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# Study on the structure and formation mechanism of 15S globulin of soybeans

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# ABSTRACT

The structure and formation mechanism of 15S globulin of soybeans were investigated. After purification, 15Srich fraction (75.6%) and 11S-rich fraction (94.3%) were obtained and further characterized with analytical ultracentrifugation (AUC) and transmission electron microscope (TEM). The results show that the molar masses of 11S and 15S are 313 kDa and 651 kDa, respectively, and the weight percentages of the monomer, dimer, and trimer in the TEM images are consistent with those of 11S, 15S, and 21S fractions measured by AUC, indicating that 15S and 21S are dimers and trimers of 11S monomer, respectively. The size of the 15S molecules is 21.1 nm  $\times$  10.4 nm, suggesting that two 11S molecules interact with each other from the side position. Moreover, the contents of subunits were determined by a combination of AUC and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with the addition of 20 mM sodium dodecyl sulfate (SDS) and 10 mM 2-mercaptoethanol (2-ME). The content of the species with a sedimentation coefficient of 4.3 S in 15S-rich fraction, which is assigned as AB-SS-AB subunits, is significantly higher than that in 11S-rich fraction with the addition of SDS; this species disappears with the addition of 2-ME, demonstrating that AB-SS-AB subunits are formed by disulfide groups from two AB subunits. These disulfide groups may locate in a non-polar interior, which is not accessible to 2-ME when the protein remains its native structure.

# 1. Introduction

Soybean proteins play an important role in food industry owing to their high nutritional value and functionality, which are usually classified into 2S, 7S, 11S, and 15S fractions (Di Giorgio, Salgado, & Mauri, 2019; Han et al., 2019; Huang, Krishnan, Pham, Yu, & Wang, 2016; Ju et al., 2020; Nishinari, Fang, Guo, & Phillips, 2014). The 11S fraction consists of glycinin that exists as a hexamer with five major subunits, where an acidic polypeptide and a basic polypeptide are linked by a disulfide bond (Badley et al., 1975; Nishinari et al., 2014). The 15S fraction is a minor component of soy proteins and its content in soluble protein isolates ranges from ~1% to 11% (Liu et al., 2017; Thanh & Shibasaki, 1976; Wolf, Babcock, & Smith, 1962). Many characters of 15S, such as solubility and dissociation, are similar to those of 11S (Wolf & Briggs, 1956, 1958). Wolf and Nelsen (1996) found that 15S was the aggregate of 11S, based on their amino acid composition and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) bands. They also showed that 15S is a dimer of 11S, according to their molar masses determined by size exclusion chromatography (SEC). However, they had difficulties to estimate the center of the 15S fraction in SEC. In SEC experiments, a series of protein standards are needed to calibrate the column, so the determination of accurate molar mass of one protein sample is difficult if the sample has a high molar mass or a different shape, when compared with the protein standards (Erickson, 2009). Besides, Wolf and Nelsen did not mention the structure of other fractions with higher sedimentation coefficients. Therefore, a method independent of standards, such as analytical ultracentrifuge, should be employed to accurately characterize the molar masses of 15S and other fractions with higher sedimentation coefficients. Moreover, transmission electron microscope (TEM) is a straightforward approach that

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provides the size and shape of the 11S and 15S. Furthermore, previous studies have shown that the content of 15S decreased to a certain degree with the addition of 2-ME or dithiothreitol (Briggs & Wolf, 1957; Wolf, 1993). However, the 15S fraction always co-existed in the 11S fraction, even with an increased concentration of 2-ME to 250 mM, while the underlying mechanism is still controversial, which needs to be further investigated. Our objective in this study was to investigate the structure and the formation mechanism of the 15S and 21S fractions by a combination of analytical ultracentrifugation (AUC) and TEM.

