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ARTICLE

Diffusion of Active Proteins into Fish Meat To Minimize Proteolytic Degradation

Patricio A. Carvajal-Rondanelli*,†,§ and Tyre C. Lanier#

†Escuela de Alimentos, Facultad de Recursos Naturales, Pontificia Universidad Católica de Valparaíso, Avenida Brasil 2950, Valparaíso, Chile, §Centro Regional de Estudios en Alimentos Saludables, CREAS, Valparaíso, Chile, and #Department of Food, Bioprocessing and Nutrition Sciences, North Carolina State University, Raleigh, North Carolina 27695

Proteases in fish muscle often cause undesired softening of intact meat pieces during refrigerated storage or slow cooking. Several food-grade proteinaceous inhibitors can overcome this softening if properly delivered to the intracellular sites where proteases are located. Fluorescence recovery after photobleaching (FRAP) and 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 (720 kDa), soybean 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 (35 min), cystatin and soybean trypsin inhibitor diffused through the cell membrane (sarcolemma) and sarcoplasm, but at a considerably slower rate (>10-fold difference) than in dilute aqueous solution. α-2-Macroglobulin did not diffuse into muscle cells within the time frame of the experiment, but did completely penetrate the cell during overnight exposure. The present study thus shows a clear dependence of D on protein inhibitor size when moving within intact skeletal muscle fibers. Low molecular weight protease inhibitors such as cystatin can be effectively diffused into intact fish muscle cells to minimize proteolytic activity and meat softening.

KEYWORDS: Protease inhibitor diffusion; fish skeletal muscle; confocal microscopy; fluorescence recovery after photobleaching; FRAP

INTRODUCTION

Proteolytic degradation of fish muscle continues post-mortem as a result of normal endogenous proteolytic activity without the counteraction of active protein synthesis. Slower cooking methods in particular can promote proteolysis and often result in undesirable softening of the cooked meat (*1*, *2*). There are many specific examples of endogenous proteolytic activity affecting the texture of fish meat or products made from it (*3*). Pacific whiting (*Merluccius* spp.) may be parasitized by *Myxosporidia* cysts, which induce high levels of cathepsin L protease that soften or even dissolve the flesh upon slow cooking (*4*, *2*). Arrowtooth flounder (*Atheresthes stomia*) exhibits a white, even-textured (when raw) fillet, which would be highly acceptable to the American market except that slower cooking methods (even conventional oven broiling) often render the meat unacceptably mushy in texture (*5*).

Several studies have explored the use of food-grade protease inhibitors, such as beef plasma, whey proteins, potato proteins, and egg albumin (*1*, *6*, *7*) as a means of preventing heat-induced degradation of fish fillet texture by endogenous proteases. Lamb-Sutton (*6*) found, however, that the minimum concentration of whole beef plasma required in the meat to achieve inactivation of proteolytic softening detracted from the flavor and appearance of the fillets. Subsequent scale-up trials involving tumbling marination of whole fillets showed inconsistent delivery of the inhibitor solution within individual fillets. Similar disappointing results for injection and soaking of fillets at commercial scale with plasma, egg white, and other commonly used food-grade protease inhibitors had been reported by McFarland (*8*).

These problems in part could be attributed to the relative impurity of the inhibitor component of such food-grade inhibitors. In the past decade several laboratories have been able to commercially fractionate and purify active protease inhibitor components such as cystatin (*9*), soybean trypsin inhibitor (*10*), and α-2-macroglobulin (*11*). Because the majority of problems with proteases affecting fish texture involve cysteine proteases, the cystatins are of greatest commercial interest. Cystatins have been successfully expressed as recombinant proteins in plants such as rice, corn, and potatoes. Chicken cystatin is a powerful protease inhibitor of lysosomal cysteine proteases. Its inhibition is extremely tight and rapid as compared to that of other cysteine protease inhibitors and plant cystatins (*12*, *13*). Several cystatin genes have been cloned and expressed in *Escherichia coli* (*14*), *Pichia pastoris* X-33, a methylotrophic yeast (*15*, *16*), and *Saccharomyces cerevisiae* (*17*), 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. Kang and Lanier (*18*) successfully infused a recombinant cystatin into arrowtooth flounder muscle

*Corresponding author (telephone +56-32-227-4218; fax +56-32-227-4205; e-mail Patricio.Carvajal@ucv.cl).

