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Differentiated Pattern of Protein Composition of Crystalline Inclusions of Newly Isolated *Bacillus thuringiensis* Strains from Silesia in Poland

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Abstract

Protein profiles of crystal delta-endotoxins were determined in twenty nine *Bacillus thuringiensis* strains-soil and phylloplane isolates – from Poland. Electrophoretic analysis revealed quantatively and qualitatively different patterns of delta-endotoxin crystal preparations of these *B. thuringiensis* strains. The crystalline parasporal inclusions of *B. thuringiensis* isolates were composed of two, three, four or five proteins. Molecular weights of these polypeptides varied from 23.4 kDa to 142 kDa. There is lack of correlation between serovars of *B. thuringiensis* strains, the morphology of crystals and the number and size of proteins in parasporal inclusions.

Key words: *B. thuringiensis*, delta-endotoxins, crystal proteins

Introduction

Parasporal inclusions produced by gram-positive bacilli *Bacillus thuringiensis* and *B. sphaericus* are the subject of intense research because of their entomopathogenic properties which are widely used in biological control of many plant pests and vectors of pathogens, *e.g.* mosquitoes and black-flies transmitting first of all malaria and other tropical diseases (Enwistle *et al.*, 1993). They are synthesized after stage II of sporulation (t2-24) and accumulate in the mother cell as crystals which can account for up to 25% of dry weight of the sporulating cells. Those crystalline inclusions, composed of one or a few proteins are named also delta-endotoxins, Cry proteins or Insecticidal Crystal Protein (ICP). Höfte and Whiteley (1989) proposed a system of nomenclature and classification of the delta-endotoxins according to their insecticidal properties and molecular relationships. Generally, Cry proteins are active against Lepidopteran (CryI of 130–140 kDa), both Lepidopteran and Dipteran (CryII of 71 kDa), Coleopteran (CryIII of 66–77 kDa) and Dipteran (CryIV of 125–145 kDa and 68 kDa) larvae.

Recent extensive screening programs have revealed numerous strains producing delta-endotoxins which do not fall into these categories. The increasingly rapid isolation and characterization of *B. thuringiensis* proteins with new pesticidal properties and a variety of sequences have resulted in a new nomenclature of Cry proteins based on hierarchical clustering using amino acid sequence identity (Crickmore *et al.*, 1998). In most cases, the insecticidal specifity, if any, has not been determined yet.

The present study was undertaken in order to establish protein patterns of newly isolated *B. thuringiensis* strains displaying different toxic activities in previous tests (Lonc *et al.*, 2001a, b, 2003).

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Experimental

Materials and Methods

Bacterial strains. The studies encompassed 29 strains from the collection of the Institute of Genetics and Microbiology at the Wrocław University (Poland). Fourteen isolates originated from the phylloplanes and soil of the Lower Silesia, mainly from the Wrocław area. These strains (Table I) were described previously by Doroszkiewicz and Lonc (1999). The rest of fifteen strains of *B. thuringiensis* (Table II) were isolated from leaves and soil of the Upper Silesia regions. The standard biochemical tests were performed for *Bacillus thuringiensis* subspecies according to Lecadet *et al.* (1999). Stock bacterial culture were maintained on brain-heart infusion agar (Difco) slants and stored at 8°C.

Table I
Bacillus thuringiensis soil and phylloplane isolates from the Lower Silesia, Poland (Doroszkiewicz and Lonc 1999)

Symbol of *B. thuringiensis* strain*	Biochemical group/ Serovar	*B. thuringiensis* subspecies	Morphology of crystal**
OpA_c1, OpP_a1, OpP_s1, OpQ3, KpC1, KpF3	I / H3[1], H18a18c[2], H42[3]	*japonensis*[1], *yoso*[2], *jinghongiensis*[3]	bp
OpS1, OpF3, OpF4, KsS1, KsA_c1	III / H2	*finitimus*	r
OpQ1	IV / H19	*tochigiensis*	c
OpF1, OpF2	V / H3a3c	*alesti*	

