A Calcium ATPase in Mosquito Larvae as a Putative Receptor for Cry Toxins

DISSERTATION

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By

Yoshio Ikeda, M.S.

The Ohio State Biochemistry Program

The Ohio State University

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Dissertation Committee:

Dr. Donald H. Dean, Advisor
Dr. Edward J. Behrman
Dr. Ross E. Dalbey
Dr. Venkat Gopalan
Abstract

Mosquitoes are vectors of several serious diseases. For example, *Aedes* is a vector of yellow fever and dengue fever. *Culex* is a vector of West Nile fever and *Anopheles* is a vector of malaria. Since there are no effective cures for these diseases, it is important to control the population of mosquitoes to reduce the occurrence of these diseases.

One of the methods to control the mosquito population is to use mosquitocidal Cry proteins. Cry proteins are synthesized by the Gram-positive bacteria *Bacillus thuringiensis* (Bt) during the sporulation stage. There are many types of Cry proteins. Some Cry proteins are toxic to Lepidoptera, whereas others are toxic to Diptera, such as the mosquito. Certain Cry proteins are toxic to larvae of certain insects. Although we know Cry proteins kill larvae of susceptible insects, we are unsure about the mechanism of toxicity of Cry proteins but it is clearly receptor mediated. The purpose of this study was to discover receptor(s) for mosquitocidal Cry proteins in the larvae of mosquito *Culex pipiens pipiens*. Discovering receptors will help us better understand the mechanism of action of Cry toxins, and aid in making more specific and effective mosquitocidal Cry proteins by protein engineering.

A strategy to define host receptors is to use photochemical-cross-linking. The advantage of using photo-cross-linking is that it can detect weak interactions between the host’s receptor and a specific Cry protein.
We attempted to use a photo-cross-linking approach involving photo-leucine, an unnatural amino acid, which can be incorporated in protein instead of leucine in mammalian cells. This photo-leucine has a diazirine group which can be activated by UV to generate a highly reactive carbene, which then, inserts into nearby molecules forming covalent bonds. Our initial attempt to synthesize photo-leucine failed because of the lack of the detail in the methods used to synthesize the photoactivatable leucine. After reviewing latest methods in the literature for the synthesis of the diazirine group, we came up with a revised strategy and successfully made photo-leucine. We attempted to incorporate photo-leucine into a Cry protein in a Bacillus subtilis strain auxotroph for leucine and methionine. The cells grew with photo-leucine, but failed to sporulate or make Cry protein even with a mixture containing both photo-leucine and leucine. As an alternative, we chose to label lysine residues of Cry protein using N-Hydroxysuccinimide (NHS)-diazi-rine.

Cry11Ba and Cry19Aa were labeled with NHS-diazi-rine. These NHS-diazi-rine labeled Cry proteins were then mixed with BBMV (Brush border membrane vesicles) proteins and activated with UV light to induce cross-linking. Cross-linked Cry19Aa proteins were pulled down using anti-Cry19Aa antibody and protein A magnetic beads. Cross-linked bands were excised from the gel and analyzed by mass spectrometry. The data from mass spectrometry showed that this cross-linked band contained a sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) as a top match. SERCA transcription level has been reported in the literature to be decreased in Cry protein-resistant larvae, providing circumstantial evidence for a role of SERCA as a receptor.
We constructed (His)$_6$-tagged Cry19Aa. Bioassays with C. pipiens larvae showed that $N$-terminally (His)$_6$-tagged Cry19Aa has three-fold lower toxicity compared to the untagged Cry19Aa, while $C$-terminally (His)$_6$-tagged Cry19Aa retained the same level of toxicity as Cry19Aa; we therefore chose to use $C$-terminally (His)$_6$-tagged Cry19Aa in pull-down assays. To verify SERCA as a binding protein of Cry19Aa, the pull-down experiment using BBMV with diazirine-modified Cry19Aa was performed. The pulled-down proteins were probed with SERCA antibody using a western blot. Although SERCA was found to be a binding partner, consistent with our earlier mass spectrometry results, firm support for this finding requires further in-depth experimentation includes controls to ascertain specificity of interaction. If SERCA is a Cry19Aa receptor, Cry19Aa might bind to SERCA from the cytoplasmic side and prevent Ca$^{2+}$ transport by blocking the formation of the aspartyl phosphate intermediate.
Dedication

Dedicated to my parents (Katsuo Ikeda and Chiyoko Ikeda) and my sister (Wakako Kazawa) who have supported and encouraged me throughout my graduate study.
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Vita

August 12, 1973… Born in Takasaki, Gunma, Japan

1997………………B.S. (Pharmaceutical Sciences), University of Tokyo, Japan

1999………………M.S. (Molecular Pharmaceutics), University of Tokyo, Japan

2003 to present ...... Graduate Teaching Associate/Graduate Research Associate,

Department of Chemistry and Biochemistry,

The Ohio State University

Publications

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Fields of Study

Major Field: Biochemistry
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List of Abbreviations

2-D 2-dimensional
A. aegypti Aedes aegypti
ALP alkaline phosphatase
An. gambiae Anopheles gambiae
APN aminopeptidase N
ATP adenosine triphosphate
BBMV Brush border membrane vesicles
BLAST Basic Local Alignment Search Tool
Bs Bacillus subtilis
Bt Bacillus thuringiensis
Bti Bacillus thuringiensis subspecies israelensis
CAT chloramphenicol acetyltransferase
CDC Centers for Disease Control and Prevention
CHAPS 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
C. p. pipiens Culex pipiens pipiens
C. p. quinquefasciatus Culex pipiens quinquefasciatus
DAPI 4′,6-diamidino-2-phenylindole
DMSO dimethyl sulfoxide
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>DPBS</td>
<td>Dulbecco’s phosphate-buffered saline</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>h</td>
<td>hours</td>
</tr>
<tr>
<td>IPG</td>
<td>immobilized pH gradient</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>KOD</td>
<td><em>Thermococcus kodakaraensis</em></td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight marker</td>
</tr>
<tr>
<td>NHS</td>
<td><em>N</em>-hydroxysuccinimide</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethanesulfonyl fluoride</td>
</tr>
<tr>
<td>PNK</td>
<td>polynucleotide kinase</td>
</tr>
<tr>
<td>RFP</td>
<td>red fluorescent protein</td>
</tr>
<tr>
<td>SB3-10</td>
<td>sulfobetaine 3-10</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarcoplasmic/endoplasmic reticulum calcium ATPase</td>
</tr>
<tr>
<td>Sf</td>
<td><em>Spodoptera frugiperda</em></td>
</tr>
<tr>
<td>SFM</td>
<td>serum-free insect media</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>SSM</td>
<td>Schaeffer’s sporulation media</td>
</tr>
<tr>
<td>supe</td>
<td>supernatant</td>
</tr>
<tr>
<td>TBAB</td>
<td>tryptose blood agar base</td>
</tr>
<tr>
<td>TGX</td>
<td>tris-glycine extended</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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Chapter 1: Introduction

1.1 Mosquitoes

There are 3,531 species of mosquitoes in the world (as of April 26, 2013) (15). Mosquitoes are vectors of several serious diseases. Examples of these mosquitoes are *Aedes, Anopheles, and Culex*.

*Aedes* is a vector of yellow fever and dengue fever. An example species of *Aedes* is *Aedes aegypti* (Figure 1A). “Aedes” means unpleasant in Greek (αιδής). The name “aedes” was introduced by Johann Wilhelm Meigen in 1818. He wrote that “aedes” means “beschwerlich (arduous)” in German (16, 17). *Aedes aegypti* was originally named as *Culex aegypti* by Fredric Hasselquist and Carl Linnaeus in 1757 (18). The name was later changed to *Aedes aegypti*. “Aegypti” refers to the finding of this mosquito in Egypt. The distribution of *Aedes aegypti* in USA is shown in Figure 2. The eggs of *Aedes aegypti*, the primary vector of dengue fever, cannot survive cold winters (less than 10 °C) (19). Dengue fever occurs in warmer regions (Figure 3) (12, 20, 21). *Aedes aegypti* typically bites people’s ankles or elbows during the day time (22). Dengue fever is caused by dengue viruses which are transmitted when an infected female mosquito sucks blood. There are four types of dengue viruses. Dengue virus is a single-stranded RNA virus which is amplified in human (21).
infected humans, it becomes infected. After infection for 8-12 days, the mosquito can transmit the virus for the rest of its life (21). There are an estimated 390 million dengue infections in 2010, of which 96 million manifested symptoms (12). The symptoms of dengue fever starts from sudden fever of 40 °C with body or head ache and facial flushing. When the patients have a fever, they should take paracetamol (Tylenol), not ibuprofen (Advil) or aspirin. Ibuprofen and aspirin promote bleeding. Then, when fever goes down, some patients can experience bleeding (dengue hemorrhagic fever). These patients need to be hospitalized to avoid death from shock or organ impairment. Death rate is 1.2 % in the Americas (21). Pregnant women should avoid mosquito bites, because dengue fever can be transmitted to their babies (either unborn or at birth) (23).

Anopheles is a vector of malaria. An example of a species of Anopheles is Anopheles gambiae (Figure 1B). “Anopheles” means hurtful in Greek [ανωφέλης, αν (negative suffix, not) + ωφέλος (help)] (24, 25). The name “anopheles” was also introduced by Johann Wilhelm Meigen in 1818. He wrote that “anopheles” also means “beschwerlich (arduous)” in German (26). “Gambiae” means that the mosquito was found in Gambia valley (around Gambia river which is the same region as the country Gambia) in West Africa (27). The distribution of Anopheles and the location of Gambia are shown in Figure 4 (3). The distribution of malaria is in Figure 5 (28). Malaria is caused by the protozoan parasites Plasmodium falciparum or Plasmodium vivax. These can be transmitted by an infected Anopheles female during blood sucking. The protozoan parasites propagate in the liver first by asexual division. They can stay in the liver for weeks or years. When the liver cells rupture, the parasites are released to the blood. The
parasites multiply in the red blood cells which will rupture and release more parasites in the blood. The symptoms of malaria (fever, chill, and hemolytic anemia) occur at this stage. Without treatment, malaria leads to death. The protozoan parasites differentiate into male and female forms in the blood. When *Anopheles* mosquito sucks blood, these male and female parasites move into the mosquito and mate. The resulting zygotes develop new asexual parasites which can be transmitted into humans (29). The number of malarial death was 1,238,000 in the world in 2010. Most of them were in Africa (1,113,000 deaths, 39% were 5 years or younger) (28).

*Culex* is a vector of West Nile fever. An example of a species of *Culex* is *Culex pipiens pipiens* (Figure 1C). “Culex” means gnat, and “pipiens” means piping sound in Latin (30). *Culex pipiens* was named by Carl Linnaeus in 1758 (31). *Culex pipiens* subspecies *pipiens* (*C. p. pipiens*, northern house mosquito) and *Culex pipiens* subspecies *quinquefasciatus* (*C. p. quinquefasciatus*, southern house mosquito) are in USA (Figure 6). West Nile fever is caused by the West Nile virus which was found in a patient in the West Nile district of Uganda in 1937. The West Nile virus is a single-stranded RNA virus, which belongs to the genus flavivirus (dengue virus also belongs to this group). Flavi comes from Latin word flavus (yellow). Yellow fever virus is also a flavivirus (32, 33). West Nile virus is maintained between infected mosquitoes and birds. Humans get the virus from infected mosquitoes, but the virus cannot replicate enough in human or horse to be infectious. Therefore a mosquito cannot get the virus from human or horse and then transmit the virus to others (34). Eighty percent of infected persons will have no symptoms. Twenty percent of them will have symptoms of West Nile fever (fever,
headache, and fatigue). Some people with West Nile fever can succumb to severe West Nile meningitis, encephalitis, or death (35). West Nile fever cases were reported annually since 1999 in USA (Figure 7, Figure 8). The death rate is about 5% of reported cases (Table 1).

The mosquito life cycle can begin with the adult female mosquito sucking blood in order for eggs to develop. She needs proteins in the blood to lay eggs. She lays eggs on the water as an egg raft (C. pipiens), as a single egg (An. gambiae) or on substrate near the water, such as on earthenware pots for storing water (A. aegypti) (36). The larvae live in water. Larvae eat particulate matter between 0.5 μm and 50 μm in size (e.g., bacteria in the water) (37). There are 4 instars in larvae. An instar is a stage of insects between each molt (38). After the 4th instar, larvae become pupae. Pupae can move but do not eat. The pupa hatches and the adult emerges from the pupal case (the picture of adult male of C. p. pipiens just emerged from its pupal case is in Figure 9). Both adult male and female needs sugar from flower or fruits for energy. Only female sucks blood for the purpose of laying eggs. Male has big antennae to hear female’s buzzing sound (Figure 9).
1.2 Bacillus thuringiensis

*Bacillus thuringiensis* (Bt) was first discovered in Japan in 1901 by Shigetani Ishiwata. He named the bacterium *Bacillus sotto*. Sotto in Japanese means sudden collapse or fainting due to shock or stroke. Aoki and Chigasaki (the Institute for Silk Growing Research in Tokyo) continued Ishiwata’s work. They observed that only an old culture of Bt killed silkworms (39).

In 1911, a German scientist, Ernst Berliner, found a related bacteria and named it *Bacillus thuringiensis* (40). The bacteria were found in the Thuringia state of Germany. In 1953, Hannay observed under the microscope that both the crystal protein and the spore were made during the late stage of the bacterial culture (Figure 10) (11). The midguts of the insects are alkaline. The crystalline protein can be dissolved in alkaline condition (11). Angus re-confirmed the experiments of Aoki who showed that a young culture was not effective but an older culture was toxic to silk worms. This crystalline protein was responsible for toxicity of the insect. Angus showed that the alkaline pH dissolved crystal protein was toxic to silkworm, whereas the spore was not toxic by feeding. Silkworms became paralyzed within 4 h after feeding on an old culture or alkaline dissolved crystal protein. The injection of an old Bt bacterial culture or spore into the body cavity of silkworm caused septicemia but not paralysis (Table 2) (7). The crystalline protein was named Cry protein (41).

There are many types of Cry proteins. Each has specific toxicity to one or more orders of insects, but for most, activity is limited to insects within one order. Order is one
of the taxonomic ranks to classify organisms (42). For example, Diptera [di (δις, two) ptera (πτερον, wing) in Greek] is the order in insects, which includes mosquito and flies (43). The order Diptera was created by Carl Linnaeus (44). Cry proteins differ in amino acid sequence, and the names of Cry proteins are based on amino acid sequence differences. For example, Cry1 and Cry2 have less than 45 % amino acid sequence identity. Cry1A and Cry1B have between 45 % to 78 % amino acid sequence identity. Cry1Aa and Cry1Ab have between 78% to 95 % amino acid sequence identity (45). As of this writing, Cry1Aa to Cry72Aa were reported (46). Although their sequences and specific toxicity towards insects are different, the structures of Cry proteins are similar. All the currently available crystal structures of Cry proteins reveal the presence of three domains (Figure 11). Therefore, the mechanism of toxicity may be similar; however, this remains to be proven (Figure 12).

There are two major proposals for mechanisms of action for Cry proteins: signal transduction (Figure 13) and the pre-pore oligomer mechanism (Figure 14) (4, 47). Initial steps are the same in both cases. Cry crystal protein is eaten by mosquito larva. The crystal is dissolved by the alkaline pH of the midgut. Cry protoxin protein is cleaved by digestion enzymes (midgut proteases) generating an active toxin (Figure 12) (48). The subsequent steps involving receptor(s) for the Cry protein and the molecular mode of action of Cry protein toxicity to insects are different in the two proposed mechanisms.

In the signal transduction mechanism, monomeric Cry protein binds to a cadherin-like receptor and transduces a signal across the membrane. The signal is delivered by a G-protein, adenylyl cyclase, and protein kinase A. Mg$^{2+}$, not Ca$^{2+}$, is needed to activate
This signal leads to cell death. Neither endocytosis of Cry protein nor apoptosis are proposed in this signal transduction model. This signal transduction mechanism was studied by transfecting insect cells (so called High five cells, cabbage looper ovary cells, Life Technologies) with a cadherin-like receptor gene (49). The oligomer Cry protein was not toxic to insect cells without cadherin-like receptors (50).

In the pre-pore oligomer mechanism, monomeric Cry protein first binds to glycosylphosphatidylinositol (GPI)-anchored aminopeptidase N (APN) or GPI-anchored alkaline phosphatase (ALP) which are abundant in the insect midgut. Subsequently, the monomeric Cry protein binds to a cadherin-like protein where α helix-1 in the domain 1 of Cry protein is cleaved proteolytically. This cleavage promotes the oligomerization of the Cry protein (pre-pore oligomer). Oligomeric Cry protein has a higher affinity than monomeric Cry protein to APN or ALP. Pre-pore oligomeric Cry protein leaves the cadherin-like protein, binds to APN or ALP, and then inserts into a membrane of midgut cell to make a pore and kills the infected cell by osmotic shock (47). Cry1Ab α helix-1 deletion mutant formed oligomers without cadherin-like protein and was toxic to the Cry protein resistant pink cotton bollworm larvae, which had the gene encoding the cadherin-like protein deleted (51).

Some receptors for Cry protein were reported. These are APN, ALP, cadherin, and glycolipids (52). Glycosylation of APN seems important for Cry protein binding (52). APN is released from the midgut of a gypsy moth larva when Cry protein was ingested (53). ALP and APN were shown to be receptors for Cry11Ba in An. gambiae larva, because the mixture of soluble Cry11Ba and ALP or APN inclusion bodies which
were overexpressed in *E. coli* reduced the toxicity compared to Cry11Ba alone (54, 55). The cadherin-like protein has cadherin repeats. When cadherin-like proteins from tobacco hornworm or *An. gambiae* were expressed in insect cells, Cry proteins (1Aa for tobacco hornworm, 4Ba for *An. gambiae*) caused the blebbing and swelling of cells and cell death (4). Glycolipids were shown to be a receptor for Cry5Ba in nematode *Caenorhabditis elegans*. Cry protein-resistant *C. elegans* is deficient in synthesizing carbohydrate chain of glycolipid (56).

Because of the high specificity of Cry proteins to insects but not to humans, Cry proteins are used as insecticides. It is also used in transgenic crops. If the mechanism of Cry protein toxicity is understood, it can be used to provide insight into making better insecticides.
Figure 1. Mosquitoes (Adult female).
(A) *Aedes aegypti*, vector of yellow fever and dengue fever.
(B) *Anopheles gambiae*, vector of malaria
(C) *Culex p. pipiens*, vector of West Nile fever
Figure 2. Distribution of *Aedes aegypti* in USA.
Figure reproduced from (9).
Figure 3. Global evidence consensus of dengue in 2010.
Figure reproduced from (12). National or subnational level of evidence consensus of dengue was shown as colors on the map. (Complete absence of dengue (green) and complete presence of dengue (red)).
Figure 4. A global map of the dominant malarial vector species.
Figure reproduced from (3). Euro. & M. East: Europe & Middle-East.
Figure 5. Cumulative probability of dying from malaria in the absence of all other causes from birth to age 80 years in 2010. Figure reproduced from (28).
Figure 6. Distribution of *Culex pipiens* in USA.
Figure reproduced from (10).
(A) *Culex pipiens* subspecies *pipiens*
(B) *Culex pipiens* subspecies *quinquefasciatus*
Figure 7. West Nile virus activity in USA in 2012 as of December 1st, 2012.
Figure reproduced from (8). Each human infection is shown as a green circle.
The numbers of cases were obtained from the CDC website (57).

