# Hazard Characterization – Intractable Proteins

November 9, 2017

# Proteins and Agricultural Biotechnology

Weight of evidence approach

Tier I – Hazard identification ← **Requires little or no protein**

- History of safe use
- Bioinformatics
- Mode of action/Specificity
- Resistance to digestion *in vitro*
- Expression level and dietary intake

Tier II – Hazard characterization

- **Acute toxicity ← Requires gram quantities**
- Repeated dose toxicity
- Hypothesis-based studies

Delaney et al., 2008. Food Chem Toxicol 46 (Suppl 2):s71-s97

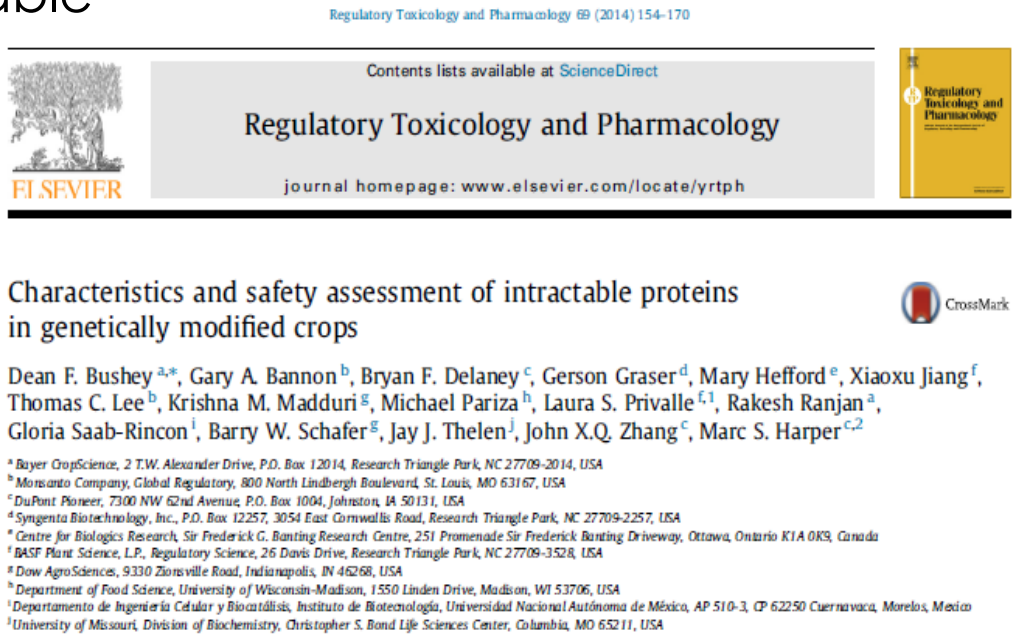
# Proteins and Agricultural Biotechnology

Some crops (will) express proteins that are difficult or impossible to isolate in quantities necessary to conduct animal trials

Characterized as Intractable

Examples:

- Membrane proteins
- Signaling proteins
- Transcription factors
- N-glycosylated proteins
- Resistance proteins
  - (R-proteins)



Regulatory Toxicology and Pharmacology 69 (2014) 154–170

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

Characteristics and safety assessment of intractable proteins in genetically modified crops

Dean F. Bushey [a,*], Gary A. Bannon [b], Bryan F. Delaney [c], Gerson Graser [d], Mary Hefford [e], Xiaoxu Jiang [f], Thomas C. Lee [b], Krishna M. Madduri [g], Michael Pariza [h], Laura S. Privalle [f,1], Rakesh Ranjan [a], Gloria Saab-Rincon [i], Barry W. Schafer [g], Jay J. Thelen [j], John X.Q. Zhang [c], Marc S. Harper [c,2]

