Insect resistance to Bacillus thuringiensis
ALTERATIONS IN THE INDIANMEAL MOTH LARVAL GUT PROTEOME

Mehmet Candas‡§§, Olga Loseva§, Brenda Oppert¶¶, Pradeepa Kosaraju§, and Lee A. Bulla, Jr.‡‡

Insect resistance to the Cry toxins of Bacillus thuringiensis (Bt) has been examined previously using a number of traditional biochemical and molecular techniques. In this study, we utilized a proteomic approach involving two-dimensional differential gel electrophoresis, mass spectrometry, and function-based activity profiling to examine changes in the gut proteins from the larvae of an Indianmeal moth (IMM, Plodia interpunctella) colony exhibiting resistance to Bt. We found a number of changes in the levels of certain specific midgut proteins that indicate increased glutathione utilization, elevation in oxidative metabolism, and differential maintenance of energy balance within the midgut epithelial cells of the Bt-resistant IMM larva. Additionally, the electrophoretic migration pattern of a low molecular mass acidic protein, which apparently altered in the Bt-resistant insect indicating that variations in amino acid content or modifications of certain proteins also are important components of the resistance phenomenon in the IMM. Furthermore, there was a dramatic decrease in the level of chymotrypsin-like proteinase in the midgut of the Bt-resistant larva, signifying that reduction of chymotrypsin activity, and subsequently decreased activation of Cry toxin in the insect midgut, is an important factor in the resistant state of the IMM. The proteomic analysis of larval gut proteins utilized in this study provides a useful approach for consolidating protein changes and physiological events associated with insect resistance to Bt. Our results support the hypothesis that physiological adaptation of insects and resistance to Bt is multifaceted, including protein modification and changes in the synthesis of specific larval gut proteins. We believe that increased oxidative metabolism may be an adaptive response of insects that undergo survival challenge and that it could mediate detoxification as well as higher rates of generalized and localized mutations that enhance their resistance and provide survival advantage. Molecular & Cellular Proteomics 2:19–28, 2003.

The insecticidal proteins (Cry toxins) produced by the soil bacterium Bacillus thuringiensis (Bt) currently are utilized commercially to safely and effectively control a wide variety of insect pests. Bt has been used in spray formulations for more than 40 years, and its insecticidal protein genes have been incorporated into several major crops where they provide a model for genetic engineering in agriculture (1). A significant threat to Bt-based insect control, however, is the potential of target insect populations to develop resistance to the Cry toxins. Although considerable fitness costs appear to be associated with the evolution of resistance to Bt in insects, laboratory selection experiments have shown a high potential for the development of resistance to Bt in a variety of insect species, demonstrating a repertoire of resistance mechanisms available to insects (2–11).

Insects that are in constant contact with Bt and its toxins most likely have a heightened innate cellular defense, and consequently they undergo physiological adaptations that result in resistance to the insecticidal action of Bt (8). Innate cellular defense mechanisms consist of nonspecific responses in both vertebrate and invertebrate organisms and are triggered by pathogen interaction. Innate defense primarily involves changes in the activity of mucosal surfaces that enhance secretion of proteolytic enzymes and antimicrobial molecules. Invertebrates such as insects rely mostly on innate cellular responses to defend themselves against invading microbes and their toxins, whereas vertebrates such as mammals additionally produce antibodies and T cells to combat microbial challenges. The entomopathogenic activity of Bt is caused primarily by the sequential action of Cry toxins ingested by susceptible insect larvae that include solubilization and activation via enzymatic processing, interactions with insect midgut epithelium, and disruption of the structural and functional integrity of the epithelium, leading to complete tissue destruction and death of the insect. Innate and adapt-

1 The abbreviations used are: Bt, Bacillus thuringiensis; DIGE, differential gel electrophoresis; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; IEF, isoelectric focusing; IMM, Indianmeal moth; IPG, immobilized pH gradient; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; NL, nonlinear; P, standard consisting of S and R proteins; R, Bt-resistant; SAAPfPNA, N-succinyl-Ala-Ala-Pro-Phe p-nitroanilide; S, Bt-susceptible; CHAPS, 3-[3-cholamidopropyl(dimethylammonio)-1-propanesulfonate; TEMED, N,N,N’,N’-tetramethylthyleylenediamine; Tricine, N-[2-hydroxy-1,1-bis(hydroxy-methyl)ethyl]glycine; V-ATPase, vacuolar ATPase.

