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ARTICLE

Single Domain Antibodies Are Specially Suited for Quantitative Determination of Gliadins under Denaturing Conditions

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Food intended for celiac patients' consumption must be analyzed for the presence of toxic prolamins using high detectability tests. Though 60% ethanol is the most commonly used solvent for prolamins extraction, 2-mercaptoethanol (2-ME) and guanidinium chloride (GuHCl) can be added to increase protein recovery. However, ethanol and denaturing agents interfere with antigen recognition when conventional antibodies are used. In the present work, a new method for gliadins quantification is shown. The method is based on the selection of llama single domain antibody fragments able to operate under denaturing conditions. Six out of 28 VHH-phages obtained retained their binding capacity in 15% ethanol. Selected clones presented a long CDR3 region containing two additional cysteines that could be responsible for the higher stability. One of the clones (named VHH26) was fully operative in the presence of 15% ethanol, 0.5% 2-ME, and 0.5 M GuHCl. Capture ELISA using VHH26 was able to detect gliadins in samples shown as negatives by conventional ELISA. Therefore, this new strategy appears as an excellent platform for quantitative determination of proteins or any other immunogenic compound, in the presence of denaturing agents, when specific recognition units with high stability are required.

KEYWORDS: Recombinant antibody fragments; camelid antibodies; immunoassays; gluten-free products; celiac disease

INTRODUCTION

Camelids possess, in addition to conventional immunoglobulins formed by the association between a heavy and a light chain, another functional class of antibodies formed by only a heavy chain, known as heavy chain antibodies (*1*, *2*). Therefore, the antigen binding site of these antibodies is limited to the variable domain of the heavy chain, called VHH. Since they consist of only one domain, VHH fragments are easier to produce by molecular engineering techniques than conventional antibodies (*3*). These VHHs are interesting antigen binding structures due to their broad biotechnological applications (*4*).

The VHH surface that corresponds to the hydrophobic contact area of the VH with the VL domain in conventional antibodies contains hydrophilic amino acid residues that ensure good solubility and high thermostability of VHH domains (*1*, *5*). Another distinct feature of VHHs is the occurrence of enlarged loops, which is thought to compensate for both the lack of an antigen-binding surface contributed by the three hypervariable loops (CDRs) of the VL domain and the absence of the VH-VL combinatorial diversity (*1*, *6*).

VHHs can efficiently bind antigens at high temperatures (*7*) or in the presence of detergents (*8*). Besides, VHHs refold efficiently after denaturation under aggressive conditions, such as the presence of detergents, urea (*8*), or low pH (*9*). These biochemical properties, not present in conventional antibodies, are particularly important for certain analytical methods, as in, for example, quantitative determination of gliadins by immunoassays. Wheat proteins (gliadins and glutenins) are known to trigger the typical intestinal damage observed in patients suffering from celiac disease (CD). This enteropathy is an immune-mediated disorder induced in genetically susceptible individuals by the ingestion of prolamins from wheat and related cereals. Celiac disease is a very common chronic gastrointestinal disorder; the prevalence in western populations is close to 1% (*10*). Once diagnosed, patients must adhere to a life-long strict gluten-free diet. Complete adherence to the diet restores the intestinal mucosa and reverts the symptoms (*11*). Therefore, patients must consume only gluten-free (natural or commercial) products, which must be analyzed to determine their gluten content.

Gluten can be defined as the rubbery mass formed by hundreds of proteins that remain when wheat dough is washed to remove starch granules and water-soluble constituents. These proteins are present in food either as monomers or linked by interchain disulfide bonds, and they are strictly insoluble in aqueous solution (*11*).

Gliadins (the protein fraction of wheat flour extracted by 60−70% ethanol) have been the most common proteins studied for the evaluation of the pathogenic mechanism of CD as well as for the assessment of gluten content in food samples aimed for

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consumption by CD patients. Quantitative immunoassays are the most commonly used techniques for gliadin determination and, therefore, for the analysis of gluten-free products (*12*). More recently, different strategies have been employed to generate conventional monoclonal antibodies against specific toxic epitopes in order to develop quantitative tests (*13*, *14*).

According to recent reports, small amounts of gluten are able to trigger histological changes in the intestinal mucosa of CD patients (*15*). Consequently, gluten-free food certification requires the use of powerful methods involving both a high detection capability as well as an efficient procedure for protein extraction from food samples. It is well-known that prolamins are strongly bound to the food matrix by covalent and noncovalent interactions. The most common extraction solvent used, 60−70% ethanol, is unable to completely recover the prolamins from food samples. To increase protein recovery during extraction, the use of guanidinium chloride (GuHCl) and 2-mercaptoethanol (2-ME) has been proposed (*16*). However, studies performed by our group demonstrated that these agents interfere with antigen binding of conventional antibodies (*17*). Thus, highly stable single domain antibodies, that would resist these denaturing extracting conditions, will serve to develop more sensitive immunoassays to detect prolamins. In this study, we report the generation of gliadin-specific VHHs and the development of quantitative immunoassays based on highly stable llama single domain antibody fragments.

MATERIALS AND METHOD

Immunization. Two young male llamas (*Lama glama*) were subcutaneously injected at days 0, 21, and 51 with 0.5 mg of gliadins (Sigma, St. Louis, MO, USA) emulsified in Incomplete Freund adjuvant (Sigma). Serum was collected prior to each boost to test the immune response against the immunogen. Twenty-four days after the third immunization, 150 mL of heparinized blood was collected for lymphocyte isolation.

