Monoclonal Antibody Sandwich Enzyme Immunoassays for Determination of Gluten in Foods

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The development and properties of two-site ("sandwich") enzyme immunoassays for gluten in foods, based on monoclonal antibodies to heat-stable ω-gliadins and related prolamins from wheat, rye, and barley, are described. The complete assay requires under 2 h when precoated microwells are used. The effects of altering component antibodies, gluten extraction conditions, and solvent on assay performance are described. Quantitative results were obtained by using a simple one-step extraction procedure with 40% ethanol over a very wide range of gluten contents (0.015–10%). The method was developed for the quantitative analysis of gluten in virtually all types of foods, whether raw, cooked, or processed.

INTRODUCTION

The low cost of wheat gluten, together with its physicochemical characteristics which enable it to bind meat fragments in processed meats, has led to widespread use of gluten or wheat flour as an extender. As such use of nonmeat proteins is regulated by food legislation, the need for monitoring of gluten additions by government agencies arises. Further, the total levels of gluten, as well as its quality in various flours, affect their suitability for use in different baked products, such as breads, cookies, cakes, and pasta (Wrigley and McMaster, 1989). Another factor leading to the need for gluten determination methods is the large number of individuals unable to tolerate wheat gluten and gluten-like proteins from rye and barley (and in some cases, oats) in their diets; the best characterized such condition is gluten-sensitive enteropathy or celiac sprue (Cooke and Holmes, 1984). However, it has proven difficult to quantify gluten for regulatory or quality-control purposes, especially after foods have been cooked or processed. Methods involving microscopy, electrophoresis, or chromatography are either tedious, not reliable, or require considerable expertise and in many cases are not quantitative (Carlson et al., 1980; McCausland and Wrigley, 1976; Hohlfield and Pietsch, 1981; Windemann et al., 1985).

Immunochemical methods for determination of gluten based on immunodiffusion in gels have also proven difficult with water-insoluble gluten proteins and are of relatively low sensitivity (Zareba, 1968; Lietze, 1969; Keyser and Mahler, 1973; Kaczkowski et al., 1985). More sensitive radioimmunoassays (RIA) and enzyme immunoassays (EIA) have been developed, based on polyclonal antisera to gliadins (the monomeric protein portion of wheat gluten) or to gliadin subfractions. The RIA of Ciclitira and Lennox (1983) was quantitative only with uncooked foods and did not detect (celiac-toxic) rye or barley prolamins.

Several groups have described sandwich EIAs for the detection of gluten in foods (Windemann et al., 1982; Meier et al., 1984; Fritschy et al., 1985; McKillop et al., 1985; Troncone et al., 1986; Freedman et al., 1987; Ayob et al., 1988). However, all of these workers have used a polyclonal antibody with specificity for either α-gliadin or total gliadin as one or (in most cases) both of the binding partners in the sandwich EIA. Antibody specificity characteristics, together with alteration of the majority of antigenic determinants in gluten following heating (Meier et al., 1984), resulted in the failure of these assays to yield quantitative results after foods have been baked or processed. Further, inappropriate antibody cross-reaction may render many of these assays either unsuitable for analysis of food containing potentially celiac-toxic prolamins from rye or barley (Freedman et al., 1987) or liable to yield false positives with maize (Troncone et al., 1986).

In our attempt to develop reliable immunoassays for gluten in cooked foods, we (Skerritt, 1985) utilized monoclonal antibodies (MAb) specific for the unusually heat-stable ω-gliadin fraction of gluten (Schofield et al., 1983). Simple qualitative (Skerritt and Smith, 1985) or quantitative (Skerritt, 1985) EIAs were developed to detect gluten proteins bound to disks prepared from a nitrocellulose solid phase, after their soaking in food extracts. While these assays provided reliable results, handling of nitrocellulose disks proved tedious, and the assay was lengthier than most microwell assays. Because of low affinities, the antibodies used in that work functioned poorly in microwell EIA (Skerritt and Martinuzzi, 1986). As a result, novel high-affinity ω-gliadin binding monoclonal antibodies were developed. In this paper, the use is described of some of these antibodies in the development of a microwell sandwich EIA for quantitation of gluten in foods.

MATERIALS AND METHODS

Monoclonal Antibodies. Methods used for the preparation and characterization of MAb to wheat gliadins and glutenins have been described in detail elsewhere (Skerritt and Underwood, 1986; Hill and Skerritt, 1989). After hybridoma recloning, selected cell lines were grown as ascites tumors in mice. Antibody from harvested ascites fluids was precipitated by ammonium sulfate and purified by hydroxylapatite adsorption chromatography (Stanker et al., 1985). Purified monoclonal antibodies were labeled with horseradish peroxidase (HRP) by a modification of the method of Nakane and Kawoi (1974); conjugates were freed of unreacted enzyme by ammonium sulfate precipitation.

In this study, 401/21 and 304/13 (both IgG_1) were used (Hill and Skerritt, 1989).

