Cross-Linking Activity of Placental F XIII$_a$ on Whey Proteins and Caseins

Fatoumata Traoré and Jean-Claude Meunier*

Laboratoire de Chimie Biologique, Institut National Agronomique Paris-Grignon, Centre de Biotechnologie Agro-Industrielle, 78850 Thiverval-Grignon, France

Human placental factor XIII (F XIII), purified from a commercial source and activated by calcium, was used to enzymatically cross-link individual whey proteins (α-lactalbumin, β-lactoglobulin, bovine serum albumin) and a mixture of total caseins and bovine serum albumin. Polymerization of DTT-reduced α-lactalbumin and β-lactoglobulin was incomplete. Saturation of the response was reached in the time range 2–3 h and the substrate concentration range 10–20 mg/mL. A heat treatment had no effect on the polymerization of α-lactalbumin by F XIII$_a$, whereas β-lactoglobulin gelled. A nonenzymatic gelation of both reduced proteins was observed above 50 mg/mL in the presence of calcium. Analysis of reaction products between BSA and caseins indicated that both proteins were polymerized, in the absence of DTT, through the formation of intermolecular cross-links. In contrast, polymerization of BSA required this reductant.

INTRODUCTION

F XIII 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 F XIII involves the formation of a $\epsilon(\gamma$-glutamyl)–lysyl bond between an acyl donor (glutamyl 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.

Chemical modification of proteins has been extensively explored to improve functional properties of food proteins as functional ingredients (Feeney and Whitaker, 1982), but concerns about safety and nutritional effects have prevented their adoption. The use of enzymes to manipulate the functional properties of proteins may circumvent concerns about safety. To date, enzymatic modification of functional properties of proteins has involved mostly hydrolysis. However, the possibility of using nonhydrolytic enzymes to improve or modify these properties under controlled and food grade conditions deserves systematic studies. Therefore, transglutaminase has potential useful applications for the manipulation of the functional and rheological properties of food proteins.

So far, cross-linking of α_{S_1}-casein, κ-casein, β-casein, β-lactoglobulin, and 11S and 7S soy globulins by guinea pig liver transglutaminase has been examined (Ikura et al., 1980; Motoki and Nio, 1983; Aboumahmoud and Savello (1990).

Recently, polymerization of caseins by placental F XIII activated by Ca^{2+} has been described (Traoré and Meunier, 1991). The goal of this paper is to report the cross-linking of α-lactalbumin, β-lactoglobulin, and bovine serum albumin (BSA) by placental F XIII$_a$ and also to describe a heteropolymerization between BSA and caseins.

MATERIALS AND METHODS

Materials. F XIII was purified from Fibrogammin according to the procedure of Traoré and Meunier (1991) including two chromatographic steps: a Blue-Sepharose CL-6B column and a phenyl-Sepharose CL-4B column. Fibrogammin (Behringwerke, Marburg, FRG) was obtained from a local pharmacy. α-Lactalbumin, β-lactoglobulin (a mixture of A and B species), caseins, and bovine serum albumin were purchased from Sigma. All other reagents were of analytical grade.

Activation of F XIII. The activation was carried out by incubation of factor XIII in the presence of 140 mM calcium (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. (1992).

The reaction was linear for 20 min at least with 6 nM F XIII$_a$. *One unit of enzymatic activity was defined as the amount of enzyme that catalyzed the formation of 1 nmol of product/min at 37 °C.*

The kinetic parameters (V and K_m) were calculated from the least-squares-fitted linear Lineweaver–Burk plots.

Protein concentration was determined according to 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 1 mL, 0.1 M Tris-HCl buffer (pH 7.6), 10 mM DTT, 5 mg/mL substrate protein, 5 mM $CaCl_2$, and 2.7 units of F XIII activated by Ca^{2+} (140 mM $CaCl_2$). Incubation was performed at 37 °C, and after 4 h, 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 F XIII$_a$.

The cross-linking between two kinds of proteins (caseins and BSA) was performed under the same experimental conditions.

Polyacrylamide Gel Electrophoresis. The cross-linkings were 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)].

