Alcohol-Soluble Proteins of Grain Sorghum

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ABSTRACT

Kafirin, the alcohol-soluble protein of grain sorghum, is a complex mixture of several protein components as revealed by disc electrophoresis in polyacrylamide gels. Disulfide groups of kafirin have been cleaved by oxidation or reduction and the products have been analyzed by gel electrophoresis and gel filtration. The electrophoretic pattern of reduced-alkylated kafirin, at pH 3.1, is similar to that of the unmodified kafirin. Gel filtration experiments on oxidized and reduced-alkylated kafirin have shown that cleavage of disulfide bonds does not greatly alter the molecular size of this protein. On reoxidation of reduced kafirin, the electrophoretic pattern and presumably the native structure of kafirin is restored. These studies have led to the conclusion that the disulfide bonds of kafirin occur predominantly in the intrachain form, analogous to the disulfide bonds of wheat gliadin.

The alcohol-soluble protein in grain sorghum represents a quantitatively significant fraction of the total endosperm protein. The proportion of this prolamin, commonly referred to as "kafirin," may range from 40 to 60% of the sorghum protein (1).

Kafirin can be extracted from sorghum endosperm meal with 60% aqueous ethanol at 60°C., in good yields (2). Further studies on the physicochemical characterization of this prolamin fraction have been hampered because of its insolubility in neutral aqueous solvents. However, disc electrophoresis in polyacrylamide gels in presence of 8*M* urea resolves kafirin into a number of protein bands (3), showing that it is a mixture of several protein components.

Considerable progress has been made in recent years in elucidating the molecular structure of gliadin and zein, the prolamins of wheat and corn, respectively (4,5,6). In these studies, valuable information on the structure of the prolamin has been obtained by modification of the disulfide groups of the protein and gel electrophoresis of the modified protein.

Disulfide bonds are believed to be responsible for certain unique rheologic properties of cereal proteins such as the viscoelastic properties of wheat gluten (4,7). Association-dissociation reactions of seed proteins, involving the disulfide bonds, are also well known (8). Reductive cleavage of the disulfide bonds of gliadin has only a slight effect on the electrophoretic pattern, and the molecular weight of gliadin is not appreciably altered. Further, reduced gliadin can be reoxidized under suitable conditions to form native gliadin (5). It was concluded from these observations that gliadin

consists of several protein components in which predominantly intramolecular disulfide bonds occur (9).

Zein has been demonstrated to be an aggregate of smaller polypeptide units linked together by intermolecular disulfide bonds (6). When zein is subjected to performic acid oxidation or reduction with sulfite, the protein migrates completely into the starch gel during electrophoresis, whereas before disulfide cleavage a part of the protein fails to migrate into the gel.

Information on the molecular structure of the alcohol-soluble proteins of grain sorghum is lacking. In the present study, disulfide groups of kafirin have been modified by oxidation or reduction and the modified protein has been studied by electrophoresis and gel filtration. Further, the reduced kafirin has been reoxidized to the disulfide form and the reduced-reoxidized protein has almost the same electrophoretic pattern as the native kafirin. These studies have demonstrated that in kafirin, disulfide bonds occur mostly in the intrachain form.

MATERIALS AND METHODS

A hybrid variety of grain sorghum (M-35-1) supplied by Agricultural Research Station, Raichur, Mysore State, was used in these experiments.

Sephadex G-100 (medium grade) and "blue dextran" were products of Pharmacia Fine Chemicals (Uppsala, Sweden). Iodoacetic acid and iodoacetamide were recrystallized to free them from iodine. Analytical-grade mercaptoethanol (Light & Co., Colnbrook, England) and urea (British Drug Houses (India) Limited) were used without further purification.

Preparation of Kafirin

Dehulled, defatted endosperm flour was obtained as described before (3). Flour (100 g.) was extracted with 1 liter of 60% (v./v.) aqueous ethanol at 60°C. for 2 hr. with intermittent shaking. After centrifugation, the supernatant was flash-evaporated to a small volume and lyophilized. This preparation contained 13.6% nitrogen (3).

