Polymerization of Meat and Soybean Proteins by Human Placental Calcium-Activated Factor XIII

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Placental $FXIII_a$ catalyzes the formation of ϵ-(γ-glutamyl)–lysine bonds within and between protein molecules. Therefore, this enzyme, activated by calcium, was used to cross-link myosin, actin and 7S globulin. High-concentration myosin and globulin solutions formed gels when they were treated by $FXIII_a$. The breaking strength of globulin gel is greater than that of myosin gel. Both gels were dissolved by various denaturants, and a SDS–PAGE experiment of the proteins, extracted by denaturants, shows that the gels were formed by species of high molecular weight and nonpolymerized proteins for globulin and only by high molecular weight species for myosin. These results indicate that gels are formed by covalent and noncovalent bonds. The covalent bonds are responsible for the formation of the species of high molecular weight, and the noncovalent ones are involved between these species.

INTRODUCTION

The demand for high-quality food proteins is increasing. To date, enzymatic modifications of functional proteins have involved mostly hydrolysis to improve the functional properties of proteins. Concerns about safety and nutritional effects have prevented the use of chemical modifications for improving functional properties of food proteins as functional ingredients. Therefore, the possibility of using nonhydrolytic enzymes to modify these properties under controlled conditions deserves systematic study. FXIII is a transglutaminase (EC 2.3.2.13) that occurs as a zymogen in plasma, placenta, and platelets (Bohn and Schwick, 1971). The reaction catalyzed by Ca^{2+}-dependent $FXIII_a$ involves the formation of a ϵ-(γ-glutamyl)–lysyl bond between an acyl donor (glutaminyl residue) and an acyl acceptor (lysyl residue). Therefore, this enzyme catalyzed conversion of soluble proteins to insoluble high molecular polymers through formation of covalent cross-links. Whitaker (1977) suggested that enzymatic cross-linking of proteins may be useful in texturization and in modification of solubility, foaming, whipping, and emulsifying properties.

Guinea pig liver transglutaminase was used to cross-link caseins, β-lactoglobulin, and soybean globulins (Ikura et al., 1980; Motoki and Nio, 1983; Aboumahmoud and Savello, 1990). Kahn and Cohen (1981) and Kurth and Rogers (1984) used bovine plasma transglutaminase activated by thrombin to cross-link meat proteins and Nonaka et al. (1989) a Ca^{2+}-independent transglutaminase derived from a microorganism to polymerize serum albumin and myosin. Placental FXIII, activated by Ca^{2+}, was used to cross-link caseins (Traoré and Meunier, 1991) and whey proteins (Traoré and Meunier, 1992). In some cases, gelation of proteins at high concentrations occurred (Nio et al., 1986).

This study investigated the conditions under which human placental $FXIII_a$ can effectively cross-link meat proteins and 7S soybean globulin to obtain either neoproteins or gels. We also report on the breaking strength of gels of globulin and on the solubility of globulin and myosin gels.
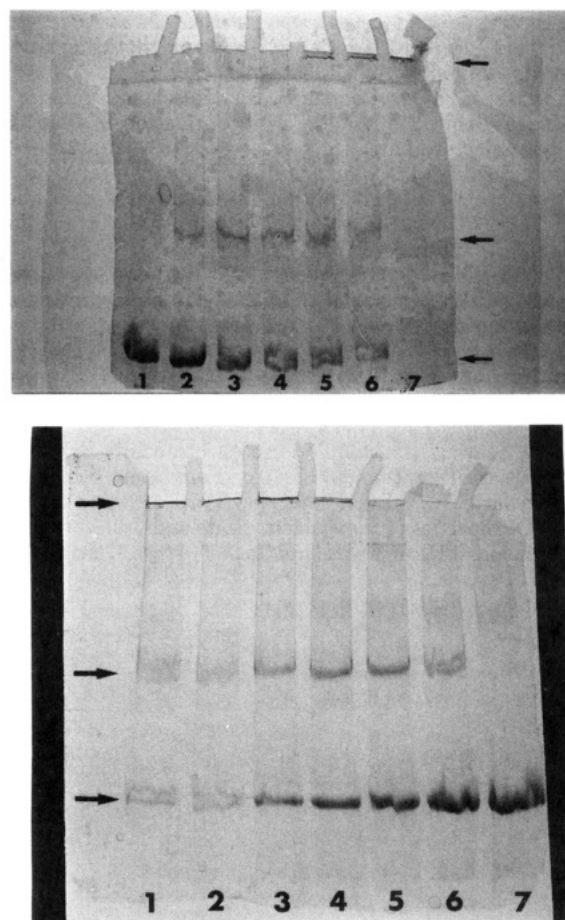

