content in *su o2* and also normal endosperm.

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Major Proteins of Soybean Seeds. Reversible and Irreversible Dissociation of β-Conglycinin

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Reversible and irreversible dissociations of β-conglycinin were investigated by ultracentrifugation, disc electrophoresis, and immunodiffusion methods. The protein had a protomer conformation (7S) at high ionic strength ($I > 0.5$) or at acidic pH (pH <4.8) and a dimer conformation (10S) at low ionic strength ($I < 0.2$) in the pH region 4.8–11.0. Rapid interconversion between the protomer (trimeric structure) and the dimer (hexameric structure) was observed in the 0.2–0.5 ionic strength region. At very low ionic strength ($I < 0.01$), the α subunit dissociated from the protein. The dissociation was reversible but may result in the generation of multiple molecular forms (B_2 to B_6 conglycinins). The quaternary structures were stable at high ionic strength. Complete reversible dissociation into subunits occurred in 5 M urea ($I = 0.01$). Reversible dissociation into monomers (3–4S) appeared at pH 12.0 ($I = 0.5$). Dissociation into polypeptides (2S) at pH 2.0 and 12.0 ($I = 0.01$) was also reversible. Irreversible dissociation at pH 13.0 may be attributable to alkaline degradation.

Oligomeric storage proteins of legume seeds show a rather complicated reaction of association–dissociation. The ability to undergo conformational changes may have a physiological significance which relates to changes in osmotic pressure (Kretovich and Smirnova, 1960). The dissociation of the storage proteins has also been suggested to occur during seed germination to make the proteins accessible to proteinase attacks before final utilization by the seedling (Catsimpoolas et al., 1968). The dissociation of a protein into its protomers is, in most cases, reversible. Further dissociation of protomer into monomers is either reversible or irreversible depending on the properties of the protein and the condition under which the dissociation and, possibly, simultaneous unfolding of the monomers take place.

On the basis of association–dissociation properties, vicilin proteins (7S globulins) from legume seeds can be divided into three types (Derbyshire et al., 1976). One type dimerizes to a 9–12S form at 0.1 ionic strength and neutral pH, the second retains a 7S form at low ionic strength, and the third is insensitive to changes of ionic strength but associates to an 18S form (probably, a tetramer of 7S) at pH values near its isoelectric point. β-Conglycinin, a major 7S soybean globulin, belongs to the first type. γ-Conglycinin from soybeans has a characteristic of the second type (Koshiyama and Fukushima, 1976). A representative of the third type is G1 protein from *Phaseolus vulgaris* seeds (Sun et al., 1974).

Conformational changes of β-conglycinin have been reported (Naismith, 1955; Roberts and Briggs, 1965; Koshiyama, 1968). It is well-known that the conversion between protomer (7S) and dimer (10S) is reversible. However, dissociation of the protomer into subunits (polypeptide chains) has been unclear. Roberts and Briggs (1965) suggested that the protomer consists of at least seven different subunits. Koshiyama (1971) proposed nine subunits in a 7S molecule. We have isolated and characterized the constituent polypeptide chains (Thanh and Shibasaki, 1977) and presented trimeric structures for six different 7S molecules of β-conglycinin (Thanh and Shibasaki, 1978a,b).

From the new view on the subunit structure we investigated the association and dissociation of β-conglycinin at various pHs, ionic strengths, and urea concentrations using ultracentrifuge, disc electrophoresis, and immunodiffusion. The present study reveals a reversible dissociation into subunits at very low ionic strength and at acidic or alkaline pH and irreversible dissociation at extreme alkaline pH values. The findings are discussed with regard to the molecular structures of β-conglycinin.

MATERIALS AND METHODS

Protein Samples. β-Conglycinin, B_1 and B_6 conglycinins, and α, α′, and β subunits were isolated and purified as described previously (Thanh and Shibasaki, 1976a,b, 1977). The proteins were freeze-dried at pH 7.0.

