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Acid Hydrolysis of Gluten Enhances the Skin Sensitizing Potential and Drives Diversification of IgE Reactivity to Unmodified Gluten Proteins

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Scope: Personal care products containing hydrolyzed gluten have been linked to spontaneous sensitization through the skin, however the impact of the hydrolysate characteristics on the sensitizing capacity is generally unknown.
Methods and Results: The physicochemical properties of five different wheat-derived gluten products (one unmodified, one enzyme hydrolyzed, and three acid hydrolyzed) are investigated, and the skin sensitizing capacity is determined in allergy-prone Brown Norway rats. Acid hydrolyzed gluten products exhibited the strongest intrinsic sensitizing capacity via the skin. All hydrolyzed gluten products induced cross-reactivity to unmodified gluten in the absence of oral tolerance to wheat, but were unable to break tolerance in animals on a wheat-containing diet. Still, the degree of deamidation in acid hydrolyzed products is associated with product-specific sensitization in wheat tolerant rats. Sensitization to acid hydrolyzed gluten products is associated with a more diverse IgE reactivity profile to unmodified gluten proteins compared to sensitization induced by unmodified gluten or enzyme hydrolyzed gluten.
Conclusion: Acid hydrolysis enhances the skin sensitizing capacity of gluten and drives IgE reactivity to more gluten proteins. This property of acid hydrolyzed gluten may be related to the degree of product deamidation, and could be a strong trigger of wheat allergy in susceptible individuals.

1. Introduction

Food allergy affects up to 10% of the population,[1,2] and has been increasing in prevalence during the last decades, particularly in Western regions.[3–5] Food allergies are more common in children compared to adults,[4] and it has been suggested that especially small children are sensitized through the skin, as allergy may evolve before the introduction of the culprit food in the children's diet and thus before the development of oral tolerance.[6,7] Supporting evidence exists from animal experiments, in which skin sensitization to different food allergens have been observed,[8–12] and where it has been demonstrated that naïve animals, without prior exposure to the given food, are more susceptible to sensitization compared to animals with previous oral food exposure.[12] Impairment of the skin barrier seems to increase the risk of food allergy,[13,14] and approximately 40% of patients suffering from atopic dermatitis (AD) exhibit food allergy

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co-morbidity.[15] Cases of allergic reactions have been observed in wheat-tolerant patients after application of cosmetic products containing wheat-derived proteins,[16–18] indicating that sensitization through the skin may even break an established oral tolerance. Specifically, a facial soap containing acid hydrolyzed gluten (Glupearl 19S) was responsible for an "outbreak" of wheat allergy in Japan, affecting more than 2100 people with no previous history of wheat allergy. In this cohort, 77% of the subjects experienced allergic symptoms after eating unmodified wheat and 25% experienced anaphylaxis.[19] Further, sensitization to acid hydrolyzed gluten correlated with sensitization to unmodified gluten and wheat,[20] supporting the break of oral tolerance and the development of allergy to unmodified wheat.

Wheat contains approximately equal amounts of gliadins and glutenins, often referred to as gluten.[21] The amino acid composition in gliadins and glutenins are characterized by a high content of glutamine and proline and by a low content of amino acids with charged side groups.[22] Gliadins are classified into three groups based on their electrophoretic mobility at low pH; α/β-gliadins (fast), γ-gliadins (intermediate), and ω-gliadins (slow). Glutenins are mainly present as polymers of individual proteins linked by disulphide bonds, and can be classified into high molecular weight (HMW) and low molecular weight (LMW) subunits based on their separation by SDS-PAGE under reducing conditions.[22,23] Gluten proteins have dynamic structures which comprise interchanging conformations and do not unfold when exposed to heating like most other proteins.[24,25] Hydrolyzing gluten proteins leads to enhanced water solubility and changes in their physicochemical properties that can be targeted and exploited in the production of consumer products. Hence, hydrolyzed gluten products are used in soap and other cosmetic products to obtain specific physicochemical product properties.[26,27] Industrial production of gluten hydrolysates involves the use of both proteolytic enzymes and strong acid treatment to obtain desired properties, such as solubilizing, emulsifying, and foaming properties. Yet, acid treatment may also cause structural modifications other than peptide bond hydrolysis, including deamidation of amino acid side chains.[28]

It is well-accepted that hydrolysis in general reduces the allergenicity of food allergens, rendering them with decreased ability to elicit allergic reactions in sensitized individuals due to the destruction of IgE binding epitopes. However, the de novo skin sensitizing capacity of gluten-derived hydrolysates, as well as the ability to break an established oral tolerance to gluten remains largely unexplored. In the present study, we characterized the physicochemical properties of five different gluten-derived products: unmodified gluten (Un Glu), enzyme hydrolyzed gluten (E Glu), and three acid hydrolyzed gluten products (Ac Glu 1–3). The skin sensitizing capacity of the various gluten products was investigated in a Brown Norway (BN) rat model of skin mediated food allergy[12] in the absence or presence of oral tolerance to wheat. The acid hydrolyzed products were found to exhibit the highest sensitizing capacity, and to drive IgE reactivity to more proteins within unmodified gluten. The acid hydrolyzed products did not break established oral tolerance to unmodified gluten, but the strong sensitizing capacity and ability to diversify IgE reactivity may be a strong trigger of wheat allergy in susceptible individuals.

2. Experimental Section

2.1. Wheat-Derived Gluten Products

Un Glu, E Glu, Ac Glu 1, and Ac Glu 2 were kindly provided by Tereos Syral (Aalst, Belgium), and Ac Glu 3 (also known as Glupearl 19S) was kindly provided by Prof. Kayoko Matsunaga, Fujita Health University School of Medicine, Aichi, Japan. Products were dissolved in sterile PBS (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4 in Milli Q water, pH 7.2) before analyses and used in animal experiments. Endotoxin content was <20 EU mg^{-1} protein in all products, measured by the Pierce LAL Chromogenic Endotoxin Quantification Kit (ThermoFisher Scientific, MA, USA), according to manufacturer's instruction.

2.2. Physicochemical Characterization of Wheat-Derived Products

2.2.1. Size Exclusion Chromatography (SEC)

Gluten samples were dissolved (5 mg mL^{-1}) in 0.1 M sodium phosphate buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 6.9) with 2% (v:v) SDS at 60 °C overnight on a rotary shaker. Native gliadin, purified as previously described,[29,30] was included as a positive control. SDS-soluble proteins were obtained after centrifugation at 7500 × *g* for 10 min. Samples were filtered before injection (0.45 µm) and separated on an ÄKTA Explorer 100 controlled by software Unicorn 5.1. A Superose 6 Prep-grade HR 30/10 column containing SDS was used (GE-Healthcare, Chicago, IL, USA), as previously described.[31]

2.2.2. 1D Gel Electrophoresis

Gluten samples were prepared for SDS-PAGE analysis by dissolving (4 mg mL^{-1}) in 2x Laemmli buffer (Bio-Rad, Hercule, CA, USA) with 5% (v:v) β-mercaptoethanol, heated for 1.5 min at 95 °C, and loaded (Un Glu 16 µg, E Glu 60 µg, Ac Glu 1, 2, 3 40 µg) on a 4–20% Mini-PROTEAN TGX Stain-Free gel (Bio-Rad). The gel was run in tris-glycine/SDS buffer (Bio-Rad). Gluten samples were prepared for native-PAGE analysis by dissolving in sample buffer without SDS and β-mercaptoethanol, and loaded on a 12% acrylamide gel (Bio-Rad). The gel was run in tris-glycine buffer (Bio-Rad). The gels were stained with Coomassie Brilliant Blue (Bio-Rad).

