TECHNISCHE UNIVERSITÄT MÜNCHEN

TUM School of Life Sciences

Investigations on the allergenicity of gluten and hydrolyzed wheat proteins in wheat-dependent exercise-induced anaphylaxis

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Vollständiger Abdruck der von der TUM School of Life Sciences der Technischen Universität München zur Erlangung einer

Doktorin der Naturwissenschaften (Dr. rer. nat.)

genehmigten Dissertation.

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Die Dissertation wurde am 13.10.2021 bei der Technischen Universität München eingereicht und durch die TUM School of Life Sciences am 16.12.2021 angenommen.

Danksagung

Als Erstes möchte ich mich bei meiner Doktormutter, Frau Prof. Dr. Katharina Scherf, für die Bereitstellung dieses sehr interessanten Themas und die in jeder Hinsicht wunderbare Betreuung bedanken. Vielen Dank für die konstruktiven Gespräche und für die Freiheit und das Vertrauen meine eigenen Ideen entwickeln und verwirklichen zu können.

Vielen Dank an Herrn Prof. Dr. Knut Brockow für die angenehme Zusammenarbeit und Betreuung der Patiententestungen an der Biederstein Klinik. Danke für das Vertrauen in meine Ideen, die konstruktiven Gespräche und die Gelegenheit als Nicht-Medizinerin mal „hinter die Kulissen“ einer Klinik blicken zu dürfen. Das war für mich eine sehr interessante Erfahrung. Julia Gebhard danke ich ganz herzlich für die sehr gute Organisation der Patienten- und Kontrolltestungen sowie für die Durchführungen der Blutabnahmen und Hauttestungen. Vielen Dank auch an Franziska Martin für die Unterstützung bei der Durchführung der basophilen Aktivierungstests und an Frau Prof. Dr. Bernadette Eberlein für Ihre Expertise beim Auswerten der BAT-Daten. Ganz besonders danke ich allen Teilnehmenden, die sich für meine Dissertation haben Blutabnehmen lassen insbesondere allen WDEIA Patientinnen und Patienten, die sich auch zu Hauttests bereit erklärten.

Den Institutsleitungen Herrn Prof. Dr. Hofmann und Frau Prof. Dr. Somoza danke ich für die Möglichkeit, dass ich meine Promotion am Leibniz-Institut für Lebensmittel-Systembiologie an der Technischen Universität München bearbeiten konnte. Allen Mitarbeiterinnen und Mitarbeitern des Leibniz-Institut für Lebensmittel-Systembiologie an der Technischen Universität München und des Lehrstuhls für Lebensmittelchemie und molekulare Sensorik danke ich für die angenehme Arbeitsatmosphäre, den wissenschaftlichen und freundschaftlichen Austausch sowie die gute Zusammenarbeit und Hilfsbereitschaft.

Herzlich bedanke ich mich bei meinen Kolleginnen und Kollegen aus dem AK Scherf, Dr. Barbara Lexhaller, Dr. Sabrina Geißlitz, Dr. Claudia Vogel, Dr. Gerold Rebholz, Sarah Jöstl, Marie-Christin Norwig, , Dr. Anke Rohleder, Dr. Marina Schopf, Dr. Darina Pronin, Tanja Schirmer, Clemens Schuster, Angelika Graßl, Stephanie Hackl, Katharina Schießer und Alexandra Axthelm für die tolle Arbeitsatmosphäre, das freundschaftliche Miteinander und den wertvollen wissenschaftlichen Austausch.

Danke an Dr. Barbara Lexhaller, Dr. Sabrina Geißlitz und Dr. Claudia Vogel von denen ich im Laufe der Promotion viel gelernt habe. Angelika Graßl danke ich für die Unterstützung bei diversen Laborversuchen. Vielen Dank an Sabine Bijewitz für das erstellen vieler Grafiken für diese Arbeit.

Danke an meine gut gelaunte Mittagsessensgruppe, Carolina Schury, Daniela Füllemann, Sarah Jöstl, Robin Maier, Valerian Kalb, Dr. Sabrina Brendel, Silvia Lang und Caterina Porcelli, die mir jeden Tag mit einer lustigen Mittagspause versüßt haben. Herzlichen Dank an Katharina N'Diaye und Carolina Schury, meine liebsten Wegbegleiterinnen schon seit dem Studium. Von ganzem Herzen danke ich Elve, Sarah und Lisa. Danke, dass ich immer auf euch zählen kann – ihr seid die Besten!

Liebsten Dank an meine Eltern, meine Schwestern und meinen Opa, die mich alle stets unterstützt und immer an mich geglaubt haben.

Vorbemerkung

Der praktische Teil dieser Arbeit wurde von Juli 2017 bis Dezember 2020 unter der Leitung von Prof. Dr. Katharina Anne Scherf am Leibniz-Institut für Lebensmittel-Systembiologie an der Technischen Universität München (bis August 2017: Deutsche Forschungsanstalt für Lebensmittelchemie, Leibniz Institut) in Freising durchgeführt.

Der klinische Teil der Arbeit wurde unter der Leitung von Prof. Dr. Knut Brockow an der Fakultät Medizin der Technischen Universität, Department für Dermatologie und Allergie am Biederstein in München durchgeführt.

Preliminary releases

Peer-review publications

Gabler AM, Scherf KA. Comparative characterization of gluten and hydrolyzed wheat proteins. *Biomolecules, **2020**, 10*(9): 1227.
Doi: 10.3390/biom10091227

Gabler AM, Gebhard J, Eberlein B, Biedermann T, Brockow K, Scherf KA. The basophil activation test differentiates between patients with wheat-dependent exercise-induced anaphylaxis and control subjects using gluten and isolated gluten protein types. *Clinical and Translational Allergy*, **2021**, e12050.
Doi: 10.1002/clt2.12050

Gabler AM, Gebhard J, Norwig MC, Eberlein B, Biedermann T, Brockow K, Scherf KA. Basophil activation to gluten and non-gluten proteins in wheat-dependent exercise-induced anaphylaxis. *Frontiers in Allergy*, **2022**, *3:822554*.
Doi: 10.3389/falgy.2022.822554

Additional publications

Gabler AM, Scherf KA. Aufklärung grundlegender Mechanismen der weizenabhängigen anstrengungsinduzierten Anaphylaxie. *47. Deutscher Lebensmittelchemikertag, Berlin, Lebensmittelchemische Gesellschaft, **2018**,* ISBN 978-3-947197-08-8

Gabler AM, Ess D, Winkler J, Brockow K, Scherf KA. Aufklärung grundlegender Mechanismen der weizenabhängigen anstrengungsinduzierten Anaphylaxie (WDEIA). *48. Deutscher Lebensmittelchemikertag, Dresden, Lebensmittelchemische Gesellschaft, **2019**.* ISBN 978-3-947197-15-6

Gabler AM, Brockow K, Scherf KA. Kofaktor-induzierte Weizenallergie. *Deutsche Lebensmittel Rundschau*, **2019**, *115*, ISSN 0012-0413

International Oral Presentations

Gabler AM, Brockow K, Scherf KA, Elucidation of the fundamental mechanisms of wheat-dependent exercise-induced anaphylaxis. *18th European Young Cereal Scientists and Technologists Workshop (EYCSTW); San Benedetto del Tronto (Italy), April 15.-17.* ***2019***

Gabler AM, Winkler J, Eberlein B, Biedermann T, Scherf KA, Brockow K, Characterization of individual sensitization profiles in patients with WDEIA using BAT with gliadins, glutenins and hydrolyzed wheat proteins. *European Academy of Allergy and Clinical Immunology (EAACI) Congress; London (United Kingdom) / Online, June 06.-08.* ***2020***

Poster presentations

Gabler AM, Scherf KA. Aufklärung grundlegender Mechanismen der weizenabhängigen anstrengungsinduzierten Anaphylaxie. (Elucidating fundamental mechanisms of wheat-dependent exercise-induced anaphylaxis). *47. Deutscher Lebensmittelchemikertag (47. German Food Chemists' Day); Berlin, 17.-19.09.****2018***

Gabler AM, Ess D, Winkler J, Brockow K, Scherf KA. Aufklärung grundlegender Mechanismen der weizenabhängigen anstrengungsinduzierten Anaphylaxie (WDEIA). (Elucidating fundamental mechanisms of wheat-dependent exercise-induced anaphylaxis). *48. Deutscher Lebensmittelchemikertag (48. German Food Chemists' Day); Dresden, 16.-18.09.****2019***

Gabler AM, Ess D, Winkler J, Brockow K, Scherf KA. Aufklärung grundlegender Mechanismen der weizenabhängigen anstrengungsinduzierten Anaphylaxie (WDEIA). (Elucidating fundamental mechanisms of wheat-dependent exercise-induced anaphylaxis). *Kongress für Ernährungs- und Sportmedizin (Congress of Nutritional and Sports Medicine); Erlangen, 22.-23.11.****2019***

Winkler JK, Gabler A, Scherf K, Kugler C, Biedermann T, Brockow K. Skin prick sensitization profiles to gluten fractions in patients with wheat-dependet exercise-induced anaphylaxis. *European Academy of Allergy and Clinical Immunology (EAACI) Congress; Online, 06.-08.06.****2020***

Table of contents

Abbreviations

ATI	α-Amylase/trypsin inhibitor
ATIs	α-Amylase/trypsin inhibitors
ATS	Allergen test solution
BAT	Basophil activation test
BG	Basophil granulocyte
BGs	Basophil granulocytes
CO-WDEIA	Conventional / classical wheat-dependent exercise-induced anaphylaxis
CD	Cluster of differentiation
DDA	Data-dependent acquisition
DIA	Data-independent acquisition
DTT	Dithiothreitol
ESI	Electrospray ionization
FACS	Fluorescence-activated cell sorting
FAb	Fluorescence-dye labeled antibody
FITC	Fluorescein isothiocyanate
FSC	Frontward scatter
Glia	Gliadins
Glut	Glutenins
GS	Glutenin subunits
GP	Gel permeation
GPT	Gluten protein type
HMW	High-molecular-weight
HPLC	High-performance liquid chromatography
HWP	Hydrolyzed wheat protein
IBAQ	Intensity-based absolute quantitation
Ig	Immunoglobulin
LC	Liquid chromatography
LMW	Low-molecular-weight
LTP	Lipid-transfer protein
mAb	Monoclonal antibody
MALDI	Matrix-assisted laser desorption/ionization

M_r	Relative molecular mass
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NCGS	Non-coeliac gluten sensitivity
NSAID	Non-steroidal anti-inflammatory drugs
OFC	Oral food challenge
PAGE	Polyacrylamide gel electrophoresis
PE	Phycoerythrin
PWG	Prolamin Working Group
RP	Reversed-phase
SDS	Sodium dodecyl sulfate
SPT	Skin prick test
SSC	Sideward scatter
TCEP	Tris(2-carboxyethyl)phosphine
TripleTOF	Triple quadrupole time-of-flight
Tris	Tris(hydroxymethyl)aminomethane
TOF	Time-of-flight
UPLC	Ultra-high performance liquid chromatography
UV	Ultraviolet
WDEIA	Wheat-dependent exercise-induced anaphylaxis

One letter code for amino acids

Alanine	A
Arginine	R
Asparagine	N
Aspartic acid	D
Cysteine	C
Glutamic acid	E
Glutamine	Q
Glycine	G
Histidine	H
Isoleucine	I
Leucine	L
Lysine	K
Methionine	M
Phenylalanine	F
Proline	P
Serine	S
Threonine	T
Tryptophan	W
Tyrosine	Y
Valine	V

1. Introduction

1.1. Allergy

1.1.1. The human immune system

The human immune system can be divided into innate and adaptive immune defense. The innate immune defense is effective from birth and operates with unspecific defense mechanisms. They are independent of previous contacts to pathogens. The adaptive immune defense is specific and operates against exogenous pathogens. This acquired immune response is triggered by cells which are part of the innate immune defense after the first contact to such an exogenous pathogen. It can be subdivided into humoral and cellular immune response [1,2].

In the cellular immune response specific immune cells identify infected, damaged or transformed cells as exogenous and eliminate them. The humoral immune defense is mediated by different proteins in body fluids such as blood. Due to the binding of antibodies to a pathogen (antigen), the antigen is identified as exogenous. As a result, the production of antibodies increases and the so-called complement system is activated. Antibodies are soluble immunoglobulins (Ig) of the blood plasma and specific defense proteins. In humans, they are divided into five main classes: IgG, IgA, IgE, IgM and IgD. Additionally, monoclonal and polyclonal antibodies are distinguished, which means they can recognize and bind one or several antigen-epitopes, respectively. In addition to their effect as a mediator, antibodies can also have a direct impact on antigens by complex formation. This leads to a direct inactivation of the antigen [1,3].

The adaptive immune response is highly specific. It is based on the selective reproduction of B-lymphocytes, which can bind the exogenous antigen. These immune cells are present in the blood circulation and display a particular antibody on their cell surface. When an antigen binds to an expressed antibody (plasma blast), the cell division of the B-lymphocyte starts. A clone is expanded and the former B-lymphocyte itself differentiates into a plasma cell (effector cell). Also, durable memory cells are generated out of plasma blasts, leading to an immunological memory. In this way the immune system can “remember” previous antigens. With renewed contact, the body is able to react much faster and with a higher number of specific antibodies against the antigen. This process is called sensitization [1,2].

1.1.2. Definition and classification

Allergic reactions are part of the adaptive immune response and represent a hypersensitivity reaction of the immune system against an antigen (allergen). At the first contact with the antigen, usually via oral, dermal or inhalative absorbtion, there is not yet an allergic reaction, because there are no specific antibodies present in the blood circulation. Thus, sensitization takes place upon first contact. With this sensitization, the body starts to produce specific antibodies against the antigen, which are present in the blood circulation afterwards. Consequently, the second and all following contacts to this antigen lead to an allergic reaction, because specific antibodies were generated. The severity of an allergic reaction as well as the symptoms in this regard are very different. Four types of allergic reactions are distinguished: Immediate-type- (Type-I-), cytotoxic-type- (Type-II-), immune-complex-mediated-type- (Type-III-) and late-type- (Type-IV-) allergy. With a share of 90 %, the immediate-type is the most common allergy type. Thereby, the allergic reaction happens within a few minutes after exposure to the antigen [1,4].

The IgE-antibodies bind to the antigen with the side of the F(ab')$_2$-fragment. With the Fc-fragment, the antibodies connect to a high-affinity FcεRI-receptor expressed on the cell surface of basophilic granulocytes and mast cells (**Figure 1, 2**). Afterwards the cell gets activated and degranulates. Mediators such as histamine, cytokines, prostaglandins, tryptase, leukotrienes, platelet activating factor and chemokines are released. As a result, blood vessels are dilated locally and their permeability is increased. In this way, more antibodies, complement system and immunoreactive cells stream from the blood into the tissue and "fight" the infection. The mediators also trigger a large number of different physical symptoms, for example swelling of mucosal membranes and a drop in blood pressure. The worst case is an anaphylactic shock [1,4,5].

The most serious event in allergology is the occurrence of an anaphylaxis. It is an acute systemic reaction, which usually happens within 1-4 hours after exposition to the causative allergen and constitutes an immediate-type-allergic reaction. In anaphylaxis, different degrees of severity are distinguished and can show respiratory, cutaneous, gastrointestinal and cardiovascular signs. They range from mild symptoms such as itching skin to serious symptoms like cardiovascular arrest, with potentially life-threatening outcome. **Table 1** shows the classification of severity and symptoms. However, not all symptoms need to occur. The classification is based on the most serious symptom [4,6].

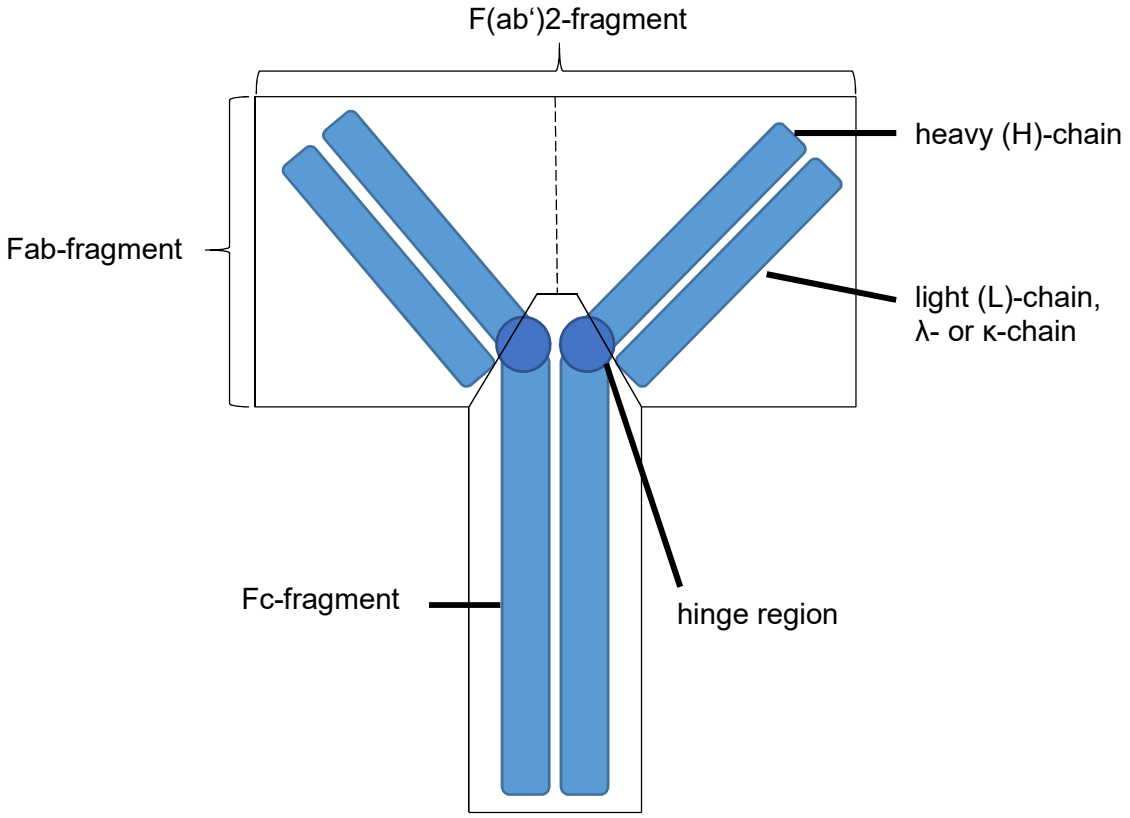

Figure 1: Scheme of an immunoglobulin E antibody (IgE). It can be divided into F(ab')2-fragment, two Fab-fragments respectively, and Fc-fragment, consisting of light and heavy chains with hinge regions. Figure modified from [5].

Peanuts, wheat, fish, chicken egg and crustaceans are the most abundant foods known to trigger an anaphylaxis. In the past years, the importance of cofactors regarding anaphylaxis was recognized. Cofactors are an additional irritation for the human body. Examples for such influencing factors are physical activity, alcohol consumption, intake of non-steroidal anti-inflammatory drugs (NSAID), emotional stress and female menstruation [4,7,8].

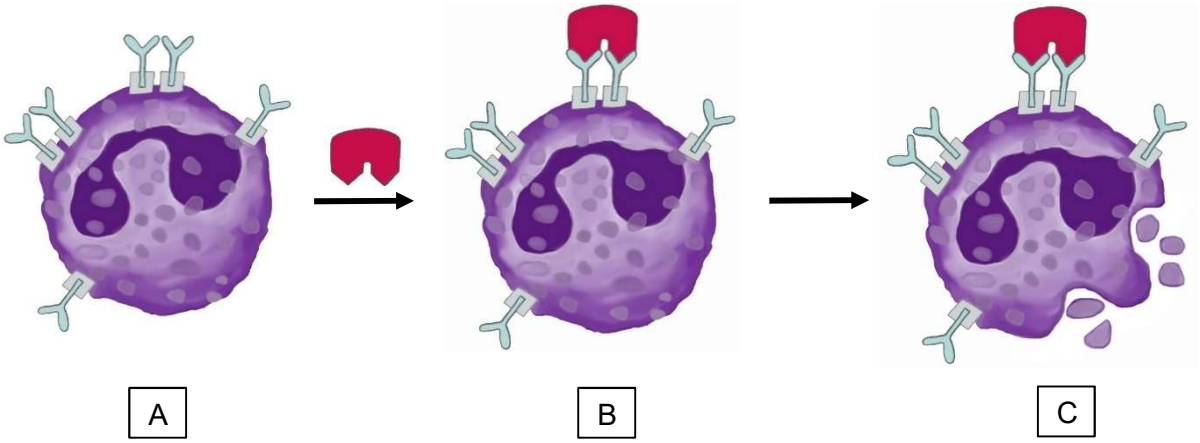

Figure 2: Schematic presentation of a basophil granulocyte (A) reacting with an allergenic substance (antigen, red). The allergen binds to the high-affinity-receptor FcεRI via cross-linking with specific IgE-antibodies (B) which results in basophil degranulation and release of mediators (C).

Table 1: Classification of anaphylaxis according to severity with corresponding symptoms, modified from Biedermann et al. [4].

		symptoms			
severity		**skin**	**gastrointestinal tract**	**respiratory tract**	**cardiovascular system**
I	low systemic reaction	itching flushing urticaria angioedema	-	-	-
II	distinctly systemic reaction		nausea cramps	rhinorrhea hoarseness dyspnoea	tachycardia hypotension arrhythmia
III	threatening systemic reaction		vomiting defecation	laryngeal edema bronchospasm cyanosis	shock unconsciousness
IV	vital organ failure		vomiting defecation	apnea	cardiovascular standstill

1.2. Wheat-dependent exercise-induced anaphylaxis

Wheat-dependent exercise-induced anaphylaxis (WDEIA) is rare, but potentially life-threatening. It is a special form of food-dependent exercise-induced anaphylaxis (FDEIA) and is classified as immediate type-I allergy [7,8]. The exact placement in the field of wheat-related disorders is shown in **Figure 3** [7,9]. WDEIA is characterized by the fact that an allergic reaction only occurs after exposition to wheat in combination with one or more cofactors [7,10,11]. The occurring symptoms correspond to the described symptoms for anaphylaxis shown in **Table 1**.

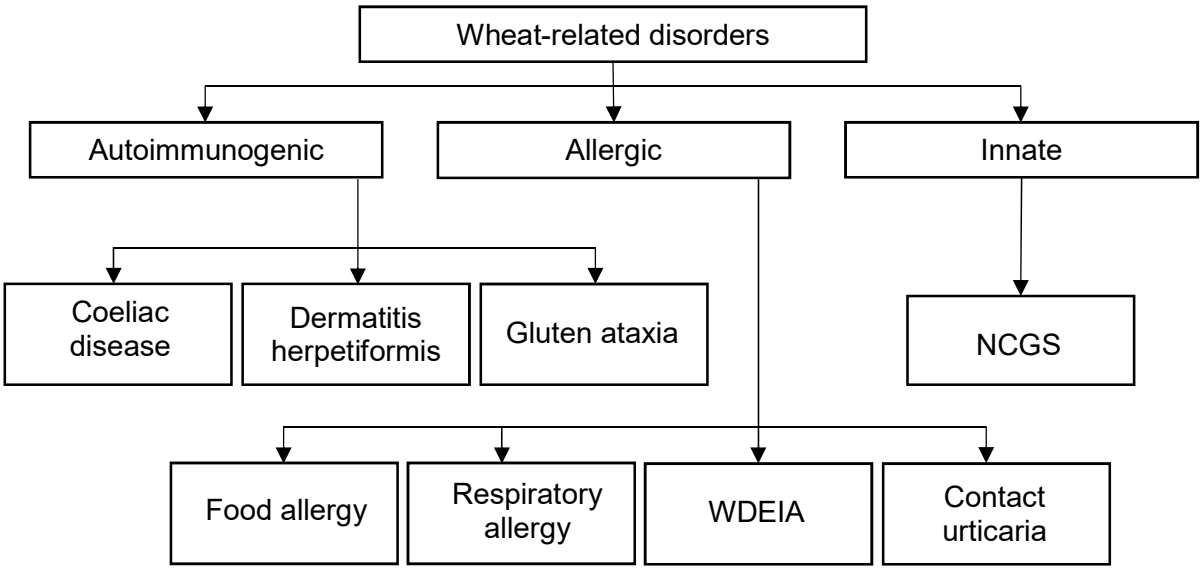

Figure 3: Spectrum of wheat-related disorders with autoimmunogenic, allergic and innate pathomechanism. NCGS = non-coeliac gluten sensitivity, WDEIA = wheat-dependent exercise-induced anaphylaxis [9,12].

1.2.1. Cofactors

The main cofactors of WDEIA are physical exercise, the intake of non-steroidal anti-inflammatory drugs (NSAID) for example acetylsalicylic acid, alcohol consumption, infectious diseases and stress. In case of physical exercise as cofactor, the interval between wheat intake and physical exercise is reported to be about 30 to 120 minutes. The intensity of the activity is variable, ranging from walking to exhausting exercise like running or cycling. Which parameters (pulse, duration, intensity, maximum oxygen uptake) have to be reached to trigger an anaphylaxis are not known yet [7,8,13].

Though WDEIA is designated as best-studied model of cofactor-induced anaphylaxis, the mechanisms how cofactors act are not completely understood. It has been reported that physical exercise disrupts the intestinal barrier function and consequently increases the bioavailability of allergens. The theory is that the permeability of the gut is increased because of physical exercise, caused by a dysregulation of the tight junctions in the intestinal epithelium. This results in a higher uptake of partially digested wheat proteins into the blood circulation. In this way more allergens are distributed in the whole body. Consequently, an IgE-mediated immune response takes place (**Figure 4**) [7,8,14].

The same theory is also suggested for other cofactors like NSAIDs, alcohol and infections. Another postulated mechanism is that physical activity as well as other cofactors lower the activation threshold of basophilic granulocytes and mast cells. That means these immune cells are IgE-activated at lower amounts of allergenic substance, compared to the usual case. Consequently, degranulation and release of mediators happen earlier [7,8,14]. In the context of cofactors, gut microbiota may play a role, too [15]. Matsuo et al. (2005) describe that physical exercise decreases the pH-value of the stomach. Therefore, the solubility of the ingested wheat proteins increases. Acetylsalicylic acid as well as alcohol lead to a similar increase in solubility. This raises the absorption later on [13,16].

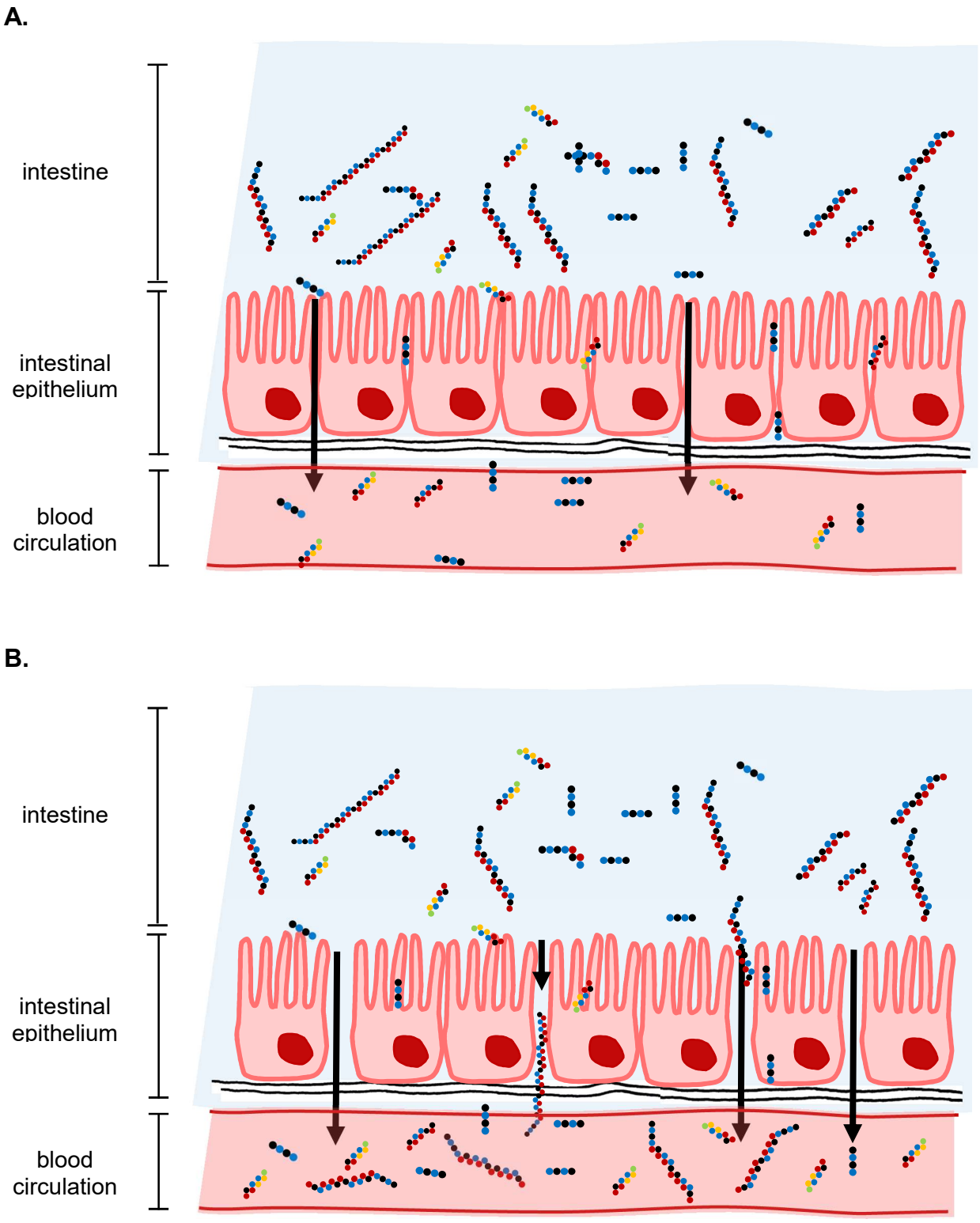

Figure 4: Possible mechanism of cofactors in the intestine of a WDEIA patient (**A**). Without cofactor: Only small digested peptides pass the intestinal epithelium and enter the blood circulation (**B**). With cofactor: Increased permeability, because of dysregulation of the tight junctions in the intestinal epithelium. Small and larger peptides pass the intestinal epithelium, in a larger quantity and enter the blood circulation.

1.2.2. Allergens

The allergic reaction is triggered by the combination of wheat proteins and cofactor. The ω5-gliadins (Tri a 19) are mostly reported to be major allergens and high-molecular-weight glutenin subunits (HMW-GS) to be minor allergens in WDEIA. About 80% of WDEIA patients show specific IgE against ω5-gliadins.[7,17,18] Reactions to other gluten protein types (GPT) from wheat are reported, like α- and γ-gliadins and low-molecular-weight glutenin subunits (LMW-GS). All GPT together build gluten, the storage protein in wheat flour [7,11,17,19].

Furthermore, Konstantinou et al. (2018) suggest that soluble wheat proteins present in the albumin/globulin fraction, like the lipid-transfer protein (LTP, Tri a 14), play a role in WDEIA.[20] Pastorello et al. (2007) and Battais et al. (2005) connected α-amylase/trypsin-inhibitors with WDEIA, potentially via IgE-cross reactions[21,22]. Matsuo et al. (2004) used synthetic peptides to identify seven allergenic epitopes within the primary sequence of ω5-gliadins (QQIPQQQ, QQLPQQQ, QQFPQQQ, QQSPEQQ,QQSPQQQ, QQYPQQQ, and PYPP) in pooled sera of 15 WDEIA-patients. Furthermore, four epitopes of them were found to be most immune reactive in WDEIA (QQIPQQQ, QQFPQQQ, QQSPEQQ, and QQSPQQQ) [17].

Wheat gluten is often used as ingredient in foods and cosmetics, because of its foaming, emulsifying, swelling and gelling properties, water-retention capacity, improvement of baking properties and texture and for protein-enrichment. In the food industry, besides bakery products, gluten is also used in various non-cereal products, in which the consumer generally does not expect the presence of gluten, for example in vegetarian food or in meat products [23,24].

Wheat gluten is poorly soluble in water. Therefore, it is treated in different chemical, biochemical and physical ways to improve solubility by modifications and/or partial hydrolysis. Hydrochloric or sulfuric acid are commonly used for acid hydrolysis and sodium hydroxide for alkaline hydrolysis. For the enzymatic hydrolysis, enzymes like trypsin, papain or pronase are used [23,25-27].

During acidic and alkaline hydrolysis deamidation takes place. In case of enzymatic hydrolysis, the deamidation depends on the enzyme used. Additionally, modifications of gluten can be carried out using physical approaches, like heat-treatment, UV-irradiation, high-pressure processing and extrusion.[23,24,28]

Hydrolysis results in the formation of proteins and peptides with lower relative molecular mass (M_r), deamidation and change of the net charge. Treatment with alkali usually does not lead to a cleavage of peptide bonds, it rather leads to the removal of intermolecular disulfide-bonds. All these processes lead to an increase of solubility, the resulting products are named hydrolyzed wheat proteins (HWP) [23,29].

Besides the patients, who react to intact proteins and belong to the group of conventional or classical WDEIA (CO-WDEIA), there is another WDEIA subgroup named HWP-WDEIA. Thereby, the patients react to HWP while tolerating intact wheat proteins [7,30]. Yokooji at al. (2013) and Hiragun et al. (2013) reported allergic reactions to HWP in cosmetics and soap in connection with WDEIA [31,32]. Chinuki and Morita (2012) examinated sera from HWP-WDEIA patients and found no or low levels of specific IgE against ω5-gliadins. These findings stand in contrast to CO-WDEIA, having specific IgE against ω5-gliadins in 80% of all cases. Additionally, they found characteristic features of facial angioedema in HWP-WDEIA patients, but not in CO-WDEIA patients [30].

Parts of the allergenic epitopes already pre-exist in native wheat protein aggregations and are laid bare through hydrolysis, but are not destroyed. Hydrolysis and deamidation might lead to the creation of new allergenic epitopes [31,32]. Also the increased solubility of HWP and the route of exposure affects their immunoreactivity. Therefore, the type and degree of hydrolysis play an essential role, because it influences the transport through natural barriers, like the skin or the small intestinal epithelium [7,31-33].

1.2.3. Diagnosis

For diagnosis of food allergy and anaphylaxis, different tools based on common guidelines are in use. First, the patient is examined and his or her clinical history is evaluated. In doing so, a lot of important basic information can be collected, ranging from exposition, symptoms, family history, severity, known risk cofactors, dietary history, to timing and chronicity [34]. Hereafter, diagnostic test approaches are carried out, like skin prick test (SPT), scratch test, atopy patch test or the intradermal test. In case of SPT, false positive test results can take place. Additionally, false negative results can happen because of the intake of antiallergenic drugs like anti-histamines [4,34]. In case of WDEIA, SPT is performed with wheat flour, gluten or wheat extracts [7,35]. The next step is the determination of specific IgE against suspected allergens. Specific IgE against wheat, gluten and ω5-gliadins are investigated for diagnosing WDEIA [7,34,35].

The golden standard to diagnose WDEIA is oral food challenge (OFC). The patient is specifically challenged with the causative food or gluten in combination with one or more cofactor(s). Infections and mental stress, as cofactors in WDEIA, are difficult to follow. The combination with NSAIDs, physical exercise and alcohol can be tested. In the challenge test, cofactor and wheat product are first tested separately, then together. The combination of both triggers WDEIA. The OFC is complicated because the amount of wheat product as well as the amount/intensity of the cofactor are individual. The test is placebo-controlled. OFC is hampered by the fact that WDEIA is not fully reproducible [7,8,35]. The execution of the challenge test is no safe procedure as it can lead to an anaphylactic shock [17]. When performing an OFC or SPT, the presence of medical professionals is crucial. Both diagnosis tools can trigger allergenic symptoms [4,34].

1.2.4. Treatment and management

In an acute situation, when anaphylaxis happens, emergency therapy is indispensable. If tolerated, it is recommended to place the patient in a supine position, elevate the legs in order to prevent blood accumulation in the lower extremities, and administer fluids. Patients are treated with anti-histamines and glucocorticoids with non-specific function to provide short-term relief from symptoms. In case of a therapy resistant circulatory shock noradrenalin is injected. Every patient suffering from the risk of anaphylaxis should take an emergency kit with them. Among other things, it always contains an adrenalin spray or adrenalin injection set [6,36-38].

