Purification and Characterization of a Specific Gliadin Component from the Wheat Cultivar Marquis[1]

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ABSTRACT

A specific gliadin component (band 50) was isolated from the wheat cultivar Marquis-K, purified, and characterized on the basis of its molecular weight, subunit composition, and isoelectric point. Purification was achieved using Sephacryl S-200 gel chromatography followed by preparative electrophoresis on a polyacrylamide gel. The preparation contains one gliadin, as shown by two-dimensional isoelectric focusing-polyacrylamide gel electrophoresis, is a monomer of molecular weight 53,250, and has an isoelectric point of 6.5.

Polyacrylamide gel electrophoresis (PAGE) of wheat gliadins has been proposed as a method for cultivar identification using the cultivar Marquis as an electrophoretic standard (Bushuk and Zillman 1978). Seed of this cultivar contains a prominent gliadin band (band 50) that can be used as a reference standard to calculate the relative mobilities of other gliadins separated by PAGE. This reference standard has been used to prepare a catalog of electrophoregram formulas of Canadian wheat cultivars (Zillman and Bushuk 1979), measure the purity of newly released cultivars (Kosmolak 1979), and study relationships between durum wheat electrophoretic patterns and gluten strength (Damidaux et al 1978, Kosmolak et al 1980). The location of reference band 50 can be ambiguous with some electrophoretic systems (Autran et al 1979). If this band is to be used as a reliable standard, it must be clearly and easily identifiable; therefore, the purification and properties of band 50 are of interest.

MATERIALS AND METHODS

Materials

Chymotrypsin (bovine) and tris (hydroxymethyl) aminomethane (TRIS) were obtained from Sigma Chemical Corp., St. Louis, MO; hemoglobin (bovine), β-lactoglobulin (bovine), serum albumin (bovine), and bromphenol blue from Nutritional Biochemical Corp., Cleveland, OH; acrylamide and *N*-*N*-methylene bisacrylamide (bis) from Eastman Kodak Co., Rochester, N.Y.; methyl green from Anachemia, Ltd., Montreal, Canada; dodecyl sodium sulfate (SDS) from Matheson Coleman and Bell, Norwood, OH; Coomassie brillant blue R250, ammonium persulfate, Bio-Rad protein assay kit, γ-globulin (bovine), *N, N, N*^{1}, *N*^{1}-tetramethylethylenediamine (TEMED), Bio-Lyte (ampholytes) from Bio-Rad Laboratories, Richmond, CA; and sephacryl S-200 (S-200) from Pharmacia, Uppsala, Sweden. Proteins were extracted from seeds of the cultivar selection Marquis-K (Kosmolak and Kerber 1980).

Protein Assay

Protein was determined using the Bio-Rad protein assay kit, as described by Bradford (1976), with γ-globulin as the standard.

Assay for Band 50

Band 50 was identified in fractions by comparison of its electrophoretic mobility with the electrophoretic mobility of band 50 present in 70% (v/v) ethanolic extracts of Marquis-K whole meal. Aliquots of the fractions were diluted with two volumes of electrophoresis buffer A (0.25% [w/v] aluminum lactate-lactic acid to pH 3.1) containing 25% (v/v) glycerine. PAGE was performed on vertical slab gels (0.15 cm thick) containing 9% (w/v) acrylamide in gel solution A (0.3% [w/v] bis, 0.02% [w/v] ascorbic acid, 0.0025% [w/v] ferrous sulfate, 0.25% [w/v] aluminum lactate-lactic acid pH 3.1, polymerized with 0.0024% [v/v] hydrogen peroxide). Electrophoresis was performed for 2 hr at 380 V constant voltage at 7° C. The gels were stained overnight in 20% (w/v) trichloroacetic acid (TCA) containing 0.01% (w/v) Coomassie blue, then photographed using Tech Pan copy film.

