Examination of the Mobilization of Storage Proteins of Wheat Kernels During Germination by High-Performance Reversed-Phase and Gel Permeation Chromatography[1]

J. E. KRUGER and B. A. MARCHYLO

ABSTRACT

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High-performance liquid chromatography (HPLC) in the gel permeation and reversed-phase modes was used to monitor changes in storage proteins of five wheat cultivars during germination. In the gel permeation mode, 0.1 M sodium phosphate buffer, pH 7.0, containing 2% SDS was used as an extractant and buffer solvent. Minimal changes occurred in the extractable storage proteins, and the major observable change was the formation of low-molecular weight species. In the reversed-phase mode, proteins extractable with 50% 1-propanol, 1% acetic acid, and 4% dithiothreitol had identical protein patterns on an Ultrapore RPSC column at any stage of germination. This indicates that proteolytic degradation of storage proteins must be extremely rapid. The similarity of chromatographic profiles from sound and germinated wheat extracts in the reversed-phase mode suggests that using this technique, the presence of sprout-damaged wheat would not complicate cultivar identification.

The mechanisms controlling the mobilization of storage protein reserves in germinating wheat are still not clearly understood. In the resting seed are endopeptidases (Hanford 1967, Grant and Wang 1972, Kruger 1973, Preston and Kruger et al 1976a), carboxypeptidases (Preston and Kruger 1976b, 1977; Kruger and Preston 1977), aminopeptidases (Kruger and Preston 1978), and many peptidases (Pett 1935). Carboxypeptidase is considered to be one of the most important of these enzymes in such cereals as barley and wheat and is largely in the endosperm (Kruger and Preston 1977), with approximately one-quarter of the endopeptidase activity (Preston and Kruger 1976a). During early germination (up to two days), these proteolytic enzymes have little effect on the mobilization of the endosperm storage reserves (Preston et al 1978). Some mechanism, such as enzyme compartmentalization, the presence of proteolytic inhibitors, or insolubilization of substrate, must prevail. From two days on, however, are rapid increases in levels of both endopeptidase and carboxypeptidase and corresponding increases in storage protein hydrolysis (Preston et al 1978). The major products formed are amino acids and peptides, and little evidence for transient polypeptide species of intermediate molecular weight has been found. The electrophoretic patterns found in barleys and their malts were very similar, which suggests that this cereal behaves in a way comparable to that of wheat (Montembault et al 1983).

Recently, we have used high-performance liquid chromatography (HPLC) in the gel permeation mode (HPGPC) to rapidly analyze changes that occurred in the salt-soluble proteins of germinating wheat (Kruger 1984). The major change observed was the progressive formation of low-molecular weight amino acids and peptides and species that adsorbed onto the column. In this study, we extended this research by using the gel permeation and reversed-phase modes of HPLC (RP-HPLC) and solvents that more fully solubilized wheat proteins, to better examine their degradation during germination. These proteins would predominantly be storage proteins, based on their solubility in the solvents used to extract them.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals used were reagent-grade, unless otherwise noted. For RP-HPLC, HPLC-grade acetonitrile, with an ultraviolet cut-off wavelength of 190 nm, was obtained from Fisher Scientific (Fair Lawn, NJ). Trifluoroacetic acid (Sequenol-grade) was obtained from Pierce Chemical Co. (Rockford, IL). Deionized water was

further purified by being passed through a Millipore super-Q system (Millipore Corp., Bedford, MA) consisting of two 0.8-μm filters, a charcoal filter, and a deionizing filter. This was followed by a final filtration through a Millipore HA 0.45-μm filter.

Wheat Samples

Selected for this study were the hard red spring (HRS) wheat cultivars, Neepawa, Glenlea, and RL 4137 (unlicensed); the soft white spring (SWS) wheat cultivar, Idaed; and the durum wheat cultivar, Wakooma.

Germination of Wheat Samples

Wheat kernels were steeped for 2 hr, spread evenly on moist blotting paper, germinated at 18°C for 1–6 days in a germination cabinet at 100% humidity, and air-dried on a bench top at 22°C. The final moisture content of the wheat was 10.5%.