SDS–Polyacrylamide Gel Electrophoresis (PAGE) and Immunoblotting. Either total protein extracts [made with 2% (w/v) SDS–10% (v/v) mercaptoethanol–0.67 M urea–16% (v/v) *N,N*-dimethylformamide] or 40% (v/v) ethanol extracts of

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various cereal grains were analyzed by SDS–PAGE [in 15% (w/v) polyacrylamide gels, 1200 V h) as described earlier (Skerritt and Underwood, 1986). In the former case, polypeptides from replicate gels were electroblotted to nitrocellulose, and specific monoclonal antibody binding was revealed by use of the MAb–peroxidase conjugate or by use of an alkaline phosphatase labeled goat anti-mouse antibody (Donovan et al., 1989).

Preparation of Meat/Gluten Blends and Flour/Starch Blends. Differing amounts of commercial vital gluten (N. B. Love Industries, Enfield, Australia), previously analyzed for protein, were blended with pure beef mince (total mass = 500 g) for 3 min at 20 °C by using a Morton (Morton Machinery Co. Ltd., Wishaw, Scotland) mixer. Samples of 100 g were then cooked in a domestic microwave (750 W) on the highest heat setting for 5 min. Gluten contents were calculated by accounting for water loss and the protein content of the gluten used.

Differing proportions of flour and prime commercial starch (0.3% protein) were blended by overnight rotation.

Cereal Antigens. The cereal standards used are described elsewhere (Skerritt and Lew, 1990). Gliadin antigens were prepared by extraction of bread wheat flour (Timgalen variety) twice with 10% NaCl (10 mL/g) followed by one extraction (10 mL/g) with either (1) 40% (v/v) ethanol, (2) 70% (v/v) ethanol, or (3) 1 M urea. Preparations were dialyzed against water and freeze-dried. While the urea preparation was homogeneous in appearance and solubility, the alcohol preparations were not. Thus, additional preparations were made by dialysis against 10 mM acetic acid instead of water and, in the case of the 70% ethanol extract, dissolution at 10 mg/mL in 50% (v/v) 2-methyl-3-propanol and lyophilization.

Extraction of Gluten from Foods. Several methods were compared for extraction of gluten from foods: (1A) vortex mixing, for 1 min each 15 min over 1 h, with mixing by inversion between vortexing: (1B) vortex mixing for 30 s only; (2) blending using an Omnimixer (Sorvall, Newtown, CT) at 50% maximal speed for 30 s, 90 s, and 3 min; and (3) high-speed (20 000 rpm) dispersion using an Ultraturrax probe (Janke & Kunkel, Dottingen, FRG). Omnimixer extraction for 30 s was used routinely unless otherwise indicated. Samples of 1 g were extracted, "as is", with 10 mL of 40% (v/v) ethanol; other extractants were used where indicated. All extractions were performed at room temperature (18–25 °C). Before use, extracts were routinely centrifuged (2500 rpm, 10 min) in a bench-top centrifuge (Hermle Z320, Gosheim, FRG), and a 100-μL aliquot of the supernatant was diluted appropriately in 1% BSA–PBS–0.05% Tween.

Gluten Determination in Flours. Gluten was washed from two flours (Timgalen bread wheat flour, 13.7% protein, and a commercial multipurpose flour, 11.3% protein) by using a Glutomatic (Falling Number AB, Stockholm, Sweden). Three replicate 10-g amounts of flour were washed for 5 min with 210 mL of water for each. Glutens obtained were freeze-dried and weighed, and nitrogen was determined by Kjeldahl analysis. The first flour contained 10.9% gluten and the second, 8.6% gluten. The flours were used to prepare standards for assessment of extractants and for calibration of the gliadin antigen standard.

Sandwich EIA. Polystyrene microwells (Immulon B, Dynatech, Chantilly, VA, or NUNC Maxisorp, Roskilde, Denmark) were coated with 1 μg/well MAb 304/13 or 401/21 in 100 μL of 50 mM sodium carbonate buffer, pH 9.6, for 60 min at 20 °C. These conditions had earlier been optimized to obtain highest signal-to-background ratios (Hill and Skerritt, 1989). Following three washes with 50 mM sodium phosphate, pH 7.2, containing 0.9% sodium chloride (PBS)–0.05% Tween 20, 150 μL/well blocking agent [1% bovine serum albumin (BSA)–0.01% sodium azide in PBS] was added for 60 min at 20 °C. Blocking agent was shaken off, and 100 μL/well gliadin antigen or food extract (diluted in 1% BSA–PBS–0.05% Tween 20) added for 30 min at 20 °C. Microwells were washed three times, and then HRP-labeled monoclonal antibody, diluted in 1% BSA–PBS–0.05% Tween 20 (100 μL/well) was added. (The dilution of conjugate that yielded an absorbance of 1.0 in indirect EIA using 2 μg/well gliadin was chosen.) Following five washes, substrate [2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) containing 0.003% hydrogen peroxide] was incubated for 10 min at 20 °C; color development was terminated by acidification and absorbances read at 414 nm.

