Resistance of *Helicoverpa armigera* to Cry1Ac toxin from *Bacillus thuringiensis* is due to improper processing of the protoxin

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The bacterium *Bacillus thuringiensis* produces ICPs (insecticidal crystal proteins) that are deposited in their spore mother cells. When susceptible lepidopteran larvae ingest these spore mother cells, the ICPs get solubilized in the alkaline gut environment. Of approx. 140 insecticidal proteins described thus far, insecticidal protein Cry1Ac has been applied extensively as the main ingredient of spray formulation as well as the principal ICP introduced into crops as transgene for agricultural crop protection. The 135 kDa Cry1Ac protein, upon ingestion by the insect, is processed successively at the N- and C-terminus by the insect midgut proteases to generate a 65 kDa bioactive core protein. The activated core protein interacts with specific receptors located at the midgut epithelium resulting in the lysis of cells and eventual death of the larvae. A laboratory-reared population of *Helicoverpa armigera* displayed 72-fold resistance to the *B. thuringiensis* insecticidal protein Cry1Ac. A careful zymogram analysis of Cry1Ac-resistant insects revealed an altered proteolytic profile. The altered protease profile resulted in improper processing of the insecticidal protein and as a consequence increased the LC50 concentrations of Cry1Ac. The 135 kDa protoxin-susceptible insect larval population processed the protein to the biologically active 65 kDa core protein, while the resistant insect larval population yielded a mixture of 95 kDa and 68 kDa Cry1Ac polypeptides. N-terminal sequencing of these 95 and 68 kDa polypeptides produced by gut juices of resistant insects revealed an intact N-terminus. Protease gene transcription profiling by semi-quantitative RT (reverse transcription)–PCR led to the identification of a down-regulated HaSP2 (*H. armigera* serine protease 2) in the Cry1Ac-resistant population. Protease HaSP2 was cloned, expressed and demonstrated to be responsible for proper processing of insecticidal protoxin. The larval population displaying resistance to Cry1Ac do not show an altered sensitivity against another insecticidal protein, Cry2Ab. The implications of these observations in the context of the possibility of development of resistance and its management in *H. armigera* to Cry1Ac through transgenic crop cultivation are discussed.

Key words: Cry 1Ac, insect, insecticide, pest management, toxin, trypsin.

INTRODUCTION

*Bacillus thuringiensis* spore crystal formulations have been used commercially in agriculture and public health insect pest management for the last thirty years very successfully. Within the last few years, the 3.6 kb gene encoding the insecticidal protein Cry1Ac has been integrated in the genomes of crop plants such as maize, soybean and cotton to offer protection against *Helicoverpa armigera* and other pests. The Cry1Ac transgenic cotton plant (event 531, Monsanto, U.S.A.) has been cultivated for the last 6–7 years in vast agricultural areas of the U.S.A., Argentina, Australia, China and India [1]. The adoption of transgenic cropping has resulted in a significant reduction in the usage and application of chemical insecticides.

The cotton bollworm, *H. armigera*, is the principal cotton pest in the old world and inflicts major losses in India, China, Pakistan, South and East Asia and parts of West Africa and Australia. Over a period of time the insect has developed resistance and cross resistance to various groups of chemical insecticides, including organochlorines, organophosphates, carbamates and synthetic pyrethroids. The insecticidal protein Cry1Ac is the most effective of all *B. thuringiensis* insecticidal proteins tested against this insect pest with an LC50 of 12–25 ng/cm2 [2]. Concerns regarding the possibility of an emergence of resistance against Cry1Ac due to failure of implementing the recommended cultivation regimen have been raised, thus questioning the sustainability of these transgenics [3,4]. Laboratory-reared populations of *H. armigera* showing resistance to Cry1Ac have been reported and they show cross resistance to other effective insecticidal proteins, e.g. Cry1Aa and Cry1Ab [5]. Isolated reports describing the emergence of resistance under laboratory conditions with different insects have identified receptor mutations or altered protease profiles in the resistant population as the basis of resistance [6–9].

Though there have been no reports of field populations of insects resistant to Cry1Ac in *H. armigera*, nevertheless, such a possibility is a definite threat [4] given that laboratory populations resistant to Cry1Ac have been isolated [8]. Since insects have developed resistance to all the different chemical insecticides used today, diverse resistance management strategies have been recommended the world over to contain the resistance development risk, which includes use of *B. thuringiensis* and non-*B. thuringiensis* crop mixtures either as pure populations in adjacent plots or as mixtures within the same plot [10]. This strategy of providing a refuge is not being strictly practiced in

Abbreviations used: BBMV, brush border membrane vesicle; HaSP, *Helicoverpa armigera* serine protease; IB, inclusion bodies; ICP, insecticidal crystal protein; IPTG, isopropyl β-D-thiogalactoside; LC50, lethal concentration for 50% mortality; ORF, open reading frame; RT, reverse transcription; UPGMA, unweighted pair group method with arithmetic mean.

