Cross-Linking of Caseins by Human Placental Factor XIII$_a$

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Human placental factor XIII, purified until of electrophoretic homogeneity from a commercial source by a simple procedure, was used to enzymatically cross-link all caseins. After 4 h of incubation at 37 °C, pH 7.6, in the presence of F XIII, whole casein and β- and κ-casein were most cross-linked. α-Casein was less sensitive to the action of F XIII$_a$. Increasing F XIII$_a$ concentration was enough to cross-link this protein. What seems important in the reactivity of caseins to F XIII$_a$ is not only the total content of glutaminyl and lysyl residues but also the specificity of F XIII$_a$ toward accessible glutaminyl residues. The difference of behavior of caseins, when subjected either to F XIII$_a$ or to guinea pig liver TGase, can be explained by a different specificity of both enzymes toward reactive glutaminyl residues. Thrombin in the activation of F XIII can be replaced by Ca^{2+}, and DTT was not required to cross-link caseins.

INTRODUCTION

The demand for high-quality food proteins is increasing. To date, with the exception of chymosin-induced coagulation of casein, enzymatic modification of functional proteins has 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.

The enzyme transglutaminase (protein-glutamine γ-glutamyl transferase, EC 2.3.2.13) is a Ca^{2+}-dependent enzyme responsible for posttranslational modifications of proteins. It allows the cross-linking of proteins like fibrin, collagen, and uteroglobulins (Bowness et al., 1987; Lynch et al., 1987). This is done by the formation of a ε(γ-glutamyl)-lysyl bond between an acyl donor (glutaminyl residue) and an acyl acceptor (lysyl residue).

Transglutaminase catalyzes conversion of soluble proteins to insoluble high molecular polymers through formation of covalent cross-links. It was suggested that enzymatic cross-linking of proteins could be employed to produce texturized food products (Whitaker, 1977). So far, cross-linking of α_{s1}-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 et al., 1983; Aboumahmoud and Savello, 1990).

Recently, polymerization of several proteins by Ca^{2+}-independent transglutaminase derived from microorganism has been described (Nonaka et al., 1989).

To our knowledge, F XIII, a transglutaminase that occurs as a zymogen in plasma, placenta, and platelets (Bohn and Schwick, 1971), has never been used to cross-link proteins. It is well-known that F XIII is inactive. It is activated by thrombin. The release of activation peptide, however, does not seem to be an absolute requirement for activation of zymogen. It has been demonstrated that at nonphysiologically high concentration (>100 mM) Ca^{2+} induces activation of plasma F XIII in the absence of any proteolysis (Credo et al., 1978). Recently, the same result was reported for the placental enzyme (Polgar et al., 1990).

This study investigated the conditions under which human placental F XIII can effectively cross-link milk proteins to obtain neoproteins. We also report on the purification of F XIII from a commercial preparation.

MATERIALS AND METHODS

Materials. Blue-Sepharose CL-6B and Phenyl-Sepharose CL-4B were purchased from Pharmacia (Uppsala, Sweden); fibrogammin was obtained from a local pharmacy. α-, β-, and κ-caseins, total casein, and thrombin (1000 NIH units/mg) were purchased from Sigma (St. Louis, MO). All other reagents were of analytical grade.

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

Activation of F XIII. The activation was carried out either by the action of thrombin or by incubation in the presence of calcium at high concentrations.

Activation by thrombin was performed by incubating enzyme (260 nM) with 160 NIH units/mL thrombin in a 100 mM Tris-HCl buffer, pH 7.5, at 37 °C for 10 min (De Backer-Royer et al., 1991). The activation by Ca^{2+} is described under Results.

Kinetic Measurements. The transfer activity was measured by the formation of ammonia. The measurement was carried out by coupling the F XIII reaction to the reaction catalyzed by glutamate dehydrogenase. The assay was performed in a 0.1 M Tris-HCl buffer, pH 7.8, containing 7 mM α-ketoglutarate, 0.4 mM NADH, 3 mM $CaCl_2$, 2 mM DDT, 10 mg/mL casein, and 0.2 mg/mL glutamate dehydrogenase.