<table>
<thead>
<tr>
<th>Year</th>
<th>West Nile fever cases USA total</th>
<th>Death USA total</th>
<th>Cases in OHIO</th>
<th>Death in OHIO</th>
</tr>
</thead>
<tbody>
<tr>
<td>1999</td>
<td>62</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2002</td>
<td>4156</td>
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<td>441</td>
<td>31</td>
</tr>
<tr>
<td>2003</td>
<td>9862</td>
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</tr>
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<td>3000</td>
<td>119</td>
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</tr>
<tr>
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<td>48</td>
<td>4</td>
</tr>
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<tr>
<td>2012</td>
<td>5245</td>
<td>236</td>
<td>119</td>
<td>7</td>
</tr>
</tbody>
</table>

Table 1. West Nile fever cases and deaths in USA and in Ohio. The numbers of cases were obtained from the CDC website (57).
<table>
<thead>
<tr>
<th></th>
<th><em>A. aegypti</em></th>
<th><em>An. gambiae</em></th>
<th><em>C. p. pipiens</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Eggs</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>Larvae</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>Pupae</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>Adult female</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>Adult male</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

Figure 9. Life cycle of mosquitoes.
Figure 10. *Bacillus thuringiensis* produces spore and crystal protein (Cry protein).

(A) First reported crystal protein (diamond shape) and spore in Bt by Hannay (Science Service Laboratory, London, Ontario, Canada in 1953). Figure reproduced from (11).

(B) Cry11Aa is produced during the sporulation stage of Bt. (1,000 X, under phase contrast microscopy).
Table 2. Angus’ experiment (7): the effect of feeding and injecting larvae of *Bombyx mori* L. with fractions of an alkali-treated culture of *Bacillus sotto*.

<table>
<thead>
<tr>
<th>Method of dosing larvae</th>
<th>By feeding</th>
<th>By injection</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Original culture</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spores and crystals</td>
<td>Paralysis within 4 h,</td>
<td>Septicemia within 12 h.</td>
</tr>
<tr>
<td>(1 x 10^5 spores per larva)</td>
<td>septicemia within 12 h.</td>
<td>No paralysis</td>
</tr>
<tr>
<td><em>Alkali-treated culture</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Spore fraction</td>
<td>No effect</td>
<td>Septicemia within 12 h.</td>
</tr>
<tr>
<td>(1 x 10^7 spores per larva)</td>
<td>Paralysis within 4 h.</td>
<td>No effect</td>
</tr>
<tr>
<td>2. Supernatant</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>3. Supernatant dialysed</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>4. Supernatant heated at 70 °C for 30 min</td>
<td>No effect</td>
<td>No effect</td>
</tr>
</tbody>
</table>

Table reproduced from (7). Angus (Laboratory of Insect Pathology, Sault Ste. Marie, Ontario, Canada in 1954) showed that alkaline dissolved crystalline protein, not spore, was toxic (causing paralysis) to silkworm.
Figure 11. Cry protein structures determined by X-ray crystallography and their specific toxicities towards insects.

Protein databank ID (6): Cry1Aa (1CIY), Cry2Aa (1I5P), Cry3Aa (1DLC), Cry3Bb (1JI6), Cry4Aa (2C9K), Cry4Ba (1W99), Cry5Ba (4D8M), Cry8Ea (3EB7). Domains are shown in different colors: domain I (magenta), domain II (cyan), domain III (green).
Figure 12. Mechanism of toxicity of Cry protein is not understood well. Cry protoxin crystal is eaten by mosquito larva. The crystal is dissolved by the alkaline pH of the midgut. Cry protoxin protein is cleaved by digestion enzymes (midgut proteases) to generate the active toxin, which kills the larva. The receptors and mechanism of uptake are still being explored.
Figure 13. Signal transduction model proposed by Bulla (University of Texas at Dallas, Richardson, Texas, USA).
Figure reproduced from (4). Cry toxin binds to cadherin receptor which activates G protein-mediated signal transduction. This signal leads to cell death. The oligomeric toxin is not toxic.
Figure 14. Pre-pore oligomer model postulated by Bravo (Universidad Nacional Autónoma de México, Cuernavaca, Morelos, México).

Figure reproduced from (47). 1. Cry protoxin is solubilized and activated by midgut proteases. 2. Cry toxin binds to GPI anchored aminopeptidase-N or alkaline phosphatase. 3. Cry toxin binds to cadherin-like receptor and α-helix 1 of domain I of Cry toxin is cleaved. 4 Cry toxin makes pre-pore oligomer and binds to GPI anchored aminopeptidase-N or alkaline phosphatase. 5. This oligomer inserts into the cell membrane, which causes cell lysis.
Chapter 2: Improved Synthesis of Photo-leucine

See appendix A.
Chapter 3: Cross-linking and Co-immunoprecipitation

3.1 Introduction

There are many methods to detect protein-protein interactions. Cross-linking is one of them. Some of the receptors of Cry proteins have been identified by ligand blot analysis using brush border membrane vesicle (BBMV) proteins and a Cry protein, but weak interactions between a Cry protein and a receptor can be difficult to detect by this method. One of the benefits using cross-linking is the ability to capture a weak-binding receptor with a covalent bond. There are many types of cross-linking reagents. We decided to use photo-leucine as the photo-cross-linking reagent (chapter 2) to capture a receptor in a short time in a desired moment, as the crosslinker can be activated by UV illumination. Co-immunoprecipitation is another method to study protein-protein interactions. By using co-immunoprecipitation, a receptor can be separated from the other BBMV proteins that do not bind the Cry protein of interest.

Some binding proteins and receptors such as aminopeptidase N and alkaline phosphatase were reported for Cry11Ba to Aedes aegypti (A. aegypti) and Anopheles gambiae (An. gambiae) (Table 3 and Table 4). A Cry19Aa receptor in Culex pipiens pipiens (C. p. pipiens) is not known. Cry19Aa is toxic to C. p. pipiens (Table 5). Therefore, Cry19Aa and C. p. pipiens are used for this experiment.
*C. p. pipiens* BBMV proteins were prepared according to English-Readdy method (58). This method uses Ca\(^{2+}\) to precipitate BBMV rapidly, as first described by Malathi et al (59). Ca\(^{2+}\) and Mg\(^{2+}\) are effective in precipitation of microsomal membrane because of their positive charge and the negative charge of membrane (60). Therefore, BBMV can be separated from microsomes by using Ca\(^{2+}\) or Mg\(^{2+}\) (60). The brush border facilitates carbohydrate and amino acid absorption. Most of the BBMV protein preparations reported so far in the Bt research field used the Mg\(^{2+}\) precipitation method, but we chose the Ca\(^{2+}\) precipitation method as it was a simpler procedure. It is worth noting that there were no differences in the results of ligand blot between Cry1Ac and *Helicoverpa armigera* (cotton bollworm) BBMV when Ca\(^{2+}\) and Mg\(^{2+}\) precipitation methods were compared (61). The Ca\(^{2+}\) precipitation method was used for analysis of *A. aegypti* BBMV proteins using 2-dimensional (2-D) gel electrophoresis in our laboratory (62). 2-D gel electrophoresis is a method to separate proteins in two dimensions using isoelectric focusing and SDS-PAGE.

The aim of the study in this chapter was to discover a Cry19Aa receptor in a *C. p. pipiens* larva using the photo-crosslinking reagent, photo-leucine. However, we were more successful in using photo-cross-linking between a diazirine-labeled Cry19Aa and *C. p. pipiens* BBMV, which was prepared by the Ca\(^{2+}\) precipitation method. Co-immunoprecipitation was included after photo-cross-linking to separate a cross-linked receptor.
3.2 Materials and Methods

3.2.1 Cry Protein Expression and Purification

Cry11Ba plasmid in 4O7 strain of *Bacillus thuringiensis* (DBT13) and Cry19Aa plasmid in 4O7 *Bacillus thuringiensis* (DBT14) were obtained from the *Bacillus* Genetic Stock Center on TBAB (tryptose blood agar base) agar plate (OSU, Columbus, OH, [http://www.bgsc.org/](http://www.bgsc.org/)). (DBT13 and DBT14 are numbers assigned by the *Bacillus* Genetic Stock Center.) Four tubes of LB (5 mL with 25 μg/mL erythromycin) were inoculated with a single colony of DBT13 (Cry11Ba) or DBT14 (Cry19Aa). Tubes were shaken at 23 °C overnight. Four 2-L flasks containing 400 mL Schaeffer’s sporulation media (SSM. Difco Nutrient Broth 8 g/L, MgSO₄·7H₂O, 0.25 g/L, KCl 1 g/L. After autoclaving, sterile CaCl₂·2H₂O, FeSO₄·7H₂O, and MnCl₂·4H₂O were added to 1 mM, 1 μM, and 10 μM, respectively (63).) with 25 μg/mL erythromycin was inoculated with the 5-mL overnight culture. The flasks were shaken at 23 °C for 4 days. Most of the cells had autolysed, as determined by analyzing cells using phase contrast microscopy. The cells were harvested at 12,429 x g (9,000 rpm with Beckman J-25 centrifuge and JA-14 rotor) at 4 °C for 10 min. The pellets were washed thrice with 100 mL of crystal wash I [0.5 M NaCl, 2 % (v/v) Triton X-100] and then were washed thrice with 100 mL of crystal wash II (0.5 M NaCl). The sample was centrifuged at 12,429 x g at 4 °C for 10 min between washes. The pellets (crystal and spore mixture) were resuspended in 80 mL Crystal wash II and 1 mM PMSF. The crystal and spore mixture was stored at 4 °C until use.
Crystal proteins were washed in water to remove NaCl from Crystal wash II prior to purifying through anion exchange column after solublization. Crystal proteins were solublized in 80 mL of 50 mM Na₂CO₃, 10 mM DTT at 37 °C for 2 h, and the suspension was centrifuged at 16,917 x g for 10 min at 4 °C. The supernatant which contained protoxin was saved. The pH of protoxin solution was adjusted to pH 8-9 with 2 M HCl. Solublized protein was digested with trypsin for Cry11Ba and chymotrypsin for 19Aa (1/20 wt/wt) at 37 °C for 1 h. Proteolysis was terminated by adding 1mM PMSF.

Activated Cry protein was loaded on a 10-mL Q-sepharose anion exchange column (GE Healthcare) which was equilibrated with 50 mM sodium carbonate, pH 10.5. After washing with the carbonate buffer, the protein was eluted with increasing amount of NaCl in the carbonate buffer. We performed 100-mL steps of 0.2 M, 0.3 M, 0.4 M, and 0.6 M. Each fraction from the flow through to 0.6 M eluate were collected in 50 mL fractions. The purity of the protein was confirmed by electrophoresing each fraction on SDS-PAGE.

The fractions with Cry protein (flow through and wash fractions for 11Ba, 0.3 M NaCl eluate fractions for 19Aa) were loaded on a Sephacryl 300 gel filtration column. The protein was eluted with 50 mM sodium carbonate (pH 10.5), 0.5 M NaCl with 15 mL per fraction. The presence of Cry protein was checked using SDS-PAGE. The fractions which contained Cry protein were combined and concentrated with Amicon Ultra-15 centrifugal filter units (EMD Millipore, molecular weight cutoff 30 kDa). The purity of Cry proteins were checked on SDS-PAGE gel [Bio-Rad 12 % TGX (Tris-Glycine extended)-precast gel] (Figure 17 and Figure 18).
The Cry protein was buffer exchanged to PBS (4 mM KH$_2$PO$_4$, 16 mM Na$_2$HPO$_4$, 115 mM NaCl, pH7.4) (64) with a PD-10 desalting column (Sephadex G-25 gel filtration column, GE Healthcare). The protein (10 nmol) in 2.4 mL PBS was mixed with NHS-diazirine (1 μmol) (Thermo Fisher Scientific) in 0.1 mL DMSO and nutated at 37 ºC overnight. NHS-diazirine is soluble in DMSO. The unincorporated label was removed using a PD-10 desalting column. The NHS-diazirine labeled Cry protein in PBS was stored at 4 ºC until further use.

3.2.2 Brush Border Membrane Vesicles Preparation

Brush border membrane vesicles (BBMV) were prepared according to the method of English and Readdy ((58); Figure 21). *C. p. pipiens* 4th instar larvae were collected on a filter paper using a Büchner funnel briefly to remove excess water. The larvae were stored at -80 ºC until further use. The larvae (2.6 g; each larva weigh 2.8 mg) were suspended in a homogenizing tube (Glas-Col) in ice cold 50 mM sucrose, 0.1 mM PMSF, 2 mM Tris-HCl pH 7.4 supplemented with a protease inhibitor cocktail (cOmplete EDTA free, Roche), and homogenized 25 strokes on ice with rotor-stator homogenizer (Tri-R Stir R) with speed level 4. Solid CaCl$_2$ was added to a final concentration of 10 mM. Ca$^{2+}$ largely binds to the membranous portion containing lipoprotein (65).

The suspension was placed on a nutator at 4 ºC for 15 min, and centrifuged at 4,250 x g for 10 min at 4 ºC to separate the endoplasmic reticulum, mitochondria, and basolateral plasma membrane to pellets (60). The supernatant was collected and
centrifuged at 27,000 x g for 10 min at 4 ºC. The pellet, which contained BBMV proteins, was resuspended in 600 μL of PBS with protease inhibitor cocktail (EDTA free). The BBMV proteins (4 mg was obtained) were put on ice and used within one day.

3.2.3 Cross-linking and Co-immunoprecipitation

BBMV proteins (500 μg) and NHS-diazirine-labeled Cry11Ba or Cry19Aa (50 μg) in PBS containing protease inhibitor cocktail (EDTA free) (up to 500 μL) were placed on a nutator at 4 ºC for 30 min. BBMV proteins and the Cry protein suspension were kept cold so that Cry protein can bind to a receptor but not insert into the membrane. This was duplicated to facilitate a comparison of with or without UV illumination reactions. To aid sample recovery, the suspension was put on the cap of a microfuge tube and placed on ice for UV cross-linking. The suspension was illuminated with a 100 W Hg lamp UV for 10 min on ice at 4 ºC. The suspension was centrifuged at 27,000 x g for 10 min at 4 ºC. Both supernatant (Supe 1) and pellet were saved. The pellet was washed thrice with 500 μL PBS with protease inhibitor cocktail (EDTA free). The suspension was centrifuged at 27,000 x g for 10 min at 4 ºC between washes. The pellet was resuspended in 500 μL PBS with protease inhibitor cocktail (EDTA free). CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate) was added to final concentration of 0.1 % (w/v) to the suspension. The suspension was solublized on ice with hand-held homogenizer.
Approximately 150 μL of Dynabeads Protein A (Life Technologies) were placed in a microfuge tube. User manual for Dynabeads Protein A was followed for the preparation of Cry19Aa antibody-protein A Dynabeads complex. Sixty μg of Cry19Aa antibody was incubated with the beads in 200 μL of PBS-T (PBS with 0.02 % (v/v) Tween 20) for 30 min at 23 °C. The bead-antibody complex was separated from unbound antibody by using a magnet. The bead-antibody complex was then washed with 200 μL PBS-T once.

CHAPS-solubilized BBMV proteins were added to the bead-antibody complex. The suspension was incubated at 23 °C overnight. The bead-antibody-Cry19Aa-receptor complex was separated from unbound BBMV proteins by using a magnet. Both the bead-antibody-Cry19Aa-receptor complex and supernatant (Supe 2 for analysis) were saved. The bead-antibody-Cry19Aa-receptor complex was washed thrice with 500 μL PBS with protease inhibitor cocktail (EDTA free). The bead-antibody-Cry19Aa-receptor complex was moved to a clean microfuge tube and antibody, Cry19A, and a receptor were dissociated from the beads by incubating the complex in 40 μL of 100 mM glycine pH 2.8 for 5 min at 23 °C. The eluate was separated from beads by using a magnet. Four μL (1/10 v/v of 40 μL elutent) of 1 M Tris pH 8 was added to the eluate to neutralize the solution. Fifty μL of 2 X SDS-PAGE sample loading buffer was added to beads, and the sample was boiled for 5 min. The beads were separated by applying the magnet and the supernatant (Beads sample) was loaded on a TGX-precast gel (Bio-Rad) along with Supe1, Supe2, and Eluate sample.
After SDS-PAGE, the gel was immersed in fixing solution (50 % (v/v) ethanol, 10 % (v/v) acetic acid) for 18 h at 23 °C for analysis by mass spectrometry. The gel was washed thrice in distilled water for 10 min at 23 °C. The gel was stained in Bio-safe Coomassie stain (Bio-Rad) (Figure 25 and Figure 35).

### 3.2.4 Mass Spectrometry Analysis

The protein bands which contained cross-linked or co-immunoprecipitated proteins were submitted to Mass Spectrometry & Proteomics facility at the Ohio State University (Columbus, OH). The band was excised, trypsinized, and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The protein fragments were searched against full Swiss Plot Database with Mascot search engine. Search parameters were as follows: peptide mass tolerance (+/- 1.8 Da), fixed modification (carbamidomethyl), variable modification (deamidated and oxidation), maximum missed cleavages (two), and fragment mass tolerance (+/- 0.8 Da). The identified proteins which have a Mascot protein score higher than 100 are listed in (Table 6 and Table 7). A Mascot ions score is based on the probability that observed MS/MS ions matching to a certain protein on the database is a random event (66-69). A Mascot ions score is written as -10 x log₁₀(P), where P is the probability of the random match of MS/MS ions (68). Mascot ions score 100 means that the probability of MS/MS ions being matched randomly to a certain protein is 10⁻¹⁰. If the matching of MS/MS ions to a certain protein is specific, the Mascot ions score will be high. A Mascot protein score is the sum of the unduplicated
Mascot ions scores for a certain protein (70). A Mascot ions score comes with an expectation value which shows the number of random matches expected to have equal or better score (68). The Mascot ions score above 51 indicated identity or extensive homology (significance threshold p < 0.05).

3.2.5 2-D Gel Electrophoresis

2-D gel electrophoresis is a useful method to separate many proteins. We followed the procedure outlined in 2-D Electrophoresis Workflow How-To Guide from Bio-Rad and Popova-Butler & Dean (62). One hundred fifty μg BBMV proteins were cleaned up using the ReadyPrep 2-D Cleanup Kit (Bio-Rad). The BBMV proteins were re-suspended in 200 μL of 2-D rehydration/sample buffer (2 M thiourea, 5 M urea, 2 % (w/v) CHAPS, 2 % (w/v) SB3-10, 65 mM DTT, and 0.2 % (w/v) Bio-Lyte ampholytes (pH 3-10, Bio-Rad)). The sample was centrifuged at 15,140 x g for 5 min at 23 ºC, and the supernatant was applied to an 11 cm IPG (immobilized pH gradient) strip pH 3-10 (Bio-Rad). The strip was rehydrated overnight (more than 12 h). The IPG strip was placed on a focusing tray and isoelectric focusing was performed using a Protean IEF cell (Bio-Rad). The focusing conditions are as follows: start voltage 0 V, final voltage 8,000 V, up to 35,000 V-hrs, rapid ramp, and 20 ºC. The focused strip was stored at -80 ºC until use. The IPG strip was equilibrated for 10 min at 23 ºC in equilibration buffer I (6 M urea, 2 % (w/v) SDS, 0.375 M Tris-HCl (pH 8.8), 20 % (v/v) glycerol, and 2 % (w/v) DTT), and then equilibrated for 10 min at 23 ºC in equilibration buffer II (6 M urea, 2 %
(w/v) SDS, 0.375 M Tris-HCl (pH 8.8), 20 % (v/v) glycerol, and 2.5 % (w/v) iodoacetamide. The IPG strip was placed on a 8-16 % Criterion Tris-HCl precast gel (11 cm IPG + 1 well, Bio-Rad), and overlaid with agarose solution (0.5 % (w/v) agarose, 25 mM Tris, 192 mM glycine, 0.1 % (w/v) SDS, 0.001 % (w/v) bromophenol blue). The electrophoresis was run at 200 V. After SDS-PAGE, the gel was immersed in fixing solution (50 % (v/v) ethanol, 10 % (v/v) acetic acid) for 18 h at 23 ºC. The gel was washed 3 times in distilled water for 10 min at 23 ºC. The gel was then stained with Bio-safe Coomassie stain (Bio-Rad) (Figure 22).

