Abstract

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Diffusion of protease inhibitors in the muscle cell

(Under the direction of Dr. Tyre Lanier)

Proteases often exist in fish muscle, arising from parasites, gut leaching, or merely normal physiology. Their activity may cause undesired softening of the meat during storage or slow cooking. Several food-grade inhibitors exist which can overcome this softening if properly delivered to the intracellular sites where proteases are located. Translational diffusion of enzyme inhibitor proteins through different subcellular compartments and intracellular architectures in the muscle cell is thus a mandatory step to effectively block the activity of such proteases.

The analyses of both macromolecule spatial distribution and fluorescence recovery after photobleaching (FRAP) by laser scanning confocal microscopy (LSCM) were used to measure the translational diffusion of fluorescein isothiocyanate (FITC)-labeled protease inhibitors into intact muscle fibers of halibut. Diffusion coefficients (D) of α-2 macroglobulin (750KDa), soy bean trypsin inhibitor (21 KDa) and cystatin (12 KDa) were measured in both muscle fibers and dilute aqueous solutions. On the time scale of the observation (35min), cystatin and soy bean trypsin inhibitor diffused through the cell membrane (sarcolemma) and sarcoplasm, but considerably more slowly (>10-fold) than in dilute aqueous solution. A hypothesis/review paper is presented which supports the theory that structuring of water within cells is a primary cause of reduced diffusivity of proteins into and within cells. α-2 macroglobulin did not diffuse at all into muscle cells within the time frame of the experiment, but did completely penetrate the cell after overnight exposure.

The present study thus shows a clear dependence of D on protein inhibitor size in intact skeletal muscle fibers. Low molecular weight protease inhibitors like cystatin can therefore be effectively diffused into intact fish muscle cells to minimize proteolytic activity and meat softening.

DIFFUSION OF PROTEASE INHIBITORS IN THE MUSCLE CELL

by

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A dissertation submitted to the Graduate Faculty of
North Carolina State University
In partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

DEPARTMENT OF FOOD SCIENCE

RALEIGH

2002

APPROVED BY:

Chairman of Advisory Committee

BIOGRAPHY

Patricio Carvajal Rondanelli was born on February 21, 1967 in Tome, Chile. He is the son of Rebeca Rondanelli and Osvaldo Carvajal. He has 3 brothers and two younger sisters; Osvaldo, Cristian, Claudio, Rebeca y Miriam. Patricio grew up in Talcahuano and went to Liceo La Asuncion where he graduated in December 1985. He began his college career at Universidad Catolica de Valparaiso in Food Engineering.   He graduated with his BS in December 1991. In January 1994, he began the graduate program under the direction of Dr. Tyre Lanier. He received a Master of Science degree in 1998. Then he continued to pursue his Ph.D under the same adviser direction.

ACKNOWLEDGMENTS

The completion of my dissertation gives me an opportunity to say thanks to America in general and some special individuals. For without their support, I certainly would not be doing what I am now.

With gladness, I express my deep gratitude to Dr Lanier. In his kindly and generous way, he gave me the full freedom to pursue my own direction of research and ideas. His encouragement, guidance and trustiness will be always appreciated. Also I would like to thank to my remaining committee members: Dr.Jeff Hinshaw, Dr. Michael Theil and Dr. Brian Farkas.

Additional thanks are extended to Professor Marta Dondero, my former adviser at the Universidad Catolica de Valparaiso in Chile. Her support and encouragement motivated me to come to the USA.

To Penny Amato, for her friendship and caring over the years and the intellectual and emotional conversations my eternal gratitude. Also I would like to thanks Rob Lombard, Alex Reiman and Natasha Evans who made my days in the lab more enjoyable. I wish to thank also Dr Nina Allen Lab staff and especially Dr Eva Johannes for training me in the use of the confocal and Carmen Lucaveche from the Department of Cell Biology at Duke University for training me to isolate and prepare muscle fibers.

To my long time dearest friend Daniel Brownstone, for his constant support, advice and love. To my two adored beauties: Aruna Radhakrishna and Rocio Lozano whom I would have married but I didn't get to them on time. To Marc Serre, Leslie Montana, Zaur Mekhtiev, Carlos Carpio, William Barfield, Chris Barefoot, Alfonso Fuminaya and Mary Harnett, many thanks for making my life fun. Also I want to say

thanks to two gone but not forgotten dear friends: Jeff Prescott and Darrin Poirier. Also to my loyal and dear friends in Chile: Roberto Munoz, Maritza Aburto, Patty Vera, and the Trujillo and Rivera families.

Finally, my gratitude goes to my dearest family in Chile and especially to my Dad, Uncles Renato and Roberto Rondanelli, Aunts Yolanda Romero and Mirta Delpiano, my dear sisters, brothers and cousins.

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A REVIEW OF PROTEASES and THEIR ACTION in SEAFOODS

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INTRODUCTION

Protease is a generic name given to those enzymes hydrolyzing the peptide bond in proteins. Proteases, including peptidases and proteinases, are polyfunctional enzymes catalyzing the hydrolytic degradation of proteins.

In the literature proteolytic enzymes, proteases and proteinases are almost-overlapping terms for the whole group of enzymes known as peptidases and is the term recommended by the International Union of Biochemistry and Molecular Biology (IUBMB). By definition, however they are slightly different, but these differences have largely been lost in current usage. Proteases compromise two groups of enzymes: the endopeptidases and the exopeptidases, which respectively cleave peptide bonds at points within the protein and remove amino acids sequentially from either N or C-terminus. The term "proteinase" is actually a synonym for endopeptidase (Barrett, 1999).

In living organisms, proteases are involved in several physiological functions, both extracellular and intracellular. Advances in analytical techniques have demonstrated that proteases conduct highly specific and selective modifications of proteins such as activation of zymogenic forms of enzymes by limited proteolysis, blood clotting and lysis of fibrin clots, and processing and transport of secretary proteins across the membranes. They are not only responsible for the complex processes involved in the normal physiology of the cell but also in abnormal pathophysiological conditions. Their involvement in the life cycle of disease-causing organisms has led them to become a potential target for developing therapeutic agents against fatal diseases such as cancer and AIDS.

Protease activity *in vivo* is controlled by several mechanisms, including by endogenous inhibitors, pH, redox potential, synthesis as a proenzyme, compartmentalization and degradation (Blankenvoorde et al., 2000). Most protease inhibitors controlling physiological processes affect the enzyme activity reversibly by binding to the protease (Hibbett, 1999).

In food technology, proteolysis is used to modify the functional and nutritional properties of food proteins. However, in some food processing examples, proteolysis by endogenous proteases (autoproteolysis) causes deterioration of organoeleptic or functional properties.

Since proteases are enzymes of metabolic as well as commercial importance, there is an extensive literature on their biochemical and biotechnological aspects. This review will review the classification of proteases and their inhibitors, then will address applications of proteases with specific emphasis on muscle proteases and their impact on meat, and then especially seafood meat quality.

CLASSIFICATION OF PROTEASES

Enzymes that hydrolyze peptide bonds can be grossly grouped into two subclasses, exopeptidases and endopeptidases, depending on where the reaction takes place in the polypeptide substrate. Exopeptidases cleave peptide bonds at the amino or carboxyl ends of the polypeptide chain, whereas endopeptidases cleave internal peptide bonds (International Union of Biochemistry, 1992).

Regardless of the source, proteases can be classified on the basis of their similarity to well-characterized proteases, such as trypsin-like, chymotrypsin-like, chymosin-like, or cathepsin-like (Barnett, 1977). They may also be classified on the basis of their sensitivity to pH, such as acid, neutral, or alkaline proteases (Neurath, 1989). They are also often classified according to their substrate specificity, response to inhibitors, or by their mode of catalysis.

The standard method of classification proposed by the Enzyme Commission (EC) of the International Union of Biochemists (IUB), is based on the mode of catalysis. This divides the proteolytic enzymes into four groups: serine, cysteine, aspartic, and metallo, the name of each class being derived from a distinct catalytic group involved in the reaction. The actives sites of serine and cysteine proteases use serine hydroxyl and cysteine thiol side groups, respectively, as the attacking nucleophiles during catalysis. Their reaction pathway entails formation of a covalent intermediate between the nucleophile and the carbonyl carbon atom of the scissile peptide bond. By contrast, the mechanism of hydrolysis for aspartic and metallo proteases does not involve a covalent intermediate because the nucleophile for both of these enzymes is a water molecule (Dunn, 1989). The different functional groups for each protease reaction mechanism affect their active pH range as well as the types of inhibitors suitable for inactivation. It should be noted that the reaction pathways in all four protease classes share common elements (Graycar, 1999):

- Binding of the substrate polypeptide into a channel or pocket on the enzyme surface.

- Nucleophilic attack on the carbonyl carbon of the scissile peptide bond by either oxygen or sulfur

- General base-assisted catalysis for removal of a proton from the nucleophile

- Stabilization of the tetrahedral transition state intermediate formed at the carbonyl carbon of the scissile peptide bond

- General acid-assisted catalysis for transfer of a proton to the amine leaving group.

Identification of the mechanistic family that a protease belongs to is the key to predicting its structure and function and may unlock strategies for inhibitor design that were previously developed for homologous members of the family. This concept was extremely valuable for the design of HIV protease inhibitors and led directly to the design of the first approved drug, Saquinavir, long before the structure of the enzyme was known (Ericson and Eissenstat, 1999).

The catalytic site of proteases is flanked on one or both sides by specificity subunits, each able to accommodate the side chain of a single amino acid residue from the substrate. These sites are numbered from the catalytic site S1 through Sn toward the N terminus of the structure and S1' through Sn' toward the C terminus. The residues

which they accommodate from the substrate are numbered P1 through Pn and P1'

through Pn' respectively (Rao et al., 1999).

Serine proteases

Serine, or alkaline proteases are so-named because they have a "super-reactive"

serine in the active site. The serine proteases are a very important family of enzymes in

animal physiology point, being involved in many critical aspects such as digestion, blood

clotting, and hormone control, to name a few (Rawlings, Barrett, 1994)

This class comprises two distinct families classified according to their structural

homology to trypsin or subtilisin. The trypsin family is the largest and includes both

mammalian and bacterial members. Some common examples are the pancreatic digestive

enzymes trypsin, chymotrypsin, and elastase; as well as the blood-clotting enzymes such

as thrombin, plasmin, and many complement enzymes. In contrast, the subtilisin family

are only found in bacteria.

The specificity of the serine proteases is usually not very high since they have

similar active sites and act through the same proteolytic mechanism. Consequently, a

single serine protease may act on various substrates although at different rates (Rawlings,

Barrett, 1994).

Serine proteases are generally active at neutral and alkaline pH, with an optimum

between pH 7 and 11. They have a broad substrate specificity. Their molecular masses

range between 18 and 35 kDa. The isoelectric points of serine proteases are generally

between pH 4 and 6. Depending on the nature of the substrate, they are either inhibited

or activated by Ca^{+2} and are generally heat labile, being readily inactivated at temperatures between 37-50C (Dahlmann et al., 1985).

These enzymes are synthesized as inactive *zymogens or pro-enzymes*, and activated when at the site of action. The activation process usually involves proteolytic cleavage, in some cases by the same enzyme, to remove a peptide (in some cases such as CT two dipeptides, but most frequently one fairly lengthy peptide from the N-terminal end).

The general 3D structure is different in the two families but they have the same active site geometry and catalysis proceeds via the same mechanism. The serine proteases exhibit different substrate specificities which are related to amino acid substitutions in the various enzyme subsites interacting with the substrate residues. Some enzymes have an extended interaction site with the substrate whereas others have a specificity restricted to the P1 substrate residue (Turk et al., 1999).

Three residues which form the catalytic triad are essential in the catalytic process i.e His 57, Asp 102 and Ser 195 (chymotrypsinogen numbering). The first step in the catalysis is the formation of an acyl enzyme intermediate between the substrate and the essential serine. Formation of this covalent intermediate proceeds through a negatively charged tetrahedral transition state intermediate and then the peptide bond is cleaved. During the second step or deacylation, the acyl-enzyme intermediate is hydrolyzed by a water molecule to release the peptide and to restore the Ser-hydroxyl of the enzyme. The deacylation which also involves the formation of a tetrahedral transition state intermediate, proceeds through the reverse reaction pathway of acylation. A water

molecule is the attacking nucleophile instead of the Ser residue. The His residue provides a general base and accept the OH group of the reactive Ser (Graycar, 1999).

Cysteine proteases

This family includes the plant proteases such as papain, ficin and bromelain, several mammalian lysosomal cathepsins, the cytosolic calpains (calcium-activated) as well as several parasitic proteases. Papain is the archetype and the best studied member of the family (Turk et al.,1997). Like the serine proteases, catalysis proceeds through the formation of a covalent intermediate and involves a cysteine and a histidine residue. The essential Cys25 and His159 (papain numbering) play the same role as Ser195 and His57 respectively. The nucleophile is a thiolate ion rather than a hydroxyl group. The thiolate ion is stabilized through the formation of an ion pair with neighboring imidazolium group of His159. The attacking nucleophile is the thiolate-imidazolium ion pair in both steps and then a water molecule is not required (McGrath, 1999).

The most important cysteine proteases in mammals are the cytoplasmic calpains and the lysosomal cathepsins. The term "cathepsins" is used to refer to intracellular proteases, mostly localized in the lysosomes, which are active at weakly acid pH values (the lysosomal pH is about 5). Lysosomes are organelles which form by pinching off from the Golgi apparatus and which contain a multitude of hydrolytic enzymes. Most of the lysosomal proteases are cysteine proteases, with the exception of cathepsin G, A,D and R. Of all lysosomal cysteine proteases, cathepsin B has been the most extensively investigated. This enzyme has been isolated from various mammalian tissues; the enzymes from different species do not differ to any great extent (Baricos et al., 1988).

Cathepsin B has seldom been detected in the extracellular medium, at least in nonpathological tissue, due to its low stability in neutral to alkaline pH (Kirschke and Barrett, 1987). It is interesting that the alkali-stable enzyme forms that have been found in the extracellular medium mostly have a higher molecular weight than the lysosomal enzymes and are mainly found in tumor tissue (Brocklehurst et al., 1987).

Comparison of the sequences of cathepsins B and H, papain and actinidin, and the known partial sequences of bromelain shows that the tertiary structures of all these proteases must be similar. One hundred and sixty-six of the amino acid residues are topologically equivalent to papain but large structural changes result from insertions in the middle part of the chain building an occluding loop. This leads to significant changes in the surface of the cleft between the domains and explains the different in specificity (Kirschke et al., 1998).

Cathepsin L differs from cathepsins B and H in that it lacks exopeptidase activity and has higher protease activity: it is the enzyme with the highest proteolytic activity in the lysosomes (Kirschke et al., 1998). Cathepsin L hydrolyzes extracellular matrix proteins such as collagen and elastin more effectively than collagenase and neutrophilic elastase, the enzymes which are better known for their activity on these substrates. Cleavage to the two chain form takes place much faster than with the other two cathepsins (Otto and Schirmeister, 1997). A further difference is that relatively large amounts (up to 40%) of procathepsin L are secreted. Cathepsin L has a broad substrate tolerance (Kirschke et al., 1998).

Cathepsin C is made up of 8 subunits, each of 24 kDa, and it cleaves dipeptides from the N terminal of peptides chains. In contrast to other cysteine proteases, cathepsin

C requires halide ions for full activity (McDonald & Barrett, 1986). Cathepsin S, a recently discovered lysosomal cysteine protease, is unevenly distributed between organs. The enzyme has been detected in high concentrations in spleen and lung. The stability of the enzyme above pH 7 is remarkable property (Pangkey et al., 2000). Cathepsin N has only been found in lymphatic cells, and at present, this enzyme is together with cathepsin K, the only known cathepsin which is tissue specific (Kirschke et al., 1998).

Cathepsin M and fructose-1,6-diphosphatase-coverting enzyme are the only lysosomal proteases that are known to be membrane bound. In contrast to other lysosomal cathepsins, both enzymes are most active at neutral pH and are stable at high pH, at least in the membrane-bound form.

The calpains are cytoplasmic cysteine proteases and have been extensively investigated. The name calpain refers to the activation of these enzymes by calcium ions and to the analogy of the cysteine proteases with papain. They occur, together with their specific endogenous inhibitors, the calpastatins, in most all mammalian and avian cell types. They are also discovered in fungi, but they have not been detected in plants. Two types of calpain have been isolated which differ in their calcium requirement. The μ-calpain requires micromolar calcium concentrations (1-100 μM) whereas m-calpain is only activated by millimolar calcium concentrations (0.1-1 mM) (Otto and Schirmeister, 1997). Calpains are cytoplasmatic enzymes, 7-30% being membrane bound. They are heterodimers, made up of a 80 kDa catalytic and 30 kDa regulatory subunits. All the calpains of a particular organism use the same regulatory subunit, and differences in specificity and calcium sensitivity arise from variation in structure of the catalytic unit.

Due to the fact that, in physiological conditions, the calcium concentration of < 1

µM cannot be sufficient to activate the calpains, the enzymes which have been isolated

are assumed to be inactive procalpains *in vivo*. The active forms, i.e. the forms that are

active at physiological calcium concentrations, have not yet been isolated, probably due

to their instability (Ono et al., 1999). µ-calpains can be activated by binding to membrane

lipids (phosphatidyl inositol). Binding to the membrane enables autocatalytic processes to

take place at the N-termini of both subunits and the calcium requirement is thereby

reduced to physiological concentrations (0.1-1 µM) (Otto and Schirmeister, 1997).

Recent results suggest that the activation of calpain corresponds to the

dissociation into subunits in the presence of calcium and that calpain functions as a

monomer of the 80 kDa subunit *in vivo* (Ono et al., 1999). Calpain preferentially cleaves

peptides with Tyr, Met, Lys or Arg at P1 and hydrophobic amino acids such as Leu and

Val at the P2 position and both isoenzymes are similar in this respect (Graycar, 1999).

However, other calpain specificities have been observed with larger proteins (Turk et al.,

1999). The pH optimum, which is neutral to weakly alkaline, corresponds to that of the

cytosol (Otto and Schirmeister, 1997)

Aspartic proteases

Aspartic proteases, commonly known as acidic proteases, are endopeptidases that

depend on aspartic acid residues for their catalytic activity.

Aspartic proteases are produced by a number of cells and tissues. These enzymes

share a high degree of similarity which involves primary structures, and most of them are

active predominantly in the acidic range of pH 2 to 4. Rennin is one exception where activity is in the pH range of 5.5 to 7.5. Their molecular masses are in the range of 30 to 45 kDa.

Most of the aspartic proteases belong to the pepsin family. The pepsin family includes digestive enzymes such as pepsin and chymosin as well as lysosomal cathepsins D and processing enzymes such as rennin, and certain fungal proteases (penicillopepsin, rhizopuspepsin, endothiapepsin). A second family comprises viral proteases such as the protease from the AIDS virus (HIV) also called retropepsin (Erickson and Eissenstat, 1999). Crystallographic studies show that these enzymes are bilobed molecules with the active site located between two homologous lobes. Each lobe contributes one aspartate residue of the catalytically active diad of aspartates. These two aspartyl residues are in close geometric proximity in the active molecule and one aspartate is ionized whereas the second one is unionized at the optimum pH range of 2-3. Retropepsins are monomeric, i.e they carry only one catalytic aspartate and then dimerization is required to form an active enzyme (Graycar, 1999).

In contrast to serine and cysteine proteases, catalysis by aspartic proteases does not involve a covalent intermediate though a tetrahedral intermediate exists. The nucleophilic attack is achieved by two simultaneous proton transfers: one from a water molecule to the diad of the two carboxyl groups and a second one from the diad to the carbonyl oxygen of the substrate with the concurrent CO-NH bond cleavage. This general acid-base catalysis, which may be called a "push-pull" mechanism, leads to the formation of a non-covalent neutral tetrahedral intermediate (Kashparov et al., 1998).

Metallo proteases

The metallo proteases include enzymes from a variety of origins, such as collagenases from higher organisms, toxins from snake venoms, and thermolysin from bacteria. They differ widely in sequence and structure, but the great majority contain a zinc atom which is catalytically active. In some cases, zinc may be replaced by another metal such as cobalt or nickel without loss of activity (Barrett, 1995).

Digestive metalloproteases, such as carboxypeptidases A and B, and thermolysin are exopeptidases. The matrix metalloproteases (MMPs) are a family of zinc endopeptidases which are responsible for the degradation of collagen in extracellular fluids. At least nine different types of MMPs have been identified including collagenases, gelatinases, stromelysins, and matrilysins. Members of this family are involved in normal connective tissue development as well as having been implicated in a range of diseases including cancer and arthritic disease (Rao et al., 1998).

The crystallographic structure of bacterial thermolysin indicates that zinc is bound by two histidines and one glutamic acid. Many metalloproteases contain the sequence His-Glu-Xaa-Xaa-His (HEXXH), which provides two histidine ligands for the zinc whereas the third ligand is either a glutamic acid (thermolysin, neprilysin, alanyl aminopeptidase) or a histidine (astacin). Other families exhibit a distinct mode of binding of the Zn atom (Graycar, 1999). The catalytic mechanism leads to the formation of a noncovalent tetrahedral intermediate after the attack of a zinc-bound water molecule on the carbonyl group of the scissile bond. This intermediate is further decomposed by transfer of the glutamic acid proton to the leaving group.

NATURAL PROTEIN PROTEASE INHIBITORS

Although necessary from a physiological point of view, proteases are potentially hazardous to their proteinaceous environment and their activity must be precisely controlled by the respective cell or organism. When uncontrolled, proteases can be responsible for serious diseases. The control of proteases is normally achieved by regulated expression/secretion and/or activation of proproteases, by degradation of mature enzymes, and by inhibition of their proteolytic activity (Otlewski et al., 1999).

Over 100 naturally occurring protein protease inhibitors have been identified so far. They have been isolated in a variety of organisms from bacteria to animals and plants. They behave as tight-binding reversible or pseudo-irreversible inhibitors of proteases preventing substrate access to the active site through steric hindrance. Their size are also extremely variable from 50 residues (e.g BPTI: Bovine Pancreatic Trypsin Inhibitor) to up to 400 residues (e.g alpha-1PI: alpha-1 Protease Inhibitor). They are strictly class-specific except proteins of the alpha-macroglobulin family (e.g alpha-2 macroglobulin) which bind and inhibit most proteases through a molecular trap mechanism.

All known naturally occurring inhibitors directed towards endogenous cognate proteases are proteins, only some microorganisms secrete small non-proteinaceous compounds which block the host protease activity. Often protease inhibitors accumulate in high quantities in plant seeds, bird eggs and various body fluids. The large concentration of protease inhibitors in mammalian and marine animal blood plasma,

where they account for more than 10% of total protein, or in plant seeds such as legumes illustrate the importance of protease/inhibitor interactions in nature and the potential of these inhibitors to regulate proteases in various biological systems.

The fact that the control of proteolysis by inhibitors is so specific makes it a valuable tool in medicine, agriculture and food technology. The human immune deficiency virus protease, the digestive systems of crop pests, and fish muscle proteases are some example of targets of study.

A general characteristic of most protein inhibitors is that they are cysteine-rich proteins. The disulfide bridges are important in the stability and active conformation structure of many of these proteins.

CLASSIFICATION OF PROTEASE INHIBITORS

Protease inhibitors can be broadly separated into two general categories based upon their spectrum of activity: the nonspecific protease inhibitors and the class-specific protease inhibitors.

Nonspecific protease inhibitors are capable of inhibiting members of all 4 classes of proteases. This class of inhibitors consists solely of the alpha macroglobulins, including human α_2-macroglobulin (α_2-M). Alpha macroglobulins are very large proteins that comprise as much as 8-10% of total serum protein. They are unique in that they have the ability to inhibit proteases from each of the 4 major classes of proteases (the aspartic, cysteine, serine, and metalloproteases). Human α_2-M is a glycoprotein composed of 4 identical subunits, each with a molecular weight of approximately 185,000. α_2-M is

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produced primarily in the liver, although other sites of production have been identified (Borth, 1992).