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The nucleotide sequence data reported for HaSP1, HaSP2, HaSP3, HaSP4, HaSP5 and HaSP6 will appear in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession numbers EF104918, EF104913, EF104914, EF104915, EF104916 and EF104917 respectively.
India and elsewhere. The general argument in not planting refugia is the wide availability of different non-transgenic host plants and other alternate host crops in the immediate vicinity of cotton fields [11]. India also has the distinction of growing large areas of cotton with “illegal” *B. thuringiensis* cotton hybrids and simulation studies have reported that development of resistance to Cry1Ac is a distinct possibility [12]. However, for all suggested resistance delay management strategies to be implemented, understanding the mode of action of Cry1Ac on target insects and also the molecular mechanism of resistance development against this toxin is important [13,14]. Here we report analysis of a laboratory reared population of *H. armigera* resistant to Cry1Ac. This resistant population displayed an altered protease profile resulting in improper processing of the Cry1Ac protoxin. Further, a specific protease, HaSP2 (*H. armigera* serine protease 2), which is capable of proper processing and activation of Cry1Ac polypeptide, among the six different groups of serine proteases from *H. armigera* midgut, displayed significantly reduced expression in the resistant insect larvae. Such resistant insect larvae do not show cross resistance to another insecticidal protein Cry2Ab, active against *H. armigera*.

**EXPERIMENTAL**

**Insects**

*H. armigera* was reared and maintained on a semi-synthetic artificial diet consisting of 300 g of chickpea flour, 10 g of wheat germ, 5.3 g of ascorbic acid, 1.7 g of sorbic acid, 3.3 g of methyl-p-hydroxybenzoate, 20 g of Wesson’s salt mixture, 2.5 g of aureomycin, 10 ml of vitamin mixture, 10 ml of methyl-p-hydroxybenzoate, 20 g of Wesson’s salt mixture, 2.5 g of formaldehyde, 48 g of yeast and 16 g of agar in 1 litre of distilled water. Larvae and pupae of cotton bollworms were collected randomly from cotton fields in and around Akola (Maharastra, India) and reared to provide a homozygous population. All the rearing procedures were carried out at 27 ± 2°C under 14:10 light/dark regime. These insects were reared in the laboratory for three generations to obtain a homozygous population. The insects were selected for resistance to Cry1Ac protoxin by releasing 400 naïve neonate *H. armigera* larvae on a diet coated with 40 ng/cm² of diet at one larva per well. After five days, the 18 survivors were transferred to a toxin-free diet and allowed to complete the life cycle. At each generation, neonate larvae were released on a diet containing 40 ng of Cry1Ac/cm² and survivors after 5 days were transferred to a toxin-free diet. This procedure was continued for 10 generations.

**Insect bioassays**

ICPs (insecticidal crystal proteins) of Cry1Ac was purified from a *Escherichia coli* strain JM 103 expressing the protoxin of Cry1Ac (cloned in pKK 223-3 expression vector). Similarly, a synthetic gene of Cry2Ab was constructed (GeneArt, Regensburg, Germany), cloned and expressed in pQE30 expression vector. The expression of Cry1Ac and Cry2Ab into the inclusion bodies of *E. coli* cells was induced by 5 mM IPTG (isopropyl β-D-thio-galactoside) and allowed to grow for 3 h following induction. Insecticidal protein concentration was estimated by densitometric scanning (Alpha System, Midlothian, VA, U.S.A.) and comparing with band intensities of BSA at different concentrations. Bioassays were conducted on neonate larvae by placing them on Cry protoxin incorporated diet in plastic vials. Cry1Ac inclusion bodies were mixed in 2 ml of distilled water and the stock was assayed in artificial diet at various concentrations such as 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5 and 10 μg/ml diet. All the assays were replicated four times and 30 larvae per treatment were used. Mortality was recorded every 24 h after treatment for 3 days. Pooled data were subject to statistical analysis. Median lethal concentration (LC₅₀, lethal concentration for 50% mortality) was calculated by Probit analysis by using the computer software Indo-Stat (Indo-Stat Services, India).

**Gut extracts**

Fifth instar 2nd day *H. armigera* larvae from the F10 generation (both resistant and susceptible populations) were chilled on ice for 10 min and midguts dissected and homogenized in 50 mM Tris pH 8.0 at 4°C. This material was further centrifuged at 17 226 g, 4°C for 15 min, and the clear supernatant was aliquoted and frozen at −80°C until further use.

**Protease activity profiles**

The protein content in the gut extract was estimated by the method of Bradford [15]. Gut extracts (5 μg/lane) were diluted in a sample buffer without 2-mercaptoethanol and resolved on SDS/12% PAGE pre-incorporated with 1% gelatin. The gel was resolved at 120 V at 10°C. After the run, the gel was incubated in a solution containing 2.5% Triton X-100 with gentle shaking for 3 h to replace the SDS with Triton X-100. The gel was then incubated in Hepes buffer (50 mM Hepes, pH 8.0, 1% Triton X-100 and 5 mM cysteine) at 37°C for 15 h. Finally, the gel was stained in 0.5% Coomassie Brilliant Blue G-250 for 3 h and destained with 10% acetic acid for several hours till the halo regions depicting protease activity appeared.

