FEB 09 2005

Position Paper: Scientific Issues Associated with the Human Health Assessment of the Cry34Ab1 Protein

Introduction

Dow AgroSciences (Dow) and Pioneer Hi-Bred International, Inc. (Pioneer) have submitted applications for FIFRA § 3 registration of the plant-incorporated protectant (PIP) *Bacillus thuringiensis* (Bt) Cry34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their production in corn. These products are intended to provide protection against western and northern corn rootworm larvae.

Reviews have been completed on product characterization and human health and can be found in memoranda dated December 6, 2004 and February 4, 2005. A preliminary safety assessment is presented in the memo dated February 4, 2005.

Since Cry34Ab1 and Cry35Ab1 are proteins, allergenic potential was considered in the safety assessment. EPA uses a weight of evidence approach suggested by scientists at the 1994 Allergenicity Conference, hosted by EPA, the Food and Drug Administration, and the U.S. Department of Agriculture, where characteristics of a protein are compared with characteristics of known allergens. More recently, this approach was outlined in the Annex to the Codex Alimentarius "Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants." Currently, no definitive tests exist for determining the allergenicity potential of proteins. EPA considers the following factors to provide assurance that a protein is unlikely to elicit an allergic reaction: 1) whether the source of the trait is associated with any reports of allergenicity; 2) amino acid sequence comparison with known allergens, both overall similarity and stepwise contiguous amino acid searches; 3) biochemical properties of the protein, including *in vitro* digestibility in simulated gastric fluid (SGF), heat stability, and glycosylation; 4) prevalence in food; and 5) specific serum screening (e.g., for proteins with sequence similarity with a known allergen). Because no single factor is fully predictive, EPA considers all of the available information in the assessment.

As part of their weight of evidence assessment, Dow and Pioneer submitted several *in vitro* digestibility studies in support of their applications for registration and tolerance exemption of Cry34Ab1 and Cry35Ab1. From analysis of these studies, as well as published studies and previous guidance from Scientific Advisory Panels, EPA has concluded that Cry35Ab1 is rapidly digested and Cry34Ab1 is moderately digested in simulated gastric fluid (SGF). Cry34Ab1 appears to digest slower than other Bt proteins that have been registered for food use and many other proteins that are not considered allergens but faster than most previously tested allergens. Dow and Pioneer also submitted data indicating that both Cry34Ab1 and Cry35Ab1 are inactivated by heat, are not glycosylated, do not have any sequence similarity to known allergens, and will only be present at low levels in food.

To further analyze the digestion of Cry34Ab1, the registrants have developed a kinetic approach

to assess protein degradation as part of their weight of evidence evaluation of Cry34Ab1. EPA is asking the Panel to comment on 1) the usefulness of the kinetic approach for moderately digestible proteins and what assay conditions are appropriate for comparing the digestion of different proteins; 2) how digestion assays should be used in the overall weight of evidence approach to allergenicity assessment; and 3) EPA's allergenicity assessment of Cry34Ab1.

Background

Pepsin digestibility is the focus of this position paper and Scientific Advisory Panel (SAP) meeting because it is the only significant issue that has arisen during the evaluation of Cry34Ab1 and Cry35Ab1. A correlation between resistance to *in vitro* digestion by the enzyme pepsin and allergenic potential has been demonstrated (Astwood, *et al.*, 1996). Therefore, pepsin digestibility is one component that is considered as part of the weight of evidence approach for assessing the allergenicity potential of proteins. The correlation between resistance to digestion and allergenicity, however, is not absolute: some allergens are rapidly digested and some non-allergens (*i.e*., proteins not known to cause allergic reactions) appear to be resistant to digestion (Fu, *et al.*, 2002).