The alpha macroglobulins are able to exert their effects on a variety of proteases because of a unique mechanism of action, referred to as the trap mechanism. The protease binds to a generic bait region on the alpha macroglobulin molecule, resulting in a conformational change in the alpha macroglobulin. This conformational change exposes a receptor-binding domain, causing rapid removal of the alpha macroglobulin-protease complex from circulation by receptor-mediated endocytosis in the reticuloendothelial system (Hibbetts et al., 1999). Even when contacting a protease in a non-living system (e.g., foodstuffs), accessibility of the bound protease to extraneous protein substrates is severely hampered due to stearic hindrance by the enveloping α_2-M molecule. One alpha macroglobulin molecule can bind to one or two molecules of protease.

Although no single specific role for the alpha macroglobulins has been identified, their major function seems to be the rapid inhibition of excess proteolytic activity due to either endogenous or exogenous proteases. Though alpha macroglobulins may be the primary inhibitor of some proteolytic enzymes, they seem to play a supportive role in the modulation of many other proteases. In addition to inhibiting proteolysis, alpha macroglobulins also can bind many nonproteolytic enzymes (Borth, 1992).

The **class-specific protease inhibitors** are each capable of inhibiting proteases from only 1 of the 4 classes of proteases listed above (Laskowski and Kato, 1980). These have lower molecular weights and higher specificity for target enzymes when compared to the alpha macroglobulins. Their higher specificity is due to specific binding sites

located within the active site of the inhibitor. This limits their sites of activity within the body but not their physiologic importance.

Thus the remaining class-specific protease inhibitors can be divided into 4 superfamilies, corresponding to the 4 classes of proteases. Most of these inhibitors abolish all enzymatic activity toward all substrates, have strictly competitive inhibitory activity, and have inhibitory sites that can each inhibit proteases belonging to only 1 of the 4 classes of proteolytic enzymes. All of these protease inhibitors prevent access of substrates to the proteases' active sites through steric hindrance. Many act in a substrate-like manner by binding directly to the active site of the protease, whereas others bind to surface sites adjacent to the actual active site. Both methods prevent interaction between the protease and substrate, and both may be reversible (Bode and Huber, 1992).

Serine Protease Inhibitors

Serine protease inhibitors comprise the largest super-family of the class-specific protease inhibitors identified to date. These inhibitors are very abundant in mammalian plasma and plant cells and play a main role in many physiologic processes (Otlewski et al., 1999). Two commonly recognized serine protease inhibitors are described in below.

Antithrombin

Antithrombin is a serine protease inhibitor involved in the coagulation cascade. It is produced in the liver and endothelial cells and is responsible for 70% of the anticoagulant activity of normal plasma. The primary target for antithrombin is serine protease thrombin, but antithrombin has some activity against all of the activated serine

protease coagulation factors. Anti-thrombin forms a complex between the active site of thrombin and the reactive site of antithrombin. In the presence of heparin, the rate of complex formation is increased approximately 2,000-fold. Thrombin or another protease binds to heparin, and brings the active site of the protease into close contact with the reactive site of antithrombin.

Trypsin soybean inhibitors

Protease inhibitors that have been isolated from soybeans are of two types: the Kunitz trypsin inhibitor (TI) and the Bowman-Birk (BB) inhibitor. The first group has an MW between 20 and 25 kDa, with a specificity directed primarily toward trypsin. The inhibitor was shown to combine tightly with trypsin. The complete amino acid sequence of the inhibitor was established by Koide et al. (1973). It consists of 181 amino acid residues and two disulfide bonds, with a reactive site at residues Arg63 and Ile64.

Soybean BB inhibitor is a single polypeptide chain of 71 amino acids including seven disulfide bounds. Its MW is about 8 kDa. The BB inhibitor is capable of inhibiting both trypsin and chymotrypsin at independent reactive sites, the trypsin reactive site being located at residues Lys16 and Ser17 and the chymotrypsin reactive site being at the residues Leu44 and Ser45. BB inhibitor has a stable conformation even after disulfide bonds are broken by heating.

Cysteine Protease Inhibitors

These inhibitors act as a protective mechanism against cysteine proteases released into circulation after cell death. Cysteine protease inhibitors also may be involved in the pathogenesis of diseases such as muscular dystrophy, Alzheimer's disease, and multiple sclerosis (Blankenvoorde et al., 2000). Synthetic cysteine protease inhibitors may have potential for use against arthritic diseases and against progression of various cancers and parasitic infections (Otto and Schirmeister,1997).

Natural Cysteine Proteins Inhibitors (Cystatins)

The cystatin superfamily contains three families of proteins that are related functionally as cysteine protease inhibitors and evolutionarily by their amino acid sequence identity. These inhibitors occur in all cells and body fluids of mammals and many lower organisms. Their function is suggested to be the protection of proteins outside the lysosomes from degradation by cysteine proteases, but also to protect active cysteine proteases outside the lysosomes from inactivation by the physiological neutral pH. The reaction of catalytically active cathepsin L with cystatin B was faster than the inactivation of cathepsin L at pH 7.4. At acidic pH values the inhibitor complex with cysteine proteases dissociates, releasing catalytically active enzymes.

The interaction of cystatins with cysteine peptidases is a reversible and tight-binding one at the active site, but without formation and cleavage of covalent bonds. Cystatins also react with their inactive carboxymethylated target enzymes.

The affinity of the cystatins to the lysosomal cysteine proteases is very high. They do not react with serine or other types of proteases (Abrahamson et al., 1991).

The cystatins are classified into three families as follows (Blankenvoorde et al., 2000):

Family 1: cystatin A, cystatin B. Synthesized without signal peptides; MW = 11-12 kDa; contain no disulfide bonds; occur intracellularly in the cytosol.

Family 2:cystatins C, D, S, SN, SA. Synthesized with signal peptides, MW = 13-14 kDa, contain disulfide bonds; are secreted and present in the body fluids.

Family 3; kininogens (other names: cysteine protease inhibitor or CPI). Exist in several forms (L kininogen, H kininogen); MW= 60-120 kDa; are glycoproteins; contain three cystatin domain, two of which are functional; occur mainly in blood plasma and in synovial and aminiotic fluids.

Cystatins have been successfully expressed as recombinant proteins in plants like rice, corn and potatoes, which may appear as suitable sources for large scale purification. In addition, several cystatin genes have been cloned and expressed in *Escherichia coli*. These cystatins, for instance oryzacystatin I (OC-I) and oryzacystatin II (OC-II), can easily be purified in large quantities and exert biological activity. In this context, it is worth emphasizing that cystatins are very stable under conditions of extreme pH and high temperatures. Recently Blankenvoorde (2000) (unpublished results) observed that egg white cystatin remains stable and biologically active in a commercial mouth rinse over a period of several months at room temperature.

In vivo, however, the degradation of cystatins could be important. For example cystatins can be inactivated by an aspartic protease while human PMN elastase rapidly

cleaves the N-terminal region of cystatin C, resulting in a decreased affinity of the truncated cystatin C for cathepsins B, H and L. On the other hand, cystatins truncated by proteolytic enzymes of *P. gingivalis* were shown to remain biologically active, and other proteases like cathepsin G and protease 4 failed to hydrolyze peptide bonds in cystatin C. In any case, to bypass *in vivo* degradation of natural cystatins, recombinant engineered cystatins could be developed which are more resistant to proteolytic degradation.

Aspartic Protease Inhibitors

The best characterized aspartic proteases from mammals (pepsin, chymosin, cathepsin D and rennin) are all inhibited by pepstatin A, a pentapeptide-like compound secreted by *Streptomyces* species. Until recently, α_2-macroglobulin was thought to be the only major inhibitor of aspartic proteases. For this reason, research into synthetic aspartic protease inhibitors is developing rapidly (Thomas et al., 1989). Synthetic inhibitors of HIV- PR have been developed and are being used with some encouraging results in the treatment of human patients infected with HIV. Within the past years many new and potent inhibitors of HIV protease have been developed (Indanivir, Saquinavir, AZT, Ritonavir, etc). They are competitive inhibitors of the HIV protease, with Ki values for the enzyme in the picomolar range. They are quite specific for the viral enzyme versus distantly-related cellular enzymes, such as pepsin, rennin and cathepsin D, and able to discriminate among them by several orders of magnitude (Erickson and Eissentat, 1999).

Metalloprotease Inhibitors

Most of the design of class specific inhibitors of metalloproteases has focused on attempts to chelate or bind the catalytic zinc atom. Synthetic inhibitors, therefore, commonly contain a negatively-charged moiety to which is attached a series of other groups designed to fit the specificity pockets of a particular protease.

Currently only one natural protein inhibitor of metallo proteases has been well characterized. This inhibitor, TIMP (tissue inhibitors of metalloproteases) inhibits members of the group of matrix metalloproteases (MMPs) secreted by connective tissue cells. The group includes collagenases, gelatinase and stromelsin. TIMPs regulate destruction of extracellular matrix. The balance between matrix metalloproteases and TIMPs seems to be an important factor in the pathogenesis of tumor invasion and arthritis (Hibbets et al., 1999).

INDUSTRIAL PROTEASES

Proteases represent one of the three largest groups of industrial enzymes and account for about 60% of the total worldwide sale of enzymes (Godfrey and West, 1996). Besides their use in detergents, they are used in the food industry for the production of cheeses, chillproofing of beer, tenderization of meat, and modification of the properties of the proteins of cereals in bread and cereal manufacture. The largest tonnage production of proteases is based on microbial sources. Recombinant DNA methods and protein engineering are today's means to develop important proteases for both food and nonfood uses.

Proteases for Detergents

Proteases are one of the standard ingredients of many detergents ranging from those used for household laundering to reagents used for cleaning contact lenses or dentures. About 13 million tons per year of proteases are added to laundry detergents, accounting for approximately 25% of the total worldwide sales of enzymes (Rao et al, 1998). The ideal detergent protease should posses broad substrate specificity to facilitate the removal of a large variety of stains due to food, blood, and other body secretions (Eriksen, 1996). Activity and stability at high pH and temperature and compatibility with other chelating and oxidating agents added to the detergents are among the prerequisites for the use of proteases in detergents. A protease is most suitable for this application if its pI coincides with the pH of the detergent solution (Olsen, 1999). Esperase and Savinase T (Novo Industry), produced by alkalophilic *Bacillus* spp, are two commercial preparations with very high isolectric points (pI 11); hence, they can withstand higher pH. Due to the present awareness for energy conservation, it is desirable to use proteases that are active at lower temperatures. A combination of lipase, amylase, and cellulase enhances the performance of proteases in this application.

All commercial proteases for detergents from the early 1990's are produced by *Bacillus* strains (Eriksen, 1996). These enzymes are usable at high pH and temperatures up to 60 C. They are all relatively non-specific serine proteases related to subtilisin. Thiol proteases cannot be used as they would be oxidized by the conditions in the wash and metalloproteases would lose their metal ion ligands. These serine proteases cleave protein chains at the C-terminal of carbonyl amino acids producing small peptides which can be readily solubilized by the surfactants.

There is currently considerable interest in developing better proteases for washing powders through protein engineering, particularly in engineering oxidation-resistance into the proteases.

Proteases in the Food Industry

<u>Dairy Industry</u>

The major application of industrially produced proteases in the dairy industry is the manufacture of cheese. The milk-coagulating enzymes fall into three main categories (i) animal rennets, (ii) microbial milk coagulants, and (iii) genetically engineered chymosin. Both animal and microbial milk-coagulating proteases belong to a class of acid aspartate proteases and have a molecular weight between 30 to 40 kDa.

Rennet extracted from the fourth stomach of unweaned calves contains the highest ratio of chymosin to pepsin activity (Wigley, 1996).

A world shortage of calf rennet due to the increased demand of cheese production has intensified the search for alternative microbial coagulants. The microbial enzymes exhibit two major drawbacks, i.e., (i) the presence of high levels of nonspecific and heat-stable proteases, which lead to the development of bitterness in cheese after storage; and (ii) a poor yield (Olsen, 1999). Extensive research in this area has resulted in the production of enzymes that are completely inactivated at normal pasteurization temperatures and contain very low levels of nonspecific proteases. In cheese-making, the primary function of proteases is to hydrolyze the specific peptide bond (the Phe105-Met106 bond) to generate para-k-casein and macropeptides. Chymosin is preferred due to its high specificity for casein, which is responsible for excellent performance in

cheesemaking. The proteases produced by GRAS (genetically regarded as safe)-cleared

microbes such as Mucor michei, Bacillus subtilis, and Endothia parasitica are gradually

replacing chymosin in cheese-making. In 1988, chymosin produced through recombinant

DNA technology was first introduced to cheese-makers for evaluation (Rao et al., 1998).

Genencor International increased the production of chymosin in *Aspergillus niger* var.

awamori to commercials levels. At present, their three recombinant chymosin products

are available and are awaiting legislative approval for use in cheese-making (Goodfrey

and West, 1996).

Baking Industry.

Wheat flour is a major component of baking processes. It contains an insoluble

protein called gluten, which determines the properties of the bakery dough. Endo- and

exoproteases from *Aspergillus oryzae* have been used to modify wheat gluten by limited

proteolysis (Chen and Hoseney, 1995). Enzymatic treatment of the dough facilitates its

handling and machining and permits the production of a wider range of products. The

addition of proteases reduces the mixing time and results in increased loaf volumes.

Bacterial proteases are used to improve the extensibility and strength of the dough.

Protein hydrolysates.

Protein hydrolysates have several applications, e.g., as constituents of dietetic and

health products, in infants formulae and clinical nutrition supplements, and as flavoring

agents. The bitter taste of protein hydrolysates is a major barrier to their use in food and

health care products. The intensity of the bitterness is proportional to the number of

hydrophobic amino acids in the hydrolysate (Olsen, 1999). The presence of a proline

residue in the center of the peptide also contributes to the bitterness. The peptidases that

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can cleave hydrophobic amino acids and proline are valuable in debittering protein hydrolysates (Pawlett and Bruce, 1996). Aminopeptidases from lactic acid bacteria are available under the trade name Debitrase. Carboxypeptidase A has a high specificity for hydrophobic amino acids and hence has a great potential for debittering. A careful combination of an endoprotease for the primary hydrolysis and an aminopeptidase for the secondary hydrolysis is required for the production of a functional hydrolysate with reduced bitterness (Lindsay, 1996).

Treatment of soy proteins with alkalis at pH 8 results in soluble hydrolysates with high solubility, good protein yield, and low bitterness. The hydrolysate is used in protein-fortified soft drinks and in the formulation of dietetic feeds (Pawlet and Bruce, 1996).

Synthesis of aspartame.

The use of aspartame as a noncaloric artificial sweetener has been approved by the Food and Drug Administration. Aspartame is a dipeptide composed of L-aspartic acid and the methyl ester of L-phenylalanine. The L-configuration of the two amino acids is responsible for the sweet taste of aspartame. Maintenance of the stereospecificity is crucial, but it adds to the cost of production by chemical methods. Enzymatic synthesis of aspartame is therefore preferred. Although proteases are generally regarded as hydrolytic enzymes, they catalyze the reverse reaction under certain kinetically controlled conditions. An immobilized preparation of thermolysin from *Bacillus thermoprotyolyticus* is used for the enzymatic synthesis of aspartame (Lindsay, 1996).

Enzymatic Tenderization of Meat

The use of the vegetable proteases papain, ficin, and bromelain for enzymatic tenderization of meat has been known for many years. These enzymes have a relatively

strong potency on muscle tissue components such as collagen and elastin. Microbial

proteases have also been shown to tenderize meat, but not to the same extent as the

vegetable proteases.

The most successful meat tenderizing enzymes are those having the ability to

hydrolyze the connective tissue proteins as well as the proteins of the muscle fibers. The

enzymes are applied to the meat by sprinkling the enzyme powder on a thin slice of meat,

dipping the meat in an enzyme solution, or injecting the enzyme solution into the meat. A

method has been developed in which the enzyme in introduced directly into the

circulatory system of the animal shortly before slaughter (Bernholdt,1975) or after

stunning the animal to cause brain death (Olsen, 1999).

Whatever method is used, the difficulty in using externally applied enzymes to

obtain an even distribution of the enzyme throughout the tissues seems to control results

of the hydrolysis reaction.

ENDOGENOUS PROTEASES IN MEAT AND SEAFOOD

Protease activity can be beneficial or deleterious in food production, processing

and preparation. An understanding of the many factors that affect enzyme activity and,

ultimately, the food quality is critical. For instance, continued activity of enzyme

postmortem in mammalian muscle can result in the degradation of meat texture. This

reaction can be desirable in red meats, because they are generally more tough in texture

and therefore must undergo this enzymatic tenderization in order to develop an

acceptable sensory quality.

But post-mortem softening of seafood (fish, mollusks and crustaceans) meats due to endogenous proteases is a serious problem. Muscle softening or mushiness can be caused by proteases endogenous to the muscle as well as by digestive or organ proteases that may seep into the muscle post-mortem. Some fish proteases are mainly activated at the moment of cooking, causing myosin degradation and subsequent textural destruction

Meat (homeotherm) Proteases

Natural meat tenderization occurs via the degradation of specific muscle proteins by endogenous proteases. As early as 24 hours after slaughter of beef, disruptions in the proteins linking the myofibrils to each other and to the sarcolemma can be observed (Taylor et al., 1995; Huff-Lonergan et al., 1996). Other changes correlated with increased tenderness include breakages within the myofibrils themselves (Ho et al., 1997). Breakages in the myofibril lead to increased fragility and fragmentation of the myofibrils (Culler et al., 1978). These breakages are associated with degradation of specific myofibrillar and cytoskeletal proteins including; titin, nebulin, desmin and troponin-T (Geesink and Koohmaraie, 1999).

Dissolution or breaks in the I-band at the position of the Z-line have been observed in conditioned meat from different species. These occur earlier in chicken (Hay et al., 1973) than in beef (Davey and Gilbert, 1969). There also appears to be a difference between the fiber types since Z-line fractures have been found to occur earlier in white than in red fibers of beef (Gann & Merkel, 1978), pork (Abbott et al., 1977) and chicken (Hay et al., 1973).

In living muscles, intracellular protein degradation is controlled, at least partly, by a number of different endogenous proteolytic systems (Goll et al., 1989). Since most of the post-mortem changes occurring in the process of meat tenderization are considered to be the result of proteolysis, proteases located inside muscle cells or cytosol can potentially be contributors to meat tenderization. It should be emphasized that the regulation of the enzymes involved in the post-mortem tenderization process is presently unclear.

Several different enzyme systems have been proposed to initiate the structural changes that are associated with meat tenderization. The lysosomal enzymes (cathepsins) have been hypothesized to play a role in postmortem proteolysis and meat tenderization because they do degrade many of the same proteins that are degraded in postmortem muscle. Many lysosomal enzymes also exhibit significant activity at pH values that are close to the pH values in post rigor meat.

Cathepsins preferentially attack myosin and actin (Gault 1992; Jiang et al., 1996). Furthermore, they can attack contractile proteins at various strategies points (Table 1) (Lawrie 1992).

Cathepsin B can rapidly degrade myosin heavy chains (Jiang et al., 1996) while cathepsin L degrades the troponins T and I, and C-protein rapidly, and degrades myosin, actin, tropomyosin, nebulin, titin, and alpha-actinin slowly. Cathepsins, especially cathepsin B and L, have pH optima which are more closely associated with the pH range (5.5~6.5) found in most postmortem skeletal muscles (Jiang et al., 1994). The fall in pH during post-mortem glycolysis weakens the walls of organelles such as lysosomes (Etherrington, 1984), which consequently causes the release of lysosomal proteases, such

as cathepsins B, H, and L (Etherrington, 1984; Jiang et al., 1994). Furthermore, when aged muscle is extended, fractures mainly appear close to the Z-lines and, though less frequently, at the junctions of A-bands and I-bands.

Calpains appear to be more important than cathepsins in typical tenderization reactions. The activity of calpain is greater in beef muscle, which show an age-dependent increase in tenderness (Koohmaraie et al., 1988), and loss of calpain activity upon prolonged storage of beef is correlated with a decrease in the tenderizing process.

Koohamaraie (1996) sustains that a protease must meet certain criteria to be considered a possible candidate for involvement in post-mortem tenderization. The protease must be endogenous to skeletal muscle cells, it must have the ability to reproduce post-mortem changes in myofibrills *in vitro*, and it must have access to myofibrils in the tissue. While μ- and m-calpains, and cathepsins D, B, H and L have all been implicated in meat tenderization (Goll et al., 1989), the calpains meet all these criteria best.

There are four observed phenomena which reveal the involvement of calpain in post-mortem muscle tenderness (Dayton et al., 1981):

1. The ultrastructural degradation of post-mortem myofibrils is quite similar to that of myofibrils treated with calpain.

2. Post-mortem myofibrillar proteins, untreated or treated with calpain have similar electrophoretical degradation patterns.

3. The Z-disk, where the calpain localized, is extremely susceptible to calpain-catalyzed hydrolysis.

4. The higher the level of calpains in muscle, the faster the rate of post-mortem tenderization.

Based on the results of numerous experiments reported by different laboratories, it can be concluded that proteolysis of key myofibrillar proteins by calpains is the underlying mechanism of meat tenderization that occurs during storage of meat at refrigerated temperatures (Goll, 1991;Koohmaraie 1994). Much evidence supports the idea that proteolysis of Z-lines by calpains causes tenderization (Jiang et al., 1991; Koohmaraie et al., 1995; Wang et al., 1993).

In spite of considerable evidence in support of the calpain proteolytic system as the underlying mechanism of post-mortem proteolysis, some doubts still exist. Some reasons for these doubts are: μ-calpain is so rapidly inactivated that it cannot account for tenderization beyond 24 to 48 h post-mortem; muscle contains twice as much calpastatin as μ-calpain activity; μ- and m-calpain are easy autolyzed.

Table 1. Endogenous Proteases Implicated with Meat Tenderization

Location	Protease	M.W	pH range	Action
Sarcoplasma	μ-calpain	110,000	6.5-7.5	Releases alpha-actitin, Z-nin
	m-calpain	110,000	6.5-7.5	Degrades desmin, connectin, nebulin, troponin T and I, tropomyosin, C- and M-proteins
Lysosome	Cathepsin B	25,000	3.5-6.5	Degrades myosin, actin, troponin T and collagen
	Cathepsin L	28,000	3.0-6.5	Degrades myosin, actin, troponin T & I, tropomyosin, alpha-actinin, and collagen
	Cathepsin D	42,000	3.0-6.0	Degrades myosin, actin, tropomyosin, troponin T & I, alpha-actinin, and collagen

Seafood (poikliotherm) Proteases

In avian and mammalian muscle, resolution of rigor is normally a rather slow process which therefore extends the postmortem aging period required for meat tenderization. On the other hand, fish muscle generally undergo very rapid softening rendering the flesh unappealing to consumers. The differences in postmortem quality changes in muscle tissues from different sources may be related to a number of factors. Whereas muscle fibers in mammals and birds are very long and interconnected by connective tissues, fish muscle contains cells which are only one layer deep and connected to each other through heat-labile connective tissue. Fish tissues therefore do not have high tensile strength due to the lack of interconnecting cells in the longitudinal direction (Hultin, 1985). Further, the higher level of some endogenous proteases in fish tissues and the relatively higher specific activities of fish proteases compared to mammalian proteases would enhance proteolysis of fish tissue (Hultin 1985; Simpson and Haard 1987).

It should be added that tissue proteases may also originate from other sources, such as those in the muscle of Pacific hake infected by *Myxosporidian* parasites and the gut (digestive enzymes) or other organ tissues.

An important example of the deleterious effect of endogenous proteases is the gel weakening phenomenon observed during cooking of surimi. Surimi is a washed, minced fish muscle which forms a thermo-irreversible elastic gel upon heating. The gel-forming ability, bland taste and color of this product has made it possible to use it as a functional

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ingredient for many different food products. For instance, surimi has been largely used as a main ingredient in formulating seafood analog products such as crab legs, scallops and shrimp. In surimi gelation, myosin plays an important role in forming the gel matrix in final products. When Pacific whiting surimi was tested with slow cooking by incubation at 60°C for 30 min prior to heat-setting at 90°C, most myosin heavy chain was degraded, and surimi did not undergo gelation even with heat setting. The resultant gel strength was nondetectable due to proteolysis of myosin.

There are many types of proteases found in fish muscle tissue. Cysteine, serine and metallo proteases are the main group of endogenous proteases found in fish muscle. Aspartyl proteases are present mainly in gastric digestive organs and are active in acid conditions (Gilberg, 1988). Their activities are controlled by specific endogenous inhibitors, activators, pH, and temperature of the environment. However, the proteolytic activity of fish and shellfish muscle varies greatly among species and with the harvesting season, gender maturation, spawning, and other variables.

