Physicochemical Properties of Aggregates of Globin Hydrolysates

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, Amichi-chuo, Ibaraki 300-03, Japan

Globin is an edible protein that is obtained in large quantity from animal blood. It can be used as an ingredient in a variety of meat products. However, globin showed a rather low solubility at neutral pH and little advantage compared with other proteins used in the food industry. The effective use of latent materials is an important research area in food science. This study succeeds in improving globin's functional properties and explaining the mechanism of gel formation. The hydrolysis of globin was performed with 0.8 M citric acid. The globin hydrolysates showed eight bands by tricine−SDS−PAGE having molecular masses that ranged from 5000 to 15 000 Da. The results of gel filtration chromatography indicated that the large aggregates were formed easily in the case of the globin hydrolysates. Physicochemical properties of aggregates of globin hydrolysates were studied by light scattering measurement and transmission electron microscope. It is clear that the aggregates were composed of two kinds of polypeptides, one of which, the β-chain, originated from the native globin and another, β-1, originated from the β-chain by cleavage between 99 (Asp) and 100 (Pro) of the β-chain through noncovalent bonding. By comparison with the position of standard protein (thyroglobulin, MW 669 000 Da), the molecular mass of the aggregate was estimated as above 700 000 Da. The aggregates of globin hydrolysate in solution approximated the thin rod-shaped model and had lengths of 130−140 nm by light scattering measurement. Electron micrography also showed the aggregates to consist of the thin rod aggregates. The molecular mass of the aggregates was determined to be 870 000 Da by light scattering measurement, indicating that the aggregate consists of 33−34 units because each unit was in the ratio of 1:1 complex of β-chain and β-1 with molecular masses of 16 000 and 10 900 Da, respectively.

Keywords: *Globin; globin hydrolysates; aggregates; light scattering; tricine−SDS−PAGE*

INTRODUCTION

Animal blood contains about 18% protein, and hemoglobin accounts for more than half of the blood protein. Most of the hemoglobin is not ultilized in the food industry because of its disagreeable color and odor. Studies of the preparation methods of decolorized globin (Tybor et al., 1975; Sato et al., 1981; Drepper and Drepper, 1979), its nutritional value (Landmann et al., 1980; Parmer et al., 1978), and the functional properties of globin, such as solubility, emulsifying properties and foaming properties, have been made by several researchers (Tybor et al., 1973, 1975; DeVuono et al., 1979; Crenwelge et al., 1974). Attempts have been made to incorporate globin into sausage meat (Caldironi et al., 1982), and a cheese-like emulsion was prepared 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 CMC column procedure for the separation of heme and globin, and the globin prepared according to their method can form a gel when heated. Decolorized globin prepared by acidified acetone (Tybor et al., 1975) showed good water-

binding properties at room temperature, and it did not form a gel after heating. However, Liu et al. (1994a) have reported that the globin made according to this method can form a gel at high globin concentration (7%).

Gel from heat-induced globin prepared according to each of the above-mentioned methods showed a low hardness and was affected easily by the bulk properties such as pH and ions and did not show good functional properties in normal gel (Liu et al., 1994a; Autio et al., 1985, 1990). In our previous studies (Liu et al., 1994b), it was found that the hydrolysis of globin by citric acid had a high solubility, and the gel formation ability induced by heating globin hydrolysates was more excellent than that of intact globin and a different type of gel was formed compared with the case of intact globin. Our results also suggested that acid hydrolysis was an efficient method of modifying and improving the new functional properties of protein by precisely controlling the hydrolysis conditions (Liu et al., 1995b).

Some works have been done on gel formation and the structural properties of globin; for example, Hayakawa et al. (1983) reported that globin heated at 90 °C formed aggregates with a highly entangled fibrous structure with a thickness of 8−10 nm. Autio et al. (1990) described the globin as more unfolded and more flexible at low pH values after CD and fluorescence analysis.

In the present paper, we first examined the hydrolyzing process of intact globin by citric acid and then characterized the constituent of the soluble aggregates obtained by hydrolyzing globin with the methods of gel filtration chromatography, tricine−SDS−PAGE, and protein sequence analysis. Further, we determined molecular masses of aggregates and the size and shape of aggregates in solution by light scattering measurement.

