Polymerization of Soybean Proteins and Spinach RuBisCO by FXIIIa with Respect to the Labeling of Reactive Glutaminyl Residues

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Calcium-activated human placental factor XIII was assayed for polymerization on some food proteins. High molecular weight polymers were visualized on SDS–PAGE. Polymerization of soybean proteins, β-conglycin, a trimer [α,α′,β], and glycinin, a hexamer of A-B dimer, depends on protein concentration, time, and the presence of DTT, although no disulfide bridge is known for β-conglycin. FXIIIa can also polymerize spinach RuBisCO, especially the L subunit. These proteins, β-conglycin, glycinin, and RuBisCO, were then assayed for incorporation of monodansylcadaverine to identify the subunit(s) containing reactive glutaminyl residues. Although all subunits can be polymerized, when purified, only the α of β-conglycin, A of glycinin, and S of RuBisCO subunits were labeled on the whole proteins. These findings could be explained by the better accessibility of the reactive glutaminyl residues from the isolated subunits to FXIIIa.

Keywords: *FXIIIa; cross-linking; soybean proteins; RuBisCO*

INTRODUCTION

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 FXIII involves the formation of an 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 weight 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.

Guinea pig liver transglutaminase was used to cross-link casein, 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), whey proteins (Traoré and Meunier, 1992), and myosin and 7S globulins from soybean (De Backer-Royer *et al.*, 1992b). In some cases, gelation of proteins at high concentrations occurred (Nio et al., 1986; De Backer-Royer *et al.*, 1992b).

In the case of factor XIIIa the presence of glutaminyl residues is not sufficient to allow the catalysis. Indeed, the efficiency with which a protein can be utilized as a substrate by transglutaminases is known to be influenced by the amino acid sequence around the glutaminyl residues (Gorman and Folk, 1980). Then the primary structure of a protein is of greater importance in assessing its ability to act as a substrate than its absolute lysine and glutamine content. Otherwise, the specificity of transglutaminases with respect to amines is weak.

Since a great number of food proteins are made up of several subunits, it is the goal of this paper to link their polymerization to the localization of the reactive glutaminyl residues among the subunits. We have chosen to perform this study on 7S and 11S soybean globulins and spinach RuBisCO.

MATERIALS AND METHODS

7S and 11S soybean globulins were purified according to the procedure of Thanh and Shibasaki (1976). The 7S and 11S subunits of globulins were prepared according to the procedures of Coates *et al.* (1985) and German *et al.* (1982), respectively. Spinach RuBisCO was a gift from Dr. Gontéro-Meunier. The S and L subunits were separated according to the method of Hubbs and Roy (1992). Monodansylcadaverine was from Sigma. All other reagents were of analytical grade. Factor XIII was purified from fibrogammin according to the procedure of Traoré and Meunier (1991). Fibrogammin (Behringwerke, Marburg, Germany) was obtained from a local drugstore.

Activation of FXIII. The activation was carried out by incubation of factor XIII in the presence of 140 mM $CaCl_2$, pH 7.5, at 37 °C for 10 min (Traoré and Meunier, 1991).

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

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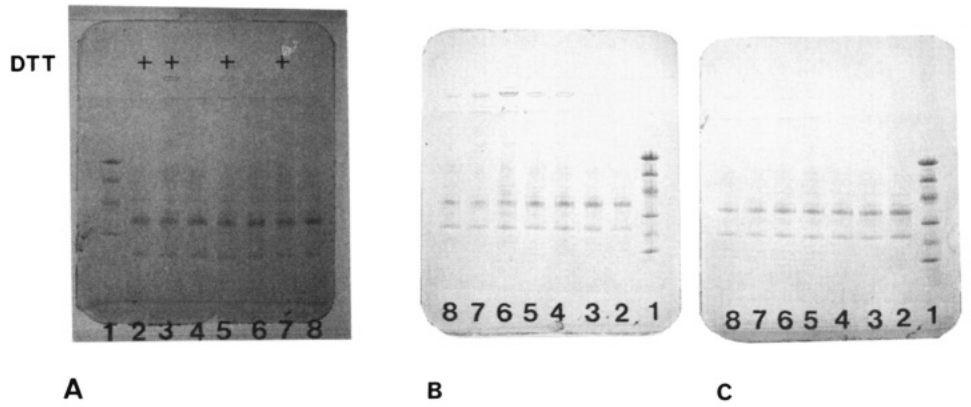