In a separate protease assay, the resolved gut extracts (5 μg/lane) were incubated with 20 ml of Tris buffer (50 mM Tris, pH 8.0, and 20 mM CaCl₂) containing 0.3% solubilized Cry1Ac protoxin for 30 min at 4°C followed by 2 h at 25°C. The gel was washed three times thoroughly with sterile water and stained with 0.5% Coomassie Brilliant Blue G-250 for 30 min. The activity profile was obtained by destaining the blot for 3 h in 10% acetic acid to observe the white halos.

**Toxin preparation**

The recombinant Cry1Ac protoxin was prepared as IB (inclusion bodies) by the method reported in [13] from a plasmid pKK223-3 bearing the Cry1Ac gene (Bacillus Genetic Stock Centre, Ohio State University, OH, U.S.A.). The amount of the toxin was quantified densitometrically by resolving the IB on SDS/PAGE. The inclusion bodies containing the toxin were solubilized in 50 mM sodium carbonate buffer, pH 10.4. This protein was purified by anion exchange chromatography and eluted with a linear gradient of 0 to 1000 mM NaCl gradient prepared in 50 mM sodium carbonate buffer, pH 10.4.

**Activation of Cry1Ac protoxin by midgut juices**

Purified Cry1Ac protoxin (2.5 μg) was incubated with midgut juices (500 μg/ml total protein) of naïve and laboratory-reared Cry1Ac-resistant *H. armigera* population in the F10 generation at 37°C. The reaction was stopped by adding 4× SDS/PAGE sample buffer containing 2-mercaptoethanol. The reaction products were resolved in a SDS/7.5% PAGE gel and stained with Coomassie Blue R-250 for 30 min. The proteins were visualized by destaining with 10% acetic acid.

**N-terminal sequencing**

Purified Cry1Ac protoxin (2.5 μg) was incubated with 0.2 μl (500 μg/ml of total protein) of midgut juices for 10 min at 37°C. The reaction was stopped by adding 4× SDS/PAGE sample
Improper protoxin activation leads to Cry1Ac resistance in H. armigera

buffer containing 2-mercaptoethanol. The reaction products were resolved on a SDS/7.5% PAGE gel and electroblotted to a nylon membrane (Millipore) using CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) buffer. The blot was stained with Comassie Blue for 10 min and washed repeatedly with methanol. The bands corresponding to 95 kDa and 68 kDa were cut and subjected to N-terminal sequencing at Iowa State University protein facility (Ames, IA, U.S.A.). The first ten amino acids were read from the proteins.

Cloning of serine proteases from H. armigera

Sequences of midgut serine proteases of H. armigera were retrieved from the GenBank®, analysed for multiple alignment by CLUSTAL W and dendrogram analysis by the UPGMA (un-weighted pair group method with arithmetic mean) method using the MacVector software (version 7.0). A protease representing each of the six groups was chosen, and gene-specific primers for the forward and reverse directions were synthesized. Midguts from 5th instar 1st day larvae were dissected in diethyl pyrocarbonate water and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer’s protocol. RT (reverse transcription) with oligo dT (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer’s protocol. RT (reverse transcription) with oligo dT primer used 5 μg of total RNA as template and Superscript II to generate the cDNA. Using the respective sets of end primers (the following primer sets were employed: HaSP1For, 5′-ATGAGCA-AATGGGTGCTGTGACTGCTGCTCACTGCC-3′ and HaSP1Rev, 5′-GAATCTCACAACGCGGACGAC3′ to yield 852 bp; HaSP2For, 5′-ATGGTCCTTCAACCACCCCTC-3′ and HaSP2Rev, 5′-AA-CACGCGTTTCACAGAT-3′ to yield 768 bp; HaSP3For, 5′-ATGGAAACTCTTTGCGCTGATCTTAAGT-3′ and HaSP3Rev, 5′-AGAAGATTGTTGATCCACTGAT-3′ to yield 885 bp; HaSP4For, 5′-ATGAGGTTTGGCTTTCTCGTGTT-3′ and HaSP4Rev, 5′-GTTAGCAGAACAAGGGCGGCTC-3′ to yield 768 bp; HaSP5For, 5′-ATGCGTATCCCTCCTTCCTCGTTGA-3′ and HaSP5Rev, 5′-CGCGTAGGATGAAATCCAGGAACTGATC-3′ to yield 762 bp; HaSP6For, 5′-ATGCCTTCCTCAGGTCTTCTCGAC3′ and HaSP6Rev, 5′-CCGCTTTAGATGAAATCCAGGAACTGATAGC-3′ to yield 769 bp), the six different proteases were cloned from the cDNA of the naïve population of H. armigera by performing 30 cycles of PCR, each of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. The PCR products from each of these 6 reactions were cloned in pGemT-easy vector and sequenced for their full length using various bio-informatic tools for the ORF (open reading frame), signal peptide region and the pro-peptide region. The mature protease region (after the pro-peptide region) was amplified by PCR using the HaSP2 cloned in pGemT-easy as template, forward primer 5′-ATGTACCCCGACCCTCAACTG-3′ (position 90–110 nucleotides), reverse primer 5′-AAGACGGTTCTTCAAGCAGAT-3′ (position 757–775 nucleotides) and the resultant PCR product cloned in pGemT-easy. This mature protein region, cloned into pGemT-easy, was digested with EcoR1, cloned at the corresponding site in the expression vector pET 32a and transformed into E. coli strain BL21(DE3). Heterologous protein expression was induced by adding 1 mM IPTG to the bacterial culture after reaching an attenuation of 0.5 at 600 nm and allowed to grow for a further 2 h. The expressed protein accumulated in the soluble fraction of E. coli and was purified by Ni-NTA (Ni²⁺-nitrilotriacetate) affinity chromatography. The bound protein was eluted with 150 mM imidazole and used for Cry1Ac protoxin activation assays.

