Insect Resistance to *Bacillus thuringiensis*

ALTERATIONS IN THE INDIANMEAL MOTH LARVAL GUT PROTEOME*

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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 is an ortholog of F_1F_0-ATPase, was considerably 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)[1] 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-

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[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; SAAPF*p*NA, *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide; S, Bt-susceptible; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; V-ATPase, vacuolar ATPase.

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 is ~100 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. *entomocidus* HD198. This Bt-resistant colony (198r) is ~100 times more resistant to the selection and is ~264 times more resistant to Cry1Ab protoxin than the susceptible colony 688s (14–16, 19).

Chemicals and Materials—Immobiline DryStrips (IPG strips) with a nonlinear (NL) pH gradient 3–10, Pharmalytes IPG buffer pH 3–10 NL, cyanine dyes Cy3 and Cy5, and Bind-silane were from Amersham Biosciences. Urea, thiourea, Tris, glycine, glycerol, SDS, CHAPS, ammonium persulfate, TEMED, and EGTA were purchased from EM Science. *N*,*N*′-Methylenebisacrylamide, CAPS, protease inhibitor mixture, dithiothreitol, iodoacetamide, and bromphenol blue were from Sigma. Coomassie Brilliant R-250 and Coomassie protein assay reagent were from Pierce. Acrylamide was from GeneMate. α-Cyano-4-hydroxycinnamic acid was from Aldrich. Polyvinyl difluoride membrane Immobilon-P and ZipTip c18 were from Millipore.

Protein Extraction—Gut brush border membrane vesicles were prepared from fourth instar larvae by Mg/EGTA precipitation and differential centrifugation (20) and kept at −80 °C until used. A protease inhibitor mixture containing 100 mM AEBSF, 80 mM aprotinin, 5 mM bestatin, 1.5 mM E-64, 2 mM leupeptin, and 1 mM pepstatin A (Novagen) was added to all buffers. Lipids were removed, and protein was precipitated with chloroform/methanol. Proteins were solubilized in lysis buffer containing 30 mM Tris-HCl, pH 8.5, 7 M urea, 2 M thiourea, and 4% CHAPS. The protein concentration in the samples was determined by a detergent-compatible Coomassie Plus protein assay reagent (Pierce).

Sample Preparation—Labeling of proteins using succinimidyl esters of propyl-Cy3 and methyl-Cy5 fluorophores was performed according to the technique described by Amersham Biosciences. The labeled extracts were mixed prior to electrophoresis.

Isoelectric Focusing (IEF)—The first separation, IEF, was performed in 3-mm Immobiline IPG strips (13 cm) with a NL pH 3–10 gradient in the IPGphor (Amersham Biosciences). For rehydration of the IPG strips, 100–300 μg of brush border membrane vesicle lysate were mixed with rehydration solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 2 mg/ml dithiothreitol, and 1% Pharmalyte 3–10 NL. The entire mixture was pipetted into the strip holder. Rehydration was performed for 12 h. Isoelectric focusing was carried out using IPGPhor (Amersham Biosciences) following a voltage step-gradient (500 V for 1 h, 1000 V for 1 h, 8000 V for 8 h).

SDS-PAGE—Prior to the second dimension (SDS-PAGE), the IPG strips containing brush border membrane vesicle proteins were placed in a solution containing 50 mM Tris-HCl buffer, pH 8.6, 30% glycerol, 2% SDS, and 0.5% dithiothreitol at 90 °C for 1 min. Following a cooling period, the strips were equilibrated in a solution containing 50 mM Tris-HCl buffer, pH 8.6, 6 M urea, 30% glycerol, 2% SDS, and 2% iodoacetamide. After equilibration, the IPG strips were applied vertically to 10% (w/v) polyacrylamide-SDS gels in low fluorescence glass plates and 1-mm spacers (Amersham Biosciences). Electrophoresis was performed in a Hoefer SE 600 gel electrophoresis unit, and the gels were run at 25 mA of constant current per gel until the bromphenol blue dye front completely migrated out of the bottom of the gels. The gels were fixed in 10% methanol, 7% acetic acid and stained by Sypro Ruby (Molecular Probes).