The presence of DTT in the assay medium was not obligatory. It can be replaced by ascorbic acid, glutathione, or cysteine. The reaction rates were respectively equal to 91%, 95%, and 90% with respect to that in the presence of DTT. Without any reductant the reaction rate was still 85% with respect to DTT.

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

Protein concentration was determined according to the method of Bradford (1976), with bovine serum albumin (Sigma highest grade) as standard.

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

Self-Polymerization of Caseins. 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 24 nM F XIII activated by thrombin (8 units) or Ca^{2+} (140 mM). Incubation was performed at 37 °C, and after 4 h, the reaction was terminated by adding 0.4 M EDTA. The control

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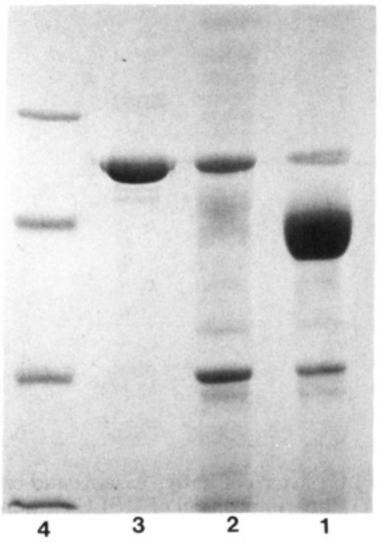


Figure 1. SDS–PAGE of purified human F XIII from fibrogammin (7.5%). (Lane 1) Fibrogammin after dialysis overnight; (lane 2) active fractions after Blue-Sepharose CL-6B column; (lane 3) active fractions after Phenyl-Sepharose CL-4B column; (lane 4) marker proteins, which include phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and carbonic anhydrase (30 kDa).

was prepared by omitting F XIII$_a$ in the mixture. The value of pH 7.6 corresponds to the optimum pH of the reaction catalyzed by F XIII$_a$ (De Backer-Royer and Meunier, 1991).

With the terminated reaction mixtures, aliquots were taken out; HPLC and SDS–PAGE experiments were carried out to detect the products polymerized through the intermolecular cross-linking catalyzed by F XIII$_a$.

Polyacrylamide Gel Electrophoresis. Horizontal slab gels with polyacrylamide in the presence of 1% SDS were used according to the method of Laemmli (1970). Test samples were immersed in boiling water before the experiment was performed. Migrations were conducted with polyacrylamide concentrations of 3% and 15% for the concentration and the separation gels, respectively.

HPLC Assay Method. Aliquots of reaction mixtures were dissolved in a 0.1 M phosphate buffer, pH 6.8, containing 0.1% SDS. The solution was then passed over a PAK 300 SW column at 0.3 mL/min. Typical elution profiles are shown in chromatograms.

RESULTS AND DISCUSSION

F XIII was purified from fibrogammin (Behringwerke, Marburg). In this commercial preparation, F XIII represents less than 5% (w/w) of the proteins. It is a "contaminant" of albumin. Fibrogammin was dissolved in a 20 mM Tris-HCl buffer, pH 7.5, containing 2 mM benzamidine, 0.1 mM DTT, and 2 mM EDTA and was dialyzed against the same buffer overnight to remove NaCl. Since albumin was bound to the aromatic anionic ligand (Cibacron blue F 3G-A) of Blue-Sepharose CL-6B by electrostatic and (or) hydrophobic interactions, we have used this dye to remove it from the fibrogammin preparation. F XIII being not bound to the Sepharose CL-6B column (1 × 20 cm), this step allowed a 5.5-fold purification of the enzyme. The active fractions were collected and submitted to a hydrophobic interaction chromatography over a Phenyl-Sepharose CL-4B (1 × 20 cm) column. Under the experimental conditions of equilibration of the column (Tris-HCl buffer) F XIII was bound to the gel by hydrophobic interactions. It was eluted by a linear gradient of ethylene glycol between 25% and 35%. The active fractions were collected, dialyzed against 20 mM Tris-HCl buffer, pH 7.5, 2 mM benzamidine, 0.1 mM DTT, and 2 mM EDTA, concentrated by ultrafiltration, and stored at −20 °C in 30% (v/v) glycerol. F XIII is homogeneous to SDS–PAGE (7.5% v/v) with a relative molecular mass of the subunit equal to 80 000 (Figure 1). The yield of the purification procedure is shown in Table I. It appears that F XIII, purified to homogeneity from this commercial preparation with a good yield (65%), is quite suitable to be used in cross-linking experiments.