Purification of Band 50

Twenty grams of seed of the cultivar Marquis-K were ground on a Udy Cyclone mill (1-mm sieve), and 19 g of the whole meal were extracted for 1 hr at 25° C with 60 ml of 70% (v/v) aqueous ethanol with continuous stirring. The resultant slurry was centrifuged at 20° C for 10 min at 20,000 × *g*, the supernate decanted, and the pellet reextracted with 60 ml of 70% ethanol. The combined supernatants were concentrated to 55 ml under reduced pressure at 35° C in a Buchler rotary evaporator. TRIS base (1 *M*) was added to give a final concentration of 10 m*M* TRIS, and the solution was centrifuged at 20° C for 10 min at 20,000 × *g*. The supernate (initial extract) was applied to an S-200 column (88 × 5 cm) equilibrated with 70% (v/v) ethanol containing 10 m*M* TRIS. Chromatography was performed using the same buffer at a flow rate of 36 ml/hr. The first 110 ml were discarded, and fractions (18 ml) were then collected and assayed for band 50. Fractions containing band 50 were pooled and concentrated under reduced pressure to 16 ml. Twelve milliliters of this solution were applied to a second S-200 column (92 × 2.6 m), equilibrated, and eluted in the same buffer system at a flow rate of 12 ml/hr. Fractions containing band 50 were concentrated as above to a volume of 3.5 ml, for preparative gel electrophoresis. Aliquots (0.5 ml) were diluted with 0.25 ml of glycerin, 0.25 ml of buffer A, and 0.01 ml of lactic acid. Methyl green was added as a marker. Preparative electrophoresis was performed on a 100-mm Bio-Rad electrophoretic apparatus (Bio-Rad Laboratories, Canada Ltd., Mississauga, Ont.) with the following modification: two teflon spacers (14 × 2 × 0.2 cm) each with a 20-gauge syringe needle embedded 2.5 cm from the lower end, replaced the Bio-Rad elution port spacers. A plug gel of 9% (w/v) acrylamide in gel solution A was formed level with the elution ports. After polymerization, buffer A containing 50% (v/v) glycerin was layered to 2 mm above the elution ports, and a separating gel solution containing 7% acrylamide in gel solution A was gently overlaid. After polymerization, the upper reservoir was filled with buffer A, and 1.0 ml of the diluted sample containing band 50 was layered onto the separating gel. A potential of 50 V was maintained until the marker dye had entered the gel, and the voltage was then increased to 380 V. Elution with half strength buffer A (flow rate 1.4 ml/min) was begun when the slower migrating component of the marker dye (blue band) was 0.5 cm from the bottom of the separating gel. Fractions (2.0 ml) containing band 50 but free of all other protein bands were pooled and concentrated to 4 ml under reduced pressure.

SDS-PAGE

Polyacrylamide gels (0.08 cm thick) containing 10% acrylamide-0.1% bis or 12.5% acrylamide-0.125% bis, and 0.31 *M* TRIS-HCl

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(pH 8.1), 0.1% (w/v) SDS and 8*M* urea were polymerized with 0.013% (w/v) ammonium persulfate and 0.06% (v/v) TEMED. An aliquot of the pooled fractions containing band 50 was dialyzed overnight against distilled water. Two hundred microliters of the resultant slurry containing precipitated protein and 200-μl aliquots containing standard proteins at a concentration of 0.2 mg/ml were adjusted to give final concentrations of 8*M* urea, 1% SDS, 2.5% (v/v) 2-mercaptoethanol, and 0.125*M* TRIS (pH 8.1). These solutions were then heated for 1 min in a boiling water bath, cooled, and aliquots applied to the gels. Bromphenol blue was used as a tracking dye. The upper electrophoresis-buffer (buffer B) contained 0.1*M* TRIS, 0.4*M* glycine, 0.1% SDS at pH 8.3, and the lower electrophoresis buffer was a 1:4 dilution of buffer B. Electrophoresis was completed when bromphenol blue had migrated to the end of the gel. Gels were fixed overnight in 20% TCA, then stained 3 hr in 0.1% Coomassie blue dissolved in ethanol–acetic acid–water (9:2:9 v/v), and destained in acetic-acid–methanol–water (7:7:86 v/v). The distance that proteins migrated into the gel was then measured with a ruler and relative migration determined by dividing the measurement by the length of the gel.

