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Characteristics of Thirty-Six *Bacillus thuringiensis* Isolates and a New Serovar of *B. thuringiensis* subsp. *kim* (Serotype H52)

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Abstract Thirty-seven strains of *Bacillus thuringiensis* were isolated from Korean soil and examined for H-antigen serotyping, toxicity, and different spectra of biological activities. The isolate HL-175 bore a specific H-antigen, different from the 51 known serotypes, a spherical δ-endotoxin crystal, and minor different biochemical characteristics. It was resistant to ampicillin, colistin, and penicillin G. Therefore, it was classified as a new serotype, H52, with the name *kim*. The other 36 isolates also produced endotoxin crystals and endospores. The crystal shape of eight strains was cuboidal while the others were bipyramidal. Biochemical characteristics of the isolates were only slightly different from the known serotypes of *B. thuringiensis*. The flagellar (H) antigens of the 36 isolates were identified as: one *colmeri* (H21), three *galleriae* (H5a,5b); two *pakistani* (H13); one *toumanoffi* (H11a,11b); and twenty-nine *kurstaki* (H3a,3b). All 36 isolates were resistant to ampicillin, colistin, penicillin, cephalothin, and chloramphenicol.

Key words: *B. thuringiensis* serotypes, *B. thuringiensis* subspecies

Bacillus thuringiensis is a rod shaped, spore-forming bacterium uniquely characterized by the production of one or more proteinaceous parasporal crystals during its sporulating cycle [10]. Because the crystals kill certain insect larvae [10], the crystals and microorganisms are important for the development of microbial insecticidal pesticides [10]. De Barjac and Bonnefoi [3, 4] showed that strains of *B. thuringiensis* can be distinguished by serotypes based on their flagellar (H) antigens. Subsequently, more than 51 serotypes of *B. thuringiensis* were reported [1, 6, 7, 8, 11, 16, 20, 21, 28, 31]. To search new serotypes and distribution of strains with different spectra of biological activities, we isolated and characterized 37 strains of *B. thuringiensis* from Korean soil. This report describes the biochemical characteristics, microscopic observations, H-antigen serotyping, antibiotic resistance patterns, and toxicity of these 37 isolates.

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Materials and Methods

Bacterial Strains and Media

The 51 known serotypes of *Bacillus thuringiensis* were obtained from IEBC Collection (W.H.O. Collaborating Centre, Pasteur Institute, France). Bacterial cells for parasporal proteinaceous crystal analysis were cultured at 28°C in a basal medium (U. G. medium) [2]. Mueller-Hinton medium (DIFCO, Detroit, U.S.A.) was used for the measurement of inhibition zones of antibiotics.

Bombyx mori and *Culex pipiens* Larvae

Bombyx mori larvae were obtained from Dr. S. P. Lee, National Institute of Agricultural Science and Technology, Suwon, Korea and *Culex pipiens* larvae were from Dr. J. C. Shim, National Institute of Health, Seoul, Korea.

Isolation of *B. thuringiensis* from Soil

Soil samples were taken from various fields planted with a variety of crops, from virgin soil, rocky soil, and forest areas. The samples were then screened for isolation of *B. thuringiensis* by the procedure described by Lee *et al.* [17, 18, 21, 22].

Confirmation of Crystal Formation

B. thuringiensis isolates were precultured in 20 ml of nutrient broth at 28°C by rotary agitation at 180 rpm overnight, and 1.0 ml of the precultures was transferred into 20 ml of U. G. media [2]. It was then cultured until sporulation at 28°C by rotary agitation at 180 rpm for 20 to

30 h. The fully mature, unlysed cells were harvested and washed twice with sterilized saline by centrifugation at 3,000 ×g for 20 min. For microscopic observation, the pellets were suspended in saline and the formation of spores and parasporal crystals was observed with a phase contrast microscope.

Biochemical Characterization and Antibiotic Susceptibility Test

Biochemical characteristics and the antibiotic susceptibility of the isolates were examined by the procedures of Lennette *et al.* [25]. Antibiotic sensitivity of *B. thuringiensis* was determined by a diffusion test with a standardized filter paper disc on Mueller-Hinton agar.

