The Vitelline Envelope of Eggs from the Giant Keyhole Limpet *Megathura crenulata*. II: Products Formed by Lysis with Sperm Enzymes and Dithiothreitol†

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ABSTRACT: The egg vitelline envelope of the marine invertebrate, *Megathura crenulata*, was lyzed either by sperm lysins A, B, C or by dithiothreitol. In each case the lysis mixture consisted of two major fractions, I and II, that could be separated by hydroxylapatite chromatography and had different electrophoretic mobilities on cellulose acetate strips. The amino acid, amino sugar, and neutral sugar compositions of fractions I and II were similar and resembled that of the intact vitelline envelope. Fractions I and II of each lysis mixture emerged in the exclusion volume of a Sepharose 6B column. A vitelline envelope fragment enzymatically formed by lysin was further degraded by dithiothreitol to form smaller fragments. A model of the vitelline envelope of the *Megathura crenulata* egg is suggested whereby the envelope is composed of polypeptide chains cross-linked by disulfide bonds and built to a large extent of closely spaced threonine residues. Most of the threonine residues are linked to carbohydrate units. Dithiothreitol dissolves the envelope by reducing disulfide bonds, whereas lysins most likely dissolve the envelope by degrading polypeptide chains.

The egg vitelline envelope of the marine invertebrate *Megathura crenulata* can be lyzed by acrosomal enzymes, termed lysins (Tyler, 1939; Krauss, 1950; Heller and Raftery, 1973). These enzymes are believed to be part of the mechanism that enables the spermatozoon to penetrate the egg and start the process of fertilization (Dan, 1967; Franklin, 1970). Three lysins, A, B, and C, have been isolated and purified from extracts of *M. crenulata* sperm (Heller and Raftery, 1973). Sperm of other marine invertebrates (Dan, 1967), amphibians (Elinson, 1971), and mammals (McRorie and Williams, 1974) have been found to carry lytic enzymes capable of lyzing the protective coats of their corresponding eggs. The vitelline envelope of *M. crenulata* can also be dissolved by disulfide reducing agents (Krauss, 1950) as has also been shown for the vitelline envelope of sea urchin eggs (Epel et al., 1970), the amphibian jelly coat (Gusseck and Hedrick, 1971), and the mammalian zona pellucida (Inoue and Wolf, 1974).

This communication describes the isolation and partial characterization of products formed by the interaction of lysins A, B, C or dithiothreitol with the egg vitelline envelope of *Megathura crenulata*.

Experimental Section

Materials. Giant keyhole limpets (*Megathura crenulata*) were purchased from Pacific BioMarine, Venice, Calif. Hydroxylapatite and Bio-Gel P-300 were from Bio-Rad Lab., Richmond, Calif., Sepharose 6B and Sephadex G-150 were from Pharmacia, Piscataway, N.J., dithiothreitol from Calbiochem, La Jolla, Calif., and alcian blue from MCB, Manufacturing Chemists, Los Angeles, Calif. All other chemicals were of reagent grade. Tris–artificial sea water was 0.01 M Tris–HCl, pH 8.6 containing 0.480 M NaCl, 0.010 M KCl, 0.026 M $MgCl_2$, 0.028 M $MgSO_4$, 0.011 M $CaCl_2$, and 0.002 M $NaHCO_3$.

Methods. The vitelline envelope was isolated as described previously (Heller and Raftery, 1973). The isolation and purification of lysins A, B, and C have been described previously (Heller and Raftery, 1973).

Lysis of the Vitelline Envelope by Lysin A, B, and C. The following procedure was used for each of the three lysins or dithiothreitol. Packed vitelline envelopes (1.0 ml) were washed three times with 10 ml of Tris–artificial sea water and suspended in 10 ml of this buffer. Lysin (1 mg) of dithiothreitol (1 mg) was added, and the mixture was incubated at room temperature for 1 h. Completion of lysis was followed by inspecting samples under a light microscope. The highly viscous solution was then heated at 70 °C for 5 min to inactivate the enzyme, made up to 30 ml with 0.01 M potassium phosphate buffer, pH 7.0, containing 0.01 M NaCl, and spun down at 43 000 *g* for 1 h. The supernatant was dialyzed against 0.01 M potassium phosphate buffer, pH 7.0, 0.01 M NaCl with three buffer changes.

Electrophoresis on cellulose acetate strips was performed on a model R-101 microzone electrophoresis apparatus (Beckman Instruments) and carried out according to the procedure described by Wardi and Allen (1972).

Hydroxylapatite Chromatography. The dialysate from the lysis reaction was applied to a hydroxylapatite column (1.5 × 25 cm) equilibrated with 0.01 M potassium phosphate buffer, pH 7.0, containing 0.01 M NaCl. The column was washed with starting buffer until all unadsorbed material was removed as monitored at 215 nm. The column was then developed with a linear gradient of starting buffer to 0.2 M potassium phosphate buffer, pH 7.0, containing 0.01 M NaCl in a total volume of 400 ml.