Figure 1. SDS–PAGE of 11S globulins subjected to FXIIIa activity (100 nM). (A) Effect of DTT and 11S concentration after 4 h of incubation: lane 1, protein markers (from the top, 94, 67, 43, and 31 kDa); lane 2, 8 mM DTT, 4.6 mg/mL 11S; lanes 3 and 4, 4.6 mg/mL 11S, FXIIIa in the presence (8 mM) and in the absence of DTT, respectively; lanes 5 and 6, 2.3 mg/mL 11S, FXIIIa in the presence (8 mM) and in the absence of DTT, respectively; lanes 7 and 8, 1.15 mg/mL 11S, FXIIIa in the presence (8 mM) and in the absence of DTT, respectively. (B) Effect of reaction time in the presence of 8 mM DTT: lane 1, protein markers (from the top, 94, 67, 43, 31, 20, and 14.4 kDa); lane 2, 4.6 mg/mL 11S; lanes 3–8, 11S subjected to FXIIIa for 1, 2, 3, 4, 6, and 10 h, respectively. (C) Effect of reaction time in the absence of DTT: lane 1, protein markers (see panel B); lane 2, 4.6 mg/mL 11S; lanes 3–8, 11S subjected to FXIIIa for 1, 2, 4, 6, 10, and 22 h, respectively. (+) indicates the presence of DTT.

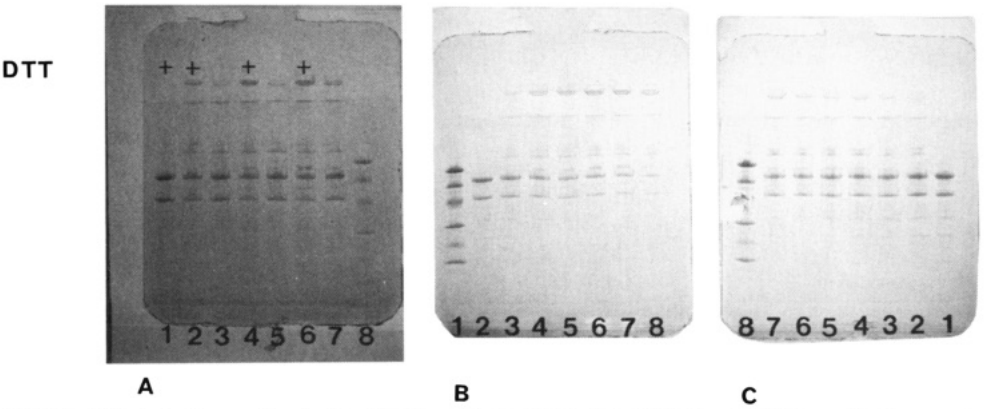


Figure 2. SDS–PAGE of 7S globulins subjected to FXIIIa activity (100 nM). (A) Effect of DTT and 7S concentration after 4 h of incubation: lane 1, 8 mM DTT, 5.04 mg/mL 7S; lanes 2 and 3, 5.04 mg/mL 7S, FXIIIa in the presence (8 mM) and the absence of DTT, respectively; lanes 4 and 5, 2.1 mg/mL 7S, FXIIIa in the presence (8 mM) and in the absence of DTT, respectively; lanes 6 and 7, 1.05 mg/mL 7S, FXIIIa, in the presence (8 mM) and in the absence of DTT, respectively; lane 8, protein markers (see legend to Figure 1B). (B) Effect of time in the presence of 8 mM DTT: lane 1, protein markers (see legend to Figure 1B); lanes 2–8, 2.1 mg/mL 7S subjected to FXIIIa for 0, 1, 2, 3, 4, 6, 10, and 22 h, respectively. (C) Effect of time in the absence of DTT: lane 8, protein markers (see legend to Figure 1B); lanes 1–7, 2.1 mg/mL 7S, subjected to FXIIIa for 0, 1, 2, 3, 4, 6, and 22 h, respectively. (+) indicates the presence of DTT).