One rationale for using digestibility assays in allergenicity assessments is the belief that proteins that are rapidly degraded in the digestive system are less likely to induce an immune response. It is generally accepted that a protein must be stable in the stomach for a sufficient period of time to sensitize an individual. Small peptides are believed to be incapable of causing allergic sensitization. However, there are reports of cases where digested proteins were capable of eliciting an allergic response in individuals who have already been sensitized (Nilsson, *et al.*, 1999). Some researchers have suggested that stability to pepsin digestion may reflect resistance to cleavage of the protein by intracellular proteases during processing for presentation to T lymphocytes and that inherent susceptibility of proteins to enzymatic digestion may influence the nature of the immune response and therefore whether allergic sensitization will develop (Dearman, *et al.*, 2002).

In Vitro Digestibility Assays

Different protocols exist for assessing a protein's susceptibility to digestion by pepsin. The conditions used in assessing *in vitro* digestibility are important because a protein can appear to be resistant to digestion or rapidly digested depending on the pH of the SGF, the ratio of pepsin to substrate protein, the concentrations of pepsin and substrate protein, the purity of the proteins, the specific activity of the pepsin, and the sensitivity of the detection method (Thomas, *et al.*, 2004). Typically, the SGF is prepared as specified in the U.S. Pharmacopeia (USP), giving a pH of 1.2 and a pepsin concentration of 3.2 mg/mL. However, the concentration of the substrate protein is not specified, and the specific activity for pepsin has only been specified in recent editions of the USP. In addition, a 2001 Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology report titled "Evaluation of Allergenicity of Genetically Modified Foods" provides a protocol for conducting digestibility assays where the pH of the SGF is 2.0, rather than 1.2. This protocol also specifies the pepsin concentration as 0.32% (w/v)

(3.2 mg/mL) and the substrate protein concentration as 2.5 mg/mL (500 µg in 200 µL SGF). The FAO/WHO protocol, however, has not been tested, so it is unknown whether or not a correlation between allergenicity and digestibility would be observed with this protocol. In addition, there is no database of digestion times for known allergens and non-allergens for comparison with new proteins tested under the conditions of this protocol. Recently, a number of organizations, companies, and government researchers published a joint study where the reproducibility of a common protocol was tested across multiple laboratories (Thomas, *et al.*, 2004). This protocol used SGF solutions of both pH 1.2 and 2.0 with a final pepsin concentration of 0.72 mg/mL (3,460 units/mg) and a ratio of 10 units of pepsin activity per µg test protein (3:1 pepsin to protein, w/w). Registrants have used a variety of conditions for testing *in vitro* digestibility of currently registered PIPs (see Table 1).

Typically, the time it takes for the test protein or its digestion fragments to become undetectable is monitored. Two approaches have been used: 1) separate reactions are set up for each of the time points and quenched by the addition of base at the appropriate time, or 2) a single reaction is set up for each replicate, and samples are removed at various time points and quenched by the addition of base. The samples are then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the protein bands on the gels are visualized either by staining the gel or using western blot analysis.

Kinetic Approach

Dow has developed a new kinetic approach for assessing a protein's *in vitro* digestibility based on estimating the rate of pepsin digestion.[1] Dow uses a protocol similar to those described above. A single reaction is set up for each replicate, and the test proteins are incubated in SGF containing pepsin at a concentration of 0.3 % (w/v), pH 1.2, at 37 °C, with constant shaking. Samples are removed at various time points and analyzed by SDS-PAGE. After the gels are stained, the relative amount of protein or protein fragment remaining at each time point is monitored by determining the band density using gel densitometry. The band density is assumed to be directly proportional to the protein concentration. The degradation over time is then assessed using a first-order (exponential) decay model. For Cry34Ab1, Dow used linear regression of the natural logarithm of the percent protein remaining versus time to determine the first-order rate constant (MRID 455845-02; Herman, *et al.*, 2003). In subsequent analyses, Dow has used non-linear regression of a 3-parameter exponential model ($[S] = [S_0]e^{-KT} + B$, where [S] is the band density at time T, $[S_0] + B$ is the Y-intercept, K is the first-order rate constant, and B is the asymptote or background estimate) to determine the first-order rate constant (MRID 463886-01). Half-lives are then calculated by dividing the natural logarithm of 0.5 by the first-order rate constant for each protein or fragment.

