Gelation of Porcine Globin by Pepsin Treatment

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The effects of pepsin treatment on the gelation of porcine globin were studied by measurements of surface hydrophobicity, extent of hydrolysis, gel strength and polyacrylamide gel electrophoresis. Gel formation occurred below pH 4.0 at 30-50°C above 3% globin concentration. After 48-h incubation at pH 3.0 and 50°C in the pepsin concentration used (0.005-1.0% (E/S)), 0.01% (enzyme-substrate ratio: E/S) pepsin gave the highest gel strength. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed several peptides with molecular weight of 4000-6000 to be present in the resultant gel after 48 h of pepsin treatment. Gel strength after 12-h incubation was highly correlated with surface hydrophobicity and markedly elevated at 2-3% hydrolysis. As longer incubation time (over 12 h) is required for maximum gel strength, the gelation of globin would thus appear to occur as follows: pepsin yields peptides from globin, which aggregate to produce a three-dimensional gel network during incubation.

Keywords: globin, gelation, pepsin treatment, surface hydrophobicity, hydrolysis

Animal blood is rich in proteins, with hemoglobin accounting for more than half. Hemoglobin has limited appeal as food material because of its disagreeable color by heme. Acidified acetone effectively removes heme from hemoglobin to give globin with low solubility in an aqueous system. Nakamura et al. (1984) improved the functional properties of globin by enzymatic and chemical modification (1984). We reported succinylation (Miyaguchi et al., 1989) or hydrochloric hydrolysis (Miyaguchi et al., 1995) to be useful for enhancing the solubility, and the emulsifying and thermal gelling of globin. Globin forms a gel during pepsin treatment (Miyaguchi et al., 1996, 1997). Peptic globin digest was investigated for emulsifying capacity (Nakamura et al., 1984) and bitter peptide formation has been studied (Aubes-Dufau et al., 1989) or hydrochloric hydrolysis (Miyaguchi et al., 1995) to be useful for enhancing the solubility, and the emulsifying and thermal gelling of globin. Globin forms a gel during pepsin treatment (Miyaguchi et al., 1996, 1997). Peptic globin digest was investigated for emulsifying capacity (Nakamura et al., 1984) and bitter peptide formation has been studied (Aubes-Dufau et al., 1989, 1995), but the gelation of globin by pepsin treatment is poorly understood. To clarify the effect of pepsin treatment on the gelation of globin, gel-strength enhancement at various pHs, temperature, and pepsin and globin concentrations, the present study was conducted. The physicochemical properties of pepsic globin digest were also studied.

Material and Methods

Preparation of porcine globin Porcine red blood cells were diluted twice with water for hemolysis. Hemoglobin solution was obtained by centrifugation at 8000×g for 30 min. Globin was prepared from hemoglobin by the acid-acetone method of Tybor et al. (1975) slightly modified by Miyaguchi et al. (1989).

Pepsin treatment of globin Globin was suspended in water, and the pH was adjusted to 2.0-5.0 by 0.1 N HCl or NaOH. Water was then added to the solution to adjust to the final protein concentration (6%). To 3 ml globin solution in a glass tube (10×50 mm), pepsin dissolved in water (1:10,000, lot M25081, Nacalai Tesque Inc., Kyoto) was added at 0.005-1.0% (E/S) enzyme content. Peptic digestion was carried out at pH 2-5 and 20-60°C for 0.5-48 h. The rheological data of a gel was obtained based on the gel strength using a rheometer (NRM-2003J, Fudoh, Tokyo) equipped with a rheoplotter (FR801, Fudoh, Tokyo) as in the previous paper (Miyaguchi et al., 1997). To prepare samples for measurements of surface hydrophobicity and hydrolysis degree and for polyacrylamide gel electrophoresis analysis, aliquots (1 ml) withdrawn at given times during proteolysis were used.