Extraction Conditions

For HPGPC, the procedure of Bietz (1984) was followed: 10 mg of finely ground sample was extracted at 60°C for 1 hr with 1 ml of
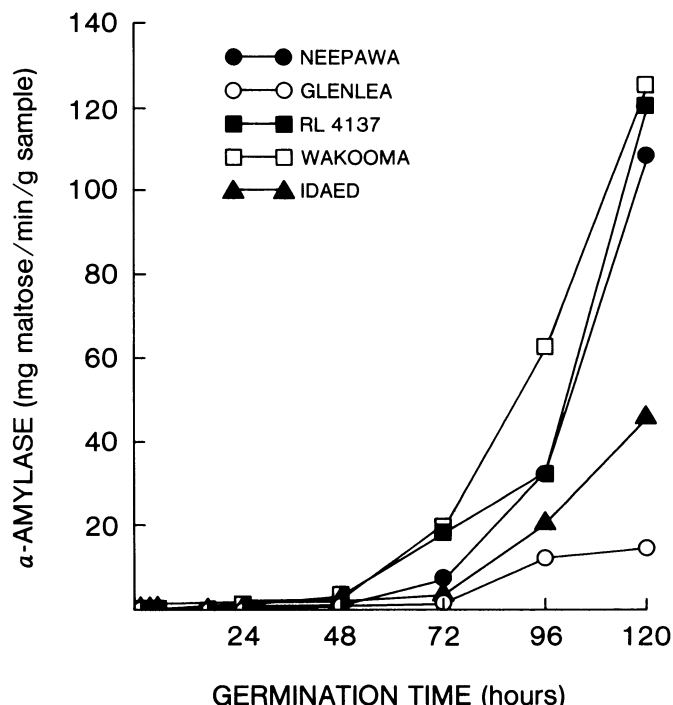

Fig. 1. Changes in α-amylase activity upon germination.

0.1M sodium phosphate buffer, pH 7.0, containing 2% sodium dodecyl sulfate. The suspension was centrifuged in an Eppendorf microcentrifuge model 5413 (Brinkman Instruments, Inc., Westbury, NY) at 8,800 × g, and 200 μl of the supernatant was immediately injected onto the HPLC.

For RP-HPLC, 100 mg of finely ground sample was extracted for 1 hr with 1 ml of 0.1M NaCl. The extract was centrifuged at 8,800 × g. The supernatant was retained for analysis, and the residue was extracted at 60°C for 1 hr with an aqueous solution containing 50% 1-propanol, 1% acetic acid, and 4% dithiothreitol (DTT); the supernatant was then contrifuged, retained, filtered through a Millipore FH 0.5-μm filter, and analyzed.