* the tree names and strains origins were used to designate isolates: K = Karkonosze National Park, O = Osola (sampling sites for leaf and soil samples); sample source: p = phylloplane, s = soil; A_c = *Acer pseudoplatanus*, C = *Corylus avellana*, F = *Fagus silvatica*, P_a = *Picea abies*, P_s = *Pinus silvestris*, Q = *Quercus robur*, S = *Sorbus aucuparia*; the numbers reflect the number of successive isolates
** bp = bipiramidal, r = rectangular, c = cuboidal

Table II
B. thuringiensis soil and leaves isolates from the Upper Silesia, Poland

Symbol of *B. thuringiensis* strains*	Biochemical group/ Serovar	*B. thuringiensis* subspecies	Morphology of crystal**
$ŚpP_s1$, $ŚpC_p5$, BpQ11, BpQ18	I/H3[1, 4, 5], H7[2], H4[3], H44[6]	*kurstaki*[1], *aizawai*[2], *keynae*[3], *sumiyoshiensis*[4], *fukuokaensis*[5], *higo*[6]	bp
ŚpQ7, BsP_s1	II/H12[7], H25[8], H30[9]	*thompsoni*[7], *coreanensis*[8], *medellin*[9]	bp
BsC1, BsC9	III/H10[10], H32[11]	*darmstandiensis*[10], *cameroun*[11]	s
BpT_x5, BpA_c4	IV/H17	*tohokuensis*	bp
BpA_c2, BsC16	V/H5	*galleriae*	s
BpT_x1	VI/H24	*neoleonensis*	s
BsC6	VII/H8[12], H35,[13] H38[14]	*ostriniae*[12], *seoulensis*[13], *oswaldocruzi*[14]	bp
$ŚpP_t3$	VIII/H3a 3c	*alesti*	bp

* the tree names and strains origins were used to designate isolates: Ś = Świerklaniec (forest), B = Brynek (park) (sampling sites for leaf and soil samples), samples source: p = phylloplane, s = soil, A_c = *Acer pseudoplatanus*, P_s = *Pinus silvestris*, Q = *Quercus robur*, P_t = *Populus tremula*, C_p = *Carpinus betulus*, C = *Cartaegus monogyna*, T_x = *Taxus baccata*, the numbers reflect the number of successive isolates.
** bp = bipiramidal, s-spherical

Media. The sporulation medium (Kaelin *et al.*, 1994) contained: glucose – 10 g, Casamino acids – 7.5 g, KH_2PO_4 – 6.8 g, $MgS0_4 \times 7H_2O$ – 123 mg, $MnSO_4 \times 4H_2O$ – 2.23 mg, $ZnSO_2 \times 7H_2O$ – 14 mg, $Fe_2(SO_4)_3$ – 20 mg, H_2O dist. – 1L, pH 7.5 was used.

Separation and purification of parasporal crystal of *B. thuringiensis* strains. The strains were grown on sporulation medium at 28°C for 5 days with shaking, giving a spore content of about 10^8 ml^{-1}. Crystals and spores of each cultures were washed twice in cold distilled water by centrifugation (30 min at 4 000 rpm) and resuspended in 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM KCl, as previously described by Lonc *et al.* (1997). Crystals were purified on discontinuous sucrose gradients (67, 72, 79 and 87%) at 15 000 rpm for 30 min. Bands were collected and washed three time in distilled water. Samples of purified crystals were solubilized in 50 mM $NaHCO_3$ (pH 10.0) for 1h at 37°C (Gill *et al.*, 1987).

SDS-PAGE. The protein composition of parasporal bodies was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was conducted essentially as described by Laemmli (1970) with a 3% stacking gel and 10% running gel. Solubilized crystal proteins were mixed with denaturation buffer (1 : 1), heated for 2 min at 100°C and then loaded onto the gel. After elecrophoresis gels were stained with Coomassie blue. The molecular masses of the parasporal body proteins were estimated by comparison with a series of protein size standards (WIDE M 4038, Sigma).

