Characterization of Oat (*Avena sativa* L.) Residual Proteins

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ABSTRACT

Oat proteins remaining in the residue following the extraction of the albumins, globulins, and prolamins were studied. The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) pattern observed for the residual proteins extracted with 7*M* urea and 2-mercaptoethanol (2-ME) was identical to that obtained with 1% SDS and 1% 2-ME, which is known to completely extract these proteins. The substitution of urea for SDS in solubilizing the oat residual proteins permitted their characterization by two-dimensional analysis (isoelectric focusing, pH 3.5–10 followed by SDS PAGE). Most of the polypeptides of the residual fraction were found to comigrate with prolamins and especially globulins. However, minor polypeptide groups did not correspond to either prolamins or globulins and these minor proteins likely represent oat glutelins. Western blot analysis of the residual proteins using anti-oat 12S immunoglobulin G (IgG) demonstrated conclusively that the major proteins of the oat residue were globulins. These results support recent reports estimating globulin levels at 70–80% of the total oat proteins and consequently, glutelin levels of less than 10%.

In relation to the other seed protein fractions, cereal glutelins have typically been difficult to completely solubilize and therefore to study. In oats, both acidic (Ewart 1968, Kim et al 1979) and basic (Draper 1973, Peterson and Smith 1976, Ma 1983) solutions have been used to solubilize the glutelin fraction. Unfortunately, these extractions are generally incomplete and some nitrogen remains in the residue. In addition, the quantity of glutelins observed is often directly related to the efficiency of the preceding extractions of the albumins, globulins, and prolamins (Shewry et al 1978). This is especially true if the insoluble residual nitrogen is also considered as glutelins (Ewart 1968, Kim et al 1979, Weiser et al 1980). Hence, different extraction schemes usually yield different proportions of glutelins present in oats. Values ranging from approximately 5% (Brohult and Sandegren 1954) to 66% (Ewart 1968, Kim et al 1979) have been reported.

Recently, the total solubilization of oat proteins was achieved with a solution containing sodium dodecyl sulfate (SDS) and 2-mercaptoethanol (2-ME) (Robert et al 1983a). After the albumins, globulins, and prolamins had been removed, this solution was also used to completely extract the remaining residual proteins, which necessarily included any glutelins. Upon SDS polyacrylamide gel electrophoresis (SDS PAGE) these residual proteins were found to consist primarily of polypeptides comigrating with prolamin and especially globulin components. The proportion of oat glutelins was then assumed to be less than 5–10%. However, SDS PAGE is limited in its resolving capacity, and the residual proteins comigrating with the prolamins and globulins could hypothetically conceal (or even be) different proteins, especially considering the large number of proteins that can be found in a seed.

In this paper we investigated in greater detail the composition of the oat residual protein fraction in order to establish the nature of its constituents.

MATERIALS AND METHODS

Sequential Extraction

Dry mature seeds of oats (*Avena sativa* L. cv. Hinoat) were provided by V. Burrows, Ottawa Research Station, Agriculture Canada. Manually dehulled seeds were ground in an electric coffee grinder and the flour proteins extracted following a typical modification of the Osborne fractionation scheme. Salt-soluble proteins were removed from 3 g of oat flour at room temperature (rt) by stirring 3 hr with 100 ml of 1*M* NaCl, 0.05*M* tris(hydroxymethyl)aminomethane (Tris) at pH 8.5. The slurry was centrifuged (15,000 × *g*, 30 min, rt) and the supernatant dialyzed for 48 hr at 4°C against several changes of distilled water to precipitate the globulins. The meal pellet was resuspended in 100 ml of 52% (v/v) ethanol and the prolamins similarly extracted. To prevent possible glutelin co-solubilization, the reducing agent 2-ME was not used in the prolamin extraction. Elevated temperatures were also avoided to minimize any adverse effects on glutelin solubility (Wilson et al 1981). Following centrifugation of the slurry (15,000 × *g*, 30 min, rt), the pellet (residue) was air-dried, and the remaining proteins were solubilized by homogenization of 0.4-g aliquots of residual flour in a glass tissue homogenizer for 20 min with 4 ml of 1% SDS or 5 ml of 7*M* urea. This extraction was followed by centrifugation (22,000 × *g*, 45 min, rt), and the supernatant was kept for analysis. The latter two extractions were performed both in the presence and absence of 1% 2-ME.

