Distribution and characterization of *Bacillus thuringiensis* from warehouse toxic to *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae)

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Abstract

Bacillus thuringiensis was isolated from 122 of 413 residue samples obtained from warehouse 58.2% of these *B. thuringiensis* isolates were toxic to Indianmeal moth, *Plodia interpunctella* (Hubner), which corrected mortality was 60% or more. 12 isolates were highly toxic to Indianmeal moth, which LC_{50} were 2.62ug/g diet to 17ug/g diet. Among them, isolates 130, 368 and 408, which belonged to serotype H_3, H_4, H_7 respectively, were more active than the other isolates and their LC_{50} were 2.62ug/g, 2.92ug/g and 3.93ug/g respectively. The isolates 130, 36 8 and 408 were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and polymerase chain reaction (PCR). The results showed that both isolates 130 and 368 contained 130－140Kda and 65Kda crystal protein while isolate 408 only contained 130－140Kda crystal protein. Isolate 130 contained the *cryIA (a)*, *cryIA (b)*, *cryIA (c)* and *cryII* gene; isolate 368 contained the *cryIA (c)* and *cryII* gene while isolate 408 contained the *cryIA (b)*, *cryIC*, *cryID* and *cry II gene*

Introduction

The Indian meal moth, *Plodia interpunctella* (Hubner), is one of the major lepidopteran pests of stored product around the world, and is widely distributed across China, causing serious losses in the stored product The formulation of *B. thuringiensis* is a major microbial insecticide and has been widely used in the control of lepidopteran pests of crops, ornamentals, forests, at least one coleopteran pest of crops and several dipteran pests that are of public health significance (McGaughey et al, 1992; Zhang Hongyu et al, 1997). Although some formulations of *B. thuringiensis* can controll infestations of Indianmeal moth in stored grain (Yu Ziniu, 1990; Zhang Hongyu et al, 1995, 1997), and very stably, it can persist without noticeable decrease in insecticidal activity for at least a year (Kinsinger and McGaughry, 1976, McGaughey et al, 1980) But there was not a commercial formulation in China yet, which was highly toxic to Indianmeal moth On the other hand, the Indianmeal moth was the first insect pest to be selected for resistance in laboratory (McGaughey et al, 1985), it will seriously threat the use of *B. thuringiensis*. Therefore, it is necessary to isolate new highly toxic *B thuringiensis* strains to overcome the resistance to *B. thuringiensis*.

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Materials and Methods

In the paper, we reported the isolation and characterization of highly toxic *B. thuringiensis* strains to Indianmeal moth from warehouse.

B. thuringiensis strains and media

B. thuringiensis HD-1 and subsp. *tenebrionis* were kindly provided by Institute of Pasteure in France and lab of microbial insecticide in Huazhong Agricultural University respectively, and the others were isolated from warehouse. All media were as previously described (Yu Ziniu et al, 1990)

Samples collection

The samples of dust, insects and stored product etc. were collected from warehouse, animal feed mill and grain manufacturing facilities by scraping about 5 grams of samples in sterile plastic bag at 4℃ The samples collected reflected the variety of residue materials present within the site (e g. settled grain dust, grains, insect webbing, bird nest and insect cadavers).

Isolation of *B. thuringiensis*

Approximately 0.5 gram of samples was suspended in 10ml of nutrient broth (0.25M sodium acetate-buffered medium), vortexed vigorously and incubated in 180 18ml test tube at 30℃ for 4h, then pasteurized at 75℃ for 10－

15 mins as described by Travers et al (1987). Insect cadavers were first homogenized in 1ml of sterile distilled water in a sterile glass issue grinder and this was followed by pasteurization as described by Meadows et al (1992) Samples were plated on nutrient agar The plates were incubated at 30℃ for 48h, and examined for colony morphology and the presence of parasporal crystals by phase-contrast microscopy. All crystal-forming colonies were removed and subcultured on L-agar and maintained for further investigations.

