Cytometry Part A 63A:108–113 (2005)

Quantification of Gliadin Levels to the Picogram Level by Flow Cytometry

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Received 16 March 2004; Revision Received 1 October 2004; Accepted 10 November 2004

Background: Celiac disease is a widely prevalent enteropathy caused by intolerance to gliadin, one of the gluten proteins. We developed two methods for the analysis of gliadin levels. Both methods use flow cytometry and rat antibodies against a 16-residue peptide of gliadin. The peptide is common to the α-, β-, γ-, and ω-gliadins.
Methods: In the one-site assay, the antigen (gliadin standard or food extract) was adsorbed on 3-μm latex particles. Sensitized particles were then incubated, in this order, with rat anti-gliadin peptide antibodies and anti-rat immunoglobulin G antibodies labeled with fluorescein isothiocyanate. In the two-site assay, the antigen was trapped on the latex particles by rat anti-gliadin antibodies and then measured by the same antibodies labeled with fluorescein.
Results: Detection limits were 1 ng/ml for the one-site assay and 10 pg/ml for the two-site assay. The two-site assay displayed gliadin at concentrations above the limit proposed by the Codex Alimentarius in 2 of 40 gluten-free products.
Conclusion: There is a growing concern that gliadin, even when present in gluten-free foods within the limit fixed by the Codex Alimentarius, over the long term may become toxic to patients with celiac disease. The techniques described in this study provide an opportunity to further decrease the acceptable limit of gliadin in gluten-free foods. © 2005 Wiley-Liss, Inc.

Key terms: gliadin level analysis; celiac disease; flow cytometry

People with celiac disease have an intolerance to a 33-residue peptide of wheat gliadin and to the related proteins of rye and barley. Oats, rice, and maize do not contain the peptide and are not toxic to the patient with celiac disease (1–3). Celiac disease has a prevalence of 1 in 100 (2) to 1 in 300 (4) and is strongly associated with DQ2 or DQ8 HLA class II haplotypes (5). Only a small fraction of the population carrying these risk haplotypes is affected (2,6). Therefore, additional factors must contribute to the development of the disease (2). The symptoms of the disease disappear when patients follow a strict gluten-free diet and reappear when gluten is reintroduced. As a result, patients must follow a lifelong gluten-free diet.

The method more commonly used to measure gliadin is enzyme-linked immunosorbent assay (7). Matrix-assisted laser desorption/ionization time-of-fly mass spectrometry has also been proposed (8). The Codex Alimentarius has established that gluten-free foods cannot contain more than 10 ng of gliadin per milligram of food product (9). This limit reflects the level of sensitivity of methods currently available for measuring gliadin. However, concern has been expressed that the threshold fixed by the codex in the long term may not be entirely safe for the patient with celiac disease (6,10,11). The two methods described in this report provide a possibility to further decrease the limit of gliadin allowed in gluten-free foods. Both methods use flow cytometry and rat antibodies against a 16-residue peptide of gliadin. In the one-site assay, latex particles are incubated with the unknown sample; the adsorbed gliadin is then measured with rat antibodies to the gliadin peptide and fluorescein-labeled goat anti-rat immunoglobulin, in that order. In the two-site assay, the same antiserum (rat anti-gliadin peptide) is used to trap the gliadin on latex particles and to label the trapped gliadin. Detection limits are 1 ng/ml for the one-site assay and 10 pg/ml for the two-site assay.

MATERIALS AND METHODS

Materials

Wheat, rye, barley, and oats cultivars included in this study were provided by the Istituto Nazionale di Cerealicoltura (Foggia, Italy); gluten-free foods were obtained from the market.

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Published online 12 January 2005 in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/cyto.a.20109

Gliadin Standards

Whole gliadin (1 mg; code G3375, Sigma-Aldrich Milan, Italy) was dissolved in 1 ml of 70% ethanol, centrifuged at $10^4 g$ (to remove undissolved material), analyzed for protein concentration by DC protein assay (Biorad, Milan, Italy), diluted in 60% ethanol (100 to 1 ng/ml), and used as standard. This gliadin (code G3375) was chosen because it compares well to the European gliadin standard (7).

