Structure of 8Sα globulin, the major seed storage protein of mung bean

Takafumi Itoh,* Roberta N. Garcia,b Motoyasu Adachi,a‡ Yukie Maruyama,a Evelyn Mae Tecson-Mendoza,b Bunzo Mikami*a and Shigeru Utsumia*‡

aFood Quality Design and Development, Graduate School of Agriculture, Kyoto University, Gokasho, Uji, Kyoto 611-0011, Japan, and bInstitute of Plant Breeding, College of Agriculture, University of the Philippines Los Baños, College, Laguna 4031, Philippines

‡ Present address: Center for Neutron Science, Tokai Research Establishment, Japan Atomic Energy Research Institute, 2-4 Shirakata, Tokai-mura, Ibaraki 319-1195, Japan.

Correspondence e-mail: sutsumi@kais.kyoto-u.ac.jp

1. Introduction

Mung beans [Vigna radiata (L.) Wilczek] are a popular food crop in Asia, South America, Australia and the USA and are similar to other legumes such as soybeans, jack beans and kidney beans. Mung bean seeds contain about 20–25% protein and are a major source of protein, especially in developing countries.

The major seed proteins of mung bean are storage globulins of the basic 7S type (~3%), vicilin type (8S; ~90%) and legumin type (11S; ~8%) (Tecson-Mendoza et al., 2001). The 8S globulins, which are the major storage globulins of mung bean, have molecular weights of about 150 kDa and consist of three homologous isoforms, 8Sα, 8Sα* and 8Sβ, which exhibit high homology (about 90% identity) to each other and have a molecular weight of about 49 kDa, indicating that the native 8S globulin consists of heterotrimers (Tecson-Mendoza et al., 2001; Bernardo et al., 2004). Like other 7S globulins, they have no disulfide linkages (Bernardo et al., 2004). Their amino-acid sequences exhibit similarity (about 68%) to soybean β-conglycinin β (a 7S globulin). It has been reported that soybean
globulins such as soybean analysis is indispensable. The crystal structures of seed storage
properties of proteins as food materials, their structural
the food utility of mung beans.
acteristics corresponding to those of soybeans and will expand
bean globulins may result in the enhancement of their char-
(Nishi et al., 2004), and
proglycinin A1aB1b (Adachi et al., 2003), jack bean canavalin (McPherson, 1980; Ko
soybean proteins have been studied (Maruyama et al., 2001, 2004; Adachi, Okuda
et al., 2004) to elucidate the structure of the
space. The protein concentration was 5 mg ml
protein and genetic engineering.
We have expressed the major isoform 8Sα of mung bean 8S
globulin in *Escherichia coli* and have successfully crystallized it (Bernardo et al., 2004). Recently, several physicochemical and functional properties of native and recombinant 8Sα
globulin have been clarified and will be described. This article describes the crystal structure of 8Sα
globulin determined by X-ray crystallography.

### Table 1
Data-collection and refinement statistics for an 8Sα globulin crystal.

<table>
<thead>
<tr>
<th>Crystal system</th>
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<tr>
<td>Space group</td>
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<tr>
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<tr>
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<td>Final model</td>
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† $R_{	ext{free}} = \sum |I - \langle I\rangle|/I \times 100$, where $I$ is the intensity of all reflections. ‡ $R$ factor = $\sum |F_o - F_c|/\sum |F_o| \times 100$, where $F_o$ is the observed structure factor and $F_c$ is the calculated structure factor. § $R_{	ext{free}}$ was calculated from a randomly chosen 10% of reflections as defined by CNS (Brünger et al., 1998).

### 2. Materials and methods

#### 2.1. Crystallization and X-ray diffraction

Recombinant 8Sα globulin was overexpressed in *E. coli*, purified and crystallized as described previously (Bernardo et al., 2004). The hanging-drop vapour-diffusion method was used to crystallize the recombinant 8Sα globulin. The hanging drop (6 μl) contained 3 μl protein solution and 3 μl reservoir solution consisting of 12% PEG 1000, 0.2 M NaCl and 0.1 M MES pH 6.0 and was equilibrated against 1 ml reservoir solution. The protein concentration was 5 mg ml$^{-1}$ and crystallization was allowed to proceed at 293 K. X-ray diffraction images of the 8Sα globulin crystal in a capillary were collected at 293 K with a Bruker Hi-Star multiwire area detector using Cu Ka radiation generated by a MacScience M18XHF rotating-anode generator and were processed with SADIE and SAINT software (Bruker, Karlsruhe, Germany) to a resolution of 2.61 Å (Table 1).

