydrophobicity was calculated as the slope of the net fluorescence versus protein concentration (mg/ml) of the samples.

Reactive Sulfhydryl Groups

Reactive sulfhydryl groups were determined according to Kim and others (2003). The isolate was diluted and homogenized for 1 minute on ice at speed 2 in a Bio-homogenizer (M133/1281-0, Bio Spec Products Inc., Bartlesville, OK) in 20 mM Tris-HCl buffer, pH 7.2 with 600 mM NaCl to 250 µg/ml. After dilution, 80 µl of 10 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) was added to the sample. This mixture was then incubated for 1 hr on ice. After incubation the sample was read at 420 nm using an Agilent 8453 diode array UV-visible spectroscopy system (Agilent Technologies Deutschland GmbH, Waldbronn, Germany). Micromolar determination of SH groups per gram in the sample were done using the following equation:

$$SH/g = [absorbance \times dilution\ factor \times 100]/[13600(mol/cm) \times sampleconcentration(mg/ml)]$$

Total Sulfhydryl Groups

Total sulfhydryl groups were determined according to Kim and others (2003) . The isolate was diluted and homogenized for 1 minute on ice at speed 2 in a Bio-homogenizer (M133/1281-0, Bio Spec Products Inc., Bartlesville, OK) in 20 mM Tris-HCl buffer, pH 7.2 with 600 mM NaCl to 250 µg/ml. After dilution, 0.5 ml of diluted isolate was mixed with 2.5 ml urea buffer. The urea buffer contained 8 M urea, 0.2 M Tris-HCl, 2% SDS, 10 mM EDTA and was adjusted to pH 8.5. After mixing with urea, 50 µl of 10 mM DTNB was added to the sample. The mixture was incubated in a water bath for 15 minutes at 40°C. After incubation the sample was read at 420 nm using an Agilent 8453 diode array UV-visible spectroscopy system (Agilent Technologies Deutschland GmbH, Waldbronn, Germany). Total sulfhydryl groups were calculated using the following equation.

$$SH/g = [absorbance \times dilution\ factor \times 100]/[13600(mol/cm) \times sample concentration(mg/ml)]$$

Myosin ATPase Activity Assay

Myosin ATPase activity was determined by the method of Perry (1955). The buffers to initiate the enzymatic hydrolysis of ATP by myosin were prepared prior to sample preparation. The reaction buffers used in this assay were 0.2 M glycine-NaOH buffer (pH 9.0), 0.1 M calcium chloride, 0.05 M ATP, sodium salt (pH 6.8) and 15% trichloroacetic acid (TCA). Liberation of inorganic phosphate was monitored in this reaction as the determinant of enzymatic function. For the estimation of inorganic phosphate the following buffers were used: 12% TCA, 10% (w/v) ammonium molybdate stock solution in 10N sulfuric acid. This ammonium molybdate stock was used in the preparation of the ferrous sulfate – ammonium molybdate reagent which was made fresh the day of analysis. For analysis of myosin ATPase activity the isolate was diluted to 1 mg/ml in Tris-HCl buffer containing 600 mM NaCl pH 7.2 and homogenized thoroughly. This was kept on ice until needed. In separate tubes for each reaction, prior to

isolate addition, the reaction buffer was prepared by adding 1.3 ml glycine-NaOH buffer, 0.2 ml calcium chloride, 0.3 ml ATP. This mixture was then incubated at 25^{o}C for 5 min to allow the temperature to equilibrate. After temperature equilibration, 0.2 ml of the individual isolate solution was added to each reaction tube with proper mixing and allowed to incubate at 25^{o}C for 5 min. After 5 min, 1 ml of 15% TCA was added to each reaction tube to stop the reaction. This was centrifuged for 10 minutes at 25,000 x g to precipitate the proteins from solution and leaving liberated phosphate from ATP in solution. Next 0.5 ml of supernatant was added to 3.2 ml of 12 % TCA with good mixing and left to stand at 25^{o}C for 10 minutes. This solution (3.0 ml) of this solution was removed and mixed with 2 ml of the ferrous sulfate – ammonium molybdate reagent and incubated at room temperature for 1 min. After 1 min of incubation the solution was transferred to a cuvette and read at 363 nm using an Agilent 8453 diode array UV-visible spectroscopy system (Agilent Technologies Deutschland GmbH, Waldbronn, Germany). The color of this reaction intensifies with time so only one reaction was done at a time to standardize the incubation time of all samples to 1 min. The results of myosin ATPase activity were assayed as relative results to the control. The control absorbance was set to 100% and the percent activity of the isolates was calculated from the absorbance of the samples relative to the control.

$$activity = pH\,treated\,sample / control\,sample$$

Protein Solubility as a Function of pH

The isolate was diluted to 10 mg/ml in DI water with thorough homogenization using a tissue homognizer (Ultra-Turrax T18, IKA Works Inc. Wilmington, NC). After dilution in water, 2 ml diluted protein was added to 2 ml buffer with good mixing. The pH range was from 1.5-12 in 0.5 increments. For pH 1.5-6.5, 2 mM citric acid buffer was used and for pH 7.0-12, 2 mM sodium phosphate buffer was used. The buffers were adjusted to the desired pH prior to

protein addition. After protein addition, the mixture was allowed to incubate on ice for 10 minutes. After incubation the protein solution was centrifuged at 3,000 x g for 10 minutes. After centrifugation, the supernatant was sampled for protein concentration using the biuret method (Torten and Whitaker 1964). Protein concentration was calculated from a standard curve based on bovine serum albumin (BSA). Percent solubility was then calculated as a percentage of an uncentrifuged control by the following equation:

$$[protein\,(mg/ml)\,\mathrm{sup}\,ernant]/[protein\,(mg/ml)\,total]$$

Protein Solubility as a Function of Salt Concentration

The isolate was diluted to 5 mg/ml in 20 mM Tris-HCl at pH 7.2 without added NaCl with thorough homogenization using an Ultra-Turrax T18. After dilution in buffer, 4 ml was taken and the appropriate amount of NaCl added to each tube to achieve 0 mM, 150 mM, 300 mM 450 mM or 600 mM concentrations. After the appropriate NaCl concentration was achieved, the samples were centrifuged at 3000 x g for 10 minutes at 5°C. After centrifugation, the supernant was collected in duplicate and soluble protein was determined according to the biuret method (Torten and Whitaker 1964). The soluble protein was compared to an uncentrifuged control. Percent soluble protein was calculated as

$$(protein\,(mg/ml)\,\mathrm{sup}\,ernant)/(protein\,(mg/ml)\,total)$$

Statistical Analysis

Experimental design, as shown in figures 1-1 – 1-4, was conducted, in duplicate, on replicate isolations as discussed above. A replicated (N=2) was determined as acceptable due to achieving an acceptable power (β=0.80, α=0.05).

One-way independent measures analyses of variance were used to examine the effects of all methods used except for the analysis of pH solubility and salt solubility which were tested

with 2-way ANOVA analysis. The Kruskal-Wallis ANOVA testing by ranks was used on data which did not pass normality. Post hoc analysis was conducted only in the presence of significant population differences. ANOVA statistical comparisons were conducted with SigmaStat, (Systat Software Inc. San Jose Ca) with a significance level of $p<0.05$. After SigmaStat completed the ANOVA analysis, the post hoc analysis recommended by SigmaStat was used. In most cases this was Tukey's test. The Holm-Sikak method was used with ATPase activity. The two-way ANOVA analysis used the Holm-Sidak pair wise comparisons. The two-way ANOVA tables and the post hoc tests are reported in appendix A.

Results

Myosin ATPase

Myosin ATPase activity was reduced by pH-shift processing (Figure 4-1). This suggests that the pH treatment leads to changes in the myosin recovered with the pH-shift isolates. Myosin ATPase activity was significantly reduced ($p<0.05$) by pH treatments 2.0, 2.5, 3.0 and 10.5. No significant differences ($p>0.05$) were seen between any of the pH-shift isolates. The isolates retained from 35% to 61% myosin ATPase activity of the raw material.

Surface Hydrophobicity

Surface hydrophobicity of the proteins in the isolates was not significantly ($p>0.05$) reduced compared to the proteins in the raw material (Figure 4-2). All isolate treatments had lower surface hydrophobicities but no main effect was determined. As with the myosin ATPase results, there were no significant differences ($p>0.05$) between any of the acid or alkali treatments.

Total Sulfhydryl Groups

When total sulfhydryls were analyzed no significant differences ($p>0.05$) were seen between any of the acid or alkali treatments within groups or between groups (figure 4-3). All

the pH treatments were not significantly different ($p>0.05$) from the control as individual treatments or as a population.

Reactive Sulfhydryl Groups

There were no significant differences ($p>0.05$) in surface sulfhydryl groups between any of the acid or alkali treatments within groups or between groups (figure 4-4). No significant differences were present between any of the pH-shift treated isolates and the control. No significant differences were present between any individual treatments or within the population.

Solubility of Proteins at Different Salt Levels

The results of the salt solubility study are shown in figure 4-5. The isolates and untreated control were compared with the Holmes-Sidak multiple pair wise comparison with a $p<0.05$. The pair wise comparison of the salt solubility is shown in appendix A. Significant differences were present between all comparisons made, both within pH treatments and within salt concentrations.

Solubility of Proteins at Different pH Values

The results of pH solubility of isolates and untreated catfish muscle are shown in figure 4-6. The isolates and untreated control were compared with the Holmes-Sidak multiple pair wise comparison with a $p<0.05$. The pair wise comparison of the pH solubility is shown in appendix A. The isolates showed significant differences ($p<0.05$) at extreme pH values but no significant differences were present between pH 4 to pH 7.5.

Discussion

The globular head of myosin has an enzymatic function where ATP is hydrolyzed to ADP and phosphate. The purpose of the enzymatic function is to break the myosin/actin bridge which is important for the muscle contraction/relaxation cycle (Berne and others 2004). Myosin ATPase activity has been reported as a good indicator of protein quality and quality of processed

fish muscle food products, such as surimi (Park, 2005). The results here for catfish show that there were no significant differences ($p>0.05$) in myosin ATPase activity between the isolate treatments. The isolate treatments had however significantly ($p<0.05$) lower ATPase activity than the control (Figure 4-1). Isolate treatments did not completely inactivate myosin ATPase activity but did reduce the activity, suggesting high and low pH treatments the proteins were subjected to modified the structure of the myosin catalytic site. The change in myosin ATPase activity has been attributed to a shifting of the sulfhydryl groups at the myosin active site (Yongsawatdigul and Park 2004). The reduction in myosin ATPase activity was highly variable as indicated from a large standard deviation (Figure 4-1). In the pH 10.5 treatment group, the myosin ATPase activity ranged from 0-62%. This range may be due to the inconsistent exposure of the myosin active site to the solvent at high pH. These inconsistencies were present in all samples with some varying more than others. The lack of significant difference ($p>0.05$) in the myosin ATPase activity (figure 4-1) may be due to the variability of the pH treated systems and differentiation between acid and alkali treatments of muscle proteins was not possible. Isolate production with either acid or alkali may or may not go through the same structural changes, however, the product is similar in that the acid or alkali treatments produce a reduction of ATPase activity which is not significantly different ($p>0.05$) among the treatment groups. This means that though there is a reduction in the ATPase activity when acid or alkali processing is used there is not a complete inactivation of myosin ATPase activity.

Surface hydrophobicity measurement is an analysis of the hydrophobic patches on the surface of a protein that bind a fluorescent probe. In this experiment, PRODAN was used as the fluorescent probe. Figure 4-2 shows no significant difference ($p>0.05$) in surface hydrophobicity between treatment groups. However, the control was higher in surface hydrophobicity than all

pH treatmented samples though no main effect was determined. Previous studies have shown that pH-shift processing increased the surface hydrophobicity of catfish myosin (Kristinsson and Hultin 2003a; Raghavan and Kristinsson 2007a, 2007b). The surface hydrophobicity of isolated catfish myosin shows pH treatment 2.5 having the greatest surface hydrophobicity followed by pH treatment 11.0 and then followed by untreated control (Davenport and Kristinsson 2003).The reduction of surface hydrophobic patches during acid and alkali treatment could be the result of structural modification during the pH readjustment to 5.5, burying the hydrophobic patches previously on the surface of the protein. However this reduction of surface hydrophobicity in the whole isolate system could also be due to micro-aggregation preventing the exposure of hydrophobic patches on the surface of the proteins.

Total sulfhydryls are the total number of -SH groups exposed after treatment in 8 M urea. Reactive sulfhydryls are the -SH groups of the protein which are exposed to the solvent (Tris buffer with 0.6M NaCl) and are able to react with DTNB. The difference between total sulfhydryls and reactive sulfhydryls is the molar content of sulfhydryl groups which are buried within the protein structure of the system. There were no significant differences ($p>0.05$) between total sulfhydryls in all treatment groups and the control. Figure 4-4 shows no –SH groups were altered during acid or alkali processing. The total sulfhydryl groups present in the samples, which may participate in gel formation, remained the same. Figure 4-3 depicts the reactive sulfhydryls measured in the control and all treatments. No significant differences ($p>0.05$) were observed between any of the treatment groups or between any of the treatment groups and the control. Due to a lack of significant differences ($p>0.05$) in total sulfhydryl and reactive sulfhydryl groups it may be concluded that sulfhydryl content was not significantly ($p>0.05$) linked to changes in gel strength (see gelation data in Chapter 3). However, structural

susceptibility during heating denaturation to expose sulfhydryl groups during thermal treatment was not assayed. Thus the molar content of disulfide bonds in the thermally set gel is currently unknown.

Salt solubility of muscle proteins is one way to assess whether functionality has been modified by pH-shift processing. Muscle proteins, myofibrillar protein in particular, are highly sensitive to changes in ionic strength and type of ions in their environment. The ability of the proteins in the isolate to enter into solution at varying concentrations of salt was tested at pH 7.0 (Figure 4-5). Myofibrillar proteins are the contractile proteins of muscle, those proteins which are salt soluble, or more specifically go into solution above an ionic strength of ~0.3. Figure 4-5 shows a reduction in solubility of the protein isolates in the presence of high salt concentrations. This reduction may be due to structural changes and/or micro-aggregation which prevent the continued solubility of these proteins under conditions which normally would solublize myofibrillar proteins. The reduction in salt solubility of isolates follows the same trend as the reduction of surface hydrophobicity. These tests provided results when compared to previous results on cod muscle protein isolates and cod myosin where surface hydrophobicity was increased while solubility was decreased (Kristinsson and Hultin 2003b) indicating a species effect of pH-shift processing. These results indicate that in catfish isolates, the protein-protein interactions are changed in such a way to prevent ionization of the protein surface thus resulting in a reduction of solubility. As stated above, the reduction in surface hydrophobicity may be due to microaggregation of misfolded proteins, thus leaving little exposed hydrophobic surface. Increased salt will solubilize muscle proteins, but it is possible the interactions between these microaggregates were too strong for the salt to overcome, thus leading to only a small increase in solubility. Due to the similarity of results observed between isolated catfish myosin (Raghavan

and Kristinsson 2007a) and whole muscle isolate, the same molecular mechanism is most likely responsible for these differences.

The modification of catfish muscle proteins by pH-shift processing resulted in isolates with different pH solubility profiles. The increased solubility of the control in the pH range 3.5-10 is most likely due to a higher amount of sarcoplasmic proteins compared to isolates. Previous work on pH-shift processing of catfish muscle (Kristinsson and others 2005b) has shown an increased retention of sarcoplasmic proteins when catfish is processed at pH 2.5 as compared to alkali processing at pH 11.0. This suggests that sarcoplasmic proteins which are largely soluble in the control co-aggregate with the myofibrillar proteins and thus become largely insoluble, explaining the different pH profile seen in the pH 3.5-10 range. The low solubility in this range would indicate that the protein isolates are more prone to aggregation than untreated control. This is likely due to the fact that the isolates are composed of only partially refolded protein structures which favor interactions with each other more than the solvent. It is also interesting to note that all isolates expect the pH 10.5 treatment, became soluble at a lower pH than the control, suggesting more protonation was needed to break the interactions between them. The pH 10.5 treatment may have been milder than the other treatment, thus giving isolates with weaker protein-protein interactions. It is also interesting to note that contrary to the results seen at low pH the isolate solubilized at lower high pH values than the control, indicating that they were more sensitive to increases in negative charges than positive charges. These data therefore suggest the pH-shift process modified the protein structure and their interactions compared to control.

Conclusions

No significant differences ($p>0.05$) were seen between total or reactive sulfhydryl groups of catfish protein isolates compared to control. No significant differences ($p>0.05$) were seen

between the surface hydrophobicity or myosin ATPase activity of the isolates but all isolate samples showed reduction in both compared to control. Significant differences ($p<0.05$) were observed in salt- and pH-dependent solubility of isolates compared to control. These results indicate that, although significant differences are not detected ($p>0.05$) in some of the commonly used tests to describe the chemical nature of protein products, isolates subjected to different pH-shift processing conditions do in fact have different properties and pH-shift processed muscle proteins may have chemical modifications which are different from traditional muscle systems. The chemical modification of catfish muscle proteins by pH-shift processing may also lead to structural and functional modification of catfish muscle proteins. Further studies are needed to determine whether the structural and functional effects when using this new technology are dictated by any chemical changes to specific amino acids during processing. With further study and characterization the formulation of custom protein isolates to provide application specific utilization may be possible.

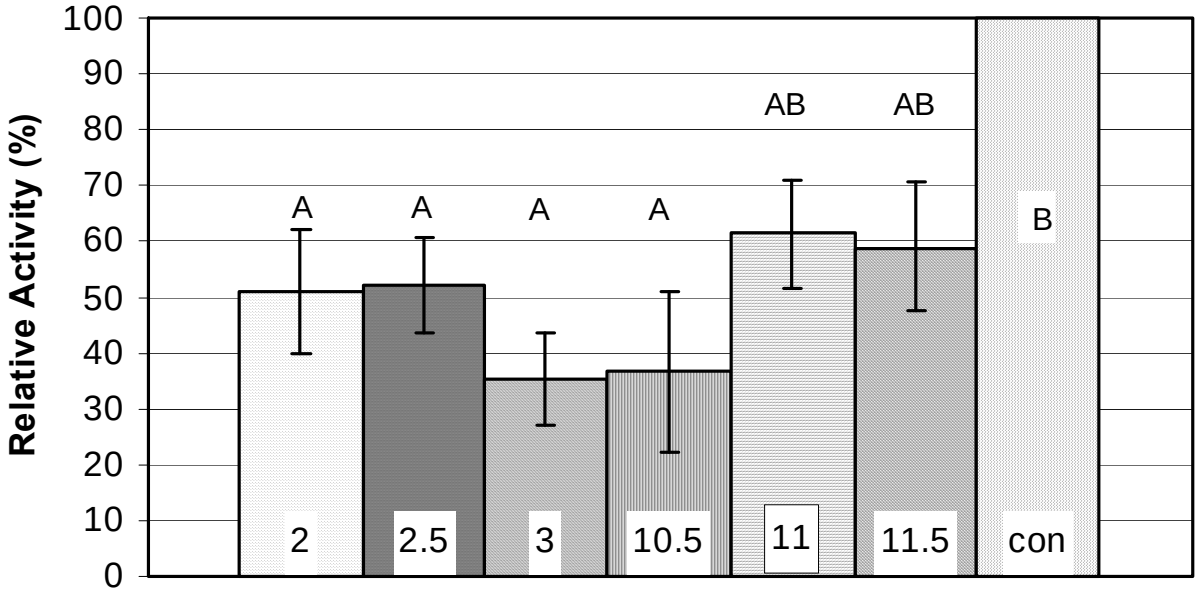

Figure 4-1: Myosin ATPase activity of catfish muscle isolates compared to a control sample. The control was normalized to 100% activity and the percentage of activity was calculated relative to the control. The significant differences present between samples ($p<0.05$) are represented by the letters above the column. Similar letters represent no significant difference ($p>0.05$) between treatments.

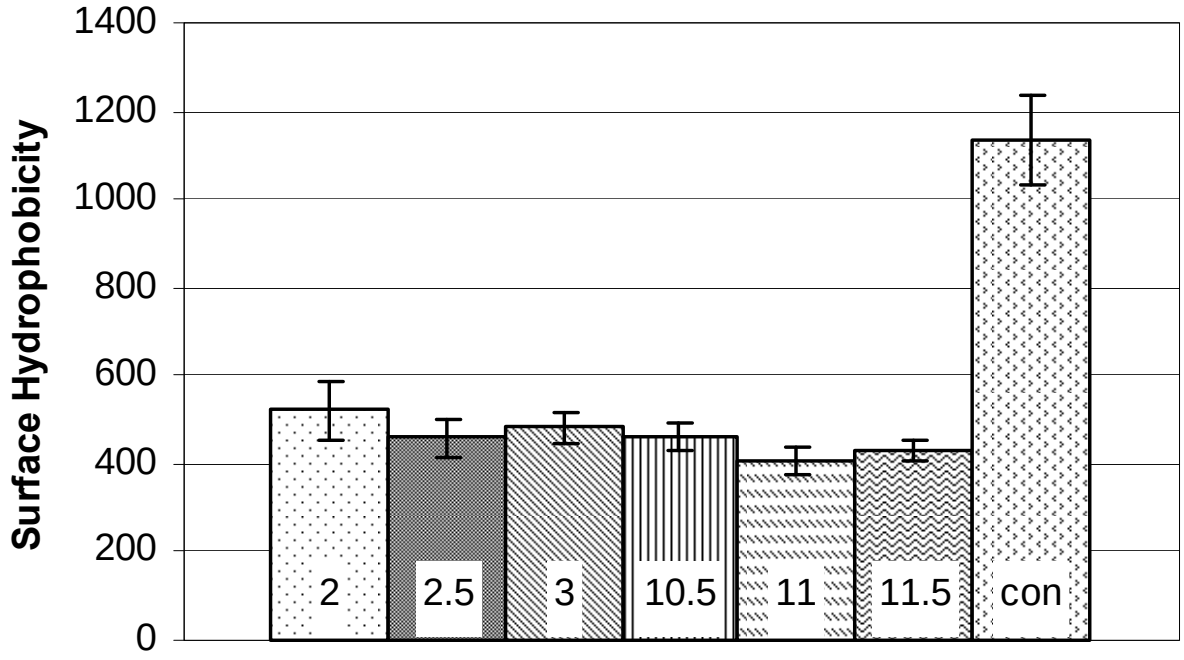

Figure 4-2: The surface hydrophobicity of catfish muscle isolates and untreated control. Hydrophobicity was assayed by developing a protein concentration curve and measuring the maximal fluorescence response of the fluorescent probe PRODAN at ~400 nm. A main effect was not determined for the sample population ($p>0.05$).

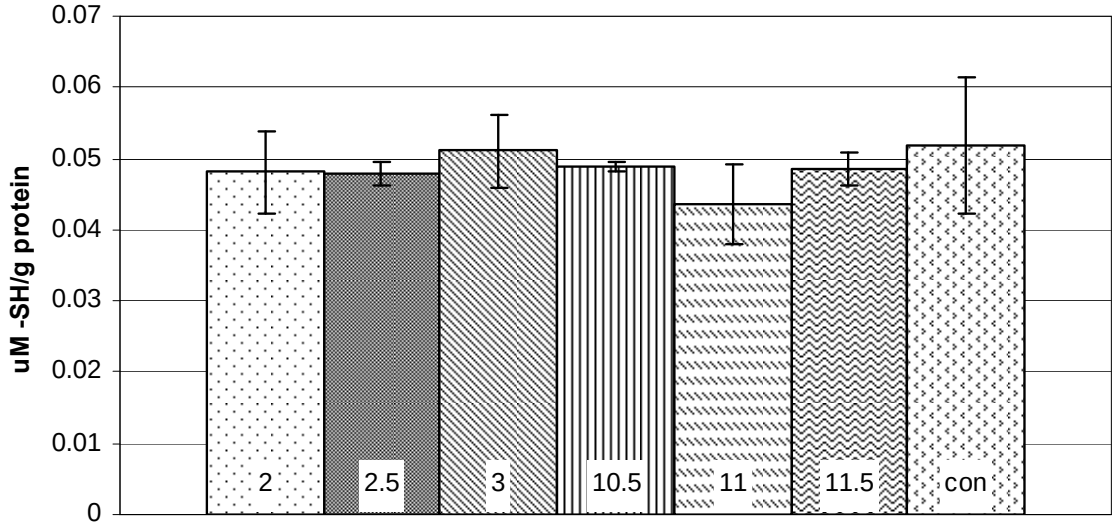

Figure 4-3: Total sulfhydryl content of catfish protein isolates compared to untreated control. Total sulfhydryl content was assayed by the reactivity of catfish proteins to DTNB after being treated for 10 min in 8M urea at 40°C. No significant differences ($p>0.05$) were present between any of the samples tested, thus it is concluded that pH-shift processing did not affect total sulfhydryl content.

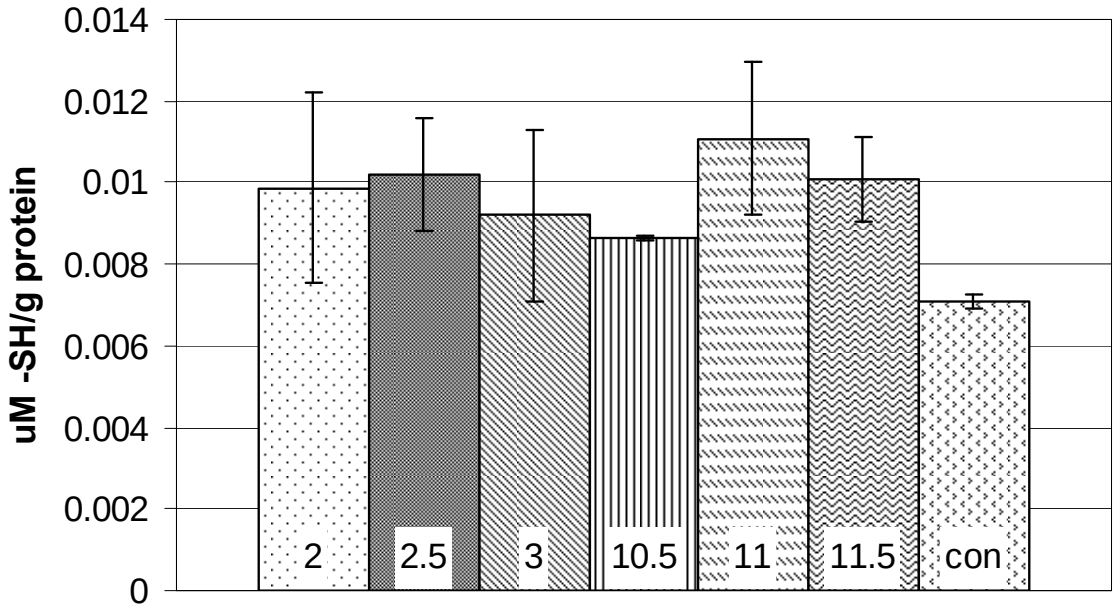

Figure 4-4: Reactive sulfhydryl content of catfish protein isolates compared to untreated control. Reactive sulfhydryl content was assayed by the reactivity of catfish muscle proteins to DTNB in T-HCl buffer. No significant differences ($p>0.05$) were present between any of the samples tested, thus it is concluded that pH-shift processing did not affect reactive sulfhydryl content.

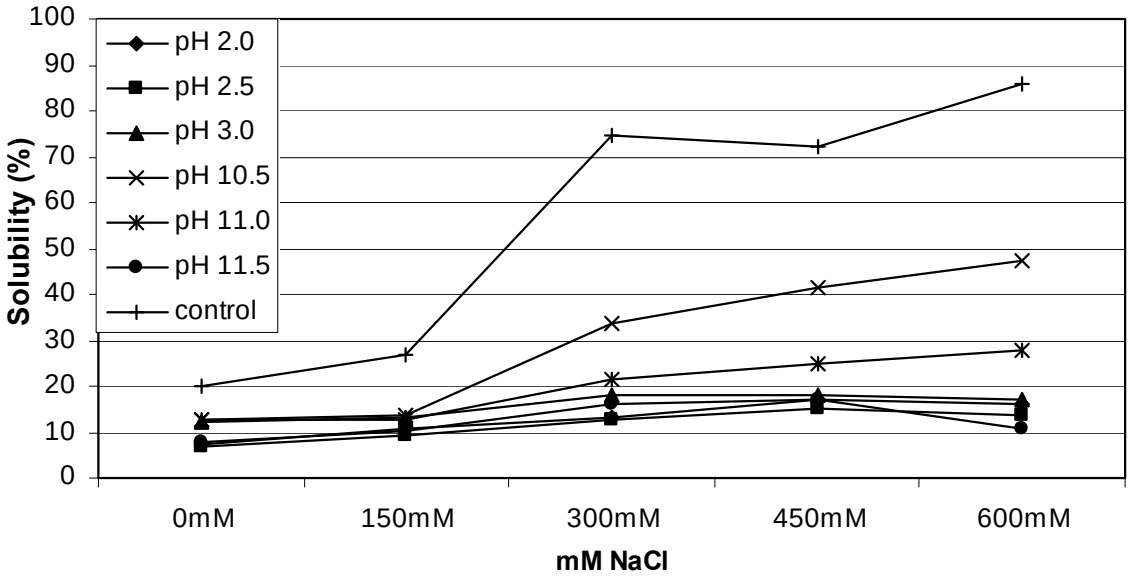

Figure 4-5: Solubility of catfish protein isolates and untreated control in varying concentrations of sodium chloride at pH 7.2 in 25 mM tris-HCl buffer. The percent solubility was determined by dividing protein concentration in the supernatant after centrifugation by the protein concentration in an uncentrifuged sample. Significant differences compared within pH treatments and salt concentrations are tabulated and reported in appendix A.

