CROSS-LINKING OF SALMON FIBRINOGEN AND FIBRIN BY FACTOR XIII AND TRANSGLUTAMINASE

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Received August 9,1973

SUMMARY: Unlike mammalian species, salmon plasma contains 2 cross-linking enzyme systems: Factor XIII and a transglutaminase which appears to be similar in its action to that described by Folk and Chung. Also, salmon plasma contains an exceedingly active protease which possesses the ability to rapidly destroy fibrinogen clottability even when the plasma is stored at a temperature of -20°C

In the past years there has been intensified interest in the cross-linking of human fibrin monomers by active Factor XIII (Laki-Lorand Factor, fibrin-stabilizing enzyme, fibrino-ligase, etc.) (1-5). We have been engaged in investigations pertaining to clotting systems of cold water fish. Our studies also extend to the mechanisms of fibrinogen and fibrin cross-linking. This report deals with results obtained utilizing fibrinogen and fibrin obtained from king salmon, Oncorhynchus tshawytscha.

Salmon blood was collected usually from the tail vein into a final volume of 0.5% trisodium citrate and maintained at ice temperature prior to centrifugation at 4°C. Two ml of the pale pink plasma from each collection vessel was clotted with approximately 10 N.I.H. units of bovine thrombin. These samples, when fresh, remained coagulated for 2-3 days when left at room temperature. Following sample removal, the plasma was stored in 150 ml aliquots at -20°C. in Teflon containers.

Highly purified fibrinogen preparations were obtained by low alcohol fractionation, 13% ethanol, at -2°C. Further purification was

carried out by a variation of the technique described by Laki (6). However, all operations were performed at 4°C. Clottability of these fibrinogen fractions was of the minimal order of 90-95%. Aliquots of both the fibrinogen, and fibrin obtained by the action of bovine thrombin, were first reduced in a mixture of SDS-mercaptoethanol-urea and subsequently electrophoresed on SDS-polyacrylamide gels, 7.5% cross-linked at pH 7.1 (7). Fig. I illustrates the results of these experiments: tube 1, purified salmon fibrinogen; and tube 2, action of bovine thrombin on purified salmon fibrinogen; i.e., fibrin. We have designated the chains of both the salmon fibrinogen and fibrin according to conventional nomenclature. It is apparent that there is a difference in the mobilities of one or two of the chains when fibrin is compared to fibrinogen. However, we do take cognizance that the designation of these chains is based on their mobilities on the gels. Tubes 1 and 2 display three distinct chains with small quantities of low and high molecular weight materials. Tubes 3 and 4 show fibrinogen and fibrin fractions obtained from salmon plasmas that have been stored at a temperature of -20°C. for periods exceeding 60 days. It is apparent that considerable breakdown of these proteins has ensued. Actually, plasmas stored at -20°C. for excessive periods or allowed to stand at room temperatures for very short periods, following thawing (24 hours or less), would not clot upon the addition of thrombin. Degraded fibrinogen fractions obtained via low alcohol fractionations from plasmas stored for prolonged periods were also non-clottable. These experiments may explain why previous investigators were unable to obtain clottable fibrinogen fractions from various fish plasmas such as garpike, bowfin and salmon (8,9). These experiments clearly demonstrate breakdown products of salmon fibrinogen, thus indicating the existence in salmon plasma of a highly active "plasmin-like" protease capable of altering or destroying fibrinogen clottability even at -20°C. All cross-linking experiments described below were performed utilizing materials obtained from plasma samples stored at -20°C. for less than 60 days.

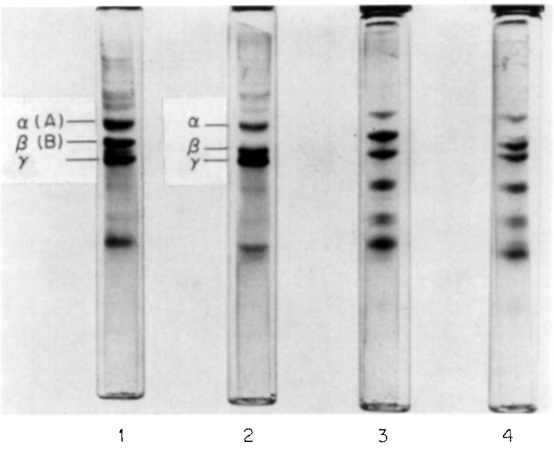

Fig. 1.