#### 2.2. Structure determination and refinement

The 8Sα globulin crystal structure was determined by the molecular-replacement method as implemented in CNS v1.1 (Brünger et al., 1998). The refined crystal structure of the recombinant soybean β-conglycinin β model was used as the probe structure (PDB code 1ipk). Model building and refinement were performed using TURBO-FRODO (AFMB-CNRS, Marseille, France) and CNS, respectively, on a Silicon Graphics Octane computer. $F_o - F_c$ and $2F_o - F_c$ maps were used to locate the correct model. Several rounds of positional and $B$-factor refinement followed by manual model building.
were performed to improve the model by increasing the data to a resolution of 2.65 Å. Water molecules were incorporated where the difference density exceeded the mean by 3.0σ or more and the 2Fo − Fc map showed a density exceeding 1.0σ. The final R factor was 19.6% for 10,853 data points in the resolution range 10.0–2.65 Å (89.1% completeness). The free R value calculated for a randomly separated 10% of the data was 25.9%. The stereo quality of the model was assessed using PROCHECK (Laskowski et al., 1993) and WHAT-CHECK (Hooft et al., 1996). Structural similarity was searched for in

the PDB (Berman et al., 2000) using DALI (Holm & Sander, 1993). The coordinates of soybean β-conglycinin β (1ipk), jack bean canavalin (1dgw), kidney bean phaseolin (2phl), soybean 11S proglycinin A1aB1b (1fxz) and oxalate decarboxylase Yvrk from Bacillus subtilis sp. 168 (1uw8) were taken from the PDB. These models were superimposed by a fitting program in TURBO-FRODO. Ribbon plots were prepared using MOLSCRIPT (Kraulis, 1991) and RASTER3D (Merritt & Murphy, 1994). The accessible surface areas (ASAs) were calculated with NACCESS (Hubbard & Thornton, 1993), which uses the algorithm of Lee & Richards (1971). The probe was taken to be a water molecule of 1.4 Å. Electrostatic surface potential was calculated using GRASP (Nicholls et al., 1991). The salt concentration was changed to 0.1 M from the default setting. The file full.crg was used for charge assignment, where the histidine residue has no charge. The cavity size was estimated by CASTp (Liang et al., 1998). The aliphatic index was estimated by the ProtParam tool from the ExPaSy Proteomics Server (Gasteiger et al., 2003).

3. Results and discussion

3.1. Crystalization and structure determination

The recombinant 8Sα globulin of mung bean is a trimer of three identical subunits, each with a molecular weight of about 49 kDa and consisting of 423 amino-acid residues without a signal peptide (Bernardo et al., 2004) (Fig. 1). 8Sα globulin crystals (0.4 × 0.3 × 0.2 mm) were obtained by the hanging-drop vapour-diffusion method as described previously (Bernardo et al., 2004). The space group was determined to be R3, with unit-cell parameters a = b = 146.6, c = 53.3 Å, and the solvent content was 47% for one subunit per asymmetric unit. Results of data collection are summarized in Table 1. The structure of the protein was determined by the molecular-replacement method using the structure of the homologous (68% identity) soybean recombinant β-conglycinin β as the starting model (Fig. 1) and was refined by the simulated-annealing and restrained least-squares methods (Table 1).

