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Monoclonal Antibodies to High Molecular weight Glutenin Subunits for Use in Measurement of Gluten in Foods

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Abstract

Background: Celiac disease (CD) is a heritable intolerance to gluten, the storage protein found in wheat, rye, and barley. Commercially available assays used to calculate gluten content in food do not measure CD toxic high molecular weight glutenins (HMWG), but rely on the calculation that gliadin multiplied by two equals total gluten, which is invalid.

Objective: We sought to generate monoclonal antibodies (mAbs), to known CD toxic HMWG subunits (HMW-GS), to develop improved assays to quantify CD toxic gluten in foods.

Design: mAbs were generated using recombinant HMW-GS as antigens. They were termed CDC-3 and CDC-7 and were characterised by dot blot assay and ELISA. A suitable mAb was used in competitive ELISA to quantify HMW-GS content in foods intended to CD suffers.

Results: Antibodies were raised against the important dough forming HMW-GS, 1Dy10 that cross-reacted with another important dough forming HMW-GS, 1Dx5. mAbs cross-reacted at high titre with HMW-GS extracted from fifteen varieties of European bread wheat. Competitive ELISA had a limit of detection of 625pg/ml, which is equivalent to 6.25x10 ppm in a food extract.

Conclusion: Our new antibodies react with a wide variety of wheat cultivars and can be used in sensitive ELISA assays for detection and quantification of CD toxic HMW-GS in foods intended to CD suffers. Summation of the food content of HMW-GS and gliadin permits improved quantitation of CD toxic gluten in nominally gluten-free foods.

Keywords: Celiac disease, Gluten, Wheat, High molecular weight glutenins, ELISA.

Introduction

Celiac disease (CD) affects 1:100 people in Northern Europe and the US. Ingestion of gluten leads to inflammation of the small intestinal mucosa, with damage to the villous architecture and malabsorption. This results in loss of nutrients and single or multiple deficiencies. Long-term complications include osteoporosis [1], increased prevalence of malignancy [2] and increased mortality [3]. The only treatment is a strict life-long gluten-free diet (GFD) with complete avoidance of wheat, rye and barley and their derivatives. The related cereal oats have been reported to be CD toxic in 5% of cases [4]. Other cereals, including maize, rice, millet and sorghum do not trigger CD. Removal of the majority of gluten from wheat flour yields purified wheat starch that is widely used as a basis for production of nominally gluten-free foods for individuals with CD.

Assays for the measurement of gluten in foods intended for those who are intolerant to gluten proteins are required. New standards allow <20ppm gluten in foods labelled gluten-free and 20-100ppm for foods labelled very low gluten [5] (WHO Codex Alimentarius 2003, amended 2008 and ratified 2015). Assays for the detection of gluten are essential for the food industry, however this is highly problematic. Until recently, several efforts have been concentrated on the detection and quantification of gliadins [6,7] but, as it is known, wheat gluten contains both gliadin and glutenin protein, both of which are CD toxic. The glutenins comprise two sub-units: the low molecular weight glutenin sub-units (LMW-GS) and the HMW-GS. These proteins have complex solubility characteristics, which previously limited purification of glutenin that was not contaminated with gliadin. Recombinant technology has enabled creation of pure HMW-GS protein fractions [8].

We previously reported the *in vivo* CD toxicity of chemically purified HMW-GS [8,9]. Immunogenicity of the single recombinant 1Dx5 and 1Dy10 HMW-GS, the two important dough-forming subunits, have been demonstrated before. Within wheat varieties, gliadin forms 58-77% of total gluten and glutenins 26-38% [10]. It was previously thought that the total gluten content of a foodstuff could be estimated by multiplying the gliadin content by two. Indeed, this system

is still universally applied, however, even based on the figures above, this calculation is flawed. Owing to the differing solubility characteristics of gluten protein fractions, huge variation in their final content occurs after processing. High performance liquid chromatography (HPLC) studies of wheat starches derived from different cultivars revealed that the ratio of the amount of glutenin to gliadin varies between 0.2x and 5.8x, mainly due to differences in processing [10]. Measurement of gliadin multiplied by two therefore potentially leads to gross under- or over-estimations of the total gluten content of foods, particularly those based on purified wheat starch.

A number of assays formats are available commercially based on two antibodies, known as the Skerritt antibody and R5 [6,11]. In both cases the total gluten content is calculated by doubling the measured "gliadin" content, however, testing of two kits based on the Skerritt antibody, demonstrated that the antibody, although raised against omega-gliadin, showed higher affinity for HMW-GS than gliadins [6]. When food samples were tested using this method and the R5 method, which does not detect glutenin [11], the results from the Skerritt method were four to ten times higher [6]. This questions the validity of the results obtained.

