# Molecular Weight and Charge Heterogeneity of Prolamins (Avenins) from Nine Oat (*Avena sativa* L.) Cultivars of Different Protein Content and from Developing Seeds

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ABSTRACT

Alcohol-soluble proteins (avenins) were extracted with 52% (v/v) ethanol (24°C) or 55% (v/v) isopropanol, 2.0% 2-mercaptoethanol (60°C) from mature seeds of nine oat (*Avena sativa* L.) cultivars ranging in total protein content from 11.9 to 19.1%. These fractions were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), isoelectric focusing (IEF), and two-dimensional analysis (IEF and SDS-PAGE). More of the major avenins were extracted with the isopropanol-mercaptoethanol mixture than with ethanol. They possessed molecular weights from 22,000 to 43,000 and pIs between pH 4.5 and 8.0. A significant degree of avenin heterogeneity existed, although variations in polypeptide patterns did not appear to correlate with differences in total seed protein content. Major avenin accumulation during seed development does not occur until two weeks after anthesis. Avenin components varied in rate and in order of appearance within the first four weeks following anthesis.

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Alcohol-soluble prolamins constitute a major storage protein fraction in cereals such as barley, corn, rye, sorghum, and wheat (Mossé 1968). Prolamins of oat (avenins), however, represent only a minor fraction relative to the seed's total protein content (Kim 1978). In view of this, the role of avenin as a storage protein may be questioned, especially given the unusually high concentration of globulins in oat (Robert et al 1983a).[3] Avenins may simply correspond to structural or metabolic proteins that are soluble in aqueous alcohol. However, the avenin fraction is notably similar to other cereal prolamins known to serve a major reserve function. For example, avenins contain unusually large amounts of glutamate and proline, but are especially low in lysine (Draper 1973, Weiser et al 1980), exhibit a significant degree of polymorphism (Kim and Mossé 1979), are present in protein bodies (Pernollet et al 1982), and are hydrolyzed upon germination (Kim et al 1979). Hence, oat prolamins are likely reserve proteins, which, throughout evolution have become (or remained) of limited quantitative importance in the oat seed. This low concentration of avenin is particularly interesting in view of the prominent role played by prolamins in determining the nutritive value of other cereal crops.

In studying 19 oat cultivars of different nitrogen content, Frey (1951) reported no significant variations in the proportions of their alcohol-soluble fraction. But qualitative changes may still exist within the avenin fraction among cultivars varying significantly in total seed protein content. Peterson and Smith (1976) showed that alcohol-soluble nitrogen accumulated in the developing oat seed beginning at four days after anthesis. At the early stages of development, however, the alcohol-soluble nitrogen may contain considerable nonprotein nitrogen, and thus not represent the avenins per se. We investigated, first, the heterogeneity of the avenins from nine oat cultivars differing markedly in protein content; and second, the rate and order of appearance of these proteins in the developing oat seed.

## MATERIALS AND METHODS

### Plant Material

The following oat cultivars were generously supplied by V. Burrows, Ottawa Research Station, Agriculture Canada: Hinoat (19.1% protein); Dal (18.1%); Sentinel (17.9%); Elgin (17.0%); Harmon (15.6%); OA-269 (15.6%); OA-424-1 (14.8%); OT-213 (14.5%); and Donald (11.9%). The growth conditions of the various cultivars and the methods of analysis of their protein content have been published (Robert et al 1983b). Seeds (cultivar Elgin) harvested at weekly intervals following anthesis were used for the developmental study. The seeds were mature and dry at the sixth and final harvest.

