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Development of a Dot-blot System to Detect Gluten in Food

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We have designed a quick, easy, and reliable enzyme immunoassay system based on dot-blot methodology, capable of determining the presence of gluten or its derivatives in food. We also describe a new solution to extract the prolamin from flours. The sensitivity of the system is less than 5 ppm using a maximum volume of 2 μl.

Keywords: *Dot-blot, gluten, coeliac disease*

INTRODUCTION

Coeliac disease, an enteropathy caused by hypersensitivity to gluten, is characterized by a shortening of intestinal vellosities and hyperplasia of the crypts, with subsequent faulty intestinal uptake (Katz & Grand, 1979). Currently, the only treatment for this disease consists of following a gluten-free diet (Van Berge-hehegouwen & Mulder, 1993). However, the dietetic prescription involves certain practical problems, such as the limitation of the consumption of numerous foods made from cereals and derivatives of wheat, oats, barley, and rye.

The development of tests capable of making specific real measurements of the gluten content in foods provides a great service to patients with coeliac disease. Different enzyme immunoassays have been developed, including different types of ELISA (Mckillop *et al*., 1985; Skerrit *et al*., 1985; Troncone *et al*., 1986; Freedman *et al*., 1987; Friis, 1988; Skerrit & Hill, 1990; Chirdo *et al*., 1995; Sorel *et al*., 1998).

The main aim of the present work was to design a quick, easy, and reliable analytical system using dot-blot methodology, capable of determining the presence of gluten or one of

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its derivatives in food. In addition, an extraction solution was also developed for solubilizing proteins responsible for the toxicity to coeliacs. The solvent doesn't interfere with the immunological methods used in this paper. The results have been compared with those of other procedures described in the literature (Sandiford *et al*., 1997).

MATERIALS AND METHODS

Samples

The samples used contained the flour of wheat, oats, rye and barley as well as different brands of manufactured products labelled gluten-free (almond sweets, D; bread sticks, E; various types of cakes, F and N; chocolate rolls, G; biscuits, H; donuts, I; breaded goods, J, M, and O; and various types of pastries, K and L; various types of noodles, P and Q; letters for soups, R; spirals for soups, S; Italian pasta, T; corn flour, U and V; and purified gliadin, Sigma, W).

Extraction of Prolamin

Flour (100 g) was defatted by stirring for 1 hr at room temperature with 500 ml chloroform and filtered, and the extraction was repeated. The defatted flour was then air-dried. Albumins and globulins were extracted by stirring the flour for 1 hr with 1000 ml of 0.5 M-NaCl at room temperature, centrifuged for 15 min at 15,000 *g*, whereupon the extraction was repeated. The pooled supernatants were retained and freeze-dried. The pelleted fraction obtained from the extraction of the albumin and globulin proteins contained the gliadin and glutenin proteins. This fraction was resuspended in water and stirred for 1 hr at room temperature to remove any residual salt and then centrifuged for 10 min at 15,000 *g* (Sandiford *et al*., 1997). The pellet fraction was stirred for 1 hr with 1000 ml of 70% (v/v) aqueous ethanol at 4°C and centrifuged. This was repeated and the supernatants containing the gliadins were pooled and dialysed against 1% (v/v) acetic acid for 60 hr prior to being freeze-dried.

Production of Antibodies

A rabbit was injected subcutaneously on different sites with 1 ml prolamin solution (1 mg prolamin/0.5 ml water), suspended in an equal volume of Freund's complete adjuvant for the first immunization. The rabbit was boosted with 1 ml prolamin solution suspended in Freund's incomplete adjuvant every two weeks. A positive reaction of the sera was obtained within 4–6 weeks. Injections with prolamin in Freund's incomplete adjuvant were continued monthly; and blood samples were taken by ear vein puncture every two weeks. After 5 months the animals were bled and the sera were kept frozen until used. The antibodies were labeled with peroxidase (Avrameas, 1969).

