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# CRODA EUROPE LTD

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

Innovation you can build on™

CRODA

# Contents

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- 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

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

# Protein Hydrolysis

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- 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)

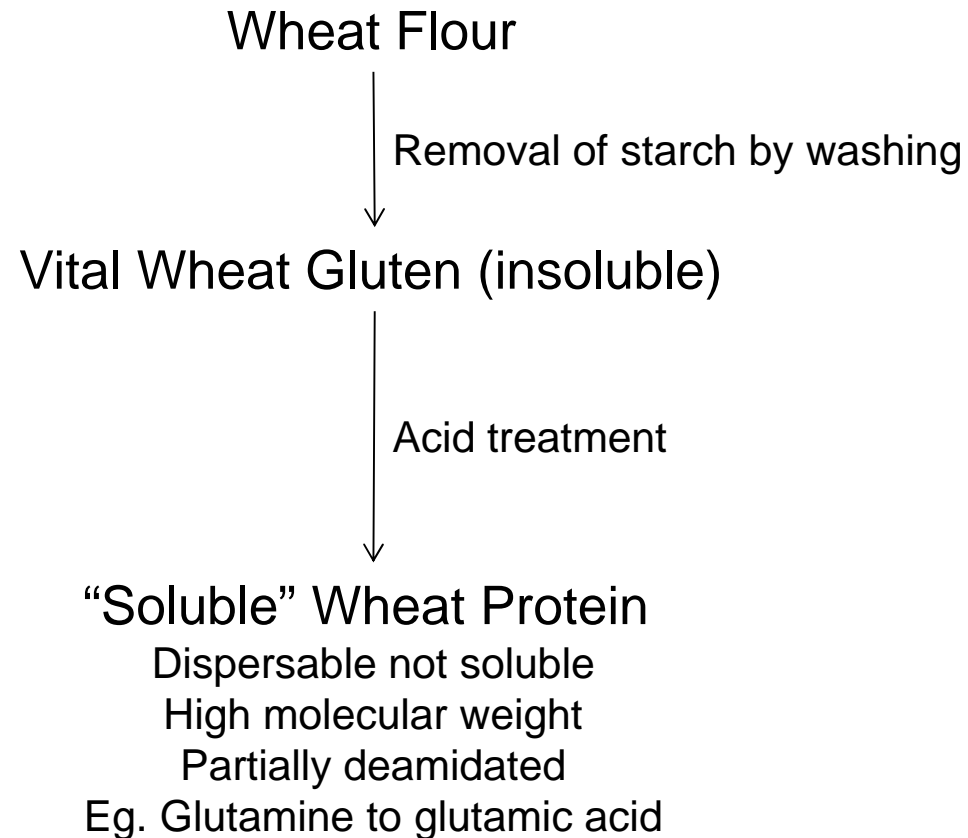