2.2.3. Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC)

Gluten samples were dissolved (1 mg mL^{-1}) in 0.11 M TFA with 5% (v:v) ACN, and filtered before injection (0.45 µm). Separation was performed on an Alliance HPLC system (Waters, Saint-Quentin-en-Yvelines, France) equipped with UV detection at 220 nm. Proteins were separated on a C_{18} column 250 mm × 4 mm, 5 µm pore (Macherey-Nagel, Düren, Germany), as previously described.[32]

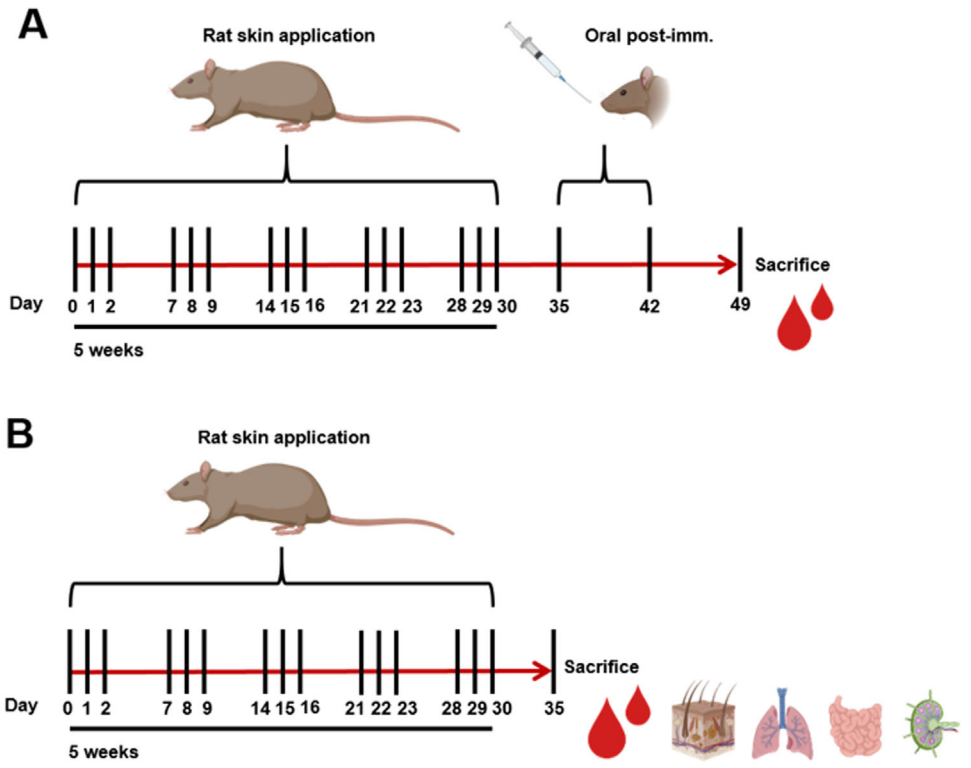

Figure 1. Animal experimental design. A) For animal experiment 1 and 3, Brown Norway rats (n = 6–8 per group) were dosed on the slightly damaged abdominal skin with either unmodified gluten (Un Glu), enzyme hydrolyzed gluten (E Glu), or one of three acid hydrolyzed gluten products (Ac Glu 1–3), or PBS as control. Rats were dosed on the skin three times per week for 5 weeks. Subsequently, rats were dosed by gavage twice with Un Glu at a 1-week interval. Blood was collected at sacrifice. Rats were bred and raised either on a diet free from wheat (Experiment 1) or a conventional rat chow containing wheat (Experiment 3). B) For animal experiment 2, Brown Norway rats (n = 6 per group) were dosed on the slightly damaged abdominal skin with Un Glu, E Glu, Ac Glu 2, or PBS as control. Rats were dosed on the skin three times per week for 5 weeks. At sacrifice blood, skin, lungs, small intestine, and mesenterial lymph nodes were collected. The figure was created with BioRender.com.

2.2.4. Deamidation Inhibition ELISA

Inhibition ELISAs for the evaluation of the extent of gluten deamidation and of the presence of residual native gliadin repeat epitopes were performed by using three complementary monoclonal antibodies, as previous described.[33,34] Briefly, ELISA plates were coated with the highly deamidated LQPEEPFPEQC peptide conjugated to BSA, with deamidated α-gliadins,[35] or with native gliadins.[29,30] Gluten samples were pre-incubated with antibodies Mab INRA-DG1,[33] MAb MCO1,[36] and MAb PQQ3B4[37] directed respectively against the high, low, or non-deamidated epitopes and subsequently added to plates. Antibody binding to coated plates was detected using horseradish peroxidase (HRP)-labeled anti-mouse IgG (170-6516, Bio-Rad). o-Phenylenediamine (P8287, Sigma-Aldrich, St. Louis, MO, USA) was used as substrate and color development was stopped with 2 M H_2SO_4.

2.3. Animals

BN rats were from the in-house breeding colonies at the National Food Institute (Technical University of Denmark) and raised on either a wheat-free (wheat naïve rats) or wheat-containing (wheat tolerant rats) diet, as previous described.[38,39] Diet and acidified water were given ad libitum. Ethical approval was provided by the Danish Animal Experiments Inspectorate (authorization no. 2015-15-0201-00553-C1). Experiments were overseen by the Animal Welfare Committee at the National Food Institute.

2.4. Dosage Regime

A previous established protocol for skin-mediated sensitization to gluten was applied in this study.[12]

Experiment 1: BN rats, raised and bred on wheat-free diet, were allocated into 16 groups of eight rats and dosed with either PBS as control or one of the five gluten products on the skin. Un Glu, E Glu, or Ac Glu 1, 2, or 3 were applied on the skin in three different doses, 5, 50, or 500 µg on their shaved and slightly damaged abdominal skin three times a week for 5 consecutive weeks. Subsequently, two post-immunizations with 50 mg of Un Glu were given by oral gavage with a 1-week interval (**Figure 1**A). Before sacrifice an ear swelling test was performed with intradermal injection of 10 µg of the skin-applied product in one ear and Un Glu in the other ear, as described elsewhere.[40] Animals were sacrificed by exsanguination using carbon dioxide as anesthesia. Blood was collected and converted into sera and stored at −20 °C until analyses.

Experiment 2: BN rats, raised and bred on wheat-free diet, were allocated into four groups of six rats and dosed with either PBS as control or 500 µg of Un Glu, E Glu, or Ac Glu 2, according to the above description, with the exception that rats were sacrificed immediately after skin application without any post-immunizations. At sacrifice blood, skin, lung, mesenteric lymph nodes, and small intestine lamina propria (LP) and Peyer's patches (PP) were collected (Figure 1B).

Experiment 3: BN rats, raised and bred on wheat-containing diet, were allocated into six groups of six rats and dosed with either PBS as control or 500 µg of Un Glu, E Glu, or Ac Glu 1, 2, or 3, and subsequently orally gavaged with 50 mg of Un Glu as described for Experiment 1 (Figure 1A).

2.5. ELISAs

2.5.1. IgG1 ELISAs

Product-specific IgG1 was analyzed by ELISA as previously described.[12] Briefly, ELISA plates were coated with the respective gluten products (Un Glu, E Glu, Ac Glu 1, 2, 3). Two-fold serial dilutions of serum samples were incubated on plates to obtain antibody Log2 titer values. IgG1 antibodies were detected using HRP-labeled mouse anti-rat IgG1 (3060-05, Southern Biotech, Birmingham, AL, USA) with 3,3′,5,5′-tetramethylbenzidine (TMB)-one (4380A, Kementec Diagnostics, Taastrup, Denmark) as substrate.

2.5.2. IgE-Capture ELISAs

Product-specific IgE was analyzed by IgE-capture ELISA as previously described.[12] Briefly, ELISA plates were coated with mouse anti-rat IgE antibody (HDMAB-123 HydriDomus, Nottingham, UK). Two-fold serial dilutions of serum samples were incubated on plates to obtain antibody Log2 titer values. Specific IgE antibodies were detected using digoxigenin (DIG)-coupled product and HRP-labeled sheep anti-DIG antibody (11633716001, Roche Diagnostics GmbH, Mannheim, Germany) with TMB-one as substrate.