3.2. Quality of the refined model

The refined model of 8Sα globulin consisted of 366 amino-acid residues (one subunit of the trimer) and eight water molecules. The electron densities of the main chain and side chain were generally very well defined in the 2Fo − Fc map, except for the five disordered regions (described below). Water molecules were also well fitted. The final overall R factor for the refined model was 19.6%, with 10,853 unique reflections in the resolution range 10.0–2.65 Å. The final free R factor was 25.9%. The final r.m.s. deviations from standard geometry were 0.008 Å for bond lengths and 1.41° for bond angles. Based on theoretical curves in the plot calculated according to Luzzati (1952), the absolute positional error was estimated to be close to 0.33 Å at a resolution of 5.0–2.65 Å. Most non-glycine residues (81.0%) lie within the most favoured regions and most other residues (18.1%) lie within the additionally allowed regions of the Ramachandran plot as

Figure 1

Amino-acid sequence alignment of 8Sα globulin and other seed storage 7S globulins obtained using ClustalW (http://align.genome.jp/). 8S, 8Sα globulin of mung bean (accession No. PRF501374A); Conβ, soybean β-conglycinin β (accession No. P25974); Canα, jack bean canavalin (accession No. PS0177); Pha, kidney bean phaseolin (accession No. AAC04316). Identical or similar amino-acid residues among the four proteins are indicated by asterisks or dots, respectively. α-Helices are indicated by black boxes and β-strands by arrows. The surrounding five amino-acid regions are disordered regions.
defined in PROCHECK (Laskowski et al., 1993). However, two residues (0.6%), Arg22 ($\varphi = -175$, $\psi = -44^\circ$) and Asp325 ($\varphi = 85$, $\psi = -16^\circ$), are in generously allowed regions and one residue (0.3%), Gln399 ($\varphi = 51$, $\psi = -65^\circ$), which exhibits well defined density in the $2F_o - F_c$ map, is in a disallowed region. Arg22 and Asp325 were observed in distorted type-I $/C_{12}$-turns, while Gln399 was located in a sharp bend of the loop near the C-terminal amino acid. The averaged $B$ factor was 52.4 Å$^2$, which is relatively high (Table 1), probably owing to the loose crystal packing of the trimer in the unit cell.

3.3. Overall $8S\alpha$ globulin structure

The overall structure of $8S\alpha$ globulin is shown as a ribbon model in Fig. 2. There is one subunit in the asymmetric unit of the crystal. The trimer consisted of three identical subunits related by a threefold axis and has approximate dimensions of $96 \times 97 \times 48$ Å, similar to other seed storage $7S$ globulins (described below; Figs. 2a and 2b). The monomer (one subunit) can be divided into two similar modules, the N- and C-terminal modules, related by a pseudo-twofold symmetry axis (Fig. 2c). Each module of the subunit consisted of a core $\beta$-barrel (jelly-roll) domain and an extended loop domain containing two helices. The secondary-structure elements were named according to the strands in soybean $\beta$-conglycinin $/C_{12}$ (Maruyama et al., 2001). Each core $\beta$-barrel domain consisted of two $/C_{12}$-sheets, $A'ABIDG$ and $JJCHEF$.

The $8S\alpha$ globulin model had five regions that could not be seen in the electron-density maps (the six N-terminal amino acids 1–6; the 20 C-terminal amino acids 404–423; 11 residues in the ‘internal I’ region, 181–191; 11 residues in the ‘internal II’ region, 214–224; nine residues in the ‘internal III’ region, 302–310) (Figs. 1 and 2). Although the total averaged $B$ factor

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Figure 2

Overall structure of $8S\alpha$ globulin. (a) The $8S\alpha$ globulin trimer is seen along a threefold axis. The three subunits are shown in red, magenta and yellow. Each subunit contains two similar modules (N- and C-terminal modules) consisting of a core $\beta$-barrel and extended loop domain. (b) View after 90° rotation around the vertical axis of (a). (c) The $8S\alpha$ globulin subunit. Colours denote secondary-structure elements (blue, $\alpha$-helices; red, $\beta$-strands; yellow, loops and coils). The broken line in the figure is the pseudo-twofold axis of the N- and C-terminal modules. (d) Intersubunit interface of $8S\alpha$ globulin. The structure is represented as a red and magenta ribbon model of $8S\alpha$ globulin subunits. In the ribbon model, yellow or blue denotes the residues participating in the hydrogen bond. Black broken lines show the hydrogen bonds of two ion pairs (Arg55–Glu311, Arg95–Glu371).
was 52.4 Å² and relatively high (Table 1), the $B$ factors at both termini of the invisible regions were higher than the mean value. The values are 79.6 Å² for the N-terminal amino acid, 75.7 Å² for internal I, 72.3 Å² for internal II, 78.0 Å² for internal III and 62.5 Å² for the C-terminal amino acid. The N-terminal amino-acid sequence and molecular size indicated by SDS–PAGE of the recombinant 8Sα globulin were consistent with the deduced amino-acid sequence from the cDNA as described previously (Bernardo et al., 2004). Therefore, the invisible regions are thought to be disordered.

