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Vol. 13(25), pp. 2484-2490, 18 June, 2014
DOI: 10.5897/AJB2014.13853
Article Number: C3A644C45437
ISSN 1684-5315
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African Journal of Biotechnology

Full Length Research Paper

Identification and cloning of two insecticidal protein genes from *Bacillus thuringiensis* strain S185

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Received 12 April, 2014; Accepted 2 June, 2014

***Bacillus thuringiensis* (Bt) is the most widely applied type of microbial pesticide due to its high specificity and environmental safety. The activity of Bt is largely attributed to the insecticidal crystal protein encoded by the *cry* genes. Different insecticidal crystal proteins of Bt have different bioactivity against distinct agricultural insect pests, and combination of these proteins not only increases insecticidal activity, but also overcomes and delays development of resistance. A Bt strain, S185, was isolated from a soil sample collected in Songfeng Shan district, Heilongjiang Province, China. Bt S185 has highly specific insecticidal activity against Coleoptera, and was determined to contain *cry8*-type genes by peptide mess fingerprint (PMF) analysis. Application of polymerase chain reaction-restricted fragment length polymorphisms (PCR-RFLP) analysis further determined the genotype due to the high homology of *cry8Ea1* and *cry8Fa1* genes. Through the full-length primers design, two insecticidal crystal protein genes *cry8Ca* and *cry8Ea* were obtained. Using prokaryotic cloning vectors, the recombinant plasmids pEB-cry8Ca and pEB-cry8Ea were transferred into expression host strain *Escherichia coli* Rosetta, thus the two genes were successfully expressed in heterologous bacteria.**

Key words: *Bacillus thuringiensis*, peptide mess fingerprint, identification, clone, insecticidal crystal protein.

INTRODUCTION

Bacillus thuringiensis (Bt) is a gram-positive soil bacterium characterized by its ability to produce crystalline inclusions named cry or cyt during sporulation (Höfte and Whiteley, 1989). These inclusions consist of proteins with highly specific insecticidal activity (Aronson et al., 1986; Whiteley and Schnepf, 1986). The insecticidal spectrum varies within the 82 different serotypes reported (Lecadet et al., 1999), and affects insects primarily from the orders Lepidoptera, Diptera and Coleoptera (Rasko et al., 2005). There are also reports of Bt active against mosquitoes (Orduz et al., 1995).

Up to August 2013, 712 cry and 38 cyt proteins have been identified, of which 49 are cry8-type proteins, which consist of 1160 to 1210 amino acids and have molecular weights of 128 to 137 kDa (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/;

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Shu et al., 2007; Yu et al., 2006). The cry8-type protein has specific insecticidal activity against a variety of Coleopteran pests such as Scarabaeidae, Curculionidae and Chrysomelidae. Many kinds of Scarabaeidae are major pests of crops and trees, due to the difficulty of prevention and control of soil-dwelling pests. Their larvae (white grub) are one of the largest underground pest groups and cause great damage to many plant species. In China, every year about 16.47 million hectares is affected by the larvae, in a serious year reaching 52.72 million hectares, with yield losses of about 20 to 40%, 933 thousand hm^2 peanuts were only planted in 2002, and there were serious occurrence area of more than 40 hm^2; there was, economic losses of 600 million yuan (Wei et al., 1985; Wang et al., 2012). Therefore, it is of great importance to isolate and clone high-activity cry8-type genes for constructing engineered bacteria and genetically modified plants for prevention and control of Coleopteran pests.

In this context, it is essential to clone and express *cry8* genes from new Bt isolates. The present study describes the identification, cloning and expression of *cry8Ca* and *cry8Ea* from a new indigenous isolate of Bt S185 strain, which has high-virulence against *Holotrichia parallela*.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

Bt

The *Bt* strain S185 used in this study was isolated from soil obtained from Songfeng Shan district, Heilongjiang Province, China. The 1 g soil sample was suspended in 15 mL sterilized distilled water and shaken. After heat treatment at 80°C for 20 min, the appropriately diluted upper-layer of the suspension was incubated on ½ Luria Broth (LB) medium (0.5% tryptone, 0.25% yeast extract and 0.5% NaCl; pH 7.0) at 30°C for three days. Colonies were examined microscopically for the presence of parasporal crystals (Hastowo et al., 1992). *Bt* strains were incubated in LB medium for three days with shaking at 30°C at 250 rpm (Maniatis et al., 1982).