2.5.3. Inhibitory IgG1 ELISAs

Inhibitory IgG1 ELISAs were performed as previously described.[12] Briefly, ELISA plates were coated with 2 µg mL^{-1} of the respective gluten products (Un Glu, E Glu, Ac Glu 1, 2, 3). Pooled serum samples were preincubated with seven 10-fold dilutions of each of the five gluten products (inhibitors) starting at a concentration of 1000 µg mL^{-1}. Afterwards, the inhibitory ELISA followed the same procedure as for detection of specific IgG1 by indirect ELISA. All analyses were done in duplicates and repeated two times.

2.6. 2D Gel Electrophoresis and Immunoblotting

2.6.1. 2D Gel Electrophoresis

Un Glu was dissolved (5 mg mL^{-1}) in 7 M urea (U5128, Merck Life Science, Milan, Italy), 2 M thiourea (T7875, Merck Life Science), and 2% (v:v) CHAPS (226947, Merck Life Science) by gentle agitation for 2 h at room temperature (RT), and centrifuged to discard possible insoluble fractions. Solubilized Un Glu was then used for IEF. Briefly, 100 µg of Un Glu protein was diluted to a final volume of 128 µL with 0.5% (v:v) ampholytes (GE17-0456-01, Merck Life Science) and 1% (w:v) DTT (D0632, Merck Life Science). The diluted sample was loaded through active rehydration overnight at 50 V on an immobilized pH gradient (IPG) strip (pH range 3–10, non-linear, GE17-6001-11, Merck Life Science). The IEF was carried out with 10 h of pre-focusing in order to slowly increase the voltage from 50 to 5000 V, and continued at 5000 V up to 80 000 VhT, limiting the current to 50 µA per strip. When the final amount of VhT was reached, focused proteins on IPG strips were either frozen at −20°, or directly reduced and alkylated with two steps of 15 min gentle agitation in equilibration buffer (6 M urea [Merck Life Science], 2% (w:v) SDS [L3771, Merck Life Science], 0.05 M tris-HCl [T1503, Merck Life Science, pH 8.8], 20% (v:v) glycerol [G5516, Merck Life Science], and 1% (w:v) DTT [D0632, Merck Life Science]), and equilibration buffer with 2.5% (w:v) iodoacetamide (I1149, Merck Life Science) instead of DTT, respectively. IPG strips were then loaded on a 12% acrylamide gel and the SDS-PAGE was run applying a current of 15 mA per gel until the Bromophenol Blue reached the front. The gels were removed from the plates and stained overnight with Coomassie Brilliant Blue (1.15444, Merck Life Science). Gels were digitalized using an Imagescanner III (GE Healthcare, Uppsala, Sweden).

2.6.2. IgE Immunoblotting

Un Glu proteins from 2D gel electrophoresis were electroblotted onto a PVDF membrane in a semidry blot apparatus (Novablot, GE Healthcare) for 1 h at a constant current of 0.8 mA cm^{-2}. The blotted membranes were incubated with pooled sera from the respective groups (Un Glu, E Glu, Ac Glu 1, 2, 3) of Experiment 1 diluted 1:10 in blocking solution (PBS with 5% (w:v) non-fat dried bovine milk (M7409, Merck Life Science) and 0.1% (w:v) Tween 20 (P1379, Merck Life Science)) for 2 h. Subsequently, the membranes were washed five times in PBS with 20% (w:v) Tween 20 and incubated with the secondary antibody (HRP-labeled-Mouse anti-Rat IgE, MA5-16813, ThermoFisher Scientific) diluted 1:3000 in blocking solution. Image analysis was performed using Progenesis SameSpots v4.5 (Nonlinear Dynamics, Newcastle, UK) and ImageMaster 2D Platinum v6.0.1 software (GE Healthcare). Spot detection, background subtraction, normalization, and spot matching were automatically performed and manually reviewed. Differentially represented immunoreactive spots among the different groups were manually excised and subjected to MS analysis and protein identification.

2.7. Mass Spectrometry (MS) Analysis

Protein spots of interest were excised from the gel. Gel plugs were washed three times under agitation (600 rpm) with first 50 µL of 50 mM ammonium bicarbonate (Sigma-Aldrich, Saint-Louis, MO, USA) and then 50 µL of 50 mM ammonium bicarbonate/ACN (Biosolve, ULC MS grade, Valkenswaard, The Netherlands) 50% (v:v). Cysteines were reduced with a 10 mM DTT

(Affymetrix, ThermoFisher Scientific, Waltham, MA, USA) solution for 40 min at 56 °C followed by alkylation with 55 mM iodoacetamide (Sigma-Aldrich) at RT in the dark. Digestion was performed overnight with 3 µL of 10 ng µL^{-1} of chymotrypsin (ThermoFisher Scientific) in 25 mM ammonium bicarbonate buffer, 5 mM $CaCl_2$ (Sigma-Aldrich), pH 8. The resulting peptides were extracted with 20 µL of aqueous solution at 1% TFA (Acros Organics, ThermoFisher Scientific), and 9 µL injected on the LC/MS system.

The LC-MS/MS analyses were performed on an Acquity M-Class UPLC (Waters Corp., Milford, MA, USA) hyphenated to a Q Exactive Plus (ThermoFisher Scientific), in nanoelectrospray positive ion mode. The trap column was a Symmetry C_{18} 5 µm (180 µm × 20 mm) and analytical column was a HSS T3 C_{18} 1.8 µm (75 µm × 250 mm) (Waters, Milford, MA, USA). Samples were loaded at 20 µL min^{-1} on the trap column in 98% solvent A during 3 min and subsequently separated on the analytical column at a flow rate of 600 nL min^{-1} with the following linear gradient: initial conditions 98% A; 5 min 93% A; 30 min 60% A; 33 min 15% A where solvent A is 0.1% formic acid in water and solvent B is 0.1% formic acid in ACN. The total run time was 60 min. The MS method is a TopN-MS/MS method where N was set to 10. The parameters for MS spectrum acquisition are: mass range from 400 to 1750 m/z; resolution of 70000; AGC target of 1e6 or maximum injection time of 200 ms. The parameters for MS2 spectrum acquisition are: isolation window of 2.0 m/z; normalized collision energy (NCE) of 27; resolution of 17500; AGC target of 1e5 or maximum injection time of 200 ms.

The data were searched against Uniprot database restricted to reviewed proteins from Viridiplantae database (December 2017, 33090 sequences) using Proteome Discoverer v 2.1.1.21 (ThermoFisher Scientific). The main parameters for the search were: 5 ppm tolerance on precursor mass and 20 mmu tolerance for MS/MS fragments, enzyme: unspecific, oxidation of methionine and deamidation of asparagine and glutamine as variable modifications and carbamidomethylation of cysteines as fixed modification.

2.8. Tissue Processing and Flow Cytometry

Abdominal skin samples were placed in PBS with dispase (1 mg mL^{-1}, Sigma-Aldrich, Saint Louis, MO, USA) and incubated overnight at RT with stirring. Subsequently, the epidermis was separated from the dermis and placed in a trypsin-EDTA buffer (Gibco, ThermoFisher Scientific) for 1 h with stirring. The cell suspension was centrifuged, counted, and re-suspended in flow cytometry staining buffer (PBS with 1% (v:v) EDTA, 5% (v:v) fetal bovine serum [FBS, Gibco, ThermoFisher Scientific]).

Small intestine LP (after the removal of PP) was cut into 5 mm pieces and washed in a wash buffer (PBS with 2% (v:v) FBS, 15 mM HEPES [Gibco, ThermoFisher Scientific]). Pieces were incubated three times for 30 min at RT with stirring in PBS with 10% (v:v) FBS, 5 mM EDTA (Gibco, ThermoFisher Scientific), 15 mM HEPES, and 1% (v:v) Penicillin-streptomycin (Gibco, ThermoFisher Scientific) in order to clear out the epithelial cells. The remaining pieces of intestine were then digested for 45 min at 37 °C in a digestion buffer (RPMI, 10% (v:v) FBS, 15 mM HEPES, 1% (v:v) Penicillin-streptomycin) containing dispase (1 mg mL^{-1}, Sigma Aldrich) and collagenase (500 µg mL^{-1}, Sigma Aldrich). Cells were recovered by centrifugation, counted, and re-suspended in flow cytometry staining buffer.