Buffers. Potassium phosphate buffer at 0.1 ionic strength ($I = 0.1$) contained 2.6 mM KH_2PO_4, 32.5 mM K_2HPO_4, and 3 mM NaN_3, pH 7.8. Buffers at various ionic strength were prepared by adding NaCl ($I > 0.1$) or dis-

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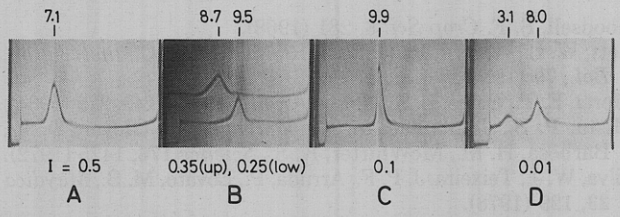

Figure 1. Ultracentrifugal patterns of β-conglycinin dissolved in standard buffer at 0.5 ionic strength (A); subsequently dialyzed against 0.35 and 0.25 (B) and 0.1 (C) ionic strength buffers; and dissolved in 0.01 ionic strength buffer (D). The protein concentration was 0.6%. Photographs were taken after 42 min of centrifugation at 55 430 rpm. Direction of sedimentation is from left to right. The $s_{20,w}$ values in svedberg units are indicated on the top of the figures.

tilled water ($I < 0.1$) to the potassium phosphate buffer. Standard buffers were the corresponding phosphate buffers which contained, in addition, 10 mM 2-mercaptoethanol.

Dissociation at Low Ionic Strength and in Urea Solution. The proteins (0.2 and 0.6% concentration) in standard buffers (I = 0.5, 0.1, and 0.01) and phosphate buffer (I = 0.01) containing 0–8 M urea were treated at 30 °C for 1 h. Dissociation of β-conglycinin with urea at 0.5 ionic strength was carried out as follows. First, the protein dissolved at 1% concentration in phosphate buffer (I = 0.5) was treated at 30 °C for 1 h. Subsequently, phosphate buffer and the buffer containing 10 M urea were added to the protein solution to prepare 0.2% protein solutions made to 0–8 M in urea. The dissociation was allowed to proceed at 30 °C for 1 h.

Dissociation at Acidic and Alkaline pHs. β-Conglycinin was dissolved at 0.2 and 0.6% concentration in deionized water (pH 7) and in 0.5 M NaCl solution and then treated at 30 °C for 1 h. Protein solution at 0.1 ionic strength was prepared by dialysis of the protein in 0.5 M NaCl against 0.1 M NaCl solution (pH 7). The protein solutions were adjusted to desired pH values with small amounts of 1 M NaOH (alkaline side) or 1 M HCl (acidic side) while stirring. After treatment at room temperature (20–22 °C) for 1 h, the samples were subjected to disc electrophoresis. Further treatment (24 h and 12 days) was carried out at 5 °C.

Analytical Methods. Standard disc electrophoresis was performed on 6.5% polyacrylamide gel. Samples (20 μL of a 0.2% solution) were incorporated in the sample gel. In a few experiments, samples were applied on top of the spacer gel. The electrode buffer contained 8 mM Tris-glycine, pH 8.3.

Ultracentrifugal analysis was carried out at 0.6% protein concentration at 20 °C with a Hitachi UCA-1 ultracentrifuge at 55 430 rpm. β-Conglycinin for sedimentation analysis at ionic strength in the range of 0.05–0.90 was treated in standard buffer (I = 0.5) at 30 °C for 1 h and subsequently dialyzed overnight against buffers at various ionic strength before analysis.

Immunodiffusion in agar gel (standard buffer, I = 0.5) was described previously (Thanh and Shibasaki, 1976a).

RESULTS AND DISCUSSION

Interconversion between Protomer and Dimer. At pH 7.6 β-conglycinin had a 7S form (protomer) at 0.5 ionic strength and a 10S form (dimer) at 0.1 ionic strength (Figure 1A,C). At intermediate ionic strength the two forms coexisted. However, mixtures of the two forms (0.6% protein concentration) exhibited single moving boundaries on ultracentrifugation although the peaks became somewhat asymmetric (Figure 1B). Iibuchi and Imahori (1978) also reported single peaks of the mixtures