### Protein Extraction

Manually dehulled groats were finely milled in an electric coffee grinder. Each oat flour sample (3 g) was stirred magnetically for 2 hr in 100 ml of solvent. Prolamins were extracted with 52% (v/v) ethanol at room temperature (Kim et al 1978) or with 55% (v/v) isopropanol, 2.0% (v/v) 2-mercaptoethanol (ME) at 60°C (Shewry et al 1978). Following extraction, the slurry was centrifuged (15,000 × *g*, 30 min, 24°C), and an equal volume of 4.0% (w/v) NaCl was added to the supernatant. The prolamins were precipitated overnight at 4°C, recovered by centrifugation (22,000 × *g*, 45 min, 4°C), dialyzed, and lyophilized. Prolamins for the developmental study were obtained after the salt-soluble proteins had been extracted with 1.0*M* NaCl, 0.05*M* tris(hydroxymethyl)aminomethane (Tris), pH 8.5 (3 g/200 ml, 3 hr, 28°C). Globulins were precipitated from the salt extract by dialysis against running water. They were recovered by centrifugation (22,000 × *g*, 45 min, 4°C) and lyophilized.

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### Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

A modified version of the Laemmli (1970) procedure was used. The resolving gel contained 14.0% acrylamide, 0.20% *N,N'*-methylene bisacrylamide (bis), 0.1% SDS, and 4.0 *M* urea in 0.375 *M* Tris/HCl buffer, pH 8.8. The stacking gel was composed of 3.8% acrylamide, 0.63% bis, 4.0 *M* urea, and 1.0% SDS in 0.125 *M* Tris/HCl buffer, pH 6.8. The running buffer was a solution containing 0.3% Tris, 1.44% glycine, and 0.1% SDS. Lyophilized protein samples (10 mg/ml, unless indicated otherwise) were dissolved in a sample buffer of 8.0 *M* urea, 1.0% SDS, 0.0 or 1.0% ME, and 0.65 *M* Tris, pH 6.8. Samples were heated in boiling water for 2.5 min before electrophoresis. Gels were run overnight at 80 V constant voltage and stained for 6 hr with 0.2% Coomassie brilliant blue R-250 (CBBR-250) in acetic acid–methanol–water (10:25:65) v/v). They were destained with acetic acid–methanol–water (10:25:65, v/v).

### Isoelectric Focusing (IEF)

IEF was performed with gels made up of 5.0% acrylamide, 0.2% bis, 6.0 *M* deionized urea, and 5.0% pH 3–10 ampholyte (Pharmacia). Protein samples (10 mg/ml, unless otherwise indicated) were solubilized in a sample buffer containing 10 m*M* glycine, 6.0 *M* urea, 1.0% ME, 1.0% Triton X-100 (pH 8.0 adjusted with Tris). The gels were prefocused at 9 W constant power for 30 min on a Pharmacia flatbed apparatus. Aliquots (25 $\mu$l) added at the anodic end were focused for 2.5 hr at 13 W. The pH gradient was measured using a surface pH electrode and pI standards. No correction was made to account for the presence of urea. Following protein fixation with 10% trichloroacetic acid for 2 hr, the gels were washed overnight with running water. They were stained for 3 hr with 0.05% CBBR-250 in acetic acid–methanol–water (10:25:65, v/v) and destained as previously mentioned.

### Two-Dimensional Analysis (IEF and SDS-PAGE)

Prolamin samples were first separated according to charge as described in the IEF section. Individual lanes were cut and equilibrated in O'Farrell (1975) buffer O (without ME or glycerol) for 45 min. Each lane was then placed on top of a second gel and resolved according to molecular weight as described in the SDS-PAGE section. The IEF lane was held in place within an additional layer of stacking gel. Following electrophoresis, the gels were fixed for 1.5 hr in 10% trichloroacetic acid (TCA), 3% sulfosalicylic acid and washed (2 × 1 hr) in 50% methanol, 10% acetic acid to remove excess ampholytes. Gels were stained for 6 hr with 0.2% CBBR-250 in acetic acid–methanol–water (10:40:50, v/v) and destained with acetic acid–methanol–water (10:40:50, v/v).