### 3.2.6 Bioassay

Six 4th instar larvae of *C. p. pipiens* were placed individually in 6 well plate (BD, Falcon non-tissue culture treated plate). The amount of the distilled tap water or Bt inclusion suspension per well was 12 mL (71). Six different concentrations of Cry11Ba or Cry19Aa Bt inclusion bodies (mixture of crystals and spores) were tested (300 ng/mL, 150 ng/mL, 75 ng/mL, 37.5 ng/mL, 18.75 ng/mL, and water only). The concentration of protoxin was determined after solubilizing Bt inclusion bodies in 50 mM sodium carbonate, 10 mM DTT at 37 ºC for 2 h. After centrifugation at 16,917 x g for 10 min, the concentration of supernatant was measured by Coomassie plus (Bradford) protein assay (Pierce). BSA was used as the reference standard. The suspension was made by serial dilution of the highest concentration using distilled tap water as the diluent. The bioassay was duplicated, so 12 larvae were tested for each concentration. The dead larvae were
counted after 24 h. If a pupa emerged, the number of the pupae was omitted from the count. LC$_{50}$ was calculated by SoftTox (SoftLabWare and WindowChem, Fairfield, CA).
3.3 Results

The *Bacillus subtilis* (Bs) leucine\textsuperscript{−} and methionine\textsuperscript{−} auxotrophic strains were obtained from the *Bacillus* Genetic Stock Center (Columbus, OH). This Bs strain grew on minimal media agar containing photo-leucine and methionine, but it mostly failed to sporulate and make Cry protein even with a mixture of 50 μg/mL photo-leucine with 10 μg/mL leucine in a chemically-defined sporulation media (72). Therefore, we chose to attach a diazirine cross-linking group to lysine residues of Cry proteins. NHS-diazirine was synthesized at first, but later purchased from Thermo Scientific. The structure of NHS-diazirine is shown in Figure 20.

Cry11Ba and Cry19Aa in Bt4O7 were obtained from the *Bacillus* Genetic Stock Center (OSU, Columbus, OH). These strains were grown in SSM until autolysis. Cry11Ba and Cry19Aa were solubilized, and activated with either trypsin (Cry11Ba) or chymotrypsin (Cry19Aa). Trypsin cleaves Cry11Ba into two protein fragments of 36 kDa and 33 kDa (Figure 16 (73)). These fragments of 11Ba remain bound together via a salt bridge (73). The activated Cry proteins were purified through anion-exchange and gel filtration chromatography. The purity of Cry proteins were checked on SDS-PAGE (Figure 17, Figure 18). Both Cry11Ba and Cry19Aa inclusion bodies which contain spores and crystal toxins were toxic to *C. p. pipiens* larvae (Table 5). When the larvae were treated with Cry11Ba or Cry19Aa, the larvae stopped moving, appearing L-shape or floating straight, or sometimes changed their colors to black, and died in 24 h (Figure 15 and Figure 41).
The purified Cry proteins were labeled with NHS-diazirine. Thiele (74) used a 200 W Hg lamp to activate half the amount of the diazirine ring (half-life) in 20 seconds (74). As we did not have access to a 200 W Hg lamp, we attempted placing the sample in a UV cuvette and placed it on a UV gel imager (Fotodyne) (Figure 19A). Half-life of diazirine ring in this condition was 20 min, as the lamp’s maximum is 300 nm and the light intensity is reduced through the filter and the cuvette. Diazirine ring has an absorption peak at 350 nm. Half-life of the diazirine ring was determined by the disappearance of the peak at 350 nm after irradiation of UV light on the photo-leucine solution in water (Figure 19C). Because of the weak activation or long time of activation, it was hard to pull down a cross-linked receptor. Finally a 100 W, 365 nm UV lamp (UVP) was obtained. This lamp can illuminate the sample directly and activate diazirine ring with a half-life of 2 min (Figure 19B, C).

To focus on the cross-linked receptor with Cry proteins, a co-immunoprecipitation was performed. Cry19Aa polyclonal antibody was custom-made through Open Biosystems from the purified Cry19Aa. This antibody also cross-reacted with Cry11Ba. The ability of antibody to pull-down Cry11Ba and 19Aa was weak, but the cross-linked proteins were pulled down (Figure 25, lane 2, band 1 and band 2). The binding between Cry proteins and C. p. picipiens BBMV proteins were performed at 4 ºC so that Cry proteins remained bound to the receptor without inserting into the membrane.

Cross-linked proteins (band 1 above 250 kDa, and band 2 a little above 100 kDa) were analyzed at the Mass Spectrometry & Proteomics facility (Columbus, OH). The results of the analysis are shown in Table 6 and Table 7. Both bands contained calcium
ATPase (Figure 26, Table 8, and Table 9), Cry11Ba (Figure 28 and Table 11), trypsin, sodium/potassium ATPase (Figure 30 and Table 12), actin (Figure 32 and Table 14), immunoglobulin γ chain. Only band 1 contained myosin (Figure 27 and Table 10), tubulin (Figure 31 and Table 13), and glyceraldehyde-3-phosphate dehydrogenase (Figure 33 and Table 15). Only Band 2 contained V-type proton ATPase (Figure 34 and Table 16). The cross-linking experiment between diazirine-modified Cry11Ba and components within C. p. pipiens BBMV showed bands in similar positions in the eluate as previously analyzed band 1 and 2 from the diazirine-modified Cry19Aa and BBMV cross-linking experiment (Figure 25 and Figure 35).
3.4 Discussion

Photo-leucine was not incorporated well or not at all into the Cry protein in *Bacillus subtilis*. Photo-leucine was developed for use in mammalian cells (74, 75). If a Cry protein can be produced in mammalian cells, Cry protein can be labeled with photo-leucine and then can be used for a cross-linking experiment.

The alternative method to label Cry protein with NHS-diazirine was successful. As Cry19Aa has 35 lysine residues, it can have more than one diazirine modification. The degree of the labeling was not determined, but we might be able to determine it by submitting the diazirine-modified Cry19Aa to electrospray ionization mass spectrometry (76).

The cross-linked proteins, band 1 and band 2 on SDS-PAGE gel, were obtained through the binding between diazirine-modified Cry19Aa and *C. p. pipiens* BBMV proteins (Figure 25). MS/MS-based proteomic analysis showed that both band 1 and band 2 contained trypsin and immunoglobulin γ chain. Trypsin was used to cleave the proteins for the analysis using mass spectrometry. Cry19Aa antibody which was used in the co-immunoprecipitation was made in the rabbit. Immunoglobulin γ chain is a part of antibody (immunoglobulin G) and it is from the organism, *Oryctolagus cuniculus* (European rabbit). These data suggest that the results of mass spectrometry are valid. The peptides were searched against full Swiss-Prot database and *Culex* did not appear in the mass spectrometry results. UniProt Knowledgebase/Swiss-Prot (reviewed) database has 539,829 entries, but *C. p. quinquefasciatus* has only 104 entries which do not include
calcium transporting ATPase (77). The genome sequence of *C. p. quinquefasciatus* was reported in 2010 (78). *An. gambiae* is a mosquito, and *Drosophila melanogaster* is a fruit fly. Their protein sequences are expected to be similar. Furthermore, the sequences of actin, V-type ATPase, glyceraldehyde-3-phosphate dehydrogenase, and tubulin are expected to be conserved among the wide range of species.

When the molecular weights of band 1 and band 2 were estimated using their migration positions in SDS-PAGE (Figure 25), the molecular weight of band 1 was between 242 kDa and 332 kDa, and the molecular weight of band 2 was between 110 kDa and 120 kDa (79). As Cry19Aa can have more than one diazirine modification, it can potentially cross-link with more than one protein. The molecular weights of identified proteins are listed on Table 6 and Table 7. There are many possible combinations of proteins in band 1. For example, the combination of calcium ATPase (110 kDa), Cry19Aa (65 kDa), sodium/potassium ATPase (80 kDa), and actin (42 kDa) can be in the range of the molecular weight of band 1 (297 kDa). Given the possibility for multiple cross-links, it is difficult to draw firm conclusions about the number of proteins in these cross-linked bands.

Cross-linking and co-immunoprecipitation experiments between Cry19Aa and *C. p. pipiens* BBMV proteins determined that calcium ATPase could be a putative receptor. According to mass spectrometry data, this calcium ATPase can be sarcoplasmic/endoplasmic reticulum type (SERCA). Although English and Readdy did not report the purity of BBMV preparation, calcium precipitation of BBMV proteins reported to include some endoplasmic reticulum (ER) proteins when compared to the
original homogenate. The percentage of ER proteins was determined by comparing specific activity of enzymes in ER. Comparison of glucose-6-phosphatase activity in the preparation of rabbit renal BBMV proteins showed 62.5% of ER contamination (80). Comparison of NADPH-cytochrome c reductase activity in the preparation of rabbit renal BBMV proteins showed 32% of ER contamination (59). It is possible then that the calcium ATPase, which we identified is from the endoplasmic reticulum origin, and not from BBMV proteins. This significance, therefore, is questionable.

Sodium/potassium ATPase was also identified in the mass spectrometry results. English of English-Readdy BBMV preparation method showed that Cry protein from Bt subspecies kurstaki was an inhibitor of sodium/potassium ATPase which was purified from a dog kidney (81). English decided to investigate sodium/potassium ATPase, because Cry protein was an inhibitor of proton/potassium ATPase, which is in the same family of cation transporting ATPase (P-type ATPase) as sodium/potassium ATPase and calcium transporting ATPase (81). Sodium/potassium ATPase, which is in the basolateral membrane, is not present in the BBMV proteins.

Actin was also identified in our cross-linking experiments. Actin is not present in BBMV proteins, but actin from Manduca sexta was shown to bind to Cry1Ac in a ligand blot of M. sexta BBMV proteins (82). This M. sexta BBMV was prepared by English-Readdy method, which was also used in our study. It was suggested that actin was a contaminant within the BBMV preparation, but actin might interact with Cry1Ac after insertion of toxin or after cell’s integrity was compromised (82, 83).
The proteins in mass spectrometry results, such as SERCA, sodium/potassium ATPase, actin, are not BBMV proteins. Ca\textsuperscript{2+} precipitation method of BBMV preparation was used in this study. Mg\textsuperscript{2+} precipitation method by Wolfersberger has been frequently used in Bt research field (84). Comparison of NADPH-cytochrome c reductase activity in the preparation of cabbage butterfly larvae BBMV proteins showed 4 % of ER contamination by Wolfersberger method (84). Ca\textsuperscript{2+} precipitation in BBMV preparation is better in removing basolateral membrane contamination than Mg\textsuperscript{2+} precipitation (85). We used the English-Readdy method, because the previous work in our lab used this method for the proteomic analysis of BBMV proteins from the midguts of the A. aegypti mosquito larvae (62). We used whole larvae instead of the midguts to prepare BBMV for this study, because of the small size of mosquito larvae and the technical difficulty of collecting the large amount of midguts. We used around 930 whole mosquito larvae for a BBMV preparation (Figure 21). We might need an even larger number of the midguts to prepare BBMV. As it took one hour for me to dissect and collect 10 midguts, it might take several weeks to prepare sufficient midguts for a BBMV preparation. If we prepare BBMV from Lepidoptera larvae such as from tobacco hornworm, we only need two to three midguts because of their large size. Using whole larvae instead of midguts might increase impurities in BBMV. The BBMV preparation from whole mosquito larvae was reported, which included a filtration through cheesecloth to remove intact remnants of the mosquito body (86). It was shown that the BBMV preparation from whole larvae enriched BBMV proteins and did not alter the binding property of Cry protein, although the degree of impurities was not reported (86). As photo-cross-linking method can
capture a binding protein that interacts only weakly, we might need more purity in BBMV preparation.

The pull-down of diazirine-modified Cry19Aa by Cry19Aa antibody was weak (Figure 24). Changing the order of the incubation did not change the result (incubation of diazirine-modified Cry19Aa and antibody first, and then with protein A magnetic beads, or incubation of the antibody and protein A magnetic beads first, then with diazirine-modified Cry19Aa). Thiele (74) suggested that photo-leucine would not interfere with antibody recognition whereas a chemical modification of lysines would. The attachment of NHS-diazirine to lysine residues of Cry19Aa might interfere with the recognition by the Cry19Aa antibody.

Mass spectrometry data showed that Cry11Ba, not Cry19Aa, was contained in the sample. The peptides matched to Cry11Ba were unique to Cry11Ba (Figure 29). As Cry11Ba and Cry19Aa were used at the same time during the experiments prior to the first cross-linking experiments, there was possibility of mislabeling of tubes between Cry11Ba and Cry19Aa. New glycerol stocks of Bt cultures producing 11Ba or 19Aa were made after each experiment. An additional experiment was started from the new glycerol stocks rather than going back to old glycerol stocks, therefore, one mislabeling might lead to continuous use of mislabeled toxin. Therefore I might have used Cry11Ba instead of Cry19Aa in the cross-linking experiment. After the mass spectrometry result, original Bt cells of Cry11Ba and Cry19Aa were obtained from Bacillus Genetic Stock Center (Columbus, OH) and used thereafter. Cry19Aa antibody recognized newly obtained Cry11Ba much better than Cry19Aa, although ability for Cry19Aa antibody to pull down
Cry11Ba was still poor. The cross-linking experiment between Cry11Ba, or Cry19Aa and *C. p. pipiens* BBMV proteins showed similar bands as in the initial experiment with Cry19Aa. Whether Cry11Ba and Cry19Aa use the same receptor was not known.

In conclusion, we identified a SERCA as well as the other proteins such as sodium/potassium ATPase and actin as binding proteins to Cry19Aa. We also highlight a shortcoming of using the photo-cross-linking method with BBMV, which most likely is contaminated with ER and basolateral membrane.
<table>
<thead>
<tr>
<th>Cry protein</th>
<th>Receptors or binding proteins</th>
<th>Methods used to identify receptors</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cry11Ba</td>
<td>Aminopeptidase-N (Binding protein)</td>
<td>Competition on BBMV binding</td>
<td>(73)</td>
</tr>
<tr>
<td>Cry11Ba</td>
<td>Alkaline phosphatase-1 (Binding protein)</td>
<td>Competition on BBMV binding</td>
<td>(73)</td>
</tr>
<tr>
<td>Cry11Ba</td>
<td>Cadherin repeat-11 (Binding protein)</td>
<td>Competition on BBMV binding</td>
<td>(73)</td>
</tr>
</tbody>
</table>

Table 3. Receptors or binding proteins for *Aedes aegypti* larvae.

<table>
<thead>
<tr>
<th>Cry protein</th>
<th>Receptors or binding proteins</th>
<th>Methods used to identify receptors</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cry11Ba</td>
<td>Alkaline phosphatase-1 (Receptor)</td>
<td>Binding on membrane bioassay with the presence of Alkaline phosphatase-1</td>
<td>(54)</td>
</tr>
<tr>
<td>Cry11Ba</td>
<td>Aminopeptidase-N-2 (Receptor)</td>
<td>Bioassay with the presence of Aminopeptidase-N-2</td>
<td>(55)</td>
</tr>
</tbody>
</table>

Table 4. Receptors or binding proteins for *Anopheles gambiae* larvae.
<table>
<thead>
<tr>
<th>Cry proteins</th>
<th>Property of toxin</th>
<th>Mosquitoes</th>
<th>Toxicity LC₅₀, ng/mL (95 % confidence limit)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>11Ba</td>
<td>Inclusion body Bt</td>
<td><em>A. aegypti</em> (4th instar)</td>
<td>15.81 (10.2–29.37)</td>
<td>(73)</td>
</tr>
<tr>
<td>11Ba</td>
<td>Soluble protoxin</td>
<td><em>A. aegypti</em> (4th instar)</td>
<td>8,053 (5,260–10,546)</td>
<td>(73)</td>
</tr>
<tr>
<td>11Ba</td>
<td>Soluble toxin</td>
<td><em>A. aegypti</em> (4th instar)</td>
<td>39,770 (31,193–50,637)</td>
<td>(73)</td>
</tr>
<tr>
<td>11Ba</td>
<td>Inclusion body Bt 4O7</td>
<td><em>C. p. pipiens</em> (4th instar)</td>
<td>61.63 (33.57-102.41)</td>
<td>This study</td>
</tr>
<tr>
<td>19Aa</td>
<td>Inclusion body Bt 4Q7</td>
<td><em>C. p. pipiens</em> (4th instar)</td>
<td>149 (103–205)</td>
<td>(87)</td>
</tr>
<tr>
<td>19Aa</td>
<td>Inclusion body Bt 4O7</td>
<td><em>C. p. pipiens</em> (4th instar)</td>
<td>74.33 (52.57-105.06)</td>
<td>This study</td>
</tr>
</tbody>
</table>

Table 5. Bioassay.

Figure 15. Bioassay of *C. p. pipiens* larvae (after 24 h). Fourth instar larvae of *C. p. pipiens* were treated with either distilled tap water or Cry11Ba inclusion bodies (300 ng/mL). The picture was taken after 24 h of the treatment. Larvae became L-shape or lied vertically, and died with Cry11Ba.
Figure 16. Trypsin digestion of Cry11Ba. Figure is reproduced from (73). Trypsin digests Cry11Ba into 2 fragments: 36 kDa (N-terminal) and 33 kDa (C-terminal).
Figure 17. Purification of Cry11Ba by anion exchange and gel filtration chromatography. Lane 1. Molecular weight marker (Bio-Rad dual color). Lane 2. Cry11Ba protoxin. Lane 3. Cry11Ba toxin. Trypsin cuts 11Ba into 2 fragments (36 kDa and 33 kDa)(Figure 16). Flow-through fraction (lane 4), and wash fraction (lane 5) from the Q-sepharose column. Lane 6. Concentrated Cry11Ba after gel filtration column.
Figure 18. Purification of Cry19Aa by anion exchange and gel filtration chromatography. Lane 1. Molecular weight marker (Bio-Rad dual color). Lane 2. Cry19Aa protoxin. Lane 3. Cry19Aa toxin. 0.3 M NaCl eluate fraction 1 (lane 4), and fraction 2 (lane 5) from the Q-sepharose column. Lane 6. Concentrated Cry19Aa after gel filtration column.
Figure 19. UV activation of the diazirine ring in photo-leucine. The time needed to activate diazirine ring was measured. The diazirine ring has a characteristic absorption peak around 350 nm. (A) Photo-leucine solution was activated in a UV cuvette which was placed on a 15 W 300 nm UV lamp (Fotodyne). It took about 20 min to activate half of diazirine ring. (B) Photo-leucine solution was placed on a petri dish on ice, which was illuminated with 100 W 365 nm UV lamp (UVP). (C) Decomposition of diazirine ring under 100 W UV lamp (UVP, condition (B)) was monitored by UV spectrum. It took about 2 min to activate half of diazirine.
Figure 20. The structure of NHS-diazirine (A), the synthesis of NHS-diazirine (B), and the structure of NHS-LC (long chain)-diazirine (C). NHS-diazirine and NHS-LC-diazirine were used to label Cry proteins. NHS-diazirine was synthesized from the first product of photo-leucine synthesis (appendix A).
Whole larvae homogenized (2.6 g ~930 larvae)

10 mM CaCl$_2$, 4 °C, 15 min

4,250 g, 10 min, 4 °C

Supernatant

Pellet (Endoplasmic reticulum, mitochondria, basolateral plasma membrane)

27,000 g, 10 min, 4 °C

Supernatant

Pellet BBMV (4 mg)

Figure 21. Diagram of English-Readdy BBMV preparation method.
Figure 22. Separation of *C. p. piriens* BBMV proteins by 2-D gel electrophoresis. MW: molecular weight marker.
Figure 23. UV cross-linking of NHS-diazirine labeled Cry11Ba and Cry19Aa (toxin only control).

