Cowpea Proteins. 2. Characterization of Water-Extractable Proteins

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Disc gel electrophoresis, gel filtration, and ultracentrifugation were used to characterize cowpea (*Vigna unguiculata*) water-extractable proteins (WEP) and water-extractable, acid-precipitated proteins (APP). After electrophoresis, the WEP showed four bands as compared to six bands in the APP. Ultracentrifuge analysis showed that the WEP contained four fractions with s_{20} values of 3.1, 8, 12.7, and 14.6, the 8S fraction being the predominant species. Fractions with molecular weights of 13 500, 20 000, 25 000, 50 000, and a large-molecular-weight fraction (>600 000) were separated from WEP on a Sephadex G-200 column. The 8–14.6S fractions were able to enter into association–dissociation reactions as evidenced by ultracentrifugation. Heating the WEP from 25 to 100 °C caused very little change in nitrogen solubility; however, electrophoresis indicated a breakdown into subunits and aggregation. Evidence from differential scanning colorimetry showed the possible formation of soluble aggregates during heating.

One of the areas to be considered in an attempt to increase the utilization of legume seeds and legume products is the physicochemical properties and functionality of the various components. One component which is of great importance in the consumption of legumes is protein.

Sefa-Dedeh and Stanley (1979) reported on conditions for optimal extraction of cowpea (*Vigna unguiculata*) proteins in different solvents. However, to fully understand the properties of these proteins, these isolated components need to be characterized. Derbyshire et al. (1976) provided a comprehensive review on the storage proteins of legume seeds. There have been numerous other reports on the characterization of legume seeds (Osborne and Campbell, 1898; Danielsson, 1949; Wolf and Briggs, 1959; Wolf et al., 1962; Adriaanse et al., 1969; Ericson and Chrispeels, 1973; Wright and Boulter, 1974). Although cowpeas are a major grain legume crop in the tropics, they are not referred to in any of these studies. The objective of this work was to characterize the water-extractable proteins (WEP) of cowpea and investigate how they are affected by ionic strength and heat.

MATERIALS AND METHODS

Materials. (*a*) *Cowpea Flour.* Cowpeas (*Vigna unguiculata*) variety "Adua Ayera" were milled as reported by Sefa-Dedeh and Stanley (1979).

(*b*) *Preparation of Protein Extracts.* Water-extractable protein (WEP) was prepared from the flour by extracting at the optimum conditions established from regression equations (Sefa-Dedeh and Stanley, 1979). The proteins were extracted for 30 min at 25 °C using a meal–solvent ratio of 0.05 (g/mL). The extract was centrifuged at 20000*g* for 20 min, dialyzed at 4 °C against distilled water for 24 h, and freeze-dried.

Water-extracted, acid-precipitated proteins (APP) were prepared by reducing the pH of the dialyzed water extract to the isoelectric point (pH 4.5) with hydrochloric acid. The precipitated proteins were centrifuged (20000*g* for 20 min) and washed once with water. The resulting protein was freeze-dried.

Salt-soluble protein was also extracted with 0.5 M sodium chloride using the conditions established from the regression equation reported by Sefa-Dedeh and Stanley (1979). After centrifuging at 20000*g* for 20 min, the extract was dialyzed against distilled water at 4 °C for 24 h. Salt-soluble proteins precipitated from solution during dialysis were separated by centrifuging at 20000*g* for 20 min. The protein was freeze-dried.

Experimental Methods. Discontinuous polyacrylamide gel electrophoresis was performed on the protein samples with a Canalco Model 1200 disc electrophoresis unit (Canalco, Rockville, MD). A 100-mg sample was dissolved in 3.0 mL of Tris–glycine buffer (pH 8.3, μ = 0.16). One gram of urea was added and the mixture filtered. A 10-μL aliquot of the solution was applied to the gel and the samples electrophoresed by applying a current of 4 ma/tube. The gels were stained after electrophoresis with aniline blue-black for 2 h and then destained with 7% acetic acid in a quick gel destainer, Model 1801 (Canalco, Rockville, MD). The gels were scanned in a Joyce–Loebl chromoscan densitometer at 620 nm to determine their electrophoretic patterns.

Fully swelled Sephadex G-200 gel was packed in a 100 × 2.5 cm glass column. Using an ISCO automatic fraction collector (ISCO, Lincoln, NE) and a flow rate of 10 mL/h, the exclusion volume was determined with blue dextran. The column was calibrated for molecular weight determination with the following proteins: aldolase, ovalbumin, chymotrypsinogen A, and ribonuclease A. The column was eluted with Tris–glycine buffer, pH 8.3, μ = 0.16. A selectivity curve for the proteins was plotted.

