Structural and Functional Properties of a Partially Purified Cowpea (*Vigna unguiculata*) Globulin Modified with Protein Kinase and Glycopeptidase

Rotimi E. Aluko, Rickey Y. Yada,* Robert W. Lencki, and Alejandro G. Marangoni

Department of Food Science, University of Guelph, Guelph, Ontario, Canada N1G 2W1

The major globulin fraction in cowpea seed was partially purified by selective ammonium sulfate precipitation and gel chromatography. The partially purified protein was enzymatically phosphorylated or deglycosylated. Phosphate content increased from 5.81 μg/mg in the untreated protein to 10.55 μg/mg in the protein kinase treated protein. Fluorescence intensity and protein solubility were significantly ($p \leq 0.05$) increased at pH 3−8 as a result of phosphorylation, while susceptibility to heat-induced coagulation was significantly ($p \leq 0.05$) decreased. At 50% deglycosylation, fluorescence intensity was significantly ($p \leq 0.05$) higher for the untreated protein than the treated protein. The intensity of the near-UV circular dichroism spectra of the deglycosylated protein was lower than that of the untreated protein at pH 3, 4, and 6, whereas the reverse was observed at pH 7 and 8. The deglycosylated protein was less soluble at pH 3, 4, 7, and 8 and was more susceptible to heat coagulation when compared to the untreated protein. Modification by protein kinase would allow use in foods where greater solubility is needed, while deglycosylation could enhance utilization in foods that require heat-induced coagulation.

Keywords: *Cowpea globulin; phosphorylation; deglycosylation; functional properties; physicochemical properties*

INTRODUCTION

Cowpea (*Vigna unguiculata*) is a popular grain legume commonly cultivated in West and East Africa as well as other developing countries, where it serves as a cheaper source of protein in place of the more expensive animal proteins (Uzogara and Ofuya, 1992). Apart from its low cost, cowpea seed contains 20−35% protein and is especially rich in lysine and other essential amino acids, except sulfur containing amino acids (Bressani et al., 1961; Uzogara and Ofuya, 1992). Previous studies of cowpea globulins have dealt with isolation and characterization of the major globulin fraction (Sefa-Dedeh and Stanley, 1979; Khan et al., 1980). However, there is the need to study how enzymatic tools can be used to modulate the structure−function properties of the major globulin protein of cowpea seeds.

Enzymatic modifications are useful tools in the study of structure−function relationships of proteins. Knowledge derived from such studies could help improve the quality of existing foods or aid the fabrication of new food products (Whitaker and Puigserver, 1982). Therefore, the purpose of modifying the structure of proteins is to create new and unique products that would possess better functional properties in food systems than the unmodified protein (Hamada, 1992). Protein kinases catalyze the phosphorylation of proteins both *in vitro* and *in vivo* and are subclassified based on the amino acid acceptor of the phosphoryl residue (Krebs, 1986). The serine and threonine kinases are the most widely used enzymes in food protein modification, since proteins containing oxygen-bound phosphate are more stable under acid conditions than the nitrogen-bound phosphate (Matheis and Whitaker, 1984). The enzymatic reaction is highly specific and results in a phosphorylated protein with higher solubility, greater water-holding capacity or greater calcium-binding capacity (Seguro and Motoki, 1990; Campbell et al., 1992; Aluko and Yada, 1995a). The increase in solubility is particularly prominent in the pH range near the isoelectric point where proteins exhibit the least solubility (Ross, 1989). The observed changes in functional properties are believed to be due mainly to the introduction of hydrophilic and negatively charged phosphoryl groups, though some influence due to subsequent conformational changes may be important (Seguro and Motoki, 1989).

Peptide:*N*-glycosidase F is a glycopeptidase ([*N*-acetyl-β-D-glucosaminyl]-L-asparagine amidohydrolase) and is obtained from extracellular secretions of the bacterium *Flavobacterium meningosepticum* (Plummer et al., 1984). The enzyme hydrolyzes a protein/peptide-bound glycosylated asparagine to aspartic acid, with the release of ammonia and an oligosaccharide containing di-*N*-acetylchitobiose at the reducing terminus (Tarentino et al., 1990).

Carbohydrates can influence the properties of their protein conjugate in diverse ways. They can provide a steric shield to inhibit penetration by large and even small molecules, or modify solubility of proteins since they are much more soluble than polypeptides (Grimaldi et al., 1985). Moreover, because of their high solubility in water, carbohydrates are preferentially accommodated on the surface of the protein where interactions with water produce frictional drag that increases the stability of the protein against denaturants (Grimaldi et al., 1985). Therefore, deglycosylation should have measurable effects on the structure and function of proteins. For example, a higher level of glycosylation has been shown to increase solubility and heat stability of β-lactoglobulin (Kitabatake et al., 1985).

