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# Safety Assessment of Hydrolyzed Wheat Protein and Hydrolyzed Wheat Gluten as Used in Cosmetics

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Status: Draft Final Report for Panel Review  
Release Date: May 16, 2014  
Panel Meeting Date: June 9-10, 2014

The 2014 Cosmetic Ingredient Review Expert Panel members are: Chairman, Wilma F. Bergfeld, M.D., F.A.C.P.; Donald V. Belsito, M.D.; Ronald A. Hill, Ph.D.; Curtis D. Klaassen, Ph.D.; Daniel C. Liebler, Ph.D.; James G. Marks, Jr., M.D.; Ronald C. Shank, Ph.D.; Thomas J. Slaga, Ph.D.; and Paul W. Snyder, D.V.M., Ph.D. The CIR Director is Lillian J. Gill, DPA. This report was prepared by Christina Burnett, Scientific Analyst/Writer, Bart Heldreth, Ph.D., Chemist CIR, and Ivan Boyer, Ph.D., Toxicologist CIR.

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## Cosmetic Ingredient Review

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Memorandum

To: CIR Expert Panel Members and Liaisons  
From: Christina L. Burnett, Senior Scientific Writer/Analyst  
Date: May 16, 2014  
Subject: Draft Final Report on Hydrolyzed Wheat Gluten and Hydrolyzed Wheat Protein

At the March 2014 meeting, the Panel was presented information by two different speakers that addressed the Type I reactions observed following exposure to hydrolyzed wheat gluten (HWG) and hydrolyzed wheat protein (HWP). Dr. Surinder P. Chahal, presented information on principles and methods of hydrolyzing proteins and characterizing the polypeptide products of hydrolysis, and presented the results of studies on the sensitization potential of HWP. Dr. Kayoko Matsunaga presented information on studies prompted by an outbreak in Japan of type 1 immediate hypersensitivity reactions to a HWG ingredient (Glupearl 19S) in facial soaps and other products. Based on the information presented, the Panel issued a tentative safety assessment on HWG and HWP with the conclusion that these ingredients are safe for use in cosmetics when formulated to restrict peptides to a weight-average MW of 3500 Da or less.

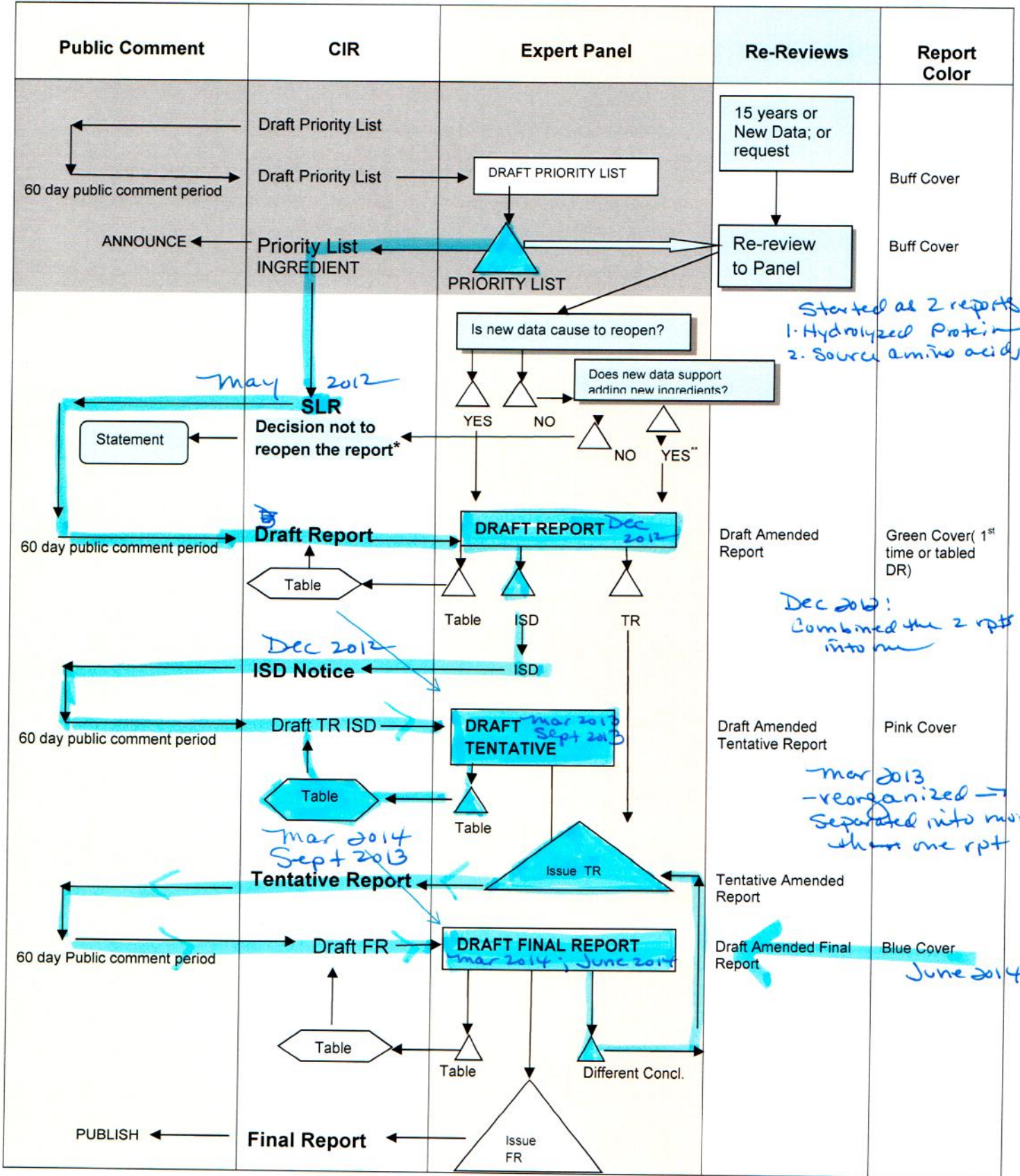
Since March, the VCRP data has been updated for 2014, and documentation was submitted by Dr. Chahal and Dr. Matsunaga (Dr. Matsunaga's submissions were translated from the Japanese, and the Japanese original and the translated versions are enclosed); no other new data have been received. Comments that were received from the Council prior to the March meeting, as well as those on the tentative safety assessment, have been considered. The comments are available for your review in this report package.

The Panel should carefully review the abstract, discussion, and conclusion of this report and issue a Final Safety Assessment.

# SAFETY ASSESSMENT FLOW CHART

*Hydrolyzed Wheat Protein*

*June 2014*



\*The CIR Staff notifies of the public of the decision not to re-open the report and prepares a draft statement for review by the Panel. After Panel review, the statement is issued to the Public.

\*\*If Draft Amended Report (DAR) is available, the Panel may choose to review; if not, CIR staff prepares DAR for Panel Review.

## **Hydrolyzed Wheat Protein and Hydrolyzed Wheat Gluten History**

**May 2012** – Scientific Literature Reviews announced separately for Source Amino Acids and Hydrolyzed Source Proteins.

**December 2012** - The CIR Expert Panel combined the 2 reports into 1 and retitled it “plant- and animal-derived amino acids and hydrolyzed proteins”. The Panel also removed the ingredient hydrolyzed spinal protein from review as it is a prohibited ingredient. The Panel requested additional data to support the safety of 75 plant- and animal-derived amino acids and hydrolyzed proteins. The additional data needed are: (1) method of manufacturing data for both plant and animal-derived amino acids and hydrolyzed proteins, especially for hydrolyzed wheat protein; and (2) composition and characterization specifications of plant and animal-derived amino acids and hydrolyzed proteins, including molecular structure and molecular weight ranges from several suppliers to determine if there is a consistency in cosmetic grade plant and animal-derived hydrolyzed proteins, especially hydrolyzed wheat protein.

**March 2013 and Post Meeting** – The Expert Panel tabled further discussion on animal- and plant-derived hydrolyzed proteins to allow CIR staff to reorganize the report and to analyze further data from Japan regarding Type 1 allergic reactions to hydrolyzed wheat protein in a soap product. The staff has decided to group hydrolyzed wheat protein and hydrolyzed wheat gluten in one report in order to facilitate consideration of the concern about hydrolyzed wheat protein in Japan and not dilute the evaluation with other unrelated ingredients. The review of the other animal- and plant-derived hydrolyzed proteins will be performed sometime in the future. Prior to being tabled, the Panel had issued an insufficient data announcement with the following data needs: (1) method of manufacturing data for hydrolyzed wheat protein; and (2) composition and characterization specifications of hydrolyzed wheat protein, including molecular structure and molecular weight ranges from several suppliers to determine if there is a consistency in cosmetic grade hydrolyzed wheat protein.

**September 2013** – The Expert Panel issued a tentative safety assessment of hydrolyzed wheat gluten and hydrolyzed wheat protein with the conclusion that these ingredients are safe for use in cosmetics when formulated to minimize peptide lengths greater than 30 amino acids. 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 Expert Panel asked that the cosmetics industry continue to provide additional data on manufacturing practices, characterization methods, and composition, including peptide size distributions, to enable better characterization of the nature and variability of these ingredients as used in cosmetic products and to enable the Expert Panel to refine its conclusion.

**March 2014** - The Panel was presented information by two different speakers that addressed the Type I reactions observed following exposure to hydrolyzed wheat gluten (HWG) and hydrolyzed wheat protein (HWP). Dr. Surinder P. Chahal, presented

information on principles and methods of hydrolyzing proteins and characterizing the polypeptide products of hydrolysis, and presented the results of studies on the sensitization potential of HWP. Dr. Kayoko Matsunaga presented information on studies prompted by an outbreak in Japan of type 1 immediate hypersensitivity reactions to a HWG ingredient (Glupearl 19S) in facial soaps and other products. Based on the information presented, the Panel issued a tentative safety assessment on HWG and HWP with the conclusion that these ingredients are safe for use in cosmetics when formulated to restrict peptides to a weight-average MW of 3500 Da or less.

**Hydrolyzed Wheat Protein and Hydrolyzed Wheat Gluten Data Profile\* – June 2014**  
**Writer, Christina Burnett**

	<b>Reported Use</b>	<b>Composition/ Impurities</b>	<b>Method of Manufacturing</b>	<b>Irritation/ Sensitization - Animal</b>	<b>Irritation/ Sensitization - Human</b>	<b>Ocular/ Mucousal</b>	<b>Case Studies</b>
<b>Hydrolyzed Wheat Gluten</b>	X		X				X
<b>Hydrolyzed Wheat Protein</b>	X	X	X	X	X	X	X

\*"X" indicates that data were available in a category for the ingredient

Search Strategy for Hydrolyzed Wheat Protein & Hydrolyzed Wheat Gluten  
(Performed by Christina Burnett and Ivan Boyer)

**February-April 2012: SCIFINDER search for Hydrolyzed Proteins (55 substances, searched under INCI names and CAS No.):**

Initial search for “adverse effect, including toxicity” yielded 18 references.

Also performed searches using the following search terms (no limits for reference type):

- “Hydrolyzed Proteins in Cosmetics” (yield = 30 references);
- “Skin Sensitization – Hydrolyzed Proteins” (yield = 1 reference);
- “Bioactive Peptides - Cosmetics” (yield = 18 references);
- “Skin Irritation – Polypeptides” (yield = 23 references);
- “Skin Sensitization – Polypeptides” (yield = 11 references);
- “Biogenic Peptides” (yield = 1 reference);
- “Bioactive Peptides – Toxicity” (yield = 28 references);
- “Bioactive Peptides – Skin Irritation” (yield = 1 reference);
- “Hydrolyzed Proteins Chicken Cells in vitro” (yield = 1 reference); and
- “Hydrolyzed Protein Irritation” (yield = 7 references).

*Many of the references were patents or efficacy reports.*

13 references were ordered.

**Search updated January 18, 2013.**

**June-July: SCIFINDER and PubMed search for Hydrolyzed Wheat and CAS Nos. 100684-25-1, 70084-87-6, 100209-50-5, and 222400-28-4**

Additional 9 references were ordered.

**Search updated January 22, 2014**

Additional 2 references were ordered, only 1 was incorporated into the report.

**Search updated April 14, 2014.**

No new pertinent information identified.

**March 17-18, 2014 CIR Expert Panel Meeting**

**Hydrolyzed Wheat Gluten and Hydrolyzed Wheat Protein**

*Presentations by Speakers to Full Panel – Monday, 17 March*

DR. BOYER: Okay, thank you. Yes, we have two speakers to address specifically, the safety assessment of hydrolyzed wheat protein, hydrolyzed wheat gluten in cosmetics, as ingredients in cosmetic products. At the last meeting, the conclusion for those ingredients was -- safe if formulated to minimize peptides longer than about 30 amino acids, and also to indicate -- the conclusion indicated that these ingredients are not safe -- not necessarily safe for products that are incidentally inhaled or applied to damaged skin, or applied to mucous membranes. And at that meeting, the panel requested that we have a couple of expert speakers come and address some of the issues, particularly related to a Type I immediate hypersensitivity reactions that have been reported in both Japan and in Europe. And we've got speakers today that can address, and particularly, the cohort in Japan, Dr. Kayoko Matsunaga, who comes to us from the Health Sciences University, and she is the Chairman of the Dermatology department there. She is a professor there. She's also the Chair of the Japanese Allergology Society's Special Committee to address hydrolysates in cosmetic products. And so she'll be able to address some of the very interesting findings that they've been able to develop over the last several years.

Our first speaker comes to us from England. He is Dr. Dass Chahal. He is a Director of the Research and Development Group at Croda, International, and he comes to us from England. And without further ado, I would invite Dr. Chahal to come up and give us his presentation and answer some questions. Thank you.

DR. CHAHAL: Good morning ladies and gentlemen. Thank you for the invitation to come and talk to you about hydrolyzed wheat proteins and allergy this morning. As Ivan's pointed out, I work for Croda. Croda has been producing and providing hydrolyzed proteins for over 40 years, approximately 42 years, so a significant level of experience there. The contents for this morning -- the presentation, as well as talking about allergy with wheat proteins -- I think it's important to understand exactly what hydrolyzed proteins are -- how they're produced. And also touch on understanding how molecular weight is measured for

these proteins, because that's important, especially if you have a target of thirty amino acids. How do you actually measure that? And then I'll go on to talk about a Croda's historical assessment of hydrolyzed wheat proteins in terms of allergic potential, and some of our previous data, and some more recent data that we've developed on this matter. And then I'd just like to touch a little bit on the CIR Expert Panel guidelines, on the use of these hydrolyzed wheat proteins, if I may.

My name is Dr. Surinder P. Chahal. I've been appointed R&D Director. I've been with the company for 27 years and probably about 22 of those years have been spent on developing hydrolyzed proteins for the cosmetic industry. There are various types of proteins that are actually available commercially, and have been for many many years. Proteins we call native proteins. These are proteins that still retain some of their inherent native structures. So generally, these are high molecular weight proteins. We then have enzyme hydrolysates. These are shorter chain peptides. Proteases are used to basically break longer chain proteins into much smaller units. And then there are also acid hydrolysates, so you can break down proteins, using strong acids. And in this case, we're talking about very short chain peptides. You can also use alkali as a means of hydrolyzing proteins, and you can generate either high molecular weight proteins intermediate or low molecular weight proteins, using an alkaline hydrolysate system.

Once you've produced these peptides, or hydrolyzed proteins, you can then also derivatize, to functionalize these materials, so they can be made more cationic to be more substantive to hair and skin. So they can be isolated to make natural soaps -- surfactants, or you can copolymerize with other monomers to produce functional ingredients and Croda does most of these. I would like to point out that Croda's led the way in hydrolyzed proteins and derivatives for many many years and so most of the industry tends to follow and do what we do here.

As I mentioned, there are three real ways of hydrolyzing a protein, so it's basically taking a natural material, which is insoluble, breaking that down using various catalysts, either acid, alkali or enzymes. Acid, you can get down to pure amino acids, so you can break the protein down all the way to individual amino acids, so when you're talking about a molecular weight of 150 daltons, for example. Alkali is a mixture of high molecular weight materials -- you could produce something that's 100,000, 125,000 daltons, all the way

down to 600 daltons. And then enzymes typically, with a standard protease, a general protease, you're probably talking anywhere between 500 daltons to about 5,000 daltons, depending on which enzyme you use. So that's a little bit about hydrolysis of proteins. Once you've hydrolyzed those materials, you can derivatize those, using cationic material, producing new materials with new functionality, quaternized, acylated, and copolymers.

It's also important to understand how these are produced from raw materials, and obviously today, my focus will be entirely on wheat. Wheat flour, from crops is obtained. You can remove the starch by washing out the carbohydrates from this wheat flour. This is an industrial, large scale operation. There's a lot of this wheat flour goes into food. That generates this native wheat gluten, which is an insoluble material, and are quite glutenous. In order for companies like Croda to actually use these materials, it needs to be in a form that's dispersible, so a lot of these companies also produce what we call isolates, and they take the wheat gluten and they treat it with either an acid or with an enzyme. And majority of the treatments over the years have been acid treatments, but nowadays, you can also obtain the soluble wheat proteins as these manufacturers call them, which are enzyme treated. Now it should be pointed out that these soluble wheat proteins are not the hydrolyzed wheat products that companies like Croda would produce. They're actually our raw materials. They're not actually soluble. They're just dispersible, so you can put them into water, and they will disperse, and form a glutenous mass. They are obviously, very high molecular weight materials, but in the case of the acid treatment, you will get some partial deamidation of the glutamine and the asparagine, and that's important to know for the rest of the slides that I'll be talking about. This partial deamidation has some impact on sensitization.

So we take, as our raw material, this insoluble, known insoluble wheat protein, and we then do the various types of hydrolysis. So we can alkaline hydrolyzed insoluble wheat protein and we can generate materials that have a very high molecular weight -- 100 to 125,000 daltons. These are very good film formers, or we can use acid hydrolysis and strong acid and pressure and we can break down the wheat protein to its individual amino acids, so we end up with a complex of 18 different amino acids -- very low molecular weight obviously.

And then you have enzyme hydrolysis, and typically most of our products are based on enzyme hydrolysis. And this generates short chain peptides with a weight average -- molecular weight of about 3,000 daltons. Okay? We can then take that enzyme hydrolysate and functionalize, so most of our products and functional products are based on this low molecular weight peptide. So we can make various cross, I've introduced some of the INCI names there, and a number of copolymers from this basic parent protein. And again as I mentioned earlier, isolate amino acids and you can generate some soaps. So that's important to understand, that the soluble wheat protein raw material obtained from our suppliers, isn't really soluble -- it's a high molecular weight material, and we have to break it down in order to produce products for the cosmetic market.

Measurement of molecular weight is also important if you're going to assign a size cut off. But measurement of molecular weight of small water soluble polymers and peptides is not easy -- it's difficult, and at best, it's approximate. And molecular weight in this industry, normally denoted as weight average molecular weight, and the methods typically used in the industry include size exclusion, HPLC, or GPC. We can use absolute molecular weight, using GPC and multi-angle laser light scatterers, or simple electrophoresis type measurements using SDS page. But I'd like to just describe the various methods and just point out that depending on the method you use, you can get a very different answer, even using SCH PLC -- if you change the column or the temperature or the standard, you can get a very very different results.

So the standard method we use -- the HPLC method, GPC, simple to run, but you can get variation. And it's an approximate method, and we basically have been using the same method without modification for about 20, 25 years -- just so we can get comparative values all the time. GPC/MALLS -- an absolute molecular weight, the results can also be influenced by the column choice in this case.

And then you have SDS-PAGE, which is a good comparative method and -- but it's dependent again on appropriate standards and it's semi-computative, whereas the other method, you can get some quantification of the various components of a distribution. Just to give you an idea, we took our enzyme hydrolyzed wheat protein with a molecular weight of approximately 3,000 daltons and we did the various molecular weight analyses. So using our standard method -- our internal method, you can see there is the

distribution, and the weight of the molecular weight is 3,147 -- so 3,000 approximately. If you then use the absolute molecular weight method -- GPC/MALLS -- here we had a couple of batches tested. The average was 2,800 daltons -- so not far away from our standard internal method -- reasonably good correlation in those two methods.

DR. LIEBLER: Excuse me, what's the lowest molecular weight you're confident in being able to measure with both this platform and the one on the previous slide?

DR. CHAHAL: Once you get down to less than 3,000, 2,000 -- it becomes very difficult. With amino acids, you can use other means of making sure that they're all amino acids. The poly-dispersity narrows quite a lot, so that also gives you an indication of the degree of hydrolysis. Okay? Did that answer your question? Okay.

The GPC electrophoresis method, the SDS-PAGE we did on that same material, and this was done at an external laboratory here in the U.S. and they ran that and they basically found nothing in that material that was above 2,000 daltons, based on their standards. So again, depending on the method you use, you can get slight variations, particular for these low molecular weight water soluble polymers. So it's not an exact science and that just needs to be understood.

I should also point out that the INCI nomenclature that's used to describe hydrolyzed proteins doesn't differentiate by molecular weight, so here in the yellow, we have two products with the same INCI name, but one is 100,000 daltons and the other is 3,000 daltons. So the INCI names that are used in the industry won't differentiate between two products with different molecular weights.

Some of the work that we've done over the years to understand proteinology, particularly hydrolyzed wheat proteinology -- we are a leading supplier of hydrolyzed wheat proteins and have been for 25 years. They've been in the market. Product safety, obviously is very important to a company like Croda, and we do a typical toxicology -- skin, eye irritation and Ames. Allergy and sensitization of hydrolyzed proteins and hydrolyzed wheat proteins has been and continues to be a difficult area to understand and assess. We can do clinical trials but it's difficult finding the right subjects to do those trials with.

Animal models can be used and are available, but as Croda is a cosmetic company, a supplier

of cosmetic ingredients, we have a non-animal testing policy and that obviously makes it very difficult for us to do those tests. There are some in vitro methods available nowadays, but these methods are not approved or validated as yet. They are being developed and hopefully ECHA in Europe will start to look at these and hopefully adopt these going forward. The latest in vitro methods are very much related to single component chemicals, for assessing those. They're not very applicable to proteins, unfortunately. But I'll talk a little bit about those as I go on.

Now proteinology concerns with hydrolyzed wheat proteins go back to the late nineties.

When wheat proteins were introduced to the market, many of our customers asked various questions regarding those. If you add a cosmetic hydrolyzed wheat protein into a cosmetic product and a person who has a wheat allergy -- a conventional wheat allergy -- are they going to react to that material? And that was a valid question. And so we did some work at that time to try and address that question. It's also good to understand that a number of multi-national manufacturers of cosmetics have their own internal guidelines and have some self-regulation when it comes to hydrolyzed proteins, and they too have their own cut offs. So that cut off, certainly with the multi-nationals, is based on an understanding that as you hydrolyze a protein down to shorter and shorter peptides, the potential for allergy is reduced to a point where it's eliminated.

And that's been a very logical assumption. So we've tried to look into these matters. So in year 2000, we did a very simple study, which was looking at our enzyme hydrolyzed wheat protein, with a molecular weight of 3,000, approximately 3,000 daltons, and just wanted to know whether it bound in vitro to human anti-gluten antibody -- just used Slot Blot and western Blot in vitro analysis, and the results were maybe indicative of the amino reactivity of this hydrolyzed wheat protein. And we use a couple of positive controls of time -- gluten, just purchased in, and our parent wheat protein, that's so-called soluble wheat protein, which we used. So both positive controls were visualized and gave a positive result, which is good. The hydrolyzed wheat protein of molecular weight 3,000, gave a negative result.

And the duplicate blot exposed to a non-immune serum gave a negative result as well, as expected. So basically the hydrolyzed wheat protein analyzed using this method, was found to be non-reactive against this anti-gluten antibody. That was our very first result, just based on this hydrolyzed

wheat protein, and we got no concerns there.

We then went on a year later to do a more extensive study, and this was a little bit different. It was in vivo. And we obtained a number of people who had an allergy, a conventional allergy to wheat. And we wanted to understand whether they elicited a Type I skin reaction to our hydrolyzed wheat proteins and a number of other proteins that we had. So the patients used for this study were wheat IgE positive individuals. The study was carried out externally at a company called PlasmaLab. The circulating IgE levels in the serums of these individuals were measures and they varied between those two units there. Their allergy pumps were primed prior to doing the testing, and six patients were used, five of which had the wheat allergy and one was a non-allergic, non-atopic control. And then we used positive and negative controls for this study. So all patients tested positive to the positive control including the control patient, so that was all good and wonderful, and all the patients tested negative to the negative control.

A little difficult to see there, but I think there's approximately about 15 different products. The majority of these are based on our short chain peptide, 3,000 dalton peptide. But there are other products there, which are alkaline hydrolysates with a molecular weight of 100,000 daltons, 125,000 daltons. These are wheat derived materials. Then we had a number of derivatives -- the amino acids, very short materials, a number of the cationic derivatives, some of our other co-polymer derivatives. And we also threw in a hydrolyzed vegetable protein in which, this case was a potato protein and also hydrolyzed oat protein -- okay, low molecular weight materials.

And as you can see there, basically, they didn't react -- so even the high molecular weight materials did not give a positive result to these patients with a conventional wheat allergy. It's important to understand that this a conventional wheat allergy and I'll talk a little bit about non-conventional wheat allergy and I'm sure Professor Matsunaga will further describe that particular wheat allergy.

DR. BELSITO: Would you call that completely negative? It looks like patient 5 was reacting to two of quaternized wheat proteins and also have this --

DR. CHAHAL: Okay, yes, we asked that question of PlasmaLabs and their response to us was that the reaction was so slight, it was probably a negative reaction.

SPEAKER: Is that individual (inaudible) clearly negative control. It wasn't like they were dramatic graphic and there were confusions as to how to read the test?

DR. CHAHAL: Yes, and these individuals were sensitized to wheat allergy, they shouldn't really have reacted to an oat protein.

DR. BELSITO: But did you know whether the patient was allergic to oat or not?

DR. CHAHAL: No, we did not know. But based on the test lab that did the work, and based on their experience, their response to us basically -- that's probably an average result, but comparing the whole issue of whether high molecular weight materials are going to be a concern or low molecular weight materials, we certainly didn't find that.

DR. BELSITO: Do you know what the molecular weight of those two quaternized proteins -- wheat proteins were --

DR. CHAHAL: Yes, the parent protein is 3,000 daltons.

DR. BELSITO: Three thousand.

DR. CHAHAL: Yes, very good. Okay. We have more recently carried out some work in Finland, and just to give you a little bit of background, the company that supplies us with the raw material -- this soluble wheat protein, or dispersible wheat protein, had an issue in Denmark where they found that a couple of people who ate this material -- this was a food product, generated an allergic response -- a Type I allergic response. They decided to take this product off the market for food applications. But they also came to Croda and said, look, we want to take this material off the market and use something else. Obviously that was a bit of a concern for us. So what we decided to do basically was, with this company who supplied the material to us, was basically say, look, if we can do the same testing on the sera from these individuals that you've done, and we can demonstrate that our products don't show any response, would you continue to supply us? And their response was yes, we'd be happy to do that, if you can show there's no response.

DR. MARKS: Would you go back to the previous slide? So you're going to say which one of these --

DR. CHAHAL: Yes, the one we -- we basically looked at all again. So we used this raw

material to make all our products, so we basically looked at all our products based on wheat. And so we did the study on the same individuals, sera from the same individuals -- group of individuals -- hospital in Helsinki, at the laboratory there, and we were required to demonstrate safety of our finished products. It was an in vitro study, using the sera from these individuals -- two types of sera used -- one was gluten hydrolysate positive, and wheat gluten negative, and the other was positive to both gluten hydrolysate and wheat gluten, and a negative control was used. Again, difficult to see there, but at the very top, number one, is our hydrolyzed, enzyme hydrolyzed wheat protein, molecular weight about 3,000, and it came back negative. The positive control was the raw material, which is at the very bottom, and that came back very positive. And there was a degree of positivity in these tests. Another product number two, which is very similar to number one, again negative. Some of our derivatives came back negative -- derivatives based on that 3,000 molecular weight material. And then we had an unusual one. We had one of our cationic materials that was negative to one sera, positive to the other. Our amino acids, which were all very, very small, 150 daltons, came back negative. There were high molecular weight wheat proteins, which previously in year 2000, for conventional wheat allergy, came back negative. In this case, this non-conventional wheat allergy which I'll talk about -- they came back positive, which was kind of expected, because these very high molecular weight materials -- 100,000, 125,000 -- came back positive. We also had a slightly unusual -- one of our copolymers came back positive on -- it was very difficult for us to understand why that should be. We also tested the enzyme used, just to make sure that wasn't allergic in any way in this test. That came back negative. There were various preservatives we would use -- they all came back negative.

But I had a question mark on a couple of those materials that were cationic and came back positive, even though that -- because they were based on this low molecular weight peptide. So we present some materials. These were all done blind at this hospital. And in this case, we again got slightly different result for our cationic materials. But my feeling was that the test method being used was giving us a false positive with cationic materials. So in this case, in this test, we also sent one of our products, where we didn't actually use a wheat protein or any protein. We just produced the poly-cationic material. So it was a protein free polyquat and that came back positive. So it just questioned the test methodology that was being used by

the hospital in Helsinki. It wasn't appropriate for cationic materials.

So, basically, on this, to conclude, we have this non-conventional wheat allergy which is related to the deamidation according to our suppliers and the testing they did -- it's related to the deamidation caused by the partial acid hydrolysis they used to produce the raw material that we used. So the 3,000 dalton peptide came back negative. It's been hydrolyzed sufficiently to reduce any allergic response. The derivatives are also negative. The cationics gave a false positive unfortunately. But the high molecular weight proteins did come back positive in this test. And as I mentioned, this indication from our supplier, that it was the acid treatment of the wheat gluten and this partial deamidation that gave rise to this non-conventional wheat allergy. And I believe this is very much the case in the studies in Japan. They also tested their enzyme treated gluten, so there's no deamidation, and the enzyme treated gluten was negative -- even the high molecular weight material. So this allergy was very specific to deamidation.

DR. BELSITO: Could you just help me understand -- I'm having trouble wrapping my brain around what you just said. You told us at the beginning that when you use acids you can go down to almost single amino acids, correct?

DR. CHAHAL: Sure.

DR. BELSITO: So why, in this acid treatment, were you getting just partial deamidation?

DR. CHAHAL: It's the -- manufacturers of these wheat isolates -- all they're trying to do is basically take something that's insoluble -- glutes -- and make it dispersible. So it's a very gentle --

DR. BELSITO: So it's the starting material that you said you start with.

DR. CHAHAL: Yes, the starting material we use, is partially acid hydrolyzed. Okay? They can use an acid, or they can use an enzyme. And that's what causes the deamidation. And in this case, you're getting about 20 percent deamidation. Not a lot.

DR. BELSITO: Enough to make it dispersible --

DR. CHAHAL: Yes.

DR. BELSITO: But still of high molecular weight.

DR. CHAHAL: Yes, yes, yes -- exactly. We have also more recently done some in vitro

testing. A company in the U.K. called Alcyomics Limited, and they use a skin testing method. It's a modification of a skin explant model, for testing desensitization. I must point out, this is a method that's in development and has been for a number of years, but according to ECA, it's still a non-validated method. But again, it will give you some indication. So it's a method basically -- you take a skin explant from an individual -- a normal individual with no allergy, take a blood sample, separate the dendritic cells and T-cells, add the test material, incubate, and then add to the skin explant, and look at visual damage that occurs to the epidermis and dermis, which is an indication of sensitization. It's graded, it's kind of -- you can see here -- Grade I on the top left there. There's no vascularization of the epidermal cells, and there's no concerns. And as you go from Grade II, bottom left, Grade III, bottom right and Grade IV, bottom right -- you can see the white area continues to grow. That's the separation of the dermis from the epidermis, and that's a response to the T-cells, a response to sensitization. So this is the marker that they use. So we did some work with this group, and we sent them two materials. One was a completely hydrolyzed wheat protein, a molecular weight of 150 daltons, and we wouldn't expect to see any sensitization with this material. And the other was the high molecular weight wheat protein that we produced, 100,000 daltons, and the results there -- we had a couple of batches of each. With the culture medium, we probably had seen no response there. We had the negative and positive control. The positive control gave a Grade III; the active control -- no response. So our hydrolyzed amino acids gave a negative response, as we would expect. The high molecular weight wheat protein in this case, gave a Grade III response, so it was showing some sensitization in this model.

So as I mentioned, it's a non-validated method, but something that is indicative. We are currently doing some more testing, for a number of products in this -- using this test, just to give us some indicative idea of the materials. And just the conclusions, from that testing. This testing was in line with the non-conventional testing that we did in Finland, in Helsinki. Just very quickly, a little bit about our understanding, and I'm sure Professor Matsunaga will certainly talk a lot more about this. I'm just going to very briefly mention -- this Glupearl 19S issue in Japan came to Croda's knowledge a couple of years ago. The JSA have done quite a lot of work in this area. It's a high molecular weight wheat protein. It's similar possibly to the raw material that we would use to actually make our hydrolyzed proteins. It's that kind of -- but

it has been acid treated -- acid pre-treated -- so it's strong acid conditions, longer hydrolysis than our raw material would have, and the higher degree of deamidation is expected for this material. And this acid treatment was determined as potentially raising the sensitization of this Glupearl 19S protein.

Animal testing was carried out on this material in Japan -- repeat skin exposure followed by an intra-peritoneal challenge, and Glupearl 19S was seen as a strong sensitizer. Our hydrolyzed wheat protein -- high molecular weight one -- 100,000 daltons, was also tested in this same model and that gave a similar response to the Glupearl 19S. So we here have two products that have deamidation, and in this particular test model, both gave a positive response. These were very high molecular weight materials. So here are the results as Croda knows them. The blue line is the Glupearl 19S that gave a positive response. The yellow line is our hydrolyzed wheat protein -- high molecular weight, partially deamidated, which also gave a positive response. The negative -- the red line, is a control. But it's interesting to point out here that our product -- a new product that we developed for this test, was based on an enzyme pre-treated soluble raw material, so it didn't have any deamidation. But it had the same high molecular weights. And that was tested in this model, and that came back negative. So again, it points to this fact that the deamidation has a part to play in this specific non-conventional wheat allergy. So, the 19S was in the soap bar that caused the hypersensitivity issues in Japan. It's different to conventional wheat allergy. It's related to this deamidation and a high molecular weight. Our high molecular weight material, similarly, partially deamidated, also reacted as a sensitizer, but if you don't deamidate, it's fine.

So finally, just moving on to the CIR Expert Panel guidelines, as they're currently written -- in the CIR report it also discussed cross reactivity to IgE in individuals pre-sensitized to wheat proteins. There is also a question mark as there is not a lot of data available on molecular weight threshold, and also, there were no real guidelines as to how you would measure molecular weight if you imposed a size. So our data, what I've shared, over the years, has shown that the conventional wheat allergy -- there's no cross reactivity to IgE with our products -- even the high molecular weight materials -- to conventional wheat allergy. We've also shown that by reducing the molecular weight of the hydrolyzed wheat proteins, there's no cross- reactivity to IgE to non-conventional wheat allergy -- this related to deamidation. So as long as you can

break them down to smaller units, the reactivity disappears. And that information was also demonstrated by a paper by Yuki Chinuki, et.al., in Japan. For molecular weight cut off, there's general consensus by experts in the field that by reducing the size of the protein, the potential for any immunogenic response is reduced or eliminated. That is well accepted. So peptides chained with 30 amino acid units are unlikely to retain their inherent native structure, and will be significantly denatured, and that's been our understanding over the years. And our investigations of peptides with a molecular weight average in the region of 3,000 daltons have been shown to be non-immunogenic. So although protein allergenicity still remains a complicated issue, the CIR Expert Panel guideline to minimize wheat peptide length greater than 30 amino acids is a robust conclusion, and it's based on available information and expert opinion.

However, there is that caveat also in the guideline. If therefore the average peptide lengths of 30 amino acids are deemed safe and acceptable, then the question is, is the following caveat required, which was -- it is not to be used on damaged skin or in products that come into contact with mucous membranes. In my opinion, if hydrolyzed wheat proteins are deemed immunogenic below a certain chain length and size, then peptides -- those peptides -- will be inherently safe, whether they're used topically or systemically. That was my opinion, but I also asked a highly respected expert in immunology, Professor Ian Kimber, who I work closely with, and asked him that very same question. I believe some of you here are aware of Professor Ian Kimber, from the U.K. So I asked him the same question, and to quote Professor Kimber -- it's probably difficult to read -- it says, "If it is established that a peptide lacks the inherent potential to stimulate an immune or allergic response, then there will be no risk of allergic sensitization, irrespective of the level of exposure. In this context, if a peptide lacks inherent sensitizing potential, there is no legitimate reason to mandate that exposure by damaged skin or mucous membranes should be restricted or prevented. In this case, there is no risk to humans, due to the lack of inherent sensitizing potential, and the absence of risk does not require protection from exposure", and that's to quote Ian Kimber on this particular question, based on the data that we have, and his understanding as an expert in the field.

So it is a recommendation that the CIR Expert Panel reconsider this caveat -- this aspect of the guideline, as it may seem unwarranted with the data that we have. So overall, 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 sensitization, as was the Danish issue. It's linked to high molecular weight deamidated proteins, very similar to the Danish issue, with the amidated proteins. But hydrolysis of these materials to low molecular weight wheat proteins eliminates potential for sensitization, as demonstrated by the Croda studies and the studies in Finland and at Alcyomics. Thank you.

DR. BELSITO: Would you just put the slide back up with your test results on those six patients -- the one nonatopic and the five atopics? You just passed it. Okay -- what were you testing here? These products -- what was the molecular weights -- 125 kilodaltons?

DR. CHAHAL: Okay, yes, the first one there -- non-quaternized wheat protein was 3,000 daltons.

DR. BELSITO: Three thousand, 100, 125 --

DR. CHAHAL: That's 100,000, 125,000 -- then we had the wheat amino acids which were 150 daltons -- just amino acids. We have the quats, which are based on the 3,000 dalton.

DR. BELSITO: So the two that were questionable were 3,000?

DR. CHAHAL: Yes.

DR. BELSITO: Daltons.

DR. CHAHAL: Three thousand daltons -- parent protein. But as I say, based on the PlasmaLab's expert opinion, those were question marks as to whether they were positive.

DR. BELSITO: That goes back to the fact that in your testing you found some of the quaternized amino acids falsely positive or --

DR. CHAHAL: There were quaternized peptides, yes, that were falsely positive in the wheat, in the Finnish study, yes -- because the test method wasn't appropriate.

DR. MARKS: Would you define more clearly for me, what you call conventional wheat allergy and non-conventional, and where that's occurred, and mechanisms as you understand it. It's a very nice presentation, thank you. You made it very clear.

DR. CHAHAL: You're welcome. I must point out that I'm a chemist, not a toxicologist or

immunologist, but by conventional, we mean people with a standard wheat intolerance for food, and --

DR. MARKS: So I'm having a little difficulty whether that wheat intolerance -- what is the mechanism? Is it IgE based? Is it some other T-cell based? Is it just a GI intolerance or does it result in an anaphylactic reaction, which presumably would be IgE mediated.

DR. CHAHAL: Yes, the testing we did for the conventional wheat allergy was on individuals who had the Type I reaction. It was IgE based.

DR. MARKS: And then, so tell me, the non-conventional? What's your concept of that?

DR. CHAHAL: Okay, this was more recent. This was the work I'm sure Professor Matsunaga will expand on, which was based on the fact that we were getting another response which was caused by this partial deamidation. And so this was a difference -- people with a conventional wheat allergy were not responding to this. But it was a new response. But it wasn't, in this case, limited to Japan. They had a couple of concerns in Denmark, to this partial deamidation.

DR. BELSITO: So just to clarify Jim's point then, these individuals, if you did an ImmunoCAP or a prick test for a standard wheat allergy, they would test negative on that test? But they tested positive to your partially deamidated wheat product?

DR. CHAHAL: So, if people with a conventional wheat allergy were tested with these products, they would test negative.

DR. BELSITO: And by conventional wheat allergy you mean, as defined by an ImmunoCap or a prick test or --

DR. CHAHAL: Yes, yes.

DR. BELSITO: And the people who were testing positive to these partially deamidated wheat proteins, if you did an ImmunoCap or a prick test for standard wheat allergy, they would test negative?

DR. CHAHAL: I don't know.

DR. MARKS: And I guess to me the important thing is, what is the phenotypic or phenotype end point, so both individuals would have an anaphylactic type response, is that correct?

DR. CHAHAL: That, in different ways, show up -- again Professor Matsunaga will cover

that, but it was a -- in the case in Japan, it was an exercise induced anaphylaxis on the -- once wheat was consumed.

DR. LIEBLER: I just have a question about deamidation. How do you assess deamidation analytically? Do you have a quantitative measure that you use and if so, does that provide any insight as to what a cutoff might be for the ability to induce an allergenic response?

DR. CHAHAL: Yes, we haven't done that work internally ourselves. We kind of rely onto our supplier and the supplier's given us that information for approximately 20 percent, using the fairly mild conditions. And my comment on the Glupearl 19S is based on, that the conditions used there are much stronger, and high temperatures, and you would expect to get a higher degree of deamidation.