Two-dimensional Gel Image Analysis—Cy3- and Cy5-derivatized 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 (Amersham Biosciences). Signal output for each dye was normalized by determining approximate exposure times. Analysis of Cy3-Cy5 image pairs and detection of protein spots with relative spot volumes was done by using ImageMaster software (Amersham Biosciences). Protein spots were identified by gel-to-gel matching with low experimental variation and with statistically significant differences in expression levels (Student's *t* test). Proteins in the S and R strains of the IMM with more than 1.4-fold difference in abundance were considered as significant. Two data sets for every protein spot in the S and R samples were compared in conjunction with a standard (P) group. The P group contained all S and R sample groups to assure the statistical consideration of every protein on each gel and to decrease gel-to-gel variation. This procedure ensured that each sample was compared with the same standard.

Peptide Mass Fingerprinting—After two-dimensional electrophoresis, the gel was stained with Sypro Ruby, and protein spots were excised using an Ettan spot picker (Amersham Biosciences). Protein spots were destained, washed, and digested overnight at 37 °C with trypsin. Peptides were extracted, purified, and mixed with matrix α-cyano-4-hydrocinnamic acid. Peptide mass spectra were acquired using a PerSeptive Biosystems Voyager DE-PRO MALDI-TOF operating in the delayed extraction reflector mode. Peptide masses were

searched against a variety of protein databases, including entries for *Drosophila melanogaster*, via the programs ProFound and MS-Fit.

Amino Acid Sequence Analysis—The transfer of proteins from the gel to polyvinyl difluoride membrane was accomplished by the method of Matsudaira (21). Electroblotting was carried out with 10 mM CAPS, pH 11.0 in 10% methanol at 50 V of constant voltage at 4 °C for 1 h. The membranes were stained with 0.1% Coomassie R-250 in 40% methanol and destained in 50% methanol. Selected spots were excised from dried membranes, and N-terminal amino acid sequencing was performed at The Macromolecular Structure, Sequencing and Synthesis Facility, Department of Biochemistry, Michigan State University.

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 10–20% two-dimensional Tricine gel (Invitrogen). 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 $CaCl_2$) 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 pI 4 and pI 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

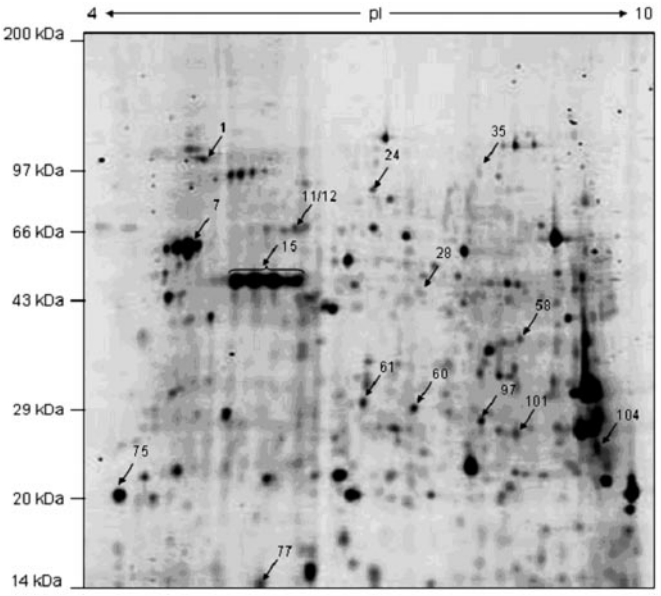

FIG. 1. **Typical two-dimensional gel image of Cy dye-labeled IMM larval midgut proteins.** Identified protein spots are indicated by *arrows*: *1*, aminopeptidase N; *7*, mitochondrial ATP synthase subunit B; *11/12*, vacuolar ATPase subunit B; *15*, actin; *24*, prophenol oxidase; *28*, phosphopyruvate hydratase; *35*, peroxinectin; *58*, cytochrome oxidase subunit I; *60*, NADH dehydrogenase subunit V; *61*, 3-dehydroecdysone reductase; *75*, F_1F_0-ATPase/susceptible variant; *77*, Ca^{2+}-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.

