Analysis of Projectin in Synchronous Lepidopteran Flight Muscles

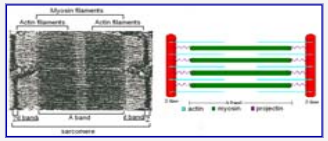
Larchinee Turner and Agnes Ayme-Southgate
Department of Biology, College of Charleston, Charleston, SC

ABSTRACT

To generate high wing beat, several insect orders have developed a novel muscle physiological system, resulting in multiple contractions per nerve impulse (asynchronous). Basal insects possess only synchronous muscles whereas asynchrony is specific to derived insects. It is expected that these two muscle types differ significantly in their protein composition, structure and abundance. One likely candidate is projectin, a large muscle protein composed of two repeated motifs, as well as unique sequences such as the PEVK domain, which is known to be an elastic region. We continue with our molecular analysis of projectin to include more diverse insect orders and correlate differences in projectin structure with muscle physiology. In this study we describe our progress for the silkworm, *Bombyx mori*, and another moth, *Manduca sexta.* We assembled most of the sequence of the projectin gene in *B. mori* from genome data and obtained partial sequence in *M. sexta* using molecular cloning techniques.

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**



Insects flight muscles are classified differently based on several properties. For example based on the ratio between muscle contraction and nerve impulses, insect flight muscles are considered as either synchronous (1:1) or asynchronous (1:>1) (**Figure 2**). In derived insects, asynchrony is possible because of the high stiffness associated with a third elastic filament, called the C-filament (Fig 1). The presence and possible function of such a filament in synchronous insects is unknown. In this study we will address this question in Lepidoptera (butterflies and moths), in particular
1) *Bombyx mori*
2)Manduca Sexta

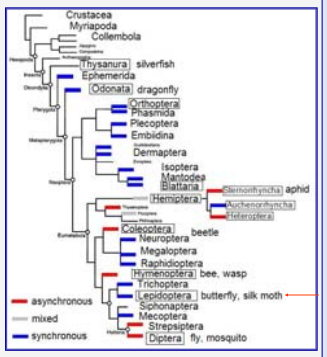


FIGURE 2: Constructed insect phylogeny with the distribution of synchronous and asynchronous muscles

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 3**). 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 asynchronous insects, the indirect flight muscles contain predominantly the shorter form. The composition and size of the PEVK domain in projectin of synchronous flight muscles of Lepidoptera is unknown.

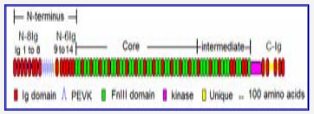


FIGURE 3: Schematic of the projectin protein and its different regions

The *Bombyx mori* genome has been sequenced. Using the information available in Genbank, the projectin gene was partially assembled. At the end of that analysis, several gaps were still present and the PEVK sequence was still very incomplete. Primers were derived from the available *B. mori's* sequence and used for *B. mori* sequencing, as well as cloning and sequencing of "its cousin" *Manduca sexta*, the tobacco hornworm.

HYPOTHESIS

Insect synchronous flight muscles with low stiffness present longer PEVK isoforms.

GENERAL PROTOCOLS

- RNA is extracted from different insects or isolated muscle tissues using Trizol (Invitrogen).
- RNA is used in Reverse Transcriptase-PCR reactions (RT-PCR; SuperScipt Platinum III, Invitrogen) with different combinations of degenerated or species-specific primers. This allows the amplification of a cDNA region of the mRNA.
- RT-PCR products are separated according to size by agarose gel electrophoresis.
- The cDNA fragments are gel-extracted, purified and cloned in the pGEM-T Easy vector (Promega) for sequencing, or in specialized vector for protein expression.

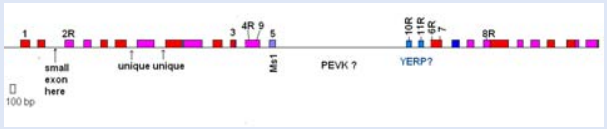


FIGURE 5: Map of Bombyx N-terminal region of the projectin gene with primers used in this study. These were used to isolate the gene for projectin from *B.mori* and *M. sexta*. The PEVK region is not conserved enough to allow for the design of degenerate primers.