Table I. Purification of Human Placental Factor XIII$_a$[a]

step	total protein, mg	total act., units	sp act., units/mg	purifn factor	yield, %
after dialysis	235.4	8476	36	1	100
Blue-Sepharose CL-6B	40.7	8075	198	5.5	95
Phenyl-Sepharose CL-4B	5.1	5465	1072	29.8	65

[a] All operations were carried out at 4 °C.

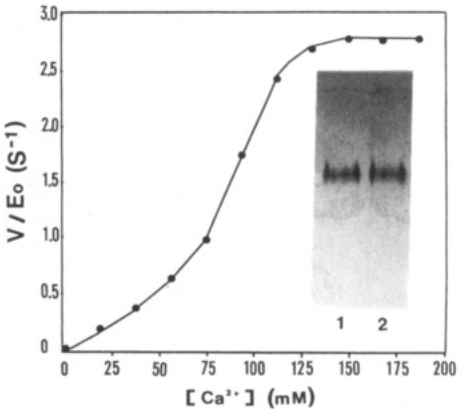


Figure 2. Activation of F XIII by calcium. F XIII (175 nM) was preincubated in a 100 mM Tris-HCl buffer, pH 7.6, at 37 °C, with varying Ca^{2+} concentrations, as indicated, for 10 min. The reaction rate was measured as described under Materials and Methods. The enzyme concentration during assay, E_0, was 6 nM. (Insert) Native PAGE of factor XIII, untreated (lane 1) and treated by 50 mM calcium (lane 2).

However, unlike guinea pig liver transglutaminase, which has been already employed to perform such a procedure with several food proteins, native F XIII is inactive. It has to be activated, either by thrombin or by calcium.

Inactive F XIII was incubated at pH 7.6 and 37 °C in the presence of Ca^{2+} at different concentrations for 10 min, and activity was assayed over aliquots. Results shown in Figure 2 indicate a sigmoidal dependence of activity on Ca^{2+} concentration. The maximum of activity was reached for about 140 mM Ca^{2+}. The time dependence of activation of F XIII by 150 mM Ca^{2+} indicates that the activity reached a plateau within 10 min and that the loss of activity at 37 °C was weak (data not shown). The pattern of a PAGE experiment of F XIII activated by Ca^{2+} shows that the mobility of Ca^{2+}-treated enzyme was not distinctly different from that of the nonactivated enzyme (Figure 2, insert). This result excludes that this activation was due to an unidentified protease active only at high Ca^{2+} concentration and present in our preparations in an amount undetectable on SDS–PAGE. It should be noted that Ca^{2+} behaved as an allosteric activator. Under the same conditions of assay, Ca^{2+}-treated F XIII and enzyme activated by thrombin exhibited the same specific activity, k_c (Table II).

Routinely, the Ca^{2+} activation of F XIII was conducted by incubating enzyme at 37 °C, pH 7.6, in the presence of 140 mM Ca^{2+} for 10 min.