Peptic globin digest characterization Surface hydrophobicity (SH) was determined according to Hayakawa and Nakai (1985), using cis-parinaric acid (CPA) as the fluorescence probe. The incubated globin with pepsin (the mixture) was heated at 100°C for 3 min to inactivate pepsin and diluted with water to various protein concentrations (0.005-0.02%). To 4 ml of the diluted solution, 20 μl CPA (3.6 mM in ethanol containing butylated hydroxytoluene) was added. Fluorescence intensity was measured with a fluorescence photometer (RF1500, Shimadzu, Kyoto). The initial slope was determined from a plot of the fluorescence intensity versus protein concentration and used as an index of the surface hydrophobicity of the peptic globin digest. The extent of hydrolysis was determined using trinitrobenzensulfonic acid (TNBS) as described by McKellar (1981). The mixture heated as previously described, was diluted to twice its volume with water. Ten microliters of the diluted solution was mixed with 2 ml 1 M potassium borate buffer (pH 9.4) and 1.0 ml 4 mM TNBS and incubated in the dark at 25°C. After 30 min, 1.0 ml 2 M monobasic sodium phosphate containing 18 mM sodium sulfate was added, and the absorbance at 420 nm was measured. L-Glycine was used to obtain the standard curve. The molar concentrations of free amino groups were expressed in glycine amino equivalents. The extent of the hydrolysis was expressed by dividing the concentration by that of the total acid hydrolysates of globin with 6 N HCl at 110°C for 24 h. Sodium dodecyl sulfate

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polyacrylamide gel electrophoresis (SDS-PAGE) was conducted as follows by the procedure of Ploug et al. (1989). One milliliter of the mixture was added to a 5 ml sample buffer that contained 12% glycerol, 2% SDS and 1% β-mercaptoethanol containing 0.01 M Tris-HCl buffer (pH 6.8). A 5-μl sample was loaded onto the top of the gel and electrophoresed at 50 V/gel for 12 h. The gel was stained overnight in 0.025% coomassie brilliant blue R-250, 10% acetic acid and 40% methanol and destained in 10% acetic acid with 30% methanol. Then molecular weight of the polypeptides was estimated using a molecular weights marker (MW Range 2512–16949, Pharmacia Biotech, Upssala, Sweden).

**Results**

*Effects of pepsin treatment on the gelation of globin*

Figure 1 shows the influence of pH on the gel strength of the globin after peptic proteolysis. Gelation occurred below pH 4.0. Gel strength increased with a reduction in pH. At pH 2.0, 12 h was required to attain maximum gel strength. The effects of incubation temperature on gel strength are shown in Fig. 2. Gelation was observed above 30°C. The gel strength increased up to 50°C. Maximum gel strength was noted at 6-h incubation at 50°C. Weak gels were formed at 60°C after 12-h incubation. Figure 3 shows the effects of pepsin concentration on gel strength. Gel was formed at all concentrations examined (0.005–1.0% (E/S)). At 0.01% pepsin, gel strength was

![Fig. 1. Effect of reaction pH on the gel strength of globin. This figure shows the effect of reaction pH on the gel strength of globin during peptic digestion. Gel strength means the breaking stress of gel. Data were obtained at 0.5, 1, 3, 6, 12, 24 and 48-h incubation. pH, temperature, pepsin concentration (pepsin-globin weight ratio) and globin concentration were proteolysis conditions. "Breaking stress" in this paper was expressed as a value analyzed with the rheoplotter. pH: ● 2.0, ○ 3.0, △ 4.0, ▲ 5.0. Temperature: 40°C, Pepsin conc.: 1.0% (E/S), Globin conc.: 6.0%.

![Fig. 2. Effect of reaction temperature on the gel strength of globin. This figure shows the effect of reaction temperature on the gel strength of globin during peptic digestion. Other terms are the same as in Fig. 1. Temperature (°C): ● 30, ○ 30, △ 40, ▲ 50, ■ 60, pH: 3.0, Globin concentration: 6.0%, Pepsin and globin conc.: See Fig. 1.

![Fig. 3. Effect of pepsin concentration on the gel strength of globin. This figure shows the effect of pepsin concentration on the gel strength of globin during peptic digestion. Other terms are the same as in Fig. 1. Pepsin conc. % (E/S): ○ 0.005, △ 0.01, ▲ 0.05, ■ 0.2, ▲ 1.0, pH: 3.0, Temperature: 50°C, Globin conc.: See Fig. 1.