Preparation of H-Antigens, Antisera, and H-Antigen Serotyping

The bacterial strains were first cultured in a basal U. G. medium with 0.7% pancreatic peptone with shaking at 28°C. During early exponential growth, an aliquot was inoculated at the agar surface of the inner small tube of a Cragie tube filled with 0.3% solution of soft nutrient agar. The bacteria migrate down the inner tube and then up the outer container. These bacteria may have good flagellar antigens, which were used for preparing H-antisera of *B. thuringiensis* as described by de Barjac [2]. H-antigen serotyping of *B. thuringiensis* strains was then determined by a cross-agglutination of the H-antigens and H-antisera among strains, using the procedure of de Barjac [2].

Bioassays

Bioassay for *Culex pipiens* or *B. mori* larvae was carried out by the procedures described by de Barjac [1, 2] and Lee *et al.* [12, 13, 19, 21, 22] with slight modifications. One or two loops of pure-cultured isolates were inoculated in 10 ml of fresh nutrient broth and then cultured at 28°C at 180 rpm overnight. Two and a half ml of the culture were transferred into 50 ml of U. G. medium and cultured again for 48 to 72 h. After pelleting the culture at 4,000 ×g for 20 min, the supernatants were discarded and the pellets were washed twice with sterilized saline by centrifugation at 4,000 ×g for 20 min. The pellets were suspended in 5 ml of saline. Then, 1.0 ml (10^8 spores/ml) of the suspended spore-crystal complex was added to 150 ml of distilled water in a disposable cup (72×80 mm) for bioassay of either *C. pipiens* 3rd instar larvae or a lump (2×2×1.5 cm^3) of semisolid food in a petri dish (2×20 cm) containing *B. mori* 3rd instar larvae. The mortality was recorded at 28°C for 48 h.

Purification of Crystals and Polyacrylamide Gel Electrophoresis

Parasporal crystals of *B. thuringiensis* were separated from spores and cellular debris by NaBr gradient centrifugation [24], dissolved in alkaline solution [26], and analyzed by 10% SDS-PAGE as described by Bollag *et al.* [9].

RESULTS AND DISCUSSION

Identification of *B. thuringiensis* Isolates

Thirty-seven strains of *B. thuringiensis* strains were isolated from Korean soil samples and examined for H-antigen serotyping, toxicity, and different spectra of biological activities. The thirty-seven isolates containing parasporal inclusion bodies (crystals) were found and named HL-101 to 181 (Table 1). There were no significant differences in shape and size between the vegetative cells of *B. thuringiensis* isolates and the known 52 *B. thuringiensis* serotypes with dimensions of 1.3-1.4×3.7-4.1 μm [1, 7, 10, 15, 21, 27]. The shape of crystals observed by phase contrast microscopy is illustrated in Table 1. Eight isolates, HL-101~105, 118, 119, and 120 (Table 1), harbored cuboidal crystals. Isolate HL-175 had a spherical crystal shape (Fig. 1) and consisted of approximately 130 and 60 kDa polypeptides as estimated by SDS-PAGE (Fig. 2). The other twenty-eight isolates had bipyramidal crystals in their cells (Table 1). These results demonstrated that the isolates had the same general

Table 1. Crystal shape of *B. thuringiensis* isolates.

Shape of crystals	*B. thuringiensis* isolates
Cuboidal	HL-101, 102, 103, 104, 105, 118, 119, 120
Roundish	HL-175
Bipyramidal	HL-106, 107, 108, 109, 110, 111, 112, 113 114, 115, 116, 117, 121, 122, 123, 124, 125 126, 127, 128, 162, 169, 173, 174, 175, 177 178, 179, 181

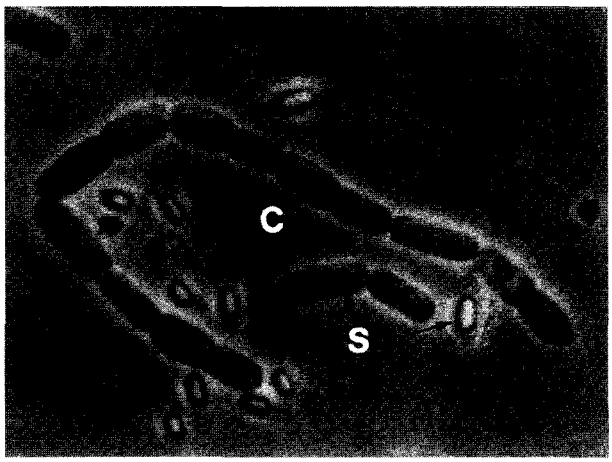

Fig. 1. Micrograph of the *B. thuringiensis* subsp. *kim* strain.
The cells grown for 48 h and at 28°C were observed by phase contrast microscopy. Arrowheads indicate spores (S) and crystals (C).