## RESULTS AND DISCUSSION

### Avenins from Different Cultivars

Figure 1 illustrates the typical SDS-PAGE patterns of the avenins obtained with the 52% (v/v) ethanol extraction from the nine oat cultivars: a, Hinoat; b, Dal; c, Sentinel; d, Elgin; e, Harmon; f, OA-269; g, OA-424-1; h, OT-213; and i, Donald. The major avenin polypeptides have molecular weights between 22,000 and 40,000. Although the same avenin constituents were observed in the presence of a reducing agent, they displayed an increase in their apparent molecular weights of approximately 1,000–2,000 (data not shown). Such an increase in the molecular weight of avenins under reducing conditions was reported previously (Bietz 1982). The higher molecular weight of the avenin components may be attributed to the breakage of intramolecular disulfide bonds, causing the polypeptides to unfold and retarding their migration. Because the avenin patterns remained unchanged, these proteins are probably not joined by intermolecular disulfide linkages. The avenin molecular weight values of 20,000–34,000, mentioned by Kim et al (1979), and of 22,000–33,000, reported by Peterson and Brinegar,[4] fall within the range we observed. Differences in these estimations may be caused by inherent variations existing among the cultivars studied. Two minor bands (approximately 15,000 and 16,000) appear consistently in the avenin SDS-PAGE patterns. These may belong to a class of prolamins similar to the low molecular weight 'A' hordeins of barley (Shewry et al 1978).

Miflin and Shewry (1979a) reported that the use of aqueous propanol (rather than aqueous ethanol) and the presence of a reducing agent can improve the efficiency of cereal prolamin extractions. Figure 2 demonstrates the typical SDS-PAGE patterns of the different oat prolamins obtained by such a combination of solvents. There was a reduction in the stain-binding capacity of the proteins, and all the avenin patterns differed. Additional polypeptides were extracted by the isopropanol-ME mixture in the majority of cases.

The SDS-PAGE results obtained in this study show that the oat prolamins display a significant degree of intraspecific polymorphism. Kim and Mossé (1979) acknowledged the utility of the avenin heterogeneity as a means of oat cultivar identification. It is interesting to note, however, the close similarity of the avenin patterns (Figs. 1 and 2) from the cultivars OT-213 (h) and Donald (i) which, as the other cultivars, are essentially unrelated (V. Burrows, *personal communication*). Although Frey (1951) reported no significant quantitative changes in the proportions of the alcohol-soluble nitrogen fraction in cultivars that varied in total protein content, it is obvious that qualitative variations can occur. The avenin polypeptide patterns for the nine cultivars listed above appear more heterogeneous than their corresponding globulin patterns (Robert et al 1983b), but it is still difficult to correlate the occurrence or intensity of particular bands with the total seed protein content. There appear, however, to be fewer distinct avenin

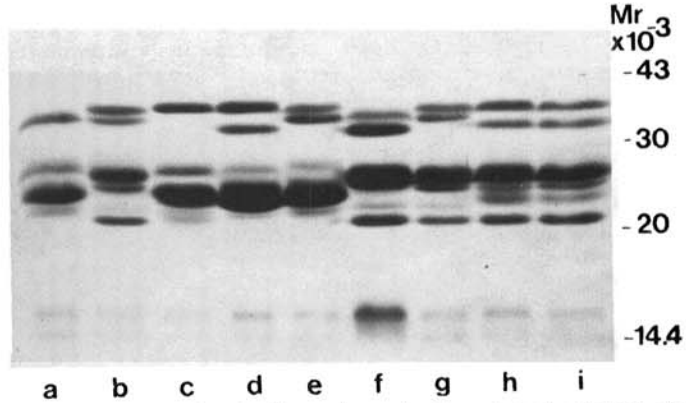


**Fig. 1.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of avenins extracted with 52% (v/v) ethanol from (in decreasing order of protein content): a, Hinoat; b, Dal; c, Sentinel; d, Elgin; e, Harmon; f, OA-269; g, OA-424-1; h, OT-213; and i, Donald. Molecular weight standards: ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000), and $\alpha$-lactalbumin (14,400).