From ‡Biological Targets, Inc., Tioga, Texas 76271, the §Center for Biotechnology and Bioinformatics and the Department of Molecular and Cell Biology, The University of Texas at Dallas, Richardson, Texas 75083, and the ¶United States Department of Agriculture, Agricultural Research Service, Grain Marketing and Production Research Center, Manhattan, Kansas 66502

Received, October 9, 2002, and in revised form, December 27, 2002

Published, MCP Papers in Press, December 31, 2002, DOI 10.1074/mcp.M200069-MCP200

This paper is available on line at http://www.mcponline.org

Molecular & Cellular Proteomics 2.1 19
Insect Resistance to *B. thuringiensis*

...ive responses such as changes in protein expression and the physiological state of the larval midgut can interrupt toxin action, rendering the gut tissue refractory to Cry toxins (8). Although decreased toxin binding by specific cadherin receptors in the insect midgut has been implicated in toxin resistance (12, 13), we believe that resistance to Bt is multifaceted involving various genetic and metabolic factors that are responsible for the development of the resistant state in an insect exposed continuously to Bt (8, 14). Therefore, we examined alterations in the larval midgut proteome of such an insect, the Indianmeal moth (IMM), to determine which proteins may be involved mechanistically in sustaining a resistant state. The IMM, *Plodia interpunctella*, is a cosmopolitan pest of stored grains and food products and is a problem in warehouses, mills, and food storage and processing plants. A Bt-resistant population of the IMM has been selected under laboratory conditions for resistance to *B. thuringiensis* subsp. *entomocidus*, strain HD198 (15). The resistant colony (688s) has been continuously reared to toxins (688s) were collected from grain storage on a farm in Riley County, Kansas (15). The colony (688s) has been continuously reared times less sensitive to this particular subspecies of Bt than the parent colony (16). Several explanations have been provided for this difference, including proteinase deficiencies, decreased toxin binding affinity, and a reduction in the number of toxin receptors (14, 17).

To accomplish the proteome analysis of the IMM, we compared the two-dimensional gel electrophoretic profiles of fluorescently labeled proteins (18) isolated from the midgut epithelium of Bt-susceptible (S) and Bt-resistant (R) IMM larvae. We found a number of changes in the levels of certain specific midgut proteins that indicate increased glutathione utilization, elevation in oxidative metabolism, and differential maintenance of energy balance within the midgut epithelial cells of the Bt-resistant IMM larva. The results of this study support our view that resistance to Bt is a complex response that can be displayed by proteomic expression profiles of midgut proteins from resistant and susceptible strains of an insect.

**EXPERIMENTAL PROCEDURES**

**Insects**—Colonies of *P. interpunctella* susceptible to *B. thuringiensis* toxins (688s) were collected from grain storage on a farm in Riley County, Kansas (15). The colony (688s) has been continuously reared on cracked-wheat diet in the laboratory. A subpopulation of the colony was selected by rearing the insect continuously on an artificial diet containing Bt subsp. *colony* was selected by rearing the insect continuously on an artificial diet in the laboratory. A subpopulation of the colony (688s) has been continuously reared to toxins (688s) were collected from grain storage on a farm in Riley County, Kansas (15). The colony (688s) has been continuously reared...
Insect Resistance to B. thuringiensis

Two-dimensional Electrophoresis Activity Blots—The two-dimensional electrophoresis activity blots were performed as described previously (17, 23, 56). Midguts from fourth instar larvae were placed in 25 μl of deionized water and frozen at −20 °C. Prior to assay, samples were thawed and centrifuged at 12,000 × g, and the supernatant was used as the source of soluble proteins. Proteins were separated in the first dimension by IEF on pH 3–10 IEF gels (Invitrogen). The gels were fixed in 3.5% sulfosalicylic and 11.5% trichloroacetic acid for 30 min. Lanes were excised, and the lane containing IEF markers was stained with Coomassie Blue (Gel-Code, Pierce). The lane containing P. interpunctella gut protein was incubated for 5 min in sample buffer and inserted into the long well of a 10% SDS-PAGE gel. Following SDS-PAGE, enzymes were electrotransferred to nitrocellulose. Nitrocellulose blots were incubated with the substrate solution N-succinyl-Ala-Ala-Pro-Phe p-nitroanilide (0.5 mg/ml in 0.1 M Tris, pH 8.1, 0.02 M CaCl₂) by placing blots in a EconoBlot (LabLogix) tray and overlaying them with 5 ml of substrate solution. The tray was covered with an EconoBlot plastic sheet and incubated with gentle rotation at 37 °C until a faint yellow color appeared. Liberated nitroanilide was diazotized for visualization by subsequent incubations of 5 min each in 0.1% sodium nitrite in 1 M HCl, 0.5% ammonium sulfamate in 1 M HCl, and 0.05% N-(1-naphthyl)-ethylenediamine in 47.5% ethanol. Membranes were placed on a plastic Gel-Bond sheet (FMC) in heat-sealed bags at −20 °C.