A 200-mg sample of cowpea protein was dissolved in 4.0 mL of Tris–glycine buffer (pH 8.3, μ = 0.16) and the mixture filtered through a Whatman No. 4 filter paper. The filtrate was loaded and chromatographed on the Sephadex G-200 column. Ten-milliliter fractions were collected at the rate of 10.0 mL/h. The elution pattern of the protein was monitored by measuring absorbance at 280 nm. The molecular weight of the fractions was estimated from the selectivity curve.

To estimate sedimentation coefficients of the proteins, 15 mg/mL solutions were prepared by dissolving the protein extracts in Tris–glycine buffer, pH 8.3 (μ = 0.16). To remove any particles the solutions were centrifuged at 5000*g* for 10 min at 4 °C. A 0.5-mL sample was analyzed by filling into a cell and centrifuging under vacuum at 20 °C at fixed speed in a Beckman Model E Analytical Ultracentrifuge using Schlieren optics; sedimentation patterns were photographed and measured at different time intervals and the sedimentation coefficients of the fractions estimated.

The influence of changing ionic strength on the sedimentation coefficient of water-soluble proteins was sim-

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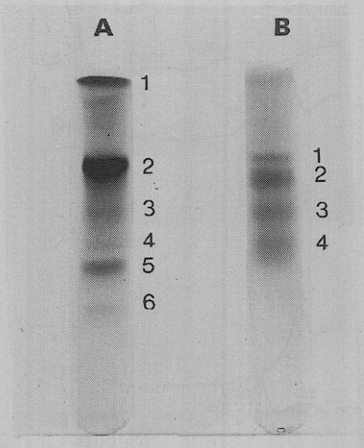

Figure 1. Disc electrophoresis patterns of cowpea proteins. (A) Water-extracted, acid-precipitated proteins. (B) Water-extracted proteins.

ilarly studied. Phosphate buffers (pH 7.0) with ionic strengths 0.05, 0.5, and 1.0 were used.

The effect of heat was studied using WEP and APP dissolved in Tris–glycine buffer (pH 8.3, μ = 0.16). Samples were heated from 25 to 100 °C and at 100 °C for 90 min. Aliquots were taken at 50, 70, and 90 °C and at different times at 100 °C (1, 5, 10, 45, and 90 min) and centrifuged at 20000*g* for 20 min to sediment any coagulum. The total nitrogen in the supernatant was determined and the remainder freeze-dried for electrophoresis and differential scanning calorimetry (DSC).

A DuPont Model 990 differential scanning calorimeter equipped with a Model 910 DSC cell base was used for thermal studies. Samples were analyzed as 15% (w/w) solutions which had been dialyzed to remove excess buffer salts. A heating rate of 5 °C/min was used; runs were performed in a nitrogen atmosphere (54 psi). A known weight of water was used in the reference pan to balance the heat capacity of the sample pan. The instrumental sensitivity was 0.005 (mcal s^{-1} $in.^{-1}$). Heats of transition (ΔH) were calculated as:

$$\Delta H = (A/MC)(60\ BE\Delta qs)$$

where A = peak area, M = sample mass, C = sample concentration, B = time base setting, E = cell calibration coefficient, Δqs = Y axis range. The transition temperature was taken as the temperature at the peak maximum.

RESULTS AND DISCUSSION

Characterization of Cowpea Proteins. Disc electrophoresis of the water-extracted, acid-precipitated proteins indicated the presence of six bands (Figure 1A), while the water-extracted proteins exhibited only four bands (Figure 1B). Proteins isolated with other solvents showed other characteristic patterns (Figure 2). The acid-precipitated proteins prepared from the water and sodium hydroxide extracts and the salt-soluble proteins show one common major band (peak 2). This may have arisen through association of the protein molecules due to the presence of ions.

Water-extracted proteins showed a pattern in the ultracentrifuge (Figure 3) similar to those reported for soybean water-soluble proteins by Wolf and Briggs (1959). Four major bands were detected with sedimentation coefficients (s_{20}) of 3.1S, 8S, 12.7S, and 14.6S, the 8S protein being the major fraction. Joubert (1957) reported the presence of a 7.3S protein in cowpeas and the presence of a 11.3S fraction was reported by Derbyshire et al. (1976).