Sample Extraction

Flour samples were extracted with ethanol directly; pasta, biscuit, and cake samples were first homogenized (2 min in a blade homogenizer). An aliquot of the sample (1 mg) was incubated (3 h at room temperature) with 1 ml of 60% ethanol, centrifuged at $10^4 g$, diluted in 60% ethanol (10^{-4} to 10^{-6}), and analyzed by flow cytometry. Artificial mix experiments were carried out by adding 10^2 ng to 1 pg of whole gliadin (Sigma-Aldrich) to 1 mg of gluten-free flour. Samples were then processed as described above.

Peptide Synthesis and Conjugation

The gliadin peptide sequence, NMQVDPSGQVQW-PQQQ, was assembled with solid-phase methodology on a Pioneer 9050 synthesis system (PE Biosystem Italia, Monza, Italy) on a 0.1-mmol scale using Fmoc chemistry (12). The purity of the solid obtained (measured by analytical reverse-phase high-performance liquid chromatography) was 85%. Determination of molecular weight (1,871.75 atomic mass units) with matrix-assisted laser desorption/ionization time-of-fly analysis and Mass-Lynx software were in agreement. This peptide was then conjugated to bovine serum albumin (BSA; Sigma-Aldrich) using glutaraldehyde (Sigma-Aldrich) (13).

Antibody Production and Absorption

Antibodies were produced in Fischer 344 rats (Harlan, Milan, Italy). Each animal received three intraperitoneal injections at 2-week intervals. Each injection consisted of 200 μg of conjugated peptide in 200 μl of phosphate buffered saline (pH 7.2; PBS) emulsified with an equal volume of complete Freund's adjuvant (first injection) or incomplete Freund's adjuvant (second and third injections). The serum was collected 2 weeks after the last injection. Antibodies to BSA and to the gliadin peptide were removed by affinity chromatography (see below).

Antibody Purification

About 100 μl of Protein G Dynabeads (Dynal Biotech, Oslo, Norway) were washed with PBS and incubated (1 h at room temperature on a rotating wheel) with 500 μl of BSA absorbed rat antibodies to the gliadin peptide (RαG16). The particles were kept at the bottom of the tube with the help of a magnet and washed with PBS, and bound antibodies were desorbed by incubation (2 min) with 30 μl of citrate buffer, pH 2 to 3. The pH was then neutralized with 4 μl of 1 M Tris buffer, pH 9, and the antibody preparation was adjusted to a concentration of 500 to 700 μg/ml.

Antibody Labeling

About 100 μl of the RαG16 antiserum were labeled directly (without previous purification) with fluorescein isothiocyanate (FITC) as described previously (14).

One-Site Assay

Latex particles (10^4, 2×10^4, 5×10^4, 10^5, and 2×10^5) with a diameter of 3 μm (code 17134, Polysciences, Warrington, PA, USA) were incubated overnight with 1 ml of gliadin standards dissolved in 60% ethanol or 1 ml of food samples extracted with 60% ethanol. Particles were washed with PBS, quenched with 2% milk blocking solution (Kirkegaard & Perry Laboratory, Gaithersburg, MD), washed again, and incubated in succession with RαG16 diluted 1:50 in PBS and anti-rat immunoglobulin G antibodies labeled with FITC ($G\alpha R^{FITC}$) diluted 1:600 in PBS. $G\alpha R^{FITC}$ was obtained from Sigma-Aldrich. Samples were analyzed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). In the analysis, a gate was set around the latex particles on the basis of their forward and side scattering characteristics. Standard markers were set by testing a negative control (where RαG16 antibodies were replaced with PBS). Data were collected with logarithmic amplification. Results are presented as the mean channel of fluorescence of the treated sample subtracted from the mean channel of the control. For each sample, data of 3,000 events were analyzed.

Two-Site Assay

About 4×10^5 latex beads (code 17134, Polysciences) were incubated overnight at 4°C on a rotating wheel with 100 μl of RαG16 purified antibody. About 2×10^4 antibody-sensitized beads were then incubated with the antigen (100 μl of gliadin standards or food extracts for 2 h at room temperature), washed with PBS, and incubated for 2 h with fluorescein-labeled RαG16 antibodies diluted 10^{-2} (50 μl/tube), washed again with PBS, and analyzed as described for the one-site assay.

Enzyme-Linked Immunosorbent Assay

The assay was carried out as described previously (15) by using RαG16 (diluted 1:50) and goat anti-rat^{HRP} (Jackson Immunoresearch Laboratories, West Grove, PA, USA) diluted 1:500.