Results

The protein content of purified parasporal crystals from twenty nine isolates of *B. thuringiensis*, analysed by SDS-PAGE, is shown in Figure 1 and Figure 2. The protein profiles of crystalline inclusions are quantatively and qualitatively different (Tables III and IV). The crystalline parasporal inclusions of *B. thuringiensis* isolates were composed of two, three, four or five proteins. Molecular weight of these polypeptides varied from 23.4 kDa to 142 kDa. Strains OpA_c1, OpP_a1, OpP_s1, OpQ3, KpC1 and KpF3 belonging to *B. thuringiensis japonensis*, *yoso* and *jinghongiensis* group produced crystals composed of two or three poplypeptides of 34.1 to 142 kDa. Crystals of OpS1, OpF3, OpF4, KsS1 and KsAc1 isolates of *B. thuringiensis finitimus* contained two, three or four proteins; their molecular weight varied from 34.7 to 139 kDa.

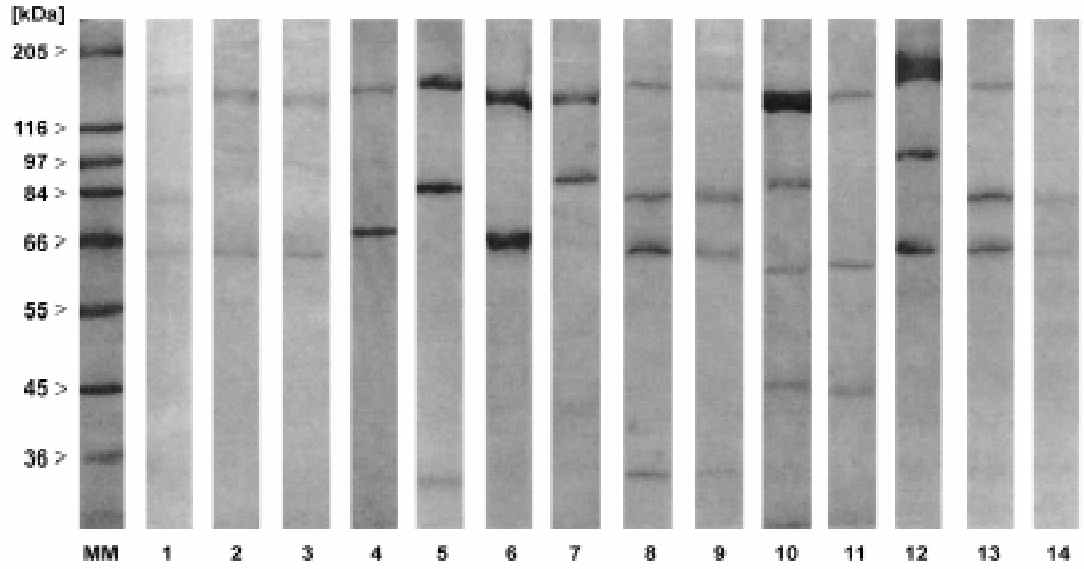

Fig. 1. Proteinogram of parasporal crystals isolated from *B. thuringiensis* strains originating from the Lower Silesia area OpA_c1 (line 1), OpP_a1 (line 2), OpP_s1 (line 3), OpQ3 (line 4), KpC1 (line 5), KpF3 (line 6), OpS1 (line 7), OpF3 (line 8), OpF4 (line 9), KsS1 (line 10), KsA_c1 (line 11), OpQ1 (line 12), OpF1 (line 13), OpF2 (line 14); MM-molecular masses of standards WIDE M 4038 (Sigma).