In case of an acute occurrence of WDEIA, it is crucial to immediately abort physical effort and allergen supply. It is necessary to pay attention to early warning signals to prevent cardiovascular symptoms which can have a potentially life-threatening outcome. The patient should carry an emergency kit, containing drugs as antihistamines, systemic corticosteroids and an adrenaline injector. It is necessary to teach the patient how to use the emergency kit for self-medication. A first aider must take an emergency call, calm down the patient, bring the patient in a stable lateral position in case of unconsciousness, secure the patient's respiratory tract and, if necessary, ventilate. After treatment of the allergenic symptoms the patient needs stationary monitoring. In case of life-threatening anaphylaxis, the patient is monitored in the intensive care unit. Moreover, to prevent further episodes of WDEIA the patient must be educated about the disease. The patients should document their diet and get an anaphylaxis management plan [4,7,34,38]. For WDEIA there is no prospective data on the optimal management plan. Depending on the severity, it is recommended to avoid exercise and NSAID administration after exposition to wheat or eliminate the exposition to the allergenic substance in total [7,8]. It is not entirely clear, if cross-reactions to rye, barley and oats occur and if other gluten-containing cereals besides wheat need to be avoided, too [7].

1.3. Analysis of gluten and hydrolyzed wheat proteins

To characterize gluten and HWP to be used in a clinical human study, it is crucial to use appropriate analytical methods with high sensitivity and specificity. Wieser et al. (1994, 1998), Kasarda (1998) and Lagrain et al. (2012) reported about the most common analysis methods for wheat proteins. Generally, the analysis is divided in three main steps. The first step is the complete extraction of gluten proteins or peptides from the sample matrix. Thereby, the extraction procedure needs to be suitable for crude gluten and HWP. In the second step, an accepted reference material is applied for calibration. The third step is the quantitation of the extracted proteins and peptides. Chromatography and electrophoresis are suitable techniques to analyze wheat proteins [39-42].

1.3.1. Extraction of gluten proteins and wheat protein hydrolysates

The first step of gluten and HWP analysis is the complete extraction out of the raw material. Gluten proteins are poorly soluble in water because of their highly complex structure with inter- and intramolecular disulfide bonds. The non-gluten proteins, namely albumins and globulins, are soluble in water or salt solution. They were pre-extracted before extracting the gluten proteins, gliadins and glutenins. Depending on the degree of hydrolysis, HWP have an increased solubility in water and are better extractable with water or salt solutions as well as aqueous alcohol solutions in comparison to gluten.[16,23,40] Gliadins are extracted with aqueous alcohol solutions containing ethanol or propanol. According to Codex Stan 118 (2015) the use of 40-70% ethanol for prolamin extraction was defined [43]. For the optimal extraction of gliadins from wheat flour Wieser et al. (1994) recommended 60% ethanol.[3] For glutenin extraction reducing conditions are required. Dithiothreitol (DTT), tris(2-carboxyethyl)-phosphine (TCEP) or 2-mercaptoethanol can be used. Because of its toxicity and unpleasant odor 2-mercaptoethanol is used less frequently. TCEP has been used for reducing gluten proteins in several studies, for example in the work of Schalk et al. (2017). In cereal chemistry, DTT is widely used as effective agent to reduce disulfide bonds [40,44,45]. This approach can also be applied to the investigation of gluten and HWP.

Summarizing the named criteria, the modified Osborne fractionation by Wieser et al. (1998) represents a reliable extraction method for wheat proteins [40]. The workflow is shown in **Figure 5**.

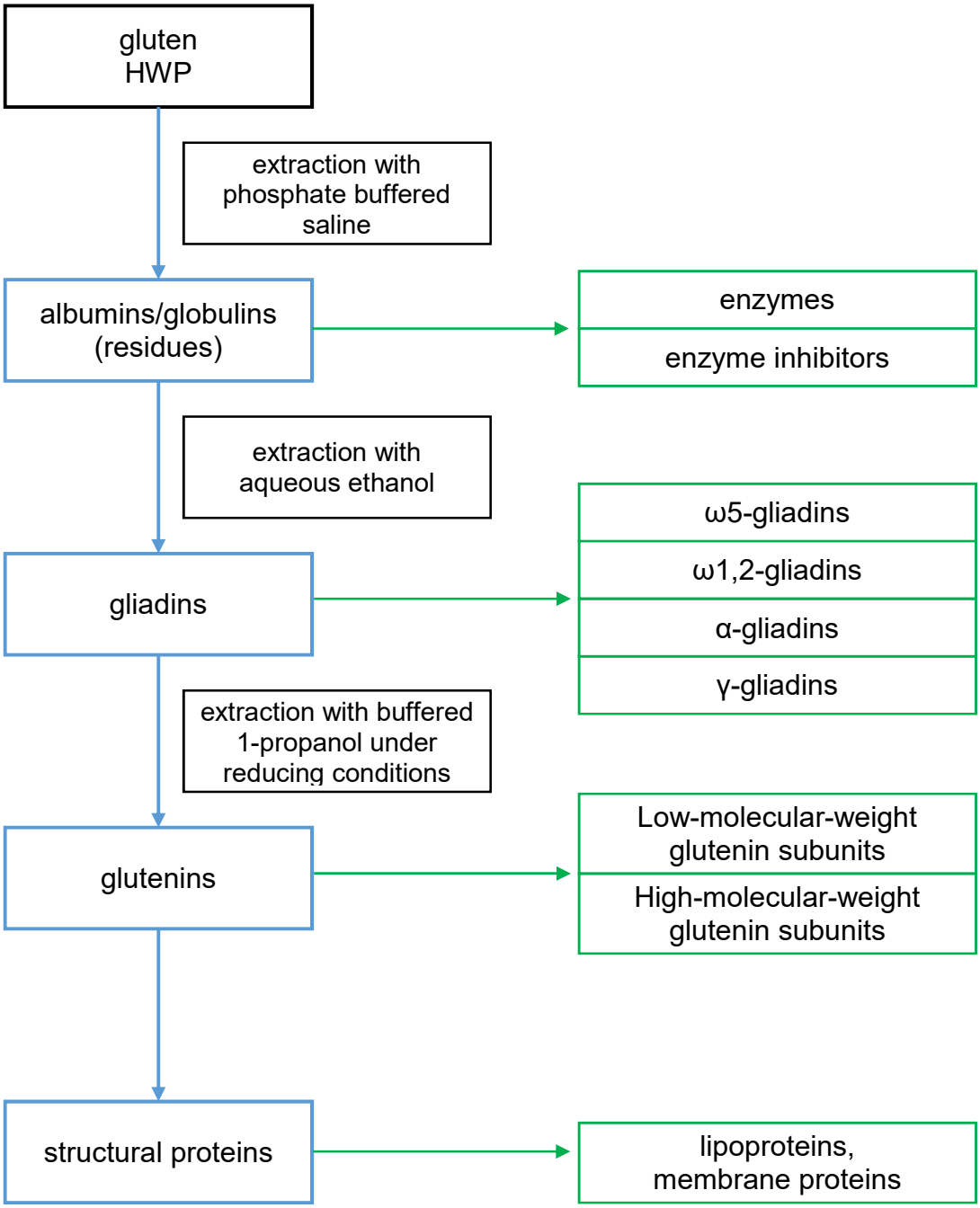

Figure 5: Procedure of the modified Osborne fractionation and overview about proteins contained in the respective fraction, adapted from [40]. Gluten or hydrolyzed wheat protein sample (HWP) is extracted stepwise with phosphate buffered saline, aqueous ethanol, and buffered 1-propanol under reducing conditions. Structural proteins remain as insoluble residue. In the albumin/globulin-fraction enzymes and enzyme inhibitors are predominant. The gliadin fraction is subdivided in ω5-, ω1,2-, α- and γ-gliadins and the glutenin fraction in low- and high-molecular-weight glutenin subunits.

1.3.2. Reference materials

A reference material is defined as “material, which is sufficiently homogeneous and stable with respect to one or more specified properties and has been established to be fit for its intended use in the measurement process” by the ISO Guide 30:2015 [46]. Reference materials are used for calibration procedures and instrument measurements. For quality assurance and to generate comparable analytical results between different laboratories and methods, standardized and well-characterized reference materials are necessary. Reference materials also play an essential role in the validation of measurements.

Gluten is a complex mixture of different proteins, with a high variation in their molecular weight. Additionally, they have a high biological variability due to modifications in the amino acid composition, because of deletion or exchange of amino acids. Furthermore, variable protein content and posttranslational modifications occur. Due to these reasons, the standardization of a gluten reference material is challenging [47].

At this time, the so-called PWG-gliadin is the only well-characterized gluten reference material. It was developed in 2006 by the Working Group on Prolamin Analysis and Toxicity. PWG-gliadin was isolated from a mixture of 28 wheat cultivars from France, Germany and UK, which are the three main wheat producing countries in Europe. Even though the PWG-gliadin reference material only represents the alcohol-soluble fraction of gluten, it is well suited to quantitate all types of wheat proteins.[48,49] PWG-gliadin is used for the calibration of reversed-phase high performance liquid chromatography (RP-HPLC) measurements, as reported by Schalk et al. (2017) and Lexhaller et al. (2017) [44,50].

1.3.3. Chromatography

For the analysis of gluten proteins HPLC has been in use since a long time. Reversed-phase (RP) and gel-permeation (GP) HPLC are widely used techniques. Gluten is separated by different hydrophobicity (RP) or different M_r (GP) of gluten protein types. Wieser et al. (1998) developed an approach for the quantitation of different wheat protein types in flour. Thereby, a procedure for gluten protein extraction and RP-HPLC measurement was described. The proteins in the flour were extracted into three fractionation steps. The first fraction contains the water- and salt-soluble albumins and globulins, the second fraction the alcohol-soluble gliadins (Glia) and the third fraction the glutenins (Glut), which are soluble under reducing conditions. The UV absorbance at 200-220 nm was used for protein detection eluting from the HPLC column. The reference material PWG-gliadin was applied for calibration (1.3.2.) [40,48].

Scherf et al. (2016) developed a quantitation method for gluten in wheat starch by GP-HPLC. The limit of detection in RP-HPLC was 300 mg/kg when using UV detection. Using laser-induced fluorescence for detection, the intensity was about 100-fold higher compared to UV detection. Consequently, it was possible to quantify lower amounts of gluten, up to 17.2 mg/g as sum of gliadins and glutenins. The described chromatographic methods are limited to the detection of gluten in raw materials because of low specificity [51,52].

1.3.4. Gel electrophoresis

Gel electrophoresis is an analytical approach to separate and analyze proteins. Depending on the research question it can consist of different materials, like agarose or polyacrylamide with different pore sizes. The electrophoresis gel is embedded in an ionic electrophoresis buffer and its pockets are loaded with protein solution. The charged proteins move in the electrophoresis gel in an electric field in relativity to their charge and molecular size. When using an approach with a polyacrylamide gel and sodium dodecyl sulfate (SDS) as denaturing agent, proteins are just separated by their M_r. The reason for this is that the detergent SDS overlays the net charge of the proteins with its strong negative charge [53].

When using a mixture of marker proteins in the electrophoresis in addition, it is possible to determine the M_r of protein bands. To visualize protein bands, the gel is stained, for example with silver nitrate or Coomassie Brilliant Blue [42,45].

SDS polyacrylamide gel electrophoresis (SDS-PAGE) is widely used in protein research. Kasarda et al. (1997), Lagrain et al. (2012) and van den Broeck et al. (2009) used the SDS-PAGE approach for the analysis of different grain proteins [41,42,45].

1.4. Proteomics-based liquid chromatography mass spectrometry

Sequential extractions are carried out for the qualitative and quantitative analysis of wheat proteins. This is commonly combined with gel-electrophoresis [42] and chromatographic techniques, like GP- [51] or RP-HPLC with UV detection [40,44]. However, these techniques are not powerful enough to detect traces of wheat proteins, because of their inadequate selectivity and specificity. Among others, modern proteomics workflows use efficient separation techniques, like ultra-high performance LC (UPLC), combined with high-resolution mass spectrometry (MS). The MS system of choice, depends on the research question. Matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) MS [48,54,55] or LC coupled to electrospray ionization (ESI) triple quadrupole TOF MS (LC-TripleTOF-MS) [42,55] are often used and are suitable to analyze gluten proteins. Mostly, the wheat proteins were extracted from the sample and subsequently hydrolyzed. The hydrolysis is typically performed using enzymatic digestions with e.g. trypsin, chymotrypsin, pepsin, other peptidases or combinations of them [56-58]. Then, the generated peptides are analyzed further by LC high resolution MS/MS techniques. The analysis of peptides is more sensitive compared to protein analysis. The targeted analysis is routinely performed with triple quadrupole MS systems [59,60]. For untargeted analysis, TripleTOF (**Figure 6**) or quadrupole ion trap MS systems can be used, either in data-dependent (DDA) or data-independent acquisition mode (DIA) [55,61,62].

The common workflow of untargeted LC-MS/MS analysis starts with the protein extraction, followed by the reduction of disulfide bonds and the alkylation of free thiol groups. Subsequent steps are the enzymatic hydrolysis, purification by solid-phase extraction (SPE) and filtration [55,58]. The resulting peptide mixture is measured with LC-high resolution MS/MS. The MS dataset is analyzed and visualized using bioinformatics software and algorithms (e.g. MaxQuant) [63] for peptide identification. Matching against protein databases (e.g. Uniprot [64]) is performed [59,65,66].

The quantitation of peptides and their corresponding proteins, can be compared relatively within the sample set by using label-free quantitation, such as the intensity-based absolute quantitation algorithm (IBAQ). In this algorithm, all intensities of the precursor ions of all detectable peptides per protein are considered and summed up. Then, this sum is divided by the number of theoretically detectable peptides to determine the IBAQ intensity of a protein [60,67].

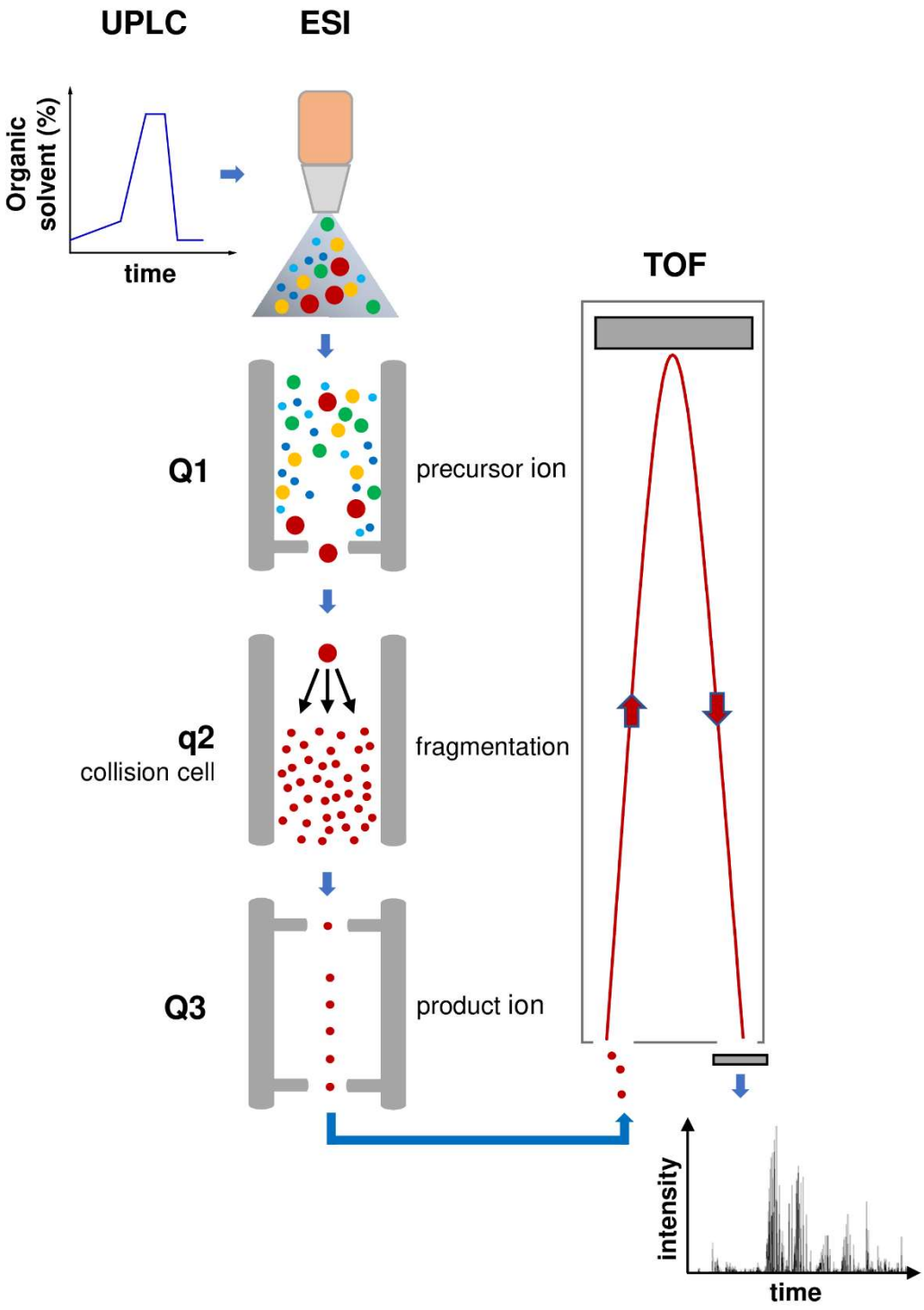

Figure 6: Ultra high performance liquid chromatography triple-quadrupole time-of-flight high resolution mass spectrometry (UPLC-TripleTOF-MS). The peptides of a sample are chromatographically separated on a UPLC column and directed to an electrospray ionization (ESI) source. The ions pass the first quadrupole (Q1) for the selection of precursor ions, fragmentation takes place in the collision cell (q2), selection of product ions in the last quadrupole (Q3). Product ions are detected in the time-of-flight (TOF) mass analyzer depending on their time-of-flight. Two-stage mirror and acceleration improve the resolution and efficiency of the measurement.

1.5. Basophil activation test coupled with fluorescence-activated cell sorting

1.5.1. Basophil granulocytes

Basophil granulocytes (BG) were first described in 1879 by Paul Ehrlich [68]. Granulocytes are divided into basophil, eosinophil and neutrophil. They have secretory granules in the cytoplasm and lobulated nuclei [69]. BGs develop in the bone marrow from hematopoietic stem cells, differentiate and mature there (hematopoiesis). Afterwards, they enter the blood circulation. BGs have a size of 5-7 µm and represent less than 1% of peripheral blood leukocytes. Their lifespan is about 60 hours. Like mast cells, they express the high-affinity IgE receptor FcεRI on the cell surface. The expression correlates with the concentration of free IgE in the blood. The IgE receptor FcεRI is activated by an IgE-antigen-complex by cross-linking. The basophil activation induced in this way results in degranulation and the release of mediators, including chemokines, cytokines and proteases. The most abundant mediator of BG, which is stored in the granules, is histamine (**Figure 2**). BGs are effector cells in IgE-mediated reactions of the adaptive immune response and mediate allergic immune reactions, thus also anaphylaxis [68,70-72]. The participation of BGs in triggering an allergic reaction is described in 1.2.

Due to the similarity of BGs to mast cells and because they are a minority among peripheral blood leukocytes, BGs had often been neglected for use in immunological studies. In the meantime, this perspective has changed. BGs have been accepted as important effector cells in the human immune system and are clinically relevant. They became important tools to study immediate-type allergies and, in addition, they are more accessible for diagnostic tests compared to mast cells [72-74].

1.5.2. Flow cytometry

Flow cytometry is widely used in clinical routine and research. The principle of flow cytometry is a hydrodynamic transport of cells (0.2-20 µm) coupled with optical detection. Immunophenotyping of certain cells is conducted based on the forward and the sideward scatter (FSC, SSC) and the fluorescence signal of cell-bound fluorescence-dye labeled antibodies (FAb). Flow cytometry enables a characterization and quantitation of intact cells and is performed as follows: A cell suspension is diluted by a sheath fluid, which consists of buffers or sodium chloride solutions, sometimes with microbicide additives. The flow speed is between 200 and 5000 events per second, depending on the analysis question. The inflow of the cell suspension is usually 0.6 to 18 mL/min. The sample stream is focused in a single-cell-sequence and passes a laser light (monochrome light source) in a right angle. The laser light is scattered by each single cell in the sample stream passing the laser light. FSC and SSC are detected. In this way an analysis of cell properties, like size and granularity is possible (**Figure 7**). Besides the scattered laser light, FAb binding to cell-surface proteins are in use. FAb are activated by the laser light and emit fluorescence signals. This improves cell type quantification or functional cell analyses. Cell counting or cell separation can be carried out using a fluorescence activated cell sorter (FACS), an advanced form of flow cytometry. Cell separation is induced by a certain fluorescence signal of cell-bound FAb, which causes pulsation of the nozzle and results in the stream breaking into small uniform drops. Each drop contains one single cell. All measured data are saved and assigned to each single cell. Simultaneously, the measured data is used to define whether the just analyzed cell is a target cell. The drop is then electrostatically charged, positive or negative depending on whether the drop contains a target cell or not. Then, the charged drop enters an electric field with charged plates and is diverted according to its charge. In this way, target cells are collected and all other cells are sorted out. The cells are separated into two populations and counted. There are cell sorters, which enable a separation in even more populations by charging the drops different polarities and strengths. This procedure takes place many thousand times per second during one sample run [75].

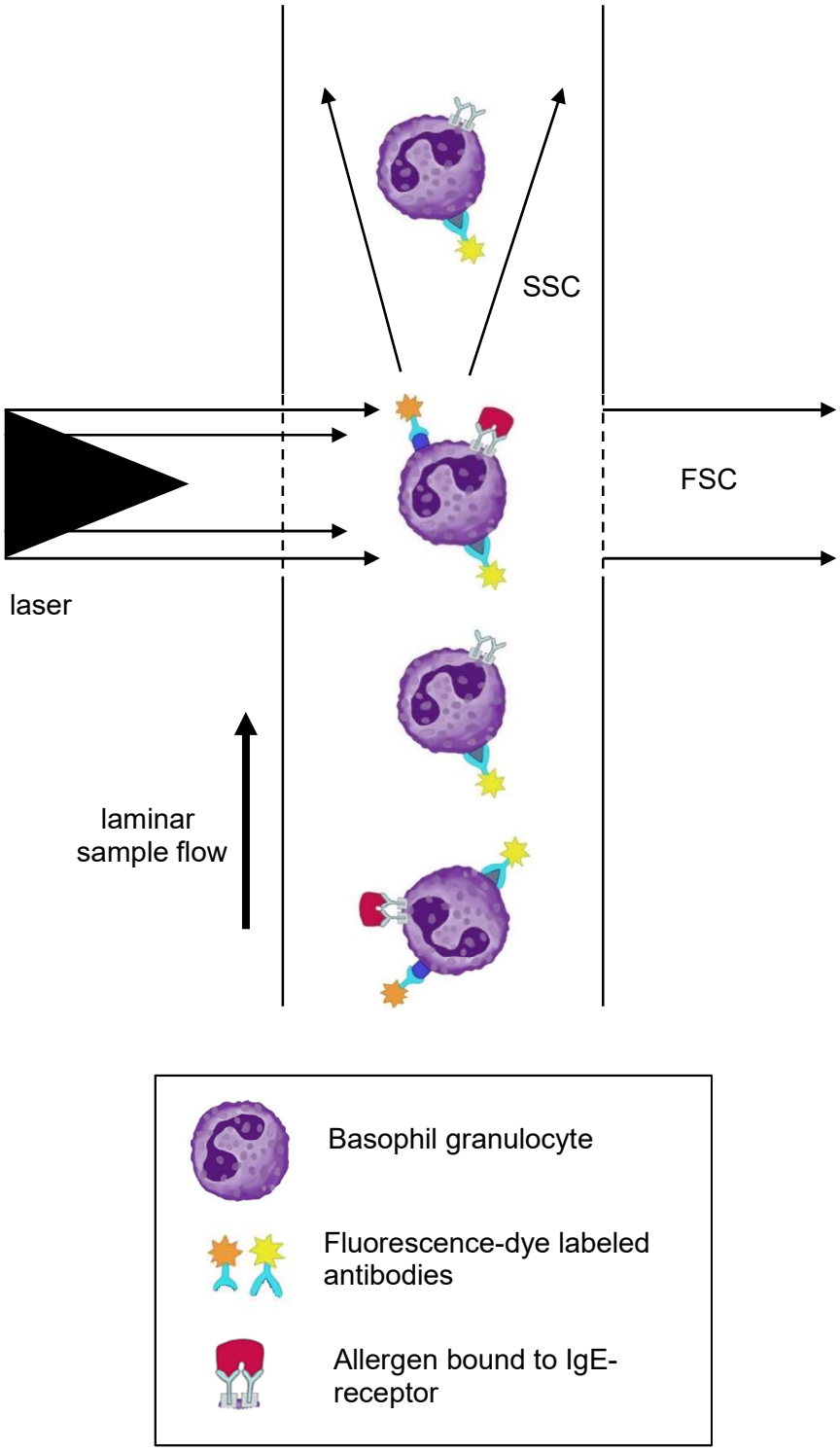

Figure 7: Schematic representation of the functionality of a fluorescence-activated cell sorter (FACS) in combination with the basophil activation test (BAT). The cell suspension in the sheath fluid contains basophil granulocytes (target cells), they move in the laminar sample flow and pass the laser light as single cells. The front- and sideward scatter (FSC, SSC) is detected. Basophil granulocytes are marked with fluorescence-dye labeled monoclonal antibodies for cell identification and detection of basophil activation, which occurs when an allergenic substance binds to the IgE-receptor.

Mono- and polyclonal antibodies fitting to certain cell-surface proteins are used to label cells with a fluorescence dye, e.g. fluorescein isothiocyanate (FITC) or phycoerythrin (PE). In the analysis of leukocytes, such as BG, a large number of usable cluster-of-differentiation (CD) antigens can be used as target for FAb [74,76].

For the analysis, it is essential to have a successful interaction of the cell-expressed-surface-protein and used FAb. Other coloring techniques include stoichiometric binding to coloring agents or fluorescence substrates, which are then metabolized by the specific cells. Additionally, the analysis of leukocytes in whole blood samples requires the depletion of erythrocytes by selective lysis. It is also necessary to enrich the leukocytes for the analysis. This is done by a centrifugation step after lysis of the erythrocytes [75].

The data from the scattered laser light and fluorescence signals are measured for each single cell. This multi-parameter measurement is represented in dot-plots, which are two-dimensional representations of determined values for a certain parameter corresponding to the intensity for the x-signal and y-signal. Each cell measurement is represented as a dot [75].

1.5.3. Basophil activation test

A new functional *in vitro* test was proposed in connection with the determination of immediate type-I allergic reactions. The Basophil Activation Test (BAT) simulates the reaction between allergen and BG *in vitro*, which is a mimicry of a potential allergic reaction in the patient's body [4,5]. Consequently, it is possible to identify whether an allergic reaction, caused by the tested allergen, would occur in the patient or not [77].

BAT requires patient blood, whole blood or isolated peripheral blood mononuclear cells. The BGs are challenged with the allergen. The resulting basophil activation is measured by FACS by monitoring specific cell-surface proteins [74].

To identify BGs and separate them from other blood cells, characteristics from scattered laser light and an identification marker, which is marked with FAb, are needed first. Eberlein et al. (2014) did a comparative study to investigate different BG identification markers for their use in BAT. The highest percentages of BGs were detected with the use of CCR3 (CD193) and IgE (FcεRI) receptors or a combination of CD203c and IgE receptors [78]. Due to the cross-linking reaction, the IgE (FcεRI) receptor is occupied by the tested allergen during the BAT [79], the use of CCR3 as identification marker is advantageous.

For instance, CD13, CD63, CD69, CD107a/b, CD164 or CD203c can be used as BG activation marker. The best experience was made with CD63 and CD203c. Therefore, commercial BAT kits are available with the use of these activation markers [79-83]. Eberlein et al. (2014) recommended the combination of CCR3 as identification marker and CD63 as activation marker for BGs in BAT [78]. CD63-upregulation is closely associated with basophil degranulation [74,84]. To perform cell analysis with FACS, the identification and activation markers are marked with specific fluorescence-dye labeled monoclonal antibodies [82].

Allergen test solutions (ATS) are solutions containing an allergenic test substance and are essential for the successful completion of a BAT. Water-soluble allergen-solutions are required as ATS. Accessible ATS range from crude extracts to purified or recombinant allergenic substances [79,85]. The BAT has been established for the diagnosis of various immediate allergies, e.g. allergies to hymenopter venom [86], antibiotics [77], omeprazole [87], analgesics, narcotics [88] and alpha-galactose [89]. In **Figure 8** the workflow for the combination of BAT and FACS is shown.

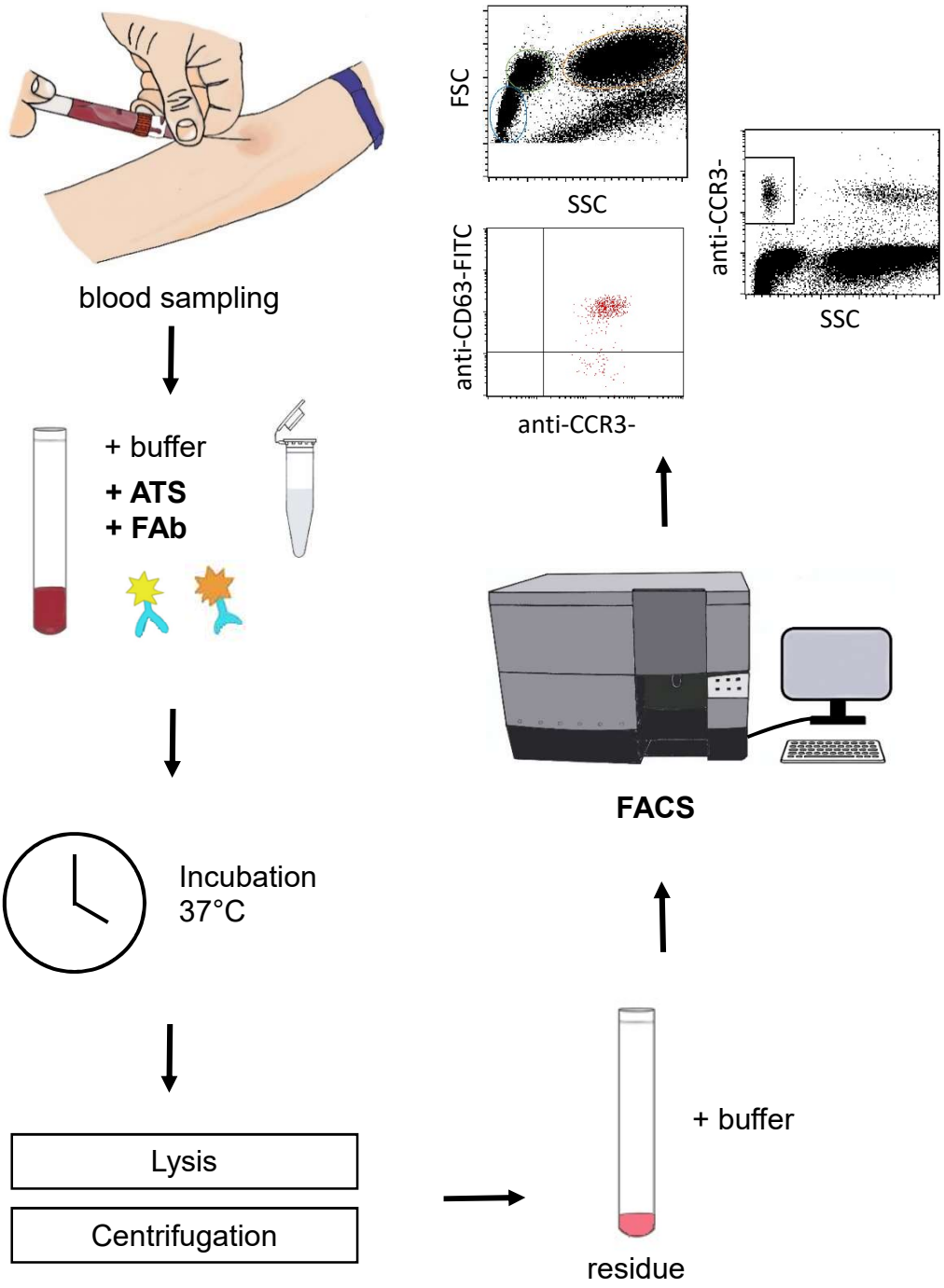

Figure 8: Workflow of Basophil Activation Test (BAT) combined with Fluorescence Activated Cell Sorting (FACS): Blood sampling is carried out. Buffer, allergen test solution (ATS) and coloring agent, consisting of two different types of fluorescence-dye labeled antibodies (FAb) are added. FAb include identification markers for the identification of basophil granulocytes (BGs) and activation markers for the determination of basophil activation, triggered by ATS. After incubation, lysis and centrifugation the supernatant is disposed. The workflow continues with the residue containing BGs. After the addition of buffer and gentle homogenization, the sample is measured with FACS. Multi-dimensional measurement is carried out with characteristics from the front and sideward scatter (FSC, SSC) and fluorescence signal of FAb (example here: anti-CCR3-phycoerythrin (PE), anti-CD63-fluorescein isothiocyanate (FITC))

1.5.4. Previous investigations on WDEIA using the Basophil Activation Test with fluorescence-activated cell sorting

There are relatively few studies investigating WDEIA with the BAT-FACS compared with the large number of studies using skin prick tests [90,91], challenge tests [7,8] [35,80], IgE measurements [17,18,90,92-94] and immunoblotting [91]. The existing studies differ in the surface marker used to determine the activation of BG. Mainly CD203c is used for this application, whereas CD63 is useful, too [30, 77,81,95-97].

Chinuki et al. (2012) investigated the upregulation of CD203c as reaction to ω5-gliadins and a HWP sample to differentiate between WDEIA patients reacting to intact gluten protein types and to HWP [30,97]. In a further study, Chinuki et al. (2020) showed that basophil activation to HWP in WDEIA patients is reduced by omalizumab treatment [96].

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2. Aim of the work

WDEIA is a potentially life-threatening allergy. Though being the best-studied model of cofactor-induced anaphylaxis a lot remains unclear at the current state. The diagnosis of WDEIA is complex and needs improvement. OFC are the golden standard, but they are hampered by difficult implementation in a routine clinical setting and reproducibility. This often leads to false negative test results. Many parameters and mechanisms of the disease are still unclear. In addition, an OFC presents a serious health risk for the patient to be diagnosed. This also applies to SPT. A diagnostic test without serious health risks, which is easy to perform and reliable, is needed. For these reasons, the aim was to establish a diagnosis test fulfilling these criteria. The measurement of specific IgE is already a good approach. However, even WDEIA patients show different specific IgE. Furthermore, the diagnosis of a patient should never be determined by one diagnostic tool alone. Consequently, the focus was set on BAT-FACS. This approach is successfully in use for the diagnosis of other immediate-type allergies and shows the potential to be useful in WDEIA diagnosis, too.

The aim of this work was to further investigate the allergenicity of gluten and HWP in the context of WDEIA to contribute to a better understanding of the underlying mechanisms and expand diagnostic possibilities. For the BAT-FACS, allergenic test solutions (ATS) containing a potential allergenic test substance, are crucial. These needed to be developed. The basis was the precise characterization of the raw materials gluten and HWP for the production of ATS. Besides others, protein and peptide characterization was carried out using SDS-PAGE, GP- and RP-HPLC and photometry. These gave information about the M_r of proteins and peptides, solubility, hydrophilicity/hydrophobicity and degree of deamidation in gluten and HWP samples. Based on the comparative characterization of gluten and HWP, a contribution to a better understanding of their different immunoreactivity was made. A selection of suitable raw materials for the ATS was possible after this characterization. Gluten from a wheat/rye translocation line was additionally included to the sample set, to test whether this can be useful as hypoallergenic food for WDEIA patients.

Further the aim was to develop well-characterized ATS containing different isolated gluten protein types, gluten and HWP to investigate WDEIA allergens and to identify suitable ATS for diagnosing WDEIA with BAT-FACS. Thereby, the compatibility of ATS with vegetative blood cells is essential. To validate the performance of the test system (developed ATS and BAT-FACS) proof-of-principle studies were performed with WDEIA patients and control subjects for the differentiation between the two groups. Based on this, recommendations can be made, which ATS is particularly suitable for diagnosing WDEIA with BAT-FACS.

