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# Development of High-sensitive Enzyme Immunoassays for Gliadin Quantification Using the Streptavidin–Biotin Amplification System

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*Optimization of three enzyme immunoassays of very high sensitivity using three anti-prolamin monoclonal antibodies (MAbs) (13B4, 11C4 and 12A1) is presented here. These MAbs are specific for those prolamins toxic for coeliac patients, as determined by immunoblotting analysis. Biotinylated MAbs were used in two of the assays. In a competitive ELISA, the binding of each biotinylated MAb to a gliadin-coated solid phase was inhibited by gliadin in the fluid phase. The best result was obtained using the biotinylated MAb13B4 (detection limit: 20 ng* $ml^{-1}$*). With regard to capture ELISA, we tested the performance of the three MAbs. In this sandwich ELISA, the MAb used for antigenic capture was the same as that used as secondary biotinylated antibody. The MAb12A1 had the best performance (detection limit: 1 ng* $ml^{-1}$*). The use of biotin-labelled gliadin in a quantitative immunoassay with a detection limit of 5 ng* $ml^{-1}$ *is also reported. This assay involves an antigenic capture using the MAb12A1 followed by a competition between biotinylated and non-biotinylated gliadin. We have found the use of the streptavidin–biotin interaction as signal amplification system to be very useful. This technique, as far as we know, has not been previously reported for gliadin quantification.*

**Keywords:** *Anti-gliadin monoclonal antibodies, gliadin quantification, ELISA*

## INTRODUCTION

Coeliac disease (CD) is one of the most frequent chronic gastrointestinal disorders in both children and adults. The highest prevalence, 1:184, was found in an Italian multi-centre study (Catassi *et al.*, 1996). CD occurs in genetically susceptible individuals as a result of

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the ingestion of food containing prolamins of wheat, barley, rye and probably oat (Marsh, 1992). Ingestion of minimal amounts of these proteins may produce the characteristic histological alterations of the small bowel mucosa (Troncone & Auricchio, 1991). Most patients show a rapid improvement of their clinical conditions when prolamins are removed from their diet. Consequently, a strict gluten-free diet (GFD) is the only possible treatment for coeliac patients (Dinari *et al.*, 1992). Clinical presentation and mucosal histology show a great variability among non-treated coeliac patients. Moreover, there is no agreement about the maximal amount of gluten that may be ingested without affecting the mucosa or producing relapse of symptoms.

Wheat proteins are widely used in the food industry to improve product quality because of their particular physicochemical properties. In some cases, these proteins can be present in minimal amounts. Wheat starch is also used as an excipient in medicines; consequently, gliadins can be found in pharmaceutical products (Miletic *et al.*, 1994).

Several methods for the analysis and quantification of prolamins have been developed using both polyclonal and monoclonal antibodies (MAbs) (Howdle & Losowsky, 1990). Nevertheless, in these assays, many drawbacks arise mainly as result of the very low solubility of prolamins in solvents compatible with antigen–antibody interaction (Skerritt & Martinuzzi, 1986). The tendency of prolamins to form macromolecular aggregates, their high homology with the resulting strong cross-reactivity and the difficulty to prepare isolated components are factors causing problems during the characterization of antibody reactivity as well as in the development of assays of high specificity and sensitivity (Festenstein *et al.*, 1987; Brett *et al.*, 1990). Furthermore, many commercial foodstuffs undergo thermal treatment during manufacturing, leading to conformational changes of proteins and changes in their immunochemical reactivity. In this case, some of the methods reported are unable to detect gliadin in processed food, as reported by Friis (1988). Using some antibodies, cross-reactivity with proteins non-toxic for coeliac patients—leading to false-positive results—have also been documented in quantitative methods (e.g. Troncone *et al.*, 1986).

It is clear that the optimization of a quantitative immunoassay that fulfils the requirements of specificity, accuracy and detectability is very difficult to achieve. Immunochemical and structural studies, as well as economical and legal reasons, and the need to ensure the composition of products and guarantee the fulfilment of GFD requirements make the development of highly sensitive analytical methods imperative.

To this end, we tested the use of the highly sensitive biotin–streptavidin system. The preparation of antigens and biotinylated MAbs is easy because biotin can be coupled under such mild conditions that generally their biological activity remains unchanged. Since streptavidin has a very high affinity for biotin (a reaction that is also multivalent) a great signal amplification is achieved (Avrameas, 1992). As far as we know, this system has not been previously used in gliadin quantification.

In this article, we show the characterization of anti-prolamins MAbs and the development of three different quantitative ELISAs of high detection limit, using both biotin-labelled anti-prolamin MAbs and biotinylated gliadin.

