BIOLOGICAL CONTROL

Insecticidal Activity of Culture Supernatants from *Bacillus thuringiensis* Berliner Strains Against *Spodoptera frugiperda* Smith (Lepidoptera: Noctuidae) Larvae

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Atividade Inseticida do Sobrenadante da Cultura de Cepas de *Bacillus thuringiensis* Berliner Contra Lagartas de *Spodoptera frugiperda* Smith (Lepidoptera: Noctuidae)

RESUMO – Recentemente, proteínas inseticidas vegetativas (Vips), identificadas no sobrenadante da cultura de certas cepas de *Bacillus thuringiensis* (B.t.), foram capazes de controlar alguns insetos-praga economicamente importantes. Como avaliação do potencial de aplicabilidade destas proteínas contra a lagarta-do-cartucho (*Spodoptera frugiperda* Smith), o inseto-praga mais importante da cultura do milho, as características e o efeito de mortalidade do sobrenadante da cultura de cinco cepas de B.t. foram investigadas. Notáveis diferenças entre as cepas foram detectadas, não somente em termos de eficiência na mortalidade dos insetos, mas também nas diferenças de mortalidade entre os sobrenadantes aquecido e não-aquecido, tratamentos estes que foram usados para distinguir a fração protéica inseticida termo-sensível de outra fração termo-estável, de natureza não-protéica (b-exotoxinas). Os padrões qualitativo, quantitativo e temporal de secreção da proteína total no meio (sobrenadante) foram avaliados por espectrofotometria e eletroforese em gel de poliacrilamida. As cepas apresentaram distintas taxas de crescimento e tempos de secreção de proteínas em relação a densidade de células na cultura. Além disso, o padrão de bandas eletroforéticas também variou de maneira cepa-específica, em ambas condições desnaturantes e não-desnaturantes. Polipeptídeos que apresentaram peso molecular semelhantes aos esperados para proteínas Vip3A identificadas anteriormente foram encontrados em cepas com alta taxa de mortalidade do sobrenadante. Os dados sugerem a possibilidade e a utilidade de pesquisar frações inseticidas de natureza protéica (do tipo Vip) em sobrenadantes de B.t., como forma para desenvolver alternativas específicas e eficientes de controle biológico que possam ser empregadas em programas de manejo integrado de *S. frugiperda* em milho tropical.

PALAVRAS-CHAVE: Insecta, controle biológico, lagarta-do-cartucho do milho, proteínas inseticidas vegetativas, genes Vip.

ABSTRACT - Novel vegetative insecticidal proteins (Vips) identified in the supernatant of *Bacillus thuringiensis* (B.t.) cultures have shown to provide ad-

equate control over a wide spectrum of economically important crop pests. To evaluate the potential applicability of these proteins against fall armyworm (*Spodoptera frugiperda* Smith) larvae, the most important insect pest for tropical maize, the characteristics and mortality effects of culture supernatants from five B.t. strains were investigated. Striking differences among strains were detected, not only in terms of efficiency in killing the insect, but also regarding to mortality effects of heated and non-heated supernatants, which were used to distinguish the heat-sensitive protein-derived insecticidal fraction from a thermostable one, with a non-protein nature (b-exotoxinas). The qualitative, quantitative and temporal patterns of total protein secretion in the medium (supernatant) were assessed through spectrophotometry and polyacrylamide gel electrophoresis. The strains showed remarkably distinct rates of growth and timing for protein secretion relative to cell density in culture. Moreover, the electrophoretic-banding patterns also varied in a strain-specific manner, both in denaturing and non denaturing conditions. Polypeptides displaying a molecular weight that is very close to the expected for previously identified Vip3A proteins were found for the strains with high supernatant-mortality ratios. The data suggest the feasibility and usefulness of searching for protein-derived (Vip-like) insecticidal fractions in B.t. supernatants as a mean of developing especific and efficient alternatives of biological control to be employed in integrated pest management programs of *S. frugiperda* in tropical maize.

KEY WORDS: Insecta, biological control, fall armyworm, vegetative insecticidal proteins, Vip genes.