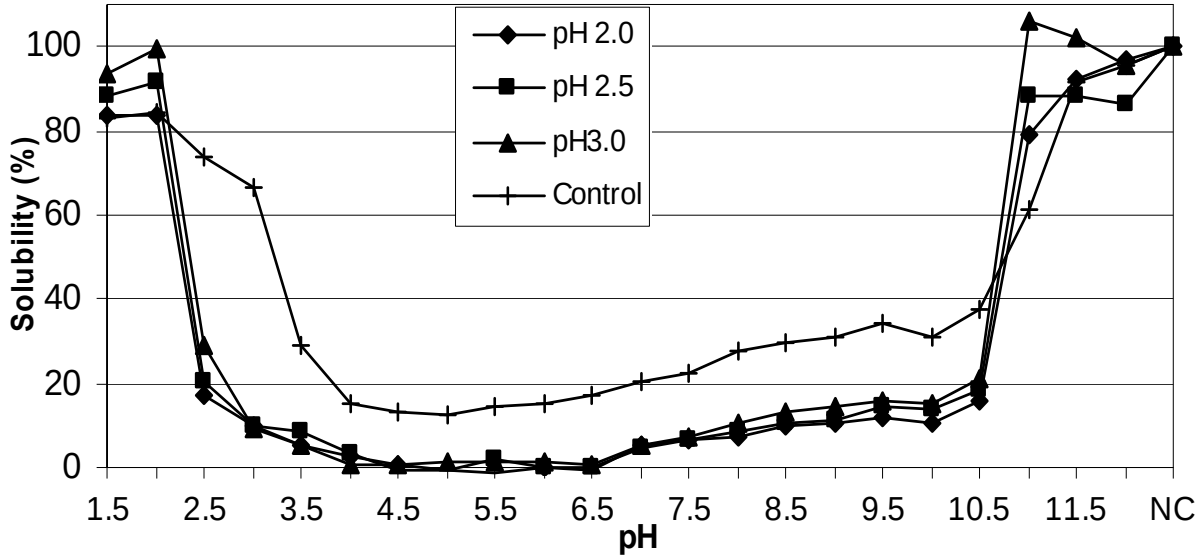

Figure 4-6: The pH solubility profile of catfish protein acid-aided isolates and untreated control from pH 1.5-12 with the non-centrifuged samples reported as the right most point on the graph. Solubility was calculated as explained in Fig. 4-5. Significant differences present between samples are shown in appendix A.

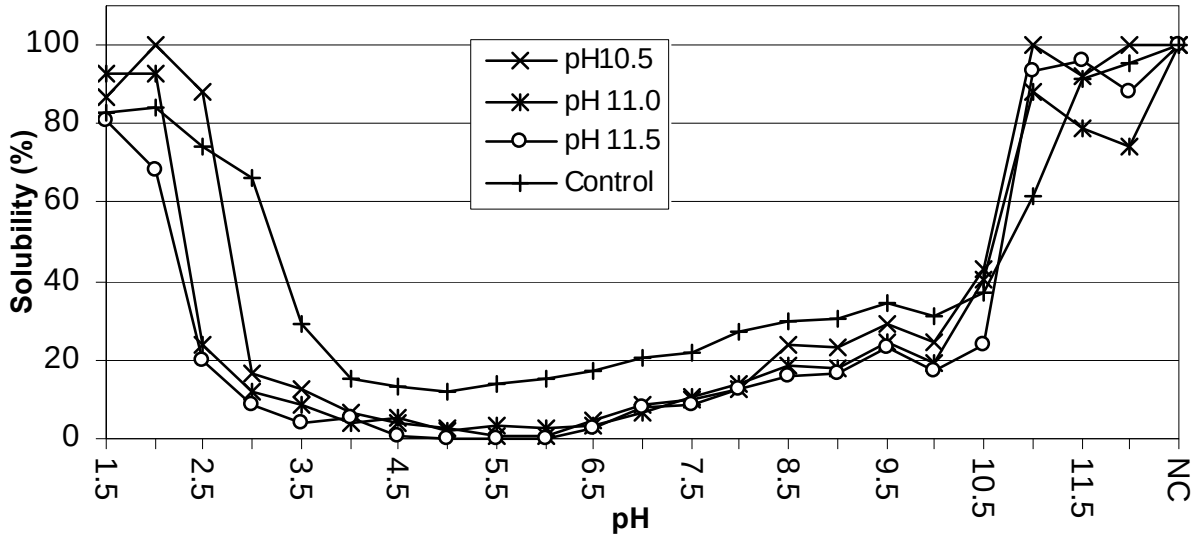

Figure 4-7: The pH solubility profile of catfish protein acid-aided isolates and untreated control from pH 1.5-12 with the non-centrifuged samples reported as the right most point on the graph. Solubility was calculated as explained in Fig. 4-5. Significant differences present between samples are shown in appendix A.

CHAPTER 5
THE EFFECT OF pH-SHIFT PROCESSING ON THE STRUCTURAL AND THERMAL PROPERTIES OF CATFISH (*Ictalurus punctatus*) PROTEIN ISOLATES

Introduction

The structure function relationship of muscle proteins is important to the application and performance of muscle based food products. The modification of protein structure from the native state is critical for meat functionality. The native structures of muscle proteins, though crucial for the function of the muscle proteins in the living system, do not exhibit functional characteristics associated with processed meat. The change in secondary structures are related to the functionality of muscle proteins, however the intermediary transition of secondary structures from native to denatured are considered to bear a more direct relationship to muscle protein functionality (Xiong 1997). Muscle based products are first influenced by the growth conditions, slaughter method and muscle type of the animal The production of functional comminuted meat products is further dictated by the processing conditions and chemical additives used. The production of comminuted meat products frequently includes the addition of salts, primarily sodium chloride. Addition of salt induces structural changes of muscle proteins due to the electrostatic interaction between proteins and the sodium and chloride ions which results in muscle fiber swelling (Xiong 1997). The swelling of muscle fibers allows for increased water retention of the meat system, and thus modifies the functional properties and ultimately the perception of the product. These changes are directed by the state of the intermediate structural characteristics of the muscle proteins. The change in intermediate structures can thus be dictated by processing conditions and chemical additives resulting in the functional properties of muscle proteins being determined by external factors (Xiong 1997).

The use of acid or alkali processing has been shown to change the structural conformations of isolated muscle proteins, specifically myosin (Kristinsson and Hultin 2003a; Raghavan and Kristinsson 2007a, 2007b), resulting in molten globular or stable intermediate protein structures.

In this study it is hypothesized that the utilization of acid-aided and alkali-aided extraction method on a warm water species (catfish) will lead to unique protein structural properties that are pH dependent. This hypothesis was investigated by determining the effect of pH shift processing at different pH on the structure and stability of catfish protein isolates produced at low and high pH treatments.

Methods

Raw Material

The raw material used in these studies was fresh catfish fillets obtained 1-3 days post harvest from a local supplier. Catfish fillets were only purchased which were determined to be within 3 days of packaging. The catfish fillets were purchased and immediately transported on ice to the laboratory and processed the same day.

Preparation of Protein Isolates

Protein isolates were prepared according to figure 2-1. Fresh fillets were initially ground in an Oster heavy duty food grinder (Niles, Ill., U.S.A.) for the preliminary disruption and collection of the muscle tissue. Following grinding, the comminuted meat was diluted 1:2 (w/v) with deionized (DI) water and homogenized in a Waring blender for two bursts of 30 seconds. Following homogenization, the resulting muscle tissue slurry was further diluted to give a final dilution ratio of 1:6 (w/v) muscle to DI water. This slurry was manually stirred with a plastic spatula to achieve good homogeneity. The pH of the slurry was adjusted according to the methods described below, using either 2N NaOH or 2N HCl as needed for the pH desired, with continuous manual mixing. Upon reaching the desired pH, insoluble material was removed by

centrifugation (Sorvall RC-5B centrifuge with a GS-3 rotor, Kendro Laboratory Products, Newtown, Conn., U.S.A.) at 10,000 x g for 20 minutes at 5^{o}C. Following centrifugation, the soluble middle layer was collected through a kitchen strainer with a mesh size of approximatly 0.25 mm to minimize contamination with other separated materials. The soluble material was readjusted to pH 5.5 as described above. After readjustment the solution was centrifuged to remove excess water and remaining soluble proteins at 10,000 x g for 20 minutes. The precipitated protein was collected by decanting the supernatant containing the unprecipitated proteins and removing it with a steel spatula. All of the precipitate from each solubilization pH was combined from the centrifuge bottles into one protein isolate. This dewatered protein isolate was further dewatered by placing the combined precipitate into cheesecloth and hand squeezing until the moisture content was below 80%. Moisture content was determined using a Cenco infrared moisture analyzer (CSC Scientific, Fairfax, Va., U.S.A.). Upon completion of manual dewatering the protein isolation was complete. Preliminary unpublished investigation of protein isolates in this laboratory found the shelf life of catfish protein isolates to be 5-7 days on ice. All protein isolates were stored on ice at the precipitation pH and used within 5 days.

Protein Concentration

Protein concentration in the isolates and the subsequent solutions was determined using the Biuret method, as described by Torten and Whitaker (1964), with of 10% w/v deoxycholic acid in water added at 10% v/v of the protein-Biuret reagent to minimize turbidity from any remaining lipids in the samples. Protein concentration was measured based on a standard curve based on BSA.

Circular Dichroism (CD)

CD was done according to Kristinsson and Hultin (2003a). The isolate was diluted and homogenized for 1 minute on ice at speed 2 in a Bio-homogenizer (M133/1281-0, Bio Spec

Products Inc., Bartlesville, OK) in 20 mM Tris-HCl buffer, pH 7.2 with 600 mM NaCl to a concentration of 10 mg/ml. This protein stock solution of 10 mg/ml was prepared and diluted to 2 mg/ml 1 hr before analysis and held on ice. For analysis of secondary structure the sample was scanned from 260-200 nm in a 0.1 cm quartz cuvette, 0.2 nm resolution scanned at 50 nm/min on a Jasco J-500C circular dichroism spectropolarimeter (Jasco Inc, Easton, MD) at room temperature. The time duration of the scan was no more than 10 minutes. Differences in total alpha helix beta structure and random coil structures present were determined from DICHROWEB using the K2D analysis program (Lobley et. al., 2002).

Isolate Susceptibility to Unfolding in Guanidine Hydrochloride (Gu-HCl)

Catfish protein isolate and the control, untreated ground catfish muscle, were treated with Gu-HCl over the range of 0-6M in 0.5 M increments. For assessment of denaturation, 222 nm was used as an indicator wavelength of alpha helical content, scanning from 220-225 nm in a 0.10 cm quartz cuvette, 0.2 nm resolution scanned at 50nm/min on a Jasco J-500C circular dichroism spectropolarimeter (Jasco Inc, Easton, MD) at room temperature. The protein concentration was 1 mg/ml. Samples were allowed to incubate for 5 minutes on ice prior to reading.

Differential Scanning Calorimetry (DSC)

DSC was conducted according to Fukushima and others (2003) on a MicroCal DSC (MicroCal, LLC, North Hampton, MA). Isolate were diluted in sample buffer (20 mM tris-HCl, pH 7.2 with 600 mM NaCl) to a protein concentration of 10 mg/ml and homogenized for 1 minute on ice at speed 2 in a Bio-homogenizer (M133/1281-0, Bio Spec Products Inc., Bartlesville, OK). The sample was degassed under vacuum for 5 min at 5^{o}C. After degassing, 0.6 ml was loaded into the sample cell, the reference cell contained sample buffer. The sample was then linearly heated at 1^{o}C/min from 5^{o}C-80^{o}C. Analysis of the data was conducted on the

software provided by the manufacturer, Origin Pro 7.5, for determination of both exothermic and endothermic events.

Susceptibility of Proteins to Transglutaminase-Induced Cross Linking

The susceptibility of pH-shift processed catfish muscle to protein cross linking was assayed using the commercial transglutaminase (TGase) Activia TI from Ajinomoto LLC (Ajinomoto Food Ingredients LLC Chicago Il). 0.2% TGase (w/w) was added to isolate diluted to 10% solids in 20mM Tris-HCl buffer, 600mM NaCl. TGase activity was assayed by using an AR2000 advanced research rheometer (TA Instrument, New Castle, DE) with a head with a flat cross-hatched polyacrylic surface and thermally controlled plate. The gap was 1000 microns and the head lowered onto the sample using the controlled speed function provided by the software. The samples were tested in oscillatory mode under controlled frequency at 0.1 Hz, and strain controlled at 0.01. TGase activity was monitored by the increase in G' over 1 hour at 30^{o}C. Activity was compared against samples with no TGase added. G' was plotted against time and the slope of the line from linear regression was used to calculate the TGase activity. The activity coefficient was calculated as the ratio of treated to untreated samples.

Statistical Analysis

Experimental design, as shown in figures 1-1 – 1-4, was conducted, in duplicate, on replicate isolations as discussed above. A replicated (N=2) was determined as acceptable due to achieving an acceptable power (β=0.80, α=0.05).

One-way independent measures analyses of variance were used to examine the effects of all methods. The Kruskal-Wallis ANOVA testing by ranks was used on data which did not pass normality. Post hoc analysis was conducted only in the presence of significant population differences. ANOVA statistical comparisons were conducted with SigmaStat, (Systat Software

Inc. San Jose Ca) with a significance level of $p<0.05$. After SigmaStat completed the ANOVA analysis, the post hoc analysis recommended by SigmaStat was Tukey's test.

Results

Circular Dichroism (CD)

pH-shift processing led to changes in secondary structure of protein isolates as seen by the CD results (figures 5-1, 5-2, 5-3). The order of secondary structure was found to be control>alkali>acid. Acid treated protein isolates had lower relative α-helix content than the control and higher relative beta structure content than the control though no main effect was observed. Alkali treated protein isolates also showed relatively lower α-helical content and higher beta structure content than the control though no main effect was observed. There were no significant differences ($p>0.05$) in secondary structure between acid and alkali treated protein isolates. No significant difference ($p>0.05$) was observed in random coil content among any treatment groups as compared with each other or with the control.

Guanidine Hydrochloride Denaturation (Gu-HCl)

Subjecting protein samples to increasing concentration of Gu-HCl gave unexpected results (figure 5-4 and 5-5). Increasing Gu-HCl to 0.5 M led to substantial protein denaturation of the control samples (70%), some additional denaturation of alkali-aided isolate samples, while acid-aided isolate was less affected. Isolates made with the pH 11.5 treatment were most affected and had fully unfolded in 0.5 M Gu-HCl. All samples demonstrated a refolding behavior in 1 M Gu-HCl, with all isolates (except the one made with the pH 3 treatment) and control having higher level of structure than untreated control at 0 M Gu-HCl. At 1.5 M Gu-HCl, control samples showed some unfolding, while acid-aided isolate samples continued to refold (figure 5-4). Alkali-aided samples were little affected going from 1 to 1.5 M Gu-HCl (figure 5-5). Going from 1.5 M to 2 M Gu-HCl led to almost complete unfolding of all samples.

Micro-Differential Scanning Calorimetry (DSC)

The thermal events observed for all pH-shift processed samples are shown in figures 5-6, 5-7 and table 5-1. The event boxes in blue (table 5-1) are endothermic events and the event boxes in red are exothermic events. The numbers listed are first the temperature and then the specific heat, normalized from an internally derived baseline using Origin software. The table boxes are grouped on a per-degree basis with samples grouped together in one row if the thermal events happen within one degree of each other.

The first event occurred at 11.8 +/- 0.09°C. The event was endothermic in all samples except pH treatment 11.5 was exothermic. The next event occurred at 20.8-21.74°C in pH treatment 10.5 and the control. Following this event a single exothermic event occurred in pH treatment 10.5 at 23.73°C. Exothermic events occurred in pH treatments 2.0, 2.5, 10.5, 11.0 and 11.5 between 28.27-29.35°C. The next event was an endothermic event in all the acid treated samples over the range of 34.79-35.26°C. The alkali treated samples had an event at 36.83-37.31°C. The control, however, had an exothermic event at 36.56°C. The pH treatments 2.5 and 3.0, had an endothermic event at 39.65°C and 39.82°C, respectively. The pH-treatments, 2.0, 10.5, 11.0, 11.5, had an exothermic event between 41.21-42.10°C. The control sample had an endothermic event at 43.47°C. An endothermic event was recorded among all the pH-treated samples occurring between 44.52-45.67°C The pH-treatments 2.0, 3.0, 10.5, 11.0, 11.5 had an endothermic event ranging from 47.69-48.34°C. The control had an exothermic event at 47.20^{o}C. In pH treatment samples 2.0, 3.0, 10,5, 11.0, 11.5 and the control, an endothermic event was recorded, while pH-treatment 2.5 had an exothermic event between 50.04-51.75°C. An exothermic event in pH-treatment 2.5 occurred at 55.71°C. The pH-treatments 11.0 and 2.0 had exothermic events at 56.83°C and 57.17°C respectively. An exothermic event occurred in pH treated samples 2.5, 10.5, 11.5 and the control between 57.82-58.81°C. The pH-treatments

3.0 and 11.5 had an endothermic event at 60.39°C and 59.61°C respectively. In pH treatment samples 2.0 and 2.5 an endothermic event occurred at 62.60°C and 63.16°C, respectively. The pH-treatments 3.0 and 10.5 had exothermic events occur at 63.63°C and 63.81°C respectively. An endothermic event occurred in the control at 64.97°C. Exothermic events occurred between 66.49-67.38°C in pH-treatments 2.0, 3.0, 10.5 and 11.5. Exothermic events occurred in pH-treatment 2.5 and the control at 68.42°C and 69.36°C respectively. Endothermic events at 71.18°C occurred in pH-treatments 2.0 and 10.5. The pH-treatment 2.0 had an exothermic event occurring at 72.88°C. The last events ranged from 74.18-76.39°C. Endothermic events occurred in pH-treatments 2.0, 2.5 and the control at 74.18°C, 75.95°C and 75.12°C, respectively. The pH-treatments 10.5 and 11.0 had exothermic events occurring at 74.83°C and 76.39°C, respectively.

Susceptibility of Proteins to Cross-linking by Transglutaminase

Susceptibility of protein isolates and control samples to transglutaminase (TGase) induced cross-linking was measured by following increases in G' of the system as a function of time and calculating the difference between final G' of the untreated and treated sample (i.e. susceptibility ratio). Figures 5-9 to 5-15 show that G' for all TGase containing samples, except control and isolates made with the pH 2.0 treatment, increased over untreated samples as time progressed however no main effect was determined. All samples however had a higher final G' for TGase samples after 1 h compared to untreated samples, resulting in a positive susceptibility ratio for all samples (figure 5-8). No significant differences ($p>0.05$) were seen in the susceptibility ratio (figure 5-8) between the acid treated samples or the alkali treated samples. No significant differences in the susceptibility ratios ($p>0.05$) were found between acid treatment and alkali treatments compared to the control.

Discussion

Circular dichroism was used as a comparative method to study the average change in the structural properties of all proteins collected during pH-shift processing. The differences observed between the average structural content of pH-shift treated samples and control samples indicate a structural transition from alpha helix to beta structure as denaturation increases. The native secondary structural content of muscle proteins followed the order of control>alkali>acid. The increase in beta structure content indicates that extreme pH used during pH shift processing induced protein unfolding at the high or low pH used to extract the proteins. After adjustment of the protein system from high or low pH to pH 5.5, refolding occurs resulting in an increase in the beta structure content of protein isolates. Previous studies have shown that during the denaturation process of proteins the beta structure secondary structure is more structurally stable than the alpha helix secondary structure and alpha helix structures may transition to beta structures before completely unfolding into a random coil (Drummy and others 2005). The structural changes induced by pH-shift processing may be species specific because, in contrast to the present study, results shown for pH adjusted cod myosin with pH-shift processing showed a minimal effect on the secondary structure of refolded myosin (Kristinsson and Hultin 2003a). However, similar to the catfish used in the present study, it was reported that acid treatment resulted in the reduction of secondary structure with isolated catfish myosin (Raghavan and Kristinsson 2007a). This indicates that the structural trends observed when testing the whole protein isolates system are specific to fish species and similar to the results obtained for isolated catfish myosin. However the differences observed between catfish myosin and cod myosin further indicate that the unfolding and refolding parameters need to determined across species and different fish may possibly impart different gel properties to the isolated protein. Though the refolding properties of catfish myosin (Raghavan and Kristinsson 2007a) showed similarities

with the results attained from the present protein isolates those results applied best to the pH 2.5 treatment. The results show that catfish myosin treated at pH 2.0 showed a larger decrease in secondary structure than treatment at pH 2.5 (Raghavan and Kristinsson, 2007a). These results are different from the effect of pH 2.0 treatment on protein isolates (figures 5-1 and 5-2) which showed that this pH did not significantly decrease ($p>0.05$) in secondary structure and in particular the alpha helix content. This means that similarities exist between the isolated individual proteins and the whole muscle system; however, the structure of protein isolates has unique properties that are not dictated exclusively by myosin.

Gu-HCl treatment is used for investigating the susceptibility of proteins to chemical denaturation. Gu-HCl as denaturant can be used to establish the chemical denaturation kinetics of a protein system. Normally one would expect a somewhat sigmoidal denaturation curve at increasing levels of Gu-HCl, but this was not seen here for the catfish proteins. Gu-HCl induced a small change in protein structure at 0.5 M for acid-aided isolates, while alkali-aided isolates were further denatured. Control became highly denatured at this low level of Gu-HCl. A further increase in Gu-HCl led to refolding (more negative response at 222 nm), followed by more unfolding (less negative response at 222 nm) as Gu-HCl increased. This data therefore does not allow for a two-state kinetic model to be established. Structural events occurring in protein folding after pH-shift processing and apparent increases in folding of protein isolates with increased Gu-HCl indicate molten globular structures are formed. However these molten globules are not unique to the isolates as the control also demonstrated apparent refolding behavior. Fan and others (1996) showed an increase in chicken liver dihydrofolate reductase activity in low concentrations of Gu-HCl with no significant change in secondary structure according to circular dichroism results of secondary structural analysis (Fan and others 1996).

The results of Fan and others (1996) and those shown here support the presence of a range of conformational isomers with similar low energy states (Kumar and others 2000) resulting in structures which have modified functional properties.

The DSC results from this study show that the thermal transitions of pH-shift processed catfish muscle differ not only from the control but are also dependent on the pH used during processing. The first temperature range which an event was recorded at was 11°C. This event occurred in all samples however pH treatment 11.5 showed an exothermic event at this low temperature compared to all other samples showing endothermic events.

The next thermal event was recorded between 20.8-21.74^{o}C in pH treatment 10.5 and the control with pH treatment 10.5 having another small event closely following at 23.73^{o}C. Major distinguishing events occurred from 34.79-35.26^{o}C in the acid treated samples and from 36.83-37.31^{o}C in alkali treated samples. In these temperature ranges endothermic events were recorded for pH-treated samples. At 36.56^{o}C, within the alkali treated range, the control had an exothermic event. Catfish muscle subjected to pH-shift processing resulted in an endothermic event occurring rather than an exothermic event. Acid processing of catfish muscle further changed the thermal sensitivity of the isolated proteins reducing the temperature, 34.79-35.26^{o}C, at which this reaction occurred. The change in the thermal response of acid treated catfish muscle may be due to the proteins co-precipitating with myosin as acid treated isolated myosin shows the first thermal event at 37^{o}C (Raghavan and Kristinsson 2007a, 2007b).

The next series of events occurred between 39.65-48.34^{o}C. The event leading this off was catfish muscle processed at pH 2.5 and 3.0 which had exothermic events occurring at 39.65^{o}C and 39.82^{o}C respectively whereas the other pH shift processed samples had endothermic events occurring between 41.22-42.1^{o}C, with the control event occurring at 43.47^{o}C. Catfish muscle

subjected to pH-shift processing exhibited a lower temperature range of thermal events, (39.65-42.1^{o}C), than the control sample, (43.47^{o}C). The control peak can be associated with the second transition of whole myosin peaks as seen for catfish myosin at pH 7.3 which exhibited a second transition peak at 44.9^{o}C (Raghavan and Kristinsson 2007b). The control at 43.47^{o}C and 47.2^{o}C showed very large exothermic peaks as seen in figure 5-6, the 4. These higher temperature peaks however did not correlate with the two myosin peaks shown by Raghavan and Kristinsson (2007b) for isolated catfish myosin which occurred at 37^{o}C and 44.9^{o}C. The first large peak at 43.47^{o}C was shifted from the pH treated samples as seen in table 5-1. The second large peak was consistent with the pH-treated samples except for pH treatment 2.5. However within these two large peaks seen in the control all the pH shift processed samples had an endothermic event between 44.52-45.66^{o}C. The range observed here would fall within an expected range with the second myosin peak shown by Raghavan and Kristinsson (2007b) with catfish myosin. Over the 39.65-42.1^{o}C temperature range Xiong (1997) reported the first transition peak of chicken breast myosin at 40^{o}C. The results of this study and those of Raghavan and Kristinsson (2007b) do not indicate that catfish myosin has the first transition at 40^{o}C but that an additional peak is observed either by a co-precipitated protein or due to the structural modification of catfish myosin during the precipitation process in the presence of other muscle proteins.

Following these series of events, between 50.04-51.02^{o}C an endothermic event occurred in all samples. This indicates that though pH-shift processing alters the structure and thermal response of muscle proteins, there is this one temperature range which is not affected by pH-shift processing and some part of myosin or another co-precipitated protein. The thermal events occurring within this temperature region have been associated with the transitions of myosin S-2 subfragment (51^{o}C) in rabbit and chicken breast, the light meromyosin chains in chicken breast

(52^{o}C) and the myosin light chains in rabbit (51^{o}C) (Xiong 1997). The thermal transitions above the 50-51^{o}C range show different patterns of thermal reactivity. These differences in the thermal events, of individual pH treatments and the control, may provide insight into the differences observed in other properties of pH-shift processed muscle proteins.

Transglutaminase (TGase) is an enzyme which promotes the formation of covalent bonds in muscle proteins. This bond is based on the cross linking of lysine and glutamine to form the dipeptide ε-(γ-glutamyl)lysine (Perez-Mateos and Lanier 2007). The formation of covalent cross links in muscle proteins in the absence of TGase are primarily by the formation of disulfide bonds (Stone and Stanley 1992).

The formation of covalent cross links in muscle proteins promotes the formation of stronger heat set gels at temperatures below the point where muscle proteins form irreversible gels, normally between 25-50°C. This increase in gel strength at low temperature is a setting effect referred to as suwari setting. The use of TGase in muscle systems has been employed to formulate muscle products from smaller pieces of meat such as scallops, steaks, shrimp and ham (Ajinomoto 2007). The use of TGase in surimi processing has been employed for the improvement of lower grade surimi or the mixture of lower grade surimi with higher grade surimi to form the gel strengths needed for appropriate product formulation.

The susceptibility of muscle products to TGase activity is based on the availability of both lysine and glutamine on the surface of the proteins to interact with the active site of TGase to form the covalent cross link. The protein motifs which facilitate TGase activity have not been characterized; however, TGase activity in muscle systems has been directly correlated with the decrease in myosin heavy chain (Perez-Mateos and Lanier 2007).

The results of this study indicate that pH treatment of catfish muscle proteins may promote interactions of the isolated proteins with TGase for all alkaline-aided isolates but not all acid-aided isolates. Thus these results indicate that structural modifications which occur with pH treatment result in the interactions of isolated proteins with TGase appear greater than those without pH treatment under most treatment conditions. Perez-Mateos and Lanier (2007) have indicated that myosin heavy chain is reduced when muscle is treated with TGase, suggesting cross-linking between myosin. The structural modifications during acid processing were more pH specific with respect to TGase treatment as treatment at pH 2.0 was not significantly different from the control.

These results, when compared with the structural results indicate that the specific changes of muscle proteins isolated at pH 2.0 are not identifiable from other acid treatments by CD but result in surface modification of the proteins as shown by TGase treatment. The high variability of pH-treatment 10.5 indicates that the refolded surface structure at this pH is not as consistent as the refolding of proteins treated under more extreme alkali conditions. The use of pH-shift processing is also species specific as the results of this study are in contrast to the results reported (Perez-Mateos and Lanier 2007) which found no increase in gel strength by TGase treatment to acid solubilization of protein isolates made from Atlantic Menhaden. The reactivity of muscle proteins with TGase indicates the accessibility of specific amino acids to TGase. Changes in the accessibility of specific amino acids indicate structural changes in the proteins directly affect the surface structure of proteins. The characterization of the specific location and proteins involved in the interaction with TGase may lead to further understanding of the protein motifs responsible for enzyme induced cross-linking and increase the understanding of pH-shift processing at specific sites within a protein or protein mixtures.