High-Performance Aqueous HPGPC

The procedure and equipment described by Kruger (1984) were used for aqueous HPGPC. Extracts were injected onto a Spherogel TSK-3000 column (Altex Scientific, Berkeley, CA), preceded by a 7.5 × 0.75-cm Spherogel TSK 2000 precolumn and a guard column packed with protein I-125 resin (Waters Associates, Milford, MA). The exclusion limit for proteins was approximately 500,000 daltons. The column buffer used was 0.1M sodium phosphate, pH 7.0, containing 2% sodium dodecyl sulphate (SDS). Column monitoring was at 280 nm with a 0–0.2 absorbance scale. Several proteins, including thyroglobulin, γ-globulin, bovine serum albumin, ovalbumin, myoglobin, and vitamin B-12, were used to
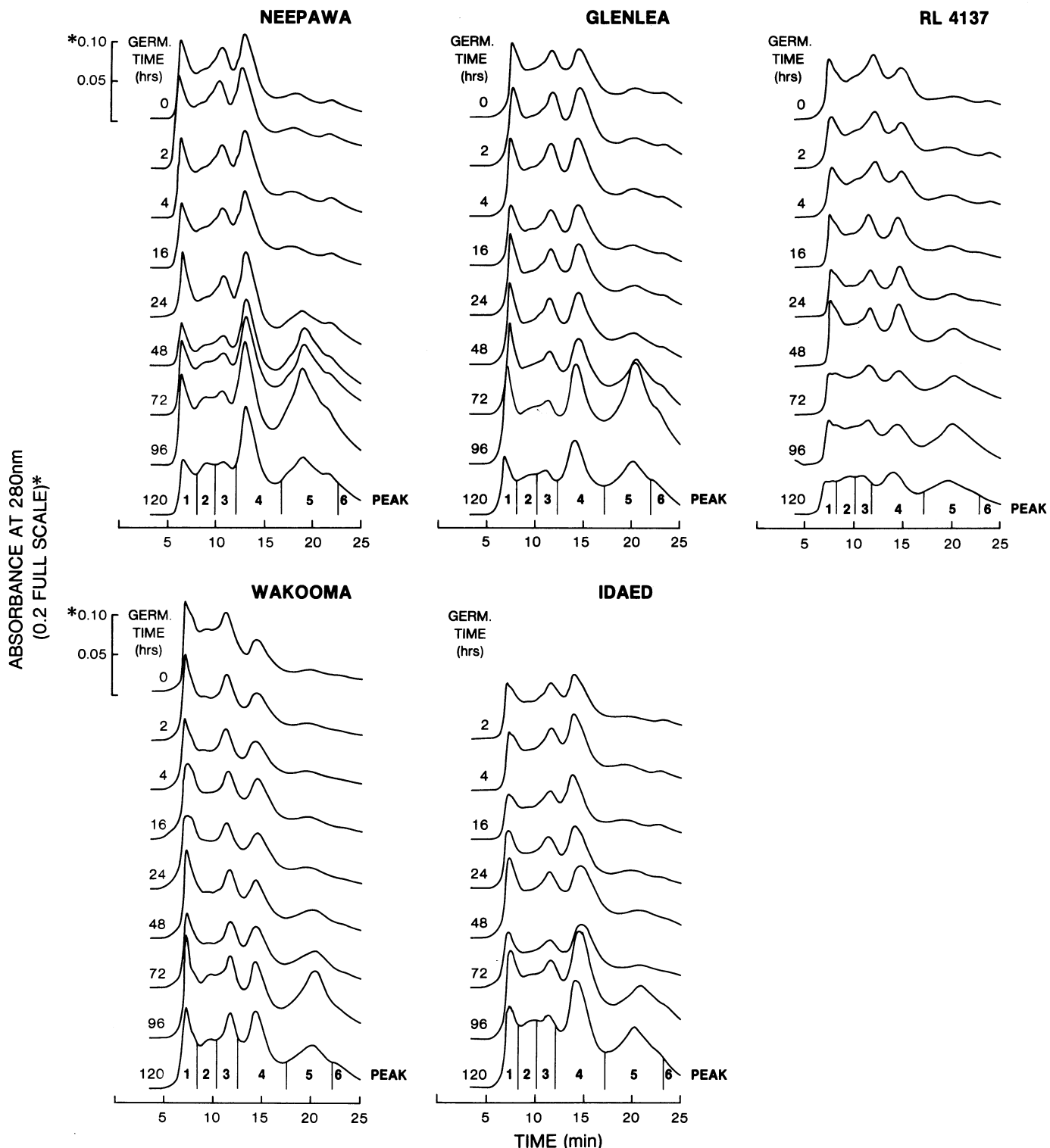

Fig. 2. Changes in HPGPC protein elution profiles for Neepawa, Glenlea, RL4137, Wakooma, and Idaed wheats upon germination.

calibrate the column.

RP-HPLC

The procedures and equipment described by Bietz (1983), Bietz et al (1984), and Marchylo and Kruger (1984) were used to conduct RP-HPLC. The column used was a 0.46 × 7.5-cm Ultrapore RPSC (Beckman Instruments, Berkeley, CA), which in some experiments was preceded by a 10-μm Aquapore RP-300 guard column (Brownlee Labs., Santa Clara, CA). Gradients were formed from two solvents consisting of 15 and 80% acetonitrile, respectively, and 0.1% trifluoroacetic acid (TFA). In some cases, the two solvents were water and 100% acetonitrile containing 0.1% TFA. Gradients and run time were chosen to optimize separations.

Alpha-Amylase Determination

Alpha-amylase was determined using the Perkin-Elmer model 191 grain amylase analyzer and β-limit dextrin substrate, as described previously (Kruger and Tipples 1981). A 5-g sample was ground with a Udy Cyclone mill (Udy Corp., Boulder, CO), and duplicate 1-g portions were analyzed.