Figure 1. SDS–PAGE of myosin subjected to $FXIII_a$ activity. (A, top) Effect of time. (Lane 1) 3 mg/mL myosin, 5 mM $CaCl_2$; (lanes 2–7) myosin subjected to 200 nM $FXIII_a$ for 1, 2, 3, 4, 5, and 6 h, respectively. (B, bottom) Effect of $FXIII_a$ concentration. (Lanes 1–6) 3 mg/mL myosin subjected for 4 h to 400, 300, 200, 150, 100, and 30 nM $FXIII_a$; (lane 7) 3 mg/mL myosin, 5 mM $CaCl_2$.

MATERIALS AND METHODS

Materials. FXIII was purified from Fibrogammin according to the procedure of Traoré and Meunier (1991). Fibrogammin (Behringwerke, Marburg, FRG) was obtained from a local pharmacy. β-casein and actin were purchased from Sigma. 7S
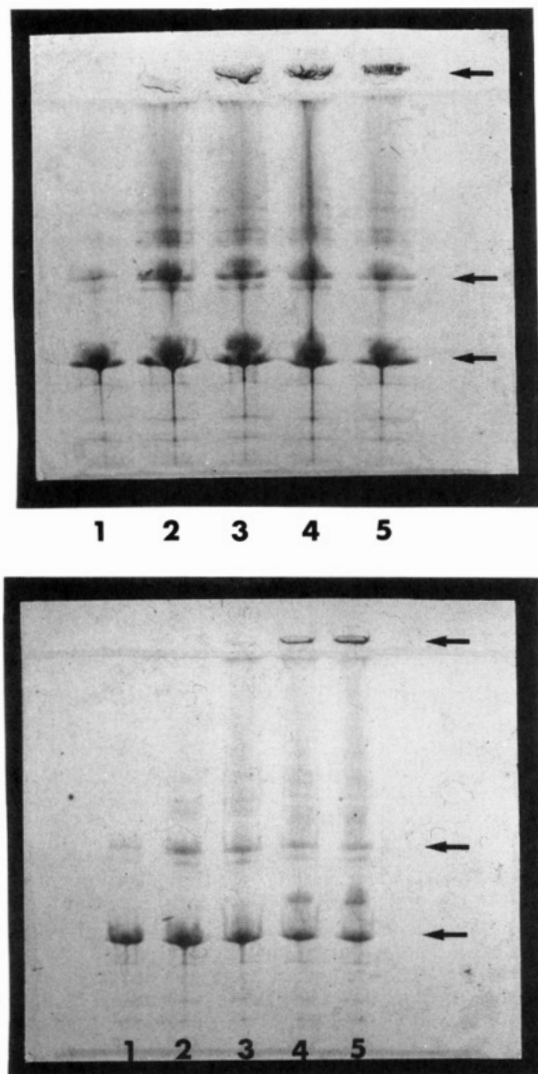
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Figure 2. SDS–PAGE of actin subjected to FXIII$_a$ activity. (A, top) Polymerization with respect to time. (Lane 1) 4 mg/mL actin, 5 mM CaCl$_2$; (lanes 2–5) actin subjected to 140 nM FXIII$_a$ for 10, 20, 40, and 60 min. (B, bottom) Polymerization with respect to FXIII$_a$ concentration. (Lane 1) 4 mg/mL actin, 5 mM CaCl$_2$; (lanes 2–5) actin subjected for 4 h to 50, 140, 230, and 340 nM FXIII$_a$.

globulin and myosin were purified according to the procedures of Thanh et al. (1975) and Perry (1955), respectively. All other reagents were of analytical grade.

