

*Full Length Research Paper*

# Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of crude extracted insecticidal crystal proteins of *Bacillus thuringiensis* and *Brevibacillus laterosporus*

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Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to determine the number and size of the insecticidal crystal proteins (ICPs) synthesized by four isolates of entomopathogenic spore-forming bacteria. Only three of the four bacteria produced ICPs with SDS-PAGE profiles, of which two were strains of *Bacillus thuringiensis* and one was a strain of *Brevibacillus laterosporus*. Large sized proteins bands were present in the range of 73 kDa to 135 kDa for the *B. thuringiensis* strains. All the three isolates had smaller proteins in the range of 13 kDa to 44 kDa. One strain of *B. thuringiensis*, NDR11, produced an unusual large protein of 200 kDa. Small proteins in the range of 24 kDa to 40 kDa were produced by *B. laterosporus*. The Cry ICPs of isolate NDR3 did not produce any electrophoretic bands, indicating that its ICP did not dissolve under the conditions used.

**Key words:** Insecticidal crystal proteins, entomopathogenic bacteria.

## INTRODUCTION

*Bacillus thuringiensis* Berliner produces various toxins, including insecticidal toxins. These toxins include delta-endotoxins, beta-exotoxins (non-protein and non-specific), alpha-exotoxins and vegetative insecticidal proteins (VIPs) (Epinasse et al., 2002; Khetan, 2001). Delta-endotoxins are the most common and most studied of these (Höfte and Whiteley, 1989). The toxins are proteins that crystallize into large structures that are visible under light microscopy (Ammons et al., 2002). These toxins have been classified into several classes, including Cry and cytolytic Cyt toxins. There are three primary ranks of Cyt toxins, and more than 70 primary ranks of identified Cry toxins. These toxins are subdivided further, based on the homology of the amino

acids of the specific genes involved (Cry or Cyt genes) (Crickmore et al., 2011). To date, these toxins have been found to be active against Diptera, Lepidoptera, Coleoptera, Mollusca and nematodes (Kumar et al., 1996). Alpha-exotoxins (phospholipase C and lecithinase C) have not been investigated as extensively as the other toxins. These toxins are toxic to insects and mice (toxicity to mice is only when they are injected with the toxin).

Only some strains of *B. thuringiensis* synthesize these toxins (Khetan, 2001). Phospholipase is a metalloprotease that degrades antibacterial proteins secreted by insects and thus prevents the antibacterial proteins from affecting germinated *B. thuringiensis* spores (Lövgren et al., 1990). VIP toxins have a similar mode of action to that of Cry and Cyt toxins, causing pores to form in the midgut epithelium of susceptible insects, which have ingested the VIP toxins. These proteins are not related to the Cry or Cyt toxins and are synthesized during the vegetative stage of bacterial

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**Table 1.** Serotype determination of the locally isolated *Bacillus thuringiensis* isolates.

Isolate	Bacterial species	Serotype
NDR1	<i>Bacillus thuringiensis</i>	<i>kenyae</i>
NDR3	<i>Bacillus thuringiensis</i>	Non-motile
NDR11	<i>Bacillus thuringiensis</i>	Strong self agglutination

Note that *Brev. laterosporus* (NDR2) had not been serotyped.

growth (Yu et al., 1997). The VIP nomenclature consists of three main classes: Vip1, 2 and 3 (Rang et al., 2005). Vip1 and 2 are toxic components of a binary toxin that is effective against Coleoptera (Warren, 1997). *B. thuringiensis* strains may also produce parasporin, which is an insecticidal crystal protein (ICP) that does not have insecticidal properties but is toxic to certain human cells. Its toxicity is limited to leukemia T-cells and does not affect healthy cells, giving it a pharmaceutical value. Toxicity has been shown to cancerous cells of the uterus and cervix (Yasutake et al., 2006). In this research, SDS-PAGE was used to get estimation on the number and size of ICPs produced by four isolates of entomopathogenic spore-forming bacteria. These results were then compared to the sizes of known ICPs in the literature.