Construction of the VHH-phage library. Mononuclear cells were isolated from llama 9204 heparinized blood by Ficoll-Hypaque (GE-Healthcare) gradient centrifugation. Total RNA was purified by TRIZOL reagent (GE-Healthcare) and subject to cDNA synthesis. To synthesize cDNA, 3 μg of RNA and 1.5 μg of oligo-dT_{30} in 7.5 μL were incubated for 10 min at 70 °C and placed on ice. 0.4 mM dNTPs, RT buffer 1X, 200 units of M-MLV RT (Promega), and 25 units of RNAsin (Promega) were added, in a total volume of 25 μL, and incubated 1 h at 42 °C. The reaction was stopped at 70 °C for 15 min.

cDNAs encoding an entire VHH domain and part of the hinge region were amplified by PCR using primers VH1Back-SfiI or VH6Back-SfiI in combination with primer Lamb7-NotI or Lamb8-NotI. Primers VHBack-SfiI anneal to VHHs N-terminal consensus sequences. Primers Lamb7-NotI and Lamb8-NotI hybridize to part of the short and long hinge region, respectively (*18*). Their sequences are as follows: VH1Back-SfiI, GCT GGA TTG TTA TTA CTC GCG GCC CAG CCG GCC ATG GCC CAG GTS MAR CTG CAG SAG TCW GG; VH6Back-SfiI, GCT GGA TTG TTA TTA TCT GCG GCC CAG CCG GCC ATG GCC GAT GTG CAG CTG CAG GCG TCT GGR GGA GG; Lamb7-NotI, G ATG GTG ATG ATG ATG TGC GGC CGC GCT GGG GTC TTC GCT GTG GTG CG; Lamb8-NotI, G ATG GTG ATG ATG ATG TGC GGC CGC TGG TTG TGG TTT TGG TGT CTT GGG. Restriction enzyme sequences are underlined. The resulting PCR fragments (approximately 450 bp long) were purified from agarose gels (GFX PCR DNA and gel band purification kit, GE-healthcare), digested sequentially with SfiI and NotI, and repurified.

For phage-display library construction, 5 μg of SfiI-NotI-digested plasmid pHEN2 (*19*) and 5 μg of digested fragment were ligated during 16 h at 16 °C. The inserts were introduced between the PelB leader signal and His-tag, in the frame with the pIII capsid protein gene in the vector pHEN2. The ligation reaction was concentrated and desalted by ethanol precipitation. Electrocompetent *E. coli* XL Blue MRF′ cells were transformed, and library size was calculated by plating aliquots on LB ampicillin agar. The VHH-phage library diversity was checked by sequencing.

Preparation of Polyclonal VHH-phages. To prepare polyclonal VHH-phages, library stock or cells preinfected with phages (eluted during panning) were grown until $A_{600nm} = 0.5$ and were then infected with at least a 10-fold excess of VCS helper phage (Promega) in the presence of 1% glucose at 37 °C. After 1 h of incubation, cells were washed by harvesting and resuspension in glucose-free fresh culture media. After growing overnight at 30 °C, VHH-phages were recovered from culture supernatant by incubation at 4 °C followed by precipitation in 4% PEG 8000 and 0.5 M NaCl. The pellet was resuspended in 1 mL of sterile PBS/100 mL of culture medium and used for panning.

Panning of the VHH-phage Library. To enrich the library for the presence of gliadin binders, panning was performed on 24 well culture plates (Hamilton). Wells were coated with 5 μg/well of gliadin during 2 h at room temperature. After blocking with 3% skim milk in PBS (SM-PBS) overnight at 4 °C, approximately 10^{13} VHH-phage particles in 1% SM-PBS were added with agitation during 2 h. Three PBS washes were performed between each step. VHH-phage binders were eluted by incubation with 100 mM glycine−HCl pH 2.2 during 10 min and immediately neutralized with 2 M Tris-HCl, pH 8.0. This procedure was followed by amplification of the eluted phages and repeated twice. At all steps, VHH-phage titers of input and output were estimated by enumeration of ampicillin resistant colonies obtained from TG1 cells infected with different VHH-phage dilutions.

Production of Individual VHH-phages. Plates with individual clones were grown in culture medium supplemented with glucose for 3 h at 37 °C with agitation, and they were superinfected with VCS helper phage for 30 min. Cells were pelleted, resuspended in the same medium without glucose, and incubated for 16 h at 30 °C. Supernatants were tested in indirect ELISA for selection. VHH-phages selected for further studies were produced in large scale, and supernatants containing the phages were incubated for 1 h on ice with PEG solution, 1:5 (20% PEG, 2.5 M NaCl). After centrifugation the pellet was resuspended in 50% glycerol in PBS and stored at −80 °C.

Indirect ELISA for Selection of VHH-phages and Assessment of VHH-phage Stability. Plates were coated overnight at 4 °C with 10 μg/mL wheat flour (cultivar Imperial) ethanolic extract in PBS, pH 7.4. All subsequent incubations were performed at 37 °C. After washing once with PBS, 0.05% Tween 20, plates were blocked for 2 h with 3% (w/v) SM-PBS. VHHs were diluted in 1% (w/v) SM-PBS and 0.05% Tween 20 (buffer A), or in buffer A containing different concentrations of ethanol, or in 2-ME/GuHCl mixtures. Dilutions were transferred into gliadin coated wells and incubated for 1 h. Plates were then washed 3 times and incubated for 1 h with anti-M13 horseradish peroxidase conjugate (BioRad, Hercules, CA, USA) diluted in buffer A. Plates were washed 3 times, and the enzyme substrate (1 mg/mL OPD; 1 μL/mL 30% H_2O_2 in citrate buffer pH 5) was added. The reaction was stopped after 15 min with 2 M H_2SO_4, and absorbance was read at 492 nm.

