CHARACTERIZATION OF ACETIC ACID SOLUBLE AND INSOLUBLE FRACTIONS OF GLUTENIN OF BREAD WHEAT

BY

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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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A MES PARENTS

JEANNETTE ET GERALD

POUR VOTRE CONFIANCE EN MOI

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AVEC AMOUR

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LIST OF ABBREVIATIONS AND ACRONYMS

Abbreviation	Definition
AAI	acetic acid insoluble
AAS	acetic acid soluble
A-PAGE	acidic polyacrylamide gel electrophoresis
AUC	acetic acid (0.1M) - urea (3M) - cetyltrimethyl ammonium bromide (0.01M)
BU	Brabender units
CV	coefficient of variation
CWES	Canada Western Extra Strong
CWRS	Canada Western Red Spring
FA	farinograph absorption
FDDT	farinograph dough development time
FN	Falling Number
Gl	Glenlea
HMW-GS	high molecular weight glutenin subunit
HPLC	high performance liquid chromatography
kDa	kiloDalton
Kp or Kt	Katepwa
LMW-GS	low molecular weight glutenin subunit
MDDT	mixograph dough development time
Mr	relative molecular mass
MU	mixograph units
NIR	near infrared reflectance
PAGE	polyacrylamide gel electrophoresis
PSI	particle size index
RT	room temperature
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TCA	trichloroacetic acid

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ABSTRACT

It has long been established that protein content and quality are important in the breadmaking potential of bread wheat cultivars. Since the importance of glutenin as a protein quality factor in breadmaking potential was demonstrated, research has focused on the possible relationship between the structure of glutenin and its functionality in breadmaking.

The objective of the present study was to determine which physicochemical properties of glutenin, related to breadmaking quality, could explain the difference in solubility of the acetic acid soluble (AAS) and acetic acid insoluble (AAI) glutenins reported to be correlated to loaf volume. The AAS and AAI fractions were obtained by modified Osborne fractionation and analyzed by electrophoresis, densitometry and HPLC. Katepwa, a good breadmaking variety, and Glenlea, an extra strong variety, were used for the study.

Varietal differences in the proportion of AAS and AAI fractions were found. The glutenin amounts of the AAS fractions were not significantly different between cultivars, whereas the gliadin content was significantly higher for Katepwa. The AAI fraction was greater in Glenlea than in Katepwa. The ratios of HMW-GS:LMW-GS were not significantly different between fractions or cultivars, except for the low ratio obtained by HPLC for the AAI fraction of Katepwa. No consistent quantitative differences in individual HMW-GS could be found between fractions. HMW-GSs 5 and 10 appeared to be present in greater amounts in the AAI fractions of both cultivars. HMW-GS 7 was present in significantly greater proportions in Glenlea fractions. Based on densitometry

and HPLC results, no consistent quantitative differences in individual HMW-GSs nor the relative proportion of HMW-GSs and LMW-GSs could explain the intervarietal variation in solubility of glutenins in 0.05M acetic acid. Gliadin interaction with glutenin appears to be a cultivar characteristic, weak flours exhibiting greater interaction than strong flours.

I. INTRODUCTION

Cereal products provide about 20% energy, 19% protein and 36% carbohydrate of the average daily American diet (Boudreau and Grégoire, 1992). World production of wheat is presently near 550 million metric tons, almost 10% more than the production of rice. In 1989, Canada was the seventh in the world, producing 24.4 million metric tons. Of this, Canada exported 10.5 million metric tons. (Boudreau and Grégoire, 1992). Wheat and related products also play a major role in the Canadian economy. For instance, in 1989 the Canadian domestic market for bakery products was valued at $1.77 billion (Anon, 1992). The quality of Canadian wheat is recognized internationally.

It has long been established that protein content and quality are important factors in the breadmaking potential of bread wheat varieties (Finney and Barmore, 1948). Since the discovery of the relationship between the solubility of glutenin and breadmaking quality, as measured by loaf volume (Orth and Bushuk, 1972), much research has focused on a structure-functionality relationship in bread wheat proteins. The presence of specific HMW-GSs, quantitative and qualitative differences in HMW-GSs and LMW-GSs and the molecular weight distribution of glutenin have been associated with quality (Graveland et al, 1985; Gupta et al, 1991; MacRitchie, 1973).

The objective of this study was to determine which physicochemical properties of glutenin, related to breadmaking quality, could explain the intervarietal variation in solubility of glutenins in acetic acid (0.05N). Proteins from two flours of very diverse breadmaking quality were extracted by the modified Osborne fractionation (Chen and Bushuk, 1970) and analyzed for quantitative differences in specific HMW-GSs and differences in the relative proportion of HMW-GS and LMW-GSs between the AAS and AAI fractions and between varieties.

II. LITERATURE REVIEW

A. Introduction

Wheat flour has the unique ability to produce leavened bread when it is mixed with water, yeast, salt and optional functional ingredients, allowed to rise, shaped and baked. All major flour constituents contribute to the final product. Lipids, particularly polar lipids, affect baking quality by interacting with proteins. Starch is important in gelatinization and retrogradation of the crumb. The proteins are responsible for the formation of a viscoelastic dough during mixing. It has long been established that protein content and quality are important in the breadmaking potential of bread wheat cultivars. Early studies showed that loaf volume was linearly related to protein content and also related to a protein quality factor (Blish and Sandstedt, 1925; Harris, 1931; Mangels, 1926; Mangels and Sanderson, 1925). The relationship between loaf volume and protein content was reported to be linear between a range of 7 to 19.3% protein (Larmour, 1931). Highest loaf volumes within a variety were generally associated with natural protein contents of 12 to 13% (Sandstedt and Ofelt, 1940). Regression of loaf volume against protein content clearly demonstrated the positive linear relationship between both variables but also showed that the varietal differences in slope were a reflection of differences in protein quality (Finney and Barmore, 1948). The search for the quality factor has been ongoing ever since.

B. Structure of Wheat Flour Proteins

Osborne (1907) classified wheat proteins into four groups based on solubility. Based on sequential extraction with water, salt solution and 70% ethanol, wheat flour proteins were classified as water soluble albumins, salt soluble globulins, alcohol soluble gliadins and glutenins, insoluble in 70% ethanol. The modified Osborne fractionation method (Chen and Bushuk, 1970) further classified glutenins into dilute (0.05N) acetic acid soluble and insoluble fractions. The albumins are low molecular weight (< 30 kDa) metabolic proteins constituting about 15% of total protein (Bushuk, 1993). Globulins, making up about 5% of total protein, vary in molecular weight from 10-98 kDa. Gliadins, forming about 40% of total protein, are single chain polypeptides of molecular weight ranging from 30 to 80 kDa and are classified as α, β, γ and ω gliadins, based on their relative mobility during electrophoresis (Bushuk, 1993). In terms of cysteine-cystine content, the α, β, and γ gliadins are sulfur-rich, whereas the ω-gliadins are sulfur-poor (Field et al, 1983a). Some 45 different gliadin components in a variety have been identified by 2D-electrophoresis (Wrigley and Shepherd, 1973). Glutenins are polymeric proteins of very high molecular weight (>1,000 kDa) made up of subunits (single-chain polypeptides) of high molecular weight (95-140 kDa) and low molecular weight (30-50 kDa). HMW-GS composition varies with wheat variety (Orth and Bushuk, 1973b) and is genetically controlled by the homeologous group 1 chromosomes. Each Glu-1 locus contains two tightly linked genes Glu-1-1 and Glu-1-2 which code for *x*- and *y*-type subunits, respectively (Ng et al, 1989). Each variety contains between three and five HMW-GS, two of which are controlled by genes on the long arm of chromosome 1D, one or two by chromosome 1B and none or one by chromosome 1A (Orth and Bushuk, 1974; Payne et al, 1980, 1981a). The genes for the major polypeptides of glutenin, the LMW-GS, are located on the short arms of chromosomes 1A, 1B and 1D.

In addition to the solubility fractions of flour proteins, gluten was also studied in the search for the biochemical basis for breadmaking quality. The earliest reported isolation of gluten from wheat flour was in 1728 by Beccari (Bailey, 1941). Gluten is the viscoelastic mass formed by washing a dough made from wheat flour under a stream of water to remove starch (Osborne, 1907). Gluten is primarily comprised of gliadin, the viscous component, and glutenin, the elastic component. Starch and lipid are also present but in much smaller quantities.

C. Wheat Protein Quality for Breadmaking

As mentioned in the introduction, the existence of a quality factor has been known for many years (Blish and Sandstedt, 1925; Finney and Barmore, 1948). Identification of the molecular basis for protein quality has been the focus of considerable research. The importance of glutenin as a protein quality factor in breadmaking potential was demonstrated by Orth and Bushuk (1972). Based on the solubility distribution of the proteins of 26 bread wheats varying in breadmaking quality, they showed that loaf volume was positively correlated (+0.85**) with residue protein or AAI glutenin and negatively correlated (-0.86**) with AAS glutenin. The variation in solubility of glutenin was attributed to variation in molecular weight. The results obtained by Orth and Bushuk (1972) were in agreement with those of Pomeranz (1965) who reported that flours of poor quality had a greater proportion of protein dispersible in 3M urea. Since then, much research has focused on the possible relationship between the structure of glutenin and its functionality in breadmaking. Reconstitution studies by Lee and MacRitchie (1971) provided evidence that the molecular weight distribution of glutenin influenced quality as measured by dough properties. Later studies provided further evidence for a solubility-

quality relationship (Field et al, 1983b; Gao and Bushuk, 1992; MacRitchie, 1987; Marchylo et al, 1990; Moonen et al, 1982; for current review refer to Schofield, 1994).

Huebner and Wall (1976) obtained two fractions of glutenin by gel filtration of AUC-extracted proteins from flours of varying quality. They reported that the ratio of glutenin I, a very high molecular weight fraction, to glutenin II, a broad spectrum of lower molecular weight glutenins, was generally higher for flours of better breadmaking performance. Flours of weak mixing and baking characteristics usually contained less unextractable protein and glutenin I.

By reconstitution with suitable amounts of high molecular weight gluten (2M urea insoluble) and whole gluten, MacRitchie (1973) was able to produce a flour of strong dough characteristics from a flour that was originally weak and very extensible. MacRitchie (1973) concluded that the strength of a flour was directly related to the molecular weight distribution of its gluten proteins. Based on reconstitution/interchange studies of paired weak and strong flours, MacRitchie (1978) reported that differences in the baking performance of bread wheats were related to the properties of the AAI glutenin. Whereas glutenins exhibited a large influence on mixing requirements and loaf volume, gliadins were reported to decrease mixing requirements but have only a small negative effect on loaf volume (MacRitchie, 1987). In a later study (Gupta et al, 1993), it was reported that the protein unextractable in 0.5% SDS was very strongly positively correlated with dough strength. The authors also reported that the unextractable protein contained a greater proportion of larger polymers and a significantly higher ratio of HMW-GS to LMW-GS than the extractable fraction.

The composition of protein fractions and hence their relationship to breadmaking quality will vary with the fractionation procedure and the starting material used. However, based on three fractionation procedures and reconstitution studies, Chakraborty and Khan (1988b) reported that, regardless of the fractionation procedure, fractions

containing larger amounts of glutenin gave the highest positive responses to loaf volume. Whereas the extent of loaf volume response was dependent on the fractionation procedure.

Since the subunit composition of glutenin varies with wheat varieties, the possibility that different combinations of subunits could account for differences in glutenin properties and hence differences in breadmaking quality has been investigated. Orth and Bushuk (1973c) reported that the presence or absence of certain HMW-GSs coded for by the D-genome had a significant effect on baking quality. Payne et al (1979) showed that the composition of the HMW-GSs was related to breadmaking quality. A strong correlation was reported between the presence of HMW-GS 1 and quality. A strong correlation between HMW-GSs 5 and 10 and baking quality was subsequently reported (Payne et al, 1981b) and later supported by Ng and Bushuk (1988). Moonen et al (1983) reported similar associations of specific HMW-GS alleles with quality, but also stated that the effects were additive. Subsequent research has shown that the proportion of the intervarietal variation in breadmaking potential that can be attributed to differences in the composition of the HMW-GSs fluctuates widely between 15% and 60% (for review see Kolster, 1992).

In addition to HMW-GS composition, the ratio of HMW-GS to LMW-GS was also an important factor in breadmaking quality. Varieties with greater dough strength were reported to have a higher HMW-GS:LMW-GS ratio (Gupta and MacRitchie, 1991). For a fixed glutenin content, an increase in the HMW-GS:LMW-GS ratio resulted in a concomitant increase in dough strength (Gupta et al, 1992). MacRitchie and Gupta (1993) investigated the effects of sulfur deficiency on protein composition and functionality. The amounts of relatively sulfur-poor HMW-GSs increased and the sulfur-rich LMW-GSs, HMW albumins and triticins decreased with decreasing sulfur level. These changes resulted in a higher HMW-GS:LMW-GS ratio, higher percentage of

unextractable polymeric protein (considered to be related to molecular weight distribution), and hence greater dough strength.

The co-migration of LMW-GS with gliadins in SDS-PAGE has made the study of these proteins difficult. The development of a 2-step SDS-PAGE method has shown significant variation of LMW-GS patterns among bread wheat varieties (Gupta and Shepherd, 1987). These authors also reported an additive effect on dough resistance when a specific LMW-GS (Glu-A3m) was present with a HMW-GS (Glu-A1b; i.e. 2*). In another study (Gupta et al, 1991), correlations between predicted and actual dough quality parameters were reported to be highest if both LMW-GSs and HMW-GSs were included.

Further support for the relationship between solubility-baking quality and glutenin properties (molecular weight, size distribution, subunit composition and ratio of HMW-GS to LMW-GS) was provided by Graveland et al (1982, 1985). Glutenins were separated into fractions varying in subunit composition and molecular weight. Glutenin I, SDS-insoluble gel protein, was the protein fraction of largest molecular weight (estimated at several million) and contained subunits in the ratio of 3 HMW-GS to 3.4 B-type-LMW-GS to 1.7 C-type-LMW-GS. Glutenin II, SDS-soluble and 70% ethanol-insoluble, had the same ratio of subunits but was of lower molecular weight than glutenin I. Glutenin III, SDS-soluble and 70% ethanol-soluble, was the lowest molecular weight fraction and contained only LMW-GS in a ratio of 3.4 B-type to 1.7 C-type. A good breadmaking wheat variety was reported to contain more glutenins overall, more glutenin I and less glutenin II than a poor quality variety.

Hamer et al (1992) reported that breadmaking quality was not only governed by the quality of the HMW-GS, but also by other factors, such as the amount of glutenin proteins. Others had previously suggested that, in addition to HMW-GS quality and composition, quantity of glutenin or HMW-GS was also important (MacRitchie, 1987; Ng

et al, 1989). Predictive models based only on HMW-GS composition alone showed no correlation (Hamer et al, 1992). Expanding these models to include other parameters related to breadmaking quality improved the positive correlation between predicted and measured loaf volume. However, much of the variation could still not be accounted for with these models, leading Hamer et al (1992) to speculate on the relative importance of other factors, including amount of glutenin proteins, protein composition, and gliadins.

Despite the fact that Kolster and Vereijken (1993) could only explain 20% of the variation in quality by differences in HMW-GS composition, the authors considered this level of variation caused by HMW-GSs to be of interest. In addition to ranking the HMW-GSs according to their effects on quality, Kolster and Vereijken (1993) also reported interactions between alleles. Such interactions would explain the lack of consistent effectiveness of scoring systems which are based on additive effects. At a constant number of HMW-GSs or at an identical HMW-GS composition, variation of the level of expression was reported. The presence of Glu-A1 alleles (1 or 2*) appeared to increase the proportion of subunits produced. And the Glu-B1 alleles were ranked according to level of expression: (7+9) = (7+8) > 7 = (6+8). The ranking of Glu-B1 alleles with respect to quantity produced was reported to be identical to their ranking for quality (Kolster, 1992). In contrast, different Glu-D1 alleles (eg. 5+10 or 2+12) produced identical amounts of subunits. The authors concluded that breadmaking quality was influenced by quantity (amount of HMW-GS) as well as quality (HMW-GS composition). Thus, the superior quality of alleles produced by Glu-A1 were probably a result of an increased proportion of HMW-GSs. Both quantity and quality appeared to be involved in the alleles of Glu-B1. And the Glu-D1 alleles appeared to exert their effect solely by intrinsic quality.

Recent studies lend further support for the importance of quantity as well as quality of HMW-GSs (Gupta and MacRitchie, 1994; Gupta et al, 1994). Alleles were

similarly ranked, additive and epistatic effects were reported, and the LMW-GSs were observed to affect quality (Gupta et al, 1994). These observations supported the conclusion by Hamer et al (1992) that Glu-1 scores, or HMW-GS composition alone, were insufficient to account for quality differences between bread wheat varieties. Allelic effects on dough strength were attributed to variation in (i) the quantity and size distribution of polymeric protein, and (ii) the amount or types of subunits produced. The relative size distribution of the polymeric protein was in turn governed by (i) the ratio of HMW-GS:LMW-GS, which depends on the quantity of individual subunits, and (ii) the polymerizing behavior of these subunits, which may be related to their basic size or structure (Gupta and MacRitchie, 1994).

D. Concluding Statement

Research to date has shown that breadmaking quality is influenced by flour protein content and protein quality. It is generally accepted that the quality factor resides with the glutenin protein, although the importance of non-glutenin proteins cannot be ignored. Glutenin quality has been related to solubility and molecular weight distribution, which in turn is related to specific HMW-GSs, HMW-GS composition, the ratio of HMW-GS:LMW-GS, specific LMW-GS alleles, and the quantity of these proteins. The positive correlation reported by Orth and Bushuk (1972) has been supported by many studies over the last two decades. Although the AAS and AAI glutenins correlate with loaf volume, the structural differences between the two fractions have not been determined. Thus, the objective of this study was to determine which, if any, of the physicochemical properties of glutenin, related to breadmaking quality, could explain the intervarietal variation in solubility of glutenins in 0.05M acetic acid solution.

III. MATERIALS

A. Wheat Samples

Sound grain of Katepwa, a cultivar of the CWRS class, and Glenlea, a cultivar of the CWES class, of the 1991 crop was obtained from the Agassiz Seed Farm. The two cultivars were chosen on the basis of their diverse flour strength.

B. Reagents and Chemicals

Acetonitrile and 1-propanol were of HPLC grade and were obtained from Burdick and Jackson (Muskegon, NJ) and BDH, Inc. (Toronto, ON), respectively. Sodium dodecyl sulfate (SDS) was of electrophoresis grade and was obtained from Bio-Rad Laboratories (Hercules, CA). Dithiothreitol was obtained from Calbiochem (LaJolla, CA). Ethanol (95%) was obtained from Commercial Alcohols Inc. (Toronto, ON). Ammonium sulfate, 85% lactic acid, methyl red and sodium chloride were of reagent grade or better and were obtained from Fisher Scientific (Fairlawn, NJ). Aluminium lactate was obtained from Fluka Chemika (Switzerland). Acrylamide was of electrophoresis grade and was obtained from Pharmacia Biotech (Uppsala, Sweden). Hydrogen peroxide (3%) was obtained from a retail pharmacy (P.S.P. DIN 00167703). Kjeldahl catalyst (TiO_2) was obtained from the Department of Plant Science, University of Manitoba (Winnipeg, MB). All other chemicals were of reagent grade or better and were obtained from Mallinckrodt Specialty Chemicals Co. (Paris, KY) or Sigma Chemical Co. (St. Louis, MO). Distilled deionized water (Milli-Q Water System ZD20 115 94; Millipore Corp., Marlborough, MA) was used in all experiments.