RESULTS

Two-dimensional Electrophoresis Gel Analysis and Identification of Specific IMM Midgut Proteins—Approximately 300 individual spots corresponding to proteins with molecular sizes ranging from 15 to 150 kDa and with isoelectric points between pl 4 and pl 10 were resolved by two-dimensional electrophoresis analysis (Fig. 1). Only the relatively abundant gut proteins of the IMM (120 spots) were selected for investigation. These protein spots were excised and digested with trypsin, and the resulting peptides were analyzed by mass spectrometry. Mass fingerprints were compared with known proteins from P. interpunctella and D. melanogaster as well as from several other insects and non-redundant protein databases. The majority of the protein peptide mass fingerprints did not match entries in the public databases indicating that there are considerable variations of the trypsin hydrolytic peptide masses for homologous proteins among organisms. Consequently, only those proteins identified are indicated by arrows in Fig. 1 and are listed in Table I.

Differential Two-dimensional Electrophoresis Analysis of Midgut Proteins from Bt-susceptible and Bt-resistant IMM Larvae—Comparison of the location and volumes associated with each spot revealed that the level of the majority of gut proteins remains virtually unchanged in the Bt-susceptible and-resistant strains (Fig. 2A, green bordered areas). The expression levels of 31 of the 120 proteins were significantly different in the S and R insects. Of the 31 differences, 20 proteins were consistently higher and 11 proteins were consistently lower in abundance in the resistant larvae (Table I and Fig. 2A, blue bordered areas and red bordered areas, respectively). The number of protein spots included in the analysis (Fig. 2B, red line) was sufficient as demonstrated by the distribution curve (Fig. 2B, blue line) and represents the accuracy of the differential analysis. In short, the sampling of protein spots in the analysis was statistically satisfactory.

Comparative Analysis of Specific Midgut Proteins in S and R IMM Larvae—The proteins identified by peptide mass fingerprinting include aminopeptidase N, mitochondrial ATP synthase subunit B; 11/12, vacuolar ATPase subunit B; 15, actin; 24, prophenol oxidase; 28, phosphopyruvate hydratase; 35, peroxinectin; 58, cytochrome oxidase subunit I; 60, NADH dehydrogenase subunit V; 61, 3-dehydroecdyson reductase; 75, F₅,F₇-ATPase/susceptible variant; 77, Ca²⁺-transporting ATPase; 97, chymotrypsin-like protein; 101, GSH transferase; 104, alcohol dehydrogenase. Masses of the protein molecular weight markers (left) and the pI range of isoelectric focusing (top) are indicated.
Insect Resistance to *B. thuringiensis*

**TABLE I**

| Spot no. | Protein ID                                | Apparent molecular mass | Apparent pl   | Spot volume abundance |
|----------|-------------------------------------------|-------------------------|---------------|-----------------------|
| 1        | Aminopeptidase N                          | 115                     | 4.6           | 1.4+                  |
| 7        | Mitochondrial ATP synthase subunit B      | 53                      | 4.7           | NC                    |
| 11/12    | Vacuolar ATPase subunit B                 | 55                      | 5.3           | 2.0+                  |
| 15       | Actin                                     | 42                      | 5.5           | NC                    |
| 24       | Peroxidase                               | 79                      | 7.0           | NC                    |
| 28       | Phosphohydrolase                          | 40                      | 7.0           | 2.1+                  |
| 35       | Peroxinectin                             | 110                     | 8.0           | 1.7–                   |
| 58       | Cytochrome oxidase subunit I              | 32                      | 8.1           | 1.9+                  |
| 60       | NADH dehydrogenase subunit V              | 32                      | 7.5           | 2.5+                  |
| 61       | 3-Dehydroecdyson reductase                | 32                      | 6.8           | 3.0+                  |
| 75       | F1F0-ATPase/resistant variant             | 20                      | 4.5           | 9.2–                  |
| 76       | F1F0-ATPase/susceptible variant           | 17                      | 4.5           | 15.3+                 |
| 77       | Ca2+-transporting ATPase                  | 13                      | 5.3           | 1.5–                  |
| 97       | Chymotrypsin-like protein                 | 30                      | 7.9           | 6.0–                  |
| 101      | GSH transferase                          | 30                      | 8.0           | 2.5+                  |
| 104      | Alcohol dehydrogenase                     | 25                      | 9.0           | 2.2–                  |

The two-dimensional DIGE analysis of larval gut protein extracts revealed two unique protein spots that represent a highly distinguishable profile for the S and R strains (Fig. 3, spots 75 and 76). Protein electroblotting and Edman sequencing of the N-terminal amino acids of the two spots revealed that the first 13 amino acids (Ala/Gly)-Asp-Ala-Pro-Lys-Asp Asp-Glu-Met-Ala-Leu-Thr-Phe) of each spot were the same, suggesting that they correspond to similar proteins in both strains but with altered migration patterns. Sequence similarity analysis for the 13-amino acid sequence using BLAST searches, combined with the molecular mass and pl of the corresponding protein spots, revealed a linkage between this IMM protein and a *D. melanogaster* gene, AAF46561, which apparently is an ortholog of mitochondrial ATP synthase subunit δ (F1F0-ATPase). The altered migration patterns of the variants (F1F0-ATPase/susceptible variant and F1F0-ATPase/resistant variant; Fig. 3, spots 75 and 76) of this IMM protein may be due to an enzymatic modification of the protein or differential expression of related genes or protein products of an alternatively spliced mRNA.

