Construction of Chimeric Insecticidal Proteins between the 130-kDa and 135-kDa Proteins of *Bacillus thuringiensis* subsp. *aizawai* for Analysis of Structure–Function Relationship

Keiko NAKAMURA, Kazuyuki OSHIE, Masatoshi SHIMIZU,
Yoji TAKADA,* Kenji OEDA
and Hideo OHKAWA

Biotechnology Laboratory, and *Agricultural Science Research Laboratory,
Takarazuka Research Center, Sumitomo Chemical Co., Ltd.,
Takatsukasa 4–2–1, Takarazuka, Hyogo 665, Japan

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Eight chimeric insecticidal protein (IP) genes were constructed between the 130-kDa and 135-kDa IP genes of *Bacillus thuringiensis* subsp. *aizawai*, and expressed in *Escherichia coli* JM103 cells. The characterization of the produced chimeric IPs indicated that the variable region (VR1) in the amino-terminal half of the IPs is responsible for the insecticidal activity against larvae of *Spodoptera litura* and *Plutella xylostella*. The carboxy-terminal half of VR1 was important for the formation of the 60-kDa active fragment in the gut juice of *S. litura* larvae. Also, combination of the other two variable regions (VR2 and VR3), which were in the central and carboxy-terminal portions of the IPs, appeared to be related to the solubility of the IPs in the gut juice.

Bacillus thuringiensis strains produce proteinaceous crystals which are toxic to insect larvae such as Lepidoptera, Diptera, and Coleoptera.¹⁾ Protein crystals of each strain contain more than one insecticidal protein (IP) with molecular masses ranging from 28 kDa to 140 kDa. In particular, lepidopteran toxin crystals, which consists of 130-kDa to 135-kDa IPs, are dissolved in an alkaline gut juice and are processed by gut-juice proteases to yield active fragments with molecular masses of approximately 60 kDa upon ingestion by susceptible insect larvae.²⁾ A number of lepidopteran toxin genes were cloned from several serotypes of *B. thuringiensis* and some of them were expressed in *Escherichia coli*^{3–11)} and *B. subtilis*.^{12,13)}

We cloned both 130-kDa and 135-kDa IP genes from *B. thuringiensis* subsp. *aizawai* IPL7, which is insecticidal against lepidopteran larvae.^{14,15)} Comparison of the primary structures deduced from the 130-kDa and 135-kDa IP genes showed that the IPs have 90.2% similarity in their total sequences and amino acid differences occur mainly in three variable

regions. Also, both 130-kDa and 135-kDa IP genes were expressed in *E. coli* cells. The 130-kDa IP produced had much higher insecticidal activities against *Plutella xylostella* and *Spodoptera litura* than the 135-kDa IP.

In this report, we constructed expression plasmids for eight chimeric IPs by exchanging fragments prepared by the cleavage at homologous sites of both 130-kDa and the 135-kDa IP genes, and expressed them in *E. coli* cells to produce chimeric IPs. The chimeric IPs produced were examined for solubility in an alkaline buffer, proteolytic processing by gut-juice proteases, and insecticidal activity against larvae of *S. litura* and *P. xylostella*. The structure–function relationship of the 130-kDa and 135-kDa IPs was also discussed.

Materials and Methods

Biochemicals. Restriction enzymes, DNA modifying enzymes, and a DNA ligation kit were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan).

Bacterial strains, plasmids and media. *Escherichia coli* strains JM109 [*recA1 endA1 gyrA96 thi hsdR17 supE44*

relA1 λ^- $\Delta(lac-proAB)$, *F'traD36 proAB lac⁺ZAM15*] and JM103 [*thi strA supE endA sbcB15 hsdR4* $\Delta(lac-pro)$, *F'traD36 proAB lac⁺ZAM15*] were obtained from Pharmacia Biochemicals Co., Ltd. (Uppsala, Sweden). The vector pKK223-4, and the expression plasmids pTB1 for the 130-kDa IP gene and pKC6 for the 135-kDa IP gene were described previously.^{14,15} *E. coli* cells carrying each of the constructed plasmids were cultivated at 37°C in L-broth supplemented with 100 µg/ml of ampicillin.