CATHEPSINS IN SEAFOODS

Lysosomes are known to harbor about 13 cathepsins which play key roles in the protein turnover *in vivo* and in the postmortem rheological changes of fish muscle. Among these lysosomal enzymes, cathepsins B, C, D, H, L, and S have been purified and characterized from fish and shellfish muscles and are the major proteases which participate in intracellular protein breakdown (Yamashita and Konagaya, 1990). Although cathepsins have activity for synthetic substrates at neutral pH, in living muscle,

the cell protects itself from this enormous hydrolytic potential since these enzymes are autolyzed or denatured in the physiological condition of the cytosol and they are also controlled by specific inhibitors in the cytosol and the extracellular space. Zeece and Katoh (1989) have reported that lysosomal membranes may lose their integrity under postmortem conditions resulting in a release of catheptic enzymes into the sarcoplasm. The fragility of the lysosomal membrane may also be enhanced upon physical abuse or upon freezing and thawing of postmortem muscle (Kolodziejska and Sikorski, 1996).

Although the muscle cathepsins generally are most active at pH 3-4, some of them retain fairly high activity up to pH 7.0. The activity of several cathepsins is, however, only negligible at low temperature. Cathepsin D could have some activity in the lowest pH range prevailing postmortem in some fish. However, it is still uncertain whether it can be regarded as a very significant factor in softening of refrigerated fish of most species. Aoki et al. (2000) detected cathepsin D activity in red or white muscle among 24 species, and no difference was seen between red- and white-flesh fish, or freshwater. This result suggested that cathepsin D was not responsible for species differences in postmortem tissue degradation. Makinodan (1996) also reported that cathepsin D did not relate to postmortem tissue degradation due to its low optimum pH. Porter et al.(1996) found extremely low activity of cathepsin D in four species Pacific whiting, arrowtooth flounder, Alaska pollack and Pacific cod.

Comparison of cathepsin activities in muscle tissues from fish species like cod, herring, sole, flounder, trout, and carp with the enzymes from mammalian muscles (e.g., rat, pig, beef, and rabbit) indicated that fish muscle contained about ten times as much as cathepsin activity as mammalian muscle (Siebert and Schmitt, 1965). It is noteworthy

that the level of proteases in muscle and other tissues is strongly influenced by several factors including the physiological state of the animal at the time of capture. During spawning migration, chum and sockeye salmon muscle were shown to have elevated levels of proteolytic activity. Cathepsins B, D, H, and L activities were 3-7 times higher in fish in spawning migration than those in feeding migration, while the activities of metabolic enzymes and the fat and protein contents decreased (Mommsen et al., 1980). In salmon muscle, elevated levels of cathepsins are considered to play an important role in the physiological changes occurring along with sexual maturation in spawning migration (Yamashita and Konagaya, 1990). Other factors that may contribute to variations in the level of cathepsin activity include: (1) the part of the fish tissue from which the muscle was taken. Muscle from the tail portion reportedly shows about twice as much activity as abdominal muscle while muscle taken from the back (near the main fin) shows intermediate activity (Siebert, 1973); and (2) the growth stage of the muscle, since small and medium size fish tend to have higher activities than large fish (Makinodan et al., 1984). These differences in levels of proteases may have a physiological significance since the turnover rate of body proteins in small and medium sized fish is generally higher than large fish.

Based on a series of studies on autolysis of chum salmon muscle, Yamashita and Konagaya (1992) suggested that cathepsin L is the main enzyme responsible for softening of the flesh even though its effect may be augmented by the activities of other cathepsins like D and E.

The most detrimental effect of autolysis was found at 55°C during heating of Pacific whiting muscle (Morrisey et al., 1993; An et al., 1994), and degradation of the

myofibrillar proteins by cathepsin L was also most severe at this temperature (approximately 90% of myosin molecules were hydrolyzed within 5 min) (Weerashinghe et al., 1994; ChangLee et al.1989). Protease activity in the muscle was reduced to less than 3% of the original activity by using the specific inhibitor of cysteine proteases, E64, indicating that the resident protease complex is mainly made up of cysteine proteases. Although numerous cysteine proteases, including cathepsins B, H and L exist in fish muscle, only cathepsin L activity was detected in surimi. The activity was found to be only 15% of the original level in fish muscle, since the washing step eliminated a large portion of the protease from the muscle (Morrisey et al., 1995). This remaining activity, however, was enough to rapidly degrade myosin and alter the rheological properties of final products during the subsequent heat process.

In Pacific whiting surimi and arrowtooth flounder muscle, the reduction of gel strength or softening of muscle tissue was attributed to degradation of myofibrillar components like myosin, actin, beta-tropomyosin/troponin T, and collagen by cathepsin L which was maximally active at 55 C (An et al., 1994). In the same report, An et al. (1994) also noted that while cathepsin L was the most active enzyme in surimi, cathepsin B was predominant in the fillets with cathepsin H showing less activity. This indicated that cathepsin L may be the principal protease contributing to texture deterioration during conventional slow cooking, while texture degradation at lower temperatures may be attributed to cathepsin B activity.

In addition to hydrolyzing myofibrillar proteins, cathepsin L was reported to have high activity against various collagens and elastin. Thus, it is presumed to cause partial disintegration of the original extracellular matrix structure, which may play an important

role in tissue softening of fish. Cathepsin L has also been associated with the production of a jelly- like softening of flounder (Toyohara et al., 1993 a) and the uncontrollable softening of Pacific hake muscle which has been parasitized by *Myxosporidia* (Toyohara et al., 1993b).

Another lysosomal cathepsin that has been recently hypothesized to be involved in softening of Arrowtooth flounder is cathepsin S (Visessanguan et al., 2001). This enzyme differs from all other lysosomal cysteine proteases in its activity and stability at neutral pH values (Bromme et al., 1993). Cathepsins B and L are optimally active under slightly acidic conditions, typically in the range of pH 5-6, and they do not have activity at pH 7.0. Moreover, purified cathepsin S hydrolyzes myosin heavy chain as well as elastin and collagen in the range of pH 5.0-8.0. The property seems to be extremely important to postmortem autolysis of fish muscle protein.

CALPAINS IN SEAFOODS

Calpains have been associated with accelerated postmortem fish and shellfish muscle autolysis. Most calpains are active at physiological pH, so it is reasonable to suspect their importance in fish softening during chilled storage.

Calpains make specific cleavages in proteins and therefore cause only limited proteolysis. But this enhances the susceptibility of the proteins to cytosolic proteases or to lysosomal proteases (Cottin et al., 1994) and increases the bulk degradation by these latter proteases. Therefore, calpains have been involved in the initiation of the muscular protein turnover by the disassembly of striated muscle myofibrils into fragments.

Studies have shown that in crustacean muscle, calpains are associated with molt-induced textural changes to the muscle and carry out non-specific generalized digestion of the myofibrillar proteins. However, vertebrate muscle calpains have been shown to be very specific, digesting primarily tropinin-T, desmin, titin and nebulin, attacking neither vertebrate actin or myosin (Koohmaraie, 1992). In contrast, fish calpains digest myosin (specifically the myosin heavy chain) to form an initial fragment with approximate molecular weight of 150 kDa (Muramoto et al., 1989). The same authors demonstrated that fish calpains were far more active at low temperatures than were mammalian calpains and that the rates of cleavage were species-specific, being most active against myosins having the lowest heat-stability. Thus, fish species adapted to colder environmental temperatures are more susceptible to calpain autolysis than those from tropical waters.

These proteases can cleave specific substrates and disrupt precise connections between cell compartments (Taylor et al., 1997). Their activities, optimum above pH 7, are time limited by autolysis (Melloni et al., 1992) and pH drop (Martinez, 1997) and thus restrained to primarily the first 24 hours postmortem (Papa et al., 1996).

Dystrophin, a subsarcolemmal actin-binding protein (ABP) located in costameric structures, ensures a link between the actin cytoskeleton and the extracellular matrix through an association with a glycoprotein complex. Papa et al.(1997) showed that dystrophin constitutes a pertinent indicator of the early proteolytic process as it is highly sensitive to calpain action. Kinetic analysis showed that 60% of dystrophin was cleaved in sea bass white muscle during the first 24 hours postmortem, and total disappearance was observed after 2 days of storage at 4 C. Moreover, these degradation kinetics were

shown to be species dependent (Kubota et al., 1996; Papa et al., 1996), and presumably related to the protease inhibitor system of the tissue (Barett et al., 1986).

Several studies point out that lipids have an important effect on calpain-membrane interactions (Melloni et al., 1996), calpain autoactivation (Elce et al., 1997), and *in vivo* sensitization to lower Ca^{+2} concentrations (Sadio et al., 1994). Bonnal et al. (2001) showed that proteolysis is strongly influenced by the muscular lipid content. In particular, comparison between low-fat diets and high-fat diets used during sea bass farming revealed a faster proteolysis rate during the first 8 hours of storage at 0 C with the high-fat diet. The authors explain that the accumulation of lipid inclusions and phospholipid micelles developing due to a fatty diet and retention of these lipids by the cytoskeletal meshwork could lead to intense translocations or activation of calpains. This would support an easier and faster degradation of the cortical cytoskeleton.

Although calpain has been identified in several fish species, including carp (Toyohara et al., 1985), tilapia and shrimp (Wang et al., 1993), as well as in tuna, croaker, red seabream and trout (Muramoto et al., 1989), little work has to date demonstrated a "cause and effect" relationship between calpain activity and instrumental measurements of texture.

METALLOPROTEASES IN SEAFOODS

Collagenolyic activity was only recently reported for fish muscle tissues, occurring in the skeletal muscle of winter flounder and the Pacific rockfish. The flounder collagenase fraction was shown to be optimally active at pH 7.5 and 40C, and was

inactivated after incubation at 65C for 30 min (Teruel and Simpson, 1995). Pacific

rockfish muscle tissue was shown to have two heat stable collagenases with molecular

weights of 47kDa and 95kDa (Bracho and Haard, 1995).

Most metalloproteases have zinc as the active metal in the catalytic site. In

mammals, metalloproteases are synthesized and secreted by connective tissues. They

degrade different kinds of collagen and other connective tissue matrix macromolecules.

These proteases are important in the turnover of the connective tissue matrix and in the

abnormal destruction of tissue proteins in some diseases.

The collagen structures may be degraded in a concerted action of different

enzymes. Initially undenatured molecules can be attacked by collagenases. The collagen

thus fragmented in specific sites can be further hydrolyzed by other proteins. The

hydrolytic changes of collagen and of other extracellulalr matrix proteins are probably to

some extent catalyzed by the heat-stable metalloproteases, identified in Pacific rockfish

muscle. Degradation at the interface between the connective tissue of the myocommata

and the muscle cell precedes any significant structural alterations within the muscle fiber

itself. Scanning electron microscopy of ice-chilled whole freshwater prawn has shown a

gradual disintegration of collagenous structures: perimysium and endomysium leading to

separation of the muscle fibers. Mizuta et al.(1991) suggested that enzymatic degradation

of nonhelical domains in collagen of the perimysium and endomysium may contribute to

the decrease in the penetration resistance of the muscle of kumera prawn stored for 24

hours at 5C.

The weakening of fish muscle in early hours postmortem at abuse temperature

results not only from enzymatic breakdown of some cytoskeleton proteins but also of

connective tissues that are responsible for the integrity of the muscle. The collagenous structures hold together the main components of the fish muscle by forming a fibrous network surrounding each muscle fiber and linking it to the myocommata. Enzymatic hydrolysis of these connective tissue structures results in breakdown of the muscle integrity and in rheological changes that decrease the suitability of the fish for different forms of utilization. In fish, type I, II, V and XI collagens have been identified so far. In the intramuscular connective tissue of fish, type I and V collagens are well-known as major and minor fibrillar components, respectively. Disintegration of the pericellular connective tissue of fish muscle was histologically observed in the spawning stage of ayu. It was also reported that before the spawning stage of ayu, the activities of various proteases had risen in its muscle tissue with a coincidental decrease in those of protease inhibitors. These results indicated that this disintegration is presumably due to degradation of the fibrillar collagens by MMPs.

Bracho and Haard (1995) identified in the skeletal muscle of Pacific rockfish two metalloproteases of 47 and 98 kDa. The activity of these enzymes was highest at pH 7-8. They were activated by calcium. However, there has been no report of fish MMPs capable of degrading type I collagen so far.

To this point, all of the *post mortem* autolytic changes described have involved changes within the muscle cell per se. However, the flesh of teleost fish is divided into blocks of muscle cells separated into "flakes" or myotomes by connective tissue called myocommata. Each muscle cell or fibre is surrounded with connective tissue which attaches to the myocommata at the ends of the cells by means of fine collagenous fibrils. During chilled storage, these fibrils deteriorate (Bremner and Hallett, 1985). More

recently, it was shown that instrumental measurements of texture of chilled trout muscle decreased as the amount of type V collagen was solubilized, presumably due to the action of autolytic collagenase enzymes (Sato et al., 1991). It is these enzymes which presumably cause "gaping" or breakdown of the myotome during long-term storage on ice or short term storage at high temperature. For Atlantic cod, upon reaching 17°C, gaping is inevitable presumably because of degradation of the connective tissue and rapid shortening of the muscle due to high temperature *rigor*.

The relatively short shelf life of chilled prawns due to softening of the tissue has also been shown to be due to the presence of collagenase enzymes (Nip et al., 1985). The source of the collagenase enzymes in prawn is thought to be the hepatopancreas (digestive organ).

PROTEASE INHIBITORS for SEAFOODS

Inhibitor additives have been widely used to control protease activity which occurs during heat processing of surimi. The most commonly used foodgrade inhibitors used are dried beef plasma protein (BPP), egg white, milk whey and a white potato extract. These additives exert various degrees of inhibition towards the proteases responsible for weak gelation of surimi. Among foodgrade inhibitors used in surimi, BPP has proven to be the most effective in both inhibiting most types of proteolytic activity and in simultaneously enhancing the gel strength of surimi. Although the mode of action of this additive has not yet been clearly elucidated, it is generally believed that α_2-macroglobulin (α_2-M) a unique, broad spectrum inhibitor (of all four classes of proteases)

and kininogen, a cysteine protease-specific inhibitor are the main inhibitors of beef plasma. The use of this protease inhibitor at 1% (w/w) concentration was shown to inhibit 78% of proteolytic activity in Pacific whiting surimi (Morrisey et al., 1993).

Egg white contains of at least two protease inhibitors, cystatin and ovomucoid. In general, these protease inhibitors have three complex forming domains which react with trypsin-like and chymotrypsin-like enzymes independently. Although food grade protease inhibitors have been widely used in surimi production, unwanted side effects have been noticed, including modified color and/or taste not inherent to surimi (Akazawa et al., 1993). The limitations of commercially available protease inhibitors has prompted effort to find alternative sources of inhibitors for surimi processing. Rice, fish blood, tomato leaves and legume seed meals have been under study as sources of cystatins.

Izquierdo-Pulido et al.(1994) partially purified OC-I from rice bran and reported that 0.14 IU of isolated OC-I per mg protein inhibited amidase activity of arrowtooth flounder by approximately 50%. Oryzacystatins isolated from different rice cultivars showed 5 to 10-fold higher protease inhibitory efficiency than bovine plasma proteins (on a weight basis) and prevented gel weakening during cooking of surimi. Application of methyl jasmonate to tomato resulted in massive accumulation of an 88-kDa cystatin (Wu and Haard, 1998). A protease inhibitor-containing extract of tomato applied to Pacific whiting surimi was highly efficient in inhibiting autolytic activity during cooking.In parallel Garcia-Carreno et al. (1996) tested various types of legume seed extracts for protease inhibition of Pacific whiting and *Merluza*, and reported that 6 out of 12 legume seed extracts tested were capable of reducing azocaseinolytic activities in fish extracts by more than 50%.

Cysteine protease inhibitors are widely distributed in nature, but their natural levels are rather low. For example, a large amount of fresh blood was required to obtain enough inhibitor protein for the characterization of human stefin A (Brzin et al., 1983) or pig L-kininogen (Lee et al., 2000). Likewise, kilogram amounts of rice seeds yielded only a microgram of oryzacystatin (Abe et al., 1987). Accordingly, it is rather difficult and time-consuming to isolate cysteine protease inhibitors directly from natural sources. A better method to obtain large amounts of purified inhibitors would seem to be via recombinant bacterial expression.

Using approved microorganisms as a major source of enzyme (inhibitor) production, this approach presents several advantages, such as controlled and uniform composition (and thus predictable safety) of the inhibitor, enhanced purity for greater effectiveness at lower use levels (thus negligible organoleptic effects), lowered allergency, and possibly cleaner labeling. In addition, the production system can easily use different types of inhibitor sources (genes) and/or microbial or plant hosts for continued yield improvement, as well as improved safety.

Abrahamson (1994) reported that human cystatin C was expressed from E. coli with relatively high yield and fully active inhibition against papain or cathepsin B. With regard to soy protease inhibitors, three inhibitors genes (L1, R1, and N2) were inserted into E. coli to express these target proteins. Among the three, both R1 and N2 showed more inhibitory activity than E-64 against western corn rootworm gut proteases. The inhibitors (12 kDa) were also reported to lose activity after heating at 100C for 15 min. This suggested that such inhibitors might be able to neutralize acid protease activity in surimi during cooking.

Kang and Lanier (1999) reported that the crude supernantant obtained by sonication of the soy-gene-cloned E. coli produced 120 times higher inhibitory activity (per gram of protein) than that of BBP against protease purified from Pacific whiting fillets. The crude soy recombinant inhibitor was able to produce Pacific whiting surimi gels of the same strength (stress value) using 10 times lesser quantity than BPP with no off-color and off-flavor problems.

Even though cystatins are useful to protect the gel strength of fish muscle during cooking, cystatin C can be inactivated by hydrolysis of the hydrophobic sites with cathepsin D, an aspartyl protease. A recombinant, glycosylated cystatin with a polymannosyl chain was shown to protect surimi from gel weakening efficiently. The recombinant cystatin is more thermostable and the gel strength of cooked herring surimi was greater than surimi gels prepared with the unglycosylated control. The suceptibility of the glycosylated cystatin to hydrolysis by cathepsin D was lower than the wild type. Thus, the glycosylation of cystatin appears to help in the conformational stability of the inhibitor against heating and proteolysis.

However, most of these recombinant cystatins were an insoluble form of inclusion bodies not accurately expressed in the bacteria cytoplasm. Solubilization of these inclusion bodies would substantially increase the running cost and limit their application. The other common problem has been the low level of expression of recombinant cystatins, despite the use of a variety of vectors (Fong et al., 1989; Kaji et al., 1990) and chemically synthesized genes, in which the codons are optimized for the bacteria.

To achieve high-level expression as well as a soluble form of recombinant cystatin, Chen et al. (2000, 2001) cloned cDNA encoding chicken cystatin into P. pastoris

X-33 expression host, a methylotrophic yeast. A large quantity of activity cystatin the soluble form was expressed. The inhibitory ability of the recombinant chicken cystatin against papain and cathepsin B and L appeared to be comparable with that of wild-type chicken cystatin. The recombinant chicken cystatin was also employed to inhibit the disintegration of mackerel surimi. The degradation of protein gels of surimi was substantially inhibited, and the gel strength of surimi proteins was much improved. The presence of recombinant cystatin (10 units/g of mince) resulted in a 40% improvement in deformation.

These results suggested that such a recombinant cystatin could inhibit the gel softening of mackerel surimi and would have high potential for use in improving seafood quality.

Cystatin inhibitor also has the potential to be diffused via injection into intact fish fillets, which have been undervalued thus far (particularly arrowtooth flounder, menhaden, and white croaker) because of their propensity to soften during oven (slow) cooking.

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Diffusivity Within the Cell: The Nature of Cell Water

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Introduction

Water is the major molecular constituent of cells, comprising about 70-80% of the cell weight (Lodish et al., 2000). Within this cellular water all diffusion-controlled reactions and solute transport take place.

The literature on the nature of cellular water is very controversial. Nearly every imaginable claim and counter claim can be found in print by reputable investigators. Most prevalent, however, has been the simplifying assumption that intracellular water is chemically and physically equivalent to the water in bulk solutions. This assumption has been used to investigate and describe a wide variety of cellular activities such as solute transport, enzyme activity, regulation of intermediary metabolism and assemble-disassembly of cellular components. It is surprising that such a paradigm still exists since, to our knowledge, no direct evidence has ever supported this view, and much has been published against it.

A more probable explanation is that most reactions in the cell occur not in solution but at interfaces (Castner & Ratner, 2002).

Consideration of Cell Architecture

The cell is the smallest unit of life capable of independently sustaining and reproducing itself. What characterizes a cell is that it has a delimiting wall or membrane separating it from its environment. Eukariotic cells range in size from about 5 to 100 μm,

whereas prokaryotic bacteria are in the range of 1-2 μm. The volume of an average mammalian cell is approximately 4 picoliter (Albert et al., 1994) within which the internal organelles occupy up to half the volume. The small, finite volume of cells and intracellular compartments means that the physiologically relevant space may be surprisingly small. The total concentration of macromolecules inside cells is very high, with proteins by far the most abundant species. As reviewed by Fulton (1982), the protein content of cells range 17 to 35% by weight. A 20% solution of protein is very crowded and significant interactions between solute molecules can be expected. How crowded a milieu the cytoplasm is can be appreciated visually from the drawings of Goodsell (1991), who has depicted the cell interior with macromolecular components at the correct concentration and drawn to scale (Figure 1).

Most of the proteins form a permeated three dimensional network of filaments known collectively as the cytoskeleton which intersects the cytoplasmic region outside the organelles.

These skeletal elements include at least three distinct filamentous components known as the microfilaments, the microtubules, and the intermediate filaments. The microfilaments are homopolymers , 8 nm in diameter, of the protein actin.

Actin is therefore a major component of the cytoskeleton network in cells. In eukaryotic cells, actin is found at high concentrations of about 4 mg/ml from which half is polymerized into filamentous microstructures (Bray and Thomas, 1975). Of course muscle cells contain an even higher content of filamentous actin, which with fibrillar myosin constitutes the bulk of the contractile system of muscle.

Although the three types of cytoskeletal filaments are often treated as independent systems, a mounting body of evidence supports the idea that they are physically interconnected.

The cytoplasm also present a significant surface area. Estimates of the surface area of the cytoskeleton have been carried out by image analysis (Gershon et al., 1986), computing an area about 100,000 μm^2 for mammalian cells. The high concentration of macromolecules inside the cell, and the large intracellular surface area presented by proteins, raises the question of whether a significant proportion of total cellular water differs from the bulk. Clegg (1987) maintains that the vast surface area of the cytoplasm could structure at least 50% of the total cell water, as this portion of the water was calculated to be within 50 Å from some protein surface. As we will see, the filamentous proteins could especially be important to water structuring.

Water association

Water is a complex fluid having unusual physical properties, such as a high boiling point, a high melting point, a high specific heat, a strong surface tension and the reluctance to dissolve non-polar solutes (Nakasako, 2001). The main causes for these unusual properties is water self-association. Each water molecule contains two donors, protons, and two acceptors, the two unshared pairs of electron of oxygen. All these four charges are located along the four arms of a tetrahedron (Albert et al., 1994).

When one of the protons comes close to one of the unshared pairs of electrons, the electrical attraction between them can result in a weak bond called hydrogen bound. Therefore, water is able to form hydrogen bounds with up to four neighbors.

Water exist in three commonly known phases (vapor, liquid and solid) which differ in hydrogen bound association. In the vapor phase thermal energy is high and molecules undergo incessant random motion. In the liquid state randomness is reduced and molecules form transient hydrogen bonds with one another. In the solid phase the four hydrogen bonds effectively immobilize each water molecule.