DR. LIEBLER: So that makes general sense. I'm just wondering how your supplier measures it.

DR. CHAHAL: Yes, I don't have that information, but it's basically a -- it shouldn't be difficult to do. It's based on the convergence of glutamine to glutamic acid and just (inaudible).

DR. LIEBLER: Yes, it's possible with the available technology now to not only quantitatively assess deamidation, but to actually look at the degree to which different peptide sequences are affected, and in the case of some of these proteins, that actually might be really important.

DR. HILL: So just one more time, because I think I'm not clear. How do you go about assessing that you're approximately 3,000 molecular weight or not, analytical chemistry-wise then?

DR. CHAHAL: We have used the same methodology for 20, 25 years -- exactly the same method, and there is some variation, depending on the methodology used, so we've done the approximate molecular weight determination which came out to about 2,800. And we've also done SDS-PAGE incidentally and that didn't find anything above 2,000, and so as I mentioned earlier, it's not an exact science. But it will give you a good indication if you can find a method that's comparable.

DR. HILL: Well, okay, so the SDS-PAGE is not going to be as sensitive as capillary electrophoresis. What kind of -- I don't even know how to ask this question, but I guess I'm wondering about the limits of detectability using that methodology. How much would it take being there, on a percentage basis,

before you could see a high molecular weight band?

DR. CHAHAL: Nowadays, you can use very, very small quantities, so in millimeters of injection.

DR. HILL: Well, yes, okay, so that's the protein which is presumably not, yes -- small peptides that are presumably below, but how much do you have to put there to see a detectable amount of -- where the molecular weights exceed, because I would think you would not have to have a large percentage of those high molecular weight impurities in there before you might still generate the hypersensitivity?

DR. CHAHAL: Again, you can use very, very small materials. We're using UV as a method of assessing these materials, so the sensitivity size -- even the shoulders on the distribution curve -- you can quantify those to some extent. But it's that same material -- that 3,000 that I talked about, which has that distribution. And that was the material that was tested.

DR. LIEBLER: So I just want to point out that the methods that are being used that you described, are sort of fine, but they're also methods that are 20, 30 plus years old. The ability of these methods to distinguish molecular species in terms of molecular weight, basically bottoms out around 3,000 or so, depending on whether -- what your gel permeation media is or the percent cross linking in your SDS-PAGE gel, and I think you probably aren't really able to respond to Ron's question that well, because it's true -- you can load a small amount of protein on SDS gel and detect it, but that doesn't really get to what he's asking and that's -- what fraction of the amount that you're loading on, that might be higher molecular weight, is undetected? So I think the answer to a lot of this these days is mass- spectrometry, and particularly MALDI mass spec would be perfectly suitable -- well, actually much better suited than any of the techniques that you measured. Do you guys use any MALDI? Or do you use it to a contract lab?

DR. CHAHAL: Yes, we have done some work in that area, in the past. I've not shared much about it today -- where we've actually generated a 20 amino acid unit peptide and then used that as a standard, in M.S. and again, we were fine with that. There weren't high molecular weight species in the (inaudible) material.

DR. HILL: Great, I mean to specifically rephrase my question, is how much material would

it take to detect a shoulder, using the methods that you're using?

DR. CHAHAL: Okay, I can't answer that.

DR. BOYER: Both of our speakers are going to be available during the team meetings, if you want to go into this in a little bit more depth, but we need -- to move on to our second speaker. Thank you very much. And Dr. Matsunaga comes to us from Fujita Health University School of Medicine. The Council has kindly provided us with a translator to facilitate the questions and answers if need be.

DR. MATSUNAGA: Good morning ladies and gentlemen. I thank you very much for inviting me to this distinguished members of the Expert Panel, CIR. Thank you. I'm going to talk about safety information, about hydrolyzed wheat proteins from Japan. I'm from Nagoya City, I live in Nagoya City and University is in Toyoake City. You know Toyota City maybe -- a famous one. Please come to Nagoya City. Our University hospital has 1,505 beds. It's the largest university hospital in Japan, and about 2,100 outpatients a day. Here is my department of dermatology and my room. Okay, I can talk today about various things, but maybe time is limited so, I will pick up with the three in 30 or 40 minutes, okay? Today I came here because I was asked by the Expert Panel of CIR if I have information that can help the Panel with this question. It will be helpful for advancing the Panel's decision -- Is there sufficient data to identify a threshold amino acid length of the peptides of hydrolyzed wheat protein or hydrolyzed wheat gluten below which there is little chance of trans-dermally inducing sensitization in healthy people or in test animals. You think as distinct from eliciting a response in already sensitized patients, which apparently is not expected to occur theoretically if the peptides are less than 30 amino acids long. Our answer is, our data suggests that there is little chance that hydrolyzed wheat proteins or hydrolyzed gluten which do not include more than 30 amino acid length can trans-dermally induce sensitization in healthy people or in test animals. I will show you the sensitization potential of the Glupearl 19S and other hydrolyzed wheat proteins down by the theoretical threshold, okay? This is hydrolyzed wheat protein for cosmetics in Japan and transdermal sensitization was done. I'm sorry but Croda Tritisol, their trade name, and Glupearl 19S, it's Katayama Chemical Company -- these two hydrolyzed wheat proteins can sensitize but several other hydrolyzed wheat proteins cannot. Transdermal sensitization was done by three day patch test, four times for four weeks and then, intra-peritoneal elicitation was done.

Zero point five percent SDS was required for sensitization to sensitize BALB/c mice with Glupearl 19S, 500 microgram in 0.5% SDS solution per pad of Glupearl 19S is used for transdermal sensitization. So repeated exposure of hydrolyzed wheat protein on the damaged skin is necessary to produce sensitization. So this is measuring hypothermia -- the body temperature of mice, and you can see Glupearl 19S here, hypothermia was seen, and also in Tritisol -- less, but there is hypothermia. But other hydrolyzed wheat proteins no reaction. And we score anaphylaxis and then Glupearl 19S -- the score was 2.7. Whereas Tritisol, you can see, 2.1, a little bit higher than, and specifically higher than gluten at 1.0. And preparation of hydrolyzed wheat proteins triggered by hydrochloric acid and heat -- you can see a hydrolyzed in 0.1N HCL under 100 degrees -- zero to 48 hours. You can see the proteins goes 0.5, 1, 3, 6, 9, 12 hours -- you can see the molecular weight decreases. And at nine hours, molecular weight less than 30 kilodaltons -- protein could not sensitize. Thirty, not three, okay? And when we see a response of patient serum IgE to Glupearl 19S fraction, Glupearl 19S immediate wheat allergy patients, serum IgE did not react to Glupearl 19S -- this one -- less than 3 kilodaltons. And there is closer activity between Glupearl 19S and Tritisol. Serum specific IgEs against Glupearl 19S Tritisol at four weeks after we did transdermal sensitization, with the two hydrolyzed wheat proteins -- this says that Glupearl 19S (inaudible). This is a sensitization with a vehicle, no reaction, and transdermal sensitization antigens, Glupearl 19S and Tritisol, you can see both give positive reactions. And Tritisol specific IgE -- that's a solid phase Tritisol -- you can see also that two positive reactions in Glupearl 19S and Tritisol. So they cross react. And we saw cross reactive responses between Glupearl 19S and the other hydrolyzed wheat proteins. These other hydrolyzed wheat proteins -- okay, you cannot remember. And when we evaluate the IgE binding activity to each hydrolyzed wheat proteins, of course Glupearl 19S positive reaction, and one, two, three, four, five -- no reaction, but from six to nine -- positive reactions. These are from the one manufacturer. It's the Croda Company, so what's the cause? This is protein contents.

DR. MARKS: Would you go back to the previous slide?

DR. MATSUNAGA: Yes?

DR. MARKS: So there are positives to molecular weight 3,000, 3,500 --

DR. MATSUNAGA: Yes, you see average molecular weight. So maybe 1,000 dalton.

This one is the number seven. Number seven is here. When we perform western blotting using patient sera, sensitized to the hydrolyzed wheat protein, Glupearl 19S, you can see, this is 100 kilodalton, so very high molecular weight proteins there, right? They reacted. And this is Tritisol, okay? And it is known to elicit an immediate hypersensitivity reaction -- two epitopes of at least eight to ten amino acid residues -- about 2,000 dalton and the spacer are connecting these two epitopes, ten amino acid residues, about 1,000 dalton -- that is at least 3,000 dalton polypeptide is essential to elicit a reaction. Here, epitope -- two epitopes and spacer -- so 3,000 dalton is essential. But we have had many patients with latex allergy, and hevein is known as a major allergen of latex allergy in those of health care workers who use natural latex gloves. The molecular weight is 4,700. So this allergen can transdermally sensitize.

We suggest approaching a polypeptide less than 3,000 dalton does not elicit an immediate hypersensitivity response. The protein does not have potency to induce immediate hypersensitivity. I have time, so I will talk. I'll continue to talk, okay?

We have learned much from an outbreak of immediate hypersensitivity to hydrolyzed wheat protein in a facial soap in Japan. This is Cha no Shizuku. It's "drop of tea", containing a hydrolyzed wheat protein, Glupearl 19S, excellent in forming foams. And the active ingredient was dipotassium glycyrrhizate -- this one, and here, hydrolyzed wheat protein powder. It's Glupearl 19S. This soap contained 0.3 percent of that. The mean molecular weight was about 50,000 dalton. It has excellent foam-ability and it was advertised on TV or internet, as good for skin care, especially for lightening skin, so that many people, many women have bought it from a mail-order house, and used. This soap was sold -- 46 million and number of clients registered -- 4.6 million, and maybe estimated 6 million measured female have used this soap and I think about 10 percent of the Japanese women have used. And the one out of 2,800, mainly women, have this wheat allergy.

I will show our first case. She was 47 year old female teacher. Her complaint was that urticaria, dyspnea and anaphylactic shock after meals. She had past history of pollinosis since 20 years ago. She had been emergency transported four times since one year ago, when she first developed urticaria dyspnea and hypertension after meals. She had two episodes of anaphylaxis when she was shoveling snow. She

started to use the soap three years ago and had noticed irritation on her face at the use of the soap since six months ago. This is a clinical manifestation of urticaria -- itchy wheals on the face and edema on the eyelids after using the soap. The patient brought this photo to us. So we have experienced a total of 54 cases at the University Hospital. Remarkable edema on the eyelid -- you see? Most of the patients are females and half of them had systemic symptoms, anaphylaxis with marked edema of the eyelids after taking foods including wheat. All have used the soap Cha no Shizuku "drops of green tea", but a few cases had not noticed itching or any symptoms on the face after cleansing their faces -- so 25% or 30% of patients did not show any contact urticaria on their faces. That's a very difficult problem for us to diagnose the first of this allergy. So, Academic Society's movement started in May, 2011. The Japanese Society of Allergology, 10,000 members, with close association of the Japanese Society of Derma Allergology and Contact Dermatitis, 500 members, established on July 4th, 2011, a special Committee for the Safety of Protein Hydrolysates in Cosmetics. I have been the Chair of this Committee. And first we made diagnostic criteria for immediate wheat allergy to the hydrolyzed wheat contained in Cha no Shizuku soap and some other products. First, the history of usage of the soap, and clinical manifestations either contact urticaria or systemic wheat allergy, and the third way, objective evidence of specific IgE or basophil activation test was positive.

Then, these patients are all over Japan -- the 47 prefectures, and now 2,107 cases are registered -- frequently seen in females, 20 to 60. Ninety-six percent of the patients were female. Only four percent were male. Because this soap was very expensive -- 1,800 yen -- maybe \$18. Is it expensive? I don't know. And the patient -- the number of cases registered now decreasing maybe 2,200 at most. And you saw the situation with the soap. Almost all of the females use for face and some used for their body, and they -- after they stopped using here and the year, stopping the use and this is symptom appearance, maybe a little bit later, so one year later or two, or one and a half year or so. And very interesting thing is clinical manifestations, during use, or after using the soap -- of course no anaphylactic shock or anaphylaxis after using -- washing their faces was seen -- only mild eyelid edema and urticarial was noticed. But after eating wheat products, 25 percent had shock, 30 percent had anaphylaxis. Totally is 55 percent had anaphylactic reactions. And this is a remarkable clinical manifestation. It's eyelid edema. Maybe while washing faces,

the hydrolyzed wheat protein is absorbed through eyelid membrane and near epidermis, membranous absorption.

According to the history of allergy, 55% had history of allergy. In Japan 40% or 50% of the women aged 40s or 50s have pollinosis. So 55% is not so high compared with the general population with allergic history. So daily washing with this hydrolyzed wheat protein contained in the soap may enhance percutaneous and through mucous membrane absorption of the antigens and sensitize. You know, class I food allergy and class II food allergy and then new percutaneous or through mucous membrane sensitization with food, and then, systemic wheat allergy occurred.

In conclusion, repeated contact with HWP in the soap induced sensitization through skin, eyelids and nasal mucosa followed by severe immediate reactions after having wheat proteins. According to the prognosis of this Glupearl 19S sensitized immediate wheat allergy, time course change of specific IgEs showed tendency of decreasing. So mean 50 percent decrease is 5.1 months. And another study, remission rate of patients with wheat allergies since the hydrolyzed wheat allergy in facial soap shown by Hiroshima University, Makiko Hiragun, shows data -- remission means patients have shown no symptoms for more than three months without any restrictions and negative in HRPT -- medium duration -- 60.6 months, so five years. But maybe in almost 95 percent of the patients really decrease and subside this allergy, but five percent patients have problem -- do not decrease the antibody or clinical manifestation. I have time? So I'll go move to recent information about antigen analysis of hypersensitivity to Glupearl 19S. Most of the patients sera have specific antigens against the glutenins, Hiragun reported and the -- you know, with antigen proteins, soluble and insoluble and now, this is a -- we are looking for gamma-gliadin and low molecular weight glutenins nowadays. We have done the western blot analysis in the Glupearl 19S wheat allergy patient. This is a figure and this is a specific part of Glupearl 19S, and conventional WDEIA with the five omega-gliadin in the exercise induced anaphylaxis patient -- they have no protein reaction here in this area here, okay? And this is the healthy first patient, healthy volunteers, and the conventional wheat allergy patients. They do not react to the Glupearl 19S at all. Only patients react to Glupearl 19S -- very specific. You can see they're all here -- positive, and this is the healthy, and conventional negative. And when we studied this, where the

antigenicity comes up from the process of antigen -- antigenic products in Glupearl 19S, this hydrolyzed wheat protein manufacturing process -- raw gluten and immediately after the addition of HCL and heating of solution, first 40 degrees, and then 50 degrees, pH 0.7, and 95 degrees Centigrade, pH 1.06. This is the four. You can see that the antigenicity comes out from number four to eight, and this is a Glupearl 19S. This is a western blot and you can see this is number four, and very smear broad stain, and we isolated responsible proteins by analysis, by a size exclusion chromatography -- identical peak of antigenicity, fraction A. This is maybe -- we think this is a part of antigenicity fraction, okay? And low molecular glutenin is proposed to be a most nominated major allergen we think. This is protein producing process, zero to four, five, six, seven, eight, Glupearl 19 and you can see this is low molecular weight glutenin, is increasing. High molecular glutenin is decreased by acid addition, and according to antigenicity, low molecular weight glutenins, gamma-gliadins increase. And deamidation advances acid as heat treatment continues, so the deamidation ratio of each protein fraction was measured by LC/MS. You can see zero to three, four, five -- acid and heat treatment increases deamidation ratio.

Finally, I would show -- you don't like animal tests. You don't do animal tests, but in Japan, we can do. We have done animal tests. It's all very important data. One animal test is, immediate type allergy test using guinea pig. This is an immediate type allergy test. Guinea pig Hartley strain, 4-5 weeks old and test sample, Glupearl 19S and gluten from wheat. Okay? The sensitization method intradermal sensitization to enhance sensitivity and topical applications sensitization to estimate actual use, and flank area was shaved and razored. The solutions were applied, and sensitization number six -- total exposure about 2.5 milligram per animal. And dorsal area was shaved and razored 2 x 3 centimeter, open 0.5 milliliter of these test samples were applied. Okay?

So initial method, P -- passive cutaneous anaphylaxis test -- intradermal applied 0.1 milliliter serum from sensitized animal into recipient animals -- passive sensitization overnight, and inject one to one mixture, 0.01 percent of test sample and Evans blue dye into dorsal venous vessel of recipient animals -- challenge -- serum from sensitized animals and test samples and Evans blue. We see the antigen binding to IgE. So results of PCA test -- Glupearl 19S sensitization method, okay? The Glupearl 19S was

positive in intradermal and topical application sensitization methods. And gluten from wheat was also positive in intradermal and topical application sensitization method. And we can see that series dilution of the Glupearl 19S had higher serum titers than gluten from wheat in both methods. So Glupearl 19S is a stronger sensitizer than gluten.

This is an acid hydrolyzed gluten from wheat. I talked before -- zero to 24 hours, then molecular weight decreases, okay? You can see the -- this positive reaction, GluPearl 19S to 5,000 dilution, but gluten to 100, and acid treatment from -- zero hour -- gluten, very likely to gluten, the final positive dilution was 1,000. And 0.5 hour the final positive dilution -- it's a very similar, at Glupearl 19S, was 2,000. And at nine hours, still 1,000. So nine hours -- a treatment for nine hours -- secondly, it's at 2,000. So sample treated for 0.5 hour has weaker sensitization potential than Glupearl 19S, but stronger potential than samples treated for zero hours -- starting hour. Sample treated for 0.5 hour and nine hour has equal potential. And cross-reactivity assessment in PCA test was done. And serum from animals sensitized by Glupearl 19S partly cross reacted with gluten from wheat. And one more mouse model of food allergy with percutaneous sensitization, followed by intake of food -- it's a new one -- and this is -- I cannot explain, maybe time is up, so we -- this Professor Hiroyuki Tanaka, (inaudible) Pharmacology, Department (inaudible) Molecules, Gifu Pharmaceutical University, have made this new method -- very good model, but we have to apply Glupearl 19S repeatedly. You can see, this is serum IgE -- once, twice, percutaneous sensitization was done, and then gradually, IgE increases. And in five weeks, peak comes. This is the same with Teshima, Dr. Teshima's data.

DR. BELSITO: Was this application under occlusion?

DR. MATSUNAGA: Under occlusion, yes.

DR. BELSITO: For twenty-four, like --

DR. MATSUNAGA: 0.5 percent SDS solution.

DR. BELSITO: Repeated application, or just once a week? That's zero, three, ten days?

DR. MATSUNAGA: No, once a week -- no, this is a -- once a week, yes. And then, a challenge of wheat -- this slide demonstrates the percutaneous sensitization with Glupearl 19S, the oral

challenge with gluten and aspirin. Challenge this needed aspirin and gluten induces anaphylactic response. And the third study, you can see that manufacturing process for Glupearl 19S, as I explained before -- one, two, four and eight. And that antigens -- you can see, this is four -- cycle one is four -- the antigenicity comes out first, and the five, six, seven eight goes up, but this one is a no reactive response, okay? So you can see antibody here -- here, so number four to eight had sensitizing potency. So acid degradation by HCL and 100 degrees or 95 degrees degraded -- these findings suggest that the gluten is degraded and has high antigenicity by acidification and hydrolysis with heat. In conclusion, these findings demonstrate that the percutaneous sensitization with Glupearl 19S and oral challenge with gluten and aspirin induces anaphylactic response and these findings suggest that the protein has high antigenicity by acidification and hydrolysis with heat during Glupearl 19S manufacturing process. And this model, mouse model is useful not only to elucidate the underlying mechanism of the food allergy caused by percutaneous sensitization, but also to evaluate antigenicity of various cosmetic components. But you cannot do animal tests, I know. Well, now we are performing the genetics study on the hydrolyzed wheat protein, sensitized immediate allergy patients in Japan. I'll be grateful if we can report original meaningful data to you all, Expert Panel of CIR, in the future. Thank you very much for your attention.

DR. BOYER: We do have a little bit of time, if you would like to ask a few questions, if we have any at this point. Otherwise, Dr. Matsunaga will be at each of the team meetings.

DR. HILL: This may require a translation. Did you get some sense for how long somebody would be need to be using this soap regularly on their face before they started to see reaction to food? I heard you say -- I just wanted to be clear.

DR. MATSUNAGA: For the shortest cases, it took only two weeks, and for the median 31.5 months, longest, three years (7.7 years).

DR. BELSITO: And did you do any studies on these individuals who were sensitized to the Glupearl and test them to hydrolyzed wheat proteins of 30 kilodaltons or less and see that they did not react to them?

DR. MATSUNAGA: So I am showing you the slide to show three groups of the degrading

the Glupearl 19S protein, so those three groups are less than 3,000 daltons in that view, and between 3,000 and 10,000 and more than 10,000. Further cases of less than 3,000, although there might be deamidation happening, there was no reaction, even though they were sensitized.

DR. LIEBLER: Do you have measurements of the deamidation for those Glupearl fractions, quantitative measurements of deamidation?

DR. MATSUNAGA: I would like to ask Mr. Nakamura to answer that. He is an expert in proteinomics.

DR. NAKAMURA: It shows the measurement of deamidation when, during the manufacturing processes, from the gluten, to Glupearl 19S. So this shows the tendency of the deamidation, of the four proteins that are included in Glupearl 19S. So this shows the ratio of deamidation, and doesn't matter -- no matter what kind of a protein, they all have -- they all show 50 percent of the deamidation, meaning, it is not depending on the kind of proteins.

DR. LIEBLER: Thank you. We'll look forward to discussing in a little more detail when we meet in the team meetings. Thank you.

DR. BOYER: Thank you very much Dr. Matsunaga, and we'll turn it back over to Lillian and to Wilma.

DR. BERGFELD: I would assume that all of these presentations will be recorded in the part of the document in the discussions today. We are now at about 10:20. Unless there are any other announcements, we'll take a small break and then assume our team meetings. And it's my understanding that Belsito team stays here? No, Marks team stays here. All right. Thank you very much. All right, break time.

(Recess)

### **Marks Team Discussion – Monday, 17 March**

DR. MARKS: First of all, I would thank the presenters on the hydrolyzed wheat protein allergy. Both presentations were excellent and I think increase our understanding. And for the Marks team, this is very timely, because some of us have short memories, so starting right off the bat with hydrolyzed wheat

protein is good. You got that joke, Ron. Okay. So, we're looking at the draft final safety assessment of hydrolyzed wheat protein and hydrolyzed wheat gluten. I now understand what the difference between hydrolyzed wheat protein is, and gluten, or wheat protein and gluten, so that was helpful in the presentation -- the first one.

As you recall, and it was just mentioned, the conclusion of this draft final safety assessment was safe for cosmetics when formulated to minimize peptide lengths greater than 30 amino acids, and we heard that approximates a molecular weight of 3,000 daltons which both presenters indicated was safe. Additionally these ingredients should not be used on damaged skin, or in products that may come in contact with mucous membranes, or maybe incidentally inhaled, and there was some question raised by the first presenter, on whether or not these restrictions had needed to occur in the conclusion.

Since our last meeting, in consideration of these -- the Council's request that the wheat protein and wheat germ protein report be reopened, and there was some discussion in the memo as to why the CIR staff disagreed with the Council and stick with, as we've done with this draft final report, just with the hydrolyzed wheat protein and hydrolyzed wheat gluten. So Tom and Ron -- I'll ask you to weigh in at this point in terms of the previous conclusion and also whether we should expand the safety assessment and reopen wheat protein and wheat germ protein and combine with this report.

DR. SHANK: I have a problem with the conclusion that we came to using the cutoff of 30 amino acids. I don't see where that comes from in our report. There's nothing in the report that shows the 30 amino acids as a cutoff. There is a French study that shows 31 kilo daltons is the cutoff. Thirty amino acids would be about four and a half kilo daltons. So I don't know where the 30 amino acids comes from, and I think we shouldn't talk about amino acid number, but we should talk about molecular weight.

DR. BOYER: It actually comes from the -- it's a theory or a hypothesis, that you need at least 15 amino acids to have a sufficient number to bind for IgE. And if you need two of those to cross link the receptors, then together with the spacer that Dr. Matsunaga mentioned, that the amino acid, in order to have any activity -- immunological activity -- needs to be at least 30 amino acids long. It's based on that reasoning.

DR. HILL: Yes, she said eight to ten, instead of 15, so she said eight to ten, eight to ten and

1,000 dalton spacer, which would be seven amino acids -- if we use 126 as an average amino acid molecular weight. So I don't know if 30 is sufficient or not, but I remember we had references that said 30. If I do eight on one end, eight on the other end, and seven in between, that's still pretty close to 30.

DR. MARKS: Is it Dr. Chahal?

DR. CHAHAL: Correct.

DR. MARKS: From a manufacturer's point of view, if we were to give guidance in the conclusion, does it matter whether we use -- I think you endorsed the conclusion of 30 amino acids, which you also said equals approximately 30, or 3,000 daltons. Does it matter in the conclusion to you, as an ingredient supplier, whether the panel would specifically say 30 amino acid units, as it presently does, or should we switch to molecular weight of 3,000 -- it should not be greater than the molecular weight of 3,000 daltons?

DR. CHAHAL: I think it would be easier for manufacturers to measure molecular weight than for them to identify an actual peptide sequences length. For wheat proteins, the -- if you look at the amino acid profile of wheat -- 30 amino acids will give you a molecular weight in fact, of 3,570, to be exact, based on the profile of amino acids. It's an average, molecular weights, and I think the current guidelines suggest that it should minimize to 30, rather than be actual 30. I think that's probably a sensible thing as well.

DR. MARKS: So with that in mind then, Ron, Ron and Tom -- particularly Ron Shank, since you made the suggestion, you would like to change the conclusion to use molecular weight as the limit, and that would be 3,000, rather than peptide length. Okay?

DR. HILL: Well that goes back to the question that I asked -- I'm going to say your name wrong if I say it -- Chahal?

DR. CHAHAL: Chahal.

DR. HILL: Okay. I asked him after the presentation, which was, how well can we measure, and we say minimum, but I think we're leaving that up to the manufacturers, with the conclusion as written. I'm just concerned to make sure that there is a good way to measure that shoulder we were talking about -- enough that we can bring it below and we don't have science to tell us what would be the threshold of that kind of sensitization response, so maybe experience will tell.

MR. NAKAMURA: It would be very difficult to measure the size of the molecule. If you want to do that, then you will have to use math. It means -- my question is, whether all the manufacturers can do that. I doubt it.

DR. SHANK: Would it be any easier to measure 30 amino acids, as opposed to 4,000 daltons?

DR. ANSELL: It should be molecular weight.

DR. SHANK: Right. I think so too.

DR. ANSELL: And not the sequent number in the sequence.

DR. SHANK: Molecular weight might be easier.

MR. NAKAMURA: Yes.

DR. HILL: Well I think what he's saying is that there are probably don't have the advanced mass spectro capability to be able to do this well, but maybe that's an opportunity for some contract labs to get in the game. I'm always good with paying more money for science and less money for certain other things.

DR. MARKS: I don't think our concern is whether they can --

DR. HILL: I know.

DR. MARKS: Scientifically if it can be done, and then that's where we're at, and so, the key here to me, was, which is the better metric to use to put a limit -- whether it's molecular weight or amino acid peptide length. We've heard that molecular weight would be a better one, and even though it will occur obviously within the discussion, that the molecular weight is an approximation. How about --

DR. BERGFELD: May I ask a question?

DR. MARKS: Sure, Wilma.

DR. BERGFELD: If you deal with a molecular weight restriction or quantification, does that get rid of the need to say anything about the deamidation or the acid hydrolysis versus enzyme versus alkaline?

DR. HILL: For me, I was struck by, I mean it took quite a bit of science in order to run down what the source of the sensitization was in this particular case. Because the message is, that I get is -- we

can have peptides in there, well above 30,000. One hundred thousand, we still won't have the problem if we didn't -- in this particular case, with this particular protein -- if we didn't see the deamidation that occurred because of acid hydrolysis. And so, I'm thinking about this in terms of going forward, with other proteins that we reviewed, that maybe 3,000 is a general thing -- we won't get this type of sensitization, but then for a manufacturer who wants to market, 100,000 -- those kinds of products and what do they need to do with other proteins besides wheat. I mean that, to me, seems like going forward -- if we aren't below 3,000, we will be on a protein by protein basis, will we not? And that's not the question on the table today, but I'm just putting that out there.

MS. BURNETT: It's a good question to pose, because you will be seeing the remaining hydrolyzed proteins in June, so keep it in mind.

DR. MARKS: So Ron specifically, would you -- Ron Shank -- that says safe for use in cosmetics when formulated to -- do you want to use that same wording? Minimize molecular weight? How would you work it?

DR. SHANK: I would base it strictly on molecular weight.

DR. MARKS: Um-hm.

DR. SHANK: I still haven't resolved in my own thinking -- certain tests show that 30,000 kilo daltons can be -- give non-allergic response. We have those data, both from your studies, and from some studies in France. And I'm just not enough of a protein chemist immunologist to handle that, but we do have those studies, and we're not discussing that and that bothers me. I don't see the need for limiting exposure to prevent damage -- or not on damaged skin, not on mucosa -- if it's not allergenic, it's not allergenic and I don't think it makes a difference if it's on damaged skin.

DR. MARKS: Could you answer my next question? I wanted to --

DR. BERGFELD: Can I ask a question before you go on? It seems that the two presentations -- there was a difference in the Professor's presentation, and because you recommended not to be applied to damaged skin.

DR. MATSUNAGA: Yes, I say no.

DR. BERGFELD: No, yes, yes, I heard that.

DR. MARKS: But was that specifically with the soap that contained the allergenic hydrolyzed protein, wheat protein -- the Glupearl 19S? Is that also, from your point of view, you would do with any hydrolyzed wheat protein, if we limit the molecular weight to 3,000 or below, would you still have concerns about exposure?

DR. MATSUNAGA: I think to elicit allergic reaction, the molecular weight of 3,000 dalton meets -- necessary.

DR. MARKS: Correct, so if we limit the hydrolyzed wheat protein to less than or equal 3,000, in your estimation, does it matter how an individual is exposed? Do we need to limit exposure, whether it's mucous membrane, inhalation, or whatever?

DR. MATSUNAGA: It's hard. I think -- molecular weight doesn't matter as far as there is no other irritancy, or no other damage or harm, such as irritancy.

DR. MARKS: And the other -- so I think we've arrived at basically, the conclusion will be -- safe is less than 3,000 molecular weight. How about the proposal by the Council, addressing reopening wheat protein and wheat germ protein including in this, so this would be a whole different -- that's been reviewed before. Do you want to do that, or do we want to deal with the hydrolyzed as a separate report as we have right now? Did I understand that -- the memo, Christina -- what the Council's proposing -- the CIR staff have felt we should not reopen those and --

DR. SHANK: We already have a document on wheat protein.

DR. MARKS: Right.

DR. SHANK: So I don't know how you would -- why you would put that in. If you want to reopen the wheat protein document, that would be a different story.

DR. MARKS: Right.

DR. SHANK: But I would not put in the whole proteins, into this report.

DR. ANSELL: We think that that could probably be handled in a discussion, that trying to, in fact -- a number of these things that we've just discussed, I think, should be carried into the discussion, but

we have -- safe as used for whole protein, yet we're limiting the size of the protein here. There should be some clarification as to how those two conclusions make sense.

DR. HILL: But her presentation made very clear how those -- that makes sense because what she showed was, if you don't do hydrolysis in such a way that you end up deamidating everything it remains safe. So two very different regimes, which is why I think mixing such apples and oranges would be a very bad idea.

DR. ANSELL: I'm sorry, I think --

DR. HILL: Her presentation made clear that --

DR. ANSELL: No, no -- I understood her presentation, but I'm not sure that everyone as clever as you, would realize how the whole proteins are safe and so we just --

DR. HILL: I'm not suggesting it should not be in the discussion. It should be in the discussion but just reopen to mix those in with this report, I think would be a very bad approach.

DR. ANSELL: We think that handling it in the discussion is just fine too.

DR. HILL: Okay, then --

DR. BERGFELD: Well, if you handle it in the discussion, do you not have to reference that particular document and talk about it somewhere other than just the discussion?

DR. MARKS: No, the previous document says it's safe.

DR. BERGFELD: I know.

DR. MARKS: Now we're just saying in this document, since the issue has arisen with Type I allergy, why that previous document -- we can say why it's safe. We don't need to reopen it.

DR. BERGFELD: You usually don't reference a discussion, that's my point. You need a reference in the document to that.

MS. BURNETT: There is a very brief reference to the wheat.

DR. BERGFELD: So you could enlarge that to --

MS. BURNETT: In the introduction, we just say that the wheat gluten report has been -- the ingredient was reviewed and found as safe, and the discussion --

DR. BERGFELD: You don't usually reference an introduction either though. I'm just trying to think where the reference could be best put, where there'd be a little discussion, a couple sentences.

MS. BURNETT: Well we can put references in the introduction, so that --

DR. BERGFELD: Can you?

MS. BURNETT: Yes, it's the discussion and conclusion.

DR. BERGFELD: Okay.

DR. MARKS: Okay, so --

MS. BURNETT: We'll find a way around it.

DR. BERGFELD: Okay.

DR. MARKS: So let's move forward with this, I think. Again, thank you for your presentations. Tomorrow I think I will be seconding a motion with a conclusion, safe with a molecular weight of less than or equal to 3,000. I guess the last part is -- this would continue to go out as a draft final summary because the conclusion has changed significantly and I guess, in my mind -- I don't know that we can move to a final. I think we had some points that were brought up this morning that need to be put in the discussion summary -- more data. So I would propose that it continue as a draft final.

DR. GILL: I would just add that I think this is a less restrictive conclusion, so would you need to see it again in a draft form?

DR. SHANK: Is it not a good idea to let industry respond -- that we have changed how the cutoff is defined? You know, if they say we can't do that, then we need to hear it?

DR. ANSELL: We are also suggesting there be a more robust discussion of the chemistry, since the molecular weight limit is so dependent on the analytical methodology, that there should be some discussion that in your lab you may, to meet that 3,000, you may actually have a 2,000 cutoff, so there should be some discussion of that and I suspect we would very much like to have a chance to review that again.

DR. GILL: So to clarify -- you'd like to see more discussion of how you -- how a lab might reach that 3,000 cutoff.

DR. ANSELL: I'm not sure so much recommendations as to the methodology, but at least a

discussion that the number is very method dependent, and so that 3,000 we're talking about is dependent on the specific methods. Other methods may get different numbers and that should be thought of.

DR. MARKS: So team members, what do you feel? Do you want to move -- and Rachel I might ask you also. Do you think it would be worthwhile delaying so that we can look at the whole report one more time, before we rule on the final safety assessment, or is this a minor enough "change in the conclusion" that we could just move forward? Tom?

DR. SLAGA: Well I think since we're in the end in the lock of the discussion, I think we should see that before that goes in.

DR. WEINTRAUB: I agree. I think there should be another opportunity to review. And one thing that I think we likely covered but I don't think we specifically articulated and that I think should be added in that discussion, is just the idea of the acid hydrolysis. Because it seems that both presentations really focused on that as the mechanism of the allergen. So in addition to looking at the weight as opposed to the number of molecules, that seems like it's another important addition.

DR. MARKS: See, what I -- and maybe you can comment -- it seemed like it was the combination of acid and heat resulted in deamidation and I guess the question -- this doesn't affect this report, but is Glupearl 19S still available worldwide, or has it been discontinued?

DR. CHAHAL: I believe it has been discontinued.

DR. MATSUNAGA: Glupearl 19S was produced in and sold in Japan, only Japan, and now -- they used to sell Glupearl 9000 like for food additives, and also, then after food additives, they -- those were very safe, so they made cosmetic grade Glupearl 19S, and then all food and cosmetic Glupearls, right now -- not for sale, so --

DR. CHAHAL: Just a point on the acid hydrolysis. You need the acid deamidation but high molecular weight as well. If you continue to break it down with acid, you will lose that.

DR. BERGFELD: Could you speak louder, I can't hear you.

DR. CHAHAL: Sorry, in terms of acid hydrolysis -- you need the acid deamidation and high molecular weight. Once you start to break it down, even if it's deamidated, in below 3,000 daltons, you

don't get any sensitization.

DR. BERGFELD: So if -- excuse me -- if you say that, is there a way of saying that in a text? That complete acid hydrolysis would be preferred?

DR. CHAHAL: I think the molecular weight answers the question.

DR. BERGFELD: Would be less allergenic and lower molecular weight.

DR. CHAHAL: Just below 3,000, 3,500, would be sufficient.

DR. BERGFELD: Okay.

DR. HILL: Not only less allergenic -- not allergenic, provided we don't get a shoulder on the size distribution.

DR. BERGFELD: So it's partial versus complete.

DR. CHAHAL: Yes.

DR. BERGFELD: Okay.

DR. MARKS: Okay, so I think we can move forward again. Thank you for your sage input. Tomorrow, presumably I'll be seconding another draft final safety assessment, with conclusion safe less than or equal to 3,000 molecular weight. They'll be no restrictions in exposure in the conclusion we would propose. Any other comments Panel Members? Anybody else, any comments? If not, I -- yes. Okay. Thank you very much.

***Belsito Team Discussion – Monday, 17 March***

DR. BELSITO: So the hydrolyzed wheat protein team has arrived, so we've been instructed to move to that, which is under hydrolyzed wheat protein. So at the September meeting we concluded that the hydrolyzed wheat gluten and hydrolyzed wheat protein are safe for use in cosmetics when formulated to minimize peptide lengths greater than 30 amino acids, and we heard this morning that we're pretty prescient to doing that. But we were also told that perhaps we should not look at restricting these products to damaged skin or products that could come into contact with mucous membranes or being incidentally inhaled, which

will probably be the major focus of our discussion. And we did recognize that if we continued with that restriction -- damaged skin, mucous membranes, and inhalation -- that we would essentially eliminate 100 currently registered products.

And we really wanted to hear a little bit more about what was happening in Japan and how these products were manufactured and allergenicity of the various products, and I would like to thank both of our speakers. I think they did a wonderful job in bringing us up to speed at what the issues were in Japan and what the issues are with deamidation and the different types of reactions.

And I guess my major comment is to go back to the handbook, and this is a continuing issue that the INCI names don't tell us a lot about the product. It's going to come up with other products that we're looking at today. And I think the International Nomenclature has to work on getting names that are specific to specific products rather than to broad categories because, quite honestly, I think it's almost a little embarrassing that the same name can be applied across a variety of different ingredients that can cause different toxicologic endpoints. But that's just -- yes?

DR. BRESLAWEC: Dr. Belsito, I want to say that the industry agrees and tomorrow the editor of the INCI Dictionary will be here and will be available to answer questions and also discuss some initiatives that we've introduced over the past year or so to update how nomenclature is assigned to make it more detailed. We have, for example, taken a category of prostaglandin-like ingredients and totally changed how they are named. We have done the same thing for certain proteins, polypeptides.

DR. EISENMANN: You will see that they have retired the general term and are working towards a more specific name.

DR. BRESLAWEC: What we are trying to do and are doing, in fact, is trying to be more current in how the names are assigned and more descriptive and this, perhaps, is one category where a change would be required.

DR. BELSITO: So I guess before we start giving any opinions, are there any other questions or issues with the presentation that any of you feel need to be clarified? I think, Dan, you had some ongoing questions that weren't quite answered?

DR. LIEBLER: My only question was about the assessment of the deamidation fraction.

In one slide the deamidation fraction was portrayed as a percentage, and I assume that is some type of measurement across the entire protein preparation. Is that a measurement of the -- well, how is that measurement obtained, the deamidation fraction?

MR. NAKAMURA: (Interpreter) Well, you use a measurement, you reach the acid. And using the genome database and it said it will include the acid in your search. Then you come up with the ratio.

DR. LIEBLER: So that provides -- I'm very familiar with that method and that provides identification, but I do not understand how it provides quantitation.

MR. NAKAMURA: (Interpreter) So you think the frequency of the peptide then you are going to be approximate.

DR. LIEBLER: Spectral count?

DR. NAKAMURA: (Interpreter) Spectral count.

DR. LIEBLER: Okay, I understand. Thank you.

DR. BELSITO: So can you interpret that for me, Dan?

DR. LIEBLER: So the question I had was how is the percentage of the deamidation reactions estimated? The deamidation reactions are conversions of glutamine to glutamate and asparagine to aspartate. So when the mass spec analyzes this mixture of peptides that's produced from the proteins, the mass spec samples the peptides that are present in the digest and records fragmentation spectra, so-called MS/MS spectra. These are then searched against a database to identify which peptides contain the unchanged glutamine and asparagine sequences and which ones contain the deamidated sequences. And so that provides identification of the different kinds of peptides in the sample. And my question was how do you go to the step of quantitative comparisons? And it turns out that when the mass spec samples a complex mixture, it is more likely to obtain multiple spectra, fingerprints, of peptides that appear in higher and higher abundance. So we refer to the term "spectral counting" to reflect that sampling phenomenon and it's a very well accepted quantitative, I guess at least rough quantitative, measure in proteomic analysis. And it's okay, I think, for the purposes of the presentation that we have. There's actually a lot of information that's potentially useful in your

datasets.