The purity of enzyme was checked by PAGE (7.5% w/v) under denaturing conditions, according to the procedure of Laemmli (1970). The transfer activity was measured by the formation of ammonia according to the procedure of De Backer-Royer *et al.* (1992a).

Cross-Linking Experiments. The standard reaction mixture contained, in a total volume of 0.4 mL, 0.1 M Tris-HCl buffer (pH 7.5), variable concentration of substrate protein, 6.2 mM $CaCl_2$, and 100 nM FXIIIa. All experiments were carried out in the presence (8 mM) and in the absence of DTT. Incubation was performed at 37 °C. At suitable times, aliquots were taken out and SDS–PAGE experiments were carried out to detect the products polymerized through the intermolecular cross-linking catalyzed by FXIIIa.

Polyacrylamide Gel Electrophoresis. Monodansylcadaverine incorporation into proteins and the cross-linking were analyzed by using vertical slab gels (NOVEX precast gels) in the presence of 1% SDS. The samples were diluted with quench buffer containing 50 mM Tris-HCl buffer (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, and 5% (v/v) 2-mercaptoethanol and heated at boiling water temperature for 3–5 min. SDS–PAGE was performed using the Tris–glycine buffer system described by Laemmli (1970) with 4–20% continuous gradient acrylamide gel. Ten micrograms of denatured protein was loaded. In some cases, electrophoresis was performed on a Pharmacia Phast system (4–20% gradient gels). Coomassie Blue staining was carried out in all cases.

Monodansylcadaverine Incorporation into Proteins. The standard reaction mixture contained, in a total volume of 0.4 mL, 0.1 M Tris-HCl buffer (pH 7.5), 6.2 mM $CaCl_2$, 100 nM FXIIIa, and 1 mM MDCd without and with DTT (8 mM). Incubation was performed at 37 °C. At suitable times, aliquots were taken out and SDS–PAGE experiments were carried out to detect subunits of proteins (RuBisCO, 7S and 11S globulins) by staining with Coomassie Blue. To detect incorporation of MDCd, photographs were performed under UV (365 nm).

RESULTS AND DISCUSSION

Polymerization of 11S and 7S Globulins. When 11S or 7S globulins were incubated in the presence of FXIII, activated by Ca^{2+}, cross-linking of the molecules occurred, leading to species with high molecular weight (Figures 1 and 2). The extent of polymerization of 11S globulins was dependent on protein concentration (Figure 1A), on reaction time (Figure 1B,C), and on the presence of DTT (Figure 1). In all cases polymerization was not complete. In contrast, 7S globulins polymerized almost completely (Figure 2B), but the extent of polymerization was also dependent on protein concentration

