Extraction, Separation, and Polymorphism of the Prolamin Storage Proteins (Secalins) of Rye

P. R. SHEWRY, S. PARMAR, and B. J. MIFLIN, Biochemistry Department, Rothamsted Experimental Station, Harpenden, Herts, England AL5 2JQ

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ABSTRACT

The amounts of secalin extracted from milled grain of rye with three alcohol/water mixtures were compared. Two sequential fractions were extracted with the solvent alone (secalin-I) and then with the solvent + 2% (v/v) 2-mercaptoethanol (secalin-II). The relative amounts of nitrogen recovered in the two fractions extracted at 20° C were greatest with 50% propan-1-ol (32.5%) and least with 60% ethanol (24%). The two fractions extracted with 50% propan-1-ol at 60° C contained more than 40% of the nitrogen, although electrophoresis showed some nonsecalin components in the secalin-II. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the fractions showed four groups of polypeptides called high molecular weight (HMW) secalins, 75,000-dalton γ-secalins, ω-secalins, and 40,000-dalton γ-secalins. Secalin-II contained mainly HMW secalins and 75,000-dalton γ-secalins. SDS-PAGE of unreduced and reduced secalin-I indicated that the HMW and 75,000-dalton γ-secalins were extracted as disulfide-linked aggregates. Fractions were also separated by electrophoresis at pH 3.2 and by three two-dimensional procedures. These showed that the 40,000- and 75,000-dalton γ-secalins were complex groups of polypeptides. Analysis of single seeds showed variation in secalin patterns within and between commercial varieties. This was not present in a homozygous population, and two-dimensional analysis of secalin from this line showed a smaller number of components.

The major storage proteins of the grain of wheat and barley are alcohol-soluble proteins or prolamins, called gliadin and hordein, respectively. These fractions have been intensively studied in relation to the quality of the grain for baking (wheat), malting (barley), and feeding to domestic animals (both cereals) (Kasarda et al 1976, Miflin and Shewry 1979a, Miflin et al 1982). Both fractions are composed of many component polypeptides that vary in their properties among different cultivars (Faulks et al 1981, Shewry et al 1979, Wrigley and Shepherd 1973). The individual components are often closely related in their amino acid compositions and sequences (Bietz et al 1977, Shewry et al 1981), and such families of polypeptides appear to be coded for by multigenic loci (Mecham et al 1978, Shewry et al 1980b), which are probably derived from the duplication and divergence of single ancestral genes.

The major storage protein in rye is also a prolamin called secalin, but this has not been widely studied, probably because rye is of lesser economic importance in the United States and Western Europe. Preston and Woodbury (1975) purified four subfractions by gel filtration and compared their amino acid compositions and migration on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). More recently, we purified and characterized four major groups of secalins and showed their relationships to storage proteins of barley and wheat (Field et al 1982, Shewry et al 1982).[1] Fractions corresponding to two of these groups were also purified and studied by Charbonnier et al (1981).

This article reports another study on the quantitative extraction, electrophoretic separation, and polymorphism of secalin fractions extracted from commercial varieties and a homozygous diploid line of rye.

MATERIALS AND METHODS

Chemicals

Stock solutions of 8 *M* urea were deionized by passage through a column of Bio-Rad AG 501 X8D mixed bed ion-exchange resin and stored before use for not more than one week at 4° C. 4-Vinylpyridine was redistilled and stored under N_2 at −15° C.

Extraction of Milled Grain

Grain of winter rye (cv. Rheidol) was milled in a Glen Creston hammer mill to pass a 0.5-mm sieve. Quantitative extraction was done by stirring duplicate 1-g samples in screw-capped polypropylene centrifuge tubes (Shewry et al 1978a) with 15-ml volumes of the following sequence of solvents: water-saturated butanol at 20° C (to extract lipids); 1.0 *M* aq. NaCl at 20° C (to extract salt-soluble proteins and nonprotein nitrogen); 50% (v/v) aq. propan-1-ol, 55% (v/v) aq. propan-2-ol or 60% (v/v) aq. ethanol at 20 or 60° C (to extract secalin-I); as above, but with the addition of 2% (v/v) 2-mercaptoethanol (to extract secalin-II); and 0.05 *M* borate buffer, pH 10, + 1% (w/v) sodium dodecyl sulfate (SDS) + 1% (v/v) 2-mercaptoethanol at 20° C (to extract glutelin).

Three 1-hr extractions were made with each solvent. The supernatants after centrifugation were bulked, and duplicate aliquots of extracts 3 and 4 were analyzed for Kjeldahl N. Extracts 3, 4, and 5 were dialyzed for 48 hr against several changes of distilled water and lyophilized.

Larger amounts of a total secalin fraction were extracted without prior extraction of salt-soluble nitrogen. Milled grain (500 g) was pre-extracted to remove alcohol-soluble components other than secalin by stirring for 1 hr with 2,500 ml of 95% (v/v) aqueous ethanol. Secalin was then extracted by stirring the residue with 2,500-ml volumes of 50% (v/v) aq. propan-1-ol + 2% (v/v) 2-mercaptoethanol at 20° C for 2 × 1 hr. After centrifugation, the supernatants were mixed with two volumes of 1.5 *M* aq. NaCl. After standing overnight at 4° C, the precipitated protein was removed by centrifugation. This was then dissolved in 8 *M* urea, dialyzed extensively against deionized water, and lyophilized. Electrophoresis of the fraction showed that it corresponded to a mixture of the secalin-I and secalin-II fractions extracted as above. The total amount of the fraction was not determined.

Proteins were reduced and pyridylethylated as described by Friedman et al (1970).

Purification of Proteins

Small amounts of γ-secalins, ω-secalins, and high molecular weight (HMW) secalins were purified as described previously (Field et al 1982, Shewry et al 1980a, 1982).

One-Dimensional Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 17.5% acrylamide gels at pH 8.9 (Shewry et al 1978a). Polyacrylamide gel electrophoresis in aluminum lactate buffer, pH 3.2 (lactate-PAGE), was done in horizontal 6-mm slab gels containing 6% acrylamide and 3 *M* urea (Shewry et al 1980a).