[a] Bayer CropScience, 2 T.W. Alexander Drive, P.O. Box 12014, Research Triangle Park, NC 27709-2014, USA
[b] Monsanto Company, Global Regulatory, 800 North Lindbergh Boulevard, St. Louis, MO 63167, USA
[c] DuPont Pioneer, 7300 NW 62nd Avenue, P.O. Box 1004, Johnston, IA 50131, USA
[d] Syngenta Biotechnology, Inc., P.O. Box 12257, 3054 East Cornwallis Road, Research Triangle Park, NC 27709-2257, USA
[e] Centre for Biologics Research, Sir Frederick G. Banting Research Centre, 251 Promenade Sir Frederick Banting Driveway, Ottawa, Ontario K1A 0K9, Canada
[f] BASF Plant Science, L.P., Regulatory Science, 26 Davis Drive, Research Triangle Park, NC 27709-3528, USA
[g] Dow AgroSciences, 9330 Zionsville Road, Indianapolis, IN 46268, USA
[h] Department of Food Science, University of Wisconsin-Madison, 1550 Linden Drive, Madison, WI 53706, USA
[i] Departamento de Ingeniería Celular y Biocatálisis, Instituto de Biotecnología, Universidad Nacional Autónoma de México, AP 510-3, CP 62250 Cuernavaca, Morelos, Mexico
[j] University of Missouri, Division of Biochemistry, Christopher S. Bond Life Sciences Center, Columbia, MO 65211, USA

# What do we know about hazardous proteins?

Many proteins exist in nature that are hazardous but most need to be administered parenterally

- Stinging
- Biting
- Injecting

Some proteins in nature cause adverse effects from oral exposure

- Phytohemagglutinin-E from (undercooked) kidney beans

Adverse effects include:

- Damage the intestinal epithelium
- Absorbed intact and produce a systemic effect

# Consideration of an *in vitro* testing method

## Goals

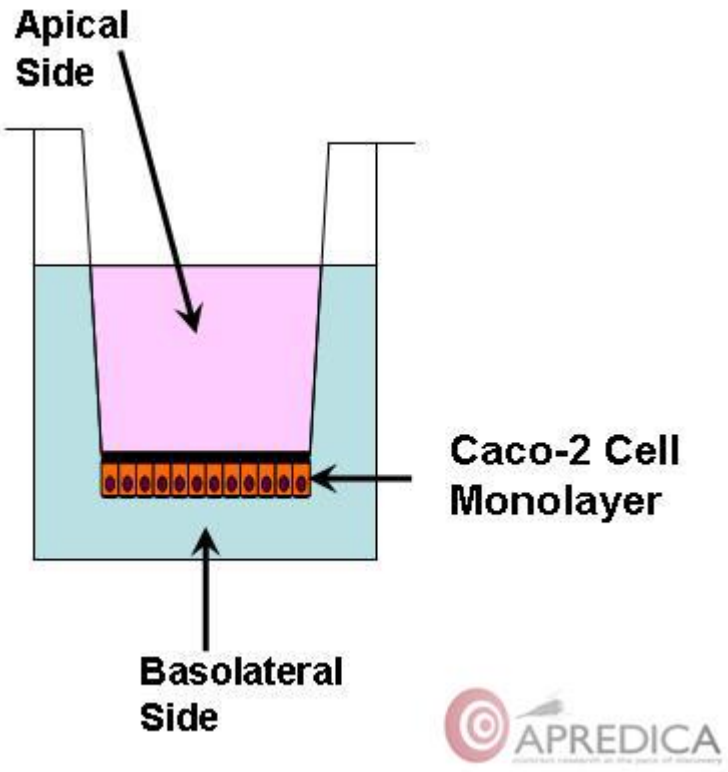
- At least as good as an animal study
- Much smaller quantity of protein
- Reduce use of laboratory animals
- Inexpensive reagents and equipment

## Human intestinal epithelial cell line monolayers

- Examples: T84, Caco-2, and HCT-8
- Derived from colon cancer
- Develop into differentiated monolayer when grown on Transwell™ insert
- Have been utilized in investigation of drug bioavailability

Hurley et al., 2016. Food Chem Toxicol 92:75-87.