RESULTS AND DISCUSSION

Cultivars of HRS, durum, and SWS wheats were germinated for one to five days, and the resultant changes in protein components were analyzed by RP-HPLC and HPGPC. Alpha-amylase activity was determined for each wheat cultivar at successive germination stages, to provide a marker for the extent of germination. As shown in Figure 1, α-amylase activities increased dramatically after a lag period of approximately 48 hr. The increase in activity varied with cultivar but increased up to 120 hr, indicating that germination was proceeding normally. Endopeptidase activities are synthesized *de novo* during germination, with the time response precisely paralleling that of α-amylase (Jacobsen and Varner 1967). The overall magnitude of changes in the α-amylase and protease activities, however, are quite different.

HPGPC of Wheat Proteins During Germination

The results of HPGPC analyses of proteins in the five wheat cultivars used in this study are shown in Figure 2. Six major components were in the elution profiles. The SDS buffer chosen for both extraction and elution of the proteins solubilizes cereal proteins such that the sizes of the resulting protein-SDS complexes are related to protein molecular weights (Bietz 1984). The molecular weight ranges of the components were: peak 1, >1,000 × 10^3 (excluded); peak 2, 173–1,000 × 10^3; peak 3, 43–173 × 10^3; peak 4, 8–43 × 10^3; and peaks 5 and 6, <8 × 10^3 (below the lower exclusion limit of the column). The very late elution time for peak 6 indicates that it contained molecular species adsorbed to the column. Variations in ratios of protein components from the different mature wheat cultivars are evident. For example, peak 4 is present in a much larger amount relative to peak 1 in Wakooma compared to Neepawa wheat. This is expected, because the cultivars varied widely genetically as well as in quality. Similar findings have been reported by Bietz (1984). Upon germination, the biggest changes were found in the progressive formation of low-molecular weight species. This corroborates a previous study in which buffer-soluble components were extracted using phosphate buffer, pH 7.0, containing 0.5 M sodium chloride and examined by HPGPC (Kruger 1984). Seven major components were present, but it was mainly the low-molecular weight entities, presumably peptides and amino acids, and an adsorbed protein component that increased progressively during germination. In this study, a substantial amount of gluten proteins, in addition to albumins and globulins, were extracted. Only minor changes occurred in the high-molecular weight species, but changes in the low-molecular weight species increased substantially. This is shown in Figure 3, in which amounts of the six components are plotted in terms of either their relative percentage or total counts (as detected by the computing integrator for a 200-μl injection) versus germination time for two of the cultivars, Neepawa and Glenlea. On a percentage basis, peaks 1 through 4 generally decrease slightly upon germination. This decrease is due to the increasing amounts of components 5 and 6. On the basis of peak areas, components 4, 5, and 6 also increase slightly. Even though the total amounts of higher-molecular weight species did not change appreciably, even small changes in the relative proportions of high-molecular weight species could have substantial effects on quality.

RP-HPLC of Germinated Wheat Proteins

RP-HPLC was used to follow changes in wheat proteins during germination. Proteins were extracted from the germinated wheats in two stages. In the first stage, albumins and globulins were extracted with a salt solution (0.1 M NaCl). Subsequently, the gluten proteins were removed at 60°C using an acqueous solvent containing 50% 1-propanol, 1% glacial acetic acid, and 4% dithiothreitol (DTT). This solvent extracted a greater percentage of gluten proteins than did other solvents compatible with RP-HPLC analysis (Kruger and Marchylo, in press). This concurs with previous work (Byers et al 1983), which showed that after salt extraction, a similar solvent (50% 1-propanol, 1% glacial acetic acid, and 1% 2-mercaptoethanol) at 60°C solubilized approximately 60% of the original seed nitrogen while leaving only 10% of the nitrogen and residue fraction in the glutenin.

