Agriculture and Agri-Food Canada | Agriculture et Agroalimentaire Canada

Allergenic Proteins of Mustard: What We Know

Janitha Wanasundara, Ph.D.
Agriculture and Agri-Food Canada
Saskatoon Research Centre

Canada

Mustard as a food allergen

- Background
  - Food allergies
  - Regulatory (Canada)
  - Protein chemistry
- Related research
- Summary

Food allergies

- An adverse immune response to a food protein

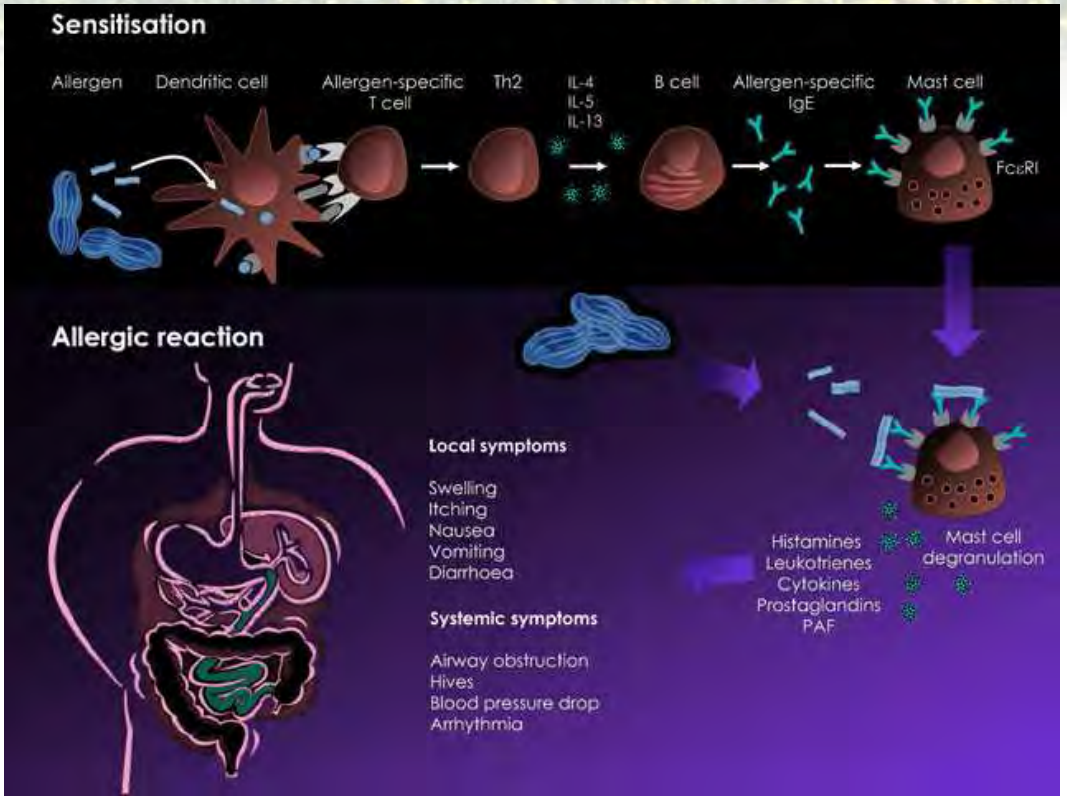
Two stages

- Sensitization

Immune system starts producing antibody in response to eating a food

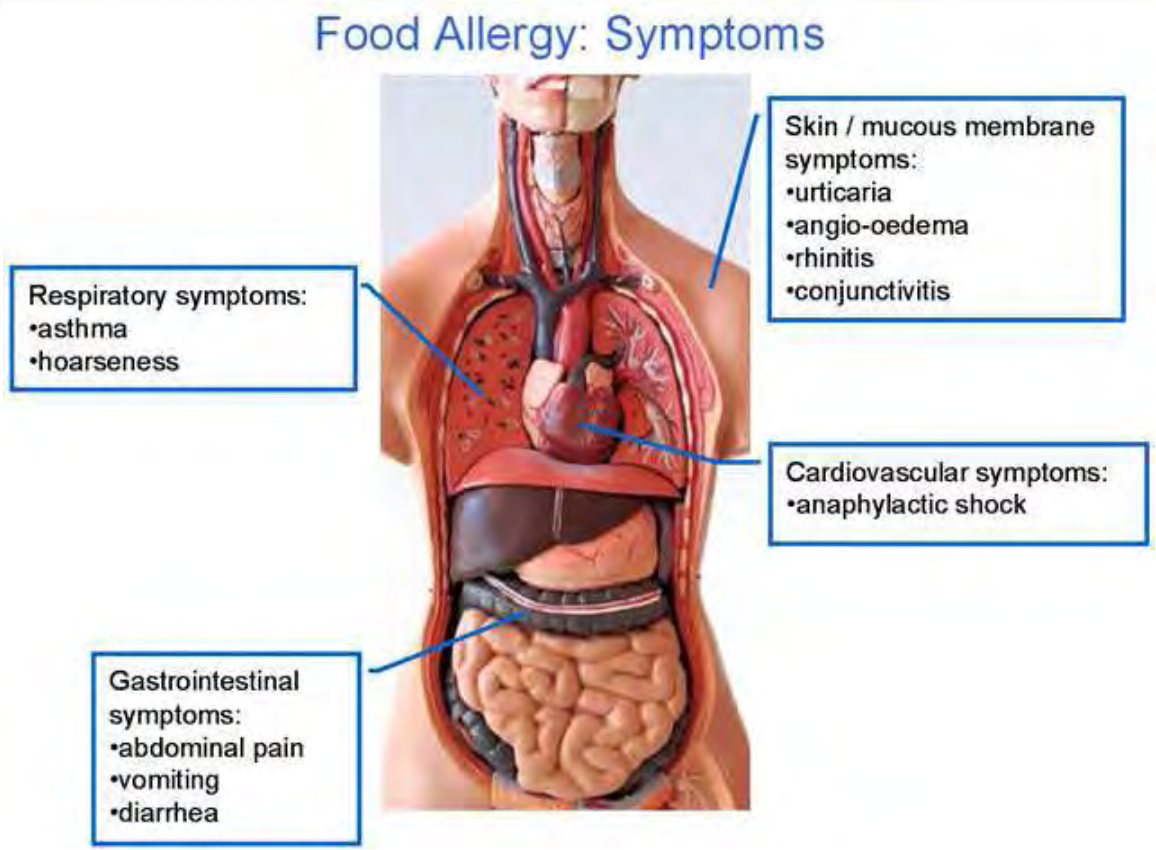
- Allergic reaction

Food is eaten for 2nd time triggering all allergic reactions.



Food allergies

- Allergic reaction is body over produces the antibody IgE causing many symptoms.



Mustard as an allergen – Regulatory background

- Eggs
- Milk
- Peanuts
- Seafood (fish, crustaceans, shellfish)
- Sesame
- Soy
- Sulphites (food additive)
- Wheat
- Mustard

In Canada
food allergies affect:
6% young children
3 - 4% adults

Mustard as an allergen – Regulatory background

Proposed regulatory amendments for allergens and allergen labeling: Canada Gazette Part I- July 26, 2008

Objectives

To minimize risk associated with inadvertent consumption of undeclared food allergens, gluten sources and sulphites

To maximize choices of safe and nutritious foods for consumers with food allergies and celiac disease.

Public comment period:

- Mustard should be added to the list and require enhanced labeling.
- Significant number of individual families and health professionals reported observations/witnessing or experiencing adverse reactions associated with consumption of mustard. Garlic and Onion

Mustard as an allergen – Regulatory background

Establishing a new allergen

- Systematic review of available scientific literature

- Pre-established criteria for assessment: Recommendations required to amend the Codex Alimantarius Commission

Mustard as an allergen - Regulatory background

Examples of pre-established criteria

Scientifically proven cause-effect relationship between exposure to the food in question and the symptoms of severe allergic reaction should exist.

Reports of systematic-type reactions following exposure to the food must be identified.

Scientific assessment of all available information on reaction from the food (prevalence data in children, adults, clinical studies).