Recombinant DNA procedure. Plasmid DNAs were prepared from *E. coli* cells by the method of Birnboim and Doly.¹⁶ Restriction DNA fragments were recovered from corresponding agarose gel slices after 0.8% agarose gel electrophoresis. Transformation of *E. coli* cells was done as described by Cohen *et al.*¹⁷

Construction of expression plasmids for chimeric IPs. The expression plasmid pTB1 for the 130-kDa IP gene was digested with *Kpn*I and *Pst*I to obtain a *Kpn*I–*Pst*I fragment (6.7 kb) which was called fragment A1. The plasmid pTB1 was also digested with *Kpn*I, *Hind*III, and *Pst*I to isolate *Kpn*I–*Hind*III (0.6 kb) and *Hind*III–*Pst*I (0.7 kb) fragments, called fragments A2 and A3, respectively. Similarly, *Kpn*I–*Pst*I (6.7 kb), *Kpn*I–*Hind*III (0.7 kb), and *Hind*III–*Pst*I (0.7 kb) fragments, called fragments C1, C2, and C3, respectively, were prepared from the expression plasmid pKC6 for the 135-kDa IP gene. The resulting DNA fragments A1 (C1), A2 (C2), and A3 (C3) were religated with a change in assortment and then introduced into *E. coli* JM109 cells to construct six expression plasmids, pACC1, pCAA1, pAAC1, pCAC1, pACA1, and pCCA1. The expression plasmids pACA1 and pCCA1 were digested by *Bam*HI and then partially digested by *Eco*RI to isolate *Bam*HI–*Eco*RI (1.1 kb) fragments, called fragments A11 and C11, respectively. The plasmids pACA1 and pCCA1 were also digested with *Eco*RI and *Eco*RV to prepare *Eco*RI–*Eco*RV (0.6 kb) fragments, called fragments A12 and C12. Similarly, a *Eco*RV–*Bam*HI fragment (6.4 kb) called fragment CA was prepared from pCCA1. Those DNA fragments were ligated in combinations of A11, C12, and CA, or C11, A12, and CA to construct two expression plasmids, pACCA1 and pCACA3, respectively. The structures of these plasmids were confirmed by restriction enzyme mapping.

Protein analysis. *E. coli* cells harboring each of the expression plasmids were grown at 37°C in L-broth to a density of 2×10^8 cells/ml. The cells were harvested from 40-µl samples of the cultures and suspended in 30 µl of a sample buffer [2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol, and 62.5 mM Tris hydrochloride (pH 6.8)], and boiled for 5 min, cooled, and sedimented at $12,000 \times g$ for 10 min. The clear supernatants obtained were analyzed by SDS–7.5% polyacrylamide gel electrophoresis (PAGE).¹⁸ Proteins were

stained with Coomassie brilliant blue.

Isolation of inclusion bodies from recombinant *E. coli* cells. Transformed *E. coli* cells (1 g wet weight) were harvested from cultures at stationary phase, resuspended in 20 ml of distilled water, and disrupted by sonication (10 min, 40 W) with a Branson Sonifier 250 after twice freeze-thaw treatments. Inclusion bodies were collected by centrifugation of the resulting cell extracts at $6,000 \times g$ for 5 min.

Solubilization of inclusion bodies in an alkaline buffer. Isolated inclusion bodies (34 µg protein) were suspended in 36 µl of 25 mM Na₂CO₃–HCl buffer (pH 9.65), and incubated at 30°C for 60 min. The suspensions were centrifuged at $10,000 \times g$ for 10 min to separate supernatant fractions (soluble fractions) from pellets (insoluble fractions). Samples of both soluble and insoluble fractions were analyzed by SDS–10% PAGE.