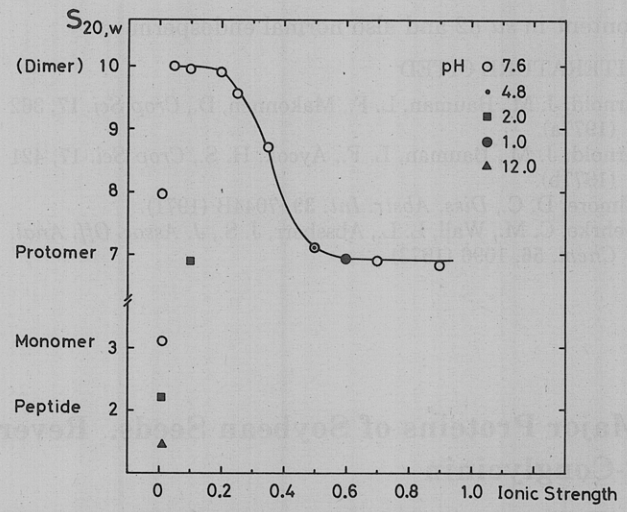

Figure 2. Conformation of β-conglycinin: sedimentation coefficient ($s_{20,w}^{0.6\%}$) of the protein at pH 7.6 as a function of ionic strength (continuous line) and dissociation into protomer, monomers, or peptides at acidic or alkaline pH.

at 0.16% protein concentration. The observation of the single peaks can be explained by the Gilbert theory (Gilbert, 1955, 1959). Roberts and Briggs (1965) and Koshiyama (1968), however, observed a double peak at the intermediate ionic strength. Since the protein concentration used by the latter authors was not given, comparison of the two results is difficult.

Figure 2 shows the change in sedimentation coefficient of β-conglycinin at mild alkaline pH (pH 7.6) with change in ionic strength. It is clear that the protomer (7S) predominates at ionic strength greater than 0.5 and the dimer (10S) at ionic strength less than 0.2. In the ionic strength region of 0.2–0.5, varying amounts of the protomer and the dimer accounted for the gradual change in sedimentation coefficient which approached 7 or 10 S as equilibrium was shifted to either side. Recently, Iibuchi and Imahori (1978) also found the gradual change in sedimentation of the protein, but in the ionic strength region of 0.2–0.8, and estimated the sedimentation coefficient $s_{20,w}^{0.16\%}$ of the protomer as 5.6 S. In our experiments, the $s_{20,w}^{0.6\%}$ of the protomer, either at alkaline pH ($I \geq 0.6$) or at acidic pH (pH 2.0, I = 0.1; and pH 1.0, I = 0.6), was 6.9 S. The different results might be attributable to the differences in variety of soybean and in the preparation of protein samples.

Reversible Dissociation at Low Ionic Strength. On ultracentrifugation, β-conglycinin dissolved in buffers at 0.01 ionic strength exhibited, together with an associated form ($s_{20,w}$ = 8.0), a slow-moving material sedimenting at 3.1 S (Figure 1D). Disc electrophoresis of the protein dissolved at various ionic strength gave identical patterns, except for the appearance of a major dissociated band (R_m 0.53) which was pronounced at 0.01 ionic strength (Figure 3A). The multiple bands (R_m 0–0.12), which are not clearly seen in the photograph, represent the associated forms (10S) of B_1 to B_6 conglycinins. The protein band at 0.53 R_m correlating to the 3S sedimenting form in Figure 1D represents, from the consideration of its sedimenting velocity and the molecular weights 42 000 and 57 000 of the subunits (Thanh and Shibasaki, 1977), a dissociated monomer (subunit).

The observation of two ultracentrifugal peaks, 3.1 S and 8.0 S, may also be explained by the Gilbert theory. In this case, a suitable explanation is a rapidly reversible dissociation in polymer ⇌ monomer reaction. The reversible dissociation would exhibit two ultracentrifugal peaks: one

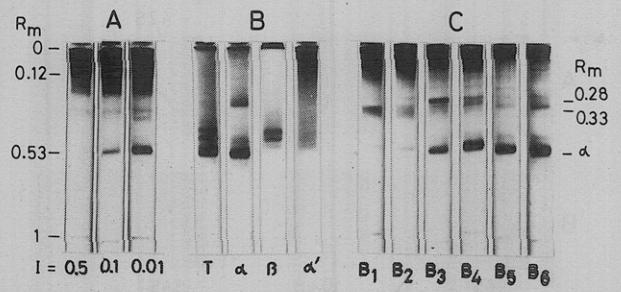

Figure 3. (A) Disc electrophoresis of β-conglycinin treated at various ionic strength. (B) Characterization of dissociated subunits on disc gel; β-conglycinin (T) and the subunits (α, α′, and β) were treated with 6 M urea before electrophoresis. (C) Dissociation of B_1 to B_6 conglycinins at low ionic strength; the freeze-dried samples were dissolved in phosphate buffer (I = 0.01, pH 7.8).