IV. METHODS

A. Varietal Purity and Homogeneity Assessment of Wheat Samples

1. Sample Preparation

Wholemeal samples of both wheats, prepared on a Udy Cyclone Mill (Udy Analyzer Co., Boulder, CO) fitted with a 1 mm sieve, were analyzed for purity of gliadin components and HMW-GS by A-PAGE and SDS-PAGE, respectively. The homogeneity of each sample was verified by A-PAGE and SDS-PAGE of 9 single seeds. Samples were prepared according to the method of Ng et al (1988).

2. Electrophoresis

A vertical slab apparatus, described by Ng et al (1988), was used for A-PAGE. Total acrylamide concentration was 6%. Electrophoresis was performed at constant current (50 mA) for 4 h. Gel temperature was maintained at 20°C by circulating water through the apparatus.

SDS-PAGE in the presence of 2-mercaptoethanol was carried out according to the method of Ng et al (1988). An SE 600-15-1.0 dual cooled vertical slab gel electrophoresis unit with 15- or 20-tooth slot formers and 1.5 mm spacers (Hoefer Scientific Instruments, San Fernando, CA) was used. A 3.0% stacking gel and 17.3% resolving gel were used at 10 mA per gel for 19 h. The wheat variety Neepawa, official grading standard of the CWRS class (Ng et al, 1988), was run with PAGE and SDS-PAGE gels as a reference standard.

B. Milling and Sample Preparation

1. Milling

Wheat samples were tempered to 15.5% moisture for 24 h at room temperature and milled into straight grade flour on a Bühler pneumatic laboratory mill (Bühler Bros., Inc., Uzwil, Switzerland). Flour yield was expressed as percent of total recovered products.

Test weight was determined using a 0.5 L Seedburo container (Seedburo Equip. Co., Chicago, IL). The result was reported on an "as is" moisture content basis in kg/hL. Particle size index (PSI), an indirect measure of grain hardness, was determined as follows. Wholemeal, prepared on a Udy Cyclone Mill (Udy Analyzer Co., Boulder, CO) fitted with a 1 mm sieve, was analyzed by NIR according to the AACC standard method 39-70 (AACC, 1983) using a Dickey-John Instalab 800 NIR product analyzer (Auburn, IL). The Falling Number value (FN Co., Huddinge, Sweden) was determined using 7 g flour (14% moisture basis) according to the AACC approved method 56-81B (AACC, 1983).

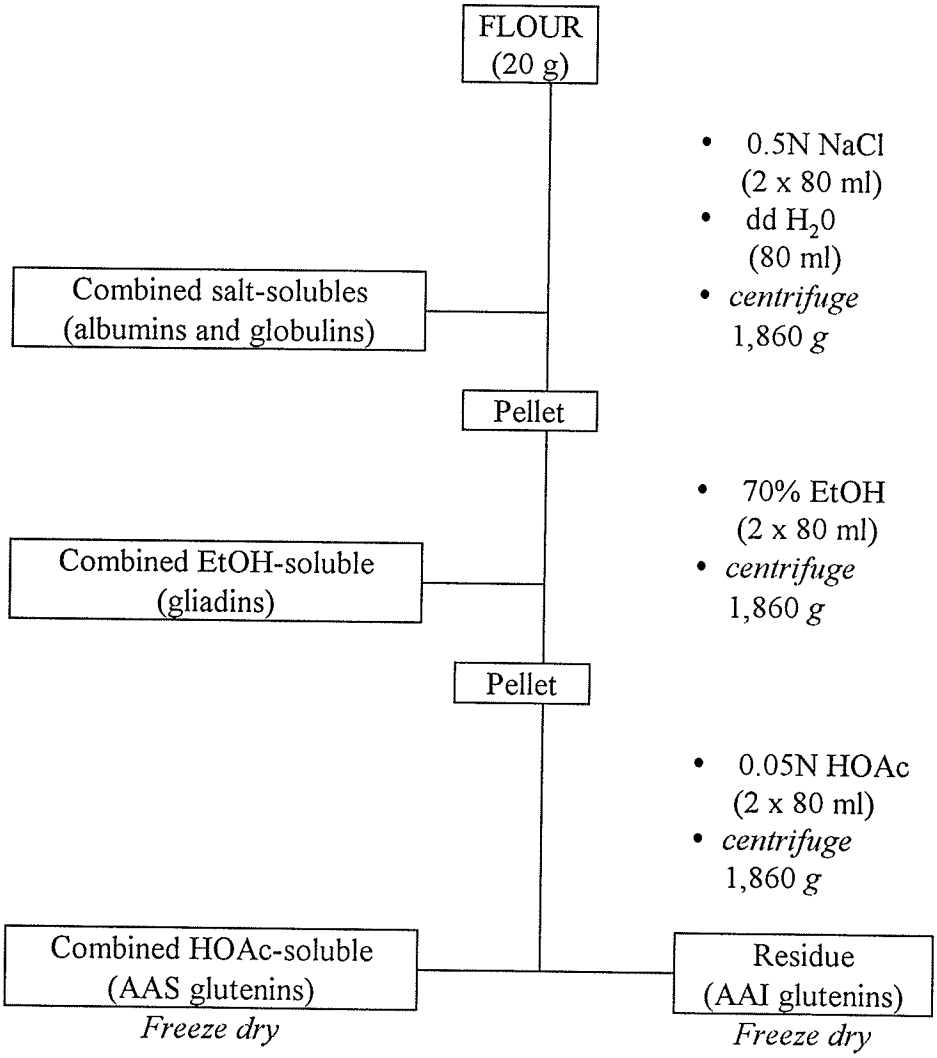
The flours were left at room temperature for two weeks to allow for natural maturing prior to sample preparation. After two weeks, the flours were stored at 4°C.

2. Modified Osborne Fractionation

A flowchart of the fractionation procedure is illustrated in Figure 1. Flour proteins were fractionated into salt soluble (0.5M sodium chloride), alcohol soluble (70% ethanol), acetic acid (0.05M) soluble and insoluble fractions according to the modified Osborne fractionation method of Chen and Bushuk (1970) with some modifications. The salt soluble fraction was not dialyzed to separate the albumins from the globulins. Fractionation was carried out at 4°C and, subsequently, at room temperature (21-23°C). The pellet remaining after the initial extraction with salt solution was cut into small

Figure 1. Flowchart of the modified Osborne fractionation procedure
(Chen and Bushuk, 1970)

✓ add fig. list


FLOUR
(20 g)
• 0.5N NaCl
(2 x 80 ml)
• dd H_2O
(80 ml)
• *centrifuge*
1,860 *g*
Combined salt-solubles
(albumins and globulins)
Pellet
• 70% EtOH
(2 x 80 ml)
• *centrifuge*
1,860 *g*
Combined EtOH-soluble
(gliadins)
Pellet
• 0.05N HOAc
(2 x 80 ml)
• *centrifuge*
1,860 *g*
Combined HOAc-soluble
(AAS glutenins)
Freeze dry
Residue
(AAI glutenins)
Freeze dry

pieces (ca 3 mm^3) to facilitate washing with water and extraction with 70% ethanol. AAS and AAI fractions were freeze-dried (Virtis Freezemobile 6 and Unitop 600L; The Virtis Company, Gardiner, NY) and stored at 4°C.

3. Purification of Acetic Acid Soluble Fraction

Purification of the AAS fraction was required to remove the majority of contaminating gliadins. A modification of the method developed by Fu and Sapirstein (1995) was used for this purpose. The AAS fraction (80 mg protein) was dissolved in 50% 1-propanol (25 ml) and was allowed to stand for 30 min with stirring. The solution was neutralized with 0.1N NaOH. A sufficient volume of 1-propanol was used to quantitatively transfer the solution from a beaker to a centrifuge tube and to produce a final 1-propanol concentration of 70%. The solution was allowed to stand 1 hour then centrifuged at room temperature for 10 min at 20,000 *g* (RC-5C Automatic Superspeed Refrigerated Centrifuge; Sorvall Instruments, DuPont Co., Newtown, CT). The residue was freeze dried and portions of the supernatant were concentrated to dryness in a SpeedVac Concentrator (Savant Instruments, Inc., Farmingdale, NY) for subsequent analyses by electrophoresis and HPLC. Protein content was determined by the micro-Kjeldahl procedure on the liquid supernatant and the freeze-dried residue, and reported on a dry basis.

C. Technological Analyses

1. Farinograph Test

Flour (50 g, 14% moisture basis) was mixed in a 50 g bowl for 15 min at 63 rpm with enough water to yield a maximum dough consistency centered at 500 BU according to the AACC approved method 54-21 (AACC, 1983). Temperature was maintained at 30°C.

2. Mixograph Test

Flour (35 g, 14% moisture basis) was mixed for 10 min at 64% water absorption according to the AACC approved method 54-40A (AACC, 1983).

D. Chemical Analyses

1. Moisture Content of Grain and Flour

A HalRoss moisture meter (Model No.919, Labtronics, Winnipeg, MB) was used to determine the moisture content of whole grain. The moisture content of flour samples was determined by the air oven method according to the AACC approved method 44-15A (AACC, 1983).

2. Ash Content of Flour

The ash content of flour samples was determined according to the AACC approved method 08-01 (AACC, 1983).

3. Protein Content of Grain, Flour and Osborne Fractions

Total nitrogen of wholemeal samples, flour samples and modified Osborne fractions was determined by the micro-Kjeldahl method (AACC 46-13). A factor of 5.7 (Tkachuk, 1969) was used to convert total nitrogen to protein content. The protein content of grain and flour samples was also determined by NIR spectroscopy according to the approved AACC method 39-10 (AACC, 1983).

E. Analyses of Osborne Acetic Acid Soluble and Insoluble Fractions

1. Electrophoresis

A vertical slab apparatus, described by Ng et al (1988) was used for A-PAGE. Total acrylamide concentration was 6%. Electrophoresis was performed at constant

current (50 mA) for 4 h. Gel temperature was maintained at 20°C by circulating water through the apparatus.

SDS-PAGE in the presence of 2-mercaptoethanol was carried out according to the method of Ng et al (1988). An SE 600-15-1.0 dual cooled vertical slab gel electrophoresis unit with 15- or 20-tooth slot formers and 1.5mm spacers (Hoefer Scientific Instruments, San Fernando, CA) was used. A 3.0% stacking gel and 17.3% resolving gel were used at 10 mA per gel for 20 h. Constant amounts of protein were loaded in each lane except for AAI fractions of Figure 9A (A-PAGE; 1.5 fold dilution).

2. Densitometry

Three replicate SDS-PAGE gels of the purified AAS and AAI fractions of Glenlea and Katepwa were scanned at 600 nm with a BioRad Video Densitometer (Model 620) and HP 3396A integrator. Constant amounts of protein were loaded in each lane. Integrated areas obtained for individual HMW-GS peaks and total integrated area were used to calculate the relative proportions of each HMW-GS. The integrated areas for the HMW-GS region and the LMW-GS region were used to calculate the ratio of HMW-GS to LMW-GS.

3. HPLC

Purified AAS and AAI fractions were prepared for HPLC according to the method of Fu and Sapirstein (1995). Flour (50 mg) and AAI fractions (5 mg protein) were extracted with 50% 1-propanol (1 ml) for 30 min, with intermittent vortexing, at room temperature, and centrifuged for 2 min at 8,800 *g*. The extraction was repeated, the supernatants discarded or kept for subsequent analyses, and the pellet washed with 50% 1-propanol. The AAS fractions (5 mg protein) and the pellets obtained from the 50% 1-propanol extracts of flour or AAI fractions were then prepared for HPLC as follows. The samples were reduced with a 0.08M Tris-HCl buffer solution (100-200 µl; pH 7.5) containing 50% 1-propanol and 1% dithiothreitol (DTT) for 1 h at 60°C, with intermittent

vortexing. The reduced protein was then alkylated with an equivalent volume of the 0.08M Tris-HCl buffer solution containing 50% 1-propanol and 14% 4-vinylpyridine for 15 min at 60°C, with intermittent vortexing. The samples were subsequently centrifuged for 5 min at 15,000 *g* and room temperature. The supernatant was syringe-filtered (0.45 μm Millex HV) into a HPLC microvial and sealed.

Samples (5 μl) were immediately analyzed on a HP1090 Liquid Chromatograph with HPLC ChemStation (DOS Series) software (Hewlett Packard), a 15 cm x 4.6 mm Zorbax 300SB-C8 column (300 Å pore size, 5 mm particle size; Chromatographic Specialties Inc., Brockville, ON), and a photodiode array detector. Flow rate was 1 $ml \times min^{-1}$; oven temperature was maintained at 50°C; a solvent gradient program of 23% to 44% acetonitrile in water containing 0.1% trifluoroacetic as organic modifier was used; total run time was 95 min. Chromatograms were recorded from the signal detected at 214 nm. Integrated areas obtained for individual HMW-GS peaks and total integrated area (minus the area attributed to ω-gliadins in the AAS glutenin samples) were used to calculate the relative proportions of each HMW-GS. The sum of the integrated areas for individual HMW-GSs and the area of the LMW-GS region were used to calculate the ratio of HMW-GS to LMW-GS.

F. Statistical Analyses

All statistical analyses were executed on a HP 9000/380 microcomputer using SAS 6.0 software (SAS Institute, 1990). ANOVA and Duncan's Multiple Range Test were performed to determine significant differences between cultivars and fractions.

All experiments were carried out at least in duplicate. Replicated results are reported as means.

V. RESULTS AND DISCUSSION

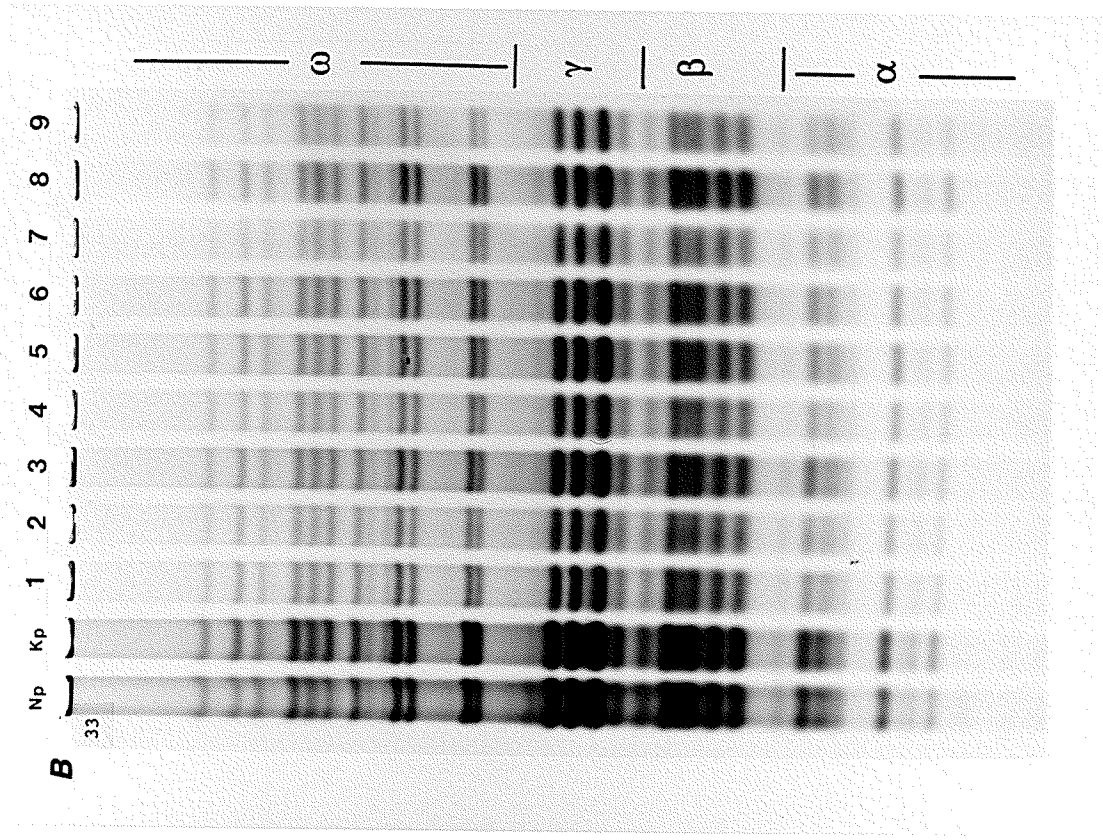
A. Varietal Purity and Homogeneity of Wheat Samples

The A-PAGE and SDS-PAGE electrophoregrams, illustrated in Figures 2 and 3, indicated that the Glenlea and Katepwa samples were pure and homogeneous. The relative mobilities of the α-, β-, γ- and ω- gliadins are indicated in Figure 2A. The slight variation of one of the α-gliadin bands of Glenlea (Figure 2A, lanes 5-8) was attributed to the presence of two biotypes in the grain sample. The HMW-GS region in the SDS-PAGE patterns is indicated in Figure 3A. There are advantages to using Neepawa as the Mr reference protein source. Neepawa flour proteins are similar in chemical and physical structure to the proteins of interest; they cover the entire range of Mrs; and they are inexpensive (Ng and Bushuk, 1987). In order of increasing mobility, the Mrs of the HMW-GS of Neepawa are: 138.2, 128.3, 114.7, 97.4 and 92.4 kDA; the Mrs of several faster moving bands, indicated by lines in Figure 3A-lane Np, are 67.5, 45.2 and 34.6 kDa (Ng and Bushuk, 1989).

B. Milling and Related Analyses

Test weight, particle size index, Falling Number value, and flour yield are reported in Table 1. Test weight, considered a rough index of flour yield, is influenced by kernel shape, uniformity of kernel size and shape, and kernel density. However, above approximately 73.4 kg/hL it has very little influence on flour yield (Halverson and Zeleny, 1988). The test weights for Glenlea (80.3 kg/hL) and Katepwa

Figure 2. A-PAGE electrophoregrams of Glenlea (A) and Katepwa (B) for varietal purity and homogeneity assessment. Np=Neepawa, Gl= Glenlea, Kp=Katepwa, 1-9 = single seeds. Boundaries for α-, β-, γ- and ω-gliadins are indicated according to Bushuk and Sapirstein (1990).