---

* Author to whom correspondence should be addressed.
† National Food Research Institute.
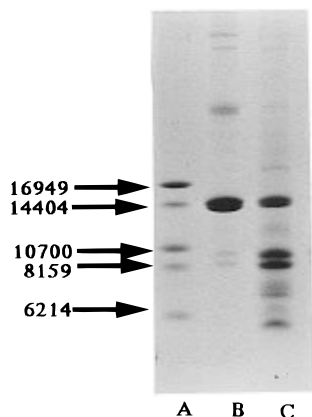‡ Ibaraki University.

2958 *J. Agric. Food Chem.,* Vol. 44, No. 10, 1996

Liu et al.

Figure 1. Tricine−SDS−PAGE patterns of globin and globin hydrolysates: (A) molecular mass markers; (B) globin; (C) globin hydrolyzates.

From these results we discuss the reasons why the gel formed by hydrolyzed globin was more excellent than intact globin.

MATERIALS AND METHODS

Materials. Globin was prepared from porcine blood cells according to the acidified acetone method (Tybor et al., 1975).

Globin Hydrolysates. After the hemoglobin was decolorized using the acid−acetone method, 0.8 M citric acid was added to the powdered globin sample to hydrolyze the globin at 95 °C for 15 min, which was then cooled to room temperature and dialyzed under flowing water with seamless cellulose tubing (pores size 10 000 Da). The dialysate was air-dried with a spray-dryer (Pulvis minispray GA-32, Yamato Science), and the sample thus obtained is the globin hydrolysate (GH) sample.

Gel Filtration. Gel filtration was carried out on a TSKgel TOYOPEARL HW-60S; column size was 40 cm × 1.6 cm. Elution was done at a flow rate of 12.5 mL/h at ambient temperature using 0.1 M citric acid buffer, pH 3.3, and the eluted protein was detected by an absorbance at 280 nm.

Light Scattering Measurements. Measurements of the intensity of the light scattering were done with a modified Ellipsometer, an automatic light scattering analyzer AEP-100 (Shimadzu Co., Ltd.), at 20 °C (Sano, 1988, 1990, 1993). The linearly polarized monochromatic incident light passed through the cylindrical scattering cell, and the light scattered at scattering angle θ was detected through the linear analyzer by a photomultiplier in a telescope arm that can rotate from 45° to 135° by a stepping motor.

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

Transmission Electron Microscopy. The aggregate solutions were diluted with 0.1 M citric acid buffer (pH 3.3), stained with 2% potassium phosphotungstate on a carbon-coated electron microscope grid, and observed with a Hitachi H-700H transmission electron microscope (Isoda et al., 1991).

RESULTS

Aggregate Formation of Hydrolyzed Globin. The hydrolysis of globin was performed with 0.8 M citric acid as described in our previous paper (Liu et al., 1994b). Figure 1 shows the tricine−SDS−PAGE patterns of globin and globin hydrolysates. The globin hydrolysates showed eight bands having molecular masses ranging from 5000 to 15 000 Da.

The self-assembling properties of globin hydrolysate were checked by gel filtration with TSKgel TOYO-PEARL HW-60S, using standard proteins of ribonuclease A (13 700 Da), bovine serum albumin (67 000 Da),
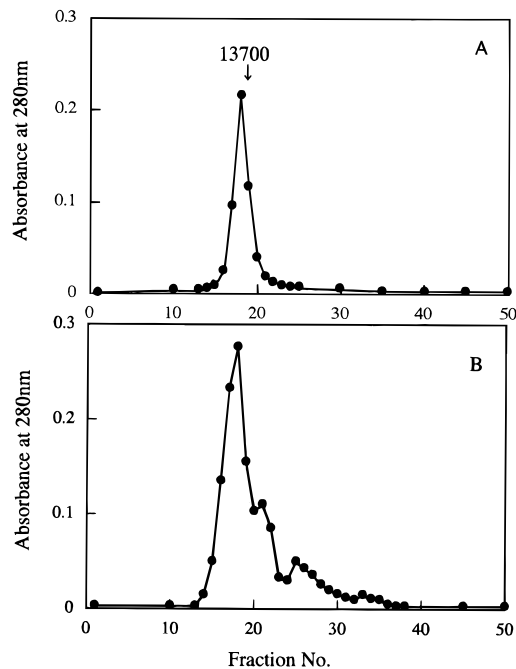

