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Molecular Cloning and Hyperexpression of a Bt Gene, *cryIAc*, in *Escherichia coli* DH5α: Production and Usage of Anti-CryIAc Antibody

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Abstract The gene coding for a Lepidoptera-specific insecticidal crystalline (or control) protein (ICP), recognized as *cryIAc*, from *Bacillus thuringiensis* subsp. *kurstaki* HD-73, was cloned into the vector pBluscript II SK-, and then transformed in *Escherichia coli* DH5α. The clone was named EBtIAc and the chimeric phagemid, as pEBtIAc. Hyperexpression of CrylAc protoxin was observed in the extract of the culture of *E. coli* harboring pEBtIAc. Crystalline protoxin was purified by differential solubility. It was dissolved in alkaline pH, and exposed to trypsin to be activated. The molecular weights of the pro- and activated toxins on SDS-PAGE were estimated to be *ca.* 130 kDa and 60 kDa, respectively. The toxicity was tested by force-feeding larvae of gypsi moth (*Lymantria dispar*) with trypsinized protoxin. Using the batch of biologically active form of the toxin as an immunogen, anti-CryIAc antiserum was raised in a New Zealand white rabbit. Immunoglobulin G was fractionated from the serum by Protein-A sepharose affinity chromatography. Immunoreactivity of the antibody was examined by dot and Western blottings. It has been found that the anti-CryIAc antibody recognized the purified toxin at a level below a nanogram in terms of quantity. Using the antibody some of Bt-corns were able to be differentiated from tons of corn kernels which were imported from America as forage crops.

Key words: *cryIAc*, Anti-CryIAc antibody, Bt-corns

Some species of *Bacillus thuringiensis* produce proteinous parasporal crystalline inclusions exhibiting toxic action with a high specificity against larvae of the phylum Insecta without known deleterious effects on humans, therefore, they are the best choice among the known biopesticides [4, 13, 21]. They are often called insecticidal control (or crystal) proteins (ICPs), Bt toxins, or even more simplified as Bt, and a gene encoding for the toxin is generally named "*cry*", or "*bt*" (for reviews, see reference [9, 11, 13, 18, 21]). Many of these genes have been cloned from various sources [1, 15, 20, 22, 27], and were modified to fit into the expression system of eukaryotes to produce transgenic crop plants [3, 5, 8, 15, 19, 23, 24]. Few transgenic Bt-crops, however, have not entirely controlled targets in the field. A transgenic Monsanto-patented Bt-cotton (distributed by Delta Pine and Land Co., U.S.A.) has failed to control cotton bollworm in the State of Texas, U.S.A. [14]. Unfortunately, no one has yet explained the reasons and consequences of this case. A group of molecular biologists at the Rural Development Administration (RDA) of the Ministry of Agriculture and Forestry in Suwon, Korea has reported that they have successfully generated a number of genetically modified organisms (GMOs) and that some of their seeds have been ready to be distributed to selected farmers. However, future developments in transgenic agriculture are now facing a serious challenge from various systems in conjunction with concerned non-governmental organizations (NGOs) around the world. Two major issues are raised by environmentalists and/or consumers relating to transgenic Bt-crops or their products. First, it is argued that GMOs contain unwanted genes and/or DNA fragments, such as antibiotic resistant determinants, bacterial or viral promoters, which could have a deleterious effect on humans. Second, demand is made to make it easy to differentiate GMOs from their wild types. Nonetheless, whether based on legitimate worries or not, the first commercial Bt-transgenic crops are facing serious protests around the world. For this reason, it may be advantageous for producers to use purified Bt toxin rather than using transgenic Bt-crops. Unfortunately, purified Bt toxins are not very stable, so it is not feasible to use them directly in the field [3, 9]. It is, therefore, a good idea to increase their stability by pegilation of Bt, or to modify the gene to produce a toxin exhibiting a stronger activity than that of the wild type. What is needed is to

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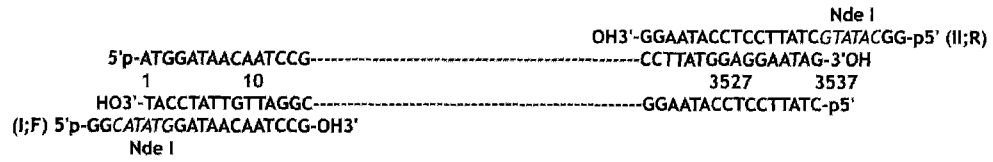