Immunoblotting

After one-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (NuPAGE Novex 4–12% Bis-Tris gels; Invitrogen, Carlsbad, CA), proteins were transferred on a 0.2-μm polyvinyl diflouride membrane (Invitrogen), incubated with RαG16 antibodies diluted 2×10^{-2}, and visualized with goat anti-rat immunoglobulin labeled with horse radish peroxidase diluted 10^{-3}.

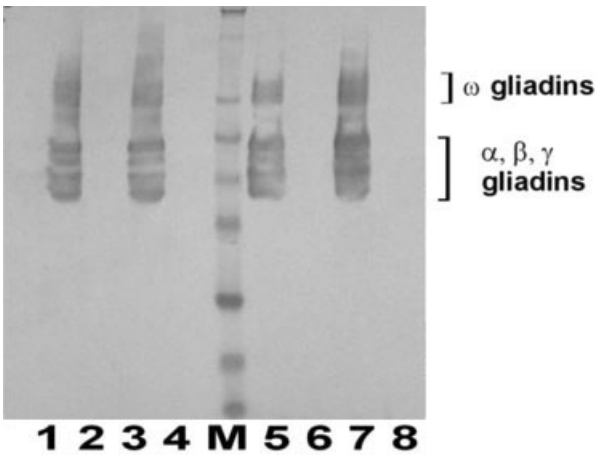

FIG. 1. Immunoblotting of α-, β-, γ-, and ω-gliadins. Lanes 1 and 3: gliadin standard (Sigma-Aldrich) at 1 and 10 mg/ml, respectively. Lanes 2, 4, 6, 8: four different gluten-free food samples. Lanes 5 and 7: flour samples at 10 and 20 mg/ml extracted with 60% ethanol. M, marker (SeeBlue Plus2 Prestained Standard, Invitrogen). The primary antibody was RαG16 and the secondary antibody was goat anti-mouse immunoglobulin G labeled with horse radish peroxidase.

Affinity Chromatography

The ligand (BSA, the peptide used as immunogen, or purified anti-gliadin antibodies) was coupled covalently to amino polystyrene particles (code 19118, Polysciences) as described by the manufacturer.

RESULTS AND DISCUSSION

Specificity of the Antiserum and Purity of the Gliadin Standard

The antibodies used in this study were obtained by immunizing rats with the 16-residue peptide NMQVDPSGQVQWPQQQ conjugated to BSA. Anti-BSA and anti-gliadin peptide antibodies were removed from the antiserum (RαG16) by affinity chromatography. Absorption with BSA was carried out to remove the reactivity for the carrier; absorption was done with the peptide to determine antibody specificity. After absorption with BSA and with the peptide, the antiserum completely lost its ability to react with wheat, barley, or rye extracts. These experiments demonstrated that, properly absorbed, the reagent was specific for the peptide used as an immunogen.

The reagent was then used to ascertain the purity of the standard used in this study. When the gliadin standard (100 ng/ml) was passed through a column containing purified RαG16 antibodies covalently bound to polystyrene beads, no protein was found in the effluent.

Immunoblotting determined that the antiserum recognizes the α-, β-, γ-, and ω-subunits (Fig. 1). The α-, β- and γ-gliadins are structurally related, whereas the ω-gliadin is structurally distinct (16). Detection of the four gliadin fractions represents an important property of the antiserum. Previous studies implicated only the α-gliadin in disease activity (17), but subsequent studies demonstrated that all gliadin fractions (including ω) are enterotoxic for patients with celiac disease (16,18–20). In addition, the ω-gliadin, being stable during heat (7), could represent a suitable target for gliadin detection in heat-processed foods.

Validation of the One-Site Assay

To determine the optimal number of latex particles per tube, different numbers of particles (10^4 to 2×10^5) were incubated with a constant volume of gliadin standards. Gliadin-sensitized beads were then incubated, in this order, with different dilutions of the primary (RαG16) and secondary ($G\alpha R^{FITC}$) antibodies. The 100-ng/ml (Fig. 2) and 1-ng/ml (data not shown) gliadin standards displayed a higher mean channel (higher fluorescence) when 2×10^4 particles/tube were used, and the primary and secondary antibodies were diluted 2×10^{-2} and 2×10^{-3}, respectively. The fluorescence intensity of the standards was further improved by fine adjustments of $G\alpha R^{FITC}$ dilution to 1.5×10^{-3}. Under these conditions, the detection limit of the assay (minimum concentration of gliadin that produced a signal clearly distinct from blank) was 1 ng/ml for wheat (Fig. 3), barley, and rye samples indifferently (data not shown). The intra-assay coefficient of variation was 1.8% to 3.2% (n = 30), and the interassay coefficient of variation was 2.2% to 5.4% (n = 10).