Activation of FXIII. The activation was carried out by incubation of factor XIII in the presence of 140 mM CaCl$_2$, pH 7.6, at 37 °C for 10 min (Traoré and Meunier, 1991).

Kinetic Measurements. The transfer activity was measured by the formation of ammonia according to the procedure of De Backer-Royer et al. (1991).

FXIII concentration was determined following the method of Bradford (1976), with bovine serum albumin as standard.

The purity of enzyme was checked by PAGE (7.5% w/v) under denaturing conditions, according to the procedure of Laemmli (1970).

Self-Polymerization Experiments. The standard reaction mixture contained, in a total volume of 200 µL, 0.1 M Tris-HCl buffer (pH 7.5), 5 mM CaCl$_2$, and variable protein and FXIII$_a$ concentrations. In the case of myosin, the protein solution also contained 0.4 M KCl to avoid myosin precipitation. Incubation was performed at 37 °C for a variable time (indicated in the figure legends). The reaction was stopped by adding 0.4 M EDTA. Aliquots were then taken out, and SDS–PAGE experiments were carried out to detect the products polymerized through the intermolecular cross-linking catalyzed by FXIII$_a$.
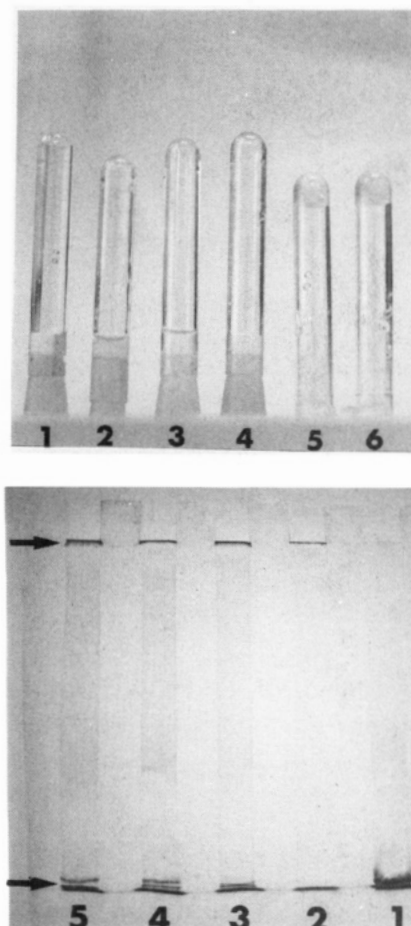

Figure 3. Gelation of myosin with respect to its concentration. (A, top) Gelation pattern. (Tubes 1 and 2) 5 mM CaCl$_2$, 10 and 40 mg/mL myosin, respectively; (tubes 3–6) 5 mM CaCl$_2$, 150 nM FXIII$_a$, and 10, 20, 30, or 40 mg/mL myosin, respectively. Incubation was done for 16 h, at pH 7 and 37 °C. (B, bottom) PAGE pattern. The content of tubes in (A) was dissolved in 50 mM or Tris-HCl buffer, pH 6.8, 2% SDS (w/v), and 5% 2-mercaptoethanol (v/v) and shaken for 1 h. The solution is therefore subjected to SDS–PAGE. (Lanes 1–5) Contents of tubes 2–6, respectively.

Polyacrylamide Gel Electrophoresis. Horizontal slab gels with polyacrylamide in the presence of 2% SDS were used for myosin cross-linking according to the procedure of Laemmli (1970). Migrations were conducted with a polyacrylamide concentration of 3%. The cross-linking of actin and globulin was analyzed by Phastsystem electrophoresis (Pharmacia) with PhastGel gradient 8–25 (continuous 8–25% gradient polyacrylamide gel) under denaturing conditions [2% SDS (w/v); 5% 2-mercaptoethanol (v/v)].