Two-Dimensional Analysis

Isoelectric Focusing (IEF)/SDS-PAGE. The protein was first separated by IEF in the 5–9 pH range in a slab gel containing 5%

[1] P. R. Shewry, E. J.-L. Lew, and D. D. Kasarda. 1980. Unpublished data.

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acrylamide and 6*M* urea (Shewry et al 1978a). The gel was sliced with a razor blade, and a strip containing the separated protein set on top of and electrophoresed into a modified Laemmli SDS gel as described by Rahman et al (1982).

IEF/Lactate PAGE. The IEF separation was performed as above and the strip inserted into a slot cast in a 6-mm thick lactate-PAGE gel containing 3*M* urea. The strip was surrounded with lactate buffer containing 6*M* urea and basic fuschin as a tracking dye. Electrophoresis was for 16 hr at 25 mA.

Lactate-PAGE/pH 9.2. This system was modified from Mecham et al (1978). A single sample was separated in a track down the center of a 22 cm×14.5 cm×6 mm thick lactate-PAGE gel, pH 3.2, containing 6*M* urea. After migration for 16 hr at 30 mA, the gel was suspended on a wire mesh in 1 L of stirred buffer, pH 9.2, containing 6*M* urea, 1.875 g of glycine, and 15.15 g of Tris. After 4 hr of equilibration, the gel was electrophoresed at 30 mA for 24 hr at right angles to the first dimension separation. The electrode buffer was pH 9.2 without urea.

Gel Staining

The lactate-PAGE, IEF/lactate-PAGE and lactate-PAGE/pH 9.2 gels were fixed and stained in 0.02% (v/v) Coomassie brilliant blue R250 in 12% (w/v) trichloroacetic acid (TCA). SDS-PAGE and IEF/SDS-PAGE gels were fixed and stained in 40% (v/v) aq. methanol containing 10% (w/v) TCA and 0.1% (w/v) Coomassie brilliant blue R250 and destained in 10% (w/v) TCA.

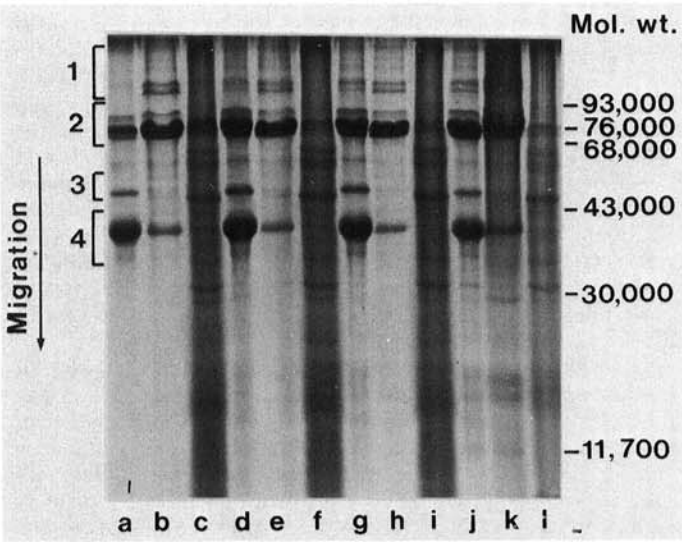

Fig. 1. SDS-PAGE of reduced and pyridylethylated secalin-I, secalin-II, and glutelin fractions from cv. Rheidol. a, d, g, j = secalin-I fractions extracted with 60% (v/v) ethanol, 55% (v/v) propan-2-ol, 50% (v/v) propan-1-ol (all at 20° C) and 50% (v/v) propan-1-ol at 60° C, respectively. b, e, h, k = secalin-II fractions extracted after secalin-I fractions a, d, g, j, respectively, with the same alcohol-water mixtures and extraction temperatures but with the addition of 2% (v/v) 2-mercaptoethanol. c, f, i, l = glutelin fractions extracted after secalin-II fractions b, e, h, k, respectively. Numbers 1–4 indicate the groups of high molecular weight secalins, 75,000-dalton γ-secalins, ω-secalins, and 40,000-dalton γ-secalins, respectively. The migration distances of molecular weight markers are also shown. These were phosphorylase B (93,000), transferrin (76,000), bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (30,000), and cytochromome c (11,700).

Single Seed Analysis

Total secalin fractions were extracted from single seeds with 50% (v/v) propan-1-ol containing 2% (v/v) 2-mercaptoethanol, reduced, pyridylethylated, and separated by SDS-PAGE as described previously (Shewry et al 1979).

RESULTS AND DISCUSSION

Extraction by Different Alcohols

Many workers extract cereal prolamins using aqueous ethanol (usually 60 or 70% v/v) at room temperature or in the cold (Kasarda et al 1976). However, gliadin is only sparingly soluble under such conditions, and the solubility is greater in aq. propan-1-ol and at higher temperatures (Dill and Alsberg 1925). We recently showed that the hordein has analogous solubility properties (Shewry et al 1980c). Similarly, these conditions give more complete extraction of the prolamins of several species, including barley, wheat, and maize (Miflin and Shewry 1979b, Miflin et al 1980). Another factor affecting prolamin extraction is the presence or absence of a reducing agent. Bishop (1939) demonstrated that the extraction of hordein is increased by the presence of bisulfite, while more recent work showed that, after extraction of prolamins with aqueous alcohol, a second fraction with prolamin-like amino acid and polypeptide compositions can be extracted using the same solvent but with the addition of a reducing agent (Landry and Moureaux 1970; Preaux and Lontie 1975; Shewry et al 1978b, 1980c). Extracting prolamins in two such sequential fractions is now common practice, and we have called these fractions prolamin-I and prolamin-II, respectively, following the nomenclature of Sodek and Wilson (1971) for the zein fractions of maize.