To establish which percentage of gliadin in the sample was detected by the method, known amounts of gliadin were added to 1 mg of gluten-free flour. The gliadin detected was at least 87% of the gliadin added to the gluten-free flour (Fig. 4). In these artificial mix experiments, 1 ng of gliadin added to 1 mg of gluten-free flour (i.e., 1 ppm) was easily detected (Fig. 5).

Two-Site Assay

A two-site (or sandwich) assay seemed the logical development of the one-site assay just described. It was assumed that latex beads sensitized with RαG16 antibodies would trap the gliadin more efficiently than would nonsensitized beads. In this case, the assay would detect gliadin at a concentration lower than 1 ng/ml (detection

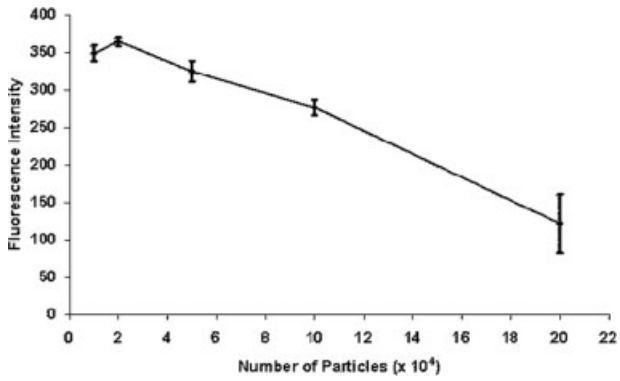

FIG. 2. One site-assay carried out with a variable number of latex beads per tube (10^4, 2×10^4, 5×10^4, 10^5, or 2×10^5). The amount of gliadin per tube was kept constant (100 ng/ml). The experiments described in the text were carried out with 2×10^4 particles/tube.

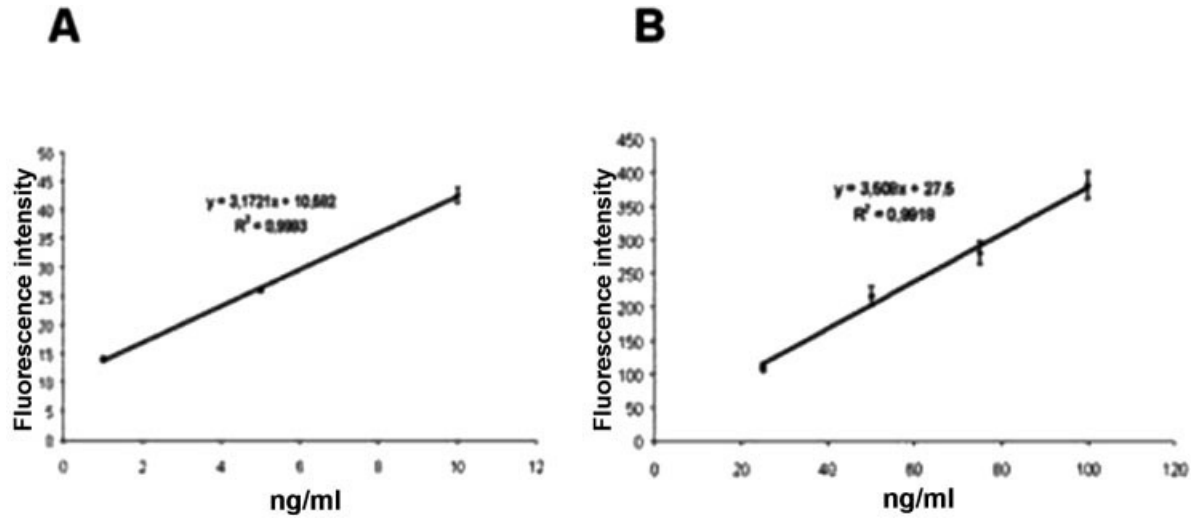

FIG. 3. Standard curve for gliadin quantification by the one-site assay. The low (1–10 ng/ml; **A**) and high (25–100 ng/ml; **B**) ranges are shown separately to visualize better the lowest (1 ng/ml) point of the curve. Each point is the average of 10 independent assays. Results are presented as fluorescence intensity of the activity tubes minus the fluorescence intensity of the control tubes (where RαG16 was omitted).