![Fig. 4. SDS-PAGE pattern of globin digested with pepsin at various concentrations. This figure shows changes in the SDS-PAGE pattern of globin digested with pepsin at various concentrations. (0–1.0% (E/S)) at pH 3.0 and 50°C for 48 h. M: Molecular weight markers.
Fig. 5. Effect of globin concentration on the gel strength of globin. This figure shows the effect of globin concentration on its gel strength during pepsin digestion. Pepsin concentration: 0.01% (E/S). Other terms are the same as in Fig. 3.

Fig. 6. Relationship between surface hydrophobicity and gel strength of the peptic globin digest. This figure shows the relationship between surface hydrophobicity and gel strength of the peptic globin digest obtained at various incubation times and contents of pepsin. pH, temperature and globin and pepsin conc. were the same as in Fig. 3. Open and closed symbols mean 0.5-6 h and 12-48 h incubation, respectively. Pepsin concentration (● 0.005%, ▲ 0.01%, ◇ 0.05%, ◆ 0.2%, ▼ 1%). Gel strengths after 12-h incubation were adequately correlated to surface hydrophobicity.

Fig. 7. Relationship between the extent of hydrolysis and gel strength of the peptic globin digest. This figure shows the relationship between the extent of hydrolysis and gel strength of the peptic globin digest obtained at various incubation times and contents of pepsin. Symbols are the same as in Fig. 6. This figure shows gel strengths after 12-h incubation increase at 2-3% hydrolysis of globin.

Discussion

Some food proteins form gels/or curds by protease treatment as shown in casein coagulation by chymosin. Soy protein forms a curd by a protease (Fuke et al., 1985). β-Lactoglobulin has been shown to gel by a protease derived from a microorganism (Otte et al., 1996). The gelation of globin by pepsin treatment has been little studied and thus was examined in detail in the present work. Gelation at this temperature may be induced by heating rather than by pepsin. Gelation occurred at acidic pH where the pepsin would be activated and the globin made more soluble. This confirms that gelation occurs by the proteolysis of globin, and globin could form a strong gel with specified a concentration of pepsin, 0.01% (E/S). Though globin was digested by various concentrations of pepsin, 0-
1.0% (E/S), some peptides with molecular weights of 4000-6000 could still be found after 48-h incubation by SDS-PAGE analysis. This was also noted for hemoglobin, and the pepsin hydrolysis reached the end point at a certain level (Aubes-Dufau et al., 1995). Adler-Nissen (1986) explains this phenomenon as due to the formation of an inhibitory peptide during hydrolysis. Gel formation occurred above 3% globin concentration. Though the gelation of the globin was observed at 7% or greater protein concentration with heating, citric globin hydrolysate requires 3% for heat-induced gelation (Ryu et al., 1994). This result suggested that limited hydrolysis would increase the gel formability of the globin, but a pepsin-induced gel may not be the same as a thermal gel in the mechanism of gelation. Gel strength was adequately correlated with the surface hydrophobicity of the peptic globin hydrolysate, thus, hydrophobic interactions between molecules may be related to the gelation of the globin. After 12 h incubation, 0.01% (E/S) pepsin promoted the gelation of globin at which time a strong gel was observed at 2-3% hydrolysis. Gelation and gel-strength enhancement may occur as follows: pepsin yields peptides from globin, which may aggregate. This aggregate may then develop into a three-dimensional gel network. A certain incubation period of more than 12-h may be required for the gelation of the peptic globin digest. This suggests that heating (below 50°C) was used not only for proteolysis but also for protein denaturation during incubation. Hydrophobic bonding may be essential for the aggregation and gelation of the globin. This possibility is supported by the finding that the gelation of food protein is due to hydrophobic interactions between molecules besides disulfide bonding (Koseki et al., 1989; Kato et al., 1990). The gelation of the globin was shown in this study to depend on pepsin treatment conditions. Though the gel may have the globin peptides induced by pepsin with a molecular weight 4000-6000, characterization of these peptides and the gelation mechanism could not be clarified in this study. We are continuing our studies to obtain further knowledge on these points.

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