Assay Precision. Within-assay precision was calculated by determination of the coefficient of variation (CV) for absorbance of the antigen (gliadin) standard curve (performed in duplicate) on nine different 96-microwell plates in 1 day. Repeatability was determined by the coefficient of variation of points on the standard curve run in triplicate on 4 consecutive days. Studies on the between-laboratory precision and repeatability of results obtained with 14 food samples, including gluten/meat blends, flours, gluten-free baking mixes, cookies, and soups, will be reported elsewhere (Skerritt and Hill, 1990b).

RESULTS

Antibody Specificity: SDS–PAGE and Immunoblotting. Both antibodies (304/13 and 401/21) bound to total grain extracts of bread and durum wheat ω-gliadins and high molecular weight glutenins (identified on the basis of their mobilities on SDS–PAGE) and to corresponding prolamins from rye and barley (Figure 1). Little reaction was noted with prolamins from other cereals. Apparent binding of these antibodies to ω-type prolamins relative to HMW glutelins was artifactually suppressed following the SDS and mercaptoethanol treatment used for SDS–PAGE, in keeping with other studies with antibodies of similar specificity (Skerritt and Underwood, 1986; Donovan et al., 1989).

Choice of Food Extractant. Earlier work has indicated that the effect of grain or food extractant on antibody cross-reaction with different prolamins was dependent on the immunoassay format used (Skerritt and Hill, 1990a). Thus, it was necessary to evaluate certain gliadin extractants (40% ethanol, 70% ethanol, 1 M urea, and 1 mM HCl) on the following criteria: (1) cereal protein cross-reaction (minimal reaction with maize but significant reaction with durum wheat, rye, and barley prolamins); (2) accuracy of results obtained with wheat starches, flour/starch, and meat/gluten blends; (3) accuracy of results after cooking, e.g., with bread crumb and meat/gluten blends; (4) reproducible extraction of gluten from samples.

Using antibody 401/21, no significant reaction (that is, EIA absorbances of <0.1) was observed with maize or the low protein (0.29%) wheat starch with any of the extractants. Use of either urea or 70% ethanol resulted in underestimation of the gluten content of a high-protein starch (0.43% protein, approximately 0.1% gluten). Further, the absorbance values produced with 1/20–1/100 dilutions of a 70% ethanol extract of this starch were also low, making it difficult to distinguish from a low-protein starch by visual inspection of colored microwells. Determination of gluten in bread crumb produced accurate results with ethanol extractants but an underestimate with 1 M urea.

A large number of flour/starch and gluten/starch blends, covering a gluten content range of 0.09–72%, were analyzed by using the four extractants, and a standard curve was prepared from extraction of a flour of known gluten content with the particular extractant under study. The "protein content" (N × 5.7) of the commercial gluten was determined at 72%; because of contamination by non-gluten protein in the commercial gluten (Skerritt, unpublished results), it is likely that the actual gluten content is slightly lower. Gluten values obtained with 40% ethanol were close to the expected values (Figure 2); in contrast, values obtained with 70% ethanol (Figure 2) or 1 M urea for samples below 0.5% gluten were slight underestimates, while those for samples over 0.5% gluten were usually overestimates. Gluten determinations made with 1 mM HCl extractant were overestimations at both high and low gluten contents.

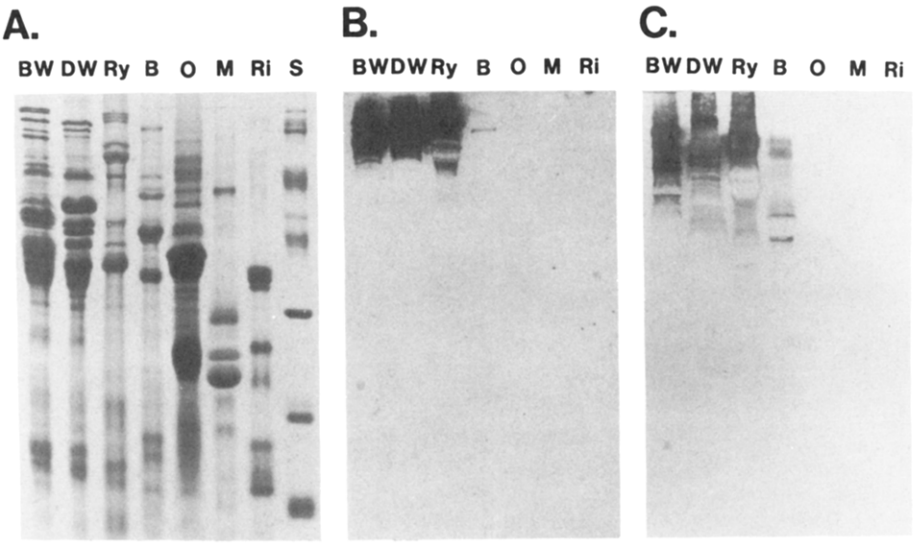

Figure 1. Specificities of 304/13 and 401/21 determined by SDS–PAGE and immunoblotting: (A) stained electrophoresis gel, total protein extracts; (B) immunoblot probed with 304/13; (C) immunoblot probed with 401/21. Cereals: BW, bread wheat; DW, durum wheat; Ry, rye; B, barley; O, oats; M, maize; R, rice. Molecular weight standards (S) are, from top, M_r 94 000, 67 000, 43 000, 30 100, 20 000, and 14 400.