So I'm satisfied that I understand how the measurements were done. I think it's a reasonable approach. It could be done even more precisely with some other methods, but this is still pretty good.

DR. BELSITO: Okay.

DR. SNYDER: So I think the methods of manufacturing impurities have to be -- are we considering the deamidation products as impurities now? I'm posing this somewhat as a question.

DR. CHAHAL: Deamidation shouldn't be considered as impurities or of any concern as long as they're hydrolyzed to below the limits that you've set.

DR. SNYDER: I guess that goes to the method of manufacturing. So what data do we have or that we know because I would assume that's part of the discussion that we want to say is that our understanding is that these will be limited. But part of the problem is that it's not -- in people who are severely allergic and particularly when you're talking about anaphylaxis, which is often a life-threatening consequence, even a very small concentration in a highly sensitized individual can be a serious consequence. So we're not talking about just developing a skin reaction here. We're talking about a very serious consequence here, so I think we have to be very clear of what we --

DR. BELSITO: Let me clarify what I heard you say, and you can tell me if this is true, that if you're getting down to fractions that are less than 30 amino acids, the deamidation products there will probably be some in that fraction, but it will not be of concern. It will not trigger the Ig immediate response because it can't cross-link. It's not long enough to cross-link the receptors.

DR. SNYDER: That's making some false assumptions because certainly those could cross-link with native proteins in the skin and serve as a size so it can cross-link. So that's not out of the realm of possibilities. I asked Dan a couple of questions during the thing because the relationship between molecular weight and the deamidation issue, are we falsely thinking that by using a size -- because one of the ways that you can hydrolyze these things is a protease and there's proteases in the skin, right? And so can they potentially be further hydrolyzed through dermal exposure?

DR. BELSITO: But if they are, though, we'd go to a smaller molecule, which would be

even less of a risk, would they not?

DR. CHAHAL: Yes. On the face of that, we've generated and suggested that if you take those short peptides and even deamidate it and use individuals that have that very same sensitization and nonconventional, there's no reaction. So you are following the fact of minimizing the size, removing a potential based on the data that was presented this morning.

DR. SNYDER: And then the only other comment I had was if we could get the data that was presented in the slide, talking about the 19S in which they clearly showed in patients that were sensitized, that if you had the 3,000 limit -- that was very solid data -- if we could have that data to put into the document because I think that's a well-recognized highly sensitive reaction to a hydrolyzed wheat protein that's very well studied and that gave me a great deal of confidence on the 3,000 because less than that --

DR. BELSITO: In getting rid of the mucous damage, et cetera. I agree.

DR. SNYDER: That to me was really compelling data for the size of anything we saw.

DR. LIEBLER: So I have a couple of additional --

DR. BELSITO: Well, wait a minute. We haven't heard the response from the Japanese.

Are we going to be able to get that specific data?

DR. BRESLAWEC: I think it's published, is it not?

DR. BELSITO: The data that we saw where the people who were sensitized to the Glupearl 19S when they looked at the different levels of response to different sized protein.

DR. SNYDER: There wasn't a reference on the slide.

DR. MASUNAGA: I will ask.

DR. SNYDER: There wasn't a reference on the slide.

DR. MASUNAGA: He's the writer.

DR. BELSITO: Okay, I would agree with you. I think it's critical that we have that data for support. But I think Ian Kimber is right on as well. If you don't have a molecule that's large enough to cross-link and elicit a reaction, then typically it takes higher levels to induce reactions than to elicit reactions. So even with damaged skin at the same concentration, assuming you're getting more in. And we saw that.

That's really not the case because people are getting sensitized through skin-only contact. So granted at higher levels, but I think --

DR. SNYDER: And quite long exposures.

DR. BELSITO: Right. We can finesse it with discussion, but that would just nail the point very solidly that here we have data specifically showing that in sensitized individuals when exposed to these hydrolyzed wheat proteins of less than 30 amino acids, there was no reactivity. And then we can explain that observation based upon the types of arguments that Ian Kimber is making.

DR. BRESLAWEC: Do you have copies of the whole presentation?

DR. MASUNAGA: I have to have the agreement with my members, colleagues, so right now I cannot say yes. But I can send you when I get the okay.

DR. BRESLAWEC: Okay. We will strive to provide copies of the entire presentation as soon as possible.

DR. BELSITO: Just one final comment. Dr. Matsunaga, if this paper -- I mean this is obviously a very important point that we need to get across to industry so we do not have these issues in the U.S. -- could your colleague at least give us a written statement of a summary of his results?

DR. MASUNAGA: Okay, tomorrow you are going to have the meeting, yes?

DR. BELSITO: Yes.

DR. MASUNAGA: So I will email to you today. I hope I make it okay.

DR. BELSITO: I'm sorry, Curt.

DR. KLAASSEN: I have a very general question and that is as most of us know, peanuts are very allergenic to some people. Have similar studies been done with peanut proteins and also showing that 30 amino acid is kind of the magical length? So, in essence, can we generalize this more than just in relationship to wheat?

DR. BELSITO: Yes.

DR. CHAHAL: There's not a lot of data in the public domain with reference to if you take any protein and you break it down at what level. There's some information out there. There's some

information on milk proteins, for example, in food as you break them down further that you remove the potential for allergenicity. I asked Professor Kimber that same question irrespective of the source of the protein, and his response was similar; that if you break them down to below a certain level, the potential for allergenicity is removed.

DR. KLAASSEN: Right.

DR. BELSITO: Dan?

DR. LIEBLER: So I have a couple of comments. One is that it's been interesting to see the data on deamidation and the relationship between deamidation and the observed adverse effect. But we really don't know if deamidation causes the effect because we haven't been able to separate deamidation from hydrolysis peptide length, so it remains a correlative relationship. And I think deamidation is probably quite prevalent in most hydrolyzed protein preparations even if it's not directly measured by the techniques that are used to characterize commercial preparations. So I think we should be careful about any speculation about the deamidation. If we discuss it at all, it could be mentioned as a possible factor, but not anything more than that.

Secondly, I think we have some confusion with the math on the molecular weights and the peptide lengths.

DR. BELSITO: Page?

DR. LIEBLER: Well, we can look for it. We have been using 30 amino acids as being 3,000. That is I think mentioned several times. But 30 amino acids if you have an average molecular weight of about 150 per amino acid residue minus 18. So I did that calculation. So a 30 amino acid peptide on average is about 4,000 molecular weight.

DR. CHAHAL: Just a clarification if I may? I did the calculation based on the amino acid profile you'll find in a wheat protein with 18 amino acids, and it worked out to 3,570 so very close.

DR. LIEBLER: You're adjusting for the frequency of some amino acids versus others, that's right. I didn't do that.

DR. CHAHAL: Yes, so in the same ballpark.

DR. BELSITO: So we're restricting it not based on weight. We're restricting it on amino

acid length.

DR. LIEBLER: Right.

DR. BELSITO: You're suggesting a weight restriction?

DR. LIEBLER: Well, a length restriction isn't actually that easy to measure. So the parameter that can be measured by the methods that you mentioned in your presentation this morning or by mass spectrometry are essentially mass measurements or surrogates for mass measurements. So I think mass measurements that are under 3,500 is probably a safe approximation of 30 amino acids in length.

DR. BELSITO: So you wanted to say in the conclusion "less than 3,500 daltons."

DR. LIEBLER: Right. I think that would be more useful guidance to industry than --

DR. BELSITO: But we just heard from Dass -- is Dass your name? -- 3,570.

DR. CHAHAL: Approximate. It's never --

DR. BELSITO: Well, we don't want to put it to the point where a safe product then gets knocked out of the ballpark because it's 3,502.

DR. LIEBLER: No, they won't ever come out like that because these are distributions of molecules and it's going to be -- when you produce a product like this, hydrolysis, you're going to have a distribution of peptide lengths. And what you really want to do is adjust for the mean and the dispersion.

DR. CHAHAL: It's a weight average measurement.

DR. LIEBLER: Yeah. And you don't want the dispersion to be too broad, but you want the mean to be -- you want the mean to be at least under 3,500. And if the top end is too broad, then a mean of 3,500 still doesn't help you because you still have a fair amount of longer material.

DR. BELSITO: So then how do we phrase that?

DR. LIEBLER: That's a good question.

DR. BELSITO: Amino 3,500 with a distribution no greater than --

DR. CHAHAL: I think your current guidelines suggest minimize anything greater than 30.

DR. LIEBLER: I think prescribing a dispersion number is probably outside of our purview, but I will also say that I'm really surprised that mass spectrometry isn't routinely employed by a major supplier

to make these measurements. I mean this is the 21st century now and that's what you guys should be doing.

DR. CHAHAL: It can be done and has been done and then you can correlate back that are easier to use. And that's what we've kind of tried to do.

DR. LIEBLER: Actually, MALDI is easier than all of the things that you described. It's not even more expensive after you count up -- anyway, off my soapbox. You would get better measurements that would keep you out of trouble.

DR. BELSITO: So let me recap where we are at this point. We're relying on Dr. Matsunaga to get permission from her colleagues to include the data showing that the smaller 3,000 dalton fraction did not elicit a response in sensitized individuals. So we're getting rid of the damaged skin, mucous membrane, aerosolized restriction, but we're also changing our conclusion for not amino acid length, but we're changing it to a weight of 3,500 daltons or less with a minimum variation about that mean?

DR. LIEBLER: Well, minimize the composition or the fraction of peptides over 3,500 molecular weight. That probably is the best way to put it.

DR. KLAASSEN: We can basically just turn it around where we have approximately 30 amino acids behind it.

DR. SNYDER: So what are the data that we're using to support 30 amino acids?

DR. BELSITO: The fact that 30 amino acids doesn't cross-link IgE receptors and cause hypersensitivity reactions. The data we have from the document that we got there before. That was the basis for our decision to limit it to 30 amino acids.

So I'm just trying to put this together. So our conclusion with this hydrolyzed wheat protein and wheat gluten is that they're "safe as used when the mean weight is 3,500 daltons or less and the ingredient is formulated to minimize peptides of greater weight?" What are we saying here?

DR. LIEBLER: So I would say instead of talking about the mean or median, I would say that "minimize the fraction of peptides of greater than 3,500 molecular weight."

DR. BELSITO: So "safe as used when the ingredient is formulized to minimize the peptides of greater than 3,500 daltons in weight."

DR. LIEBLER: That's fine.

DR. BELSITO: Paul, you had some problems in there?

DR. SNYDER: Well, minimize. Does that mean 20 percent, 30 percent? Do we need to -- I think this is a life-threatening thing. I think we need to have some pretty rigid guidelines. I think if we know that 3,500, I would prefer some stronger language, but I don't know what "minimize" means. Does it mean less than 10 percent?

DR. BRESLAWEC: When you minimize to under 3,500 daltons, what does that mean for you?

DR. CHAHAL: One of the slides I'm going to show you for some methods for measurement, you said the polydispersity was fairly narrow and that's the weight averages with 3,000. What you would find in the shoulder may be 4,000, 5,000, but you may be talking a few percent. But it's that material that was tested.

DR. BELSITO: That material meaning the material that you're talking about was the material that was tested by our Japanese guests?

DR. CHAHAL: No, it was material that we tested and analysis done.

DR. BELSITO: So perhaps it would be nice if we could see if there was further chemical characterization on the 3,000 dalton peptide that was tested by --

DR. CHAHAL: We did the test --

DR. BELSITO: Oh, okay.

DR. SNYDER: I think we just want to have some data to ensure that the methodologies currently used in practice do, in fact, limit the amount of material as a contaminant was greater than 3,500.

DR. BRESLAWEC: What you have said suggests to me that when you formulate an ingredient, you formulate it so 95 percent or so is less than 3,500.

DR. CHAHAL: Yes, it also depends on the method you use to measure. We saw that as well.

DR. SNYDER: The issue is methodology. So I just think that we want to give clear

guidance and we want to give clear competence in what we're stating. That's just what we have to do. I think those two methodologies has to be picked up a little bit into what we're saying, how we understand what is happening actually in practice that's going into formulation.

DR. BELSITO: So I guess my question to you as I try to sort through what you're saying is are we saying that this is still insufficient pending additional information on that methodology?

DR. SNYDER: I think that we saw presentations, but we didn't actually see data. I would like to have a couple of examples of what the size distribution was of material that was actually used in ingredients.

DR. BELSITO: So are you comfortable with going safe and seeing that data later or are you wanting, since you're concerned about --

DR. SNYDER: I don't like the language minimized without any limit on minimize. I don't like that.

DR. MASUNAGA: Minimize? What you mean by minimize.

DR. BELSITO: So if we knew what the distribution was of the product that was used in Dr. Nakamura's studies, we would have a better sense of where we wanted industry to have their cutoffs.

DR. SNYDER: So in the soap, the 19S, what percentage of that ingredient was above 3,500?

DR. BELSITO: All of it.

DR. SNYDER: It was all of it.

DR. BELSITO: It was huge.

DR. MASUNAGA: No, no, no. What did you say? 0.3, 0.3 ingredients.

DR. BELSITO: It was a large molecular weight, but the molecular weight of the Glypure-19S is --

DR. MASUNAGA: Oh, average which is 50,000.

DR. BELSITO: 50,000, right. It's a very big molecule.

DR. LIEBLER: See, the Glupearl is -- this is the Glupearl tail wagging the hydrolyzed

wheat dog here, is it not?

DR. BELSITO: Well, you're from Tennessee.

DR. LIEBLER: No, I mean all humor aside, this protein product, this preparation, is driving the discussion here. And we have tried to rationalize the results based on molecular weight and perhaps deamidation. It's quite possible that neither of those factors explain what happened; that there was something else that happened with that soap and that protein preparation in those individuals.

DR. MASUNAGA: Actually in Japan, they are trying to make a regulation to regulate to make it a standard that would be only up to 60 percent of the molecular weight of 10,000 or more. So to me, 3,000 is very small, but it is safe. It is very possible that they will be able to measure in Japan and that is one factor. So I think there is a question whether you should put the 3,000 molecular weight first or to apply to everything. And in Japan we might have more.

DR. LIEBLER: So I think being able to measure deamidation will be valuable, but I think we will only be able to draw conclusions about deamidation if we actually do studies with chemically defined deamidated peptides or proteins administered in the appropriate model systems to control for the difference between deamidation and molecular weight. Those experiments I think have not been done yet.

DR. MASUNAGA: No.

DR. LIEBLER: Yes, I think this is a very interesting line of investigation, but it's too soon for us to incorporate this into our recommendations. I think at best we can point to the potential role of deamidation, the interplay of deamidation, molecular weight, and modification of critical epitopes.

DR. MASUNAGA: You can make a best document, but you cannot make complete document I think.

DR. LIEBLER: That's right.

DR. BELSITO: Okay, any other points? Okay, getting back to my last question to you, Paul, are you comfortable tomorrow with saying "safe as used when the ingredient is formulized to a mean peptide weight of 3,500 daltons or less" Mean or median?

DR. MASUNAGA: No mean.

DR. BELSITO: How do you want that phrased?

DR. KLAASSEN: I wouldn't say either.

DR. BELSITO: How exactly do you want that conclusion phrased going to weight? Or is it better to go back to the 30 amino acid and let industry figure out what that weight would be?

DR. SNYDER: Do they have any animal data to support the distribution that will not elicit in an animal model or any kind of test system that we can put some science behind? Because I think we understand where the cutoff is, but I don't know --

DR. BRESLAWEC: What they're saying is the cutoff that they're looking at is 10,000.

DR. SNYDER: 10,000, I know.

DR. BRESLAWEC: So if we're looking at a 3,500 cutoff, then I think we have a little more flexibility with what the term "minimize" means.

DR. SNYDER: Well, but we don't have the data to understand what they're basing the 10,000 on.

DR. BRESLAWEC: Well, I think Croda has --

DR. EISENMANN: Croda's the main supplier, so their data -- he says their tail is whatever size it is, that's probably pretty good with industry.

DR. SNYDER: If we can have the data and put it into the methods of manufacturing in the chemistry section, I'm fine. I think we just have to -- we need more data, robust data.

DR. CHAHAL: It's terminology typically used, so measuring molecular weight is the weight average.

DR. BELSITO: You need to speak up a little.

DR. CHAHAL: The terminology normally used for molecular weight measurement is weight average molecular weight.

DR. BELSITO: Okay, so "safe as used when the ingredient is formulized to minimize peptides of greater than 3,500 daltons weight average?"

DR. CHAHAL: That would be the correct terminology in molecular weight measurements.

DR. BELSITO: So greater than 3,500 daltons as a mean molecular weight measurement?

How should we phrase that?

DR. BRESLAWEK: "Minimize to a weight average of 3,500 daltons."

DR. BELSITO: "To minimize to the peptides to a weight average of 3,500 daltons or less."

DR. SNYDER: I like that a lot better than minimizing a negative.

DR. BELSITO: So "safe as used when the ingredient is formulated to minimize the peptides to a weight average of 3,500 daltons or less."

DR. LIEBLER: How about instead of minimize, restrict because minimize doesn't mean the same thing in that sentence anymore. It's the wrong word.

DR. BELSITO: "To restrict." Okay, that's more restrictive. I like that.

DR. SNYDER: That was actually the word I was looking for.

DR. CHAHAL: It will depend on the method of measurement to some extent as well.

DR. BELSITO: Right, we understand that.

DR. CHAHAL: And with a weight average of 3,500 you will have a shoulder.

DR. BELSITO: Yes, and we were hoping, as Paul suggested, you could provide us with a little bit more analytical chemistry, showing us exactly the weight distribution of what your hydrolyzed wheat protein with a weight average of 3,500 daltons would look like.

DR. LIEBLER: Right.

DR. CHAHAL: I can do that.

DR. BELSITO: Then my final question to you, Paul, is we got some promises out there. Are you comfortable going with a safe as used tomorrow? Or are you saying it's insufficient pending receipt of the data from Japan and from industry?

MS. BECKER: You also have the option of tabling it.

DR. BELSITO: I don't want to table it.

MS. BECKER: Or you can act on it the next time. Insufficient suggests that there's data out there that you absolutely need.

DR. BELSITO: I'm following it.

DR. SNYDER: And I would defer to Lillian. I can live with either or. I'm not going to hold a document if I believe the data's there. I think both presentations were very well done, and I have a lot of confidence in the science behind them. It's just that I'd like to see some of that actually brought in, incorporated into the document.

DR. BELSITO: Okay, so then -- I don't even know if I'm doing this. I am doing this.

MS. BURNETT: I can give you the difference in terms of how it sets up. If you give us -- if you say safe as, it goes out again for review as another tentative announcement. We'd have to have it posted by next week for comment. If we don't the data and then tabling it, we could still bring it back in June, but it won't go out for the public comment as of next Friday. That would be the difference time-wise for us in how the document's presented.

DR. BELSITO: So I think that what I'm hearing from you, Paul, and Curt and Dan, if you're in agreement with this, I'm going to go as a "safe as used when the ingredient is formulated to restrict the peptides to a weight average of 3,500 daltons or less." And that conclusion was based on the assumption that we'd be getting some data from Japan that would come into the document, some data from industry that would show us what a hydrolyzed wheat protein with a weight average of 3,500 daltons, what that dispersion around that weight average is in terms of weights. Okay? Everyone's comfortable with that? Any comments?

DR. SNYDER: Just, Don, I want to make sure that we -- whatever weight you put in the document you put a unit because there's a lot of them. We go between daltons and kilodaltons -- some with daltons and some no units. So just because this is such a critical part of the document, we need to make sure that we capture all those.

DR. BELSITO: Okay. Any other comments? If not, thank you so much. This has really been very informative for us and has really answered a lot of our questions and concerns.

Okay, so save that puppy and go back to where we were. Maybe try to knock off one ingredient before lunch? So the tocopherols, I mean I think they're doable in 15 minutes.

So at the December meeting we reopened the safety assessment on the -- oh, wait a minute.

There is one more issue that we need to discuss that will probably chew up 15 minutes. It doesn't involve the other hydrolyzed wheat protein and that was PCPC's request that we go back and review the wheat --

DR. EISENMANN: I was just a little concerned that you have no limit. So I don't know that you have to open it because it's not an important ingredient to the industry. But it's just -- to me that it's something that's out there and I don't know that you want to deal with it or not.

DR. BELSITO: I didn't want to deal with it.

DR. EISENMANN: Okay, that's fine.

DR. BELSITO: So then let's go to tocopherols.

DR. SNYDER: I guess I didn't want to deal with it either.

DR. BELSITO: Did you?

DR. SNYDER: No.

DR. EISENMANN: Well, if it's not an important ingredient to the industry, so we can just --

DR. BELSITO: Well, it was a good thought.

***Full Panel Meeting - Tuesday, 18 March***

DR. BERGFELD: Thank you. Moving on to the next ingredient, which is wheat protein, Dr. Belsito presenting.

DR. BELSITO: Yes. This is the safety assessment of hydrolyzed wheat protein and hydrolyzed gluten. And we very much appreciate the presentations that we had from Dr. Matsunaga yesterday and also from the representative from Croda. Based on those, we felt that we could go with the safe as used when the ingredient is formulated to restrict the peptides to a weight average of 3,500 daltons or less. We would like to be able to incorporate the data that we saw that when you took sensitized individuals and challenged them at that level they did not react, and Dr. Matsunaga was going to look into the availability of us to have that data in the report. We also asked that Croda provide us with some specifications as to if the weight average was 3,500 daltons, what would be the spectrum of other hydrolyzed wheat proteins. And I

believe we have that information here which I will ask all of my panel members to see if they agree. But I think it's a pretty narrow and safe range, particularly since we've heard that you could actually go up to weight averages of 5,000. Was that correct with the Japanese -- 10,000 daltons?

So I would like to make that motion, safe as used when the ingredients formulated to restrict the peptides to a weight average of 3,500 daltons or less. We chose weight because we were told it's very hard -- it's a much easier measurement than to do amino acid numbers.

DR. MARKS: Second.

DR. BERGFELD: Any other discussion regarding the wheat protein?

Seeing none, I want to thank the presenters. You certainly helped us move to this conclusion. All those in favor please indicate by raising your hands.

Thank you. Unanimous.

(Motion passed)

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## Safety Assessment of Hydrolyzed Wheat Protein and Hydrolyzed Wheat Gluten as Used in Cosmetics

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The 2014 Cosmetic Ingredient Review Expert Panel members are: Chairman, Wilma F. Bergfeld, M.D., F.A.C.P.; Donald V. Belsito, M.D.; Ronald A. Hill, Ph.D.; Curtis D. Klaassen, Ph.D.; Daniel C. Liebler, Ph.D.; James G. Marks, Jr., M.D.; Ronald C. Shank, Ph.D.; Thomas J. Slaga, Ph.D.; and Paul W. Snyder, D.V.M., Ph.D. The CIR Director is Lillian J. Gill, DPA. This report was prepared by Christina Burnett, Scientific Analyst/Writer, Bart Heldreth, Ph.D., Chemist CIR, and Ivan Boyer, Ph.D., Toxicologist CIR.

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### **Cosmetic Ingredient Review**

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## **ABSTRACT**

The Cosmetic Ingredient Review (CIR) Expert Panel reviewed the product use, formulation and safety data on hydrolyzed wheat protein and hydrolyzed wheat gluten, which function as skin and hair conditioning agents. The Panel determined that data from clinical and laboratory studies was sufficient to demonstrate that these ingredients will not elicit Type 1 immediate hypersensitivity reactions in sensitized individuals, and will not induce sensitization when the polypeptide lengths of the hydrolysates do not exceed 30 amino acids. The Panel concluded that hydrolyzed wheat gluten and hydrolyzed wheat protein are safe for use in cosmetics when formulated to restrict peptides to a weight-average MW of 3500 Da or less.

## **INTRODUCTION**

This safety assessment is of hydrolyzed wheat protein (HWP) and hydrolyzed wheat gluten (HWG), which are each mixtures of amino acids and peptides of varying lengths derived from wheat sources. These ingredients function as skin and hair conditioning agents in personal care products. The CIR Expert Panel (Panel) previously reviewed the safety of  $\alpha$ -amino acids, animal- and plant-derived amino acids, hydrolyzed collagen, hydrolyzed corn protein, and *Triticum Vulgare* (wheat) gluten and concluded that these ingredients are safe for use in cosmetic products.<sup>1-7</sup>

## **CHEMISTRY**

The ingredients in this group are interrelated because they each are prepared from wheat proteins by partial hydrolysis to yield cosmetically acceptable raw materials. The definitions of these ingredients are presented in Table 1. Wheat gluten typically represents about 85% of wheat protein, and consists of the water-insoluble fraction of wheat proteins, including gliadins and glutenins.<sup>8</sup> The remaining 15% of wheat proteins consists of water-soluble, non-gluten proteins, including albumins and globulins.

These protein derivatives are prepared by subjecting wheat proteins to enzymatic (e.g., papain hydrolysis) or other chemical hydrolyses (e.g., acid, alkaline, or steam hydrolysis). The resulting polypeptide-, oligopeptide-, and peptide-containing products are used as conditioning agents in hair and skin products. Methods used to manufacture protein hydrolysates typically yield broad molecular weight (MW) distributions of peptides, 500-30,000 daltons (Da); however, certain enzymes, such as papain, can routinely yield narrower distributions of 500-10,000 Da.<sup>9-11</sup> For example, if the average MW of an amino acid is assumed to be 135 Da, then, under the broader distribution figures, these ingredients are approximately 4 to 220 amino acids in length (approximately 4 to 74 amino acids in length under the narrower distribution).<sup>12</sup>

## **Method of Manufacturing**

A supplier reported that HWP (MW = 350 Da) may be prepared by both alkaline and enzyme hydrolysis.<sup>13</sup> These processes occur for several hours until the desired MW is reached. The final product is a 25% water solution of HWP. Summary information that includes this data along with additional data from other suppliers can be found in Table 2.

HWP contained in a facial soap that is associated with anaphylaxis reactions in Japan was produced from gluten by partial hydrolysis with hydrogen chloride at 95°C for 40 min.<sup>14</sup> The MW of the main band of HWP, as determined with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), was 40,000-50,000 Da, which was larger than the main band in gluten.

Water insoluble ("vital") wheat gluten is prepared by washing wheat flour to remove the starch.<sup>15</sup> The gluten remaining is treated with acid to partially deamidate the proteins, which renders them dispersible ("soluble") in water. The resultant proteins have relatively high MWs, which can be hydrolyzed by acid, alkali or protease treatment to yield water soluble proteins, polypeptides, or amino acids, depending on the method and the extent of the hydrolysis. Polypeptides can then be derivatized by quaternization or copolymerization.

There is no standard method for measuring the MWs of the small polypeptides that can be produced by hydrolyzing gluten, for example.<sup>15</sup> The MWs typically are measured by Size-Exclusion High Pressure Liquid Chromatography / Gel Permeation Chromatography (SEHPLC/GPC), GPC / Multi-Angle Laser Light Scattering (MALLS), or SDS-PAGE, and are expressed as weight-average MWs.

## **Impurities**

A supplier of HWP (MW = 350 Da) reported levels of heavy metals and arsenic at  $\leq 5$  ppm and 0.5 ppm, respectively.<sup>13</sup>

## USE Cosmetic

The HWP and HWG addressed in this safety assessment function primarily as hair conditioning agents and skin conditioning agents (miscellaneous) in cosmetic formulations.<sup>16</sup> An additional function may include film formers (HWP).

Table 3 presents the current product-formulation data for HWP and HWG. According to information supplied to the Food and Drug Administration (FDA) by industry as part of the Voluntary Cosmetic Registration Program (VCRP), HWP has the most reported uses in cosmetic and personal care products, with a total of 1077; approximately half of those uses are in non-coloring hair products.<sup>17</sup> HWG has a total of 78 uses in cosmetic and personal care products with about half of the uses reported to be hair tints.

In the Personal Care Products Council's (Council) use concentration survey, HWP had a wide maximum use concentration range of  $2.0 \times 10^{-5}$  to 1.7%, with the 1.7% reported in rinse-off non-coloring hair products.<sup>18</sup> HWG had a maximum use concentration range of 0.005% to 0.09%, with 0.09% reported in eye makeup preparations.

HWP is used in cosmetic sprays, including aerosol and pump hair spray products and hair tonics. HWG and HWP may also be used in spray face and neck skin care products and skin fresheners – use in this fashion cannot be confirmed. When used in cosmetic sprays, these ingredients could possibly be inhaled. The maximum concentration of these ingredients reported to be used in a spray product is 0.5% (HWP) in a pump hair spray. In practice, 95% to 99% of the droplets/particles released from cosmetic sprays have aerodynamic equivalent diameters  $>10 \mu\text{m}$ , with propellant sprays yielding a greater fraction of droplets/particles  $<10 \mu\text{m}$  compared with pump sprays.<sup>19,20</sup> Therefore, most droplets/particles incidentally inhaled from cosmetic sprays would be deposited in the nasopharyngeal and bronchial regions and would not be respirable (i.e., able to enter the lungs) to any appreciable amount.<sup>21,22</sup>

HWP and HWG are not restricted from use in any way under the rules governing cosmetic products in the European Union.<sup>23</sup>

## Non-Cosmetic

The FDA determined that the use of peptones as direct food substances is generally recognized as safe (GRAS). These GRAS peptones are defined as “the variable mixture of polypeptides, oligopeptides, and amino acids that are produced by partial hydrolysis of casein, animal tissue, soy protein isolate, gelatin, defatted fatty tissue, egg albumin, or lactalbumin (whey protein) (21 CFR §184.1553).

The FDA defines the term “protein” to mean any  $\alpha$ -amino acid polymer with a specific defined sequence that is greater than 40 amino acids in size.<sup>24</sup> The FDA considers a “peptide” to be any polymer composed of 40 or fewer amino acids.

The FDA requires allergen labeling when major allergens are included in food. The major allergens include wheat, milk, egg, fish, Crustacean shellfish, tree nuts, peanuts, and soybeans.<sup>25</sup>

## TOXICOKINETICS

No published toxicokinetics studies on HWP and HWG were identified by a literature search for these ingredients and no unpublished data were submitted.

## TOXICOLOGICAL STUDIES

The proteins that serve as the sources of the HWPs and HWGs that are addressed in this safety assessment are found in the foods we consume daily. The potential systemic effects, other than sensitization, from possible absorption of these substances through the skin would not be expected to be different from those of oral exposures and are, thus, not discussed in detail in this report. This assessment focuses on evaluating the potential for these ingredients to cause sensitization reactions and irritation. Data from the previous safety assessment on  $\alpha$ -amino acids support that mixtures of amino acids would not likely be irritants or sensitizers.

## GENOTOXICITY

No published genotoxicity studies on HWP and HWG were identified by a literature search for these ingredients and no unpublished data were submitted.

## **CARCINOGENICITY**

No published carcinogenicity studies on HWP and HWG were identified by a literature search for these ingredients and no unpublished data were submitted.

## **IRRITATION AND SENSITIZATION**

*[From the CIR Safety Assessment of  $\alpha$ -amino acids]<sup>1</sup>: Cysteine HCl and methionine were used as negative controls in in vitro assays to predict potential skin irritants. In separate dermal and ocular studies, arginine (up to 5%), aspartic acid (up to 0.2%), cysteine (up to 5%), glycine (up to 2%), magnesium aspartate (up to 0.1%), serine (up to 0.3%) and tyrosine (up to 1%) did not produce any adverse effects in rats, guinea pigs, or mouse skin models. Glutamic acid was used as a negative control in an in vitro study to identify skin sensitizers. Products containing amino acid ingredients at concentrations up to 2.784% were not dermal irritants or sensitizers in HRIPT studies. In several validation studies for in vitro phototoxicity assays, histidine was used as a negative control. Neither magnesium aspartate up to 0.5% nor 1% tyrosine was phototoxic in assays using yeast.*

### **Irritation**

#### ***Dermal – Non-Human***

In a primary dermal irritation study in 6 New Zealand white rabbits, acid- and enzyme-hydrolyzed HWP was not a primary skin irritant (primary skin irritation score = 0.50; a score of 5+ indicates a primary dermal irritant).<sup>26</sup> The 25% aq. solution (MW = 350 Da) was applied for 24 h to 2.5 cm<sup>2</sup> sites that were clipped, abraded, and occluded.

#### ***Dermal - Human***

HWP was non-irritating in a human irritation patch test performed in 42 subjects.<sup>27</sup> The HWP was tested at 25% aq. solution (MW = 350 Da), and the subjects received a single dermal dose under occlusive conditions for 48 h.

#### ***Ocular – Non-Human***

In an ocular irritation study in 6 albino rabbits, HWP (25% aq. solution, MW = 350 Da) was not a primary eye irritant.<sup>28</sup>

### **Sensitization**

#### ***Dermal - Non-Human***

The possibility of a transdermal pathway for sensitization to gluten and acid-hydrolyzed HWP was studied using BALB/c mice.<sup>14</sup> The HWP was supplied by a manufacturer in Japan and was produced from gluten by partial hydrolysis with hydrogen chloride at 95° C for 40 min. The resultant HWP had a MW of approximately 40,000-50,000 Da. The 7-week-old female mice were shaved and tape-stripped 10 times to remove the stratum corneum, and were then exposed to HWP and gluten (500 µg/mouse), with and without sodium dodecyl sulfate (SDS), or to HWP (20-500 µg/mouse), with SDS, via transdermal patches for 3 to 4 cycles (each cycle consisting of 3 days with the patch on followed by 4 days without the patch), 3 days/week. Active systemic anaphylaxis (ASA) was then induced by intraperitoneal injection of HWP or gluten, respective of the material used during the transdermal exposure. Rectal temperature, scores of anaphylactic responses, and plasma histamine levels were measured. Dose-dependent production of IgE and IgG1 were observed. The i.p. injection of HWP caused ASA in the mice exposed transdermally to HWP, with decreased rectal temperatures, increased anaphylaxis scores, and increased plasma histamine levels. The i.p injection of gluten clearly induced ASA in the mice transdermally exposed to gluten in the presence of SDS, but not in the absence of SDS. When compared to the vehicle control group, the content of HWP-specific IgE and IgG1 was significantly increased in the HWP groups with and without SDS and in the gluten-with-SDS group; IgE in the gluten-without-SDS group was barely increased. The serum content of gluten-specific IgE was significantly increased in the gluten-with-SDS group and both HWP groups, but barely increased in the gluten-without-SDS group, when compared to the vehicle-control group. The serum content of gluten IgG1 with and without SDS and HWP without SDS were also significantly increased, but there were individual differences in the gluten-without-SDS group that showed that SDS had an important role in sensitization by transdermal exposure. Following elicitation of the immediate hypersensitivity reactions, harvested splenocytes were re-stimulated with HWP for 72 h. The secretion of IL-4, IL-5, and IL-10 was increased while that of IL-2 and interferon (IFN)- $\gamma$  was

significantly decreased, demonstrating that transdermal sensitization with HWP was associated with a T helper 2 response.

### ***Dermal - Human***

In an occlusive human repeated insult patch test (HRIPT) of 52 subjects, no dermal irritation or sensitization was observed in response to HWP (25% aq. solution, MW = 350 Da) when applied at a volume of 0.2 ml under a 20 mm<sup>2</sup> Webril patch.<sup>29</sup>

A study of sensitization to protein hydrolysates in hair-care products was performed in 3 groups of Finnish patients.<sup>30</sup> The first group, which consisted of 11 hairdressers with hand dermatitis, submitted to scratch and prick tests with 22 trademarked protein hydrolysates, including 2 HWP trademarked hydrolysates (specific chemical characteristics not provided). The second group was comprised of 2160 consecutive adults with suspected allergic respiratory disease: they were subjected to skin prick tests with hydroxypropyl trimonium hydrolyzed collagen, hydrolyzed collagen and/or hydrolyzed milk protein. The third group of 28 adult patients with atopic dermatitis was also tested with 1 to 3 of the hydrolysates tested in group 2 via a skin prick test. Positive reactions were seen in a total of 12 patients (all female with atopic dermatitis) to the hydroxypropyl trimonium hydrolyzed collagen, hydrolyzed collagen and/or hydrolyzed milk protein. No adverse reactions to the HWP trademarked hydrolysates were observed.<sup>30</sup>

### ***Type 1 Hypersensitivity***

There have been several reports of Type 1 (i.e., immediate) hypersensitivity reactions to personal care products that contain HWP or HWG, as summarized below. It has been hypothesized that an allergen must have at least 2 IgE-binding epitopes, and each epitope must be at least 15 amino-acid residues long, to trigger a Type 1 hypersensitivity reaction.<sup>31</sup> Type 1 responses can be elicited in sensitized patients when pairs of IgE molecules against a specific allergen are bound to receptors on the surface of mast cells and other cells that mediate immune reactions. The binding of an allergen molecule to two receptor-bound IgE molecules results in the crosslinking of the pair of IgE molecules. The cross-linking of sufficient numbers of IgE pairs bound to the receptors on the surface of a mast cell results in degranulation of the mast cell and the release of vasoactive amines, which are responsible for the Type 1 reaction.

The sera from 5 European patients were studied to determine the reactivity of IgE with hydrolyzed gluten.<sup>32</sup> In 4 of the patients, immediate contact hypersensitivity to HWP (IHHWP) manifested as urticaria in response to either dermal contact with HWP (2 patients) or the ingestion of processed foods containing HWP (2 patients), without sensitivity to traditional wheat food products. The fifth patient (control) exhibited conventional wheat-dependent exercise-induced anaphylaxis (CO-WDEIA) in response to ingesting traditional wheat food products without exhibiting sensitivity to HWP.

The IgE reactivity of sera from the IHHWP patients and the CO-WDEIA patient was characterized using extracts of 4 hydrolyzed gluten preparations (enzymatically- or acid-hydrolyzed), total unmodified wheat protein (UWP), and UWP fractions (i.e., albumins/globulins, gliadins, and glutenins, including high-MW glutenin subunits [HMW-GS] and low-MW glutenin subunits [LMW-GS]). The IgE cross-reactivity of the sera was examined from one IHHWP patient with the extracts of one HWP preparation and UWP. Finally, the relative molecular size distributions of two HWP preparations (one the product of acid hydrolysis with a low degree of deamidation and the other the product of enzymatic hydrolysis) was characterized, and the binding of IgE in the serum of one IHHWP patient was determined using the separated polypeptide fractions of two HWP preparations.

The results showed reactivity of serum IgE from the IHHWP patients, especially with the albumins/globulins fraction and less so with the gliadins and LMW-GS fractions, but not with the HMW-GS fraction of UWP. Reactivity of serum IgE from one of the IHHWP patients was observed with the  $\omega$ 5-gliadin of UWP; this patient distinctly exhibited exercise-induced allergic reactions (urticarial) to ingestion of HWP in processed foods. Reactivity of serum IgE from the CO-WDEIA patient was observed with  $\omega$ 5-gliadin and LMW-GS fractions, but not with the HMW-GS fraction of UWP.

Binding patterns of serum IgE from the IHHWP patients to HWP preparations varied by IHHWP patient and by HWP preparation, but in no case did the IgEs bind to HWP polypeptides less than 31,000 Da. The binding of serum IgE to UWP or to the albumins/globulins fraction of UWP was partially inhibited by HWP. However, the binding of serum IgE to HWP was almost completely inhibited by UWP or HWP. Based on these results, the authors suggested that almost all of the epitopes in the HWP preparation tested were also available in UWP. The molecular-size profiles of two of the HWP preparations ranged from <5,000 Da to > 1,000,000 Da, and both preparations contained substantial amounts of high-MW constituents. Binding of IgE in the serum of the IHHWP patient was greatest to the highest MW fractions of both of these HWP preparations (400,000 Da to 1,000,000 Da),

weaker to intermediate molecular-weight fractions (30,000 Da to 400,000 Da), and faint or undetectable to the lowest molecular-weight fractions (< 31,000 Da).