## MATERIALS AND METHODS

### Preparation of Protein Extracts

Protein extracts were prepared from wheat, barley, rye, triticale, oat, soy, rice and maize flour extracted with 70% (v/v) aqueous ethanol (10 ml $g^{-1}$, 2 h at room temperature) after removal of the albumin-globulin fraction using 0.15 M-NaCl (10 ml $g^{-1}$, four times for 1 h each, at room temperature). Samples were centrifuged at 10 000 × *g*, 15 min at 8°C after each incubation step. The prolamin fraction (ethanol 70% soluble proteins) was obtained as the supernatant of the last centrifugation step.

**Preparation of Gliadin Standard Solution**

Gliadin (Sigma Chemical Co) was dissolved in 70% aqueous ethanol at approximately 2 mg $ml^{-1}$. The solution was first filtered through filter paper (Whatman no. 1) and then through a 0.22-$\mu$m Nucleopore membrane (Millipore, MA, USA). A clear and stable gliadin solution was obtained using this procedure. Kjeldahl's method was employed to determine the protein concentration in the standard solution.

**Production of Anti-prolamin MAbs**

BALB/c mice (8 weeks old) were intraperitoneally immunized with commercial gliadin (Sigma Chemical Co., MO, USA) in phosphate-buffered saline (PBS) with Freund's complete adjuvant (0.2 ml, 50 $\mu$g protein). They were later inoculated every 3 weeks during 4 months with gliadin prepared in Freund's incomplete adjuvant (0.1 ml, 20 $\mu$g $ml^{-1}$). Serum samples were analyzed after 7–10 days of the last booster. The mouse with the highest antibody titre was chosen for fusion. MAbs were derived by somatic cell hybridization of NSO myeloma cells with spleen cells from a hyperimmune mouse as described by Kohler and Milstein (1975). Supernatant screening was performed with an indirect ELISA using plates coated with gliadin standard solution (10 $\mu$g $ml^{-1}$). The isotypes of the MAbs obtained were determined by double diffusion of culture supernatant with commercial anti-isotype sera. MAbs 11C4, 12A1 and 13B4 were purified from the corresponding ascitic fluid by anion exchange fast performance liquid chromatography (FPLC) using a Mono Q HR5/5 column (LKB-Pharmacia, Upsala, Sweden). Purity was analyzed by SDS-PAGE and isoelectrofocusing.

**Preparation of Biotin-labelled MAb**

MAbs were biotin conjugated according to the method of Harlow and Lane (1988). Briefly, purified MAbs were dissolved and dialyzed against 0.1 M-sodium borate buffer, pH 8.8. A solution of each MAb (1 mg $ml^{-1}$) was incubated with *n*-hydroxysuccinimido-biotinamidocaproate ester (Sigma) (100 $\mu$g of ester/mg of MAb), at room temperature for 4 h. Reaction was stopped by adding 1 M-$NH_4Cl$ (20 $\mu$l/250 $\mu$g of ester). Unconjugated biotin was eliminated by chromatography using a P-10 column (Pharmacia). Finally, the biotinylated MAb was lyophilized and stored at $-20$°C.

**Preparation of Biotin-labelled Gliadin**

Commercial gliadin (Sigma) was dissolved in 0.001 M-NaOH and then filtered through a nitrocellulose membrane (0.45 $\mu$m). This solution was dialyzed against 0.1 M-sodium borate buffer, pH 8.8, and was biotin conjugated as described previously.

**Sequential Competitive ELISA**

A competitive assay using MAbs was performed according to a similar procedure to that described by Chirdo *et al.* (1995). Polystyrene strips (Maxisorp, Nunc, Denmark) were coated for 16 h at 4°C with 50 $\mu$l/well of gliadin in PBS solutions (10 $\mu$g $ml^{-1}$). Wells were washed with PBS containing 0.05% (v/v) Tween 20 (PBS-T) and non-specific binding sites were blocked by incubating with 200 $\mu$l/well of 3% (w/v) dry skimmed milk in PBS for 2 h at 37°C. After each incubation step, wells were washed with PBS-T solution. Gliadin standard solutions and biotin-labelled MAbs were dissolved in PBS-T containing 1% (w/v) of dry skimmed milk (diluent solution). Solutions containing equal volumes of antigen and antibody solutions were mixed and incubated, for 2 h at 37°C in plastic tubes (preincubation). Then, 50 $\mu$l/well of each sample were incubated for 30 min at 37°C in the gliadin-coated wells. After washing, plates were incubated with conjugated streptavidin-alkaline phosphatase (Sigma) (1 h at 37°C). After three cycles of washing with PBS-T and one with substrate buffer (10% diethanolamine, 0.01% $MgCl_2$, pH 9.8), colour reaction was developed with 50 $\mu$l/well of *p*-nitrophenylphosphate (PNPP)(1 mg $ml^{-1}$). After 30 min,

the colour reaction was stopped with 0.1 M-EDTA (50 μl/well). Optical density was determined at 405 nm ($OD_{405}$). Absorbance values were transformed by logit function (Tijssen, 1985).