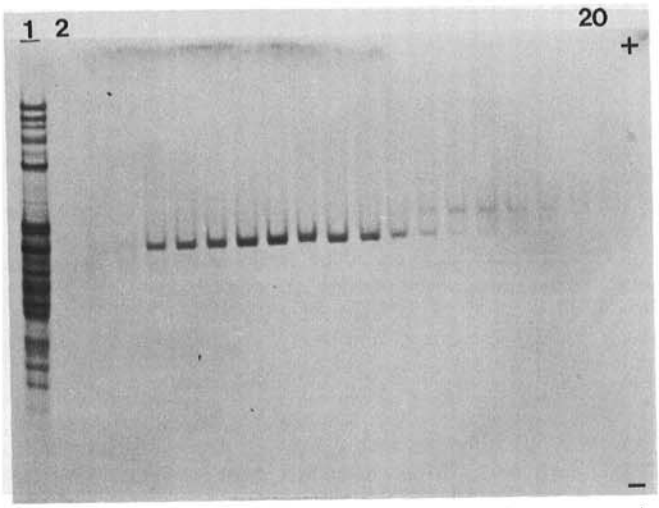

Fig. 1. Electrophoregrams of fractions following preparative polyacrylamide gel electrophoresis. 1 = Marquis-K (70% ethanol extract); 2–20 = fractions 44–62; fractions 47–53 were pooled for recovery of band 50.

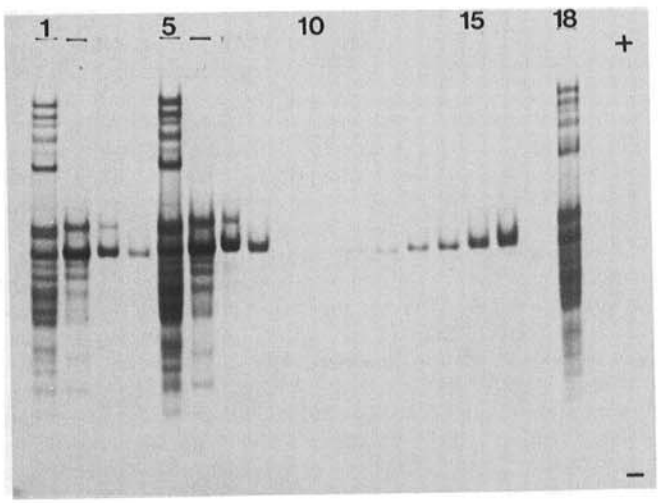

Fig. 2. Electrophoregrams of fractions containing band 50 at progressive stages of purification. 1, 5, 18 = Marquis-K (70% ethanolic extract); 2, 6 = pooled fractions (63–70) after first S-200 column; 3, 7 = pooled fractions (57–59) after second S-200 column; 4, 8 = pooled fraction (47–53) after preparative polyacrylamide gel electrophoresis; and 10–16 = increasing amounts (0.19, 0.38, 0.76, 1.5, 2.3, 3.0, 5.0 μg protein, respectively) of purified band 50.