Additional considerations – can it be a hidden source of allergen in prepackaged foods – in the context of labeling.
e.g. spices, hydrolyzed proteins

Mustard as an allergen - Regulatory background

Review – Is mustard a priority allergen?

42 scientific publications considered.

- Occurrence of mustard food allergies in children and adults - Canadian case reports support.
- 3rd or 4th most food allergen – European data (France)
- Recognized allergen by International Union of Immunological Societies (mustard among the list of 14 allergens, 2009)
- Credible cause-effect relationship – Supported by positive double blind, placebo-controlled, food allergy challenge (DBPCFC). 1.6 mg MP Spain, 2003; 0.8 mg MP France, 2005
- Report of severe systemic reactions following exposure to small amounts.
- Allergenic protein – resistant to heat and digestive enzymes
- Can be a hidden allergen when in mixtures and preparations – high thermal stability of allergen

Mustard as an allergen - Regulatory background

Sufficient scientific evidence to fulfill all Canadian Criteria for addition of mustard to the list of priority food allergens in Canada.

Health Canada recommended:

Mustard must be added to the list of food allergens requiring enhanced labeling.

Proposed amendments to the Food and Drug Regulations on labeling of food allergens – decision should reflect accordingly.

Final regulations in Canada Gazette Part II- 2010

www.hc-sc.gc.ca

food & nutrition>food safety>food allergy & intolerances

Mustard as an allergen – Protein chemistry

- Mustard – *Brassicaceae*/*Cruciferae* species plants.
  - *Brassica juncea*: Brown and Oriental mustard
  - *Sinapis alba*: Yellow (white) mustard

- Primary use of mustard – based on glucosinolate type and content, isothiocyanates
- Yellow mustard – based on glucosinolates, mucilage and protein

  Variety of uses

Where we find mustard?

Condiment and Spice – Whole or cracked seed, dry mixes, ground, wide range of products

Paste – Ground seeds, water, vinegar, additives pungency, consistency

Ground flour: Heat treated (de-heated) mustard flour, DHMF

- *S. alba*, used for its' protein and polysaccharides
- Myrosinase inactivated by heat (110-180° C); no "bite"
- Binder, Emulsifier, Oil & Water absorber, Flavour enhancer, Thickener, Antioxidant & Antimicrobial agent

Where is enzyme inactivated mustard?