## MATERIALS AND METHODS

Standard microbiological techniques were followed in the laboratory for the routine microbiological assays which include sterilization, aseptic techniques and culture preparation (Prescott et al., 1999; Wheelis and Segel, 1979).

### Cultures

Bacterial isolates were obtained from diseased insects such as *Tenebrio molitor*, Coleoptera adults and larvae of *Schizonycha* spp. collected in sugarcane producing areas in KwaZulu-Natal, (KZN), and insect rich environments such as compost, grain dust from grain storage bins and *T. molitor* cultures. These bacterial isolates were isolated and identified previously using biochemical tests as described by Thiery and Frachon (1997) (Du Rand, 2010). They have also been subjected to tests in bioassays to determine toxicity. The four isolates tested were found to display toxicity towards Coleoptera (Du Rand, 2010).

*B. thuringiensis* isolates were sent to Dr Michio Ohba (Graduate School of Agriculture, Kyushu University, Hakozaki 6-10-1, Higashi-ku, Fukuoka, B12-8581) in Japan for serotyping (Table 1). A standard method similar to that described by Thiery and Frachon, (1997) was used.

### Protein preparation for SDS-PAGE

Two techniques were used for the protein preparations: 1) whole cell preparation for SDS-PAGE and 2) crude protein extraction.

### Whole cell preparation for SDS-PAGE

Bacterial isolates (NDR1, 2, 3 and 11) were cultured in 100 ml nutrient broth (Biolab, Biolab Diagnostics, 259 Davidson Road,

Wadeville, Johannesburg) for 5 days at 30°C in a shaker water bath (250 rpm), or until cell lysis. Cell suspensions of 1 ml was placed in 2 ml Eppendorff tubes and were centrifuged at 10,000 x *g* for 15 min in a centrifuge (Hermle, Labotec, SA) and washed twice with distilled water. The supernatant was discarded and the pellet was resuspended in 0.5 M KOH for 3 h. Samples were boiled for 2 min after which 200 µl treatment buffer was added along with glycerol and saturated bromothymol blue. The samples were stored at -20°C until further use. This method was adapted from Luo and Adang (1994).

### Crude protein extraction for SDS-PAGE

Isolates were cultured in 100 ml nutrient broth for 5 days at 30°C in a shaker water bath. The cell suspensions were sonicated for 15 min. Aliquots of 1 ml of the sonicated cell suspensions were centrifuged at 10,000 x *g* for 15 min and washed twice to remove unwanted cell debris. The supernatant was discarded and the pellet was re-suspended in 0.5 M KOH for 3 h. The pellet of the isolate NDR3 was also re-suspended in 3.3B M NaBr (López-Meza and Ibarra, 1996). The suspensions were centrifuged at 27,000 x *g* for 15 min, after which the supernatant was kept and the pellet discarded. The supernatant was then boiled for 2 min in a treatment buffer (5 ml 2-mercaptoethanol, 12.5 ml 4X Tris-Cl/SDS, pH 6.8, 5 drops of bromophenol, 30 ml 10% SDS and 10 ml glycerol). The samples were stored at -20°C until further use. This method was adapted from Luo and Adang (1994).

