Analysis Of Projectin PEVK Isoforms And Biomechanics Of Dragonfly Flight Muscles

Sean Bear, Agnes Ayme-Southgate, Ruud Schilder* and Jim Marden*
Department of Biology, College of Charleston, Charleston, SC
** Dept. of Biology, Penn State University, State College, PA*

ABSTRACT

Insect flight muscles are extraordinary in their diversity at the anatomical, physiological and molecular levels; in particular muscle stiffness can be very different across insect orders. In derived insects a large part of the elasticity of flight muscles is attributed to the protein, projectin. The molecular characterization of projectin is available for several derived insects and reveals a highly conserved modular organization, including a variable elastic PEVK domain. If projectin is indeed responsible for a portion of the muscle stiffness, distinct PEVK isoforms and unique combinations of such isoforms could confer small differences in muscle mechanical properties. We explore whether such a correlation exists by obtaining molecular data for individual dragonfly, L. pulchella, where flight muscle biomechanics data are already available. Individual muscles were used for semi-quantitative RT-PCR reactions. We determine the ratio of the different projectin PEVK forms using capillary electrophoresis, fluorescence measurement of peak intensity and statistical analysis.

BACKGROUND

Every striated muscle in vertebrates and invertebrates is composed of bundles of muscle fibers containing myofibrils. The sarcomeres are the repeated unit of myofibrils. They are bound on each side by the Z band and contain regular arrangements of actin thin filaments and myosin thick filaments. **Figure 1**

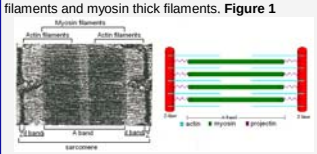
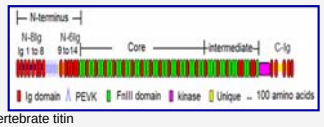


FIGURE 1: Striated muscle model Muscle tissue sample and simplified diagram depicting sarcomere composition.

Mechanical properties of muscles such as maximum work output are often associated with properties of the myofibrils, in particular passive and active stiffness. Many insect flight muscles are relatively stiff, such as muscles from bees, flies, and dragonflies. The elasticity and high resting stiffness of muscles has been associated in derived insects with a third elastic filament (Fig 1) called the connecting or C-filaments. The presence and possible function of a C-filament in basal insects, such as dragonflies is unknown.

One of the major components of C-filaments is the protein projectin. It is an extremely large protein at ~1 MgDa. Projectin has now been characterized in several insects, and is composed of 39 repeating Ig and FnIII domains, which are known to be involved in protein-protein interactions. Projectin also contains a unique sequence known as the PEVK domain towards the NH_2-terminus (**Figure 2**). This domain is characterized by an usually elevated percentage of four specific amino acids: Proline (P), Glutamic acid (E), Valine (V), and Lysine (K). This segment of the gene undergoes complex alternative splicing events, generating PEVK domains of different lengths. In derived insects, the indirect flight muscles contain predominantly the shorter form.

FIGURE 2: Schematic of the projectin protein and its different regions



The same domain is present in the vertebrate titin protein, and in vertebrate both the unusual amino acid composition and the variable length of the PEVK domain have been shown to correlate with different mechanical behavior and muscle physiological properties as shown in the model presented in **Figure3**. When a muscle is stretched the conformation of the PEVK domain changes to enable the total length change. With the same amount of stretch (i.e. force) a short PEVK would generate a higher tension than a long PEVK sequence.

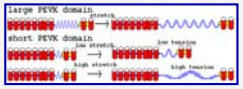
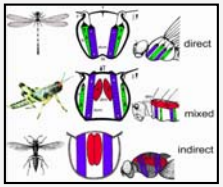


FIGURE 3: PEVK mechanical behavior and muscle stiffness

Dragonflies have direct synchronous flight muscles, ike the basalar muscles.

FIGURE 4: Schematic of three types of insect flight muscles based on attachment of the muscles to the wing base: direct, indirect or mixed



HYPOTHESIS

Insect flight muscles with high resting stiffness contain predominantly but not exclusively a short PEVK sequence, whereas insect muscles with low stiffness present longer isoforms. Variation in the relative concentration of various PEVK isoforms is correlated with differences in the mechanical properties of flight muscles

In this study we will investigate the possible correlation between qualitative and quantitative distribution of PEVK isoforms in basalar flight muscles from individual dragonflies for which flight mechanical data are available.

GENERAL PROTOCOLS

❑ RNA is extracted from the basalar muscles of individual *Lilbellula pulchella* using Trizol (Invitrogen). The samples were provided by Dr. Jim Marden who had previously obtained the mechanical flight data for each insects (Figure 6, right).

RNAs are used in Reverse Transcriptase-PCR reactions (RT-PCR; SuperScript Platinum III, Invitrogen) with different combinations of primers.. We used a duplex approach, i.e. each RT-PCR reaction is done with 2 sets of primers, one for the PEVK and one for the kinase domain of projectin as the internal control. Two different primer sets selectively amplify different PEVK isoforms (Figure 5, below) Each forward primer (Lp5 or Lp6) has a fluorescent dye attached to it (blue in Figure 8, right). The kinase forward (kin1) has a different dye attached so we can distinguish it from the PEVK (green in Figure 8, right).