[1]The kinetic approach to pepsin digestion was developed by scientists at Dow AgroSciences. Applications by both Dow and Pioneer for registration of their Cry34Ab1 and Cry35Ab1 products rely on these data. Because Dow scientists developed this approach, the explanation of the kinetic approach only mentions Dow.

Dow has asserted that first-order decay is predicted based on enzyme theory as long as the pepsin concentration is high and the substrate concentration is low ($<< K_m$). Dow has also stated that the first-order rate constant determined under these conditions is equal to V_{max}/K_m, which is a measure of the inherent efficiency of an enzymatic reaction. As long as first-order conditions are met, first-order rate constants and half-lives are unaffected by changes in protein substrate concentration. Therefore, first-order rate constants can be used to predict relative digestion efficiencies for proteins, even if protein concentrations are varied among experiments. In addition, Dow has stated that the enzyme concentration is saturating when the USP concentration of pepsin (0.32%) is used, making the rate constants relatively insensitive to changes in enzyme concentration (Herman, *et al.*, 2005). Dow has stressed that the kinetic approach should improve the accuracy of determining pepsin digestibility, and relying on the disappearance of a protein band on a gel to measure digestibility of proteins as an endpoint may be problematic. The disappearance results depend on a number of factors including the affinity of different proteins for the dye or antibody used to visualize the gel, the amount of protein loaded on the gel, type of gel and dye used, and development time.

EPA's Assessment of the Kinetic Approach

A method for assessing pepsin digestion, such as the kinetic approach, that is not dependent on detection method may be an improvement over relying on the substrate disappearance endpoint in cases where digestion does not appear to be rapid. However, EPA is confident that the endpoint method that has been used for assessing digestion of previously registered proteins has been adequate. In addition, caution is warranted in interpreting results and comparing the kinetic rates of pepsin digestion of different proteins determined using the kinetic approach. Many of the same factors that affect the endpoint method can also affect the digestion rates such as enzyme activity, purity of protein substrate, concentrations of pepsin and substrate, pH, temperature, and whether or not the reaction mixture is shaken or stirred. Also, the digestion rates calculated using Dow's approach depend on the fit to first-order kinetics. Pepsin hydrolysis is a multi-step/multi-reaction process, and the kinetics may not be simple to predict.

Pepsin has been shown to hydrolyze proteins using different mechanisms, depending on reaction conditions (Choisnard, *et al.*, 2002) and presumably depending on the protein substrate. One pepsin molecule can degrade one protein substrate molecule at a time (i.e., the one-by-one or processive mechanism); a pepsin molecule can also move from one protein substrate molecule to another, cleaving as it goes, generating intermediate peptide products (i.e., the zipper mechanism); or it can use a mechanism that is in between these two extremes. Choisnard *et al.* (2002) demonstrated that at pH 4.5, pepsin hydrolyzed native hemoglobin using the one-by-one mechanism, while pepsin hydrolysis of denatured hemoglobin proceeded by the zipper mechanism. It is unclear whether the rate of decay of the starting substrate would follow first order kinetics regardless of the mechanism. It likely depends on what the rate-limiting step of the reaction is, which may depend on reaction conditions and the protein substrate. Also, during pepsin-catalyzed hydrolysis of many proteins, intermediate digestion fragment peptides are formed. Presumably, in some cases, these fragments could compete with starting substrate and inhibit the rate of decay of starting substrate; the decay rate might not follow first order kinetics

for these cases.