Because time is a limiting factor for the execution of a diagnostic test in clinical routine, an approach for fast and simple ATS preparation was investigated. A proof-of-principle study with WDEIA patients and control subjects should be implemented to evaluate the quality of the test system (developed ATS and BAT-FACS). Proteomics-based untargeted UPLC-Triple-TOFMS analysis was carried out for the exact characterization of the ATS. For high-resolution MS a Triple-Quadrupol TOF system was used, followed by dataset analysis and visualization with bioinformatics tools.

3. Results

3.1. Comparative characterization of gluten and hydrolyzed wheat proteins

A sample set consisting of six vital gluten samples, two treated gluten samples and seven commercially available HWP was investigated by different methods to assess differences and similarities in molecular properties. First, the crude protein content was determined by the combustion method of Dumas. To study the solubility of the different samples, the modified Osborne fractionation was carried out and proteins were quantitated using RP-HPLC. Furthermore, Angelika Miriam Gabler developed a GP-HPLC method to separate small proteins/peptides into certain mass ranges using marker proteins. SDS-PAGE was performed to characterize M_r of proteins and peptides in gluten and HWP.

Angelika Miriam Gabler evaluated all HPLC data and calculated the protein content in the Osborne fractions (RP-HPLC) and the percentage of proteins/peptides in different mass ranges (GP-HPLC). All data for RP-, GP-HPLC and photometric ammonium determination was summed up and interpreted by Angelika Miriam Gabler. Therefore, she visualized the data and performed statistical data analysis. Angelika Miriam Gabler wrote the manuscript, designed all figures and revised the manuscript according to the reviewer comments.

No in-depth comparative studies characterizing solubility, hydrophilicity/hydrophobicity and M_r distribution of proteins and peptides in gluten and HWP were available. This work closed this gap. Based on the identified molecular differences found between gluten and HWP, this work helps to explain the different immunoreactivity, and thus also the allergenicity of HWP compared to native gluten. It was shown in the present work that depending on the degree of hydrolysis and deamidation, each HWP can differ highly from another HWP. Consequently, the potential immunoreactivity can vary strongly and this is important to consider.

biomolecules

MDPI

Article

Comparative Characterization of Gluten and Hydrolyzed Wheat Proteins

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Received: 21 July 2020; Accepted: 19 August 2020; Published: 24 August 2020

Abstract: Hydrolyzed wheat proteins (HWPs) are widely used as functional ingredients in foods and cosmetics, because of their emulsifying and foaming properties. However, in individuals suffering from celiac disease or wheat allergy, HWPs may have a modified immunoreactivity compared to native gluten due to changes in molecular structures. Although a variety of HWPs are commercially available, there are no in-depth comparative studies that characterize the relative molecular mass (M_r) distribution, solubility, and hydrophilicity/hydrophobicity of HWPs compared to native gluten. Therefore, we aimed to fill this gap by studying the above characteristics of different commercial HWP and gluten samples. Up to 100% of the peptides/proteins in the HWP were soluble in aqueous solution, compared to about 3% in native gluten. Analysis of the M_r distribution indicated that HWPs contained high percentages of low-molecular-weight peptides/proteins and also deamidated glutamine residues. We also found considerable differences between the seven HWPs studied, so that each HWP needs to be studied in detail to help explain its potential immunoreactivity.

Keywords: celiac disease; gel electrophoresis; gliadin; gluten; high-performance liquid chromatography (HPLC); hydrolyzed wheat proteins; wheat allergy

1. Introduction

Wheat gluten is the viscoelastic mass that remains when starch and other water-soluble components are washed out of wheat dough [1]. According to Codex Standard 163–1987, wheat gluten consists of >80% protein, 5–10% lipids, and residues of starch and non-starch polysaccharides [2]. The term "gluten" describes a mixture of over 100 different proteins with a mono-, oligo-, or polymeric structure. Oligomeric and polymeric proteins consist of monomers linked by interchain disulfide bonds. Gluten proteins contain high amounts of glutamine (Gln/Glu: 37.1 mol-% in gliadins, 30.1 mol-% in glutenins) and proline (16.6 mol-% in gliadins, 11.9 mol-% in glutenins) as well as low amounts of amino acids with charged side chains, such as lysine (0.8 mol-% in gliadins, 2.1 mol-% in glutenins) [1,3]. According to the so-called Osborne fractionation, gluten proteins can be divided into two protein fractions depending on their solubility, the gliadins and the glutenins. Gliadins are soluble in 60% ethanol, whereas glutenins remain insoluble [4,5]. Furthermore, gluten proteins can be subdivided by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or reversed-phase high-performance liquid chromatography (RP-HPLC) into gluten protein types: α-, γ-, ω1,2-, and ω5-gliadins and high- and low-molecular-weight glutenin subunits (HMW-GS and LMW-GS) [5–7].

Gliadins consist of mostly monomeric and glutenins of oligomeric and polymeric proteins. Gluten is responsible for the unique baking properties of wheat flour, by building a viscoelastic gluten

network [1,7]. Wheat gluten is often used as an ingredient to improve and standardize the baking properties of wheat flours [1,8].

Furthermore, gluten is also used in various non-cereal products, in which the consumer generally does not expect gluten to be present. For example, wheat gluten is used as binding or protein-enriching agent in meat products or even cosmetics [9,10]. To improve wheat gluten solubility, it is treated in different chemical and biochemical ways, leading to modifications and/or partial hydrolysis. Deamidation of proteins takes place during hydrolysis with acid or alkali. In the case of enzymatic hydrolysis, the deamidation depends on the enzyme used [8–10]. Hydrolysis leads to the formation of proteins or peptides with lower relative molecular masses (M_r) and deamidation to changes of the net charge. Both processes increase the solubility of gluten. Treatment with alkali removes intermolecular disulfide bonds, but usually, it does not lead to a cleavage of peptide bonds [8,11]. Sodium hydroxide is typically used for alkaline hydrolysis; hydrochloric acid or sulfuric acid for acidic hydrolysis; and enzymes like papain, trypsin, and pronase for enzymatic hydrolysis [8,12,13]. Furthermore, gluten can be modified by physical means, like high-pressure processing, heat treatment, extrusion, and UV irradiation [8,14,15].

Wheat is one of the most common foods that may cause adverse reactions, such as celiac disease, non-celiac gluten sensitivity, and wheat allergy. Celiac disease is a chronic small intestinal immune-mediated enteropathy in genetically predisposed individuals, caused by the ingestion of gluten. Its estimated prevalence is about 1% of the population worldwide, with 0.7% reported based on biopsy-confirmed cases and 1.4% reported based on seroprevalence [16]. Wheat allergy has a prevalence of 0.2–1% and is defined as an adverse immune response, with intestinal and extraintestinal symptoms occurring within minutes or hours after exposition [17]. Thereby, the exposition triggering the immunoglobulin E (IgE)-mediated allergy can be through inhalation, skin contact, or oral ingestion. Wheat allergy can be classified into immediate food allergy, wheat-dependent exercise-induced anaphylaxis (WDEIA), respiratory allergy, and skin allergy. Gluten proteins and inhibitors in wheat have been identified to play a key role in wheat allergy [17,18]. WDEIA is a cofactor-triggered wheat allergy and is subdivided into conventional-WDEIA (CO-WDEIA) and hydrolyzed wheat protein-WDEIA (HWP-WDEIA). In CO-WDEIA, patients react to native gluten proteins, whereas in HWP-WDEIA, patients are sensitized to HWP but tolerate native gluten. This underlines that hydrolysis affects the immunoreactivity of wheat proteins [19]. Allergic reactions to soap and cosmetics containing HWPs were reported [20,21]. Parts of the relevant immunoreactive epitopes are already pre-existent in native wheat protein aggregations and are laid bare through hydrolysis but not destroyed. Besides, new epitopes may be created through hydrolysis and simultaneous deamidation [20,21]. Additionally, the increased solubility of the HWP and the route of exposure also affects the immunoreactivity of HWP compared to the native form. Consequently, the degree and type of hydrolysis play an important role, because this could affect the transport through natural barriers, such as the small intestinal epithelium or the skin [19–22].

Gluten is used as the starting material for the production of HWP, which may also be called partially hydrolyzed gluten to better reflect this. There are various methods of how gluten can be hydrolyzed, including chemical, biochemical, and physical approaches, leading to highly variable preparations of HWP. In most cases, the functional properties of the HWP were described as emulsifying or foaming [8,23]. Additionally, the sensory properties were analyzed as well as the use of HWP as a nutritional additive. When characterizing the proteins and peptides in HWP, biochemical analyses, such as enzyme-linked immunosorbent assays (ELISAs) or Western blots, are widely used [23–30]. However, there are only few studies on commercially available HWP compared to native gluten. Gessendorfer et al. (2009) prepared three enzymatically hydrolyzed prolamins from wheat, rye, and barley and characterized the resulting HWP using ELISA, SDS-PAGE, and RP-HPLC [31]. Wieser and Scherf developed a well-characterized HWP for diagnosis and clinical investigations of wheat-related disorders [32], but comprehensive comparative investigations on commercially available HWP are

missing. Additionally, the detection of modified gluten proteins can be done with ELISA or polymerase chain reaction (PCR) [9,33–35].

Considering the differences between HWP and native gluten in terms of immunoreactivity, it is reasonable to hypothesize that various treatments cause changes in the molecular structures of gluten. Consequently, there is a need to characterize commercial HWP in comparison to native gluten. In the present study, gluten and commercially available HWP products were analyzed for their crude protein contents, solubility, and M_r distribution of the proteins and peptides as well as the contents of free ammonium as an indicator for deamidation.

2. Materials and Methods

2.1. Reagents and Materials

All reagents and chemicals were from Merck (Darmstadt, Germany), Sigma-Aldrich (Darmstadt, Germany), Serva (Heidelberg, Germany), Applichem (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany), Honeywell (Offenbach, Germany), and J.T. Baker (Arnhem, Netherlands) in analytical grade or higher. Ultra-pure water for HPLC was purified with an Arium 611VF water purification system (Sartorius, Goettingen, Germany). Prolamin Working Group (PWG)-gliadin was used for calibration, which is well suited to quantitate all types of different wheat proteins [5,7,36]. Wheat gluten and HWP were obtained from Hermann Kröner GmbH (Ibbenbueren, Germany), Sigma-Aldrich (Darmstadt, Germany), Mühle Schlingemann (Waltrop, Germany), Tereos (Lille, France), Solabia Group (Pantin, France) Manildra Group (Gladesville, Australia), Golden Peanut GmbH (Garstedt, Germany), Reform- und Muehlenbaeckerei Boesen GmbH (Langenfeld, Germany), and Tate & Lyle (Aalst, Belgium). Seven HWP (HWP 1–7), six gluten samples (G1–6), and two treated gluten samples (G7, G8) were purchased. G7 is described as denatured wheat protein with a high protein content and G8 as a slightly textured product from wheat protein. Protein/peptide markers used for the gel-permeation HPLC were purchased from Sigma-Aldrich (Darmstadt, Germany): β-amylase from sweet potato (200 kDa), albumin from bovine serum (66 kDa), carbonic anhydrase from bovine erythrocytes (29 kDa), cytochrome c from horse heart (12.4 kDa), α-lactalbumin (14 kDa), and glutathione (0.3 kDa). Furthermore, a gluten peptide (1.9 kDa) was received from Genescript Biotech (Piscataway Township, NJ, USA).

2.2. Determination of Crude Protein Contents

The crude protein content of all samples was determined by the combustion method according to Dumas (ICC Standard Method No.167). The samples were analyzed using a Leco TruSpec Nitrogen Analyzer (LECO, Mönchengladbach, Germany) to determine the nitrogen content after combustion at 950 °C. Ethylenediaminetetraacetic acid (EDTA) was used for calibration. The nitrogen content was multiplied by the factor 5.7 to calculate the crude protein content (wheat protein, ICC No. 105/2) [7].

2.3. Stepwise Fractionation According to Solubility

The stepwise fractionation according to solubility was performed according to the modified Osborne method by Wieser et al. (1998) [5]. Three fractions A, B, and C were received. Fraction A is soluble in aqueous salt solution (0.4 mol/L NaCl, 0.067 mol/L Na_2HPO_4/KH_2PO_4, pH 7.6), fraction B is soluble in 60% ethanol (*v*/*v*), and fraction C is soluble in 1-propanol/0.05 mol/L Tris/HCl, pH 7.5 (50% (*v*/*v*), with 2 mol/L (*w*/*v*) urea and 0.06 mol/L dithiothreitol, DTT) (glutenin extraction solution).

The gluten samples G1–G8 (20 mg) were first extracted with 3 × 0.5 mL aqueous salt solution to obtain fraction A by vortex mixing for 2 min, followed by stirring at room temperature for 30 min and centrifugation (25 min, 3750× *g*, 20 °C). The corresponding three supernatants each were united and filled up with the extraction solution to a volume of 2 mL. The residue was discarded.

In a separate experiment, the gluten samples G1–G8 (20 mg) were extracted with 3 × 1.5 mL 60% ethanol (*v*/*v*) by 2 min vortex mixing, 30 min stirring at room temperature, and centrifugation

(25 min, 3750× *g*, 20 °C). The three supernatants were combined, and the volume was adjusted to 5 mL with 60% ethanol. To calculate the content of fraction B, the content of fraction A was deducted from the 60% ethanol extract after the RP-HPLC measurement. To extract fraction C, the residue was extracted three times with 1.5 mL of glutenin extraction solution by vortex mixing for 2 min followed by stirring at 60 °C under nitrogen atmosphere for 30 min and centrifugation (25 min, 3750× *g*, 20 °C). The supernatants were united and filled up with the extraction solvent to a volume of 5 mL. All extracts were filtered (0.45 µm) and each sample was analyzed in triplicate (n = 3) [5,7].

HWP1–HWP7 were extracted three times with 0.5 mL aqueous salt solution to obtain fraction A. The residue was subsequently extracted three times with 0.5 mL 60% ethanol (*v*/*v*) to obtain fraction B. Then, the residue was extracted three times with 0.5 mL of solution C. All steps were carried out exactly as described above, except that the final volumes were 2 mL for fractions A, B, and C, respectively. All extracts were filtered (0.45 µm) and each sample was analyzed in triplicate (n = 3) [5,7].

2.4. Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC)

Protein composition and contents in fractions A, B, and C were analyzed by RP-HPLC on a Jasco XLC instrument (Jasco, Gross-Umstadt, Germany) using a C_{18} column at 60 °C (Acclaim™ 300, C_{18}, 2.1 × 150 mm, 300 nm, 3 µm, Thermo Fisher Scientific, Braunschweig, Germany). The elution solvents were 0.1% trifluorocacetic acid (TFA) in ultra-pure water (A) and 0.1% TFA in acetonitrile (B). The flow rate was 0.2 mL/min with the following gradient: 0 min 0% B, 0.5 min 24% B, 20 min 56% B, 20.1–24.1 90% B, 24.2–30.0 min 0% B. 2) [5,7]. For G 1–8, the injection volume was 20 µL for fraction A and C and 10 µL for fraction B. For HWP 1–8, it was necessary to vary the injection volumes, because of the different protein contents in the fractions (1–20 µL). The absorbance at 210 nm was detected. PWG-gliadin was dissolved in 60% ethanol (*v*/*v*) to a concentration of 2.5 mg/mL and used for external calibration (50, 37.5, 25, 12.5, and 5 µg absolute) [36]. The limit of quantitation was estimated by injecting different amounts of PWG-gliadin (12.5, 5.00, 3.75, 2.50, 1.25, 0.50, and 0.25 µg absolute). The protein content of PWG-gliadin was 93.1 g/100 g. The software Jasco Chrompass was used for data analysis (version 1.2).

2.5. Gel-Permeation High-Performance Liquid Chromatography (GP-HPLC)

To analyze the M_r of proteins and peptides in HWP and treated gluten samples, HWP1–HWP7 and G7,8 were extracted by stepwise fractionation according to solubility. Three fractions I, II, and III were received. Fraction I, soluble in aqueous salt solution (0.4 mol/L NaCl, 0.067 mol/L Na_2HPO_4/KH_2PO_4, pH 7.6), corresponds to fraction A in Section 2.3. Fraction II, soluble in 60% ethanol, corresponds to fraction B in Section 2.3. Fraction III is a mixture of ultra-pure water and acetonitrile (50/50, *v*/*v*) and corresponds to fraction C in Section 2.3. Each fraction was extracted three times. Fraction I and II were extracted from HWP (20.0 mg or 100 mg) as described for fractions A and B in Section 2.3 for HWP. Fraction III was extracted from the residue three times with 0.5 mL of ultra-pure water and acetonitrile (50/50, *v*/*v*) by vortex mixing for 2 min followed by stirring at room temperature for 30 min and centrifugation (25 min, 3750× *g*, 20 °C). The corresponding three supernatants were united and filled up with the respective extraction solvent to a volume of 2 mL. All extracts were filtered (0.45 µm) and each sample was analyzed in triplicate (n = 3) [5].

For chromatographic analysis, two GP-HPLC systems were used. For larger molecules, a GP-HPLC system according to Scherf et al. (2016) was used (system 1) [36]. A BioSep-SEC-s3000 (300 × 4.6 mm, 29 nm, 5 µm, Phenomenex) was used on a Jasco HPLC Extrema (Jasco, Gross-Umstadt, Germany) at 20 °C. Elution solvents A and B were used with a flow rate of 0.3 mL/min and an isocratic gradient composition of 50% A and 50% B. Because of different protein/peptide contents in the different fractions, the injection volumes were between 1 and 20 µL. The absorbance at 210 nm was detected. To separate smaller molecules, a second GP-HPLC system was used with a BioBasic SEC-60 column (150 × 7.8 mm, 6 nm, 5 µm, Thermo Scientific) on the same Jasco HPLC Extrema at 20 °C (system 2). The elution solvents were 0.1% TFA in ultra-pure water (A) and 0.1% TFA in acetonitrile (B). The flow rate was

1 mL/min with an isocratic gradient composition of 70% A and 30% B for a duration of 12 min. Because of the different protein/peptide contents in the different fractions, the injection volumes were between 5 and 20 µL.

The absorbance at 210 nm was detected [37]. The software ChromNAV was used for data analysis (Jasco Deutschland GmbH, Pfungstadt, Germany). Proteins and peptides with known M_r were measured to mark integration areas for a certain M_r range. β-Amylase from sweet potato (200 kDa), albumin from bovine serum (66 kDa), carbonic anhydrase from bovine erythrocytes (29 kDa), and cytochrome c from horse heart (12.4 kDa) were used for system 1. α-Lactalbumin (14.2 kDa), a gluten peptide (1.9 kDa), and glutathione (0.3 kDa) were used for system 2. The area under the curve (AUC) was integrated in each section and calculated as the percentage of the total area.

2.6. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to Lagrain et al. (2012) [6]. G1–G8 and HWP1–HWP7 were characterized by SDS-PAGE on a homogenous NuPAGE 10% polyacrylamide Bis-Tris gel (10 × 1 mm wells) (Invitrogen, Carlsbad, CA, USA). A mixture of proteins was used as a size standard (PageRuler^{TM} Unstained Protein Ladder, Thermo Fisher Scientific). All samples (1.5 mg each) were mixed with 1 mL of extraction buffer (200 g/L sucrose, 59.7 g/L Tris-HCl, 40 g/L SDS, 0.3 g/L EDTA, 0.4 g/L Coomassie blue, 0.1 g/L phenol red, 0.11 mmol/L HCl) containing 7.72 g/L DTT. After incubation for 12 h, the samples were heated to 60 °C for 10 min and centrifuged (5 min, 5000× *g*, 20 °C). Two different running buffers were used. The MOPS running buffer consisted of 20.9 g/L 3-(N-morpholino) propane sulfonic acid (MOPS), 12.1 g/L Tris-HCl, 2 g/L SDS, 0.6 g/L EDTA, and 0.77 g/L DTT as the reducing agent. The MES running buffer consisted of 20.9 g/L 2-(N-morpholino) ethane sulfonic acid (MES), 12.1 g/L Tris-HCl, 2 g/L SDS, 0.6 g/L EDTA, and 0.77 g/L DTT, as the reducing agent. The running time was 30 min at 115 mA and 200 V. The protein bands were fixed with 12% (*w*/*w*) trichloroacetic acid for 30 min, stained with Coomassie blue for 30 min, and destained first with methanol/glacial acetic acid/water (50/10/40, *v*/*v*/*v*), and then with methanol/glacial acetic acid/water (10/10/80, *v*/*v*/*v*). The gels were scanned using the Gel Doc^{TM} EZ Imager (Bio-Rad Laboratories, Munich, Germany) and the Image Lab software (Bio-Rad Laboratories, Munich, Germany). The images were converted to grayscale [6,7].

2.7. Contents of Free Ammonium

For the determination of free ammonium in G1–G8 and HWP1–HWP7, 50 mg of sample (n = 3) were weighed into a 50-mL two-neck round-bottom flask with a 25-mL dropping funnel with a vacuum equalizer and a vacuum receiver. Glass wool was inserted into the vacuum receiver and wetted with 200 µL sulphuric acid (0.5 mol/L). Vacuum was created using a vacuum pump and 5 mL of boric acid/sodium hydroxide buffer (pH 10) were added via the dropping funnel. Ammonia was expelled for two hours while stirring at room temperature. The glass wool was then transferred into a 50-mL volumetric flask and the vacuum receiver was washed with water. The measurement was performed by photometry [38]. For this purpose, the samples were mixed with 4 mL each of the following solutions: (a) 130 g/L sodium salicylate, 130 g/L trisodium citrate dihydrate, and 970 g/L disodium-tacanonitrosyl-(III)-ferrate-dihydrate dissolved in water; (b) 0.032 g/L sodium hydroxide and 0.002 g/L sodium dichloro-isocyanurate dissolved in water. The flask was then filled up with water to a volume of 50 mL and left to stand for 1 h at room temperature. The measurement was carried out according to DIN ISO 11732 with a UV-2401 spectrophotometer PC (Shimadzu, Neufahrn, Germany) at 655 nm. An external calibration line was prepared using ammonium sulfate [38].

2.8. Statistical Analysis

Statistical analysis was carried out with the use of Origin 19 (OriginLab Cooperation, Northampton, MA, USA) and SigmaPlot 14 (Systat Software GmbH, Erkrath, Germany).

3. Results

3.1. Determination of Crude Protein Contents

The untreated (G1–G6) and the treated gluten samples (G7–G8) showed comparable crude protein contents with 703.2–855.1 and 731.6–765.0 mg/kg, respectively (Table 1). The HWP had a wider range and HWP5 (650 mg/g) had the lowest and HWP7 (898 mg/g) the highest crude protein content. Still, the mean values of gluten samples and HWP were quite similar (G1–G8: 773 ± 45.3 mg/g; HWP1–HWP7: 756 ± 67.9 mg/g; total: 765 ± 57.6 mg/g). HWP5 and G4 were significantly different from each other.

Table 1. Contents of fractions A, B, and C and their sum (SUM), crude protein and free ammonium in the gluten samples (G1–G8) and hydrolyzed wheat proteins (HWP1–HWP7). Values are given as means (n = 3) and different capital letters indicate significant differences between the samples in each column (one-way ANOVA, Tukey's post hoc test, $p < 0.05$).

Sample	Fraction A	Fraction B	Fraction C	SUM	Protein Content	Free NH_4^+
	[mg/g]	[mg/g]	[mg/g]	[mg/g]	[mg/g]	[mg/g]
HWP 1	400.7 ^{G}	<2.3	<4.7	400.7	737.4 ^{A,B}	4.12 ^{A,B}
HWP 2	569.4 ^{J}	25.9 ^{A}	<4.7	595.3	752.6 ^{A,B}	0.59 ^{A,B}
HWP 3	493.6 ^{I}	<2.3	<4.7	493.6	764.9 ^{A,B}	5.00 ^{B}
HWP 4	158.2 ^{E}	237.2 ^{E}	142.5 ^{B}	537.9	737.3 ^{A,B}	0.39 ^{A,B}
HWP 5	443.4 ^{H}	118.3 ^{B}	15.4 ^{A}	577.1	649.9 ^{A}	0.13 ^{A,B}
HWP 6	280.0 ^{F}	150.2 ^{C}	162.2 ^{B,C}	592.4	750.2 ^{A,B}	0.15 ^{A,B}
HWP 7	45.8 ^{D}	423.1 ^{G}	146.5 ^{B}	615.4	898.8 ^{B}	0.56 ^{A,B}
G 1	18.4 ^{C}	410.9 ^{G}	191.8 ^{C,D}	621.1	766.2 ^{A,B}	0.11 ^{A,B}
G 2	15.1 ^{B}	509.6 ^{I}	226.6 ^{D}	751.3	824.5 ^{A,B}	0.05 ^{A}
G 3	14.8 ^{C}	460.6 ^{H}	212.9 ^{D}	688.3	778.1 ^{A,B}	0.12 ^{A,B}
G 4	11.0 ^{A}	505.6 ^{I}	212.9 ^{D}	729.5	855.1 ^{B}	0.05 ^{A}
G 5	21.1 ^{C}	394.2 ^{G}	182.4 ^{C}	597.7	757.3 ^{A,B}	0.09 ^{A,B}
G 6	21.6 ^{C}	427.6 ^{G}	208.9 ^{D}	658.1	703.2 ^{A,B}	0.11 ^{A,B}
G 7	14.8 ^{B}	321.3 ^{F}	331.3 ^{E}	667.4	765.0 ^{A,B}	0.10 ^{A,B}
G 8	10.9 ^{A}	183.7 ^{D}	336.9 ^{E}	531.5	731.6 ^{A,B}	0.10 ^{A,B}
VC *	2.24	2.29	3.13	-	6.14	5.14

* Variation coefficient [%]: median of all relative standard deviations per analysis.

3.2. Stepwise Fractionation According to Solubility and RP-HPLC

The contents of fractions A, B, and C in gluten samples and HWP were determined according to the modified Osborne fractionation, combining extraction and RP-HPLC analysis [4,7]. The RP-HPLC chromatograms of the three fractions from gluten (G1) and HWP2 are shown as examples in Figure 1 and those of all other samples are available as Supplementary Material Figures S1–S13. Fraction B consists of gliadins, whereas fraction C contains glutenins. The contents of fraction B were between 321.3 and 505.6 mg/g and fraction C between 182.4 and 331.3 mg/g. The contents of fraction B in G3, G7, and G8 were significantly different to each other and to other gluten samples as well as HWP (Table 1). Residues of albumins and globulins, present in fraction A, were determined (11.0–21.6 mg/g). They are mostly washed out during the gluten extraction process of wheat gluten but not completely. The content of fraction A was similar for gluten samples in most cases. The contents of the three fractions were similar for G8 (A: 10.9 mg/g, B: 183.7 mg/g, C: 336.9 mg/g) compared to the untreated gluten samples (G1–G6). G7 (A: 14.8 mg/g, B: 321.3 mg/g, C: 331.3 mg/g) had a similar content of fraction C but significantly different contents of fraction A and B in relation to G1–G6 and G8 and all HWP (except fraction A of G2).

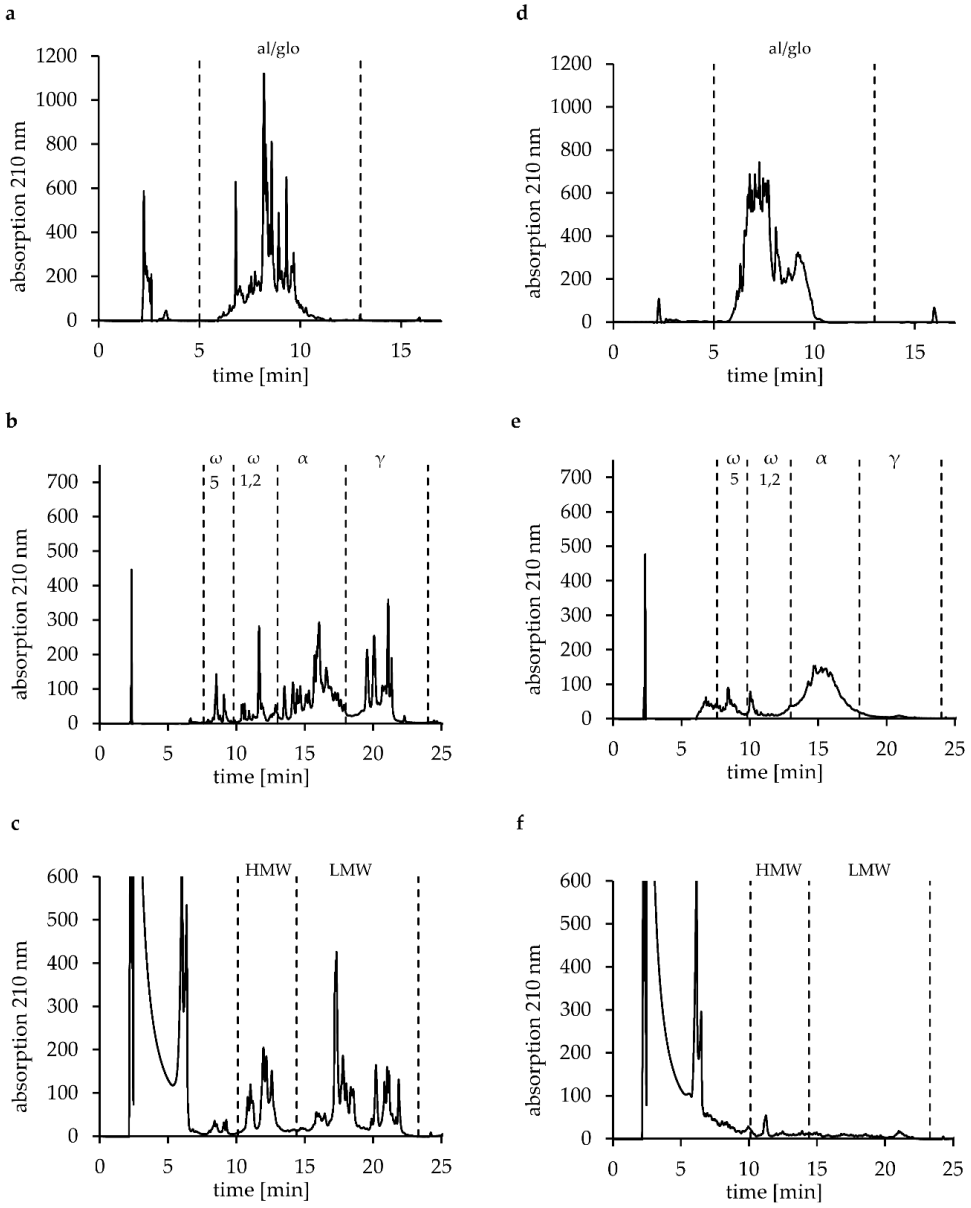

Figure 1. Reversed-phase high-performance liquid-chromatography: Chromatograms of the three fractions, soluble in aqueous salt solution (fraction A), soluble in 60% ethanol (fraction B), and soluble in glutenin extraction solution (fraction C) of gluten G1 (**a**–**c**) and hydrolyzed wheat protein HWP2 (**d**–**f**). Albumins and globulins (fraction A: **a**,**d**), gliadins subdivided into ω5-, ω1,2-, α-, and γ-gliadins (fraction B: **b**,**e**), and glutenins subdivided into high-molecular-weight- (HMW-) and low-molecular-weight- (LMW-) glutenin subunits (fraction C: **c**,**f**).

The contents of fraction A from all HWP (45.8–569.4 mg/g) were significantly higher compared to gluten G1–G8. This results from the modification process where gluten proteins are degraded to smaller proteins, which are consequently more soluble in aqueous solutions. Furthermore, the contents of fraction A from HWP1–HWP7 were significantly different to each other. For HWP, the contents were between 25.9 and 423.1 mg/g for fraction B and between 15.4 and 162.2 mg/g for fraction C. For HWP1 and HWP3, the contents of fraction B were lower than 2.3 mg/g. This value was estimated by injecting decreasing amounts of PWG-gliadin and checking the linearity of the calibration curve. For HWP1, HWP2, and HWP3, the contents of fraction C were lower than 4.7 mg/g. HWP2 and HWP5 had a significantly lower content of fraction B than the other HWP and G1–G8.

3.3. Gel-Permeation High-Performance Liquid Chromatography (GP-HPLC)

To have a closer look at the different HWP and modified gluten samples, fractions I, II, and III were analyzed by GP-HPLC to determine the M_r distribution of the proteins and peptides using two chromatographic systems (I and II). After the extraction of fractions I and II according to the modified Osborne fractionation, fraction III was extracted with a water-acetonitrile mixture (50/50, *v*/*v*). It was impossible to use the glutenin extraction solution, as commonly used in the modified Osborne fractionation combined with RP-HPLC, because it generated a broad interfering signal in the GP-HPLC chromatogram at a retention time of 6.9 min in system I. The chromatograms of the three fractions analyzed by the two GP-HPLC systems are shown in Figure 2 for HWP2 and the quantitative results are presented in Tables 2 and 3. The GP-HPLC chromatograms of all other HWP samples and of G7 and G8 are available as Supplementary Material Figures S14–S29.

Table 2. Gel-permeation HPLC (system I): Analysis of fractions I, II, and III of treated gluten (G7 and G8) and hydrolyzed wheat proteins (HWP1–HWP7) according to their relative molecular mass distribution. Areas within each fraction were set by marker substances. 1: 200–66 kDa; 2: 66–29 kDa; 3: 29–14 kDa; 4: < 14 kDa. Values are given as means (n = 3) and different capital letters indicate significant differences between the samples in each column (one-way ANOVA, Tukey's post hoc test, $p < 0.05$). "-" integration not possible, because the area under the curve was not different to baseline.

Sample	Fraction I				Fraction II				Fraction III			
	1	2	3	4	1	2	3	4	1	2	3	4
	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]
HWP 1	-	-	-	100.0 ^{B}	-	-	-	-	-	-	-	-
HWP 2	-	1.5 ^{A}	6.5 ^{D}	92.0 ^{A,B}	-	17.0 ^{B}	32.6 ^{A,B,C}	50.4 ^{C}	-	-	-	-
HWP 3	-	-	-	100.0 ^{B}	-	-	-	-	-	-	-	-
HWP 4	-	2.3 ^{A,B}	5.0 ^{C}	92.7 ^{A,B}	14.0 ^{C}	16.1 ^{A,B}	26.2 ^{A,B,C}	43.7 ^{B,C}	60.2 ^{A,B}	14.3 ^{A,B}	9.8 ^{A,B}	15.7 ^{A,B}
HWP 5	11.6 ^{A}	10.6 ^{B}	11.6 ^{F}	66.2 ^{A}	39.5 ^{F}	14.2 ^{A}	9.7 ^{A}	36.6 ^{A,B,C}	52.0 ^{A,B}	14.3 ^{A,B}	8.3 ^{A,B}	25.4 ^{A,B}
HWP 6	7.3 ^{B}	9.7 ^{A,B}	15.6 ^{G}	67.4 ^{A}	31.6 ^{E}	15.2 ^{A,B}	16.4 ^{A,B}	36.8 ^{A,B,C}	63.5 ^{B}	13.4 ^{A,B}	7.8 ^{A}	15.3 ^{A,B}
HWP 7	-	4.0 ^{A,B}	8.8 ^{E}	87.2 ^{A,B}	31.0 ^{D,E}	16.9 ^{B}	24.6 ^{A,B,C}	27.5 ^{A}	61.3 ^{A,B}	12.2 ^{A}	11.7 ^{A,B}	14.8 ^{A}
G 7	-	-	3.5 ^{B}	96.5 ^{A,B}	11.3 ^{B}	15.1 ^{A,B}	39.5 ^{B,C}	34.1 ^{A,B}	30.7 ^{A,B}	12.1 ^{A}	37.6 ^{A,B}	19.6 ^{A,B}
G 8	-	-	2.0 ^{A}	98.0 ^{A,B}	5.8 ^{A}	14.7 ^{A,B}	42.9 ^{C}	36.6 ^{A,B,C}	11.7 ^{A}	15.3 ^{B}	45.0 ^{B}	28.0 ^{B}
CV * [%]	8.8	4.9	4.9	0.7	2.8	1.3	3.6	2.1	3.3	2.3	6.5	5.5

* Variation coefficient: median of all relative standard deviations per analysis.