Serological tests

H-serotyping based on *B. thuringiensis* flagellar antigens was performed by the agglutination method as described by De Barjac(1981). Dilution of specific antisera raised against the flagellar antigens determining the 57 H-serotypes of *B. thuringiensis* were to serotype motile bacterial suspensions of the unknown *B. thuringiensis* strains

Bioassays

To prepare the sporulated culture, all *B thuringiensis* isolates were cultured in 50ml liquid nutrient agar medium until sporulation was completed The cultures were harvested by centrifugation at 6000 rpm for 15mins and washed two times with cold 1M NaCl and three times with distilled water, then resuspended in 5ml distilled water, and 1ml suspension was centrifugated at 10000 rpm for 5mins and dried until weight didn't change during 4h at 37℃ to determine the dry weight

The preparetions were bioassayed in a larval diet of cracked wheat fortified with wheat germ, yeast, honey and glycerin as described by McGaughey et al (1985). For primary screening tests, a spore-crystal suspension of isolates was diluted 10-fold and mixed with diet at a rate of 1ml/5g and fed to 15 two-instar larvae. The mortality was recorded.

After 48h at 25℃ 60 − 70% rh, L D = 12 12. Bioassays were replicated three times Isolates were considered toxic if they caused 60% or more corrected mortality in a bioassay. The samples with 100% mortality were diluted 100-fold and mixed with the larval diet at a rate of 1ml/5g to achieve the highest dosage, the serial 1 2 dilutions were made for 4 lower doses, then, 15 two-instar were added to each infested jar. The infested jar were held at 25℃, 60 − 70% rh, the mortality was recorded after 48h The mortality in the treated samples was corrected for that in control The concentrations killing 50% of the larvae (LC_{50}) were calculated

Characterization of the isolates with highly toxic to Indianmeal moth

Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed according to the method described by Sambrook et al (1989). Oligonucleotide primers used in this PCR analysis were synthesized and supplied by Sangon, and listed in Table 1. *B thuringiensis* isolates were grown in 5ml LB medium and incubated at 28℃ with vigorous shaking for approximatly 5h, then plasmid DNA for PCR analysis was prepareed from *B thuringiensis* culture according to the method described by Sambrook et al(1989). Finally, the washed DNA pellets were resuspended in 20ul of sterile distilled water PCR amplification were conducted in 25ul reaction volume which consisted of 1ul of DNA template and primer mixtures with 0.01nM each, 3ul of dNTP mixtures with 1.25 mM and 1U of Taq polymerase PCR amplification was performed for 25 cycles with denaturation of template DNA at 94℃ for 1 min, annealing templetes and oligonucleotide primers at 52℃ for 2mins and extension of PCR products at 72℃ for 3 mins PCR products were analyzed by 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light

Results

Isolation of *B. thuringiensis*

Total of 122 out of 413 samples collected yielded *B thuringiensis* and belonged to serotype H_3, H_4, H_5, H_7, H_8, H_{10}, H_{23}, H_{27} etc.

Distribution of toxicity to Indianmeal moth

Table 2 showed that the distribution of corrected mortality for the isolates separated into groups according to the method described by meadows et al (1992) Each value represents a 5% step in mortality to Indianmeal moth in table 2. The 122 of isolates belonged into different corrected mortality group 58.2% of 122 isolates were toxic to Indianmeal moth, which their corrected mortality were more than 60%. Among them, 12 isolates were distributed in the 90% to 100% corrected mortality group, and were highly toxic to Indianmeal moth

The results also showed that the different isolates belonging to the same serotype had different toxic level to Indianmeal moth The frequency of toxicity was the greatest in isolates belonging to H_7, a total of 100% of isolates toxic to Indianmeal moth while the frequency of isolates belonging to H_8 was the lowest, which only 12.5% of isolates was toxic to Indianmeal moth And 76.92%, 52 27%, 51 72% of isolates, which belonged to H_3, H_4, H_7 respectively, were toxic to Indianmeal moth

Some isolates with 100% corrected mortality in the primary screening tests were evaluated for LC_{50} The results showed that their LC_{50} were 2.62 to 17 ug/g diet while LC_{50} of standard *B thuringiensis* strain HD-1 was 14.3ug/g. Among them, isolates 130, 368 and 408, which

LC_{50} were 2.62ug/g, 2.96ug/g and 3.93ug/g (Table 3) respectively, were more active against Indianmeal moth than the other isolates.