Ultracentrifuge analysis of legume seed proteins generally shows the presence of a 7 to 8S fraction and an 11 to 12S fraction. The 7S fraction is referred to as vicilin and the 11 to 12S fraction is referred to as legumin (Danielsson, 1949). Water-extracted proteins from cowpeas thus contain both vicilin and legumin. It is interesting to note that the 8S fraction of the water-soluble protein did not appear after preparing isoelectric-precipitated protein from the water extract (Figure 3C). This confirms the report by Danielsson (1949) that the 8S fraction of peas (*Pisum sativum*) showed no precipitation at any pH. Although the 8S fraction was in the water extract (Figure 3B), when the pH of the extract was lowered to 4.5 only the 12S fraction precipitated, leaving the 8S fraction in solution, which explains its absence from the ultracentrifuge pattern (Figure 3C). The cowpea water-extracted proteins were shown to have both a heavier component (14.6S) and a lighter component (3.1S). The salt-soluble proteins showed the presence of a predominant 8.6S fraction (Figure 3D), a 2.7S and 12.1S fraction.

The data from the ultracentrifuge analysis confirm the reports that legume proteins are made up of two major components with sedimentation coefficients of 7 to 8S and 11 to 12S, respectively.

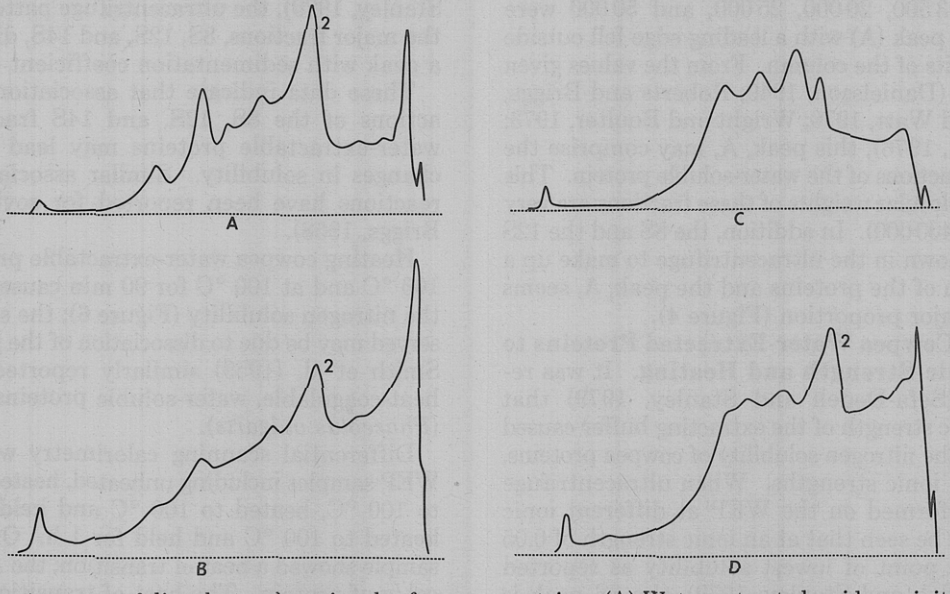

Figure 2. Densitometer scans of disc electrophoresis gels of cowpea proteins. (A) Water-extracted, acid-precipitated proteins. (B) Sodium hydroxide extracted, acid-precipitated proteins. (C) Water-extracted proteins. (D) Salt-soluble proteins.

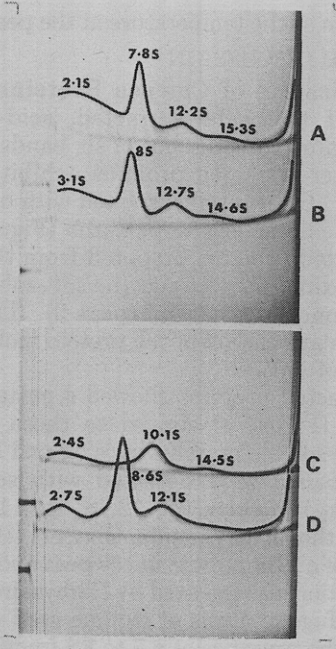

Figure 3. Ultracentrifuge patterns of cowpea proteins. (A, B) Water-extracted proteins. (C) Water-extracted, acid-precipitated proteins. (D) Salt-soluble proteins. Rotor speed: A, B, 56 123 rpm; C, D, 56 100 rpm.

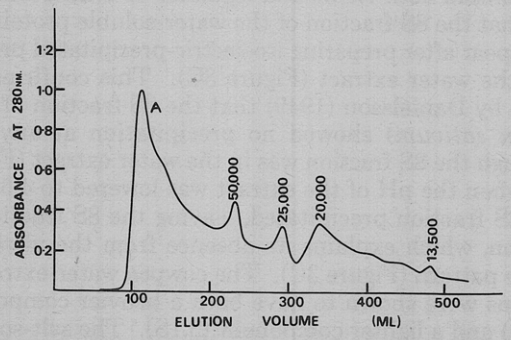

Figure 4. Elution profile of water-extractable proteins on Sephadex G-200 column. (Numbers over peaks indicate approximate molecular weights estimated from the selectivity curve.)