To test the NHS diazirine label of Cry11Ba or Cry19Aa, the diazirine labeled Cry11Ba and Cry19Aa were illuminated with UV light. Only diazirine-labeled toxins formed oligomers upon exposure of UV light (lanes 4 and 9). MW: molecular weight marker.
Figure 24. Immunoprecipitation of Cry19Aa.
Diazirine-modified Cry19Aa was immunoprecipitated with increasing amount of Cry19Aa antibody (2 μg, 10 μg, and 20 μg). MW: molecular weight marker. Supe: supernatant.
Figure 25. Cross-linking and Co-immunoprecipitation between diazirine-modified Cry19Aa and C. p. pipiens BBMV proteins. Cross-linked receptors are expected to be in the eluate fraction with UV illumination (lane 2). When the eluate fractions (lanes 2 and 6) were compared, two additional bands (bands 1 and 2) appeared. These bands were submitted to mass spectrometry for analysis.

Table 6. Mass spectrometry analysis of band 1 to identify Cry19Aa binding proteins in *C. p. pipiens*.
Band 1 from Figure 25 was excised out of the gel and analyzed by mass spectrometry at the OSU CCIC Proteomics Facility.
Top matches above score 100 are shown. Number of distinct sequences: number of peptides which have different sequences. Sequence coverage: percentage of amino acid sequence of a protein which is matched by peptides. Molecular weight of *C. p. pipiens or C. p. quinquefasciatus* proteins are shown except for Cry11Ba, trypsin, and Ig. For Cry11Ba, trypsin, and Ig, molecular weight of the proteins are from the organisms on the table. Molecular weight was calculated from amino acid sequence by using ProtParam tool (Swiss Institute of Bioinformatics, (88)).

<table>
<thead>
<tr>
<th>Proteins identified</th>
<th>Organism</th>
<th>Mascot Score</th>
<th>Number of matched peptides</th>
<th>Number of distinct sequences</th>
<th>Sequence coverage (%)</th>
<th>Molecular weight (kDa)</th>
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<tbody>
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<td>Calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type</td>
<td><em>Anopheles gambiae</em></td>
<td>962</td>
<td>36</td>
<td>16</td>
<td>17</td>
<td>110</td>
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<td>Myosin heavy chain, muscle</td>
<td><em>Drosophila melanogaster</em></td>
<td>644</td>
<td>22</td>
<td>12</td>
<td>10</td>
<td>219</td>
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<td>Cry11Ba</td>
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<td>612</td>
<td>30</td>
<td>13</td>
<td>28</td>
<td>69</td>
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<td>Trypsin</td>
<td><em>Sus Scrofa</em></td>
<td>474</td>
<td>20</td>
<td>5</td>
<td>25</td>
<td>25</td>
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<td>Sodium/potassium-transporting ATPase subunit alpha</td>
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<td>Tubulin alpha chain testis specific</td>
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<td>Actin, acrosomal process isoform</td>
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<tr>
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<td>5</td>
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<td>Ig gamma chain C region</td>
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<td>Glyceraldehyde-3-phosphate dehydrogenase testis specific</td>
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<td>2</td>
<td>4</td>
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Table 7. Mass spectrometry analysis of band 2 to identify Cry19Aa binding proteins in *C. p. piperiens*. Band 2 from Figure 25 was excised out of the gel and analyzed by mass spectrometry at the OSU CCIC Proteomics Facility. Top matches above score 100 are shown. Number of distinct sequences: number of peptides which have different sequences. Sequence coverage: percentage of amino acid sequence of a protein which is matched by peptides. Molecular weight of *C. p. piperiens* or *C. p. quinquefasciatus* proteins are shown except for Cry11Ba, trypsin, and Ig. For Cry11Ba, trypsin, and Ig, molecular weight of the proteins are from the organisms on the table. Molecular weight was calculated from amino acid sequence by using ProtParam tool (Swiss Institute of Bioinformatics, (88)).

<table>
<thead>
<tr>
<th>Proteins identified</th>
<th>Organism</th>
<th>Mascot Score</th>
<th>Number of matched peptides</th>
<th>Number of distinct sequences</th>
<th>Sequence coverage (%)</th>
<th>Molecular weight (kDa)</th>
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<tr>
<td>Trypsin</td>
<td><em>Sus Scrofa</em></td>
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<td>42</td>
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<td>Cry11Ba</td>
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<td>8</td>
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<td>5</td>
<td>30</td>
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<td>Actin, muscle (fragments)</td>
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<td>17</td>
<td>42</td>
</tr>
<tr>
<td>Sodium/potassium-transporting ATPase subunit alpha</td>
<td><em>Drosophila melanogaster</em></td>
<td>159</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td>80</td>
</tr>
<tr>
<td>Probable V-type proton ATPase subunit B</td>
<td><em>Caenorhabditis elegans</em></td>
<td>152</td>
<td>3</td>
<td>2</td>
<td>6</td>
<td>55</td>
</tr>
</tbody>
</table>
Figure 26. Peptide coverage of calcium-transporting ATPase from mass spectrometry. (A) from band 1. Sequence coverage is 17 %. (B) from band 2. Sequence coverage is 15 %. Matched peptides are shown in red.
<table>
<thead>
<tr>
<th>Residues</th>
<th>Mascot ions score</th>
<th>Expectation value</th>
<th>Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>36 – 47</td>
<td>75</td>
<td>0.00017</td>
<td>K.YGPNELPAEEGK.T</td>
</tr>
<tr>
<td>110–127</td>
<td>78</td>
<td>3.5e-6</td>
<td>R.NAESAIEALKEYEPEMGK.V + Oxidation (M)</td>
</tr>
<tr>
<td>174–188</td>
<td>106</td>
<td>1.8e-7</td>
<td>R.IDQSLTGESVSVIK.H</td>
</tr>
<tr>
<td>189–197</td>
<td>56</td>
<td>0.0014</td>
<td>K.HTDAVPDPR.A</td>
</tr>
<tr>
<td>205–217</td>
<td>40</td>
<td>0.025</td>
<td>K.NILFSGTNVAAGK.A</td>
</tr>
<tr>
<td>236–245</td>
<td>53</td>
<td>0.003</td>
<td>R.TEMSETEEIK.T + Oxidation (M)</td>
</tr>
<tr>
<td>236–251</td>
<td>70</td>
<td>0.00074</td>
<td>R.TEMSETEEIKTPLQQK.L + Deamidated (NQ); Oxidation (M)</td>
</tr>
<tr>
<td>252–261</td>
<td>57</td>
<td>0.012</td>
<td>K.LDEFGEQLSK.V</td>
</tr>
<tr>
<td>352–364</td>
<td>89</td>
<td>2e-6</td>
<td>K.TGTLTTNQMSVSR.M + Oxidation (M)</td>
</tr>
<tr>
<td>436–450</td>
<td>68</td>
<td>0.0001</td>
<td>K.VGEATETALIVLAEK.L</td>
</tr>
<tr>
<td>481–488</td>
<td>50</td>
<td>0.035</td>
<td>K.EFTLEFSR.D</td>
</tr>
<tr>
<td>620–628</td>
<td>51</td>
<td>0.034</td>
<td>R.VIVITGDNK.A</td>
</tr>
<tr>
<td>712–727</td>
<td>100</td>
<td>6.5e-8</td>
<td>K.KAEIGIAMGSGTAVAK.S + Oxidation (M)</td>
</tr>
<tr>
<td>713–727</td>
<td>91</td>
<td>1.2e-6</td>
<td>K.AEIGIAMGSGTAVAK.S + Oxidation (M)</td>
</tr>
<tr>
<td>823–835</td>
<td>60</td>
<td>0.0011</td>
<td>K.ADEGLISGWLFFR.Y</td>
</tr>
<tr>
<td>972–984</td>
<td>79</td>
<td>6.3e-5</td>
<td>K.FSLPVVLLDEILK.F</td>
</tr>
</tbody>
</table>

Table 8. The Mascot ions scores and the expectation values of the matched peptides with distinct sequences to calcium-transporting ATPase from band 1. When there were duplicated sequences of peptides, the one with the higher Mascot ions score is shown in the table.
<table>
<thead>
<tr>
<th>Residues</th>
<th>Mascot ions score</th>
<th>Expectation value</th>
<th>Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>36 – 47</td>
<td>70 0.0002</td>
<td></td>
<td>K.YGPNELPAEEGK.T</td>
</tr>
<tr>
<td>110 – 119</td>
<td>59 0.0091</td>
<td></td>
<td>R.NAESAIIEALK.E</td>
</tr>
<tr>
<td>110 – 127</td>
<td>83 6e-7</td>
<td></td>
<td>R.NAESAIIEALKKEYEPMGK.V + Oxidation (M)</td>
</tr>
<tr>
<td>174 – 188</td>
<td>112 4.3e-8</td>
<td></td>
<td>R.IDQSIILTGESVSVIK.H</td>
</tr>
<tr>
<td>205 – 217</td>
<td>94 2.5e-6</td>
<td></td>
<td>K.NILFSGTNVAAGK.A</td>
</tr>
<tr>
<td>234 – 251</td>
<td>41 0.017</td>
<td></td>
<td>K.IRTEMSETEIEIKTPLQQK.L + Oxidation (M)</td>
</tr>
<tr>
<td>236 – 245</td>
<td>57 0.0046</td>
<td></td>
<td>R.TEMSETEIEK.T + Oxidation (M)</td>
</tr>
<tr>
<td>236 – 251</td>
<td>53 0.035</td>
<td></td>
<td>R.TEMSETEIEIKTPLQQK.L + Deamidated (NQ); Oxidation (M)</td>
</tr>
<tr>
<td>252 – 261</td>
<td>60 0.0073</td>
<td></td>
<td>K.LDEFGEQLSK.V</td>
</tr>
<tr>
<td>334 – 351</td>
<td>88 4e-7</td>
<td></td>
<td>R.SLPSVETLGCTSVICSDK.T</td>
</tr>
<tr>
<td>352 – 364</td>
<td>82 8e-6</td>
<td></td>
<td>K.TGTLTTNQMSVSR.M + Oxidation (M)</td>
</tr>
<tr>
<td>465 – 471</td>
<td>52 0.044</td>
<td></td>
<td>R.SSAICVR.Q</td>
</tr>
<tr>
<td>629 – 636</td>
<td>48 0.011</td>
<td></td>
<td>K.ATAEAICR.R</td>
</tr>
<tr>
<td>712 – 727</td>
<td>56 0.0003</td>
<td></td>
<td>K.KAEIGIAMGSGTAVAK.S + Oxidation (M)</td>
</tr>
<tr>
<td>713 – 727</td>
<td>93 2.8e-6</td>
<td></td>
<td>K.KAEIGIAMGSGTAVAK.S + Oxidation (M)</td>
</tr>
<tr>
<td>823 – 835</td>
<td>60 0.0015</td>
<td></td>
<td>K.ADEGLISGWLFRR.Y</td>
</tr>
</tbody>
</table>

Table 9. The Mascot ions scores and the expectation values of the matched peptides with distinct sequences to calcium-transporting ATPase from band 2. When there were duplicated sequences of peptides, the one with the higher Mascot ions score is shown in the table.
Figure 27. Peptide coverage of muscle myosin heavy chain from mass spectrometry. From band 1. Sequence coverage is 10%. Matched peptides are shown in red.
Table 10. The Mascot ions scores and the expectation values of the matched peptides with distinct sequences to muscle myosin heavy chain from band 1. When there were duplicated sequences of peptides, the one with the higher Mascot ions score is shown in the table.

<table>
<thead>
<tr>
<th>Residues</th>
<th>Mascot ions score</th>
<th>Expectation value</th>
<th>Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>976 – 990</td>
<td>59</td>
<td>0.012</td>
<td>R.NLNEIAHQDELINK.L + Deamidated (NQ)</td>
</tr>
<tr>
<td>1024–1039</td>
<td>78</td>
<td>5.6e-6</td>
<td>K.AKLEQTLDELEDLSER.E</td>
</tr>
<tr>
<td>1060–1070</td>
<td>79</td>
<td>9.5e-5</td>
<td>K.LTQEAVALDLR.N</td>
</tr>
<tr>
<td>1141–1164</td>
<td>56</td>
<td>0.00087</td>
<td>R.ELEELGERLEEAGGATSAQIELNK.K + Deamidated (NQ)</td>
</tr>
<tr>
<td>1396–1409</td>
<td>88</td>
<td>1.6e-5</td>
<td>R.LAEAEETIESLNYQ.K</td>
</tr>
<tr>
<td>1420–1433</td>
<td>97</td>
<td>1.5e-6</td>
<td>R.LSTEVEDLQLEGDR.A</td>
</tr>
<tr>
<td>1459–1471</td>
<td>53</td>
<td>0.011</td>
<td>K.VDDLAAELDSQK.E + Deamidated (NQ)</td>
</tr>
<tr>
<td>1503–1520</td>
<td>40</td>
<td>0.016</td>
<td>K.NLADVEKDLDQIEGGR.N</td>
</tr>
<tr>
<td>1532–1556</td>
<td>83</td>
<td>9.2e-6</td>
<td>R.LEAEDELQAALEEAEEALEEQENK.V</td>
</tr>
<tr>
<td>1616–1633</td>
<td>100</td>
<td>7.8e-7</td>
<td>K.KLEADINEEIEALDANK.A</td>
</tr>
<tr>
<td>1728–1748</td>
<td>76</td>
<td>0.00016</td>
<td>R.KLESELQTLHSDLIDELLNEAK.N + Deamidated (NQ)</td>
</tr>
<tr>
<td>1880–1894</td>
<td>96</td>
<td>5.6e-7</td>
<td>R.QIEEAEEIAALNLAK.F</td>
</tr>
</tbody>
</table>
Figure 28. Peptide coverage of Cry11Ba from mass spectrometry.
(A) from band 1. Sequence coverage is 28 %. (B) from band 2. Sequence coverage is 14 %.
Matched peptides are shown in red.
Table 11. The Mascot ions scores and the expectation values of matched peptides with distinct sequences to Cry11Ba from band 1 (A) and from band 2 (B). When there were duplicated sequences of peptides, the one with the higher Mascot ions score is shown in the table.
Figure 29. CLUSTAL O (1.1.0) multiple sequence alignment of Cry11Ba and Cry19Aa. Matched peptides from band 1 are shown in red.
Figure 30. Peptide coverage of sodium/potassium-transporting ATPase from mass spectrometry. (A) from band 1. Sequence coverage is 8 %. (B) from band 2. Sequence coverage is 3 %. Matched peptides are shown in red.
Table 12. The Mascot ions scores and the expectation values of the matched peptides with distinct sequences to sodium/potassium-transporting ATPase from band 1 (A) and from band 2 (B). When there were duplicated sequences of peptides, the one with the higher Mascot ions score is shown in the table.
Figure 31. Peptide coverage of tubulin alpha chain (A) and beta chain (B) from mass spectrometry. (A) and (B) both from band 1. Sequence coverage for (A) and (B) is both 15%. Matched peptides are shown in red.
<table>
<thead>
<tr>
<th>Residues</th>
<th>Mascot ions score</th>
<th>Expectation value</th>
<th>Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>65 – 79</td>
<td>75</td>
<td>0.00032</td>
<td>R.AVFVDLEPTVVDEVR.T</td>
</tr>
<tr>
<td>230 – 243</td>
<td>96</td>
<td>2.6e-6</td>
<td>R.LIGQIVSSITASLR.F</td>
</tr>
<tr>
<td>244 – 264</td>
<td>83</td>
<td>5e-5</td>
<td>R.FDGALNVDLTEFQTNLVPYPR.I + Deamidated (NQ)</td>
</tr>
<tr>
<td>353 – 370</td>
<td>38</td>
<td>0.047</td>
<td>K.VGINYQPPTVVPGGLAK.V + Deamidated (NQ)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Residues</th>
<th>Mascot ions score</th>
<th>Expectation value</th>
<th>Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>63 – 77</td>
<td>65</td>
<td>0.0028</td>
<td>R.AVLVDLEPGTMSVR.S + Oxidation (M)</td>
</tr>
<tr>
<td>242 – 251</td>
<td>56</td>
<td>0.018</td>
<td>R.FPGQLNADLR.K</td>
</tr>
<tr>
<td>283 – 297</td>
<td>73</td>
<td>0.00041</td>
<td>R.ALTVPETLQMFDAK.N + Oxidation (M)</td>
</tr>
<tr>
<td>337 – 350</td>
<td>50</td>
<td>0.0016</td>
<td>K.NSSYFVEWIPNVK.T</td>
</tr>
<tr>
<td>363 – 379</td>
<td>89</td>
<td>1.1e-5</td>
<td>K.MSATFIGNSTAIQELFK.R + Deamidated (NQ); Oxidation (M)</td>
</tr>
</tbody>
</table>

Table 13. The Mascot ions scores and the expectation values of the matched peptides with distinct sequences to tubulin alpha chain (A) and beta chain (B). Both are from band 1. When there were duplicated sequences of peptides, the one with the higher Mascot ions score is shown in the table.
Figure 32. Peptide coverage of acrosomal isoform actin (A) and muscle actin (B) from mass spectrometry. (A) is from band 1. (B) is from band 2. Sequence coverage for (A) and (B) is both 17%. Matched peptides are shown in red.

(A) 1 MCDEDVAALV VDNAGGMCKA GFAGDDAPRA VFPSIVGRPR HQGVMOVGMQ
51 KDSYVGDEAQ SKRGILTLKY PIEHGVNTNW DDEMEKIHHT FYNELRVAPE
101 EHPVLLTEAP LNPKANREKM TQIMFETFNT PAMYVAIQAV LSYASGR
151 GIVLDSGDGV SHTVPIYEGY ALPHAILRLD LAGRDLTDYL MKILTERGYS
201 FTTAAREIV RDIKEKLCVV ALDFEHMITT AASSSLEKS YELPDQVIT
251 IGNERFRCPFE AMFQPSFLGM EACGHETTF NSIMKCDVPC RDKLYANTVL
301 SGGSTMPFGI ADRMQKEIGA LAPSMTKI IAPPERKYSV WIGGSIASL
351 STFQWMWISK QEFDESQPSI VHACKF

(B) 1 AGFAGDDAPR AVFPSIVGRP RDAYVGDEAQ SKRGILTLKI APEESPVLLT
51 EAPLNPKTGI VLDTGDYVT THPIYEGYCL PHAILRLDLA GDLTAYLTK
101 GYSFVVTAER EIVRSYELPD GQVITIGNER CDIDIRK DLF ANNVLSGGT
151 MYPGIDREI TALAPPTIKI KIIAPPERKE EYDESGPGIV HR
<table>
<thead>
<tr>
<th>Residues</th>
<th>Mascot ions score</th>
<th>Expectation value</th>
<th>Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 – 29</td>
<td>72</td>
<td>0.00044</td>
<td>K.AGFAGDDAPR.A</td>
</tr>
<tr>
<td>52 – 62</td>
<td>35</td>
<td>0.025</td>
<td>K.DSYVGDEAQSK.R</td>
</tr>
<tr>
<td>149–178</td>
<td>86</td>
<td>2e-5</td>
<td>R.TTGIVLDSGDGVHSHTVPIYEGYALPHAILR.L</td>
</tr>
<tr>
<td>240–255</td>
<td>95</td>
<td>4.8e-7</td>
<td>K.SYELPDGQVITIGNER.F</td>
</tr>
</tbody>
</table>

Table 14. The Mascot ions scores and the expectation values of the matched peptides with distinct sequences to acrosomal isoform actin from band 1 (A) and muscle actin from band 2 (B). When there were duplicated sequences of peptides, the one with the higher Mascot ions score is shown in the table.
Figure 33. Peptide coverage of glyceraldehyde-3-phosphate dehydrogenase from mass spectrometry.
From band 1. Sequence coverage is 4 %. Matched peptides are shown in red.