The present investigation was carried out to determine the effects of enzymatic phosphorylation and

* Author to whom correspondence should be addressed [telephone (519) 824-4120, extension 8915; fax (519) 824-6631; e-mail ryada@uoguelph.ca].

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deglycosylation on some physicochemical and functional properties of the major globulin fraction of cowpea seed.

MATERIALS AND METHODS

Cowpea seeds were obtained from the Institute for Agricultural Research, Ahmadu Bello University, Zaria, Nigeria, and prepared into flour as earlier reported (Aluko and Yada, 1995b). The catalytic subunit of rabbit muscle cyclic adenosine monophosphate-dependent protein kinase, adenosine triphosphate (ATP), and endoglycosidase F preparation (6 units/vial) containing peptide:*N*-glycosidase F activity were obtained from Sigma Chemical Co. (St. Louis, MO).

Partial Purification of the Major Cowpea Globulin. A modified method of Khan et al. (1980) was used to prepare and partially purify the major globulin of cowpea as follows. Cowpea flour was extracted with 10 volumes of 0.1 M sodium phosphate buffer, pH 8.0, containing 0.4 M NaCl, by stirring the slurry on a magnetic stirrer (70% maximum speed) for 30 min at room temperature. The slurry was centrifuged at 23000*g* for 20 min and the supernatant filtered through Whatman number 1 paper to remove particulate matter. Solid ammonium sulfate was added to the filtrate up to 80% saturation and stirred at 4 °C for 2 h and centrifuged at 23000*g* and 4 °C for 20 min. The precipitate was discarded while the supernatant was brought up to 100% ammonium sulfate saturation, stirred at 4 °C for 2 h, and centrifuged at 23000*g* and 4 °C for 20 min (Khan et al., 1980). The precipitate was dissolved in distilled water and dialyzed against distilled water until the water insoluble globulins precipitated. The dialyzate was centrifuged at 23000*g* and 4 °C for 20 min and the precipitate freeze-dried.

The freeze-dried precipitate (25 mg) was dissolved in 10 mL of the extraction buffer and applied onto a gel filtration column (2.5 × 90 cm) packed with Sepharose CL-6B. The column had been previously equilibrated with the 0.1 M sodium phosphate buffer, pH 8.0, containing 0.05% (w/v) sodium azide as an antimicrobial agent. The protein was eluted at room temperature using a flow rate of 11.4 mL/h and 5.2 mL fractions collected; protein content of the fractions were estimated by measuring their absorbance at 280 nm in a UV-visible recording spectrophotometer (Model UV-260, Shimadzu Corporation, Kyoto, Japan). Fractions 73−76 contained in the major peak (fractions 71−78) were pooled together, dialyzed against distilled water, freeze-dried as the partially purified globulin, and stored desiccated at −20 °C.

Protein Kinase Reaction. The partially purified globulin was dissolved in 0.2 M sodium phosphate buffer, pH 7.0, containing 2 mM $MgCl_2$ and 5 mM dithiothreitol. Preliminary studies were carried out to determine optimum phosphorylation conditions. The effects of substrate, enzyme and ATP concentrations, and time were determined at 37 °C using a reaction volume of 0.5 mL in 1.5 mL Eppendorf micro tubes. The reactions were terminated with the addition of 0.5 mL of 12.5% trichloroacetic acid (TCA) and the precipitates recovered by centrifugation at 16000*g* for 10 min. The precipitates were washed once with 0.5 mL of 12.5% TCA and centrifuged at 16000*g* for 10 min, and the amount of phosphate in the precipitates was determined according to the method of Chen et al. (1956). K_m and V_{max} values were calculated according to the method of Leatherbarrow (1993).

To prepare the phosphorylated globulin used for determination of physicochemical and functional properties, the following optimum reaction conditions were used from the optimization procedure: 6.7 protein kinase units/mg substrate protein; 4 mg/mL substrate protein concentration; 80 μM ATP; 60 min (reaction time); 37 °C. For the untreated protein, the enzyme was omitted. Total reaction volume was 15 mL for either the untreated or phosphorylating conditions. The reaction was stopped by transferring the reactants to an ice-bath and adding 10 mL of 0.4 M EDTA, pH 8.0. After 1 h in the ice-bath, the untreated and enzyme-containing protein solutions were dialyzed against distilled−deionized water for 48 h with eight water changes and the dialyzates freeze-dried and stored desiccated at −20 °C. Phosphate contents were determined according to the method of Chen et al. (1956). The phosphate used in the prior isolation and reaction steps did not affect the final analysis.