Electrophoresis

Aliquots of residual protein extracts were added to an equal volume of electrophoresis sample buffer (8*M* urea, 1% SDS, 0.65*M* Tris, pH 6.8) in the presence or absence of 1% 2-ME. SDS PAGE was carried out in 12 and 14% acrylamide gels as described previously (Robert et al 1983a).

Two-dimensional (2-D) analysis consisting of isoelectric focusing (IEF) in a pH 3.5–10 range (Ampholines, LKB) followed by SDS PAGE was performed as reported by Robert et al (1983b). To promote the detection of the oat prolamins and residual proteins, the gels were silver stained (Bio-Rad Silver Stain Kit, bulletin 1089).

Western Blot Analysis

Antibodies were raised in New Zealand white rabbits against oat 12S globulins which had been obtained by isoelectric point precipitation and purified by three cycles of sucrose density gradient centrifugation (Adeli and Altosaar 1984). IgG was isolated by affinity chromatography on Protein A Sepharose (Tan-Wilson et al 1976, Matlashewski 1983).

Anti-oat 12S IgG was labelled with ^{125}I (low pH, high concentration, New England Nuclear) according to a modification of the procedure used by Greenwood et al (1963) as described by Robert et al (1984a). The specific activity was approximately 3μCi/μg of IgG. Immediately following SDS PAGE, protein transfer was carried out electrophoretically for 24 hr at 135 mA as reported by Towbin et al (1979). The nitrocellulose sheet (0.45 μm, BA85, Schleicher and Schuell) was preincubated for 1 hr at 40°C in 50 ml of 3% bovine serum albumin and 3% normal rabbit serum in phosphate buffered saline (0.14*M* NaCl, 2.7 m*M* KCl, 8.1 m*M* $Na_2HPO_4 \cdot 7H_2O$, 1.5 m*M* KH_2PO_4, pH 7.4). After washing in phosphate buffered saline, the sheet was then challenged with the ^{125}I-labeled antibody for 6 hr, washed, dried, and exposed to Kodak X-Omat AR film as outlined by Robert et al (1985a).

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RESULTS AND DISCUSSION

To perform IEF for 2-D analysis of the oat residual proteins, an extracting solution without SDS had to be found that would also provide the extensive solubilization of these proteins previously obtained with this detergent (Robert et al 1983a). The SDS PAGE patterns of the 7*M* urea and 1% SDS extracts of the oat residual proteins are shown in Figure 1. If globulins make up a significant fraction of the residual proteins, a major group of polypeptides should comigrate with standard unreduced oat globulins (lane a, mol wt 52,000–70,000) in the absence of 2-ME. This was apparently the case with the 1% SDS extract (lane b), but the situation was not as clear with the 7*M* urea extract (lane c). This difference seems to be caused by a higher level of protein aggregation in the urea extract limiting protein entry into the gel and resolution. If the residual proteins found at molecular weights 52,000–70,000 in the SDS extract include globulins, the latter should dissociate into the individual α-(33,000–43,000 mol wt) and β-(20,000–25,000 mol wt) globulin subunit groups upon addition of 2-ME. Indeed, a predominance of polypeptides comigrating with the standard α and β oat globulin subunit groups (lane h) was observed for the SDS extract under reducing conditions (lane f). There was a concomitant decrease in the amount of the higher molecular weight proteins. The urea extract (lane d) similarly displayed proteins comigrating with the reduced globulin subunits despite the low amount of 52,000–70,000 mol wt proteins in the unreduced extract (lane c). In the presence of 2-ME, the SDS PAGE patterns of the urea and SDS extracts were essentially identical. This was true whether the reducing agent was added to the final sample as above or was originally present in the extracting solution (lanes e and g, respectively). These two extracts were therefore assumed to be equivalent, and the urea (+ 2-ME) extract was used for 2-D analysis.