### Capture ELISA

Polystyrene strips (Maxisorp, Nunc) were coated for 16 h at 4°C with 50 μl/well of MAbs in PBS solutions (20 μg $ml^{-1}$). Wells were washed and blocked as previously described; then gliadin standard solutions of different concentrations were incubated for 1 h at 37°C. After washing, wells were incubated with the appropriate dilution of biotin-labelled MAb (in each assay, the same MAb used in the capture) for 1 h at 37°C. Incubation with conjugated streptavidin–alkaline phosphatase and the colour were developed as already described. Results were expressed as optical density at 405 nm (percentage of maximal binding) versus log of gliadin concentration.

### Competitive ELISA Using Biotin-labelled Gliadin

Polystyrene strips (Maxisorp, Nunc) were coated for 16 h at 4°C with 50 μl/well of MAbs in PBS solution (20 μg $ml^{-1}$). Wells were washed and blocked as previously described. Calibration curves were prepared using gliadin standard solutions diluted in a solution containing biotin-labelled gliadin (1 μg $ml^{-1}$) and incubated for 1 h at 37°C. Incubation with conjugated streptavidin–alkaline phosphatase and the colour were developed as already described. Results were expressed as logit function versus log of gliadin concentration.

### Immunoblotting Analysis of Protein Extracts

Protein extracts were separated by SDS-PAGE (10–15% polyacrylamide gradient concentration) according to Laemmli (1970). Proteins were transferred to nitrocellulose sheets according to Towbin (1979). Membranes were then blocked by incubating with a solution of 1% (w/v) dry skimmed milk in TBS (0.05 M-Tris–HCl, 0.150 M-NaCl, pH 7.4) for 2 h at 37°C. After washing, membranes were incubated with the purified MAbs at the appropriate dilution in 0.5% (w/v) of dry skimmed milk in TBS for 1 h at 37°C. After washing, membranes were incubated with conjugated rabbit anti-mouse immunoglobulin peroxidase (Dako, Denmark) (1 h at 37°C). After three cycles of washing with TBS, the colour was developed with 4-chloro-1-naphthol and $H_2O_2$.

### Immunoblotting from Proteins Resolved by A-PAGE

A prolamin extract from wheat flour was separated by PAGE at pH 3.1 (A-PAGE) according to Lafiandra and Kasarda (1985). Proteins were transferred to nitrocellulose membranes using an acidic buffer (0.1 M-lactic acid) for 1 h at 250 mA. Western blot analysis was performed as described above.

## RESULTS

### Characterization of MAb Specificity

Thirty hybridomas secreting anti-prolamin MAbs were obtained (18 $IgG_1$, nine IgM, two $IgG_3$ and one IgA). In this study, three $IgG_1$ MAbs termed 13B4, 12A1 and 11C4 were selected according to their pattern of recognition as analyzed by immunoblotting.

Figure 1 shows the immunoblotting obtained with the ethanol 70% soluble protein fractions of wheat, barley, rye, triticale, oat, soy, rice and maize. It can be seen that MAbs 11C4 and 12A1 exhibit a similar pattern of reactivity. Both MAbs have a broad recognition pattern and react with components of most groups of prolamins from wheat, barley, rye and triticale. These MAbs recognize α-, β- and γ-gliadins, and two intense bands from the ω-gliadin region (50–55 kDa) (lane 1). In lane 2, (barley prolamins), two groups of bands are detected: one close to 50 kDa, corresponding to C-hordeins, and the other in the region

