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A Novel *Bacillus thuringiensis* Crystal Protein Cry51Aa1 with Preferentially Anti-cancer Activity

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Cry51Aa1, a novel crystal protein comprised of 309 amino acid residues with a predicted molecular weight of 34 kDa, was purified from *Bacillus thuringiensis* strain F14-1. After trypsin digestion, we observed three clear smaller bands of Cry51Aa1 protein upon separation by gel. In addition, this digested Cry51Aa1 protein had no obvious toxicity to several types of insect larvae and hemolytic activity human erythrocyte. However, the activated Cry51Aa1 could exhibit cytocydal activity against to MDA-MB-231, HCT116, CaCo2 and U87 tumor cells, while no cytotoxic activity was found in Cry51Aa1 treated PC3, HepG2 and LO2 cells. Importantly, MDA-MB-231 cells showed the most sensitivity to activated Cry51Aa1 within all the cell lines assessed. These observations referred to the presence of a new non insecticidal toxin of Cry51Aa1, which should be a new parasporin protein.

Key words: *Bacillus thuringiensis*, Cry51Aa1, Anticancer activity, Preferential cytotoxicity, MDA-MB-231.

Bacillus thuringiensis (Bt), a spore-forming anaerobic gram-positive bacterium belonging to the *Bacillus cereus* group, can produce various crystal (Cry) proteins which are widely used in engineered agriculture for their natural cytotoxicity to insects. Wherease, non-insecticidal Cry proteins were isolates in natural environments[1, 2]. It's worth to note that the non-insecticidal Cry proteins often account for more than 90% of the natural populations from soils [3, 4] and phylloplanes [5]. This raises a survey for the biological roles of these non-insecticidal proteins.

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A subgroup of Cry proteins, classified as parasporin (PS), have been shown to exhibit cytocidal activity preferential for human cancer cells of various origins, which is a novel biological activity of Cry proteins undiscovered before. Besides, there is no insecticidal and hemolytic activity in PS proteins [6-9]. Until now, 19 PS proteins, which are divided for 6 groups, have been identified and studied, even though they have different cytotoxicity spectra and mechanisms underlying their cancer cell-killing action. PS1Aa1 has been demonstrated with high cytotoxicity against HeLa, HL-60 and MOLT-4 cells, but without toxicity to Jurkat T, A549, Caco2 cells [10]. It seems that the anticancer effect is mediated by PS1Aa1 induced Ca^{2+} level increase and cell apoptosis, though PS1Aa1 is not a membrane pore-forming toxin [11]. PS2Aa1 shows extremely high toxicity to Sawano, MOLT-4, HL-60, Jurkat T and HepG2 cells,

which is possibly due to its damage to cell plasma membrane permeability [12]. Relatively, PS3Aa1 has relatively narrow cytotoxicity spectra, as it only shows toxicity to HL-60 and HepG2 cells [13]. PS4Aa1 induces cell swelling, nuclear shrinkage and cell burst, which also reveals a non-apoptotic effect, and protein structure analysis suggests that PS4 is a unique ^{2}-pore-forming toxin with a cholesterol-independent activity [14, 15]. PS6Aa1, another newly identified parasporin prtotein, shows higher toxicity to HepG2 and HeLa cells than that to Caco2 and HC cells [16]. Though PS5Aa1 was added in the parasporin nomenclature, no result was published to elucidate its cytocidal activity. Therefore, six groups of PS proteins has been established, but we cann't exclude the exist of more parasporin-like proteins which could be promising in the application of cancer therapy.

In this study, we investigated the cytotoxic activity of Trypsin digested crystal protein Cry51Aa1 in insect and mammalian cells. We found that Cry51Aa1 protein converted into three main bands after digestion and speration on SDA-PAGE, which showed neither hemolytic activity nor cytotoxicity to Lepidoptera or nematode. However it showed predominant inhibition to the survival of several kinds of mammalian cancer cells, especially breast cancer cell MDA-MB-231, while non-digested Cry51Aa1 has no effect to any studied cells.