Comparisons of the alcohol-soluble, reduced proteins in ungerminated and 96-hr-germinated wheats from the five cultivars are shown in Figure 4. As reported by Bietz et al (1984), noticeable differences were found in the protein patterns of the different cultivars. For a particular cultivar, however, the sound and germinated samples were almost identical in the number of protein components as well as the relative ratios between them. The major difference was the appearance in the germinated samples of two small and extremely hydrophobic components which eluted later than any of the other proteins (approximately 49–51.5 × 10^2 sec). Various other stages of germination were examined, and in all cases, the protein patterns were identical to that of the ungerminated control, except for minor differences. Increases in hydrolytic reaction products could be discerned, however, by analysis of salt extracts. Typical chromatograms for salt and alcohol extracts of sound and 96-hr-germinated Glenlea and Wakooma wheats are compared in Figure 5. In general, a larger percentage of these components eluted in the through peak,
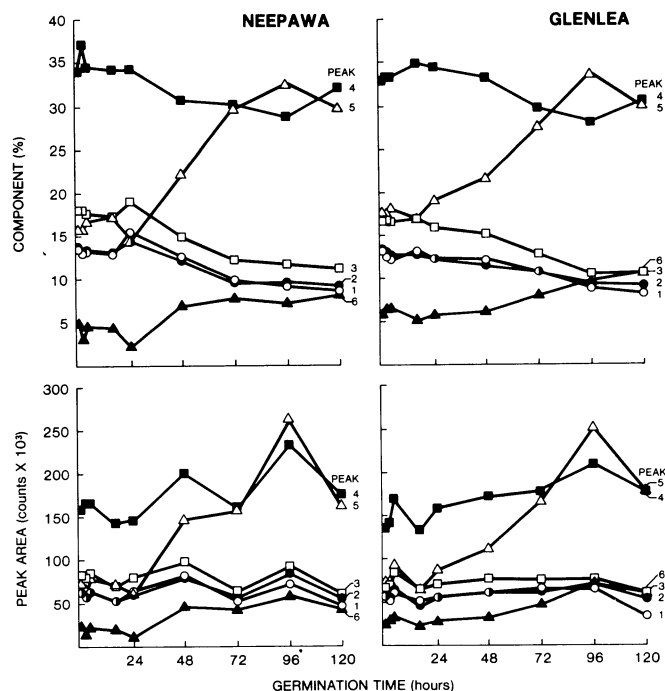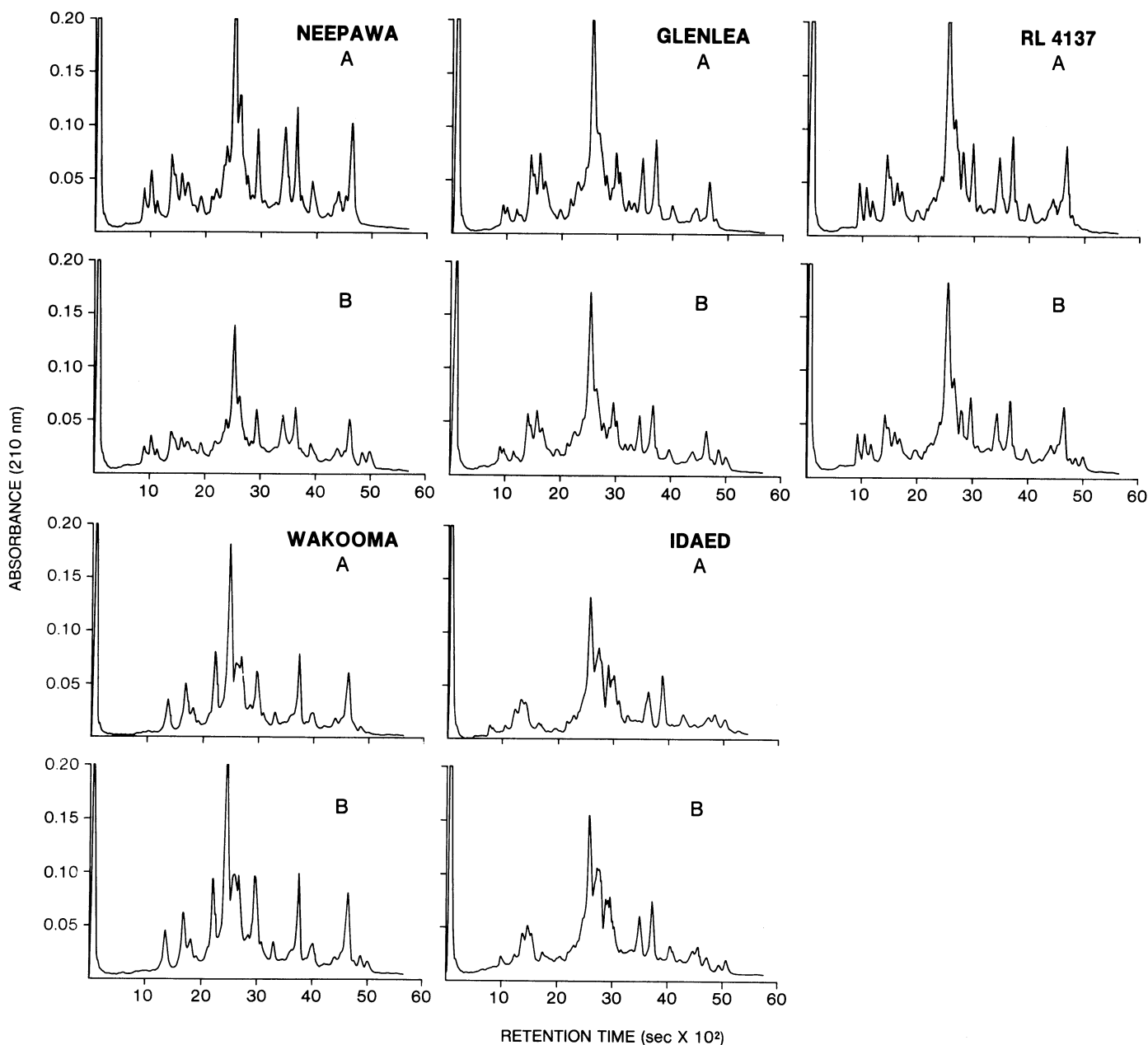