Figure 2. Gel filtration of globin and globin hydrolysates: (A) globin; (B) hydrolysates (immediately after globin was hydrolyzed). Standard protein: 13 700 ribonuclease A.
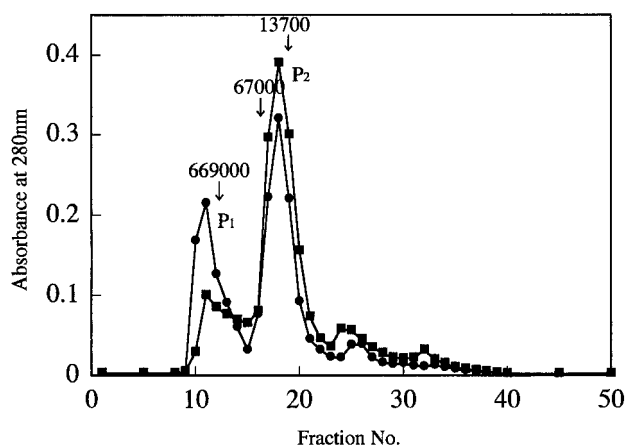

Figure 3. Gel filtration of globin hydrolysates. Standard proteins were ribonuclease A(13 700), bovine serum (67 000), and thyroglobulin (669 000). (●) The hydrolysates were allowed to stand for 24 h after globin was hydrolyzed. (■) The hydrolysates were allowed to stand for 10 h after globin was hydrolyzed.

and thyroglobulin (669 000 Da). In the case of intact globin, a sharp monomer peak was detected at fraction 18 (see Figure 2A). When gel filtration of the hydrolysates was done immediately after the globin was hydrolyzed, the position of the main peak was the same as in intact globin, except for the peaks of the lower molecular masses (Figure 2B). This is in accordance with the tricine−SDS−PAGE patterns. As shown in Figure 3, chromatography of the samples standing for 10 and 24 hr at 30 °C showed a new peak formed by self-assembly that grew with increasing standing time. On the contrary, the other peaks assigned to the lower molecular mass peptides became smaller with time. These results indicated that the large aggregates were formed easily in the case of the globin hydrolysate. From a comparison with the position of standard protein (thyroglobulin, 669 000 Da), the molecular mass of the aggregates was estimated as above 700 000 Da. On the other hand, when globin hydrolysates were treated with
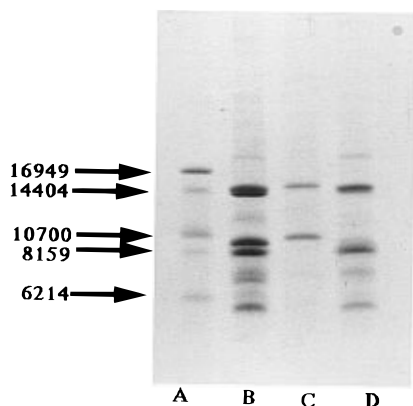
Physicochemical Properties of Globin Hydrolysates

J. Agric. Food Chem., Vol. 44, No. 10, 1996 **2959**

Figure 4. Tricine−SDS−PAGE patterns of each fraction from gel filtration: (A) standard proteins; (B) globin hydrolysates; (C) fraction P1 from gel filtration of globin hydrolysates; (D) fraction P2 from gel filtration of globin hydrolysates.
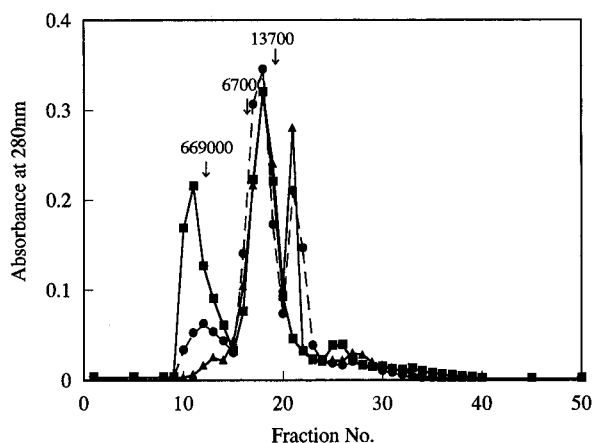

Figure 5. Gel filtration of the hydrolysates treated with 8 M urea and β-ME: (●) hydrolysates; (■) hydrolysates treated with 8 M urea; (▲) hydrolysates treated with 8 M urea + β-ME.