<table>
<thead>
<tr>
<th>Residues</th>
<th>Mascot ions score</th>
<th>Expectation value</th>
<th>Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>332 – 352</td>
<td>54</td>
<td>0.00037</td>
<td>K.LTGMAFRVPTPNVSVDLTCR.L</td>
</tr>
<tr>
<td>339 – 352</td>
<td>81</td>
<td>6.9e-5</td>
<td>R.VPTPNVSVDLTCR.L</td>
</tr>
</tbody>
</table>

Table 15. The Mascot ions scores and the expectation values of the matched peptides with distinct sequences to glyceraldehyde-3-phosphate dehydrogenase from band 1.
When there were duplicated sequences of peptides, the one with the higher Mascot ions score is shown in the table.
Figure 34. Peptide coverage of probable V-type proton ATPase subunit B from mass spectrometry.
From band 2. Sequence coverage is 6%. Matched peptides are shown in red.

<table>
<thead>
<tr>
<th>Residues</th>
<th>Mascot ions score</th>
<th>Expectation value</th>
<th>Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>76 – 90</td>
<td>97</td>
<td>3e-7</td>
<td>K.AVVQVFEGTSGIDAK.N</td>
</tr>
<tr>
<td>302 – 317</td>
<td>53</td>
<td>0.023</td>
<td>R.GFPGYMYTDLATIYER.A + Oxidation (M)</td>
</tr>
</tbody>
</table>

Table 16. The Mascot ions scores and the expectation values of the matched peptides with distinct sequences to probable V-type proton ATPase subunit B from band 2. When there were duplicated sequences of peptides, the one with the higher Mascot ions score is shown in the table.
Figure 35. Cry11Ba and C. pipiens BBMV cross-linking and co-immuno precipitation. Cross-linking and co-immuno precipitation experiment was repeated with diazirine-modified Cry11Ba. There were bands (lane 1) in the similar positions as there observed with diazirine-modified Cry19Aa (Figure 25, lane 2). MW: molecular weight marker. Supe: supernatant.
Chapter 4: Is SERCA in the BBMV Preparations the Binding Partner for Certain Cry Proteins?

4.1 Introduction

Calcium ATPase was found through a binding study with Cry19Aa to be a possible receptor for Cry19Aa. Three experiments were performed to see whether calcium ATPase is a receptor for Cry19Aa. The first one was to make a (His)$_6$-tagged Cry19Aa in order to affinity purify the candidate receptor. The second one was to express calcium ATPase in insect cells. If calcium ATPase is expressed and purified, it can be used to verify the binding between Cry19Aa and calcium ATPase. Calcium ATPase can also be used with the Cry19Aa in a bioassay to verify the functionality of the receptor. If the mixture of calcium ATPase and Cry19Aa reduces the toxicity compared to Cry19Aa alone, this would suggest that the calcium ATPase is a receptor. The third experiment was to detect calcium ATPase in the photo-cross-linking experiment by western blot analysis to verify the mass spectrometric proteomic results (Figure 44).

The Bulla group at the University of Texas performed a protein study to compare midgut proteins between a Bt subspecies entomocidus resistant strain of Indian meal moth larvae and a susceptible wild type larvae (89). By comparing proteins by 2-D electrophoresis, they determined that the calcium-transporting ATPase (apparent
molecular mass 13 kDa because of protein degradation during the process) was expressed at a 1.5-fold lower level in resistant larvae than susceptible larvae. Since this study addressed calcium regulation in the plasma membrane, this protein may be a plasma membrane calcium-transporting ATPase (89).

The Després group at Université de Grenoble compared the transcription level in whole larvae between Bt subspecies *israelensis* (Bti) resistant strain of mosquito *A. aegypti* larvae and susceptible wild type larvae (90, 91). By sequencing short 20-mer-tagged cDNA libraries, Després and coworkers showed that the calcium transporting ATPase (AAEL 006582, sarcoplasmic/endoplasmic reticulum calcium ATPase, SERCA) was transcribed 4.2-fold lower in Bti-resistant larvae than wild type (90). The Després group also analyzed the transcription level in the midgut between Bti resistant strain of mosquito *A. aegypti* larvae and susceptible wild type larvae. There are 3 splicing variants encoded by the SERCA gene of *A. aegypti* larvae (A, B, and C). Out of these, the AAEL006582-RC SERCA C was transcribed 1.1-fold lower, and AAEL006582-RA SERCA A was transcribed 1.9-fold higher in Bti resistant larvae (91). One of the plasma membrane calcium-transporting ATPase (pmca3) was transcribed 1.74-fold higher in Bti resistant larvae (91). These results showed that calcium ATPase may be linked to the Cry protein resistant process and calcium ATPase could be a candidate receptor for Cry protein.

Cry19Aa is not toxic to *A. aegypti*, but is toxic to *C. p. pipiens* and *C. p. quinquefasciatus*. Bti produces 4 major Cry proteins, Cry4A, Cry4B, Cry11A, and CytA. The resistant larvae of *C. p. quinquefasciatus* to these 4 Cry proteins were still
susceptible to Cry19Aa (92). Therefore, the mechanism of action of Cry19Aa and its receptor can be different from those of Cry proteins in Bti.

SERCA is a membrane protein in the sarcoplasmic reticulum or endoplasmic reticulum. For one ATP hydrolyzed, SERCA transports 2 Ca\(^{2+}\) ions from the cytosol to the lumen of sarcoplasmic reticulum or endoplasmic reticulum while transporting 2 H\(^+\) in the opposite direction (Figure 36), (1). SERCA removes Ca\(^{2+}\) ions from the cytosol so that muscle can relax. Humans have 3 types of SERCA proteins (SERCA1, SERCA2, and SERCA3) (93). A mutation in the SERCA1 gene causes Brody myopathy, whose symptoms are painless muscle cramping and stiffening after exercise. Muscles relax after few minutes of rest (94). A decrease in the amount of the SERCA2a protein is related to heart failure (93).

Calcium ATPase may be involved in a resistance to Cry protein toxicity. When the insect larva eats a Cry protein, it is paralyzed. This is reasonable as SERCA is involved in the relaxation of muscle. Therefore, the paralysis of the insect can be related to SERCA. Paralysis of silkworm by Cry protein occurs in 4 h, which is a slow process (Table 2). If Cry19Aa binds to SERCA, it might be a later event in the mechanism of toxicity. Cry19Aa may have a novel type of receptor or binding protein. The possibility of SERCA interacting with the Cry19Aa protein was examined.
4.2 Materials and Methods

4.2.1 Plasmid Extraction

Cry11Ba in 4O7 *Bacillus thuringiensis* (DBT13), Cry19Aa in 4O7 *Bacillus thuringiensis* (DBT14) were obtained from the *Bacillus* Genetic Stock Center on TBAB agar plate (OSU, Columbus, OH, http://www.bgsc.org/). LB (5 mL with 25 μg/mL erythromycin) was inoculated with a single colony of DBT13 (Cry11Ba) or DBT14 (Cry19Aa). Tubes were shaken at 23 °C overnight. One hundred mL BHI (brain heart infusion media) with 25 μg/mL erythromycin was inoculated with a 5-mL overnight culture of Cry11Ba or 19Aa. The flasks were shaken at 23 °C for 4 h. The cells were harvested at 12,429 x g at 10 min, 4 °C. The plasmids were extracted with the Qiagen miniprep kit except that the cells were lysed by incubating at 37 °C for 30 min with 50 μL of lysozyme (50 mg/mL) in lysis buffer (P1 buffer). The purified shuttle vector plasmid pH315 with Cry11Ba or Cry19Aa was transformed into *E. coli* DH5α competent cells for isolating plasmid at a later time. The plasmids were extracted with the Qiagen miniprep kit.

4.2.2 Incorporation of (His)$_6$-tag to Cry19Aa

Mutagenesis primers were designed to incorporate a (His)$_6$ tag within the Cry19Aa protein. Forward and reverse primers have (His)$_3$ coding sequences at their 5′
ends. The reverse primer has \((GTG)_3\), while the forward primer has \((CAT)_3\); such an approach helped avoid annealing of the two primers which would have occurred if we used the same codon for all six positions. When the plasmid was amplified and ligated, successful mutagenesis should be in a \((CAC)_3(CAT)_3\) at the insertion site thus adding a \((\text{His})_6\) tag (Figure 39). \((\text{His})_6\) tag insertion sites were chosen by aligning known crystal structures of activated Cry toxins (Cry1Aa, Cry3A, Cry4Aa, and Cry4Ba) and Cry19Aa by Clustal Omega (95). The similarity of Cry protein structures suggest that Cry19Aa can be cleaved by trypsin or chymotrypsin at a similar position as the other Cry proteins (Figure 11). Primers used to make \((\text{His})_6\) tag Cry19Aa are listed in Table 17. Primers were obtained from Sigma-Aldrich (St. Louis, MO). The melting temperature of the primers was calculated using the Oligo Analyzer program provided by Integrated DNA Technologies (96).

Primers (300 pmol) were phosphorylated in a 50 μL reaction with T4 polynucleotide kinase (PNK; 10 U, NEB), T4 PNK reaction buffer, 1 mM ATP at 37 °C for 30 min. The enzyme was heat inactivated at 65 °C for 20 min. The phosphorylated primers were stored at 4 °C. These primers (at 6 μM) were used directly in the PCR reaction.

The pHT315-Cry19Aa plasmid was amplified with phosphorylated primers to introduce a \((\text{His})_6\) tag. KOD hot start polymerase (Novagen, Toyobo) was used for PCR using the KOD-Plus-Mutagenesis kit (Toyobo) (97, 98). The recipe and the reaction conditions are listed in Table 18. Two μL DpnI (10 U/μL, NEB) were added to the reaction and incubated at 37 °C for 1 h to digest the template DNA. Ten μL of this sample
was loaded on a 1 % (w/v) agarose gel in TAE buffer to visualize the PCR products. The gel was stained in ethidium bromide solution and visualized on UV transilluminator (Fotodyne) (Figure 40).

The DNA was column purified using a Nucleospin gel and PCR Clean-up kit (Clontech). The concentration of the PCR product of the N1-(His)₆-Cry19Aa plasmid was 6 ng/μL. Five μL of the cleaned PCR product was ligated by T4 DNA ligase following instructions in the quick ligation kit (NEB). Two μL of the ligation reaction was added to 50 μL chemically competent ER2925 (NEB, methylation- deficient strain of E. coli) cells for transformation. Unmethylated plasmid is needed to transform Bt. Colonies and the transformants were selected on LB plus ampicillin (100 μg/mL) agar. After incubation at 37 ºC for 18 h, a few colonies (2~3) were obtained. Five mL of LB media containing ampicillin (100 μg/mL) was inoculated with a single colony. Overnight culture at 37 ºC was used to make a glycerol stock and also to purify the plasmid utilizing the Qiaprep Spin Miniprep kit (Qiagen). The presence of a (His)₆ tag coding sequence within the N1-(His)₆-Cry19Aa gene was confirmed by DNA sequencing at the Plant-Microbe Genomics Facility (OSU, Columbus OH) or GENEWIZ (South Plainfield, NJ). The primers which were used for DNA sequencing are listed in Table 17.

4.2.3 Bt Transformation

Bt transformation was performed as described elsewhere (99).
4.2.4 Cry Protein Expression

*Bacillus thuringiensis* 4Q7 (plasmidless strain of *Bacillus thuringiensis* subspecies *israelensis*) was obtained from the *Bacillus* Genetic Stock Center on TBAB agar plate (OSU, Columbus, OH, [http://www.bgsc.org/](http://www.bgsc.org/)). Two tubes of LB (5 mL with 25 μg/mL erythromycin) were inoculated with a single colony of Cry19Aa, N1-(His)$_6$-Cry19Aa or Cry19Aa-(His)$_6$-C in 4Q7. A tube of LB (5 mL) was inoculated with a single colony of 4Q7. Tubes were shaken at 23 °C overnight. Two 2-L flasks containing 400 mL SSM with 25 μg/mL erythromycin was inoculated with this 5-mL overnight culture of Cry19Aa, N1-(His)$_6$-Cry19Aa or Cry19Aa-(His)$_6$-C in 4Q7. A 2-L flask containing 400 mL SSM were inoculated with the 5-mL overnight culture of 4Q7. The flasks were shaken at 23 °C for 4 days. Most of the cells had autolysed as determined by analyzing cells using phase contrast microscopy. The cells were harvested at 12,429 x g at 4 °C for 10 min. The pellets were washed thrice with 100 mL of crystal wash I [0.5 M NaCl, 2 % (v/v) TritonX-100] solution and then were washed thrice with 100 mL of crystal wash II (0.5 M NaCl) solution. The cells were centrifuged at 12,429 x g at 4 °C for 10 min between washes. The pellets were resuspended in 40 mL Crystal wash II solution containing 1 mM PMSF. The crystal and spore mixture was stored at 4 °C until further use.
4.2.5 Bioassay

Six 4th instar larvae of *C. p. pipiens* were placed individually in 6 well plate (BD, Falcon non-tissue culture treated plate). The amount of the distilled tap water or Bt inclusion suspension per well was 12 mL. Six to seven different concentrations of Cry19Aa, N1-(His)_6-Cry19Aa and Cry19Aa-(His)_6-C isolated from Bt inclusion bodies (mixture of crystals and spores) were tested as well as 4Q7 (spores only). The concentrations were as follows: Cry19Aa (180 ng/mL, 90 ng/mL, 45 ng/mL, 22.5 ng/mL, 11.25 ng/mL, and water only); N1-(His)_6-Cry19Aa (950 ng/mL, 475 ng/mL, 237.5 ng/mL, 118.8 ng/mL, 59.38 ng/mL, 29.69 ng/mL, and water only); Cry19Aa-(His)_6-C (126 ng/mL, 63 ng/mL, 31.5 ng/mL, 15.75 ng/mL, 7.88 ng/mL, and water only); 4Q7 (1,000 ng/mL, 300 ng/mL, 150 ng/mL, 75 ng/mL, 37.5 ng/mL, 18.75 ng/mL, and water only). The concentration of protoxin was determined by solubilizing Bt inclusion bodies in 50 mM sodium carbonate, 10 mM DTT at 37 °C for 2 h. After centrifugation at 16,917 x g for 10 min, the concentration of supernatant was measured by the Coomassie plus (Bradford) protein assay (Pierce). BSA was used as the reference standard. The suspension was made by serial dilution of the highest concentration using distilled tap water as the diluent. The dead larvae were counted after 24 h. If a pupa emerged, the number of the pupae was omitted from the count. LC_{50} was calculated by the SoftTox program (SoftLabWare and WindowChem, Fairfield, CA).
4.2.6 Rearing Mosquitoes

Three types of mosquitoes were reared in the mosquito room in our laboratory. They are *An. gambiae*, *A. aegypti* (100), and *C. p. pipiens*. The mosquito room has a photoperiod of alternating 14 h of light and 10 h of dark, with a temperature of 28 °C and 85 % humidity. Cow blood used for the adult female to lay eggs was obtained from the veterinary medical center of the Ohio State University (Columbus, OH).

4.2.7 Baculovirus Transfection

Sf9 cells from *Spodoptera frugiperda* (fall armyworm) [Sf9 cells adapted in Sf-900 III SFM (serum free insect media), Lot 1119777, Life Technologies] were grown as a suspension culture in 60 mL of Sf-900 III SFM (Life Technologies) in a 250 ml flask at 25 ºC. BaculoDirect C-Term Expression Kit (Life Technologies) was used for transfection and expression of SERCA gene in Sf9 cells.

The pENTR/SD/D-TOPO Cloning Kit with One Shot TOP10 Chemically Competent *E. coli* (catalog number K2420-20, Life Technologies) was used to clone the SERCA gene. This pENTR vector was used to integrate SERCA gene into the linear baculovirus genome by LR recombination for 18 h at 23 ºC. The presence of the gene was checked with PCR. pENTR CAT (chloramphenicol acetyltransferase, which was provided in the TOPO cloning kit) was used as a positive control as well. The protocol described within the Baculodirect baculovirus expression system manual was followed.
Sf9 cells (passaged 9 times, $9.2 \times 10^5$ cells/mL, 99% viability) in Sf-900 III SFM media were plated at a density of $8 \times 10^5$ cells/well in a 6-well plate. Ten mL of plating medium was prepared by mixing 1.5 mL Grace’s insect medium supplemented with 10% FBS (fetal bovine serum) and 8.5 mL of Grace’s insect medium unsupplemented. After the attachment of the insect cells, they were washed with 2 mL of plating media, and then with 2 mL of Grace’s insect medium unsupplemented. Transfection was performed in 2 mL of Grace’s insect medium unsupplemented. Transfection mixture A ($8 \mu$L of cellfectin II reagent in 100 μL of Grace’s insect medium unsupplemented) and transfection mixture B ($10 \mu$l of LR recombination in 100 μL of Grace’s insect medium unsupplemented) were made. Mixture A and B were combined and incubated at 23 °C for 30 min. After 30 min, the transfection mixture was added dropwise to the cell. The cells were incubated for 5 h at 23 °C. The transfection mixture was removed and 2 ml of Sf-900 III SFM and 100 μM ganciclovir was added. The cells were incubated for 4 days. No sign of protein expression of SERCA was detected through western blot (Figure 51).

4.2.8 Plasmid Transfection

pPac-PL-DsRed plasmid which has a red fluorescent protein gene from *Discosoma* sp. under an actin 5C promoter was a kind gift from Sathiya Manivannan (a graduate student in the laboratory of Prof. Amanda Simcox, Department of Molecular Genetics, Ohio State University, Columbus, OH USA). The procedures described within the cellfectin II manual for plasmid transfection were followed.
Sf9 cells (passaged 9 times, 7.4 x 10^6 cells/mL, 98 % viability) in Sf-900 III SFM media were plated at 8 x 10^5 cells/well in 2 mL Sf-900™ III SFM in a 6-well plate. Sterile coverslip was placed in the well to grow the cells on the coverslip for observation under microscope later. Transfection mixture A (8 μl of cellfectin II reagent in 100 μl of Grace’s insect medium unsupplemented) and transfection mixture B (1 μg of pPac-PL-DsRed plasmid in 100 μl of Grace’s insect medium unsupplemented) were made. Each was vortexed and incubated at room temperature for 20 min (a control without plasmid was included as negative control.) Mixture A and B were combined and incubated at 23 ºC for 10 min. After 10 min, the transfection mixture was added dropwise to the cell. The cells were incubated for 56 h at 23 ºC without changing the media.