SDS-gel electrophoretic patterns of reduced salmon fibrinogens and fibrins. 1. Salmon fibrinogen; 2. salmon fibrin; 3. salmon fibrinogens from plasmas stored in excess of 60 days; 4. thrombin action on salmon fibrinogen of tube 3.

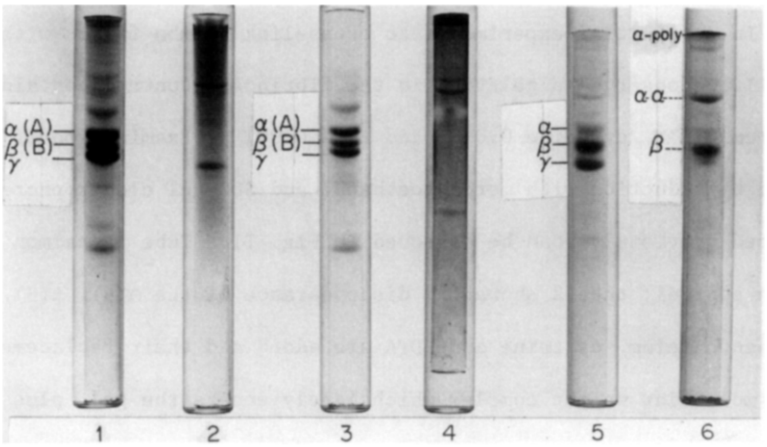

Fig. 2.

SDS-gel electrophoretic patterns of reduced and cross-linked salmon fibrinogen and bovine fibrinogen and fibrin. 1. salmon fibrinogen; 2. salmon fibrinogen, Ca^{++}, Cys, EDTA; 3. bovine fibrinogen, Ca^{++}, Cys, EDTA; 4. bovine fibrinogen, Ca^{++}, Cys, EDTA, guinea pig liver transglutaminase; 5. bovine fibrin; 6. bovine fibrin, Ca^{++}, Cys, EDTA, Factor XIII.

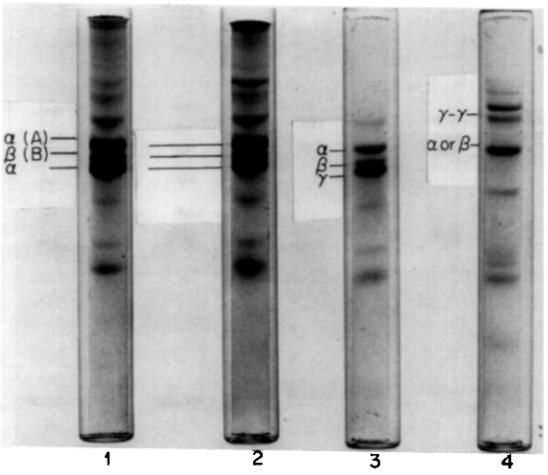

Fig. 3.

SDS-gel electrophoretic patterns of reduced salmon fibrinogens and cross-linked fibrin obtained from DFP-treated plasmas. 1. salmon fibrinogen; 2. salmon fibrinogen, Ca^{++}, Cys, EDTA; 3. salmon fibrin; 4. salmon fibrin, Ca^{++}, Cys, EDTA, Factor XIII.

In our initial experiments to cross-link salmon fibrin with Factor XIII, we observed a gelation in the fibrinogen control containing only calcium 0.01M, cysteine 0.01M, and EDTA 0.001M. Examination of this phenomenon by reduction with mercaptoethanol and SDS-gel electrophoresis as described previously, can be observed in Fig. II. Tube 1, salmon fibrinogen control; tube 2 shows the disappearance of the α(A), β(B), and γ bands when calcium, cysteine and EDTA are added and their replacement by a high molecular weight complex which barely enters the gel, plus a slight quantity of transient material. The effect appears to be complete within 2 hours but the gelation is observable with 2 minutes. The possibility that small residual quantities of thrombin impurities in the fibrinogen fractions might be activating residual inactive Factor XIII appeared unlikely since the cross-linking pattern was unlike that which is observed when active Factor XIII cross-links human fibrinogen and human and bovine fibrins (10, 3, 11) It appeared more likely that the salmon plasma already possessed an active

transglutaminase of the variety described by Folk and Chung (12). Thus, it was not possible to discern the mode of cross-linking of salmon fibrin via Factor XIII under the existing experimental conditions.