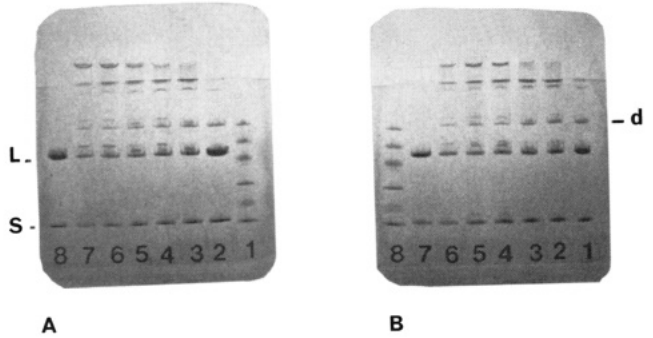


Figure 3. SDS–PAGE of spinach RuBisCO subjected to FXIIIa activity (100 nM). (A) Effect of time in the presence of 8 mM DTT: lane 1, protein markers (see legend to Figure 1B); lanes 2–7, 2 mg/mL RuBisCO subjected to FXIIIa for 0 and 30 min and 1, 4, 14, and 24 h; lane 8, 2 mg/mL RuBisCO incubated alone for 24 h. (B) Effect of time in the absence of DTT: lanes 1–6, 2 mg/mL RuBisCO subjected to FXIIIa for 0 and 30 min and 1, 4, 14, and 24 h; lane 7, 2 mg/mL RuBisCO incubated alone for 24 h; lane 8, protein markers (see legend to Figure 1B). d indicates L dimers.

(Figure 2A), on reaction time (Figure 2B,C), and on the presence of DTT (Figure 2). Therefore, the best conditions of polymerization were obtained at 22 h, in the presence of 8 mM DTT. The improvement of polymerization of 11S globulins by DTT can be attributed to the reduction of the disulfide bridges between the A and B subunits, leading to the unfolding of the protein chain and the unmasking of the glutaminyl group. This unfolding may also lead to partial aggregation of the disordered molecules, with masking of reactive residues. Therefore, only the molecules with unmasked glutaminyl residues cross-link to high molecular weight products. On PAGE run, aggregates dissociate to molecules, while the cross-linked molecules did not obviously. This hypothesis, already given (Traoré and Meunier, 1992) for β-lactoglobulin and α-lactalbumin, could explain the incomplete polymerization of 11S globulins with respect to time. It is unknown if 7S globulins have disulfide bridges, but the α-subunit has three sulfydryl groups. The positive effect of DTT can mean that this subunit possesses one disulfide bridge.

The formation of high molecular weight polymers when 7S globulin was treated by FXIIIa confirms that the gelation of this protein was linked to the cross-linking of the globulin molecules (De Backer-Royer *et al.*, 1992b). The K_m and V_{max} for 7S globulin, calculated from the least-squares-fitted linear Lineweaver–Burk plots, were 10^{-5} M and 0.3 s^{-1}, respectively. Both values were less than that determined for the different caseins (Traoré and Meunier, 1991), when the specificity constant, k_c/K_m (3×10^4 s^{-1} M^{-1}) was of the same order of magnitude as that of total casein (3.8×10^4 s^{-1} M^{-1}).

Polymerization of RuBisCO. When subjected to FXIIIa, RuBisCO was partially polymerized (Figure 3), since now high molecular weight polymers did not enter into the gel. DTT did not improve the cross-linking; therefore, it was not necessary. The band corresponding to the L subunit was strongly reduced, while that of the S subunit remained constant. The L subunit was therefore preferentially polymerized. It was noted that the L subunit dimerized at the start of the run. On the time course of FXIIIa action, the L dimer disappeared.

Incorporation of MDCd into 7S and 11S Globulins and RuBisCO. Monodansylcadaverine was incorporated into the glutaminyl residues of 7S and 11S globulins and RuBisCO thanks to FXIIIa. Since these proteins are made up of several subunits, MDCd allowed us to label a particular subunit to check which subunits have a reactive carboxamide. Results of SDS–PAGE experiments shown in Figure 4 indicate that α subunit of 7S globulins was labeled, while for 11S globulins the A subunit (acidic) was fluorescent (Figure 5). No incorporation was found in other subunits [α′ and β for 7S globulins, a trimer [α,α′,β], and B (basic) subunits for 11S proteins, a hexamer of A-B dimer]. Only the large (L) subunit of RuBisCO was labeled by MDCd (Figure 6), in agreement with results of Figure 3. These results mean that α, A, and L subunits contain reactive glutaminyl residues. Results of Figures 3 and 6 can be explained by the reaction of a reactive Gln residue of RuBisCO L subunit against a Lys residue of another L subunit to give rise to a polymer of L subunit, whereas S subunits hidden in the core of the molecule did not react. In contrast, the polymerization of 7S and 11S globulins involved the whole molecule since apparently the subunits disappeared at the same level.