MATERIALS AND METHODS

Reagents

Trypsin (Sigma, St Louis, MO, USA) and BCA protein assay reagent kit (Beyotime Institute of Biotechnology, Haimen, China) were purchased from their respective company. Phenyl methyl sulfonyl fluoride (PMSF) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Biosharp (Hefei, China).

Bacterial Strains culture and crystal protein purification

Bacillus thuringiensis strain F14-1, which can produce crystal protein Cry51Aa1, was presented by Professor Sun in State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan, China. Strain F14-1 was cultivated on ICPM agar plates for more than 48 h at 28°C. When 80~90% of the cells reached sporulation monitored by microscopy, cultures were harvested. After washing three times in 1 M NaCl and resuspending in distilled water, parasporal inclusion bodies were purified by technique described previously [17].

Mammalian cells culture conditions

The human MDA-MB-231 (human breast adenocarcinoma), HCT116 and Caco2 (human colon cancer), U87 (glioma), HepG2 (hepatic cancer), PC3 (prostate cancer) and LO2 (human embryo liver cell) cell lines (from CCTCC, Wuhan) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Life Technologies, Shanghai, China) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin in 5% CO_2 at 37 °C.

Protein separation and treatment

For separation of proteins, the crystal suspension was added to gel-loading buffer (2% sodium dodecyl sulfate, 10% glycerol, 5% mercaptoethanol, 0.05% bromophenol blue, 125 mM Tris–HCl (pH 6.8) and incubated at 100 °C for 10 min. After centrifugation for 3 min, the supernatant was analyzed by SDS-PAGE. The gels were stained with Coomassie blue R-250 (Sigma).

The purified crystal protein Cry51Aa1 was solubilized with carbonate buffer (pH 10.0) at 37 °C for 1 h. Solubilized proteins were treated with 2.5mg/ml trypsin (pH 8.0) at 37 °C for 60 min, and then phenyl methyl sulfonyl fluoride was added to reach a final concentration of 1 mM to stop the proteolytic reaction. The sample was then dialyzed against 50 mM Tris (pH 9.0) overnight at 4°C. Protein concentration was determined by BCA protein assay reagent kit.

Insecticidal activity test

Following the procedure described previously [18], toxicity of Cry51Aa1 protein was assessed against second-instars larvae of *Bombyx mori* and second-stage juvenile of *Caenorhabditis elegans*. Briefly, fresh mulberry leaves were cut to the same size and brushed with the same volume of protein solution to be tested. After drying at room temperature, the leaves were placed in individual petri dishes. For each set, twenty-five second-instars larvae were placed onto two individual leaves. Larval mortality was monitored and the number of live larvae was counted 6 days later.

Hemolytic activity

Hemolytic activity of activated and non-activated Cry51Aa1 proteins against human erythrocytes were analyzed as the published method [19]. Briefly human erythrocytes were washed twice with 20 mMTris-buffered saline (pH 8.0) followed by centrifugation at 800 g. Then resuspend the pellet and adjust the concentration to 2% (v/v) in Tris-buffered saline. The diluted Cry51Aa1 protein was mixed with an equal volume of erythrocyte suspension in 96-well plate. After incubation at 27 ºC for 18 h, the plate was centrifuged at 800g for 10min, and absorbance of each well was measured at 540 nm. Each assay was repeated three times. Human blood sample collection and use is approved by the Clinical Research Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology.

Cytotoxicity assays

The cytotoxic activity of Cry51Aa1 protein on different cell lines was measured by the MTT method. Briefly, each well of the 96-well microtest plat was added with 90 µl of cell suspension containing 1×10^4 cells and incubated at 37 °C for 24 h. Next, Cry51Aa1 protein solution was added to each well and the plate was incubated at 37 °C for 24 h. Then, 200 µl of the MTT reagent (0.5 mg/ml) was added to each well. After incubation at 37 °C for 4 h, cell media was removed and 150 µl dimethyl sulfoxide was added to each well. Absorbance of the dissolved solution in each well was recorded at 590 nm with a reference filter of 620 nm. The degree of cytotoxicity (CT) in this assay was determined as described previously [17].

