UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

MEMORANDUM MAR 10 1994

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

SUBJECT: SAB Review of Mammalian Toxicity Studies Submitted by Monsanto Agricultural Company in Support of the Registration for Transgenic Potato Plants Expressing the Delta-Endotoxin from *Bacillus thuringiensis* var. *tenebrionis* (Submission No.: S450750; ID No.: 000524-UTU DP Barcode No.: D195921).

TO: Phillip Hutton/Willie Nelson (PM 18)
Insecticide-Rodenticide Branch
Registration Division (H7505C)

FROM: Cindy Schaffer, Microbiologist C. Schaffer
Biological Pesticides Section
Science Analysis Branch
Health Effects Division (H7509C)

THROUGH: Roy Sjoblad, Ph.D, Section Head
Biological Pesticides Section
Science Analysis Branch
Health Effects Division (H7509C)

AND: Bill Burnam, Branch Chief
Science Analysis Branch
Health Effects Division (H7509C)

ACTION: SAB has been asked to review the mammalian toxicology data submitted in support of the registration of Monsanto Agricultural Company's transgenic potato plants expressing the delta-endotoxin from *Bacillus thuringiensis* var. *tenebrionis* as the active ingredient.

CONCLUSION: SAB accepts the toxicology data as submitted.

DATA REVIEW RECORD

Product Name: Transgenic Potato Plants expressing the delta-endotoxin from *Bacillus thuringiensis* var. *tenebrionis*
ID No: 000524-UTU
Chemical ID: 006432
Submission No: S450750
MRID No: 429322-16 Dose Characterization.
429322-17 Acute Oral Toxicity Test in Mice.
429322-18 In-Vitro Digestibility.

(1)

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SUMMARY OF DATA SUBMITTED:

Dose Characterization: The *B.t.t.* proteins were determined to be stabile and the dosing concentrations were determined to be 74.9 mg/ml, 14.62 mg/ml and 7.4 mg/ml.
CLASSIFICATION: ACCEPTABLE

Acute Oral Toxicity (152A-10): *B.t.t.* protein was not toxic by oral gavage when mice were dosed with up to 5220 mg/kg body weight.
CLASSIFICATION: ACCEPTABLE- TOX CATEGORY IV

In-Vitro Digestibility: The 68 kD and 55 kD *B.t.t.* proteins degraded within 30 seconds in simulated gastric fluid when analyzed by western blot and were not active against Colorado potato beetles after degradation. The 68 kD *B.t.t.* protein degraded to 55 kD within 2 hours of incubation in simulated intestinal fluid. The 55 kD form remained unchanged after 14 hours of incubation and retained its bioactivity and western blot results.

DATA EVALUATION REPORT

Reviewed by: Cindy Schaffer, Microbiologist, SAB/HED
Secondary Reviewer: John Kough, Ph.D., Biologist, SAB/HED

STUDY TYPE: Dose Characterization
MRID NO: 429322-16
TEST MATERIAL: *B.t.t.* protein
SYNONYMS:
PROJECT NO: 92-01-37-11
SPONSOR: Monsanto Company, St. Louis MO
TESTING FACILITY: Monsanto Company/Agricultural Group, St. Louis Mo
TITLE OF REPORT: Colorado Potato Beetle (CPB) Active *Bacillus thuringiensis* subsp. *tenebrionis* Protein Dose Formulation, Dose Confirmation, and Dose Characterization for Albino Mice Acute Toxicity Study (ML-92-407).
AUTHOR(S): Paul B. Lavrik, David E. Bartnicki and Steve R. Sims
STUDY COMPLETED: 31 August 1993
CONCLUSION: The *B.t.t.* proteins were determined to be stable and the dosing concentrations were determined to be 74.9 mg/ml, 14.62 mg/ml and 7.4 mg/ml.
CLASSIFICATION: ACCEPTABLE

I. STUDY DESIGN

Test Material: *B.t.t.* protein, batch no.: 5192101, lot no. 5002046, was isolated and generated from *Escherichia coli* containing the plasmid pMON5450. The purity of the dosing solution was determined to be greater than 95%.

Methods: The test (*B.t.t.*) and control (bovine serum albumen, BSA) substances were administered in two equal 1 ml doses [administered 4 hours apart]. The test substance was prepared in concentrations of 78 mg/ml, 15 mg/ml and 7.5 mg/ml (equivalent to a targeted dose of 500, 1000 and 5220 mg/kg mouse body weight); while the BSA was only prepared at the highest dose, 78 mg/ml. To ascertain the concentration and stability of the test substance, BSA control and *B.t.t.* protein standards; SDS-PAGE analysis, the bicinchoninic acid (BCA) protein assay and integrated optical density (OD) methods were utilized. Biological activity of the test substance was evaluated by the Colorado potato beetle diet bioassay. Each sample was re-analyzed post gavage one week following the mouse acute toxicity study to assure stability of the test material.

II. RESULTS:

A. *B.t.t.* Concentrations:

Test material - Pre-Gavage:
The concentration of the test material proteins were determined to be 74.9 mg/ml, 14.62 mg/ml and 7.40 mg/ml based on mass weight, 73.97 mg/ml, 11.56 mg/ml and 8.09 mg/ml based on the BCA protein assay and 71.44 mg/ml, 13.77 mg/ml and 8.34 mg/ml based on OD. The target doses were 78 mg/ml, 15 mg/ml and 7.5 mg/ml, respectively.

Test material - Post Gavage:
The concentration of the test material proteins were determined to be 74.9 mg/ml, 14.62 mg/ml and 7.40 mg/ml based on mass weight, 67.41 mg/ml, 14.65 mg/ml and 7.44 mg/ml based on the BCA protein assay and 73.87 mg/ml, 15.68 mg/ml and 9.02 mg/ml based on OD. The target doses were 78 mg/ml, 15 mg/ml and 7.5 mg/ml, respectively.

BSA control - Pre-Gavage:
The concentration of the BSA control was determined to be 72.36, 71.02 and 90.71 based on the mass weight, BCA protein assay and OD, respectively. The target BSA control concentration was 75 mg/ml.

BSA control - Post-Gavage:
The concentration of the BSA control was determined to be 72.36, 60.97 and 82.66 based on the mass weight, BCA protein assay and OD, respectively. The target BSA control concentration was 75 mg/ml.

B.t.t. protein standard - Pre-Gavage:
The concentration of the protein standard was determined to be 1.22 mg/ml, 1.23 mg/ml and 1.18 mg/ml based on the mass weight, protein assay and OD, respecitively. The target *B.t.t.* protein standard was determined to be 1.26 mg/ml.

B.t.t. protein standard - Post-Gavage:
The concentration of the protein standard was determined to be 1.22 mg/ml, 1.06 mg/ml and 0.93 mg/ml based on the mass weight, protein assay and OD, respecitively. The target *B.t.t.* protein standard was determined to be 1.26 mg/ml.

B. Insect Bioassay, Colorado potato beetle LC_{50} ug/ml

The *pre-gavage* bioactivity of the *B.t.t.* proteins were determined to be 0.39 - 0.69, 0.46 - 0.71, 0.30 - 1.36, and 0.32 - 0.54 for the target concentrations of 78 mg/ml, 15 mg/ml, 7.5 mg/ml and the *B.t.t.* protein standard, respectively.

The *post-gavage* bioactivity of the *B.t.t.* proteins were determined to be 0.69 - 1.11, 0.58, 0.17 - 1.81, and 0.92 - 1.46 for the target concentrations of 78 mg/ml, 15 mg/ml, 7.5 mg/ml and the *B.t.t.* protein standard, respectively.

C. SDS-PAGE analysis:
The SDS-PAGE gel showed stability of the *B.t.t.* proteins from pre to post gavage.

III. SAB DISCUSSION:
The *B.t.t.* proteins were determined to be stable and the dosing concentrations were determined to be 74.9 mg/ml, 14.62 mg/ml and 7.4 mg/ml.

DATA EVALUATION REPORT

Reviewed by: Cindy Schaffer, Microbiologist, SAB/HED
Secondary Reviewer: John Kough, Ph.D., Biologist, SAB/HED

STUDY TYPE: Acute Oral Toxicity-Mice (152A-11)
MRID NO: 429322-17
TEST MATERIAL: *Btt* protein
SYNONYMS: Bacillus thuringiensis var. tenebrionis
PROJECT NO: 92170/ ML-92-407
SPONSOR: Monsanto Company, St. Louis, MO
TESTING FACILITY: Monsanto Environmental Health Laboratory, St. Louis, MO
TITLE OF REPORT: Acute Oral Toxicity Study of *B.t.t.* Protein in Albino Mice.
AUTHORS(S): Mark W. Naylor
STUDY COMPLETED: 2 March 1993
CONCLUSION: *B.t.t.* protein was not toxic by oral gavage when mice were dosed with up to 5000 mg/kg body weight.
CLASSIFICATION: ACCEPTABLE- TOX CATEGORY IV

I. STUDY DESIGN

Test Material: The microbial pest control agent (MPCA) is *Bacillus thuringiensis* var. *tenebrionis* (Btt) protein, approximately 90-95% pure, in a 0.05M Na Carbonate buffer solution, pH 10.5. Each test animal received two 1 ml doses, 66.66 ml/kg, four hours apart (at various concentrations, see below) by oral gavage.

Test Animals: Fifty male and fifty female CD-1 albino mice were obtained from Charles River Laboratories, Inc., Portagem NI. The males weighed between 28.3g and 34.4 g and females weights ranged from 22.6 g to 27.5 g at the beginning of the study.

Methods: The animals were assigned to one of five groupsof ten animals of each sex as follows: The MV1, FV1 groups were dosed with the carbonate buffer vehicle; the MV2, FV2 groups were dosed with 5000 mg/kg BSA + carbonate buffer at a total dose of 75 mg/ml; group T-1 (M1, F1) had a total dose of 500 mg/kg at a concentration of 7.5 mg/ml; group T-2 (M2, F2) was treated with 1000 mg/kg total dose Btt protein at a concentration of 15 mg/ml; and group T-3 (M3, F3) had a total dosage of 5220 mg/kg at a concentration of 78.3 mg/ml. The mice were randomly weighed on the day of initial dosing, day 6 and at termination (day 7 - 8). Treated animals were observed for signs of toxicity twice daily. Food consumption was noted on days 1 through 6. Male and female mice in the treatment groups were sacrificed on days 7 and 8, respecitvely. The

animals were examined by necropsy for any macroscopic abnormalities at which time samples of the aorta, adrenals, kidney, brain, liver, lungs, spleen, submaxillary and mesenteric lymph nodes, caecum, colon, duodenum, esophagus, eyes, femur with joint, gall bladder, gross lesions, heart, ileum, jejunum, muscle, ovaries, pancreas, pituitary, prostate, rectum, salivary gland, sciatic nerve, seminal vescicles, skin, spinal chord, sternum and marrow, stomach, testes with epididymus, thymus, thyroid/parathyroid, trachea, uterus and urinary bladder were collected for pathology and frozen.

II. RESULTS

A. Body Weights:

An average of 50% of the males and 78% of the females, including vehicle controls exhibited a slight loss (~0.5 g on average) in body weight during the study.

B. Clinical Observations:

No abnormalities were noted in any mouse during the study.

C. Food Consumption:

Group	*Substance*	*Target Dose*	*Average gm/day*
Vehicle Control	0.5M CO_3 buffer	66.66 ml/kg	♂ 5.87
			♀ 6.30
	BSA	5000 mg/kg	♂ 5.52
			♀ 5.68
Test Group	Btt protein	500 mg/kg	♂ 5.40
			♀ 6.39
		1000 mg/kg	♂ 5.20
			♀ 5.57
		5000 mg/kg	♂ 5.20
			♀ 7.27

D. Deaths:

One female vehicle control died day 1 of the study; and one male treated with 500 mg/kg Btt protein was found dead day 3 of the study. The registrant stated that the cause of female death was attributed to the gavage procedure.

E. Necropsy observations:

Enlarged Submaxillary Lymph Nodes: (1) MV1
(3) MV2
(2) M1
(3) M2
(3) M3
(1) FV2

Enlarged Spleen:	(1) MV2
	(1) M1
Foci on Liver:	(2) FV2
Yellow/Tan Mass on Liver:	(1) FV1
Cyst on Ovary:	(1) F1
Atrophied Ovaries:	(1) F1

F. Pathology:

Histopathology was not performed on any animal during the study.

III. SAB DISCUSSION:

No significant signs of toxicity or mortality related to dosing with the test material was observed. Enlarged submaxillary lymph nodes were noted in the BSA and Btt treated animals. The registrant stated that "This is a common, spontaneous finding in mice of this strain and neither it nor any other gross finding was considered related to treatment." SAB concurs with the registrants findings.

DATA EVALUATION REPORT

Reviewed by: Cindy Schaffer, Microbiologist, SAB/HED
Secondary Reviewer: John Kough, Ph.D., Biologist, SAB/HED

STUDY TYPE:	In-Vitro Digestibility
MRID NO:	429322-18
TEST MATERIAL:	68 kD and 55 kD *B.t.t.* proteins from *Escherichia coli* containing the plasmid pMON5450, batch No.: 5192101
PROJECT NO:	92-01-37-16
SPONSOR:	Monsanto Company, St. Louis MO
TESTING FACILITY:	Monsanto Company/Agricultural Group, St. Louis Mo
TITLE OF REPORT:	Assessment of the Metabolic Degradation of the Colorado Potato Beetle (CPB) Active Protein in Simulated Mammalian Digestive Models.
AUTHOR(S):	Pamela J. Keck, Steven R. Sims and David E. Bartnicki
STUDY COMPLETED:	31 August 1993
CONCLUSION:	The 68 kD and 55 kD *B.t.t.* proteins degraded within 30 seconds in simulated gastric fluid when analyzed by western blot and were not active against Colorado potato beetles after degradation. The 68 kD *B.t.t.* protein degraded to 55 kD within 2 hours of incubation in simulated intestinal fluid. The 55 kD form remained unchanged after 14 hours of incubation and retained its bioactivity and western blot results.
CLASSIFICATION:	ACCEPTABLE

I. STUDY DESIGN

Test Material: Two *B.t.t.* proteins were used in this study: a 68 kD protein isolated from *E. coli* containing the plasmid pMON5450 (lot number 5192101; and a trypsinized version of the 68 kD protein: a 55 kD protein, lot number 5214569. The purity of the dosing solution was determined to be greater than 95%.

Methods: Preparations of the *B.t.t.* proteins were diluted to 2μg protein/ml in simulated gastric (SGF) or simulated intestinal fluid (SIF). The SGF contained 3.2 g pepsin in 2.0 g NaCl/liter adjusted to a pH of 1.2 with HCl. The SIF contained 10 g pancreatin/liter phosphate buffer, pH 7.5. Each sample was incubated with either SGF or SIF for a distinct period of time as follows and were evaluated by western blot and bioassay with Colorado potato beetle (CPB). Six aliquots of each *B.t.t.* protein were removed from the gastric fluid at 0, 15, 30 seconds, 1, 2, and 10 minutes and

evaluated by western blot and bioassay. Since the pilot studies in the SIF demonstrated that the 68 kD protein converted to the 55 kD protein within 30 seconds and did not further degrade after an additional 16 hours in SIF, the timepoints were determined to be 0, 30 seconds, 2 and 15 minutes, 2 hours and overnight.

Samples of the SGF were neutralized by the addition of sodium carbonate to a pH of approximately 7 and placed on ice. The SIF samples were terminated by freezing on dry ice for the bioassay or by the addition of SDS-PAGE sample buffer for the western blots. The bioassay samples were prepared by spiking 1 μg *B.t.t.* protein/ml CPB diet. The level of bioactivity was evaluated by correlating the CPB mortality with *B.t.t.* alone versus the bioactivity of the SGF or SIF sample containing a 10 minute incubation with the *B.t.t.* proteins corrected for bioactivity of the SGF or SIF reagents alone.

II. RESULTS:

A. Western Blots: SGF: Both the 68 kD and 55 kD *B.t.t.* proteins rapidly degraded (within 30 seconds) in simulated gastric fluid (see attached).
SIF: The majority of the 68 kD protein degraded to 55 kD within 2 hours of incubation in simulated intestinal fluid. Additional degradation of the 68 kD band, or the resulting 55 kD band, was not seen by 14 hours post incubation. The 55 kD protein band did not degrade in SIF during the 14 hour incubation period (see attached).

B. Bioassay: SGF: Bioactivity was determined to be at background levels after ten minutes of incubation with SGF in both the 68 kD and 55 kD proteins.
SIF: Bioactivity remained unchanged when both the 68 kD and 55 kD proteins were exposed to SIF for 14 hours.

III. SAB DISCUSSION:
The study's results are consistent with the conclusion that the simulated conditions of the human gastric fluid would rapidly degrade the *Btt* protein.

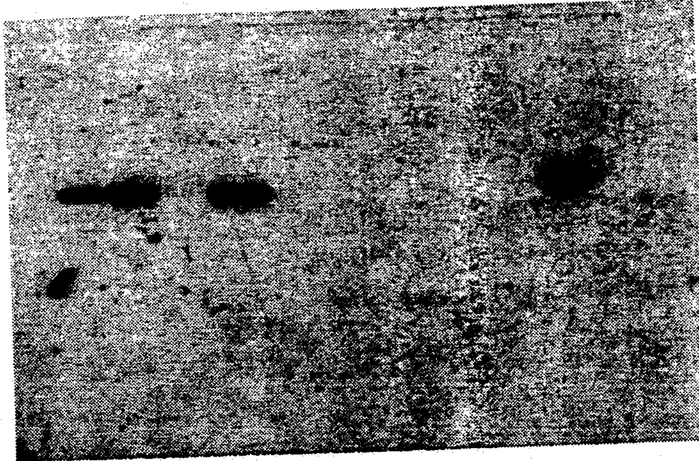

Figure 1. Western blot analysis of the 68 kD *B.t.t.* protein in simulated gastric fluid (SGF). All samples were diluted to 1 ng/well based on the initial amount of 68 kD *B.t.t.* protein/sample. One ml of SGF was spiked with 2 µg of 68 kD *B.t.t.* protein; 50 µl aliquots were removed over time and quenched with 15 µl of 0.2 M sodium carbonate, pH 11.4, and 65 µl of western sample buffer (2X Laemmli buffer) was subsequently added and the entire contents boiled for 5 minutes. This gel represents the results of one of the triplicate samples. Lane 1 is 0.5 ng of *B.t.t.* in buffer (50 mM sodium carbonate, pH 10.5), and lanes 2 and 10 are 1 ng of *B.t.t.* protein in buffer; lanes 3 and 5 are SGF alone, lane 4 is the *B.t.t.* protein added to quenched SGF, lanes 6-9 are samples of the *B.t.t.* protein after exposure to SGF for 30 seconds, 60 seconds, 2 and 10 minutes, respectively.

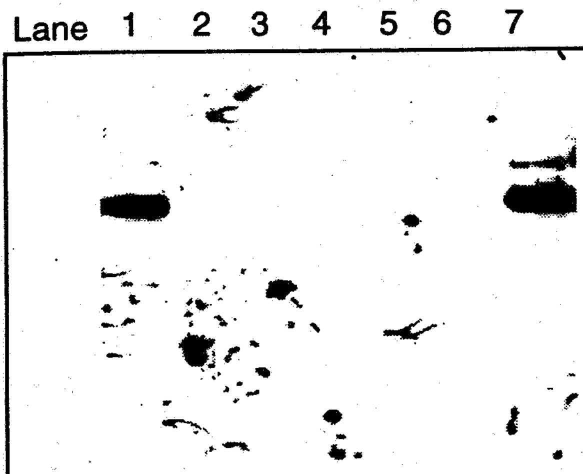

Figure 2. Western blot analysis of the 55 kD *B.t.t.* protein in simulated gastric fluid (SGF). All samples were diluted to 1 ng/well based on the initial amount of 55 kD *B.t.t.* protein/sample. One ml of SGF was spiked with 2 µg of 55 kD *B.t.t.* protein; 50 µl aliquots were removed over time and quenched with 15 µl of 0.2 M sodium carbonate, pH 11.4 and 65 µl of western sample buffer (2X Laemmli buffer) was subsequently added and the entire contents boiled for 5 minutes. This gel represents the results of one of the triplicate samples. Lanes 1 and 7 are 1 ng of 55 kD *B.t.t.* protein in quenched SGF; lane 2 is SGF alone; lanes 3-6 are samples of the 55 kD *B.t.t.* protein which were exposed to the SGF for 30 seconds, 60 seconds, 2 minutes and 10 minutes, respectively.