Peptide:*N*-glycosidase F Reaction. A preliminary experiment conducted to determine the optimum deglycosylation rate involved variations in the substrate concentration (1, 2, 3, and 4 mg/ml), sodium thiocyanate (0, 0.5, 1.0, 1.5, and 2.0 M), duration of enzyme reaction (24 and 48 h), and enzyme concentration (225, 450, and 900 milliunits/mg globulin). The results showed that the highest deglycosylation level was obtained with 2 mg/mL globulin, 0.5 M sodium thiocyanate, 24 h reaction time, and 450 milliunits of enzyme/mg globulin. Therefore, to prepare deglycosylated globulin used for determination of physicochemical and functional properties, a 2 mg/mL protein solution was made in 0.1 M sodium phosphate buffer, pH 7.5, containing 0.5 M sodium thiocyanate (NaSCN) and 0.02% (w/v) sodium azide. At time zero, 20 μL of 0.1 M phenylmethanesulfonyl fluoride (protease inhibitor) per mL of reaction volume was added followed by 450 milliunits of *N*-glycosidase F per milligram substrate protein, in a total reaction volume of 20 mL. An untreated sample containing all the above except the enzyme was also prepared. The untreated and treated protein solutions were incubated at 37 °C for 24 h in a water bath after which they were transferred to an ice bath. The cooled solutions were then dialyzed against distilled water for 48 h, freeze-dried, and stored desiccated at −20 °C. The amount of residual sugars in the untreated and treated proteins were determined by the colorimetric method of Dubois et al. (1956) with mannose as standard. Determinations of residual sugars were carried out on duplicate samples with two determinations per replicate sample.

Gel Electrophoresis. Native and SDS-PAGE were run on 8−25% gradient gels using 2 mg/mL protein solutions on a PhastSystem Separation and Control and Development Units according to the manufacturer's instructions (Pharmacia LKB, Montreal, PQ). For native-PAGE, freeze-dried protein samples were dispersed in 0.1 M sodium phosphate buffer, pH 8.0, centrifuged at 16000*g* for 5 min and 1 μL applied to each lane. Samples were prepared for SDS-PAGE by mixing the freeze-dried proteins with 5% SDS in Tris-glycine buffer, pH 8.0, containing 10% (v/v) 2-mercaptoethanol followed by heating in boiling water for 5 min. The samples were cooled to room temperature, centrifuged at 16000*g* for 5 min and 1 μL applied to each lane. The gels were then stained with Coomassie Brilliant Blue G-250.

Physicochemical Properties. No determinations were performed at pH 5.0 since the native and enzymatically modified proteins were mostly insoluble (<1% solubility). Aromatic hydrophobicity (ARH) and maximum intrinsic fluorescence intensity (I_{max}) were determined as previously described (Aluko and Yada, 1995b).

Circular dichroism spectra were measured using a JASCO J-600 spectropolarimeter (Japan Spectroscopic Co. Ltd., Tokyo, Japan) under constant nitrogen flush. Near-UV CD spectra was recorded from 240−320 nm with 0.2−0.3 mg/mL protein solutions. Far-UV CD spectra was recorded from 190−250 nm using 0.05−0.1 mg/mL protein solutions. A 1 cm pathlength quartz cell was used for the near-UV CD while 0.1 cm pathlength was used for far-UV CD measurements. The spectra were reported in terms of mean residue ellipticity, θ, which was calculated according to the method of Schmid (1989). The proportions of the secondary structure fractions (α-helix, β-sheet, β-turn, random) were determined using the Jasco protein secondary structure estimation program (Japan Spectroscopic Co.) based on the method of Chang et al. (1978).

Functional Properties. Protein solubility was determined according to the method of Aluko and Yada (1995b), while the method of Voutsinas et al. (1983) was used to determine heat coagulability.

Statistical Analysis. Analyses were done in duplicate with two determinations per replicate. Duncan's multiple range tests were performed according to the appropriate SAS (1990) method.

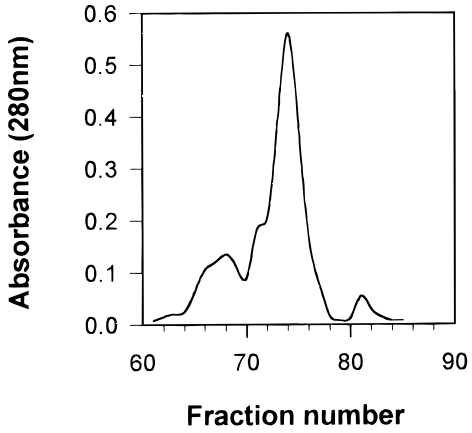

Figure 1. Sepharose CL-6B gel filtration chromatogram of crude cowpea globulin from ammonium sulphate precipitation. A 25 mg sample in 10 mL of 0.1 M sodium phosphate buffer, pH 8.0, containing 0.4 M NaCl and 0.05% sodium azide was applied onto the column (2.5 × 90 cm) which was previously equilibrated with the same buffer. The sample was eluted with the phosphate buffer and 5.2 mL fractions collected. Fractions 1−59 are not shown because they had absorbance values close to zero.

