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FROM BIOASSAYS TO *DROSOPHILA* GENETICS: STRATEGIES FOR CHARACTERIZING AN ESSENTIAL INSECT NEUROHORMONE, BURSICON*

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We describe the molecular analysis and cellular expression of the insect peptide neurohormone, bursicon. Bursicon triggers the sclerotization of the soft insect cuticle after ecdysis. Using protein elution analyses from SDS gels, we determined the molecular weight of bursicon from different insects to be approximately 30 kDa. Four partial peptide sequences of *Periplaneta americana* bursicon were obtained from purified nerve cord homogenates separated on two-dimensional gels. Antibodies produced against one of the sequences identified the cellular location of bursicon in different insects and showed that bursicon is co-produced with crustacean cardioactive peptide (CCAP) in the same neurons in all insects tested so far. Additionally, using the partial peptide sequences, we successfully searched the *Drosophila* genome project for the gene encoding bursicon. With *Drosophila* as a tool, we can now verify the function of the sequence using transgenic flies. Sequence comparisons also allowed us to verify that bursicon is conserved, corroborating the older data from bioassays and immunohistochemical analyses. The sequence of bursicon will enable further analysis of its function, release, and evolution.

Keywords: Insect cuticle – neuropeptide – protein purification – ecdysis – immunocytochemistry

INTRODUCTION

The majority of insect neuropeptides modulate neuronal and behavioral functions. This article profiles the neuropeptide, bursicon, which is involved in regulating biochemical events during cuticle hardening. Bursicon is released from a small subpopulation of neurons (see Results) into the hemolymph and triggers the sclerotization of the newly formed cuticle after an insect has shed its old one at the end of the molting cycle. We have indications that it also fulfills an identical function in crustaceans. The mechanism as to how this triggering process works is not yet known, but we will show in this article that the progress in characterizing bursicon since its discovery in 1962 by Fraenkel promises that this question will be resolved in the near future.

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Bursicon is absolutely necessary for insect survival. We have stressed in earlier publications that an interruption in the action of bursicon results in a soft outer cuticle. Cuticular hardening not only provides protection for the insect, but also a framework for muscle attachment and the ability for the insect to walk, crawl and fly. Interference with bursicon release or its actions will, therefore, likely result in the death of the animal. In addition, the sequential timing of cuticular-related events needs to be incredibly accurate. For example, if the new cuticle hardens before the old one is completely removed, it will be trapped within the confines of a cuticle too small and, therefore, perish. If its wings are not fully inflated before its wing cuticle hardens, it will be left with crumpled wings and incapable of flight.

Bursicon has resisted its characterization for many years. Fraenkel and Hsiao [7] and Cotrell [2] showed in parallel using a bioassay (the ligated fly bioassay using the blowfly *Sarcophaga bullata*) that if the hemolymph of freshly ecdysed flies is injected into the hemocoel of other flies that have been ligated at the neck and therefore remain white and soft, this will lead to the tanning (sclerotization and melanization) of their cuticle. They concluded that it must contain a factor, bursicon (coined by Fraenkel [6]), which triggers tanning. Fraenkel and Hsiao [7] also showed that this factor is different from ecdyson, and Fraenkel et al. [9] showed later by protein digestion that bursicon must be a peptide. Fraenkel and Hsiao [8] also showed that homogenates of the CNS of different insects injected into ligated flies lead to tanning, supporting the hypothesis that both the receptor for bursicon and the peptide may be conserved among different insects.

Several early attempts to purify bursicon failed. The only information known until recently was an estimate of its molecular size. In *Sarcophaga bullata* (blowfly), *Periplaneta americana* (cockroach), and *Manduca sexta* (moth), its molecular size was estimated by several groups to be between 20 kDa and 60 kDa [9, 20, 22, 25]. In this article we give an overview of our efforts and successes in using protein biochemistry, protein microsequencing, antibody production, molecular biology, and *Drosophila* genetics to finally characterize the elusive bursicon.

