Aggregates and Gel Network Structure of Globin Hydrolysates

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A gel with excellent functional properties was prepared successfully using the hydrolysates of globin. In the present study, the structures of intermediate aggregates and gel network were observed directly with an electronic microscope. It was shown clearly that the intermediate aggregates were in a thin rod shape with a length of 130−140 nm, which was in good accordance with the results of the light scattering obtained in a previous study. The diameter of intermediate aggregates was 4−5 nm. Each unit of the intermediate aggregate was composed of β-chain and peptide β-1 in a ratio of 1:1. Its molecular weight was 26922 Da, and it had a diameter of 4.1 nm. The thin rod-shaped aggregates were formed with units through the hydrophobic interaction. The length of intermediate aggregate was >30−33 times the diameter. Furthermore, the cross-linked structure formed by peptide α-1 and the thin rod-shaped aggregates was also confirmed by the photography of the electronic microscope. These results supported the model proposed in previous papers as proper to depict exactly the formation and structure of the gel network of globin hydrolysates.

Keywords: *Globin hydrolysates; aggregate; peptide α-1; electronic microscope*

INTRODUCTION

Globin, an animal protein material, has been experimentally incorporated into sausage meat (*1*). Using globin as an ingredient, a cheese-like emulsion has been prepared (*2*). However, some disadvantageous properties such as low solubility at neutral pH and unattractive color and odor have limited its application in the food industry. To solve these problems, many researchers have worked on globin decolorization (*3*, *4*), nutrition (*5*, *6*), and functional properties such as solubility, emulsifying properties, and foaming properties (*3*, *7*−*9*). Although the abilities of protein to form a gel and to provide a structural matrix for holding water are very important in the food industry (*10*−*12*), very few studies have been done on the gelling properties of globin, primarily because proteins were significantly altered during the processes of heme and globin separation and, as a result, gelation does not take place. Autio et al. (*10*) used a CMC−precipitation method to separate heme and globin, and the gel formation of the obtained globin has been reported to be excellent, occurring at 4.5%. Other studies were performed on the gel formation and structural properties of globin. For example, Hayakawa et al. (*12*) reported that aggregates with highly entangled fibrous structure and having a thickness of 8−10 nm were formed by heating globin heated at 90 °C.

Globin hydrolysates having better solubility and ability in gel formation than intact globin have been successfully prepared with citric acid in our previous studies (*13*−*16*). The globin hydrolyzed with 0.8 M citric acid showed eight kinds of peptides of globin hydrolysates by tricine−SDS-PAGE, with molecular weights ranging from 5000 to 15000 Da. Intermediate aggregates of globin hydrolysates with a length of 130−140 nm were measured by transmission electronic microscope and the light scattering method. The molecular weight of the intermediate aggregates was estimated as 870000 Da by gel filtration and the light scattering method (*13*).

Furthermore, a highly hydrophilic peptide α-1 originated from the α-chain was isolated with hydrophobic chromatography. According to light scattering, small-angle X-ray scattering (SAXS), and CD spectral methods, the formation of aggregates (oligomers) from peptide α-1 depended on the temperature and the concentration of peptide α-1 (*14*, *16*). One unit of aggregates of peptide α-1 was composed of seven to nine molecules of peptide α-1. The secondary structure of peptide α-1 did not change even after the aggregates were formed. We also observed the aggregation process of globin hydrolysates and the transformation process from intermediate aggregates to soft gel in the presence of peptide α-1 with the quasi-elastic light scattering method (*15*). On the basis of these results, the gel formation of globin hydrolysate was supposed to be divided into two steps. The first step is mainly the formation process of intermediate aggregates (in thin rod shapes) of globin hydrolysate. The second step was the formation process of early gel network structure by entangling peptide α-1 among thin rod-shaped aggregates, and then the gel was formed finally. During these processes about eight molecules of peptide α-1 combined with each other and behaved as a cross-linker entangling among the thin rod-shaped aggregates of globin hydrolysates to facilitate the formation of gel network (*15*). A temporary gel formation model for globin hydrolysates was proposed in our previous papers (*15*), but the final matrix structure of gel remained to be verified by experiments. For practical applications, the character of the matrix structure is of importance to the food industry. If the matrix structural character were well understood, it would be of great help to determine what kinds of food the globin hydrolysates can be added to and how to apply them in the food industry.

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In the present study, the structures of intermediate aggregates and gel formed by globin hydrolysate were investigated with an electronic microscope. The matrix structural character of the gel was shown clearly by the electronic microscope, which supported our temporary model for the gel formation process.

MATERIALS AND METHODS

Globin. Porcine hemoglobin was decolorized according to the acid−acetone method (*3*).