# Hydrolysed Protein Derivatives

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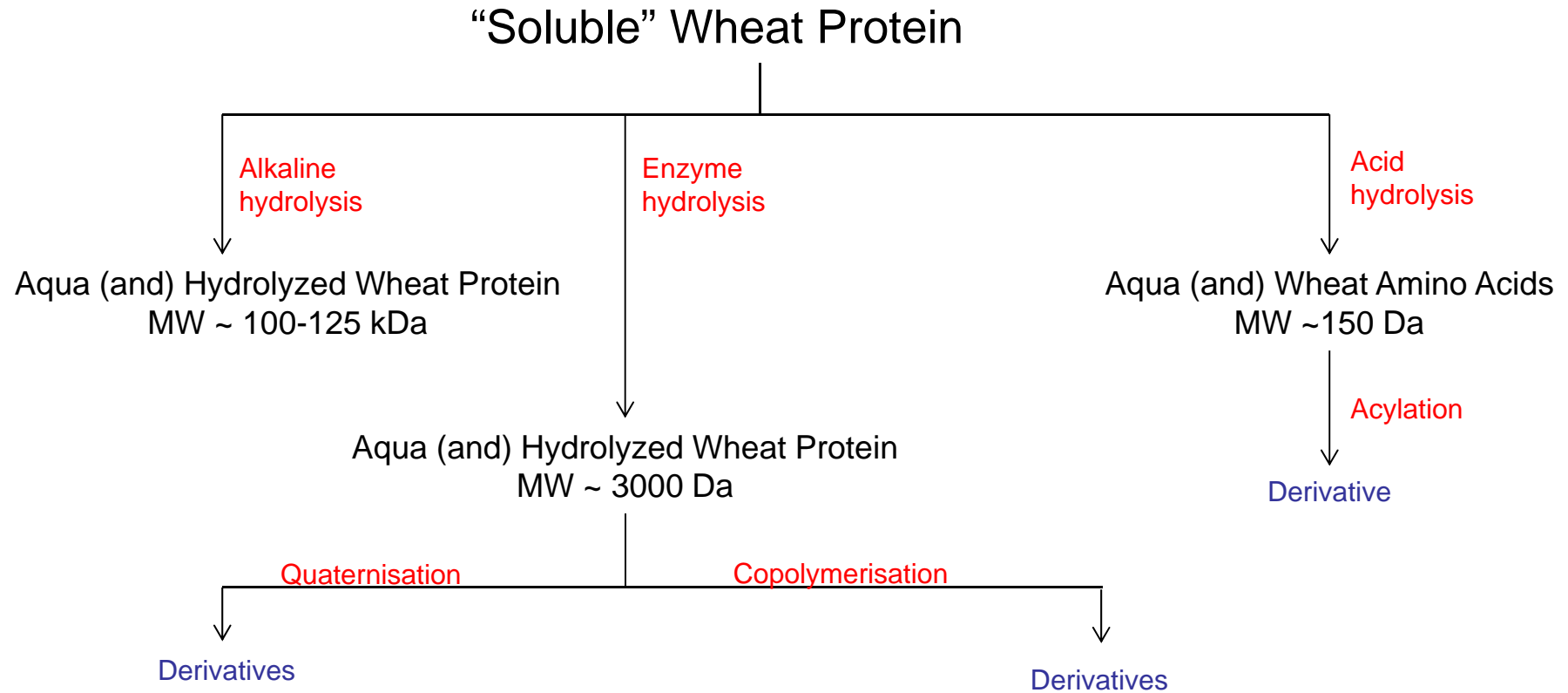
- What are they?
- These are protein products that have been hydrolysed and then modified in some way to alter their functionality
- **Quaternised proteins**
- **Acylated Proteins**
- **Co-polymers**

# Wheat protein isolate

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# Hydrolysed wheat proteins & derivatives



# Measurement of molecular weight ( $M_w$ )

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

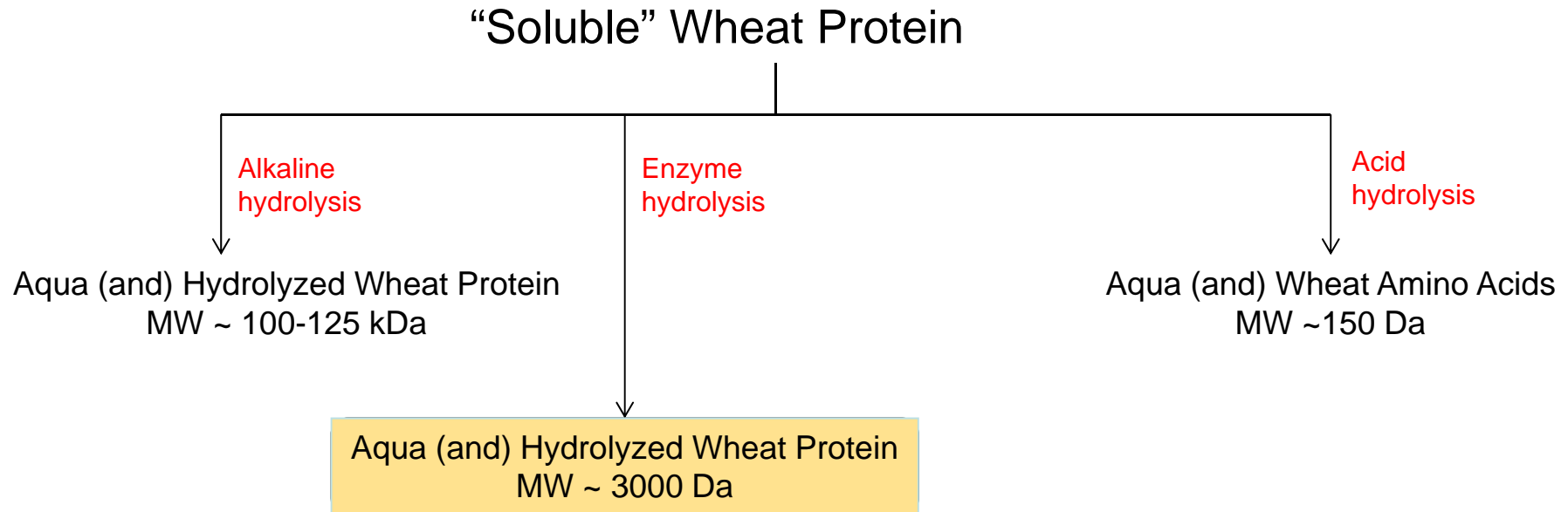
# Measurement of molecular weight (Mw)

