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# Study of a New Gliadin Capture Agent and Development of a Protein Microarray as a New Approach for Gliadin Detection

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## Abstract

Celiac disease is an immune-mediated enteropathy precipitated by the ingestion of gluten-containing foods in genetically predisposed children and adults. After a positive diagnosis for celiac disease, the only available treatment is to adopt a gluten-free diet, and evaluation of the absence of gluten in foods is crucial for the health of celiac patients. In the present study, a recombinant glutamine-binding protein (GlnBP) from *Escherichia coli* showed its ability to recognize peptides deriving from digested wheat flour. GlnBP and the commercially available 4F3 monoclonal antibody, raised against a region of the α-gliadin peptide 33-mer from wheat, demonstrated the ability to detect gliadin extracted from wheat flour. Recombinant GlnBP and 4F3 monoclonal antibody were used as new capture agents for the development of a protein chip able to detect gluten in foods. The protein microarray system has proven to detect the presence of gliadin in a range of concentrations between 500 and 5 ppm.

**Keywords:** Celiac disease; Gluten; Gliadin; Protein microarray; Glutamine-binding protein; Monoclonal antibody

## Introduction

Celiac disease and wheat IgE-dependent allergy are diseases triggered by gluten present in wheat and by similar proteins in other grains such as rye, barley, kamut, spelt, and, in some cases, oats, but also by non-gluten proteins (e.g. amylase-trypsin inhibitors in baker´s asthma) [1]. Celiac disease can be considered as an autoimmune disease in which the gluten-triggered immune system produces damage in the intestine [2].

After a positive diagnosis for celiac disease, the only treatment so far available is to adopt a strict gluten-free diet, which allows the intestinal mucosa to recover functionality [3]. In order to protect celiac patients through a strict gluten-free diet, it is important to make early diagnosis, especially for the prevention of complications that could derive from celiac disease, such as autoimmune diseases (thyroiditis, diabetes, etc.), cancer (intestinal lymphoma) and other diseases with a strong social impact (fractures due to osteopenia, infertility, abortions, etc.) [4-6]. For these reasons, it is very important to promote help for celiac people through periodic checks of food sold as gluten-free, which may contain gluten contamination as a result of production processes.

The European Union, the World Health Organization and the Codex Alimentarius require reliable and sensitive detection methods for toxic prolamin measurement in foods, in order to certify gluten-free products [7]. The Codex Alimentarius Commission allows a maximum gluten level of 20 ppm (mg/kg) in total for gluten-free food, based on the food as sold or distributed.

There are several commercially available test kits based on the ELISA method for gliadin and/or gluten detection. These methodologies are based on a variety of antibodies able to detect gluten with high sensitivity. Three monoclonal antibodies named PN3, 401.21 and R5 have been widely employed in various gluten detection systems. The variability in measurement of gluten content detected by different commercial gluten assays is strongly dependent on the reactivity of these antibodies, which are directed against different epitopes and fractions of gluten [8]. Antibody PN3 was raised against a 19-mer (LGQQQPFPPQQPYPQPQPF) synthetic peptide of A-gliadin ('Aggregable' α-gliadin) which had been demonstrated to cause mucosal damage in celiac patients [9-11]. MAb 401.21, developed by Skerritt and Hill [12], has been recommended for analytical assays of gluten by the Association of Official Analytical Chemists (AOAC). This monoclonal antibody reacts mainly with ω-gliadins and, to a small degree, with α- and γ-gliadins. Since this antibody mainly recognizes ω-gliadin, the quantification of gluten by ELISA could be underestimated [13].

Currently, the Type-1 method for determining gluten content in so-called "gluten-free" foods that is accepted by the Codex Alimentarius Commission is an R5 antibody-based ELISA combined with a cocktail extraction solution [14]. The monoclonal R5 antibody, raised against the ω-type of rye prolamins (ω-secalins), is directed against epitopes such as QQPFP, QQQFP, LQPFP, and QLPFP occurring in CD-toxic amino acid sequences of prolamins from wheat (gliadins), rye (secalins), and barley (hordeins) [1,14,15]. R5-mAb reacts strongly with α- and γ-gliadins and with proteins of an apparent molecular weight of about 50,000 and 75,000 and higher (probably ω-gliadins) [8].

Cross-reactivity of the monoclonal R5 antibody has been observed with non-toxic proteins, such as soy protein and lupine. For soybeans, this problem has been partially solved, using an aqueous ethanolic solution containing, 2-mercaptoethanol and guanidine hydrochloride [16]. A disadvantage of R5 antibody-based ELISA is its overestimation of the amounts of hordein in contaminated foods made from oats and barley, because the calibration was performed towards wheat prolamin [17].

In addition to the variability of antibody recognition, the efficiency

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of an immunochemical test is generally affected by several factors: reference material used, ELISA method (sandwich or competitive) and the gluten extraction method [18].

One limitation of the ELISA sandwich method is its inability to measure hydrolysed prolamins. Indeed, according to the Prolamin Working Group, the sandwich R5 ELISA method is suitable for detecting contamination from intact gluten [19]. For this reason, the competitive R5 ELISA assay is more efficient than the sandwich method.

Most sensitive methods for quantifying gliadin and glutenin in wheat grain have been based on analysis by mass spectrometry (MS) [20]. These methods help identify wheat gluten-specific peptides as markers suitable for high-sensitivity detection of gluten traces in so-called "gluten-free" foods (approximately 1 ppm of digested gliadin) [21,22]. Although these techniques are highly specific and sensitive, they are time-consuming and require specialized personnel.