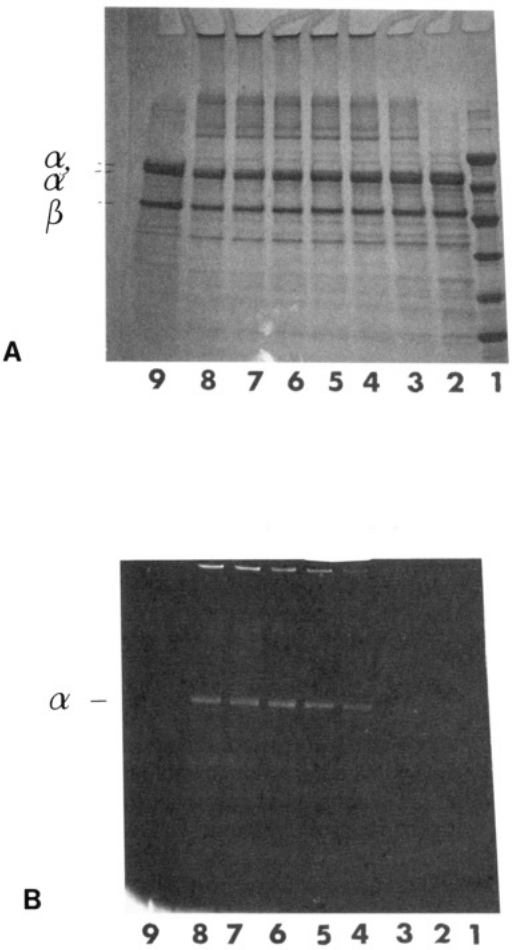
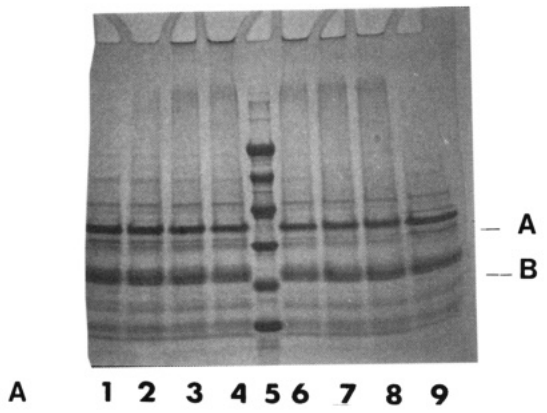


Figure 4. Incorporation of MDCd into 7S globulins; comparison between SDS–PAGE revealed by staining and under UV. (A) SDS–PAGE of incorporation revealed by Coomassie blue staining; effect of reaction time: lane 1, protein markers (see legend to Figure 1B); lanes 2–8, 1.68 mg/mL 7S globulins and 1 mM MDCd (in the presence of 8 mM DTT), subjected to FXIIIa for 0, 1, 3, 5, 8, 17, and 26 h, respectively; lane 9 is a control (lane 8 without FXIIIa). (B) SDS–PAGE of incorporation revealed under UV (365 nm); same legend as above.