We suggest it is important to develop sensitive assays to quantify glutenins and gliadins separately, using antibodies that do not cross-react between the two groups of proteins in order to assess correctly toxic gluten content in foods for individuals with CD. There have been some reports of generation of mAbs to HMW-GS. A polyclonal antiserum to HMW-GS 2 of wheat cultivar Highbury was shown to react with related subunits from other wheat cultivars. However, omega-gliadins from certain cultivars reacted more strongly with the antiserum than the HMW-GS immunogen [6], resulting in different outcomes for given food samples. Mills et al previously described two mAbs [12]. These were specific for the 1A and 1D encoded X-type, but not the Y-type HMW-GS of wheat. Mills and colleagues also generated mAbs to glutenin proteins for use in assessment of gluten quality. Wang and colleagues [13] reported the development of a mAb raised against 1B encoded X- and Y-type HMW-GS, which also bound HMW storage proteins in rye and barley, but not oats.

Mitea et al [7] have developed a mAb to a T cell stimulatory epitope in HMWG. However, their mAb only reacts with wheat and rye, but not *triticalle*, barley or oats. The epitope recognised by this mAb showed a high degree of overlap with a known T cell stimulatory epitope within wheat HMW-GS [14]. Li and colleagues [15] developed four mAbs using HMW-GS from spelt wheat. Two cross-reacted only with LMW but not HMW-GS, a third cross-reacted strongly with LMW-GS and only weakly to HMW-GS and the fourth cross-reacted only with certain X-type subunits.

We wished to raise mAbs to known CD toxic HMW-GS, both X- and Y-type, to develop improved assays for quantification of CD toxic gluten in foods for affected individuals.

Materials and methods

Antigens

Recombinant HMW-GS (rHMW-GS): Recombinant 1Dx5 HMW-GS was expressed in yeast and recombinant 1Dy10 HMW-GS in maize [16]. The proteins were enriched from their host matrices by a specific precipitation procedure and purified by preparative reversed-phase HPLC. Analytical HPLC, N-terminal sequencing and mass spectrometry confirmed purity and identity. The recombinant material was purified from the vector and then partially digested with pepsin and trypsin [16] to make them soluble in physiological solutions.

Chemically purified HMW-GS (cpHMW-GS): To limit the requirement for recombinant HMW-GS, chemically purified HMW-GS were prepared from wheat flour, as previously described by Wieser [16]. This method was previously used to produce material that was contaminated with <0.2% gliadin and LMW-GS [7].

rHMW-GS for immunisation: Peptide mixtures of rHMW-GS produced by pepsin-trypsin digestion were conjugated to Tubercullin Purified Protein Derivative (PPD). PPD was obtained in the form of Heaf Multiple Puncture test solution (Evans Vaccines, Liverpool, UK). The liquid form was precipitated by the addition of four volumes of acetone, and the PPD bound protein recovered by centrifugation, at 9,000g for 10 minutes.

The PPD was then air-dried, weighed and the molar quantity calculated (MW 10,000). Peptide was weighed out to provide a molar ratio of PPD: peptide of 2:1. For 1Dy10 this was assumed to be 20,000. The 1Dy10 used was a pepsin- trypsin digest with Frazer's Fraction III that when subjected to similar digestion yielded a peptide mixture with an average MW of 20,000 [16]. The peptide was then mixed with PPD and dissolved in PBS to give a concentration of 2mg PPD/ml. Glutaraldehyde was added to a concentration of 0.05% and the mixture placed in the dark, with stirring for 24 hours. Ammonium chloride was added to provide a 0.1 M solution; stirring was continued for a further 30 minutes. The conjugate was precipitated with acetone at -70°C for one hour. The suspension was centrifuged at 9,000g for 10 minutes, the supernatant poured away and the precipitate allowed to dry. The resultant dried precipitate was weighed and resolubilised in PBS at a final concentration of 1mg/ml conjugate.

Gliadins: Dr. H Wieser [17] provided Prolamin Working Group (PWG) (European Standard gliadin). Gliadin from wheat cultivar Kolibri was prepared as described [18]. Gliadin was purchased from Sigma (G3375). A pepsin-trypsin digest of gliadin was prepared (Sigma P0609 and T4019), according to the method of Bolte [19].

Extraction of HMW Prolamins from foods: This was achieved using a patented extraction buffer (60% glycerol (v/v), 0.1% TCEP (w/v) in Tris-HCl pH7.4, details of which are given in Patente Nacional 200802393 por "Procedimientos Para La Extracción De Prolaminas Tóxicas Del Gluten En Celiaquía"). The mixture was then vortexed for 30 seconds.

A mixture of 100mg of food/ml of buffer was incubated for 7 minutes in a thermos-mixer at 1,400rpm and 85°C. This was followed by centrifugation for 3 minutes at 12,000rpm. Extractions were performed twice. For the initial experiments, the samples were diluted 1:250 and for the subsequent experiments 1:100 in PBS.

Other cereal prolamins: Pure milled flour from rye (variety Rheidol), barley (variety Porter) and oats (Variety Peniarth) were obtained from Plant Breeding International, Trumpington, UK. Dr. F Janssen of the Regional Food Inspection Service, Zuppen, Netherlands, kindly donated whole grains of rice, maize, millet and sorghum. They were extracted with the patented extraction buffer described above.

Pure grains of various European wheat cultivars, all of which have been used for bread making were kindly provided by A. B. Semper (Oslo), as follows.