The molecular weight of the water-extracted proteins were estimated by gel filtration on Sephadex G-200 column (Figure 4). Four major peaks with molecular weights of approximately 13 500, 20 000, 25 000, and 50 000 were detected. A fifth peak (A) with a leading edge fell outside the exclusion limits of the column. From the values given in the literature (Danielsson, 1949; Roberts and Briggs, 1965; Pusztai and Watt, 1970; Wright and Boulter, 1973; Derbyshire et al., 1976), this peak, A, may comprise the 8S and the 12S fractions of the water-soluble protein. This is because the molecular weights of these fractions are very large (140 000 to 400 000). In addition, the 8S and the 12S fractions were shown in the ultracentrifuge to make up a major proportion of the proteins and the peak, A, seems to comprise a major proportion (Figure 4).

Reactions of Cowpea Water-Extracted Proteins to Changes in Ionic Strength and Heating. It was reported earlier (Sefa-Dedeh and Stanley, 1979) that changing the ionic strength of the extracting buffer caused large changes in the nitrogen solubility of cowpea proteins, especially at low ionic strengths. When ultracentrifuge analysis was performed on the WEP at different ionic strengths, it may be seen that at an ionic strength of 0.05 (Figure 5A), the point of lowest solubility as reported earlier (Sefa-Dedeh and Stanley, 1979), the 8S peak is absent; instead, a new peak with sedimentation coefficient

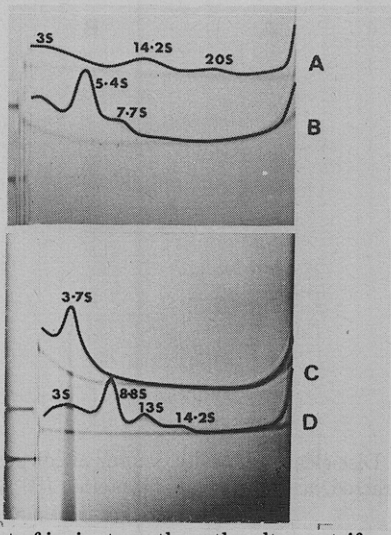

Figure 5. Effect of ionic strength on the ultracentrifuge patterns of cowpea water-extractable proteins. Ionic strength: (A) 0.05, (B) 0.50, (C) 1.00, (D) 0.16. Rotor speed: 56 100 rpm.

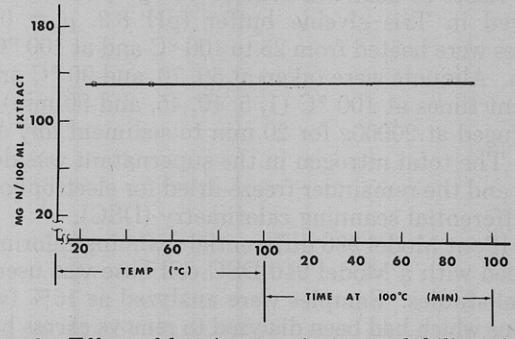

Figure 6. Effect of heating on nitrogen solubility of water-extractable cowpea proteins.

(s_{20} = 20) appears. This may be due to the association of the molecules resulting in the low solubility reported at this ionic strength. With an increase in ionic strength to 0.5 (Figure 5B), there is the reappearance of the 8S fraction and a new fraction with a sedimentation coefficient of 5.4S. It seems that the large fractions (12S, 14S, and part of the 8S fractions) dissociate at this ionic strength resulting in an increase in solubility. At ionic strength 1.0 (Figure 5C), when the highest solubility was reported (Sefa-Dedeh and Stanley, 1979), the ultracentrifuge patterns show that all the major fractions, 8S, 12S, and 14S, dissociated to form a peak with sedimentation coefficient (s_{20}) of 3.7S.

These data indicate that association–dissociation reactions of the 8S, 12S, and 14S fractions of cowpea water-extractable proteins may lead to the observed changes in solubility. Similar association–dissociation reactions have been reported for soybeans (Wolf and Briggs, 1958).

Heating cowpea water-extractable proteins from 25 to 100 °C and at 100 °C for 90 min caused little change in the nitrogen solubility (Figure 6); the slight increase observed may be due to dissociation of the protein molecules. Smith et al. (1959) similarly reported the absence of heat-coagulable, water-soluble proteins in kidney beans (*Phaseolus vulgaris*).

