CRODA EUROPE LTD

CIR EXPERT PANEL MEETING – MARCH 17, WASHINGTON DC
HYDROLYSED WHEAT PROTEINS AND ALLERGY
Contents

- Hydrolysis of proteins and what this means and how it is achieved.
- Measurement of molecular weight of hydrolysed proteins.
- Croda’s assessment of its hydrolysed wheat proteins for allergy potential
  - Previous data
  - Recent data
  - Recent in-vitro data
- CIR Expert Panel Guidelines on use of hydrolysed wheat proteins
Types of Proteins

- Native Proteins
- Enzyme Hydrolysates
- Acid Hydrolysates
- Alkaline Hydrolysates
- Quaternised Proteins
- Acylated Proteins
- Protein Copolymers
Protein Hydrolysis

➢ To convert a protein that is insoluble into a protein ingredient that is soluble

- **Acid** (eg. hydrochloric acid)
- **Alkali** (eg. sodium hydroxide)
- **Enzymes** (eg. protease)
Hydrolysed Protein Derivatives

- What are they?

- These are protein products that have been hydrolysed and then modified in some way to alter their functionality

- Quaternised proteins
- Acylated Proteins
- Co-polymers
Wheat protein isolate

Wheat Flour
- Removal of starch by washing

Vital Wheat Gluten (insoluble)
- Acid treatment

“Soluble” Wheat Protein
- Dispersable not soluble
- High molecular weight
- Partially deamidated
- Eg. Glutamine to glutamic acid
Hydrolysed wheat proteins & derivatives

“Soluble” Wheat Protein

- Alkaline hydrolysis
  - Aqua (and) Hydrolyzed Wheat Protein
    - MW ~ 100-125 kDa
  - Aqua (and) Hydrolyzed Wheat Protein
    - MW ~ 3000 Da

- Enzyme hydrolysis
  - Aqua (and) Hydrolyzed Wheat Protein
    - MW ~ 3000 Da

- Acid hydrolysis
  - Aqua (and) Wheat Amino Acids
    - MW ~150 Da
  - Derivative

- Quaternisation
  - Derivatives

- Copolymerisation
  - Derivatives
Measurement of molecular weight (Mw)

- Mw measurement of small water soluble polymers and peptides is difficult and at best approximate.
- Mw is usually denoted as weight average molecular weight.
- Methods typically used include:
  - SEHPLC/GPC
  - Absolute Mw using GPC/MALLS
  - SDS-PAGE
Measurement of molecular weight (Mw)

- **SEHPLC/GPC**
  - Simple to run method.
  - Significant variance based on columns used.
  - Appropriately sized exclusion media has to be used.
  - Standards used can give significant variance.
  - Temperature and eluents will impact results.

- **GPC/MALLS**
  - Results also influenced by column choice and exclusion media.

- **SDS-PAGE**
  - Good comparative method
  - Dependant on appropriate standards
  - Semi-quantitative.
Measurement of molecular weight (Mw)

- Method comparison using Aqua (and) Hydrolyzed Wheat Protein.
  - Enzyme hydrolysed low molecular weight version.

“Soluble” Wheat Protein

- Alkaline hydrolysis
  - Aqua (and) Hydrolyzed Wheat Protein
    - MW ~ 100-125 kDa

- Enzyme hydrolysis
  - Aqua (and) Wheat Amino Acids
    - MW ~150 Da

- Acid hydrolysis
  - Aqua (and) Hydrolyzed Wheat Protein
    - MW ~ 3000 Da
Measurement of molecular weight (Mw)

- SEHPLC/GPC

Mw = 3,147 Da
Measurement of molecular weight (Mw)

- GPC/MALLS

Refractive index (blue) and 90° Light scattering (red) for Analysis 3

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number Average (Mn)</th>
<th>Weight Average (Mw)</th>
<th>Polydispersity (Mw/Mn)</th>
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<tbody>
<tr>
<td>Hwp5</td>
<td>1375</td>
<td>2517</td>
<td>1.83</td>
</tr>
<tr>
<td>Hwp6</td>
<td>1417</td>
<td>3082</td>
<td>2.18</td>
</tr>
<tr>
<td>Average</td>
<td>1394</td>
<td>2800</td>
<td>2.01</td>
</tr>
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</table>
Measurement of molecular weight (Mw)

SDS-PAGE

<table>
<thead>
<tr>
<th>Sample</th>
<th>Use Conc.</th>
<th>Intact Protein Detected &gt;2.0 kDa?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqua (and) Hydrolyzed Wheat Protein.</td>
<td>0.025%</td>
<td>No</td>
</tr>
<tr>
<td>Aqua (and) Hydrolyzed Wheat Protein.</td>
<td>0.025%</td>
<td>No</td>
</tr>
<tr>
<td>Aqua (and) Hydrolyzed Wheat Protein.</td>
<td>0.025%</td>
<td>No</td>
</tr>
</tbody>
</table>

3 different batches used.
INCI nomenclature and molecular weight

- INCI nomenclature does not differentiate by molecular weight.