The ASAs of one subunit and the trimer, using a probe radius of 1.4 Å, were 18 470 and 39 714 Å², respectively. 28.3% of the ASA of the subunit is used for formation of the trimer. The association of subunits is dominated by the extended loop domain and the $\beta$-sheet (Fig. 2). The nonpolar atoms comprise 67.8% of the intersubunit interface. The number of residues participating in the hydrophobic interactions (C—C contacts <4 Å) were 42 in the N-terminal module and 34 in the C-terminal module, respectively. 20 hydrogen bonds (<3.2 Å) were found. In particular, two ion pairs among these interactions were observed between two modules (Arg55–Glu311, 2.7 Å; Arg95–Glu371, 3.2 Å) (Fig. 2d).

### 3.4. Structural comparison

The 8Sα globulin structure consisted of two jelly-roll folds and exhibited similarity to four other reported seed storage 7S globulin structures that have amino-acid sequence similarities to 8Sα globulin, i.e. soybean β-conglycinin α’c (Maruyama et al., 2004), β-conglycinin β (Maruyama et al., 2001), jack bean canavalin (McPherson, 1980) and kidney bean phaseolin (Lawrence et al., 1994). The amino-acid sequence similarities between 8Sα globulin and these 7S globulins are about 68% for α’c and β, about 52% for canavalin and about 58% for phaseolin (Fig. 1). In addition to seed storage 7S globulins, this basic fold is common to 11S globulins [soybean proglycinin A1aB1b (Adachi et al., 2001) and mature glycinin A3B4 (Adachi, Kanamori et al., 2003)], plant germin (an Mn-binding protein with oxalate oxidase and superoxide dismutase activities; Woo et al., 2000), plant auxin-binding protein (Woo et al., 2002) and the bacterial oxalate decarboxylase YvrK (Anand et al., 2002) in the SCOP database (http://scop.mrc-lmb.cam.ac.uk/scop; Murzin et al., 1995).

Fig. 3(a) shows the superimposition of 8Sα globulin on other 7S globulins. The r.m.s. deviation was 0.6 Å for the superimposition of 355 common Cα atoms of β-conglycinin β, 0.8 Å for 343 Cα atoms of jack bean canavalin and 0.9 Å for 342 Cα atoms of kidney bean phaseolin calculated by the RIGID program implemented in TURBO-FRODO. These structures had almost similar topologies, consisting of the
two core β-barrels and extended loop domains. The N- or C-terminal amino acids are disordered in other 7S globulins (Fig. 3a). Internal I is in the extended loop region of the N-terminal module, participating in the intersubunit interface (Fig. 2), and was longer than those of any other 7S globulins (Fig. 1). This site is also disordered in β-conglycinin β. However, in phaseolin and canavalin, this site exists as a short loop (Fig. 3a). Internal II is in the region connecting the N- and C-terminal modules near the two core β-barrels and does not take part in trimer formation (Fig. 2). This loop is also disordered in the 7S globulins, except for β-conglycinin β. Superimposition of this site was not well fitted in comparison with that of the overall structure (Fig. 3a). Therefore, this site is highly flexible within these globulins. Internal III is a neighbour of the loop near the N-terminal amino acid (Fig. 2). Although this site exists as a short loop and a turn in canavalin, this site is also disordered in other globulins (Fig. 3a).