Gel Formation by FXIII$_a$. The protein solutions were prepared with 0.1 M Tris-HCl buffer containing 5 mM CaCl$_2$ with the concentrations and pHs indicated in the figure legends. Aliquots (200 µL) of the protein solutions were taken out and put into the small test tubes. FXIII$_a$ was added to these solutions at a concentration indicated in the figure legends, followed by incubation at 37 °C for variable time periods. Protein gel formation was confirmed by standing the test tubes on their heads.

Breaking Strength of Gels. Gels were formed in test tubes of 13-mm diameter by the method described previously. The breaking strength was measured by an Instron 1122 (Instron, Buc, France) and expressed as the strength, in Newtons, required to break the surface of the gel with a circular plunger (5-mm diameter) at a velocity of 12 cm/min at 22 °C.

Solubility of Gels in Denaturants. The myosin and globulin gels were formed in test tubes according to the method just described. They were diluted 10-fold (v/v) in either 100 mM Tris-HCl buffer, pH 7, 25 °C, 8 M urea, 6 M guanidinium
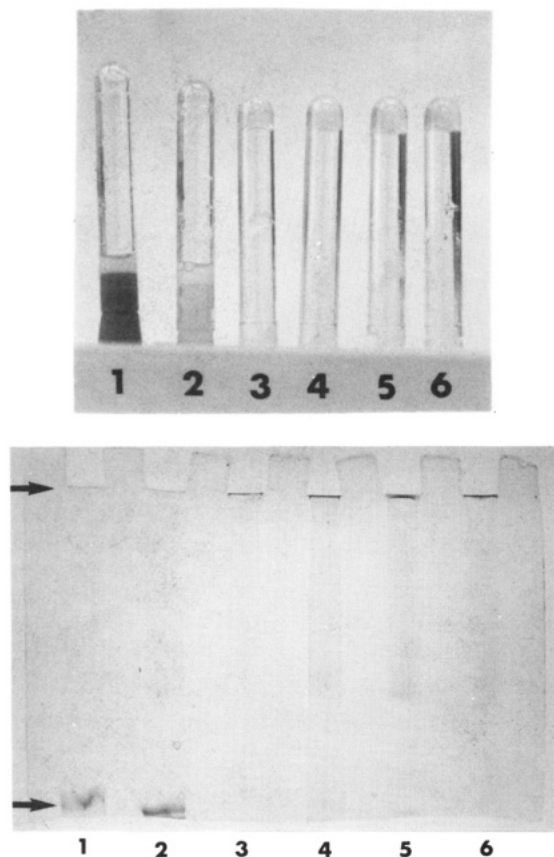
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Figure 4. Gelation of myosin with respect to pH. (A, top) Gelation pattern. (Tubes 1 and 2) 40 mg/mL myosin, 5 mM $CaCl_2$, pH 6 and 9, respectively; (tubes 3 and 4) 40 mg/mL myosin, 5 mM $CaCl_2$, 300 nM $FXIII_a$, pH 6 and 7, respectively; (tubes 5 and 6) 40 mg/mL myosin, 5 mM $CaCl_2$, 300 nM FXIIIa, pH 8 and 9, respectively. (B, bottom) PAGE pattern. The content of tubes was dissolved in 50 mM Tris-HCl buffer, pH 6.8, 2% SDS (w/v), and 5% 2-mercaptoethanol (v/v) and shaken for 1 h, and the solution was subjected to SDS–PAGE. (Lanes 1–6) Contents of tubes 1–6, respectively.

hydrochloride, or 2% (w/v) SDS. The mixtures were energetically shaken for various time periods. The concentrations of solubilized proteins were determined using the BCA method (Smith et al., 1985).