The fall armyworm, *Spodoptera frugiperda* Smith, is one of the most important pests for tropical maize, causing up to 34% reduction in the overall productivity of this crop in Brazil (Cruz *et al.* 1996). The control of fall armyworm is mostly performed by means of chemical insecticides, a traditional system that has negative side effects to human and animal health, as well as to the environment as a whole. The biological control of insect pests by directly employing natural enemies, or substances derived from them, appear as an alternative to minimize the impact of chemical pesticide applications (Valadares-Inglis *et al.* 1998). A proper understanding of the ecological mechanisms involved in the interactions between pests and their natural enemies is desirable to reduce costs in crop production, to prevent chemical intoxication of workers, and to preserve the environment and public health.

Insecticidal d-endotoxins, effective against *Spodoptera spp.* and other insects but innocuous to animals and humans, are the constituents of parasporal crystal inclusions produced during the sporulation phase of the soil bacterium *Bacillus thuringiensis*, or 'B.t.' (Bravo 1997). These compounds have been used in the formulation of commercial bioinsecticides, which have shown to provide adequate and consistent levels of biological control of fall armyworm (Höfte & Whiteley 1989, Knowles 1994, Valadares-Inglis *et al.* 1998). However, problems related to (i) the reduced persistence of pure endotoxins over the plant during enough time for an efficient entomocidal action, (ii) the difficult access of the insect to the endotoxins as a consequence of their physical-chemical properties, and (iii) the yet high costs of production for these bioinsectides, are all responsible for a not very wide diffusion of such system of biological control (Koziel *et al.* 1993, Valadares-Inglis *et al.* 1998). Therefore, in order to improve

the efficiency of insecticidal formulations and so to reduce manufacturing costs, it is still necessary to search for new toxins and strains, and to test different combinations of them (Bohorova *et al*. 1997).

Novel B.t. insecticidal proteins, recently isolated from the culture supernatant of certain strains in the vegetative growth phase (vegetative insecticidal proteins, or Vips), have provided adequate levels of control against a wider spectrum of economically important crop pests when compared to the d-endotoxins (Estruch *et al*. 1996, Yu *et al*. 1997). The polypeptides corresponding to Vip activities have a molecular weight of ~88 kDa and are sensitive to high temperatures (Estruch *et al*. 1996). This characteristic is important to help differentiate the protein-derived activity from another form of thermostable insecticidal action that is also present in the supernatant, the b-exotoxins (Bond *et al*. 1971, Estruch *et al*. 1996). In this work, the potential applicability of this novel type of protein-derived insecticidal activity secreted in the culture supernatant was assessed for five B.t. strains against tropical fall armyworm. Possible differences in insecticidal action among the strains, and between distinct fractions of their respective supernatants were investigated, as well as whether the presence of particular peptides might correlate with high insecticidal activity.

Material and Methods

Bacterial strains and culturing conditions. Five B.t. strains were used in these experiments; two – 'T09' and 'HD125' - were considered as being 'foreign', since they were obtained from the collections of the Institute Pasteur (France) and the U.S. Department of Agriculture, respectively. The remaining three strains, '344', '520B' and '606A', belong to a collection of strains obtained from Brazilian soils and under the supervision of the Applied Biology Group - NBA - of the National Maize and Sorghum Research Center - CNPMS / EMBRAPA. They were chosen for these studies due to a different performance regarding the biological effect of their parasporal crystal inclusions (d-endotoxins) on *S. frugiperda* larvae (Valicente *et al*., in press). To insure fresh and standardized bacterial cultures for the experiments, clumps of cells from –80ºC stocks of each strain were pre-inoculated in 20 ml of medium (using 125 ml-flasks) and grown for about 12 hours at 30ºC, at 300 rpm, in a rotatory shaker. Twenty ml from each of these so called 'pre-cultures' were then inoculated in 80 ml of the same medium (in 250 ml-flasks) and incubated in the same conditions for 16 hours. The bacterial cells were cultivated in a modified version of PMB medium (Bohorova *et al*. 1996) with the following composition per liter: peptone 5.0 g, meat extract 3.0 g, yeast extract 2.0 g, $MgSO_4$ 0.3 g, $FeSO_4$ 0.02 g, $ZnSO_4$ 0.02 g, and $MnSO_4$ 0.02 g. In order to estimate the amount of cells in culture at a certain time, the optical density (O.D.) was taken by spectrophotometry at a wavelength of 600 nm, by removing an 1-ml aliquot from the culture for direct measurements.