HWP1 and HWP3 both contained only proteins/peptides with M_r below 14 kDa in fraction I. No peaks were detectable anymore in fractions B and C of the modified Osborne fractionation (Table 1) and consequently, fractions II and III in the GP-HPLC system I also showed no AUC. This clearly shows that HWP1 and HWP3 were extensively hydrolyzed. The M_r distribution of HWP2 showed 92.0% of proteins/peptides with an M_r below 14 kDa, 6.5% with an M_r of 14–29 kDa, and 1.5% with an M_r of 66–29 kDa in fraction I. In fraction II, the distribution of M_r changed, but 50.4% of the proteins/peptides were still below 14 kDa. No signals were detected in fraction III of HWP2, as expected from fraction C. All other HWP4–HWP7 as well as G7 and G8 displayed signals above the estimated threshold in all three fractions (Table 2). HWP5 and HWP6 showed quite similar M_r distributions in fractions I, II, and III (Table 2). HWP4 and HWP7 contained no proteins/peptides with M_r 200–66 kDa in fraction I. Furthermore, they had low percentages for M_r 29–66 kDa (2.3–4.0%) and M_r 14–29 kDa (5.0–8.8%) but high percentages for M_r < 14 kDa (87.2–92.7%) in fraction I. Both fractions II and III of HWP7 had similar percentages in M_r as HWP6. Fraction II of HWP4 showed a different M_r distribution compared to the other samples, whereas fraction III was comparable to HWP6 and HWP7. The treated gluten samples G7 and G8 were comparable in the percentages of M_r in all fractions.

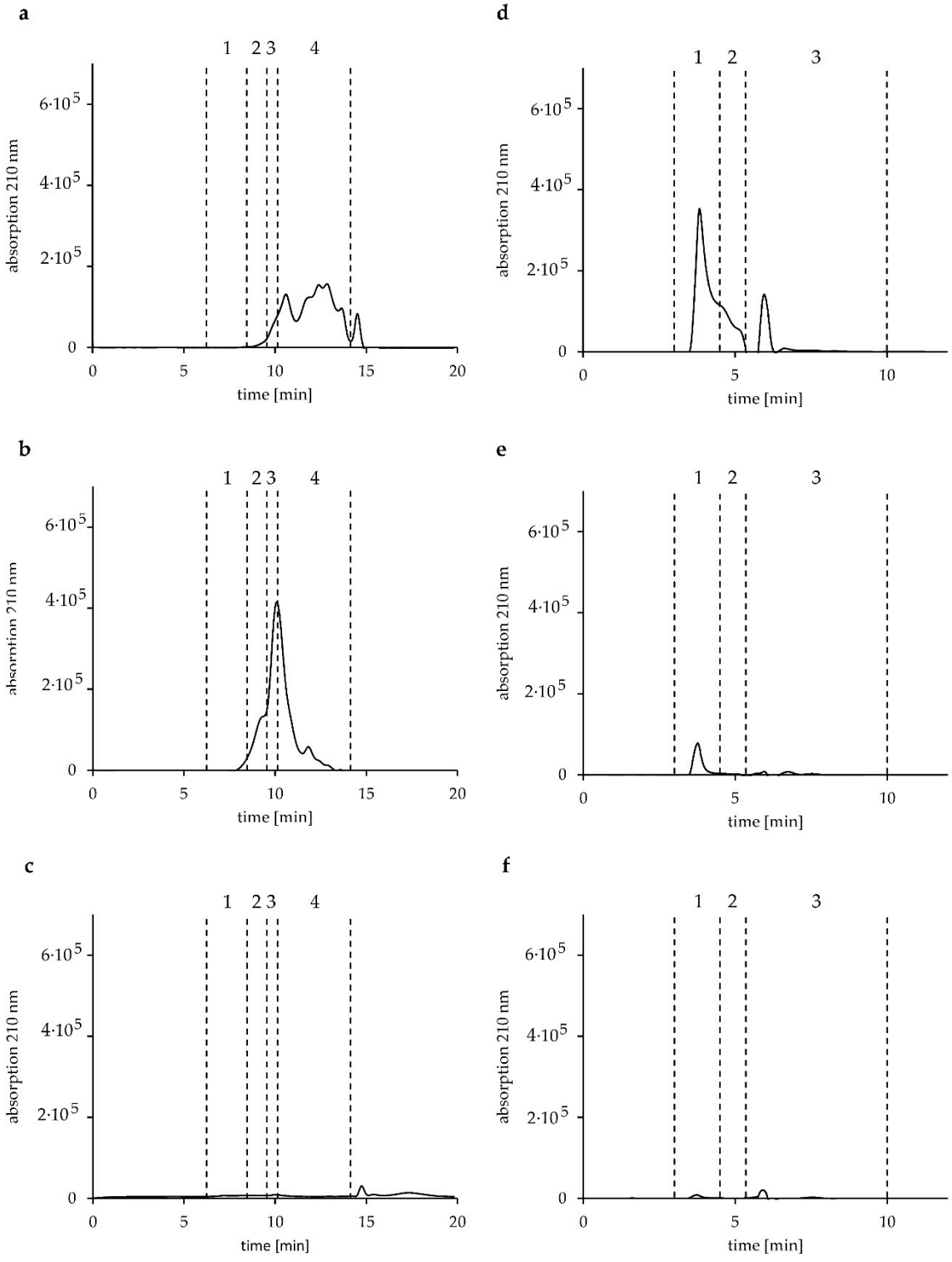

Figure 2. Gel-permeation high-performance liquid-chromatography: Chromatograms of the three fractions, soluble in aqueous salt solution (fraction I: **a**,**d**), soluble in 60% ethanol (fraction II: **b**,**e**), and soluble in acetonitrile/water (50/50, *v*/*v*) (fraction III: **c**,**f**) of HWP2 in two different systems. System I (**a**–**c**) is subdivided into the following ranges of relative molecular masses: M_r 200–66 kDa (1), M_r 66–29 kDa (2), 29–14 kDa (3), < 14 kDa (4). System II (**d**–**f**) is subdivided into the following ranges of relative molecular masses: $M_r \geq 14$ kDa (1), M_r 14–2 kDa (2), $M_r < 2$ kDa (3).

Table 3. Gel-permeation HPLC (system II): Analysis of fractions I, II, and III of treated gluten (G7 and G8) and hydrolyzed wheat proteins (HWP1-HWP7) according to their relative molecular mass distribution. Areas within each fraction were set by marker substances. 1: ≥ 14 kDa; 2: 14–2 kDa; 3: < 2 kDa. Values are given as means (n = 3) and different capital letters indicate significant differences between the samples in each column (one-way ANOVA, Tukey's post hoc test, $p < 0.05$). "-" integration not possible, because the area under the curve was not different to baseline.

Sample	Fraction I			Fraction II			Fraction III		
	1	2	3	1	2	3	1	2	3
	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]
HWP 1	16.8 ^{B}	33.9 ^{G}	49.3 ^{F}	-	-	-	-	-	-
HWP 2	66.3 ^{E}	18.8 ^{F}	14.9 ^{B}	100.0	-	-	-	-	-
HWP 3	3.9 ^{A}	39.1 ^{H}	57.0 ^{G}	-	-	-	-	-	-
HWP 4	75.3 ^{I}	13.7 ^{E}	11.0 ^{A}	100.0	-	-	100.0	-	-
HWP 5	73.7 ^{G,H,I}	11.5 ^{D}	14.8 ^{B}	100.0	-	-	-	-	-
HWP 6	74.2 ^{H,I}	4.5 ^{B,C}	21.3 ^{C}	100.0	-	-	100.0	-	-
HWP 7	70.5 ^{F}	5.6 ^{C}	23.9 ^{D}	100.0	-	-	100.0	-	-
G 7	53.3 ^{D}	2.5 ^{A}	44.2 ^{E}	100.0	-	-	100.0	-	-
G 8	31.6 ^{C}	-	68.4 ^{H}	100.0	-	-	100.0	-	-
CV * [%]	2.0	2.9	2.6	0.0	-	-	0.0	-	-

* Variation coefficient: median of all relative standard deviations per analysis.

The GP-HPLC system II was established to have a closer look at proteins/peptides with low M_r (Table 3). As expected from the Osborne fractionation (Table 1), HWP1 and HWP3 only showed baseline values in fractions II and III. The M_r distribution in fraction I of HWP1 and HWP3 was significantly different to all other samples and to each other. HWP1 had significantly higher percentages of proteins/petides with $M_r \geq 14$ kDa but lower percentages of proteins/petides with $M_r < 2$ kDa compared to HWP3. In fraction I, the M_r of HWP2 was mostly ≥ 14 kDa (66.3%), whereas the percentage of peptides with an $M_r < 2$ kDa was similar to that of HWP5. HWP4 and HWP5 as well as HWP6 and HWP7 showed comparable M_r distributions in fraction I, respectively. Considering fractions II and III, HWP1 and HWP3 only had baseline values, indicating that the samples were completely soluble in aqueous salt solution. HWP2 and HWP5 showed no peaks in fraction III, but fraction II contained 100% of proteins/peptides with $M_r \geq 14$ kDa.

When comparing the results from RP-HPLC and GP-HPLC analyses, we found 15.4 mg/g of fraction C in HWP5 after extraction/RP-HPLC analysis (Table 1) but no signal above the baseline after extraction/GP-HPLC analysis. This discrepancy can be explained with the change of extraction solvents for both analyses. Apparently, the glutenin extraction solution with DTT used in combination with RP-HPLC (fraction C) is more efficient at extracting the proteins/peptides from the sample than the water-acetonitrile mixture used for GP-HPLC (corresponding fraction III). The difference was small and only apparent for HWP5, because it had such low contents of fraction C/III. HWP4, HWP6, and HWP7 had 100% of proteins/peptides with $M_r \geq 14$ kDa in fractions II and III, respectively, as did G7 and G8. The M_r distribution of G7 and G8 was significantly different from HWP1–HWP7 in fraction I. G8 was the only sample with no AUC in the range of M_r 14–2 kDa. G7 and G8 were also significantly different to each other, which is according to expectations, because they were treated in different ways.

3.4. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Two different reducing buffer systems, using MES and MOPS, were tested for SDS-PAGE. Overall, the gels looked similar regarding band patterns, but the bands were sharper with MOPS compared

to MES. Consequently, the SDS-PAGE gels with MOPS running buffer are discussed in the following (Figure 3). The protein marker contained proteins with 15 and 10 kDa, but these bands were not separated at the end of the gel, so that all bands in this range were designated as $M_r \leq 15$ kDa.

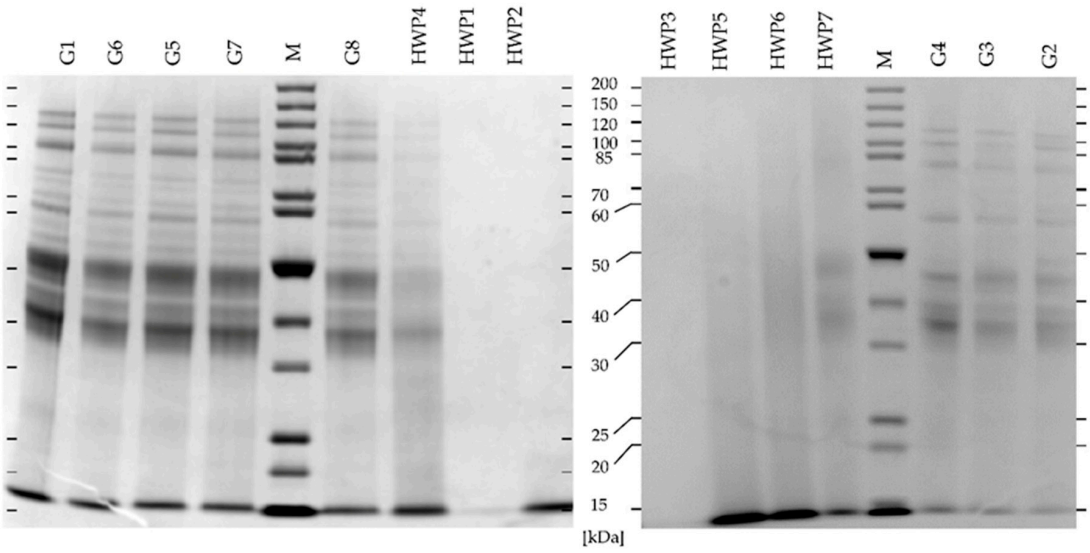

Figure 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of gluten samples (G1–G8) and hydrolyzed wheat proteins (HWP1–HWP7). Protein marker 3.5 µg, samples 15 µg.

Gluten protein types have different M_r, ranging from HMW-GS with 65–90 kDa, to LMW-GS with 30–50 kDa and to gliadins with 28–55 kDa. Among gliadins, the M_r ranges are 49–55 kDa for ω5-gliadins, 39–44 kDa for ω1,2-gliadins, and 28–39 kDa for α- and γ-gliadins. Veraverbeke et al. and Lagrain et al. reported that the M_r of HMW-GS is overestimated to 80–120 kDa in SDS-PAGE due to aggregation effects This is visible in all gluten samples (G1–G8), with three characteristic bands of HMW-GS in this range. In general, the native gluten samples (G1–G6) showed the typical protein bands of the different gluten protein types. A protein band with $M_r \leq 15$ kDa was also present in each native gluten sample (G1–G6), which results from residues of albumins and globulins (see also Section 3.2, fraction A) [1,6,7,39].

The treated gluten samples G7 and G8 showed the same protein bands as the native gluten samples G1–G6, indicating that the treatment did not cause extensive changes in the protein composition. In contrast, the HWP were completely different. Depending on their degree of hydrolysis, they showed more or fewer protein bands. HWP4 and HWP7 showed protein bands in the range of 30–50 kDa and approximately 60 and 85 kDa, but they were much more blurred compared to G1–G8. HWP5 and HWP6 showed only blurred lanes with no discernible protein bands. HWP2, HWP5, and HWP6 showed one band at $M_r \leq 15$ kDa, which was also visible in G1–G8, as well as HWP4 and HWP7. HWP1 and HWP3 showed no protein bands at all, indicating that they had been extensively hydrolyzed.

3.5. Contents of Free Ammonium

The content of free ammonium in a sample is an indicator for hydrolysis under drastic conditions, like highly concentrated mineral acids and high temperature, or the use of deamidating enzymes, such as transglutaminases [9]. The native gluten samples (G1–G6) had low contents of free ammonium (0.05–0.12 mg/g) (Table 1), as did the treated gluten samples (G7–G8: 0.10 mg/g). However, the values were higher (0.13–5.00 mg/g) for HWP. This was expected and depends on the production process. HWP1 and HWP3 had the highest contents of free ammonium (4.12 and 5.00 mg/g, respectively). G2 and HWP3 had significantly different contents of free ammonium.

As shown with RP-HPLC, GP-HPLC, and SDS-PAGE, HWP1 and HWP3 were both extensively hydrolyzed wheat protein samples. These two samples may have been processed via hydrolysis with mineral acid under heating, which is a very common procedure in the food industry. As shown above, HWP2 has also been extensively hydrolyzed. In contrast to HWP1 and HWP3, the ammonium content of HWP2 was low (0.59 mg/g) and this could be an indicator that HWP2 might have been hydrolyzed enzymatically, with a long duration [9].

4. Discussion

Gluten and HWP showed similarities in the crude protein contents (about 765 mg/g) and these contents were expected for gluten [1]. This shows that the treatment of the HWP did not change the crude protein content, except for HWP 5. The content of free ammonium was used as an indicator for deamidation. Consequently, higher values were expected for the HWP compared to gluten and the results supported the expectation, because HWP had average contents of 1.56 mg/g and gluten of 0.09 mg/g. No exact contents have been reported in the literature so far, but it is known that deamidation may take place during hydrolysis and is also carried out intentionally to achieve the desired functional properties, such as increased solubility [8,9].

The most remarkable differences between gluten and HWP were the contents of fraction A of the modified Osborne fractionation. HWP (on average: 341.6 mg/kg) showed significantly higher contents than gluten (on average: 16.0 mg/kg), according to expectations. Crude gluten is treated in chemical and biochemical ways to improve solubility. Consequently, a higher percentage of proteins or peptides is soluble in salt solution and does not require organic solvents or reducing agents anymore to become soluble, like intact gliadins and glutenins do. The extent of the increase in solubility and thus contents of fraction A depend on the type and degree of processing. Consequently, with increased contents in fraction A, HWP had decreased contents in fraction B and C. Among others, Kanerva et al. (2011) and Wu et al. (1976) described a noticeably increased solubility of HWP compared to gluten [9,28].

In agreement with the RP- and GP-HPLC results, differences were also visible between gluten and HWP using SDS-PAGE. While gluten showed typical protein bands, the HWP showed less or even no protein bands, because of protein degradation. Generally, the protein bands were weaker in HWP than in gluten, which was expected. Wieser et al. (2018) showed a change in protein bands in SDS-PAGE relative to the duration of hydrolysis [32]. However, the degree of hydrolysis and the presence of protein bands in SDS-PAGE is not only dependent on the duration but also on the type of hydrolysis. Chemical hydrolysis is usually harsher than enzymatic digestion [8,12,32,40].

Interestingly, in SDS-PAGE, a protein band was found at $M_r \leq 15$ kDa in G1–G8 and in most HWP samples, except HWP1 and HWP3. In the GP-HPLC system I, this is also clearly visible in area 4 of fraction I ($M_r < 14$ kDa), which had the highest percentage in the fraction of these samples. Additionally, in the GP-HPLC system II, the highest percentage was present in area 1 of fraction I ($M_r \geq 14$ kDa) for HWP2, 4–7 and G7. This indicates the presence of proteins with M_r around about 14 kDa or lower. This may result from, e.g., α-amylase/trypsin-inhibitors that have a M_r of about 12–16 kDa, but further analyses are required to unambiguously identify these proteins [41].

HWP1 and HWP3 did not show any protein bands in SDS-PAGE, which indicated the status of total hydrolysis. This is also visible in their GP-HPLC measurements, showing M_r lower than 14 kDa in both systems. Such an extensive hydrolysis can be carried out with the use of 0.5–1 mol/L hydrochloric acid and boiling [9,28].

Furthermore, the HWP were different from each other in many cases. The contents of fractions A and B were significantly different in every HWP. Additionally, differences in M_r using GP-HPLC were visible. It was predictable that HWP were different from each other, because many different approaches for gluten hydrolysis are in use like treatment with chemicals [13] and enzymes or high-pressure processing and UV irradiation [8].

The differences between native gluten and HWP are likely to result in difficulties regarding gluten analysis, important for people with wheat-related disorders, who need to avoid gluten. The identified differences between HWP support the allegation that their determination is challenging [9,33,35].

In general, it should be noted that the differences between gluten and HWP need to be considered when developing analytical methods, e.g., because sample preparation is affected due to different solubility. Another point is that reference materials used for calibration may need to be adapted or that different assay formats may be necessary, i.e., a competitive ELISA as opposed to a sandwich format [31,35]. Regarding diagnostic approaches, the immunoreactivity might differ greatly, as the differences found at the molecular level suggest. The same applies to different HWP, because their properties are highly variable. The in-depth characterization of the samples allowed us to select particularly interesting HWP samples showing a low or a high degree of hydrolysis for further work to characterize the sensitization profiles in wheat allergic patients. Testing the levels of gluten immunogenic peptides arising after the ingestion of gluten or HWP in patients' urine or stool samples would be very interesting to assess potential differences in bioaccessibility, bioavailability, and uptake vs. excretion ratios [42].

How the identified molecular differences influence the mechanisms of celiac disease and wheat allergy is hard to say. On the one hand, hydrolysis uncovers immunoactive epitopes in the proteins and potentially generates new ones by deamidation. In addition, the increased solubility of HWP may have an influence on the bioavailability and digestibility in the body. On the other hand, hydrolysis can also destroy immunoactive epitopes, because of extensive protein degradation [19–22].

5. Conclusions

Commercially available HWP and gluten samples were characterized according to their crude protein content, solubility, and M_r of the proteins and peptides as well as the content of free ammonium as indicator for deamidation. Differences in the protein composition, solubility, and M_r distribution between HWP and native gluten were expected and found, especially for the solubility of HWP and gluten in aqueous salt solution. Additionally, all analyzed HWP were significantly different from each other. This shows that the molecular characteristics of HWP generally are highly variable and that these are likely to cause differences in the immunoreactivity of the products. These findings highlight that the exact characterization of HWP products is very important to establish relationships between protein structure and immunoreactivity for patients suffering from wheat-related disorders. It is necessary to pay attention to the molecular differences between gluten and HWP, especially for the development of analytical or diagnostic methods.

Supplementary Materials: The following are available online at http://www.mdpi.com/2218-273X/10/9/1227/s1, Figures S1–S13: Reversed-phase HPLC analysis of G2 to G8 as well as HWP1 and HWP3 to HWP7, Figure S14–S21: Gel-permeation HPLC analysis of HWP1, HWP3 to HWP7 and G7 and G8, Figure S22–S29: Gel-permeation HPLC analysis of HWP1, HWP3 to HWP7 and G7 and G8.

Author Contributions: Conceptualization, K.A.S.; Formal analysis, Investigation, Methodology, A.M.G.; Resources, Supervision, K.A.S; Visualization, Writing—original draft, A.M.G.; Writing—review and editing, K.A.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Acknowledgments: The authors would like to thank Ms. Angelika Graßl and Ms. Stefanie Hackl (Leibniz-LSB@TUM) for their excellent technical assistance. We acknowledge support by the KIT-Publication Fund of the Karlsruhe Institute of Technology.

Conflicts of Interest: The authors declare no conflict of interest.

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3.2. The basophil activation test differentiates between patients with wheat-dependent exercise-induced anaphylaxis and control subjects using gluten and isolated gluten protein types

A proof-of-principle study of the basophil activation test for the diagnosis of WDEIA with ATS from gluten and GPT was designed and carried out. Based on the analysis in 3.1., a representative gluten sample was selected. Angelika Miriam Gabler isolated single GPT (ω5-, ω1,2-, α-, γ-gliadins, HMW-/LMW-GS) from the representative gluten sample, with the use of the modified Osborne fractionation, dialysis, precipitation methods, preparative RP-HPLC and lyophilization. She developed a preparation method using enzymatic digestion with pepsin to increase the solubility of gluten proteins in order to generate ATS. These ATS are usable in BAT on vegetative blood cells to study the allergenicity of gluten and isolated GPT for WDEIA patients and control subjects. Furthermore, a human study was accomplished on twelve WDEIA patients and ten control subjects to assess the allergenicity of the developed ATS in the context of WDEIA. Thereby, Angelika Miriam Gabler carried out the BAT experiments on the study participants.

Angelika Miriam Gabler evaluated the study data. To investigate the individual sensitization profiles to GPT in WDEIA patients, different parameters were studied including $CD63^+$ basophils, $CD63^+$ basophils/anti-FcεRI ratio and area under the curve of dose response curves from patients and controls as response to ATS. Receiver operating characteristic curves were generated to assess the discriminability of patients and controls in BAT with each ATS. The test sensitivity and specificity were determined. Angelika Miriam Gabler compared the results from BAT to clinical routine diagnostics using Spearman’s correlation test. Angelika Miriam Gabler developed the methodology and carried out the investigations. She analyzed the data and designed figures and tables. She wrote the manuscript draft and did the revisions according to the reviewer comments.

This work showed that BAT with the use of ATS from ω5-gliadin and HMW-GS is a helpful tool to supplement WDEIA clinical routine diagnostics. The $CD63^+$ basophils turned out to be the best parameter for the excellent differentiation between WDEIA patients and control subjects. Additionally, this study expands the knowledge of individual sensitization profiles in WDEIA patients to single GPT.

Received: 13 March 2021 | Revised: 17 May 2021 | Accepted: 21 July 2021

DOI: 10.1002/clt2.12050

RESEARCH

The basophil activation test differentiates between patients with wheat-dependent exercise-induced anaphylaxis and control subjects using gluten and isolated gluten protein types

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Funding information

Bundesministerium für Bildung und Forschung, Grant/Award Number: 01EA2106A; Buehlmann Laboratories AG; H.+W. Winkler Stiftung "Hilfe für den Menschen"; Karlsruher Institut für Technologie, Grant/Award Number: KIT-Publication Fund; Deutsche Forschungsgemeinschaft (DFG; German Research Foundation), Grant/Award Number: CRC 1371 P06; Luxembourg National Research Fund (FNR), Grant/Award Number: project C17/BM/11656090 - DFG DACH-Lead-Agency BI696/12-1

Abstract

Background: Oral food challenge using gluten and cofactors is the gold standard to diagnose wheat-dependent exercise-induced anaphylaxis (WDEIA), but this procedure puts patients at risk of an anaphylactic reaction. Specific IgE to ω5-gliadins as major allergens and skin prick tests to wheat may yield negative results. Thus, we designed a proof-of-principle study to investigate the utility of the basophil activation test (BAT) for WDEIA diagnosis.

Methods: Different gluten protein types (GPT; α-, γ-, ω1,2- and ω5-gliadins, high-molecular-weight glutenin subunits [HMW-GS] and low-molecular-weight glutenin subunits [LMW-GS]) and gluten were used in different concentrations to measure basophil activation in 12 challenge-confirmed WDEIA patients and 10 control subjects. The results were compared to routine allergy diagnostics. Parameters analyzed include the percentage of $CD63^+$ basophils, the ratio of $\%CD63^+$ basophils induced by GPT/gluten to $\%CD63^+$ basophils induced by anti-FcεRI antibody, area under the dose-response curve and test sensitivity and specificity.

Results: GPT and gluten induced strong basophil activation for $\%CD63^+$ basophils and for $\%CD63^+$/anti-FcεRI ratio in a dose-dependent manner in patients, but not in controls ($p < 0.001$, respectively). BAT performance differed from acceptable (0.73 for LMW-GS) to excellent (0.91 for ω5-gliadins) depending on the specific GPT as evaluated by the area under the receiver operating characteristic curve. Patients showed individual sensitization profiles. After determination of the best cut-off points, ω5-gliadins and HMW-GS showed the best discrimination between patients and controls with a sensitivity/specificity of 100/70 and 75/100, respectively.

Conclusion: This study shows the alternative role of BAT in better defining WDEIA and the causative wheat allergens. The best BAT parameters to distinguish

Katharina Anne Scherf and Knut Brockow share co-senior authorship.

WDEIA patients from controls were %CD63$^+$ basophil values for ω5-gliadins and HMW-GS.

KEYWORDS
ω5-gliadin, basophil activation test, gluten, wheat allergy, wheat-dependent exercise-induced anaphylaxis (WDEIA)

1 | INTRODUCTION

Wheat-dependent exercise-induced anaphylaxis (WDEIA) is a rare, but potentially life-threatening cofactor-induced wheat allergy. ω5-gliadins and high-molecular-weight glutenin subunits (HMW-GS) are most often reported as major allergens, but reactions to other gluten protein types (GPTs) from wheat, like low-molecular-weight glutenin subunits (LMW-GS), α- and γ-gliadins were also described. All GPT together constitute gluten, the storage proteins in wheat flour.[1-4]

WDEIA diagnosis is challenging, because of the variety of possible allergenic wheat proteins and the combination with a cofactor. Skin prick tests (SPTs) and specific IgE (sIgE) to wheat may be negative. Even sIgE to ω5-gliadins are only positive in about 80% of WDEIA patients, indicating that other GPT also play a role in WDEIA. Wheat product and exercise challenge failed to induce symptoms in the majority of patients despite a clear history.[1,4,5] Thus, oral food challenge with gluten, that has a protein content of 70%–80% compared to the 8%–15% in wheat flour, combined with cofactors is often needed to overcome non-responsiveness.[6,7]

A new approach to complement WDEIA diagnosis is the basophil activation test (BAT) combined with florescence-activated cell sorting. Stimulation with an allergen-containing solution (allergen test solution [ATS]) induces upregulation of the expression of cell surface proteins, such as CD63 or CD203c. BAT-derived parameters such as the percentage of basophils that respond to a given dose of the ATS or the area under the curve (AUC) of a dose-response curve have been shown to be sensitive biomarkers corresponding to the clinical severity of anaphylactic reactions.[8,9] The BAT has been established for the identification of different immediate allergies, like allergy against wheat,[8,10] hymenoptera venom,[11] and alpha-galactose.[12]

The aim of this proof-of-principle study was to investigate the utility of the BAT to improve WDEIA diagnosis and to determine individual sensitization profiles in WDEIA patients to different isolated and well-characterized GPT and gluten.

2 | METHODS

2.1 | Study population

The following exclusion criteria were considered in the selection of participants to avoid potential confounding factors and/or health risk to any of the participants: Pregnancy/lactation; systemic intake of corticosteroids (cortisone) 3 weeks and/or antihistamines (anti-pruritic drugs) 1 week before the start of the test; intake of laxatives, anti-diarrhea drugs, thyroid hormone preparations, antibiotics, immunosuppressive drugs, analgesic drugs (aspirin, NSAIDs, etc.) taking psychotropic drugs and certain blood pressure medications (ACE inhibitors, ß-blockers); serious internal diseases (gastrointestinal, neurological, cardiovascular, rheumatic diseases, celiac disease, cancer, kidney diseases, acute infections, etc.); bronchial asthma.

A total of 23 participants were consecutively recruited from the medical center (15 f, 8 m, 25–76 years). Twelve of them were patients with a history of WDEIA based on positive oral food challenge, SPT, sIgE, and clinical history (5 f, 7 m, 26–60 years, Table 1). Provocations had been done with 8–32 g gluten intake as described.[7,13] Some patients, depending on their history, were given increasing doses of cofactors (500–1000 mg of ASA ± 10–20 ml of 95% ethanol; Braun, Melsungen, Germany diluted with 200 ml of black currant-flavored water) 30 min before gluten challenge and standardized aerobic and anaerobic exercise was undertaken 30–60 min after gluten ingestion. Eleven individuals without a history of any wheat-related disorder were included in the study as controls (10 f, 1 m, 25–76 years, Table 2).

The study protocol was approved by the ethics committee of the Technical University of Munich and all participants gave written informed consent before being included in the study.

2.2 | Skin prick test

SPT was carried out on the forearm with the following substances: wheat flour, gluten, isolated LMW-GS, HMW-GS, and gliadins. A 10% histamine-dihydrochloride solution (ALK-Abello, Hørsholm, Denmark) was used as positive and isotonic sodium chloride solution (Fresenius Kabi Deutschland GmbH) as negative control.[7] Details of the production and characterization of GPT can be found in the Supporting Methods.

2.3 | Serum sIgE and total IgE

Serum sIgE and total IgE levels were measured by ImmunoCap and Phadia 250 (Thermo Fisher Scientific). Serum sIgE levels of the following allergens were determined: *Dermatophagoides pteronyssinus* (d1), timothy grass (g6), birch pollen allergen (Bet v 1, t215), wheat flour (f4), rye flour (f5), gluten (f79), gliadin (f98), ω5-gliadin (Tri a 19, f416), and lipid-transfer protein (Tri a14, f433).

TABLE 1 Characteristics of WDEIA patients, diagnosed by oral food challenge: sex, age, BMI, atopic dermatitis, total IgE (KU/L), sIgE (KU/L) against wheat flour, rye flour, gluten, gliadins, ω5-gliadin, lipid-transfer protein, *dermatophagoides pteronyssinus*, timothy grass, birch pollen allergen, cofactor, symptoms, severity according to Messmer and Ring and involved organ systems

						sIgE											
Patients	**Sex**	**Age**	**BMI**	**AD**	**Total IgE**	**WF**	**RF**	**G**	**Glia**	**ω5**	**Tri a 14**	**DP**	**TG**	**Bet v1**	**Cofactor**	**Symptoms**	**Severity**
1	F	45	19	-	203.0	0.72	0.66	0.65	0.29	8.12	<0.10	0.28	0.37	<0.10	Exercise, NSAID, stress, mental strain	i, f, u, g, gi, v	II (skin, gut)
2	M	39	21	-	97.3	0.32	0.73	0.80	1.10	3.47	<0.10	0.34	<0.10	<0.10	Exercise	i, f, u, g	I (skin)
3	M	42	28	RCA, A	105.0	0.83	0.82	0.73	0.87	3.97	<0.10	<0.10	0.82	<0.10	Exercise, stress, mental strain	i, u, r, un, d	III (skin, respiratory tract, CVS)
4	M	60	32	RCA	50.0	0.47	1.19	2.08	1.53	4.98	<0.10	<0.10	<0.10	0.32	Exercise, stress	i, u, un	III (skin, CVS)
5	M	48	29	-	95.0	0.32	0.26	0.28	0.13	1.52	<0.10	0.22	<0.10	<0.10	Exercise	i, f, un	III (skin, CVS)
6	M	54	24	RCA, A	319.0	1.44	3.40	5.89	4.67	10.70	<0.10	0.17	0.60	<0.10	Exercise, NSAID, stress, cold, heat, mental strain	i, u, h	II (skin, CVS)
7	F	47	37	-	95.3	0.14	0.16	0.22	<0.10	0.32	<0.10	0.25	<0.10	<0.10	Exercise, NSAID, alcohol	u, r, un	III (skin, respiratory tract, CVS)
8	M	55	27	RCA	368.0	2.70	2.98	7.38	5.10	11.40	<0.10	0.9	2.49	5.15	Exercise, alcohol, cold, heat	i, f, u, un	III (skin, CVS)
9	F	52	23	-	172.0	0.55	1.51	2.41	2.25	5.04	<0.10	<0.10	3.55	0.75	Exercise, AA, alcohol	i, f, u, r, g, un, h	III (skin, respiratory tract, CVS)
10	F	54	29	-	90.3	0.47	1.14	1.26	0.64	4.09	<0.10	<0.10	0.17	<0.10	AA, stress, cold, mental strain	i, f, u	I (skin)
11	M	56	29	RCA, AE	2193.0	5.95	7.04	11.70	11.00	30.20	<0.10	15.60	>100	<0.10	AA	i, f, u, r, g, v	II (skin, gut)
12	F	26	23	-	82.6	0.21	0.45	<0.10	<0.10	<0.10	<0.10	0.38	1.46	0.20	Exercise	i, f, u, g	I (skin)

Abbreviations: A, asthma; AA, acetylsalicylic acid; AD, atopic disease; AE, atopic eczema; Bet v1, birch pollen allergen; BMI, body mass index; CVS, cardiovascular system; DP, *dermatophagoides pteronyssinus*; f, flush; g, globus sensation; G, gluten; gi, gastrointestinal complaints; Glia, gliadins; h, hypotension; i, itching; NSAID, non-steroidal anti-inflammatory drugs; OAS, oral allergy syndrome; r, respiratory distress; RCA, allergic rhinoconjuctivitis; RF, rye flour; sIgE, specific immunoglobulins; TG, timothy grass; Tri a 14, lipid-transfer protein; u, urticaria; un, unconsciousness; v, vomiting; WF, wheat flour; ω5, ω5-gliadin.

2.4 | Preparation of BAT ATSs

Gliadins were extracted from wheat gluten using 60% aqueous ethanol. After dialysis and lyophilization, the gliadin fraction was separated into ω5-, ω1,2-, α-, and γ-gliadins by preparative reversed-phase high-performance liquid-chromatography. The glutenins were extracted from the residue after gliadin removal using 50% aqueous propanol, 60°C and reducing conditions. The HMW-GS and LMW-GS were obtained by sequential precipitation with 40% and 80% acetone, respectively.[14-16] Details of the production and characterization of the GPT can be found in the Supporting Methods.

GPT or gluten (15 mg) and 0.6 ml pepsin solution (0.6 mg/ml pepsin solved in 0.01 mol/L hydrochloric acid, enzyme-substrate ratio of 1/25) were incubated for 120 min at 37°C. The digest was stopped by adjusting the pH value to 7.0 with sodium hydrogen carbonate solution (50 mg/ml). The solution was filtered (0.45 µm) and the protein/peptide concentrations were measured at 205 nm by a micro volume UV/VIS spectrophotometer NanoDrop One (Thermo Fisher Scientific). If necessary, the sample solution was diluted with water to a concentration of 4 mg/ml. Further dilutions were made, to receive the following concentrations: 2.0, 0.8, 0.4, and 0.08 mg/ml. A pepsin-control was prepared in the same way, but without gluten proteins. ATS were prepared and stored at −20°C in aliquots until use in BAT.