RESULTS AND DISCUSSION

Partial Purification of the Major Globulin Protein. Protein recovery of the crude globulin by ammonium sulfate precipitation was approximately 45.5%. The elution pattern for the gel chromatography of crude globulin preparation is shown in Figure 1. One major peak (fractions 71−78) was obtained along with two minor peaks (fractions 61−70 and 80−84). About 80% of protein in the crude globulin was recovered in the major globulin peak. Fractions 73−76 of the major peak were freeze-dried as the partially purified globulin and analyzed by native and SDS gel electrophoresis, and the results are shown in Figure 2. The native gel pattern of the crude protein (A, lane 2) showed 1 major protein band (apparent molecular weight 321 kDa) in addition to 1 minor band of molecular weight 91 kDa. Only the major protein band was observed for the partially purified protein on native gel (A, lane 3). The apparent molecular weight (321 kDa) calculated for the major band on the native gel is similar to the 320000 Da reported by Derbyshire et al. (1976) in storage proteins of legume seeds.

The SDS-PAGE results showed that the partially purified protein was made up of two major polypeptide chains with relative molecular weights of 55 and 50 kDa while there were five low molecular weight polypeptide chains (25−35 kDa) and four higher molecular weight polypeptides (≥61 kDa) as minor bands. The work of Khan et al. (1980) revealed the presence of two major (58 and 52 kDa) and 1 minor (63 kDa) polypeptide chains as components of the major globulin fraction of cowpea proteins. The crude protein, however, in addition to the two major polypeptides found in the partially purified protein, had several (11) other minor bands with molecular weights ranging from 25−85 kDa. From densitometric scans, the minor polypeptide bands represented 58.8% of the crude globulin, while they decreased to 38.6% in the partially purified globulin. The SDS-PAGE results would suggest that the partially purified globulin is an oligomeric protein, which is consistent with the report of Derbyshire et al. (1976). According to Derbyshire et al. (1976), the presence of 56 and 53 kDa polypeptides in a legume protein is indicative of a 7S globulin. Therefore, the predominance

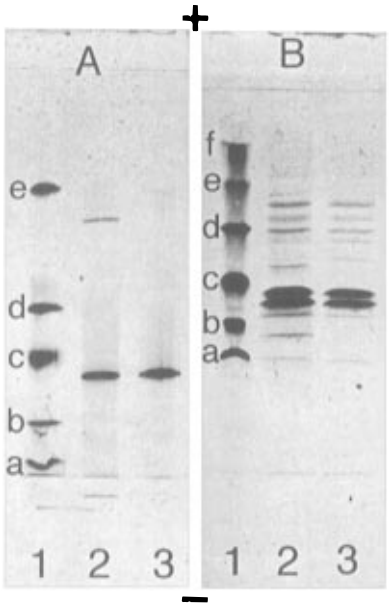

Figure 2. Native (A) and SDS (B) gel electrophoresis patterns for crude and partially purified cowpea globulin (fractions 73−76 from Figure 1). Approximately 2 μg of protein was applied to each lane. **Native gel.** Lane 1, standard proteins: a, thyroglobulin (669000 Da); b, ferritin (440000 Da); c, catalase (232000 Da); d, lactate dehydrogenase (140000 Da); e, bovine serum albumin (67000 Da). Lane 2, crude globulin. Lane 3, partially purified globulin. **SDS gel.** Lane 1, standard proteins: a, phosphorylase b (94000 Da); b, bovine serum albumin (67,000); c, ovalbumin (43000 Da); d, carbonic anhydrase (30000 Da); e, soybean trypsin inhibitor (20100 Da); f, α-lactalbumin (14400 Da). Lane 2, crude globulin; Lane 3, partially purified globulin.

of the 55 and 50 kDa polypeptides (61% from the densitometric scan) in the currently isolated protein would suggest that the partially purified globulin consists mostly of the 7S protein, though other non-7S proteins could be present. It is possible that the 321 kDa protein (typical of an 11S globulin) obtained on native gel (Figure 2) contains, in part, the dimerized form of 7S protein, since at the low ionic strength used in this study, the 7S protein can migrate as an 11S protein (Derbyshire et al., 1976). Moreover, it is recognized that a single band on native gel is not indicative of protein purity, since on native gels proteins migrate not only according to their molecular weights, but also their charge density (Dunn, 1989).