- **Processed Meat products**

  Emulsifies fat
  Binds water and fat - reduce cooking loss (4%)
  Increases peelability of sausages
  Reduces lipid oxidation of products –preserves flavour
  Replaces, milk protein ingredients
  Reduces production cost

- **Sauces and Mayonnaise**

  Emulsifies fat
  A thickener – replaces polysaccharide gums
  Replaces egg yolk powder
  Increases smoothness and texture - high liquid absorption capacity
  A natural replacer for tomato solids

Mustard as an allergen – Protein chemistry

International Union of Immunological Societies (IUIS) Allergen nomenclature sub-committee

List of allergens

- Plante Magnoliopsida
  - Brassicales
    - *Brassica juncea* : Bra j 1
    - *Brassica napus* : Bra n 1
    - *Brassica oleracea :* Bra o 3
    - *Brassica rapa* : Bra r 1, Bra r 2
    - *Sinapis alba* : Sin a 1, Sin a 2, Sin a 3, Sin a 4

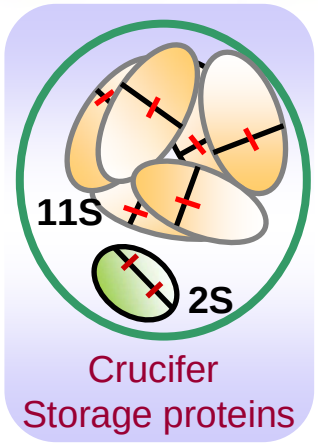
2S albumin

11S globulin

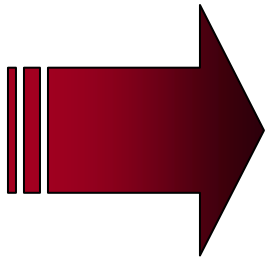
ns-LTP

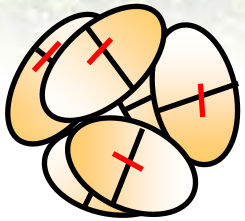
Profilin
Peach, melon

Mustard as an allergen - Protein chemistry



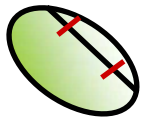
Crucifer
Storage proteins





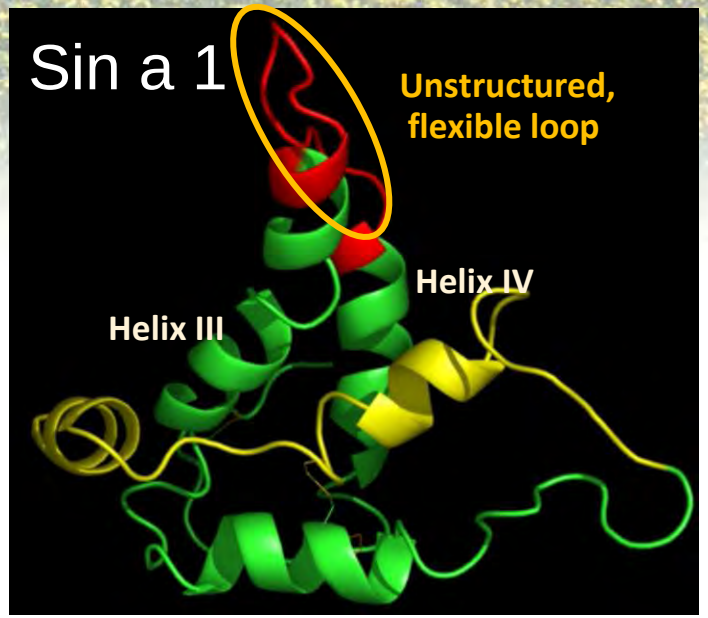
11S, Cruciferin
Cupin superfamily
A Hexamer
Large molecule
300-330 kDa
Structure & amino acid composition different from 2S albumin (Napin)

2S, Napin
Prolamin superfamily
A Monomer/Dimer
Small molecule 13-18 kDa
Different biological activities



Polymorphic proteins codified by multigene families

Mustard as an allergen - Protein chemistry



- Low molecular weight napin; 14.1 kDa
- Two polypeptide chains:
  2 inter- and 2 intra- chain S-S bonds
- Primary structure:
  …C..C/..CC..CXC..C….C… motif
  High in Pro & Gln, one Tyr
- Secondary structure:
  5 helices, right handed super helix
- Tertiary structure
  similar to LTPs, cereal α–amylase and trypsin inhibitors
- Recognized epitopes:
  solvent exposed
  strong possibility to be in the hypervariable region

Mustard as an allergen - Protein chemistry

Sin a1	Identity	Similarity
Bra j 1	86%	91%
Bra n 1	92%	94%

Bra n 1

Sin a 1

Bra j 1

```
P01090 2                                                              QQ 62
P01091 2                                                              QQ 18
P27740 2                                                              QQ 62
P17333 2                                                              QQ 62
P80208 2SS3_BRANA -------------------------------------SAGPFRIPKCRKEFQQAQHLRACQQ 25
P15322 ALL1_SINAL -------------------------------------PAGPFRIPKCRKEFQQAQHLRACQQ 25
P80207 ALL1_BRAJU --------------------------------------AGPFRFPRCRKEFQQAQHLRACQQ 24
P24565 2SSI_BRANA -----------------------------------------QPQKCQREFQQEQHLRACQQ 20
                                                            :*::**** ***:****

2SS2_BRANA WLHKQAMQSGGGPSWTLDGEFDFEDDMENPQGPQQRPPLLQQCCNELHQEEPLCVCPTLKGA 124
2SS1_BRANA WLHKQAMQSGGGPSWTLDGEFDFEDDMEK-QGPQQRPPLHQQYCNELQQEEPLCVCPTLKGA 79
2SSB_BRANA WLHKQAMQSGSGPSWTLDGEFDFEDDMENPQGPQQRPPLLQQCCNELHQEEPLCVCPTLKGA 124
2SS4_BRANA WLHKQAMQSGSGPSWTLDGEFDFEDDMENPQGPQQRPPLLQQCCNELHQEEPLCVCPTLKGA 124
2SS3_BRANA WLHKQAMQSGSGP-----------------QGPQQRPPLLQQCCNELHQEEPLCVCPTLKGA 70
ALL1_SINAL WLHKQAMQSGSGPSWTLDDEFDFEDDMENPQGPQQRPPLLQQCCNELHQEEPLCVCPTLKGA 87
ALL1_BRAJU WLHKQAMQSGSGP---------------QPQGPQQRPPLLQQCCNELHQEEPLCVCPTLKGA 71
2SSI_BRANA WIRQQLAGSPFQS-----------------GPQEGPWLREQCCNELYQEDQVCVCPTLKQA 64
           *:::*    *    .                ***: * *  :* **** **: :******: *
```