limit of the one-site assay). Moreover, gliadin, in particular the ω-fraction (21), contains repetitive sequences (16). If the antiserum detected a recurrent epitope on the gliadin molecule, then the same reagent (RαG16) could work as a binding antibody (immobilized on the latex beads) and a detector antibody (in a fluorescein-labeled form). The assay developed accordingly and displayed the capacity to detect gliadin at 10 pg/ml (Fig. 6). The intra-assay coefficient of variation was 2.3% to 7.4% (n = 15), and the interassay coefficient of variation was 3.4% to 9.2% (n = 5).

In artificial mix experiments, the assay detected 85% to 90% of the gliadin added to the flour (data not shown).

This proteomic two-site assay performs better than the genomic real-time polymerase chain reaction assay recently described, which detects 160 pg/ml of gliadin (22).

In this technique, the gliadin in the sample is detected by the monoclonal antibody R5 directly conjugated with an oligonucleotide probe. The probe, coupled with a reporter and a quenching dye, is degraded by the 5′-3′ exonuclease activity of DNA polymerase and the reporter dye freed from the quenching dye. Thus the fluorescence intensity is proportional to the amount of polymerase chain reaction product formed.

Food Analysis by the One-Site Assay

The next objective was to determine whether gliadin was present in flour and gluten-free food samples above the limit imposed by the Codex Alimentarius. This limit, 10 ng of gliadin per milligram of food product, was out-

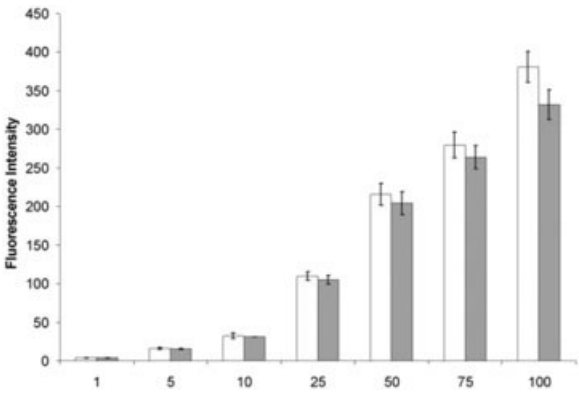

FIG. 4. Recovery of added gliadin in an artificial mix. Gliadin standards at 1, 5, 10, 25, 50, 75, and 100 ng/ml (white bars) and percentage of gliadin recovered from artificial mixes containing 1, 5, 10, 25, 50, 75, and 100 ppm (gray bars). Recovered gliadin was at least 87%. Error bars represent the standard deviation of 30 determinations.

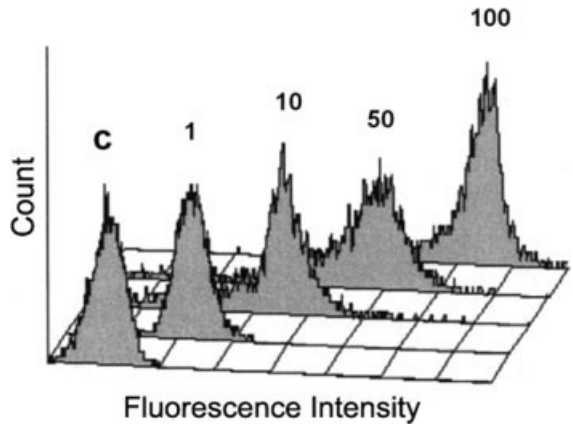

FIG. 5. Gliadin recovery in artificial mix of gliadin with gluten-free flour. One part per million of product (1 ppm) was easily detected by the one-site assay. C, control (no gliadin added); 1, 10, 50, 100, flow cytometric profiles of 1 mg of gluten-free flour mixed with 1, 10, 50, and 100 ng of gliadin and then extracted with 1 ml of 60% ethanol. Numerical range, 10^0 to 10^4. Type of scale, logarithmic.

side the range of the two-site assay. For this reason, the less sensitive one-site assay was used.

Gliadin was detected at a concentration higher than 10 ng/ml in 6 of 45 gluten-free food samples and was totally absent only in seven. The analysis was extended to 15 products in which gliadin was expected to be absent (processed cheese products, fruit juice, sausage, mortadella, and salami). Surprisingly, two of these products showed a gliadin concentration above 10 ng/ml and the remaining 13 showed a concentration of 1.5 to 8.3 ng/ml.