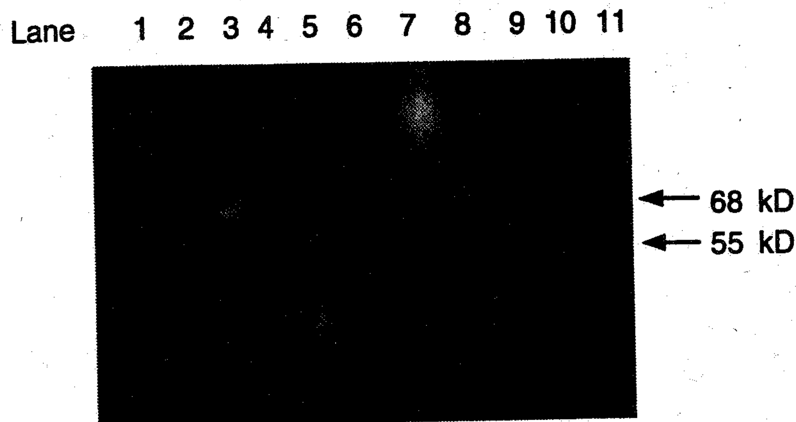

Figure 4. Western blot analysis of the 68 kD *B.t.t.* protein incubated in simulated intestinal fluid (SIF). All samples were diluted to 1 ng/well based on the initial amount of 68 kD *B.t.t.* protein/sample. One milliliter of SIF was spiked with 2 µg of 68 kD *B.t.t.* protein and 50 µl aliquots were removed and quenched with 50 µl of boiling western sample buffer (2X Laemmli buffer) and boiled again for 5 minutes. This gel represents the results seen in one of the triplicate samples. Lanes 1 and 11 are 1 ng of the 68 kD *B.t.t.* protein in buffer, lanes 2 and 3 are SIF at the initial and 14 hour timepoints, respectively; lanes 4 and 5 are samples of SIF that were quenched prior to the addition of the *B.t.t.* protein (lane 4: boiling 2X western sample buffer was added to SIF and boiled for 5 minutes and then the 68 kD *B.t.t.* protein was added; in lane 5, SIF was removed and boiled and then the 68 kD *B.t.t.* protein added followed by the addition of boiling 2X western sample buffer and the entire mixture boiled again for 5 minutes); lanes 6-10 are the samples of 68 kD protein which were exposed to SIF for 30 seconds, 2 minutes, 15 minutes, 2 hours and 14 hours, respectively.

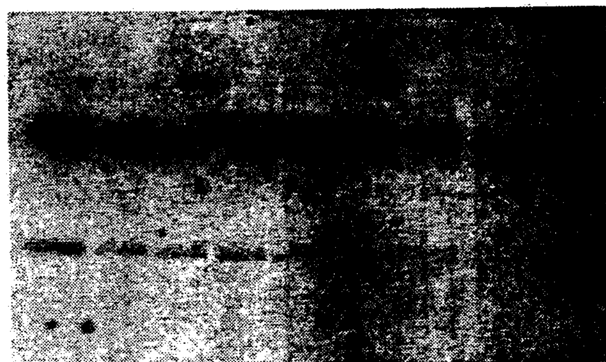

Figure 5. Western blot analysis of the 55 kD *B.t.t.* protein incubated in simulated intestinal fluid (SIF). One ml of SIF was spiked with 2 µg of 55 kD *B.t.t.* protein and 50 µl aliquots were removed and quenched with 50 µl of western sample buffer (2X Laemmli buffer) and boiled. All samples were diluted to 1 ng/well based on the initial amount of 55 kD *B.t.t.* protein/sample. This gel represents the results observed in one of the duplicate samples. Lanes 1 and 2 are 1 ng of *B.t.t.* protein in SIF that were quenched prior to the addition of the *B.t.t.* protein (lane 1 the SIF was removed and boiled, the *B.t.t.* protein added and then boiling 2X western sample buffer was added, the mixture was again boiled for 5 minutes; lane 2 the SIF was removed, 2X boiling western sample buffer added, the mixture boiled again and then the *B.t.t.* protein added) lane 3 is the 55 kD *B.t.t.* protein in 50 mM sodium carbonate, pH 10.5 buffer; lanes 4-8 are samples of 55 kD protein which were exposed to the SIF for 30 seconds, 2 minutes, 15 minutes, 2 hours and 14 hours, respectively; lane 9 is the same as lane 1. Note: the band in lane 4 appears smeared and this resulted during the transfer process indicated by the duplicate gel run for this analysis.