Study Title

Assessment of the *In vitro* Digestive Fate of *Bacillus thuringiensis* var. *kurstaki* HD-73 Protein

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STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA §10(d)(1) (A), (B), or (C).

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STATEMENT OF COMPLIANCE

This study meets the requirements for 40 CFR Part 160 and 21 CFR Part 158.

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QUALITY ASSURANCE STATEMENT

This signed statement indicates that the NPD Quality Assurance Unit has monitored this study and reviewed the study data and final report. These reviews indicate that the final report accurately presents the raw data as developed during the study.

Dates of reviews as well as dates that findings were reported to testing facility management and the study director are listed below.

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ABBREVIATIONS

C	Centigrade
E. coli	*Escherichia coli*
Fig.	Figure
h	hours
M	molar
mg	milligram
min	minute
ml	milliliter
ng	nanogram
PAGE	polyacrylamide gel electrophoresis
s	second
SDS	sodium dodecyl sulfate
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
SOP	Standard Operating Procedure
TCA	trichloroacetic acid
µg	microgram
µl	microliter
B.t.k.	*Bacillus thuringiensis* var. *kurstaki*
var.	variety, synonymous with subspecies
TBW	tobacco budworm
ELISA	enzyme-linked immunosorbent assay
TCA	trichloroacetic acid

SUMMARY

Cotton, *Gossypium hirsutum*, has been genetically engineered to be resistant to selected insect pests (Lepidoptera). Resistance was accomplished by the insertion of a gene from *Bacillus thuringiensis* var. *kurstaki* (*B.t.k.*) which encodes for the production of a protein insecticidal to Lepidoptera larvae but safe to mammals, birds, fish and beneficial insects. A second gene encoding neomycin phosphotransferase II (NPTII) was also introduced as a marker to enable selection of cells containing the *B.t.k.* gene. Larvae of Lepidopteran pests are the most important insect pests impacting successful cotton production. Cotton typically requires numerous chemical insecticide treatments for Lepidoptera control. The cotton varieties containing the *B.t.k.* gene will significantly reduce chemical insecticide use in cotton and, therefore, provide a major benefit to cotton growers and the environment.

Prior to the commercialization of this insect resistant cotton variety, data and information were produced to demonstrate that this cotton variety is equivalent to current cotton varieties in composition and agronomic performance and that the proteins expressed by the inserted genes cause no adverse effect when consumed by domestic or wild animals and beneficial insects. Exposure to humans is not expected since protein is not present in the processed cotton fiber or oil at detectable levels, and cottonseed meal is not used in human foods. Areas of investigation included: molecular characterization of the introduced genes; biochemical characterization of the expressed *B.t.k.* protein; estimation of the levels of the *B.t.k.* protein in cotton products; safety of the *B.t.k.* protein to mammals and beneficial insects; and the environmental fate of the *B.t.k.* protein.

The purpose of this study was to assess the degradation of the *B.t.k.* HD-73 protein using *in vitro* mammalian digestion models consisting of simulated gastric and intestinal fluids. Simulated digestion fluids are widely used as models of animal digestion. They have been used: to investigate the digestibility of plant and animal proteins and food additives; to assess protein quality; and to assess bioavailability of pharmaceuticals. The *B.t.k.* HD-73 protein is converted to a lower molecular weight bioactive core upon exposure to trypsin (tryptic core), a previously described characteristic of this and other *Bacillus thuringiensis* insecticidal proteins. The degradation of *B.t.k.* HD-73 to its tryptic core and the subsequent degradation of this tryptic core were assessed by western blot analysis. In addition, dissipation of the *B.t.k.* HD-73 protein bioactivity in these digestion models was assessed by insect bioassay.

B.t.k. HD-73 protein -- purified from *Escherichia coli*, characterized and demonstrated to be equivalent to the *B.t.k.* HD-73 protein expressed in insect resistant cotton plants -- was incubated at approximately 37°C in simulated gastric and intestinal fluids. At defined incubation time periods the digestions

were terminated and the levels of *B.t.k.* HD-73 protein and its tryptic core were measured by western blot analysis. The dissipation of the bioactivity of the *B.t.k.* HD-73 protein was assessed by incorporating *B.t.k.* HD-73 protein digestion solutions into artificial insect diet and measuring its affect on the mortality of tobacco budworm (*Heliothis virescens*). The initial concentration of *B.t.k.* HD-73 protein in digestion solutions that were evaluated by western blot was approximately 2 µg/ml. For samples evaluated for bioactivity, final *B.t.k.* HD-73 protein concentrations of approximately 0.75, 7.5 and 75 µg/ml were used.

After approximately 30 seconds incubation in gastric fluid, no intact *B.t.k.* HD-73 protein was detected by western blot analysis and less than 50% of the tryptic core (formed by the degradation of intact *B.t.k.* HD-73 protein) was detected. Greater than 90% of the *B.t.k.* HD-73 protein bioactivity against tobacco budworm dissipated at all three concentrations tested after approximately 5 minutes of incubation in gastric fluid. This bioactivity assay used does not distinguish between *B.t.k.* HD-73 protein and its tryptic core since both are comparably active.

In simulated intestinal fluid, the *B.t.k.* HD-73 protein readily degraded to its tryptic core; no *B.t.k.* HD-73 protein was detected by western blot analysis after a 30 minute incubation. The tryptic core, formed from added *B.t.k.* HD-73 protein, did not degrade significantly after approximately a 21 hour incubation in intestinal fluid as detected by western blot analysis. This result is consistent with the bioassay which also indicated no significant degradation of activity against tobacco budworm after approximately a 21 hour incubation in intestinal fluid.

The results of this study establish that the *B.t.k.* HD-73 protein and its associated functional activity will readily degrade upon exposure to gastric fluid in the mammalian digestive tract. In intestinal fluid, the *B.t.k.* HD-73 protein readily degrades to its tryptic core; this tryptic core remains intact and bioactive for at least 21 hours. This result is as expected; the tryptic core of this and other *Bacillus thuringiensis* insecticidal proteins have been shown to be relatively resistant to trypsin digestion.

INTRODUCTION

The purpose of this study was to assess the degradation of the *Bacillus thuringiensis* var. *kurstaki* HD-73 [Cry IA(c)] (Hofte and Whitely, 1989) protein (abbreviated as *B.t.k.* HD-73) and its biological activity using *in vitro* mammalian digestion models. Degradation of the *B.t.k.* HD-73 protein was assessed using western blot analysis. Degradation of the biological activity of the *B.t.k.* HD-73 protein was assessed using an insect bioassay. The *B.t.k.* HD-73 protein, expressed in cotton plants, confers resistance to Lepidopteran insect damage. The *B.t.k.* HD-73 degrades in the presence of trypsin to form a trypsin-resistant core protein (called tryptic core in this study) and this tryptic core is the insecticidally active component of the *B.t.k.* HD-73 protein (Bielot *et al.*, 1989).