The lungs, PP, and the mesenteric lymph nodes were removed and crushed to obtain a single-cell suspension using a grinder. After lysis of the red blood cells from the lung using Red Blood Cell Lysing Buffer Hybri-Max (Sigma Aldrich) for 5 min, cells were washed, filtered using a 40 µm cell strainer, counted, and re-suspended in flow cytometry staining buffer.

Cells from all the organs were stained with the following antibodies for 20 min in the dark at 4 °C, and analyzed on a CANTO II (BD Biosciences, San Jose, CA, USA): CD3-Percpefluor710 (clone eBioG4.18 (G4.18); eBiosciences, Waltham, MA, USA), CD4-PEcy7 (clone OX-35), CD25-BV421 (clone OX-39), CD8-BV510 (clone OX-8; BD Biosciences, San Jose, CA, USA), and ZOMBIE NIR (Biolegend, San Diego, CA, USA) for the viability. The data were analyzed using FlowJo (Treestar, Ashland, OR, USA).

2.9. Statistical Analysis

Statistical analysis was performed using GraphPad Prism v8.0.2 (GraphPad Software Inc., San Diego, CA, USA). Differences between groups were evaluated using non-parametric Kruskal-Wallis tests followed by Dunn's multiple-comparisons test. Dose-responses and correlations were evaluated using Spearman's rank correlation test. *p*-values below 0.05 were considered statistically significant.

3. Results

3.1. Acidic and Enzymatic Hydrolysis Lead to Distinct Changes in Physicochemical Properties of Gluten

SEC analysis (**Figure 2**A) showed that Un Glu contained complexes larger than 1000 kDa, as well as a large amount of complexes between 100 and 1000 kDa, likely corresponding to polymers of HMW and LMW glutenins. Monomers and small complexes constituted the largest proportion of Un Glu with MW in the range of 30–100 kDa, whereas only a very low amount of proteins could be observed with molecular weight (MW) below 30 kDa. In contrast to Un Glu, E Glu contained no high MW fractions and the monomeric part was switched towards low MW peptides mostly detected within the range of 5–30 kDa. The three Ac Glu products had very similar MW protein distributions, with monomeric proteins primarily in the range of 30–100 kDa. SDS-PAGE analysis under reducing conditions (Figure 2B) showed that Un Glu was composed of individual proteins within the range of 12 to 120 kDa, with bands in the area from 37 to 50 kDa, corresponding to α/β, γ-gliadins, and LMW glutenin subunits, being the most pronounced. E Glu showed a greater range of specific peptides, with a high density of bands within the low MW area (<30 kDa), whereas the three Ac Glu products were found to create a smear of peptides with sizes primarily between 10 and 50 kDa. Thus, the Ac Glu products were found to have a distinct peptide composition than the E Glu product, with a different pattern of low MW peptides.

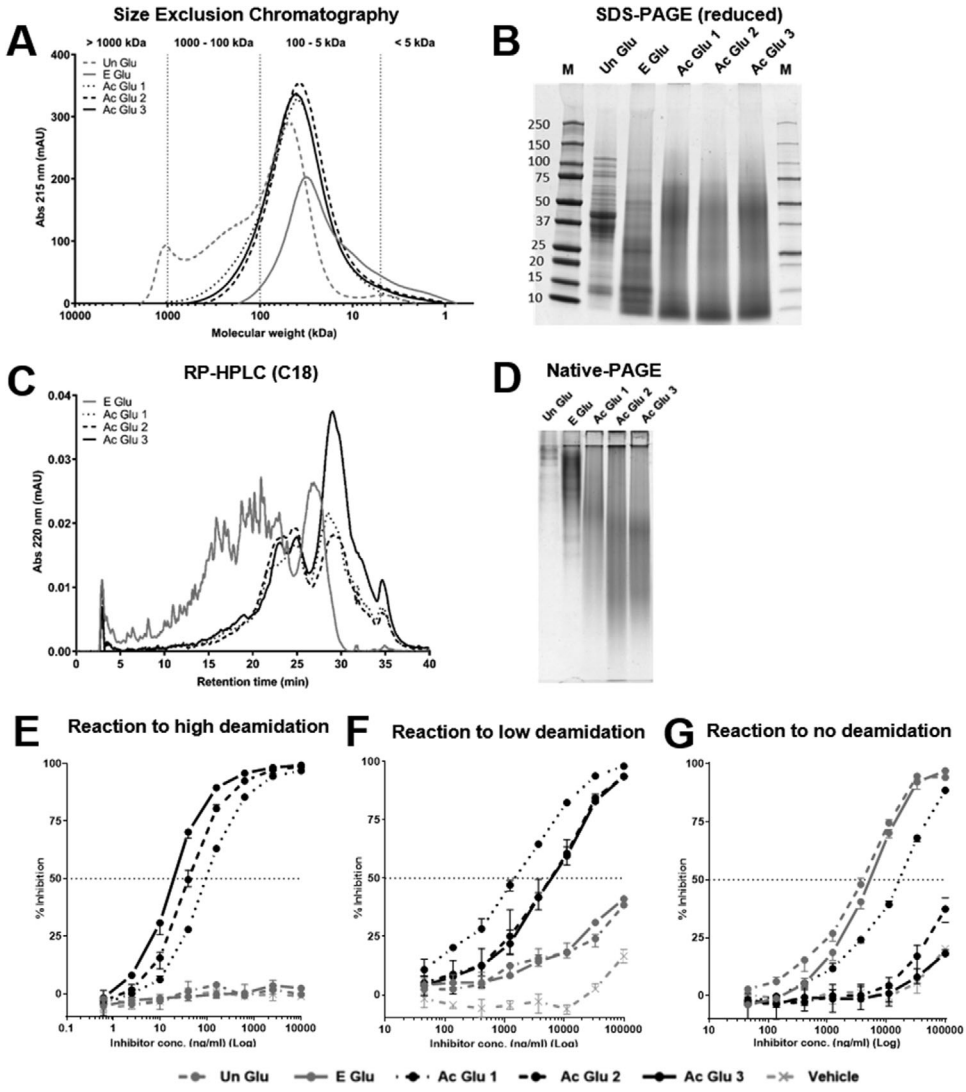

Figure 2. Physicochemical characterization of gluten products. Physicochemical analysis of unmodified gluten (Un Glu), enzyme hydrolyzed gluten (E Glu), and acid hydrolyzed gluten (Ac Glu 1–3). A) Molecular size distribution determined by Size Exclusion Chromatography. B) SDS-PAGE analysis under reduced conditions. C) Solubility and hydrophobicity determined by reverse-phase high-performance liquid chromatography (RP-HPLC). D) Native-PAGE analysis. E–G) Inhibition of ELISA detecting the ability of each product to inhibit reactions between antibody-antigen pairs detecting known high-, low-, and non-deamidated gluten-derived epitopes. Marker (M).

RP-HPLC analysis (Figure 2C) showed that E Glu contained a high amount of peptides with low hydrophobicity, eluting between 5 and 25 min, whereas the Ac Glu products were composed of peptides with a higher hydrophobicity that mainly eluted between 20 and 35 min. A high peak at 30 min was observed only for Ac Glu 3, making it stand out from the Ac Glu 1 and 2, indicating that Ac Glu 3 exhibited the highest hydrophobicity. Un Glu could not be analyzed with RP-HPLC due to its large amount of insoluble compounds. Native gel analysis under non-reducing conditions (Figure 2D) showed that Un Glu had the slowest migration into the gel due to few charged residues and the large sizes of proteins. E Glu had a higher mobility compared with Un Glu and migrated longer into the gel due to its composition of smaller peptides as a result of hydrolysis. The three Ac Glu products migrated further into the gel with the fastest migration of Ac Glu 2 and 3, stressing that the proteins and peptides in the Ac Glu products had more charged side chains when compared to E Glu.