Dow and Pioneer have submitted two comparison studies using a number of allergens and non-allergens (i.e., proteins that are not known to cause allergic reactions) to test the kinetic approach. The first submitted study (MRID 461239-20, reviewed in the memorandum from R. Edelstein to M. Mendelsohn dated August 17, 2004) was designed for a different purpose and used conditions (pH 1.2 and 2.0; pepsin concentration: 0.72 mg/mL; test protein concentration: 0.25 mg/mL; pepsin: test protein ratio: 3:1, w/w) that did not allow comparison with the previously submitted digestion study on Cry34Ab1. Most of the proteins digested either too quickly or too slowly for their digestion rates to be determined. Of the digestions that were analyzed kinetically, some demonstrated good fit to first-order kinetics, while some had poor fit. The study showed a correlation between resistance to pepsin digestibility and allergenicity. The second comparison study (MRID 463886-01) used the same conditions that were used in the digestibility studies on Cry34Ab1 (pH 1.2, pepsin concentration: 0.32%, test protein concentration: 0.002 mM, pepsin: test protein ratio of approximately 20:1, mol/mol). In this second comparison study, half-lives for the proteins tested ranged from < 30 seconds to > 60 minutes. Allergens tended to be more stable in SGF than non-allergens, but a strong correlation between digestion rate and allergenicity was not observed for the set of proteins tested (seven allergens and eight non-allergens). However, to determine half-lives using Dow's protocol, the test protein must be stable enough in SGF for it to be measured over several time points. Therefore, the non-allergens tested in this study were those known to digest slower than many other previously tested non-allergens. The data fit well to a first-order decay model, except for early time points, and half-lives calculated using initial substrate concentrations that differed by 5-fold were fairly consistent.

While Dow's kinetic approach is less dependent on detection method than the end-point method typically used for assessing pepsin digestibility, it is only applicable to proteins with moderate digestibility. Because of the high enzyme and low substrate concentrations used, many test proteins are digested so quickly that they are undetectable at the first time point. In addition, the method depends on the fit to first-order decay. While the digestion of a number of proteins appears to fit the model, in the comparison study described above, early time points for several of the proteins were omitted to obtain a good fit to the model.

Dow has stated that the first-order rate constant obtained under these conditions is equal to V_{max}/K_m. It is well-known that the first-order rate constant from a substrate concentration-time profile is equal to V_{max}/K_m when the initial substrate concentration is much less than K_m, and catalytic quantities of enzyme are used (Segel, 1975). However, under conditions of high enzyme concentration, although it appears pseudo-first-order kinetics is still predicted (Schnell and Mendoza, 2004; Schnell and Maini, 2000; Tzafriri, 2003), it is unclear whether the first-order rate constant equals V_{max}/K_m. The standard assumptions (e.g., steady-state assumption: $d[ES]/dt \simeq 0$) used to derive rate equations for enzyme reactions do not apply under conditions of high enzyme concentration (Segel, 1975; Schnell and Mendoza, 2004; Schnell and Maini, 2000; Tzafriri, 2003). Different assumptions must be made to derive rate equations under these conditions. In addition, Dow did not determine K_m values for any of the proteins tested.

Therefore, it is unknown whether the concentrations used are much less than the K_m values for each of the proteins.

EPA's Assessment for Cry34Ab1 and Cry35Ab1

Data have been submitted demonstrating the lack of mammalian toxicity at high levels of exposure to pure Cry34Ab1 and Cry35Ab1 proteins. Three acute oral toxicity studies on Cry34Ab1 and Cry35Ab1 in mice were submitted: 1) Oral toxicity of Cry34Ab1 alone (MRID 452522-07); 2) Oral toxicity of Cry35Ab1 alone (MRID 452522-08); and 3) Oral toxicity of Cry34Ab1 and Cry35Ab1 combined (MRID 452522-09). All animals survived the two-week studies, and no treatment-related effects were observed. The results of these studies demonstrate the safety of the proteins at levels well above maximum possible exposure levels that are reasonably anticipated in the crops.