Conclusions

This study demonstrated that pH shift processing decreases the alpha helical content of protein isolates, increases the structural flexibility and modified the temperatures and types of thermal events catfish muscle proteins underwent. These results support our hypothesis that pH-shift processing will affect the structure and thermal reactivity of catfish muscle proteins and those effects were unique to the pH used during processing. It may be concluded that pH-shift processing affects catfish muscle protein secondary structure. This modification of secondary structure may lead to the modification of the thermal reactivity and the susceptibility of protein isolates to enzymatic modification and ultimately the modification of the thermal gelation properties of catfish muscle proteins. Additionally the use of enzyme treatment, specifically TGase, may provide an effective method of analysis for determining the modification of the surface structure of muscle proteins and provide insight to the mechanisms involved in the interaction and gelation of a highly complex material.

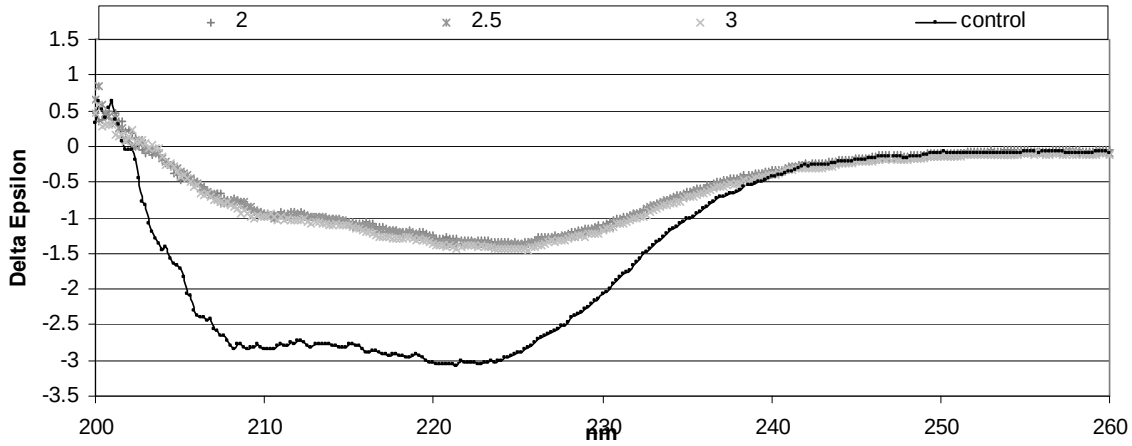

Figure 5-1: Circular dichroism of catfish protein isolate made with acid processing and untreated catfish muscle recorded from 200-260nm. Delta epsilon units reported were obtained from molar ellipiticity. Samples were scanned in 25 mM Tris-HCl and 600 mM NaCl, pH 7.2. The treatment pH is shown at the top.

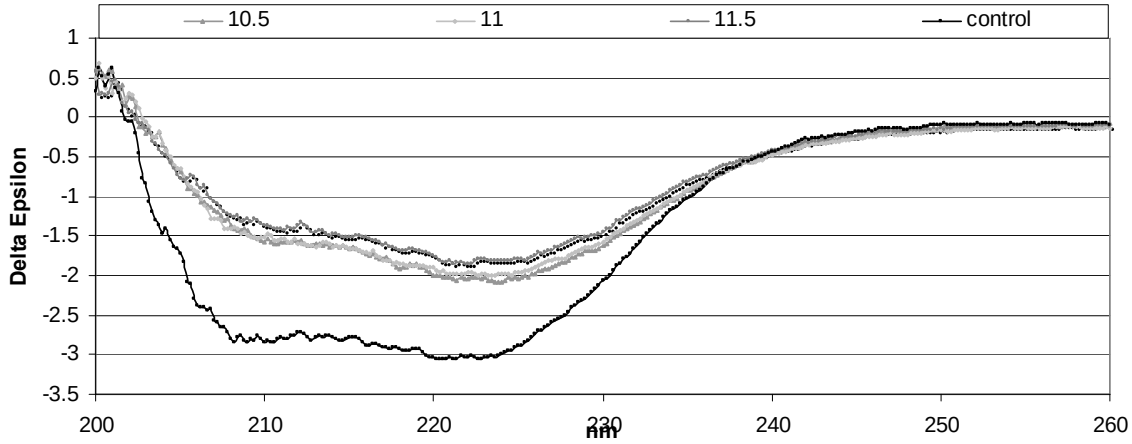

Figure 5-2: Circular dichroism of catfish protein isolate made with alkali processing and untreated catfish muscle recorded from 200-260nm. Delta epsilon units reported were obtained from molar ellipiticity. Samples were scanned in 25 mM Tris-HCl and 600 mM NaCl, pH 7.2. The treatment pH is shown at the top.

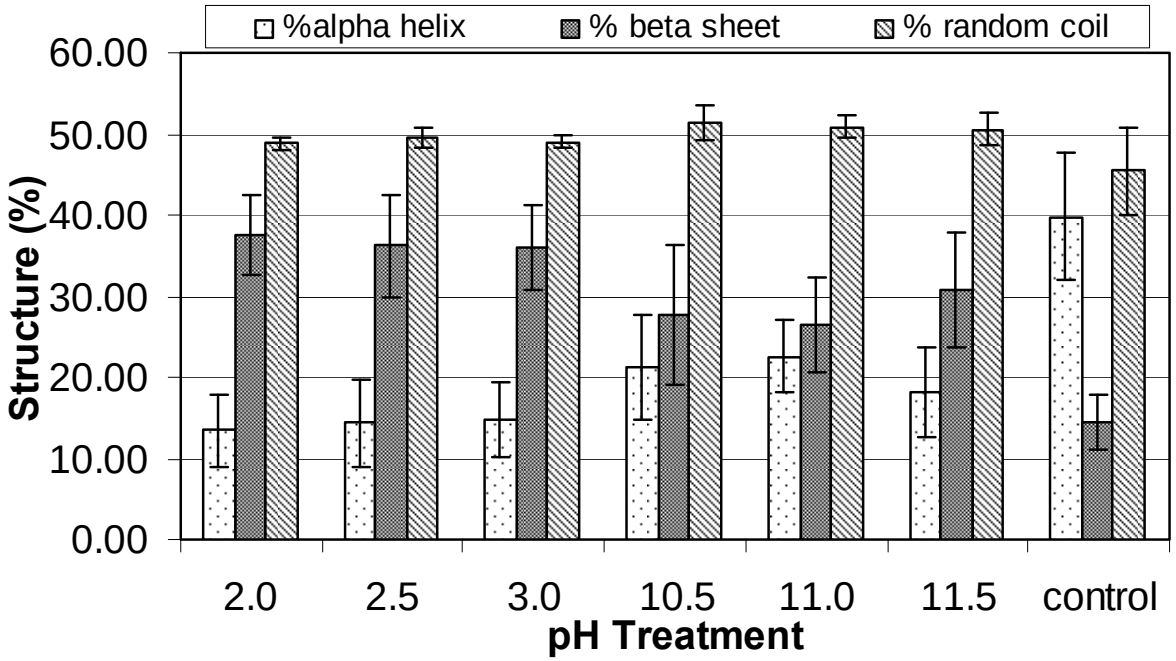

Figure 5-3: Level of apparent α-helix, β-structureand random coil in catfish protein isolates and untreated control. Percent structure was calculated from Dichroweb using the K2D model. The K2D model did not require a protein reference set and provided a secondary structural model without requiring data points below 200 nm. No significant differences ($p>0.05$) were present between any of the samples for all structures.

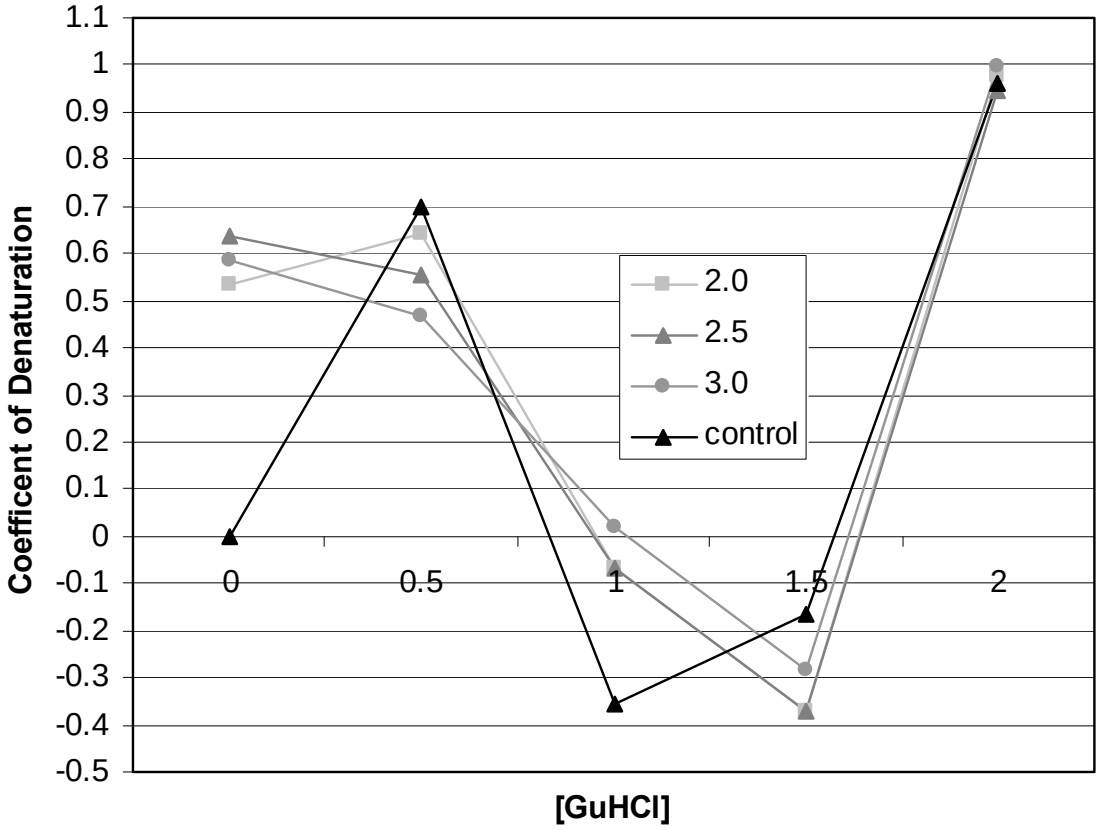

Figure 5-4: Effect of Gu-HCl (0-2 M) on apparent structure of low pH processed catfish protein isolates and untreated control. GuHCl treatment used 222 nm as the indicator wavelength of alpha helix content. The change in structural content was determined by whole muscle control in 0 M GuHCl having 100% native or 0% denatured structure. 100% denatured structure was calculated from the response at 222 nm of the control in 6 M GuHCl. The two state denaturation model was then used to calculate the fraction of denaturation of each sample.

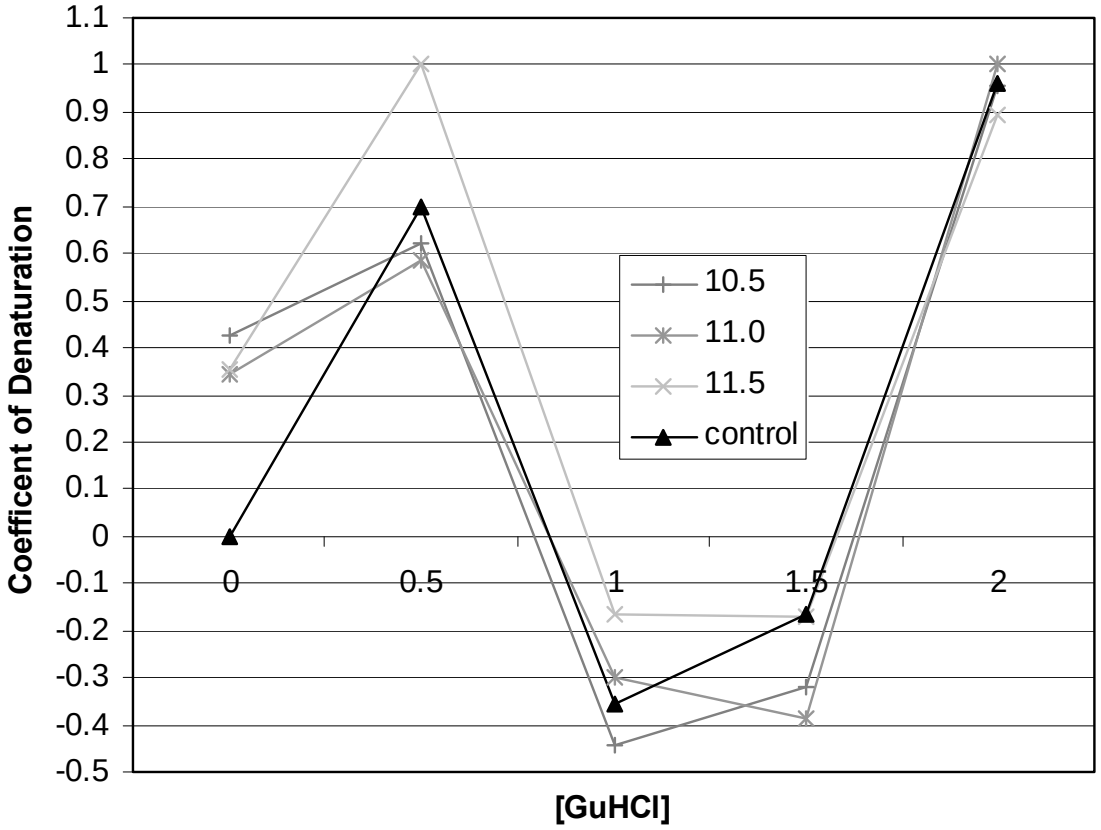

Figure 5-5: Effect of Gu-HCl (0-2M) on apparent structure of high pH processed catfish protein isolates and untreated control. GuHCl treatment used 222 nm as the indicator wavelength of structural content. The change in structural content was determined by whole muscle control in 0 M GuHCl having 100% native or 0% denatured structure. 100% denatured structure was calculated from the response at 222 nm of the control in 6 M GuHCl. The two state denaturation model was then used to calculate the fraction of denaturation of each sample.

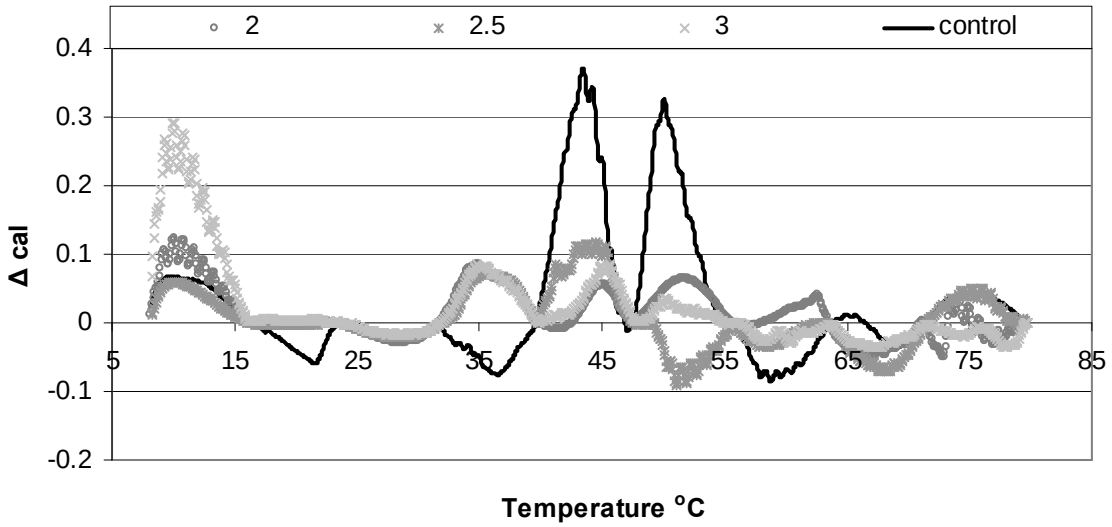

Figure 5-6: DSC thermograms of low pH treated catfish protein isolates and untreated control. The thermograms represented here are the average of 4 replicate scans from two separate isolations. The curves were smoothed together by 3 point averaging. Average smoothing was repeated 4 times to obtain the smoothed graph. The black line is the untreated control.

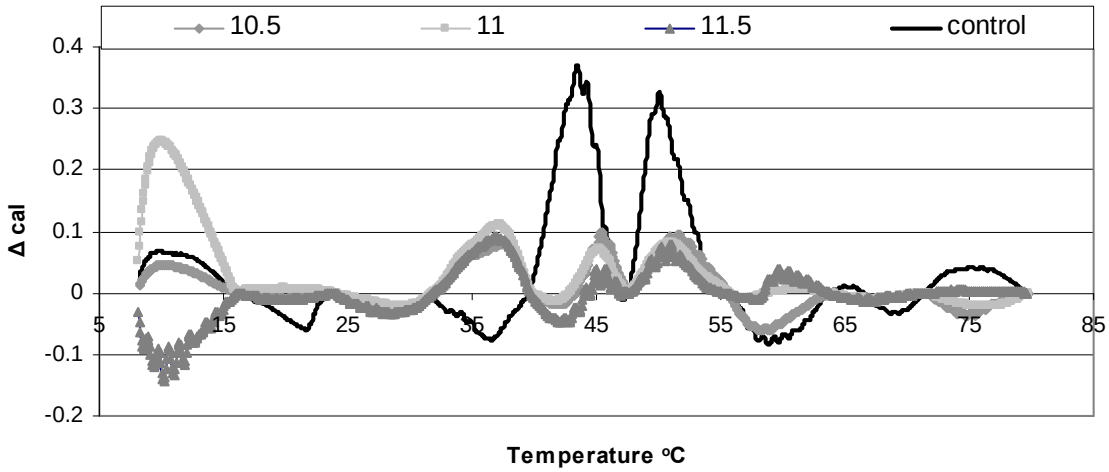

Figure 5-7: DSC thermograms of high pH treated catfish protein isolates and untreated control. The thermograms represented here are the average of 4 replicate scans from two separate isolations. The curves were smoothed together by 3 point averaging. Average smoothing was repeated 4 times to obtain the smoothed graph. The black line is the untreated control.

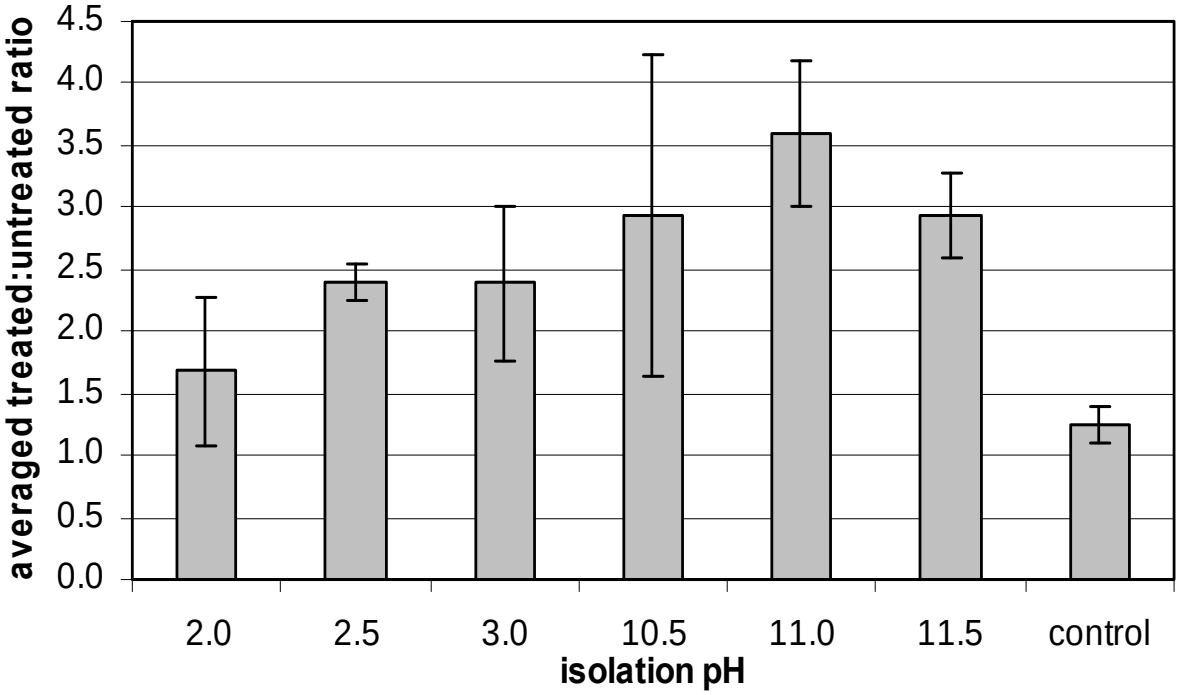

Figure 5-8:The susceptibility of catfish protein isolates and untreated control to enzymatic treatment with transglutaminase (TGase). Measurement of TGase activity was the relative increase in the storage modulus between 0.02% TGase treated and untreated incubated at 30°C for 1 hr. A main effect was not determined for the sample population ($p>0.05$).

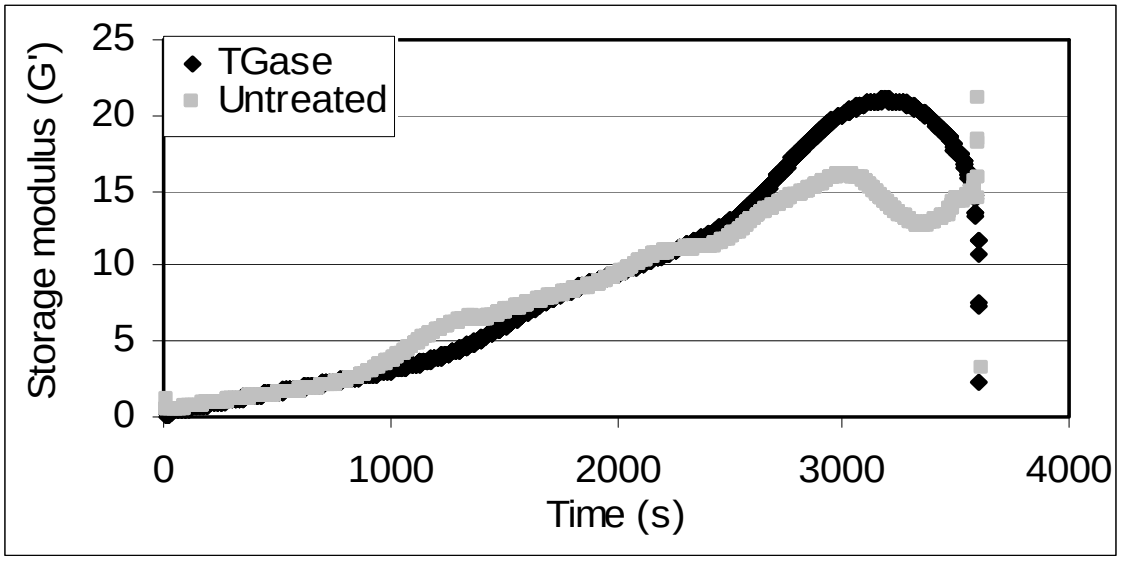

Figure 5-9: Compiled and smoothed rheogram of pH treatment 2.0 with (black) and without (gray) TGase treatment. Protein pastes were prepared at 10% solids in 20 mM tris-HCl buffer with 0.6 M NaCl, pH 7.2. Samples were tested at 30°C for 1 hour. Rheograms were combined and smoothed using TA rheology advantage data analysis software.

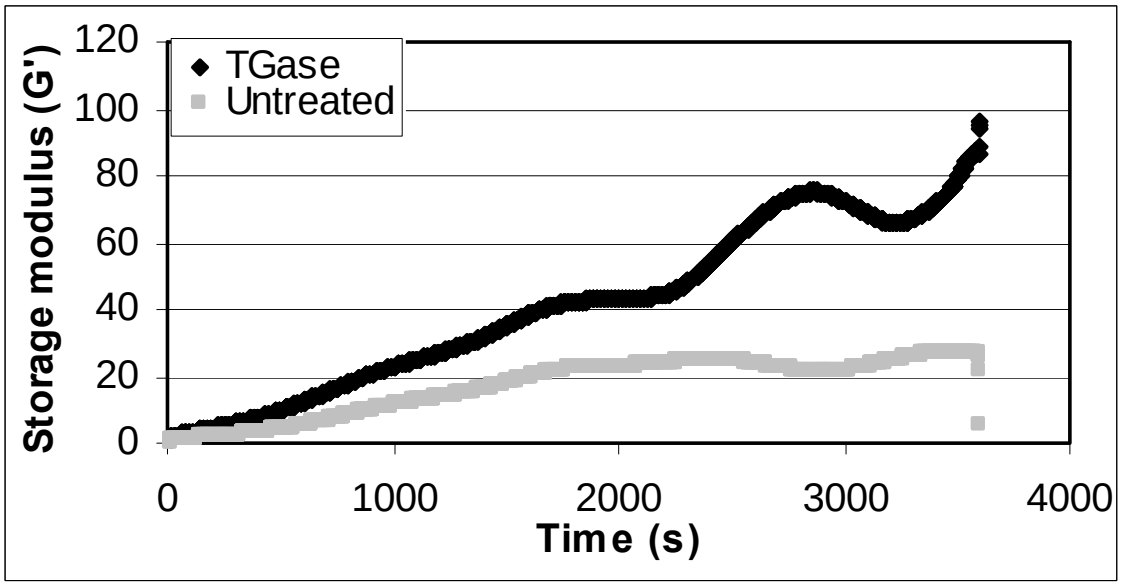

Figure 5-10: Compiled and smoothed rheogram of pH treatment 2.5 with (black) and without (gray) TGase treatment. Protein pastes were prepared at 10% solids in 20 mM tris-HCl buffer with 0.6 M NaCl, pH 7.2. Samples were tested at 30°C for 1 hour. Rheograms were combined and smoothed using TA rheology advantage data analysis software.

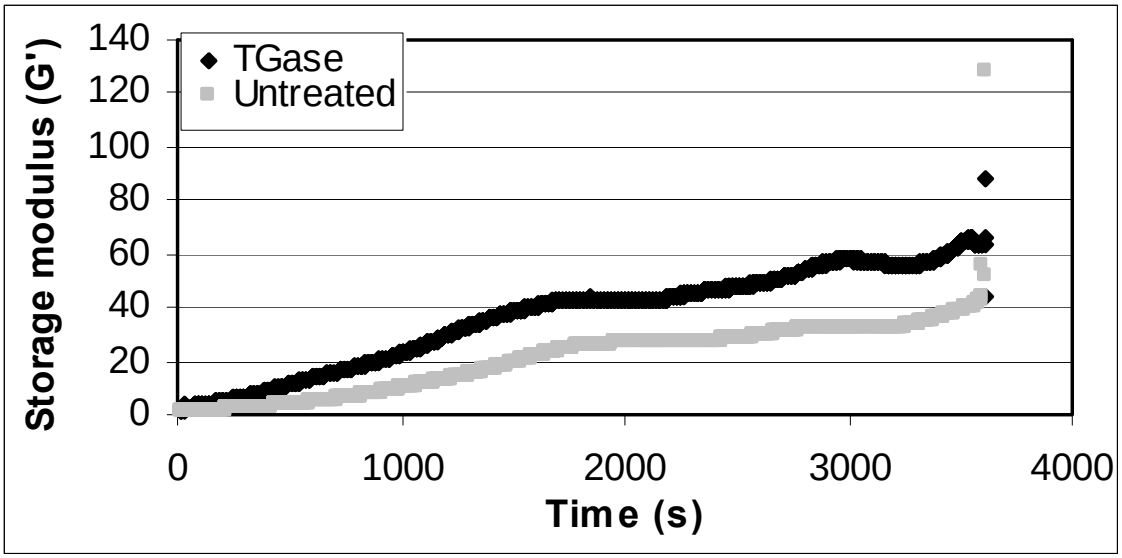

Figure 5-11: Compiled and smoothed rheogram of pH treatment 3.0 with (black) and without (gray) TGase treatment. Protein pastes were prepared at 10% solids in 20 mM tris-HCl buffer with 0.6 M NaCl, pH 7.2. Samples were tested at 30°C for 1 hour. Rheograms were combined and smoothed using TA rheology advantage data analysis software.

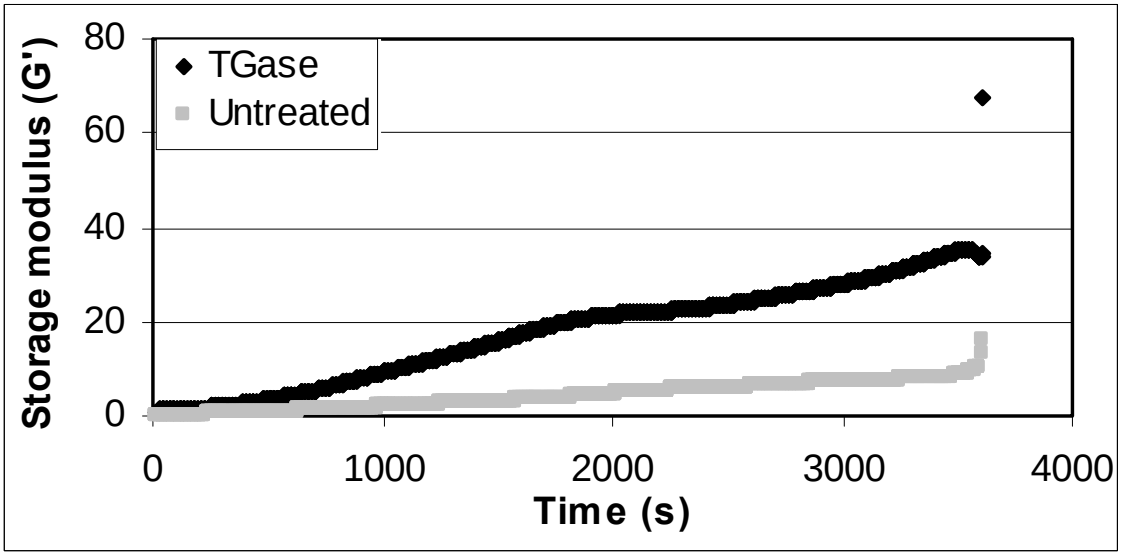

Figure 5-12: Compiled and smoothed rheogram of pH treatment 10.5 with (black) and without (gray) TGase treatment. Protein pastes were prepared at 10% solids in 20 mM tris-HCl buffer with 0.6 M NaCl, pH 7.2. Samples were tested at 30°C for 1 hour. Rheograms were combined and smoothed using TA rheology advantage data analysis software.

