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Comments to the Scientific Advisory Panel Evaluating Cry34Ab1/35Ab1, March 1-2, 2005, Docket No. OPP-2004-0395

Center for Food Safety (CFS)[a] appreciates the opportunity to comment on the allergenicity assessment of Cry34Ab1, and more generally on allergenicity risk assessment for genetically engineered (GE) crops at EPA.[b] CFS also commends EPA for conducting this SAP as part of continuing efforts to better understand the risks of GE crops, and thanks the panelists for taking time from their busy schedules to provide this public service. Our comments are in three parts: 1) a general introduction, 2) some background on food allergenicity assessment for GE foods, and especially the gastric stability assay, and 3) our assessment of the potential allergenicity of Cry34 and whether EPA should register this protein.

We disagree with EPA's preliminary finding that Cry34Ab1 is unlikely to be an allergen. We believe, as discussed in more detail below, that available data suggest that EPA cannot conclude with a "reasonable certainty" (the legal standard) that no harm will occur if Cry34/Cry35 corn is registered. In particular, Cry34 is as stable to digestion as many important food allergens, and other data relied on by EPA are insufficient to override the digestive stability data. For example, although EPA bases its judgment in part on low levels of Cry34 in corn, the protein is found at over 3,000 fold higher concentration than a previous SAP found unacceptable for Cry9C from StarLink corn. EPA's contention that *Bacillus thuringiensis* is non-allergenic is unsupported, and contradicted by published studies that suggest otherwise. The heat inactivation data is insufficient without showing that the protein is not only inactivated, which may merely mean it is denatured, but also degraded to peptides that are too small to cause sensitization.

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[a] The Center for Food Safety is a not-for-profit public interest group located in Washington, DC, that works to protect the safety of people and the environment on issues concerning genetic engineering and other new and emerging technologies, and by the support of sustainable technologies. www.centerforfoodsafety.org

[b] I will use Cry34 and Cry34Ab1 interchangeably in my comments

In a situation such as exists with Cry34Ab1, where the level of hazard is not precisely known, the regulatory decision will reflect whether EPA takes a cautious approach, to ensure that the public is adequately protected, or puts the public at some risk in order to allow commercialization. In making such a decision, the potential benefits should be considered. Several products and approaches are already on the market or available to farmers that adequately control rootworm, and Cry34/Cry35 does not substantially improve the situation. Therefore, it would be especially irresponsible to allow this corn rootworm product on the market under the existing circumstances.

An alternative to rejecting Cry34Ab1 outright would be for the SAP to recommend additional tests that could be dispositive concerning allergenicity. We are not aware of currently available methods that could clearly resolve Cry34Ab1 allergenicity, and if the SAP could advance this issue, for Cry34Ab1 as well as future GE proteins, they would perform a significant service. We feel that it is important to point out that the issue of inadequate allergenicity testing has been present for as long as GE foods have been commercially produced, almost 10 years, with little progress. This is due in significant part to the limited resources devoted by the agencies to solving this problem, notwithstanding this and other SAP, and it therefore represents a significant failing of the current GE regulatory system. For example, as far as we know, there has been relatively little research funding devoted by either EPA or FDA to improving allergenicity testing.

There has also been little government effort to validate and adopt the recommendations of respected international bodies such as the FAO/WHO. For example, even though the digestive stability test has been widely accepted and used since the mid 1990s, only recently has there been multiple-lab testing to standardize and validate this assay.[1] As a consequence, as noted in the table provided by EPA at the end of its position paper, reaction conditions for the digestive assay have varied widely. In particular, pepsin-to-test-protein ratios have been up to several thousand fold higher than those recommended by the FAO/WHO, despite data by TJ Fu of FDA and others that show that such high levels of pepsin could make some allergens appear to be unstable, and therefore to wrongly look like non-allergens.[2 3] And although industry efforts to begin to standardize testing procedures are commendable, regulatory agency direction is needed to ensure that adequately protective methods that the public can trust are developed. In other words, it is not enough that the tests simply work in the narrow sense and are reproducible, but that the best tests are used – i.e. tests that are adequately protective of the public. This requires the active participation of the regulatory agencies charged with public protection.