8 M urea, gel filtration, (Figure 5) showed that the aggregates became samller in the presence of 8 M urea. This result suggested that hydrophobic interaction among polypeptides was essential for the formation of aggregates.

To identify the constituent of the aggregates, the fraction of aggregates was analyzed with tricine−SDS−PAGE. As shown in Figure 4, it is clear that the aggregates were composed of two kinds of polypeptides, one of which was β-chain originated from intact globin and the other was peptide β-1 originated from β-chain of globin by cleavage between 99 (Asp) and 100 (Pro) by protein sequencer (Liu et al., 1995b), which suggested that the aggregates were formed from β-chain and β-1 peptide combined specifically through noncovalent bonding.

Light Scattering. Tertiary structure of the aggregates in solution was measured with a light scattering method (Sano et al., 1988, 1990, 1993). Figure 6 shows the light scattering pattern of the aggregates, in which KC/R_θ is plotted against $\sin^2(\theta/2)$. Here K is the optical constant, C the concentration, θ the scattering angle, and R_θ the reduced scattering intensity observed at the scattering angle θ. Considering the effect of the internal interference factor, we derived the equation

$$KC/R_\theta = 1/MP(\theta) + 2A_2c \qquad (1)$$

where $P(\theta)$ is the particle scattering factor, M the molecular mass, and A_2 the second virial coefficient.
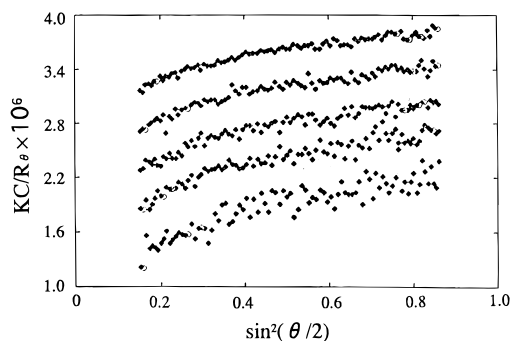

Figure 6. Angular dependence of light scattering for the aggregates of globin hydrolysates in 0.1 M citric acid buffer, pH 3.3.
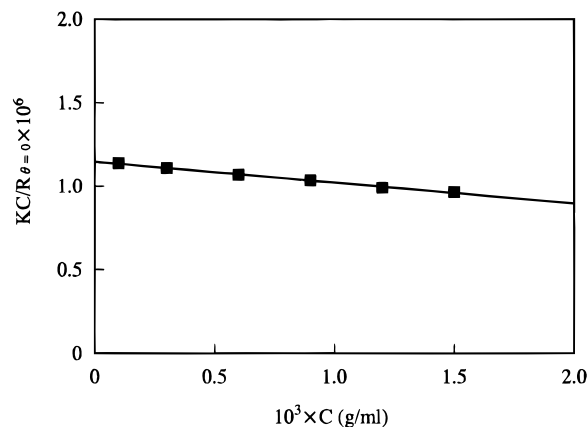

Figure 7. Concentration dependence of inverse scattering intensity extrapolated to $\theta = 0$ for the aggregates of globin hydrolysates in 0.1 M citric acid buffer, pH 3.3.

According to eq 1, a plot of $KC/R_{\theta \to 0}$ values extrapolated to $\theta \to 0$ against each C, as shown in Figure 7, gives molecular mass from the intercept of the ordinate and the second virial coefficient from the inclination of the straight line. The values obtained were $M_w = 872\,200$ Da and $A_2 = -6.075 \times 10^{-5}$ mL·mol/g.

The M_w obtained from gel filtration was more than 700 000 Da by comparison with the standard protein (thyroglobulin, 669 000 Da). The M_w obtained by light scattering method was in accordance with that obtained from gel filtration. Therefore, it was estimated that the aggregate was formed by 33−34 units, each unit being in the ratio of 1:1 complex of β-chain and β-1 of molecular masses of 16 000 Da and 10 900 Da, respectively.