Soft spring wheats: Sverno, Ring, Rang, Draband, Pompe

Hard spring wheats: Dragon, Kadet

Soft winter wheats: Folke, Hilder, Portal, Solid Kosack

Hard winter wheats: Holme, Stark

Spelt wheat: Dinkle

These were hand-milled using a pestle and mortar and the milled flours extracted (1mg/ml) as above. Wheat starches A, B and C, representing acceptable, borderline and unacceptable gluten content were obtained from the Gluten Plate Test Kit (Transia Ltd) and were similarly extracted.

Production of Monoclonal Antibodies

A colony of Balb-C mice taking a GFD was established, in order to provide enhanced serum immune response to injected gluten [20]. The murine diet was provided by Harlan UK, Oxford (Code TD.94158). The mice were "primed" by subcutaneous injection of BCG vaccine and four weeks later immunised with a subcutaneous injection of 100µg conjugated peptide in Freund's complete adjuvant. Four weeks later the mice were immunised with subcutaneous injections of peptide carrier conjugate in Freund's incomplete adjuvant. Seven to ten days later a blood sample was tested for IgG class antibody. If titres of >1:3000 were found, an intravenous injection of 50µg of the conjugate was given. Three to five days after the IV injection, the mouse was culled and the spleen removed. Murine splenocytes were obtained from the spleen and fused with the immortal myeloma cells (P3X), using standard methods [21]. The resultant hybridomas were tested for IgG antibody production against rHMW-GS. Limiting dilution clones were screened for IgG antibody production from cloned positive hybridomas. Supernatants were purified with 50% ammonium sulphate precipitation.

Dot Blot Assays: Semi-quantitative dot blot assays were initially used, to examine the cross-reactivity of our mAbs with other cereals. Rye, barley, oats, maize, millet, rice, buckwheat and wheat starch were extracted at 100mg/ml, blotted on to nitro cellulose and tested using the method of Freedman [22]. mAbs were used at 1:80; anti-mouse alkaline phosphatase (Sigma A4132) was diluted 1:2000. The substrate was BCIP/NBT (Sigma B5655). The nitrocellulose sheets were air-dried and photographed immediately.

Indirect Enzyme Linked-Immunosorbant Assay (ELISA): Murine sera and the generated mAbs were tested with indirect ELISA. The plates were coated with Kolibri or PWG gliadin at 50µg/ml in 40% ethanol. Carbonate/bicarbonate buffer, pH 9.6 was used to coat with HMW-GS 1Dx5 and 1Dy10, (25µg/ml). To screen the mAb supernatants against fifteen wheat varieties, the extracts described above were used directly to coat ELISA plates. Mice sera where diluted to 1:400 and titred out to 1:102,400. Antibody supernatants from fusions were tested undiluted. Concentrated antibodies that had been precipitated from the culture supernatants were tested at 1:100-1:400 dilution and titred down to 1:25,600-1:102,400. Following 45 minutes incubation of the sera or supernatants at 37°C, the bound antibody was detected using rabbit anti-mouse IgG, alkaline phosphatase conjugated (Sigma A4132) at 1:2000. This was again incubated for 45 minutes at 37°C, followed by detection with para-nitro-phenol solution (Sigma 7998). The optical density was read at 405nm.

SDS-PAGE and Western-Blotting: Samples of European Standard gliadin and Kolibri gliadin were separated on SDS-PAGE under reducing conditions and subjected to Western-blotting. Blots were either stained with Memcode protein stain or probed with a different mAb that recognises the CDC-5 immunodominant gliadin peptide [18], or with newly generated antibody CDC-3 that recognises HMW-GS 1Dy10.

Quantitative Competitive ELISA: mAbs CDC-3 and CDC-7 generated against HMW-GS 1Dy10 were used to develop competition ELISAs. These required an initial immobilisation of HMW-GS as a coating antigen, with competition for binding to antibody between glutenins, in the sample or standard, and the coating HMW-GS. In our initial experiments, we used 1Dx5 HMW-GS as the coating and competing antigens. Checkerboard titrations were used to determine the optimum conditions. When chemically purified HMW-GS (cpHMW-GS) were obtained as described above, efforts were made to develop a new assay with these as the coating and competing antigens. Preliminary experiments were carried out to compare the optimised competition assay using r1Dx5 HMW-GS and new alternatives using r1Dy10 HMW-GS and cpHMW-GS. Competition assays were developed in one and two-step indirect and direct formats. In the one-step indirect format, pre-incubation of standard HMW-GS, CDC-7 and anti-mouse HRP together for 60 minutes was followed by incubation on the plate for a further 60 minutes. The substrate was then incubated separately. In the two-step indirect format, pre-incubation of standard HMW-GS and CDC7 for 60 minutes was followed by incubation on the plate for 60 minutes. The anti-mouse HRP was then incubated separately on the plate for 60 minutes, followed by substrate.

The final optimized assay emerging from checkerboard titration was a one-step indirect competition assay as follows:

Coating antigen cpHMW-GS: 0.507µg/ml in 0.05 M carbonate-bicarbonate buffer pH9.6 (Sigma C3041) for 60 minutes at 37°C.

Pre-incubation of standard cpHMW-GS as competition antigen from 200ng/ml to 0ng/ml, antibody CDC-7 at 0.923µg/ml and commercial anti-mouse HRP (Sigma A-9044) at a dilution of 1:10,000, in PBS-tween 20 0.05% (Sigma P-3563) for 60 minutes at room temperature.