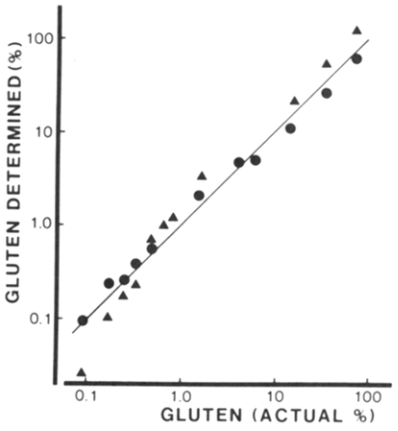

Figure 2. Determination of gluten in flour–starch and gluten–starch blends using 40% (●) and 70% (▲) ethanol extractants.

Values obtained for raw meat/gluten blends (containing 0.17–5.4% gluten) were, however, generally similar for 40% and 70% ethanol extracts. Values obtained with 70% ethanol were 102 ± 21% of 40% ethanol values (n = 5). After cooking, values obtained with 40% ethanol extraction were not significantly altered (112 ± 9%, n = 6), but those obtained with 70% ethanol were grossly elevated (390 ± 100%, n = 5) compared with the values obtained for raw meat/gluten blends extracted with the same solvent. In contrast, when 1 mM HCl was used as an extractant, gluten values for raw meat/gluten blends were markedly elevated over actual gluten contents. Gluten values with 1 M urea were similar to those obtained with 40% ethanol, and results were not markedly affected by cooking (106 ± 14% of raw, n = 6).

Antibody Cross-Reaction with Proteins from Different Cereals. The four extractants, plus 10 mM acetic acid, were first investigated for extraction of immunoreactive protein from related cereals by using 401/21 as both solid-phase bound and labeled antibody (Table I). Extractant had significant effects on the detection limit of the assay; 70% ethanol yielded the lowest sensitivity, while 10 mM acetic acid, 1 M urea, and 40% ethanol enabled more sensitive detection. Forty percent ethanol gave the most desirable cereal protein cross-reaction profile for a test for celiac-toxic gluten in foods. With 40% ethanol, durum wheat extracts were equally potent to bread wheat extracts (rather than less potent, as with 1 M urea and 1 mM HCl), and rye extracts were also equally potent (rather than being more potent as with the other extractants, apart from 1 mM HCl). While barley is likely less toxic than wheats or rye, significant detection was still obtained. In contrast, when 304/13 was used, another high-affinity ω-gliadin binding antibody, a very strong reaction was obtained with rye proteins but very little with barley proteins, rendering this combination less suitable for use in gluten determination. SDS–PAGE analysis of 40% ethanol-extracted proteins from the major cereals is shown in Figure 3. The full complement of α, β, γ, and ω-gliadins (M_r 30 000–70 000) was extracted from bread and durum wheats as well as lower molecular weight CM-proteins (M_r 14 000–16 000). In rye, these lower molecular weight proteins were extracted, as well as γ-secalins of M_r 40 000 and 75 000. Relatively little protein was extracted from barley—only small amounts of M_r 14 000–16 000 proteins, C-hordeins, and some β- or γ-hordeins. Maize proteins were detected very weakly, but significantly, by extraction with 70% ethanol or 10 mM acetic acid. Taken together, these results suggested that 40% ethanol was the most suitable extractant for quantitative determination of gluten.

Apart from providing inaccurate results with flour/starch and gluten/meat blends containing low amounts of gluten, variability in gluten data was obtained with high-gluten samples (such as flours) when 1 mM HCl was used as the extractant. Low EIA absorbance values were usually associated with a visible gliadin aggregate or "scum" seen on the surface of the extract after centrifugation. This effect was sometimes noted after homogenization of food/flour samples, but never after gentle hand mixing or vortex mixing. As hand mixing did not enable quantitative recovery of gluten from processed foods, assessment of 1 mM HCl was discontinued. Cross-reaction of the 401/21 combination was further investigated by using various wheat protein fractions (Figure 4). Albumin and globulins reacted very weakly, while gliadins and glutenins were most potent; as discussed earlier (Hill and Skerritt, 1989) the potency difference between gliadins and glutenins is exaggerated by use of alkaline solvent for the latter.

Analysis of a selection of wheat-based foods using these

Table I. Effect of Cereal Grain Extractant on Gluten Determination in Cereals

extractant	bread wheat, EC_{50} detection limit			cross-reaction relative to bread wheat = 100						
	1/diln[b]	1/diln	ng/mL	durum	rye	barley	oats	maize	rice	millet
1. Antibody Combination 401/21–401/21										
ethanol (40%)										
potency[c]	6200	45000	130	89	150	6	–	–	–	–
maximum[d]				105	121	50	–	–	–	–
ethanol (70%)										
potency	2800	19000	310	87	200	2	–	0.01	–	–
maximum				98	90	37	–	10	–	–
urea (1 M)										
potency	5600	40000	160	42	200	0.3	–	–	–	–
maximum				97	110	27	–	–	–	–
HCl (1 mM)										
potency	3800	29000	190	46	66	0.2	–	–	–	–
maximum				90	77	21	–	–	–	–
acetic acid (10 mM)										
potency	15000	60000	130	92	330	0.01	–	–	–	–
maximum				97	175	41	–	20	–	–
2. Antibody Combination 304/13–304/13										
ethanol (40%)										
potency	10000	60000	100	70	410	0.2	–	–	–	–
maximum				93	120	20	–	–	–	–