2.5 | Basophil activation test

For quantitative determination of in vitro basophil activation, Flow CAST (Buehlmann Laboratories AG) was used, as described previously.[12] Venous blood was collected from participants in EDTA tubes and used immediately. The blood samples were gently homogenized at room temperature (RT). Per measurement, 50 µl of ATS (concentration 4.0–0.2 mg/ml), 100 µl stimulation buffer, 50 µl blood and 20 µl staining reagent were gently mixed by hand in polystyrene tubes. The staining reagent consisted of anti-CD63-fluorescein-isothiocyanate and anti-CCR3-pycoerythrin monoclonal antibodies (mAb). The tubes were then incubated for 25 min at 37°C. By addition of 2 ml lysis reagent and standing for 5 min in the dark at RT, the stimulation was stopped. The tubes were centrifuged at 500 × *g* for 5 min. The supernatant was decanted and the residue was resuspended in 200 µl of wash buffer by gentle mixing. Highly specific anti-FcεRI mAb and N-formyl-methionyl-leucyl-phenylalanine were used as positive controls. To determine the background value, stimulation buffer alone was used. The flow cytometric analysis was performed using a FACSCalibur system (Becton-Dickinson Immunocytometry System) with a 488 nm, 15 mW and a 635 nm, 10 mW argon laser. Basophils were gated as low side scatter CCR3/side scatter^{low}. CCR3 was used to identify basophils and CD63 as basophil activation marker, both marked with fluorescence-dye-labeled mAb. BD CellQuest (Becton-Dickinson Immunocytometry System) was used for data analysis. In each measurement, ≥450 basophil granulocytes (BG) were counted. The upregulation of the basophil activation marker CD63 by the tested ATS reflects the induced basophil activation.[11,12,17,18]

2.6 | Determination of different BAT parameters

The basophil activation (%CD63$^+$ basophils) was calculated by the percentage of CD63-expressing BG relative to the total number of counted BG in each measurement. The %CD63$^+$ basophils/anti-FcεRI ratio is defined as the quotient of the maximum percentage of activated %CD63$^+$ basophils, induced by an IgE-dependent stimulus, and the percentage of activated basophils triggered by the anti-FcεRI mAb as positive control.

2.7 | Statistical analysis

Statistical analysis was performed with SigmaPlot 14 (Systat Software GmbH) and Origin 19 (OriginLab Corporation). Statistical significance was tested by one-way ANOVA and Dunn's post hoc test. Receiver operating characteristic (ROC) analyses were carried out to estimate the discriminatory ability of the investigated parameters. Therefore, the area under the ROC curve, was used as further characteristic. The optimized cut-off of basophil activation (%) for best selectivity and specificity was determined from the ROC curve. Correlations between BAT results (maximum %CD63$^+$ basophils, %CD63$^+$/FcεRI ratio), diameter of wheals and erythema in SPT, severity (grouped comparisons I, II, III) and sIgE values were analyzed using Spearman's correlation test.

3 | RESULTS

3.1 | Study population

Twelve patients (7 m, 5 f; age range: 26–60 years; median age: 48 years) with a clinical history of WDEIA and positive challenge test were included in the study (Table 1). The control population consisted of 11 controls without a clinical history of any wheat-related disorder; six subjects were atopic. One subject with atopy was excluded, because of non-responsiveness to the positive control anti-FcεRI mAb in the BAT. Therefore, 10 controls were analyzed further (1 m, 9 f; age range: 25–76 years; median age: 44 years) (Table 2).

3.2 | SPT, serum sIgE and total IgE

A SPT is classified as positive when the diameter of the wheal, caused by the test substance, is greater or equal than the diameter of the wheal of the negative control with 3 mm added. Patients did not show wheals for the negative control, and they all showed a distinct allergic reaction to the positive control. There were positive responses to gluten and gliadins in SPT in all patients (Table 3, Figure 1). In case of wheat flour, HMW-GS and LMW-GS positive results were obtained with only two exceptions. Patients p7 and p12 were the only ones showing negative results to some of the test

TABLE 2 Characteristics of controls: sex, age, atopic dermatitis, total IgE (KU/L), sIgE (KU/L) against wheat flour, rye flour, gluten, gliadins, ω5-gliadin, lipid-transfer protein, *dermatophagoides pteronyssinus*, timothy grass, birch pollen allergen

					sIgE								
Controls	Sex	Age	AD	Total IgE	WF	RF	G	Glia	ω5	Tri a 14	DP	TG	Bet v1
1	F	76	-	82.7	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
2	F	71	RCA, OAS	38.9	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	15.9
3	F	27	RCA, OAS	44.2	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	6.16	5.30
4	F	26	-	6.7	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
5	F	25	-	5.7	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
6	F	32	RCA, A	49.8	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	2.62	0.86	1.72
7	F	26	-	15.0	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
8	F	51	-	3.5	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
9	M	54	RCA	28.0	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	2.87	1.86	<0.10
10	F	55	RCA	960.0	1.95	1.68	0.19	<0.10	<0.10	<0.10	0.33	2.36	>100

Abbreviations: A, asthma; AE, atopic eczema; AD, atopic disease; Bet v1, birch pollen allergen; DP, *dermatophagoides pteronyssinus*; G, gluten; Glia, gliadins; OAS, oral allergy syndrome; RCA, allergic rhinoconjuctivitis; RF, rye flour; sIgE, specific immunoglobulins; TG, timothy grass; Tri a 14, lipid-transfer protein; ω5, ω5-gliadin; WF, wheat flour.

TABLE 3 Skin prick test results for WDEIA patients (p) to wheat flour, gluten, gliadins and HMW-GS/LMW-GS

	Wheat flour		Gluten		Gliadins		LMW-GS		HMW-GS		Histamine		NaCl	
Patients	W	E	W	E	W	E	W	E	W	E	W	E	W	E
1	**4.0**	13.0	**6.5**	14.0	**7.0**	18.0	**4.5**	9.0	**4.5**	11.0	6.0	8.0	0.0	0.0
2	**10.5**	15.0	**9.0**	15.0	**7.0**	10.0	**9.0**	16.0	**12.5**	18.0	6.0	9.0	0.0	2.0
3	**6.5**	9.0	**6.0**	17.0	**7.0**	15.0	**7.5**	18.0	**7.0**	17.0	7.0	10.0	0.0	0.0
4	**6.0**	7.0	**7.0**	10.0	**7.0**	8.0	**10.0**	15.0	**10.0**	13.0	6.0	8.0	0.0	0.0
5	**6.0**	9.0	**4.0**	14.0	**4.0**	5.0	**7.0**	20.0	**5.0**	20.0	6.0	8.0	0.0	2.0
6	**6.0**	15.0	**6.0**	14.0	**4.5**	14.0	**9.0**	23.0	**7.0**	19.0	6.0	13.0	0.0	0.0
7	2.5	5.0	**5.5**	11.0	**3.0**	5.0	**4.5**	9.0	**5.5**	8.0	6.0	11.0	0.0	0.0
8	**7.0**	18.0	**12.0**	31.0	**8.5**	24.0	**12.0**	24.0	**6.0**	17.0	6.0	19.0	0.0	2.0
9	**5.0**	18.0	**3.5**	10.0	**7.5**	15.0	**3.0**	9.0	**5.0**	16.0	6.0	21.0	0.0	0.0
10	**5.5**	22.0	**4.4**	21.0	**5.0**	23.0	**5.5**	25.0	**5.5**	22.0	4.0	15.0	0.0	0.0
11	**8.0**	18.0	**6.5**	21.0	**12.0**	27.0	**11.0**	28.0	**8.5**	25.0	6.0	21.0	0.0	2.0
12	1.0	2.0	**3.0**	7.0	**3.5**	6.0	1.5	2.0	2.5	4.0	5.0	18.0	0.0	1.0
Range	1.0–12.0	2.0–25.0	3.0–15.0	5.0–35.0	3.0–11.0	5.0–32.0	1.0–18.0	1.0–30.0	2.0–13.0	3.0–30.0	3.0–7.0	7.0–30.0	-	0.0–2.0
Median	5.8	12.6	6.1	15.4	6.3	14.2	7.0	16.5	6.6	15.8	5.8	13.4	0.0	0.8

Note: The results are the median of a double determination $n = 2$ (except WDEIA patient 4, $n = 1$). Isotonic sodium chloride was used as negative (NaCl) and a 10% histamine solution as positive control (histamine). The diameters for wheals and erythema were documented in mm. A result is classified as positive (marked in bold), when the diameter of the wheal, caused by a test substance, is greater or equal than the diameter of the wheal caused by the negative control with 3 mm added. The range and median of all patients' results per test substance is documented.

Abbreviations: E, erythema; HMW, high-molecular weight glutenin subunits; LMW-GS, low-molecular weight glutenin subunits; W, wheals; WDEIA, wheat-dependent exercise-induced anaphylaxis.

substances (p7: wheat flour, p12: wheat flour, LMW-GS, HMW-GS). There were no significant differences in wheal or erythema diameter between wheat flour, gluten, gliadins, HMW-GS, and LMW-GS between the patients ($p > 0.05$).

Significantly higher values were found for total IgE and sIgE against *Dermatophagoides pteronyssinus*, timothy grass, wheat flour, rye flour, gluten gliadins, and ω5-gliadins in patients compared to controls ($p < 0.05$), respectively. No significant differences between

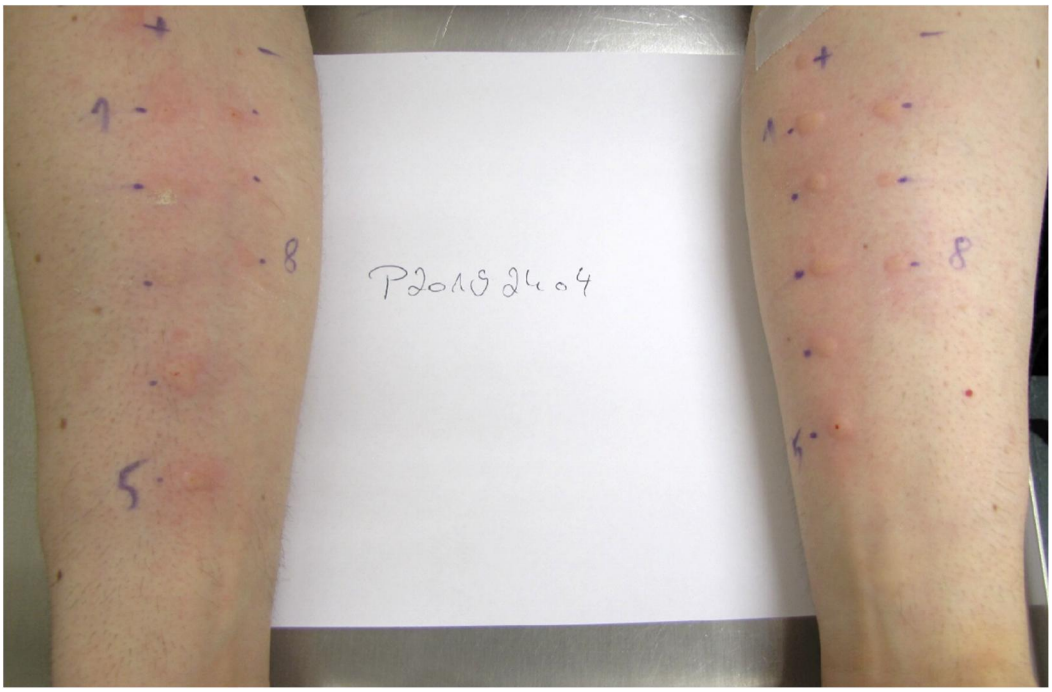

FIGURE 1 Exemplary skin prick test (SPT) results for wheat-dependent exercise-induced anaphylaxis patient 3. The following SPT substances were applied: +: histamine positive control, −: sodium chloride negative control, 1: gluten, 2: strongly hydrolyzed wheat protein, 3: slightly hydrolyzed wheat protein, 4: low-molecular-weight glutenin subunits, 5: high-molecular-weight glutenin subunits, 6: gliadins, 7: special gluten sample (significantly lower ω5-gliadin content), 8: wheat flour

patients and controls were found for sIgE against birch pollen allergen (Bet v 1) and lipid-transfer protein (Tri a14) ($p > 0.05$). For details see Tables 1 and 2.

3.3 | Evaluation of the response induced by ATSs in BAT

The induced allergenic response to the ATS was evaluated by three parameters used in BAT: %CD63$^+$ basophils, %CD63$^+$/anti-FcεRI ratio and AUC of dose-response curves. The ATS made from gluten and GPT induced basophil activations in patients with WDEIA. The basophil activation was dose-dependent up to a maximum of 71.3 %CD63$^+$ basophils in case of ω5-gliadins, 61.5% for gluten, 59.8% for LMW-GS, 53.7% for γ-gliadins, 50.7% for HMW-GS, 49.2% for α-gliadins, and 37.3% for ω1,2-gliadins. Significant differences between patients and controls were found for each ATS at every concentration tested ($p < 0.001$) (Figure 2). There were no significant differences in the background values for patients (range: 0.4%–1.7% CD63$^+$ basophils, median: 0.9% CD63$^+$ basophils) and controls (range: 0.4%–3.1% CD63$^+$ basophils, median: 1.1% CD63$^+$ basophils). The %CD63$^+$/anti-FcεRI ratio was significantly higher for gluten and all GPT for patients compared with controls at most concentrations ($p < 0.001$) (Figure S1). The dose-response curves were generated from the values of %CD63$^+$ basophils. The AUC as evaluation parameter combines the triggered allergic response (%CD63$^+$ basophils) and all tested doses in one. The AUC of ω5-gliadins, α-gliadins, and HMW-GS were significantly higher in patients compared to controls ($p < 0.001$), but there were no significant differences for ω1,2-gliadins, γ-gliadins, LMW-GS, and gluten ($p > 0.05$). For patients, median AUC values were 56.5 (range: 3.8–232.2) for ω5-gliadins, 34.7 (range: 0.4–163.7) for α-gliadins, and 24.0 (range: 3.7–102.9) for HMW-GS. Table 4 shows the AUCs of dose-response curves of gluten and GPT for patients and controls.

3.4 | ROC curves

The ROC curve describes how accurately the test can distinguish patients from controls. The greatest AUC values for concentration-independent ROC curves were determined for %CD63$^+$ basophils as characteristic, for ω5-gliadins (0.908), HMW-GS (0.867), and gluten (0.850) (Table 5; Figure 3). Concentration-independent ROC curves were generated from the maximum values for %CD63$^+$ basophils out of all tested concentrations for each single ATS in patients and controls. The optimal discrimination threshold (cut-off) for %CD63$^+$ basophils, when a basophil activation is classified as "allergen response" to a ATS, was determined for best sensitivity and specificity of concentration-independent ROC curves (Table 5). Concentration-dependent ROC curves showed best results at 4.00 mg/ml for ω5-gliadins and HMW-GS, 2.00 mg/ml for LMW-GS and gluten as well as 0.8 mg/ml for ω1,2-, α- and γ-gliadins. More information about ROC curves and results for concentration-dependent ROC curves are presented in the Supporting Information (Tables S1 and S2; Figure S2).

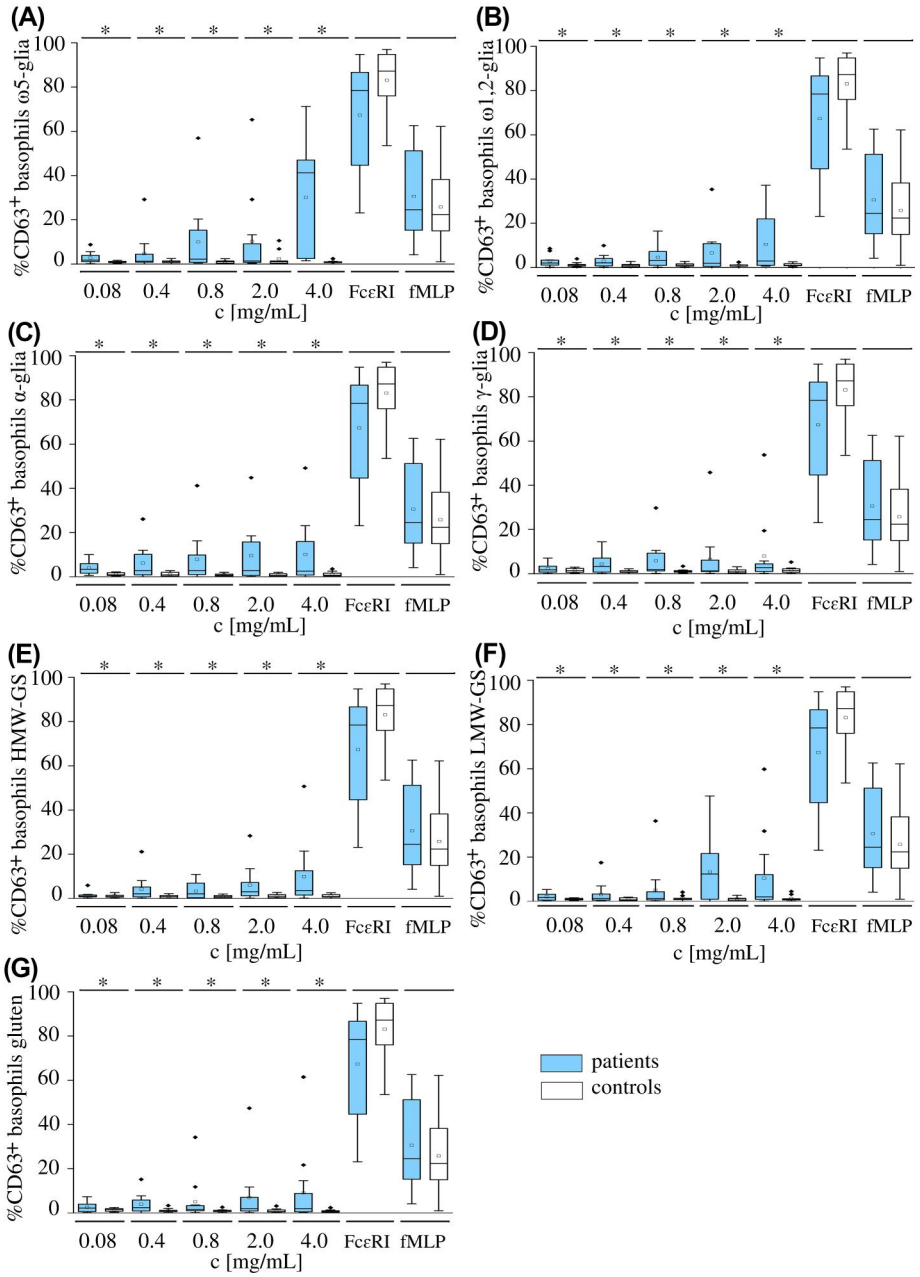

FIGURE 2 Dose-dependent basophil activation (%CD63$^+$ basophils) in wheat-dependent exercise-induced anaphylaxis patients (blue) and controls (white) using allergen test solutions from gluten protein types ω5-gliadins (A), ω1,2-gliadins (B), α-gliadins (C) and γ-gliadins (D), high-molecular-weight glutenin subunits (E) and low-molecular-weight glutenin subunits (F), and gluten (G) at concentrations of 4.0, 2.0, 0.8, 0.4, and 0.08 mg/ml and the positive controls anti-FcεRI monoclonal antibody and N-formyl-methionine-leucyl-phenylalanine (fMLP). Significant differences between patients and controls are indicated by asterisks (one-way ANOVA, Dunn's post hoc test, $p < 0.001$). Diamonds indicate individual outliers beyond the interquartile range

4 | DISCUSSION

In this proof-of-principle study we show that BAT with gluten and GPT solutions is a very promising tool to better define WDEIA. Gluten and GPT induced strong basophil activation in a dose-dependent manner in patients at most allergen concentrations, but not in controls. This is important, because the necessity of challenge tests together with one or several cofactors makes the diagnosis of WDEIA challenging. SPT and sIgE are routine diagnostic measures to detect sensitization, but SPT and sIgE to wheat flour extracts and even sIgE to ω5-gliadins may give negative results in WDEIA. Alternative in vitro methods to confirm the diagnosis are needed.[7]

Our results are in agreement with a study by Chinuki et al. who measured basophil CD203c expression to differentiate between classical WDEIA with IgE primarily directed against ω5-gliadins and a new WDEIA subtype caused by hydrolyzed wheat protein (HWP_A)

TABLE 4 Area under the dose-response curve (%CD63$^+$ basophils by concentration of the allergen test solutions [mg/ml]) from patients and controls for gluten and ω5-, ω1,2-, α-, and γ-gliadins and HMW-GS/LMW-GS

	Gluten	ω5-gliadins	ω1,2-gliadins	α-gliadins	γ-gliadins	HMW-GS	LMW-GS
p1	44.4	114.2	44.0	54.4	10.6	28.0	46.9
p2	12.6	4.9	24.9	39.4	18.7	35.2	41.1
p3	2.8	3.8	4.2	7.9	7.3	17.2	40.7
p4	5.4	7.0	6.6	8.4	9.0	19.0	20.5
p5	2.8	6.5	2.6	2.6	3.7	3.7	3.1
p6	44.8	80.8	48.7	63.8	49.0	49.7	80.7
p7	1.7	6.5	3.0	0.4	0.8	0.9	2.3
p8	3.0	44.3	1.1	1.9	3.3	1.3	0.5
p9	170.4	232.2	108.1	163.7	156.2	102.9	172.1
p10	9.4	50.7	3.1	3.7	4.0	8.4	4.3
p11	9.8	67.2	12.4	11.8	7.9	8.7	9.3
p12	7.5	59.8	56.6	58.1	29.9	12.9	24.6
Range (p)	1.7–170.4	3.8–232.2	1.1–108.1	0.4–163.7	0.8–156.2	0.9–102.9	0.5–172.1
Median (p)	26.2	56.5	26.3	34.7	25.0	24.0	37.2
c1	3.6	3.8	2.1	1.3	2.5	1.7	2.4
c2	3.8	3.3	1.5	6.9	8.1	1.9	6.5
c3	6.3	3.8	5.9	6.2	6.6	8.4	6.7
c4	4.4	4.2	4.7	4.4	5.0	7.2	5.2
c5	2.1	2.2	3.4	1.4	3.0	4.1	3.8
c6	9.8	16.3	9.9	8.3	11.2	7.4	9.4
c7	3.3	3.9	5.4	3.7	5.0	4.4	6.3
c8	1.6	2.7	2.3	2.2	3.5	0.6	1.5
c9	3.1	18.3	2.8	1.8	5.8	4.6	3.7
c10	1.4	2.0	3.3	1.5	1.2	1.5	1.6
Range (c)	1.4–9.8	2.0–18.3	1.5–9.9	1.3–8.3	1.2–11.2	0.6–8.4	1.5–9.4
Median (p)	3.9	6.1	4.1	3.8	5.2	4.2	4.7

Abbreviations: c, control; HMW-GS, high-molecular-weight glutenin subunits; LMW-GS, low-molecular-weight glutenin subunits; p, patient.

ATS	AUC	Cut-off (%CD63$^+$ basophils)	Sensitivity (%)	Specificity (%)
ω5-gliadins	0.908	1.8	100	70
HMW-GS	0.867	3.0	75	100
Gluten	0.850	2.8	75	90
α-gliadins	0.792	3.4	67	90
ω1,2-gliadins	0.758	2.6	67	80
γ-gliadins	0.750	3.1	67	80
LMW-GS	0.725	2.0	75	70

Abbreviations: ATS, allergen test solutions; AUC, area under the ROC curve; HMW-GS, high-molecular weight-glutenin subunits; LMW-GS, low-molecular-weight glutenin subunits; ROC, receiver operating characteristic.

TABLE 5 Patient and control data from concentration-independent ROC curves for ATSs from gluten and ω5-, ω1,2-, α-, and γ-gliadins and HMW-GS/LMW-GS with AUC and optimal discrimination threshold for %CD63$^+$ basophils (cut-off), when a basophil activation is classified as "allergen response" to an ATS

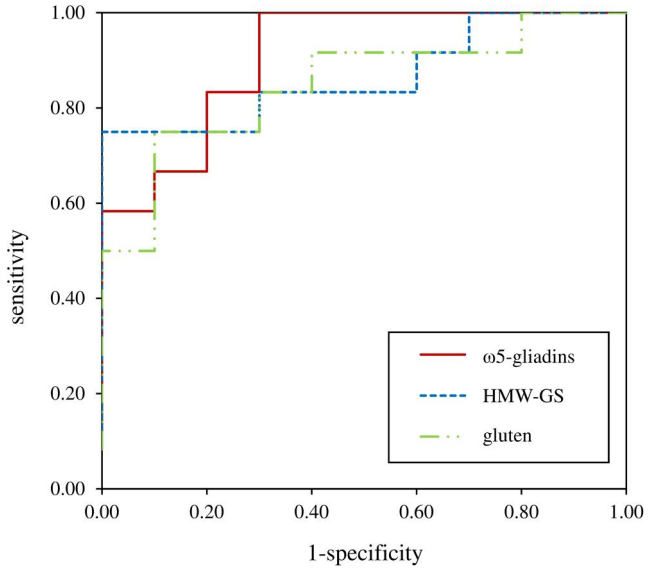

FIGURE 3 Concentration-independent receiver operating characteristic curves for ω5-gliadins, high-molecular-weight glutenin subunits (HMW-GS), and gluten, which had the highest sensitivity and specificity. The maximum basophil activation %CD63$^+$ basophils out of all tested concentrations for each single allergen test solution in patients and controls was taken to generate ROC curves

present in a soap in Japan. Significant enhancement of CD203c expression was observed with ω5-gliadins in patients with classical WDEIA and with HWP_A in patients sensitized by the soap, but not vice versa.[10] However, this study is of limited value for clinical routine, as (1) they only tested five patients in each group, but no controls, (2) used only ω5-gliadins and HWP_A, and (3) measured CD203c, a different basophil activation marker.

CD63 and CD203c are both in use as activation markers in BAT, while CD63 is the most common one. The upregulation of CD63 is closely associated with basophil degranulation induced by allergen stimuli.[19] Hoffmann et al. (2016) reported that the upregulation of CD203c also occurs to non-degranulation stimuli, which is not the case for CD63.[20] Eberlein et al. (2015) recommended the combination of CCR3 as identification marker for basophils and CD63 as activation marker for basophils in BAT and this is the setup also used in the present study.[21]

In BAT, only water-soluble allergen solutions can be tested in patient's blood. Chinuki et al. used aqueous, ethanolic, and alkaline extractions to generate ATS for BAT, but without further protein characterization.[22]

In the present study, a well-characterized representative gluten sample was used to isolate single GPT, α-, γ-, ω1,2-, and ω5-gliadins as well as HMW- and LMW-GS. Detailed information about the basophil activation in patients to GPT were obtained.[23] The challenge of poor solubility of gluten proteins in aqueous solutions was overcome by increasing their solubility and accessibility via partial hydrolysis with pepsin.[24]

The highly specific anti-FcεRI mAb that imitates bridging of the receptor by an allergen has been used as a positive control in BAT for numerous years. Rubio et al. analyzed %CD63$^+$ basophils and the %CD63$^+$/anti-FcεRI ratio after incubation with milk protein for prediction of the outcome of an oral challenge test. They reported a significant correlation between the %CD63$^+$/anti-FcεRI ratio and the outcome of the oral challenge test, depending on the ingested dose and reaction severity in patients with food allergy.[25]

In addition, Santos et al. found a correlation between the %CD63$^+$/anti-FcεRI ratio and the reaction severity during oral food challenge to peanuts.[26] In disagreement, there were no correlations between BAT results (maximum %CD63$^+$ basophils, %CD63$^+$/anti-FcεRI ratio), diameter of wheals and erythema in SPT, severity of symptoms (grouped comparisons I, II, III) and sIgE values (Spearman correlation test, $p > 0.05$) in our study.

Basophil activation (%CD63$^+$ basophils) and %CD63$^+$/anti-FcεRI ratio were significantly higher in patients for all tested GPT and gluten in most concentrations compared to controls. Additionally, a helpful characteristic is the AUC of the dose-response curve, which combines basophil activation and sensitivity. In our study, the AUC of dose-response curves of ω5- and α-gliadins and HMW-GS were significantly higher in patients compared to controls.

Calculating the AUC of ROC curves gives information about the discriminability of patients from controls depending on different parameters. BAT performance differed between GPT and gluten, with only acceptable results for α-gliadins, γ-gliadins, LMW-GS, and ω1,2-gliadins, but excellent results for ω5-gliadins (AUC ROC: 0.908), HMW-GS (AUC ROC: 0.867) and gluten (AUC ROC: 0.850). Sensitivity and specificity of basophil activation to these substances at optimal cut-off in WDEIA patients as compared to atopic and nonatopic control subjects were good for ω5-gliadins (sensitivity: 100%, specificity: 70%), HMW-GS (sensitivity: 75%, specificity: 100%), and gluten (sensitivity: 75%, specificity: 90%) respectively. The maximum %CD63$^+$ basophils turned out to be the best parameter to differentiate between patients and controls, with significant differences for all tested allergens. It is conspicuous that sensitivity and specificity were higher for ω5-gliadins, HMW-GS and gluten than for ω1,2-, α-, and γ-gliadins, and LMW-GS, because ω5-gliadins and HMW-GS have previously been identified as most relevant allergens in patients with WDEIA.[1-4]

Matsuo et al. recommended to determine sIgE against epitopes of ω5-gliadins and HMW-GS in combination for WDEIA diagnosis.[27] Based on our results, we can also confirm this recommendation for their use in BAT. Other allergenic GPT were less important in our study.[2,4] BAT identified the sensitization profile of WDEIA patients to be particularly directed against ω5-gliadins and HMW-GS, but in individual patients also against α-gliadins, γ-gliadins, LMW-GS, and ω1,2-gliadins. For example, two patients (p6, p9) showed high responses to LMW-GS 59.8 (p9) and 31.8 (p6) %CD63$^+$ basophils, concentration 4.0 mg/ml).[1-4]

One limitation of our study is the comparatively small number of WDEIA patients and controls. Due to the very low prevalence of

WDEIA overall, a single-center study such as ours can only include a certain number of individuals from the surrounding area. Our main intent was to identify the most suitable ATS for use in BAT from the panel of different gluten and GPT preparations tested. Now that we have identified ω5-gliadins, HMW-GS and gluten as most promising ATS, further work with more WDEIA patients from multiple centers is needed to put the cut-off levels on a broader basis and include WDEIA patients with negative SPT, WDEIA patients with positive SPT, but positive challenge only with cofactors as well as individuals who are sensitized (wheat IgE-positive), but clinically tolerant as proven by oral challenge.

According to the results of the proof-of-principle study we showed the potential of the BAT as alternative to routine SPT and sIgE measurements in WDEIA diagnosis. The BAT turned out to be promising to study the allergenicity of different GPTs, which becomes only possible after special preparation to increase water solubility, as required for BAT. Our findings indicate the use of %CD63$^+$ basophils as best parameter to discriminate between patients and controls and highlight the allergenicity particularly of ω5-gliadins and HMW-GS for WDEIA.

ACKNOWLEDGMENTS

This work was supported by the authors' institutes, by research and development grants from the German Federal Ministry of Education and Research (BMBF), project ABROGATE (funding number: 01EA2106A) and by Deutsche Forschungsgemeinschaft (DFG; German Research Foundation) CRC 1371 P06; bilateral funding from Luxembourg National Research Fund (FNR) project C17/BM/11656090 - DFG DACH-Lead-Agency BI696/12-1. We acknowledge support by the KIT-Publication Fund of the Karlsruhe Institute of Technology. We thank Franziska Martin for her excellent technical support, Buehlmann Laboratories AG (Schönenbuch, Switzerland) for supporting by reduced reagent costs, the H.+W. Winkler Stiftung "Hilfe für den Menschen" for their financial support of research on diagnosis and management of patients with WDEIA as well as everyone who participated in the study.

CONFLICT OF INTEREST

B. Eberlein received methodological and technical support from Buehlmann Laboratories AG (Schönenbuch, Switzerland). The other authors declare no competing interests.

AUTHOR CONTRIBUTIONS

Conceptualization: Angelika Miriam Gabler, Julia Gebhard, Bernadette Eberlein, Tilo Biedermann, Katharina Anne Scherf, and Knut Brockow; Formal analysis, investigation, and methodology: Angelika Miriam Gabler and Julia Gebhard; Data curation: Angelika Miriam Gabler, Julia Gebhard and Bernadette Eberlein; Funding acquisition, resources, and Supervision: Bernadette Eberlein, Tilo Biedermann, Katharina Anne Scherf, and Knut Brockow; Visualization: Angelika Miriam Gabler; Writing—original draft: Angelika Miriam Gabler; Writing—review & editing: Julia Gebhard, Bernadette Eberlein, Tilo Biedermann, Katharina Anne Scherf, and Knut Brockow. All authors read and approved the final manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Gabler AM, Gebhard J, Eberlein B, Biedermann T, Scherf KA, Brockow K. The basophil activation test differentiates between patients with wheat-dependent exercise-induced anaphylaxis and control subjects using gluten and isolated gluten protein types. *Clin Transl Allergy*. 2021;e12050. https://doi.org/10.1002/clt2.12050

3.3. Basophil activation to gluten and non-gluten proteins in wheat-dependent exercise-induced anaphylaxis

In this work Angelika Miriam Gabler developed easily accessible ATS from gluten and HWP for WDEIA clinical routine diagnosis for BAT and carried out a proof-of-principle study. Based on the results of part 3.1., raw materials for the preparation of ATS were selected: A representative gluten sample, gluten from a wheat/rye translocation line with 89% lower content of ω5-gliadins in comparison to the representative gluten, slightly and extensively hydrolyzed wheat protein. She applied the ATS in a BAT human study on twelve WDEIA patients and ten control subjects to investigate their allergenicity in WDEIA. Angelika Miriam Gabler carried out the BAT experiments on blood samples from the study participants and evaluated the study data. Additionally, she compared the BAT parameters %CD63$^+$ basophils and %CD63$^+$ basophils/anti-FcεRI ratio to routine diagnostics. ROC curves were created to investigate the potential of the test approach to distinguish between patients and controls. The test sensitivity and specificity were determined, which were excellent for the slightly hydrolyzed wheat protein sample. It was shown, that BAT-FACS is an alternative tool for WDEIA clinical routine diagnosis.

For a better understanding of the allergenic responses in WDEIA patients, Angelika Miriam Gabler characterized the tested ATS using SDS-PAGE, RP-/GP-HPLC and proteomics-based high resolution UPLC-TripleTOF-MS. The MS dataset was analyzed and visualized using bioinformatics software and algorithms for peptide identification and matching against a protein database of *Triticum aestivum*, by Angelika Miriam Gabler. The identified proteins were relatively quantitated using the IBAQ algorithm. Angelika Miriam Gabler developed the methodology and carried out the investigations. She analyzed the data and visualized them in figures and tables. She wrote the manuscript draft and did the revisions.

The results from the proteomics-based UPLC-TripleTOF-MS analysis together with the determined BAT results indicated that non-gluten proteins are also relevant allergens in the context of WDEIA besides gluten since high percentages of ATIs were determined in the ATS.

In addition, yet unidentified allergenic epitopes need to be present in the ATS because the identified proteins were searched for known WDEIA epitopes and only one epitope was found. Consequently, the known epitopes cannot explain the allergenic basophil activations triggered by these ATS in WDEIA patients. ATIs were not often reported in the context of WDEIA. Since WDEIA patients responded intensely to these ATS, the relevance and allergenicity of ATIs in WDEIA should be investigated further.

frontiers in Allergy

ORIGINAL RESEARCH
published: 25 February 2022
doi: 10.3389/falgy.2022.822554

Basophil Activation to Gluten and Non-Gluten Proteins in Wheat-Dependent Exercise-Induced Anaphylaxis

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Specialty section:
This article was submitted to Allergens, a section of the journal Frontiers in Allergy

Received: 25 November 2021
Accepted: 26 January 2022
Published: 25 February 2022

Citation:
Gabler AM, Gebhard J, Norwig M-C, Eberlein B, Biedermann T, Brockow K and Scherf KA (2022) Basophil Activation to Gluten and Non-Gluten Proteins in Wheat-Dependent Exercise-Induced Anaphylaxis. Front. Allergy 3:822554. doi: 10.3389/falgy.2022.822554

Wheat-dependent exercise-induced anaphylaxis (WDEIA) is a cofactor-induced wheat allergy. Gluten proteins, especially ω5-gliadins, are known as major allergens, but partially hydrolyzed wheat proteins (HWPs) also play a role. Our study investigated the link between the molecular composition of gluten or HWP and allergenicity. Saline extracts of gluten (G), gluten with reduced content of ω5-gliadins (G-ω5), slightly treated HWPs (sHWPs), and extensively treated HWPs (eHWPs) were prepared as allergen test solutions and their allergenicity assessed using the skin prick test and basophil activation test (BAT) on twelve patients with WDEIA and ten controls. Complementary sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), high-performance liquid chromatography (HPLC), and mass spectrometry (MS) analyses revealed that non-gluten proteins, mainly α-amylase/trypsin inhibitors (ATIs), were predominant in the allergen test solutions of G, G-ω5, and sHWPs. Only eHWPs contained gliadins and glutenins as major fraction. All allergen test solutions induced significantly higher %CD63$^+$ basophils/anti-FcεRI ratios in patients compared with controls. BAT using sHWPs yielded 100% sensitivity and 83% specificity at optimal cut-off and may be useful as another tool in WDEIA diagnosis. Our findings indicate that non-gluten proteins carrying yet unidentified allergenic epitopes appear to be relevant in WDEIA. Further research is needed to clarify the role of nutritional ATIs in WDEIA and identify specific mechanisms of immune activation.