# 2. Materials and methods

# 2.1. Materials

Low-temperature defatted soybean flour was purchased from Harbin High-Tech Co., Ltd. (Harbin, China). The soy flour contained more than 50% of protein and less than 2% of oil according to the manufacturer's specifications. 2-ME, N,N'-methylenebis(acrylamide) (MBA), and N,N, N', N'-tetramethylethylenediamine (TMEDA) were purchased from Aladdin Reagent Company (Shanghai, China). Sodium chloride (NaCl), potassium dihvdrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>), dipotassium hvdrogen phosphate trihydrate (K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O), hydrochloric acid (HCl), Coomassie brilliant blue R250, acetic acid, ethanol, acrylamide, ammonium persulfate, sodium dodecyl sulfate (SDS), and tris(hydroxymethyl)aminomethane (Tris) were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Concanvalin A-Sepharose 4B medium was purchased from GE Healthcare (Piscataway, NJ, USA). Electrode buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3), 5× protein loading buffer without reducing agent [50% (v/v) glycerol, 10% (w/v) SDS, 0.05% (w/v) bromophenol blue, 313 mM Tris-HCl, pH 6.8], and 2× protein loading buffer [20% (v/v) glycerol, 3% (w/v) DTT, 4% (w/v) SDS, 0.02% (w/v) bromophenol blue, 100 mM Tris-HCl, pH 6.8] were purchased from Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China). Pre-stained color protein marker was purchased from Beyotime Biotechnology (Shanghai, China). All the chemicals used in this study were of analytical grade.

# 2.2. Isolation of glycinin

The 11S fraction was obtained from soybean flour following a method previously described with some modifications (Thanh & Shibasaki, 1976). An aliquot of 7.0 g of soybean flour was dispersed in 210 mL of 63 mM Tris-HCl buffer (pH 7.9) containing 10 mM 2-ME. After stirred at room temperature for 1 h, the dispersion was centrifuged at 8, 000 rpm for 25 min using a 5810R centrifuge (Eppendorf, Germany). The obtained supernatant was adjusted to pH 6.6 with 2 M HCl. Then, the slurry was stored in a refrigerator at 4 °C overnight, followed by centrifugation at 8,000 rpm for 25 min. The obtained precipitate was washed for three times with the extraction buffer at pH 6.6. The precipitate was dissolved in a standard buffer (pH 7.6) with an ionic strength of 500 mM containing 33 mM K<sub>2</sub>HPO<sub>4</sub>, 2.6 mM KH<sub>2</sub>PO<sub>4</sub>, 400 mM NaCl, 0.02% (w/v) NaN<sub>3</sub>, and 10 mM 2-ME. After the solution was centrifuged, the supernatant was dialyzed in a dialysis bag with molecular weight cut-off (MWCO) of 100 kDa against the standard buffer for 48 h. The crude 11S solution was stored in the refrigerator at 4  $^\circ$ C until further use.

#### 2.3. Purification of glycinin

In order to remove the 7S fraction, a Concanvalin A-Sepharose 4B column was used according to the method reported by Kitamura et al. (Kitamura, Okubo, & Shibasaki, 1974). An aliquot of 3.0 mL Concanvalin A-Sepharose 4B medium was packed in a 9 mm (inner diameter) column with a 20 mM Tris-HCl buffer (pH 7.4) containing 500 mM NaCl and equilibrated with the standard buffer at room temperature. Then, 4.0 mL of crude 11S solution with a concentration of 19.0

mg·mL<sup>-1</sup> was loaded, and 11S was eluted with the standard buffer. The absorbance of fractions was monitored at a wavelength of 280 nm. Subsequently, 1.0 mL of each fraction was collected in one tube until the absorbance returned to the baseline. The concentration of 11S fraction was determined based on the absorbance (0.84 Abs =  $1.0 \text{ mg·mL}^{-1}$ ) at a wavelength of 280 nm and a path length of 10 mm (Wolf, 1993). SEC was performed based on an ÄKTA primer system (GE, USA). An aliquot of 1.0 mL of protein sample with a concentration of 44.0 mg·mL<sup>-1</sup> was loaded at a rate of 1.0 mL/min into a Superdex200 16/600 column. The temperature of the column was maintained at 10 °C. The column was eluted with the standard buffer, and 0.6 mL of each fraction was collected in one tube. The absorbance of the fractions at a wavelength of 280 nm was measured.