Globin Hydrolysates. Globin powder was dissolved in 0.8 M citric acid and hydrolyzed at 95 °C for 15 min. Then the sample was cooled to room temperature and dialyzed with flowing water using seamless cellulose tubing (pore size 10000 Da). The dialysate was air-dried with a spray-dryer (Pulvis minispray-GA-32, Yamato Science), and the resulting sample was named globin hydrolysates (GH).

Analysis of Amino Acid Composition. The amino acid composition of globin hydrolysates was analyzed according to an HCl hydrolyzing method. The hydrolyzations were carried out at 110 °C for 24 and 72 h. The amino acid composition was analyzed by amino acid analyzer (L-8500, Hitachi Co., Ltd., Kyoto, Japan).

Preparation of Intermediate Aggregates of Globin Hydrolysates. The intermediate aggregates of globin hydrolysates were prepared by gel filtration. The gel filtration was carried out on a TSKgel Toyopearl HW-60S (Tosoh Corp., Tokyo, Japan), with a column size of 40 cm × 1.6 cm. Elution was performed at a flow rate of 12.5 mL/h at ambient temperature with 0.1 M citric acid buffer (pH 3.3). The proteins eluted were monitored by measuring the absorbance at 280 nm. Fraction P_1 was used as the intermediate aggregates sample (*13*).

Gel Preparation. Solutions (total protein concentration of 5%) with different ratios of intermediate aggregates of globin hydrolysate (fraction P_1 collected from gel filtration) and peptide α-1 (GHP 1 fraction obtained form hydrophobic chromatography) were heated at 90 °C for 15 min and then cooled at ambient temperature for over 1 h to form a gel. The gel hardness was expressed as the value of breaking stress and measured with a rheometer (Nrm20031, Fudo Industrial Co., Ltd., Kyoto, Japan).

Transparency Measurement. Solutions with different protein concentrations were heated at 90 °C for 15 min and then placed into a cell (light path length of 1.0 cm). The transparency measurements of the gel were carried out with a Shimadzu UV-1200 spectrophotometer at 700 nm.

Transmission Electronic Microscope. The globin hydrolysates and the intermediate aggregate solutions were diluted with 0.1 M citric acid buffer (pH 3.3). A small drop of sample was applied to a carbon-coated copper electron microscope grid and then stained with uranyl acetate. Specimens were observed with a Hitachi H-700H transmission electron microscope at an accelerating voltage of 80 kV (*17*).

RESULTS AND DISCUSSION

Structure of Intermediate Aggregates. In our previous studies, we have succeeded in preparing a gel from the hydrolysates of globin, which have better functional properties than intact globin (*14*). To make clear the mechanism of gel formation, at first we analyzed the globin hydrolysates with tricine−SDS-PAGE and then examined the properties of all peptides existing in the globin hydrolysates. As reported in our previous paper (*13*) the globin hydrolysates comprised mainly eight kinds of peptides; they could form aggregates (intermediate aggregates) at room temperature, and the molecular weight of the aggregate was >700000 Da by gel filtration. Intermediate aggregates (P_1 fraction) were formed by two kinds of peptides (globin β-chain and the peptide β-1) combined with noncovalent bond (*13*). However, the size and the shape of the intermediate aggregates remained unknown. Because the size and shape of the intermediate aggregates were the primary factors governing the structure of the gel network, the molecular weight and the shape of the intermediate aggregates were measured with a light scattering method. The weight-average molecular weight obtained was 872200 Da. Furthermore, the shape and size of intermediate aggregates were analyzed, and it was found that they were in a thin rod shape with a length of 130−140 nm (*13*).

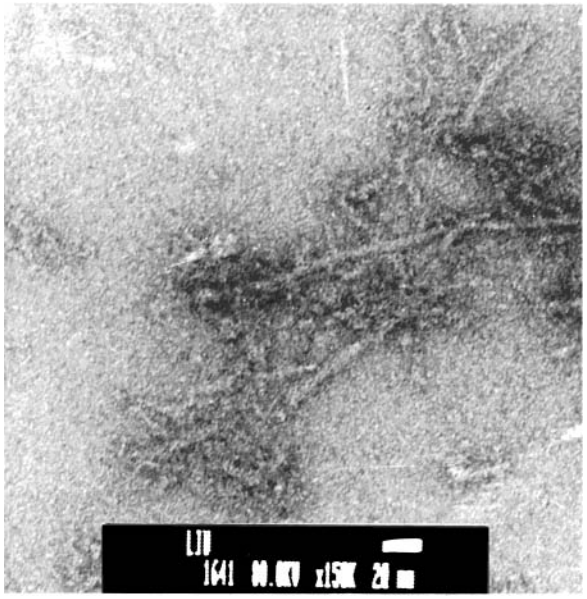

Figure 1. Electronic micrograph of the thin rod-shaped aggregates of globin hydrolysates with negative staining. The aggregates were dissolved in 0.1 M citric acid buffer (pH 3.3) and stained with uranyl acetate on a carbon-coated electronic microscope grid.