Supernatant collection and total protein extraction. The supernatants from the strains were collected by centrifugation of the whole 80 ml-cultures above, split in 40-ml cap-tubes, and spun in a fixed-angle rotor (SL-50T, Sorvall® Super T 21 centrifuge) at 30,500 x *g* for 15 min at 4ºC. The liquid was withdrawn carefully to avoid disturbing the pellet of cells, collected in a separate container, and filtered by gravity through 3MM paper to assure complete removal of clumps of cells that might be still present. Total protein was extracted and purified according to a established protocol (Estruch *et al*. 1996), which is summarized as follows: the total protein fraction was precipitated with ammonium sulfate at a 70% final concentration on ice, centrifuged at approximately 20,000 x *g* for 12 min, resuspended in 10 ml of 20mM Tris-HCl (pH 7.5), dialized in one liter of the same buffer (exchanged three times) at 4ºC, adjusted to pH 4.5 using sodium citrate at pH 2.5, left at room temperature for 40 min, spun down at ~20,000 x g for 12 min, and finally

resuspended in 500 ml of the same buffer. The protein was quantified by the method of Bradford (1976).

Polyacrilamyde gel electrophoresis. The protein composition of each supernatant was assessed by polyacrylamide gel electrophoresis, under both denaturing and non-denaturing conditions (SDS- and Native-PAGE). Seven mg of total protein per strain were resuspended in loading buffer with SDS + b-mercaptoethanol, boiled for 2 min, loaded into vertical mini-gel (BioRad®), and ran for 3.5 hours at 65 V. The stacking gel was 6.0% acrylamide / 0.125 M Tris-HCl (pH 6.8) / 0.1% SDS, and the resolving gel was 12.5% acrylamide / 0.5 M Tris-HCl (pH 8.3) / 0.1% SDS (Laemmli 1970). The gels were afterwards stained by a sensitive silver-based technique, as described in Blum *et al*. (1987). For non-denaturing (native) PAGE, the overall gel and running conditions were the same as for SDS-PAGE, except that SDS and b-mercaptoethanol were eliminated from the stacking and resolving gels, as well as from the loading buffer. In order to differentiate the thermosensitive insecticidal portion (Vip) of the secreted B.t. proteins from thermostable b-exotoxins present in the supernatant of each strain (Bond *et al*. 1971, Estruch *et al*. 1996), the native-PAGE was performed applying heated (1-2 min at 95ºC) and non-heated protein samples for each strain. The gel pH (8.8) is appropriate for proteins with an acidic net charge, as in the case of Vips.

Insect rearing and toxicity bioassays. For each strain, the heating of its culture supernatant (20 min at 95ºC, then ice for 5 min) was used to distinguish between the effects of the thermosensitive insecticidal portion (Vip) and the thermostable fraction (Estruch *et al*. 1996) on the mortality of *S. frugiperda* larvae. Two days-old larvae of fall armyworm were obtained from ovipositions of field-sampled female adults reared on artificial, semi-solid diet cubes (~1 cm^3), at 28ºC in a 14 h light : 10 h dark conditions. The diet composition, which was the same used for all toxicity experiments, was as follows (for every liter of water): cooked-bean grains 123.6 g, wheat germ 59.3 g, live beer-brewing yeast 38.0 g, ascorbic acid 3.82 g, nipagin 2.36 g, sorbic acid 1.23 g, agar 15.35 g, formaldehyde 3.1 g, phosphoric acid 0.131 ml, and propionic acid 1.3 ml. Diet cubes were soaked in 5 ml of either heated or non-heated supernatants and applied to larvae, which were maintained individually in closed 50-ml disposable containers (one diet cube per container). Mortality was computed every three days during the whole larval period. Control larvae were reared in diets soaked in heated and non-heated pure culture medium. The supernatants used for the toxicity bioassays were collected and processed from 16-18 h-grown bacterial cultures. To evaluate statistically the difference in mortality between the non-heated and heated aliquots of the supernatant from a given strain, a chi-square (χ^2) analysis was performed based on a 2x2 contingency table, on a per-strain basis; the null hypothesis considered was that there was no difference in larvae mortality between non-heated and heated aliquots of a given supernatant. The χ^2 values and the respective probabilities of the differences as being due to randomness ('P'; 1 d.f.) are shown in Fig. 1 for the strains with a minimum mortality ratio of 0.2. The number of larvae assayed per strain were 94 for T09, 93 for HD125, and between 43 and 48 for the strains 344, 520B, 606B, and for the control.

