

Characterization of Low-Molecular-Weight Protein with High Affinity for Flour Lipid from Two Wheat Classes¹

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ABSTRACT

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A low-molecular-weight protein (S protein) fraction with a high affinity for flour polar lipid was isolated from flours of one hard and one soft wheat variety. The fraction was characterized by lipid content and composition, acidic polyacrylamide gel electrophoresis (PAGE), gradient gel sodium dodecyl sulfate PAGE (SDS-PAGE), and reversed-phase high-performance liquid chromatography (RP-HPLC). The content of this protein in the two wheat varieties was approximately the same. Lipid contents and compositions of the two preparations were similar. PAGE

and SDS-PAGE patterns were similar for both varieties, but RP-HPLC patterns were quite different. Defatting the flour did not affect the yield of S protein or the PAGE and SDS-PAGE patterns. The two major components of the S protein fraction were isolated by Sephadex G-50 gel-filtration chromatography and characterized by electrophoresis, amino acid analysis, and RP-HPLC. PAGE, SDS-PAGE, and amino acid analysis results did not show any significant intervarietal differences, but the S protein fractions appeared distinctly different upon RP-HPLC.

Numerous reports have implicated interactions between flour proteins and lipids with breadmaking potential of wheat flour (Hoseney et al 1970, Chung et al 1978, MacRitchie 1980, Pomeranz 1980, Frazier et al 1981, Bushuk et al 1984). Generally, lipid-binding ability has been attributed to glutenin or gliadin (Olcott and Mecham 1947; Ponte et al 1967; Hoseney et al 1970; Chung and Tsen 1975; Bekes et al 1983; Zawistowska et al 1984; Zawistowska et al 1985a,b). Recently it was shown (Zawistowska et al 1985a) that a low-molecular-weight protein fraction tentatively named "S" protein, constituting about 5% of total flour protein, was associated with as much as 20% of flour polar lipid. Because flour polar lipids may positively affect loaf volume (Chung et al 1982, Bekes et al 1986), the high affinity of S protein for polar flour lipids suggested that this protein may be important in bread dough functionality. Accordingly, S protein fractions from two wheat varieties of different baking potential were compared by electrophoresis, in terms of lipid content and composition, and by reversed-phase high-performance liquid chromatography (RP-HPLC). Additionally, S protein fractions were prepared from partially and totally defatted flour samples in order to investigate the effect, if any, of flour lipid on yield and composition of S protein. The two major components of S protein were also isolated by Sephadex G-50 gel-filtration chromatography and partially characterized. Results are described and discussed in this article.

MATERIALS AND METHODS

Chemicals

Molecular weight reference proteins, Coomassie Brilliant Blue R250, 2-mercaptoethanol, and 3-methylaminopropionitrile were obtained from Sigma (St. Louis, MO). Acrylamide, bisacrylamide, and sodium dodecyl sulfate (SDS) were of electrophoresis grade and were obtained from Bio-Rad (Richmond, CA). All other chemicals were of analytical reagent grade.

Preparation of Flours

The wheat varieties used in this study were Neepawa, a hard red spring wheat, and Chile, a soft white spring wheat. Wheats were grown in 1983 under identical conditions in western Canada. A Buhler experimental mill was used to mill grain into flour. Flour

yield, protein ($N \times 5.7$), and ash contents were 72, 15.7, and 0.44% for Neepawa and 66, 14.9, and 0.50% for Chile, respectively. Protein and ash contents, expressed on dry bases, were determined by approved AACC methods (AACC 1983).

Defatting

Completely and partially defatted flours were prepared by extraction with water-saturated butanol and *n*-hexane, respectively (Bekes et al 1983).

Preparation of Glutens

Gluten was hand washed in a stream of distilled water from doughs mixed in air to peak development in a farinograph; washing was continued until water was clear. Wet gluten was freeze-dried, ground by mortar and pestle, and stored at 4°C.

Preparation of Gliadin

Unde-fatted Neepawa flour was extracted with 70% ethanol with agitation using a Buchler vortex-evaporator for 15 min, centrifuged at $27,000 \times g$ for 10 min, and immediately used for HPLC separation.

Fractionation of Gluten

Gluten was fractionated by ammonium sulfate precipitation of the acetic acid-soluble proteins dissolved in AUC solvent (0.1M acetic acid, 3M urea, and 0.01M cetyltrimethylammonium bromide) (Wasik and Bushuk 1974). Three concentrations of ammonium sulfate (7.8, 14.5, and 20.2%, w/v) yielded four fractions, three precipitates P₁, P₂, P₃, and a supernatant (S) fraction. Each precipitate was separated by centrifugation at $20,000 \times g$ for 15 min. The S fraction was concentrated on a model 8200 Amicon standard cell using YM5 membrane (5,000 mol wt cut off), dialyzed against deionized water, and freeze-dried. Gluten fractionation was performed in two replications, and results were averaged. A *t* test was calculated at the 0.05 level of significance for dry matter distribution in ammonium sulfate fractions.