Fig. 1. Primer binding regions of the gene, *cryIAc*, for PCR cloning.
The primer I (F) binds to the 5' region of the gene, whereas the primer II (R) binds to the 3' end of the gene.

have a Bt-gene cloned in a stable and easily manipulable form. In this research, a Bt gene (*cryIAc* of *Baccilus thuringgiensis* subsp. *kurstaki* HD-73) amplified by PCR, was recombined into a prokaryotic cloning/expression vector, pBluscript II SK-, and then transformed into *E. coli* DH5α. Hyperexpression of the *cryIAc* gene was examined, and its toxicity was confirmed by using the forced-feeding method. Using this polypeptide as an immunogen, anti-CryIAc polyclonal antibody was raised in a rabbit to differentiate GMOs from natural agro-products grown all around the world including Korea.

Biosamples, Cloning Vehicles and Major Protocols

Bacillus thuringiensis subsp. *kurstaki* HD-73 was grown to sporulation, and a 75 kb long plasmid known to contain *cryIAc* was fractionated from the culture by sucrose gradients [7, 20]. The plasmid was used as a template DNA for gene amplification. The host for gene cloning was *Escherichia coli* DH5α (*supE44 ΔlacU169 [Φ80lacZΔM15] hsdR17 recA1 endA1 gyrA96 thi-1 relA1*). A phagemid, pBluescript II SK- was used as a cloning vector. It was transformed in the *E. coli* DH5α, from which the phagemid was obtained by using the alkaline lysis method, followed by equilibrium ultracentrifugation in CsCl-ethidium bromide density gradient [17, 25]. The prepared DNA was quantified by a spectrophotometer (Beckman DU-65, U.S.A.), and stored at −80°C. For nucleotide sequence analysis, *E. coli* SOLR (Stratagene, U.S.A.), and helper phage M13KO7 (International Biotechnologies Inc., U.S.A.: 1×109 pfu/ml) were used to obtain single strand DNAs. Some U.S.-imported corn (*Zea mays*) kernels obtained from a forage company were grown in a controlled growth chamber (HB-301L Hanback Scientific Co., Korea: 25°C, 12-h-dark and 12-h-light cycle) for three weeks as reported [18]. Korean endemic corn kernels provided by RDA, were grown similarly as the above for the control (None GMO [Non Bt-corn]). Total proteins extracted from three-week old corn leaves were used for immunological identification of any ICP(s) which were expressed during the growth period of imported corns [25].

A gene, *cryIAc*, was cloned into a *Sma*I-restricted pBluescript II SK- by PCR using a 75 kb plasmid for the template DNA [2, 12, 17]. A forward (**I**) and a reverse (**II**) primers were designed for PCR to amplify the entire open reading frame (ORF) of the gene, *cryIAc*. The DNA primers were chemically produced by a DNA synthesizer (Applied Biosystems PCR-MATE Model 391, U.S.A.) [17]. *Nde*I site flanks are in each 5'-end of primer I (**F** [forward: 5'p-GG-*CATATG*-GATAACAATCCG-3'OH]) and primer II (**R** [reverse]: 5'P-GG-*CATATG*-CTATTCCTCC-ATAAG-3OH). The 5'-end of the primers were kinased when in use [17]. Their names and nucleotide sequences along with annealing positions to the template are shown in Fig. 1. The PCR-amplified *cryIAc* DNA was digested with *Nde*I and filled-in 5'-protrudings to produce a blunt-ended fragment. Meanwhile the vector, pBluescript II SK-, was digested with *Sma*I. The two blunt-ended DNAs were ligated and the recombinant phagemid was transfected into *E. coli* DH5α. Transformants were selected by α-complementation on LB-ampicillin (100 mg/ml) agar plates. The clone was named EBtIAc and the chimeric phagemid, pEBtIAc. Nucleotide composition of pEBtIAc, has been determined by the method of Sanger *et al.* [26]. To obtain template DNA for the sequencing, *E. coli* SOLR cell was transformed by pEBtIAc. A single strand DNA was prepared by an aid of a helper phage, M13KO7, and primed by SK or T3-primer. Medium contained IPTG (200 mg/ml) were collected by centrifugation (4,000 ×g, 10 min, 4°C), from which IPCs (pro- and activated-forms) were obtained as before [18, 19]. The solubilization buffer for ICP contained 100 mM Na_2CO_3, 20 mM DTT, pH 10.5. A number of transformed cells of EBtIAc at mid-exponential growth phase (A_{600} of 0.5) in LB digestion conditions were employed to activate the protoxin. A satisfactory result was obtained when the protoxin was incubated at 37°C for 3 h in the solubilizetion buffer containing 2% trypsin. Protein concentration was determined by following the method of Bradford [6]. Polyacrylamde gel (12.5%) electrophoresis was performed under denaturing conditions with SDS (0.1%) where a vertical slab gel unit was used with 1.5 mm spacer arms (Hoeffer Scientific, U.S.A.) [6, 18, 25]. Desired quantity of protein was loaded into a well, and supplied with a constant power (5 volts/cm). After the initial run, the gel was stained with Coomassie Brilliant blue, and a duplicate gel was used for blotting. Under these experimental conditions a good resolution of the pro- or activated toxin was obtained.