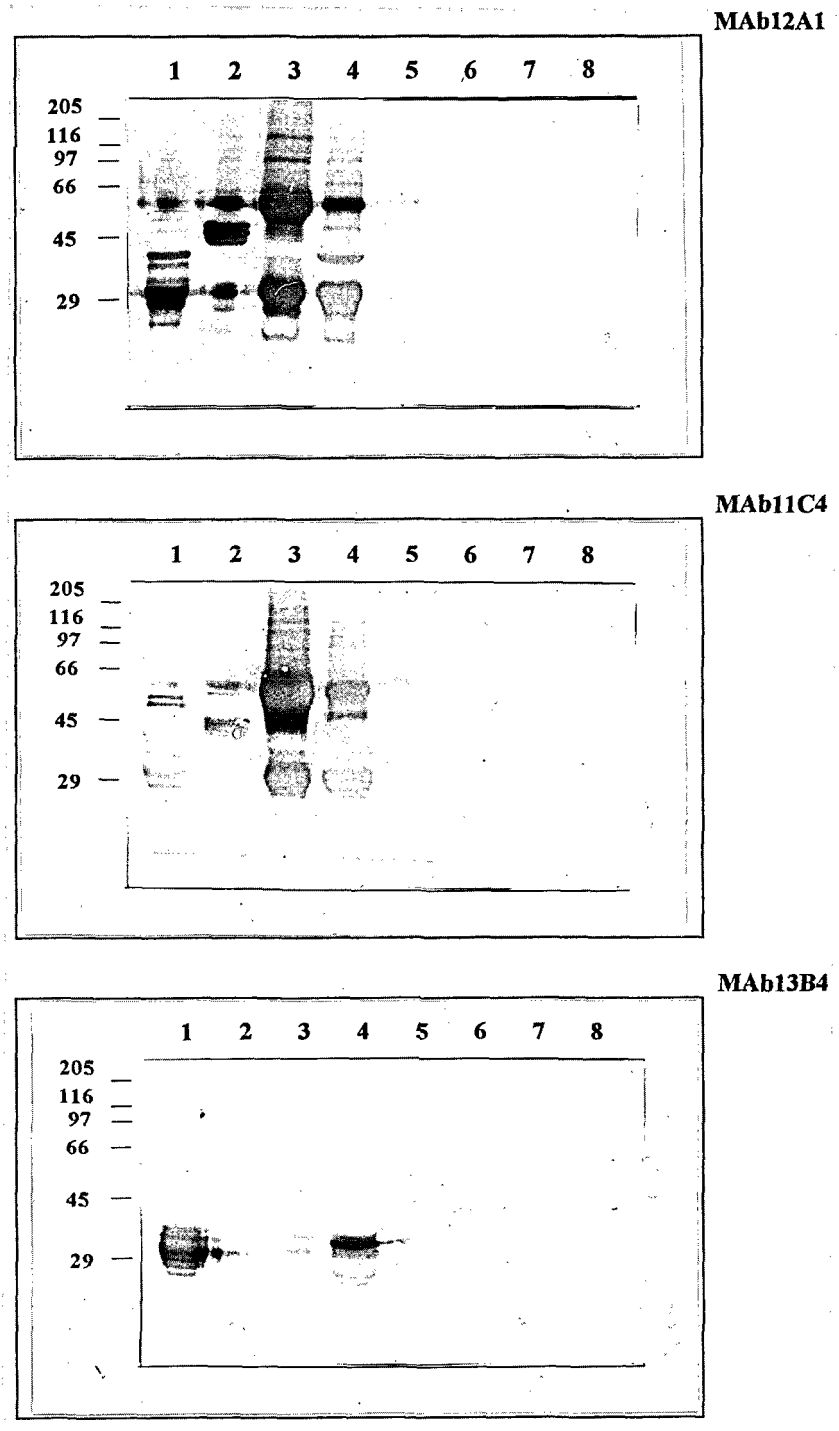


FIG. 1. Immunoblotting *analysis after* SDS-PAGE separation. Protein fractions (ethanol 70% soluble) from wheat (lane 1), barley (lane 2), rye (lane 3), triticale (lane 4), oat (lane 5), soy (lane 6), rice (lane 7) and maize (lane 8) were separated by SDS-PAGE (10–15% acrylamide gradient concentration) and revealed with MAb 12A1, 11C4 and 13B4.

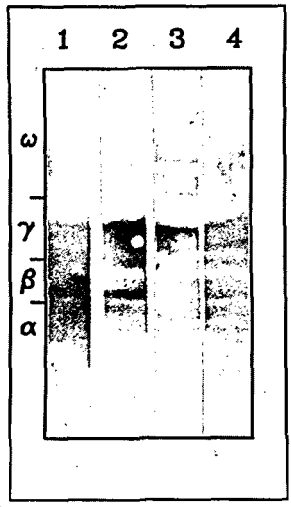


FIG. 2. Immunoblotting analysis after A-PAGE separation. Gliadin fractions separated by acid-PAGE and revealed with MAb13B4 (lane 1), MAb11C4 (lane 2), MAb12A1 (lane 3) and rabbit anti-prolamin serum (lane 4).

of 40 kDa, corresponding to B-hordeins. A reactivity in the region of 50–40 kDa and 30 kDa is observed in rye proteins (lane 3), corresponding to $\omega$-secalins and $\gamma$-secalins. Both MAbs show reactivity against triticale proteins (lane 4), mainly in the homologous components of wheat and rye. MAb11C4 also reacts slightly with some oat components (lane 5).

MAb13B4 exhibits high reactivity against wheat and triticale proteins (lanes 1 and 4, respectively). Intense bands can be observed in the regions of $\alpha$-, $\beta$- and $\gamma$-gliadins, on the contrary, a weak reactivity with barley and rye prolamins is observed (lanes 2 and 3, respectively).

The results show that these three MAbs react with proteins from those cereals toxic for coeliac patients, whilst reactivity against proteins that do not induce CD such as soy, rice and maize was undetectable.

