STRUCTURAL CHARACTERIZATION OF THE FLIGHT MUSCLE OF MAN DU CA
SEXTA

BY

YU-SHU CHENG

DEPARTMENT OF BIOLOGICAL AND CHEMICAL SCIENCES

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Approved _________________________

Adviser

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<tr>
<td>λ</td>
<td>Wave length</td>
</tr>
<tr>
<td>x</td>
<td>The distance between zero-order and first-order lines</td>
</tr>
<tr>
<td>h</td>
<td>The height from muscle preparation to screen</td>
</tr>
<tr>
<td>Sin θ</td>
<td>Angle of diffraction</td>
</tr>
<tr>
<td>d</td>
<td>Separation between the planes of the crystal</td>
</tr>
<tr>
<td>pCa</td>
<td>Negative log of available calcium concentration</td>
</tr>
<tr>
<td>C</td>
<td>Celsius</td>
</tr>
<tr>
<td>SL</td>
<td>Sarcomere length</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine-N,N,N’,N’-tetraacetic acid</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometer</td>
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ABSTRACT

There are several properties of the flight muscle *Manduca sexta* that are interesting. In its physiological characteristics, like mammalian skeletal and cardiac striated muscle, it is a synchronous muscle. However, it is much more similar structurally to the more widely known asynchronous insect flight muscles of *Drosophila* and *Lethocerus*. The goal of the thesis is to study the structural characteristics and perform mechanical studies of dorsal and ventral muscle of *Manduca* flight muscle. In pursuit of this goal, a secondary purpose was to develop better storage conditions for skinned insect muscle which can maintain muscle structure and function.

Maximum active force as a function of time in storage was compared for storing at 4°C without glycerol and storing at -80°C with 75% glycerol. The maximum force values were almost the same during days 0-4. However, the muscle stored at -80°C with 75% glycerol gave higher active force in high calcium (pCa 4.5) solution over a longer time in storage than muscle stored at 4°C. Both ventral and dorsal muscles can deliver 40-50% of original active force for up to 21 days in storage.

X-ray diffraction experiments were done to compare the response of chemically skinned *Manduca* flight muscle to temperature and to the induction of rigor. The lattice spacing (d₁₀) decreased and intensity ratio I₂₀/I₁₀ increased when the temperature of the relaxing solution bathing the solution increased from 10°C to 40°C. Lattice spacing (d₁₀) decreased, but the I₂₀/I₁₀ intensity ratio increased slightly while concentration of dextran increased from 1% to 6%. Six percent dextran was insufficient to restore the *in vivo* lattice spacing.
CHAPTER 1
INTRODUCTION

Muscles, the basic tissues of animals that allow them to move, represent one of the most sophisticated machines inside of the body. The motion not only allows body movement but also support the life support systems such as heart beating. The muscles in different animals are not all identical in structure, even though they appear to generate force by the same molecule mechanism.

![Classification of the major muscle types](image)

Figure 1.1 Classification of the major muscle types. (Adapted from J. Squire 1981)

The above list which is not exhaustive but include the main types used in muscle research to date. All types of muscle fibers produce movement by contraction; these muscles share many common structural features and have the same basic mechanism to generate force. However, the diversity of muscle structures over the animal kingdom might cause distinct ways of force generation in different species. In order to generate force, muscle has specific structural proteins arranged regularly and the arrangement of the actin and myosin myofilaments differ across the animal kingdom.
The flight muscle of the Hawk moth, *Manduca sexta*, is a synchronous, non-fibrillar muscle. Very little is known about the structure and function of this kind of muscle compared to the much better studied asynchronous fibrillar insect flight muscles, vertebrate skeletal and cardiac muscles. One of the goals of the research reported here is to use *Manduca sexta* insect flight muscle to study the structural basis of changes using laser light diffraction and X-ray diffraction *in vitro*.

1.1 **Muscle Physiology**

There are three types of muscle, skeletal muscle, cardiac muscle and smooth, in the human body (Figure 1.1). A single striated muscle bundle contains hundreds of muscle fibers; it has thousands of myofibrils which made from many of sarcomeres. There are easily to distinguish by their appearance under the light microscope. Skeletal muscle and cardiac muscle are striated muscles which have similarities in intracellular structure and the physiological responses to high concentration of calcium solution. All of the insect muscles are striated. There are two types of insect flight muscle, synchronous muscle and asynchronous muscle. In synchronous muscle, the frequency of muscle contraction is faster than the nerve frequency of stimulation (Josephson, 2005). Synchronous muscle elicits a muscle contraction in response to one nervous action potential.
Figure 1.2 Structural organizations in a typical vertebrate skeletal muscle. (Adapted from C.R. Bagshaw 1982)

Figure 1.2 illustrates the levels of structure in skeletal muscle which is made up of many multi-single fibers; each single fiber of the diameter approximately 1-2 µm and length approximately 20-100µm. Each fiber is made up of parallel bundles of myofibrils each of which is made up of repeating blocks called sarcomeres, which are the smallest and basic structural units exist in muscle. The structure of the sarcomere is composed of thick filaments and thin filaments as shown blow.

Figure 1.3 Electron microscope image of striated muscle sarcomere. (Adapted from H.E. Huxley, 1967)
The details of the sarcomeric structure of striated muscle as seen by electron microscopy are shown in figure 1.3. One sarcomere is the distance between two neighboring Z-band (or called Z-Disk) which shown as a series of dark lines under electron microscope. The I band is the lighter region on either side of the Z-bands is made up of actin filaments. The A band, a darker region extending the entire length of the thick filaments arranged in a hexagonally array with inter-digitating thin filaments. The think filament is composed of a bundle the protein bundle with its enzymatic head part exposed on the surface where it is free to interact with the thin filaments. (Hasselbach, 1953).

Figure 1.4 Schematic of Basic Structure of Sarcomere. (Adapted from Bloom and Fawcett 1975)

The sliding filament model of contraction, states that the arrays of thick and thin filaments slide past each other without appreciable change in length (H.E. Huxley and Hanson, 1954). Actin and myosin interact and slide relative to each other during muscle contraction, see Figure 1.4. The myosin molecule is an ATP-dependent motor protein; it has a head-tail structure where the tails self-assemble into the thick filament backbones
and the heads can attach and detach to the actin filaments. Myosin heads binds an ATP that allowing it disassembles with actin filament when the muscle started contraction. Then myosin will connect to another site of while ATP is hydrolyzed (Webb and Trentham 1983). Releasing the phosphate group, myosin hand will move toward H-band where located the central of one sarcomere.