An initial comparison was made of the amounts of secalin (determined as secalin N) extracted by aqueous solutions of 60% (v/v) ethanol, 50% (v/v) propanol-1-ol, and 55% (v/v) propan-2-ol. Two sequential fractions were extracted in the absence and presence of 2% 2-mercaptoethanol (Table I). The concentrations of the three alcohols were selected because they are optimal for the extraction of hordein (Shewry et al 1980c). When the extractions were made at 20° C, the amount of N extracted without a reducing agent was least with 60% ethanol (16%) and greatest with 50% propan-1-ol (24%). This order of efficiency is similar to that previously reported for hordein (Shewry et al 1980c). When the extraction temperature was increased to 60° C, an additional 10% of the total grain N was extracted by the most efficient solvent, 50% propan-1-ol. Further extraction of the meal at 20° C with the same three solvents but containing 2% 2-mercaptoethanol removed a further 8% of the total N. A similar fraction extracted with 50% (v/v) propan-1-ol + 2% 2-mercaptoethanol at 60° C accounted for about 6%. Addition of the amounts extracted with and without mercaptoethanol (secalin-I and II) gave values for total secalin ranging from 24 to 40% according to the alcohol used.

To evaluate the nature of the proteins extracted by the different solvents, the fractions were reduced by treatment with 2-mercaptoethanol, pyridylethylated, and separated by SDS-PAGE (Fig. 1). Four groups of polypeptides were observed. We previously

TABLE I
Recovery of Nitrogen in the Secalin-I and Secalin-II Fractions Extracted by Different Alcohols[a]

Solvent	Temperature of Extraction (%)	Percent Total Nitrogen		
		Secalin-I	Secalin-II	Secalin-I + II
60% (v/v) ethanol	20	16.1	7.9	24.0
55% (v/v) propan-2-ol	20	20.6	8.4	29.0
50% (v/v) propan-1-ol	20	24.2	8.3	32.5
50% (v/v) propan-1-ol	60	34.2	6.3	40.5

[a] The secalin-I fraction was extracted by the solvent alone and the secalin-II fraction by the solvent + 2% (v/v) 2-mercaptoethanol. The total N content of the seed meal was 13.4 mg/g (dry basis). Electrophoretic separations of these fractions are shown in Figs. 1, 2, and 3.

purified components from each of these groups, established their homology with the prolamins of wheat, and accordingly called them HMW secalins (group 1 in Fig. 1), 75,000-dalton (75k) γ-secalins (group 2), ω-secalins (group 3), and 40,000-dalton (40k) γ-secalins (group 4) (Field et al 1982, Shewry et al 1982).[2] The apparent molecular weights of the HMW secalins were above 100,000 daltons and of the ω-secalin band approximately 52,500. Comparison of the SDS-PAGE patterns of secalin-I fractions extracted by the three solvents showed that 60% ethanol extracted relatively less of the 75k γ-secalins and only traces of HMW secalins (Fig. 1a,d,g,j). More of both of these groups were extracted by 50% propan-1-ol, especially when the extractions were made at 60° C (Fig. 1g,j).

SDS-PAGE of the secalin-II fractions showed that they contained mainly 75k γ-secalins and HMW bands with only traces of the 40k γ-secalins and ω-secalins (Fig. 1b, e, h, k). The HMW bands were most intense in the secalin-II fraction extracted with 60% ethanol + 2-mercaptoethanol, which is consistent with the virtual absence of these bands from the corresponding secalin-I fraction (Fig. 1a, b). The secalin-II fraction extracted by propan-1-ol + 2-mercaptoethanol at 60° C gave an intensely stained background, indicating the presence of nonsecalin material (Fig. 1k). The presence of secalin polypeptides in the meal after the three series of extractions at 20° C was demonstrated by SDS-PAGE of a residual protein (glutelin) fraction extracted with SDS and 2-mercaptoethanol at pH 10 (Fig. 1c, f, i). The main contaminant was 75k γ-secalins, notably in the fraction from the meal previously extracted with 60% ethanol (Fig. 1c). Only traces of these components were present in the fraction from meal extracted with 50% propan-1-ol at 60° C (Fig. 1, l). HMW secalins were not present in great amounts in the glutelin fractions, indicating that they were efficiently extracted in the presence of 2-mercaptoethanol, even by 60% ethanol at 20° C. This is in contrast to wheat and barley, in which the homologous HMW prolamins are only efficiently extracted in the presence of reducing agent + 1% acetic acid.[3]

Although the SDS-PAGE separations (Fig. 1) showed that polypeptides other than these four major groups were present in the secalin-I and secalin-II fractions, they were found only in trace amounts and were not studied further.

Effect of Reduction and Alkylation on SDS-PAGE Patterns

The protein fractions separated in Fig. 1 were reduced and alkylated before separation. This prevents the formation of disulfide bonds and was used to ensure that the gel patterns represented individual subunits (the primary gene products) rather than a mixture of subunits and ill-defined aggregates. To determine whether some subunits were extracted as disulfide-linked aggregates, the four secalin-I fractions were separated by SDS-PAGE without reduction (Fig. 2a–d), after reduction (Fig. 2e–h), and after reduction and alkylation (Fig. 2i–l). The different preparation procedures had no apparent effects on either the migration distance or the relative intensity of the ω-secalin band, which is consistent with the reported absence of cysteine from these components.[4] The 40k γ-secalin band migrated slightly faster in the unreduced samples (Fig. 2a–d), but the relative intensity of this component was approximately the same, regardless of the preparation procedure. The faster migration may have resulted from the presence of intramolecular disulfide bonds in the component polypeptides. This would be expected to result in a more compact conformation that would pass more readily through the gel matrix. The greatest difference between the patterns of the unreduced and reduced samples was in the HMW region. Although some protein was present in the 75k region in the unreduced samples, this band was of relatively low intensity, and many additional bands of increasing molecular weight were also present (Fig. 2a–d). On reduction, these bands disappeared, and a concomitant increase occurred in the 75k band (Fig. 2e–h). This suggests that most of the additional bands in the unreduced sample were disulfide-linked aggregates of the 75k γ-secalin. Some of these bands ran in the same positions as reduced HMW secalins. Consequently, we were unable to conclude whether the HMW secalins were also aggregated. However, at least one HMW secalin band (Fig. 2h) appeared to be absent from the corresponding unreduced sample (Fig. 2d), suggesting that they probably were aggregated. These separations cast doubt on the identity of the doublet immediately above the 75k γ-secalin band (Fig. 2a). This doublet was not present in a purified 75k γ-secalin fraction (Shewry et al 1982), nor was its relative intensity affected by reduction (Fig. 2).