The Sf9 cells were washed with 2 mL of DPBS twice. The cells were fixed on coverslip with 4 % (v/v) formaldehyde for 2 min at 37 ºC. The cells were washed twice with 2 mL of DPBS for 2 min at 23 ºC. The cells were incubated with 1μg/mL of Hoechst 33342 (Thermo Scientific) to stain the DNA. The cells were washed twice with 2 mL of DPBS. Prolong gold antifade reagent (Life Technologies) was used to mount the coverslip. The cells were observed under a confocal microscope (Olympus FV1000, Campus Microscopy and Imaging Facility OSU) with DAPI (4’,6-diamidino-2-phenylindole) and RFP image acquisition settings. (X 200 magnification) (Figure 52). When the percentage of the cells which expressed DsRed was calculated, transfection efficiency was around 3 %.
4.3 Results

The placement of (His)$_6$ tag in Cry19Aa was designed by aligning amino acid sequences of known crystal structures of activated Cry toxins (Cry1Aa, Cry3A, Cry4Aa, and Cry4Ba) and Cry19Aa by Clustal Omega (95) (Figure 37 and Figure 38), since a crystal structure of Cry19Aa is not known. Cry4Ba toxin is shorter than the other Cry toxins at the N-terminus. Cry toxin can be activated by trypsin or chymotrypsin. Trypsin cleaves a protein on the C-terminal side of lysine or arginine, whereas chymotrypsin cleaves a protein on the C-terminal of phenylalanine, tyrosine, or tryptophan. (His)$_6$ tag insertion sites were chosen so that activated Cry19Aa toxin gives similar length to Cry4Ba (N2-(His)$_6$-Cry19Aa) or other Cry toxins (N1-(His)$_6$-Cry19Aa) (Figure 37). There is no trypsin or chymotrypsin cleavage site at the C-terminus of Cry19Aa (Figure 38).

We first attempted cloning Cry19Aa into pET45b vector (Novagen), which enables insertion of a (His)$_6$ tag at the N-terminus. Although the protoxin of N1-(His)$_6$-Cry19Aa and N2-(His)$_6$-Cry19Aa were expressed, chymotrypsin digested the protoxin completely. Activated N1-(His)$_6$-Cry19Aa and N2-(His)$_6$-Cry19Aa toxins were not obtained. The sizes of the crystals of this N1-(His)$_6$-Cry19Aa and N2-(His)$_6$-Cry19Aa were much smaller than those of wild type Cry19Aa when they were observed under phase contrast microscopy. The folding of the protein might not be proper because of the deletion of the N-terminal amino acids before (His)$_6$ tag. Subsequently, we attempted inserting (His)$_6$ tag in pHT315 vector which has both Cry19Aa and ORF2 (Figure 39).
ORF2 helps promote crystal formation of the Cry19Aa. Without ORF2, expression of
Cry19Aa is low (5).

(His)$_6$ tag insertion was accomplished by PCR mutagenesis (Figure 39 and Table
18). The presence of the (His)$_6$ tag sequence was confirmed by DNA sequencing (Plant-
Microbe Genomics Facility, OSU, Columbus, OH).

Bt was transformed with Cry19Aa, N1-(His)$_6$-Cry19Aa, N2-(His)$_6$-Cry19Aa, and
Cry19Aa-(His)$_6$-C. The expression level of N2-(His)$_6$-Cry19Aa was very low, therefore
N1-(His)$_6$-Cry19Aa and Cry19Aa-(His)$_6$-C, which were expressed at high amounts in Bt
4Q7, were used. The amounts of solubilized protoxin obtained from 800 mL SSM culture
were 104 mg (wild type Cry19Aa), 38 mg (N1-(His)$_6$-Cry19Aa), and 64 mg (Cry19Aa-
(His)$_6$-C). Although the expression levels were decreased in (His)$_6$ tag variants of
Cry19Aa, crystals were observed during sporulation. The chymotrypsin activation of
protoxin gave activated toxin, and the presence of (His)$_6$ tag after activation was
confirmed by western blot with anti-(His)$_6$ antibody (Figure 42). (His)$_6$ tag Cry19Aa
were purified through Talon cobalt resin (Clontech, Figure 43). Some N1-(His)$_6$-Cry19Aa
was also in the wash fraction. We inserted (His)$_6$ tag after trypsin digestion site for N1-
(His)$_6$-Cry19Aa, but we digested with chymotrypsin. The binding of (His)$_6$ tag to the
resin might be weak because amino acids before (His)$_6$ tag might interfere with the
binding. We used chymotrypsin because trypsin digests Cry19Aa into two fragments (40
kDa and 25 kDa, (87)).

Bioassays against C. p. pipiens larvae were conducted for Cry19Aa, N1-(His)$_6$-
Cry19Aa, Cry19Aa-(His)$_6$-C, and 4Q7 Bt (Table 19). The toxicity of Cry19Aa-(His)$_6$-C
was similar to Cry19Aa wild type. Toxicity of N1-(His)$_6$-Cry19Aa reduced three-fold compared to Cry19Aa and Cry19Aa-(His)$_6$-C. None of the larvae died with 4Q7 Bt spore suspension which does not contain a Cry protein; therefore toxicity in the spore suspension was from Cry19Aa, not from the spores or 4Q7 Bt cells.

The cross-linking study using diazirine-modified Cry19Aa-(His)$_6$-C was unsuccessful (Figure 45). SDS-PAGE showed that most of the proteins visible on the gel were on the beads. Incubation of beads and BBMV proteins for 15 h might have been too long or we should have used higher concentration of imidazole to elute the proteins. Also, most of diazirine-modified Cry19Aa-(His)$_6$-C was in the supernatant. This observation likely reflects that only a small portion of the Cry protein bound to BBMV proteins or alternatively that the bound receptors were released into the solution.

To capture receptor candidates, BBMV proteins were solubilized first and then cross-linked with diazirine-modified Cry19Aa-(His)$_6$-C or LC (long chain) diazirine-modified Cry19Aa-(His)$_6$-C (Figure 20 and Figure 46). Long chain diazirine-modified Cry protein might be able to capture more proteins than the diazirine-modified counterpart. If Cry protein makes protein complexes with receptors, LC diazirine might capture proteins associated with the Cry protein receptor. LC diazirine might insert into membrane lipid, therefore facilitating capture of membrane protein receptors. The disadvantage of LC diazirine is that it might capture a non-binding protein. This trial, incubation time with magnetic beads was reduced to 30 min. This reduction in time was possible because of the high affinity of Cry19Aa-(His)$_6$-C to cobalt magnetic beads. There were very weak bands in the eluate. These bands might contain cross-linked Cry19Aa-(His)$_6$-C to a
binding protein from BBMV. When the molecular weights of bands were estimated using the migration position on the SDS-PAGE gel in Figure 46, the molecular weight of protein band in lane 3 was 251 kDa, and the molecular weight of protein band in lane 7 was between 344 kDa and 385 kDa. Further analyses, such as mass spectrometry or western analysis with a SERCA antibody, are necessary to determine whether this is a binding protein.

To test whether the SERCA antibody reacts with cross-linked protein, cross-linking experiments in Chapter 3 were repeated. SERCA antibody (Monoclonal anti-SERCA1, C-terminus, Sigma S 1189) was used. Sigma listed that this antibody was against sarcoplasmic reticulum from rabbit skeletal muscle and that it will react with human, canine, and rabbit SERCA (101). Dr. Naresh Bal (Dr. Muthu Periasamy’s laboratory, Department of Physiology and Cell Biology, the Ohio State University, Columbus, OH USA) suggested that this antibody would react with SERCA from mosquito, because this antibody recognizes a highly conserved region (residue 506-1001) which is exposed to cytosol from sarcoplasmic/endoplasmic reticulum (101). *C. p. pipiens* SERCA has 73% amino acid sequence identity with a rabbit SERCA which was used in X-ray crystallography (Figure 36 and Figure 50). *C. p. pipiens* BBMV proteins and diazirine-modified Cry19Aa or diazirine-modified Cry19Aa-(His)_6-C were mixed and illuminated with UV. Cry 19Aa was co-immunoprecipitated with Cry19Aa antibody and protein-A magnetic beads. Cry19Aa-(His)_6-C was co-immunoprecipitated with cobalt magnetic beads. After SDS-PAGE, proteins were transferred to PVDF membrane and probed with SERCA antibody. BBMV proteins or Cry19Aa with BBMV proteins
were reacted with SERCA antibody (Figure 44). SERCA was not detected in lanes for Cry19Aa-(His)$_6$-C co-immunoprecipitation, partly because most of the BBMV proteins were in the beads fraction which was not included in this analysis (Figure 45).

We attempted to overexpress SERCA protein in insect cells using the baculoviral expression system. Glycosylation is sometimes needed for Cry protein to recognize a receptor. A protein which is expressed in an insect cell will get a simple glycosylation in the cell. The glycosylated SERCA which is overexpressed in insect cell would have a higher chance to be recognized by Cry protein.

SERCA cDNA was cloned from total RNA which was extracted from whole larvae of *C. p. pipiens*. The sequence of SERCA cDNA from *C. p. pipiens* contained 18 base insertions compared to that of *C. p. quinquefasciatus* (Figure 47 and Figure 48). This cDNA codes for 1,001 amino acids of SERCA from *C. p. pipiens* (Figure 49). The 18-base insertion corresponds to a 6-amino acid insertion, but SERCA from *A. aegypti* has the same 6-amino acid insertion (Figure 49). The insertion is therefore not an artifact from PCR amplification.

Western blot analysis using SERCA antibody or (His)$_6$ antibody did not show the presence of SERCA or SERCA-(His)$_6$-C. Amplification of baculovirus by infecting insect cells for a 2$^{nd}$ time (amplification sample) also did not give a detectable amount of SERCA (Figure 51). Changing transfection reagents, using a different cell line (Sf21), and increasing transfection time did not improve this result.

To assess the transfection efficiency, pPac-PL-DsRed plasmid which has actin 5C promoter linked to the red fluorescent protein was transfected to Sf9 cells using cellfectin
II. The transfection efficiency was estimated by observing expression of red fluorescent protein under confocal microscope. The transfection efficiency was around 3 % (Figure 52). Sathiya Manivannan (a graduate student in the laboratory of Prof. Amanda Simcox, Department of Molecular Genetics, Ohio State University, Columbus, OH USA) transfected this plasmid into Drosophila S2 cells using Effectene (Qiagen) with a transfection efficiency of 25 %. This result suggests that the transfection efficiency using cellfectin II to Sf9 cells is low.
4.4 Discussion

SERCA was identified as a possible Cry protein receptor through a binding study between *C. pipiens* BBMV proteins and Cry19Aa in Chapter 3. SERCA is an important pump which removes Ca^{2+} from the cytosol and helps to make muscle relax after contraction. Cry protein is known to cause paralysis to insects. *C. p. pipiens* larvae became L-shaped and stopped moving 4 h after treatment with Cry11Ba or Cry19Aa (Figure 41). The cause of paralysis might be due to SERCA inhibition by Cry protein. This hypothesis necessitates entry of Cry protein into the cell and then interact with the ER-localized SERCA. While cry protein can be inserted into membrane (102), its movement inside the cell by endocytosis is not known.

To improve pull down of Cry19Aa, N-terminal and C-terminal fusions of (His)$_6$ tag to the Cry19Aa was made. The incorporation of (His)$_6$ tag into the Cry19Aa sequence worked and produced a stable protoxin and an activated toxin. Western blot analysis against (His)$_6$ tag showed that the (His)$_6$ tag was indeed retained within the toxin after activation with chymotrypsin (Figure 42). Bioassay measurement showed that Cry19Aa-(His)$_6$-C was toxic to the *C. p. pipiens* larvae, while N1-(His)$_6$-Cry19Aa was less toxic. (His)$_6$ tag might change the conformation of N1-(His)$_6$-Cry19Aa or affect the mechanism of toxicity. The important domains of Cry protein in receptor recognition are domain II or domain III located on the C-terminal side. If receptor binds to Cry protein, the C-terminal (His)$_6$ tag might be buried by the receptor.
Mass spectrometry results showed that SERCA was in BBMV preparations; it is conceivable that BBMV preparations were contaminated with some ER proteins. SERCA antibody reacted with both BBMV proteins and cross-linked proteins (Figure 44). Whether SERCA antibody cross-reacts with another plasma membrane calcium ATPase or just reacts with the ER SERCA is unclear. The structures of SERCA and plasma membrane calcium ATPase are predicted to be similar (103). The amino acid sequence identity after residue 245 of *C. p. pipiens* SERCA and *C. p. quinquefasciatus* plasma membrane calcium transporting ATPase 2 is 30%, therefore the identified protein in mass spectrometry is likely to be SERCA.

SERCA bands were detected in the eluate fraction in the diazirine-modified Cry19Aa cross-linking experiment (Lane 3, Figure 44). The positions of SERCA bands on the western blot were at the same positions as band 1 and band 2 which were analyzed by mass spectrometry (Lane 2, Figure 25). Although SERCA was found to be a binding partner, consistent with our earlier mass spectrometry results, firm support for this finding requires further in-depth experimentation includes controls to ascertain specificity of interaction.

There is a possibility that Cry11Ba binds to SERCA as Cry11Ba was detected in mass spectrometry with SERCA. If the cross-linked proteins are separated by 2-D SDS-PAGE, it might be possible to see whether SERCA and Cry11Ba are cross-linked or separate. If SERCA protein is expressed in insect cell, we can test whether SERCA binds to Cry11Ba or Cry19Aa. Then, we can also perform competition binding assay between
Cry11Ba or Cry19Aa and BBMV proteins in the presence of SERCA to test whether binding of SERCA to Cry11Ba or Cry19Aa is specific.

Baculoviral expression of SERCA was not successful. One reason was that the transfection efficiency of the virus might be low based on transfection of the pPac-PL-DsRed plasmid. The transfection efficiency of pPac-PL-DsRed was 3 % (Figure 52).

There might not be enough SERCA protein expression to be detected by western blot. It would have been preferable to verify baculoviral expression of SERCA-DsRed fusion protein rather than expression of DsRed through plasmid, because the expression of SERCA can be estimated by observing DsRed under confocal microscope. The cloning of DsRed gene to the pENTR/SD/D-TOPO vector using Top10 E. coli competent cells failed. The colonies were grown on LB plus kanamycin agar after transformation, but those colonies failed to grow on either LB plus kanamycin agar or liquid media. The cells most likely lost the plasmid. If the cells retained the plasmid, they could have grown under kanamycin using kanamycin resistant gene on the plasmid. The transformation of E. coli DH5α cells with pPac-PL-DsRed plasmid also failed. It was found that E. coli JM103 competent cells could retain pPac-PL-DsRed plasmid, and these cells grew after inoculated to LB plus ampicillin agar or media. Incorporating DsRed gene to pENTR/SD/D-TOPO vector might be possible by using JM103 strain instead of TOP10, because JM103 can retain the plasmid with DsRed gene.

If baculoviral transfection of SERCA gene is successful, it might be possible to obtain a viral plaque which baculovirus makes after transfection. Since this viral plaque contains baculovirus, thus the virus can be amplified by transferring the plaque to new
Sf9 cells. We can also estimate a transfection efficiency of baculovirus by counting the number of plaques.

Sodium/potassium ATPase and SERCA are members of P-type (phosphorylated intermediate-type) ATPases (104). English showed that Cry protein from Bt subspecies *kurstaki* was a reversible inhibitor of sodium/potassium ATPase which was purified from a dog kidney (81). As SERCA antibody for rabbit, human, and canine also reacted with mosquito BBMV proteins, the structures of dog SERCA and mosquito SERCA might be similar. The structure of sodium/potassium ATPase is shown in Figure 53. The structures of sodium/potassium ATPase and SERCA are similar (Figure 53B). The mechanism of ion transport of SERCA was shown in Figure 54. The mechanism of ion transport of sodium/potassium ATPase is expected to be similar to SERCA (13). English suggested that the Cry protein blocked Na\(^+\) transport from the cytoplasmic side and blocked the formation of the aspartyl phosphate intermediate (Figure 54, step 3). Similarly, Cry19Aa might bind to SERCA from the cytoplasmic side and prevent Ca\(^{2+}\) transport by blocking the formation of the aspartyl phosphate intermediate.

In conclusion, we constructed (His)\(_6\)-tagged Cry19Aa. Bioassays with *C. pipiens* larvae showed that N-terminally (His)\(_6\)-tagged Cry19Aa has three-fold lower toxicity compared to the untagged Cry19Aa, while C-terminally (His)\(_6\)-tagged Cry19Aa retained the same level of toxicity as Cry19Aa; we therefore chose to use C-terminally (His)\(_6\)-tagged Cry19Aa in pull-down assays. To verify SERCA as a binding protein of Cry19Aa, the pull-down experiment using BBMV with diazirine-modified Cry19Aa was performed. The pulled-down proteins were probed with SERCA antibody using a western
blot. Although SERCA was found to be a binding partner, consistent with our earlier mass spectrometry results, firm support for this finding requires further in-depth experimentation includes controls to ascertain specificity of interaction. If SERCA is a Cry19Aa receptor, Cry19Aa might bind to SERCA from the cytoplasmic side and prevent Ca^{2+} transport by blocking the formation of the aspartyl phosphate intermediate.
Figure 36. X-ray crystal structure of SERCA with 2 Ca\(^{2+}\) (PDB 1SU4) (1).

SERCA has 10 transmembrane helices and transports 2 Ca\(^{2+}\) from cytosol to lumen for each ATP hydrolyzed. Two H\(^+\) is transported from lumen to cytoplasm in exchange.
CLUSTAL O(1.1.0) multiple sequence alignment

Cry1Aa1CIY
Cry3A1DLC
Cry4Aa2C9K
Cry4Ba1W99
Cry19Aa

MHYYGNRNEYDILNASSNSNYKDWLNVCEGYHIENPR

Cry1Aa1CIY
-------
IETGYT-PIDISLSLTQFLLSE--FVGAGFVLGLVDIWGIF--GPSQWDAF
Cry3A1DLC
-ATTKDVIOKGIS-VVGDLLGVVGFPGGG----ALVSFYTNFLNTIWPS----EDPKWAF
Cry4Aa2C9K
---3ELSAYTVVGTWL--TGFVTFPLGLALIGFG--TLIPVLFPQAQ-DQSNLNSDF
Cry4Ba1W99
---------------------------------------------------
Cry19Aa
EASVRAEGKGLG--IVS----TIVGFFGGS--IILDITILFYQISEL

N1-(His)_6-Cry19Aa  R-(His)_6 AGLGK. ....  Cry proteins will be activated by either trypsin or
N2-(His)_6-Cry19Aa  Y-(His)_6 QISEL. ....  chymotrypsin.