Statistical analysis

The results at least from two independent experiments were analyzed by two-tailed Student's *t*-test using Prism (GraphPad, San Diego, CA, USA). All data are represented as mean value ± S.E.

RESULTS

Cry51Aa1 protein analysis of *Bacillus thuringiensis* F14-1 strain

The purified crystal protein of strain F14-1 was analyzed by SDS-PAGE and a predominant 34 kDa peptide band was found (Fig. 1A). The gene sequence of this crystal protein Cry51Aa1 has been sumiteed to GenBank (DQ836184.1) in 2006 and at that time its detailed function was still unclear. Parasporal proteins capable of exhibiting their activity to either insects or cancer cells must undergo a proteolytic treatment in the presence of proteases. Therefore, we used different concentrations of trypsin to treat Cry51Aa1 protein and found 2.5 mg/ml trypsin could completely digested the primary Cry51Aa1 protein. Under the trypsin treatment condition described in methods, three small clear bands about 30 kDa, 25 kDa and less than 25 kDa were observed by SDS-PAGE as shown in Fig.1B. Particularly, the smallest band named Cry51Aa1-T3 is relatively more stronger

Table 1. Analysis of the cleaved proteins of Cry51Aa1 by MS spectrometry

Observed band	Position in pro-Cry51Aa1	Cleavage site	Theoretical (Da)
Cry51Aa1-T1	41-309	Lys37	29520.7869
	41-307	Lys37/Lys307	29287.5629
	7-278	Lys10/Lys282	29834.0399
	24-291	Lys23/Lys291	29382.4769
	24-290	Lys23/Lys290	29236.2879
Cry51Aa1-T2	41-277	Lys40/Lys277	25853.6319
	54-291	Arg53/Lys291	26103.9379
	54-290	Arg53/Lys290	25957.7489
	24-263	Lys23/Arg263	25853.5449
Cry51Aa1-T3	92-309	Lys91	23780.3659
	93-309	Lys92	23134.1769
	93-307	Lys92/Lys307	23400.9529
	41-263	Lys40/Arg263	24043.6359
	54-273	Arg53/Arg273	23895.4329

Table 2. The cytotoxicity degree classification of activated Cry51Aa1

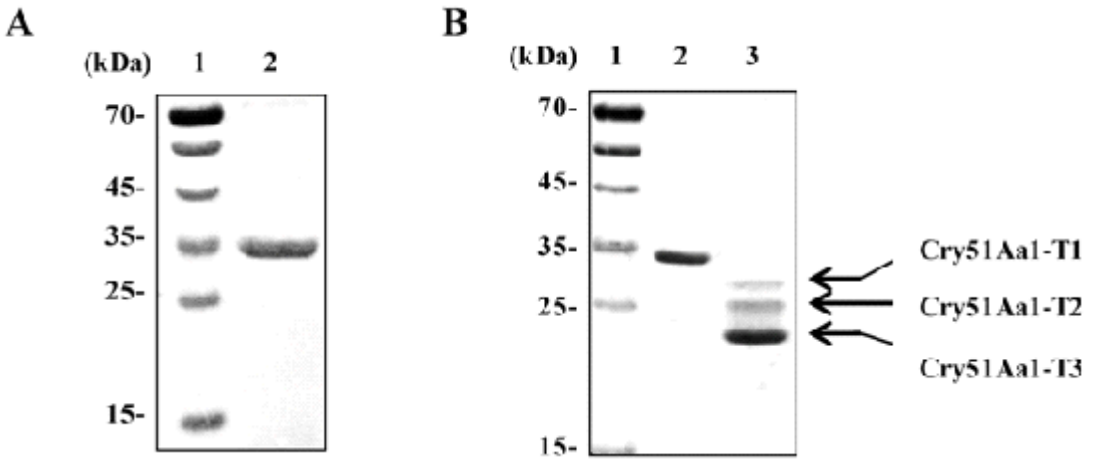
Cry51Aa1(μg/ml)	MDA-MB-231	HCT116	CACO2	PC3	LO2	HepG2	U87
1	+	±	±	-	-	-	-
10	++	+	+	-	-	-	+
20	+ ++	+ +	+ +	-	-	-	++
100	+ + +	+ +	+ +	-	-	/	/