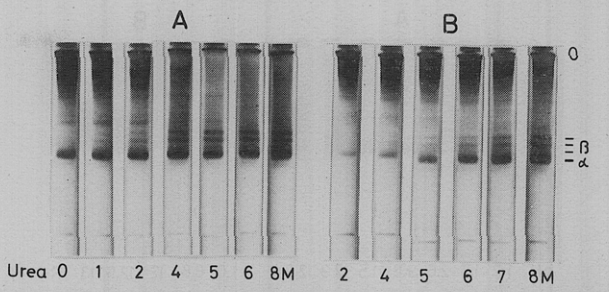

Figure 4. Dissociation of β-conglycinin with urea. The protein was treated in phosphate buffers containing urea at 0.01 ionic strength (A) and 0.5 ionic strength (B). The direction of migration in the disc gels is from the top. Dissociated subunits are identified at the left of Figure B.

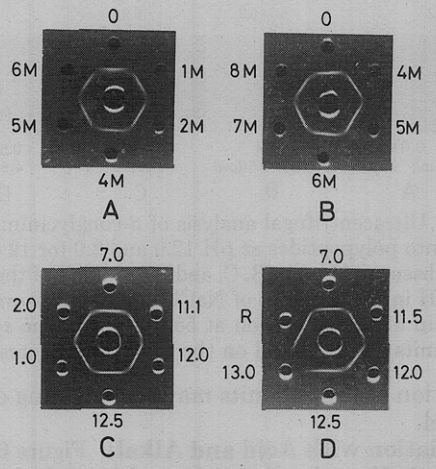

Figure 5. Double gel immunodiffusion of β-conglycinin treated with 0–8 M urea at 0.01 ionic strength (A) and 0.5 ionic strength (B) and treated in water at various pH for 12 days (C and D). The gel contained 1% agar in standard buffer (I = 0.5). The center wells contained antisera to the native β-conglycinin. The peripheral wells contained the treated samples at 2 mg/mL concentration. Loss of the reactivity appears at pH 13.0; however, neutralization of the freshly prepared alkaline peptides restored the reactivity (well R).

is the monomer (subunit) and the other, a mixture of the "dimer" and monomer.

Characterization of the dissociated monomer is shown in Figure 3B. Protein samples (β-conglycinin and the isolated subunits) were dissolved in phosphate buffer containing 6 M urea (I = 0.01, pH 8.0) and subsequently subjected to electrophoresis in the absence of urea. β-Conglycinin dissociated into four bands. One major band at 0.53 R_m was identical with the band from α subunit and with the dissociated monomer in Figure 3A. The other bands were identical with those from β subunit. The α′ subunit gave no bands but a broad zone in the 0–0.5 R_m region. The observation of α and β bands and the identification of the α band with the dissociated monomer suggest that the α and β polypeptide chains, upon removal of urea by electrophoresis, may refold to a definite structure, rather than random structures at various steps of refolding which would lead to broadening of protein zones on disc gels.

Since freeze-dried samples were used in this investigation, the dissociation had obviously taken place in the preparation processes, during dialysis of the protein solution against water before freeze-drying. However, the dissociation was reversible: ultracentrifugal patterns of the protein run at 0.5 and, subsequently, at 0.1 ionic strength showed no dissociated subunits (Figure 1A,C). Without prior treatment at high ionic strength (I = 0.5), the protein dissolved directly in buffer (I = 0.1) gave a minor band of α subunit (Figure 3A). This indicates that reassociation of the subunit is favored at high ionic strength.

Figure 3C shows the dissociation of B_1 to B_6 conglycinins which were dissolved directly in low ionic strength buffer (I = 0.01). The conglycinins of group A (B_1 and B_2) exhibited no dissociated α monomer, whereas the conglycinins of groups B and C (B_3 to B_6) gave the α band. The protein bands in the 0.28–0.33 R_m region may represent dissociated intermediates. One of these bands was identical with an α aggregate (0.28 R_m) in Figure 3B. The others have not identified.

The reversible dissociation at low ionic strength may lead to molecular rearrangements, i.e., the generation of several molecular conglycinins from one molecular species. The freeze-dried samples of B_3 and B_4 conglycinins ($\alpha\alpha'\beta$ and $\alpha_2\beta$, respectively) dissolved in standard buffer (I = 0.5) showed multiple conglycinins (B_2 to B_6) on disc gels. During the isolation of the six conglycinins in the previous report (Thanh and Shibasaki, 1976b), the dissociation was not likely to occur since the proteins, from extraction to final purification, had been kept in buffers ($I \geq 0.05$). No interconversion between the conglycinins had been observed on repeated chromatography and on electrophoresis. The six conglycinins are, therefore, the isomers of β-conglycinin that exist in soybean-meal extracts, rather than a result of manipulation after extraction.