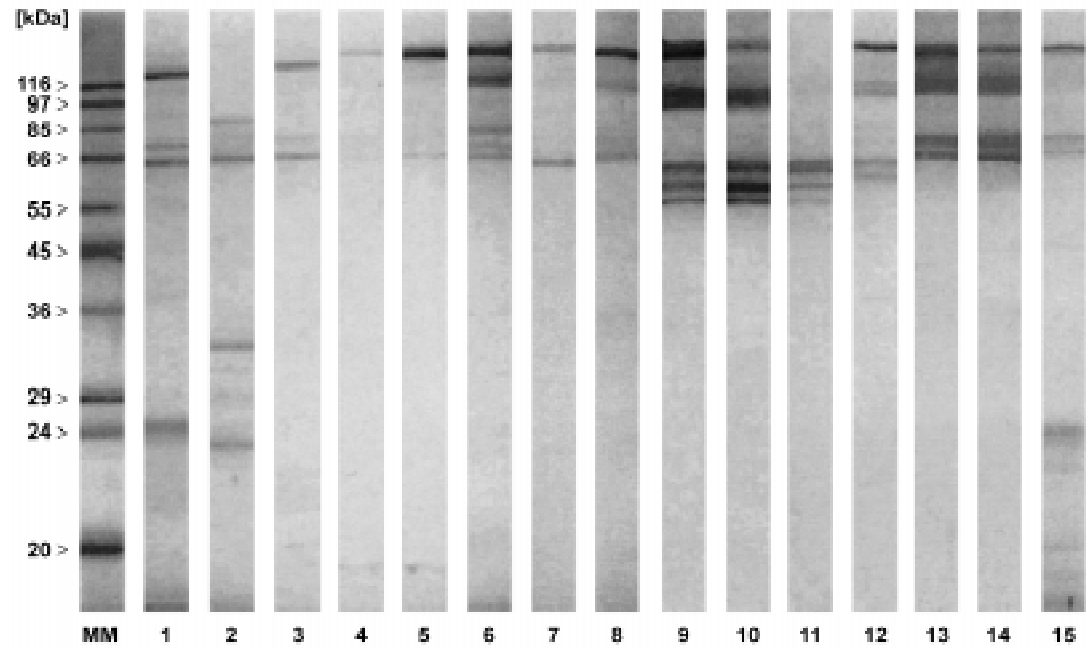

Fig. 2. Electrophoregram of crystalline inclusions of *B. thuringiensis* isolates from the Upper Silesia regions BpT_x5 (line 1), $ŚpC_p5$ (line 2), BpQ11 (line 3), BsB9 (line 4), BsC1 (line 5), BsC6 (line 6), BsC16 (line 7), BpA_c2 (line 8), $ŚpP_s1$ (line 9), BpQ18 (line 10), BpT_x1 (line 11), BsP_s1 (line 12), ŚpQ7 (line 13), $ŚpP_t3$ (line 14), BpA_c4 (line 15); MM-molecular masses of standard WIDE M 4038 (Sigma).

Table III
Molecular weight of delta-endotoxins in parasporal inclusions of *B. thuringiensis* isolates from the Lower Silesia

Number	Symbol of *Bacillus thuringiensis* strain	Subspecies / Biochemical type	Molecular weight of delta-endotoxins (kDa)
1.	OpA_c1	*B. thuringiensis japonensis*, *yoso, jinghongiensis* / I	136.3; 80; 63.6
2.	OpQ3	*B. thuringiensis japonensis*, *yoso*, *jinghongiensis* / I	136.3; 68
3.	OpP_a1	*B. thuringiensis japonensis*, *yoso*, *jinghongiensis* / I	136.3; 64.4
4.	OpP_s1	*B. thuringiensis japonensis*, *yoso*, *jinghongiensis* / I	133.6; 62.9
5.	KpC1	*B. thuringiensis japonensis*, *yoso*, *jinghongiensis* / I	142; 86.2; 34.1
6.	KpF3	*B. thuringiensis japonensis*, *yoso*, *jinghongiensis* / I	136.3; 66.2
7.	OpS1	*B. thuringiensis finitimus* / III	130.1; 66.2
8.	OpF3, OpF4	*B. thuringiensis finitimus* / III	139.2; 82; 64.4; 34.7
9.	KsS1	*B. thuringiensis finitimus* / III	133.6; 86.2; 60.5; 45
10.	KsA_c1	*B. thuringiensis finitimus* / III	133.6; 61.3; 44.4
11.	OpQ1	*B. thuringiensis tochigiensis* / IV	147.9; 100.2; 63.6
12.	OpF1, OpF2	*B. thuringiensis alesti* / V	136.3; 80; 63.6; 80

