Quasi-elastic Light Scattering Study on the Globin Hydrolysate Gel Formation Process

Xin Qi Liu,*,† Masami Yonekura,‡ Masakazu Tsutsumi,‡ and Yoh Sano†

National Food Research Institute, Tsukuba City, Ibaraki 305, Japan, and Faculty of Agriculture, Ibaraki University, Amimachi-chuo, Ibaraki 300-03, Japan

To investigate the aggregation process of globin hydrolysates treated with 0.8 M citric acid and the transformation process of the resulting aggregates to gel mediated by addition of peptide α-1, the interaction between the aggregates of the globin hydrolysate and peptide α-1 was examined through the determination variation in diffusion coefficient *D* with reaction time by quasi-elastic light scattering technique. The results revealed that in the case of the aggregates of the globin hydrolysate alone the diffusion coefficient *D* showed no change, but in the case of the mixture containing the aggregates of the globin hydrolysates and peptide α-1 (the mixing ratio is 7:3, and the final total concentration is 20 mg/mL) the diffusion coefficient decreased sharply with reaction time. On the basis of these results a gelation model for the globin hydrolysates was presented as follows: initially, 8 molecules of the randomly coiled peptide α-1 are involved in the initial complex of the globin hydrolysate and peptide α-1; next, the cross-linked structure was constructed by the interaction between peptide α-1 and the rod-shaped aggregates; and then a network structure was formed, and a gel was finally formed.

Keywords: *Globin; globin hydrolysates; aggregates; peptide α-1; quasi-elastic light scattering; circular dichroism*

INTRODUCTION

Animal blood contains about 18% protein and hemoglobin, accounting for more than half of the blood protein. Most of the hemoglobin is not utilized in the food industry because of its unattractive color and odor. Studies of the preparation methods of decolorized globin (Tybor et al., 1975; Sato et al., 1981; Drepper et al., 1979), nutritional qualities (Landmann et al., 1980; Parmer et al., 1978), and functional properties of globin such as solubility, emulsifying properties, and foaming properties were made by several researchers (Tybor et al., 1973, 1975; Penteado et al., 1979; Crenwelge et al., 1974). Attempts have been made to incorporate globin into sausage meat (Caldironi et al., 1982), and a cheeselike emulsion was prepared by using globin as an ingredient (Knapp et al., 1978). Although the abilities of proteins to form a gel and to provide a structural matrix for holding water applications are useful in the food industry, very few studies have been done on the gelling properties of globin (Autio et al., 1985, 1990; Hayakawa et al., 1983), primarily because most of the processes developed for heme and globin separation alter the proteins to such an extent that gelation does not take place. Sato et al. (1981) and Autio et al. (1985) have presented a carboxymethylcellulose (CMC) column procedure for the separation of heme and globin, and the globin prepared according to their methods can form a gel when heated. Decolorized globin prepared by acidified acetone (Tybor et al., 1975) showed acceptable water-binding properties at room temperature, and it did not form a gel after heating. However, Liu et al. (1994a) were first to report that the globin made according to this method can form a gel at a high globin concentration (7%).

Heat-induced globin gel prepared according to each of the above-mentioned methods had a low hardness, was affected easily by many factors such as pH and ions, and lacked adequate functional properties (Liu et al., 1994a; Autio et al., 1985, 1990). Some attempts have been made at gel formation and to study the structural properties of globin; for example, Hayakawa et al. (1983) have reported that when globin was heated at 90 °C, a highly entangled fibrous structure of aggregate could be formed with thickness of 8−10 nm. Autio et al. (1990) have observed that globin was more unfolded and more flexible at low pH with CD and fluorescence spectra. In our previous studies (Liu et al., 1994b), it was found that the globin hydrolysates prepared by 8 M citric acid had a high solubility, the ability to form a gel is greater than that of intact globin, and the type of gel formed is different from that of intact globin. Our results also suggested that acid hydrolysis was an effective method for modifying and improving new functional properties of protein by precisely controlling the hydrolytic conditions (Liu et al., 1995b). We also reported that a molecular mass of about 870 000 Da aggregate was built up by the *β*-chain of globin and peptide α-1, which had a length of 130−140 nm and a thin rod shape. In solution, the aggregates exist in monodispersive state and could not form a gel by self-polymerization between the aggregates (Liu et al., 1996).