Preparation of Alpha Kafirin

Kafirin (5 g.) was extracted with 100 ml. of 95% ethanol for 2 hr. at 25°C. with mechanical shaking. The suspension was centrifuged and the residue was re-extracted twice with 100-ml. portions of the solvent. The supernatants from the three extractions were combined and the solvent was removed by flash evaporation at 30°C. Alpha-kafirin was dried in vacuum desiccator over phosphorus pentoxide. It had 10.8% nitrogen and was a pale-yellow hygroscopic material.

Performic Acid Oxidation

Protein samples were oxidized with performic acid by the procedure of Hirs (10). Protein (100 mg.) was dissolved in 2 ml. of 98% formic acid and 8 ml. of performic acid was added. Oxidation was allowed to proceed for 20 min. at 25°C. The reaction mixture was then diluted with 5 vols. of water and formic acid was removed by flash evaporation. The residue was suspended in water and lyophilized.

Reduction with Mercaptoethanol

Cleavage of disulfide bonds by reduction was performed essentially as

described by Crestfield *et al.* (11). To 150 mg. of protein in a screw-capped vial, 4.8 g. urea, 0.5 ml. of EDTA solution (50 mg. disodium EDTA per ml.), and 6.0 ml. of tris buffer, pH 8.6 (5.23 g. tris and 9.0 ml. of 1.0*N* HCl diluted to 30 ml. with water) were added and the protein was dissolved. The solution was flushed with nitrogen and 0.1 ml. of mercaptoethanol was added, and the vial was tightly closed. Reduction was allowed to proceed for 4 hr. at 30°C.

At the end of the reaction period ⅓ volume of the reduced sample was transferred to a vial, flushed with nitrogen, and stored at –20°C. until required for electrophoretic analysis.

To another one-third portion, 100 mg. of iodoacetic acid (dissolved in 1 ml. of 0.05*N* NaOH) or 100 mg. of iodoacetamide was added for alkylating the SH groups of reduced protein. The alkylating reagent added was slightly more on a molar basis than the amount of mercaptoethanol present in the reaction mixture. After 20 min. of reaction with alkylating agent, the solution was diluted with 5 vols. of water, dialyzed against water, and lyophilized. The products will be referred to as: reduced-carboxymethylated (RCM-) protein and reduced-carboxamidomethylated (RCAM-) protein, respectively.

Reoxidation of Reduced Protein

The remaining aliquot of the reduced protein was divided into two equal halves and used for reoxidation of sulfhydryl to disulfide (5). One portion was dialyzed without further dilution (protein concentration was approximately 1.5%) against 6*M* urea for 12 hr. in the cold and then for 2 days against several changes of distilled water. Another portion of the reduced protein was diluted tenfold (protein concentration was about 0.15%) with 6*M* urea, dialyzed for 12 hr. against 6*M* urea, and then against distilled water. The reduced-reoxidized kafirin was obtained as a dry powder on lyophilization.

Gel Filtration

Approximately 30 mg. of protein was dissolved in 50% (v./v.) acetic acid and the absorbance of the protein solution was measured at 280 millimicrons in a Beckman DU spectrophotometer. The protein solution was applied to a 1.6 by 35-cm. column of Sephadex G-100 which was previously equilibrated with 50% acetic acid and eluted with the same solvent. Fractions of 3.5 ml. were collected at a flow rate of 7 ml. per hr. Protein concentration in each fraction was determined by measuring the absorbance at 280 millimicrons. Recovery of protein in column eluates was calculated by summing up absorbance of each fraction under the "peak" area. Void volume of the Sephadex column was determined with the use of a solution of blue dextran (12). Contents of the tubes corresponding to the peak fractions were pooled, dialyzed against water, and lyophilized. The protein samples thus recovered were analyzed by disc electrophoresis in polyacrylamide gels.