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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 (*19*). Toyohara and others (*20*, *21*) showed that the heat-stable proteases in threadfin bream were inhibited by STI, whereas Yongsawatdigul et al. (*22*) showed that STI successfully inhibited proteolysis in tilapia.

MacDonald and Lanier (in Hamann et al., 1990) (*7*) 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 classes of proteases (*23*). Lamb-Sutton (*6*) found that commercially purified α-2-macroglobulin and a commercially made dried beef plasma product containing α-2-macroglobulin inhibited the inherent heat-stable proteolytic activity in surimi gels made from Atlantic menhaden, Pacific whiting, Alaska pollock, and arrowtooth flounder. A plasma fraction enriched in α-2-macroglobulin also seemed to firm chunks of arrowtooth flounder during subsequent cooking at a much lower total protein concentration in the diffusing solution than was required using beef plasma solution.

In all of these previous studies, the effects of protease inhibitors were measured on the finished product after their delivery and the proteins themselves were not tracked. The aim of this research was to measure whether effective delivery of protease inhibitors into meats could be achieved and to characterize the influence of the medium (meat fibers) on their molecular diffusion.

MATERIALS AND METHODS

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 North Carolina 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 (*24*, *25*). Throughout the experiment, isolated tissue was superfused with Ringer's solution. Halibut strips were used because their fibers are bigger than those of many fishes and easier to isolate. To cryoprotect the strips for freezing, they were incubated for at least 30 min in each of four successive solutions containing 0.5, 1.0, 1.5, and 2 M sucrose, respectively. Following equilibration with 2 M sucrose, the strips were cut parallel to the fiber orientation and trimmed to a diameter of approximately 1.5 mm and a length of 10−15 mm. They then were rapidly frozen in liquid nitrogen and then stored at −80 °C. For thawing, the strips were transferred directly into a solution containing 2 M sucrose. Subsequently, the strips were again soaked successively for 30 min each in solutions of 1.5, 1, and 0.5 M sucrose and finally held in Ringer's solution. From these strips, single fibers were isolated using fine tweezers and a stereomicroscope (Bausch and Lomb). To further mitigate any deleterious effects of freezing, we treated the muscle fibers in medium containing 0.4% polyvinylpyrrolidine to restore membrane lipid fluidity to normal levels (*26*). 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 are not changed due to this freezing procedure (*27*).

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, 1 mm deep, held 200 μL, sufficient volume to ensure that the fibers were completely surrounded by solution. Fiber ends were glued in place with cyanoacrylate glue. Test solutions were added and removed with a plastic transfer pipet with a narrow tip. All solutions were adjusted to pH 7.2 at the experimental temperature of 22 °C for real-time confocal microscopy.

Protease Inhibitors. We monitored the diffusion of three proteins: (1) a recombinant cystatin (cystein protease inhibitor) of plant (soy) origin produced in our laboratory (MW ∼ 12 kDa; (*18*)); (2) α-2-macroglobulin (Sigma, MW ∼ 720 kDa), a protease inhibitor capable of irreversible binding and therefore inhibiting a wide variety of proteases; and (3) soybean trypsin inhibitor (STI; Sigma, MW ∼ 21 kDa), a potent chymotrypsin inhibitor that has been studied extensively for its ability to prevent carcinogenesis in both in vivo and in vitro systems (*28*).

Fluorescent Labeling of Protease Inhibitors. Protease inhibitors were labeled with fluorescein isothiocyanate (FITC, isomer I, Sigma) according to methods outlined by Hermanson (*29*). FITC was dissolved in DMSO and then mixed with the test protein solutions dissolved in 100 mM sodium carbonate buffer (pH 9.0) and reacted overnight in the dark. Excess label was then removed from the protein solution by gel filtration (Sephadex G-25, Sigma). The fluorophore/protein solution was diluted with Ringer's solution, labeling was evaluated spectrophotometrically at pH 7.2, and the average molar ratios of dye to protein subunit were 1.0, 1.1, and 1.5 mol/mol for FITC−cystatin, FITC−STI, and FITC−α-2-macroglobulin, respectively. All fluorescently labeled proteins were adjusted to the same intensity using ultrafiltration membranes (Amicon, Beverly, MA) to concentrate the protein solutions to their final absorbance.

Protein Spatial Distribution. Confocal images were collected from a Leica inverted microscope attached to a Leica confocal scanning unit (TCS SP; Leica, Wetzlar, Germany) using a 40×, 1.25 NA oil immersion lens. For time-lapse imaging of FITC-labeled proteins, images were taken every 5 min with each plane being averaged two times. FITC was excited by an argon ion laser at 488 nm with emission recorded at 500−550 nm. The microscope had the ability to record the differential interference contrast (DIC) 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, Rochester, NY).

