Investigation of an *In Vitro* Method for Protein Hazard Characterization

Bryan Delaney, PhD, DABT, Fellow, ATS

DuPont Pioneer
Johnston, Iowa
bryan.delaney@pioneer.com
515.535.7086

Conflict of Interest Statement

- I have no conflict of interest.
- Funding for all of the studies presented here was provided by DuPont Pioneer.
- Studies at MGH were conducted as contract research funded by DuPont Pioneer.

Agricultural Biotechnology

Many crops produced using biotechnology express proteins from a non-native source

- Insect resistance → Cry proteins from *Bacillus thuringiensis*
- Herbicide tolerance → CP4 EPSPS from Agrobacterium
- Disease resistance → Viral coat proteins

Agricultural Biotechnology

Proteins are tested for safety before commercialization

- Weight of evidence approach
- Tier I – Hazard identification
  - History of safe use
  - Bioinformatics
  - Mode of action/Specificity
  - Resistance to digestion *in vitro*
  - Expression level and dietary intake
  - Tier II – Hazard characterization

Tier II – Hazard Characterization

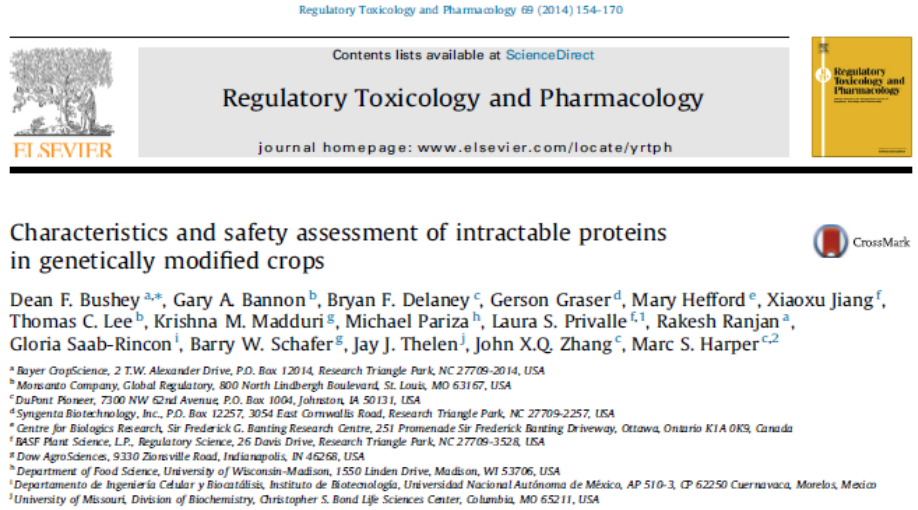
- Acute toxicity
- Repeated dose toxicity
- Hypothesis-based studies

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

Agricultural Biotechnology

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

- Characterized as Intractable proteins
- Includes:
  - 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

Agricultural Biotechnology

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 characterizatio

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

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 exist in nature that cause adverse effects from oral exposure

- Undercooked kidney beans (Phytohaemagglutinin-E)

Adverse effects include:

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

Consideration of an *In Vitro* Testing Method

Goal

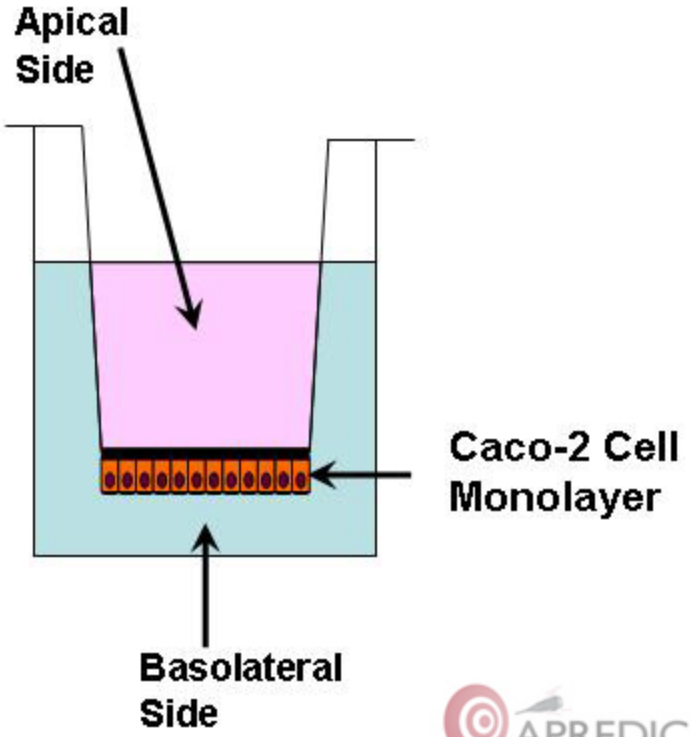
- At least as good as an animal study
- Much smaller quantity of protein
- Reduce use of laboratory animals
- Inexpensive reagents and equipment

Consideration of an *In Vitro* Testing Method

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

Consideration of an *In Vitro* Testing Method



Addition of known protein toxins to apical side:

- Cytotoxicity
  - LDH
  - MTT
- Monolayer integrity
  - Transepithelial Electrical Resistance(TEER)
  - [^{3}H]-Inulin or FITC-Inulin
  - HRP

Consideration of an *In Vitro* Testing Method

Outline

- Proof of concept investigation
- Effect of digestive enzymes
- Primary human polarized small intestinal epithelial barriers
- Intractable proteins

Consideration of an *In Vitro* Testing Method

Outline

- **Proof of concept investigation**
- Effect of digestive enzymes
- Primary human polarized small intestinal epithelial barriers
- Intractable proteins

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)

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

Proof of Concept Investigation

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

Consideration of an *In Vitro* Testing Method

Outline

- Proof of concept investigation
- **Effect of digestive enzymes**
- Primary human polarized small intestinal epithelial barriers
- Intractable proteins

Effect of Digestive Enzymes

Design

Hazardous proteins:

- Phytohaemagglutinin E (PHA-E)
- Concanavalin A (Con A)
- Wheat germ agglutinin (WGA)
- Melittin (Mel)

Innocuous proteins:

- Bovine serum albumin (BSA)
- β-Lactoglobulin (β-Lg)
- Fibronectin (Fib)
- Rubisco (Rub)

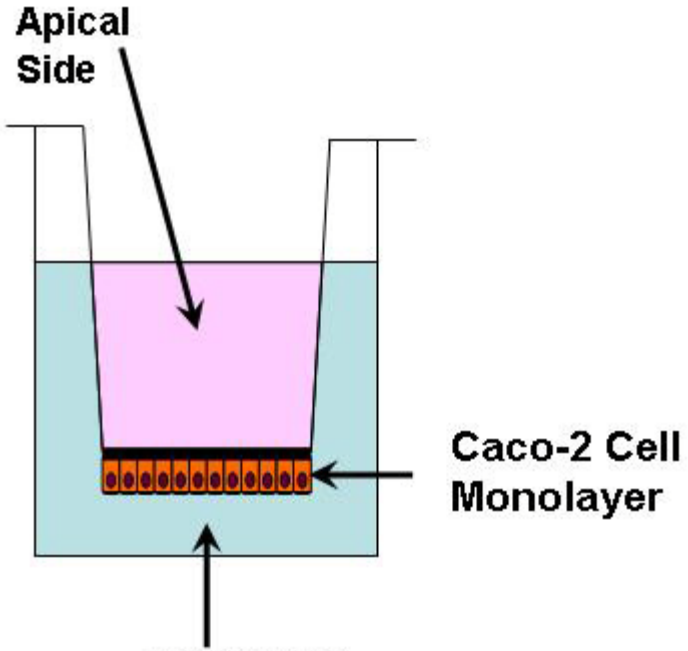
Effect of Digestive Enzymes

Design

Symbol	Treatment	Enzymes	Exposure	Stop
I	None	None		
G	SGF	Pepsin	37 C, 1 hr	NaOH to pH 7.5
GL	SGF → Lyophilized/Suspended	Pepsin	37 C, 1 hr	NaOH to pH 7.5
S	Sequential	Pancreatin	37 C, 2 hr	100 C, 10 min
SL	Sequential → Lyophilized/Suspended	Pancreatin	37 C, 2 hr	100 C, 10 min