MATERIALS

Test substance. The test substance for this study was the *B.t.k.* HD-73 protein that was purified from *Escherichia coli* (Lot Number 5025385) to 67% purity by ELISA (Volume 5) and shown to be equivalent to the *B.t.k.* HD-73 protein expressed in insect resistant cotton plants (Volume 4). Concentrated stock solutions of *B.t.k.* HD-73 protein were prepared from solid material dissolved in buffer (10 mM sodium carbonate/bicarbonate, pH 10.5).

Control substance. The control substance for this study was the buffer used to prepare the *B.t.k.* HD-73 protein solutions (10 mM sodium carbonate/ bicarbonate, pH 10.5, prepared by dilution from a 1 M stock solution).

Reference substance. Incubated samples were referenced to unincubated (time = 0) samples. Analytical standards of the *B.t.k.* HD-73 protein and the tryptic core of the *B.t.k.* HD-73 protein were used in the western blot analysis for reference to the digestion samples. Two types of standards were used because the *B.t.k.* HD-73 protein was known to degrade to the biologically-active tryptic core upon exposure to trypsin (Bietlot *et al.*, 1989), a component of the simulated intestinal fluid used in this study.

Test system. The test systems for this study were simulated gastric fluid and simulated intestinal fluid prepared as described in SOP Number GEN-PRO-055-00. Simulated digestion solutions were used in this *in vitro* study to simulate digestion, simplify analysis of protein degradation and increase reproducibility relative to *in vivo* systems. These digestion fluids were used within 24 hours of preparation, stored at approximately 4°C and assayed for protease activity before use. The test system incubation tubes were clearly

marked using a permanent marking pen with unique alphanumeric codes reflecting the treatments.

Test system justification. *In vitro* studies with simulated digestion solutions are widely used as models of animal digestion. Simulated digestion solutions have been used to investigate the digestibility of plant proteins (Nielson, 1988, Marquez *et al.*, 1981), animal proteins (Zikakis *et al.*, 1977), and food additives (Tilch and Elias, 1984); to assess protein quality (Akeson and Stahmann, 1964); to study digestion in pigs and poultry (Fuller, 1991); to measure tablet dissolution rates to assess bioavailability for pharmaceuticals (Alam *et al.*, 1980); and to investigate the controlled-release properties of experimental pharmaceuticals (Doherty *et al.*, 1991). The method of preparation of the simulated digestion solutions to be used in this study is described in the United States Pharmacopeia (USP, 1989), a frequently cited reference for *in vitro* digestion studies.

Reagents. Pepsin (porcine, Product Number P-7000), pancreatin (porcine, Product Number P-1500), and hemoglobin (bovine, Product Number H-2625) were obtained from Sigma Chemical Company (St. Louis, MO). Resorufin-labeled casein (Product Number 1080733) was obtained from Boehringer Mannheim Corporation (Indianapolis, IN).

METHODS

Assessment of gastric and intestinal fluid activity. Pepsin activity was measured in simulated gastric fluid (SGF) according to SOP Number GEN-PRO-057-00 by measuring spectrophotometrically the increase in supernatant absorbance at 280 nm following TCA precipitation of SGF incubations with hemoglobin. Protease activity in simulated intestinal fluid (SIF) was measured according to SOP Number GEN-PRO-058-00 by measuring spectrophotometrically the increase in supernatant absorbance at 574 nm following TCA precipitation of SIF incubations with resorufin-labelled casein.

Addition of Test Substance. Aliquots of purified *B.t.k.* HD-73 protein was added to temperature-equilibrated (approximately 37°C) digestion solutions by pipetting from concentrated *B.t.k.* HD-73 protein stock solutions. This method of administration of the test substance was selected to provide reproducible dosing of small amounts of protein. One ml volumes of digestion fluids were incubated in 15 ml plastic test tubes with the following exceptions: 1) a 0.1 ml incubation volume in a 1.5 ml plastic test tube were used for the t = 0 samples of *B.t.k.* HD-73 protein in SGF and SIF in Part I; and 2) a 0.1 ml incubation volume in a 1.5 ml plastic test tube was used for the whole incubation samples for both SGF and SIF in Part I. These treatments used a reduced volume to

keep final quenched sample volumes compatible with equipment used for aliquots sampled from the 1-ml digestion incubations. The digestion solutions were thoroughly mixed immediately after the addition of the *B.t.k.* HD-73 protein.

Incubation. All digestion solutions were incubated at approximately 37°C in a shaker-incubating water bath (Model G76, New Brunswick Scientific Co, Edison, N.J.). Incubation solutions were agitated continuously throughout the incubation period with the following exceptions: agitation was interrupted briefly to facilitate sampling; and no agitation occurred between sampling intervals that were less than 2 minutes. In the latter case, incubation samples were agitated manually just prior to sampling.

Part I. Assessment by western blot analysis. In this part, the *B.t.k.* HD-73 protein was added to digestion fluids to a final concentration of approximately 2 µg/ml. This dose was selected based on the detection range of the western blot analysis. The *B.t.k.* HD-73 protein was incubated for five time intervals (including t = 0) up to 20 minutes in SGF and up to 21 hours in SIF. For one-ml incubation volumes, 50 µl aliquots of the incubation solution were removed and the digestion terminated. For SGF incubations, termination was by neutralization with 0.2 M sodium carbonate (15 µl sodium carbonate per 50 µl digestion solution.) For SIF incubations, termination was by 1:1 dilution with protein-denaturing 2X SDS-PAGE sample buffer (Laemmli, 1970) followed by heating to about 100°C for approximately 5 minutes. Neutralized SGF incubation samples were diluted 1:1 with 2X SDS-PAGE sample buffer and heated to approximately 100°C for approximately 5 minutes. For 0.1-ml incubation solutions, the entire incubation solution was terminated, diluted with 2X SDS-PAGE sample buffer and heated as described for aliquots from one-ml incubations. All samples were stored at approximately -80°C until analyzed for *B.t.k.* HD-73 content by western blot analysis. Western blotting was completed according to SOP Number BtC-PRO-002-02 (Volume 3, Appendix 3)

Part II. Assessment by insect bioassay. In this part, the amount of degradation of *B.t.k.* HD-73 protein bioactivity over a fixed time interval in digestion fluids was assessed using the tobacco budworm (*Heliothis virescens*) (TBW). The *B.t.k.* HD-73 protein was added to digestion fluids to final concentrations of 0.75, 7.5 and 75 µg/ml. These concentrations were selected based on the sensitivity of the TBW assay. These samples were incubated for a single time interval -- 5 min for SGF and 20.7 hr for SIF -- approximately corresponding to one time interval used in Part I. The one-ml incubation volumes were terminated by neutralization with sodium carbonate for SGF and by freezing on dry ice for SIF. The *B.t.k.* HD-73 protein content of these samples was also assessed by western blot analysis to allow a direct comparison with the TBW bioassay results. 50 µl samples were taken from

each incubation mixture in Part II and the *B.t.k.* HD-73 protein concentration estimated by western blot analysis as described for Part I. Terminated digestion samples to be evaluated for TBW activity were stored at approximately -80°C. On the day of the TBW assay, performed according to SOP Number BUG-PRO-022-02, samples were thawed, diluted to 6 ml with distilled water, brought to approximately 30 ml with TBW and poured into tray wells. Individual TBW larvae were added to each well (24 larvae per sample), incubated 7 days at approximately 28°C and scored for mortality.