Further, a highly hydrophilic peptide α-1 was isolated from hydrophobic chromatography. We have studied its properties by a light scattering method and CD spectra (Liu et al., 1997). The results showed that peptide α-1 associates reversibly, these properties depending on the concentration of peptide α-1 and temperature. We examined the gel formation ability by mixing peptide α-1 and the rod-shaped aggregates and observed that the gel thus produced had a hardness increasing with

* Author to whom correspondence should be addressed (telephone +81-298-38-8031; fax +81-298-38-8082; e-mail liuxinqi@nfri.affrc.go.jp).

† National Food Research Institute.

‡ Ibaraki University.

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peptide α-1 concentration. We supposed that peptide α-1 may behave as a cross-linker between aggregates of globin hydrolysate and a facilitator in the formation of gel network.

To test the hypothesis that the transformation process of these aggregates to gel was mediated by peptide α-1, we examined the interaction between the aggregates of the globin hydrolysates and peptide α-1 through detecting the variation in the diffusion coefficient *D* with reaction time by a quasi-elastic light scattering technique. The gel formation of the globin hydrolysates could therefore be divided into the following two steps on the basis of our results. The first step is mainly the formation process of rod-shaped aggregates made up by globin *β*-chain and peptide α-1; the second step is the formation process of early gel network structure induced by the entanglement of peptide α-1 around rod-shaped aggregates, and then the gel was finally formed.

MATERIALS AND METHODS

Materials. Globin was prepared from porcine blood cells using Tybor's (1975) acidified acetone method with a little modification (Liu et al., 1995a).

Globin Hydrolysates. The decolorized powder globin was hydrolyzed with 0.8 M citric acid at 95 °C for 15 min, then cooled to room temperature, and dialyzed against flowing water with Seamless Cellulose Tubing (molecular cutoff size 10 000 Da). The dialysate was air-dried with a spray-dryer (Pulvis minispray-GA-32, Yamato Science, Tuskuba, Japan), and the resulting sample was designated globin hydrolysate (GH) sample.

Preparation of the Aggregate of the Globin Hydrolysates. The 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); the column size was 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), and the protein eluted was monitored by measuring the absorbance at 280 nm. P_1 fraction was used as the aggregate sample (Liu et al., 1996).

Preparation of Peptide α-1. The hydrophobic chromatography was carried out on butyl-Toyopearl 650S (Tosoh). The column (14 mm × 190 mm) was equilibrated with 0.1 M citric acid buffer containing 0.3 M NaCl. Then the column was eluted with solutions A−E: solution A, citric acid buffer containing 0.3 M NaCl; solution B, citric acid buffer containing 0.15 M NaCl; solution C, citric acid buffer without NaCl (the pH of all buffers was 3.2); solution D, distilled water; solution E, 0.05 N NaOH. The GHP_1 fraction eluted from solution C was used as peptide α-1 sample (Liu et al., 1997).

Quasi-elastic Light Scattering Measurement. The diffusion coefficients of solutions were measured with the quasi-elastic light scattering instrument of a light scattering analyzer ELS-800 (Ohtsuka Co. Ltd., Tokyo, Japan) at 20 °C (Niki et al., 1994; Sano, 1987, 1993). The light source was a He−Ne laser of 632.8 nm, and the scattering angle was 90°. The autocorrelater was connected to the light scattering analyzer, and the autocorrelation function of the photomultiplier photocurrent was automatically analyzed by standard computer fitting techniques to obtain the translational diffusion coefficient of each solution of globin hydrolysate. The experiments were accumulated over 100 times for the same samples and repeated 3 times. The date was very reproducible.

The sample solution was diluted to the desired concentrations with 0.1 M citric acid buffer (pH 3.3) and made optically clean by filtering with a Millipore (Germany) filter (pore size, 0.45 μm).

RESULTS

Association Process of Globin Hydrolysates. Globin was hydrolyzed at 95 °C for 15 min and placed at room temperature. The variations in diffusion coefficient *D* of the globin hydrolysates were monitored with the quasi-elastic light scattering method at various concentrations and reaction times. The experiments were accumulated over 100 times for the same samples and repeated 3 times. The data were very reproducible. The results obtained are shown in Figure 1. From this figure, it is observed that the diffusion coefficient *D* became smaller with reaction time; especially in the first 4 h a sharper decrease could be observed at the intermediate concentration, but an appreciable change appeared after that. On the other hand, the diffusion coefficient *D* decreased rapidly with increasing globin hydrolysate concentration.