Effect of Digestive Enzymes

Design



Measurement at 24 and 48 hr:

- Cytotoxicity
  - Neutral red uptake
- Monolayer integrity
  - FITC dextran (70 kDa)
- Tight junction integrity
  - TEER
  - TRITC-dextran (4.4 kDa)
- Light microscopy

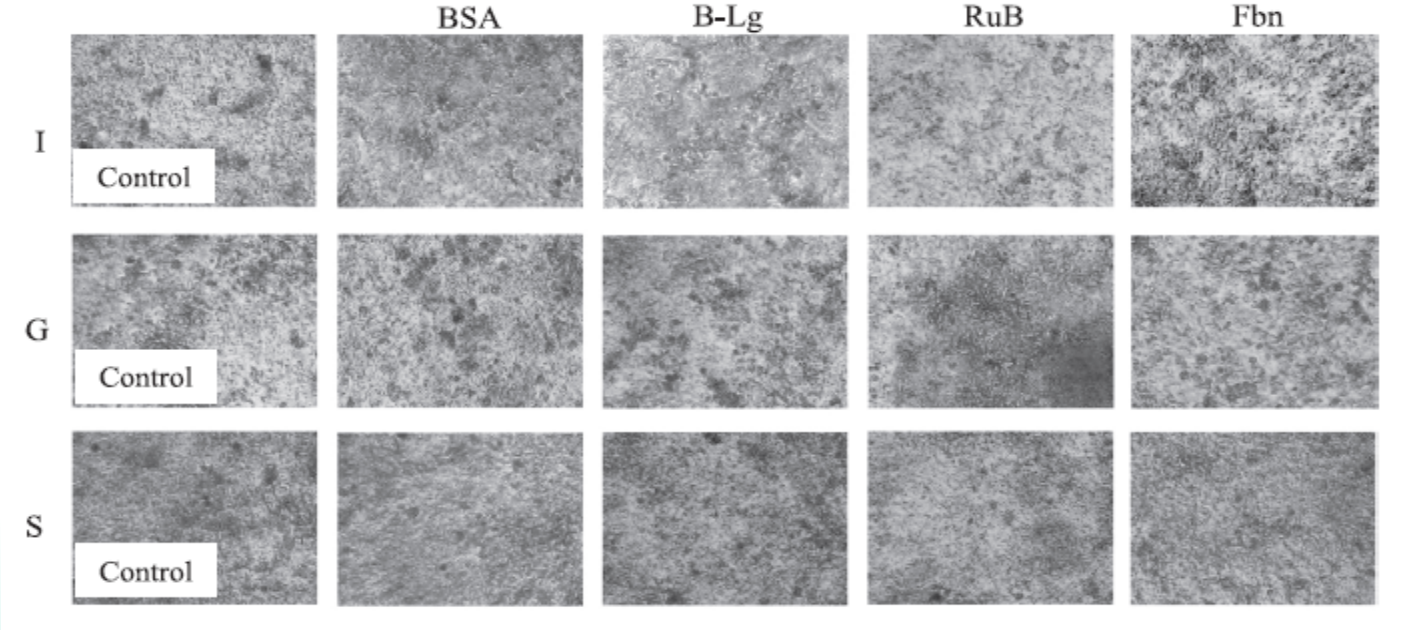
Effect of Digestive Enzymes

Effects on monolayers (selected)