Keywords: allergy, amylase/trypsin inhibitor, basophil activation test, gluten, proteomics, wheat

INTRODUCTION

Wheat-dependent exercise-induced anaphylaxis (WDEIA) is a cofactor-induced wheat allergy. It is generally considered to be rare. In Japanese adolescents, the prevalence of food-dependent exercise-induced anaphylaxis predominantly to wheat was 0.017%. However, as these patients tolerate wheat in the absence of association with cofactors, WDEIA may not be recognized in many patients and they are often given the diagnosis of idiopathic anaphylaxis instead (1–3).

Patients with WDEIA may react to intact gluten proteins and/or partially hydrolyzed wheat proteins (HWPs) (4–6). Besides others, Yokooji et al. and Hiragun et al. reported allergic reactions in patients with WDEIA to HWPs in facial soap (6, 7). HWPs are made of gluten subjected to chemical or enzymatic partial hydrolysis to obtain foaming and emulsifying properties for use in foods and cosmetics (6, 8, 9). Depending on the treatment, HWPs differ significantly from one another regarding their functional properties and molecular composition (8, 10–12). Partial hydrolysis may lead to exposure of pre-existent allergenic epitopes otherwise buried within protein aggregates or to the formation of new epitopes, e.g., through deamidation (6, 7). The increase in solubility of HWPs compared with native gluten also affects allergen passage through the skin or the small intestine (6, 7, 13).

About 80% of patients with WDEIA have specific IgE (sIgE) against ω5-gliadins, the major allergens in WDEIA (14), but sensitization to other wheat gluten proteins, such as high- and low-molecular-weight glutenin subunits (HMW-GS and LMW-GS) or α- and γ-gliadins has also been reported (15–20). Water- or salt-soluble non-gluten proteins, such as lipid-transfer proteins (LTPs) associated with baker's asthma, were also suggested to play a role in WDEIA (21–23). Pastorello et al. found sIgE against α-amylase/trypsin inhibitors (ATIs) in WDEIA patients' sera (24), but the role of nutritional non-gluten proteins as causative agents for WDEIA is currently underexplored.

Approaches to diagnose WDEIA include clinical history, skin prick test (SPT), measurement of sIgE against ω5-gliadins, and oral gluten challenge combined with cofactor as golden standard (4). Due to the risk of a serious anaphylactic reaction during the challenge tests, there is a need to establish alternatives. The *in vitro* basophil activation test (BAT) using well-defined allergen test solutions (ATSs) may be suitable, because basophil activation is directly related to the allergenicity of a test substance (25–28). The BAT is already used to diagnose and investigate IgE-mediated allergies, e.g., allergy against antibiotics (29) or bee and wasp venom (30). Schwager et al. evaluated the allergenic potential of natural and recombinant peanut oleosins using the BAT on peanut-allergic and peanut-sensitized patients in comparison with a control group. A complex cocktail of 12 antibodies was used to identify basophils. The activation marker of identified basophils was CD63 (31). The same group improved the BAT workflow for reliable results with a time saving approach to make it suitable for clinical routine. *Inter alia*, they compared the approach of Schwager et al. with a simplified approach using CD63 (activation marker) and CD203c and FcεRIα (identification markers). As they found no significant differences between the results of both strategies, they showed that the necessary simplification to make BAT applicable in clinical routine is possible and reliable. Furthermore, Behrends et al. used different peanut allergens in the BAT, such as oleosins and defensins, Der p 2, Bet v 1, Ara h 8, Ara h 14, and Ara h 15 (31, 32). One important aspect of both studies is the application of single peanut allergens in the BAT. These were either isolated and purified from raw and in-shell roasted peanuts or recombinantly expressed in *Escherichia coli* (31, 32). The robust and optimized BAT setup using these single allergens allowed the differentiation between peanut-allergic and peanut-sensitized individuals (32).

Mehlich et al. tested alpha-gal sensitized patients in comparison with healthy controls for their basophil reactivity to commercial alpha-gal allergens and pork kidney extract. Thereby, CCR3 was assessed as an identification marker and CD63 as an activation marker for basophils. Similar to the peanut-BAT, they were able to differentiate between patients with alpha-gal syndrome and asymptomatic alpha-gal sensitization within the sensitized patient group using BAT (33).

Chinuki et al. used the BAT to examine the allergenicity of a HWP product in 10 WDEIA patients. The HWP had been produced by acid hydrolysis, but further details on its molecular composition were not provided (5, 34).

We already demonstrated that BAT using CCR3 as identification marker and CD63 as activation marker for basophils allowed the discrimination of patients with WDEIA from controls. ATSs made from peptic hydrolysates of ω5-gliadins, HMW-GS and total gluten showed the best sensitivity and specificity at optimal cut-off (20). Although these three peptic hydrolysates work very well in BAT, one drawback of using those ATSs is that they cannot be easily prepared in routine clinical practice, because the procedure involves elaborate gluten fractionation and digestion (20).

Therefore, we aimed to provide aqueous ATS from gluten samples with different molecular properties that can be easily made for use in BAT. We included four ATSs to cover a wide range of variability in molecular composition. These ATSs were prepared as saline extracts from one representative sample of wheat gluten (G) and of slightly hydrolyzed wheat proteins (sHWPs) and extensively hydrolyzed wheat proteins (eHWPs) selected from our previous work (10). The fourth sample was produced from flour of wheat variety Pamier, a wheat/rye translocation line with an 89% lower content of ω5-gliadins (G-ω5; 2.40 mg/g), the main allergen in WDEIA, in comparison with representative gluten (G; 22.3 mg/g) (35). If G-ω5 truly induced lower allergenic responses, products made of this variety might be nutritionally beneficial for patients with WDEIA. We combined allergenicity assessment using SPT and BAT with the characterization of allergenic proteins in the ATS to identify which proteins are present in those saline ATSs.

METHODS

Materials

Gluten and HWPs were from Hermann Kröner GmbH (Ibbenbüren, Germany), Tate & Lyle PLC (London, UK), and Manildra Group (Gladesville, Australia). G in the present study corresponds to G1, G-ω5 to G4, sHWP to HWP7, and eHWP to HWP3 (7). All reagents and chemicals were from Sigma Aldrich (Darmstadt, Germany), Merck (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany), Honeywell (Offenbach, Germany), J. T. Baker (Arnhem, The Netherlands), and Fresenius Kabi Deutschland GmbH (Bad Homburg, Germany). Water was purified with an Arium 611VF water purification system (Sartorius, Goettingen, Germany). Pepsin (from porcine mucosa,

10 FIP U/mg), trypsin (from bovine pancreas, TPCK treated, 10.000 BAEE U/mg protein), α-chymotrypsin (from bovine pancreas, TLCK-treated, ≥40 U/mg protein), and thermolysin (from *Geobacillus stearothermophilus*, 30–175 U/mg protein) were purchased from Sigma Aldrich (Darmstadt, Germany) and Merck (Darmstadt, Germany).

Allergen Test Solutions

To prepare saline ATS for BAT, the sample (eHWP: 25 mg, sHWP: 100 mg, G: 100 mg, and G-ω5: 100 mg) was weighed into a 2 ml tube followed by addition of glass beads for better homogenization and 1 ml 0.9% isotonic NaCl solution. The suspension was homogenized by vortex mixing for 1 min, stirring for 20 min at room temperature, and ultrasonic treatment for 3 min. After centrifugation (2,300 × *g*, 15 min, 20°C), the supernatant was filtered (0.45 μm, regenerated cellulose, GE Healthcare, Chicago, IL, USA) and the protein/peptide concentrations measured at 205 nm by a micro volume UV/VIS spectrophotometer NanoDrop One (Thermo Fisher Scientific, Carlsbad, CA, USA). The ATS from eHWP was diluted 1:5 (v/v) with 0.9% isotonic NaCl solution to adjust protein/peptide concentrations of all ATS for BAT experiments.

Several supernatants of G, G-ω5, and sHWP were prepared, pooled, and lyophilized for ultra-performance liquid chromatography (UPLC)-TripleTOF-MS analysis. The lyophilized powder was carefully homogenized with mortar and pestle and weighed into 2 ml tubes (6 mg). eHWP was used directly (4 mg), because it was completely soluble in isotonic NaCl solution.

Study Population

Twelve patients with a clinical history of WDEIA based on positive oral food challenge (5 women, 7 men, 26–60 years, median age: 48 years) and 10 individuals without a history of any wheat-related disorder were included in the study as healthy controls (9 women, 1 male, 25–76 years, median age: 44 years). Five of the control subjects were atopic. Further details on the study population are reported in Gabler et al. (20). The study protocol was approved by the ethics committee of the Technical University of Munich and all participants gave written informed consent before being included in the study.

Skin Prick Test

Skin prick test was carried out on the forearm with gluten (G, G-ω5) and hydrolyzed wheat proteins (eHWPs and sHWPs). Histamine dihypochloride solution (10%) from ALK-Abello (Hørsholm, Denmark) served as a positive control and isotonic NaCl solution from Fresenius Kabi Deutschland GmbH (Bad Homburg, Germany) as a negative control. The SPT was defined as positive, when the wheal diameter caused by the tested substance was ≥3 mm larger than the diameter of the negative control (4).

Basophil Activation Test

Flow CAST (Bühlmann Laboratories AG, Schönenbuch, Switzerland) was used for quantitative determination of *in vitro* basophil activation, as described previously (20). Anti-FcεRI-mAb and *N*-formyl-methionyl-leucyl-phenylalanine were used as positive controls. Flow cytometry was performed using a FACSCalibur system (Becton-Dickinson Immunocytometry System, Heidelberg, Germany) with a 488 nm, 15 mW and a 635 nm, 10 mW argon laser. Basophils were gated as low side scatter CCR3/side scatter^{low}. CCR3 was used as identification marker for basophils and CD63 as basophil activation marker, labeled with anti-CCR3-phycoerythrin mAb and anti-CD63-fluorescein-isothiocyanate, respectively. BD CellQuest (Becton-Dickinson Immunocytometry System) was used to analyze the data. At least 450 basophils were counted per measurement (13, 28). The following BAT parameters were studied: basophil activation (%CD63$^+$ basophils) expressed as percentage of basophil granulocytes expressing CD63 divided by the total number of counted basophil granulocytes per single measurement and %CD63$^+$ basophils/anti-FcεRI ratio as quotient of the basophil activation (%CD63$^+$ basophils) triggered by ATS and by the anti-FcεRI mAb as positive control (33).

Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis

Sodium dodecyl-sulfate polyacrylamide gel electrophoresis was carried out according to Lagrain et al. (36). In brief, lyophilized ATS (G, G-ω5, sHWP) and eHWP (used directly, because of complete solubility in isotonic NaCl) were incubated with reducing extraction buffer for 12 h at room temperature, heated to 60°C for 10 min and centrifuged (5,000 × *g*, 20°C, 5 min). A homogeneous NuPAGE 10% polyacrylamide Bis-Tris gel (10 mm × 1 mm wells) (Invitrogen, Carlsbad, CA, USA) was used with a MOPS running buffer. The PageRuler Unstained Protein Ladder served as a molecular mass (M_r) standard (Thermo Fisher Scientific). The running time was 30 min at 200 V and 115 mA. Protein bands on the gel were fixed with 12% trichloroacetic acid (w/w) (30 min), stained with Coomassie blue (30 min) and destained in two steps. The gels were scanned using the Gel Doc EZ Imager (Bio-Rad Laboratories, Munich, Germany) and the Image Lab software (Bio-Rad Laboratories) (10, 37).

Gel Permeation HPLC

Two different gel permeation (GP)-HPLC systems, previously reported by Gabler at al. and Scherf et al. were used to analyze the M_r distribution of proteins and peptides in the ATS compared with protein markers of known M_r (10, 38). Measurements were performed on a Jasco HPLC Extrema (Jasco, Gross-Umstadt, Germany). A BioSep-SEC-s3000 column (300 mm × 4.6 mm, 29 nm, 5 μm, Phenomenex, Aschaffenburg, Germany) was used for protein separation with an isocratic gradient (50:50, 0.1% trifluoroacetic acid (TFA) in ultrapure water/0.1% TFA in acetonitrile) with a flow rate of 0.3 ml/min at 20°C. Chromatography was carried out on a BioBasic SEC-60 column (150 mm × 7.8 mm, 6 nm, 5 μm, Thermo Fisher Scientific) with an isocratic gradient (70:30, 0.1% TFA in ultrapure water/0.1% TFA in acetonitrile) at a flow rate of 1.0 ml/min for small proteins/peptides. The injection volume was 3–5 μl.

Reversed-Phase HPLC

The protein/peptide concentration of the ATS was analyzed according to Gabler et al. using reversed phase (RP-)HPLC on a Jasco XLC instrument (Jasco) using a C_{18} column at 60°C (Acclaim 300, C_{18}, 2.1 mm × 150 mm, 300 nm, 3 µm, Thermo Fisher Scientific). The elution solvents were 0.1% TFA in ultrapure water (A) and 0.1% TFA in acetonitrile (B) at a flow rate of 0.2 ml/min. Gradient elution was performed: 0 min 0% B, 0.1–0.5 min 24% B, 0.6–15 min 56% B, 15.1–19.1 min 90% B, 19.2–35.0 min 0% B. The injection volume was 20 µl. Prolamin Working Group (PWG)-gliadin was used for external calibration (10, 39).

Ultra-Performance Liquid Chromatography (UPLC)-TripleTOF-MS

Reduction and Alkylation

Lyophilized ATS from G, G-ω5, and sHWP as well as eHWP were dissolved in 320 µl of TRIS-HCl buffer (0.5 mol/L, pH 8.5) and 320 µl 1-propanol. For reduction, 50 µl of Tris-(2-carboxyethyl)-phosphine (TCEP) solution (22 mg/ml TCEP in TRIS-HCl buffer) were added and the samples shaken for 30 min at 60°C under nitrogen. After cooling, 100 µl of chloroacetamide (CAA)-solution was added for alkylation (34 mg/ml CAA in TRIS-HCl buffer). The samples were shaken for 45 min at 37°C in the dark. The solutions were evaporated to dryness (37, 40).

Enzymatic Digestion

Different protein digestions were carried out: pepsin + trypsin (PT), pepsin + chymotrypsin (PC), pepsin + trypsin + chymotrypsin (PTC), trypsin + chymotrypsin (TC), and thermolysin (TLY). Digestion was performed by adding pepsin [750 µl, 0.2 mg/ml in 0.15 mol/L HCl, pH 2, enzyme/substrate (E:S) ratio of 1:20 (w/w)] to the alkylated residues and shaking for 60 min at 37°C. After the peptic digest, the pH was adjusted to 6.5 with PBS (50 mmol/L). Then, trypsin and/or chymotrypsin [E:S of 1:20 for T or C, E:S of 1:40 for TC (w/w)] were added and the samples were hydrolyzed for 120 min at 37°C. For TC digestion, TC was added to the alkylated residues [1 ml, 0.12 mg/ml T/C in 0.1 mol/L TRIS-HCl-buffer, E:S of 1:50 (w/w)] followed by incubation for 16 h at 37°C. The digestions were stopped by heating for 10 min at 95°C (37, 40). TLY digestion [E:S of 1:20 (w/w)] was carried out in TRIS-HCl $CaCl_2$ buffer (0.2 mol/L TRIS, 0.5 mmol/L $CaCl_2 \cdot 2H_2O$, pH 6.5) at 37°C for 16 h. The reaction was stopped with formic acid (FA) (41–43).

Solid Phase Extraction

Enzymatic digests were purified by solid phase extraction (SPE) using 100 mg Discovery DSC-18 cartridges (Supelco, Bellefonte, PA, USA). After activation with methanol, equilibration with 80/20 (v/v) acetonitrile 0.1% FA in water and washing with 2/98 (v/v) acetonitrile/0.1% FA the cartridges were loaded with sample and washed again. Elution was carried out using 40/60 (v/v) acetonitrile/0.1% FA in the first step and 80/20 (v/v) acetonitrile/0.1% FA in the second. Both eluates were united and evaporated to dryness. The residues were dissolved in 500 µl 0.1% FA and filtered immediately before UPLC-TripleTOF-MS analysis (40, 44).

UPLC-TripleTOF-MS

The UPLC-TripleTOF-MS analysis was performed using an UPLC system ExionLC coupled to a TripleTOF 6600 MS (SCIEX, Darmstadt, Germany). A bioZen peptide PS-C18 column (100 mm × 2.1 mm, 10 nm, 1.6 µm) (Phenomenex) was used. Peptides (injection volume 10 µl) were separated using linear gradient elution (0–65 min 5% B to 100% B, 65–69 min 100% B, 69–70 min 100% B to 5% B, 70–75 min 5% B; solvent A: 0.1% FA in water, solvent B: 0.1% FA in acetonitrile) with a flow rate of 0.35 ml/min at 40°C. The MS was operated in positive electrospray ionization mode and the following settings: ion spray voltage 5,500 eV, source temperature 550°C, heating gas 0.45 MPa, nebulizing gas 0.38 MPa, curtain gas 0.24 MPa.

The MS was operated in information-dependent acquisition (IDA) mode. The mass-to-charge range for MS1 was 350–1,800, using an accumulation time of 250 ms, collision energy of 10 V, and a declustering potential of 80 V. The IDA criteria for the precursor ion included intensity of >100 counts/s and the resolution was set to 0.5 Da. MS2 spectra of the 20 most abundant compounds were recorded in a mass-to-charge range of 350–1,800, using an accumulation time of 40 ms, collision energy of 35 V, declustering potential of 80 V, and a collision energy spread of 5 V. Instrument control and data acquisition were performed with Analyst TF software (v 1.7.1., SCIEX).

Analysis of UPLC-TripleTOF-MS Data

The raw data were analyzed against the proteome of *Triticum aestivum* (UniprotKB, download 08/2019) using the proteomics software MaxQuant (version 1.6.3.4) (45). The search parameters including specific and unspecific digestion are reported in **Supplementary Table 1**. All other parameters were kept as default settings. The intensity based absolute quantitation (iBAQ) algorithm implemented in MaxQuant was used to estimate wheat protein abundances in the ATS. A total sum normalization of protein iBAQ intensities between sample measurements was performed to correct for different total protein injection amounts (37, 40).

Statistical Analysis

A statistical analysis was performed with Origin 2020 (OriginLab Cooperation, Northampton, MA, USA) and SigmaPlot 14 (Systat Software GmbH, Erkrath, Germany). One-way ANOVA with Dunn's *post-hoc* test ($p < 0.05$) was used to identify significant differences between the ATS analyzed by HPLC, SPT, and BAT. Receiver operating characteristic (ROC) analyses were carried out to estimate how well BAT parameters, such as area under the ROC curve (AUC) distinguished between patients and controls. The optimized discrimination threshold (cut-off) for the %$CD63^+$ basophils/anti-FcεRI ratio was determined based on the ROC curve for best selectivity and specificity.

RESULTS

Allergenicity of Gluten and HWP for Patients With WDEIA

Skin Prick Test

As expected, all patients with WDEIA showed sensitizations to the positive control (wheal and erythema mean diameter (W/E): 5.8 and 13.4 mm), but none to the negative control (0 mm). A positive reaction was triggered in all patients with WDEIA for G (W/E 6.1 and 15.4 mm), in 11 of 12 patients for sHWP (W/E 5.8 and 13.7 mm), in 10 of 12 patients for G-ω5 (W/E 3.8 and 7.0 mm), and in 9 of 12 patients for eHWP (W/E 6.2 and 11.1 mm) (**Figure 1** and **Supplementary Table 2**). Large interindividual differences were observed that resulted in wide ranges of minimal and maximal diameter for each substance, ranging from 0.5 to 16.5 mm for W and from 2.0 to 31.0 mm for E overall. There were no significant differences ($p > 0.05$) in mean wheal diameter between the four substances, even if G, sHWP, and eHWP triggered wheals that were comparable in size with those of the positive control and about 60% larger compared with G-ω5. The mean erythema diameter caused by G, sHWP, and eHWP was also similar to that of the positive control. The erythema following SPT with G was significantly higher ($p < 0.05$) than that with G-ω5, but all other pairwise comparisons were not significantly different from one another.

Basophil Activation

All ATS for gluten and HWP induced basophil activation in the blood of patients with WDEIA, except for p5, p7, and p8 (**Figure 2** and **Supplementary Figures 1–8**). As already observed in the SPT, the responses were highly individual, e.g., with blood from patients p1 and p6 showing the highest basophil activation for eHWP, p9 for sHWP and eHWP, and p11 for G and G-ω5. Contrary to expectations, G-ω5 did not lead to lower basophil activation in comparison with G in general. The basophil activations (%CD63$^+$ basophils) of patients were in a range between 0.2 and 63.0% (median: 9.4%) for G, 0.6–82.6% (median: 11.6%) for G-ω5, 0.4–72.7% (median: 8.2%) for eHWP, and 2.2–80.0% (median: 23.1%) for sHWP. Significant differences in %CD63$^+$ basophils between patients and controls were found for sHWP ($p < 0.05$), but not for G, G-ω5, and eHWP. In contrast, patients showed significantly higher %CD63$^+$ basophils/anti-FcεRI ratios compared with controls with all ATS ($p < 0.05$) (**Figure 3**). Consequently, the %CD63$^+$ basophils/anti-FcεRI ratio was used as characteristic parameter for further investigations.

There were no significant differences ($p > 0.05$) in patient %CD63$^+$ basophils/anti-FcεRI ratios between the four different ATS (median G = 0.113, G-ω5 = 0.178, eHWP = 0.130, and sHWP = 0.408), *inter alia*, due to high interindividual variability. The %CD63$^+$ basophils/anti-FcεRI ratios were low for all ATS in controls (median: G: 0.018, G-ω5: 0.016, eHWP: 0.069, and sHWP: 0.019). The ROC curves generated for all ATS from the %CD63$^+$basophils/anti-FcεRI ratio of patients and controls revealed that BAT with sHWP gave the highest AUC (0.925) with excellent sensitivity (100%) and specificity (83%) to discriminate between patients with WDEIA and controls (**Supplementary Figure 9** and **Supplementary Table 3**).

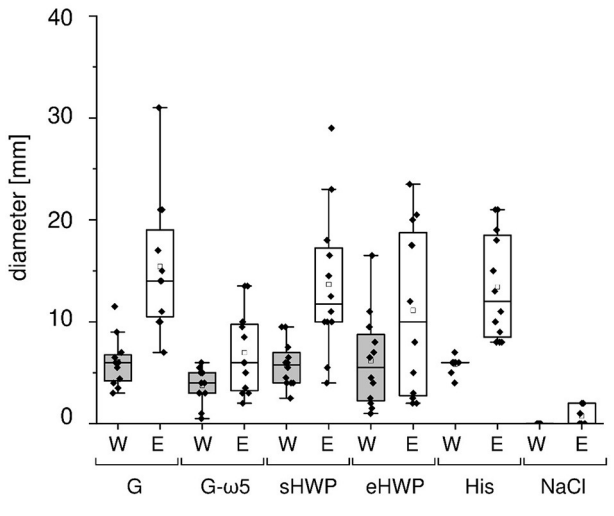

FIGURE 1 | Skin prick test results of patients with wheat-dependent exercise-induced anaphylaxis. Allergen test solutions from gluten G and G-ω5 and from slightly and extensively hydrolyzed wheat proteins sHWP and eHWP were used, as well as histamine dihypochloride (10%) solution (His) as positive control and isotonic sodium chloride solution as negative control (NaCl). The diameter of the wheals (W) and erythema (E) were documented in mm. A double determination was performed for each patient ($n = 2$), except patient 4 ($n = 1$). The data for gluten G were added for comparison and were already reported in Gabler et al. (20).

Identification of Allergenic Proteins in the Test Solutions

Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis

In SDS-PAGE, all protein bands from the ATS had M_r below or equal to 60 kDa (**Figure 4**). The lack of larger and hydrophobic proteins, such as HMW-GS was expected, because the ATSs were aqueous extracts of G, G-ω5, and sHWP or were completely soluble in water as in case of eHWP. The band pattern of G and G-ω5 was similar with bands at 60, 57, 47, and 37 kDa and three additional ones at 52, 40, and 27–24 kDa for G-ω5. Bands with M_r about 60 kDa typically belong to ω-gliadins and the additional band at 52 kDa in G-ω5 is likely to be from ω-secalins. The other bands in the range from 37 to 47 kDa can be assigned to gliadins and LMW-GS (46). While eHWP showed a weak and blurred band at 20–27 kDa and its main band at 10–16 kDa, sHWP had only one band at 10–16 kDa. This indicates that proteins were degraded through hydrolysis in sHWP and eHWP. The most intense protein band in all ATSs was at M_r 10–16 kDa and this range corresponds to non-gluten proteins of the water-/salt-soluble albumin/globulin fraction, such as grain softness proteins, puroindolines, purothionins (Tri a 37), non-specific lipid-transfer protein (Tri a 14), and ATIs (Tri a 15, Tri a 28, Tri a 29, Tri a 30, and Tri a 40), many of them already known as allergens (47).

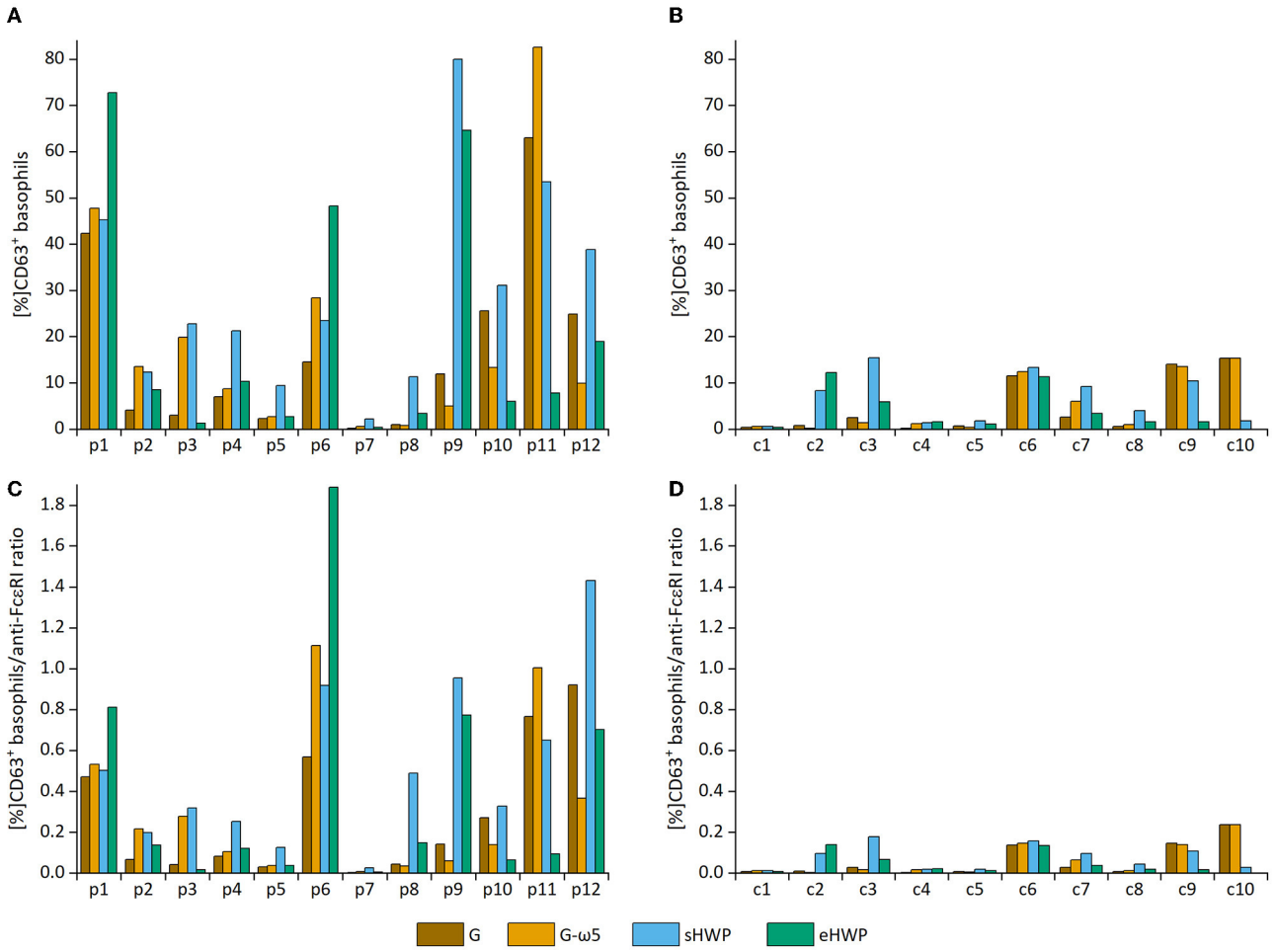

FIGURE 2 | Basophil activation in individual patients and controls. Allergen test solutions from gluten G and G-ω5 and from slightly hydrolyzed wheat proteins (sHWPs) and extensively hydrolyzed wheat proteins (eHWP) were used. **(A)** %CD63$^+$ basophils from patients p1–p12, **(B)** %CD63$^+$ basophils from controls c1–c10, **(C)** %CD63$^+$ basophils/anti-FcεRI ratio from patients p1–p12, **(D)** %CD63$^+$ basophils/anti-FcεRI ratio from controls c1–c10.

High-Performance Liquid Chromatography

Gel permeation- and RP-HPLC analyses were carried out to obtain further information complementary to SDS-PAGE on the M_r distribution of the proteins in the ATS and their hydrophobicity profile. Both GP-HPLC systems showed that there were high percentages of proteins with M_r of about 14 kDa present in the ATS (**Figure 5**). More than 68% of all proteins in the four ATS had a M_r about or below 14 kDa, according to system I suitable for a M_r range from < 14 to 200 kDa (G: 81.9%, G-ω5: 68.3%, sHWP: 84.4%, and eHWP: 92.2%) (**Supplementary Figure 10**). System II suitable for a M_r range from < 2 to ≥ 14 kDa confirmed that over 75% of proteins in the ATS had a M_r about 14 kDa (G: 85.6%, G-ω5: 91.5%, sHWP: 83.3%, and eHWP: 75.7%) (**Supplementary Figure 11**). These results corresponded well to the protein band pattern on the SDS-PAGE gel.

The RP-HPLC chromatograms of the G and G-ω5 ATS showed the typical hydrophobicity profile of the albumin/globulin fraction. In contrast, the peaks in the chromatograms of sHWP and eHWP could not be clearly assigned to any reference chromatogram of intact wheat proteins, again indicating protein degradation (**Supplementary Figure 12**). The protein concentrations of the ATS used for the BAT experiments determined by RP-HPLC were 2.10 mg/ml (G), 2.05 mg/ml (G-ω5), 3.96 mg/ml (sHWP), and 3.00 mg/ml (eHWP). Higher protein concentrations were not achievable with this preparation procedure for G, G-ω5, and sHWP, because of limited solubility. The concentration range of the four ATS, in which the allergenic basophil activation was triggered, was not directly comparable between the ATS. The concentrations were not set in a specific range, but resulted from preliminary tests, which were primarily intended to exclude non-specific activations in the control group while triggering specific activations in patients.

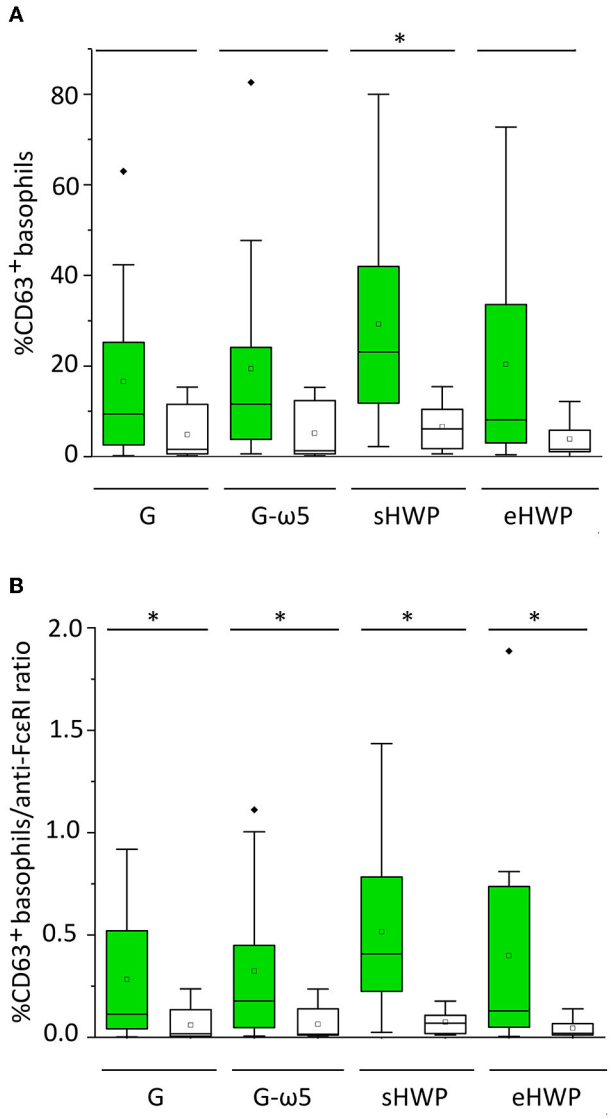

FIGURE 3 | Basophil activation in patients (green) and controls (white). Allergen test solutions from gluten G and G-ω5 and from sHWPs and eHWPs were used. **(A)** %CD63$^+$ basophils, **(B)** %CD63$^+$ basophils/anti-FcεRI ratio. Significant differences between patients and controls are indicated by asterisks (one-way ANOVA, Dunn's *post-hoc* test, $p < 0.05$). There were no significant differences between patients' reactivity to different ATS. Diamonds indicate individual outliers outside the first or third quartiles. Squares represent the mean and lines the median. Whiskers mark the 1.5 interquartile range. The box corresponds to the range in which the middle 50% of the data are located.

Proteomics-Based Untargeted Liquid-Chromatography Mass Spectrometry of the ATS

While SDS-PAGE and HPLC already provided valuable information on the identities of the proteins in the ATS, untargeted UPLC-TripleTOF-MS of different enzymatic digests of the ATS was performed to identify the specific proteins in the ATS and their proportions. Different enzyme combinations were used to maximize protein identifications and avoid bias, because gluten proteins, and especially ω5-gliadins, are known to be resistant to cleavage with P, T, or C (40). Of the PT, TC, PTC, PC, and TLY digestions used (**Figure 6** and **Supplementary Figure 13**), PT turned out to be the most suitable, because percentages of identified proteins in the ATS were the highest in comparison with other digestions. Consequently, the peptides and corresponding proteins identified in the ATS after PT digestion are reported in **Supplementary Tables 4–11**, using both specific and unspecific digestion mode for data evaluation.

The identified proteins in the ATS made from gluten samples G and G-ω5 contained 96.6 and 99.3% of ATIs, such as ATI-types CM1, CM2, CM3, CM16, 0.28, and 0.53 in G and CM2, CM3, CM16, and 0.19 in G-ω5. ATIs are soluble in aqueous salt solutions whereas gluten, by definition, remains mostly insoluble. Therefore, it appears reasonable that ATIs were enriched during ATS preparation with isotonic NaCl solution. Small proportions of LMW-GS and α-gliadins were present in G, as well as α- and ω-gliadins in G-ω5.

The solubility of sHWP in aqueous solutions was comparable with that of gluten samples (10) as was the composition of the ATS. It consisted of 88.8% of ATIs, such as ATI-types CM1, CM2, CM3, CM16, 0.19, and 0.28, as well as a slightly higher proportion of 3.7% of gluten proteins (LMW-GS, gliadins) compared with G and G-ω5. In contrast, eHWP contained 70.2% of gluten proteins, with 58.2% gliadins (α-, β-, γ-gliadins) and 12.0% glutenins (LMW-GS). This difference can be explained by the fact that eHWP was strongly hydrolyzed and completely soluble in aqueous solutions. ATIs (CM1 and CM3) only represented 16.0% of proteins in eHWP and the remaining 13.8% were other proteins, such as enzymes and uncharacterized proteins. The investigations using TC, PTC, PC, and TLY digestions showed some variation in protein composition compared with the PT digestion, but the overall picture of identified protein groups in the ATS was similar (**Supplementary Figure 13**).