Overall, the authors concluded that most IgE epitopes in UWP are conserved in HWP produced by industrial hydrolysis processes, and the production of new epitopes in the hydrolysates does not appear to contribute substantially to the differences in allergic responses in IHHWP patients compared with CO-WDEIA patients. Additionally, epitopes in UWP appear to be destroyed in HWP polypeptides less than about 30,000 Da. Analysis of HWP fractions under non-reducing, non-dissociating conditions suggested that differences in allergic responses between IHHWP patients and CO-WDEIA patients may be attributable to hydrolysis-induced re-organization in HWP of epitopes that already exist in UWP; re-organization through entanglements, S-S bond interchanges, or non-covalent interactions among the HWP polypeptides may produce relatively soluble, high-MW polypeptide aggregates that can present multiple epitopes efficiently to trigger allergic responses to HWP.<sup>32</sup>

In a Japanese study, wheat protein hydrolysates that were produced by enzymatic hydrolysis had higher concentrations of peptides with MWs greater than 1,050 Da, compared with those produced by acid hydrolysis, which had extremely low concentrations of peptides with MWs greater than 1,050 Da.<sup>33</sup> Investigation of the reactivity of these 2 types of hydrolysates revealed that the acid hydrolysates rarely inhibited IgE binding whereas enzymatic hydrolysates clearly inhibited the binding of IgE to wheat proteins.<sup>33</sup> IgE of patients that had Type 1 hypersensitivity to HWP through percutaneous and/or rhinoconjunctival exposure to a facial soap containing HWP (40,000-50,000 Da) reacted with high-MW polypeptide aggregates.<sup>34</sup> However, an in vitro elicitation test using IgE from different categories of wheat-allergic patients (including patients sensitized to commercial HWP produced by acid hydrolysis, pediatric patients with food allergy to native wheat, adult patients exhibiting WDEIA, and non-atopic healthy adults) revealed that glens acid-hydrolyzed to various extents retained the ability to activate mast cells in patients sensitized by exposure to commercial acid-hydrolyzed HWP.<sup>35</sup>

A study was performed comparing 5 Japanese women exhibiting both contact allergy (rhinoconjunctival reactions) to HWP (40,000-50,000 Da) in a facial soap and WDEIA reactions to eating "normal wheat products" such as bread, pasta, and pastries (referred to as HWP-WDEIA patients) with 18 Japanese women exhibiting CO-WDEIA reactions.<sup>36</sup> The authors distinguished the 5 Japanese HWP-WDEIA patients from European patients exhibiting IHHWP (see study summarized above), some of whom also exhibited allergic reactions to foods containing HWP, but none with allergic reactions to eating "normal wheat products."

Positive skin prick tests were obtained for HWP in all 5 of the HWP-WDEIA patients, in contrast to the CO-WDEIA patients. Sera from HWP-WDEIA patients exhibited statistically-significantly elevated IgE reactivity with HWP, compared to reactivity with each of the wheat-protein fractions (i.e., albumins/globulins, gliadins, and glutenins). In contrast, sera from CO-WDEIA patients exhibited statistically-significantly elevated reactivity with the gliadins fraction of wheat proteins, compared to reactivity with HWP.

Sera from the HWP-WDEIA patients exhibited statistically-significantly elevated IgE reactivity with HWP, gluten, wheat flour, and each of the wheat-protein fractions, and statistically-significantly reduced reactivity with recombinant  $\omega$ 5-gliadin, compared to sera from CO-WDEIA patients. Based on these results, the authors suggested that sensitization of HWP-WDEIA patients to components of the gliadins fraction other than  $\omega$ 5-gliadin may help explain the elevated reactivity of sera from HWP-WDEIA patients with the complete gliadins fraction.

Pre-incubation of sera from HWP-WDEIA patients with HWP completely inhibited IgE reactivity with wheat extracts, but pre-incubation with wheat extracts did not inhibit reactivity with HWP. Conversely, pre-incubation of sera from CO-WDEIA patients with HWP only weakly inhibited reactivity with wheat extracts, while pre-incubation with wheat extracts strongly inhibited reactivity with HWP. Based on these results, the authors suggested that the reactivity of sera from CO-WDEIA patients with HWP is attributable to IgE-binding epitopes that survive the hydrolysis of wheat proteins.

Overall, the authors concluded: (1) HWP-WDEIA is a clinical phenotype distinct from CO-WDEIA, as well as from the contact sensitivity to HWP observed in European patients that do not exhibit sensitivity to ingesting "normal wheat products," (2) the use of a facial soap containing HWP caused both primary contact dermal / rhinoconjunctival sensitization to HWP and, secondarily, WDEIA sensitization to ingested wheat proteins in the HWP-WDEIA patients, and (3) sensitization to gliadins other than  $\omega$ 5-gliadin (e.g.,  $\omega$ 1-2-gliadin and  $\gamma$ -gliadin) may be more important than sensitization to  $\omega$ 5-gliadin in the pathogenesis of HWP-WDEIA, compared with the pathogenesis of CO-WDEIA.<sup>36</sup>

In another study, the allergic reactions of a group of Japanese patients diagnosed with HWP-WDEIA were found likely the result of sensitization primarily through percutaneous and/or rhinoconjunctival exposures to HWP (acid-hydrolyzed UWP; 40,000-50,000 Da) in a facial soap.<sup>8</sup> The authors noted that, by 2010, more than 1300 patients who had used the soap exhibited facial angioedema after use, tested positive for sensitivity to the HWP in skin-prick tests and positive for serum IgE reactivity with the HWP, and developed WDEIA reactions in response to

eating natural UWP. Angioedema predominated in the HWP-WDEIA patients, especially angioedema of the eyelids, in contrast to the urticarial wheals predominating in CO-WDEIA patients. The onset of allergic reactions in the HWP-WDEIA patients typically was 1 month to 5 years after starting to use the soap. Many of these patients developed WDEIA in response to eating wheat food products at about the same time as, or subsequent to, the onset of urticarial reactions to the soap.

About half of the HWP-WDEIA patients tested positive in skin-prick tests for sensitivity to wheat and bread. Almost all of the HWP-WDEIA patients tested positive in skin-prick tests for sensitivity to solutions of the soap or the HWP in the soap, in contrast to CO-WDEIA patients, none of whom exhibited sensitivity to these solutions. Only about 7% of HWP-WDEIA patients exhibited serum IgE reactivity with  $\omega$ 5-gliadin, compared to 80% of CO-WDEIA patients. Reactivity with  $\omega$ 5-gliadin among the few positive HWP-WDEIA patients was substantially weaker than the corresponding reactivity among the CO-WDEIA patients. About 17% of HWP-WDEIA patients exhibited serum IgE reactivity with  $\omega$ 5-gliadin and/or HMW-GS, compared to about 94% of CO-WDEIA patients. On the other hand, 70% or more HWP-WDEIA patients exhibited serum IgE reactivity with wheat protein or gluten, compared to only 30% to 40% of CO-WDEIA patients. Sera from HWP-WDEIA patients exhibited IgE binding to HWP polypeptides and to water-soluble and water-insoluble constituents of UWP, but not to purified  $\omega$ 5-gliadin. In comparison, serum IgE from CO-WDEIA patients bound to  $\omega$ 5-gliadin, as well as to the water-soluble and water-insoluble constituents of UWP, but not to the polypeptides of the HWP preparation. Pre-incubation of sera from the HWP-WDEIA patients with solutions of the HWP preparation resulted in concentration-dependent inhibition of the binding of IgE to HWP polypeptides. HWP, but not purified  $\omega$ 5-gliadin, up-regulated the CD203c (an ecto-enzyme on the cell membranes of basophils and mast cells) in HWP-WDEIA patients. However,  $\omega$ 5-gliadin, but not the HWP, up-regulated CD203c in cells from CO-WDEIA patients.

The authors suggested that (1) the hydrophilic constituents of HWP may play an important role in percutaneous and/or rhinoconjunctival sensitization to HWP, (2) production of HWP by acid hydrolysis of UWP will yield charged terminal amino- and carboxyl-groups that increase the water solubility of the HWP, compared to that of UWP, and (3) the surfactants in a soap product will likely facilitate the dermal penetration of the HWP polypeptides, and thereby help to increase the likelihood of sensitization through percutaneous/rhino-conjunctival exposures in people using such products.<sup>8</sup>

Recommendations have been made to individuals with known protein hypersensitivity to minimize dermal exposure to botanical ingredients such as HWP and to not use products that have these constituents that can be incidentally inhaled.<sup>37</sup> Additionally, it has been recommended that manufacturers of personal care products not use known or suspected allergens (including constituents of plants known to produce Type 1 hypersensitivity reactions or of plants that are in the same phylogenetic families as these plants) in products that may be incidentally inhaled (e.g., sprays, shampoos or shower gels, and, presumably, loose powder products as well).

Research on Type 1 hypersensitivity reactions in Japan to products containing HWP is ongoing, as reported by the Japanese Society of Allergology's Special Committee for the Safety of Protein Hydrolysates in Cosmetics. Current developments are available at: [http://www.jsaweb.jp/modules/en/index.php?content\\_id=11](http://www.jsaweb.jp/modules/en/index.php?content_id=11).

The outbreak in Japan of Type 1 immediate hypersensitivity reactions to a HWG in facial soaps and other products was attributed mainly to the use of a popular soap product (*Cha no shizuku*) containing 0.3% of a HWG called Glupearl 19S (trade name). Glupearl 19S has an average MW of about 50,000 Da.<sup>38</sup> There are presently more than 2100 registered cases of this type of sensitivity across Japan. Data from 547 patients indicated that the signs of sensitization typically appeared 31.5 months (median) after starting to use the soap. The clinical manifestations of sensitization to Glupearl 19S include eyelid edema and contact urticaria during or after using the soap in many, but not all, of the patients. Eating foods containing wheat ingredients caused anaphylactic reactions in about 55% of the patients, including anaphylactic shock in about 25%. Clinical and experimental evidence indicates that the patients have systemic reactions to ingested wheat products because they have been sensitized through percutaneous or permucosal (i.e., through the ocular or nasal mucosae) absorption of Glupearl 19S.<sup>38,39</sup>

Wheat gluten hydrolysates prepared by acid hydrolysis at high temperatures (95°C or 100 °C) for 0 to 48 hours have weight-average MWs ranging from < 3000 Da to > 10,000 Da, depending on the duration of the hydrolysis.<sup>38</sup> Regardless of the duration, all of the hydrolysates are about 50% deamidated by the treatment. Glupearl 19S and hydrolyzed gluten preparations prepared by acid-hydrolysis at 100 °C for 0.5 h exhibited a sensitization potential through dermal exposure in an in vivo mouse model, but gluten that was more extensively hydrolyzed under these conditions for 9 hours exhibited weak sensitization potential in this model.<sup>38,39</sup> The MWs of the hydrolysate preparations, determined by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE), were  $\leq$  70,000 Da after 0.5-h acid hydrolysis and  $\leq$  30,000 Da after 9-h hydrolysis. Glupearl 19S and other gluten hydrolysates that were acid-hydrolyzed (at 100 °C) for 0, 0.5, 1, 2, 3, 6, 9, 12, 24, or 48 hours were fractionated by size (i.e., three fractions: < 3000 Da, 3000-10,000 Da, and > 10,000 Da fractions) using

ultrafiltration spin columns without sodium dodecyl sulfate (SDS) to avoid the effects of SDS binding to the polypeptides, which can reduce the accuracy of MW estimates.<sup>38,39</sup> The fractions were then tested for IgE responses (using serum IgE from an HWP-sensitized patient) by the IgE crosslinking-induced luciferase expression (EXiLE) method. The results showed that the  $\leq 3000$  Da fractions of Glupearl 19S and the other hydrolysate preparations did not elicit an IgE response, and the 3000-10,000 Da fractions elicited only a weak response, in contrast to both Glupearl 19S and the  $> 10,000$  Da fraction. The authors concluded that the elicitation of a type 1 hypersensitivity response depends on the presence of an epitope with a MW  $\geq 3000$  Da.<sup>39</sup>

Wheat gluten rendered dispersible by mild acid hydrolysis was further hydrolyzed enzymatically to different extents to yield HWP preparations, including:  $\sim 150$  Da,  $\sim 3000$  Da,  $\sim 100,000$  to  $125,000$  Da preparations.<sup>15</sup> Some of the  $\sim 150$  Da,  $\sim 3000$  Da, and  $\sim 100,000$  to  $125,000$  Da were derivatized to yield quaternized peptides, copolymers, or acylated derivatives. The polypeptides of the  $\sim 3000$  Da MW preparation did not bind to human anti-gluten (specifically, anti-gliadin) antibodies in vitro in slot blot and western blot analyses, indicating the absence of reactivity of this preparation, in contrast to the gluten and dispersible-gluten preparations that were used as positive controls. All of the 6 wheat IgE-positive patients with conventional wheat allergy tested negative in skin-prick tests with the  $\sim 150$  Da,  $\sim 3000$  Da,  $\sim 100,000$  Da, and  $\sim 125,000$  Da hydrolysate preparations and a number of derivatives, most of which were derivatives of the  $\sim 3000$  Da preparation.

Several Danish individuals developed allergic reactions to a dispersible (i.e. rendered "soluble" by mild acid hydrolysis) wheat protein that was used in food products as an emulsifier.<sup>15</sup> This protein was hydrolyzed enzymatically to produce  $\sim 150$  Da,  $\sim 3000$  Da, and  $\sim 100,000$  to  $125,000$  Da preparations, each of which was tested by Immunospot<sup>®</sup> or IgE binding using sera from these patients. The  $\sim 150$  Da and  $\sim 3000$  Da preparations and their derivatives yielded negative results, in contrast to the  $\sim 100,000$  to  $125,000$  Da preparation. The authors concluded that the allergic responses in these patients are associated with the partial deamidation of the peptides by acid hydrolysis, and the hydrolysis to  $\sim 3000$  Da removes the potential for eliciting an allergic response.

Thus, the results of several studies indicate that hydrolysates of gluten with weight-average MWs  $< 3000$  Da exhibit no potential to elicit hypersensitivity reactions in sensitized individuals, in contrast to Glupearl 19S and other hydrolysates with weight-average MWs  $> 10,000$  Da.<sup>15,38</sup> Duplicate analysis (GPC MALLS) of two samples of the  $\sim 3000$  Da hydrolysates that were negative in the in vitro and in vivo studies described above indicated that  $\sim 3\%$  of the molar mass of the preparation exceeded  $3500$  Da, and  $\sim 2\%$  exceeded  $4000$  Da.<sup>40</sup> The authors note that the analyses of the low MW preparations are at the extreme of the sensitivity of the method used.

The experimental results support the hypothesis that a polypeptide must be at least 30 amino acids long to have the two IgE-binding epitopes required to elicit Type 1 hypersensitivity reactions.<sup>38</sup> The weight-average MW of the amino acids of wheat protein and wheat gluten is about  $119$  Da.<sup>15</sup> Thus, polypeptides from wheat protein or wheat gluten that are 30 amino acids long will have a weight-average MW of about  $3570$  Da.<sup>15</sup> It follows that polypeptides with weight-average MWs of  $3500$  Da or less do not have the properties required to induce Type 1 hypersensitivity.<sup>15,38</sup>

### **Phototoxicity**

No published phototoxicity studies on HWP and HWG were identified by a literature search for these ingredients and no unpublished data were submitted.

### **CASE STUDIES**

A case of WDEIA in a non-atopic 40-year-old woman was reported in Japan.<sup>8</sup> The patient developed facial wheals and nasal discharge while using an HWP- (Glupearl 19S) containing facial soap (*Cha no shizuku*) over the course of a year (HWP =  $40,000$  - $50,000$  Da). Additionally, she suffered multiple episodes of eyelid edema after eating bread or while working or walking during an 11-month period prior to diagnosis. Skin prick tests were positive with a solution of the soap or the HWP, but negative with wheat or bread. The patient also tested positive for WDEIA after ingesting wheat and aspirin together (aspirin, like exercise, is a well-known trigger of allergic reactions). SDS-PAGE and western blotting analyses showed that serum IgE from this patient reacted with polypeptides, which ranged from  $15,000$  to  $250,000$  Da, in a HWP preparation and with both the water-soluble and water-insoluble fractions of UWP, but not with  $\omega 5$ -gliadin.

An additional 3 cases of WDEIA were reported by the same researchers in Japan.<sup>41</sup> The 3 female patients had used the same brand of soap that contained HWP ( $40,000$ - $50,000$  Da). Skin prick tests revealed positive reactions to a 0.1% solution of the soap in physiological saline and to 0.1% HWP in physiological saline. Western blotting of the patients' sera IgE yielded positive reactions with the HWP. The researchers concluded that WDEIA was attributable to cross reactivity to wheat protein induced by HWP exposures in these patients.

A 51-year-old Japanese woman had been using a facial soap containing HWP (40,000-50,000 Da) daily for several years.<sup>42</sup> Approximately 3 months after she started to use the soap, she began to develop angioedema on the eyelids and urticarial rash on the face. She experienced similar episodes many times over a 5-year period when eating wheat-containing food followed by mild exercise, with clinical signs limited to her face. Five years after her initial use of the soap containing HWP, she had an anaphylactic reaction after ingesting normal wheat products and was suspected of having WDEIA. She had no history of atopic dermatitis, food hypersensitivities, or dry skin. The patient developed eyelid angioedema, dyspnea, and a generalized urticarial rash on her entire upper extremity following a skin prick test with the HWP from the soap diluted 1:10,000. An IgE test for wheat and gluten yielded 0.36 UA/ml and 0.40 UA/ml, respectively. Serum  $\omega$ -5 gliadin-specific IgE antibody titers were within normal limits. The patient did not have a mutation in human filaggrin (FLG), a defect that may disrupt skin barrier function.

In another case study, a 42-year-old woman reported an intense burning sensation over her face, neck, and scalp several hours after applying a moisturizing cream that contained HWP.<sup>43</sup> Specific chemical characteristics of the HWP were not provided. Patch testing with the diluted ingredients of the moisturizing cream resulted in a positive reaction (D2+, D4+) to 50% aq. HWP. No reactions were observed from skin prick testing to standardized wheat extract or contact-urticaria testing with HWP.

Contact urticaria was reported in a 46-year-old woman.<sup>44</sup> The patient developed the clinical signs after applying an eyelid cream and a body moisturizer that contained HWPs for 3 months prior to consulting her physician. Strong positive reactions were observed from the preserved food, wheat gluten that was in the food, the cosmetic creams, and HWP in open application tests and skin prick tests. Further investigation revealed that the HWPs in the cosmetic creams were from the same manufacturer as the gluten in the preserved food. Specific chemical characteristics of the HWP were not provided.

A 27-year-old woman was reported to have a pruritic, erythematous, urticarial rash that became increasingly more intense after subsequent use of a moisturizing body cream that contained HWP.<sup>45</sup> The wheat hydrolysate was not characterized in this study. Skin prick tests with common inhalant allergens, natural rubber latex, and cereal grains, including wheat, were negative. Also negative were the results of prick tests with a series of 21 protein allergens from plant and animal sources that included hen's egg, cow's milk, milk casein, almond, silk protein, aloe gel, papaya fruit, and hydrolyzed collagen. Total serum IgE was slightly elevated. The individual components of the body cream tested negative in an open application test, but a skin prick test was positive (8 mm) to HWP. Further IgE testing revealed that binding occurred specifically to wheat hydrolysate.

In another case study, a 64-year-old woman was reported to have itchy, erythematous, edematous lesions on the eyelids, face, and neck following use of a moisturizing cosmetic cream.<sup>46</sup> The patient was patch tested with the (GEIDC) standard and cosmetics series, the cosmetic cream, and the individual ingredients of the cream. Positive reactions (++) were observed to nickel sulfate, the cosmetic cream (tested neat), and to the HWP ingredient of the cream (10% aq.). Open testing with the HWP (10% aq.) was negative at 30 min. Specific chemical characteristics of the HWP were not provided.

A 23-year-old man with no history of atopy was reported to have a rash that occurred immediately after application of a face cream.<sup>47</sup> The rash included highly pruritic wheals on the face and neck accompanied by bilateral palpebral edema. Other systemic symptoms were not observed. The patient reported a similar reaction previously to a sunscreen and did not report food-induced symptoms or intolerance. A nonblinded skin test with the face cream was negative. Patch testing with the cosmetics True Test panel and the patient's own personal care products resulted in a positive (++) reaction to the patient's face cream at 48 and 96 h; all other readings were negative. Patch testing with the components of the face cream resulted in a positive (++) reaction to 1% HWP in water at 48 and 96 h. Testing in 10 control subjects yielded negative results. The patient underwent further prick tests with flours and cereals, with positive results reported for malt (5 x 4 mm), cereal mix (7 x 5 mm), oats (5 x 5 mm), and hydrolyzed wheat extract (18 x 14 mm). Total IgE was 136 U/ml (reference range = 1-100 U/ml). Results of specific IgE testing to buckwheat, rice, oats, barley, rye, corn, common millet, soy, and wheat were negative. Specific chemical characteristics of the HWP were not provided.

In a case study of a 3-year-old girl with a history of moderate atopic dermatitis, eczema-like skin eruptions were observed following use of an emollient containing HWP.<sup>48</sup> Scaly erythematous lesions were observed on her knees. No evidence of contact urticaria was observed. Closed patch tests with the European standard series and the emollient were positive (+) for the emollient on days 2 and 3. Additional patch tests with the individual components of the emollient yielded positive results (++) for palmitoyl-HWP on days 2 and 3. Prick test, open test, and open patch test for palmitoyl-HWP were negative, as were prick test and radioallergosorbent test with wheat. Specific chemical characteristics of the HWP were not provided.

Two cases of reactions to HWP were reported in hairdressers.<sup>49</sup> In the first case, the patient, a 23-year-old female with no history of atopy who had been employed as a hairdresser for 2 years, developed watery rhinitis,

conjunctivitis, dyspnea, angioedema of the eyelids, asthma-like symptoms at work, contact urticaria, and burning and tingling of the hands and soles when exercising after consumption of wheat-containing foods following long-term use of sprayable hair conditioner and another hairspray that contained laurimonium hydroxypropyl HWP. In the second case, the patient, a 22-year-old female with a history of atopic eczema who had been employed as a hairdresser for 6 months, developed urticarial wheals, work-related sneezing, nasal itching, watery rhinitis, and generalized urticarial and eyelid edema when exercising after consumption of wheat-containing foods following use of spray products also containing laurimonium hydroxypropyl HWP. The exercise-induced symptoms ceased after the second hairdresser switched to a grain-free diet. Skin prick tests with the common aeroallergen series and natural rubber latex were performed with standardized extracts, histamine hydrochloride, and diluent controls. Prick testing was also conducted with wheat, oat, barley and rye flours, gliadin, hair-bleaching agents, paraphenylenediamine, and the products containing HWP and the individual ingredients. Open skin applications tests were performed with the products containing HWP, and specific inhalation challenge or nasal provocation tests were performed with one of the products or the HWP ingredient.

Both patients had strong positive skin prick tests and urticarial reactions in the open skin tests to the products containing HWP. Of the ingredients in these products, laurimonium hydroxypropyl HWP gave a strong positive reaction in the skin prick test while the remaining ingredients caused no reactions. Three atopic and 4 healthy volunteers were negative to the same HWP. Additionally, the patients were skin-prick-test negative to wheat flour, persulfate salts, and paraphenylenediamine. Occupational asthma was diagnosed in the first patient based on a specific inhalation challenge test with one of the products. This patient also had a rhinitis reaction with itching and marked watery rhinorrhea. In the second patient, nasal provocation with HWP caused marked rhinorrhea with swelling of nasal mucosa. Nasal provocation with HWP in 2 volunteers was negative.<sup>49</sup>

### SUMMARY

HWG and HWP function primarily as skin and hair conditioning agents in personal care products. These protein derivatives are prepared by subjecting wheat proteins to acidic, enzymatic or other chemical, partial hydrolyses.

HWP has the most reported uses in cosmetic and personal care products, with a total of 1077; approximately half of those uses are in non-coloring hair products. HWG has 78 reported uses, with about half of the uses reported to be in hair tints.

In the Council's use concentration survey, HWP had a wide maximum use concentration range of  $2.0 \times 10^{-5}$  to 1.7%, with the 1.7% reported in rinse-off non-coloring hair products. HWG had a maximum use concentration range of 0.005% to 0.09%, with the 0.09% reported in eye makeup preparations.

The FDA determined the use of peptones as direct food substances are GRAS.

Ocular and dermal irritation studies of HWP found this ingredient not to be a significant irritant.

In a study of the transdermal pathway for sensitization to gluten and acid-hydrolyzed HWP (40,000 - 50,000 Da) with and without SDS in BALB/c mice, the i.p. injection of HWP caused ASA, with decreased rectal temperatures, increased anaphylaxis scores, and increased plasma histamine levels. The i.p injection of gluten clearly induced ASA in the presence of SDS, but not in the absence of SDS. The content of HWP-specific IgE and IgG1 was significantly increased in the HWP groups with and without SDS and in the gluten-with-SDS group; IgE in the gluten-without-SDS group was barely increased. The serum content of gluten-specific IgE was significantly increased in the gluten-with-SDS group and both HWP groups, but barely increased in the gluten-without-SDS group. The serum content of gluten IgG1 with and without SDS and HWP without SDS were also significantly increased, but there were individual differences in the gluten-without-SDS group that showed that SDS had an important role in sensitization by transdermal exposure. The secretion of IL-4, IL-5, and IL-10 was increased while that of IL-2 and interferon (IFN)- $\gamma$  was significantly decreased, demonstrating that transdermal sensitization with HWP was associated with a T helper 2 response.

A HRIPT study of HWP (MW = 350 Da) concluded that this ingredient was not a dermal irritant during the induction phase or sensitizer during the challenge phase of the study.

Multiple cases of allergic reactions, including Type 1 immediate hypersensitivity reactions, were reported in individuals who had used personal care products that contained HWP, most of which were to a facial soap in Japan that contained HWP of 40,000-50,000 Da from acid hydrolysis of gluten at high temperatures. Several studies have been conducted to characterize the cause, manifestations, and mechanisms of these reactions, including tests of serum IgE binding and reactivity to wheat protein, wheat-protein fractions, and HWP and HWG prepared using acid- and/or enzyme-hydrolysis methods yielding products with varied polypeptide size profiles. Hydrolysates with weight-average MWs < 3000 exhibit no potential to elicit hypersensitivity reactions in sensitized individuals, in contrast to hydrolysates with weight-average MWs >30,000 Da. Experimental results support the hypothesis that

polypeptides with weight-average MWs of 3500 Da or less do not have the potency required to induce Type 1 hypersensitivity.

### **DISCUSSION**

The HWP and HWG ingredients discussed in this safety assessment are protein hydrolysates consisting of polypeptides with average MWs ranging from approximately 500 Da to greater than 30,000 Da, depending on the extent of the hydrolysis. The Panel reviewed data from a raw materials manufacturer and information presented by experts on the potential for exposures to HWP and HWG in cosmetic products to cause Type 1 immediate hypersensitivity reactions. Traditional human repeat insult patch tests (HRIPT) and related tests do not assess the ability of a substance to cause Type 1 reactions.

Production processes involving high-heat acid hydrolysis of wheat protein or wheat gluten can yield partially deamidated high-MW polypeptides with substantial potential to sensitize individuals through percutaneous and permucosal exposures, especially in formulations that contain surfactants. Studies have shown that hydrolysates with weight-average MW of approximately 3000 Da or less exhibit no potential to elicit hypersensitivity reactions in sensitized individuals, in contrast to hydrolysates with weight-average MWs >10,000 Da. Substantial experimental results support the theory that a polypeptide must be at least 30 amino acids long (i.e., MW about 3570 Da, assuming 119 Da/amino acid) to have the two IgE-binding epitopes needed to elicit Type 1 hypersensitivity reactions. Thus, polypeptides with MWs less than 3500 Da do not have the properties required to induce Type 1 hypersensitivity.

The Panel discussed the issue of incidental inhalation exposure to HWP or HWG in aerosol and pump hair spray products. There were no inhalation toxicity data identified or provided. HWP and HWG reportedly are used at concentrations up to 0.5% (HWP) in cosmetic products that may be aerosolized. The Panel noted that 95% – 99% of droplets/particles produced in cosmetic aerosols would not be respirable to any appreciable amount. Coupled with the small actual exposures expected in the breathing zone, the absence of the potential for polypeptides less than 3500 Da from HWP or HWG to induce sensitization, and the generally non-irritating nature of these ingredients, the available information indicates that incidental inhalation would not be a significant route of exposure that might lead to local respiratory or systemic effects. A detailed discussion and summary of the Panel's approach to evaluating incidental inhalation exposures to ingredients in cosmetic products is available at <http://www.cir-safety.org/cir-findings>.

The Panel also addressed concerns about pesticide residues and heavy metals that may be present in botanical ingredients. They emphasized that the cosmetics industry should continue to use the necessary procedures to limit these impurities in the ingredients before blending into cosmetic formulations.

### **CONCLUSION**

The CIR Expert Panel concluded that hydrolyzed wheat gluten and hydrolyzed wheat protein are safe for use in cosmetics when formulated to restrict peptides to a weight-average MW of 3500 Da or less.

**TABLES****Table 1.** Definitions and functions of the ingredients in this safety assessment.<sup>16</sup> (The italicized text below represents additions made by CIR staff.)

<b>Ingredient CAS No.</b>	<b>Definition</b>	<b>Function</b>
Hydrolyzed Wheat Gluten 100684-25-1	Hydrolyzed Wheat Gluten is the <i>partial</i> hydrolysate of Triticum Vulgare (Wheat) Gluten derived by acid, enzyme or other method of hydrolysis.	Hair Conditioning Agent; Skin-Conditioning Agent-Misc.
Hydrolyzed Wheat Protein 70084-87-6 100209-50-5 222400-28-4	Hydrolyzed Wheat Protein is the <i>partial</i> hydrolysate of wheat protein derived by acid, enzyme or other method of hydrolysis.	Film formers; Hair Conditioning Agent; Skin-Conditioning Agent - Misc.

**Table 2.** Summary of information from suppliers of hydrolyzed wheat protein.\*<sup>50</sup>

<b>Source</b>	<b>Method of Manufacture</b>	<b>Molecular Weight</b>	<b>Nitrogen Content</b>	<b>Gluten Content</b>
1 product defatted wheat germ	3 products enzyme hydrolysis	1 product average MW = 350 Da	1 product 12-15% nitrogen	1 product "gluten-free"
	1 product alkaline and enzyme hydrolysis	1 product average MW = 2200 Da		1 product < 100 ppm gluten
				1 product about 50 ppm gluten

\* Information includes data summarized in Anonymous, 2012.<sup>13</sup>

**Table 3.** Frequency and concentration of use for hydrolyzed wheat gluten and hydrolyzed wheat protein according to duration and type of exposure.<sup>17,18</sup>

	Hydrolyzed Wheat Gluten		Hydrolyzed Wheat Protein	
	# of Uses	Conc. of Use (%)	# of Uses	Conc. of Use (%)
<b>Totals<sup>1</sup></b>	<b>75</b>	<b>0.005-0.09</b>	<b>1069</b>	<b>0.00002-1.7</b>
<b><i>Duration of Use</i></b>				
Leave-On	11	0.005-0.09	519	0.00006-1
Rinse-Off	61	0.005-0.01	542	0.00002-1.7
Diluted for (Bath) Use	3	NR	8	0.00002
<b><i>Exposure Type</i></b>				
Eye Area	1	0.09	60	0.01-0.9
Incidental Ingestion	NR	NR	20	0.008-0.03
Incidental Inhalation-Spray? <sup>2,6</sup>	6	0.005	287	0.00006-0.4
Reported Spray <sup>3</sup>	NR	NR	NR	0.0003-0.5 <sup>a</sup>
Incidental Inhalation-Powder? <sup>4,6</sup>	7	NR	150	0.00006
Reported Powder <sup>5</sup>	NR	NR	NR	0.05
Dermal Contact	21	0.01-0.09	384	0.00002-1
Deodorant (underarm)-Spray? <sup>2</sup>	NR	NR	NR	NR
Reported Spray <sup>3</sup>	NR	NR	NR	NR
Reported as Not Spray <sup>3</sup>	NR	NR	NR	NR
Hair - Non-Coloring	16	0.005	522	0.003-1.7
Hair-Coloring	38	NR	91	0.002-0.3
Nail	NR	NR	30	0.002-0.04
Mucous Membrane	12	NR	122	0.00002-0.1
Baby Products	3	NR	2	NR

NR = Not reported

1. Because each ingredient may be used in cosmetics with multiple exposure types, the sum of all exposure types may not equal the sum of total uses.

2. It is possible these products may be sprays, but it is not specified whether the reported uses are sprays.

3. Use in a spray product has been reported in response to a survey conducted by the Council.

4. It is possible these products may be powders, but it is not specified whether the reported uses are powders.

5. Use in a powder product has been reported in response to a survey conducted by the Council.

6. Not specified whether a powder or a spray, so this information is captured for both categories of incidental inhalation.

a. 0.03-0.05% in aerosol hair sprays; 0.0003-0.5% in pump hair sprays; and 0.002-0.02% in spray tonics, dressings, and other hair grooming aids.

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**2014 FDA VCRP Raw Data**

01A - Baby Shampoos	HYDROLYZED WHEAT GLUTEN	2
01B - Baby Lotions, Oils, Powders, and Creams	HYDROLYZED WHEAT GLUTEN	1
02B - Bubble Baths	HYDROLYZED WHEAT GLUTEN	2
02D - Other Bath Preparations	HYDROLYZED WHEAT GLUTEN	1
03G - Other Eye Makeup Preparations	HYDROLYZED WHEAT GLUTEN	1
05A - Hair Conditioner	HYDROLYZED WHEAT GLUTEN	3
05F - Shampoos (non-coloring)	HYDROLYZED WHEAT GLUTEN	8
05I - Other Hair Preparations	HYDROLYZED WHEAT GLUTEN	3
06B - Hair Tints	HYDROLYZED WHEAT GLUTEN	38
10A - Bath Soaps and Detergents	HYDROLYZED WHEAT GLUTEN	8
10E - Other Personal Cleanliness Products	HYDROLYZED WHEAT GLUTEN	1
12A - Cleansing	HYDROLYZED WHEAT GLUTEN	1
12C - Face and Neck (exc shave)	HYDROLYZED WHEAT GLUTEN	6
01C - Other Baby Products	HYDROLYZED WHEAT PROTEIN	2
02B - Bubble Baths	HYDROLYZED WHEAT PROTEIN	7
02D - Other Bath Preparations	HYDROLYZED WHEAT PROTEIN	1
03A - Eyebrow Pencil	HYDROLYZED WHEAT PROTEIN	1
03B - Eyeliner	HYDROLYZED WHEAT PROTEIN	1
03C - Eye Shadow	HYDROLYZED WHEAT PROTEIN	1
03D - Eye Lotion	HYDROLYZED WHEAT PROTEIN	9
03E - Eye Makeup Remover	HYDROLYZED WHEAT PROTEIN	3
03F - Mascara	HYDROLYZED WHEAT PROTEIN	22
03G - Other Eye Makeup Preparations	HYDROLYZED WHEAT PROTEIN	23
04C - Powders (dusting and talcum, excluding aftershave talc)	HYDROLYZED WHEAT PROTEIN	1
05A - Hair Conditioner	HYDROLYZED WHEAT PROTEIN	159
05B - Hair Spray (aerosol fixatives)	HYDROLYZED WHEAT PROTEIN	18
05C - Hair Straighteners	HYDROLYZED WHEAT PROTEIN	4
05D - Permanent Waves	HYDROLYZED WHEAT PROTEIN	6
05E - Rinses (non-coloring)	HYDROLYZED WHEAT PROTEIN	1
05F - Shampoos (non-coloring)	HYDROLYZED WHEAT PROTEIN	137
05G - Tonics, Dressings, and Other Hair Grooming Aids	HYDROLYZED WHEAT PROTEIN	123
05H - Wave Sets	HYDROLYZED WHEAT PROTEIN	9
05I - Other Hair Preparations	HYDROLYZED WHEAT PROTEIN	65
06A - Hair Dyes and Colors (all types requiring caution statements and patch tests)	HYDROLYZED WHEAT PROTEIN	74
06B - Hair Tints	HYDROLYZED WHEAT PROTEIN	1

06C - Hair Rinses (coloring)	HYDROLYZED WHEAT PROTEIN	10
06D - Hair Shampoos (coloring)	HYDROLYZED WHEAT PROTEIN	5
06G - Hair Bleaches	HYDROLYZED WHEAT PROTEIN	1
07A - Blushers (all types)	HYDROLYZED WHEAT PROTEIN	1
07B - Face Powders	HYDROLYZED WHEAT PROTEIN	5
07C - Foundations	HYDROLYZED WHEAT PROTEIN	12
07E - Lipstick	HYDROLYZED WHEAT PROTEIN	20
07F - Makeup Bases	HYDROLYZED WHEAT PROTEIN	2
07I - Other Makeup Preparations	HYDROLYZED WHEAT PROTEIN	12
08A - Basecoats and Undercoats	HYDROLYZED WHEAT PROTEIN	6
08B - Cuticle Softeners	HYDROLYZED WHEAT PROTEIN	2
08C - Nail Creams and Lotions	HYDROLYZED WHEAT PROTEIN	1
08E - Nail Polish and Enamel	HYDROLYZED WHEAT PROTEIN	8
08F - Nail Polish and Enamel Removers	HYDROLYZED WHEAT PROTEIN	3
08G - Other Manicuring Preparations	HYDROLYZED WHEAT PROTEIN	10
10A - Bath Soaps and Detergents	HYDROLYZED WHEAT PROTEIN	72
10E - Other Personal Cleanliness Products	HYDROLYZED WHEAT PROTEIN	22
11A - Aftershave Lotion	HYDROLYZED WHEAT PROTEIN	4
11G - Other Shaving Preparation Products	HYDROLYZED WHEAT PROTEIN	1
12A - Cleansing	HYDROLYZED WHEAT PROTEIN	22
12C - Face and Neck (exc shave)	HYDROLYZED WHEAT PROTEIN	66
12D - Body and Hand (exc shave)	HYDROLYZED WHEAT PROTEIN	28
12F - Moisturizing	HYDROLYZED WHEAT PROTEIN	34
12G - Night	HYDROLYZED WHEAT PROTEIN	14
12H - Paste Masks (mud packs)	HYDROLYZED WHEAT PROTEIN	12
12I - Skin Fresheners	HYDROLYZED WHEAT PROTEIN	2
12J - Other Skin Care Preps	HYDROLYZED WHEAT PROTEIN	24
13B - Indoor Tanning Preparations	HYDROLYZED WHEAT PROTEIN	1
13C - Other Suntan Preparations	HYDROLYZED WHEAT PROTEIN	1

*Fiscal 2012 Annual Report*

Health and Labour Sciences Research Grant  
(Immune-Allergic Disorders Prevention and Treatment Research Project)  
A Study on the Condition and Pathology of Anaphylaxis in Adults  
Substudy Report

### Hydrolyzed Wheat Proteins Sensitization and Challenge Study

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**Abstract:**

In recent years, a large number of cases of wheat allergy have been reported in those who have had long-term use of facial soap containing hydrolyzed wheat proteins (HWPs). This issue has become a major social problem. In this study project, we have established a mouse model of percutaneous sensitization and investigated the percutaneous sensitization potentials of Glupearl 19S (an HWP present in a facial soap, *Cha no Shizuku*) and related compounds with the experimental model since the last fiscal year. In the present fiscal year, we further compared the percutaneous sensitization potentials of Glupearl 19S and its starting material gluten, and investigated the effect of hydrolysis on the percutaneous sensitization potential of gluten. The study reproduced the results confirming that Glupearl 19S exhibits a potent percutaneous sensitization potential and demonstrated the utility of this experimental model. The results also indicated that gluten exhibits an increased percutaneous sensitization potential after undergoing hydrolysis to a certain extent but showed no percutaneous sensitization potential after further hydrolysis.

Furthermore, to investigate the potential of HWP to elicit cross-reactivity, we conducted an HWP sensitization test with HWPs that have been acid-hydrolyzed over different time durations and an *in vitro* humanized mast cell activation test (EXiLE) with serum samples from pediatric patients with a conventional wheat allergy (PedWA). In HWP-sensitized serum samples, acid-hydrolysis increased the potential for gluten to elicit a response, and further acid-hydrolysis resulted in no apparent attenuation of such a potential. In the serum samples from PedWA patients, however, the acid-hydrolysis of gluten, depending on the treatment duration, rapidly attenuated such a potential to elicit a response. In addition, we fractionated a number of HWPs by size under non-denaturing conditions using ultrafiltration and used the EXiLE test to analyze the potential of each of the fractions to elicit an IgE response. The results showed that fractions with a molecular weight of  $\leq 3$ kDa exhibit no potential of inducing an IgE response.

**A. Objectives**

Reports in the past few years have indicated that a large number of long-term users of a facial soap containing HWPs (*Cha no Shizuku* [Drop of Tea] soap by Yuuka Co., Ltd.) developed allergic symptoms after consuming wheat products. That number has reached 1,769 as of January 20, 2013, according to an epidemiological survey by a Special Committee of the Japanese Society of Allergology. Some of these patients showed serious symptoms, and the issue has become a major social problem. Potential pathways for sensitization by ingredients in the facial soap include percutaneous absorption and mucosal absorption (through the ocular and nasal mucosae).

This research group is responsible for the HWP sensitization and challenge study in animal and cell models, which began in fiscal 2011 and continued into fiscal 2012. This research is part of the basic research in "A Study on the Current Condition and Pathology of Anaphylaxis in Adults," which by and large has been conducted to advocate the processing methods, concentrations, and methods of use of cosmetics and quasi drugs containing food proteins with lower antigenicity. The primary goal is to establish a mouse percutaneous sensitization test procedure using Glupearl 19S, which comprises of

acid-hydrolysates of wheat proteins present in *Cha no Shizuku* soap; to compare the percutaneous sensitization potential between Glupearl 19S and its starting material gluten; to investigate the percutaneous sensitization potentials of HWPs other than Glupearl 19S; and to examine changes in sensitization potential of gluten as it undergoes acid-hydrolysis over different time durations. In addition, the secondary goal is to employ an *in vitro* sensitization test that uses humanized mast cells to investigate changes in the potential of Glupearl 19S to elicit a response as it undergoes acid-hydrolysis over different time durations and to assess the molecular size of hydrolysates that exhibit a potential to elicit a response.