Quantitative RT–PCR

For comparing the relative transcript abundance of each of the above six different proteases between the naïve and laboratory-reared Cry1Ac-resistant H. armigera populations of the F10 generation, they were subjected to quantitative RT–PCR analysis. The amount of RNA in both the treatments was normalized by amplifying the β-actin gene in each treatment to equal intensity after 20 cycles of RT–PCR using the one step RT–PCR kit (Qiagen GmbH, Hilden, Germany). The amplification regimen was as follows: reverse transcription at 43 °C for 35 min, an initial activation step at 95 °C for 15 min followed by 20 PCR cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s and extension at 72 °C for 1 min. Using this normalized amount of RNA as template, the transcripts of all the six different proteases in the Cry1Ac-resistant and naïve populations were probed by amplifying their gene products. The primers used for amplification were the same as those used for cloning these proteases as described above. Before loading on to 1% agarose gels, the RT–PCR products were treated with 1 μg of RNase (Qiagen) at 37 °C for 10 min to eliminate template RNA, since it obliterates the accurate estimation of the β-actin gene product in the gel. The gels were photographed with Polaroid 667 black-and-white print film and scanned for net intensity of each RT–PCR product using the software Kodak 1D, version 2.0.

Expression and purification of HaSP2

The sequence of HaSP2 was analysed using various bio-informatic tools for the ORF (open reading frame), signal peptide region and the pro-peptide region. The mature protease region (after the pro-peptide region) was amplified by PCR using the HaSP2 cloned in pGemT-easy as template, forward primer 5′-ATGATCCCAGACCCGGCTCACTG-3′ (position 90–110 nucleotides), reverse primer 5′-AAGACGGTTCTTCAAGCAGAT-3′ (position 757–775 nucleotides) and the resultant PCR product cloned in pGemT-easy. This mature protein region, cloned into pGemT-easy, was digested with EcoR1, cloned at the corresponding site in the expression vector pET 32a and transformed into E. coli strain BL21(DE3). Heterologous protein expression was induced by adding 1 mM IPTG to the bacterial culture after reaching an attenuation of 0.5 at 600 nm and allowed to grow for a further 2 h. The expressed protein accumulated in the soluble fraction of E. coli and was purified by Ni-NTA (Ni²⁺-nitrilotriacetate) affinity chromatography. The bound protein was eluted with 150 mM imidazole and used for Cry1Ac protoxin activation assays.

RESULTS

Development of resistance by H. armigera to Cry1Ac

H. armigera naïve 1st instar larvae were selected on an artificial diet containing Cry1Ac protoxin. After ten generations, the larvae were able to tolerate 72-fold more Cry1Ac protoxin (in terms of their LC₅₀ dose) than the susceptible larvae (Table 1). The resistance dose is reflected more in the LC₅₀ values than in the LC₉₀ values, suggesting that the larvae become much more adapted to survive an ever increasing dose of the selection pressure. Lindig blot-analysis of BBMV (brush border membrane vesicle) proteins from the Cry1Ac-resistant and susceptible population with activated Cry1Ac did not show any differences between them (Supplemental Figure S1 at http://www.BiochemJ.org/bj/419/bj4190309add.htm).

Table 1: Median lethal dosages of Cry1Ac in H. armigera neonates

<table>
<thead>
<tr>
<th>Sr. Number</th>
<th>Generation</th>
<th>LC₅₀ dose (μg/ml)</th>
<th>LC₉₀ dose (μg/ml)</th>
<th>FL limit (LC₉₀/FL)</th>
<th>Slope (± S.E.M)</th>
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<tbody>
<tr>
<td>1</td>
<td>F1 (selected)</td>
<td>0.47</td>
<td>5.66</td>
<td>0.31–0.72</td>
<td>1.19 (0.31)</td>
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<tr>
<td>2</td>
<td>F2 (selected)</td>
<td>0.74</td>
<td>4.61</td>
<td>0.54–1.00</td>
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<td>3</td>
<td>F3 (selected)</td>
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<td>5.9</td>
<td>0.71–1.34</td>
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<td>4</td>
<td>F4 (selected)</td>
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<td>7.41</td>
<td>0.65–1.41</td>
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<td>5</td>
<td>F5 (selected)</td>
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<td>0.87–1.48</td>
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<td>6</td>
<td>F6 (selected)</td>
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<td>10.83</td>
<td>1.23–2.22</td>
<td>1.57 (0.18)</td>
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<tr>
<td>7</td>
<td>F7 (selected)</td>
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<td>14.89</td>
<td>0.99–2.06</td>
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</tr>
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<td>8</td>
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<td>2.27–4.51</td>
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</tr>
<tr>
<td>10</td>
<td>F10 (selected)</td>
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<td>33.51</td>
<td>2.46–5.32</td>
<td>1.32 (0.60)</td>
</tr>
</tbody>
</table>

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Activity profiles with (A) gelatin as substrate, bands P1 to P4 are protease bands seen in susceptible insects; and (B) with Cry1Ac 135 kDa protoxin as substrate. Lane 1, midgut extract from naïve larvae; and lane 2, midgut extract from Cry1Ac-resistant larvae. CP1 to CP4 are bands seen in resistant insects.