Dissociation in Urea Solutions. Roberts and Briggs (1965) and Koshiyama (1970) reported a gradual dissociation of β-conglycinin in urea solutions. The dissociation into subunits could be followed easily by disc electrophoresis (Figure 4). At 0.01 ionic strength, pH 8.0, the dissociation occurred from 1 M urea concentration where, along with α subunit, β subunit was observed (Figure 4A). The protein dissociated completely into subunits at urea concentrations equal to or greater than 5 M.

The quaternary structure of the protein was rather stable at 0.5 ionic strength (Figure 4B). It is likely that urea up to 4 M did not disrupt the structure. At low urea concentration (2–5 M), gradual changes in sedimenting velocity of β-conglycinin (Koshiyama, 1970) may be explained by a rapid reversal of dissociation. This dissociation could not be observed on disc gels since complete reassociation may take place during electrophoresis. Urea more than 5 M promoted the dissociation which is obviously seen in Figure 4B.

The dissociation in urea solutions was reversible. Upon removal of urea by dialysis against buffers, the subunits recombined to form β-conglycinin (Thanh and Shibasaki, 1978b). Direct application of the urea-treated samples on immunoplates also yielded the recovery of immunological reactivity (Figure 5A,B). This suggests that refolding and

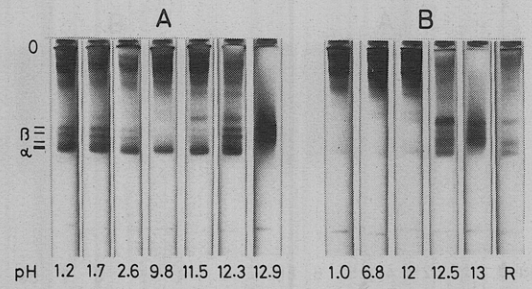

Figure 6. Dissociation of β-conglycinin at extreme pH: (A) the protein was dissolved in water, treated at the indicated pH for 24 h, and subjected to disc electrophoresis; (B) the dissociation in the presence of 0.5 M NaCl, (R) neutralization of the freshly prepared alkaline peptides.

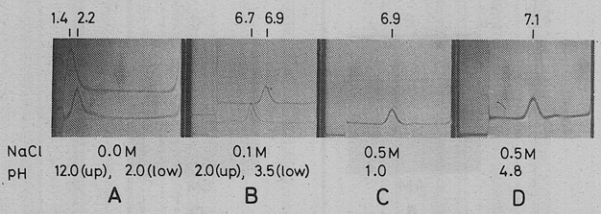

Figure 7. Ultracentrifugal analysis of β-conglycinin: (A) dissociation into polypeptides at pH 12.0 and 2.0 for 12 days at 5 °C in the absence of NaCl; (B, C, and D) stability of the 7S form at acidic pH in the presence of NaCl. Photographs were taken after 60 min of centrifugation at 55 430 rpm. The $s_{20,w}^{0.6\%}$ in svedberg units are indicated on the top of the figures.

reassociation of the subunits may occur during diffusion in agar gel.

Dissociation with Acid and Alkali. Figure 6A shows the effect of pH on the dissociation of β-conglycinin at low ionic strength. The protein dissolved in water was adjusted to the indicated pH value by addition of HCl or NaOH. This resulted in a small change of ionic strength in the pH region of 2–12. The electrophoresis patterns in the pH region of 3–11 were similar; no dissociated β subunit was observed. At lower pH (pH <3), the dissociation was time-dependent: β subunit was observed after 24 h of treatment. However, the dissociation was not complete even after 12 days of treatment. The protein treated at pH 2.0 for 12 days exhibited dissociated subunits of 2.2 S (Figure 7A, lower pattern). Alkali-induced dissociation occurred at pH 11.5. At pH 12.0 the alkaline polypeptides had a sedimentation coefficient of 1.4 S (Figure 7A, upper pattern). Unfolding of the polypeptides at pH 2.0 and 12.0 is assumed to account for the low sedimentation coefficients obtained.