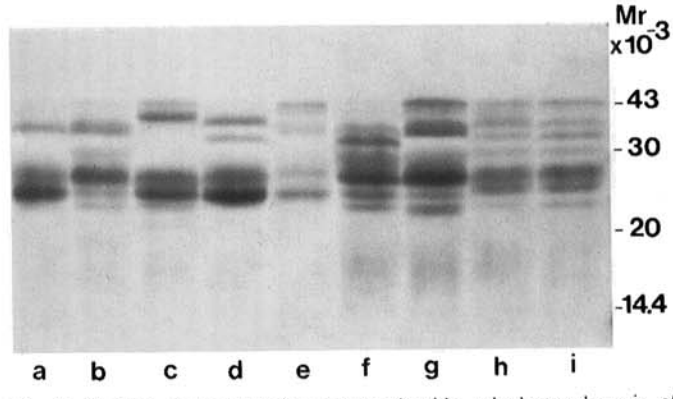


**Fig. 2.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of avenins extracted with 55% (v/v) isopropanol, 2.0% 2-mercaptoethanol from (in decreasing order of protein content): a, Hinoat; b, Dal; c, Sentinel; d, Elgin; e, Harmon; f, OA-269; g, OA-424-1; h, OT-213; and i, Donald. Molecular weight standards: ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000), and $\alpha$-lactalbumin (14,400).

[4] See n. 3 above.

components in the cultivars with higher protein content (eg, compare Hinoat (a) and Donald (i), Fig. 2).

Since the isopropanol-ME mixture provided a more complete extraction of oat prolamins, the remaining experiments involved only avenin obtained with this solvent. Figure 3 represents the typical polypeptide distribution of the nine different avenin preparations focused within a pH range of 3–10. Isoelectric focusing revealed an even greater avenin heterogeneity than was evident from the SDS-PAGE analysis or from previous reports. Such a considerable degree of charge heterogeneity has also been observed with prolamins from barley (Shewry et al 1978), corn (Righetti et al 1977), rye (Shewry et al 1982), and wheat (Kasarda et al 1976). The majority of the avenin bands were found between pH 4.5 and 8.0. The different patterns were quite similar, in spite of the large number of bands present and the inherent differences among the various cultivars. For example, major bands were found consistently in all the cultivars (Fig. 3, arrow and bracket). Some bands appeared to be relatively more intense in some cultivars than in others (Fig. 3, bars). This may indicate a differentiated mode of biosynthetic regulation of these particular polypeptides, but it does not appear to be correlated to the total protein content of the seed. Actually, contrary to the previous gels (Figs. 1 and 2), cultivars with the higher protein contents displayed the larger number of avenin bands.

Differences in the banding patterns between the cultivars OT-213 (h) and Donald (i) that were not obvious upon SDS-PAGE became apparent with the higher resolution of IEF. Hence, even though SDS-PAGE of oat prolamins may be a suitable method of cultivar identification in most cases, combining it to IEF data would be more reliable.

Cultivars Elgin and OA-424-1 represent the major variations in avenin patterns displayed by the cultivars studied. Two-dimensional gels (IEF and SDS-PAGE) of their avenins (Figs. 4 and 5, respectively) show the corresponding molecular weight and pI of each band observed upon the one-dimensional separations. Both cultivars have a similar spot (A) of molecular weight 28,000 corresponding to the major IEF band to which the arrow points in Fig. 3. Similarly, the (B) region in Figs. 4 and 5 displays some common features. Spots within this region have molecular weights between 35,000 and 43,000 and include the major IEF doublet bracketed in Fig. 3. In cultivar OA-424-1, the more basic component of the doublet also gives rise to a major low molecular weight spot (C).

Oat prolamins, like those from barley, corn, rye, and wheat, involve a large number of polypeptides found within a limited range of molecular weights and charge distribution. Bietz (1982) reported the close homology in the *N*-terminal amino acid sequence of avenin components. Different allelic forms and post-translational modifications could account for some of the avenin heterogeneity we have observed. Nonetheless, part of this heterogeneity likely results from the divergent evolution of duplicated single ancestral genes. Evidence in other cereals showing persistent polymorphism in homozygous populations (Miflin and Shewry 1979b, Shewry et al 1983) and messenger RNA microheterogeneity (Marks and Larkins 1982) favors such a possibility.