MAb reactivity was also analyzed by immunoblotting after A-PAGE separation (Figure 2). Although the three MAbs recognize $\alpha$-, $\beta$- and $\gamma$-gliadins, significant differences can be observed. MAb13B4 (lane 1) reacts weakly with some $\omega$-gliadins. MAb11C4 (lane 2) recognize $\alpha$-, $\beta$- and $\gamma$-gliadins, but in a more restricted way. Mab12A1 (lane 3) reacts strongly with some $\gamma$- and $\omega$-gliadins. Lane 4 shows the reactivity of a rabbit anti-prolamin serum that recognizes components of all groups of gliadins (lane 4) (Chirdo *et al.*, 1995).

## Optimization of Three Different ELISAs for Gliadin Determination

*Sequential competitive ELISA.* To optimize a sequential competitive ELISA using immobilized gliadin, a titration assay was performed (Figure 3(a)). The working dilution biotinylated MAb was established as the one that produces 80–90% of maximal binding, corresponding to: 13B4 = 1/3200, 11C4 = 1/200 and 12A1 = 1/1600.

In the quantitative assay, calibration curves were calculated from $OD_{405}$ values transformed by the logit function (Figure 3(b)). Assay using the MAb13B4 yielded the best results. This assay presents a broad range of linear response and a high sensitivity, parameters of the assay are as follows: 50% inhibition (logit $p = 0$): 120 ng $ml^{-1}$ and a detection limit of 20 ng $ml^{-1}$ ($t$-test, confidence: 99%). MAbs 11C4 and 12A1 led to assays

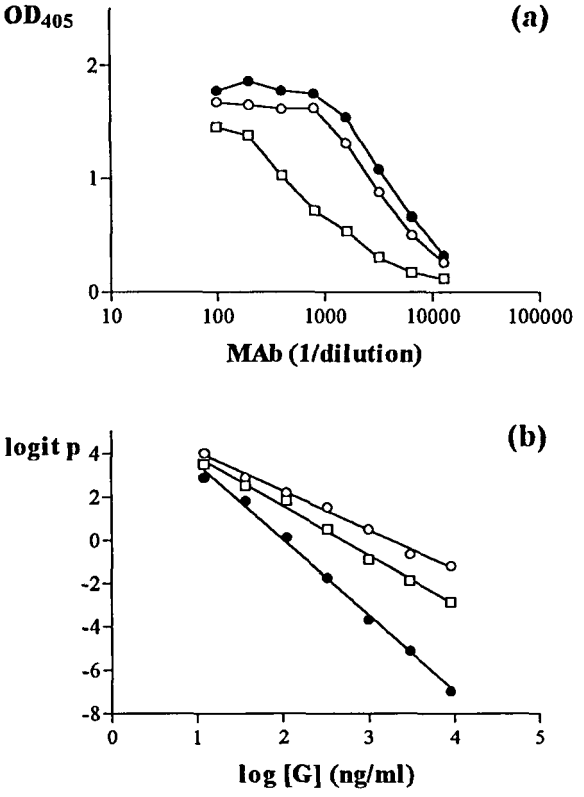


FIG. 3. Competitive ELISA. (a) Titration curves of biotinylated MAbs in plates coated with gliadin (10 $\mu$g ml$^{-1}$). (b) Calibration curves. [G]: gliadin concentration (range: 12 ng ml$^{-1}$–9 $\mu$g ml$^{-1}$). MAb: 13B4 (●), 12A1 (○), 11C4 (□). Symbols represent the mean value of triplicate experiments.

with lower sensitivity and detectability (50% inhibition: 500 ng ml$^{-1}$ and 1.4 $\mu$g ml$^{-1}$, respectively) and present a low slope in the line of the linear regression.

*Capture ELISA.* This capture design involves the use of the same MAb for both antigenic capture and as the secondary biotinylated antibody. At first, we analyzed the concentration of MAbs used for coating. Figure 4(a) shows that the adequate coating concentration of MAbs is 20 $\mu$g ml$^{-1}$. Under this condition and using gliadin at 10 $\mu$g ml$^{-1}$ (saturant concentration), it can be seen that MAb12A1 leads to higher $OD_{405}$ values (approximately 1.5) than those obtained with MAb11C4. No positive response was observed when MAb13B4 was used.

Figure 4(b) shows the calibration curve in capture ELISA using a 20 $\mu$g ml$^{-1}$ MAb solution for coating. The best result was obtained using MAb12A1. The sigmoid curve shows a working range of 0.3 to 50 ng ml$^{-1}$, and a detection limit of 1 ng gliadin/ml ($t$-test, confidence: 99%). The assay using the MAb11C4 presents a lower detectability as judged by its shifting to higher gliadin concentrations.