Escherichia coli

The Institute of Plant Protection, Chinese Academy of Agricultural Sciences supplied *E. coli* JM109 as the cloning host and *E. coli* Rosetta as the expression host. The strains were grown in LB medium at 37°C for 12-16 h.

Plasmids

pMD19-T (TaKaRa Biotechnology Company) was used as cloning vector. pEB vector, an improvement of pETblue-2 vector was used as the expression vector modified by CL Shu in 2009 (Changlong et al., 2009).

Microscopy

Optical microscopy observation

The tested strains were dyed with carbol fuchsin after cultivation on solid ½ LB medium at 30°C for 2 dayd, and then examined under an oil immersion objective (Gundersen et al., 1988).

Electron microscopy observation

Spores and crystals of *Bt* S185 were collected by centrifugation at 12 000 rpm for 10 min. The pellet was washed three times with distilled water, and suspended in phosphate buffer containing 3% glutaraldehyde, then dehydrated in dilute ethanol-propylene oxide series and embedded in an Epon resin mixture (Sangon Ltd. China). The sample was photographed by a New Bio-TEM electron microscope (Hitachi Ltd. Japan) operating at an accelerating voltage of 80 kV, after undergoing ultrathin sectioning by a Reichert ultramicrotome (Leika Aktiengesellschaft. Wien Austria) and staining with uranyl acetate and lead citrate.

Plasmid DNA extraction

Bt strain plasmid DNA extraction was as described by Song et al. (2003). The *E. coli* strain plasmid was prepared and further purified by a plasmid kit (Axygen Products), used according to the manufacturer's instructions. Plasmid profiles were determined for each strain by electrophoresis through 0.7% agarose gel.

Protein analysis

Bt S185 was grown with shaking at 180 rpm at 30°C in ½ LB medium until sporulation was complete, as determined by microscopy. The spores and crystals were harvested by isoelectric point deposition (Guo et al., 2005).

Recombinant *E. coli* cells were grown overnight at 37°C in LB containing ampicillin (amp). Bacterial cells were added to 200 ml LB/amp (200 μg/ml) medium and shaken at 180 rpm at 25°C to an OD600 of 0.6 to 0.7 (Srimonta et al., 2012). Expression was induced by adding 0.1 mM isopropyl-β-d-thiogalactoside (IPTG) at low temperature (20°C) for 6 h. The cells were gathered by centrifugation at 8 000 rpm for 10 min at 4°C. For experiments, the cells were resuspended in TE buffer (20 mM Tris, 1 mM EDTA, pH 8.0) and lysed using ultrasonic waves, repeated the 3 / 3 s intervals until the clock reached 10 min. The supernatant was collected and the precipitate was suspended in TE buffer; both stored at −20°C.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 8% gel as described by Laemmli (1970). The molecular weights were estimated by comparison with the protein ladder (GenStar Biosolutions Co. Ltd).

Protein samples used for PMF were derived from SDS-PAGE. The enzymatic digestion was performed as described by Fernandez et al. (1998) and Gharahdaghi et al. (1999). Mass spectrometric analysis was done by the Beijing Genomics institution Co. Ltd.

Cloning and expressing of insecticidal crystal protein gene.

Total DNA of S185 was isolated as described by Iizuka et al. (1981). Primers were generated based on the published sequence of *cry8Ca* and *cry8Ea* genes. The primer sequences used in this study were as follows (SangonCo.Ltd):
cry8C5:5'ATGAGTCCAAATAATCAAAATG3';
cry8C3:5'TTACTCTTCTTCTAACACGAGTTC3';
cry8E5:5'ATGAGECCAAATAATCAAAATG3';
cry8E3:5'TTACTCTACGTCAACAATCAATTC3'.

PCR was carried out for 30 cycles under the following conditions: 94°C for 5 min, 94°C for 30 s, 52°C for 60 s, 72°C for 2 min and 72°C for 10 min. Restriction digestion and ligation were carried out as per the manufacturer's instructions (TaKaRa Biotechnology