The particle scattering factor $P(\theta)$ was determined experimentally by eq 1, and the results are shown with solid circles in Figure 6. The particle scattering factor was theoretically calculated for various kinds of models such as sphere, rod, and random coil. We tried many models to explain the agreement between scattering data and the theoretical values. The rod-shaped model was much more suitable in this case. For a system of long rods with the most probable molecular mass distribution, the theoretical scattering factor is given as

$$P(Q) = (2/QL_w) \arctan(QL_w/2) \qquad (2)$$

where L_w is the weight-averaged length of the rods and $Q = 4\pi n_0 \sin(\theta/2)/\lambda$. Theoretical values are shown in Figure 8 by changing the length of the rods from 100 to 250 nm. The experimental values were in good agreement with the theoretical values of $L_w = 130-140$ nm.
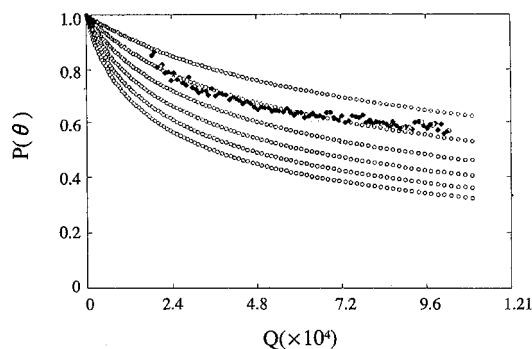
2960 *J. Agric. Food Chem.,* Vol. 44, No. 10, 1996

Liu et al.

Figure 8. Particle scattering factor $P(\theta)$ vs Q (nm^{-1}). Solid circles show the expermental data. The theoretical curve as thin rod-shaped model is shown with open circles, the length of which ranges from 100 to 250 nm; interval is 30 nm.

Figure 9. Transmission electron micrographs of the aggregates of globin hydrolysates with negative staining. The aggregates were dissolved in 0.1 M citric acid buffer, pH 3.3.

This suggests that the aggregates of globin hydrolysates in solution approximated the thin rod-like model of the length 130−140 nm.

The electron micrograph also showed that the aggregates consisted of the thin rod aggregates, as shown in Figure 9. This result is in accordance with the light scattering data.

DISCUSSION

In our previous studies, gel was formed by heating at 90 °C for 15 min in the solution of intact globin (7 mg/mL); the gel showed very low hardness (Liu et al., 1994a). To improve the functional properties of gel formed by globin, we hydrolyzed the globin with citric acid at 95 °C, which gave globin hydrolysates. The globin hydrolysates showed higher solubility and better gel formation properties than intact globin (Liu et al., 1994b). Under our experimental conditions, it was observed that six kinds of new peptides were produced (Figure 1) and each constituent content can be changed by controlling the experimental conditions.

The process of aggregate formation of globin hydrolysates was monitored by gel filtation (Figures 2 and 3). The quantity of the aggregates gradually increased with standing time. It was reported that larger aggregates can be produced by self-assembly of each aggregate when the globin gel formed, and new peaks tending to higher molecular mass would appear on gel filtration (Hayakawa et al., 1983; Koseki et al., 1989). However, in the case of globin hydrolysates the height of the aggregate peaks, that is, the quantity of the aggregates
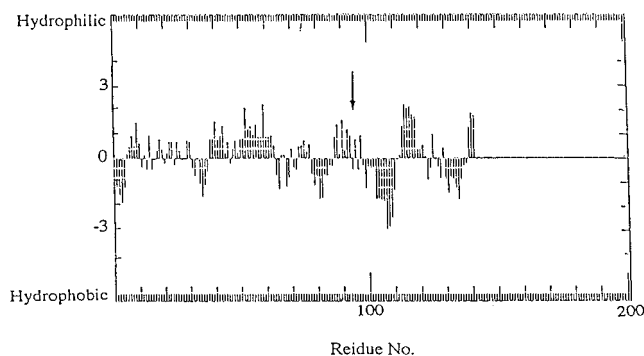

Figure 10. Hydrophilic and hydrophobic indices of intact globin β-chain calculated from amino acid sequence data according to the method of Kyte et al. (1982).

presented, changed. The change of the molecular mass of the aggregates has not been observed through the gel filtration method. The approximate molecular mass was above 700 000 Da by comparison with standard protein (thyroglobulin, 669 000 Da). These results indicated that the aggregates were produced by the interaction between constituents, but the aggregates formed did not tend to the higher molecular mass component in the case of globin hydrolysates.