# 2.4. Electrophoresis

SDS-PAGE was performed using on an electrophoresis apparatus (Tanon EPS-300, Tanon Science & Technology Co. Ltd., Shanghai, China) with 12% (w/v) separating gel and a 4% (w/v) stacking gel according to previous literature (Laemmli, 1970). A mixture of the protein solution and  $2\times$  protein loading buffer at a volume ratio of 1:1 was heated to 90 °C for 15 min, to obtain a final protein concentration of 1.0 mg·mL<sup>-1</sup>. Then, the mixture was cooled to room temperature and centrifuged at 8,000 rpm for 1 min. In order to study the interactions between two 11S molecules in one 15S molecule, the protein solution was mixed with  $5 \times$  protein loading buffer without reducing agents at a volume ratio of 1:4. Then, 2-ME was added into the mixture without heating, to cleave disulfide bonds. An aliquot of 20 µL of the treated samples and 10 µL of the pre-stained color protein marker were loaded into gel lanes. Electrophoresis was performed at a voltage of 80 V for 120 min. The gel was stained in an aqueous solution containing 10% (v/v) acetic acid, 45% (v/v) ethanol, and 0.25% (w/v) Coomassie brilliant blue R-250, and then destained in an aqueous solution containing 8% (v/v) acetic acid and 25% (v/v) ethanol.

# 2.5. AUC

Sedimentation velocity experiments were carried out with a Proteomelab XL-A analytical ultracentrifuge (Beckman Coulter, USA) using a four-hole An-60 Ti analytical rotor (Gao, Wu, & Ye, 2016; Si et al., 2019; Wang, Fan, Ye, Liu, & Zhang, 2018). An aliquot of 410 µL of standard buffer as the reference and 400 µL of protein solution (0.6  $mg \cdot mL^{-1}$ ) were loaded into a double-sector cell. A centerpiece with a path length of 12 mm was used. The speed of rotor was 35,000 rpm during the characterization of the 11S and 15S fractions and 58,000 rpm during the measurement of the subunits. The operation temperature of rotor was 20 °C. The time dependence of the absorbance at different radial positions was monitored at a wavelength of 280 nm by an UV-Vis absorbance detector, and the data were analyzed by the software SEDFIT (version 15.01b) using c(s) or  $c(s, f/f_0)$  model to obtain the sedimentation coefficient distribution (Brown & Schuck, 2006; Lebowitz, Lewis, & Schuck, 2002; Schuck, 2000). Viscosity and density of the buffer solution were calculated by the Sednterp software (Laue, Shah, Ridgeway, & Pelletier, 1992). The partial specific volume value of 0.73  $\text{cm}^3 \cdot \text{g}^{-1}$  reported by Badley et al. (1975) was used. Each experiment was repeated at least twice.

# 2.6. TEM

The protein images were taken under a Tecnai Spirit microscope (FEI, USA) operated at 120 kV, to observe the structure. For TEM analysis, each copper mesh covered with a pre-coated carbon film was subjected to a hydrophilic treatment. The edge of the copper mesh was held by tweezers, and 2.5  $\mu$ L of the protein solution at ~5  $\mu$ g·mL<sup>-1</sup> was dropped on the side of the copper mesh. After the protein solution was dropped on the carbon film for 90 s, a filter paper was used to absorb the

protein solution from the edge of the copper mesh. A drop of 2% (w/v) uranyl acetate (UA) dye solution was dropped on the sample, and immediately absorbed with a filter paper. Then, another drop of UA solution was dropped on the sample, and the sample was further dyed for 90 s. Excess dye solution was removed using a filter paper from the edge of the copper mesh, and the sample was air-dried at room temperature. The completely dried samples were observed under electron microscope.

#### 3. Results and discussion

# 3.1. SDS-PAGE analysis

In this study, we followed the methods reported by Thanh et al. (1976) and Kitamura et al. (1974) to purify the 11S fraction. In brief, 11S and 7S fractions can be separated based on their different solubility at various pH values and binding ability with concanavalin A of Concanvalin A-Sepharose 4B medium. Fig. 1 shows the SDS-PAGE profiles of buffer extractable protein by alkali extraction (Lane 2), crude 11S after acid precipitation (Lane 3), and purified 11S after removing 7S by Concanvalin A-Sepharose 4B column (Lane 4). Buffer extractable sovbean protein (Lane 2) contains  $\alpha'$ ,  $\alpha$ , and  $\beta$  subunits of 7S and acidic (A) and basic (B) subunits of 11S (Liu et al., 2017; Shen et al., 2020). The molar masses of the subunits of both 7S and 11S, estimated in comparison with protein standards, are close to the values described previously (Hu, Cheung, Pan, & Li-Chan, 2015; Mozafarpour, Koocheki, Milani, & Varidi, 2019). It is clear that the content of 7S decreases dramatically, whereas small amounts of 7S is still present (Lane 3). Lane 4 shows the absence of  $\alpha'$ ,  $\alpha$ , and  $\beta$  subunits of 7S, suggesting that 7S was completely removed.