For diffusion of fluorescent components into the muscle fibers, the respective fluorescently labeled molecules were immersed in Ringer's solution. All solutions were adjusted to pH 7.2 at the experimental temperature of 23 °C for real time confocal microscopy. For diffusing out experiments, we used the same Ringer's solution but without the addition of the test 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 min 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's solution. Then a second round of scanning was carried out as the molecules diffused out of the fibers and into fresh Ringer's solution.

Diffusion Measurements. *Fluorescence Recovery after Photobleaching* (*FRAP*). To measure the diffusion rates of the inhibitor proteins, FRAP was performed on FITC-labeled proteins in free solution (Ringer's) as well as those already diffused into fibers. The 488 nm line of a 100 mW argon ion laser was used for sample bleaching and fluorescence excitation. The recovery of fluorescence at the bleached spot, which indicates diffusion of the surrounding unbleached molecules into that area, was monitored at 520 nm using the time lapse/bleach function on a Leica TCS NT. Typical settings for bleach and recovery scans were 0.3% of the maximum laser power for 0.5−2 s, followed by a time series of up to 50 recovery images obtained typically over 0.5−170 min. For slowly diffusing samples (high molecular weight macromolecules), four or more images were collected at each time point (recovery curves). Images were taken from the center of the fiber using a 40× 1.25 NA oil immersion lens and an optical zoom of 1.0. Diffusion of FITC-labeled proteins in free Ringer's solution was measured on a regular slide with a coverslip sealed with paraffin.

Data Analysis of FRAP. Diffusion coefficients and mobile fractions of protease inhibitors were obtained by fitting photobleaching data to a solution of Fick's equation by Tong and Anderson (*30*, *31*):

$$\frac{F(t)}{\mathrm{Fi}} = f_{\mathrm{m}}\left[\sum_{n=0}^{\infty}\frac{(-K_{\mathrm{b}})^n}{n!}\left(1+n(1+2t/\tau_{\mathrm{D}})\right)^{-1}\right] + (1-f_{\mathrm{m}})\frac{F(0^+)}{\mathrm{Fi}} \quad (1)$$

F is the fluorescent emission, Fi is the measured value of F before the photobleaching step, and $F(0^+)$ is the fluorescent signal immediately after

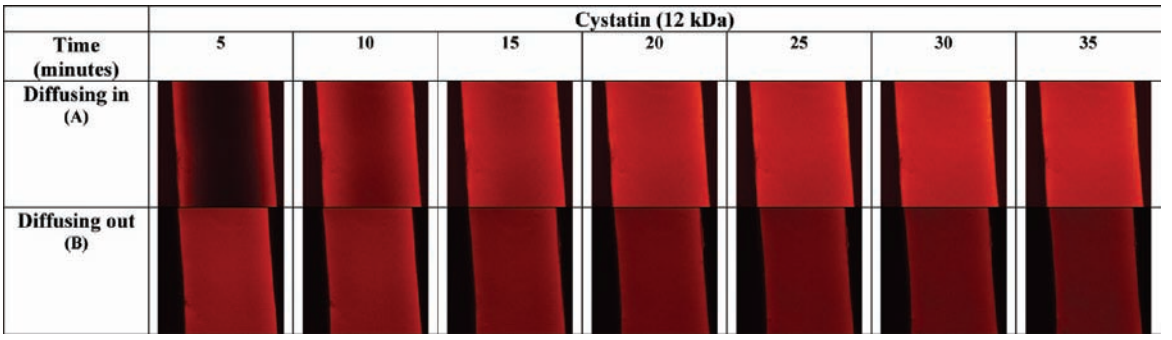

Figure 1. Confocal images of a longitudinal optical section through the core of an intact muscle fiber incubated for 35 min in (**A**) FITC−cystatin in Ringer's solution or (**B**) pure Ringer's solution following diffusion of FITC−cystatin into the fiber.