A further possibility to detect gliadin was proposed by De Stefano et al. [23], who showed that a periplasmic protein of *Escherichia coli*, named glutamine-binding protein (GlnBP), is capable of binding some amino acid sequences of gliadin with high affinity. GlnBP is a monomeric protein, consisting of 224 amino acid residues (26 kDa), responsible for L-glutamine transport across the bacterial cytoplasmic membrane. Mass spectrometry experiments have shown that GlnBP specifically binds an amino acid sequence as XXQPQPQQQQQQQQQQQL, present in toxic prolamins [24]. Since this sequence is present only in toxic prolamins, GlnBP could be used as a molecular probe for gliadin detection. Using this protein as a molecular probe, De Stefano et al. [23] have developed a silicon-based optical biosensor for detecting gliadin peptides.

In the present work, recombinant GlnBP and the 4F3 monoclonal antibody (raised against a 33-mer peptide of wheat α-gliadin) were tested for their binding capacity to gliadins or peptide derivatives in order to evaluate their potential in developing an ultrasensitive biosensor for gliadin detection in food. Moreover, this is the first time that a protein microarray technology has been applied for gluten detection. The protein microarray could be an innovative approach for quantitative analysis of gluten in foods, characterized by high performance and relatively easy-to use procedures.

## Materials and Methods

### Strains, media and vectors

The *Escherichia coli* strain DH5α was used in all DNA manipulations. The *E. coli* strain employed for homologous expression was the BL21(DE3) strain and the plasmid used for protein expression in bacteria was the pET32 vector (Merck KGaA, Germany). Bacterial cultures were grown in LB medium (Sigma, USA) supplemented, when required, with 50 μg/L of ampicillin. Bacterial strain maintenance was achieved by plating onto LB medium supplemented with 2% (w/v) agar.

### Gene amplification and expression in *E. coli*

The gene encoding the periplasmic GlnBP was amplified from the *E. coli* strain DH5α genome by PCR using Platinum DNA polymerase (Life Technologies, USA), the primer pairs GlnBP-F, 5′-AAAGGATCCAAGTCTGTATTAAAAGTTTCA-3′ and GlnBP-R, 5′-AAAAAGCTTTTTCGGTTCAGTACCGAACCA-3′ (sequences respectively containing *Bam*HI and *Hind*III restriction sites are underlined). The PCR conditions were: 94°C, 3 min (once); 94°C, 30 s; 50°C, 15 s; 72°C, 45 s (35 cycles); 72°C, 10 min (once). The PCR amplicon was purified from agarose, digested with *Bam*HI and *Hind*III, and ligated with a similarly digested pET32 vector by using the T4 DNA ligase. *E. coli* strain DH5α was transformed with the ligation mixture and recombinant clones were identified by plating onto LB medium containing ampicillin (50 μg/ml). Recombinant plasmid DNA was extracted as described by Bleve et al. [25] and submitted to sequence analysis. DNA sequence was determined using the Dye Terminator Cycle Sequencing Ready Reaction with AmpliTaq DNA polymerase (Applied Biosystems, USA) and run on an automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer, Applied Biosystems, USA). The sequence data were analyzed by the Chromas software (version 1.45). The pET-GlnBP plasmid was used to transform the *E. coli* BL21(DE3) strain. The recombinant *E. coli* colonies were inoculated in 100 ml-LB medium containing ampicillin. Cultures were incubated at 37°C. When the turbidity of the culture was $OD_{600nm}$=0.8 protein expression was induced by the addition of 1 mmol/L IPTG and the culture was incubated at 25°C overnight with shaking at 200 rpm.

### Protein purification

Bacterial cells were collected by 10 min centrifugation at 5000×g and suspended in lysis buffer (50 mmol/L sodium phosphate, pH 8.0, 0,3 mol/L sodium chloride, 10 mmol/L imidazole and protease inhibitor) to a final volume of 30 ml. Cells were lysed in a French Press (Thermo Electron Corporation, USA) and the lysate was centrifuged at 4°C for 10 min at 5500×g to remove cell debris then applied to a 1.5 ml HIS-Select Nickel Affinity gel (Sigma, USA) pre-equilibrated with lysis buffer according to the manufacturer's instructions. The column was washed with 30 column volumes of wash buffer (50 mmol/L sodium phosphate, pH 8.0, 0.3 mol/L sodium chloride, 10 mmol/L imidazole). The GlnBP was eluted from the column using 5 volumes of elution buffer (50 mmol/L sodium phosphate, pH 8.0, 0.3 mol/L sodium chloride, 250 mmol/L imidazole). The purified protein was collected in five fractions each of 0.5 ml.

### Polyacrylamide gel electrophoresis and western blot assays

The polyacrylamide gel electrophoresis assay was performed at 140 V and 25-30 mA in sodium dodecyl sulfate (SDS) Tris/glycine buffer in BioRad MiniProtean II gel electrophoresis equipment [26]. Protein concentration was determined using the Protein Assay dye reagent (Bio-Rad, USA) with bovine serum albumin as standard.

Western blots were performed as previously described [27] using a mouse anti-His monoclonal antibody (Sigma, USA) or a mouse gliadin peptide monoclonal antibody (4F3) (Thermo Fisher Scientific, USA) at dilutions of 1:5000 and a goat anti-mouse IgG conjugated with peroxidase (Bethyl Laboratories, USA) at dilutions of 1:10000.