with each spot revealed that the level of the majority of gut proteins remains virtually unchanged in the Bt-susceptible and -resistant strains (Fig. 2*A*, *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. 2*A*, *blue bordered areas* and *red bordered areas*, respectively). The number of protein spots included in the analysis (Fig. 2*B*, *red line*) was sufficient as demonstrated by the distribution curve (Fig. 2*B*, *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, vacuolar ATPase subunit B, actin, prophenol oxidase, phosphopyruvate hydratase, peroxinectin, cytochrome oxidase subunit I, NADH dehydrogenase subunit V, 3-dehydroecdysone reductase, Ca^{2+}-transporting ATPase, chymotrypsin-like protein, GSH transferase, and alcohol dehydrogenase. Spot volume comparisons (Fig. 3) and relative changes in the levels of the identified proteins (Fig. 4 and Table I) indicate subtle as well as pronounced differences in the midgut proteome of the S and R IMM larvae.

TABLE I

Changes in the levels of proteins identified in the midgut epithelium of IMM larvae resistant to Bt

NC, no change; +, increase; −, decrease.

Spot no.	Protein ID	Apparent molecular mass	Apparent pI	Spot volume abundance
		kDa		
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	Prophenol oxidase	79	7.0	NC
28	Phosphopyruvate hydratase	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-Dehydroecdysone reductase	32	6.8	3.0+
75	F_1F_0-ATPase/susceptible variant	20	4.5	9.2−
76	F_1F_0-ATPase/resistant variant	17	4.5	15.3+
77	Ca^{2+}-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 pI 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 (F_1F_0-ATPase). The altered migration patterns of the variants (F_1F_0-ATPase/susceptible variant and F_1F_0-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 Proteinases 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 SAAPF*p*NA as a substrate (Fig. 5). The molecular mass as well as the pI 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 pI of the chymotrypsin-like proteinases (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 (F_1F_0-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-

