

Quantification of Gliadin by Flow Cytometry

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Gliadin is a heterogeneous group of alcohol-soluble wheat storage proteins, comprising $\approx 50\%$ of the gluten proteins (Campbell et al 1987; Fido et al 1997). Gliadin influences important baking properties of wheat, in particular loaf volume (Weegels et al 1994; Khatkar et al 2002) and water absorption (Dong et al 1992). Gliadin has been used also as a tool for wheat varietal identification (Bietz et al 1984). More importantly, gliadin, along with the prolamins of rye, barley and possibly of spelta wheat (Kasarda and D'Ovidio 1999), is responsible for the pathogenesis of the celiac disease. This food intolerance has a prevalence of 1/200 to 1/300 (Hin et al 1999) and is strongly associated with DQ2 and DQ8 HLA class II haplotypes (Sollid 2000). The symptoms of the disease disappear when patients follow a strict gluten-free diet and reappear when gluten is introduced again. Thus, treatment of celiac disease relies exclusively on a gluten-free diet.

At present, ELISA is used frequently to ascertain the absence of toxic prolamins in gluten-free foods (Sorell et al 1998). We investigated the possibility of using ELISA for flow cytometry. The applications of this technique are becoming increasingly numerous. However, flow cytometry can only analyze single cells. To mimic a cell population in this study, the gliadin in test samples was extracted with 70% ethanol, adsorbed on latex particles, coated with rat anti-gliadin antibodies, labeled with fluoresceinated goat anti-rat immunoglobulins, and finally measured by flow cytometry. The same approach has been used before to detect toxins (Evidente et al 1997), antibodies (Presani et al 1989; Iannelli et al 1998), and viruses (Iannelli et al 1996), which are all not visible to the laser in their natural state. The method described in this study can detect all cereal prolamins (gliadin, secalin, hordein, and avenin) potentially toxic to the celiac patient with a sensitivity of 5 ppm (5 ng of gliadin added to 1 mg of maize flour), introducing the condition to further reduce the amount of gluten allowed in gluten-free foods.

MATERIALS AND METHODS

Materials

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

Gliadin Standards

Whole gliadin (Sigma, Milan, Italy) was dissolved in 70% ethanol (1 mg/1 mL), centrifuged at $10^4 \times g$ (to remove undissolved material), analyzed for protein concentration by DC protein assay

(Biorad, Milan, Italy), diluted in 70% ethanol (100 ng/mL – 5 ng/mL) and used as standard.

Sample Extraction

Flour samples were extracted with ethanol directly; pasta, biscuit and cake samples were homogenized first (2 min in a blade homogenizer). An aliquot of the sample (1 mg) was incubated (3 hr at room temperature) with 1 mL of 70% ethanol, centrifuged at $10^4 \times g$, diluted in 70% ethanol (10^{-2} – 10^{-5}), and analyzed by flow cytometry. Artificial mix experiments were conducted by adding 100 ng – 5 ng of whole gliadin to 1 mg of maize flour. Samples were then processed as described above.

Antibodies

Antibodies were produced in four Fischer 344 rats (Harlan, Italy). Each animal received two intraperitoneal injections at two-week intervals. Each injection consisted of 300 μ g of whole gliadin emulsified with 300 μ L of complete Freund's adjuvant. Blood samples were collected two weeks after the last injection and the antisera were pooled. The specificity of antibodies to gliadin (referred to as R α G) was established by absorption (1 mL of the antiserum diluted 5×10^{-3} was incubated at room temperature for 3 hr with 150 ng of gliadin and then centrifuged at $10^4 \times g$ for 5 min).

Flow Cytometric Assay

Latex particles (2.5×10^4 , 5×10^4 and 10^5) with a 3 μ m diameter (Polysciences, Milan, Italy) were incubated overnight with 1 mL of gliadin standards dissolved in 70% ethanol or with 1 mg of food sample extracted with 1 mL of 70% ethanol. Thus, incubation was conducted in 70% ethanol. Particles were washed with phosphate buffered saline (PBS pH 7.2), quenched with 2% milk-blocking solution (Kirkegaard & Perry), washed again, and