The sequences of the identified proteins in the ATS were analyzed for known WDEIA epitopes (15, 48, 49). Only the epitope QQPGQ was identified two times in an ω-gliadin (Uniprot accession: C0KEH9) present in G-ω5. All other identified proteins in the ATS contained none of the known WDEIA epitopes.

DISCUSSION

We expected to see differences in allergenicity to patients with WDEIA between G and G-ω5, because G-ω5 was gluten from a wheat/rye-translocation line (35) that contains a significantly lower amount of ω5-gliadins. SPT results showed that wheal and erythema diameters caused by G-ω5 were the lowest compared with other substances, but the differences were not significant except for the comparison of erythema diameter between G and G-ω5. However, the BAT %CD63$^+$ basophils/anti-FcεRI ratio was similar for G and G-ω5 with almost identical median values and ranges, as were all parameters derived from the ROC curves. It was reasonable to assume that gluten with a significantly lower

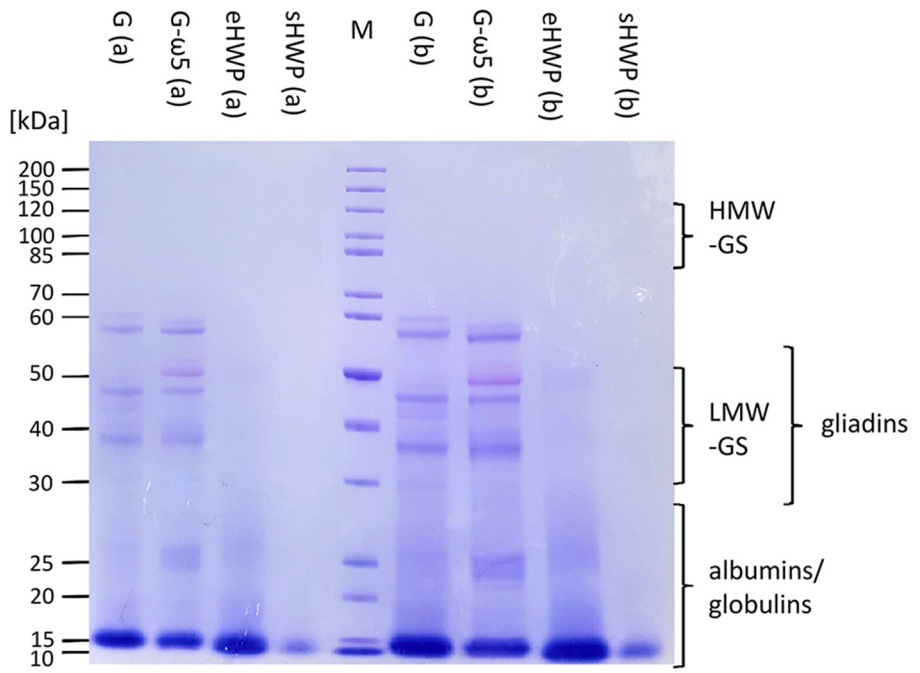

FIGURE 4 | Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of allergen test solutions. Gluten G and G-ω5 as well as sHWPs and eHWPs were analyzed. Protein marker (M) 3.5 μg, samples 5.3 μg (a), and 15.0 μg (b). HMW-GS, high-molecular-weight glutenin subunits, LMW-GS, low-molecular-weight glutenin subunits. The albumin/globulin fraction may consist of, e.g., grain softness proteins, puroindolines, purothionins (Tri a 37), non-specific lipid-transfer protein (Tri a 14), and amylase/trypsin-inhibitors (Tri a 15, Tri a 28, Tri a 29, Tri a 30, and Tri a 40) (40).

ω5-gliadin content would trigger lower basophil activations in patients than representative gluten, since ω5-gliadins are considered to be the main allergen of WDEIA (14). In several cases, it appeared as if even the opposite was the case, because stronger basophil activities occurred for G-ω5 in comparison to G in p1, p2, p3, p4, p6, and p11. These results indicate that other allergenic proteins need to be relevant and present in the ATS.

Altenbach et al. used transgenic wheat with reduced content of ω5-gliadins and assessed its allergenicity in sera of eleven patients with WDEIA using a two-dimensional immunoblot analysis. Seven out of eleven patients showed reduced levels of immunoglobulin E (IgE) reactivity to ω5-gliadins using transgenic wheat, but the same sera showed IgE reactivities to other gluten proteins at the same time. Additionally, sera from three patients generally had the highest IgE reactivity not to ω5-gliadins, but to HMW-GS, α-gliadins, and non-gluten proteins. They concluded that this transgenic wheat line was not beneficial for the nutrition of patients with WDEIA because of the complexity of the immune response in the participating patients with WDEIA. Without knowing to which wheat protein groups, a patient with WDEIA is sensitized, it is too risky overall to consume transgenic wheat. Even if the ω5-gliadin content is reduced therein, other wheat proteins were shown to trigger IgE reactivity in patients with WDEIA (50). Our findings support their conclusion and still leave a wheat-free diet and/or avoidance of cofactors as the only safe option for patients with WDEIA. Further, the identified proteins in ATS from G and G-ω5 both contained over 96% of ATIs (non-gluten proteins) and only very low proportions of gliadins, so that a potential difference in ω5-gliadin content was most likely negligible.

We expected to find LTPs in the aqueous ATS, as they are known to be soluble and to cause basophil activity in patients. Pastorello et al. described three cases of exercise-food challenge confirmed patients with WDEIA. They identified a 9 kDa LTP as the allergenic protein in these patients by immunoblotting. Simultaneously, these patients showed no reactivity to the gliadin and glutenin fractions (23). The protein band of the albumin/globulin fraction (10–15 kDa) of G-ω5 and sHWP in the SDS-PAGE gel of the lyophilized ATS suggested that LTPs may be present. However, no LTPs were identified with the proteomics UPLC-TripleTOF-MS approach (PT-digestion), but high percentages of ATIs. In our previous study, the same WDEIA patient cohort was tested for sIgE against LTP. All patients showed negative results (< 0.1 KU/L; LTP/Tri a 14) (20).

Based on the heterogeneous molecular properties of sHWP and eHWP, we expected differences among the parameters investigated, but we did not find any significant differences in SPT or $CD63^+$ basophils/anti-FcεRI ratio. The only parameters that differed were those derived from the ROC curves indicating that sHWP yielded higher sensitivity/specificity (100%/83%) compared with eHWP (75%/70%) to discriminate between patients and controls. Due to a lack of studies so far, it remains unclear how degree and type of protein hydrolysis affect the allergenicity of gluten in WDEIA. Hydrolysis to a certain

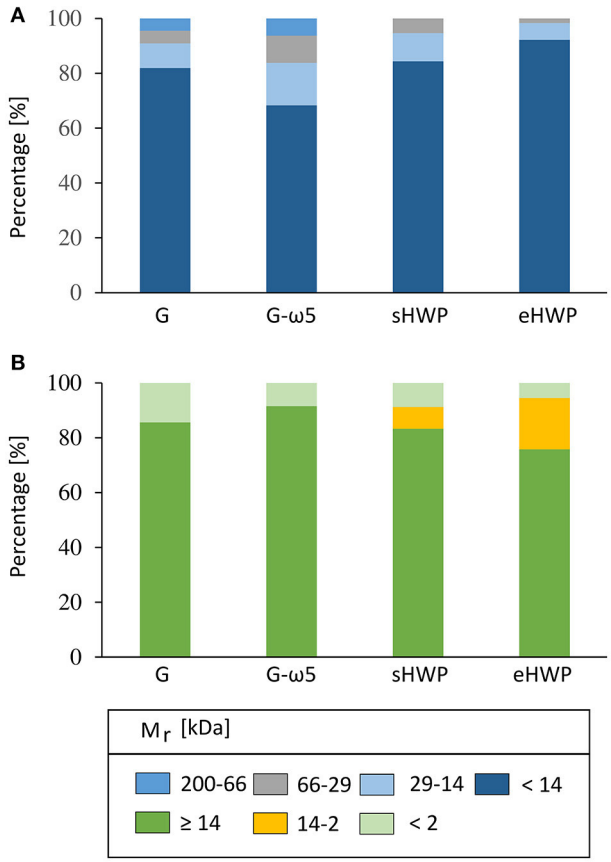

FIGURE 5 | Gel-permeation high-performance liquid chromatography (GP-HPLC). Relative molecular mass distribution (M_r) [%] of the allergen test solutions from gluten G and G-ω5 and from sHWPs and eHWPs. Areas within each fraction were set by marker substances. Two different systems were used. **(A)** 1: 200–66 kDa; 2: 66–29 kDa; 3: 29–14 kDa; 4: <14 kDa. GP-HPLC; **(B)** 1: ≥14 kDa; 2: 14–2 kDa; 3: <2 kDa.

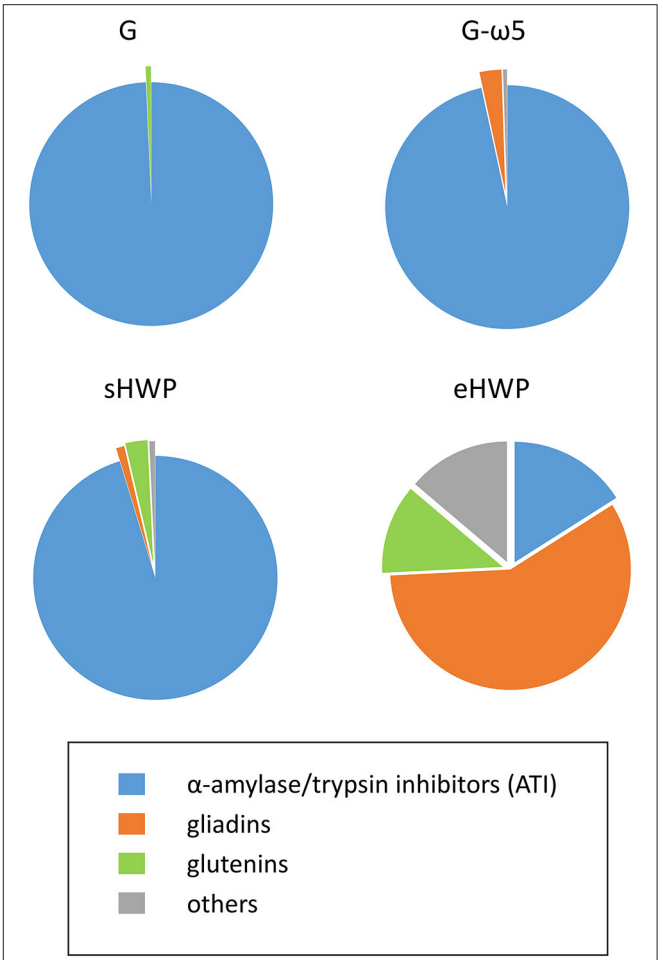

FIGURE 6 | Percentages of protein groups in allergen test solutions. Groups include α-amylase/trypsin inhibitors (ATI, blue), gliadins (orange), glutenins (green), and others (gray) in allergen test solutions from gluten G and G-ω5 and from sHWPs and eHWPs. Percentages were determined using the intensity based absolute quantitation (iBAQ) algorithm with ultra-performance liquid chromatography (UPLC)-TripleTOF-MS data (protein score > 40) in specific digestion mode (pepsin + trypsin). Details on specific proteins are available in **Supplementary Tables 4–11**.

degree may increase the allergenicity by exposing epitopes or generating new ones (6, 7, 51). Beyond that degree, continued hydrolysis is expected to decrease allergenicity, because epitopes are degraded.

Neither SPT nor BAT revealed clear differences between gluten samples (G, G-ω5) and HWP (sHWP, eHWP) in terms of allergenicity. SDS-PAGE and GP-HPLC revealed that all ATS contained high percentages of proteins with M_r 10–16 kDa (SDS-PAGE) and about 14 kDa (GP-HPLC). UPLC-TripleTOF-MS analysis showed that high percentages of ATIs were present and their M_r correspond exactly to this mass range. These findings raise the question, whether ATIs are implicated not only in baker's asthma, but also in WDEIA. Until now, the main focus was on gluten proteins, such as ω5-gliadins and HMW-GS as major WDEIA allergens (19, 48, 52), though there are reports that ATIs may also play a role in WDEIA (24). IgE immunoblotting with patients' sera showed reactions to ATIs present in wheat protein fractions and ATI-types CM1, CM3, CM16, and 0.19 were identified in the allergenic fraction (24), similar to our results.

The sequences of all identified proteins in the ATS were analyzed for known WDEIA epitopes (15, 48, 49). Only one known epitope (QQPGQ) was identified, indicating that other epitopes appear to be relevant in WDEIA which are currently unknown. Western blotting using patient samples can be used in further studies to support their identification.

Sandiford et al. and Battais et al. reported possible cross-reactive epitopes between gliadins and ATIs (53, 54). Pastorello et al. used wheat flour within their study, which naturally contains ATIs, whereas we used gluten. By definition, gluten is poorly soluble in water and salt solutions, but residues of the soluble albumin/globulin fraction still remain in the gluten polymer, even after extensive washing to remove starch and other flour constituents (38, 55).

Besides ATIs, small percentages of gliadins and/or LMW-GS were present in the ATS from G, G-ω5, and sHWP. Overall, these three ATS showed a high degree of similarity regarding protein composition and solubility, again confirming that sHWP was only slightly hydrolyzed.

The basophil activation triggered by eHWP was according to expectations, because over 70% of proteins were gliadins and glutenins, but rendered soluble due to more extensive hydrolysis compared with sHWP. As gluten proteins are already known as relevant allergens in WDEIA, basophil activation in patients was anticipated (4, 48). Chinuki et al. reported a HWP product in soap, which was produced by acid hydrolysis and triggered allergic reactions in patients with WDEIA (5, 19). Apparently the degree of hydrolysis was enough to solubilize all wheat proteins in water, but not enough to significantly destroy allergenic epitopes in eHWP.

The %CD63$^+$ basophils/anti-FcεRI ratio was identified as an appropriate BAT parameter to differentiate reactivity to ATS between patients with WDEIA and control subjects. The discriminability estimated from the AUC of each ROC curve varied between gluten samples and HWP, with the best results for sHWP (AUC ROC: 0.925). Specificity (83%) and sensitivity (100%) of %CD63$^+$ basophils/anti-FcεRI ratio using sHWP at optimal cut-off were very good. Another advantage is that ATS preparation was easy and fast and did not require complex extractions or enzymatic digestions, as in the case of gluten isolates (20, 34, 55). We found no correlations between BAT results (CD63$^+$ basophils, %CD63$^+$ basophils/anti-FcεRI ratio), diameter of wheals and erythema in SPT, sIgE, or disease severity. This is understandable regarding the levels of sIgE, because only clinical routine IgE determination for WDEIA was available. As mainly ATIs were identified in the ATS (G, G-ω5, and sHWP), the allergic reactions to these are found here and those did not appear to be related to sIgE against wheat flour, gluten, gliadin, ω5-gliadin, or LTP. Further insights into possible correlations between BAT and sIgE levels could be gained by measurements of sIgE against ATI types. One possible reason for the lack of correlation between BAT results and disease severity may be that basophil granulocytes are only one part of the whole allergic reaction that has many other influencing factors (e.g., mast cells) (56).

Three of the twelve patients (p5, p7, and p8) showed low basophil activations to G, G-ω5, sHWP, and eHWP in general. P8 showed low basophil activations to the ATS assessed here, but showed a high reactivity to ω5-gliadins in our previous study (20). Patients p5 and p7 had low basophil reactions in the present and in our previous study (20). The IgE positive control showed a basophil activation in both cases, but the basophil granulocytes did not react to the allergens tested in either case. The exact reasons remain unclear at this point in time, but warrant further investigations.

In our previous study, we investigated the basophil activity to isolated ω5-gliadins in the context of WDEIA with the same patients and controls as in the present study. The BAT parameter %CD63$^+$ basophils was identified as the best parameter in this case to differentiate between patients and controls. Thereby, the ATS from isolated ω5-gliadins showed a test sensitivity of 100%, a specificity of 90%, and an AUC ROC of 0.975 at a concentration of 4 mg/ml (20). In comparison with G, G-ω5, and eHWP, the results for ω5-gliadins were better, but comparable with sHWP (sensitivity 100%, specificity 83%, and AUC ROC 0.925). As we identified high amounts of ATI in the ATS of sHWP, this underlines the result of the present study, that non-gluten proteins carrying yet unidentified allergenic epitopes appear to be relevant in WDEIA.

One acknowledged limitation of our study is the comparatively small number of patients with WDEIA and controls. The main reason is that the prevalence of WDEIA is very low overall and the participants were only recruited from the surrounding area of one specialized center. Our main intent was to identify the causative proteins in the ATS first, before we continue studies with more patients with WDEIA from several centers.

In conclusion, we found differences in allergenicity of gluten and HWP samples with varying molecular composition in individual patients with WDEIA using SPT and BAT. The %CD63$^+$ basophils/anti-FcεRI ratio was the most promising parameter to distinguish patients from controls. The procedure to prepare ATS from sHWP is easy and can be performed even in routine clinical practice to help establish BAT as another option to complement the WDEIA diagnosis. Since the ATS made of G, G-ω5, and sHWP predominantly contained ATIs and only small concentrations of gluten proteins, more research is needed to clarify the role of non-gluten proteins in WDEIA and identify specific mechanisms of immune activation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee, Technical University of Munich. The patients/participants provided their written informed consent to participate in this study.

AUTHORS CONTRIBUTIONS

AG, JG, M-CN, BE, TB, KS, and KB: conceptualization. AG, JG, and M-CN: formal analysis, investigation, and methodology. AG, JG, and BE: data curation. BE, TB, KS, and KB: funding acquisition, resources, and supervision. AG: visualization and writing—original draft. JG, M-CN, BE, TB, KS, and KB: writing—review and editing. All authors read and approved the final manuscript.

FUNDING

This work was supported by the authors' institutes, by research and development grants from the German Federal

Ministry of Education and Research (BMBF), project ABROGATE (funding number: 01EA2106A), and the Deutsche Forschungsgemeinschaft (DFG; German Research Foundation) CRC 1371 P06; bilateral funding from Luxembourg National Research Fund (FNR) project C17/BM/11656090—DFG DACH-Lead-Agency BI696/12-1.

ACKNOWLEDGMENTS

We thank Franziska Martin and Angelika Grassl for excellent technical support, Bühlmann Laboratories AG (Schönenbuch, Switzerland) for supporting by reduced reagent costs, the H.+W. Winkler Stiftung Hilfe für den Menschen for their financial support of research on diagnosis and management of patients with WDEIA as well as everyone who participated in the study. We acknowledge support by the KIT-Publication Fund of the Karlsruhe Institute of Technology.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/falgy.2022.822554/full#supplementary-material

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Conflict of Interest: BE received methodological and technical support from Bühlmann Laboratories AG (Schönenbuch, Switzerland).

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

4. General discussion

4.1. Discussion

4.1.1. Comparative characterization of gluten and hydrolyzed wheat proteins

Six vital gluten samples (G1-6), two treated gluten samples (denaturated (G7), slightly textured (G8)) and seven commercially available HWP (HWP1-7) were investigated in terms of their molecular properties: crude protein content, solubility, hydrophilicity/hydrophobicity, the content of free ammonium as an indicator for deamidation during production and the M_r distribution of proteins and peptides. The background of the comparative characterization was that molecular differences were expected both between gluten and HWP and between the single HWP. Molecular differences indicate differences in the immunoreactivity and consequently in the allergenicity [1-3]. Gluten and HWP were confirmed to be significantly different in 3.1. Additionally, significant differences in the molecular properties were found between all HWP. Various approaches for gluten hydrolysis are reported in literature making it obvious that various HWP result depending on the procedure [4-6].

On average, the crude protein content of the gluten samples (773 mg/g) and HWP (756 mg/g) were similar. Wieser et al. (2018) reported a crude protein contents in gluten of 718.8 mg/kg and 707.1 mg/g in enzymatically hydrolyzed gluten. This shows that the treatment of gluten to produce HWP did not influence the crude protein content [7]. The content of free ammonium was considered as indicator for deamidation. Because deamidation may take place as side reaction during the hydrolysis of gluten to HWP, higher amounts of free ammonium were anticipated in HWP than in gluten. Deamidation is also carried out in a targeted manner to increase solubility [1,4]. This hypothesis was verified because the content of free ammonium in HWP (1.56 mg/g) was many times higher than for gluten (0.09 mg/g). So far, no exact contents have been reported in literature.

The most outstanding difference between gluten and HWP was the content of proteins/peptides soluble in aqueous salt solution (fraction A). As expected, all HWP showed significantly higher contents in fraction A in comparison to gluten samples (RP-HPLC). In the production of HWP, gluten is treated with chemical, biochemical and/or physical approaches to increase solubility. As a result, a higher proportion of proteins/peptides are soluble in aqueous solutions. The use of reducing agents or organic solvents to increase solubility, as it is the case for intact gluten, is not necessary anymore [4,7]. Besides others, Wu et. al. (1976) and Kanerva et al. (2011) reported a clearly increased solubility of HWP in comparison to gluten [1,8]. Exemplary RP-HPLC chromatogramms for a gluten and a HWP sample are shown in **Figure 9**.

In agreement with RP-HPLC, the molecular differences between gluten and HWP were evident in SDS-PAGE. Gluten samples showed typical protein bands, while HWP showed decreased amounts or even no protein bands, which is due to protein degradation. It was expected that in SDS-PAGE less protein bands are present in HWP than in gluten. Wieser et al. (2018) reported a correlation of the duration of hydrolysis and the change in visible protein bands [7]. Besides the duration, the type of hydrolysis used also affects the detectable protein bands in SDS-PAGE. Hydrolysis with chemicals, e.g. concentrated acid, is usually more extensive than enzymatic hydrolysis [4,7,9,10]. Remarkably, a protein band at $M_r \leq 15$ kDa was detected in the treated gluten samples and in most HWP. These findings were confirmed by GP-HPLC and designated the presence of proteins with this M_r, e.g. α-amylase/trypsin inhibitors (M_r 12-16 kDa) [11].

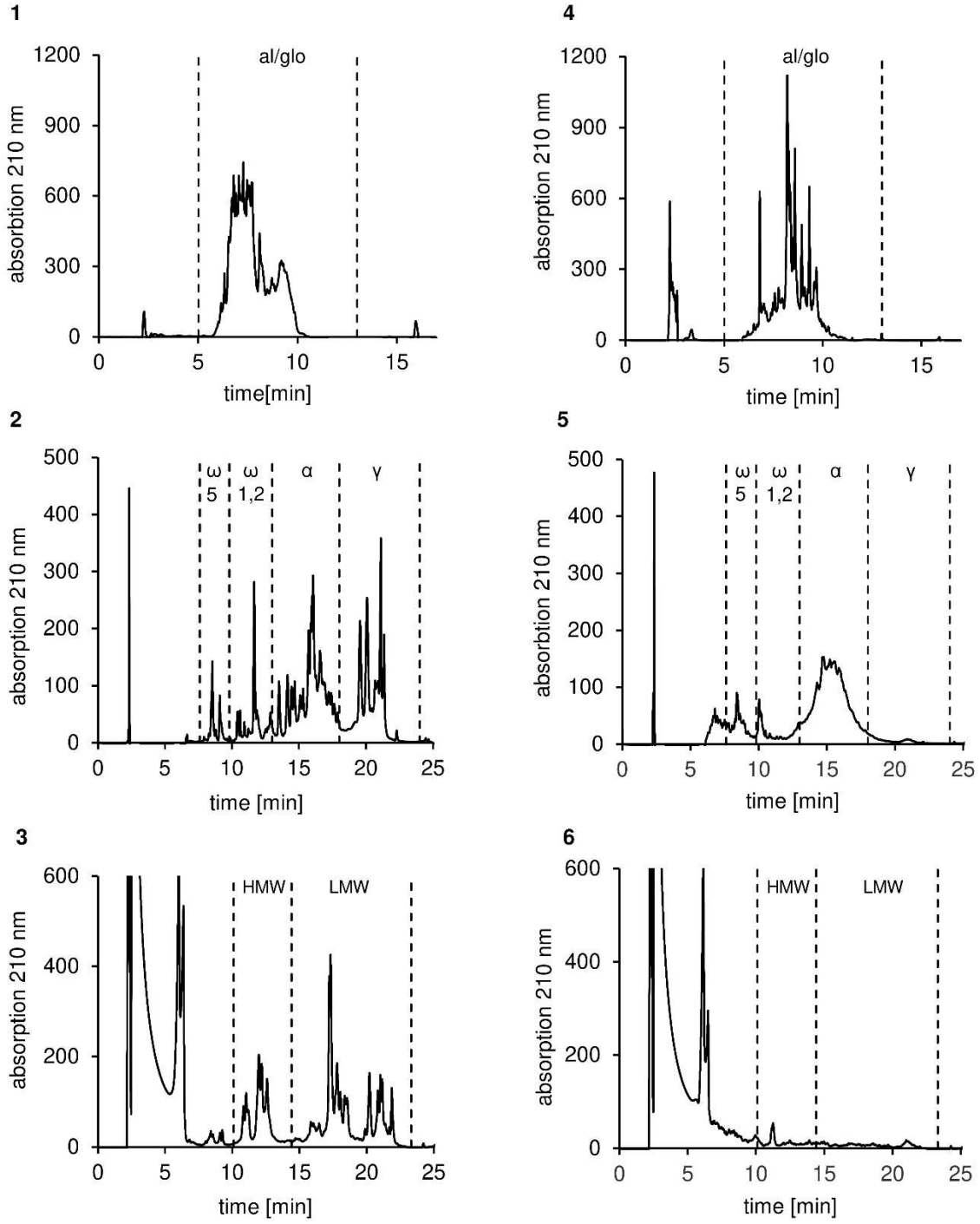

Figure 9: Reversed-phase high-performance liquid chromatograpy: Chromatogramms of the three fractions: Fraction I: soluble in aqueous salt solution (1, 4), fraction II: soluble in 60% ethanol (2, 5), fraction III: soluble in reducing buffer/propanol mixture (3, 6) of gluten G1 (1-3) and extensively hydrolyzed wheat protein HWP 2 (4-6). Albumins and globulins are conained in fraction I, gliadins in fraction II and glutenins in fraction III. Gliadins are subdivided into α-, γ-, ω1,2-, ω5-gliadins and glutenins in high- and low-molecular weight glutenin subunits (HWP-/LMW-GS). Detection of the absorption at 210 nm.

Residues of AlGlo were already found by RP-HPLC analysis in case of the treated gluten samples. In case of HWP, a reason for this protein band might be the presence of small proteins resulting from protein degradation. All HWP examined were different from each other. In SDS-PAGE, depending on the degree of hydrolysis, protein bands were either similar to gluten or less visible or even not present. In two HWP (HWP1, 3), no protein bands were detectable. GP-HPLC analysis showed the presence of M_r ≤ 14 kDa. This showed that these HWP were extensively hydrolyzed. In RP-HPLC all HWP had significantly different contents in the salt-soluble (fraction A) and alcohol-soluble fraction (fraction B). The content of proteins in the reducing-buffer propanol-mixture (glutenin-extraction solution, fraction C) was significantly lower than in gluten samples and also different among HWP. In three HWP (HWP1, 2, 3), there were no proteins detectable in this fraction. It was expected that HWP would differ greatly from each other because the great variability of different hydrolysis approaches in use in HWP production results in different products with different functionalities [1,4,8,12].

Within this study (3.1.), gluten samples and HWP were comparatively characterized due to their molecular properties. It was demonstrated that HWP are highly variable and are likely to trigger different immune responses. This highlights the importance of accurate characterization of HWP products to establish correlations between immunoreactivity and protein structure in the context of wheat-releated disorders. Furthermore, it is crucial to pay attention to the molecular differences between HWP and gluten for the development of diagnostics and analytical methods.

4.1.2. Allergic response

On the basis of the results from 3.1., gluten samples and HWP were selected to investigate the allergenicity of gluten and HWP in the context of WDEIA and to improve the clinical diagnosis of WDEIA (3.2, 3.3).

Table 2: Overview of the sample set, selected from the characterized raw materials (see 3.1.) for the production of allergenic test solutions (ATS) and their application in the basophil activation test on patients with wheat-dependent exercise-induced anaphylaxis and control subjects to investigate their allergenicity.

Raw material	Sample name	ATS
Representative wheat gluten	G 1	Isolated gluten subfractions: α-, γ-, ω1,2- and ω5-gliadins HMW,LMW-GS
		Pepsin-digested gluten (Gp)
		Saline extract from representative gluten (G)
Gluten with reduced ω5-gliadin content*	G 4	Saline extract from gluten (G-ω5)
Extensively HWP	HWP 2	Saline HWP extract (eHWP)
Slightly HWP	HWP 7	Saline HWP extract (sHWP)

* from wheat variety Pamier, a wheat/rye translocation line with 89% lower content of ω5-gliadins, the main allergen in WDEIA [13,14]

As allergenicity is linked to the activation of BG, BAT-FACS was chosen to measure the basophil activation in human whole blood as response to the tested substance [15,16]. The collection of patient's blood is done with the addition of anticoagulants, e.g. heparin or ethylenediaminetetraacetic acid (EDTA). The basophil reactivity decreases over time, consequently BAT-FACS needs to be carried out within four hours after blood sampling for best viability and functionality of BG [17].

Mukai et al. (2017) reported that a storage of blood samples at 4°C allows a later implementation of BAT-FACS after up to 24 hours [18]. All BAT-FACS experiments conducted in 3.2. and 3.3. were performed immediately after blood collection.

CD63 was used as basophil activation marker, which is most common together with CD203c. CD63 up-regulation is closely associated with the degranulation of BG induced by allergen stimuli [19]. A remarkable difference between CD63 and CD203c is that the up-regulation of CD203c also happens to non-degranulation stimuli, which does not occur for CD63 [20]. Eberlein et al. (2014) recommended the combined use of CCR3 as identification marker and CD63 as activation marker for BGs in BAT-FACS [21]. This marker combination was used in the present studies (cf. 3.2., 3.3.). Besides others, Laguna et al. (2018) used this marker combination to investigate immediate allergic reactions to omeprazole [22].

The highly specific anti-FcεRI mAb was used as immunologic positive control in all BAT-FACS experiments. It imitates the bridging of the FcεRI-(IgE-) receptor by an allergen and has been in use as positive control in BAT-FACS for many years [23]. As widely used non-immunologic positive control, N-formyl-methionyl-leucyl-phenylalanine was used to verify that the BG are alive and prove that basophil activation is possible. The basophil activation thereby is triggered by chemotaxis [24-26].

In 3.1., a representative gluten sample was identified (G1) that made it possible to isolate single GPT (ω5-, ω1,2-, α-, γ-gliadins and HMW-/LMW-GS) and examine their allergenicity in WDEIA separately (3.2). BAT-FACS requires water-soluble ATS because the test is applied on vegetative blood cells. This was a challenge because gluten proteins are poorly soluble in aqueous solutions. Organic solvents or reducing agents, which are usually used to increase the solubility of gluten, cannot be used in BAT-FACS ATS because they would damage or destroy the vegetative blood cells [27]. Consequently, it was expected that special preparations are necessary to generate ATS from gluten and GPT to make them suitable for BAT-FACS. An enzymatic digestion using pepsin turned out to be useful for increasing the solubility of gluten proteins in water. Wieser et al. (2018) also used pepsin for the preparation of a defined gluten hydrolysate for clinical investigations and diagnosis of wheat hypersensitivities [7].

Chinuki et al. (2020) applied aqueous, ethanolic and alkaline extracts from wheat proteins as ATS but without characterizing the contained proteins [28].

A blank sample consisting of pepsin digestion medium without gluten proteins was also examined in the present study to exclude unspecific basophil activations to the pepsin digestion medium, which was used to prepare ATS from gluten and GPT. The medium was observed to be well compatible with the blood cells. The generated ATS from gluten (Gp) and GPT were tested in five different concentrations (0.08, 0.4, 0,8, 2.0, 4.0 mg/mL) on blood taken from patients and controls. The aim of this proof-of-principle study, shown in 3.2., was to investigate the utility of this approach for WDEIA diagnosis and identify individual sensitization profiles in WDEIA-patients to Gp and isolated GPT.

A further hypothesis was that differences in the molecular composition of HWP and gluten samples cause differences in their allergenicity to WDEIA patients. To investigate the link between heterogeneous molecular properties and allergenicity, further ATS were generated. To cover a wide range of variability in molecular composition representative gluten (G, G1), gluten from a wheat/rye translocation line (G-ω5, G4) with 89% lower content of ω5-gliadins and a slightly (sHWP, HWP7) and extensively HWP (eHWP, HWP 2) were included. Thereby, a simple and fast extraction method was developed for the preparation of ATS from these samples (3.3). In terms of clinical routine elaborative preparations of ATS are not feasible. These ATS are faster to prepare using saline than the ATS reported in 3.2. [13,14,29]. Saline is easily accessible as an infusion solution in hospitals, where diagnosis of WDEIA is carried out. Furthermore, saline is cell compatible, which is essential for ATS in BAT-FACS. As saline infusion solution was used, no saline blank was applied in the study.

ATS from gluten, GPT and HWP were applied in BAT-FACS on 22 participants in total, 12 WDEIA patients and 10 control subjects (3.2, 3.3.). As test parameters the basophil activation (%$CD63^+$ basophils), the %$CD63^+$ basophils/anti-FcεRI ratio were studied and in case of Gp and GPT the area under the curve (AUC) of dose-response curves in addition, which combines basophil activity and sensitivity.

The basophil activation (%CD63$^+$ basophils) was identified as most promising parameter for Gp and GPT as significantly higher values were observed in patients in comparison to controls at every concentration tested. Mostly, patients showed basophil activations in a dose-dependent manner.

For the saline ATS from gluten and HWP the %CD63$^+$ basophils/anti-FcεRI ratio showed significantly higher values in patients in comparison to controls, while the parameter %CD63$^+$ basophils only showed significanty different results for sHWP. Significant differences in BAT-FACS parameters between patients and controls were expected. They are crucial for sensitivity and specificity of a diagnostic test. In **Figure 10** the results for the basophil activation (%CD63$^+$ basophils) triggered by all tested ATS are shown seperately for patient- and control-group.

Strong basophil activations to ω5-gliadins in patients were expected and occurred in BAT-FACS (1.9-71.3% CD63$^+$ basophils), because they are the major allergens in WDEIA. The responses to other GPT were anticipated to be highly individual in patients. This was the case. Also, HMW-GS noticeably triggered basophil activations (0.8-50.7% CD63$^+$ basophils), which is understandable, because it is often described as minor allergen in WDEIA [2,7, 27,29]. The AUC of the dose-response curve were significantly higher in patients compared to controls in case of the ω5-, α-gliadins and HMW-GS.

The ranges of the basophil activations triggered by the saline ATS in patients were large, G-ω5 (0.6-82,6% CD63$^+$basophils), sHWP (2.2-80.0% CD63$^+$basophils), eHWP (0.4-72.7% CD63$^+$basophils) and G (0.2-63.0% CD63$^+$basophils). Unexpectedly, G-ω5 induced a similar or even higher allergic reaction compared to G. G-ω5 contains 89% less ω5-gliadins than the representative gluten G. A reduced content of the reported major allergen in WDEIA, was expected to make a remarkable difference in allergenicity. That suggests that other wheat proteins play an essential role, too.