Fractionation of S Fraction

Gel-filtration chromatography of S protein fractions was performed on Sephadex G-50 in AUC solvent. Column size was 2.6 \times 90 cm, flow rate was 12 ml/hr, and 3-ml fractions were collected. Protein content of the eluate was monitored at 280 nm by an LKB Uvicord SII ultraviolet absorbance monitor. Appropriate fractions were pooled, dialyzed against distilled water, and freeze-dried. Ovalbumin (mol wt 45,000) and trypsinogen (mol wt 24,000) were used to calibrate the column to facilitate estimation of molecular weights of S protein peaks. Percentages of eluted fractions were estimated from peak areas of the fractions collected. Areas from three separations were averaged; the experimental error was $\pm 3\%$.

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Lipid Analysis

For lipid determination, 60 mg of S fraction was extracted at 40°C by shaking with three portions of water-saturated butanol (ratio of solvent to sample in each extraction was 12.5:1, v/w). The first extraction was performed overnight, and subsequent extractions for 1 hr. Extracts were clarified by filtration, by centrifugation at 12,000 × g for 20 min, or both. Filtrates were combined and evaporated with a stream of nitrogen at 40°C. The dry residues were extracted with chloroform, filtered, and chloroform was evaporated with a stream of nitrogen at 40°C. Lipid content was determined gravimetrically from the residue remaining after solvent evaporation. Total lipid was fractionated by silicic acid column chromatography into neutral lipid (eluted with chloroform) and polar lipid (eluted with acetone followed by methanol) (Kates 1972). Lipid analyses were performed in triplicate and averages are reported.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed on 6.3% gels in aluminum lactate buffer at pH 3.1 (Bushuk and Zillman 1978), except that run time was decreased from 5 to 2 hr.

Sodium dodecyl sulfate (SDS)-PAGE was done on an LKB 2001 vertical electrophoresis unit (Zawistowska and Bushuk 1986) using 10–18% gradient gels and the discontinuous buffer system (Laemmli 1970). Proteins were dissolved in 62.5 mM Tris-HCl, pH 6.8, containing 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol. Samples were heated for 1.5 min in a boiling water bath to ensure complete unfolding of proteins and were cooled before application to the gel. Molecular weights were estimated from migration distances of bovine serum albumin (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), beta-lactoglobulin (18,400), and lysozyme (14,300).

Amino Acid Analysis

Amino acid compositions were determined on an LKB 4151 Alpha Plus automatic amino acid analyzer. Samples were hydrolyzed in 6N HCl for 16 hr at 121°C under vacuum, evaporated to dryness, dissolved in 0.2M sodium citrate buffer, pH 2.2, and filtered before analysis in duplicate. Cysteine and methionine were determined as cysteic acid and methionine sulfone following performic acid oxidation (Hirs 1967). Tryptophan was not determined. Values for aspartic and glutamic acids include asparagine and glutamine, respectively.

RP-HPLC

RP-HPLC was performed on a large-pore (300Å) SynChropak RP-P (C₁₈) column (4.1 × 250 mm) (Bietz 1983, Bietz and Cobb 1985). The system consisted of a Waters M6000A and M45 solvent-delivery system controlled by a model 660 solvent programmer, a WISP 710A automatic sample injector, and a model 450 variable-wavelength detector. For analysis, 0.5 mg of sample was dissolved in 1 ml of AUC solvent. Samples (50–100 μl) were analyzed using a linear gradient of 25–50% acetonitrile containing 0.1% trifluoroacetic acid over 50 min (total run time 60 min) at 1.0 ml/min and 70°C. Detection was at 210 nm using detector settings of 0.2–0.4 absorbance units full-scale. Data were recorded on a Houston Instruments Omniscribe recorder (10 mV full-scale).

RESULTS AND DISCUSSION

Characterization of S Protein

Distribution of fractions from ammonium sulfate precipitation is shown in Table I. Defatting had little effect on the dry matter distribution among the four fractions. Only for Chile it was found that defatting with *n*-hexane affected the amount of P₂ as compared with control flour, and there was a significant difference in amount of P₃ in *n*-hexane defatted and totally defatted sample. All other differences in dry material distribution among ammonium sulfate fractions were not significant. The S fraction constituted approximately 12% of the acetic acid-soluble gluten protein. Similar yields were obtained previously (Zawistowska et al 1985a) with the cultivar Neepawa (from different crop year) and by Frazier et al (1981) for an unspecified U.S. hard red spring wheat variety.