**Function-based Activity Profiling of Chymotrypsin-like Proteases in IMM Larval Gut**—An important factor in the mechanism(s) of physiological adaptation and resistance of insects to Bt is the altered capacity to process protoxin to Cry toxin (17). Because chymotrypsin is involved in the activation of Cry toxins, i.e. conversion of protoxin to activated toxin, chymotrypsin activity profiling was performed with gut extracts from S and R larvae using two-dimensional gels overlaid with SAAPFpNA as a substrate (Fig. 5). The molecular mass as well as the pl of the chymotrypsin-like protein that was determined by two-dimensional DIGE (Table I, spot 97) could be readily correlated to in-gel substrate hydrolysis (Fig. 5). Hydrolysis of the substrate was observed in a region corresponding to the location of proteins with molecular mass of 25-30 kDa (Fig. 5, lower arrows) as well as in the stacking gel (Fig. 5, upper arrows). The pl of the chymotrypsin-like proteases (Fig. 5, lower arrows) ranged from slightly acidic to basic in the S strain and from slightly acidic to neutral in the R strain. The two-dimensional activity blots reveal that the decrease in alkaline chymotrypsin-like activity is selective in the R strain.

**DISCUSSION**

The present study demonstrates that two-dimensional DIGE analysis of larval gut proteins and peptide mass fingerprinting are useful in consolidating protein changes and physiological events associated with insect resistance to Bt. The mechanisms underlying physiological adaptation and development of resistance to Bt as well as to other biological and environmental challenges appear to involve a repertoire of evolutionary mechanisms at the molecular and cellular levels (22). The results of our proteome analysis demonstrate that variation in the accumulation levels as well as modifications of specific proteins are components of the physiological adaptation complex harbored by the resistant IMM population. The two-dimensional DIGE analysis revealed a unique protein with altered electrophoretic mobility that represents a potential diagnostic marker for susceptible and resistant populations of the IMM (Figs. 1–4, spots 75 and 76; Table I). The 13-amino acid N-terminal sequences of the variants of this protein from the Bt-susceptible and -resistant larvae are identical. We identified the protein, using its 13-amino acid N-terminal sequence in a BLAST search, as an ortholog of *Drosophila* gene AAF46561, which apparently is related to mitochondrial ATP synthase (F1F0-ATPase) subunit δ. Differential modification of this protein in the Bt-resistant IMM reflects a change in the gene or in the protein that may be part of the adaptive re-
response involved in resistance. Such a modification may be a beneficial mutation related to modulation of oxidative metabolism and differential energy requirement of the Bt-resistant larva. Alternatively, the modification may be the result of a deleterious mutation that has occurred in the insect during adaptation. The implication(s) of this specific protein modification in the resistant state of this insect is yet to be determined. Nevertheless, the pronounced alteration in the electrophoretic migration pattern of this protein demonstrates that alternative gene splicing or protein modification is involved in resistance of IMM to Bt. Indeed transposon-mediated disruption of a specific gene has been linked to insect resistance to Bt (12). Likewise, changes in protein glycosylation also have been shown in both insect and nematode resistance to Bt (24, 25). Therefore, characterization of the F$_1$F$_0$-ATPase subunit δ, which exhibits such a prominent difference in the Bt-susceptible and resistant strains, is underway.

Although the biological significance of any modification of the F$_1$F$_0$-ATPase in insect resistance is not known, we believe that development of insect resistance to Bt most likely involves increased oxidative metabolism and enhancement of cellular stress responses that render the insect refractory to Bt and its insecticidal Cry toxins. For example, the amount of GSH transferase (Figs. 1–4, spot 101; Table I) was 2.5 times higher in the R IMM, suggesting an increase in the use of reduced GSH and possible changes in the redox environment in the midgut epithelium of the R IMM larva. GSH transferases constitute a family of inducible enzymes that are important for detoxification reactions and protecting insect cells from insecticides (26). Higher levels of GSH transferases have been found in chemical insecticide-resistant insect strains together with increased levels of carboxylesterases and cytochrome p450s that are responsible for metabolic resistance to insecticides (27, 28). In addition to an increase in a GSH transferase, two-dimensional DIGE analysis of S and R IMM larval midgut proteins revealed noticeable increases in the levels of cytochrome c oxidase subunit I (Figs. 1–4, spot 58; Table I) and NADH dehydrogenase subunit 5 (Figs. 1–4, spot 60; Table I), which reflects an increased oxidative metabolism in the midgut of the resistant insect.