1.2 Muscle Regulation

![Diagram of myosin cross-bridges with actin.](image)

Figure 1.5 Schematic of myosin cross-bridges with actin. (Wakabayashi et al. 2001)

There are many regulatory proteins involved in muscle contraction. ATP hydrolysis on the myosin head is responsible for conformation changes. Figure 1.5 shows tropomyosin protein surrounding on the surface of the actin filament. Troponin molecules is protein complex which has three subunits, troponin I, troponin C and troponin T, these subunits can bind to tropomyosin protein to form a complex.

During muscle activation, a neuron will transmit an action potential to stimulate sarcoplasmic reticulum to release the calcium ions. Calcium ions will allow tropomyosin
to move on the actin filament to expose the myosin binding site, and then myosin head will attach to actin filament form cross-bridges structure. In addition, myosin heads attach actin filaments tightly in the rigor state when ATP is not bound and hydrolyzed.

Moreover, calcium ions binds with troponin-C which is one of the motor proteins located on thin filament, regulating the conformation of myosin bind site on the actin filament to be free (Takeda, *et al.*, 2003). Therefore, muscle contraction is a controlled procedure, directional mechanical work via modulation of free calcium ion concentrations and ATP-dependent regulations.

1.3 The Sliding Filament Theory

The sliding filament model of contraction is a concept which explains the mechanism of muscle shortening during muscle contraction (Huxley and Hanson, 1953). More precisely, the lengths of thin and thick filament do not change but slide to each other during the muscle contraction, which means the sarcomere length will change (Fig 1.6).

![Figure 1.6 Muscle activation between the thick filament and thin filament during the muscle contraction. (Adapted from Gordon *et al*, 1965)](image-url)
The mechanism of force produced is via cyclic interactions between the thick filament and thin filaments. The myosin heads will be powered by ATP hydrolysis to run the muscle contraction cycle (Fig 1.7). Myosin is an ATP dependent protein which binds and unbinds the thin filaments while undergoing conformational changes that pull the thin filaments relative to the thick filament. After detachment and hydrolyzing ATP, a head will bind to a new site on actin located closer the plus end of the actin filament. ATP therefore provides the energy source for the conformational change and, hence, muscle contraction. The ATP molecule, in turn, is synthesized from ADP and Pi using the free energy of oxidation of glucose and fatty acids.
1.4 Insect Flight Muscle

Muscle, especially vertebrate muscle, is a very well-studied tissue but there are many remaining mysteries. Insect flight muscle has similar physiological properties to vertebrate muscle despite some significant structural differences. Moreover, compared to human tissues or vertebrate animal tissues, insect flight muscle is easier to obtain, which not only makes it easier to study but allows for comparisons between invertebrate and vertebrate muscle.

Flight muscles in insects can adopt one of two kinds of contraction mechanisms. The flight muscle of the Hawkmoth, *Manduca sexta*, is a synchronous muscle and an emerging model system for structure and function studies of muscle. There are very few structural and functional studies of *Manduca* flight muscle in the literature but *Manduca* is a widely used organism for integrative biology. Synchronous insect muscle powers flight by a direct coupling of contraction to the neuronal activation. Asynchronous muscles use the phenomenon of delayed stretch activation to decouple activation from calcium release, permitting highly frequency wing movements (Josephson, et al., 2000)

Synchronous flight muscle has to release relatively large amounts of \( \text{Ca}^{2+} \) with every muscle contraction, and take it up with every relaxation. \( \text{Ca}^{2+} \) diffusion and uptake with repeating muscle contraction and relaxation cycles will represent a significant amount of the overall energy budget of the muscle. While there have been many studies of asynchronous muscles, primarily from the giant water bug, *Lethocerus*, and the fruit fly, *Drosophila*, there have been very few structure function studies of synchronous insect flight muscle. Such studies may be very useful in comparative studies with mammalian skeletal and cardiac muscle, which are also synchronous.
Current investigations of *Manduca* flight muscle focus on the dorsolongitudinal muscle (DLMs) which control the wing-beats during the insect flight. The DLM’s are separated into 5 subunits that differ in the properties depending on whether they are dorsally located or ventrally located (Figure 1.8). The ventral muscle fibers are longer than the dorsal muscle fibers. There are functional differences between the ventral and dorsal muscles. There is a temperature gradient in the *Manduca* flight muscle so that higher temperatures in the ventral muscle in the interior of the animal lead to reduced contraction times (George, 2010). Generally speaking, temperature is a critical factor affecting muscle contraction rate *in vivo*, the rate will increased during higher temperature in invertebrates and vertebrates (Josephson, 1984)
1.5 Muscle Storage

Many physiological experiments are done on membrane intact muscle, either isolated from the animal or done in situ. These experiments have the advantage of being the most physiological but maybe difficult to interpret due to the complexity of the system. Muscles where the membranes have been removed using detergent ("skinned muscles") are often used as a model system where one has direct experimental access to the myofilaments. This allows precise knowledge of the calcium concentration, ionic strength, pH and so on but can no longer be considered truly physiological. To make progress, both kinds of experiments are necessary. At the beginning of these experiments we did not have well-established protocols for making skinned Manduca sexta flight muscle preparations.

Experience so far (Jiangmin Liu, MS thesis IIT) shows that the best results are obtained when muscles are taken from still living moths and used for experimentation directly after the skinning protocol. However, it is often difficult to arrange to obtain live moths at the appropriate times for either mechanical or X-ray experiments. It would be highly desirable, therefore to find preparation and storage conditions that can maintain the structural and physiological functions as close as possible to freshly prepared muscle.

Previous research on Manduca sexta insect flight muscle (Mengjie Liu, MS thesis IIT), suggests some avenues to explore for muscle fiber storage conditions. Our goal here is to figure out storage conditions that will be work best for experiments that may take place several days after the initial dissection. This can be evaluated by a combination of functional (maximum calcium activated force) and structural measurements (light and X-ray diffraction).
Several sets of muscle fibers were evaluated by measuring the maximum calcium activated force after storage at low temperature for various time periods. Our goal was to find storage conditions which could maintain the structure and function of muscle fibers for at least 14 days. The first conditions we tried is to store the muscle in skinning solution at 4°C without glycerol and Triton. A problem with this approach is that muscle proteins will degrade under 4°C environment over a period of days, even in the presence of protease inhibitors. Another approach is storing the muscle fibers in -80°C with 75% glycerol that may be compared with 4°C without added glycerol. However, this approach may still destroy the integrity of sarcomere units in muscle and its ability to generate force (Kawai, 1990). It also appears that the results may depend on the type of muscle filament arrangement in different species and the muscle structural order. For example, the insect muscle of Lethocerus indicus has very high structural order, and may be stored at -80°C with 75% glycerol for years without affecting sarcomeric structure and force generation ability.