Activity profiles of *H. armigera* midgut

Proteins from midgut extracts of *H. armigera* 5th instar 2nd day larvae of Cry1Ac-resistant (10th generation) and susceptible population were assayed for their ability to hydrolyse gelatin. Midgut proteins were resolved in SDS/12% PAGE and the gel was subjected to an activity blot using gelatin as substrate. In all the protease activity profiles, care was taken to resolve equal amounts of protein. Differences were observed in the activity profiles between Cry1Ac-resistant and normal insects (Figure 1). Four halo regions representing proteolytic dissolution of gelatin corresponding to the range of 25–35 kDa were clearly detected in susceptible insects. The expression and mobility profiles of these proteolytic halos were reduced in the Cry1Ac-resistant insects. Besides these, higher-molecular-mass proteins with gelatin-hydrolysing abilities were also detected. These high-molecular-mass proteins were more abundant in Cry1Ac-resistant insects (Figure 1A).

Similar activity blots, where polypeptide Cry1Ac was used as the substrate, revealed that susceptible insects had four major halo regions acting on Cry1Ac. The expression of two of the faster-migrating proteases in resistant insects are totally abolished, whereas the upper two that move slowly are greatly reduced (Figure 1B, proteases marked by arrows). Interestingly, two different halo regions that are induced in resistant insects are not observed in susceptible insects (Figure 1B). Taken together, these results clearly demonstrate that insects resistant to Cry1Ac have an altered protease profile with respect to both Cry1Ac and gelatin.

Preliminary characterization of the nature of the protease involved in gelatin and Cry1Ac degradation by specific protease inhibitors, leupeptin and PMSF tentatively classified them as serine proteases (results not shown).

**Activation of Cry1Ac protoxin by *H. armigera* midgut juices**

Subsequent to observing an altered protease activity profile between resistant and susceptible insects, we studied the ability of these midgut proteases to process Cry1Ac protoxin (Figure 2, lane 2, band 1) to an activated form. Commercial trypsin was able to cleave the protoxin to a 65 kDa activated Cry1Ac toxin (Figure 2, lane 3, band 2). Gut juice from the susceptible insect population (25 ng total protein) could properly cleave and activate Cry1Ac to its reported 65 kDa form (Figure 2, lane 4, band 3) while a 68 kDa band is also seen (Figure 2, lane 4, band 4), whereas the gut juices from resistant insects (25 ng total protein) failed to appreciably cleave the protoxin (Figure 2, lane 5). On incubating Cry1Ac protoxin with a higher concentration (100 ng total protein) of gut juice from resistant insects for 10 min, a 95 kDa polypeptide (Figure 2, lane 6, band 5) was formed in addition to the 68 kDa band (Figure 2, lane 6, band 6), and a fully activated toxin of 65 kDa is also visible, but at very low amounts below the 68 kDa band (Figure 2, lane 6). On prolonged incubation of Cry1Ac protoxin with the gut juice of resistant insects (for 30 min or more), the proportion of the 95 kDa protein is reduced, whereas the proportion of the 68 kDa protein increased (results not shown).

Furthermore, to demarcate the origin of the 95 and 68 kDa polypeptides, we analysed their respective N-terminal sequence. The analysis revealed that the 95 and 68 kDa polypeptides were not proteolytically processed from the N-terminus and retained the sequence of the protoxin. A cartoon in Supplemental Figure S2 (http://www.BiochemJ.org/bj/419/bj4190309add.htm) depicts these results.

**Cloning of serine proteases from *H. armigera***

The above results suggested that serine proteases (similar to trypsin and chymotrypsin) of sizes between 25–35 kDa are
Improper prototoxin activation leads to Cry1Ac resistance in H. armigera

Response of Cry1Ac resistant and susceptible H. armigera to Cry1Ac transgenic cotton plants (Event S31)

The response of Cry1Ac-susceptible and -resistant H. armigera neonates were similar when assayed on non-transgenic cotton Mech-162. However, marked differences were observed when the larval populations were assayed against Cry1Ac transgenic Mech-162 B. thuringiensis (BollGard I). The mean percentage mortality in the B. thuringiensis-susceptible strain was 95.5%, whereas it was 42.2% in the Cry1Ac-resistant population (Table 2). Thus the laboratory-reared Cry1Ac-resistant H. armigera population was able to withstand the dose of Cry1Ac expressed in Cry1Ac transgenic cotton plants of Mech 162 B. thuringiensis (BollGard I). Although the LC₅₀ dose of the Cry1Ac-resistant population increased 72-fold against Cry1Ac, the same population showed increased susceptibility against another bioactive insecticidal protein, Cry2Ab, present in BollGard II (Table 2) and there was no significant difference in their LC₅₀ values with respect to Cry2Ab. Furthermore, when Cry1Ac protoxin-resistant insects were fed purified trypsin-activated toxin, the magnitude of resistance came down from approx. 72-fold to almost complete loss of the resistance phenotype. The results indicated that there is insignificant difference in the toxicity of activated Cry1Ac in the resistant and susceptible strain of H. armigera and the toxicity is nearly the same.