Electrophoresis and Immunoblotting

The samples were boiled (5 min) in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer under reducing conditions and electrophoresed on SDS −12.5% PAGE gels (Laemnli, 1970). The individual water/salt-insoluble proteins were separated by SDS-PAGE (PhastGel System equipment, Pharmacia LKB, Piscataway, NJ, USA) using a 12.5% acrylamide gel (PhastGel). Standard proteins as molecular-weight markers for SDS-PAGE were Phosphorylase B (97,400), bovine-serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), trypsin inhibitor (21,500), and α lactalbumin (14,200). Together with the prolamin extracted using the Sandiford *et al*. (1997) method, as described above, samples were also extracted with a solution composed of 40% ethanol (v/v), 0.01% acetonitrite, and 10.3 ppm antifoam A, all dissolved in carbonate buffer (0.1 M) at pH 9.6. To 0.25 g of the flours, 1 ml of the above solution was added and

stirred manually for 5 min and, after being decanted, the supernatant was collected. As a gliadin control, Gliadin was used (Sigma). After electrophoresis the separated prolamin was transferred from unstained gels to nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, USA) by means of PhastTransfer (Pharmacia LKB, Piscataway, NJ, USA) following standardized procedures (Towbin *et al*., 1979). The effectiveness of the transference was verified by staining the membranes with a red Ponceaux solution (0.2%, w/v, in 3%, w/v, trichloroacetic acid). The nitrocellulose strips were incubated with a polyclonal anti-gliadin antibody labeled with peroxidase or with a rabbit anti-gliadin antibody marked enzymatically for 30 min at 37°C, followed by three washings with TPBS (PBS, 0.1 M, pH 7.2 + Tween 20 0.3%). As a substrate, 3.3 tetrahydroclorhydric diaminobenzidine, 0.5 mg/ml 0.1 M Tris-HCl, pH 7.4, and hydrogen peroxide diluted 1:5000, was incubated for 20 min at room temperature in darkness. Afterwards, the reaction was stopped by washing generously with distilled water.

Dot-ELISA

The nitrocellulose membrane was balanced in PBS and left to dry for 15 min at room temperature. Next, it was sensitized with the prolamin extracted from the food products, using the solution developed, and left to dry at room temperature. Afterwards blocked for 30 min with TPBS (PBS, 0.1 M, pH 7.2; Tween 20 at 0.3% [v/v]), to which 0.5% [w/v] gelatine was added. This was followed by washing with TPBS and thorough incubation with the rabbit anti-gliadin antibody marked enzymatically at a dilution of 1/800 for 30 min, and by three washings with TPBS and incubation with the substrate for 10 min at room temperature in a solution of 0.5 mg/ml 4-Cloro-1 naphthol in 12% (v/v) buffer Tris-saline, pH 7.4, containing 0.1% H_2O_2 (30%, v/v). The reaction was stopped with distilled water (Rogan *et al*., 1991). The dot-ELISA analysis of the quantity of gliadin contained in the manufactured products was made by the program Quantiscan v1.25, these being prepared as sample patterns of 20, 10 and 5 ppm of gliadin.

Statistical Treatment

The results were processed by the GraphPad Instat program to calculate the straight line of the regression and statistically analysed.

RESULTS

Dot-ELISA

Figure 1 shows the dot-ELISA performed with the 19 food products labelled "gluten-free". Table 1 lists the gliadin content (ppm) in each sample, most being less than 10 ppm and the sensitivity being less than 5 ppm.

Electrophoresis

Figure 2 shows the SDS-PAGE profiles of the soluble prolamin in alcohol/water under reducing conditions, showing the patterns of gliadin, secalin, hordein, and avenin. All the lanes show a heterogeneous mixture of proteins with molecular weights of 90–14 KDa, while, with the Sandiford method, the number of bands for the different prolamin proved less.

Immunoblotting

Figure 3 presents the results for the immunoblot of the prolamin from wheat, oats, barley, rye, and commercial gliadin when exposed to an anti-gliadin polyclonal antibody labeled with HRP.

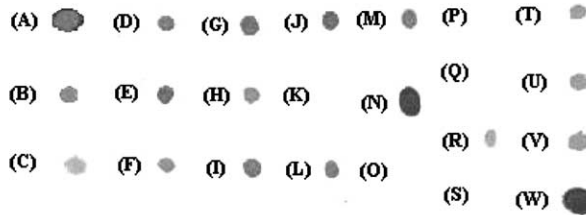

FIG. 1. Results of dot-ELISA in the food products assayed. (A) 20 ppm, (B) 10 ppm, (C) 5 ppm, (D), (E), (F), (G), (H), (I), (J), (K), (L), (M), (N), (O), (P), (Q), (R), (S), (T), (U) and (V), samples of food origin

Lane E and F show different bands between molecular weights 30 and 43 KDa, these also being present in barley and rye but being hardly visible in oats. In addition, a high-molecular-weight band appeared in all the prolamin.