Fig. 3. Change in component (%) and peak area upon germination for molecular weight components shown for Neepawa and Glenlea wheat in Figure 2.

indicating that they were more hydrophilic in nature than were the gluten components and the gradient of acetonitrile was altered to better resolve them. As a consequence, resolution of the alcohol-soluble proteins is diminished. The germinated wheat contained considerably more salt-soluble nitrogenous material than did the sound wheat, as indicated by increased total area on chromatograms and a different chromatographic elution profile. Small changes were observed upon standing within chromatograms for the germinated wheat salt extracts, indicating that emphasis should not be placed on the number of relative amounts of the salt-soluble protein components observed by RP-HPLC.

RP-HPLC or HPGPC is an exceedingly rapid method for analyzing the nature of proteins in cereals. A complete analysis of the separation of proteins by reversed-phase chromatography on the basis of hydrophobicity is obtained in less than 2 hr, whereas molecular weight profiles were obtained in 1 hr. Selected solvents were compatible with each mode of separation and maximized protein solubility. However, different amounts and types of proteins could be solubilized by the different solvents. The surprising finding of this study was that the gluten proteins soluble

in propanol-acetic acid-DTT retained their integrity throughout germination, with all five cultivars. Furthermore, no additional major components were created. With HPGPC, profiles of the high-molecular components did not change dramatically, although there was a progressive increase in the amount of low-molecular weight species. The appearance of predominantly low-molecular weight compounds is in agreement with previous studies (Kruger 1984, Preston et al 1978), which determined that hydrolytic products resulting from enzymic degradation of proteins were predominantly amino acids with peptides remaining at the same level. These studies also suggested that the carboxypeptidases in germinated wheat (Preston and Kruger 1976a,b, 1977; Kruger and Preston 1977) very rapidly hydrolyzed the wheat endosperm proteins once limited endoproteolysis had taken place. The present study provides further evidence of the rapidity of this transformation, as almost no additional alcohol-soluble protein or peptide species were detectable by RP-HPLC upon germination. The relative proportions of components also did not change, indicating that preferential hydrolysis of individual proteins does not occur. Explaining the mechanism of this phenomenon is

Fig. 4. RP-HPLC separation of proteins from A) sound and B) 96-hr-germinated Neepawa, Glenlea, RL4137, Wakooma, and Idaed wheats. Chromatographic conditions: run time, 105 min. A buffer: acetonitrile:water (15:85) containing 0.1% TFA. B buffer: acetonitrile:water (80:20) containing 0.1% TFA. Gradient: 20–58% B. 25-µl injection.