Small chain

Large chain

```
2SS2_BRANA SKAVKQQIQQQGQQQGK----QQMVSRIYQTATHLPKVCNIPQVSVCPFQKTMPGPSY    178
2SS1_BRANA SKAVKQQIQQQEQQQGK-----QQMVNRIYQTATHLPKVCNIPQVSVCPFQKTMPGPSY   133
2SSB_BRANA SKAVKQQIQQQGQQQGK-----LQMVSRIYQTATHLPKVCKIPQVSVCPFQKTMPGPSY   178
2SS4_BRANA SKAVKQQVRQQQGQQGQQ---LQQVISRIYQTATHLPKVCNIPQVSVCPFQKTMPGPSY   180
2SS3_BRANA SRAVKQQVRQQQGQQGQQ---LQQVISRIYQTATHLPKVCNIPQVSVCPFQKTMPGPS-   126
ALL1_SINAL SKAVKQQVRQQLGQQGQQGPHLQHVISRIYQTATHLPKVCNIRQVSVCPFKKTMPGPS-   146
ALL1_BRAJU SKAVKQQIRQQGQQQGQQGQQLQHEISRIYQTATHLPRVCNIPRVSICPFQKTMPGPS-   130
2SSI_BRANA AKSVRVQG-----QHGP------FQSTRIYQIAKNLPNVCNMKQIGTCPFIAIPFFP--   111
           :::*: *      *:*        .**** *.:**.**:: ::. ***
```

Hypervariable region

Research – Yellow mustard

- Quantification of allergenic napin protein
  Sin a 1 and Bra j 1 – Immunochemical

- Effect of low pH and heat treatment on detection of Sin a 1 – Model study

- Pepsinolysis of napin and cruciferin – *In vitro*

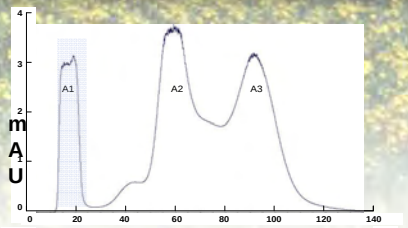
Research – Yellow mustard

- Commercial kits – total mustard protein, not specific
- Purified Sin a 1 and Bra j 1
- Rabbit pAb developed against purified protein
- S-ELISA, LOD 20 ppm

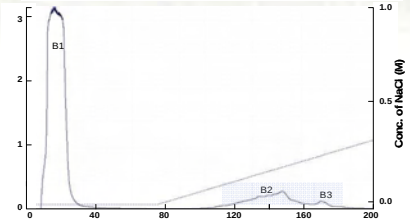
Purification and identity confirmation of Sin a 1

Protein extract

Size Exclusion Chromatography (SEC):
Sephadex G-25
Tris-HCl (50 mM, pH 8.5, 1 M NaCl)

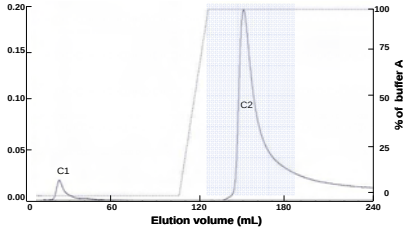


Cation Exchange Chromatography (CEC):
*Resource S*Buffer A: Tris-HCl (50 mM, pH 8.5, 5 mM EDTA, 28 mM $NaHSO_3$)Buffer B: Tris-HCl (50 mM, pH 8.5, 1 M NaCl, 28 mM $NaHSO_3$)

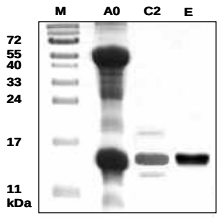


Hydrophobic Interaction Chromatography (HIC):
*Phenyl Sepharose 6*Buffer A: Tris-HCl (50 mM, pH 8.5, 5 mM EDTA, 28 mM $NaHSO_3$)

Buffer C: Tris-HCl (50 mM, pH 8.5, 5 mM EDTA, 28 mM $NaHSO_3$, 0.85 M Na_2SO_4)

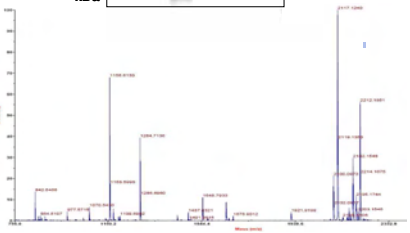


Electroelution
model 422 Electro-Eluter
Lane M, Molecular marker; Lane A0, YM proteins extract; Lane C2, Protein fractions containing napin peak from HIC; Lane E, Protein from electroelution, after S-S band reduction without β-ME