Digestion of chimeric IPs in a larval gut juice of *Spodoptera litura*. Fourth-instar larvae of *S. litura* were nipped with a forceps and vomited matters were centrifuged at $10,000 \times g$ for 10 min to collect a gut-juice fraction. Inclusion bodies (17 µg protein) were suspended in 18 µl of 25 mM Na₂CO₃–HCl buffer (pH 9.65) and incubated at 30°C with 2 µl of the gut-juice fraction, which was diluted five-fold with the carbonate buffer. The boiled sample buffer (20 µl) was added to each of the reaction mixtures to stop digestion, and then half of the volume of each reaction mixture was analyzed by SDS–10% PAGE. Purification of a 60-kDa fragment from a digestion mixture of the 130-kDa IP was done as described by Nagamatsu *et al.*¹⁹

Insecticidal activity. Chimeric IPs prepared from recombinant *E. coli* cells were assayed for insecticidal activity against larvae of *S. litura* and *P. xylostella*. For tests against *S. litura*, 1 ml of a suspension of each of the chimeric IPs (1 mg or 0.3 mg protein per ml of distilled water) was added to 13 g of an artificial diet, which was then fed to ten of the fourth-instar larvae at 25°C. Mortality was monitored after 3 days. For tests against *P. xylostella*, a punched cabbage leaf with a diameter of 5 cm was dipped into a protein suspension of chimeric IPs (2 or 0.2 µg protein per ml of distilled water), and then dried at room temperature. Ten of the third-instar larvae were fed on the leaf disk at 25°C, and mortality was monitored after 4 days.

Results

Construction of expression plasmids for chimeric IPs

Figure 1 illustrates the overall strategy for construction of expression plasmids for chimeric

Fig. 1. Construction of Expression Plasmids for Chimeric IPs.

The expression plasmids pTB1 and pKC6 for the 130-kDa and 135-kDa IP genes were divided into three fragments by digestion at *KpnI*, *HindIII*, and *PstI* sites. The three fragments of pTB1 were exchanged with the corresponding fragments of pKC6, and then religated to yield six chimeric expression plasmids pACC1, pCAA1, pAAC1, pCAC1, pACA1, and pCCA1. The expression plasmids pACC1 and pCAA1 were constructed by exchanging the *BamHI*–*EcoRI* fragment and *EcoRI*–*EcoRV* fragment of the plasmid pCCA1 with the corresponding fragments of the plasmid pACA1. Hatched and open boxed regions indicate the coding regions for the 130-kDa and 135-kDa IPs, respectively. Black and dotted arrows show the *tac* promoter and *rrnB* transcription terminator, respectively. Thin lines represent the vector plasmid pKK223-4. Restriction enzyme sites are shown as follows: B, *BamHI*; E, *EcoRI*; H, *HindIII*; K, *KpnI*; P, *PstI*; V, *EcoRV*.

ic IPs. Six chimeric IP genes were constructed by exchanging three fragments and two sub-fragments prepared by cleavage at homologous sites of both 130-kDa IP and 135-kDa IP genes. Both pTB1 and pKC6 were cleaved at *KpnI*, *HindIII*, and *PstI* sites, to divide the coding regions into three fragments. The resulting fragments, *PstI*–*KpnI*, *KpnI*–*HindIII*, and *HindIII*–*PstI*, included the variable regions VR1, VR2, and VR3, respectively. Thus,

the 130-kDa and 135-kDa IPs divided into the three segments were called AAA and CCC, respectively. The three prepared DNA fragments were religated with a change in assortment to yield six expression plasmids, pACC1, pCAA1, pAAC1, pCAC1, pACA1, and pCCA1, for chimeric IPs called ACC, CAA, AAC, CAC, ACA, and CCA, respectively. Both pACA1 and pCCA1 were cleaved at the *EcoRI* site to divide the amino-terminal A and

C segments into two portions. The resulting *Bam*HI-*Eco*RI and *Eco*RI-*Eco*RV fragments of the plasmid pCCA1 were exchanged with the corresponding fragments of the plasmid pACA1 to yield two expression plasmids, pACCA1 and pCACA3, for chimeric IPs called ACCA and CACA, respectively. In the constructed expression plasmids, the chimeric IP genes were each inserted between the *tac* promoter and the *rrnB* terminator in the correct orientation.

