Proceedings of the 28th Meeting

WORKING GROUP
on PROLAMIN ANALYSIS and TOXICITY

Edited by
Peter Koehler
German Research Centre for Food Chemistry

September 25 - 27, 2014
Nantes, France

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Deutsche Forschungsanstalt für Lebensmittelchemie /
German Research Centre for Food Chemistry
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Impressum

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Scientific Organisation
Prof. Dr. Peter Koehler
Deutsche Forschungsanstalt für Lebensmittelchemie
Lise-Meitner-Str. 34, 85354 FREISING, GERMANY
Phone: +49 8161 712928; Fax: +49 8161 712970
Email: peter.koehler@tum.de

Hosts
Dr. Sandra Denery & Olivier Tranquet
INRA
Rue de la Géraudière BP 71627
44 316 Nantes Cedex 3, France
Phone: +33 2 40 67 50 27, Fax: +33 2 40 67 50 25
E-mail: olivier.tranquet@nantes.inra.fr

Cover picture and picture of participants
Thomas Mothes

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Lise-Meitner-Strasse 34, 85354 Freising
Phone: +49 8161 712928; Fax: +49 8161 712970
dfa@lrz.tum.de / www.dfal.de

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Preface

The 28th meeting of the Working Group on Prolamin Analysis and Toxicity (PWG) was held in Nantes (France) from 25th to 27th of September, 2014. The hosts were Olivier Tranquet, Laurie Brard, Colette Larré, and Sandra Denery from the allergy research group at INRA in Nantes. Among the 13 members of the PWG, nine participated in person, two were replaced by colleagues from their institutes, and two were not able to participate. Because of a strike of the Air France pilots, several people were not able to get to Nantes. The audience comprised 60 persons that included one invited speaker and guests from coeliac societies, academia, and industry. The latter was represented by starch producers, manufacturers of gluten-free foods, and producers of test kits for the analysis of gluten in food as well as for diagnostic antibody assays.

As agreed by the PWG in 2013, a symposium was held on antigen receptors in coeliac disease comprising two presentations of recognised experts in this field. In addition, two presentations were dedicated to research on coeliac disease and wheat allergy in France. The analytical and clinical sessions featured in total 14 presentations covering aspects from gluten analysis, gluten modification by technology or breeding, serology, pathochemistry, and detection of gluten fragments in human stools.

I would like to express my gratitude to all participants for their contributions and discussions during the meeting. Furthermore, I would like to express my warm thanks to the local organising team, in particular Laurie Brard and Olivier Tranquet for having organised a perfect meeting. This book would not have been possible without the help of Katharina Scherf who made an excellent job in proofreading of the manuscript. Last but not least, I would like to express my appreciation to all friends, colleagues, and sponsors for their ongoing support of the meeting.

Freising, April 2015 Peter Koehler

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1 Executive summary

Among the topics of the meeting were antigen receptors in coeliac disease, analytical issues of gluten, diagnosis of coeliac disease, and further aspects of the pathomechanism.

Analytical session

The analytical session was comprised of nine presentations that mainly addressed analytical methods for gluten quantitation, and it appears that progress is being made in the analysis of partially deamidated gluten. Two papers addressed breeding and genetic initiatives on reducing coeliac activity of wheat. One presentation was dealing with reduction or abolishment of coeliac toxicity by chemical oxidation.

Clinical session

Five presentations were given in the clinical session. The first part of the session addressed the use of serology in the diagnosis of coeliac disease. Another two topics were related to the pathomechanism of coeliac disease, and finally, there was one presentation on the presence of coeliac-active gluten peptides in human stool samples. Two extra presentations apart from the regular clinical session gave a comprehensive overview on the research activities in France related to coeliac disease and wheat allergy.

Symposium: Antigen receptors in coeliac disease

The symposium included two presentations of recognized experts in this field of research. The presentations impressively showed the progress that has been made in relation to explaining the role of T cells in the pathomechanism and to using them as diagnostic tools in coeliac disease. An exciting new technique, referred to as mass cytometry (“CyTOF”), has a tremendous potential for mapping multiple markers of coeliac and related diseases.

28th Meeting of the Working Group on Prolamin Analysis and Toxicity (PWG), Nantes, France September 25 – 27, 2014

2 List of Participants

GROUP MEMBERS

Prof. Dr. Carlo Catassi
(not attending), substituted by
Dr. Simona Gatti
Università Politecnica delle Marche
Facoltà di Medicina e Chirurgia
Istituto di Clinica Pediatrica
Via Corridoni 11
60123 ANCONA, ITALY
Phone: +39 349 2235 447
Fax: +39 071 36281
Email: simona.gatti@hotmail.it

Prof. Dr. Fernando G. Chirdo
Laboratorio de Investigación en el
Sistemma Immune (LISIN)
Facultad de Ciencias Exactas
Universidad Nacional de La Plata
cc 711
(1900) LA PLATA, ARGENTINA
Phone: +54 221 421 0 497, 423 0121,
423 5 333 (Int 45)
Fax: +54 221 422 6947
Email: fchirdo@biol.unlp.edu.ar

Prof. Dr. Paul J. Ciclitira
King´s College London
(Division of Diabetes and
Nutritional Sciences)
The Rayne Institute (KCL)
St Thomas´ Hospital
Westminster Bridge Road
LONDON SE1 7EH, UK/ENGLAND
Phone: +44 207 620 2597;
207 188 2494
Fax: +44 207 261 0667
Email: mila.labar_weintrop@kcl.ac.uk
(secretary)
Email: paul.ciclitira@kcl.ac.uk

Prof. Dr. Conleth Feighery, MD
University of Dublin, Department of
Immunology, St. James´s Hospital
James´s Street
DUBLIN 8, IRELAND
Phone: +353 1 896 3432
Fax: +353 1 454 5609
Email: con.feighery@tcd.ie

Dr. Luud Gilissen
(not attending), substituted by
Dr. René Smulders
Plant Research International (PRI)
Wageningen University
Droevendaalsesteeg 1
6708 PB WAGENINGEN,
THE NETHERLANDS
Phone: +31 317 480840
Email: rene.smulders@wur.nl

Prof. Dr. Peter Koehler
Deutsche Forschungsanstalt
für Lebensmittelchemie
Lise-Meitner-Straße 34
85354 FREISING, GERMANY
Phone: +49 8161 712928
Fax: +49 8161 712970
Email: peter.koehler@tum.de

Prof. Dr. Frits Koning
Leiden University Medical Centre, E3-Q
Department of Immunohaematology
and Bloodbank
Albinusdreef 2
2333 ZA LEIDEN,
THE NETHERLANDS
Phone: +31 715 266673
Fax: +31 715 265267
Email: fkoning@lumc.nl

Prof. Dr. Knut Lundin
Oslo Universitetssykehus
HF Rikshospitalet
Postboks 495
N-0424 OSLO, NORWAY
Phone: + 47 909 80325
Fax: +47 2307 2410
Email: k.e.a.lundin@medisin.uio.no

Prof. Dr. Thomas Mothes
Universitätsklinikum Leipzig A.ö.R.
Institut für Laboratoriumsmedizin,
Klinische Chemie und Molekulare
Diagnostik
Liebigstraße 27
04103 LEIPZIG, GERMANY
Phone: +49 341 97 22251
Fax: +49 341 97 22379
Email: mothes@medizin.uni-leipzig.de

Prof. Dr. Dr. Detlef Schuppan
I. Medizinische Klinik und Poliklinik
Universitätsmedizin der Johannes
Gutenberg-Universität Mainz
Institut für Translationale Medizin
Langenbeckstraße 1
55131 MAINZ, GERMANY
Phone: +49 6131 177355/
177356/177104
Fax: +49 6131 177357
Email: detlef.schuppan@unimedizin-
mainz.de

Prof. Dr. Martin Stern
Universitätsklinik für Kinder- und
Jugendmedizin
Hoppe-Seyler-Straße 1
72076 TÜBINGEN, GERMANY
Phone: +49 7071 2983781
Fax: +49 7071 295477
Email: martin.stern@med.uni-
tuebingen.de

Prof. Dr. Riccardo Troncone
Department of Pediatrics and European
Laboratory for the Investigation of
Food-Induced Diseases
University of Naples "Federico II"
Via Pansini, 5
80131 NAPLES, ITALY
Phone: +39 081 7463383
Fax: +39 081 5469811
Email: troncone@unina.it

HOSTS

Dr. Sandra Denery
INRA
Rue de la Géraudière BP 71627
44316 NANTES CEDEX 3, FRANCE
Phone: +33 2 40675137
Fax: +33 2 40675025
E-mail: sandra.denery@nantes.inra.fr

Dr. Olivier Tranquet
INRA
Rue de la Géraudière BP 71627
44316 NANTES CEDEX 3, FRANCE
Phone: +33 2 40675027
Fax: +33 240675025
E-mail: olivier.tranquet@nantes.inra.fr

INVITED SPEAKER

Prof. Dr. Nadine Cerf-Bensussan
Imagine, Institute of Genetic Diseases
Laboratoire d'Immunité Intestinale
Boulevard du Montparnasse
75015 PARIS, FRANCE
Phone: +33 142754288
Fax: +33 142754223
Email: nadine.cerf-bensussan@inserm.fr

GUESTS

Mr. Gunnar Adås
Fria Gluten Free
Fältspatsgatan 12
42130 VÄSTRA FRÖLUNDA,
SWEDEN
Phone: +46 317341330
Fax: +46 317341335
Email: gunnar@fria.se

Mrs. Tova Almlöf
Semper AB
Semper AB Box 1101
SE 17222 SUNDBYBERG, SWEDEN
Phone : +46 850593100
Email: Tova.Almlof@semper.se

Mrs. Sofia Beisel
Deutsche Zöliakiegesellschaft e.V.
Kupferstr 36
70599 STUTTGART, GERMANY
Phone: +49 711 45998115
Fax: 49 711 479981-50
Email: sofia.beisel@dzg-online.de

Ms. Barbara Bidan
Fleury Michon
Rue de Pierre Brune
85110 CHANTONNAY, FRANCE
Email: bidan.barbara@fleurymichon.fr

Dr. Marie Bodinie
INRA
Rue de la Géraudière BP 71627
44316 NANTES CEDEX 3, FRANCE
Phone: +33 2 40675000
Email: marie.bodinier@nantes.inra.fr

Mr. Gregory Bouchaud
INRA
Rue de la Géraudière BP 71627
44316 NANTES CEDEX 3, FRANCE
Phone: +33 2 40675000
Email: gregory.bouchaud@nantes.inra.fr

Dr. Markus Brandt
Ernst Böcker GmbH & Co KG
Ringstrasse55-57
32423 MINDEN, GERMANY
Phone: +49 571 837990
Fax: +49 571 8379920
Email: markus.brandt@sauerteig.de

Mrs. Laurie Brard
INRA
Rue de la Géraudière BP 71627
44316 NANTES CEDEX 3, FRANCE
Phone: +33 2 40675039
Fax: +33 2 40675043
Email: wgpat2014meeting@nantes.inra.fr

Mrs. Ilona Bruins Slot
RIKILT Wageningen UR
Institute of Food Safety
Akkermaalsbos 2
6708 WB WAGENINGEN
THE NETHERLANDS
Phone: +31 317 480388
Email: ilona.bruinsslot@wur.nl

Mr. Henrik Dahlquist
Fria Bröd AB
Fältspatsgatan 12
421 30 VÄSTRA FRÖLUNDA,
SWEDEN
Phone: +46 70 654 600
Fax: +46 317341335
Email: Henrik.Dalquist@fria.se

Mrs. Hertha Deutsch
Österreichische Arbeitsgemeinschaft
Zöliakie
Anton Baumgartner Straße 44/C5/2302
1230 VIENNA, AUSTRIA
Phone: +43 166 71887
Email: hertha.deutsch@utanet.at

Mr. Elias Diaz Ramiro
Instituto Nacional des Consumo
Centro de Investiagción y Control
de la Calidad
Avenida Cantabria, 52
28042 MADRID, SPAIN
Phone: +34 918224744
Email: elias.diaz@consumo-inc.es

Dr. Clyde Don
Foodphysica
Vogelwikke 12
6665 HP DRIEL, THE NETHERLANDS
Phone: +31 622 543047
Email: clyde.don@foodphysica.com

Dr. Jean Dunne
University of Dublin
Department of Immunology,
St. James´s Hospital
James´s Street
DUBLIN 8, IRELAND
Phone: +353 1 416 2921
Fax: +353 1 454 5609
Email: dunnej1@tcd.ie

Mr. Lukas Frank
Romer Labs Divison Holding GmbH
Technopark 1
3430 TULLN, AUSTRIA
Tel: +43 2272 615 33 13155
Fax: +43 2272 615 33 13111
Email: lukas.frank@romerlabs.com

Dr. Gyöngyvér Gell
MTA Centre of Agricultural Research
Department of Applied Genomics
Brunszvik 2
2462 MARTONVÁSÁR, HUNGARY
Phone: +36 225 69521
Fax: +36 225 69514
Email: gell.gyongyver@agrar.mta.hu

Dr. Thomas Grace
Bia Diagnostics
294 N. Winooski Ave, Ste 116A
VT 05401 BURLINGTON, USA
Phone: +1 802 5400148
Fax: +1 802 5400147
Email: thomasgrace@biadiagnostics.com

Mrs. Robin Grace
Bia Diagnostics
294 N. Winooski Ave, Ste 116A
VT 05401 BURLINGTON, USA
Phone: +1 802 5400148
Fax: +1 802 5400147
Email: robin@biadiagnostics.com

Mrs. Gertrud Granel
Association of German Cereal
Processors and Starch Producers
(VDGS e.V.)
Johannesstraße 37
53225 BONN, GERMANY
Phone: +49 30 8892368915
Fax: +49 30 88923689 19
Email: granel@vdgs.org

Dr. Reka Haraszi
Campden BRI
Station road
CHIPPING CAMPDEN,
GL55 6LD, UK
Phone: 00441386842240
Email: reka.haraszi@campdenbri.co.uk

Dr. Angéla Juhász
MTA Centre of Agricultural Research
Department of Applied Genomics
Brunszvik 2
2462 MARTONVÁSÁR, HUNGARY
Phone: +36 225 69531
Fax: +36 225 69514
Email: juhasz.angela@agrar.mta.hu

Dr. Päivi Kanerva
Department of Food and
Environmental Sciences
University of Helsinki
Agnes Sjöbergin katu 2
P.O. Box 66
00014 HELSINKI, FINNLAND
Phone: +358 405 084 064
Fax: +358 9 191 58475
Email: paivi.kanerva@helsinki.fi

Dr. Katharina Scherf
Deutsche Forschungsanstalt für
Lebensmittelchemie
Lise Meitner-Strasse 34
85354 FREISING, GERMANY
Phone: +49 8161712927
Fax: +49 8161712970
Email: katharina.scherf@Lrz.tum.de

Dr. Colette Larre
INRA
Rue de la Géraudière BP 71627
44316 NANTES CEDEX 3, FRANCE
Phone: +33 240675000
Fax: +33 240675025
Email: colette.larre@nantes.inra.fr

Mrs. Stelle Lindeke
R-Biopharm AG
An der neuen Bergstraße 17
64297 DARMSTADT, GERMANY
Phone: +49 6151 810225
Fax: +49 6151 8102734
Email: s.lindeke@r-biopharm.de

Dr. Jussi Loponen
Fazer Group
Fazerintie 6
FI-01230 Vantaa
P.O. Box 4
00941 HELSINKI, FINNLAND
Phone: +358 40 7329772
Email: jussi.loponen@fazer.com

Ms. Roberta Luppi
INRA
Rue de la Géraudière BP 71627
44316 NANTES CEDEX 3, FRANCE
Phone: +33 240675000
Fax: +33 240675025
Email: roberta.luppi@nantes.inra.fr

Dr. Edurne Simon Magro
University of the Basque Country
Paseo de la Universidad, 7
01006 VITORIA-GASTEIZ, SPAIN
Phone: +34 945-01 30 69
Fax: +34 945-01 30 14
Email: edurne.simon@ehu.es

Dr. Mª Carmen Mena Valverde
National Center of Biotechnology,
CSIC
28049 MADRID, SPAIN
Phone: +34 915854670
Fax: +34 915854506
Email: mcmena@cnb.csic.es

Ms. Kathryn Miller
Coeliac UK
3RD Floor, Apollo Center,
Desborough RD,
HIGHWYCOMBE
HPII 2QW, UK
Phone: +44 01494796130
Email: kathryn.miller@coeliac.org.uk

Dr. Luisa Novellino
Associazione Italiana Celiachia
Via Caffaro, 10
16124 GENOVA, ITALY
Phone: +39 010 8449406
Email: l.novellino@celiachia.it

Director Gavin O'Connor
European Commission,
Institute for Reference Materials and Measurements,
Standards for Food Biosciences
Retieseweg 111
B-2440 GEEL, BELGIUM
Phone: +32 14 571812
Email: Gavin.O'CONNOR@ec.europa.eu

Dr. Lea Pollak
Croatian National Institute of Public Health
Rockefellerova 7
10000 ZAGREB, CROATIA
Phone: +385 01048630266
Fax: +385 14863365
Email: lea.pollak@hzjz.hr

Mrs. Catherine Remillieux-Rast
Association Française des Intolérants au Gluten (AFDIAG)
Rue de Venise 23
78740 VAUX-SUR-SEINE, FRANCE
Phone: +33 681270911
Fax: +33 130993668
Email: c.remillieux_rast@yahoo.fr

Mr. Nermin Sajic
EuroProxima B.V.
Beijerinckweg 18
6827 BN ARNHEM, THE NETHERLANDS
Phone: +31 26 3630364
Fax: +31 263645111
Email: nermin.sajic@europroxima.com

Dr. Juan Ignacio Serrano-Vela
Asociacion de Celiacos de Madrid
Calle Lanuza 19-bajo
28028 MADRID, SPAIN
Phone: +34 917130147
Fax: +34 917258059
Email: nachoserrano@celiacosmadrid.org

Dr. Tuula Sontag-Strohm
Department of Food and Environmental Sciences
University of Helsinki
Agnes Sjöbergin katu 2
P.O. Box 66
00014, HELSINKI, FINLAND
Phone: 358 9 19158230
Fax: +358 9 191 58475
Email: tsontag@mappi.helsinki.fi

Dr. Jean-Yves Thebaudin
GGF
Route de Thennes
80110 MOREUIL, FRANCE
Phone: +33 685949878
Fax: +33 322330561
Email: contact@c-viatis.com

Mrs. Pauline Titchener
Neogen Europe Ltd.
The Dairy School, Auchincruive
KA6 5HW AYR, SCOTLAND, UK
Phone: +44 1292 525 600
Fax: +44 1292 525 601
Email: p.titchener@neogeneurope.com

Dr. Thomas Weiss
R-Biopharm AG
An der neuen Bergstrasse 17
64297 DARMSTADT, GERMANY
Phone: +49 6151 8102 186
Fax: +49 6151 8102734
Email: t.weiss@r-biopharm.de

Mr. Johannes Wolf
Universitätsklinikum Leipzig A.ö.R.
Institut für Laboratoriumsmedizin,
Klinische Chemie und Molekulare
Diagnostik
Liebigstraße 27
04103 LEIPZIG, GERMANY
Phone: +49 341 97 22379
Fax: +49 341 97 22455
Email: johannes.wolf@medizin.uni-
leipzig.de

3 Programme

THURSDAY, September 25, 2014

20:00 Arrival of Prolamin Group and all participants
Informal get-together with dinner
Welcome by Colette Larré (INRA)
Location: Hotel Mercure "Ile de Nantes", Nantes

FRIDAY, September 26, 2014

09:00 Opening of the meeting (Peter Koehler)

09:15 Allergy to wheat: Clinical data, allergens and epitopes (Dr. Sandra Denery, Nantes, France)

09:55 THE PROLAMIN WORKING GROUP RESEARCH REPORTS

- Analytical reports (Chirdo, Ciclitira, Feighery, Gilissen, Koehler, Koning, Lundin, Mothes, Schuppan, van Eckert; guests)

11:15 Coffee break

11:30 Analytical research reports (continuation)

13:00 Lunch

14:15 Analytical research reports (continuation)

15:00 THE PROLAMIN WORKING GROUP RESEARCH REPORTS

- Clinical reports (Catassi, Chirdo, Ciclitira, Feighery, Koning, Lundin, Mothes, Schuppan, Troncone; guests)

17:00 Coffee break

17:30 THE PROLAMIN WORKING GROUP EXECUTIVE MEETING (members only)

20:00 Joint dinner of all participants
Location: Restaurant "La Passagère" Baron Lefevre, Nantes

SATURDAY, September 27, 2014

09:00	**SYMPOSIUM** **Antigen receptors in coeliac disease** (Chair: Prof. Dr. Martin Stern, Tuebingen, Germany)
09:05	**Molecular basis for T cell receptor recognition of HLA-DQ-gluten in coeliac disease** (Prof. Dr. Frits Koning, Leiden, The Netherlands)
09:50	**Direct visualization of gliadin specific T cell in diagnosis of coeliac disease** (Prof. Dr. Knut Lundin, Oslo, Norway)

10:35 Coffee break

11:05 Mechanisms of loss of immune tolerance to gluten in coeliac disease (Prof. Dr. Nadine Cerf-Bensussan, Paris, France)

11:50 Discussion of current developments concerning gluten analysis, clinical and legal aspects

Statements by participating organisations, representatives from industry and guests

- Outline: Action plan PWG 2015

13:00 Lunch and Farewell

Afternoon

- Extra time for informal meeting and additional PWG executive meeting concerning action plan

SUNDAY, September 28, 2014

Departure of the PWG

4 Analytical research reports

4.1 Complementarity of native and deamidated gluten detection with R5 and INRA-DG1 mAbs

Olivier Tranquet, Roberta Lupi, Colette Larré, Sandra Denery

INRA, UR1268 Biopolymers, Interactions, Assemblies, Nantes, France

Introduction

Diversification of gluten applications was achieved through the production of water-soluble gluten also named wheat isolates. Deamidation, one of the methods for this purpose, may be obtained with either chemical (acid or alkali) or enzymatic treatment and lead to the conversion of glutamine and asparagine into glutamic and aspartic acids. These types of products can be found in both cosmetics and food.

Since the 2000s, severe allergic reactions to deamidated gluten (DG) have been reported in individuals although they were tolerant to native wheat protein [1]. In Japan, five women, who exhibited a particular and severe food allergy to wheat after sensitisation by a soap containing acid-hydrolysed gluten, were described in 2011 [2]. In 2013, more than 1,300 people had been sensitised by the same soap and exhibited severe symptoms after wheat ingestion [3]. Management of these allergies is extremely difficult both for patient and food manufacturers. Therefore, for clinical purposes a wheat isolate was proposed for skin-prick-tests [4]. More recently, the identification of several epitopes linked to this allergy drove to conclude to a new type of allergy specifically elicited by DG [5]. One of these epitopes (QPEEPFPE), derived from a repetitive sequence of ω2- and γ-gliadins, was shown to be dominant.

Most of the gluten detection methods rely on antibodies that target domains of gliadins containing repetitive sequences. These glutamine-rich domains are likely to be modified by deamidation. Kanerva et al. demonstrated that analytical methods based on R5, on Skerritt's or on G12 monoclonal antibodies (mAbs) were not efficient for recognising DG [6]. To fill this gap, mouse monoclonal antibodies were produced against the peptide LQPEEPFPEQC. They were characterised by immunochemical methods with purified gliadins. mAb INRA-DG1 specifically bound deamidated gluten with high affinity and without any reaction to native wheat gluten. A competitive ELISA assay to detect deamidated gluten was developed with mAb INRA-DG1 [7]. Analysis of native and deamidated glutens with R5 and INRA-DG1 competitive assay suggested a good complementarity of these two antibodies for gluten detection whatever its deamidation status.

In this work, we have detailed this complementarity at the epitopic level and evaluated the compatibility of the INRA-DG1 competitive assay with an extraction procedure designed for native gliadins.

Materials and methods

Pepscan investigation

Pepscan was carried out according to Denery et al. [5] with peroxidase-conjugated R5 mAb taken out of RIDASCREEN® Gliadin competitive kit (R-Biopharm, Germany) and diluted 1/20 in 2.5% (w/v) skimmed dried milk and 5% (w/v) sucrose in tris-buffered saline TBS.

Competitive INRA-DG1 ELISA

Microtiter plates (Maxisorp, NUNC, Roskilde, Denmark) were coated with LQPEEPFPEQC conjugated to BSA in 10 mmol/L carbonate-bicarbonate buffer (pH 9.6) and left overnight at 4 °C. 5 g of rice flour samples were suspended in 10 mL of phosphate-buffered saline (PBS) or of Mendez cocktail [8] and incubated 40 min at 50 °C, then 40 mL of PBS or of a 80% ethanol solution was added and incubated for 1h at RT. Supernatants were collected by centrifugation at 2,500 *g* and further diluted 1/20 in PBS containing 0.1% skimmed milk prior incubation with INRA-DG1 mAb (crude supernatant diluted at 1/4,000) for 2 h at 37 °C. Then, 100 µL of antibody sample solution was added to plates coated in BSA-LQPEEPFPEQC and incubated for 1 h. After washing, bound mAbs were revealed by incubation with horseradish peroxidase anti-mouse IgG (170-6516, Bio-Rad, 1/3,000 dilution) and orthophenylenediamine (Sigma) as the substrate. Colour development was stopped with 100 µL of 2 mol/L H_2SO_4 and the absorbance was read at 492 nm. Inhibition of antibody binding was expressed as the percentage of the maximal response obtained with antibody in the absence of the competitor according to the following formula:

% inhibition = 1 - (sample OD/ blank sample OD)

Industrially deamidated gluten (heated under acidic conditions) was used as standard and for rice flour contamination. Spiked rice flour: 5 g of rice flour was spiked with 100 µg of deamidated gluten.

Results and discussion

In our recent work, we have shown that R5 and INRA-DG1 exhibited complementary reactivity toward native and deamidated glutens [7]. INRA-DG1 reactivity on peptides from the repetitive sequences of ω2- or γ-gliadins deamidated at different levels was determined by the pepscan technique and the epitopic sequences of INRA-DG1 mAb were found to be Q/EPQ/EEPFPE. The same pepscan membrane was used to compare the reactivity of R5 mAb with the INRA-DG1 mAb (Fig. 1). Peptides 2 to 9 corresponded to the Ala scan of the peptide QPQQPFPQ from native ω2- or γ-gliadin (peptide 1). Analysis of the R5 reactivity on these nine peptides revealed a minimal consensus epitope of R5 as QQA/PFP. In accordance with Osman et al. [9], Pro in position 2 or 5 and Gln in the first or in the last position could be replaced by Ala without impacting R5 binding. In addition, the capacity of both antibodies (INRA-

DG1 and R5) to bind to the same peptide with its Gln more or less substituted by Glu (Fig. 1 lane 10 to 24) was examined. Although R5 was tolerant to Gln/Ala substitution in the first or in the last position of peptide 1, the Gln/Glu substitution at these positions reduced the R5 binding (peptide 10 and 13). This reduction was strengthened with a double substitution, since the R5 binding was almost fully abolished with peptide 16. All other Gln/Glu substitutions were detrimental for R5 binding.

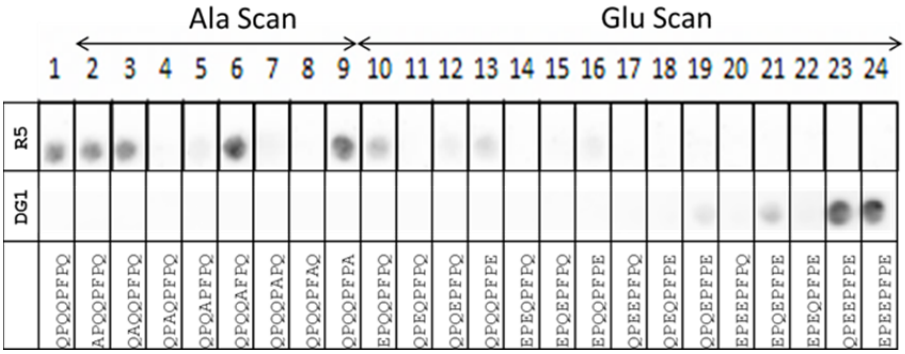

Figure 1. *Reactivity of INRA-DG1 and R5 mAbs on octapeptides synthesised on cellulose membrane. 1 original peptide; Ala Scan: 2 - 9 Ala substitution at each amino acid position; Glu Scan: 10 - 24 Gln/Glu substitutions*

This result obtained at the epitopic level confirmed that R5 and INRA-DG1 mAbs did not bind the same epitopes and that there was no overlap between them.

Numerous tests for the detection of gluten are available; those using the R5 antibody are recommended by the Codex Alimentarius. The analysis of several wheat isolates showed that they could be more or less deamidated and in a number of cases, they escaped detection by R5 kit. Faced with a sample with a putative gluten contamination, the analyst cannot know whether the gluten is deamidated or not.

Simultaneous analysis of the sample with R5 and INRA-DG1 mAbs was considered and the compatibility of the extraction buffer used in the R5 methods was tested in the INRA-DG1 competitive ELISA. Native gliadins are usually solubilised in ethanol with or without a preliminary extraction step in solutions containing denaturant and reducing agents such as in the Mendez cocktail. On the other hand, DG was extracted in aqueous buffer such as PBS. Rice flour samples spiked with DG at 20 mg/kg were extracted in PBS, in Mendez cocktail + ethanol or in Mendez cocktail + PBS. Samples were then analysed in the INRA-DG1 competitive assay and inhibition percentages were calculated (Fig. 2). Whatever the extraction conditions, no cross-reaction was observed with unspiked rice flour samples. Analysis of samples spiked at 20 mg/kg resulted in significant inhibition (Fig. 2). The test allowed the determination of the DG content, the results varied slightly according to extraction conditions. Recovery rates were 100%, 107%, and 129% for extraction in PBS, in Mendez cocktail + EtOH, or in Mendez cocktail + PBS, respectively.

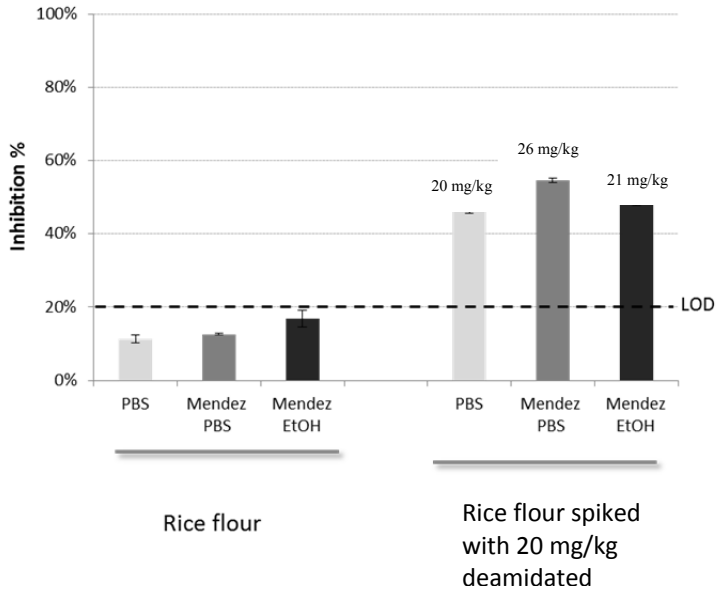

Figure 2. *Detection of deamidated gluten in rice flour spiked at 20 mg/kg after extraction with PBS or with cocktail Mendez. LOD, Limit of detection*

Conclusions

Among the gliadins, R5 mAb bound ω- and γ2-gliadins strongly [10]. Deamidation of gluten induced Gln/Glu substitution and impaired its detection by R5. In this study, we have shown the impact of Gln/Glu substitution on R5 binding to peptides deduced from the repetitive sequences of ω- and γ2-gliadins, highlighting that Gln/Glu substitutions inside the R5 epitopes and in the flanking amino acids are detrimental. On the contrary, INRA-DG1 is able to detect these deamidated epitopes pointing out the interest of INRA-DG1 mAb to complete the tools available for reliable gluten detection.

Aknowledgment

We thank Valerie Echasserieau and Manon Beltrando for their technical assistance and INRA Transfer for its funding.