Since Cry34Ab1 and Cry35Ab1 are proteins, allergenic potential was also considered. Several *in vitro* digestibility studies were submitted. In the first study (MRID 452422-12), Cry34Ab1 and Cry35Ab1 were incubated in SGF (pepsin concentration: 0.3 % (w/v); pH 1.2; 37 °C) with a pepsin to protein substrate ratio of approximately 20:1, mol/mol (equivalent to 60:1, w/w for Cry34Ab1 and 17:1, w/w for Cry 35Ab1). Samples taken at 1, 5, 7, 15, 20, 30, and 60 minutes were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot. Cry35Ab1 was no longer visible at the five-minute time-point using both SDS-PAGE stained with Coomassie Brilliant Blue and western blot detection. Cry34Ab1 was visible on the stained gel for the 15-minute sample, but not in later sample time points. In the western blot analysis, Cry34Ab1 was visible in the 20-minute sample, but not in later sample time points. In conclusion, this first study showed that Cry34Ab1 was digested within 30 minutes and Cry 35Ab1 was digested within 5 minutes in SGF under the conditions of the study.

Because Cry34Ab1 appeared to be somewhat resistant to SGF in the study described above, Dow submitted a second study on the *in vitro* digestibility of Cry34Ab1 in SGF (MRID 455845-02). The digestion was performed under the same conditions as the previous study except that reaction mixtures were shaken during incubation, and samples were analyzed at 1, 2, 3, 5, 7.5, 10, 15, and 20 minutes. The previous study on pepsin digestibility of Cry34Ab1 and Cry35Ab1, as well as other pepsin digestibility studies used in allergenicity assessments, focused on the time required for the protein to become undetectable, and therefore, the results are dependent on the detection limit of the analytical method used. In this second study, Dow determined the rate of pepsin digestion of Cry34Ab1 by measuring the relative amounts of Cry34Ab1 at each of the time points based on SDS-PAGE densitometry estimates. Under the conditions of the study, the rate of decay fit a first-order model (with respect to Cry34Ab1 concentration), and Dow estimated the DT_{50} (half-life) and DT_{90} (time until 90% decay) to be 1.9 minutes and 6.2 minutes, respectively. In this experiment, Cry34Ab1 was visible on gels and blots in 15 minute time point samples but not in 20 minute time point samples.

In the comparison study where Dow and Pioneer used the kinetic approach to assess the digestibility of a number of allergens and non-allergens under the same conditions as those used

in the digestibility studies on Cry34Ab1 (MRID 463886-01), two allergens and two non-allergens were shown to digest similarly to Cry34Ab1. From the digestibility studies that Dow submitted as well as published studies, and previous guidance from Scientific Advisory Panels, EPA has concluded that Cry35Ab1 is rapidly digested and Cry34Ab1 is moderately digested in SGF. Cry34Ab1 appears to digest slower than other Bt proteins that have been registered for food use and many other proteins that are not considered allergens but faster than most previously tested allergens.

Submitted studies on heat stability of the Cry34Ab1 and Cry35Ab1 proteins demonstrate that these proteins are inactivated when heated for 30 minutes at 90 °C and 60 °C, respectively (MRIDs 453584-01, 455845-01, 458086-01, and 458602-01). A comparison of amino acid sequences of Cry34Ab1 and Cry35Ab1 with known allergens showed no overall sequence similarities or homology at the level of 8 contiguous amino acid residues (MRID 452422-05). Cry34Ab1 and Cry35Ab1 expressed in corn were shown not to be glycosylated (MRID 461239-06). Expression level analysis indicated that the proteins are present at relatively low levels in corn; on a dry weight basis, Cry34Ab1 is present at a concentration of approximately 70 ng/mg in grain, and Cry35Ab1 is present at a concentration of approximately 1 ng/mg in grain (MRID 461239-04). In addition, *Bacillus thuringiensis* is not considered to be an allergenic source. EPA has concluded that the weight of the evidence indicates that the Cry34Ab1 and Cry35Ab1 proteins are unlikely to be food allergens.

Cry34Ab1 appears to be moderately digested in SGF, rather than rapidly digested. Considering all of the available information– Cry34Ab1 originates from a non-allergenic source, has no sequence similarity with known allergens, is not glycoslyated, is inactivated by heat, is moderately digested in SGF, and will only be present at low levels in food– EPA has concluded that Cry34Ab1 is unlikely to be a food allergen.