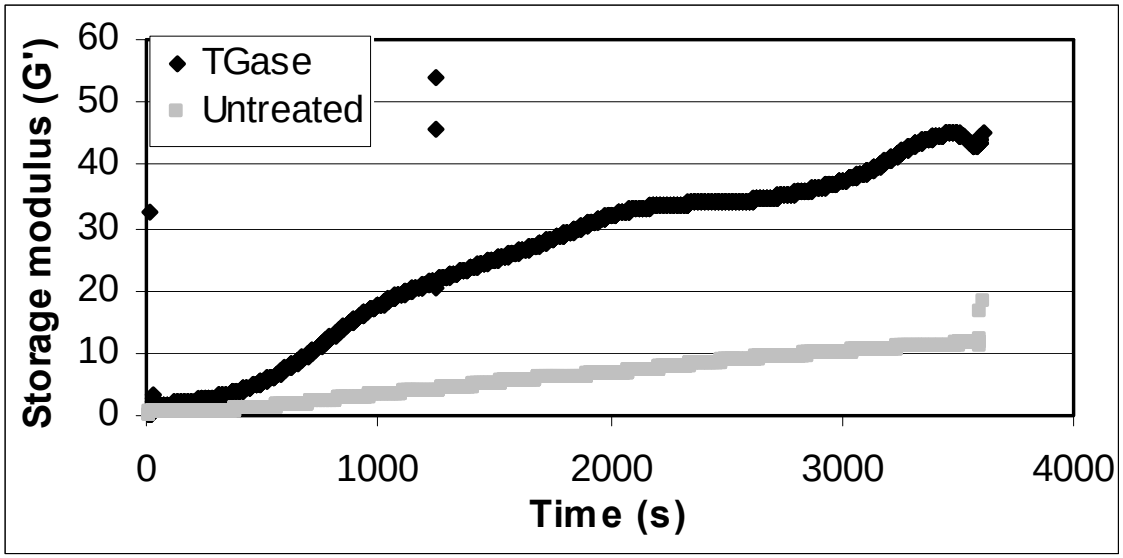

Figure 5-13: Compiled and smoothed rheogram of pH treatment 11.0 with (black) and without (gray) TGase treatment. Protein pastes were prepared at 10% solids in 20 mM tris-HCl buffer with 0.6 M NaCl, pH 7.2. Samples were tested at 30°C for 1 hour. Rheograms were combined and smoothed using TA rheology advantage data analysis software.

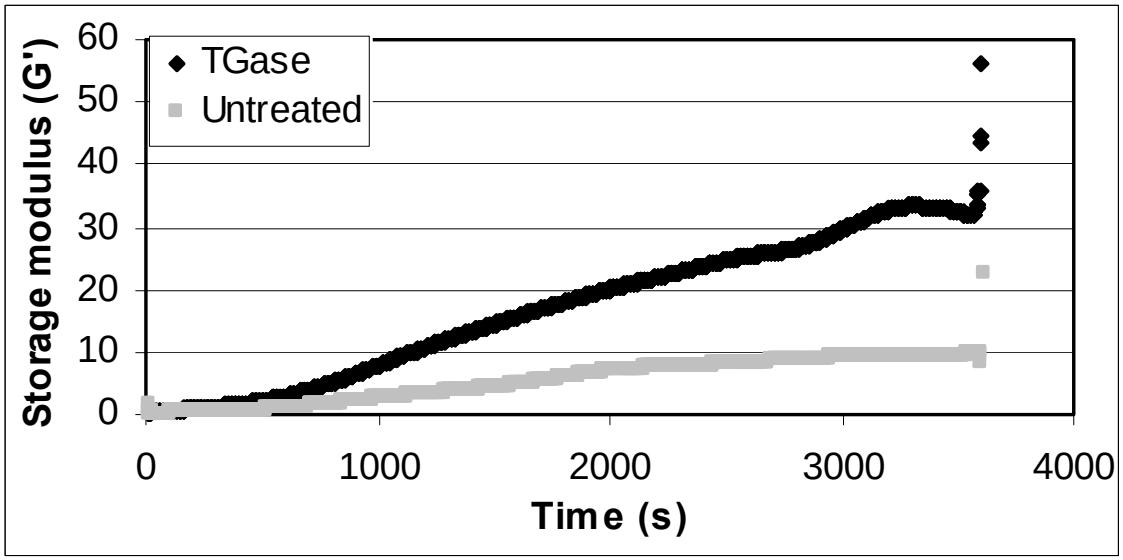

Figure 5-14: Compiled and smoothed rheogram of pH treatment 11.5 with (black) and without (gray) TGase treatment. Protein pastes were prepared at 10% solids in 20 mM tris-HCl buffer with 0.6 M NaCl, pH 7.2. Samples were tested at 30°C for 1 hour. Rheograms were combined and smoothed using TA rheology advantage data analysis software.

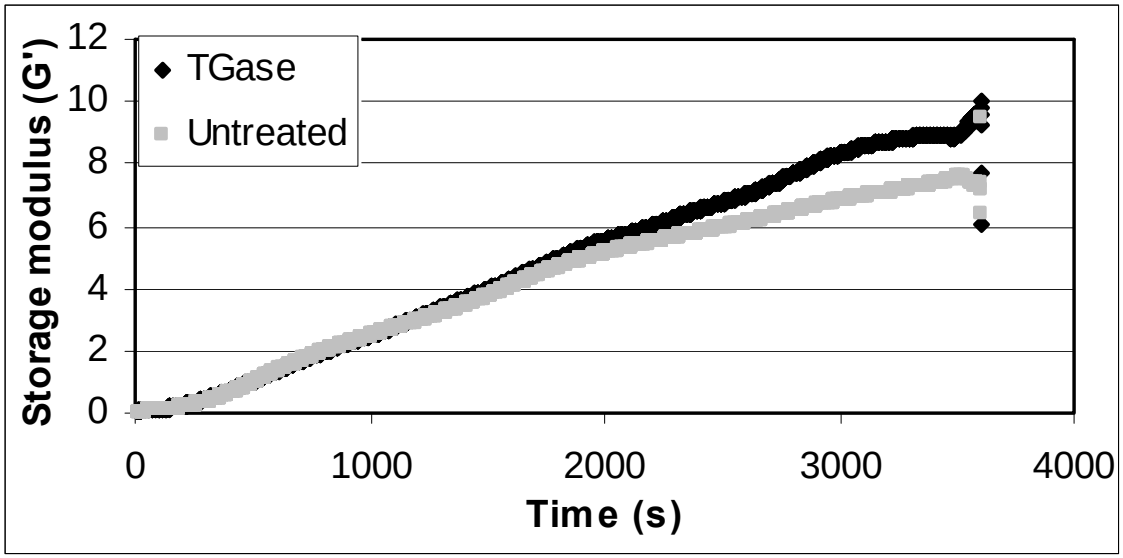

Figure 5-15: Compiled and smoothed rheogram of the control with (black) and without (gray) TGase treatment. Protein pastes were prepared at 10% solids in 20 mM tris-HCl buffer with 0.6 M NaCl, pH 7.2. Samples were tested at 30^{o}C for 1 hour. Rheograms were combined and smoothed using TA rheology advantage data analysis software.

Table 5-1: Thermal events of catfish protein isolates and untreated control as recorded by DSC. The smoothed transposed data was used to generate this peak table. The columns are separated into samples and the rows are thermal events. Thermal events are paired to ~1oC. Blue represents an endothermic event, orange represents an exothermic and yellow denotes no event was present.

pH 2.0		pH 2.5		pH 3.0		pH 10.5		pH 11.0		pH 11.5		Control	
T (°C)	tCP	T (°C)	tCP	T (°C)	tCP	T (°C)	tCP	T (°C)	tCP	T (°C)	tCP	T (°C)	tCP
11.79	0.1044	11.82	0.0476	11.80	0.2234	11.84	0.0386	11.76	0.2021	11.84	-0.1160	11.79	0.0622
						20.80	-0.0103					21.74	-0.0600
						23.73	-0.0007						
28.55	-0.0285	28.27	-0.0215			29.35	-0.0297	29.14	-0.0214	28.54	-0.0342		
34.80	**0.0852**	**35.26**	**0.0722**	**34.79**	**0.0845**								
						37.31	**0.0803**	**37.25**	**0.1111**	**36.83**	**0.0908**	**36.56**	**-0.0758**
		39.65	0.0076	39.82	0.0010								
41.22	-0.0091					41.85	-0.0188	41.94	-0.0133	42.10	-0.0484		
												43.47	0.3714
45.19	0.0563	44.52	0.1175	45.64	0.0838	45.42	0.0985	45.34	0.0719	45.67	0.0398		
47.70	0.0007			47.84	0.0018	47.77	0.0007	47.69	0.0019	48.34	0.0155	47.20	-0.0118
51.75	0.0645	51.09	-0.0907	51.08	0.0238	51.66	0.0960	50.93	0.0817	51.02	0.0776	50.04	0.3260
		55.71	-0.0028					56.83	-0.0108	57.82	-0.0101		
57.17	-0.0047	58.62	-0.0355			58.15	-0.0623					58.81	-0.0844
				60.39	-0.0284					59.61	0.0392		
62.60	0.0400	63.16	0.0003	63.63	-0.0024	63.82	-0.0013						
												64.97	0.0109
66.49	-0.0522			67.11	-0.0354	67.38	-0.0143			67.22	-0.0140		
		68.42	-0.0710									69.36	-0.0339
71.18	0.0020					71.18	0.0001						
72.88	-0.0502												
74.18	0.0396	75.95	0.0502			74.83	-0.0385	76.39	-0.0214			75.12	0.0424

CHAPTER 6
THE EFFECT OF HEATING RATE ON pH-SHIFT PROCESSED CATFISH (*Ictalurus punctatus*) MUSCLE PROTEINS

Introduction

The functional characteristics of muscle proteins are determined by the structural conformation of the proteins in raw muscle and the intermediate structures of proteins during the transition from the native to denatured state (Xiong 1997). One of the ways to dictate the structural transition of muscle proteins from the native state to the non-native state is by the application of heat (Phillips and others 1994). Temperatures above the denaturation temperature are usually required to achieve the structural intermediates necessary which result in the formation of a gel network of proteins (Phillips and others 1994). Muscle proteins denature over a wide temperature range. Thermal transitions points of isolated myosin are in the range of 40^{o}C-67^{o}C (Xiong 1997). Muscle proteins show increases in gel strength when subjected to thermal treatment., However, at medium temperatures, or about 35-55^{o}C, muscle proteins have exhibited weakening in gel strength which is thought to be due to the disassociation of the helix-coil of myosin. Above this temperature range strengthening of protein-protein interactions occur, and gel strength increases again, This dissociation is thought to disrupt the gel network as it is forming but further increases in temperature result in the reformation of the gel network resulting in the final set form (Xiong 1997). Thus certain structural and conformational changes of muscle proteins during thermal processing are required to achieve desirable functionalities (Xiong 1997). The modification of thermal processing by altering the heating rate has been shown to affect the functional properties of comminuted muscle protein products (Riemann and others 2004a). For example, slower heating rates allows progressive, sequential unfolding of proteins leading to a finer texture and increased water holding capacity of muscle proteins (Xiong 1997).

The objective of this study was to investigate the effect of various heating rates on the gel forming ability of protein isolates made from acid and alkali processed catfish protein isolates.

Methods

Heating rate gelation studies were conducted at North Carolina State University (Raleigh, NC) in the laboratory of Dr. Tyre Lanier.

Production of Protein Isolates

Fresh channel catfish was obtained for the study from a local supplier. Protein isolates were produced as outlined in Figure 2-1: pH 2.5 for the acid-aided process and pH 11.0 for the alkali-aided process. Precipitation following filtration was preformed at pH 5.5. Removal of insoluble materials was accomplished by screening the material 3 times through progressively tighter mesh. After precipitation of the clarified muscle protein homogenates chiffon cloth was used for dewatering protein paste to less than 78% moisture.

Protein Composition

Protein isolates were assayed for moisture in a drying oven for 24hrs. Protein content was assumed to be 97% dry weight (Kristinsson and others 2005b).

Torsion

Torsion tubes, 10 cm in length and 1.8 cm in diameter were filled with protein paste at 78% moisture and subsequently heated from 10°C to a 70°C internal endpoint, either rapidly by a cylindrical microwave (Riemann and others 2004b), at 20 or 98°C/min to test rapid heating effects, slowly at 1°C/min by immersion in a programmable water bath or placed directly in an 70°C water bath for 15 minutes. Temperatures were measured by a fiber optic probe (Riemann and others 2004b). Upon reaching 70°C, rapidly heated samples were held for 0 or 20 min prior to rapid cooling by immersion in ice water for sufficient internal cooling to <10°C for ~30 min. Samples were held static in a cylindrical microwave applicator (length 16 cm, radius 12.5 cm)

and heated using power settings calculated previously. After reaching a final temperature of 70°C, this temperature was maintained in gels during the subsequent holding period by utilizing feedback software (Riemann and others 2004b).

Rheology

Rheological changes (storage modulus, G') of pastes/gels at 78% moisture were non-destructively and continuously measured as pastes were heated, held and cooled using a 40 mm, 4 degrees slope cone and plate attachment of a constant stress, small strain rheometer (Stresstech, Rheologica instruments AB, Lund, Sweden). Oscillation parameters were those used in Riemann et al 2004. Heating conditions were at either 20°C/min, the most rapid heating rate possible for this apparatus, 0.5°C/min, 1°C/min, 2°C/min or 5°C/min to an endpoint temperature of 70°C followed by holding for 0 or 20 min prior to cooling at 5°C/min to 10°C.

Statistical Analysis

Preliminary investigation of the affect of heating rate on pH-shift processed catfish protein isolates was conducted on a single isolation of catfish fillets. The fillets used in this study were obtained from two lots of fillets shipped to a local supplier. Torsion studies were conducted on four to six cooked gels. Each gel was sampled 2 to 4 times. Rheological studies were conducted in duplicate.

The heating rate study was analyzed with Graph Pad QuickCalcs online calculator software (Graph Pad Software Inc. San Diego Ca). Analysis used an unpaired t-test with a manual bonferoni correction, overall significance of $p<0.05$ with the individual significant p-values of $p<0.004$.

Results

Torsion

The torsion stress and strain results were obtained by thermally treating catfish muscle isolated at both acid (2.5) and alkali (11.0) pH by two heating methods: microwave heating at a heating rate of 20°C per minute with (20/20) and without (20/0) a 20 minute hold, a heating rate of 98°C per minute with a 20 minute hold (98/20) and water bath heating rates of 0.5°C (0.5 water), 1.0°C (1 water), and 10°C (10 water) per minute. Rapid heating provided total process times of 3 - 23 and 21 min, respectively, for 20°C with and without 20 minute holding and 98°C per minute with a 20 minute hold. The stress and strain are shown in figures 6-1 and 6-2, respectively.

The stress results showed that alkali treatments were significantly higher ($p<0.05$) than acid treatment. Alkali processing showed an order of 20/20>1 water>0.5 water>98/20>10 water>20/0. Significant differences were not present ($p>0.05$) between alkali treated samples 20/0 and 10 water. Significant differences were not present ($p>0.05$) 0.5 water and1 water when compared to 20/20 and 98/20 however significant differences were present between 98/20 and 20/20. Acid processing showed an order of 0.5 water>1 water>98/20>20/20>10 water>20/0. Significant differences were not present ($p>0.05$) between acid treated samples 20/0, 20/20 and 10 water. Significant differences were not present ($p>0.05$) between acid treated samples 98/20 and 1 water. Acid treatment 20/20 was not significantly different ($p>0.05$) from 1 water however it wsa significantly different ($p<0.05$)from 98/20. Significant differences were present ($p<0.05$) between the acid treated sample 0.5 water and all other acid treated samples.

The strain results showed that alkali treated catfish protein was significantly higher ($p<0.05$) than acid treatment 10 water. The strain results showed alkali treated catfish protein 20/0 had significantly higher ($p<0.05$) strain values than acid treated catfish protein 20/20, 98/20,

0.5 water and 10 water. The alkali treated isolate showed an order of 20/20>0.5 water>20/0>1 water>98/20>10 water. Acid treatment showed an order of 1 water=20/20>98/20>0.5 water>10 water>20/0.

Rheology

The rheograms shown in figures 6-4 - 6-10 show the gel formation process of varied heat treatments. During the heating phase all rapidly heated samples, both acid and alkali, followed almost exactly the same heating pattern (figure 6-10) with differentiation between the gels occurring at either the onset of holding or cooling, depending on the treatment. The acid sample, though not significant ($p>0.05$), did not reach as high a G' during high temperature holding as the alkali treatment. Acid treated catfish muscle not held at high temperature exhibited a reduction in G' on cooling at 32^{o}C. No decrease on cooling was observed for the acid sample held at high temperature or the alkali treated samples. The acid and alkali samples heated at 0.5^{o}C per minute did not follow the same gelation pattern on heating or cooling after heating ~30^{o}C (figure 6-6, 6-9). During the heating phase of the samples heated at 0.5^{o}C the G' decreased until ~30^{o}C. After this decrease the G' began to increase in both the acid and alkali treated samples. The alkali treated sample increased at a greater rate than the acid treated sample and did not exhibit a major decline in G' after it began to increase at 30^{o}C. The acid treated sample increased in G' until reaching the maximum G' of the heating and cooling regime at ~42^{o}C. Above ~42^{o}C the acid treated sample decreased in G' until the start of the cooling phase at 70^{o}C.

The rheology results shown figure 6-3 of this study show that prior to heating there were no significant differences ($p>0.05$) between any of the samples treated under acid or alkali conditions. After heating at 70°C prior to either holding or cooling, the G' of alkali isolate heated at 20°C per minute with no hold was significantly higher ($p<0.05$) than acid treated

isolate heated under the same conditions. After holding at 70°C no significant differences ($p>0.05$) were shown between any of the samples, before holding or after holding. No significant differences ($p>0.05$) were observed based on heating regimen within acid or alkali treated samples. When comparing acid treatment to alkali treatment heating at 20°C per minute with or without holding alkali treated protein isolates had a significantly higher ($p<0.05$) G', however, acid treated isolate held for 20 minutes at 70°C did not show significant differences ($p>0.05$) between any of the acid treated or alkali treated samples.

Discussion

Structural shifting from the native state to intermediate states results in modifications of muscle protein functionality. The transition of muscle proteins from native to intermediate states may be induced by the heating method applied to a muscle protein paste . Heating rate has shown to affect the functional properties of muscle proteins.

Isolates heated at a high rate with holding at high temperature, had a significantly higher ($p<0.05$) stress than rapidly heated samples with no holding at high temperature. The increase gel strength of isolates treated held at high temperature may allow the requisite time for muscle proteins to unfold into a conformation of higher functionality. Previous studies on high temperature holding with rapid heating rates showed muscle proteins improving in gel strength during short high temperature holding times. These results follow previously published studies showing that rapid heating rates with no holding produce gels with lower strength than those cooked at slower rates (Riemann and others 2004). The application of high temperature holding for alkali treated catfish muscle proteins followed the results reported by Riemann and others (2004) showing an increase in gel strength with increased high temperature holding on rapidly heated comminuted muscle pastes (Riemann and others 2004). The acid processed catfish isolate, however, did not follow this same trend with high temperature holding of rapidly heated

gels increasing gel strength. Heating at 20°C/min with or without holding did not significantly affect ($p>0.05$) the gel strength of acid treated catfish muscle. However the very high heating rate of 98°C/min with 20 min holding resulted in significant increases ($p<0.05$) in gel strength. The increased gel strength of rapidly heated gels were significantly lower than gels cooked at the slowest heating rate of 0.5°C/min ($p<0.05$) but were not significantly lower than gels cooked at 1°C/min heating rate ($p>0.05$).

The rheological results from this study indicate that during rapid heating, the high heating rate has the predominating effect on the mechanism of gelation of pH-shift processed muscle as seen by the similar patterns of gel formation in figure 6-10. The modification of catfish muscle proteins by pH-shift processing changing the gel rigidity on cooling, indicate that the modified protein structures alter the hydrogen bond formation associated with the cooling phase of gelation (Lanier 2000). The effect of high temperature holding on the gel structure after cooling may be due to increased covalent linkages formed at high temperature (Lanier 2000) or the application of enough energy into the muscle protein matrix to allow for a more complete denaturation of muscle proteins resulting in a more favorable structural rearrangement (Xiong 1997) which promotes the formation of hydrogen bonds (Lanier 2000).

The proposed equivalence point calculation (Riemann and others 2004a) for the determination of functional performance of heated muscle pastes does not apply to acid processed catfish muscle as the equivalent points in the heated products did not result in equivalent improvements in gel strength. The lack of improvement with the equivalent point method as proposed may be due to the modification of the protein structure during acid processing, which resulted in larger decreases in alpha helical structure and thus reduced the

overall reactive potential of acid produced catfish isolate as shown in chapter 3 with the reduction in gel strength of catfish muscle processed at pH 2.5.

Conclusions

These results show that acid and alkali processing form gels with different gel strengths at fracture regardless of the rate heat is applied. However during rapid heating the effect on gel formation, as shown by the rheology results, indicates that the predominant effect on gel formation is the rate heat is applied. Using the equivalent point method, rapid heating with extended high temperature holding for equal thermal input to the system, for the production and analysis of comminuted muscle products produced with alkali processing was shown to be effective whereas the structural modification of acid processing may not allow for the use of rapid heating to produce thermally set gels. The further modification of gel properties and the functional performance of muscle proteins subjected to pH-shift processing by modulating the rate of heat application as shown here may increase the future utilization and application of pH-shift processed protein isolates by expanding the range of textural properties achievable and reducing the required cook time for high quality comminuted muscle products.

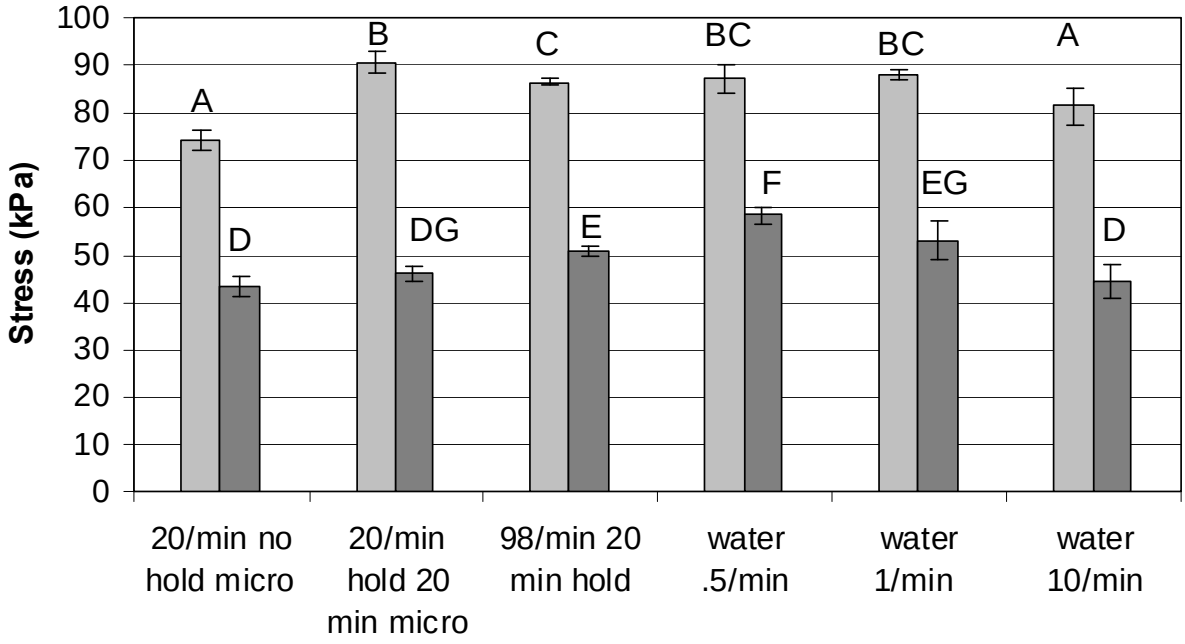

Figure 6-1: Stress response of acid-aided (pH 2.5) in dark grey and alkali-aided (11.0) in light grey catfish protein isolates (pH 11.0) recorded on the Torsion Gelometer. The microwaved samples were heated at 20°C/min with no high temperature holding (20/min no hold micro) or with a 20 minute high temperature hold time (20/min hold 20 min micro) and at 98°C/min with a 20 minute hold (98/min 20 min hold) for both acid- and alkali-aided isolates. The lower heating rates were achieved in a water bath. The 0.5°C/min (water .5/min) and 1°C/min (water 1/min) heating rates were done using a computer controlled water bath. The 10°C/min (water 10/min) heating rate was achieved by preheating a water bath to 70°C and placing the torsion tubes directly into the hot water bath for 15 minutes. Gel diameter was 1 cm and the machine rotated the gel at 2.5 RPM. The significant differences present between samples ($p<0.05$) are represented by the letters above the column. Similar letters represent no significant difference ($p>0.05$) between treatments.

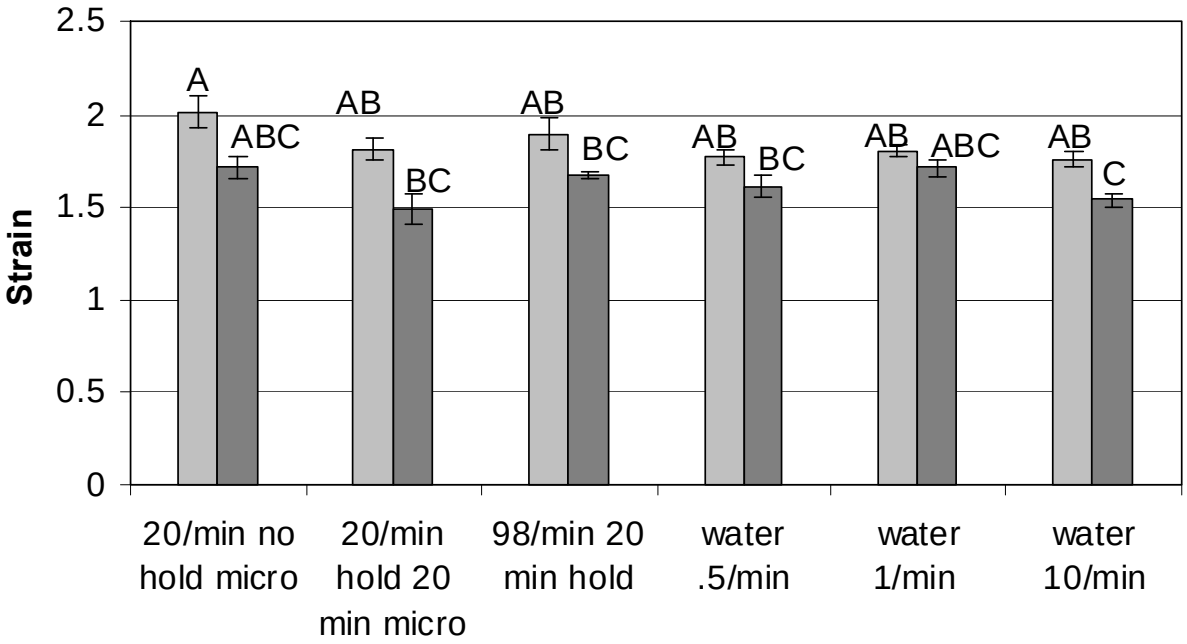

Figure 6-2: Strain response of acid-aided (pH 2.5) in dark grey and alkali-aided (11.0) in light grey catfish protein isolates (pH 11.0) recorded on the Torsion Gelometer. The microwaved samples were heated at 20°C/min with no high temperature holding (20/min no hold micro) or with a 20 minute high temperature hold time (20/min hold 20 min micro) and at 98°C/min with a 20 minute hold (98/min 20 min hold) for both acid- and alkali-aided isolates. The lower heating rates were achieved in a water bath. The 0.5°C/min (water .5/min) and 1°C/min (water 1/min) heating rates were done using a computer controlled water bath. The 10°C/min (water 10/min) heating rate was achieved by preheating a water bath to 70°C and placing the torsion tubes directly into the hot water bath for 15 minutes. Gel diameter was 1 cm and the machine rotated the gel at 2.5 RPM. The significant differences present between samples ($p<0.05$) are represented by the letters above the column. Similar letters represent no significant difference ($p>0.05$) between treatments.