Table IV
Molecular weight of delta-endotoxins in parasporal inclusions of *B. thuringiwnsis* isolates from the Upper Silesia, Poland

Number	Symbol of *Bacillus thuringiensis* strain	Subspecies/biochemical type	Molecular weight of delta-endotoxins (kDa)
1	$ŚpC_p5$	*B. thuringiensis kurstaki, B. thuringiensis aizawai, B. thuringiensis fukuokaensis, B. thuringiensis higo*/ I	88; 65,5; 32,8; 23,4
2	$ŚpP_s1$, BpQ18	*B. thuringiensis kurstaki, B. thuringiensis aizawai, B. thuringiensis fukuokaensis, B. thuringiensis higo*/ I	142; 102; 74; 66; 64
3	BpQ11	*B. thuringiensis kurstaki, B. thuringiensis aizawai, B. thuringiensis fukuokaensi, B. thuringiensis higo*/ I	136; 74; 65
4	ŚpQ7, BsP_s1	*B. thuringiensis thompsoni, B. thuringiensis coreanensis, B. thuringiensis medellin*/ II	142; 102; 72; 64
5	BsC1, BsC9	*B. thuringiensis darmstandiensis, B. thuringiensis cameroun*/ III	138; 65,5
6	BpT_x5	*B. thuringiensis tohokuensis*/ IV	132; 74; 65,5; 24,6
7	BpA_c4	*B. thuringiensis tohokuensis*/ IV	142; 72; 66; 24
8	BsC16	*B. thuringiensis galleriae*/ V	142; 64
9	BpA_c2	*B. thuringiensis galleriae*/ V	142; 98; 64
10	BpT_x1	*B. thuringiensis neoleonensis*/ VI	74; 66; 64
11	BsC6	*B. thuringiensis ostriniae, B. thuringiensis seoulensis, B. thuringiensis oswaldocruzi*/ VII	138; 102; 74; 65,5
12	$ŚpP_t3$	*B. thuringiensis alesti*/ VIII	142; 102; 72; 64

An identical protein pattern (34.7, 64.4, 82.0 and 139.2 kDa) was characteristic of phylloplane strains OpF3 and OpF4 (*B. thuringiensis alesti*). Two other strains of *B. thuringiensis alesti*: OpF1 and OpF2 had crystals composed of only 63.1, 80,0 and 136.3 kDa. Different protein profiles had parasporal inclusions of *B. thuringiensis alesti* $ŚpP_t3$ isolated from the Upper Silesia region. Four proteins with estimated molecular masses of *ca* 142, 102, 72 and 64 kDa were detected as the main components of these crystals. The same protein profile of crystals had two other strains of *B. thuringiensis* ŚpQ7 and BsP_s1 belonging to *B. thuringiensis thompsoni, coreanensis* and *medellin* group. Four strains with biochemical characters of *B. thuringiensis kurstaki*, *aizawai*, *fukuokaenensis* and *higo* isolated from phylloplane from the Upper Silesia area produced crystals which contained three, four or five delta-endotoxins of 23.4 to 142 kDa. Identical protein profiles of inclusions had two strains BsC1 and BsC9 belonging to *B. thuringiensis darmstandiensis* and *cameroun*