RESULTS AND DISCUSSION

When α-lactalbumin or β-lactoglobulin (at 5 mg/mL) was incubated for 4 h in the presence of DTT and 2.7 units of F XIII activated by 140 mM Ca^{2+}, cross-linking of the molecules occurred, leading to species of high molecular weight (Figure 1). This polymerization was not complete, unlike that of caseins (Traoré and Meunier, 1991).

* To whom correspondence should be addressed.

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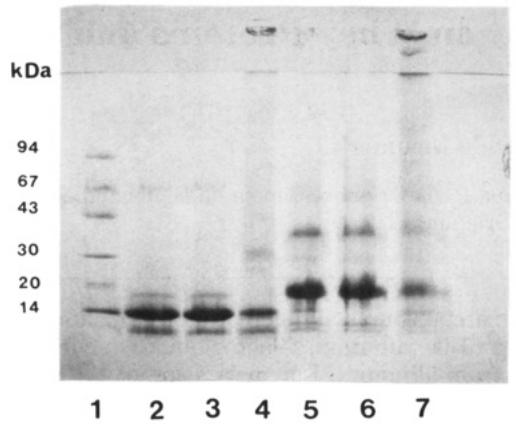

Figure 1. SDS–PAGE of β-lactoglobulin or α-lactalbumin subjected to F XIII$_a$ activity. (Lane 1) Standard proteins: phosphorylase *b* (94 kDa); bovine serum albumin (67 kDa); ovalbumin (43 kDa); carbonic anhydrase (30 kDa); soybean trypsin inhibitor (20 kDa); α-lactalbumin (14.4 kDa). (Lane 2) 5 mg/mL α-LA. (Lane 3) α-LA subjected to F XIII$_a$ without reductant (DTT). (Lane 4) α-LA subjected to F XIII$_a$ in the presence of 10 mM DTT. (Lane 5) 5 mg/mL β-LG. (Lane 6) β-LG subjected to F XIII$_a$ without reductant (DTT). (Lane 7) β-LG subjected to F XIII$_a$ in the presence of 10 mM DTT.

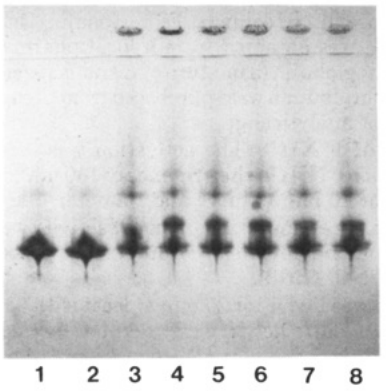

Figure 2. Influence of the cystein concentration on the cross-linking of β-lactoglobulin. (Lane 1) 10 mg/mL β-LG. (Lane 2) β-LG, 10 mM calcium. (Lanes 3–8) β-LG subjected to F XIII$_a$ in the presence of 10, 20, 30, 40, 60, and 80 mM of cysteine, respectively.

Increasing F XIII concentration (up to 800 nM) did not improve the degree of polymerization. DTT, in the standard incubation medium, could be replaced by cysteine or by cysteine and glutathione for α-lactalbumin or β-lactoglobulin, respectively. For instance, in Figure 2, we show the influence of the cysteine concentration on the polymerization of β-lactoglobulin. It appeared that a 20 mM cysteine concentration is sufficient to give the optimum polymerization, but when the reductant was omitted, no cross-linking occurred (Figure 1). By leading to a partial unfolding, the reductant may have enhanced cross-linking by unmasking lysyl and (or) reactive glutaminyl residues of both proteins. Instead, 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 glutamine residues (Gorman and Folk, 1980). The poor reactivity of α-LA and β-LG to F XIII$_a$ does not necessarily mean that a great lot of glutaminyl residues are buried in the molecule but that the reactive ones (i.e., those with the suitable amino acid sequence around them) are not accessible to the enzyme.