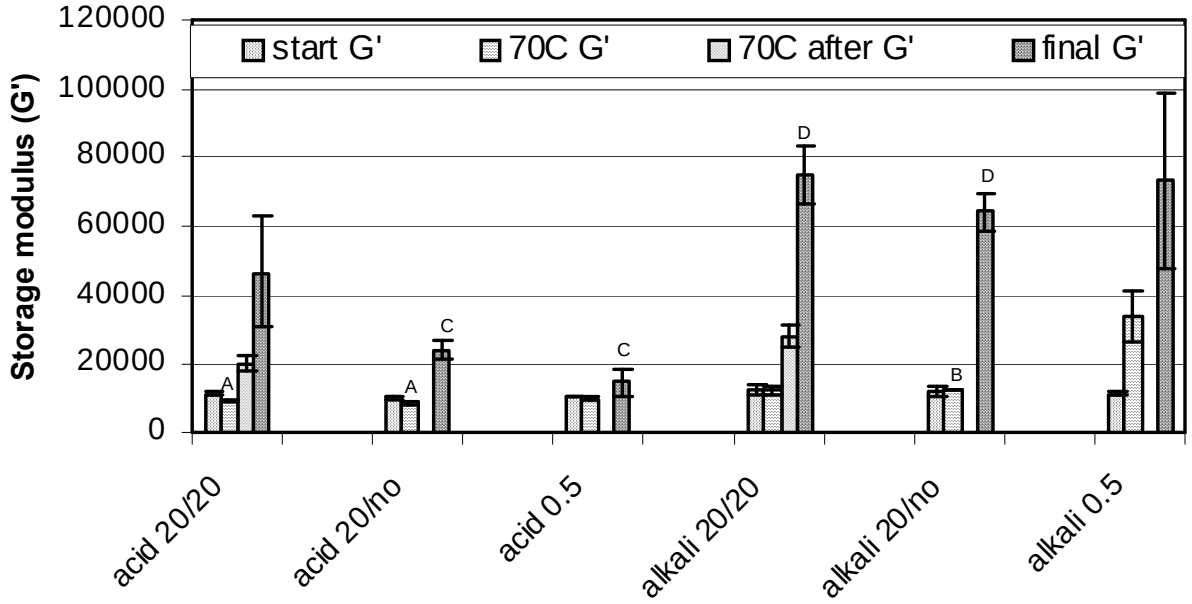

Figure 6-3: Rheological response of acid aided (pH 2.5) and alkali aided catfish protein isolates (pH 11.0) recorded on a ATS controlled stress rheometer. The first dashed column is starting G' at loading prior to any thermal treatment. The second dashed column is G' after heating to 70°C at the heating rate stated. The third dotted column is G' after holding for 20 min at 70°C, shown only in 20 minute hold samples. The fourth dotted column is G' after cooling to 1°C at 10°C/min. The thermal treatments for both acid- and alkali-aided isolates were heating at 20°C/min with (acid 20/20, alkali 20/20) or without (acid 20/0, alkali 20/0) 20 minutes holding at 70°C and heating at 0.5°C/min (acid 0.5, alkali 0.5). The significant differences ($p<0.05$) are denoted by letters above the column. The letters a and b represent the significant differences ($p<0.05$) between samples after heating to 70°C. The letters c and d represent the significant differences ($p<0.05$) between samples after heating and cooling to 5°C but before holding or cooling. No other significant differences were present between the samples.

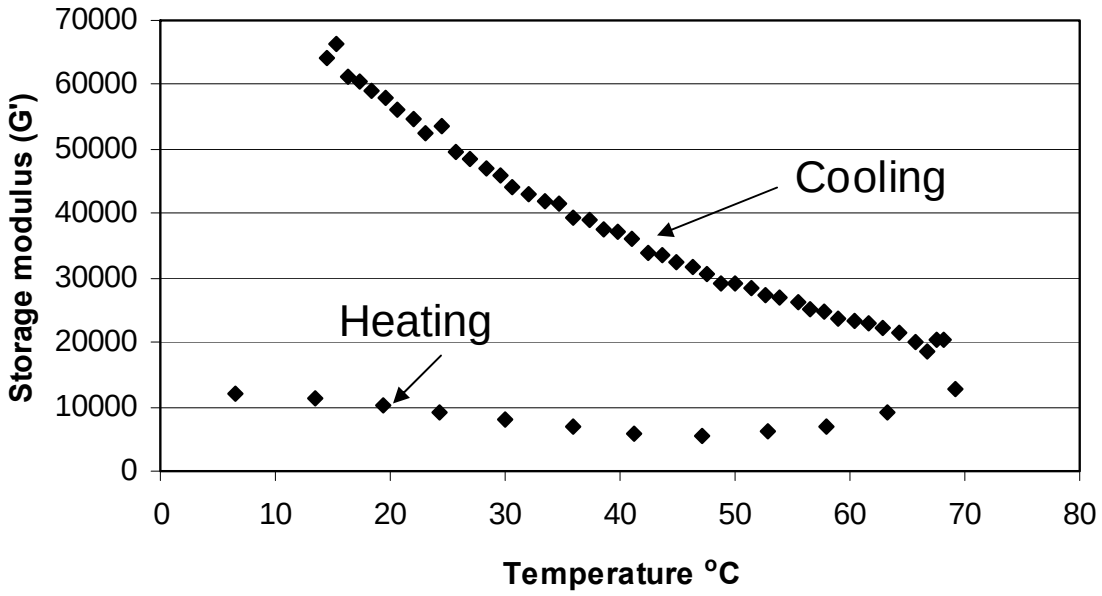

Figure 6-4: Rheogram of alkali treated catfish muscle heated at 20°C/minute with no high temperature holding. Duplicate samples at 78% moisture were heated in the presence of 2% added NaCl at pH 7.2 and averaged. The sample was tested under a controlled stress of 100 pa at a frequency of 0.1 Hz.

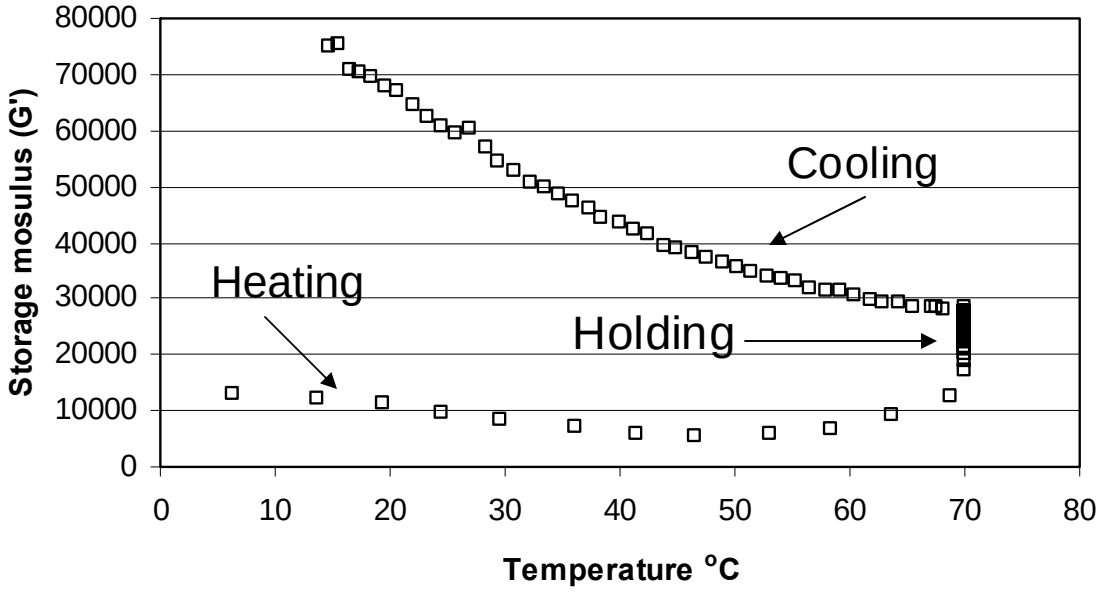

Figure 6-5: Rheogram of alkali treated catfish muscle heated at 20°C/minute with 20 minutes high temperature holding. Duplicate samples at 78% moisture were heated in the presence of 2% added NaCl at pH 7.2 and averaged. The sample was tested under a controlled stress of 100 pa at a frequency of 0.1 Hz.

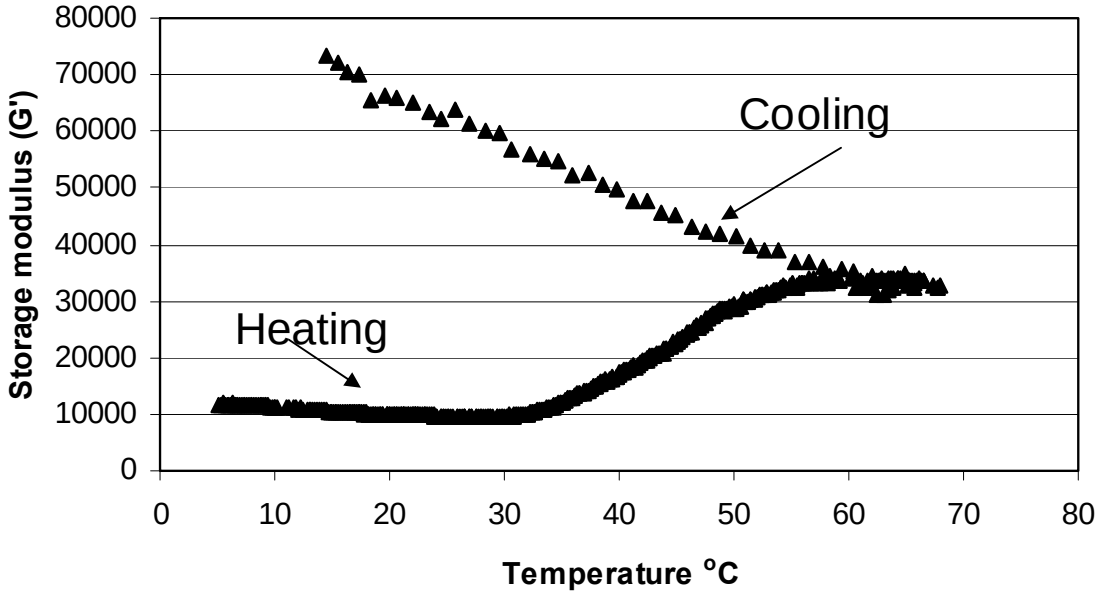

Figure 6-6: Rheogram of alkali treated catfish muscle heated at 0.5°C/minute with no high temperature holding. Duplicate samples at 78% moisture were heated in the presence of 2% added NaCl at pH 7.2 and averaged. The sample was tested under a controlled stress of 100 pa at a frequency of 0.1 Hz.

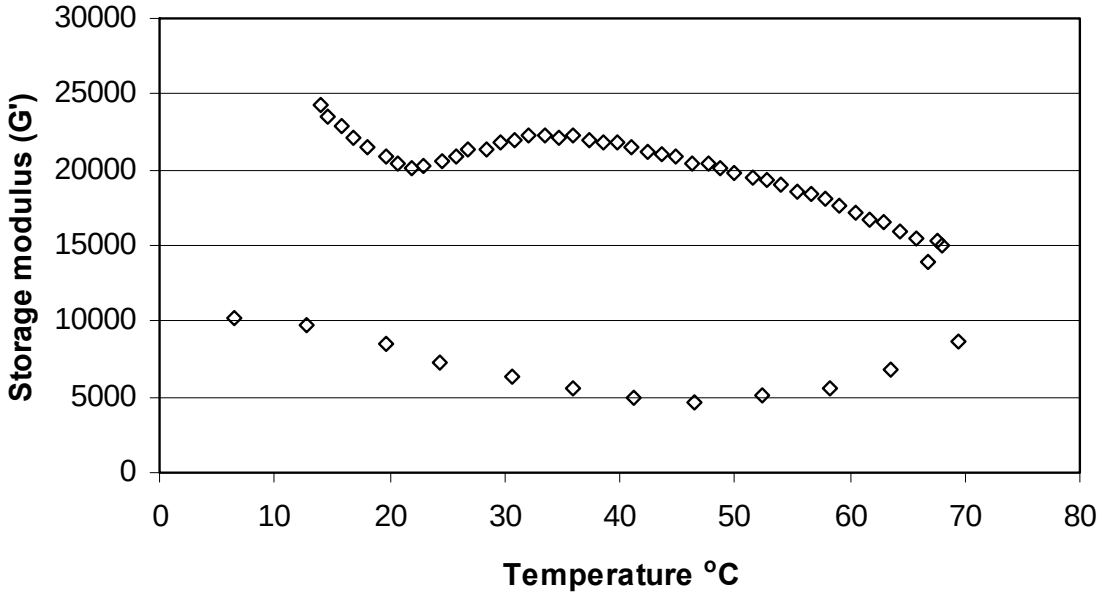

Figure 6-7: Rheogram of acid treated catfish muscle heated at 20°C/minute with no high temperature holding. Duplicate samples at 78% moisture were heated in the presence of 2% added NaCl at pH 7.2 and averaged. The sample was tested under a controlled stress of 100 pa at a frequency of 0.1 Hz.

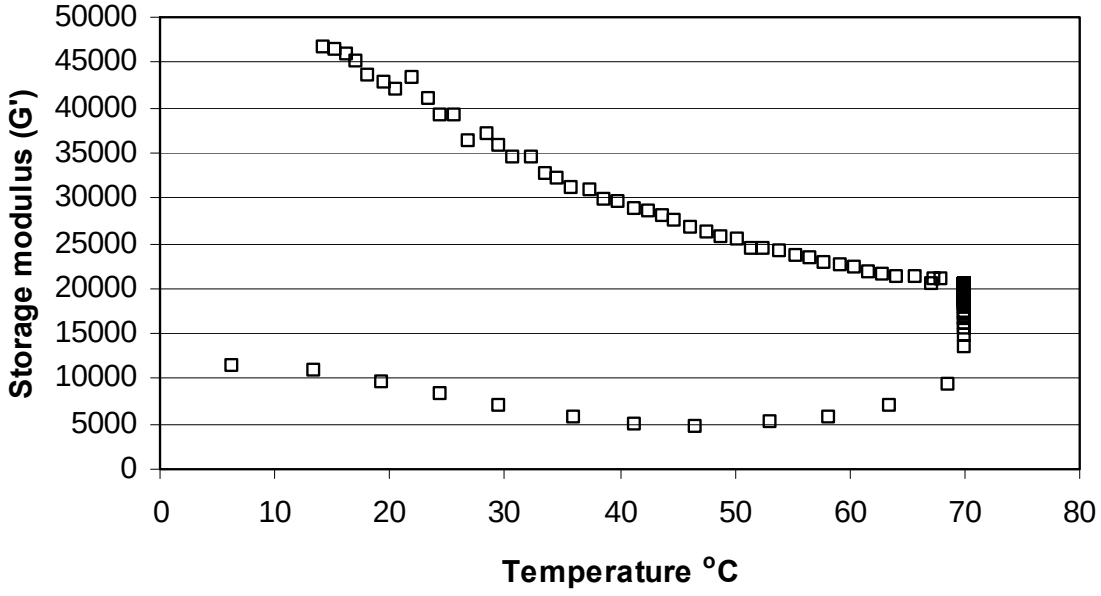

Figure 6-8: Rheogram of acid treated catfish muscle heated at 20°C/minute with 20 minutes high temperature holding. Duplicate samples at 78% moisture were heated in the presence of 2% added NaCl at pH 7.2 and averaged. The sample was tested under a controlled stress of 100 pa at a frequency of 0.1 Hz.

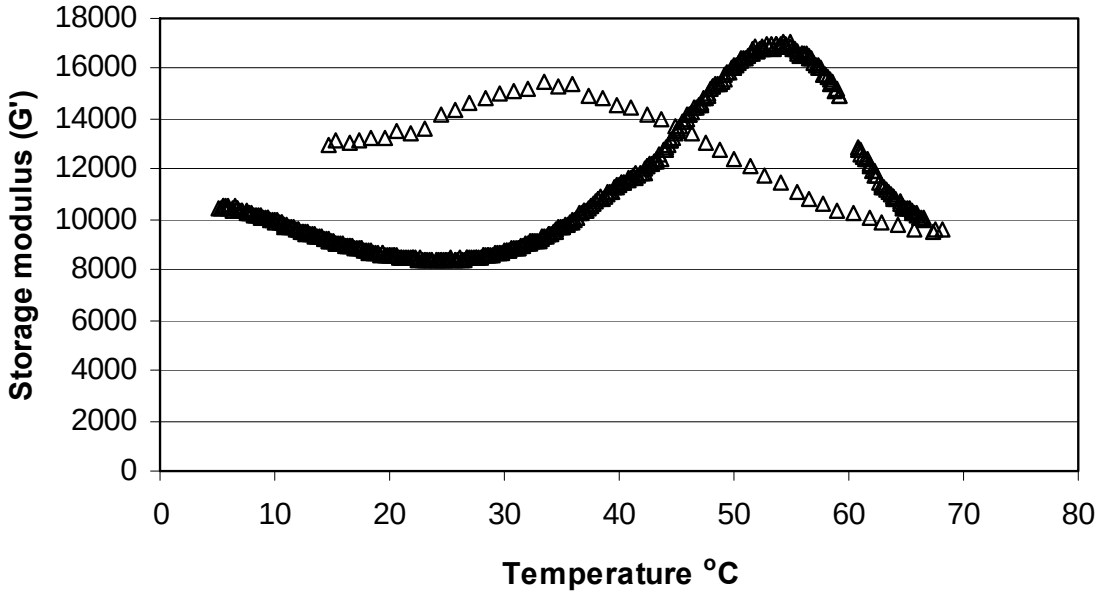

Figure 6-9: Rheogram of acid treated catfish muscle heated at 0.5°C/minute with no high temperature holding. Duplicate samples at 78% moisture were heated in the presence of 2% added NaCl at pH 7.2 and averaged. The sample was tested under a controlled stress of 100 pa at a frequency of 0.1 Hz.

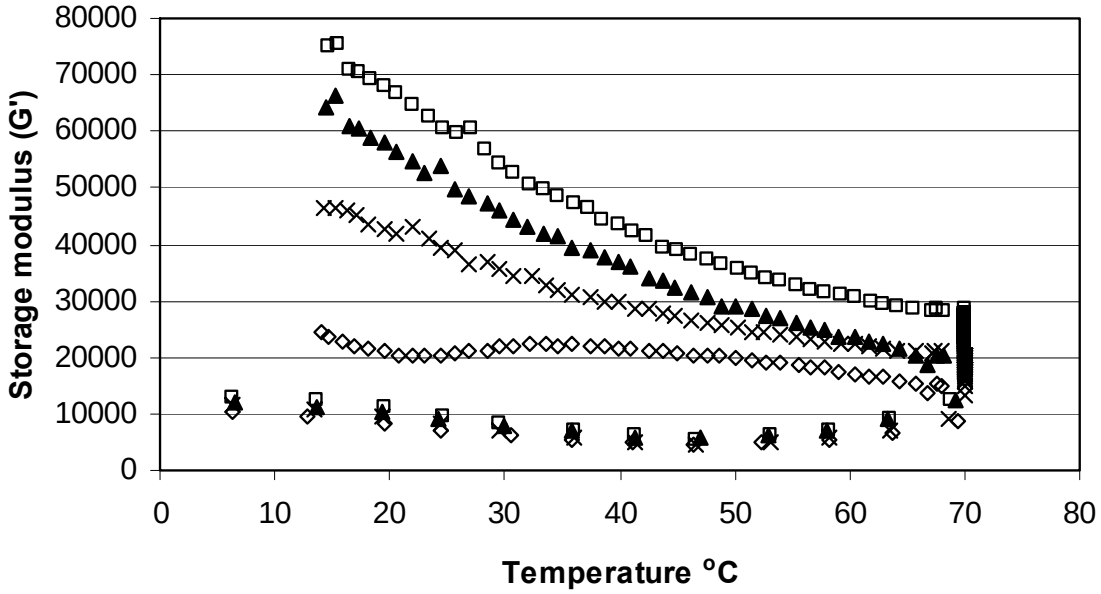

Figure 6-10: Rheograms of acid and alkali treated catfish muscle heated at 20°C/minute with and without 20 minutes high temperature holding. Duplicate samples at 78% moisture were heated in the presence of 2% added NaCl at pH 7.2 and averaged. Acid treated samples heated at 20°C with no hold (◊), acid treated samples heated at 20°C with a 20 minute high temperature hold (×). Alkali treated samples heated at 20°C with no hold (▲), alkali treated samples heated at 20°C with a 20 minute high temperature hold (□). The sample was tested under a controlled stress of 100 pa at a frequency of 0.1 Hz.

CHAPTER 7
GENERAL DISCUSSION

The results of these studies show that pH-shift processing leads to structural modification of catfish muscle proteins. The structural modification of catfish muscle proteins appears to be responsible for the modification in solubility of catfish muscle proteins but does not lead to significant differences in the chemical properties tested. The structural modification of catfish muscle proteins by pH-shift processing was found to lead to the change in the thermal and enzymatic sensitivity of catfish muscle proteins ultimately leading to the modifications observed in the physical properties of thermally treated catfish muscle proteins. By using pH-treatment on catfish muscle proteins, different functional properties are exhibited based on the type of pH-treatment used. The modification of these functional properties and the use of one or more of the pH-treatments studied provide a protein product with a wide variety of physical properties possibly leading to the custom formulation of muscle proteins products. The custom formulation of muscle products may be accomplished by the utilization of one or more pH-processing techniques studied here. For example using one pH treatment a soft gel could be formed, but a hard elastic gel with another pH treatment. The results of this study indicate that further investigation is needed to fully understand the mechanisms responsible for the changes in the physical properties observed, for example studying other chemical properties of muscle proteins not investigated here and the effect of multiple proteins on the refolding of mechanisms of muscle proteins.

Chemical analysis of pH-shift processed catfish muscle proteins showed no differences between reactive or total sulfhydryl groups. These results therefore show that pH-shift processing does not affect the sulfhydryl content of catfish muscle prior to thermal treatment. The change in surface hydrophobic groups did not show any significant difference ($p>0.05$) in

the type of pH-shift processing used, however untreated catfish muscle proteins indicated higher surface hydrophobicity than pH-shift processed catfish muscle. The lower surface hydrophobicity for isolates was unexpected, as a higher hydrophobicity would have been expected since proteins were only partially refolded. The most plausible explanation is that microaggregates of proteins formed when the bulk of hydrophobic groups were buried from the solvent. The decrease in myosin ATPase activity by pH-shift processing indicates that the active site on the myosin head was modified. Reduction in myosin ATPase has been attributed to modification in the SH group in the active site of the myosin head as previously concluded (Yongswawatdigul and Park 2002). Since no significant difference was seen in the reactive and total sulfhydryl groups the reduction in myosin ATPase activity may be due to a structural shift at the active site of the myosin head as no conclusive data was shown on the modification of SH groups. The change in solubility of catfish muscle proteins by pH-shift processing shows that pH-shift processing does reduce the solubility in both sodium chloride and modify the pH solubility profile of muscle proteins. The reduction in solubility, especially for low pH treatments, indicates that though sarcoplasmic proteins are retained during low pH treatment (Kristinsson and others 2005b) the solubility of sarcoplasmic proteins is reduced by low pH treatment. High pH treatment also retains sarcoplasmic proteins, but to a lesser degree than low pH treatment (Kristinsson and others 2005b) however the solubility results shown here indicate that high pH treatment does not reduce the solubility of sarcoplasmic proteins as drastically as low pH treatment. The reduction in solubility of the isolates is supported by the structural data which show only partial refolding after pH-treatment and thus presumably structures more prone to protein-protein interactions.

The rheological properties of catfish muscle proteins show that pH-shift processing reduces the temperature at which the phase change from a more viscous state to a gel like material occurs. The reduction in temperature as shown by the phase angle to the onset of the thermal transition of pH-shift processed catfish muscle proteins does not correlate with an increase in gel rigidity but the results as shown in the storage modulus indicate that with the correct pH salt combination increases in gel rigidity are possible. The range of differences seen within all the pH treated samples provides a textural schematic of what pH shift processing can do for muscle proteins.

The structural changes induced by pH-shift processing suggest that a molten globular form is induced after pH-shift processing. The modification of the protein structure into a molten globular form is presumed to be responsible for the chemical and physical changes seen in pH-shift processed catfish muscle. The changes in structure also cause the muscle proteins to be more flexible as treatment with low concentrations of guanidine hydrochloride induced an increased response at 222 nm which may indicate the reformation of alpha helix structure in pH-shift processed muscle proteins. The structural changes due to pH-shift processing caused a change in thermal sensitivity as seen by the DSC data. The shifting of thermal events in both type and temperature show the important effect of pH-shift processing on muscle proteins as thermal treatment is the most common processing treatment to muscle proteins. This molten globular form of the muscle proteins after pH-shift processing is attributed to the changes seen in the chemical and functional properties of the protein isolates. The modifications of thermal events show that the reactivity of catfish muscle proteins to thermal treatment undergoes different transitions and reactions at different temperatures. The change in structure of catfish muscle proteins also increased the susceptibility to enzymatic cross-linking. The increase in

enzymatic sensitivity indicates that pH-shift processing changes the structure of the muscle proteins to promote interaction with transglutaminase.

The change in the thermal treatment regime of pH-shift processed catfish muscle showed that pH-shift processed catfish muscle can be modified functionally by the thermal treatment regime. Further investigation into the cooking methods used in pH-shift processed proteins may lead to an increased range of textural properties and reduced processing times. The reduced processing times may lower energy requirements and reduce the cost of processing.

This new understanding of pH-shift processing allows the production of meat based products designed with specific physical properties. This is possible due to the range of gel textures generated by the different pH processing parameters. In addition the combination of gels produced with multiple isolates may provide a variety of textures in a single product which is more closely related to meat and fillets. While the studies presented here show that pH-shift processed catfish muscle proteins undergo structural changes which lead to changes in the physical properties, future research is needed to determine the mechanisms for transforming pH-shift produced isolates into manufactured fillets.