Figure 37. Rationale underlying choice of N-terminal (His)_6 tag insertion site for Cry19Aa.
Multiple sequence alignment of Cry19Aa with known crystal structures of Cry proteins (Cry1Aa, Cry3A, Cry4Aa, and Cry4Ba). Because of
the similarity of Cry protein structures, Cry19Aa is predicted to be cleaved at a similar position as the other Cry proteins. This similar
position was chosen to insert (His)_6 tag.
Cry1Aa1CIY NQGNFS---ATM-----SSGSNLQSGSFRTV---GFTTPFNFSNGSSV--FTLSAHVFNS
Cry3A1DLC NQYYFD---KTI-----NKGDLTLYNSFNLA---SFSTPFELSGNNL-----QIGVTGLSA
Cry4Aa2C9K AELG-MALNPTFS---GTDYNLKYKDFQYL---EFSNEVKFAPNQ--NISLVFNRSDVYT
Cry4Ba1W99 SRGTTISTESTFSRPNNIPTDLKYEEFRYKDPFDAIVMRLLSNQ--LITIAIQPIETNS
Cry19Aa IQQ----------*QIHNISPTYGAFSYLESFTITTTENIFDLTMEVTPYGRQFVED
                 : :*::*::*::*

Cry1Aa1CIY GNEVYIDRIEFVPAEVTFEAEDLER
Cry3A1DLC GDKVYIDKIEFIPVN----------
Cry4Aa2C9K NTTVLIDKIEFPLITR----------
Cry4Ba1W99 NNQVIIIDRIEIIPITOSVLDE-----
Cry19Aa IPSLILDKIEFPTN----------
                 : :*::*::*::*

ORF2 helps crystallization of Cry19Aa

Figure 38. Rationale underlying choice of C-terminal (His)_6 tag insertion for Cry19Aa. Multiple sequence alignment of Cry19Aa with known crystal structures of Cry proteins (Cry1Aa, Cry3A, Cry4Aa, and Cry4Ba). Because of the similarity of Cry protein structures, Cry19Aa is predicted to be cleaved at a similar position as the other Cry proteins. Cry19Aa does not have trypsin or chymotrypsin digestion site at the C-terminus. ORF2 which is considered to be similar to C-terminal domain of the other Cry proteins are coded after Cry19Aa (5).
Figure 39. Strategy for (His)$_6$ tag insertion into Cry19Aa. (His)$_6$ tag was inserted into the plasmid encoded Cry19Aa protein by PCR amplification of plasmid with primers which have (His)$_3$ coding sequence at the 5’ sides.
<table>
<thead>
<tr>
<th>Primers</th>
<th>Primer sequence</th>
<th>Tm (melting temperature)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward 19Aa N1 (His)&lt;sub&gt;6&lt;/sub&gt;</td>
<td>cat cat cat GCT GGA CTT GGT AAG GGA TTA GG</td>
<td>57.1 ºC</td>
</tr>
<tr>
<td>Reverse 19Aa N1 (His)&lt;sub&gt;6&lt;/sub&gt;</td>
<td>gtt gtt gtt TCT AAC GCT TGC TTC TCT AGG ATT TTC</td>
<td>56.7 ºC</td>
</tr>
<tr>
<td>Forward 19Aa N2 (His)&lt;sub&gt;6&lt;/sub&gt;</td>
<td>cat cat cat CAA ATT TCA GAG CTA CTT TGG C</td>
<td>52.4 ºC</td>
</tr>
<tr>
<td>Reverse 19Aa N2 (His)&lt;sub&gt;6&lt;/sub&gt;</td>
<td>gtt gtt gtt GTA AAA CAA TCC AAT TGT ATC TAA GAT AAT AG</td>
<td>51.5 ºC</td>
</tr>
<tr>
<td>Forward 19Aa C (His)&lt;sub&gt;6&lt;/sub&gt;</td>
<td>cat cat cat TGA TAC CAT TCA CAG GAA ATA TGA GG</td>
<td>54.6 ºC</td>
</tr>
<tr>
<td>Reverse 19Aa C (His)&lt;sub&gt;6&lt;/sub&gt;</td>
<td>gtt gtt gtt GTG AGT TGG GAG GAA TTC GAT TTT ATC</td>
<td>54.1 ºC</td>
</tr>
<tr>
<td>Sequence primer for 19Aa N (His)&lt;sub&gt;6&lt;/sub&gt;</td>
<td>ATG CAT TAT TAT GGG AAT AGG AAT G</td>
<td>51.3 ºC</td>
</tr>
<tr>
<td>Sequence primer for 19Aa C (His)&lt;sub&gt;6&lt;/sub&gt;</td>
<td>AGA GCA GAT TCA GGT TTA ACT ATG</td>
<td>52.6 ºC</td>
</tr>
</tbody>
</table>

Table 17. Primer sequences for insertion of a (His)<sub>6</sub> tag to Cry19Aa and their melting temperatures. Melting temperatures of primers which anneal to Cry19Aa (without catcatcat or gtggtggtg) were calculated using Oligo Analyzer, Integrated DNA Technologies (96).
### PCR reaction components

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume added</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 X KOD buffer</td>
<td>5 μL</td>
<td>1 X</td>
</tr>
<tr>
<td>25 mM MgSO₄</td>
<td>3 μL</td>
<td>1.5 mM</td>
</tr>
<tr>
<td>dNTPs (2 mM each)</td>
<td>5 μL</td>
<td>0.2 mM each</td>
</tr>
<tr>
<td>Sterile distilled water</td>
<td>Add to adjust total reaction volume to 50 μL</td>
<td></td>
</tr>
<tr>
<td>Forward phosphorylated primer (6 μM)</td>
<td>2.5 μL</td>
<td>0.3 μM</td>
</tr>
<tr>
<td>Reverse phosphorylated primer (6 μM)</td>
<td>2.5 μL</td>
<td>0.3 μM</td>
</tr>
<tr>
<td>Template DNA (50 – 150 ng/μL)</td>
<td>1 μL</td>
<td></td>
</tr>
<tr>
<td>KOD Hot Start DNA Polymerase (1 U/μL)</td>
<td>1 μL</td>
<td>0.02 U/μL</td>
</tr>
<tr>
<td>Final volume</td>
<td>50 μL</td>
<td></td>
</tr>
</tbody>
</table>

### PCR reaction steps

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature</th>
<th>Length of time</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94 ºC</td>
<td>2 min</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>98 ºC</td>
<td>10 s</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Tm – (5~10 ºC)</td>
<td>30 s</td>
<td>Tm = melting temperature of the part of primer which anneals to template. N1 and N2-(His)₆-Cry19Aa(48 ºC used) Cry19Aa-(His)₆-C (52 ºC used)</td>
</tr>
<tr>
<td>4</td>
<td>68 ºC</td>
<td>X min</td>
<td>X is the length of the template in kb. (1 min/kb). pHT315-Cry19Aa is 10.1 kb, so 10 min was used.</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td>Return to step 2, Y times. Y is the length of the template in kb. (1 time/kb). pHT315-Cry19Aa is 10.1 kb, so 10 cycles were used.</td>
</tr>
<tr>
<td>6</td>
<td>4 ºC</td>
<td>18 h</td>
<td></td>
</tr>
</tbody>
</table>

Table 18. PCR recipe and reaction conditions for mutagenesis.
The recipe on the top was taken from the KOD Hot Start DNA Polymerase kit (Novagen, Toyobo) (97). The reaction conditions on the bottom are from the KOD-Plus-Mutagenesis Kit (Toyobo) (98).
Figure 40. Stained 1 % (w/v) agarose gel shown the amplification of (His)$_6$ tag Cry19Aa by PCR. pHT315-Cry19Aa plasmid was amplified with the (His)$_6$ tag mutagenesis primer using KOD hot start polymerase. Ten μL of PCR reactions were loaded on a 1 % (w/v) agarose gel and stained with ethidium bromide. The DNA bands were visualized by UV light. Lane 1: 1 kb DNA marker (Life Technologies). Lane 2: N1-(His)$_6$-Cry19Aa. Lane 3: N2-(His)$_6$-Cry19Aa. Lane 4: Cry19Aa-(His)$_6$-C.
<table>
<thead>
<tr>
<th>Cry proteins</th>
<th>Property of toxin</th>
<th>Mosquitoes</th>
<th>Toxicity LC$_{50}$, ng/mL (95% confidence limit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cry19Aa</td>
<td>Inclusion body Bt 4Q7</td>
<td><em>C. p. pipiens</em> (4$^{th}$ instar)</td>
<td>80.92 (53.77–144.20)</td>
</tr>
<tr>
<td>N1-(His)$_6$-Cry19Aa</td>
<td>Inclusion body Bt 4Q7</td>
<td><em>C. p. pipiens</em> (4$^{th}$ instar)</td>
<td>232.48 (155.35–363.49)</td>
</tr>
<tr>
<td>Cry19Aa-(His)$_6$-C</td>
<td>Inclusion body Bt 4Q7</td>
<td><em>C. p. pipiens</em> (4$^{th}$ instar)</td>
<td>74.45 (51.23–131.11)</td>
</tr>
<tr>
<td>None</td>
<td>Inclusion body Bt 4Q7</td>
<td><em>C. p. pipiens</em> (4$^{th}$ instar)</td>
<td>Not toxic at 1,000 ng/mL</td>
</tr>
</tbody>
</table>

Table 19. Bioassay of (His)$_6$-Cry19Aa to *C. p. pipiens* 4$^{th}$ instar larvae.

Figure 41. *C. p. pipiens* larvae 24 h after treatment with water (left) and with Cry19Aa (right). The picture was taken through dissecting microscope (X 10). The larva at left is alive. The larvae at the right are dead (above) or L-shaped and barely moving (bottom).
Figure 42. Expression of (His)$_6$ tag Cry19Aa. Ten μg each of protein was separated by SDS-PAGE. The gel was stained with Bio-safe Coomassie stain (Bio-Rad) (A). The proteins shown in panel (A) were transferred to a PVDF membrane and used to detect (His)$_6$-tag by western analysis (B). The primary antibody (Sigma) and goat anti-mouse antibody (Bio-Rad) was used at 1:3000 dilution. The proteins were detected using the chemiluminescense kit (Bio-Rad). The positions of molecular weight marker (precision plus protein dual color standards, red at 75 kDa and 25 kDa, Bio-Rad) were manually marked on the X-ray film by overlaying the film on the PVDF membrane. MW: molecular weight marker.
Figure 43. Purification of Cry19Aa fused with a (His)_6 tag at the N- or C-termini using the Talon cobalt resin (Clontech). N1-(His)_6-Cry19Aa and Cry19Aa-(His)_6-C were bound to the Talon resin and eluted with 150 mM imidazole. MW: molecular weight marker.
Figure 44. Western blot of diazirine-modified Cry19Aa and *C. p. pipientis* BBMV proteins probed with a SERCA antibody. *C. p. pipientis* BBMV proteins and diazirine-modified Cry19Aa or diazirine-modified Cry19Aa-(His)_6-C were mixed and illuminated with UV light, co-immunoprecipitated with Cry19Aa antibody and protein-A magnetic beads. After SDS-PAGE, proteins were transferred to a PVDF membrane and probed with SERCA antibody. SERCA positive control (lane 1) was exposed for 10 s, while the other lanes were exposed on a film for 1 min. The positions of molecular weight marker (precision plus protein dual color standards, red at 75 kDa and 25 kDa, Bio-Rad) were manually marked on the X-ray film by overlaying the film on the PVDF membrane. MW: molecular weight marker. Supe: supernatant.
Figure 45. Cross-linking diazirine-modified Cry19Aa-(His)<sub>6</sub>-C to proteins in C. p. pypiens BBMV.
Note the magnetic beads might have absorbed BBMV proteins. Protein bands were not visible in eluate and supe 2 fractions. MW: molecular weight marker. Supe: supernatant.
Figure 46. Cross-linking diazirine or LC-diazirine-modified Cry19Aa-(His)_6-C to detergent solubilized BBMV proteins. CHAPS solubilized BBMV proteins were cross-linked with diazirine or LC (long chain)-diazirine-modified Cry19Aa-(His)_6-C. A faint band visible on lane 3 and 7 might contain cross-linked proteins.
Figure 47. DNA sequence of SERCA from *C. p. pipiens*.
SERCA cDNA was cloned from RNA extracted from whole larvae of *C. p. pipiens*.
Figure 48. The sequence alignment of first 240 bases of SERCA from *C. p. pipiens* (P) and *C. p. quinquefasciatus* (Q) by BLAST (105). The cloned sequence of SERCA from *C. p. pipiens* has an 18-base insertion compared to the reported mRNA sequence from *C. p. quinquefasciatus* (NCBI reference sequence number XM_001868153.1). The results of the entire base alignment: Identity 2960/3006 bases (98 %), gap 18/3006 bases.
Figure 49. SERCA amino acid sequence from \textit{C. p. piciens}.

(A) SERCA amino acid sequence was obtained by translating DNA sequence (Figure 47) using EMBOSS transseq program (106). 109.8 kDa.

(B) Part of the sequence alignment of SERCA from \textit{C. p. piciens} (P) and \textit{C. p. quinquefasciatus} (Q) by BLAST (105). \textit{C. p. piciens} has a 6-amino acid insertion. The results of the entire alignment: Identities 988/1001 (99\%), gap 6/1001.
GenBank: EDS26297.1 (SERCA from \textit{C. p. quinquefasciatus}).

(C) Part of the sequence alignment of SERCA from \textit{C. p. piciens} (P) and \textit{A. aegypti} (Ae) by BLAST (105). Both \textit{C. p. piciens} and \textit{A. aegypti} have the same 6-amino acid insertion. The results of the entire alignment: Identities 937/1001(94\%), gap 2/1001.
GenBank: EAT41828.1 (SERCA from \textit{A. aegypti}).
CLUSTAL 2.1 multiple sequence alignment

1SU4rabbit      MEAAHSKSTEEAYFVGSSEGTGTLQPKQVRHLKYGHNLEPAEESKSLWELVIREQFDL 60
    cpipiens      MEQCHKTVECYGFVRFSDEKGLTQPKQVEKQYKGTPLAEQGKTGWQELQFDL 60
    **:**   :.**:**    :.**:**   :.**:**    :.**:**    :.**:**    :.**:**

1SU4rabbit      LVRILLALACISPFLAVAFGEETITAFEPVFVILLILANAYVQQRNANGAENAI 120
    cpipiens      LVRILLALACISPFLAVAFPLTFVQSTIAGVFVILLILANAYVQQRNANGAENAI 120
    **:**   :.**:**    :.**:**    :.**:**    :.**:**    :.**:**    :.**:**

1SU4rabbit      EYPEMKQVRADKSRQVQTARSDPVGDEVEVQKPADYRILRSNKKLSTTVQIQSIL 180
    cpipiens      EYPEMKQVRADKSRQVQTARSDPVGDEVEVQKPADYRILRSNKKLSTTVQIQSIL 180
    **:**   :.**:**    :.**:**    :.**:**    :.**:**    :.**:**    :.**:**

1SU4rabbit      TGESVROKHTEFVPSQPVQACQKNNFLSTGNSAGRAGLIVAVEGRHFTQRQOMA 240
    cpipiens      TGQSVROKHTEFVPSQPVQACQKNNFLSTGNSAGRAGLIVAVEGRHFTQRQOMA 240
    **:**   :.**:**    :.**:**    :.**:**    :.**:**    :.**:**    :.**:**

1SU4rabbit      ATEQ体会到QKLDEFGVLIKSVLISICAVAVLINVIGNHNDPVPVGSRIAGAAYFYKIAV 299
    cpipiens      ATEQ体会到QKLDEFGVLIKSVLISICAVAVLINVIGNHNDPVPVGSRIAGAAYFYKIAV 299
    **:**   :.**:**    :.**:**    :.**:**    :.**:**    :.**:**    :.**:**

1SU4rabbit      ALAANFAPEGLPAPVTICLALGTRRMAEKNAIVRSLPSVEVTICSTVSICSDKGTLTTNQ 360
    cpipiens      ALAANFAPEGLPAPVTICLALGTRRMAEKNAIVRSLPSVEVTICSTVSICSDKGTLTTNQ 360
    **:**   :.**:**    :.**:**    :.**:**    :.**:**    :.**:**    :.**:**

1SU4rabbit      MSVCKMFIDKVDGDFCSLNEFSITGSTYAPEGEVLKNDKPIRSGQFDGLVELATICALC 420
    cpipiens      MSVSRMFDKVDGDFCSSFLNEFSITGSTYAPEGEVLKNDKPIRSGQFDGLVELATICALC 420
    **:**   :.**:**    :.**:**    :.**:**    :.**:**    :.**:**    :.**:**

1SU4rabbit      KEFTLEFSRDRKSMVYCSPAKSSRAAVGNMVEAGPEVIRDNCVYVSTREMGP 540
    cpipiens      KEFTLEFSRDRKSMVYCSPAKSSRAAVGNMVEAGPEVIRDNCVYVSTREMGP 540
    **:**   :.**:**    :.**:**    :.**:**    :.**:**    :.**:**    :.**:**

1SU4rabbit      LGLPEALIPVQLLWVNLVTDGLPATALGFNPPDLDIMDRPPRSPKEPLISGWLFFRYMAT 840
    cpipiens      LGLPEALIPVQLLWVNLVTDGLPATALGFNPPDLDIMDRPPRSPKEPLISGWLFFRYMAT 840
    **:**   :.**:**    :.**:**    :.**:**    :.**:**    :.**:**    :.**:**

1SU4rabbit      GGYVGAATVGAAWWFMYAEDQPGVYQLTHFMQCTEDHPEEGLEDCEIFAPEPMTA 900
    cpipiens      GGYVGAATVGAAWWFMYAEDQPGVYQLTHFMQCTEDHPEEGLEDCEIFAPEPMTA 900
    **:**   :.**:**    :.**:**    :.**:**    :.**:**    :.**:**    :.**:**

1SU4rabbit      ALDLTQWLMVLKISLPVIGLDEILKFIARNYLEG-------- 994
    cpipiens      PLNAEEWITVMKFSLPVVLLDEILKLVARRISDEVTKKWE 1001
    **:**   :.**:**    :.**:**    :.**:**    :.**:**    :.**:**    :.**:**

Figure 50. Amino acid sequence alignment of SERCA from rabbit and C. p. pipiens.
Sequence was aligned using clustalW2 program (EMBL-EBI). The sequence of rabbit SERCA is from PDB1SU4 in Figure 36. The amino acid identity was 73 %.

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Figure 51. Immunoblot analysis to examine the expression of SERCA in Sf9 cells. (A) Western analysis to check for expression of SERCA. Lane 1 (kind gift from Dr. Sanjaya Sahoo, Dr. Periasamy’s laboratory). The primary anti-SERCA antibody was at 1:2,500. (B) Western analysis to check for expression of (His)$_6$-tag SERCA. (His)$_6$-YidC positive control, 61 kDa, lane 13, (kind gifts from Drs. Lu Zhu and Ross Dalbey). The primary anti-(His)$_6$ antibody (from Sigma) was used at 1:3,000. Secondary Goat-anti mouse HRP antibody was used at 1:3,000 dilution. The blot was detected using HRP chemiluminescence. These positive controls reacted with its antibody and were located at the expected sizes. SERCA-(His)$_6$-C, SERCA, and (His)$_6$-CAT were not detected. The positions of molecular weight markers (precision plus protein dual color standards, red at 75 kDa and 25 kDa, Bio-Rad) were manually marked on the X-ray film by overlaying the film on the PVDF membrane. MW: molecular weight. Transf.: transfection.
Figure 52. Transfection of DsRed into Sf9 cells.
pPac-PL-DsRed plasmid (kind gift from Mr. Sathiya Manivanann, Department of Molecular Genetics, OSU) was transfected into Sf9 cells using the cellfectin II reagent. The cells were fixed with 4 % (v/v) formaldehyde. The cells were stained with Hoechst 33342 (DNA stain) and observed with a confocal microscope. The transfection efficiency, based on expression of DsRed, was around 3 %. The number of the cells which expressed DsRed (24 cells) was divided by the total number of the cells (773 cells). This gave 3.1 %.
Figure 53. X-ray crystal structure of sodium/potassium ATPase.
Figure reproduced from (2). (A) X-ray crystal structure of sodium/potassium ATPase from shark. (B) α domain of sodium/potassium ATPase (yellow) is similar to SERCA (lime).
Figure 54. Ion transport mechanism of SERCA.
Figure reproduced from (13). 1. Two Ca\(^{2+}\) binds to SERCA from the cytoplasmic side. 2. ATP binds to the N (nucleotide binding)-domain. 3. Formation of an aspartyl phosphate intermediate at the P (phosphorylation)-domain. 4. The A (actuator)-domain rotates and Ca\(^{2+}\) is released to the lumen and two or three H\(^+\) binds from the lumen. 5. Dephosphorylation of the aspartyl phosphate. 6. H\(^+\) is transported to the cytoplasmic side.
References


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40. Berliner E (1915) Über die Schlaffsucht der Mehlmottenraupe (Ephestia Kühniella Zell.) und ihren Erreger Bacillus thuringiensis n. sp. *Zeitschrift für Angewandte Entomologie* 2:29-56.