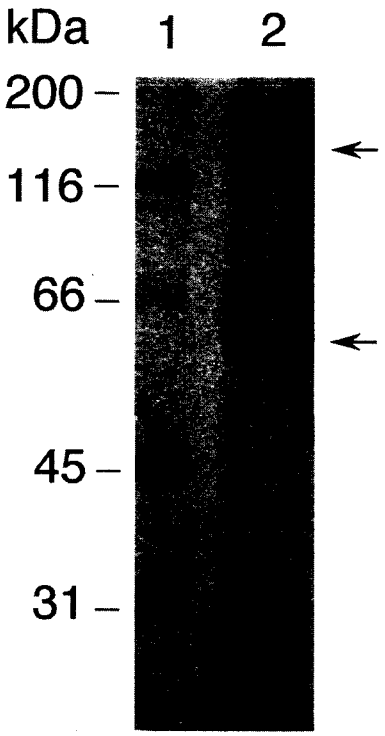

Fig. 2. Electrophoretogram of crystal proteins from the HL-175 strain.
The lysate of the HL-175 strain grown for 48 h at 28°C was analyzed by 10% SDS-PAGE. Lanes 1, standard molecular markers; 2, lysate of the HL-175. Arrows indicate the two crystal protein bands, 130 and 60 kDa, respectively.

shape of δ-endotoxin crystals as the already known serotypes of *B. thuringiensis* do [2, 14, 18, 20, 21, 22, 29, 30].

Biochemical characteristics of the 37 isolates were examined as shown in Table 2. Minor differences in biochemical reactions existed among the strains. The 37 isolates were gram-positive rods and were motile (Table 2). Only the HL-101 isolate was negative in the methyl-red test; the other 36 strains were positive. All isolates showed positive reactions for the VP test, nitrate reduction, gelatin hydrolysis, and acid production from glucose. Catalase production was positive except for the two isolates, HL-169 and 175. Urease production was positive for 30 isolates. β-Hemolysis reaction was negative only in the HL-175; all of the others were positive. Maltose utilization was positive except in HL-174. Twenty isolates produced arginine decarboxylase. Production of lysine and ornithine decarboxylases was positive only in isolate HL-175. Five isolates were positive in the utilization of cellobiose, seven isolates were positive in the utilization of raffinose, and 21 isolates were positive in the utilization of salicin. Only the HL-113 isolate used starch. These biochemical characteristics revealed that the isolates had general properties similar to the already known serotypes of *B. thuringiensis* [3, 4, 16, 17, 18, 20, 21, 22, 23, 30].

All 37 strains were negative in the production of indole, hydrogen sulfide, phenylalanine deaminase, and gas from glucose, and negative in the utilization of adonitol, arabinose, citrate, dulcitol, inositol, lactose, manitol,

Table 2. Biochemical characteristics of *B. thuringiensis* isolates.

Characteristics examined	Biochemical reactions												
	HL-101	102	103	104	105	106	107	108	109	110	111	112	BTK
Gram stain/motility/VP reaction/ nitrate reduction/gelatin hydrolysis/β-hemolysis	+	+	+	+	+	+	+	+	+	+	+	+	+
Methyl-red reaction	-	+	+	+	+	+	+	+	+	+	+	+	+
Productions of													
- indole/H_2S/phe deaminase /lys, arg, ornithinedecarboxylases	-	-	-	-	-	-	-	-	-	-	-	-	-
- catalase/oxidase /acid from glucose	+	+	+	+	+	+	+	+	+	+	+	+	+
- urease	+	+	-	-			+	+	+	+	+	+	+
- gas from glucose	-	-	-	-	-	-	-	-	-	-	-	-	-
Utilizations of													
- adonitol/arabinose/citratel /dulcitol/inosito/lactose/manitol /rhamnose/sorbitol/starch/xylose	-	-	-	-	-	-	-	-	-	-	-	-	-
- cellobiose	-	-	-	-	-	+	+	-	-	-	-	+	+
- esculin/maltose	+	+	+	+	+	+	+	+	+	+	+	+	+
- raffinose	+	+	+	+	+	+	+	-	-	-	-	-	+
- salicin	-	-	-	-	+	+	+	+	-	-	-	-	+
- sucrose	-	-	+	-	-	-	-	-	-	-	-	-	-

BTK: Known serotype of *B. thuringiensis* subsp. *kurstaki* strain.(+): positive reaction, (-): negative reaction.