At ionic strength greater than 0.1, the quaternary structure of the protein was stable in a wide pH range of 2–11. Disc electrophoresis (Figure 6B) showed no dissociation at acidic side up to pH 2.0 (I = 0.1) or pH 1.0 (I = 0.6). Complete dissociation was observed at pH 12.5. The protein kept a 7S form in acidic solutions (Figure 7B,C,D).

β-Conglycinin treated at pH equal to or less than 12.5 regained the immunological reactivity (Figure 5C,D). Uncomplete dissociation of the protein may account partly for this reactivity. However, the reactivity of the subunits completely dissociated at pH 12.5 can be explained only by the refolding and reassociation during diffusion. The dissociation is considered, therefore, reversible. Irreversible dissociation, as judged by the loss of immunological reactivity, occurred at pH 13.0 (Figure 5D). Neutralization of the alkaline peptides treated at pH 13.0 for 24 h at 5 °C gave no reactivity. However, shorter treatment time (15 min) permitted, upon neutralization, recovery of the reactivity.

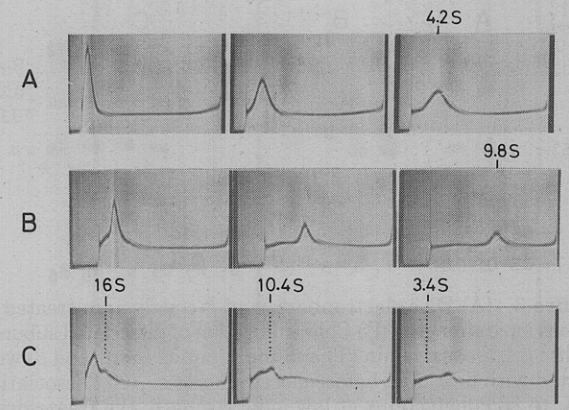

Figure 8. Ultracentrifugal patterns of β-conglycinin treated at pH 12.0 in 0.5 M NaCl solution (A); subsequently dialyzed against 0.5 and then 0.1 ionic strength phosphate buffers (B); treated at pH 12.5, 0.5 M NaCl and dialyzed against the phosphate buffers (C). Photographs were taken at intervals of 18 min (A and B) and 9 min (C) after reaching 55 430 rpm. Protein concentration was 0.6%.

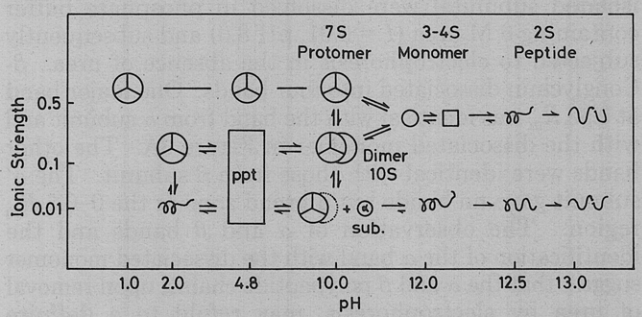

Figure 9. Schematic representation of the conformational changes of β-conglycinin at various pH and ionic strength, the reversible dissociation into subunits at alkaline pH and at low ionic strength, and the irreversible dissociation at extreme alkaline pH. (ppt) isoelectric precipitation.

Ultracentrifugal analysis also revealed the reversal of dissociation at alkaline pH in the presence of 0.5 M NaCl. B-Conglycinin underwent conformational changes at pH 12.0 (Figure 8A). The dissociated subunits (4S) may readily recombine during electrophoresis at pH 8.3 and, therefore, are not seen in Figure 6B (pattern at pH 12). Dialysis of the subunits against buffers (I = 0.5 and 0.1, pH 7.6) yielded a 10S form ($s_{20,w}$ = 9.8) of the native protein (Figure 8B). The dissociation at pH 12.5 was partially reversible. At the extreme pH, the reassociated 10S form was recovered in low yield, together with a 3S form and a 16S aggregate (Figure 8C).

Association and Dissociation Phenomena. Figure 9 shows a schematic representation of the conformation of β-conglycinin at various pH and ionic strength. The representation of the molecular structures is based on the foregoing results and the trimeric and hexameric structures proposed for the protomer (7S) and "dimer" (10S) of the protein (Thanh and Shibasaki, 1978a).