Insect toxicity assays were performed as described below. Trypsinized protoxin produced by the recombinant phagemid, pEBtIAc, was subjected to larvae of gypsi moth

(*Lymantria dispar*) obtained from RDA in the Ministry of Agriculture, Suwon, Korea. Concentrated Stock solution of the toxin was diluted serially in a phosphated buffered saline (8 mM Na_2PO_4, 2 mM KH_2PO_4, 150 mM NaCl, pH 7.4), and 2 µl of the diluted solution was administered *per so* through the mouth with an aid of a Hamilton syringe. Controls were fed on 2 µl of buffer solution only. Immediately after the injection, each of the larvae was kept in a cup for 5 days on an artificial diet that was obtained from RDA, and then LC_{50} (fifty *per cent* lethal concentration) of the toxin was determined as previously described [1]. Ten larvae were used per concentration of the toxin in the motility analysis.

Immunization procedures were basically the same as before [16, 25]. Trypsinized protoxin of CryIAc (200 µg in 200 µl) was used as an immunogen. The IgG fraction of the serum was isolated for blottings. Dot immunoassay was employed to measure the titration of antbody, while western blotting was employed to identify a specific protein (*cryIAc* gene product) reacted with anti-CryIAc antibody. Immune complexes were detected on the blots by the application of goat anti-rabbit IgG horseradish peroxidase conjugate (GAR-HRP, a secondary antibody) with horse radish peroxidase substrates (1-chloro-4-naphthol and hydrogen peroxide) system.

Construction of Recombinant *cryIAc* in the Vector, pBluescript II SK-

To place *cryIAc* under the full control of the cloning/expression vehicle, pBluescript II SK-, only the ORF of the gene [3,537 bp: 3534 bp (1,178 AAs x 3 bp) + 3 bp (termination codon, TAG)] was amplified by PCR with forward and reverse primers containing ***Nde*I** restriction sequence, 5'-GG**CATATG**-3'. Therefore, the amplified DNA fragment was 3,550 bp long (ORF 3.537 bp + forward primer 5 bp + reverse primer 8 bp) (Fig. 2B, lane 2). Only the five nucleotides, GG**CAT**, of the forward primer (5'-GG**CAT*ATG***-3') contributed to the formation of the 3,550 bp fragment, since it contained ***ATG*** coding for the first amio acid (Met) of the gene, *cryIAc*. The PCR amplified DNA was digested by *Nde*I and filled the 3'-protruding ends in appropriate nucleotides to produce 3,542-bp long DNA fragment with blunt ends (Fig, 2A: **T**-ORF-**GATA**-). Then, it was ligated to Sma I-linearized pBluescript II SK- (2,958 bp long). As a result, 3'-end ("**A**") of the manipulated 3,542 bp long DNA fragment has been positioned next to the 715th nucleotide ("*G*") of the vector, followed by "**T**", and by the vector's 716th nucleotide ("C") of upper strand of the duplex (Fig. 2A). The circular recombinant phagemid was 6.500-bp long (Fig. 2B, lane 3), and named as pEBtIAc (Fig. 2C). The 716th nucleotide of the original