This poor reactivity is consistent with the low specific activity (0.44 and 1.80 s^{-1}) and the relatively high Michaelis constant values (0.13 and 0.25 mM) for α-LA and β-LG,

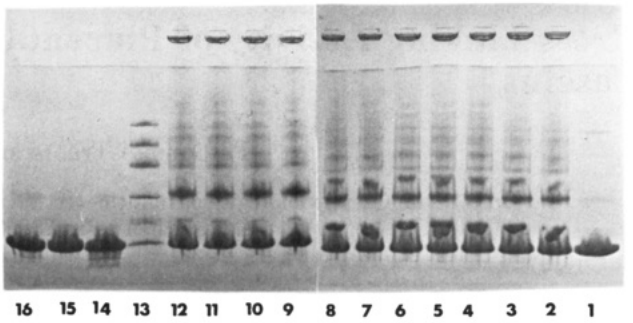

Figure 3. Influence of time on the polymerization of α-lactalbumin. (Lane 1) 5 mg/mL α-LA. (Lanes 2–12) α-LA subjected to 200 nM F XIII$_a$ in the presence of 40 mM cysteine for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 19 h, respectively. (Lane 13) Standard proteins. See legend to Figure 1. (Lane 14) α-LA, cysteine. (Lane 15) α-LA, calcium. (Lane 16) α-LA, cysteine, calcium.

respectively. It follows that the k_c/K_m values (the specificity constant) are lower than those for the different caseins, which appear to be better substrates to F XIII$_a$ (Traoré and Meunier, 1991).

To explain the incomplete polymerization of β-LG and α-LA, we have hypothesized that reductant unfolded proteins in the incubation medium, leading to a partial aggregation of the disordered molecules, with masking reactive residues. Therefore, only the molecules with unmasked glutaminyl residues cross-link to high molecular weight products. On the PAGE run, aggregates dissociate to β-LG and α-LA molecules, while the cross-linked molecules did not, obviously. These latter products were the results of F XIII action, since α-LA (or β-LG) plus the reductant (without F XIII$_a$) does not polymerize (data shown for α-LA, Figure 3, lane 15).

Heating of α-lactalbumin for 20 min at 85 °C, before treatment by F XIII$_a$, did not give rise to polymerization. Since β-lactoglobulin polymerized (below 40 mg/mL) or gelled (above 40 mg/mL) when heated (data not shown; Dalgleish, 1990), it was not possible to check whether heating can be used to replace the reductant; it is clear that the heat treatment did not replace the reductant in the polymerization process of α-lactalbumin, although the heat treatment must induce unfolding of the protein with an unmasking of reactive glutaminyl and lysyl residues. Unfolding also unmasks hydrophobic regions of protein, resulting in aggregation of molecules to minimize hydrophobic regions contacting water molecules. In this process, glutaminyl and lysyl residues would become again hidden.

α-Lactalbumin (5 mg/mL) polymerization was studied with respect to time, cysteine being the reductant. After an incubation of 2 h, the extent of polymerization was constant and a network of filaments appeared in the reaction tubes (Figure 3). The treatment of β-LG (10 mg/mL) by F XIII$_a$ in the presence of cysteine gave the same pattern: the polymerization was optimum after 2 or 3 h. When F XIII$_a$ was omitted from the incubation mixture containing either α-LA or β-LG plus cysteine and Ca^{2+}, no high molecular weight species were observed and, at the protein concentrations used (5–10 mg/mL), the mixture was cloudy. This beginning of precipitation was not due to the action of F XIII$_a$.

To increase the extent of polymerization and to obtain gelation of these proteins, their concentration in the standard mixture was increased during a 4-h incubation. When the concentration of β-lactoglobulin was increased (up to 100 mg/mL), we observed a protein precipitation due to calcium, when the reductant was omitted. In the presence of a reductant, polymerization occurred up to 25 mg/mL β-LG, without gelation (Figure 4, lanes 4 and 5);