# Consideration of an *in vitro* testing method




**Addition of known protein toxins to apical side:**

- Cytotoxicity
  - LDH release
  - MTT
- Monolayer integrity
  - TEER
  - [$^3$H]-Inulin or FITC-inulin
  - HRP

Hurley et al., 2016. Food Chem Toxicol 92:75-87.

# Proof of concept investigation

Comparison of effects following addition of innocuous or known hazardous proteins

- Hazardous proteins
  - Streptolysin O (SLO)
  - Clostridium difficile toxin A (ToxA)
  - Clostridium difficile toxin B (ToxB)
  - Lymphotoxin (LT)
  - Lysteriolysin O (LLO)
  - Mastoparan (Mast)
  - Melittin (Mel)
- Innocuous proteins
  - Bovine serum albumin (BSA)
  - Porcine serum albumin (PSA)
  - Fibronectin (Fib)
  - Rubisco (Rub)

Hurley et al., 2016. Food Chem Toxicol 92:75-87.

| 24 hr | Cytotoxicity | | Monolayer Integrity | | |
|---|---|---|---|---|---|
| | LDH | MTT | [$^3$H]-Inulin | HRP | TEER |
| | T84/Caco2/HCT-8 | T84/Caco2/HCT-8 | T84/Caco2/HCT-8 | T84/Caco2/HCT-8 | T84/Caco2/HCT-8 |
| Toxin | | | | | |
| SLO | N/N/N | N/N/N | N/N/N | N/N/N | N/N/N |
| ToxA | N/N/N | N/N/N | Y/Y/Y | Y/N/N | Y/Y/Y |
| ToxB | N/N/N | N/N/N | Y/Y/Y | Y/Y/Y | Y/Y/Y |
| LT | N/N/N | N/N/N | N/N/N | N/N/N | Y/N/N |
| LLO | N/Y/Y | N/N/N | N/Y/N | N/N/N | N/N/N |
| Mast | Y/Y/Y | Y/Y/N | Y/Y/Y | Y/Y/N | Y/Y/Y |
| Mel | Y/Y/Y | Y/Y/Y | Y/Y/Y | Y/Y/Y | Y/Y/Y |
| Dietary | | | | | |
| BSA | N/N/N | N/N/N | N/N/N | N/N/N | N/N/N |
| PSA | N/N/N | N/N/N | N/N/N | N/N/N | N/N/N |
| Fib | N/N/N | N/N/N | N/N/N | N/N/N | N/N/N |
| Rub | N/N/N | N/N/N | N/N/N | N/N/N | N/N/N |

Hurley et al., 2016. Food Chem Toxicol 92:75-87.

# Proof of concept investigation

## General summary

- Known hazardous proteins damaged monolayers
  - TEER was the most sensitive indicator
- None of the tested innocuous proteins damaged monolayers

Food and Chemical Toxicology 92 (2016) 75–87

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox


### An experimental platform using human intestinal epithelial cell lines to differentiate between hazardous and non-hazardous proteins

Bryan P. Hurley [a, b, *], Waheed Pirzai [a], Alex D. Eaton [a], Marc Harper [c], Jason Roper [d], Cindi Zimmermann [c], Gregory S. Ladics [d], Raymond J. Layton [c], Bryan Delaney [c]

[a] Mucosal Immunology & Biology Research Center, Massachusetts General Hospital, CNY 114 (114-3503), Charlestown, MA, 02129, United States
[b] Department of Pediatrics, Harvard Medical School, Boston, MA, 02129, United States
[c] DuPont Pioneer, 8325 NW 62nd Avenue, Johnston, IA, 50131, United States
[d] DuPont Haskell, 1090 Elkton Road, Newark, DE, 19714, United States