Results and Discussion

The number of cells in culture was initially evaluated as a proper parameter for the standardization of culture conditions, such that cultures from the five strains were allowed to grow until a narrow optical density (O.D.) range of 0.9-1.1, at 600 nm, and their total protein content was estimated directly from the supernatant. The results indicated that cultures from different B.t. strains behave differently regarding the total amount of protein secreted in the medium relative to the density of cells (Table 1). The strain 606B grew faster,

reaching the above specified O.D. after 5 h of culture, whereas 520B and 344 were slower, taking 6 h and 40 min to do so. Nevertheless, the former showed the lowest amount of total protein in the supernatant, in contrast to the latter two, which had a supernatant with the highest protein content (Table 1). A possible explanation for this trend would be that the amount of proteinaceous material present in the medium, which might be explained by higher rates of protein absorption than secretion by the cells, at initial stages of culture growth. This phenomenon also occurred for the other four strains, although at lower optical densities. Further studies on the whole profile of protein secretion through culturing

Table 1. Incubation time required and estimation of the total protein content of the supernatant from five B.t. strains, which were grown up to a 0.9 to 1.1 values of optical density at 600 nm.

B.t strains	Incubation time (h:min)	Total protein estimate[1] (O.D. at 595 nm)	Difference to control
Medium (control)	-	0.424	-
606B	5:00	0.332	-0.092
HD125	6:00	0.535	0.111
T09	6:30	0.547	0.123
520B	6:40	0.630	0.206
344	6:40	0.619	0.195

[1]Estimated by the method of Bradford (1976) that measures absorbance of light by protein-dye complexes at a wavelength of 595 nm.

strains display a specific physiological trade-off between cell division and protein metabolism, with the secretion of proteins appearing to be inversely proportional to the growth rate, at least at those initial stages. This consequently suggests that cell number is not an appropriate standard to describe a similar physiological state among different B.t. cultures. Moreover, the fact that the strains also demonstrated a distinct rate of growth further disapproves O.D. at 600 nm as the standardizing parameter because, when performing comparative studies with B.t. strains, the culturing process should be inconveniently interrupted at different times.

The difference in the total amount of protein in the supernatant (O.D. at 595 nm) relative to the control (pure medium) yielded a negative value for the 606B strain (Table 1). This seems to indicate a decrease in the total time are currently underway for these and other B.t. strains in order to assess thoroughly the relationships between cell density, growth rate and protein secretion in the supernatant (medium).

The range of O.D. at 600 nm chosen for these studies (between 0.9 and 1.1) aimed to collect the supernatant at the logarithmic phase of culture growth, which is when the secretion of Vips is expected to initiate (Estruch *et al*. 1996). Since cell density has not shown to be a good parameter for a proper standardization of the physiological conditions of B.t. cultures, the 'culturing time' was then employed, based upon two arguments: first, the secretion of Vips extends farther during the culturing process, even after reaching the sporulation phase (Estruch *et al.* 1996); and second, a minimally measurable amount of protein is accumulated for all strains after

reaching the stationary phase (data not shown), thus allowing comparisons among distinct supernatants collected at a same time, in terms of their insecticidal activity. Hence, standardized 16 to 18-h grown cultures from all strains had their crude supernatant collected and used for biological assays, in which the mortality of fall armyworm was evaluated on feeding experiments using artificial diets (Fig. 1).