Specificity of F XIII Activated by Thrombin or by Ca^{2+}. The specificity constant, k_c/K_m, of the reaction

Table II. Specificity of Factor XIII Activated either by Thrombin or by Ca^{2+}

protein	k_c, s^{-1}	K_m, mM	$k_c/K_m \times 10^{-3}$, s^{-1} M^{-1}
α-casein[a]	1.61	0.10	16
α-casein[b]	3.30	0.22	15
β-casein[a]	6.06	0.034	178
β-casein[b]	12.00	0.063	190
κ-casein[a]	4	0.05	80
κ-casein[b]	8.9	0.10	89
total casein[a]	5	0.13	38
total casein[b]	5.26	0.14	37.50

[a] Specificity with Ca^{2+}-treated factor XIII. [b] Specificity with factor XIII activated by thrombin.

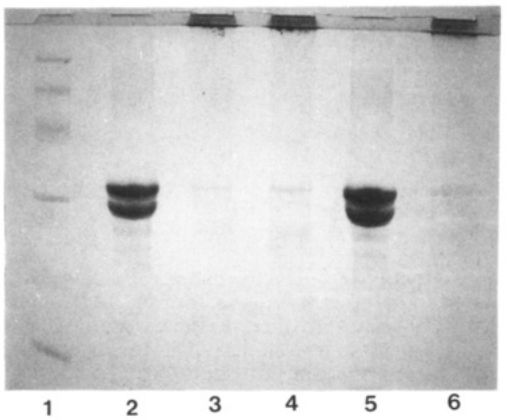


Figure 3. SDS–PAGE of caseins subjected to F $XIII_a$ activity. (Lane 1) Standard proteins are phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14.4 kDa); (lane 2) caseins; (lane 3) caseins subjected to 24 nM F XIII activated by 8 units of thrombin; (lane 4) caseins subjected to F XIII activated by 140 mM Ca^{2+}; (lane 5) caseins heated for 25 min at 50 °C; (lane 6) caseins denatured by heating and subjected to F XIII activated by 140 mM Ca^{2+}.

catalyzed by activated F XIII was calculated for α-, β-, and κ-caseins and total caseins. In each case, the substrate behaved as an acyl donor and acyl acceptor. The results are given in Table II. In all cases, the kinetics were Michaelian. For total caseins, there is no significant difference between the kinetic parameters whatever the procedure of activation (either thrombin or Ca^{2+}). For α-, β-, and κ-caseins, the k_c and K_m values for the reaction catalyzed by F XIII activated by thrombin were 2-fold higher and lower, respectively, than those concerning the reaction catalyzed by Ca^{2+}-activated F XIII, so that the k_c/K_m values were of the same order of magnitude. This means that Ca^{2+}-activated F XIII can be used instead of the enzyme treated by thrombin. That is what was done.

Self-Polymerization of Caseins. When whole caseins were incubated in the presence of F XIII, activated either by thrombin or by Ca^{2+}, cross-linking of the molecules occurred, leading to species of high molecular weight (Figure 3). Heating of caseins at 50 °C for 25 min before incubation with F XIII did not modify the pattern (Figure 3). The same results were obtained at 65 °C for 30 min (data not shown). With another enzyme (transglutaminase from guinea pig liver), heat treatment of dairy proteins reduced cross-linking of whey proteins (Aboumahmoud and Savello, 1990).

The products of the enzymatic reaction were analyzed by HPLC. HPLC elution patterns for untreated casein and thrombin-activated, F XIII treated casein (Figure 4A) and for untreated casein and Ca^{2+}-activated, F XIII treated casein were superimposed (Figure 4B). It is evident that the major quantity of casein was cross-linked. Superimposition of HPLC patterns for thrombin- and Ca^{2+}-activated enzyme shows that the degree of cross-linking was independent on the activation procedure (Figure 4C). The heating of caseins did not improve or prevent the cross-linking (Figure 4D).