Control of Bias. Three replicate digestion incubations were prepared for each treatment. SGF and SIF samples without *B.t.k.* HD-73 protein added were prepared to assess whether these digestion fluids interfere with the analytical methods used in this study. Buffer plus *B.t.k.* HD-73 protein (no SGF or SIF) treatments were prepared to allow an estimate of the recovery of the *B.t.k.* HD-73 protein and its bioactivity from the digestion solutions. In Part I, a sample was prepared in which the entire digestion solution was quenched and diluted with SDS-PAGE sample buffer to allow an assessment if the 50 µl aliquots sampled from the 1 ml digestion solutions are representative of the entire digestion solution.

Data analysis. For Part I, *B.t.k.* HD-73 protein concentration for each assay sample was estimated by visual comparison of the sample band intensity to the band intensity of two concentrations of the *B.t.k.* HD-73 protein and tryptic fragment standards. The mean *B.t.k.* HD-73 protein concentration was calculated from the three replicates of each treatment. The degradation of the *B.t.k.* HD-73 protein was reported as a percentage of *B.t.k.* HD-73 protein degraded at each time interval relative to the estimated *B.t.k.* HD-73 protein concentration in the unincubated treatment.

For Part II, the *B.t.k.* HD-73 bioactivity of each assay sample was reported as TBW mortality, calculated as the percentage of TBW larvae that died after the TBW larvae incubation period relative the initial number of larvae added to the insect diet. The mean and standard error of TBW mortality was calculated for the three replicates from each treatment.

RESULTS

Part I. Assessment of digestion by western blot analysis

Gastric fluid. *B.t.k.* HD-73 protein degraded rapidly in SGF (Figure 1). No *B.t.k.* HD-73 protein was detected after 30 seconds incubation, the earliest time point tested (lane 8). The western blot procedure used can typically be used to quantitate *B.t.k.* HD-73 protein levels down to 2 ng/well and tryptic core levels down to 0.5 ng/ml. The tryptic core of *B.t.k.* HD-73 protein was

more than 50% degraded by SGF incubation for 30 seconds (lane 8); it was not detectable after 7 min (lane 10). SGF did not produce bands by western blot analysis that overlap with those produced by *B.t.k.* HD-73 or tryptic core proteins at t = 0 (lane 5) or after 7 min incubation (data not shown). Recovery of *B.t.k.* HD-73 protein from SGF was approximately 100% (lane 7 versus lane 6). These data confirm the validity and specificity of this western blot assay for these analyses.

The digestion samples were prepared for western blot analysis to provide approximately 10 ng *B.t.k.* HD-73 protein/ lane if no *B.t.k.* HD-73 protein degradation occurred during the incubations. The data in Figure 1 suggests a higher level of *B.t.k.* HD-73 protein than expected was used in this experiment (compare lane 6 to lane 2). The *B.t.k.* HD-73 standard used for this western provided a lower signal than freshly prepared standards; the *B.t.k.* HD-73 protein dose to SGF was likely near the expected level of 2 µg/ml. The low standard response for *B.t.k.* HD-73 protein did not affect the interpretation of this western blot as band intensities were compared to t = 0 (lane 7) to assess the degree of degradation.

No *B.t.k.* HD-73 or tryptic core of *B.t.k.* HD-73 proteins were detected after 20 min incubation in SGF in the whole incubation sample control (lane 12). This is a similar result to that obtained with the 50 µl aliquot withdrawn from the approximately one ml incubation sample (lane 11).

Intestinal fluid. *B.t.k.* HD-73 protein degraded rapidly to its tryptic core in SIF and this tryptic core remained stable up to 20.8 hours in SIF (Figure 2), as expected. No *B.t.k.* HD-73 protein was detected after 30 min incubation, the earliest time point tested (lane 8). The *B.t.k.* HD-73 protein was nearly completely converted to its tryptic core within 30 min incubation in SIF (lane 8) and the level of the tryptic core remained approximately constant after 20.8 hr incubation (lane 11). SIF did not produce bands by western blot analysis that overlap with those produced by *B.t.k.* HD-73 or tryptic core proteins at t = 0 (lane 5) or after 20.8 hr incubation (data not shown). These data confirm the validity and specificity of this western blot assay for these analyses. Recovery of *B.t.k.* HD-73 protein from SIF was less than 50% (lane 7 versus lane 6). This was attributed to *B.t.k.* HD-73 protein degrading during the quench procedure. An alternate quench procedure, boiling the sample 5 min before adding the 2X SDS-PAGE sample buffer (Protocol Amendment Number 1, Appendix 2), was tried and produced similar results. It appears the *B.t.k.* HD-73 protein is extremely labile in SIF; the quench procedures tried were not successful with this particular protein.

The level of *B.t.k.* HD-73 protein in digestion samples before incubation in SIF (lane 6) was higher than expected when compared to the *B.t.k.* HD-73 standard

(lane 2). This is likely due the use of standard containing less *B.t.k.* HD-73 protein that expected, as noted for Figure 1. The level of tryptic core of *B.t.k.* HD-73 protein formed from the added *B.t.k.* HD-73 protein (lanes 8 through 12) are similar to the tryptic core standards. This suggests the original dose was close to the 2 µg/ml level. The low standard response for *B.t.k.* HD-73 protein did not affect the interpretation of this western blot as band intensities were compared to t = 0 (lane 6) to assess the degree of degradation. The 50 µl aliquots taken from *B.t.k.* HD-73 protein incubations in SIF after 20.8 h incubation (lane 11) were found to be representative of the whole digestion solution by comparison to a sample incubated for 20.8 h in which the entire incubation sample was quenched and diluted with SDS-PAGE sample buffer (lane 12).

Part II. Assessment of digestion by bioactivity

Investigation of potential assay interference. No TBW mortality above the water control was attributable to the incorporation of SGF and SIF into the diet for TBW at the levels used in this study (1 ml SGF or SIF/ 30 ml insect diet) (Table 1). Incubated SGF and SIF also did not increase TBW mortality (Table 1). This result supports the appropriateness of the TBW assay to assess the dissipation of *B.t.k.* HD-73 bioactivity in SGF and SIF.