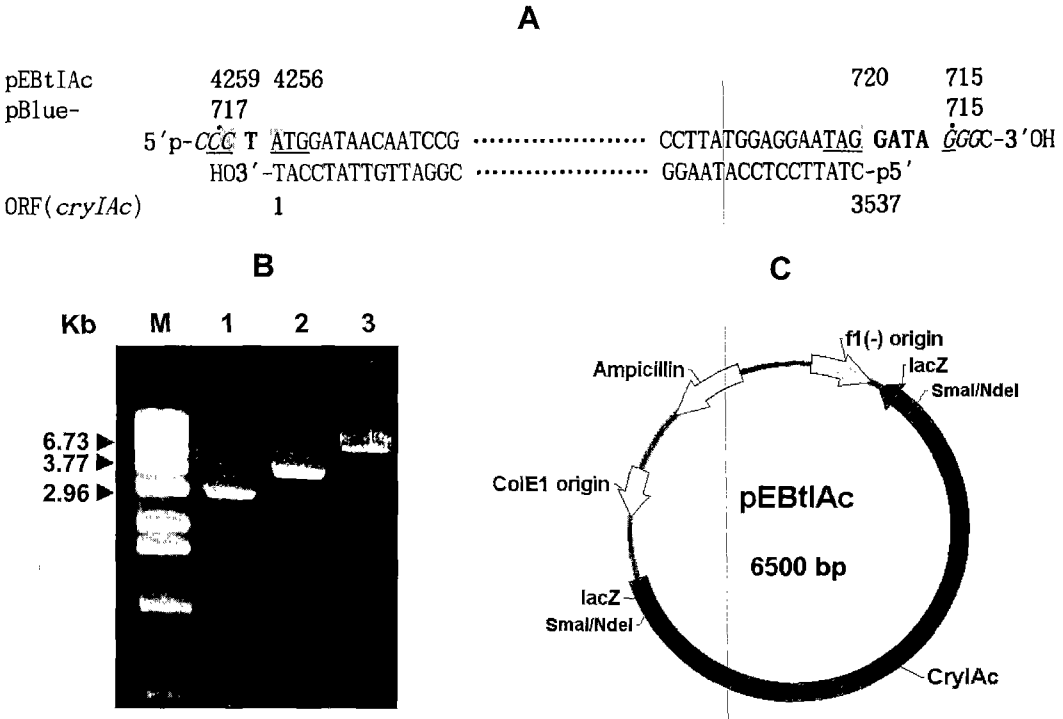

Fig. 2. Construction of recombinant phagemid, pEBtIAc.

(A) Linear map of pEBtIAc: ORF of *cryIAc* produced by PCR was cloned into *Sma*I-cut pBluescript II SK- (See the text for details: PBlue- means pBluescript II SK-). (B) Agarose gel electrophoresis patterns of manupulated DNA fragments. Lane M: DNA size markers. Lane 1: *Sma*I-cut pBluescript II SK- (2,958 bp). Lane 2: PCR-amplified ORF of *cryIAc* gene (3.550 bp). Lane 3: Linearized pEBtIAc (6,500 bp) isolated from the clone, EBtIAc. (C) Circular map of pEBtIAc: Manipulated ORF of *cryIAc* restricted with *Nde*I (3,548 bp) was ligated to *Sma*I-cut pBluescript II SK- (2,958 bp) of *cryIAc* to a 6,500 long circular phagemid, pEBtIAc.

vector (pBlue- in Fig. 2A) became the 4,258th nucleotide of pEBtIAc in Fig. 2A. Cloning of the gene, *cryIAc*, was achieved as pEBtIAc that was transformed into *E. coli* DH5α. A transformant selected on an appropriate medium has been named "EBtIAc" from which the phagemid, pEBtIAc, was successfully recovered (Fig 2B, lane 3). The nucleotide composition of the gene was confirmed, and the sequence revealed its identity to the reported [27]. It is, thus, safe to say that the recombinant gene, pEBtIAc, was not subject to gene rearrangements, and topologically sound. This construct also in flame in respect to the direction of *lacZ* expression (Fig 2C), since the codon "CCG" (from 717th- to 715th-nucleotide of pBlue-; for Proline) was substitutrd by the codon "CCT" (from 4,259th-[same as 717th-nucleotide of pBlue-] to 4257th-nucleotide of pEBtIAc; for Proline), from which the start codon, ATG (4256th-4354th), of *cryIAc* was succeeded (Fig. 2A).