Alkylation of the samples had little further effect on the SDS-PAGE patterns of the fractions, with the exception that the 75k γ-secalin band was resolved into two bands when alkylated (Fig. 2e–h, i–l).

Lactate-PAGE of Secalin Fractions

Electrophoresis at low pH in aluminum lactate buffer, either in starch gels or polyacrylamide gels, is the most widely used system for the separation of wheat gliadins (Kasarda et al 1976) and has been used by previous workers to separate secalin (Bernard et al 1977; Charbonnier et al 1981; Shepherd 1968, 1973; Shepherd and Jennings 1971). We therefore used lactate-PAGE at pH 3.2 to separate the secalin-I and secalin-II fractions prepared using the least (60% ethanol at 20° C) and most (50% propan-1-ol at 60° C) efficient of the four extraction procedures (Fig. 3).

The unreduced secalin-I fraction (Fig. 3a, e) was separated into a major group of fast-migrating bands (area A in Fig. 3a) and a series of slower bands (area B). These separations are similar to ones previously reported by other workers (Bernard et al 1977; Charbonnier et al 1981; Shepherd 1968, 1973; Shepherd and Jennings 1971). On reduction (Fig. 3b, f), the mobility of the fastest group decreased, but its relative intensity apparently was not affected. Major changes also occurred in band pattern in the slow-migrating region with the elimination of many of the slowest bands

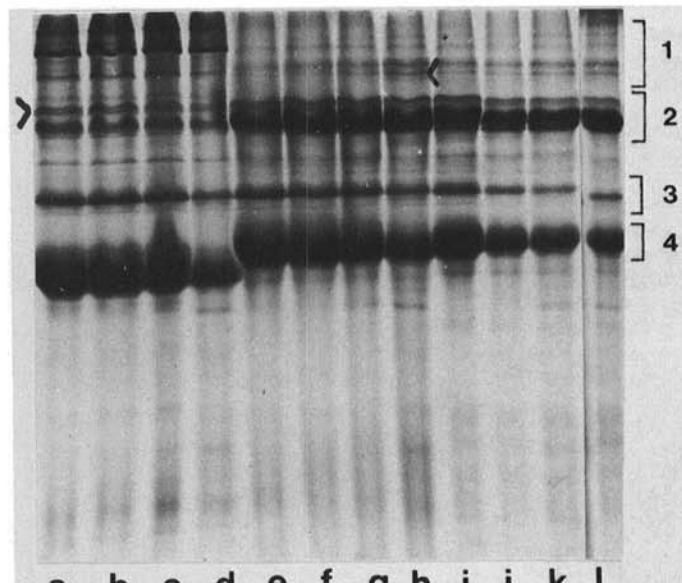

Fig. 2. SDS-PAGE of nonreduced (a–d), reduced (e–h), and reduced and pyridylethylated (i–l) secalin-I fractions extracted from cv. Rheidol with 60% (v/v) ethanol at 20° C (a, e, i), 55% (v/v) propan-2-ol at 20° C (b, f, j), 50% (v/v) propan-1-ol at 20° C (c, g, k), and 50% (v/v) propan-1-ol at 60° C (d, h, l). Numbers 1–4 indicate the groups of high molecular weight secalins, 75,000-dalton γ-secalins, ω-secalins, and 40,000-dalton γ-secalins, respectively. Track 1 was run on a separate gel from tracks a–k; hence the slight difference in relative migration of the ω-secalins. The band indicated by an arrow in track a may not be a 75k γ-secalin. The band indicated by an arrow in track h is an HMW secalin, which is absent from the corresponding unreduced sample (track d).

[2] P. R. Shewry, E. J.-L. Lew, and D. D. Kasarda. 1980. Unpublished data.

[3] P. R. Shewry. 1980. Unpublished data.

[4] P. R. Shewry, E. J.-L. Lew, and D. D. Kasarda. 1982. Unpublished data.

and increases in the relative intensity of faster components. The effects of reduction suggested that the fast- and slow-migrating groups were 40k and 75k γ-secalins, respectively, and this was confirmed by comigration with purified preparations of these groups of polypeptides. The decrease in speed of migration of the 40k γ-secalins is probably due to the adoption of a less compact conformation on reduction, whereas the very slow bands eliminated on reduction are probably disulfide-linked aggregates of the 75k γ-secalins. Alkylation (Fig. 3c, g) resulted in faster migration of both groups of components, which is consistent with addition of basically charged pyridylethyl groups to the cysteine residues.

Whereas the fast- and slow-migrating groups (A and B, respectively, in Fig. 3) were separated into several bands in the unreduced samples, only a single broad band was present in each region in the reduced and the reduced and alkylated samples. Further analyses by two-dimensional procedures showed that each of these bands was composed of several components with similar mobilities. The resolution of these two groups was not improved by changes in the electrophoretic conditions. The same two major groups of components were also present in the reduced and pyridylethylated secalin-II fractions, but relatively less of the fast-migrating group (45k γ-secalins) was present in the fraction extracted with 50% propan-1-ol at 60° C, which is consistent with the SDS-PAGE separations of these fractions (Fig. 1b, k).

The migration behavior of the ω and HMW secalins was studied, using fractions prepared from reduced and pyridylethylated total secalin. A fraction containing total ω-secalins comigrated with the leading edge of the slow-migrating group (B); a second fraction containing an HMW component (which gave a single band on SDS-PAGE) comigrated with the trailing edge (results not shown).