Optimization of the Protein Kinase Reaction. The effects of time, ATP, and enzyme and substrate concentration on the rate of incorporation of phosphate into the partially purified globulin were determined and reaction conditions optimized accordingly. The progress curve for the reaction was linear up to 60 min, corresponding to the initial velocity region of the reaction. ATP concentration became saturated above 60 μM. The velocity versus enzyme concentration curve was linear up to 5 units/mg globulin. A preliminary kinetic analysis of cowpea globulin phosphorylation was performed, and a K_m value of 1.12 μg/mg globulin and V_{max} of 13.2 μg phosphate/mL were obtained. The phosphate content of the phosphorylated protein used for determination of physicochemical and functional properties was 10.55 μg/mg, a significant ($p \leq 0.05$) 81% increase when compared with the value of 5.81 μg/mg obtained for the untreated protein.

Level of Deglycosylation. The deglycosylated protein had a residual carbohydrate content of 0.96% (w/w), compared to the 1.94% (w/w) found in the untreated

Table 1. Fluorescence Intensity Maxima (I_{max}) and Aromatic Hydrophobicity (ARH) of Untreated and Phosphorylated Partially Purified Cowpea Globulin[a]

	I_{max}		ARH	
pH	untreated	phosphorylated	untreated	phosphorylated
3	40.20[d]	44.55[a]	3203.85[b]	4039.30[a]
4	34.95[f]	37.65[e]	1395.80[d]	1884.60[c]
6	36.80[e]	41.30[c]	285.20[f]	365.90[f]
7	33.45[g]	43.25[b]	551.50[ef]	368.70[f]
8	41.60[c]	43.40[b]	742.40[e]	378.40[f]

[a] Mean of two determinations. For each attribute, means with different letters are significantly different ($p \leq 0.05$).

Table 2. Fluorescence Intensity Maxima (I_{max}) and Aromatic Hydrophobicity (ARH) of Untreated and Deglycosylated Partially Purified Cowpea Globulin[a]

	I_{max}		ARH	
pH	untreated	deglycosylated	untreated	deglycosylated
3	51.25[a]	45.95[c]	2964.20[a]	2767.30[b]
4	42.30[e]	40.20[f]	1314.09[c]	854.96[d]
6	44.60[d]	40.90[f]	285.26[f]	94.21[g]
7	48.85[b]	45.20[cd]	413.26[e]	317.92[ef]
8	46.20[c]	45.05[cd]	389.57[e]	357.57[e]

[a] Mean of two determinations. For each attribute, means with different letters are significantly different ($p \leq 0.05$).

protein. Thus, about 50% of the carbohydrate on the globulin was removed by the *N*-glycosidase. This is not surprising since complete removal of carbohydrate from most glycoproteins is difficult to achieve (Tarentino and Plummer, 1982). The presence of carbohydrate moieties on the partially purified globulin would suggest that it is made up mostly of the 7S protein.

Fluorescence Emission Properties. Table 1 shows the I_{max} and ARH fluorescence emission spectra properties of the untreated and phosphorylated proteins at pH 3−8. There was no significant ($p > 0.05$) difference in wavelength of maximum fluorescence (λ_{max}) between the untreated and phosphorylated proteins, with mean values of 343.9 and 343.7 nm, respectively. The maximum fluorescence intensity (I_{max}) of the phosphorylated proteins was significantly ($p \leq 0.05$) higher than that of the untreated protein at pH 3−8, which is an indication that in the phosphorylated protein the aromatic groups are less exposed to the polar environment than in the untreated protein. Decreased interactions between aromatic amino groups and polar solvents have been reported to be responsible for higher fluorescence intensity in proteins, since the rate of fluorescence quenching by polar radicals is reduced (Demchenko, 1986). The result suggests that introduction of negatively charged phosphate groups in the phosphorylated protein caused the hydrophobic groups to move farther into the protein inner core in order to avoid the increased polarity of the protein surface, when compared to the untreated protein.

Aromatic hydrophobicity (ARH) determined with the 1-anilino-8-naphthalenesulfonate (ANS) probe was significantly ($p \leq 0.05$) higher at pH 3.0 and decreased as the pH increased (Table 1); similar results were obtained for a cowpea globulin isolate (Aluko and Yada, 1995b). Increased binding of ANS at low pH values have also been reported for biological proteins such as toxins and anticoagulants (Sanyal et al., 1995). It has been shown that ANS binds very strongly to proteins in the molten globule state, much more than it binds to the unfolded or native state (Ptitsyn and Semisotnov, 1991). Therefore, the higher ARH of the phosphorylated protein at pH 3 and 4 may be due to the presence of a molten globule state compared to the untreated protein.