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- **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.

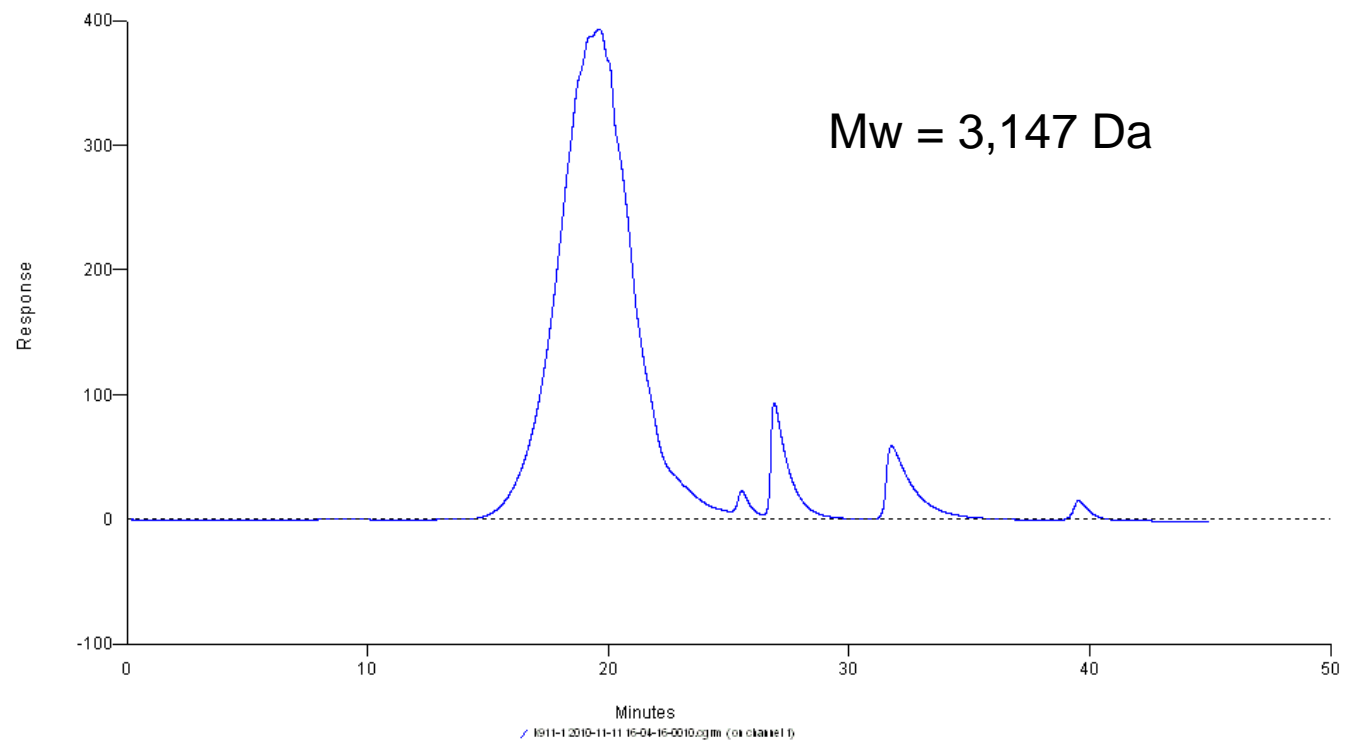
# Measurement of molecular weight (Mw)