++++, extremely high; +++, high; ++, moderate; +, low; ±, very low or –, non-toxic /, not detected

than others, implying that Cry51Aa1 is more inclined to be digested to this form which may contribute by potential key residues recognized by trypsin. Combined with the predicted digestion sites in Cry51Aa1 and calculated sizes of their resulted bands, we grouped these information responsible for the predicted digested-Cry51Aa1 protein fragments (Table 1). As there were many potential recognition sites which can be cleaved Cry51Aa1 into fragments, we calculated percentage of each site according to the times of their occurrence in Table 1. The residues of Lys40 and Arg53 were most likely to be digested in protein sequence of Cry51Aa1. In addition, Arg263 ranked the second level within all the candidate digestion sites, suggesting that the Cry51Aa1 fragment from position 41 to 263 could be the most abundant band after digestion, which is constent with our observation in Fig. 1B. Further investigation on the Cry51Aa1 trpsin digestion map is needed.

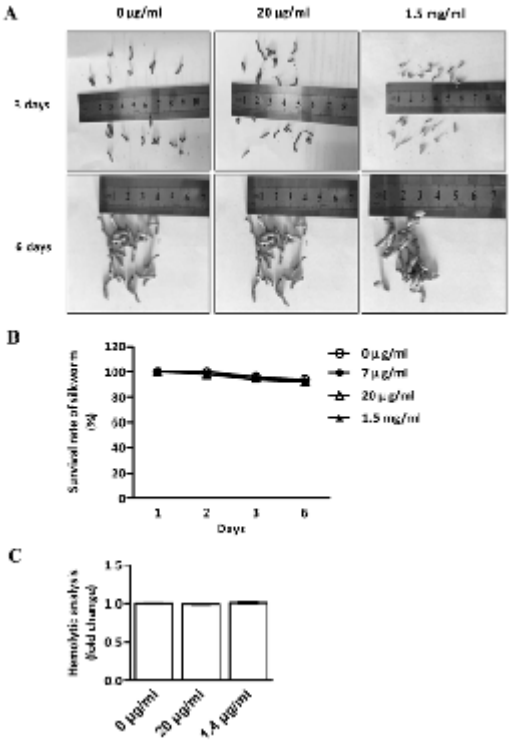
Insecticidal and hemolytic activity analysis of Cry51Aa1

We first examined the *in vivo* cytotoxity of Cry51Aa1 to insects and found that Cry51Aa1 had no obvious toxicity to second-instar larvae of *Bombyxmori* and second-stage of *Caenorhabditi selegans*. The representative images of *Bombyxmori* larvae treated with Cry51Aa1 were shown in Fig.2A. The survival rates of silkworm larvae in the prensence of Cry51Aa1 protein was measured and the results showed that silkworm survival was not significantly affected by Cry51Aa1 treatmentm, even in the highest concentration of 1.5 mg/ml as showed in Fig. 2B. The non-insecticidal activity suggested that Cry51Aa1 may rarely contribute to control agricultural insect pests. We then checked its hemolytic activity to human erythrocytes and found that the activated Cry51Aa1 showed no hemolytic activity (Fig. 2C). Since the structure