Two-Dimensional Analysis

Several two-dimensional separation procedures were evaluated for their ability to give improved resolution of the 40k and 75k groups of γ-secalins. Isoelectric focusing was used as the first dimension of two systems, the second dimensions being SDS-PAGE (Fig. 4a) and lactate-PAGE (Fig. 4b). Both systems gave partial resolution of the two groups into several components. Although the degree of resolution was approximately the same with both systems, we found that the IEF/SDS-PAGE procedure was easier to use and gave more reproducible separations. The two groups of γ-secalins showed great variation in their isoelectric points, with more alkaline components present in the 40k group. The ω-secalins and HMW prolamins were only minor components of the total secalin fraction used for these separations and, as a consequence, only traces of these can be seen in the IEF/SDS-PAGE separations. A series of diffuse slow-migrating spots possibly corresponded to some of the HMW components in the IEF/lactate PAGE separation (Fig. 4b). We also attempted to develop a two-dimensional system using lactate PAGE as the first dimension and SDS-PAGE as the second but were unable to obtain good resolution, despite using a range of conditions. The third two-dimensional system used was the two-pH (3.2 and 9.2) system

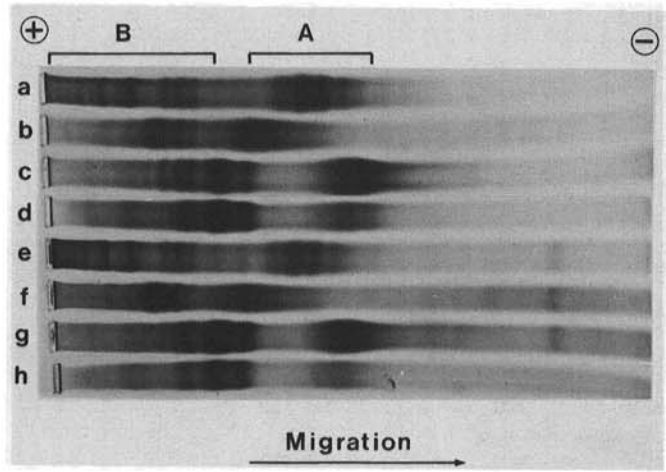

Fig. 3. Lactate-PAGE (pH 3.2) of secalin fractions extracted from cv. Rheidol with 60% (v/v) ethanol at 20° C (a–d) and 50% (v/v) propan-1-ol at 60° C (e–h). a, e = nonreduced secalin-I; b, f = reduced secalin-I; c, g = reduced and pyridylethylated secalin-I; d, h = reduced and pyridylethylated secalin-II (extracted with 2% 2-mercaptoethanol). The fast- and slow-migrating groups of components (A and B) are predominantly 40k and 75k γ-secalins, respectively.

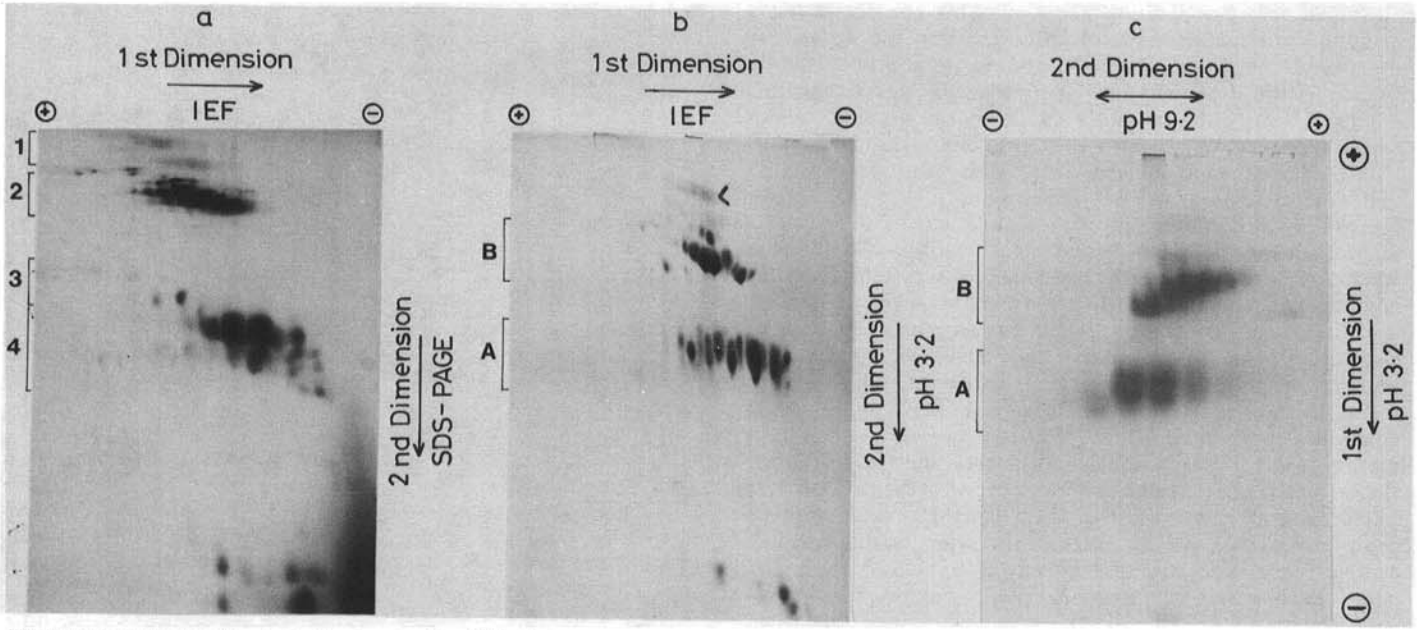

Fig. 4. Two-dimensional analysis of reduced and pyridylethylated total secalin from cv. Rheidol. **a,** IEF (pH range 5–9) followed by SDS-PAGE. Numbers 1, 2, 3, and 4 indicate the groups of high molecular weight components, 75,000-dalton γ-secalins, ω-secalins, and 40,000-dalton γ-secalins, respectively. **b,** IEF (pH range 5–9) followed by lactate-PAGE (pH 3.2). **c,** Lactate-PAGE (pH 3.2) followed by pH 9.2 electrophoresis. The groups of fast- and slow-migrating components (A and B, respectively) in the lactate-PAGE dimensions of b and c correspond to those indicated in Fig. 3. The arrow in 2b indicates a series of polypeptides that probably correspond to HMW secalin components.

of Mecham et al (1978), modified to prevent precipitation of the protein by the presence of 6 *M* urea in the gel and in the buffer used to equilibrate the gel between dimensions. Even with these modifications, the resolution was poor (Fig. 4c).