**B. Methods**Preparation of antigen suspensions

Katayama Chemical Industries Co., Ltd. supplied Glupearl 19S. Gluten (Sigma G5004) and Glupearl 19S powder, respectively, were suspended in 1M Tris (pH 11.4) to make 100 mg/mL and left standing at room temperature overnight to yield the stock suspensions. The stock suspensions were diluted 10-fold with PBS and adjusted to a pH near 8 before use for percutaneous sensitization.

The acid-hydrolysis of gluten for (i) animal experiments was achieved by first diluting the gluten stock suspension to a final gluten concentration of 40 mg/mL and adjusting to pH 1 with 1 N hydrochloric acid, and then heating on a heating block at 100°C for 0.5, 1, 2, 3, 6, 9, 12, 24, and 48 hours. At each time point, a sample of the suspension was neutralized with 1.5 N sodium hydroxide solution to stop the hydrolysis reaction, and then diluted with PBS to a final gluten concentration of 10 mg/mL. The 0-hour hydrolysis sample was prepared by adding the gluten stock suspension to a pre-neutralized 1 N hydrochloric acid solution to a concentration of 40 mg/mL without heating. The acid-hydrolysis of gluten for (ii) cell experiments was achieved by adding the stock suspension to 0.1 N hydrochloric acid to a concentration of 1 mg/mL and then heating on a heating block at 100°C for 0.5, 1, 3, 6, 9, 12, 24, and 48 hours. At each time point, a sample of the suspension was neutralized with 0.1 N sodium hydroxide solution to stop the hydrolysis reaction. The 0-hour hydrolysis sample was prepared by adding the gluten stock suspension to a pre-neutralized 0.1 N hydrochloric acid solution to a concentration of 1 mg/mL without heating.

The extent of hydrolysis was checked by SDS electrophoresis with 10%–20% or 15%–25% acrylamide gel (DRC Co., Ltd.).

#### Simple fractionation of HWP suspensions by ultrafiltration

The acid-hydrolyzed HWPs prepared as described above and Glupearl 19S (0.5 mg/mL) were fractionated with ultrafiltration spin columns (Microcon/Amicon Ultra, Millipore) into 10 kDa high-molecular-weight (HMW) fraction and 3 kDa low-molecular-weight (LMW) fraction, and each fraction was returned to its original volume. For convenience, concentrations of HWPs after fractionation were expressed in terms of pre-fractionated HWP-equivalent.

#### Percutaneous sensitization experiment in mice

Animals used were Japan SLC-supplied 7-week old female BALB/c mice given the MF feed (Oriental Yeast Co., Ltd.). A total of 5 to 10 animals were assigned to each group. The skin on the dorsal-lateral area of each animal was shaved when the animal reached 8-week old (Day 0). The antigen suspension was applied on the shaved skin with a patch to sensitize the animal percutaneously for 3 days beginning on the next day (Days 1–3). The patches applied on the shaved skin were prepared by cutting patch tester TORII (Torii Pharmaceutical Co., Ltd.) into 2 cm x 2 cm squares, and the pads were soaked with 50 µL of the antigen suspension (500 µg of protein). The patch testers were taped with surgical tape to protect the patch. Animals also wore the Elizabethan collar to prevent the patch from peeling off. After 3 days of sensitization, the patches were removed (Day 4). Animals were given a 4-day rest period subsequently. After the 7-day routine (i.e. a 3-day sensitization period followed by a 4-day rest)

was repeated four times, the sensitized animals were tested for antigen-specific IgE antibodies and IgG1 antibodies by ELISA. On Day 25, animals were challenged intraperitoneally (i.p.) with 1 mg/100 µL of the sensitizing antigen to induce allergic response. At 30 minutes post-i.p., the rectal temperature in the animals was measured to determine the change. Further, animals were observed for anaphylactic signs, which were scored according to the criteria provided in Table 1. At 30 minutes post-challenge, whole blood samples were collected from animals under anesthesia for measurements of plasma histamine concentration by Histamine EIA Kit (SPI-BIO).

#### *Experiment 1*

Table 2 presents a list of the sensitizing antigens; Fig. 1, the sensitization schedule. Groups of 8 animals each were studied to investigate the percutaneous sensitization potentials of Glupearl 19S and gluten. Further, to investigate the effect of the coexistence of a surfactant at sensitization, 1 group of animals received an antigen suspension spiked with sodium lauryl sulfate (SDS) to a final concentration of 0.5%.

#### *Experiment 2*

Table 2 presents a list of the sensitizing antigens; Fig. 1, the sensitization schedule. To confirm the percutaneous sensitization potential of the alkaline-hydrolyzed wheat protein HWP-D (acquired through the Japan Cosmetic Industry Association), which exhibited a relatively potent sensitization potential in the study conduct last fiscal year, a retest was conducted on groups of 10 animals each.

#### *Experiment 3*

Table 2 presents a list of the sensitizing antigens; Fig. 1, the sensitization schedule. The percutaneous sensitization potentials of acid-hydrolysates of gluten that have been hydrolyzed over different durations were investigated. The acid-hydrolysis of gluten was performed as directed above. Neutralized suspensions were spiked with SDS to a final concentration of 0.5% and used as antigen in the sensitization by patch. The unhydrolyzed gluten (A 0h), hydrolyzed gluten that showed a SDS-PAGE pattern the same as that by Glupearl 19S (A 0.5h), and hydrolyzed gluten that showed a SDS-PAGE pattern with practically no protein bands of  $\geq 30$  kDa (A 9h) were tested as antigens in groups of 5 animals each.

#### *In vitro* allergic response challenge test with humanized mast cells

The humanized mast cells used were a cultured rat mast cell line (RS-ATL8 cells) stably transfected with a transcription factor NF-AT-controlled firefly luciferase-expressing reporter gene and a human FcεRI gene.<sup>6,7</sup> The cells were seeded at  $5 \times 10^4$  cells/50 µL per well in a 96-well white clear bottom plate, sensitized with 100-fold diluted HWP, added with the PedWA (pediatric patients with a conventional wheat allergy) serum or the serum from a patient with a conventional wheat-dependent exercise-induced anaphylaxis (CO-WDEIA), and incubated overnight. The wells were washed 3 times with PBS using a cell washer (Tecan HydroSpeed).

Next, the cells were stimulated with different HWP antigens suspended in MEM medium supplemented with 10% inactivated fetal bovine serum and incubated for 3 hours. Then a homogenous substrate One-GLO™ (Promega) was added, with the emitted luminescence measured with a luminometer EnVision (PerkinElmer). The measurements were conducted in duplicates, and cell activations were expressed as relative values, with the luminescence from the cells given no antigen stimulation as 1.

#### Statistical Analysis

(i) Animal study data were tabulated with Microsoft Excel and analyzed with the IBM SPSS Statistics software by the Dunnett's test versus the vehicle (V) group and the Tukey's multiple comparison test, with the significant level set to  $p < 0.05$ .

(ii) The changes in luciferase expression in the cell experiment were analyzed by the Steel's method based on changes relative to the luminescence from the negative control, the serum sample of a healthy human subject.

#### (Ethical considerations)

Efforts were made to minimize suffering of animals in percutaneous sensitization and blood sampling, and animal care/management were carried out in accordance with the institute's regulations. These experiments were conducted after approvals by the animal ethics review committee of the National Institute of Health Sciences.

### C. Results

#### (a) Percutaneous sensitization study in mice

##### *Experiment 1*

The percutaneous sensitization potentials of Glupearl 19S and gluten as well as the effect of a surfactant (SDS) at sensitization were investigated.

Fig. 2-1 shows the levels of mouse serum antigen-specific antibodies at 4 weeks post-sensitization. A and B show the results for Glupearl 19S-specific IgE and IgG1, respectively. In both the group sensitized with a combination of SDS and Glupearl 19S (HS) and the group sensitized with Glupearl 19S alone (H), blood IgE and IgG1 levels increased significantly compared with the vehicle group (V). In addition, C and D show the results for gluten-specific IgE and IgG1, respectively. The blood IgE and IgG1 levels increased significantly in the group sensitized with a combination of SDS and gluten (GS) compared with those in the V group. In the group sensitized with gluten alone (G), no significant increase in IgG1 level was noted. Furthermore, the significant response to Glupearl 19S seen in the GS group (A, B) and the significant response to gluten seen in both the HS and H groups (C, D) indicated the presence of cross-reactivity between Glupearl 19S and gluten.

Fig. 2-2 shows the anaphylactic responses after intraperitoneal antigen challenge. A shows the

changed in rectal temperature at 30 minutes post-challenge. The body temperature at 30 minutes post-challenge decreased greatly by a mean of 3.5 degrees in the HS group and by a mean of 2.7 degree in the H group compared with that in the V group. Further, a decrease of 2.1 degree in body temperature was noted even in the GS group. The difference from the body temperature in the V group was significant in all 3 groups. Meanwhile, no body temperature drop was noted in the G group. B shows the blood histamine concentrations at 30 minutes post-challenge. In the H group that showed a great decrease in body temperature, the blood histamine concentration increased greatly. However, in the HS group, which had the greatest decrease in body temperature, no increase in histamine concentration was noted (this will be discussed later). In the GS and G groups, no marked increase was noted in histamine concentration. C shows the scores of anaphylactic signs 30 minutes post-challenge. The mean score was high (3.25) in the HS group and 2.25 in the H group. Meanwhile, the score was 1.0 in the GS group and low (0.5) in the G group.

##### *Experiment 2*

HWP-D, one of the HWPs other than Glupearl 19S that were studied in fiscal 2011, was retested.

Fig. 3-1 shows the levels of antigen-specific antibodies generated at 4 weeks post-sensitization. In both the Glupearl 19S group (19S) and HWP-D group, the percutaneous sensitization markedly increased the blood levels of antigen-specific IgE and IgG1 at 4 weeks post-dose.

Fig. 3-2 shows changes in body temperature 30 minutes post-challenge (A), scores of anaphylactic signs (C), and blood histamine concentrations at 30 minutes post-challenge (B). Body temperatures decreased greatly (A) by a mean of 2.5 degrees in the 19S group and by a mean of 2.2 degrees in the HWP-D group compared with those in the V group. The blood histamine concentration at 30 minutes post-challenge increased greatly in both the 19S group and the HWP-D group (B) compared with that in the V group. The scores of anaphylactic signs were also high in both groups: a mean of 3.0 in the 19S group and 2.8 in the HWP-D group (C). A, B, and C all showed an equivalence between the 19S group and the HWP-D group.

Fig. 3-1 shows increased blood levels of sensitizing antigen-specific IgE and IgG1 at 4 weeks post-sensitization. The extent of reactivity exhibited by the respective sensitizing antigen-specific antibodies against the other antigen was evaluated by ELISA. The results are shown in Fig.3-3. A shows the results of an ELISA test with Glupearl 19S as the solid-phase antigen, and B shows those with HWP-D as the solid-phase antigen. As with Fig. 3-1, A shows a reactivity in the Glupearl 19S group but also a reactivity in the HWP-D group as well; significant differences were noted compared with the V group. B also shows that a reactivity was noted in the Glupearl 19S group beside the HWP-D group; again, significant differences were noted compared with the V group. These results suggested the presence of

cross-reactivity between Glupearl 19S and HWP-D.

### *Experiment 3*

Acid-hydrolysates of gluten, similar to Glupearl 19S, were prepared to investigate their percutaneous sensitization potentials.

Fig. 4-1 shows the SDS-PAGE pattern with respect to time, as gluten underwent hydrolysis. At 0.5 hour, the SDS-PAGE pattern was similar to that of Glupearl 19S, with a broad smear in the molecular weight  $\leq 70$  kDa region. As time progressed, the bands in the high-molecular-weight region disappeared, and migrated to the low-molecular-weight region. Based on this pattern, the non-hydrolyzed gluten (A0h), the 0.5-hour hydrolysates (A0.5h), which exhibited a pattern similar to that of Glupearl 19S, and the 9-hour hydrolysates (A9h), which is mostly comprised of  $\leq 30$  kDa, were isolated; their percutaneous sensitization potentials in mice were studied.

Fig. 4-2 shows the levels of antigen-specific antibodies produced at 4 weeks post-sensitization. Increases in antigen-specific IgE levels were noted in the Glupearl 19S (19S) and the A0.5h groups (A). Furthermore, increases in IgG1 levels were noted in the 19S, the A0.5 h, and the A0h groups (B).

Fig. 4-3 shows the anaphylactic responses after intraperitoneal antigen challenge. As shown in A, the rectal temperature decreased by a mean of 4.8°C in the 19S group (compared to the V group at 30 minutes post-challenge). In the A0.5h group, an equally large decrease of 4.4°C was noted. At the same time, the decrease was 1.2°C in the A0h group, 1.8°C in the A9h group, showing no significant deviation from the V group. The blood histamine concentration at 30 minutes post-challenge increased substantially in the 19S group, and increased significantly in the A0.5h group (B). C shows the scoring system to indicate anaphylactic signs. The mean score was high at 3.0 in both the 19S and A0.5h groups. The score was slightly lower, however, at 1.6 in both the A0h and A9h groups.

### (b) *In vitro* allergic challenge test with humanized mast cells

#### (i) SDS electrophoretic analysis of changes in molecular weight of acid-hydrolyzed gluten

Acid-hydrolyzed gluten was analyzed by 15–25% SDS electrophoresis. For non-hydrolyzed gluten, a large number of bands were observed in the 30 - 40 kDa region. Upon initiation of hydrolysis, the bands were observed in a broader region, from low (several kDa) to high molecular weight (several hundred kDa). Subsequently, the mean molecular weight decreased as time progressed (Fig. 5-1A; as presented in the fiscal 2011 report). After 12 hours of hydrolysis, almost all hydrolysates were of apparent molecular weight of  $\leq 10$  kDa. However, note the mobility in SDS electrophoresis is generally premised on the condition that the amount of bound SDS (negative charge) per unit weight is constant.<sup>8</sup>

#### (ii) Analysis of IgE response to acid-hydrolyzed gluten using serum samples of various wheat allergy

### patients

IgE responses to the above-mentioned hydrolyzed gluten and Glupearl 19S were analyzed using serum samples of various wheat allergy patients and a humanized mast cell line.<sup>4</sup> Specifically, serum samples were collected from 8 healthy subjects, 10 patients sensitized by Glupearl 19S (HWP), 8 pediatric wheat food allergy (pedWA) patients, and 9 conventional wheat-dependent exercise-induced anaphylaxis (CO-WDEIA) patients. RS-ATL8 cells were sensitized overnight with serum samples diluted 100-fold. After rinsing, the cells were stimulated with 0-, 1-, 6-, and 12-hour acid-hydrolyzed HWPs and Glupearl 19S at 37°C for 3 hours. The responses observed are shown in Fig. 5-1B. With the serum samples from HWP patients, no response to the non-hydrolyzed gluten was noted, but responses to hydrolyzed gluten were marked and attenuated along with the duration of hydrolysis. The responses to Glupearl 19S were also strong. Meanwhile, with the serum samples from PedWA patients, the response to the un-hydrolyzed gluten was the strongest, and the response attenuated along with the duration of hydrolysis. The PedWA serum also showed a response to Glupearl 19S. Lastly, with the serum samples from CO-WDEIA patients, other than a weak response to the un-hydrolyzed gluten, no responses to the HWPs were detected.

#### (iii) Analysis of the relationship between various HWP molecular weights using ultrafiltration and IgE response

As stated above, there is a broad distribution of apparent molecular weights of acid-hydrolyzed HWPs and Glupearl 19S, from several kDas to hundreds of kDas (Fig. 5-1A). Generally, as hydrolysis progresses, the molecular weights of antigens decrease, making them unable to crosslink with the IgE on mast cells.<sup>9</sup> To investigate (by the EXiLE method) which fractions of hydrolysates the serum IgE from HWP patients would react to, Glupearl 19S was fractionated into different molecular-weight fractions by consecutive fractionation with 10-kDa and 3-kDa cut-off ultrafiltration spin columns.

As shown in Fig. 5-2, the IgE from this HWP patient (42-year old female) showed a response of  $\geq 2$ -fold the background to a mere 10 fg/mL of Glupearl 19S. The responses to the  $>10$  kDa fraction differed very little from those to the whole Glupearl 19S. With the 3–10 kDa fraction, the concentration of antigen required for activation increased substantially compared with that of the pre-fractionated Glupearl 19S. With the  $<3$  kDa fraction, no responses were detected. Please note that all concentrations shown here have been converted to values of the pre-fractionated Glupearl 19S-equivalent.

Next, the serum sample from the same patient was used to analyze 0-, 1-, 6-, and 12-hour acid-hydrolysates of gluten that have been separated into the 3 kDa and 10 kDa fractions. No responses to the un-hydrolyzed gluten were detected. The IgE responses to gluten hydrolysates decreased as the duration of hydrolysis increased (Fig. 5-3). No

responses were detected against the  $\leq 3$  kDa fractions just as in the test against Glupearl 19S.

#### D. Discussion

##### (a) Percutaneous sensitization study in mice

To investigate the causal relationship between the use of *Cha no Shizuku* soap and wheat food allergy, the percutaneous sensitization study in mice has been conducted since the last fiscal year. Sensitization to mice was achieved after 3 to 4 (3 to 4 weeks) repeated sensitizations applied on the skin with patches soaked with Glupearl 19S, as demonstrated by allergic signs (anaphylaxis) induced by a subsequent intraperitoneal injection of the antigen. These results are consistent with those reported by other groups: that is, sensitizations by food proteins can occur percutaneously.<sup>10-12</sup> In the present fiscal year, the study conducted in the last fiscal year was repeated with a larger number of mice per group to improve the reliability of the study, and additional studies were also conducted to study the subject more extensively.

The study conducted last fiscal year in groups of 5 animals each showed that Glupearl 19S and gluten exhibit percutaneous sensitization potentials and that the co-presence of a surfactant promotes sensitization. The study (Experiment 1) was repeated; this time with groups of 8 mice each to improve the reliability of experiment conditions. The results showed that Glupearl 19S clearly exhibits a percutaneous sensitization potential, with a peritoneal injection inducing potent signs of anaphylaxis in sensitized animals, and that gluten also sensitized the animals in the presence of SDS, but the subsequent signs of anaphylaxis were weaker than those seen in Glupearl 19S-sensitized animals. These results were practically the same as those obtained in the study conducted in the last fiscal year, demonstrating that this experimental model is very useful for investigating percutaneous sensitization potentials of proteins. Further, as shown in Fig. 2-1, the study also yielded an interesting finding: a cross-reactivity between Glupearl 19S and gluten. The results also showed that SDS promotes sensitizations, which may be attributable to an increased cutaneous permeability for protein antigens or an adjuvant effect on the cutaneous immune system. As shown in Fig. 2-2, the blood histamine levels remained low despite marked decreases of body temperature and signs of anaphylaxis in the HS group. While no apparent reasons are known, one possible hypothesis is that when sensitization progresses well, the mast cells may be stimulated to some extent and begin to release histamine gradually in the latter half of the sensitization process, making the post-challenge increase in blood histamine level more moderate.

In fiscal 2011, HWPs other than Glupearl 19S were screened for their percutaneous sensitization potentials, and the results showed that HWP-D, an alkaline-hydrolyzed wheat protein, exhibits a sensitization potential slightly weaker than that

shown by Glupearl 19S. Experiment 2 in the present fiscal year was conducted to confirm the percutaneous sensitization potential of HWP-D by repeating the test under conditions to improve reliability, using groups of 10 mice each. The results showed the generation of specific antibodies and signs of anaphylaxis after a peritoneal challenge, confirming that HWP-D exhibits a percutaneous sensitization potential that is almost equivalent to that of Glupearl 19S. Further, as shown in Fig. 3-3, a cross-reactivity between the two was observed.

Glupearl 19S is gluten hydrolysates prepared by treating it with heat under acidic conditions. In Experiment 3, acid-hydrolysis of gluten was performed by the same procedure over different durations to provide samples hydrolyzed to various degrees to consider their percutaneous sensitization potentials. The 0.5-h hydrolysates (A0.5h) showed an SDS-PAGE pattern similar to that of Glupearl 19S and exhibited a sensitization and an anaphylaxis-inducing potential that is comparable to Glupearl. In contrast, the un-hydrolyzed gluten (A0h) and extensively hydrolyzed 9-h hydrolysates (A9h) exhibited weak sensitization and anaphylaxis-inducing potential. These results showed that Glupearl 19S or A0.5h, which has been acid-hydrolyzed to some extent, exhibits a sensitization potential greater than that of gluten, which is their starting material, and that the more extensively hydrolyzed gluten hydrolysates such as A9h have a relatively low sensitization potential. In Glupearl 19S and A0.5h, hydrolysis has changed the gluten structure slightly, causing typically non-epitope sequences to behave like the epitope, possibly giving the gluten hydrolysates an increased antigenicity.

##### (b) In vitro challenge test with humanized mast cells

The results of SDS electrophoresis showed a very broad distribution of acid- and heat-treated gluten hydrolysates, with molecular weights ranging from several kDa to several hundred kDa (Figure. 5-1A). However, as stated above, note that the mobility in SDS electrophoresis does not always reflect the actual molecular weight. Specifically, any substantial change to the surface electrical charge on a protein will result in a change to the amount of the negatively-charged SDS molecules bound to the protein, which often results in a change of mobility on electrophoresis.<sup>13</sup>

When proteins are heated in the presence of an acid, the reactions that can occur include cleavages of the peptide bonds and deamidation of glutamine and asparagine residues.<sup>14</sup> Gluten is a protein having a very high glutamine content. Thus, the deamidation of glutamine residues into glutamate residues in the gluten molecule will result in many negative charges. These negative charges make binding of the SDS molecules more difficult, which may have contributed to the change in mobility on electrophoresis or the apparent molecular weight. Therefore, it is important to know the molecular weight (molecular size) of non-denatured gluten hydrolysates.

Various HWPs were fractionated by size with a simple technique using ultrafiltration membrane without using SDS. The fractions were then analyzed for IgE response by the EXiLE method. The results indicated that the  $\leq 3$  kDa fraction of both Glupearl 19S and acid-hydrolyzed gluten exhibits no potential to induce IgE responses (Fig. 5-1, 5-3B). As cleavages of the peptide backbone progress with hydrolysis, the antigen molecule has become less effective for inducing a crosslink with IgE. The results from SDS electrophoresis alone indicated that the fraction of peptides with an apparent molecular weight of  $\leq 3$  kDa increased with the extent of hydrolysis, which is consistent with the results of IgE response. Both the EXiLE method and the size fractionation by ultrafiltration spin columns can be conducted without denaturing the peptides. The two procedures combined make it possible to analyze the molecular size of antigen under a near physiological condition, using the IgE response as an indicator.

## E. Conclusions

The mouse model of percutaneous sensitization used in the study last fiscal year was again used in a study focused on Glupearl 19S. The study results obtained reproduced results showing that Glupearl 19S exhibits a strong percutaneous sensitization potential and demonstrated the utility of this experimental model. The results also showed that HWP-D, one of the HWPs, exhibits a percutaneous sensitization potential as well as a cross-reactivity with Glupearl 19S. Further, the results of studies on acid-hydrolysates of gluten indicated that gluten exhibits an increased percutaneous sensitization potential after undergoing hydrolysis to a certain extent but showed no percutaneous sensitization potential after further hydrolysis. In addition, the *in vitro* challenge study of serum samples from patients who were *Cha no Shizuku* soap-users also showed that Glupearl 19S has a higher inducing potential than gluten and that the epitope present in proteins of a relatively high molecular weight of  $\geq 3,000$  is involved in the induction.

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## F. Health Risk Information

None

## G. Publications

### 1. Research paper publication

1) Adachi R, Nakamura R, Sakai S, Fukutomi Y, Teshima R. Sensitization to acid-hydrolyzed wheat protein by transdermal administration to BALB/c mice, and comparison with gluten. *Allergy* 2012; **67**:1392-1399.

- 2) Nakamura R, Nakamura R, Adachi R, Itagaki Y, Fukutomi Y, Teshima R. Evaluation of Allergenicity of Acid-Hydrolyzed Wheat Protein Using an *in vitro* Elicitation Test. *Int Arch Allergy Immunol* 2012; **160**: 259-264.
- 3) Teshima R: HWP-induced allergies. *Farumashia* 2013; **49(2)**; 116- 120

## 2. Conference presentation

- 1) Adachi R, Nakamura R, Sakai S, Fukutomi Y, Teshima R. Study on percutaneous sensitization by HWP in a mouse model. The 24th Spring Meeting of Japanese Society of Allergology (May 2012)
- 2) Nakamura R, Nakamura R, Adachi R, Itagaki Y, Fukutomi Y, Teshima R. A comparison of IgE binding potentials and elicitation potentials of acid-hydrolyzed wheat. The 24th Spring Meeting of Japanese Society of Allergology (May 2012)
- 3) Nakamura R, Nakamura R, Adachi R, Itagaki Y, Matsunaga K, Fukutomi Y, Teshima R. An evaluation by *in vitro* activation test of cross-reactivity of IgE from patients sensitized by soap containing acid-hydrolyzed wheat. The 24th Spring Meeting of Japanese Society of Allergology (May 2012)
- 4) Nakamura R, Nakamura R, Sakai N, Adachi R, Itagaki Y, Fukutomi Y, Teshima R. Analysis of IgE responses in serum samples from patients sensitized with soap containing acid-hydrolyzed wheat. The 19th Annual Meeting of the Japanese Society of Immunotoxicology (September 2012)
- 5) Adachi R, Nakamura R, Sakai N, Fukutomi Y, Teshima R. A comparative analysis of percutaneous sensitization potentials of HWPs with a mouse model. The 62nd Annual Meeting of Japanese Society of Allergology (November–December 2012)
- 6) Kitano T, Yamashita H, Adachi R, Teshima R, Fukutomi Y, Matsunaga K, Inagaki N, Tanaka H. Effects of oral load of antigen on mice systemically sensitized with hydrolyzed wheat powder. The 62nd Annual Meeting of Japanese Society of Allergology (November–December 2012)
- 7) Nakamura R, Nakamura R, Sakai N, Adachi R, Fukutomi Y, Teshima R. Analysis of effects of molecular size on allergenicity of HWPs by the EXiLE method. The 85th Annual Meeting of the Japanese Biochemical Society (December 2012)
- 8) Adachi R, Nakamura R, Sakai S, Fukutomi Y, Teshima R. Sensitization to acid-hydrolyzed wheat protein by transdermal administration to BALB/c mice. 52<sup>nd</sup> Society of Toxicology Annual Meeting and ToxExpo (2013. 3)

## H. Intellectual Property Filing and Registration (including any pending filling and registration)

1. Patent  
None

2. Utility model registration  
None

3. Other

## 【平成 24 年度報告書】

厚生労働科学研究費補助金（免疫アレルギー疾患等予防・治療研究事業）  
「成人独自のアナフィラキシーの実態と病態に関する研究」  
分担研究報告書

## 加水分解小麦の感作性・惹起性に関する研究

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## 研究要旨：

近年、加水分解小麦（HWP）を含有する洗顔石鹸の長期使用により小麦アレルギーを発症する事例が数多く報告され、社会的に大きな問題となっている。本研究では、昨年度より、マウスを使用する経皮感作試験系を構築し、この試験系を用いて、茶のしずく石鹸に含有されていた加水分解小麦タンパク質であるグルパール 19S 及び関連物質の経皮感作性について検討を進めてきた。本年度は、引き続きこの試験系を用い、グルパール 19S とその原料であるグルテンの経皮感作性の比較、加水分解がグルテンの経皮感作性に与える影響等について、更なる検討を行った。その結果、グルパール 19S が強い経皮感作性を有する点について再現性のある結果が得られ、本試験系の有用性が示された。また、グルテンの加水分解がある程度進行した状態では経皮感作性が増大し、さらに加水分解が進行すると経皮感作性は消失することが示された。

また、HWP の交叉惹起能の検討として、酸加水分解時間の異なる HWP を用いて HWP 感作および従来型の小児小麦アレルギー患者（PedWA）血清による *in vitro* ヒト化マスト細胞活性化（EXiLE）試験を行ったところ、HWP 感作血清では酸加水分解によりグルテンの惹起能が増大し、酸加水分解が進んでもその惹起能は減弱しにくい傾向にあったが、PedWA 患者血清では、グルテンの酸加水分解の処理時間に応じて惹起能は速やかに減弱した。また、限外ろ過膜を用い、各種 HWP を非変性下でサイズ分画し、それぞれの画分の IgE 応答性を EXiLE 法により解析したところ、分画分子量 3kDa 以下の画分には IgE 反応性を誘導する能力がないことが示された。

## A. 研究目的

近年、加水分解小麦（HWP）を含有する洗顔石鹸（茶のしずく：株式会社悠香販売）の長期使用により、小麦製品を摂取した際にアレルギー症状を呈する事例が数多く報告されている。日本アレルギー学会特別委員会の疫学調査における平成 25 年 1 月 20 日現在の症例登録数は 1,769 例に達している。これらの患者には重篤な症例も多く、社会的に大きな問題となっている。洗顔石鹸の成分により感作される経路としては、皮膚から吸収される経皮感作、あるいは目や鼻の粘膜から吸収される経粘膜感作が考えられる。

私共のグループでは、「成人独自のアナフィラキシーの実態と病態に関する研究」の基礎部門の中で、より抗原性の低い食物タンパク質由来の化粧品、並びに医薬部外品の加工方法、濃度、使用方法の提唱をすることを大きな目的とし、平成 24 年度は、平成 23 年度に続き、動物、細胞モデルを用いる加水分解小麦の感作性並びに惹起能に関する研究を担当した。茶のしずくに含まれていた小麦酸加水分解物であるグルパール 19S を用いてマウスを用いる経皮感作試験の手法を確立すること、グルパール 19S

とその原料であるグルテンの経皮感作性の比較、グルパール 19S 以外の加水分解小麦タンパク質の経皮感作性の検討、並びにグルテンの酸加水分解の経時変化による感作能の変化の検討を行うことを第一の目標として、また、ヒト化マスト細胞を用いる *in vitro* 惹起試験を用いて、グルパール 19S 並びに酸加水分解の経時変化による惹起能の変化の検討、並びに惹起能を持つ分子サイズについても検討することを第二の目標とした。

## B. 研究方法

## 抗原懸濁液の調製

グルパール 19S は片山化学工業株式会社より入手した。グルテン（Sigma G5004）およびグルパール 19S 粉末を 100 mg/mL となるよう 1M Tris (pH 11.4) に加えて懸濁し、終夜室温に静置してストック懸濁液を作製した。経皮感作にはストック懸濁液を PBS で 10 倍希釈し、pH を 8 付近に調整したものをを用いた。

グルテンの酸加水分解については、(i)動物実験用には、グルテンのストック懸濁液に、グルテン終濃度 40 mg/mL かつ pH1 となるように 1N 塩酸を加え、

100℃のヒートブロック上で、0.5, 1, 2, 3, 6, 9, 12, 24, 48 時間加熱した。所定の時間経過後、1.5N 水酸化ナトリウム水溶液を加えて中和し加水分解反応を停止した後、グルテン終濃度 10 mg/mL となるように PBS にて希釈した。分解0時間のサンプルは、1N 塩酸を予め中和した溶液中にグルテンストック懸濁液を 40 mg/mL 加え、加熱は行わずに調製した。(ii)細胞試験用には、グルテンの酸加水分解には、0.1N 塩酸中にストック懸濁液を 1 mg/ml となるよう加え、100℃のヒートブロック上で 0.5、1、3、6、9、12、24、48 時間加熱した。所定の時間経過後、0.1N 水酸化ナトリウム水溶液で中和し、加水分解反応を停止した。0 時間の試料は、0.1 N 塩酸を予め中和した溶液中にグルテンストック懸濁液を 1 mg/ml となるよう加え、加熱は行わなかった。

加水分解の進行は、10-20%または、15-25%アクリルアミドゲル (D.R.C.株式会社) を用いた SDS 電気泳動により確認した。

#### 加水分解小麦 (HWP) 懸濁液の限外ろ過による簡易分画

前述のごとく作製した酸加水分解 HWP およびグルパール 19S (0.5mg/ml) を、限外ろ過スピンカラム (Microcon/Amicon Ultra, ミリポア) を用いて、分画分子量 10kDa または 3kDa で高分子量側 (HMW) と低分子量側 (LMW) とに分画し、それぞれ元の容量までメスアップした。このとき、分画後の各種 HWP の濃度は、便宜上分画前の HWP 相当量として表すこととした。

#### マウスを用いた経皮感作実験

動物は、7 週齢の雌性 BALB/c マウスを日本エスエルシーより購入し、MF 飼料(オリエンタル酵母工業株式会社)を給餌した。1 群中の匹数は 5-10 匹とし、8 週齢時に背面片側を剃毛し (Day 0)、翌日より 3 日間抗原懸濁液を剃毛部に貼付して経皮感作を行った (Day 1-3)。抗原懸濁液の貼付には、パッチテスター「トリー」(鳥居薬品株式会社)を 2 cm 角に切り取ったものを用い、パッド部に 50  $\mu$ L の抗原懸濁液 (500  $\mu$ g of protein) を浸潤させ剃毛部に貼付した。パッチテスターの上からサージカルテープを巻いてパッチを保護し、さらにマウスの首にエリザベスカラーを装着してパッチの剥脱を防いだ。3 日間の感作後にパッチを外し (Day 4)、その後 4 日間休ませるという操作を 1 クールとし、4 クールの感作後、抗原特異的 IgE 抗体及び IgG1 抗体を ELISA 法で測定した。アレルギー反応の惹起は Day 25 に、感作抗原 1 mg/100  $\mu$ L を腹腔内投与 (i.p) して行った。i.p. 後 30 分間、マウスの直腸内体温変化の測定を行った。また、アナフィラキシー症状を観察し、Table 1 の基準に従ってスコアリングした。惹起 30 分後に麻酔下で全血を採取し、血漿中ヒスタミンの濃度を、Histamine EIA Kit (SPI-BIO) にて測定した。

#### 【実験 1】

感作抗原を Table 2 に、感作スケジュールを Fig. 1 に示す。1 群の匹数を 8 匹として、グルパール 19S

及びグルテンの経皮感作性について検討を行った。また、感作時の界面活性剤の共存効果を調べるため、ラウリル硫酸ナトリウム (SDS) を終濃度 0.5% となるように抗原懸濁液に添加する群も設定した。

#### 【実験 2】

感作抗原を Table 2 に、感作スケジュールを Fig. 1 に示す。前年度の研究において比較的強い感作性を示したアルカリ加水分解小麦タンパク質である HWP-D (日本化粧品工業連合会を通して入手) について、1 群の匹数を 10 匹とし、経皮感作性を確認するための再試験を行った。

#### 【実験 3】

感作抗原を Table 2 に、感作スケジュールを Fig. 1 に示す。分解時間を変化させた酸加水分解グルテンを抗原とし、経皮感作性について検討した。グルテンの酸加水分解は上述のとおりに行った。中和した懸濁液に SDS を終濃度 0.5% となるように添加し、貼付抗原とした。1 群の匹数を 5 匹として、未分解グルテン (A0h)、グルパール 19S と同様の SDS-PAGE パターンを示したもの (A0.5h)、および加水分解が進み、SDS-PAGE で 30 kDa 以上のタンパク質バンドがほぼ消失しているもの (A9h) を抗原として選択した。

#### ヒト化マスト細胞を用いた in vitro アレルギー反応惹起試験

ヒト化マスト細胞として、転写因子 NF- $\kappa$ B の制御下にホタルルシフェラーゼを発現するレポーター遺伝子およびヒト Fc  $\epsilon$  RI 遺伝子を安定的に導入したラット培養マスト細胞株 (RS-ATL8 細胞) を用いた<sup>6,7)</sup>。同細胞をクリアボトム白色 96 ウェルプレートに  $5 \times 10^4$  cells/50  $\mu$ l ずつ播種し、100 倍希釈した HWP 感作、小児小麦食物アレルギー患者 (PedWA) または従来型の運動誘発性小麦アレルギー患者 (CO-WDEIA) 血清を添加して終夜培養した。細胞洗浄機 (Tecan HydroSpeed) を用いて PBS によりウェルを 3 回洗浄後、10% の非働化ウシ胎児血清を含む MEM 培地に懸濁した各 HWP 抗原溶液 により 37℃ インキュベータ中で 3 時間細胞を刺激し、ホモジニアス型基質液 One-GLO™ (Promega) を添加して発光量をルミノメータ EnVision (PerkinElmer) により測定した。測定は duplicate で行ない、細胞の活性化は、抗原未刺激時の発光量を 1 とする相対値で表した。

#### 統計解析

(i) 動物データは Microsoft Excel により集計し、IBM SPSS Statistics ソフトウェアを用いて、V 群を基準とした Dunnett の検定および各群間の Tukey の多重検定を行い、 $p < 0.05$  を有意とした。

(ii) 細胞実験におけるルシフェラーゼ発現の変化は、健常人血清をネガティブコントロールとする発光量の相対変化を Steel 法により解析した。

#### (倫理面への配慮)

マウスへの経皮感作、採血においては、動物の苦痛を最小限に留めるように努め、動物飼育・管理に

当たっては研究所の利用規定に従った。本実験は、国立医薬品食品衛生研究所動物倫理審査委員会の承認を得てから行った。

## C. 研究結果

### (a) マウスを用いた経皮感作試験

#### 【実験 1】

グルパール 19S 及びグルテンについて、経皮感作性、及び感作時の界面活性剤(SDS)の効果について検討を行った。

Fig.2-1 には、感作 4 週後のマウス血清中の抗原特異的抗体についての検討結果を示す。A、B はそれぞれグルパール 19S 特異的な IgE 及び IgG1 についての検討結果を示している。SDS 共存下グルパール 19S 感作群(HS)、グルパール 19S のみの群(H)の両群とも、血中 IgE、IgG1 が Vehicle 群(V)と比較して有意に増加していた。また C、D はそれぞれグルテン特異的な IgE 及び IgG1 についての検討結果を示している。SDS 共存下グルテン感作群(GS)では V 群と比較して血中 IgE、IgG1 が有意に増加した。グルテンのみの群(G)では IgG1 のみ有意な増加が見られた。また、GS 群でもグルパール 19S に対する有意な反応性が見られること(A、B)、HS 群及び H 群でもグルテンに対する有意な反応性が見られること(C、D)から、グルパール 19S とグルテンの間には交差反応性があることが示された。

Fig.2-2 には、抗原腹腔内投与によるアナフィラキシー反応惹起後の応答を示す。A は惹起後 30 分間の直腸内体温の変化を示している。30 分後、HS 群では V 群と比較して平均 3.5 度、H 群では 2.7 度と、体温の大きな低下が見られた。また GS 群でも 2.1 度低下しており、これらの 3 群では V 群との間に有意な差があった。一方 G 群では体温低下は見られなかった。B は惹起 30 分後の血中のヒスタミン濃度を示している。体温が大きく低下した H 群では血中ヒスタミン濃度が大きく増大していた。しかし、体温低下が最も大きかった HS 群では、ヒスタミン濃度の増大は見られなかった(この点に関しては後述する)。GS 群及び G 群では、顕著なヒスタミン濃度の増大は見られなかった。C は惹起後 30 分間のアナフィラキシー症状のスコアリングの結果である。HS 群では平均 3.25 と高いスコアであり、また H 群でも平均 2.25 であった。一方、GS 群では 1.0、G 群では 0.5 と低いスコアであった。

#### 【実験 2】

23 年度において検討したグルパール 19S 以外の加水分解小麦タンパク質のうち、HWP-D について再試験を行った。

Fig.3-1 には、感作 4 週後の抗原特異的抗体産生の検討結果を示す。グルパール 19S 群(19S)、HWP-D 群ともに、4 週間の経皮感作により血中の抗原特異的 IgE 及び IgG1 の顕著な増加が見られた。

Fig.3-2 には、惹起後 30 分間の体温変化(A)、アナフィラキシー症状のスコアリング(C)、及び 30 分後の血中ヒスタミン濃度(B)を示す。体温低下については、19S 群では V 群と比較して平均 2.5 度、HWP-D

群では平均 2.2 度と、大きな体温低下が見られた(A)。惹起 30 分後の血中ヒスタミン濃度は、19S 群、HWP-D 群ともに、V 群と比較して大きく増大していた(B)。アナフィラキシー症状のスコアリングについても、19S 群では平均 3.0、HWP-D 群では平均 2.8 という高いスコアであった(C)。A、B、C のいずれでも、19S 群と HWP-D 群は同程度の結果を示した。