Table 1. Characteristics of PCR Primers used.

Primer	Sequence	gene recognized	Product Size(bp)
Cry-I			
3'-primer	5'-ATCACTGAGTCGCTTCGCATGTTTGACTTTCTC		
5'-primer	5'-TCGAATTGAATTGTTCCGGCAGAAGTA	*cryIA(a)*	724
	5'-GAATTGCTTTCATAGGCTCCGTC	*cryIA(b)*	236
	5'-TCACTTCCCATCCGACACTCTACC	*cryIA(c)*	487
	5'-GTCAACCTTATGATCACCTGGGCTTC	*CryIB*	830
	5'-CAACCTCATTTGGTGCACCTTC	*CryIC*	288
	5'-GGTACATTTAGATATTCACAGCCAC	*CryID*	414
	5'-CTTAGGGATAAATGTAGTACAG	*CryIE*	883
	5'-CCGGTGACCCATTAACATTCCAATC	*CryIF*	388
Cry-II			
3'-primer	5'-GGACCACCTCGTGCCCGGATA		
5'-primer	5'-CAGATACCCTTGCTCGTGTAA	*CryII*	1070
cry-III			
3'-primer	5'-CATCTGTTGTTTCTGGAGGCAAT		
5'-primer	5'-CGTTATCGCAGAGAGATGACTTTAAC	*CryIII*	595

Table 2. Distribution of Corrected Mortality of Indianmeal moth Caused by *B thuringensis* Isolates.

Corrected Mortality(%)	H_3	H_4	H_5	H_7	H_8	H_{10}	H_{23}	H_{27}	AA*	Total
0 – 4					2					2
5 – 9					1					1
10 – 14		2			1					3
15 – 19		2	2						1	5
20 – 24	1	3	1						2	7
25 – 29		2								2
30 – 34		3	2							5
35 – 39		2					1			3
40 – 44	2	1	2					1		6
45 – 49		2								2
50 – 54		1	4		1		1			7
55 – 59		3	3		2					8
60 – 64		1	2	2	1		2			8
65 – 69	4	9	2			2			2	19
70 – 74	2	5	4						2	13
75 – 79	1	6								7
80 – 84	2	1		2		2				7
85 – 89			2	3						5
90 – 94			4	2						6
95 – 100	1	1	1	3						6
Total	13	44	29	12	8	4	4	1	7	122

* AA auto-agglutinable

Table 3. Characteristics of Isolates with highly Toxic to Indianmeal moth.

Strain NO	LC_{50} (ug/g)	95% FL#		Serotype	Cry Protein composition	Genotype
		Lower	Upper			
130	2.96	1.51	4.41	H_3	~135KDa, 65KDa	*cryIA(a)*, *cryIA(b)*, *cryIA(c)*, *cryII*
368	2.62	1.86	3.38	H_4	135KDa, 65KDa	*cryIA(c)*, *cryII*
408	3.93	2.73	5.15	H_7	~135KDa	*cryIA(b)*, *cryIC*, *cryID*, *cryII*

FL: Fiducial Limits

Characterization of isolates with highly toxic to Indianmeal moth

SDS-PAGE of 3 isolates that were active against Indianmeal moth were presented in Figl. The results showed that both isolates 130 and 368 contained ~135Kda and 65Kda crystal proteins, and were similar with standard *B. thuringiensis* strain HD-1, while isolate 408 only contained ~ 135Kda crystal protein. The PCR product profiles of isolates identified were shown in Fig. 2 ~ 4. Isolate 368 had the *cryIA(c)* and *cryII* gene which codes for crystal toxin; isolate 408 had the *cryIA(b)*, *cryIC*, *cryID* and *cryII* gene and isolate 130 had the *cryIA(a)*, *cryIA(b)*, *cryIA(c)* and *cryII* gene while contrl *B. thuringiensis* HD-I had *cryIA(a)*, *cryIA(b)*, *cryIA(c)* and *cryII*. All isolates identified didn't have *cryIII* gene (data not shown).