Production and Purification of Soluble VHH Monomers. To obtain soluble VHH in high yield, VHH DNAs were cloned into a

pAB1 expression vector (*20*). For cloning purposes, VH6Back-SfiI (previous mentioned) and Lamb9-*Eco*RI primers were used. Lamb9-*Eco*RI contains an *Eco*RI restriction site after the c-myc tag−2xStop codons sequence. The lamb9-*Eco*RI primer sequence is ACG GCC AGT GAA TTC TTA TTA CCC ATT CAG ATC C. The *Eco*RI sequence is underlined. After SfiI and *Eco*RI digestion, the purified PCR fragments were ligated into the predigested pAB1 and then used to transform *E. coli* HB2151 cells. After 4 h of induction with 1 mM IPTG, the expressed proteins were extracted from the periplasmic space through osmotic shock, passing from 500 mM to 125 mM sucrose solution in the buffer 200 mM Tris-HCl, 0.5 mM EDTA, pH 8. Further purification of soluble protein was achieved on a Hi-Trap chelating HP column (GE-healthcare). The column was washed with 10 mM imidazole (Sigma), 0.3 M NaCl in sodium phosphate pH 8.0, and elution was done using 250 mM imidazole in the same buffer. Further enrichment of soluble protein for affinity measurements was achieved on a cation exchanger MonoS column (GE-healthcare). The column was equilibrated with 50 mM MES (Sigma) pH 5.2, and elution was performed by adding 1 M NaCl in the same buffer. The purity of the preparation was checked by SDS-PAGE and Western blot, which showed one band of approximately 20 KDa, the expected molecular weight.

Gliadin Standards. Three different sources of gliadins were used in this study:

(1) Gliadin standard solution (1 mg gliadin/mL) was prepared using commercial gliadin (Sigma) in ethanol 60% and quantified by Kjeldahl′s method (*21*).
(2) European Working Group in Prolamin Analysis and Toxicity (PWG) standard was prepared as indicated (*22*), to obtain 1 mg gliadin/mL in 60% ethanol.
(3) Wheat, barley, rye, soy, maize, and rice ethanolic extracts were prepared by incubating the respective flour with 60% ethanol after albumin and globulin extraction with 0.15 M NaCl.

Kinetic Analysis by Surface Plasmon Resonance. Kinetic analyses of the gliadin-VHH interactions were determined using an IAsys Biosensor instrument. VHH26 was immobilized in 10 mM sodium acetate pH 5.3 on a carboxymethylated dextran layer. A coupling efficiency of 150 arc. sec. was obtained. For kinetic constant determinations, diluted solutions of different gliadins in PBS, 0.05% Tween 20, 15% ethanol (final concentration) were analyzed. Binding traces were recorded for at least five different concentrations, taking as the concentration the total protein concentration of each sample. Association and dissociation rate constants were calculated using the FASTFIT software. For binding surface regeneration, 15% ethanol was used.

Quantitative Determination of Gliadins by Sequential Competitive ELISA. Plates were coated overnight at 4 °C with 1 μg/mL gliadin standard in PBS pH 7.4. All subsequent incubations were performed at 37 °C. After washing once with PBS, 0.05% Tween 20, plates were blocked for 2 h with 3% (w/v) SM-PBS. Standard gliadin or sample extracts were preincubated in 1.5 mL plastic tubes for 2 h with an appropriate dilution of antigliadin VHH-phages at a 1:1 ratio, diluted in 1% (w/v) SM-PBS containing 0.05% Tween 20 (buffer A). Then, samples were transferred into gliadin-coated wells and incubated for 30 min. Plates were then washed three times and incubated for 1 h with anti-M13-HRP (General Electric) diluted in buffer A. Plates were washed three times, and the enzyme substrate (1 mg/mL OPD; 1 μL/ml H_2O_2 in citrate buffer pH 5) was added. The reaction was stopped after 15 min with 2 M H_2SO_4, and absorbance was read at 492 nm. The limit of detection was calculated according to the *t* test (confidence = 99%) using a logit p transformation (*23*).

Quantitative Determination of Gliadins by Capture ELISA. Plates were coated overnight at 4 °C with soluble VHH26 (5 μg/mL in PBS pH 7.4). All subsequent incubations were performed at 37 °C. After washing once with PBS-0.05% Tween 20, plates were blocked for 2 h with 3% (w/v) SM-PBS. Standard gliadin was serially diluted in buffer A, transferred into VHH26 coated wells, and incubated for 1 h. Plates were then washed 3 times, incubated for 1 h with biotinylated antigliadin monoclonal antibodies (mAbs 1B4, 2A1, or 3B4) (*24*), washed again, and further incubated for 1 h with avidin-alkaline phosphatase. Plates were washed 3 times, and the enzyme substrate (*p*-nitrophenyl phosphate in diethanolamine buffer pH 9.8) was added. The reaction was stopped after 30 min with EDTA, and absorbance was read at 405 nm. The limit of detection was calculated according to the *t* test (df = 7, confidence = 99%) (*23*). This procedure was also used with each of the mAbs as capture antibody and VHHs fragments as secondary antibody. Detection was performed using anti-His mAb-HRP (Sigma).

RESULTS

Library Construction and VHH-phage Selection. Two llamas were immunized with a whole-gliadin commercial preparation, and the specific humoral response was analyzed by indirect ELISA. The peripheral blood sample of the immunized animal with higher antigliadin titers was used for construction of a VHH-phage display library (Supporting Information Figure 1). A library composed of 8×10^7 single clones was obtained, and its quality was checked by sequencing 15 randomly chosen clones, which showed all different VHH sequences, demonstrating the variability and the good quality of the library.