Fig. 3(b) shows the superimposition of 8Sα globulin on soybean proglycinin A1aB1b and oxalate decarboxylase YvrK from B. subtilis sp. 168, which also exhibited a high degree of similarity to 8Sα globulin in the PDB (Berman et al., 2000) as observed using DALI (Holm & Sander, 1993). The r.m.s. deviation was 1.8 Å for the superimposition of 306 common Cα atoms of A1aB1b and 1.8 Å for 235 Cα atoms of YvrK. YvrK is a manganese-dependent enzyme that catalyzes the conversion of oxalate to formate and carbon dioxide and has a hexameric conformation consisting of two trimers on top of one another, differing from mung bean 8S globulins. The overall structures of their subunits resemble each other well. However, some differences exist in the extended loop region located in an intersubunit interface of the N-terminal module and the N-terminal amino-acid residues (Fig. 3b). Although the extended loop regions were superimposed well in 7S and 8S globulins (Fig. 3a), these regions were not well fitted in 8Sα globulin, 11S glycine and YvrK. In YvrK only, the loop located in the N-terminal amino-acid residues is longer and protrudes to the outside (blue Cα model in Fig. 3b). The manganese and four manganese-binding residues (three histidines and one glutamate) of YvrK are not observed in the 8Sα globulin structure. These residues are not conserved in the other seed storage globulins. Furthermore, to the best of our knowledge, there are no reports of any metalloenzymatic activity of these seed storage globulins.

3.5. Molecular surface and physicochemical properties

Owing to their useful physicochemical properties, such as gel-forming and

**Figure 4**
The molecular surface of (a) 8Sα globulin and (b) soybean β-conglycinin β. Structures are represented as white molecular-surface models. Hydrophobic residues are green. The electrostatic potential surface of (c) 8Sα globulin and (d) soybean β-conglycinin β. Structures are represented as white molecular-surface models. The electrostatic potential surface are drawn in the range from −15k_B T (red) to +15k_B T (blue), where k_B is Boltzmann’s constant and T is the absolute temperature (K).
emulsifying abilities, various seed storage globulins are important for utilization as food materials (Utsumi, 1992; Utsumi et al., 1997). The surface hydrophobicity of a protein is significantly related to its solubility (Nakai & Li-Chan, 1988). Solubility is one of the most important features for the physicochemical properties of these globulins. The ASA ratio of hydrophobic residues (Ala, Val, Leu, Ile, Pro, Met, Phe, Tyr and Trp) on the molecular surface of the trimer was 4957/39714 Å² Å⁻² (12.5%; Fig. 4a). This value was very close to that of soybean β-conglycinin β trimer (5494/40755 Å² Å⁻²; 13.5%; Fig. 4b), with which mung bean 8S globulin α has high amino-acid sequence and structural similarities (Figs. 1 and 3a). Therefore, the globulins would be expected to exhibit similar hydrophobic properties. Indeed, both exhibited similar retention times on hydrophobic (butyl or phenyl Sepharose) column chromatography (data not shown). The globulin is soluble in salt solution but not in water. 8Sα globulin and soybean β-conglycinin β were soluble in the examined pH range at high ionic strength (μ = 0.5). This is because electrostatic interactions between polypeptides are suppressed by the presence of the salt. However, their solubilities differ under alkaline conditions (pH > 7) at low ionic strength (μ = 0.08) (data not shown). 8Sα globulin is highly soluble at pH > 7 and μ = 0.08, while soybean β-conglycinin β is almost insoluble under these conditions. Considering the close similarity in surface hydrophobicity (Figs. 4a and 4b), the difference in the solubility under alkaline conditions at low ionic strength may arise from the difference in charge–charge interactions and not the hydrophobic interactions of protein–protein contacts through the molecular surface. Figs. 4(c) and 4(d) show the electrostatic potential on the molecular surface. In the case of 8Sα globulin, although one surface was covered by mainly negative potentials, the other was not mainly covered by positive potential (Fig. 4c). On the other hand, positive or negative potential was closely distributed at the centre of each surface of β-conglycinin β (Fig. 4d). It is thought that the predominantly and concentrated electrostatic potential (positive or negative) surfaces lead to charge–charge interactions in β-conglycinin β. Owing to this undesirable interaction through the molecular contacts, β-conglycinin β would precipitate under alkaline conditions (pH > 7) and low ionic strength. Although the effect of the disordered regions discussed above cannot be determined, the internal II disordered region (214–224), which exists on the surface and is visible in β-conglycinin β, might affect the solubility.