Isolelectric Focusing (IEF)

Gel electrofocusing was performed using a modification of the method of Wrigley and Shepherd (Bietz and Wall 1972). Glass tubes (100 × 3 mm i.d.) were filled to 67 mm with a mixture containing 5% (w/v) acrylamide, 0.1% (w/v) bisacrylamide 8*M* urea, 0.8% (w/v) Bio-Lyte carrier ampholites (pH 6–8), 0.8% (w/v) Bio-Lyte (pH 3–10), 0.8% (w/v) Bio-Lyte (pH 7–9) 5% glycerin, 0.013% (w/v) ammonium persulfate, and 0.06% (v/v) TEMED. After polymerization, gels were overlaid with 15 μl of 2*M* urea containing 0.8% (w/v) Bio-Lyte (pH 3–10), using 0.2% (v/v) sulfuric acid as upper (anode) eletrolyte and 0.4% (v/v) ethanolamine as lower (cathode) electrolyte. Aliquots (200 μl) of either purified band 50 or the initial extract diluted with electrophoresis buffer A were mixed with 100 mg urea and 10 μl Bio-Lyte (pH 3–10) and applied between the top of the gels and the overlay solution. Electrophoresis was performed at 20° C for 24 hr at a maximum current of 0.25 mA per tube and a maximum potential at 450 V. The pH of some IEF gels was measured across 1-cm regions with a LKB multiphor pH surface electrode upon completion of electrofocusing. Electrofocused gels were either stored at −20° C for further electrophoresis or washed in 20% (w/v) TCA for 4 hr and stained in 0.01% (w/v) Coomassie blue as described.

Separation was performed in the second dimension by embedding the electrofocused gel in 2-ml 6% acrylamide in gel solution A, above a 9% acrylamide gel as described under "Assay for Band 50," or embedded in a 2-ml 5% acrylamide gel above a 10% acrylamide gel as described under "SDS-PAGE."

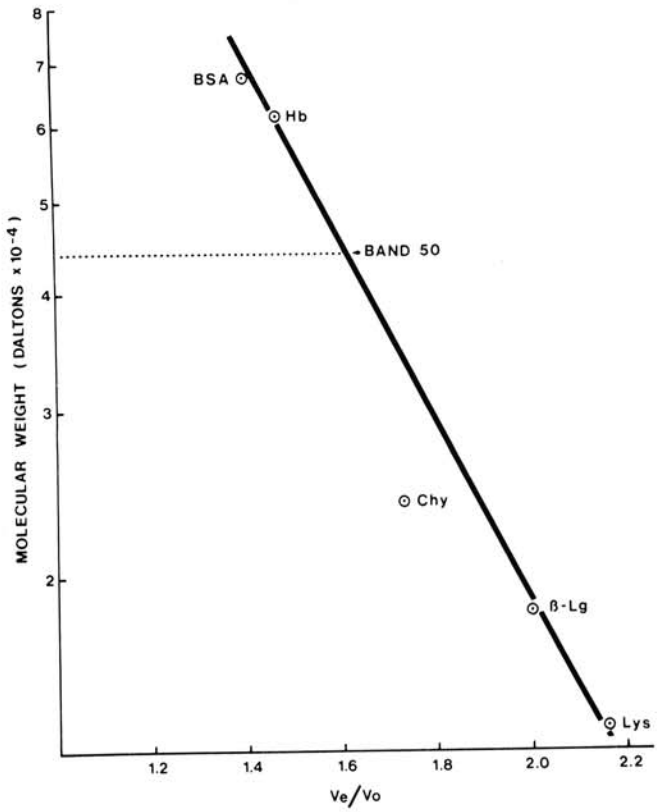

Fig. 3. Molecular weight determination of purified band 50 by chromatography on Sephacryl S-200. The column (82.5 × 1.2 cm) equilibrated in 0.4*M* NaCl/10 m*M* TRIS (pH 7.5), was calibrated using blue dextran 2,000. BSA = serum albumin (mol wt 68,000); Hb = hemoglobin (mol wt 62,000); Chy = chymotrysin (mol wt 24,000); β-Lg = β-lactoglobulin (mol wt 18,400); Lys = lysozyme (mol wt 13,900). Initial extract (0.4 ml) was chromatographed on the column equilibrated in 10 m*M* TRIS base 70% ethanol at a flow rate of 3.2 ml/hr, collecting 1.6 ml fractions. V_e = Elution volume (ml); V_o = void volume (ml).