Lipid content determination of the S fraction of nondefatted flours showed the S fraction of Neepawa to contain 22.6% (± 1.0) lipid, and that of Chile, 24.6% (± 0.9). Most of the lipid was polar lipid. The S fraction of Neepawa contained 3.8% (± 0.1) neutral lipid and 18.8% (± 1.0) polar lipid, whereas that of Chile contained 3.9% (± 0.1) neutral lipid and 20.7% (± 1.1) polar lipid. The total lipid content for Neepawa was similar to the result obtained for Neepawa from a different crop year (Zawistowska et al 1985a). However, the proportion of neutral to polar lipid obtained for the two Neepawa samples was different. The reason for this is unknown, but it could be due to environment. An effect of

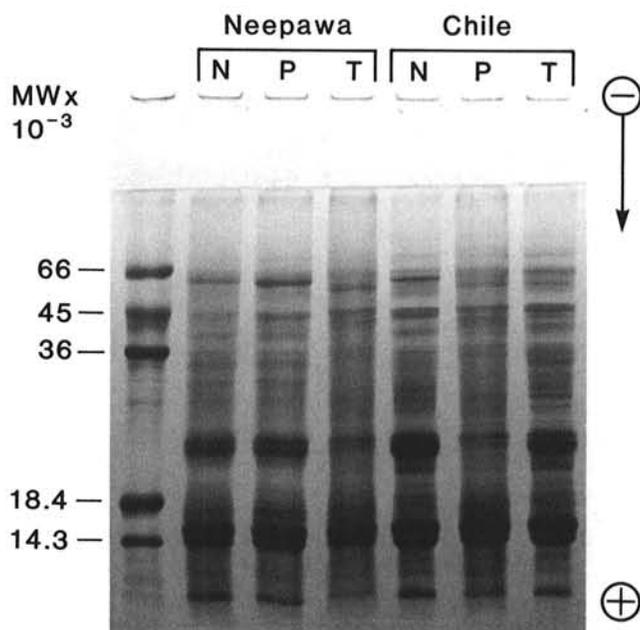


Fig. 1. Gradient (10–18%) sodium dodecyl sulfate-polyacrylamide gel electrophoretic patterns of S fractions from Neepawa and Chile flours. N, nondefatted; P, partially defatted; and T, totally defatted. Pattern on left is of molecular weight reference proteins.

TABLE I
Dry Matter Distribution in Fractions of Acetic Acid-Soluble Gluten Prepared by Ammonium Sulfate Precipitation^a

Fraction	Neepawa			Chile		
	Nondefatted (%)	Partially Defatted (%)	Totally Defatted (%)	Nondefatted (%)	Partially Defatted (%)	Totally Defatted (%)
Precipitate						
P ₁	50.6 (±2.7)	51.1 (±1.3)	51.6 (±2.1)	56.9 (±2.8)	47.1 (±2.6)	54.1 (±1.7)
P ₂	34.5 (±1.4)	34.0 (±1.7)	33.9 (±1.1)	28.9 (±1.8)	38.0 (±2.1)	32.2 (±0.8)
P ₃	2.4 (±0.6)	2.2 (±0.1)	3.1 (±0.3)	2.0 (±0.4)	3.0 (±0.3)	1.5 (±0.3)
Supernatant						
S	12.5 (±0.6)	12.7 (±0.7)	11.4 (±0.6)	12.2 (±0.7)	11.9 (±0.6)	12.2 (±0.7)

^a Means from two replications are given in the table; standard deviations are in parentheses.

environment on lipid content and composition has been reported (Fisher et al 1964, 1966; Bekes et al 1986).

SDS-PAGE showed that all six S protein preparations were quite heterogeneous (Fig. 1). Each pattern was dominated by two major bands characterized by molecular weights of approximately 14,000 and 16,000. Additional numerous minor bands were observed throughout the fractionation range for all samples of both varieties. Visual evaluation of relative band intensities showed no major quantitative changes caused by defatting flours with the exception of S protein of the partially defatted Chile flour. For this sample, intensity of bands that correspond to gel-filtration fraction II SDS-PAGE subunits (see below) is much lower than that of the same band of the other S protein fractions. It might be that the protein of this fraction was partially extracted by *n*-hexane during defatting of flour. No similar observation, however, was made for S protein from partially defatted Neepawa flour. This fact could be explained by different protein-lipid interactions in these two wheat varieties.