Acid treatment causes deamidation of asparagine and glutamine amino acid residues within gluten.[26,27] The level of deamidation was assessed by means of ELISAs, as the ability of each product to inhibit reactions between antibody-antigen pairs detecting known high-, low-, and non-deamidated gluten-derived epitopes, respectively. The three Ac Glu products were all able to fully inhibit the binding of antibodies specific for highly deamidated epitopes in contrast to Un Glu and E Glu, demonstrating the absence of highly deamidated epitopes in Un Glu and E Glu (Figure 2E). It was found that Ac Glu 3 was the most deamidated product followed by Ac Glu 2, which was followed by Ac Glu 1. These results were supported by analyses of the capacity to inhibit reactions toward low-deamidated epitopes,

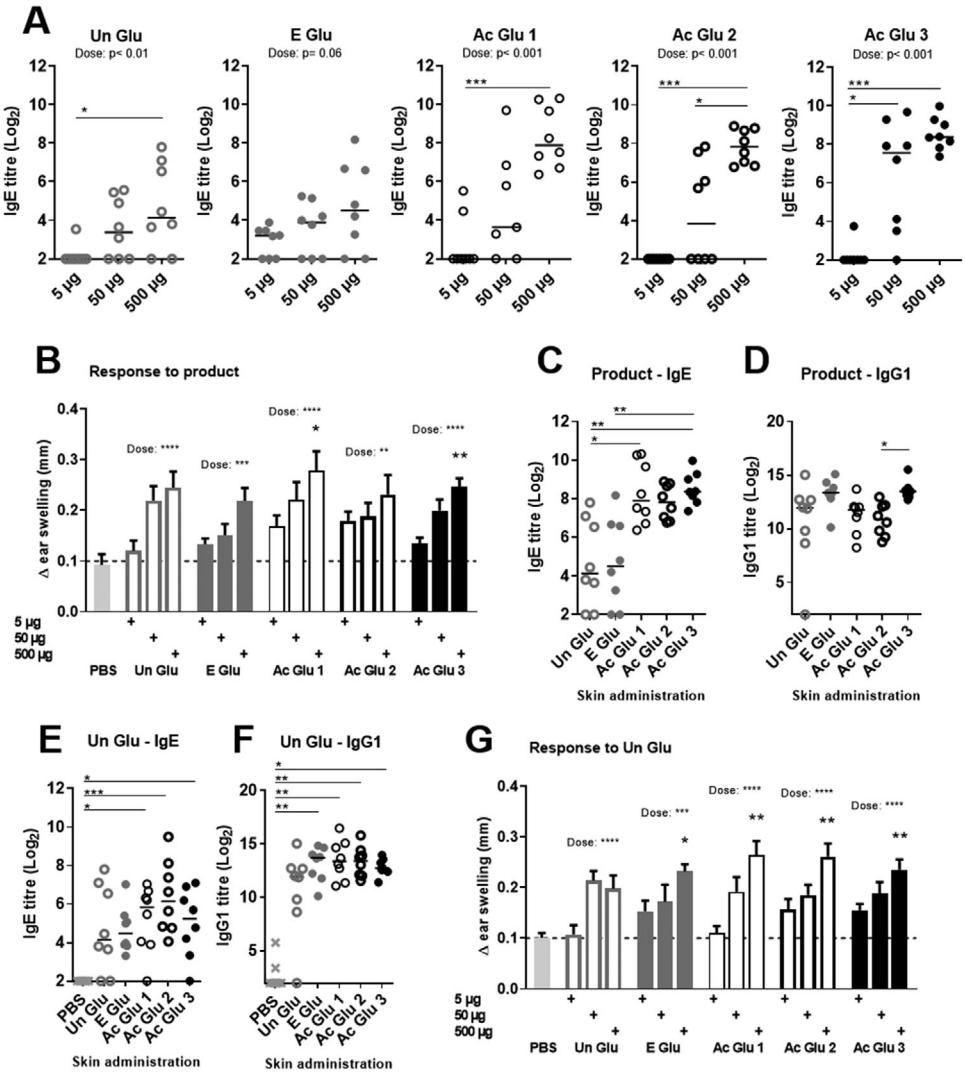

Figure 3. Sensitization to gluten products via the skin in wheat-naive rats. Unmodified gluten (Un Glu), enzyme hydrolyzed gluten (E Glu), acid hydrolyzed gluten (Ac Glu 1–3), or PBS (control) were applied on the slightly damaged abdominal skin of wheat-naïve Brown Norway rats (three times per week for 5 weeks; $n = 8$ per group). Subsequently, rats were dosed by gavage two times with Un Glu at a 1-week interval. A) Serum product-specific IgE after skin application of 5, 50, or 500 µg product. B) The clinical ear swelling response to intradermal injection of product after skin application of 5, 50, or 500 µg product. C–D) Serum product-specific IgE and IgG1 after skin application of 500 µg product. E–F) Serum Un Glu-specific IgE and IgG1 after skin application of 500 µg product. G) The clinical ear swelling response to intradermal injection of Un Glu after skin application of 5, 50, or 500 µg product. Each symbol represents an animal and horizontal bars indicate median value. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

demonstrating that Ac Glu 1 contained a higher inhibitory capacity than Ac Glu 2 and Ac Glu 3 (Figure 2F). Antibody binding to non-deamidated epitopes was fully inhibited by Un Glu and E Glu, with Ac Glu 1 showing a bit lower inhibitory capacity, and Ac Glu 2 only demonstrated a weak capacity to inhibit binding to non-deamidated epitopes, whereas Ac Glu 3 showed no inhibitory capacity at all (Figure 2G).

3.2. Gluten Products Induced Skin Sensitization in a Dose-Dependent Manner

Wheat-naïve BN rats were used to evaluate the skin sensitizing capacity of the five different gluten products by application of the products on slightly damaged abdominal skin three times per week for 5 weeks, followed by two oral administrations with Un Glu to simulate gluten ingestion after skin exposure (Figure 1A). All five gluten products induced a dose-dependent sensitization as measured by the levels of product-specific IgE in serum (**Figure 3**A). The clinical relevance of the sensitization to the gluten products was reflected in the allergic reactions mounted to the individual products in the ear swelling test, which correlated with the dose applied on the skin (Figure 3B), demonstrating the development of functional product-specific IgE. When comparing results from the highest doses, it was suggested that the deamidated Ac Glu products induced a stronger sensitization than Un Glu and E Glu (Figure 3C; non-deamidated vs deamidated products, Mann–Whitney U test, $p < 0.0001$), although

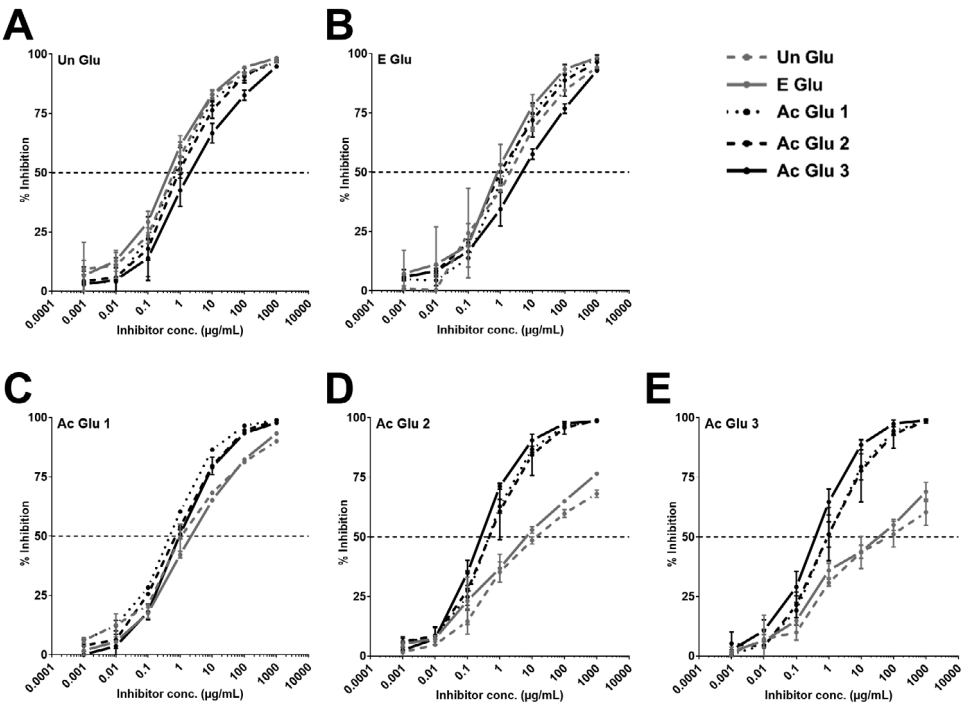

Figure 4. IgG1 cross-reactivity of gluten products after skin sensitization in wheat-naïve rats by means of inhibitory ELISA. Inhibitory ELISAs examining the capacity of gluten products to inhibit the binding between specific IgG1 antibodies and the products they were raised against in wheat-naïve rats. Plates were coated with A) unmodified gluten (Un Glu), B) enzyme hydrolyzed gluten (E Glu), C) acid hydrolyzed gluten 1 (Ac Glu 1), D) acid hydrolyzed gluten 2 (Ac Glu 2), or E) acid hydrolyzed gluten 3 (Ac Glu 3). Results are expressed as the percentage inhibition against the concentration of inhibitor.