Comparison of midgut epithelial proteins in both S and R IMM larvae also revealed a noticeable increase in the level of aminopeptidase N (Figs. 1–4, spot 1; Table I). Previously, increased levels of mRNA for aminopeptidase-like genes were observed in R IMM (23). Similarly, we reported higher amino-peptidase activity in the larval midgut of a Colorado potato beetle strain that had developed substantial resistance to the Cry3Aa toxin of B. thuringiensis subsp. tenebrionis (8). Aminopeptidase N is an ectoenzyme associated primarily with the microvillar membrane (brush border) of midgut cells. The enzyme is implicated in scavenging amino acids as well as degrading terminal peptides. Significantly, aminopeptidase N functions in the synthesis of GSH by providing cysteinylglycine as exogenous precursor (29, 30). Probably the increase in the level of aminopeptidases in toxin-resistant insects is required to replenish the GSH pool in the R IMM larval midgut cells that have a relatively higher utilization of this tripeptide.
Insect Resistance to *B. thuringiensis*

Fig. 3. Selected areas of two-dimensional gels and corresponding protein spot volumes for specific proteins in the S and R strains of the IMM. Protein spot migration patterns of Cy3- and Cy5-labeled proteins were detected in gels using a 2920 2D-Master Imager equipped with excitation-emission filters at 540/590 nm for Cy3 dyes and 620/680 nm for Cy5 dyes (see “Experimental Procedures”). Analysis of Cy3-Cy5 image pairs and detection of protein spots with relative spot volumes were accomplished using ImageMaster software (Amersham Biosciences). Specific proteins were identified by MALDI-TOF mass spectrometry analysis. F$_1$F$_0$-ATPase was identified by BLAST similarity search analysis. 2-DE, two-dimensional electrophoresis.

antioxidant than the S cells. In fact, a 2.5-fold increase in the level of NADH dehydrogenase subunit V (Figs. 1–4, spot 60; Table I) lends further support to the fact that there is an increase in the ribonucleotide-dependent reduction processes, including reduction of GSSG to GSH in the midgut epithelium of the R IMM larva. Such a trend in the levels of these particular enzymes typifies increased GSH utilization, which generally is compensated by elevated GSH synthesis as well as ribonucleotide-dependent reduction of GSSG. Apparently, change in the intracellular redox state of midgut epithelial cells in the R IMM larva is involved in up-regulation of aminopeptidase N, and the resulting increased aminopeptidase N activity contributes to an enhanced immune state. Furthermore, the R IMM larva contained increased V-ATPase
Insect Resistance to *B. thuringiensis*

**Fig. 4.** Relative levels of specific midgut proteins in the S and R larvae. Comparative quantitative changes in the individual proteins were identified by mass spectrometry analysis. Negative spot volume ratios reflect decreased amounts in the R strain.

(Figs. 1–4, spot 11/12; Table I). This enzyme is present in the apical membrane of goblet cells and represents the primary energy source for secretion and absorption by serving as an H⁺/K⁺ transporter across the insect midgut epithelium (31–36). During starvation as well as larval molt of insect larvae, the level of V-ATPase decreases (36). This decrease has been shown to be regulated by ecdysteroids (36) and may accommodate the lower energy required in the midgut tissue during starvation and molting. Perhaps, an increased V-ATPase level, which accompanies the Bt-resistant state of the IMM larva, reflects the elevated cellular energy profile across the midgut epithelium that may be necessary to combat toxin stress and consequently to maintain resistance at the tissue level. Indeed a similar increase in the level of phosphopyruvate hydratase (Figs. 1–4, spot 28; Table I), an enzyme that catalyzes the formation of a high energy metabolic intermediate, phosphoenol pyruvate (37), supports the notion that the Bt-resistant larval midgut has an elevated cellular energy profile and consequently a higher oxidative metabolism. This pattern suggests a biochemical basis for an increased oxidative metabolism and elevated responses to stress in the resistant state. In fact, in the R IMM larva, the level of 3-dehydrodysone reductase (Figs. 1–4, spot 61; Table I), which is an important enzyme for the inactivation of ecdysteroid in insect hemolymph (38, 39), was 3 times higher than the Bt-susceptible strain. Apparently, Bt resistance in the IMM involves changes in certain biochemical modulators, such as ecdysteroids, that have broad influence in the insect larva. These changes that accompany the resistant state in the IMM could be actuated by mutations in the key regulators or changes in their expression or their modulated functions, which in turn are transmitted genetically.