Fig.3-1 では、感作 4 週後の血中で、それぞれの感作抗原に特異的な IgE 及び IgG1 が増加したことを示した。そこで、それぞれの感作抗原に対して産生された抗体が、もう一方の抗原に対してどの程度の反応性を有するかという点に関して、同様に ELISA を用いて検討した。結果を Fig.3-3 に示す。A はグルパール 19S を ELISA の固相抗原とした場合、B は HWP-D を固相抗原とした場合の結果である。A では、Fig.3-1 との場合と同じくグルパール 19S 群で反応性が見られているが、HWP-D 群でも同様に反応性が見られ、V 群との間に有意な差があった。B の場合も、HWP-D 群のみでなく、グルパール 19S 群でも反応性が見られ、やはり V 群との間に有意な差があった。これらの結果から、グルパール 19S と HWP-D との間には交差反応性があることが示された。

#### 【実験 3】

グルパール 19S と同様のグルテン酸加水分解物を調製し、その経皮感作性について検討した。

Fig.4-1 にはグルテンの加水分解による SDS-PAGE パターンの経時的な変化を示す。加水分解 0.5 時間では、SDS-PAGE のパターンはグルパール 19S と類似しており、70 kDa 以下に広くスメアなパターンを示した。その後時間とともに高分子量側のバンドが消失し、低分子量側に移行した。このパターンをもとに、未分解グルテン(A0h)、グルパール 19S とパターンが類似している 0.5 時間分解物(A0.5h)、及びほぼ 30kDa 以下にまで分解されているもの(A9h)を取り上げ、マウスに対する経皮感作能を検討した。

Fig.4-2 には、感作 4 週後の抗原特異的抗体産生の検討結果を示す。IgE については、グルパール 19S 群(19S)だけでなく、A0.5h 群でも抗原特異的 IgE の増加が見られた(A)。また IgG1 については、19S 群、A0.5h 群に加えて A0h 群でも増加が見られた(B)。

Fig.4-3 には、抗原腹腔内投与によるアナフィラキシー反応惹起後の応答を示す。A に示すように、惹起 30 分後、19S 群では V 群と比較して平均 4.8 度低下した。A0.5h 群でも 4.4 度と大きな低下が見られた。一方 A0h 群では 1.2 度、A9h 群では 1.8 度であり、V 群との間に有意な差は見られなかった。惹起 30 分後の血中ヒスタミン濃度については、19S 群で大きく増大した他、A0.5h 群でも有意な増大が見られた(B)。C にはアナフィラキシー症状のスコアを示す。19S 群、A0.5h 群はともに平均 3.0 と高いスコアであったが、A0h 群、A9h 群はともに 1.6 とやや低いスコアであった。

### (b) ヒト化マスト細胞を用いた in vitro 惹起試験

### (i) 酸加水分解グルテンのSDS電気泳動による分子量変化の解析

酸加水分解グルテンを 15–25% SDS 電気泳動により解析したところ、未分解グルテンでは 30–40kDa 付近を中心に多くのバンドが観察されたが、加水分解が始まると低分子(数kDa)から高分子(数百kDa)までブロードに分布し、その後時間の経過とともに平均分子量は小さくなった(Fig. 5-1A; 昨年度報告済み)。分解 12 時間後の時点では、ほぼすべてが 10kDa 以下の見かけの分子量を示した。ただし、SDS 電気泳動における泳動度は、一般に単位重量あたりに結合する SDS の重量(負電荷)が等しいことを前提としていることに注意を要する<sup>8)</sup>。

### (ii) 各種小麦アレルギー患者血清を用いた酸加水分解グルテンへの IgE 反応性の解析

上記のごとく加水分解したグルテンおよびグルパール 19S について、各種の小麦アレルギー患者血清およびヒト化マスト細胞株を用いて IgE の反応性を解析した<sup>4)</sup>。具体的には、健常人 8 名、グルパール 19S 感作患者(HWP) 10 名、小児小麦食物アレルギー患者(PedWA) 8 名、成人通常型小麦依存性運動誘発アナフィラキシー患者(CO-WDEIA) 9 名である。これらの患者血清を 100 倍希釈して RS-ATL8 細胞を一晩感作し、洗浄後、100 ng/ml の 0, 1, 6, 12 時間加水分解した酸加水分解 HWP およびグルパール 19S により 37°C で 3 時間刺激を行なった。すると、Fig. 5-1B に示したような応答を示した。すなわち、HWP 患者では分解前のグルテンには応答しないが、分解後のグルテンには顕著に反応し、その後時間とともに反応性が減弱した。グルパール 19S にも強く反応した。一方、PedWA 患者では、分解前のグルテンに最も強く反応し、分解の時間に応じて反応性が減弱した。グルパール 19S にも反応した。そして、CO-WDEIA 患者では、分解前のグルテンに弱い反応性が認められたが、HWP には全く反応しなかった。

### (iii) 限外ろ過を用いた各種HWPの分子量とIgE応答性との関係の解析

上述した通り、酸加水分解HWPおよびグルパール 19S の見かけの分子量は数kDaから数百kDaまで幅広く分布する(Fig. 5-1A)。一般に、加水分解が進行すると、抗原の分子量が低下し、マスト細胞上のIgEを架橋することができなくなると考えられている<sup>9)</sup>。そこで、グルパール 19S を 10kDa および 3kDa の分画分子量を持つ限外ろ過スピンカラムにより連続的に分画し、どの画分にHWP患者血清IgEとの反応性があるかをEXiLE法により調べた。

Fig. 5-2 に示した通り、この HWP 患者(42 歳女性)の IgE はわずか 10 fg/ml のグルパール 19S にもバックグラウンドの 2 倍以上の反応を示した。10kDa 以上の画分の反応性は、グルパール 19S 全体の反応性と大差がなかった。一方、3–10kDa の画分では、活性化に必要な抗原濃度は分画前に比べて大幅に増加した。そして、3kDa 未満の画分は、全く反応しなかった。なお、ここで示した濃度は、すべて分画前のグルパール 19S に換算した値であることに注意さ

りたい。

次に、同じ患者血清を用い、0, 1, 6, 12 時間酸処理したグルテンをそれぞれ 3kDa および 10kDa で分画したところ、未分解グルテンでは応答性はなく、分解後に現れた IgE 応答性は、分解時間の経過とともに減少していった(Fig. 5-3)。このとき、グルパール 19S と同様に、3kDa 以下の画分には応答性が認められなかった。

## D. 考察

### (a) マウスを用いた経皮感作試験

本研究では、茶のしづく石鹼の使用と小麦摂取によるアレルギー発症との因果関係を検討するため、昨年度よりマウスを用いた経皮感作試験を行ってきた。その結果、マウスの皮膚にグルパール 19S を浸潤させたパッチを貼付するという感作を 3-4 回(3-4 週)繰り返すことにより感作を成立させ、抗原の腹腔内投与によりアレルギー症状(アナフィラキシー)を誘発することが可能であることを示した。これらの結果は、食物由来タンパク質による感作が経皮的に起こりうるという他のグループの報告とも矛盾しない<sup>10-12)</sup>。本年度は、昨年度行った検討に関して、1 群あたりのマウス匹数を増やしたより信頼性の高い系での再試験、また、昨年の試験結果に新たな結果を加えることにより試験内容を充実させるための試験等を行った。

グルパール 19S 及びグルテンについては、昨年度 1 群 5 匹で検討を行い、グルパール 19S 及びグルテンが経皮感作能を有すること、及び界面活性剤の共存により感作が促進されること等を示した。今回の【実験 1】では、1 群のマウス匹数を 8 匹に設定し、より信頼性の高い実験条件で再試験を行った。その結果、グルパール 19S が明らかな経皮感作能を有し、感作後の腹腔内投与により強いアナフィラキシー症状を誘発すること、グルテンでは SDS 共存下で感作が進行するが、アナフィラキシー症状の程度はグルパール 19S と比較して弱いことを示した。これらの結果は昨年の試験結果とほぼ同様であり、本試験系がタンパク質の経皮感作能を検討する上で非常に有用な系であることが明らかとなった。また、Fig. 2-1 に示すように、グルパール 19S とグルテンの間には交差反応性があるという興味深い結果を得ることもできた。SDS については感作を促進する効果が見られたが、この原因としては、抗原であるタンパク質の皮膚透過性を増大させる、あるいは皮膚の免疫系に対してアジュバントとしての作用を示すという可能性が考えられる。Fig. 2-2 では、HS 群について、顕著な体温低下やアナフィラキシー症状が見られているにも関わらず、血中ヒスタミン濃度が低いという結果となった。この理由は明らかではないが、1 つの仮説として、感作が非常に順調に進行した場合、感作過程の後半では経皮的惹起がある程度起こり、肥満細胞からのヒスタミン遊離が緩やかに進行しているため、最終的な惹起時に血中ヒスタミン濃度があまり増大しないという可能性が考えられる。

23 年度においては、グルパール 19S 以外の加水

分解小麦についても経皮感作性に関するスクリーニング的な検討を行ったが、その中で、アルカリ処理による加水分解小麦タンパク質である HWP-D はグルパール 19S よりやや弱いながらも感作性を示すという結果が得られた。本年度の【実験 2】では、HWP-D の経皮感作能について明確にするため、1群のマウス匹数を 10 匹に設定し、より信頼性の高い実験条件で再試験を行った。その結果、特異的抗体産生、腹腔内投与惹起後のアナフィラキシー症状ともに、HWP-D はグルパール 19S とほぼ同等の作用を示し、経皮感作能を有することが明らかとなった。また、Fig.3-3 に示すように、両者の間には交差反応性が見られた。

グルパール 19S はグルテンを酸性条件下加熱することによって調製された加水分解物である。そこで【実験 3】では、同様の方法でグルテンの酸加水分解を行い、分解時間を変えることにより分解の程度の異なる分解物を調製し、その経皮感作性について検討した。その結果、グルパール 19S と同様の SDS-PAGE パターンを示す 0.5 時間分解物(A0.5h)はグルパールとほぼ同等の感作性及びアナフィラキシー誘発性を示したのに対し、未分解グルテン(A0h)及び分解が進んだ 9 時間分解物(A9h)では弱かった。この結果から、酸加水分解がある程度進行したグルパール 19S あるいは A0.5h は、原料であるグルテンよりも高い感作性を有すること、また、より分解が進んだ A9h はそれ程高い感作性を有していないことが示された。グルパール 19S や A0.5h では、加水分解により構造がやや変化し、通常グルテンではエピトープとはならない配列がエピトープとなることにより、高い抗原性を獲得するのではないかと考えられる。

#### (b) ヒト化マスト細胞を用いたin vitro惹起試験

SDS電気泳動によると、酸と加熱により加水分解したグルテンは、見かけの分子量が数kDaから数百kDaまで非常に広く分布するようにみえた (Fig. 5-1A)。しかし、前述の通り、SDS電気泳動における泳動度は、必ずしも実際の分子量を反映しないおそれがある。具体的には、タンパク質の表面電荷に大きな変化が起これば、負に帯電したSDS分子のタンパク質への結合量が変化し、結果として泳動度の違いとして観察される例がしばしばある<sup>13)</sup>。

タンパク質を酸の存在下で加熱したときに起こる反応としては、ペプチド結合の開裂のほか、グルタミンやアスパラギン残基の脱アミド化がある<sup>14)</sup>。グルテンはグルタミンを非常に多く含むタンパク質であるため、グルタミン残基の脱アミド化が起こりグルタミン酸残基へと変化すれば、多くの負電荷を持つことになる。これによりSDS分子が結合しにくくなり、泳動度すなわち見かけの分子量に変化が起こる可能性が考えられる。従って、非変性下での分子量(分子サイズ)を知ることも重要である。

今回、限外ろ過膜を用い、各種 HWP を SDS を加えない非変性下で簡便にサイズ分画し、それぞれの画分の IgE 反応性を EXiLE 法により解析した。その結果、グルパール 19S、酸加水分解グルテンのい

れも、分画分子量 3kDa 以下の画分には IgE 反応性を誘導する能力がないことが示された (Figs. 5-2, 5-3B)。加水分解が進行し、ペプチド結合主鎖の開裂が進むことにより、抗原分子が IgE の架橋を十分に誘導することができなくなったためとみられる。SDS 電気泳動の結果を見るかぎり、見かけ上 3kDa 以下のペプチドは加水分解の経過とともに増えてくるが、本結果は IgE 反応性の結果と矛盾しなかった。限外ろ過スピンカラムによるサイズ分画と EXiLE 法はともに非変性下で実施できるため、両者を組み合わせることにより、より生理的な条件に近い形で IgE 反応性を指標とした抗原の分子サイズの解析が可能になると思われた。

## E. 結論

昨年度検討を開始したマウス経皮感作試験系を用い、グルパール 19S を中心に検討を行った。その結果、グルパール 19S が強い経皮感作性を有する点について再現性のある結果が得られ、本試験系の有用性が示された。その他の加水分解小麦タンパク質の 1 種である HWP-D についても経皮感作性が見られ、またグルパール 19S との間には交差反応性があることも示された。また、グルテン酸加水分解物を用いた試験より、加水分解がある程度進行した状態では経皮感作性が増大し、さらに加水分解が進行すると経皮感作性は消失することが示された。また、茶のしずく患者血清を用いた in vitro 惹起試験においても、グルパール 19S はグルテンに比べ高い惹起能を有し、分子量 3000 以上の比較的高分子のタンパク質に存在するエピトープが惹起に関係していることが示唆された。

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## F. 健康危険情報

なし

## G. 研究発表

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## H. 知的財産権の出願・登録状況(予定を含む)

### 1. 特許取得

なし

### 2. 実用新案登録

なし

### 3. その他

なし

**Table 1. Scoring Criteria for Signs of Anaphylaxis**

Score 0	No signs
1	Scratching mouth, ear, nose, head, etc.; scratching the ear canal with the hind leg
2	Hypoactivity, faster breathing, isolation from other specimens, swelling around the eye, nose or mouth, piloerection
3	Stationary for >1 minute, laying down in a prone position, wheezing, breathing difficulty, cyanosis around the mouth or tail, transient convulsion
4	No response to whisker touching, decreased or no response to stimuli, unconsciousness, trembling, convulsion
5	Death

**Table 2. Sensitizing Antigens****Experiment 1 (5 groups of 8 animals each)**

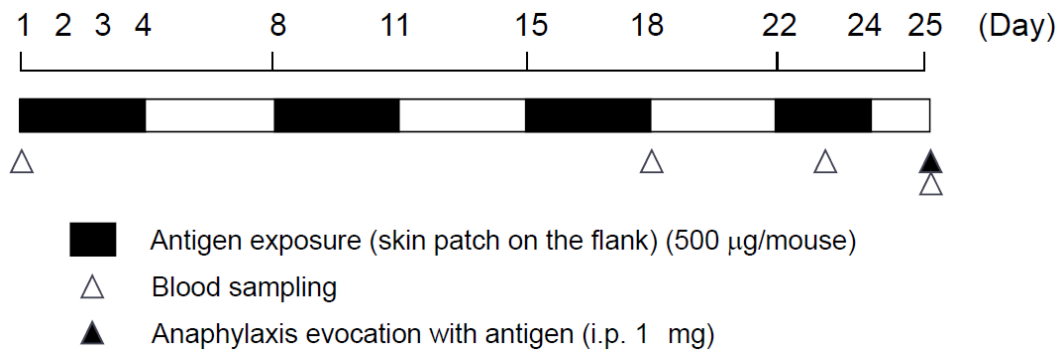
Group	Sensitizing Compound	Sensitizing Antigen Dose	Route of Challenge	Challenging Compound
V	PBS + 0.5% SDS	-	i.p. (1 mg)	Glupearl 19S
HS	Glupearl 19S + 0.5% SDS	500 µg	i.p. (1 mg)	Glupearl 19S
H	Glupearl 19S	500 µg	i.p. (1 mg)	Glupearl 19S
GS	Gluten + 0.5% SDS	500 µg	i.p. (1 mg)	Gluten
G	Gluten	500 µg	i.p. (1 mg)	Gluten

**Experiment 2 (3 groups of 10 animals each)**

Group	Sensitizing Compound	Sensitizing Antigen Dose	Route of Challenge	Challenging Compound
V	PBS + 0.5% SDS	-	i.p. (1 mg)	Glupearl 19S
19S	Glupearl 19S + 0.5% SDS	500 µg	i.p. (1 mg)	Glupearl 19S
HWP-D	HWP-D + 0.5% SDS	500 µg	i.p. (1 mg)	HWP-D

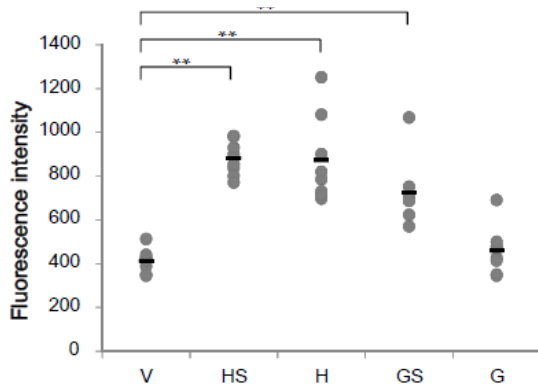
**Experiment 3 (5 groups of 5 animals each)**

Group	Sensitizing Compound	Sensitizing Antigen Dose	Route of Challenge	Challenging Compound
V	PBS + 0.5% SDS	-	i.p. (1 mg)	Glupearl 19S
19S	Glupearl 19S + 0.5% SDS	500 µg	i.p. (1 mg)	Glupearl 19S
A0h	0-h acid-hydrolyzed gluten + 0.5% SDS	500 µg	i.p. (1 mg)	0-h acid-hydrolyzed gluten
A0.5h	0.5-h acid-hydrolyzed gluten + 0.5% SDS	500 µg	i.p. (1 mg)	0.5-h acid-hydrolyzed gluten
A9h	9-h acid-hydrolyzed gluten + 0.5% SDS	500 µg	i.p. (1 mg)	9-h acid-hydrolyzed gluten

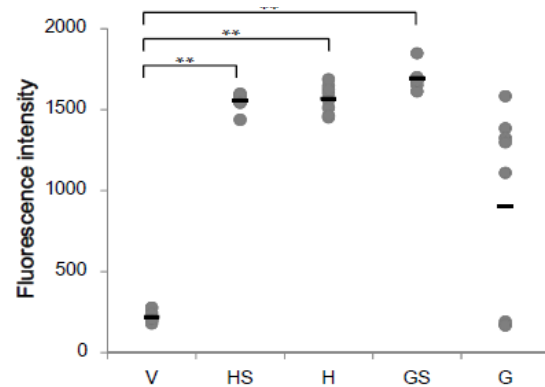


**Figure 1. Percutaneous Sensitization Schedule**

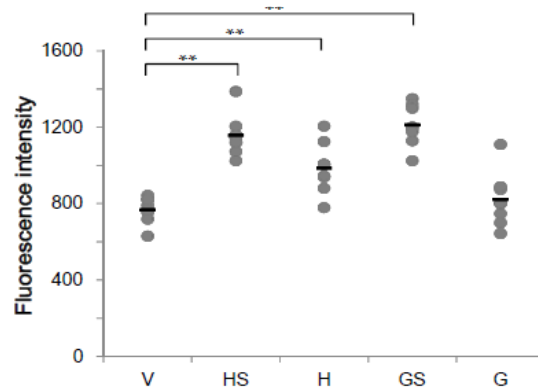
**A. Glupearl 19S-specific IgE**



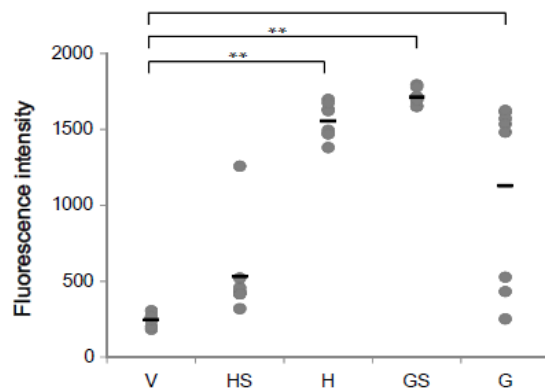
**B. Glupearl 19S-specific IgG1**



**C. Gluten-specific IgE**



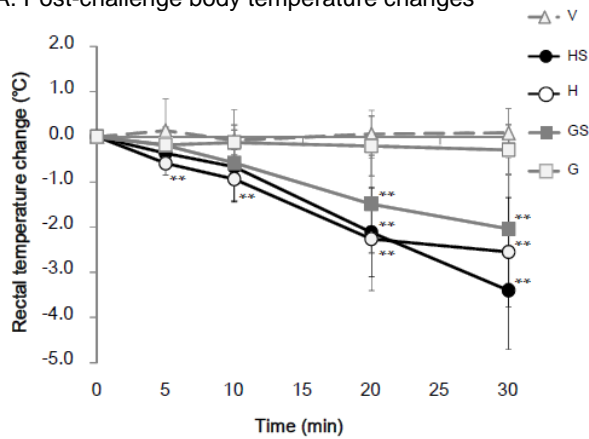
**D. Gluten-specific IgG1**



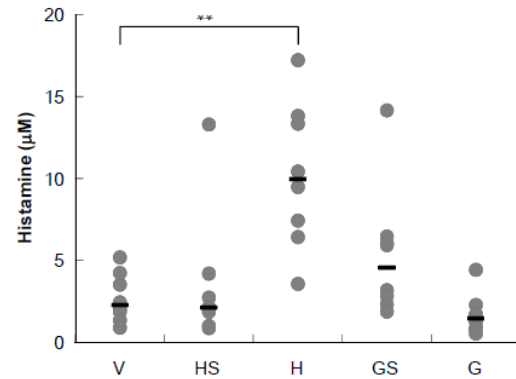
**Figure 2-1 Antigen-specific Antibodies Production 4 Weeks after Percutaneous Sensitization (Day 23) with Glupearl 19S and Gluten**

See Table 2 for antigens used to treat each group. Dots denote the data of individual animals; bars indicate the group means. \*\*p < 0.01

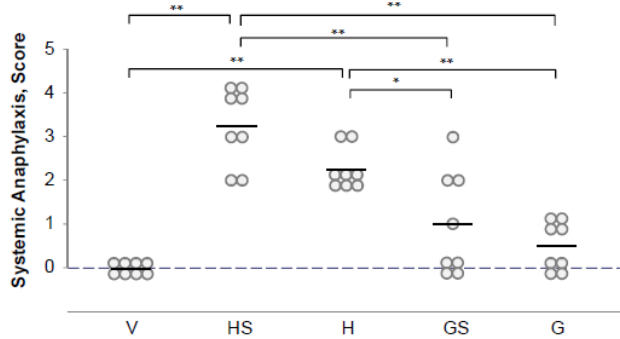
A. Post-challenge body temperature changes



B. Plasma histamine level 30 min. post-challenge



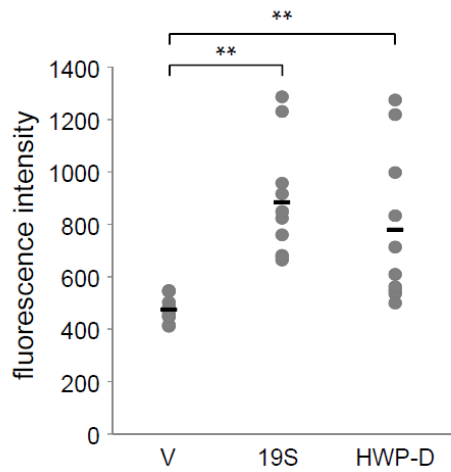
C. Score of signs of anaphylaxis



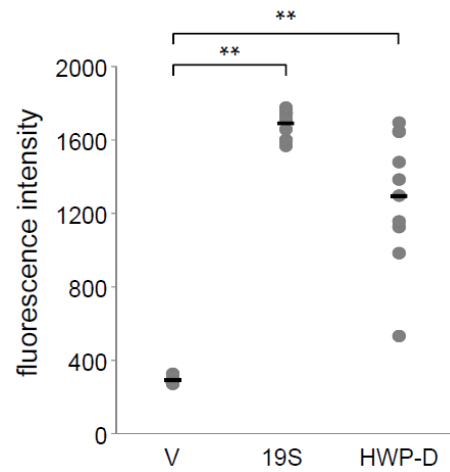
**Figure 2-2. Anaphylactic Responses in Mice Percutaneously Sensitized with Glupearl 19S and Gluten**

A: Body temperatures shown are the mean  $\pm$  SD for each group. B, C: Dots denote the data of individual animals; bars indicate the group means. \*p < 0.05, \*\*p < 0.01

A. Antigen-specific IgE



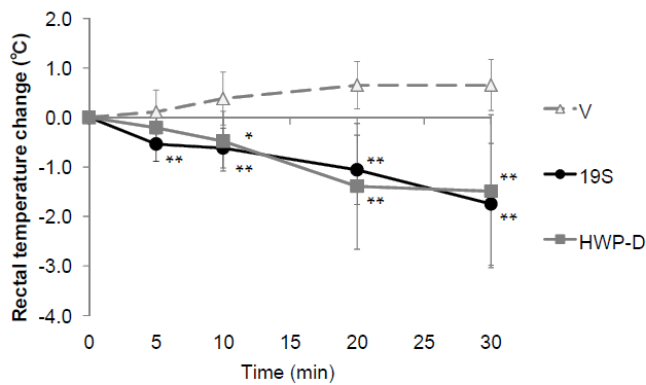
B. Antigen-specific IgG1



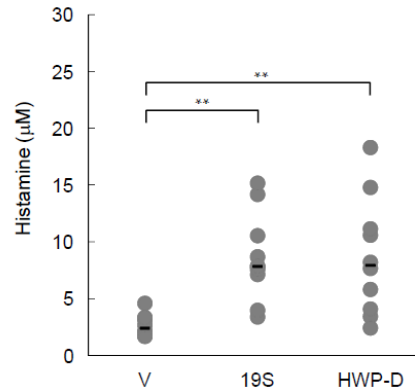
**Figure 3-1. Antigen-specific Antibodies Production 4 Weeks after Percutaneous Sensitization (Day 23) with Glupearl 19S and HWP-D**

See Table 2 for antigens used to treat each group. Dots denote the data of individual animals; bars indicate the group means. \*\*p < 0.01

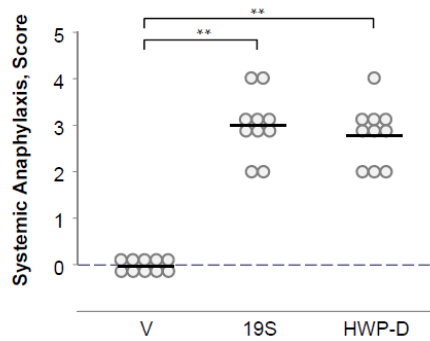
A. Post-challenge body temperature changes



B. Plasma histamine level 30 min. post-challenge

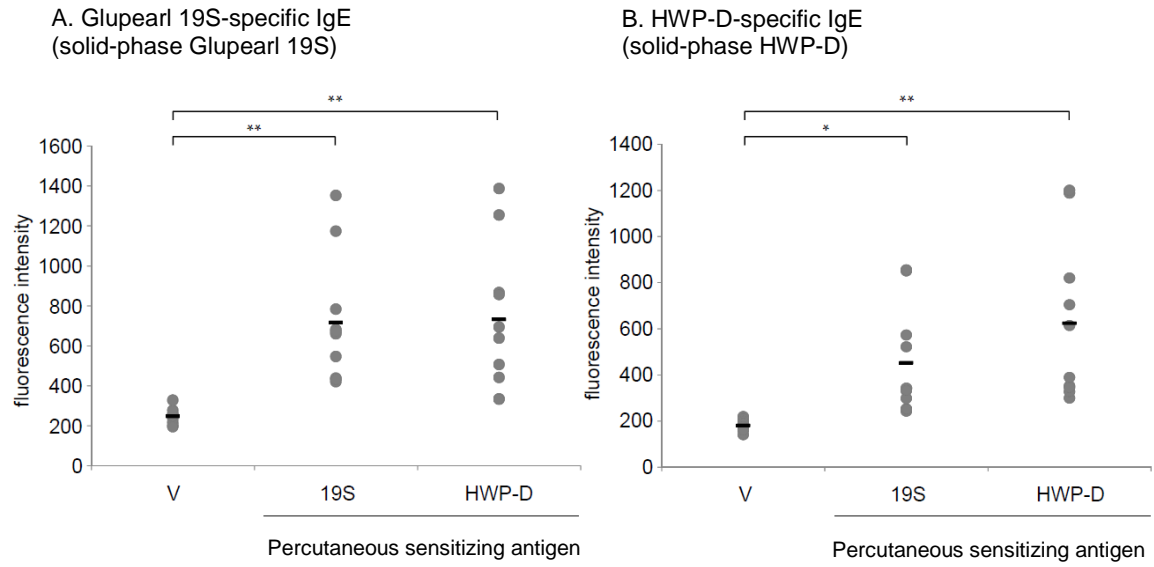


C. Score of signs of anaphylaxis



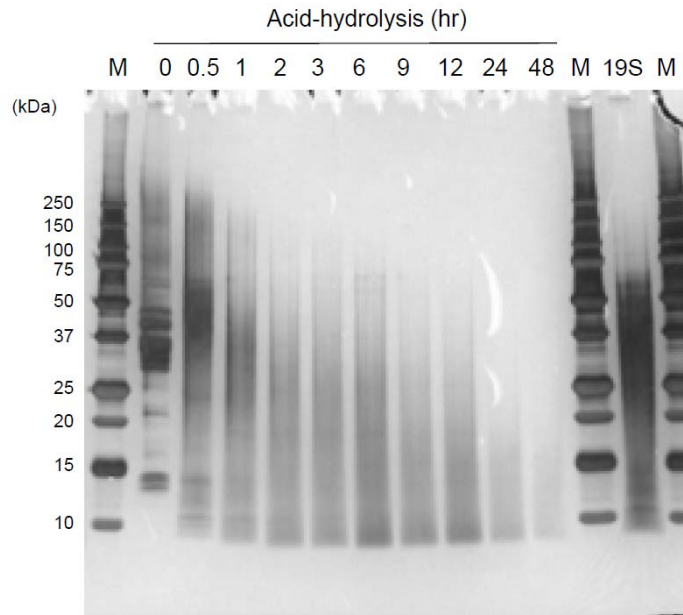
**Figure 3-2. Anaphylactic Responses in Mice Percutaneously Sensitized with Glupearl 19S and HWP-D**

A: Body temperatures shown are the mean  $\pm$  SD for each group. B, C: Dots denote the data of individual animals; bars indicate the group means. \*p < 0.05, \*\*p < 0.01

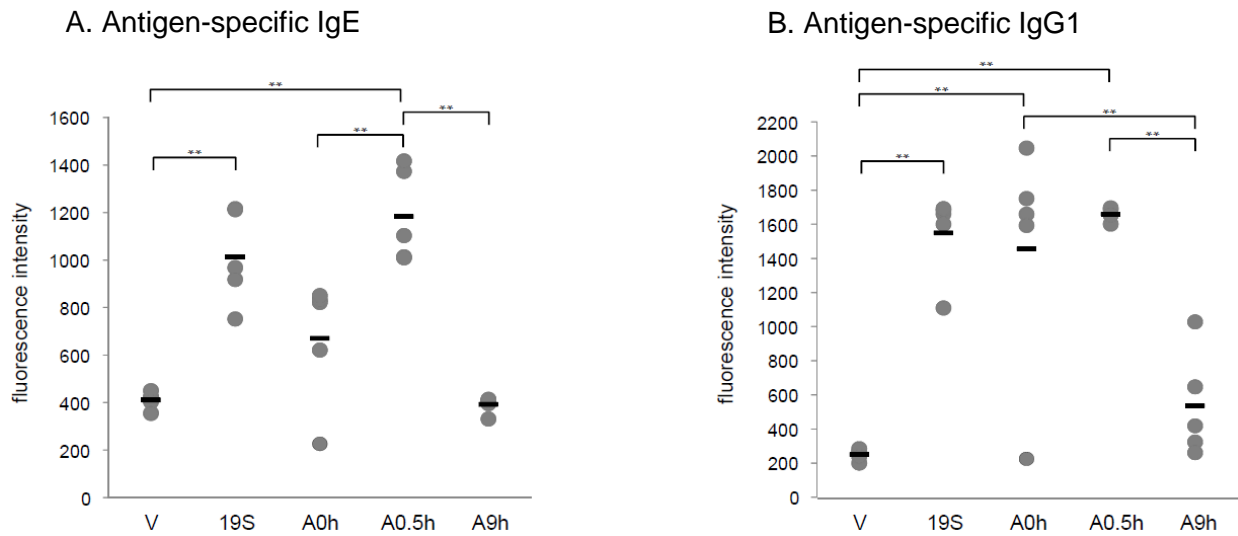


**Figure 3-3. Cross-reactivity between Glupearl 19S and HWP-D**

Dots denote the data of individual animal; bars indicate the group means. \*\*p < 0.01



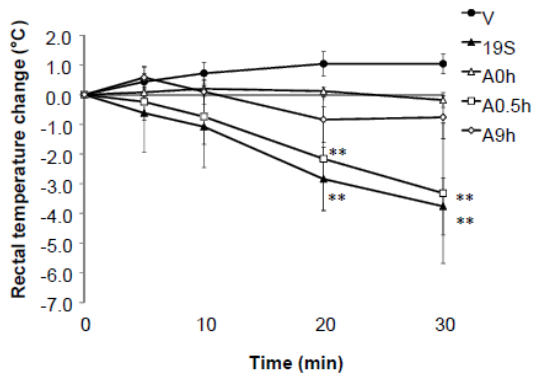
**Figure 4-1 SDS-PAGE of Acid-hydrolysates of Gluten**  
 Pattern of 10–20% polyacrylamide gel silver staining



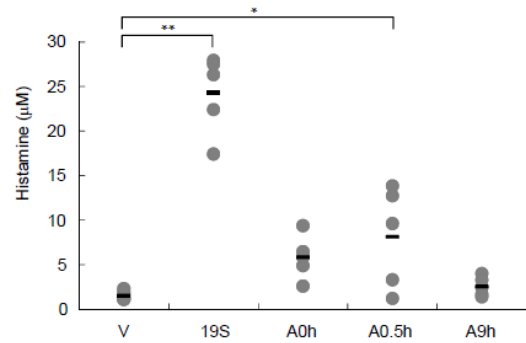
**Figure 4-2. Antigen-specific Antibodies Production 4 Weeks after Percutaneous Sensitization (Day 23) with Acid-hydrolysates of Gluten**

See Table 2 for antigens used to treat each group. Dots denote the data of individual animals; bars indicate the group means. \*\* $p < 0.01$

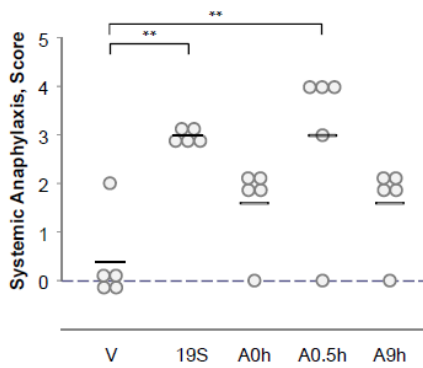
A. Post-challenge body temperature changes



B. Plasma histamine level 30 min. post-challenge



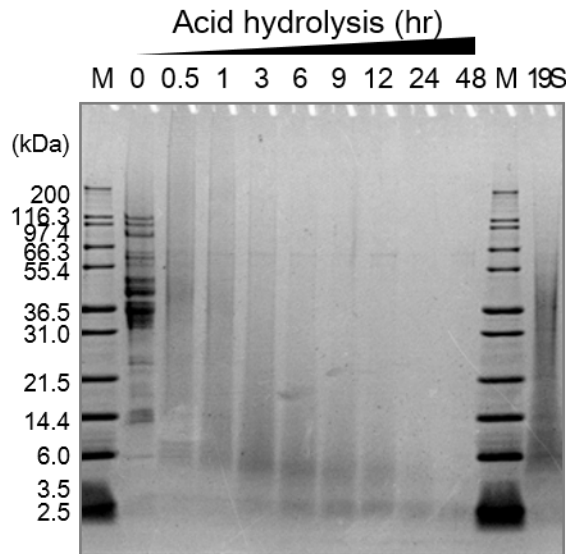
C. Score of signs of anaphylaxis



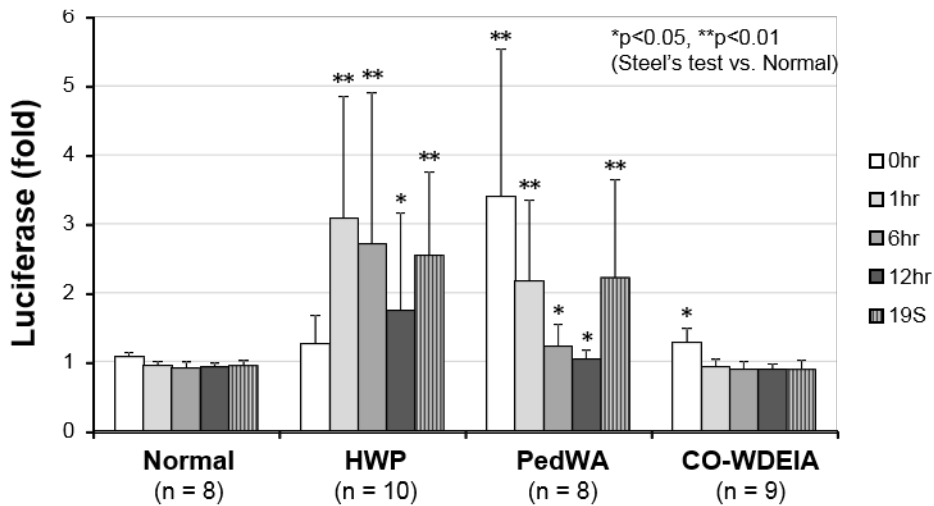
**Figure 4-3. Anaphylactic Responses in Mice Percutaneously Sensitized with Acid-hydrolysates of Gluten**

A: Body temperatures shown are the mean  $\pm$  SD for each group. B, C: Dots denote the data of individual animals; bars indicate the group means. \* $p < 0.05$ , \*\* $p < 0.01$

**A: Changes in molecular weights of acid-hydrolysates of gluten**

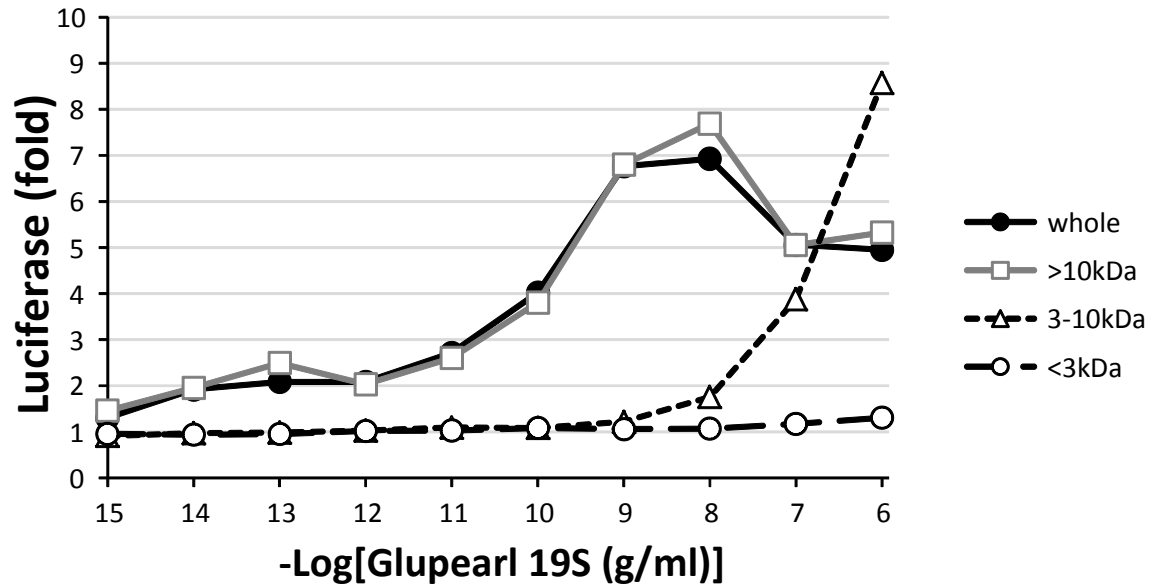


**B: Differences in response to HWP among groups of various wheat allergy patients**



**Fig 5-1. Differences in Response to HWP among Groups of Various Wheat Allergy Patients**

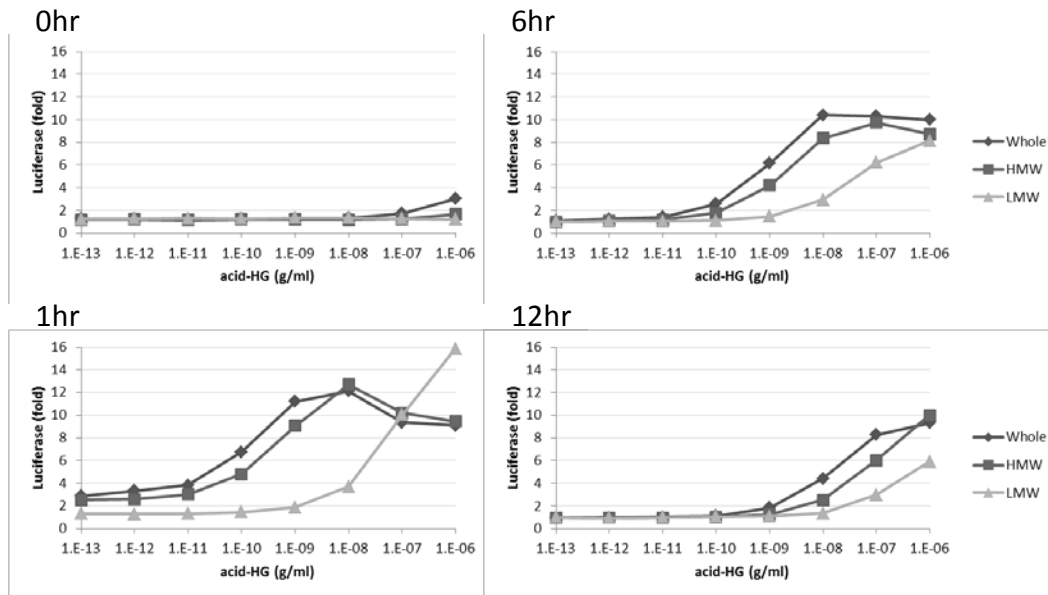
(A) CBB-stained SDS electrophoresis gel of wheat gluten hydrolysates prepared by heating at 100°C for 0, 1, 6, and 12 hours in the presence of 0.1 N HCl. (B) EXiLE test of ser samples from healthy subjects (normal), HWP patients, PedWA patients, and CO-WDEIA subjects. The antigen concentration was set at 100 ng/mL. The term "19S" denotes Glupearl 19S.