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4.2 Collaborative study on gluten detection using the RIDA®QUICK Gliadin dipstick

Katharina Scherf[1], Peter Koehler[1], Thomas Weiss[2], Steffen Uhlig[3], Markus Lacorn[2]

[1] Deutsche Forschungsanstalt für Lebensmittelchemie, Leibniz Institute, Freising, Germany
[2] R-Biopharm AG, Darmstadt, Germany
[3] QuoData GmbH, Dresden, Germany

Introduction

Currently, the only known effective treatment for coeliac disease (CD) is a lifelong gluten-free diet, which should contain less than 20 mg gluten per day to prevent a relapse of intestinal symptoms [1]. The Codex Alimentarius and legislation in the United States, Canada, and Europe endorse a threshold of 20 mg gluten/kg for gluten-free foods [2]. To guarantee the safety of gluten-free products for CD patients, specific and sensitive analytical methods are needed. Immunologic methods based on specific antibodies are recommended for the determination of gluten in foods. The R5 monoclonal antibody primarily recognises the epitope QQPFP, which is present in gliadins, secalins, and hordeins and occurs in many CD-toxic or -immunogenic peptides [3]. Sandwich and competitive ELISA formats based on the R5 antibody were successfully validated as AACC International Approved Method 38-50.01 for intact gluten [4] and 38-55.01 for partially hydrolysed gluten [5], respectively. Additionally, the R5 Sandwich ELISA is endorsed by the AOAC International as Official Method of Analysis first action status 2012.01 and laid down as a Codex Type 1 Method for the analysis of gluten [6].

Immunochromatographic assays, available as lateral flow devices or dipsticks, provide rapid qualitative results indicating the presence or absence of the substance to be determined. The RIDA®QUICK Gliadin dipstick based on the R5 antibody is intended to be used as a swab test of potentially contaminated surfaces or to check for gluten contamination of raw materials after ethanol extraction or of processed materials after cocktail extraction [7].

Following the guidelines for validation of qualitative binary chemistry methods of the AOAC International Stakeholder Panel on Alternative Methods (ISPAM) [8], an international collaborative study was set up to validate the R5 dipstick (RIDA®QUICK Gliadin, R7003, R-Biopharm) for qualitative gliadin/gluten detection in raw and processed food materials as an AACC International Approved Method.

Materials and methods

The R5 dipstick test kit for the detection of gliadin in raw and processed food (RIDA® QUICK Gliadin, R7003, R-Biopharm) was used in this study together with the cocktail solution for part B of this collaborative test (cocktail (patented), R7006, R-Biopharm). Two sets of assay controls (R7010 and R7012, R-Biopharm) for use with the ethanol extraction or the cocktail extraction were also provided.

Participating laboratories

Eighteen laboratorie (designated A to W) participated in the collaborative study: one each in Argentina, Austria, Belgium, Canada, Finland, Hungary, Ireland, Italy, Sweden, Switzerland, and the United Kingdom; three in Germany and four in the United States. All laboratories had previous experience with immunological tests and were advised to use a clean, separate room due to the possibility of gluten contamination. The laboratories were given four weeks each to perform the analyses for part A and for part B.

Samples and sample presentation

Due to the two different extraction protocols, the collaborative test was split into two parts (A and B) with four samples and ten replicates, respectively. All concentrations were determined using the RIDASCREEN® Gliadin R7001 (R-Biopharm) (Tab. 1).

Table 1. *Samples for part A (unprocessed samples with ethanol extraction) and part B (processed samples with cocktail extraction) of the collaborative study.*

Part A		**Part B**	
Sample	**Gliadin content [mg/kg][1]**	**Sample**	**Gliadin content [mg/kg] [1]**
Maize flour	0.9[2]	Cookie	0.2[2]
Maize flour	2.4[2]	Maize snack	3.2
Maize flour	5.5	Maize snack	6.7
Maize flour	9.4	Maize snack	23.6

[1] Quantitative values determined by RIDASCREEN® Gliadin R7001 (R-Biopharm). [2]Below the limit of quantitation (2.5 mg gliadin/kg) of the RIDASCREEN® Gliadin; values extrapolated according to [4]

All samples were checked for homogeneity using the RIDASCREEN® Gliadin R7001 (R-Biopharm) according to the IUPAC recommendations for proficiency tests [9]. Ten blinded replicates for each sample [8] were provided to each participating laboratory. The samples were marked with a laboratory-specific letter (A to W), an “E” for ethanol extraction (part A) or a “C” for cocktail extraction (part B) and a randomised number from 1 to 40.

Method and qualitative evaluation

The method and a training video were provided to each laboratory with the instructions to follow the method as written with no deviations. All results obtained by visual inspection after comparison to an evaluation card had to be recorded in a ready-

to-use Excel sheet. First, possible sources of contamination, such as containers and surfaces, the cocktail solution, the 60 or 80% ethanol solution, and the dilution buffer were checked for gluten contamination using the dipsticks. Then, two sets of assay controls with known concentrations were analysed to ensure proper familiarisation with the test. Finally, each blind-coded sample (in total 80 samples) was extracted once and analysed according to the instructions. Each sample had to be marked either as positive, negative or invalid. In case of an invalid result (missing control line or incomplete target line), retesting of the sample was requested.

Results and discussion

The results for each laboratory and sample are shown in Tab. 2 (part A with ethanol extraction) and Tab. 3 (part B with cocktail extraction).

Table 2. *Numbers of total and positive samples detected in part A using the R5 dipstick with ethanol extraction (data from all 18 participating laboratories).*

		Sample 1 (negative)	**Sample 2 (low)**	**Sample 3 (medium)**	**Sample 4 (high)**
mg gliadin/kg		**0.9**	**2.4**	**5.5**	**9.4**
Lab code	**total**	**positive**	**positive**	**positive**	**positive**
A	10	0	10	10	10
B	10	0	10	10	10
D	10	0	10	10	10
E	10	0	10	10	10
F	10	0	10	10	10
G	10	0	10	10	10
H	10	0	10	10	10
I	10	0	9	10	10
L	10	0	10	10	10
M	10	0	9	8	10
N	10	0	10	10	10
O	10	0	10	10	10
P	10	0	10	10	10
R	10	0	10	10	10
S	10	0	9	10	10
T	10	0	10	10	10
U	10	0	10	10	10
W	10	2	10	10	10
Sum	180	2	177	178	180
POD		0.01	0.98	0.99	1.00

POD: probability of detection (averaged over all 18 laboratories)

Fourteen out of 18 laboratories reported neither false positives nor false negatives after ethanol extraction. From the remaining four laboratories, one found two false positives, three found one false negative for the low concentration, and only one laboratory reported two false negatives for the medium concentration. Averaged over all 18 laboratories, this resulted in an extremely low probability of detection (POD) of 0.01 for the negative sample and very high POD values of 0.98, 0.99, and 1.00 for the low-, medium-, and high-concentrated samples, respectively.

The cocktail extraction procedure yields a fourfold higher dilution compared to the ethanol extraction. As expected, the sample with the low gliadin concentration showed higher variability than after ethanol extraction. Despite this difference, the data provided by 9 out of 18 laboratories revealed no false negatives or false positives.

Table 3. *Numbers of total and positive samples detected in part B using the R5 dipstick with cocktail extraction (data from all 18 participating laboratories).*

		Sample 1 (negative)	**Sample 2 (low)**	**Sample 3 (medium)**	**Sample 4 (high)**
mg gliadin/kg		**0.2**	**3.2**	**6.7**	**23.6**
Lab code	**total**	**positive**	**positive**	**positive**	**positive**
A	10	2	7	10	10
B	10	1	10	10	9
D	10	0	9	10	10
E	10	0	1	10	10
F	10	0	10	10	10
G	10	0	10	10	10
H	10	0	10	10	10
I	10	0	9	10	10
L	10	0	8	10	10
M	10	0	10	10	10
N	10	0	10	10	10
O	10	0	10	10	10
P	10	0	10	10	10
R	10	0	10	10	10
S	10	0	0	10	10
T	10	0	9	10	10
U	10	0	1	10	10
W	10	0	10	10	10
Sum	180	3	144	180	179
POD		0.02	0.80	1.00	0.99

POD: probability of detection (averaged over all 18 laboratories)

Only two laboratories found three false positive results in total, which is nearly the same rate as for the ethanol extraction procedure. For the low-concentrated sample, the reported detection rate was either 70 to 100% or 0 to 10% for individual laboratories. This was very interesting, because this implies that the visual inspection resulted in a clear individual cut-off colour value for a positive sample as opposed to a hypothetical detection rate of around 50%. As for part A after ethanol extraction, the average POD for the negative sample after cocktail extraction was very low (0.02). For the low-, medium-, and high-concentrated samples the POD values were calculated as 0.80, 1.00, and 0.99, respectively. Fig. 1 shows a graphical representation of the raw data for parts A and B. The POD in 10% increments is plotted against the gliadin concentration. The larger the circles are, the more laboratories are included, and the line connects the average POD values (n = 18).

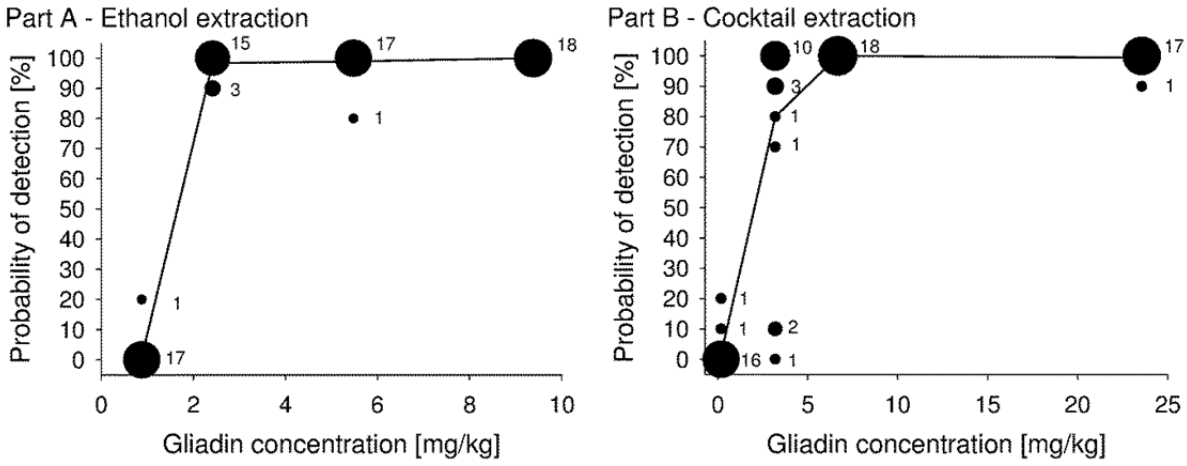

Figure 1. *Bubble plots representing the number of labs per probability of detection (POD) level and gliadin concentration for part A (ethanol extraction) and part B (cocktail extraction). The numbers next to the circles indicate the number of labs and the line connects the average POD values (n = 18)*

Conclusions

The results of part A (ethanol extraction) and part B (cocktail extraction) of the collaborative study with 18 laboratories show that the R5 dipstick RIDA® QUICK Gliadin is capable of qualitatively detecting low levels of gliadin in unprocessed as well as processed samples. The results substantiate the suitability of the test and can be used to submit the report to AACC International and AOAC International for evaluation and addition to the approved methods.

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4.3 AOAC Performance Tested Method℠ 061403 - Validation of AgraStrip® Gluten G12 assay for the detection of gluten in food and surfaces

Lukas Frank[1], Adrian S. Rogers[2], Scott Radcliffe[3], Meredith Sutzko[3], Zheng Jiang[3], Denise Freitag[4], Christy Swoboda[4]

[1] Romer Labs Division Holding GmbH, Tulln, Austria
[2] Romer Labs UK Ltd, Runcorn, United Kingdom
[3] Romer Labs Inc, Newark, DE, USA
[4] Romer Labs Inc, Union, MO, USA

Introduction

Gluten describes the main group of proteins in wheat, rye, and barley and consists of prolamins (in wheat: gliadin, rye: secalin, barley: hordein) and glutelins (in wheat: glutenin) occurring in the same ratio. Due to its physicochemical characteristics, gluten is used in food products as a binder, to help dough rise, and to give dough a more appetising texture [1]. Coeliac disease is a disorder of the small intestine resulting in malabsorption and inflammation. In persons who are genetically susceptible, it is caused by an immune reaction to gliadin, which cannot be enzymatically degraded to amino acids in the intestine due to its high proline and glutamine content. The only effective treatment is a lifelong gluten-free diet [1]. According to Codex Alimentarius Standard 118-1979, "gluten-free" products must comply with gluten levels (including prolamin fractions from rye, barley, and oats) below 20 mg/kg and "foods specially processed to reduce gluten content" must comply with levels between 20 and 100 mg/kg [2]. Utilisation of a reliable and accurate screening method for gluten-free ingredients, foods, and processing equipment will ensure safety of food products for coeliac sufferers.

Romer Labs® developed an immunochromatographic lateral flow assay for the qualitative detection of gluten in raw ingredients, processed foods, finished food products, and environmental surfaces using the G12 antibody developed by Belén Morón [3]. The G12 antibody targets a 33-mer peptide, which is resistant to enzymatic digestion and heat denaturation, as well as being the fragment of the gliadin protein, to which coeliac disease sufferers react, making it a reliable analytical marker. This study was performed to validate the AgraStrip® Gluten G12 assay method under the guidance of the AOAC Performance Tested MethodsSM (PTM) program against AOAC Official Method of Analysis SM 2012.01.

Materials and methods

Validation study

This validation study was conducted according to the AOAC Approved Final AgraStrip Gluten G12 Validation Outline v6.2 (February 3, 2014), prepared for Romer Labs by the AOAC Research Institute PTM program. Parameters tested include: cross-reactivity, interference, incurred samples, food matrixes, environmental surfaces, product consistency, stability, test kit variation, and robustness [4].

Preparation of validation materials

(a) Gluten-free food matrixes included Bob's Red Mill White Rice Flour, Ener-G Foods Gluten-Free Tapioca Loaf, Lucy's Gluten-Free Sugar Cookies, So Delicious Coconut Milk Vanilla Bean Ice Cream (package states gluten-free), and Chatfield's Double Dark Semi-Sweet Chocolate Chips (package states manufactured in a dedicated gluten-free facility).

(b) Reference materials used were gliadin produced by the Prolamin Working Group (PWG), protein content = 91.4%, gliadin content = 88.2%, and wheat gluten produced by Sigma-Aldrich, Cat. No. G5004, Lot No. SLBD0196V, protein content = 84.0%, gliadin content as measured by AOAC Official Methods of Analysis℠ (OMA) 2012.01 = 32.6% (Note: during extraction for OMA 2012.01, it was observed that the wheat gluten tended to agglutinate and adhered to the sides of the extraction vial; therefore, it is unlikely that all gliadin in the wheat gluten can be accurately measured in such a concentrated standard by this method).

Spiking of the test samples was performed using purified gliadin (88.2% gliadin by certificate of analysis) obtained from the PWG, or wheat gluten standard (WGS) obtained from Sigma-Aldrich (84.0% protein by certificate of analysis). PWG gliadin was dissolved at 1 mg/mL by weight in 60% ethanol (EtOH), which was calculated to be a working stock solution at 882 mg/kg gliadin. WGS was suspended at 1 mg/mL by weight in melted clarified cocoa butter, calculated to be a working stock suspension at 840 mg/kg gluten.

(c) Spiked samples were calculated using dilution factors to achieve the final concentrations indicated by AOAC Final AgraStrip Gluten G12 Validation Outline for each spike material and test matrix. Rice flour, bread, and cookies were spiked with gliadin, which was first dissolved to 1 mg/mL in 60% EtOH. This was further diluted into an amount of 60% EtOH calculated to saturate 1/10 of the final bulk matrix sample, then added to the small matrix portion, and EtOH was allowed to evaporate at room temperature. The small spiked portion was then ground by mortar and pestle to separate granules and gradually mixed homogeneously by adding 1/10 final bulk volume unspiked matrix at a time, then remixing, until final bulk volume was achieved. Ice cream, chocolate, and rice flour for stability, precision, repeatability, and robustness were spiked with Sigma-Aldrich WGS. Because gluten so readily binds even small amounts of water, the gluten was suspended at 1 mg/mL in cocoa butter.

Cocoa butter, when melted into a liquid fat, provided a water-free diluent, and it was possible to incorporate the WGS into the matrix while retaining a coating of fat around the WGS granules to protect them from binding water in the matrix. Cocoa butter originates from chocolate, and thus was appropriate for spiking into that matrix. Because cocoa butter has properties similar to coconut oil, being a solid at room temperature, it was also deemed appropriate as a spike diluent for ice cream. From the 1 mg/mL WGS stock, a 1/10 dilution was made before spiking a calculated amount into 1/10 the final bulk volume of melted ice cream or chocolate. Additional melted ice cream or chocolate was added 1/10 the final bulk volume at a time, then remixed, until the final bulk volume was achieved. The chocolate was then frozen to -20 °C and ground. Rice flour was spiked in a similar method, while kept warm to keep the cocoa butter from setting fully, then the final bulk volume allowed to cool to room temperature and ground once more. All spike methods were tested prior to performing the validation study, and homogeneity testing of five samples from each spiked matrix demonstrated a homogeneously distributed spike. This testing, however, did not take into account the amount of time required to aliquot the full number of samples needed for the study, during which the spike in ice cream gradually was exposed to enough water in the matrix that it began to agglutinate as well as adhere to tube and container walls, pipet tips, etc. This homogeneity testing, while taken from representative areas around the bulk sample, could not necessarily detect small “hot spots” of concentrated spike material within the bulk.

(d) The incurred matrix study, performed by an independent laboratory, involved a spiking strategy similar to that of Romer Labs, although the entire gluten-free bread mix matrix was saturated with a more dilute solution of gliadin in 60% EtOH, and allowed to dry 18 h before baking. Thirty mg/kg gliadin was spiked into the bread mix prior to baking in order to achieve a final 15 mg/kg gliadin concentration (30 mg/kg gluten) in the baked bread, per AOAC Final AgraStrip Gluten G12 Validation Outline V6.2 (Tab. 2 and 5) [4]. The bread mix was prepared and baked following the manufacturer’s instructions, cooled for 1 h, then dried 18 h using a LabConco freeze-dry system and homogenised by mortar and pestle.

(e) For environmental surface testing, a 25 cm^2 area of a stainless steel coupon was spiked with the 1 mg/mL stock solution of gliadin in 60% EtOH by pipetting a volume onto the surface calculated to spike to the concentrations indicated in AOAC Final AgraStrip Gluten G12 Validation Outline V6.2. The coupon was then covered and incubated at room temperature until the EtOH fully evaporated before swabbing.

Methodology

The test procedure was carried out as described in the manufacturer’s package insert. Analyst 1 spiked sample matrixes in bulk, aliquotted to tubes and extracted, then blind-coded and randomised extracts. Analyst 2 then ran and read the blinded extracts, after which results were decoded and analysed. Where indicated in the protocol, a third analyst tested cross-reactivity and food matrix samples using reference method AOAC

OMA 2012.01. Note: Previous studies have documented that results of AOAC OMA 2012.01 have a relative standard deviation (RSD) ranging from 22 to 52% depending on the gliadin concentration of the sample [5].

Results and discussion

Cross-reactivity study

Thirty-eight gluten-free labelled food samples were screened, unspiked, against the AgraStrip Gluten G12 assay at the 20 mg/kg threshold for potential cross-reactivity as well as for quantitative gliadin content using AOAC OMA 2012.01 (LOD = 2.5 mg/kg). The results (not shown) indicate no cross-reactivity with any of the screened compounds. The authors note that soya flour, at 8.1 mg/kg gliadin, had a higher level of contamination than desired for this study. Four different brands of soya flour were tested by AOAC OMA 2012.01, all having similar or higher levels of contamination, and the brand screened against AgraStrip Gluten G12 represents the brand with the lowest level of contamination.

Interference study

Each of the 38 samples screened in the cross-reactivity study was spiked with 5 mg/kg gliadin and assayed with AgraStrip Gluten G12 at the 5 mg/kg and 20 mg/kg threshold levels. At 5 mg/kg, all spiked compounds returned a positive result, and at 20 mg/kg, all compounds were negative (data not shown). The authors noted that soya flour, which with the 8.1 mg/kg contamination level plus the 5 mg/kg gliadin spike was calculated to have a gliadin concentration of 13.1 mg/kg, had a noticeably brighter test line at the 5 mg/kg threshold than the other compounds screened, but was still negative at the 20 mg/kg threshold.

Incurred samples study

The independent laboratory prepared “Bob’s Red Mill Homemade Wonderful” gluten-free bread mix in unspiked and 30 mg/kg (target 15 mg/kg after baking) gliadin spike portions. The bread mix ingredients are as follows: stone-ground garbanzo bean flour, potato starch, corn starch, sweet white sorghum flour, tapioca flour, evaporated cane juice, fava bean flour, xanthan gum, active dry yeast, potato flour, sea salt (magnesium carbonate as flowing agent), guar gum, and soy lecithin. The yeast package was bloomed in warm milk for 5 min, then added to bread mix, along with one egg, ¼ cup melted butter, and one teaspoon cider vinegar. The ingredients were mixed, placed into a baking pan, then covered and allowed to rise for 30 min. The bread was next baked in a Hot Point model R3787W oven at 375 °F (approximately 190 °C) for 50 min and then allowed to cool on the bench for 1 h. The cooled bread was freeze-dried, homogenised, and tested with AgraStrip Gluten G12. Tab. 1 shows that unspiked samples all returned a negative result. For the spiked samples, at 30 mg/kg gluten, the

5 and 10 mg/kg thresholds returned all positive results, while the 20 mg/kg threshold returned all negative results.

Table 1. *Independent laboratory incurred sample study results.*

Matrix	Gliadin Spike Concentration	AgraStrip® Detection Threshold (mg/kg Gluten)	N	Candidate			Avg AOAC OMA 2012.01 Results (mg/kg Gliadin) N=3
				x	POD_C	95% CI	
Incurred Bread	0 mg/kg	5	30	0	0	0.00-0.11	**<2.5**
		10	30	0	0	0.00-0.11	
		20	30	0	0	0.00-0.11	
	15.0 mg/kg	5	30	30	1	0.89-1.00	**15.0**
		10	30	30	1	0.89-1.00	
		20	30	0	0	0.00-0.11	

Food matrix testing

The following food matrixes were pre-screened by AOAC OMA 2012.01 for gliadin content: white rice = 0.0 mg/kg, gluten-free cookies = 0.18 mg/kg, gluten-free bread = 0.08 mg/kg, gluten-free ice cream = 0.15 mg/kg, and gluten-free chocolate = 0.05 mg/kg. For each of the 5, 10, and 20 mg/kg threshold levels, the aforementioned food matrixes (n = 30) were spiked with 0, 3, 8, 15, and 25 mg/kg gliadin (rice flour, cookies, bread) or WGS (ice cream, dark chocolate), then assayed with AgraStrip Gluten G12. As well, three replicates of each spike level were screened for gliadin content using AOAC OMA 2012.01. The independent laboratory also performed a food matrix test using rice flour.

For rice flour tested by the independent laboratory, shown in Tab. 2, the 0, 3, 8, 15, and 25 mg/kg gliadin spikes returned gliadin concentrations at <2.5, 2.9, 7.5, 15.2, and 23.0 mg/kg, respectively. At 0 and 3 mg/kg gliadin spikes, AgraStrip Gluten G12 had POD of 0.00 at all three gluten thresholds. At 8 mg/kg gliadin spike, the POD was 1.00, 0.10, and 0.00 at the 5, 10, and 20 mg/kg gluten thresholds, respectively.

At 15 mg/kg gliadin spike, the PODs were 1.00, 1.00, and 0.10 at the 5, 10,and 20 mg/kg gluten thresholds, respectively. At 25 mg/kg gliadin spike, POD was 1.00 at all three thresholds.

For rice flour, shown in Tab. 3, the 0, 3, 8, 15, and 25 mg/kg gliadin spikes returned gliadin concentrations at <2.5, 3.6, 5.0, 13.5, and 23.5 mg/kg, respectively. At 0 mg/kg gliadin (0 mg/kg gluten) spike, AgraStrip Gluten G12 had a POD of 0.00 at all three gluten thresholds. At 3 mg/kg gliadin (6 mg/kg gluten), PODs were 0.40, 0.00, and 0.00 at the 5, 10, and 20 mg/kg thresholds, respectively. At 8 mg/kg gliadin (16 mg/kg gluten) spike, there were PODs of 1.00, 0.43, and 0.10 at the 5, 10, and 20 mg/kg gluten thresholds, respectively. At 15 and 25 mg/kg gliadin (30 and 50 mg/kg gluten) spikes, POD was 1.00 at all three thresholds.

Table 2. *Independent laboratory food matrix testing results for rice flour.*

Matrix	Gliadin Spike Concentration	AgraStrip® Detection Threshold (mg/kg Gluten)	N	Candidate			Avg AOAC OMA 2012.01 Results (mg/kg Gliadin) N=3
				x	POD_C	95% CI	
Rice Flour	0 mg/kg	5	30	0	0.00	0.00-0.11	<2.5
		10	30	0	0.00	0.00-0.11	
		20	30	0	0.00	0.00-0.11	
	3 mg/kg	5	30	0	0.00	0.00-0.11	2.9
		10	30	0	0.00	0.00-0.11	
		20	30	0	0.00	0.00-0.11	
	8 mg/kg	5	30	30	1.00	0.89-1.00	7.5
		10	30	3	0.10	0.03-0.26	
		20	30	0	0.00	0.00-0.11	
	15 mg/kg	5	30	30	1.00	0.89-1.00	15.2
		10	30	30	1.00	0.89-1.00	
		20	30	3	0.10	0.05-0.30	
	25 mg/kg	5	30	30	1.00	0.89-1.00	23.0
		10	30	30	1.00	0.89-1.00	
		20	30	30	1.00	0.89-1.00	

Table 3. *Food matrix testing results for gliadin-spiked rice flour.*

Matrix	Gliadin Spike Concentration	AgraStrip® Detection Threshold (mg/kg Gluten)	N	Candidate			Avg AOAC OMA 2012.01 Results (mg/kg Gliadin) N=3
				x	POD_C	95% CI	
Rice Flour	0 mg/kg	5	30	0	0.00	0.00-0.11	<2.5
		10	30	0	0.00	0.00-0.11	
		20	30	0	0.00	0.00-0.11	
	3 mg/kg	5	30	12	0.40	0.25-0.58	3.6
		10	30	0	0.00	0.00-0.11	
		20	30	0	0.00	0.00-0.11	
	8 mg/kg	5	30	30	1.00	0.89-1.00	5.0
		10	30	13	0.43	0.27-0.61	
		20	30	3	0.10	0.03-0.26	
	15 mg/kg	5	30	30	1.00	0.89-1.00	13.5
		10	30	30	1.00	0.89-1.00	
		20	30	30	1.00	0.56-0.86	
	25 mg/kg	5	30	30	1.00	0.89-1.00	23.5
		10	30	30	1.00	0.89-1.00	
		20	30	30	1.00	0.89-1.00	

For gluten-free cookies, shown in Tab. 4, the 0, 3, 8, 15, and 25 mg/kg gliadin spikes returned gliadin concentrations at 0.3, 2.7, 8.3, 11.7, and 17.8 mg/kg, respectively. At 0 mg/kg gliadin (0 mg/kg gluten) spike, AgraStrip Gluten G12 had a POD of 0.00 at all three gluten thresholds. At 3 mg/kg gliadin (6 mg/kg gluten), the PODs were 0.93,

0.87, and 0.00 at the 5, 10, and 20 mg/kg thresholds, respectively. At 8 mg/kg gliadin (16 mg/kg gluten) spike, there were PODs of 1.00, 1.00, and 0.13 at the 5, 10, and 20 mg/kg gluten thresholds, respectively. At 15 mg/kg gliadin (30 mg/kg gluten) spike, there were PODs of 1.00, 1.00, and 0.97 at the 5, 10, and 20 mg/kg thresholds, respectively. At 25 mg/kg gliadin (50 mg/kg gluten) spike, the POD was 1.00 at all three thresholds.

Table 4. *Food matrix testing for gliadin-spiked gluten-free cookies.*

Matrix	Gliadin Spike Concentration	AgraStrip® Detection Threshold (mg/kg Gluten)	N	Candidate			Avg AOAC OMA 2012.01 Results (mg/kg Gliadin) N=3
				x	POD_C	95% CI	
Cookies	0 mg/kg	5	30	0	0.00	0.00-0.11	0.3
		10	30	0	0.00	0.00-0.11	
		20	30	0	0.00	0.00-0.11	
	3 mg/kg	5	30	28	0.93	0.79-0.98	2.7
		10	30	26	0.87	0.70-0.95	
		20	30	0	0.00	0.00-0.11	
	8 mg/kg	5	30	30	1.00	0.89-1.00	8.3
		10	30	30	1.00	0.89-1.00	
		20	30	4	0.13	0.05-0.30	
	15 mg/kg	5	30	30	1.00	0.89-1.00	11.7
		10	30	30	1.00	0.89-1.00	
		20	30	29	0.97	0.83-1.00	
	25 mg/kg	5	30	30	1.00	0.89-1.00	17.8
		10	30	30	1.00	0.89-1.00	
		20	30	30	1.00	0.89-1.00	

For gluten-free bread, shown in Tab. 5, the 0, 3, 8, 15, and 25 mg/kg gliadin spikes returned gliadin concentrations at <2.5, 2.3, 7.6, 13.4, and 18.8 mg/kg, respectively. At 0 mg/kg gliadin (0 mg/kg gluten) spike, AgraStrip Gluten G12 had a POD of 0.00 at all three gluten thresholds. At 3 mg/kg gliadin (6 mg/kg gluten), the PODs were 1.00, 0.43, and 0.00 at the 5, 10, and 20 mg/kg thresholds, respectively. At 8 mg/kg gliadin (16 mg/kg gluten) spike, there were PODs of 1.00, 0.93, and 0.10 at the 5, 10, and 20 mg/kg gluten thresholds, respectively. At the 15 mg/kg gliadin (30 mg/kg gluten) spike, there were PODs of 1.00, 1.00, and 0.97 at the 5, 10, and 20 mg/kg thresholds, respectively. At the 25 mg/kg gliadin (50 mg/kg gluten) spike, the POD was 1.00 at all three thresholds.

Table 5. *Food matrix testing for gluten-free bread.*

Matrix	Gliadin Spike Concentration	AgraStrip® Detection Threshold (mg/kg Gluten)	N	Candidate			Avg AOAC OMA 2012.01 Results (mg/kg Gliadin) N=3
				x	POD_C	95% CI	
Bread	0 mg/kg	5	30	0	0.00	0.00-0.11	<2.5
		10	30	0	0.00	0.00-0.11	
		20	30	0	0.00	0.00-0.11	
	3 mg/kg	5	30	30	1.00	0.89-1.00	2.3
		10	30	13	0.43	0.27-0.61	
		20	30	0	0.00	0.00-1.00	
	8 mg/kg	5	30	30	1.00	0.89-0.11.	7.6
		10	30	28	0.93	0.79-0.98	
		20	30	3	0.10	0.03-0.26	
	15 mg/kg	5	30	30	1.00	0.89-1.00	13.4
		10	30	30	1.00	0.89-1.00	
		20	30	29	0.97	0.83-1.00	
	25 mg/kg	5	30	30	1.00	0.89-1.00	18.8
		10	30	30	1.00	0.89-1.00	
		20	30	30	1.00	0.89-1.00	

For gluten-free ice cream the 0, 3, 8, 15, and 25 mg/kg WGS spikes returned gliadin concentrations at <2.5, 6.2, 21.4, 38.2, and 30.4 mg/kg, respectively. At 0 mg/kg WGS spike, AgraStrip Gluten G12 had a POD of 0.00 at all three gluten thresholds. At 3 mg/kg WGS, the PODs were 1.00, 0.93, and 0.00 at the 5, 10, and 20 mg/kg thresholds, respectively. At 8 mg/kg WGS spike, there were PODs of 1.00, 1.00, and 0.33 at the 5, 10, and 20 mg/kg gluten thresholds, respectively. At 15 and 25 mg/kg WGS spike, the POD was 1.00 at all three thresholds.

For gluten-free chocolate the 0, 3, 8, 15, and 25 mg/kg WGS spikes returned gliadin concentrations at <2.5, 4.4, 5.6, 13.2, and 32.9 mg/kg, respectively. At 0 mg/kg WGS spike, AgraStrip Gluten G12 had a POD of 0.00 at all three gluten thresholds. At 3 mg/kg WGS, the PODs were 0.10, 0.00, and 0.00 at the 5, 10, and 20 mg/kg thresholds, respectively. At 8 mg/kg WGS spike, there were PODs of 0.93, 0.23, and 0.03 at the 5, 10, and 20 mg/kg gluten thresholds, respectively. At 15 mg/kg WGS spike, there were PODs of 1.00, 1.00, and 0.57 at the 5, 10, and 20 mg/kg thresholds, respectively. At 25 mg/kg WGS spike, the POD was 1.00 at all three thresholds.