Gastric fluid. The bioactivity of the *B.t.k.* HD-73 protein dissipated readily in SGF (Table 2); 91 to 100% of the initial bioactivity was dissipated due to incubation of 7.5 and 75 µg/ml *B.t.k.* HD-73 protein for 5 min in SGF. This result is consistent with the western blot analysis results for Part I. Furthermore, no *B.t.k.* HD-73 protein was detected by western blot analysis of the same 5 min incubation samples tested for TBW mortality (data not shown). The unincubated TBW mortality for the 0.75 µg/ml dose was too low to provide an assessment of the dissipation of *B.t.k.* HD-73 protein bioactivity.

Intestinal fluid. The bioactivity of the *B.t.k.* HD-73 protein was stable for up to 20.7 h incubation in SIF (Table 3). This result is also consistent with the results obtained by western blot analysis in Part I. Western blot analysis of the same samples used for bioactivity measurement show conversion of *B.t.k.* HD-73 protein to its tryptic core and then stability of the tryptic core in SIF (data not shown). The TBW bioassay does not distinguish between the *B.t.k.* HD-73 and tryptic core proteins.

DISCUSSION

B.t.k. HD-73 protein degrades readily in gastric fluid. This degradation corresponds to a comparable dissipation of the insecticidal activity. Upon incubation in SIF, the *B.t.k.* HD-73 protein rapidly degrades to the tryptic core. As expected (Chroma and Kaplan, 1990), this tryptic core is stable for up to 20.8 hours in SIF. The insecticidal activity of the *B.t.k.* HD-73 protein, known to be due to the formation of a trypsin-stable core (Bielot *et al.*, 1989), is also stable in SIF for up to 20.8 hours.

Both analytical methods used in this study, western blot analysis and TBW activity, were appropriate to assess degradation or dissipation of the *B.t.k.* HD-73 protein due to their sensitivity, selectivity and lack of significant interferences from SGF and SIF. Recoveries of added *B.t.k.* HD-73 protein or bioactivity were high from SGF by both western blot analysis and TBW mortality. Recovery of *B.t.k.* HD-73 protein from SIF as assessed by western blot was low (less than 50%) due to the rapid degradation of the *B.t.k.* HD-73 protein during the standard quench procedure, and even when a more rigorous quench procedure was tried. Recovery of *B.t.k.* HD-73 protein bioactivity, which is due to the tryptic core, was high as assessed by TBW mortality. The TBW assay does not distinguish between the *B.t.k.* HD-73 protein and its tryptic core.

B.t.k. HD-73 protein purified from *Escherichia coli* is an appropriate test material for this study. It has been shown to be equivalent to the protein expressed in insect resistant cotton plants (Volume 4). The use of a purified sample of *B.t.k.* HD-73 protein allows a direct assessment of the stability of this protein in digestion fluids without the complication of additional components in the test material that could influence protein degradation.

There is no likely avenue for human consumption of insect resistant cotton products containing the *B.t.k.* HD-73 protein. Most animal feed prepared from cotton is prepared from processed cottonseed meal which contains no detectable *B.t.k.* HD-73 protein (Volume 3, Appendix 8). Incidental consumption of insect resistant cotton plants by mammals would provide relatively small amounts of the *B.t.k.* HD-73 protein to the digestive system; line 531 insect resistant cotton plants express the *B.t.k.* HD-73 protein at estimated levels of 1.6 and 0.9 ppm in leaves and seeds, respectively (Volume 3, Appendix 2). If consumed, these levels would be diluted by the addition of digestion juices, and then likely further diluted by the consumption of other feeds. In this study, *B.t.k.* HD-73 protein levels were added to digestion fluids at levels up to 75 ppm and shown to rapidly degrade in SGF.

There is no human oral exposure to the *B.t.k.* HD-73 protein due to its expression in insect resistant cotton plants. The results of this study support the safety of the *B.t.k.* HD-73 protein to mammals by establishing that substantial degradation of *B.t.k.* HD-73 protein will occur in the stomach before it reaches the intestine. Furthermore, mammals do not have *B.t.k.* protein receptors in the intestine (Hofmann, *et al.*, 1988; Sacchi *et al.*, 1986). Finally, this combination of rapid degradation of the *B.t.k.* HD-73 protein and the lack of receptors in mammals are consistent with the acute gavage study (Volume 15) which established the safety of the *B.t.k.* HD-73 protein after mammalian consumption.

CONCLUSIONS

The results of this study establish that the *B.t.k.* HD-73 protein and its associated insecticidal activity will readily degrade upon exposure to gastric fluid in the mammalian digestive tract. The stability of the insecticidally active tryptic core of the *B.t.k.* HD-73 protein for 20.8 hours in intestinal fluid was expected; the tryptic fragment of this and other *Bacillus* insecticidal proteins have been shown to be relatively resistant to digestion by trypsin, a key protease in intestinal fluid (Guyton, 1981).

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1989. The United States Pharmacopeia, Vol. XXII, NF XVII. United States Pharmacopeial Convention, Inc., Rockville, MD. pp.1788-1789.

Table 1. Effect of simulated gastric and intestinal fluids on tobacco budworm mortality.

SGF and SIF samples were incubated at approximately 37°C and quenched as described under "Methods". Incubated and non-incubated SGF and SIF were incorporated into the diet of TBW at 1 ml digestion fluid/ 30 ml insect diet. Data represents the mean and standard error ( ) of TBW larval mortality from three replicate samples each evaluated with 24 larvae.

Treatment		TBW % mortality	
Water control	t = 0 min	5.6	(1.4)
Buffer control	t = 0 min	1.4	(1.4)
SGF	t = 0 min	5.6	(1.4)
	t = 5 min	0.0	(0.0)
SIF	t = 0 min	1.4	(1.4)
	t = 20.7 h	1.4	(1.4)

Table 2. Dissipation of *B.t.k.* HD-73 protein bioactivity in simulated gastric fluid.

Three levels of purified *B.t.k.* HD-73 protein were incubated in SGF for 5 min at approximately 37°C, quenched by neutralization with sodium carbonate and incorporated into TBW diet. Data represent the mean and standard error ( ) of three replicated digestion samples, each assayed for mortality with 24 larvae.