Questions for the Panel

Protocols for Digestibility Assays

1) Dow has stated that enzyme kinetic theory predicts first order kinetics for pepsin hydrolysis under conditions of high enzyme and low substrate concentrations and has demonstrated that the rate of substrate disappearance under these conditions follows first-order kinetics for a number of proteins. However, for several proteins, initial time points were omitted to achieve a good fit to the model. Dow states that the data were not included "based on theoretical considerations, which include: potential zero-order or mixed order kinetics due to high substrate concentration, possible presence of denatured and highly digestible protein contaminating the native protein preparation, or the possibility of an initial burst phase or transient phase preceding the first-order phase of digestion (Schnell and Maini, 2000; Milgrom et al., 1998)."

The Panel is requested to comment on whether the explanation justifies omitting early time points or whether the poor fit of early time points indicates a problem with the model.

2) Dow has asserted that first-order decay is predicted based on enzyme theory as long as the pepsin concentration is high and the substrate concentration is low ($<< K_m$) and that the first-order rate constant determined under these conditions is equal to V_{max}/K_m. Dow has also stated that as long as first-order conditions are met, first-order rate constants and half-lives are unaffected by changes in substrate protein concentration and that first-order rate constants can be used to predict relative digestion efficiencies for proteins even if the protein concentration is varied among experiments. In addition, Dow has stated that at the USP concentration for pepsin of 0.32%, the enzyme concentration is saturating and can also be varied between experiments without affecting the first-order rate constant.

The Panel is asked to comment on these statements. How much can the pepsin or protein substrate concentrations vary without affecting the kinetics of pepsin digestion and first-order rate constants?

3) Typically, for comparing the *in vitro* digestibility of different proteins, researchers have used fixed concentrations of pepsin and substrate protein on a weight basis (mg/mL) rather than adjusting for molecular weight of the substrate protein, presumably because larger proteins likely have more potential pepsin cleavage sites. However, Dow states that "while multiple pepsin-labile sites may occur within a protein, a single site is often responsible for limiting digestion rates, and thus the number of molecules, rather than total weight, is most often more influential in determining the kinetics that describe decay."

The Panel is asked to comment on Dow's statement. To compare the rate of pepsin digestion of different proteins, is it more appropriate for the concentration of test protein to be constant on a weight basis (mg/mL) or a mole basis (mol/L)?

4) Typically, researchers have looked at the effect of pepsin to substrate ratio rather than concentrations on digestion (Karamac, *et al.*, 2002). How do varying the ratios and/or concentrations affect the rate of hydrolysis?

5) Different assays exist for determining pepsin activity. A pepsin activity assay based on measuring the trichloracetic acid-soluble products of pepsin hydrolysis of hemoglobin is provided in USP, 2004 under the entry for pepsin. However, the entry in USP, 2004 for "gastric fluid, simulated" references the Food Chemicals Codex for pepsin activity, which provides an assay that measures pepsin digestion of egg albumen.

The Panel is asked to comment on the appropriateness of using a fixed concentration of pepsin versus using a fixed specific activity of pepsin in digestibility protocols. How would the use of different pepsin activity assays affect the measured pepsin activity units?

6) Typically, scientists have used SDS-PAGE with staining or western blot analysis for monitoring digestion reactions. HPLC is also sometimes used.

The Panel is asked to comment on the pros and cons of the different methods that could be used for monitoring digestion reactions.

7) Some researchers have used one digestion reaction and removed aliquots at various times for monitoring, while others have set up separate reactions for each of the time points.

What are the pros and cons of these approaches?

8) Under the current protocol, Dow's kinetic approach is only applicable to moderately digestible proteins (i.e., using Dow's protocol, many proteins digest too quickly and some too slowly to obtain an adequate number of data points for quantitative kinetic analysis).