Since fluorescence was found at the top of the lanes for 11S and 7S globulins, high molecular weight species were also made up between MDCd and globulins. To explain this result, we hypothesize that all cadaverine molecules are not dansylated; in that case, cadaverine



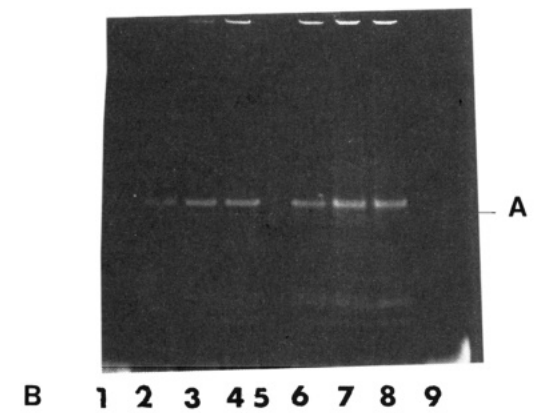
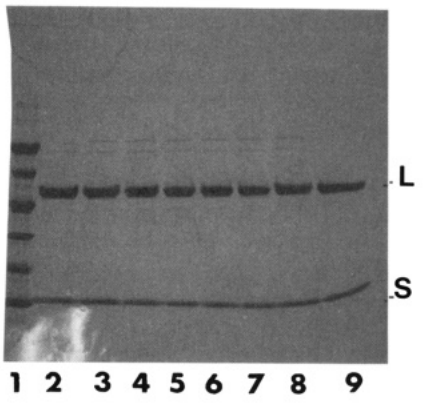


Figure 5. Incorporation of MDCd into 11S globulins; comparison between SDS–PAGE revealed by staining and under UV. (A) SDS–PAGE of incorporation revealed by Coomassie blue staining; effect of reaction time: lane 5, protein markers (see legend to Figure 1B); lanes 1–8 (except 5), 4.6 mg/mL 11S globulins and 1 mM MDCd (in the presence of DTT), subjected to FXIIIa for 0, 1, 3, 5, 8, 17, and 26 h, respectively; lane 9 is a control (lane 8 without FXIIIa). (B) SDS–PAGE of incorporation revealed under UV (365 nm); same legend as above.



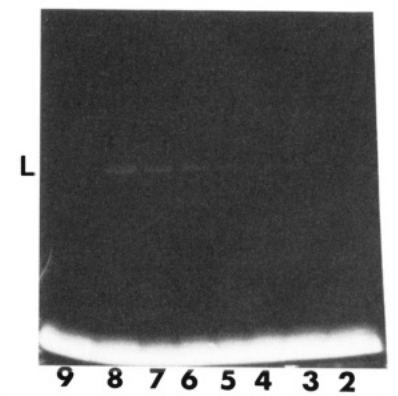


Figure 6. Incorporation of MDCd into spinach RuBisCO; comparison between SDS–PAGE revealed by staining and under UV. (A) SDS–PAGE of incorporation revealed by Coomassie blue staining; effect of reaction time: lane 1, protein markers (see legend to Figure 1B); lanes 2–8, 1.3 mg/mL RuBisCO, 1mM MDC (in the presence of 8 mM DTT), subjected to FXIIIa for 0, 1, 3, 5, 8, 17, and 26 h, respectively; lane 9 is a control (lane 8 without FXIIIa). (B) SDS–PAGE of incorporation revealed under UV (365 nm); same legend as above, except that the numbering is reversed. At the bottom, the big band was that of free monodansylcadaverine.

molecules could link α subunits of 7S and A subunits of 11S globulins, respectively. Another explanation is that α or A subunits were partially labeled; under these conditions isopeptidic bonds could be made up between nonlabeled glutaminyl residues of α and lysyl residues for α′ or β subunits for 7S globulins and between nonlabeled glutaminyl residues of A and lysyl residues of B for 11S globulins.

Monodansylcadaverine L subunit of RuBisCO did not give rise to high molecular weight species (no fluorescence at the top of the wells, Figure 6B), while native RuBisCO polymerized. To account for these results, one can put foward that all of the reactive glutaminyl residues were labeled by MDCd so that none was allowed to react with lysyl residues of the L or S subunits. The labeling of the L subunit of spinach RuBisCO agrees well with the results of Margosiak *et al.* (1990), who identified the large subunit of RuBisCO as a substrate for transglutaminase in *Medicago sativa* (alfalfa).