[a] Data shown are geometric means of 2–10 determinations using antibody 401/21. [b] Bread wheat extract dilution (diln) yielding 50% of maximal absorbance. [c] Determined at the extract dilution yielding an absorbance of 0.1 (– = <0.01%). [d] Maximum absorbance (or absorbance at a 1/40 cereal extract dilution) relative to a maximum absorbance on bread wheat standard curve (– = <10%).

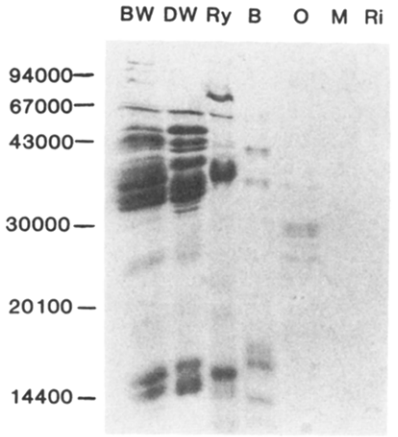

Figure 3. Analysis by SDS–PAGE of cereal grain proteins extracted by 40% ethanol. Symbols as for legend to Figure 1.

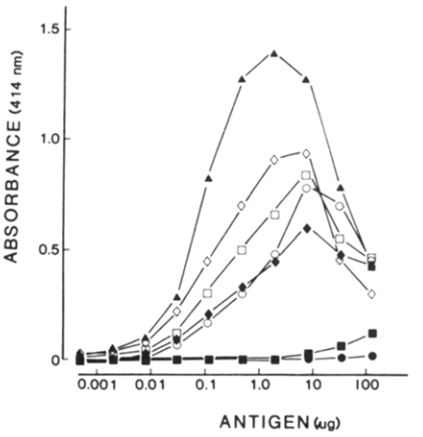

Figure 4. Cross-reaction of 401/21 in sandwich ELISA with wheat protein fractions: (●) albumin; (■) globulin; (▲) glutenin; (◇) purified ω-gliadin, gliadin extracted with (□) 40% ethanol, (○) 70% ethanol, and (◆) 1 M urea.

antibody combinations gave acceptable results for 401/21, but results obtained with 304/13 used as both solid-phase bound and labeled antibody tended to be a little low (Table II). Therefore, 401/21 was used in further work.

Effect of Extraction Method. The effects of extraction method were examined by using a large set of food samples; results obtained with key samples of known gluten content are shown (Tables III and IV). Vortex mixing, either for 30 s only or repeatedly over 1 h, produced similar results. Certain samples such as ground meats, spiked by us with high amounts of gluten (e.g., 1.9% gluten determined, 3% actual), and confectionery (caramel, 0.3% gluten determined, 1.3% actual) gave inaccurate results. Use of the Omnimixer for 30 s (only) appeared optimal. Longer mixing, for 90 s or 3 min, caused only slight heating of the samples but decreased gluten values obtained with high-gluten samples (Table V). Gluten concentration response curves obtained with bread wheat flour were superimposable with those obtained with standard gliadin antigen when either vortexing or 30-s (but not longer) homogenization with an Ultraturrax or Omnimixer was used. Possibly the antigens determined in this assay are sensitive to shear denaturation or shear-induced aggregation. Comparison of results obtained with homogenization using a blade-type device (Omnimixer) or a high-frequency homogenization with a shaft-type device (Ultraturrax) (Table IV) showed that while both produced quite reasonable extraction of gluten, values obtained by using the Ultraturrax were 10–15% higher on average.

In the assay shown (Table III) analysis of uncentrifuged samples produced accurate results, except for an underestimate of gluten content of a flour and an overestimate with a soup sample. Between-assay reproducibility was lower when samples were not centrifuged.

Choice of Antigen Standard. Experiments were performed to determine an antigen standard that (1) gave quantitatively correct results for gluten in foods over a wide range of EIA product absorbances, (2) could be stored lyophilized or reconstituted for over 12 months, and (3) when lyophilized, was readily soluble in the extracting solution used for food samples. Initial experiments with preparations 1–3 (see Materials and Methods) showed that

Table II. Quantitation of Gluten in Wheat Flour Containing Foods Using Various Antibody Combinations[a]

	antibody combination	
solid-phase bound	304/13	401/21
enzyme-labeled	304/13	401/21
starches		
HB, 0.25% protein	nd[b]	nd
F, 0.29% protein	nd	nd
NN, 0.37% protein	0.064	0.052
pregel, 0.41% protein	0.049	0.060
maize		
starch, 0.50% protein	nd	nd
meal, 8.5% protein	nd	nd
cake flours		
plain flour	5.4	7.2
self-rising flour	8.1	7.5
bread crumb	7.4	11.0
cookie	3.4	4.8
gluten-free bread mixes		
1	0.055	0.061
2	0.11	0.14
3	0.016	0.015
4	0.18	0.22
processed meat		
1	nd	nd
2	1.0	1.4

[a] Data shown are percent gluten by weight. [b] nd, not detectable (<0.01% gluten).