Therefore, we believe that a crucial function of this SAP goes beyond the immediate recommendations about the possible allergenicity of Cry34Ab1, and includes recommendations for improving GE allergenicity assessments in general. This is well within the purview of the SAP. And although the SAP cannot solve the bigger problems concerning the inadequacies and lack of standards of current allergenicity testing, it can point the way. Ultimately, unless EPA and its sister agencies take more seriously the need to develop better procedures and improve the rigor of allergenicity and other GE safety testing, the public cannot be expected to have confidence in pronouncements about the safety of these crops. We hope the SAP will appreciate, and not miss, this opportunity to assist EPA with the larger issue of improving its risk assessment process, as well as advising EPA on the potential allergenicity of Cry34.

Background

The immediate issue for the SAP is the potential allergenicity of Cry34, and more specifically, the importance of gastric digestion assay data. The gastric stability assay has been widely accepted as an important part of allergenicity assessments of GE foods beginning around the mid 1990s. Several papers further supported the assay beginning around 1996, and continuing through the FAO/WHO consultation in 2001 that resulted in acceptance by the Codex Alimentarius.[4] [5]

Although certainly imperfect, it should be kept in mind that for proteins without significant previous human exposure, as is likely the case for Cry34, there are no accepted tests that are more reliable for determining potential allergenicity. For example, although sequence homology searches can be very useful when a match is found, they typically say very little about allergenicity for proteins such as Cry34 that have not been food constituents, and are not similar to food proteins or known allergens. In other words, there is no reason to believe that known (and sequenced) allergens represent the range of possible protein sequences that are capable of producing an allergic reaction. In fact, unless we know that the currently available sequences of allergens and IgE epitopes represent a substantial proportion of the possible allergen sequences, negative results in a sequence homology or similarity search tell us little about the likelihood that a novel protein will become an allergen. Therefore, it would not be surprising for novel proteins that have not been in the food supply to prove allergenic, even without a match to the sequence of a known allergen.

Similarly, loss of function due to processing, such as by heating, does not mean that a protein will necessarily be rendered non-allergenic. For example, some milk allergens can have either conformational or linear epitopes, where the latter may reflect sensitization to the denatured form of the protein. [6] [7] For example, Vila et al. found that: "Specific IgE antibodies against linear (denatured) as well as conformational (native) milk proteins were determined by probing dot-blots with patients' sera." [8] More generally, loss of function may simply mean that the protein is denatured rather than degraded into short peptides, and could therefore still be allergenic. Abundance of the protein in food has also been used in predicting the likelihood of allergenicity, since most (but not all) food allergens are typically plentiful proteins. But the recent SAP on StarLink concluded that even a proposed tolerance of 20 PPB was not acceptable because a lower limit for sensitization could not be determined.[9] We note that the FAO/WHO consultation did not rely upon either heat stability or protein abundance in its recommended allergenicity determination. The FAO/WHO specifically noted that "…allergens can sensitize susceptible individuals at less than milligram levels, possibly at less than microgram levels," and "Thus, level of expression cannot yet be incorporated into the assessment of the allergenicity of genetically modified foods." [10] Animal models, another potentially useful approach, are not yet considered reliable predictors of food allergenicity**.**

Finally, there have been several instances where food allergens have been found to be unstable in the gastric assay, as well as some instances where supposed non-allergenic proteins

have been stable. This may demonstrate that the correlation between gastric stability is imperfect, and makes interpretation more difficult, but does not invalidate the assay. Food allergens also show a range of digestion times, which also complicates the interpretation of results. In addition, it is not clear that the proteins have always been adequately "classified" prior to the assay. For example, if stability is correlated with allergenicity because the protein must reach immune tissue in the intestines for sensitization to occur, then oral allergy syndrome allergens may not fit the model because sensitization may occur through the respiratory homologue of the food allergen.[c] Similarly, if the food is always eaten in a cooked form, which degrades the GE protein or makes it more susceptible to digestion, then using "raw" protein in the assay may not be expected to fit the model. Considering these or other factors may lead to refinements of the assay, and any guidance that the Panel can provide to EPA that could improve the assay would be welcome.