RESULTS AND DISCUSSION

Purification of Band 50

Because gliadins are soluble in 70% ethanol but only sparingly soluble in water or dilute salt solution, the whole meal was extracted with 70% ethanol to enrich the proteins present in the gliadin fraction relative to other major seed proteins. This initial extract was then chromatographed on an S-200 gel column (equilibrated in 70% ethanol) to separate band 50 from the other proteins in the gliadin fraction on the basis of molecular weight. Under these conditions, all of the gliadins bound to the S-200 gel and could not be eluted. The addition of 10 m*M* TRIS to the developing solvent prevented binding. The fractions containing band 50 also contained several other proteins having mobilities similar to band 50. After the second chromatography on an S-200 column, fractions containing band 50 appeared to be free of all but one other protein (band 44). We removed band 44 from band 50 using preparative PAGE (Fig. 1).

Electrophoregrams of aliquots from the initial extract, pooled fractions after the first and second S-200 chromatography separations, and pooled fractions after preparative PAGE are shown in Fig. 2. An effective purification was achieved after the preparative electrophoresis step. A range of concentrations (0.19–5.0 μg protein) of the pooled fractions after preparative PAGE was also included to assess the purity of band 50. The limit of detection of band 50 in the gel assay system was below 0.19 μg protein. Aliquots containing 5 μg protein had no detectable levels of contaminants. This indicated that the fraction containing band 50 was at least 95% homogeneous with respect to other gliadins. The yield of band 50 in this purification system was 380 μg protein/g whole meal, representing 1.6% of the 70% ethanol soluble protein.

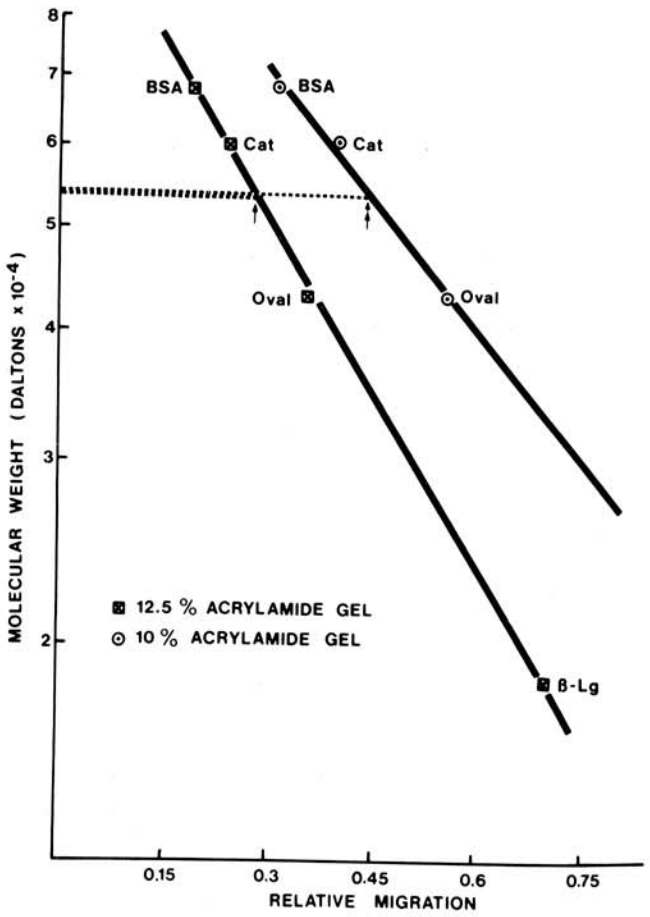

Fig. 4. Molecular weight determination of purified band 50 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins used for calibration: serum albumin (BSA, mol wt 68,000); catalase (Cat, mol wt 60,000); ovalbumin (Ova, mol wt 43,000, chymotrysin (Chy, mol wt 24,000); β-lactoglobulin (β-Lg, mol wt 18,400).