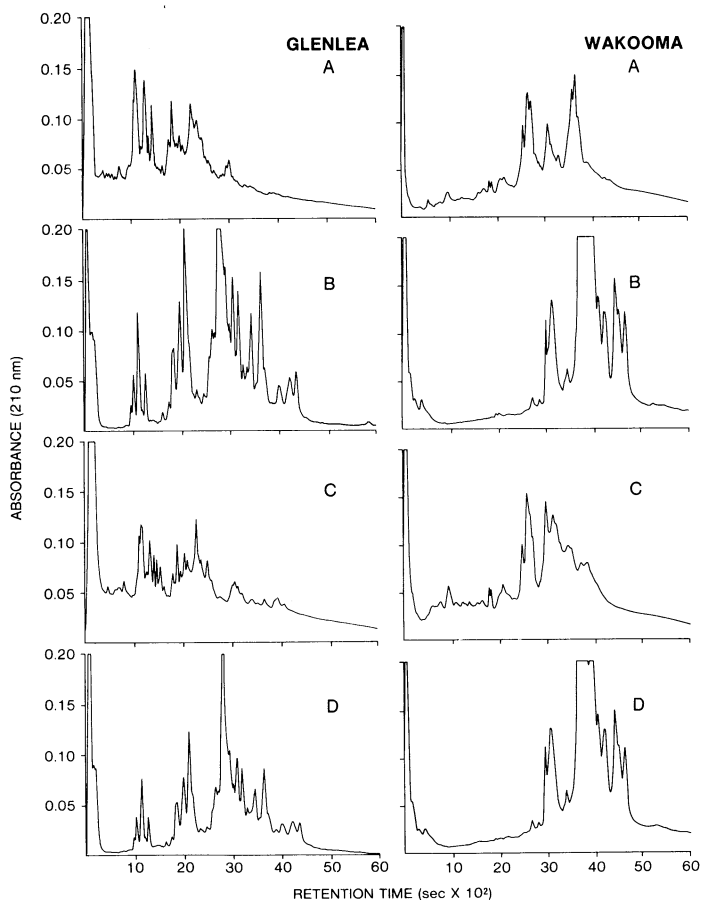**Fig. 5.** RP-HPLC of proteins from sound and 96-hr-germinated Glenlea and Wakooma wheats. A) sound wheat, salt-soluble proteins. B) sound wheat, protein-soluble in 50% 1-propanol, 1% acetic acid, and 4% DTT after removal of salt-soluble proteins. C) 96-hr-germinated wheat, salt-soluble proteins. D) 96-hr-germinated wheat, protein-soluble 50% 1-propanol, 1% acetic acid, and 4% DTT after removal of salt-soluble proteins. Chromatographic conditions: run time, 105 min. A buffer: water containing 0.1% TFA. B buffer: 100% acetonitrile containing 0.1% TFA. Gradient: 10–70% B. Injection volumes: salt-soluble proteins, 200-μl sound, 100-μl 96-hr-germinated. Alcohol-soluble proteins, 25 μl.

difficult. One possible explanation is that the proteins in wheat are initially insolubilized or compartmented in such a manner that minimal proteolysis occurs during early germination (up to 2 days). As germination proceeds, increases in endopeptidases occur along with limited interaction between enzyme and substrate. Intermediate products from this localized reaction would be broken down immediately to predominately amino acids by carboxypeptidase action. Because only a small part of the storage proteins are degraded at any time, the bulk remains untouched and would give the same patterns as found in the mature seed by RP-HPLC analysis. Until the wheat was extensively degraded, there would always be a preponderance of undegraded reserve protein relative to transient polypeptides, such that the latter would only be detected with difficulty.

The finding of similar chromatographic protein patterns for sound and germinated wheat has practical implication for cultivar identification using RP-HPLC techniques. It indicates that field-sprouted wheat should not interfere or complicate the unique protein patterns found for particular wheat cultivars. Caution should be taken, however, until it has been shown that all cultivars behave similarly.

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