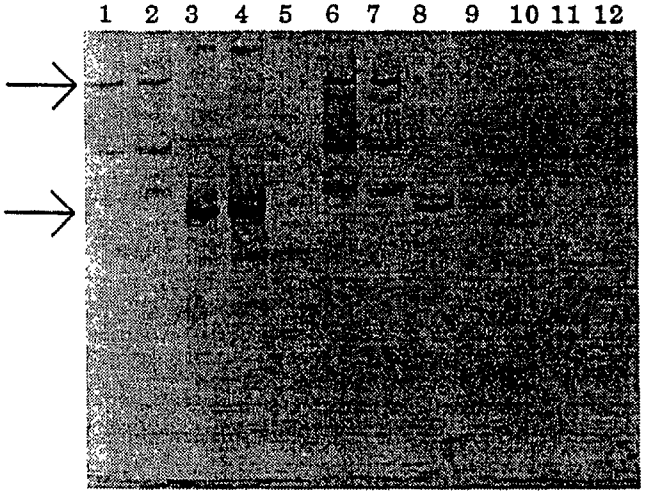
B.t.k. HD-73 (µg/ml)	Digestion fluid	Incubation time (min)	TBW % mortality	
0.75	Buffer control	0	1.4	(1.4)
	SGF	0	8.3	(4.2)
	"	5	0.0	(0.0)
7.5	Buffer control	0	33.3	(4.2)
	SGF	0	38.9	(7.4)
	"	5	0.0	(0.0)
75	Buffer control	0	83.3	(6.4)
	SGF	0	79.2	(6.4)
	"	5	6.9	(5.0)

Table 3. Dissipation of *B.t.k.* HD-73 protein bioactivity in simulated intestinal fluid.

Three levels of purified *B.t.k.* HD-73 protein were incubated in SIF for 20.7 hr at approximately 37°C, quenched by freezing on dry ice and incorporated into TBW diet. Data represent the mean and standard error ( ) of three replicated digestion samples, each assayed for TBW mortality with 24 larvae.

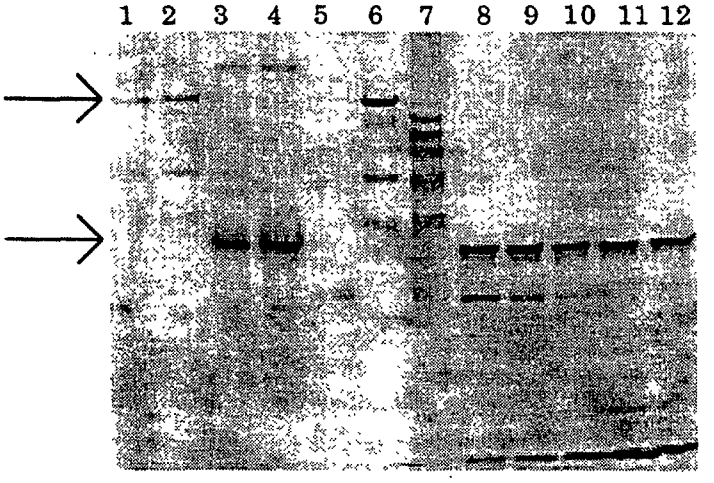
B.t.k. HD-73 (µg/ml)	Digestion fluid	Incubation time (hr)	TBW % mortality	
0.75	Buffer control	0	1.4	(1.4)
	SIF	0	11.1	(6.1)
	"	20.7	8.3	(4.8)
7.5	Buffer control	0	33.3	(4.2)
	SIF	0	36.1	(7.4)
	"	20.7	59.7	(6.1)
75	Buffer control	0	83.3	(6.4)
	SIF	0	98.6	(1.4)
	"	20.7	100.0	(0.0)

Figure 1. Western blot analysis of *B.t.k.* HD-73 protein degradation in simulated gastric fluid.



Western blot shown is one of three replicate digestions. *B.t.k.* HD-73 protein was added to SGF to a final concentration of approximately 2 µg/ml, incubated at approximately 37°C, quenched by neutralization with 0.2 M sodium carbonate and evaluated by western blot analysis. Lanes 1 and 2 are *B.t.k.* HD-73 protein standard added at 5 and 10 ng/lane, respectively. Lanes 3 and 4 are tryptic core of *B.t.k.* HD-73 protein standard added at 5 and 10 ng/lane, respectively. Lane 5 is SGF plus buffer at t = 0 min. Lane 6 is buffer plus *B.t.k.* HD-73 protein at t = 0 min. Lanes 7 through 11 are *B.t.k.* HD-73 protein after 0, 0.5, 2, 7 and 20 min incubation in SGF, respectively. Lane 12 is SGF plus *B.t.k.* HD-73 after 20 min incubation where the entire incubation sample was quenched and prepared for western blot analysis. The gel shown is gel number G3 from 6/1/93.

Figure 2. Western blot analysis of *B.t.k.* HD-73 protein degradation in simulated intestinal fluid.



Western blot shown is one of three replicate digestions. *B.t.k.* HD-73 protein was added to SIF to a final concentration of approximately 2 µg/ml, incubated at approximately 37°C, quenched by heating to approximately 100°C in 2X SDS-PAGE sample buffer and evaluated by western blot analysis. Lanes 1 and 2 are *B.t.k.* HD-73 protein standard added at 5 and 10 ng/lane, respectively. Lanes 3 and 4 are tryptic core of *B.t.k.* HD-73 protein standard added at 5 and 10 ng/lane, respectively. Lane 5 is SIF plus buffer at t = 0 min. Lane 6 is buffer plus *B.t.k.* HD-73 protein at t = 0 min. Lanes 7 through 11 are *B.t.k.* HD-73 protein after 0, 0.5, 1.9, 3.8 and 20.8 hr incubation in SIF, respectively. Lane 12 is SIF plus *B.t.k.* HD-73 after 20.8 hr incubation where the entire incubation sample was quenched and prepared for western blot analysis. The gel shown is gel number I3 from 6/1/93.

APPENDIX 1

SOP List

SOP List

GEN-PRO-055-00	Preparation of simulated gastric and intestinal fluid
GEN-PRO-057-00	Assay for pepsin activity in simulated gastric fluid
GEN-PRO-058-00	Assay for protease activity in simulated intestinal fluid
BtC-PRO-002-02	Procedure for western blotting: Alkaline phosphatase development
BUG-PRO-022-02	Diet incorporation for measurement of insecticidal activity of purified *Bacillus thuringiensis* endotoxin proteins and endotoxin proteins expressed in cotton seed

APPENDIX 2

Protocol
Study Number 92-01-36-22
Experiment Number 92-427-728

Experimental Protocol#

Study #: 92-01-36-22
Experiment #: 92-427-728

Study Title: Assessment of the *in vitro* digestive fate of *Bacillus thuringiensis* var. *kurstaki* HD-73 protein

Sponsor:
The Agricultural Group of Monsanto Company
New Products Division
700 Chesterfield Parkway North
St. Louis, MO 63198

Primary Testing Facility:
The Agricultural Group of Monsanto Company
New Products Division
700 Chesterfield Parkway North
St. Louis, MO 63198

Study Director:
Joel E. Ream
Research Specialist
The Agricultural Group of Monsanto Company
700 Chesterfield Parkway North - GG4K
St. Louis, MO 63198
(314) 537-6678

Approved By:

Sponsor:

Date: 5/24/1993

R. L. Fuchs, Ph.D.
Associate Fellow, Regulatory Science
GG4G, (314) 537-6438
The Agricultural Group of Monsanto Company

Study Director:

Date: 5/25/93

J. E. Ream
Research Specialist, Regulatory Science
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The Agricultural Group of Monsanto Company

Reviewed by:

Quality Assurance:

Date: 5/25/93

K. F. Yount
A3NF, (314) 694-2309
Sr. Quality Assurance Auditor
Monsanto Company

Quality Control:

Date: 5/25/93

R. S. King
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GLP/QC Coordinator
The Agricultural Group of Monsanto

Statistical Support:

Date: 5/25/93

L. R. Holden, PhD
BB5F, (314) 537-6885
Science Fellow, Statistics
The Agricultural Group of Monsanto

1. Purpose:

The purpose of this study is to assess the degradation of the *Bacillus thuringiensis* var. *kurstaki* HD-73 [Cry IA(c)][1] protein (abbreviated as "*B.t.k.* HD-73") and its biological activity using *in vitro* mammalian digestion models. Degradation of the *B.t.k.* HD-73 protein will be assessed using western blot analysis. Degradation of the biological activity of the *B.t.k.* HD-73 protein will be assessed using an insect bioassay. The *B.t.k.* HD-73 protein, expressed in cotton plants, confers resistance to Lepidopteran insect damage.

2. Proposed Experimental Start Date: May, 1993

3. Proposed Experimental Termination Date: June, 1993

4.0 Test, Control & Reference Substances:

4.1 Test Substance

The test substance for this study will be the *B.t.k.* HD-73 protein that has been purified from *E. coli* (Lot Number 5025385). Concentrated stock solutions of *B.t.k.* HD-73 protein will be prepared from solid material dissolved in buffer (10 mM sodium carbonate/bicarbonate, pH 10.5). A description of the preparation of the stock solutions of the test substance will be documented and included with the study.

4.2 Control Substance

The control substance for this study will be the buffer used to prepare the *B.t.k.* HD-73 protein solutions (10 mM sodium carbonate/ bicarbonate, pH 10.5, prepared by dilution from a 1 M stock solution).

4.3 Reference Substance

Incubated substances will be referenced to unincubated (time = 0) samples. Analytical standards of the full-length *B.t.k.* HD-73 protein and the tryptic fragment of the full-length *B.t.k.* HD-73 protein will be used in the western blot analysis for reference to the digestion

samples. Two standards will be used because the full-length *B.t.k.* HD-73 protein is known to degrade to a biologically-active tryptic fragment upon exposure to trypsin, a component of the simulated intestinal fluid used in this study.

5. Test System:

The test systems for this study will be simulated gastric fluid and simulated intestinal fluid prepared as described in The United States Pharmacopeia[2]. Simulated digestion solutions will be used in this *in vitro* study to simulate digestion, simplify analysis of protein degradation and increase reproducibility relative to *in vivo* systems. These digestion fluids will be used within 24 hours of preparation, stored at approximately 4°C and assayed for protease activity according to applicable SOPs prior to use. The test system incubation tubes will be clearly marked using a permanent marking pen with unique alphanumeric codes reflecting the treatments.

6. Test System Justification:

In vitro studies with simulated digestion solutions are widely used as models of animal digestion. Simulated digestion solutions have been used to investigate the digestibility of plant proteins[3,4], animal proteins[5] and food additives[6]; to assess protein quality[7]; to study digestion in pigs and poultry[8]; to measure tablet dissolution rates to assess bioavailability for pharmaceuticals[9]; and to investigate the controlled-release properties of experimental pharmaceuticals[10]. The method of preparation of the simulated digestion solutions to be used in this study is described in the United States Pharmacopeia, a frequently cited reference for *in vitro* digestion studies.

7.0 Experimental:

7.1 Experimental Summary

This study will be conducted in two parts:

Part I. Assessment of degradation of *B.t.k.* HD-73 protein as detected by western blot analysis

Part II. Assessment of degradation of *B.t.k.* HD-73 protein bioactivity as detected by insect bioassay

The *B.t.k.* HD-73 protein will be added to simulated gastric fluid (SGF) and simulated intestinal fluid (SIF), incubated at approximately 37°C, the reaction terminated, and the *B.t.k.* HD-73 content of the incubation solutions evaluated by western blot analysis (Part I) or insect bioactivity (Part II). Part I will consist of 17 separate treatments with 3 replicate digestion incubations per treatment (total samples = 51). Part II will consist of 20 separate treatments with three replicate digestion incubations per treatment (total samples = 60). A list of all treatments, incubation labels and sample labels is attached (Attachment #1).

7.2 Addition of Test Substance

Aliquots of purified *B.t.k.* HD-73 will be added to temperature - equilibrated (approximately 37°C) digestion solutions by pipetting from concentrated *B.t.k.* HD-73 stock solutions. This method of administration of the test substance was selected to provide reproducible dosing of small amounts of protein. One-ml volumes of digestive fluids will be incubated in 15-ml plastic test tubes with the following exceptions: 1) a 0.1-ml incubation volume in a 1.5-ml plastic test tube will be used for the t = 0 samples of *B.t.k.* HD-73 protein in SGF and SIF (incubation samples GP-0 and IP-0, Part I); and 2) a 0.1-ml incubation volume in a 1.5-ml plastic test tube will be used for the whole incubation samples for both SGF and SIF (incubation samples GPW and IPW, Part I). These treatments use a reduced volume to keep final quenched sample volumes compatible with equipment used for aliquots sampled from the 1-ml digestion incubations. The digestion solutions will be thoroughly mixed immediately after the addition of the *B.t.k.* HD-73 protein.

7.3 Incubation

All digestion solutions will be incubated at approximately 37°C in a shaker-incubating water bath. Incubation solutions will be agitated continuously throughout the incubation period with the following exceptions: agitation may be interrupted briefly to facilitate sampling; and no agitation will occur between sampling intervals that are less than 2 minutes. In the latter case, incubation samples will be agitated manually just prior to sampling.

7.4 Part I. Assessment by Western Blot Analysis

In this part, the *B.t.k.* HD-73 protein will be added to digestion fluids to a final concentration of approximately 2 µg/ml. This dose was selected based on the detection range of the western blot analysis. The *B.t.k.* HD-73 protein will be incubated for five time intervals (including t = 0) with a total duration not to exceed 20 minutes in SGF and 24 hours in SIF. For one-ml incubation volumes, 50-µl aliquots of the incubation solution will be removed and the digestion terminated. For SGF, incubation termination will be by neutralization with 0.2 M sodium carbonate (15µl sodium carbonate per 50 µl digestion solution.) For SIF, incubation termination will be by 1:1 dilution with protein-denaturing 2X SDS-PAGE sample buffer[11] followed by heating to about 100°C for approximately 5 minutes. Neutralized SGF incubation samples will be diluted 1:1 with 2X SDS-PAGE sample buffer and heated to approximately 100°C for approximately 5 minutes. For 0.1-ml incubation solutions, the entire incubation solution will be terminated, diluted with 2X SDS-PAGE sample buffer and heated as described for aliquots from one-ml incubations. All samples will be stored at approximately -80°C until analyzed for *B.t.k.* HD-73 content by western blot analysis. Western blot analysis will be conducted according to SOP.