Genetic Variation in Secalin Patterns

Rye is a species that usually outcrosses; consequently, most varieties are mixtures of genotypes. To look for genetic variation in secalin, we extracted total secalin fractions from single seeds of cv. Rheidol and separated them by one-dimensional SDS-PAGE (Fig. 5a–j). This showed the presence of variation in the band patterns of all four groups of secalin polypeptides. When the same procedure was used to examine single seeds of a range of cultivars bred and grown in northern Europe, an even wider range of variation was apparent, as shown by the selection of patterns in Fig. 5k–u. Notably, relatively greater amounts of ω-secalins were present in the fractions extracted from single seeds than in those prepared from milled whole grain (Fig. 1). This is probably due to incomplete extraction of the γ-secalins by the single seed procedure. Because of the intravarietal heterogeneity, the analysis of secalin polypeptide patterns is of limited potential value for the varietal identification of single seeds of rye, which is in contrast to the situation with wheat and barley (Autran 1975, Shewry et al 1979).

Analysis of Homozygous Grain

Some of the polymorphism present in secalin fractions extracted from milled grain samples (Fig. 4) may be due to genetic variation in the population. To determine the extent of polymorphism in homozygous grain, we analyzed seed from a line produced by pollen culture followed by chromosome doubling to give homozygous diploid plants. This line (MPI 209) was generously supplied by G. Wenzel of the Max-Planck-Institut, Köln. SDS-PAGE of fractions from single seeds (Fig. 6a) confirmed that the sample was genetically homogeneous. Two-dimensional analysis (IEF/SDS-PAGE) of a fraction prepared from milled grain (Fig. 6b) showed several polypeptides in the 40k and 75k γ-secalin regions. These were more clearly resolved than in the corresponding separation of secalin from the heterogeneous variety Rheidol (Fig. 4a). The poor resolution of the Rheidol fraction probably was not due to deficiencies in the electrophoretic procedure but to the presence of many components with similar properties that were derived from different lines in the mixed population. The fraction from MPI 209 was also relatively rich in ω-secalins and HMW secalins, and several polypeptides corresponding to these can be recognized on the two-dimensional gel (Fig. 6b). The factors controlling the relative amounts of the different prolamin groups in rye are not known, but in barley and wheat the relative amounts of the S-poor C hordein and ω-gliadin fractions (which are homologous with ω-secalin) are affected by the N and S status of the plants (Kirkman et al 1982, Wrigley et al 1980).

GENERAL DISCUSSION

Total secalins (secalin-I + II) accounted for slightly less than 40% of the total N in the sample of Rheidol used for these experiments. An exact value cannot be given because SDS-PAGE analysis showed that the most effective solvent (50% propan-1-ol + 2-mercaptoethanol at 60° C) also extracted nonsecalin polypeptides, whereas the other solvents left some residual secalins in the glutelin fraction. The secalin fraction is composed of four major groups of polypeptides that exhibit great variation in electrophoretic properties between genotypes. Of the electrophoretic systems used, SDS-PAGE gave the best separations of the four major groups and the two-dimensional IEF/SDS-PAGE system of the component polypeptides of these groups. Lactate-PAGE gave reasonably good resolution of the unreduced secalin-I fractions, but the separation

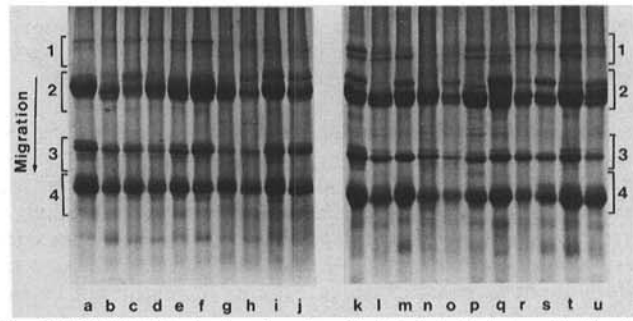

Fig. 5. SDS-PAGE of reduced and pyridylethylated total secalin fractions extracted from single seeds of different cultivars of rye. a–j, u = Rheidol; k = Gazelle; l = Otello; m = Animo; n = Greenfold; o = Dominant; p = Rhyader; q = Lovaszpatonai; r = Parana; s = Tetragorzow; and t = Ashill Pearl. 1, 2, 3, and 4 indicate the groups of high molecular weight secalins, 75,000-dalton γ-secalins, ω-secalins, and 40,000-dalton γ-secalins, respectively.