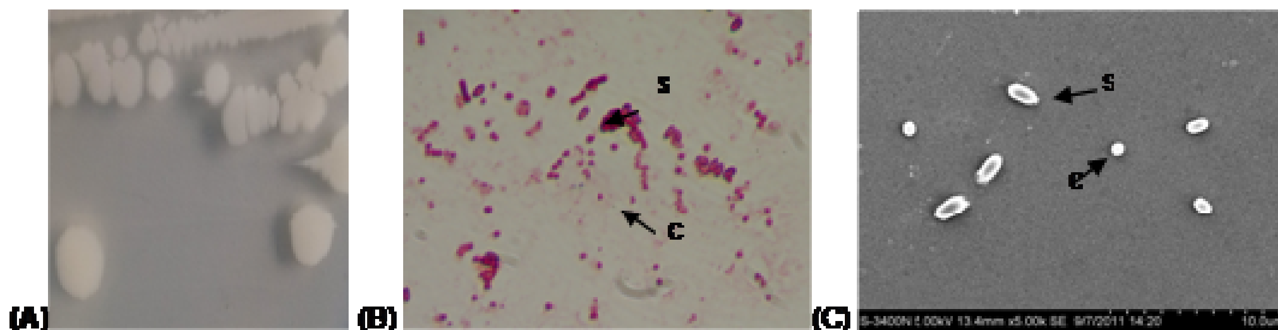


Figure 1. (A)The colony form of S185, Optical (B) and scanning electron (C) microscope image of *Bt* S185 strain. S, Spores; C, crystals.

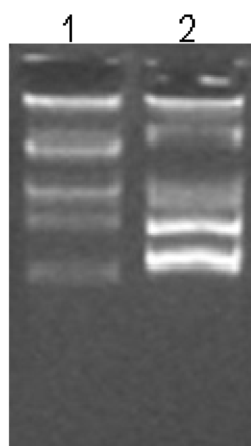


Figure 2. Plasmid DNA pattern of S185 and *Bt* subsp. *kurstaki* HD-73. Lane 1, *Bt* S185; lane 2, HD-73. The DNA fragments were electrophoresed on 0.7% agarose gel.

Company). Preparation of *E. coli* competent cells was as per the standard procedure (Sambrook et al., 1989). Vectors were inserted with approximately 3.7-kb target fragments, and then transformed into the *E. coli* host performed as described previously by Lenin et al. (2001). Positive recombinant-clones were selected by blue-white selection.

Insect toxicity assay

The toxicity of purified *Bt* S185 crystal was tested on larvae of *H. parallela, H. oblita* and *Anomala corpulenta* Motschulsky. Bioassays were performed in 24-well culture plates with 2-cm^2 wells. Purified crystal protein of S185 was suspended in distilled water to a final concentration of 10 g/L. Serial dilutions of crystals were tested by bioassay against *H. parallela* larvae. Bioassays were repeated three times. Larval mortality was recorded after 48 h, and concentrations causing 50% mortality were determined by Probit analysis. Lethal concentrations were expressed per cm^2 of surface.

GenBank accession number

The nucleotide sequence data published in this paper, *cry8Ca* and *cry8Ea*, were assigned GenBank accession numbers ADB54826 and JQ837282, respectively.

RESULTS

Optical and electron micrograph observation of *Bt* S185

After incubation for 48 h in ½ LB medium, *Bt* S185 formed a single milky colony with neat edges. The colony was of a thin circular or nearly circular disk somewhat thicker in the center (Figure 1A). The vegetative masses of the colony were long rod-shapes under the oil immersion objective. However, the spores were stick-like and the crystals were spherical (Figure 1B and C).

Plasmid and protein profile analysis of *Bt* S185

The plasmid profile of *Bt* S185 showed five bands, which was found to be clearly different from *Bt* subsp. *kurstaki* HD73 (Figure 2), especially in regard to the small plasmid bands. Protein profile analysis of *Bt* S185 showed a 130 kDa protein band (Figure 3).

Insect toxicity assays and the physiological and biochemical characteristics of S185

The corrected mortality rate of *Bt* S185 for larvae of *H. parallela*, *H. oblita* and *A. corpulenta* Motschulsky were all >50%. Analysis of the data showed that the highest mortality rate of different concentrations of S185 protein against larva of *H. parallela* was 85.1%. Purified S185 protein against *H. parallela* larvae had LC_{50} of 0.335 μg/ml, 95% limiteds (0.141-0.518 μg/ml).