"Soluble" Wheat Protein

- Alkaline hydrolysis: Aqua (and) Hydrolyzed Wheat Protein. MW ~ 100-125 kDa
- Enzyme hydrolysis: Aqua (and) Hydrolyzed Wheat Protein. MW ~ 3000 Da
- Acid hydrolysis: Aqua (and) Wheat Amino Acids. MW ~ 150 Da

- There is no standard method for measuring molecular weight.
Hydrolysed wheat proteins and allergy

- Croda is a leading global supplier of hydrolysed wheat proteins for cosmetic use.
- Product safety is extremely important and standard toxicity testing is carried out for all new product introductions and includes skin irritation, eye irritation and AMES.
- Allergy/sensitisation of hydrolysed wheat proteins has been and continues to be difficult to assess:
  - Clinicals – finding the right subjects, different modes of sensitisation
  - Animal models available; non-animal testing issues.
  - In-vitro methods; no approved/validated methods for sensitisation.
Croda data on allergy testing

- Potential allergy concerns relating to the use of hydrolysed wheat proteins go back to the late 90’s.
- Related primarily to people with food intolerance to wheat.
  - What if they used a cosmetic containing a hydrolysed wheat protein?
- Some multinationals produced their own internal guidelines on hydrolysed proteins based on molecular weight
  - (eg. 2000 Da or 3000 Da upper limits).
  - Based on the assumption that the greater the degree of hydrolysis, the lower the potential for allergenicity – a very logical assumption.
To determine whether Aqua (and) Hydrolyzed Wheat Protein (Mw 3000) binds in-vitro to a human anti-gliadin antibody.

Method – Slot Blot and Western Blot in-vitro analysis.

Result may be indicative of the immunoreactivity of this hydrolysed wheat protein.

Positive controls used:
- Gliadin (Sigma)
- Parent wheat protein r/m used to make the above hydrolysed wheat protein.
Results

- **Slot Blot**
  - Both positive controls were visualised by the human anti-gliadin antibody (+ve result).
  - The hydrolysed wheat protein was not visualised by the human anti-gliadin antibody (-ve result).
  - Duplicate blot exposed to a non-immune human serum (non-specific control antibody) was negative.

- **Western Blot**
  - The hydrolysed wheat protein analysed by Western Blot was also found to be non-reactive.

- **Conclusion**
  - Slot Blot and Western Blot analysis confirmed that low molecular weight Aqua (and) Hydrolyzed Wheat Protein was not recognised by a human anti-gliadin antibody.
External in-vivo study - 2001

- To evaluate a range of hydrolysed proteins and derivatives, using the Prick Test, to determine if they elicit a Type I skin reaction.
  - Patients used for the study were wheat IgE positive individuals
  - Circulating IgE levels in serum were determined; IgE titres for these patients varied between 12.9 to 46 units.
  - Six patients were used for the testing, one of which was a non-allergic and non-atopic control
  - Positive and negatives controls were used.
  - All patients tested +ve to the positive control, including the control patient. All patients tested –ve to the negative control.
## Results