7.5 Part II. Assessment by Insect Bioassay

In this part, the amount of degradation of *B.t.k.* HD-73 protein bioactivity over a fixed time interval in digestion fluids will be assessed using the tobacco budworm *Heliothis virescens* (TBW) assay. The *B.t.k.* HD-73 protein will be added to digestion fluids to final concentrations of 0.75, 7.5 and 75 µg/ml. These concentrations were selected based on the sensitivity of the TBW assay. These samples will be incubated for a single time interval (two time points including t = 0). The incubation time interval will be chosen based on the results of Part I; it will be approximately the same as one of the time intervals from Part I. This interval will be chosen based on the following criteria: the time interval is one that indicates substantial degradation of the *B.t.k.* HD-73 protein by western blot analysis, or the time interval is the longest one used in Part I if no substantial degradation is observed. The one-ml incubation volumes will be terminated by neutralization with sodium carbonate for SGF and by freezing on dry ice for SIF. 50-µl samples will be taken from each incubation mixture in Part II and the *B.t.k.* HD-73 protein concentration estimated by western blot analysis. Terminated

samples to be evaluated for TBW activity will be stored at approximately -80°C. On the day of the TBW assay, samples will be thawed, incorporated into artificial TBW diet and assayed for activity against TBW according to applicable SOPs.

7.6 Control of Bias

Three replicate digestion incubations will be prepared for each treatment.

For Part I and Part II, the following treatments will be prepared to assess whether the SGF and/or SIF solutions are interfering with the analytical methods used in this study:

SGF + buffer (- *B.t.k.* HD-73 protein)
SIF + buffer (- *B.t.k.* HD-73 protein)

To estimate the recovery of added *B.t.k.* HD-73 from SGF and SIF in Part I and Part II, the following treatments will be compared:

Buffer + *B.t.k.* HD-73 protein (t = 0)
SGF/SIF + *B.t.k.* HD-73 protein (t = 0)

For Part I, to assess whether the 50-µl aliquots sampled from the 1-ml digestion solutions are representative of the entire digestion solution, the following treatments will be compared:

SGF/SIF + *B.t.k.* HD-73 protein, time interval #4 (50-µl aliquot of 1-ml incubation sample)
SGF/SIF + *B.t.k.* HD-73 protein, time interval #4 (0.1-ml incubation sample in which entire incubation sample is prepared for western blot analysis)

7.7 Proposed Statistical Methods

For Part I, *B.t.k.* HD-73 protein concentration for each assay sample will be estimated by visual comparison of the sample band intensity to the band intensity of two concentrations of the *B.t.k.* HD-73 protein standard. The mean *B.t.k.* HD-73 protein concentration will be reported from the three replicates of each treatment. Degradation of *B.t.k.* HD-73 protein will be reported as a percentage of *B.t.k.* HD-73

protein degraded at each time interval relative to the estimated *B.t.k.* HD-73 protein concentration in the unincubated treatment.

For Part II, the *B.t.k.* HD-73 bioactivity of each assay sample will be reported as TBW mortality, calculated as the percentage of TBW larvae that died after the TBW larvae incubation period relative the initial number of larvae added to the insect diet. The mean and standard error of TBW mortality will be calculated for the three replicates from each treatment. Degradation of *B.t.k.* HD-73 protein bioactivity will be related to the percent change in TBW mortality within the digestion incubation time interval tested.

8. Records to be Maintained

Records specific to this study that will be maintained are:

Stock solution preparation
Dosing solution preparation
Dosing descriptions
Incubation interval data
Western blot gel loading forms
Original western blots and electronically-scanned copies
TBW assay sample data forms
Sample transfer forms
Statistical analyses

9. Study Conduct Statement:

This experiment shall be conducted in accordance with the protocol. Any change, revision or deviation from this protocol will be documented properly and communicated to the Study Director immediately. (If the Study Director is unavailable, deviations will be communicated to the GLP/QC Coordinator who will inform the Study Director as soon as possible.) All specimens will be identified clearly with the Study and Experiment numbers and a unique identifier. All data generated during the conduct of this study will be recorded directly and promptly using indelible ink, preferably black. Entries, at least once per page, will be identified with the protocol number, dated on the day of entry and signed or initialed by the person entering the information. Data generated by automated data collection systems will include the protocol number, initials or signature of the individual responsible for the direct data input and the date. Any change in data entries will be made so as not to obscure the

original entry, will indicate the reason for the change, will be dated and the responsible individual will be identified.

10. Key Personnel / Co-investigators:

Steven R. Sims	Research Specialist	Monsanto Company
John N. Leach	Research Technician	Today's Temporary

11. Confidentiality:

No raw data, worksheets, data or other information summaries, reports, or other information related to this study may be revealed or released to any third party without prior notification and authorization of the Agricultural Group of Monsanto.

12. GLP Compliance:

This experiment will be conducted in compliance with the United States EPA FIFRA Good Laboratory Practice Regulations (40 CFR Part 160), FDA Good Laboratory Practice Regulations (21CFR Part 58) and OECD Good Laboratory Practice Standards and principles.

13. References:

1. Hofte, H. & Whiteley, H. R. 1989. Microbiological Reviews 53: 242-255.
2. The United States Pharmacopeia, Vol. XXII. 1990. United States Pharmacopeial Convention, Inc. Rockville, MD, pp. 1788-1789.
3. Nielson, S. S. 1988. Cereal Chem. 65: 435-442.
4. Marquez, U. M. L. & F. M. Lajolo. 1981. J. Agric. Food Chem. 29: 1068-1074.
5. Zikakis, J. P., Rzucidlo, S. J. & Biasotto, N. O. 1977. J. Dairy Science 60: 533-541.
6. Tilch, C. & Elias, P. S. 1984. Mutation Research 138: 1-8.
7. Akeson, W. R. & Stahmann, M. A. 1964. J. Nutrition 83: 257-261.
8. Fuller, M. F., *ed.* 1991. *In vitro digestion for pigs and poultry*, C.A.B. International, U.K.
9. Alam, A. S., Hagerman, L. M. & Imondi, A. R. 1980. Arch. Int. Pharmodyn. Ther. 247: 180-189.
10. Doherty, A. M., *et al.* 1991. J. Med. Chem. 34: 1258-1271.
11. Laemmli, U. K. 1970. Nature 227: 680-685.