Until a more reliable means of determining allergenicity is found, simulated digestive stability should remain a fundamental part of allergenicity determinations. That said, how should this assay be applied? Although the kinetic approach proposed by DowAgro may eventually have merit in some circumstances, the EPA position paper points to several weaknesses of this approach. More basically, although the work by DowAgro is a good start, this kinetic assay has not been adequately validated. Such validation requires testing by several labs of the same proteins and samples, and varying the assay conditions to determine how the assay can be done in the most reproducible and accurate manner, as well as determining how best to interpret the results. This has not been done with the kinetic approach. There has been considerably more experience with the visible-endpoint measurement approach that has always been previously used to determine stability. In addition, there have been recent attempts to begin validating endpoint measurement and reaction conditions.[11] Therefore, regardless of what the panel decides about the concept of using a kinetic approach in measuring stability, the current results of the kinetic measurement of stability should not be accepted. Instead, the endpoint data for Cry34 should be carefully considered. I briefly considered those original data for the Cry34 Experimental Use Permit in April 2003, and include that analysis as Appendix A.

Potential Allergenicity of Cry34

EPA, in its position paper, concludes on page 7 that "Cry34Ab1 is unlikely to be a food allergen." We disagree with EPA's assessment for several reasons. It is very difficult, due the limitations of currently available tests, to determine with any precision how likely it is for Cry34Ab1 to become an allergen. None of the available data can safely exclude the possibility of allergenicity, while some data support that possibility. We therefore believe that the available data suggest that there is a reasonable possibility that Cry34Ab1 could become an allergen. Unless additional dispositive data can be produced, we believe that prudence and reasonable caution demand that crops containing Cry34Ab1 in the edible portion are not approved.

First, Cry34 is as stable to simulated gastric digestion, or more stable, than several known food allergens, and more stable than most non-allergen food proteins. Recognizing that there is some variation in the visible endpoints for various food allergens, probably due in part to

[c] The physiological meaning of stability has not been determined, but only hypothesized.

variations in protein purification, gel electrophoresis and protein staining,[12] Cry34 survives digestion (visible at 15 min - 20 min, but not at 30 min) similar to several known food allergens. These allergens include the major peanut allergen Ara h 2 (20-60 min), egg ovalbumin (20-60 min) (Thomas, K. op. cit.), egg conalbumin (15 min), bovine milk casein (15 min), bovine serum albumin (15), soybean lectin (15 min), soybean glycinin (15 min), soybean Gly m Bd 30K (8 min), and peanut lectin (8 min) (Metcalfe et al., op. cit.). Alpha-lactalbumin was stable for only 2 min (Metcalfe et al., op. cit). Similar results can be found in Fu et al. (op. cit) and other references. Several major allergens are not "highly" stable, so there does not appear to be a clear correlation between the importance of the allergen and the magnitude of its stability. Put another way, it does not seem valid to consider what EPA calls "moderate" stability of Cry34 as an indicator of lower probability of allergenicity.

EPA considers several other parameters in arriving at its conclusion that Cry34Ab1 will not become an allergen. In particular, EPA comments that because Cry34Ab1 is inactivated by heat, is not glycosylated, has no sequence similarity or homology with known allergens, and is present at relatively low levels in corn kernels, and that *Bacillus thuringiensis* in not considered an allergenic source, it is unlikely to be an allergen. We have addressed some of these issues generally in the "Background" section above.

EPA claims that *Bacillus thuringiensis* is not an allergen, in contradiction to available evidence. In particular, Bernstein et al. found preliminary evidence that *B. thuringiensis*, and more specifically, spore preparations from *B. thuringiensis*, may be allergenic.[13] Although clinical symptoms were not present, important indicators of allergy including *B. thuringiensis* spore-specific IgE and positive scratch tests were demonstrated. In addition, other data suggest that Cry proteins may act as potent adjuvents. While not proof that *B. thuringiensis* or Cry proteins are allergens, these data are suggestive that they could be. Unfortunately, EPA has done no follow-up studies to address the potential allergenicity of Cry proteins. In addition, there are limited data on any significant previous exposure of the public to Cry34, a necessary prerequisite to allergy. EPA is apparently therefore incorrect when it claims on page 7 of its position paper that Cry34Ab1 "originates from a non-allergenic source," since the evidence to substantiate this claim is lacking.