#### 3.2. Ultra-centrifugal analysis

Fig. 2 shows the normalized sedimentation coefficient distributions c (s) of the products at different purification stages, measured by



**Fig. 2.** Normalized sedimentation coefficient distributions of soybean proteins at different purification stages. Buffer extractable protein (black curve); crude 11S after acid precipitation (red curve); purified 11S after removing 7S (blue curve) by Concanvalin A-Sepharose 4B column. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

analytical ultracentrifugation sedimentation velocity at a rotor speed of 35,000 rpm and analyzed using the software program SEDFIT (Lebowitz et al., 2002; Schuck, 2000). For buffer extractable protein dialyzed against the standard buffer for 48 h, four major peaks, with the sedimentation coefficients of 2.7 S, 6.6 S, 11.1 S, and 16.4 S, are observed, which were 2S, 7S, 11S, and 15S fractions, respectively, described in a previous study (Nishinari et al., 2014). Their corresponding standard sedimentation coefficient ( $s_{20, w}$ ) values are 3.0 S, 7.4 S, 12.3 S, and 18.2 S, respectively. The measured contents of these compositions are



Fig. 1. SDS-PAGE pattern of soybean proteins at different purification stages. Lanes 1 and 5: Protein molecular weight standards; Lane 2: Buffer extractable protein; Lane 3: Crude 11S after acid precipitation; Lane 4: Purified 11S after removing 7S by Concanvalin A-Sepharose 4B column.

summarized in Table 1. For crude 11S after acid precipitation and re-dissolved in standard buffer, the contents of 2S and 7S decrease from 28.4% to 2.7% and 24.1% to 3.7%, respectively. At the same time, the contents of 11S and 15S increase from 41.1% to 84.5% and 4.6% to 7.1%, respectively. These values are similar to those reported by Thanh et al. (1976) and Wolf et al. (1996) who used a similar method. Moreover, for the protein purified by Concanvalin A-Sepharose 4B column, *c* (*s*) shows that the contents of the 2S and 7S fractions decrease to a much lower level. However, 1.1% of 2S and 1.5% of 7S are still present, as shown in Table 1. Considering that Fig. 1 clearly shows the complete removal of glycoprotein 7S (Lane 4), the presence of 2S and 7S is presumably attributed to the existence of the subunits and half-molecule of 11S (Thanh & Shibasaki, 1976).

#### 3.3. SEC

Fig. 3 shows the SEC profile of the purified 11S after removing 7S by Concanvalin A-Sepharose 4B column. The SEC profile contains two peaks, corresponding to the 15S-rich and 11S-rich fractions, since AUC result (Fig. 2) shows that this protein sample mainly contains 11S and 15S. The normalized sedimentation coefficient distributions of the fraction *a* at 52.6 mL and fraction *b* at 61.0 mL are shown in Fig. 4. For fraction a (15S-rich fraction), three peaks with sedimentation coefficients of 11.1 S, 16.4 S, and 21.4 S are present, and the corresponding contents are 11.2%, 75.6%, and 12.8%, respectively. In this study, the fraction with the sedimentation coefficient of 21.4 S is designated as 21S. The content of 15S (75.6%) is higher than the highest content of 15S-rich fraction (64.2%) purified by Wolf and Nelsen (1996). For fraction *b*, only one major peak with a sedimentation coefficient of 11.1 S is present, and its content is as high as 94.3%. The molar mass of 11S is calculated as 313 kDa, by using c(s) model in SEDFIT, which is consistent with the value reported by Badley et al. (1975). Because there are three species in fraction a and the frictional coefficient ratios of these species can be different,  $c(s, f/f_0)$  model was used to fit the data. The calculated molar masses of 15S and 21S are 651 kDa and 933 kDa, respectively, the former of which is in range of 500-770 kDa that was reported previously (Achouri et al., 2010; Hou & Chang, 2004; Marcone, Bondi, & Yada, 1994; Wolf, 1970; Wolf & Nelsen, 1996). The molar mass measured by AUC is supposed to be more accurate, since protein standards are not used for AUC experiments and no interaction between the 15S fraction and the column is present. The result clearly demonstrates that 15S is a dimer of 11S. Moreover, the molar mass of 21S is about three times heavier than that of 11S, further indicating that 21S might be a trimer of 11S.