The wavelength of maximum fluorescence emission (λ_{max}) also did not differ significantly ($p > 0.05$) between the untreated and deglycosylated proteins, with mean values of 344.7 and 344.3 nm, respectively. However, the untreated protein displayed significantly ($p \leq 0.05$) higher total fluorescence intensity (I_{max}) at pH 3−7, when compared to the deglycosylated protein (Table 2). The decreased I_{max} of the deglycosylated protein would indicate greater exposure of some of its aromatic amino acid residues to the solvent than those of the untreated protein, since the degree of fluorescence quenching increases with increased hydration (Sanyal et al., 1993). The I_{max} result of the deglycosylated protein is in contrast to that obtained for the phosphorylated protein, which would indicate that removal of carbohydrate moieties caused exposure and/or reduced interactions between the aromatic amino acid residues, whereas phosphorylation had the opposite effect. The deglycosylated protein had significantly ($p \leq 0.05$) lower ARH at pH 3−6, when compared to the untreated protein (Table 2). The result indicates that deglycosylation caused a reduction in the number of surface hydrophobic clusters, since the latter are the binding sites for ANS (Ptitsyn and Semisotnov, 1991). The ARH result is consistent with the I_{max} result which indicated reduced interaction between the aromatic groups and increased interaction with the polar environment.

Circular Dichroism. There were no significant ($p > 0.05$) differences between the untreated and phosphorylated globulins in the proportions of secondary structure fractions obtained from far-UV CD spectra at pH 3−8. The mean values for α-helix, β-sheet, β-turn, and random structures were, respectively, 6.1, 74.6, 7.3, and 25.4%, for both untreated and phosphorylated globulins. The results showed a predominance of the β-sheet conformation at all the pH levels for both the untreated and phosphorylated proteins. Similar results were obtained for the deglycosylated globulin. The results suggest that neither the introduction of phosphate groups nor removal of carbohydrate moieties was strong enough to cause a change in the secondary structure of the cowpea globulin. Previous reports have also shown that mild treatments are not effective in changing the secondary structure of proteins (Rao et al., 1991; Luo et al., 1995).

The near-UV CD spectra of the untreated and phosphorylated proteins at pH 3.0 are shown in Figure 3. Above pH 3.0, there were no substantial differences in the intensities of the spectra when the untreated protein was compared to the phosphorylated protein. The result would suggest a loss in the tertiary structure of the protein at pH 3.0 as a result of phosphorylation (Strickland, 1974). Since a total loss of tertiary structure (zero near-UV CD intensity) was not obtained and there was no change in secondary structure, it could be postulated that the phosphorylated protein existed as a molten globule (MG) at pH 3.0 (Eubanks and Creighton, 1991; Luo et al., 1995). In the MG state, the protein assumes a conformation that is intermediate between the native and the completely unfolded states; secondary structure remains unchanged, but the tertiary structure of the MG differs from that of the native protein (Ptitsyn and Semisotnov, 1991). A molten globule state has also been observed for the globulin from *Amaranthus hypochondriacus* (Marcone, 1996).

The near-UV CD spectra for untreated and deglycosylated proteins are shown in Figure 4, parts A, B, and C, for pH 3, 6, and 8, respectively. The spectra at

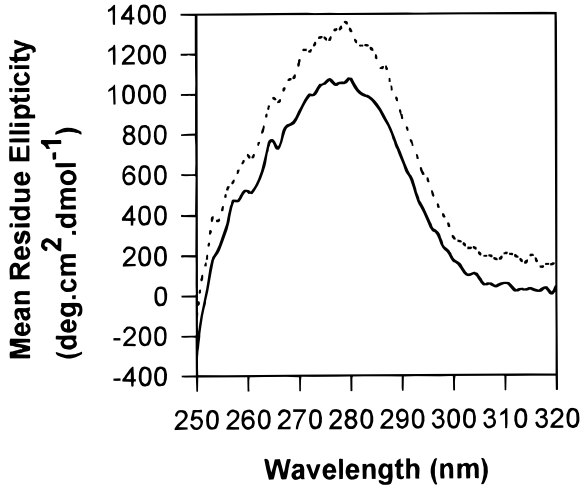

Figure 3. Near-UV CD spectra at pH 3.0 of untreated (···) and phosphorylated (—) partially purified cowpea globulin. The spectra each represent an average of four scans for protein concentrations ranging from 0.2 to 0.3 mg/mL.