Please comment on the usefulness of the kinetic approach for proteins that are not rapidly degraded.

Allergenicity Assessment

9) The 2001 FAO/WHO report and 2003 Codex guidelines both recommend using in vitro digestibility in assessing the allergenicity potential of a protein. The FAO/WHO report provides a "decision tree" approach, while the Codex guidelines suggest a weight of evidence approach. Codex guidelines state "resistance of a protein to degradation in the presence of pepsin under appropriate conditions indicates that further analysis should be conducted to determine the likelihood of the newly expressed protein being allergenic," and "it should be taken into account that a lack of resistance to pepsin does not exclude that the newly expressed protein can be a relevant allergen." The Codex guidelines, however, don't specify how a protein should be further evaluated if it is "resistant" to degradation, and "resistant" is not defined.

a) What weight should *in vitro* digestibility studies be given in the overall assessment compared with other criteria such as sequence homology?

b) The Panel is asked to comment on the appropriateness of setting acceptable/unacceptable limits for digestibility in assessing the safety of a protein.

10) Stable digestion fragments are often formed during pepsin digestion of proteins, and Dow has used the kinetic approach to estimate the half-lives of several digestion fragments.

Please comment on the significance of the rate of digestion of protein fragments for allergenicity assessments.

Cry34Ab1 Assessment

11) Cry34Ab1 appears to be moderately digested in SGF, rather than rapidly digested. Considering all of the available information– Cry34Ab1 originates from a non-allergenic source, has no sequence similarity with known allergens, is not glycoslyated, is inactivated by

heat, is moderately digested in SGF, and will only be present at low levels in food– EPA has concluded that Cry34Ab1 is unlikely to be a food allergen.

Please comment on the Agency's conclusions regarding the allergenicity of Cry34Ab1.

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Table. Comparison of Pepsin Digestibility Studies

Protein	MRID	pH	Pepsin:Protein ratio[a] (w/w)	Pepsin:Protein ratio[a] (mol/mol)	Disappearance Time (Western Blot)	Disappearance Time (SDS-PAGE)	Digestion Fragment Disappearance Time
Cry1Ac	431452-14	1.2	1600:1	3370:1	<30 sec		<7 min (western)
Cry1Ac	43995-03	1.2	352:1	740:1	<2 min (<1% remaining)		
Cry1Ac	43995-03	1.2	3.5:1	7.4:1	<5 min (<1% remaining)		
Cry1Ab	433236-06	1.0-1.2	3:1	10:1	<2 min		
Cry1Ab	433236-06	1.0-1.2	0.007:1	0.027:1	<5 min		
Cry1Ab	451144-01	1.5	15:1	56:1	<15 min	<15 min	
PAT	439995-03	1.2	93:1	61:1	trace 2 min		
PAT	439995-03	1.2	0.93:1	0.61:1	<5 min		
PAT	458084-16	1.2	6.5:1	4.3:1	<30 sec	<30 sec	
Cry1F	455423-18	1.2		2:1	<1 min	<1 min	
VIP3A	458358-06	1.0-1.2 (not measured)	1.3:1	1.5:1	immediate disappearance		
Cry9C	451144-01	1.5	15:1		<1 hour	<1 hour (<10% remaining at 30 min)	
Cry9C	451144-02	1.2	32:1		<1 hour	<1 hour	
Cry9C	442581-08	2.0	Not given	Not given	>4 hours	>4 hours	
Cry2Ab2	449666-03	1.2	20:1[b]		<15 sec (<2% remaining)	<15 sec	
Cry3Bb1	454240-06	1.2	20:1[b]		<60 sec (<1% remaining)	<15 sec	<8 min
Cry35Ab1	452422-12	1.2	17:1	20:1	<5 min	<5 min (<3% remaining)	
Cry34Ab1	452422-12	1.2	60:1	20:1	<30 min (<0.38% remaining)	<20 min	

Cry34Ab1	455845-02	1.2	60:1	20:1	<15 min (<0.38% remaining)	<10 min	