caution should be noted when comparing different IgE assays with possible different sensitivities. In comparison, the immune system produced similar levels product-specific IgG1 (Figure 3D; non-deamidated vs deamidated products, Mann–Whitney *U* test, $p = 0.37$).

The sensitization to E Glu and Ac Glu products was found to mediate cross-reactivity to Un Glu, as demonstrated by the induction of Un Glu-specific IgE in serum (Figure 3E). All products were found to induce similar levels of Un Glu-specific IgG1 (Figure 3F). The level of cross-reactivity to Un Glu was reflected by the clinical reactivity to Un Glu, which correlated with the dose of gluten product applied to the skin (Figure 3G), demonstrating the functionality of the Un Glu cross-reactive IgE and their relevance in clinical gluten allergy.

3.3. The Gluten Products Exhibit Distinct Antibody-Binding Profiles

In order to compare the epitope profiles of the different gluten products, inhibitory ELISA was performed to examine the capacity of the different products to inhibit the binding between specific IgG1 antibodies and the products they were raised against in the wheat-naïve BN rats. All five gluten products were able to fully inhibit the binding between Un Glu and antibodies raised against Un Glu (**Figure 4**A), indicating that all products contain epitopes similar to the epitopes on the intact native Un Glu, and hence that these epitopes are preserved even after hydrolysis. Likewise, all products were able to fully inhibit the binding between E Glu and antibodies raised against E Glu (Figure 4B). On the contrary, Un Glu and E Glu were not capable of fully inhibiting the binding between the Ac Glu products and the antibodies raised against these three Ac Glu products (Figure 4C–E). Greatest inhibition was observed towards Ac Glu 1, followed by Ac Glu 2 and Ac Glu 3. These findings demonstrate the development of new epitopes by acid hydrolysis of gluten, and that the level of new epitopes correlates with the degree of deamidation.

IgE immunoblot was performed in order to visualize the IgE protein binding profile developed in response to gluten proteins during the skin sensitization in the wheat-naïve BN rats. A 2D gel was run with Un Glu (**Figure 5**A), blotted and incubated with sera from wheat-naïve BN rats sensitized with Un Glu (Figure 5B), E Glu (Figure 5C), or Ac Glu 1–3 (Figure 5D -F.). Sensitization to the different gluten products was found to give rise to distinct IgE binding profiles, with more immunoreactive spots visualized for E Glu and Ac Glu compared to Un Glu, and more for Ac Glu than for E Glu. A total of six areas on blots of Un Glu were found (Figure 5B–F) and proteins identified (Table S1, Supporting Information). LC-MS/MS analysis identified spot 1, 2, and 3, as being glutenins (spot 1 as HMW subunit, and spots 2 and 3 as LMW subunit), spot 4 as being GTP-binding protein and actin, whereas spot 5, and spot 6 were identified as α/β-gliadins. An overriding identification of areas 4 and 5 for BN rats sensitized with Ac Glu, stresses a different sensitization pattern for Ac Glu compared to Un Glu and E Glu, suggesting that Ac Glu drives "diversification" of IgE epitopes leading to sensitization to more proteins.

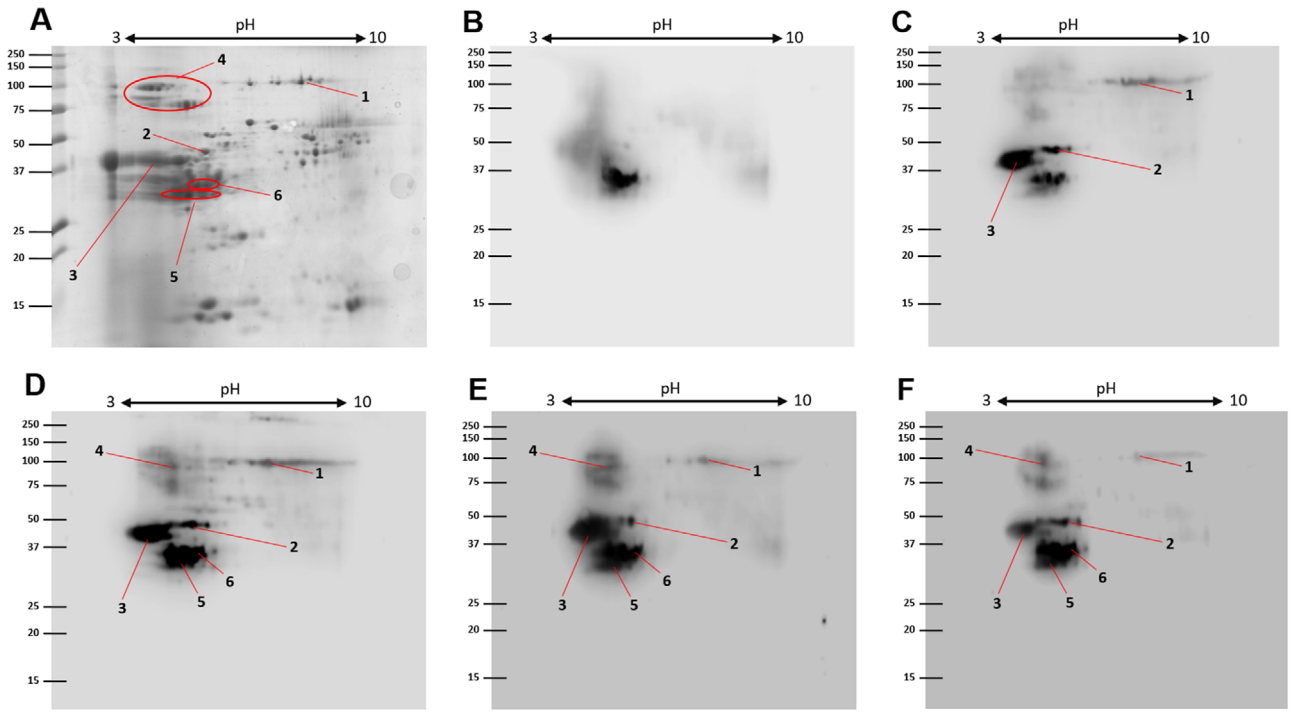

Figure 5. IgE reactivity to unmodified gluten proteins after skin sensitization to different gluten products in wheat-naïve rats. Visualization of the IgE protein binding pattern to unmodified gluten developed in response to gluten product sensitization via the skin in wheat-naïve Brown Norway rats. A) 2D gel of unmodified gluten (Un Glu). Blot incubated with sera from rats dosed with B) Un Glu, C) enzyme hydrolysed gluten (E Glu), D) acid hydrolyzed gluten 1 (Ac Glu 1), E) acid hydrolyzed gluten 2 (Ac Glu 2), F) acid hydrolysed gluten 3 (Ac Glu 3). Immunoreactive spots are marked with numbers 1–6.