APPENDIX
STATSTICAL TABLES

One Way Analysis of Variance
Kruskal-Wallis One Way Analysis of Variance on Ranks
Cook Loss

Group	N	Missing	Median	25%	75%
CLpH2	4	0	1.611	1.331	1.890
CLpH2.5	4	0	2.251	2.072	2.430
CLpH3	4	0	0.942	0.189	1.695
CLpH10.5	4	0	1.242	0.971	1.512
CLpH11	4	0	1.036	0.952	1.119
CLpH11.5	4	0	1.765	1.158	2.372
CL control	4	0	1.766	1.301	2.030

H = 7.621 with 6 degrees of freedom. (P = 0.267)

One Way Analysis of Variance
Press Test

Group Name	N	Missing	Mean	Std Dev	SEM
PLpH2	4	0	9.802	1.032	0.516
PLpH2.5	4	0	9.101	2.149	1.075
PLpH3	4	0	6.949	1.242	0.621
PLpH10.5	4	0	8.347	1.798	0.899
PLpH11	4	0	6.170	1.183	0.592
PLpH11.5	4	0	6.271	1.481	0.741
PLcontrol	4	0	10.187	0.745	0.373

Source of Variation	DF	SS	MS	F	P
Between Groups	6	66.830	11.138	5.337	0.002
Residual	21	43.830	2.087		
Total	27	110.660			

One Way Analysis of Variance
Kruskal-Wallis One Way Analysis of Variance on Ranks
Fold Test

Group	N	Missing	Median	25%	75%
FpH2	4	0	3.750	3.000	4.750
FpH2.5	4	0	2.500	2.000	3.000
fpH3	4	0	4.000	3.000	5.000
fpH10.5	4	0	5.000	5.000	5.000
FpH11	4	0	5.000	5.000	5.000
FpH11.5	4	0	5.000	5.000	5.000
Fcontrol	4	0	5.000	5.000	5.000

H = 20.016 with 6 degrees of freedom. (P = 0.003)

One Way Analysis of Variance
Kruskal-Wallis One Way Analysis of Variance on Ranks
Transglutaminase treatment

Group	N	Missing	Median	25%	75%	
TGASE2	4	0	1.707	0.716	2.635	
TGASE2.5	4	0	2.306	2.191	2.608	
TGASE'3	4	0	2.598	1.410	3.369	
TGASE10.5	4	0	3.783	1.444	10.555	
TGASE11	4	0	4.031	2.899	4.290	
TGASE11.5	4	0	2.863	2.526	3.346	
TGASECONTROL	4	0	1.049	1.016	1.112	

H = 11.121 with 6 degrees of freedom. (P = 0.085)

One Way Analysis of Variance
Kruskal-Wallis One Way Analysis of Variance on Ranks
Rheology with salt, 80°C

Group	N	Missing	Median	25%	75%	
REHWS80CG'2	4	0	3103.000	2179.500	4052.000	
REHWS80CG'2.5	4	0	2151.500	2042.000	2323.000	
REHWS80CG'3	4	0	1232.000	1071.000	1393.000	
REHWS80CG'10.5	4	0	4516.500	4194.500	4838.000	
REHWS80CG'11	4	0	7151.500	4971.500	10221.000	
REHWS80CG'11.5	4	0	4815.500	4468.500	5874.000	
REHWS80CG' CONTROL	4	0	2224.500	2122.500	2320.000	

H = 23.488 with 6 degrees of freedom. (P = <0.001)

One Way Analysis of Variance
Kruskal-Wallis One Way Analysis of Variance on Ranks
Rheology without salt, 80°C

Group	N	Missing	Median	25%	75%	
REHNS80CG'2	4	0	3900.500	2296.000	5560.500	
REHNS80CG'2.5	4	0	1522.500	1365.500	1750.000	
REHNS80CG'3	4	0	8551.000	7724.500	10739.000	
REHNS80CG'10.5	4	0	8353.000	8053.500	9306.500	
REHNS80CG'11	4	0	13270.000	9532.000	16600.000	
REHNS80CG'11.5	4	0	14050.000	13055.000	15400.000	
REHNS80CG' CONTROL	4	0	6379.500	5375.500	7099.000	

H = 23.675 with 6 degrees of freedom. (P = <0.001)

One Way Analysis of Variance
Kruskal-Wallis One Way Analysis of Variance on Ranks
Rheology with salt after cooling to 5°C

Group	N	Missing	Median	25%	75%
REHSALTG'2	4	0	10487.500	6824.500	15360.000
REHSALTG'2.5	4	0	6843.500	6458.500	7463.000

REHSALTG'3	4	0	3018.500	2532.000	3505.000
REHSALTG'109.5	4	0	14825.000	13920.000	15545.000
REHSALTG'11	4	0	14190.000	11350.000	17260.000
REHSALTG'11.5	4	0	19810.000	18385.000	24010.000
REHSALTG' CONTROL	4	0	7688.000	7521.500	8113.000

H = 22.379 with 6 degrees of freedom. (P = 0.001)

One Way Analysis of Variance
Kruskal-Wallis One Way Analysis of Variance on Ranks
Rheology without salt after cooling to 5^{o}C

Group	**N**	**Missing**	**Median**	**25%**	**75%**	
REHG'2	4	0	12315.000	8375.500	19625.000	
REHG'2.5	4	0	5190.500	3929.000	6506.500	
REHG'3	4	0	35500.000	29775.000	44675.000	
REHG'109.5	4	0	31550.000	30275.000	35610.000	
REHG'11	4	0	51540.000	37565.000	63940.000	
REHG'11.5	4	0	57295.000	55460.000	63645.000	
REHG' CONTROL	4	0	21855.000	18650.000	24095.000	

H = 24.214 with 6 degrees of freedom. (P = <0.001)

One Way Analysis of Variance
Kruskal-Wallis One Way Analysis of Variance on Ranks
Torsion strain

Group	**N**	**Missing**	**Median**	**25%**	**75%**	
TORSTRAIN2	8	0	1.325	1.255	1.410	
TORSTRAIN2.5	8	0	1.175	1.140	1.295	
TORSTRAIN3	8	0	1.085	0.970	1.150	
TORSTRAIN10.5	8	0	1.395	1.020	1.605	
TORSTRAIN11	8	0	1.560	1.380	1.715	
TORSTRAIN11.5	8	0	1.575	1.525	1.660	
TORSTRAIN CONTROL	8	0	1.250	1.025	1.435	

H = 23.804 with 6 degrees of freedom. (P = <0.001)

One Way Analysis of Variance
Torsion stress

Group Name	**N**	**Missing**	**Mean**	**Std Dev**	**SEM**	
TORSTRESS2	8	0	84.790	11.315	4.001	
TORSTRESS2.5	8	0	73.833	5.836	2.063	
TORSTRESS3	8	0	89.440	18.116	6.405	
TORSTRESS10.5	8	0	99.359	14.489	5.123	
TORSTRESS11	8	0	127.396	18.656	6.596	
TORSTRESS11.5	8	0	108.529	13.181	4.660	
TORSTRESS CONTROL	8	0	41.960	4.667	1.650	

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Between Groups	6	35383.363	5897.227	33.189	<0.001
Residual	49	8706.538	177.684		

Total	55	44089.901

One Way Analysis of Variance
Kruskal-Wallis One Way Analysis of Variance on Ranks
Punch Test Jelly Strength

Group	N	Missing	Median	25%	75%	
PTJS2	9	0	6292.500	3196.050	8430.250	
PTJS2.5	9	0	4636.400	4177.200	5239.200	
PTJS3	9	0	3872.600	3259.250	4764.600	
PTJS10.5	7	0	6374.400	4860.575	7720.000	
PTJS11	6	0	6153.500	5349.600	7173.200	
PTJS11.5	7	0	7174.400	6230.325	7675.150	
PTJS CONTROL	9	0	1728.000	1546.425	2127.375	

H = 32.504 with 6 degrees of freedom. (P = <0.001)

One Way Analysis of Variance
Kruskal-Wallis One Way Analysis of Variance on Ranks
Punch Test Distance

Group	N	Missing	Median	25%	75%	
PTD2	9	0	7.600	7.150	12.925	
PTD2.5	9	0	6.700	6.500	8.600	
PTD3	9	0	6.700	6.350	7.125	
PTD10.5	7	0	8.300	7.975	9.325	
PTD11	6	0	8.000	7.700	8.500	
PTD11.5	7	0	7.900	7.600	8.950	
PTD CONTROL	9	0	9.100	7.175	10.250	

H = 19.340 with 6 degrees of freedom. (P = 0.004)

One Way Analysis of Variance
Kruskal-Wallis One Way Analysis of Variance on Ranks
Punch Test Force

Group	N	Missing	Median	25%	75%	
PTF2	9	0	491.000	455.000	819.500	
PTF2.5	9	0	618.000	605.000	688.250	
PTF3	9	0	578.000	513.750	661.750	
PTF10.5	7	0	768.000	576.000	877.500	
PTF11	6	0	791.500	701.000	853.000	
PTF11.5	7	0	876.000	608.250	938.500	
PTF CONTROL	9	0	192.000	186.750	222.750	

H = 30.333 with 6 degrees of freedom. (P = <0.001)

One Way Analysis of Variance
Kruskal-Wallis One Way Analysis of Variance on Ranks
Reactive Sulfhydryl Groups

Group	N	Missing	Median	25%	75%	
RSG2	4	0	0.00973	0.00584	0.0139	
RSG2.5	4	0	0.0104	0.00784	0.0126	
RSG3	4	0	0.00927	0.00555	0.0128	
RSG10.5	4	0	0.00858	0.00857	0.00872	
RSG11	4	0	0.0109	0.00784	0.0143	
RSG11.5	4	0	0.0101	0.00830	0.0119	
RSG CONTROL	4	0	0.00706	0.00677	0.00738	

H = 5.409 with 6 degrees of freedom. (P = 0.493)

One Way Analysis of Variance
Kruskal-Wallis One Way Analysis of Variance on Ranks
Total Sulfhydryl Groups

Group	N	Missing	Median	25%	75%
TSGpH2	4	0	0.0313	0.0303	0.0335
TSGpH2.5	4	0	0.0363	0.0294	0.0388
TSGpH3	4	0	0.0322	0.0318	0.0333
TSGpH10.5	4	0	0.0293	0.0224	0.0356
TSGpH11	4	0	0.0312	0.0301	0.0345
TSGpH11.5	4	0	0.0344	0.0234	0.0456
TSGcontrol	4	0	1.766	1.301	2.030

H = 11.209 with 6 degrees of freedom. (P = 0.082)

One Way Analysis of Variance
Kruskal-Wallis One Way Analysis of Variance on Ranks
Surface Hydrophobicity

Group	N	Missing	Median	25%	75%
SHpH2	4	0	534.735	413.460	632.420
SHpH2.5	4	0	452.370	387.580	529.155
SHpH3	4	0	495.025	435.430	531.250
SHpH10.5	4	0	472.125	410.955	509.775
SHpH11	4	0	398.455	361.400	456.695
SHpH11.5	4	0	431.795	388.550	474.115
SHcontrol	2	0	1131.840	989.180	1274.500

H = 8.701 with 6 degrees of freedom. (P = 0.191)

One Way Analysis of Variance

ATPase activity

Group Name	N	Missing	Mean	Std Dev	SEM
ATPpH2	4	0	51.014	22.191	11.095
ATPpH2.5	4	0	51.858	17.213	8.606
ATPpH3	4	0	35.211	16.573	8.286
ATPpH10.5	4	0	36.729	28.726	14.363
ATPpH11	4	0	61.346	19.634	9.817
ATPpH11.5	4	0	59.045	22.770	11.385
ATPcontrol	4	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	6	11271.662	1878.610	4.709	0.003
Residual	21	8377.625	398.935		
Total	27	19649.287			

One Way Analysis of Variance

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%	
CDRAN2	5	0	0.480	0.480	0.495	
CDRAN2.5	5	0	0.480	0.480	0.515	
CDRAN3	5	0	0.480	0.480	0.502	
CDRAN10.5	5	0	0.490	0.480	0.560	
CDRAN11	5	0	0.500	0.487	0.528	
CDRAN11.5	5	0	0.490	0.480	0.520	
CDRAN CONTROL	5	0	0.510	0.338		0.538

H = 2.266 with 6 degrees of freedom. (P = 0.894)

One Way Analysis of Variance

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%	
CDA2	5	0	0.0800	0.0725	0.220	
CDA2.5	5	0	0.0800	0.0650	0.255	
CDA3	5	0	0.0800	0.0800	0.250	
CDA10.5	5	0	0.150	0.108	0.340	
CDA11	5	0	0.210	0.150	0.320	
CDA11.5	5	0	0.110	0.0975	0.302	
CDA CONTROL	5	0	0.310	0.292		0.563

H = 12.623 with 6 degrees of freedom. (P = 0.049)

One Way Analysis of Variance
Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%	
CDB2	5	0	0.430	0.285	0.448	
CDB2.5	5	0	0.440	0.232	0.455	
CDB3	5	0	0.430	0.248	0.440	
CDB10.5	5	0	0.370	0.1000	0.412	
CDB11	5	0	0.290	0.153	0.368	
CDB11.5	5	0	0.400	0.178	0.412	
CDB CONTROL	5	0	0.130	0.0950	0.183	

H = 12.285 with 6 degrees of freedom. (P = 0.056)

Two Way ANOVA of pH Solubility data

Two Way Analysis of Variance
General Linear Model
Dependent Variable: Solubility

Source of Variation	DF	SS	MS	F	P
Sample pH	22	796410.028	36200.456	472.098	<0.001
Treatment pH	6	15843.955	2640.659	34.437	<0.001
Sample pH x Treatment pH	132	32574.525	246.777	3.218	<0.001
Residual	479	36729.665	76.680		
Total	639	883137.566	1382.062		

Main effects cannot be properly interpreted if significant interaction is determined. This is because the size of a factor's effect depends upon the level of the other factor.

The effect of different levels of Sample pH depends on what level of Treatment pH is present. There is a statistically significant interaction between Sample pH and Treatment pH. (P = <0.001)

Power of performed test with alpha = 0.0500: for Sample pH : 1.000
Power of performed test with alpha = 0.0500: for Treatment pH : 1.000
Power of performed test with alpha = 0.0500: for Sample pH x Treatment pH : 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05
Comparisons for factor: **Treatment pH within 1.5: No Significant Differences**

Comparisons for factor: **Treatment pH within 2**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
10.500 vs. 11.500	31.839	5.142	0.000	0.002	Yes
3.000 vs. 11.500	31.523	5.091	0.000	0.003	Yes
11.000 vs. 11.500	24.463	3.951	0.000	0.003	Yes
2.500 vs. 11.500	23.433	3.784	0.000	0.003	Yes

Comparisons for factor: **Treatment pH within 2.5**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
con vs. 2.000	57.028	9.210	0.000	0.002	Yes
con vs. 11.500	53.758	8.682	0.000	0.003	Yes
con vs. 2.500	53.306	8.609	0.000	0.003	Yes
con vs. 11.000	49.815	8.045	0.000	0.003	Yes
con vs. 3.000	44.938	7.258	0.000	0.003	Yes
con vs. 10.500	35.612	5.751	0.000	0.003	Yes
10.500 vs. 2.000	21.416	3.459	0.001	0.003	Yes
10.500 vs. 11.500	18.146	2.931	0.004	0.004	Yes

Comparisons for factor: **Treatment pH within 3**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
con vs. 11.500	57.549	9.294	0.000	0.002	Yes
con vs. 3.000	57.127	9.226	0.000	0.003	Yes
con vs. 2.500	56.663	9.151	0.000	0.003	Yes
con vs. 2.000	56.613	9.143	0.000	0.003	Yes
con vs. 11.000	54.283	8.767	0.000	0.003	Yes
con vs. 10.500	49.738	8.033	0.000	0.003	Yes

Comparisons for factor: **Treatment pH within 3.5**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
con vs. 11.500	24.689	3.987	0.000	0.002	Yes
con vs. 3.000	23.942	3.867	0.000	0.003	Yes
con vs. 2.000	23.548	3.803	0.000	0.003	Yes
con vs. 11.000	20.575	3.323	0.001	0.003	Yes
con vs. 2.500	20.255	3.271	0.001	0.003	Yes

Comparisons for factor: **Treatment pH within 4: No Significant Differences**

Comparisons for factor: **Treatment pH within 4.5: No Significant Differences**

Comparisons for factor: **Treatment pH within 5: No Significant Differences**

Comparisons for factor: **Treatment pH within 5.5: No Significant Differences**

Comparisons for factor: **Treatment pH within 6: No Significant Differences**

Comparisons for factor: **Treatment pH within 6.5: No Significant Differences**

Comparisons for factor: **Treatment pH within 7: No Significant Differences**

Comparisons for factor: **Treatment pH within 7.5: No Significant Differences**

Comparisons for factor: **Treatment pH within 8**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
con vs. 2.000	20.053	3.239	0.001	0.002	Yes
con vs. 2.500	18.845	3.043	0.002	0.003	Yes

Comparisons for factor: **Treatment pH within 8.5**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
con vs. 2.000	19.850	3.206	0.001	0.002	Yes
con vs. 2.500	19.338	3.123	0.002	0.003	Yes

Comparisons for factor: **Treatment pH within 9**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
con vs. 2.000	19.903	3.214	0.001	0.002	Yes
con vs. 2.500	19.273	3.113	0.002	0.003	Yes

Comparisons for factor: **Treatment pH within 9.5**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
con vs. 2.000	22.572	3.645	0.000	0.002	Yes
con vs. 2.500	19.634	3.171	0.002	0.003	Yes

Comparisons for factor: **Treatment pH within 10**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
con vs. 2.000	20.492	3.309	0.001	0.002	Yes

Comparisons for factor: **Treatment pH within 10.5**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
10.500 vs. 2.000	26.817	4.331	0.000	0.002	Yes
10.500 vs. 2.500	24.607	3.974	0.000	0.003	Yes
11.000 vs. 2.000	24.195	3.907	0.000	0.003	Yes
11.000 vs. 2.500	21.985	3.551	0.000	0.003	Yes
10.500 vs. 3.000	21.658	3.498	0.001	0.003	Yes
con vs. 2.000	21.241	3.430	0.001	0.003	Yes
10.500 vs. 11.500	19.350	3.125	0.002	0.003	Yes
11.000 vs. 3.000	19.036	3.074	0.002	0.004	Yes
con vs. 2.500	19.031	3.073	0.002	0.004	Yes

Comparisons for factor: **Treatment pH within 11**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
3.000 vs. con	44.686	7.217	0.000	0.002	Yes
10.500 vs. con	38.914	6.285	0.000	0.003	Yes
11.500 vs. con	32.067	5.179	0.000	0.003	Yes
3.000 vs. 2.000	27.165	4.387	0.000	0.003	Yes
11.000 vs. con	27.067	4.371	0.000	0.003	Yes
2.500 vs. con	26.711	4.314	0.000	0.003	Yes
10.500 vs. 2.000	21.393	3.455	0.001	0.003	Yes

Comparisons for factor: **Treatment pH within 11.5**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
3.000 vs. 11.000	23.241	3.753	0.000	0.002	Yes

Comparisons for factor: **Treatment pH within 12**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
10.500 vs. 11.000	26.405	4.264	0.000	0.002	Yes
2.000 vs. 11.000	23.213	3.749	0.000	0.003	Yes
3.000 vs. 11.000	21.632	3.494	0.001	0.003	Yes
con vs. 11.000	21.485	3.470	0.001	0.003	Yes

Comparisons for factor: **Treatment pH within NC: No Significant Differences**

Comparisons for factor: **Sample pH within 2**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
NC vs. 5.500	101.286	16.358	0.000	0.000	Yes
NC vs. 5.000	100.893	16.294	0.000	0.000	Yes
NC vs. 6.500	100.387	16.213	0.000	0.000	Yes
NC vs. 6.000	99.892	16.133	0.000	0.000	Yes
NC vs. 4.500	99.343	16.044	0.000	0.000	Yes
12.000 vs. 5.500	98.441	15.898	0.000	0.000	Yes
12.000 vs. 5.000	98.048	15.835	0.000	0.000	Yes
12.000 vs. 6.500	97.543	15.753	0.000	0.000	Yes
NC vs. 4.000	97.352	15.722	0.000	0.000	Yes
12.000 vs. 6.000	97.047	15.673	0.000	0.000	Yes
12.000 vs. 4.500	96.499	15.585	0.000	0.000	Yes
NC vs. 7.000	94.696	15.294	0.000	0.000	Yes
NC vs. 3.500	94.664	15.288	0.000	0.000	Yes
12.000 vs. 4.000	94.507	15.263	0.000	0.000	Yes
11.500 vs. 5.500	93.528	15.105	0.000	0.000	Yes
NC vs. 7.500	93.296	15.067	0.000	0.000	Yes
11.500 vs. 5.000	93.134	15.041	0.000	0.000	Yes
NC vs. 8.000	92.704	14.972	0.000	0.000	Yes
11.500 vs. 6.500	92.629	14.960	0.000	0.000	Yes
11.500 vs. 6.000	92.133	14.880	0.000	0.000	Yes
12.000 vs. 7.000	91.852	14.834	0.000	0.000	Yes
12.000 vs. 3.500	91.819	14.829	0.000	0.000	Yes
11.500 vs. 4.500	91.585	14.791	0.000	0.000	Yes
12.000 vs. 7.500	90.451	14.608	0.000	0.000	Yes
NC vs. 3.000	90.370	14.595	0.000	0.000	Yes
NC vs. 8.500	89.956	14.528	0.000	0.000	Yes
12.000 vs. 8.000	89.860	14.512	0.000	0.000	Yes
11.500 vs. 4.000	89.593	14.469	0.000	0.000	Yes
NC vs. 10.000	89.377	14.434	0.000	0.000	Yes
NC vs. 9.000	89.227	14.410	0.000	0.000	Yes
NC vs. 9.500	88.232	14.250	0.000	0.000	Yes

12.000 vs. 3.000	87.525	14.135	0.000	0.000	Yes
12.000 vs. 8.500	87.111	14.069	0.000	0.000	Yes
11.500 vs. 7.000	86.938	14.041	0.000	0.000	Yes
11.500 vs. 3.500	86.905	14.035	0.000	0.000	Yes
12.000 vs. 10.000	86.532	13.975	0.000	0.000	Yes
12.000 vs. 9.000	86.383	13.951	0.000	0.000	Yes
11.500 vs. 7.500	85.537	13.814	0.000	0.000	Yes
12.000 vs. 9.500	85.387	13.790	0.000	0.000	Yes
1.500 vs. 5.500	85.196	13.759	0.000	0.000	Yes
2.000 vs. 5.500	85.133	13.749	0.000	0.000	Yes
11.500 vs. 8.000	84.946	13.719	0.000	0.000	Yes
1.500 vs. 5.000	84.803	13.696	0.000	0.000	Yes
2.000 vs. 5.000	84.740	13.686	0.000	0.000	Yes
1.500 vs. 6.500	84.297	13.614	0.000	0.000	Yes
2.000 vs. 6.500	84.235	13.604	0.000	0.000	Yes
NC vs. 10.500	83.864	13.544	0.000	0.000	Yes
1.500 vs. 6.000	83.802	13.534	0.000	0.000	Yes
2.000 vs. 6.000	83.739	13.524	0.000	0.000	Yes
1.500 vs. 4.500	83.253	13.445	0.000	0.000	Yes
2.000 vs. 4.500	83.191	13.435	0.000	0.000	Yes
NC vs. 2.500	83.119	13.424	0.000	0.000	Yes
11.500 vs. 3.000	82.611	13.342	0.000	0.000	Yes
11.500 vs. 8.500	82.197	13.275	0.000	0.000	Yes
11.500 vs. 10.000	81.619	13.181	0.000	0.000	Yes
11.500 vs. 9.000	81.469	13.157	0.000	0.000	Yes
1.500 vs. 4.000	81.262	13.124	0.000	0.000	Yes
2.000 vs. 4.000	81.199	13.114	0.000	0.000	Yes
12.000 vs. 10.500	81.019	13.085	0.000	0.000	Yes
11.500 vs. 9.500	80.474	12.997	0.000	0.000	Yes
12.000 vs. 2.500	80.274	12.964	0.000	0.000	Yes
11.000 vs. 5.500	80.186	12.950	0.000	0.000	Yes
11.000 vs. 5.000	79.793	12.887	0.000	0.000	Yes
11.000 vs. 6.500	79.287	12.805	0.000	0.000	Yes
11.000 vs. 6.000	78.792	12.725	0.000	0.000	Yes
1.500 vs. 7.000	78.606	12.695	0.000	0.000	Yes
1.500 vs. 3.500	78.574	12.690	0.000	0.000	Yes
2.000 vs. 7.000	78.544	12.685	0.000	0.000	Yes
2.000 vs. 3.500	78.511	12.680	0.000	0.000	Yes
11.000 vs. 4.500	78.243	12.636	0.000	0.000	Yes
1.500 vs. 7.500	77.206	12.469	0.000	0.000	Yes
2.000 vs. 7.500	77.143	12.459	0.000	0.000	Yes
1.500 vs. 8.000	76.615	12.373	0.000	0.000	Yes
2.000 vs. 8.000	76.552	12.363	0.000	0.000	Yes
11.000 vs. 4.000	76.252	12.315	0.000	0.000	Yes
11.500 vs. 10.500	76.105	12.291	0.000	0.000	Yes
11.500 vs. 2.500	75.360	12.171	0.000	0.000	Yes

1.500 vs. 3.000	74.280	11.996	0.000	0.000	Yes
2.000 vs. 3.000	74.217	11.986	0.000	0.000	Yes
1.500 vs. 8.500	73.866	11.929	0.000	0.000	Yes
2.000 vs. 8.500	73.803	11.919	0.000	0.000	Yes
11.000 vs. 7.000	73.596	11.886	0.000	0.000	Yes
11.000 vs. 3.500	73.564	11.881	0.000	0.000	Yes
1.500 vs. 10.000	73.287	11.836	0.000	0.000	Yes
2.000 vs. 10.000	73.224	11.826	0.000	0.000	Yes
1.500 vs. 9.000	73.138	11.812	0.000	0.000	Yes
2.000 vs. 9.000	73.075	11.802	0.000	0.000	Yes
11.000 vs. 7.500	72.196	11.660	0.000	0.000	Yes
1.500 vs. 9.500	72.142	11.651	0.000	0.000	Yes
2.000 vs. 9.500	72.079	11.641	0.000	0.000	Yes
11.000 vs. 8.000	71.605	11.564	0.000	0.000	Yes
11.000 vs. 3.000	69.270	11.187	0.000	0.000	Yes
11.000 vs. 8.500	68.856	11.120	0.000	0.000	Yes
11.000 vs. 10.000	68.277	11.027	0.000	0.000	Yes
11.000 vs. 9.000	68.128	11.003	0.000	0.000	Yes
1.500 vs. 10.500	67.774	10.945	0.000	0.000	Yes
2.000 vs. 10.500	67.711	10.935	0.000	0.000	Yes
11.000 vs. 9.500	67.132	10.842	0.000	0.000	Yes
1.500 vs. 2.500	67.029	10.825	0.000	0.000	Yes
2.000 vs. 2.500	66.966	10.815	0.000	0.000	Yes
11.000 vs. 10.500	62.764	10.136	0.000	0.000	Yes
11.000 vs. 2.500	62.019	10.016	0.000	0.000	Yes

Comparisons for factor: **Sample pH within 2.5**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
NC vs. 4.500	100.668	16.258	0.000	0.000	Yes
NC vs. 5.000	100.529	16.235	0.000	0.000	Yes
NC vs. 6.500	100.132	16.171	0.000	0.000	Yes
NC vs. 6.000	99.828	16.122	0.000	0.000	Yes
NC vs. 5.500	97.908	15.812	0.000	0.000	Yes
NC vs. 4.000	96.841	15.640	0.000	0.000	Yes
NC vs. 7.000	95.503	15.424	0.000	0.000	Yes
NC vs. 7.500	93.288	15.066	0.000	0.000	Yes
2.000 vs. 4.500	92.150	14.882	0.000	0.000	Yes
2.000 vs. 5.000	92.010	14.860	0.000	0.000	Yes
2.000 vs. 6.500	91.614	14.796	0.000	0.000	Yes
NC vs. 8.000	91.497	14.777	0.000	0.000	Yes
NC vs. 3.500	91.370	14.756	0.000	0.000	Yes
2.000 vs. 6.000	91.310	14.747	0.000	0.000	Yes
NC vs. 3.000	90.419	14.603	0.000	0.000	Yes
NC vs. 8.500	89.444	14.445	0.000	0.000	Yes
2.000 vs. 5.500	89.390	14.437	0.000	0.000	Yes
11.000 vs. 4.500	88.759	14.335	0.000	0.000	Yes

11.500 vs. 4.500	88.737	14.331	0.000	0.000	Yes
1.500 vs. 4.500	88.725	14.329	0.000	0.000	Yes
11.000 vs. 5.000	88.619	14.312	0.000	0.000	Yes
11.500 vs. 5.000	88.597	14.309	0.000	0.000	Yes
NC vs. 9.000	88.597	14.309	0.000	0.000	Yes
1.500 vs. 5.000	88.586	14.307	0.000	0.000	Yes
2.000 vs. 4.000	88.323	14.264	0.000	0.000	Yes
11.000 vs. 6.500	88.223	14.248	0.000	0.000	Yes
11.500 vs. 6.500	88.201	14.245	0.000	0.000	Yes
1.500 vs. 6.500	88.189	14.243	0.000	0.000	Yes
11.000 vs. 6.000	87.919	14.199	0.000	0.000	Yes
11.500 vs. 6.000	87.897	14.195	0.000	0.000	Yes
1.500 vs. 6.000	87.886	14.194	0.000	0.000	Yes
2.000 vs. 7.000	86.985	14.048	0.000	0.000	Yes
12.000 vs. 4.500	86.669	13.997	0.000	0.000	Yes
12.000 vs. 5.000	86.529	13.975	0.000	0.000	Yes
NC vs. 10.000	86.409	13.955	0.000	0.000	Yes
12.000 vs. 6.500	86.133	13.911	0.000	0.000	Yes
11.000 vs. 5.500	85.999	13.889	0.000	0.000	Yes
11.500 vs. 5.500	85.977	13.885	0.000	0.000	Yes
1.500 vs. 5.500	85.965	13.883	0.000	0.000	Yes
12.000 vs. 6.000	85.829	13.861	0.000	0.000	Yes
NC vs. 9.500	85.294	13.775	0.000	0.000	Yes
11.000 vs. 4.000	84.932	13.717	0.000	0.000	Yes
11.500 vs. 4.000	84.910	13.713	0.000	0.000	Yes
1.500 vs. 4.000	84.899	13.711	0.000	0.000	Yes
2.000 vs. 7.500	84.770	13.690	0.000	0.000	Yes
12.000 vs. 5.500	83.909	13.551	0.000	0.000	Yes
11.000 vs. 7.000	83.594	13.500	0.000	0.000	Yes
11.500 vs. 7.000	83.572	13.497	0.000	0.000	Yes
1.500 vs. 7.000	83.560	13.495	0.000	0.000	Yes
2.000 vs. 8.000	82.979	13.401	0.000	0.000	Yes
2.000 vs. 3.500	82.852	13.381	0.000	0.000	Yes
12.000 vs. 4.000	82.842	13.379	0.000	0.000	Yes
2.000 vs. 3.000	81.901	13.227	0.000	0.000	Yes
NC vs. 10.500	81.654	13.187	0.000	0.000	Yes
12.000 vs. 7.000	81.504	13.163	0.000	0.000	Yes
11.000 vs. 7.500	81.379	13.143	0.000	0.000	Yes
11.500 vs. 7.500	81.357	13.139	0.000	0.000	Yes
1.500 vs. 7.500	81.345	13.137	0.000	0.000	Yes
2.000 vs. 8.500	80.925	13.069	0.000	0.000	Yes
2.000 vs. 9.000	80.079	12.933	0.000	0.000	Yes
11.000 vs. 8.000	79.588	12.853	0.000	0.000	Yes
11.500 vs. 8.000	79.566	12.850	0.000	0.000	Yes
1.500 vs. 8.000	79.554	12.848	0.000	0.000	Yes
11.000 vs. 3.500	79.461	12.833	0.000	0.000	Yes