# 3.4. TEM

TEM images of fractions *a* and *b* are shown in Fig. 5. For fraction *b*, Fig. 5A and 5B clearly show the presence of 11S monomer. Previously, Badley et al. (1975) stated that 11S monomer is a hollow cylinder formed by two hexagonal rings consisted of twelve subunits, and the size measured by TEM is approximately  $10.0 \times 10.0 \times 7.0$  nm. In this study, the sizes of protein particles were measured by the software ImageJ and the results are summarized in Table 2, except for a few large aggregates, with sizes larger than 35 nm, which might be formed during the preparation of TEM samples. The thickness and average diameter of the 11S

# Table 1

Composition of soybean proteins at different purification stages, measured by analytical ultracentrifugation.

Sample	Component/%						
	28	7S	11S	15S	>185		
Buffer extractable protein	28.4	24.1	41.1	4.6	1.8		
Crude 11S after acid precipitation	2.7	3.7	84.5	7.1	2.0		
Purified 11S after removing 7S	1.1	1.5	88.4	7.9	1.1		



**Fig. 3.** SEC elution profile of purified 11S after removing 7S by Concanvalin A-Sepharose 4B column. The absorbance of the fractions were monitored at a wavelength of 280 nm. The elution volumes of fractions a and b are 52.6 and 61.0 mL, respectively.



**Fig. 4.** Normalized sedimentation coefficient distributions of fractions *a* and *b* obtained by SEC in Fig. 3.

monomers are (7.4  $\pm$  0.6) nm and (10.2  $\pm$  1.1) nm, respectively, which is consistent with those reported by Badley et al. (1975). For fraction a, in addition to 11S monomers, 11S dimers with the size of  $10.4 \times 21.1$ nm are also present, which further clearly indicates that 15S is the dimer of 11S, as suggested by Wolf and Nelsen (1996). Moreover, our result shows that the two 11S monomers interact with each other from the side position. Previously, Tulloch and Blagrove (1985) observed a similar structure for 18S cucurbitin sample isolated from pumpkin seed. Furthermore, some trimers with linear, angular, and triangular configurations are also observed (Fig. 5C and D). The weight percentages of all the particles were statistically analyzed and shown in Fig. 6. The result shows that the weight percentage distributions of monomers, dimers, and trimers are consistent with the sedimentation coefficient distributions (Fig. 4), indicating that they correspond to 11S, 15S and 21S fractions, respectively. Scheme 1 shows the schematic representation of the formation of 15S and 21S.



**Fig. 5.** Transmission electron microscopy micrographs of fractions *a* and *b* obtained by SEC of purified 11S after removing 7S. (A) and (B): Fraction *b*; (C) and (D): Fraction *a*. Scale bar represents 50 nm.

# Table 2

The measured sizes of molecules, calculated from transmission electron microscopy.

Fraction	Particle type		Measured aspect	Dimension(nm)	Angle(°)	Particle number
Ь	Monomer		Thickness	$7.4\pm0.6$	_	17
			Diameter	$10.2 \pm 1.1$	-	263
а	Monomer		Diameter	$10.2\pm1.2$	-	37
	Dimer		End-to-end length	$21.1\pm1.7$	-	48
			Width	$10.4\pm1.2$	-	96
	Trimer	Linear	End-to-end length	$31.1\pm0.9$	-	4
			Width	$10.4\pm0.9$	-	12
		Angular	Angle	_	$111 \pm 15$	2
		Triangular	Edge length	$21.5\pm1.9$		3

# 3.5. Interactions between two 11S molecules in each 15S molecule

Although Derbyshire, Wright, and Boulter (1976) speculated that 15S in legumes is linked by disulfide bonds, and Wolf (1993) stated that 15S is disulfide-linked polymer, Briggs and Wolf (1957) found the presence of 15S under 2-ME up to 250 mM. To study the types of the interactions between two 11S molecules in one 15S molecule, 11 mM N-ethylmaleimide (NEM) was used to block sulfhydryl groups to prevent the formation of new disulfide-linked polymers after removing 2-ME (Sun & Arntfield, 2012). Fig. 7 shows the normalized sedimentation coefficient distributions of fractions *a* and *b* after the addition of 11 mM NEM, and subsequent dialyzing against a sodium phosphate buffer with an ionic strength of 100 mM, containing 33 mM Na<sub>2</sub>HPO<sub>4</sub>, 2.6 mM