3.4. Skin Sensitization Induces Gut Immune Responses

The impact of skin application of the gluten products, and hence sensitization via the skin, on T cell numbers in skin, lung, mesenteric lymph nodes, and small intestine LP and PP was analyzed by flow cytometry in wheat-naïve BN rats (Figure 1B). Both the skin and small intestine LP showed an increase in CD3+CD4+ helper T cell numbers in response to skin application of gluten products compared to the PBS control group (**Figure 6**A–C), whereas CD3+CD8+ T cell numbers were unchanged. No differences were observed for any of the two T cell populations in the lung, PP and the mesenteric lymph nodes (data not shown). The increase in T cell numbers was independent of the gluten product applied on the skin, indicating that changes in T cell-mediated immune responses were not driving the differences in product allergenicity reflected by the specific IgE levels.

3.5. Deamidation Promote Sensitization in Animals with Oral Tolerance to Wheat

Sensitization experiments were repeated in BN rats on a wheat-containing diet to determine the role of established oral tolerance to gluten (Figure 1A). Among the five gluten products, only Ac Glu 3 was found to significantly induce product-specific sensitization via the skin in animals with oral tolerance to wheat (**Figure 7**A). However, we found that the level of deamidation in Ac Glu products (Ac Glu 3>2>1) correlated with the ability to induce product-specific IgE (Spearman's rank correlation test, $p = 0.007$). In animals with oral tolerance to wheat, none of the products were able to induce cross-reactivity to Un Glu (Figure 7B). The skin exposure to gluten products were shown to have a statistically significant effect on levels of product-specific and Un Glu-specific IgG1 levels for only the Ac Glu 3 sensitized wheat-tolerant rats (Figure 7C,D).

4. Discussion

The skin plays a crucial role as a protective barrier between the host and the external environment. In recent years much focus has been drawn to the skin as a site of allergen exposure both in relation to food allergy sensitization[41–43] and desensitization.[44,45] In this study, the skin as route of sensitization was investigated with the main purpose of comparing five physicochemically different gluten products in a rat model. An unmodified, an enzyme hydrolyzed and three different acid hydrolyzed gluten products were compared in a dose-response study evaluating the allergenicity, immunogenicity, and cross-reactivity. The enzyme hydrolyzed product was found to contain smaller peptides with a distinct peptide size distribution profile compared to the acid hydrolyzed gluten products, which all contained a smear of peptides of different sizes. This observation is likely attributable to the relative specific nature of enzyme-mediated hydrolysis compared to the more promiscuous acid-mediated hydrolysis. Furthermore, the acid hydrolysates were found to contain varying degrees of deamidation, which was associated with epitope profiles that were distinct from the unmodified and enzyme hydrolyzed gluten products. This demonstrates

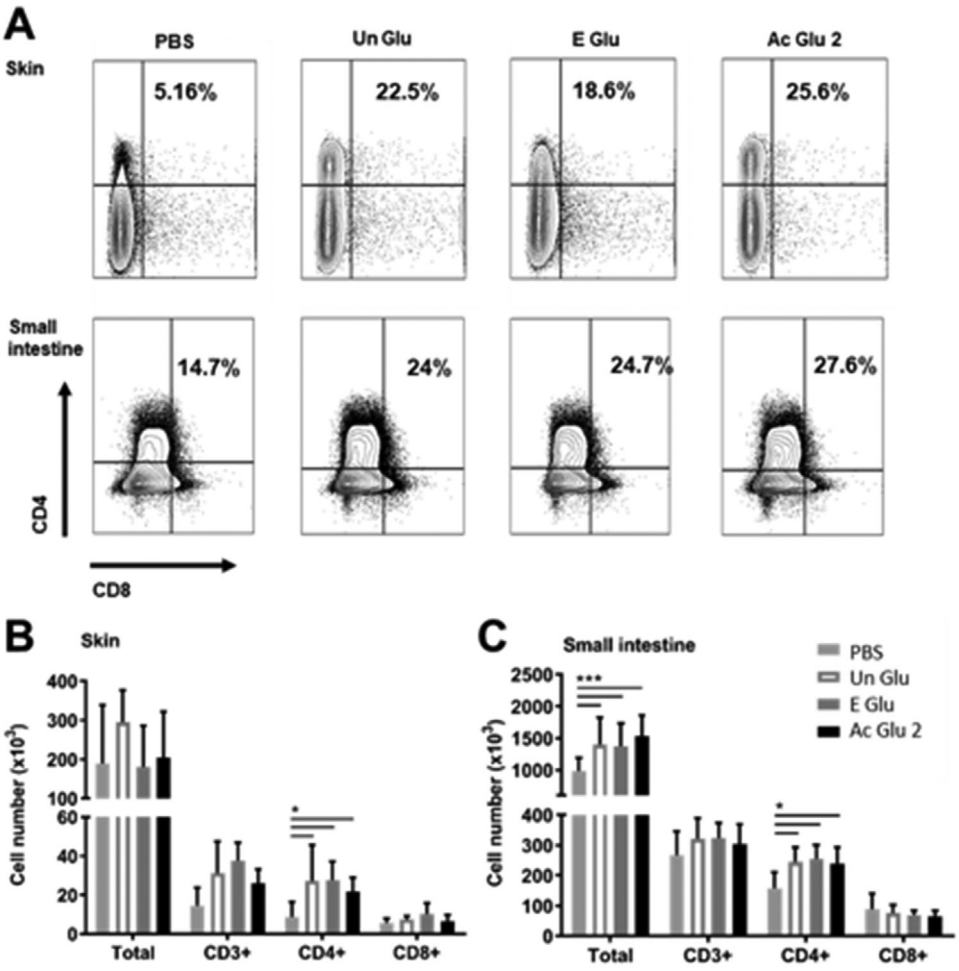

Figure 6. T cell composition in the skin and small intestine following skin sensitization to gluten products in wheat-naïve rats. The impact of skin application of different gluten products on T cell numbers in the skin and small intestine lamina propria in wheat-naïve rats (three times per week for 5 weeks; $n = 6$ per group) by flow cytometry. A) T cell populations evaluated by flow cytometry of helper (CD4) and cytotoxic (CD8) T cell markers within the CD3+ T cell population. B-C) Absolute cell numbers within the tissue samples. $*p < 0.05$, $***p < 0.001$.

that acid hydrolysis of gluten induces the generation of new epitopes that are not present in native gluten, which is in line with our previous studies of sensitization to gluten products via the intraperitoneal and oral routes in BN rats.[39,46] Although new epitopes were generated by acid hydrolysis, all products were found to retain epitopes present in unmodified gluten protein.

Unmodified gluten and all hydrolyzed gluten products were able to induce dose-dependent sensitization via the skin in wheat-naïve rats. Sensitization was associated with product-specific ear swelling, indicating the development of a clinically defined allergic response to the products. The acid hydrolyzed products were found to exhibit the greatest intrinsic sensitizing capacity, as these products induced the highest levels of product-specific IgE compared to unmodified and enzyme hydrolyzed gluten. However, it should be noted, that the increased sensitization was not associated with increased clinical relativity in the ear swelling test, which could be due the sensitivity of this outcome measure. The high sensitizing capacity of acid hydrolyzed products can probably not be explained by the capacity of the products to penetrate the skin and stimulate immune responses, as all products induced similar levels of product-specific IgG1. This finding indicates that the cutaneous immune system recognizes all products to a similar extend, but the acid hydrolyzed products exhibit specific properties that enhance sensitization via the skin when compared to unmodified and enzyme hydrolyzed products. Interestingly, only the most deamidated of the acid hydrolyzed products was capable of inducing sensitization via the skin in the rats with oral tolerance to wheat. These results indicate that the skin sensitization capacity of acid hydrolysates is associated with the degree of product deamidation, and thus correlates with the amount of new epitopes generated and the depletion of native unmodified gluten epitopes. The increased allergenicity of acid hydrolyzed gluten products is supported by previous animal studies addressing the sensitization capacity via the skin[12,47] and intraperitoneal[35,39,46,48] routes. However, only few studies have investigated the oral sensitizing capacity of hydrolyzed gluten, where our group has previously reported that wheat-naïve BN rats could be orally sensitized to enzyme hydrolyzed gluten, but not unmodified or acid hydrolyzed gluten products (without the use of adjuvants).[46] Furthermore, BN