Conclusion and Discussion

The research results showed that most *B. thuringiensis* isolates (58.2%) from warehouse were toxic to Indianmeal moth, which corrected mortality was more than 60%. 12 isolates were highly toxic to Indianmeal moth, which LC_{50} were 2.62 to 17ug/g diet. Among them, isolates 130, 368 and 408 belonging to H_3, H_4, H_7 respectively were more active to indianmel moth than the others, their LC_{50} were 2.62ug/g, 2.96ug/g and 3.93ug/g respectively. Isolate 130 and 408 contained ~135Kda and 65Kda while isolate 408 only contained ~135Kda crystal protein. Isolate 130 had the *cryIA(a)*, *cryIA(b)*, *cryIA(c)* and *cryII* gene; isolate 368 had *cryIA(c)* and *cryII* gene and isolate 408 had the *cryIA(b)*, *cryIC*, *cryID* and *cryII* gene.

The results suggested that diversity of toxicity to Indianmeal moth was evident in *B. thuringiensis* isolates from warehouse. The mortality level of 122 isolates were distributed from 0 to 100% corrected mortality, and there was not a large corrected mortality group, so repeated isolations of one strain or a small number of strains that caused substantially different mortality levels didn't occur as suggested by Meadow et al (1992). The results also suggested that serotypes of the *B. thuringiensis* strains didn't directly reflect their insecticidal activity although certain relationships were established between the entomocidal parasporap crystal and the flagellar antigen as suggested by Bourque et al (1993) and Kou et al (1996).

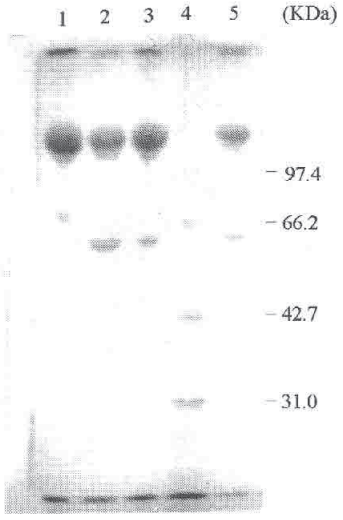

Fig.1. Cry protein profiles of *B. thuringiensis* isolates. Lanes: 1. isolate408; 2. isolate368; 3. isolate130; 4. molecular mass marker; 5. HD-1

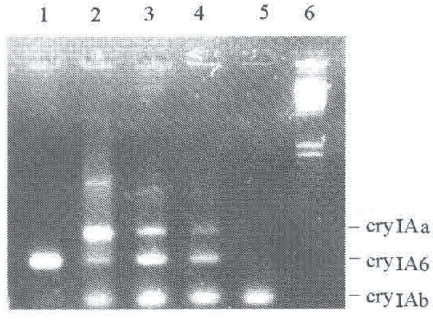

Fig.2. PCR product profiles using the mixed. *cryIA-type* primer. Lanes: 1. isolate368; 2. isolate135; 3. HD-1; 4. isolate130; 5. isolate408; 6. HindIII-digested

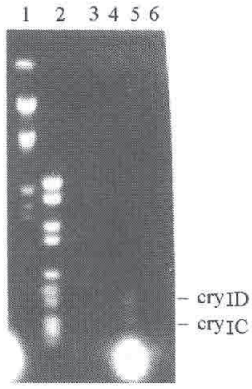

Fig.3. PCR product profiles using the mixed. *CryIB*, *cryIC*, *cryID*, *cryIE*, *cryIF* primers Lanes:1. EcoRI-and HindIII-digested; 2. DNA ladder;3. isolate130;4. isolate368; 5. isolate408;6. isolateHD-1.

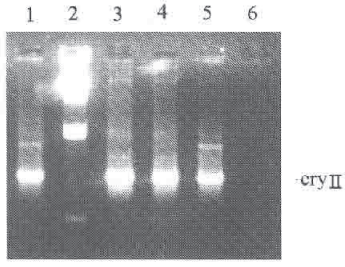

Fig.4. PCR product profiles using the cryII. primer. Lanes:1. isolate130;2. HindIII-digested; 3. isolate368; 4. isolate408;5. isolateHD-1; 6. negative-control (no DNA)

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