Rice flour spiked with WGS at 10 000 mg/kg was also tested to determine whether the assay could experience hook effect at high contamination levels. Results indicate positive results at all three thresholds.

Environmental surface testing

Both the independent laboratory and the authors prepared 25 cm^2 stainless steel coupons as unspiked with n = 5, fractional recovery spike of 3.5 µg gliadin (7 µg gluten) with n = 30, and high level spike at 17.7 µg gliadin (35.4 µg gluten) with n = 5.

Both laboratories recorded AgraStrip Gluten G12 results as all negative for the unspiked coupons, and all positive for the high level (data not shown). For fractional recovery, the independent laboratory recorded 19/30 positive, and the authors recorded 18/30 positive.

Further testing

Intra- and inter-assay variation, stability, and robustness studies were carried out as well but did not show any significant conspicuities, thus justifying the positive AOAC –RI approval (data not shown).

Conclusions

The results of this study demonstrate that the AgraStrip Gluten G12 test kit will neither cross-react with a broad spectrum of gluten-free food samples, nor will those samples interfere with a positive result. Environmental surface spikes were recovered as expected, demonstrating no false-positive results, fractional recovery just below the LOD, and recovery at high spike concentration. The assay demonstrated consistent results between different production lots, as well as between different kits within the same lot. Ongoing stability studies show that the AgraStrip Gluten G12 kit is stable over a period of three months, as well as over a period of 50 days during accelerated stability at 42 °C. Results of the robustness study indicated that shortening the protein extraction time is not advisable, nor is halving the amount of extraction buffer used, as this effectively doubles the gluten concentration in the extract. A 5 min extraction, over-filling the extraction buffer, and varying the AgraStrip incubation time did not significantly affect the test outcome. Food matrix testing indicates the AgraStrip Gluten G12 assay is capable of detecting the presence of 5, 10, and 20 mg/kg gliadin spike levels in gluten-free rice flour, cookies, and bread at the respective 5, 10, and 20 mg/kg assay thresholds. Per the validation outline, gliadin was spiked at 0, 3, 8, 15, and 25 mg/kg levels, though it comprises only approximately 50% of the total protein content of gluten. The AgraStrip Gluten G12 antibodies detect the presence of gluten through a gliadin epitope; however, the assay is designed to reflect gluten content (while AOAC OMA 2012.01 measures gliadin content). Therefore, food matrix testing, where gliadin was spiked at 3, 8, 15, and 25 mg/kg, represents gluten concentrations of approximately 6, 16, 30, and 50 mg/kg in the AgraStrip Gluten G12 assay. During incurred matrix testing, no spike recovery at the 20 mg/kg threshold was observed, despite the sample having measured at approximately 15 mg/kg gliadin or 30 mg/kg gluten. Because AOAC OMA 2012.01 has a RSD ranging from 22 to 52% the range for the amount of gliadin in the baked bread allows for the possibility that the amount of gliadin in the baked bread could have been below the 20 mg/kg threshold.

The AgraStrip Gluten G12 assay has been observed to perform reliably when testing heat-treated samples, due to the highly stable sequential epitope, which the G12 antibody detects. The high gliadin concentrations observed during the gluten-free ice cream testing reflect the difficulty of spiking complete WGS into a food matrix

containing water. The gluten particles drew water in, bloomed, and tended to adhere to tube walls and pipet tips, as well as agglutinate. This agglutinative effect caused by the water present in ice cream likely produced heterogeneity of gluten concentrations observed with that matrix. A gliadin spike into the ice cream likely would have produced better homogeneity. Because a fat, cocoa butter, was used to suspend and dilute the WGS for chocolate, and chocolate had far less water content, better homogeneity was achieved, and the results more accurately reflect detection of the spike levels of WGS, despite the high levels of tannins present in 70% cocao dark chocolate tested. Still, at the 8 mg/kg threshold in chocolate, two false-negative results were observed. These results were due to the chocolate hardening in the bottom of the extraction tube, before it could be fully mixed with the extraction buffer. These results do not indicate a failure of the assay to detect gluten, but rather reflect the challenge of spiking and manipulating, within such a large study, a matrix having physical properties of chocolate. Over the entire study, no false-positive results were observed in any blank sample. The AgraStrip Gluten G12 test kit is a consistent, stable, robust, and accurate lateral flow assay for the qualitative detection of gluten in raw ingredients, processed food, finished food products, and environmental surfaces.

Abbreviations

N	Number of test portions
x	Number of positive test portions
POD_C	Candidate method confirmed positive outcomes divided by the total number of trials
95% CI	Confidence Intervals

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4.4 Proposal for developing a hordein standard for barley-based products

Päivi Kanerva, Xin Huang, Hannu Salovaara, Tuula Sontag-Strohm

University of Helsinki, Helsinki, Finland

Introduction

Immunological ELISA assays are used for the quantification of residual gluten in gluten-free products. These assays are based on the assumption that the protein fractions that are harmful for people with coeliac disease are similar in wheat, barley, and rye. However, this is not true. Each cereal has its own composition of proteins.

Protein content of wheat and barley is about the same being around 12 - 14%, whereas the protein content in rye is somewhat lower, being about 10%. The protein fraction, which is harmful for coeliacs, accounts for about 60 - 80% of the total protein content of wheat, barley, and rye. This protein fraction is called gluten. Gluten contains multiple proteins that are named gliadins and glutenins in wheat, hordeins in barley, and secalins in rye.

The quantification of gluten is typically based on a gliadin reference material, which is not suitable for the quantification of barley hordeins [1]. Despite similarities, gliadins are not directly comparable to hordeins and secalins, and several differences are known. Therefore, we consider it necessary to develop a separate reference material for the quantification of barley prolamins from gluten-free products with possible barley contaminations, such as oat products.

Prolamins of wheat, barley, and rye

Codex Standard 118 for "foods for special dietary use for persons intolerant to gluten" describes gluten as the protein fraction, to which some persons are intolerant and that is insoluble in water and 0.5 mol/L NaCl [2]. Half of the gluten proteins are considered to be prolamins, which are defined as the fraction from gluten that can be extracted by 40 - 70% of ethanol. Gluten contains multiple proteins, which are grouped by their size and composition. All of them are soluble in aqueous alcohol solution and contain high amounts of the amino acids proline and glutamine. Some of these proteins, however, need reduction to render them soluble in aqueous alcohol, because they form high-molecular-weight (HMW) polymers stabilised by disulphide bonds. Examples for these are glutenins in wheat and D-hordeins in barley. These proteins are often considered as glutelins, but due to the homology of the proteins extracted in aqueous alcohols with or without reduction, it would be better to call all of them prolamins as suggested by Shewry and Tatham [3]. In wheat, low-molecular-weight (LMW) glutenin subunits are actually very similar to gliadins based on their amino and sequence and molecular weight. The main difference between gliadins and LMW

glutenins is the lower solubility of LMW glutenin subunits in aqueous alcohols without reduction. There are, however, differences in the amino acid compositions of HMW glutenins of wheat when compared to gliadins.

Gluten proteins are often divided into monomers and polymers based on differences in their tendency to form aggregates. Wheat α-, γ- and ω-gliadins are regarded as monomers, while HMW and LMW glutenins form polymers. In barley, only C- and γ-hordeins exist as monomers, while B- and D-hordeins form polymers. In rye, the protein types are γ40k- and ω-secalins, and γ75k- and HMW-secalins, respectively. The relative ratios of these monomeric and polymeric proteins have been calculated and are shown in Tab. 1.

In the Codex Standard for gluten-free products, the prolamins are considered to be the monomeric fraction, whereas the polymeric fraction (glutenins) is not solubilised or not recognised by the antibody. Therefore, prolamin contents are multiplied by two to get the gluten content of the sample. If we look at the ratios between theoretical amounts of monomeric and polymeric proteins in different cereals, we get different numbers for each cereal.

Table 1. *Theoretical ratios of monomeric and polymeric gluten protein types.*

	Monomeric gluten protein types	**Polymeric gluten protein types**	**Theoretical ratio between monomeric and polymeric**
Wheat	α-, γ- and ω-gliadins **together about 50%**	LMW and HMW glutenins, some γ-gliadins, **together about 50%**	1:1
Barley	C-hordeins and some γ-hordeins **together about 20%**	B- and D-hordeins and some γ-hordeins **together about 80%**	1:4
Rye	γ40- and ω-secalins **together about 40%**	γ75- and HMW secalins **together about 60%**	2:3

However, in practice, considerable amounts of polymeric proteins are extracted by aqueous alcohol without reduction (Fig. 1). This can be seen especially with barley and rye. In barley, based on the solubility, C- and part of the B-hordeins would belong to prolamin group, while the remaining B-hordeins and D-hordeins would be glutelins. It depends on the extraction conditions, which proportion of B-hordeins is extracted into alcohol solution without reduction and how many of them need reduction of disulphide bonds before being solubilised. In rye, all secalin groups (γ-40k-, γ-75k-, ω- and HMW-secalins) are soluble to a certain extent in aqueous alcohols, while γ-75-

and HMW-secalins, which have a higher molecular weight, need reducing conditions to become alcohol-soluble.

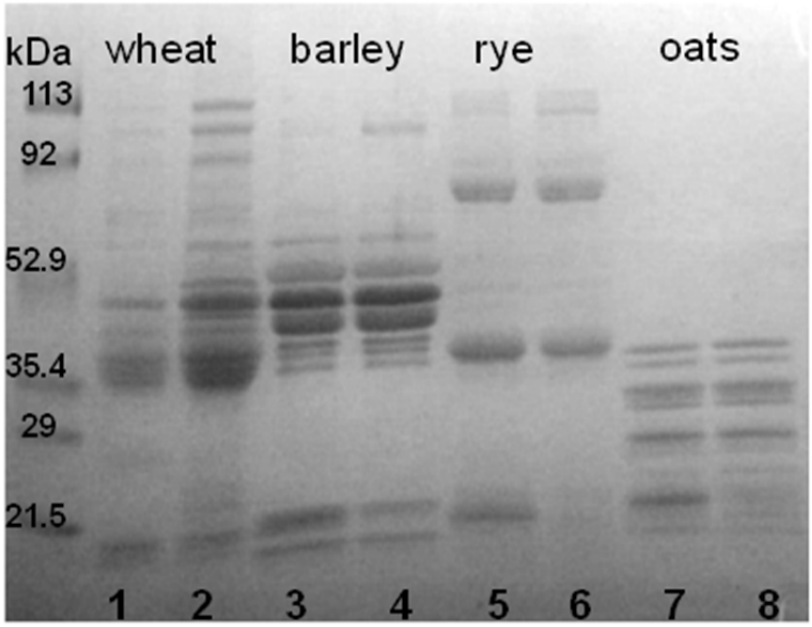

Figure 1. *SDS-PAGE separation of gluten proteins of wheat, barley, rye, and oats under reducing conditions. Samples were extracted with 60% ethanol (odd numbers) or with 60% ethanol and 1% DTT (even numbers)*

Therefore, dividing gluten proteins into two fractions, prolamins and glutelins, is not practical. Since proteins in these groups share high similarity with each other, the proportion of proteins falling into each group varies and creates unnecessary error, when the total gluten content is calculated. Instead, these proteins could be considered as one group, in which all proteins can be considered harmful for coeliacs. Reference materials and assays for gluten quantification should aim to detect all gluten protein types.

Separate reference materials to each harmful cereal

High complexity of gluten proteins makes it very difficult to develop antibodies that could recognise all protein types. Antibodies bind to different prolamin types with different affinities, which evidently causes inaccuracies in analyses. One way to minimise these inaccuracies would be the development of separate reference materials for products containing gluten from different cereals. There is already a separate reference material for products containing hydrolysed proteins [4].

We suggest that separate gluten reference materials should be produced for each cereal. These reference materials should contain all gluten proteins types of that cereal so that the material would represent the gluten composition of flour as close as possible. It would be important to study the recognition of antibodies towards all gluten protein types, so that the gluten content is measured directly and no multiplication factors are needed. The different reactivity of antibodies with gluten

protein types and the variability between cultivars with respect to the gluten composition have an effect on the results. Therefore, it is important to investigate this variance and determine how strongly the results are affected.

When using separate reference materials for gluten detection for each coeliac-active cereal, we will face a problem with products with an unknown source of contamination. Contamination can come from one or more sources and choosing the right reference material is challenging. Another method is needed to find out the source of contamination and the reference material should be decided on this basis. If several sources are present, the reference material with the lowest reactivity would offer the safest way to avoid false-negative results.

Conclusions

We are suggesting a specific reference material for barley-based products and products with a possible barley contamination. The reference material should contain all gluten protein types so that they would resemble the total gluten content in flour. The reference material should be produced from carefully selected barley cultivars that would represent the entire of range compositions.

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4.5 Development of wheat varieties with reduced contents of coeliac-immunogenic epitopes through conventional and GM strategies

Marinus J.M. (René) Smulders[1], Aurélie Jouanin[1,2], Jan Schaart[1], Richard G.F. Visser[1], James Cockram[2], Fiona Leigh[2], Emma Wallington[2], Lesley A. Boyd[2], Hetty C. van den Broeck[3], Ingrid M. van der Meer[3], Luud J.W.J. Gilissen[3]

[1] *Wageningen UR, Plant Breeding, Wageningen, The Netherlands*
[2] *NIAB, Cambridge, UK*
[3] *Wageningen UR, Bioscience, Wageningen, The Netherlands*

Abstract

Cereals, especially wheat, may cause several food-related diseases, of which gluten intolerance (coeliac disease, CD) is the best defined: specific immunogenic epitopes, nine amino acid-long peptide sequences, have been identified from various gluten proteins. These may activate T cells, causing inflammation of the small intestine and a wide variety of other symptoms. Here, we review several breeding-related strategies aiming at reduction or elimination of such epitopes from wheat, including variety selection, re-synthesis of hexaploids, deletion of specific chromosomal fragments, RNA-interference, mutagenesis and genome editing using CRISPR/Cas9. The related issue of genetic modification (GM) is discussed. These strategies should lead to wheat food products to be used in gluten-free diets for diagnosed CD individuals and/or to strongly reduce the burden from immunogenic gluten to the non-diagnosed CD population.

Introduction

Cereals may cause allergies and intolerances after consumption in some people. The prevalence of IgE-mediated allergy to wheat (and cereals in general) is very low, although wheat contains many immunogenic proteins, however, without clinical relevance [1,2]. Individuals that express the human leukocyte antigen (HLA-) DQ2 and/or DQ8 can become intolerant to gluten proteins from wheat, rye, and barley and may develop coeliac disease (CD), a chronic inflammation of the small intestine. This leads to a variety of symptoms ranging from bowel to skin, bone, nerve, and muscle complaints. The prevalence of CD is 1 - 3% of the general population worldwide. Strict gluten-free consumption is currently the only remedy.

Recently, a new condition, separate from CD called ‘gluten sensitivity’ or ‘wheat sensitivity’ has arisen, even though no medical connection to gluten consumption or particular wheat-specific compounds has been made. Based on the frequency of people with clinical bowel complaints (irritable bowel syndrome, IBS) that seem to improve on a gluten-free diet, the prevalence of this non-celiac wheat sensitivity (NCWS)

might be 6% of the population [2]. As well as individuals diagnosed with CD or IBS, others have actively decided to avoid wheat and gluten, however, often for (self-diagnosed) reasons without clear medical motives, but rather for personal (health-related) preference.

Avoiding consumption of gluten from wheat, rye, and barley is in practice difficult to do. Several strategies have, therefore, been considered to reduce the incidence of cereal-related diseases, with major focus on CD [2]. Strategies include the reduction of the number of CD epitopes in gliadins and glutenins in wheat, or the reduction of the levels of these proteins in wheat grains. Two groups of people that may especially benefit from such gluten-free or reduced-gluten food products are individuals not yet diagnosed with CD (which is the majority of CD sufferers), and the potential patients who may develop CD or IBS after prolonged and abundant wheat and gluten consumption. Here, we review several of these strategies, based on variety selection, re-synthesis of hexaploids, deletion of specific chromosomal fragments, RNA interference, mutagenesis, and genome editing.

In search of low CD-toxic wheat accessions and varieties

Gene-bank wheat collections around the world contain modern and old varieties of hexaploid and tetraploid wheat varieties, as well as landraces (locally adapted/grown wheat populations that pre-date modern breeding approaches), wild emmers (*Triticum dicoccoides*) and wild diploid species that are related to the ancestors of cultivated tetraploid and hexaploid wheat. Gene-banks are a useful source of wheat germplasm for analysing the relationships between individual gluten genes, proteins, or epitope variants and CD immunogenicity, and may contain wheat lines that can be identified as less harmful.

It is not straightforward to make wheat CD-safe, i.e., to develop wheat varieties with fewer or no immunogenic gliadin and glutenin epitopes. Commercial wheat varieties are tetraploid or hexaploid, and gliadins, which carry the most immunogenic epitopes, are encoded by large gene families. However, the epitopes are now reasonably well characterised. In 2012, a list of well-defined CD epitopes was published [3] (twenty-four HLA-DQ2 restricted epitopes and seven HLA-DQ8 restricted epitopes), including six epitopes from α-gliadins and 11 from γ-gliadins, complemented by two ω-gliadin epitopes, three low-molecular-weight (LMW)-glutenin, and two high-molecular-weight (HMW)-glutenin epitopes (only DQ8-restricted). As over 90% of CD patients are HLA-DQ2 positive, the HLA-DQ8 epitopes play a minor role in the development of CD [4]. All epitopes are nine amino acids long, forming the peptide fragment that fits into the DQ2 and DQ8 receptor-grooves, from where the epitopes are presented to the T cells.

At the genomic level, the occurrence of CD epitopes varies between gliadin genes (each locus comprising of multiple gene copies), between homoeologous loci, and between wheat varieties and species. The α-gliadin gene sequences from the D genome contribute most to CD immunogenicity, while those from the B genome contribute the least [5-8]. For γ-gliadins, the highest number of CD epitopes has also been found in

the genes residing on the D genome [9]. Less sequence information is available for ω-gliadins. The involvement of ω-gliadins in CD immunogenicity has been recognized recently from cross-reactivity of T cells with rye and barley epitopes [10].

Screening with monoclonal antibodies (mAbs) revealed limited gliadin genetic diversity in modern wheat varieties [11]. This study also demonstrated an increased presence of Glia-α1 epitopes and a reduced presence of Glia-α3 epitopes in modern wheat varieties compared to landraces and older varieties. CD patients respond more violently to Glia-α1 epitopes than to Glia-α3 [12, 13]. The increase in Glia-α1 may be the result of wheat breeding during the last decades [14]. Some old hexaploid wheat varieties have been identified with relatively low mAb response, as have two lines derived from a heterogeneous tetraploid durum wheat landrace [15]. In addition, analysis of nine landraces of farro wheat (*T. turgidum ssp. dicoccum*) revealed three landraces causing negligible proliferation of T cell lines from CD patients (comparable to the negative control), whereas the other landraces studied showed intermediate to very high responses [16].

Diploid einkorn wheat (*T. monococcum*, A genome) is an ancient wheat species with good nutritional characteristics and bread making quality. Food products made from the variety ‘Monlis’ have been shown to be tolerated by CD patients, revealing similar absence of CD toxicity-related symptoms as found for rice [17]. New combinations of gluten genes can be made by crossing and selection at the diploid level, although this is a tedious procedure. The increasing incidence of CD during recent decades would justify that reduced levels of CD epitopes become a new quality trait in wheat breeding [14].

The utility of screening wheat seed material using epitope-specific mAbs is, however, limited. The specificity of mAbs varies, and is not exactly the same as that of the major histocompatibility complex (MHC) as the mAbs only recognise peptide sequences of four to six amino acids, which is substantially shorter than the nine amino acid-long epitope sequences. Therefore, the identification of wheat genotypes with reduced CD toxicity using mAbs should be regarded as a preliminary screen, with selected lines being rescreened with more accurate methods. In this regard, we have previously examined the CD immunogenic potential of tetraploid durum wheat by deep sequencing of the N-terminal region of α-gliadin transcripts, which includes the repetitive domain with potential CD epitopes, from grains at two developmental stages [18]. A direct approach would be to use quantitative proteomics to identify the gliadins in mature grains, as it determines the exact amino acid sequence and the amount of the proteins produced during grain development. This is difficult, because of the diversity of gluten genes in any single wheat variety, and the relative insensitivity of these proteins to the proteolysis step necessary for analysis. However, methods have now been developed that enable identification and quantification of specific CD epitopes in chymotryptic gluten digests [35].

Reconstituting hexaploid bread wheat: synthetic hexaploids

The α-gliadins encoded on the D genome of bread wheat possess the highest CD-immunogenic potential [6,7], and different varieties are quite similar in this respect. Overall, the genetic variation in the D genome of bread wheat is much lower than that present in the A and B genomes. This suggests that the hybridisation of *T. turgidum* (AB genome) with *Aegilops tauschii* (D genome) to form *T. aestivum* (ABD genome), hexaploid bread wheat, involved only a few *Ae. tauschii* genotypes, resulting in a strong genetic bottleneck. This has been supported by several studies that show high levels of genetic diversity among wild *Ae. tauschii* accessions [19, 20]. In order to introduce new quality characteristics into bread wheat, *T. turgidum spp. durum* has been hybridised with genetically diverse *Ae. tauschii* accessions, followed by chromosome doubling, to produce new, synthetic hexaploid wheat (SHW), amongst others at CIMMYT (Mexico) and at NIAB (UK). We are now screening *Ae. tauschii* accessions that are low in immunogenic gliadins to produce customised SHW that may result in wheat-based products that are safer for CD patients (Schaart et al., in prep).

Deleting entire loci

Wheat deletion lines lack part of chromosomes. A set of hexaploid wheat deletion lines of the variety 'Chinese Spring' (http://www.k-state.edu/wgrc/Germplasm/Deletions/del_index.html) was used to test the effects of individual deletions on the reduction of CD epitopes and on changes in baking-technological properties. A line, in which the short arm of chromosome 6D (6DS) is missing, was analysed. This deletion had eliminated the 6D α-gliadin locus and resulted in strongly decreased mAb responses against Glia-α1 and Glia-α3 epitopes, along with a clearly visible loss of particular protein bands in one-dimensional gel electrophoresis [21]. It also led to a significant change in dough mixing properties and dough rheology, with the dough becoming stiffer and less elastic. Dough quality could be improved or restored with oat prolamins (avenins) [14]. In contrast, the deletion of D genome ω-gliadins, γ-gliadins, and low molecular weight (LMW)-glutenin subunits on chromosome 1DS removed some epitopes, but retained technological properties [21]. As large chromosome deletion lines often grow poorly due to the loss of many genes, deletion lines are useful as model systems, but are not applicable in commercial breeding programs.

RNAi

Since glutenins are most important for baking quality and gliadins contain most of the CD epitopes, scientists have been exploring ways to mutate, delete or silence just the gliadin genes. Two research groups have successfully silenced gliadin expression using RNA interference (RNAi). Becker et al. [22] silenced α-gliadins, eliminating 20 different storage proteins from the grains. Gil-Humanes et al. [23] also effectively down-regulated various gliadins in bread wheat. Using T-cell tests, they found a 10 to 100-fold reduction of DQ2 and DQ8 epitopes in α-gliadins, γ-gliadins, and ω-gliadins. Indeed, total gluten extracts of three transgenic wheat lines failed to elicit T cell

responses [23]. Baking-technologically relevant parameters were also tested, with down-regulation of γ-gliadins resulting in an increase of other gluten proteins, but with little or no effect on dough strength, or gluten and starch properties [24-26].

A different approach used RNAi to suppress the DEMETER (DME) homoeologues in wheat [27]. The DME genes encode a 5-methylcytosine DNA glycosidase, which demethylates the promotor regions of gliadins and LMW glutenins in the wheat endosperm. This demethylation is essential for activation of the genes during endosperm development. Transformed plants showed a high degree of suppression in DME gene transcript abundance, with >75% reduction in the amount of immunogenic prolamins.

Such RNAi wheat lines, as long as their agronomic properties and yields are acceptable, may become candidates for the production of wheat-based products for 'gluten-free' or 'low-in-gluten' diets. However, it should be noted that a line with an RNAi construct stably integrated into the genome is considered a GM plant. The costs of the regulatory process of GM plants worldwide prevents these lines from being developed into commercial varieties. Additionally, growth of GM crops is banned in many countries, providing a further disincentive to commercially produce GM wheat varieties for CD patients.

Mutation breeding

Mutation breeding is based on the induction of random mutations followed by selection of plants carrying mutations in the target gene(s). The mutation-selection process is called Targeting Induced Local Lesions in Genomes (TILLING). It can be performed on populations, in which mutations have been induced chemically, for example by ethyl methane sulfonate (EMS) treatment of seed, by gamma-radiation or by fast neutrons. Products from mutation breeding are exempted from GM regulation in the EU. The question of whether the number, or nature of the mutations is sufficient to have a significant effect on the number of CD epitopes expressed has never been studied in detail. Mutation breeding in a polyploid plant is challenging, as a mutation of a single locus usually does not produce a phenotype. Fewer CD epitopes in the multigene gliadin families would also not produce a different phenotype, so we intend to screen the relevant part of the gliadin genes using next generation sequencing (NGS) approaches [18].

EMS mutation primarily results in G/C to A/T nucleotide transitions. Applying EMS to bread wheat may, therefore, create point mutations in α-gliadin or γ-gliadin genes, some of which would disrupt CD epitopes or result in truncation of the predicted proteins (stop-codon mutation). At UC Davis (USA), a tetraploid wheat EMS population exists for *T. turgidum* cv. 'Kronos' [28]. Recently, the 'Kronos' TILLING population has been sequenced following exome capture [29], allowing mutants to be identified bioinformatically. We intend to screen the exome capture sequence data to identify and prioritise mutations in gliadin genes. The next step will be to confirm the gliadin expression profiles in developing grains of selected mutants or offspring

thereof, at mRNA [18] and/or protein level, followed by combining selected mutant homoeologues into a common genetic background by crossing, and removal of background mutation by back-crossing to the Kronos parent.

Gamma irradiation produces reactive oxygen species (ROS) that cause DNA oxidative damage or DNA single/double strand breaks. When double strand breaks are repaired by Non-Homologous End Joining (NHEJ), which is an error-prone process, this may cause base-pair substitutions, large deletions [30] or even inversions [31]. These can mutate (inactivate) epitopes, remove whole gliadin genes or even multiple genes. We plan to screen an existing population of gamma-irradiated hexaploid wheat of the cultivar 'Paragon' (made at the John Innes Centre, Norwich, UK) for (i) the loss of genes using a quantitative DNA method and (ii) the occurrence of smaller mutations and the expression of the related genes.

Genome editing

CRISPR/Cas9 is technically a relatively easy technique to perform genome editing, i.e., the induction of mutations and deletions at specific, targeted locations within the genome [32]. This requires the introduction of a construct that contains the code for a nuclease, Cas9 and a guide RNA that targets the gene sequence to be altered. The nuclease will generate double strand breaks, which will be repaired by NHEJ in some plants introducing indels and other errors [33]. In wheat, this technology can mutate genes on the three homoeologous chromosomes simultaneously [34]. Since gliadins are grouped at single loci on chromosome groups 1 and 6, double strand breaks simultaneously generated in genes located close to each other within the same locus could lead to deletion of the intervening gene copies. We want to explore this method, and determine whether this system can mutate or remove gliadin genes/epitopes and thus, can contribute to lowering the level of CD epitopes in wheat. Since CRISPR/Cas9 constructs would be transformed into wheat, the approach used here is considered GM under the current European legal framework. However, as the GM CRISPR/Cas9 construct can be removed by segregation in subsequent generations, the offspring may be considered non-GM in other parts of the world, where the product of GM is considered rather than the process used to generate it.

Alternative targeted mutagenesis towards synthetic hexaploids

The strategies described above may also be combined. For instance, TILLING and CRISPR/Cas9 could be implemented in diploid *Ae. tauschii* to remove CD epitopes and/or α-gliadins. Selected offspring, with fewer α-gliadin gene copies or fewer immunogenic epitopes, could then be hybridised with the tetraploid hypoimmunogenic landraces [15, 16] to create a synthetic hexaploid safer for CD patients.

Conclusions

'Reduced CD toxicity' (through a combination of selection of germplasm, mutation breeding, and/or genetic modification) is predicted to become a global breeders' aim in

the near future. Although breeding of bread wheat varieties exclusively through crossing and selection will not result in varieties that are completely coeliac-safe, selection can consider the allelic blocs or clusters and pyramid those carrying the lowest numbers of epitopes. Gene transcript sequencing and proteomics are useful tools for efficient diagnosis of the selected lines regarding quantification of CD toxicity [18,35]. For practical application of such new less- or non-CD-toxic wheat varieties in food products, it will be necessary to develop separate production chains from field to consumer, to avoid contamination with regular high CD-toxic wheat varieties. Such developments may have a strong impact on reducing the incidence of gluten-related disorders in the general population.

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4.6 Potential of wild wheat relatives in coeliac research

Gyöngyvér Gell[1], Krisztina Kovács[1] and Angéla Juhász[1]

[1] Agricultural Institute, CAR, HAS, Martonvásár, Hungary

Introduction

Prolamins constitute the main endosperm storage proteins in wheat, barley, rye, and maize, while globulins are the predominant storage proteins in oat and rice, accounting for about 70 - 80% of the total protein [1]. The classification method of Shewry et al. [2] divides them into three distinct groups, namely the sulphur (S)-rich, S-poor prolamins, and high-molecular-weight (HMW) glutenins. α- and γ-gliadins are the main protein families with respect to coeliac disease (CD) [3,4]. These proteins contain a number of T-cell stimulatory epitopes, mostly in their repetitive regions [5-7]. To date, the only effective treatment of CD is a lifelong gluten-free diet for people suffering from CD.

Wild *Aegilops* species are excellent resources for pre-breeding, serve as valuable sources of biotic and abiotic stress resistance, can serve as excellent sources for enhanced micronutrient content, and may be beneficial in chronic disease prevention. Next to the quality-related issues, several studies are focused on the determination of the toxic potential of wheat genome donors. Most of these studies were focusing on single protein families, such as α- or γ-gliadins [8-10]. Based on several studies [8-12] a large amount of genetic variation exists among diploid wheat species, including the ancestors of bread wheat, in CD-triggering prolamins. It is, therefore, worthwhile to screen for *Aegilops* genotypes with reduced CD activity in a complex way.

Materials and methods

Different *Triticum* and *Aegilops* seeds were derived from the Cereal Gene Bank of the Department of Plant Genetic Resources and Organic Breeding, Martonvásár. Crude protein contents were determined using the Dumas method in triplicates.

In case of total protein extracts, proteins were extracted with SDS buffer followed the protocol Dupont and co-workers 2011 [13], which extracted a greater percentage of protein from wheat flour than other methods and facilitated removal of starch.

After the electrophoresis, the proteins were transferred to ImmobilonP PVDF membrane and IgA based immunoblot were carried out. Patient's sera suffering from CD obtained from Dr. Gábor Veres (1st Department of Pediatrics, Semmelweis University of Medicine) and Dr. Korponay-Szabó Ilma (Heim Pál Children's Hospital, Coeliac Centre). In this study, sera of two CD-negative, of three CD-positive patients on a gluten-free diet and of five CD-positive patients on a normal diet were used for

the estimation of the toxic potential of the total protein extracts of the seeds of the wheat relatives.

Immunodetection by ELISA was made with two different commercially available ELISA kits. The R5 Ridascreen Gliadin (R-Biopharm, Germany) sandwich enzyme immunoassay and AgraQuant Gluten G12 (Romer Labs, Austria) sandwich enzyme assays were used to determine the toxic peptide content of prolamin extracts of the investigated genotypes. The analysis was performed according to the manufacturers´ instructions. For each genotype, calculated gliadin contents determined by the ELISA assays were normalised by the protein contents. Values obtained for the hexaploid cultivar 'Chinese Spring' were used as a measure of toxic R5 and G12 peptide content and obtained gliadin contents were normalised against the value of the control line 'Chinese Spring'. These relative values were used to compare toxic protein contents of the investigated genotypes.

The storage protein sequences of the investigated genotypes were retrieved from the UniProt database (http://www.uniprot.org/) and analysed with the ProPepper software (propepper.net), searching linear epitopes. The toxic peptides, resulting from the bioinformatics analyses, served as a basis of the motif list, which was created with CLC Genomic Workbench 3.6.5. The motif search on the aligned storage proteins resulted in the epitope map of the selected genotypes. To simulate gastrointestinal digestion, proteins were analysed for potential cleavage sites using the Expasy PeptideCutter tool [14]. Endopeptidases such as trypsin, pepsin (pH 1.3), and chymotrypsin were involved simultaneously in the *in silico* digestion analysis, and epitopes that were resistant to the enzymatic cleavage were identified.