On the other hand, the major forces involved in the aggregate formation through the interaction between constituents were cleaved by urea and β-mercaptoethanol (β-ME) treatment, as shown in Figure 5. The aggregate peak was decreased sharply by urea treatment; upon further treatment with β-ME, the peak disappeared and was not detectable. This result suggested that the noncovalent bonding may be important for the formation of aggregates through the interaction between constituents and that the disulfide bridge did not play an important role in the aggregate formation, because globin has only one thiol group per monomer and no disulfide bonds (Braunitzer et al., 1978; Hayakawa et al., 1983). It may be speculated that this thiol group contributes to the initial complex formation at the early stages.

The composition of the aggregates was analyzed by tricine−SDS−PAGE (Figure 4). It was found that the aggregates were formed by two kinds polypeptides, one of which was the β-chain originated from intact globin and the other was β-1 peptide originated from the β-chain of globin by cleavage between 99 (Asp) and 100 (Pro) using protein sequencer. The hydrophilic and hydrophobic indices of intact globin were calculated according to the method of Kyte et al. (1982) and are shown in Figure 10, where the arrow indicates the cleavage point (between 99 and 100). Intact β-chain of globin had high hydrophobic range between 105 and 146; the range was cleaved by hydrolysis to obtain β-1 peptide (from 1 to 99). These results suggested that the larger aggregates were formed by β-chain and β-1 peptide specifically combined. The molecular masses of β-chain and β-1 peptide were 16 000 and 10 900 Da, respectively. The molecular mass of the aggregates was determined as above 700 000 Da by gel filtration and as 870 000 Da by light scattering measurements, which indicated that the aggregates consist of about 33−34 units, and each unit is in the ratio of 1:1 complex of β-chain and β-1.

The thin rod-shaped aggregates were formed by β-chain and β-1 peptides by noncovalent bonding, whose length was 130−140 nm. Hayakawa et al. (1983) have reported that intact globin heated at 90 °C formed

fibrous "macro-aggregates" (higher aggregates in this paper), and these fibrous macro-aggregates formed a three-dimensional network leading to a gel. In our previous paper (Liu et al., 1994a) it was reported that the gels were very soft and sometimes took a sol state. In the present studies of globin hydrolysate, the higher aggregate formed by the combination of thin rod particles could not be observed. We found that the transformation process of thin rod-shaped aggregates to gel depended greatly on the presence of the hydrolysate fragment β-1 peptide. β-1 peptide was obtained by cleaving between 94 (Asp) and 95 (Pro) in β-chain of globin (Liu et al., 1995b), and it had a much higher hydrophobicity. In the presence of β-1 peptide, the size of the aggregates increased gradually with time. This result suggested that β-1 peptide plays a role as a bridge between the aggregate particles in the transformation process of the aggregates to gel. Therefore, it is considered that the thin rod aggregates did not tend to form highly entangled fibrous aggregates and only in the presence of β-1 peptide did they form a regular three-dimensional network leading to a gel; the gel formed became harder than intact globin gel (Liu et al., 1994b, 1995a), which suggested that the globin hydrolysate was useful to control gel strength.

Due to the good water-holding properties, investigation of using the globin gel to control the hardness (or softness) of sausage meat, ham, and traditional proteinaceous foods such as surimi-kamaboko may contribute to food processing.

ACKNOWLEDGMENT

LITERATURE CITED

Autio, K.; Lyytikainen, H.; Malkki, Y.; Kanko, S. Penetration studies of blood globin gels. *J. Food Sci.* **1985**, *50*, 615–617.

Autio, K.; Saito, M.; Kohyama, K.; Nishinari, K. Globin protein gelation: the effect of pH and temperature. *Food Hydrocolloids* **1990**, *4* (2), 87–93.

Braunitzer, G.; Schrank, B.; Stangl, A.; Scheithauer, U. Hemoglobins, XXI: Sequence analysis of porcine hemoglobin. *Hoppe-Seyler's Z. Physiol. Chem.* **1978**, *359*, 137–146.

Caldironi, H. A.; Ockerman, H. W. Incorporation of blood protein into sausage. *J. Food Sci.* **1982**, *47*, 405–408.