Lying somewhere between ordinary liquid water and ice is the so-called structured or interfacial water. In this discussion, the term "interface" includes the surface of a relatively immobile biomacromolecule as well as that of a solid. The water molecules at an interface experience different local interactions compared to the bulk water molecules that are distant from the interface. The basic reason for the difference is readily understood. A water molecule at an interface experiences, on one "side," other water molecules to which it potentially can form hydrogen bonds with the characteristic tetrahedral angles. On its other "side," the water molecule experiences the molecular structure of substrate material that has different geometrical characteristics. Consequently the interactions of water molecules with this material are different from those with the neighboring water molecules on its former side. The different interactions lead to a difference in the motions as well as the arrangement and bonding characteristics compared to a bulk water molecule. In structured water the molecules are linked, but less regularly than in ice. Because the hydrogen bonds are bent (Pauling, 1959), structured

water density is higher than of ice, allowing molecules to crowd together more closely (Garrigos, 1993; Pollack, 2001).

There are at least two types of interfacial water, depending on whether the interfacial surface is hydrophobic or hydrophilic. Hydrophobic surfaces induces to a strong self-association of water molecules or clathrate formation. That excludes association with, and solubilization of non-polar solutes (Israelachvili and Pashley, 1982).

The second type of interfacial water is the one near hydrophilic surfaces. Charged and polar elements of hydrophilic surfaces react strongly with dipolar water, which defeats water's tendency to self-associate (Vogler,1998). The reaction creates the layers of adherent water (Israelachvili and Pashley, 1982). Thus, hydrophilic surfaces will induce stratification, whereas hydrophobic surfaces will induce clathrate formation.

The cell interior contains surfaces of every conceivable ilk some water of each type is anticipated: clathrate, interfacial and bulk. On the other hand, a single macromolecular species dominates the cytoplasm: protein.

Polarized Multilayer Theory

The most extensive alternative view of the nature of cell water is that formulated by Gilbert Ling about 50 years ago (Ling, 1951) called the association-induction hypothesis. Since that time he and his associates have carried out a vigorous research program to test and modify the hypothesis, the published work being so voluminous that

a complete listing of papers would be prohibitive. Some key references are Ling (1962, 1984, 1992).

Ling maintained that virtually all cell water assumes a dynamic structure different from that of normal liquid water. This departure originates from interaction of cellular water with a matrix of fully-extended protein chains. The carboxyl (CO) and imino (NH) groups of these fully extended proteins offer properly spaced, alternating negatively and positively-charged sites. Together, these charged sites adsorb a layer of (oppositely-oriented) water molecules or dipoles. The water molecules thus oriented and polarized in turn polarize and orient a second layer of water molecules and this continues until virtually all cell water is oriented and polarized (Figure 2).

Ling based the polarized multilayer (PM) theory on the earlier studies of deBoer and Zwikker (1929) and Bradley (1936), which described multilayer adsorption of polar gases on appropriately charged surfaces, and on the dipole nature of water. The idea that charged surfaces attract dipoles had been very controversial, but has been given support by several critical observations.

In recent years three types of observations have given strong support to the PM theory. The first is that polished quartz surfaces placed in a humid atmosphere will adsorb films of water up to 600 molecules thick (Pashley & Kitchener, 1979). The second important set of observations are those of Israelachvili and colleagues (Israelachvili and McGuiggan, 1988; Israelachvili and Wennestron, 1997) who measured the force required to displace solvents sandwiched between parallel mica surfaces. They observed that the closer the surfaces approached, the higher was the force required to separate them. The overall behavior was largely classical, but the force separation relation was not purely

monotonic; that is, the force did not decrease directly as a function of the intervening

distance. Superimposed on the anticipated monotonic response was a series of regularly

spaced peaks and valleys (Figure 3). The spacing between peaks was equal to the

diameter of the sandwiched molecules. This correspondence held no matter what fluid

was involved. Thus, the force oscillations appeared to be in some way related to a

layering of molecules between surfaces.

      The Israelachvili experiments confirmed molecular layering near charged surfaces

while the Pashley & Kitchener experiments implied that many such layers of dipolar

water can adhere to one another to create multilayers (Pollack, 2001). When two

polymeric surfaces are in close proximity to one another, water in the intervening space

thus acts to bond these surfaces together. Israelachvili points out that this bound water

layer has little to do with water self-association interactions at charged surfaces but rather

is completely dominated by surface-water interactions (Israelachvili and Wennerstrom,

1996). It is this water-surface interaction that deprives bound-water molecules from a

nearest-neighbor hydrogen-bonded association (Toney et al., 1994).

      Recent experiments using a carbon nanotube tipped AFM penetrating hydrated

ionized domains (surfaces) showed similar layering of water molecules (Jarvis et al.,

2000;Cho and Sigmund, 2002). The force increased as the nanotube needle penetrated

through the successive water layers. Penetration through each successive layer was

signaled by a rise and then a sharp fall in the force.

      Ling conjectured that when water assumes the dynamic structure of polarized

multilayers, its physicochemical properties would be expected to differ from these of

normal liquid water. In essence water suffers motional (rotational and translational) restrictions.

Ling attempted to capture the physicochemical properties of the polarized multilayer water on the reduction in solvency of various solutes via calculation of the equilibrium distribution coefficient, q, which is directly related to the partition ratio:

$$q = [S]_{in}/[S]_{ex}$$

where $[S]_{in}$ and $[S]_{ex}$ are the equilibrium solute concentrations in the cell or model (usually a protein-containing dialysis bag) and in the external bathing solution, respectively. A plot of $[S]_{in}$ against $[S]_{ex}$ yields a straight line with a slope equal to q.

The equilibrium distribution coefficient, q, varies with each solute (S) according to its size and as a function of the physical state of the solvent water (Ling, 1993; Mentre, 2001). In the case where structuring of water occurs, q would assume a value of less than unity. This would infer that the structured aqueous phase was less solvent for a given solute than a bulk aqueous phase. This solute would therefore be partially excluded from the more structured water, which is organized in space and time such that its entropy is less than that of pure bulk water (Ling, 1993; Hazlewood, 1995).

Thermodynamic Description of PM theory

Solute distribution within structured water, such as exists in cells or select dialysis model systems, versus that in normal liquid water is a special case of solute distribution between two different solvents (Tong et al., 1996).

The partition or q-value of a solute is an expression of an equilibrium phenomena. As such, the q-value of the solute is determined by the standard free energy difference (ΔG) of the solute in the two phases. The energy change in transferring a solute from the external normal aqueous medium to inside a living cell or dialysis bag containing polarized water involves two components, a volume component and a surface component:

(i) Volume component: Since water molecules in the state of polarized multilayers are held together more strongly due to the mutual polarization, the energy needed to excavate a hole in the polarized water is greater than the energy gained in filling the hole left behind in the external normal liquid water. Furthermore, the larger the probe molecule, the larger the size of the excavated hole; the larger the excavated hole, the greater the difference between the energy spent and gained. The greater the energy difference, the greater the degree of exclusion of the probe molecule from the polarized water (q<1).

(ii) Surface component: If the probe molecules contain exposed polar groups of such a steric and electronic configuration that they can interact more strongly with water molecules and with more water molecules in the dynamic-polarized-water structure than in the external liquid water, this strong interaction will create an energy difference in

favor of the probe molecules being preferentially accumulated within the dialysis bag (more structured water). This results in a partition or q-value equal to or above unity (q>1). On the other hand, if the steric and electronic configurations of the exposed groups of the probe molecules do not fit, or fit less well, into the dynamic structure of polarized water in the bag than in the external medium, then the energy difference is unfavorable for the accumulation of the solute inside the dialysis bag. This drives the partition or q-value toward below unity.

The entropy change involved in moving a solute from the external normal liquid water to the phase containing polarized water reflects primarily the alteration in the degree of motional freedom of the solute, translational and rotational. Since water molecules in the state of polarized multilayers are more tightly held to immediately-neighboring water molecules, and directly or indirectly immobilized through intervening polarized water molecules to the fixed negative and positive sites, the solute molecules are subject to greater translation and rotational motional restriction in polarized water than in normal liquid water. Again, the larger the probe molecules, and the more complex the structure, the greater is the loss of entropy, especially rotational entropy.

Model (Protein) Studies

Equilibrium dialysis was used to test the hypothesis that fully extended proteins more effectively reduce the solvency of bulk-phase water for probe molecules and ions (Ling et al.,1980 and Ling and Ochsenfled,1983). A protein solution in a dialysis bag was

immersed in a solution containing a high concentration of radioactively-labeled $NaSO_4$ at 25°C. After diffusion equilibrium had been reached, the equilibrium concentration of Na^+ in the bag, and that the external solution, were determined and their ratio calculated and expressed mathematically by equation (1). Table 1 shows the partition values of labeled Na^+ in solutions of native proteins at 20% concentration after equilibrium dialysis in 1.5M Na-labeled Na_2SO_4 solution at neutral pH. Eleven native globular proteins had minimal effects on the solvency of water; in all cases, the partition values for Na^+ were close to unity, indicating unaltered, or weakly-altered solvency of the bulk water. In sharp contrast, gelatin effectively reduced the solvency of water for Na^+ (partition value < 1).

Ling explained that the great difference in water solvency between gelatin and the native globular proteins was due to the unique amino acid composition of gelatin/collagen. This consists of monotonically repeating triplets of the sequence Gly-X-Y, where X is often proline and Y is often hydroxyproline. Neither of these residues carries a proton on its backbone of N atoms, and hence cannot form hydrogen bonds required to hold together α-helical or β-pleated-sheet conformation (Voet and Voet, 1990). Further, glycine constitutes the largest percentage (33%) of the amino acid residues of gelatin. Glycine is one of the strongest "helix breakers", therefore the NH and CO groups closest to a glycine residue in a protein have little tendency to form α-helical structures. In native collagen, some interchain hydrogen bonds are formed among the 3 intertwining collagen chains; after denaturation, these interchain hydrogen bonds are broken. When dissolved in water, gelatin thus contains large segments of its polypeptide chains in the fully extended state. This gives gelatin the ability to polarize or structure bulk water in multilayers, thus reducing solvency for Na^+ and other solutes.

The absence of tertiary-structure-stabilizing disulfide bridges (-S-S-) also enhances the fully extended conformation. In contrast, the polypeptide chains of the nine other more normal native proteins do not contain large proportions of non-helix-forming amino acid residues. As a result, their backbone NH and CO groups are largely engaged in α-helical and other intramolecular hydrogen bonds and, as such, are unable to interact with the bulk phase water. Native proteins which are without effect on the solvency can become affective after exposure to a concentrated solution of urea (or guanidine HCl). Both denaturants are well known for their ability to open up the secondary structure of native proteins, thereby transforming these folded native proteins into a fully extended conformation (Ling and Ochsenfeld, 1989).

Ling and Hu (1988) showed plots of the equilibrium concentration of various non-electrolytes in dialysis bags containing 39% native bovine hemoglobin against their concentrations in the external bathing solutions. In each case, the distribution curve was a straight line, and the slopes of the straight lines, which equals the q-value of that solute in the water in the bags, did not deviate much one from another. In a similar plot of the same set of non-electrolytes in 18% NaOH-denatured bovine hemoglobin, the distribution curves were straight lines also. However in this case the slopes, or q-values were widely different.

When the two sets of q-values were plotted against the molecular weights of the non-electrolytes, Figure 4 was obtained. Note that here the q-values of all nonelectrolytes in 39% native hemoglobin are close to one, indicating that water in the 39% native hemoglobin solutions has solvency close to that of normal liquid water for all the solutes

listed. In contrast, most of the q-values from the NaOH-denatured hemoglobin solutions decrease steadily with increasing molecular weight.

The minimal effect of native hemoglobin on the solvency of all the non-electrolytes studied confirmed that proteins with the backbone NH and CO groups locked in alpha-helical and other intramacromolecular H bonds do not react, or react weakly with the bulk phase water. As a result the solvency of water in the 39% native hemoglobin solution was not different from that in the dilute salt solution outside the bags, as revealed by the unchanging q-value close to unity.

In contrast, the model of NaOH-denatured hemoglobin showed pronounced change of the solvency of the bulk-phase water. In the altered water, the distribution of most of the non-electrolytes studied followed the "side rule": low q-values for small molecules with low molecular weights, all essentially along a continuous line.

As compared with extended or fibrous proteins, globular proteins may be considered to be weakly hydrated. From consideration of the hydration events observed by a variety of experimental techniques and from X-ray diffraction data of native globular proteins, it is known that water molecules do adsorb to the protein surfaces with relatively long residence times. But these bind mostly on just the backbone carbonyls (CO groups of the amide in the protein backbone), since all other adsorption sites, such as backbone NH groups, are occupied or locked into the folded secondary or tertiary structure (Careri and Peyrard, 2001). Also, the exchange rate of water molecules from the backbone carbonyl group of the protein surface to the bulk water is still quite high. Thus it can be assumed that buildup of a polarized multilayer of structured water on the

surface of globular proteins would be impeded by the more limited and widely distributed loci available for interaction with water.

Water Structuring in the Presence of Certain Solutes

Typically globular proteins are very unstable at low concentration in water solution. It is thus a common practice to stabilize globular proteins by adding solutes such as sugars and polyols at high concentration (0.5M or higher).

Over the past three decades a great body of experimental data has been generated concerning the preferential interactions of various types of solutes with various globular proteins (Timasheff 1998). The preferential interactions have been shown to depend on protein type, solvent type, solvent concentration, temperature, pH and solution composition.

Preferential interactions generally refer to the redistribution of solvent (water) in the immediate vicinity of the protein surface. The observed interactions may be neutral (unchanged concentration of solute or water at the immediate vicinity of the protein as compared to the bulk), preferential binding (excess of solute over the bulk solvent composition) or preferential exclusion (deficiency of solvent in the vicinity of the protein). The last corresponds to an excess of water; that is, to preferential hydration of the protein surface. This would seem to coincide with a structuring of water in the immediate vicinity of the protein surface, since the particular solutes involved cannot penetrate this hydration layer to interact with the protein.

Low molecular weight compounds such as sugars, glycerol, polyols (sorbitol, mannitol), some amino acids, methyl amines, and some salts which induce salting out of proteins are compounds that stabilize globular proteins by opposing any conformational change. There are two thermodynamic consequences of adding these compounds to an aqueous globular protein solution: the first is the increase of surface tension, which is identical for the addition of all solutes at identical osmolality, and is the same whether in pure water or in a protein solution.

The second consequence will be the perturbation of the chemical potential of the solute by the protein. This in turn perturbs the chemical equilibrium in the domain of the protein. To restore the chemical equilibrium, the chemical potential of the solvent in the protein domain must be changed. This can be accomplished by adjusting the concentration of the solvent in the protein domain. In the case of sugars and polyols, or any other of compounds that raise the surface tension of water, the interaction between the solvent and the protein domain will be unfavorable. As a consequence two events will take place simultaneously: some of the solute will be excluded from the immediate domain of the protein surface (Figure 5) and the water in this domain will change its physical-chemical properties, becoming more dense or more structured. The solute diffusivity in this new domain will be reduced and a partition effect will take place.

Fully extended proteins, such as gelatin or antifreeze glycoproteins, have the same physico-chemical effect on the water domain immediate to the protein surface, only this is significantly stronger (Ben, 2001).

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Cellular Studies

The first property of polarized water examined in some detail by Ling was the solvency for various solutes in muscle cells. This study offered insight into why Na^+, sugars and free amino acids are as a rule found in low concentrations in intracellular water as compared to in the surrounding medium.

Ling et al. (1993) studied intracellular-extracellular partitioning of 21 uncharged solutes (non-electrolytes) including sugars, alcohols, and other electrolytes that ranged in mass between 32 and 900 Daltons. Each solute was radio-labeled, placed in an incubating solution containing a muscle cell, and the concentration inside the muscle cell was measured during holding for up to six days. Temperature was kept at 0°C to ensure that the cell did not appreciably metabolize any of these solutes.

The main finding was that the inside to outside partition ratio diminished as the molecular size of the solute increased; i.e., exclusion from the cell was based on size. Smaller solutes entered the cell rather easily with a steady-state partition coefficient near 1.0. Larger ones encountered more difficulty, partition coefficients dropping progressively down to 0.08. Of the 21 solutes studied, 14 fell on a single curve (Figure 6). For those solutes, exclusion depended uniquely on molecular weight or, rather, on their effective solute volume. Such behavior within the cell could be explained by water structuring according to the PM theory.

Lee and Timasheff (1981) showed that most such compounds increase the surface tension of water and are totally excluded from protein surfaces. As a result several of

these, such as sucrose and some other sugars and polyols, have been commonly used as protein stabilizers.

For the remaining seven solutes the partition coefficient also increased with molecular size, but the curve shifted slightly to the right. The rightward shift implied that this class of solutes diffused through the cell relatively more easily. At least five of the seven solutes in question (ethylene glycol, glycerol, dimethyl sulfoxide, acetamide, propanediol) are also recognized stabilizers of protein structure (against the denaturing effects of heat, freezing, pressure, etc.). However, Timasheff (1998) maintained that these particular compounds actually penetrate to the protein surface (Timasheff, 1998) and he assumed that the protein hydration in solutions of these compounds is the same as in pure water. Though they preferentially bind to the protein surface, they nontheless stabilize the native protein structure because their preferential binding to the denatured protein is less than that to the native form (Timasheff, 2002).

Several workers have noted the ease with which compounds having these properties diffuse easily within the cell, and therefore concluded that cellular water is no different in structure from bulk water (Hill, 1930; Ernst, 1963). It is apparent, however, that these latter compounds diffuse within cellular water as if it more resembled bulk water due to their inclusion, rather than exclusion from the water structure.

Tracer Diffusion Studies

In recent years, tracer diffusion has been an established means of probing the architecture of complex polymer matrices and the cytoplasm.

Data are commonly presented in terms of D_w, the diffusion coefficient of the molecule in a reference buffer (aqueous solution); D_{matrix}, the diffusion coefficient in the matrix; D_{matrix} / D_w, the relative diffusion coefficient; and R_h, the hydrodynamic radius of the molecule. The relationship of D_{matrix} / D_w to R_h depends on the architecture of the matrix in question. In a Newtonian fluid, molecule diffusion simply obeys the Stokes-Einstein equation:

$$D = kT/6\pi\eta R_h,$$

where D is the diffusion coefficient and η is the fluid viscosity. Thus, if the cytoplasm were a Newtonian fluid, then D_{cell} / D_w, with D_{cell} being the diffusion coefficient in the cytoplasm, would be independent of R_h.

Luby-Phelps and coworkers have extensivly studied the diffusion of inert tracers in the cytoplasm of the Swiss 3T3 cells and protein solutions by the fluorecent recovery after photobleaching (FRAP) technique (Luby-Phelps et al., 1987). To overcome the possibility of either specific or nonspecific interactions of proteins with the intracellular structures, and to understand how the intracellular medium hindered diffusion, inert macromolecules such as dextrans and Ficolls were employed. Ficolls are highly branched molecules that approximate a hard sphere much more closely than dextrans, which are flexible, long chains that do not have a well-defined size or shape (Larm et al., 1971).

The ratios (D_{cell} / D_w) for both types of macromolecules in the cytoplasm of living cells were not constant, but strongly size dependent, confirming that cytoplasm cannot be

regarded as a simple Newtonian fluid (Figure 7a). In order to rule out the effect of a high

concentration of protein in the cytoplasm, the authors measured the diffusion coefficients

of the same tracers in concentrated solutions of globular proteins (24% ovalbumin and

26% BSA) at physiological conditions. In contrast to the diffusion of Ficoll and dextran

in cytoplasm, their diffusivity in these concentrated protein solutions did not appear to be

size dependent (Figure 7b). By this criterion, concentrated protein solutions behaved as

Newtonian fluids, albeit of much higher viscosity than water. The authors suggested that

the diffusion of tracer particles in cytoplasm is hindered by the cytoskeletal network and

associated macromolecules and organelles.

Measurements of the intracellular diffusion coefficient of small molecules

indicate that the viscosity of the aqueous portion of the cytoplasm generally ranges two-

to at most six-fold that of water (Mastro et al., 1984; Jacobson and Woijcieszyn, 1984;

Luby-Phelps et al.,1988.). Larger particles, such as proteins and dextrans, diffuse much

more slowly than could be accounted for by this relatively small increase in cytosolic

viscosity (Luby-Phelp et al., 1988).

Several recent studies using tracer diffusion in cells have found markedly reduced

diffusion coefficients compared to the values in aqueous solution and thus support

Lubby-Phelp's work (Mastro et al., 1984; Hou et al., 1990; Popov and Poo, 1992; Kao et

al,1993;Arrio-Dupont et al., 1996, 2000; Papadopulus et al., 2000). Unfortunately, all

these authors neglected to account for changes in the physical properties of the

intracellular water and thus concluded that the diffusion of tracers in the cytosol was

exclusively dominated by enhanced viscosity, steric effects or binding of the diffusing

species to elements of the cytoskeleton.

7. Water mobility measurements

To acquire more insight into the diffusional behavior of molecules in the cytoplasm, it is be desirable to account for the differing mechanisms which affect molecular mobility.

The diffusion behavior of higher molecular weight components (dextrans and Ficoll) are not good indicators of the dynamics of cell water since such diffusion is affected by several factors, such as percolation, obstructions, viscosity etc. A better approach is to measure the motional properties of the water molecule. The diffusional properties of the water molecules are directly related to the physical state of the cellular liquid and the water bath constitutes the basic surrounding environment for diffusion of solutes. Therefore, a characterization of the dynamics of the water medium can be predictive of intracellular molecular mobility in general.

Nuclear magnetic resonance (NMR) techniques have the important advantage that motion of the water molecules can be directly monitored (Stilbs et al., 1983). Pulse proton NMR methods are particularly sensitive to diffuse motions of the water molecule. In varying the delay between the applied gradient magnetic fields, the motional behavior of water can be studied on a timescale that typically varies in the millisecond to second range. NMR techniques have been extensively used to monitor water diffusion in a wide variety of cells, tissues and whole organisms (Pfeuffer et al., 1998; Vogt et al., 2000). The apparent diffusion coefficient of water found in these studies is typically two to four times lower than the corresponding diffusion constant in pure water (Table 2).

The reason for the slower diffusion of water in cells has two possible interpretations. The first is that all of the water in the cell is structured, in multiple layers emanating from the surfaces of proteins. The self-diffusion coefficient of this structured water would therefore be expected to be lower than for water molecules in the pure-bulk aqueous form. The second interpretation is that most of the water in the cell has the self-diffusion coefficient of pure water but that the slower water diffusion-rate is due to obstructions and compartments encountered by the water molecules during the measurement.

The problem with using NMR is that, even under the most optimal conditions available, a relatively long time is needed to measure diffusion over distances comparable to the dimensions of a cell. Thus the reduction of the water diffusion coefficient could reflect encounters of water molecules with physical barriers in the cell rather than altered rotational mobility, making precise interpretation of NMR data difficult (Cameron et al., 1997;Luby-Phelps, 2000).

Quasi-elastic neutron scattering (QENS) techniques have also been utilized to measure the water dynamics in the cell (Trantham et al.,1984; Rorschach et al., 1987; Clegg, 1984). QENS methods are sensitive to water dynamics that occur on a picosecond timescale. As a consequence, no time is available for the molecules to make large scale translational motions and the influence of physical encounters with other physical barriers is greatly reduced (Middenforf, 1984). Unfortunately, because the samples must be held under non-physiological conditions for several days, the use of this technique has been limited to a few hardy cell and tissue types, such as the brine shrimp Artemia and frog sartorious muscle (Rorschach et al., 1973; Trantham et al., 1984).

The data from both QENS and NMR (Table 2) reveal that the water diffusion rate in cells is less than half that of pure water. As the results from the QENS method are made in such a way as to minimize obstructions that water might encounter, the water diffusion rate provides evidence that most, if not all, of the water in these cells has water self-diffusion properties that differ from that of pure 'bulk' water.