11.500 vs. 3.500	79.439	12.829	0.000	0.000	Yes
1.500 vs. 3.500	79.427	12.828	0.000	0.000	Yes
NC vs. 2.500	79.397	12.823	0.000	0.000	Yes
12.000 vs. 7.500	79.289	12.805	0.000	0.000	Yes
11.000 vs. 3.000	78.510	12.679	0.000	0.000	Yes
11.500 vs. 3.000	78.488	12.676	0.000	0.000	Yes
1.500 vs. 3.000	78.476	12.674	0.000	0.000	Yes
2.000 vs. 10.000	77.890	12.579	0.000	0.000	Yes
11.000 vs. 8.500	77.534	12.522	0.000	0.000	Yes
11.500 vs. 8.500	77.512	12.518	0.000	0.000	Yes
1.500 vs. 8.500	77.501	12.516	0.000	0.000	Yes
12.000 vs. 8.000	77.498	12.516	0.000	0.000	Yes
12.000 vs. 3.500	77.371	12.496	0.000	0.000	Yes
2.000 vs. 9.500	76.776	12.399	0.000	0.000	Yes
11.000 vs. 9.000	76.688	12.385	0.000	0.000	Yes
11.500 vs. 9.000	76.666	12.382	0.000	0.000	Yes
1.500 vs. 9.000	76.654	12.380	0.000	0.000	Yes
12.000 vs. 3.000	76.420	12.342	0.000	0.000	Yes
12.000 vs. 8.500	75.444	12.184	0.000	0.000	Yes
12.000 vs. 9.000	74.598	12.048	0.000	0.000	Yes
11.000 vs. 10.000	74.499	12.032	0.000	0.000	Yes
11.500 vs. 10.000	74.477	12.028	0.000	0.000	Yes
1.500 vs. 10.000	74.466	12.026	0.000	0.000	Yes
11.000 vs. 9.500	73.385	11.852	0.000	0.000	Yes
11.500 vs. 9.500	73.363	11.848	0.000	0.000	Yes
1.500 vs. 9.500	73.351	11.846	0.000	0.000	Yes
2.000 vs. 10.500	73.136	11.811	0.000	0.000	Yes
12.000 vs. 10.000	72.409	11.694	0.000	0.000	Yes
12.000 vs. 9.500	71.295	11.514	0.000	0.000	Yes
2.000 vs. 2.500	70.878	11.447	0.000	0.000	Yes
11.000 vs. 10.500	69.745	11.264	0.000	0.000	Yes
11.500 vs. 10.500	69.723	11.260	0.000	0.000	Yes
1.500 vs. 10.500	69.711	11.258	0.000	0.000	Yes
12.000 vs. 10.500	67.655	10.926	0.000	0.000	Yes
11.000 vs. 2.500	67.488	10.899	0.000	0.000	Yes
11.500 vs. 2.500	67.465	10.896	0.000	0.000	Yes
1.500 vs. 2.500	67.454	10.894	0.000	0.000	Yes
12.000 vs. 2.500	65.398	10.562	0.000	0.000	Yes

Comparisons for factor: **Sample pH within 3**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
11.000 vs. 6.500	105.648	17.062	0.000	0.000	Yes
11.000 vs. 4.000	105.532	17.044	0.000	0.000	Yes
11.000 vs. 4.500	105.332	17.011	0.000	0.000	Yes
11.000 vs. 5.500	105.046	16.965	0.000	0.000	Yes
11.000 vs. 5.000	104.667	16.904	0.000	0.000	Yes

11.000 vs. 6.000	104.523	16.881	0.000	0.000	Yes
11.500 vs. 6.500	101.939	16.463	0.000	0.000	Yes
11.500 vs. 4.000	101.823	16.444	0.000	0.000	Yes
11.500 vs. 4.500	101.622	16.412	0.000	0.000	Yes
11.500 vs. 5.500	101.336	16.366	0.000	0.000	Yes
11.000 vs. 3.500	101.122	16.331	0.000	0.000	Yes
11.000 vs. 7.000	100.984	16.309	0.000	0.000	Yes
11.500 vs. 5.000	100.958	16.305	0.000	0.000	Yes
11.500 vs. 6.000	100.814	16.282	0.000	0.000	Yes
NC vs. 6.500	99.583	16.083	0.000	0.000	Yes
NC vs. 4.000	99.467	16.064	0.000	0.000	Yes
NC vs. 4.500	99.267	16.032	0.000	0.000	Yes
2.000 vs. 6.500	99.155	16.014	0.000	0.000	Yes
11.000 vs. 7.500	99.148	16.012	0.000	0.000	Yes
2.000 vs. 4.000	99.039	15.995	0.000	0.000	Yes
NC vs. 5.500	98.981	15.985	0.000	0.000	Yes
2.000 vs. 4.500	98.838	15.962	0.000	0.000	Yes
NC vs. 5.000	98.602	15.924	0.000	0.000	Yes
2.000 vs. 5.500	98.552	15.916	0.000	0.000	Yes
NC vs. 6.000	98.458	15.901	0.000	0.000	Yes
2.000 vs. 5.000	98.174	15.855	0.000	0.000	Yes
2.000 vs. 6.000	98.030	15.832	0.000	0.000	Yes
11.500 vs. 3.500	97.413	15.732	0.000	0.000	Yes
11.500 vs. 7.000	97.275	15.710	0.000	0.000	Yes
11.000 vs. 3.000	96.948	15.657	0.000	0.000	Yes
11.000 vs. 8.000	95.706	15.457	0.000	0.000	Yes
11.500 vs. 7.500	95.438	15.413	0.000	0.000	Yes
12.000 vs. 6.500	95.157	15.368	0.000	0.000	Yes
NC vs. 3.500	95.057	15.352	0.000	0.000	Yes
12.000 vs. 4.000	95.041	15.349	0.000	0.000	Yes
NC vs. 7.000	94.919	15.329	0.000	0.000	Yes
12.000 vs. 4.500	94.841	15.317	0.000	0.000	Yes
2.000 vs. 3.500	94.629	15.283	0.000	0.000	Yes
12.000 vs. 5.500	94.555	15.271	0.000	0.000	Yes
2.000 vs. 7.000	94.490	15.260	0.000	0.000	Yes
12.000 vs. 5.000	94.176	15.210	0.000	0.000	Yes
12.000 vs. 6.000	94.032	15.186	0.000	0.000	Yes
11.500 vs. 3.000	93.239	15.058	0.000	0.000	Yes
11.000 vs. 8.500	93.210	15.053	0.000	0.000	Yes
NC vs. 7.500	93.083	15.033	0.000	0.000	Yes
1.500 vs. 6.500	92.943	15.010	0.000	0.000	Yes
1.500 vs. 4.000	92.827	14.992	0.000	0.000	Yes
2.000 vs. 7.500	92.654	14.964	0.000	0.000	Yes
1.500 vs. 4.500	92.627	14.959	0.000	0.000	Yes
1.500 vs. 5.500	92.341	14.913	0.000	0.000	Yes
11.500 vs. 8.000	91.997	14.858	0.000	0.000	Yes

1.500 vs. 5.000	91.963	14.852	0.000	0.000	Yes
1.500 vs. 6.000	91.818	14.829	0.000	0.000	Yes
11.000 vs. 9.000	91.790	14.824	0.000	0.000	Yes
11.000 vs. 10.000	90.920	14.684	0.000	0.000	Yes
NC vs. 3.000	90.883	14.678	0.000	0.000	Yes
12.000 vs. 3.500	90.631	14.637	0.000	0.000	Yes
12.000 vs. 7.000	90.493	14.615	0.000	0.000	Yes
2.000 vs. 3.000	90.455	14.609	0.000	0.000	Yes
11.000 vs. 9.500	90.202	14.568	0.000	0.000	Yes
NC vs. 8.000	89.641	14.477	0.000	0.000	Yes
11.500 vs. 8.500	89.500	14.454	0.000	0.000	Yes
2.000 vs. 8.000	89.212	14.408	0.000	0.000	Yes
12.000 vs. 7.500	88.657	14.318	0.000	0.000	Yes
1.500 vs. 3.500	88.418	14.280	0.000	0.000	Yes
1.500 vs. 7.000	88.279	14.257	0.000	0.000	Yes
11.500 vs. 9.000	88.080	14.225	0.000	0.000	Yes
11.500 vs. 10.000	87.211	14.085	0.000	0.000	Yes
NC vs. 8.500	87.145	14.074	0.000	0.000	Yes
2.000 vs. 8.500	86.716	14.005	0.000	0.000	Yes
11.500 vs. 9.500	86.493	13.969	0.000	0.000	Yes
12.000 vs. 3.000	86.457	13.963	0.000	0.000	Yes
1.500 vs. 7.500	86.443	13.961	0.000	0.000	Yes
NC vs. 9.000	85.725	13.845	0.000	0.000	Yes
2.000 vs. 9.000	85.296	13.775	0.000	0.000	Yes
12.000 vs. 8.000	85.215	13.762	0.000	0.000	Yes
NC vs. 10.000	84.855	13.704	0.000	0.000	Yes
11.000 vs. 10.500	84.770	13.690	0.000	0.000	Yes
2.000 vs. 10.000	84.427	13.635	0.000	0.000	Yes
1.500 vs. 3.000	84.244	13.605	0.000	0.000	Yes
NC vs. 9.500	84.137	13.588	0.000	0.000	Yes
2.000 vs. 9.500	83.709	13.519	0.000	0.000	Yes
1.500 vs. 8.000	83.001	13.405	0.000	0.000	Yes
12.000 vs. 8.500	82.719	13.359	0.000	0.000	Yes
12.000 vs. 9.000	81.299	13.130	0.000	0.000	Yes
11.500 vs. 10.500	81.061	13.091	0.000	0.000	Yes
1.500 vs. 8.500	80.505	13.002	0.000	0.000	Yes
12.000 vs. 10.000	80.429	12.989	0.000	0.000	Yes
12.000 vs. 9.500	79.711	12.873	0.000	0.000	Yes
1.500 vs. 9.000	79.085	12.772	0.000	0.000	Yes
NC vs. 10.500	78.705	12.711	0.000	0.000	Yes
2.000 vs. 10.500	78.276	12.642	0.000	0.000	Yes
1.500 vs. 10.000	78.216	12.632	0.000	0.000	Yes
1.500 vs. 9.500	77.498	12.516	0.000	0.000	Yes
11.000 vs. 2.500	77.093	12.451	0.000	0.000	Yes
12.000 vs. 10.500	74.279	11.996	0.000	0.000	Yes
11.500 vs. 2.500	73.384	11.852	0.000	0.000	Yes

1.500 vs. 10.500	72.065	11.639	0.000	0.000	Yes
NC vs. 2.500	71.028	11.471	0.000	0.000	Yes
2.000 vs. 2.500	70.600	11.402	0.000	0.000	Yes
12.000 vs. 2.500	66.602	10.756	0.000	0.000	Yes
1.500 vs. 2.500	64.389	10.399	0.000	0.000	Yes
2.500 vs. 6.500	28.555	4.612	0.000	0.000	Yes
2.500 vs. 4.000	28.439	4.593	0.000	0.000	Yes
2.500 vs. 4.500	28.238	4.560	0.000	0.000	Yes
2.500 vs. 5.500	27.952	4.514	0.000	0.000	Yes
2.500 vs. 5.000	27.574	4.453	0.000	0.000	Yes
2.500 vs. 6.000	27.430	4.430	0.000	0.000	Yes
2.500 vs. 3.500	24.029	3.881	0.000	0.000	Yes
2.500 vs. 7.000	23.890	3.858	0.000	0.000	Yes

Comparisons for factor: **Sample pH within 10.5**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
12.000 vs. 6.000	99.546	16.077	0.000	0.000	Yes
11.000 vs. 6.000	99.493	16.068	0.000	0.000	Yes
12.000 vs. 5.500	99.449	16.061	0.000	0.000	Yes
11.000 vs. 5.500	99.396	16.053	0.000	0.000	Yes
NC vs. 6.000	99.200	16.021	0.000	0.000	Yes
NC vs. 5.500	99.103	16.005	0.000	0.000	Yes
2.000 vs. 6.000	99.087	16.003	0.000	0.000	Yes
2.000 vs. 5.500	98.990	15.987	0.000	0.000	Yes
12.000 vs. 5.000	97.840	15.801	0.000	0.000	Yes
11.000 vs. 5.000	97.787	15.793	0.000	0.000	Yes
NC vs. 5.000	97.493	15.745	0.000	0.000	Yes
2.000 vs. 5.000	97.381	15.727	0.000	0.000	Yes
12.000 vs. 4.500	96.262	15.546	0.000	0.000	Yes
11.000 vs. 4.500	96.209	15.538	0.000	0.000	Yes
NC vs. 4.500	95.915	15.490	0.000	0.000	Yes
2.000 vs. 4.500	95.802	15.472	0.000	0.000	Yes
12.000 vs. 6.500	95.542	15.430	0.000	0.000	Yes
11.000 vs. 6.500	95.489	15.421	0.000	0.000	Yes
NC vs. 6.500	95.195	15.374	0.000	0.000	Yes
2.000 vs. 6.500	95.083	15.356	0.000	0.000	Yes
12.000 vs. 4.000	93.501	15.100	0.000	0.000	Yes
11.000 vs. 4.000	93.448	15.092	0.000	0.000	Yes
NC vs. 4.000	93.154	15.044	0.000	0.000	Yes
2.000 vs. 4.000	93.041	15.026	0.000	0.000	Yes
12.000 vs. 7.000	91.919	14.845	0.000	0.000	Yes
11.000 vs. 7.000	91.866	14.836	0.000	0.000	Yes
NC vs. 7.000	91.572	14.789	0.000	0.000	Yes
2.000 vs. 7.000	91.459	14.771	0.000	0.000	Yes
11.500 vs. 6.000	91.095	14.712	0.000	0.000	Yes
11.500 vs. 5.500	90.998	14.696	0.000	0.000	Yes

12.000 vs. 7.500	90.445	14.607	0.000	0.000	Yes
11.000 vs. 7.500	90.391	14.598	0.000	0.000	Yes
NC vs. 7.500	90.098	14.551	0.000	0.000	Yes
2.000 vs. 7.500	89.985	14.533	0.000	0.000	Yes
11.500 vs. 5.000	89.389	14.436	0.000	0.000	Yes
11.500 vs. 4.500	87.810	14.181	0.000	0.000	Yes
12.000 vs. 3.500	87.760	14.173	0.000	0.000	Yes
11.000 vs. 3.500	87.707	14.165	0.000	0.000	Yes
NC vs. 3.500	87.413	14.117	0.000	0.000	Yes
2.000 vs. 3.500	87.300	14.099	0.000	0.000	Yes
11.500 vs. 6.500	87.091	14.065	0.000	0.000	Yes
1.500 vs. 6.000	86.172	13.917	0.000	0.000	Yes
1.500 vs. 5.500	86.075	13.901	0.000	0.000	Yes
11.500 vs. 4.000	85.049	13.736	0.000	0.000	Yes
1.500 vs. 5.000	84.465	13.641	0.000	0.000	Yes
12.000 vs. 3.000	83.841	13.540	0.000	0.000	Yes
11.000 vs. 3.000	83.788	13.532	0.000	0.000	Yes
NC vs. 3.000	83.494	13.484	0.000	0.000	Yes
11.500 vs. 7.000	83.468	13.480	0.000	0.000	Yes
2.000 vs. 3.000	83.381	13.466	0.000	0.000	Yes
1.500 vs. 4.500	82.887	13.386	0.000	0.000	Yes
1.500 vs. 6.500	82.167	13.270	0.000	0.000	Yes
11.500 vs. 7.500	81.993	13.242	0.000	0.000	Yes
1.500 vs. 4.000	80.126	12.940	0.000	0.000	Yes
11.500 vs. 3.500	79.308	12.808	0.000	0.000	Yes
1.500 vs. 7.000	78.544	12.685	0.000	0.000	Yes
12.000 vs. 9.000	77.209	12.469	0.000	0.000	Yes
11.000 vs. 9.000	77.156	12.461	0.000	0.000	Yes
1.500 vs. 7.500	77.070	12.447	0.000	0.000	Yes
NC vs. 9.000	76.862	12.413	0.000	0.000	Yes
2.000 vs. 9.000	76.749	12.395	0.000	0.000	Yes
12.000 vs. 8.500	76.447	12.346	0.000	0.000	Yes
11.000 vs. 8.500	76.394	12.338	0.000	0.000	Yes
NC vs. 8.500	76.100	12.290	0.000	0.000	Yes
2.000 vs. 8.500	75.988	12.272	0.000	0.000	Yes
12.000 vs. 10.000	75.645	12.217	0.000	0.000	Yes
11.000 vs. 10.000	75.592	12.208	0.000	0.000	Yes
11.500 vs. 3.000	75.389	12.175	0.000	0.000	Yes
NC vs. 10.000	75.298	12.161	0.000	0.000	Yes
2.000 vs. 10.000	75.186	12.143	0.000	0.000	Yes
1.500 vs. 3.500	74.385	12.013	0.000	0.000	Yes
12.000 vs. 8.000	88.031	11.608	0.000	0.000	Yes
11.000 vs. 8.000	87.977	11.601	0.000	0.000	Yes
NC vs. 8.000	87.684	11.562	0.000	0.000	Yes
2.000 vs. 8.000	87.571	11.548	0.000	0.000	Yes
12.000 vs. 9.500	71.218	11.502	0.000	0.000	Yes

11.000 vs. 9.500	71.165	11.493	0.000	0.000	Yes
NC vs. 9.500	70.871	11.446	0.000	0.000	Yes
2.000 vs. 9.500	70.758	11.428	0.000	0.000	Yes
1.500 vs. 3.000	70.466	11.380	0.000	0.000	Yes
11.500 vs. 9.000	68.757	11.104	0.000	0.000	Yes
11.500 vs. 8.500	67.996	10.981	0.000	0.000	Yes
11.500 vs. 10.000	67.194	10.852	0.000	0.000	Yes
11.500 vs. 8.000	79.579	10.494	0.000	0.000	Yes
1.500 vs. 9.000	63.834	10.309	0.000	0.000	Yes
1.500 vs. 8.500	63.072	10.186	0.000	0.000	Yes
11.500 vs. 9.500	62.766	10.137	0.000	0.000	Yes
1.500 vs. 10.000	62.270	10.057	0.000	0.000	Yes
12.000 vs. 2.500	62.049	10.021	0.000	0.000	Yes
11.000 vs. 2.500	61.996	10.012	0.000	0.000	Yes
NC vs. 2.500	61.702	9.965	0.000	0.000	Yes
2.000 vs. 2.500	61.590	9.947	0.000	0.000	Yes
1.500 vs. 8.000	74.656	9.844	0.000	0.000	Yes
1.500 vs. 9.500	57.843	9.342	0.000	0.000	Yes
12.000 vs. 10.500	57.394	9.269	0.000	0.000	Yes
11.000 vs. 10.500	57.341	9.261	0.000	0.000	Yes
NC vs. 10.500	57.047	9.213	0.000	0.000	Yes
2.000 vs. 10.500	56.934	9.195	0.000	0.000	Yes
11.500 vs. 2.500	53.598	8.656	0.000	0.000	Yes
11.500 vs. 10.500	48.942	7.904	0.000	0.000	Yes
1.500 vs. 2.500	48.674	7.861	0.000	0.000	Yes
1.500 vs. 10.500	44.019	7.109	0.000	0.000	Yes
10.500 vs. 6.000	42.153	6.808	0.000	0.000	Yes
10.500 vs. 5.500	42.056	6.792	0.000	0.000	Yes
10.500 vs. 5.000	40.446	6.532	0.000	0.000	Yes
10.500 vs. 4.500	38.868	6.277	0.000	0.000	Yes
10.500 vs. 6.500	38.148	6.161	0.000	0.000	Yes
2.500 vs. 6.000	37.497	6.056	0.000	0.000	Yes
2.500 vs. 5.500	37.400	6.040	0.000	0.000	Yes
10.500 vs. 4.000	36.107	5.831	0.000	0.000	Yes
2.500 vs. 5.000	35.791	5.780	0.000	0.000	Yes
10.500 vs. 7.000	34.525	5.576	0.000	0.000	Yes
2.500 vs. 4.500	34.213	5.525	0.000	0.000	Yes
2.500 vs. 6.500	33.493	5.409	0.000	0.000	Yes
10.500 vs. 7.500	33.051	5.338	0.000	0.000	Yes
2.500 vs. 4.000	31.452	5.079	0.000	0.000	Yes
10.500 vs. 3.500	30.366	4.904	0.000	0.000	Yes
2.500 vs. 7.000	29.870	4.824	0.000	0.000	Yes
2.500 vs. 7.500	28.395	4.586	0.000	0.000	Yes
9.500 vs. 6.000	28.329	4.575	0.000	0.000	Yes
9.500 vs. 5.500	28.232	4.559	0.000	0.000	Yes
9.500 vs. 5.000	26.622	4.300	0.000	0.000	Yes

10.500 vs. 3.000	26.447	4.271	0.000	0.000	Yes
2.500 vs. 3.500	25.711	4.152	0.000	0.000	Yes
9.500 vs. 4.500	25.044	4.045	0.000	0.000	Yes
10.500 vs. 8.000	30.637	4.040	0.000	0.000	Yes
9.500 vs. 6.500	24.324	3.928	0.000	0.000	Yes
10.000 vs. 6.000	23.901	3.860	0.000	0.000	Yes
10.000 vs. 5.500	23.804	3.844	0.000	0.000	Yes
8.500 vs. 6.000	23.099	3.731	0.000	0.000	Yes
8.500 vs. 5.500	23.002	3.715	0.000	0.000	Yes
9.000 vs. 6.000	22.338	3.608	0.000	0.000	Yes
9.500 vs. 4.000	22.283	3.599	0.000	0.000	Yes
9.000 vs. 5.500	22.241	3.592	0.000	0.000	Yes
10.000 vs. 5.000	22.195	3.585	0.000	0.000	Yes

Comparisons for factor: **Sample pH within 11**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
NC vs. 5.000	97.821	15.798	0.000	0.000	Yes
NC vs. 6.000	97.578	15.759	0.000	0.000	Yes
NC vs. 6.500	96.964	15.660	0.000	0.000	Yes
NC vs. 5.500	96.675	15.613	0.000	0.000	Yes
NC vs. 4.000	95.928	15.493	0.000	0.000	Yes
NC vs. 4.500	94.476	15.258	0.000	0.000	Yes
NC vs. 7.000	93.276	15.064	0.000	0.000	Yes
NC vs. 3.500	91.691	14.808	0.000	0.000	Yes
1.500 vs. 5.000	90.686	14.646	0.000	0.000	Yes
1.500 vs. 6.000	90.443	14.607	0.000	0.000	Yes
2.000 vs. 5.000	90.333	14.589	0.000	0.000	Yes
2.000 vs. 6.000	90.090	14.550	0.000	0.000	Yes
1.500 vs. 6.500	89.829	14.507	0.000	0.000	Yes
NC vs. 7.500	89.585	14.468	0.000	0.000	Yes
1.500 vs. 5.500	89.540	14.461	0.000	0.000	Yes
2.000 vs. 6.500	89.476	14.451	0.000	0.000	Yes
2.000 vs. 5.500	89.187	14.404	0.000	0.000	Yes
1.500 vs. 4.000	88.793	14.340	0.000	0.000	Yes
2.000 vs. 4.000	88.441	14.283	0.000	0.000	Yes
NC vs. 3.000	88.039	14.218	0.000	0.000	Yes
1.500 vs. 4.500	87.341	14.106	0.000	0.000	Yes
2.000 vs. 4.500	86.988	14.049	0.000	0.000	Yes
NC vs. 8.000	86.317	13.940	0.000	0.000	Yes
11.000 vs. 5.000	86.268	13.932	0.000	0.000	Yes
1.500 vs. 7.000	86.141	13.912	0.000	0.000	Yes
11.000 vs. 6.000	86.025	13.893	0.000	0.000	Yes
2.000 vs. 7.000	85.788	13.855	0.000	0.000	Yes
11.000 vs. 6.500	85.411	13.794	0.000	0.000	Yes
11.000 vs. 5.500	85.122	13.747	0.000	0.000	Yes
1.500 vs. 3.500	84.555	13.656	0.000	0.000	Yes

11.000 vs. 4.000	84.375	13.627	0.000	0.000	Yes
2.000 vs. 3.500	84.203	13.599	0.000	0.000	Yes
11.000 vs. 4.500	82.923	13.392	0.000	0.000	Yes
1.500 vs. 7.500	82.450	13.316	0.000	0.000	Yes
NC vs. 9.000	82.287	13.289	0.000	0.000	Yes
2.000 vs. 7.500	82.097	13.259	0.000	0.000	Yes
11.000 vs. 7.000	81.723	13.198	0.000	0.000	Yes
NC vs. 8.500	81.163	13.108	0.000	0.000	Yes
1.500 vs. 3.000	80.904	13.066	0.000	0.000	Yes
NC vs. 10.000	80.674	13.029	0.000	0.000	Yes
2.000 vs. 3.000	80.551	13.009	0.000	0.000	Yes
11.000 vs. 3.500	80.137	12.942	0.000	0.000	Yes
1.500 vs. 8.000	79.182	12.788	0.000	0.000	Yes
2.000 vs. 8.000	78.829	12.731	0.000	0.000	Yes
11.000 vs. 7.500	78.031	12.602	0.000	0.000	Yes
11.500 vs. 5.000	76.935	12.425	0.000	0.000	Yes
11.500 vs. 6.000	76.693	12.386	0.000	0.000	Yes
11.000 vs. 3.000	76.486	12.352	0.000	0.000	Yes
11.500 vs. 6.500	76.079	12.287	0.000	0.000	Yes
NC vs. 2.500	75.905	12.259	0.000	0.000	Yes
11.500 vs. 5.500	75.790	12.240	0.000	0.000	Yes
NC vs. 9.500	75.254	12.154	0.000	0.000	Yes
1.500 vs. 9.000	75.151	12.137	0.000	0.000	Yes
11.500 vs. 4.000	75.043	12.119	0.000	0.000	Yes
2.000 vs. 9.000	74.799	12.080	0.000	0.000	Yes
11.000 vs. 8.000	74.764	12.074	0.000	0.000	Yes
1.500 vs. 8.500	74.028	11.956	0.000	0.000	Yes
2.000 vs. 8.500	73.676	11.899	0.000	0.000	Yes
11.500 vs. 4.500	73.591	11.885	0.000	0.000	Yes
1.500 vs. 10.000	73.539	11.877	0.000	0.000	Yes
2.000 vs. 10.000	73.186	11.820	0.000	0.000	Yes
11.500 vs. 7.000	72.391	11.691	0.000	0.000	Yes
12.000 vs. 5.000	71.763	11.590	0.000	0.000	Yes
12.000 vs. 6.000	71.520	11.551	0.000	0.000	Yes
12.000 vs. 6.500	70.907	11.451	0.000	0.000	Yes
11.500 vs. 3.500	70.805	11.435	0.000	0.000	Yes
11.000 vs. 9.000	70.733	11.423	0.000	0.000	Yes
12.000 vs. 5.500	70.618	11.405	0.000	0.000	Yes
12.000 vs. 4.000	69.871	11.284	0.000	0.000	Yes
11.000 vs. 8.500	69.610	11.242	0.000	0.000	Yes
11.000 vs. 10.000	69.120	11.163	0.000	0.000	Yes
1.500 vs. 2.500	68.770	11.106	0.000	0.000	Yes
11.500 vs. 7.500	68.699	11.095	0.000	0.000	Yes
12.000 vs. 4.500	68.418	11.050	0.000	0.000	Yes
2.000 vs. 2.500	68.417	11.049	0.000	0.000	Yes
1.500 vs. 9.500	68.119	11.001	0.000	0.000	Yes

2.000 vs. 9.500	67.766	10.944	0.000	0.000	Yes
12.000 vs. 7.000	67.218	10.856	0.000	0.000	Yes
11.500 vs. 3.000	67.154	10.845	0.000	0.000	Yes
12.000 vs. 3.500	65.633	10.600	0.000	0.000	Yes
11.500 vs. 8.000	65.432	10.567	0.000	0.000	Yes
11.000 vs. 2.500	64.351	10.393	0.000	0.000	Yes
11.000 vs. 9.500	63.701	10.288	0.000	0.000	Yes
12.000 vs. 7.500	63.527	10.260	0.000	0.000	Yes
12.000 vs. 3.000	61.981	10.010	0.000	0.000	Yes
11.500 vs. 9.000	61.401	9.916	0.000	0.000	Yes
11.500 vs. 8.500	60.278	9.735	0.000	0.000	Yes
12.000 vs. 8.000	60.259	9.732	0.000	0.000	Yes
11.500 vs. 10.000	59.788	9.656	0.000	0.000	Yes
NC vs. 10.500	59.669	9.637	0.000	0.000	Yes
12.000 vs. 9.000	56.229	9.081	0.000	0.000	Yes
12.000 vs. 8.500	55.106	8.900	0.000	0.000	Yes
11.500 vs. 2.500	55.019	8.886	0.000	0.000	Yes
12.000 vs. 10.000	54.616	8.821	0.000	0.000	Yes
11.500 vs. 9.500	54.369	8.781	0.000	0.000	Yes
1.500 vs. 10.500	52.534	8.484	0.000	0.000	Yes
2.000 vs. 10.500	52.181	8.427	0.000	0.000	Yes
12.000 vs. 2.500	49.847	8.050	0.000	0.000	Yes
12.000 vs. 9.500	49.196	7.945	0.000	0.000	Yes
11.000 vs. 10.500	48.115	7.771	0.000	0.000	Yes
11.500 vs. 10.500	38.783	6.264	0.000	0.000	Yes
10.500 vs. 5.000	38.152	6.162	0.000	0.000	Yes
10.500 vs. 6.000	37.909	6.122	0.000	0.000	Yes
10.500 vs. 6.500	37.295	6.023	0.000	0.000	Yes
10.500 vs. 5.500	37.006	5.977	0.000	0.000	Yes
10.500 vs. 4.000	36.260	5.856	0.000	0.000	Yes
10.500 vs. 4.500	34.807	5.621	0.000	0.000	Yes
12.000 vs. 10.500	33.611	5.428	0.000	0.000	Yes
10.500 vs. 7.000	33.607	5.428	0.000	0.000	Yes
10.500 vs. 3.500	32.022	5.172	0.000	0.000	Yes
10.500 vs. 7.500	29.916	4.831	0.000	0.000	Yes
10.500 vs. 3.000	28.370	4.582	0.000	0.000	Yes
10.500 vs. 8.000	26.648	4.304	0.000	0.000	Yes
NC vs. 12.000	26.058	4.208	0.000	0.000	Yes
10.500 vs. 9.000	22.618	3.653	0.000	0.000	Yes
9.500 vs. 5.000	22.567	3.645	0.000	0.000	Yes
9.500 vs. 6.000	22.324	3.605	0.000	0.000	Yes