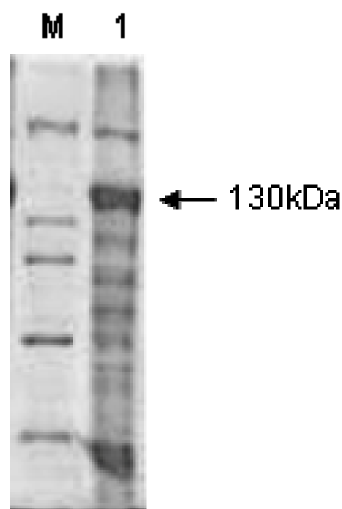
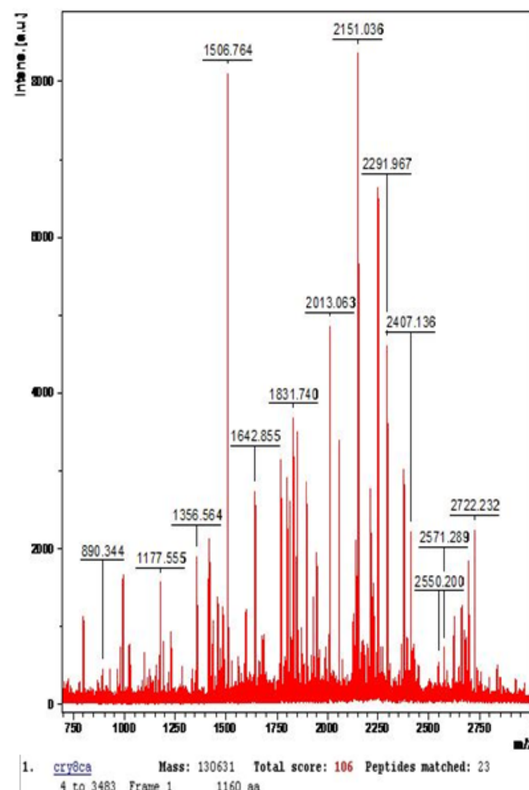


Figure 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile of parasporal inclusion proteins from S185. Lane 1, S185 parasporal inclusion protein M: molecular weight marker. SDS was performed on an 8% gel. Spore crystal mixture obtained from the 20 ml culture was suspended in 200 ml of sterile distilled water. 5 µl of each sample was analyzed.



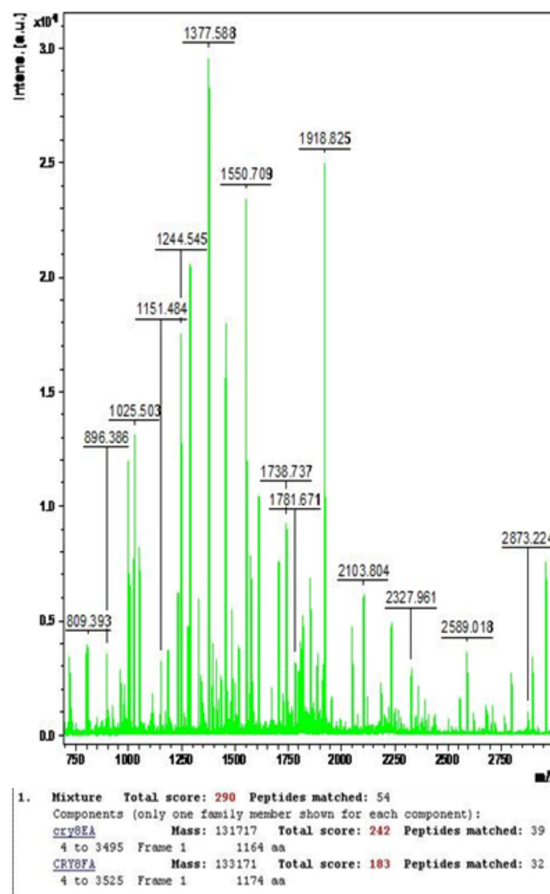


Figure 4. PMF analysis of S185 crystal protein.

Peptide mass fingerprint and PCR-RFLP analysis of *cry*-type genes from *Bt* S185

With the homology comparison of the peptide fragment in the NCBI database, PMF of *Bt* S185 crystal protein preliminarily showed that the crystal protein produced by S185 strain contained *cry8Ca*-type and *cry8Ea*-type or *cry8Fa*-type genes (Figure 4). Using PCR-RFLP of *cry8*-type genes from S185, the primers for full-length genes were based on the encoding region of the known *cry8Ca* and *cry8Ea* genes in GenBank. With the strategy described in the Materials and Methods, a 3.7-kb fragment of *cry8Ca* and the same amount of *cry8Ea* were isolated from *Bt* S185. As *cry8Ea* has very high homology to *cry8Fa* and a close score was obtained with the PMF, PCR-RFLP was used to make a distinction. Analysis by DNAMAN showed that the full-length *cry8Ea* had two *Eco*RI site, 1148 and 2056 respectively. The completely digested *cry8Ea* fragments were 893, 908 and 1288 bp. However, the full-length *cry8Fa* had two *Eco*RI sites on 1572 and 2235, thus the completely digested fragments would be 663, 893 and 1572 bp. Therefore, enzyme action by *Eco*RI will distinguish *cry8Ea* from *cry8Fa*. There were obvious bands at 900 and 1200 bp on the restriction enzyme map instead of 663 bp (Figure 5), thus *cry8Fa* was not contained in the Bt S185 strain.