As noted above, the amount of Cry34Ab1 in corn kernels cannot be relied upon to eliminate the possibility of Cry34 allergenicity. The position paper states that Cry34Ab1 is present at about 70 ng/mg in corn kernels. Although Cry34 concentration is lower than for most food allergens, it is over 3,000-fold higher than the 20 PPB that the second StarLink SAP found unacceptably high for setting a tolerance, because a minimal level for sensitization could not be established.[14] It is ironic that DowAgro used an ubiquitin promoter to produce the more stable Cry34Ab1, which causes production in the kernel where the protein is not needed to control corn rootworm, while using a wheat root-preferential promoter for the less stable Cry35Ab1, which produces 70-fold less Cry35Ab1 in the kernels than Cry34Ab1. Perhaps one recommendation of the SAP could be to produce new Cry34/Cry35 corn with low or no expression of Cry34Ab1 in the kernel.

The second StarLink SAP determined that Cry9C had a moderate possibility of being allergenic, and a low possibility of actually producing allergy due to limited public exposure. Although Cry9C was stable to heat as well as digestion, while Cry34 may be somewhat less

stable to digestion and is inactivated on heating, on the other hand StarLink was present only as a low-level contaminant (and the proposed tolerance was only to allow for elimination from food supply through dilution, rather than to allow continued planting) while Cry34 is being proposed as an indefinite addition to the food supply, with ongoing exposure levels considerably higher than were likely the case for Cry9C.

CFS believes that the possible consequences of a commercialized Cry34Ab1 allergen should be considered in the context of the food and the crop. Corn is usually not an allergenic food. Therefore it can often be recommended as an alternative source of protein for individuals, and especially children, who have multiple food allergies, or who are more prone to develop food allergies. This is not the case for several important sources of vegetable protein such as soybeans, nuts, peanuts, and wheat, which are all prominent food allergens. Therefore, we should take special care to avoid turning corn into a more common food allergen.

There is no pressing need for a corn rootworm product containing Cry34/Cry35. Although the current available products have some drawbacks, Cry34/Cry35 corn is unlikely to produce significant improvements over current products. There are insufficient data to suggest substantially higher yield, and it does not improve nutrition. For all of these reasons, EPA should not approve the registration of Cry34Ab1.

Therefore, on balance, we believe that Cry34Ab1 has a similar possibility of becoming a food allergen as did Cry9C. EPA correctly found that for StarLink, a "low" possibility of becoming a food allergen was sufficient to deny a tolerance (or exemption from tolerance), and it should do the same in the case of Cry34Ab1. As noted above, it is impossible to accurately quantify the allergenic potential of Cry34Ab1, or to put "too fine a point" on this analysis, which is precisely why EPA should take a reasonably cautious approach in keeping Cry34Ab1 off the market unless more definitive data can be produced dismissing the possibility of allergenicity.

Submitted by,

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Appendix A

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Attention: Docket ID Number OPP-2002-0350

April 7, 2003

Comments to EPA re: Dow Agro Request for a Tolerance for Corn Rootworm Transgenic Corn Containing Cry34Ab1/Cry35Ab1

Center for Science in the Public Interest (CSPI) submits the following comments recommending that EPA deny DowAgro Science's request for a food tolerance or an exemption from tolerance for its new rootworm-protected corn containing Cry34Ab1/Cry35Ab1 (hereafter Cry34/35). This exemption would allow DowAgro to incorporate Cry34/35 corn into the food supply when grown under an experimental use permit (EUP). We make our recommendation based on simulated gastric digestion (SGD) data submitted to EPA by DowAgro indicating that Cry34Ab1 may become a food allergen if allowed into the food supply. Furthermore, due to the limitations of current allergencity tests, we highly recommend that EPA present the allergencity data for Cry34/35 to independent experts on allergenicity, such as the Scientific Advisory Panel, before making a decision on the full registration of Cry34/35 in the future.