Figure 4 shows the pattern of bands of the gliadins for an antibody obtained from rabbit and marked with HRP (lane B) and a commercial anti-gliadin antibody.

DISCUSSION

The great problem of working with gluten proteins is the difficulty with solubilization (Singh & Macritchie, 1989). The aggregation of proteins, assumed to be the result of disulphide cross-linking, and its apolar and ionic character increases its molecular weight and reduces solubility (Wrigley & Bietz, 1988). Depending on the solubility of the proteins that form part of the wheat flour, these can be classified as: albumins, water-soluble; globulins, soluble in saline solutions; glutenins, partially soluble in diluted acids or alkaline

TABLE 1. Gliadin content in each sample (ppm), (A) 20 ppm, (B) 10 ppm, (C) 5 ppm, (D–W) samples assayed

Sample	Net volume	Background	Colorimetric density	Concentration (ppm)
A	27591.00	1083.00	26508	20
B	23193.00	1083.00	22110	10
C	14883.00	1083.00	13800	5
D	15955.00	768.00	15187	5
E	23633.00	918.00	22715	14
F	18723.00	768.00	17955	9
G	18884.00	765.00	18119	14
H	18740.00	768.00	17972	9
I	23417.00	864.00	22553	14
J	27997.00	1026	26971	18
K	–	–	–	–
L	20661.00	867.00	19794	11
M	10463.00	672.00	9791	1
N	33707.00	972.00	32735	25
O	–	–	–	–
P	–	–	–	–
Q	–	–	–	–
R	14699.00	720.00	13979	4
S	–	–	–	–
T	13353.00	816.00	12537	3
U	15488.00	918.00	14570	5
V	18050.00	1026.00	17024	7
W	36488.00	1026.00	35462	28

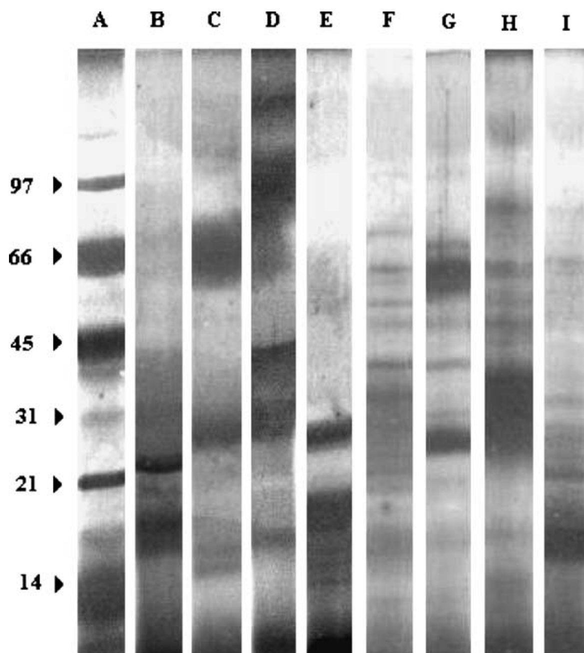

FIG. 2. (A) Standard of molecular weights, (B) Avenin extracted by method B, (C) Secalin extracted by method B, (D) Hordein extracted by method B, (E) Gliadin extracted by method B, (F) Avenin extracted by method A, (G) Secalin extracted by method A, (H) Hordein extracted by method A, (I) Gliadin extracted by method A (method A: Sandiford *et al*. (1997); method B: extracting solution).

solutions; and gliadins, which constitute the alcohol-soluble fraction of wheat gluten. Wheat is philogenetically close to barley, rye and oats (Shewry *et al*., 1992), and prolamin are mainly responsible for the damage caused to the intestinal mucosa (Howdle *et al*., 1984;

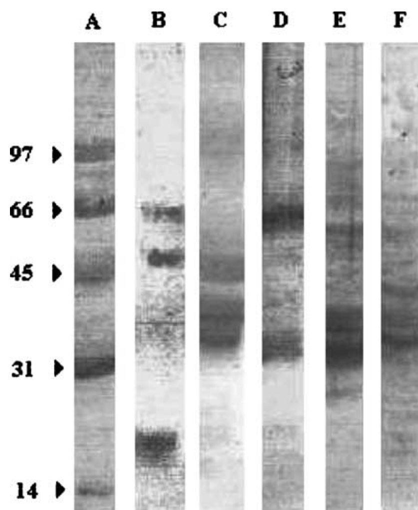

FIG. 3. (A) Standard of molecular weights, (B) Avenins, (C) Hordeins, (D) Secalins, (E) Gliadina, (F) Commercial gliadin.