		4.4kDa dextran		70kDa dextran		TEER		Viability
		µg/cm²		µg/cm²		Ohms*cm²		% NRU
		24 h	48 h	24 h	48 h	24 h	48 hs	48 h
Controls	I	0.6 (0.1)	0.8 (0.1)	ND	0.006 (0.002)	423 (65)	508 (65)	100 (10)
	SGF	0.8 (0.1)	1.1 (0.1)	ND	ND	531 (146)	465 (132)	93 (10)
	SGIF	1.5 (0.5)	2.0 (0.3)	ND	ND	2386 (451)	1071 (218)	101 (21)
BSA 1000 µg/mL	I	0.6 (0.3)	0.7 (0.3)	ND	ND	755 (42)	626 (157)	98 (4)
	SGF	0.5 (0.2)	0.7 (0.1)	ND	ND	1243 (408)	1464 (489)	101 (8)
	SGIF	1.8 (0.6)	2.0 (0.7)	ND	ND	2989 (674)	1283 (300)	108 (19)
PHA-E 1000 µg/mL	I	1.7 (0.8)	14.4 (1.6)	**0.65 (0.64)**	**6.4 (3.5)**	419 (169)	51 (11)	112 (11)
	SGF	3.7 (1.0)	13.1 (5.0)	**1.32 (0.61)**	**4.0 (1.4)**	330 (78)	197 (65)	109 (3)
	SGIF	5.0 (1.4)	2.4 (0.7)	**0.87 (0.16)**	**1.2 (0.4)**	832 (220)	1321 (132)	113 (11)
Mlt 500 µg/mL	I	16.8 (2.9)	39.9 (3.9)	**15.6 (1.0)**	**24.9 (0.7)**	11 (2)	4 (2)	17 (11)
	SGF	0.5 (0.1)	0.7 (0.1)	0.01 (0.01)	0.01 (0.01)	589 (152)	2991 (465)	98 (2)
	SGIF	1.1 (0.5)	1.5 (0.4)	0.03 (0.01)	0.03 (0.02)	1784 (341)	1692 (77)	96 (3)