Acidic PAGE of all S protein preparations (Fig. 2) revealed four major and at least three minor bands of medium mobility, and minor bands throughout the high-mobility range. Analogous

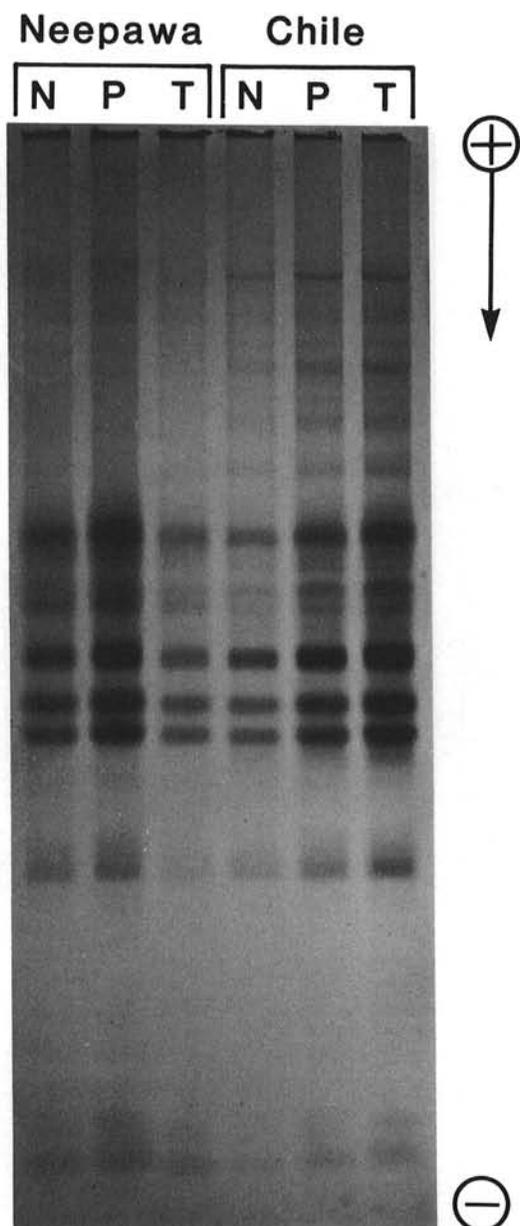


Fig. 2. Acidic polyacrylamide gel electrophoretic patterns of S fractions from Neepawa and Chile. N, nondefatted; P, partially defatted; and T, totally defatted.

patterns for the two varieties were similar, and defatting of the flours did not change electrophoregrams.

RP-HPLC of S fractions from nondefatted Neepawa and Chile flours, however, showed substantive qualitative and quantitative differences between the two varieties (Fig. 3). For example, peaks a, b, h, l, p, r, and s are present in the chromatogram of Neepawa but absent in that of Chile, and Chile has additional peaks g and o. Major quantitative differences are also evident, for example, peak e is much larger in Neepawa, whereas peaks m and n are much larger in Chile.

Comparison of chromatograms of nondefatted, partially defatted, and totally defatted flours of each variety (results not shown) revealed quantitative differences in some peaks, possibly indicating selective interactions of specific proteins with flour lipids.

RP-HPLC comparison of S fraction and 70% aqueous, ethanol-soluble proteins (mainly gliadin) from nondefatted Neepawa flour (Fig. 4) showed that most S fraction peaks elute between 11 and 30 min, whereas major gliadin peaks elute from 25 to 45 min. Thus, most S protein components are less hydrophobic than most gliadins. In this respect, S proteins resemble many wheat albumins and globulins (Bietz 1983, Bietz et al 1984). This observation is consistent with results that showed the low-molecular-weight S