<table>
<thead>
<tr>
<th>Products</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Positive Control</td>
<td></td>
</tr>
<tr>
<td>Negative Control</td>
<td></td>
</tr>
<tr>
<td>Aqua (and) Hydrolyzed Wheat Protein. Mw ~ 3000 Da</td>
<td></td>
</tr>
<tr>
<td>Aqua (and) Hydrolyzed Wheat Protein. Mw ~ 100 KDa</td>
<td></td>
</tr>
<tr>
<td>Aqua (and) Hydrolyzed Wheat Protein. Mw ~ 125 Kda</td>
<td></td>
</tr>
<tr>
<td>Aqua (and) Wheat Amino Acids</td>
<td></td>
</tr>
<tr>
<td>Aqua (and) Hydroxypropyltrimonium Hydrolyzed Wheat Protein</td>
<td></td>
</tr>
<tr>
<td>Aqua (and) Lauryldimonium Hydroxypropyl Hydrolyzed Wheat Protein</td>
<td></td>
</tr>
<tr>
<td>Aqua (and) Cocodimonium Hydroxypropyl Hydrolyzed Wheat Protein</td>
<td></td>
</tr>
<tr>
<td>Aqua (and) Steardimonium Hydroxypropyl Hydrolyzed Wheat Protein</td>
<td></td>
</tr>
<tr>
<td>Aqua (and) Hydrolyzed Wheat Protein/PVP Crosspolymer</td>
<td></td>
</tr>
<tr>
<td>Aqua (and) Hydrolyzed Wheat Protein PG-Propyl Silanetriol</td>
<td></td>
</tr>
<tr>
<td>Aqua (and) Laurdimonium Hydroxypropyl Hydrolyzed Wheat Protein (and)</td>
<td></td>
</tr>
<tr>
<td>Laurdimonium Hydroxypropyl Hydrolyzed Wheat Starch</td>
<td></td>
</tr>
<tr>
<td>Hydroxypropyltrimonium Hydrolyzed Wheat Protein (and)</td>
<td></td>
</tr>
<tr>
<td>Hydroxypropyltrimonium Hydrolyzed Wheat Starch</td>
<td></td>
</tr>
<tr>
<td>Aqua (and) Hydrolyzed Vegetable Protein</td>
<td></td>
</tr>
<tr>
<td>Aqua (and) Hydrolyzed Oats</td>
<td></td>
</tr>
</tbody>
</table>

Patient 6 - was a non-allergic and non-atopic control
One patient reacted very slightly to 3 products - considered insignificant by the test house.
All wheat derivatives are based on Aqua (and) Hydrolyzed Wheat Protein. Mw ~ 3000 Da
Danish allergy to wheat in food products.

- A very small group of people in Denmark showed allergy to a “soluble” wheat protein used in food products as an emulsifier.
- The “soluble wheat” protein in question was used to produce high and low molecular weight peptides and amino acids.