Sepharose 6B Chromatography. The lysis products emerging from the hydroxylapatite column were each concentrated on a PM-10 Amicon Diaflo membrane to about 1 ml and applied to a Sepharose 6B column (1.5 × 96 cm)

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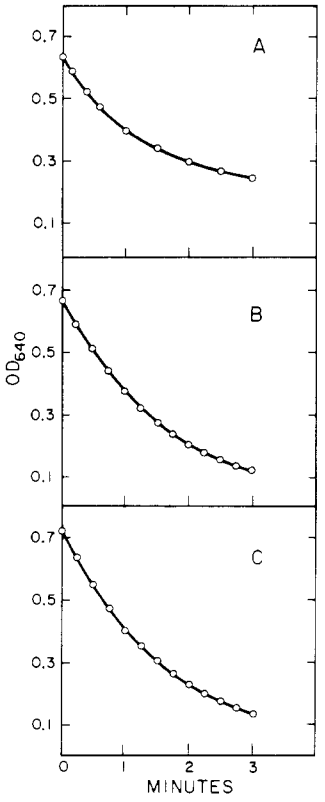

FIGURE 1: Decrease in turbidity of vitelline envelope suspension following the addition of lysin: (A) lysin A, E_0 20 μg/ml; (B) lysin B, E_0 2.5 μg/ml; (C) lysin C, E_0 5 μg/ml.

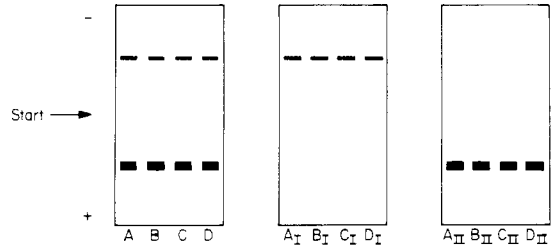

FIGURE 2: Cellulose acetate electrophoresis of the lyzed vitelline envelope. A, B, C, and D correspond to the vitelline envelope lysis with either lysin A, B, C, or dithiothreitol, respectively. AI, BI, CI and DI stand for fraction I of the hydroxylapatite chromatography of each lysis reaction. AII, BII, CII, and DII stand for fraction II of the hydroxylapatite chromatography of each lysis reaction (Figure 3).

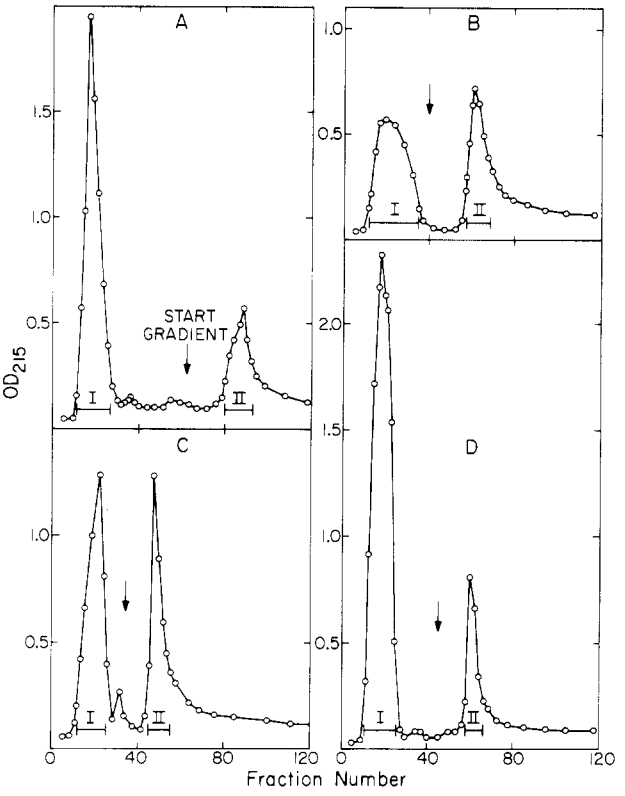

FIGURE 3: Hydroxylapatite chromatography of the lyzed vitelline envelope. The column (1.5 × 25 cm) was equilibrated with 0.01 M potassium phosphate buffer, pH 7.0, containing 0.01 M NaCl. Fraction volume was 2.5 ml. A, B, C, and D stand for the vitelline envelope lysis with either lysin A, B, C or dithiothreitol, respectively.

equilibrated with 0.01 M potassium phosphate buffer, pH 8.0, containing 0.5 M NaCl.

Amino acid, amino sugar, and neutral sugar analyses were carried out as described before (Heller and Raftery, preceding article). Sulfhydryl groups were assayed by the Ellman method (1959). Amino groups were assayed by the ninhydrin method (Duggan, 1957). Reducing sugar was assayed by the Park–Johnson ferricyanide method (Spiro, 1966).

Results

Lysis of the Vitelline Envelope with either Lysins A, B, C or Dithiothreitol. Incubation of the vitelline envelope with either lysins A, B, C or dithiothreitol resulted in each case in complete dissolution with the formation of highly viscous solutions. An indication of the envelope dissolution is the decrease in turbidity at 640 nm of a vitelline envelope suspension following the addition of the lysins (Figure 1). Electrophoresis of these lyzed mixtures on cellulose acetate strips at pH 8.6 revealed two bands when the strips were stained with the carbohydrate stain alcian blue (Figure 2). No bands could be detected when the strips were stained with the protein stain Ponceau S. Attempts to electrophorese the mixtures on polyacrylamide gels proved fruitless, presumably due to the high viscosity and/or high molecular weights of the reaction products.