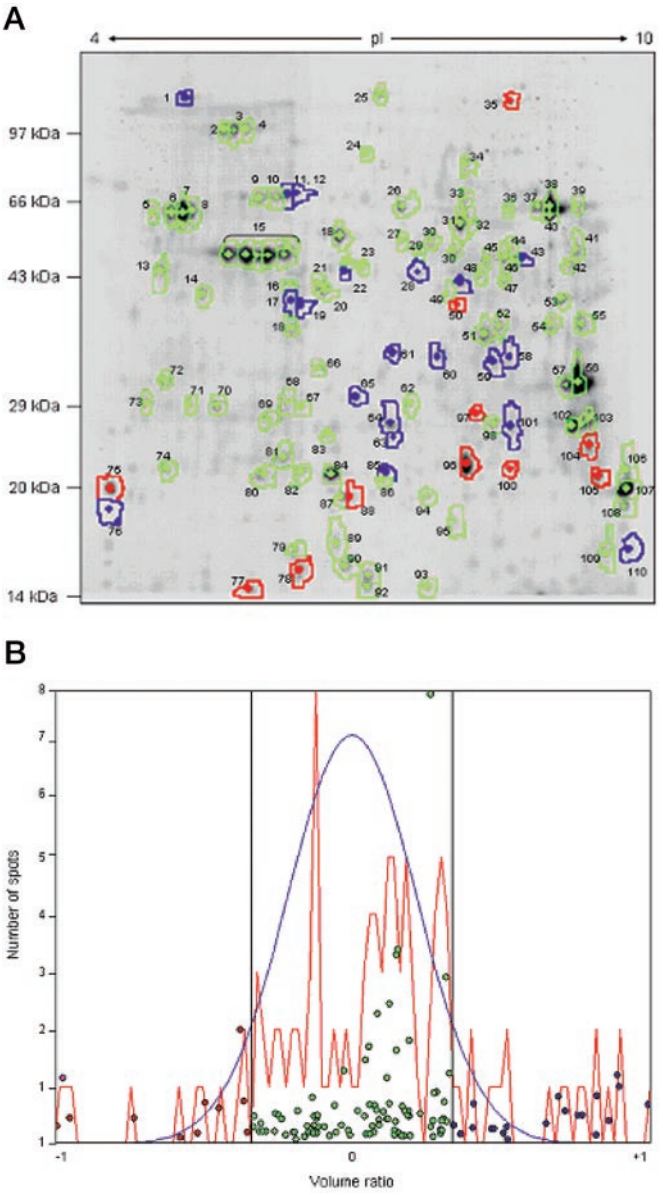

FIG. 2. **Two-dimensional DIGE of midgut proteins from S and R IMM larvae.** *A*, the volumes of protein spots, which correspond to protein abundance, were compared in Bt-susceptible and Bt-resistant larvae. Spot areas with *blue borders* indicate proteins with increased amounts in the resistant insect, and those with *red borders* indicate proteins with decreased amounts in the resistant strain. *Green bordered areas* demark protein spots with no change in the susceptible and resistant strains (see also Table I). Molecular masses (in kilodaltons) of marker bands (*right*) and pI ranges (*top*) are indicated. *B*, histogram of midgut protein abundance in Bt-susceptible and Bt-resistant IMM larvae. The *blue circles* represent increased proteins, and *red circles* represent decreased proteins. The *green circles* represent unchanged proteins. The *blue curve* represents the distribution of all the protein spots included in the analysis. The *red line* indexes clusters of protein spots that show quantitative changes.

sponse 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_1F_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_1F_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 aminopeptidase 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