RESULTS AND DISCUSSION

Polymerization of Myosin and Actin. When myosin or actin was incubated in the presence of FXIII, activated by Ca^{2+}, cross-linking of the molecules occurred, leading to species of high molecular weight (Figures 1 and 2). The extent of polymerization of myosin was dependent on time (Figure 1A) and on enzyme concentration (Figure 1B). In contrast, actin did not polymerize completely (Figure 2). Indeed, after a while (~20 min), the polymerization was stopped (Figure 2A), and for a constant incubation time (4 h), the increase of enzyme concentration did not improve the polymerization process (Figure 2B). That was confirmed by an incubation of 4 h (data not shown; same pattern as lane 5, Figure 2A) and by a 2-fold increase in the enzyme concentration with respect to lane 5, Figure 2B (data not shown; same pattern as lane 5, Figure 2B). DTT was not a prerequisite for the cross-linking of both proteins. In its presence, the same pattern was obtained (data not shown); i.e., in the particular case of actin the polymerization was not improved. These results were not surprising since actin and myosin contain 5 and 18 sulfhydryl groups, respectively, but no cystine. The identity of the patterns, obtained either in the presence
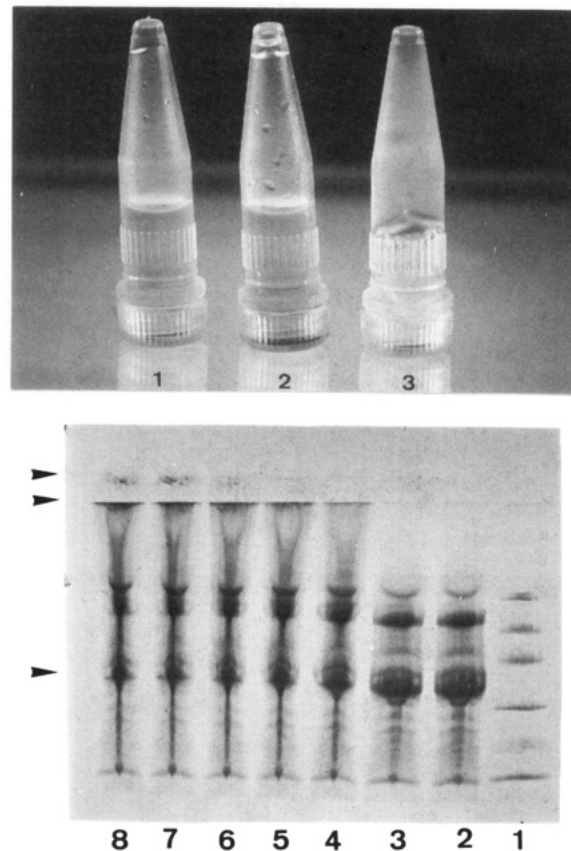

Figure 5. Gelation of globulins. (A, top) Gelation pattern. 7S globulins (60 mg/mL) were subjected to 10 mM $CaCl_2$ (tube 2) and 10 mM $CaCl_2$ and 333 nM $FXIII_a$ (tube 3). Photographs were made after an overnight incubation at 37 °C. (B, bottom) PAGE pattern. The content of tubes was dissolved in 50 mM Tris-HCl buffer, pH 6.8, 2% SDS (w/v), and 5% 2-mercaptoethanol (v/v) and shaken for 30 min. The solution was then subjected to SDS–PAGE. (Lane 1) Standard proteins are phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14.4 kDa); (lane 2) content of tube 3; (lanes 3–7) contents of tube 3, but after only 0, 30 min, or 1, 2, and 4 h of incubation, respectively; (lane 8) content of tube 3.

of DTT or in its absence, means that the polymers detected in the gel could not have been formed by disulfide bonds.

The fact that $FXIII_a$ polymerized partially actin, whereas microbial transglutaminase did not (Nonaka et al., 1989), is possibly due to a difference in the specificity of both enzymes toward glutaminyl residues. Indeed, the efficiency with which a protein can be utilized as a substrate by transglutaminase is known to be influenced by the amino acid sequence adjacent to the reactive glutaminyl residues (Gorman and Folk, 1980). The same explanation was also put forward to account for the difference of behavior of $FXIII_a$ and guinea pig liver transglutaminase with respect to caseins (Traoré and Meunier, 1991).