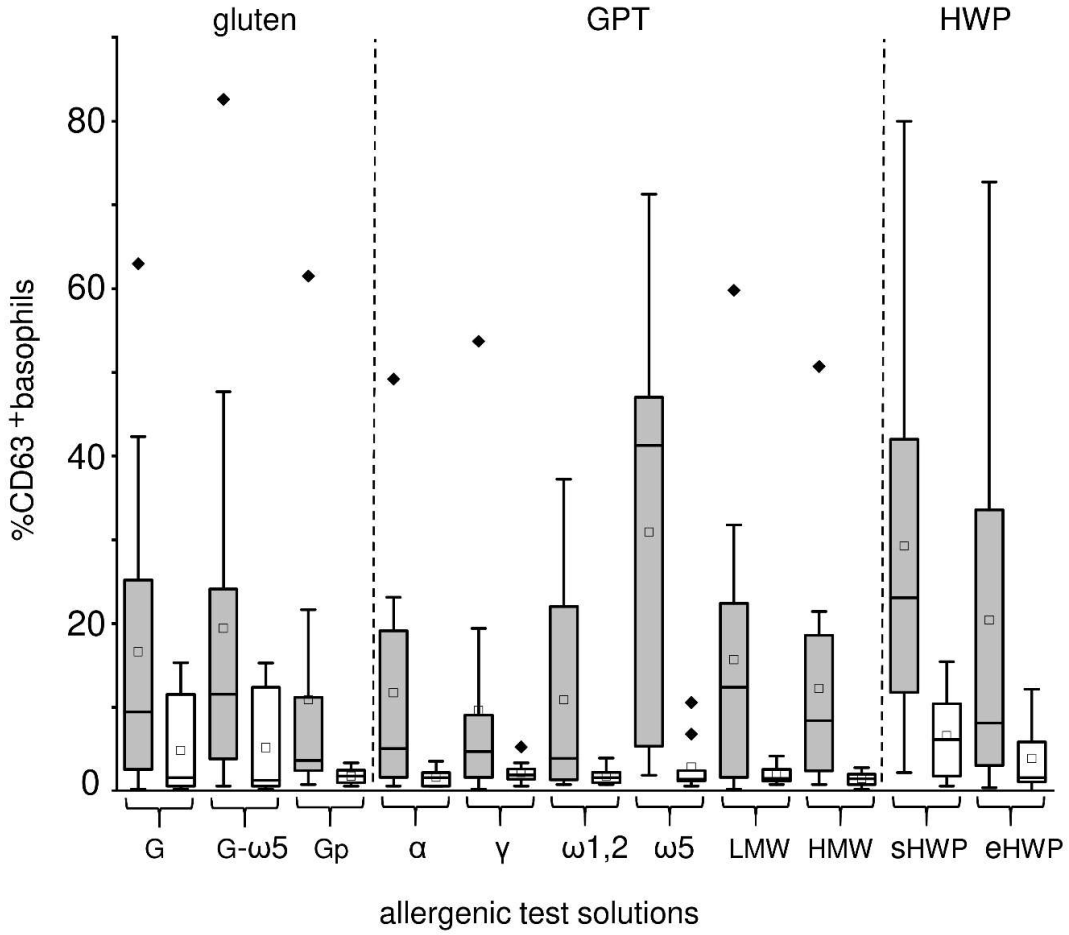

Figure 10: Basophil activations (%CD63⁺ basophils) triggered by allergenic test solutions from gluten samples (saline extract from representative gluten (G), saline extract from gluten with reduced content of ω5-gliadins (G-ω5) and pepsin digested representative gluten (Gp)), gluten protein types (GPT; α-, γ-, ω1,2, ω5-gliadins, high- and low-molecular weight glutenin subunits) and saline extracts from slightly and extensively hydrolyzed wheat proteins (sHWP, eHWP).

Slightly HWP (sHWP) caused higher basophil activations in patients in comparison to extensively HWP (eHWP). Also sHWP showed significant differences for the basophil activation and the %CD63⁺ basophils/ anti-FcεRI ratio between patients and controls while eHWP only showed significant differences for the basophil activation. This point of view indicated that slight hydrolysis of wheat proteins may lead to a higher allergenic potential than extensively hydrolyzed wheat proteins.

The AUC of receiver-operating-characteristic (ROC) curves gives information about the distinguishability of patients and controls depending on different parameters. The closer the calculated value for AUC of ROC curves is to 1, the better is the distinguishability [24]. For Gp and GPT the %CD63$^+$ basophils was the parameter showing the most significant differences between patients and controls, consequently the AUC ROC curve was calculated for this parameter with and without depending on ATS concentration. In the concentration-independent calculation, the maximum %CD63$^+$ basophils of all five ATS concentrations per test substance is taken into account. The concentration-independent calculation provided the best results. Acceptable results were received for α-, γ-, ω1,2-gliadins and LMW-GS and excellent results were found for ω5-gliadins (0.908), HMW-GS (0.867) and gluten (0.850). At the optimal discrimination threshold, determined from the ROC curve, specificity and sensitivity were good for ω5-gliadins (sensitivity: 100%, specificity: 70%), HMW-GS (sensitivity: 75%, specificity: 100%) and gluten (sensitivity: 75%, specificity: 90%) (0.850). It is noticeable that ω5-gliadins, HMW-GS and gluten showed the best result for the tested BAT-FACS parameters as well as for sensitivity and specificity in comparison to α-, γ-, ω1,2-gliadins and LMW-GS. These results were anticipated, because ω5-gliadins and HMW-GS are the most relevant allergens in WDEIA and gluten also contains ω5-gliadins and HMW-GS [2,30,31]. Matsuo et al. (2005) recommended to quantify specific IgE against ω5-gliadins and HMW-GS in combination for the diagnosis of WDEIA [29]. The proof-of-principle study (3.2.) showed that BAT-FACS with gluten and GPT, after digestion with pepsin to increase their solubility, is an alternative tool for WDEIA diagnosos. The results of the study indicate the use of ATS from ω5-gliadins and HMW-GS in BAT-FACS and to use maximum %CD63$^+$ basophils as parameter for the best discrimination between patients and controls.

For saline ATS, the %CD63$^+$ basophils/anti-FcεRI ratio was identified as best parameter to distinguish between patients and controls, consequently this parameter was used to generate ROC curves. The AUC of ROC curves was excellent for sHWP (0.925) and the best among all ATS in 3.2 and 3.3, with a sensitivity of 100% and specificity of 83% at the optimal discrimination threshold. Results for G (0.775, sensitivity: 92%, specificity: 70%) and G-ω5 (0.783, sensitivity: 92%, specificity: 60%) were acceptable. The eHWP proved to be less suitable (0.692, sensitivity: 75%, specificity: 70%).

In conclusion, the best results were determined for ω5-gliadins (concentration-dependent ROC at 4 mg/mL; AUC of ROC curve: 0.975 sensitivity 100 %, specificity 90 %; concentration-independent ROC: AUC of ROC curve: 0.908, sensitivity: 100%, specificity 70%), sHWP (AUC of ROC curve: 0.925, sensitivity 100%, specificity 83%). This indicates that these ATS are highly allergenic for WDEIA patients. Test specificity and sensitivity derived from the ROC analysis as well as AUC of ROC curves were the most promising parameters to estimate WDEIA allergenicity. This results show the alterative role of the used BAT-FACS approach in WDEIA diagnosis and tool to study the causative wheat allergens. Furthermore, the results of the saline ATS indicate the involvement of other wheat proteins in the allergic reaction, besides the major allergens ω5-gliadins.

Since CO-WDEIA and HWP-WDEIA are distinguished, differences in the individual sensitization profiles of WDEIA-patients were expected. Patients with HWP-WDEIA are sensitized to HWP and patients with CO-WDEIA are sensitized to gluten and GPT, especially ω5-gliadins [2]. In 3.2 and 3.3, no exact differentiation in two groups was visible. At the optimal discrimination threshold above which basophil activation is classified as “allergenic”, all tested WDEIA patients showed allergenic reactions to ω5-gliadins (100%). However, 83% of these patients showed allergenic reactions to sHWP, too. This is in contrast to Chinuki et al. (2012). They reported in their study that patients with HWP-WDEIA did not show allergic basophil activations to ω5-gliadins and conversely, no allergic basophil activations were observed in patients with CO-WDEIA to the HWP studied. They used a different activation marker (CD203c) in the BAT. When measuring specific IgE to ω5-gliadins in these patients, they also observed cross reactions. HWP-patients had specific IgE against wheat and gluten (native). One HWP-WDEIA patient even had specific IgE against ω5-gliadins, while showing no allergic basophil activation to ω5-gliadins in BAT. The reason for this was described to be unclear [32].

Since only few studies are available that use BAT-FACS to diagnose WDEIA, a direct comparison is hampered. Additionally, the available studies do not go into detail about their test sensitivity and specificity [32]. When comparing the test accuracy with BAT-FACS approaches to diagnose other allergies, the sensitivity and specificity proved to be of high quality. Eberlein et al. (2010) used the BAT-FACS approach to diagnose allergy to antibiotics and reported 55% sensitivity and 80% specificity [25]. Laguna et al. (2018) reported 74% sensitivity and 100% specificity for the allergy to omeprazole diagnosed using BAT-FACS [22].

The development of BAT-FACS ATS from gluten, GPT and HWP and the implementation out of the two proof-of-principle studies on patients and controls were successful. It is very practical that ATS from sHWP showed great test accuracy, as the ATS can be quickly prepared in comparison to ATS from GPT such as ω5-gliadins. If the approach of the proof-of-principle BAT-FACS study leads to the epansion of clinical diagnosis tools for WDEIA, the application of ATS from sHWP will be very appropriate.

4.1.3. Identification of allergenic peptides in the saline allergenic test solutions from gluten and hydrolyzed wheat proteins

The results of the basophil activation caused by saline ATS in 4.1.2. suggest the involvement of other wheat proteins in the allergic reaction, besides the major allergens ω5-gliadins. Therefore, the saline ATS (G, G-ω5, sHWP, eHWP) were characterized in detail using SDS-PAGE, RP- and GP-HPLC and proteomics-based UPLC-TripleTOF-MS for a better understanding of the contained proteins in the ATS.

The RP-HPLC chromatograms of G and G-ω5 showed the typical hydrophobicity profile of the albumin/globulin fraction. In contrast, the peaks in the chromatograms of sHWP and eHWP could not be clearly assigned to any reference chromatogram of intact wheat proteins, indicating protein degradation. The protein concentrations of the ATS used for the BAT experiments determined by RP-HPLC were 2.10 mg/mL (G), 2.05 mg/mL (G-ω5), 3.96 mg/mL (sHWP) and 3.00 mg/mL (eHWP) [7,33].
The protein concentrations of the ATS were different. For G, G-ω5 and sHWP no higher protein concentrations were achievable with this preparation procedure which is because of solubility. The concentrations were not set similar and in a specific range because the allergenic basophil activation was not diectly comparable between different ATS. The used concentrations resulted from preliminary experiments where a lot of different dilutions were tested, which intended to trigger high basophil activations in patients while excluding non-specific BG activations resulting from cell iritation due to overload with substance. This corresponds to the discriminability of the patient and control group which has a direct influence on the test specificity and sensitivity [24].

SDS-PAGE and GP-HPLC provided consensual information on the M_r distribution of proteins in the ATS and their hydrophobicity profile. All protein bands in the SDS-PAGE gel from the ATS had M_r below or equal to 60 kDa. The lack of hydrophobic and larger proteins such as HMW-GS was expected, because the ATS were aqueous extracts of gluten (G, G-ω5) and sHWP or were completely soluble in water as in case of eHWP. The most intense protein band in all ATS was at M_r 10-16 kDa. This range corresponds to non-gluten proteins of the water-/salt-soluble albumin/globulin fraction, such as non-specific lipid-transfer protein (Tri a 14), grain softness proteins, puroindolines, purothionins (Tri a 37) and ATIs (Tri a 15, Tri a 28, Tri a 29, Tri a 30, Tri a 40). Many of them are already known as allergens [12, 33-36]. Both GP-HPLC systems showed that there were high percentages of proteins with M_r of about 14 kDa present. These results are in agreement to the protein band pattern on the SDS-PAGE gel.

Additionally, ATS were characterized by proteomics-based untargeted UPLC-TripleTOF-MS in the data-dependent acquisition mode. Data analysis was performed using bioinformatics tools on the basis of protein databases. The proteins contained in the ATS were identified with protein database searches and the percentage of the identified protein relative to the total protein content in ATS was calculated using the IBAQ algorithm [35,37,39]. The results are shown in **Figure 11** (data evaluation using the specific digestion mode for trypsin and pepsin).

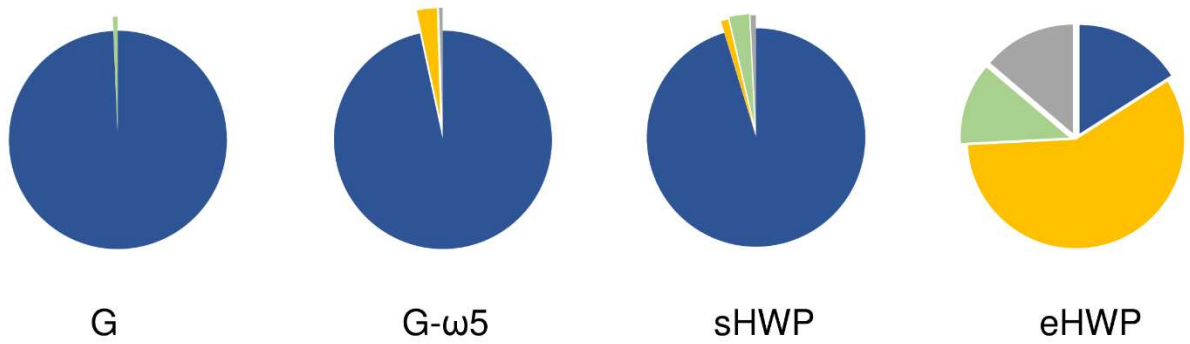

Figure 11: Percentages of protein groups in allergenic test solutions identified by proteomics-based untargeted UPLC TripleTOF-MS, determined using the intensity-based absolute quantitation (IBAQ) algorithm on basis of a protein database (protein score >40, specific digestion mode: pepsin + trypsin).

It was shown that G, G-ω5 and sHWP were similar regarding the percentages of gluten and non-gluten proteins. This is caused by the similar solubility of the raw materials in the saline extraction solution used for the preparation of ATS. Based on the mild hydrolysis used in the production of sHWP, the solubility in aqueous-solutions was just slightly increased in comparison to gluten.

The identified proteins in G and G-ω5 contained 96.6% and 99.3% of ATIs, including ATI-types CM1, CM2, CM3, CM16, 0.28 and 0.53 in G and CM2, CM3, CM16 and 0.19 in G-ω5. ATIs are soluble in aqueous salt solutions whereas gluten, by definition, remains mostly insoluble [27]. Therefore, it appears reasonable that ATIs were enriched during ATS preparation with saline solution. Small proportions of α-gliadins and LMW-GS were present in G, as well as α- and ω-gliadins in G-ω5. The ATS sHWP mainly consisted of ATIs (88.8%) including ATI-types CM1, CM2, CM3, CM16, 0.19 and 0.28. A slightly higher proportion of gluten proteins (LMW-GS, gliadins) was present (3.7%) compared to G and G-ω5. These findings match with the results found in SDS-PÁGE and GP-HPLC. In contrast, eHWP contained 70.2% of gluten proteins, with 58.2% gliadins (α-, β-, γ-gliadins) and 12.0% LMW-GS. ATIs (CM1 and CM3) only represented 16.0% of proteins in eHWP. The remaining 13.8% were other proteins such as enzymes and uncharacterized proteins. This difference to the other three ATS can be explained by the fact that eHWP was strongly hydrolyzed and completely soluble in aqueous solutions. All ATS had in common that in SDS-PAGE the largest protein band was at 10-16 kDa and in GP-HPLC the largest percentage of proteins was at approximately 14 kDa. The reasons therefore were different. G, G-ω5 and sHWP contained high percentages of ATIs, while eHWP contained degraded gluten proteins resulting from extensive hydrolysis.

It is reasonable to assume that eHWP would consequently induce the highest basophil activation in WDEIA patients when containing the highest percentage of gluten proteins compared to the other ATS, but this was not the case [2,29]. The %$CD63^+$ basophils was not significantly different between G, G-ω5, sHWP and eHWP. The sequences of the identified proteins in the ATS were analyzed for known WDEIA epitopes [29,30,40]. Only one known epitope QQPGQ was identified thereby. These findings indicate that yet unidentified allergenic epitopes appear to be relevant in WDEIA. Sandiford et al. (1997) and Battais et al. (2005) reported possible cross-reactive epitopes between ATIs and gliadins [41,42].

Pastorello et al. (2007) found that ATIs are important allergens in wheat and may play a role in WDEIA, too. They documented responses to ATIs in the albumin/globulin fraction of wheat flour and to ATIs present as residues in the gliadin and glutenin fraction in the immunoblotting [43].

In the present study gluten was used as raw material for ATS (3.2., 3.3.). By definition, gluten is not soluble in water and salt solutions. Residues of the soluble albumin/globulin fraction still remain in the gluten polymer, even after extensive washing to remove starch and other flour constituents [7,34]. These residues were already determined in 3.1. G and G-ω5 were comparable with the albumin/globulin fraction of Pastorello et al. (2007). They found IgE bindings to the contained ATI residues in the gliadin fraction in two of six WDEIA patients, but not to the gliadins. ATI types CM2 and CM16 were identified therein. In the albumin/globulin fraction they identified different ATI-types: CM1, CM3, CM16 and 0.19 [36]. Besides others, these ATI-types were detected in the saline ATS from gluten (G, G-ω5) with the proteomics-based UPLC-TripleTOF-MS approach, too.

An additional explanation, why no known WDEIA epitopes were found in eHWP is that the strong hydrolysis, during the manufacturing process of the raw material, had degraded the gluten proteins to such an extent that allergenic epitopes had been destroyed [4,5].

It was expected that low amounts of gluten proteins are present in the generated ATS from G and G-ω5 because a saline extraction was used where gluten proteins are poorly soluble. On the other hand, decreased basophil activities were expected for G-ω5 in comparison to G in patients, because of the lower content of ω5-gliadins in G-ω5, which is the major allergen in WDEIA [2,30]. If this were the case, flour from transgenic wheat with reduced ω5-gliadin content could be used as a hypoallergenic food. However, no significant difference was estimated between G and G-ω5 for the basophil activity. Further, the identified proteins in ATS from G and G-ω5 both contained over 96% of ATIs (non-gluten proteins) and only very low proportions of gliadins, so that a potential difference in ω5-gliadin content in the raw material was most likely negligible.

Altenbach et al. (2020) assessed the allergenicity of transgenic wheat with reduced content of ω5-gliadins on the sera of eleven WDEIA patients. Two-dimensional immunoblotting was used. In seven of eleven patients reduced levels of IgE reactivity to ω5-gliadins in transgenic wheat were seen but the same sera showed IgE reactivities to other gluten proteins at the same time, too. Moreover, sera from three patients generally showed the greatest IgE reactivity not to ω5-gliadins, but α-gliadins, HMW-GS and non-gluten proteins. Altenbach et al. concluded that this transgenic wheat is not beneficial for the nutrition of WDEIA patients, because of the determined complexity of the immune response in the tested patient group. Thus, it is not reasonable to use this transgenic wheat flour as a hypoallergenic food. Even if the content of ω5-gliadins is lower therein other wheat proteins were shown to trigger an allergenic IgE reaction in WDEIA patients [44]. Our findings are in agreement with their conclusion and still leave a wheat-free diet and/or avoidance of cofactors as the only safe option for WDEIA patients [2,13].

It was hypothesized that the ATS from pepsin-digested gluten Gp (3.2.) and HWP samples (sHWP, eHWP) might trigger similar basophil activations. The ATS result from hydrolyzed wheat proteins, sHWP and eHWP during the manufacturing process of the raw material (hydrolysis method is unknown) and Gp during the preparation of ATS (enzymatic hydrolysis). In agreement with the hypothesis, the basophil activations triggered by eHWP and Gp were not significantly different from each other. But sHWP showed significantly higher basophil activations in relation to Gp in all tested concentrations (0.08-4.0 mg/mL, 3.2.). As a result of the enzymatic digestion during the ATS preparation of Gp, the solubility was increased and high percentages of gluten proteins were present in the ATS in contrast to sHWP and similar to eHWP (3.3.). In sHWP high percentages of ATIs (non-gluten proteins) were identified (88.8%). This significant difference underlines that yet unidentified epitopes are present in non-gluten-proteins. Further research is needed to clarify the role of ATIs in the context of WDEIA and identify specific mechanisms of immune activation.

ω5-gliadins and the sHWP, containing ATIs to 88.8%, and were the most allergenic ATS in this work. Both triggered high basophil activations in WDEIA patients, thereby they were not significantly different from each other. They showed significantly higher values for %CD63$^+$ basophils and %CD63$^+$ basophils/anti-FcεRI ratio for patients in comparison to the control group. Resulting from this, both show the best test sensitivity and specificity (3.2. 3.3.).

In conclusion, both proof-of-principle studies (3.2., 3.3.) showed the potential of BAT-FACS in the field of WDEIA diagnostics. Thereby, ATS from ω5-gliadins and sHWP were identified to be most appropriciate in the BAT-FACS approach and is a promising alternative tool to diagnose WDEIA. It was also shown that previously unidentified WDEIA epitopes need to be present in wheat proteins and that non-gluten proteins, specifically ATIs, also appear to play a role in the context of WDEIA.

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5. Summary

In the spectrum of wheat-related disorders, WDEIA is defined as immediate type-1 allergy. It is rare, but potentially life-threatening and caused by the exposition to wheat proteins and the presence of one or more cofactors. Patients suffer from anaphylactic reactions affecting the gastrointestinal and respiratory tracts, the skin and the cardiovascolar system with different severity. GPT, the storage proteins of wheat, are the most commonly reported WDEIA allergens. Thereby, the ω5-gliadins are known as major allergens and the HMW-GS as minor allergens. Reactions to other GPT and HWP are also reported. The golden standard to diagnose WDEIA is still an OFC, which puts patients at risk of anaphylaxis.

The aim of this work was to further investigate the allergenicity of gluten and HWP in the context of WDEIA to contribute to a better understanding of the underlying mechanisms and expand diagnostic possibilities.

First gluten and HWP were comparatively characterized (SDS-PAGE, content of free ammonium and RP-/GP-HPLC). Based on the results, products were selected from which ATS were prepared in order to investigate the BAT-FACS as a possible alternative diagnostic tool. Thereby, anti-CCR3-PE-mAb (BG identification marker) and anti-CD63-FITC-mAb (BG activation marker) were used. Second, patient studies with control subjects were carried out. The results showed that the BAT-FACS is very promising to supplement WDEIA routine diagnosis, as especially the ATS from ω5-gladins and sHWP showed very good test sensitivity and specificity. Individual senzitization profiles of the patients were generated after isolation of single GPT and their application as ATS in the BAT-FACS study.

Gluten from a wheat/rye translocation line (G-ω5, 89% reduced content of ω5-gliadins in comparison to representative gluten G) was also applied in the BAT-FACS study to evaluate whether this could possibly be used as a hypoallergenic food. However, allergenic basophil activations in patients were documented, consequently this product is not recommended as hypoallergenic food for WDEIA patients. Our findings still leave a wheat-free diet and/or avoidance of cofactors as the only safe option for WDEIA patients.

Further on, fast and easily preparable ATS, usable in clinical routine diagnosis where elaborate preparations are unwanted, were generated and sucessfully tested on patients and controls. Thereby, a detailed characterization of the contained proteins in the ATS was performed (SDS-PAGE, RP-/GP-HPLC and proteomics-based untargeted high-resolution UPLC-TripleTOF-MS). These results together with the determined BAT results indicated that non-gluten proteins are also relevant allergens in the context of WDEIA since high percentages of ATIs were determined in the ATS (G, G-ω5, sHWP).

In addition, yet unidentified allergenic epitopes need to be present in the fast preparable ATS (G, G-ω5, sHWP, eHWP) because the identified proteins were searched for known WDEIA epitopes and only one epitope was found. Consequently, the known epitopes cannot explain the allergenic basophil activations triggered by these ATS in WDEIA patients.

6. Zusammenfassung

Innerhalb des Spektrums der weizenbedingten Erkrankungen wird die WDEIA als unmittelbare Typ-1-Allergie definiert. Sie ist selten, aber potenziell lebensbedrohlich und wird durch die Exposition gegenüber Weizenproteinen und das Vorhandensein eines oder mehrerer Kofaktoren verursacht. Die Patienten leiden unter anaphylaktischen Reaktionen, die den Magen-Darm-Trakt, die Atemwege, die Haut und das kardiovaskuläre System in unterschiedlichem Ausmaß betreffen. Glutenprotein Typen (GPT), die Speicherproteine des Weizens, sind die am häufigsten beschriebenen WDEIA-Allergene. Dabei sind die ω5-Gliadine als Hauptallergene und die HMW-GS als Nebenallergene bekannt. Es wird auch über Reaktionen auf andere GPT und hydrolysiertes Weizenprotein (HWP) berichtet. Der goldene Standard für die WDEIA Diagnose ist nach wie vor eine orale Provokationstestung mit dem verursachenden Lebensmittel oder Gluten, die die Patienten dem Risiko einer Anaphylaxie aussetzt.

Ziel dieser Arbeit war es, die Allergenität von Gluten und HWP im Kontext der WDEIA zu untersuchen, um zu einem besseren Verständnis der zugrunde liegenden Mechanismen und Erweiterung der diagnostischen Möglichkeiten beizutragen.

Zum einen wurden Gluten und HWP vergleichend charakterisiert (SDS-PAGE, Gehalt an freiem Ammonium und RP-/GP-HPLC). Auf dieser Grundlage wurden Produkte ausgewählt, aus denen allergene Testlösungen (ATS) hergestellt wurden, um den basophilen Aktivierungstest kombiniert mit fluoreszenz-aktivierter Zellsortierung (BAT-FACS) als alternative Diagnosemöglichkeit für WDEIA zu untersuchen. Dabei wurden Anti-CCR3-phycoerythrin-monoklonale Antikörper (Identifikationsmarker für Basophile Granulozyten) und Anti-CD63-Fluoresceinisothiocyanat-monoklonale Antikörper (Aktivierungsmarker für Basophile Granulozyten) verwendet. Es wurden anschließend Patientenstudien mit Kontrollpersonen durchgeführt. Die Ergebnisse zeigten, dass der BAT-FACS eine vielversprechende Ergänzung zur Routinediagnostik der WDEIA darstellt, da insbesondere die ATS aus ω5-Gladinen und je sHWP je eine sehr gute Testsensitivität und -spezifität aufwiesen. Durch die Isolierung einzelner GPT und deren Verwendung als ATS in der BAT-FACS-Studie wurden individuelle Sensibilisierungsprofile der Patienten erstellt.

Gluten aus einer Weizen/Roggen-Translokationslinie (G-ω5, 89% reduzierter Gehalt an ω5-Gliadinen im Vergleich zu repräsentativem Gluten G) wurde ebenfalls in der BAT-FACS-Studie eingesetzt, um zu evaluieren, ob dies möglicherweise als hypoallergenes Lebensmittel verwendet werden könnte. Es wurden jedoch allergene basophile Aktivierungen bei Patienten dokumentiert, so dass dieses Produkt nicht als hypoallergenes Lebensmittel für WDEIA-Patienten zu empfehlen ist. Unsere Ergebnisse zeigen, dass eine weizenfreie Ernährung und/oder die Vermeidung von Kofaktoren die einzige sichere Option für WDEIA-Patienten ist.

Weiterhin wurden schnelle und einfach zuzubereitende ATS generiert, die in der klinischen Routinediagnostik eingesetzt werden können, wo aufwendige Zubereitungen unerwünscht sind. Diese ATS wurden erfolgreich an Patienten und Kontrollen getestet. Dabei wurde eine detaillierte Charakterisierung der in den ATS enthaltenen Proteine durchgeführt (SDS-PAGE, RP-/GP-HPLC und Proteom-basierte nicht-zielgerichtete hochauflösende UPLC-TripleTOF-MS). Diese Ergebnisse deuten zusammen mit den ermittelten BAT-FACS Ergebnissen darauf hin, dass auch Nicht-Gluten-Proteine relevante Allergene im Rahmen der WDEIA sind, da hohe Mengen an α-Amylase-Trypsin-Inhibitoren in den ATS bestimmt wurden (G, G-ω5, sHWP).

Darüber hinaus zeigte sich, dass in den einfach-herstellbaren ATS (G, G-ω5, sHWP, eHWP) nicht identifizierte allergene Epitope vorhanden sein müssen, da die identifizierten Proteine auf bekannte WDEIA-Epitopen analysiert wurden und lediglich eines der bekannten Epitope gefunden wurde. Folglich können die allergenen basophilen Aktivierungen bei den WDEIA-Patienten nicht durch das Vorhandensein bekannter WDEIA-Epitope erklärt werden.

7. Future research

Future research on the characterization of gluten and HWP as well as the improvement of WDEIA diagnosis can be classified into different topics.

The proteome composition of wheat is influenced by genetics and environmental factors. Consequently, the characterization of a higher number of gluten and HWP samples from different cultivars of species grown in different years would provide a more complete evaluation of the proteome variability.

It is well known that wheat-related diseases are also linked to other gluten-containing cereals, like rye and barley. Thus, it would be interesting to evaluate the allergenicity of these cereals with respect to WDEIA.

Furthermore, it needs to be clarified which hydrolysis procedure decreases or increases the allergenicity regarding CO-WDEIA and HWP-WDEIA in detail. Experiments using different chemical, biochemical and physical approaches should be used to hydrolyze gluten and determine the allergenicity of the resulting products.

This work showed that large proportions of ATI-types are contained in some ATS that trigger high basophil activations in WDEIA patients. Consequently, it is necessary to investigate the role of ATIs in the context of WDEIA in depth, as this has received little attention so far. One possibility would be to isolate or purchase single ATI types from wheat and test their allergenicity on WDEIA patients and controls in BAT-FACS. The preparation of ATS from ATI-types would be easier compared to gluten proteins because ATIs are water soluble. The approach used in 3.3 of producing ATS with saline infusion solution would be useful, as the ATIs are solved therein.

On basis of the the proof-of-principle studies in this work, more participants need to be assessed with the developed approach to verify the utility of this BAT-FACS approach to supplement WDEIA diagnostics.

The results of this work have shown great potential for future research and raised many new questions about the role of gluten proteins and HWP in WDEIA.

8. Appendix

8.1. Comparative Characterization of Gluten and Hydrolyzed Wheat Proteins

8.1.1. Bibliographic data

Title	Comparative Characterization of Gluten and Hydrolyzed Wheat Proteins
Authors	Angelika Miriam Gabler, Katharina Anne Scherf
Journal	Biomolecules
Publication year:	2020
Volume:	10
Issue:	1227
DOI:	10.3390/biom10091227
Hyperlink:	www.mdpi.com/2218-273X/10/9/1227

8.1.2. Publication reprint permission

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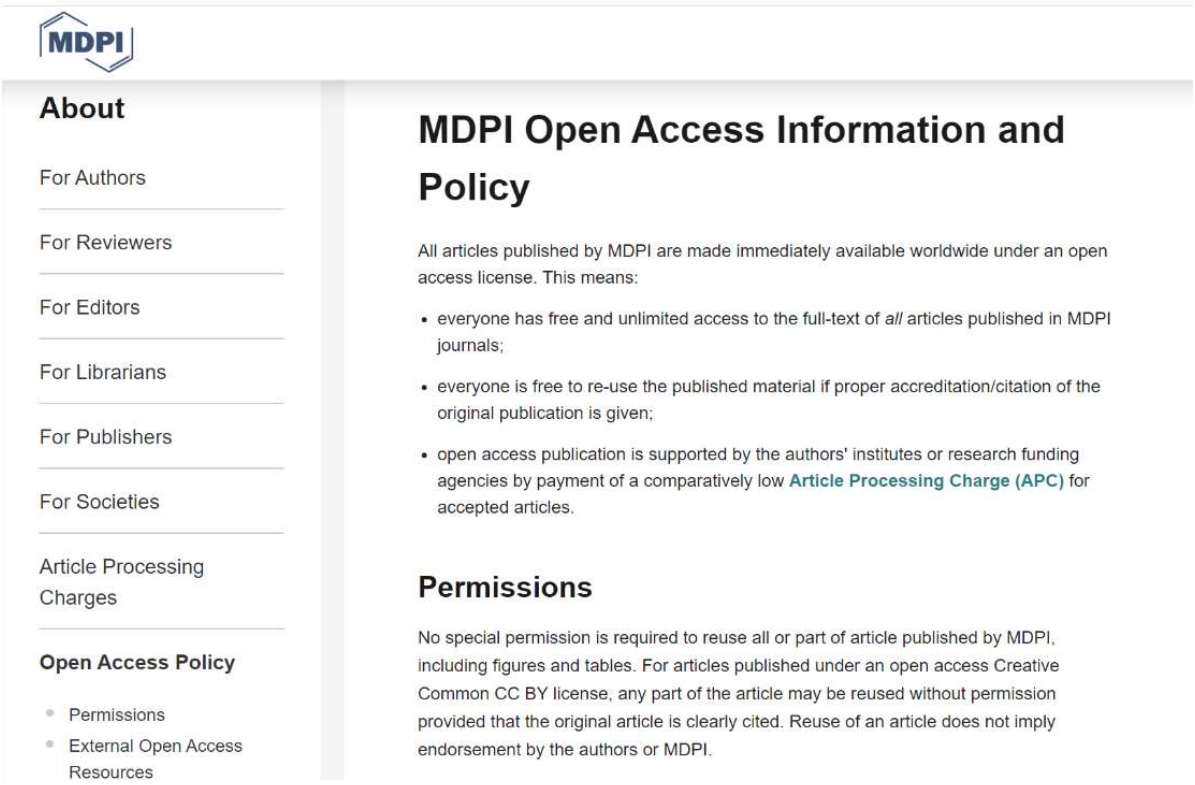

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8.2. The basophil activation test differentiates between patients with wheat-dependent-exercise induced anaphylaxis and control subjects with the use of gluten and isolated gluten protein types

8.2.1. Bibliographic data

Title	The basophil activation test differentiates between patients with wheat-dependent-exercise induced anaphylaxis and control subjects with the use of gluten and isolated gluten protein types
Authors	Angelika Miriam Gabler, Julia Gebhard, Bernadette Eberlein, Tilo Biedermann, Katharina Anne Scherf, Knut Brockow
Journal	Clinical and Translational Allergy
Publication year:	2021
Volume:	e12050
DOI:	10.1002/clt2.12050
Hyperlink:	www.onlinelibrary.wiley.com/doi/full/10.1002/clt2.12050

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8.3. Basophil activation to gluten and non-gluten proteins in wheat-dependent exercise-induced anaphylaxis

8.3.1. Bibliographic data

Title	Basophil activation to gluten and non-gluten proteins in wheat-dependent exercise-induced anaphylaxis
Authors	Angelika Miriam Gabler, Julia Gebhard, Marie-Christin Norwig, Bernadette Eberlein, Tilo Biedermann, Knut Brockow, Katharina Anne Scherf
Journal	Frontiers in Allergy
Publication year:	2022
Volume:	3
Article:	822554
DOI:	10.3389/falgy.2022.822554
Hyperlink:	www.frontiersin.org/articles/10.3389/falgy.2022.822554/full

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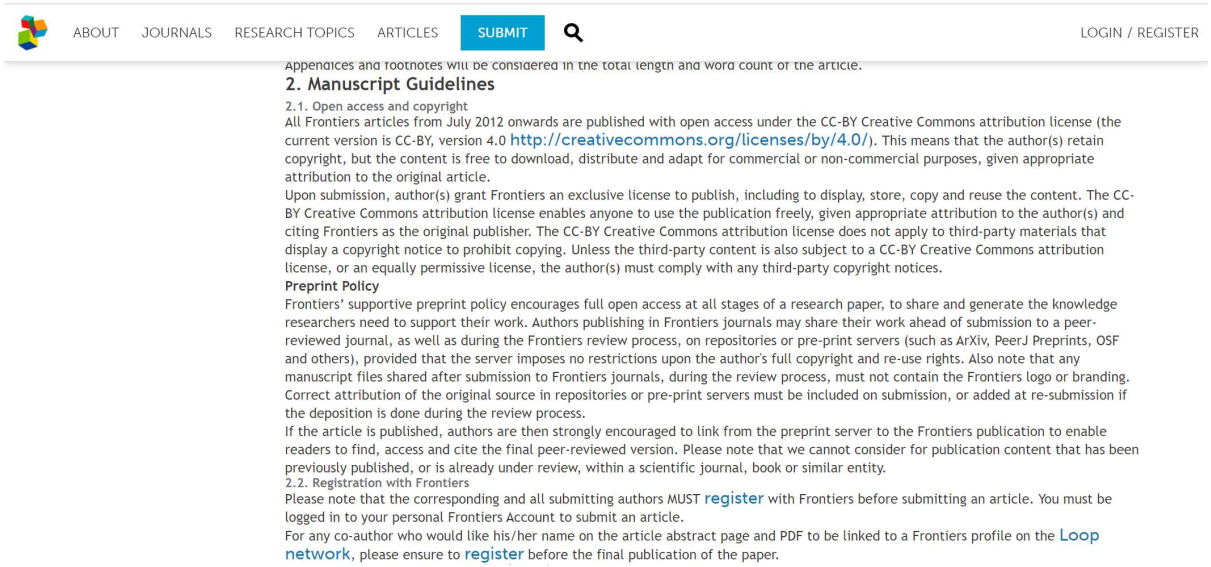

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