Table III. Determination of Gluten (Percent) in Foods: Effect of Extraction Technique and Time[a]

	extraction method			
	vortex mixer	Omnimixer		
sample	4 × 30 s	30 s	180 s	30 s
centrifuged	yes	yes	yes	no
maize starch	nd[b]	nd	nd	nd
wheat starch				
0.29% protein	nd	0.01	nd	nd
0.32% protein	0.031	0.035	0.033	0.028
0.43% protein	0.085	0.10	0.068	0.11
flour (10% gluten)	8.5	8.9	4.6	5.7
gluten-free bread mix	0.17	0.17	0.20	0.26
cooked meats				
no gluten	nd	nd	nd	nd
with 1.1% gluten	1.2	1.1	0.72	1.1
soups				
no gluten	nd	nd	nd	0.035
with 0.1% gluten	0.11	0.10	0.093	0.18

[a] Antibody combination 401/21–401/21 and 40% ethanol extractant used. [b] nd, not detectable.

the antigens dissolved most readily in the solvent used for their original preparation e.g., the 1 M urea extracted antigen dissolved readily in 1 M urea at 0.5–5 mg/mL but required sonication to be dissolved in aqueous ethanol. Standard curves for each antigen were thus compared with those made by using 1 M urea, 1 mM HCl, and 40% or 70% ethanol extraction of a flour of known gluten content. Curves were parallel when the solvent for antigen preparation and flour extraction were the same; the urea solvent and extractant standard curves were steeper than the other curves (Figure 5). Further, while the antigen curve and the flour extract curves were superimposable for 40% and 70% ethanol, the urea antigen standard was considerably less potent than gliadin from freshly urea extracted flour (not shown). This could result from either denaturation during drying of the dialyzed urea extract or an effect of the salt pretreatment on gliadin extraction by urea. Seventy percent ethanol extracted gliadin, made homogeneous by 2-methyl-3-propanol treatment, was of similar potency to untreated 70% ethanol extracted gliadin but did not readily dissolve in 40% ethanol.

Table IV. Determination of Gluten (Percent) in Foods: Comparison of High-Speed Homogenization Using an Omnimixer and an Ultraturrax

	homogenizer		
sample	1/dilution tested	Omnimixer	Ultraturrax
wheat starch			
0.29% protein	50	nd[a]	nd
0.32% protein	50	0.031	0.037
0.42% protein	50	0.083	0.085
flour			
8.6% protein	2500	5.5	6.2
13.7% protein	2500	9.9	12.0
cookie	2500	2.5	2.9
cooked meak with gluten			
1	50	0.22	0.26
2	50	0.30	0.33
3	500	1.8	1.9

[a] nd, not detectable.

Table V. Gluten Content of Selected Foods Measured by Sandwich ELISA

food	1/dilution tested	EIA absorbance	gluten, %
starches			
1 (0.24% total protein)	50	0.06	nd[a]
2 (0.32% total protein)	50	0.34	0.03
3 (0.43% total protein)	50	0.78	0.12
baking mixes labeled as gluten-free			
1	50	0.08	nd
2	50	1.05	0.30
3	50	0.33	0.03
breakfast cereals labeled as gluten-free			
rice-based	50	0.03	nd
granola	50	0.71	0.09
baked foods			
bread-crumbing mix	2500	0.85	7.3
sweet cookie	2500	0.64	3.7
cracker	2500	0.62	3.5
crispbread	2500	0.33	1.5
baby foods			
beef-based, no thickener	50	0.00	nd
chicken-based, flour thickener	50	1.10	0.40
processed meats/small goods			
cooked beef product 1	500	0.03	nd
cooked beef product 2	500	0.73	1.00
cooked beef product 3	2500	0.95	10
ham sausage	500	0.38	0.40
salami	500	0.02	nd
soups, vegetable-based			
1	50	0.02	nd
2	50	1.08	0.39
confectionery			
caramel A	50	0.05	nd
caramel B	50	0.08	nd
caramel X	500	0.83	1.4
chocolate	500	0.22	0.20
other food protein sources			
lentils	50	0.00	nd
soy flour	50	0.00	nd
egg	50	0.00	nd
milk powder	50	0.00	nd

[a] nd, not detectable (<0.016%).

Dialysis of 40% ethanol extracted gliadin against 10 mM acetic acid did not alter its potency, but considerably improved its solubility. Over the range 16 ng–1 μg/well, acid-dialyzed gliadin gave 97 ± 2% the absorbances obtained for undialyzed gliadin. Meredith (1965) and Lookhart (1987) also noted that mild acid treatment enhanced gliadin solubility. Once reconstituted in 40% ethanol, acid-dialyzed gliadin was very stable. Indeed, no loss of activity has been noted after 1 year of storage at 4 °C or after 50 days at 37 °C (not shown).