Gelation of Myosin. When myosin was incubated for 16 h at pH 7, 37 °C, in the presence of $FXIII_a$ (150 nM) and calcium, the solutions turned into gels. The gels retained the initial shapes even on inversion, as shown in Figure 3. However, the solutions did not gel, even after 16 h of incubation, unless $FXIII_a$ was added. The proteins extracted from gels formed by $FXIII_a$ were analyzed by SDS–PAGE (Figure 3B). The monomer fractions of protein diminished and polymers appeared. The gelation was well linked to the cross-linking of myosin molecules by $FXIII_a$. The gelation was observed at myosin concentrations greater than or equal to 30 mg/mL. The gelation

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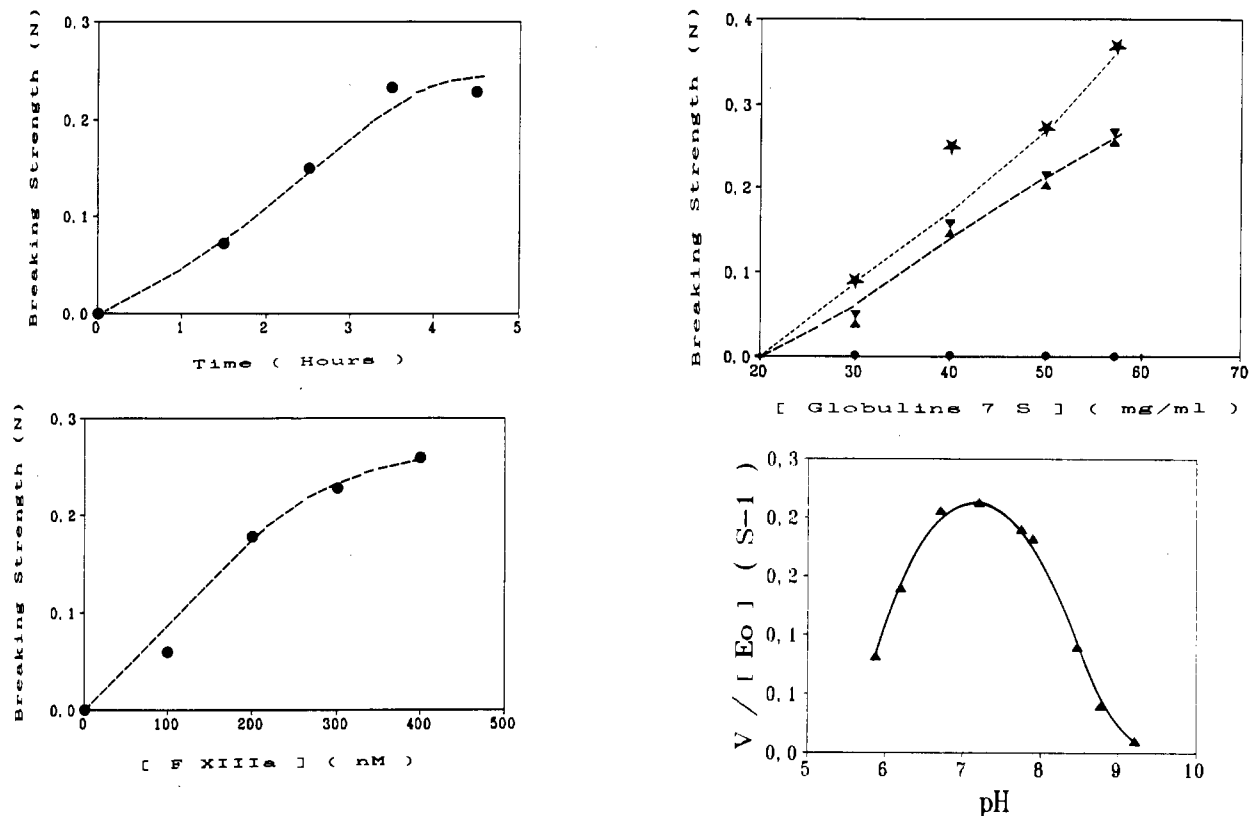*J. Agric. Food Chem.*, Vol. 40, No. 11, 1992 **2055**