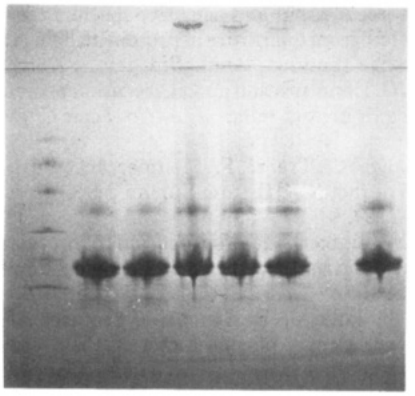

Figure 4. Effects of increasing β-LG concentration on its cross-linking. (Lane 1) Standard proteins. See legend to Figure 1. (Lane 2) 100 mg/mL β-LG. (Lane 3) β-LG, 10 mM calcium. (Lanes 4–7) 10, 25, 50, and 75 mg/mL β-LG, respectively, subjected to 2.7 units of F XIII$_a$ in the presence of 20 mM cysteine.

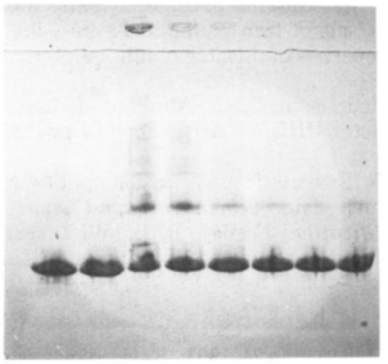

Figure 5. Influence of concentration of α-LA on its cross-linking. (Lane 1) 5 mg/mL α-LA. (Lane 2) α-LA, 10 mM calcium, 20 mM cysteine. (Lanes 3–8) 5, 10, 25, 50, 75, and 100 mg/mL α-LA, respectively, subjected to 2.7 units of F XIII$_a$ in the presence of 20 mM cysteine.

at 50 and 75 mg/mL β-LG, gelation was observed (Figure 4, lanes 6 and 7). We can see that the content of high molecular weight products decreased from lanes 4 to 7. At 50, and especially at 75 mg/mL of β-LG, gelation occurred without cross-linking. This was confirmed by the occurrence of gelation in a control which contained β-lactoglobulin (100 mg/mL), Ca^{2+}, and the reductant (DTT, e.g.). In another control, β-lactoglobulin and Ca^{2+}, no gelation was observed; instead, the protein precipitated. Increasing β-lactoglobulin concentration gave rise to a competition between enzymatic and nonenzymatic processes. The nonenzymatic gelation might involve the masking of the reactive glutaminyl residues. We believe that the formation of gels reported by Tamimoto and Kinsella (1988) was not the result of action of transglutaminase but, as in our study, was due to a nonenzymatic process. Reduced β-lactoglobulin gelled in the presence of calcium and in the absence of F XIII$_a$ at concentrations greater than or equal to 50 mg/mL. For α-lactalbumin, we observed the same behavior (Figure 5). However, instead of a complete gelation (like β-LG), we observed the formation of a network of filaments at an αLA concentration above 25 mg/mL. This network appeared also in the absence of F XIII$_a$.

BSA (at 5 mg/mL), treated by F XIII$_a$, polymerized only in the presence of DTT (Figure 6). With increasing concentration (up to 100 mg/mL), a gelation appeared with the occurrence of high molecular weight species

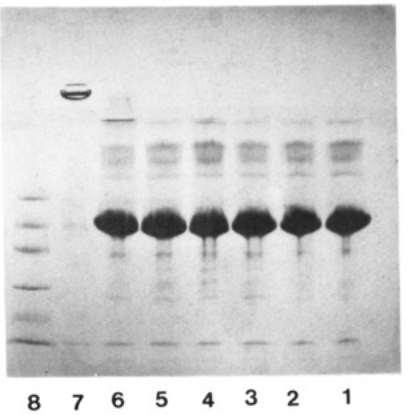

Figure 6. SDS–PAGE of bovine serum albumin subjected to F XIII$_a$ activity. (Lane 1) 5 mg/mL BSA. (Lane 2) BSA, 10 mM calcium. (Lane 3) BSA, calcium, 20 mM cysteine. (Lane 4) BSA, calcium, 10 mM DTT. (Lane 5) BSA, calcium, 2.7 units of F XIII$_a$. (Lane 6) BSA, calcium, 2.7 units of F XIII$_a$, 20 mM cysteine. (Lane 7) BSA, calcium, 2.7 units of F XIII$_a$, 10 mM DTT. (Lane 8) Standard proteins. See legend to Figure 1.