B
33
Np
Kp
1
2
3
4
5
6
7
8
9
ω
γ
β
α

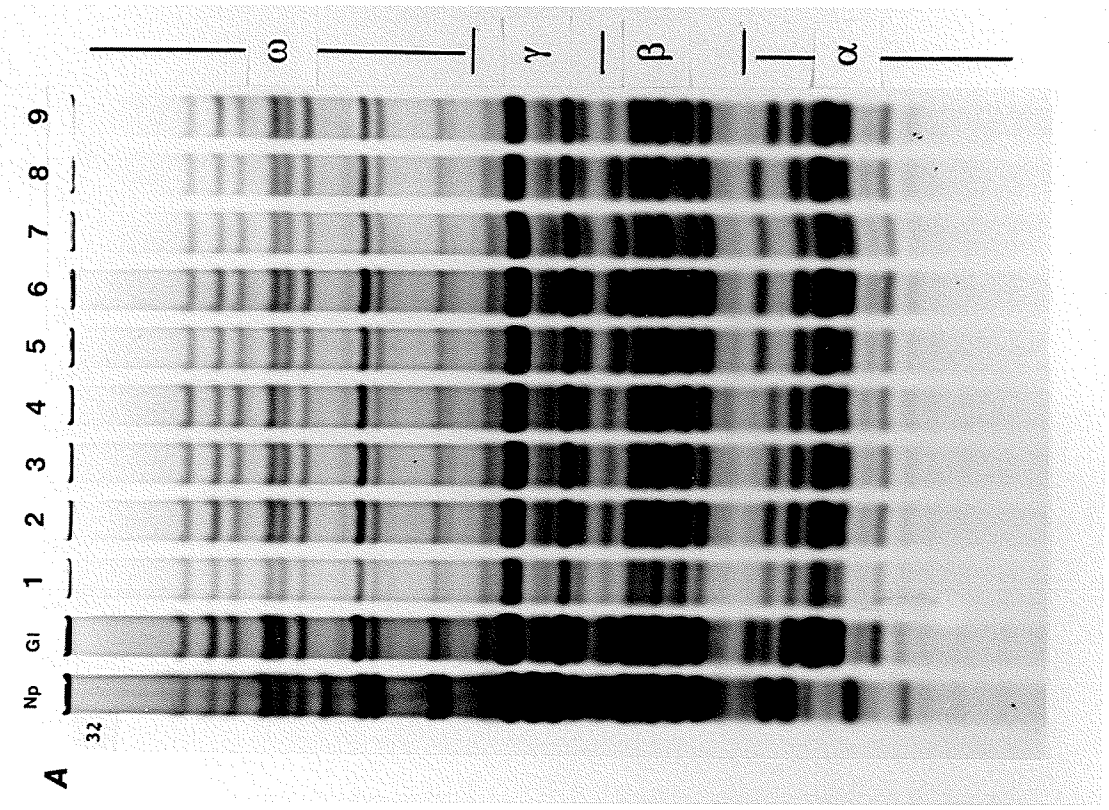
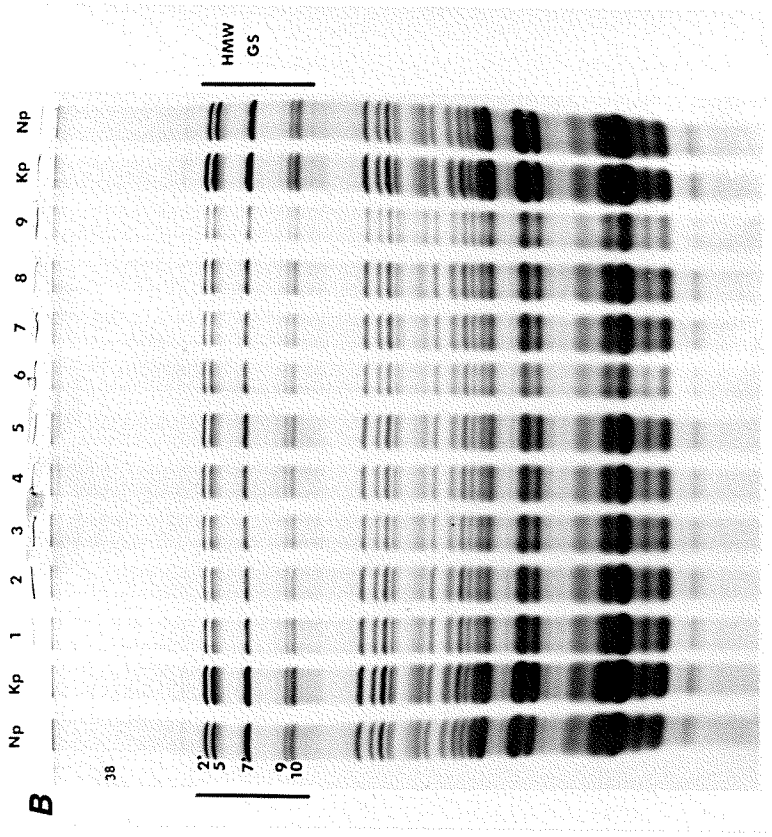

A
32
Np
Gl
1
2
3
4
5
6
7
8
9
ω
γ
β
α

Figure 3. SDS-PAGE electrophoregrams of Glenlea (A) and Katepwa (B) for varietal purity and homogeneity assessment. Np=Neepawa, Gl= Glenlea, Kp=Katepwa, 1-9 = single seeds.


B
38
Np
Kp
1
2
3
4
5
6
7
8
9
Kp
Np
HMW
GS
2*
5
7*
9
10

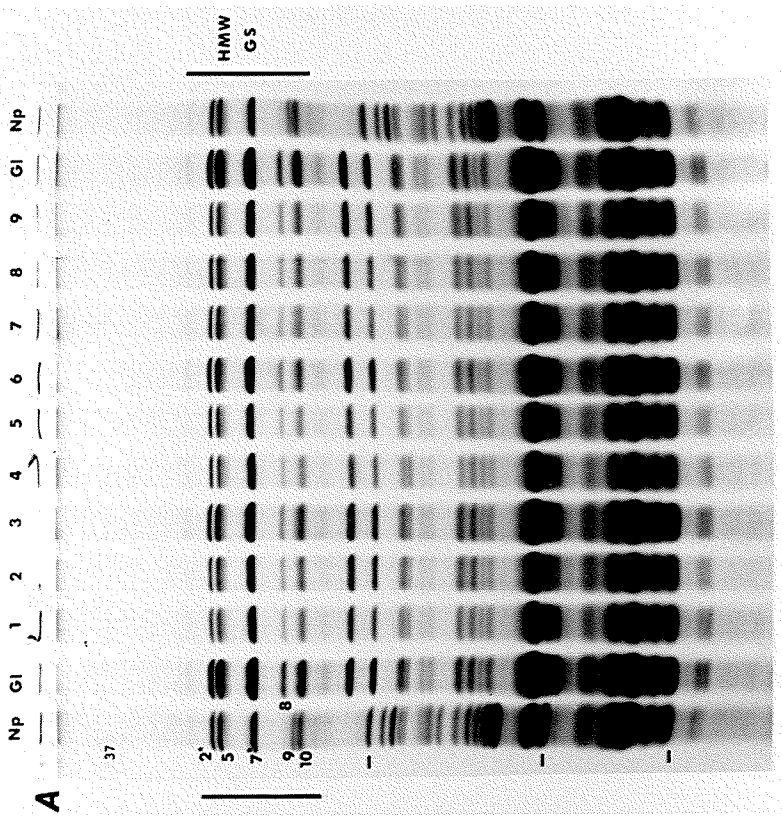

A
37
Np
Gl
1
2
3
4
5
6
7
8
9
Gl
Np
HMW
GS
2*
5
7*
8
9
10

TABLE 1. Test Weight, Particle Size Index, Falling Number Value, and Flour Yield of Glenlea and Katepwa Samples

Wheat	Glenlea	Katepwa
Test Weight (kg/hl)	80.3	79.4
PSI (%)	50.2	49.5
FN Value (s)	484	484
Flour Yield (%)[1]	75.2	72.4

[1] Flour yield as % of total recovered products.

(79.4 kg/hL) were well above this value. PSI values for Glenlea (50.2) and Katepwa (49.5) were similar and in the lower range of hard wheat classes (Williams and Sobering, 1986). FN values were identical (484 s) for both varieties and well above the acceptable range of 200-300 for wheats used in the production of leavened bread (Mailhot and Patton, 1988). Samples were therefore considered to be sound, and suitable for the proposed research.

Protein, moisture, and ash contents of Glenlea and Katepwa flours are reported in Table 2. Protein contents for Glenlea (13.6%) and Katepwa (13.3%) were relatively similar, thus minimizing any effects on analyses due to differences in protein quantity. Protein, moisture and ash contents were within acceptable ranges for bread wheats used for leavened products (Mailhot and Patton, 1988).

Technological tests were performed at the outset to ensure that the samples selected had physical dough properties representative of each variety. Farinograph and mixograph curves are presented in Figures 4 and 5 respectively and reflect the expected characteristics typical of each variety based on experience at the Grain Research Laboratory (Marchylo et al, 1992a) and the University of Manitoba. Farinograph dough development time and mixograph mixing time to peak (Table 3) were clearly longer for the CWES variety Glenlea (23.0, 5.1 min, respectively) than for the CWRS variety Katepwa (6.5, 2.4 min, respectively). Mixing tolerance index and mixograph peak height (Table 3) provided further evidence for the extra strong properties of Glenlea (10 BU, 600 MU versus 15 BU, 580 MU for Katepwa).

TABLE 2. Protein, Moisture and Ash Contents (%) of Glenlea and Katepwa flours *

	Flour	
Determination	Glenlea	Katepwa
Protein	13.6	13.3
Moisture	13.3	13.7
Ash	0.42	0.45

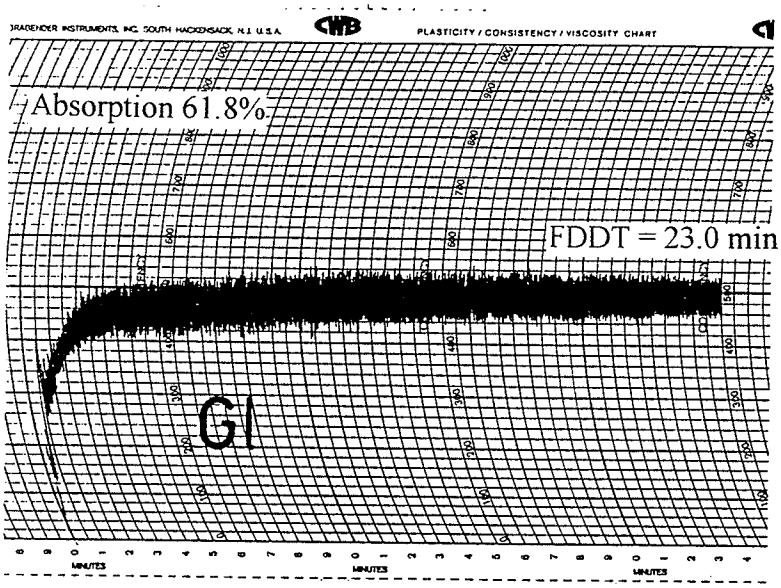
* Values reported on a 14% moisture basis.

TABLE 3. Farinograph and Mixograph Data for Glenlea and Katepwa Flours

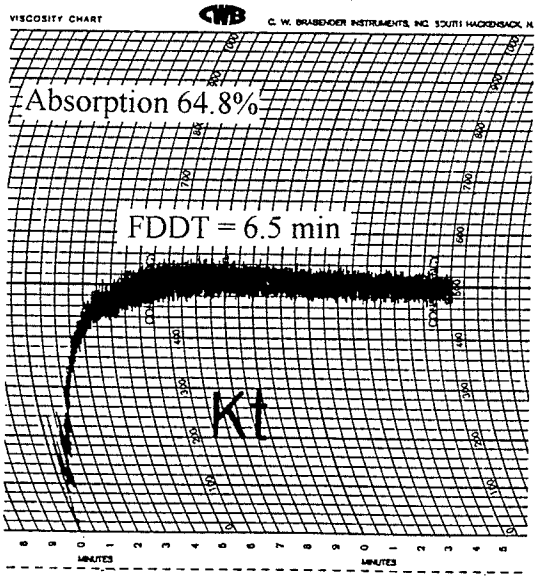
	Glenlea	Katepwa
Farinograph:		
Farinograph absorption (%)	61.8	64.8
Dough development time (min)	23.0	6.5
Mixing tolerance index (BU)	10	15
Mixograph[1]:		
Mixing time to peak (min)	5.1	2.4
Peak height (MU)	600	580

[1] 64% absorption

Figure 4. Farinograph curves for Glenlea (Gl) and Katepwa (Kt)



B
U



Time (minutes)

Figure 5. Mixograph curves for Glenlea (Gl) and Katepwa (Kt)

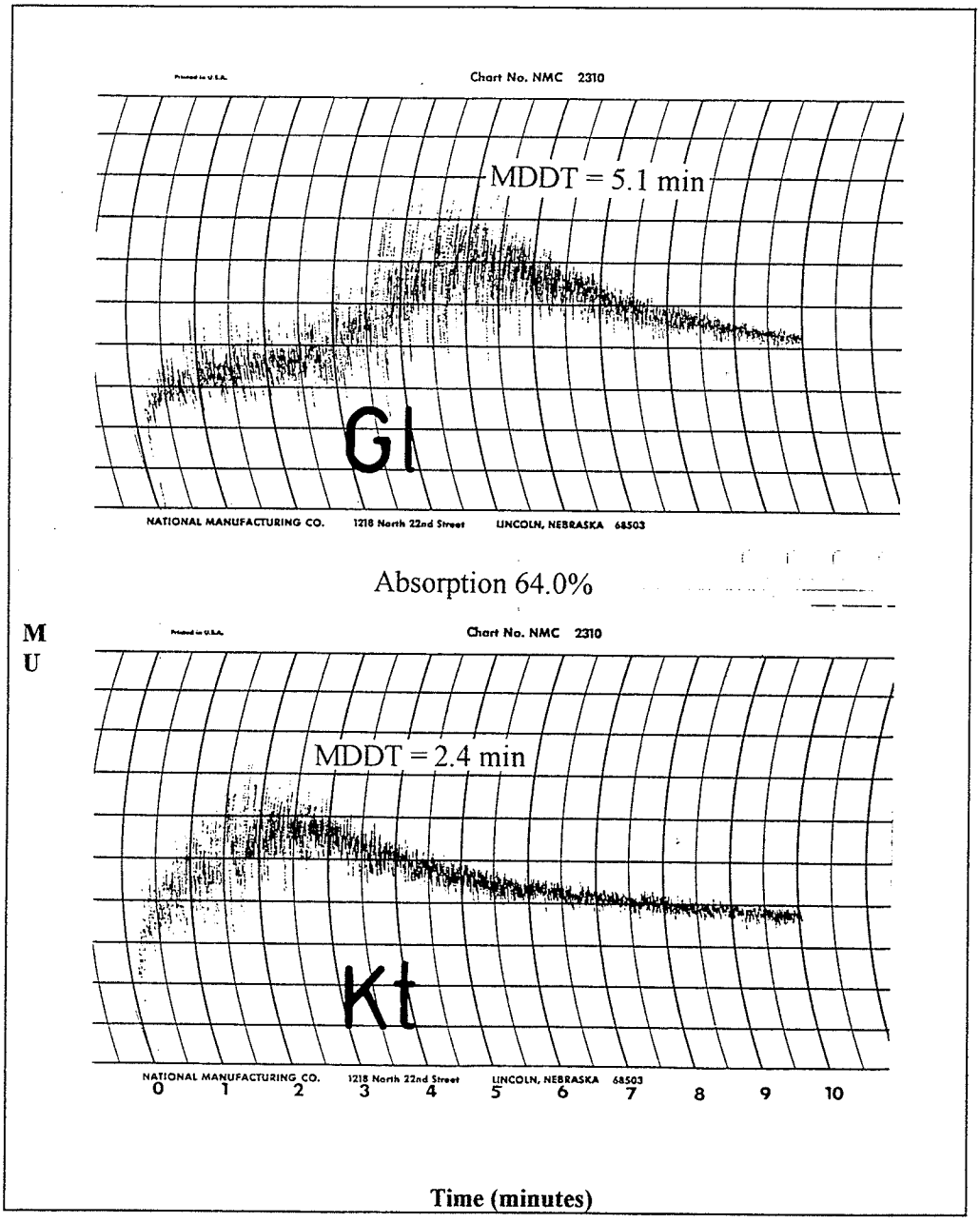


Chart No. NMC 2310
MDDT = 5.1 min
Gl
NATIONAL MANUFACTURING CO. 1218 North 22nd Street LINCOLN, NEBRASKA 68503
Absorption 64.0%
M
U
Chart No. NMC 2310
MDDT = 2.4 min
Kt
NATIONAL MANUFACTURING CO. 1218 North 22nd Street LINCOLN, NEBRASKA 68503
0 1 2 3 4 5 6 7 8 9 10
Time (minutes)

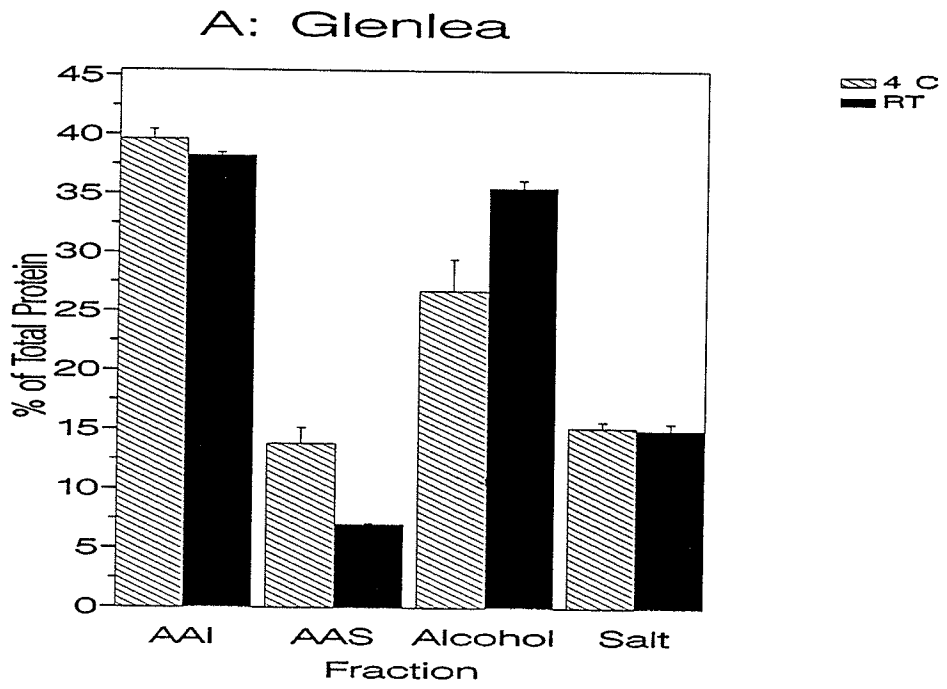
C. Sample Preparation

1. Modified Osborne Fractionation

The modified Osborne fractionation was initially performed at 4°C. However, A-PAGE of the modified Osborne fractions obtained by fractionation at 4°C indicated substantial contamination by gliadins of the AAS fraction (results not shown). Much less contamination was observed in the AAI fraction. The fractionation was thus repeated at room temperature to increase the extractability of gliadins with 70% ethanol (Byers et al, 1983). Comparison of the solubility distributions obtained by fractionation at 4°C and room temperature for each variety (Figures 6 and 7; also tabulated in Tables 4 and 5 in Appendix I) indicated a significant shift of protein away from the AAI and in particular the AAS fractions to the ethanol soluble fraction for both Glenlea and Katepwa with increasing temperature. Analysis of the A-PAGE and SDS-PAGE electrophoregrams (Figures 8 and 9) indicated that gliadins, particularly ω-gliadins, were extracted more efficiently at the higher temperature but still persisted to a great extent in the AAS fraction of both varieties. Katepwa appeared to contain more gliadin contamination than Glenlea in both the AAS and AAI fractions and at both extraction temperatures. However, comparison of the intensities of the HMW-GS bands suggested that the solubility of glutenin in 70% ethanol and 0.05N acetic acid solution was also enhanced at the higher extraction temperature. It is known that reduced and polymeric glutenin are partially soluble in 70% ethanol (Bietz and Wall, 1973; Graybosch and Morris, 1990) and that varietal differences exist (Huebner and Bietz, 1993). Thus, the increased solubility at higher temperature may have resulted in underestimation of glutenin contents in AAS and AAI fractions. The relative amounts of protein in each Osborne solubility fraction are comparable to those obtained by Chakraborty and Khan (1988a).