MATERIAL AND METHODS

The methods used to yield the results as described here have been elaborated in detail in our earlier publications about bursicon. The following procedures have been used:

1. Ligated fly bioassay [12, 13, 15, 18]
2. Protein purification [17, 19]
3. Elution of bioactive bursicon from SDS-polyacrylamide gels [15, 18, 19]
4. Two-dimensional (2-D) SDS polyacrylamide gel electrophoresis (PAGE) and Western blotting [18, 19]
5. Protein microsequencing [14]
6. Immunocytochemistry [14, 19]
7. *In situ* hybridization (Dewey et al., in preparation)

RESULTS

Bursicon of meal beetle larvae *(Tenebrio molitor)* was first purified to homogeneity by protein purification methods and high performance liquid chromatography (HPLC) technology [15]. *T. molitor* larvae are available commercially by the kg, and large amounts of raw material are necessary for the purification of a rare protein. After a 108,000X purification two protein bands in SDS gels remained, and only the approximately 30 kDa band contained bursicon activity. Bursicon activity was tested in this and all other experiments using the ligated fly bioassay. The elution of bursicon from the SDS gel required reestablishing the bioactivity of the eluted bursicon by separating the protein from SDS through (trichloroacetic acid) TCA precipitation, resuspension in saturated urea, and dilution of the urea solution in phosphate buffer, which allowed the protein to renature. This procedure was a critical step in the protein purification scheme [15, 17, 19].

Determination of the molecular mass of bursicon

The gel elution procedure allowed us to identify the molecular mass of bursicon in insects other than *T. molitor*, i.e. from *Calliphora erythrocephala*, *Gryllus bimaculatus*, *Locusta migratoria*, *P. americana* and *M. sexta* [17]. The ventral nervous system of these insects was dissected and homogenized, and then bursicon was partially purified and run on an SDS gel. The gel was cut into slices and aligned with prestained marker proteins. Each gel piece was subjected to protein elution. From the first four insects only the gel pieces comprising a molecular weight between 28–32 kDa contained bursicon activity. These results supported the bioassay data from Fraenkel and Hsiao [8] that bursicon of distantly related insects may be a similar protein. These data do not allow, however, for the prediction of similarity in the amino acid sequence of bursicon in these species. There were sufficient differences between the cricket and cockroach bursicon, e.g. to predict sequence differences in these two species (see Discussion in [17]). Surprisingly, we were unable to show the existence of *M. sexta* bursicon in the ligated fly bioassay. This is evidence that the sequence of this species may be appreciably different from that of the other species tested. Another outcome from these experiments was that the nervous system of *P. americana* seemed to contain far more bursicon than that of the other species. Dissecting nervous systems for homogenization instead of homogenizing whole animals comprises thus, an at least 160X purification step, avoiding the application of a cocktail of poisonous protease inhibitors to suppress the activity of gut proteases during homogenization of whole animals. For our further work we therefore acquired cockroaches from S.C. Johnson & Son Inc., Racine WI, USA by the thousands free of charge, froze them, and dissected the nervous systems from thawed animals.

First microsequencing attempts and detection of Cu, Zn superoxide dismutase

Since bursicon activity seemed to comigrate in SDS gels with an approximately 30 kDa protein band, homogenates of 450 cockroach ventral nerve cords were purified approximately 43,000X using HPLC technology and run on one SDS gel. The 30 kDa protein band was excised and microsequenced [19]. Four partial peptide sequences containing 10–16 amino acids were identified. All four could be aligned with Cu, Zn superoxide dismutase (SOD) of *Drosophila virilis* with an identity of 60%, 75%, 90% and 92% (Swiss protein base).

It seemed unlikely to us that bursicon could be identical to SOD, an enzyme that catalyzes the removal of superoxides, which are byproducts of cell metabolism [10]. Could SOD, a 32 kDa protein, have been co-purified with bursicon and inadvertently sequenced because of its high abundance? To test this hypothesis we ran two-dimensional gels in the hope of separating the two proteins due to differences in their isoelectric points.