When recombinant GlnBP was used in Western blot, after the blocking step, the membrane was subjected to washing for 10 min in 1X TS buffer (0.05 mol/L Tris-Cl pH 7.4, 0.2 mol/L NaCl) and then incubated for 1 h under agitation with the purified recombinant Glutamine-Binding Protein (2.5% (w/v) milk powder, 1X TS, 7 μg/μl GlnBP, in distilled water). The membrane was then washed four times (10 min each) in 1X TS buffer and subjected to incubation with the anti-His antibody.

### Digestion of gliadin, wheat and corn flour

An amount of 20 mg of gliadin (Sigma, USA) or of 400 mg of wheat and corn flour were added to 1 ml of 100 mmol/L TrisHCl pH 8. Then trypsin (Sigma, USA), chymotrypsin (Sigma, USA) and

trypsin+chymoptrypsin were added to a final concentration of 0.1 mg/ml to a final volume of 1 ml.

After incubation for 10 min at 37°C or at 25°C, the samples were cooled on ice and centrifuged at 5000×g for 5 min. The supernatant was collected, boiled, freeze-dried and stored at -20°C. Total proteins dissolved in the supernatant were quantified using the Protein Assay dye reagent (Bio-Rad, USA).

### Protein microarray

Untreated gliadin, chymotrypsin digested gliadin and BSA (negative control) were spotted in triplicate onto epoxysilane-coated Nexterion slides (Slide E, Schott, Germany) using a robotic printing SpotArray 24 system (Perkin Elmer, USA) with a concentration of 500, 50 and 5 µg/ml in 0.1 mol/L $NaHCO_3$ buffer (pH 8.5). The proteins were immobilized by covalent binding between the epoxy groups on the slide surface and the amine and hydroxyl groups of the proteins. The printed slides were incubated in the dark at room temperature and 60% humidity for 2 h. The printed slides were then kept dry at 4°C until use.

The protein chips were incubated in slight agitation on a shaker at room temperature for 1 h in PBS containing glycine (0.5 mol/L) and bovine serum albumin (BSA, 1%, w/v) to block non-specific binding. Hybridization was performed, in phosphate-buffered saline (PBS) for 1 h in dark and at room temperature, with fluorescently labeled proteins: GlnBP (recombinant) and 4F3 monoclonal antibody at the concentration of 0.1 mg/ml. GlnBP and 4F3 were labeled with Alexa-555 protein labeling kit (Molecular Probes, Life Technologies, USA). The remaining excessive free dye was removed by gel filtration (Biogel P-6, Bio-Rad, USA) according to the manufacturer's instructions. After hybridization, the slides were washed with PBS+BSA (0.1%, w/v), dried in a centrifuge at 500×g for 2 min and subsequently scanned with Array Scanner 428 (Affymetrix, USA). Fluorescence intensities were analyzed using ScanAlyse software (Eisen Lab).

## Results

### Recombinant Glutamine-Binding Protein expression and purification

To isolate the Glutamine-Binding Protein-coding gene from *E. coli*, a DNA-PCR-based strategy was used. Isolation of the gene was carried out by PCR with the aim of adding six histidine codons to its 5' and 3'-terminus. By using total DNA extracted from *E. coli* cultured cells as template, the DNA amplified fragment, denoted as *GlnBP*, was cloned in the pET32 vector. The subsequent nucleotide sequence analysis showed that the cloned Glutamine-Binding Protein gene was identical to the permease of the periplasmic glutamine-binding protein (*ECs0889*) gene (GenBank: EU903664.1). The sequence results were confirmed by sequencing five independent clones, thus excluding the possibility of nucleotide differences being due to mistakes caused by Taq polymerase-driven amplification. The isolated *GlnBP* gene sequence consists of an open reading frame of 759 bp in length, which potentially codes for a protein of 249 amino acids with an estimated molecular mass of 27.4 kDa.

Bacterial cells of *E. coli* BL21(DE3) transformed with the pET-*GlnBP* vector were grown and subjected to induction and then lysed by French Press. Glutamine-Binding Protein purification was carried out by affinity chromatography using optimized conditions. The Glutamine-Binding Protein produced by recombinant bacteria was studied by electrophoresis on an SDS-PAGE (Figure 1). A major band

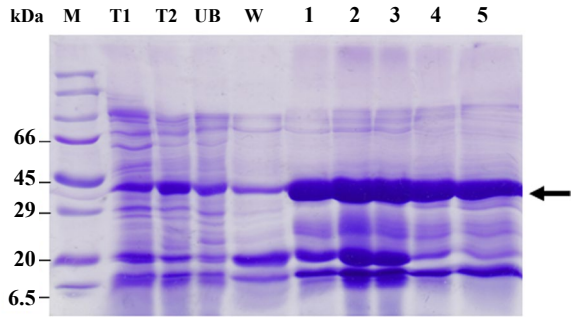


**Figure 1:** SDS-PAGE analysis of the recombinant Glutamine Binding Protein in *E. coli* and purified by affinity chromatography. The Glutamine Binding Protein is evident *(arrow)* in the samples obtained after elution. *M,* molecular markers (expressed in kDa), *T1* and *T2,* cell lisates after 6 and 10 hours induction; *UB,* portion of cell lysate not bound to the affinity resin; *W,* column wash buffer to eliminate aspecificities; *lanes 1-5,* fractions 1-5 eluted from the affinity column.