DISCUSSION

Insecticides constitute a major ingredient in modern crop husbandry, especially in tropical regions. Chemical insecticides constitute nearly 90% of the total insecticide market, the rest being biopesticides. Insecticidal proteins produced by strains of B. thuringiensis are the most successful biopesticide presently used in commercial agriculture, especially against lepidopteran
One candidate gene from each of the six protease groups identified in Figure 3 was cloned from the Indian population of *H. armigera* and sequenced full-length. The ORF of these genes were translated to their respective protein sequences which were compared by multiple alignment analysis among themselves. The protein residues that are conserved are shaded. The gene sequences of these protease genes have been deposited in the Genbank® as described in the Results section.

**Table 2. Response of neonate *H. armigera* strains to *B. thuringiensis* and non-*B. thuringiensis* cotton**

Ten neonate larvae were released in a leaf disc of the respective treatment and each treatment was replicated ten times. The mortality data were analysed by Student’s *t* test and values superscripted with different letters are significantly different at 95 % confidence limits. *H. armigera* naïve population maintained without selection pressure for 10 generations. **H. armigera** maintained following 10 generations of selection on Cry1Ac.

<table>
<thead>
<tr>
<th>Variety for bioassay</th>
<th>Cry1Ac-susceptible population*</th>
<th>Cry1Ac-resistant population**</th>
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</thead>
<tbody>
<tr>
<td>MECH-162</td>
<td>5.55&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.44&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>MECH-162 BolGard I</td>
<td>95.55&lt;sup&gt;b&lt;/sup&gt;</td>
<td>42.22&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Mech 162 BolGard II</td>
<td>97.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>67.9&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**Table 3. Median lethal doses of Cry1Ac in *H. armigera* neonates**

*H. armigera* naïve population maintained without selection pressure for 10 generations. **H. armigera** maintained following 10 generations of selection on Cry1Ac.

<table>
<thead>
<tr>
<th>H. armigera mortality</th>
<th>Cry1Ac-susceptible population*</th>
<th>Cry1Ac-resistant population**</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC&lt;sub&gt;50&lt;/sub&gt; to Cry2Ab</td>
<td>128.1 ng/ml</td>
<td>135 ng/ml</td>
</tr>
<tr>
<td>LC&lt;sub&gt;50&lt;/sub&gt; to trypsin-activated Cry1Ac</td>
<td>0.18 ng/ml</td>
<td>0.22 ng/ml</td>
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</table>

**Figure 4. Protein sequence alignment of different proteases from the larval midgut of *H. armigera* populations used in the present study**

One candidate gene from each of the six protease groups identified in Figure 3 was cloned from the Indian population of *H. armigera* and sequenced full-length. The ORF of these genes were translated to their respective protein sequences which were compared by multiple alignment analysis among themselves. The protein residues that are conserved are shaded. The gene sequences of these protease genes have been deposited in the Genbank® as described in the Results section.

**Figure 5. Differential expression of serine protease genes in the larval midgut of *H. armigera* from a naïve population and a population resistant to Cry1Ac**

The relative expression of these genes was established by relative RT–PCR and normalized using their β-actin transcripts. The primers used for amplifying the different protease genes are described in the Experimental section. The gel was photographed with a Polaroid 667 black-and-white film and scanned for its mean intensity for each of the RT–PCR products using the software KODAK 1D, version 2.0. M, markers; R, Cry1Ac-resistant; S, Cry1Ac-sensitive.

Insects. Following ingestion by susceptible larvae, insecticidal proteins are solubilized in the alkaline gut environment and subsequently proteolytically activated in releasing an active core toxin polypeptide that binds to specific receptors, leading to pore formation and the eventual death of the insect. The

*cotton bollworm, *H. armigera*, is a cosmopolitan, polyphagous agricultural pest and is effectively controlled by the ICP Cry1Ac. Transgenic cotton plants bearing the 3.6 kb *cry1Ac* gene (Event
In the last 4 years [17], there have been a number of reports of laboratory-reared populations of insect pests such as Heliothis virescens, Manduca sexta, Lymantria dispar, Plutella xylostella and Plodia interpunctella developing resistance to Cry1Ac [18]. Studies directed towards understanding the basis of this resistance reveal changes in the expression profile of the cadherin protein and reduced binding of the toxin to midgut epithelium. Mutations in the cadherin gene resulting in shorter transcripts have been reported from Heliothis virescens and Pectinophora gossypiella [6,7]. Other studies have reported changes in the activity profiles of midgut proteases between resistant and naive insects [19]. Development of resistance has been directly correlated with the loss in function of a major gut protease in Plodia interpunctella [9]. There have been two reports on the development of resistance by *H. armigera* to Cry1Ac. One of them reports a mutation in the cadherin gene, whereas the other reports changes in the activity of midgut proteases belonging to the class esterase [8,20].