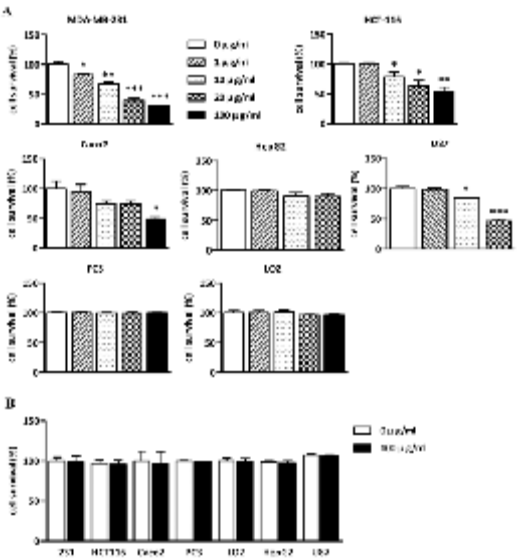
A. Cry51Aa1 Protein of F14-1 detected by SDS-PAGE, harboring a 34-kDa peptide and its sequenced N-terminal sequence. B. Cry51Aa1 protein was digested with 2.5 mg/ml trypsin for 1hour at 37 °C followed by SDS-PAGE analysis

Fig. 1. Cry51Aa1parasporal inclusion protein and its digestionby trypsin



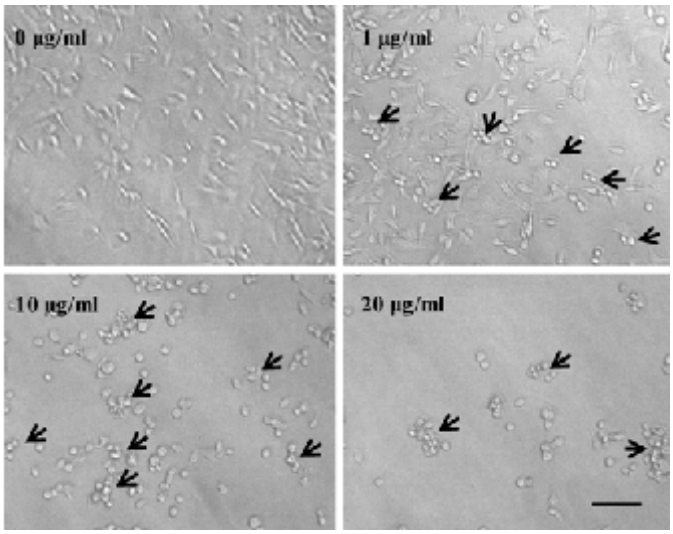
A.Photography of larvae of silkworm (*Bombyx mori*) fed with Cry51Aa1 protein. B. Survival rate curves of Cry51Aa1 treated-silkworm larvae. C. Hemolytic activity of Cry51Aa1 to normal human erythrocytes was measure at 540 nm absorbance.

Fig. 2. Insecticydal and hemolytic analysis of digested Cry51Aa1



A. Cytotoxicity was measured by MTT method. Trypsin-treated Cry51Aa1 was added to cultured MDA-MB-231, HCT-116, Caco2, PC3,U87,hepG2 or LO2 cells at different final concentrations (1, 10, 20, and 100 µg /ml). After incubated at 37°C for 24 h, cells were harvested and viability was measured. Values are shown as the mean and standard deviation of triplicate measurements. B. Cells were treated with none-activated Cry51Aa1 followed by MTT assay.

Fig. 3. Cytotoxity analysis of activated Cry51Aa1 against several mammalian cells in vitro



MDA-MB-231 cells were treated with digested Cry51Aa1 at indicated concentrations for 24 h followed by optical imaging under a microscope. Scale bar, 50 µm.

Fig. 4. Morphological observation of MDA-MB-231 cells treated with Cry51Aa1

analysis revealed that Cry51Aa1 is a paprasporin protein (data not shown), it rasied the question of whether this protein has anti-cancer cell activity.