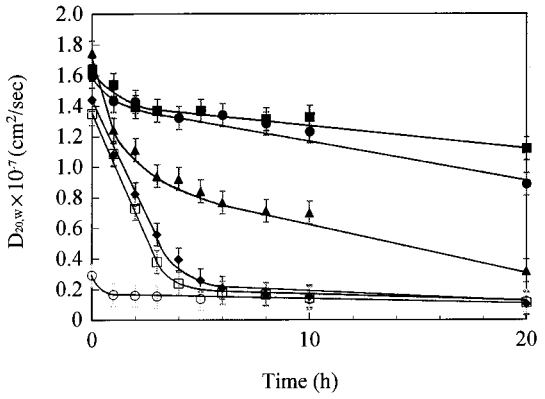

Figure 1. Time dependence of apparent diffusion coefficients changes of globin hydrolysates standing for various reaction times after pro-heating at 95 °C for 15 min. Concentration of globin hydrolysates: (■) 10 mg/mL, (●) 15 mg/mL, (▲) 20 mg/mL, (◆) 25 mg/mL, (□) 35 mg/mL, (○) 45 mg/mL. Mean values and their standard deviations are shown with each symbol and error bars.

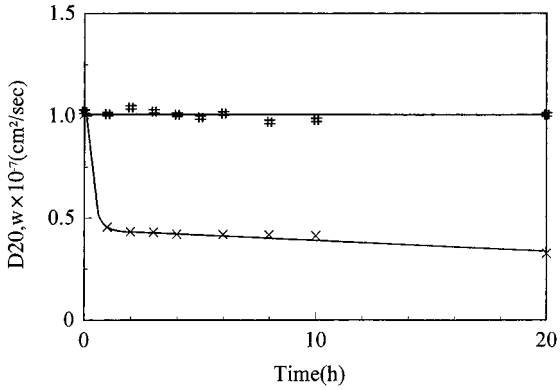

Figure 2. Apparent diffusion coefficient of aggregates (P_1 fraction) after addition of peptide α-1 (GHP_1 fraction). The samples were pro-heated at 95 °C for 15 min. (#) Aggregates (P_1 fraction); (×) aggregates (P_1 fraction) + peptide α-1 (GHP_1 fraction).

Initial Complex Formation of Peptide α-1 and the Globin Hydrolysate Aggregates. The aggregates (P_1 fraction) of the globin hydrolysates alone (20 mg/mL) and the mixture of peptide α-1 (GHP_1 fraction) and the aggregates (P_1 fraction) [mixing ratio is 3:7 for the situation near the same condition as in the whole hydrolysate globin shown already in the previous paper (Liu et al., 1997), and the total concentration is 20 mg/mL] were heated at 90 °C for 15 min and then allowed to stand at room temperature. The variations in diffusion coefficient *D* with reaction time are shown in Figure 2. No change in diffusion coefficient *D* was observed in the case of aggregates (P_1 fraction) alone, indicating that the association between aggregates did not happen.

However, the diffusion coefficient *D* decreased rapidly in the case of the mixture of peptide α-1 (GHP_1 fraction) and the aggregates (P_1 fraction) in the first hour, following a gentle decline suggesting that peptide α-1 (GHP_1 fraction) may play a role in the formation of cross-linked structure between aggregates, the initial step of which was very fast.

DISCUSSION

A gelation occurred in the mixture of the aggregates of the globin hydrolysates and peptide α-1; however, the mechanism remained to be resolved. For this reason, we examined the properties of all peptides contained in the globin hydrolysates as a first attempt. The globin hydrolysates were analyzed with tricine−SDS−PAGE. As reported in our previous paper (Liu et al., 1996), the globin hydrolysates were comprised mainly of 8 kinds of peptides, and a molecular weight of above 700 000 Da of polymer aggregates was generated when they were placed at room temperature by the gel filtration (Liu et al., 1996). We have speculated that the size and the shape of the intermediate aggregates were the primary factors governing the structure of the gel network. The intermediate aggregates were combined by two kinds of peptides (globin *β*-chain and peptide α-1) with noncovalent bond (Liu et al., 1996). However, we have not yet obtained more detailed data on the size and the shape of the aggregates (P_1 fraction). We therefore measured the molecular mass and the shape of the aggregates (P_1 fraction) with the light scattering method. The value for the weight-average molecular mass was obtained as 870 000 Da. The shape and size of aggregates (P_1 fraction) were analyzed by comparing the theoretical and experimental curves, and it was found that the aggregates (P_1 fraction) of globin hydrolysates were in thin rod shapes with a length of 130−140 nm; these results were also supported by electron microscope observations (Liu et al., 1996). The aggregates (P_1 fraction) of the globin hydrolysates in a monodisperse form were also proved by the quasi-elastic light scattering intensity as shown in Figure 2, but further polymerization between aggregates (P_1 fraction) could not be observed.