Additionally, a decrease in the level of calcium-transporting ATPase (Figs. 1–4, spot 77; Table I) in the midgut epithelium of the R IMM larva indicates possible alterations in calcium ion influx/exflux through the plasma membrane. Calcium-transporting ATPase is a ubiquitous eukaryotic protein that participates in numerous cellular regulatory processes, including intracellular calcium mobilization, vesicle trafficking, and receptor turnover (40, 41). The decreased level of this enzyme indicates alteration in calcium homeostasis in the midgut of the resistant IMM. Alternatively, calcium-transporting ATPase is subject to down-regulation during infection (42) or inactivation when cells experience an elevated oxidative environment (43). Thus, the increased oxidative metabolism that apparently is characteristic of the resistant state of the IMM may be involved in the down-regulation of this particular protein.

The alimentary tract is one of the largest organs in an insect, and therefore, it is important in growth and development. The midgut tissue is highly specialized, and the physiological state of this tissue is critical to nutrient uptake (44). Furthermore, it is the front line of defense against potential pathogens (45). Hence, factors related to the growth and development of insects, especially those involved in maintaining gut integrity, influence the action of Cry toxins. The pH of the gut environment, distribution and processing of toxin in the gut lumen, proteinase activity, and the capacity of the gut tissue to respond to or withstand toxin stress are important determinants in the entomopathogenicity of Bt. For example, insect lethality for a given amount of toxin depends on the gut surface area-to-volume ratio (13). This ratio decreases during the growth and development of larvae and results in a dilution of toxin molecules because of the increase in the midgut lumen volume as well as an increased number of receptor molecules in the midgut (13, 46). Additional mechanisms that may contribute to resistance to Bt include decreased toxin binding, changes in the glycosylation of epithelial cell surface molecules, midgut pH, and composition of the proteolytic enzyme.

**Fig. 5.** Chymotrypsin activity in midgut extracts from S and R strains of the IMM. Function-based activity profiles for chymotrypsin-like enzymes in midgut extracts from the S and R strains were measured in two-dimensional gels overlaid with SAAPFPNA as a substrate. Arrows point to the pink areas of substrate hydrolysis. The molecular mass as well as the pI of the chymotrypsin-like protein correlates with the protein identified by two-dimensional DIGE (Table I, spot 97). Molecular masses (in kilodaltons) of marker bands (right) and pI ranges (bottom) are indicated.
complex in the midgut (8, 47–54). We observed a significant decrease in an alkaline (pI 7.9) chymotrypsin-like proteinase (Figs. 1–4, spot 97; Table I) in the R IMM. Furthermore, alkaline chymotrypsin-like activities were observed only in the susceptible strain, not in the resistant strain (Fig. 5). These findings corroborate previous reports of proteinase deficiencies in this particular resistant insect (17, 55, 56). Reduction in alkaline chymotrypsin activity may be a significant factor in resistance by decreasing the activation of Cry toxins, which normally is mediated in an alkaline midgut environment (57–59). Protection from the toxin in such a manner, coupled to enhanced resistance within the midgut epithelial tissue, apparently renders the insect immune to Bt and its toxins.

Insect innate immunity to potential pathogens and parasites involves an array of reactions that include proteolytic cascades that regulate coagulation and melanization of hemolymph, production of reactive intermediates of oxygen and nitrogen, and secreted antimicrobial peptides (60–65). One of the proteins identified in our study is prophenol oxidase (Figs. 1–4, spot 24; Table I), which is an essential component of the biochemical pathway in melanization and hemolymph coagulation (66). There was no change in the amount of this protein in the midguts of S and R IMM larvae. Perhaps prophenol oxidase-related reactions are important in the larval midgut during early responses and adaptation of the susceptible insect to Bt and exposure to its toxins. Such early responses that include prophenol oxidase and related proteolytic cascades could provide immediate protection to the larva by strengthening the midgut epithelium and enhancing cellular defenses in the tissue. Once the insect reaches a resistant state, acute responses might be replaced by adaptive changes and enhanced stress responses that render IMM larvae immune to Bt and Cry toxin action.

There was a noticeable decrease in the level of peroxinectin (Figs. 1–4, spot 35; Table I), which is an important cell adhesion protein with peroxidase activity and is involved in interactions with extracellular superoxide dismutase and integrin (67–69). The peroxidase and cell adhesion functions of peroxinectin are regulated by prophenol oxidase and are believed to contribute to the generation of cellular antimicrobial activity (67–69). Why the level of peroxinectin in the midgut epithelium of the R IMM larva is decreased while that of prophenol oxidase is unchanged is unknown. However, disruption of a specific cadherin, which most likely is involved in cell adhesion in larval midgut epithelial tissue, has been linked to Cry toxin resistance in the cotton budworm Heliothis virescens (12). Therefore, it would be interesting to know whether the decreased level of peroxinectin in the R IMM is related to cell adhesion and Bt resistance.