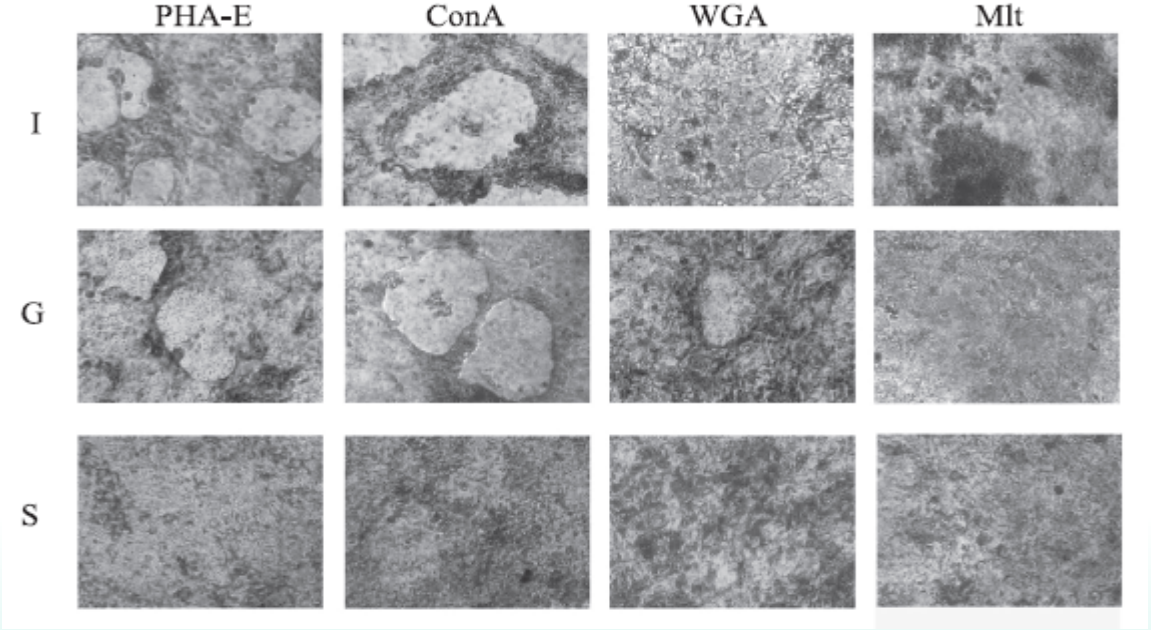
Effect of Digestive Enzymes

Light microscopy



Effect of Digestive Enzymes

Light microscopy

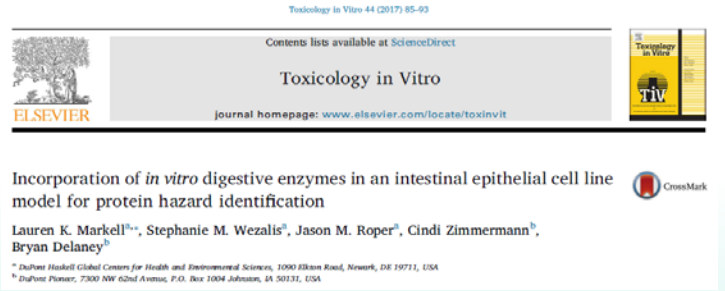


Effect of Digestive Enzymes

Summary

- First level bullet No effects from innocuous proteins +/- digestive enzymes
- Hazardous proteins that **were completely degraded** in the presence of digestive enzymes did **NOT** alter monolayer integrity
- Hazardous proteins that **resisted degradation** in the presence of digestive enzymes DID alter

  monolayer integrity



Consideration of an *In Vitro* Testing Method

Outline

- Proof of concept investigation
- Effect of digestive enzymes
- **Primary human polarized small intestinal epithelial barriers**
- Intractable proteins

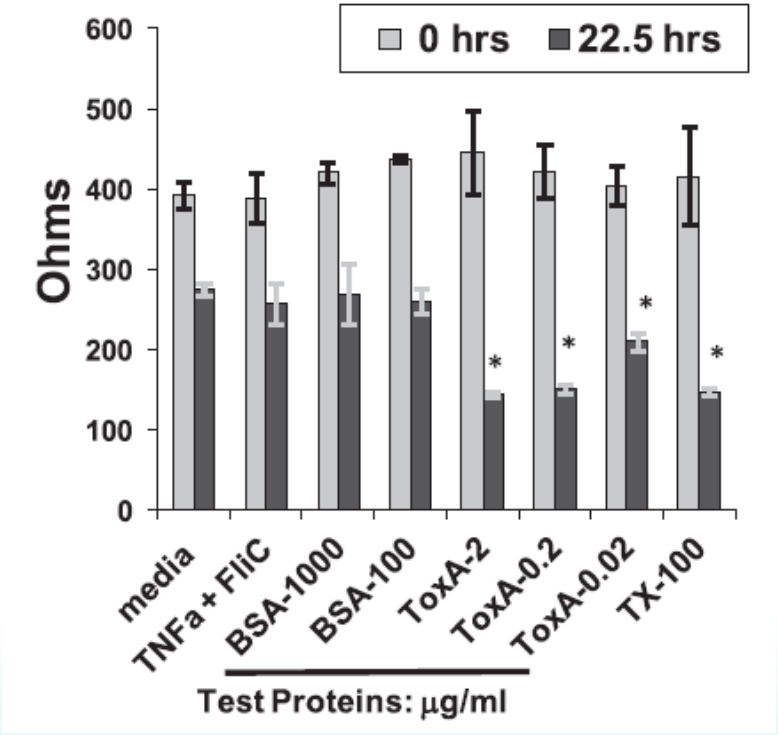
Primary Human Polarized Small Intestinal Epithelial Barriers