Figure 6. Breaking strength of globulin gels. (A, top left) Breaking strength as a function of time. The gels were formed under the conditions indicated in Figure 5, lane 6, except for the incubation time. (B, bottom left) Breaking strength with respect to the FXIII$_a$ concentration. The gels were formed under the conditions indicated in Figure 5, lane 6, for a 4.30 min incubation time with variable FXIII$_a$ concentration. (C, top right) Breaking strength as a function of the globulin concentration at three pH values. The gels were formed under the conditions indicated in Figure 5, lane 6. (★) pH 6.04; (✗) pH 7.10; (●) pH 8.01. (D, bottom right) pH dependence of FXIII$_a$ with 7S globulin. Buffers were 0.1 M MES between pH 5.90 and 7.20, 0.1 M Tris-HCl between pH 7.75 and 8.80, and 0.1 M glycine at pH 9.20. The globulins and FXIII$_a$ concentrations were 0.86 mg/mL and 100 nM, respectively.

of myosin (40 mg/mL) by FXIII$_a$ (300 nM) was studied at four pH values (6, 7, 8, and 9). In all cases, myosin gelled (Figure 4A). The SDS–PAGE pattern of proteins extracted from gels shows that complete cross-linking of myosin occurred in any case (Figure 4). No significant differences in the cross-linking were observed with respect to pH. Unfortunately, since the gels were soft, it was not possible to study their breaking strength.

Gelation of 7S Globulin. A solution of 7S globulin (60 mg/mL) containing FXIII$_a$ and calcium turned into gel after an overnight incubation (Figure 5, tube 3). The simultaneous presence of Ca^{2+} and FXIII$_a$ was necessary to the gelation. This process was linked to the cross-linking of the globulin molecules (Figure 5B).

Since the protein solutions treated by FXIII$_a$ gave firm gels, the breaking strength of the gels was studied under different conditions. Figure 6 shows the time course of the breaking strength of a solution of globulin (60 mg/ mL) at pH 6.04. Within 5 h, it reached its maximum value. The value of the breaking strength of a gel, obtained after 4 h of incubation, depended on the FXIII$_a$ concentration, confirming the enzymatic nature of the gel (Figure 6B). As shown in Figure 6C, the gelation depended on the pH values. At pH 8.01, no gelation occurred. At pH 6.04, the breaking strength was a little bit greater than that at pH 7.10. This result is not consistent with the pH dependence of FXIII$_a$ (Figure 6D). Indeed, the enzyme is fully active at pH 8; however, gelation was not observed at this pH value. To explain this discrepency, one could put forward that neo-proteins, made at pH 8, did not gel because physicochemical conditions are not met to build the network (e.g., hydrogen bonds may be less numerous at

pH 8 than at pH 7). The pH dependence of the gelation of globulin is similar to that reported by Nio et al. (1986).

Under the experimental conditions of gelation of 7S globulin, β-casein did not gel. However, the mixture globulin-β-casein (50 mg/mL each) treated by FXIII$_a$ (300 nM) at pH 7.01 for 4 h at 37 °C turned into gel. While the breaking strength of globulin was 0.2 N, the breaking strength of the mixture gel was greater, 0.47 N. The addition of β-casein to globulin improved the gel firmness. On a breaking strength scale of food products, globulin and the mixture gels stand between yogurt and a cheese, like Camembert. The mixture myosin–globulin (35 mg/ mL each) gelled at pH 7 (incubation for 6 h at 37 °C). But like gelation of myosin, the gel was soft and no measure of the breaking strength was possible.