group. Their crystals had two polypeptides of 138 and 65.5 kDa. Both strains were isolated from soil of the same area. *B. thuringiensis* strains of two subspecies: *B. thuringiensis tohokuensis* and *B. thuringiensis galleriae* had delta-endotoxins that can vary in number and in size from around 24 to142 kDa. Different protein patterns of crystals were stated in strains BpT_x5 and BpA_c4 belonging to *B. thuringiensis tohokuensis*. Molecular weight of four delta-endotoxins included 132, 74, 65.5 and 24.6 kDa in BpT_x5 strain and 142, 72, 66 and 24 kDa in BpA_c4 strain. Crystals of BpA_c2 and BsC16 isolates belonging to *B. thuringiensis galleriae* were composed of two or three proteins of 64 to 142 kDa. The unique protein pattern of crystalline inclusions was characteristic for three strains of different serovars of *B. thuringiensis*: BpT_x1, BsC6 and OpQ1. Crystals of OpQ1 isolate belonging to *B. thuringiensis tochigiensis* contained three delta-endotoxins of 63.6, 100,2 and 147,9 kDa. Also proteins of 64, 66 and 74 kDa were present in parasporal inclusion of BpT_x1 isolate of *B. thuringiensis neoleonensis*. Four delta-endotoxins of 138, 102, 74 and 65.5 kDa were found in soil isolate BsC6 belonging to *B. thuringiensis ostriniae*, *seoulensis* and *oswaldocruzi* group.

It can be seen that in 29 analyzed *B. thuringiensis* strains both large (from 130.1 to 147.9 kDa) and/or medium (from 60.5 to 102 kDa) delta-endotoxins occurred. A great diversity of delta-endotoxin profiles was observed by SDS-PAGE methods. Some profiles indicated components lower than 60 kDa, and others showed multiple components or components ranging between 138 and 64 kDa. Components higher than 142 kDa appeared much less frequently and were detected in the crystals of OpQ1 isolate. Small proteins of 23.4–45.0 kDa were found in eight isolates, namely KpC1, OpF3, OpF4, KsS1, KsA_c1, $ŚpC_p5$, BpT_x5 and BpA_c4 originating from different sample sources and areas. These components were isolated from strains belonging to various serovars: *B. thuringiensis kustaki*, *B. thuringiensis finitimus*, *B. thuringiensis japonensis* and *B. thuringiensis tohokuensis*. Analysis of the protein composition of crystalline inclusions of newly isolated *B. thuringiensis* strains from Poland indicate that two or more subgroup of the same serotype may possess several crystals with different protein profiles of delta-endotoxins.

Discussion

The previous entomopathogenic activity of *B. thuringiensis* strains from the Wrocław collection encouraged us to attempt a closer characteristics of their parasporal inclusions. The inclusions are a potential source of new, insecticidal proteins which in the future may join the world's collection of delta-endotoxins, or even become directly useful in biological control of pest insects or vectors of infectious and invasive diseases. It is in the line with COST 862 programm "Bacterial toxins for insects control" (http://cost.cordis.lu/src/cso_informatio.cfm).

Our investigation indicated that crystals of twenty nine *B. thuringiensis* isolates possessed quantatively and qualitatively different patterns of delta-endotoxins. In addition there is no correlation between serovar *B. thuringiensis* strain and type of crystalline inclusion produced. Several isolates producing crystals of the same morphology belong to different serovars. Often the typical bipiramidal parasporal inclusions were synthesised by many various *B. thuringiensis* strains, including *B. thuringiensis japonensis*, *B. thuringiensis kurstaki*, *B. thuringiensis thompsoni*, *B. thuringiensis tohokunensis*, *B. thuringiensis ostriniae*, and *B. thuringiensis alesti.* Other *B. thuringiensis* isolates formed spherical, rectangular or cuboidal crystalline inclusions. Also the same crystals of various serovars of *B. thuringiensis* strains were morphologically indistinguishable. These phenomenon were observed also by many authors (Benintende *et al.*, 1997; Lecadet *et al.*, 1999; Martin *et al.*, 1989; Ohba *et al.*, 1986; Ohba *et al.*, 1989; Ohba *et al.*, 1992; Wassano *et al.*, 1997). The presence of bipiramidal or cuboidal parasporal inclusions may indicate Lepidoptera-specific *B. thuringiensis* strains. However, isolates belonging to the anti-Lepidoperan strains of *B. thuringiensis darmstandiensis*, *B. thuringiensis galleriae* and *B. thuringiensis finitimus* produced another type of crystals, particularly spherical and rectangular. This result may indicate that amino acid composition of Cry proteins and the number of protoxins in the crystal determine the shape as well as insecticidal properties.