Results and discussion

Different *Triticum* and *Aegilops* genotypes were investigated in our study using bioinformatics, proteomics, and immunomics. Several other studies have focused on the allergen behaviour and toxic nature of the storage proteins of different cereal species, like *Aegilops tauschii, Triticum monococcum,* and *Triticum urartu* gliadins [1-5]. Most of these studies were focused on individual protein subclasses, primarily the alcohol-soluble fraction of the seed storage proteins. In our case, all of the major prolamin subclasses were analysed with bioinformatics methods, in this way we can get more information on the toxic potential of storage proteins. ELISA-based gluten tests are widely accepted for the determination of gluten contamination in gluten-free and low gluten food samples. Depending on the antibodies used, the assays are measuring a specific protein type, a single peptide motif or a peptide set specific for a group of gluten proteins. Recent *in silico* studies proved that all the Osborne fractions may contain proteins with toxic epitopes [15-16], however, their number and expressed amount varies. Both the epitope mapping analyses and the ELISA assays results confirm that epitope content of species from the same ploidy level show high variability. While species, related to the A and M genomes (*T. urartu, T. monococcum, Ae. comosa*), have the lowest R5 and G12 epitope contents, the U and B genomes in

diploid species show higher R5 epitope contents. Even though the highest number of epitopes was found in the cysteine mutant ω-gliadins and some γ-gliadins of *T. aestivum*, these sequence variations were less frequent.

T. monococcum, T.urartu, and Ae.comosa were identified to have significantly lower gliadin content as per ELISA methods using antibodies against confirmed toxic epitopes. (Fig. 1). Because of the contrary results of the ELISA assay, a broad range of bioinformatics analyses and epitope mapping were made based on the publicly available prolamin sequences. High diversity in the toxic epitope content and distribution between the investigated species was found. γ-Gliadins showed the highest pattern diversity, with 11 different epitope patterns in *T. aestivum*, 8 in *Ae. tauschii* and 7 in *T. monococcum*. More detailed information about the ELISA assays and the bioinformatics analyses can be found in Gell et al. [17], recently published.

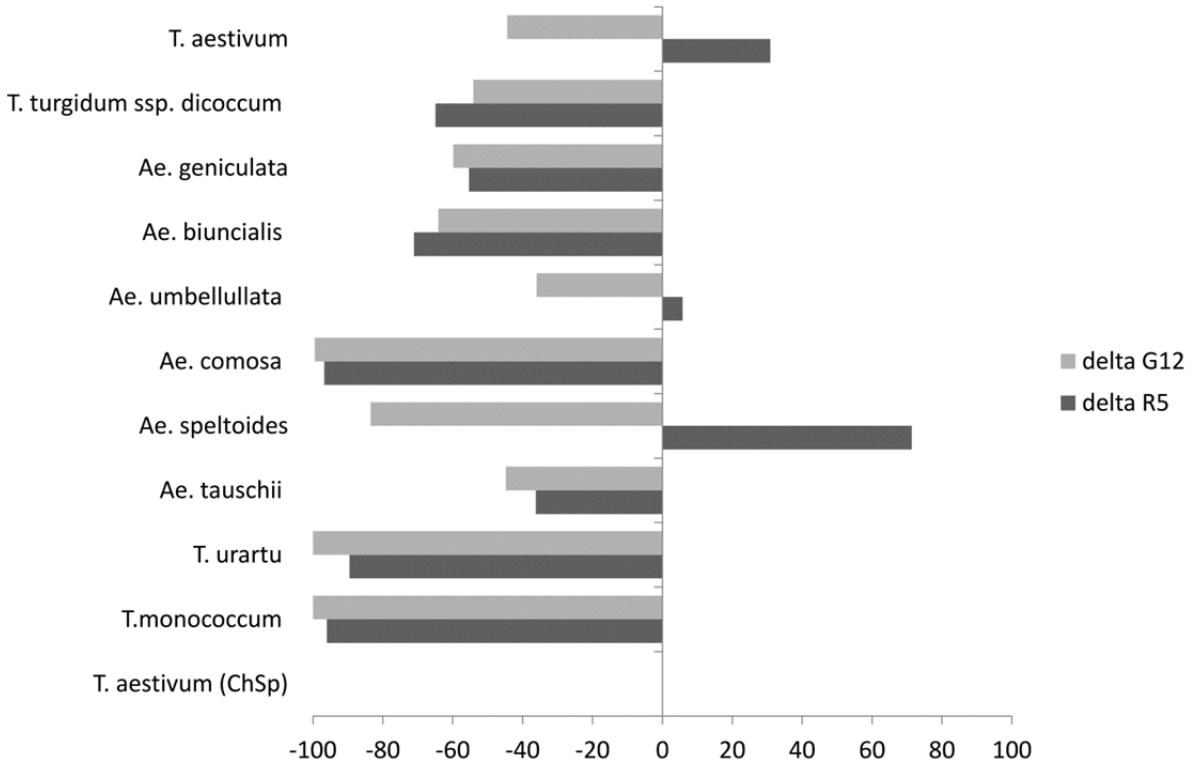

Figure 1. *Comparison of the toxic epitope content of different genotypes. Relative values were obtained by normalising the obtained gliadin contents from G12 and R5 ELISAs against the value of cv. 'Chinese spring' (= 0)*

The results of the immunoblots with human sera derived from CD patients strengthened the results of the bioinformatics analyses. In all of the investigated genotypes, strong CD-associated proteins with all of the individual sera were found (Fig. 2). There were differences between the blot pattern of *Triticum* and *Aegilops* species. In contrast to the ELISA assay results, *Ae. urartu* was the one of the most immunoreactive species. The ELISA assays, performed in this study, were used to characterise selected species with different genomic backgrounds. However, the high

variation in epitope frequencies of sequences within the same species, obtained by bioinformatics analyses, highlighted the importance of analysis at the genotypic or accession level. Identification of new prolamin alleles of various wheat species and wild relatives is of great importance in order to find germplasm suitable to grow in different environmental conditions and also to find breeding sources suitable for special end-use requirements of consumers with gluten sensitivity. The joint sequential and immunoanalytical study of cereals and wild wheat relatives has both analytical and clinical implications. First of all, our results provide further confirmation of the considerable variability in expression of CD-specific peptides of cereals with different genetic backgrounds. Besides, the results show further variability depending on the antibody used. These results highlighted the fact that the reliability of immmunoanalytical results may be dependent on the variety of the sample and the method used. Today, the ELISA methodology is used to determine whether food products, intended for CD patients, contain gluten below the regulated threshold levels of 20 and 100 mg/kg gluten for gluten-free and low gluten foods, respectively. Thus, based on our results, it would be important to reveal, how this variability affects the analytical results in this low mg/kg range. This is even more important for the species that are showing lower ELISA gliadin values, or none at all, as in the case of *T. monococcum*.

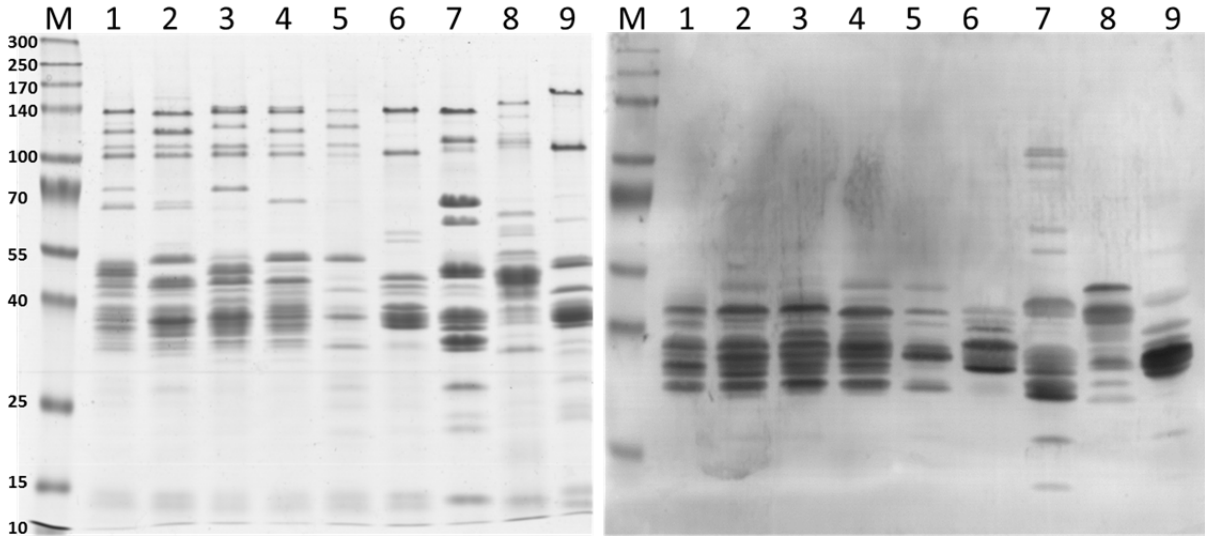

Figure 2. *SDS-PAGE of the investigated genotypes and immunoblot with IgA of selected prolamin extracts of genotypes with CD patients´ sera. 1. T. aestivum - Chinese Spring; 2. T. aestivum – Bánkúti; 3. T. aestivum – Spelt1; 4. T. aestivum – Spelt2; 5. T. turgidum ssp. dicoccum – Fehér tönke; 6. Aegilops tauschii - MVGB-589; 7. T. urartu - MVGB-115; 8. Aegilops speltoides – MVGB 1321; 9. Aegilops speltoides – MVGB 1147: M. Marker proteins*

Conclusions

In this study, G12 and R5 commercially available ELISA test kits were used. There were strong differences between the results of these kits and between the different species, too. Based on our epitope mapping analyses there is a significant number of sequences that do not contain the toxic peptides used for monoclonal antibody production (R5 – QQPFP; G12 - QPQLPY). Additionally, the R5 peptide was also present in the m-type LMW glutenins that were not represented in the gliadin standard used for calibration of the R5 mAb-specific ELISA assay. Hence, the tests using R5 and G12 mAbs are underestimating the gluten content of the analyzed samples. The level of this underestimation strongly depends on the expressed amounts of these peptides, which is a factor influenced by the types and number of unique proteins and their expression level. Based on the results of the bioinformatics and immunomics analyses, some diploid and tetraploid species were identified that have significantly lower gliadin content as per ELISA methods using antibodies against confirmed toxic epitopes. Independently from the ploidy level, none of them is suitable for patients suffering from CD due to the high toxic epitope content and strong immunoreactive behaviour.

Due to the evolving time of ancestral grasses and the monophyletic origin of the prolamin gene family, it is hard to believe to find any safely edible cereals in the *Pooideae* subfamily for patients suffering from CD. However, a reduced toxic peptide content of diploid and tetraploid species and genotypes would already delay the induction of the disease in young children.

In summary, our results can only serve as a pilot study to investigate the epitope content of wheat-related species. Some of the analysed accessions seem to contain remarkably lower levels of gliadin compared to bread wheat. However, these levels are still higher than the 20 mg/kg gluten level, which is required for the gluten-free declaration of a product. Still, these species can be ideal candidates for breeding new varieties with a lower toxic epitopes content, this way providing new dietary supplements for CD patients. However, before that point could be reached, their potentially lower toxicity must be confirmed by *in vitro* or *in vivo* studies. Additionally, variability in allergen content and effect of environmental changes on allergen protein content of the different species should be determined. Also, effects of food processing and subsequent gastrointestinal digestion on the toxic epitope content, on their biological reactivity, and on detectability are important.

Acknowledgement

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4.7 ProPepper - A database for identification of peptide and epitope composition of cereal grain proteins

Angéla Juhász[1], *Réka Haraszi*[2], *Csaba Maulis*[3]

[1] *Applied Genomics Department, CAR, HAS, Martonvásár, Hungary*
[2] *Campden BRI, Chipping Campden, UK*
[3] *Enterpreneur developer, Budapest, Hungary*

Introduction

Gluten proteins, especially their prolamin content, are responsible for severe health problems, like coeliac disease (CD) and partially for wheat allergy (WA). Prolamin proteins, like high- and low-molecular-weight glutenins, α-, γ-, and ω- gliadins share high degree of sequence similarity, based on which the precise identification of unique alleles is really challenging. The detection of gluten proteins is extremely important not only due to their direct effect on end-use quality but also for food safety reasons. Variability of grain composition of cereal genotypes leads to methodological problems in food allergen research and genotype selection in breeding for quality. Due to the high sequence homology in seed protein sequences of cereal species, the exact identification of proteins that are involved in cereal allergy and gluten intolerance, their genotypic frequency, variability and stability are not known due to limitations in the used methodology. High-resolution methods such as mass spectrometry (MS) require accurate molecular quantitative relationships in order to relate the detection of peptide mass to their protein sources. Quantitative relationships between prolamin peptide biomarkers and the final gluten/prolamin content are difficult to establish due to genotypic and environmental variability. Additionally, their special amino acid composition, the significantly higher portion of proline and glutamine residues, has led to poor digestibility by trypsin, one of the most commonly used enzyme in MS-based proteomics. Many of these peptides, identified from different prolamin types, were proven to be immunoreactive in patients suffering in CD or wheat allergies. In order to assist peptide biomarker search, a database (ProPepper™, popepper.net) was developed that contains members of the prolamin superfamily proteins identified from *Poaceae* species, peptides obtained with multi-enzyme *in silico* digestion as well as linear epitopes responsible for wheat-related food disorders.

Materials and methods

The ProPepper database contains three sets of databases (proteins, peptides, and epitopes) that are cross-connected. 2072 complete protein sequences, all members of the prolamin superfamily, isolated from different *Poaceae,* were retrieved from the Uniprot database. Sequences were aligned for a precise identification of the protein types. Misannotations were corrected and information related to chromosomal

location, origin and allele were added using Genbank information and published results. Chromosomal origin of proteins, retrieved from *Triticum* species, were blasted against the wheat genome survey sequence database.

An application, the Protein Digestion Multi Query, was also developed for *in silico* digestion of the protein dataset. Enzymes, like trypsin, pepsin (pH 1.3), and chymotrypsin (low specificity), were applied in different combinations allowing simultaneous application of the enzymes. By the *in silico* digestion of the protein dataset currently 25303 unique peptide sequences provide the peptide database in Propepper as a result of 408 655 unique digestion events with only these three enzymes.

The third component is the epitope database that contains 43 CD-related core epitopes and the integration of 414 gluten-related T-cell epitopes is in progress.

These three datasets are related by unique IDs in order to analyse prolamin characteristics at species and genotype level; to identify peptides resistant to gastrointestinal enzymes; to identify peptides suitable for MS-based marker analyses; and to identify epitopes at unique protein or peptide level. Some examples of the use of epitope dataset and peptide dataset are presented below.

Results and discussion

Epitope module

The ProPepper database and analysis platform is suitable to provide answers related to the epitope content of unique genotypes, to determine differences in the toxicity of the different species, as well as to determine prevalence of unique epitopes or peptides in the different prolamin protein families. In order to analyse the toxicity of unique genotypes, either protein sequences isolated from the asked genotype or the allelic composition of Glu-1, Glu-3, Gli-1, and Gli-2 loci should be known. For example, genotypes with partial or complete allelic identity to cv. ‘Chinese Spring’ show the epitope characteristics of ‘Chinese Spring’.

Two species, *Triticum aestivum* and *Aegilops tauschii* both with a significant amount of protein sequence data, were used to compare the core epitope content in *Poaceae* species. Protein sequences of the different prolamin types were analysed separately. Prevalence of sequences without core epitopes was determined as well as epitope density measured. Although α-gliadins are considered to be the primary cause of gluten toxicity, our results have confirmed that epitopes are common in all the prolamin protein types (Fig. 1). However, while about 90% of the γ-gliadins in *T. aestivum* contain at least one core epitope, the number of protein sequences without core epitopes is higher in *Ae. tauschii*. On the other hand, in *T. aestivum* only about 50% of high-molecular-weight glutenin subunits (HMW-GS) contain core epitopes. These toxic peptides are more frequent in the *Ae. Tauschii* HMW-GS. When the count of unique core epitopes contains all three gliadins groups, generally a larger number of

core epitopes was identified in *Ae. tauschii* sequences compared to the bread wheat sequences. In case of low-molecular-weight glutenin subunits (LMW-GS) the prevalence of epitopes is significantly higher in bread wheat sequences than in *Ae. tauschii* sequences. These differences can serve as an indicator to the different toxicity of the A, B, and D genomes but can also be related to the underutilised allele sources present in *Ae. tauschii*.

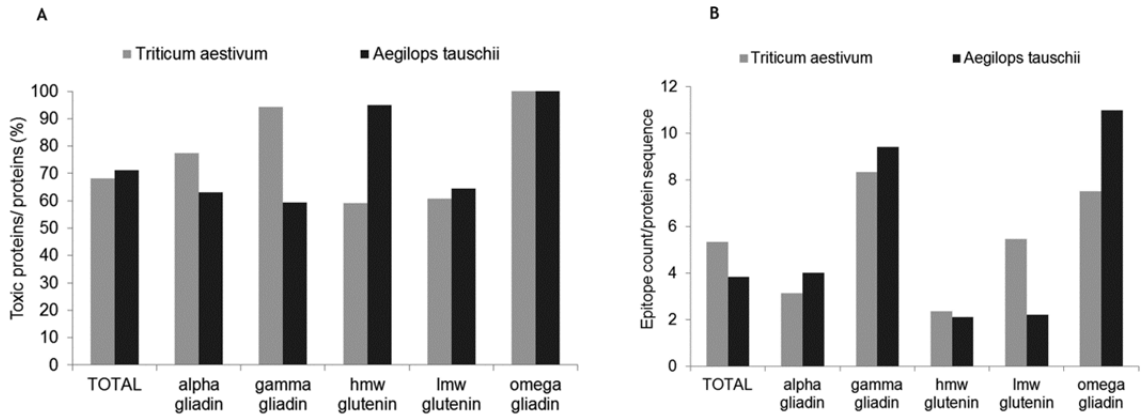

Figure 1. *Differences in the prevalence and frequency of known core epitopes in prolamin protein types of bread wheat (T. aestivum) and Ae. tauschii. A- Ratio of protein sequences with core epitopes in the different prolamin types; B - Number of epitopes present in a sequence*

Additional analysis was performed to determine the prevalence and frequency of the QQPFP peptide monoclonal antibody (mAb) R5 in the different prolamin types and cereal species. Six *Aegilops* species with different genome composition and four *Triticum* species were used in the analysis. Prolamin types were analysed separately. Based on our results, the R5 monoclonal antibody is specific for four different prolamin types: LMW-GS, α-, γ-, and ω-gliadins. The presence of the QQPFP peptide was identified in 25% of LMW-GS, 80% of α-gliadins, and nearly all γ- and ω-gliadins (Tab. 1). This may result in an underestimation of the gliadin content, when the R5 mAb is used. ELISA kits working with R5 mAb were developed to be specific on gliadins. However, our bioinformatics analyses have confirmed that the 25% of LMW-GS that contain this peptide belong to the m-type subunits, which are common in all wheat genotypes and their expressed amount is responsible for the majority of the LMW-GS proteins. These results strongly confirm that, although the number and distribution of CD-related core epitopes show a significant variability in the different prolamin types and in the different species, their presence is common in all prolamin types. Therefore, gliadin specific ELISAs working with mAbs need markers that are present in all the gliadin sequences but are absent in glutenin sequences.

Table 1. *Prevalence (%) of the R5 peptide (QQPFP) in the different prolamin types of Aegilops and Triticum species.*

R5 (QQPFP)	HMW-GS	LMW-GS	α-Gliadin	γ-Gliadin	ω-Gliadin
Ae. comosa	0	22.2	41.7	100	-
Ae. umbellulata	0	60	83.3	100	-
Ae. tauschii	-	24.1	95.5	100	100
Ae. speltoides	0	25	71.4	100	-
T. monococcum	0	0	86.9	100	-
Ae. biuncialis	-	-	-	-	-
Ae. geniculata	-	0	-	-	-
T. turgidum	0	0	100	92.3	-
T. aestivum	0	29.5	80.5	98.6	83.3
T. urartu	0	0	83.3	100	-

MS module

The Propepper database can be a useful tool in the design and evaluation of MS-based proteomics workflow, when analysing cereals for prolamin proteins and peptides. The application of Propepper is not entirely replacing the experimental phase of such workflow but can be useful, when expert knowledge of prolamins is not available. This concerns the design of a digestion method prior to MS analysis, targeted multiple reaction monitoring (MRM) method developments or the data processing of a mass spectrum and the identified masses. The *in silico* digested peptide content of the Propepper helps to select target peptides and the best enzymes, when optimising a digestion method, as well as to identify the specificity of the identified peptides by a search engine or data processing algorithm. Since the peptides in Propepper were blasted for specificity, the initial workflow for a targeted MRM approach in order to establish a candidate peptide marker list can be achieved by Propepper.

In a discovery work, the list of identified masses from a mass spectrum needs to be related to a peptide sequence and a protein source. This information is in a database that is selected by the user and contains protein sequences most frequently in fasta format. The quality of this database is very important. The database size and the specificity of the data entries are all influencing the final scores during the matching process. This score is usually optimised for trypsin digestions, so in case of other enzyme(s) used; the meaning of this score is limited. Propepper offers the opportunity to relate peptide masses to a genotype or species via the identification of individual peptides and its protein source even at allelic level (Fig. 2).

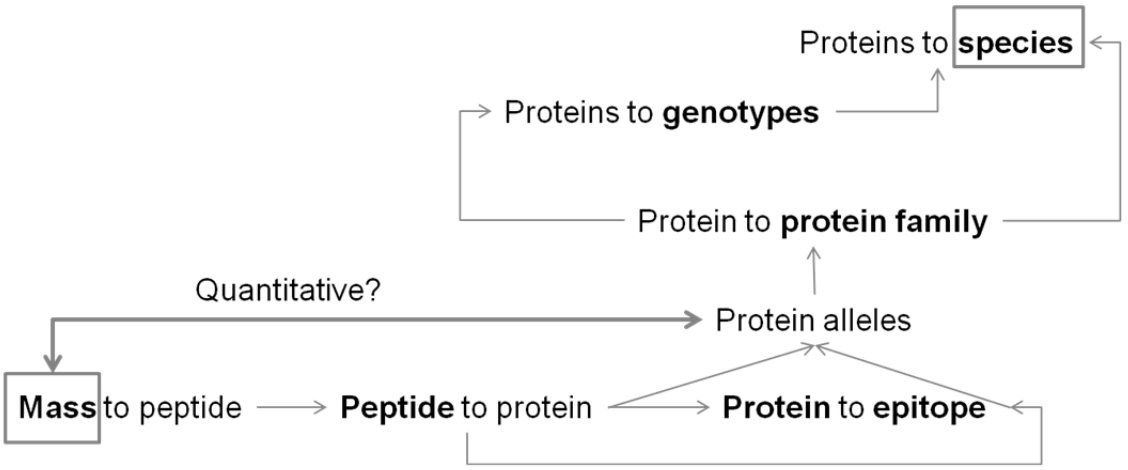

Figure 2. *Relationships among data that can be obtained from the Propepper database*

Monoisotopic mass information entered to Propepper will show all related connections to potential peptide sequences, digestion events, proteins, and genotypes. By entering the mass in the relevant search field of the Propepper database, the hits appear real time. Then, by the selection of the peptide, the relevant sequence and other annotated information will be available. Fig. 3 shows the first step of such mass search and a summary of the results obtained from Propepper.

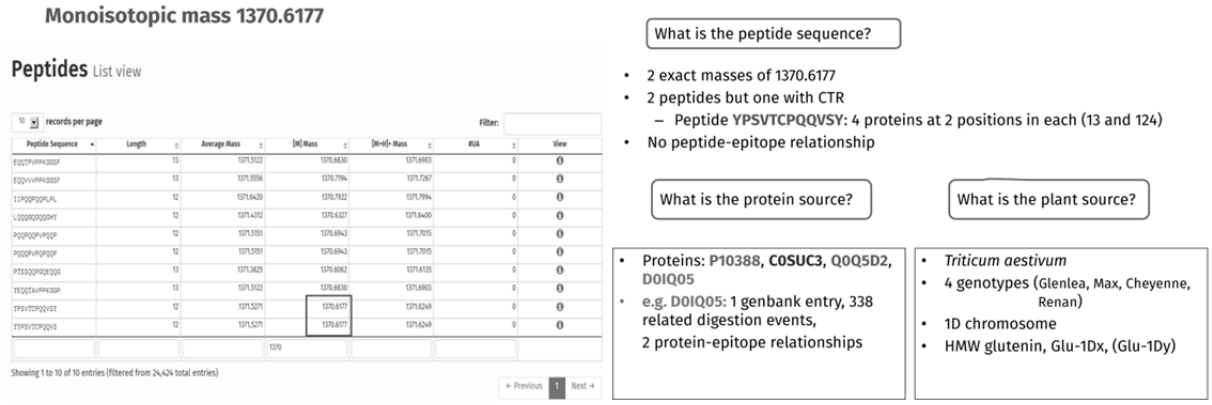

Figure 3*. Case study of how to use peptide mass entries in the Propepper database to establish its relevance to peptides, proteins, genotypes, and species*

Conclusions

Propepper is a regularly maintained, manually curated expert database of prolamin peptides, epitopes, and proteins that combines the knowledge of 5+ well-known and acknowledged databases. It provides a great tool for proteomics, MS, and clinical experts that are dealing with prolamins, this unique and complex protein family.

Currently linear T-cell-related epitopes are included in the database, mostly connected with CD-specific symptoms. Incorporation of linear B-cell-related epitopes will also

enhance our understanding about gluten peptides involved in cereal related food disorders.

The quantitative relationships of peptides and proteins are still missing and would provide valuable information to monitor quantitative changes in peptides of genotypes and species using quantitative mass spectrometry.

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4.8 Optimisation of proteomic protocols for the analysis of gluten

M. Carmen Mena[1], Manuel Lombardía[1], Sergio Ciordia[1], Juan P. Albar[1†], Alberto Paradela[1]

[1] Proteomics Facility, Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas, Madrid, Spain
[†]Deceased

Introduction

The accepted technique for the analysis of gluten in food samples is the R5 ELISA assay [1,2], but immunological techniques present the common disadvantages associated to antibody specificity and sensitivity. Applying proteomics to this field is of great interest to complement other techniques and to achieve the maximum accuracy in the analysis of gluten in foods.

There are several difficulties associated to the use of proteomics in the analysis of prolamins and glutelins, including the limited number of sequences of wheat, barley, and rye that are available in public databases. Appropriate sample preparation procedures are also essential for a correct analysis of samples.

Prolamins are a complex mixture of proteins that are more difficult to analyse than other proteins by standard mass spectrometry (MS) methods of analysis [3]. For example, the high number of different gluten proteins is a rate-limiting step in proteomics workflows. On the other hand, enzymatic digestion of proteins by endoproteases is a key step in protein identification by MS techniques. Trypsin, the enzyme mainly used for this purpose, cleaves C-terminal to lysine and arginine, but these cleavage points are not appropriate for generating peptides of an optimum length in the case of gluten proteins due to their low percentage of these amino acids [4] . For that reason, the main objective of this study is to optimise different protocols using proteases with different specificities to obtain a different set of gluten fragments in order to achieve a complete characterisation of gluten proteins.

Materials and methods

We used the Working Group on Prolamin Analysis and Toxicity (PWG) gliadin standard (2 mg/mL in 60% ethanol/water (v/v)). This standard is obtained from a mixture of 28 wheat cultivars representative of the European wheat-producing countries [5]. Flour from seeds of wheat (*Triticum aestivum*) was also used. To obtain a fine homogenised powder, seeds were thoroughly ground with an IKA A11 analytical mill (IKA®, Staufen, Germany).

Proteins from wheat and PWG-gliadin standard were extracted, precipitated, and total protein concentration was determined. Some of gluten proteins are aggregated in their native form. For that reason and in order to get the maximum recovery of proteins, we performed a denaturation step with guanidine. Tris(2-carboxyethyl)phosphine) (TCEP) was used for reduction of disulphide bonds. Afterwards, gluten proteins were extracted with 60% ethanol/water (v/v). Before digestion and in order to use the suitable amount of enzyme to digest the samples it is essential to quantify the total amount of proteins. For this purpose, we used the Pierce 660 nm Protein Assay, as this method is compatible with most of the reagents used for protein extraction, including detergents and reducing agents. Furthermore, we used iodoacetamide for alkylation of sulphhydryl groups to prevent reformation of disulphide bonds.

In this study, we digested the samples with different proteases: trypsin (proteomics grade, from porcine pancreas Sigma, T6567) that cleaves C-terminal to lysine (K) and arginine (R); chymotrypsin (sequencing grade, from bovine pancreas, Roche), which cleaves C-terminal to large hydrophobic residues (tyrosine (Y), tryptophan (W), phenylalanine (F)) and leucine (L); and pepsin (from porcine gastric mucosa, Sigma, P6887), which cleaves at the amino side of phenylalanine (F) and leucine (L). Trypsin and chymotrypsin digestions were performed using a 1:10 enzyme:protein ratio at 37 °C overnight (12 - 16 h) on a shaker. Pepsin digestion was performed using a 1:20 enzyme:protein ratio at 37 °C for 6 h on a shaker. In addition, we performed a combined digestion with two enzymes using first trypsin at a 1:10 enzyme:protein ratio at 37 °C overnight (12 - 16 h) on a shaker followed by chymotrypsin at a 1:20 enzyme:protein ratio at 37 °C for 2.5 h on a shaker. Finally, a combined triple digestion was performed, combining trypsin and chymotrypsin as detailed above, followed by a final digestion step using pepsin at a 1:40 enzyme:protein ratio at 37 °C for 1 h on a shaker.

The resulting peptide mixtures were desalted and cleaned tryptic peptides from in-solution digestion were evaporated to dryness. An aliquot of each digested sample was subjected to triplicate nano LC ESI-MS/MS analysis using a nano liquid chromatography system (Eksigent Technologies nanoLC Ultra 1D plus, AB SCIEX, Foster City, CA) coupled to a high speed Triple TOF 5600 Q-TOF mass spectrometer (AB SCIEX , Foster City, CA) with a duo spray ionisation source. The C_{18} nano-column was a silica-based reversed-phase column and for this analysis, a trap column was included switched on-line with the nano-column. The flow rate and gradient elution were optimised for these samples. MS and MS/MS spectra, obtained for individual samples in unprocessed form, were converted to Mascot generic form files using PeakView software v.1.1, followed by a search against the *Triticum* National Center for Biotechnology Information (NCBI) database (2014_July - 43799 protein sequences), using a licensed version of Mascot v.2.5.0. (www.matrixscience.com; Matrix Science, London, UK) as search engine.

Results and discussion

The extraction of gluten in wheat, barley, and rye standards is more affordable than in processed foods. 60% ethanol/water is particularly useful to extract prolamins, which are the alcohol-soluble proteins of gluten (wheat gliadins, barley hordeins, and rye secalins). In addition, under reducing conditions solutions together with the prolamins, the main extractions are the polymeric hight-molecular-weight (HMW-) and low-molecular-weight (LMW-) glutenin fractions and their equivalents in barley and rye [6]. In this study, we analysed all the fractions.

We have compared the number of gluten proteins identified using different proteases and their combinations. Trypsin and chymotrypsin digestions were performed according to proteomics literature. Pepsin digestion required a shorter incubation time and a lower enzyme: substrate ratio than digestions with trypsin and chymotrypsin to avoid a complete digestion of peptides, but even under these conditions, we observed that proteins were extensively digested, resulting in very short fragments amenable for identification of most of the proteins (Tab. 1 and Tab. 2). Therefore, pepsin digestion, using the described conditions, is not the method of choice for gluten identification by MS techniques.

As expected, wheat contains a greater number of different total proteins (Tab. 1) than those found in the PWG-gliadin reference (Tab. 2). Nevertheless, we observed in the PWG standard a significant number of glutenins and other proteins indicating that even though the standard is processed in order to obtain only the gliadin fractions of gluten, there are other types of proteins that remain in the standard (Tab. 2).