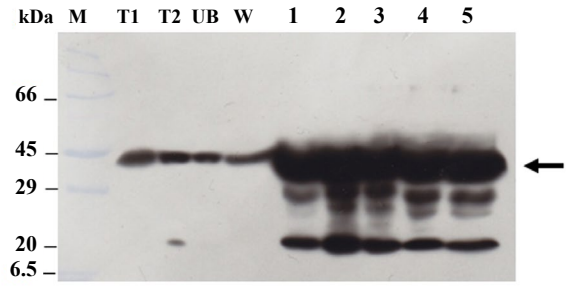


**Figure 2:** Western blot analysis of the recombinant Glutamine Binding Protein in *E. coli* purified by affinity chromatography. *M,* molecular markers (expressed in kDa), *T1* and *T2,* cell lisates after 6 and 10 hours induction; *UB,* portion of cell lysate not bound to the affinity resin; *W,* column wash buffer to eliminate aspecificities; *lanes 1-5,* fractions 1-5 eluted from the affinity column. Glutamine Binding Protein was revealed by a commercial anti-histidine antiserum.

at about 43 kDa was observed after purification, deriving from the molecular weight of GlnBP and the polypeptides added by the pET vector sequences in-frame expressed with the *GlnBP* gene. The purified protein was probed in Western blot analysis with an antiserum to histidine-tag and the assay gave a clear-cut reaction indicating that the 43 kDa band corresponds to the recombinant protein (Figure 2). In addition, smaller proteins were detected, which indicated possible degradation forms of the recombinant protein.

Fractions corresponding to the purified protein were pooled and the total protein concentration amounted to 7.4 mg/ml.

### Recombinant Glutamine-Binding Protein and 4F3 monoclonal antibody binding assays

The purified protein was then used to perform the evaluation of its binding capacity towards peptides derived from a gliadin extract and to test its binding capacity towards gliadin peptides in wheat flour and possible cross-reactivity with other flours.

To evaluate the ability of GlnBP to bind gliadin peptides, gliadin (Sigma) was digested by the enzymes trypsin and chymotrypsin, separately or in combination, to mimic the effect of digestion that gliadin undergoes in the human gastro-intestinal tract. Figure 3 shows a schematic representation of the detection method used to reveal the ability of GlnBP to bind gliadin peptides. In order to perform this assay,

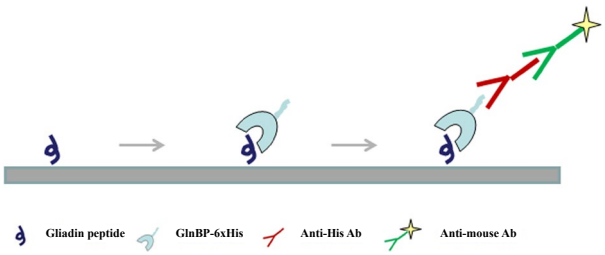


**Figure 3:** Schematic representation of gliadin peptide detection method by the use of purified GlnBP.

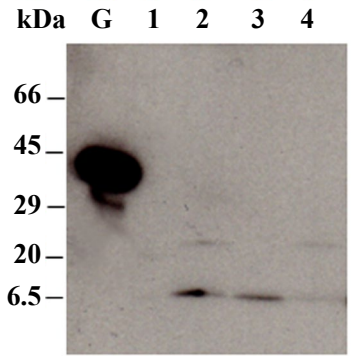


**Figure 4:** Western Blot analysis of gliadin treated with chymotrypsin detected by recombinant GlnBP. *G*, recombinant GlnBP; *1*, gliadin incubated in buffer for 10 min at 25°C; *2*, gliadin treated with chymotrypsin for 10 min at 25°C; *3*, gliadin treated with chymotrypsin for 10 min at 37°C; *4*, gliadin incubated in buffer for 10 min at 37°C. Recombinant GlnBP was probed against an anti-histidine antiserum.

it was necessary to set up the conditions for GlnBP protein binding. The optimum conditions for GlnBP binding to the immobilized proteins on PVDF membrane were as follows: incubation of the membrane for 1 h under stirring with a concentration of purified GlnBP equal to 110 µg/ml in the presence of a buffer containing 2.5% (w/v) low fat milk. Among all the tested conditions, the purified GlnBP was able to recognize gliadin peptides after both treatment with chymotrypsin for 10 min at 25°C and 37°C, thus also indicating that the recombinant protein was in an active form (Figure 4).

The ability of recombinant GlnBP to bind peptides deriving from wheat flour was assayed. For this purpose, samples of wheat flour (400 mg) were digested by the enzymes trypsin and chymotrypsin, independently or in combination at 37°C or at 25°C for 10 min, which preliminary analyses indicated as the minimum time required obtaining a good level of wheat flour digestion. After 10 min trypsin digestion, the dissolved protein/peptides resulted 9.4 ± 0.8 mg/ml and 7.7 ± 0.4 mg/ml (at 37 or 25°C, respectively). Using chymotrypsin, the dissolved total protein/peptides resulted 9.8 ± 1.1 mg/ml and 7.6 ± 1.2 (after 10 min at 37 and 25°C, respectively). When trypsin and chymotrypsin were used in combination, the dissolved total protein/peptides resulted 12.0 ± 1.6 mg/ml and 11.2 ± 1.4 mg/ml (after 10 min at 37 or 25°C, respectively).

The purified GlnBP was able to detect the presence of gliadin peptides in wheat flour subjected to both treatments. Indeed, GlnBP recognized gliadin peptides in wheat flour digested separately with trypsin or chymotrypsin and in the trypsin+chymotrypsin combination after incubation at 37°C, as well as in the case of untreated wheat flour incubated at 37°C, as control (Figure 5A). When the assay was carried out at 25°C, the GlnBP was able to bind peptides in wheat flour samples treated with chymotrypsin and trypsin+chymotrypsin, as well as in the sample resulting from the simple incubation of the flour at 25°C without the use of enzymes, as control (Figure 5B). At both temperatures (37°C and 25°C), in untreated wheat flour samples, GlnBP was able to bind peptide fragments of molecular weight comprised between about 6.5 to about 45 kDa (Figure 5A and 5B).