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



# Measurement of molecular weight (Mw)

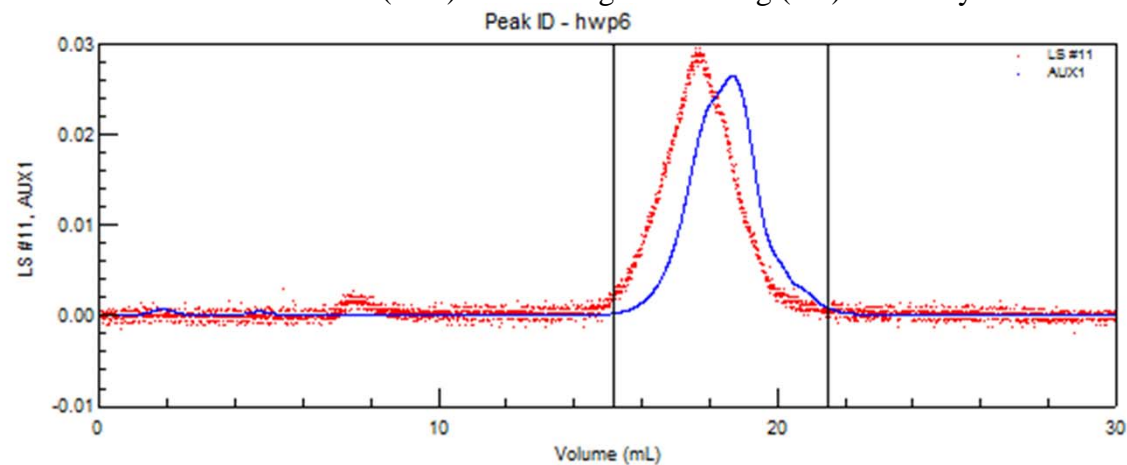
- SEHPLC/GPC



# Measurement of molecular weight (Mw)

- GPC/MALLS

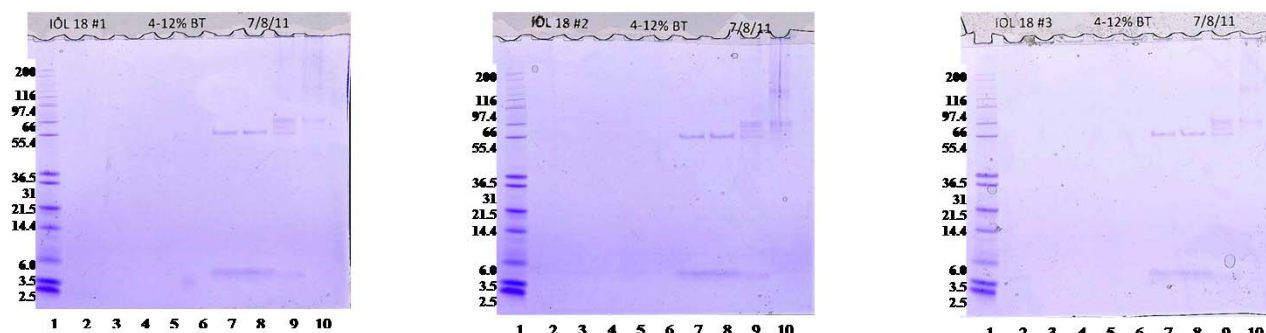
Refractive index (blue) and 90° Light scattering (red) for Analysis 3



Sample	Number Average (Mn)	Weight Average (Mw)	Polydispersity (Mw/Mn)
Hwp5	1375	2517	1.83
Hwp6	1417	3082	2.18
<b>Average</b>	<b>1394</b>	<b>2800</b>	<b>2.01</b>