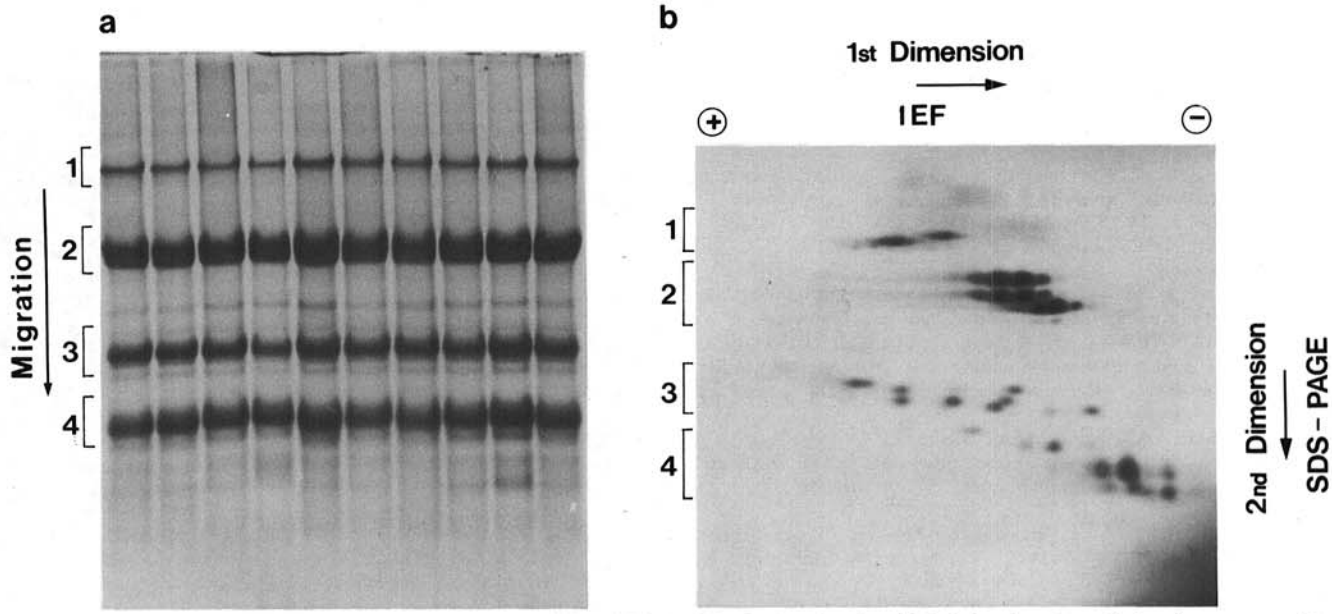

Fig. 6. Analysis of secalin fractions from homozygous line MPI 209. **a,** SDS-PAGE of reduced and pyridylethylated total secalin fractions extracted from single seeds. **b,** Two-dimensional analysis (IEF followed by SDS-PAGE) of a reduced and pyridylethylated total secalin fraction extracted from milled whole grain. 1, 2, 3, 4 indicate the groups of high molecular weight secalins 75,000-dalton γ-secalins, ω-secalins, and 40,000-dalton γ-secalins, respectively.

of the reduced and reduced and alkylated fractions was poor.

The two groups of γ-secalins are similar in their migration on SDS-PAGE, both under nonreducing and reducing conditions, to the two major groups purified by Preston and Woodbury (1975). Comparison of their amino acid compositions (Shewry et al 1982) confirms this identity. In a more recent study of the nonaggregated secalins, Charbonnier et al (1981) purified and characterized fractions corresponding to the ω-secalins and the 40k γ-secalins. Preston and Woodbury (1975) and Charbonnier et al (1981) also purified groups of low molecular weight (LMW) components that were present in relatively small quantities in their extracts. These groups differed, however, in their amino acid compositions; lysine represented 2.8 mol % of the fraction of Preston and Woodbury (1975) but was absent from those of Charbonnier et al (1981), who noted that their compositions were similar to those of the LMW gliadins isolated by Salcedo et al (1979). Preston and Woodbury (1975) also purified a further fraction that had an amino acid composition similar to their LMW fraction, eluted between the 40k γ-secalins and LMW components on gel filtration (apparent mol wt 27,000) but comigrated with the 40k γ-secalins on SDS-PAGE. Neither we nor Charbonnier et al (1981) have identified components with similar properties.

The biochemical basis for the difference in the aggregation behavior of the two groups of γ-secalins is not known. Chemical analysis shows great similarity in their amino acid compositions (notably 2.5 and 1.8 mol % cysteine in the 40k and 75k groups, respectively), N-terminal amino acid sequences (identical major residues at 17 out of 20 positions) and C-terminal amino acids (histidine in both) (Shewry et al 1982). The difference in aggregation may be determined by the presence or the absence of cysteine residues in conformations favorable for the formation of intermolecular disulfide bonds or by the specific action of enzyme systems responsible for the formation of such bonds. In either case, the primary structure (amino acid sequence) will be important, as this, together with environmental effects, will be responsible for the conformation and will provide recognition sites for the enzyme systems. These possibilities are being investigated in our laboratory.

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LITERATURE CITED

AUTRAN, J.-C. 1975. Nouvelle possibilités d'identification des variétés françaises de blé par électrophorèse des gliadines du grain. Ind. Aliment. Agric. 9-10:1075.

BERNARD, M., AUTRAN, J.-C., and JOUDRIER, P. 1977. Possibilités d'identification de certains chromosomes de siègle à l'aide de marqueurs biochimiques. Ann. Amelior. Plant. 27:355.

BIETZ, J. A., HUEBNER, F. R., SANDERSEN, J. E., and WALL, J. S. 1977. Wheat gliadin homology revealed through N-terminal amino acid sequence analysis. Cereal Chem. 54:1070.

BISHOP, L. R. 1939. The proteins of barley with special reference to hordein. M.Sc. thesis. University of Birmingham, U.K.

CHARBONNIER, L., TERCÉ-LAFOGUE, T., and MOSSÉ, J. 1981. Rye prolamins: Extractability, separation, and characterization. J. Agric. Food Chem. 29:968.

DILL, D. B., and ALSBERG, C. L. 1925. Preparation, solubility, and specific rotation of wheat gliadin. J. Biol. Chem. 65:279.

FAULKS, A. J., SHEWRY, P. R., and MIFLIN, B. J. 1981. The polymorphism and structural homology of storage polypeptides (hordein) coded by the *Hor-2* locus in barley (*Hordeum vulgare* L.). Biochem. Genet. 19:841.

FIELD, J. M., SHEWRY, P. R., MARCH, J., and MIFLIN, B. J. 1982. The purification and characterization of homologous high molecular weight storage proteins from grain of wheat, rye and barley. Theor. Appl. Genet. 62:329.

FRIEDMAN, M., KRULL, L. H., and CAVINS, J. F. 1970. The chromatographic determination of cystine and cysteine residues as S-β-(4-pyridylethyl) cysteine. J. Biol. Chem. 245:3868.