Separation of bursicon and SOD by 2-D gel analysis

Two-dimensional SDS-PAGE combined with elution experiments helped to identify the unique protein spots containing bursicon activity. To determine whether the SOD spots in 2-D gels were identical to the bursicon spots, Western blot analyses using anti-SOD antibodies were performed. Since antibodies against an insect SOD were not available, we produced polyclonal antibodies against two partial SOD sequences in rabbits and tested their specificity for SOD of *P. americana* [19]. As Figs 1 and 2 show, this analysis provided proof that bursicon appeared in four electrophoretic forms between pH 5.3–5.9 and that these spots were not identical to those of SOD. Bursicon activity could only be eluted from gel slices in this pH region (Fig. 1). The SOD spots as detected by Western blotting using the anti-SOD antisera (Fig. 2B) appeared at higher pH values, close to pH 7 and as approximately 15 kDa and 30 kDa monomeric and dimeric forms, respectively.

Protein blotting in combination with a glycosylation assay (Biorad) indicated, in addition, that bursicon may not be glycosylated (Fig. 2C). However, the abundance of protein in the bursicon spots may have been too low for the detection of glycosylation in this assay. The monomeric form of SOD, however, seemed to be weakly glycosylated, similar to other proteins that seemed to be in low abundance as the silver-stained gel in Fig. 2A suggests.

All 2-D gels for the detection of bursicon were run under non-reducing conditions, because our earlier studies showed that bursicon activity was highly reduced if one-dimensional SDS gels were run under reducing conditions, i.e. if 2-mercaptoethanol was used in the sample buffer [15]. If 2-D gels were run under reducing conditions in the second dimension, no bursicon spots could be detected in the 30 kDa region after silver staining. Instead, spots at about 15 kDa appeared at isoelectric points

identical to the bursicon active spots at 30 kDa (Fig. 3A, B). This experiment suggested that bursicon must be a dimer.

Partial peptide sequences of bursicon

The production of antisera against *P. americana* SOD enabled us to identify the unique bursicon spots in 2-D gels and provided the basis for finally purifying bursi-