Analytical Studies

Chromatography of the initial extract on the S-200 column had no effect on the subsequent electrophoretic mobility of band 50, indicating that it did not dissociate into subunits under these conditions. Thus, chromatography on S-200 was used to determine the molecular weight of band 50.

Figure 3 illustrates the V_e/V_o of standard proteins plotted against the log of their molecular weights (Andrews 1965). Band 50, eluted at V_e/V_o of 1.62, which corresponds to a molecular weight of 44,000. Gel chromatography of band 50 was in 70% ethanol, containing 10 m*M* TRIS, but the calibration of the column was in an aqueous solvent because the standards were insoluble in 70% ethanol containing 10 m*M* TRIS. This may result in some error in the determination of the molecular weight of band 50.

A denaturing system containing urea, SDS, and 2-mercaptoethanol (which dissociates proteins into their constituent subunits), showed that band 50 contained only one protein. The calculated molecular weight of this protein when determined in a 10 and a 12.5% acrylamide gel was 53,500 and 53,000, respectively (Fig. 4). These values and the value of 44,000 daltons obtained under nondenaturing conditions using chromatography on S-200 indicates that band 50 does not have subunits. The best estimate of this protein's molecular weight is 53,250 (Bietz and Wall 1972, Weber and Osborn 1969).

When band 50 was subjected to isoelectric focusing (IEF), only one protein band was evident and was present in the region of the gel having a pH of 6.5, indicating a pI of 6.5 for this protein. Two-dimensional IEF-PAGE of band 50 resulted in one protein spot with either the pH 3.1 gel or the SDS-PAGE gel used in the second dimension, indicating that band 50 was free of all other proteins present in the 70% ethanolic extract of Marquis-K whole meal (Fig. 5).

The purification of this protein permits its use as a reference standard in other electrophoretic systems such as starch gels containing urea, gradient polyacrylamide gels, and two-dimensional gel systems in which the identity of Marquis band 50 is obscured by other proteins in the total gliadin extract (Autran et al 1979). The purification procedure described here should also be applicable to the purification of other gliadins, thereby enabling chemical and physical comparisons to be made between gliadins with identical electrophoretic mobilities but isolated from different varieties.

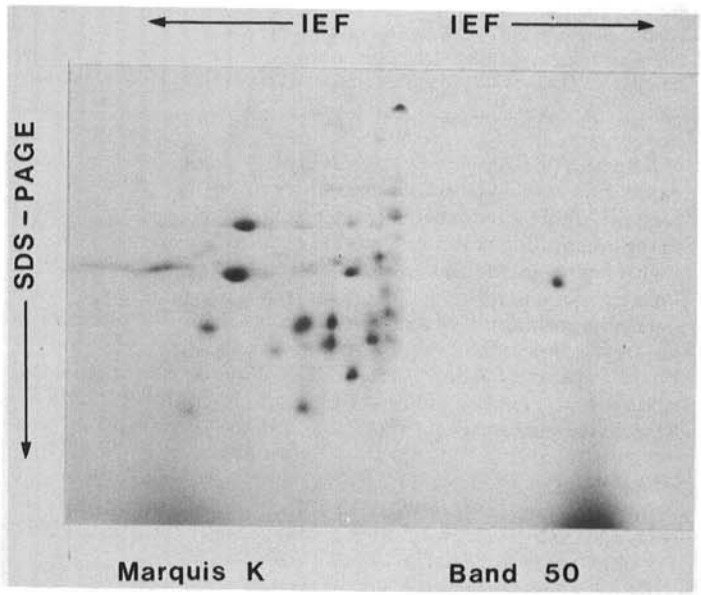

Fig. 5. Two-dimensional isoelectric focusing-polyacrylamide gel electrophoresis (IEF-PAGE) of Marquis-K (70% ethanolic extract) and purified band 50. First dimension IEF. Second dimension sodium dodecyl sulfate-PAGE; both IEF gels applied to same gel with low pH ends towards center.

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