Thermal stability is also one of the most important physicochemical properties of these globulins related to food functions such as heat-induced gelation. The thermal stability of 8Sα globulin indicated by DSC analysis is Tm = 350.6 K at ionic strength μ = 0.5 and pH 7.6 (Table 2) (data not shown). For soybean β-conglycinin isoflavones, these values under the same conditions are 351.7 K for α, 350.4 K for αc, 355.8 K for α′, 364.4 K for α′c and 363.9 K for β (Maruyama et al., 1999). The difference in thermal stability between β-conglycinin α′c, which consists only of the core region without the long N-terminal extension region (141 residues), and β has been accounted for by a combination of several structural features, such as cavities, number of hydrogen bonds, intersubunit salt bridges, surface hydrophobicity, number of proline residues and loop region (Maruyama, Maruyama et al., 2004). The Tm values of deletion mutants αc (deletion of the N-terminal extension region; 125 residues) and α′c (deletion of the N-terminal extension region; 141 residues) are very close to those of the α and α′ subunits (Table 2; Maruyama et al., 1999). Thus, the thermal stabilities of β-conglycinins are conferred by the core regions. The cavity, which is inside the molecule and which decreases the thermal stability of the 8Sα globulin, was 6441 Å³ in volume. This value is higher than that for any other β-conglycinin (5463.5 Å³ for α′c and 4753.7 Å³ for β; Table 2). Furthermore, the order of these values (8Sα globulin > α′c > β) is correlated with their respective thermal stability values (8Sα globulin < α′c < β). The numbers of hydrogen bonds and salt bridges of 8Sα globulin are greater than those of β (Table 2). Although the hydrogen bonds and salt bridges are thought to be important features for stable packing, this is not consistent with the DSC experimental data. The surface hydrophobicities were very close to each other as described above. The number of proline residues, which decreases the entropy of the denatured structure, is 19 in the 8Sα globulin subunit (Table 2). Although α and α′ have a higher number of proline residues than any other globulin, they do not exhibit the highest thermal stability. Also, αc and α′c have a similar number of proline residues to 8Sα globulin and β. Thus, the proline residues in the N-terminal extension region of α or α′ have little effect on thermal stability. Shorter loops have been shown to be another stabilizing feature (Maruyama, Maruyama et al., 2004; Chakravarty & Varadarajan, 2002). The effect of disordered regions cannot be discussed completely. The long disordered (or flexible) regions of the seed storage globulins (the N-terminal extension region of β-conglycinin and the hypervariable regions of 11S glycgin) have little influence on their thermal stabilities, as described above (Maruyama et al., 1999; Prak et al., 2005). For soybean β-conglycinins and mung bean 8Sα globulin, there are long disordered regions (internal I, II and III) which are rich in charged residues, e.g. glutamic acid and lysine residues, and the amino-acid sequences of the regions are almost conserved between the proteins (Fig. 1). This might affect the thermal

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† There are no usable three-dimensional structures. ‡ DSC measurement at ionic strength (μ = 0.5) and pH 7.6 (Maruyama et al., 1999). § Cavitory volume was calculated by CASTp (Liang et al., 1998). † The values in parentheses represent the number of salt bridges of the intersubunit. ‡ This value was calculated using the ProtParam tool (Gasteiger et al., 2003).
stability a little. The influence of the water molecules on the thermal stability of the storage proteins cannot be discussed here, because the resolutions of the seed storage globulin structures, at around 2.6 Å, are generally too low to observe the water molecules in the model (Maruyama et al., 2001; McPherson, 1980; Ko et al., 1993; Lawrence et al., 1994). To discuss the contribution of water molecules to the difference in the thermal stability among β-conglycinins and 8Sa globulin, higher resolution will be needed. Furthermore, the aliphatic index, which is the relative volume occupied by aliphatic side chains (Ala, Val, Ile and Leu), has been shown to explain the difference in the thermal stability of several 11S globulins (Molina et al., 2004). According to this report, the higher the aliphatic index, the higher the thermal stability. However, this trend is not exhibited among β-conglycinin and 8S globulins (Table 2).

4. Conclusion

The refined 8Sa globulin structure consisted of one subunit of the biological trimer and two jelly-roll folds. There were five invisible regions. The overall structure, with the exception of these disordered regions, very much resembled those of other invisible regions. The overall structure, with the exception of the biological trimer and two jelly-roll folds. There were five 8S globulins (Table 2).

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