In a subsequent step, binding specificity of recombinant GlnBP was assayed against gliadin peptides from wheat flour and against the non-toxic peptides produced following chymotrypsin digestion of corn flour, a safe cereal for celiac patients. This test was also performed using the monoclonal antibody 4F3 (Thermo), raised using the immunogenic synthetic peptide corresponding to residues K (58) LQPFPQPELPYPQPQ (73) of wheat α-gliadin peptide.

For the assay, wheat flour and corn flour were treated with the enzyme chymotrypsin for 10 min at room temperature (25°C). Western blot analysis revealed that GlnBP and 4F3 monoclonal antibody were able to detect the presence of gliadin peptides in both wheat flour samples, untreated and digested with chymotrypsin for 10 min at 25°C (Figure 6A and B). As expected, treatment with the enzyme led to the release of various peptides, of molecular weights varying from 60 to 20 kDa in the treated sample revealed by the 4F3 antibody and of 6.5 kDa in the treated sample revealed by GlnBP. R5 antibody (R-Biopharm) was chosen as the standard control for the

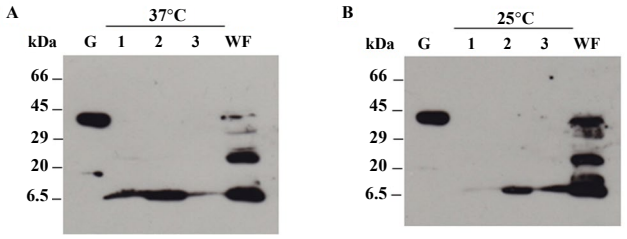


**Figure 5:** Western Blot analysis of wheat flour treated with trypsin, chymotrypsin and trypsin+chymptrypsin detected by recombinant GlnBP. A: *G*, recombinant GlnBP; wheat flour digested at 37°C for 10 min with *1)* trypsin, *2)* chymotrypsin and *3)* trypsin+chymotrypsin; *WF*, wheat flour incubated in buffer for 10 min at 37°C. B: *G*, recombinant GlnBP; wheat flour digested at 25°C for 10 min with *1*) trypsin, *2*) chymotrypsin and *3*) trypsin+chymotrypsin; *WF*, wheat flour incubated in buffer for 10 min at 25°C. Recombinant GlnBP was probed against an anti-histidine antiserum.

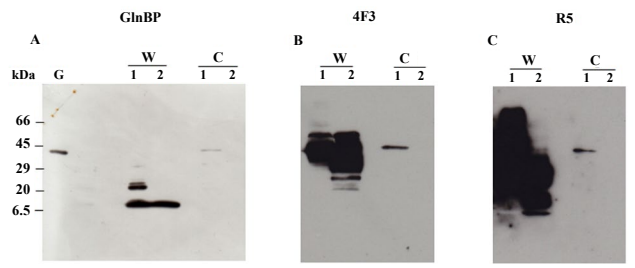


**Figure 6:** Western Blot carried out on wheat and corn flour after digestion with chymotrypsin at 25°C for 10 min. A: *G*, recombinant GlnBP; *W1* (wheat flour) and *C1* (corn flour) incubated in buffer at 25°C for 10 min; *W2* (wheat flour) and *C2* (corn flour) digested with chymotrypsin at 25°C for 10 min, revealed by the recombinant GlnBP. B: *W1* (wheat flour) and *C1* (corn flour) incubated in buffer at 25°C for 10 min; *W2* (wheat flour) and *C2* (corn flour) digested with chymotrypsin at 25°C for 10 min, revealed by the 4F3 monoclonal antibody. C: *W1* (wheat flour) and *C1* (corn flour) incubated in buffer at 25°C for 10 min; *W2* (wheat flour) and *C2* (corn flour) digested with chymotrypsin at 25°C for 10 min, revealed by the R5 antibody.

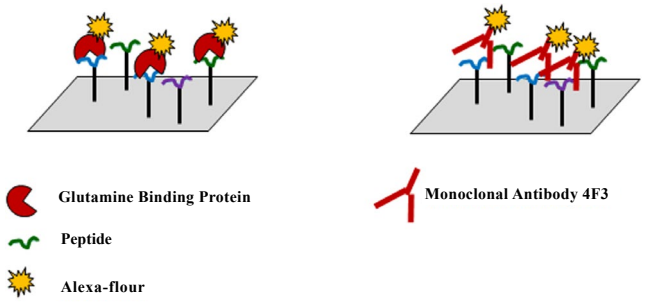


**Figure 7:** Schematic representation of the protein microarray method for gliadin peptides detection, by the use of purified recombinant GlnBP and monoclonal antibody 4F3.