1.6 Laser Light Diffraction

Laser diffraction is one of the methods used to monitor the sarcomere length of muscle fiber (Cleworth and Edman, 1972). Light diffraction will depend on the three dimensional arrangement of diffraction planes created by the sarcomere patterns within a given muscle which may pose difficulties in interpreting sarcomere length measurements from laser diffraction. So, the Bragg condition is only fulfilled for a subset of these domains that can then contribute to the recorded signals.
The magnified diffraction patterns can be imaged on a screen some distance away from the muscle. The distance between pairs of diffraction lines can be measured and converted to sarcomere length using the wavelength of laser and the incidence angle of the laser beam according to Bragg’s law, (Brenner 1985).

1.7 X-ray Diffraction Patterns form *Manduca sexta* Muscle

X-ray diffraction is the most powerful method to analyze the tertiary structure of crystalized proteins at the atomic level. X-ray fiber diffraction of muscle is more limited but it may be used on both skinned muscle *in vitro* and intact muscle *in vivo*, that is, under near physiological conditions. Such experiments have told us much of what we know of the molecular series of events during muscle contraction.

Since the pioneering work of Huxley, 1953 many investigators have been investigating the structure and function of the molecules that located in the muscle sarcomeres (Huxley, 2004). The ability to obtain two-dimensional X-ray patterns from contracting muscle, first demonstrated in 1964, allowed observation of the actin and myosin changes in muscle during contraction with substantial shortening (Huxley, 1965). X-ray experiments have been complemented by an important new type of evidence, that from single molecule studies where, the sliding of actin directionally in the presence of ATP over substrates coated with myosin molecule (Yanagida, 1985). The arrangements of actin and the α-helical substructure of para-myosin filaments of molluscan smooth muscle in early experiments using X-ray film (Millman and Elliott, 1972).

The X-ray diffraction patterns of muscle arises from the organized arrangement of the myosin and actin filaments in the sarcomere, and have been to study low angle X-ray
muscle fiber diffraction pattern from living insect during resting and tethered flight (Irving and Maughan, 2000). Even though myosin and actin are difficult to crystallize, they assemble naturally into the muscle fiber structure. The packing of thick and thin filaments is similar in that the thick filaments are located on the lattice points of a hexagonal array. Insect flight muscle differs from vertebrate striated muscle in that, in insects, six thin filament fibers are arranged on a hexagonal pattern surrounding each thick filament. The arrangement of thick filaments and thin filaments in insect flight muscle is shown in Figure 1.9. The big round dots and small round dots represent thick filaments and thin filaments respectively.

![Figure 1.9 Schematically filament lattice of insect flight muscle. (Adapted from J. Squire 1981)](image)

The strongest reflection in the X-ray diffraction pattern of insect flight muscle, is called the 1,0 reflection from the 1,0 lattice plane on the equator. The 1,0 plane contains one thick filament and one thin filament per crystallographic unit cell. The second strongest reflection is the third one named the 2,0 reflection which comes from the 2,0 plane which contains one thick filament and three thin filaments per crystallographic unit
cell. There is also a weak reflection between 1,0 and 2,0 equatorial reflections which is called the 1,1 (Figure 1.9)

![Figure 1.10 The cross section of a myofibril. (Adapted from Irving 2005)](image)

Figure 1.10 The cross section of a myofibril. (Adapted from Irving 2005)

![Figure 1.11 Illustration of Bragg’s Law derivation. (Adapted from J. Squire 1981)](image)

Figure 1.11 Illustration of Bragg’s Law derivation. (Adapted from J. Squire 1981)

X-ray diffraction form 3-D crystals could be thought of in terms of the reflections of X-rays from successive crystal planes and their subsequent interference. Bragg’s law describes how the peaks of scattered intensity that are observed at the Bragg angle θ, corresponding to the angle of scattering, and the integer number of wavelengths (Figure...
The intensity of the 1,0 and 2,0 peaks are high in *Manduca sexta* flight muscle. Spacing and intensities from these reflections may be measured easily. However, the intensity of the 1,1 peak may be much harder to measure accurately because this reflection is so much weaker than 1,0 peak and 2,0 peak. In sum, the ratio of the 2,0 and 1,0 reflection intensities may be used to assess the movement of cross-bridges in the A-band region of the muscle. Myosin heads have moved away from the thick filaments backbone if the intensity of the ratio increases, and vice versa.

### 1.8 Scope of the Thesis

The work in this thesis is organized around the following two aims:

**Aim1** - the goal is to evaluate and compare different kinds of storage conditions to determine those that can preserve *Manduca sexta* muscle fibers structural and physiological functions for the longest time. The dorsal and ventral parts of the DLM show different mechanical properties and so are examined separately. Our assay is to use the maximum active force of muscle fiber and see how it changes with time in storage. This functional assay is supplemented by laser diffraction and in some cases X-ray diffraction as structural assays.

**Aim2** - the goal here is to study the relationship of muscle spacing and crossbridge disposition in skinned muscle as a function of temperature. A second goal is to see what concentration of dextran, an inert polymer that is too big to enter the myofilament lattice, can reverse the swelling the myofilaments experience upon so skinning so that the *in vivo* lattice spacing is restored. Muscles will be bathed in a series of dextran solutions and the lattice spacing measured by X-ray diffraction. These changing conditions may also affect the distribution of crossbridges in the sarcomere. Any such changes will be assessed by
measuring the $I_{20}/I_{10}$ equatorial intensity ratio from all X-ray patterns. In order to assess the maximum amounts the intensity ratio can change with binding of myosin heads to actin, we will collect X-ray diffraction patterns from muscles in nucleotide-free rigor solution where all heads will be strongly bound to the thin filament.
CHAPTER 2
MATERIALS AND METHODS

2.1 Sample Preparation

2.1.1 Solution preparation. There are three types of solutions that were used in mechanics experiments. The pH values of these solutions are adjusted to 6.8 using KOH at 22°C. The composition of above chemicals are shown in Table 2.1, all numbers are presented in mM:

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Relaxing solution</th>
<th>Pre-activating solution</th>
<th>pCa 4.5 solution</th>
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<tbody>
<tr>
<td>MOPS</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>NaN₃</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>MgAc₂·4H₂O</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Na₂ATP</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>EGTA</td>
<td>5</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td>HDTA</td>
<td>--</td>
<td>4.5</td>
<td>--</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>--</td>
<td>--</td>
<td>4.95</td>
</tr>
</tbody>
</table>

Pre-activating solution contains, in addition to the other components, 1,6-diaminohexane- N,N’,N’ tetra-acetic acid (HDTA). The electron charge and diffusion coefficient of HDTA and EGTA are the same. However, HDTA has dramatically lower stability constant for Ca²⁺ than EGTA (Moisescu and Thieleczek 1978)
pCa 4.5 solution added additional 4.95 mM Ca\(^{2+}\) which allows the muscle to generate maximum force when the muscle is bathed in the solution. The three solutions are prepared and calculated by online software which is shown as follow:

http://www.stanford.edu/~cpatton/CaMgATPEGTA-NIST.htm

EGTA has a higher affinity for Ca\(^{2+}\) compared to Mg\(^{2+}\). Thus, the amount of metal ions which added will not equal to the amount of free ions available in the solution.

The components of rigor solution are 20mM MPOS and 20mM EDTA, the pH, adjusted to 6.8 by NaOH.

Dextran is a large inert polymer that in solution is commonly used to osmotically compress the lattice of thin and thick filaments in order to restore the lattice spacing observed \textit{in vivo} when doing X-ray and mechanics experiment. In vertebrate muscles, the concentration of Dextran required is typically between 3 and 4\% (Millman, 1998). In order to determine what this value is for \textit{Manduca} DLM, we used five different concentration of dextran solution in the experiment: 1\%, 2\%, 3\%, 4\% and 6\%.

\section*{2.1.2 Preparation of Chemically Skinned \textit{Manduca sexta} Flight Muscle.} \textit{Manduca sexta} is an agricultural pest and it is illegal to ship live moths across state lines. Moths are shipped therefore, with the heads severed, courtesy of Dr. T. Daniel at the University of Washington. The muscles in the thoraces could stay alive for 2-4 days when the storage temperature is 4\(^\circ\)C. To avoid contamination of the muscle fibers, it is required to remove the wings, legs and abdomen via scissors prior to dissection. Then the hairs located on the thorax also need to be removed. The thorax is cut longitudinally into two equal halves. The ventral and dorsal muscle bundles are dissected from the thorax halves using surgical scissors and fine forceps. Individual muscle bundle specimens were
approximately 3mm in length and 1mm thick. Muscle bundles were then treated with skinning solution with 1% Triton X-100 and a protease inhibitor cocktail pill (Roche) per 50mL at 4°C for 10-12 hours. The protease inhibitor cocktail prevents protein degradation within the muscle fiber. It will help maintain organization of the muscle structure and maximum force generation. Triton is a non-ionic detergent that has also been used extensively to solubilize membranes and to isolate membrane-bound proteins in a variety of animals. Triton X-100, however, has been shown to cause damage of tegument and muscle in *Schistosoma Mansoni* (Depenbusch et al, 1982). So, skinning times need to be carefully controlled. In mechanical experiments, the sarcomere length of ventral muscles and dorsal muscles are stretched to 3.3 µm, assumed to be in the working range of the living muscle, prior to doing the experiment.

2.1.3 **Storage of Skinned Muscle Fibers.** Temperature is one of the factors which affect the protein degradation process, even though protease inhibitors are present in the solutions bathing the muscles. In general, muscles will last longer when stored at lower temperatures. Ice crystals formed during freezing, however, may cause the crystal structure of proteins to change; the process is irreversible and unavoidable phenomenon in nature. Ice crystal formation can be minimized by using glycerol as a cryoprotectant. Two types of storage methods were compared in this thesis. One was simply to store the skinned muscle fibers at 4°C in the skinning solution without Triton and glycerol. Another was to store the muscle fiber at -80°C in skinning solution with 75% glycerol, but without added Triton-X100. Glycerol is using to prevent ice crystal formation in freezing temperature. However, previous studies indicated that it is very important to wash out 75% glycerol from the muscle fibers prior to experiments on the day of
measurement. In order to make sure glycerol goes into muscle fiber prior to low temperature storage at -80°C, muscles should be bathed in the skinning solution described above and incubated at 4°C for two hours with rotation. Before the experiment started, the muscle was removed from the -80°C freezer, and the muscle was washed with skinning solution for 40 minutes each time, and repeat the washes three times before starting the experiment.
2.2 Mechanical Experiments

2.2.1 Sarcomere Length Measurement. Laser diffraction is one of the most commonly used methods used to estimate the sarcomere length of muscle. Muscle fibers were illuminated by a 10mW helium-neon laser beam, wave-length of 632.8nm (Uniphase model 106-2) as the laser light source. The beam is focused on the muscle fibers, and then the resulting diffraction pattern projected onto a screen located approximately 28 cm above the sample. The patterns are recorded using a scientific grade solid state camera (COHU) and digital frame grabber (Scion FG Capture). In order to increase the intensity and improve the quality of diffraction pattern, it is necessary to adjust the angle of the mirror between the longitudinal axis of the fibers and the laser beam. The incident light of laser beam will be diffracted from the alternating A and I bands in the sarcomeres, to produce a straight through zero order band width and symmetrically placed higher order bands on either side on the screen, see Fig 2.1. The distance between the zero-order band and the first-order band is used to estimate the sarcomere length (SL). This distance is measured via Mitutoyo electronic calipers from mm, and then converted to SL into µm by the following formula:

\[
SL = \frac{\lambda}{\sin(Tan^{-1}\frac{x}{h})}
\]

\(\lambda\) is the laser wave length, \(x\) is the distance between zero-order and first-order reflection on the screen and \(h\) is the distance from the muscle fibers to the screen.
2.2.2 Force measurement. There are several instruments for the experiment of force measurement in the Irving lab. Either independent force transducers (Aurora Scientific Inc., series 400A) and or the dual-mode servo-motor controller (Aurora Scientific Inc., 300B series) may be used to produce an output voltage proportional to the force. Force and muscle length output signals were digitized either using a digital/analog controller system (Aurora Scientific Inc, model 600A) or using National Instruments A/D, D/A hardware (National Instruments, cDAQ-9178) with a custom LabVIEW (National Instrument, 8.5.1) computer program. This allowed following force generation and length changes as a function of time after changing buffer solutions to these containing various amounts of calcium. In the experiments where maximum active force is measured as described below, the data was recorded at 3.3 \(\mu\)m SL in various solutions. The muscle fibers were mounted on hooks using cellulose nitrate glue and lowered into the top of a custom-made plastic was bath in the chamber. The schematic of chamber is shown blow, the inner space volume is approximately 1cm\(^3\); it is more economical to use than the
previous chambers used in the Dr. Irving lab. Moreover, a window can apply provide a live view of the muscle during the force measurement experiment.