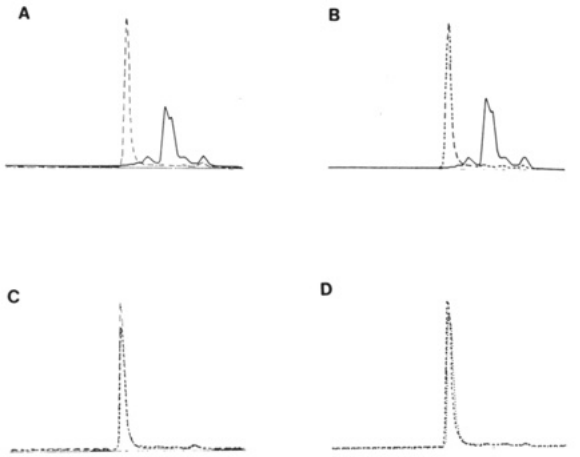


Figure 4. HPLC pattern of native caseins and caseins treated by F $XIII_a$. (A) Superimposition of HPLC elution patterns for untreated casein (—) and thrombin-activated, F XIII treated casein (- - -); (B) superimposition of HPLC elution patterns for untreated casein (—) and Ca^{2+}-activated, F XIII treated casein (–•–); (C) superimposition of HPLC elution patterns for Ca^{2+}-activated (–••–) and thrombin-activated (- - -) F XIII treated casein; (D) superimposition of HPLC elution patterns for caseins subjected to F XIII activated by Ca^{2+}, before (- - -) and after (···) treatment at 50 °C for 25 min.

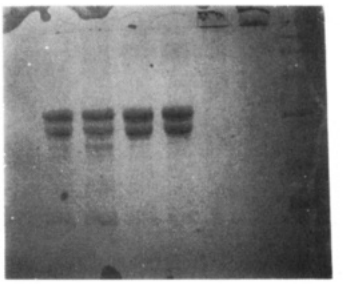
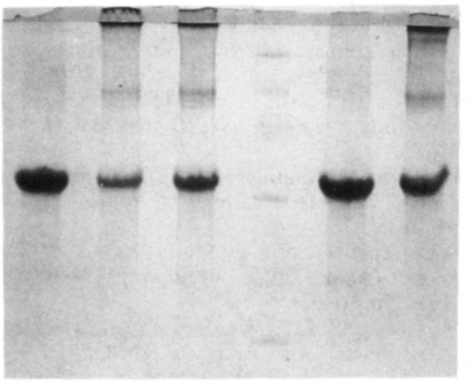


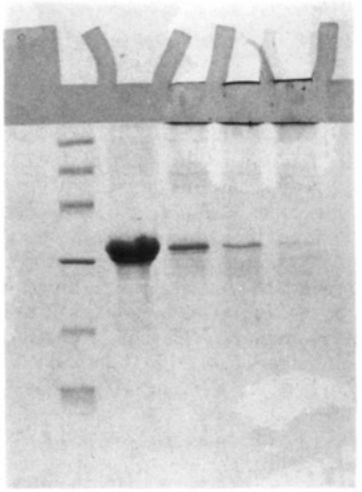
Figure 5. Comparison of the SDS-PAGE patterns of caseins subjected to F $XIII_a$ activity in the presence and in the absence of reductant. (Lane 1) Native caseins; (lane 2) native caseins plus 5 mM Ca^{2+}; (lane 3) native caseins plus 10 mM DDT; (lane 4) native caseins plus 10 mM DTT plus 5 mM Ca^{2+}; (lane 5) caseins subjected to 24 nM F XIII (activated by 140 mM Ca^{2+}) in the presence of 5 mM Ca^{2+}; (lane 6) caseins subjected to 24 nM F XIII (activated by 140 mM Ca^{2+}) in the presence of 10 mM DTT and 5 mM Ca^{2+}; (lane 7) standard proteins, see legend to Figure 3.