The assay was used to detect gliadin in flour and food samples after treatment of extracts at 120°C for 30 min. The thermal process decreased gliadin concentration by only 10% to 15%. Because heat treatment during food processing does not affect gliadin toxicity (23), the capacity of the assay to identify heat-treated gliadin is significant.

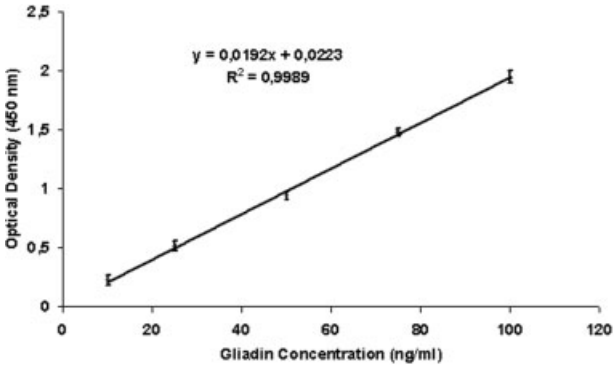

FIG. 7. Standard curve for gliadin quantification by the enzyme-linked immunosorbent assay. Gliadin was adsorbed on the wells and detected with RαG16 and goat anti-rat labeled with horse radish peroxidase, in that order. Detection limits are 10 ng/ml for the enzyme-linked immunosorbent assay and 1 ng/ml for the one-site assay.

Relative Importance of Variables Involved in Assays

RαG16 antibodies in a standard enzyme-linked immunosorbent assay format (Fig. 7) displayed a detection limit of 10 ng/ml (vs. the 1-ng/ml detection limit of the corresponding one-site assay). In our experience, the use of beads is best exploited in association with the flow cytometer. Therefore, these two variables were not analyzed individually. Depending on the analytical sensitivity required, the researcher may decide to use the one-site assay (with a detection limit of 1 ng/ml) or the two-site assay (with a detection limit of 10 pg/ml). Thus the main variable responsible for the improvements displayed by the methods described in this report over the current ones is the combined use of beads and flow cytometry.

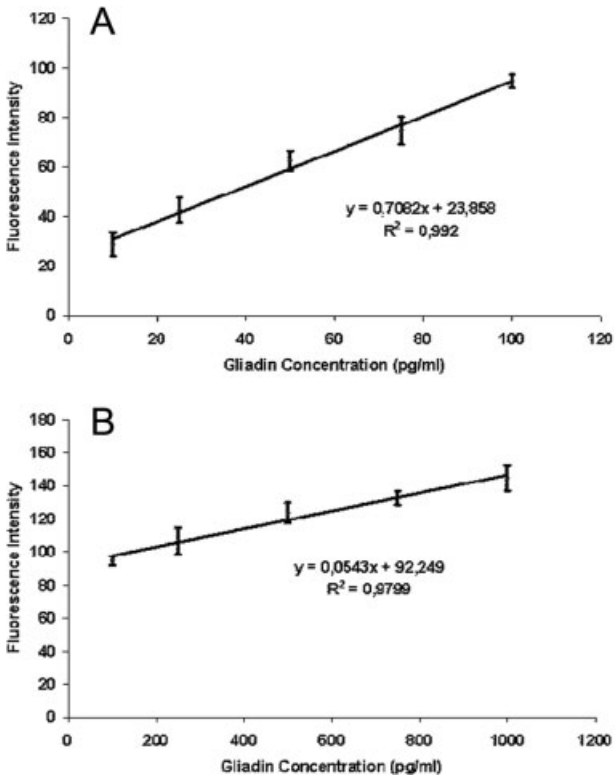

FIG. 6. Standard curve for gliadin quantification by the two-site assay. The low (10–100 pg/ml; **A**) and high (100–1,000 pg/ml; **B**) ranges are shown separately to visualize better the lowest (10 pg/ml) point of the curve. Each point is the average of 10 independent assays. Results are presented as fluorescence intensity of the activity tubes minus the fluorescence intensity of the control tubes (where gliadin was omitted).

ACKNOWLEDGMENTS

We thank two anonymous referees for their constructive criticism and Roberto Di Matteo for animal immunizations.

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