NaH<sub>2</sub>PO<sub>4</sub>, and 0.02% (w/v) NaN<sub>3</sub> without 2-ME for 48 h. The result shows that small amounts of 3S and 7S are formed in both fractions. These 3S and 7S molecules should be the subunits and half-molecules of 11S, due to the dissociation of 11S when dialyzed in the buffer with a lower ionic strength. Approximately 12.6% 15S is formed in fraction *b*, presumably because NEM failed to block all the sulfhydryl groups of 11S. The percentage of 15S in fraction *a* decreases to 42.1%, and the percentage of 11S increases, suggesting the dissociation of 15S into 11S when dialyzed in the buffer, which is consistent with the results reported by Wolf and Nelsen (1996).

SDS and 2-ME have been widely used to explore the interactions inside protein molecules, since hydrophobic interactions can be disrupted by SDS, and disulfide bonds can be cleaved by 2-ME (Hou, He, &

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Fig. 6. The weight percentage (%) of soybean proteins with different structures, calculated from TEM. (A): Fraction *a* (15S-rich fraction); (B): Fraction *b* (11S-rich fraction).



Scheme 1. Schematic diagram of the formation of dimers and trimers with different structures.



**Fig. 7.** Normalized sedimentation coefficient distributions of fractions *a* and *b* with the addition of 11 mM N-ethylmaleimide, followed by dialyzing against a sodium phosphate buffer with an ionic strength of 100 mM for 48 h.

Wang, 2020; Hu et al., 2013; Rao & Rao, 1979). Rao and Rao (1979) found that the 12S protein of mustard seed would completely dissociate with the addition of 0.5% (w/v) SDS. In this study, after the addition of



**Fig. 8.** Normalized sedimentation coefficient distributions of fractions a and b under various conditions. Black curve: Fraction a treated with 20 mM SDS; Blue curve: Fraction b treated with 20 mM SDS; Red curve: Fraction a treated with both 20 mM SDS and 10 mM 2-ME; Green curve: Fraction b treated with both 20 mM SDS and 10 mM 2-ME. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

NEM and dialysis, fractions a and b were treated with 20 mM SDS and 10 mM 2-ME, followed by AUC analysis. Fig. 8 shows the normalized sedimentation coefficient distributions of fractions a and b under various conditions. For fractions a and b treated with 20 mM SDS (black and blue curves), four peaks with sedimentation coefficients of 1.0 S, 2.2 S, 3.0 S, and 4.3 S are observed in both fractions. These fractions are designated as 1.0S, 2.2S, 3.0S, and 4.3S fractions, respectively. The percentage of 4.3S in fraction a (7.0%) is higher than that in fraction b (2.9%). Using c  $(s, f/f_0)$  model in SEDFIT, the molar masses of 3.0S and 4.3S are calculated as 73.9 kDa and 145 kDa, respectively. The molar mass of 3.0S is higher than the theoretical molar mass of AB subunit (54-64 kDa), presumably due to the binding of SDS molecules with the subunits (Martin, Bos, & Vliet, 2002; Reynolds & Tanford, 1970; Ruiz-Henestrosa, Martinez, Patino, & Pilosof, 2012). However, the molar mass ratio of 4.3S to 3.0S is  $\sim$ 2.0, indicating that the 4.3S is a dimer of 3.0S. For fractions a and b treated with both 20 mM SDS and 10 mM 2-ME (red and green curves), a peak (2.2 S) and a shoulder peak (2.0 S) is found in both fractions with insignificant difference.

SDS-PAGE was performed under the same conditions (Fig. 9) to verify the results shown in Fig. 8. After treated with 20 mM SDS, fractions *a* (Lane 3) and *b* (Lane 4) exhibit a major band of 52 kDa and minor bands of 34 kDa and 30 kDa, consist of SS-linked acidic and basic subunits (AB) and acid subunits (A), respectively. Light bands of ~100 kDa and >130 kDa are also present, which might be SS-linked AB subunits (AB-SS-AB) (Tan, Ngoh, & Gan, 2014; Wolf, 1993). The content of AB-SS-AB subunits in Lane 4 is slightly richer than that in Lane 3. With the addition of 2-ME, both fractions *b* (Lane 8) and *a* (Lane 9) mainly exhibit the bands for A and B subunits, while the band of AB-SS-AB subunits disappears, suggesting that the disulfide bond between ABs in the AB-SS-AB subunits was cleaved by 2-ME. Therefore, the peaks with sedimentation coefficients of 2.0 *S*, 2.2 *S*, 3.0 *S*, and 4.3 S (Fig. 8) correspond to B, A, AB subunits and AB-SS-AB subunits, respectively.