Production of chimeric IPs in *E. coli*

E. coli JM103 cells were transformed with each of the constructed expression plasmids. The transformants were cultivated at 37°C for 15 hr and then analyzed by SDS-PAGE. The intensive bands with molecular masses ranging from 130 kDa to 135 kDa as estimated from the constructed expression plasmids were found in the recombinant *E. coli* strains (Fig. 2A, lanes 2 to 11). No protein bands corresponding to molecular masses from 130 kDa

to 135 kDa were found in the control cells (JM103/pKK223-4) (Fig. 2A, lane 1). The bands with molecular masses from 130 kDa to 135 kDa were reacted with anti-IP antibodies raised against the protein crystals consisting of the 130 and 135-kDa IPs of the *aizawai* IPL7 strain (data not shown). Under optimal culture conditions, the chimeric IPs amounted to approximately 20–30% of the total cellular protein, as measured by densitometric scanning of the SDS-PAGE gels (data not shown). The molecular masses of the chimeric IPs, AAC, CAA and CAC, which contained a 26 amino acid deletion, were estimated to be about 130 kDa on the basis of the mobility on the SDS-PAGE gels. Similarly, the molecular masses of the other chimeric IPs such as ACA, ACC, CCA, ACCA, and CACA were approximately 135 kDa. Judging from intensity of the protein bands in Fig. 2A, the 135-kDa IPs, CCC, ACA, ACC, CCA, and CACA, were produced to slightly higher extents in the recombinant *E. coli* cells than the

Fig. 2. Polyacrylamide Gel Analysis of Chimeric IPs Produced in Recombinant *E. coli* Cells.

A. *E. coli* JM103 cells harboring each of the expression plasmids were cultivated at 37°C for 15 hr, and cell extracts were analyzed by SDS-7.5% PAGE. Proteins were stained with Coomassie brilliant blue. Lanes: 1, JM103/pKK223-4; 2, JM103/pTB1; 3, JM103/pKC6; 4, JM103/pAAC1; 5, JM103/pACA1; 6, JM103/pACCA1; 7, JM103/pCCA1; 8, JM103/pCAA1; 9, JM103/pCAC1; 10, JM103/pACCA1; 11, JM103/pCACA3. B. Inclusion bodies of the chimeric IPs were prepared from cultured recombinant *E. coli* cells, and 4.5-μg samples of protein preparations were analyzed by SDS-7.5% PAGE. Proteins were stained with Coomassie brilliant blue. Lanes: 1, AAA; 2, CCC; 3, AAC; 4, ACA; 5, ACC; 6, CCA; 7, CAA; 8, CAC; 9, ACCA; 10, CACA.

130-kDa IPs such as AAA, AAC, CAA, and CAC, and the 135-kDa IP ACCA.

When observed under a phase contrast microscope, the cultured *E. coli* cells carrying each of the expression plasmids contained inclusion bodies at stationary phase. The inclusion bodies were isolated from the cultured recombinant *E. coli* cells by the combination of sonication and centrifugation, and then the proteins in the inclusion bodies were analyzed by SDS-PAGE (Fig. 2B). Densitometric scanning showed that the inclusion body preparations contained the corresponding chimeric IPs with approximately 80% purity (data not shown).

Solubility in an alkaline buffer of inclusion bodies

Solubilization under non-reducing alkaline conditions of IP crystals seems to be the first step for the activation processes in insect guts. Therefore, the inclusion bodies prepared from the cultured recombinant *E. coli* cells were examined for solubility in 25 mM Na_2CO_3 -HCl buffer (pH 9.65), the pH of which was adjusted to that of the gut-juice fraction prepared from

S. litura larvae. After incubation of the inclusion bodies in the alkaline buffer, the reaction mixtures were centrifuged to separate soluble fractions from insoluble precipitates. Both fractions were analyzed by SDS-PAGE. As shown in Fig. 3, the inclusion bodies of ACC and CCC were solubilized to the degrees of 85–90%. On the other hand, the inclusion bodies of ACA and CCA were rather insoluble under the same conditions and were recovered to degrees of less than 5% in the soluble fractions. These results indicated that the inclusion bodies of the chimeric IPs with the structure of XXC (X implies A or C) were soluble, but those of the chimeric IPs with the structure of XCA were rather insoluble in the alkaline buffer. The inclusion bodies of AAA, CAA, AAC, and CAC containing the central A segment showed intermediate solubility in the alkaline buffer. Both AAA and CAA were solubilized to extents of 50–55%, but AAC and CAC had slightly less solubility than AAA and CAA. These results suggested that the combination of the central C segment and the carboxyl-terminal C or A segment is important for determination of solubility of the inclusion

Fig. 3. Solubilization in an Alkaline Solution of Inclusion Bodies of Chimeric IPs.