Assay Sensitivity. The sensitivity (limit of detection)

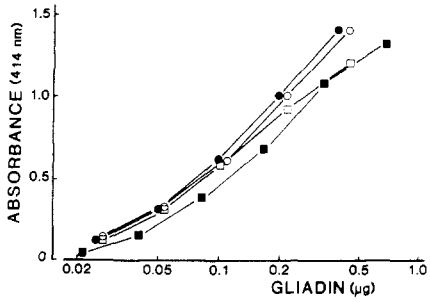

Figure 5. Comparison of antigen standard curves (solid symbols) and bread wheat extract dilution curves, prepared by using a flour of known gluten content (open symbols) for 40% ethanol (●, ○) and 1 M urea (■, □) extractants.

of the assay was defined as the gliadin concentration yielding an EIA absorbance of 0.1 above gliadin-free "blank" wells. Typically this concentration was 0.010–0.015 μg gliadin/well (0.10–0.15 μg of gliadin/mL). By use of a 1/5 dilution of food extracts, 0.001–0.0015% (10–15 μg/g) gluten is just detectable in foods. All (commercially prepared) wheat starches contain traces of gluten, and wheat-derived starches could readily be distinguished from maize or potato starches when 1/5–1/10 extract dilutions were used. When 1/50 extract dilutions were used, only starches unacceptable for use in gluten-free diets (containing more than 0.02% gluten) gave appreciable color (>0.2 absorbance unit) in the assay.

Determination of Gluten in Flours. For these experiments, three composite flours were analyzed from each of nine wheat varieties (Cook, Eagle, Halberd, Oxley, Rac 557, Miskle, Kialta, Meteor, and Wilgoyne) at three flour protein levels—approximately 9%, 11%, and 13%. Varietal effects on binding of the 401/21 capture–tag combination were minimal: at the lower protein level, 6.0–9.8% gluten was detected, and at the high-protein level, 10.0–13.6% gluten was detected. A significant correlation between flour protein content and gluten content was obtained ($r = 0.628$, $n = 27$, $P < 0.001$).

Analysis of Foods. A wide variety of foods was analyzed; gluten present was calculated by reference to a standard curve for gliadin (Figure 6A) by using the formula

$$\text{gluten (\% by mass of original sample)} = \text{gliadin (μg/mL)} \times \text{dilution factor}/500$$

which takes into account the 10 mL/g extraction ratio and the assumption that 50% of the protein in gluten is gliadin (Huebner, 1970; Kent, 1975). These analyses (Table V), together with results from analysis of flour/starch, gluten/starch, and gluten/meat blends and a range of other food samples (Skerritt and Hill, unpublished results) containing known amounts of gluten, demonstrate that reliable quantification of gluten is possible over several orders of magnitude (0.016–50%) of total gluten.

A number (18) of enzymically modified glutens were also tested in the assay, but meaningful gluten estimations could not be made, as the shape of the dilution curves for modified gluten extracts differed markedly from that of the gliadin standard. Nevertheless, some preparations were detectable at as great extract dilutions as (undigested) gluten, while other modified glutens were detected rather weakly. No gluten was detected in a range of lager beers, while an ale and a stout were positive for gluten (product absorbance >0.1 at dilutions above 1/100 and 1/250, respectively). Again, as the shape of the dilution curve differed from that of the gliadin standard, meaningful gluten determinations could not be made. Finally, one caramel confection (caramel X) thickened with

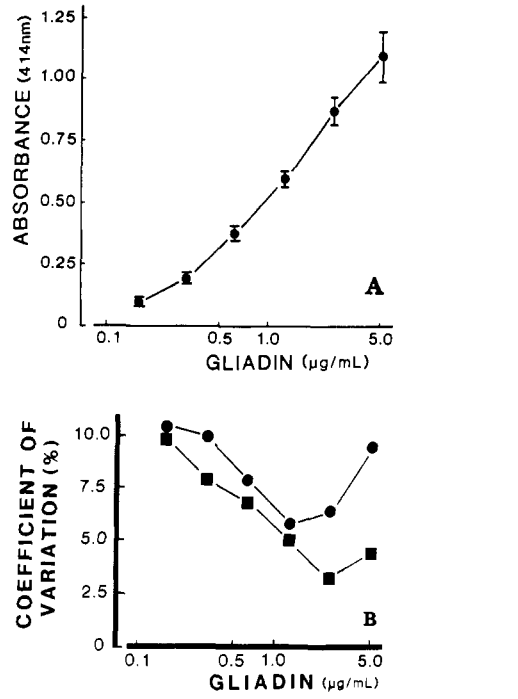

Figure 6. (A) Mean standard curve for gluten determination in foods. Standard deviations of the mean for 12 assays are shown. (B) Coefficients of variation for repeatability (between assay, ■) and reproducibility (within assay, ●) of ELISA product absorbance values obtained with gliadin standards.

(approximately 20%) wheat flour had high levels of gluten detectable, while two caramel preparations, known to have virtually no gluten (prepared from a wheat starch of 0.25–0.29% protein), did not produce an absorbance response.