The above experiments were repeated utilizing bovine fibrinogen, 95-97% clottable, Factor XIII-free, and guinea pig liver transglutaminase. The results are demonstrated in Fig. II: tube 3, bovine fibrinogen control in the presence of calcium, cysteine and EDTA; tube 4, same as tube 3 with the addition of guinea pig liver transglutaminase. As can be seen in tube 4, similar results have occurred in the cross-linking pattern compared to tube 2; only a high molecular weight complex has barely entered the gel. This result is in complete agreement with the action of guinea pig liver transglutaminase on human fibrinogen first reported by Chung (13; Chung, et al., 14) and recently by Schwartz, et al. (10). Tube 5 demonstrates the α, β and γ chains of bovine fibrin in the presence of calcium, cysteine and EDTA; tube 6 is the same as tube 5 with the addition of thrombin-activated Factor XIII, illustrating the classic resulting cross-linked γ-γ dimer, α-polymer, and uncrossed-linked β chains (11).

In order to demonstrate the existence of cross-linking by Factor XIII in salmon plasma, it was necessary to remove or inactivate the residual transglutaminase. We found it possible to inactive transglutaminase activity in our highly purified salmon fibrinogen fractions by first incubating the salmon plasma in the cold, 4°C., with relatively large quantities of pure diisopropylphosphorofluoridate (DFP), 50μg/200ml plasma, for 18 hours prior to fractionation. Highly purified fibrinogen fractions were obtained as described earlier in this publication. Fig. III illustrates the results of cross-linking of salmon fibrin with thrombin-activated Factor XIII. Tube 1 contains salmon fibrinogen; tube 2, salmon fibrinogen plus Ca^{++}, cysteine and EDTA; tube 3, salmon fibrin; tube 4, salmon fibrin, Ca^{++}, cysteine, EDTA, plus Factor XIII. Tube 2, in contrast to tube 2 in Fig. II, clearly demonstrates the absence of cross-linking. However, when transglutaminase

was added to the ingredients of tube 2, a cross-linking pattern similar to tube 2, Fig. II, was obtained. It can be seen in tube 4, Fig. III, that cross-linking of the γ chain has occurred yielding the γ-γ dimer. Either the α or β chains have cross-linked into a high molecular weight material barely entering the gel. Exactly the same results were obtained by residual Factor XIII inherent in the fibrinogen fractions when active Factor XIII was omitted from the contents of a mixture containing the same ingredients as tube 4, only the time factor of cross-linking was increased. Current investigations will show which of these chains have cross-linked.

Presently we do not know whether or not the DFP treatment inactivates the salmon plasma transglutaminase directly or if a protease-like enzyme responsible for the transglutaminase activation is inhibited. We are aware that the relative large quantities of DFP utilized may contain a contaminant which is capable of inhibiting SH-dependent enzymes, such as has been shown to be the case with the DFP inhibition of papain (15,16).

Our experiments clearly demonstrate that salmon plasma contains two cross-linking enzyme systems: (1) a transglutaminase and (2) a classic type Factor XIII. From an evolutionary viewpoint, it is of interest that an invertebrate, lobster, contains only a transglutaminase-like enzyme while salmon, a vertebrate, contains 2, a transglutaminase and a Factor XIII, the latter which in higher vertebrates becomes dominant in about 400 million years.

Preliminary investigations with lamprey fibrinogen have shown essentially a cross-linking pattern similar to Factor XIII cross-linking. However, the action of bovine thrombin on lamprey fibrin (via bovine thrombin) indicates no cross-linking by residual Factor XIII.

Presented in part at the annual meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N.J., April 15-20, 1973.

ACKNOWLEDGMENT

We wish to express our thanks to Drs. Folk and Chung for generous quantities of guinea pig liver transglutaminase and Factor XIII.

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