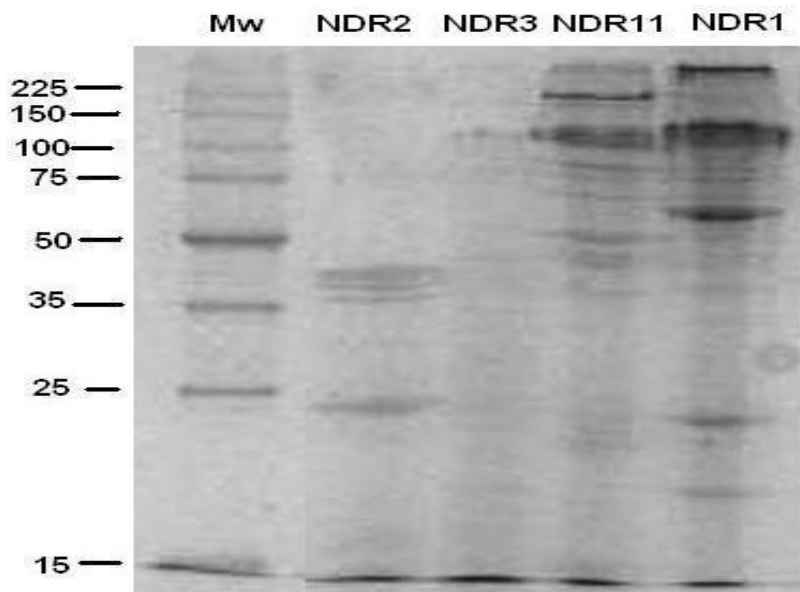
### SDS-PAGE

The ICPs compositions of the *B. thuringiensis* and *Brev. laterosporus* isolates were determined by SDS-PAGE. A method described by Hames and Rickwood (1990) was used. The samples were run at 125 V for 110 min using a vertical Bio-Rad mini-Protean II cell with a 3% stacking gel and a 12.5% running gel. The gels were stained for 24 h with Coomassie Blue stain. Molecular weight markers from Promega (Whitehead Scientific, SA) were used to estimate the molecular weight of the ICPs.

## RESULTS

### SDS-PAGE of complete spores and insecticidal crystal proteins and protein preparation 1

Three crystal producing isolates, NDR1, 2 and 11, generated visible bands when SDS-PAGE analysis was conducted on complete spores and ICPs of insecticidal crystal producing isolates, incubated for 5 days at 30°C.



**Figure 1.** SDS-PAGE analysis of complete spores and ICPs of insecticidal crystal producing isolates, incubated for 5 d at 30°C; Note: Mw = a band of molecular weight markers.

#### ***Isolate NDR1 (B. thuringiensis)***

Three major bands and seven minor bands were formed. The major bands sizes were estimated to be 135, 110 and 63 kDa. The minor bands sizes were estimated at 93, 85, 75, 68, 38, 23 and 20 kDa (Figure 1).

#### ***Isolate NDR2 (Brev. laterosporus)***

Four minor bands were observed. The bands had estimated sizes of 43, 40, 38 and 24 kDa (Figure 1).

#### ***Isolate NDR3 (B. thuringiensis)***

The extract from this isolate formed poor bands, which were not visualized using SDS-PAGE (Figure 1).

#### ***Isolate NDR11 (B. thuringiensis)***

Two major bands and several minor bands were observed. All the bands were of high molecular weight proteins. The major band sizes were estimated at 200, 135 and 110 kDa. Minor bands were estimated at, 100, 85, 75, 63, 52, 47, 43 and 38 kDa (Figure 1).

#### **SDS-PAGE of crude extracted, alkaline solubilised protein crystals, and protein preparation 2**

Three isolates were analyzed using this technique, NDR2 (*Brev. laterosporus*), NDR1 and 11 (*B. thuringiensis*) (Figure 2). The ICPs of isolate NDR3 did not dissolve in

alkali in the first analysis, using an extraction of complete spores and ICPs; hence isolate NDR3 was not included in the second SDS-PAGE gel analysis. No purification steps were undertaken.

#### ***NDR1 (B. thuringiensis)***

Five protein bands were observed for isolate NDR1. The sizes of the minor bands were estimated at 85, 68, 23 and 20 kDa, respectively. The major band size was estimated at 150 kDa (Figure 2).