The problem of overlapping solubilities of wheat protein classes was evident in

Figure 6. Comparison of % total protein for modified Osborne fractions obtained at 4°C and room temperature for Glenlea (A) and Katepwa (B)


A: Glenlea
4 C
RT
% of Total Protein
0
5
10
15
20
25
30
35
40
45
AAI
AAS
Alcohol
Salt
Fraction

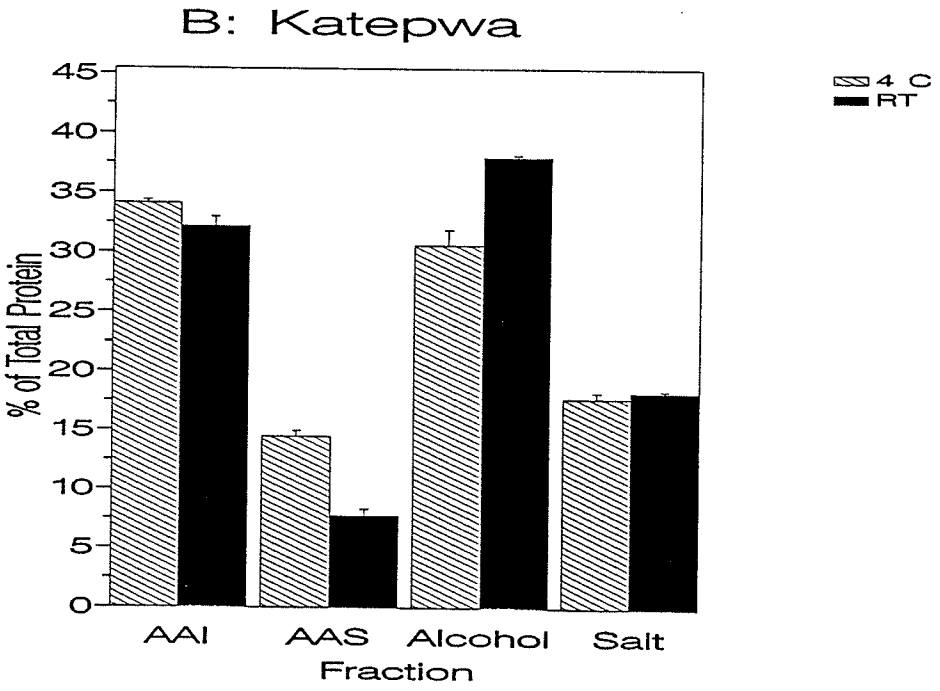
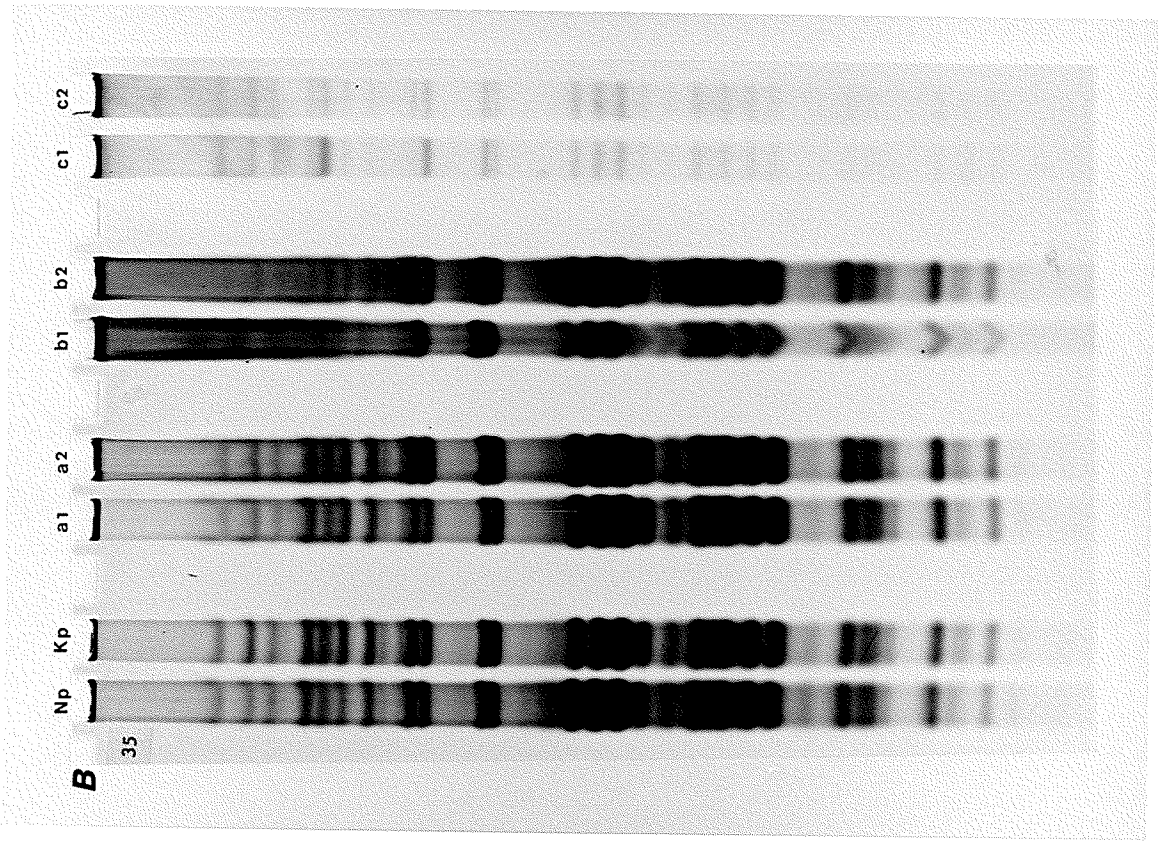

B: Katepwa
4 C
RT
% of Total Protein
0
5
10
15
20
25
30
35
40
45
AAI
AAS
Alcohol
Salt
Fraction

Figure 7. A-PAGE electrophoregrams of modified Osborne fractions of Glenlea (A) and Katepwa (B) obtained at 4°C and room temperature. Np=Neepawa, Gl=Glenlea, Kp=Katepwa, 1 = 4°C, 2 = room temperature, a = ethanol-soluble fraction, b = AAS fraction, c = AAI fraction.


c2
c1
b2
b1
a2
a1
Kp
Np
B
35

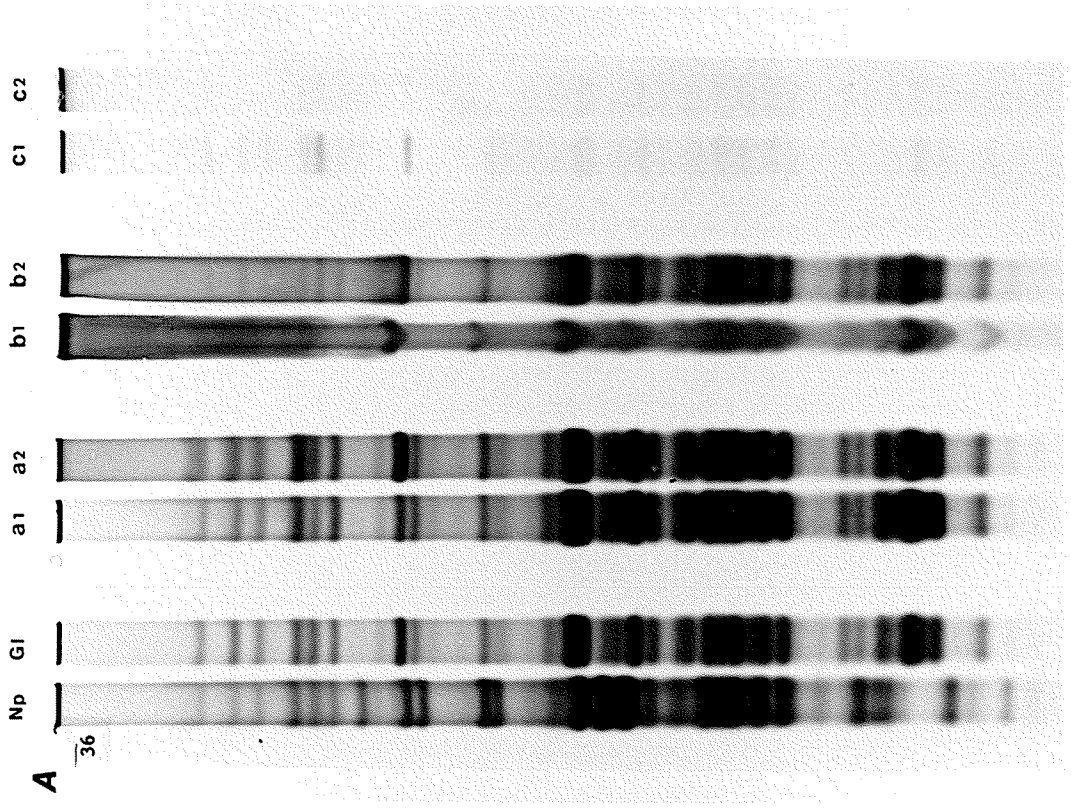

c2
c1
b2
b1
a2
a1
Gl
Np
A
36

Figure 8. SDS-PAGE electrophoregrams of modified Osborne fractions of Glenlea and Katepwa obtained at 4°C and room temperature. Np=Neepawa, Gl= Glenlea, Kp=Katepwa, a = 4°C, b = room temperature, 1 and 4 = ethanol-soluble fraction, 2 and 5 = AAS fraction, 3 and 6 = AAI fraction

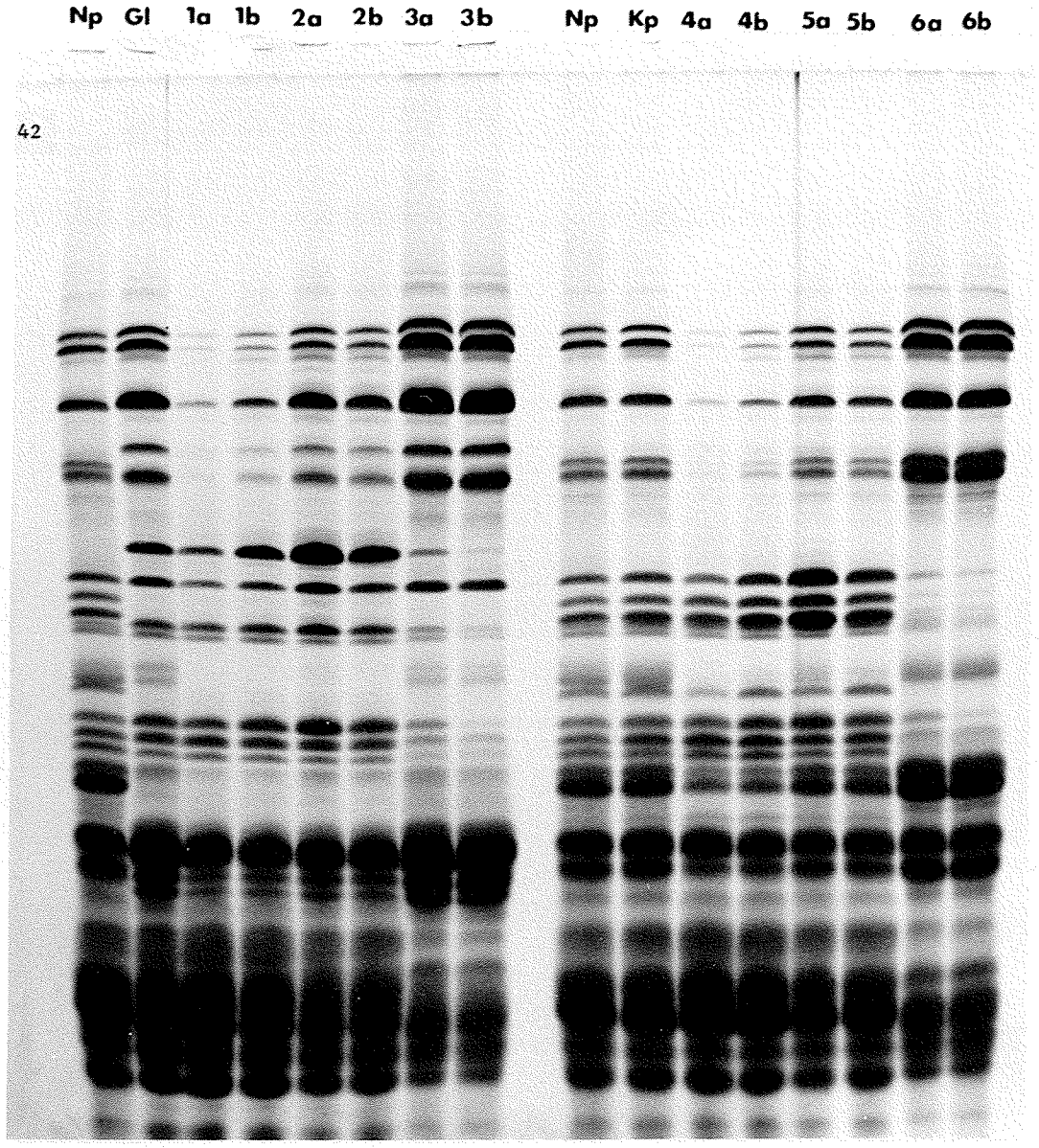

Np
Gl
1a
1b
2a
2b
3a
3b
Np
Kp
4a
4b
5a
5b
6a
6b
42

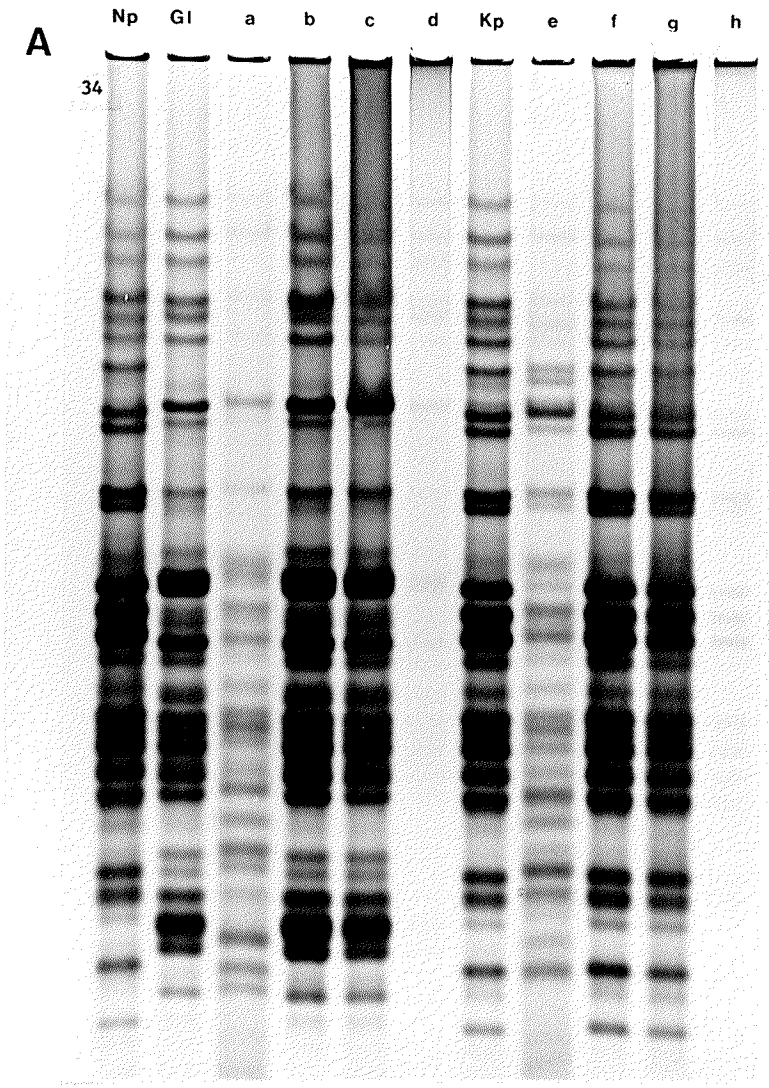
Figure 9. Gliadin bands were present in all modified Osborne fractions and glutenin bands were present in the ethanol soluble, AAS and AAI fractions, illustrating the challenge inherent in quantitation and analysis of total gliadins or glutenins. SDS-PAGE patterns of AAS fractions under non-reducing conditions of both Glenlea (Fig.10, lane a2) and Katepwa (Fig.10, lane c2) clearly showed that gliadin contamination was significant. Hence, despite the improvement due to higher extraction temperature, purification of the AAS fractions was necessary before further electrophoretic/densitometric and HPLC analyses of the glutenins. Comparison of the SDS-PAGE patterns under reducing and non-reducing conditions for the AAS and AAI fractions (Figure 10) indicated that the AAI fractions for both cultivars contained relatively little gliadin contamination. Accordingly, these AAI fractions were analyzed without additional purification. It was noted that Katepwa appeared to have more contamination in its AAI fraction (Fig.10, d2) than did Glenlea (Fig.10, b2).

The protein solubility distributions (Figure 11) were similar in range to other studies using the modified Osborne fractionation (Chakraborty and Khan, 1988a; Orth and Bushuk, 1972). The AAS fraction represented a substantially lower proportion of total protein (5.6-8.7%) when compared to the ethanol soluble (38.1-38.2%) and AAI (29.7-34.6%) fractions. Comparison of the protein solubility distribution between Glenlea and Katepwa indicated a significant varietal difference in the amounts of salt soluble, AAS and AAI protein fractions. There was no significant varietal difference between ethanol soluble fractions. Katepwa contained significantly more salt soluble protein (18.2%) than Glenlea (16.3%). Glenlea contained a significantly lesser amount of AAS protein (5.6%) and a significantly greater amount of AAI protein (34.6%) than Katepwa (8.7% and 29.7%, respectively). These results are in general agreement with those of Orth and Bushuk (1972) and are consistent with the reported correlations of the AAS and AAI

Figure 9. A-PAGE (A) and SDS-PAGE (B) electrophoregrams of room temperature modified Osborne fractions of Glenlea (Gl) and Katepwa (Kp).

(A): A-PAGE : lanes a,e = salt-soluble; b,f = ethanol-soluble; c,g = AAS; d,h = AAI; and Np = Neepawa.