![Diagram of chamber used for force measurements](image)

**Figure 2.2** Three-dimensional diagram of chamber used for force measurements.

### 2.2.3 Maximum Active Force Measurement Protocol

In order to generate maximum force, muscle fibers have to be soaked in a solution containing a relatively high calcium concentration of 4.95mM CaCl$_2$. However, the muscle fiber may be damaged during the process of changing solution from relaxing solution to pCa 4.5 solutions directly. Thus, pre-activating solution play an important role in prevents muscle fiber damage before adding pCa 4.5 solutions. In the beginning of this protocol, each muscle fiber should be stretched to the same length, 3.3µm, which was used when soaking in relaxing solution. When the muscle is stretched to a sarcomere length of 3.3µm at relaxing solution, the force detected is the passive force by generated by cytoskeletal elements such as titin like proteins (Chen-Ching Yuan, MS thesis IIT). To measure maximum calcium-activate force, the bath solution is changed in the following order: relaxing solution, pre-activating solution, then to pCa4.5 solution. The force output
as the solution series is changed is the total force generated by the muscle fibers. In order to calculate active force, the passive force measured prior to stimulation need to be deducted from total force. Furthermore, the length and force of SL will be recorded and monitored by the program from the beginning to the end of experiment.

2.3 X-ray Diffraction Experiments

2.3.1 Experiment Apparatus. Small-angle X-ray diffraction patterns were performed using the small-angle instrument on the BioCAT beamline 18ID at the Advanced Photon Source, Argonne National Labs Illinois. The energy of the X-ray beam was set to 12keV which corresponds to a wavelength of 0.103 nm. The size of the beam is approximately 0.15mm and 0.05mm (horizontal and vertical) at the focus position which was at the detector plane. A CCD dictator (MAR 165, Rayonix Inc., Evanston IL) was used to record the X-ray diffraction patterns in the experiment. The muscle fibers were mounted between the hooks inside of a chamber which is shown in Figure 2.3 and Figure 2.4.

Figure 2.3 Three-dimensional graphic of chamber for X-ray diffraction experiment.
Figure 2.4 Schematic front view of chamber used for the X-ray diffraction experiments.

The protocol for collecting X-ray diffraction patterns is to first mount the fibers between the hooks in the chamber using nitrocellulose glue and then fill up the inner space of chamber with the desired solutions and then start shoot the X-ray beam though the MYLAR® (0.001”) windows of the chamber to hit the muscle fibers and the diffraction pattern is recorded using the CCD dictator. Multiple X-ray exposures in the same place will damage the muscle fibers. So it is necessary to move the muscle either vertically or horizontally between each X-ray exposure.

2.3.2 X-ray Experimental protocol. Freshly skinned muscle fiber bundles were stored in relaxing solution before the X-ray experiment. Then the muscle fibers were mounted on the hooks in the chamber which described above. There are two sets of solutions used for the X-ray experiments described in this thesis. One is to use one of five different concentrations of dextran in relaxing solution, 1%, 2%, 3%, 4% and 6%, at room temperature (20°C). Another is to examine muscles at seven different temperatures for the relaxing solution, 10°C, 15°C, 20°C, 25°C, 30°C, 35°C, and 40°C by X-ray diffraction.
We also compared muscles in relaxing solution to those in rigor solution. The exposures time for low-angle X-ray diffraction pattern was set to 1 second, and the X-ray pattern detected using a MAR 165 CCD-based X-ray detector.

2.3.3 Data Analysis. Analysis of X-ray diffraction patterns required using several different programs. In order to measure the intensity of the equatorial reflection from the X-Ray diffraction pattern between the muscles the first step uses the program FIT2D (v12.077, http://www.esrf.eu/computing/scientific/FIT2D/). The first step is to convert the equatorial part of the two dimensional diffraction patterns to an intensity projection along the equator using a the Projection tool in the FIT2D program. The results of the “projection” tool can be saved to Ascii files in “chiplot” format. When doing projections it is important to ensure the highest points on each pair of peaks should be the same height, which ensures that the projection axis is parallel to the equatorial axis. If the heights of the peaks are not equal, it is an indication that the axis of the pattern was not chosen correctly.

There are two methods of measurement which we using by FIT2D. First one is to measure the distance between peaks in the X-Ray diffraction pattern using the Distance tool. Second one is named projection which shown in Figure 2.5.
First you draw a long axis of the box around the diffraction spots as shown in Figure 2.5. This box will produce a projection of the intensity along a line through the equator which is then saved into an ASCII format “Chiplot” file. The custom program chiplot.analyze.py is used to separate the data in the chiplot file into the left and right side of the patterns showing the intensity peaks. There should be two obviously peaks per side of the chiplot from *Meaduca sexta* muscle sample. Next, using FITYK (Windows version 0.9.8) allows fitting gaussian functions to the peaks. The function used functional forms for the background and constraints on the reflection positions and widths. In order to fitting the functions to the peaks, the function which we used is “Pearson7”. This function, int eh form accepted by Fityk is as follows:

Define Pearson73peaks(area1=\((height*fwhm)/.5\),
area2=\((height*fwhm/1)/1\),area3=\((height*fwhm/2)/1\),s10=center,
sigmac=1, sigmad=fwhm/2.5, sigmas=0.3, s0=.1,shape=2)  =
Pearson7A((area1/sqrt(sigmac^2+sigmad^2+sigmas^2)))*(1-sigmas),
s10,sqrt(sigmac^2+sigmad^2+sigmas^2),shape)+Pearson7A((area2/sqrt(sigmac^2+(sigm
ad*1.732^2+(sigmas*1.732^2)^2)*(1-sigmas), (s10+s0)*1.732-s0, sqrt(sigmac^2+(sigmad*1.732)^2+(sigmas*1.732^2)^2),shape)+
Pearson7A((area3/sqrt(sigmac^2+(sigmad*2.0)^2+(sigmas*2.0^2)^2))*(1-sigmas),
(s10+s0)*2.0-s0,sqrt(sigmac^2+(sigmad*2.0)^2)+
(sigmas*2.0^2)^2),shape)

The parameters in this function are adjusted using the Marquart-Levenberg algorithm to obtain the best fit function. The smooth background can be either removed using the baseline tool in Fityk or using the convex hull algorithm incorporated into chiplot.analyze.py. A properly subtracted baseline should result in a plot similar to that shown in Figure 2.6. The fit data is shown in Figure 2.7.