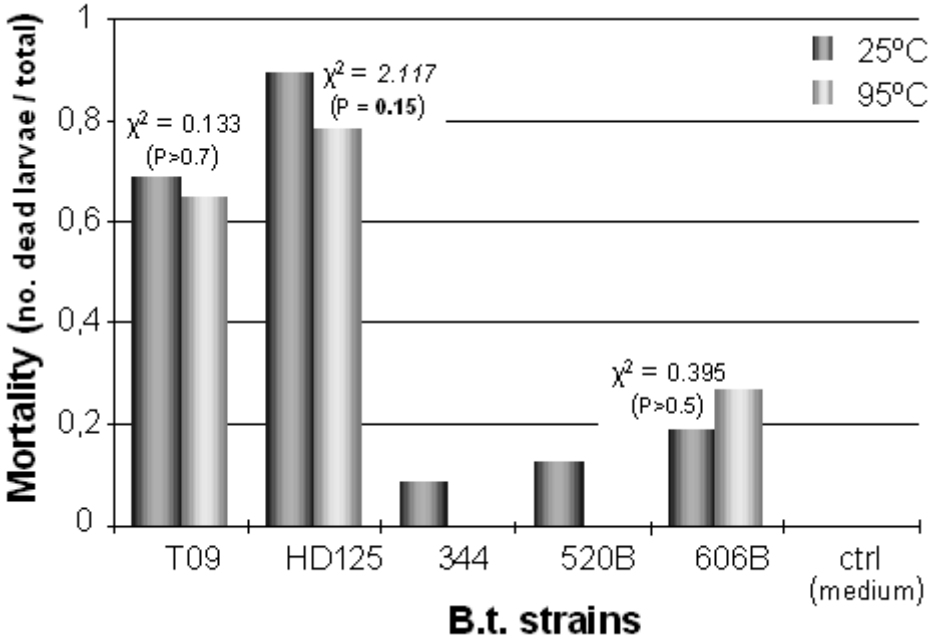

Figure 1. Mortality of *S. frugiperda* during the larval period on laboratory feeding experiments, in which artificial diets were soaked in non-heated and heated supernatants collected from five *B. thuringiensis* strains cultured up to 16 - 18 h. ('χ^2' = chi-square value of the difference of deaths between non-heated and heated supernatant, per strain; 'P' = probability of those differences being due to randomness).

Remarkable differences in mortality ratios are readily observed from the graph, with the strains HD125 and T09 showing higher insecticidal activity on their supernatants when compared to the other three strains. Heating the supernatant at 95ºC before applying to the larvae generally caused a drop in the activity of the supernatant, but did not eliminate it, suggesting that there are in fact two forms of insecticidal action in the supernatant: a conspicuous one, which was responsible alone for a ratio of mortality in the 0.65-0.78 range (T09 and HD125), and appeared to be thermostable (probably b-exotoxins – Bond *et al*. 1971), since it remained active after extensive heating. The other, thermally sensitive, was assessed by the difference between 'total' and 'heated' activities (Fig.1). As pointed out earlier, it is very likely that such a thermal-sensitive portion of the supernatant correspond to a protein-like (Vip) insecticidal activity (Estruch *et al*. 1996).

In order to address the statistical significance of the differences between non-heated

and heated supernatants on a per-strain basis, a *chi-square* (χ^2) analysis was performed (see Methods). Although none of the χ^2 values obtained for the differences shown in the graph strictly indicated a statistical significance at the conventional level (i.e. $P<0.05$; 1 d.f.), if one considers lowering the stringency for statistical significance, then the results might suggest that the strain HD125 would in fact contain a protein-like insecticidal action ($P=0.15$). Adding support to this statement, feeding experiments using the purified total protein from the medium (see below) revealed a detectable mortality effect only for the strain HD125 (data not shown), confirming the trend observed from the previous experiment. Another important conclusion that can be drawn from the data is that supernatant-insecticidal activity in B.t. strains is not associated with the same effect produced by the parasporal crystal inclusions. With exception of 606B, all strains used in this work had shown previously to have a very high crystal-like activity against *S. frugiperda* larvae, with mortality ratios of 1.0 (Valicente *et al*., in press); here, only the supernatant from HD125 and T09 were more effective, yet showing a lower mortality ratio ranging between 0.65 and 0.90. It is important to mention, though, that at least part of the differences in supernatant mortality observed among strains could have been possibly due to distinct strain-specific profiles of total and Vip protein secretion, both in a qualitative and quantitative manner, as we were limited to culturing time as the standardizing parameter for these experiments (see above). Nevertheless, these results seemed sufficient to demonstrate the feasibility of searching for efficient supernatant insecticidal proteins based on their heat-dependent activities.