#### ***NDR2 (B. laterosporus)***

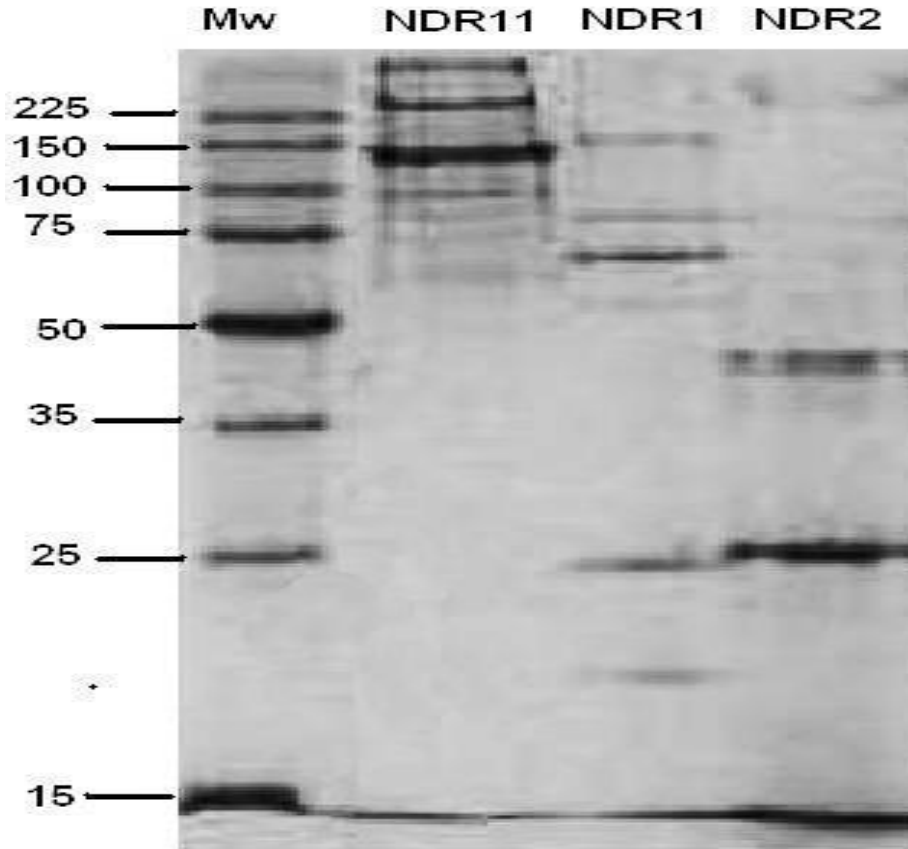
Three major bands were observed. The sizes were estimated between 40, 38 and 24 kDa (Figure 2).

#### ***NDR11 (B. thuringiensis)***

Two major bands were observed for isolate NDR11 and were estimated to be 135 and 110 kDa. Faint minor bands were also observed. Minor bands were observed at 100, 85 and 63 kDa (Figure 2).

## **DISCUSSION**

SDS-PAGE was used to determine the number of different proteins present in each of the isolate's ICPs.



**Figure 2.** SDS-PAGE analysis of crude extracted, alkaline solubilisation of ICPs from insecticidal crystal producing isolates.

Two analyses were carried out, using (1) whole cell preparations (Figure 1) and (2) crude protein extractions (Figure 2). The two analyses were undertaken to reduce the possibility of the SDS-PAGE analysis missing any protein bands due to a specific extraction protocol (Figure 2). Only three of the four isolates generated protein bands in both analyses (Figures 1 and 2). Isolate NDR3 did not produce convincing bands (Figure 1), which indicated that its ICP's did not dissolve under the conditions used here. Several bands were missing for the *B. thuringiensis* strains in the crude protein extraction method when compared with the whole cell preparation method (Figures 1 and 2). Large sized proteins bands were missing for isolate NDR1, whereas smaller molecular weight (MW) sized protein bands were missing for NDR11 (Figures 1 and 2). This demonstrated that the crude extraction method had eliminated some proteins and only contained proteins present in the ICPs. The whole cell preparation thus contained proteins that do not form part of the ICPs. All the bands were present in both the whole cell and crude extraction method for the strain of *Brev. laterosporus* (NDR 2), although the 43-kDa protein band was weak in the crude extraction gel. 1 and NDR11 shared common bands estimated at 135, 110, 75

and 68-kDa (Figure 1). This indicated that these two isolates may share common toxins in their ICPs. The large MW protein bands greater than 80-kDa, are typical of lepidopteran specific toxin sizes (Höfte and Whiteley, 1989). Both 1 and NDR11 had large MW protein bands that fell within lepidopteran specific sized toxins. *B. thuringiensis* subsp. *kenyae* is known to produce Cry1 and Cry2 toxins and these toxins are larger than 130-kDa and it was thus not surprising to find these sized protein bands (Höfte and Whiteley, 1989; Masson et al., 1992; Van Frankenhuyzen and Nystrom, 2002). Cry1 and Cry2 toxins are also toxic to certain species of Coleoptera (Van Frankenhuyzen and Nystrom, 2002).