❑ RT-PCR products are separated according to size by agarose gel electrophoresis to test the validity of the reaction (Figure 7, right)

❑The reaction products are sent to the GENEWIZ company for AFLP analysis using capillary electrophoresis and fluorescence measurement of peak intensity. AFLP (Amplified Fragment Length Polymorphism) is widely used to distinguish DNA size variants due to mutation, allelism, or, in our case, alternate splicing.

❑ Dr. Marden's lab performed the bivariate statistical analysis to test the correlation between their data on flight mechanics and our molecular evaluation of PEVK splice variants. (Figure 9, right)

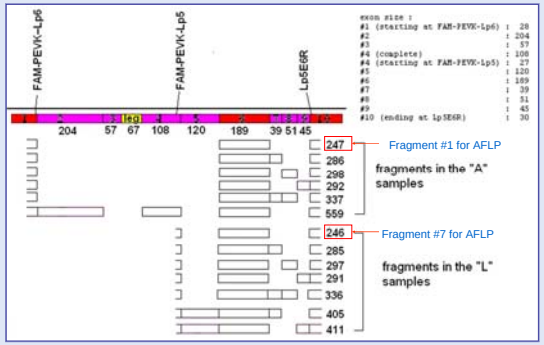


Figure 5: *L. pulchella* PEVK region. This schematic indicates the known exons of the PEVK segment and the position of the primers used. Regions coded red indicate constitutive exons and regions coded pink indicate facultative exons. Constitutive exons are always present in the transcript; there is some variability regarding facultative exons which are only present in some individuals. The yellow "leg" region indicates an exon that has only been amplified in the leg. The two highlighted fragments are the most abundant products, which were further quantified by AFLP

We would like to thank the entire Southgate Lab and the INBRE and HHMI Programs for their support.

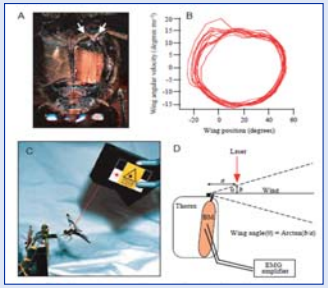


Figure 6: analysis of PEVK isoforms (A) The basalar muscle of the mesothorax exposed by removal of a portion of the cuticle and the underlying air sacs. (B) Sample trace of wing position and velocity from a recording made using a laser distance sensor on the base of the forewing. (C) Photograph showing a dragonfly attached by its ventral thorax to a narrow glass beam (not visible) that extends from a strain gauge. (D) Diagram showing the geometry of the basalar muscle attachment to the base of the forewing, an approximation of the EMG recording electrodes and the method used to calculate wing position from the laser distance sensor. *From Marden et al, 2001*

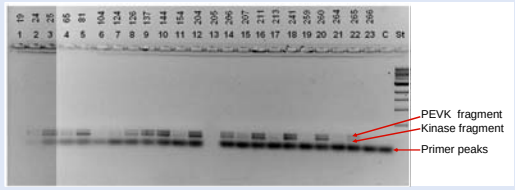
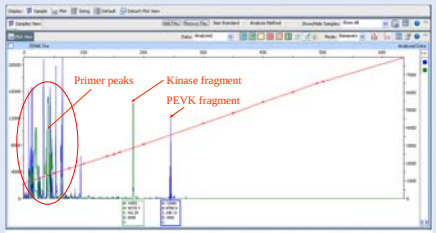


Figure 7: analysis of PEVK isoforms 1.2% agarose-TBE gel of RT-PCR reactions on *L. pulchella* RNA samples from 12 individuals using Lp6-Lp6R and kin1-kin2R primer sets. This figure demonstrates the nature of the duplex reaction as both the PEVK region and Kinase region fragments are present.



Dye/Sample Peak	Size	Height	Area in Point	Area in BP	Data Point	Begin Point	Begin BP	End Point	End BP
B, 123	244	4833	43389	3684	3582	3567	243.001	3584	244
G, 93	182	14363	115573	10135	2859	2839	180.493	2889	185

Figure 8: chromatogram from AFLP and example of peak analysis This chromatogram indicates the signal strength of the fluorescent tags associated with the PEVK and kinase fragments. The kinase fragment as an internal control represents 100% of the projectin transcripts. Information regarding peak intensity can be interpreted to provide data regarding the relative amount of PEVK variants in each individual.

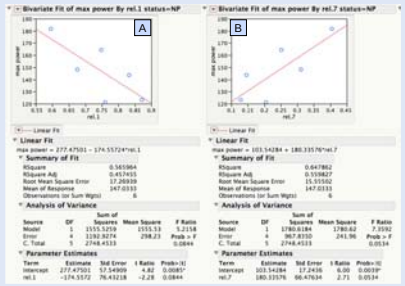


Figure 9: analysis of PEVK isoforms Simple linear regression analysis on the relative abundance of 2 of the splice forms and muscle power output. Significance of effects of power was tested using ANOVA (for regression, i.e. basically testing whether the slope is different from 0; at an alpha = 0.05), using JMP software (SAS Institute). Graphs display a possible correlation between maximum work output during flight and relative abundance of PEVK variants. (A) Slope of curve demonstrates a positive correlation of the short PEVK variant (fragment #1). (B) Slope depicts an inverse correlation between power output and relative abundance of the long PEVK isoform (fragment #7). Fragments are referenced in Figure 5.