Inclusion bodies of chimeric IPs were prepared from cultured recombinant *E. coli* cells as described in Materials and Methods, and incubated in 25 mM Na_2CO_3 -HCl buffer (pH 9.65) at 30°C for 60 min. Both supernatant and pellet fractions were prepared by centrifugation and then analyzed by SDS-10% PAGE. Lanes: 1 and 2, ACC; 3 and 4, CCC; 5 and 6, AAA; 7 and 8, CAA; 9 and 10, AAC; 11 and 12, CAC; 13 and 14, ACA; 15 and 16, CCA; lanes 1, 3, 5, 7, 9, 11, 13, and 15, for the supernatants; lanes 2, 4, 6, 8, 10, 12, 14, and 16, for the pellets.

Fig. 4. Processing of the 130-kDa IP in the Gut Juice of *Spodoptera litura* Larvae.

The 130-kDa IP (AAA) produced in the *E. coli* JM103/pTB1 cells was purified and incubated with the gut juice of *S. litura* larvae as described in Materials and Methods. The reaction mixtures corresponding to 8.5 μ g of the AAA were analyzed by SDS-10% PAGE. Lanes: 1, AAA; 2, 3, 4, 5, 6, and 7, AAA incubated with the gut juice for 2, 30, 60, 120, 240, and 480 min, respectively; 8, the gut-juice fraction.

bodies of the chimeric IPs in the alkaline buffer.

Processing of chimeric IPs in a larval gut juice

The inclusion bodies of the 130-kDa IP (AAA) produced in the JM103/pTB1 cells were incubated with the gut-juice fraction prepared from *S. litura* larvae in the alkaline buffer (pH 9.65). As shown in Fig. 4, the parental AAA was rapidly digested to yield a major polypeptide with the molecular mass of 62 kDa after 2 min of incubation. The polypeptide was then converted to a 60-kDa fragment during further incubation. The 60-kDa fragment was maintained during 8 hr incubation. The 60-kDa fragment was purified and confirmed to be as toxic as the parental AAA against *S. litura* larvae. In addition, the amino-terminal five amino acid residues of the purified 60-kDa fragment were identical to the sequence from amino acid residue 29 to 33 of the parental AAA (data not shown).

The chimeric IPs produced in the recombinant *E. coli* cells were also incubated with

Fig. 5. Proteolytic Digestion of Chimeric IPs.

Chimeric IP preparations and reaction conditions were the same as described in the footnotes of Fig. 4. Chimeric IP preparations were each incubated with the gut juice for 45 min, and then analyzed by SDS-10% PAGE. Lanes: 1 and 2, AAA; 3 and 4, AAC; 5 and 6, ACA; 7 and 8, ACC; 9 and 10, CCC; 11 and 12, CCA; 13 and 14, CAA; 15 and 16, CAC; 17 and 18, CACA; 19 and 20, ACCA; 21, the gut-juice fraction. 1, 3, 5, 7, 9, 11, 13, 15, 17, and 19, incubated without the gut juice; 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20, incubated with the gut juice.

the gut juice in the alkaline buffer. Samples of the reaction mixtures were analyzed by SDS-PAGE before and after incubation. As shown in Fig. 5, the chimeric AAC, ACA, and ACC (AXX) containing the amino-terminal A segment yielded nearly the same amount of 60-kDa to 62-kDa fragments as those from the parental AAA (Fig. 5, lanes 2, 4, 6, and 8). On the other hand, the 60-kDa to 62-kDa fragments were hardly found with CCC, CCA, CAA, and CAC, (CXX), which contained the amino-terminal C segment (Fig. 5, lanes 10, 12, 14, and 16). The chimeric CACA produced 60-kDa to 62-kDa fragments to a extent similar to that of the parental AAA, while the other chimeric ACCA showed faint bands (Fig. 5, lanes 18 and 20). Thus, the chimeric IPs containing the carboxy-terminal half of the amino-terminal A segment yielded nearly the same amount of the 60-kDa to 62-kDa fragments as those from the parental AAA. Therefore, this segment seemed to be closely related to the formation of the 60-kDa to 62-kDa fragments from the IPs.