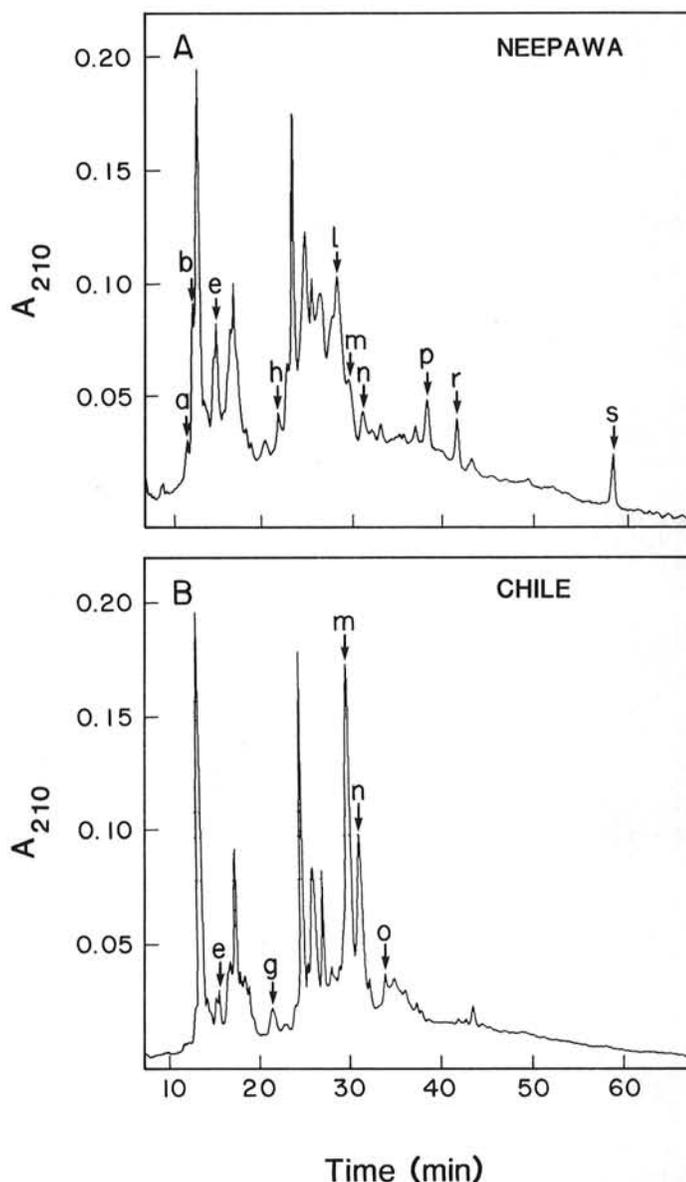


Fig. 3. Reversed-phase high-performance liquid chromatographic patterns of S fractions from nondefatted flours of Neepawa (A) and Chile (B).

protein components are also present in flour albumins/globulins prepared by Osborne fractionation (Zawistowska and Bushuk 1986). That study also shows that low-molecular-weight S protein components are soluble in 0.1M NaCl. NaCl solubility, and the surface hydrophobicities deduced from RP-HPLC, indicate that S proteins are globulin-like. Globulins are reported in gluten preparations, but few have been characterized (Kruger 1970, Nimmo et al 1968, Redman and Fisher 1968).

The chromatogram of Neepawa S fraction (Fig. 4A) contained six major peaks, c, e, f, i, j, and l. Some of these peaks (e.g., c, i, and l) were also present in gliadin (Fig. 4B), which is consistent with electrophoresis results that showed S protein bands in PAGE electrophoregrams of gliadins (Zawistowska et al 1985a).

Fractionation of S Protein Preparations

Because SDS-PAGE, PAGE, and RP-HPLC showed that S fractions were quite heterogeneous, they were further fractionated to better compare their major low-molecular-weight components. Gel-filtration chromatograms for S fractions from nondefatted flours contained three major subfractions (Fig. 5). Subfraction I eluted at the void volume, subfraction II eluted at a volume equivalent to a molecular weight of about 40,000, and subfraction III, a very broad peak, at a molecular weight below 24,000 (eluted after trypsinogen). Percentages of subfraction III were

significantly different: of the total S protein, 22% was in subfraction III of Neepawa and 38% in subfraction III of Chile.

Characterization of Gel-Filtration Subfractions

Gel-filtration subfractions of S fractions were compared by SDS-PAGE and PAGE. SDS-PAGE (Fig. 6) showed a clear separation based on molecular size. Subfractions III showed only two strong bands at approximately mol wt 14,000 and 16,000, whereas subfractions I and II contained many components with molecular weights above 16,000. Interestingly, the SDS-PAGE in reducing conditions of Sephadex G-50 gel filtration subfraction II shows molecular weight of the major components (25,000 and lower) to be approximately one half of the molecular weight estimated by gel filtration (Fig. 5), where subfraction II eluted at about 45,000. These results suggest that the gel-filtration fraction may be a dimer of the main component in SDS-PAGE.

PAGE patterns of Neepawa subfractions I, II, and III (Fig. 7) were also quite different, although there was some overlap. Subfractions III of each variety exhibited three major and five minor bands of the same relative mobilities.