Competition: The pre-incubated mix was added to the coated

plate and competition was allowed to occur for 60 minutes at room temperature.

Development: The colour was developed by the addition of 3,3',5,5'-Tetramethylbenzidine (TMB) (Sigma T0440) for 60 minutes at RT and stopped with dilute H_2SO_4. The plates were read on a Perkin Elmer Wallac Victor 2 Counter Plate Reader at 450nm.

Compatibility Studies: We tested the compatibility of the developed assay with the optimized extraction buffer described above at various dilutions from very low (1:3, 1:5, 1:10), to those that would be typically used in the final ELISAs (1:50 and 1:100).

Reproducibility: The assay was tested for its reproducibility, in one and two-step indirect formats with peptic-tryptic digestive HMWG peptides.

Standard curves (n=24 assays, 12 per plate on 2 plates) were constructed from 200-0ng/ml, using the determined optimised conditions.

Analysis of real samples: Real samples were supplied by a number of companies and by the faculty of pharmacy Universidad del País Vasco. The extraction of samples was carried out as above; 75mg of sample was added to 750µl of the optimized patented extraction solution.

Results

Production of chemically purified HMW-GS for use as standards: The material was subjected to SDS-PAGE under reducing conditions, and the results are shown in Figure 1. Wheat albumins (i), globulins (ii) and gliadins (iii) from different cultivars are shown in the first three panels with molecular weight markers. In panel (iv) chemically purified HMW-GS are shown with molecular weight markers. Only the typical bands for HMW-GS were seen with no visible bands in the region of gliadin or LMW-GS, so that the material was considered suitable for use as a standard in our assay.

Results of Immunisation with rHMW-GS and Polyclonal Serology: Six mice immunized with HMW-GS 1Dy10 had serum IgG titres to the antigen 1:25,000-1:200,000. Sera from two mice were tested for reaction with 1Dy10 and compared to 1Dx5 and PWG gliadin. There was full cross-reaction of the two murine sera (A & B) with 1Dx5, and a notable partial reaction with PWG gliadin, in both cases. The results are shown in Table 1. Fusions were performed using spleenocytes from 1Dy10-immunised mice and that generated the production of the antibodies CDC-3 and CDC-7.

Table 1: Titres of two immunised mice sera to gliadin and HMW-GS

Titre to:	PWGg	1Dy10	1Dx5
Mouse A	1:50,000	1:200,000	1:200,000
Mouse B	1:25,000	1:200,000	1:200,000

Characterisation of Monoclonal Antibodies CDC-3 and CDC-7 to HMW-GS

Indirect ELISA: The antibodies displayed a strong cross-reaction with rHMW-GS 1Dx5, and a partial but significant reaction with PWG gliadin, the gel electrophoresis of which reveals that it contains approximately 30% HMWG; this data is not shown. In the case of each of the fifteen wheat cultivars tested, antibody CDC-3 had titres in between 1:6400 and 1:25,600 and CDC-7 between 1:1600 and 1:25,600.

Dot-blot assays: Figure 2 shows that antibody CDC-3 clearly reacts with proteins extracted from the flours of rye, barley and oats and from wheat starches B and C. There was no cross-reaction with extracts of maize, millet, sorghum or buckwheat. A slight reaction was seen with rice; this is probably due to a non-specific reaction secondary to the large quantity of protein loaded onto the paper. The results for CDC-7 were almost identical.