Crenwelge, D. D.; Dill, C. D.; Tybor, P. T.; Landmann, W. A. A comparison of the emulsification capacities of some protein concentrates. *J. Food Sci.* **1974**, *39*, 175–177.

De Vuono C. Penteado, M.; Lajolo, F. M.; Pereira dos Santos, N. Functional and nutritional properties of isolated bovine proteins. *J. Sci. Food Agric.* **1979**, *30*, 809–815.

Drepper, G.; Drepper, K. Verfahren zur herstellung neuer eiweiβprodukte aus schlachttierblut fur nahrungsmittel (A method of manufacturing new protein products from animal blood for use in food and feed). *Fleischwirtschaft* **1979**, *59*, 1252–1257.

Hayakawa, S.; Suzuki, Y.; Nakamura, R.; Sato,Y. Physicochemical characterization of heat-induced soluble aggregates of bovine globin. *Agric. Biol. Chem.* **1983**, *47* (2), 395–402.

Isoda, S.; Kita, Y.; Donkai, N.; Sano, Y.; Wegner, G. Dependence of DC-resistivity on morphology in polypyrroles. *Bull. Inst. Chem. Res. Kyoto Univ.* **1991**, *69*, 331–341.

Knapp, F. W.; Schmidt, R. H.; Mauldin, W. J.; Ahmed, E. M. Evaluating cheese-like emulsions from animal blood proteins and whey solids. *J. Food Prot.* **1978**, *41*, 257–258.

Koseki, T.; Fukuda, T.; Kitabatake, N.; Doi, E. Characterization of linear polymers induced by thermal denaturation of ovalbumin. *Food Hydrocolloids* **1989**, *3* (2), 135–148.

Kyte, J.; Doolittle, R. F. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **1982**, *157*, 105–132.

Landmann, W. A.; Dill, C. W.; Young, C. R. Nutritive value of globin-amino acid and complementary globin-cereal mixtures. *J. Nutr.* **1980**, *110*, 2254–2262.

Liu, X. Q. (Shinki Ryu); Yonekura, M.; Tsutsumi, M. Gel formation of globin prepared by acid-acetone method and globin hydrolyzates. *Nippon Shokuhin Kogyo Gakkaishi* **1994a**, *41* (3), 178–183 (in Japanese).

Liu, X. Q. (Shinki Ryu); Yonekura, M.; Tsutsumi, M. Gel properties of globin hydrolyzates. *Nippon Shokuhin Kogyo Gakkaishi* **1994b**, *41* (3), 196–201 (in Japanese).

Liu, X. Q. (Shinki Ryu); Yonekura, M.; Tsutsumi, M. Aggregates formation of globin hydrolyzates by their association. *Nippon Shokuhin Kogyo Gakkaishi* **1995a**, *42* (8), 562–568 (in Japanese).

Liu, X. Q. (Shinki Ryu); Yonekura, M.; Tsutsumi, M. Restrictive hydrolysis of globin by citric acid. *Nippon Shokuhin Kogyo Gakkaishi* **1995b**, in press (in Japanese).

Parmer, E. L.; Surak, J. G.; Knapp, F. W. Nutritional evaluation of porcine globin using tetrahymena pyriformis strain E. *J. Food Sci.* **1978**, *43*, 499–501.

Sano, Y. Optical anisotropy of bovine serum albumin. *J. Colloid Interface Sci.* **1988**, *124*, 403–406.

Sano, Y. Intrinsic optical anisotropy of southern bean mosaic virus. *J. Colloid Interface Sci.* **1990**, *139*, 14–19.

Sano, Y. Degree of linear polarization method for determination of intrinsic optical anisotropy of southern bean mosaic virus. *Biopolymers* **1993**, *33*, 69–74.

Sato, Y.; Hayakawa, S.; Hayakawa, M. Preparation of blood globin through carboxymethyl cellulose chromatography. *J. Food Technol.* **1981**, *16*, 81–91.

Tybor, P. T.; Dill, C. W.; Landmann, W. A. Effect of decolorization and lactose incorporation on the emulsification capacity of spray-dried blood protein concentrates. *J. Food Sci.* **1973**, *38*, 4–6.

Tybor, P. T.; Dill, C. W.; Landmann, W. A. Functional properties of proteins isolated from bovine blood by a continuous pilot process. *J. Food Sci.* **1975**, *40*, 155–159.