The data in Table 2 were further evaluated by plotting the water diffusion rate versus the percentage of water for each of the systems (Figure 8). Clearly, the diffusion rate of pure water is greater than two-fold faster than in the cellular systems, as measured by either NMR or by QENS. There is also a significant positive linear regression fit (P<0.001) of the cellular systems versus the water content. The change in D as a function of water content may be due to either an increase in physical obstructions or due to the possibility that water molecules further away from the protein surface are somewhat more mobile than the water molecules that are closer to the protein surface. Extrapolation of the cellular water diffusion data to 100% water indicates a water-self-diffusion value that is roughly half that of pure water (1,300 μm^2/s). Of interest is the fact that the data from either QENS or NMR, analyzed separately, give a similar intercept value when extrapolated to 100% water. Thus both methods of analysis led to a similar conclusion: on the average, the self-diffusion coefficient of water in these living cellular systems deviates significantly from that expected of pure water. And, in the case of QENS data, this deviation is not likely to be accounted for by physical obstructions to diffusion other than to the water itself (Rorschach et al., 1987). The fact that the extrapolation to the 100% water axis on Fig. 7 still shows significantly slower water self-diffusion than bulk water indicates that most, if not all, of the water in these cells is slowed in its motion as

compared to bulk water. One explanation for slowed water motion in cells is suggested

by the observations that a large fraction of cellular water has perturbed osmotic and

motional properties (osmotically unresponsive). This can be accounted for by a few

layers (in the range of 2-8) of structured water molecules slowed in motion by about two-

fold compared to water molecules in the pure bulk state. Under this condition any cell

that contains less than 75 to 80% water by mass might be expected to have all of its water

in a state that differs from pure bulk water.

Antifreeze Proteins

A prediction of the PM hypothesis of Ling is that proteins which are organized

into repeating units which yield a more regular charge spacing should thus structure the

most water. The paradigm that comes to mind is nature's antifreeze proteins, which

presumably mediate their freeze resistance by structuring many layers of vicinal water

molecules, thereby inhibiting transition of water into the ice state (Pollack, 2002).

Biological antifreeze molecules constitute a diverse class of proteins found in

Arctic and Antarctic fish, as well as in amphibians, trees, plants, and insects. These

compounds are unique in that they have the ability to inhibit the growth of ice by

lowering the freezing point of solutions noncolligatively and consequently are essential

for the survival of organisms inhabiting environments where sub-zero temperatures are

routinely encountered (Yeh and Feeny, 1996). These antifreeze proteins are 100 times

more effective than any other type of antifreeze molecule (DeVries et al, 1970; Ben,

2001). This unusual ability is attributed only to biological antifreeze molecules (Ben, 2001).

There are two types of biological antifreeze molecules, the antifreeze proteins (AFPs) and the antifreeze glycoproteins (AFGPs). AFPs are divided into four types, each possessing a very different primary, secondary and tertiary structure. In contrast, AFGPs are subject to considerably less structural variation. A typical AFGP is composed of a repeating tripeptide unit (threonyl-alanyl-alanyl) in which the secondary hydroxy group of the threonine residue is glycosylated with the disaccharide β-D-galactosyl-(1,3)-α-D-N-acetylgalactosamine (Yeh and Feeny, 1996) (Figure 9).

The exact mechanism whereby these molecules inhibit ice crystal growth at the molecular level remains a source of intense debate. Researchers have long proposed that the binding of AFGPs to the ice surface likely involves hydrogen bonding between the polar groups of the saccharide residue (the hydroxyl groups) and the ice surface. However, studies have demonstrated that the number of potential hydrogen bonds between the antifreeze molecule and the ice surface appears to be insufficient to explain the observed tight binding of AFGPs to ice. Modeling studies have looked at all possible binding configurations, and in the best case only two hydroxy groups per disaccharide are in position to form hydrogen bonds with the ice surface (Ben, 2001).

A consistent problem with elucidating the molecular mechanism of action for AFGPs and AFPs is that the ice-water interface has not been well characterized. In fact, the interface itself is probably not an abrupt transition as typically represented in static models since the most recent evidence shows the loss of organized ice structure at the interface to be fairly gradual, occurring over approximately ten angstroms (Hayward and

Haymet, 2001). This is a problem, especially when attempting to "map" possible interactions between AFGP and the ice surface.

As a way of testing how the AFP from winter flounder aligns with the ice-crystal surface Haymet et al. (1998) prevented the hydrogen bonding (replaced polar with non-polar residues) and tested to see if this mutant protein would also inhibit ice growth. His experiments showed no change. The modified AFP also had the antifreeze effects.

To further investigate this result, with negates the hydrogen-bond theory, Dalal et al. (2001) undertook a "molecular dynamic" simulation, a computational approach which puts molecules together and sets them in motion to go about their natural biochemical arragements while recording what happens.

Most prior AFP simulations have represented the interaction of AFP molecules with ice alone, in the absence of liquid water. With computational practicality as a major consideration, excluding the water has been an acceptable approximation since it supports the hydrogen-bond theory.

Dalal et al. (2001), for the first time, represented the full ice-water interface with no imposed limit on the interactions. The first step was to allow the ice-water interface to find its natural equilibrium between solid and liquid. After this preliminary step, itself a major computation, the author included the protein and collected data from 510 picoseconds of simulated molecular dynamics, more than twice as long as prior studies. The results support Haymet's experimental findings refuting the hydrogen-bond theory, and showing that accurate simulations of this interaction depend on realistically including the ice-water interface. The authors concluded that it is vital that any alternative

hypothesis account for the fact that the proteins interacts with water molecules in between liquid and crystal form.

In terms of the PM theory, these proteins may owe their antifreeze properties to traits similar to those of gelatin: an extended repeating peptide chain and the consequential long-range polarization of water in the ice/protein interface. Considerable evidence that the antifreeze glycoproteins exist in an extended state has already been collected (Feeney et al., 1978; Yeh and Feeney, 1996). Furthermore, the antifreeze protein binding sites are relatively flat and engage a substantial proportion of the protein's surface in ice binding (Jia and Davies., 2002). The antifreeze proteins from sculpin and northern flounder appear to have high helical content; however, the high helical content may not be the state these proteins assume in their natural functional state.

Another important functional aspect of antifreeze proteins pointed out by DeVries (DeVries et al., 1970) called attention to the fact that a large volume of water affected antifreeze protein performance.

Evidence for Structuring of Water in Foods

Water is the major component of most foods, wherein the proper amount, locations, and orientation of water profoundly affect the physical, chemical and microbiological properties and thus the structure, appearance, taste and stability. However, various foods with the same water content can differ in stability and physical properties. Thus water content alone is not an adequate indicator of food stability and water organization (Fennema, 1996).

The most widely used measure of the availability of water in foods is water activity (a_w), the equilibrium vapor pressure of the product. This concept has been associated with rates of chemical reactions, physical changes and microbial growth. Its importance for predicting food stability has been widely accepted although it has been recognized that a_w is not always a reliable predictor. A_w, being an equilibrium dynamic descriptor, is quite inappropriate in many product situations because the measured physicochemical properties are time-dependent. The measured a_w relies on the existence of a liquid/vapor dynamic equilibrium, defined by equal rates of evaporation and condensation. Such an equilibrium is assumed to be re-established rapidly when one of the experimental variables (temperature, composition) is changed. In liquid systems, this assumption is always valid, because the diffusion rates of water molecules are high compared to the time scale of the thermodynamic measurement (vapor pressure) (Frank, 1991).

The situation is completely different when the substrate is a more solid food such as meats or gel systems. For such complex foods the use of a_w is inappropriate, since the water in the product is not at equilibrium with the vapor above it, and the product components are unlikely to be in internal equilibrium. The a_w concept, based on dilute systems, becomes quite inappropriate for application to structured-water food systems.

Evidence has suggested that the molecular mobility (rotational and/or translational mobility) of water is related to important diffusion-limited properties of foods which contain a substantial content of amorphous, hydrophilic molecules (carbohydrates and proteins), ranging in size from monomers to large polymers (Fennema, 1996). When a food is cooled and/or reduced in moisture content (such as by

freezing or drying), some components are converted to a glassy state. The molecular

mobility of the components is greatly reduced and the food become more stable. Slade

and Levine (1991) applied such "food polymer science" concepts to describe the

relationship between molecular mobility (in terms of the glass transition temperature,

Tg') and food stability. Molecular mobility of food systems as measured by Tg' can be

useful for predicting the physical properties, quality, stability, and safety of food systems.

Thus Tg' is often regarded as a useful indicator of the temperature below which food will

be well protected from deteriorative reactions that are diffusion-limited (Slade and

Levine, 1991).

The textural or rheological properties, as well as textural softening of food

products can be explained based on glass transition theory. However, it has not yet been

conclusively proven that glass transition is a better alternative than the concept of water

activity as a predictor of microbial growth or chemical reaction rates.

One problem is that the glass transition cannot be considered as an absolute

threshold for cessation of molecular mobility. Transport of water and other small

molecules take place even in the glassy state at a significant rate, resulting in effective

exchange of water in multi-domained foods or sensitivity to oxidation of encapsulated

materials. Denaturation of myosin storage under the glass transition temperature has

been also detected (Carvajal and Lanier, 1999).

One of the most surprising findings has been that the glass transition temperature

of meats is much higher than many other food systems. Apparent Tg' values of mackerel,

cod and beef were between –11 to –13 °C. More surprising was the finding that gelatin

and collagen fractions from mackerel yielded apparent Tg' values as high as –7°C (Brake

and Fennema, 1999). In contrast, there is a considerable body of evidence that most

hydrated globular proteins undergo a glass like transition at -93 to –53°C. This includes

studies of molecular motion as monitored by ESR spin labels (Steinhoff et al., 1989),

phosphorescence (Strambini and Gabellieri, 1984), and neutron scattering (Doster et al.,

1989).

Thus fully extended proteins exhibit a higher glass transition temperature,

indicating that these systems reach the arrested metastable state more easily than globular

proteins. Globular proteins show glass transition temperatures closer to that of bulk water

(about -113°C) (Bizzarri and Cannistraro, 2002), indicating that water at the interface

with these proteins associates weakly.

Bulk water in cells, and in extended protein systems such as gelatin, likely exists

in the state of polarized multilayers. We suggest that during rapid cooling of these

systems the amorphous state of the water (glass transition temperature) is more rapidly

attained since this water is already of greatly restricted mobility.

Practical Consequences of the PM theory

A colleague of Ling, Raymond Damadian, realized that it was possible to map

images of living cells and tissues by nuclear magnetic resonance (NMR) because of the

variations in water structure among them. In particular, water structure in cancer cells

appeared to differ from that in normal cells such that NMR mapping might be used to

detect cancer without surgery or any other invasive process. Thus was born the concept

of magnetic resonance imaging (MRI) (Damadian et al., 1971), a technique that has not

only revolutionized diagnostic medicine but also found application in fields such as molecular biology and even food science.

As further applied to food science, PM theory offers a more plausible explanation of why certain proteins with an extended structure, such as are prevalent in meats, gelatin, and many food gels, cause the structuring of water more than most native globular proteins. Evidence for this lies in the higher glass transition temperatures measured for these food systems.

With particular reference to the subject of this dissertation, PM theory would predict that cytoplasmic protein constituents within cells impose motional restrictions on rotational and translational diffusion of water, and thereby also restrict diffusion of solutes and macromolecules into and within cells. This is a restraint to diffusion in addition to that imposed by the tortuosity that results from tight packing of cytoskeletal elements of the cell, by high viscosity due to elevated protein concentration, and by any binding of solutes or macromolecules that may occur to cellular constituents such as membranes, etc..

Conclusions

We have summarized a few examples of experiments and relevant new research that strongly support the PM hypothesis originated by Ling.

Our choices have been influenced by research that indicates alternative roles for water in cell dynamics and water behavior at protein interfaces. We believe that the evidence is compelling that the solvent properties of a very large part of the cytoplasmic

water differs distinctly from that of the bulk liquid surrounding the cell. We also believe that the evidence is sufficient to suggest that long held misconceptions regarding the aqueous compartments of the cell should be abandoned if we are ever to understand the structure and properties of water in cells and its effects on solute transport. The PM multilayer theory can also be extended to other protein systems to explain phenomena related to protein stability and function that are the great importance for the pharmaceutical and food industries.

This approach must also be extended to work with product formulations more closely resembling actual commercial products; i.e., with reduced meat contents and containing added fillers such as starches/water (low fat products) or finely divided fats (standard products). Such composite products could well perform differently from the simple meat gels evaluated in this study.

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	Concentration of medium (M)	Water content (%)	q-value
Globular Proteins			
Egg Albumin	1.5	82.1	1.000
α-Chymotrpsinogen	1.5	82.7	1.004
Fibrinogen	1.5	82.8	1.004
γ-Globulin	1.5	82.0	1.004
Hemoglobin	1.5	73.7	0.923
β-Lactoglobulin	1.5	82.6	0.991
Lysozyme	1.5	82.0	1.009
Pepsin	1.5	83.4	1.031
Ribonuclease	1.5	79.9	0.984
Fibrous Proteins			
Gelatin	1.5	57.0	0.537
	0.1	84.0	0.890

From Ling et al., (1980) and Ling and Ochsenfled (1983).

Table 1: q-values of Na^+ in water containing native globular proteins and gelatin.

Method	System	D ($\mu m^2/s$)	Percent water	Reference
NMR	Pure "bulk"water	2,400	100	Haner et al., 1989
	Bovine lens ;			
	Nuclear homogenate	670.0	58	Cameron et al., 1997
	Cortex homogenate	970.0	75	
	Rabbit lens	840.0	70	
	Rabbit lens	660.0	52	Neville et al., 1974
QENS	Pure "bulk' water	2,200	100	Roroschach et al., 1987
	Artemia cysts	696.0	55	Trantham et al., 1984
	Artemia cysts	750.0	58	Clegg, 1984
	Skeletal muscle frog*	1000.0	82	Rorschach et al., 1987
	C phycocyanin	480.0	15.9	

*Measured at 3°C.

Table 2.Water self-diffusion in biological systems at 20C as measured by nuclear

magnetic resonance (NMR) or quasi-elastic neutron scattering (QENS).

(Cameron et al., 1997)
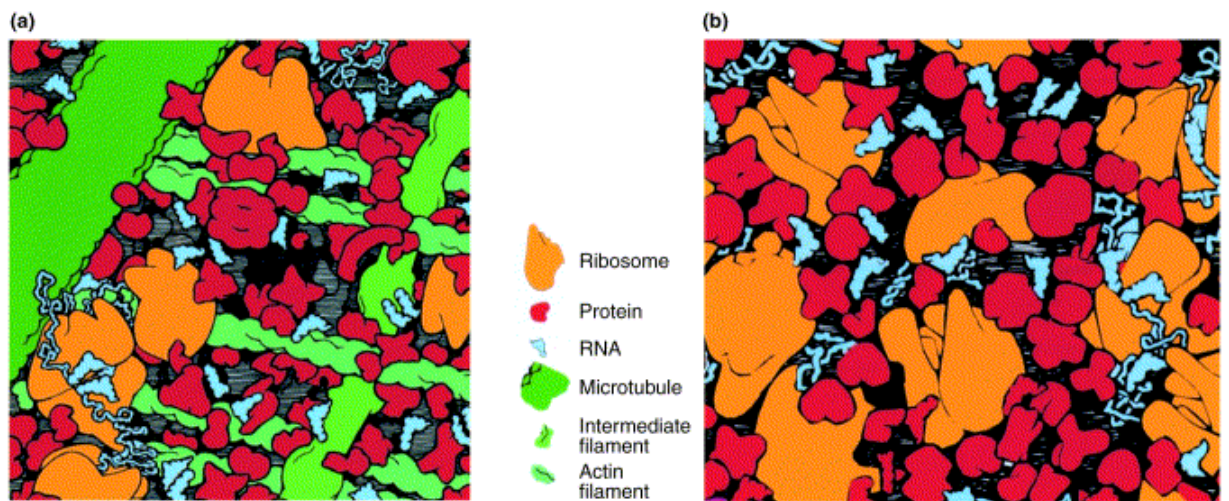
Figure 1. Schematic view of the interior of an (a) eukaryotic and (b) E. coli cells. All components are drawn to scale at the correct concentration. Each square illustrates the face of a cube of cytoplasm with an edge 100 nm in length. Small solutes are omitted from the drawing. Reprinted (Goodsell, 1991).
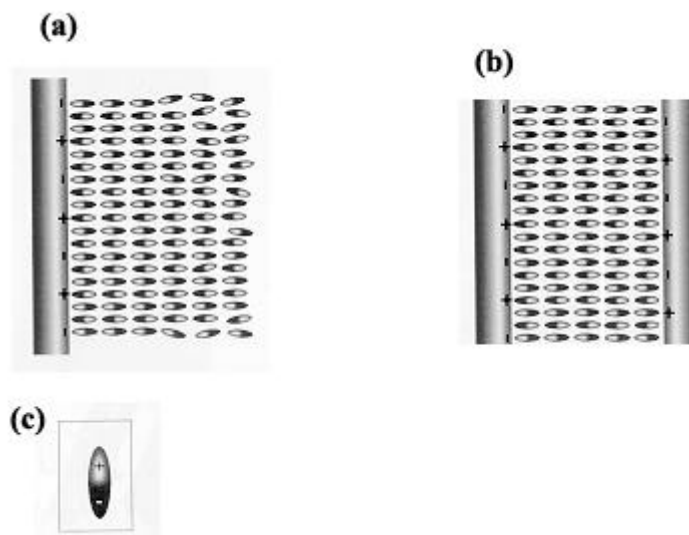
Figure 2: Effect of charged site distribution on the formation of dynamic structure of polarized multilayers of water molecules: (a) surface with alternating positive and negative sites (b) surfaces placed face to face (type with greatest stability) (c) water dipole. Adapted from Ling, 1992.
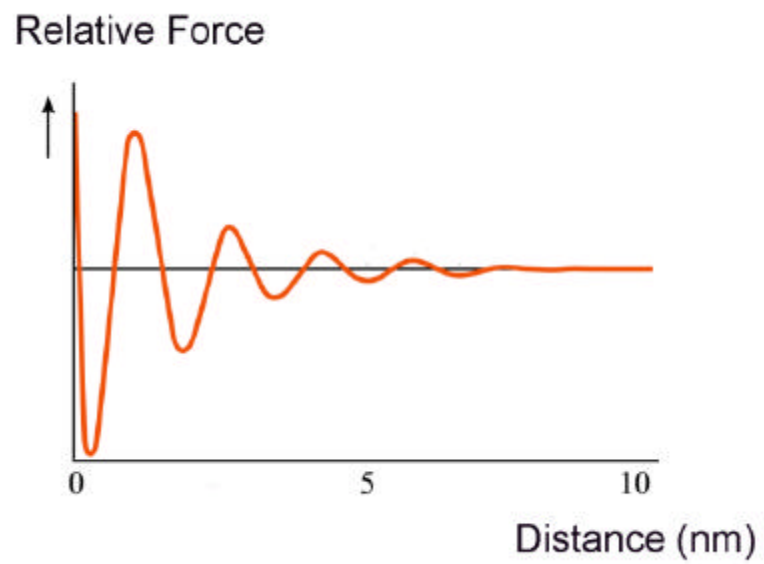
Figure 3: Effect of separation on force between closely spaced mica plates. After Horn and Israelachvili (1981)
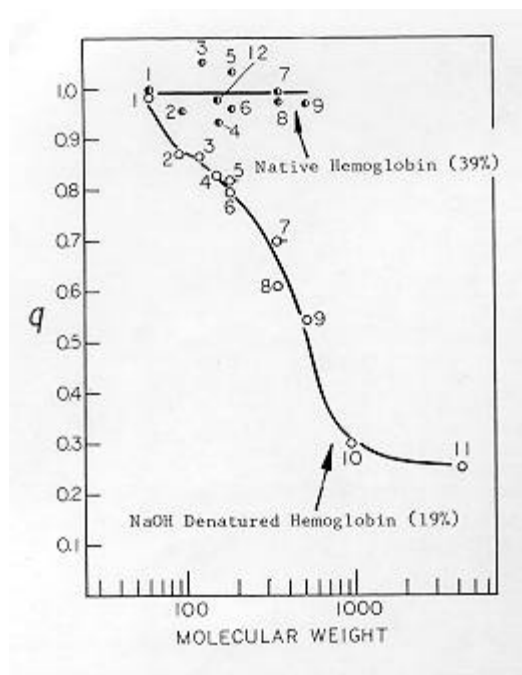
Figure 4: The relation between the q-value of the solutes, and the molecular weights of the solutes in 39% of native hemoglobin at neutral pH (half filled circles) and the 19% solution of NaOH-denaturated hemoglobin at alkaline pH (empty circles). (1) ethyl glycol, (2) glycerol, (3)erythritol, (4) xylitol, (5)sorbitol, (6)D-manitol, (7)sucrose, (8)trehalose, (9)raffinose, (10)inulin, (11)PEG 4000, (12)D-xylose. After Ling and Hu, 1988.
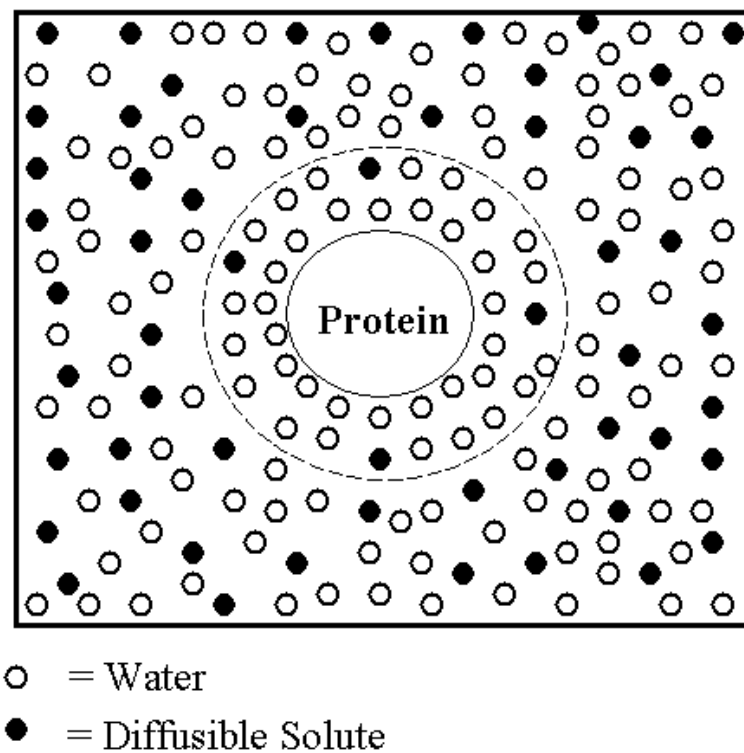
Figure 5: Schematic representation of preferential hydration of the protein surface
domain and solute exclusion, at dialysis equilibrium. (from Timasheff, 1989).
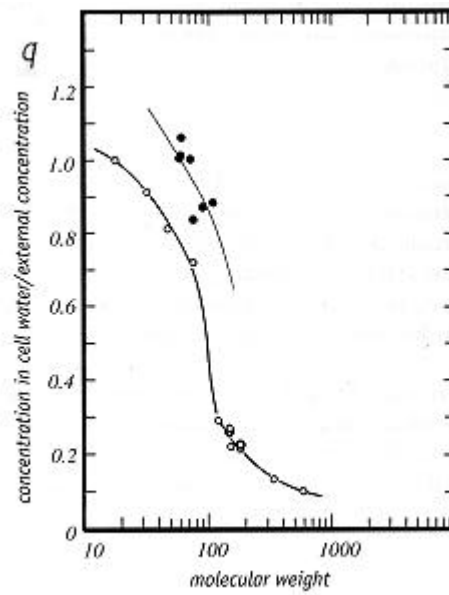
Figure 6. Experimental partioning of solutes of different size between inside and outside the cell. After Ling et al., (1993)
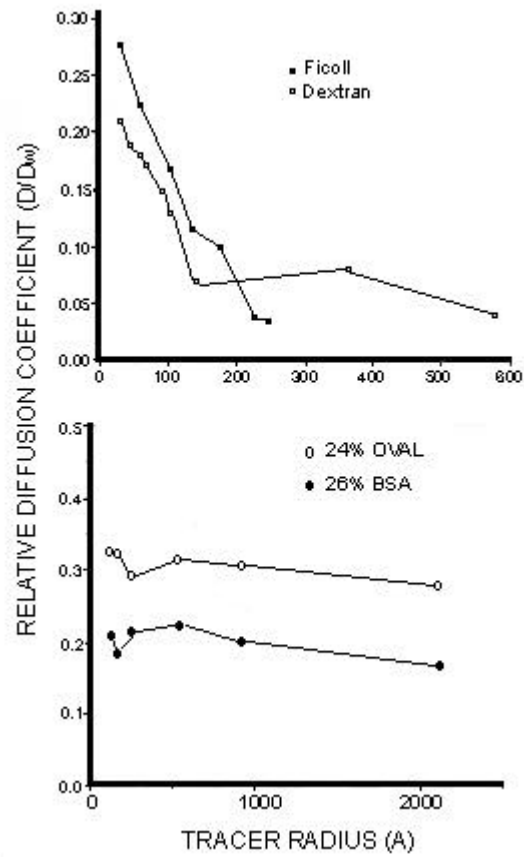
Figure 7: (a) The relative diffusion coefficient of size-fractionated dextran and Ficoll tracer particles in Swiss3T3 cells. (b) The relative diffusion coefficient of the same tracers in concentrated solutions of proteins (24% ovalbumin and 26% bovine serum albumin (BSA)). Luby-Phelps et al., 1987.
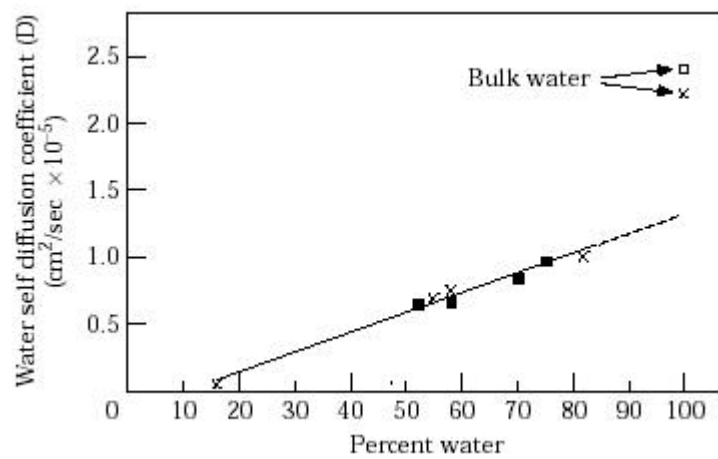
Figure 8: Water self diffusion for several biological systems (data from Table 2) as measured by quasi-elastic neutron scattering (Quens, X on the figure) or nuclear magnetic resonance (NMR, closed squares) plotted against the percent water in the specimen (Cameron et al., 1997)
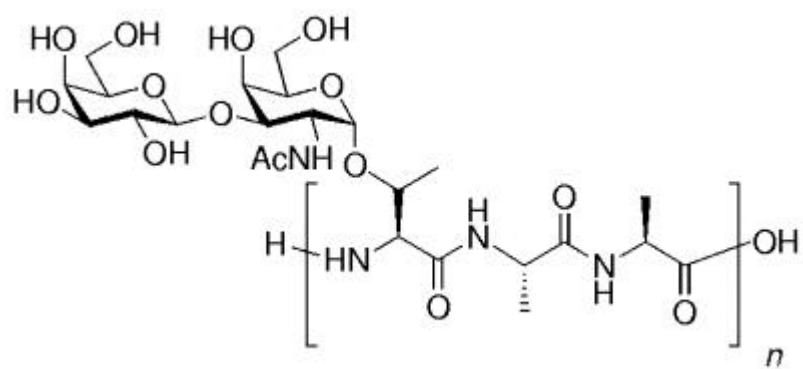
Figure 9: Chemical structure of a typical antifreeze glycoprotein (AFGP)

Manuscript 3

Diffusion of Active Proteins into Fish Meat to Minimize Proteolytic Degradation

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INTRODUCTION

Meat Degradation by Proteases

      Acceptability of seafoods is primarily determined by their texture when the flavor is bland or otherwise acceptable. Soft or mushy seafood texture is especially unpalatable to Western consumers. An experienced person can readily select raw seafoods which are likely to exhibit good odor and flavor once cooked, but the texture which a seafood will exhibit upon cooking is much more unpredictable in the raw state. This is because freshness and species of the fish are not always the primary determinants of texture. Variation in the content of proteolytic enzymes can also lead to textural variations in the prepared seafood that are difficult to detect in the uncooked meat.

      Protein degradation occurs in fish muscle postmortem due to the continued activity of the inherent proteolytic enzymes, without the counteraction of protein synthesis. The physiological changes occurring upon fish death also promote muscle autolysis because the muscle can no longer maintain homeostasis of temperature, pH, salt concentrations, and cellular integrity required for enzyme compartmentalization. Storage conditions may reduce enzyme activity, but chilling and freezing only retard the proteolytic activity. Once the product has been thawed and the material returned to a more favorable temperature, the proteolytic activity continues to act on the fish muscle.

      There are many specific examples of endogenous proteolytic activity affecting the texture of fish muscle or products made from it (Kinoshita et al., 1990). Hakes from the Pacific coast, including Pacific whiting (*Merluccius* spp.), may be parasitized by Myxosporidia cysts which, although possibly invisible to the eye, induce high levels of

cathepsin L protease and soften or even dissolve the flesh upon slow cooking (Okada et al.,1981; An et al., 1981). Arrowtooth flounder (*Atheresthes stomia*), now a fish in great abundance and even considered a nuisance fish in the North Pacific (about 5.7 billion individuals or 65% of the flatfish biomass in the Gulf of Alaska, excluding halibut; Holmes, 1991; Greene and Babbitt, 1990), produces white, even textured (when raw) fillets which would be highly acceptable to the American market except that slower cooking methods often render them unacceptably mushy in texture. Cathepsin has also been implicated in this textural defect (Wasson, 1992). Parasitization leading to textural defects in cooked salmon, similar to that occurring in hakes and whiting, has recently become a major problem for producers and processors of farmed (mariculture) salmon (Calkins, 1997).

A measure of control can be obtained by careful attention to cooking temperature (Wasson, 1992). The temperature should be kept well below the active range of the enzyme (50C to 60C; An et al., 1996) prior to cooking of the meat, then very rapidly cooked to an inactivating temperature, thus avoiding much of the protein degradation that would otherwise occur. However, this greatly limits the cooking methods that can be employed and thus the versatility of the seafood.

Food Grade Protease Inhibitors

Lamb-Sutton (1995) began to explore the use of food grade protease inhibitors, such as beef plasma, whey proteins, potato proteins and egg albumin (Hamann et al, 1990; Wasson, 1992), as a means of preventing heat-induced degradation of fish fillet texture by endogenous proteases. While her initial trials with diffusing solutions of such

inhibitors into small chunks of Arrowtooth flounder showed great promise, the plasma concentration required in the meat in order to achieve inactivation of proteolytic softening produced off colors and flavors. Also, scaled up trials with tumbling marination of whole fillets showed inconsistent delivery of the inhibitor solution throughout fillets. Similar disappointing results for injection and soaking of fillets at commercial scale with plasma, egg white and other food-grade materials containing protease inhibitors were reported by McFarland (1990).

Two problems were identified with this former approach. First, the active inhibitor in plasma and other inhibitor-containing materials was in relatively low concentration, and thus much inactive protein material had to be delivered along with this active component. This limited the equilibrium concentration of the active inhibitor component and simultaneously delivered undesirable components into the meat that adversely affected taste and color. A more concentrated and purified source(s) of inhibitor was needed for diffusion into fish meat. Secondly, no work was conducted to assure an even spatial distribution of protein inhibitors throughout the subcellular compartments and intracellular architectures in the cell sarcoplasm, a mandatory step to effectively block the activity of endogenous proteases which are primarily active against the cellular myofibrillar proteins (Gault, 1992; Jiang et al., 1996; Koohamaraie, 1996).

In the last decade several laboratories have been able to commercially fractionate and purify active protease inhibitor components such as cystatin (Nakai, 1999), soybean trypsin inhibitor (Lawrence and Koundal, 2002) and α-2-macroglobulin. Because the majority of problems with proteases affecting fish texture involve cysteine proteases, the cystatins (cysteine protease inhibitors) are of greatest commercial interest. Cystatins

have been successfully expressed as recombinant proteins in plants like rice, corn and potatoes. Chicken cystatin is a powerful protease inhibitor of lysosomal cysteine proteases (Ki= 5 10^{-12}M). Its inhibition is extremely tight and rapid as compared to other cystein protease inhibitors and plant cystatins (Turk and Bode 1991;Turk et al., 1997). Several cystatin genes have been cloned and expressed in *Escherichia coli* (Kang and Lanier, 1999a), and *P. pastoris* X-33, a methylotrophic yeast (Chen et al., 2000, 2001) which can be easily purified in large quantities. Recombinant cystatin could thus be commercially produced by fermentation to yield a very potent and pure food-grade protease inhibitor.

In this context it is worth emphasizing that cystatins are very stable under conditions of extreme pH and high temperatures (Nakai, 1999). For example, DSC measurements have confirmed that chicken cystatin behaves as a thermophilic protein; that is, it is very stable. From pH 4.0 to 7.7 no thermal denaturation was observed by DSC at temperatures up to 115C (Zerovnik et al., 1997). Thus it can likely resist thermal treatments such as cooking and be effective as a protease inhibitor.

Furthermore, recombinant engineered cystatins could be developed which are even more resistant to proteolytic degradation. Cystatin is of lower molecular weight than most beef plasma components, and this should ease its penetration into muscle. Recently Kang and Lanier (1999b) successfully infused a recombinant cystatin into Arrowtooth flounder muscle chunks by injection to achieve reduction of proteolytic activity during cooking, resulting in firming of the meat.

The Kunitz-type soybean trypsin inhibitor (STI) can inhibit trypsin and chymotrypsin (serine proteases), important animal digestive enzymes (Song and Suh,

1998). Toyohara and Shimizu (1988) showed that the heat-stable proteases in threadfin-bream were inhibited by STI. The inhibitory effects of soybean trypsin were subsequently studied on oval-filefish meat gels that were softened by myofibrillar proteases (Toyohara et al., 1990). They found that soybean trypsin inhibitor inhibited the breakdown of myosin heavy chain (MHC) at 50C; however, it was not effective at 65C. Recently, Yongswatdigul et al. (2000) showed that STI successfully inhibited proteolysis in tilapia, addressing the issue that serine proteases may be in part responsible for proteolytic activity postmortem in this fish.

MacDonald and Lanier (in Hamann et al., 1990) proposed that a likely protease inhibiting agent in beef plasma and dried egg white was α-2-macroglobulin, a large molecular weight protein (725 kDa) known to inactivate proteases in all four of classes of proteases (Starkey and Barret, 1977). Lamb-Sutton (1995) found that purified α-2-macroglobulin and a crude plasma fraction containing this inhibited the inherent heat-stable proteolytic activity in surimi gels made from Atlantic menhaden, Pacific whiting, Alaska pollock and arrowtooth flounder as evidenced by SDS-PAGE analysis of protein integrity and gel mechanical properties by torsion testing. The crude plasma fraction enriched in α-2-macroglobulin also seemed to firm chunks of arrowtooth flounder during cooking at the optimum temperature for proteolytic activity (near 60C), at a much lower total protein concentration in the diffusing solution than was required for a similar effect using beef plasma solution.

Diffusion of Protease Inhibitors into Fish Meat

To achieve an even distribution of protease inhibitor within intact muscle tissues, immersing the meat in a solution of the inhibitor has been the method of choice, sometimes after scoring or injection of the meat to increase the surface area of exposure for solute intake.

Physical proximity is an essential requirement for molecular interaction to occur. We accept it as indisputable that random thermal motion of molecules remains as prerequisite for the formation and dissociation of enzyme-substrate/inhibitor and other specific intermolecular complexes (Agutter and Taylor, 1996;Albert et al., 1998).

Diffusion theory

Molecules in solution undergo Brownian motion under thermal bombardment from their surroundings, which causes them to move even when not subject to an external force (Bloomfield, 2000). This thermal motion, called diffusion, is random in magnitude and direction. Diffusion is also observed when a concentration gradient is set up in solution or when a barrier between solutions at two different concentrations is removed. The tendency toward equalization of concentrations is attributable at the macroscopic level to a gradient of concentration; but at the molecular level it can be understood as the random motion of molecules, with more molecules in the concentrated region of solution available to move randomly into the more dilute solution.

The microscopic aspect of diffusion theory, which holds that random thermal motion of molecules in liquids are responsible for macroscopically observable time-dependent changes was provided not by the classical thermodynamics, but the kinetic theory. Einstein succeeded, ultimately, by proving an account of Brownian motion. First explicitly described by Robert Brown in 1928, who observed under a light microscope, in

aqueous suspension of minute pollen grains a rapid, continuous, short range motion of small particles that "..arose neither from currents in the fluid nor from its gradual evaporation, but belong to the particle itself." This phenomenon had remained something of a thorn in the flesh of 19th century physics and had certainly resisted explanation in terms of classic thermodynamics.

At the macroscopic level, the diffusion coefficient, D, is classically defined as and measured by the decrease with time (t) of the concentration gradient (dc/dt) of the substance (Fick's law):

$$\partial c/\partial t = D\,\partial^2 c/\partial x^2$$

Einstein in 1905 derived the following relationship from the solution to Fick's second law for a relationship between the root mean square displacement, r, the diffusion coefficient D, and the time, over which the displacement of the species is measured, for unbounded diffusion in three dimension in an isotropic medium:

$$\langle r^2 \rangle = 6Dt$$

For a two dimentional analog the above equation becomes:

$$\langle r^2 \rangle = 4Dt$$

The five assumptions implicit in this solution are:

1. The time over which measurement is made is many orders of magnitude greater than the duration of the average Brownian motion.

2. The solvent activity is constant at all planes through the direction of movement across which measurement takes place, i.e., the solution is homogeneous and infinitely dilute.

3. Molecular bombardments of the particle by solvent are equally probable from all directions.

4. The system is unstirred (stationary). There is no bulk solvent flow; solvent molecules move only (i) by random thermal motion and (ii) in exchanging places with particles.

5. There are not significant particle-particle interactions, i.e., individual particles migrate independently.

With the development of modern techniques such as dynamic laser light scattering, nanosecond fluorescence depolarization measurements, fluorescent recovery after photobleaching, fluorescence correlation spectroscopy and NMR, the study of diffusion has become much easier than with other older techniques and has made measurements of diffusion properties more convenient, precise, and refined. With the availability of the diffusion data, several new models of diffusion, new concepts, as well as modifications or improvements of the existing theories have appeared in the literature in the last decade (Masaro, 1999). At the same time, development of new theoretical and computational tools have enabled calculation of the properties of complex, realistic molecular models (Masaro, 1999;Bloomfield, 2000).

Crossing the cell membrane

Central to the more popular views of living cell is the postulate that the membrane is the primary, if not sole controller of the asymmetric distribution of solutes. In this manner, the plasma membrane of eukaryotic cells is impermeable to the vast majority of peptides and proteins (Wadia & Dowdy, 2002). However this dogma has been shown to

be untrue since over the years different studies have revealed the permeability of various cells to these macromolecules (Peptides:Egglenton and Egglenton, 1933; Proteins: Zierler, 1958; McLaren et al., 1960; Dawson, 1966; Ryber, 1968).

The subject has received a great deal of interest a decade ago when Green and Frankel independently demonstrated that the nuclear transcription activator protein (Tat) from the HIV-1 virus was able to enter cells when added to surrounding media (Green et al., 1988; Frankel et al., 1988). Subsequently, several other proteins with the same capabilities have been identified, including the Drosophila homeotic transcription factor ANTP and the herpes simplex virus type 1 (HSV-1) VP22 transcription factor (Elliot and O'hare, 1997; Joliot et al., 1991). The process by which peptide or protein motifs cross the cellular plasma membrane is called transduction (Hawiger, 1999).

The identification of short basic peptides sequences from these proteins that confer cellular uptake has led to the recent identification and synthesis of numerous new protein transduction domains (PTD) (Wender et al., 2000; Futaki et al., 2001; Ho et al., 2001). Significantly, when covalently crosslinked to full-length proteins, these PTDs are capable of delivering biologically active proteins in excess of 700 kDa to a wide variety of cell types (Bonetta, 2002).

Protein transduction occurs in a rapid, concentration-dependent fashion that appears to be independent of receptors and transporters and instead is thought to target the lipid bilayer components of the cell membrane. Thus in principle all mammalian cell types should be susceptible to protein transduction (Schawarze et al., 1999; Bonetta, 2002). Although there is a limited homology between these PTDs, the rate of cellular

uptake has been found to strongly correlate to the number of basic residues present, specifically the number of arginine residues (Wender et al., 2000; Futaki et al., 2001)

Unfortunately, the idea that PTDs react with the cell membrane does not have any foundation since none of the studies cited above have done specific research on the interaction of PTDs and cell membranes in vivo. Their results and conclusions are based on experiments performed by incubating cells with fluorescently labeled proteins and following how long it takes for equilibration of these macromolecules within the cell.

Intracellular barriers

Even though the cytoplasm is classically described by the dilute solution paradigm, theories that view the cytoplasm as playing the dominant role in the control of cellular interior have existed for more than a century (Ling, 1984).

Indeed, several studies have indicated that the diffusion rate of molecules species in the cellular cytoplasm is significantly reduced relative to rates in dilute solutions and that the corresponding diffusion coefficient show a marked dependence on molecular weight (Luby-Phelps et al., 1988; Arrio-Dupont et al, 1996, 2000). Such observations could indicate that the intricate fibrous network acts as a molecular sieve that slow down the translational motions of molecules according to their dimension and form. However, Ling (1992) sustained that the impaired diffusion is consistent with water structuring. Additionally, over a half-century of research has clearly concluded that many metabolic pathways and their component enzymes are restricted to specific cell compartments, and numerous soluble enzymes show intracellular binding to specific intracellular sites (Clegg 1992;Wallimann et al., 1998).

Next to a possible variation in diffusional mechanisms with size and conformation of the molecular solute, different obstructive paths may manifest themselves on distinct timescales (Luby-Phelps, 2000). On the shortest timescale, typically in the picosecond range, the translocation of the solute is minimal and its local diffusive behavior is predominantly influenced by the solvent medium. For longer time intervals, the translational movement of the particles is such that interaction with the cytoskeleton become prominents. Carvajal and Lanier (2003) have done an extended review of the different factors affecting solute diffusion in the cell.

The aim of this thesis is to study both the potential delivery of protease inhibitors and to characterize the influence of the medium on molecular diffusion in the muscle fiber. To accomplish this goal, the spatial distribution and the diffusion coefficient of several protease inhibitors are determined.

The method adopted should be suitable for examing noninvasively living cells under physiological conditions. Moreover, a high spatial resolution is mandatory to resolve molecular diffusion on the subcellular level. Here we employ a series of advanced optical microscopic techniques that comply with all these demands.

Imaging Diffusion Processes

Confocal Microscopy.

Laser Scanning Confocal Microscopy (LSCM) is a relatively new form of optical microscopy. It has been extensively used in the biological sciences for over a decade, but its application in food science in not nearly as extensive. By using a point-source rather than whole field illumination, a higher spatial resolution can be obtained if light is

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subsequently detected through a confocal pinhole. The power of the confocal apparatus is the rejection of out-of-focus radiation, thereby improving the resolution in the axial direction considerably. If raster scanning is employed, three-dimensional images with microscopic resolution can be reconstructed from the collected data. The practical implementation of the confocal microscope coincided with the introduction of the laser as the sample illumination source. The outstanding collinearity of the lightrays that make up the laser beam enables a true point illumination in the object plane of the microscope. The three dimensional submicrometer resolution enables refined imaging of cellular structures (Sheppard and Shotton, 1996; Pawley and Centonze, 1998).

With light confocal microscopy, structures can be resolved with resolutions of typically a few hundred nanometers. Although many subcellular objects can by visualized with the optical microscope, structural compositions that manifest themselves on a nanometer scale remain invisible. In this regard, the confocal microscope is still inferior to electron microscopes, which can attain resolutions of 0.1 nm (Joy and Pawley, 1992). However, electron microscopy requires drastic sample preparation steps that disqualify the technique for live cell imaging. Similar problems are faced in soft X-ray microscopy. Although X-ray microscopes can be used to examine hydrated cells with high resolution (~30 nm), applications are limited to samples which are rapidly frozen to cryogenic temperatures (Joy and Pawley, 1992).

One of the other main advantages is the minimal sample preparation required. Specimens can be viewed "as-is" with the LSCM without a prior dehydration step, embedding in paraffin, fixation, or complicated sectioning. Typically sample thickness used is in the order of millimeters, but sections as thin as 2-3 μm may also be used. Not

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only does this save time, but also physiological processes and physical structure can be monitored undisturbed and free of artifacts caused by complicated preparation (Vodovotz et al., 1996).

The distinctive abilities of optical and generation of three dimensional images provide LSCM with many advantages in the study of muscle fiber. Optical cross sections of a fiber can be obtained nondestructively within a minute, and can be quantified using image analysis rather easily. These valuable features provided by LCSM bring great convenience to the measurement of fluorecently labeled protein spatial distribution, the process of protein diffusion, and even the location of proteins in the muscle fiber.

The superb advances achieved in light microscopy have allowed researchers to detect isolated single molecules on coverslips and in biological samples (Schmidt et al.,1996). Next to this, the compelling ability to selectively image specific molecules of interest allows a detailed inspection of the dynamic distribution of functional biomolecules in cells. Due to the development of numerous fluorescent markers and the continuous progress in labeling protocols (Haugland, 1996), an extended spectrum of biomolecular species can be displayed in the fluorescence microscope.

Not surprisingly, virtually all optical investigations of intracellular diffusion have been based on fluorescence methods. The most direct way to tailor diffusional properties of intracellular compounds is to track the pathways of single fluorescent particles during a given observation time. Single-particle tracking has for instance been used to study membrane physics (Saxton and Jacobson, 1997), viscosity of the cytoplasm (Yamada et al., 2000), and protein diffusion in cytoplasm and nucleus (Goulian and Simon, 2000).

Fluorescence Recovery after Photobleaching.

The mobility (diffusion) of a fluorescent protein can be assessed using a specific type of photobleaching technique called fluorescence recovery after photobleaching (FRAP). In this technique fluorescent molecules in a small region of the cell are irreversibly photobleached using a high powered laser beam and subsequent movement of surrounding non-bleached fluorescent molecules into the photobleached area is recorded at low laser power (Ellenbeerg and Lippincott, 1998).

Two kinetic parameters of a protein can be discerned from quantitative studies that use FRAP: the mobile fraction, f_m, which is the fraction of fluorescent proteins that can diffuse into the bleached region during the time course of the experiment, and the diffusion constant, D.

A typical FRAP curve, which provides information on D and f_m is shown in Figure 1. The mobile fraction provides a measure of the extent to which the fluorescent protein can move within the cells. It is determined by calculating the ratio of the final to the initial fluorescence intensity in the bleached region, corrected for the amount of fluorescence removed during photobleaching. When the mobile fraction is less than 100%, some fluorescent molecules might be irreversibly bound to a fixed/anchored substrate. Alternatively, non-diffusional factors, such as diffusion barriers or discontinuities within the structure where a protein localizes, might be responsible for the reduced mobility.

The diffusion constant, D, is obtained by plotting the recovery of relative fluorescence intensity within the bleached region as a function of time, and fitting this recovery curve by appropriate equations.
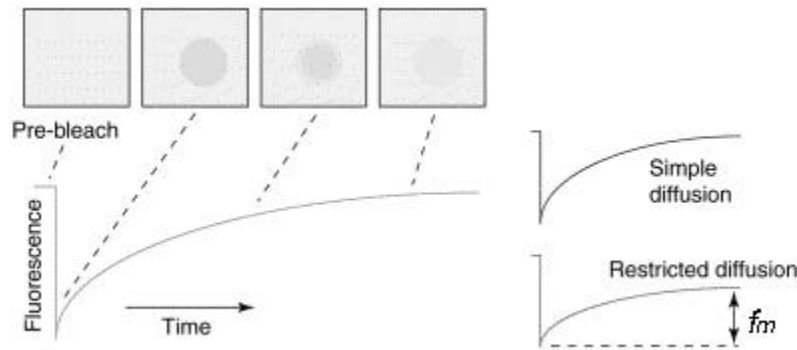
Figure 1: Spot bleaching showing fluorescent recovery into a circular bleached region by inward diffusion of unbleached fluorophores. (Right) Fluorescent recovery curves for simple and restricted diffusion: fm is the fractional recovery of the photobleached signal (Verkman, 2002).

Confocal microscopy offers many advantages over the standard fluorescent microscopy to perform FRAP. Confocal microscopy allows fluorescence detection not only on the sample's surface but also at an arbitrary depth inside the sample with a high spatial resolution without any interference of fluorescence from out-of-focus levels of the sample as encountered in a conventional microscope (Cutts et al., 1995; Meyvis et al., 1999).

In the present work, protein diffusion into and within muscle fibers was studied by two techniques using the LSCM, qualitative analysis of fluorophore spatial distribution in muscle fibers in the diffusion process, as well as the quantitative analysis

of fluorescent recovery after photobleaching (FRAP). By using the LSCM, we not only could obtain the diffusion coefficients simply and quickly, but also obtained information about the dynamics of the protein mobility into and within muscle fibers.

MATERIALS AND METHODS

Overview of experimental plan

Various fluorescently labeled protease inhibitors were diffused into isolated intact fish muscle fibers and the dynamics of the diffusion was followed by confocal microscopy.

Treatments yielding satisfactory diffusion of protease inhibitors as assessed by confocal microscopy were then quantitatively evaluated by measuring the diffusion coefficients and solute mobility within the cytoplasm of the muscle cell. Such techniques will be needed to model the diffusion process and compare effectiveness of treatments in the future.

Fish muscle fiber preparation and mounting

Fresh Pacific halibut (*Hippoglossus stenolepis*) was obtained from a local distributor (Fresh Catch Seafood, Durham NC). The fish were previously caught in the Gulf of Alaska and kept in ice before arriving in NC within 72 h of capture.

Small strips of white skeletal muscle were isolated from the anterior epaxial region from each of two fish samples and prepared according to a method described by Brenner (Brenner, 1983;Yu and Brenner, 1989). Throughout the experiment, isolated

tissue was superfused with Ringer solution (Langfeld et al, 1991). Halibut strips were used because its fibers are bigger and easier to isolate. Sucrose was added as a cryoprotectant in order to store the strips at –80C without any detectable structural damage. In short, before freezing, the strips were incubated for at least 30 min each in solution containing 0.5 M, 1.0 M, 1.5 M and 2 M sucrose. After equilibration with 2 M sucrose, the strips were cut into sections of about 1-1.5 mm in length and rapidly frozen in liquid nitrogen and stored at –80 C. For thawing, the strips were transferred directly into a solution containing 2 M sucrose. Subsequently, the strips were again incubated successively for 30 min each in solution with 1.5 M, 1 M, 0.5 M sucrose and finally kept in Ringer solution. From these strips, single fibers were isolated. In order to further mitigate the deleterious effects of freezing we treated the muscle fibers in media containing 0.4% polyvinylpyrrolidine to restore membrane lipid fluidity to normal levels (James et al., 1999). Several studies using x-ray diffraction and mechanical methods have been carried out to demonstrate that parameters such as equatorial intensities, isometric force, shortening velocity, fiber ATPase activity, and fiber stiffness revealed no changes due to this freezing procedure (Kraft et al., 1995).

In this study, for all exchange experiments, unfixed intact fibers were used. For confocal microscopy, the fibers were mounted in specially made slides having a circular opening (15 mm diameter) cut from the center. The bottom of this circular chamber was sealed with a greased coverslip placed below the opening in the slide. The chamber, 1mm deep, can hold 200 μL, volume enough to ensure that the fibers were completely surrounded by solution. For adding the fluorescently labeled proteins quickly, new solution could be sucked through the chamber while the fiber remained in place on the

microscope stage. All solutions were adjusted to pH 7.2 at the experimental temperature of 22C for real-time confocal microscopy.

Macromolecules to be diffused into muscle fibers

Protease inhibitors

We monitored the diffusion of four proteins: (1) a recombinant cystatin (cystein protease inhibitor) of plant (soy) origin produced in our laboratory (see method below) and one of chicken origin (Sigma) (RSC; MW ~11 KDa); (2) alpha-2-macroglobulin (Sigma), a large (720KDa) protease inhibitor capable of irreversible binding and therefore inhibiting a wide variety of proteases; (3) Soybean trypsin inhibitor (STI; Sigma) (21KDa), a potent chymotrypsin inhibitor that has been studied extensively for its ability to prevent carcinogenesis in both in vivo and in vitro systems; (4) R-Phycoerythrin (RPE), a 240 kDa autofluorescent protein. RPE (Molecular Probes), which was obtained as an ammonium sulfate precipitate, was dialyzed against 100mM sodium phosphate, 150mM sodium chloride (pH 7.2) at 4C with multiple changes of the dialysis solution. The RPE solution was then spun through a 0.2 μm filter and stored at 4C.

Production of recombinant soy cystatin (RSC) by E. coli fermentation

E. coli containing the soy cystatin gene were isolated from a single colony on agar plates by streaking. Following preparation of an overnight preculture from the isolated colony, the culture is diluted 1:100 in LB broth for bulk incubation at 37C. When an A_{600} of 0.5 is obtained, the bulk culture was quickly cooled to room temperature on ice, and

isopropyl β-D-thiogalactoside (IPTG, 0.4 mM) was introduced for the accumulation of

recombinant protein during continued incubation overnight at 37C.

The cells thus cultured were harvested by centrifugation (8,000 x g) for 30 min

and sonicated after suspension in 20 mM potassium phosphate buffer (pH 6.0),

containing 10 mM sodium azide (buffer A). Following separation of cell debris by the

same centrifugation, the protein precipitated between 20-80% saturation of ammonium

sulfate was pooled, dissolved in buffer A and adjusted to 20% ammonium sulfate. The

resulting solution was loaded onto a phenyl sepharose CL-4B column (2.5 x 30 cm)

which is equilibrated with buffer A containing 20% ammonium sulfate (buffer B). After

the sample was loaded, the column is washed with buffer B until A_{280} is below 0.05. RSC

bound to the column is eluted by buffer A and collected into a fraction collector with 6.5

ml in each tube. The fractions having high readings at A_{280} were pooled and assayed for

protein content and inhibitory activity.

Dextrans

In order to validate the FRAP technique we initally worked with FITC-labeled

dextrans having mean molecular weights similar to those of the protease inhibitors

selected for this study (Sigma FD 10S, FD 20DS, FD 250DS, FD 500DS). We diffused

the FITC-dextrans into aqueous solutions and muscle fibers in the same manner as we

would later do with the protease inhibitors, for two main reasons.   First, there is a

precedent for the use of such molecules to study diffusion in biological systems (Luby-

Phelps et al., 1987). Second, the structural properties of dextrans in solution have been

identified (Ogston and Woods, 1953). Dextrans are neutral molecules and very water

soluble. Consequently dextrans do not interact significantly with cellular components

(Luby-Phelps, 1987). However, after having succeeded in obtaining similar results to

published literature, the experimental work was subsequently carried out only with

proteins.

Fluorescent labeling of protease inhibitors

Protease inhibitors were labeled with fluorescein isothiocyanate (FITC, Isomer I,

Sigma) by methods outlined by Hermanson (1996). Briefly, the method includes

activating functional groups of the dissolved proteins by increasing the pH using

phosphate, bicarbonate or borate buffers. Fluorescein isothiocyanate (FITC), modified by

the manufacturer (Molecular Probes, Eugene, OR) to contain a negative functional group,

is then added and allowed to react in the dark for 1-2 hr before stopping the reaction with

1/10 volume of 1.5M hydroxylamine. Excess label was then removed from the protein

solution by gel filtration (Sephadex G-25, Sigma). The fluorophore/protein labeling was

evaluated spectrophotometrically at pH 7.2, and the average molar ratio of dye to protein

subunit was 1.0, 1.1 and 1.5 mol/mol for FITC-cystatin, FITC-STI and FITC-2-

macroglobulin, respectively. All fluorescently labeled proteins and dextrans were

adjusted to the same intensity. Ultrafiltration membranes (Amicon, Beverly, MA) were

used to concentrate the protein solutions to their final absorbance.

Visualization of protein diffusion and distribution within fish muscle fibers

Protein spatial distribution

Confocal images were collected in a Leica inverted microscope attached to a Leica confocal scanning unit (TCS SP: Leica,Wetzlar, Germany). A range of interchangeable lenses were available for this microscope, including a 63x, 1.2 NA water immersion lens. For time-lapse imaging of FITC, images were taken every 5 minutes with each plane being averaged two times. FITC was excited by an argon ion laser at 488 nm with emission recorded 500 to 550 nm. The microscope has the ability to record the DIC (differential interference contrast) image simultaneously with the fluorescence confocal images. The images were collected by a Hamamatsu cooled Color CCD camera (Hamamatsu, Japan).

All images were processed using Adobe Photoshop 5.0 by applying standard methods of image optimization such as contrast and brightness adjustments. All images were enhanced equally and printed on a Kodak 8670 PS thermal printer (Eastman Kodak, New York, USA)

For diffusion of fluorescent components into the muscle fibers the respective fluorescently labeled molecules were immersed in Ringer solution. All solutions were adjusted to pH 7.2 at the experimental temperature of 23C for real time confocal microscopy. For diffusing out unbound fluorescently labeled molecules we used the same Ringer solution but without adding the tested molecules.

When following the spatial distribution of macromolecules, no reagents were added to reduce photobleaching. The observation time at each point was short (<20 s), and the intensity of the exciting light was kept as low as possible. Scanning occurred every 5 minutes during the first 35 min of incubation period with the fluorescently labeled molecules. After this time of incubation the fibers were rinsed three times in

Ringer solution without the fluorescently labeled molecules. And a second round of scanning was followed.

Fluorescence recovery after photobleaching

FRAP was performed by using the timelapse/bleach function on a Leica TCS NT (Leica,Wetzlar, Germany). This function conducts photobleaching and records fluorescence recovery automatically according to pre-set time intervals. Seven recording series were available to allow various time intervals during photobleaching and fluorescence recovery. The 488-nm line of a 100-mW argon ion laser was used for sample bleaching and fluorescence excitation. Emitted light was monitored at 520 nm. Typical setting for prebleach and recovery scans were 0.3% of the maximum laser power. Images were obtained with a 40 x 1.25NA oil objective and an optical zoom of 1.0. The depth at which diffusion measurements were taken was at the center of the sample. The radius of the bleaching spot was measured from the first image after photobleaching. The mean fluorescence intensity of recovery was measured by making a small circle at the center of the photobleached spot in each image. The minimum bleach scan was 0.5 s, although typical times were 2 s. For recovery, the program reset the instrument to the prebleach configuration and a time series of up to 50 recovery images was collected, tipically over 0.5-170 min. For slowly diffusing samples (cells or high molecular weight macromolecules), 4 or more images were collected at each time point (recovery curves) (Figures 7 and 9). Since the distribution histograms per recovery curve were fairly close to normal distribution, group averages were compared by the two-tailed student's t test (Anexo).

Protein diffusion in aqueous solution

Diffusion coefficients of protease inhibitors and dextrans in free solution were measured for diluted solutions in Ringer solution, placed as a thin layer between a glass slide and a coverslip. The edges of the coverslip were sealed with paraffin. The diffusion rate of a smaller proteins and dextrans could be decreased by adding various sucrose concentrations to increase the viscosity of the medium. The values were then extrapolated to the buffer viscosity.

Data analysis of FRAP

We used the technique known as "spot" FRAP (Axelrod et al., 1976; Simon et al., 1988) to measure the diffusion coefficient of the macromolecules. A laser beam (λ=488) was focused as a circular region of characteristic radius ω onto the cell. An optical cut-off filter (λ=515) was used to select the appropriate wavelength for the maximum fluorescence emission (520 nm) from the fluorophore. Because the cells were thin (< 1mm) and transparent, the spot radius was constant throughout the thickness of the cell. The experiment was started by amplifying the intensity of the incident beam by several orders of magnitude for a short time (typically 0.5 sec) and cutting back the power to the normal monitoring level. Switchinng between modes required 1.6 s, which was fast compared to the time scale of fluorescence recovery observed. The short pulse of high intensity radiation bleached some of the fluorescent molecules (typically 20-70%) so they became inactive with respect to the fluorescent signal. Because these fluorophore molecules were bound to mobile macromolecules that continually diffuse within the cell,

the fluorescence signal from the illuminated spot recovered with time. By tracking this recovery and modeling it as a diffusion process, we determined the self-diffusion coefficient D for the macromolecule.

The modeling of FRAP has two parts (Axelrod et al., 1976; Tong and Anderson, 1996). First, the photobleaching step is assumed to be a first-order reaction (with respect to the labeled solute concentration). Account must be taken of the gaussian nature of the intensity (I) of the beam:

$$I(r) = (2P_o/\pi\omega^2)\exp(-2r^2/\omega^2) \tag{3}$$

where r is the radial distance from the center of the beam, P_o is the total power of the laser beam, and ω is the beam radius. The second part is to model the recovery by Fick's second law of diffusion:

$$\frac{\delta C}{\delta t} = D \frac{1}{r} \frac{\delta}{\delta r}\left[\frac{\delta C}{\delta r}\right] \tag{4}$$

Where C is the concentration of the labeled macromolecule within the sample and D is the diffusion coefficient of the macromolecule. The measured fluorescence emission F is given by

$$F(t) = (q/A) \int_0^\infty C(r,t) \, I(r) 2\pi r dr \tag{5}$$

where q is the product of the quantum efficiencies of light absorption, emission, and detection and A is the attenuation factor of the laser beam. Neither q nor A is required to

determine D, because the fluorescence signals are always normalized by the initial value Fi before photobleaching. The following expression is obtained by solving Eq 4 and substituting the result and Eq 3 into Eq 5 (Axelrod et al., 1976):

$$F(t) = Fi \sum_{n=1} \frac{(-K_b)^n}{n!} (1+n \, (1 + 2t/\tau_D))^{-1} \qquad (6)$$

$$F(0^+) = Fi \, \frac{(1-\exp(-K_b))}{K_b} \qquad (7)$$

Where Fi the measured value of F before the photobleaching step and $F(0^+)$ is the fluorescence signal immediately after the photobleaching step. The diffusion time is related to beam radius and diffusion coefficient by

$$\tau_D = \gamma \omega^2/4D \qquad (8)$$

where τ_D is the characteristic diffusion time and γ is a correction factor

The two unknown parameters, τ_D and K_b, can be determined by fitting Eq. 6 to the data F versus t. This approach assumes total recovery of the fluorescence signal after long times (F → Fi as t → ∞).

Over the time scale of the experiment (on the order of minutes), it is often not possible to get a good fit of the data to Eq. 6 because not all of the macromolecules appear to be mobile. To improve the fit, we assumed that only a fraction f_m of the labeled macromolecules were mobile and the fraction $(1-f_m)$ did not diffuse. The mobile and immobile fractions were assumed to have equal probability of being bleached. The mobile fraction is related to the measured fluorescence before and immediately after photobleaching and at "infinite" time (t>>τ_D) after recovery by the following expression:

$$f_m = \frac{F(\infty) - F(0^+)}{Fi - F(0^+)} \qquad (9)$$

Equation 6 is replaced by the following:

$$\frac{F(t)}{Fi} \;=\; \sum_{n=1} \frac{(-K_b)^n}{n!}\,(1+n\,(1 + 2t/\tau_D))^{-1} \;+\; (1-fm)\,\frac{F(0^+)}{Fi} \tag{10}$$

Where K_b is defined in Eq 6. The first term represent the fluorescence of the mobiles species, which is time dependent, and the second term represents the fluorescence of the immobile species, which is constant. Now three parameters are used to fit the data for F versus t: τ_D, K_b, and fm. The diffusion coefficient for the macromolecule is obtained from the best fit value of τ_D and Eq 8. Simulations using parameters values appropriate for our experiments indicate that truncating the series of Eq. 10 after n=8 describes the curve F(t) to within 1% of the characteristic time τ_D for a Monte Carlo simulation with 2% random fluctuations in the simulated data.

Parameters were estimated by non-linear regression analysis model fitting the data to equation 10 using Sigma Plot (v 5.0) (Jandel Scientific) program.

RESULTS and DISCUSSION

Spatial distribution of proteins

Diffusion Into the Muscle Cell

Figures 2-5A show time series of confocal images of intact muscle fibers recorded during diffusion of the labeled protease inhibitors of various molecular weights, as well as that for a naturally fluorescent protein. Images show optical sections through the center of the fiber. The fluorescent intensity reflects the amount of fluorescent protein present in the examined optical section. Diffusion occurred under physiological conditions in real time at 23C. Special attention was paid to maintain equal experimental conditions regarding the composition of solutions, temperature, and flow.

Diffusion of cystatin and STI (Figures 2A and 3A) resulted in a rapid increase of fluorescent intensity homogeneously within the sarcoplasm. Faster equilibration was attained by STI (between 10 to 15 min) than by cystatin (between 15 to 20 min) wherein fluorescent intensity did not significantly change after these respective times. Diffusion of these lower molecular weight proteins (11 and 21 kDa) into the sarcoplasm did not appear to be restricted by the cell membrane.

R-phycoerythrin (240 kDA) diffused more slowly than cystatin or STI and no homogeneous fluorescence was established during the course of the experiment (65min). Diffusion of R-phycoerythrin resulted in an increase of fluorescence intensity only in the outer layers of the muscle fiber, indicating either binding or accumulation of this component by the cytoplasm.

α-2-macroglobulin was excluded from the sarcoplasm of the muscle fiber. Even after 35 min of incubation only the sarcomeres in the extreme outer layers of the fiber accumulated (FITC)-α-2-macroglobulin, a 720kDa protein.

Intracellular Binding of Proteins

Subsequent to diffusion of these proteins into muscle cells, their binding to intracellular components can be assessed by carefully exchanging the medium with fresh Ringer solution containing no protein (Figures 2-5B) to induce an outward diffusion.

Cystatin was displaced from the cell sarcoplasm, as revealed by the disappearance of fluorescence, in 15 to 20 min (Figure 2B). However, only a marginal loss in fluorescence occurred for soybean trypsin inhibitor (Figure 3B), indicating a strong binding of STI to structural proteins in the muscle cell. This binding was homogeneous within the cell even after 35min.

R-phycoerythrin showed a similar pattern of mobility in diffusing out of the cell as it had in diffusing into the cell (Figure 4B), indicating that the accumulation of this protein at the surface was not related to cytoplasmic binding of the protein.

For α-2-macroglobulin a dramatic drop in fluorescence intensity was seen immediately after the medium exchange (Figure 5B). This indicated that the development of fluorescence observed during diffusion in was merely an accumulation at the immediate surface of the fiber. This is also revealing as to the great difficulty with which this molecule is able to move in into the sarcoplasm.

Any effects of bleaching during the test scans with the confocal laser scanning microscope were ruled out by comparison with other areas of the fibers that had not been scanned previously.

Diffusion of Proteins in Aqueous Medium

Diffusion studies of FITC labeled proteins were first carried out in aqueous solutions to validate the FRAP technique and also in order to later compare with diffusion rates measured within the cell.

The cystatin solution recovered most rapidly following photobleaching as compared to the other proteins of higher molecular weight, photobleached for the same time interval (1s; Figure 6).

According to FRAP recovery curves for the three FTIC-labeled protein (Figure 7), fluorescence intensity eventually returned to near initial levels, indicating that essentially all the labeled proteins were mobile in aqueous solution.

The D values obtained from these FRAP experiments at 23C (Table 1) are in excellent agreement with the diffusion coefficients measured by others using unlabeled molecules by other techniques.

These data indicated that translational diffusion of these proteins in aqueous solution was not affected by fluorescence labeling and showed the present FRAP technique to be appropriate for protein diffusion measurements.

Table 1. Protein diffusion coefficients in aqueous solutions.

Macromolecule	Molecular weight	D_{water} (μm^2/s)±SE	D literature (μm^2/s)
Cystatin	11 kDa	$110.9_{\pm}$ 5.0	NA
Soybean trypsin inhibitor	21 kDa	$92.7_{\pm}3.4$	99*
α-2-macroglubulin	720kDa	$24.9_{\pm}$ 2.3	25**

*CRC Handbook of Biochemistry (1968)
** http://www.foresight.org/Nanomedicine/Ch03_0.html
NA: not available

Mobility of Proteins in Muscle Cells

Muscle fibers were incubated for 12 hours at 4C in FITC-labeled protein solutions prior to photobleaching measurements. Within this period of incubation all the proteins reached an equilibrium distribution within the muscle fiber. In the time series of fluorescence images of muscle fibers before and after a circular spot of ~ 4 μm was bleached (Figure 8), the darkened zone produced by the bleach pulse was progressively filled in with unbleached labeled protein as it diffused into the bleached zone.

As compared with the photobleaching data obtained in aqueous solution (Figure 7), the recoveries of photobleaching within the cells were slower and incomplete, particularly for the 720 kDa FITC α-2-macroglobulin (Figure 9). The diffusion coefficients measured within muscle fibers at 23C for the three protease inhibitors ranged between 1.5 to 10 μm^2/s (Table 2). These values for cystatin and STI are about one tenth that of the respective D in dilute protein solution (Table 1). Alpha-2-macroglobulin,

which has a larger mass, exhibited a diffusivity in the cell sarcoplasm that was decreased to 1/17 of its value in dilute solution.

Table 2. Protein diffusion coefficients in muscle cells.

	Molecular weight (kDa)	$D_{water} \pm SE$	$D_{cell} \pm SE$	Mobile fraction (%)	D_{cell}/D_{water}
Cystatin	11	110.9± 5.0	9.7± 0.6	86.2	1/11
Soybean trypsin inhibitor	21	92.7±3.4	9.5± 0.4	68.3	1/10
α-2-macroglobulin	720	24.9± 2.3	1.5±0.2	31.7	1/17

Incomplete recoveries of the fluorescence intensity were observed over the time scale of the FRAP experiments (Figures 8, 9). The apparent mobile fraction was included in Equation 10 to account for partial recovery of the fluorescence. While cystatin and soybean trypsin inhibitor exhibited the same diffusion coefficient in the muscle cell their mobile fractions were quite different; only 68% of the total population of FITC-soybean trypsin inhibitor molecules were mobile. This supports our previous finding that STI binds extensively to structural components within the muscle cell.

In our case, although STI and cystatin exhibited different diffusivities in aqueous solution in accordance with their molecular weight, once inside the cell sarcoplasm these proteins moved at the same rate. This suggests that factors other than just molecular weight affect the mobility of protein molecules within the cell.

Arrio-Dupont et al. (1996) suggested that diffusing proteins may interact with cellular structures by specific binding and non-specific interactions due to their electrostatic charges.

Three factors have been discussed in the literature as likely being mainly responsible for a low translational diffusion of macromolecules within the cell: high viscosity of the intracellular medium, steric hindrance and/or reversible binding (Kao et al.,1993). As we have previously discussed in the second manuscript, none of these offer a sufficient explanation of the observed decrease in diffusivity of inert macromolecules within cells. The intracellular diffusion of proteins that have hydrophobic domains and/or ionizable surface groups might be even slower than for inert tracer particles, due to binding interactions with intracellular components. In fact, fluorescent analogs of almost

every protein ever studied by FRAP, including many that have no known binding sites inside cells, diffuse very slowly within intact cells. In general, the cytoplasmic diffusion coefficient has not been well correlated with the radius (molecular weight) of the protein and a significant immobile component has been evident in the FRAP recovery curves (Wojcieszyn et al., 1981; Wang et al., 1982; Jacobson and Wojcieszyn, 1984; Luby-Phelps et al., 1986; Arrio-Dupont et al., 1996).

One widely studied exception, the Green Fluorecent Protein (GFP; Swaminathan et al., 1997) recovers at about the rate predicted for an inert spherical tracer of the same size - two to four times slower than in dilute aqueous solution. The crystal structure of GFP was recently reported to be a dimer 'beta can' (cylindrical, flat ended) structure with very few ionizable or hydrophobic groups on its surface, which might explain its apparent lack of binding (Ormo et al., 1996; Yang et al., 1996). On the other hand, the Kunitz soybean trypsin inhibitor consists of a single polypeptide chain crosslinked by two disulfide bridges which presents a highly hydrated surface (Song and Suh, 1998). This fact could justify the persistence fluorescent intensity during diffusing out and the high immobile fraction calculated in our FRAP studies.

Chicken cystatin conformation seems to resemble GFP conformation since it consists of a 5 stranded antiparallel beta-sheet wrapped around a five-turn alpha-helix (Turk and Bode, 1991). This beta barrel conformation seems to give a inert surface character to protein molecules and allowing them to diffuse more freely (having less constraints).

Literature on inhibition of proteolytic events within the cytoplasm is very scarce. Most of the research has been focused on diffusion within model aqueous systems (Roberts et al.,1995).