# Measurement of molecular weight (Mw)

- SDS-PAGE

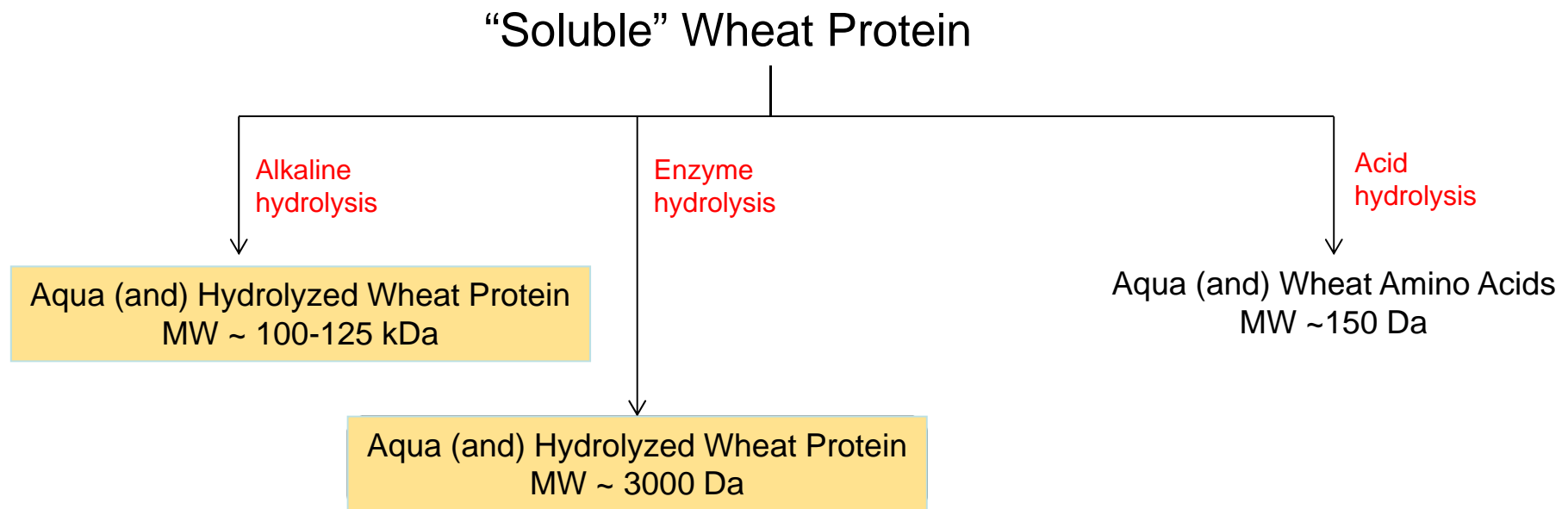


Sample	Use Conc.	Intact Protein Detected >2.0 kDa?
Aqua (and) Hydrolyzed Wheat Protein.	0.025%	No
Aqua (and) Hydrolyzed Wheat Protein.	0.025%	No
Aqua (and) Hydrolyzed Wheat Protein.	0.025%	No

3 different batches used.

# INCI nomenclature and molecular weight

- INCI nomenclature does not differentiate by molecular weight.



- There is no standard method for measuring molecular weight.

# Hydrolysed wheat proteins and allergy

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- 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

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- 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.

## Internal in-vitro study - 2000

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- 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

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- **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

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- 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