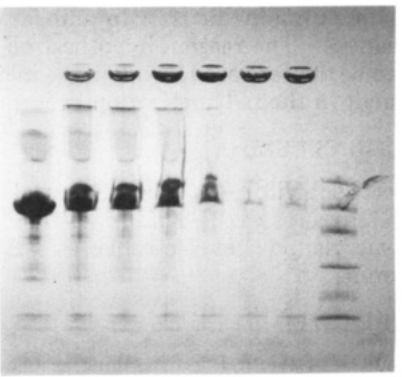

Figure 7. Effects of increasing BSA concentration on its polymerization. (Lane 1) 100 mg/mL BSA, 10 mM DTT. (Lanes 2–7) 100, 75, 50, 25, 10, and 5 mg/mL BSA, respectively, subjected to 2.7 units of F XIII$_a$ in the presence of 10 mM DTT. (Lane 8) Standard proteins. See legend to Figure 1.

(Figure 7). Since BSA (100 mg/mL) in the presence of Ca^{2+} and DTT (in the absence of F XIII$_a$) gelled, the gelation was not enzymatic. In contrast to β-LG and α-LA, the gelation was not concomitant to the masking of reactive residues, so that partial polymerization of BSA can occur.

Total casein is not known to be extensively polymerized by F XIII$_a$ (Traoré and Meunier, 1991). When casein was mixed with BSA, a heteropolymerization occurred. In Figure 8, it is clear that new polymers are made up when lane 7 is compared to lane 6, since in lane 7 the BSA stain had considerably decreased. DTT was not needed, while BSA alone did not polymerize in its absence. Cysteine did not replace DTT in the polymerization process of BSA (Figure 6). DTT, being a stronger reductant than cysteine, must have induced a more unfolded structure with the unmasking of both active glutaminyl and lysyl residues. The finding that BSA copolymerized with casein without DTT indicated that on the native BSA molecule there is (are) one or several reactive glutaminyl or lysyl residue(s) but not the simultaneous presence of reactive glutaminyl **and** lysyl residues.

A heat treatment (80 °C, 5 min) of BSA led to its polymerization (formation and dimer and tetramer). Pretreatment at 80 °C for 10 min, before treatment by F XIII$_a$ without DTT, gave rise to high molecular weight species in a very small amount. In the presence of a reductant (DTT), the extent of polymerization of BSA not heated

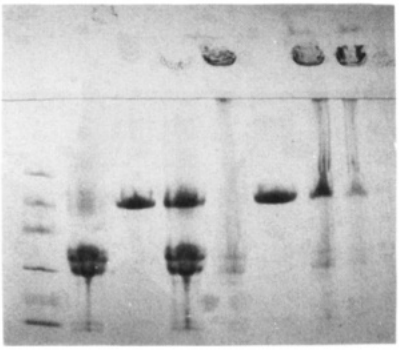

Figure 8. Cross-linking reaction between bovine serum albumin and caseins. (Lane 1) Standard proteins. See legend to Figure 1. (Lane 2) 5 mg/mL caseins. (Lane 3) 5 mg/mL BSA. (Lane 4) 5 mg/mL caseins, 5 mg/mL BSA. (Lane 5) Caseins subjected to F $XIII_a$. (Lane 6) BSA subjected to F $XIII_a$. (Lane 7) Caseins and BSA subjected to F $XIII_a$. (Lane 8) Caseins and BSA subjected to F $XIII_a$ in the presence of DTT.

was much higher. Thus, heating cannot replace the reductant in the unfolding of BSA to unmask active Gln and Lys residues. The reason, hypothesized for α-lactalbumin, can be put forward to explain the inefficiency of heat treatment in the polymerization process of BSA.

ABBREVIATIONS USED

β-LG, β-lactoglobulin; α-LA, α-lactalbumin; DTT, dithiothreitol; F XIII, factor XIII; F $XIII_a$, activated factor XIII; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin.

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