Mass fingerprinting
Voyager-DE STR MALDI instrument

Protein identification was searched against NCBI using MASCOT



Research - Level of 2S allergenic protein

Sin a 1 content of yellow mustard as determined by sandwich-ELISA (Shim & Wanasundara 2008)

YM	Total protein (mg/g)[c]	Sin a 1 (mg/g)[c]	Sin a 1 content as % of total soluble protein[d]
AC Base	244.3±11.8	1.80±0.29	0.737
AC Pennant	324.5 ±9.2	2.32±0.28	0.715
Andante	388.7±19.7	2.94±0.50	0.756
HS3	176.4±11.2	0.82±0.09	0.465
HS4	181.8±13.5	0.82±0.08	0.451
HS5	209.6±13.8	1.08±0.12	0.515

Bra j 1 content of brown & oriental mustard as determined by sandwich-ELISA
(Shim & Wanasundara, unpublished)

Mustard	Total protein (mg/g)[c]	Bra j 1 (mg/g)[c]	Bra j 1 content as % of total soluble protein[d]
AC Vulcan (OM)	208.1±20.1	0.59±0.05	0.283
Duchess (BM)	279.1±19.7	1.00±0.09	0.360
Canola quality (OM)	306.3 ±28.7	0.63±0. 05	0.206

Research – Effect of low pH and heat treatment

- Dehulled mustard flour – spice

  Salad dressings and sauces: pH 4.0 and acetic acid

- Deheated mustard flour - binder/filler

  Meat and bakery applications: pH 6-7, various additives, heat treatment

- Dehulled yellow mustard flour (YMF) - commercial product
- Sin a 1 using pAb-Sin a 1