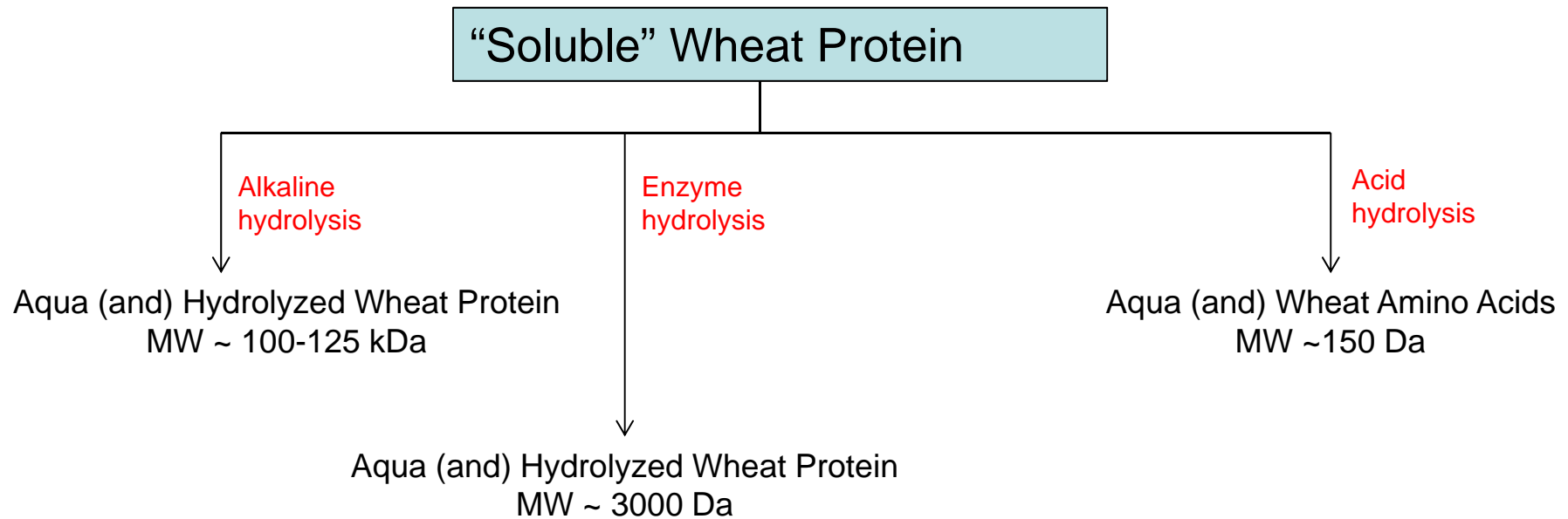
Products	Patients					
	1	2	3	4	5	6
Positive Control	Positive	Positive	Positive	Positive	Positive	Positive
Negative Control	Negative	Negative	Negative	Negative	Negative	Negative
Aqua (and) Hydrolyzed Wheat Protein. Mw ~ 3000 Da	Negative	Negative	Negative	Negative	Negative	Negative
Aqua (and) Hydrolyzed Wheat Protein. Mw ~ 100 KDa	Negative	Negative	Negative	Negative	Negative	Negative
Aqua (and) Hydrolyzed Wheat Protein. Mw ~ 125 Kda	Negative	Negative	Negative	Negative	Negative	Negative
Aqua (and) Wheat Amino Acids	Negative	Negative	Negative	Negative	Negative	Negative
Aqua (and) Hydroxypropyltrimonium Hydrolyzed Wheat Protein	Negative	Negative	Negative	Negative	Negative	Negative
Aqua (and) Lauryldimonium Hydroxypropyl Hydrolyzed Wheat Protein	Negative	Negative	Negative	Negative	Negative	Negative
Aqua (and) Cocodimonium Hydroxypropyl Hydrolyzed Wheat Protein	Negative	Negative	Negative	Negative	Negative	Negative
Aqua (and) Steardimonium Hydroxypropyl Hydrolyzed Wheat Protein	Negative	Negative	Negative	Negative	Negative	Negative
Aqua (and) Hydrolyzed Wheat Protein/PVP Crosspolymer	Negative	Negative	Negative	Negative	Negative	Negative
Aqua (and) Hydrolyzed Wheat Protein PG-Propyl Silanetriol	Negative	Negative	Negative	Negative	Negative	Negative
Aqua (and) Laurdimonium Hydroxypropyl Hydrolyzed Wheat Protein (and) Laurdimonium Hydroxypropyl Hydrolyzed Wheat Starch	Negative	Negative	Negative	Negative	Slight Reaction	Negative
Hydroxypropyltrimonium Hydrolyzed Wheat Protein (and) Hydroxypropyltrimonium Hydrolyzed Wheat Starch	Negative	Negative	Negative	Negative	Slight Reaction	Negative
Aqua (and) Hydrolyzed Vegetable Protein	Negative	Negative	Negative	Negative	Negative	Negative
Aqua (and) Hydrolyzed Oats	Negative	Negative	Negative	Negative	Slight Reaction	Negative

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

Positive	Positive
Negative	Negative
Slight Reaction	Slight Reaction

# 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



# Danish allergy to wheat in food products.

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- 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

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- 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