Electrophoretic analysis revealed great differentiation of protein patterns of the purified delta-endotoxins from *B. thuringiensis* isolates. Most of them appear to belong to Cry1 class, associated with effects against pest moths. It is the largest group of medium (*ca*. 81 kDa) and large-sized (130–142 kDa) proteins. There are about 135 Cry proteins (45%) among the 300 delta-endotoxins described to date (http://www.biols.susx.ac.uk/home/ Neil_Crickmore). In our investigations the crystals of 27 out of 29 *B. thuringiensis* isolates possessed the large-sized proteins of 130.1 to 147.9 kDa. These molecular weight are also characteristic of numerous anti-Lepidopteran toxins of Cry1 category, *eg.* Cry1A (Lee *et al.*, 1991), Cry1B

(Brizzard *et al.*, 1988; Kuo *et al.*, 2000), Cry1C (Honee *et al.*, 1988), Cry1F (Chambers *et al.*, 1991), Cry1I (Choi *et al.*, 2000; Tailor *et al.*, 1992) or Cry1K (Koo *et al.*, 1995). Several of them were found in parasporal inclusions of *B. thuringiensis berliner*, *B. thuringiensis kurstaki*, *B. thuringiensis entomodiscus*, *B. thuringiensis alesti*, *B. thuringiensis aizawai, B. thuringiensis morrisoni* and *B. thuringiensis wuhanensis* (Brizzard *et al.*, 1988; Chambers *et al.*, 1991; Choi *et al.*, 2000; Entwistle *et al.*, 1993; Kuo *et al.*, 2000; Lee *et al.*, 1991). However, the proteins of 130–140 kDa can be similar in size to the Cry4 Diptera-specific proteins (Höfte *et al.*, 1989) and the Cry3 Coleoptera-specific proteins (Ceron *et al.*, 1995; Wasano *et al.*, 1997). Whereas the medium-sized proteins (64 to 102 kDa) were identified in all the presently tested strains. The molecular masses of these proteins can correspond to various classes of known Cry proteins, *eg.* Cry2 specificity against Lepidoptera and Dipteran larvae (Espinasse *et al.*, 2002; Honguy *et al.*, 2000; Nichols *et al.*, 1989), Cry3 and Cry5 toxicity against Coleopteran or Coleopteran and Lepidopteran larvae (Crickmore *et al.*, 1998; Espinasse *et al.*, 2002; Lopez-Meza *et al.*, 1996; Shin *et al.*, 1995) or Cry10 and Cry11 subclasses activity against Dipteran larvae (Delécluse *et al.*, 1995; Saitoh *et al.*, 1998; Wirth *et al.*, 2000). The size of delta-endotoxins of 70 to 105 kDa may indicate also the proteins of Cry4 class (Kawalek *et al.*, 1995; Tabashnik, 1992). A few crystalline inclusions of eight *B. thuringiensis* isolates classified to four biochemical groups, including *B. thuringiensis tohokuensis*, *B. thuringiensis kurstaki*, *B. thuringiensis finitimus* and *B. thuringiensis japonensis* were composed of the small components of polypeptides of 23.4 to 45 kDa. The size of these proteins may indicate the Cyt proteins (25–30 kDa) (Cheong *et al.*, 1997; Gill *et al.*, 1987; Georghiou *et al.*, 1997; Ragni *et al.*, 1996; Thomas *et al.*, 1983) or anti-coleopteran delta-endotoxins of Cry3 and Cry35 subclasses (39–55 kDa) (Ellis *et al.*, 2002; Entwistle *et al.*, 1993; Kim *et al.*, 2000).

In the nearest future the immunological relationships of separated components of the crystals from *B. thuringiensis* isolates will be analysed by Western blot. Distribution of *cry* genes of *B. thuringiensis* strains isolated from Silesia also will be determined by PCR method. This investigation will complete the data on Cry proteins present in parasporal inclusions of *B. thuringiensis* strains originating from Poland. This way the genetic relationships of genes encoding ICPs will be compared with the data presented by Święcicka *et al.* (2005).

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