Differential scanning calorimetry was performed on WEP samples including unheated, heated to 90 °C, heated to 100 °C, heated to 100 °C and held for 15 min, and heated to 100 °C and held for 1 h. Only the unheated sample showed a heat of transition; the ΔH value was 3.33 cal/g of protein. The heat of transition was 83 °C. All others yielded only a base line. These data indicate that

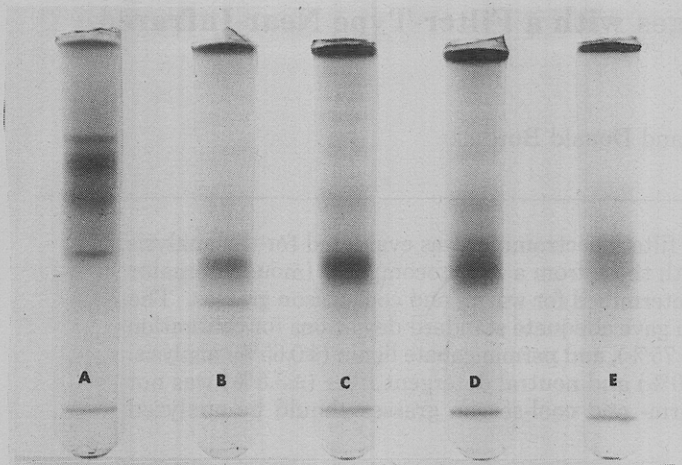

Figure 7. Effect of heating at 100 °C, at times specified, on the disc electrophoretic patterns of cowpea water-extractable proteins: (A) unheated, (B) 1 min, (C) 5 min, (D) 45 min, (E) 90 min.

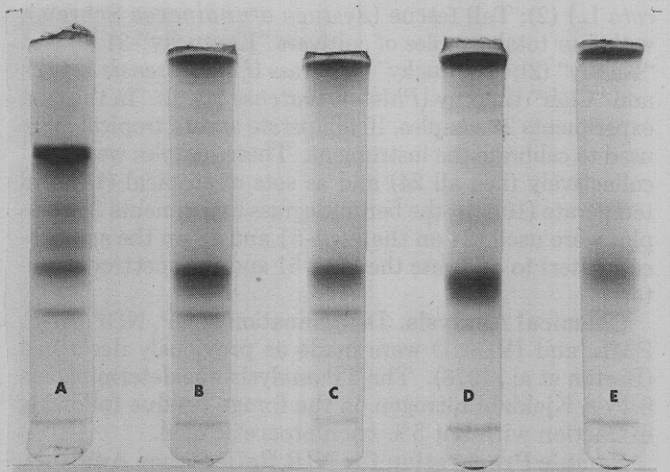

Figure 8. Effect of heating at 100 °C, at times specified, on disc electrophoretic patterns of cowpea water-extracted, acid-precipitated proteins: (A) unheated, (B) 1 min, (C) 5 min, (D) 45 min, (E) 90 min.

the water-soluble cowpea proteins are particularly sensitive to heat and undergo thermal dissociation and unfolding at temperatures lower than that observed for plant proteins such as soy (Armstrong et al., 1979). Thus it appears that the action of heat on this fraction is to produce water-soluble denatured proteins.

The formation of soluble aggregates in the 11S protein of soybeans heated at 100 °C has been reported (Wolf and Tamura, 1969). They concluded that heating disrupts the quaternary structure of the 11S protein and separated the subunits into a soluble and, later, an insoluble fraction. Similar conclusions were reached by Armstrong et al. (1979).

Disc electrophoresis of the heated cowpea protein samples indicated that aggregation of the molecules occurred as evidenced from the presence of a fraction that did not move from the top of the gels (Figures 7 and 8). In addition to these aggregates, the formation of intermediate fractions occurred (Figure 7, 5 min; Figure 8, 45 min). The fact that there was little change in the total nitrogen of the cowpea proteins during heating shows that the aggregates formed may be soluble and thus are not removed by centrifugation.

CONCLUSIONS

Cowpea water-extractable proteins possess some characteristics similar to that of soybean. They are comprised of four fractions with sedimentation coefficients (s_{20}) of 3.1, 8, 12.7, and 14.6. These fractions are able to enter into association–dissociation reactions which affect their solubility. The absence of heat-coagulable protein in these protein extracts may have practical implications. For example, they may be suitable for use in food systems with a liquid base such as in beverages and vegetable milk preparations.

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