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“Soluble” Wheat Protein
```

```
<table>
<thead>
<tr>
<th>Hydrolysis Type</th>
<th>Protein Type</th>
<th>Molecular Weight (kDa/Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline hydrolysis</td>
<td>Aqua (and) Hydrolyzed Wheat Protein</td>
<td>~100-125</td>
</tr>
<tr>
<td></td>
<td>Aqua (and) Hydrolyzed Wheat Protein</td>
<td>~3000</td>
</tr>
<tr>
<td>Enzyme hydrolysis</td>
<td>Aqua (and) Hydrolyzed Wheat Protein</td>
<td>~3000</td>
</tr>
<tr>
<td>Acid hydrolysis</td>
<td>Aqua (and) Wheat Amino Acids</td>
<td>~150</td>
</tr>
</tbody>
</table>
```
Danish allergy to wheat in food products.

- Croda tested the hydrolysed wheat proteins, hydrolysed wheat protein derivatives and amino acids produced using the “soluble” wheat protein, on sera from sensitised individuals in Denmark.
IgE binding capacity of wheat products

- Immunospot or in-vitro IgE binding to wheat samples.
- Sera used:
  - A. Gluten hydrolysate-IgE positive and wheat/gluten-IgE negative serum pool (GH+/G-)
  - B. Gluten hydrolysate-IgE positive and wheat/gluten-IgE positive serum pool (GH+/G+)
  - C. IgE negative control serum (NS)
# Results

<table>
<thead>
<tr>
<th>Sample</th>
<th>Product</th>
<th>A: GH+/G-</th>
<th>B: GH+/G+</th>
<th>C: NS</th>
<th>Comment.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Aqua (and) Hydrolyzed Wheat Protein. Mw ~ 3000 Da</td>
<td>neg</td>
<td>neg</td>
<td>neg</td>
<td>no IgE binding</td>
</tr>
<tr>
<td>2</td>
<td>Cropeptide W</td>
<td>neg</td>
<td>neg</td>
<td>neg</td>
<td>no IgE binding</td>
</tr>
<tr>
<td>3</td>
<td>Aqua (and) Hydrolyzed Wheat Protein PG-Propyl Silanetriol</td>
<td>neg</td>
<td>neg</td>
<td>neg</td>
<td>no IgE binding</td>
</tr>
<tr>
<td>4</td>
<td>Aqua (and) Hydroxypropyltrimonium Hydrolyzed Wheat Protein</td>
<td>neg</td>
<td>neg</td>
<td>neg</td>
<td>no IgE binding</td>
</tr>
<tr>
<td>5</td>
<td>Aqua (and) Hydrolyzed Wheat Protein/PVP Crosspolymer</td>
<td>neg</td>
<td>neg</td>
<td>neg</td>
<td>no IgE binding</td>
</tr>
<tr>
<td>6</td>
<td>Aqua (and) Wheat Amino Acids</td>
<td>neg</td>
<td>neg</td>
<td>neg</td>
<td>no IgE binding</td>
</tr>
<tr>
<td>7</td>
<td>Aqua (and) Hydrolyzed Wheat Protein. Mw ~ 100 KDa</td>
<td>positive</td>
<td>positive</td>
<td>neg</td>
<td>IgE binding (A&gt;B)</td>
</tr>
<tr>
<td>10</td>
<td>Aqua (and) Hydrolyzed Wheat Protein. Mw ~ 125 Kda</td>
<td>positive</td>
<td>positive</td>
<td>neg</td>
<td>IgE binding (A&gt;B)</td>
</tr>
<tr>
<td>13</td>
<td>Protease Enzyme</td>
<td>neg</td>
<td>neg</td>
<td>neg</td>
<td>no IgE binding</td>
</tr>
<tr>
<td>14</td>
<td>Preservative Potassium Sorbate</td>
<td>neg</td>
<td>neg</td>
<td>neg</td>
<td>no IgE binding</td>
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<tr>
<td>15</td>
<td>Preservative Phenoxyethanol</td>
<td>neg</td>
<td>neg</td>
<td>neg</td>
<td>no IgE binding</td>
</tr>
<tr>
<td>16</td>
<td>Preservative Euxyl K300</td>
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<td>neg</td>
<td>neg</td>
<td>no IgE binding</td>
</tr>
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<td>17</td>
<td>Preservative Vantocil</td>
<td>neg</td>
<td>neg</td>
<td>neg</td>
<td>no IgE binding</td>
</tr>
<tr>
<td>18</td>
<td>Preservative EDTA/Propylene Glycol</td>
<td>neg</td>
<td>neg</td>
<td>neg</td>
<td>no IgE binding</td>
</tr>
<tr>
<td>19</td>
<td>Wheat Protein r/m for products above. Positive Control</td>
<td>positive</td>
<td>positive</td>
<td>neg</td>
<td>IgE binding (A&gt;B), the highest IgE binding.</td>
</tr>
</tbody>
</table>
Conclusions

- Hydrolysis of “soluble” wheat protein to Aqua (and) Hydrolyzed Wheat Protein – Mw 3000 Da removes potential for allergic response.
- Derivatives also negative.
- In these studies, Aqua (and) Hydrolyzed Wheat Protein – Mw 100 kDa and 125 kDa gave a positive result.
- Indication was that this allergy was linked to the acid treatment of wheat gluten – partial deamidation.
Other in-vitro testing

- A human skin test for immunogenicity, sensitivity and potency assessment.
- Modification of skin explant model for testing allergic reactions and contact sensitivity.
- Non-validated method.

Blood Sample → Recover DC/T-cell fraction → Add test material and incubate

Look for visual histopathological changes and grade I-IV. Vacuolisation of epidermal cells

Add to skin explant

If the protein is antigenic it will activate an immune response (T-cells) which in turn cause the skin damage

Innovation you can build on™
Skin damage - grading

**Grade I** skin damage showing very mild vacuolisation of epidermal cells

**Grade II** skin damage showing diffuse vacuolisation of epidermal cells

**Grade III** skin damage showing cleft formation between the epidermis and dermis caused by confluent vacuolar damage to basal keratinocytes

**Grade IV** skin damage showing the complete separation of the epidermis and dermis
Results

- **Materials used.**