pH 4.0 and 7.0 were similar to the spectra shown for pH 3.0 and 8.0, respectively (data not shown). The intensities of the spectra were lower at pH 3, 4, and 6 for the deglycosylated protein than for the untreated protein. Since a decrease in near-UV CD spectra is associated with a change in the tertiary structure from a compact to a loose structure (Strickland, 1974), it can be implied that deglycosylation caused a loss in tertiary structure of the protein. Similar change in tertiary structure of phosphorylated globulin at pH 3.0 was also observed (Figure 3). However, the magnitude of the differences in near-UV CD spectra between untreated and modified proteins was greater for the deglycosylated protein than the phosphorylated protein. It is possible, therefore, that deglycosylation (removal of carbohydrate moieties and addition of carboxyl groups) was a more severe form of treatment than phosphorylation (addition of phosphate groups), at least at the levels of modifications obtained in this work. At pH 7 and 8, the deglycosylated protein, however, showed a more intense spectra (Figure 4C), suggesting a more ordered structure than the untreated protein (Strickland, 1974). The increase in tertiary structure of the deglycosylated protein at pH 7 and 8 may have been as a result of its increased interaction with the solvent, resulting in a more ordered protein structure than the untreated protein. This is because at neutral pH and above, there is an increased charge effect resulting from ionization of the deglycosylation-added carboxyl groups which would favor increased protein−solvent interactions.

Protein Solubility and Heat Coagulability. Solubility and heat coagulability of the untreated and phosphorylated proteins are shown in Table 3. Except at pH 7.0, the phosphorylated protein had significantly ($p \leq 0.05$) higher solubility than the untreated protein. The improvement in solubility can be attributed to introduction of negatively charged phosphate groups onto the protein molecule. The solubility of a protein has been reported to be a manifestation of the equilibrium between protein-solvent and protein−protein interactions (Kinsella et al., 1985). Therefore, addition of hydrophilic phosphate groups shifts the equilibrium away from protein−protein interactions and enhances solubility of the protein. The increased solubility of the phosphorylated protein when compared to the untreated protein is in agreement with the I_{max} result shown in Table 1, i.e., increased interactions between the aromatic residues and decreased exposure of the residues to the polar solvent as a result of phosphorylation. Similar results showing improved protein solubility as a result of enzymatic phosphorylation have been reported for soybean protein isolate (Campbell et al., 1992; Shih and Campbell, 1993) and cowpea protein isolate (Aluko and Yada, 1995a).

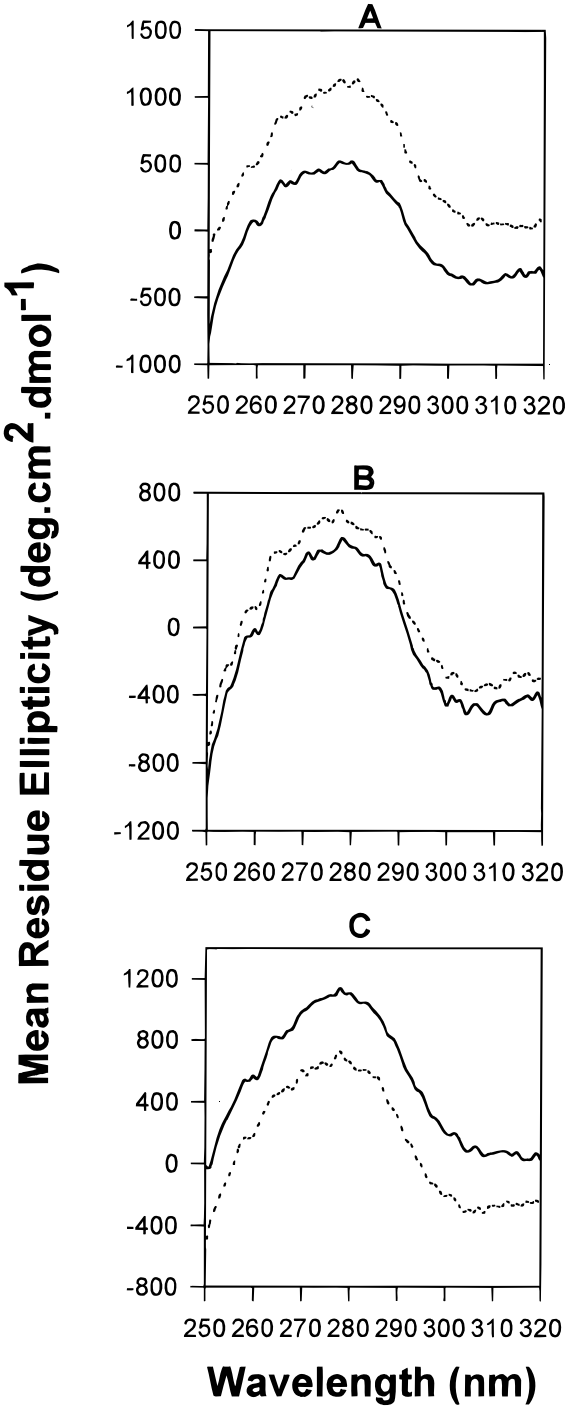

Figure 4. Near-UV CD spectra at pH 3.0 (A), 6.0 (B), and 8.0 (C) of untreated (···) and deglycosylated (—) partially purified cowpea globulin. The spectra each represent an average of four scans for protein concentrations ranging from 0.2 to 0.3 mg/mL.