To select specific binders, approximately 10^{13} VHH-phage particles from the library were used in each of three consecutive panning rounds using commercial gliadins. Nonspecific binding was evaluated in wells without antigen. The enrichment during the panning procedure was estimated by determining the specific binding of randomly chosen clones from each round by VHH-phage ELISA. As expected, due to positive selection, the proportion of binders increased during successive panning rounds (6%, 21%, and 40%, respectively, for the first, second, and third rounds) (Supporting Information Table 1). Twenty-eight VHH-phages from the third panning round were further evaluated. The biological activity of each clone was evaluated by titration curves in indirect VHH-phage ELISA. For further studies, a working dilution of each VHH-phage was chosen based on the dilution producing an optical density of about 1.5 in indirect phage-ELISA.

Binding Capacity in the Presence of Ethanol. Prior to quantitative immunoassay in gluten-free food certification, the gliadin fraction is conventionally extracted from food samples with 60% aqueous ethanol, a solvent that commonly interferes with the binding of conventional antibodies.

To characterize the biological activity of VHH-phages in the presence of ethanol, specific binding was analyzed by indirect VHH-phage ELISA on gliadin-coated wells. As expected, binding decreased with higher ethanol concentrations, but six selected VHH-phages (clones 11, 13, 19, 26, 33, and 36) were more resistant among the 28 clones tested. Since clones 11, 19, 33, and 36 correspond to the same sequence (see below, sequencing analysis), only the reactivity of VHH-phages 13, 26, and 33 was depicted in **Figure 1**. Gliadin recognition in the presence of 15% ethanol remained about 80% of the maximum (compared to control samples with no ethanol added) for the six selected

VHH-phages. Exceptionally, the VHH26-phage keeps 92% of the reactivity in 15% ethanol. Clones with a reactivity below 80% of the maximum for this condition were not selected. The evaluation of the specific binding at different ethanol concentrations showed that selected VHH-phages are biologically active at 15% ethanol.

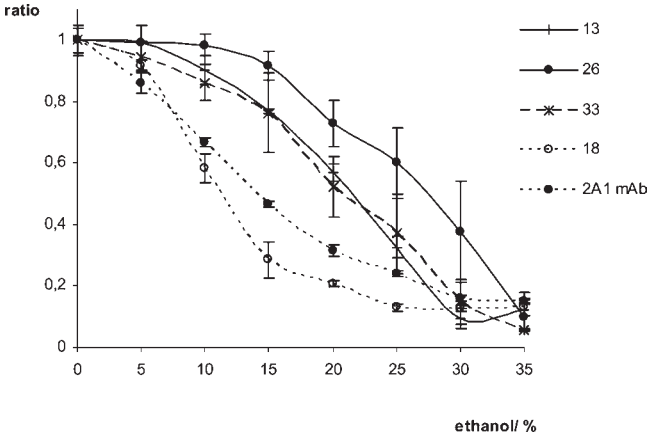

Figure 1. Interference by ethanol in the VHH-phage specific binding. The reactivity of different VHH-phages was evaluated by indirect phage-ELISA in gliadin-coated wells. VHH-phages were diluted in buffer containing ethanol at concentrations ranging from 0% up to 35%. Clone 18 is depicted as an example of nonstable VHH-phages. The reactivity of the 2A1 monoclonal antibody is depicted as an example of conventional antibodies. Results are expressed as the optical density of the sample relative to control (VHH-phage without ethanol addition)(arbitrary units represented as ratio = 1). Duplicate results from one representative experiment are shown.

Assessment of 2-ME and GuHCl Interference in VHH-phage Binding. As mentioned above, extraction buffers containing denaturing and reducing agents have been proposed to increase prolamin recovery from food samples. Particularly, the use of 60% ethanol, 2% 2-ME, 2 M GuHCl has been recommended (*16*). However, these agents disturb protein structure and can cause severe interference in the quantitative immunoassays when conventional antibodies are used (*17*). The effect of these agents on the specific binding of VHH-phages was analyzed by indirect phage-ELISA in the presence of increasing 2-ME and GuHCl concentrations (**Figure 2**). Binding of the VHH-phages of clones 11, 13, 19, 26, 33, and 36 was more resistant to aggressive conditions than the cases other tested clones. Only clones 13, 26, and 33 are represented in **Figure 2A** because the others were repeated clones (see below, sequencing analysis). There were no relevant differences in the binding ability among clones 13, 26, and 33 in the presence of denaturing conditions. The specific binding of clone VHH26 was 50% of the maximum at 0.5% 2-ME, 0.5 M GuHCl (**Figure 2B**). Therefore, based on the stability in ethanol, 2-ME, and GuHCl, VHH-phages 13, 26, and 33 were selected for further studies. As a comparison, the effect of denaturing agents on an antigliadin mAb from our panel is shown in **Figure 2C**. The fact that ethanol resistant VHH-phages were also resistant to 2-ME and GuHCl may indicate shared structural features which confer the ability of antigen