Gel Electrophoresis

Disc electrophoresis in polyacrylamide gels (7.5% acrylamide) in presence of 8*M* urea was carried out as described earlier (3). For the electrophoretic

analysis of mercaptoethanol-reduced protein samples, mercaptoethanol was included in electrode tray buffers at a concentration of 0.005*M*. Since the polymerization of sample gel was inhibited in presence of mercaptoethanol, application of the reduced protein samples for electrophoresis was modified in the following manner. Sucrose was added to the sample solution to form a highly viscous solution, and this solution was layered over the gels under the buffer with a syringe. Tris-glycine buffer, pH 8.6 (buffer A), was used with pH 9.5 gel system and aluminum lactate-lactic acid buffer, pH 3.1 (buffer D) was used with pH 4.3 gel system (3). Bromphenol blue and methyl green were used as tracking dyes with pH 9.5 gel and pH 4.3 gel, respectively.

RESULTS

Gel Electrophoresis of Modified Kafirin

Disc-electrophoretic patterns of native kafirin and disulfide-modified kafirins, in pH 9.5 gel with buffer A and pH 4.3 gel with buffer D, are shown in Fig. 1. The alcohol-soluble proteins of sorghums are poorly resolved in pH 9.5 gel, but good separation of components could be achieved in pH 4.3 gel (Fig. 1, A_1 and D_1).

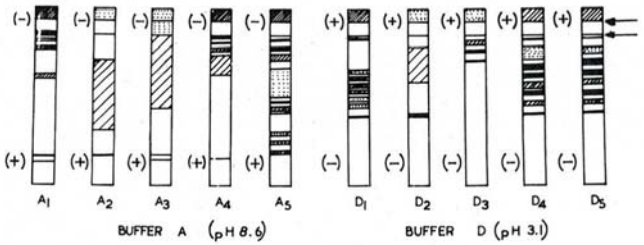

Fig. 1. Disc electrophoresis patterns of native (A_1 and D_1), oxidized (A_2 and D_2), reduced (A_3 and D_3), RCAM- (A_4 and D_4), and RCM-kafirin (A_5 and D_5), in buffers A and D, respectively. The lines corresponding to the upper and lower arrows indicate the boundaries between sample gel and spacer gel and between spacer gel and lower gel, respectively.

Performic acid-oxidized and mercaptoethanol-reduced kafirins show an indistinct pattern on electrophoresis in polyacrylamide gels in buffer A. In this buffer, the oxidized and reduced proteins show a wide diffuse band with considerable trailing. Two distinct fast-moving bands appear in the same position in both the native and the reduced kafirins, and only one of these fast-moving bands is seen in the oxidized kafirin (Fig. 1, A_1, A_2, and A_3). The mobility of oxidized and reduced kafirins in buffer D is much less than in buffer A (Fig. 1, D_2 and D_3). The reduced kafirin shows three distinct slow-moving bands in buffer D.

It was thought that alkylation of the sulfhydryl groups of reduced kafirin might improve the electrophoretic separation of the components. Therefore, the reduced kafirin fraction was alkylated with iodoacetic acid or iodoacetamide. Alkylation with the latter reagent (RCAM-protein) does not alter the net charge on the protein, but RCM-protein will carry more negative charges. Figure 1 (A_4, A_5, and D_4, D_5) shows the electrophoretic patterns of RCAM-kafirin and RCM-kafirin in buffers A and D, respectively. Three

slow-moving bands, one fast-moving band, and a diffuse band of intermediate mobility are seen in the pattern of RCAM-kafirin, in buffer A. However, in this buffer, RCM-kafirin shows nine distinct bands, three of which have slow mobility; the rest are fast-moving components. The number and mobilities of the electrophoretic components in both RCAM-kafirin and RCM-kafirin are quite similar when the samples are run at pH 3.1 (buffer D).