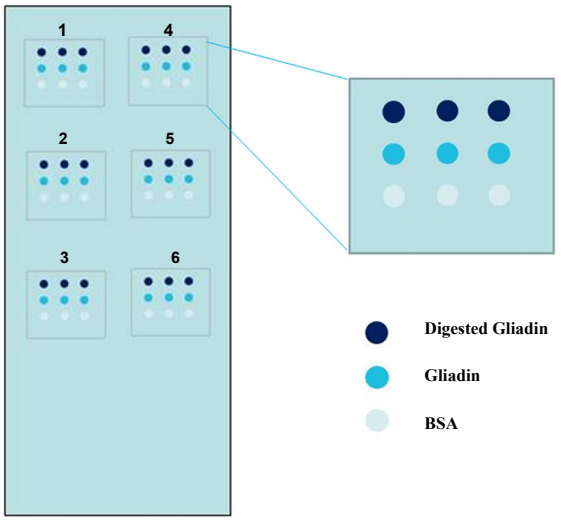


**Figure 8:** Schematic diagram of the protein microarray. Each subarray was spotted in triplicate with gliadin digested with chymotrypsin (Digested Gliadin) and with untreated gliadin (Gliadin), incubated in buffer for 10 min at 25°C. BSA was used as negative control (BSA). The proteins were immobilized in three different concentrations 500 µg/ml (subarray 1 and 4), 50 µg/ml (subarray 2 and 5) and 5 µg/ml (subarray 3 and 6).

experiment, since it is unequivocally recognized as the best antibody for the detection of gluten and commonly used in the ELISA test accepted by the Codex Alimentarius. R5 antibody showed the same behavior of 4F3 antibody, but it revealed a different peptide recognition pattern, ranging from about 30 to 6.5 kDa (Figure 6C). It is worthwhile noting that after prolonged exposure time, all three detection systems showed a slight cross-reactivity with peptides from the corn flour sample, not treated with chymotrypsin. However, in such conditions, GlnBP showed a lower level of cross-reactivity than the two tested monoclonal antibodies (Figure 6A-6C).

## Protein microarray for the detection of gliadin peptides

A protein microarray was prepared to test GlnBP recombinant and the 4F3 monoclonal antibody for the sensitive detection of gliadin peptides on a miniaturized system (Figure 7).

Peptides resulting from chymotrypsin digested gliadin (10 min at room temperature, named digested gliadin) and untreated gliadin were deposited on a microarray glass slide in order to develop a protein chip. The deposition (10 nl per spot of 100 µm diameter) was performed by a robotic system according to the diagram shown in Figure 8. The microarray consisted of 6 subarrays each containing spots in triplicate of digested gliadin, untreated gliadin and BSA (negative control).

In each subarray digested gliadin, untreated gliadin and BSA were deposited at three different concentrations, i.e. 500 µg/ml (subarrays 1 and 4), 50 µg/ml (subarrays 2 and 5) and 5 µg/ml (subarrays 3 and 6) according to the diagram shown in Figure 8. Since the liquid volume deposited for each spot on the chip corresponded to 10 nl, the total amount of protein/peptide deposited was 5 ng, 0.5 ng and 0.05 ng, respectively for the three concentrations (500, 50 and 5 µg/ml).

The recombinant GlnBP is predominantly able to bind gliadin peptides obtained by chymotrypsin treatment at the three tested

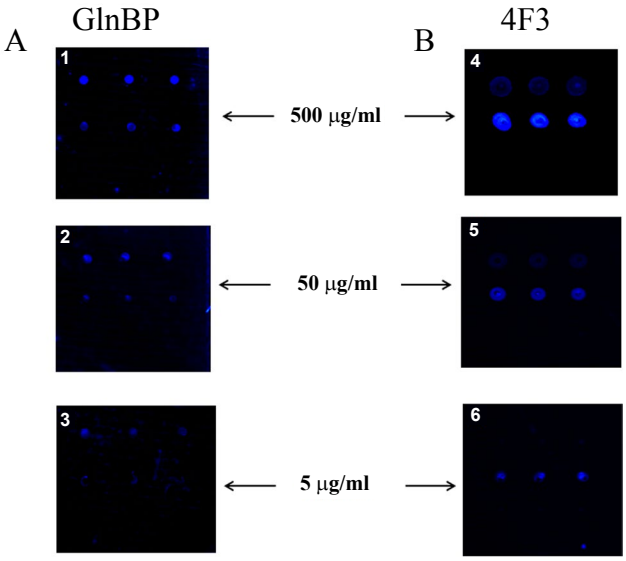


**Figure 9:** Acquired image by array scanner of subarrays where digested and untreated gliadin were immobilized in triplicate according to the diagram in Figure 8 at the concentration of 500 µg/ml (subarrays 1 and 4), 50 µg/ml (subarrays 2 and 5) , and 5 µg/ml (subarrays 3 and 6). The subarrays were incubated with A) recombinant GlnBP and B) 4F3 monoclonal antibody both labeled with Alexa-Fluor 555.

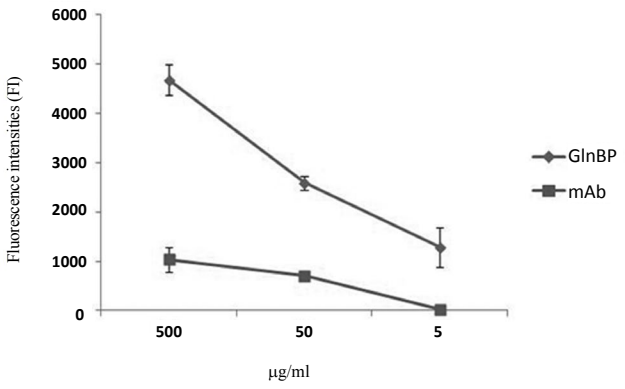


**Figure 10:** Fluorescence intensity analysis (FI) of the subarrays (shown in Figure 9) spotted with chymotrypsin digested gliadin at decreasing concentrations (500, 50 and 5 µg/ml) and incubated with the 4F3 monoclonal antibody and recombinant GlnBP both labeled with Alexa Fluor 555. Error bars indicate the standard deviations around the mean values obtained from the analysis of the spots in triplicate.