Table 1. *Total number of proteins and number of gluten proteins (glutenins or gliadins) identified in wheat, using different enzymes for digestion of proteins.*

Digestion[a]	Total no. of proteins	No. of gluten proteins			% Gluten/Total
		Gliadins	Glutenins	Gluten	
T	349	35	28	63	18,1
C	164	53	19	72	43,9
P	37	8	6	14	37,8
T+C	226	63	30	93	41,2
T+C+P	244	65	28	93	38,1

[a] T= Trypsin, C= Chymotrypsin, P= Pepsin

The percentage of gluten proteins identified in the wheat extracts changes according to the enzyme used. According to our results, the use of chymotrypsin gives more information than trypsin, and there is a higher number of identified proteins with any of the enzyme combinations (Tab. 1). Other studies have used chymotrypsin for identifying gluten proteins and provided interesting results [7].

Fig. 1 represents the number of common and unique proteins found in wheat extracts digested with different enzymes. It is important to notice that each method generates complementary information and therefore, the use of more than one method in parallel is useful for a more exhaustive analysis of the sample. Quite unexpectedly, low overlap between the set of proteins identified using either trypsin or chymotrypsin was found, probably due to the very different residue specificity (Fig. 1).

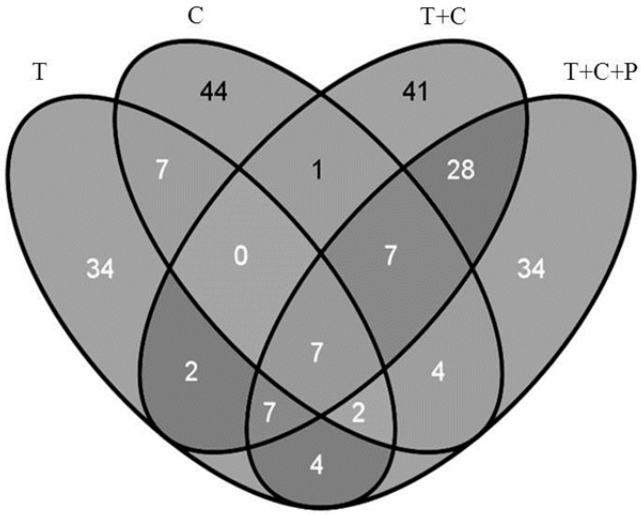

Figure 1. *Comparative analysis of the numbers of unique and common different gluten proteins identified in wheat using different enzymes for digestion of proteins. T= Trypsin, C= Chymotrypsin, P= Pepsin*

The percentage of gluten proteins identified in the PWG-gliadin reference was higher than in the wheat extract using any of the enzymes. Approximately 80% of the proteins identified using chymotrypsin, alone or in combination with other proteases, correspond to gluten proteins. However, our results indicate that there are other proteins present in this standard.

Table 2. *Total number of proteins and number of gluten proteins (glutenins or gliadins) identified in PWG-gliadin, using different proteolytic experimental strategies.*

Digestion[a]	Total no of proteins	No. of gluten proteins			% Gluten/Total
		Gliadins	Glutenins	Gluten	
T	301	82	54	136	45,2
C	213	115	53	168	78,9
P	14	6	2	8	57,1
T+C	184	98	50	148	80,4
T+C+P	181	99	47	146	80,7

Digestion[a]: T= Trypsin, C= Chymotrypsin, P= Pepsin

As described in Fig. 1 for wheat extracts, results summarized in Fig. 2 show that the use of different proteases produces complementary information.

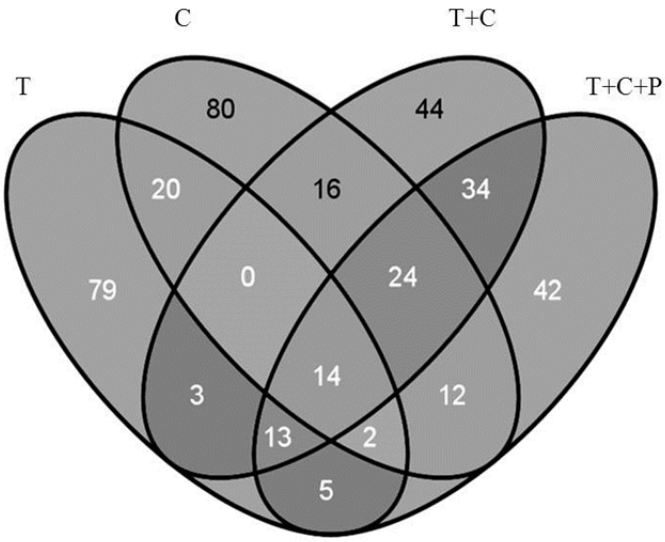

Figure 2. *Comparative analysis of the numbers of unique and common different gluten proteins identified in PWG-gliadin using different enzymes for digestion of proteins. T= Trypsin, C= Chymotrypsin, P= Pepsin*

As shown in Tab. 1 and 2, the use of proteases other than trypsin increases the number of identified proteins including those related with toxicity for coeliacs such as α/β and ω-gliadins and LMW-glutenins. According to our results, the most suitable method in order to get the maximum coverage of proteins is the use of a combination of more than one digestion protocol.

Conclusions

Even though digestion with chymotrypsin yields a higher number of identified gluten proteins, trypsin also provides interesting information. Thus, there are unique proteins identified with every type of enzyme used for digestion. Apart from pepsin, the other proteases studied (trypsin, chymotrypsin, and their combinations) are useful for proteomic analysis of gluten proteins. The combination of trypsin and chymotrypsin is the digestion method of choice to identify a high number of gluten proteins in wheat by MS techniques. In addition, there are other proteins different from gliadins in the PWG-gliadin reference.

In conclusion, in order to achieve the maximum information of the sample, it should be useful to use more than one digestion method in parallel. Depending on the protease used, the sequence coverage obtained is different.

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4.9 Oxidation of 33-mer peptide

Xin Huang, Päivi Kanerva, Hannu Salovaara, Tuula Sontag-Strohm

University of Helsinki, Helsinki, Finland

Introduction

Prolamins have relatively high resistance to breakdown by gastric and pancreatic enzymes. Shan et al. [1] digested α-gliadin, using gastric and pancreatic enzymes, and analysed the resulting peptides with liquid chromatography and mass spectrometry. A peptide of 33 amino acids (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF, α2-gliadin, residue 56-88) remained intact, while most other fragments were cleaved into small peptides. This peptide, the 33-mer, has three epitopes, PFPQPQLPY, PYPQPQLPY (two copies), PQPQLPYPQ (three copies) that stimulate coeliac disease- (CD) active T cells. It is anticipated that the breakdown of 33-mer into small peptides can reduce its toxicity.

A proline-endopeptidase from *Flavobacterium Meningosepticum* was reported to be a potent candidate for a peptidase therapy for CD because of its post-proline specificity [1]. In addition, endogenous proteases isolated from germinated seeds [2], were able to degrade peptide PQPQLPYPQPQLPY, which is the repetitive part of 33-mer. However, oxidation by reactive oxygen species (ROS) is another mechanism for protein degradation. Free radical-mediated oxidation often occurs in food and biological systems. Then, hydroxyl radical (·OH), a very strong ROS, can be formed through a Fenton-type reaction, where hydrogen peroxide reacts with a reduced transition metal (e.g. Fe^{2+}, Cu^{+}) [3]. Protein oxidation can cause fragmentation of polypeptide chains, generation of protein-protein cross-links, and oxidation of amino acid side chains [4].

Uchida et al. [5-6] and Kato et al. [7] studied metal-catalysed oxidation of proline-containing peptides and collagen. It was reported that oxidation of proline-containing peptides in a Cu^{2+}/H_2O_2 system not only caused oxidative modification of proline residues, but also triggered oxidative cleavage of proline peptide bonds, and the generation of 2-pyrrolidone compounds. Collagen has a triple helical structure and a repeated sequence of glycine-X-Y, where X and Y are often proline and hydroxyproline. Wheat and barley prolamins also contain repeating sequences with proline residues, and this suggests that breakdown of repeating sequences could occur in prolamins as well as in collagen.

The aim of our study was to examine the non-enzymatic oxidation of the model 33-mer peptide under different oxidation conditions. Our hypothesis was that the 33-mer can be degraded by non-enzymatic oxidation and that oxidative modification of CD-active epitopes can reduce the toxicity of the 33-mer peptide.

Materials and methods

The reaction solution contained 2 mg/mL 33-mer peptide and 0.05 mmol/L $CuSO_4$ or $FeSO_4$ in milli-Q water (pH 4.5). The reaction was initiated by adding freshly prepared 50 mmol/L hydrogen peroxide or ascorbic acid. Incubation temperature was 37 °C. The reaction was terminated by adding 1 mmol/L EDTA.

The reaction mixture was analysed by size-exclusion chromatography (SEC). SEC columns Superdex Peptide 10/300 GL and Superdex 200 10/300 (GE Healthcare Biosciences AB, Uppsala, Sweden) were combined in a model 1200 HPLC system. A competitive ELISA was used for the 33-mer immunological activity test (Ridascreen Gliadin Competitive R7021, R-Biopharm, Darmstadt, Germany). Dityrosine cross-links were characterised by luminescence spectrometer. The measurement was upon fluorescence excitation at 284 nm, and the intensity was recorded at emission 410 nm [8]. Protein carbonyl group was measured with the DNPH method described by Reznick and Packer [9].

Results and discussion

Size distribution changes of the 33-mer peptide after metal-catalysed oxidation were observed by SEC analysis. The most extensive decrease in size of the 33-mer was found, when oxidative treatment was given using Fe^{2+}/H_2O_2 (Fig. 1, oxidation of Cu^{2+}/H_2O_2, Fe^{2+}/ascorbic acid, and Cu^{2+}/ascorbic acid not shown). A broad peak was observed in Fe^{2+}/ H_2O_2 oxidation of 33-mer peptide, indicating modification of 33-mer structure. Some small fragments and aggregates were formed. Fe^{2+}/H_2O_2 oxidation of the 33-mer was chosen for immunological activity test. The R5 antibody mainly recognises the epitope QQPFP, but also recognises the epitopes LQPFP and QLPYP. After 4 h of incubation, the immunological activity against R5 antibody decreased to 40% of its original value, and remained 18% after 24 h of oxidation (Fig. 2). Production of cross-links through dityrosine linkages was observed in Fe^{2+}/H_2O_2 oxidation of 33-mer (Fig. 3). With the initiation of the tyrosine radical by hydroxyl radicales, dityrosine is likely to be formed, resulting in intra- and intermolecular cross-links. Hydroxyl radicals can also attack aromatic side chain of phenylalanine, adding a hydroxyl group to the ring structure. During 4 h of oxidation, carbonyl groups were readily formed through Fe^{2+}/H_2O_2 oxidation of the 33-mer. Carbonyl groups formation from protein oxidation mainly originates from the amino acids proline, lysine, arginine, and threonine. Side-chain modification of proline residues by hydroxyl radicals generates glutamate-5-semialdehyde. Considering the amino acid composition of the 33-mer, the high content of proline residues mostly contributed to the formation of carbonyl groups.

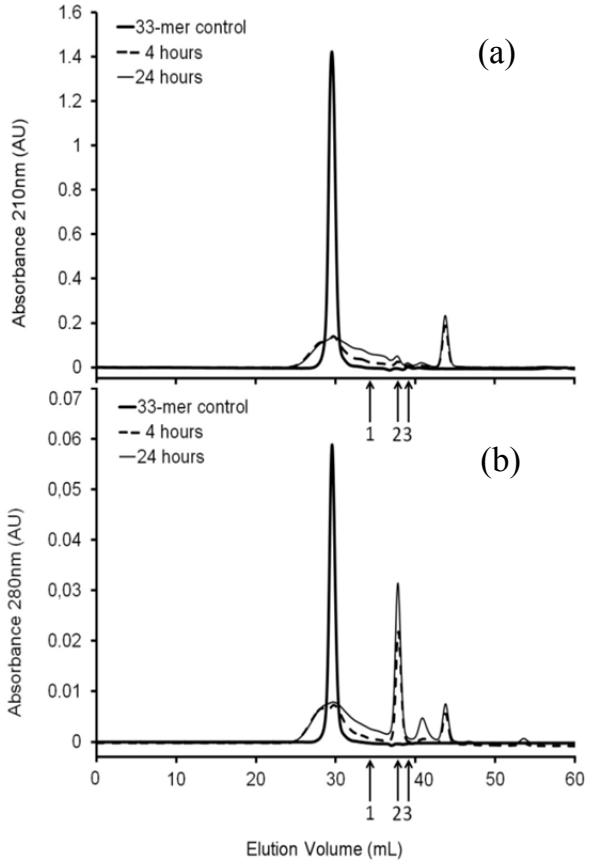

Figure 1. *Size-exclusion chromatography of Fe^{2+}/H_2O_2 oxidation of 33-mer peptide. (a) Absorbance 210 nm; (b) absorbance 280 nm. Arrows 1, 2 and 3 indicate the elution volume of molecular markers 1085.2, 356.4 and 146.2 g/mol*

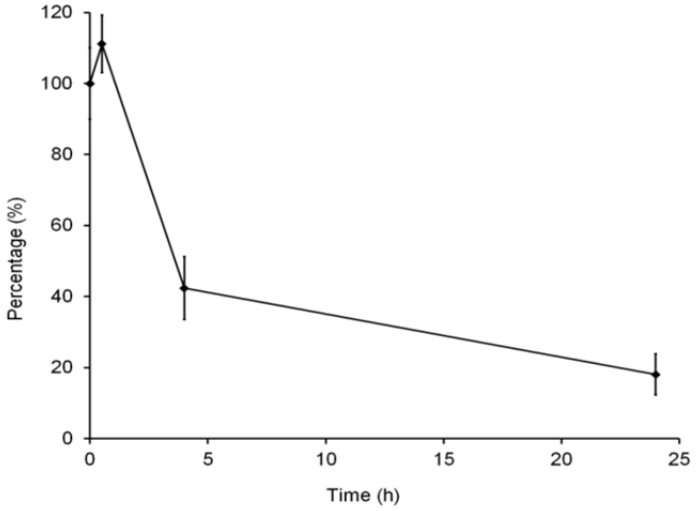

Figure 2. *R5 competitive enzyme-linked immunosorbent assay (ELISA) of Fe^{2+}/H_2O_2-catalysed 33-mer oxidation. Intact 33-mer control contains 100% activity against R5 antibody. R5 recognition of oxidised 33-mer is shown as percentage of the initial 33-mer activity. Error bars indicate the standard deviation of four measurements*

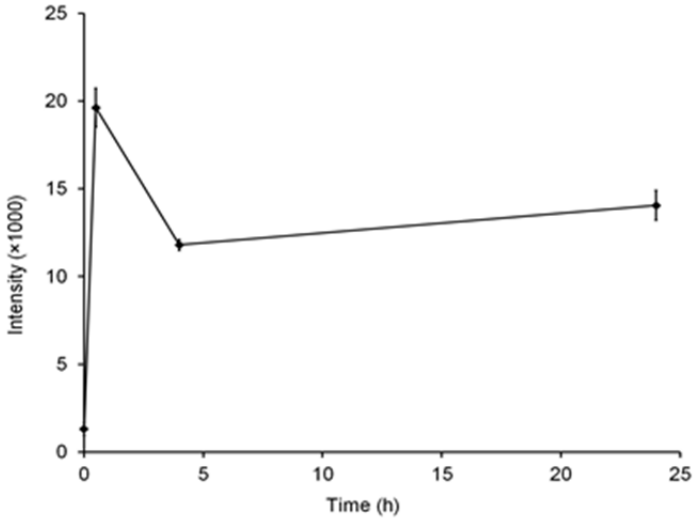

Figure 3. *Putative production of dityrosine linkages in Fe^{2+}/H_2O_2 oxidation of the 33-mer peptide over time*

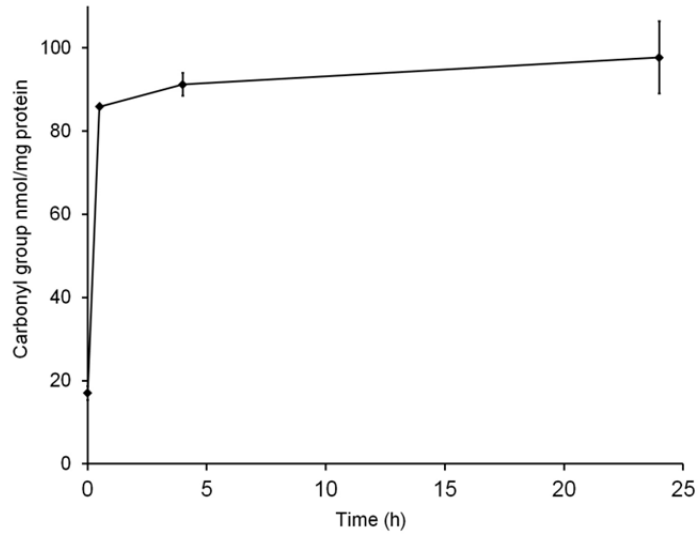

Figure 4. *Yields of carbonyl groups in Fe^{2+}/H_2O_2 oxidation of 33-mer peptide over time*

Conclusion

In our study, we showed that a model 33-mer peptide from prolamin can be modified by metal-catalysed oxidation. In Fe^{2+}/H_2O_2-induced oxidation, the immunological activity of 33-mer peptide decreased to 18% of its initial level after 24 h of oxidation, when measured by the R5 competitive ELISA test for CD-active epitopes. Carbonyl groups and dityrosine cross-links were readily formed indicating proline and tyrosine modification. This also changed the structure of the CD-active epitopes. Oxidation can

be further studied in the modification of cereal prolamin proteins, such as wheat gliadin and barley hordein, to reduce their CD-immunological activities.

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4.10 Production of gluten-free beer by using malt extract with high peptidase activity

Verena Knorr, Herbert Wieser, Peter Koehler

Deutsche Forschungsanstalt für Lebensmittelchemie, Leibniz Institut, Freising, Germany

Introduction

In Germany, about 500,000 people (approximately 100,000 diagnosed and 400,000 silent cases) suffer from coeliac disease (CD), which is associated with damage of the intestinal mucosa and subsequently leads to malabsorption and deficiency symptoms. CD is triggered by the ingestion of storage proteins of wheat (gliadins and glutenins), rye (secalins), and barley (hordeins), which are called gluten in the field of CD [1]. Coeliacs have to adhere to a strict lifelong gluten-free diet. For example, they cannot drink conventional beer and have to consume surrogates made from gluten-free cereals or pseudocereals [2]. However, these products do not meet the German beer law ("Reinheitsgebot") and mostly differ from barley-based beers in terms of aroma and taste. It has been known for a long time that gluten is massively degraded by endogenous peptidases in germinating cereals. Preliminary experiments have shown that extracts from germinated cereals were able to degrade gluten in malt drink to a concentration below 20 mg gluten/kg, which is required by the Codex Alimentarius and European legislation (regulation 41/2009) to justify a gluten-free claim [3]. Thus, cereal malt and products thereof might be used to detoxify gluten-containing, cereal-based beverages. Therefore, the aim of this study was the production of malt with optimised peptidase activity, which should be used for degrading gluten during beer production to yield a product with a gluten content below 20 mg/kg and quality parameters comparable to conventional beer.

Materials and methods

First, the conditions during germination of barley (time, temperature, water content of the grains) were systematically altered by means of Response Surface Methodology (RSM) to obtain malt with the highest possible peptidase activity as well as good suitability for brewing. The gluten-specific peptidase activity of aqueous malt extracts was determined by RP-HPLC using two coeliac-active substrate peptides (PQPQLPYPQPQLPY; P1 from α-gliadin and SQQQFPQPQQPFPQQP; P2 from γ-hordein) [4,5] and quantifying the degree of degradation over time. In addition, the influence of kilning and of temperature on the peptidase activity of the aqueous extract were examined. Then, an enzyme-active malt extract was prepared by concentrating the aqueous solution at 50 °C under reduced pressure. The concentrated malt extract was used for the degradation of the gluten content of wort, and the effects of the pH-

value, incubation time, temperature, and the extent of concentration of the extract were studied. Finally, the process was up-scaled and the resulting beer was analysed for its gluten content (R5 competitive ELISA) and sensory properties.

Results and discussion

The results of the RSM showed that endogenous malt peptidases efficiently degraded coeliac-active peptides, partly yielding fragments with a length of less than nine amino acids. The enzyme activity of the malt was significantly increased compared to the raw material and optimised by altering the germination parameters. High temperatures of up to 80 °C during kilning only caused a small decrease of the endogenous peptidase activity of the malt. The aqueous malt extract tolerated temperatures of up to 50 °C without loss of activity. By removing water under reduced pressure at 50 °C and using a high water-to-grist ratio, the activity of the malt extract was further increased. However, concentrating not only increased the peptidase activity but also the gluten content of the extract. After adding the concentrated extract to cold wort, gluten was most efficiently degraded at high temperatures and high pH, however, the enzymes were active for a longer time at low temperatures. During incubation of wort with 10% malt extract (water-to-grist ratio of 1 : 2.5; 38% Brix) at 50 °C, the gluten content decreased below 20 mg/kg after 24 h (threshold for gluten-free foods) and below the limit of quantitation of the ELISA method (10 mg/kg) after 36 h (Fig. 1).

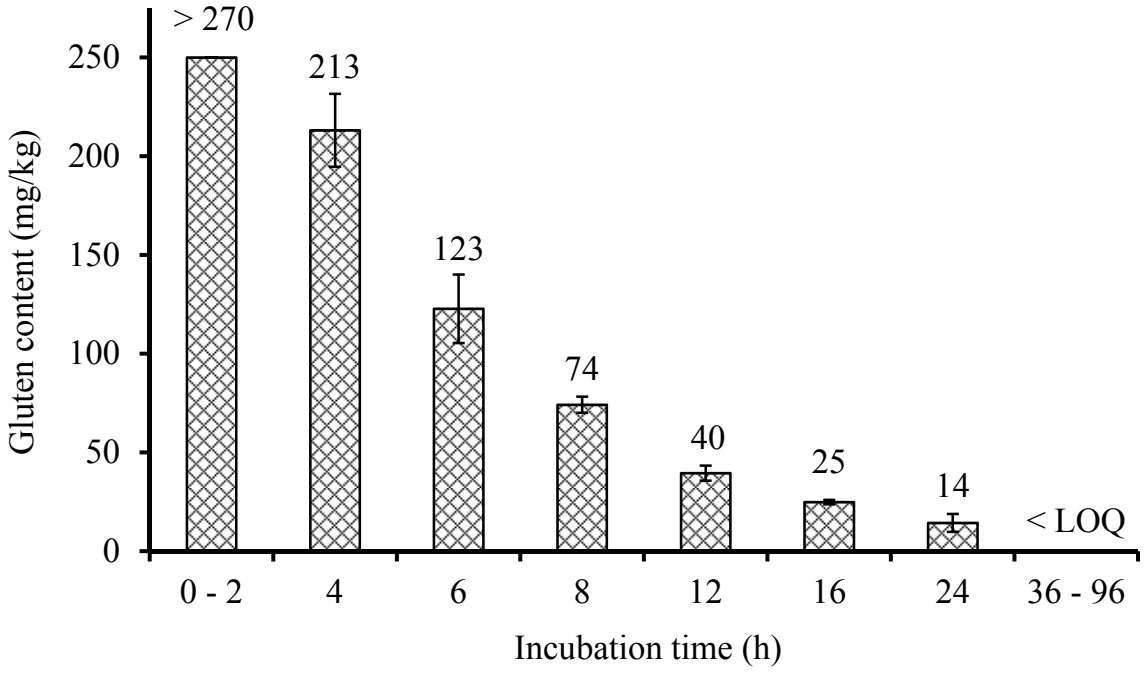

Figure 1. *Decrease of the gluten content of wort after addition of malt extract with high peptidase activity as affected by the incubation time (0 - 96 h). Gluten was quantitated by means of a competitive R5 ELISA. > 270: Concentration beyond the working range of the method; < LOQ: Concentration below the limit of quantitation of the method (10 mg gluten/kg)*

Finally, a malt extract (40% Brix, water-to-grist-ratio 1 : 2.5) was prepared and 10% were added to wort. After an incubation time of 24 h at 50 °C followed by fermentation, the gluten content of the beer was below 10 mg gluten/kg compared to 86.4 ± 9.0 mg/kg in the control without added malt extract. Sensory evaluation of the beers according to DLG (Fig. 2) showed that the peptidase-treated beer could not be distinguished from the reference beer and was more popular than a gluten-free millet beer. However, the extract-treated beer had a lower foam stability than the reference.

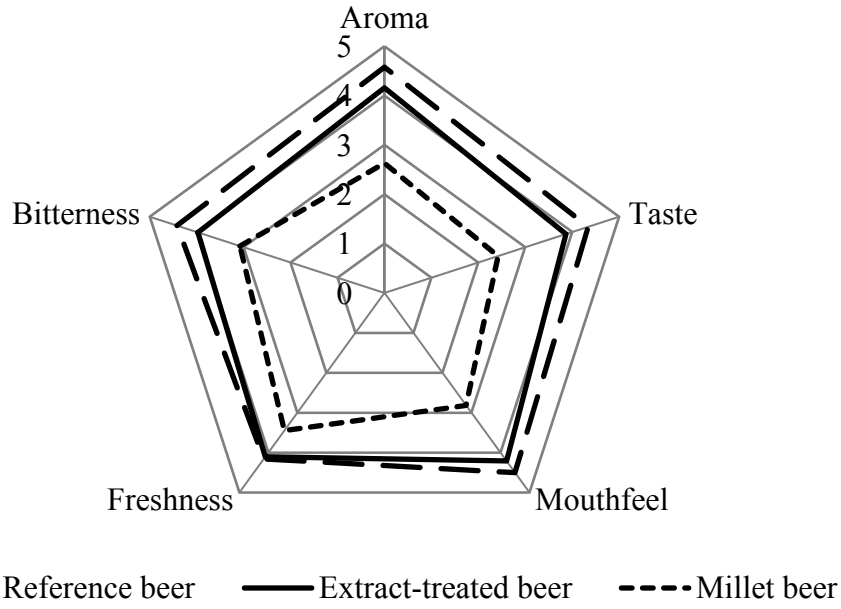

Figure 2. *Results of the sensory evaluation of beers. Reference beer was produced without addition of malt extract; extract-treated beer was produced by adding 10% concentrated malt extract with high peptidase activity; millet beer was commercially available. Rating: 0 = unsatisfactory; 5 = no deviation from quality requirements*

Conclusions

It is possible to produce gluten-free beer according to the German "Reinheitsgebot". Malt with high peptidase activity can be obtained by optimising the germination of barley grains. Shelf life and peptidase activity can be increased by concentrating an aqueous extract of the malt. A mixture of concentrated malt extract with high peptidase activity and conventional wort enables the production of gluten-free beer with a gluten content below 20 mg/kg. The sensory properties of this gluten-free beer are comparable to conventionally produced beer.

Acknowledgement

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5 Clinical research reports

5.1 The prospective multicentre trial of antibody diagnostics in paediatric coeliac disease (AbCD) - blinded status report after registration of 437 children

Thomas Mothes[1], *Johannes Wolf*[1], *David Petroff*[2], *Dirk Hasenclever*[3]

[1] *Institute for Laboratory Medicine, Clinical Chemistry and Molecular Diagnostics, Medical Faculty of the University and University Hospital, Leipzig, Germany*
[2] *Clinical Trial Centre, University of Leipzig, Germany*
[3] *Institute for Medical Informatics, Statistics and Epidemiology, University of Leipzig, Leipzig, Germany*

Introduction

The European Society of Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) recently published new guidelines for the diagnosis of coeliac disease (CD) [1]. One important question asked in the new guidelines was "In which patients can the diagnosis of CD be made without duodenal biopsies?" The guidelines recommend that "in children and adolescents with signs or symptoms suggestive of CD and very high anti-tTG titres with levels exceeding 10 times the upper limit of normal (> 10 x ULN) the likelihood for villous atrophy (Marsh 3) is high. In this situation, the paediatric gastroenterologist may discuss with the parents and patient (as appropriate for age) the option of performing further laboratory testing (EMA, HLA) in order to make the diagnosis of CD without biopsies. Antibody positivity should be verified by EMA from a blood sample drawn at a separate occasion to the initial test in order to avoid false-positive serology results due to mislabelling of blood samples or other technical mistakes. If EMA testing confirms specific CD antibody positivity in this second blood sample, the diagnosis of CD can be made and the child started on a GFD. It is advisable to check for HLA types in patients diagnosed without small intestinal biopsy to reinforce the diagnosis of CD."

The guidelines went on to say that the performance in clinical practice should be evaluated prospectively. A prospective international multicentre biopsy-controlled trial on antibody diagnostics in paediatric coeliac disease (AbCD, German Clinical Trial Register ID: DRKS00003854) [2] was started recently (inclusion of the first participant October 18, 2012) to evaluate the proposed algorithm to diagnose CD. Here, we present a status report. Our analysis is blinded, i.e., data on symptoms, antibodies, and histology are not correlated with diagnoses.

Basic trial flow

The basic flow of the AbCD trial is as follows: After registration of the patient according to the inclusion criteria (children and adolescents scheduled for duodenal biopsy as by standard clinical practice with the primary aim to confirm or refute CD), there are several visits. At the first visit, the reasons for biopsy are documented and, if there were already prior antibody tests, the results of these tests are recorded.

At the next visit, a blood sample is taken and endoscopy is performed. The results of the histological assessment of the biopsy at the trial centre are documented. The blood samples are shipped for assay of antibodies (IgA- and IgG-antibodies against tissue transglutaminase [aTTG], deamidated gliadin peptides [aDGP], and endomysium [EMA], as well as total IgA). The antibody assays are performed applying the tests of EUROIMMUN in Dassow, Germany (without knowledge of histology or of clinical symptoms). The slides with the tissue sections are sent for blind reference histology to the Departments of Pathology of the Clinical Centre "Sankt Georg" and of the University Hospital (Leipzig, Germany). In case of marked differences between the local (trial centre) and the central pathology, there is a third histological assessement in the Department of Pathology of the University Hospital Dresden (Germany). There is feedback to the trial centres within 3 months, before the patient is seen next in the hospital for follow-up (visit 3).

Visit 3 takes place about 3 months after endoscopy. If the patient is on a gluten-free diet, another blood sample is taken and analysed. In most cases, a diagnosis can already be made after 3 months. If the paediatric gastroenterologist is not sure about the diagnosis at this time, we wait for a fourth visit.

Twelve children's hospitals (2 from England, 2 from Austria, and 8 from Germany) participate in the trial (see acknowledgments).

Current status

Four hundred and thirty-seven patients were registered by July 4, 2014. Of them, 376 children already underwent endoscopy. For most of the remaining children, the documentation was not yet complete. From the patients, who had already had an endoscopy, 262 children had already finished the trial. Of the remaining children, in 113 cases, the follow-up was still missing. From the regularly finished patients, 180 had CD and 78 had no CD (prevalence 69.8%). In 4 cases, the diagnosis remained unclear.

Of the 376 patients who already underwent endoscopy, 359 had a prior antibody test (with test results available already before the start of the AbCD trial). Endoscopy was performed in 77% of the cases due to the results of these previous antibody tests. Among these tests, the assay of IgA-aTTG was most often used to select children for endoscopy. About 60% of patients were selected for biopsy due to positive IgA-aTTG and more than 70% of patients due to positive IgA-aTTG and / or IgA-EMA tests.

The next two most common causes for endoscopy were abdominal pain (60%) and diarrhea (31%). The low frequency of children with failure to thrive (17%), weight loss (13%), and short stature (10%) was striking.