Amino acid compositions of these two subfractions III (Table II) showed no significant differences. The most abundant amino acids were cysteine (12%), glutamic acid (11%), proline (10%), glycine (9%), and basic amino acids (9%). These compositions differ from

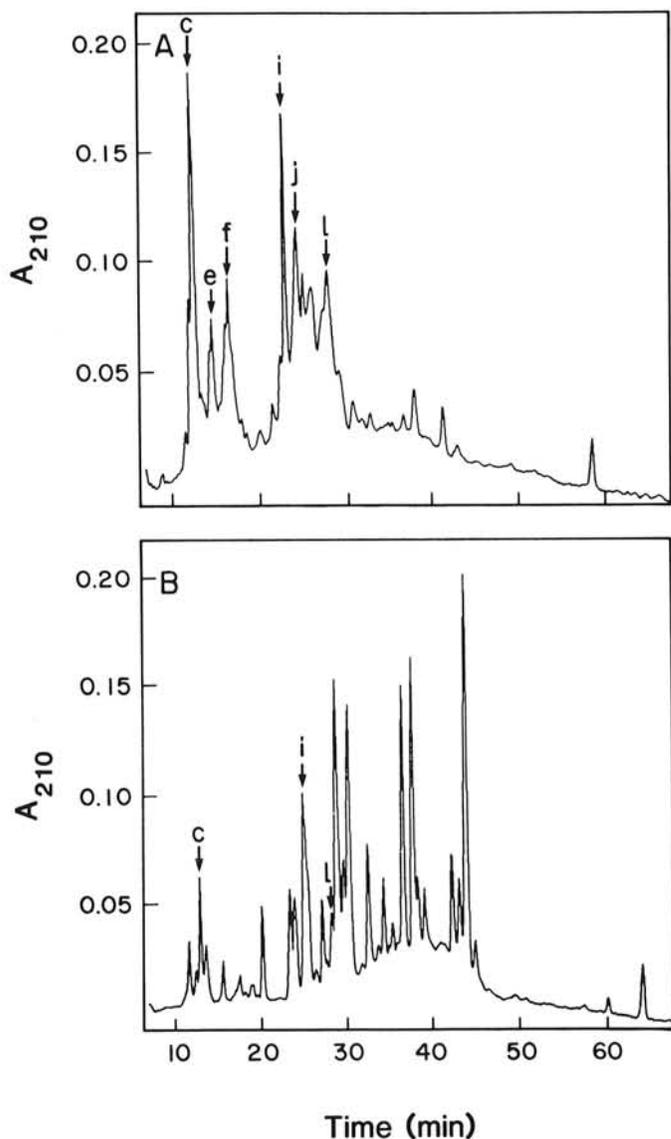


Fig. 4. Reversed-phase high-performance liquid chromatographic patterns of S fraction (A) and gliadin (B) from nondefatted Neepawa flour. Peaks characterized by identical retention times are indicated by the same letter.

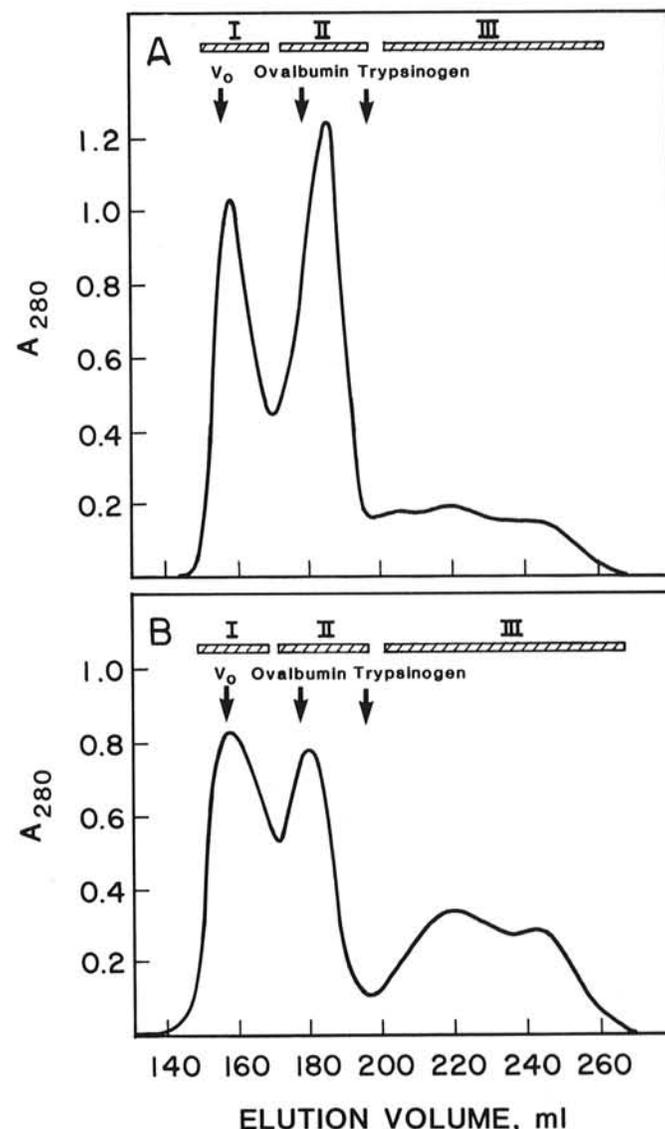


Fig. 5. Sephadex G-50 elution profiles of S fractions from nondefatted Neepawa (A) and Chile (B) flours. Arrows indicate void volume (V_o) and elution volumes for ovalbumin (45,000) and trypsinogen (24,000).