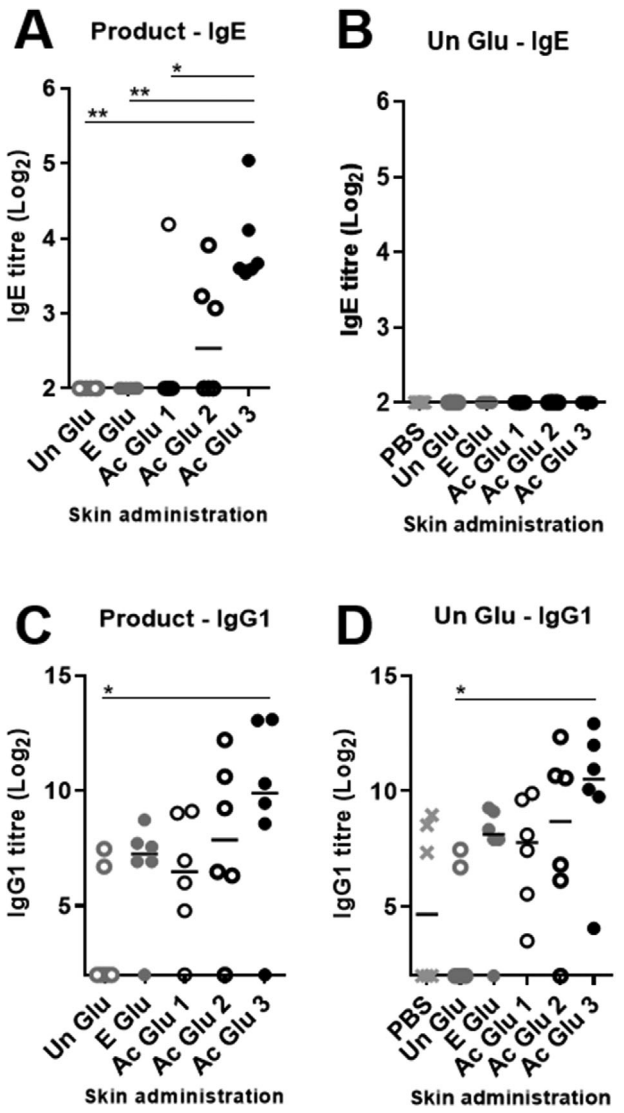

Figure 7. Sensitization to gluten products via the skin in wheat-tolerant rats. Unmodified gluten (Un Glu), enzyme hydrolyzed gluten (E Glu), acid hydrolyzed gluten (Ac Glu 1–3), or PBS (control) were applied to the slightly damaged abdominal skin of wheat-tolerant Brown Norway rats (three times per week for 5 weeks; $n = 6$ per group). Subsequently, rats were dosed by gavage twice with Un Glu at a 1-week interval. A) Serum product-specific IgE after skin application of 500 µg product. B) Serum Un Glu-specific IgE after skin application of 500 µg product. C) Serum product-specific IgG1 after skin application of 500 µg product. D) Serum Un Glu-specific IgG1 after skin application of 500 µg product. Each symbol represents an animal and horizontal bars indicate median value. $*p < 0.05$, $**p < 0.01$.

rats with oral tolerance to wheat could not be sensitized to unmodified or hydrolyzed gluten products via the oral route.[39] These findings highlight differences in the underlying mechanisms of sensitization via the skin and oral routes, which may depend on the specific physicochemical nature of the sensitizing allergen.

All hydrolyzed gluten products were able to promote cross-reactivity and clinical reactivity to unmodified gluten through the skin in wheat-naïve rats. However, the products were not able to break tolerance to unmodified gluten in rats with oral tolerance to wheat. This finding was unexpected, as the Ac Glu 3 was the same gluten product used in the acid hydrolyzed gluten-containing facial soap that was responsible for at least 2111 cases of wheat allergy in Japan,[19] where several patients experienced allergic symptoms after ingestion of food products containing unmodified wheat. It is important to notice, that only an estimated <0.1% of soap users developed allergic symptoms, and that symptoms usually first appeared after 1 year of use.[20] These findings support the cutaneous allergenicity of acid hydrolyzed gluten products, but indicates that the risk of wheat allergy and break of oral tolerance to wheat may be largely explained by individual underlying risk factors. Indeed, a recent genome-wide association study of the Japanese population found that allergy induced by the soap containing acid hydrolyzed gluten was associated with variations in the HLA-DR and RBFOX1 genes indicating a role for alterations in antigen recognition by T cells.[20]

The acid hydrolyzed gluten products were found to promote sensitization to more gluten proteins, as shown by a more diverse IgE reactivity pattern in 2D immunoblots. It could be speculated that this property may explain the ability of acid hydrolyzed gluten to break oral tolerance in humans after skin exposure. Indeed, wheat allergy induced by skin sensitization to acid hydrolyzed gluten may represent a specific allergy phenotype, as it is neither associated with sensitization to ω-5 gliadin found in wheat-dependent exercise-induced anaphylaxis (WDEIA) nor with filaggrin loss-of-function mutations commonly associated with other food allergies.[20] Deamidation by acid changes glutamine and asparagine into their corresponding acids, glutamate, and aspartate,[26,27] giving rise to more unique epitopes within the product. Furthermore, acid hydrolysis induces a wide variety of peptides that may stimulate immune responses. This increased antigenic diversity may increase the risk for cross-reactive IgE and break of tolerance, as the products contain a mixture of native and closely resembling epitopes, for which oral tolerance may be weak or incomplete. However, this hypothesis may not explain the increased cutaneous allergenicity of the acid hydrolyzed products in the absence of oral tolerance to wheat. We speculate, that acid hydrolysis promotes physicochemical modifications that serves as a type-2 immune adjuvant. Acid treatment of proteins can promote a wide variety of chemical modifications beyond hydrolysis and deamidation, including oxidation and amino acid stereoisomerization.[49,50] The immune stimulatory properties of such chemical modifications remain largely unexplored.

The skin sensitization to gluten products was found to drive the expansion of helper T cells in both the skin and small intestine LP, but not in the lungs or mesenteric lymph nodes. This finding suggests an immunological cross-talk between the skin and intestine, which could play a role in the expression of food allergy. Indeed recent studies have demonstrated the expansion of intestinal type-2 immune cells and increased elicitation of food allergy in models of AD-like skin damage and inflammation.[42,51] We did not observe differences in skin helper T cell expansion between the gluten products, indicating that differences in T cell related immune responses within the skin cannot explain the differences in product allergenicity.

In conclusion, this study found that all five gluten products were able to sensitize through the skin in a dose-dependent manner in the absence of oral tolerance to wheat. The acid hydrolyzed gluten products exhibited increased sensitizing capacity, which may correlate with the degree of deamidation. Skin sensitization to acid hydrolyzed gluten promoted sensitization to more proteins present in gluten. This diversification of IgE reactivity by acid hydrolyzed gluten products could be a trigger of wheat allergy in susceptible individuals.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

A.-S.R.B. and L.C. share first authorship. K.L.B. conceived the study. K.L.B., A.-S.R.B., L.C., and C.B.M. designed and conducted the animal studies. A.-S.R.B., C.V., and S.D.P. analyzed product physicochemical properties. A.-S.R.B. and L.C. performed serum antibody analyses. C.P., P.R., and G.M. performed IgE blotting and MS/MS analyses, respectively. L.C., S.B., D.A., and G.B. performed flow cytometry analysis. A.-S.R.B., L.C., J.M.L., and K.L.B. analyzed and presented the data. A.-S.R.B. and J.M.L. drafted the manuscript and K.L.B. revised it. All authors made substantial intellectual contributions, reviewed the manuscript critically, and approved the final version of the manuscript.

Data Availability Statement

Data available on request from the authors.

Keywords

Brown Norway rats, deamidation, hydrolysis, skin sensitization, wheat allergy

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