During the course of this study, we developed a laboratory population of *H. armigera* showing 72-fold resistance to the insecticidal protein Cry1Ac. The midgut protease profile between the naive and resistant populations showed characteristic differences with the loss in activity of two major protease polypeptides hydrolysing gelatin in resistant insects. When presented with Cry 1Ac as substrate, the two populations revealed contrasting protease profiles. In susceptible insects, there were four major Cry1Ac-hydrolysing protease zones, whereas in resistant insects, activity of two of these zones is abolished and two new Cry1Ac-metabolising zones are recruited. The Cry1Ac protoxin is proteolytically processed by the midgut trypsin proteases, both from the N-terminus and the C-terminus, to yield a 65 kDa active toxin [21]. The exact nature of this reaction is not fully understood and there has been no report of a single trypsin protease being implicated in this process. It is believed that the correct activation of the protoxin is one of the most important and essential steps for proper insecticidal activity. Precise cleavage of the first 28 amino acids from the N-terminus is absolutely essential for optimum biological activity. It has been observed that Cry1Ac protoxins not proteolytically cleaved at the N-terminus result in 25-fold less toxicity than the properly activated toxins lacking the first 28 amino acids [22]. N-terminal analysis of the 95 and 68 kDa polypeptides of the Cry1Ac protoxin after processing by resistant *H. armigera* gut juices revealed that in both polypeptides the first 28 amino acids of the protoxin have not been cleaved. Hence it appears that a quantitative change in N-terminal proteolytic processing of Cry1Ac is a major contributing factor in resistance. Earlier studies have reported that activated Cry1Ac with an intact N-terminus binds more tightly with the receptors in the BBMV of insect guts as compared with the precisely activated form [22]. Such preferential binding probably results in the competing out of precisely processed active toxin from the viable toxin-binding sites on the receptor molecule by the imprecisely processed and less potent toxin molecules, thus manifesting the resistance phenotype. Taken together, these results with the earlier observation that resistant insects have a faster degradation of the activated toxins than the naive population clearly establishes an important role for the protoxin-activating step in the resistance development process.

Analogous resistance development processes have been reported earlier from Plodia interpunctella and Plutella xylostella resistant to Cry1Ac [19,23]. Such resistant *Plodia. interpunctella* were reported to lack a major protease that activates the *B. thuringiensis* protoxin [9]. Cry1Ac-resistant *Plutella xylostella* showed more than 15000-fold resistance to full-length Cry1Ac solubilized protoxin, with only a 94-fold resistance to the activated 65 kDa toxin [23]. These results clearly argue for a defect in toxin activation as a major resistance mechanism in lepidopteran insects against Cry1Ac. Another study on *H. armigera* with resistance to Cry1Ac reported that resistant insects displayed higher levels of enzyme esterase [20]. These upregulated esterases were also shown to bind tightly to Cry1Ac insecticidal proteins. Thus changes in activity of midgut-associated serine proteases appear to be common physiological mechanism in lepidopteran insects developing resistance to the midgut-acting *B. thuringiensis* insecticidal proteins.

Action of insecticidal proteins upon ingestion depends on two major facilitating events, activation by a midgut protease and recognition by the specific receptor for docking and eventual lysis of the midgut epithelium. In this context, aberration of any of the two events would result in reduced toxicity of the ingested protein. Earlier experiences with chemical insecticides and gut protease inhibitors have shown that the gut protease transcriptome and proteome is not a static entity, but rather is able to modulate the activity of various proteases both collectively and individually under different conditions [24,25].

*H. armigera* has been extensively studied for its proteolytic enzymes in the midgut and it has been reported that serine proteases, such as trypsin and chymotrypsin, contribute maximally to the proteolytic activity [15]. The amino acid sequence of proteases from the *H. armigera* midgut fall into six different groups. Since there is very little divergence between sequences of proteases within a group, it was not possible to design primers that could clearly differentiate one from the other. Hence, one protease was selected from each group and analysed for its transcript abundance by semi-quantitative RT–PCR. The transcript of the protease HaSP2 was substantially reduced in the resistant insect population, whereas those of the other five proteases did not show significant changes. Earlier reports also indicate that the protease HaSP2 shows altered expression in *H. armigera* populations insensitive to the soybean trypsin inhibitor [16].

Further studies to understand the specific role of this protease in Cry1Ac activation were performed to determine its specific ability to hydrolyse Cry1Ac protoxin (135 kDa) to the activated form. The heterologously expressed HaSP2 cleaved Cry1Ac protoxin to its activated 65 kDa core. Hence it is tempting to assign the function of proteolytic activation of the Cry1Ac protoxin in the larval midgut to protease HaSP2, although we cannot rule out other proteases also being implicated.