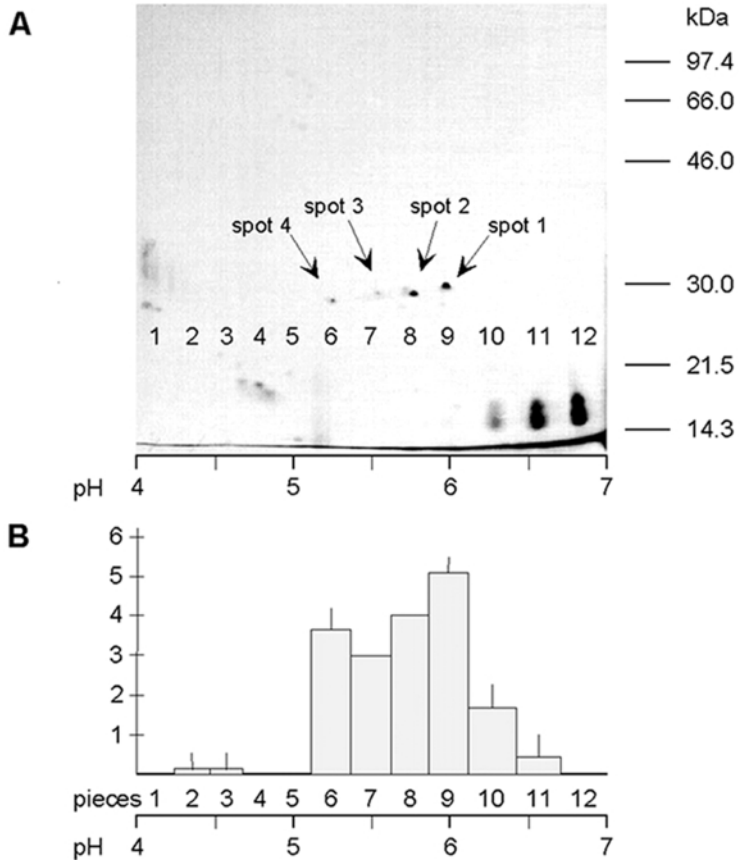

Fig. 1. Two-dimensional SDS PAGE of bursicon active *P. americana* purified nerve cord homogenate run under non-reducing conditions. *A:* A silver-stained gel shows four protein spots (spot 1–4 from left) at approximately 30 kDa. *B:* Histogram denoting bursicon activity as tested in the ligated fly bioassay. Pieces 1–12 were cut in the molecular weight range between 28–32 kDa from a copper stained two-dimensional gel run in parallel with the gel shown in A. The widths of the columns show the pH range of each piece. Proteins were eluted from each piece and tested for bursicon activity. Pieces cut from the region that contained the three large spots at 15 kDa showed no activity, i.e. a score similar to buffer controls (score = 0.2)

con. The bursicon spots with the highest staining intensity (using Coomassie-brilliant blue as a stain) were excised, and the spots from seven gels were pooled for microsequencing. We dissected 2825 ventral nerve cords of *P. americana*. The protein from

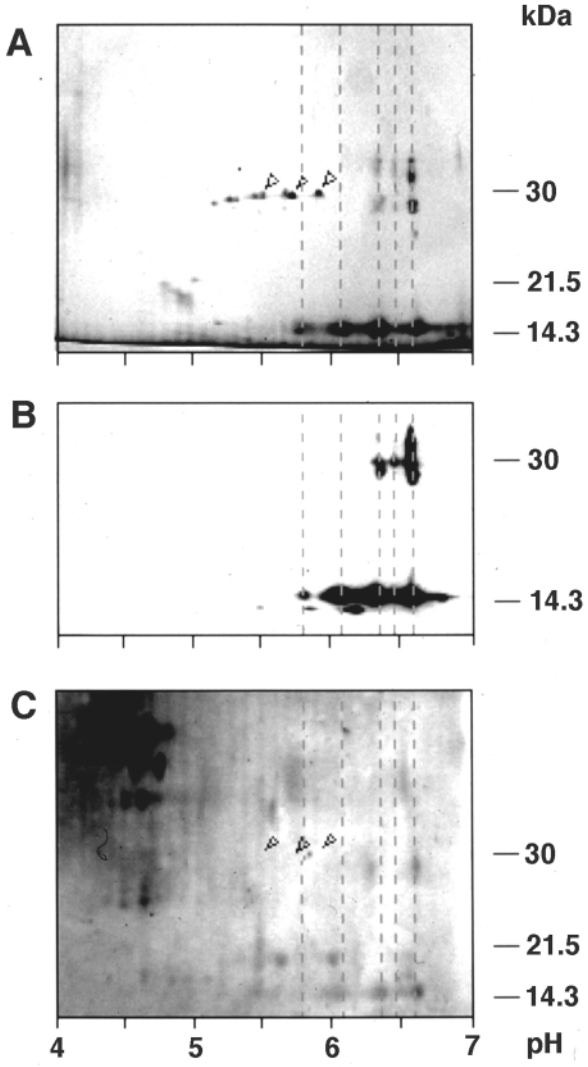

Fig. 2. Separation of bursicon and SOD in two-dimensional gels and blots. *A:* Silver stained gel as in Fig. 1. Arrowheads point to bursicon active spots 1–3 of Fig. 1A. *B:* Western blot of another two-dimensional gel using an antiserum against *Drosophila virilis* SOD. The antiserum detects only the predicted forms of SOD at 15 kDa and above and below 30 kDa between pH 6.4–6.6. The bursicon active spots were not detected. *C:* Glycosylation assay on another protein blot using an immun blot kit for Glycoprotein detection (Bio-Rad). The bursicon active spots (arrowheads) were not labeled, indicating that bursicon is not glycosylated