  - Aqua (and) Wheat Amino Acids. Mw ~150Da
  - Aqua (and) Hydrolyzed Wheat Protein. Mw ~100kDa

- **Results:**

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>Response grades</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture Medium</td>
<td>I</td>
</tr>
<tr>
<td>Culture Medium</td>
<td>I</td>
</tr>
<tr>
<td>Aqua (and) Hydrolyzed Wheat Protein. Mw ~100 kDa</td>
<td>III</td>
</tr>
<tr>
<td>Aqua (and) Hydrolyzed Wheat Protein. Mw ~100 kDa</td>
<td>III</td>
</tr>
<tr>
<td>Aqua (and) Wheat Amino Acids</td>
<td>I</td>
</tr>
<tr>
<td>Aqua (and) Wheat Amino Acids</td>
<td>I</td>
</tr>
<tr>
<td>0.1µM DNBC – Positive Control</td>
<td>III</td>
</tr>
<tr>
<td>0.0001% Triton-X – Negative Control</td>
<td>I</td>
</tr>
</tbody>
</table>
Conclusion

- Two materials tested and both derived from the same partially deamidated wheat protein r/m.
- One extensively hydrolyzed – Aqua (and) Wheat Amino Acids.
- The other partially hydrolyzed – Aqua (and) Hydrolyzed Wheat Protein. Mw ~100 kDa.
- The extensively hydrolyzed wheat protein has no sensitisation potential.
- The partially hydrolyzed high molecular weight wheat protein gave a sensitisation response.
CIR Expert Panel – guidelines for HWP’s

- The CIR Expert Panel concluded that hydrolysed wheat gluten and hydrolysed wheat protein are safe in cosmetics when formulated to minimize peptide lengths greater than 30 amino acids (approximately 3.3 kDa). Additionally, these ingredients should not be used on damaged skin or in products that may come into contact with mucous membranes or may be incidentally inhaled.

- The CIR report also discusses:
  - Cross-reactivity of IgE in individuals pre-sensitized to wheat proteins.
  - That no data is available on Mw threshold below which sensitisation would not be induced in pre-sensitised individuals.

[The CIR guideline does not specify a method for measuring MW]…
Cross-reactivity

- Our data has shown that there is no cross-reactivity to IgE in individuals with conventional wheat allergy.
- We have also shown that by reducing the molecular weight of the hydrolysed wheat proteins, there is no cross-reactivity to IgE in individuals with the non-conventional wheat allergy related to deamidated wheat protein.
- The latter has also been demonstrated by Yuko Chinuki et al (JSA).
MW cut-off for HWP’s

- There is general consensus by experts in the field that by reducing the size of the protein the potential for an immunogenic response is reduced and eliminated.
- Peptide chains with 30 AA units are unlikely to retain their inherent native structure and will be significantly denatured.
- Our investigations with peptides of weight average molecular weight 3000Da have been shown to be non-immunogenic.
- Although protein allergenicity remains a complicated issue, the CIR Expert Panel guideline to minimize wheat peptide lengths greater than 30 AA units is robust, based on information available and expert opinion.
CIR Expert Panel – guidelines for HWP’s

- If therefore average peptide lengths of 30 AA’s are deemed safe and acceptable, is the following caveat required in the guideline?
  - Additionally, these ingredients should not be used on damaged skin or in products that may come into contact with mucous membranes or may be incidentally inhaled.

- If hydrolysed wheat proteins are deemed non-immunogenic below a certain chain length/size, then the peptides will be inherently safe whether they are used topically or systemically.

- Prof. Ian Kimber (a highly respected protein immunologist) was asked for his opinion on this matter. His comment was:
CIR Expert Panel – guidelines for HWP’s

“If it is established that a peptide lacks the inherent potential to stimulate an immune or an allergic response, then there will be no risk of allergic sensitisation irrespective of the level of exposure. In this context, if a peptide lacks inherent sensitising potential there is no legitimate reason to mandate that exposure via damaged skin or mucus membranes should be restricted or prevented. In this case there is no risk to humans due to the lack of inherent sensitising potential, and the absence of risk does NOT require protection from exposure.”

Ian Kimber
Professor of Toxicology
University of Manchester. UK

- It is a recommendation that the CIR Expert Panel reconsider this aspect of the guideline as it seems unwarranted.
Overall conclusions

- Croda results have shown no cross-reactivity to conventional wheat sensitized individuals – even with high molecular weight wheat proteins.
- The Japanese sensitization is different to conventional sensitisation.
  - Linked to high molecular weight deamidated wheat protein.
  - Similar to the Danish food issue with deamidated wheat protein.
  - Hydrolysis to lower molecular weight wheat proteins eliminates potential for sensitization as demonstrated by the Croda studies.
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