Although the quantity of prolamins is much lower in oat, they do not demonstrate a reduced degree of polymorphism when compared to that observed in cereals where these proteins occur in greater proportions. Thus, extensive heterogeneity does not seem to be either a consequence or a requirement for a major storage protein role in cereals, but rather a result of relatively low selective pressures. In addition, barley, rye, and wheat contain a high concentration of prolamin and a comparatively small amount of globulins, whereas the situation is reversed in oat. Yet these four cereals are phylogenetically related. This may denote the genetic flexibility inherent to the storage protein system and hence the feasibility of manipulations aimed at improving the nutritional quality of cereal proteins without the loss of important agronomic properties.

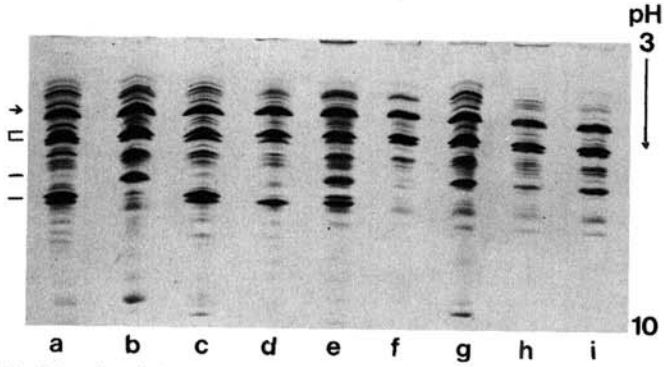


**Fig. 3.** Isoelectric focusing (pH 3–10) of avenins from (in decreasing order of protein content): a, Hinoat; b, Dal; c, Sentinel; d, Elgin; e, Harmon; f, OA-269; g, OA-424-1; h, OT-213; and i, Donald. Arrow and bracket indicate common major bands; bars indicate important variations among cultivars.

### Developmental Appearance of Avenins

Avenins from developing Elgin seeds were not visible one week after anthesis (data not shown) and were barely detectable after two weeks; the typical avenin SDS-PAGE pattern was evident thereafter (Fig. 6). Some polypeptides varied in relative intensity, especially between the second and fourth weeks. Lower molecular weight polypeptides (22,000, 16,000, 15,000) became evident only at the fourth week (Fig. 6, arrows).

The same extracts were also subjected to IEF (Fig. 7). Similar to the SDS-PAGE patterns, there is very little protein evident at two

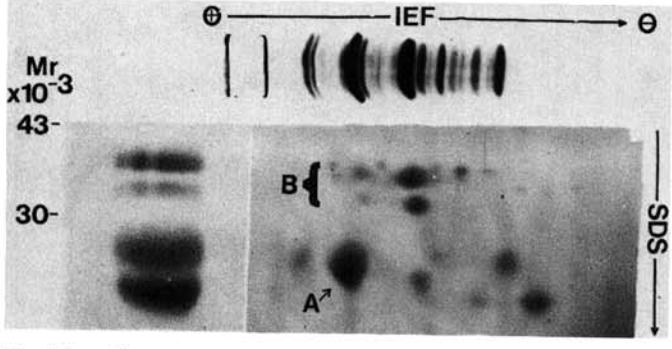


**Fig. 4.** Two-dimensional analysis (IEF, pH 3–10 followed by SDS-PAGE) of avenins from the cultivar Elgin. Molecular weight standards: ovalbumin (43,000) and carbonic anhydrase (30,000). **A** and **B** refer to polypeptides discussed in the text.