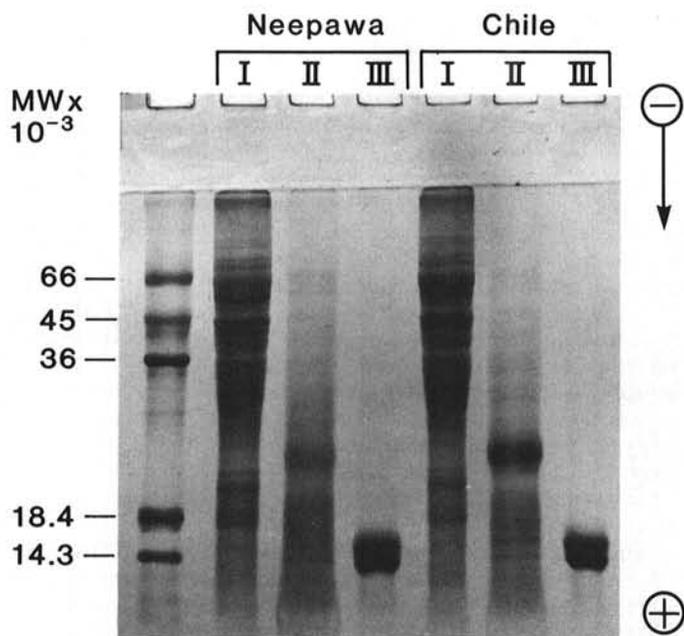


Fig. 6. Gradient (10–18%) sodium dodecyl sulfate-polyacrylamide gel electrophoretic patterns of subfractions I, II, and III from Sephadex G-50 gel filtration of S fractions from nondefatted Neepawa and Chile flours. Pattern on left is of reference proteins.

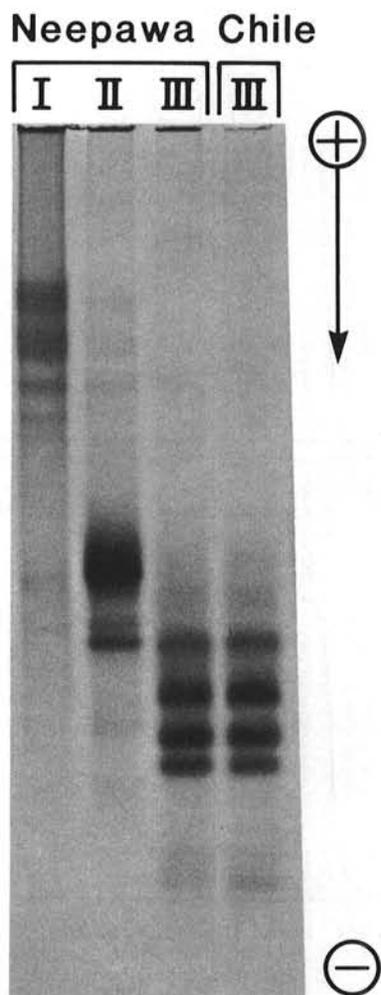


Fig. 7. Polyacrylamide gel electrophoretic patterns of S protein subfractions from Sephadex G-50 gel filtration of S fractions from nondefatted Neepawa and Chile flours.

those of gliadin and glutenin, having relatively higher contents of basic amino acids, aspartic acid, and threonine, and lower contents of glutamic acid and proline.

Subfractions III of Neepawa and Chile S proteins were further compared by RP-HPLC (Fig. 8). Whereas PAGE revealed eight bands in each sample (and SDS-PAGE only two bands), RP-HPLC resolved many more components (22 for Neepawa and 24 for Chile). These results clearly indicate greater heterogeneity among S proteins than was revealed by PAGE or SDS-PAGE; apparently conservative variation exists among hydrophobic amino acid residues of proteins that are otherwise identical in size and charge. Such variants are evident upon RP-HPLC, but not by PAGE or SDS-PAGE. The resulting chromatograms (Fig. 8) also had major quantitative and qualitative differences, suggesting that S proteins may differ among varieties. In contrast it has been generally accepted, based on electrophoresis (Wrigley et al 1982), that albumin and globulin compositions of common wheat varieties are similar.