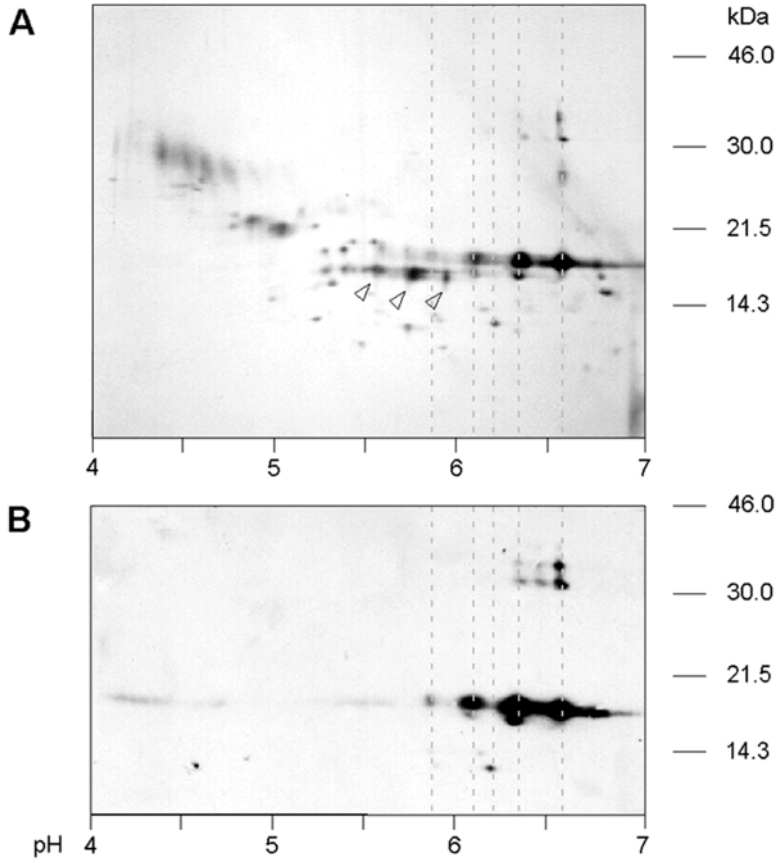

Fig. 3. Two-dimensional SDSPAGE of bursicon active nerve cord homogenates run under reducing conditions. *A:* In the silver-stained gel the bursicon spots at 30 kDa have disappeared. Instead, spots appear now at about 15 kDa at pH positions predicted for the bursicon spots. *B:* A western blot of a gel also run under reducing conditions and incubated with the anti-SOD antiserum show only labeling of the different forms of SOD

bursicon spot #2 (pH 5.7/30 kDa; see Fig. 1) was microsequenced by the Harvard Microchemistry Laboratory. The amino acid sequence of five peptides was obtained (Table 1).

Cellular expression of bursicon in different insects

Immunizing and boosting two rabbits produced antibodies against the sequence GT27 (Table 1). After testing the antisera for their specificity using an ELISA, the serum of one rabbit (S115) was used for immunocytochemistry. We had shown ear-

lier that a pair of anterior bilateral cells in the thoracic ganglia of cricket *G. bimaculatus* is immunoreactive to crustacean cardioactive peptide (CCAP). These cells are homologues to the CCAP-immunoreactive (ir) neurons in other insects [3, 4]. These neurons display a Tyndall effect in older animals and contain bursicon as shown by single cell bioassays for bursicon [18]. In *P. americana* CCAP-ir cells appeared in an

Table 1
Partial amino acid sequences of bursicon

HPLC peak	Sequence	Confidence
GT21	LTQEGQASMEVK	high
GT27	DGSSYLQVSGSK	reasonable for G in 2nd position and S in 4th position, otherwise high confidence
GT15, GT33	IWQMER	high
GT51	EASVNNV	low