When DTT was omitted from the incubation medium (Figure 5), cross-linking always occurred. In contrast, the presence of DTT was necessary to cross-link caseins and whey proteins by guinea pig liver transglutaminase (Ikura et al., 1980; Tanimoto and Kinsella, 1988; Aboumahmoud and Savello, 1990). A conformational change of these proteins, caused by reduction of disulfide bonds, was needed for them to be substrates for polymerization. We interpret the absence of requirement of DTT in the polymerization of caseins by placental F $XIII_a$ by a different specificity of this enzyme, with respect to TGase, toward the glutaminyl residues. The efficiency with which a protein can be utilized as a substrate by transglutaminase is known to be influenced by the amino acid sequence around the reactive glutaminyl residues (Gorman and Folk, 1980). Thus, the primary structure of a protein is of greater



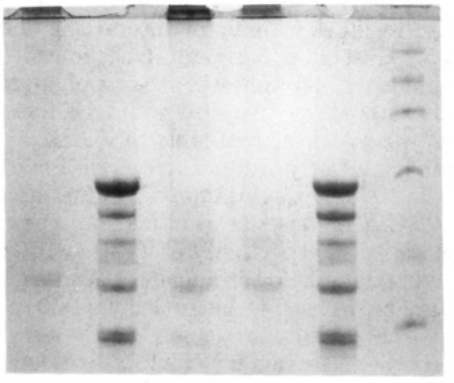
1 2 3 4 5 6

Figure 6. SDS–PAGE of α-casein subjected to F XIII$_a$ activity. (Lane 1) α-Casein; (lanes 2 and 3) α-casein subjected to 24 nM F XIII activated either by thrombin or by Ca^{2+}, respectively; (lane 4) standard proteins, see legend to Figure 3; (lane 5) α-casein heated for 25 min at 50 °C; (lane 6) heated α-casein treated with F XIII activated by Ca^{2+}.



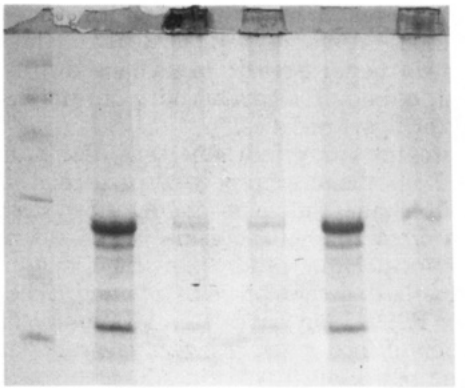
1 2 3 4 5

Figure 7. SDS–PAGE of α-casein subjected to F XIII$_a$ activity: effect of F XIII$_a$ concentration. (Lane 1) Standard proteins, see legend to Figure 3; (lane 2) α-casein; (lane 3) α-casein subjected to 24 nM F XIII activated by Ca^{2+}; (lane 4) α-casein subjected to 48 nM F XIII activated by Ca^{2+}; (lane 5) α-casein subjected to 96 nM F XIII activated by Ca^{2+}.



6 5 4 3 2 1

Figure 8. SDS–PAGE of β-casein subjected to F XIII$_a$ activity. (Lane 1) Standard proteins, see legend to Figure 3; (lane 2) β-casein; (lane 3) β-casein subjected to 24 nM F XIII activated by thrombin; (lane 4) β-casein subjected to F XIII activated by Ca^{2+}; (lane 5) β-casein heated at 50 °C for 25 min; (lane 6) heated β-casein subjected to F XIII activated by Ca^{2+}.



1 2 3 4 5 6

Figure 9. SDS–PAGE of κ-casein subjected to F XIII$_a$ activity. (Lane 1) Standard proteins, see legend to Figure 3; (lane 2) κ-casein; (lane 3) κ-casein subjected to 24 nM F XIII activated by thrombin; (lane 4) κ-casein subjected to F XIII activated by Ca^{2+}; (lane 5) κ-casein heated for 25 min at 50 °C; (lane 6) heated κ-casein subjected to F XIII activated by Ca^{2+}.

importance in assessing its ability to act as a substrate than its absolute lysine and glutamine content. It is obvious that the possibility to cross-link proteins in the absence of DTT is an advantage in food science.