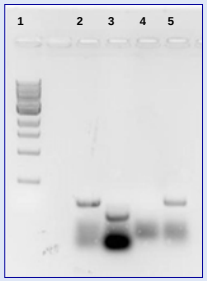


FIGURE 6: Gel electrophoresis of *B. mori* RT-PCR.
Lane 1: Kb ladder
Lane 2: Bm5E1-2R
Lane 3: Bm5E3-4R
Lane 4: Bm5E9-6R
Lane 5: Bm5E9-10R

Larval RNA was used for these reactions. The resulting bands in lanes 2, 3, and 5 were cut and cloned into the pGEM-T easy vector. Sequences are now available

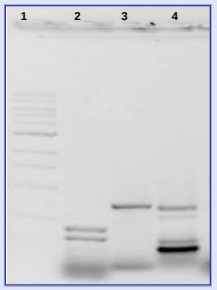


FIGURE 7: Gel electrophoresis of *Manduca* larval telson RT-PCR.
Lane 1: Kb ladder
Lane 2: Bm5E3-4R
Lane 3: Bm5E9-6R
Lane 4: Bm5E9-10R

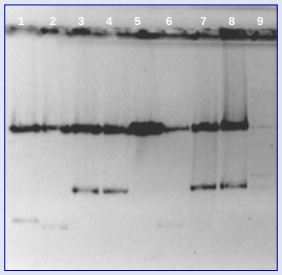


FIGURE 8: Gel electrophoresis of Bombyx plasmid DNA
Lane 1: Bm5E9-10R M1
Lane 2: Bm5E9-10R M2
Lane 3: Bm5E9-10R B1
Lane 4: Bm5E9-10R B2
Lane 5: Bm5E9-10R S1
Lane 6: Bm5E9-10R S2
Lane 7: Bm5E9-6R 1
Lane 8: Bm5E9-6R 2
Lane 9: Kb Ladder

Plasmid samples were purified using a PureYield miniprep kit by Promega. DNA of chosen plasmid sample was quantified by spectrophotometry and sent for sequencing at the Grice Marine Laboratory's Molecular Core Facility

Bombyx mori

Manduca sexta

FIGURE4: pictures of Bombyx and Manduca: larva and adults with citation

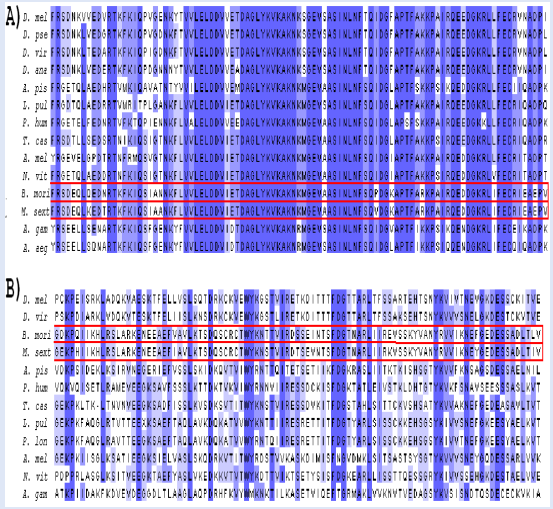


FIGURE9 : CLUSTALW analysis.
The partial amino acid sequence in B. mori and M. sexta projectin N-terminus N8Ig domain was compared to various insect orders using CLUSTALW alignment software. Higher percentage identity between amino acids is indicated through darker color coding.
A) N-Ig#1 and 2, B) N-Ig #8.
The two moth sequences are boxed in red. They conform to the general pattern: a high degree of conservation at the beginning of the protein, as opposed to a very low conservation in the region just before the extensible PEVK domain (Ig#8). Ig#1 and #2 are proposed to be embedded in the Z-line of the sarcomere, to provide anchoring support. This could account for the higher conservation between amino acids in Ig#1 and #2, which is needed to maintain a secondary/tertiary structure/conformation for attachment to other proteins. Ig#8 is closer to the PEVK domain, and could also be involved in domain unfolding during muscle stretch, therefore the ability to unfold is posibile with maintaining secondary/tertiary conformation (through conserved amino acids) is less important.
The insects whose sequences are aligned are:

- Diptera = D.mel: *Drosophila melanogaster*, D.pse: *D. pseudoobscura*, D.vir: *D. virilis*, D.ana: *D. annanassae*, A.gam: *Anopheles gambiae*, A.aeg: *Aedes aegyptii*,
- Lepidoptera = B.mori: *Bombyx mori* (silkworm), Msext: *Manduca sexta* (tobacco hornworm)
- Coleoptera = Tcas: *Tribolium castaneum*
- Hymenoptera = Amel: *Apis mellifera*, Nvit: *Nasonia vitripennis*
- Hemiptera = Apis: *Acyrthosiphon pisum*
- Phthiraptera = Phum: *Pediculus humanus*
- Odonata = Lpul: *Libellula pulchella*, Plon: *Pachydiplax longipennis*

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