In Figure 4(b), we have compared the performance of ELISA results using the biotinylated MAbs and the commercial assay (Gluten assay Kit, Cortecs Diagnostic, UK). According to producers, the commercial assay has a detection level of 160 ng gliadin/ml. It can be seen that the curve obtained using the commercial kit appears at a higher gliadin concentration, showing a lower detectability than the capture ELISA using the MAb12A1.

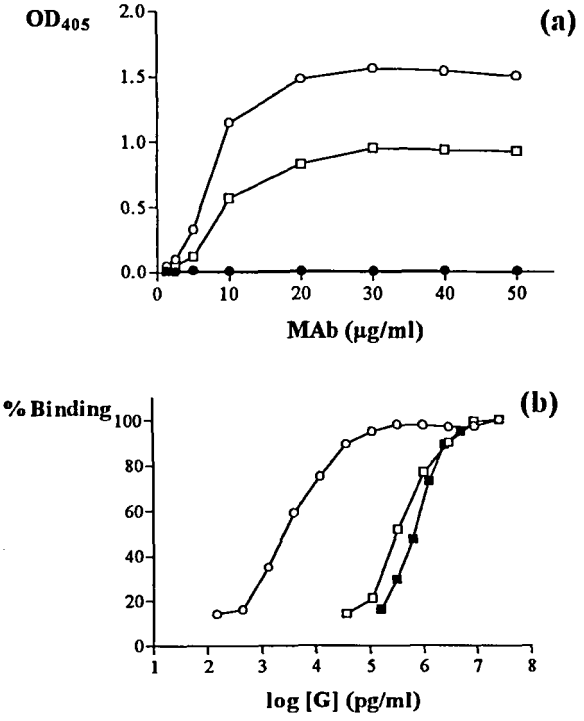


FIG. 4. Capture ELISA. (a) ELISA results using different MAb concentration for coating. (b) Calibration curves. [G]: gliadin concentration (range: 150 pg $ml^{-1}$–27 $\mu g\,ml^{-1}$). MAb: 13B4 (●), 12A1 (○), 11C4 (□), Commercial Kit (Gluten assay Kit, Cortecs Diagnostic, UK) (■). Symbols represent the mean value of triplicate experiments.

*Competitive ELISA using biotin-labelled gliadin.* To test the use of biotinylated gliadin as a marker for the antigen–antibody interaction, we performed a competitive ELISA. To this end, plates were separately coated with the three MAbs under study, then biotinylated gliadin and non-labelled gliadin were allowed to compete for the antibody binding sites in the solid phase. Finally, the reaction was developed with streptavidin-alkaline phosphatase.

Figure 5(a) shows that all MAbs efficiently captured the biotinylated gliadin. After determining the appropriate biotinylated gliadin concentration (1 $\mu g\ ml^{-1}$), a competitive ELISA was performed. In this assay, the concentration of both MAbs and biotinylated gliadin are critical in achieving a high detectability assay. The results shown in Figure 5(b) show that the assay using the MAb12A1 presents the best performance with 50% inhibition at 40 ng gliadin/ml and a detection limit of 5 ng gliadin/ml. MAb13B4 presents a slightly lower detectability (50% inhibition = 110 ng gliadin/ml). The assay using the MAb11C4 shows only poor detectability (50% inhibition = 900 ng gliadin/ml).

## DISCUSSION

Several methods based on polyclonal antibodies (PAbs) and MAb technology have been used in the immunochemical analysis of prolamins (Ellis *et al.*, 1989; Skerritt & Lew, 1990) and in the quantification of gliadin in foodstuffs manufactured for coeliac patients (Howdle & Losowsky, 1990). Some manufactured products (food and medicines) contain small amounts of prolamins, thus, high detectability methods are necessary to certify that the resulting products are gluten free.

Among the different methods published, Skerritt and Smith (1985) developed a method