The distribution of antibody data (IgA-aTTG versus IgG-aDGP) is shown in Fig. 1. The percentage of patients with antibody concentrations in the "grey zone" was 18.3% for IgA-aTTG and 43.2% for IgG-aDGP. We define the "grey zone" here to be

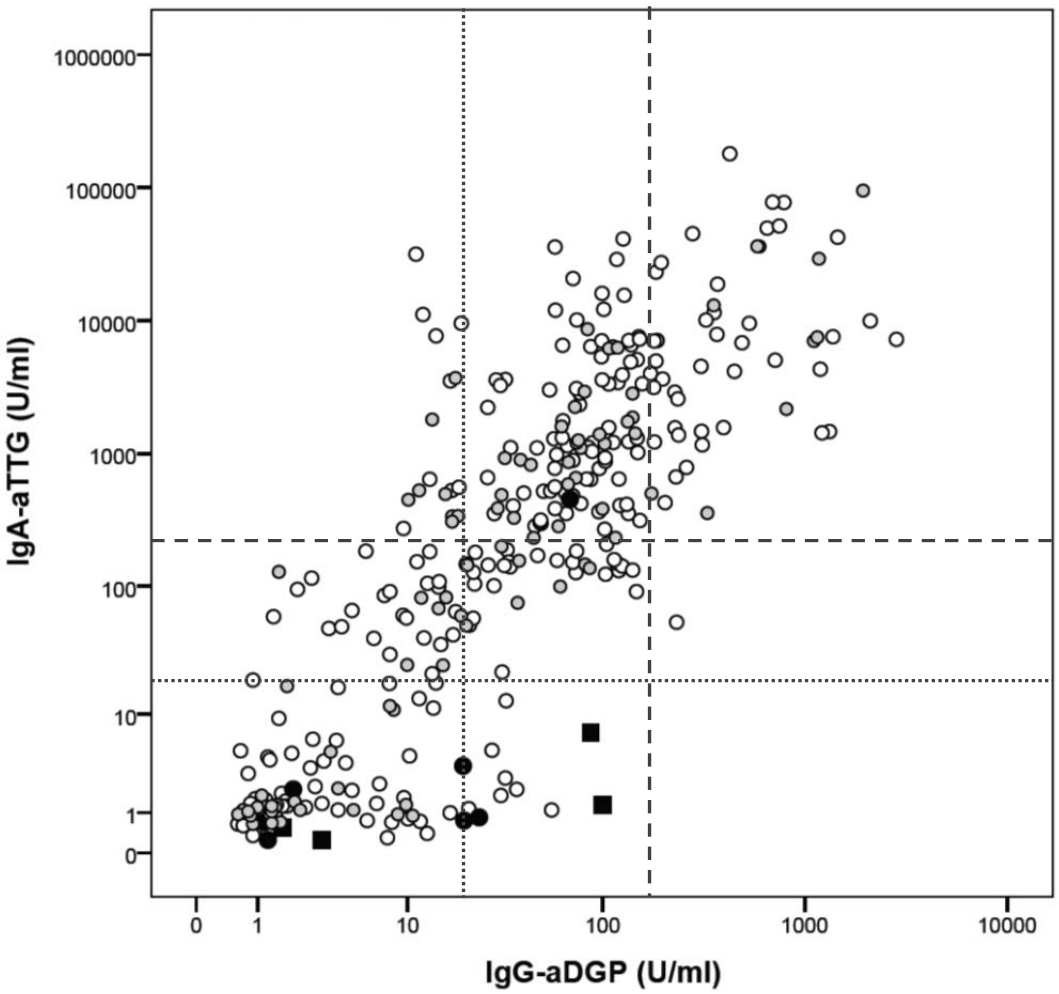

Figure 1. *Concentration of IgA-antibodies against tissue transglutaminase (IgA-aTTG) and of IgG-antibodies against deamidated gliadin peptides (IgG-aDGP) from 345 patients included hitherto into the AbCD trial. Open circles: IgA-competent patients, filled black circles: partial IgA deficiency (IgA < age specific cut-off but > 50 mg/l), filled black squares: selective IgA-deficiency (IgA < 50 mg/l); filled grey circles: hitherto unknown IgA-status. The dotted lines indicate the company cut-offs and the dashed lines indicate the 10 x company cut-offs (10 x ULN)*

the range between the company cut-off and 10 x ULN. The percentage of patients in the grey zone was higher than decribed in our recent retrospective trial [3]. Reasons for that may be different recruiting strategies, higher prevalence or higher number of unclear cases in the prospective trial. Half of the patients had their follow-up visit after

a little bit more than 3 months and more than 90% of the patients within 5 months. There was a clear tendency for a decrease in antibody concentrations during follow-up. There were a few children, in whom the antibodies did not decrease, but instead even increased up to 5- or 6-fold, presumably due to lack of a proper gluten-free diet.

The histology reports were complete from 309 patients. In 13 cases, we found a marked difference (compatible versus not compatible with CD) between local and reference histology. Therefore, a second reference histology was necessary. In 10 of these 13 cases, a marked difference between the estimate of the second and the third pathologist was found. That is why a final joint evaluation of the second and third pathologist was performed. In 12 of 309 cases, the differences were relevant for diagnosis. There were 9 cases, in which the first assessment did not suggest CD, but the reference histology did. And there were 3 cases, in which first assessment pointed to CD, but the reference histology did not.

Conclusions

About half of the total number of AbCD patients has already been recruited. In 69% of patients, the final diagnosis of CD was made. This – at first glance – pretends a high pre-test probability. However, the main reason for inclusion into the trial was previous antibody testing. This means a strong preselection of patients by prior IgA-aTTG and IgA-EMA testing. The high proportion of patients, who have already had an IgA-aTTG or an IgA-EMA test, reflects a current clinical reality. The strong preselection is a reason for the high prevalence of CD patients. This high prevalence does not reflect a true pre-test probability (i.e., before measurement of antibodies), which is probably much lower [4-6]. The diagnostic properties of the tests and diagnostic algorithms should ideally be robust enough to be reliable even without prior tests.

In the current data, we see less clearly distinct groups of patients with high and low antibody values than with retrospective patient selection. There were important differences between local and central histology evaluations. In about 4% of cases, the differences were relevant for diagnosis. The last patients are expected to be recruited by the end of 2015.

Acknowledgments

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Hospital, Halle, Germany), A. Krahl (Children's Hospital "Prinzessin Margaret", Darmstadt, Germany), M. Heiduk (Department of Paediatrics, Helios Hospital, Plauen, Germany), W.-D. Huber (University Children's Hospital, Vienna, Austria), to the reference pathologists V. Wiechmann (Department of Pathology of the Clinical Centre "Sankt Georg", Leipzig, Germany), D. Aust (Department of Pathology, University Hospital, Dresden, Germany), A.-K. Höhn (Department of Pathology, University Hospital, Leipzig, Germany), and to B. Teegen and A. Jahnke (EUROIMMUN Medizinische Labordiagnostika AG, Niederlassung Dassow) for antibody assays. This trial is part of a project funded by the European Regional Development Fund.

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5.2 WIESLAB® Celiac hs Screen: detection of autoantibodies against coeliac disease-specific peptide antigens

Norman Händel[1], Christian Spranger[2], Johannes Wolf[3]

[1] University Children's Hospital, Leipzig, Germany
[2] "Sankt Georg" Children's Hospital Leipzig, Leipzig, Germany
[3] Institute for Laboratory Medicine, Molecular Diagnostics and Clinical Chemistry, University of Leipzig, Leipzig, Germany

Introduction

The current European guidelines [1] estimated the performance of IgG antibody tests against deamidated gliadin peptides (IgG-DGP) to be inferior to that of IgA anti-tissue transglutaminase (IgA-tTG) and IgA antibodies against endomysium diagnosing paediatric coeliac disease (CD). Contrary, the British Society of Gastroenterology recommended IgG anti-DGP assays in diagnosis of adult CD in their current guidelines [2]. Conclusive studies on the predictive power of IgG-DGP tests are still rare. Possibly IgG-DGP assays provide an additional benefit for a reliable diagnosis of CD.

WIESLAB® Celiac hs Screen test is a novel microplate-based assay for the detection of IgG-DGP. The synthetic peptide sequences mimic deamidated gluten peptides recognised by T cells in the initial immune response of CD. More than 70 peptides were screened for reactivity and the most suitable peptide sequences were selected as antigens for the test.

Previous studies with WIESLAB® Celiac hs Screen test revealed sensitivities and specificities of 85 to 87% and 94 to 98% for CD, respectively [data not published]. However, inclusion of CD patients on a gluten-free diet and lacking information on histology in the control group were strong limitations of the study.

Therefore, we have retrospectively assessed the performance of Celiac hs Screen in a collection of sera of patients with intestinal disorders or CD-associated diseases [3,4] and compared the results with data of two commercially available tests (IgA-tTG and IgG-DGP, Phadia) for diagnosis of CD. We hypothesised that a double positive test result (IgA-tTG and IgG-DGP) could render biopsies unnecessary for diagnosing CD.

Materials and methods

We analysed the performance and predictive power of the WIESLAB® Celiac hs Screen test (Euro Diagnostica AB, Malmö, Sweden) performed in sera of 276 children and adolescents (123 boys and 153 girls, mean age 7.8 years, range 0.8 - 17.9 years).

The serum samples included 69 sera of CD patients and 207 disease controls resulting in a prevalence of about 25%. The samples were collected consecutively between 2006 and 2012 in Leipzig (University Children's Hospital Leipzig and "Sankt Georg" Children's Hospital Leipzig, Germany).

All patients were biopsied during upper gastrointestinal endoscopy and a detailed histology report (including information of either number of intraepithelial lymphocytes or their count with respect to cut-off) was available. Blood samples were taken on a gluten-containing diet not more than three months before and not later than three weeks after biopsy. Selective IgA deficiency (sIgAD) was found in two controls and three CD patients. There were six children with type 1 diabetes mellitus (four CD patients and two controls). Furthermore, the control group comprised 12 patients with either Crohn's disease or ulcerative colitis.

Antibodies were measured (blinded to the diagnosis and histological data) in the lab of Euro Diagnostica AB in Malmö applying the WIESLAB® Celiac hs Screen test and two further ELISAs (IgA-TTG and IgG-DGP of Phadia, Uppsala, Schweden), respectively.

Concerning a single and double test strategy, we assume the diagnosis is reliable, if positive predictive value (PPV) and negative predictive value (NPV) are simultaneously at least 95% with a lower limit for the confidence interval ($CI_{95\%}$) of 90%. PPV and NPV were calculated depending on prevalence (10% and 50%, respectively) using Bayes' formula. A maximum prevalence of 10% is assumed as pre-test probability of patients without any prior antibody test [5].

Receiver Operating Characteristic (ROC) analyses were performed with SPSS 20.

Results and discussion

The IgA-tTG test had the best combination of sensitivity and specificity (highest accuracy) as well as of $PPV_{10\%}$ and $NPV_{50\%}$ (Tab. 1). However, the sensitivity was lower in comparison to that of the WIESLAB® Celiac hs Screen (0.91 to 0.94). The WIESLAB® Celiac hs Screen test possessed also the highest $NPV_{50\%}$ but the lowest specificity and $PPV_{10\%}$.The Phadia test for IgG-DGP showed intermediate results but also a low $PPV_{10\%}$ of only 0.54.

We believe that there are several reasons for the low specificity of the WIESLAB® Celiac hs Screen test. The main reason seems to be the choice of a low cut-off (> 3 U/mL). Therefore, we performed a ROC analysis. The analysis (AUC = 0.962) disclosed that a cut-off of > 5 U/mL would increase the accuracy. Applying this higher cut-off would not significantly influence sensitivity and NPV but would improve specificity and PPV markedly (Tab.1).

The most prominent feature of the WIESLAB® Celiac hs Screen is its high sensitivity also at a cut-off of 5 U/mL. Hence, the question arises if this test is able to pick up CD patients, who are negative for IgA-tTG. In total, six CD patients were not recognised

by the IgA-tTG test but three of them were positive for WIESLAB® Celiac hs Screen (Tab. 2).

Table 1. *Performance and predictive power of WIESLAB® Celiac hs Screen at different cut-offs in comparison to two often used CD-specific antibody tests.*

	Tests			
	WIESLAB® Celiac hs Screen (> 3 U/mL)	**WIESLAB® Celiac hs Screen (> 5 U/mL[a])**	**Phadia IgG-DGP (>10 U/mL)**	**Phadia IgA-tTG (>10 U/mL)**
TP + CD	65	64	57	63
TN + no CD	167	187	197	206
TP + no CD	40	20	10	1
TN + CD	4[b]	5	12	6[c]
Sensitivity	0.94 (0.85-0.98)	0.93 (0.83-0.97)	0.83 (0.71-0.90)	0.91 (0.81-0.96)
Specificity	0.81 (0.75-0.86)	0.90 (0.85-0.93)	0.95 (0.91-0.97)	0.99 (0.97-1.00)
Accuracy	0.84 (0.79-0.88)	0.91 (0.88-0.94)	0.92 (0.89-0.95)	0.97 (0.95-0.99)
$PPV_{10\%}$	0.35 (0.26-0.44)	0.52 (0.41-0.62)	0.65 (0.54-0.77)	0.96 (0.90-1.00)
$NPV_{50\%}$	0.93 (0.90-0.97)	0.93 (0.89-0.96)	0.85 (0.80-0.89)	0.92 (0.88-0.96)

[a] Increased cut-off determined by ROC-analysis. [b] 1xCD with sIgAD and 1xCD with CVID. [c] 2x CD with sIgAD and 1xCD with CVID. Numbers in brackets display the 95% Wilson confidence interval. CD, coeliac disease; PPV, positive predictive value; NPV, negative predictive value; TP, test positive; TN, test negative; CVID, common variable immunodeficiency

One has a selective sIgAD. Interestingly, we found two further CD patients, which were IgA competent but with a negative result of IgA-tTG. It should be mentioned that these patients would be detected similarly by the IgG-DGP assay of Phadia (Tab. 2).

In our opinion, these IgA-tTG-negative CD patients are often underrepresented or not mentioned in most studies due to the pre-selection by IgA-tTG assays. Moreover, these patients would have been overlooked by the ESPGHAN guidelines, which recommend omitting assays based on the detection of IgG-DGP. According to several studies, the proportion of IgA-tTG-negative CD patients may be as high as 8% [6] or even 24% [7].

Is it possible to diagnose or exclude CD without confirmatory endoscopy? For that, the ESPGHAN recommended to apply the tenfold cut-off of the upper limit (10xULN) for the IgA-tTG test, and confirmatory testing by IgA-EMA and HLA-typing [1].

Table 2. *CD patients with a negative IgA-tTG result.*

	Age (y)	Gender	Histology	Phadia IgA-tTG (U/mL)	WIESLAB® Celiac hs Screen (U/mL)	Phadia IgG-DGP (U/mL)	IgA
1	10.8	F	Marsh 3C	0.6	0.8	0.9	normal
2	8.9	M	Marsh 3A	0.1	1.3	0.4	CVID
3	1.7	M	Marsh 3A	0.0	2.2	2.3	sIgAD
4	9.0	F	Marsh 3B	3.7	17.0	25.0	normal
5	3.9	F	Marsh 3A	5.8	29.0	23.0	normal
6	10.1	F	Marsh 3C	0	112	50	sIgAD

CVID, Common variable immunodeficiency; sIgAD; selective IgA deficiency

Different from the ESPGHAN guidelines, we hypothesise that, when combining IgA-tTG and WIESLAB® Celiac hs Screen, the reliable criteria concerning PPV and NPV for a diagnosis without biopsy are fulfilled (Tab. 3). This is true for both the low and the high cut-off of WIESLAB® Celiac hs Screen. Using the low cut-off of only 3 U/mL, the number of needless biopsies would be almost be doubled. Therefore, the higher threshold value should be used. Using a combination of the two Phadia tests, the results are comparable (Tab. 3).

To the best of our knowledge, there are currently only two reports on a two-test procedure for diagnosis and exclusion of CD without endoscopy. Bürgin-Wolff et al. [8] calculated a PPV and NPV of 0.96 and 0.98, respectively, but regarded a two-test strategy as unsuitable due to a high number of false-positives (5 of 119 controls). Instead, the authors recommended a three- or four-test strategy with predictive values up to 1.00. Unfortunately, the authors did not report the influence of prevalence and did not calculate confidence intervals.

A second retrospective study [5] of more than 1000 children and adolescents (including 376 CD patients) revealed that, if combining IgA-tTG and IgG-DGP, a double negative result reliably excludes CD and a double positive result reliably confirms the diagnosis of CD up to a prevalence of 17%. A low prevalence (≤ 10%) has to be assumed, if patients are selected without any prior antibody test. However, at specialised gastroenterologists´ practices (with prior positive antibody test), higher prevalences have to be considered.

Conclusions

The WIESLAB® Celiac hs Screen test at company cut-off possesses high sensitivity but low specificity. At higher cut-off (5 U/mL), the specificity increases without significant loss in sensitivity. A combination of the WIESLAB® Celiac hs Screen with the IgA-tTG assay (two-test strategy) results in high PPV and high NPV at prevalences < 10% and > 50%, respectively. Hence, the two-test strategy could render biopsies

unnecessary for diagnosis in a large part of CD patients. The retrospective nature, the size of study population, and the strategy of patient selection (on the basis of prior IgA-tTG testing) do not allow a final assessment. Therefore, the analysis should be validated in a further large prospective trial.

Table 3. *Two-test strategy for obtaining reliable test results to avoid biopsies.*

	Tests		
	WIESLAB® Celiac hs Screen (> 3 U/mL) + Phadia IgA-tTG	**WIESLAB® Celiac hs Screen (> 5 U/mL) + Phadia IgA-tTG**	**Phadia IgG-DGP + Phadia IgA-tTG**
TP + CD	62	61	54
TN + no CD	167	188	196
TP + no CD	1	1	1
TN + CD	3	3	3
unclear test[a]	43	23	22
Sensitivity	0.90 (0.79-0.95)	0.88 (0.78-0.95)	0.78 (0.66-0.87)
Specificity	0.81 (0.75-0.86)	0.91 (0.86-0.94)	0.95 (0.90-0.97)
$PPV_{10\%}$	0.95 (0.90-1.00)	0.95 (0.90-1.00)	0.95 (0.90-1.00)
$NPV_{50\%}$	0.994 (0.98-1.00)	0.95 (0.92-0.98)	0.995 (0.99-1.00)
to be biopsied	15.2%	8.33%	7.97%

[a] Controls and CD patients with only one positive test. Numbers in brackets display the 95% Wilson confidence interval. CD, coeliac disease; PPV, positive predictive value; NPV, negative predictive value; TP, test positive; TN, test negative

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5.3 Intraluminal p31-43 gliadin peptide induces enteropathy

Romina Araya[1], Jennifer Jury[2], Elena F. Verdu[2], Fernando Chirdo[1]

[1] *Instituto de Estudios Inmunológicos y Fisiopatológicos (IIFP-CONICET/UNLP), Buenos Aires, Argentina*
[2] *Farncombe Institute, McMaster University, Hamilton, Canada*

Introduction

Coeliac disease (CD) pathogenesis depends on the activation of gluten specific Th1 cells in susceptible individuals, who carry the HLA-DQ2 and/or HLA-DQ8 predisposing alleles [1,2]. The mechanisms, involved in the exacerbated expansion of Th1 cells, have been largely characterized during the last decades. Most of our knowledge of CD pathogenesis was based on the evaluation of small pieces of duodenal biopsies, which account for chronic lesions. Consequently, the initial steps of the mucosal damage are poorly understood [3].

Animal models are very useful tools to evaluate different steps in disease mechanisms and test new therapeutic strategies. Different complex models using genetically modified mice have been reported to assess CD pathogenesis [4]. The usefulness of different proposed animal models resides in mimicking certain steps of disease pathogenesis. Most studies focused on the analysis of the chronic phase of the disease, but few have evaluated the initial steps leading to mucosal damage or the precipitating factors of the disease.

Epidemiological studies support the association of enteric infections with the subsequent development of inflammatory or functional gastrointestinal disease [5,6]. Viral infections, in particular enterovirus such as rotavirus, have been suggested to increase the incidence of CD [7,8]. Case reports have also suggested the association of enteric infections with CD onset [9]. Currently, the underlying mechanisms that support this association are poorly understood. However, it is well known that innate immune activation generates a rapid and strong anti-viral response, which essentially involves the production of Type I IFNs and other components of the inflammatory response. The link among enterovirus infection and CD raises the hypothesis that Type I IFNs may be involved in loss of tolerance to gluten through the ability to promote inflammation and a strong Th1 response [10].

Poly I:C, a synthetic ligand, reproduces the innate effects of dsRNA and it is used to mimic viral infections. Previous studies have proposed that intraperitoneal poly I:C induces enteropathy. These models used parenteral poly I:C administration and mucosal damage seemed to be specific for TLR3 activation [11-13]. The underlying mechanisms proposed for intestinal damage include NK cells [14] and CD8$\alpha\alpha^+$ IELs

with critical participation of IL-15 [11,12]. A recent study, however, has proposed that neither NK cells, IELs or IL-15 are involved in the induction of intestinal pathology [13]. Even when enteropathy was clear, none of the studies evaluated the effect of poly I:C directly injected in the lumen. Understanding the early and delayed consequences of immune activation by enteric viral infections or dsRNA, has important implications for post-infectious inflammatory and functional consequences [6]. To assess the local effects of different stimuli present in the intestinal lumen, we have recently developed an animal model based on the intraluminal administration of poly I:C [15]. This model showed that transient mucosal damage has physiological consequences after subsequent gluten challenge.

Aim

The aim of our study was to develop a murine model of enteropathy based on the intraluminal administration of gliadin peptide p31-43 and to evaluate the mechanism associated to mucosal damage.

Materials and methods

Six to eight-week-old C57BL/6 mice were intraluminaly treated with Poly I:C (4), p31-43 (5) or PBS (5) and sacrificed after 12 h. In some experiments, we used a non-related peptide (NRP) derived from human thyroid peroxidase (TPO). Small intestinal samples were collected and fixed with formalin. Paraffin-embedded sections (5 μm) were obtained, re-hydrated and stained with H&E for further morphometric analysis. Sections were processed for immunofluorescence microscopy studies, using anti-Ki67 antibody (Novus Biologicals) and the proper secondary antibody (Invitrogen), to evaluate cell proliferation in crypts. Furthermore, the *In Situ* Cell Death Detection Kit (Roche) was used to evaluate cellular death in the small intestine from p31-43 or PBS-treated mice. For the statistical analysis, an unpaired t-test was performed.

Results and discussion

Development of a murine model of enteropathy by intraluminal administration of poly I:C

We developed a model of enteropathy based on the intraluminal injection of poly I:C, a synthetic ligand of TLR3, as well as two cytoplasmic receptors, RIG-I and MDA5, in wild-type mice (C57BL/6).

Briefly, the intraluminal administration of poly I:C to C57BL/6 mice produced severe enteropathy. At 12 h post poly I:C, we observed shortening and widening of villi, edema, and increased cell infiltration in the lamina propria (LP). Using H&E staining, edema, dilated capillaries, and interstitial spaces were observed in intestinal villi. The evaluation of the villous height/crypt depth ratio (V/C), the most used parameter to assess histological changes at the small intestinal mucosa, showed a statistically significant reduction of V/C ratio in poly I:C treated mice compared to those receiving

PBS. Furthermore, the number of IELs was increased after poly I:C treatment (Fig. 1). At 72 h post poly I:C treatment, a partial recovery of V/C ratio was observed, while the number of IELs remained still altered (not shown). As expected, the surgical procedure altered intestinal histology. However, this change was transient and control mice exhibited a faster recovery after treatment.

We performed additional studies to validate this model of enteropathy [15], which allows the assessment of the mechanisms locally induced in the proximal small intestine by luminal stimuli, as it is the case for viral infections or dietary antigens.

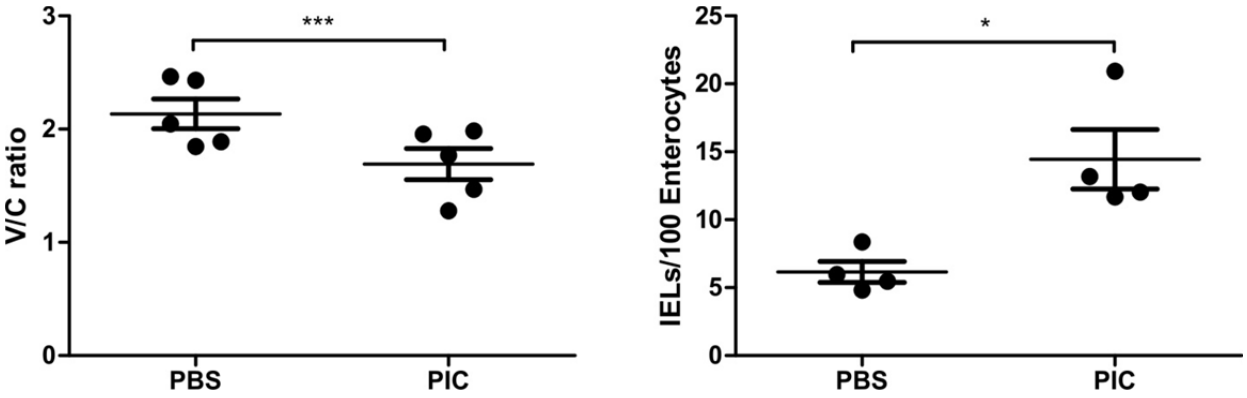

Figure 1. *Model of small intestinal enteropathy based on intraluminal administration of Poly I:C. Evaluation of histological parameters in poly I:C- and PBS-treated C57Bl/6 mice. V/C ratio and IELs counting were determined 12 h post poly I:C or PBS treatment. Stats: Unpaired t-test, *p<0.05, ***p<0.001*

Intraluminal administration of p31-43 peptide induces enteropathy

Based on the model described above, we tested whether the p31-43 gliadin peptide triggers mucosal damage when injected at the lumen of the proximal small intestine of wild-type mice.

As controls, we used C57BL/6 mice treated with PBS as control for the surgical procedure, as well as a peptide with an unrelated sequence (NRP) to rule out unspecific effects of a peptide fragment inoculated in the lumen.

The assessment of histological parameters showed that p31-43 induced a severe enteropathy. The V/C ratio was reduced, while the number of IELs was increased in p31-43-treated mice (Fig. 2). At 12 h post p31-43 treatment, we observed shortening and widening of villi, increased cell infiltration in the lamina propria (LP), and edema indicated by an increase of interstitial space in villous tips (not shown). On the other hand, PBS and NRP, did not present any histological impairment. At 72 h post p31-43, there was a partial recovery of V/C ratio and IELs counting, but both parameters remained statistically significantly altered compared with PBS or NRP-treated mice (not shown). As it was described previously, the surgical procedure altered intestinal

histology [15]. However, this change was transient and PBS and NRP control mice presented a faster recovery after treatment.

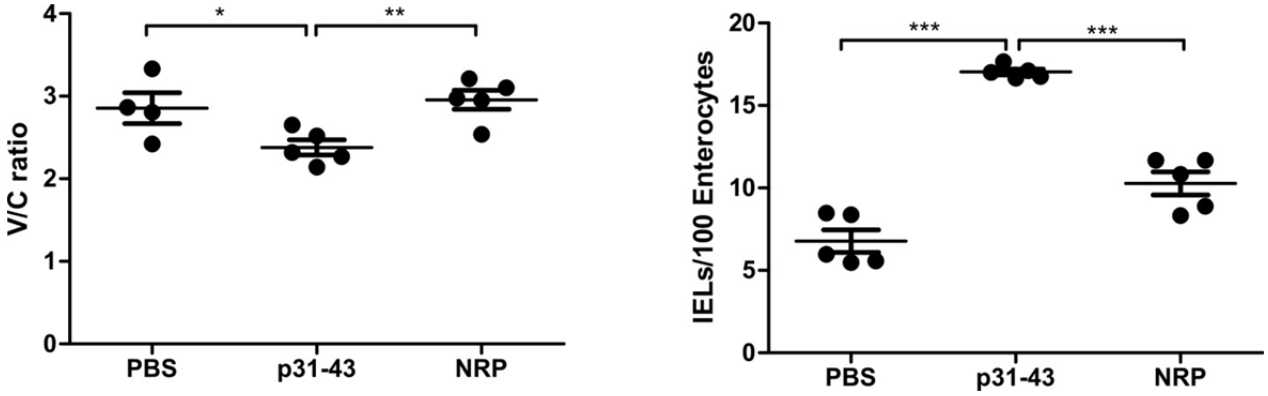

Figure 2. *p31-43 induces enteropathy. Evaluation of histological parameters in p31-43, PBS- and NRP-treated C57BL/6 mice. V/C ratio and IELs counting were determined after 12 h after intraluminal injection of p31-43, NRP or PBS. Stats: Unpaired t-test, *p<0.05, ***p<0.001*

p31-43 induces cellular death and cell crypt proliferation

Small intestinal damage is normally accompanied by increased cell death and in parallel, a regenerating process that involves crypt hypertrophy. Since we were interested in evaluating mechanisms of mucosal damage involved in this enteropathy model, we assessed cell death and proliferation.

When we evaluated cellular death by the TUNEL technique in small intestinal sections from p31-43- and PBS-treated mice 12 h post-treatment, a strong increase of the number of $TUNEL^+$ cells mainly in the lamina propria of p31-43-treated mice was observed in comparison with PBS-control mice (Fig. 3A). Furthermore, $TUNEL^+$ cells were also observed in the epithelial layer of the small intestine of p31-43-treated mice, which were not observed in control mice (not shown). Statistically significant differences were observed, when the number of $TUNEL^+$ cells was automatically determined in p31-43- and PBS-treated mice (Fig. 3A).

Next, we assessed the proliferative response in the intestinal mucosa induced by p31-43. To this end, we used the Ki67 marker, which is a nuclear protein associated with cellular proliferation. The number of $Ki67^+$ cells was determined in the crypts 12 h post-treatment. Intraluminal administration of p31-43 induced a strong proliferative response in the crypts (Fig. 3B).

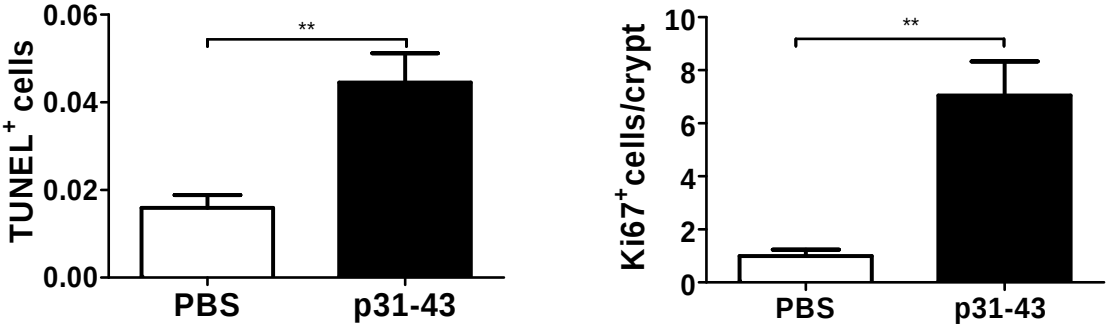

Figure 3. *p31-43 induces crypt proliferation and cellular death in the small intestine. Cell death (TUNEL$^+$ cells) and proliferative response (Ki67$^+$ cells) were evaluated in intestinal sections of C57BL/6 mice 12 h after intraluminal administration of p31-43 or PBS. Unpaired t-test, **p<0.01*

Altogether, these results show that p31-43 treatment produced a significant increase of cell death in the lamina propria and in the enterocytes with a strong proliferative response in the crypts.

Conclusions

In this study, we show the development of a murine model of enteropathy in wild-type mice based on the intraluminal administration of p31-43. This is the first model describing the *in vivo* effects of p31-43 in wild-type mice, which reproduces some of the features found in active CD.

This work highlights the relevance of an early induction of innate immunity mechanisms by p31-43. Further investigations should be done to dissect the signalling pathways implied in p31-43-mediated damage *in vivo*.

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5.4 T lymphocytes also react with tissue transglutaminase

Ross Comerford[1,2,3], Christian Coates[1,2], Greg Byrne[1,2], Jacinta Kelly[1,2,3], Conleth Feighery[1,2]

[1] Department of Immunology, St James's Hospital, Dublin 8, Ireland
[2] Institute of Molecular Medicine, Trinity College Dublin, Dublin 8, Ireland
[3] National Children's Research Centre, Our Lady's Hospital For Sick Children, Crumlin, Dublin 12, Ireland

Introduction

The principal autoantigen of coeliac disease (CD) is the ubiquitously expressed, multifunctional enzyme tissue transglutaminase (tTG) [1]. The demonstration of IgA autoantibodies directed to this self-protein is an integral component in the diagnosis of CD [2]. tTG is also intimately involved in CD pathogenesis through the modification of gluten peptides by deamidation, which facilitates their presentation to the immune system via HLA-DQ2 or DQ8 molecules [3,4].

In addition to their reactivity with gliadin peptides, gliadin-specific T cells have been speculated to provide T-cell help to tTG-specific B cells, resulting in the anti-tTG response characteristic of CD. This 'hapten-carrier' theory, proposed by Sollid, was based upon the fact that T cells specific for tTG had never been isolated, and the gliadin-dependent nature of the anti-tTG response [5]. Preliminary findings from our group indicated that T cells specific for tTG could be detected in CD patients and in some control individuals, in a HLA-DQ and HLA-DR restricted manner [6]. The existence of tTG-specific T cells was further confirmed in a recent paper by Ciccocioppo et al., who described the presence of T cells, mostly CD4 positive, that proliferated to tTG in a HLA-DQ2-restricted manner [7].