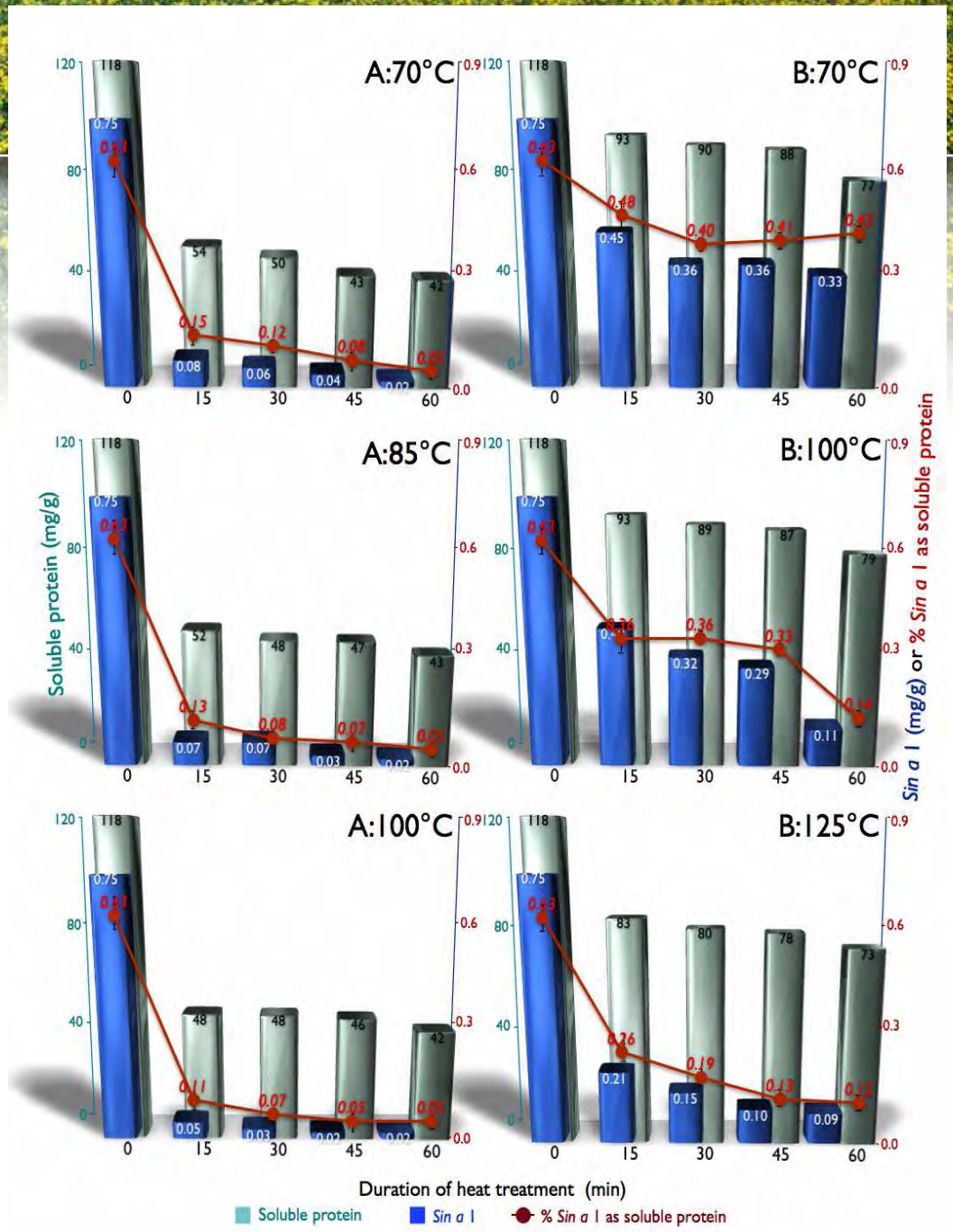
Research – Effect of heat

Commercial YMF

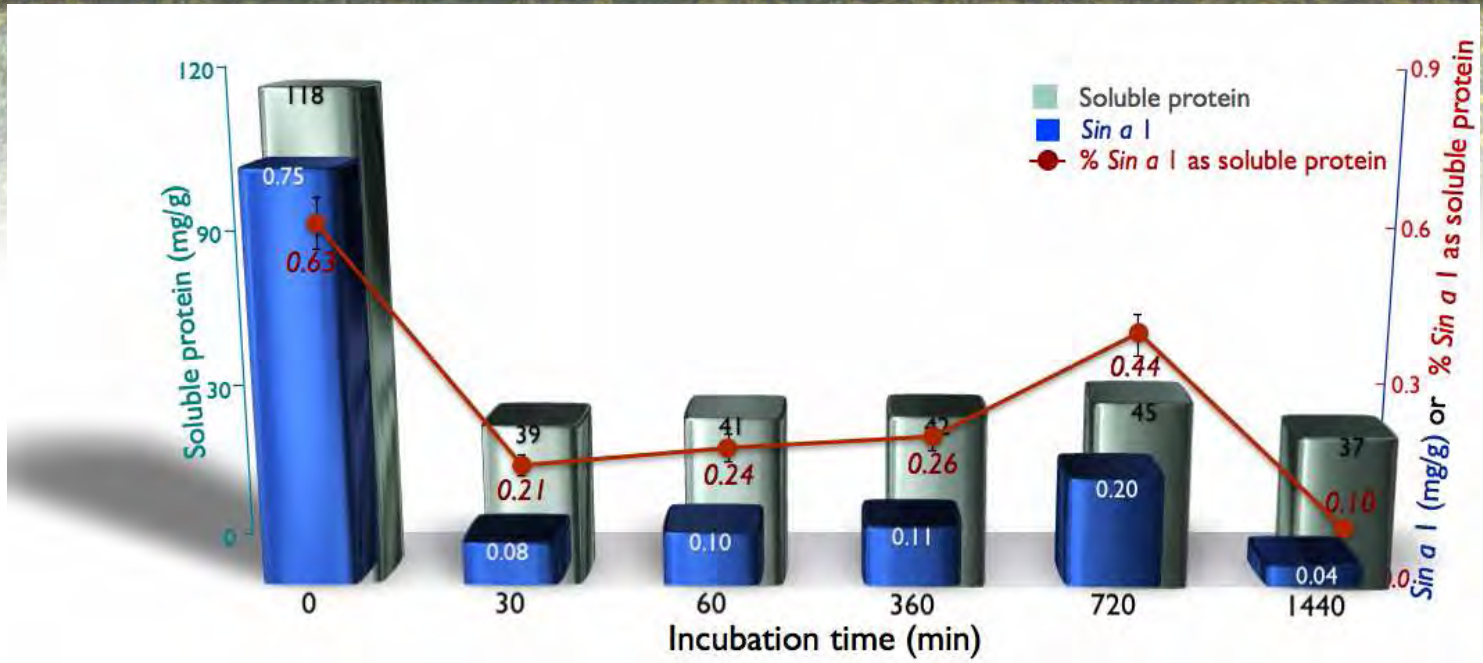
A: Heat treatment of slurry (1:20, w:v)

B: Heat treatment of dry powder

- All samples were freeze dried after treatment
- Extraction were with PBS (pH 7.4).