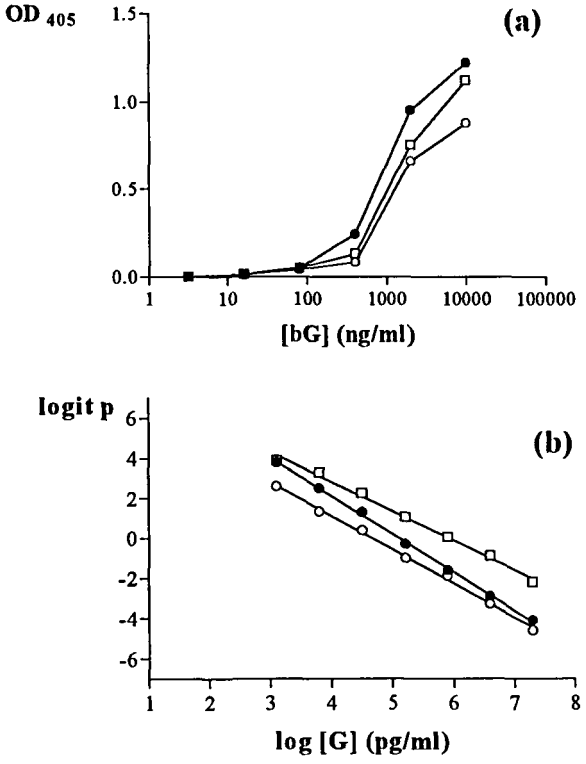


FIG. 5. Competitive ELISA using biotinylated-gliadin. (a) Binding analysis of biotinylated gliadin (bG) to MAbs in solid phase (20 $\mu g\ ml^{-1}$). (b) Calibration curves. [G]: gliadin concentration (range: 1.2 ng $ml^{-1}$–20 $\mu g\ ml^{-1}$), compited with biotinylated gliadin at 1 $\mu g\ ml^{-1}$. MAb: 13B4 (●), 12A1 (○), 11C4 (□). Symbols represent the mean value of triplicate experiments.

based on MAbs that allows the evaluation of thermally treated samples, but has only limited sensitivity. Troncone *et al.* (1986) developed a capture ELISA with PAbs that exhibits a high detectability, but it showed cross-reactivity with non-toxic prolamins from rice and maize. Friis (1988) using PAbs developed a competitive ELISA which in spite of being able to detect up to 10 ng gliadin/ml cannot be used on thermally processed food.

The $\omega$-gliadins, involving only 10–15% of total gliadins, are the most heat-resistant prolamins. An anti-$\omega$-gliadin MAb was used to develop a test (detection level of 160 ng gliadin/ml) able to detect gliadin in thermally treated food (Skerritt & Hill, 1990a). Nevertheless, in the determination of gliadin in very-low gluten content products or in food having partially lost protein reactivity, the use of high-affinity antibodies, able to detect components from most gliadin groups, as well as totally or partially denatured proteins, would be more adequate (Millis *et al.*, 1995).

Ellis *et al.* (1994) reported a capture ELISA of high sensitivity (15 ng gliadin/ml) that detects, with different affinity, prolamins of wheat, barley, rye and oat. We have recently published the optimization of a sequential competitive ELISA using anti-gliadin PAbs, specific for toxic prolamins, that presents very high detectability (1 ng gliadin/ml), and can be used to quantify gliadin even in processed food (Chirdo *et al.*, 1995, Rumbo *et al.*, 1996).

In the study presented here, we chose three MAbs (13B4, 11C4 and 12A1), according to their specificity as characterized by immunoblotting. MAbs 12A1 and 11C4 recognize most of the gliadins and glutenins, as well as several hordeins and secalins. Conversely,

MAb13B4 shows an intense reactivity against gliadins, but a very low one against prolamins from barley and rye (Figure 1). Immunoblotting analysis after A-PAGE separation showed that the three MAbs detect components of all groups of gliadins. MAb12A1 shows a marked reactivity against several $\omega$-gliadins, while MAbs 13B4 and 11C4 react slightly with these proteins (Figure 2).

Analysis of MAb reactivity by immunoblotting indicates that these three MAbs are specific for toxic cereal proteins, whereas they do not react with proteins harmless to CD.

Wheat prolamins present wide regions of repetitive sequences (Shewry & Tatham, 1990) which induce a strong humoral immune response and determine the cross-reactivity of these proteins (Festenstein *et al.*, 1987; Brett *et al.*, 1990). It can be concluded, from the immunoblotting analysis (Figures 1 and 2), that the three MAbs under study recognize repetitive sequences. Different specific anti-prolamin sera and MAbs reported by other authors, and most of the MAbs from our panel (not shown), indicate that, irrespective of the strategy employed in the immunization (crude gliadins, purified fractions or synthetic peptides) or in the screening procedure, most of the antibodies prepared have a broad recognition pattern and react with different repetitive sequences (Skerritt & Underwood, 1986; Festenstein *et al.*, 1987; Ellis *et al.*, 1989; Brett *et al.*, 1990; Skerritt & Hill, 1990b; Millis *et al.*, 1995).

Gliadin purification is cumbersome, making immunoblotting the method most commonly used to study the reactivity of anti-prolamin MAbs (Skerritt & Underwood, 1986; Skerritt, 1988). Nevertheless, it must be considered that experimental conditions influence MAbs' reactivity assignation, for instance, at high concentrations, low-affinity interactions can be detected, variations in MAb concentration can substantially modify the antibody recognition pattern (Donovan *et al.*, 1989). MAb reactivity can also be lost when proteins are separated under denaturing conditions (SDS-PAGE with reducing agents). This was observed with anti-gliadin MAbs, particularly with MAbs that detect S-poor prolamins (Brett *et al.*, 1990).

To develop immunoassays of high sensitivity, the avidin–biotin system has been used (Avrameas, 1992). The simple procedure to conjugate biotin to antigens and antibodies, the high biotin–avidin association constant and the resulting large amplification of the enzymatic signal makes it possible to have a highly sensitive method. We tested the use of this amplification system by biotin conjugation of the three MAbs under study and commercial gliadin, and three different ELISA methods were thus optimized.