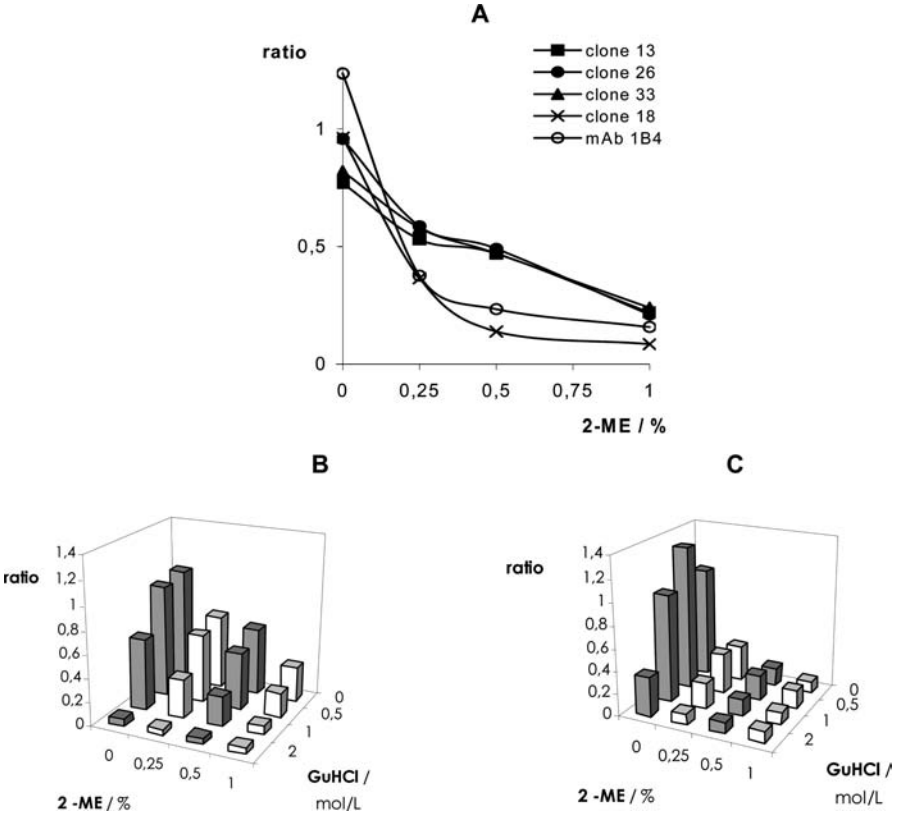

Figure 2. Binding interference by guanidinium chloride and 2-mercaptoethanol. (**A**) Analysis of the binding of selected VHH-phages (13, 18, 26, 33) and antigliadin 1B4 murine mAb to gliadin in 0.5 M GuHCl and different concentrations of 2-ME. Clone 18 is depicted as an example of nonstable VHH-phages. According to sequence analysis, clone 33 is the same as clones 11, 19, and 36 (see below). (**B**) Binding of the VHH26-phage to gliadin in different combinations of GuHCl and 2-ME concentrations. (**C**) Binding of antigliadin 1B4 murine mAb to gliadin in different combinations of GuHCl and 2-ME concentrations. Results are expressed as OD ratio relative to the control sample (VHH-phage dilution without additives) (arbitrary units represented as ratio = 1). Duplicate results from one representative experiment are shown.

Clone	FR1	CDR1	FR2	CDR2
	20	30	40 50	60
13	GGGLVQAGGSLRLSCVAS	GRTFSSYT	MGWFRQAPGKEREFVGA	VSWGGDT
33	....	....	....	....
26	....	I	.A.............A.	S......

Clone	FR3	CDR3	FR4
	70 80 90 100	110 120	
13	YYADSVE-GRFTVSRVNAKNTVNLQMNSLKPEDAAVYYC	AENDNY**C**SGYG**C**FDPMS---YDY	WGQG
33	......K................................	....	....
26	......K........H......Y................	............Y.Y.G......	..P.

Figure 3. Sequence analysis of three selected VHH clones. Sequence alignment of three postpanning VHH clones (13, 26, and 33). Uncommon cysteines are represented in bold. Numbering and CDR designations are according to the IMGT numbering system (*25*).

binding under conditions that normally would result in protein unfolding or denaturation. Considering the ability to specifically interact with the antigen in the presence of ethanol, GuHCl, and 2-ME, the VHH26-phage showed the highest apparent stability.

Sequence Analysis of Selected VHHs. The sequence analysis of the six selected VHH-phages (clones 11, 13, 19, 26, 33, and 36) revealed that four of them (clones 11, 19, 33, and 36) correspond to the same sequence, making a total of three unique clones. Clones VHH13 and VHH33 differ from each other in only one residue, and clone VHH26 differs from clones 13 and 33 in ten residues—three of them located at the CDR3 (**Figure 3**). It is noteworthy that all selected VHH clones show a large CDR3 with similar length (20 amino acids). It is known that VHHs can have disulfide bonds formed between Cys23 and Cys104. The three ethanol resistant clones showed two extra cysteines in CDR3, not present in any prepanning clone, separated by only four residues between them. These two additional cysteines in the same position of the CDR3, presumably forming an internal disulfide bridge, could decrease the flexibility of this long loop. The sequences of the three highly stable VHH clones were deposited in the GenBank, with accession numbers from FJ155928 to FJ155930.

Altogether, the sequence analysis showed that the antigliadin VHH-phages selected in the presence of harsh conditions (i.e., ethanol, GuHCl, and 2-ME) all have very similar CDR3 sequences, indicating that highly stable recognition could result as consequence of a limited structural solution.

VHH-phage Reactivity Is Specific for Wheat Prolamins. Methods for gluten-free food certification require a specific detection of toxic prolamins. To avoid false positive results, antibodies used in quantitative assays must not crossreact with harmless proteins from other vegetables frequently employed in food manufacturing.

To evaluate the cross-reactivity of the selected clones, sequential competitive assays were performed on plates coated with 10 μg/mL of the whole prolamin fraction (wheat ethanolic extract). The binding of each VHH-phage was set to compete with that of prolamins from ethanolic extracts, either from wheat, barley, rye, maize, soy, or rice. Only wheat extract competed for VHH26-phage binding to coated gliadin; meanwhile extracts from the other plants did not affect the VHH26-phage recognition even at protein concentrations as high as 10 μg/mL (**Figure 4**). The other two selected clones showed similar results (data not shown). So, although prolamins from wheat, barley, and rye have characterized sequences with high homology and, consequently, cross-reactivity, selected VHH-phages only reacted with wheat proteins, suggesting that the recognized epitopes are only present in gliadins. These epitopes might be located in a region unique for α-gliadins which is not present in γ- or ω-gliadins, since these last two contain many homologous sequences in barley and rye.