Polymerization of Subunits of Globulins and RuBisCO. 7S globulin was split into its subunits, and α, α′, and β subunits were purified. We obtained α, α′, and β subunits in a good purity. Now the three subunits polymerized (Figures 7 and 8) in contrast to the lone labeling of the α subunit by MDCd. This result could be explained by the unmasking of active glutaminyl residues of α' and β subunits in the course of the dissociation of the trimer α,α′,β. DTT does not have a sensitive effect on the FXIIIa action.

In Figure 9, we show the effect of FXIIIa on the acidic subunit of 11S globulins. It can be seen that HMW polymers appeared at the top of the wells, indicating a partial polymerization of this subunit. DTT did not improve this polymerization since a disulfide bridge occurs only between the A and B subunits and not inside the A subunit. DTT improved obviously the polymerization of 11S globulin (Figure 1B).

Figure 10 shows the polymerization of the S subunit of RuBisCO, although this subunit was not labeled by MDCd. This result agrees well with the 3D structure of RuBisCO: the S subunits are in the core of the molecule, and the dissociation allowed the unmasking of its reactive glutaminyl residues. It also appears that DTT improved slightly the polymerization. Due to its insolubility, the L subunit could not be submitted to the action of FXIIIa.

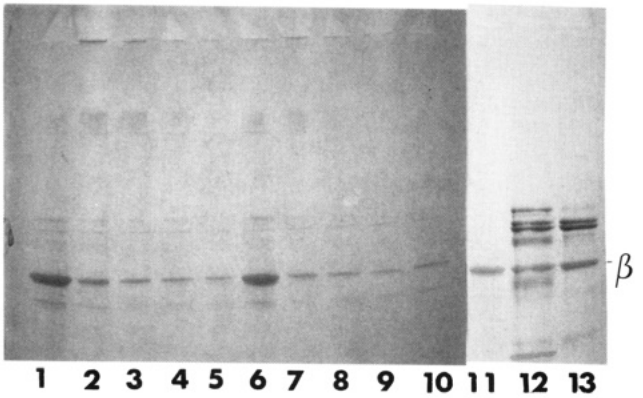
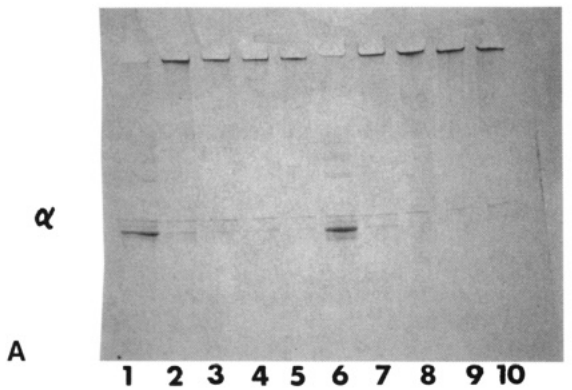


Figure 7. SDS–PAGE of β-subunit of 7S globulins subjected to FXIIIa (100 nM); lanes 1–5, 2 mg/mL β-subunit, subjected to FXIIIa action (without DTT) for 0 and 30 min and 1, 2, and 4 h; lanes 6–10, 2 mg/mL β-subunit subjected to FXIIIa action in the presence of 8 mM DTT for 0 and 30 min and 1, 2, and 4 h; lane 11, 2 mg/mL β-subunit incubated alone for 4 h; lane 12, protein markers and 7S globulins; lane 13, 2 mg/mL 7S globulins.