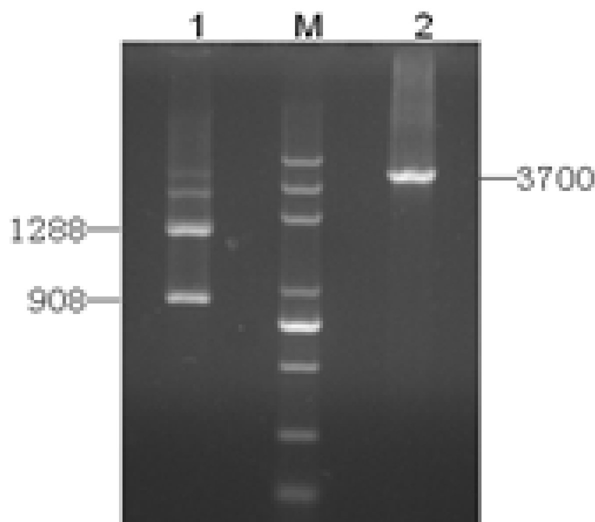


Figure 5. *B. thuringiensis* strain S185 after polymerase chain reaction (PCR)-restriction fragment length polymorphism analysis. S185 PCR products and PCR-RFLP patterns (using cry8Ea-specific primers) Lane 1, PCR-RFLP patterns of *cry8Ea*, digested with endonucleases *Eco*RI; lane 2, PCR products of *cry8Ea*.

Cloning and expression of the crystal protein genes

The recovered PCR product was separately selected and cloned into pEB vector as stated; termed the recombinant plasmids pEB-8Ca and pEB-8Ea. By sequencing the recombinant plasmids pEB-8Ca and pEB-8Ea, the overall length sequences *cry8Ca* and *cry8Ea* were obtained. The predicted amino acid sequence of the cloned gene was analyzed to identify any similarities to other known protein sequences of cry and cyt toxins on NCBI. The cry8Ca protein contained 1160 amino acids and had a molecular weight of 130.25 kDa. The cry8Ca protein was acidic, its isoelectric point was 4.85 and its amino acid sequence shared 99% similarity with that of three other cry8Ca proteins. This protein therefore belongs to a new class of *Bt* crystal proteins, named cry8Ca by the *B. thuringiensis* Delta-endotoxin Nomenclature Committee. In addition, the cry8Ea crystal protein showed 100% sequence identity to the cry8Ea1 crystal protein and contained 1140 amino acids with a molecular weight of 128.97 kDa. This protein was also acidic and its isoelectric point was 4.85. These two sequences are also assigned in the GenBank database.

The recombinant plasmids pEB-8Ca and pEB-8Ea were transformed into the expression host *E. coli* Rosetta, after being transformed into *E. coli* JM109 for propagation. Expression of the toxin proteins of *E. coli* were induced by addition of IPTG and were collected by centrifugation. SDS-PAGE showed that proteins of molecular mass of 130.25 and 128.97 kDa were highly expressed in *E. coli* by pEB vector (Figures 6 and 7).

DISCUSSION

In recent years, the serious damage caused by Coleopteran pests has increased attention on developing new insecticidal crystal proteins against them. As reported, cry8-type toxins are insecticidal to a number of Coleopteran pests, especially certain species of scarabs (Michaels et al., 1996; Ogiwara et al., 1995; Sato et al., 1994; Shin-ichiro et al., 2003; Yamaguchi et al., 2008). Various insecticidal crystal proteins against Coleoptera have been patented, such as cry8Aa1, cry8Ba1, cry8Ca2, cry8Da2, cry8Ea1, cry8Fa1, cry8Ga1 cry8Ha1 and cry8Na1 (Abad et al., 2002; Feng et al., 2008; Michaels et al., 1996; Song et al., 2007), and some of them have already been used for development of biological pesticides and research on transgenic plants (Feng et al., 2008; Bixby et al., 2007). Thus, new *cry8*-type genes have great application potential and broad application prospects.