Insecticidal activity of chimeric IPs

The insecticidal activity of the chimeric IPs

prepared from the cultured recombinant *E. coli* cells was tested against larvae of *S. litura* and *P. xylostella*. Table I shows the averaged values of several repeated experiments. The chimeric IPs containing the amino-terminal A segment (AXX) showed nearly the same insecticidal activities as those of the parental AAA against both *S. litura* and *P. xylostella*, although AAC was slightly less active against *S. litura* than the other three IPs (AAA, ACC, and ACA). The chimeric IPs with the amino-terminal C segment (CXX) showed the insecticidal activity against *P. xylostella* similar to that of the parental CCC, while CAA and CAC were less active against *S. litura* than the others (CCC and CCA). The chimeric CACA and ACCA had much lower activities against *S. litura* than ACA and CCA. On the other hand, both CACA and ACCA showed nearly the same activities against *P. xylostella* as those of ACA and CCA, respectively. These results indicated that the amino-terminal A segment is responsible for the insecticidal activity against both *S. litura* and *P. xylostella*, and the carboxy-terminal half of the amino-terminal A segment is important for the insecticidal activity against *P. xylostella*. The

Table I. INSECTICIDAL ACTIVITIES OF CHIMERIC IPs PRODUCED IN RECOMBINANT *E. coli* CELLS

Chimeric IP ^a	% Mortality			
	<i>Spodoptera litura</i> ^b		<i>Plutella xylostella</i> ^c	
	Dose		Dose	
	0.3 mg/ml	1 mg/ml	0.2 µg/ml	2 µg/ml
AAA (130 kDa)	60	90	30	100
ACC	50	100	40	100
AAC	10	60	10	80
ACA	50	100	30	70
CCC (135 kDa)	10	90	10	20
CCA	0	90	20	20
CAA	0	30	20	30
CAC	0	0	10	20
<u>CACA</u>	0	10	30	80
<u>ACCA</u>	0	0	20	20

^a Each of chimeric IPs produced in recombinant *E. coli* cells was partially purified as described in Materials and Methods.

^b Counted 3 days after treatment of 10 larvae per sample.

^c Counted 4 days after treatment of 10 larvae per sample.

amino-terminal segment covered the entire 60-kDa active fragment.

Discussion

Figure 6 is a comparison of the amino acid sequences deduced from the 130-kDa and 135-kDa IP genes of the *aizawai* strain. The overall similarity in the total amino acid sequences was 90.2%. Amino acid differences, deletions, and insertions occurred mainly in the three variable regions, VR1, VR2, and VR3. VR1 was from amino acid residues 206 to 455 of the 135-kDa IP, which contained 68 amino acid differences in the 250 amino acid residues. VR2 from amino acid residues 788 to 819 contained a 26 amino acid insert and one amino acid difference. VR3 from amino acid residues 1,061 to 1,137 contained a 4 amino acid deletion and 15 amino acid differences in the 77 amino acid residues. The 130- and 135-kDa IPs contained 12 and 17 cysteine residues, respectively. Particularly, the 26 amino acid insert in the VR2 region derived from the 135-kDa IP contained 4 cysteine residues.