If the residual proteins with molecular weights equivalent to the α-and β-globulin subunit groups are really globulins, they should also exhibit the respectively acidic and basic charge distribution typical of these subunits (Brinegar and Peterson 1982a, Walburg and Larkins 1983). The 2-D analysis might also reveal different proteins comigrating with the globulins or prolamins which were not evident upon one-dimensional SDS PAGE. Figure 2 represents 2-D analyses of the globulins, prolamins, and proteins remaining in the residue following the extraction of the albumins, globulins, and prolamins. The great majority of the residual polypeptides of molecular weights equivalent to those of the globulin subunit groups (Fig. 2C, brackets) also displayed the characteristic charge distribution of these subunits (Fig. 2A). With silver staining the lighter color of some protein spots (e.g., α subunits) is mainly attributed to the large amount of protein present. Major protein spots found between the α- and β-globulin subunit groups consisted primarily of prolamins (Fig. 2B). There were, however,

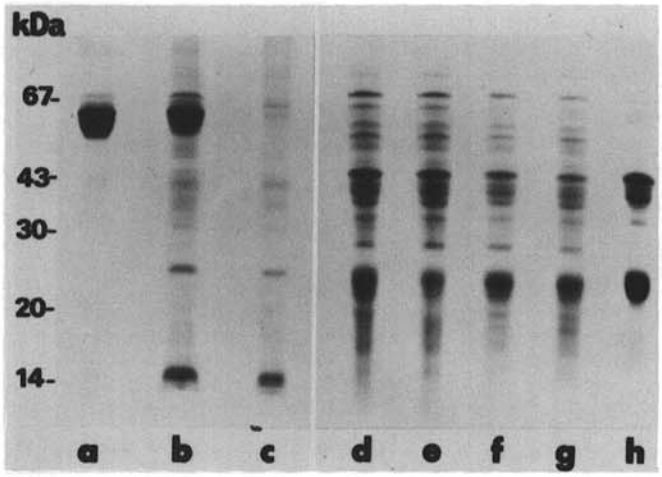

Fig. 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of: **(a)** standard oat globulin extracted and electrophoresed (− 2-mercaptoethanol, 2-ME); oat residual proteins extracted with **(b)** 1% SDS (− 2-ME), **(c)** 7*M* urea (−2-ME), and electrophoresed (−2-ME); oat residual proteins extracted with **(d)** 7*M* urea (− 2-ME), **(e)** 7*M* urea (+ 2-ME), **(f)** 1% SDS (− 2-ME), **(g)** 1% SDS (+ 2-ME), and reduced before electrophoresis; **(h)** standard oat globulin extracted and electrophoresed (+ 2-ME). Molecular weight markers: bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400).