(B): SDS-PAGE: lanes 1,5 = salt-soluble; 2,6 = ethanol-soluble; 3,7 = AAS; 4,8 = AAI; and Np = Neepawa


A
Np
Gl
a
b
c
d
Kp
e
f
g
h
34

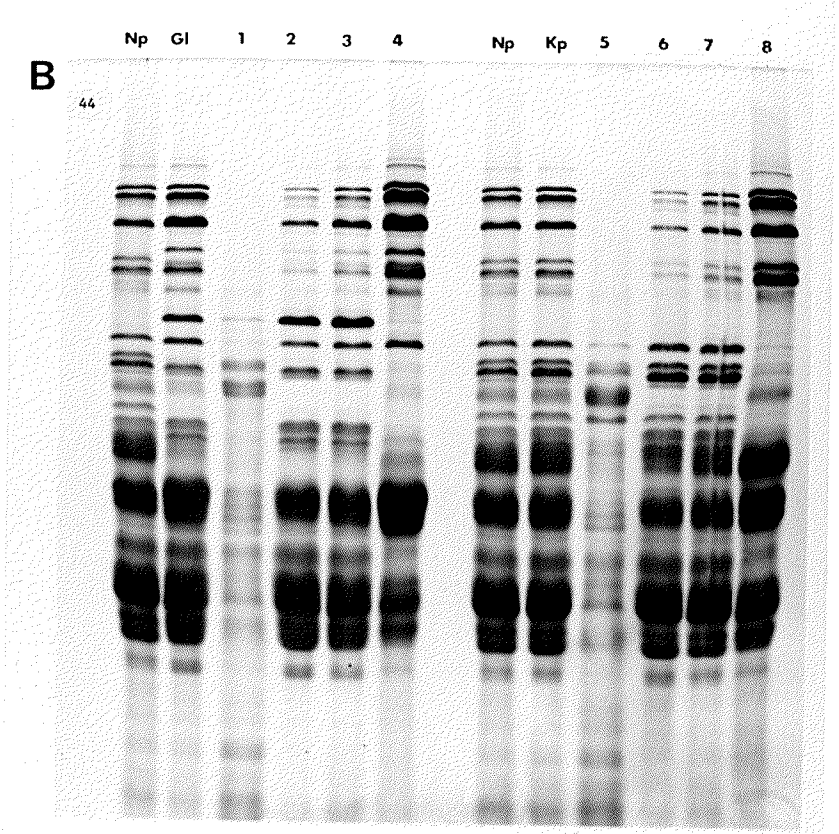

B
Np
Gl
1
2
3
4
Np
Kp
5
6
7
8
44

Figure 10. SDS-PAGE electrophoregram of reduced and unreduced AAS and AAI fractions of Glenlea and Katepwa . Np=Neepawa; Gl=Glenlea; a1,b1 = reduced AAS, AAI fractions; a2,b2 = unreduced AAS, AAI fractions; Kp=Katepwa; c1,d1 = reduced AAS, AAI fractions; c2,d2 = unreduced AAS, AAI fractions

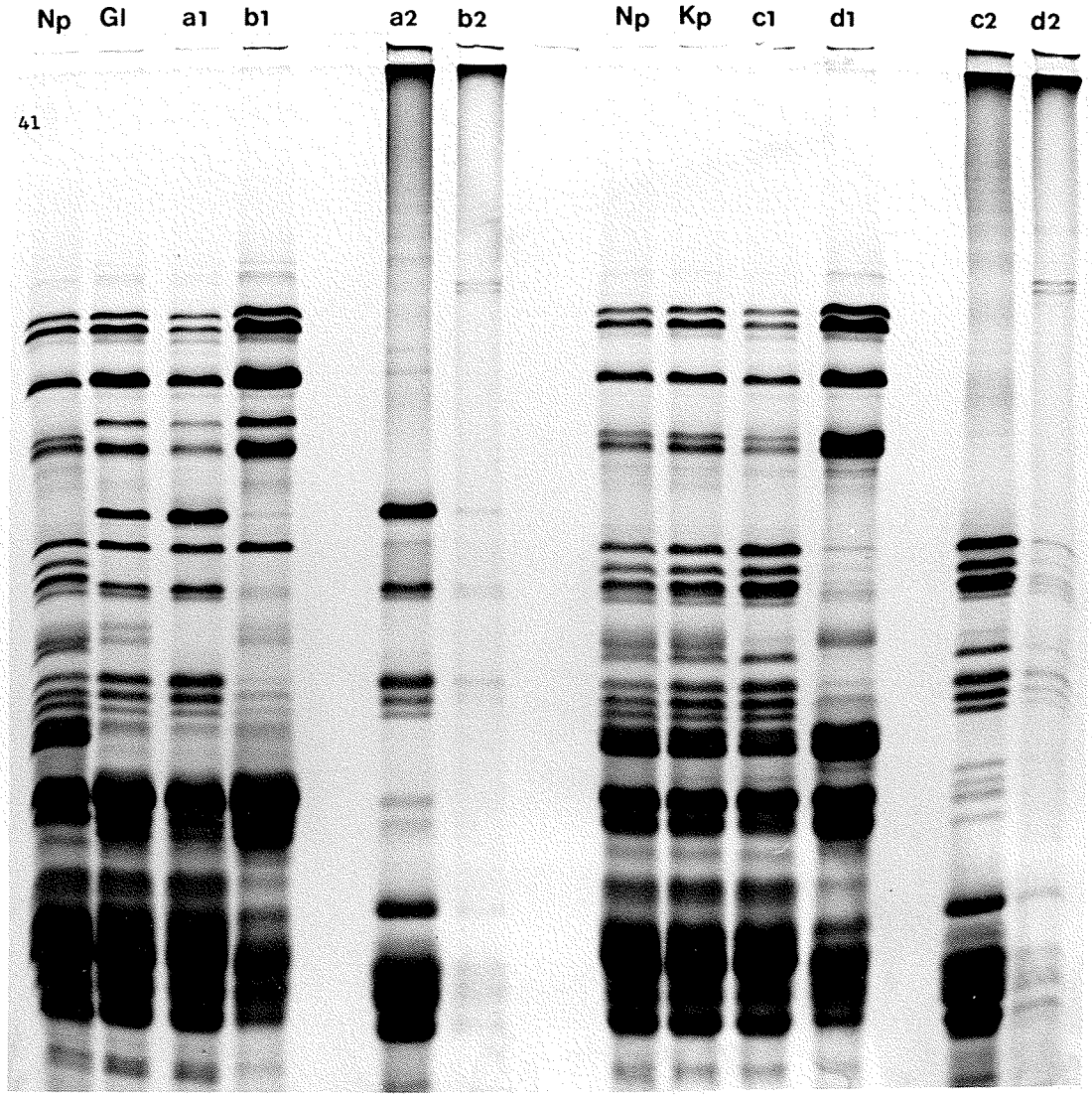
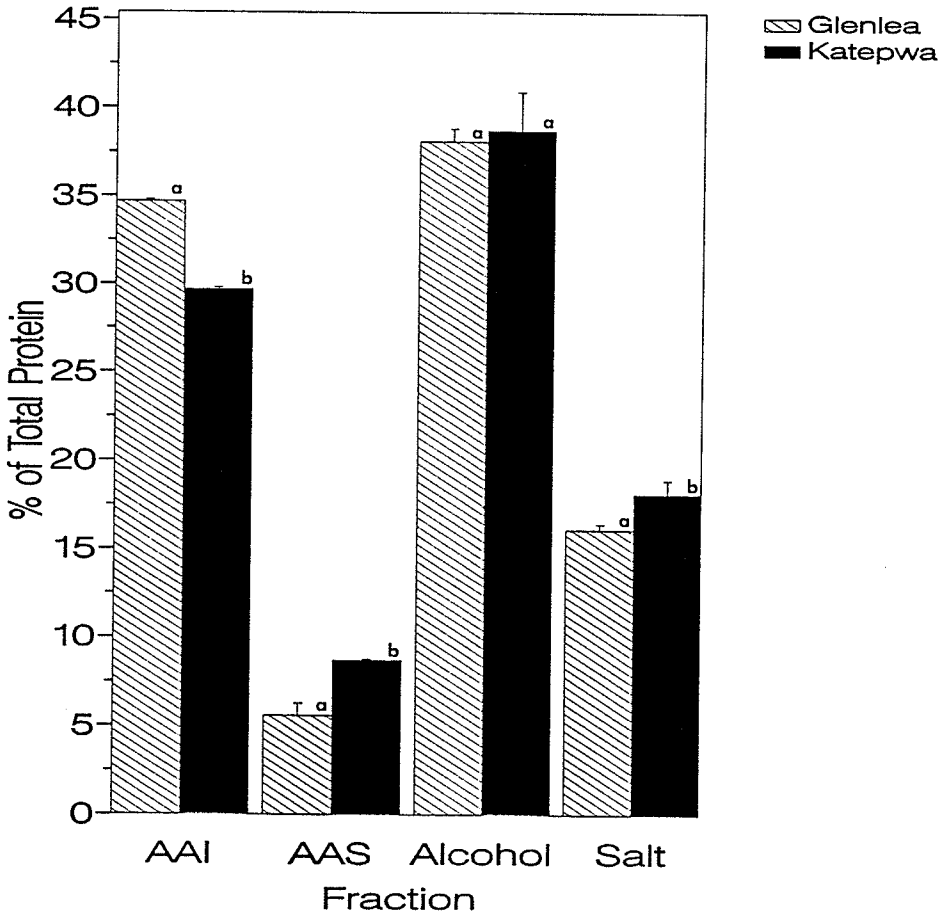

Np
Gl
a1
b1
a2
b2
Np
Kp
c1
d1
c2
d2
41

Figure 11. Comparison of % of total protein for Glenlea and Katepwa flour fractions obtained by modified Osborne fractionation at room temperature.
(Letters within a fraction indicate significant difference; $\alpha = 0.01$)


45
40
35
30
25
20
15
10
5
0
% of Total Protein
a
b
a
b
a
a
a
b
AAI
AAS
Alcohol
Salt
Fraction
Glenlea
Katepwa

fractions with breadmaking potential. The extra strong properties of Glenlea are likely a reflection of its molecular weight distribution being shifted to the higher end (hence lower glutenin solubility), whereas the less strong variety Katepwa probably has a molecular weight distribution that is shifted somewhat lower than Glenlea's. Protein recoveries were very good for both Glenlea (94.6%) and Katepwa (94.8%).

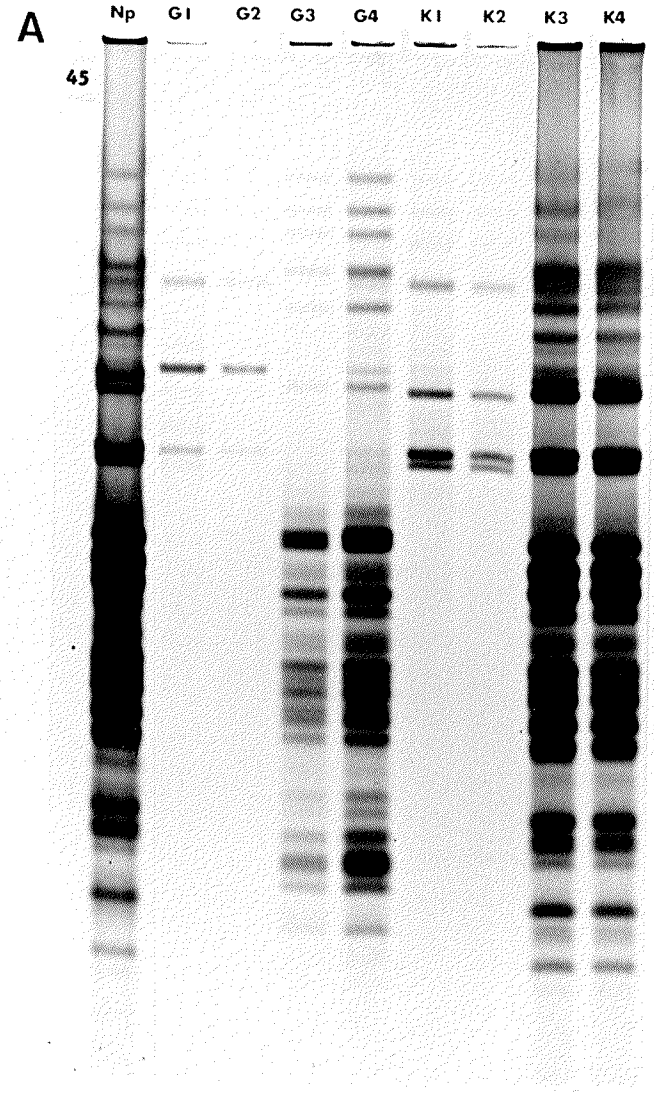
2. Purification of Acetic Acid Soluble Fraction

Burnouf and Bietz (1989) reported that acidic 70% ethanol could solubilize more protein than neutral 70% ethanol. This appeared to be the case for AAS fractions dispersed in 50% and also 70% 1-propanol. The 70% 1-propanol precipitation method of Fu and Sapirstein (1995) required modification due to the low pH (5.2-5.3) of the 50% 1-propanol protein dispersion. The ineffective separation of glutenins and gliadins by precipitation was apparent from the A-PAGE and SDS-PAGE electrophoregrams (Figure 12) of the precipitates and supernatants obtained after 30 min and overnight treatment. Increasing the propanol concentration to 75% was more effective (Figure 13), but glutenin bands were still detected in the SDS-PAGE patterns of the supernatants.

Neutralization of the 50% 1-propanol protein solution prior to 70% 1-propanol precipitation was successful in separating the AAS glutenin polymers from the gliadins. This is evident in the PAGE electrophoregrams (Figure 14) of the precipitates and the supernatants. Precipitation of the neutral 70% 1-propanol supernatant by increasing the propanol concentration to 75% caused precipitation of more ω-gliadins and other gliadins, but there was no evidence of the presence of HMW glutenins (Figure 15).

The purified glutenin obtained by 70% 1-propanol precipitation was freeze-dried and ground to a fine powder with a dental amalgamator (WIG-L-BUG, Chicago, IL) as done previously by others (Gupta and MacRitchie, 1991). Recoveries of protein in the fractionation of the gliadin and glutenin in the AAS fraction were excellent at 99.6% and

Figure 12. A-PAGE (A) and SDS-PAGE (B) electrophoregrams of 70% 1-propanol precipitates and supernatants of AAS fractions of Glenlea and Katepwa. Np=Neepawa; Gl=Glenlea; G1,G2 = overnight, 30 min precipitates; G3,G4 = overnight, 30 min supernatants; Kp=Katepwa; K1,K2 = overnight, 30 min precipitates; K3,K4 = overnight, 30 min supernatants.


A
Np
G1
G2
G3
G4
K1
K2
K3
K4
45

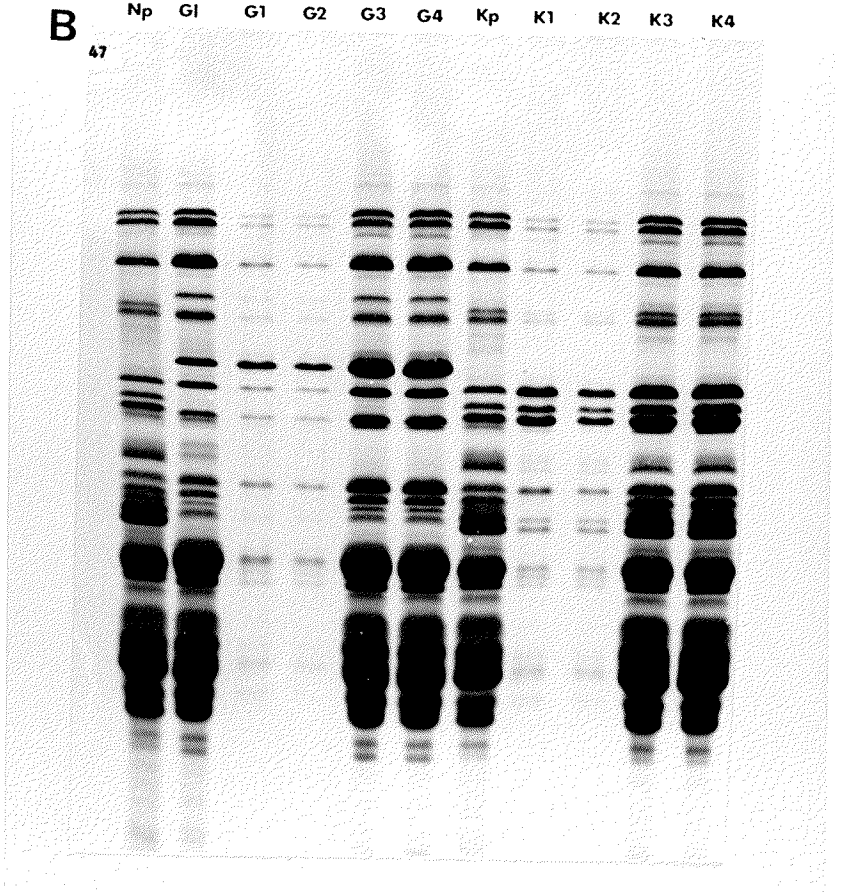

B
Np
GI
G1
G2
G3
G4
Kp
K1
K2
K3
K4
47

Figure 13. A-PAGE (A) and SDS-PAGE (B) electrophoregrams of 75% 1-propanol precipitates (P) and supernatants (S) of AAS fractions of Glenlea (Gl) and Katepwa (Kp)

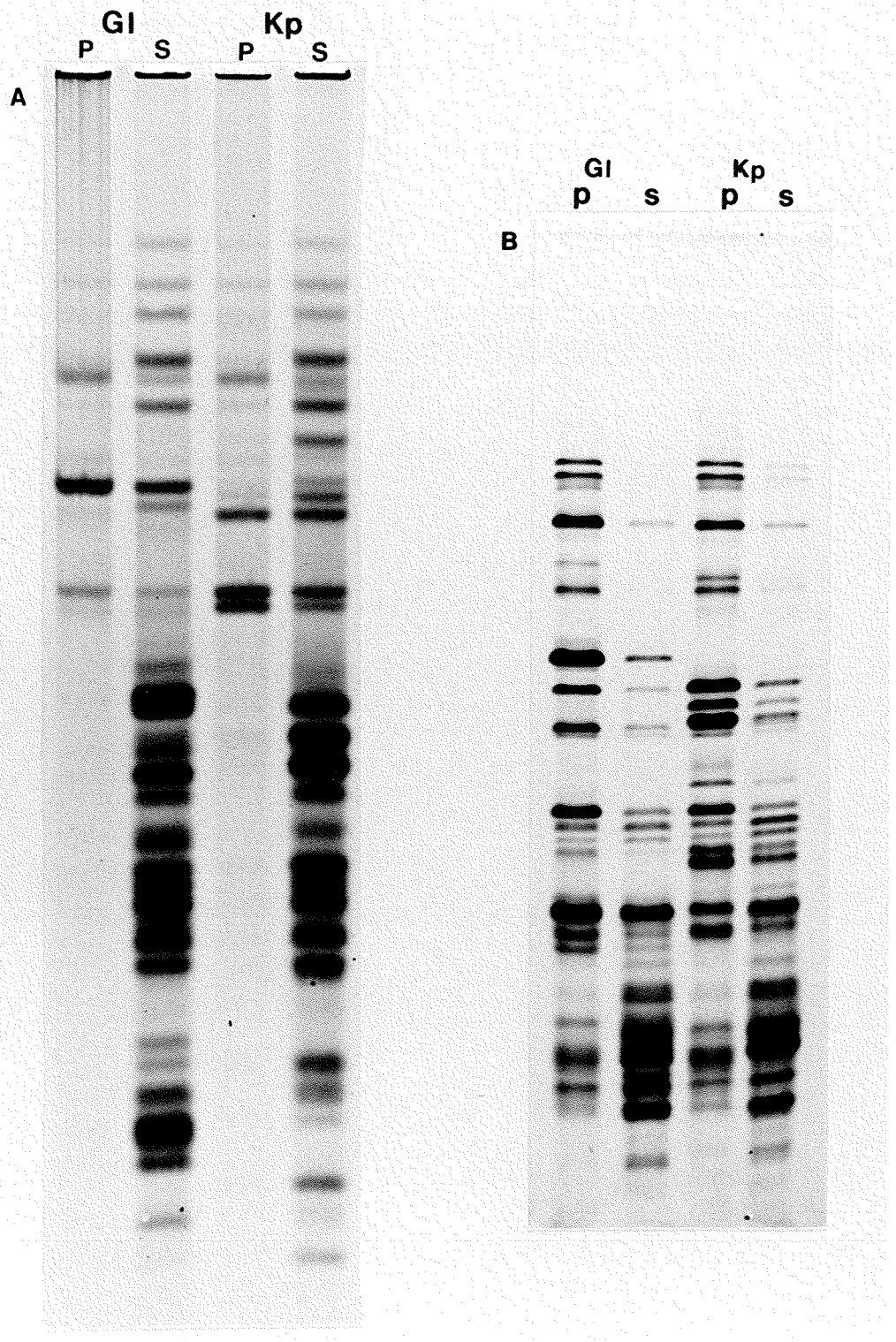

A
Gl
Kp
P
S
P
S
B
Gl
Kp
p
s
p
s

Figure 14. A-PAGE (A) and SDS-PAGE (B) electrophoregrams of 70% 1-propanol precipitates (p) and supernatants (s) of AAS fractions of Glenlea (Gl) and Katepwa (Kp) *after neutralization.*