Attachment 1: Incubation and Assay Sample Labels

Part I. Assessment by Western Blot Analysis

Incubation sample label*	Assay sample label*	Description
BP	BP-0	Buffer + protein, t = 0 min
GB	GB-0	SGF + buffer, t = 0 min
	GB-4	SGF + buffer, time interval #4
GP-0	GP-0	SGF + protein, t = 0 min
GP	GP-1	SGF + protein, time interval #1
	GP-2	SGF + protein, time interval #2
	GP-3	SGF + protein, time interval #3
	GP-4	SGF + protein, time interval #4
GPW	GPW-4	SGF + protein, whole incubation sample control, time interval #4
IB	IB-0	SIF + buffer, t = 0 hr
	IB-4	SIF + buffer, time interval #4
IP-0	IP-0	SIF + protein, t = 0 hr
IP	IP-1	SIF + protein, time interval #1
	IP-2	SIF + protein, time interval #2
	IP-3	SIF + protein, time interval #3
	IP-4	SIF + protein, time interval #4
IPW	IPW-4	SIF + protein, whole incubation sample control, time interval #4

* An additional numeral (A, B or C) will be added to reflect replicate digestion solutions.

SGF = simulated gastric fluid; SIF = simulated intestinal fluid

Attachment 1 (continued)

Part II. Assessment by Insect Bioassay

Incubation sample label*	Description
B-0	Buffer, t = 0
G-0	SGF, t = 0
G-1	SGF, time interval #1
I-0	SIF, t = 0
I-1	SIF, time interval #1
B-L-0	Buffer + 0.75 µg/ml *B.t.k.* HD-73, t = 0
G-L-0	SGF + " , t = 0
G-L-1	SGF + " , time interval #1
I-L-0	SIF + " , t = 0
I-L-1	SIF + " , time interval #1
B-M-0	Buffer + 7.5 µg/ml *B.t.k.* HD-73, t = 0
G-M-0	SGF + " , t = 0
G-M-1	SGF + " , time interval #1
I-M-0	SIF + " , t = 0
I-M-1	SIF + " , time interval #1
B-H-0	Buffer + 75 µg/ml *B.t.k.* HD-73, t = 0
G-H-0	SGF + " , t = 0
G-H-1	SGF + " , time interval #1
I-M-0	SIF + " , t = 0
I-M-1	SIF + " , time interval #1

* An additional numeral (A, B or C) will be added to reflect replicate digestion solutions

SGF = simulated gastric fluid; SIF = simulated intestinal fluid

Attachment 2: Abbreviations

B.t.k.	*Bacillus thuringiensis* var. *kurstaki*
E. coli	*Escherichia coli*
°C	degrees Centigrade
SOP	Standard Operating Procedure
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
µg	microgram
µl	microliter
ml	milliliter
SDS	sodium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis
TBW	tobacco budworm
GLP	Good Laboratory Practice
QC	Quality Control
EPA	Environmental Protection Agency
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
OECD	Organization for Economic Cooperation and Development

Treatment codes:

B	buffer
P	protein (= *B.t.k.* HD-73 protein)
G	gastric fluid
I	intestinal fluid
W	whole incubation sample
L	low dose
M	medium dose
H	high dose

Attachment 3: Study co-investigator acknowledgement of study protocol

Study: Assessment of the *in vitro* digestive fate of *Bacillus thuringiensis* var. *kurstaki* HD-73 protein

Signature: Steven R. Sims Date: 5/25/93

Steven R. Sims
Monsanto Agricultural Group, New Products Division
(314) 537-6549

Signature: John Leach Date: 5/24/93

John N. Leach
Today's Temporary, contracted to
Monsanto Agricultural Group, New Products Division
(314) 537 7460

Monsanto
The Agricultural Group

Protocol Amendment
SOP Reference: GEN-POL-005

New Products Division - Regulatory Sciences

Study Number: 92-01-36-22

Amendment #: 1
Date change implemented: 06/17/93

Experiment's affected by this amendment:

92-427-728

Page No/s. &/or Section/s: p.6, sect. 7.4 originally stated:

For SIF, incubation termination will be by 1:1 dilution with protein-denaturing 2X SDS-PAGE sample buffer followed by heating to about 100 C for approximately 5 minutes.

This section is amended as follows:

If necessary, an alternative quench procedure will be used for SIF incubations. For this alternative procedure, the SIF incubation solution will be terminated by heating at approximately 100 C for five minutes before the addition of 2X SDS-PAGE sample buffer and heating as described originally. This alternative quench will be used if the results using the original quench procedure described suggest it is not optimal. In this case, only the BP-0, IP-0 and IP-1 treatments will be repeated using the alternative quench procedure.

Reason for amendment:

Initial results from Part I. suggest a low recovery of the full-length B.t.k.HD-73 protein from intestinal fluid as observed by comparison of the BP-0 and IP-0 treatments. There appears to be a rapid degradation of the full-length B.t.k. HD-73 protein towards the tryptic fragment during the initial assay termination steps.

This change will impact the Study in the following ways:

This change may provide data supporting a high recovery of the full-length B.t.k. HD-73 protein. This would enhance this study. The single time point incubation treatment will allow a comparison of the results using the alternative quench to those using the original quench procedure.

Signature of Approval:

Study Director: Joel E. Rean Date: 6-17-93

Signatures of Acknowledgement:

Sponsor: [signature] Date: 6/18/93
Quality Assurance: Marcia E. Strauss Date: 6/30/93
Not Applicable for this Protocol _____ Date: _____
Not Applicable for this Protocol _____ Date: _____
Not Applicable for this Protocol _____ Date: _____

CC:

Monsanto
The Agricultural Group

New Products Division - Regulatory Sciences

Protocol Amendment
SOP Reference: GEN-POL-005

Study Number: 92-01-36-22

Amendment #: 2
Date change implemented: 06/17/93

Experiment's affected by this amendment:
92-427-728

Page No/s. &/or Section/s: p.11, Attach #1, Part II originally stated:
Incubation Sample Labels:

B-0, ... I-1

B-L-0, ... I-L-1

B-M-0, ... I-M-1

B-H-0, G-H-0, G-H-1, I-M-0, I-M-1

This section is amended as follows:
Incubation Sample Labels:

B-0, ... I-1

B-L-0, ... I-L-1

B-M-0, ... I-M-1

B-H-0, G-H-0, G-H-1, I-H-0, I-H-1

Reason for amendment:
The indicated sample labels in the protocol were incorrect.

This change will impact the Study in the following ways:
No impact; correct labels were placed on appropriate samples.

Signature of Approval:

Study Director: _____ Date: 6-25-93

Signatures of Acknowledgement:

Sponsor: _____ Date: 6/29/93
Quality Assurance: _____ Date: 6/30/93
Not Applicable for this Protocol _____ Date: _____
Not Applicable for this Protocol _____ Date: _____
Not Applicable for this Protocol _____ Date: _____

CC: