Friends of the Earth

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COMMENTS ON THE APPLICATION BY

PIONEER HI-BRED RSA

AND

DOW AGROSCIENCE SOUTHERN AFRICA

FOR COMMODITY CLEARANCE OF GENETICALLY MODIFIED 59122 MAIZE

TO THE NATIONAL DEPARTMENT OF AGRICULTURE, SOUTH AFRICA

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Friends of the Earth U.S. (FoE) appreciates the opportunity to offer comments for consideration by South Africa's National Department of Agriculture as it decides whether or not to grant commodity clearance to genetically modified 59122 Bt maize by Dow and Pioneer.

Friends of the Earth has taken an active interest in the human health assessment of "plant-incorporated pesticides" (PIPs) since contamination of the world's food supply by StarLink corn in 2000-2001. We submitted extensive comments to two Scientific Advisory Panels (SAPs) advising the U.S. Environmental Protection Agency (EPA) on the StarLink corn episode, and to the EPA concerning the re-registration of Bt crops in the U.S. in 2001. Our comments on the re-registration dealt in great detail with the allergenicity assessment of Cry proteins, in particular their digestive stability,[1] an area in which we have developed considerable expertise.

As you may know, the U.S. EPA is currently considering an application from Dow/Pioneer to register the Cry34/35 insecticidal proteins that are expressed in Herculex RW in the U.S.[2] The following comments are adapted from testimony we gave to a Scientific Advisory Panel convened by the EPA to address troubling evidence concerning the digestive stability of Cry34Ab1.[3] Digestive stability is considered by allergists to be a prime indicator of allergenicity (though no single parameter is predictive).

Summary Comments:

The Cry34Ab1 protein produced by Dow/Pioneer's Herculex RW (maize 59122) has a medium to high likelihood of causing food allergies in South African consumers. Friends of the Earth reaches this conclusion based on the following evidence:

1) There is strong evidence that Bt insecticidal toxins in general are allergenic; thus, Cry34Ab1 comes from a likely allergenic source;

2) Cry34Ab1 possesses "moderate digestive stability" in *in vitro* tests conducted by Dow/Pioneer; under the more gut-similar conditions stipulated by international allergy experts for such *in vitro* tests, Cry34Ab1 would be seen to possess ***great*** digestive stability approaching that of StarLink corn's Cry9C;

3) Dow/Pioneer did not use internationally accepted test protocols to assess whether Cry34Ab1 has structural similarity to known allergens, another key test for allergenicity, while it is known that other Cry proteins do possess such similarity;

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[1] FREESE, B. (2001). A Critique of the EPA's Decision to Reregister *Bt* Crops and an Examination of the Potential Allergenicity of *Bt* Proteins. Adapted from Comments of Friends of the Earth to the EPA, Docket No. OOP-00678B, Dec. 9, 2001. http://www.foe.org/safefood/comments.pdf

[2] As you may know, under the U.S. regulatory framework, the EPA has jurisdiction over plants genetically engineered to produce incorporated pesticides. Interestingly, the EPA registers and, strictly speaking, regulates only the plant-incorporated pesticide, not the whole plant.

[3] "Scientific Issues Associated with the Human Health Assessment of the Cry34Ab1 Protein," FIFRA Scientific Advisory Panel to the EPA, March 1st & 2nd, 2005. Docket Number: OPP-2004-0395. http://www.epa.gov/scipoly/sap/.

4) Dow/Pioneer did not measure the degree of ***breakdown***, if any, of Cry34Ab1 upon exposure to heat (but rather only "inactivation"); heat stability is considered another indicator of allergenicity;

5) Maize is a staple in the diet of many South Africans, who will therefore be exposed to much higher levels of Cry34Ab1 than consumers in the U.S. or elsewhere, raising the likelihood of allergic sensitization and reaction to this protein.

Recommendation:

Herculex RW is not approved for commercial cultivation or consumption anywhere in the world, and may never be. Because of the strong suggestive evidence of allergenicity cited above, and Dow/Pioneer's failure to follow internationally accepted test protocols to better answer the allergenicity question, commodity clearance of Herculex RW would pose an unnecessary and completely avoidable risk to the health of South Africans.

We therefore urge the National Department of Agriculture to reject Dow/Pioneer's bid for commodity clearance of maize 59122. At the very least, we urge the Department to defer a decision pending completion of its own independent safety assessment of maize 59122 conducted in accordance with the internationally accepted protocols of a 2001 expert consultion of the Food and Agriculture and World Health Organizations.[4] These protocols, besides representing the best thinking of the world's leading allergists, has been endorsed by Kraft Foods, the largest food company in the United States.[5]

Comments:

Before addressing issues specific to human health assessments of PIPs (Section II) and Cry34Ab1 (section III), we will first discuss the larger context and history of EPA regulation in this area.

I. Inadequacies in the EPA's Regulatory Framework[6]

1) Inappropriate statutory authority: Under the Coordinated Framework for Biotechnology, the EPA regulates "plant-incorporated pesticides" under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA). FIFRA is a statute designed for chemicals, and is ill-suited to the very different situation presented by plant genetic engineering. Chemical insecticides are applied externally and can be washed off whole food, PIPs are embedded in the plant matrix and cannot. Insecticides are discrete, stable and readily quantifiable chemical compounds, while PIPs, as products of living organisms, are subject to variation in level and modification

[4] FAO-WHO (2001). "Evaluation of Allergenicity of Genetically Modified Foods," Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology, Jan. 22-25, 2001. http://www.fao.org/es/ESN/food/pdf/allergygm.pdf

[5] See http://www.kraft.com/responsibility/quality_food_biotechnology.aspx.

[6] For a fuller discussion, see Freese and Schubert (2004). "Safety Testing and Regulation of Genetically Engineered Foods," *Biotechnology and Genetic Engineering Review,* Vol. 21, Nov. 2004.

by cellular processes. Insecticides do not propagate themselves, while PIP-generating transgenes can, through cross-pollination or seed dispersal. Most importantly, plants to which insecticides are applied are not normally subject to unintended changes in composition, while the crude, highly mutagenic transformation procedures[7] used to splice pesticidal genes into plants can always be expected to change the plant's makeup in unpredictable ways. In short, FIFRA forces the false view of transgenic crops as equivalent to the conventional parent crop plus the transgenic additives. Accordingly, EPA explicitly disavows authority over any aspects of the GE crop beyond its incorporated pesticide, including any unintended effects of transformation (which are supposedly, but far from adequately, addressed by FDA).[8]

One sign of the inadequacy of this inappropriate statutory framework is indicated by the belated discovery by academic scientists, five years after commercialization, that hybrids derived from several Bt corn events exhibit markedly increased lignin levels in stalk tissue.[9] Both EPA and FDA missed this. This and other unintended effects discovered in transgenic crops[10] suggest the need for metabolic profiling or whole-food animal feeding trials rather than an exclusive focus on the transgenic protein. FoE believes that the failure of regulatory agencies to consider, much less address, such issues is attributable in part to the inappropriate, chemical-oriented framework of current statutes, which have no place for them.

2) Surrogate proteins used in testing: Biotechnology companies rarely test the transgenic protein actually produced in their engineered crops. Instead, they make use of bacterially-generated surrogate proteins that may differ in important respects from the plant-produced one. These surrogates are generated by transforming a bacterium (usually *E. coli*) with the same genetic construct used to engineer the plant. Due to frequent fragmentation of genetic constructs in the plant GE process, yielding truncated genes/protein products and fusion proteins, as well as differences in post-translational processing between plant and bacteria, the plant-produced protein will almost always differ from the bacterial surrogate, and the results of tests conducted on the latter may not reflect the toxicology of the plant-produced protein that is consumed. A National Academy of Sciences committee that conducted an exhaustive review of Bt crops recommended that: "Tests should preferably be conducted with the protein as produced in the plant." If surrogates are nonetheless used: "The EPA should provide clear, scientifically justifiable criteria for establishing the biochemical and functional equivalency when registrants request permission to test non-plant-expressed

[7] For a comprehensive review, see: Wilson, A. et al (2004). "Genome Scrambling – Myth or Reality? Transformation-Induced Mutations in Transgenic Crop Plants," EcoNexus, Technical Report, Oct. 2004, http://www.econexus.info

[8] EPA PIP (2001). Regulations under the Federal Insecticide, Fungicide, and Rodenticide Act for Plant-Incorporated Protectants (Formerly Plant-Pesticides). *Federal Register*, Vol. 66, No. 139, July 19, 2001, 37771 – 37817. http://www.epa.gov/scipoly/pips.htm

[9] SAXENA, D. AND STOTZKY, G. (2001). *Bt* Corn Has a Higher Lignin Content than Non-*Bt* Corn. *American Journal of Botany* **88**(9), 1704-1706.

[10] KUIPER, H.A.., KLETER, G.A., NOTEBORN, H..P,J.M., KOK, E.J. (2001). Assessment of the food safety issues related to genetically modified foods. *The Plant Journal* **27**(6), 503-528; HASLBERGER, A.G. (2003). Codex guidelines for GM foods include the analysis of unintended effects. *Nature Biotechnology* **21**(7) , 739-741.

proteins in lieu of plant-expressed proteins."[11] Four years later, and the EPA has still failed to do this, even though its scientific advisers have proposed such 'test substance equivalence' criteria.[12] Corporate test substance equivalence studies conducted thus far for currently registered Bt crop PIPs have not met the SAP's criteria.[13] We have not had the opportunity to determine whether the Cry34/35 proteins used by Dow in its digestive stability and other tests are bacterial surrogates, or if so, whether they meet the SAP's test-substance equivalence standards.

3) Limited range of endpoints: The EPA considers only the potential of the novel PIP (or its bacterial surrogate) to be acutely toxic or allergenic (see 4 below). Yet proteins can have numerous other effects that require evaluation.[14] For instance, proteins can be anti-nutrients, like avidin, which binds biotin and thus causes vitamin B deficiency upon chronic oral exposure. Proteins like lactoferrin have immunomodulatory activity. Proteins like lysozyme and lactoferrin have bactericidal properties in some situations, while lactoferrin may actually promote the growth of certain pathogenic bacteria by supplying them with needed iron in others.[15] Improperly folded proteins are implicated in brain-wasting prion diseases, and are even thought to be the actual infectious agent. Transgenic proteins that differ in subtle respects from the "same" protein in its native version can elicit destructive immune system responses, as is thought to be the case with recombinant human erythropoietin generated in certain E. coli systems, which is implicated in over 100 cases of red blood cell aplasia.[16] Small peptide breakdown products of proteins have been shown to have teratogenic and other effects, as have unusual amino acid analogs. Clearly, the FDA needs to broaden its range of endpoints beyond toxicity and allergenicity.

4) No data requirements: Despite release of its Plant-Incorporated Protectant rule in 2001, the EPA has still failed to establish data requirements specific to PIPs. Applicants are referred to a decade-old guidance (1994) that devotes just four short paragraphs to testing for human health effects.[17] This Statement of Policy merely recommends acute oral toxicity tests in rodents and *in vitro* digestibility tests on the plant pesticide. No other endpoints or decision criteria are mentioned. Protocols are not specified even for these two recommended tests.

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[11] *Genetically Modified Pest-Protected Plants: Science and Regulation*. Committee on Genetically Modified Pest-Protected Plants, National Research Council, National Academy of Sciences, Washington, DC: National Academy Press, 2000. http://books.nap.edu/catalog/9795.html

[12] "Mammalian Toxicity Assessment Guidelines for Protein Plant Pesticides," FIFRA Scientific Advisory Panel to the EPA, SAP Report No. 2000-03B, September 28, 2000.

[13] Freese (2001), op. cit.

[14] For a general discussion, see "Mammalian Toxicity Assessment Guidelines for Protein Plant Pesticides," FIFRA Scientific Advisory Panel to the EPA, SAP Report No. 2000-03B, September 28, 2000.

[15] Recombinant avidin has been generated in corn, recombinant lactoferrin and lysozyme in rice. See Freese, B., Hansen, M., Gurian-Sherman, D. (2004). "Pharmaceutical Rice in California," Friends of the Earth, Center for Food Safety, Consumers Union, Environment California. http://www.foe.org/camps/comm/safefood/biopharm/index.html; Weinberg, E.D. (2001). "Human lactoferrin: a novel therapeutic with broad spectrum potential," *J. Pharmacy & Pharmacology* 53(10), pp. 1303-10. http://munstermom.tripod.com/HumanLactoferrin2001.htm.

[16] Freese, B (2003). "Comments on draft guidance for industry: Drugs, biologics and medical devices derived from bioengineered plants for use in humans and animals," Friends of the Earth, Jan. 2003, pp. 23-25. http://www.foe.org/biopharm/commentsguidance.pdf

[17] EPA STATEMENT OF POLICY (1994). Proposed Policy; Plant-Pesticides Subject to the Federal Insecticide, Fungicide, and Rodenticide Act and the Federal Food, Drug, and Cosmetic Act, *Federal Register*, Vol. 59, No. 225, November 23, 1994. http://www.pestlaw.com/x/fedreg/1994/EPA-19941123A.html

II. Past Human Health Assessments of Bt Crops

The first versions of Bt potatoes, corn and cotton were introduced in 1995 and 1996. EPA re-registered most of these Bt crop PIPs in 2001, plus one new corn PIP (Cry1F). As discussed above, EPA's 1994 guidance recommends only acute toxicity and digestive stability tests. In the context of the re-registration review process in 2001, EPA ostensibly expanded the allergenicity assessment to include tests on PIPs for heat stability and amino acid homology to known allergens. However, these additional data were for the most part not collected from PIP registrants prior to re-registration of Bt corn PIPs (for 7 years) and a Bt cotton PIP (5 years) in 2001. In addition, the EPA largely failed to consider suggestive evidence of PIP allergenicity that either appeared in published studies or was unearthed from unpublished corporate submissions in the period from 1996 to 2001. Appendix 1 summarizes the available data as of December 2001.

III. Allergenicity Assessment of Cry34Ab1

EPA's stated criteria for its allergenicity assessment of Cry34Ab1 are: 1) Whether source of the gene is associated with allergic reactions; 2) amino acid sequence comparison to known allergens; 3) heat stability; 4) digestive stability; 5) glycosylation; 6) prevalence in food; and 7) specific serum screening.[18]

1) Allergenicity of source: In its position paper, EPA states that "*Bacillus thuringiensis* is not considered to be an allergenic source." Yet allergic symptoms including allergic rhinitis, angioedema, dermatitis, pruritus, swelling, erythema with conjunctival injection, exacerbations of asthma, angioedema and rash have been reported in farm workers and others exposed to *Bt* spraying operations.[19] Bernstein *et al.* demonstrated that purified Cry protein extracts of *Bt* microbial pesticides containing Cry1Ab and Cry1Ac elicited positive skin tests and IgE antibody responses in two farm workers exposed to these toxins by the inhalational, dermal and possibly oral routes. Positive skin tests and the presence of IgE antibodies in serum are considered indicators of allergenicity. Though Bernstein *et al.* did not observe allergic reactions in these workers, they note that the workers were tested after only 1 to 4 months of exposure, and that "clinical symptoms would not be anticipated unless there was repeated long-term exposure…" In addition, they note that the "healthy worker effect" might have skewed their results – that is, susceptible farm workers might have associated their allergic symptoms with *Bt*, sought other employment to avoid exposure, and hence not been included in their study.

   EPA is aware of this study. Scientific advisers to the Agency recommended use of the reagents developed by Bernstein et al to be used for skin testing and serologic evaluation of Bt

[18] For EPA's position paper, see: http://www.epa.gov/scipoly/sap/2005/march/positionpaper.pdf.
[19] Bernstein et al (1999). "Immune responses in farm workers after exposure to Bacillus thuringiensis pesticides," Environmental Health Perspectives 107(7), pp. 575-82.

protein exposed individuals.[20] There is also a series of studies by Vazquez et al documenting immunogenic, though not allergic, responses to Cry1Ac, a version of which is used in Bt cotton, and which is very similar to the Cry1Ab in the major Bt corn events.[21] Thus, EPA's assumption that Bt is not allergenic is not supported by the evidence, and is weakened by the Agency's failure to follow up on suggestive evidence of allergenicity.

2) Amino acid homology to known allergens: EPA reports that Dow submitted a study that showed no overall sequence similarities or homology at the level of 8 contiguous amino acid residues to known allergens. This choice of 8-AA sequences was recommended in 1996.[22] Since then, a number of refinements and alterations have been recommended: for instance, allowance of substitution of chemically similar amino acids in the 8-AA sequence,[23] and comparisons based on identity of 6 rather than 8 contiguous amino acids (FAO-WHO 2001). It would seem advisable to conduct another study following the FAO-WHO protocol, particularly in view of the suggestive evidence of allergenicity of Bt spore preparations described above.

3) Heat stability: EPA states that Dow and Pioneer have submitted data indicating that Cry34Ab1 is "inactivated by heat." This presumably refers to loss of insecticidal activity as measured by bioassay in target insect species. The problem with using insecticidal activity as the parameter of heat stability is the implicit assumption that the insecticidal mode of action is relevant to potential allergenicity, and that loss of insecticidal activity somehow correlates with loss of allergenic potential. This assumption does not appear to be warranted, since it is the size of the breakdown fragments, not (loss of) insecticidal activity, which is of interest for allergenic potential. Loss of insecticidal activity could involve nothing more than (partial) denaturation, with little or no breakdown of the protein's primary amino acid structure. This probably explains why a background paper to FAO-WHO 2001 recommends techniques (HPLC, SDS-PAGE) to directly measure the size of fragments resulting from the heating process, and does not mention bioassays at all.[24] The potential relevance of this issue

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[20] "Bt Plant-Pesticides Risk and Benefits Assessments," FIFRA Scientific Advisory Panel to the EPA, March 12, 2001, p. 76. Available at: http://www.epa.gov/scipoly/sap/2000/october/octoberfinal.pdf

[21] VÁZQUEZ-PADRÓN, R.I., MORENO-FIERROS, L., NERI-BAZÁN, L., DE LA RIVA, G.A. AND LÓPEZ-REVILLA, R. (1999a). Intragastric and intraperitoneal administration of Cry1Ac protoxin from *Bacillus thuringiensis* induces systemic and mucosal antibody responses in mice. *Life Sciences* **64**(21), 1897-1912; VÁZQUEZ-PADRÓN, R.I., MORENO-FIERROS, L., NERI-BAZÁN, L., DE LA RIVA, G.A. AND LÓPEZ-REVILLA, R. (2000a). Characterization of the mucosal and systemic immune response induced by Cry1Ac protein from *Bacillus thuringiensis* HD 73 in mice. *Brazilian Journal of Medical and Biological Research* **33**, 147-155; VÁZQUEZ, R.I., MORENO-FIERROS, L., NERI-BAZÁN, L., DE LA RIVA, G.A. AND LÓPEZ-REVILLA, R. (1999b). *Bacillus thuringiensis* Cry1Ac protoxin is a potent systemic and mucosal adjuvant. *Scandinavian Journal of Immunology* **49**, 578-584; VÁZQUEZ, R.I., GONZALES-CABRERA, J., GARCIA-TOVAR, C., NERI-BAZÁN, L., LÓPEZ-REVILLA, R., HERNANDEZ, M., MORENO-FIERRO, L. AND DE LA RIVA, G.A. (2000b). Cry1Ac protoxin from *Bacillus thuringiensis* sp. *kurstaki* HD73 binds to surface proteins in the mouse small intestine. *Biochemical and Biophysical Research Communications* **271**, 54-58.

[22] METCALFE, D.D., ASTWOOD, J.D., TOWNSEND, R., SAMPSON, H.A., TAYLOR, S.L. AND FUCHS, R.L. (1996). Assessment of the Allergenic Potential of Foods Derived from Genetically Engineered Crop Plants. *Critical Reviews in Food Science and Nutrition* **36**(S), S165-186.

[23] Gendel (1998). "The use of amino acid sequence alignments to assess potential allergenicity of proteins used in genetically modified foods," Advances in Food and Nutrition Research 42, pp. 45-62; "Mammalian Toxicity Assessment Guidelines for Protein Plant Pesticides," FIFRA Scientific Advisory Panel to the EPA, SAP Report No. 2000-03B, September 28, 2000.

[24] Helm, Ricki M. (2001). "Stability of known allergens (digestive and heat stability)," Working Paper Biotech 01/07 for the Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology, January 22-25, 2001.

and the need for proper assessment of Cry34Ab1's heat stability based on breakdown fragment size rather than loss of insecticidal activity is indicated by heat stability tests conducted on Cry1Ab and Cry9C that employed SDS-PAGE, which revealed that both proteins possessed significant thermostability.[25]

4) Digestive stability: Both *in vivo* and *in vitro* test systems have been suggested, though simple *in vitro* tests are far more common in practice. A recent Scientific Advisory Panel recommends use of both methods as part of a decision-tree approach: "…the stability of introduced Bt-pesticidal gene products in the gastrointestinal tract should be tested by *in vitro* simulation of gastric and intestinal digestion and *in vivo*."[26]

   Test systems which attempt to mimic physiological conditions much more closely than simple *in vitro* tests in acidic pepsin solutions have also been proposed. For instance, Minekus et al.[27] have developed a model that "simulates to a high degree the physiology of the stomach and small intestine of monogastric animals and man."[28] This model takes account of factors such as temperature, pH, saliva, gastric and intestinal secretions (electrolytes, enzymes, co-factors, bile, and pancreatic juice), as well as gastric and intestinal mixing. "The model was developed as an alternative for human and animal experiments and validated successfully in comparison to *in vivo* experiments with human volunteers and fistulated pigs and calves for the digestion of proteins."[29]

   If the EPA nonetheless chooses to base its assessments only on simple *in vitro* pepsin tests, then it would seem advisable that they be designed to be more representative of the range of human gastric conditions, for instance taking account of the antacid effect of food. Human gastric pH is typically 1 – 2 under fasting conditions, rising to a value of over 5 during a meal; this variation is augmented by considerable variation among individuals and in a given person over time.[30] A working paper for the FAO-WHO 2001 expert consultation recommends several pepsin tests at pH values of "1.0, 1.5, 2.0, 4.0 and 6.0 due to the pH variation in the stomach following a meal."[31] It should be noted that all but one of the digestive stability tests listed in EPA's table were conducted at pH values of 1.0 to 1.5, more characteristic of fasting pH. The use of such acidic conditions for digestive stability testing of novel proteins has been criticized by a leading expert on Bt proteins and GM crop safety testing, Dr. Hubert Noteborn: "The continual setting of the pH value of 1.2 does not mimic accurately the kinetics of the physiological events in the human stomach."[32]

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[25] Noteborn, H. (1998). "Assessment of the Stability to Digestion and Bioavailability of the LYS Mutant Cry9C Protein from Bacillus thuringiensis serovar tolworthi," submitted to the EPA by AgrEvo, EPA MRID No. 447343-05.

[26] EPA Scientific Advisory Panel, "Bt Plant-Pesticides Risk and Benefits Assessments," March 12, 2001, p. 75. Available at: http://www.epa.gov/scipoly/sap/2000/october/octoberfinal.pdf

[27] Minekus, M. et al (1995). "A multicompartmental dynamic computer-controlled model simulating the stomach and small intestine," ALTA 23: 197-209. http://altweb.jhsph.edu/publications/journals/atla/atla23_2/atla23_2b.htm See also: http://www.pharma.tno.nl/Product.cfm?PShID=372&DivID=7

[28] See Helm (2001), op. cit., p. 6

[29] Ibid., p. 6

[30] Thomas, K. et al (2004). "A multi-laboratory evaluation of a common in vitro pepsin digestion assay protocol used in assessing the safety of novel proteins," *Regulatory Toxicology and Pharmacology* 39: 87-98.

[31] Helm (2001), p. 10.

[32] Dr. Hubert Noteborn, SAP member, as quoted in the transcript to: "Assessment of Scientific Information Concerning StarLink Corn," FIFRA Scientific Advisory Panel, SAP Report No. 2000-06, December 1, 2000. p. 399.

Both of Dow's tests on Cry34Ab1 were conducted at this unrepresentative pH = 1.2. Would milder pH values change the reported results of roughly 10 to 30 minutes' disappearance time? Thomas et al (2004) (cited above) report "no appreciable difference" in pepsin digestion times at pH = 1.2 versus pH = 2.0 for a group of allergens and non-allergens. Interestingly, however, this study included only one transgenic protein (PAT, well-known to be readily digestible, and even this was a bacterial surrogate). Surprisingly, not a single Cry protein was included, even though the digestion assay protocol being evaluated is specifically intended for use in testing recombinant proteins in genetically modified crops. Such transgenic proteins could certainly have been obtained, as the majority of the paper's authors are affiliated with agricultural biotechnology companies.

Of possibly more relevance to the case of Cry34Ab1, and EPA assessment of Cry proteins in general, are data sets on the digestive stability of two other Cry proteins: Cry1Ab and Cry9C. Cry1Ab is the most common transgenic protein in Bt corn; different versions are generated by Monsanto's MON810 and Syngenta's Bt11 events, while an earlier version no longer registered in the US, Event176, also produced Cry1Ab. Cry9C is the insecticidal toxin produced in StarLink corn, which is also no longer registered.

Consider the test results for Cry1Ab listed by the EPA in its position paper, reproduced below, plus those for two studies the Agency left out:

Protein	MRID	pH	Pepsin : Protein ratio (w/w)	Disappearance time (Western blot)
Cry1Ab	433236-06	1.0-1.2	3 : 1	< 2 min.
Cry1Ab	433236-06	1.0-1.2	0.007 : 1	< 5 min.
Cry1Ab (tryptic core)	434392-01	1.2	1600 : 1	2 min. (> 90% degraded)
Cry1Ab	451144-01	1.5	15 : 1	< 15 min.
Cry1Ab5	447343-05	2.0	20 : 1	60-120 min.[33] (90% degraded)

The three tests at pH = 1.0 – 1.2 differed by more than five orders of magnitude in pepsin : protein ratio, yet the disappearance times vary by only several-fold. Even if only the first two test results are considered (conducted as part of the same study, and so presumably comparable), the effect of pepsin : protein ratio is small. A fourth test, conducted with more pepsin than either of the first two but at the milder pH of 1.5, yielded a 3-7 fold longer disappearance time of < 15 minutes. A fifth test, conducted at pH = 2.0 rather than pH = 1.5 and at a slightly greater pepsin : protein ratio than the fourth test, yielded results indicating moderately greater stability. Even if not precisely comparable, these test results show clearly that Cry1Ab stability increases with pH value, while it bears much less relation to pepsin : protein ratio.

[33] Noteborn (1998), op. cit. The percentage of the parent band remaining over time, as measured by scanning densitometer, was as follows: 2 min.: 41% remaining; 15 min.: 21%; 30 min.: 21%; 60 min.: 11%; 120 min.: 9%.

Finally, in a study of cattle fed Bt corn (Event 176, Cry1Ab) for 4 weeks, ELISA tests of samples from various parts of the GI tract revealed "remarkable amounts of Bt toxin," and the protein was detected in the faeces as well.[34]

Cry9C digestion assay results reported by the EPA, plus one study the Agency left out, are reproduced below:

Protein	MRID	pH	Pepsin : protein ratio (w/w)	Disappearance time (Western blot)
Cry9C	451144-02	1.2	16 : 1	< 1 hour
Cry9C	451144-01	1.5	15 : 1	< 1 hour
Cry9C	451144-02	2.0	16 : 1	No degradation after 4 hours
Cry9C	447343-05	2.0	20 : 1	78% remaining after 2 hours
Cry9C	442581-08	2.0	not given	No degradation after 4 hours

While Cry9C is digestible within one hour at either pH 1.2 or 1.5, it remains largely or completely undigested at 2 – 4 hours. Note that the pepsin : protein ratio was nearly the same for the first two tests at the lower pH values and two of the tests conducted at pH = 2.0. As for Cry1Ab, pH appears to be the controlling factor influencing the digestibility of Cry9C. Aventis agrees: "The stability of the Cry9C protein in SGF is extremely sensitive to pH. Acid pH of 1.2 produces digestion of the Cry9C protein, when incubated with pepsin, in 30-60 minutes. No digestion of Cry9C is observed in SGF at pH = 2.0."[35]

The digestive stability tests reported by the EPA for Cry34Ab1 indicated "moderate stability" even at pH = 1.2. It remains an open question whether Cry34Ab1 would exhibit greater stability at pH = 2.0, but the results for Cry1Ab and Cry9C at least suggest this possibility. In any case, at pH = 1.2, Cry34Ab1's disappearance time (20-30 minutes) is quite similar to that of Cry9C's (30-60 minutes). If the 60:1 pepsin : protein ratio used in Dow's test were dropped to the 15:1 ratio used for Cry9C, it might resemble the latter still more. EPA should have Dow repeat the assay at pH = 2.0 and a pepsin : protein ratio of 1.3:1 (in accordance with the FAO-WHO 2001 protocol) to gain a better idea of how the protein behaves at a pH value at least somewhat more representative of the range of human gut acidity.

EPA criticizes the FAO-WHO 2001 protocol on the grounds that it "has not been tested, so it is unknown whether or not a correlation between allergenicity and digestibility would be observed with this protocol." The assumption seems to be that the Agency's past assessments and/or Dow's kinetic approach ***are*** based on such an established correlation. This is not the case. As we have seen, all of the EPA's allergenicity assessments of PIPs have been made on the basis of digestibility tests conducted under varying combinations of

[34] Einspanier, R., Lutz, B., Rief, S., Berezina, O., Zverlov, V., Schwartz, W., Mayer, J. (2004). "Tracing residual recombinant feed molecules during digestion and rumen bacterial diversity in cattle fed transgene maize," *European Food Research and Technology,* 218(3): 269-73.

[35] van der Klits, R-J (2000). "Comparison of the *in vitro* digestibility based upon pH of the endotoxin Cry9C derived from *Escherichia coli* and *Bacillus thuringiensis,* Aventis CropScience, MRID 451144-02.

pH, pepsin : test protein ratio, and probably other factors as well. On the other hand, Dow's proposed kinetic approach is novel, and can in no way be considered "validated" on the basis of merely two "comparison studies using a number of allergens and non-allergens." One of these comparison studies was conducted with a 20-fold lesser pepsin : test protein ratio (3:1 (w/w)) than the studies on Cry341Ab (60:1 (w/w)), leading the EPA to conclude that this study "did not allow comparison with the previously submitted digestion study on Cry34Ab1." While the second comparison study ***was*** conducted under the same conditions as the Cry34Ab1 test, "a strong correlation between digestion rate and allergenicity was not observed for the set of proteins tested…"

In any case, as argued above, the primary purpose of a digestibility assay should be to simulate human gut conditions so as to provide a measure, however crude, of the dwell time of the protein and sizeable fragments in the human GI tract, not establish some sort of abstract correlation between digestibility measured under conditions not typical of the human gut and the allergenicity of selected allergens.

The EPA's position paper implies that Dow's digestive stability tests measured breakdown of both the whole Cry34Ab1 protein and any digestion fragments, but the EPA did not specify a minimal fragment size. FAO-WHO 2001 advises that a fragment as small as 3.5 kD could be allergenic. Thus, "disappearance" should be clearly defined and assessed as breakdown to fragments < 3.5 kD. The potential relevance of this issue is indicated by a pepsin digestion study on Cry1Ab (not cited above), which found that the protein was degraded only to 15 kD fragments after 2 hours at pH = 2.[36]

4) Prevalence: While it is true that Cry34/35 is present in Dow/Pioneer's corn at levels lower than is typical of many food allergens, FAO-WHO 2001 states that "level of expression cannot yet be incorporated into the assessment of the allergenicity of genetically modified foods" because "…allergens can sensitize susceptible individuals at less than milligram levels, possibly at less than microgram levels."

The available evidence suggests that Cry34Ab1 comes from a potentially allergenic source and possesses substantial digestive stability approaching that of Cry9C. While amino acid homology comparisons have not turned up matching sequences to known allergens, the protocol employed was based on a allergenicity testing scheme dating back to 1996, and since that time refinements and alterations have been suggested to increase its sensitivity; the test should be repeated according to FAO-WHO 2001 standards, with consideration given to allowing substitution of biochemically similar amino acids, as suggested by FDA scientist Steven Gendel. Likewise, it is still not clear whether or to what extent heat treatment breaks down the Cry34Ab1 protein due to the apparent failure to measure breakdown size with appropriate test procedures.

On balance, FoE disagrees with EPA's preliminary assessment that Cry34Ab1 is unlikely to be an allergen, and believes there is not adequate evidence to conclude with a "reasonable certainty" that Cry34/35 corn will not cause harm if introduced into the commercial food supply. Thus,

[36] Noteborn et al (1995). "Safety assessment of the *Bacillus thuringiensis* insecticidal crystal protein CRYIA(b) expressed in transgenic tomatoes," in Engel, et al (eds.), American Chemical Society Symposium Series 605, Washington, DC, pp. 134-47; see Freese (2001), op. cit. for analysis.

based on the available evidence, we urge the EPA to reject Dow/Pioneer's petition to register Cry34Ab1 as a PIP.

IV. Conclusion

While continued research to find better means to evaluate novel proteins for potential allergenicity is certainly desirable, the fact is that Bt crops are being widely grown and consumed right now, and new varieties like Dow/Pioneer's are in the pipeline. Thus, there is a pressing need to apply the best available testing scheme to protect public health. Given the lack of predictive parameters of allergenicity, we believe a cautious approach is called for, one which errors (since errors are inevitable with the current state of knowledge) on the side of protecting public health rather than the commercial interests of PIP applicants.

FoE sees many compelling reasons for the EPA to require plant-incorporated pesticide applicants to follow ***single, standardized protocols*** for all tests relating to the human health assessments of PIPs, including digestive stability. Such standardized protocols would ensure equable treatment of all applicants and permit collection of comparable data that should be of great use in future refinements to allergenicity assessments of novel proteins. Such refinements, however, should not be introduced on an *ad hoc* basis, in response to an individual applicant's desire to register a particular product, but rather only as the result of a considered assessment of a wide range of data as well as past protocols. With respect to digestive stability, in the long-term we would like to see more sophisticated testing methodologies, such as those cited above, which make a real effort to simulate human GI conditions. Until then, we support the FAO-WHO 2001 protocol.

FoE recommends that EPA adopt the FAO-WHO 2001 allergenicity assessment protocol as ***prescriptive*** for all applicants for several reasons:

1) It represents the best thinking of leading allergy experts, who took pains to consider and refine the previous allergenicity assessment protocols developed over the past decade.

2) It is the only allergenicity assessment scheme that provides the detailed test protocols required for a badly needed standardized approach to allergenicity assessments. In particular, we support the specification of pH = 2 for digestive stability testing as better reflective of the range of human gastric pH values (e.g. following consumption of food) rather than the extremely acidic pH = 1.2 (fasting) used by Dow.

3) FAO-WHO 2001 was agreed to by an *international* panel of experts, and as such answers to the frequent demand of biotechnology companies for international harmonization of biotech-related regulatory standards. The fact that certain interests might not like this particular internationally accepted protocol should be no argument against it.

4) Finally, there is a broad movement in both American society and the American food industry towards healthier, safer foods and more stringent measures to ensure food safety. One sign of this is the recent endorsement of the FAO-WHO 2001 allergenicity assessment protocol by Kraft Foods, America's largest food company.
(See http://www.kraft.com/responsibility/quality_food_biotechnology.aspx.)

Appendix 1

In October of 2001, the EPA re-registered the entire class of Bt crops: 3 varieties of corn and one of cotton (Bt potatoes were originally given an unlimited registration). The Agency was supposed to undertake a thorough re-assessment of the potential health and environmental impacts of Bt crops before reaching a decision on re-registration, taking account of the most current scientific information and the recommendations of its scientific advisors. The following table summarizes the deep flaws in the Agency's allergenicity assessment of Bt crops. The three parameters – digestive & heat stability, structural similarity to known allergens - are those chosen by the EPA itself (EPA BRAD Human Health Assessment). The Agency either failed to collect relevant studies ("NONE"), accepted defective studies ("INADEQUATE"), or ignored independent studies demonstrating potential allergenicity ("RED FLAG").

This table (slightly modified) is excerpted from "A Critique of the EPA's Decision to Re-Register Bt Crops and an Examination of the Potential Allergenicity of Bt Proteins," by Bill Freese for Friends of the Earth, December 9, 2001, available at www.foe.org/safefood/comments.pdf.

Summary of Available Data for Allergenicity Assessment

Company Crop Bt protein	Digestive Stability	Heat Stability	Amino Acid Sequence Homology
Monsanto Yieldgard Corn Cry1Ab	**RED FLAG** Digestive stability similar to (though lesser than) that of StarLink Cry9C (1)	**RED FLAG** Heat stability comparable to that of StarLink Cry9C (2)	**RED FLAG** Matches w/ vitellogenin, an egg-yolk allergen, "warrant additional evaluation" (3)
Syngenta Bt 11 Corn Cry1Ab	**RED FLAG** Digestive stability similar to (though lesser than) that of StarLink Cry9C (1)	**RED FLAG** Heat stability comparable to that of StarLink Cry9C (2)	**RED FLAG** Matches w/ vitellogenin, an egg-yolk allergen, "warrant additional evaluation" (3)
Monsanto BollGard Cotton Cry1Ab/Ac	**INADEQUATE** Flawed study shows degradation in 2-7 minutes (4)	**INADEQUATE** Only shown to be "inactive" in processing study (5)	**RED FLAG** Cry1Ab/Ac has the same vitellogenin-matching subsequences as Cry1Ab (3, 6)
Mycogen & Pioneer Herculex Corn Cry1F	**INADEQUATE** Test conditions not specified by EPA (7)	**INADEQUATE** Only shown to be "inactive" in bioassay after 30 min. at 75° & 90°C (5)	**OK** Though more stringent test would be desirable (8)
Monsanto NewLeaf Potato Cry3A	**INADEQUATE** Test conditions not specified by EPA (7)	**NONE** (9)	**RED FLAG** Amino acid sequences found in which 7-10 matched β-lactoglobulin, a milk allergen (10)

Notes to Human Health Assessment Table

(1) "The Cry1Ab protein was digested at a similar, if slightly faster, rate than the E. coli-derived Cry9C protein in simulated gastric fluid." (Aventis CropScience 2000, "Cry9C Protein: The Digestibility of the Cry9C Protein by Simulated Gastric and Intestinal Fluids," study submitted to the EPA by Aventis CropScience, p. 17). In another study, Noteborn (1998) found that it took two hours to achieve > 90% degradation of Cry1Ab(5) in SGF (165 μg/ml SGF, pH = 2.0) Noteborn (1998), p. 21, Annex 1 – Table 1, p. 31. See note (2) for full Noteborn citation.

(2) "Studying the Cry1Ab5 protein a relatively significant thermostability was observed which was comparable to that of the Lys mutant Cry9C protein." Noteborn (1998). "Assessment of the Stability to Digestion and Bioavailability of the LYS Mutant Cry9C Protein from Bacillus thuringiensis serovar tolworthi," study submitted to the EPA by AgrEvo, p. 22

(3) "…the initial alignment between Cry1A(b) and vitellogenin located subsequences in which 9 to 11 amino acids were identical (82% similarity). Realignment indicated that these regions contained stretches of 11 biochemically similar and 12 evolutionarily similar amino acids (100% similarity over 11 or 12 amino acids." "For example, the similarity between Cry1A(b) and vitellogenin might be sufficient to warrant additional evaluation." Gendel, Steven M. "The use of amino acid sequence alignments to assess potential allergenicity of proteins used in genetically modified foods," Adv. in Food and Nutrition Research , Vol. 42, 1998, pp. 58-60. The EPA apparently did not consider this study in its reassessment of Cry1Ab corn. The Agency states merely that companies did not submit structural comparisons: "Amino acid homology comparisons for Cry1Ab, Cry1Ac and Cry3A against the database of known allergenic and toxic proteins were not submitted." (EPA BRAD 2001, p. IIB2)

(4) Monsanto conducted this study under conditions that proved extremely favorable to rapid digestion of the Cry1Ab/Ac hybrid protein: pH = 1.2, 2 μg test protein / ml SGF. Experts now recommend testing with much higher concentrations of test protein at a milder pH (pH = 2.0).

(5) "Inactive" here means "unable to kill insects" in bioassays, which provide little or no information about degradation of the protein into amino acids and small peptides, which is what should have been measured (e.g. by HPLC or SDS-PAGE)

(6) "Cry1A(c) has the same sequence as Cry1A(b) in the region involved, and therefore produced the same alignments, but this was not considered an independent alignment because the proteins are closely related." Gendel, Steve, p. 59. (See note (3) for citation)

(7) EPA fails to cite the pH value of SGF. If test conducted at pH = 1.2, it should be repeated at pH = 2.0. See note (4).

(8) Many experts recommend a more stringent test than one based on 8 contiguous amino acids.

(9) "No heat stability studies were available for Cry3A." EPA BRAD 2001, p. IIB2.

(10) "First, the initial alignment between Cry3A and β-lactoglobulin located subsequences in which 7 of 10 amino acids matched exactly. Realignment with both the evolutionary and biochemical matrices indicated that the intercalary amino acids were similar, meaning that the alignment was 100% similar over 10 amino acids." Gendel, Steve, pp. 58-59. See note (3) for citation. The EPA apparently did not consider this study in its reassessment of Bt crops, stating merely that "additional amino acid sequence homology" data are needed to "complete product database" for Cry3A NewLeaf potatoes. EPA BRAD 2001, Table B1, p. IIB3.