An alternate explanation is based on the theory of Ling (Ling, 1992) that water (being 80% in the muscle fiber) is totally structured by the cytoskeleton proteins. The mobility of water is thus reduced by the formation of polarized multilayers induced by relatively close contact with protein surfaces. Thermodynamically, this reduced mobility of water translates into a reduced chemical activity for cellular water as compared with bulk water. The solvent properties of this perturbated water may also differ from bulk water in a solute dependent manner, thereby explaining completely the observed reduced

diffusion coefficients of the various proteins in the present experiments (Carvajal and Lanier, 2003).

CONCLUSIONS

We observed that the equilibration of protein molecules which bind with high affinity to sarcomeric sites is not slowed down significantly compared with molecules of approximately the same size, but with no high affinity for binding. Larger molecules exhibited more difficulty in diffusing throughout the sarcomeres and tend to accumulate in the periphery of the muscle cell, showing a large gradient in fluorescent intensity in the core of the fiber. Cystatin and soybean trypsin inhibitor appeared to easily enter the cell by passive diffusion with little resistance. The hindered diffusion noted between bulk water (aqueous solution) and within the cell seems to be clearly occur in the sarcoplasm.

Thus, passive diffusion of smaller molecules of active proteins like cystatin and soybean trypsin inhibitor into intact muscle and muscle cells seems entirely feasible from a commercial standpoint. It will, however, be necessary to study the permeation of these protease inhibitors into intact muscle tissue and observe whether any resistance to diffusion is offered by the intercellular spaces. Although the alpha-2-macroglobulin cannot diffuse rapidly into the cell it will be of great interest to study its permeation through the intracellular spaces. α-2- macroglobulin may yet be capable of reducing the interchange of proteases between cells since this inhibitor can inhibit proteases of all classes. Studies to enhance the mobility of proteases by chemical or physical methods are also needed.

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FIGURE 2: Confocal images of longitudinal optical sections through the core of intact muscle fibers incubated for 35min with (A) FITC-cystatin in Ringer solution (B) Ringer solution
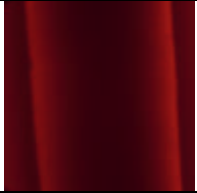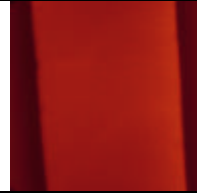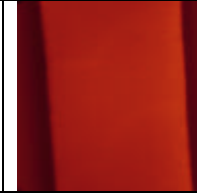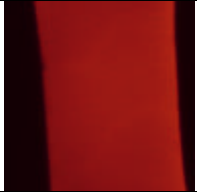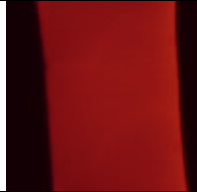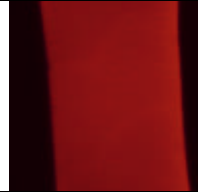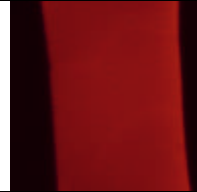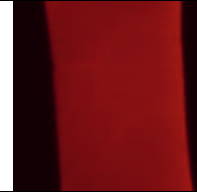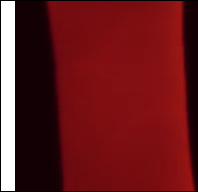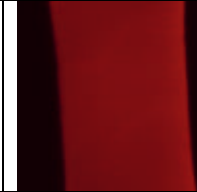
Soybean trypsin inhibitor (21 kDa)						
Time (minutes)						
5	10	15	20	25	30	35

FIGURE 3: Confocal images of longitudinal optical sections through the core of intact muscle fibers incubated for 35min with (A) FITC-soybean trypsin inhibitor in Ringer solution (B) Ringer solution

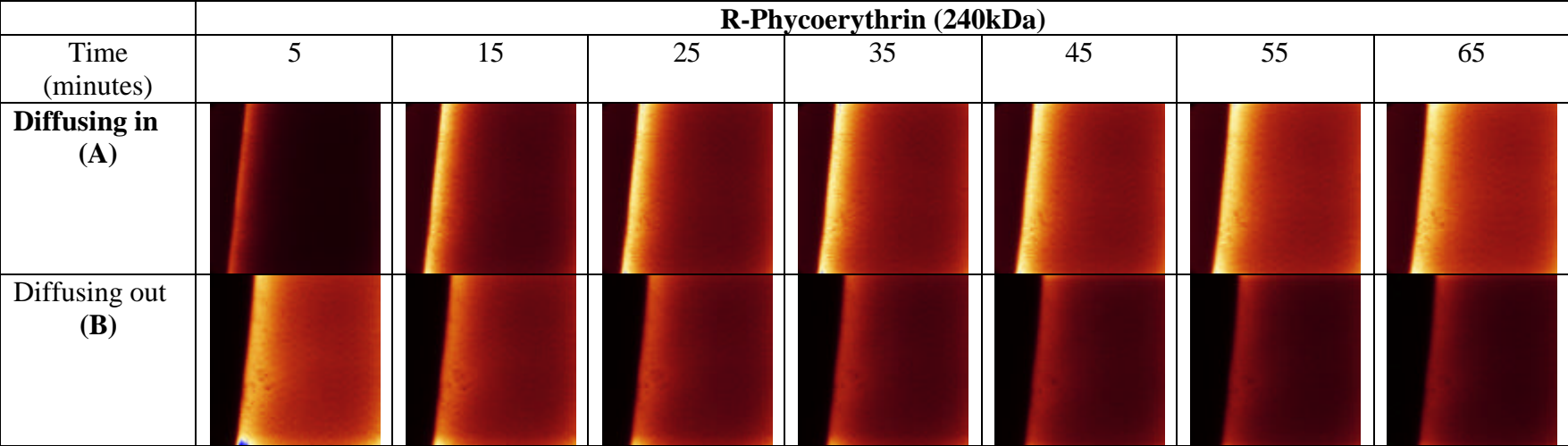| Time (minutes)	R-Phycoerythrin (240kDa)						
	5	15	25	35	45	55	65
Diffusing in (A)							
Diffusing out (B)							

FIGURE 4: Confocal images of longitudinal optical sections through the core of intact muscle fibers incubated for 55min with (A) R-Phycoerythrin in Ringer solution (B) Ringer solution

α-2-macroglobulin (720 kDa)							
Time (minutes)	5	10	15	20	25	30	35
Diffusing in (A)							
Diffusing out (B)							

FIGURE 5: Confocal images of longitudinal optical sections through the core of intact muscle fibers incubated for 35min with (A) FITC-α-2-macroglobulin in Ringer solution (B) Ringer solution
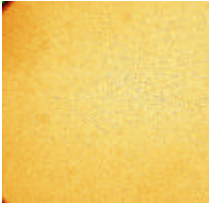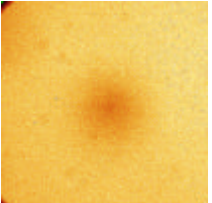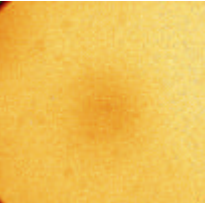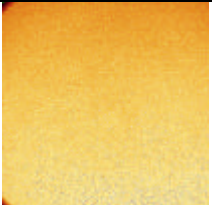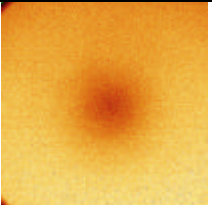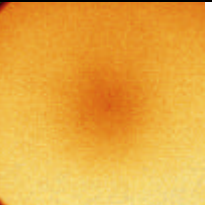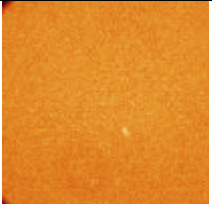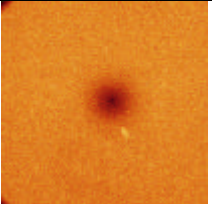
	Before	Right after	Next	Infinite
Cystatin (10kDa)				
Soybean trypsin inhibitor (21kDa)				
α-2-macroglobulin (720kDa)				

FIGURE 6: Confocal images before and after spot bleaching of labeled protein solutions
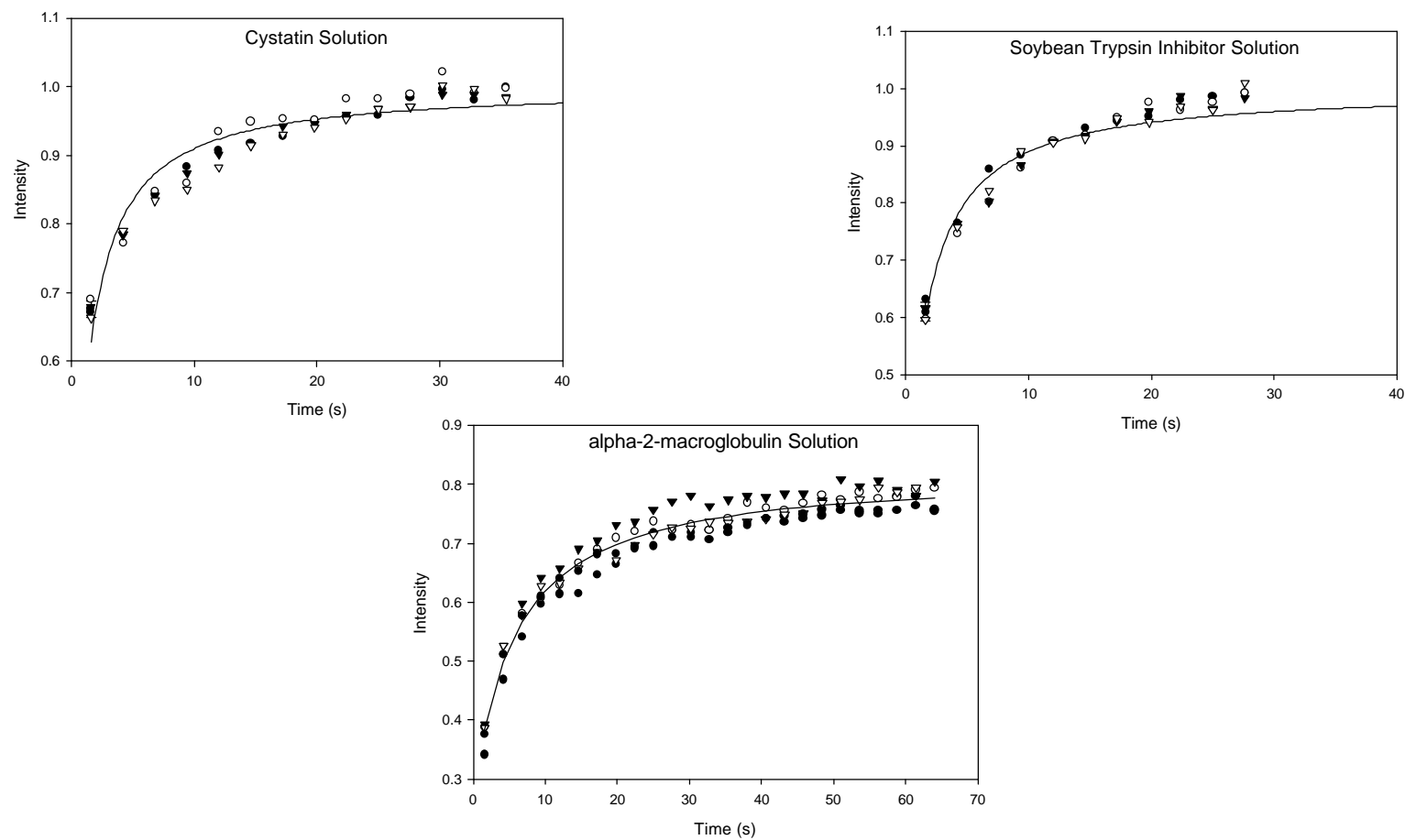
FIGURE 7: Experimental FRAP curves recorded for fluorescently labeled proteins and their respective best-fit curves in Ringer solution.

	Before	Right after	Next	Infinite
Cystatin (10kDa)				
Soybean trypsin inhibitor (21kDa)				
α-2-macroglobulin (720kDa)				
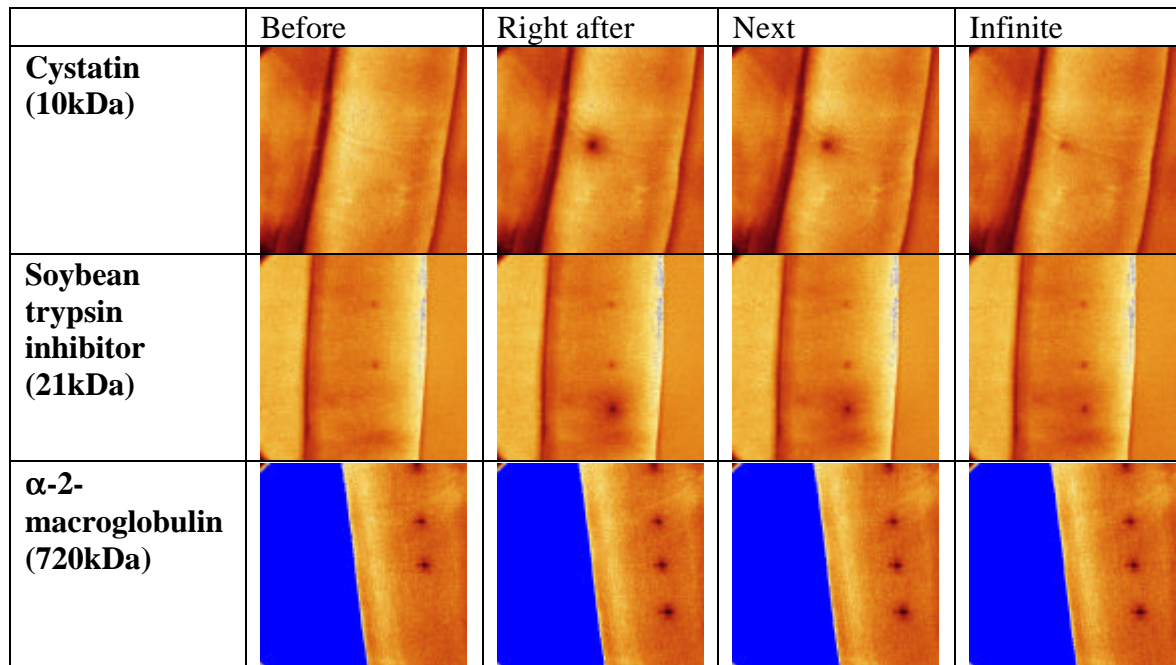
FIGURE 8: Confocal images before and after spot bleaching in muscle fibers labeled with FITC-protein solutions

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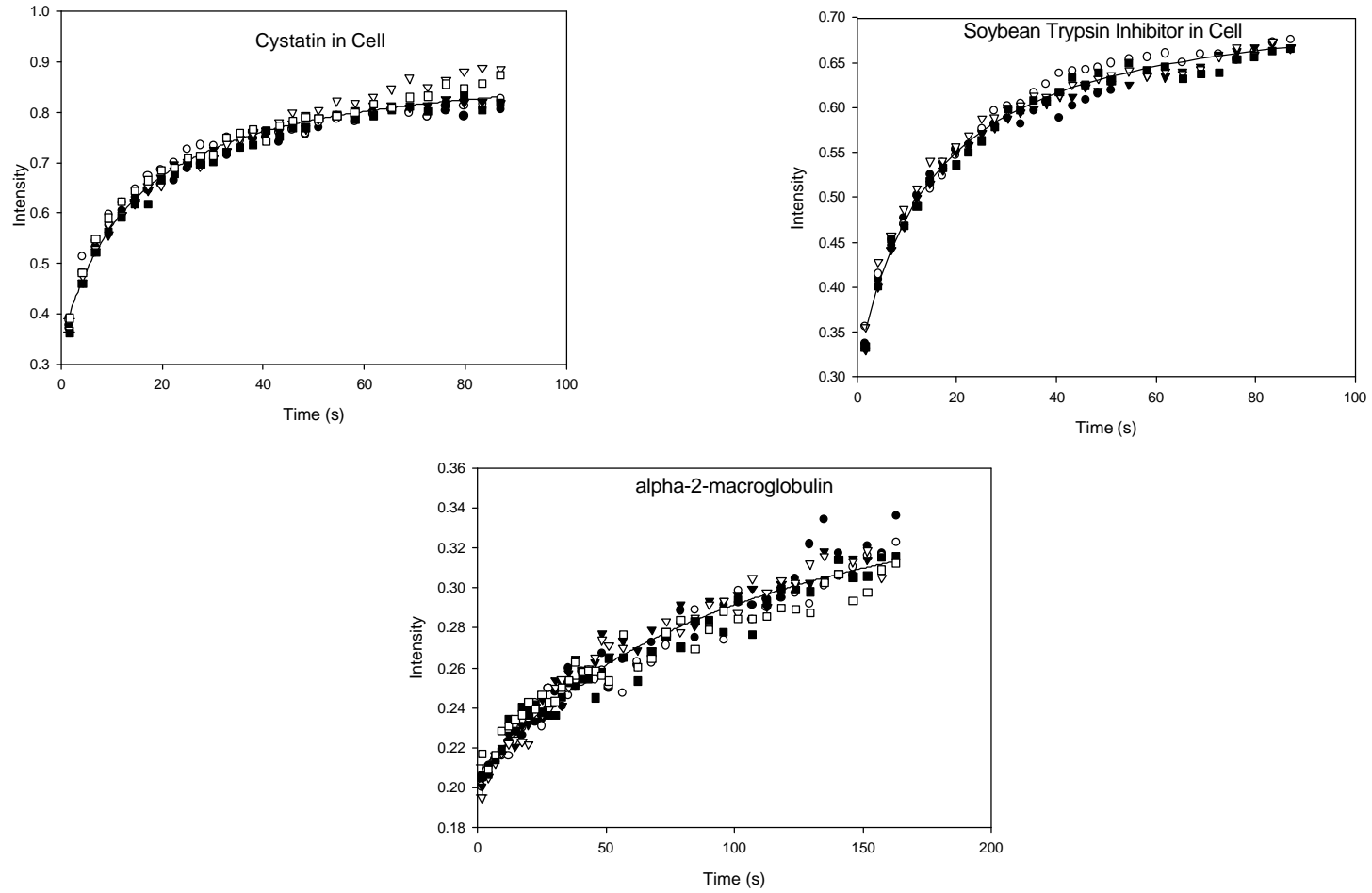
FIGURE 9: Experimental FRAP curves recorded for fluorescently labeled proteins and their respective best-fit curves in muscle fibers

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OUTPUT STADISTICAL RESULTS

Nonlinear Regression

[Variables]
x = {col(1),col(1),col(1),col(1)}
y = {col(7,1,size(col(1))),col(8,1,size(col(1))),col(9,1,size(col(1))),col(10,1,size(col(1)))}
[Parameters]
a = 2 ' {{previous: 2.02278}}
b = 1 ' {{previous: 0.99382}}
[Equation]
f=1- a/(2+2*x/b)+a^2/(factorial(2)*(3+4*x/b))-a^3/(factorial(3)*(4+6*x/b))+a^4/(factorial(4)*(5+8*x/b))-
a^5/(factorial(5)*(6+10*x/b))+a^6/(factorial(6)*(7+12*x/b))-a^7/(factorial(7)*(8+14*x/b))+a^8/(factorial(8)*(9+16*x/b))-
a^9/(factorial(9)*(10+18*x/b))+a^10/(factorial(10)*(11+20*x/b)) -a^11/(factorial(11)*(12+22*x/b))+
a^12/(factorial(12)*(13+24*x/b))-a^13/(factorial(13)*(14+26*x/b))+a^14/(factorial(14)*(15+28*x/b))-
a^15/(factorial(15)*(16+30*x/b)) +a^16/(factorial(16)*(17+32*x/b))-
a^17/(factorial(17)*(18+34*x/b))+a^18/(factorial(18)*(19+36*x/b))-
a^19/(factorial(19)*(20+38*x/b))+a^20/(factorial(20)*(21+40*x/b))-
a^21/(factorial(21)*(22+42*x/b))+a^22/(factorial(22)*(23+44*x/b))-
a^23/(factorial(23)*(24+46*x/b))+a^24/(factorial(24)*(25+48*x/b))-
a^25/(factorial(25)*(26+50*x/b))+a^26/(factorial(26)*(27+52*x/b))-
a^27/(factorial(27)*(28+54*x/b))+a^28/(factorial(28)*(29+56*x/b))-
a^29/(factorial(29)*(30+58*x/b))+a^30/(factorial(30)*(31+60*x/b))
fit f to y
[Constraints]
a>1

b>0
[Options]
tolerance=0.000100
stepsize=100
iterations=100

Cystatin Solution

R = 0.97020411   Rsqr = 0.94129601   Adj Rsqr = 0.94020890

Standard Error of Estimate = 0.0222

	Coefficient	Std. Error	t	P
a = K	2.0228	0.2881	7.0214	<0.0001
b= τ_D	0.9938	0.2747	6.8581	<0.0001

Analysis of Variance:

	DF	SS	MS	F	P
Regression	1	0.4270	0.4270	865.8694	<0.0001
Residual	54	0.0266	0.0005		
Total	55	0.4536	0.0082		

Soybean trypsin inhibitor

R = 0.97818443   Rsqr = 0.95684479   Adj Rsqr = 0.95581728

Standard Error of Estimate = 0.0236

	Coefficient	Std. Error	t	P
a= K	4.0837	1.0618	3.8461	0.0004
b= τ_D	1.1886	0.2279	5.6158	<0.0001

Analysis of Variance:

	DF	SS	MS	F	P
Regression	1	0.5191	0.5191	931.2312	<0.0001
Residual	42	0.0234	0.0006		
Total	43	0.5425	0.0126		

Alpha-2-macroglobulin solution

R = 0.97916607   Rsqr = 0.95876619   Adj Rsqr = 0.95808464

Standard Error of Estimate = 0.0200

	Coefficient	Std. Error	t	P
a= K	3.5955	0.2539	14.1602	<0.0001
b= τ_D	4.4253	0.4359	10.1515	<0.0001
c =fm	0.7567	0.0060	125.1869	<0.0001

Analysis of Variance:

	DF	SS	MS	F	P
Regression	2	1.1249	0.5624	1406.7425	<0.0001
Residual	121	0.0484	0.0004		
Total	123	1.1733	0.0095		

Cystatin in Muscle Cell

R = 0.98715210   Rsqr = 0.97446928   Adj Rsqr = 0.97418079

Standard Error of Estimate = 0.0182

	Coefficient	Std. Error	t	P
A=K	2.7323	0.0846	32.3098	<0.0001
b= τ_D	11.4105	0.6716	16.9910	<0.0001
c =fm	0.8619	0.0077	111.6755	<0.0001

Analysis of Variance:

	DF	SS	MS	F	P
Regression	2	2.2288	1.1144	3377.9118	<0.0001
Residual	177	0.0584	0.0003		
Total	179	2.2872	0.0128		

Soybean Trypsin Inhibitor in Muscle Cell

R = 0.99192802   Rsqr = 0.98392119   Adj Rsqr = 0.98372863

Standard Error of Estimate = 0.0105

	Coefficient	Std. Error	t	P
a=K	3.0162	0.0554	54.4547	<0.0001
b= τ_D	11.5806	0.5444	21.2726	<0.0001
c=fm	0.6832	0.0042	141.6785	<0.0001

Analysis of Variance:

	DF	SS	MS	F	P
Regression	2	1.1264	0.5632	5109.6704	<0.0001
Residual	167	0.0184	0.0001		
Total	169	1.1448	0.0068		

Alpha-2-macroglobulin in Muscle Cell

R = 0.97478723   Rsqr = 0.95021014   Adj Rsqr = 0.94978458

Standard Error of Estimate = 0.0072

	Coefficient	Std. Error	t	P
a=K	4.7937	0.0376	127.4104	<0.0001
b= τ_D	74.2421	6.8757	10.7977	<0.0001
c=fm	0.3173	0.0083	26.7069	<0.0001

Analysis of Variance:

	DF	SS	MS	F	P
Regression	2	0.2331	0.1165	2232.8759	<0.0001
Residual	234	0.0122	0.0001		
Total	236	0.2453	0.0010		