Research - Effect of low pH

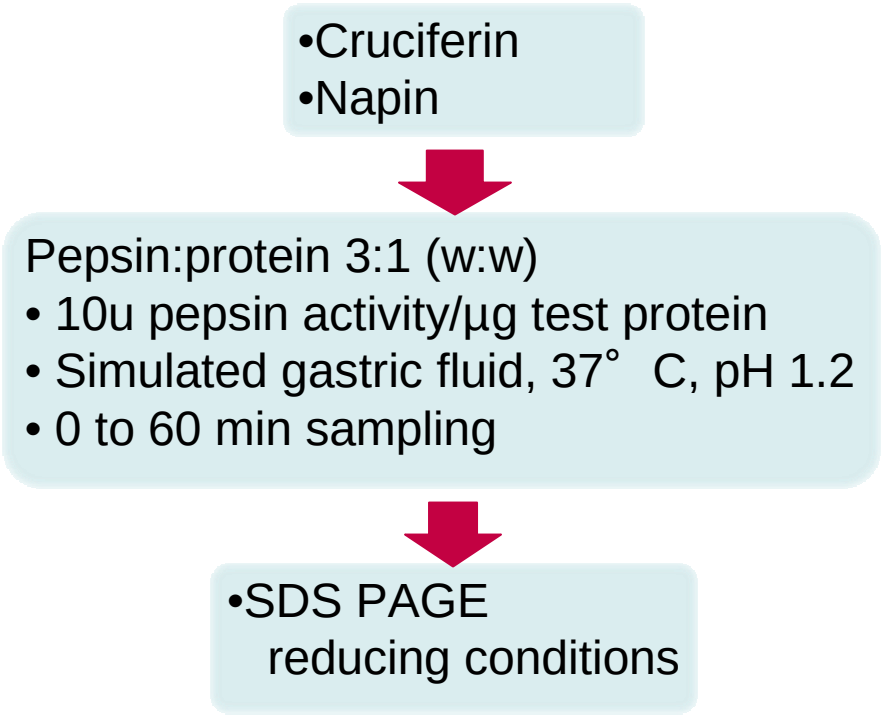


Commercial YMF

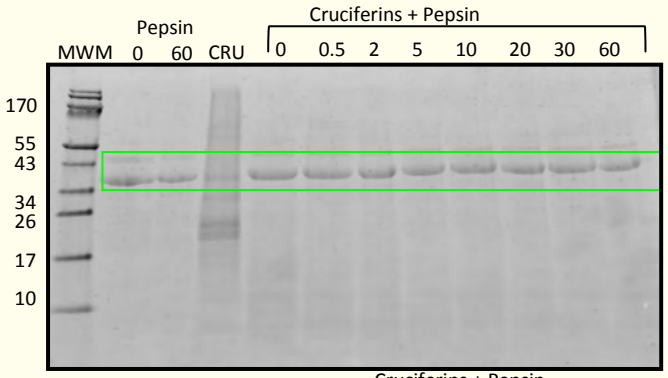
- Incubated in acetic acid (4%, v/v) solution at RT.
- After incubation samples were freeze dried and extractions were prepared in PBS buffer at pH 7.4

Research - *In vitro* digestion stability

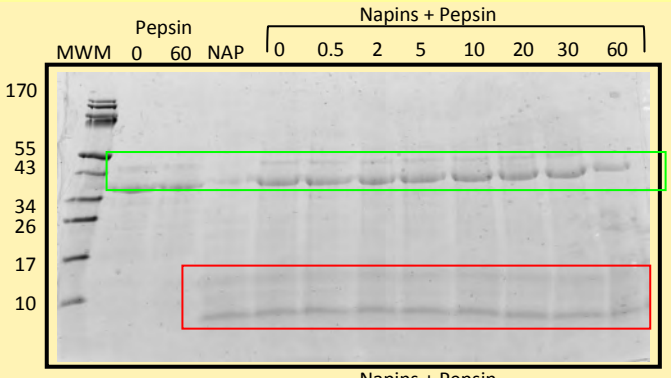
- Assess digestion stability of cruciferin and napin
- *In vitro* pepsin digestion assay for safety of novel proteins
- For food allergens (*Thomas et al.* 2004, Regulatory Toxicology & Pharmacology, 39:87-98)

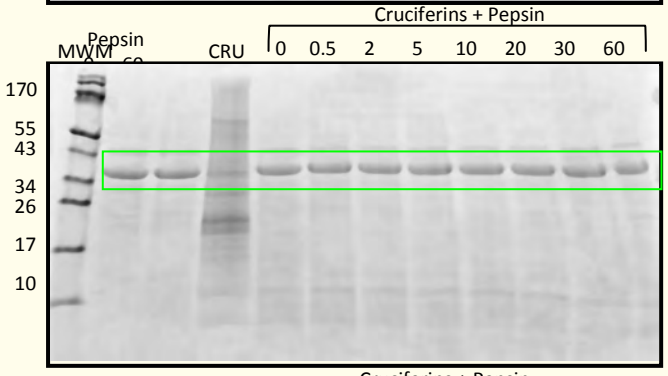


Research – *B. juncea* - cruciferin and napin

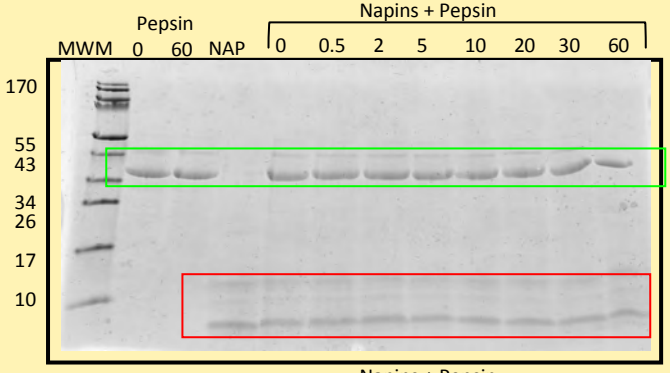


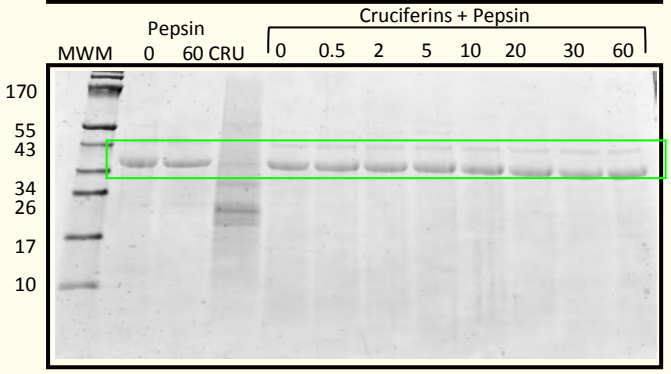
AC Vulcan (oriental)