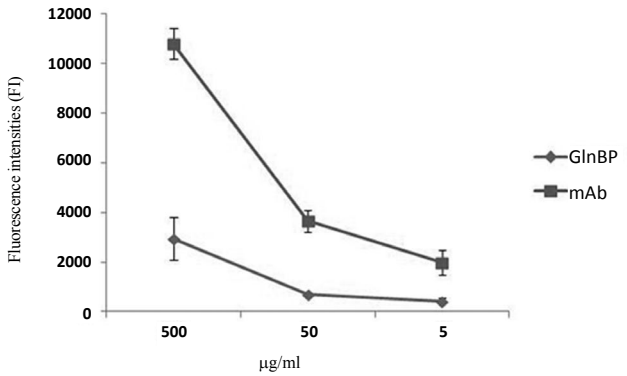


**Figure 11:** Fluorescence intensity analysis (FI) of the subarrays (shown in Figure 9) spotted with untreated gliadin at decreasing concentrations (500, 50 and 5 µg/ml) and incubated with the 4F3 monoclonal antibody and recombinant GlnBP both labeled with Alexa Fluor 555. Error bars indicate the standard deviations around the mean values obtained from the analysis of the spots in triplicate.

concentrations, whereas a weak signal was obtained with undigested gliadin (Figures 9-11). By contrast, 4F3 antibody was unable to bind digested gliadin with the same affinity as GlnBP, but showed a higher affinity to the gliadin merely incubated at 25°C for 10 min (Figures 9-11). Both recombinant GlnBP and 4F3 antibody were able to detect the presence of gliadin antigens in a range of concentrations between 5-500 g/ml, corresponding to 5-500 ppm.

## Discussion

Glutamine-binding protein (GlnBP) and the 4F3 monoclonal antibody were tested for their ability to bind gluten or its derivatives and for their potential use in the development of miniaturized and ultrasensitive biosensors suitable for detecting gliadin in food.

Currently, the most widely used and officially recognized systems for detecting gluten in foods are based on the use of antibodies in immunoassays. Several commercially available ELISA kits are based on antibodies such as: a) the mouse monoclonal antibody developed against 401.21 ω-gliadins [28]; b) the PN3 antibody developed against a synthetic 19-mer peptide (LGQQQPFPPQQPYPQPQPF) of α-gliadins [9]; c) the R5 antibody developed against an ω-secalin extract [29]; d) the G12 monoclonal antibody raised against the α-gliadin 33-mer toxic peptide, as detection antibody, together with the A1 antibody as capture agent [30]. These antibodies have high specificity towards classes of gliadins, glutenins, hordeins, secalins and avenins, but they may have cross-reactivity against flours not containing gluten and none of them completely solves the problem of gluten detection in foods [18]. GlnBP can perform as well as R5 and 4F3 monoclonal antibodies, since it revealed only slight cross-reactivity against undigested corn flour.

The search for new gluten capture agents is also necessary, since the antibodies that have so far been developed recognize only some of the epitopes that can stimulate T cells [31]. Toxicity of gluten is not fully understood, which is partly due to the fact that gluten is a complex mixture of proteins. ELISA available methods are valuable tools in the definition of gluten-free foods [18]. The GlnBP has been chosen in this study due to its potential to be used alone or in combination with existing antibodies, especially to improve detection of peptides extracted from processed foods.

The GlnBP, is a monomeric protein located in the periplasmic space of *E. coli*. This protein binds glutamine and poly-glutamine residues with high affinity and is responsible for the first step in the active transport of L-glutamine across the bacterial cytoplasmic membrane. The protein consists of two globular domains linked by two similar peptides. The crystallographic data indicate that the two domains perform large movements when the ligand is bound [32]. Considering that gluten proteins are rich in glutamine residues, De Stefano et al. [23] and Staiano et al. [24] have shown that the GlnBP is able to bind with high affinity to amino acid sequences characteristic of gluten, such as gliadin, a complex mixture of proteins toxic to coeliacs. However, the possibility that glutamine-binding protein could recognize other specific target sequences in proteins other than alpha-gliadins has to be considered. For this reason, further studies need to be performed by mass spectrometry approaches.

The gene coding for GlnBP, modified at its 3' and 5' termini with six codons encoding histidine residues, was cloned and homologously expressed in *E. coli*. The resulting purified protein was biologically active when tested for its ability to specifically bind standard gliadin extract and wheat flour.

A new approach was developed in order to obtain-in a relatively short time and at temperature conditions close to those of the environment-the release of gliadin peptides from complex structures present in flour samples or baked products, mimicking gastro-intestinal digestion by the use of trypsin and/or chymotrypsin, as proposed by previous studies [33,34].

Analogously to the data reported by De Stefano et al. [23], recombinant GlnBP was able to specifically bind digested gliadin. Furthermore, GlnBP was able to recognize gliadin peptides deriving from wheat flour treated with chymotrypsin or trypsin/chymotrypsin at room temperature for 10 min in a manner comparable to those produced by treating the flour with trypsin or chymotrypsin at 37°C for the same time.

Although the results here reported are only qualitative, the analysis of gliadin and gliadin peptides released after digestion of wheat flour will be carried out in future in order to set up a device that can perform quantitative analysis. Currently, in the preparation of samples for ELISA assays, approved as official methods by the AOAC, the two critical steps are protein extraction from the food matrix, especially in the case of processed foods, and solubilization of purified proteins. The AOAC ELISA assays on ω-gliadin and R5 ELISA kits employ cocktail solutions containing ethanol, 2-mercaptoethanol and guanidine hydrochloride for extracting prolamins from cooked or uncooked food and for increasing gluten solubility, and they require the use of laboratory equipment and not less than 4-5 hours to obtain a result. Today, there is a pressing need for a simplified procedure for treating fresh or processed food matrices which i) provides maximum solubilization of prolamins in aqueous solution in the shortest possible time (maximum 10 min), without using substances that are dangerous or harmful to human health, ii) does not require the use of specific laboratory equipment and experienced staff, iii) requires safe handling of samples without any form of leakage and avoiding contamination and contact with the operator.