The six isomers (B_1 to B_6) of β-conglycinin seem not to differ from one another in the association–dissociation behaviors at ionic strength equal to or greater than 0.1. At its isoelectric point (pH 4.8) β-conglycinin keeps a protomer conformation ($s_{20,w}$ = 7.1) in 0.5 M NaCl solution (I = 0.5) and precipitates at lower ionic strength. The protomer is stable in the pH region 1–11.5, at ionic strength greater than 0.5, and in the acidic region (pH 2–4) at ionic strength less than 0.2. The dimer is predominant in the pH region 5–11 and at ionic strength less than 0.2. Both forms, dimer and protomer, exist at pH 7.6 and intermediate ionic strength, and at pH 4.8 and low ionic

strength.

The monomers (subunits) are dissociated reversibly from the protomer or the dimer at pH 11.5–12.0. They may undergo conformational changes before extensively unfolding at pH 12.5. At extreme alkaline pH (pH 13) alkaline degradation may account for the irreversible dissociation. At ionic strength less than 0.01, reversible dissociation into subunits occurs even at mild alkaline or mild acidic pH. In the alkaline region, the dissociation of B_3 to B_6 conglycinins gives α subunit. In the acidic region both α and β subunits are dissociated from β-conglycinin.

Dissociation of β-conglycinin with urea has been found to accompany simultaneously the destruction of internal structure of the protein, which led to the suggestion that the subunits were very compactly and complicatedly folded on the formation of the gross structure (Koshiyama, 1971). However, in the present study the ability of the protein to undergo dissociation at low ionic strength and at physiological pH suggests that the subunits can exist as organized monomers in equilibrium with the trimer and hexamer within soybean seeds. Thus, the dissociation may not require an extensive destruction of the secondary and tertiary structures of the protein.

Contrary to a report of Koshiyama (1968) we found that the dissociation at alkaline pH was reversible. The dissociation coincided with the ionization of tyrosine residues in β-conglycinin. Tyrosine and the electrostatic interactions that are disrupted at alkaline pH due to unionization of ϵ-amino group of lysine (pK = 10.53) and guanidine group of arginine (pK = 12.48) are considered to contribute to the interaction between the subunits. Tyrosine residues are likely to be present in the subunit contact region—therefore, exposed to the media during dissociation—rather than being buried in the interior of the subunits, because their ionization at alkaline pH was not time-dependent (Koshiyama, 1971). In a similar manner, most of the tyrosine residues of conarachin (78%), which ionized with apparent pK of 11.2 at low ionic strength, were proposed to be located at the interfaces of the subunits, stabilizing the quaternary structure of the protein (Yotsuhashi, 1973).

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Protein Fractions from Five Varieties of Grain Sorghum: Amino Acid Composition and Solubility Properties

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Classic protein fractions 1, 2, 3, and 4 (albumins, globulins, prolamins, and glutelins, respectively) were obtained from five varieties of sorghum that differed in endosperm/pericarp structure and in tannin content. Amino acid profiles and protein distribution of isolated fractions showed some differences among varieties. The chemical scores varied from 9 to 91. Methionine, cysteine, isoleucine, and leucine were most limiting in fractions 1 and 2. Fractions 3 and 4 were most deficient in lysine in all five varieties. Leucine/lysine ratio was above 20 in all of the fractions 3 except for that in a high-tannin variety; the ratio of all of the other fractions was below 4. Fractions 1 and 2 extracted from the high-tannin variety contained less protein than those from the other varieties. The comparable extent of essential amino acid deficiencies and excesses in these fractions is given.

Grain sorghum, *Sorghum bicolor* (L.) Moench, is an important food crop in many parts of the world and is a major feed grain produced in the United States. Cultivated sorghums include five races: bicolor, caudatum, durra, guinea, and kafir (Schecter and Dewet, 1975). The physical and chemical characteristics of the grain which vary among varieties, account for differences in biological values and availability of nutrients. Recent studies on several varieties suggested that the efficiency of grain fed to ruminants is related, in part, to both peripheral and internal structures of the seed (Sullins, 1972; Sullins and Rooney, 1974) and to the presence of polyphenolic compounds (Axtell, 1976). Only a small amount of nutritional data of sorghum fed to humans, however, has been reported.

The amino acid composition of the total protein in sorghum is similar to that of corn and other cereals, where lysine is the most limiting amino acid (Wall and Blessin, 1970). Low content of lysine is attributed to the high content of prolamin (fraction 3) in most normal varieties. The biological value of sorghum is further reduced by the presence of metabolic inhibitors and by chemical inter-

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