Another protein that exhibited a decrease (2.2-fold) in the R IMM larva is alcohol dehydrogenase (Figs. 1–4, spot 104; Table I). At the present time, the connection between decreased alcohol dehydrogenase and adaptation to Cry toxin stress is not clear, although there may be a linkage between metabolic adaptation and the physiological state of Bt resistance. Likewise, a significant decrease in protein content and alcohol dehydrogenase activity has been reported for Droso phila subjected to high temperature stress (35°C), but currently there is no simple explanation for the involvement of alcohol dehydrogenase in stress resistance (70).

Peptide mass fingerprinting, which involves mass spectrometry coupled with bioinformatics and relies on current databases containing amino acid sequences of proteins, is a useful proteomic technique for protein identification. When judiciously applied, this technique can yield important information pertaining to proteins that share considerable cross-species identity and functionality (71). Currently there are few gene sequences available for the IMM and other important insect pests. With increased availability of genome information and predicted proteomes of insects, a comprehensive understanding of the resistant state of insects and their genomic and proteomic expression profiles will be facilitated. Furthermore, comparative proteomic analysis of susceptible and resistant insects should help elucidate innate responses and adaptive changes that contribute to insect immunity. Specifically, two-dimensional DIGE analysis of the IMM and other insects will be invaluable in understanding how insects adapt to or resist environmental challenges. Moreover, assessment of proteomic differences, along with biochemical and genetic changes associated with insect resistance to Bt, will be helpful in designing strategies to circumvent the emergence of resistance in target insect populations and in managing the long term efficacy of Bt-based biopesticides. Overall the results of this study support our hypothesis that insect resistance to Bt is multifaceted and may reconcile the various distinct findings reported in the literature (8, 9, 14). Whether there are commonalities in the development of insect resistance to chemical and biological pesticides remains to be determined. Nevertheless, overlapping biochemical and genetic factors that potentially lead to cross-resistance could present a serious threat to effective control of agriculturally, medically, and industrially important insects. Thus, identification of proteins involved in resistance to chemical or biological agents should provide new targets for designing new effective insecticides.

Acknowledgments—We thank Philippe Fournier, Paul Shirk, Karl Gordon, Xia Qing-you, and Emmanuelle d’Alençon for help in confirming the BLAST search results.

* This research was supported by United States Department of Agriculture Grant USDAACSEES 97-35302-4781 and United States Agency for International Development Grant 263-0240-G-00-6014-00 (to L. A. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