Taking into consideration that part of the contrasting effects in the mortality of fall armyworm, on laboratory feeding experiments, observed among strains could have been due to either less protein accumulated in the supernatant of certain strains at a given time (quantitative component), or to a distinct pattern of polypeptides secretion (qualitative component), the total protein secreted in the medium of four cultures showing contrasting effects of mortality (T09, HD125, 520B, 344) was purified, and its composition analyzed through polyacrylamide gel electrophoresis (Figs. 2 and 3). Although a small difference in the total quantity of protein recovered by the extraction procedure was indeed noted between strains, the qualitative differences in the peptides present in the supernatants seemed more remarkable.

The electrophoretic profile under denaturing conditions (SDS-PAGE) showed a very specific banding pattern for each strain (Fig. 2), in sharp contrast to a similar peptide composition exhibited by those same strains when the crystal (d-endotoxin) fraction was purified and electrophoresed (Valicente *et al*., in press). This result suggests, indeed, that the differences in the protein-derived (heat-labile) insecticidal fraction among supernatants is probably caused by a higher or lower secretion of Vip-like peptides, which is a strain-specific component. To strengthen this hypothesis, bands with the approximately 80-kDa molecular weight expected for Vip3A proteins (Estruch *et al*. 1996) were identified only in the two strains with higher supernatant activity (arrows - Fig. 2). Considering the potential of preliminarily screening for Vip activities using simple protein extraction and SDS-PAGE, further work with more strains is being now performed in order to confirm if the presence / absence of those bands can correlate with effective supernatants.

To further investigate the possibility of using peptide band(s) as a marker for significant Vip-like activity, the protein composition of each supernatant was also assessed by native-PAGE (Fig. 3). As it can be noticed, the electrophoretic pattern of the non-heated samples appeared to be also different among the four strains, despite the similar mortality effects of the T09 and HD125. Moreover, in the heated samples, several bands disappeared relatively to the non-heated counterpart, indicating that they belong to the heat-sensitive fraction of the total supernatant protein.

The presence of some thermally labile

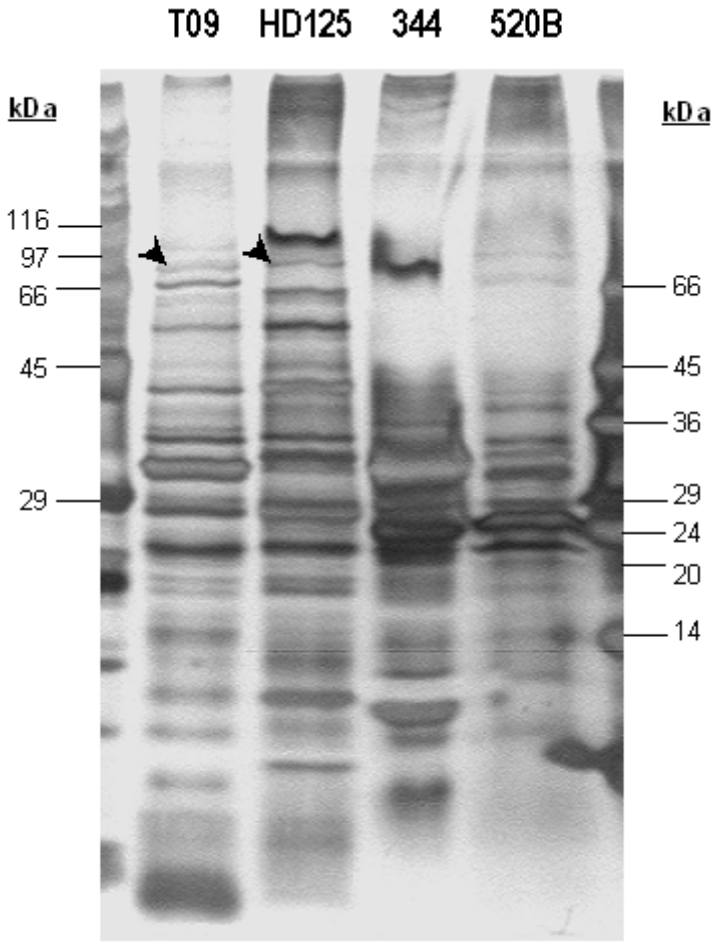

Figure 2. SDS-PAGE (12.5% gel) of total protein extracted from 16-h culture supernatant of four *B. thuringiensis* strains showing contrasting effects in the mortality of fall armyworm.