# Effect of digestive enzymes

## General summary

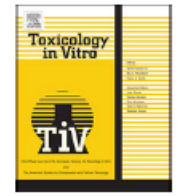
- No effects from innocuous proteins +/- digestive enzymes
- Cytotoxic proteins that **were completely degraded** in the presence of digestive enzymes did not alter monolayer integrity
- Cytotoxic proteins that **resisted degradation** in the presence of digestive enzymes **DID** alter monolayer integrity

Toxicology in Vitro 44 (2017) 85–93

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit




### Incorporation of *in vitro* digestive enzymes in an intestinal epithelial cell line model for protein hazard identification

Lauren K. Markell[a,*], Stephanie M. Wezalis[a], Jason M. Roper[a], Cindi Zimmermann[b], Bryan Delaney[b]

[a] DuPont Haskell Global Centers for Health and Environmental Sciences, 1090 Elkton Road, Newark, DE 19711, USA
[b] DuPont Pioneer, 7300 NW 62nd Avenue, P.O. Box 1004 Johnston, IA 50131, USA

# Primary human polarized small intestinal epithelial barriers

## General summary

- *C. difficile* toxin A altered monolayer integrity at comparable doses observed with cell line monolayers
- Innocuous protein (BSA) did not damage monolayers at any concentration

Food and Chemical Toxicology 106 (2017) 70–77

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox


### Primary human polarized small intestinal epithelial barriers respond differently to a hazardous and an innocuous protein

A.D. Eaton [a], C. Zimmermann [b], B. Delaney [b, 1], B.P. Hurley [a, *, 1]

[a] Department of Pediatrics, Mucosal Immunology & Biology Research Center, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA
[b] DuPont Pioneer, Johnston, IA, USA

# Intractable proteins

**Table 1**
Proteins and controls.

| Protein/toxin | Abbreviation | Category | Vendor* | Range tested |
|---|---|---|---|---|
| Bacteriorhodopsin | BRh | Transmembrane | Sigma-Aldrich | 0.01–10 μg/ml |
| Human c-MET | MET | Signaling | Antibodies-online.com | 0.01–10 μg/ml |
| Follistatin | FST | Signaling glycoprotein | Antibodies-online.com | 0.005–5 μg/ml |
| Activating transcription factor 2 | ATF2 | Transcription Factor | Antibodies-online.com | 0.01–10 μg/ml |
| **Control** | **Abbreviation** | **Category** | **Vendor*** | **Range tested** |
| Assay media | (−) | (−) control | Invitrogen | (−) |
| TritonX-100 | TX-100 | (+) control[a,b] | Sigma-Aldrich | 0.1% |
| *Clostridium difficile* Toxin A | ToxA | Enterotoxin | List Laboratories | 2 μg/ml |
| Flagellin + TNFα | FliC + TNFα | (+) control[c] | Enzo Life Sci. & eBioscience | 0.1 μg/ml each |

| | | Overall Hazard Analysis | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Protein | [Range] | Cytotoxicity | | Disruption of Barrier | | | Inflammation | |
| | μg/ml | LDH | MTT | Inulin | HRP | TEER | IL-8 | IL-6 |
| ToxA | 2 | - | + | + | + | + | + | - |
| BRh | 0.01-10 | - | - | - | - | - | - | - |
| c-MET | 0.01-10 | - | - | - | - | - | - | - |
| FST | 0.005-5 | - | - | - | - | - | - | - |
| ATF2 | 0.01-10 | - | - | - | - | - | - | - |
| | | - | no hazard detected | | + | hazard detected | | |

# Intractable proteins

## General summary

- Various types of intractable proteins were tested in human intestinal epithelial cell monolayers
- None of the tested proteins altered membrane integrity

Food and Chemical Toxicology 98 (2016) 262–268

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox


### Polarized monolayer cultures of human intestinal epithelial cell lines exposed to intractable proteins - *In vitro* hazard identification studies