Table 2. Continued.

Characteristics examined	Biochemical reactions											
	HL-113	114	115	116	117	118	119	120	121	122	123	124
Gram stain/motility/VP and methyl-red /nitrate reduction /gelatin hydrolysis /β-hemolysis	+	+	+	+	+	+	+	+	+	+	+	+
Productions of												
- indole/H_2S/phe deaminase /lys, ornithine decarboxylase /gas from glucose	-	-	-	-	-	-	-	-	-	-	-	-
- arg decarboxylase	-	-	-	-	-	+	+	+	+	+	+	+
- urease	+	+	+	+	+	-	-	-	+	+	-	+
- catalase/acid from glucose /oxidase	+	+	+	+	+	+	+	+	+	+	+	+
Utilizations of												
- adonitol/arabinose/cellobiose /citrate/dulcitol/inositol/lactose /manitol/rhamnose/raffinose /sorbitol/sucrose/xylose	-	-	-	-	-	-	-	-	-	-	-	-
- esculin/maltose	+	+	+	+	+	+	+	+	+	+	+	+
- salicin	+	-	-	-	-	+	-	-	+	+	-	-
- starch	+	-	-	-	-	-	-	-	-	-	-	-

(+): positive reaction, (-): negative reaction.

Table 2. Continued.

Characteristics examined	Biochemical reactions												
	HL-125	126	127	128	162	169	173	174	175	177	178	179	181
Gram stain/VP reaction/methyl-red reaction/nitrate reduction /gelatin hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+
β-hemolysis/motility	+	+	+	+	+	+	+	+	-	+	+	+	+
Productions of													
- indole/H_2S/phe deaminase /gas from glucose	-	-	-	-	-	-	-	-	-	-	-	-	-
- catalase	+	+	+	+	+	-	+	+	-	+	+	+	+
- lys/orn decarboxylase	-	-	-	-	-	-	-	-	-	-	-	-	-
- oxidase	+	+	+	+	+	+	-	+	+	-	-	-	+
- arg decarboxylase/urease /acid from glucose	+	+	+	+	+	+	+	+	+	+	+	+	+
Utilizations of													
- adonitol/arabinose/citrate /dulcitol/inositol/lactose/ manitol/rhamnose /sorbitol/starch/raffinose /xylose/sucrose	-	-	-	-	-	-	-	-	-	-	-	-	-
- esculin/maltose	+	+	+	+	+	+	+	+	-	+	+	+	+
- cellobiose	-	-	-	-	+	+	-	-	-	-	-	-	-
- salicin	+	+	+	+	+	+	+	+	+	+	+	+	+

(+): positive reaction, (-): negative reaction.

rhamnose, sorbitol, and xylose. Strain HL-103 used only sucrose. These results indicated that the isolates had general biochemical characteristics similar to the already known serotypes of *B. thuringiensis* [3, 4, 17, 18, 20, 21, 22, 23, 30].

Thirty-seven isolates were tested against eleven antibiotics. The 37 isolates showed common resistance to ampicillin, colistin, and penicillin G. Thirty-six strains were resistant to carbenicilloin and cephalothin except for HL-175.

Table 3. Serological classification of *B. thuringiensis* isolates.

Isolates	H-serotype	Subsp.	Agg titer[a]
HL-101	H3a,3b	*kurstaki*	1:25,600
102	H21	*colmeri*	1:25,600
103	H5a,5b	*galleriae*	1:25,600
104	H13	*pakistani*	1:25,600
105	H13	*pakistani*	1:25,600
106~117	H3a,3b	*kurstaki*	1:25,600
118	H11a,11b	*toumanoffi*	1:25,600
119	H5a,5b	*galleriae*	1:25,600
120	H5a,5b	*galleriae*	1:25,600
121128	H3a,3b	*kurstaki*	1:25,600
162, 169	H3a,3b	*kurstaki*	1:25,600
173, 174	H3a,3b	*kurstaki*	1:25,600
175	H52	*kim*	1:25,600
177~179	H3a,3b	*kurstaki*	1:25,600
181	H3a,3b	*kurstaki*	1:25,600

a. Homologous agglutination titer.