Together with the AOAC reference R5 monoclonal antibody, both recombinant GlnBP and the 4F3 monoclonal antibody were able to detect gliadin, extracted from wheat flour at room temperature for 10 min, with low levels of cross-reactivity in corn flour, which does not contain gluten. These findings indicated that treatment at room temperature can be conceivable for the development of a quick

protocol for sample treatment, which does not require special attention for sample preparation.

Among the numerous methods proposed for gliadin detection, mention may be made of systems based on SDS-PAGE in combination with immunoblotting, immunoelectrophoresis, mass spectroscopy and instrumental analytical techniques, such as MALDI/TOF-MS and ES-MS combined with high performance liquid chromatography (HPLC) [21,35,36]. Due to its high sensitivity and small sample requirement, MS is an important tool for analysis and identification of markers deriving from gliadins, glutenins and their proteolytic degradation of mixtures of peptides [37]. Although methods based on MS proteomic approaches can produce accurate quantification of gluten in foods at trace amounts, revealing a sensitivity limit comparable to that of ELISA tests (1 ppm) [38], they cannot be miniaturized. Moreover, approaches based on HPLC and capillary electrophoresis have been widely used to analyze prolamins in foods and for the quantitative determination of gliadin [39,40]. Recent studies have evaluated the use of flow cytometry to detect levels of gliadin equal to 10 pg/mL [41]. However, the most commonly used methods for measuring gliadin are based on immunological procedures, including immunoblotting and ELISA, using monoclonal or polyclonal antibodies against various components of gliadin [35,40,42-44]. The ELISA sandwich method provides high sensitivity and specificity and allows the quantitative analysis of prolamins of wheat (gliadin), rye (secalin) and barley (hordein) in raw and processed foods for values up to 2.5 mg/kg of gliadin (which corresponds to 5 mg/kg of gluten). The detection limits of the ELISA kits available vary from 2 to 10 mg/kg of gliadin. There are also several commercial systems for the immune-chromatographic detection of gluten traces in food products which take between 5 and 15 min to provide a result. These systems have detection limits corresponding to 2.5 mg/kg of gliadin (5 mg/kg of gluten).

In recent years, the miniaturization of biosensors and their integration into functional biochip microarrays enable simultaneous detection of several analytes, the diagnosis or predisposition to disease, as well as the identification of custom drug response profiles.

In this study, preliminary results were produced concerning the development of a protein chip for the detection of gluten in foods using two different capture agents, recombinant GlnBP and the 4F3 monoclonal antibody. For the development of the protein chips, optimal conditions for the immobilization and maintenance of gliadin protein and peptide structures were established. As expected, GlnBP demonstrated the ability to preferentially bind gliadin peptides produced by gliadin digestion. It could be hypothesized that gliadin digestion produced peptides available for GlnBP recognition, whereas these peptides are not available for GlnBP in intact gliadin. Furthermore, although the antibody maintained a slight binding capacity for gliadin peptides, it bound with greater affinity to undigested gliadin. For the first time, the good correspondence between the fluorescence intensity resulting from immobilized gliadin or gliadin peptides and the capture agents demonstrated that a protein microarray system can detect this kind of interaction. Its dose-response curves prove that the protein microarray system, which uses GlnBP or 4F3 antibody, can detect the presence of gliadin in a concentration range between 500 and 5 ppm, the latter value corresponding to the 20 ppm limit of gliadin content necessary to describe a food as gluten-free. An optical system that uses GlnBP deriving from recombinant *E. coli* as the capture molecule for gliadin, has been proposed by De Stefano et al. [23]. These authors developed an optical biosensor based on silicon for detecting traces of gliadin, demonstrating a linear response for concentrations ranging between 2 and 8 μmol/L. By LC-MS method used for gluten quantitation, although specific peptides can be selectively detected and concentrations exactly determined, peptide yield depends on the composition of the gluten sample, on the efficiency of the enzymatic digestion and on processing of the food. Although the presented approach shows the same drawbacks described above, as it detects gluten via peptides, and further studies to measure the possible conversion of peptides into gluten concentration is needed, the proposed system has the potential to be improved as a quantitative method and in an easy-to-use portable device so as to allow even a non-specialized user to perform a food analysis in a limited period of time.

The approach described in this study is preparatory for future analyses of the glutamine-binding protein specificity towards different gluten protein types from wheat, rye, and barley. Experiments are now under way to test different protein types (glutenin and gluten proteins) from rye and barley, and, after improving the extraction method, to test the system using real samples (bakery foods, pasta, pizza, etc.).

## Conclusions

In conclusion, GlnBP and the 4F3 monoclonal antibody could be two good candidates as capture agents for gliadin and gliadin-derived peptides. For the first time, a protein microarray has now been developed as an innovative, high-performance and relatively easy-to-use system for quantitative detection analysis of gluten in foods. This system was chosen due to its potential i) to simultaneously analyze the same sample using different capture agents raised against several toxic prolamins (gliadin, glutenins, hordeins, secalins) and their derived peptides and ii) to be easily miniaturized so as to produce a portable system.

Further experiments are now under way to optimize an easy and rapid extraction method for gluten-derived peptides from processed foods and to set up the microarray system in order to quantitatively detect these molecules.

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