Designations of the sequences are the numbers of peptide peaks eluted from a reverse-phase HPLC column after trypsin digestion

identical position in the thoracic ganglia to that of crickets. In the abdominal ganglia 2–4 large lateral CCAP-ir somata could also be identified. The single cell bioassay showed that they also contained bursicon [14]. The antiserum S115 exclusively labeled the neurons that were also CCAP-ir in *G. bimaculatus*, *T. commodus* (an Australian cricket), and *P. americana*. This was shown by parallel incubation of adjacent 12-µm paraplast sections with either anti-CCAP antiserum or S115 (Fig. 4). Furthermore, neurons in *M. sexta* that were identified as containing bursicon by Taghert and Truman [26] were also labeled [14]. These results support the hypothesis that the sequence GT27 is a partial bursicon sequence and must share some homology between the four insect species. The S115 antisera also labeled a few neurons in the brain of the three species. These cells were distinct from other CCAP-ir somata. A similar result was obtained in *D. melanogaster*. In the central nervous system of the third larval instar, a cluster of 3–4 cells was labeled in each brain hemisphere. Further tests need to be carried out to show whether bursicon is detected in those neurons, because they could not be tested for bursicon activity.

The use of the partial peptide sequences of P. americana *to reveal the gene encoding bursicon*

In this paragraph we will report in short about our progress in discovering the gene encoding bursicon. A detailed report is in progress. The four partial peptide sequences from *P. americana* (Table 1) were used in a protein BLAST search against

the *D. melanogaster* genome database. The sequence *CG13419*, which encodes a protein with a high similarity to these peptide sequences, was identified. Sequences GT15/33, GT27 and GT51 are 100%, 67% and 60% identical, respectively, and GT21 is 50% similar to the deduced amino acid sequence of *D. melanogaster*. The properties of the deduced protein with an approximately 15 kDa molecular weight indicate that we found the sequence of the bursicon monomer. Our sequence information has been used to show that an *Anopheles gambiae* partial nucleotide sequence has a high similarity to CG13419 [24], supporting earlier findings from bioassays [8] and immunocytochemistry ([14]; using the antiserum S115 against the partial sequence GT27) that bursicon is conserved among insects. Experiments using transgenic *D. melanogaster* support our conclusions that the *CG13419* gene encodes bursicon. We will report about these experiments in detail elsewhere.