bands common to T09 and HD125, but not to 344 and 520B, may be associated with Vip-like activities against fall armyworm, since the migration of potential Vip protein(s) through the gel was insured by its(their) acidic pI. However, the analysis of more strains is certainly required to confirm this hypothesis, since the non-lethal supernatant of the 344 and 520B strains also showed heat-labile bands, although not necessarily with the same electrophoretic pattern of migration (Fig. 3). Bands that decreased their intensity relatively to the non-heated counterpart also correspond to thermally sensitive peptides, for which a longer heating is probably needed for complete disappearance. By contrast, bands with

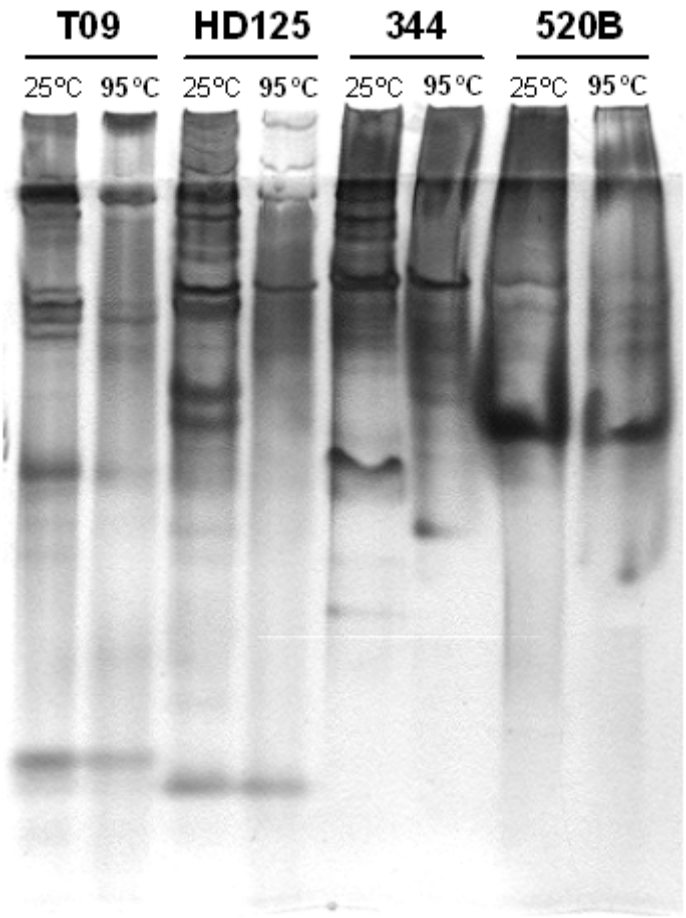

Figure 3. Native-PAGE (12.5% gel) of non-heated (25ºC) and heated (95ºC) samples of total protein extracted from 16-h culture supernatant of four *Bacillus thuringiensis* strains showing contrasting effects in the mortality of fall armyworm.

a similar intensity that were present in both heated and non-heated samples, in a single strain, may either belong to a more thermostable fraction of the total protein secreted by the B.t. cells in the supernatant, or simply represent minimal peptide subunits produced by the heat-derived denaturing procedure.

The results presented here certainly stimulate further evaluation of supernatants from other B.t. strains against fall armyworm (and perhaps other important insect-pests in maize – Estruch *et al*. 1996), in the search for even more efficient Vip activities as alternative bioinsecticides to be used in IPM programs.

A collection of over a thousand other 'domestic' strains, isolated from Brazilian soils (Valicente *et al*., in press), are currently under assessment of their Vip contents and activity against *S. frugiperda* larvae. The composition of genes and secreted proteins of these and other B.t. strains is currently being characterized in greater detail, not only with the aim of pinpointing the specific Vip(s) responsible for the protein-derived biological activity of B.t. culture supernatants against tropical races of *S. frugiperda*, but also to better dissect the protein composition in the supernatant of B.t. strains effective in the control of *S. frugiperda* larvae, as well as to help isolate the Vip gene(s) possibly related to those larval-controlling effects.

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