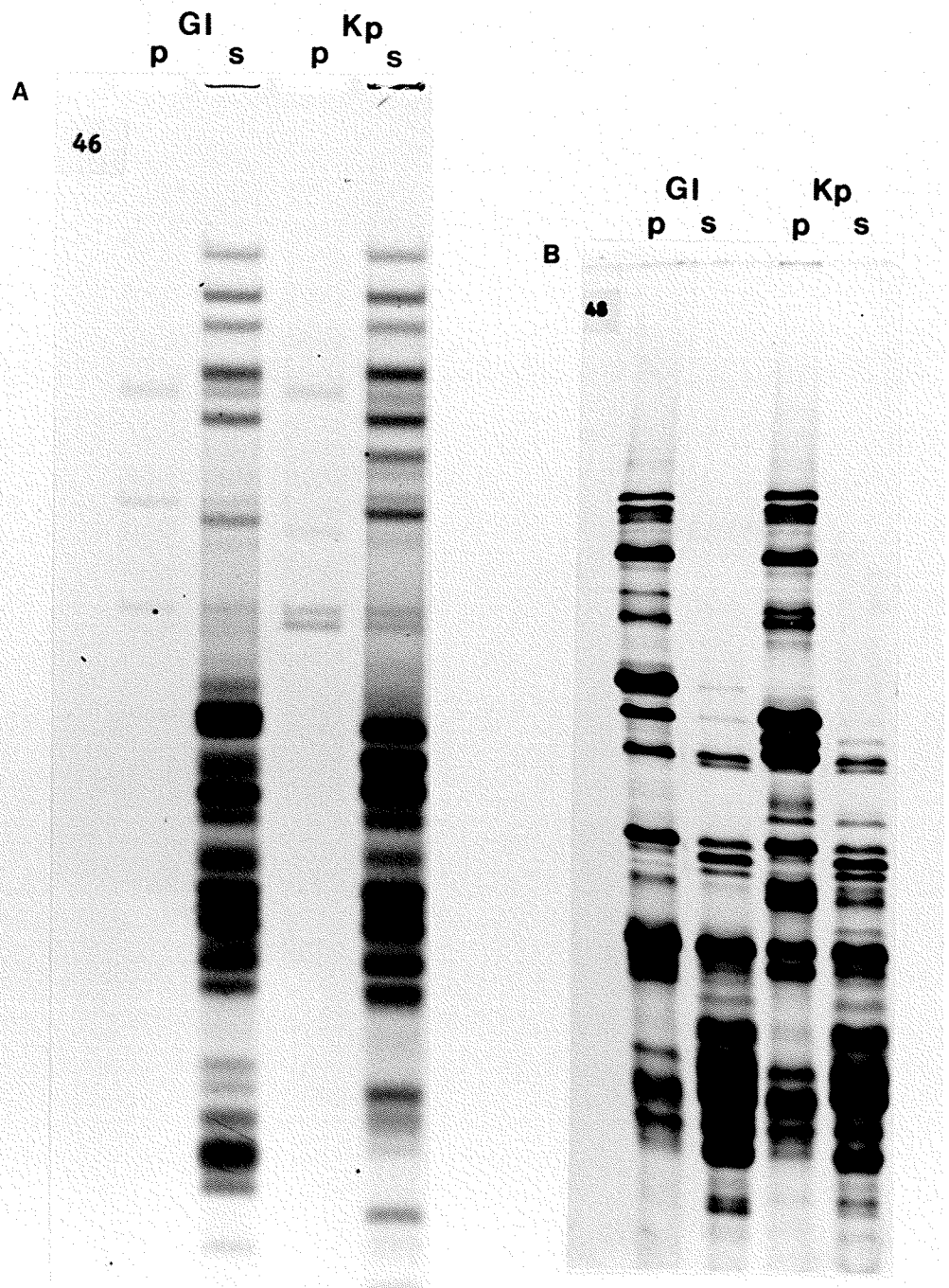
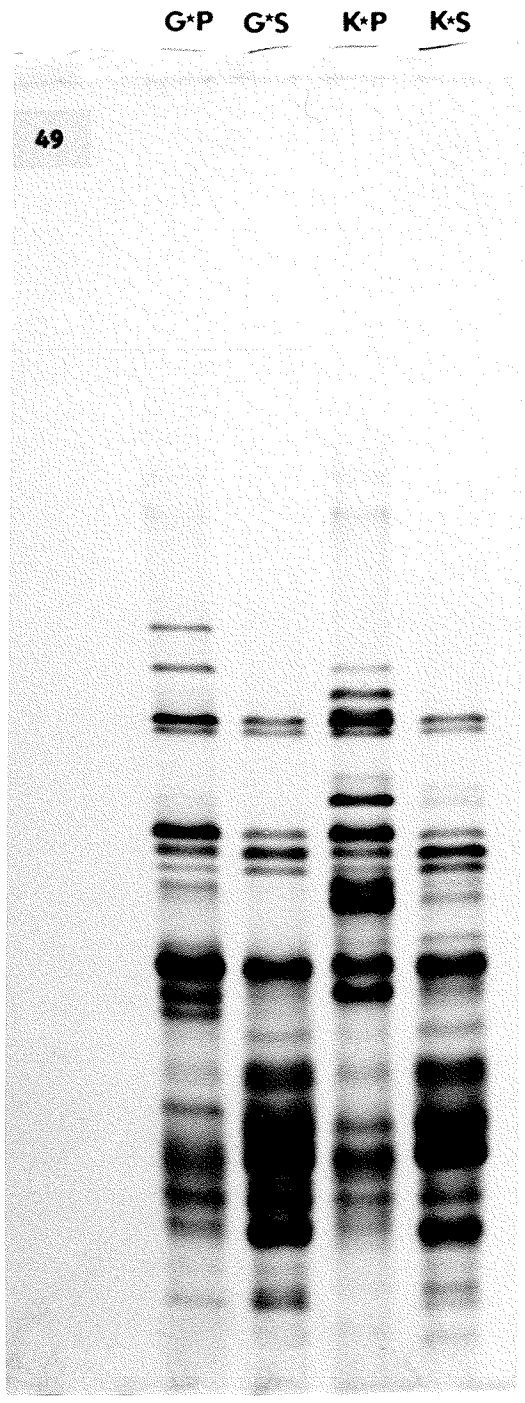

Gl
p s
Kp
p s
A
46
Gl
p s
Kp
p s
B
48

Figure 15. SDS-PAGE electrophoregram of precipitates (-P) and supernatants (-S) of Glenlea (G-) and Katepwa (K-) obtained by increasing the 70% 1-propanol supernatant concentration to 75%


G*P
G*S
K*P
K*S
49

96.9% for Glenlea and Katepwa, respectively (Table 6). Nitrogen determination on the 70% 1-propanol supernatants and the freeze-dried precipitates revealed that Glenlea had significantly more propanol insoluble protein (glutenin) and less soluble protein (gliadin) than did Katepwa (Table 6) on a % basis of total AAS protein. However, when comparing the propanol fractions on the basis of % total flour protein, the cultivars differed only in the percentage of the propanol soluble (gliadin) fraction and not in the percentage of propanol insoluble (glutenin) protein (Table 7).

Figure 16 illustrates the cultivar differences in the AAS fractions. Katepwa contains slightly more glutenin (3.82%) than Glenlea (3.52%) in the AAS fraction, although the difference is not statistically significant. Despite this slightly higher value, Katepwa has less glutenin overall (32.5%) than Glenlea (38.1%), based on total AAS and AAI glutenins. The significant difference in the gliadin portion, however, suggests that the negative correlation of the AAS fraction with loaf volume reported by Orth and Bushuk (1972) may not be entirely related to the relative contents of soluble glutenin. The varying amounts of gliadin may be the real factor causing the negative correlation. The presence of contaminating albumins, globulins and gliadins in the AAS fraction and in other fractions employing different extraction procedures has been reported (Bietz and Wall, 1973; MacRitchie et al, 1991; Orth and Bushuk, 1973a). The weaker wheat cultivar (Katepwa) appears to contain more gliadin that is complexed with glutenin. In the modified Osborne fractionation, this gliadin is not extracted by 70% ethanol but is extracted by 0.05N acetic acid together with some glutenin. In the stronger cultivar, less gliadin is complexed with glutenin and hence relatively more is extracted by the 70% ethanol. Hence, the AAS fraction contains less gliadin. These observed differences in gliadin complexing with glutenin between flours of widely different strength and baking performance may provide a clue to the structure-functionality role of glutenins.

TABLE 6. Percentage Total AAS Protein and % Recovery of AAS Fractions of Glenlea and Katepwa After Precipitation in 70% 1-propanol

Fraction	Glenlea	Katepwa
70%-1-propanol-soluble (%)	36.6 (5.1)a[1]	53.0 (3.6)b
70%-1-propanol-insoluble (%)	63.0 (5.1)a	43.9 (5.4)b
% recovery of AAS protein	99.6	96.9

[1] Values assigned different letters within each fraction are significantly different ($\alpha = 0.01$)
CV within parentheses

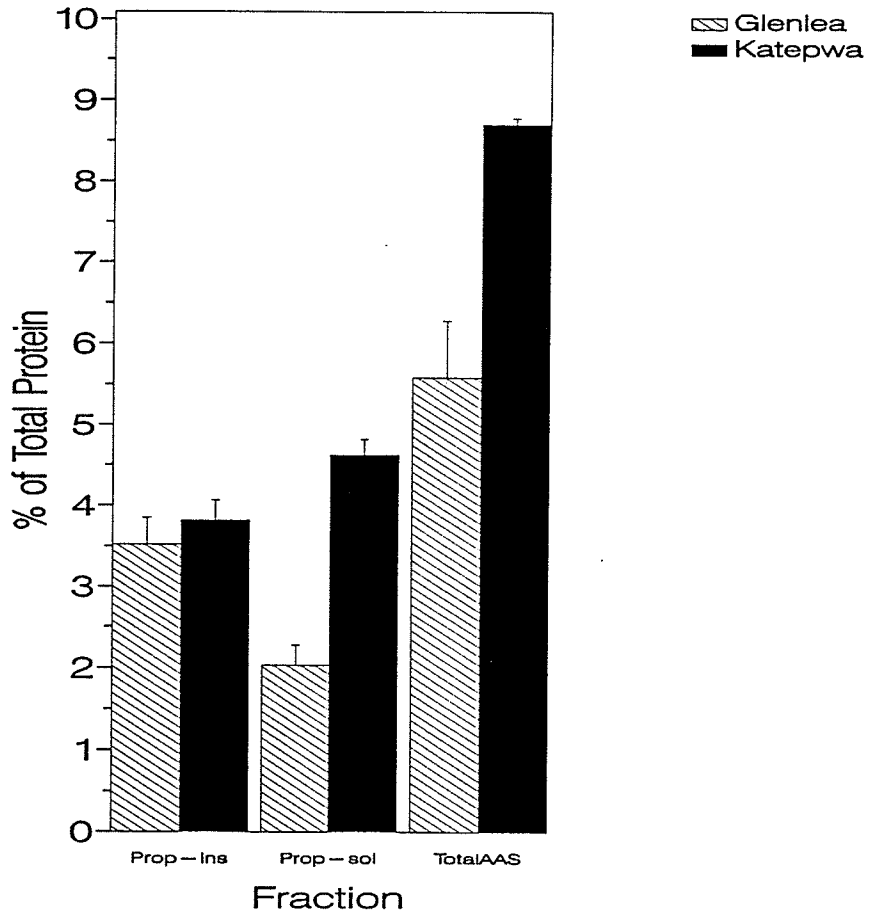
TABLE 7. Percentage Total Flour Protein and % Recovery Represented by 70%-1-propanol Soluble and Insoluble Fractions of AAS Protein from Glenlea and Katepwa

Fraction	Glenlea	Katepwa
70%-1-propanol-insoluble	3.52 (9.4)a[1]	3.82 (6.5)a
70%-1-propanol-soluble	2.04 (11.8)a	4.62 (4.3)b
Total AAS protein	5.58 (12.5)a	8.70 (0.9)b
% recovery	99.6	96.9

[1] Values assigned different letters within each fraction are significantly different ($\alpha = 0.01$)
CV within parentheses

Figure 16. % of total protein represented by 70% 1-propanol-soluble and insoluble fractions of AAS protein of Glenlea and Katepwa

indicate significance in figure


Glenlea
Katepwa
% of Total Protein
0
1
2
3
4
5
6
7
8
9
10
Prop – Ins
Prop – sol
TotalAAS
Fraction

D. Analyses of Acetic Acid Soluble and Insoluble Fractions

1. Electrophoresis/Densitometry

Results of triplicate densitometric analyses (Appendix II) are tabulated in Table 8. Based on these results, there were no significant quantitative differences in specific HMW-GS between fractions or between cultivars, except for HMW-GS 7. Subunit 7 only exhibited a cultivar difference, the content being higher in Glenlea than in Katepwa. Differences between AAS and AAI fractions within each cultivar were not significant. Nor were there any significant differences in the ratios of HMW-GS:LMW-GS between AAS and AAI fractions or between cultivars.

Experimental results clearly indicated substantial variation between replicates, with coefficients of variation ranging from 2.4% to 67.6%, with an average of 17.2%. Variation between replicates may be sufficient to mask actual significant differences between fractions or between cultivars. Many more replicates would be required to improve the accuracy (Kolster et al, 1992). The presence of some ω-gliadins in the SDS-PAGE patterns increased the total area attributed to LMW-GS and thus resulted in an under-estimate of the ratio of HMW-GS to LMW-GS in the AAS fractions. The relative proportions of total HMW-GSs were consistently lower for the AAS fractions when compared to the values obtained by HPLC. Another possible source of error, as pointed out by Kolster et al (1992), is the assumption that the degree of stain binding is constant for all subunits and that staining intensity is linearly related to the amount of protein applied. Relative proportions of HMW-GSs 2* and 10 were consistently lower than values obtained by HPLC and vice versa for subunits 7, 8 and 9.

Despite the lack of significance, certain trends were noted. There appeared to be more of subunit 10 in both Katepwa fractions compared to Glenlea fractions. Of

Table 8. Relative Amounts (%)[1] of HMW-GS and LMW-GS and Ratios of HMW- to LMW- GS in AAS and AAI Fractions of Glenlea and Katepwa Obtained by Densitometry

	Glenlea		Katepwa	
Subunit or Region	AAS	AAI	AAS	AAI
HMW-GS 2* (ns)	15.8 (3.8)	14.6 (10.3)	16.7 (6.0)	14.9 (29.5)
HMW-GS 5 (ns)	19.9 (6.0)	22.1 (5.4)	19.2 (5.2)	22.4 (30.4)
HMW-GS 7	37.2 (4.0)a	35.0 (3.4)ab	29.6 (2.4)bc	24.5 (26.5)c
HMW-GS 8/9 (ns)	9.4 (14.9)	7.9 (7.6)	9.2 (13.0)	8.2 (29.3)
HMW-GS 10 (ns)	17.7 (13.6)	20.4 (19.6)	25.4 (8.3)	29.9 (67.6)
Total HMW-GS (ns)	28.8 (14.9)	38.4 (16.1)	21.6 (19.4)	39.0 (39.2)
LMW-GS region	68.2 (6.6)ab	58.9 (11.2)ab	75.7 (6.3)a	56.9 (25.6)b
Ratio of HMW:LMW-GS (ns)	0.42 (21.4)	0.66 (25.8)	0.29 (24.1)	0.68 (77.9)

[1] Means of triplicates; CV in parentheses;
Values with the same letter are not significantly different (α=0.05); (ns) = no significant difference

particular interest was the observation that both cultivars had greater amounts of subunits 5 and 10 in the AAI fraction than in the AAS fraction. The ratio of HMW-GS to LMW-GS was greater for the AAI fraction of both cultivars.

2. HPLC

Results of trial HPLC analyses of 70% 1-propanol precipitated AAS fractions without neutralization (results not shown), suggested that as the propanol concentration was raised from 50% to 70% in the slightly acidic protein dispersions, differential precipitation of glutenin was occurring. Presumably, the higher Mr polymers precipitated at lower concentrations than the lower Mr polymers. The acidic conditions, even at 70% 1-propanol, prevented the precipitation of the glutenin of lowest Mr. This phenomenon could account for the contrasting ratios of HMW-GS:LMW-GS observed between the HPLC data obtained from AAS fractions prepared without neutralization (results not shown) and the densitometry data.

Agreement between densitometry data and HPLC data was only slightly improved when the HPLC separations were repeated. Peak areas obtained from HPLC chromatograms by integration of neutralized, propanol precipitated AAS-glutenin and AAI fractions are tabulated in Table 9. An example of an integrated chromatogram is included in Appendix III-A. The use of normalized peak areas (i.e. % of total area) is reported to improve precision (Scanlon et al, 1989). Based on hydrophobic interaction chromatography, the ω-gliadins are the most hydrophilic of the gliadins and glutenins (Magnus and Khan, 1992). The ω-gliadins eluted earlier than the HMW-GS and LMW-GS in the HPLC separation of the purified AAS fractions. Thus, their contribution to integrated area was obviated in the HPLC analysis. HPLC results should also provide more accurate results than the densitometric scans, provided that integration specifications are judiciously chosen and samples are successfully and efficiently extracted, reduced and

Table 9. Relative Amounts (%) of HMW-GS and LMW-GS and Ratios of HMW-GS to LMW-GS ~~for~~ in AAS and AAI Fractions of Glenlea and Katepwa Obtained by HPLC[1]

	Glenlea		Katepwa	
Subunit or Region	AAS	AAI	AAS	AAI
HMW-GS 2*	12.4 (1.1)$_a$	11.7 (2.4)$_b$	14.8 (0.0)$_c$	13.1 (2.2)$_d$
HMW-GS 5	18.6 (0.38)$_a$	21.8 (0.65)$_b$	26.2 (4.6)$_c$	24.6 (0.29)$_c$
HMW-GS 7/7*	43.0 (2.6)$_a$	40.4 (0.18)$_b$	29.6 (2.4)$_c$	29.8 (1.4)$_c$
HMW-GS 8/9 (ns)	12.6 (15.2)	11.6 (1.8)	14.1 (3.5)	14.4 (0.49)
HMW-GS 10	13.4 (4.2)$_a$	14.6 (2.9)$_{ab}$	15.6 (5.4)$_b$	18.1 (5.1)$_c$
Total HMW-GS	34.8 (0.61)$_a$	34.5 (0.41)$_a$	35.4 (2.2)$_a$	30.2 (0.94)$_b$
LMW-GS region	65.2 (0.32)$_a$	65.5 (0.22)$_a$	64.6 (1.2)$_a$	69.8 (0.41)$_b$
Ratio of HMW:LMW -GS	0.535 (1.32)$_a$	0.525 (1.35)$_a$	0.545 (3.89)$_a$	0.435 (1.62)$_b$
ω-gliadin (%) in AAS[2]	13.9 (7.1)	n/a	20.8 (7.1)	n/a

[1] Means of duplicates; CV in parentheses

Values with the same letter are not significantly different (α=0.05); (ns) = no significant difference

[2] Exluded from calculations of relative amounts of AAS glutenin

alkylated (Scanlon et al, 1989). In addition, resolution, reproducibility and column stability are reported to be superior for the Zorbax column used in this study. Hydrolysis of the bonded silica phase under high temperature and low pH is minimized with the presence of steric iso-propyl groups (Marchylo et al, 1992b).