Design

- Primary cells are not transformed = $$$
- Heterogeneous cell population (not just epithelial cells)
- Comparison of BSA and *C. difficile* toxin A
  - TEER
  - FITC-Inulin flux
  - HRP flux
  - MTT conversion
  - LDH release

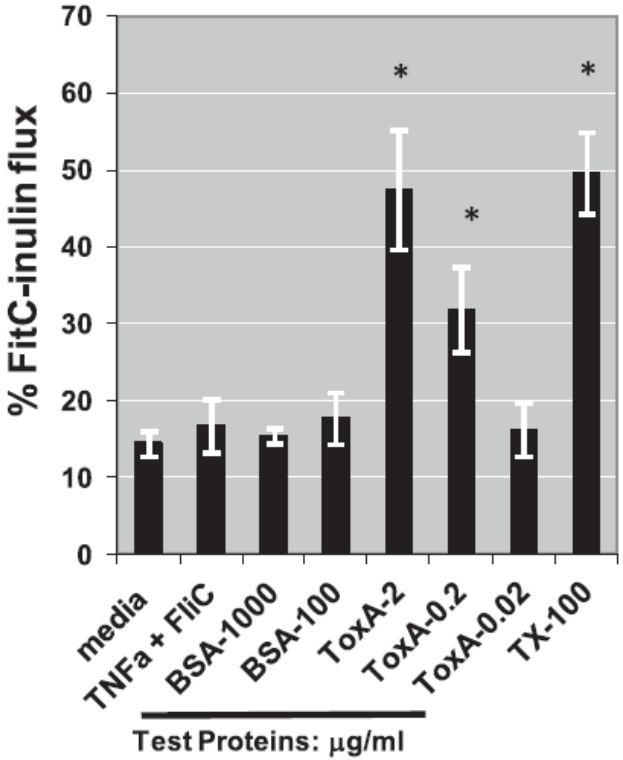
Primary Human Polarized Small Intestinal Epithelial Barriers

TEER



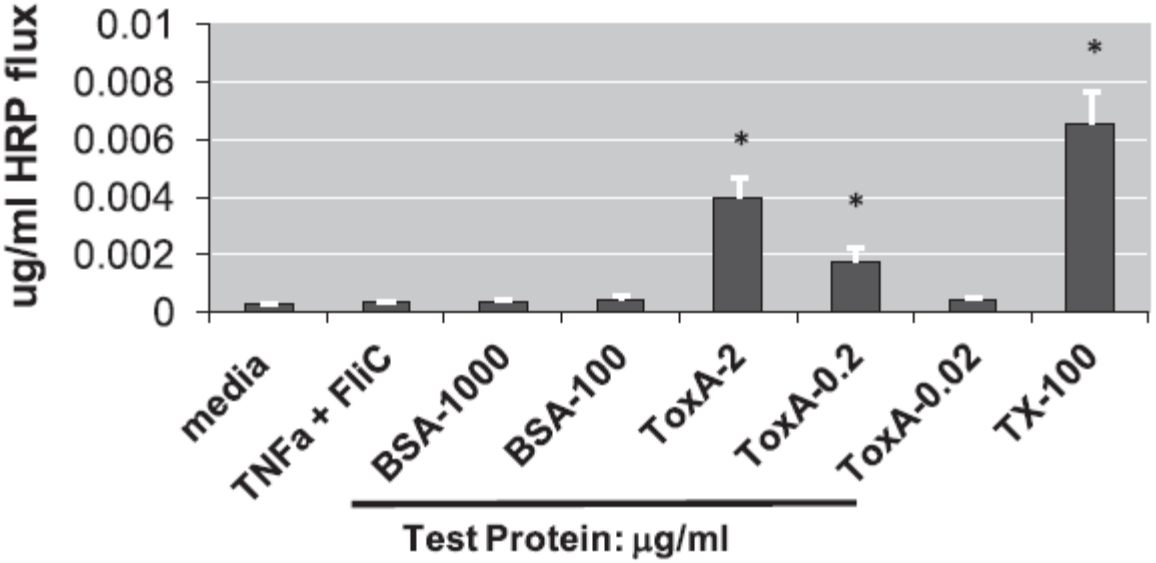
Primary Human Polarized Small Intestinal Epithelial Barriers