Figs. 8 and 9 show the presence of AB-SS-AB subunits in fractions a and b with the addition of SDS, indicating that the AB-SS-AB subunits are not formed by hydrophobic interaction. Moreover, the AB subunits in both fractions disappear with the addition of 2-ME, and the percentages of B (2.0 S) and A (2.2 S) increase due to the cleavage of the disulfide bond between A and B. Meanwhile, the AB-SS-AB subunits also disappear, suggesting that the AB-SS-AB subunits are also formed by disulfide bonds. Besides, we found the percentage of AB-SS-AB subunits (4.3 S) (fraction a containing 6.95%, fraction b containing 2.90%; Fig. 8) is

approximately one-sixth of that of 15S (fraction a containing 45.47%, fraction b containing 13.01%; Fig. 7). It is well known that 11S exists as a hexamer, consisting of six AB subunits. If two 11S molecules form one 15S molecule via one subunit AB of the six subunits of each 11S, the percentage of AB-SS-AB should be one-sixth of all subunits after adding 20 mM SDS. Therefore, we can speculate that each 15S molecule might be formed via AB-SS-AB subunits between 11S molecules. Scheme 2 shows the schematic representation of the dissociation of 15S protein after the addition of 20 mM SDS and 10 mM 2-ME. However, the disulfide groups in AB-SS-AB subunits should be buried in a non-polar microenvironment (in the interior of the 15S molecule), indicating that these disulfide groups are not easily accessible to reducing agents such as 2-ME. Otherwise, for example, if the disulfide bonds are formed via the relatively exposed sulfhydryl groups of 11S molecules, they will be easily reduced by 2-ME, and the 15S molecule will be changed to two 11S molecules. It is the reason that the percentage of 15S is as high as 6-8%, even with the addition 250 mM 2-ME (Briggs & Wolf, 1957).

# 4. Conclusion

The 15S-rich fraction with 75.6% purity of 15S component and 11Srich fraction with 94.3% purity of 11S component were obtained and studied by a combination of AUC and TEM. The results show that the molar masses of 11S and 15S are 313 kDa and 651 kDa, and the molecular size is 21.1 nm  $\times$  10.4 nm, respectively, demonstrating that 15S is the dimer of 11S, and the two 11S monomers interact with each other from the side position. Moreover, the 21S is the trimer of 11S, which has linear, angular, and triangular types of configurations. The interactions between two 11S molecules in one 15S molecule was investigated using 20 mM SDS and 10 mM 2-ME. After the addition of 20 mM SDS, the AUC and SDS-PAGE show that the content of the species with a sedimentation coefficient of 4.3 S in the 15S-rich fraction is higher than that in the 11Srich fraction, which is speculated to be disulfide-linked AB subunits (AB-SS-AB). The AB-SS-AB subunits disappears after the addition of 10 mM 2-ME in both fractions. We speculate that each 15S molecule might be formed via the disulfide groups in AB-SS-AB subunits between 11S molecules, and these disulfide groups in AB-SS-AB subunits may locate in a non-polar interior of the protein. Therefore, reducing agents such as 2-ME cannot cleave these disulfide groups, and the 15S fractions are always present in the soy protein solutions containing reducing agents.



**Fig. 9.** SDS-PAGE patterns of fractions *a* and *b* under various conditions and buffer extractable protein. Lane 1: Molecular weight standards; Lane 3: Fraction *b* treated with 20 mM SDS; Lane 4: Fraction *a* treated with 20 mM SDS; Lane 6: Buffer extractable protein; Lane 8: Fraction *b* treated with 20 mM SDS and 10 mM 2-ME; Lane 9: Fraction *a* treated with 20 mM SDS and 10 mM 2-ME.



Scheme 2. Schematic diagram of the dissociation of 15S with the addition of 20 mM SDS and 10 mM 2-ME.

# Author contribution

Xiaodong Ye conceived the project. Kang Ni and Yating Gao performed the experiments. Xiaodong Ye, Kang Ni and Yating Gao contributed to data interpretation and manuscript preparation.

#### Declaration of competing interest

The authors declare no conflict of interest.

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