Heat coagulability was significantly ($p \leq 0.05$) reduced as a result of phosphorylation, except at pH 6.0. The increased heat stability of the phosphorylated protein may also be attributed to interaction with water as a result of the increased level of phosphate groups. Such increased interactions could have reduced the

Table 3. Protein Solubility (PS) and Heat Coagulability (HC) of Untreated and Phosphorylated Partially Purified Cowpea Globulin[a]

	PS (%)		HC (%)	
pH	untreated	phosphorylated	untreated	phosphorylated
3	77.73[e]	97.16[b]	1.22[e]	0.00[f]
4	75.81[e]	89.83[d]	11.88[b]	7.65[c]
5	0.11[h]	0.94[h]	nd	nd
6	16.13[g]	22.06[f]	98.02[a]	98.70[a]
7	95.26[bc]	94.57[c]	2.89[d]	0.00[f]
8	97.27[b]	99.65[a]	0.00[f]	0.00[f]

[a] Mean of two determinations. For each functional property, means with different letters are significantly different ($p \leq 0.05$). nd = not determined.

Table 4. Protein Solubility (PS) and Heat Coagulability (HC) of Untreated and Deglycosylated Partially Purified Cowpea Globulin[a]

	PS (%)		HC (%)	
pH	untreated	deglycosylated	untreated	deglycosylated
3	73.59[b]	70.59[c]	4.88[e]	13.01[cd]
4	71.02[c]	65.07[d]	14.64[cd]	22.96[b]
5	0.14[f]	0.27[f]	nd	nd
6	20.20[e]	18.78[e]	99.10[a]	99.52[a]
7	75.20[b]	70.86[c]	10.25[de]	16.52[c]
8	77.42[a]	74.00[b]	12.69[cd]	14.91[cd]

[a] Mean of two determinations. For each functional property, means with different letters are significantly different ($p \leq 0.05$). nd = not determined.

tendency for protein−protein interactions required for heat-induced coagulation. Decreased heat coagulability of the phosphorylated globulin is also in agreement with decreased exposure of its aromatic groups as shown by the intrinsic fluorescence emission properties, I_{max} (Table 1), since the tendency for protein−protein interactions through hydrophobic bonds is reduced, when compared to the untreated globulin where the hydrophobic groups are more exposed. The higher heat stability of the phosphorylated protein is ideal for utilization in heat-based beverages, e.g., as a coffee whitener (Kinsella, 1982).

Table 4 shows protein solubility and heat coagulability of the untreated and deglycosylated cowpea globulins. Removal of carbohydrate resulted in a protein with significantly ($p \leq 0.05$) lower solubility than the untreated protein, except at pH 5 and 6. The observed decrease in solubility is in agreement with a reduction in intrinsic fluorescence (I_{max}) shown in Table 2 which suggested an increased exposure of the aromatic amino acid residues to the solvent, a condition that does not favor protein−solvent interactions (Kinsella et al., 1985). On the other hand, the untreated protein was more heat stable than the deglycosylated protein. Removal of carbohydrate moieties from the globulin seemed to have increased protein−protein interactions more than protein−solvent interactions. The lower heat stability of the deglycosylated protein is an indication that the presence of carbohydrate moieties on proteins reduces the potential for protein−protein interactions that could lead to aggregation and/or precipitation, as was previously reported for β-lactoglobulin (Kitabatake et al., 1985). The present results are consistent with the suggestions of Grimaldi et al. (1985) that carbohydrate groups of proteins, through interactions with water, produce frictional drag that increases the stability of the protein against denaturants. In addition, the significant ($p \leq 0.05$) increase in heat lability as a result of deglycosylation is supported by the observed reduction in tertiary structure (Figure 4, parts A and B), since greater penetration of the structure by water is favorable to heat-induced coagulation (Kinsella et al., 1985).

Conclusion. Addition of charge through phosphorylation increased interactions between the cowpea globulin and the polar environment, leading to increased solubility, decreased heat stability, and decreased exposure of the hydrophobic groups. On the other hand deglycosylation which involved both removal of sugar molecules and addition of charge led to increased exposure of the hydrophobic groups and hence decreased solubility of the globulin. The results suggest that the increased protein−protein interactions as a result of loss of sugar groups was greater than the increased protein−solvent interactions that arose from the introduction of carboxyl groups.

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