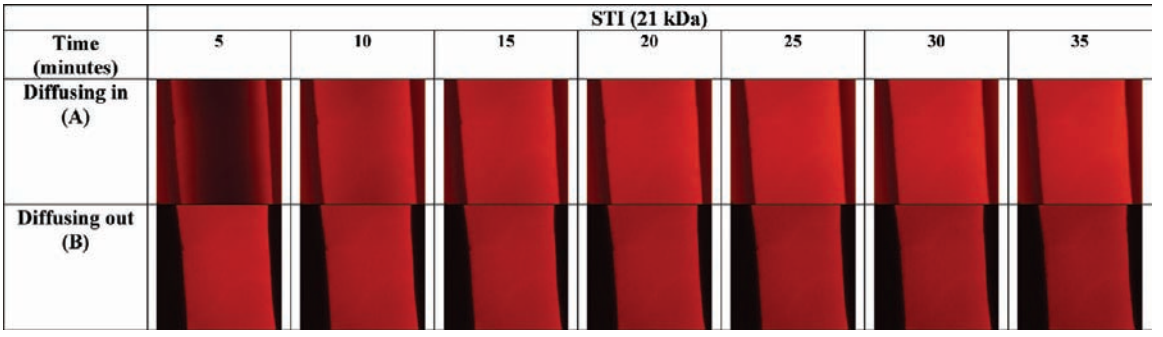

Figure 2. Confocal images of a longitudinal optical section through the core of an intact muscle fiber incubated for 35 min in (**A**) FITC−soybean trypsin inhibitor in Ringer's solution or (**B**) pure Ringer's solution following diffusion of FITC−STI into the fiber.

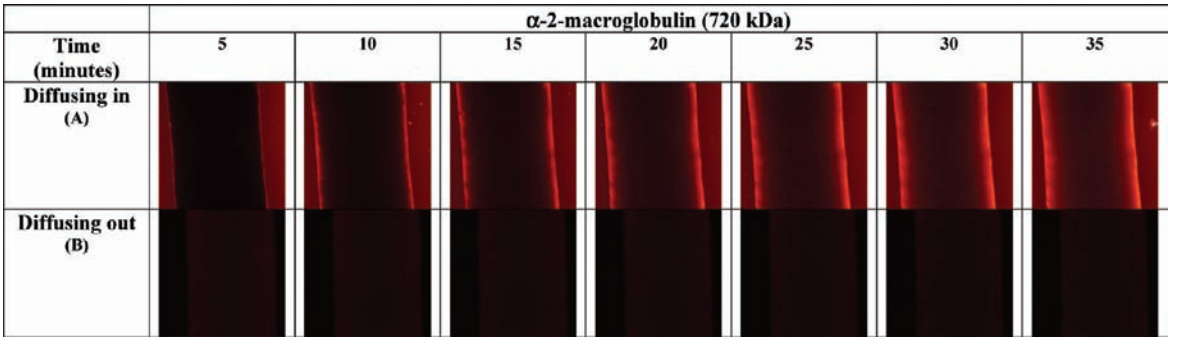

Figure 3. Confocal images of a longitudinal optical section through the core of an intact muscle fiber incubated for 35 min in (**A**) FITC−α-2-macroglobulin in Ringer's solution or (**B**) pure Ringer's solution following diffusion of FITC−α-2-macroglobulin into the fiber.

the photobleaching step. The first term represents the fluorescence of the mobile species, which is time dependent, and the second term represents the fluorescence of the immobile species, which is constant. Three parameters were used to fit the data for F versus t: the diffusion time (τ_D), the constant K_b, and the mobile fraction of molecules (f_m). The diffusion coefficient for the macromolecule was obtained from the best-fit value of τ_D and eq 2 that relates to the beam radius (ω).

$$\tau_D = \omega^2/4D \quad (2)$$

Simulations using appropriate parameter values indicated that truncating the series of eq 1 after $n = 8$ described 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 (*30*). Parameters were estimated by nonlinear regression using Sigma Plot software (v 5.0) (Systat Software, Inc.).

RESULTS AND DISCUSSION

Spatial Distribution of Proteins. *Diffusion into the Muscle Cell.* **Figures 1A**, **2A**, and **3A** show time series of confocal images of intact muscle fibers recorded during diffusion of the labeled protease inhibitors of various molecular weights into fibers. Images show the optical section at 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 23 °C. Special attention was given to maintain equal experimental conditions regarding the composition of solutions, temperature, and flow. Any effects of photobleaching during the test scans were ruled out by comparison with other areas of the fibers that had not been scanned previously.