Other antibiotics (amikacin, chloramphenicol, clyndamycin, erythromycin, gentamycin, kanamycin, tetracycline, tobramycin, vancomycin) were sensitive to the 37 strains. These results indicated that the isolates had antibiotic resistance similar to the already known serotypes of *B. thuringiensis* [17, 18, 20, 22].

B. thuringiensis strains were differentiated and classified according to the H-antigen of the cells [1, 2, 5]. To classify the new isolates, the homologous and heterologous titres among the H-antigens and antisera of *B. thuringiensis* isolates and the known serotypes were measured. The H-antisera of the new isolates had a high titration value of 1:25,600 only against their homologous H-antigens (Table 3). The H-antigen of the isolate, HL-175, did not cross-react with the antisera of the 51 other serotypes known at that time, whereas the H-antigens of the 51 known serotypes did not react with an antiserum specific for HL-175. This indicated that the isolate had to be a new serotype of *B. thuringiensis*. Therefore, the new serotype was designated as serotype H52 for the HL-175 strain and named *kim*. The other 36 isolates were serologically identified as; one *B. thuringiensis* subsp. *colmeri* (serotype H21) [11] for the HL-102 strain; three *B. thuringiensis* subsp. *galleriae* (serotype 5a,5b) [3, 6] for the HL-103, HL-119, and HL-120 strains; two *B. thuringiensis* subsp. *pakistani* (serotype H13) [8] for the HL-104 and HL-105 strains; one *B. thuringiensis* subsp. *toumanoffi* (serotype H11a,11b) [10] for the HL-118 strain; and 29 *B. thuringiensis* subsp. *kurstaki* (serotype H3a,3b) [6]. Data presented in Table 3 show that *B. thuringiensis* subsp. *kurstaki* (serotype H3a,3b) is the predominant serotype detected in the soil of Korea. These data demonstrated that the isolates could be classified into six serotypes according to the H-antigens of the cells. Burges [10] reported that *B. thuringiensis* subsp. *kurstaki* (49%), *galleriae* (45%), and *dakota* (5.7%) strains are predominantly present in the United States.

Table 4. Toxicity *B. thuringiensis* isolates against *Bombyx mori* 3rd instar larvae.

Isolates/ subspecies	No. of larvae tested	No. of dead at hour(s)						Mortality (%)
		1	2	3	4	8	12	
Control	20	0	0	0	0	0	0	0
BTK	20	14	3	3	-	-	-	100
HL-102c	20	0	0	0	0	0	0	0
103g	20	0	0	0	0	0	0	0
119g	20	0	0	0	0	0	0	0
120g	20	0	0	0	0	15	5	100
104p	20	0	0	0	0	0	0	0
105p	20	0	0	0	0	0	0	0
118t	20	0	0	0	0	0	0	0
101k	20	0	0	0	0	0	0	0
106k	20	7	5	2	3	3	-	-
107~109k	20	16	3	1	-	-	-	100
110k	20	18	2	-	-	-	-	100
111k	20	17	3	-	-	-	-	100
112k	20	12	3	3	2	-	-	100
113~116k	20	17	3	-	-	-	-	100
117k	20	12	4	4	-	-	-	100
121k	20	13	3	2	1	1	-	100
122k	20	14	3	0	0	3	-	100
123k	20	16	1	1	2	-	-	100
124k	20	13	1	1	0	31	3	100
125k	20	12	3	0	0	4	1	100
126k	20	15	2	1	0	2	-	100
127k	20	13	3	0	0	3	1	100
128k	20	15	2	1	0	2	-	100
162k	20	17	2	1	-	-	-	100
169k	20	6	12	0	0	0	2	100
173k	20	10	10	-	-	-	-	100
174k	20	6	10	0	0	0	4	100
177~181k	20	10	10	-	-	-	-	100
175kim	20	3	5	6	0	0	6	86

BTK, *B. thuringiensis* subsp. *kurstaki* strain known; c, *colmeri;* g, *galleriae;* k, *kurstaki;* p, *pakistani;* t, *toumanoffi.* Control: H_2O.