As already emphasized, the use of transgenic plants expressing the Cry1Ac protein has revolutionized the field of insect pest management for the last few years. This ICP is toxic to most of the important lepidopteran insect pests of major agricultural crops such as *Heliothis virescens*, *H. armigera*, *Pectinophora gossypiella*, *Earias vitella* and *Plutella xylostella*. The Cry1Ac protein expressed in these transgenic plants is in the form of the 135 kDa protoxin [26]. The insect consumes this protoxin, which is then proteolytically activated in the insect gut to the more active 65 kDa toxin form. Several studies, including the present study, have demonstrated that development of resistance to this ICP is mediated by changes in the protease profile of resistant insects leading to altered protoxin processing [9,19,20,22,23,27]. The major practice suggested and assiduously implemented in growing transgenic *B. thuringiensis* crops is to grow a 20 % refuge population of non-transgenic crop plant so that the chances of a homozygous resistant population is greatly reduced. Furthermore, much of this practice is modelled on the basis of a change in the site of the receptor in the resistant insects. A recent report has indicated that *Helicoverpa zea* has developed field-level...
resistance against Cry1Ac on transgenic crops in the U.S.A. [28]. An alternate approach to manage possible resistance to Cry1Ac in target insects is to deploy structurally divergent insecticidal proteins with a different mechanism of action. A number of studies have reported the ability of insects resistant to Cry1Ac to acquire cross resistance to structurally similar insecticidal proteins such as Cry1Aa, Cry1Ab, Cry1C etc. It has also been shown that insecticidal proteins such as Cry2Ab, Cry1F and Cry9 retain their ability to kill Cry1Ac-resistant insects, although instances of insects developing resistance to both Cry1Ac and Cry2Ab have been reported [29,30]. Importantly, our results reveal that an H. armigera population showing 72-fold resistance to Cry1Ac protoxin (which has reduced HaSP2 protease activity) remains susceptible to trypsin-activated Cry1Ac and to Cry2Ab, indicating that activation of Cry2Ab is not affected in Cry1Ac-resistant insects. This result gives further credence to the earlier observation of improper activation of the Cry1Ac protoxin as a reason for resistant phenotype and the effectiveness of gene pyramiding (such as BollGard II) with two different Cry proteins offering effective protection against development of resistance to Cry1Ac.

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SUPPLEMENTARY ONLINE DATA

Resistance of *Helicoverpa armigera* to Cry1Ac toxin from *Bacillus thuringiensis* is due to improper processing of the protoxin

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**Figure S1** Ligand–blot analysis of Cry1Ac-resistant and -susceptible BBMV to Cry1Ac

Membrane preparations (10 μg of protein) from Cry1Ac-susceptible and -resistant *H. armigera* 5th instar larvae were subjected to SDS/7.5 % PAGE and transferred on to a nitrocellulose membrane. After blocking the membranes in 3 % BSA contained in PBS, they were incubated with 200 ng/ml activated Cry1Ac toxin for 1 h. The blot was then washed three times with PBS and overlaid with Cry1Ac rabbit polyclonal antibodies for 1 h. After three washes with PBS, they were overlaid with alkaline phosphatase-conjugated anti-rabbit secondary antibodies. The blot was developed with Nitro Blue Tetrazolium after washing three times with PBS. Lane 1, Cry1Ac-susceptible insect; lane 2, Cry1Ac-resistant insect; lane 3, Bio-Rad pre-stained marker with molecular masses to the right in kDa.

**Figure S2** Imprecise processing of Cry1Ac protoxin by *H. armigera* larval midgut proteases

The 95 and 68 kDa proteins resolved in Figure 2(B) were transferred to nylon membrane and sequenced for the first 10 amino acids from the N-terminus. The 95 and 68 kDa polypeptides retained the N-terminal sequence of the protoxin whereas the 68 kDa protein from the Cry1Ac-susceptible insect has the activated toxin form starting from the 28th amino acid.

**Figure S3** Heterologus expression of midgut serine protease HaSP2 and its ability to activate Cry1Ac protoxin

(A) The gene encoding HaSP2 was cloned in-frame to an N-terminal fusion protein in the vector pET32(b) and transformed into BL21(DE3) *E. coli* cells. Such transformed cells were induced with IPTG for 3 h, which resulted in the expression of HaSP2 protein in the soluble fraction of *E. coli* cells, shown in lane 1. Lane 2, uninduced cells; lane 3, protein molecular-mass marker (Bio-Rad low-molecular-mass); lane 4, Ni-NTA affinity chromatography purified protein. (B) The heterologously expressed HaSP2 protein was purified by affinity chromatography using Ni-NTA resin and 50 μg of this protein was incubated with 10 μg of Cry1Ac protoxin overnight at 37°C. The reaction was stopped by adding 4× SDS/PAGE sample buffer and the samples were resolved by SDS/7.5 % PAGE. The protein bands were visualised by staining with Coomassie Blue. Lane 1, BioRad broad range pre-stained marker; lane 2, HaSP2 purified protein; lane 3, Cry1Ac protoxin incubated with the purified protease; lane 4, Cry1Ac protoxin. The activated form of Cry1Ac (65 kDa) is indicated by an arrow. Molecular masses are shown to the left in kDa.

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The nucleotide sequence data reported for HaSP1, HaSP2, HaSP3, HaSP4, HaSP5 and HaSP6 will appear in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession numbers EF104918, EF104913, EF104914, EF104915, EF104916 and EF104917 respectively.

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