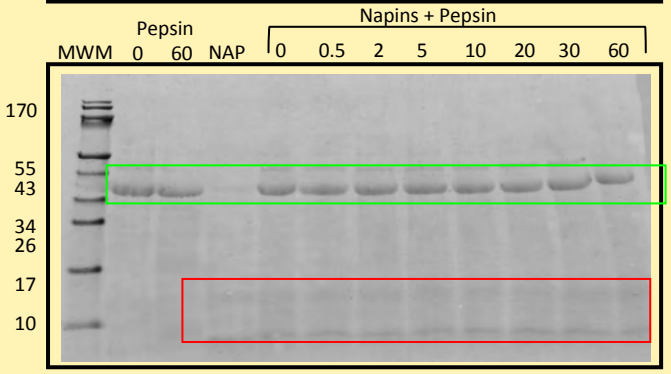


Duchess (brown)

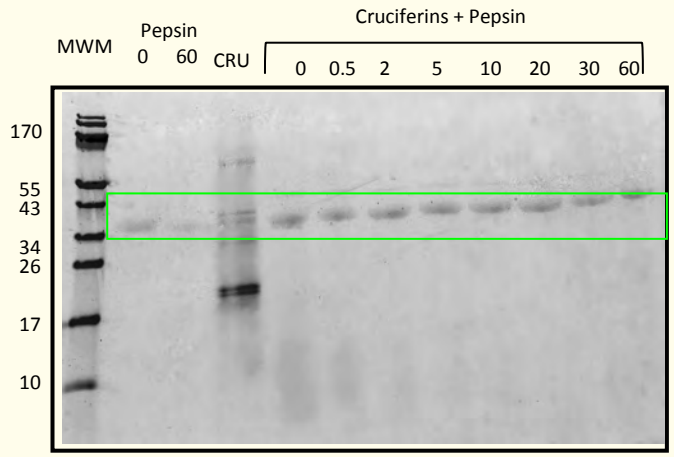




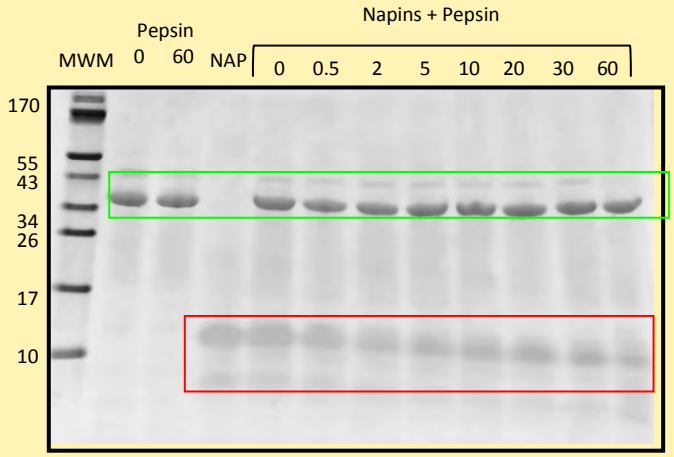
Canola quality

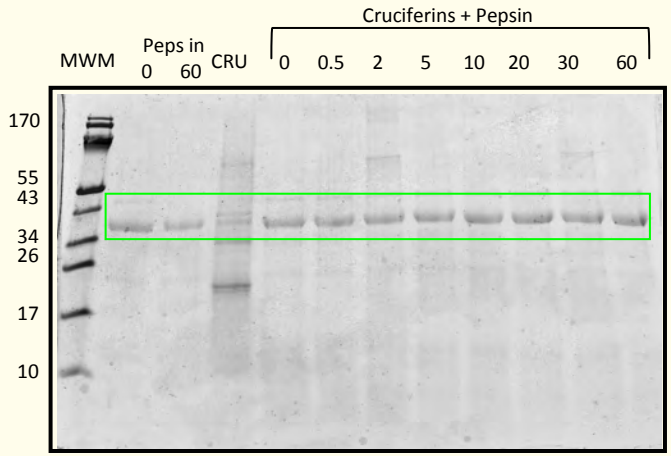


Research - *S. alba* - cruciferin and napin

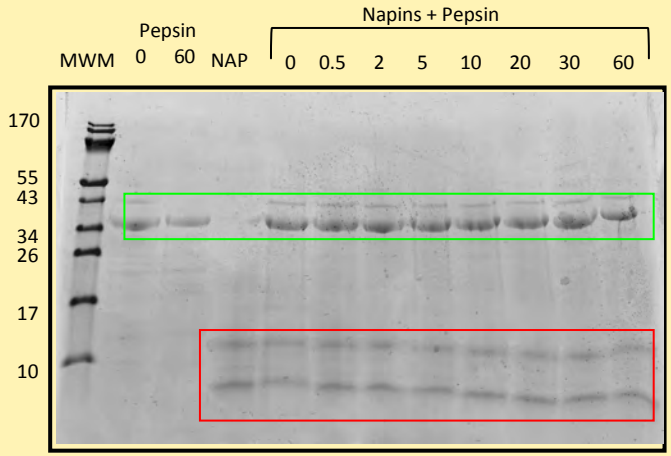


AC Pennant (yellow)





Andante (yellow)



What do not know

- Exposure levels of allergenic proteins in different mustard seed types?
- Whether food processing conditions modify the allergenicity of mustard?
- Do individuals with other food allergies need to be careful with mustard?
- Is it possible to develop mustard with low (zero) levels of allergenic proteins?

Summary

- Mustard (brown, oriental and yellow) will be recognized as a priority food allergen in Canada.
- Most potent allergenic protein of mustard is in the napin fraction.
- We need more understanding on the fate of mustard allergens due to food processing practices.

Acknowledgements

- Saskatchewan Mustard Development Commission
- Agriculture Development Fund, SK
- Agriculture and Agri-Food Canada

Thank You !

Canada