In conclusion, our studies demonstrated that two widely different wheat varieties, Neepawa and Chile, contain similar amounts of S protein (about 5% of total flour protein), which binds

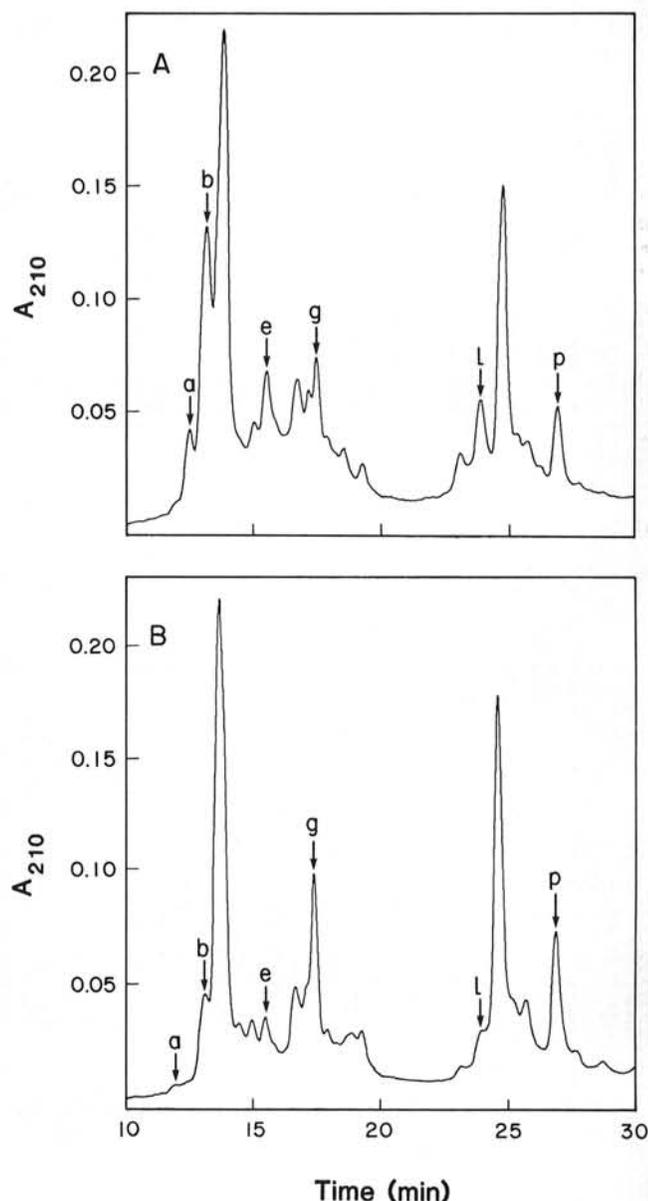


Fig. 8. Reversed-phase high-performance liquid chromatographic patterns of S protein Sephadex G-50 gel filtration subfractions III from nondefatted Neepawa (A) and Chile (B) flours. Arrows indicate peaks having identical retention times on both chromatograms.

TABLE II
Amino Acid Composition of Subfractions III of S Protein Fractions
from Nondefatted Neepawa and Chile Flours^a

Amino Acid	Neepawa (mol %)	Chile (mol %)	Nearest Integer
Alanine	6.1	6.0	6
Arginine	3.9	4.4	4
Aspartic acid ^b	7.1	7.0	7
Cysteine	12.1	11.8	12
Glutamic acid ^c	11.4	11.3	11
Glycine	9.4	9.3	9
Histadine	2.3	2.2	2
Isoleucine	2.6	2.7	3
Leucine	7.2	7.5	7
Lysine	2.9	2.7	3
Methionine	3.0	3.2	3
Phenylalanine	2.3	1.8	2
Proline	9.6	10.0	10
Serine	6.0	6.0	6
Threonine	4.6	4.8	5
Tyrosine	3.7	3.5	4
Valine	5.8	5.8	6

^a Mole percent of the total protein; tryptophan not determined.

^b Includes asparagine.

^c Includes glutamine.

substantial amounts of polar flour lipid. S fraction proteins of the two varieties analyzed by PAGE and SDS-PAGE appeared similar but differed significantly upon RP-HPLC. Gel-filtration chromatography demonstrated that the two S fractions differed in contents of the low-molecular-weight subfraction III. Chile (the poor quality variety containing slightly more lipid) contained nearly twice as much subfraction III as Neepawa. Neepawa and Chile subfractions III were similar as analyzed by PAGE and SDS-PAGE, but significantly different by RP-HPLC. Further studies are needed to determine which S protein components specifically bind polar lipid and to compare a larger number of wheat varieties of different baking quality to confirm or reject the hypothesis that S proteins affect breadmaking quality through their high affinity for polar flour lipids.

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