Electrophoresis of Reduced-Reoxidized Kafirin

Mercaptoethanol-reduced kafirin was reoxidized at two different protein concentrations as described under "Methods." This reoxidized kafirin was electrophoresed in buffer D, and the patterns of native, reduced, and reduced-reoxidized kafirins are shown in Fig. 2. The electrophoretic pattern of

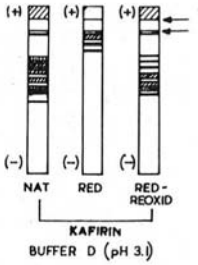

Fig. 2 (left). Disc electrophoresis patterns of native (NAT), reduced (RED), and reduced-reoxidized (RED-REOXID) kafirin of grain sorghum, in buffer D.

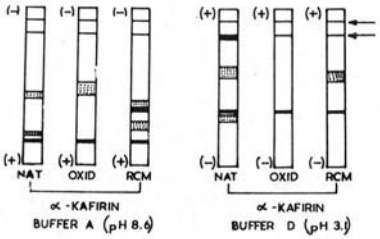

Fig. 3 (right). Disc electrophoresis patterns of native (NAT), oxidized (OXID), and reduced-carboxymethylated (RCM-) alpha kafirin, in buffers A and D.

reduced-reoxidized kafirin is similar to that of unmodified kafirin, showing that on reoxidation the electrophoretic mobility of most of the original protein components in the alcohol-soluble proteins of grain sorghum are restored. The electrophoretic patterns of reoxidized kafirin are exactly similar, whether reoxidation of reduced protein is done at 1.5 or 0.15% protein concentration. Reoxidation of reduced kafirin at a protein concentration higher than 1.5% could not be carried out, since kafirin is insoluble in urea buffers at higher protein concentrations.

Electrophoresis of Alpha Kafirin

About 15% of the kafirin fraction of grain sorghum is soluble in 95% ethanol and this component has been designated as alpha kafirin, in analogy with the alpha zein of corn prolamin. However, alpha zein constitutes a major portion (about 80%) of the prolamin of corn, whereas alpha kafirin represents a minor component of sorghum prolamin. The alpha kafirin shows fewer electrophoretic components than the original kafirin fraction (Fig. 3). In buffer A, the two major components of alpha kafirin have fast mobilities, and two faint bands are also visible. At pH 3.1, alpha kafirin shows a distinct fast-moving band, a faint fast-moving band, and a wide diffuse band of intermediate mobility. The performic acid-oxidized alpha kafirin shows a fast-moving band and a diffuse band with intermediate mobility in buffer A, whereas the reduced-carboxymethylated (RCM-) alpha kafirin shows two distinct and two diffuse bands when run in this buffer.

On the other hand, oxidized alpha kafirin shows a single band in buffer D, and reduced-alkylated alpha kafirin shows two bands (Fig. 3).

The electrophoretic pattern of the beta kafirin (kafirin of sorghum insoluble in 95% ethanol) in buffer D is similar to that of the original kafirin except that the intensities of two of the fast-moving protein components is reduced relative to that of the original kafirin fraction.

Gel Filtration

The elution patterns of kafirin, oxidized kafirin, and RCM-kafirin from

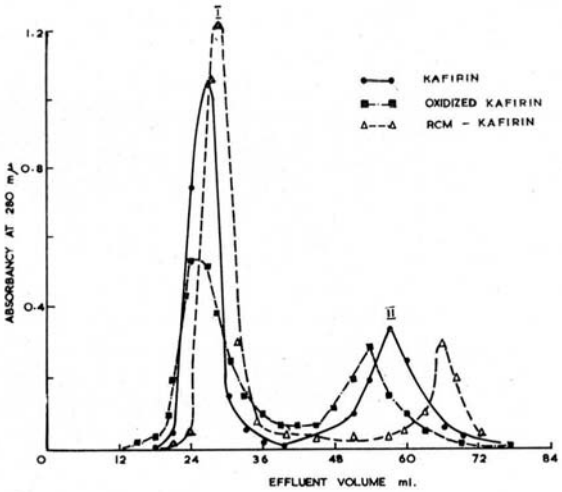

Fig. 4. Gel filtration on Sephadex G-100 (1.6 by 35-cm. column) of sorghum kafirin, performate-oxidized kafirin, and RCM-kafirin.