Diffusion of cystatin and STI (**Figures 1A** and **2A**) resulted in a rapid increase of fluorescent intensity homogeneously within the sarcoplasm. Faster equilibration was attained by STI (between 10 and 15 min) than by cystatin (between 15 and 20 min) wherein fluorescent intensity did not significantly change after these respective times. Diffusion of these lower molecular weight proteins (12 and 21 kDa) into the sarcoplasm did not appear to be restricted by the cell membrane. Conversely, α-2-macroglobulin

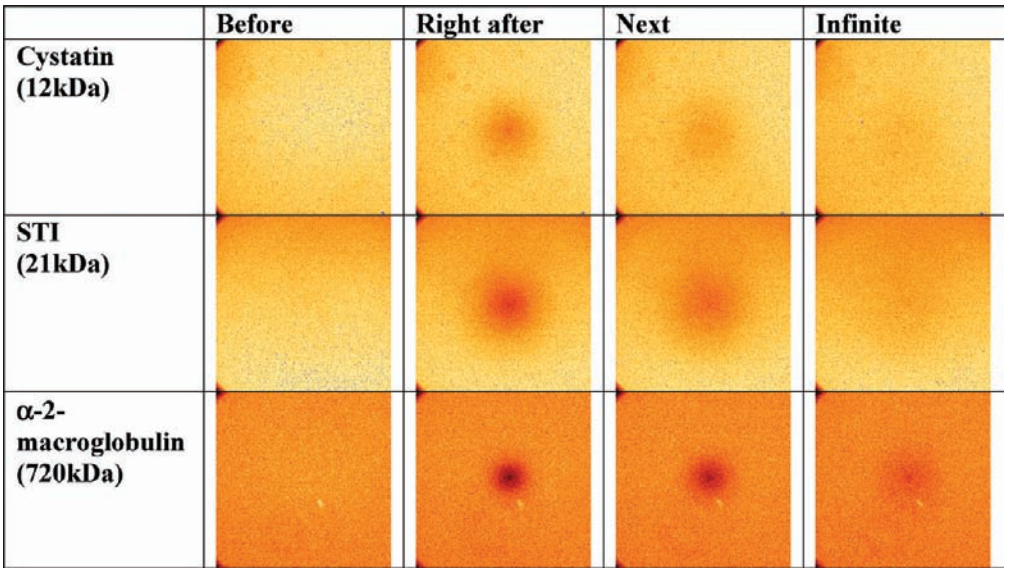

Figure 4. Confocal images before and after spot bleaching of labeled protein solutions.

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 720 kDa protein (**Figure 3A**).

Intracellular Binding of Proteins. Subsequent to diffusion of these proteins into muscle cells, their binding to intracellular components was assessed by carefully exchanging the protein-containing medium with fresh Ringer's solution (**Figures 1B**, **2B**, and **3B**) to induce an outward diffusion. Cystatin was easily displaced from the cell sarcoplasm, as revealed by the disappearance of fluorescence, in 15−20 min (**Figure 1B**). However, only a marginal loss in fluorescence occurred for STI (**Figure 2B**), indicating a strong binding of STI to structural proteins inside the muscle cell. This binding was homogeneous within the cell even after 35 min.

For α-2-macroglobulin a dramatic drop in fluorescence intensity was seen immediately after the medium exchange (**Figure 3B**). This indicated that the development of fluorescence observed during inward diffusion was merely an accumulation at the immediate surface of the fiber, revealing the great difficulty for this molecule to move across the sarcolemma and into the sarcoplasm within the test time. During a longer incubation of 12 h, however, α-2-macroglobulin was able to penetrate into and disperse within the muscle cell (**Figure 6**).

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 to later compare with diffusion rates measured within the cell. The cystatin solution recovered fluorescence most rapidly following photobleaching as compared to the other proteins of higher molecular weight, photobleached for the same time interval (1 s; **Figure 4**).

According to FRAP recovery curves for the three FTIC-labeled proteins (**Figure 5**), fluorescence intensity eventually returned to near initial levels, indicating that essentially all of the labeled proteins were mobile in aqueous solution. The *D* values obtained from these FRAP experiments at 23 °C (**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.

Mobility of Proteins in Muscle Cells. Muscle fibers were incubated for 12 h at 4 °C in FITC-labeled protein solutions prior to photobleaching measurements. Within this period of incubation, all of 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 6**), the darkened zone produced by the bleach pulse was progressively filled in with surrounding labeled protein (unbleached) as it diffused into the bleached zone.

As compared with the photobleaching data obtained in aqueous solution (**Figure 4**), the recoveries of photobleaching within the cells were slower and incomplete, particularly for the 720 kDa FITC−α-2-macroglobulin (**Figure 7**). The diffusion coefficients measured within muscle fibers at 23 °C for the three protease inhibitors ranged between 1.5 and 10 $\mu m^2/s$ (**Table 2**). These values for cystatin and STI are about $1/_{10}$ that of the respective *D* in dilute protein solution (**Table 1**). α-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.