Eight chimeric IP genes were constructed by exchanging three fragments and two subfragments prepared by cleavage at the homologous sites of the 130-kDa IP (AAA) gene with the

corresponding fragments of the 135-kDa IP (CCC) gene, and were expressed in *E. coli* cells. Although the production levels of most of the chimeric IPs were nearly the same as those of the parental AAA and CCC, the chimeric IPs containing the central C segment (XCX) were produced at slightly higher levels than the chimeric IPs containing the central A segment (XAX). Huber *et al.*²⁰⁾ reported that the cysteine residues in the IPs of subsp. *kurstaki* HD-1 are involved in crystal formation. Thus, this may suggest that the additional four cysteine residues (794, 800, 812, and 814) located in the 26 amino acid insert in the VR2 region of the central C segment are important for stability in the recombinant *E. coli* cells of the chimeric IPs, probably due to formation of intrachain disulfide bonds. The chimeric IPs with different combinations of the central and carboxy-terminal segments showed differences in the solubility in the alkaline buffer under non-reducing conditions. The chimeric IPs with the structure of XCC were soluble and the IPs with XCA were rather insoluble. Couche *et al.*²¹⁾ reported that solubilization of the crystal proteins of *B. thuringiensis* subsp. *israelensis* required disulfide cleavage, either in the presence of a reducing agent such as mercaptoethanol or at pH 11.75 or greater. It is, there-

Fig. 6. Comparison of the Amino Acid Sequences of Both 130-kDa and 135-kDa IPs of *B. thuringiensis* subsp. *aizawai*.

Boxes show the entire primary structures of both 130 and 135-kDa IPs, and vertical lines in the 135-kDa IP indicate the amino acid residues which are different from those of the 130-kDa IP sequence. Gaps show deleted regions. Arrowheads indicate location of cysteine residues. VR1, VR2, and VR3 indicate the variable regions found in the amino acid sequences between the 130-kDa and 135-kDa IPs. Numerals indicate amino acid residues of the 135-kDa IP. N, Amino-terminus; C, carboxy-terminus.

fore, presumable that the solubility of the chimeric IPs is related to combination of the cysteine residues located in the VR2 and VR3 regions.

Digestion of the parental AAA with the gut juice prepared from *S. litura* larvae produced a 60-kDa active fragment. Nagamatsu *et al.*¹⁹⁾ isolated a trypsin-resistant toxic peptide with the molecular mass of 58 kDa from the IP of *B. thuringiensis* subsp. *dendrolimus*. Höfte *et al.*⁹⁾ also purified a polypeptide with the molecular mass of 60 kDa from the IP of *B. thuringiensis* subsp. *berliner* 1715. The amino-terminal five amino acid residues of the 60-kDa fragment derived from the parental AAA was identical to those of the reported trypsin-resistant peptides.^{9,19)} The comparison of digestion profiles of the chimeric IPs with the gut juice of *S. litura* larvae suggested that the amino-terminal A segment, particularly, the carboxy-terminal half of VR1 ranging from positions 333 to 456 is important for formation of the 60-kDa active fragment in the gut juice.

Comparison of the insecticidal activities of the chimeric IPs against larvae of *S. litura* and *P. xylostella* suggested that VR1 of the amino-terminal A segment is responsible for the activity. In particular, the carboxy-terminal half of VR1 was important for the activity against *P. xylostella*, although the region was not sufficient for the activity against *S. litura*. Also, the chimeric IPs with different combinations of VR1 (ACA, CCA, CACA, ACCA) had different insecticidal specificities. So, VR1 appeared to be important in establishing the insecticidal specificity of the IPs. In addition, the chimeric IPs with different combinations of VR2 and VR3 derived from the parental AAA and CCC were different to some extent in the insecticidal activity against *S. litura*. Thus, VR2 and VR3, which were outside of the 60-kDa active fragment, may be related to activation processes in the gut juice.

Recently, Ge *et al.*²²⁾ reported the construction of chimeric IPs between *B. thuringiensis* subsp. *kurstaki* HD-1 IP and *B. thuringiensis* subsp. *kurstaki* HD-244 IP. Comparison of the insecticidal activity against

Bombyx mori of the chimeric IPs produced in *E. coli* cells suggested that the *B. mori* specificity domain of the HD-1 IP is in the amino-terminal portion of the hypervariable region (positions 332 to 450). This observation agreed well with our results showing that VR1, in the 60-kDa active fragment, is important for insecticidal activity of the chimeric IPs.

Elucidation of the structure-function relationships related to VR1, VR2, and VR3 appears to be useful for design of engineered IPs with improved insecticidal activity, insecticidal spectra, and stability.

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