Cytotoxicity analysis of Cry51Aa1 protein toward different cell lines

An extend screening of Cry51Aa1 protein's cytotoxicity to different human cell lines was commenced after no insecticidal and hemolytic activity of Cry51Aa1 was found as described above. The results showed that digested Cry51Aa1 had no cytotoxic activity to PC3, HepG2 or LO2 cells, but significantly inhibited the survival of MDA-MB-231, HCT-116, Caco2 and U87 cells (Fig. 3A). Basing on literature criteria [17], we classified the cytotoxic degrees of Cry51Aa1 to the cell lines studied in Table 2. It showed that only MDA-MB-231 responded to Cry51Aa1 at 1 µg/ml and was most effectively affected at 20 µg/ml of the protein among all the cell lines, implying that MDA-MB-231 was the highest sensitive cell type used here. It's note worthy that neither live cancer cell HepG2 nor nomal live cell LO2 was affected by treatment of digested Cry51Aa1, which indicates the effect of Cry51Aa1 could be tissue specific. Additionally, we treated the cells with non-activated Cry51Aa1 and no cytotoxic effect was found in all the cell lines at the highest concentration of 100 µg/ml protein (Fig. 3B), suggesting that the digested bands after activation process through trypsin digestion rather than the primary Cry51Aa1 protein has the cancer cell-killing function.

Morphological alterations of MDA-MB-231 cells upon treatment with Cry51Aa1

Our finding indicated that MDA-MB-231 cells are sensitive to activated Cry51Aa1 protein, but how this protein preferentially influenced MDA-MB-231 cells is unclear. By observation under microscope, we found that the cells became rounding even under the treatment of 1 µg/ml of the Cry51Aa1 as shown in figure 4 mared by black arrows. The number of round-shaped cells increased in a dose dependent manner followed by cellular clusters. The detailed mechanisms of the Cry51Aa1's effects on MDA-MB-231 cells remain further investigation.

DISCUSSION

Non insecticidal *Bacillus thuringiensis* delta endotoxin is called parasporin which is well known for their ability to recognize and kill cancer cells [20]. The present study was the first to evaluate the cytocidal effect of Cry51Aa1, a parasporal protein from *Bacillus thuringiensis* strain F14-1. The three novel features of Cry51Aa1 (non-insecticidal, non-hemolytic and cancer cell-killing) suggest that Cry51Aa1could possibly be a new parasporin protein. Furthermore, marked differences are evident in the anti-cancer cytotoxicity spectra between Cry51Aa1 and other parasporins. Cry51Aa1 has been found to present a preferential cytotoxicity towards breast cancer cell MDA-MB-231, while all the other four known parasporins (PS1Aa1, PS2Aa1, PS3Aa1, PS4Aa1) were demonstrated to be cytotoxic to HepG2 cells [7], which couldn't be affected by Cry51Aa1 at all. Though we also found obvious cytotoxic effect of Cry51Aa1 on U87 and HCT116 cells, but it's not certain whether other parasporins have the same effects or not. However as the same as PS2Aa1 and PS4Aa1PS Cry51Aa1 could partly cytotoxic to Caco2 cells [7], suggesting some similarities might still exist within these toxins.

Of particular interest is that MDA-MB-231 is extremely sensitive to Cry51Aa1 since the treatment of activated Cry51Aa1 low to 1 µg/ml concentration could exhibit activity against MDA-MB-231 rather than other cell types (Table 2 and Fig. 4). The mechanism of this selective cytotoxicity is still unknown. In the prevailed opinion, a specific receptor involved in the toxicity of bacterial toxins has been evidenced for decades [21-27]. We determined the subcellular localization of digested Cry51Aal in MDA-MB-231 cells through confocal immunofluorescent imaging. It should be noted that rhodamine-labeled Cry51Aa1 protein could enter into cells and locate in nucleus (Data not shown), which implies that Cry51Aa1 could exhibits its function via binding to specific receptor followed by internalization. Identification of the target receptor using active fragment of Cry51Aa1 protein remains further investigation. Therefore, cloning and expression of digested band within Cry51Aa1 protein will be greatly helpful to explore the underlying mechanism by which Cry51Aa1 kills cancer cells, as well as for its future application in breast cancer therapy.

CONCLUSIONS

In summary, Cry51Aa1 parasporal crystal protein which purified from a *Bt* strain F14-1, has no obvious insecticidal and hemolytic activity, but exhibits cytotoxicity towards to several types of cancer cells, especially breast cancer cell MDA-MB-231.Taken together, these results suggest that Cry51Aa1could be a new member of parasporin protein family and may be a promising agent in breast cancer therapy.

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