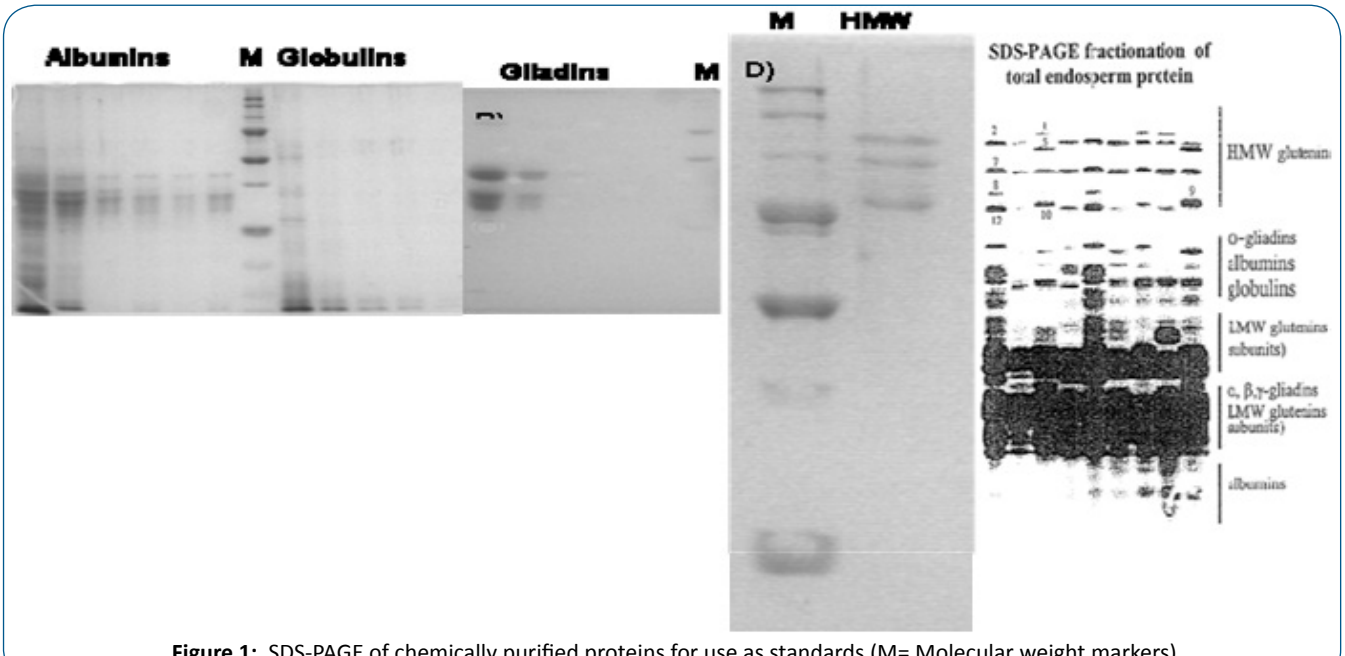

Figure 1: SDS-PAGE of chemically purified proteins for use as standards (M= Molecular weight markers)

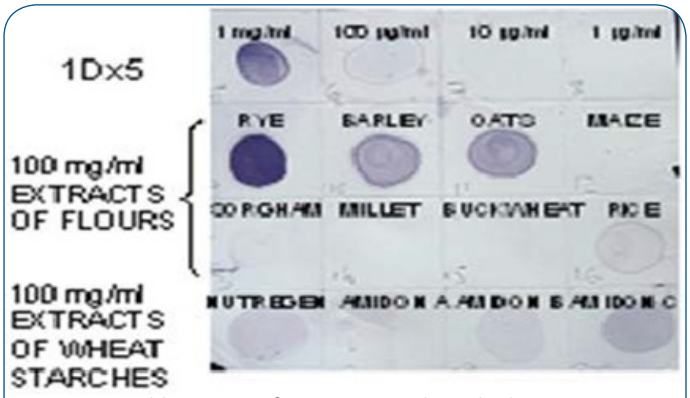

Figure 2: Dot blot assay of various cereals with the anti-HMW-GS CDC-3. Flour extracts and BSA were extracted at 20mg/ml. Amidon wheat starches A, B and C were extracted at 50mg/ml

Immunoblotting: In order to assess whether the ELISA reaction of antibodyCDC-3 with PWG gliadin was due to shared or similar epitopes between gliadin and HMW-GS, or whether the PWG gliadin was contaminated with HMW-GS, we performed SDS-PAGE and Western- blotting.

Figure 3 clearly shows the presence of HMW-GS as a contaminant in PWG gliadin, and it was to this only that the anti-Dy10 monoclonal antibody, CDC-3, reacted. By contrast an anti-gliadin antibody CDC-6 (that reacts to gliadin but not HMWG) was used. CDC-6 reacted only to those LMW bands on the gel attributable to gliadins. Interestingly there was no CDC-3 reactive HMW material present in the Kolibri gliadin sample.

Although the latter was rather badly degraded, the Kolibri gliadin sample appeared to comprise only material that reacted with CDC-6, an anti-alpha gliadin antibody, although this appeared to contain some HMW material.

Development of a Competitive ELISA for Measurement of HMW-GS of Wheat

Optimisation Coating Antigen and Competing Antigens: Checkerboard titration for CDC-7 revealed the optimal conditions for assays based on use of r1Dx5, r1Dy10 or cpHMW-GS as coating and competing antigens. The results obtained are shown in Table 2. Indirect competition assays employed the r1Dx5, r1Dy10 or cpHMW-GS that were optimally coated on to the microtitre plate at 0.675µg/ml, 4µg/ml and 0.507µg/ml respectively. CDC-7 was used at a concentration of 3.691µg/ml, 1.845µg/ml and 0.923µg/ml for 1Dx5, 1Dy10 and cpHMW-GS respectively. Anti-mouse-HRP was used at 1:10,000 in all cases. In direct competition assays, the 1Dx5, 1Dy10 or cpHMW-GS were optimally coated on to the microtitre plates at 0.675µg/ml, 8µg/ml and 4.056µg/ml respectively. CDC-7 was labelled and used at 7.382µg/ml for r1Dx5 and 14.764µg/ml for r1Dy10 and cpHMW-GS. The limit of detection (LOD) was less than 10ng/ml in all cases; however, a much lower concentration of CDC-7 could be used with cpHMW-GS as coating and competing antigen in an indirect assay.

Incubation times: Pre-incubation and incubation times were studied with cpHMW-GS as coating and competing antigens in an indirect competition assay, using optimised conditions for this antigen. The results obtained are presented in Table 3. 60 minutes incubation was necessary for good values of maximum absorbance (A_{max}), the LOD being slightly better with a 60 minutes pre-incubation time.