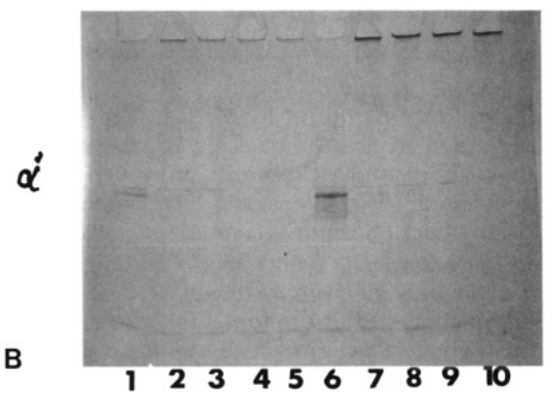


Figure 8. SDS–PAGE of α- and α′-subunits of 7S globulins subjected to FXIIIa (100 nM). (A) SDS–PAGE of α-subunit: 2 mg/mL α-subunit, subjected to FXIIIa action for 0, 1, 4, 10, and 20 h, without DTT (lanes 1–5) and in the presence of DTT (lanes 6–10). (B) SDS–PAGE of α′-subunit: 2 mg/mL α′-subunit, subjected to FXIIIa action for 0, 1, 4, 10, and 20 h without DTT (lanes 1–5) and in the presence of DTT (lanes 6–10).

In conclusion, only one subunit type of 7S and 11S globulins and RuBisCO is capable of incorporating MDCd. That means that reactive glutaminyl residues are either not present in all of the subunits or are masked by the 3D structure. For 7S globulin and RuBisCO the active glutaminyl residues of α′, β, and S subunits are masked. We were not able to obtain B subunits of 11S globulin and L subunits of RuBisCO due to their high insolubility in buffer. Therefore, we cannot decide whether they contain active glutaminyl residues.

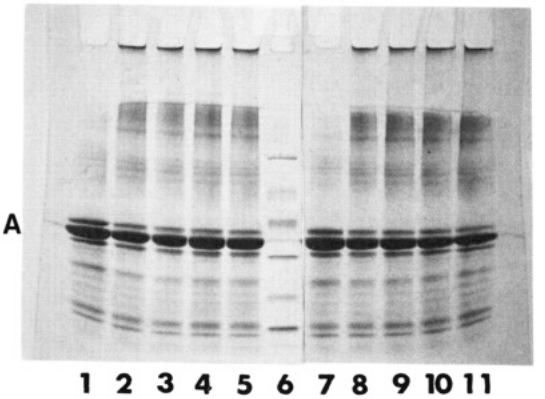


Figure 9. SDS–PAGE of acidic (A) subunit of 11S globulins subjected to FXIIIa (100 nM); effect of reaction time: lanes 1–5, 2.35 mg/mL acidic subunit, subjected to FXIIIa action for 0, 1, 4, 10, and 30 h, respectively; lane 6, protein markers (see legend to Figure 1B); lanes 7–11, like lanes 1–5 but in the presence of 8 mM DTT.

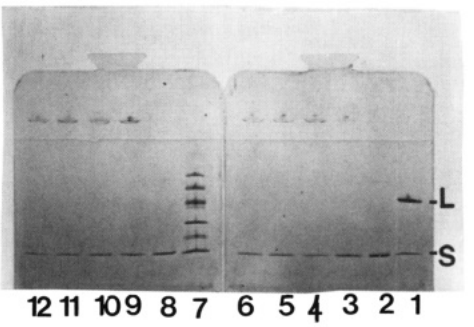


Figure 10. SDS–PAGE of the small (S) subunit of spinach RuBisCO subjected to FXIIIa (100 nM): 0.14 mg/mL S subunit, subjected to FXIIIa action for 0, 1, 4, 14, and 21 h in the absence of DTT (lanes 2–6) and in the presence of DTT (lanes 8–12); lane 1, 0.14 mg/mL S subunit incubated alone for 21 h; lane 7, protein markers (see legend to Figure 1B).

ABBREVIATIONS USED

FXIII, factor XIII as proenzyme; FXIIIa, activated FXIII; MDCd, monodansylcadaverine; DTT, dithiothreitol; HMWP, high molecular weight polymers; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; RuBisCO, ribulose 1,5-bisphosphate carboxylase/oxygenase; 11S globulin, glycinin; 7S globulin, β-conglycin; S, small subunit; L, large subunit.

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