The HMW-GSs elute in order of increasing hydrophobicity: 10, 8 (Glenlea) and 9 (Katepwa, Neepawa), 5, 7 (Glenlea) and 7* (Katepwa, Neepawa), and 2* (Kawka et al, 1992; Marchylo et al, 1992a). Subunit 7 of Glenlea is distinguished from subunit 7* of Katepwa because the HPLC retention times are different, despite the similar relative mobilities in SDS-PAGE. Comparison of elution times was not possible because of differences in the conditions used by others.

Same day reproducibility was excellent; no changes in peak retention times or signal patterns were apparent. Qualitative reproducibility between replicates of different days was also good, with only a slight shift in the baseline. Coefficients of variation were generally below 5% for all replicates. HMW-GS 8 in the AAS fraction of Glenlea (CV 15.2%) and HMW-GS 10 in the AAS (CV 5.4%) and AAI (CV 5.1%) fractions of Katepwa were the only exceptions. Significant differences in relative amounts of individual HMW-GSs between the AAS and AAI fractions were found. But the only consistent difference was for HMW-GS 2* which was present in slightly greater quantity in the AAS fraction of Glenlea and Katepwa. Subunit 5 was significantly greater in the AAI fraction of Glenlea but not of Katepwa. Similarly, subunit 7 was significantly greater in the AAS fraction of Glenlea but not of Katepwa. No quantitative differences for subunit 8 and 9 were found between fractions or cultivars. Subunit 10 was significantly greater in the AAI fraction of Katepwa but not of Glenlea. Intercultivar differences were significant for all HMW-GSs except subunits 8 and 9. Katepwa contained significantly greater quantities of HMW-GSs 2*, 5 and 10 in both fractions. Glenlea contained a

significantly larger amount of subunit 7 in both fractions. The ratio of HMW-GS:LMW-GS was similar for all fractions except the AAI fraction of Katepwa, which was substantially lower. Significant differences between HMW-GS content or HMW-GS:LMW-GS ratio were only found between the AAS and AAI fractions of Katepwa.

Comparison of the HPLC data with that obtained from densitometry showed some similarities and differences. Both methods produce similar distribution patterns for the HMW-GS contents in both fractions and both varieties. A portion of the rather consistent variation could be ascribed to the variation in staining intensity of different bands in SDS-PAGE. The higher quantity of HMW-GSs 5 and 10 in the AAI fractions observed in the densitometry data is significant in the HPLC data for Glenlea but only for subunit 10 of Katepwa. The significantly higher amount of subunit 2* in the AAS fraction as determined by HPLC was also apparent in the densitometry data although the difference was not found to be significant. Quantitative differences in individual HMW-GSs between the AAS and AAI fraction were inconsistent and not always significant and hence, precluded conclusions regarding the influence of specific subunits on solubility with any reasonable certainty. The only consistent observation was that HMW-GS 7 (Glenlea) was present in greater amounts than 7* (Katepwa). HPLC data indicated that Katepwa contained significantly greater amounts of subunits 2*, 5 and 10, but densitometry data did not support this observation. Subunits 8 and 9 were quantitatively similar for both varieties and both methods. As for the ratio of HMW-GS:LMW-GS, densitometry indicated higher ratios for the AAI fractions of both varieties, although this difference was not statistically significant. However, HPLC data showed no difference in the ratio between the AAS and AAI fractions of Glenlea and the AAS fraction of Katepwa. The ratio was significantly lower for the AAI fraction of Katepwa only. Thus, on the basis of

the results obtained by HPLC and densitometry the HMW- to LMW-GS ratio could not explain differences in solublity of glutenin in dilute acetic acid.

VI. GENERAL DISCUSSION

The objective of this study was to determine, in terms of fundamental molecular properties, why some wheat glutenin is soluble in 0.05N acetic acid solution while the rest is insoluble in this solvent. This characteristic of glutenin appears relevant to the molecular basis of breadmaking quality since Orth and Bushuk (1972) observed that for a group of 26 wheat cultivars grown in four locations in western Canada, the amount of the soluble glutenin in the flour was inversely correlated with loaf volume (an index of baking quality) whereas the amount of insoluble glutenin was directly correlated.

Consistent with the earlier results of Orth and Bushuk (1972) and more recent results of Chakraborty and Khan (1988a), the present study showed that the weaker flour (Katepwa) contained more AAS protein than the stronger flour (Glenlea), 8.7% and 5.6%, respectively. Also as reported earlier, Katepwa contained less AAI protein than Glenlea, 29.7% and 34.6% respectively. Earlier studies interpreted these results as reflecting a substantive difference in the solubility of glutenin as it was assumed that the preceding extraction of the flour with 70% ethanol solution had removed all of the gliadin and that the residue, which was further fractionated by extraction with acetic acid solution, contained mostly glutenin.

Analysis of the AAS protein in the present study by A-PAGE showed that this fraction contained substantial quantities of gliadin along with glutenin. The amount of gliadin in this fraction appears to be genotype dependent. The actual values obtained were 4.62% and 2.04% for Katepwa and Glenlea, respectively. When the amount of protein in the AAS fraction was adjusted for the gliadin content, the difference in the glutenin

content of the two AAS fractions was relatively small but the fraction of the weaker cultivar Katepwa contained slightly more (3.82%) glutenin than the fraction of the stronger cultivar Glenlea (3.52%). When the amount of glutenin was adjusted for the content of co-precipitated ω-gliadins, this small difference was practically eliminated. On the basis of the small number of samples (two), the difference between the cultivars was not statistically significant. Examination of a larger number of wheat cultivars of more widely diverse quality than that covered by the two samples used in the present study would be helpful in clarifying what appears to be a discrepancy between the published results and the results obtained in this study. The implication of the presence of a genotypically-dependent amount of gliadin in the AAS fraction in the functional properties of flour will be discussed later.

In the context of the objective of this study, the results showed some interesting, albeit not large, differences between AAS and AAI glutenin. SDS-PAGE patterns showed that the two glutenin fractions were the same in terms of presence of bands in the HMW- and LMW-GS regions. Densitometric analysis of the SDS-PAGE patterns showed only minor quantitative differences in the relative amounts of the GSs in the AAS and AAI glutenins. The AAS glutenin contained slightly less subunits 5 and 10 than the AAI glutenin. It should be recalled that these two subunits are controlled by genes on the 1D chromosome and according to several authors (Orth and Bushuk, 1973c; Payne et al, 1981; Ng and Bushuk, 1988), they are especially important to breadmaking quality of wheat. Also, the ratio of HMW-GSs:LMW-GSs calculated from the densitometric data, though not significant, was higher for the AAI fraction than for the AAS fraction for both cultivars. This data is generally consistent with solubility as the glutenin molecules of higher molecular mass would be less soluble.

HPLC comparison of AAS and AAI glutenin showed similar trends to those obtained by SDS-PAGE and densitometry but there were some substantive differences. HPLC results showed that the AAS glutenin of both cultivars contained more of subunit 2* than did the AAI glutenin; densitometry of SDS-PAGE patterns did not show this difference. Differences in the amounts of other HMW-GSs were observed for each cultivar but these were not consistent when the results for the two cultivars were compared. Also, the HPLC ratio of HMW-GSs:LMW-GSs was very similar for the AAS and AAI fractions for Glenlea; the ratio was higher for the AAI fraction than for the AAS fraction for Katepwa. The reason for the discrepancy between the HPLC and SDS-PAGE/densitometry results is not known and warrants further investigation.

In summary, this study has shown that the AAS and AAI glutenins are quite similar in subunit composition and content. Small differences were observed which appear to be consistent with the solubility of the two fractions of glutenin but may not be sufficient to account for the observed difference in solubility in acetic acid solution. It appears therefore that the previously-reported (Orth and Bushuk, 1972; Gupta and MacRitchie, 1991) explanation of the variability in glutenin solubility based on molecular weight is plausible. Proof of this hypothesis must await the development of a method for accurate determination of the molecular weight of polymeric glutenin molecules. Other properties of glutenin such as concentration of intermolecular disulfide crosslinks, secondary and tertiary structure, etc. may contribute to its solubility but there is no clear evidence to support this speculation.

Essentially all of the alcohol-insoluble gliadin was extracted by the 0.05N acetic acid solution used in the next step of fractionation. The alcohol insolubility of some of the gliadin is attributed to its strong aggregation with glutenin. Furthermore, it appears that the proportion of gliadin that is not extracted depends on the genotype. In the present

study, the AAS fraction of Katepwa, the weaker variety, contained substantially more gliadin than the same fraction of Glenlea, the extra strong variety.

The genotype specific aggregation of gliadin and glutenin may have implications in the functional properties of wheat flour doughs during mixing. It is generally believed (see for example Khan et al, 1989; Lonkhuijsen et al, 1992) that during dough mixing, gliadin and glutenin aggregate to form the viscoelastic gluten. The time required for this aggregation (i.e. the dough development time in the mixograph or the farinograph) would depend on the degree of intrinsic interaction between the two proteins in wheat flour. Cultivars that have more aggregated gliadin-glutenin in the flour (i.e. a higher gliadin content in the AAS fraction) would require less mixing for optimum development and vice versa. This explanation is plausible for the results obtained in the present study for two diverse wheat cultivars. It remains to be confirmed by similar experiment on a larger number of wheat cultivars covering a wider range of dough mixing properties.

A possible molecular basis of the large difference in dough mixing properties of the two cultivars used in this study deserves further comment. The two cultivars have the same 1D HMW-GSs, 5 + 10, which are considered more important to breadmaking quality than the other HMW-GSs. Also both have the 1A HMW-GS, 2*. They differ in the 1B HMW-GSs; Glenlea has 7 + 8 whereas Katepwa has 7* + 9. Glenlea contains relatively more of the subunit 7 than Katepwa. Subunit 8 is similar to subunit 9 but is slightly higher in molecular mass. The implication of the small difference in HMW-GS composition in the observed difference in dough properties of the two cultivars studied remains to be discovered by future research.

VII. SUMMARY

1. The present study showed that the weaker flour (Katepwa) contained more AAS protein than the stronger variety (Glenlea). Katepwa also contained less AAI protein than Glenlea. This varietal difference was in accord with research correlating the fractions to breadmaking quality (Orth and Bushuk, 1972, and other references cited herein).

2. There were significant intervarietal differences in gliadin content of the AAS fraction but the amount of glutenin was similar. When the amount of protein in the AAS fraction was corrected for gliadin content and co-precipitated ω-gliadin content, the difference in the glutenin content of the two AAS fractions was relatively small. Further research with a larger number of wheat cultivars of diverse breadmaking quality would help to clarify the discrepancy between published results and the results obtained in this study.

3. Densitometric results showed only minor quantitative differences in the relative amounts of the GSs. The AAS fraction contained slightly less HMW-GSs 5 and 10 than the AAI fraction. The ratio of HMW-GSs:LMW-GSs was higher for the AAI fraction than for the AAS fraction. HPLC results showed similar trends but there were some appreciable differences. HPLC results showed that the AAS glutenin contained more of subunit 2* than did the AAI glutenin. Also, the ratio of HMW-GSs:LMW-GSs was similar in both fractions for Glenlea, but higher in the AAS fraction than in the AAI fraction for Katepwa. The discrepancy in the results obtained between the two methods warrants further investigation.

4. The proportion of HMW-GS 7 in Glenlea was significantly greater than that of 7* in Katepwa. This cultivar difference was observed for both AAS and AAI fractions.

5. This study has shown that the AAS and AAI glutenins are quite similar in subunit composition and content. The small differences observed appear to be consistent with the solubility of the two fractions but may not be sufficient to account for the observed difference in solubility in acetic acid solution.

6. The alcohol insolubility of some of the gliadin appears to be genotype-dependent and is attributed to its strong aggregation with glutenin. Cultivars that have more aggregated gliadin-glutenin (i.e. higher gliadin content in the AAS fraction) would required less mixing time for optimum dough development. Similar experiments on a larger number of wheat cultivars of diverse dough mixing properties would help to confirm this observation.

7. Glenlea and Katepwa have similar HMW-GS compositions. It is plausible that the larger quantity of subunit 7 and the lesser degree of gliadin-glutenin interaction, as defined by gliadin content in the AAS fraction, could account for part of the stronger dough properties of Glenlea. How the small differences in HMW-GS composition, or possible differences in the LMW-GS composition, can give rise to the large difference in dough properties remains to be discovered by further research.

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APPENDICES

APPENDIX I. Solubility Distribution and Recovery from Modified Osborne Fractionation at 4°C and Room Temperature

TABLE 4. Percentage Total Protein* and % Recovery for Glenlea and Katepwa Flour Fractions Obtained by Modified Osborne Fractionation at 4°C

	Flour	
	Glenlea	Katepwa
	% of total protein	% of total protein
Salt-soluble	15.2 (3.3)a	17.7 (2.8)a
Alcohol-soluble	26.8 (10.1)a	30.6 (4.2)b
Acetic acid-soluble	13.8 (9.4)a	14.5 (3.4)b
Residue	39.6 (2.0)a	34.1 (0.9)b
Recovery	95.4	96.9

* Values with the same letter within each fraction are not significantly different (α=0.05)
CV within parentheses

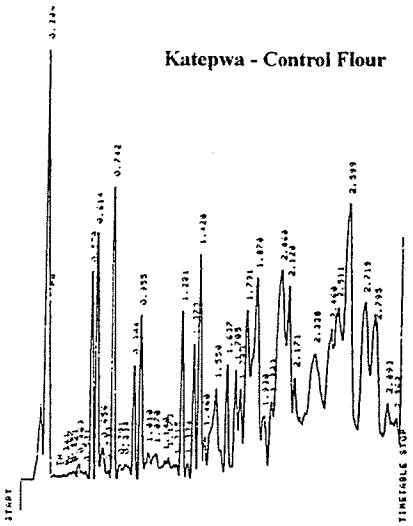
TABLE 5. Percentage Total Protein* and % Recovery for Glenlea and Katepwa Flour Fractions Obtained by Modified Osborne Fractionation at Room Temperature

	Flour	
	Glenlea	Katepwa
Fraction	% of total protein	% of total protein
Salt-soluble	15.0 (4.0)a	18.2 (1.1)a
Alcohol-soluble	35.6 (1.7)a	38.0 (0.5)b
Acetic acid-soluble	7.0 (1.4)a	7.8 (7.7)b
Residue	38.2 (0.8)a	32.2 (2.5)b
Recovery	95.8	96.2

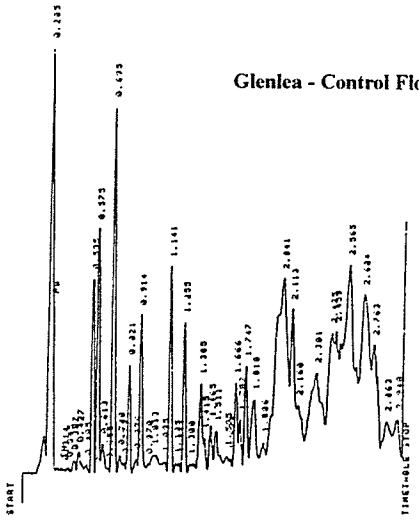
* Values with the same letter within each fraction are not significantly different (α=0.05)
CV within parentheses