KASARDA, D. D., BERNARDIN, J. E., and NIMMO, C. C. 1976. Wheat proteins. Page 158 in: Advances in Cereal Science and Technology. Vol. I. Y. Pomeranz, ed. Am. Assoc. Cereal Chem., St. Paul, MN.

KIRKMAN, M. A., SHEWRY, P. R., and MIFLIN, B. J. 1982. The effect of nitrogen nutrition on the lysine content and protein composition of barley seeds. J. Sci. Food Agric. 33:115.

LANDRY, J., and MOUREAUX, T. 1970. Heterogeneité des glutelines du grain de mais: Extraction selective et composition en acides amines des trois fractions isolées. Bull. Soc. Chim. Biol. 52:1021.

MECHAM, D. K., KASARDA, D. D., and QUALSET, C. O. 1978. Genetic aspects of wheat gliadin proteins. Biochem. Genet. 16:831.

MIFLIN, B. J., BYERS, M., FIELD, J. M., and FAULKS, A. J. 1980. The isolation and characterization of proteins extracted from whole milled grain, gluten and developing protein bodies of wheat. Ann. Technol. Agric. 29:133.

MIFLIN, B. J., FIELD, J. M., and SHEWRY, P. R. 1982. Cereal storage proteins and their effects on technological properties. Page 255 in: Seed Proteins. J. Daussant and J. Mossé, eds. Academic Press, London.

MIFLIN, B. J., and SHEWRY, P. R. 1979a. The synthesis of proteins in normal and high lysine barley seed. Page 239 in: Recent Advances in the Biochemistry of Cereals. D. L. Laidman and R. G. Wyn Jones, eds. Academic Press, London.

MIFLIN, B. J., and SHEWRY, P. R. 1979b. The biology and biochemistry of cereal seed prolamins. Page 137 in: Seed Proteins Improvement in Cereals and Grains Legumes. Vol. I. IAEA, Vienna.

PREAUX, G., and LONTIE, R. 1975. The proteins of barley. Page 89 in: The Chemistry and Biochemistry of Plant Proteins. J. B. Harborne and C. F. Van Sumere, eds. Academic Press, New York.

PRESTON, K. R., and WOODBURY, W. 1975. Amino acid composition and subunit structure of rye gliadin proteins fractionated by gel filtration. Cereal Chem. 52:719.

RAHMAN, S., SHEWRY, P. R., and MIFLIN, B. J. 1982. Differential protein accumulation during barley grain development. J. Exp. Bot. 33:717.

SALCEDO, G., PRADA, J., and ARAGONCILLO, C. 1979. Low MW gliadin-like proteins from wheat endosperm. Phytochemistry 187:725.

SHEPHERD, K. W. 1968. Chromosomal control of endosperm proteins in wheat and rye. Page 86 in: Proc. 3rd Int. Wheat Genet. Symp. Aust. Academy of Sciences, Canberra, Australia.

SHEPHERD, K. W. 1973. Homoeology of wheat and alien chromosomes controlling endosperm protein phenotypes. Page 745 in: Proc. 4th Int. Wheat Genet. Symp. E. R. Sears and L. M. S. Sears, eds. Agric. Exp. Stn. Univ. of Missouri, Columbia.

SHEPHERD, K. W., and JENNINGS, A. C. 1971. Genetic control of rye endosperm proteins. Experientia 27:98.

SHEWRY, P. R., AUTRAN, J.-C., NIMMO, C. C., LEW, E. J.-L. and KASARDA, D. D. 1980a. N-terminal amino acid sequence homology of storage protein components from barley and a diploid wheat. Nature 286:520.

SHEWRY, P. R., ELLIS, J. R. S., PRATT, H. M., and MIFLIN, B. J. 1978a. A comparison of methods for the extraction and separation of hordein fractions from 29 barley varieties. J. Sci. Food Agric. 29:433.

SHEWRY, P. R., FAULKS, A. J., PICKERING, R. A., JONES, I. T., FINCH, R. A., and MIFLIN, B. J. 1980b. The genetic analysis of barley storage proteins. Heredity 44:383.

SHEWRY, P. R., FIELD, J. M., KIRKMAN, M. A., FAULKS, A. J., and MIFLIN, B. J. 1980c. The extraction, solubility and characterization of two groups of barley storage polypeptides. J. Exp. Bot. 31:393.

SHEWRY, P. R., FIELD, J. M., LEW, E. J.-L., and KASARDA, D. D. 1982. The purification and characterization of two groups of storage proteins (secalins) from rye (*Secale cereale* L.). J. Exp. Bot. 33:261.

SHEWRY, P. R., HILL, J. M., PRATT, H. M., LEGGATT, M. M., and MIFLIN, B. J. 1978b. An evaluation of techniques for the extraction and separation of hordein and glutelin from barley seed and a comparison of the protein composition of Bomi and Risφ 1508. J. Exp. Bot. 29:677.

SHEWRY, P. R., LEW, E. J.-L., and KASARDA, D. D. 1981. Structural homology of storage proteins coded by the *Hor-1* locus of barley (*Hordeum vulgare* L.). Planta 153:246.

SHEWRY, P. R., PRATT, H. M., FAULKS, A. J., PARMAR, S., and MIFLIN, B. J. 1979. The storage protein (hordein) polypeptide pattern of barley (*Hordeum vulgare* L.) in relation to varietal identification and disease resistance. J. Natl. Inst. Agric. Bot. 15:34.

SODEK, L., and WILSON, C. M. 1971. Amino acid composition of proteins isolated from normal, opaque-2 and floury-2 corn endosperms by a modified Osborne procedure. J. Agric. Food Chem. 19:1144.

WRIGLEY, C. W., DU CROS, D. L., ARCHER, M. J., DOWNIE, P. G., and ROXBURGH, C. M. 1980. The sulfur content of wheat endosperm proteins and its relevance to grain quality. Aust. J. Plant Physiol. 7:755.

WRIGLEY, C. W., and SHEPHERD, K. W. 1973. Electrofocusing of grain proteins from wheat genotypes. Ann. N.Y. Acad. Sci. 209:154.

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