FITC-Inulin flux



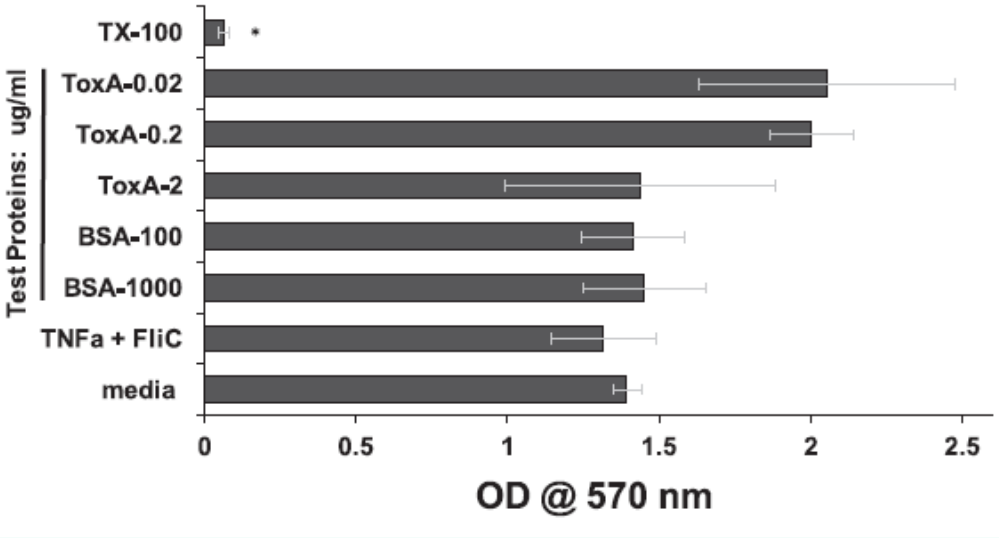
Primary Human Polarized Small Intestinal Epithelial Barriers