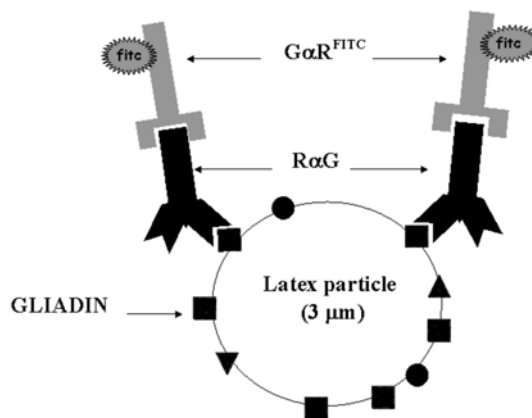


Fig. 1. Following extraction of gliadin with 70% ethanol, samples were incubated with latex particles. Gliadin (■), a major gluten protein, adsorbed passively on the latex surface, along with other unrelated proteins (●, ▲). Gliadin was then detected specifically through its interaction with rat anti-gliadin (R α G) and goat anti-rat immunoglobulin antibody labeled with fluorescein-isothiocyanate (G α R^{FITC}).

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incubated in succession with RαG (diluted 5×10^{-3} in PBS) and goat anti-rat immunoglobulin antibodies labeled with fluorescein isothiocyanate (referred to as GαR^{FITC}). The optimal dilution of GαR^{FITC} (2.5×10^{-3} in PBS) was established in the course of preliminary titration experiments. GαR^{FITC} was obtained from Sigma. Gliadin adsorbed on latex beads reacted with RαG antibodies and these, in turn, with GαR^{FITC} (Fig. 1). Samples were then analyzed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). In the analysis, a gate was set around the latex particles on the basis of forward (FSC) and side (SSC) scattering characteristics. FSC measured the size and SSC measured the granularity (internal fine structure) of the particles. Each assay included negative controls for PBS in place of RαG antibodies; preimmune rat serum in place of RαG; and 0 ng/mL gliadin point. Results are presented as the mean channel of fluorescence of the treated sample subtracted by the mean channel of the more fluorescent among the negative controls (rat preimmune serum in place of RαG). Logarithmic units were transformed into linear channels as described previously (Iannelli et al 1996). For each sample, the data of 3,000 events were analyzed.

RESULTS AND DISCUSSION

Preliminary experiments established the optimal number of latex particles per tube. The 100 ng/mL (high) and 5 ng/mL (low) gliadin standards both displayed a higher mean channel (higher fluorescence) when 2.5×10^4 particles per tube were used. All the experiments used this number of particles per tube.

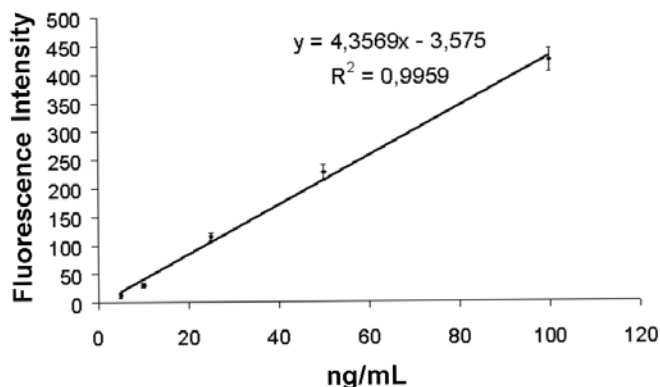


Fig. 2. Standard curve for gliadin determination by flow cytometry. Each point of the curve is the average of eight independent assays. Results are presented as fluorescence intensity of the treated samples minus the fluorescence intensity of control tubes containing rat preimmune serum instead of RαG.

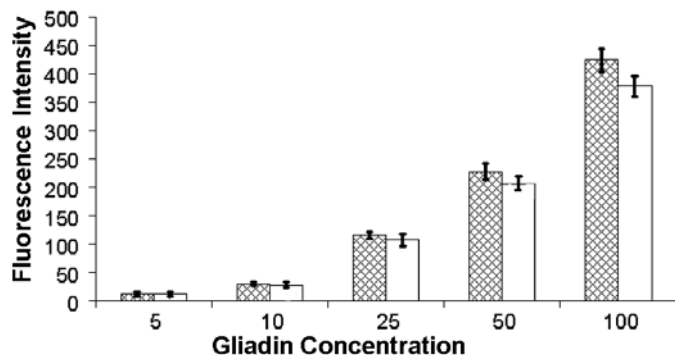


Fig. 3. Gliadin standards at 5, 10, 25, 50, 100 ng/mL (filled columns) and amount of gliadin recovered from artificial mixes at 5, 10, 25, 50, 100 ppm (clear columns). Comparison of the two sets of data shows that at least 88% of gliadin is recovered. Error bar of each column represents the standard deviation of 24 determinations.