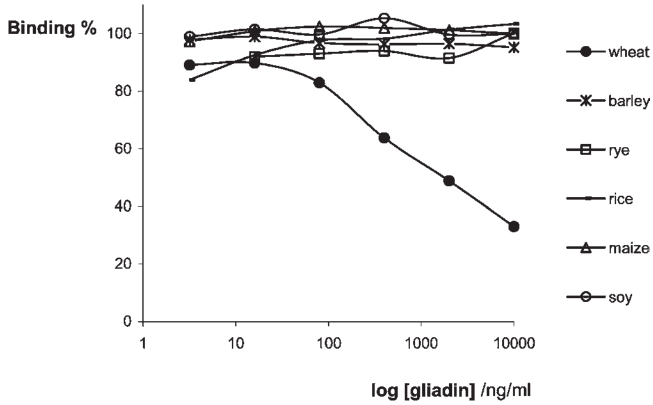

Figure 4. VHH-phage reactivity is specific for wheat prolamins. Sequential competitive phage-ELISA was performed in gliadin-coated wells. Binding of VHH26 clone competed with different protein concentrations of ethanolic extracts from wheat, barley, rye, maize, soy, or rice. Duplicate results from one representative experiment are shown.

Kinetics of the Soluble VHH26/Gliadins Interaction. To assess the affinity constants of the VHH26/gliadins interaction, a soluble VHH26 fragment was produced and purified to homogeneity (Supporting Information Figure 2).

To determine the kinetic parameters, the VHH26 fragment was immobilized on a carboxy-dextran cuvette. Different concentrations of soluble gliadins were evaluated in 15% ethanol. The affinity of the VHH interaction with three different sources of antigens, commercial gliadin (Sigma), ethanolic extract from wheat flour of Oasis cultivar, and the PWG standard (*22*), was evaluated in an IAsys biosensor. Since the prolamin fraction is a complex mixture of proteins with molecular mass ranging from 30 up to 75 kDa, the affinity constants could not be calculated because the exact molar concentration of the interacting antigens was unknown. In order to make possible a comparison, 30 kDa was used as representative molecular weight for gliadins. Based on this estimation, the apparent on-rates for the three kinds of gliadins ranged from 1.2 to 2.4 $\times$ 10^4 M^{-1} s^{-1}, whereas the dissociation rates ranged from 1.2 to 2.1 $\times$ 10^{-2} s^{-1}, resulting in dissociation constants next to the 10^{-7} M range (Supporting Information Table 2). The three kinds of gliadins showed similar kinetic constants for binding to VHH26; thus, it can be concluded that VHH26 recognizes an equivalent distribution of epitopes in these three sources of gliadins.

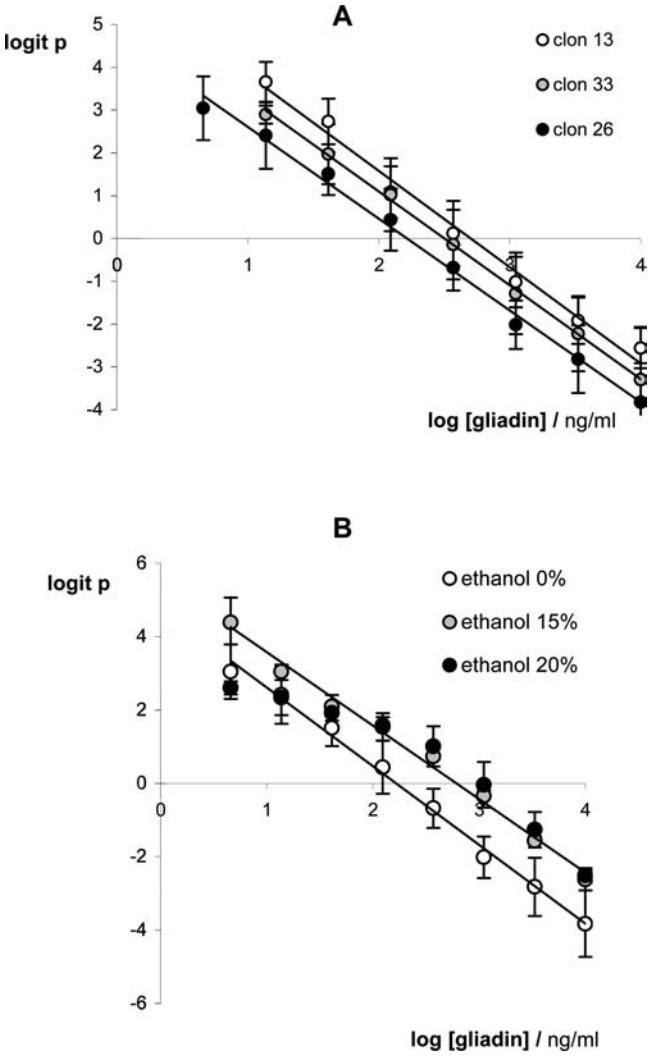

Figure 5. Performance of the quantitative assay by competitive ELISA using VHH-phages. (**A**) Quantitative determination of gliadins with a VHH-phage. Gliadin calibration curves for VHH26 (black), VHH33 (gray), or VHH13 (white). Mean values of triplicate results are shown. (**B**) Interference of ethanol on the quantitative assay. Standard curves for VHH26 were prepared in PBS (white), 15% ethanol (gray), or 20% ethanol (black). The results are the mean value of eight independent curves (two independent assays each, with one consisting of four independent curves).