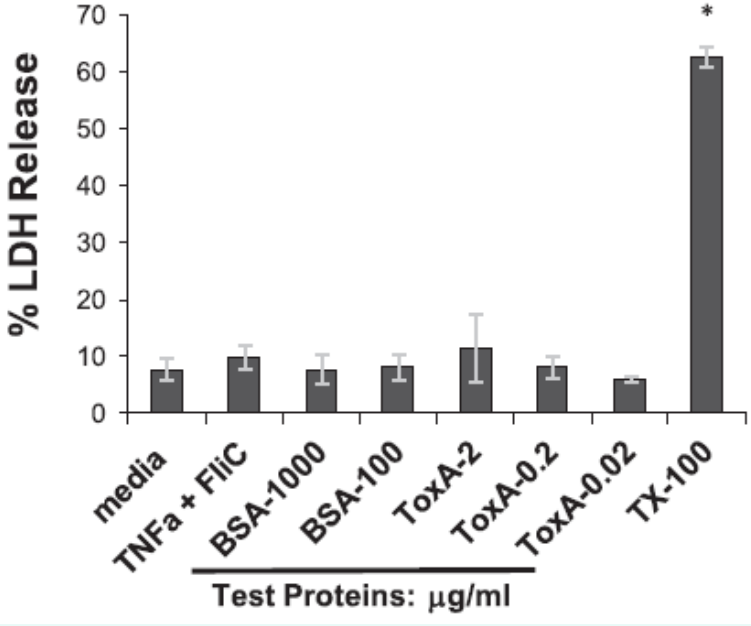
HRP flux



Primary human polarized small intestinal epithelial barriers

Viability

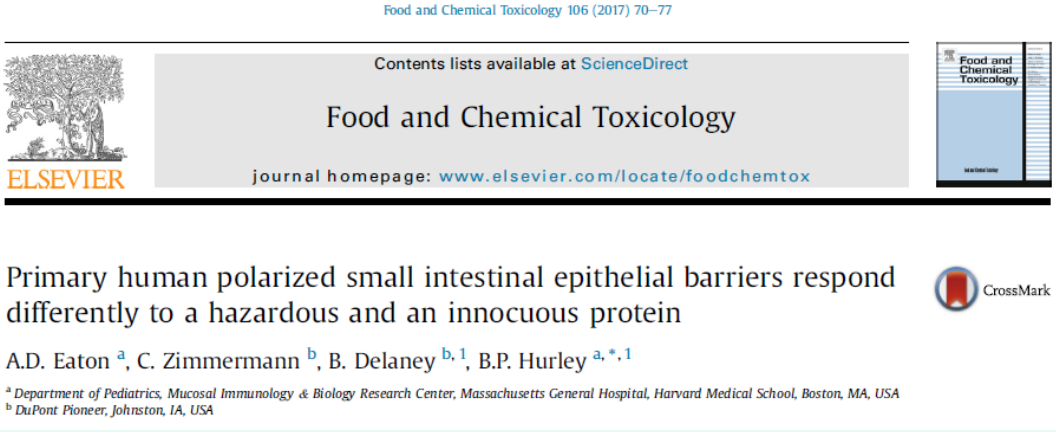




Primary Human Polarized Small Intestinal Epithelial Barriers

Summary

- *C. difficile* toxin A altered monolayer integrity at comparable doses 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

Consideration of an *In Vitro* Testing Method

Outline

- Proof of concept investigation
- Effect of digestive enzymes
- Primary human polarized small intestinal epithelial barriers
- **Intractable proteins**

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

Intractable Proteins

		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

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 for protein hazard characterization:

- Human intestinal epithelial cell line monolayers appear to respond differently to hazardous and non-hazardous proteins
- Effect of digestive enzymes can be incorporated
- Results in cell lines correlate with primary cell monolayers
- May be useful for intractable proteins

References (all available open access)

- Bushey DF, Bannon GA, Delaney B, Graser G, Hefford M, Jiang X, Lee TC, Madduri KM, Pariza M, Privalle LS, Ranjan R, Saab-Rincon G, Schafer BW, Thelen JJ, Zhang JXQ, and Harper MS. 2014. Characteristics and safety assessment of intractable proteins in genetically modified crops. *Regulatory Toxicology and Pharmacology* 69:154-170.

- Hurley BP, Pirzai W, Eaton AD, Harper M, Ladics G, Roper J, Zimmermann C, Layton RJ, and Delaney B. 2016. An experimental platform using human intestinal epithelial cell lines to differentiate between hazardous and non-hazardous proteins. *Food and Chemical Toxicology* 92:75-87.

- Hurley BP, Eaton AD, Zimmermann C, and Delaney B. 2016. Polarized monolayer cultures of human intestinal epithelial cell lines exposed to intractable proteins - *In vitro* hazard identification studies. *Food and Chemical Toxicology* 98:262-268.

- Eaton AD, Hurley BP, Zimmermann C, and Delaney B. 2017. Primary human intestinal epithelial barriers respond differently to a hazardous and an innocuous protein. *Food and Chemical Toxicology* 106 (Part A):70-77.

- Markell LK, Wezalis SM, Roper JM, Zimmermann C, and Delaney B. 2017. Incorporation of *in vitro* digestive enzymes in an intestinal epithelial cell line model for protein hazard identification. *Toxicology In Vitro* 44:85-93.

Acknowledgements

DuPont Pioneer

Cindi Zimmermann
Marc Harper
Ray Layton

MGH/Harvard Medical School

Bryan Hurley
Waheed Pirzai
Alex Eaton

DuPont Haskell Global Centers for Health and Environmental Sciences

Jason Roper
Stephanie Wezalis
Lauren Markell
Greg Ladics