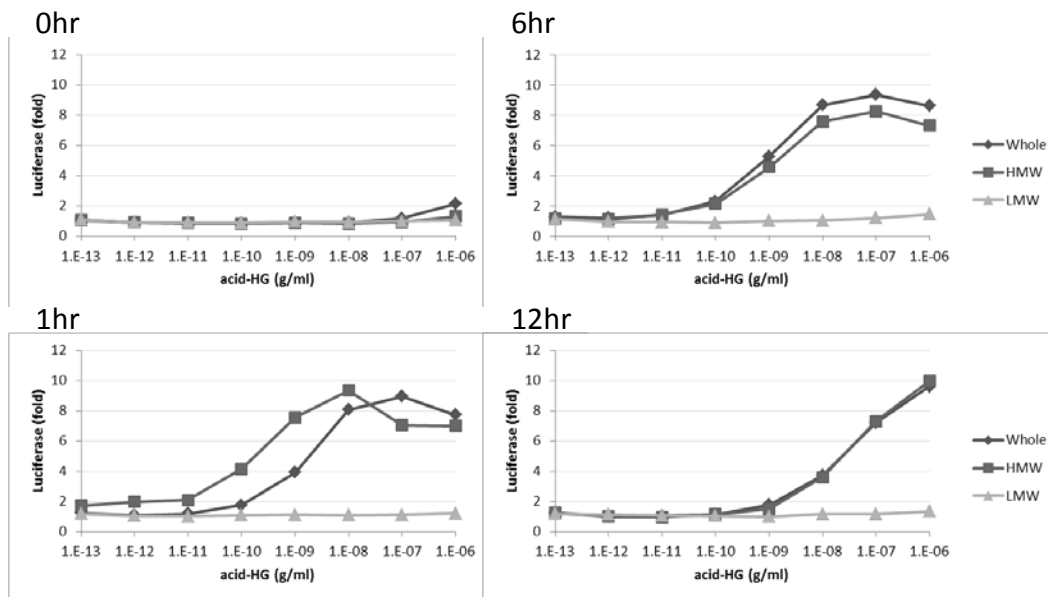
**Fig 5-2. Glupearl 19S fractions and IgE Responses**

Glupearl 19S was fractionated with 10- and 3-kDa cut-off ultrafiltration spin columns into 3 fractions: >10 kDa, 3–10 kDa, and <3 kDa. The responsiveness of RS-ATL8 cells sensitized with the serum from an HWP patient (42-year old female) was investigated. The concentrations shown are expressed in equivalent concentration of the pre-fractionated Glupearl 19S. The potential of the >10 kDa fraction to activate mast cells was practically the same as that of the pre-fractionated Glupearl 19S. The 3–10 kDa fraction also showed a weak activity. The <3 kDa fraction, on the other hand, elicited no response from the mast cells.

**A: EXiLE responses to fractions separated by 10-kDa cut-off ultrafiltration**



**B: EXiLE responses to fractions separated by 3-kDa cut-off ultrafiltration**



**Fig 5-3. Fractions of Acid-hydrolysates of Gluten and IgE Responses**

Gluten was suspended in 0.1 N HCl to a concentration of 1 mg/mL and heat at 100°C for 0, 1, 6, and 12 hours and fractionated with a 10-kDa (A) or a 3-kDa (B) cut-off ultrafiltration spin column. The pre-fractionated gluten is denoted as “whole,” the residual solution on top of the ultrafiltration filter as “HMW,” and the filtrate as “LMW.” The responsiveness of RS-ATL8 cells sensitized with the serum from an HWP patient (42-year old female) was investigated. The concentrations shown are expressed in equivalent concentration of the pre-fractionated gluten. As with Glupearl 19S, the ≤3 kDa fraction elicited no response.

**Table 1. アナフィラキシー症状のスコアリング**

Score 0	症状なし
1	口、耳、鼻、頭などを掻く、後ろ足で耳の穴を掻く
2	活動低下、呼吸が速くなる、1匹だけ離れている、眼・鼻・口の周囲の腫脹、立毛
3	1分以上動かない、うつぶせで横たわる、ゼーゼーと息を切らす、呼吸困難、口の周囲や尾のチアノーゼ、一過性の痙攣
4	ひげに触れても反応しない、刺激に対する反応の低下・無反応、意識消失、震え、痙攣
5	死亡

**Table 2. 感作抗原****実験1 (1群8匹 x 5群)**

群名	感作検体	感作抗原量	惹起方法	惹起検体
V	PBS + 0.5%SDS	-	i.p. (1 mg)	グルパール19S
HS	グルパール19S + 0.5%SDS	500 µg	i.p. (1 mg)	グルパール19S
H	グルパール19S	500 µg	i.p. (1 mg)	グルパール19S
GS	グルテン + 0.5%SDS	500 µg	i.p. (1 mg)	グルテン
G	グルテン	500 µg	i.p. (1 mg)	グルテン

**実験2 (1群10匹 x 3群)**

群名	感作検体	感作抗原量	惹起方法	惹起検体
V	PBS + 0.5%SDS	-	i.p. (1 mg)	グルパール19S
19S	グルパール19S + 0.5%SDS	500 µg	i.p. (1 mg)	グルパール19S
HWP-D	HWP-D + 0.5%SDS	500 µg	i.p. (1 mg)	HWP-D

**実験3 (1群5匹 x 5群)**

群名	感作検体	感作抗原量	惹起方法	惹起検体
V	PBS + 0.5%SDS	-	i.p. (1 mg)	グルパール19S
19S	グルパール19S + 0.5%SDS	500 µg	i.p. (1 mg)	グルパール19S
A0h	0h酸加水分解グルテン + 0.5%SDS	500 µg	i.p. (1 mg)	0h酸加水分解グルテン
A0.5h	0.5h酸加水分解グルテン + 0.5%SDS	500 µg	i.p. (1 mg)	0.5h酸加水分解グルテン
A9h	9h酸加水分解グルテン + 0.5%SDS	500 µg	i.p. (1 mg)	9h酸加水分解グルテン

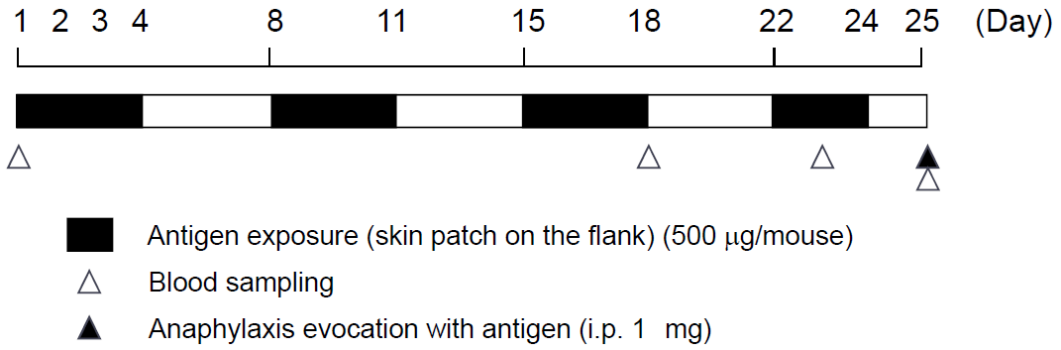
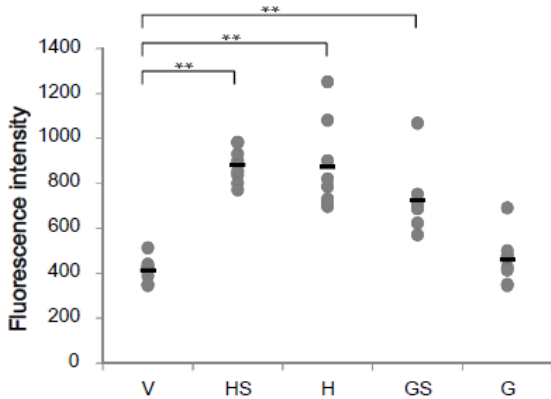
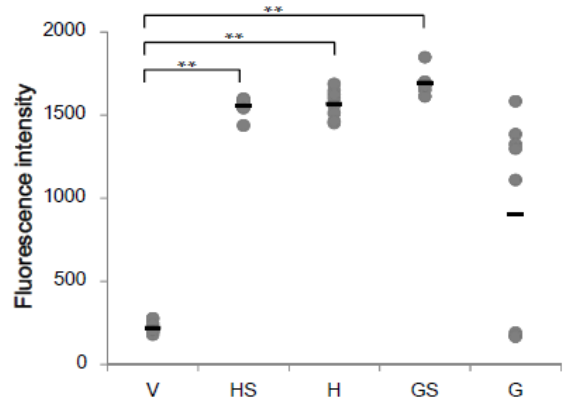


Figure 1. 経皮感作試験スケジュール

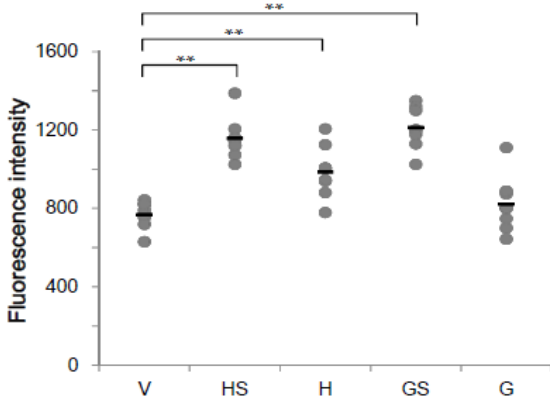
A. グルパール19S特異的IgE



B. グルパール19S特異的IgG1



C. グルテン特異的IgE



D. グルテン特異的IgG1

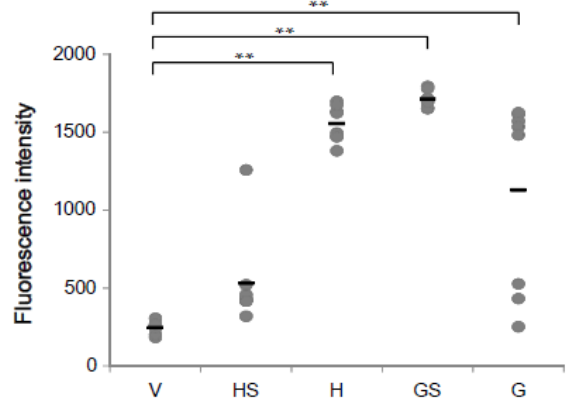
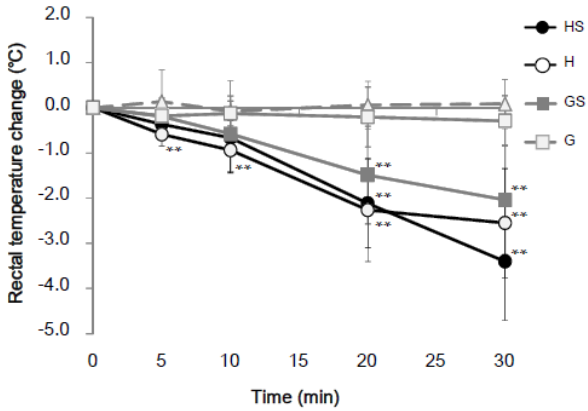
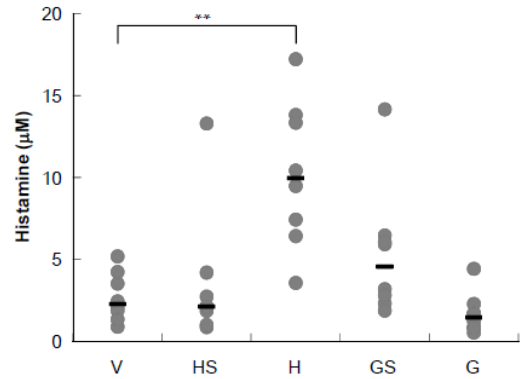


Figure 2-1 グルパール19Sおよびグルテン経皮感作4週後(Day 23)の抗原特異的抗体産生  
 各群の処理抗原についてはTable2に示す。ドットはマウス各個体のデータを、バーは各群の平均値を示す。  
 \*\*p<0.01

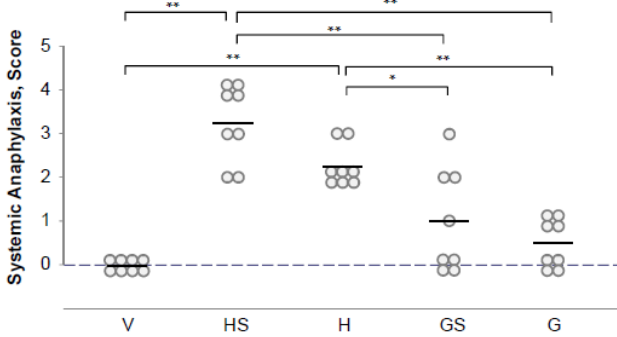
A. 惹起後の体温変動



B. 惹起30分後の血漿中ヒスタミン濃度



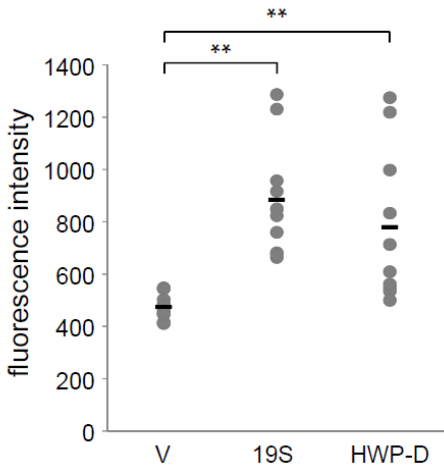
C. アナフィラキシー症状のスコアリング



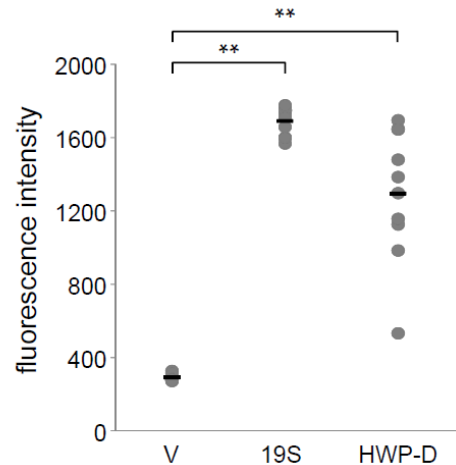
**Figure 2-2. グルパール19Sおよびグルテン経皮感作マウスのアナフィラキシー反応惹起**

A:各群の体温データをMean±S.D.で示す。B, C:ドットはマウス各個体のデータを、バーは各群の平均値を示す。\* $p < 0.05$ , \*\* $p < 0.01$

A. 抗原特異的IgE



B. 抗原特異的IgG1



**Figure 3-1 グルパール19S及びHWP-D経皮感作4週後(Day 23)の抗原特異的抗体産生**

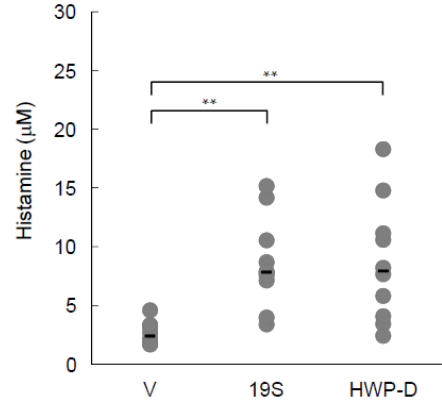
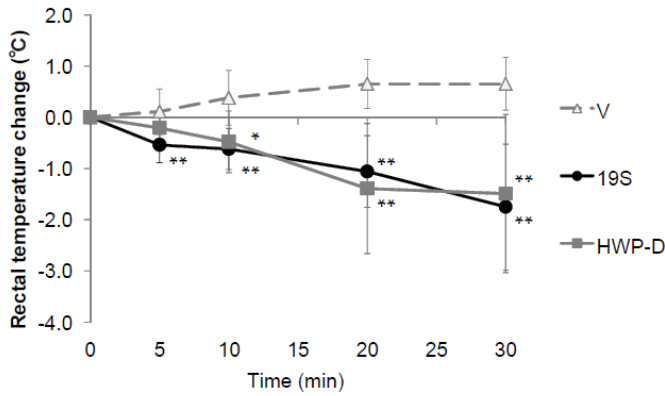
各群の処理抗原についてはTable2に示す。ドットはマウス各個体のデータを、バーは各群の平均値を示す。  
\*\* $p < 0.01$

### A. 惹起後の体温変動

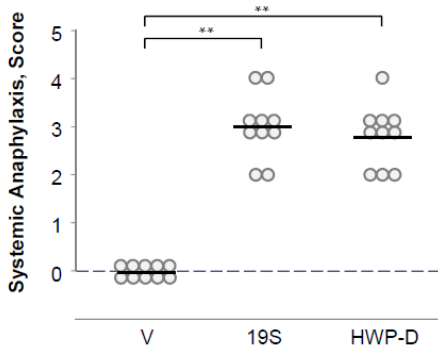
Distributed for comment only -- do not cite or quote

### B. 惹起30分後の血漿中ヒスタミン濃度

Distributed for Comment Only -- Do Not Cite or Quote



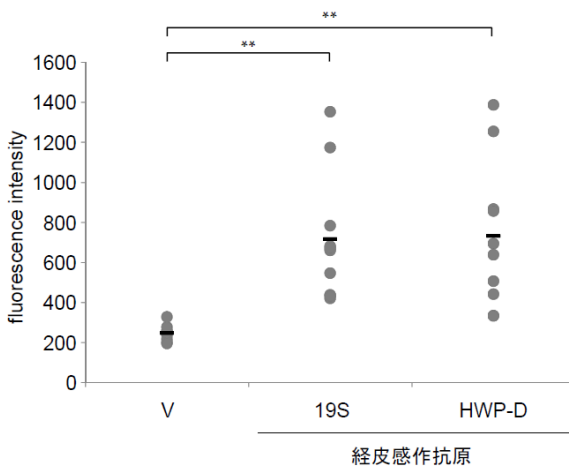
### C. アナフィラキシー症状のスコアリング



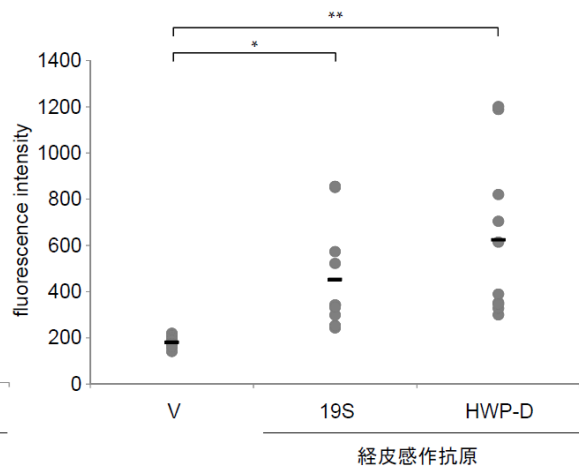
## Figure 3-2 グルパール19SおよびHWP-D経皮感作マウスのアナフィラキシー反応惹起

A:各群の体温データをMean±S.D.で示す。B, C:ドットはマウス各個体のデータを、バーは各群の平均値を示す。\* $p < 0.05$ , \*\* $p < 0.01$

### A. グルパール19S特異的IgE (グルパール19Sを固相化)

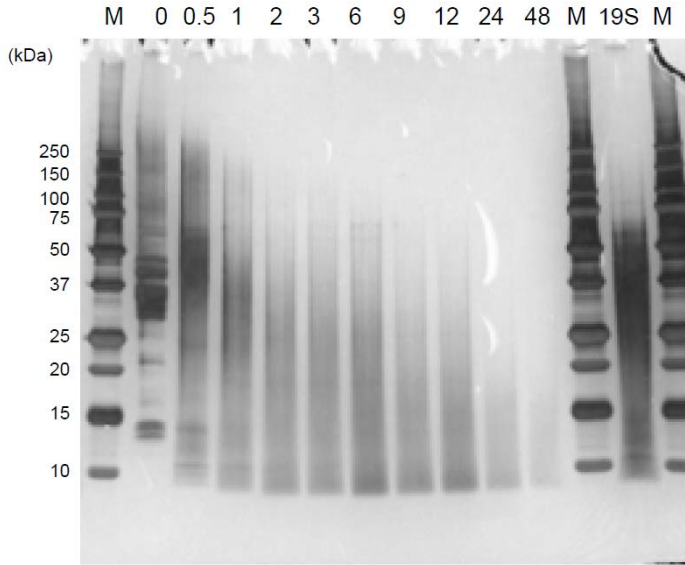


### B. HWP-D特異的IgE (HWP-Dを固相化)



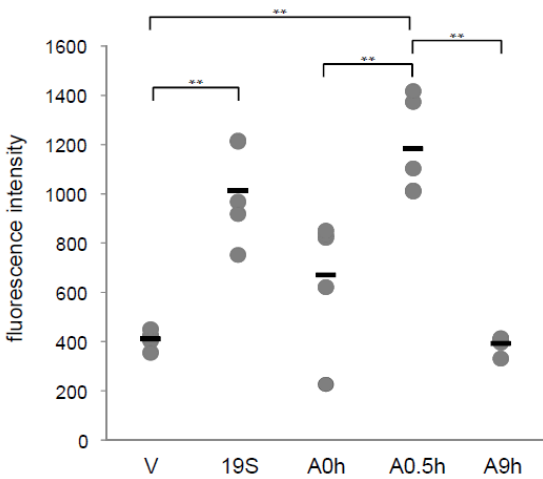
## Figure 3-3 グルパール19SおよびHWP-Dの交差性の検討

ドットはマウス各個体のデータを、バーは各群の平均値を示す。\*\* $p < 0.01$

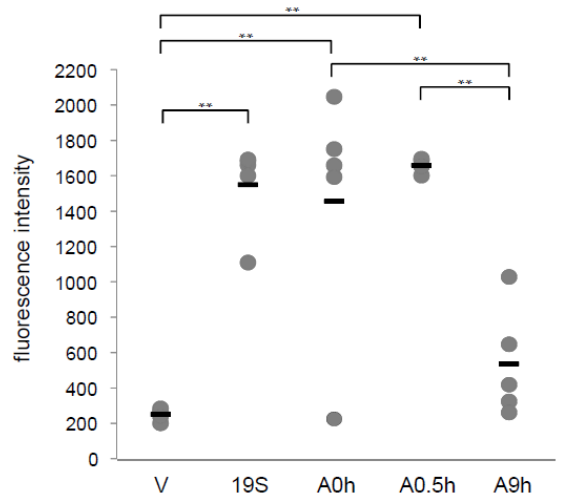


**Figure 4-1 グルテン酸加水分解物のSDS-PAGE**  
10-20%ポリアクリルアミドゲルの銀染色パターン

**A. 抗原特異的IgE**



**B. 抗原特異的IgG1**

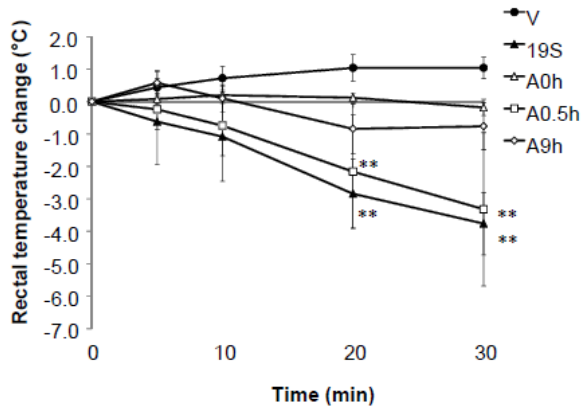


**Figure 4-2 グルテン酸加水分解物経皮感作4週後(Day 23)の抗原特異的抗体産生**

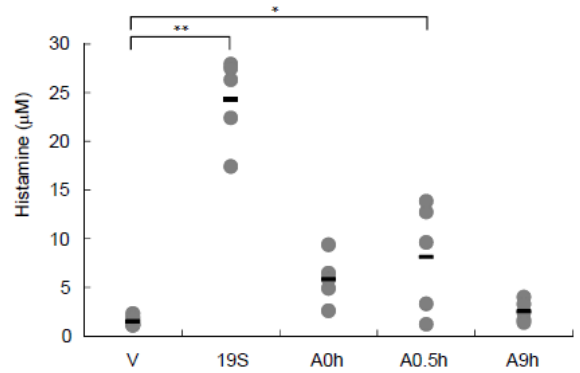
各群の処理抗原についてはTable2に示す。ドットはマウス各個体のデータを、バーは各群の平均値を示す。

\*\*p<0.01

A. 惹起後の体温変動



B. 惹起30分後の血漿中ヒスタミン濃度



C. アナフィラキシー症状のスコアリング

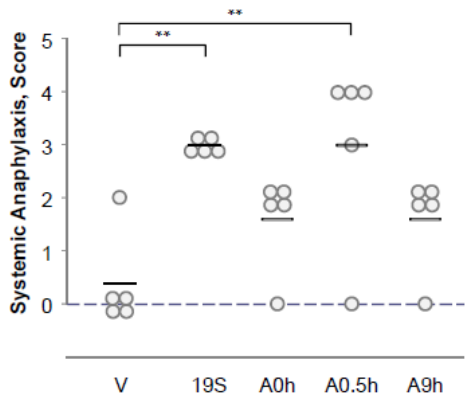
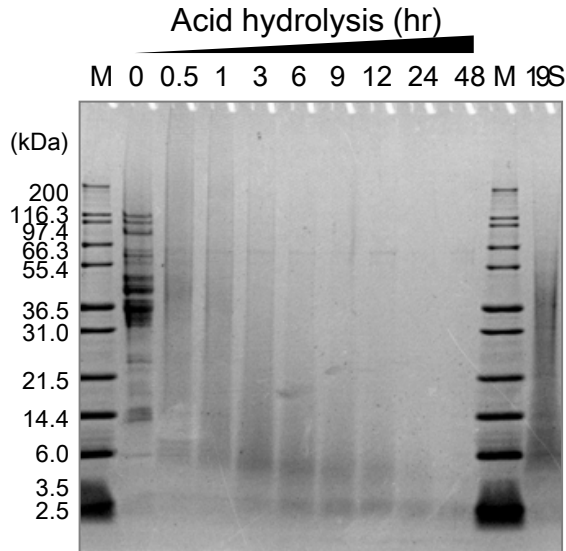


Figure 4-3 グルテン酸加水分解物経皮感作マウスのアナフィラキシー反応惹起

A:各群の体温データをMean±S.D.で示す。B, C:ドットはマウス各個体のデータを、バーは各群の平均値を示す。

\*p<0.05, \*\*p<0.01

## A: 酸加水分解グルテンの分子量変化



## B: 小麦アレルギー患者群によるHWPへの応答性の違い

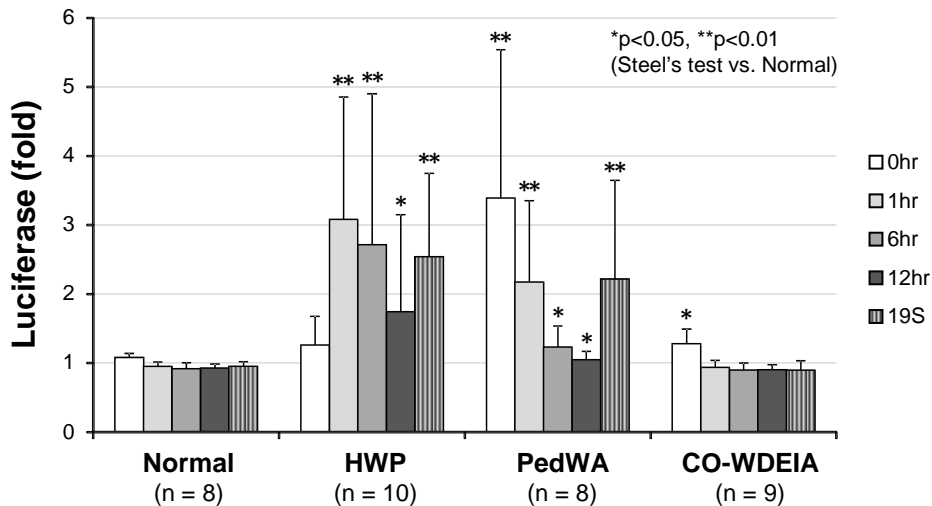


Fig 5-1. 小麦アレルギー患者群による酸加水分解HWPへの応答性の違い  
 (A) 小麦グルテンを0.1N HCl存在下で0、1、6、12時間100°Cに加熱して加水分解し、15-25% SDS電気泳動を行なった際のCBB染色像。(B) 健常人(Normal)、HWP患者(HWP)、小児小麦食物アレルギー患者(PedWA)、成人通常型小麦依存性運動誘発アナフィラキシー患者(CO-WDEIA)におけるEXILE試験を行なった。抗原濃度は100ng/mlに固定した。19Sはグルパール19Sを指す。

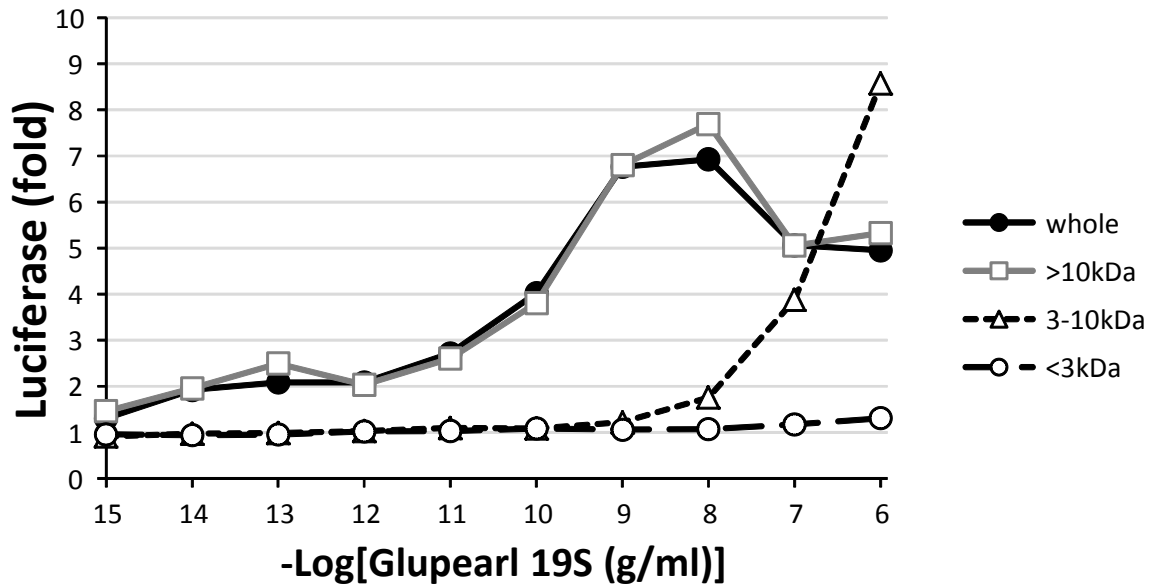
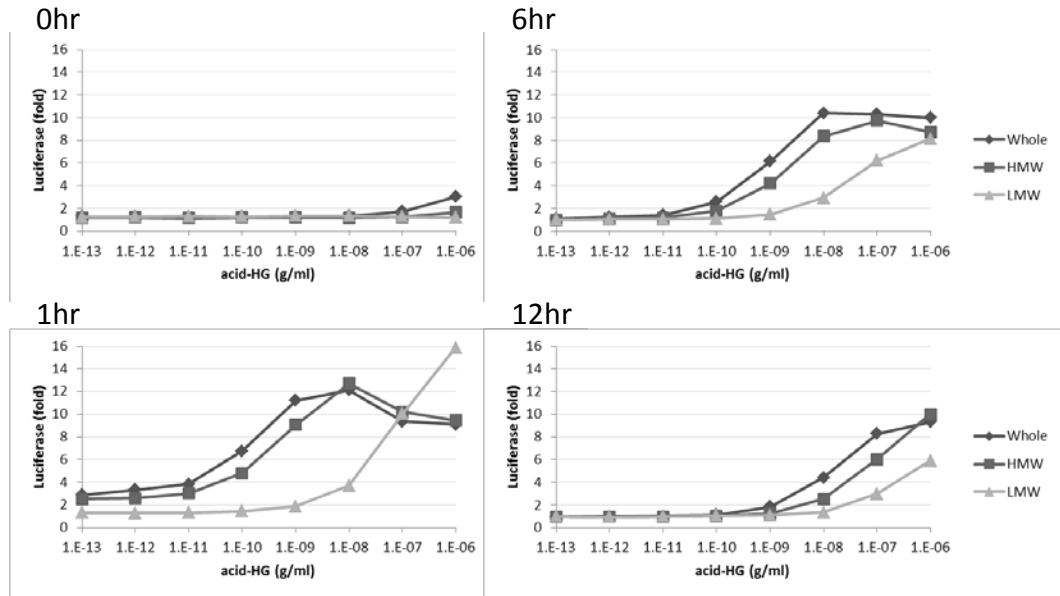


Fig. 5-2. グルパール19Sの分画とIgEの応答性

10kDaおよび3kDa分画分子量を持つ限外ろ過スピンカラムにより、グルパール19Sを>10kDa, 3-10kDa, <3kDaの3分画に分け、HWP患者(42例女性)血清で感作したRS-ATL8細胞の応答性を調べた。濃度は、分画前のグルパール19S相当量で表している。10kDa以上の画分には分画前とほぼ同等のマスト細胞活性化能があったが、3-10kDaの画分にも弱いながら活性があることが分かった。一方、3kDa以下の画分にはマスト細胞は応答しなかった。

## A: 分画分子量10kDaの限外ろ過の各画分へのEXiLE応答



## B: 分画分子量3kDaの限外ろ過の各画分へのEXiLE応答

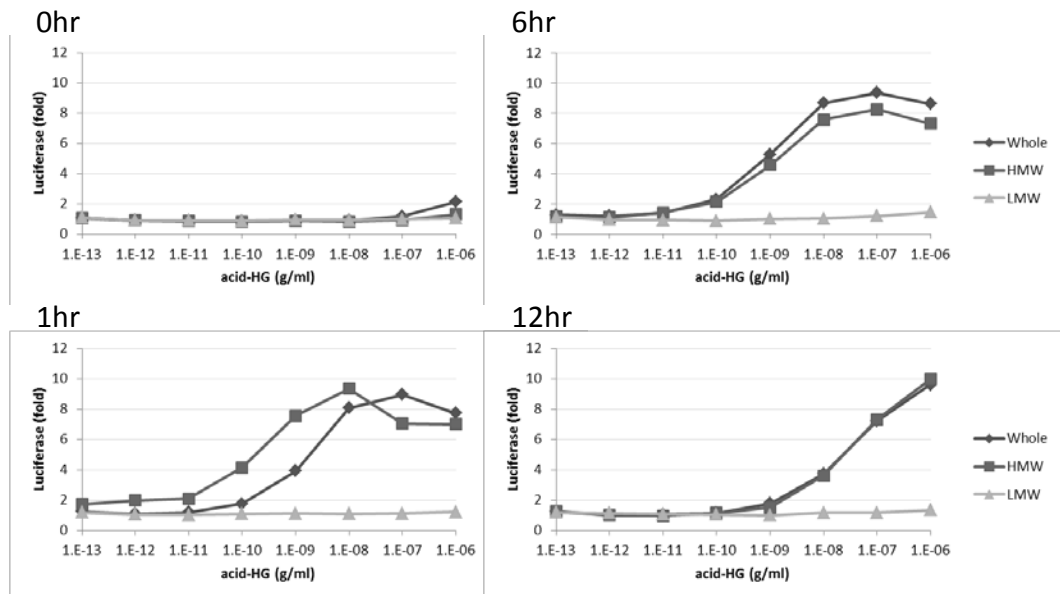


Fig. 5-3. 酸加水分解グルテンの分画とIgEの応答性

グルテンを0.1N HClに1mg/mlで懸濁し、100°Cで0, 1, 6, 12時間加熱したものを、(A) 10kDaまたは(B) 3kDa分画分子量を持つ限外ろ過スピナラムにより分画した。ここでは、分画前をwhole、限外ろ過膜上段に残った溶液をHMW、ろ液をLMWと記す。HWP患者(42歳女性)血清で感作したRS-ATL8細胞の応答性を調べた。濃度は、分画前のグルテン相当量で表している。グルパール19Sの場合と同様に、3kDa以下の画分には応答性が認められなかった。

Sakai S, Adachi R, Nakamura R, et al. 2014. Molecular profile analysis of allergenic acid hydrolyzed wheat protein. Poster presentation sponsored by National Institute of Health Sciences (Tokyo, Japan), National Institute of Technology and Evaluation (Tokyo, Japan), and Sagamihara National Hospital (Sagamihara, Japan).