Figure 2.6 Baseline removed in FITYK program.
It is sometimes necessary to manually adjust the peak parameters after the fitting process to fit the data more accurately. The last step is to output the peak parameters to excel, also that the intensities values from the X-ray diffraction of muscle can be analyzed. These procedures result in tables of both the intensities and positions of equatorial reflections from the muscle samples.
CHAPTER 3
RESULTS

Small angle X-ray fiber diffraction patterns from striated muscle can show structural details that cannot be observed by naked eyes or others methods. It can supply detailed structural information that can be related to the function of muscle. So freshly skinned muscle without any glycerol typically yields more and clearer meridional reflections and layer lines, and also more equatorial reflections, such as the 3.0. These features are diminished in muscles stored in glycerol. (Mengjie Zhang, M.S. thesis IIT, 2012)

In Figure 3.1 shows a 2D X-ray diffraction pattern from ventral dorsal longitudinal muscle of *Manduca sexta* which bathed in rigor solution. The pattern shows very clear layer lines, and also more equatorial reflections.

![Figure 3.1 X-ray 2D Images of Manduca Sexta Ventral Muscle in rigor solution.](image)
3.1 Storage Time-Force Relationship

Both ventral and dorsal muscles, stored either at 4°C without glycerol and -80°C with 75% glycerol were used to study maximum force as a function of storage time. The muscle samples were attached to the hooks on the apparatus using cellulose nitrate glue. For each condition we repeated each experiment 3 times and calculated the mean values. (Individual muscle samples are not large enough to do more sets.) The sarcomere length was setting at 3.3um for measure maximum force when bathing bathed in pCa 4.5 solutions. The relationship of maximum force vs. time in storage was measured over a 35 day period. The ventral and dorsal parts of the insect flight muscle were measured separately and compared.

Figure 3.2 shows that there are no large differences in maximum active force between the dorsal and ventral parts of flight muscle when the maximum active force of muscles samples storage were stored under at 4°C without glycerol over a 14 day period. After 5 days of storage under at 4°C without glycerol, the maximum active force will drop dramatically on day 6, and then the maximum active force decreases smoothly from day 6 to day 14.
The muscle samples could maintain more maximum active force when stored at \(-80^\circ C\) with 75% Glycerol. It did not change so much during Day 1 to Day 5 (Fig 3.2), similar to muscle stored at \(4^\circ C\) without glycerol. However, the force did not drop
dramatically at day 6 unlike figure 3.2. It is keep approximately 70% of origin maximum active force from day 6 to day 9, then decrease to approximately 50% of origin maximum active force from day 10 to day 14.

When both storage conditions are compared, it appears that there is some degradation of the muscle after 4-5 days even at -80°C in 75% glycerol but the degree of degradation is less at low temperature than at high temperature. Protease inhibitors are present under both conditions so it is not clear whether the decay at longer time is due to proteolysis or to some other process. *Lethocerus* IFM is much more stable at low temperature than *Manduca* muscle. It is possible that some soluble component in *Manduca* muscle is required for stable structure that is washed out during the skinning process but what this component could be is completely unknown.

![Graph showing force (mN) per area (mm^2) over time (Day) for ventral and dorsal muscle](image)

Figure 3.4 Comparison of maximum active force of *Manduca sexta* ventral and dorsal insect muscle after storage 4°C without glycerol during 35 days.
Figure 3.5 Comparison of maximum active force of *Manduca sexta* ventral and dorsal insect muscle after storage -80°C with 75% glycerol during 35 days.

In Figure 3.4 and Figure 3.5, the maximum active force measurement experiments were extended to day 35. Every 7 days force was measured from dorsal and ventral muscle fibers at day 21, day 28 and day 35. There were still no force change after day 14 when the muscle storage in 4°C without glycerol. However, the force still has approximately 50% of maximum active force before day 21. Then decrease to 10% after day 28, and the force was the same with figure 3.4 after day 35 in dorsal and ventral muscle fibers. Force was reasonably stable at 40-50% of maximum right after skinning between day 6 – day 21.
3.2 Temperature studies

3.2.1 Relationship between lattice spacing and temperature Figure 3.6, figure 3.7 and figure 3.8 show $d_{10}$ lattice spacing as function of temperature from different muscle fiber. The lattice spacing was measured from X-ray patterns using FIT2D. Comparison both ventral and dorsal muscle in the same plot in Figure 3.8 from Manduca muscle. Temperature was varied using a circulating water bath and heater to control the temperature inside of the experiential chamber. There were three samples used for each point and that the error bars represent the standard error of the mean.

![Graph showing lattice spacing (d10) as a function of temperature in ventral muscle.](image)

Figure 3.6 Lattice spacing ($d_{10}$) as a function of temperature in ventral muscle.
Figure 3.7 Lattice spacing ($d_{10}$) as a function of temperature in ventral muscle.

Figure 3.8 Comparison spacing of lattice ($d_{10}$) as a function of temperature in both dorsal and ventral muscles.

Figure 3.6 and Figure 3.7 indicate that lattice spacing in both ventral and dorsal muscles start off at 65.4nm and 59.2nm in $d_{10}$. Lattice spacing shrinks smoothly as the temperature increases. The muscle lattice appears to be unstable above 40$^\circ$C where the standard error increased. The $d_{10}$ lattice spacing in ventral muscle is approximately 14%
lower at 40°C then at 10°C in the ventral muscle, and the d10 lattice spacing decreases approximately 16% at 40°C from the spacing at 10°C in dorsal muscle fiber. Moreover, it indicates the lattice spacing will shrink to 51.3nm when the ventral muscle fiber bathed in rigor solution which shown in figure 3.6, and the lattice spacing will shrink to 49.5nm when the dorsal muscle fiber bathed in rigor solution which shown in Figure 3.6.

Figure 3.8, compares dorsal and ventral muscle in the same plot, which appears there were highly significant difference in lattice spacing at 10°C and 15°C (t-test, P<0.01). It was a significant difference of lattice spacing at 20°C (t-test, P<0.05). However, there were no significant differences in lattice spacing due to temperature gradient increased after 25°C (t-test, P>0.05).