Isolate NDR11 also had a very unusual crystal protein estimated at 200 kDa (Figure 1). Benintende et al. (1999) had a similar finding of a non-toxic 200 kDa protein from *B. thuringiensis* subsp. *Kenyae*. 1 and NDR11 also had protein bands between 80 and 73 kDa which are the sizes of typical Coleopteran specific toxins (Figures 1 and 2) (Höfte and Whiteley, 1989). One common band estimated at 38kDa was shared by 1, 2 and NDR11 (Figure 1). This indicated that these three isolates had a toxin with the same MW (Figure 1). Isolate NDR2 had four low MW bands and the 24 kDa band could possibly

be a Cyt toxin because these toxins are smaller MW proteins, usually less than 30 kDa in size (Du et al., 1999; Höfte and Whiteley, 1989). Low MW toxins were also found to be present in NDR1 ICPs. Low MW toxins such as 13, 14 and 44 kDa are typical of binary toxins (Ellis et al., 2002; Höfte and Whiteley, 1989; Khetan, 2001). Large proteins, greater than 137 kDa, were disregarded because the largest Cry toxins were 137 kDa in size (Hofte and Whiteley, 1989). Protein bands larger than 137 kDa were assumed to contain either undissolved ICPs, cellular debris or non-toxic proteins (Benintende et al., 1999). This was more evident in Figure 1 than Figure 2. This may be attributed to the fact that Figure 1 is of an SDS-PAGE of the whole pellet, rather than the SDS-PAGE of isolated ICPs, as shown in Figure 2. Isolate NDR2 was a strain of *Brev. laterosporus* and little research has been done on the crystal producing strains of this species. This is mainly because there has been a general consensus that *Brev. laterosporus* toxins are not as effective as those of strains of *B. thuringiensis* (Zahner et al., 1999). Toxins of *B. thuringiensis* are effective against Lepidoptera, Diptera, Coleoptera and even nematodes (Kumar et al., 1996; Wei et al., 2003).

In contrast, little has been documented on the host range and effectiveness of the toxins of *Brev. laterosporus* and the potential of this species as a biological control agent. Alkaline conditions (KOH) and the ionic solution of NaBr did not dissolve the protein crystals of isolate NDR3 (results not shown). Du et al. (1994) also found that the proteins of some isolates of *B. thuringiensis* did not dissolve easily, and resorted to extreme alkaline conditions to dissolve some *B. thuringiensis* protein toxins. Alternatively, the ICPs of isolate NDR3 may need other means or enzymes to break it down, in order for individual proteins. Further research needs to be conducted to establish the requirements for release and solubilization of isolate NDR3's ICP. Numerous questions remain to be answered as to the type of toxins present in the isolates screened here, and which of these specific proteins are toxic towards Coleoptera. For example, the types of toxins present in the ICPs of *Brev. laterosporus* are still unknown. Furthermore, SDS-PAGE only provides a determination of the number of proteins present in a sample, and their specific sizes. However, it does not allow the determination of the type of protein, the potency of the protein toxin, or which insects that the toxin may be effective against. Some of the toxins discovered here may be new toxins. However, SDS-PAGE could not be used to determine this. Protein sequencing would be required to determine this.

## Abbreviations

**ICPs**, Insecticidal crystal proteins; **VIPs**, vegetative insecticidal proteins; **SDS- PAGE**, sodium dodecyl

sulphate polyacrylamide gel electrophoresis; **KZN**, kwazulu-natal; **MW**, molecular weight.

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