Sephadex G-100 columns are shown in Fig. 4. Since kafirin was insoluble in aqueous solvents but soluble in 50% acetic acid, this solvent was used for the gel filtration of kafirin (11). It was considered that the use of 50% acetic acid, which minimizes aggregation of protein by preventing protein-protein interactions, would be desirable in achieving fractionation of the strongly aggregating seed proteins (8,13). It is known that aggregation of insulin is prevented in solutions containing high concentrations of acetic acid (14).

Protein recoveries from the Sephadex columns varied from 85 to 98% of that applied on the column. About 60% of the native kafirin applied to Sephadex G-100 columns was eluted in peak I immediately after the void volume of the column. Thus, nearly two-thirds of the kafirin fraction was excluded from the gel; it consists of protein components with a molecular weight in the range of 150,000 (exclusion limit of Sephadex G-100) or higher. Another 30% of the protein was retarded on the Sephadex column and was eluted in peak II. The material eluted in this peak consists of low-molecular-weight protein components. This peak II material is straw-yellow in color and is perhaps associated with pigments (1).

Performic acid-oxidized kafirin and RCM-kafirin show somewhat similar elution patterns to that of unmodified kafirin on Sephadex column chromatography (Fig. 4). Again, 60 to 65% of the modified protein was recovered in the peak I fractions, and 30 to 35% of the protein applied to the column was eluted in peak II.

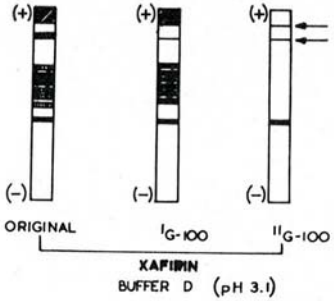

Fig. 5. Disc electrophoresis patterns of original kafirin, and peak I_{G-100} and peak II_{G-100} fractions of kafirin from Sephadex G-100 column chromatography (Fig. 4).

Figure 5 shows the electrophoretic patterns of the protein components eluted in peak I and peak II fractions (I_{G-100} and II_{G-100}) when unmodified kafirin was subjected to gel filtration. Peak I and peak II materials, obtained on chromatography of RCM-kafirin on Sephadex G-100, were also analyzed by electrophoresis. The electrophoretic patterns of I_{G-100} from unmodified kafirin and RCM-kafirin are quite similar to that of the corresponding unfractionated protein samples. However, the fraction eluted in peak II shows a single fast-moving protein band.

The resolving power of Sephadex G-100 columns for protein components of the prolamin fraction, when 50% acetic acid is used as the eluting solvent, is obviously poor. Although high concentrations of acetic acid are effective in preventing aggregation of proteins like insulin, it appears that aggregation of the protein units in the kafirin complex into high-molecular-weight components occurs in presence of 50% acetic acid. This aggregation behavior of the alcohol-soluble protein may be due to hydrophobic or polar interactions and not dependent on disulfide bond formation.

These large aggregates, with a molecular weight of 150,000 or higher, are excluded from the Sephadex gel. Therefore, separation of the individual protein components by gel filtration chromatography has not been achieved.

DISCUSSION

Kafirin is a major protein fraction of sorghum endosperm. The disulfide groups of this protein fraction have been modified by oxidative or reductive cleavage, and the modified protein has been analyzed by electrophoresis in polyacrylamide gels and by gel filtration.