Comparisons for factor: **Sample pH within 11.5**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
NC vs. 5.500	100.248	16.190	0.000	0.000	Yes
NC vs. 5.000	99.849	16.126	0.000	0.000	Yes

NC vs. 6.000	99.816	16.120	0.000	0.000	Yes
NC vs. 4.500	99.122	16.008	0.000	0.000	Yes
NC vs. 6.500	97.396	15.730	0.000	0.000	Yes
11.500 vs. 5.500	96.012	15.506	0.000	0.000	Yes
NC vs. 3.500	95.804	15.472	0.000	0.000	Yes
11.500 vs. 5.000	95.614	15.442	0.000	0.000	Yes
11.500 vs. 6.000	95.580	15.436	0.000	0.000	Yes
11.500 vs. 4.500	94.887	15.324	0.000	0.000	Yes
NC vs. 4.000	94.510	15.263	0.000	0.000	Yes
11.000 vs. 5.500	93.694	15.132	0.000	0.000	Yes
11.000 vs. 5.000	93.296	15.067	0.000	0.000	Yes
11.000 vs. 6.000	93.262	15.062	0.000	0.000	Yes
11.500 vs. 6.500	93.160	15.045	0.000	0.000	Yes
11.000 vs. 4.500	92.569	14.950	0.000	0.000	Yes
11.500 vs. 3.500	91.568	14.788	0.000	0.000	Yes
NC vs. 3.000	91.306	14.746	0.000	0.000	Yes
NC vs. 7.500	91.246	14.736	0.000	0.000	Yes
11.000 vs. 6.500	90.842	14.671	0.000	0.000	Yes
11.500 vs. 4.000	90.274	14.579	0.000	0.000	Yes
11.000 vs. 3.500	89.250	14.414	0.000	0.000	Yes
12.000 vs. 5.500	88.361	14.270	0.000	0.000	Yes
12.000 vs. 5.000	87.963	14.206	0.000	0.000	Yes
11.000 vs. 4.000	87.956	14.205	0.000	0.000	Yes
12.000 vs. 6.000	87.929	14.201	0.000	0.000	Yes
NC vs. 8.000	87.329	14.104	0.000	0.000	Yes
12.000 vs. 4.500	87.236	14.089	0.000	0.000	Yes
11.500 vs. 3.000	87.070	14.062	0.000	0.000	Yes
11.500 vs. 7.500	87.010	14.052	0.000	0.000	Yes
12.000 vs. 6.500	85.510	13.810	0.000	0.000	Yes
11.000 vs. 3.000	84.752	13.688	0.000	0.000	Yes
11.000 vs. 7.500	84.692	13.678	0.000	0.000	Yes
NC vs. 8.500	84.336	13.620	0.000	0.000	Yes
12.000 vs. 3.500	83.918	13.553	0.000	0.000	Yes
NC vs. 9.000	83.518	13.488	0.000	0.000	Yes
11.500 vs. 8.000	83.093	13.420	0.000	0.000	Yes
NC vs. 10.000	82.628	13.344	0.000	0.000	Yes
12.000 vs. 4.000	82.624	13.344	0.000	0.000	Yes
1.500 vs. 5.500	80.914	13.068	0.000	0.000	Yes
11.000 vs. 8.000	80.775	13.045	0.000	0.000	Yes
1.500 vs. 5.000	80.516	13.003	0.000	0.000	Yes
1.500 vs. 6.000	80.482	12.998	0.000	0.000	Yes
11.500 vs. 8.500	80.100	12.936	0.000	0.000	Yes
NC vs. 2.500	79.848	12.896	0.000	0.000	Yes
1.500 vs. 4.500	79.789	12.886	0.000	0.000	Yes
12.000 vs. 3.000	79.419	12.826	0.000	0.000	Yes
12.000 vs. 7.500	79.359	12.817	0.000	0.000	Yes

11.500 vs. 9.000	79.282	12.804	0.000	0.000	Yes
11.500 vs. 10.000	78.392	12.660	0.000	0.000	Yes
1.500 vs. 6.500	78.063	12.607	0.000	0.000	Yes
11.000 vs. 8.500	77.783	12.562	0.000	0.000	Yes
11.000 vs. 9.000	76.964	12.430	0.000	0.000	Yes
NC vs. 9.500	76.623	12.375	0.000	0.000	Yes
1.500 vs. 3.500	76.471	12.350	0.000	0.000	Yes
NC vs. 10.500	76.397	12.338	0.000	0.000	Yes
11.000 vs. 10.000	76.074	12.286	0.000	0.000	Yes
11.500 vs. 2.500	75.612	12.211	0.000	0.000	Yes
12.000 vs. 8.000	75.442	12.184	0.000	0.000	Yes
1.500 vs. 4.000	75.177	12.141	0.000	0.000	Yes
NC vs. 7.000	92.065	12.140	0.000	0.000	Yes
11.000 vs. 2.500	73.295	11.837	0.000	0.000	Yes
12.000 vs. 8.500	72.450	11.701	0.000	0.000	Yes
11.500 vs. 9.500	72.388	11.691	0.000	0.000	Yes
11.500 vs. 10.500	72.161	11.654	0.000	0.000	Yes
1.500 vs. 3.000	71.972	11.624	0.000	0.000	Yes
1.500 vs. 7.500	71.912	11.614	0.000	0.000	Yes
11.500 vs. 7.000	87.829	11.582	0.000	0.000	Yes
12.000 vs. 9.000	71.631	11.569	0.000	0.000	Yes
12.000 vs. 10.000	70.742	11.425	0.000	0.000	Yes
11.000 vs. 9.500	70.070	11.316	0.000	0.000	Yes
11.000 vs. 10.500	69.843	11.280	0.000	0.000	Yes
11.000 vs. 7.000	85.511	11.276	0.000	0.000	Yes
2.000 vs. 5.500	68.296	11.030	0.000	0.000	Yes
1.500 vs. 8.000	67.995	10.981	0.000	0.000	Yes
12.000 vs. 2.500	67.962	10.976	0.000	0.000	Yes
2.000 vs. 5.000	67.898	10.966	0.000	0.000	Yes
2.000 vs. 6.000	67.865	10.960	0.000	0.000	Yes
2.000 vs. 4.500	67.171	10.848	0.000	0.000	Yes
12.000 vs. 7.000	80.178	10.573	0.000	0.000	Yes
2.000 vs. 6.500	65.445	10.569	0.000	0.000	Yes
1.500 vs. 8.500	65.003	10.498	0.000	0.000	Yes
12.000 vs. 9.500	64.737	10.455	0.000	0.000	Yes
12.000 vs. 10.500	64.510	10.418	0.000	0.000	Yes
1.500 vs. 9.000	64.184	10.366	0.000	0.000	Yes
2.000 vs. 3.500	63.853	10.312	0.000	0.000	Yes
1.500 vs. 10.000	63.295	10.222	0.000	0.000	Yes
2.000 vs. 4.000	62.559	10.103	0.000	0.000	Yes
1.500 vs. 2.500	60.515	9.773	0.000	0.000	Yes
1.500 vs. 7.000	72.731	9.591	0.000	0.000	Yes
2.000 vs. 3.000	59.354	9.586	0.000	0.000	Yes
2.000 vs. 7.500	59.294	9.576	0.000	0.000	Yes
1.500 vs. 9.500	57.290	9.252	0.000	0.000	Yes
1.500 vs. 10.500	57.063	9.216	0.000	0.000	Yes

2.000 vs. 8.000	55.377	8.943	0.000	0.000	Yes
2.000 vs. 8.500	52.385	8.460	0.000	0.000	Yes
2.000 vs. 9.000	51.567	8.328	0.000	0.000	Yes
2.000 vs. 10.000	50.677	8.184	0.000	0.000	Yes
2.000 vs. 7.000	60.113	7.927	0.000	0.000	Yes
2.000 vs. 2.500	47.897	7.735	0.000	0.000	Yes
2.000 vs. 9.500	44.672	7.215	0.000	0.000	Yes
2.000 vs. 10.500	44.445	7.178	0.000	0.000	Yes
NC vs. 2.000	31.951	5.160	0.000	0.000	Yes
11.500 vs. 2.000	27.716	4.476	0.000	0.000	Yes
11.000 vs. 2.000	25.398	4.102	0.000	0.000	Yes
10.500 vs. 5.500	23.851	3.852	0.000	0.000	Yes
9.500 vs. 5.500	23.624	3.815	0.000	0.000	Yes
10.500 vs. 5.000	23.453	3.788	0.000	0.000	Yes
10.500 vs. 6.000	23.419	3.782	0.000	0.000	Yes
9.500 vs. 5.000	23.226	3.751	0.000	0.000	Yes
9.500 vs. 6.000	23.192	3.746	0.000	0.000	Yes
10.500 vs. 4.500	22.726	3.670	0.000	0.000	Yes
9.500 vs. 4.500	22.499	3.634	0.000	0.000	Yes

Comparisons for factor: **Sample pH within con**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
NC vs. 5.000	87.813	14.182	0.000	0.000	Yes
NC vs. 4.500	86.677	13.998	0.000	0.000	Yes
NC vs. 5.500	85.814	13.859	0.000	0.000	Yes
NC vs. 6.000	84.784	13.693	0.000	0.000	Yes
NC vs. 4.000	84.722	13.683	0.000	0.000	Yes
12.000 vs. 5.000	83.240	13.443	0.000	0.000	Yes
NC vs. 6.500	82.837	13.378	0.000	0.000	Yes
12.000 vs. 4.500	82.104	13.260	0.000	0.000	Yes
12.000 vs. 5.500	81.241	13.121	0.000	0.000	Yes
12.000 vs. 6.000	80.211	12.954	0.000	0.000	Yes
12.000 vs. 4.000	80.149	12.944	0.000	0.000	Yes
NC vs. 7.000	79.483	12.837	0.000	0.000	Yes
11.500 vs. 5.000	79.070	12.770	0.000	0.000	Yes
12.000 vs. 6.500	78.264	12.640	0.000	0.000	Yes
11.500 vs. 4.500	77.933	12.586	0.000	0.000	Yes
NC vs. 7.500	77.915	12.583	0.000	0.000	Yes
11.500 vs. 5.500	77.071	12.447	0.000	0.000	Yes
11.500 vs. 6.000	76.040	12.281	0.000	0.000	Yes
11.500 vs. 4.000	75.978	12.271	0.000	0.000	Yes
12.000 vs. 7.000	74.911	12.098	0.000	0.000	Yes
11.500 vs. 6.500	74.093	11.966	0.000	0.000	Yes
12.000 vs. 7.500	73.342	11.845	0.000	0.000	Yes
NC vs. 8.000	72.652	11.733	0.000	0.000	Yes
2.000 vs. 5.000	72.059	11.638	0.000	0.000	Yes

NC vs. 3.500	71.116	11.485	0.000	0.000	Yes
2.000 vs. 4.500	70.922	11.454	0.000	0.000	Yes
1.500 vs. 5.000	70.827	11.439	0.000	0.000	Yes
11.500 vs. 7.000	70.740	11.425	0.000	0.000	Yes
NC vs. 8.500	70.105	11.322	0.000	0.000	Yes
2.000 vs. 5.500	70.060	11.315	0.000	0.000	Yes
1.500 vs. 4.500	69.691	11.255	0.000	0.000	Yes
NC vs. 9.000	69.324	11.196	0.000	0.000	Yes
11.500 vs. 7.500	69.172	11.171	0.000	0.000	Yes
2.000 vs. 6.000	69.029	11.148	0.000	0.000	Yes
2.000 vs. 4.000	68.968	11.138	0.000	0.000	Yes
NC vs. 10.000	68.885	11.125	0.000	0.000	Yes
1.500 vs. 5.500	68.828	11.116	0.000	0.000	Yes
12.000 vs. 8.000	68.079	10.995	0.000	0.000	Yes
1.500 vs. 6.000	67.798	10.949	0.000	0.000	Yes
1.500 vs. 4.000	67.736	10.939	0.000	0.000	Yes
2.000 vs. 6.500	67.082	10.834	0.000	0.000	Yes
12.000 vs. 3.500	66.543	10.747	0.000	0.000	Yes
1.500 vs. 6.500	65.851	10.635	0.000	0.000	Yes
NC vs. 9.500	65.660	10.604	0.000	0.000	Yes
12.000 vs. 8.500	65.533	10.584	0.000	0.000	Yes
12.000 vs. 9.000	64.751	10.457	0.000	0.000	Yes
12.000 vs. 10.000	64.312	10.387	0.000	0.000	Yes
11.500 vs. 8.000	63.908	10.321	0.000	0.000	Yes
2.000 vs. 7.000	63.729	10.292	0.000	0.000	Yes
NC vs. 10.500	62.623	10.114	0.000	0.000	Yes
1.500 vs. 7.000	62.498	10.093	0.000	0.000	Yes
11.500 vs. 3.500	62.372	10.073	0.000	0.000	Yes
2.000 vs. 7.500	62.161	10.039	0.000	0.000	Yes
2.500 vs. 5.000	61.723	9.968	0.000	0.000	Yes
11.500 vs. 8.500	61.362	9.910	0.000	0.000	Yes
12.000 vs. 9.500	61.087	9.866	0.000	0.000	Yes
1.500 vs. 7.500	60.929	9.840	0.000	0.000	Yes
2.500 vs. 4.500	60.586	9.785	0.000	0.000	Yes
11.500 vs. 9.000	60.581	9.784	0.000	0.000	Yes
11.500 vs. 10.000	60.142	9.713	0.000	0.000	Yes
2.500 vs. 5.500	59.724	9.645	0.000	0.000	Yes
2.500 vs. 6.000	58.693	9.479	0.000	0.000	Yes
2.500 vs. 4.000	58.632	9.469	0.000	0.000	Yes
12.000 vs. 10.500	58.050	9.375	0.000	0.000	Yes
11.500 vs. 9.500	56.917	9.192	0.000	0.000	Yes
2.000 vs. 8.000	56.897	9.189	0.000	0.000	Yes
2.500 vs. 6.500	56.746	9.165	0.000	0.000	Yes
1.500 vs. 8.000	55.666	8.990	0.000	0.000	Yes
2.000 vs. 3.500	55.361	8.941	0.000	0.000	Yes
2.000 vs. 8.500	54.351	8.778	0.000	0.000	Yes

1.500 vs. 3.500	54.130	8.742	0.000	0.000	Yes
3.000 vs. 5.000	54.057	8.730	0.000	0.000	Yes
11.500 vs. 10.500	53.880	8.702	0.000	0.000	Yes
2.000 vs. 9.000	53.570	8.652	0.000	0.000	Yes
2.500 vs. 7.000	53.393	8.623	0.000	0.000	Yes
2.000 vs. 10.000	53.131	8.581	0.000	0.000	Yes
1.500 vs. 8.500	53.120	8.579	0.000	0.000	Yes
3.000 vs. 4.500	52.920	8.547	0.000	0.000	Yes
1.500 vs. 9.000	52.338	8.453	0.000	0.000	Yes
3.000 vs. 5.500	52.058	8.407	0.000	0.000	Yes
1.500 vs. 10.000	51.899	8.382	0.000	0.000	Yes
2.500 vs. 7.500	51.825	8.370	0.000	0.000	Yes
3.000 vs. 6.000	51.027	8.241	0.000	0.000	Yes
3.000 vs. 4.000	50.966	8.231	0.000	0.000	Yes
2.000 vs. 9.500	49.906	8.060	0.000	0.000	Yes
11.000 vs. 5.000	49.193	7.945	0.000	0.000	Yes
3.000 vs. 6.500	49.081	7.927	0.000	0.000	Yes
1.500 vs. 9.500	48.674	7.861	0.000	0.000	Yes
11.000 vs. 4.500	48.056	7.761	0.000	0.000	Yes
11.000 vs. 5.500	47.194	7.622	0.000	0.000	Yes
2.000 vs. 10.500	46.869	7.569	0.000	0.000	Yes
2.500 vs. 8.000	46.562	7.520	0.000	0.000	Yes
11.000 vs. 6.000	46.163	7.455	0.000	0.000	Yes
11.000 vs. 4.000	46.101	7.445	0.000	0.000	Yes
3.000 vs. 7.000	45.727	7.385	0.000	0.000	Yes
1.500 vs. 10.500	45.637	7.370	0.000	0.000	Yes
2.500 vs. 3.500	45.025	7.272	0.000	0.000	Yes
11.000 vs. 6.500	44.216	7.141	0.000	0.000	Yes
3.000 vs. 7.500	44.159	7.132	0.000	0.000	Yes
2.500 vs. 8.500	44.015	7.108	0.000	0.000	Yes
2.500 vs. 9.000	43.234	6.982	0.000	0.000	Yes
2.500 vs. 10.000	42.795	6.911	0.000	0.000	Yes
11.000 vs. 7.000	40.863	6.599	0.000	0.000	Yes
2.500 vs. 9.500	39.570	6.391	0.000	0.000	Yes
11.000 vs. 7.500	39.295	6.346	0.000	0.000	Yes
3.000 vs. 8.000	38.896	6.282	0.000	0.000	Yes
NC vs. 11.000	38.621	6.237	0.000	0.000	Yes
3.000 vs. 3.500	37.359	6.034	0.000	0.000	Yes
2.500 vs. 10.500	36.533	5.900	0.000	0.000	Yes
3.000 vs. 8.500	36.349	5.870	0.000	0.000	Yes
3.000 vs. 9.000	35.568	5.744	0.000	0.000	Yes
3.000 vs. 10.000	35.129	5.673	0.000	0.000	Yes
12.000 vs. 11.000	34.048	5.499	0.000	0.000	Yes
11.000 vs. 8.000	34.031	5.496	0.000	0.000	Yes
NC vs. 3.000	33.756	5.452	0.000	0.000	Yes
11.000 vs. 3.500	32.495	5.248	0.000	0.000	Yes

3.000 vs. 9.500	31.904	5.152	0.000	0.000	Yes
11.000 vs. 8.500	31.485	5.085	0.000	0.000	Yes
11.000 vs. 9.000	30.704	4.959	0.000	0.000	Yes
11.000 vs. 10.000	30.265	4.888	0.000	0.000	Yes
11.500 vs. 11.000	29.877	4.825	0.000	0.000	Yes
12.000 vs. 3.000	29.183	4.713	0.000	0.000	Yes
3.000 vs. 10.500	28.867	4.662	0.000	0.000	Yes
11.000 vs. 9.500	27.040	4.367	0.000	0.000	Yes
NC vs. 2.500	26.090	4.214	0.000	0.000	Yes
10.500 vs. 5.000	25.190	4.068	0.000	0.000	Yes
11.500 vs. 3.000	25.013	4.040	0.000	0.000	Yes
10.500 vs. 4.500	24.054	3.885	0.000	0.000	Yes
11.000 vs. 10.500	24.003	3.876	0.000	0.000	Yes
10.500 vs. 5.500	23.191	3.745	0.000	0.000	Yes
2.000 vs. 11.000	22.866	3.693	0.000	0.000	Yes
10.500 vs. 6.000	22.161	3.579	0.000	0.000	Yes
9.500 vs. 5.000	22.153	3.578	0.000	0.000	Yes
10.500 vs. 4.000	22.099	3.569	0.000	0.000	Yes

Two Way ANOVA of Salt Solubility data

Two Way Analysis of Variance
Dependent Variable: Solubility

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Treatment pH	6	5.995	0.999	436.432	<0.001
Salt Conc	4	2.018	0.505	220.381	<0.001
Treatment pH x Salt Conc	24	2.021	0.0842	36.777	<0.001
Residual	244	0.559	0.00229		
Total	278	10.612	0.0382		

Main effects cannot be properly interpreted if significant interaction is determined. This is because the size of a factor's effect depends upon the level of the other factor.

The effect of different levels of Treatment pH depends on what level of Salt Conc is present. There is a statistically significant interaction between Treatment pH and Salt Conc. (P = <0.001)

Power of performed test with alpha = 0.0500: for Treatment pH : 1.000
Power of performed test with alpha = 0.0500: for Salt Conc : 1.000
Power of performed test with alpha = 0.0500: for Treatment pH x Salt Conc : 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **Salt Conc within 2**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
450M vs. 0M	0.0953	3.982	0.000	0.005	Yes
600M vs. 0M	0.0883	3.691	0.000	0.006	Yes

Comparisons for factor: **Salt Conc within 2.5**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
450M vs. 0M	0.0814	3.405	0.001	0.005	Yes
600M vs. 0M	0.0676	2.824	0.005	0.006	Yes

Comparisons for factor: **Salt Conc within 3: No Significant Differences**

Comparisons for factor: **Salt Conc within 10.5**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
600M vs. 0M	0.345	14.420	0.000	0.005	Yes
600M vs. 150M	0.334	13.954	0.000	0.006	Yes
450M vs. 0M	0.286	11.971	0.000	0.006	Yes
450M vs. 150M	0.275	11.505	0.000	0.007	Yes
300M vs. 0M	0.210	8.759	0.000	0.009	Yes
300M vs. 150M	0.198	8.294	0.000	0.010	Yes
600M vs. 300M	0.135	5.660	0.000	0.013	Yes
450M vs. 300M	0.0768	3.211	0.001	0.017	Yes
600M vs. 450M	0.0586	2.449	0.015	0.025	Yes

Comparisons for factor: **Salt Conc within 11**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
600M vs. 150M	0.153	6.406	0.000	0.005	Yes
600M vs. 0M	0.151	6.308	0.000	0.006	Yes
450M vs. 150M	0.121	5.059	0.000	0.006	Yes
450M vs. 0M	0.119	4.961	0.000	0.007	Yes
300M vs. 150M	0.0886	3.706	0.000	0.009	Yes
300M vs. 0M	0.0863	3.608	0.000	0.010	Yes
600M vs. 300M	0.0646	2.700	0.007	0.013	Yes

Comparisons for factor: **Salt Conc within 11.5**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
600M vs. 0M	0.105	4.223	0.000	0.005	Yes
450M vs. 0M	0.0941	3.934	0.000	0.006	Yes
300M vs. 0M	0.0831	3.474	0.001	0.006	Yes
600M vs. 150M	0.0818	3.302	0.001	0.007	Yes
450M vs. 150M	0.0713	2.981	0.003	0.009	Yes

Comparisons for factor: **Salt Conc within control**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
600M vs. 0M	0.659	27.559	0.000	0.005	Yes

600M vs. 150M	0.590	24.647	0.000	0.006	Yes
300M vs. 0M	0.547	22.883	0.000	0.006	Yes
450M vs. 0M	0.522	21.839	0.000	0.007	Yes
300M vs. 150M	0.478	19.972	0.000	0.009	Yes
450M vs. 150M	0.453	18.927	0.000	0.010	Yes
600M vs. 450M	0.137	5.720	0.000	0.013	Yes
600M vs. 300M	0.112	4.675	0.000	0.017	Yes
150M vs. 0M	0.0697	2.912	0.004	0.025	Yes

Comparisons for factor: **Treatment pH within 0M**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
control vs. 2.500	0.131	5.463	0.000	0.002	Yes
control vs. 2.000	0.124	5.200	0.000	0.003	Yes
control vs. 11.500	0.120	5.025	0.000	0.003	Yes
control vs. 3.000	0.0758	3.168	0.002	0.003	Yes
control vs. 10.500	0.0729	3.048	0.003	0.003	Yes

Comparisons for factor: **Treatment pH within 150M**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
control vs. 2.500	0.174	7.281	0.000	0.002	Yes
control vs. 11.500	0.167	6.984	0.000	0.003	Yes
control vs. 2.000	0.160	6.692	0.000	0.003	Yes
control vs. 11.000	0.142	5.924	0.000	0.003	Yes
control vs. 3.000	0.136	5.684	0.000	0.003	Yes
control vs. 10.500	0.131	5.494	0.000	0.003	Yes

Comparisons for factor: **Treatment pH within 300M**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
control vs. 2.500	0.619	25.884	0.000	0.002	Yes
control vs. 2.000	0.615	25.726	0.000	0.003	Yes
control vs. 11.500	0.585	24.435	0.000	0.003	Yes
control vs. 3.000	0.567	23.688	0.000	0.003	Yes
control vs. 11.000	0.531	22.190	0.000	0.003	Yes
control vs. 10.500	0.411	17.172	0.000	0.003	Yes
10.500 vs. 2.500	0.208	8.712	0.000	0.003	Yes
10.500 vs. 2.000	0.205	8.553	0.000	0.004	Yes
10.500 vs. 11.500	0.174	7.262	0.000	0.004	Yes
10.500 vs. 3.000	0.156	6.515	0.000	0.004	Yes
10.500 vs. 11.000	0.120	5.018	0.000	0.005	Yes
11.000 vs. 2.500	0.0884	3.694	0.000	0.005	Yes
11.000 vs. 2.000	0.0846	3.536	0.000	0.006	Yes

Comparisons for factor: **Treatment pH within 450M**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
control vs. 2.500	0.572	23.898	0.000	0.002	Yes
control vs. 2.000	0.552	23.056	0.000	0.003	Yes

control vs. 11.500	0.549	22.930	0.000	0.003	Yes
control vs. 3.000	0.542	22.648	0.000	0.003	Yes
control vs. 11.000	0.473	19.792	0.000	0.003	Yes
control vs. 10.500	0.309	12.916	0.000	0.003	Yes
10.500 vs. 2.500	0.263	10.981	0.000	0.003	Yes
10.500 vs. 2.000	0.243	10.140	0.000	0.004	Yes
10.500 vs. 11.500	0.240	10.014	0.000	0.004	Yes
10.500 vs. 3.000	0.233	9.732	0.000	0.004	Yes
10.500 vs. 11.000	0.164	6.876	0.000	0.005	Yes
11.000 vs. 2.500	0.0982	4.105	0.000	0.005	Yes
11.000 vs. 2.000	0.0781	3.264	0.001	0.006	Yes
11.000 vs. 11.500	0.0751	3.138	0.002	0.006	Yes
11.000 vs. 3.000	0.0683	2.856	0.005	0.007	Yes

Comparisons for factor: **Treatment pH within 600M**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
control vs. 2.500	0.722	30.198	0.000	0.002	Yes
control vs. 2.000	0.695	29.067	0.000	0.003	Yes
control vs. 3.000	0.685	28.633	0.000	0.003	Yes
control vs. 11.500	0.675	27.257	0.000	0.003	Yes
control vs. 11.000	0.578	24.165	0.000	0.003	Yes
control vs. 10.500	0.387	16.187	0.000	0.003	Yes
10.500 vs. 2.500	0.335	14.011	0.000	0.003	Yes
10.500 vs. 2.000	0.308	12.880	0.000	0.004	Yes
10.500 vs. 3.000	0.298	12.445	0.000	0.004	Yes
10.500 vs. 11.500	0.288	11.618	0.000	0.004	Yes
10.500 vs. 11.000	0.191	7.978	0.000	0.005	Yes
11.000 vs. 2.500	0.144	6.033	0.000	0.005	Yes
11.000 vs. 2.000	0.117	4.902	0.000	0.006	Yes
11.000 vs. 3.000	0.107	4.467	0.000	0.006	Yes
11.000 vs. 11.500	0.0968	3.911	0.000	0.007	Yes

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BIOGRAPHICAL SKETCH

Matthew Paul Davenport was born in Lexington, KY, and moved to Gainesville, FL two years later where he was raised. Matthew attended Gainesville High School graduating in 1998. During the last year and a half of high school, Matthew started working as a laboratory technician for Dr. Carlotta Grooves at the University of Florida in the College of Veterinary Medicine, Department of Basic Sciences; the focus of that work was on environmental toxicology. After graduation from Gainesville High School, Matthew attended Greenville College in Greenville, Illinois, graduating in 2002 with a Bachelor of Arts degree in biology. While at Greenville College Matthew began work in the area of food science, investigating carbon monoxide processing of yellow fin tuna and the effect of consumption on the consumer. This project was done in collaboration with the Food Science and Human Nutrition Department of the University of Florida. After graduation from Greenville College, Matthew began work in the Food Science and Human Nutrition Department at the University of Florida as a laboratory technician for Dr. W. Steven Otwell during the summer of 2002. Matthew began his graduate work in the fall of 2002 under the tutelage of Dr. Hordur G. Kristinsson. During his matriculation at the University of Florida, Matthew joined Alpha Zeta, agricultural honors fraternity, and Phi Tau Sigma, food science honors fraternity, where he served as vice president from 2005-2006 and president from 2006-2007. Upon graduation Matthew hopes to contribute to the scientific community, both academic and industrial through his pursuit of knowledge in the sciences, specifically food science.