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

# Conclusions

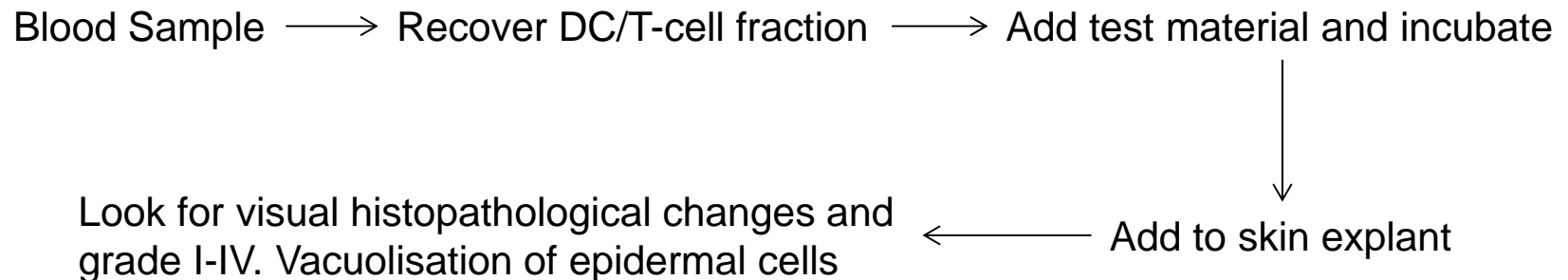
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- Hydrolysis of “soluble” wheat protein to Aqua (and) Hydrolyzed Wheat Protein – Mw 3000 Da removes potential for allergic response.
- Derivatives also negative.
- In these studies, Aqua (and) Hydrolyzed Wheat Protein – Mw 100 kDa and 125 kDa gave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

## Other in-vitro testing

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- 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.

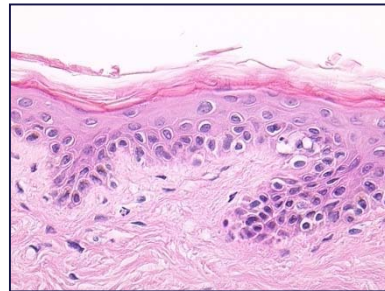


If the protein is antigenic it will activate an immune response (T-cells) which in turn cause the skin damage

# Skin damage - grading

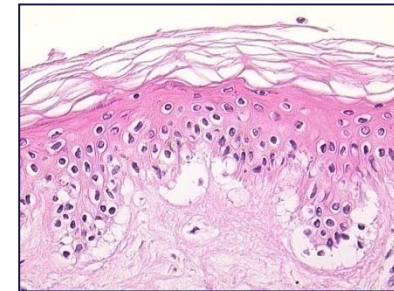
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**Grade I** skin damage showing very mild vacuolisation of epidermal cells



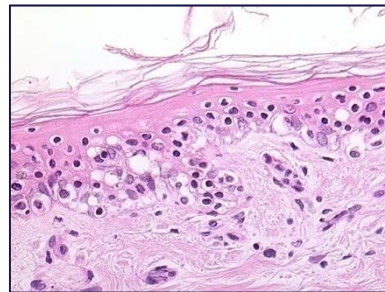
**Grade I**

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



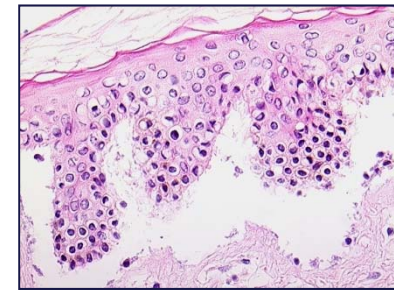
**Grade III**

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



**Grade II**

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



**Grade IV**

# Results

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

	Response grades	
Culture condition	ALC091	ALC092
Culture Medium	I	I
Culture Medium	I	I
Aqua (and) Hydrolyzed Wheat Protein. Mw ~100 kDa	III	III
Aqua (and) Hydrolyzed Wheat Protein. Mw ~100 kDa	III	III
Aqua (and) Wheat Amino Acids	I	I
Aqua (and) Wheat Amino Acids	I	I
0.1µM DNCB – Positive Control	III	III
0.0001% Triton-X – Negative Control	I	I

# Conclusion

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- 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

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- 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

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- 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 HWVP's

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- 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 HWVP's

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- 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

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*“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

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- 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.

# Non-warranty

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