Incubation Temperatures: Pre-incubation and incubation temperatures were tested to obtain the final optimum conditions. The results are presented in Table 4 and show that using a

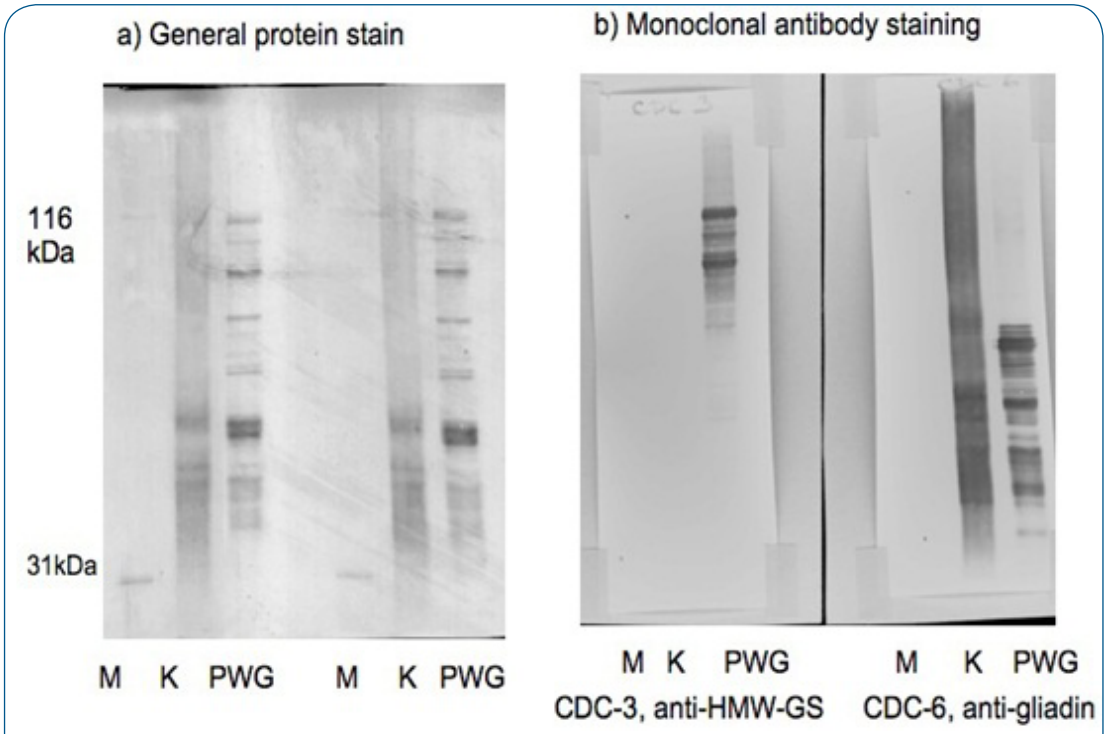

Figure 3: SDS-PAGE and subsequent blots immunostainings of Kolibri and European standard gliadin with a) general protein stain and b) monoclonal antibodies (M= Molecular weight markers; K= Kolibri; PWG= European standard gliadin)

Table 2: Results obtained with direct and indirect competition assays with the various HMW-GS and CDC-7

Coating	Dx5-GS		Dy10-GS		Pure HMW-GS	
	Direct	Indirect	Direct	Indirect	Direct	Indirect
A_{max}	0.51±0.01	2.85±0.03	0.99±0.01	2.19±0.01	1.45±0.01	1.22±0.05
A_{min}	0.09±0.00	0.54±0.01	0.12±0.00	0.31±0.00	0.23±0.01	0.29±0.01
Hillslope	-1.01±0.02	-2.02±0.11	-0.80±0.02	-1.05±0.01	-0.95±0.05	-1.17±0.23
EC_{50} (ng/ml)	25.2±0.14	22.93±0.67	31.35±1.33	13.14±0.12	15.44±1.53	14.39±3.64
LOD (ng/ml)	2.35±0.92	7.24±0.34	1.51±0.41	1.55±0.03	2.46±0.52	6.23±0.52
R^2	0.10±0.00	0.10±0.00	0.10±0.01	0.10±0.00	0.10±0.00	0.98±0.01

Table 3: Results of indirect competition assays carried out at different incubation and pre-incubation times (A_{max}= Maximum absorbance; A_{min}= Minimum absorbance; **LOD=** limit of detection)

	60 min incubation time				30 min incubation time				15 min incubation time			
	LOD ng/ml	R^2	A_{max}	A_{min}	LOD ng/ml	R^2	A_{max}	A_{min}	LOD ng/ml	R^2	A_{max}	A_{min}
60min pre-inc	0.5±0.19	0.1±0.00	1.65±0.03	0.23±0.01	0.38±0.17	0.10±0.00	1.14±0.03	0.11±0.00	6.20±0.50	0.99±0.00	0.95±0.00	0.05±0.01
30min pre-inc	1.73±0.32	0.10±0.00	1.73±0.00	0.20±0.04	3.06±0.52	0.10±0.00	1.06±0.02	0.15±0.01	5.1±0.36	0.99±0.00	1.02±0.01	0.01±0.02
15min pre-inc	1.86±0.48	0.10±0.00	1.41±0.02	0.26±0.01	4.05±1.23	0.99±0.00	1.11±0.02	0.09±0.00	4.27±0.67	0.99±0.00	0.84±0.01	0.09±0.01