Currently, peptide mass fingerprinting technology is used less in insecticidal gene identification. This method can quickly detect the insecticidal protein of a strain by directly comparing the similarity of peptides, and is an effective way to explore novel insecticidal protein genes with high reliability and ease of operation. However, because the current *Bt* insecticidal protein peptide database is incomplete, the peptide mass fingerprinting cannot distinguish high homology insecticidal proteins. In the present study, the peptide mass fingerprinting could not distinguish any cry8Ea or cry8Fa toxins that *Bt* S185 contained, so PCR-RFLP and sequencing were used for further identification of insecticidal protein genes. The combination of peptide mass fingerprinting, PCR-RFLP and sequencing technique successfully cloned and expressed two novel insecticidal protein genes from a high-activity insecticidal *Bt* strain.

Conflict of Interests

The author(s) have not declared any conflict of interests.

ACKNOWLEDGMENTS

We are grateful to the Institute of Plant Protection, HeBei CangZhou Academy of Agricultural Sciences for bioassay. This study was supported by grants from the National High Technology Research Development Program (863 Program) (Grant No. 2011aa10a203), National Natural Science Foundation for Genetically Modified Organisms Breeding Major Program (Grant No. 2014ZX0800913B-002), Project for National Basic Science Personnel Training Foundation (Grant No. J1210069), Scientific Research Foundation for the Doctoral Program of Northeast Agricultural University (Grant No. 2010RCB54), the open fund of State Key Laboratory of Biology for Plant Diseases and Insect Pests

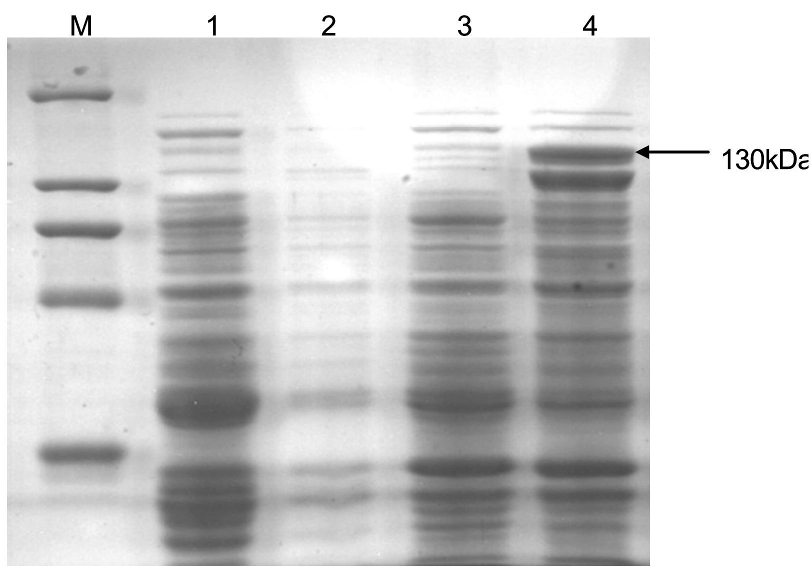


Figure 6. The SDS-PAGE profile of cry8Ea1 proteins from *E. coli* Rosetta. Lane 1, empty gsensor pEB; lane 2, induced empty gsensor pEB; lane 3, *cry8Ea1* in Rosetta without induced; lane 4, *cry8Ea1* in Rosetta with induced.

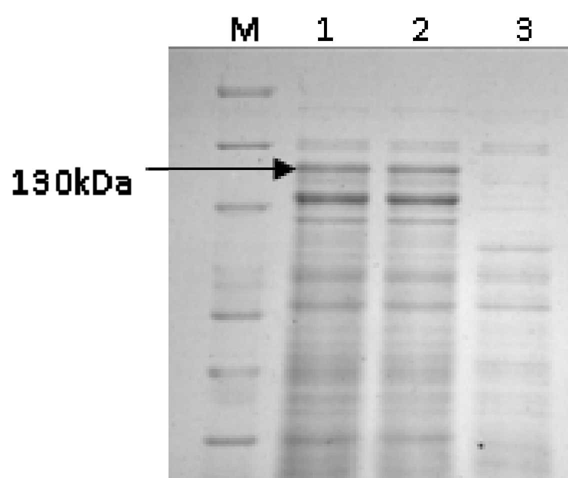


Figure 7. The SDS-PAGE profile of cry8Ca4 proteins from *E. coli* Rosetta. Lane 1, supernatant of *cry8Ca4* in Rosetta; Lane 2, sediment of *cry8Ca4* in Rosetta; Lane 3, empty gsensor pEB.

(SKLOF201405) and the open fund of Key Laboratory of Ministry of Education of soybean biology.

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