105. The National Center for Biotechnology Information (NCBI) (accessed on March16 2013) BLAST (basic local alignment search

Appendix A: Reprint of the paper, Yoshio Ikeda and E. J. Behrman “Improved Synthesis of Photo-leucine”.

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Improved Synthesis of Photo-leucine

Yoshio Ikeda and E. J. Behrman
Ohio State Biochemistry Program and the Department of Biochemistry,
Ohio State University, Columbus, Ohio, USA

Abstract: An improved and reproducible synthesis of photo-leucine [3-(3-methyl-3-diazirinyl)-alanine] is presented.

Keywords: Carbene; Diazirine; Photo-cross-linking reagent; Photo-leucine

INTRODUCTION

Photo-leucine [3-(3-methyl-3-diazirinyl)-alanine] (7), shown in Scheme 1, is a leucine analog with two useful properties: (a) it can be incorporated randomly into proteins in place of leucine by the ribosomal machinery, and (b) it can be decomposed photolytically by irradiation at 350 nm to generate a carbene leading to cross-linking with molecules within a range of a few Ångstroms, thereby providing a valuable tool for structural analysis.\textsuperscript{[1-3]} The lifetime of this carbene is not known, but related structures have lifetimes in the nanosecond range.\textsuperscript{[4]}

Outlines of its synthesis have been published\textsuperscript{[3,5]} but our attempts to follow the literature descriptions were hampered by a lack of detail. We give here a reproducible and detailed procedure for the synthesis of this important molecule. This procedure should be applicable with little modification to the synthesis of other alpha-amino acids that contain the diazirine functional group (Scheme 1).

Syntheses have been reported very recently of two photo-amino acids, both of which avoid the low yield resolution step used here.

Received in the USA March 23, 2008
Address correspondence to E. J. Behrman, Department of Biochemistry,
Ohio State University, 484 West 12th Avenue, Columbus, OH, 43210. E-mail: behrman.1@osu.edu
Improved Synthesis of Photo-leucine

\[
\text{Scheme 1. Synthesis of photo-leucine.}
\]

Vila-Perelló et al.\textsuperscript{[6]} synthesized photo-methionine and MacKinnon et al.\textsuperscript{[7]} reported a procedure for Boc-photo-leucine.

**EXPERIMENTAL**

All reagents and solvents were of commercial grade and were used without further purification. IR spectra were taken on a Nicolet Impact model 410.

3-(3-Methyl-3-diazirinyl)propanoic Acid (2) (CA 25055-86-1)

This procedure is expanded and modified from Ref. 8 and 9.

Levulinic acid (1) (Aldrich) (7.02 g, 59.2 mmol), dissolved in 450 mL of methanol, was put in a 1-L, three-necked, round-bottomed flask in a hood. The flask was insulated with sand contained in an ice bucket. Liquid ammonia was introduced to the flask by cooling ammonia gas with an ethanol/dry ice condenser attached to the side neck of the flask. The other necks were open to the air. The solution was mixed with a magnetic stirrer, while liquid ammonia was added until the volume of the mixture increased by about 20%. Then hydroxylamine-O-sulfonic acid (Aldrich) (11.79 g, 101.1 mmol, 1.7 eq) was added in 10 portions over 1 h. A white precipitate of ammonium sulfate formed gradually. Liquid ammonia was continually added to maintain the volume. The suspension was stirred for an additional 3 h. Then the addition of ammonia was stopped, and the sand bath was removed. The suspension was stirred for 18 h at room temperature to evaporate the excess ammonia.

The reaction mixture was filtered on a Büchner funnel and then by gravity to remove the ammonium sulfate, which was washed with methanol. The solvent was removed using a rotary evaporator. The residue was dissolved in methanol (30 mL) and cooled to 5°C, and then triethylamine (11.4 mL, 81.4 mmol, 1.4 eq) was added. The mixture was stirred for 15 min at room temperature. The solvent was again removed on a rotary evaporator. This step converts the ammonium salt of the product to the
triethylamine salt. If ammonia remains in the mixture, explosive nitrogen triiodide will be formed in the next step.[8]

After evaporation, no smell of ammonia was detected. The residue was dissolved in methanol (300 mL) and cooled to 5°C. Triethylamine (60 mL, 428 mmol, 7.2 eq) was added. Solid iodine was added with stirring at a rate of about 0.5–1 g/min until the red color of iodine remained. A total of 15.03 g (59.2 mmol, 1.0 eq) of iodine was added. The mixture was concentrated by rotary evaporation. The time between adding the last portion of iodine and evaporation was about 40 min. Both shorter and longer times gave lower yields.

The residue was partitioned between 100 mL of CH₂Cl₂ and 50 mL of 3% Na₂CO₃. Changing the solvent from dichloromethane to diethyl ether did not remove iodine completely in the first extraction. The organic layer was again extracted with 2 × 50 mL of 3% Na₂CO₃. The combined water layers were washed with 2 × 50 mL of CH₂Cl₂. The water layer was acidified to pH < 2 with concentrated HCl on ice. The acidified water layer was extracted with 3 × 50 mL of CH₂Cl₂. The combined organic layers containing 2 and iodine were washed with 100 mL of water and then 100 mL 20% NaCl. The organic layer was dried with magnesium sulfate. Evaporation of the organic layer gave 6.14 g of a red oil. The product was at least 93% pure by proton NMR. The yield was about 50%. Triethylamine and some iodine were removed in this extraction.

To remove the remaining iodine, the red oil was partitioned between 50 mL of diethyl ether and 25 mL of 3% Na₂CO₃. The time between the first extraction and second extraction is important, as the remaining iodide ion and the product can reform iodine. The organic layer was again extracted with 2 × 25 mL of 3% Na₂CO₃. The combined water layers were washed with 2 × 25 mL of diethyl ether. The water layer was acidified to pH < 2 with concentrated HCl on ice. The acidified water layer was extracted with 3 × 25 mL of CH₂Cl₂. The combined organic layers were washed with 50 mL of water and then 50 mL of 20% NaCl. The absence of iodine was checked with starch. The organic layer was dried with magnesium sulfate. Evaporation of the organic layer gave 3.14 g (24.5 mmol) of 3-(3-methyl-3-diazirinyl)propanoic acid (2) as a yellow oil in 33–41% yield.

¹H-NMR (CDCl₃, TMS) (600 MHz), δ 1.05 (3 × H-5, s), 1.73 (2 × H-3, t, J = 7.7 Hz), 2.25 (2 × H-2, t, J = 7.7 Hz) and 11.55 ppm (1 × COOH, broad). ¹³C-NMR (CDCl₃, TMS) (150 MHz), δ 19.47 (methyl), 24.92 (C-4), 28.42 (C-2), 29.18 (C-3), and 178.8 ppm (COOH). These values correspond closely to those of Refs. 8 and 10. IR (neat) νmax 836, 932, 1068, 1226, 1292, 1387, 1417, 1440, 1586 (diazirine), 1713, 2300–3600 (broad) cm⁻¹. UV: λmax = 347 nm (ε = 62 M⁻¹ cm⁻¹), 363 nm (ε = 50 M⁻¹ cm⁻¹) in methanol. Compare with Refs. 8 and 10.
Improved Synthesis of Photo-leucine

2-Bromo-3-(3-methyl-3-diazirinyl)propanoic Acid (4) (CA 851960-79-7)

This procedure is expanded and modified from Refs. 3 and 11.

3-(3-Methyl-3-diazirinyl)propanoic acid (2) (2.56 g, 20 mmol) in 2.0 mL of CCl₄ was put into a 50-mL round-bottomed flask. A water-cooled condenser equipped with a drying tube was attached to the flask. Thionyl chloride (9.47 g, 5.81 mL, 79 mmol, 3.95 eq) was added in a hood. The reaction mixture was stirred in a 65°C water bath for 30 minutes. HCl and SO₂ were evolved. The reaction mixture was cooled to room temperature. The completion of chlorination was confirmed by the IR spectrum in which the carbonyl group of 2 at 1713 cm⁻¹ disappeared and a new absorption of the acid chloride (3) appeared at 1796 cm⁻¹ [IR spectrum of the reaction mixture: IR (neat) νmax 689, 765, 788, 909, 961, 1036, 1232, 1285, 1340, 1388, 1407, 1447, 1582 and 1586 (diazirine), 1796 (carbonyl), 2866, 2927, 2960 cm⁻¹].

Finely ground N-bromosuccinimide (4.31 g, 24 mmol, 1.2 eq) was added to the reaction mixture. Then 10 mL of CCl₄ was added, followed by two drops of 48% aqueous HBr. The reaction mixture was stirred and heated in a 70°C water bath for 10 minutes. A red-orange suspension formed.

The reaction mixture was then heated to 80°C until the reaction mixture became light yellow (around 1.5 h). The color of the reaction mixture changed successively from red orange to light orange, yellow orange, yellow, to light yellow. After that, the color of the reaction mixture became darker yellow. It is important to remove the reaction mixture from the water bath before the color of the reaction mixture changes to dark brown to achieve a good yield. Di-bromination products increased with time. When the reaction mixture was kept at 85°C for 4 h, the color of the reaction mixture became dark brown. The carbonyl peak of di-bromo product around 1814 cm⁻¹ became larger. IR (neat) νmax 738, 829, 914, 1056, 1092, 1183, 1222, 1236, 1286, 1363, 1387, 1426, 1588 (diazirine), 1714 (carbonyl of 2), 1739 (carbonyl of 4), 1814 (carbonyl of dibromo product), 2350-3600 (broad) cm⁻¹.

The reaction mixture was transferred to a 100-mL round-bottomed flask and rinsed with CCl₄. Bromine, SOCl₂, and CCl₄ were removed by rotary evaporation. A yellow-white solid and an orange liquid remained. The mixture was filtered on a Büchner filter and then by gravity. Both filtrates were washed with CCl₄. The CCl₄-soluble material was collected. The filtrate was evaporated, yielding 5.40 g of a brown oil (crude α-Br 3).

Crude α-Br 3 (5.40 g) was put into a 250 mL three-necked flask. The amount of acetone and 1 M NaHCO₃ was calculated as if the bromination reaction had proceeded with 100% yield (20 mmol). Acetone (38 mL) was added to the flask, which was cooled to around 10°C. Then
48 mL of 1 M NaHCO₃ was added to the reaction mixture dropwise over 45 min to form a brown suspension.

The reaction mixture was acidified to pH < 2 with concentrated HCl on ice. The water layer was extracted with 3 × 15 mL of CH₂Cl₂. The combined organic layers were washed with 50 mL of water and then 50 mL of 20% NaCl. The organic layer was dried with magnesium sulfate. Evaporation of the organic layer gave a brown oil (3.19 g).

There were two carbonyl peaks in the IR spectrum at 1717 (starting material, 2), and 1741 cm⁻¹ (mono-bromo product, 4). The intensities of the carbonyl absorptions of 2 and 4 were about equal. The carbonyl absorption of the dibromo product at 1814 cm⁻¹ was very small, implying a yield of about 50%.

Attempted purification on a silica column resulted in a large loss of the product 4, so the crude mixture was used in the next amination reaction without further purification, relying on the greater reactivity of 4 than 2 with ammonia.

IR (neat) ν max 739, 830, 913, 1068, 1092, 1159, 1222, 1237, 1269, 1365, 1388, 1428, 1588 (diazirine), 1717 (carbonyl of 2), 1741 (carbonyl of 4), 2350–3600 (broad) cm⁻¹.

3-(3-Methyl-3-diazirinyl)-DL-alanine (5) (CA 851960-84-4)

This procedure is expanded and modified from Ref. 3.

Impure 2-bromo-3-(3-methyl-3-diazirinyl)propanoic acid 4 (1.24 g) obtained from the previous step, was divided between two 38-mL-capacity pressure tubes (Ace Glass) equipped with silicone O-rings. FEPFE O-rings (fluoroelastomer) melted during the reaction. Ammonia-saturated methanol (made by adding NH₃ gas to methanol at 4°C) (16.7 mL) was put into each tube on ice in a hood. Aqueous 29% NH₄OH (3.3 mL) was added at 5°C. The screw caps of the pressure tubes were tightened. The tubes were then placed in a water bath behind an explosion protection shield and heated in the water bath at 55°C for 5 days. The thermal stability of photo-leucine is not known but analogous structures have large Ea values in the 30 kcal/mol range. The water bath was cooled to 5°C. The caps were opened on ice behind the explosion protection shield in the hood. The tubes were left in the hood overnight to evaporate the NH₃. The brown solution was put into a 100-mL round-bottomed flask and dried by rotary evaporation to give 1.67 g of crude 5 as a brown solid.

A cation exchange column (15 g of Dowex 50 W × 8–200, H⁺ form) was used for purification. The cation exchange column was prepared by adding 100 mL of 3 N HCl and then washing with distilled H₂O (around
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250 mL) until the pH of the eluate from the column became neutral. Crude 5 (1.67 g) was dissolved in a few milliliters of distilled H₂O. The solution was acidified to pH < 2 with concentrated HCl on ice. The sample was applied to the column. The column was washed with 10 bed volumes (10 × 30 mL = 300 mL) of distilled H₂O. The sample was eluted with 2 N NH₄OH (about 200 mL) in 15 mL portions. Each fraction was checked by silica thin-layer chromatography (TLC, water–methanol–acetonitrile = 1:1:4). DL-photo-leucine (5) was detected with a ninhydrin reagent containing s-collidine. The recipe for the ninhydrin reagent containing collidine is 50 mL of 0.1% ninhydrin in ethanol, 2 mL of s-collidine [2,4,6-trimethylpyridine], 15 mL of acetic acid. Fractions 21–26, showing green-brown spots on TLC, were pooled and placed in a 250 mL round-bottomed flask after filtration by gravity. The solution was dried by rotary evaporation. The residue was put into a 100 mL round-bottomed flask with several milliliters of distilled H₂O. The solution was again dried by rotary evaporation and then by using P₂O₅ under vacuum overnight. The yield was 0.42 g of DL-photo-leucine (5) (2.9 mmol) as a brown solid, 37% yield from [3-(3-methyl-3-diazirinyl)-propanoic acid] (2) or 15% overall from 1. The purity of DL-photo-leucine [3-(3-methyl-3-diazirinyl)-DL-alanine] (5) was more than 90% as estimated from the ¹H NMR spectrum.

¹H-NMR (D₂O, HOD) (800 MHz), δ 1.07 (3 × H-5, s), 1.72 (1 × H-3, dd, J = 8.2 Hz, 15.6 Hz), 2.04 (1 × H-3', dd, J = 5.5 Hz, 15.6 Hz) and 3.69 ppm (1 × H-2, dd, J = 5.5 Hz, 8.1 Hz). ¹³C-NMR (D₂O) (150 MHz), δ 18.40 (methyl), 23.71 (C-4), 36.05 (C-3), 51.01 (C-2), and 173.63 ppm (COOH). Compare with Ref. 3. IR (nujol) ν max, 856, 944, 971, 996, 1034, 1099, 1139, 1191, 1224, 1271, 1323, 1358, 1416 (carboxyl), 1511 (carboxyl), 1583 (diazirine), 1632, 2161 (characteristic peak for amino acid), 2300–3700 (broad, NH₃⁺) cm⁻¹

N-Acetyl-3-(3-methyl-3-diazirinyl)-DL-alanine (6) (CA 851960-88-8)

This procedure is expanded and modified from Ref. 3.

3-(3-Methyl-3-diazirinyl)-DL-alanine (5) (0.20 g, 1.4 mmol) was dissolved in 5.5 mL of 1 N NaOH at 5°C in a 50-mL round-bottomed flask in a hood to form a brown solution. Ice (about 4 g) was added in the flask. Acetic anhydride (0.39 mL, 4.1 mmol, 2.9 eq) was added dropwise to the mixture at 5°C. The reaction mixture was shaken vigorously for 45 min at 5°C.

The reaction mixture was acidified to pH < 2 with concentrated HCl on ice. The acidified water layer was extracted with 3×20 mL of ethyl acetate. The combined organic layers were washed with 40 mL of water
and then 40 mL of 20% NaCl. The organic layer was dried with magnesium sulfate. Evaporation of the organic layer gave a yellow oil and white solid, 0.13 g (0.7 mmol), 50% yield.

The aqueous fractions containing recovered 5 were again acetylated to yield an additional 0.07 g (0.38 mmol) of 6. The combined yield of \( N \)-acetyl-3-(3-methyl-3-diazipyrinyl)-DL-alanine (6) was 78%. Its purity was more than 97% as estimated from the \( ^1H \) NMR spectrum.

\( ^1H \)-NMR (D\(_2\)O, HOD) (600 MHz), \( \delta = 1.00 \) (3 × H-5, s), 1.62 (1 × H-3, dd, \( J = 4.9 \text{ Hz}, 15.2 \text{ Hz} \)), 2.01 (3 × acetyl H, s), 2.05 (1 × H-3', dd, \( J = 4.6 \text{ Hz}, 15.2 \text{ Hz} \)) and 4.25 ppm (1 × H-2, dd, \( J = 4.5 \text{ Hz}, 10.2 \text{ Hz} \)). IR (neat) \( \nu_{\text{max}} \) 795, 982, 1046, 1164, 1242, 1318, 1381, 1436 (CONH), 1557 (diazipyrine), 1622, 1694 (carbonyl), 1929, 2350-3079 (broad), 3345 (NH) cm\(^{-1}\).

3-(3-Methyl-3-diazipyrinyl)-L-alanine (7) (CA 851960-91-3)

This procedure is expanded and modified from Ref. 3.

\( N \)-Acetyl-3-(3-methyl-3-diazipyrinyl)-DL-alanine (6) (0.20 g, 1.1 mmol) was dissolved in 21.8 mL of distilled water in a 50-mL round-bottomed flask to form a yellow solution. The pH was adjusted with 29% NH\(_4\)OH to pH 7.5. Porcine kidney acylase I (Sigma, grade II) (35.3 mg) was added to the reaction mixture to form a opalescent yellow solution. Porcine kidney acylase I is reported to be sensitive to autoxidation with a half-life of 36 h in air\textsuperscript{[13]} However, we find that reaction under nitrogen did not improve the yield. The reaction mixture was kept at 30°C in a water bath. Reaction reached completion in one h. Rates of deacetylation of \( N \)-acetyl-L-amino acids by porcine kidney acylase I vary over a 250-fold range depending on the nature of the R-group.\textsuperscript{[13,14]} Photo-leucine is evidently a poor substrate. Additional acylase I (9.0 mg) was added to the reaction mixture. When the reaction was followed by the ninhydrin colorimetric method described in Refs. 13 and 15, the reaction conditions were varied slightly. The reaction conditions were varied slightly. 2 M K\(_2\)CO\(_3\) was used to adjust the pH to 7.5 instead of NH\(_4\)OH to avoid interference with ninhydrin. The reaction was carried out at 38°C instead of 30°C. Aliquots of 0.2 mL were assayed. The resulting violet color was read with a Klett-Summerson photoelectric colorimeter using a 560-nm filter.

The reaction mixture was acidified to pH < 2 with concentrated HCl on ice. The water layer was extracted with 3 × 20 mL diethyl ether. The combined organic layers containing \( N \)-acetyl-3-(3-methyl-3-diazipyrinyl)-D-alanine were saved. The acidified water layer containing 7 was filtered on a Büchner funnel and then by gravity, and then it was evaporated to give 0.14 g of a white solid and yellow liquid.
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Purification was identical to that described for racemic compound. The yield was 0.03 g of L-photo-leucine (7) (0.21 mmol) as an off-white solid, 19% yield.

IR (nujol) \( \nu_{\text{max}} \)

- 852, 1112, 1192, 1324, 1361, 1411 (carboxyl), 1519 (carboxyl), 1584 (diazirine), 1604, 2350–3500 (broad, \( \text{NH}_2^+ \)) cm\(^{-1}\).

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