Table 4: Results obtained in an indirect competition assay (60 minutes pre-incubation and incubation time) at different pre-incubation and incubation temperatures (A_{max}= Maximum absorbance; A_{min}= Minimum absorbance; **LOD=** limit of detection)

	22ºC incubation				4ºC incubation				37ºC incubation			
	LOD ng/ml	R^2	Amax	Amin	LOD ng/ml	R^2	Amax	Amin	LOD ng/ml	R^2	Amax	Amin
22°C Pre-inc	0.62±0.12	0.1±0.00	2.42±0.02	0.19±0.02	0.68±0.158	0.99±0.005	1.52±0.01	0.13±0.00	0.33±0.07	0.10±0.0	1.45±0.01	0.19±0.01
4°C Pre-inc	0.82±0.15	0.1±0.0	3.27±0.01	0.17±0.00	0.57±0.15	0.1±0.00	1.33±0.03	0.15±0.01	0.42±0.11	0.1±0.00	1.50±0.01	0.21±0.00
37°C Pre-inc	0.68±0.32	0.1±0.00	2.21±0.05	0.20±0.02	4.60±0.23	0.10±0.00	1.30±0.01	0.11±0.01	0.66±0.22	0.1±0.00	1.28±0.00	0.19±0.00

60 minutes pre-incubation and incubation time at 22°C gave optimal results in terms of LOD and A_{max} in both cases.

Reproducibility: The results in Table 5 demonstrate high intra- and inter-plate reproducibility of the optimised assay.

Compatibility of the assay with extraction buffer: The results demonstrate that the assay formats are highly compatible with the extraction buffer used, even at low dilutions, including 1:10, demonstrating the applicability of the assay to the detection of real samples. Similar results were obtained using a one (Figure 4a) or two-step indirect assay (Figure 4b).

Real sample Analysis

Wheat starches: Five samples obtained commercially and used in the production of gluten free foods had various HMW-GS subunits content (1, 17, 30, 42 and 51ppm)

Samples from gluten-free food manufacturers: The results from products with a competitive ELISA made from naturally gluten-free ingredients are shown in Table 6. While the majority of products were of low glutenin content, two of the products, corresponding to Dr. Schar vanilla wafers and Oleander onion snacks, had a HMW-GS in excess of 10ppm and would be

Table 5: Analysis of reproducibility of developed assay (A_{max}= Maximum absorbance; A_{min}= Minimum absorbance; **LOD=** limit of detection)

	PLATES (n=12)	LOD (ng/ml)	EC_{50} (ng/ml)	A_{max}	A_{min}	Hillslope	R^2
Intraplate	**1**	0.47±0.21	4.67±1.06	1.62±0.13	0.17±0.07	-0.843±0.19	0.99±0.00
	2	0.38±0.18	4.57±0.42	1.65±0.04	0.16±0.03	-1.05±0.11	0.10±0.00
Interplate	**AVERAGE (n=2)**	0.42±0.2	4.60±0.79	1.64±0.11	0.15±0.05	-0.945±0.18	0.10±0.02

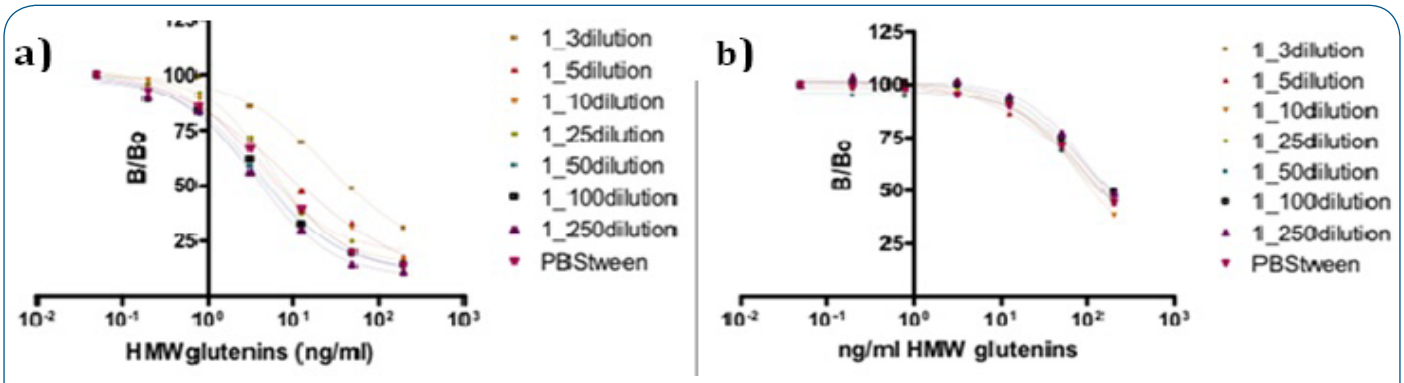

Figure 4: ELISA demonstrationof the compatibility in the assay with the extraction buffer at various dilutions in a) the one-step assay and b) the two-step assay (%B/BO= percentage per bound)