Toxicity

By bioassay against insect larvae, the new subspecies *B. thuringiensis* subsp. *kim* (HL-175 isolate; serotype H52) showed high toxicity to both *B. mori* larvae (Table 4) and *C. pipiens* larvae (Table 5). There was 100% mortality of *B. mori* larvae in 12 h at a dose of 10^8 spores per 6 cm^3, and of *C. pipiens* larvae in 48 h at a dose of 10^8 spore per 150 ml. The isolates HL-101 (*kurstaki*), HL-102 (*colmeri*), HL-103 (*galleriae*), HL-104 and HL-105 (*pakistani*), HL-118 (*toumanoffi*), and HL-119 (*galleriae*) were not toxic against *B. mori* 3rd instar larvae, but the other *B. thuringiensis* subsp. *kurstaki* strains showed very high toxicity against the larvae. In 1 h feeding, 50-90% mortality

Table 5. Toxicity of *B. thuringiensis* isolates against *Culex pipiens* larvae.

Isolates/ subspecies	No. of larvae tested	No. of dead larvae at 48 h	Mortality (%)
Control	30	0	0
BTI	30	30	100
HL-102c	30	26	86
103g	30	15	50
119g	30	30	100
120g	30	29	97
104p	30	10	33
105p	30	12	40
118t	30	26	86
101k	30	22	73
106k	30	27	89
107k	30	28	93
108k	30	27	89
109k	30	26	86
110k	30	25	82
111k	30	26	86
112k	30	25	82
113k	30	19	63
114~116k	30	26	86
117k	30	22	73
121k	30	18	60
122k	30	26	86
123k	30	20	67
124k	30	30	100
125k	30	22	73
126k	30	23	77
127k	30	27	90
128k	30	18	60
162k	30	30	100
169k	30	26	86
173k	30	30	100
174k	30	29	97
177~181k	30	30	100
175kim	30	30	100

BTI: *B. thuringiensis* subsp. *israelensis* known; BTK: *B. thuringiensis* subsp. *kurstaki* known; c. *colmeri*; g, *galleriae*; k, *kurstaki*; p, *pakistani*; t, *toumanoffi*. Control: H_2O.

of the *B. mori larvae* was shown. In particular, the *B. thuringiensis* subsp. *kurstaki* isolates (HL-107 to HL-111, HL-113 to HL-116, HL-123, HL-162) were the most active against *B. mori* larvae (Table 4). The isolates showed 33-100% toxicity against the *C. pipiens* 3rd instar larvae. The isolates HL-119 (*galleriae*), HL-124 (*kurstaki*), HL-162 (*kurstaki*), HL-173 (*kurstaki*), HL-175 (*kim*), and HL-177 to HL-181 (*kurstaki*) showed 100% lethality to the larvae in 48 h. The isolates HL-101 (*kurstaki*), HL-102 (*colmeri*), HL-103 (*galleriae*), HL-104 (*pakistani*), and HL-105 (*pakistani*) did not show toxicity against *B. mori* 3rd instar larvae, but those strains were moderately toxic to the *C. pipiens* larvae in 48 h. Also, the two strains, HL-118 (*toumanoffi*) and HL-119 (*galleriae*), did not show any toxicity against the *B. mori*, but they showed 86 and 100% mortality, respectively, to the *C. pipiens* larvae at the dose in 48 h. The HL-119 (*galleriae*) showed 100% mortality to the *C. pipiens* larvae in 48 h. Based on the data shown in Tables 4 and 5, we could not predict toxicity from serotypes or crystal morphology [10, 17, 22, 29]. The variable toxicity of *B. thuringiensis* to different taxonomic groups of insect larvae may most likely be due to different mechanisms to activate the crystal and also due to different gut conditions of the insects.

By SDS-PAGE analysis, the solubilized crystal proteins of the *B. thuringiensis* serotype H52 were estimated to be 130 and 60 kDa (Fig. 2). The apparent molecular weight of the 130 kDa protein was similar to crystal proteins of other *B. thuringiensis* strains [9, 12], and the fragments of 60, 43, and 29 kDa might be different from other *B. thuringiensis* strains [9].

In conclusion, the isolate HL-175 bore specific H-antigen, and had minor differences in biochemical characteristics and antibiotic resistances. Therefore, it was classified as a new serotype H52 with the name *kim*. The predominant serotype of the isolates was serotype H3a,3b (*B. thuringiensis* ser. *kurstaki*), which was highly toxic to *B. mori* larvae and moderately toxic to *C. pipiens* larvae.

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