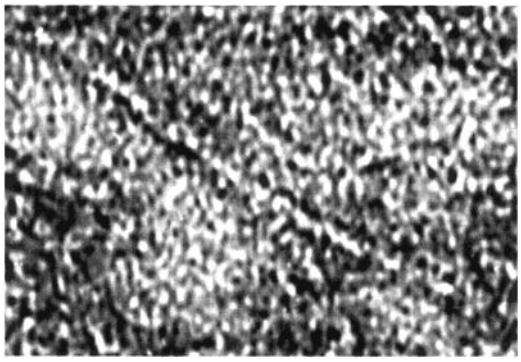

Figure 2. Electronic micrograph of the intermediate aggregates that existed in a thin rod-shaped structure.

The detailed structure and diameter of intermediate aggregates were still unclear. Therefore, we used an electronic microscope to observe the structure of intermediate aggregates. The electron micrograph showed clearly intermediate aggregates were in thin rod shapes with a length of 130−140 nm and a diameter of 4−5 nm (Figure 1). This result was in good accordance with our previous results obtained from the light scattering method (*13*). Furthermore, we analyzed the electron micrograph of the intermediate aggregates with image processing software. As shown in Figure 2, a string of globular entities was visible, implying that the thin rod-shaped aggregates were formed by the unit of intermediate aggregates and extended in a linear manner.

Table 1. Calculation of the Apparent Specific Volume of Globin Hydrolysates

amino acid residue	W_i (% by wt of residue)	V_i (specific vol of residue)	V_iW_i (% by vol of residue)
half-cystine	1.08	0.63	0.68
aspartic acid	11.86	0.59	7.00
threonine	2.97	0.70	2.08
serine	5.77	0.63	3.64
glutamic acid	7.76	0.66	5.12
glycine	7.89	0.64	5.05
alanine	10.72	0.74	7.93
valine	9.58	0.86	8.24
methionine	0.49	0.75	0.37
leucine	13.40	0.90	12.06
tyrosine	1.54	0.71	1.09
phenylalanine	5.62	0.77	4.33
tryptophan	1.19	0.74	0.88
histidine	6.05	0.67	4.05
lysine	6.71	0.82	5.50
arginine	3.45	0.70	2.42
proline	3.29	0.76	2.50

$\sum W_i = 99.37$ $\sum W_iV_i = 72.94$

$\sum W_iV_i/\sum W_i = V_p = 0.734$ mL/g

To make clear the length and diameter of the intermediate aggregates, we analyzed the amino acid compositions of globin hydrolysates. The results of the amino acid compositions of globin hydrolysates are shown in Table 1. The apparent specific volumes of globin hydrolysates can be estimated quite reliably from the amino acid composition.

$$V_p = \sum W_iV_i/\sum W_i = 0.734 \text{ cm}^3/\text{g}$$

It was confirmed by gel filtration chromatography and tricine−SDS-PAGE that a unit of intermediate aggregate was composed of two kinds of polypeptide of globin hydrolysates. One was β-chain ($M_w = 16035$ Da) originating from the intact globin, and the other was β-1 ($M_w = 10887$ Da) originating from β-chain by cleavage of the β-chain between 99 (Asp) and 100 (Pro). The ratio of β-chain to peptide β-1 in one unit of the intermediate aggregate was 1:1 (*13*). Then the one unit molecular weight of intermediate aggregate was expected to be 26922 Da. Therefore, the size of one unit of intermediate aggregate could be calculated from the equation

$$MV_p/N_A = 4\pi/3(d/2)^3$$

where M is the molecular weight of one unit and N_A is Avogadro's number. The diameter of a unit is given by $d = 41$ **Å**.