Hyperexpression of pEBtIAc (*cryIAc*) in the Clone, EBtIAc

Total proteins and CryIAc protoxin were purified as described in this text. The protoxin was treated with trypsin to be activated, and were then subjected to SDS-PAGE. Analysis of the protein profile was shown in Fig. 3. It is clear that the major constituent of protein was a polypeptide of *ca.* 130 kDa (lanes 2, 3). The molecular mass (*Mr*) of the pEBtIAc product was the right size of CryIAc protoxin that was reported elsewhere [1, 27]. This constituent was not present in the wild (Fig. 3, lane 1), implying that the gene, *cryIAc*, was fairly well expressed in the clone, EBtIAc. The *Mr* of the trypsin-digested CryIAc, *ca.* 60 kDa, seemed to be slightly lower than previously observed, 66 kDa (Fig. 3, lane 4). It could be due to the differences of experimental conditions, such as concentration of the enzyme, pH, reaction temperature, *etc.* Some studies showed poor expression of Bt genes in *E. coli* HB101 [27], whereas others reported on a good expression of the same genes in *E. coli* HB101 [1, 10], or DH5α [19]. The reasons for the differences are not quite clear, but, they might be due to differences in the regulatory system (especially host RNA polymerase binding site) of a vector, gene copy number among recombinant clones, and so on [1, 10, 19, 27]. Insect toxicity of the ICP was assayed by force-feeding larvae of gypsi moth on purified CryIAc protoxin. By triplicate of assay, LD_{50} of 5 (ng/ul) was obtained.

Nucleotide composition of pEBtIAc, an apparent *Mr* and insect toxicity of the gene product, led us to conclude that *cryIAc* gene cloning led us to believe our success.

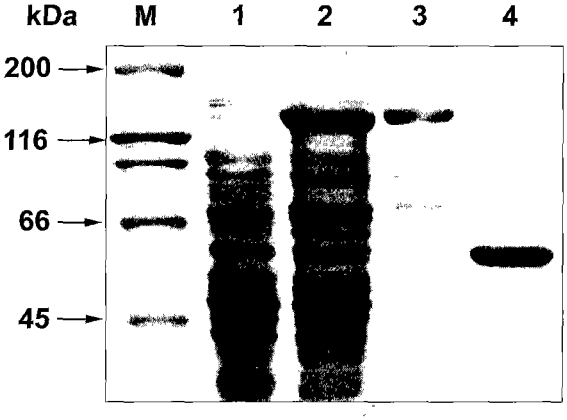

Fig. 3. SDS-PAGE pattern of CryIAc.
Total proteins, purified pro- and trypsinized toxins were separated on a SDS-polyacrylamide gel (12 per cent), and stained with Coomassie Blue. Lane M: High molecular weight protein markers (*Msr* were marked with horizontal arrows next to appropriate bands in figures [unit - kDa]). Lane 1: Total proteins from wild-type. Lane 2: Total proteins from EBtIAc. Lane 3: Protoxin. Lane 4: Trypsinized toxin.

Production and Application of Anti-CryIAc Antibody for Screening Bt-Corn

Biologically active form of CryIAc toxin was used as an immunogen for the production of anti-CryIAc antibody in rabbits. Titration of anti-CryIAc antibody was accomplished by dot and Western blottings. CryIAc protoxin as the antigen was serially diluted from a quantity that was 8 ng to 0.5 ng. Experiments showed that anti-CryIAc antibody detected the activity of activated CryIAc to a quantity below a nanogram (Fig. 4A). But control experiment with pre-immune serum did not react with CryIAc protoxin

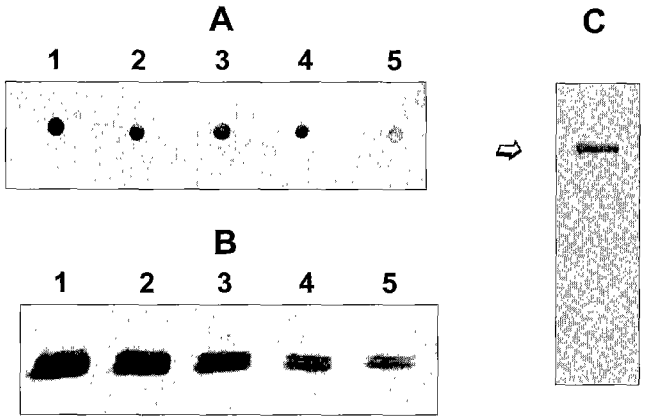

Fig. 4. Titration of anti-CryIAc antibody by blot immunoassay.
(A). Dot blot: Five CryIAc spots with different concentrations were applied onto nitrocellulose membrane for titration of the rabbit anti-CryIAc antibody. The antigen-antibody complexes were visualized by the application of the GAR-HRP system. 1: 8 ng, 2: 4 ng, 3: 2 ng, 4: 1 ng, 5: 0.5 ng. (B). Western Blot: Different amounts of CryIAc were resolved by SDS-PAGE, and transferred onto nitrocellulose membrane. The blot was treated with the antibody as above. 1: 10 ng, 2: 5 ng, 3: 2.5 ng, 4: 1.25 ng, 5: 0.625 ng. (C). Western Blot: Total proteins fractionated from EBtIAc were resolved by SDS-PAGE, and transferred onto nitrocellulose membrane. The blot was treated with the antibody as above. The major band indicated by a horizontal open arrow is the right size of CryIAc protoxin (ca. 130 kDa).