Table 6: HMW-GS content (ppm) of various foods made from naturally gluten-free ingredients

Manufacture	HMW-GS (ppm)	Items
Maizena	0.327±0.254	Corn flour sauce thickener
	1.851±0.388	Corn flour
Hacendado	1.4±1.51	Cheese chips
	1.796±0.052	Potato sticks
	2.1±0.13	Milled black pepper
	4.959±0.74	Yeast powder
Sanavi	3.49±0.07	Rosceli Nata
	5.558±0.774	Gluten-free Pasta
Glutafin	5.5±0.4	Gluten-free biscuit
Dr. Schar	6.726±0.66	Fette crocanti
	14.595±2.244	Vanilla Wafers
Oleander	12.72±2.06	Onion snack

Table 7: The glutenin content (ppm) of products based on reduced gluten-free ingredients (wheat starch)

Manufacture	Product	Gluten content (ppm)
Glutafin	White mix	12.74±2
Odlums	Titamyl white flour	4.43±0.42

expected to contain in excess of 20ppm total gluten once gliadin and LMW-GS had been included. Thus these products could not be considered gluten-free. However, according to the new legislation, they could be labelled as very low gluten.

Discussion

We have raised mAbs against 1Dy10 HMW-GS that react with both this and the other important dough forming HMW-GS 1Dx5. Both of these HMW-GS have been shown to be CD toxic [9]. Reactions have been demonstrated with glutenin from fifteen varieties of bread wheat, suggesting that the epitopes detected are widely distributed. Rye and barley are known to have HMW prolamins, which are comparable to the HMW-GS of wheat, known as the HMW secalins and hordeins correspondingly. Oats prolamins are also known to contain sequences that correspond to the HMW prolamins of wheat, rye and barley. Given the strong cross-reactivity of our antibodies with all four cereals, it seems likely that a common sequence is being recognised.

Little is known about CD activating sequences within HMW prolamins. It is impossible to know whether these common sequences are CD toxic or not. A Dutch group have developed a mAb to the T cell stimulatory epitope in HMW glutenin, however, their mAb only reacts with wheat and rye [7] whereas our mAb reacts with wheat, rye, barley and oats.

Our results, both from dot assays and quantitative ELISA, indicate that purified wheat starches are contaminated with HMW-GS, emphasizing the importance of measuring these toxic proteins in foods based on wheat starch but intended for consumption by celiac patients. A number of real food samples from named manufacturers were analysed. The majority had low glutenin content but HMW-GS was detectable in almost all the samples, even those based on naturally gluten-free ingredients. This emphasises the importance of a 20ppm limit for foods labelled as gluten-free. Two of the samples based on wheat free ingredients had a significant content of HMW-GS.

Another set of samples came from the reference laboratory at the Universidad del País Vasco. These were gluten-free products

Table 8: Results of the HMW-GS content (ppm) in "gluten-free" samples from the reference laboratory at Universidad del País Vasco

<20ppm gluten		>100 ppm gluten		>20 ppm gluten	
Item	**HMW-GS**	**Item**	**HMW-GS**	**Item**	**HMW-GS**
Rice flour	0.00	**Crisps**	135.48±19.58	**Yeast**	34.66±3.77
Food colorant	0.00	**Cookies**	222.6±14.66		
Corn flakes	0.60±0.43	**Host**	353±16.67		
Beer	0.73±0.09	**Dried fruits**	573±68.59		
Fruit muesli	2.09±0.19				
Crisps	2.66±0.99				
Nachos	2.67±0.23				
Spaghetti	3.89±0.27				
Cream cake	4.06±1.11				
Strawberry cookies	5.33±0.21				
Corn flakes	7.47±1.20				
Chocokrispies	7.87±0.72				
Soya cookies	8.434±0.85				
Rice flour (second)	9.16±1.08				
Chocolate rice	10.55±2.23				
Powdered chili	10.49±0.86				
Soya fat	14.94±1.32				
Maize flour	4.82±0.41				

intended for consumption by CD patients. We were unable to obtain either the names of the products, or a list of their ingredients. Still, some contained very considerable amounts of HMW-GS, for example gluten-free Communion Hosts with 353ppm glutenin and mixed dried fruits with 573ppm glutenin.

European standard gliadin was prepared by ethanolic extraction of various wheat strains [15]. The considerable contamination of this material with HMW-GS indicates that the latter proteins are partially ethanol soluble. It must therefore be expected that any gliadin material prepared in this way may be contaminated with HMW-GS.

This has important implications for the future of cocktail ELISAs intended for the measurement of gliadins and glutenins. If the gliadin standard is contaminated with glutenin this could skew the results, resulting in an inaccurate estimation of the total gluten content. Appropriate standard material will be required for the development of accurate assays for measurement of all CD toxic gluten.

Conclusion

We conclude that the current quantitation of gluten content in gluten-free foods of multiplying the gliadin content by two provides incorrect estimations, and that the individual quantification of HMW-GS as well as gliadin in gluten-free foods increases the accuracy of gluten contamination of commercially available gluten-free foods for individuals with CD.

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