In Figures 6–9, we present the SDS-PAGE patterns of the products of the cross-linking of α-, β-, and κ-caseins, respectively. All of these proteins were able to polymerize, whatever the procedure of activation used (thrombin or calcium). κ- and β-caseins were more susceptible to F XIII cross-linking than α-casein, since α-casein completely polymerized at a higher F XIII$_a$ concentration (Figure 7). α-Casein polymerized with a greater extent when it was mixed with other caseins in the whole casein (Figure 3). β- and κ-caseins completely polymerized when the F XIII$_a$ concentration was increased twice with respect to the concentration used in Figures 8 and 9 (data not shown).

To explain the lesser reactivity of α-casein, two hypotheses are possible. At first, the k_c/K_m values and the extent of polymerization were well correlated to the Q/K ratio (Gln/Lys) values of casein: 0.8, 1.7, and 2.1 for α-, κ-, and β-caseins, respectively. The higher is the content of glutaminyl residues with respect to lysyl residues, the higher would be the reactivity of caseins. But such a hypothesis cannot explain why the specificity of guinea pig liver TGase over caseins was different from that of F XIII$_a$. Indeed, the lesser reactivity was observed with κ-casein (Ikura et al., 1980), α- and β-caseins being fully active. As told above, a different specificity of both enzymes toward reactive glutaminyl residues might explain this observation. The lesser reactivity to F XIII$_a$ of α-casein, with respect to β- and κ-caseins, may be explained by a combination of these two factors: the absolute lysyl and glutaminyl content and the specificity toward accessible glutaminyl residues. Native α-casein is known to form a soluble polymer by self-association (Payens and Vreeman, 1982). This fact might also explain the lesser reactivity of α-casein, if reactive glutaminyl and lysyl residues are buried inside the polymer. Since the polymerization was not improved when the reaction was carried out in the presence of 0.005% SDS (to compensate the slow inactivation of F XIII$_a$ by SDS, aliquots of enzyme were added at suitable time intervals) and since an increase in the F XIII$_a$ concentration is sufficient to improve it, this explanation can be rejected.

When α-casein is in the mixture of caseins, it must keep its disordered structure (Herskovitz, 1960) with reactive

glutaminyl residues capable of interacting with β- and κ-caseins. β-Caseins at 5 mg/mL from micelles (Payens and Vreeman, 1982) however they polymerized. An explanation is that reactive glutaminyl residues are at the surface of the micelles, accessible to F $XIII_a$.

κ-Casein has a fairly ordered structure (Loucheux-Lefebvre et al., 1978) with glutaminyl residues accessible to F $XIII_a$. κ-Casein was less reactive to guinea pig liver TGase, because the specificity of TGase with respect to glutaminyl residue is lower and not because glutaminyl residues are buried inside the molecule, as hypothesized by Ikura et al. (1980).

Heating of α-caseins at 50 °C for 25 min did not improve polymerization (Figure 6, lane 6), although heat treatment must induce unfolding of the protein with an unmasking of active glutaminyl and lysyl residues. But unfolding unmasks also hydrophobic regions of protein with, as a consequence, an aggregation of molecules to avoid contact of the hydrophobic regions with water molecules. Under this process, glutaminyl and lysyl residues would become again hidden. As for β- and κ-caseins (Figures 8 and 9, lane 6), heat treatment had no effect (no decrease in polymerization). A more drastic heat treatment, 65 °C for 30 min, gave the same results (data not shown). The heat treatment did not lead to a masking of reactive glutaminyl and (or) lysyl residues.

The present study indicates that placental F XIII, activated by calcium, can polymerize caseins. An incubation of 4 h under the conditions described was optimum for most cross-linking of caseins. DTT was not a prerequisite for polymerization. The functional properties of polymerized casein proteins remain to be studied. Placental F $XIII_a$ may have potential applications for the production of food materials with new functional properties and better nutritive values.

ABBREVIATIONS USED

DTT, dithiothreitol; F XIII, factor XIII; F $XIII_a$, activated factor XIII; EDTA, ethylenediaminetetraacetic acid tetrasodium salt; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; kDa, kilodalton; TGase, transglutaminase.

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