The oxidized and reduced kafirins show indistinct electrophoretic patterns in alkaline buffer (pH 8.6). The protein components with slow mobilities are markedly affected by oxidation or reduction of the disulfide bonds. Changes in mobilities resulting from oxidation may be ascribed to the addition of negative sulfonic acid groups to the protein (6). However, some of the observed changes may have resulted from destruction of the amino acids tryptophan, tyrosine, and histidine in the protein by performic acid oxidation (15), leading to conformational changes. Reduction with mercaptoethanol cleaves the disulfide bonds and essentially linear molecules are formed. The SH groups formed on reduction tend to be reoxidized to the disulfide form. The indistinct electrophoretic patterns observed with the reduced protein at alkaline pH may reflect this instability of SH groups in the reduced protein.

To overcome these difficulties, the reduced kafirin was alkylated with iodoacetamide or iodoacetic acid. The reduced alkylated kafirins show almost similar electrophoretic patterns and the same number of components as that of unmodified kafirin, at pH 3.1. However, RCM-kafirin shows several fast-moving bands at pH 8.6, in contrast with the considerably lower mobilities of the components in native kafirin and RCAM-kafirin at this pH. This difference in the mobilities of the RCAM- and RCM-kafirin, at alkaline pH values, may be ascribed to the presence of negatively charged S-carboxymethyl groups in RCM-kafirin.

Cleavage of intermolecular disulfide bonds between peptide chains will result in fragmentation of the protein, and an increase in the number of electrophoretic components can be expected. On the other hand, rupture of the intramolecular disulfide bonds should cause little or no change in molecular weight or electrophoretic pattern, provided the net charge on the protein remains the same. Since the electrophoretic pattern of the reduced-alkylated kafirin is similar to that of the native protein, it would appear that the protein components in the prolamin fraction of sorghum contain mostly intramolecular disulfide bonds. In this respect, they resemble the gliadin fraction of wheat gluten (5).

Experiments on the gel filtration behavior of oxidized and reduced kafirins on Sephadex G-100 also show that cleavage of the disulfide bonds of kafirin does not lead to a reduction in molecular size of the protein, since the elution profile of the disulfide-modified kafirin is similar to that of the native kafirin.

Evidence in support of the conclusion that kafirin contains predominantly intramolecular disulfide bonds is also adduced from the experiments on the reoxidation of reduced kafirin. Reoxidized-reduced kafirin has almost the same electrophoretic pattern as that of native kafirin. Therefore, oxidation of reduced kafirin has restored the original electrophoretic properties of the protein. This is possible only if the majority of the disulfide bonds are intramolecular (5). The alcohol-soluble protein of grain sorghum has a low cystine content (2), and perhaps the cystine residues in this protein fraction are involved mostly in intramolecular disulfide bonding.

Beckwith et al. (5) have reported that reoxidation of reduced gliadin at high protein concentration (5%) gave a product which appeared to contain predominantly intermolecular disulfide bonds. It is possible that reoxidation of reduced kafirin at a high protein concentration (5% or more) will favor intermolecular disulfide bond formation, and the resulting product may not resemble native kafirin in its properties.

A noteworthy feature of the studies on the reversible reduction and reoxidation of the kafirin fraction of sorghum is that these investigations were done on a material which has been demonstrated to be a mixture of several distinct electrophoretic components (3). Yet, on reoxidation of reduced kafirin, the electrophoretic properties of the various components are restored. Earlier studies by Beckwith *et al.* (5) demonstrated that the restoration of native structure of most of the components in the heterogeneous gliadin complex occurs on reduction and reoxidation of wheat gliadin.

Although it has been shown that reoxidized kafirin has the same electrophoretic mobility as that of native kafirin, it is not a conclusive demonstration of complete restoration of the original structure of kafirin complex. It is necessary to determine several physical properties of the reoxidized protein, namely, molecular weight and shape, viscosity, and optical rotatory dispersion (5), and also the immunological properties of the reoxidized kafirin (16).

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