Furthermore, the globin hydrolysates consisting of at least three components (GHP_1, GHP_2, GHP_3) having different hydrophobicities, were found by hydrophobic chromatography. The main component of the highly hydrophilic fraction GHP_1 was peptide α-1 (Liu et al., 1997). The light scattering and CD spectra of peptide α-1 indicated that peptide α-1 associated reversibly depending on the concentration of peptide α-1 and temperature (Liu et al., 1997). In solution peptide α-1 existed in two types: the monomeric form and the random coiled form, which was made up of 7−9 molecules of peptide α-1 at 20 °C. However, the aggregates (oligomer) of peptide α-1 were formed only by 6 molecules at 50 °C, which was smaller than that formed at 20 °C (Liu et al., 1997). For peptide α-1, which is in a random coiled state which makes it easy to bind with the thin rod-shaped aggregates (P_1 fraction), we suggested that peptide α-1 was concerned in the formation of cross-linked structure (Liu et al., 1997). Thus, we presented the following gel formation mechanism based on the results described above:

globin → globin hydrolysates → aggregates → cross-linked structure (combination of the aggregates with peptide α-1) → **network → gel**

We supposed that these steps are not independent and progress simultaneously. The interactions between these steps and the interactions between the components of the gel were studied by determining the variations in diffusion coefficient *D* with reaction time using quasi-elastic light scattering (Figure 1). An increase in the globin hydrolysate concentration reduced the diffusion coefficient *D* rapidly. After standing for 20 h, the amount of the aggregates of the globin hydrolysates rose, but the diffusion coefficient *D* decreased. Because the frequency of collision between particles increases consistently with concentration, the aggregates are formed more easily at high concentration of the globin hydrolysates, and then the diffusion coefficient *D* decreases rapidly. In the case of low concentration of globin hydrolysates such as 10 and 15 mg/mL, the decrease of diffusion coefficient *D* was relatively fast at the initial step and became slower after 2 h, suggesting that in these cases a rather long time is essential to form the network or it is impossible to form a network. A great decrease in diffusion coefficient *D* was found under a high concentration of the globin hydrolysates (above 25 mg/mL) at the beginning and then remained almost constant after 5 h. It can be explained that the complexes can be formed rapidly when the concentration was high (above 25 mg/mL), and the initial network structure was constructed in a short time (within 5 h).

In the intermediate concentration, a relatively great change of diffusion coefficient *D* was observed during the first 4 h. The diffusion coefficient *D* in this concentration became smaller rapidly during the first 4 h, indicating that the aggregates were formed quickly; in higher concentration the early network structure of the gel may have already been constructed in this step. We also determined the changes of diffusion coefficient *D* with the aggregates (P_1 fraction) produced by globin hydrolysates; the results (Figure 2) showed that in the case of aggregates (P_1 fraction) alone there are no changes in the diffusion coefficient *D*; however, in the case of mixture of aggregates (P_1 fraction) and peptide α-1 (mixing ratio 7:3; final concentration 20 mg/mL) the value dropped more sharply than did that of the whole globin hydrolysates mixture, indicating that because the rod-shaped aggregates (P_1 fraction) have been already formed, it is not necessary to take time for this formation process. Thus, the network of gel constructed by the interaction of the aggregates (P_1 fraction) and peptide α-1 can be formed within 1 h. From the results described above (shown in Figure 3), we concluded that the gel formation of whole globin hydrolysate mixture can be divided into two steps. The first step was mainly the formation of the rod-shaped aggregates (P_1 fraction), which originated from low molecular weight peptides (globin *β*-chain and peptide *β*-1). In this step the oligomer formation of peptide α-1 was also involved. The second step was the formation process of early network structure based on the interaction between rod-shaped aggregates (P_1 fraction) produced at the first step and peptide α-1. The transformation from the aggregates (P_1 fraction) to network may be affected by the concentration of aggregates (P_1 fraction). The formation of network structure probably began in the first step when a certain amount of aggregates (P_1 fraction) was accumulated, and this process became gradually dominant in the second step.