Quantitative Assay Using the VHH26-phage. To evaluate the ability of selected VHH-phages to be used in a quantitative immunoassay, a sequential competitive phage-ELISA was optimized. Calibration curves for clones VHH13, VHH33, and VHH26 showed similar behavior, though the best results were observed for VHH26. The slope of the standard curve was similar for the three clones, and the regression analysis showed an $r^2 > 0.99$. Particularly, the detection limit determined with 99% confidence for clone VHH26 was 3.5 ng gliadin/mL (the detection limits for assays using phage-VHH13 and phage-VHH33 were 24 ng/mL and 15 ng/mL, respectively) (**Figure 5A**).

Additionally, the effect of ethanol on the calibration curve for VHH26-phage was assessed by performing the preincubation step of the sequential competitive ELISA in the presence of 15% or 20% ethanol. As expected from our previous results, the presence of 15% ethanol did not modify the calibration curve, though a slight shift to higher gliadin concentration was observed (**Figure 5B**). However, the dose−response curve was altered when the assay was performed in the presence of 20% ethanol.

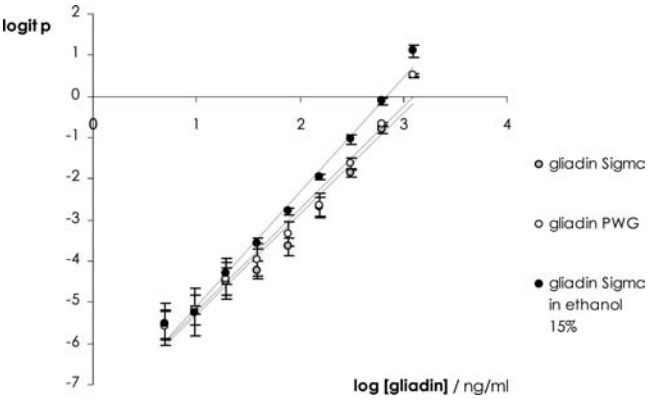

Figure 6. Quantitative determination of gliadins by capture ELISA with soluble VHH26. Standard curves were prepared with commercial gliadin (Sigma) (gray), PWG gliadin (white), or commercial gliadin (Sigma) in buffer containing 15% ethanol (black). The results are the mean value of four independent assays.

Interference by 20% ethanol was evident at low gliadin concentrations, as linearity was lost for concentrations below 100 ng gliadin/mL.

Quantitative Immunoassays with Soluble VHH26. Following the same procedure as above, a sequential competitive ELISA was performed using soluble VHH26 instead of VHH26-phage. This assay failed to produce a quantitative assay, probably due to the low affinity of the gliadin−VHH26 interaction as determined by surface plasmon resonance.

To investigate the use of soluble VHH26 in a quantitative capture ELISA, two formats were evaluated. First, we used an antigliadin mAb from our panel (1B4, 3B4, or 2A1) for antigen capture and the soluble VHH26 as secondary antibody, followed by detection with anti-His HRP conjugated antibody. Though all three mAbs were able to capture soluble gliadin, only the 3B4 mAb showed a detection limit of 40 ng gliadins/ml.

In a second approach, soluble VHH26 was used as capture antibody and the antigliadin biotinilated mAbs (2A1, 3B4, or 1B4) as secondary antibodies. Only the pair VHH26-3B4 biotinilated mAb produced a high performance quantitative assay. The assay using VHH26-3B4 showed a detection limit of 10 ng gliadins/mL. Importantly, the calibration curve for the gliadin standard solution prepared with commercial gliadin overlapped with that prepared using the European PWG standard (**Figure 6**). When the performance of this quantitative assay was evaluated in the presence of 15% ethanol, no change was observed in the slope of the regression analysis, demonstrating that the assay is fully operative under these conditions.

To evaluate the performance of the quantitative test using soluble VHH26 in capture ELISA, 19 different commercial food samples were analyzed. The VHH26 capture ELISA and the competitive ELISA, based on conventional antibodies and routinely used in our laboratory for gluten-free food certification (*21*), produced equivalent results for most of the samples (**Table I**). Remarkably, VHH26 capture ELISA was able to detect gliadins in two of the samples which were negative for the competitive ELISA (limit of detection: 1 ppm).

DISCUSSION

Gliadins, the most studied proteins in the pathogenic mechanism of CD, are able to trigger an innate response in the intestinal mucosa as well as to cause a specific activation of lamina propria T lymphocytes. It is now clear that both fractions, gliadins and glutenins, contain toxic peptides (*10*, *11*). Though different

Table I. Gliadin Determination in Commercial Food Samples by VHH26 Capture ELISA and Competitive ELISA Using Conventional Antibodies[a]

food sample	competitive ELISA	VHH ELISA
tapioca	nd	nd
rice biscuit	nd	nd
cocoa	nd	nd
marmalade	nd	nd
jelly	nd	nd
sausage	nd	nd
vanilla biscuit	**nd**	**0.4**
almond biscuit	**nd**	**0.6**
cured shoulder of pork	1	1.7
cinnamon	1	3
pizza spices	30	40
chorizo	3	3
vanilla biscuit	1	2
ice cream	200	220
spicy pork sausage	180	77
paprika	540	630
vegetable stock	16	12
chicken stock	11	17
fine herbs stock	26	11

[a] Analysis of gluten content in 19 commercial food samples by ELISA using VHH26 soluble fragment (limit of detection 0.24 ppm) and by competitive ELISA using conventional antibodies (limit of detection: 1 ppm). Results were expressed as ppm. nd = not detectable. Sample dilutions were 1:20 and 1:4 for competitive ELISA and VHH ELISA, respectively.