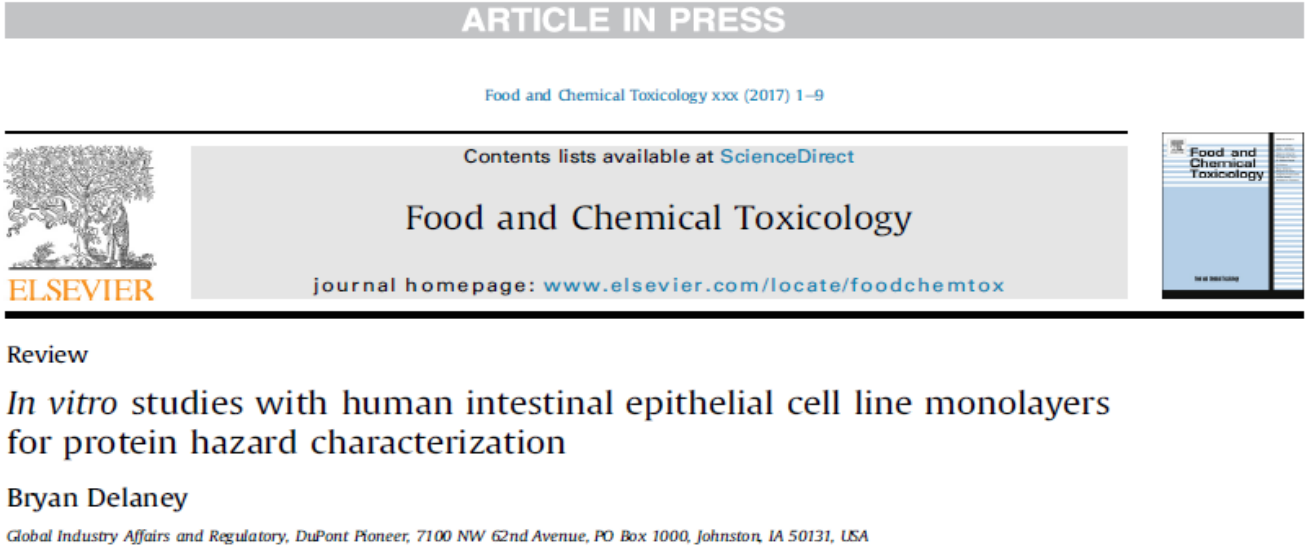
Bryan P. Hurley [a, b, *], Alex D. Eaton [a], Cindi Zimmermann [c], Bryan Delaney [c]

[a] Mucosal Immunology & Biology Research Center, Massachusetts General Hospital, 55 Fruit Street, Jackson 1402, Boston, MA, 02114, USA
[b] Department of Pediatrics, Harvard Medical School, Boston, MA, USA
[c] DuPont Pioneer, 8325 NW 62ndAvenue, Johnston, IA, 50131, USA

# Conclusions

*In vitro* testing with human intestinal epithelial cell line monolayers

- Respond differently to hazardous and non-hazardous proteins
- Role of digestive enzymes can be useful
- No obvious advantage to using primary monolayers
- May be useful for intractable proteins



ARTICLE IN PRESS

Food and Chemical Toxicology xxx (2017) 1–9

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Review

## *In vitro* studies with human intestinal epithelial cell line monolayers for protein hazard characterization

Bryan Delaney

*Global Industry Affairs and Regulatory, DuPont Pioneer, 7100 NW 62nd Avenue, PO Box 1000, Johnston, IA 50131, USA*

# Acknowledgements

DuPont Pioneer

- Cindi Zimmermann
- Marc Harper
- Ray Layton

Massachusetts General Hospital/Harvard Medical School

- Bryan Hurley
- Waheed Pirzai
- Alex Eaton

DuPont Haskell Global Centers for Health and Environmental Sciences

- Jason Roper
- Lauren Markell
- Stephanie Wezalis