The aim of this study was to further investigate the presence of tTG-specific T cells in the periphery of CD patients and healthy controls. Such a finding is in keeping with reports that T cells specific for other autoantigens have been detected in healthy subjects [8,9]. Peripheral blood samples were challenged with tTG and proliferative responses measured. tTG-reactive T-cell lines were generated from responsive individuals and both the intracellular and secreted cytokines IFN-γ, IL-10, IL-17A, and IL-21 were measured, in tandem with the measurement of proliferation of these cells.

Materials and methods

Study subjects

CD patients and controls were recruited from the Departments of Gastroenterology and Immunology, St James's Hospital Dublin. Patients with CD were subdivided according

to their treatment status and included 33 patients with untreated CD, 65 patients with treated CD and 54 healthy control subjects.

Proliferation studies

The proliferation of fresh peripheral mononuclear cells (PBMC) was investigated in the following manner. Cells were separated by density gradient centrifugation and cultured in RPMI medium with 5% autologous serum in a round-bottomed 96-well microtitre plate. Wells containing medium and cells only were added in order to measure background proliferation, and used to calculate the stimulation index (SI) based on ^{3}H-thymidine incorporation. The tTG antigens used were guinea pig tTG (gp tTG, Sigma), erythrocyte tTG (tTG^{ery}, Inova Diagnostics), and recombinant human tTG (rh tTG, produced in SF9 insect cells, Zedira), at a concentration of 10 μg/mL. In some further experiments, a further recombinant tTG product was employed [10].

Cells were cultured for 6 days and for the last 18 h of culture 0.5 μCi of ^{3}H-thymidine (PerkinElmer) was added. The stimulation index (SI) was calculated by dividing the mean of the three cpm values for each antigen by that of the unstimulated wells, with an SI of 2 or greater being considered positive. In experiments measuring the proliferation of tTG-sensitised T-cell lines, the incubation time was shortened to 72 h. MHC restriction was investigated using PBMCs from 16 of the CD patients and two normal controls. PBMCs were cultured with gp tTG in the presence or absence of (i) a γ2A anti-HLA-DR (L243, Becton and Dickinson); (ii) a γ2A non-specific isotype matched control antibody (Becton and Dickinson); (iii) a γ1 anti-HLA-DQ (SPV-L3, a gift from Dr. Hergen Spits, University of Amsterdam, Amsterdam, The Netherlands), or (iv) a γ1 non-specific isotype matched control antibody (Becton and Dickinson). Proliferation was measured as described above and results were calculated on the basis of percentage inhibition.

T-cell line generation

For the generation of tTG-specific T-cell lines, 1x10^{6} PBMCs from CD patients or control individuals in RPMI medium were incubated with tTG^{ery} at 10 μg/mL. After 5 days in culture, 20 IU/mL of recombinant human IL-2 (Sigma) was added to each well. The cells were then cultured for a further 7 days, receiving 20 IU/mL IL-2 on day 9. In order to expand the T-cell lines, further stimulation with tTG^{ery} was performed on days 14 and 28 of culture, using irradiated autologous PBMCs as antigen-presenting cells.

These cell suspensions were then returned to culture for a further 72 h, with proliferation then measured by ^{3}H-thymidine incorporation. Cellular phenotype (CD4 or CD8 positivity) as well as intracellular cytokine production was determined by flow cytometric analysis. Cultures for intracellular cytokine staining had 10 ng/mL phorbol 12-myristate 13-acetate (Sigma), 1 μg/mL ionomycin (Sigma), and 10 μg/mL Brefeldin A (Sigma) added for the final 10 h of culture.

Flow cytometry

Intracellular cytokine production and the phenotype of samples of T cells from each T-cell line in response to re-stimulation with tTG was assessed. The panel of antibodies used was CD3-APC-efluor780, CD4-APC, CD8-PECy5, and IFN-γ-FITC (eBioscience, San Diego, CA). Cells were gated on forward/side scatter, CD3, and aqua fluorescent reactive dye (Invitrogen) exclusion, which permitted the measurement of live cells only. Quadrants for all parameters were set using fluorescence minus one (FMO) controls. For all flow cytometric analysis, $5x10^5$ cells from each sample were measured.

Measurement of cytokines by ELISA

The supernatants, removed weekly from each T-cell line and media-only controls, were analysed for the presence of IFN-γ, IL-10, IL-4, IL-17A, and IL-21 using ELISA MAX™ kits (BioLegend, San Diego, CA).

Statistical analysis

Differences in proliferation in response to tTG between patient and control groups were evaluated for statistical significance using the Mann-Whitney test, with the paired t-test used to identify significant effects of MHC blocking on proliferative responses to tTG. The level of significance was set at 0.05.

Results and discussion

Lymphocyte proliferation assays

Positive proliferative responses to four different sources of tTG, including two recombinant proteins, were observed not only in patients with CD but also in some healthy control subjects (Fig. 1). Responses to gp tTG were observed in 80% of untreated coeliac patients, compared to 40% of treated coeliac patients and 38% of control subjects (Fig. 1a). Furthermore, the overall SI response of the untreated CD patients was significantly higher than both the treated CD patients ($p = 0.0042$, Mann-Whitney test) and the normal control subjects ($p=0.0063$, Mann-Whitney test). For the tTG^{ery} antigen, lymphocyte proliferation was again more frequently detected in untreated CD patients (45%), with 35% of treated CD patients and 24% of controls responding (Fig. 1b). A similar pattern of response to the recombinant human tTG preparation (rh tTG) was noted, with positivity detected in the majority of untreated CD patients (Fig. 1c). Although the level of proliferation was low in the majority of subjects, these findings strongly suggest the presence of T cells in the circulation, which specifically react with tTG.

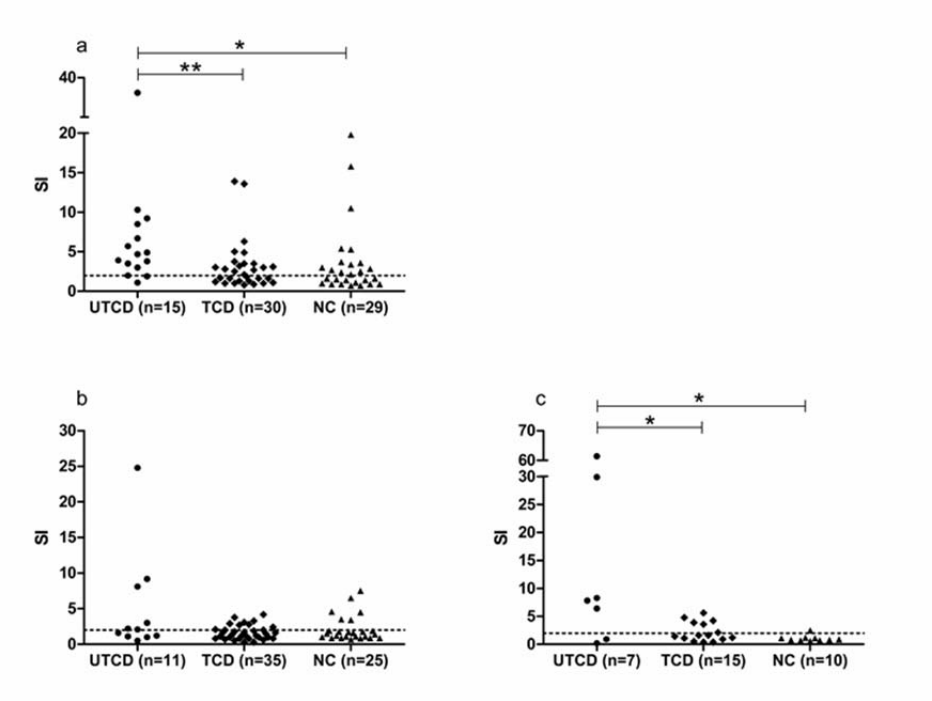

Figure 1. *Proliferative responses to guinea pig, erythrocyte, and recombinant human tTG. Proliferation of PBMCs from normal controls (NC), untreated CD patients (UTCD), and treated CD patients (TCD) to guinea pig (a), erythrocyte (b), and recombinant (c) human tTG*

MHC blocking studies

In MHC blocking studies, the inhibitory effect of added monoclonal anti-HLA-DR or anti-HLA-DQ to gp tTG-stimulated PBMC was investigated in 18 individuals (16 CD and 2 normal control subjects) with known proliferative responses to this antigen. Both anti-MHC II antibodies caused marked inhibition of proliferation and this was particularly so with the anti-HLA-DR antibody (results not shown). This finding is also good evidence that antigen-presenting cells, employing MHC class II molecules, were involved in the tTG activation of T cells.

T-cell line generation

Three polyclonal T-cell lines were generated by initial stimulation of PBMC with tTG^{ery} and fortnightly re-stimulation of the cell line thereafter (Fig. 2a). The T-cell line CD01 was generated from a male of 51 years, who had biopsy-confirmed CD, and was on a strict gluten-free diet for ten years, as evidenced by serial tTG/EMA negativity. The T-cell lines CT01 and CT02 were generated from two non-CD controls, males of 32 and 56 years, respectively. All three individuals were selected, because they gave a positive proliferative response to tTG^{ery} in ^{3}H-thymidine incorporation assays. The T-cell lines established were predominantly CD4 positive, the percentage of which increased over the duration of the culture period for CD01 and CT01 (Fig. 2b).

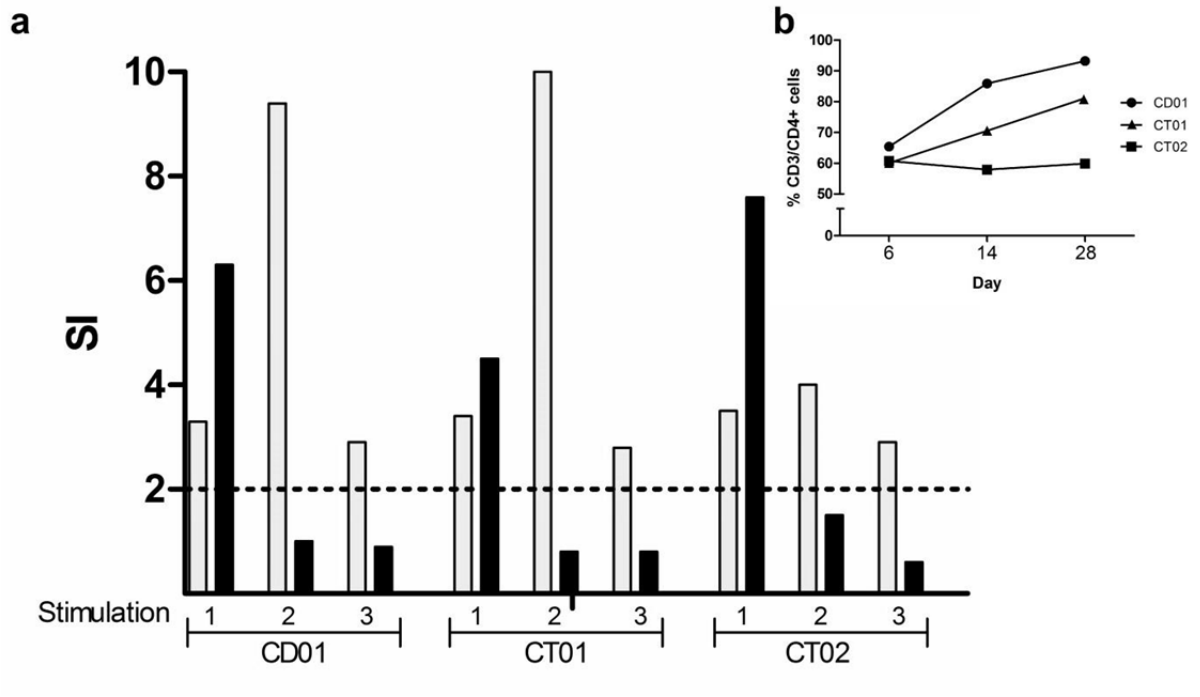

Figure 2. *The proliferative responses and phenotype of cells from each T cell line per round of stimulation. (a) The proliferation of samples of cells from each T cell line in response to erythrocyte tTG (grey), and PPD (black) was measured by 3H Thymidine incorporation. Stimulations 1, 2, and 3 represent days 6, 14, and 28 of culture. (SI= stimulation index, the dashed line indicates the cut-off for positivity). (b) Shows the percentage of CD4+ T cells contained in each cell line at days 6, 14, and 28 of culture*

In order to confirm the antigen specificity of the T-cell lines, re-stimulation with tTG^{ery} was performed, with responsiveness measured by 3H-thymidine incorporation. At each re-stimulation (days 14 and 28), a proliferative response to tTG^{ery} was detected, with the strongest response being the second stimulation for each cell line. Although the cell lines had been established using tTG^{ery}, after the first stimulation samples of cells from each cell line had the ability to proliferate in response to the SF9 insect cell-derived rh tTG antigen at levels equivalent to tTG^{ery}.

Intracellular IFN-γ production by T cells from the tTG-specific T-cell lines

Intracellular IFN-γ production in response to stimulation with tTG^{ery} was a feature of the three T-cell lines (Fig. 3a). The strongest increase of CD3/IFNγ+ cells in response to tTG exposure was seen at stimulation 2 (day 14) for each of the T-cell lines. Similar levels of cytokines were produced, when rh tTG was substituted for tTG^{ery} (Fig. 3b).

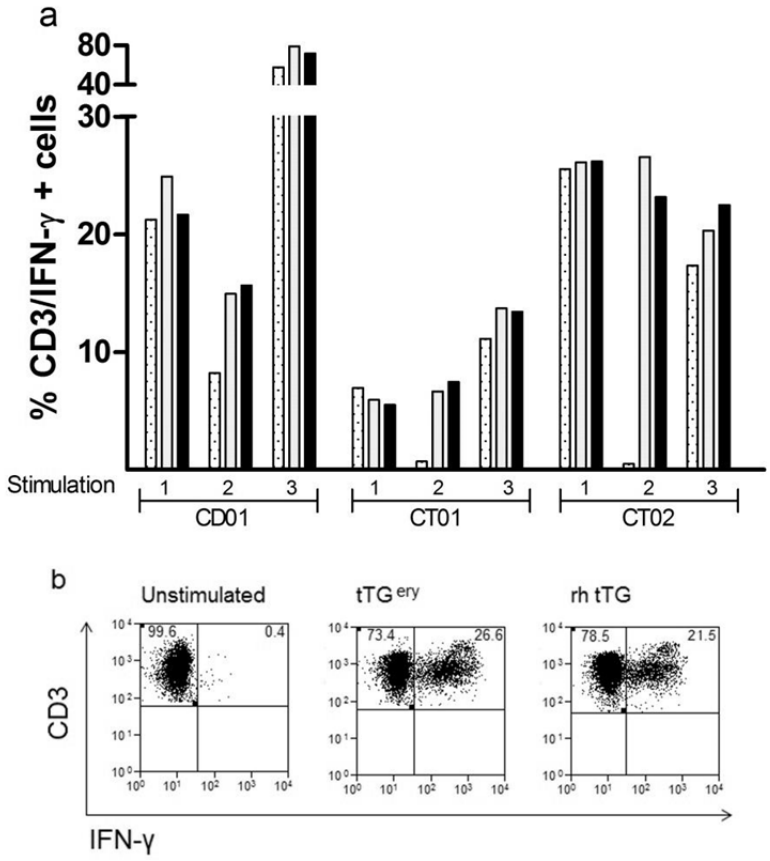

Figure 3. *Intracellular IFN-γ production by tTG-specific T cells. The production of IFN-γ by T cells from each cell line was measured by intracellular cytokine staining (a). Grey bars = erythrocyte tTG, black bars recombinant human tTG. Background levels (dotted white bars) were calculated using unstimulated PBMCs (stimulation 1), or cells from each cell line and feeder cells only (stimulations 2 and 3). Stimulations 1, 2, and 3 represent days 6, 14, and 28 of culture. The data generated from such a typical experiment is shown in (b), which details stimulation 2 of the CT02 T-cell line*

Cytokine secretion by tTG-specific T-cell lines

ELISA measurement of secreted cytokines revealed that IFN-γ was the dominant cytokine secreted by each T-cell line, with the highest levels detected in the culture supernatants of the CD01, the treated CD patient-derived cell line (Fig. 4a). This reached a peak in the week following the first re-stimulation with tTG^{ery}. In this cell line, IL-10 secretion was detected in the first two weeks of culture only, with IL-17A and IL-21 secretion observed throughout the culture period.

The first control-derived T-cell line, CT01, produced lower levels of IFN-γ and high levels of IL-10 and IL-17A, all of which reached a maximal level following the first re-stimulation of the cell line with tTG^{ery} (Fig. 4a,b,c). The second control-derived T-cell line, CT02, produced moderately large amounts of IFN-γ, IL-10, and high levels of IL-21, all of which peaked after the first re-stimulation with tTG^{ery} (Fig. 4a,b,d); this

cell line produced no IL-17A (Fig. 4c). No IL-4 was detected in the culture media of any of the three tTG-specific T-cell lines (data not shown).

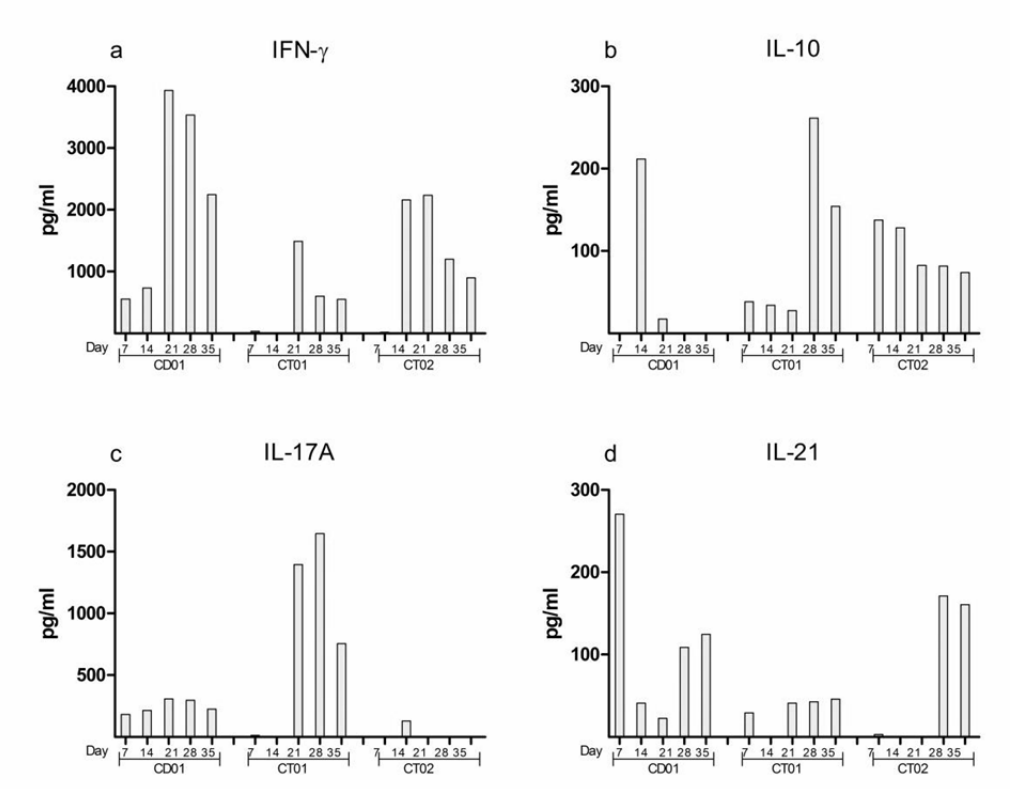

Figure 4. *Cytokine secretion by the tTG-specific T-cell lines. Samples of each culture supernatant were removed weekly and analysed for the presence of secreted cytokines by ELISA. On days 14 and 28, supernatants were removed prior to the re-stimulation of each cell line*

Conclusions

This study describes the isolation of tTG-specific T cells from the peripheral blood of treated CD patients and in some normal control subjects. This finding is in keeping with many publications demonstrating the presence of autoantigen-reactive T cells responding to a range of self-antigens. When these T cells were maintained in longterm culture, a range of cytokines were produced with IFN-γ being particularly prominent. Although not yet explored, it is conceivable that tTG-reactive T cells play a role in the pathogenesis of the coeliac lesion.

Acknowledgements

This research was funded by the Children's Medical Research Foundation, Dublin, Ireland. The authors acknowledge the kind gifts of erythrocyte transglutaminase from Walter Binder, Innova Diagnostics, San Diego, California, and the monoclonal antibody SPV-L3 from Dr. Hergen Spits, University of Amsterdam.

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5.5 Detection of gluten immunogenic peptides (GIP) in stools as a method of monitoring the gluten-free diet in children

Simona Gatti[1], Alice Guazzarotti[1], Sara Quattrini[1], Tiziana Galeazzi[1], Carlo Catassi[1]

[1] Università Politecnica delle Marche, Ancona, Italy

Introduction

Methods of evaluating the compliance to the gluten-free diet (GFD) include clinical, serological and histological tests, but currently a specific, non-invasive and standardised method is lacking. A recent study has shown that a significant part of α-gliadin 33-mer (33Eps) is resistant to gastrointestinal (GI) digestion [1]. The G12 and A1 monoclonal antibodies (mAbs) against the main immunogenic epitope of the α-gliadin 33-mer, already proven to successfully detect toxic peptides in food samples [2-3], have recently been tested to quantify immunogenic peptides in faeces. A G12 competitive ELISA test has been shown to easily quantify traces of gluten in faeces and furthermore a recent study [1] has demonstrated that: 1. the faecal amount of gluten reflects the ingested quantity; 2. gluten peptides become undetectable after 3-4 days of GFD and appear on day 3 during a gluten challenge.

We aimed to investigate the clinical usefulness of the new fecal test in children with coeliac disease (CD) and to compare this new method with traditional methods of evaluating the adherence to the GFD.

Materials and methods

CD children on a GFD for at least 6 months, healthy children on a normal diet and healthy controls on a GFD for one week were enrolled. A 3-day food diary (including report of quantities and brands of all the ingredients) was used to monitor the diet before enrollment. According to the diary, three classes of contamination risks (no evidence of contamination, possible risk of contamination, clear evidence of contamination) were identified. Furthermore, we evaluated the overall adherence to the standards suggested by the Italian Coeliac Society on the supply, preparation, and consumption of the GF foods, using a 16-point questionnaire. Evaluating the percentage of correct responses, adherence to the national standards was scored in three classes: excellent (> 80%), intermediate (60-80%) and poor (< 60%). Gastrointestinal symptoms were evaluated through the Gastrointestinal Symptoms Rating Scale (GSRS) [4] and coeliac serology (tTG IgA and DGP IgG antibodies) was collected within 1 month from the enrollment. The competitive ELISA iVYLISA GIP (Biomedal Diagnostic, Sevilla) designed to detect and quantify gluten immunogenic peptides (essentially peptides related to the 33-mer) and based on the G12 antibody

was used to analyse the stool samples collected after 3 days of food-record. The kit contains six standards (100, 50, 25, 12.5, 6.25, 3.12 ng/mL GIP) and the lower quantitation limit of the assay is 312 ng GIP/g sample (for a sample dilution of 1:10). Correlations between symptoms, food diary, and questionnaire analysis were analysed.

Results and discussion

Seventy-two CD children (mean age: 10.63 ys, SD: 4.78 ys), 16 controls on a normal diet (mean age: 7.97 ys, SD: 4.66 ys) and 4 healthy volunteers (medical doctors already trained on the GFD) following a GFD for at least one week were enrolled. Demographical and clinical data are outlined in Tab. 1.

Table 1. *Demographical and clinical features of the study group.*

Patients	**Age, years (mean ± SD)**	**Sex**	**GFD, years (mean ± SD)**	**National Celiac Society members (%)**
CD on a GFD (N=72)	10.63 (± 4.78)	20 M 52 F	3.43 (± 2.84)	67.27
Controls on a normal diet (N=16)	9.43 (± 6.45)	8 M 8 F	-	-
Controls on a GFD (N=4)	29.2 (± 3.2)	4 F	-	-

In CD children, the mean GFD duration was 3.43 ys (SD: 2.84 ys). Evaluation of the compliance to the GFD (including serological, 3-day food diary and questionnaire results) is summarised in Tab. 2.

Table 2. *Evaluation of adherence to the GFD measured by serological data, 3-day food diary and questionnaire.*

Adherence to the GFD	**Serology (IgA tTG and/or IgG DGP)**	**3-day food record**	**16-point questionnaire (based on the standards suggested by the National Coeliac Society)**
Good	Negative: 64%	No risk of contamination: 34%	Excellent adherence: 53%
Poor	Positive: 36%	Possible risk of contamination: 56% Evidence of contamination: 10%	Intermediate adherence: 37% Low adherence: 10%

Overall 47% of CD children were found to have detectable amounts of gluten in stools compared to 100% of controls on a normal diet. Mean GIP values in the CD group were significantly lower compared to the controls (Fig. 1).

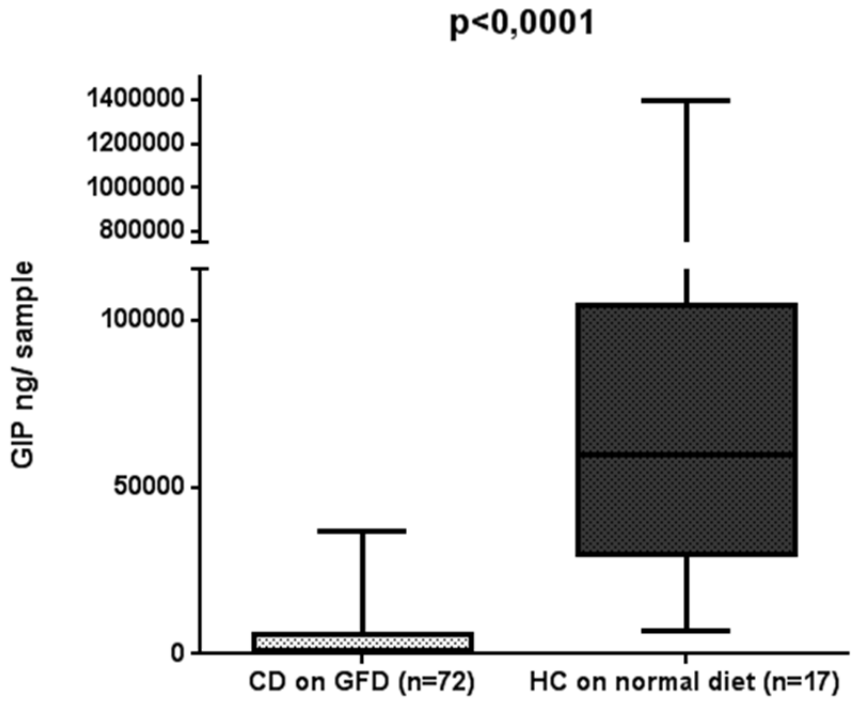

Figure 1. *Comparison between fecal GIP levels (ng/g sample) of CD children on a GFD and healthy controls (HC) on a normal diet*

No significant correlation was found between GIP levels and adherence to the diet (measured by the diary and the questionnaire). Both GI symptoms measured by the GSRS score and levels of "coeliac autoantibodies" were found to be positively correlated with GIP values (Fig. 2).

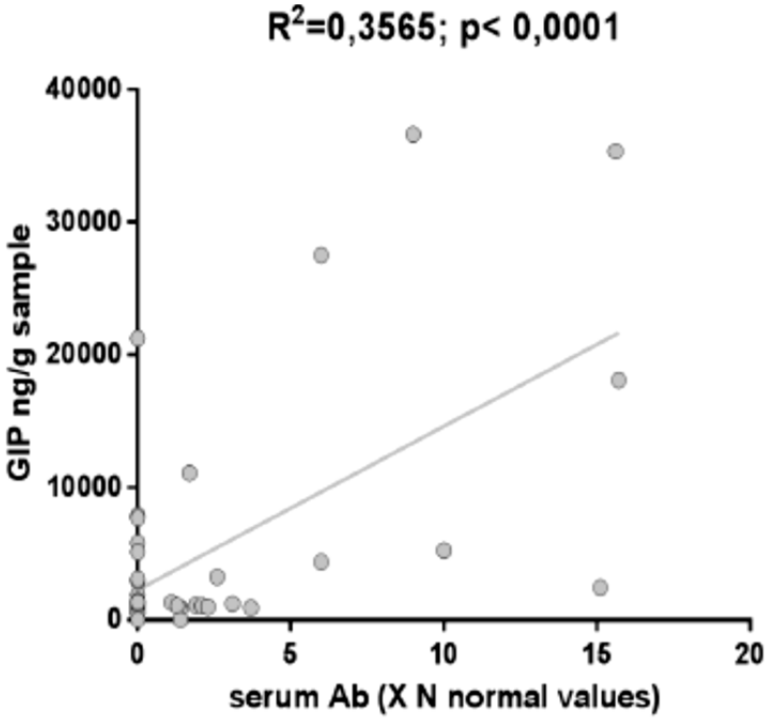

Figure 2. *Positive correlation between faecal GIP levels and serum antibodies in coeliac patients*

Analysing serial samples collected from the group of healthy volunteers during 7 days of GFD, further results were obtained: 1. In some subjects faecal gluten disappeared more slowly than expected (more than 3 days, as previously described [1]) 2. Some subjects continued to eliminate gluten despite the GFD, 3. Levels of GIP in stools can vary more quickly than previously thought (Fig. 3).

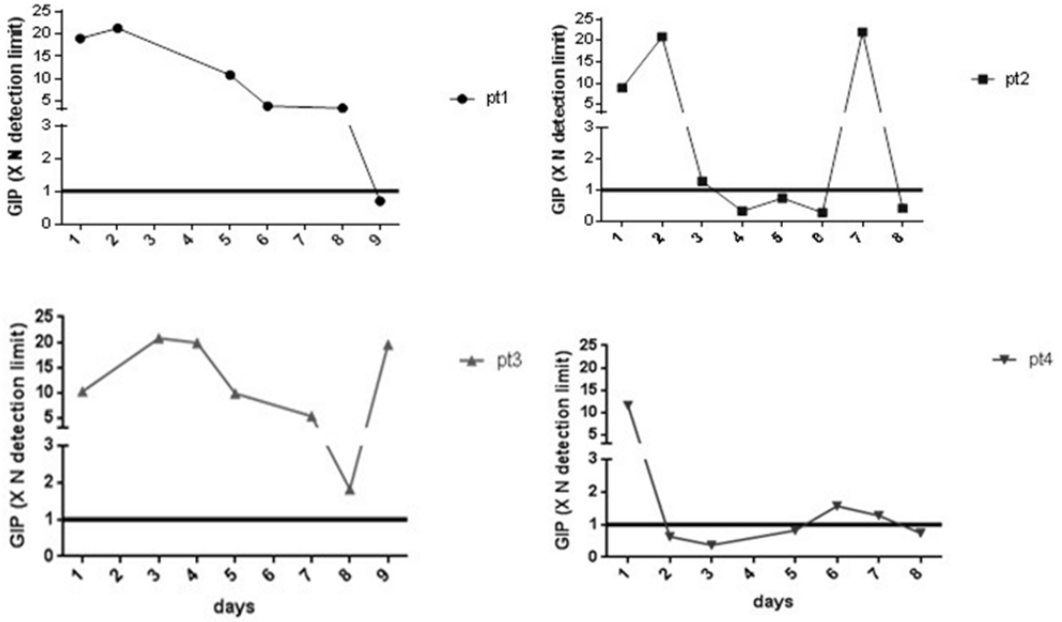

Figure 3. *Time to elimination of ingested gluten in the 4 healthy controls well-trained on the GFD*

These final results raised some questions about the specificity of the test and the need to consider possible confounding environmental factors occurring during sample collection or the analysis.

Conclusions

The iVYLISA GIP test is a non-invasive, very sensitive, and promising test to assess the compliance to the GFD, especially in children. Our results show that a high percentage of CD children have detectable traces of gluten in faeces. This may indicate incomplete adherence to the GFD and furthermore, we found a significant correlation with both clinical and serological data. Our preliminary findings need to be replicated in other centres and possibly compared to a larger group of healthy controls. However, the presence of gluten in control samples (collected from well-trained subjects on the GFD) could reflect a low sensitivity of the test. The technique itself is not particularly challenging, but the analysis is quite long (5 - 6 hours) and can present some minimal technical problems.

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6 Antigen receptors in coeliac disease

6.1 Molecular basis for T-cell receptor recognition of HLA-DQ-gluten in coeliac disease

Frits Koning

Department of Immunohematology and Blood Transfusion
Leiden University Medical Centre, Leiden, The Netherlands

Introduction

Gluten-specific T cells are characteristic for coeliac disease (CD) [1]. Here, I briefly describe the latest observations related to the actual T-cell receptor repertoire that is associated with this disease-causative T-cell response. In addition, a novel technological advance allows system-wide analysis of the immune system with unprecedented resolution. I briefly describe this technology and the implications it may have for the discovery of tissue- and disease-specific immune signatures.