Incomplete recoveries of the fluorescence intensity were observed over the time scale of the FRAP experiments (**Figures 6** and **7**). The apparent mobile fraction was included in eq 1 to account for partial recovery of the fluorescence. Whereas cystatin and STI exhibited the same diffusion coefficient within the muscle cell, their mobile fractions were quite different; only 68% of the total population of FITC−STI molecules was 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.

Three main factors have been discussed in the literature as likely being responsible for a low translational diffusion of macromolecules within the cell: high viscosity of the intracellular medium, steric hindrance, and/or reversible binding (*32*, *33*). None of these offer a sufficient explanation of the observed decrease in diffusivity of inert macromolecules within cells, however (*34*). Arrio-Dupont et al. (*35*) also suggested that diffusing proteins may interact with cellular structures by specific binding and nonspecific interactions due to their electrostatic

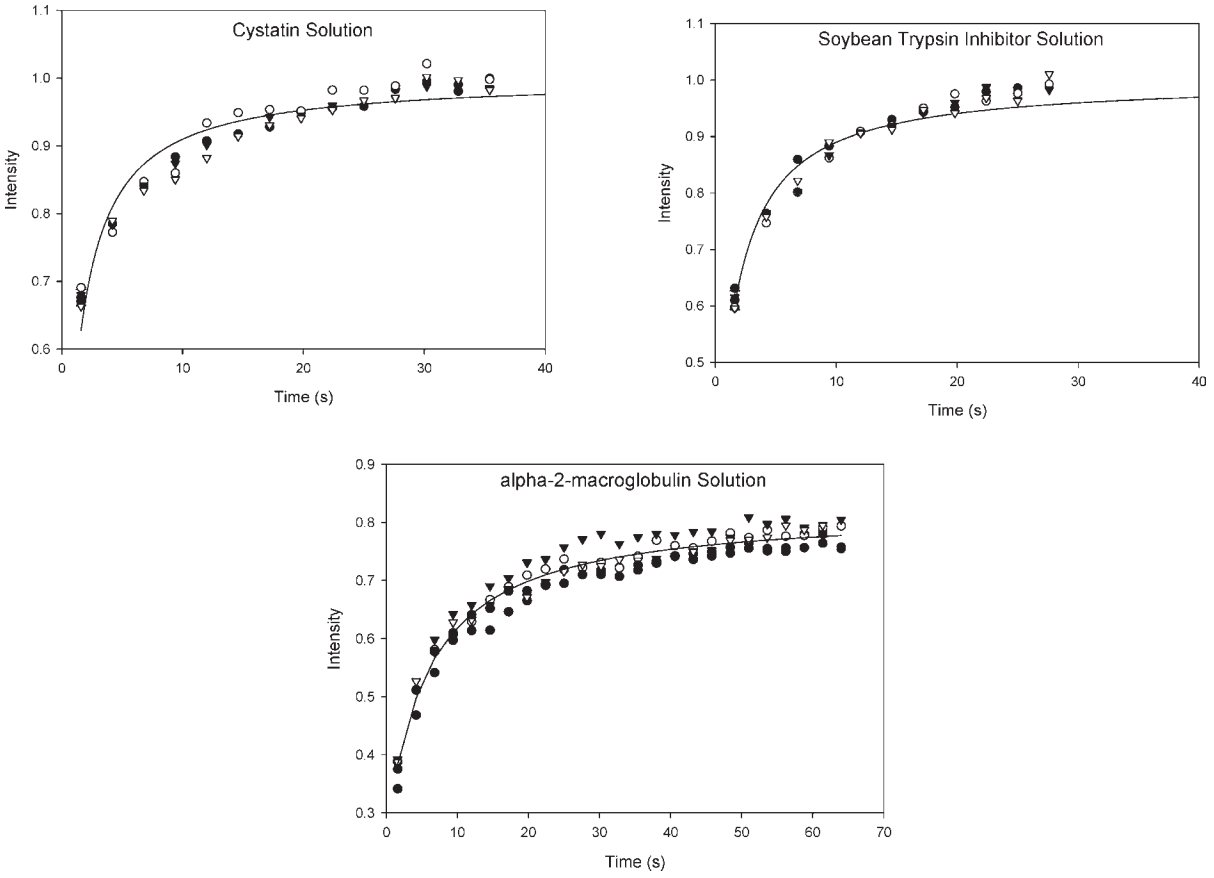

Figure 5. Experimental FRAP curves recorded for fluorescently labeled proteins and their respective best-fit curves in Ringer's solution.