# CRODA EUROPE LTD

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

# 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)

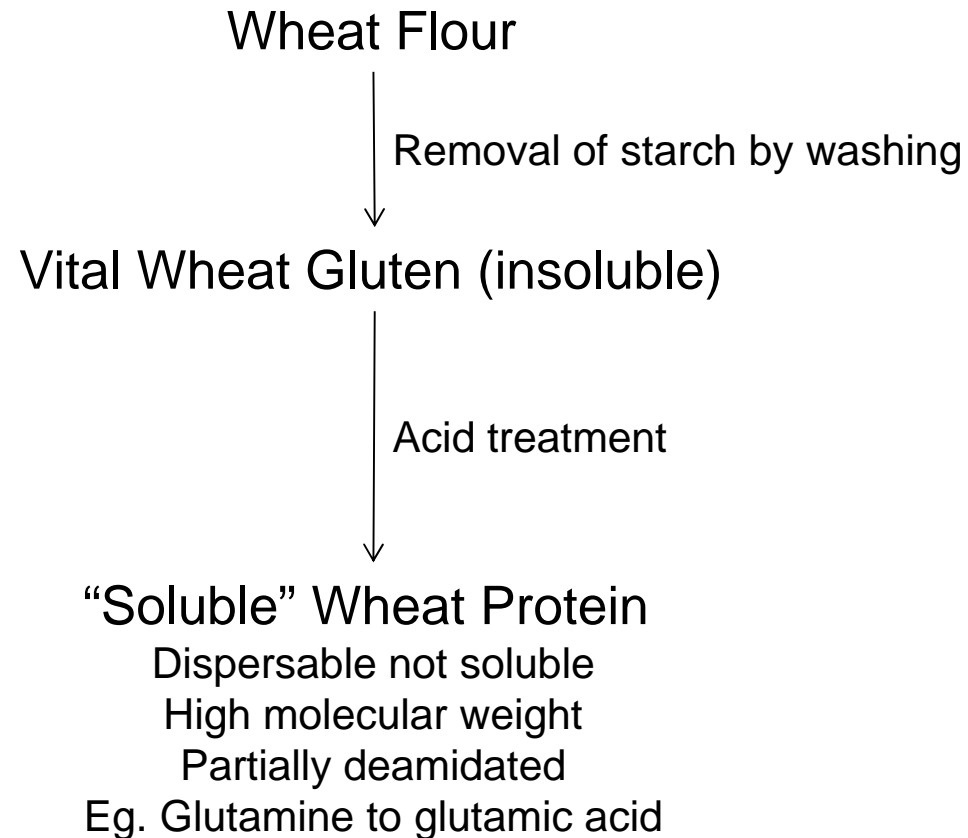
# Hydrolysed Protein Derivatives

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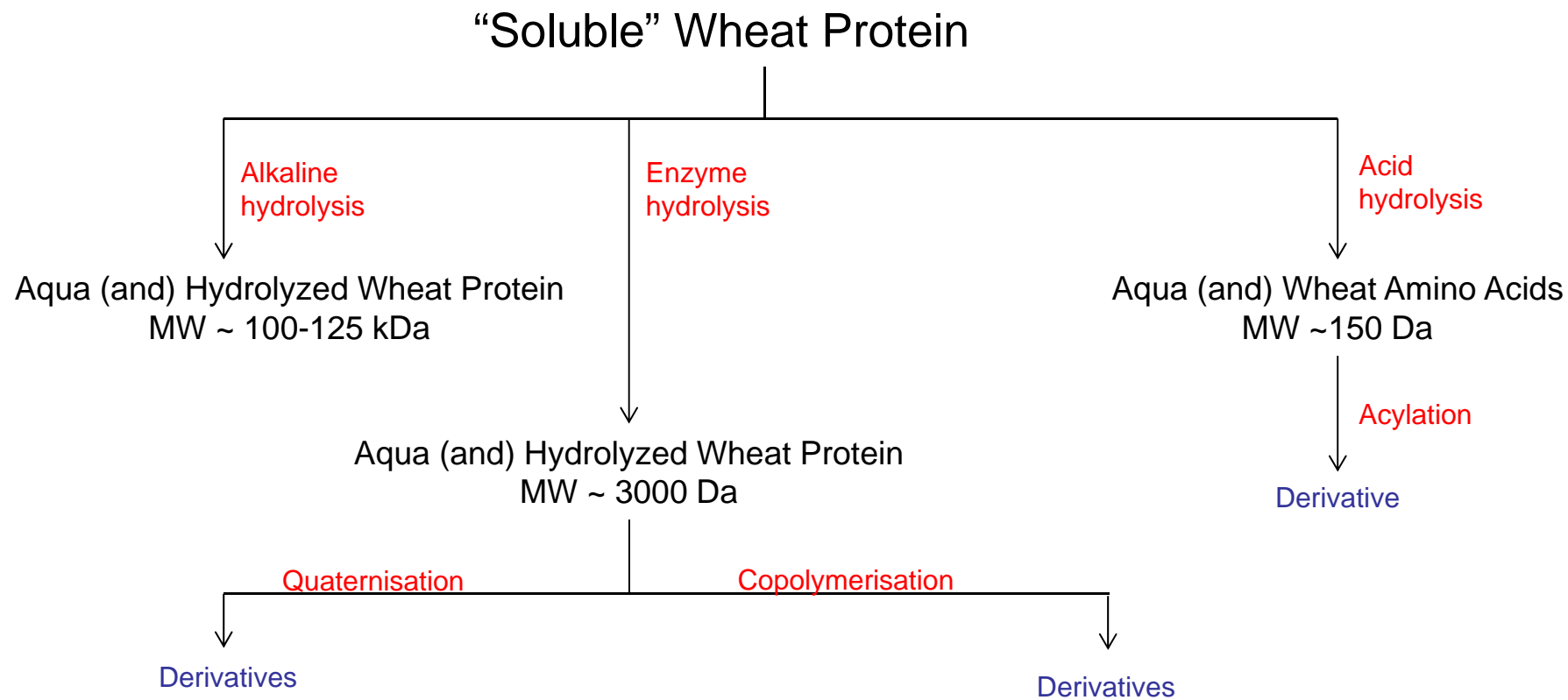
- What are they?
- These are protein products that have been hydrolysed and then modified in some way to alter their functionality
- **Quaternised proteins**
- **Acylated Proteins**
- **Co-polymers**

# 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

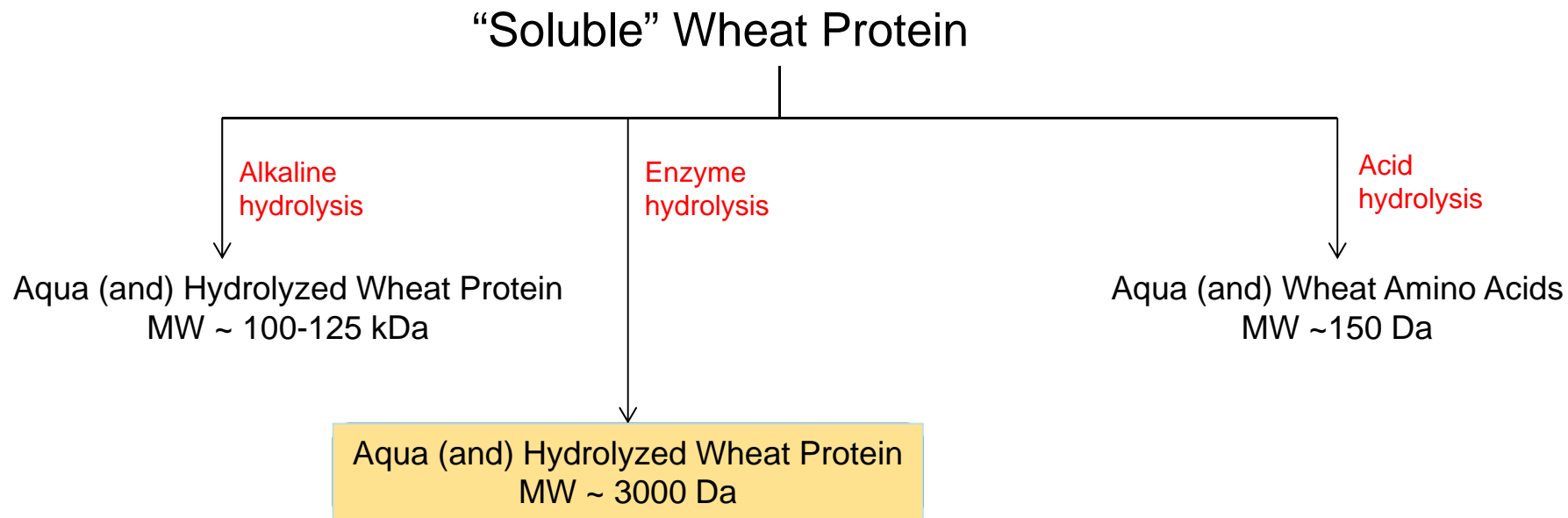
# Measurement of molecular weight (Mw)

---

- **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.

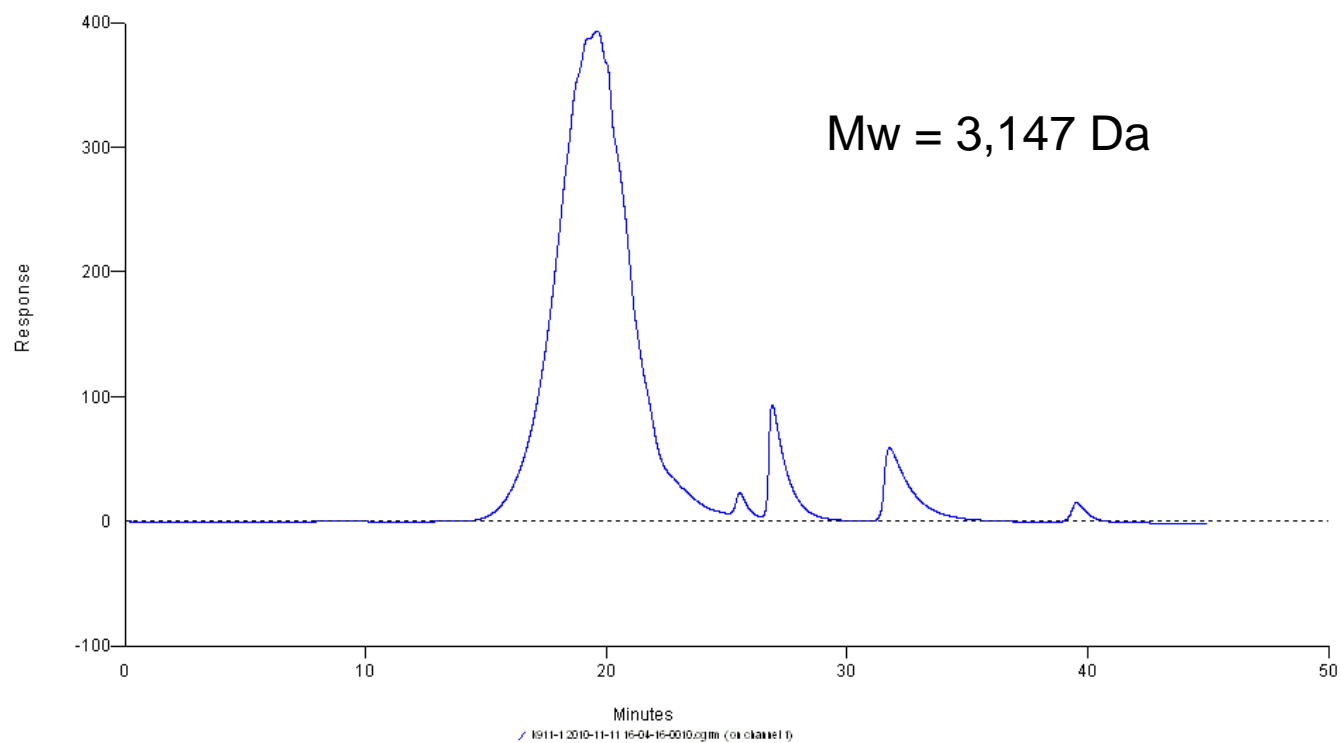
# Measurement of molecular weight (Mw)

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



# Measurement of molecular weight (Mw)

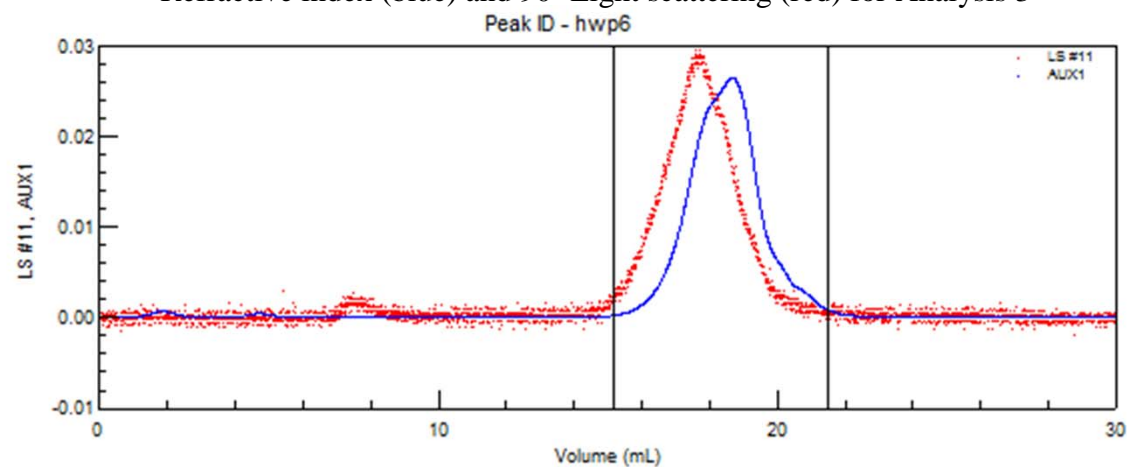
- SEHPLC/GPC



# Measurement of molecular weight (Mw)

## ■ GPC/MALLS

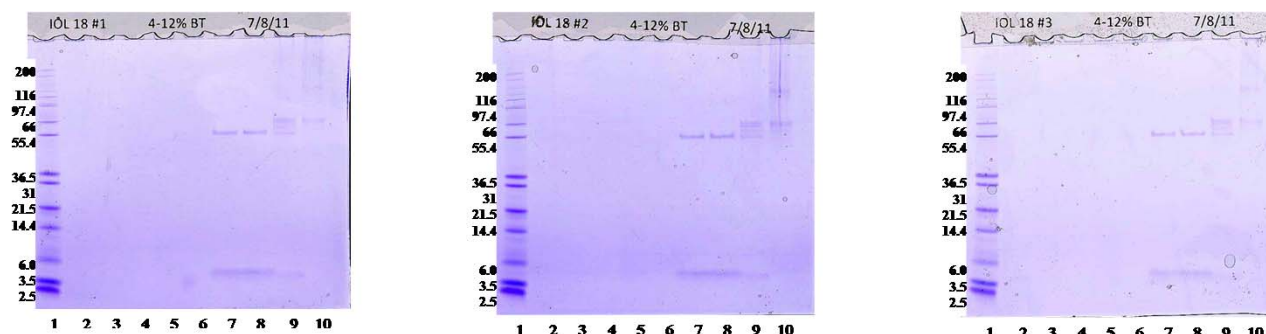
Refractive index (blue) and 90° Light scattering (red) for Analysis 3



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

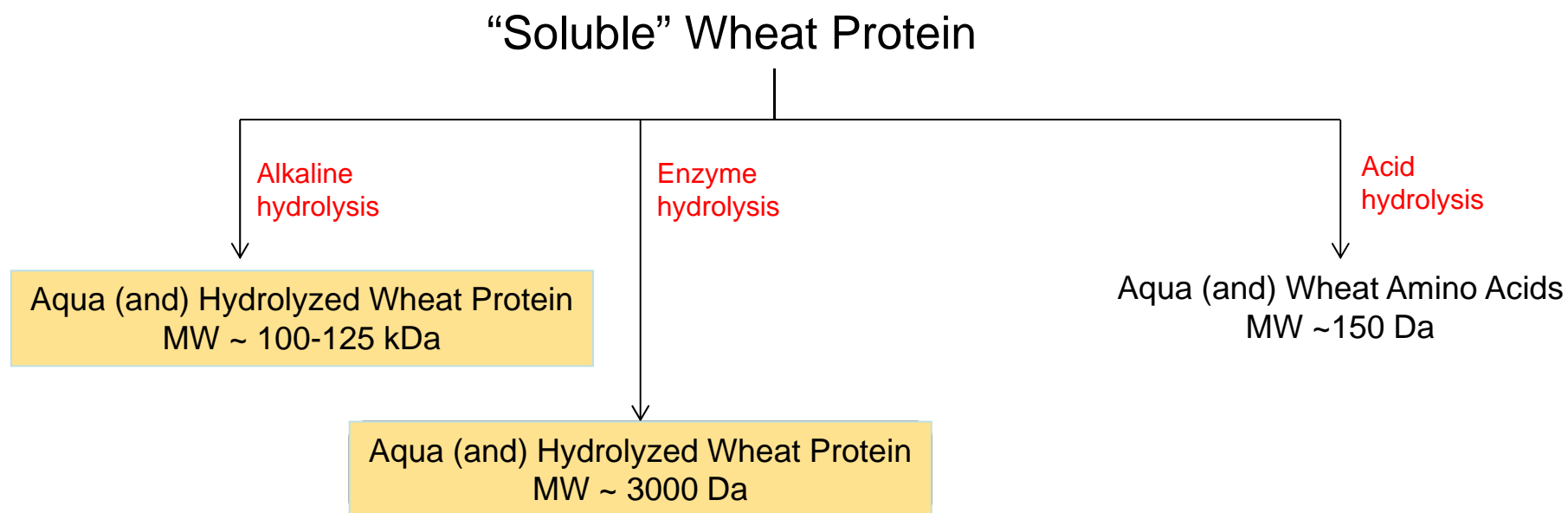


Sample	Use Conc.	Intact Protein Detected >2.0 kDa?
Aqua (and) Hydrolyzed Wheat Protein.	0.025%	No
Aqua (and) Hydrolyzed Wheat Protein.	0.025%	No
Aqua (and) Hydrolyzed Wheat Protein.	0.025%	No

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

---

- 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

---

- 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

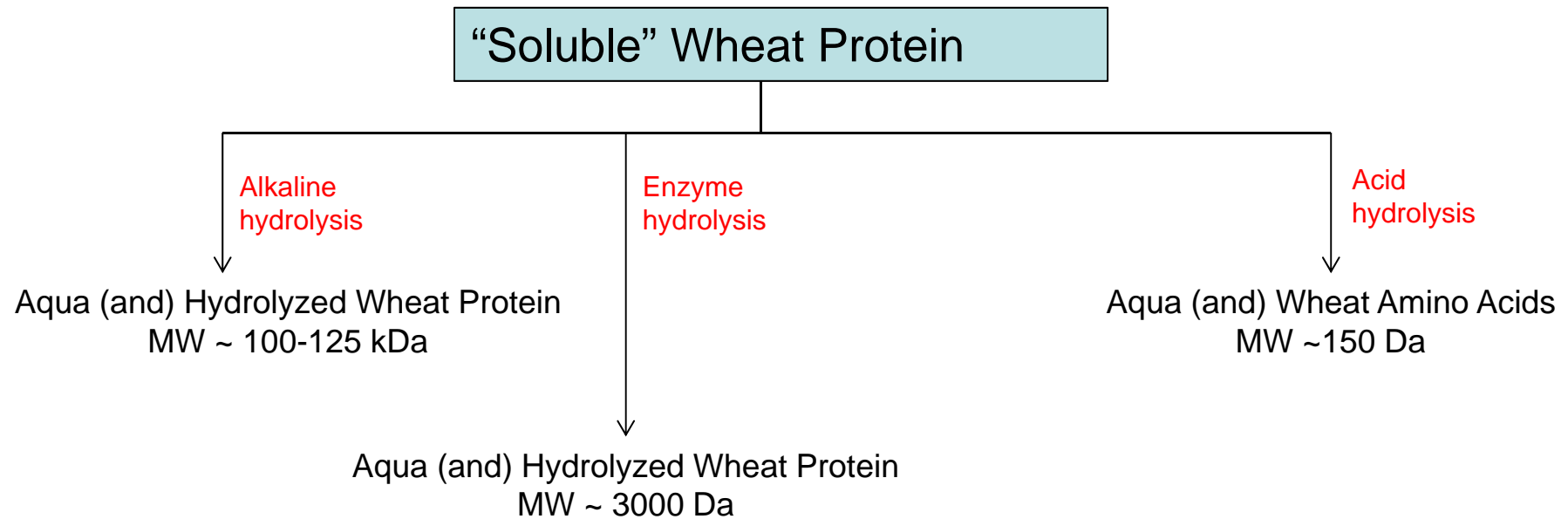
Products	Patients					
	1	2	3	4	5	6
Positive Control	Positive	Positive	Positive	Positive	Positive	Positive
Negative Control	Negative	Negative	Negative	Negative	Negative	Negative
Aqua (and) Hydrolyzed Wheat Protein. Mw ~ 3000 Da	Negative	Negative	Negative	Negative	Negative	Negative
Aqua (and) Hydrolyzed 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  
 Negative  
 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

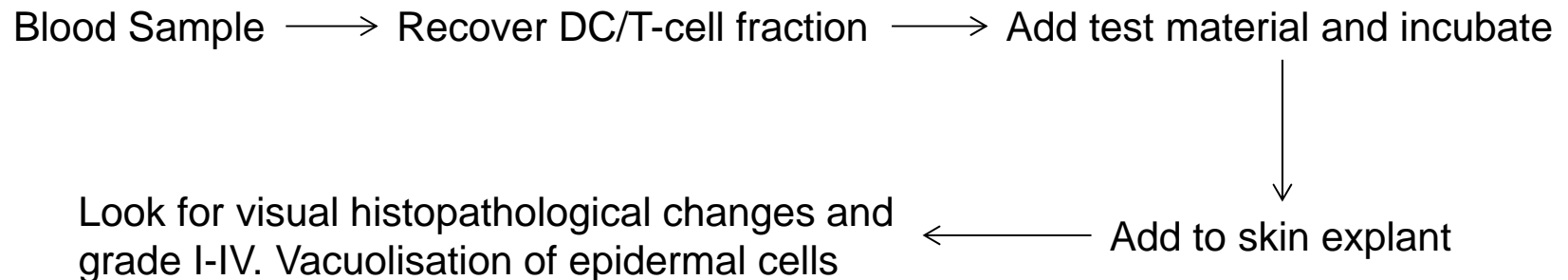
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- Hydrolysis of “soluble” wheat protein to Aqua (and) Hydrolyzed Wheat Protein – Mw 3000 Da removes potential for allergic response.
- Derivatives also negative.
- In these studies, Aqua (and) Hydrolyzed Wheat Protein – Mw 100 kDa and 125 kDa gave 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.

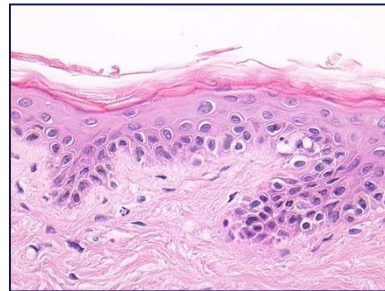


If the protein is antigenic it will activate an immune response (T-cells) which in turn cause the skin damage

# Skin damage - grading

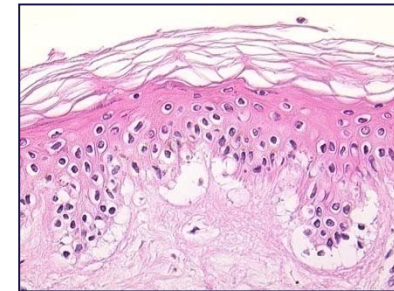
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**Grade I** skin damage showing very mild vacuolisation of epidermal cells



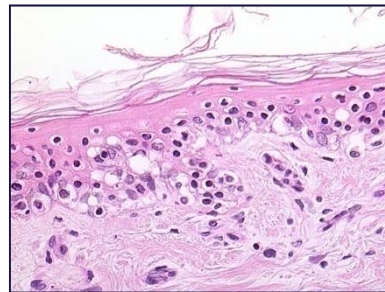
**Grade I**

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



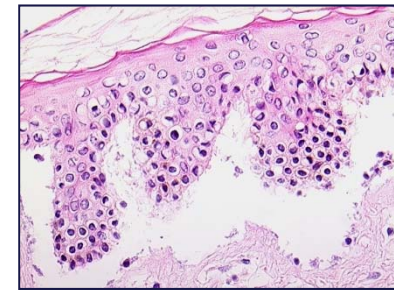
**Grade III**

**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:

Culture condition	Response grades	
	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

---

- 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 HWVP'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 HWP'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

---

The information in this publication is believed to be accurate and is given in good faith but no representation or warranty, express or implied, as to its completeness or accuracy is made. The test results described herein, some of which were obtained by third parties, are given in good faith and are believed to be accurate and reproducible but no representation or warranty, express or implied, is made with respect to its completeness or accuracy.

# Technical Report

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## Molecular Weight Analysis of Hydrolysed Wheat Protein

Date: 30/04/2014

Request: Internal

Author: Neil James

### **Summary**

Samples of Croda's enzyme-hydrolysed wheat protein product have been analysed externally for molecular weight using GPC MALLS. Weight-average and number-average molecular weights have been determined, as well as cumulative weight fractions for the samples to show the percentages above and below specific molecular weight cut-offs.

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## 1 INTRODUCTION

Samples of Croda's enzyme-hydrolysed wheat protein product (INCI – Aqua (and) Hydrolyzed Wheat Protein) were sent for external GPC MALLS analysis to determine molecular weight and the cumulative weight fraction at specific molecular weight cut-offs.

## 2 METHOD

Analysis was carried out by the Centre for Water Soluble Polymers at Glyndwr University. Two samples of enzyme-hydrolysed wheat protein were supplied by Croda (batch numbers 851376 and 866846). GPC MALLS, using refractive index to determine concentration, was used to analyse the samples. Each sample was run in duplicate.

## 3 ANALYSIS

The results of the analysis are summarised in Tables 1 and 2. Table 1 shows the weight-average molecular weight values (Mw), the number-average molecular weight values (Mn) and the polydispersity values. Mw and Mn values are stated in Daltons (Da).

Sample		Run 1	Run 2
851376	Mn	925.9	825.6
	Mw	1262	1320
	Polydispersity (Mw/Mn)	1.364	1.599
866846	Mn	1390	1042
	Mw	1643	1321
	Polydispersity (Mw/Mn)	1.182	1.267

**Table 1: Molecular weight values**

Table 2 shows the cumulative weight fraction results for the two samples. An average of the four results at each molecular weight cut-off has been taken to determine the percentage above the specified cut-off.

Sample	Cumulative Weight Fraction					
	851376		866846		Average	% Fraction above Molar Mass
	Run 1	Run 2	Run 1	Run 2		
Molar Mass						
1500	0.7220	0.7082	0.5190	0.7000	0.6623	33.77
2000	0.8516	0.8189	0.7457	0.8525	0.8172	18.28
2500	0.9215	0.8868	0.8803	0.9280	0.9042	9.59
3000	0.9570	0.9277	0.9456	0.9639	0.9486	5.15
3500	0.9760	0.9519	0.9751	0.9815	0.9711	2.89
4000	0.9866	0.9672	0.9889	0.9903	0.9833	1.68
4500	0.9926	0.9774	0.9951	0.9950	0.9900	1.00

**Table 2: Cumulative weight fraction results**

It should be noted that low molecular weights such as those seen for these samples are at the extreme of sensitivity for GPC MALLS.

#### 4 CONCLUSIONS

The hydrolysed wheat protein supplied for this analysis (Aqua and Hydrolyzed Wheat Protein) was exactly the same product on which Croda has developed immunogenicity data (as shared with the CIR Expert Panel). The product in every test was demonstrated not to give rise to an antigenic or immunogenic response. The CIR Expert Panel agreed to a weight average MW cut-off of 3500 Daltons but requested data on how much material would actually exceed the cut-off value from a normal distribution. The results above, in table 2, indicate that there would be 2.89% above the 3500 cut-off and 97.11% below 3500.

It must be pointed out that there will be some variation at these low molecular weights, as the sensitivity of testing is near its limit. We used the most appropriate method to generate this data. From Croda's immunogenicity testing, these small levels above 3500 do not cause any allergenic issues.

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# Investigation of Hydrolyzed Wheat Protein Solution Using Gel Permeation Chromatography

April 2014 – M. Evans

## **Aims and Objectives**

Samples of hydrolyzed wheat protein solution were received for investigation. A GPC/MALLS system was used to determine molecular mass distribution.

## **Equipment - GPC system**

The GPC system used comprised of the following component parts:

Inline eluent degasser

Pump

Rheodyne valve fitted with 200 µl manual injection loop.

Dawn DSP Laser Photometer (multi-angle laser light scattering detector).

Optilab DSP interferometric refractometer (refractive index detector)

Column: GE Healthcare Superose 12 GL 10/300 (Mw Range 1,000-300,000)

Eluent: 100 mM Sodium Acetate/Acetic Acid Buffer pH 4.5 plus 50mM NaCl

Flow rate: 0.5 ml/min (filtered by vacuum through a 0.22 µm GS membrane before use)

Sample solutions were filtered through a 0.45 µm PTFE syringe filter upon injection.

The analysis was carried out at normal laboratory temperature.

Analysis software – Astra 4.90.07

## Procedure - Gel Permeation Chromatography

0.4g of sample was made up to 12g with eluent. The dilute sample was filtered using a 0.45 micron syringe filter and 200 $\mu$ l injected onto the GPC column by means of a Rheodyne injection valve and loop. Samples were run in duplicate.

### GPC Results

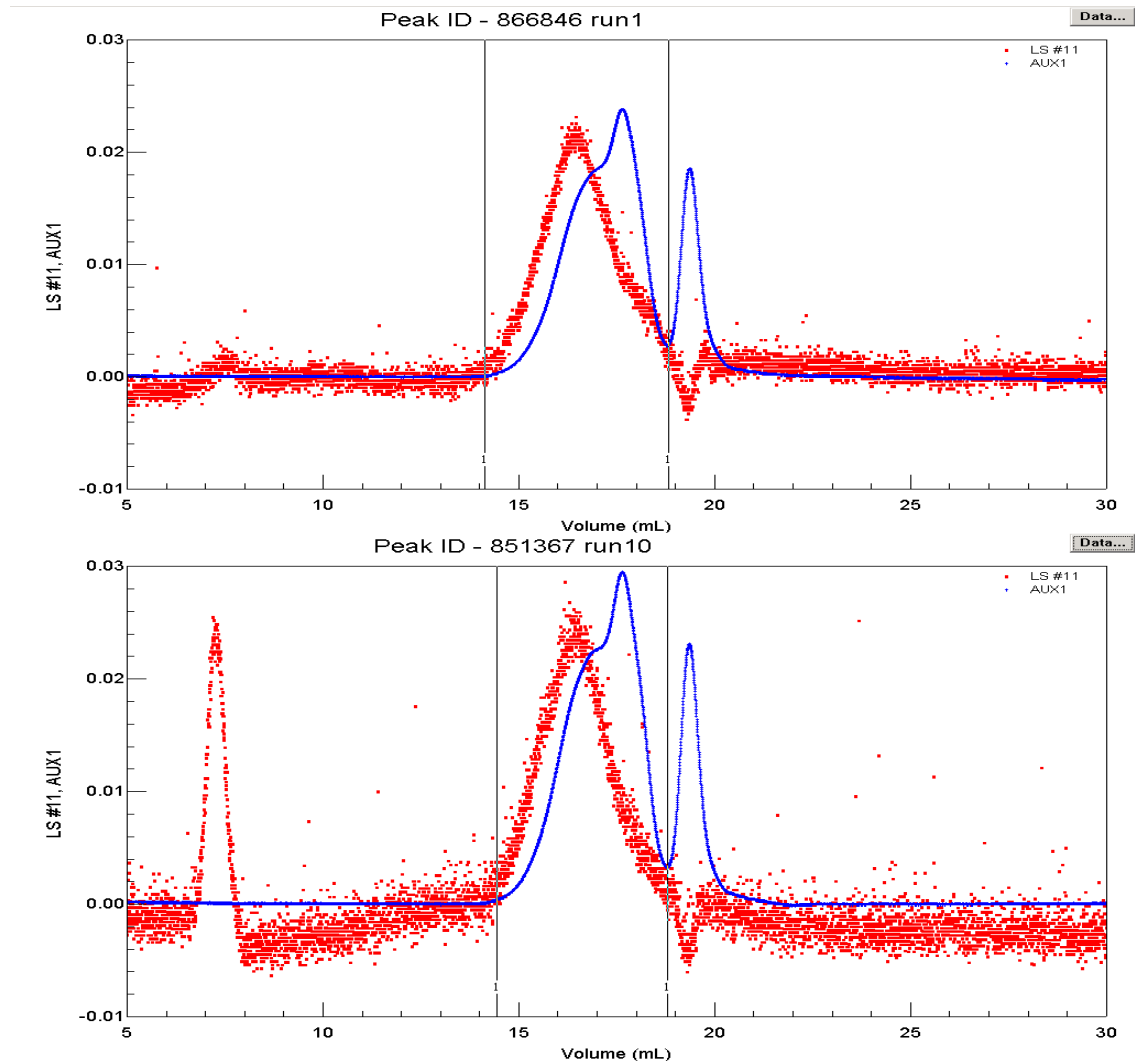
The analysis is complicated due to the presence of other compounds that influence refractive index in the sample matrix and an unknown initial sample concentration.

The dn/dc value of the sample was undetermined and so a 100% mass recovery calculation was used. The injected mass was calculated assuming 20% protein content concentration by mass. A Zimm plot was used for analysis.

The weight average (Mw) and number average (Mn) are reported in Table 1 and the elution profiles are shown in figure 1.

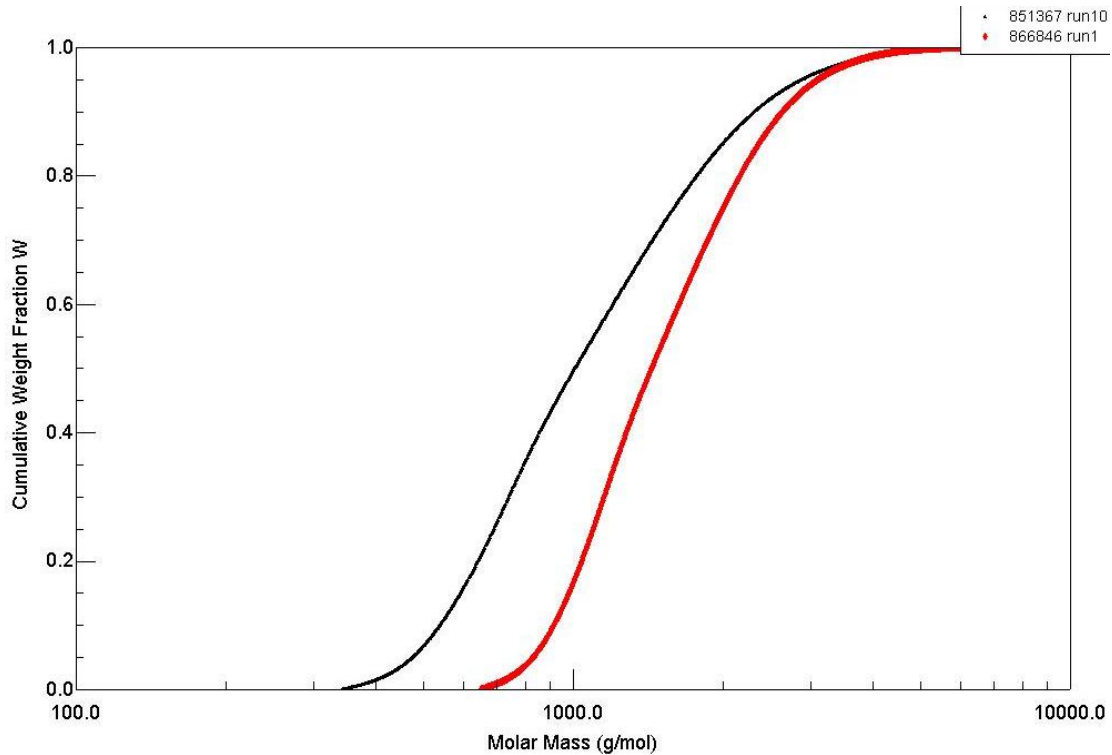
**Table 1:** Molecular weight distribution data - reported error shown in brackets

Sample	Run 1 20%	Polydispersity (Mw/Mn)	Run 2 20%	Polydispersity (Mw/Mn)
851376 Mn	925.9 (2.4%)	1.364 +/-0.042	825.6 (2.6%)	1.599 +/-0.054
Mw	1262 (1.9%)		1320 (2.2%)	
866846 Mn	1390 (2.1%)	1.182 +/-0.031	1042 (1.6%)	1.267 +/-0.025
Mw	1643 (1.5%)		1321 (1.3%)	



**Figure 1:** Refractive index (blue) and light scattering (red) sample profile.

Astra software was used to produce a plot of cumulative weight fraction vs molecular mass (a first order fit was applied to the light scattering data). An example of the resultant plot is shown in figure 2 and a summary of the data is shown in table 2.



**Figure 2:** Cumulative weight fraction vs molecular mass for the samples: 851367 – black, 866846 – red

**Table 2:** Summary of cumulative plot data

Molar Mass	Cumulative Weight Fraction			
	851376 Run1	Run2	866846 Run1	Run2
1500	0.722	0.7082	0.519	0.7
2000	0.8516	0.8189	0.7457	0.8525
2500	0.9215	0.8868	0.8803	0.928
3000	0.957	0.9277	0.9456	0.9639
3500	0.976	0.9519	0.9751	0.9815
4000	0.9866	0.9672	0.9889	0.9903
4500	0.9926	0.9774	0.9951	0.995

Signed: *M Evans* M. Evans (Analyst)

Date: 25<sup>th</sup> April 2014



## Memorandum

**TO:** Lillian Gill, D.P.A.  
Director - COSMETIC INGREDIENT REVIEW (CIR)

**FROM:** Halyna Breslawec, Ph.D.  
Industry Liaison to the CIR Expert Panel

**DATE:** March 10, 2014

**SUBJECT:** Comments on the Draft Report: Safety Assessment of Hydrolyzed Wheat Protein and Hydrolyzed Gluten as Used in Cosmetics Prepared for the March 17-18, 2014 meeting

### Key Issues

Abstract, Discussion - Please revise the following sentence (similar sentences occur in the Abstract and Discussion - this one is from the Abstract): "The Panel determined that data on the elicitation of Type I hypersensitivity reactions in sensitized individuals were adequate to support the safety of these ingredients with peptide length distributions not exceeding 30 amino acids." This sentence is misleading as it suggests that information on Type I reactions is supporting safety. It is information that indicates that smaller peptides do not bind IgE and that lower molecular weight Hydrolyzed Wheat Protein does not cause sensitization that supports safety, not the "data on the elicitation of Type I hypersensitivity reactions in sensitized individuals."

Chemistry - As common methods used to measure protein molecular weight are not precise, a brief discussion of methods used would be helpful. Some general information on the amino acid composition of wheat proteins would also be helpful. Composition information may help suggest why an issue has been observed with some Hydrolyzed Wheat Proteins used in cosmetic products but not other hydrolyzed proteins used in cosmetic products.

Table 3, Cosmetic Use section- Do not state that the Council "confirmed" that some uses are sprays and powders. Footnote 3 is not correct. THE COUNCIL DID NOT "CONFIRM" THAT CERTAIN USES WERE SPRAYS OR POWDERS. The Council just reports the results of the survey. Use of the word "confirmed" is very misleading. It suggests that there was independent confirmation of the form of the product. This did not happen. This survey was conducted in 2012, and there was no additional effort to "confirm" that the information reported by the companies was correct. In the Cosmetic Use section

change: “The maximum concentration of HWP confirmed to be used...” to “The maximum concentration of HWP reported to be used...”

Summary - The Summary should include some information about the results of the studies that have been conducted.

#### Additional Comments

Method of Manufacturing - What was the molecular weight of gluten as measured by SDS-PAGE (reference 14)?

Toxicological Studies - What are the toxicities of wheat protein and wheat gluten following oral exposure? If there are no adverse effects associated with wheat protein and wheat gluten following oral exposure, the following sentence does not make sense: “Toxicities from dermal exposure, other than irritation and sensitization would not be expected to be different from oral exposures and as such not of concern by the Panel.” This sentence also does not make sense if sensitization is the only adverse effect following oral exposure - as the information in the report suggests that there are differences in sensitization following oral and dermal exposure.

Sensitization, Dermal - Non-Human - The doses used in the mouse study are not clear (reference 14). It says 500 µg/mouse for the induction exposures, but it also says there was “dose-dependent production of IgE and IgG1. If only one dose was used, how could the production of IgE and IgG1 be dose-dependent? Perhaps they used multiple i.p. challenge doses? The i.p. doses should also be stated.

Type 1 Hypersensitivity, 1<sup>st</sup> and 11<sup>th</sup> paragraphs - Please be more specific and change “ IgE epitopes” to “ IgE-binding epitopes”.

Discussion - It should also be noted that the mouse study showed a response to gluten with SLS but not without SLS treatment.



## Memorandum

**TO:** Lillian Gill, D.P.A.  
Director - COSMETIC INGREDIENT REVIEW (CIR)

**FROM:** Halyna Breslawec, Ph.D.  
Industry Liaison to the CIR Expert Panel

**DATE:** April 24, 2014

**SUBJECT:** Comments on the Tentative Report: Safety Assessment of Hydrolyzed Wheat Protein and Hydrolyzed Gluten as Used in Cosmetics

### Key Issues

In the Chemistry section, it would be helpful to include information on the relative proportions of amino acids in wheat protein, e.g., wheat protein is relatively high in glutamine. This may also provide some insight into why it is appropriate to use a value of 117 Da/amino acid when determining the MW cut-off for these ingredients.

Discussion - Please revise the following sentence: "Traditional human repeat insult patch tests (HRIPT) and related tests do not elicit Type 1 reactions." Perhaps the CIR Expert Panel is trying to state: "Traditional human repeat insult patch tests (HRIPT) and related tests do not assess the ability of a substance to cause Type 1 reactions."

### Additional Comments

Abstract - MW is missing from the conclusion in the abstract.

Introduction - Rather than stating that the related ingredients are "safe for use in cosmetic ingredients", it should state "safe for use as cosmetic ingredients" or safe for use in cosmetic products".

Method of manufacturing - "or amino acids" in the last sentence of the third paragraph is misplaced (it currently states: "which can be derivatized by quaternization or copolymerization, or amino acids,"

Type 1 Hypersensitivity - Please revise the last sentence of this section. Rather than stating that peptides with weight-average MWs of 3500 Da or less do not have the "potency" required to induce Type 1 hypersensitivity, it should state that they do not have the "properties" needed to induce Type 1 hypersensitivity.

Summary - The mouse study that showed transdermal sensitization to Hydrolyzed Wheat Protein and gluten (in the presence of SDS) should be mentioned in the Summary.

Table 3 - The preparation of the use tables needs to be more transparent. The current protocol should be publically available.

Are skin fresheners going to be considered both potential spray and powder products (as is done for Hydrolyzed Wheat Protein)?

The use information provided by the Council has a face powder (makeup product) reported to contain 0.05% Hydrolyzed Wheat Protein. Why is this product in the Incidental Inhalation-Powder? row, rather than the Reported Powder row?

The use information provided by the Council reported a not spray face and neck skin care product containing 0.06% Hydrolyzed Wheat Gluten. Why is this product presented in the Incidental Inhalation-Spray? row - the product is not a spray product.

When there are no uses in deodorant products reported, why is it necessary to have 3 rows all containing NR?