APPENDIX II. Densitometric Scans of SDS-PAGE Electrophoregrams - Replicate 1

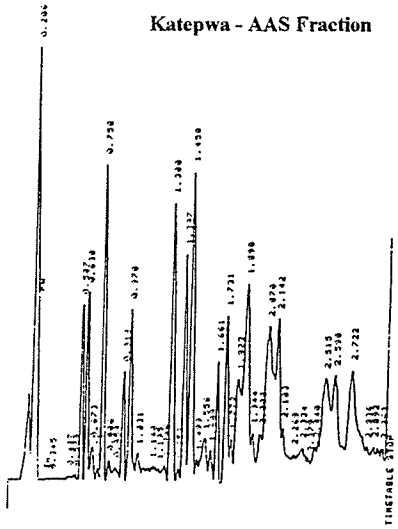
Katepwa - Control Flour



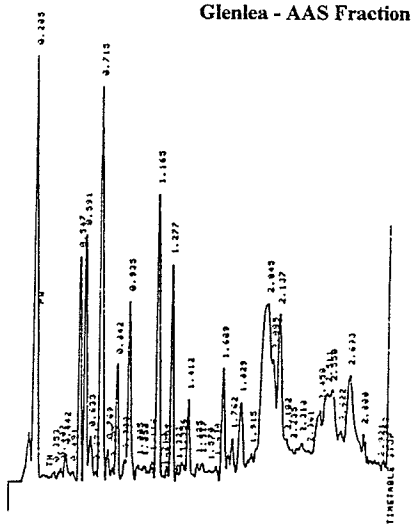
Glenlea - Control Flour



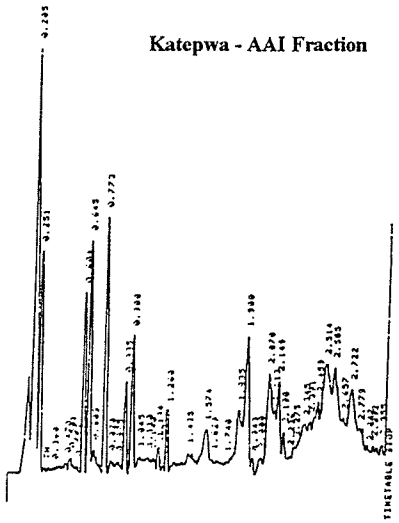
Katepwa - AAS Fraction



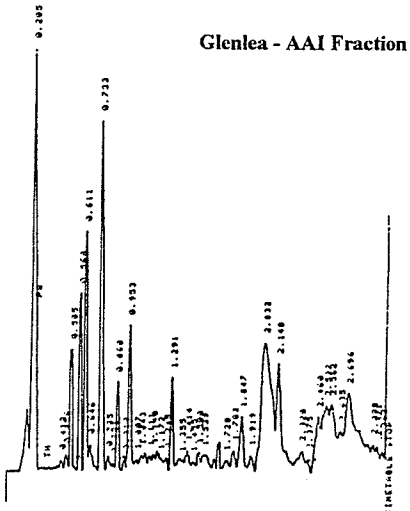
Glenlea - AAS Fraction



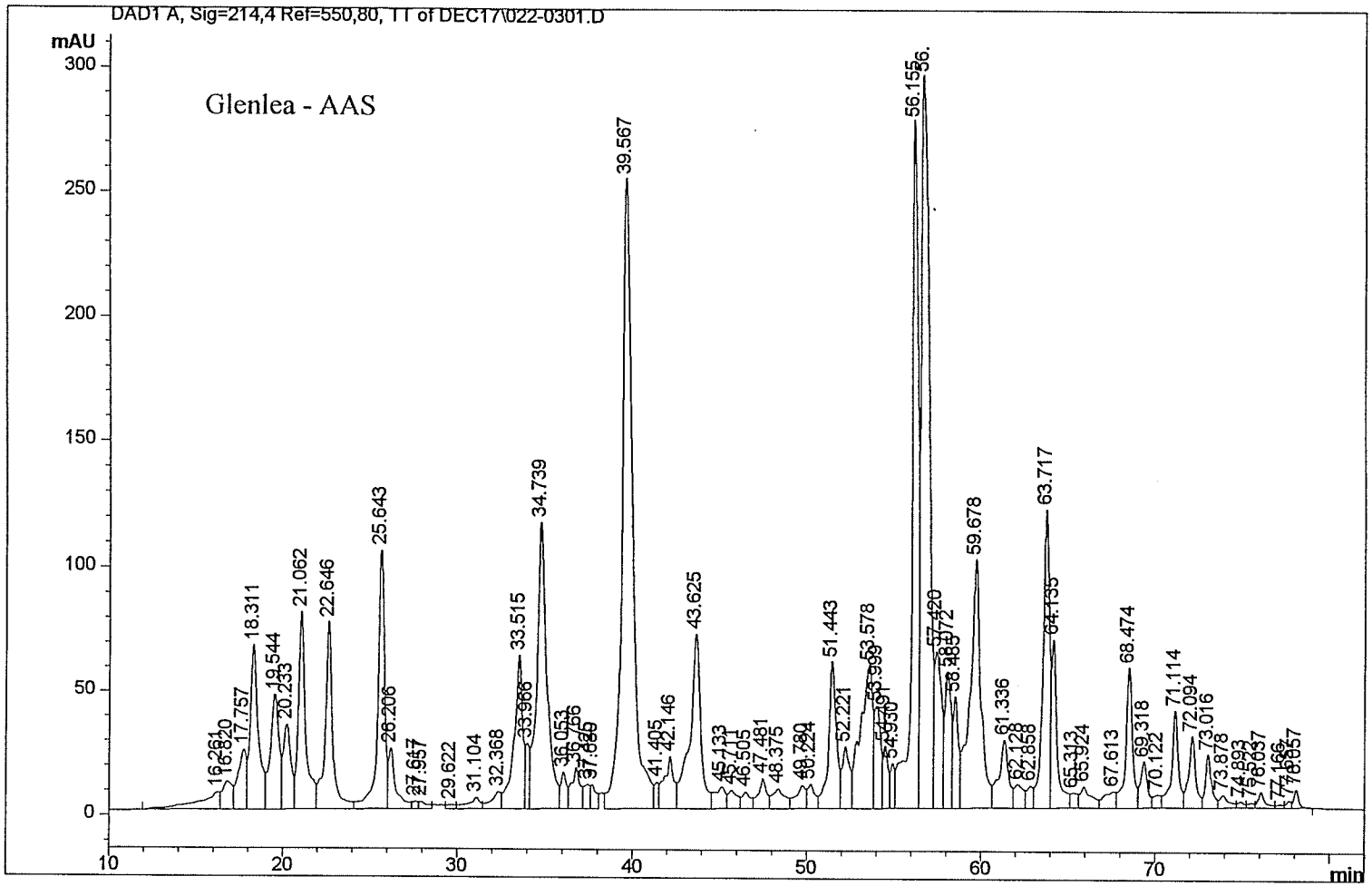
Katepwa - AAI Fraction



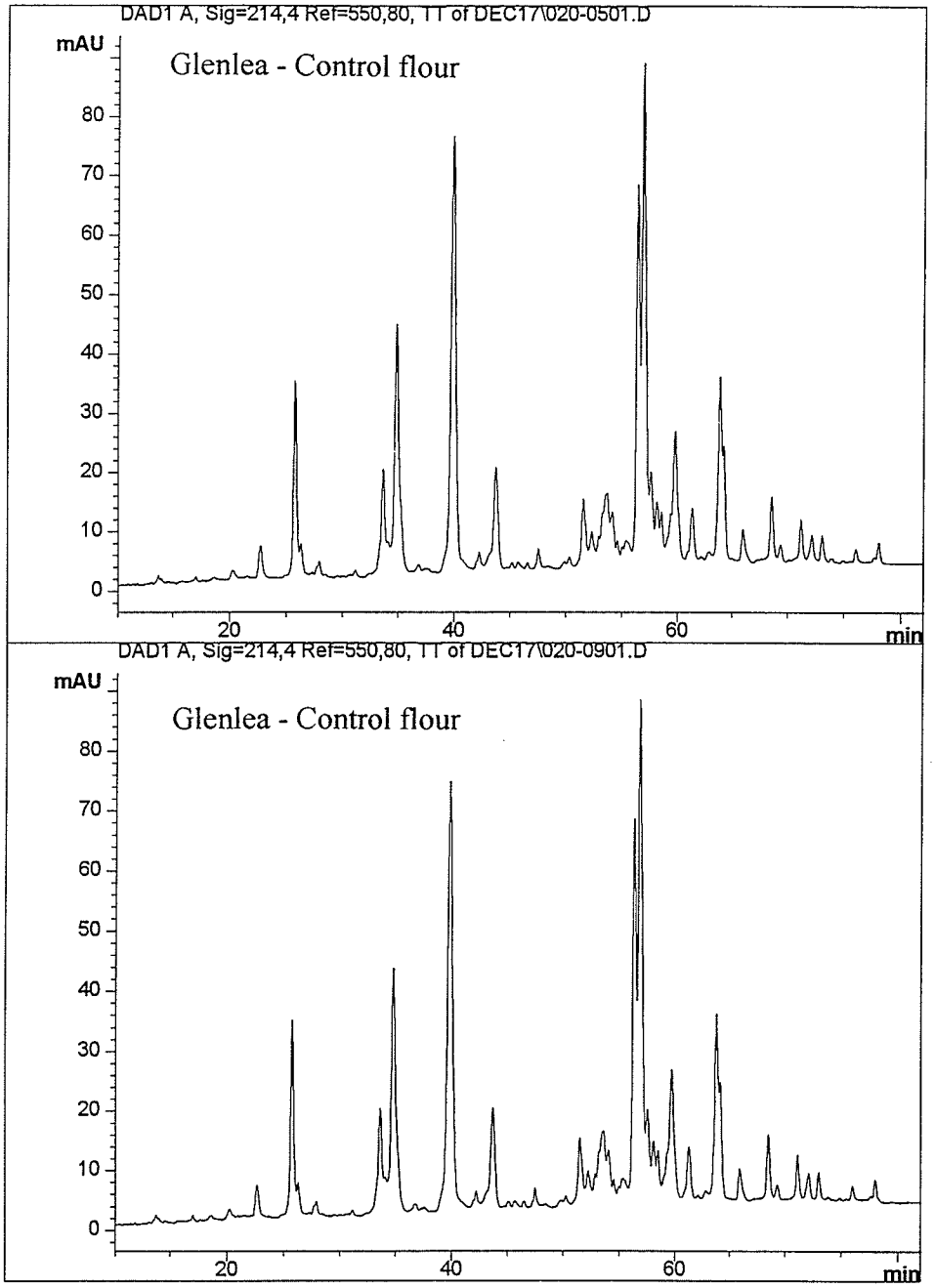
Glenlea - AAI Fraction



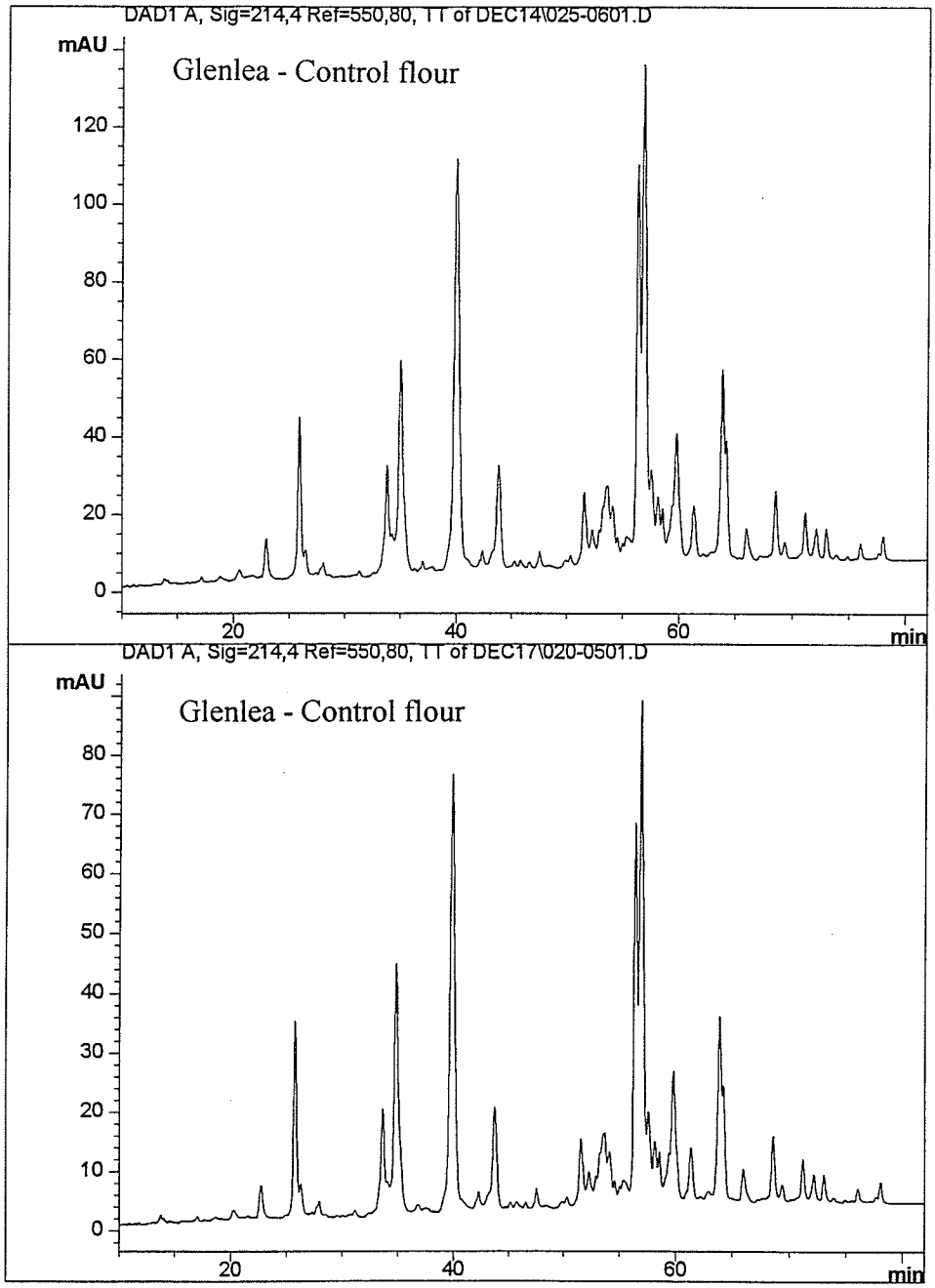
APPENDIX III. HPLC Chromatograms



Sample integration



Same Day Reproducibility



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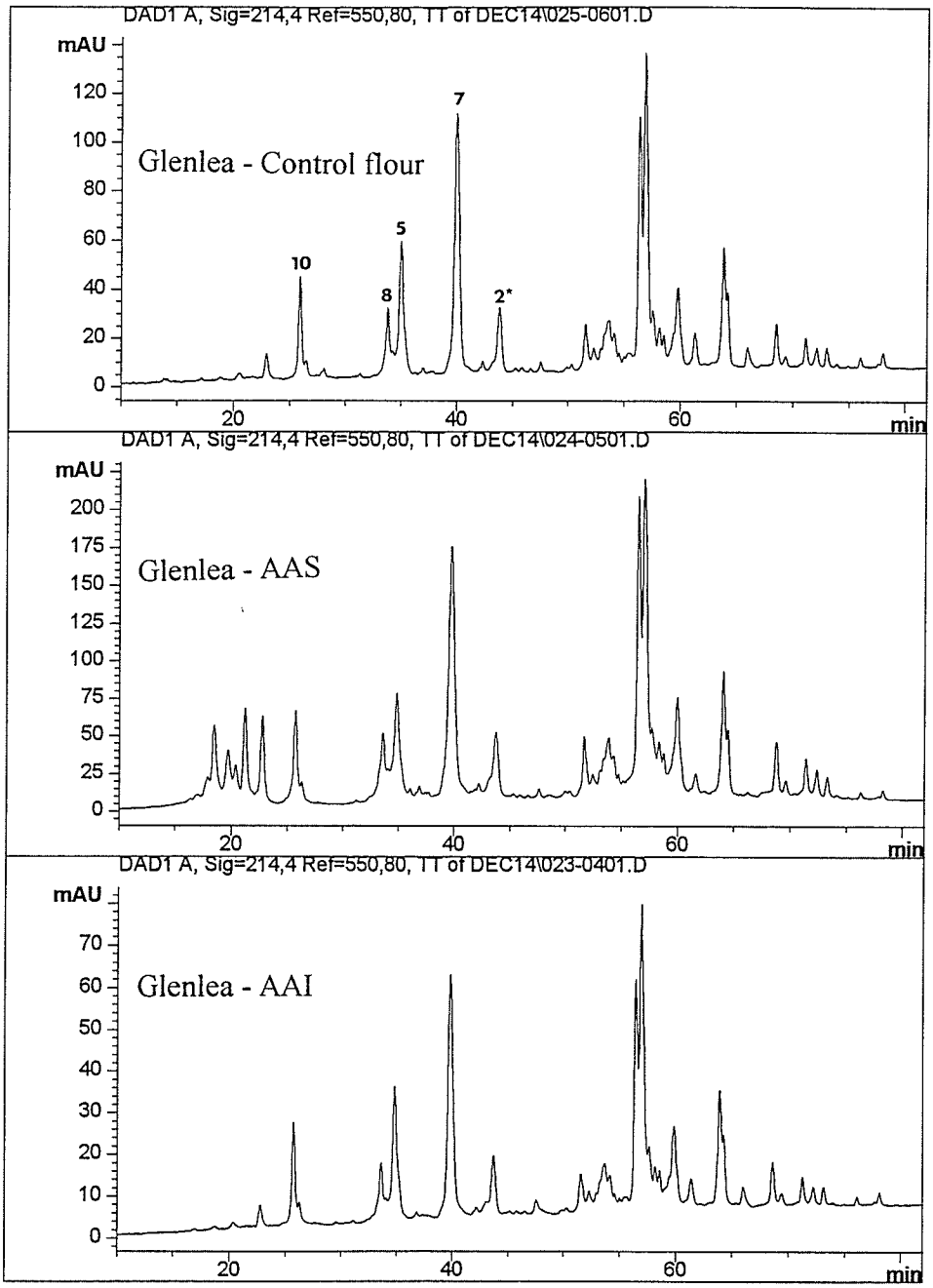
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mAU
Glenlea - Control flour
10
8
5
7
2*
min
DAD1 A, Sig=214,4 Ref=550,80, TT of DEC14\024-0501.D
mAU
Glenlea - AAS
min
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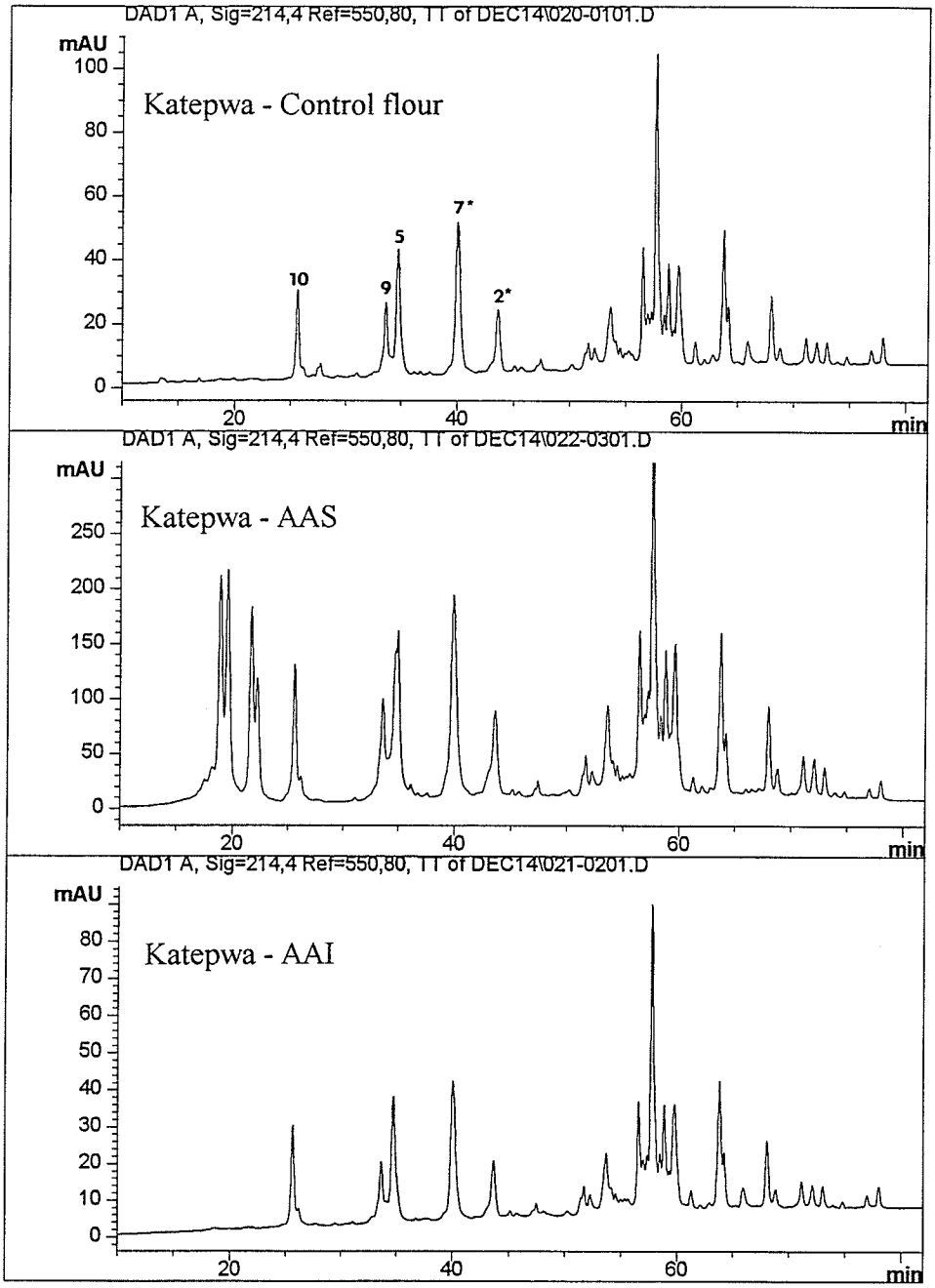
Glenlea - AAI
min

Replicate 1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mAU
Katepwa - Control flour
10
9
5
7*
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DAD1 A, Sig=214,4 Ref=550,80, TT of DEC14\022-0301.D
mAU
Katepwa - AAS
min
DAD1 A, Sig=214,4 Ref=550,80, TT of DEC14\021-0201.D
mAU
Katepwa - AAI
min

Replicate 1