∥ To whom correspondence should be addressed. Tel.: 972-883-4226; Fax: 972-883-4215; E-mail: bulla@utdallas.edu.
membrane calcium-transporting ATPase expression. J. Infect. Dis. 186, 372–378
43. Stepniak, K., Zajdel, A., Wilczok, A., Wilczok, T., Grzelak, A., Mateja, A., Sozymski, M., and Bartosz, G. (2000) Dopamine-melanin protects against tyrosine nitration, tryptophan oxidation and Ca²⁺-ATPase inactivation induced by peroxynitrite. Biochim. Biophys. Acta 1523, 189–195
44. Anderson, E., and Harvey, W. R. (1966) Active transport by the Cercopia midgut. II. Fine structure of the midgut epithelium. J. Cell Biol. 31, 107–134
45. Loeb, M. J., Martin, P. A. W., Hakim, R. S., Goto, S., and Takeda, M. (2001) Regulation of cultured midgut cells after exposure to sublethal doses of toxin from two strains of Bacillus thuringiensis. J. Insect Physiol. 47, 599–606
46. Candas, M., Brian R. Francis, B. R., Griko, N. B., Midboe, E. G., and Bulla, L. A., Jr. (2002) Proteolytic cleavage of the developmentally important cadherin BT-R in the midgut epithelium of Manduca sexta. Biochemistry 41, 13717–13724
47. Van Rie, J., McGaughey, W. H., Johnson, D. E., Barnett, B. D., and Van Mellaert, H. (1990) Mechanism of insect resistance to the microbial insecticide Bacillus thuringiensis. Science 247, 72–74
48. Ferre, J., Escriche, B., Bel, Y., and Van Rie, J. (1999) Biochemistry and genetics of insect resistance to Bacillus thuringiensis insecticidal crystal proteins. FEMS Microbiol. Lett. 132, 1–7
49. Sayyed, A. H., Haward, R., Herrero, S., Ferre, J., and Wright, D. J. (2000) Genetic and biochemical approach for characterization of resistance to Bacillus thuringiensis toxin Cry1Ac in a field population of the diamondback moth, Plutella xylostella. Appl. Environ. Microbiol. 66, 1509–1516
50. Marroquin, L. D., Elyassnia, D., Griffiths, J. S., Feitelson, J. S., and Aroian, R. V. (2000) Bacillus thuringiensis (Bt) toxin susceptibility and isolation of resistance mutants in the nematode Caenorhabditis elegans. Genetics 155, 1693–1699
51. Griffiths, J. S., Whitacre, J. L., Stevens, D. E., and Aroian, R. V. (2001) Bt toxin resistance from loss of a putative carbohydrate-modifying enzyme. Science 293, 860–864
52. Sayyed, A. H., Gati, R., Kouskoura, T., Wright, D. J., and Crickmore, N. (2001) Susceptibility of a field-derived, Bacillus thuringiensis-resistant strain of diamondback moth to in vitro-activated Cry1Ac toxin. Appl. Environ. Microbiol. 67, 4372–4373
53. Oppert, B. (1999) Protease interaction with Bacillus thuringiensis insecticidal toxins. Arch. Insect Biochem. Physiol. 42, 1–12
54. Oppert, B., Kramer, K. J., Johnson, D. E., Maclntosh, S. C., and McGaughey, W. H. (1994) Altered protoxin activation by midgut enzymes from a Bacillus thuringiensis resistant strain of Plodia interpunctella. Biochem. Biophys. Res. Commun. 198, 940–947
55. Oppert, B., Kramer, K. J., Johnson, D., Upton, S. J., and McGaughey, W. H. (1996) Luminal proteinases from Plodia interpunctella and the hydrolysis of Bacillus thuringiensis Cry1Ac(c) protoxin. Insect Biochem. Mol. Biol. 26, 571–583
56. Bulla, L. A., Kramer, K. J., and Davidsson L. I. (1977) Characterization of the entomocidal parasporal crystal of Bacillus thuringiensis. J. Bacteriol. 130, 375–383
57. Bulla, L. A., Kramer, K. J., Cox, D. J., Jones, B. L., Davidsson, L. I., and Lookhart, G. L. (1981) Purification and characterization of the entomocidal protoxin of Bacillus thuringiensis. J. Biol. Chem. 256, 3000–3004
58. Andrews, R. E., Jr., Bibilos, M. M., and Bulla, L. A., Jr. (1985) Protease activation of the entomocidal protoxin of Bacillus thuringiensis subsp. kurstaki. Appl. Environ. Microbiol. 50, 737–742
59. Meister, M., Hetru, C., and Hoffmann, J. A. (2000) In Origin and Evolution of the Vertebrate Immune System Current Topics in Microbiology (Du Pasquier, L., and Litman, G. W., eds) Vol. 248, pp. 17–36, Springer-Verlag, Berlin
60. Lowenberger, C. (2001) Innate immune response of Aedes aegypti. Insect Biochem. Mol. Biol. 31, 219–229
61. Vass, E., and Nappi, A. J. (2001) Fruit fly immunity. BioEssays 23, 860–864
62. Vass, E., and. Nappi, A. J. (2001) Fruit fly immunity. BioEssays 23, 860–864
63. Vass, E., and. Nappi, A. J. (2001) Fruit fly immunity. BioEssays 23, 860–864
64. Muta, T., and Iwanaga, S. (1996) The role of hemolymph coagulation in innate immunity. Curr. Opin. Immunol. 8, 41–47
65. Gillespie, J. P., Kanost, M. R., and Trenczek, T. (1997) Biological mediators of insect immunity. Annu. Rev. Entomol. 42, 611–643
66. Lavine, M., and Strand, M. (2002) Insect hemocytes and their role in immunity. Insect Biochem. Mol. Biol. 32, 1295–1309
67. Johansson, M. W., and Soderhall, K. (1988) Isolation and purification of a cell adhesion factor from crayfish blood cells. J. Cell Biol. 106, 1795–1803
68. Johansson, M. W. (1999) Cell adhesion molecules in invertebrate immunity. Dev. Comp. Immunol. 23, 303–315
69. Sritunyalucksana, K., Wongpisuvisantti, K., Johansson, M. W., and Soderhall, K. (1991) Peroxinecin, a cell adhesive protein associated with the proPO system from the black tiger shrimp, Penaeus monodon. Dev. Comp. Immunol. 25, 353–363
70. Oudman, L., Van Delden, W., Kamping, A., and Bijlsma, R. (1992) Interaction between the Adh and α Gpdh loci in Drosophila melanogaster: adult survival at high temperature. Heredity 68, 289–297
71. Lester, P. J., and Hubbard, S. J. (2002) Comparative bioinformatic analysis of complete proteomes and protein parameters for cross-species identification in proteomics. Proteomics 2, 1392–1405