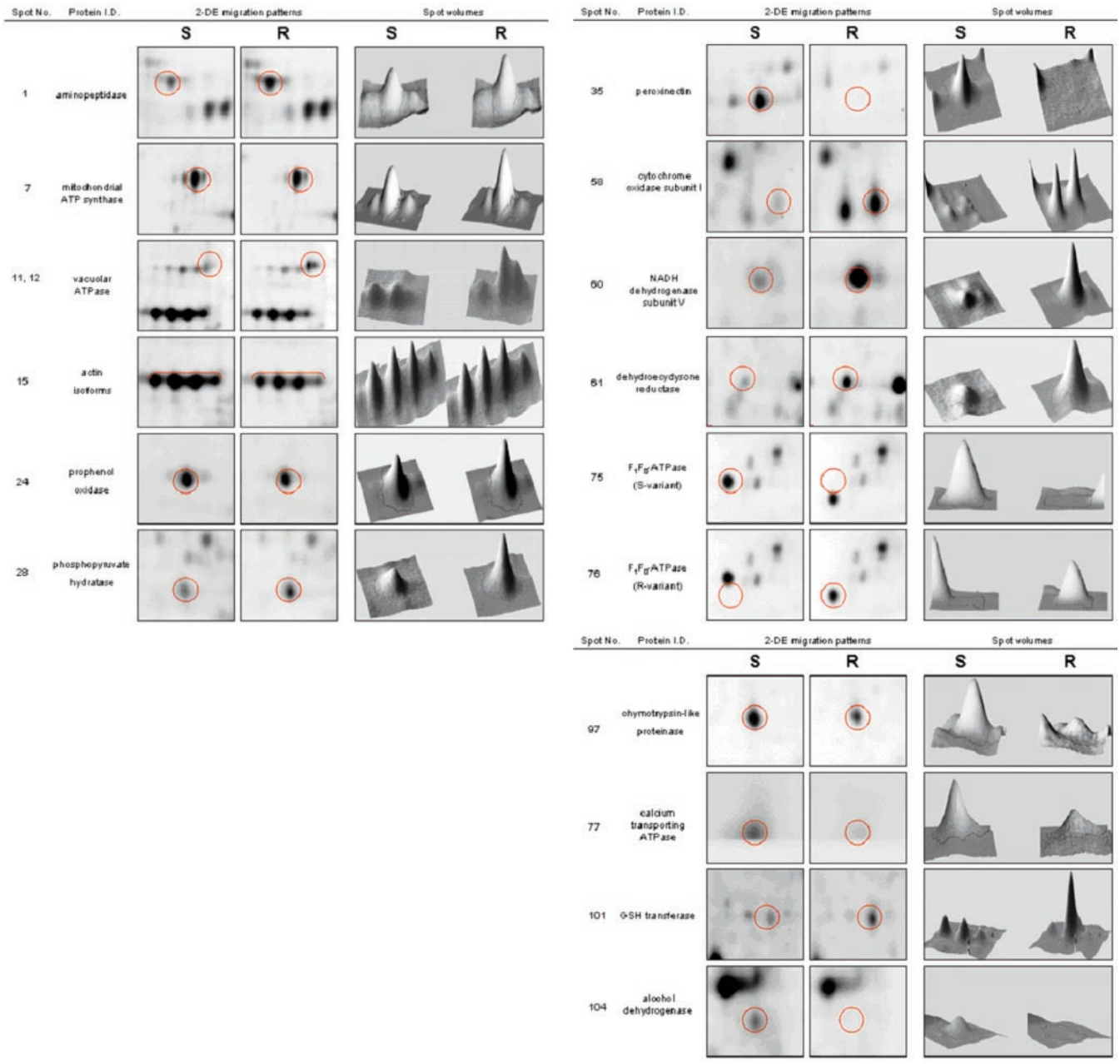

FIG. 3. **Selected areas of two-dimensional gels and corresponding protein spot volumes for specific proteins in the S and R strains of the IMM.** Protein spot migration patterns of Cy3- and Cy5-labeled proteins were detected in gels using a 2920 2D-Master Imager equipped with excitation-emission filters 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_1F_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

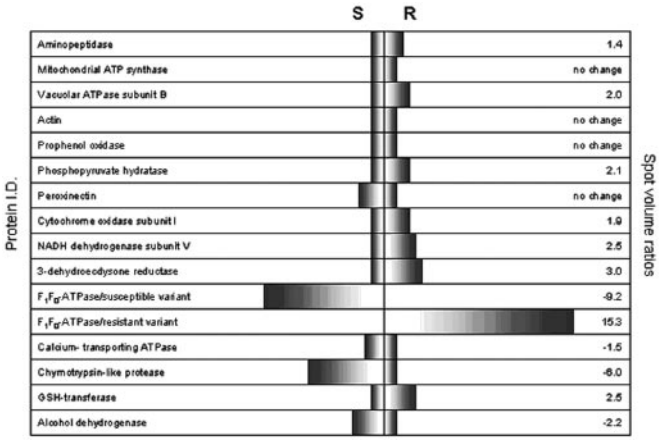

FIG. 4. **Relative levels of specific midgut proteins in the S and R larvae.** Comparative quantitative changes in the individual proteins were identified by mass spectrometry analysis. Negative spot volume ratios reflect decreased amounts in the R strain.

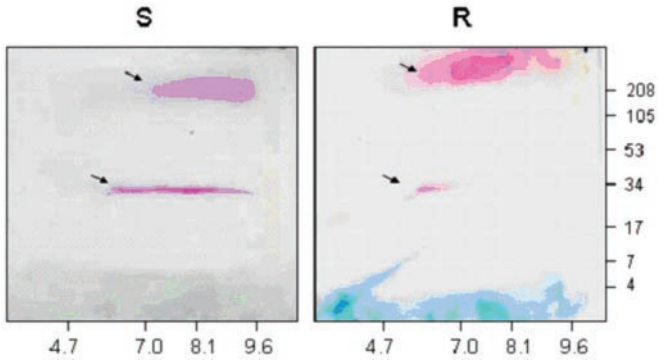

FIG. 5. **Chymotrypsin activity in midgut extracts from S and R strains of the IMM.** Function-based activity profiles for chymotrypsin-like enzymes in midgut extracts from the S and R strains were measured in two-dimensional gels overlaid with SAAPF*p*NA as a substrate. *Arrows* point to the *pink areas* 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.

(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-dehydroecdysone 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 a 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/efflux 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

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 *Drosophila* 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.

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