alternative therapeutic strategies have been proposed, the strict gluten-free diet is still the only treatment able to revert the symptoms and keep the integrity of the mucosal tissue. CD has a high individual variability, which poses difficulties to determine the tolerable amount of toxic proteins. The Codex Alimentarius Committee on Nutrition and Food for Special Dietary Use, the international commission ruling the norms for special foods, has been working on the Standard for Foods for Special Dietary Use for Persons Intolerant to Gluten. The use of two different levels of maximal gluten content for gluten-free products is currently under evaluation: naturally gluten-free products should not exceed 20 ppm, while those specially processed to reduce the gluten content have a maximum limit of 100 ppm (*26*). The limit of 20 ppm makes it necessary to use specific and high-detectability techniques. For the last 20 years, several analytical strategies have been proposed to increase the reliability of the ELISA tests used in the certification of gluten-free food (*12*, *16*, *21*, *27*, *28*). To this end, the complete extraction of gliadins from food samples is required. However, the extraction of gliadins is very far from being complete when conventional extraction solvent (60% ethanol) is used. It can be improved by adding reducing and disaggregating agents to this solvent and working at the temperature 50 °C (*29*). This extraction procedure has been recommended for the quantitative ELISA developed by Mendez's group (*16*), which has been ring-tested (*30*) and suggested as the Type I method to the Codex Alimentarius Commission (*26*).

It is known that addition of 2-ME and GuHCl in the extraction buffer increases gliadin recovery from food samples. We have demonstrated in previous work that this complemented extraction buffer interferes with conventional antibody binding, leading to false estimation of gliadin content. This effect was observed with rabbit antigliadin polyclonal antibodies and with two different antigliadin murine monoclonal antibodies in different assay platforms (*17*). In some cases, this interference could be avoided by diluting the sample prior to analysis, but this strategy reduces the detection capability of the test.

To develop a quantitative assay able to work under denaturing conditions, we took advantage of the use of llama antibody fragments, due to their particularly high physicochemical stability compared with that of conventional antibodies (*7*).

These llama antibody fragments are composed of only the heavy variable domain known as VHH, the smallest natural fragment with binding capacity. VHH domains are remarkably soluble and have been shown to retain their capacity for specific binding after adverse conditions, such as low pH (*9*), or even during stringent conditions, such as high temperatures (*7*) or the presence of detergents (*8*).

Since gliadin determination by immunoassays is performed on samples extracted with 60% ethanol, the main aim of this development was to select VHH-phages which retain their binding capacity in the presence of ethanol. Those VHH-phages would improve the detectability of gliadins in a quantitative detection method because they can be used in less diluted samples in order to avoid ethanol interference. To this end, we analyzed the binding of 28 VHH-phages at different ethanol concentrations to select clones resistant to gliadin−solvent extractions, showing that 21% (6 from 28 clones) of assayed VHH-phages retain their binding capacity in 15% ethanol (**Figure 1**).

The quantitative assay using conventional antibodies needs at least 20-fold dilution of the ethanolic extracts prior to the immunoassay (*16*, *21*, *30*); meanwhile, the assay with VHH-phages can be performed in 4-fold diluted samples. As consequence, the use of the VHH26 fragment in gliadin quantification allows a 5 times increment in the detection capability compared with the test using conventional antibodies.

The clones selected for ethanol resistance also showed increased apparent stability to other chemical denaturants such as 2-ME and GuHCl (**Figure 2**), indicating that the long CDR3 with an internal disulfide bridge (**Figure 3**) would stabilize this antibody fragment against different harsh chemical conditions.

Further studies are needed to confirm that the extra disulfide bridge in the long CDR3 of VHH26 is responsible for its remarkable apparent resistance. But the fact that the three selected VHH-phages share this feature suggests that this stabilizing bond is important in maintaining the rigidity of the loop and allowing antigen recognition under stringent conditions. Remarkably, Jesper et al. (*31*) isolated a VHH with the same two cysteines (separated by four residues) in the CDR3 using heat denaturation to select aggregation-resistant VHHs. It is noteworthy that soluble VHH26 showed similar trends of stability as its respective VHH-phage. In particular, VHH26 also works in the presence of 15% ethanol, indicating the feasibility of using phage-ELISA to select specific VHH clones with particular physicochemical characteristics.

The performance of gluten determination by VHH26 capture ELISA was evaluated on commercial food samples. VHH26 capture ELISA produced equivalent results for 17 out of 19 samples (6 negative and 11 positive) when compared with those obtained by competitive ELISA using conventional antibodies (*21*) (**Table I**). Remarkably, two samples were detected as positive only by VHH26 capture ELISA. Their gluten content was below the limit of detection for the conventional ELISA and was therefore considered as negative. The increment in the power of detection was, in part, due to the higher ethanol resistance of the VHH26 clone, which allows working with sample dilutions of 1:4 instead of the 1:20 required for ELISA using conventional antibodies. In addition, the kinetics analysis by surface plasmon resonance showed that VHH26 had similar recognition of gliadins from different mixtures of cultivars (PWG standard) as well as from individual varieties. This is an important fact for gluten determination in food, since results obtained by this method

would be less influenced by the presence of gluten from different sources, as commonly occurs in food analysis. VHHs also offer an extraordinary opportunity for manipulation by molecular techniques, creating new fragments with higher affinity. The relatively low affinity (about 100 nM) of the selected VHH26 can be improved by using standard *in vitro* affinity maturation procedures or, alternatively, by producing bivalent VHHs (*32*). Furthermore, VHHs' stability can also be increased by introduction of an additional disulfide bond (*33*).

In this work, quantitative immunoassays based on a gliadin-specific, highly stable, llama single domain antibody fragment were described. This strategy appears as an excellent platform for quantitative determination of proteins or any other immunogenic compound in the presence of denaturing agents. Single domain antibodies resistant to harsh conditions broaden the field of application of conventional immunoassays.

Supporting Information Available: Figures showing the antibody response against gliadins by immunized llamas and the SDS-PAGE results, and tables showing panning of the library and kinetic analysis of the VHH26/gliadins interaction. This material is available free of charge via the Internet at http://pubs.acs.org.

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