3.2.2 Relationship between intensity $I_{20}/I_{10}$ and temperature Figure 3.9, Figure 3.10 and Figure 3.11 show $I_{20}/I_{10}$ as a function of temperature from the same experiment as shown above that lattice spacing of ventral and dorsal part of Manduca flight muscle.

![Ventral muscle](image)

Figure 3.9 The ratio of $I_{20}/I_{10}$ as a function of temperature in the ventral muscle.
In Figure 3.9, lattice shrinkage is accompanied by an approximately 14% increase in $I_{20}/I_{10}$ going from 10°C to 35°C, then the ratio decreases again to 1.14 at 40 °C in the ventral part of *Manduca* muscle. In Figure 3.10, lattice shrinkage in dorsal muscle is
accompanied by an approximately 28% increase in $I_{20}/I_{10}$ at 40°C. However, the standard error is much larger, suggesting instability, after 35°C. The intensity ratios, however, were much higher when the dorsal and ventral muscle fibers were bathed in the rigor solution. A possible explanation of the intensity changes with increasing temperature may be that ATP is consumed more rapidly and some of the muscles are going into rigor. But because the changes with rigor are so much larger, it seems likely that this may not be the case.

Figure 3.11 compares dorsal and ventral muscle in the same plot. Interestingly, there is no significant difference in ratios from 15°C to 40°C, but there is a significant difference at 10°C and 30°C (t-test, P<0.05).
3.3 Osmotic Compression studies

3.3.1 Relationship between lattice spacing and temperature Figure 3.12, Figure 3.13, and Figure 3.14 show lattice spacing as function of concentration of dextran from different muscle fibers.

![Graph showing lattice spacing as a function of dextran concentration in ventral muscle](image)

Figure 3.12 Spacing ($d_{10}$) as a function of concentration of dextran in the ventral muscle.

The lattice spacing decreased significantly with increasing dextran concentration in the dorsal part of muscle. Figure 3.9 shows that lattice spacing decreases from 61.3nm in 1% dextran to 66.2nm in 6% dextran a 7.3% decreased in d10 lattice spacing in ventral muscle.
Figure 3.13 Spacing ($d_{10}$) as a function of concentration of dextran in the ventral muscle.

Figure 3.14 Comparison lattice spacing ($d_{10}$) as a function of concentration of dextran in both dorsal and ventral muscles.

In Figure 3.13, the lattice spacing was decrease significantly 55.8nm in 1% dextran to 50.9nm in 6% dextran, an 8.7% decrease in $d_{10}$ from 1% to 6% in the dorsal part of the dorsal longitudinal muscle.
In Figure 3.14, comparing dorsal and ventral muscle in the same plot, the interesting thing is, that lattice spacings were significantly different at all concentrations of dextran from 1% to 6% (t-test, P<0.01)
CHAPTER 4
DISCUSSION

4.1 Overview

*Manduca sexta* flight muscle is synchronous, with a single muscle action potential eliciting each contraction. The contractile rates of these muscles are temperature dependent during the insect flight (McCrea and Heath, 1971). Temperature gradients in locomotor muscle would necessarily imply a gradient in the functional roles played by regions within single muscle (George, 2011). Previous work showed that there indeed appears to be a gradient in functional roles between cooler dorsally located muscles and warmer, ventrally located muscles (George, 2011). An important biological question is whether or not these differences are due solely to temperature or are there structural and functional differences between dorsally and ventrally located muscles? Research on skinned muscle may help answer this question. Before such research is meaningful we needed to find storage conditions that yielded stable preparations in terms of force output. The second question addressed in this thesis is how does sarcomeric structure, as assessed by lattice spacing and I_{20}/I_{10} intensity ratio, change with temperature in skinned DLM and does it differ in dorsally and ventrally muscle? A third question addressed in this thesis was what concentration of dextran is required to restore the *in vivo* d_{10} lattice spacing and does it differ between dorsal and ventral muscle? The fourth question addressed what is the range of intensity ratios one might expect from totally relaxed muscle where no myosin heads are attached to actin to muscle in rigor mortis where all the myosin heads may be expected to be bound to actin.
4.2 Storage of skinned *Manduca* flight muscle

In both ventral and dorsal muscle stored either at 4°C without glycerol or -80°C with 75% glycerol, the length laser diffraction patterns did not show any obvious changes during a 14 days period at a 3.3 μm sarcomere length. However, there are huge drop of maximum force generation ability in 4°C without glycerol after day 5, so that there is very little force response from day 6 to day 14 while bathed in pCa 4.5 solution. On the other hand, maximum force generation ability in the muscles stored at -80°C with 75% glycerol dropped 40% from day 6 to day 9, and to 50%-60% from day 10 to day 14. Even though low temperature inhibits function protein degradation, there is still a significant reduction in force generation ability in the muscle fiber. Protease inhibitors can prevent the functional protein degradation and maintain functional protein activity but the presence of inhibitors were not sufficient to prevent a reduction in force. The X-ray diffraction experiments, however, indicate that it appears to be possible to store prepared *Manduca sexta* insect flight muscle for at least 14 days at -80°C with 75% glycerol with good structural preservation enabling a large class of future experiment not requiring fresh muscle samples.

According to earlier experiments (Jiangmin Liu, MS thesis IIT), the average sarcomere length of dorsal muscle at slack length is 3.7μm, and the average sarcomere length of ventral muscle at slack length is 3.2μm SL. However, we were able to obtain a SL of 3.3μm in both ventral and dorsal muscles in the experiments in this thesis. This discrepancy may result from using a different instrument, or by using a different skinning procedure than is now being used, even though we used the same method to calculate and measure the SL in muscle.
4.3 Changes in sarcomere structure with temperature

Since chemical reactions in general are faster at higher temperatures one might expect myosin cross-bridges would generate larger forces at higher temperature; the phenomenon provides an opportunity to study the structural function (Irving, 2005). Moreover, the actin and myosin are arranged in a very regular helical manner (Huxley, 1967). Furthermore, the intensity of the X-ray reflections is sensitive to the distribution of mass in the plane perpendicular of the myosin and actin filament. Here, we used X-ray diffraction patterns from ventral and dorsal parts of Manduca flight muscle to characterize the structural changes at different temperatures.