Because the weight-average molecular weight of intermediate aggregates was 872200 Da as obtained from the light scattering method (*13*), the intermediate aggregate were estimated being formed by ~33 units (872200 Da/26922 Da). As it has been reported that the aggregates were in thin rod shapes (*13*), the length of intermediate aggregates could be calculated by using the following equation:

$$41(\text{Å}) \times 33(\text{units}) = 135.3 \text{ nm}$$

This result was in good agreement with that obtained from the light scattering method. On the basis of the results discribed above, we postulated a model for the formation of intermediate aggregates in Figure 3. Each unit of the intermediate aggregate was composed of β-chain and peptide β-1 in a ratio of 1:1 and had a molecular weight of 26922 Da and a diameter of 4.1 nm. It has been proven that β-chain and peptide β-1 had high hydrophobicity by hydrophobic chromatography (*13*). Every unit combined with one another with hydrophobic interaction and extended in a linear manner to form thin rod-shaped aggregates. This temporary model was supported by electronic microscope observation. In Figure 1, it was shown that the intermediate aggregates were in thin rod shapes with a length of 130−140 nm and a diameter of 4−5 nm, and the length of the intermediate aggregate was ~30 times its diameter. This was in good coincidence with light scattering results and the calculated value described above.

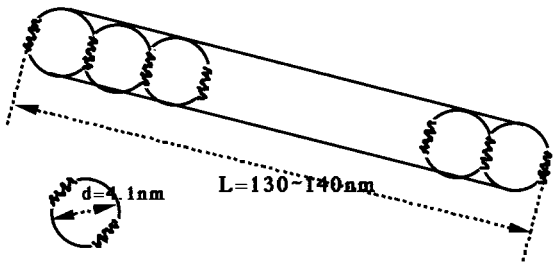

Figure 3. Model for the formation of intermediate aggregates of globin hydrolysates.

The formation of fibrous structures by globular proteins has been intensively studied. Koseki et al. (*18*) and Doi and Kitabatake (*19*) examined the formation of linear polymers of ovalbumin by the light scattering method. Ovalbumin polymers were described by a wormlike cylinder model with $M_L = 1.6 \times 10^3$ Å^{-1}, $d =$ 120 Å, and $q = 230$ Å, where M_L, d, and q are the molecular weight per unit contour length, diameter, and persistence length, respectively. In our previously published paper (*13*), the shape and size of intermediate aggregates were analyzed by comparing the theoretical calculation and experimental curves of light scattering. The attempt to theoretically calculate the parameter of diameter effect was made, but no sound results were obtained, possibly because the diameter was too small compared to its length; therefore, the light scattering intensity was very weak, and its effect on light scattering was negligible.

Structure of Gel Network. It is interesting that the intermediate aggregates of globin hydrolysates showed neither further association among the aggregates nor tendency to form a gel. We have proven that the intermediate aggregates of the globin hydrolysate existed in a state of monomer by quasi-elastic light scattering method (*13*, *15*). To form a network of the gel, the cross-link combination should take place between intermediate aggregates of globin hydrolysates. Using hydrophobic chromatography, we isolated a highly hydrophilic polypeptide α-1 originated from the α-chain. By adding peptide α-1 to intermediate aggregates, a gel was formed. The hardness of the gel could be improved by increasing the concentration of peptide α-1, implying that peptide α-1 played an important role in the formation of gel (*15*).

The light scattering and CD spectra of peptide α-1 indicated that peptide α-1 had properties of association and dissociation depending on the concentration of peptide α-1 and temperature (*14*). Peptide α-1 existed in two types in solution at 20 °C: a monomeric form and a random coiled form, which was made up of seven to nine molecules of peptide α-1. The dissociation of the peptide α-1 aggregates occurred at higher temperature.

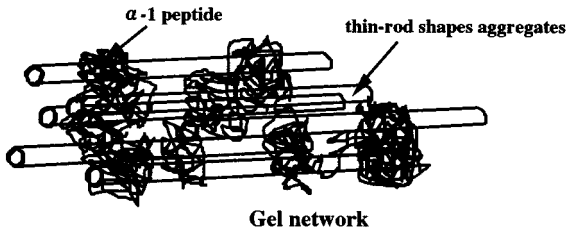

Figure 4. Schematic model for the formation gel network.