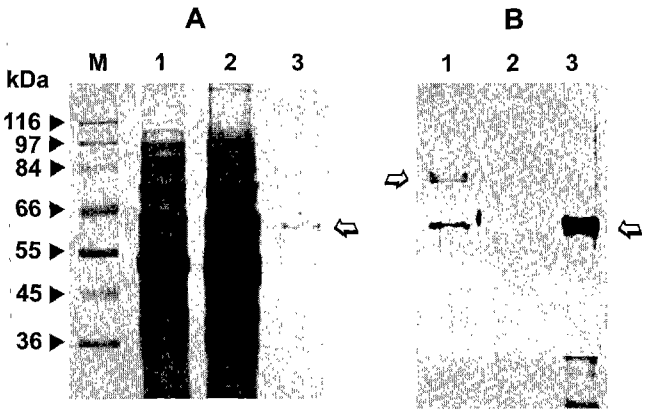

Fig. 5. SDS-PAGE and immunoblot analysis of CryIAc in corn leaves.

Total proteins (200 ug per lane) were extracted from the three-week-old imported and Korean endemic corn leaves, respectively, and they were resolved on SDS polyacrylamide gel. One of the duplicated gels was stained with Coomassie Blue (panel A) or subjected to immunoblotting with anti-CryIAc antibody (panel B). The antigen-antibody complexes were visualized by the application of the GAR-HRP system. (A) Lane M: Protein high molecular weight markers (*Msr* were marked with solid horizontal solid wedges next to appropriate bands [unit - kDa]). Lane 1 - Total proteins from imported corn leaves. Lane 2: Total proteins from Korean endemic corn leaves. Lane 3 - Trypsinized CryIAc. (B) Lane 1: Total proteins from imported corn leaves reacted with the antibody; the band indicated by a horizontal open arrow seems to be undegraded toxin. Lane 2: Total proteins from Korean endemic corn leaves reacted with the antibody; no band appeared. Lane 3: Trypsinized CryIAc reacted with the antibody indicated by a horizontal open arrow.

when dilution was used. Similar data were obtained from Western blot immunoassay (Fig. 4B). Also total proteins obtained from the transformant, EBtIAc, were analyzed by SDS-PAGE. The ICP among total proteins was seen by western blot process that used an anti-CryIAc antibody (Fig. 4C). The data suggest that anti-CryIAc antibody showed some specificity for detecting CryIAc protoxin. This identification system was employed to screen Bt-corns from the U. S. A. as a forage crop during the year of 2000. Two protein bands reacted with anti-CryIAc antibody were observed in the imported corn (Fig. 5B, lane 1), but not with Korean endemic corn (Fig. 5B, lane 2). It has been reported that a company in the U.S.A. produced genetically modified corn having a truncated *bt* gene whose product was slightly heavier (*ca.* 70 kDa) than an activation domain [18]. The band (*ca.* 70 kDa) indicated by an open arrow in the lane 1 (Fig. 5B), could thus be considered as the product of the truncated *bt* harboring in the imported corn. The other one whose size is slightly smaller (*ca.* 60 kDa) than the former on the blot, seems to be a slightly degraded form of the gene product. Therefore, it can be further used to screen *cryIAc*-transgenic organisms (for instance, imported corn) at the translational level.

The recombinant phagemid clone, pEBTIAc, mass production of the toxin, CryIAc along with anti-CryIAc actibody will be good assets for ICP in this country. In particular, pEBtIAc that could be used to generate site-directed mutant(s) of the *cryIAc* gene producing more toxic biopesticide with ease. The activated toxin might be spread directly onto target pests (e.g. large white butterfly larvae on leaves of *Brassica* spp.) during their hatching season. This seems to be the best way to lessen environmentalists' concern about a plausible drift of *bt* gene(s) from GMOs.

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