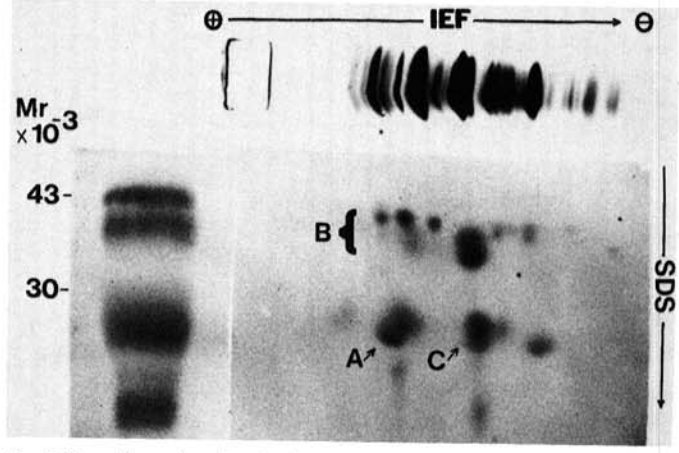


**Fig. 5.** Two-dimensional analysis (IEF, pH 3–10 followed by SDS-PAGE) of avenins from the cultivar OA-424-1. Molecular weight standards: ovalbumin (43,000) and carbonic anhydrase (30,000). **A, B,** and **C** refer to polypeptides discussed in the text.

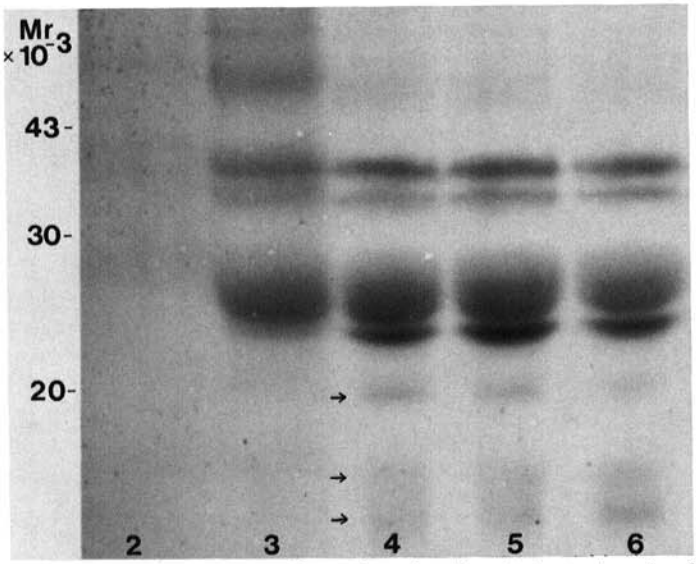


**Fig. 6.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of avenins from developing Elgin seeds harvested weekly following anthesis. Numbers 2–6 correspond to weeks post-anthesis. Because of different proportions of nonprotein material found in the protein extracts, the 25-μl aliquots analyzed from harvest 2 contained 50 mg/ml, harvest 3 40 mg/ml, and harvests 4–6 10 mg/ml of protein sample. Molecular weight standards: ovalbumin (43,000), carbonic anhydrase (30,000), and soybean trypsin inhibitor (20,000). Arrows indicate polypeptides that became apparent at the fourth harvest.