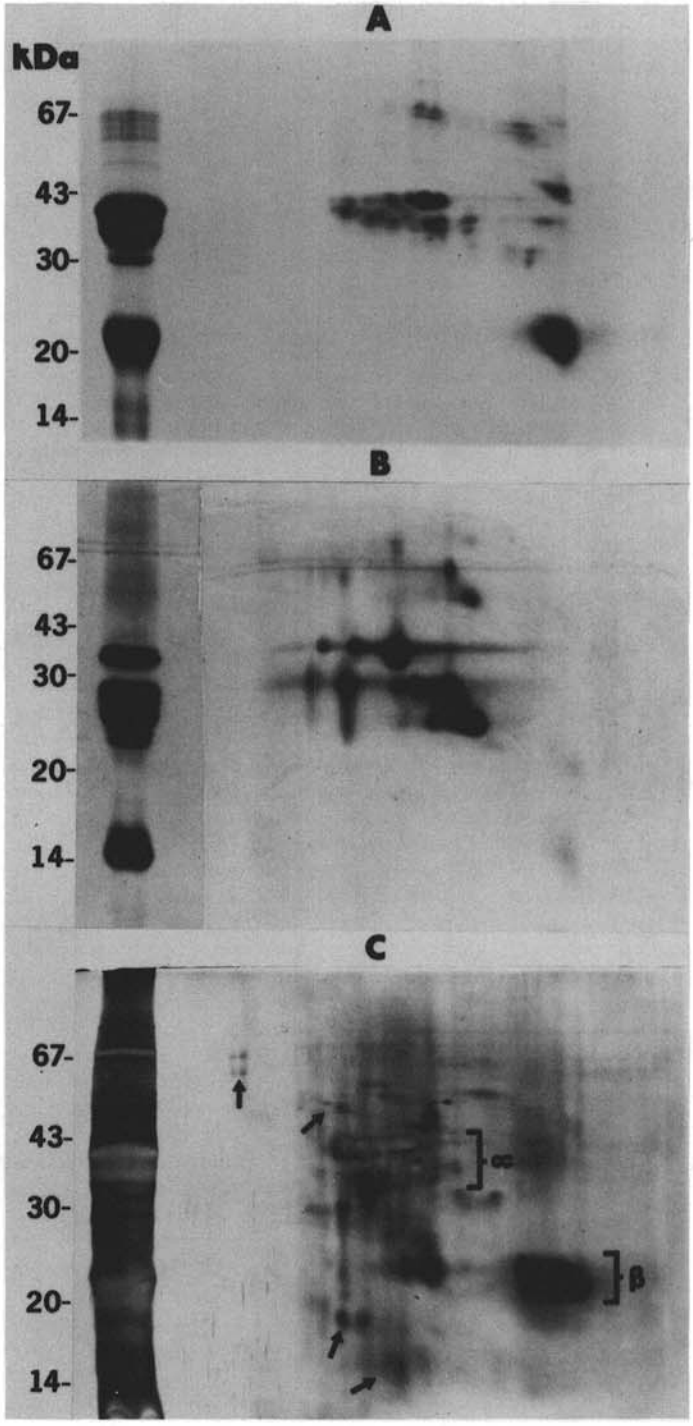

Fig. 2. Two-dimensional analysis (isoelectric focusing, pH 3.5–10 followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis) of Hinoat **(A)** globulins, **(B)** prolamins, and **(C)** residual proteins. Molecular weight markers: bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400). Brackets denote α- and β-globulin subunits, and arrows point to polypeptides, which are probably glutelins.