We find that SGD data for Cry34/35 presented in MRID 455845-02 and MRID 452422-12 indicate that Cry34Ab1 should be considered to be stable according to accepted scientific literature, and therefore disagree with Dow Agro's interpretation of instability of Cry34Ab1. While digestive stability does not prove that a protein will become a food allergen it has been

accepted by international groups of experts on allergencity as indicating a reasonable likelihood of allergenicity (Astwood et al., Metcalfe et al., United Nations Food and Agriculture Organization/World Health Organization). Therefore, EPA cannot determine with reasonable certainty that Cry34Ab1 will not cause allergic reactions when consumed.

DowAgro presents two sets of data concerning SGD which found that intact Cry34Ab1 could be detected for 20-30 minutes using sensitive detection methods (Western blot), or that 90% of Cry34Ab1 was digested after 6.2 minutes using less sensitive detection methods (Coomassie-stained SDS-PAGE gel). DowAgro discounts the 20-30 minute stability data by claiming that the detection method (Western blot) was more sensitive than in SGD for previous Cry proteins registered by EPA. Based on their calculation of 90% digestion of Cry34Ab1 after 6.2 minutes, DowAgro concludes that Cry34Ab1 is not stable.

DowAgro's interpretation of SGD data is not in accord with currently accepted standards for several reasons. First, literature such as that cited by DowAgro on SGD finds that several food allergens were digested in as few as two to eight minutes (Astwood et al., Metcalfe et al.). By that criterion, detection of Cry34Ab1 after 6.2 minutes would indicate stability, not instability (see Astwood et al., Table 1).

Furthermore, the cited literature uses the longest time-point where SGD test protein can be detected as a measure of stability, not the rate of digestion used by DowAgro to determine 90% digestion. For example, Astwood et al. use the longest time-point for which food allergens can be observed as their measure of stability. Like DowAgro, they use Coomassie-stained SDS-PAGE gels to detect undigested allergens. Cry34Ab1 is visible, even under the poorly reproduced gel pictures found in MRID 455845-02 and MRID 452422-12, for up to 20 minutes, and clearly visible at 7.5 to10 minutes in most of the gels.[14]

In addition, DowAgro uses more than three-fold higher proportion of pepsin-to-test-protein (Cry34Ab1) in its SGD assay compared to Astwood et al., which may make Cry34Ab1 appear to be less stable than it would if carried out according to the literature. Recent experiments clearly demonstrate that the proportion of pepsin to allergen can change the apparent stability of the allergen (T.J. Fu and Fu et al.). More recent protocols recommend even lower pepsin to test protein proportions (United Nations Food and Agriculture Organization/World Health Organization). These more recent protocols also recommend detecting SGD test protein using silver staining or colloidal gold which is more sensitive than Coomassie staining and may approach the sensitivity of the Western blots used by DowAgro that showed 20-30 minute stability of Cry34Ab1.

We recognize that the limited dietary exposure, both in amount and duration, that would be caused by granting a temporary tolerance for Cry34/35 makes the likelihood of allergic reaction low. However, it is a chance that should not be taken. DowAgro could continue to grow Cry34/35 corn under an EUP on a crop-destruct basis if other safety criteria are found to be acceptable. Therefore, it would be especially imprudent to approve the requested tolerance.

Finally, the dilemma presented by the current lack of tests that would more clearly determine the potential allergencity of a protein new to the food supply is obvious in the example

of Cry34/35. This example emphasizes the crucial need for the development of more definitive tests and, in their absence, the delineation of acceptable standards for performing and interpreting currently available tests such as SGD. Such standards are provided by the FAO/WHO expert consultation on food allergy assessment for GE proteins (United Nations Food and Agriculture Organization/World Health Organization), and EPA should adopt those standards. Until such measures are taken, EPA will continue to face situations that may challenge the credibility of its decisions, and hence public confidence in GE technology.

Sincerely,

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