Solubility of Myosin and Globulin Gels in Denaturants. The solubility of myosin gel in several denaturants with respect to shaking time is shown in Figure 7. Within 90 min, in 6 M guanidinium hydrochloride and 2% SDS, the gels were entirely dissolved; 8 M urea is less efficient in this process (50% dissolution within 90 min). The solubilization of globulin gel in urea, SDS, or guanidinium hydrochloride was easier: within 5–10 min, the gel was completely dissolved (Figure 7B). The SDS–PAGE patterns of both gels, dissolved in 2% SDS, show a mixture of monomers and polymers (Figure 5B) for globulin and only high molecular weight species for myosin at 300 nM FXIII$_a$ (Figure 4B). It is concluded that these gels were formed by covalent and noncovalent bonds. The covalent bonds [ε-(γ-glutamyl)–lysine)] are responsible for the formation of species of high molecular weight. At a critical concentration (and at pH lower than 8 for

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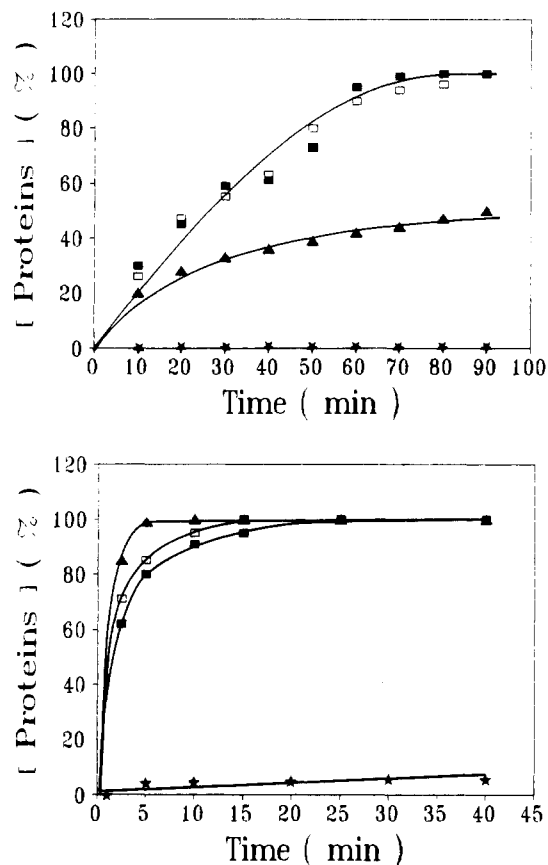
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Figure 7. Kinetics of dissolution of myosin and 7S globulin gels in several denaturants. (A, top) Myosin gel (tube 4, Figure 4, 200 μL) was diluted 10-fold in 100 mM Tris-HCl buffer, pH 7, 25 °C (★), in 8 M urea (▲), in 6 M guanidinium chloride (■) or in 2% SDS (□) and then vigorously shaken for various time periods. The concentration of solubilized proteins was determined according to the BCA method (Smith et al., 1985). The initial concentration in myosin is taken for 100%. (B, bottom) Globulin gel was treated under the same conditions as above. (★) Tris-HCl buffer; (▲) urea; (□) SDS; (■) guanidinium hydrochloride.

globulin), these species gelled (burying nonpolymerized monomers and species of low molecular weight in the case of globulin). The bonds between the aggregates are hydrogen and hydrophobic bonds. The denaturants cleaved the links and liberated the aggregates into the solution.

Our results are different from those of Nio et al. (1986). These authors found that the gels were not dissolved by all denaturants tested, but they studied the gelation of αS_1 casein by guinea pig liver transglutaminase and shook the gels only for 10 min; they decided solubility only by looking at the gels without performing protein dosage. So, they inferred that the αS_1-casein gels were formed by covalent bonds without involvement of noncovalent bonds. Had increased shaking time and a more sensitive method than visual inspection been used to detect solubilization of gels, dissolution may have been seen. On the other hand, it is difficult to reconcile the claim of the absence of solubilization of gel in denaturants (including SDS) and the SDS-PAGE pattern of gel proteins extracted by SDS (Figure 4, Nio et al., 1986).

In conclusion, investigations concerning the action of human placental $FXIII_a$ on myosin and globulin led to the

definition of experimental conditions of gel formation, without the requirement of a reductant.

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