Table 1. Protein Diffusion Coefficients of FITC-Labeled Protease Inhibitors in Aqueous Solution

macromolecule	mol wt (kDa)	D_{water} ($\mu m^2/s$) ± SE	D lit. ($\mu m^2/s$)
cystatin	12	110.9 ± 5.0	NA[a]
soybean trypsin inhibitor	21	92.7 ± 3.4	99[b]
α-2-macroglobulin	720	24.9 ± 2.3	25[c]

[a] NA, not available. [b] CRC Handbook of Biochemistry (1968). [c] http://www.foresight.org/Nanomedicine/Ch03_0.html.

charges. Thus, 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 analogues 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 many FRAP recovery curves (*35*−*39*).

One widely studied exception, the green fluorescent protein (GFP), recovers at about the rate predicted for an inert spherical tracer of the same size, that is, 2−4 times slower than in dilute aqueous solution (*40*). 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 (*41*, *42*). In contrast, the Kunitz STI used in the present study consists of a single polypeptide chain cross-linked by two disulfide bridges, which presents a highly hydrated surface (*19*). This fact could justify the persistent fluorescent intensity during diffusing out and the high immobile fraction calculated in our FRAP studies. The conformation of chicken cystatin seems to resemble GFP's conformation because it consists of a five-stranded antiparallel β-sheet wrapped around a five-turn α-helix (*12*). This β-barrel conformation seems to give an inert surface character to protein molecules, allowing them to diffuse more freely (having fewer constraints).

An alternate explanation for reduced diffusion within cells compared to that in free solution is based on the theory of Ling (*43*) 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 perturbed 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.

Conclusions. We observed that the equilibrium diffusion rate of protein molecules which bind with high affinity to sarcomeric sites is not slowed significantly compared with molecules of approximately the same size, but without an affinity for binding. Extremely large molecules exhibited more difficulty in diffusing into the sarcomeres and thus tended to accumulate at the periphery of the muscle cell during short incubation times. Cystatin and soybean trypsin inhibitor appeared to easily enter the cell by passive diffusion with little resistance. The hindered diffusion within the cell as compared to that noted in bulk water (aqueous solution) seems to mainly occur within the sarcoplasm and is likely associated with structuring of water by the sarcoplasmic proteins.

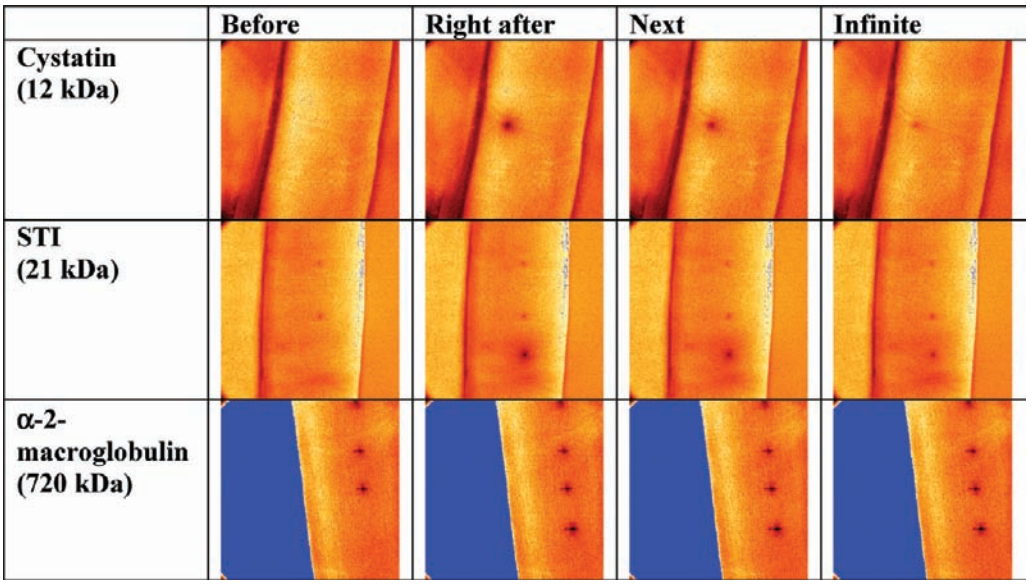

Figure 6. Confocal images before and after spot bleaching in muscle fibers containing FITC-labeled protein.