Dilution 5×10^{-3} of the antiserum allowed detection of wheat, barley, rye, and oats prolamins. At this dilution, the antiserum, on absorption with gliadin, had negligible capacity to react with barley, wheat, oats, and rye. The antiserum was thus specific for gliadin. Under these conditions, the sensitivity of the assay was 5 ng/mL (Fig. 2). The mean intraassay coefficient of variation range was 1.9–2.3 ($n = 24$), the mean interassay coefficient ($n = 8$) range was 3.3–5.2. The range (linear portion of curve) was 5–100 ng/mL. Gliadin was still detected after treatment of the extract at 120°C for 30 min (the thermal process reduced prolamins concentration by $\approx 15\%$). Analysis of the artificial mix of gliadin with gluten-free flour easily detected 5 ppm of gliadin. In the course of these artificial mix experiments, at least 88% of added gliadin was recovered (Fig. 3).

To analyze flour and gluten-free food samples, flour from at least three cultivars of each species of cereals were analyzed (Table I). Gliadin concentration ($\mu\text{g}/\text{mg}$ of sample) was 15.4–19.2 in *Triticum durum*, 28–51.8 in *T. aestivum*, 3.8–7.2 in barley, 4.4–16.1 in rye, and 0.3–1.2 in oats. Among gluten-free products (30 flour samples and 30 uncooked pasta samples), one tested positive. Rice and maize (two cultivars each) tested negative.

The Codex Alimentarius Commission (Denery-Papini et al 1999) recommends setting the limit of gluten concentration in gluten-free foods at 20 ppm. However, no data are available as to the long-term tolerance of small doses of gliadin by the celiac patient (Stern et al 2001). In this context, the possibility given by the assay to further reduce the above threshold looks interesting. The monoclonal R5 (Osman et al 2001) recognizes specifically the sequence of wheat, barley, and rye prolamins inducing the celiac disease. Together, the present method and the reagent R5 can improve significantly the quality control of gluten-free foods, not only in terms of sensitivity, but also of specificity. Recently, the prolamins working group has made available a reference gliadin preparation (Stern 2003). The reproducibility of the present technique (interassay CV 3.3–5.2), along with the above reagent, are a good basis for a much needed standardization of the serologic test for the celiac disease (Stern et al 2000).

TABLE I
Gliadin Content of 23 Cultivars of Five Cereal Species^a

Species	Gliadin Content ($\mu\text{g}/\text{mg} \pm \text{SD}$)
<i>Triticum aestivum</i>	
Victo	28 \pm 2.0
Golia	34 \pm 3.1
Manital	50.7 \pm 2.2
Forge	51.8 \pm 2.3
Freccia	51.1 \pm 2.6
Francia	42.1 \pm 2.6
Rye	
Rapid	15.7 \pm 1.2
Canovus	10.5 \pm 1.4
Avant	16.1 \pm 1.2
Danko	4.4 \pm 0.7
Oats	
Croata	0.3 \pm 0.2
Argentin	0.4 \pm 0.2
Marisa	0.8 \pm 0.8
Prevision	1.2 \pm 1.0
Rogar8	0.4 \pm 0.5
<i>Triticum durum</i>	
Simeto	15.4 \pm 1.5
Ofanto	15.9 \pm 1.0
Ciccio	19.2 \pm 2.4
Barley	
Puffin	7.2 \pm 1.7
Arone	6.3 \pm 2.1
Aliseo	5.8 \pm 1.2
Anellio	4.9 \pm 1.1
Diomede	3.8 \pm 1.1

^a Each value is the average of two independent measurements.

The assay can find application also in the field of cereal chemistry. In combination with antibodies specific for single gliadin fractions, the procedure may help to define, without ambiguity, the influence of gliadin on the technological properties of wheat flour. Of course, the method has a limitation in the high cost of the cytometer (far higher than that of any ELISA plate reader), a condition that will prevent many laboratories from adopting the method.

In conclusion, the assay described here can measure gliadin with a sensitivity of 5 ppm. Additional useful features of the technique are gliadin detection in heat-processed food products (120°C for 30 min); recovery of up to 88% of gliadin present in the sample; use of unchanged marker settings throughout the experiments, leading to optimized and uniform test conditions for all samples.

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