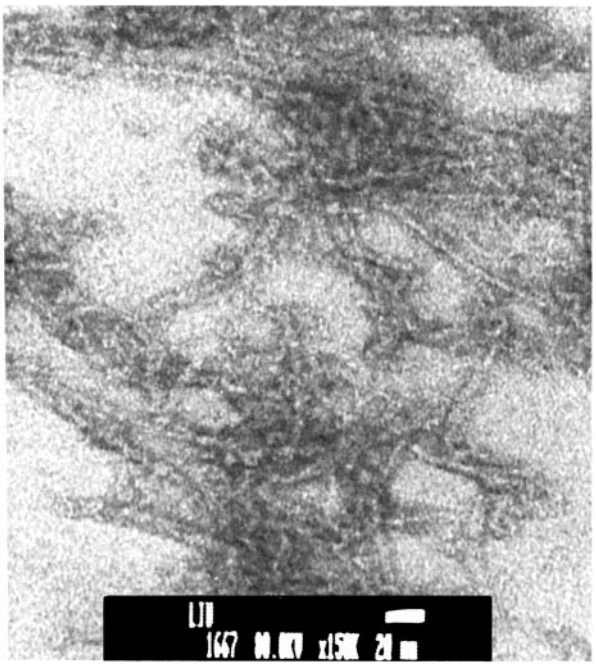

Figure 5. Electronic micrograph of gel network of globin hydrolysates with negative staining. The gels of globin hydrolysates were dissolved in 0.1 M citric acid buffer (pH 3.3) and stained with uranyl acetate on a carbon-coated electronic microscope grid.

When peptide α-1 was added to the solution of intermediate aggregates, the peptide α-1 bound to the intermediate aggregates and formed a cross-linked structure (*15*). On the basis of the results obtained from quasi-elastic light scattering, we proposed that the following two steps were involved in the process of gel formation. The first step was mainly the formation of thin rod-shaped intermediate aggregates. The second step was the formation of early gel network structure by entangling peptide α-1 with thin rod-shaped intermediate aggregates, and then the gel was formed finally (*15*). The temporary model for the gel formation of globin hydrolysates is shown in Figure 4. It is presented on the basis of only our previous experiment results. Further investigation is needed to support it. By using an electronic microscope, the structure of the gel network could be observed clearly. As shown in Figure 5, many thin rod-shaped aggregates were observed clearly, supporting the results obtained from light scattering (*13*). Furthermore, the cross-linked structure formed with peptide α-1 and thin rod-shaped aggregates was also confirmed by the photography of the electronic microscope, supporting the temporary model proposed in our previous papers as proper to be employed to depict exactly the formation and structure of the gel network of globin hydrolysates (Figures 4 and 5).

Hayakawa et al. (*12*) studied the structure properties of globin by electronic microscope observation. Their results showed that globular aggregates were formed spontaneously with the unfolding of globin molecules, and subsequently fibrous macroaggregates were formed via extension and association of globular aggregates. Finally, the fibrous macroaggregates formed a three-dimensional gel network under the conditions of higher protein concentrations and higher temperature. Therefore, it was clear that the gel they obtained was different from that obtained by globin hydrolysates. In our previous studies (*13*−*16*), the gel formed by intact globin showed lower solubility, hardness, and transparency. The amount of globin needed to form a gel was >7%, whereas that of globin hydrolysates was 3.0%, indicating that the gel formation ability of globin hydrolysates was better than that of intact globin under our experimental conditions. The gel formation ability of the globin and globin hydrolysates and the transparency of resulting gels are compared in Table 2.

Table 2. Comparison of Globin and Globin Hydrolysates on Gel Formation and Transparence

	protein concn (%)					
	2.0	3.0	4.0	5.0	6.0	7.0
globin						
gel formation (pH 4.0)						+[a]
breaking stress (g)						2.0
transparence (700 nm T%)						15
globin hydrolysates						
gel formation (pH 4.0)		+	+	+	+	+
breaking stress (g)		1.7	5.0	10.8	13.3	21.6
transparence (700 nm T%)		91	78	60	45	35

[a] +, gel was formed.

In general, the hardness and transparency of a gel are affected by the water-holding ability, protein concentration, and interaction force of molecules because the isoelectric point of intact globin is in the range of neutral pH. Therefore, intact globin showed a lower solubility over a wide pH range. Furthermore, the hydrophobic bonding between globular aggregates of intact globin is the major interaction force in the formation of a three-dimensional gel network, and the gel formed showed a poor water-holding capability. Therefore, a high concentration of protein is required for gel formation.

The globin hydrolysates had higher solubility and showed better ability in gel formation than intact globin. In the case of globin hydrolysates, because peptide α-1 has a high hydrophilicity it easily formed the matrix structure, which could entrap many water molecules in the spaces of the gel network. The abilities of proteins to form a gel and to provide a structural matrix for water holding are very useful in the food industry. It is very useful to exploit so-called soft gels in the food industry to control the texture of foods. Study of gel-controlling technology is of great help to improve the physical texture (or softness) of sausage meat, ham, and traditional proteinaceous foods such as surimi-kamaboko and may contribute to food processing. Using a spiral structural peptide such as peptide α-1 as a gel junction, the functional properties of gel are improved. Therefore, more economical and more nutritionally acceptable food products are expected to be produced by adding globin hydrolysates to food products.

The present study suggested that acid hydrolysis was an effective method to modify and improve functional properties of protein by precisely controlling the hydrolytic conditions.

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