Assay Precision. Assay precision, determined as the coefficient of variation for absorbance values of the antigen standard curve, over nine separate standard curves run simultaneously, was good (coefficients of variation, 5.9–10.5%, Figure 6B); precision was greatest for the middle values on the standard curve. Between-assay repeatability four different days) had a CV of 3.3–10.0% for absorbance values (Figure 6B), indicating good repeatability. The mean standard curve from 12 consecutive assays is shown in Figure 6A.

DISCUSSION

The method described in this paper is the first quantitative microwell EIA suitable for gluten determination in all types of foods (i.e., raw, cooked, and processed). No false positives were seen. The only false negatives identified were samples containing low amounts of gluten together with a polyphenol-containing substance such as cocoa, coffee, or hops. Such interference was noted earlier by developers of a polyclonal antibody based test for gliadin in uncooked foods (Windemann, 1987) and has been attributed to interactions with polyphenols inhibiting gliadin extraction; it was reduced by these workers by addition of dry casein to the food before extraction of the mixture in 3 M urea in 70% ethanol. We have developed a simpler method for reducing or eliminating these effects by use of polyphenol-binding additives to the 40% ethanol extractant (Skerritt and Hill, unpublished results).

Choice of food extractant was critical in obtaining quantitatively correct results, those obtained with 40% ethanol being more accurate. However, particular examples in which 70% ethanol produced inaccurate results were for cooked meat/gluten blends and, to a lesser extent, flour/starch and gluten/starch blends. Ethanol

(70%) has been favored as the gliadin extractant for most groups that have developed EIAs for food analysis (Fritschy, 1985; Troncone et al., 1986; Freedman et al., 1987; Ayob et al., 1988). Detection of gluten was also appreciably less potent with 70% ethanol than 40% ethanol, reinforcing gel electrophoresis results which showed that relatively more ω-gliadin is extracted by 40% ethanol. The lower ethanol concentration has also been shown to be a slightly superior extractant for total gliadin (Meredith, 1965). The other groups that have used 70% ethanol as an extractant have used broader specificity antibodies and have not attempted to estimate gluten recoveries from spiked samples (Fritschy et al., 1985; Freedman et al., 1987; Ayob et al., 1988).

High-speed homogenization for 30 s was a simple and reliable method for extraction of gluten. However, use of a single extraction step and 10 volumes of extractant as in this sample would not be expected to quantitatively extract all gluten from food samples. Reextraction of the residue from centrifugation with 10 mL of 40% ethanol per gram of food removed a further 1.0% gluten from a high-gluten flour (11% gluten), 0.46% gluten from a low-gluten flour (5.9% gluten), 0.40% gluten from a meat containing 2.7% gluten, 0.38% gluten from cookies (3.6% gluten), 0.13% gluten from a soup (2.0% gluten), and 0.01% gluten from a starch (0.11% gluten). Thus, a further 7–15% gluten was removed by a second extraction. However, when the two extracts were combined before analysis, determined gluten values were slightly too high, for samples such as starches, flour, cookies, and meats, where the actual gluten contents were known. This is because the antigen standard used was prepared by a single extraction and was calibrated with respect to flours prepared similarly. Increasing the extraction volume to 20 or 50 mL/g did not significantly increase the amount of gluten removed by a single extraction. Therefore, in view of the requirement for the gluten extraction step to be as simple as possible, a single extraction was used routinely.

Apart from yielding reliable results with cooked foods, MAbs offer several other advantages over polyclonal antisera for gluten detection in foods. There exists a potentially unlimited supply of antibodies of consistent affinity and specificity; problems such as occasional unwanted cross-reactions with maize prolamins will not occur. Appropriate use of the assay described in this paper will provide a reliable means of gluten determination for the following.

(1) Starch quality can be monitored. Kjeldahl analysis can only measure total nitrogen content of starches. It is, however, the actual gluten content that determines suitability of a starch for special dietary foods (for gluten intolerance) or baby foods or as a filler in pharmaceuticals.

(2) Process monitoring can be done in wheat starch/gluten manufacture. The assay can analyze effluent (monitoring gluten losses) and wet or dry glutens and starches.

(3) The total gluten content of flours or baked products can be rapidly measured without the need for gluten washing, Kjeldahl analysis, or subtraction of the approximate protein contents of nonflour components in baked goods. Thus, gluten content can be modified in the development of better baked goods of various types.

(4) The method is suitable for testing both uncooked product mixes and baked or processed products including processed meats. This can help development of optimized product formulations. Meat products can be checked to ensure they fulfil meat and nonmeat protein content regulations.

(5) The gluten content of foods for gluten-intolerant individuals (celiacs) and of other types of foods, where there is an advantage in labeling them as being gluten-free, e.g., baby and infant foods, some health foods, and pharmaceuticals, can be determined.

The antibodies (and products of related clones) described in this paper and the overall techniques form the basis of a commercially available (Medical Innovations Ltd., Queensland, Australia) antibody-based kit for gluten detection in foods. With certain proprietary modifications, enabling stabilization of antibody, solid phase, and enzyme conjugates, the method has recently been tested successfully in 15 laboratories in official interlaboratory collaborative trials (Skerritt and Hill, 1990b).

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