The gluten-specific T-cell response

CD is characterised by the presence of gluten-specific CD4 T cells in the lamina propria of the affected organ: the small intestine [1]. Typically, such T cells respond to modified gluten peptides bound to the disease-predisposing HLA-DQ2 or HLA-DQ8 molecules. The modification of gluten peptides involves the conversion of one or more glutamine residues in gluten peptides into the negatively charged glutamic acid residues and this is driven by the activity of the enzyme tissue transglutaminase [2,3]. The introduction of one or more negative charges in gluten peptides facilitates the binding to HLA-DQ2 or HLA-DQ8 and is thus a likely key step towards the development of full-blown disease. Although it is not exactly known where this modification takes place, it is likely to be in the affected tissue itself, as tissue tranglutaminase is released upon stress and tissue damage.

Gluten is well known to be a complex mixture of α-, γ-, and ω-gliadins and low-molecular-weight and high-molecular-weight-glutenins. Immunogenic peptides have been identified in all of these proteins, the majority of which is restricted by HLA-DQ2, the major disease-predisposing allele [4]. Such HLA-DQ2-restricted epitopes are usually found in proline-rich regions in gluten proteins, protecting them from enzymatic degradation in the gastrointestinal tract [5]. In contrast, HLA-DQ8 epitopes are fewer and generally more sensitive to degradation [5]. Thus, it is likely that relative to HLA-DQ8, HLA-DQ2 will present a larger repertoire and higher numbers of gluten peptides and this may underlie the larger disease risk conferred by this HLA-DQ allele [6].

Despite the fact that many antigenic gluten epitopes exist, some appear more relevant, because they are immunodominant, i.e., recognized by T cells from the majority of patients. There are now several publications documenting that the T-cell receptor repertoire, specific for such immunodominant gluten peptides, is biased, as it is dominated by the expression of particular T-cell receptor Vβ- and/or Vα-gene segments [7-9]. The potential T-cell receptor repertoire is estimated to exceed 10^{15}, allowing the generation of a vastly diverse T-cell compartment to cope with the plethora of surrounding pathogens. The chance that two randomly selected T cells express a similar T-cell receptor is, therefore, essentially zero. Nevertheless, T cells specific for immunodominant gluten peptides, isolated from unrelated patients, were found to express (nearly) the same T-cell receptor Vβ and/or Vα chains, indicating strong selection and expansion of T cells expressing such receptors in patients with coeliac disease. While in HLA-DQ2 positive patients such T cells often express the T-cell receptor TRBV7-2 gene segment [7,9], in HLA-DQ8-associated disease the TRBV9 gene segment is overrepresented [8]. Strikingly, in such T-cell receptors a non-germline encoded arginine residue is present in either the CDR3α or CDR3β region. As the CDR3 regions are generated during the recombination process, the presence of the non-germline encoded arginine is thus the result of the recombination process and a further indication that there is strong selection for particular T-cell receptors in the pathogenesis of CD. Structural studies show that this arginine plays a critical role in the interaction between the gluten-specific T-cell receptors and the HLA-DQ-gluten complexes, as it is positioned directly above the HLA-bound gluten peptides, where it makes contact with both the HLA-DQ molecule and the bound gluten peptide [8,9]. Moreover, these structures reveal the molecular basis for the selective usage of particular TRBV gene segments, because germline encoded residues in the V-regions were found to make critical interactions with HLA-DQ-gluten as well.

What remains to be established is the T-cell receptor repertoire specific for the less immunodominant gluten peptides and their role in the disease process. An important issue is whether such T cells are merely bystanders and relatively harmless or by themselves sufficient for the induction of disease symptoms. In other words, if the T cells specific for the immunodominant gluten epitopes, would be eliminated, would that constitute a cure or would the remaining repertoire of T cells still lead to disease upon gluten consumption? If the former is the case, novel strategies to eliminate T cells to immunodominant gluten peptides might constitute a cure for CD.

For details please see references 7-9.

High-dimensional analysis of the mucosal immune system

Cells of the immune system play a crucial role in the defense against pathogens as they survey tissues for the presence of abnormalities, such as infection by bacteria/viruses. However, there are many disease conditions, where the immune system does not function properly. These include conditions, where the T-cell response is wrongly

directed, e.g., where T cells destroy healthy cells in autoimmune diseases including type 1 diabetes, rheumatoid arthritis, and CD.

The analysis of cell surface protein expression patterns by fluorochrome-based flow cytometry has contributed greatly to our understanding of cellular heterogeneity and cellular differentiation for cell types within the hematopoietic system and beyond. Flow cytometry has become a benchmark technology for single cell analysis in both diagnostics and research. Each gradual technical improvement that has resulted in an ability to measure a larger number of cellular markers in parallel, has been adopted by the research community very rapidly. In practice, however, this technique has reached a technical plateau, as the spectral overlap between fluorochromes limits the number of markers that can simultaneously be monitored. To escape this, plateau flow cytometry has now been combined with mass spectrometry, termed mass cytometry. In mass cytometry, the fluorochrome tags are replaced by a series of rare earth elements (e.g., lanthanides), which are attached to antibodies through metal-chelator coupling reagents [10]. Cells are labelled by incubation in a cocktail of tagged antibodies; as the cells flow through the instrument, they are atomised at 5500 K, and the released tags are identified and quantitated by time-of-flight mass spectrometry (MS). Rates are reasonable, at 1000 cells/s. The beauty of the approach stems from three factors: the precision of MS detection, which eliminates overlap between tags; the number of detectable markers (36 at present but up to 100 in the future); and the absence of background noise.

We have now developed a 32 antibody panel that has been specifically designed to delineate the heterogeneity of all immune subsets in the intestinal mucosa. Our preliminary data demonstrate that this initial antibody panel can be applied to cell suspensions isolated from intestinal biopsies of patients with Crohn's disease, CD and controls. Due to the multidimensionality of the resulting data, the results cannot be analysed effectively with conventional flow cytometry tools (FACSDiva, FlowJo), but require dedicated, often cloudbased, software packages, which are all operational. Together, these methods make it possible to characterise the composition of the mucosal immune system at unprecedented resolution. Thus, with dedicated antibody panels it is feasible to obtain comprehensive information on the composition and characteristics of the immune system in health and disease. Our preliminary results indicate that both tissue- and disease-specific immune signatures can be detected. Further optimisation of this approach may lead to the identification of disease-associated biomarkers and improved diagnostics and prognostics.

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7 Statements by participating organisations, representatives from industry, and guests

7.1 Current regulatory status on gluten-free claims

Johan De Meester[1]

[1] Cargill R&D Centre Europe, Vilvoorde, Belgium

Introduction

Codex Standard 118-1979, revised in 2008, sets a standard for foods for special dietary use for persons intolerant to gluten. Similarly and applying from 1st January 2012 onwards, Regulation (EC) 41/2009 sets compositional and labelling standards for foods claiming to have low gluten content, the allowed claims being "gluten-free" and/or "very low gluten." Gluten-free products can be foods specially produced, prepared and/or processed to meet the special dietary needs of people intolerant to gluten, as well as conventional products, but in all cases their gluten content shall not be higher than 20 parts per million (mg/kg) gluten.

"Very low gluten" claim foods must be specially prepared and/or processed to meet the special dietary needs of people intolerant to gluten. These are foods containing ingredients made from wheat, rye, barley, oats, or their crossbred varieties that have been specially processed to remove gluten. Very low gluten food must contain no more than 100 mg/kg gluten. Very low gluten food may also bear a gluten-free claim when meeting the 20 mg/kg threshold.

The EU legislator has recasted the current legislation on food for particular nutritional uses, which resulted in a transfer of the current regulatory standard for very low gluten and gluten-free claims under the scope of the Food Information Regulation (EU) 1169/2011.

In the following paragraphs, an update is provided on the significant legislative changes and steps that were required to transfer the provisions on gluten-free claims from Regulation (EC) 41/2009 to the Food Information Regulation (EU) 1169/2011.

Also attention is provided to compare the gluten-free claims in different geographies with the current applicable gluten-free provisions in relation to the Codex Standard 118-1979 (rev. 2008). The goal was to provide an overview, in which geographies gluten-free claims are introduced in line with the Codex Standard 118-1979 (rev. 2008).

Steps taken by the EU Commission on recasting gluten-free claims

Commission Regulation (EC) No 41/2009 of 20 January 2009 [1], concerning the composition and labelling of foodstuffs suitable for people intolerant to gluten sets out

harmonised rules on the information that is provided to consumers on the absence or reduced presence of gluten in food. In 2012, the EU Commission proposed to recast Directive 2009/39/EC [2], which establishes the legislative framework for foodstuffs for particular nutritional uses, so-called 'dietetic foods'.

The EU Commission envisaged thus to cover foodstuffs for persons, who are gluten-intolerant under the regulation for particular nutrition. If gluten-free claims would no longer be regulated under Regulation (EC) 41/2009, they would need to be compliant with the provisions of the Regulation (EC) No 1924/2006 [3], which is for healthy people following a nutritional profile. A number of stakeholders did not perceive this as the way forward [4], since the HNC Regulation is not the right legislation for addressing the needs of people with particular nutritional requirements. Coeliac patients but also any category of consumers would not profit from such a proposed recasting of the legislation. Coeliac patients require a certain diet to remain healthy.

On 29 February 2012, the EP ENVI Committee voted to support significant amendments on the regulation for particular nutrition. With regard to food, suitable for people intolerant to gluten, the Committee proposed that these statements should be regulated solely by the Food Information Regulation (EU) 1169/2011 [5].

Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers sets out rules on information to be provided for all food, including non prepacked food, on the presence of ingredients, such as gluten-containing ingredients, with a scientifically proven allergenic or intolerance effect in order to enable consumers, particularly those suffering from a food allergy or intolerance, such as gluten intolerance, to make informed choices, which are safe for them.

Regulation (EU) No 609/2013 of the European Parliament and the Council of 12 June 2013 [6] on food intended for infants and young children, food for special medical purposes, and total diet replacement for weight control introduces a new legal status for labelling of foodstuffs suitable for people intolerant to gluten.

Directive 2009/39/EC and Regulation (EC) No 41/2009 will be repealed by Regulation (EU) No 609/2013 of the European Parliament and of the Council of 12 June 2013 on food intended for infants and young children, food for special medical purposes, and total diet replacement for weight control. Nevertheless, consumers should continue to be appropriately informed and not misled or confused, when information on the absence or reduced presence of gluten in foods is provided by food business operators after the repeal of Regulation (EC) No 41/2009. To this end, it is necessary to transfer the existing rules under a different legal framework. In this context, Regulation (EU) No 609/2013 foresees that for the sake of clarity and consistency, the rules on the use of the statements 'gluten-free' and 'very low gluten' should be regulated under Regulation (EU) No 1169/2011.

The provisions concerning these products shall be transferred to Regulation (EU) No 1169/2011 on the provision of food information to consumers, guaranteeing

consumers the same levels of protection offered by the current Regulation (EC) No 41/2009 concerning the composition and labelling of foodstuffs suitable for people intolerant to gluten.

The European Commission shall, therefore, firstly adopt a delegated act to modify Chapter 3 of Article 36 of Regulation (EU) No 1169/2011. This modification shall enable the Commission, via an implementation act in a subsequent phase, to establish the requirements applicable to the information on the absence or reduced presence of gluten in food products.

Article 36(2) of Regulation (EU) No 1169/2011 requires that information, provided by food business operators, shall not mislead the consumer, shall not be ambiguous or confusing for the consumer and shall, where appropriate, be based on the relevant scientific data. To that effect, Article 36(3) of that Regulation requires the Commission to adopt implementing acts on the application of the requirements referred to in paragraph 2 of the same Article in certain specific cases identified therein.

In order to ensure that consumers are appropriately informed and not misled or confused by information, provided by food business operators on a divergent basis, Article 36(4) of that Regulation provides for the possibility for the Commission to supplement, by means of delegated acts, Article 36(3) with additional cases of provision of food information, for which the Commission shall adopt implementing acts on the application of the requirements referred to in Article 36(2).

This Delegated Regulation amends Article 36(3) of Regulation (EU) No 1169/2011 by supplementing it with the addition of a new letter (d): 'information on the absence or reduced presence of gluten in food'.

Following its adoption, the Commission intends to prepare an implementing act on the basis of amended Article 36(3) of Regulation (EU) No 1169/2011 in order to transfer the rules of Commission Regulation (EU) No 41/2009 under the new legislative framework of Regulation (EU) No 1169/2011.

Commission Delegated Regulation (EU) No 1155/2013 of 21 August 2013 [7], amending Regulation (EU) No 1169/2011 of the European Parliament and of the Council on the provision of food information to consumers as regards information on the absence or reduced presence of gluten in food.

The Commission has subsequently published the Commission Implementing Regulation (EU) No 828/2014, of 30 July 2014 [8], on the requirements for the provision of information to consumers on the absence or reduced presence of gluten in food, establishing the criteria that enable the use of the terms “gluten-free” and “very low gluten” in food. These statements could also include "suitable for people intolerant to gluten" or "suitable for coeliacs". In the case of foods specifically produced, prepared and/or processed to meet special nutritional requirements of the coeliac community, the use of additional expressions such as "specifically formulated for people intolerant to gluten" or "specifically formulated for coeliacs" is possible.

This Regulation shall be applicable from 20 July 2016, when Regulation (EC) No 41/2009, of 20 January 2009, concerning the composition and labelling of foodstuffs suitable for people intolerant to gluten, will be abrogated. Also Parnuts Framework Directive 2009/39 will be abolished on 20 July 2016.

Gluten-free claims in other geographies

Codex Standard for foods for special dietary use for persons intolerant to gluten 118-1979 (rev. 2008) [9] foresees in contradiction to Commission Regulation (EC) No 41/2009 of 20 January 2009 a number of analytical details. Codex 118-1979 (rev. 2008) points out that the antibody, used for gluten analysis in foods, should react with cereal protein fractions toxic for persons intolerant to gluten – and not cross-react with other cereal proteins or other constituent of foods/ingredients. The detection limit should be appropriate according to the state of the art. It should be 10 mg gluten/kg or below. Reference is made to the enzyme-linked immunoassay (ELISA) R5 Mendez method.

In other geographies, also limits are set for gluten-free and low in gluten. In a number of geographies and countries, regulatory references are found, which establish levels for gluten-free and low in gluten. In Tab. 1, some more details are summarised.

Only in Europe we have found provisions in regulations that are mirroring the gluten-free and low in gluten limits as set out in the Codex Standard. Canada and USA have also a limit on gluten-free of 20 mg/kg or less.

Table 1. *Limits for gluten-free and low in gluten in different geographies.*

Geography	Regulatory reference	Gluten-free [mg/kg]	Low in gluten [mg/kg]
Codex	STAN 118-1979 - rev. 2008	20	20 - 100
Argentina	Disposición 2574/2013	10	
Australia (FSANZ)	Issue 103, Standard: 1.2.8 Clause 16	< 3	20
Canada (FDR)	Section B.24.018	20	
Europe	Regulations 41/2009 & 1169/2011	20	100
USA	FDA Final Rule 05 August 2013	20	

Division 24 of the Food and Drug Regulations (FDR) from Health Canada sets out specific regulations that apply to "Foods for Special Dietary Use". As of August 4, 2012, section B.24.018 of the Food and Drug Regulations will state that: “It is prohibited to label a food in a manner likely to create an impression that it is a gluten-free food, if the food contains any gluten protein or modified gluten protein, including any gluten protein fraction, referred to in the definition "gluten.” [10,11].

In August 2004, the FDA has provided for a clear regulatory framework on allergens referred to as FALCPA (Food Allergen Labeling and Consumer Protection Act - Public Law 108-282, Title II) [12]. In 2007, the FDA outlined a proposed definition for “gluten-free” and solicited feedback. So it took the FDA almost a decade to

provide for the necessary direction on the labelling of gluten-free products. On 5th August 2013, FDA issued a final rule to define the term “gluten-free” for voluntary use in the labelling of foods. Establishing a definition of the term “gluten-free” and uniform conditions for its use in food labelling will help ensure that American consumers with coeliac disease are provided truthful and accurate information with respect to foods labelled gluten-free. Gluten-free labelling (< 20 mg/kg) for foods is accepted to carry the label “gluten-free,” “no gluten”, “free of gluten”, or “without gluten” without special preference of the wording for the specific labelling. The final rule issued by FDA gave a deadline of compliance by 5th August 2014 [13].

Argentina has in Disposición 2574/2013 defined 'gluten-free' as gluten in food below 10 mg/kg. This decree is part of the foods code in Argentina (Artículo 1383 del Código Alimentario Argentino).

Australia and New Zealand have two limits described in the Food Safety Australia New Zealand code (FSANZ). Gluten-free products should have less than 3 mg/kg of gluten, which is the limit of detection of the R5 antibody method. Products low in gluten in Australia and NZ should be not more than 20 mg/kg.

Conclusions

The EU Commission has established in Regulation (EU) No. 609/2013 that the rules on the use of the statements ‘gluten-free’ and ‘very low gluten’ should be in the future regulated under the FIR, in particular under Article 21 of the FIR on the labelling of certain substances or products causing allergies or intolerances listed in Annex II to the FIR. Regulation (EU) No. 609/2013 also sets out that the legal acts to be adopted pursuant to Regulation (EU) No. 1169/2011, which are to transfer the rules on the use of the statements ‘gluten-free’ and ‘very low gluten’, as contained in Regulation (EC) No. 41/2009, must ensure at least the same level of protection for people that are intolerant to gluten as currently provided for under Regulation (EC) No. 41/2009. That transfer of rules should be completed before 20 July 2016.

Furthermore, the EU Commission must consider, according to Recital 41 of Regulation (EU) No. 609/2013, how to ensure that those, who are intolerant to gluten, are adequately informed of the difference between a food that is specially produced, prepared and/or processed in order to reduce the gluten content of one or more gluten-containing ingredients and other food that is made exclusively from ingredients naturally free of gluten.

At the international level, the Codex Standard for foods for special dietary use for persons intolerant to gluten not only addresses the use of ‘gluten-free’ labels, but it also sets requirements for the use of the statement ‘this food is by its nature gluten-free’. An equivalent provision must be adopted in the EU before 20 July 2016.

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7.2 Towards the production of reference materials for food allergen and gluten-free analysis for improved food safety management

Peter Koehler[1], Lívia Hajas[2], Sándor Tömösközi[2], Roland Poms[3]

[1] *Deutsche Forschungsanstalt für Lebensmittelchemie, Leibniz Institut, Freising, Germany*
[2] *Budapest University of Technology and Economics, Department of Applied Biotechnology and Food Science, Budapest, Hungary*
[3] *MoniQA Association, Vienna, Austria*

Introduction

It is one of the aims of MoniQA - Monitoring and Quality Assurance in the Total Food Supply Chain (www.moniqa.org) - the global food safety network registered as MoniQA Association, to provide guidelines for method validation, reference materials and to assess the reliability of methods through validation studies and proficiency testing schemes. In 2013, MoniQA initiated a task force on the development of reference materials for food allergen and gluten analysis. The task force is an international group comprised of several standardisation organisations (SDOs), industry representatives, policy makers, test kit providers, method developers, analytical companies, as well as representatives from research groups. The chairman of the Working Group on Prolamin Analysis and Toxicity (PWG) is part of the group. Three aims are in the focus of the task force: (i) To develop, prepare, and provide well-characterised commodity materials for the further production of incurred reference materials, spiked samples, and extracts; (ii) to generate and publish a guidance document on the special requirements and production of allergen reference materials; (iii) to seek general acceptance of the reference materials and the guideline by the international community (e.g., EU authorities, Codex Alimentarius, AOAC International). Priority commodities were identified as being gluten, milk, egg, peanut, hazelnut, and soy. This short overview reports the current status of the production of gluten reference materials.

Materials and methods

Grain material was sourced from all over the world to get a broad spectrum of genetic and technological variants. Twenty-one wheat, two rye, and four barley varieties from Australia, Austria, Canada, China, Germany, and Hungary were compiled. The grains were milled into white flour on a laboratory mill (Quadrumat Junior, Brabender, Germany) and characterised (Fig. 1). Among the different methods were NIR spectrometry (basic composition), analysis of the crude protein content (N x 5.7)

according to the method of Dumas, determination of the wet and dry gluten content after washing of the dough by a glutomatic, one-dimensional SDS-PAGE to determine the composition of protein subunits, and extraction/RP-HPLC to quantitate the composition of protein fractions and protein types. The ELISA response of flours will be determined using different test kits. Model matrices incurred with selected individual varieties will be prepared and analysed using several ELISA kits.

In parallel with collecting and characterising the varieties, a preliminary experiment was performed to estimate the effects of genetic and environmental variability of wheat on the ELISA measurements. Three flour mixtures were prepared (mixture 1: Mv-Magvas, Mv-Mazurka, Mv-Verbunkos, Yumai-34, Dekan from the harvest year 2011; mixture 2: same as mixture 1 but from the harvest year 2012; mixture 3: Mv-Magvas, Bezostaja-1, Hereward, Soissons, Glenlea from the harvest year 2012) and compared to flour from one wheat variety (Mv-Magvas, harvest years 2011 and 2012, respectively). The ELISA responses were determined in unprocessed and processed model matrices using RIDASCREEN® Gliadin (R7001, R-Biopharm AG), AgraQuant® Gluten Assay (COKAL0248, Romer Labs), and AgraQuant® Gluten G12 Assay (COKAL0200, Romer Labs).

Results and discussion

The idea of the initiative was to produce incurred materials, extracts at different gluten concentrations, and model foods, e.g., a rice cookie with exactly specified gluten content. For this purpose, a selection of grain samples should be milled to obtain minimally processed starting materials (flours), which had to be thoroughly characterised for as many as possible ingredients, in particular for their gluten content. The intended workflow is shown in Fig. 1.

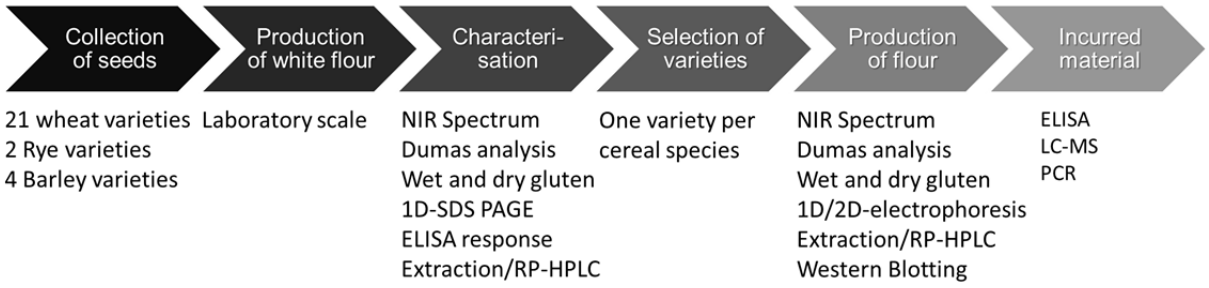

Figure 1. *Schematic representation of the workflow for the development of reference materials for gluten analysis*

Challenges and questions had to be faced in the beginning. For example, should one or a mixture of varieties be used for flour production, was the geographic origin important, was white flour or whole grain flour more suitable? White flour is stable and appropriate for gluten detection, whereas whole grain flour additionally contains putative allergens from the outer layers (e.g., aleurone), but is less stable. Therefore, periodic quality control analysis and stability testing is required during storage.

Defined amounts of wheat flours made from one variety or from a mixture of five varieties as well as PWG gliadin were added to a cookie recipe. PWG-gliadin was used as a reference material. The blends of dry raw materials as well as baked cookies were analysed using three ELISA kits and the recoveries based on the nominal gliadin concentrations were determined. The nominal gliadin concentration was calculeted according to the weight of added flours to the cookie recipe and the gluten content of flour determined by the Glutomatic System. The results are shown in Fig. 2. Gliadin recovery was strongly dependent on the ELISA kit used for analysis. Whereas Kit A and Kit B yielded comparable results, Kit C provided gluten concentrations that were approximately 3.5 times higher than the target values. However, the experiment also showed that there were only small differences in the ELISA response between samples from one variety and mixtures of varieties. Based on this result, it can be assumed that it is sufficient to use only one variety as the basis for reference material production. The first criterion for the selection of a variety was based on SDS-PAGE. A suitable variety should have five high-molecular-weight glutenin subunits (HMW-GS) and a typical pattern of ω-gliadins. No significant differences were identified in the band pattern of low-molecular-weight glutenin subunits (LMW-GS) and gliadins. The second criterion was the RP-HPLC analysis. Typical gliadin and glutenin patterns as well as a typical gliadin/glutenin ratio were considered. Based on the results, it will be decided whether one variety or a mixture will be used for further work.

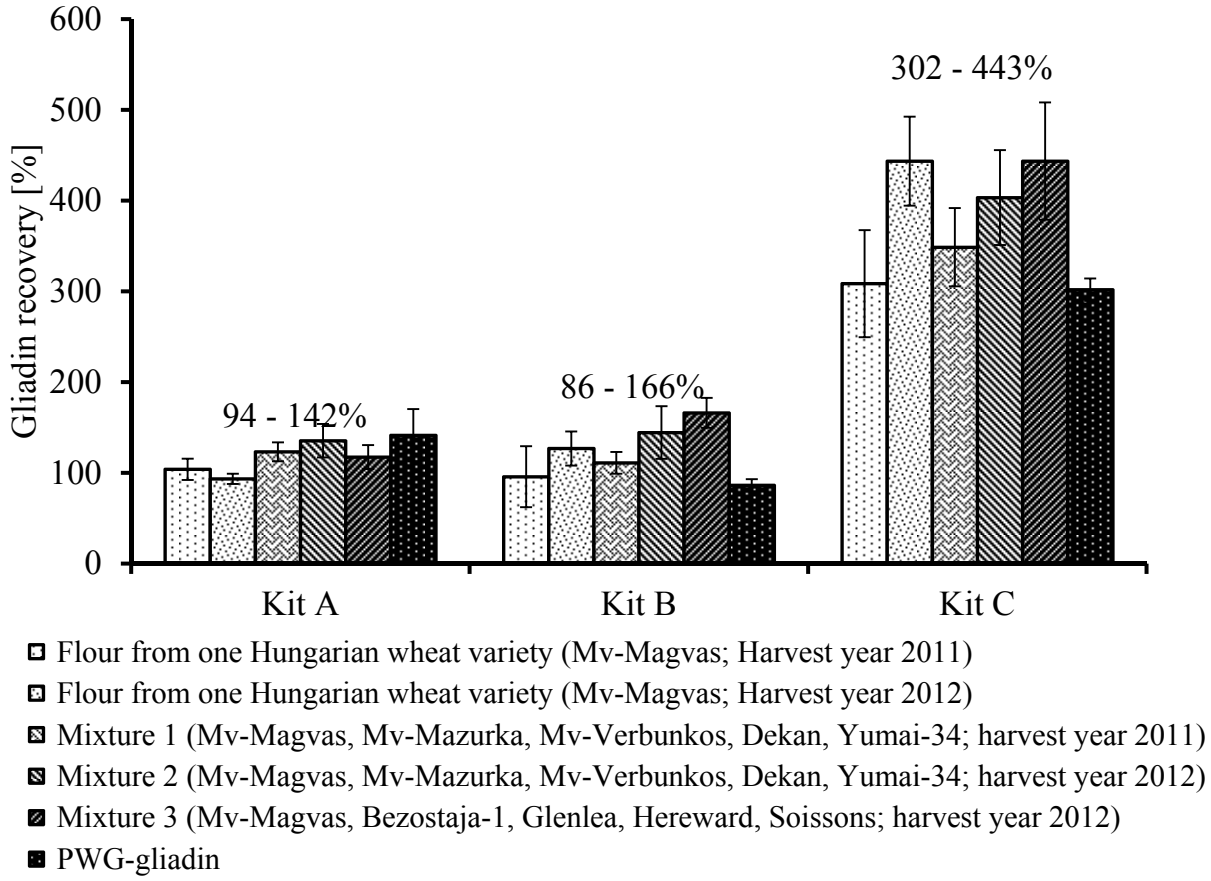

Figure 2. *Recovery of gliadins from different wheat flours and flour mixtures in the blend of dry ingredients of cookie recipe using three different ELISA kits*

Outlook

Rye and barley varieties for reference material production will be identified as described for wheat. A rice cookie recipe will be finalised in the first half of 2015 so that incurred reference materials with given gluten content can be prepared. This will be characterised in parallel by the different research institutes involved in the initiative. The first samples for research/trial purposes (small quantities) should be available through MoniQA Association in 2016. Publication of a mini-review and opinion paper as well as a guidance document on best practice production of food allergen reference materials is targeted for late 2015.

7.3 Industrial view on the approval of novel methods for gluten quantitation

Gunnar Adås, Henrik Dahlquist

Fria Gluten Free, Västra Frölunda, Sweden

There are ongoing discussions to approve further methods of analysis for the determination of the gluten content in addition to the existing Mendez R5-ELISA. There is a need for other methods to be approved for different reasons.

As a food producer, we want to highlight the possible consequences of changed or altered approved methods of analysis. If the results of novel methods do not correspond to the results of existing methods, the basis for decision-making in the industry will become more difficult and sometimes confusing. The food industry depends on long-term and stable regulations. Our worry is that the approval of novel methods might lead to future changes of the legal regulations.

Like many other food producers, Fria Gluten Free uses gluten-free wheat starch as the main ingredient in gluten-free bread. As analysed by the Mendez R5-ELISA, the gluten content is well below 20 mg/kg complying with the threshold and the analytical method required for a gluten-free claim. Consumption of food in line with this regulation has been agreed to be safe for persons with coeliac disease.

Our statement: Do not recommend any novel methods for quantitation of the gluten content, unless they have been tested using bread baked with gluten-free wheat starch and the results are comparable to those obtained with the Mendez R5-ELISA.

8 Perspectives and action plan of the PWG

Peter Koehler

Deutsche Forschungsanstalt für Lebensmittelchemie, Leibniz Institut, Freising, Germany

The Prolamin Working Group executive meeting and joint discussion held on 26th of September 2014 led to the decisions and statements outlined below.

Action plan

I. Analytical

- Peter Koehler is responsible for the PWG gliadin reference material (Peter.Koehler@tum.de).
- PWG gliadin will continue being the reference material supported by the group. Material for five to ten years is still in stock.
- Plans for research towards new reference materials have been discussed:
  - Isolation of protein reference materials from reference flours of the MoniQA initiative (end 2015/beginning 2016).
  - Possible project proposal in the Horizon 2020 program of the EU on reference materials based on synthetic peptides or proteins.
  - Dr. Roland Poms (MoniQA) will be invited to give a presentation on reference materials at the 2015 meeting.
- Collaborative studies on gluten quantitation in collaboration with AOACI and AACCI will continue.

II. Clinical

- At the 2015 meeting, a symposium on "Innate immunity and coeliac disease" will be held. Speakers will be Bana Jabri, Riccardo Troncone, and Detlef Schuppan.

III. Members, Policy

- Dr. Renate van Eckert (Wellington, New Zealand) will leave the group.
- Prof. Nadine Cerf-Bensussan has been identified as a potential new member of the group.
- The website will be updated (Fernando Chirdo).
- This printed, citable book (print run: 300 copies with ISBN number) was made possible by funding of Dr. SCHÄR GmbH/Srl, (Burgstall, BZ, Italy) and by the help of Mrs. Anneliese Stoiber and Dr. Gaby Andersen, Deutsche Forschungsanstalt für Lebensmittelchemie (Freising, Germany). It will be distributed among leaders of opinion in gluten analysis and clinical medicine.

Next meeting: 2015

We are very pleased to announce the venue for our meeting in 2015:

Tulln, Austria

Host:

Mag. Simone Schreiter
Romer Labs Division Holding GmbH
Technopark 1, 3430 Tulln, Austria
Phone: +43 664 8842 7934
Fax: +43 2272 615 3313111
E-mail: simone.schreiter@romerlabs.com

Time: October 8 – 10, 2015

Focus of the meeting:

- Innate immunity and coeliac disease
- Gluten quantitation (immunochemical/non-immunochemical)
- Gluten reference materials for analytical and clinical studies

The meeting will be limited to 50 participants and attendance is by invitation only. Invitations will be sent by April 2015. Registration deadline will be June 15, 2015.

For registration please contact:

Simone Schreiter
(address: see above)

Very special thanks to the hosts for this kind invitation!

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