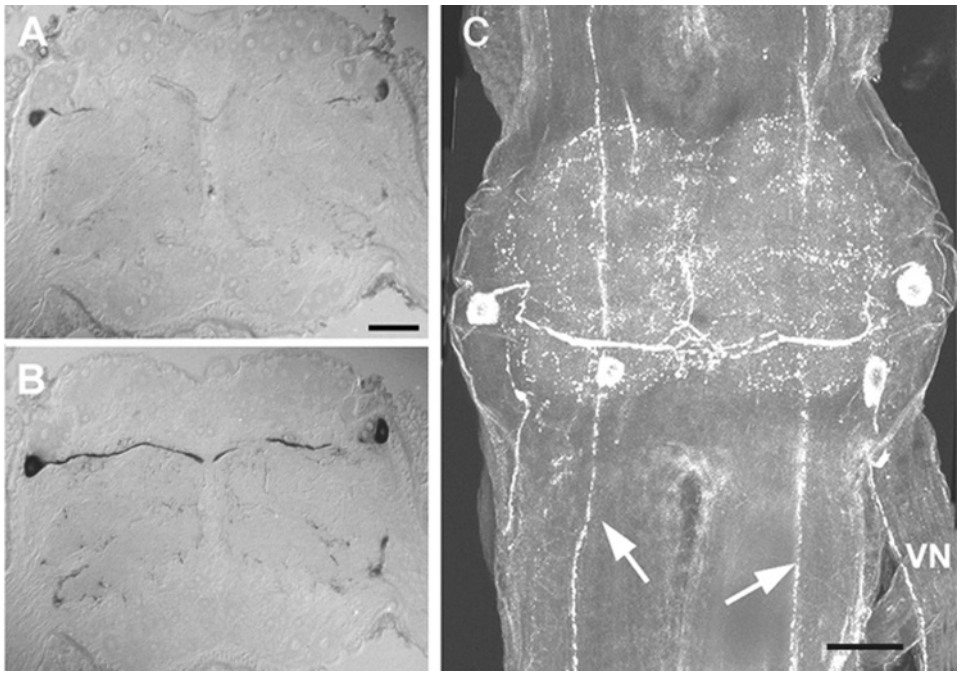

Fig. 4. Labeling of bursicon-containing somata in the ventral nerve cord of *P. americana* using the antiserum S115 against a partial bursicon sequence. *A, B:* Demonstration of colocalization of crustacean cardioactive peptide (A; CCAP) and bursicon (B) in consecutive 14 µm sections through the 2nd thoracic ganglion using DAB. *C:* Wholemount of first unfused abdominal ganglion; visualization of bursicon detection by Cy3. Arrows mark stained fibers projecting between ganglia. Scale bars = 100 µm

DISCUSSION

Bursicon has resisted its molecular analysis for many years. It has taken us more than seventeen years to finally gain knowledge of the nucleotide sequence encoding bursicon. Even though the protein is very stable as it retains its activity through heating to 80^0 C and precipitation by TCA and acetone, it is difficult to isolate it by chromatographic methods. For example, it was not possible to separate bursicon from SOD by ion exchange chromatography on a reversed-phase HPLC [19]. The *D. melanogaster* genome project made it finally possible to verify that the partial amino acid sequences of *P. americana* bursicon are highly identical to a gene with a previously unknown function. We can now extend our analysis of bursicon's function to a new level of investigation.

Since we know the nucleotide sequence of bursicon we are aiming to express the recombinant bioactive protein. Work is in progress to express bursicon in a bacterial expression system using the pET28 vector, which contains both a T7 and a HIS-tag (Novagen). We were able to express a monomeric protein of the correct size, but the dimerization of this monomer to a bioactive dimeric bursicon provides a new challenge. A bioactive recombinant protein will be important for conducting ligand-receptor studies to test whether the receptor DLGR2, a glycoprotein hormone receptor, is the receptor for bursicon as suggested by Baker and Truman [1]. The *D. melanogaster rickets* mutant shows defects in tanning and other postsecdysal behavior and shows deficiencies in the gene encoding DLGR2.

The sequence information will be used for the production of new antisera and monoclonal antibodies. We are now able to select highly immunogenic and conserved sequences for synthesizing peptides for immunizations. One of our main goals is to solve the puzzle of why CCAP and bursicon, which have sequential actions during ecdysis, are produced and stored in the same neurons in the ventral nervous system of many insects. With high-affinity antisera it will be possible to test at which phase during ecdysis bursicon is released into the blood circulation. Three possibilities exist. First, the hormones could be released at the same time, but the downstream events triggered by bursicon may have a longer latency than the initiation of the eclosion motor program triggered by CCAP. Alternatively, there may be sequential release of CCAP (a nonapeptide) and bursicon (a 30 kDa protein) from neurosecretory endings, a potentially novel finding in any system. Finally, although bursicon is thought not to be released into the hemolymph until after CCAP-induced ecdysis behavior is completed [23, 27], perhaps small amounts of hormone are indeed released prior to ecdysis. In this process, neurons that are consistently labeled with S115 [14] in the brain of different insects might be involved. In *M. sexta*, one hint that a portion of bursicon may indeed be released into the blood prior to adult ecdysis is that wing plasticization (induced by bursicon) occurs *before* the insect sheds its old cuticle [22]. Since *M. sexta* has been the model insect for the control of molting and ecdysis, this species seems ideal for tackling the above questions.

This outline, regarding some projects utilizing the sequence information of bursicon, emphasizes that the elucidation of the bursicon sequence will significantly

impact our understanding of bursicon's role in the neuroendocrine control of molting. This information could be applied, in the long run, to the development of novel strategies in insect pest management.

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