Figure 4.1 Lattice spacing in Manduca living flight muscle. (George et al, 2013)
Figure 4.1, shows published lattice spacing ($d_{10}$) and equatorial intensity ratio ($I_{20}/I_{10}$) data from intact, electrically stimulated ventral and dorsal muscle of *Manduca sexta* in 25°C and 35°C while being sinusoidally oscillated (George, 2013). These data indicate the range of physiological lattice spacing and intensity ratios one might expect in living muscle at different temperatures. These data may be compared and contrasted with the data on skinned muscle presented in Chapter 3. Figure 4.1 shows that lattice spacing is smaller in 25°C muscle than in 35°C. Moreover, muscle at 25°C showed a stable intensity ratio during mechanical oscillation and electrical stimulation. The similar response of both dorsal and ventral muscle at 25°C supports the notion that dorsal muscle’s contractile dynamics are simply a response to lower temperature and not to regional specializations in dorsal muscle.

The lattice spacing ($d_{10}$) of both ventral and dorsal skinned muscles shown in Figure 3.5 and Figure 3.6 are higher than those shown in Figure 4.1 at 25°C and 35°C. Lattice spacing is known to swell when the plasma membrane is removed by chemically skinning. The lattice spacing swelling about 18% at 25°C, 10% at 35°C in ventral muscle, and approximately 10% at 25°C in dorsal muscle after comparing Figure 3.5, Figure 3.6 and Figure 4.1. Moreover, lattice spacing ($d_{10}$) was related with intensity ratio of muscle in Figure 4.1, the intensity ratios ($I_{20}/I_{10}$) shown in Figure 3.8 and Figure 3.9 was somewhat higher than Figure 4.1 at 25°C and 35°C.

In Figure 3.5 and Figure 3.6, the lattice spacing ($d_{10}$) decreased in both ventral and dorsal muscles when the temperature increases. $d_{10}$ decreased 14%-16% in both ventral and dorsal muscles as temperature increased from 10°C to 40°C. The lattice
spacings in skinned muscle are approximately 60 nm at 25°C, 62 nm at 35°C for ventral muscle and 55.6 nm at 25°C going to 52.2 at 35°C for dorsal muscle in Figure 3.5 and Figure 3.6. However, in intact muscle, the lattice spacing are approximately 50 nm in dorsal muscle at 25°C, 49 nm in ventral muscle at 35°C, and 49 nm in ventral muscle at 25°C. Even though lattice spacings are substantially larger in skinned muscles, the differences with temperature from 25°C to 35°C are similar in magnitude (~4-5%) in skinned and intact muscle. The intensity ratio increased 28% in ventral muscles, and increased 14% from 10°C to 35°C in dorsal muscle. From 25°C to 35°C, the changes are 1% for dorsal muscle and 25% for ventral muscle which can be compared to the ~60% change in intact ventral muscle. Not that I_{20}/I_{10} in intact ventral at 35°C is ~ 1.1 whereas in skinned muscle it is higher ~1.9.

In summary, the results which shown above are, the lattice spacing (d_{10}) will decrease and intensity I_{20}/I_{10} will increase with an increase in muscle temperature in both skinned and living muscle.

4.4 Effect of lattice compression with dextran

Here the goal was to find out what concentration of dextran restored the in vivo lattice spacing which shown in figure 4.1. It appears that there was no significant difference in lattice spacing through the contraction cycle for muscle at 25°C or 35°C. However, there was a highly significant difference in lattice spacing (d_{10}) of skinned dorsal and ventral muscles when the muscle bathed in 1% to 6% dextran solution as shown in figure 3.11 and figure 3.12 (n=3, t-test, P<0.001).
Unfortunately, we did not find out what we had hoped in this *in vitro* study. Most others studies, in both vertebrate and invertebrate muscles, report that 3-4% dextran is sufficient to restore the *in vivo* spacing. Here even 6% dextran was not sufficient. However, we did not attempt experiments above 6% dextran in this thesis. In the results shown in Figure 3.11 and Figure 3.12, these curves seem flat from 4% to 6% dextran. However, it may or may not have other behaviors at concentrations higher than 6% dextran. Regardless, the discrepancy between our experiment and others in the literature is puzzling. It may be that *Manduca* flight muscle is much stiffer radially than other muscles. A more likely alternative explanation is that intact *Manduca* DLM might operate at a much longer sarcomere length than used here (3.3 µm). Lattice spacing is known to decrease as a function of sarcomere length (Irving et al., 2000). Intensity ratio in cardiac muscle is also known to decrease with sarcomere length consistent with our observation that $I_{20}/I_{10}$ is much higher in skinned ventral muscle than in intact at 35°C. (Farman, 2011)

4.5 Conclusion

Muscles stored at -80°C with 75% glycerol can generate more force longer than those stored at 4°C. However, force is reduced to ~50% after 5 days even at low temperature. This indicates that once the muscles fibers skinned, they should be used as early as possible, based on the reduced force generation after 4 days at either 4°C without glycerol and -80°C with 75% glycerol. It is possible that that the protease inhibitors are not adequately preventing proteolysis even in -80 °C. This could be checked with PAGE and other protease inhibitor strategies could be tried.

The X-ray experiments show that in both dorsal and ventral muscle, lattice spacing
goes down and $I_{20}/I_{10}$ goes up with increasing temperature but the behavior show significant differences between the two muscles when examined at the same sarcomere length and the same temperature. This suggests that there may indeed be structural differences between the two muscles. Similarly, lattice spacing decreases with increasing dextran concentration in both dorsal and ventral muscle. While the slope of this relationship is similar in dorsal and ventral muscle, the lattice spacings are very different suggesting that dorsal muscle is much more compressible than ventral muscle. This also supports the idea that ventral and dorsal muscles have structural differences that may relate to their different functions.

This work have a number of limitations that can be addressed in future experiments, For the X-ray experiments, studying temperature osmotic compression, the sarcomere length was set to just above slack length but were not measured. The SL of the muscles should be set to known values before we start the experiment. Because it appears that in vivo muscle might be at a much longer sarcomere length than 3.3 µm, we should measure both lattice spacing ($d_{10}$) and intensity ($I_{20}/I_{10}$) in different sarcomere length of ventral and dorsal muscles. Secondly the osmotic experiments should be extended to higher concentrations of dextran (over 6%) in the future experiment. To study and figure out the real physiological condition in vivo; third, not only add more concentration of dextran plots in the future experiment, but also needs to measure the intensity ratio as a function of dextran concentration which can compared with the response of lattice spacing to the dextran gradient. All these studies would benefit from electron microscopy measurements of in vivo sarcomere length and filament dimensions.
BIBLIOGRAPHY