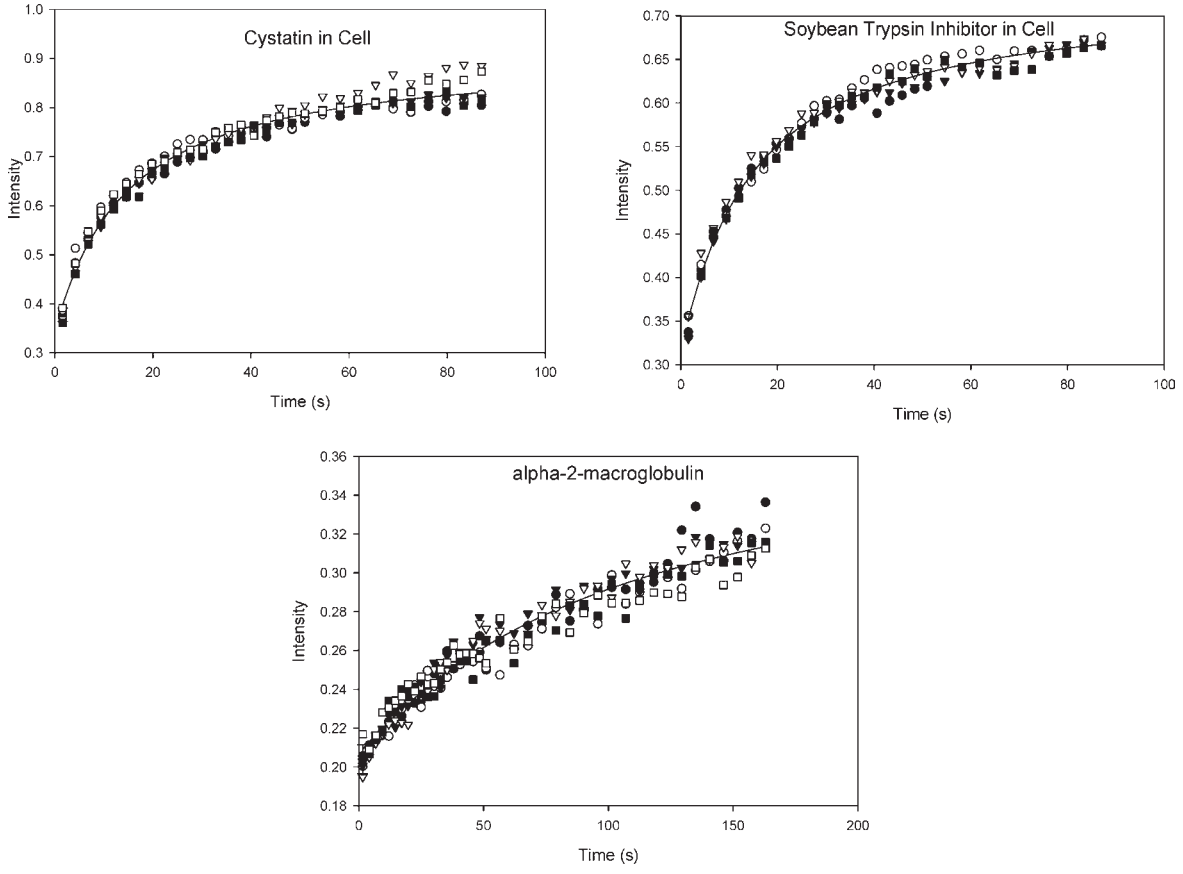

Figure 7. Experimental FRAP curves recorded for fluorescently labeled proteins in muscle fibers and their respective best-fit curves.

Table 2. Protein Diffusion Coefficients of FITC-Labeled Protease Inhibitors in Muscle Cells (Fibers)

macromolecule	mol wt (kDa)	$D_{water} \pm$ SE	$D_{cell} \pm$ SE	mobile fraction (%)	D_{cell}/D_{water}
cystatin	12	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

Thus, passive diffusion of smaller molecules of active proteins such as cystatin and soybean trypsin inhibitor into intact muscle and muscle cells seems entirely feasible from a commercial application 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 α-2-macroglobulin cannot diffuse rapidly into the cell, it should be of interest to study its permeation through the intracellular spaces. α-2- Macroglobulin may yet be useful in treating meats to reduce the interchange of proteases between cells because this inhibitor can inhibit proteases of all classes.

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