Sequential competitive ELISAs were performed using the three biotinylated MAbs. Using the MAb13B4, a high detectability quantitative assay was obtained (Figure 3(b)). Its detection limit is 20 ng gliadin/ml, corresponding to 2 mg gluten/100 g of dry product (at 1:50 sample dilution). A competitive assay using an anti-$\omega$-gliadins MAb (detection limit = 25 mg gluten/100 g) was reported by Hill and Skerritt (1990). On the contrary, the other two MAbs (11C4 and 12A1) produced assays with a lower detectability.

Compared with a simple inhibition ELISA, the sequential competitive ELISA involves an extra incubation step which leads to an increase in the detection capability of the test. This effect was analyzed using anti-gliadin PAbs in our previous work (Chirdo *et al.*, 1995). This assay presents a detection limit of 1 ng gliadin/ml corresponding to 0.1 mg gliadin/100 g of dry product at 1/50 sample dilution.

When capture ELISA was performed, it was observed that the MAb12A1 produced the best results. This assay presented a calibration curve with a broad range of lineal response (0.3–50 ng $ml^{-1}$) and a detection limit of 1 ng gliadin/ml (0.05 mg gluten/100 g at 1:50 of dry product sample dilution). The capture ELISA adopted as official method by the AOAC (Skerritt & Hill, 1991) has a detection limit of 160 ng gliadin/ml (Figure 4(b)).

An interesting result was observed when MAb13B4 was used in capture ELISA (Figure 4(a)). Even though this MAb led to the best result in the competitive assay, it showed a poor performance in a capture ELISA. In some cases, the lack of biological activity of the MAbs could result from structural changes and denaturation due to hydrophobic interactions with the plastic surface (Jitsukawa *et al.*, 1989). Almost a 90% decrease in activity was observed

with some MAbs, whereas in other cases, the reactivity was completely lost (Butler *et al.*, 1993). Nevertheless, we could not find significant differences when biotinylated gliadin was captured by any of the three MAbs (Figure 5(a)). In spite of the fact that MAb13B4 is as able as the other two in capturing antigen, it proved to be useless for sandwich ELISA. This result suggests that conformational changes due to adsorption to the solid phase are not the reason why the MAb13B4 cannot be used in capture ELISA. According to the immunoblotting results, MAb13B4 recognizes epitopes localized in repetitive sequences present in several prolamins. Since this capture ELISA involves the use of the same antibody in the capture as well as secondary biotinylated-antibody, results may be explained by considering that MAb13B4 recognizes an epitope present at low density or epitopes not easily reached for antibody interaction.

Since it was possible to use biotinylated gliadin as a marker of binding of the anti-prolamins MAbs (Figure 5(a)), we developed a competitive ELISA using plates coated with a MAb for antigenic capture. The competition between biotinylated and non-biotinylated gliadin was then determined. Figure 5(b) shows a broad range of linear response achieved with the three MAbs. The best result was obtained using MAb12A1. The parameters of the assay indicate that this assay shows a 50% inhibition at 40 ng gliadin/ml and a detection limit of 5 ng gliadin/ml, corresponding to 0.5 mg gluten/100 g of dry product (at 1:50 sample dilution). A lower detectability was obtained with MAbs 13B4 and 11C4.

The assay described, employing biotinylated gliadin, can be useful for immunochemical studies of prolamins, since native labelled-gliadin can compete with extracts of prolamins, gliadin submitted to different conditions (e.g. thermal treatments) or with prolamin peptides.

## CONCLUSIONS

In this study, we have characterized the reactivity of three anti-prolamin MAbs (13B4, 12A1 and 11C4). Results show that these three MAbs are specific for protein toxic for coeliac patients while they do not react against proteins from vegetables non-related to CD, such as soy, rice and maize.

Furthermore, we have shown the use of the streptavidin–biotin amplification system employing both biotinylated MAbs and biotin-labelled gliadin. To our knowledge, this is the first report about the use of this amplification system in gliadin quantification.

Three different ELISAs using the MAbs 13B4, 12A1 and 11C4 were optimized. Even though each MAb recognizes repetitive sequences, when they were employed in different ELISA, designs led to quite different results.

The three optimized ELISAs present high detectability. Two of these assays employed biotinylated MAbs. In competitive ELISA, the best result was achieved by using MAb13B4 (detection limit = 20 ng $ml^{-1}$). In capture ELISA, the MAb12A1 yielded the best performance (detection limit = 1 ng $ml^{-1}$).

We also show the use of biotinylated gliadin in a competitive assay on plates coated with a MAb. Using the MAb12A1, up to 5 ng gliadin/ml could be detected.

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