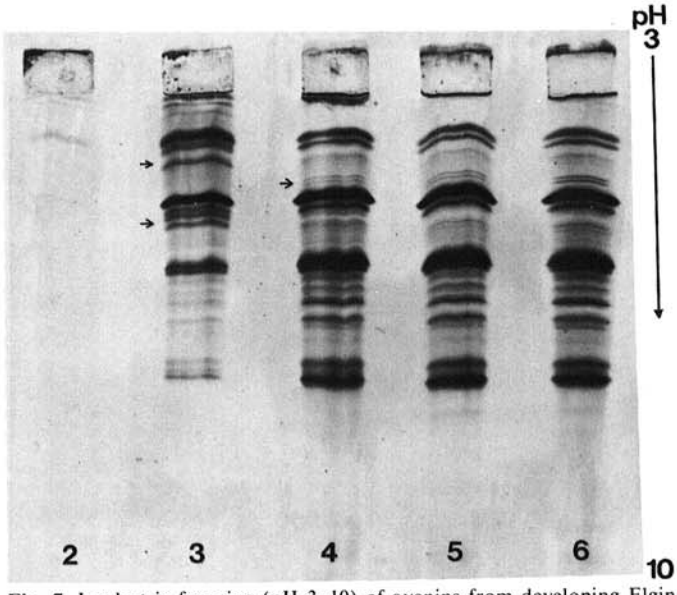


**Fig. 7.** Isoelectric focusing (pH 3–10) of avenins from developing Elgin seeds harvested at weekly intervals following anthesis. Numbers 2–6 correspond to weeks post-anthesis. Because of different proportions of nonprotein material found in the protein extracts, the 25-μl aliquots analyzed from harvest 2 contained 35 mg/ml, harvest 3 25 mg/ml, and harvests 4–6 10 mg/ml of protein sample. Arrows indicate significant variations occurring in avenin polypeptide patterns between harvests 3 and 4.

weeks post-anthesis. Variations in the avenin patterns of the third and fourth weeks were again apparent (Fig. 7, arrows).

These findings persisted upon repeated analysis and suggest that major avenin biosynthesis does not begin until two weeks post-anthesis and that some differentiation exists in the rate and order of appearance of the different avenin constituents. Analogous results have been reported for barley (Rahman et al 1982) and wheat prolamins (Mecham et al 1981). Robert et al (1983b), using SDS-PAGE, evidenced the differential accumulation of the different oat globulin polypeptides. Figure 8 represents the IEF patterns of

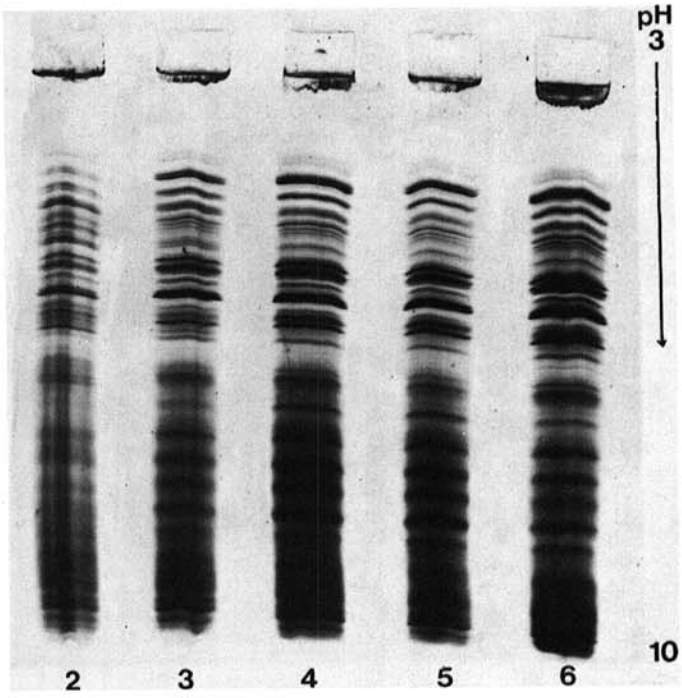


**Fig. 8.** Isoelectric focusing (pH 3–10) of globulins from developing Elgin seeds harvested at weekly intervals following anthesis. Numbers 2–6 correspond to weeks post-anthesis.

globulins obtained from the same seeds used for the avenin extraction. Although little globulin is apparent at one week post-anthesis (data not shown), it becomes apparent that significant globulin biosynthesis starts approximately one week before that of the avenins.

## CONCLUSION

The mixture of isopropanol and ME appears to be a more appropriate solvent for extensive extraction of oat prolamins than the commonly used aqueous ethanol solution. Even though avenins are known to constitute a minor seed protein fraction, these were shown by SDS-PAGE and IEF to involve considerable polypeptide heterogeneity. Nonetheless, the majority of avenin constituents was found within limited ranges of molecular weights (22,000–43,000) and isoelectric points (pI 4.5–8.0). Similar to oat globulins, the avenin fraction displayed some differences in rate and sequence of appearance of its components. The relatively low final concentration of prolamins in oat may partly be due to the fact that major avenin synthesis begins approximately one week after that of the globulins.

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