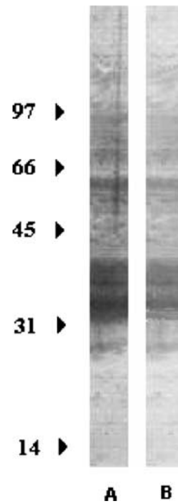

FIG. 4. (A) Rabbit anti-gliadin antibodies marked with peroxidase, (B) Rabbit anti-gliadin antibodies marked with commercial peroxidase (Sigma).

Davidson & Bridges, 1987; Marsh, 1992), although there are discrepancies concerning the toxicity of the oat sample (Anand *et al*., 1978).

A combination of different techniques to obtain the gliadin fraction is used by some authors (Huebner & Bietz, 1983), who modified the Osborne fraction to later isolate the gliadin fraction by chromatographic exclusion by molecular size.

The results show that of the 19 foods labeled gluten-free, only one had a quantity of gliadin exceeding 20 ppm (sample N). The sensitivity of Dot-ELISA, less than 5 ppm using the extracting solution to obtain prolamin and using a maximum volume of 2 μl, enabled the detection, in the food products, of gliadin levels lower than the range permitted by the World Health Organization (20 ppm) in foods labeled gluten free (Codex Standards for 'Gluten-free Foods', 1986). Similar assays have been developed by different authors (Skerrit & Smith, 1985), who designed an ELISA assay using monoclonal antibodies as the revealing antibody; however, this technique was not capable of detecting trace quantities in gluten-free foods, the sensitivity of the assay being 20 ppm. Afterwards, a similar ELISA was developed, which presents less sensitivity at 10 ppm (Freedman *et al*., 1987). The sensitivity of both diagnostic methods proved lower than under our working conditions.

The use of the Immunoblot corroborated that the antiserum obtained as well as proteins extracted or acquired commercially can be used with the dot-ELISA technique developed. The greater intensity of the reaction found for the gliadin extracted from industrial flour (lane E) vs. the previously purified commercial gliadin (lane F) indicated that the use of the solution was less denaturing for the protein.

The different prolamin (avenins, hordeins, secalins, and gliadins) extracted by a previously developed method (Sandiford *et al*., 1997), and the extracting solution presented remarkable differences with respect to the number of bands rendered by the two extraction methods, showing, under our working conditions, bands of different molecular weight in all the prolamin assayed, which were completely absent in the extraction made with the Sandiford method.

In the case of gliadins, bands of molecular weights of 30–91 KDa appeared, with up to 7 bands with different molecular weights appearing, these being absent when the method was different. In addition, low-molecular-weight bands (14–30 KDa), were visible, representing proteins described with an inhibitory function against α-amylase/protease, which appear in

the soluble fraction of these flours (Bushuk & Zillman, 1978). When the extraction was made in the oat flours, bands of very low molecular weight (14–18 KDa) were visible, plus a wider band of approximately 30 KDa. In the extraction made under our conditions, up to 5 bands of molecular weight between 40 and 75 KDa were visible, in addition to a band of 18 KDa. In the pattern found for the secalins, regardless of the extraction method used, two large bands appeared with molecular weights of 30 and 66 KDa. Nevertheless, bands of different molecular weights could be seen, present under only our working conditions (a band of 40 KDa and another of 45 KDa); also, another two bands were appreciable, one of 31 and another of 68 KDa, confirming the high resolution of the solvent used. In the case of the pattern of bands found for the hordeins, the result was very similar to that for secalins, presenting bands of 45, 47 and 62 KDa.

This assay can be used as a quick, easy, and sensitive method, to detect gluten in food or dietetic food products.

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