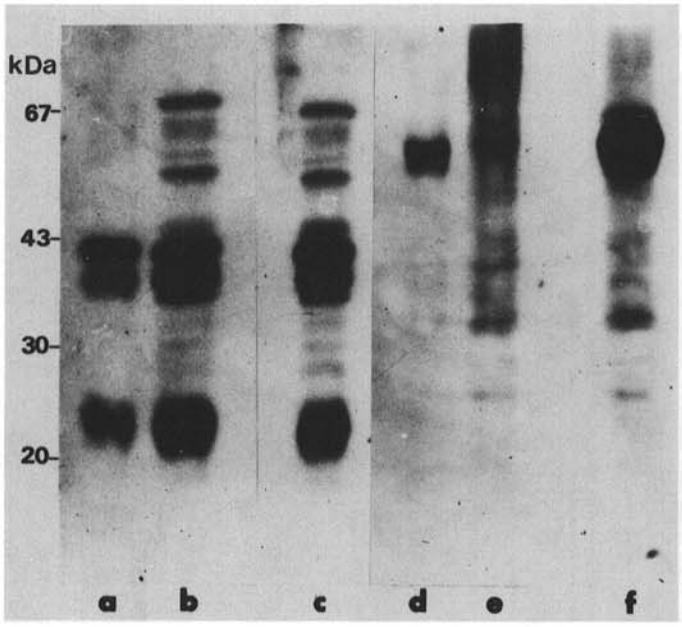

Fig. 3. Western blot analysis of oat protein extracts using ^{125}I-labeled anti-12S oat IgG: **(a)** standard oat globulin in the presence of 2-mercaptoethanol (+ 2-ME); oat residual proteins extracted with **(b)** 7*M* urea (+ 2-ME), **(c)** 1% sodium dodecyl sulfate (SDS) (+ 2-ME), and **(d)** standard oat globulin (− 2-ME); oat residual proteins extracted with **(e)** 7*M* urea (− 2-ME) and **(f)** 1% SDS (− 2-ME). Molecular weight markers: bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30.000), and soybean trypsin inhibitor (20,000).

small clusters of protein spots that did not comigrate with the 2-D pattern of either the globulins or the prolamins (Fig. 2C, arrows). By process of elimination, these proteins are likely true candidates for the glutelin fraction (albumins, being water soluble, are not likely to be still present in significant amounts in the residue).

Although very slight, the possibility still exists that the residual proteins comigrating with the globulins were glutelins with similar molecular weight and charge distributions. Convergent evolution brought about by functional constraints for the biosynthesis, assembly, packaging, and hydrolysis of these proteins might cause such a similarity. To investigate this possibility, the residual proteins from oats were challenged with ^{125}I-labeled anti-oat 12S IgG. The α- and β-globulin subunits are the building blocks of the 12S holoprotein (Peterson 1978). Results presented in Figure 3 show that the residual proteins comigrating with globulins evidently possessed antigenic determinants recognized by the antibody. The antibody hybridized selectively to the reduced standard globulin subunits (lane a) and also cross-reacted with polypeptides of identical molecular weights in both the urea (lane b) and SDS (lane c) extracts. These polypeptides can, therefore, be considered globulins. Interestingly, the antibody also recognized polypeptides in the 60,000 mol wt region within these extracts. The individual oat globulin subunits are known to arise from the post translational cleavage of precursors of approximately 60,000 mol wt (Matlashewski et al 1982, Brinegar and Peterson 1982b, Walburg and Larkins 1983, Adeli and Altosaar 1983, Adeli et al 1984). The polypeptides recognized by the antibody in this region may correspond to uncleaved globulin precursors having a reduced salt solubility.

With the SDS extract, in the absence of 2-ME, the radioactive signal shifted from the subunit groups to the 52,000–70,000 mol wt region (lane f). This supports the hypothesis that the latter proteins are the result of the disulfide association of the globlulin subunits (as in lane d). With the urea (− 2-ME) extract, a smear of radioactivity was detected in the high molecular weight region (lane e), again suggesting poor resolution due to protein aggregation. The fact that a reducing agent is necessary to properly resolve the globulins present in the urea extract may partly explain their insolubility during the preceding salt extraction and consequently their occurrence in the residue. In addition to protein aggregation, interactions with cell constituents (membrane, lipids, phenolics . . .) may also account for the insolubility of some globulins in a salt solution. An extreme example of this phenomenon occurs in rice, where 12S globulin-like proteins are almost insoluble in salt solutions and are found largely as residual proteins (Zhao et al 1983, Robert et al 1985b).

The finding that prolamins, and to a greater extent globulins, contribute the major portion of the oat residual proteins indicates that quantities previously reported for these protein fractions following Osborne fractionation were likely underestimates. The value of approximately 70–80% recently attributed to the globulin proportion of the total oat seed protein seems to reflect more closely the actual situation (Robert et al 1983a, Colyer and Luthe 1984). Therefore, oat glutelins must consequently account for less than 5–10% of the total seed proteins.

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