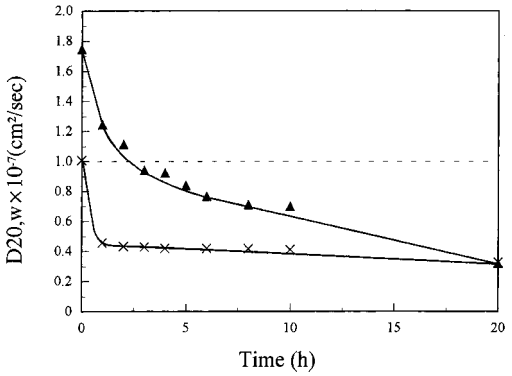

Figure 3. Apparent diffusion coefficients of whole globin hydrolysates mixture and rod-shaped aggregates (P_1 fraction) after addition of peptide α-1. The samples were pro-heated at 95 °C for 15 min. (▲) Whole globin hydrolysates (GH) (see ▲ 20 mg/mL line of Figure 1); (×) aggregates (P_1 fraction) + peptide α-1 (GHP_1 fraction) (see × aggregates + peptide α-1 line of Figure 2).

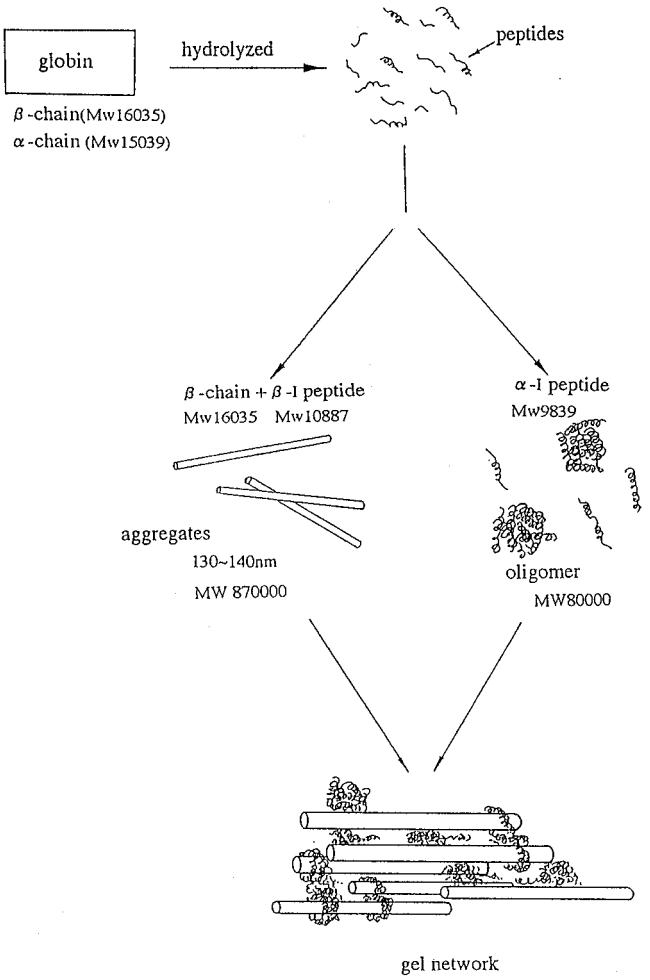

Figure 4. Schematic model of gel formation of whole globin hydrolysates.

On the results described above, the schematic model of gel formation of globin hydrolysates is presented in Figure 4.

At first, the globin hydrolysates containing mainly eight kinds of peptides were obtained by hydrolyzing the globin with 8 M citric acid. The globin hydrolysates were composed of undigested globin α-chain, β-chain, peptide α-1 originated from globin α-chain with a molecular mass of 9 839 Da, and peptide β-1 from globin β-chain with a molecular mass of 10 887 Da.

When the globin hydrolysates were cooled to room temperature after heating, a rod-shaped aggregate (P_1 fraction) with a length of 130−140 nm and a molecular mass of about 870 000 Da was formed by a noncovalent bond between the globin β-chain and peptide β-1 originated from the globin β-chain. Peptide α-1 originated from globin α-chain has a high hydrophilicity and shows the properties of association and dissociation depending on concentration and temperature. The hypothetical gel formation mechanism of the globin hydrolysates was presented as follows: initially, the randomly coiled polymers were produced by about 8 molecules of peptide α-1; next, the cross-linked structures were constructed between the lengths of 130−140 nm rod-shaped aggregates; then the network was gradually formed; and the gel was finally formed.

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