Chromatography of each of the lysis mixtures on hydroxylapatite resulted in separation into two fractions, I and II (Figure 3). Fractions, AI, BI, CI, and DI (Figure 3) corresponded to the positively charged band on cellulose acetate electrophoresis, whereas fractions AII, BII, CII, and DII corresponded to the negatively charged band (Figure 2). In order to estimate the molecular weights of the lysis products, the various fractions emerging from the hydroxylapatite chromatography (Figure 3) were applied to a Sepharose 6B column. All the lysis fractions (AI, AII, BI, BII, CI, CII, DI, DII) emerged in the exclusion volume of the column, suggesting high molecular weight species. The recovery of fractions AII, BII, CII, and DII from the Sepharose column was about 30% as compared with almost quantitative recovery of fractions AI, BI, CI, and DI from the column. Elution of a Sepharose column containing fraction AII with 0.2 M galactose did not yield any more material. A similar recovery (30%) was achieved when AII was applied to Sephadex G-150 and Bio-Gel P-300 columns. Fractions AII, BII, CII, and DII tended also to stick to Amicon Diaflo membranes.

The conclusion from these experiments was that the action of the lysins or dithiothreitol on the vitelline envelope was to break the original structure at only a few selected

Table I: Amino Acid Composition of Vitelline Envelope Fractions Formed by Lysis with Either Lysin A, B, C or Dithiothreitol.

Amino Acid	Vitelline Envelope	Mol %							
		AI	AII	BI	BII	CI	CII	DI	DII
Lysine	1.2	1.2	1.0	1.3	1.0	1.5	1.3	1.2	1.3
Histidine	1.5	1.6	1.4	1.5	1.4	1.5	1.5	1.6	1.4
Arginine	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.4	0.3
Aspartic acid	1.4	1.0	1.6	1.0	2.0	1.2	1.5	1.1	1.5
Threonine	61.0	62.8	62.5	62.3	61.4	61.6	60.5	62.8	60.8
Serine	1.4	1.1	1.6	1.1	1.3	1.3	1.5	1.1	1.0
Glutamic acid	1.1	0.7	1.3	0.8	3.4	0.8	1.5	0.6	2.1
Proline	16.6	17.1	15.9	16.8	14.0	17.0	16.5	17.7	17.2
Hydroxyproline	4.6	4.5	4.2	4.8	3.5	4.1	4.9	4.3	3.9
Glycine	0.6	0.2	1.0	0.2	1.3	0.3	0.7	0.1	0.8
Alanine	2.6	2.5	1.9	2.3	3.2	2.5	2.7	2.3	2.2
Half-cystine[a]	1.3	1.3	1.4	1.5	1.4	1.5	1.5	1.1	1.5
Valine	0.5	0.2	0.3	0.2	0.2	0.3	0.4	0.2	0.3
Methionine[a]	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Isoleucine	1.4	1.4	1.3	1.4	1.3	1.4	1.5	1.4	1.5
Leucine	3.7	3.6	3.4	4.0	3.8	4.1	3.0	3.8	3.9
Tyrosine	0.2	0.1	0.3	0.1	0.1	0.2	0.2	0.1	0.1
Phenylalanine	0.2	0.1	0.3	0.1	0.1	0.1	0.2	0.1	0.1

[a] Determined as cysteic acid and methionine sulfone following oxidation with performic acid.

Table II: Relative Molar Proportions of the Vitelline Envelope Fractions Formed by Lysis with Either Lysin A, B, C or Dithiothreitol.

	Vitelline Envelope	Mol %							
		AI	AII	BI	BII	CI	CII	DI	DII
Total amino acids	37.3	37.9	37.8	37.0	36.6	37.8	40.1	35.5	40.5
Threonine	22.7	23.8	23.6	23.0	22.5	22.3	24.3	22.3	24.6
Galactosamine	22.1	22.3	22.6	21.5	20.8	23.0	20.4	22.7	20.9
Neutral sugar	40.6	39.8	39.6	41.4	42.4	39.2	39.5	41.8	37.6
Fucose	15.9	17.7	14.9	17.3	17.8	14.5	17.9	18.4	8.2
Galactose	24.7	22.1	24.7	24.1	24.6	24.7	24.6	23.4	29.4

sites. The fragments of each lysis mixture, i.e., AI and AII, could be separated on hydroxylapatite, had different electrophoretic mobilities on cellulose acetate, and had high molecular weights. The electrophoretic and chromatographic methods on the other hand were not sensitive enough to determine whether fragments like AI, BI, CI, or DI were identical (due to cleavage of the structure at the same site in each case) or just shared an overall similarity, masking the fact that the lysins and dithiothreitol actually cleave the envelope structure at different sites.

Amino Acid Composition. Table I shows the amino acid composition of the various fractions that emerged from the hydroxylapatite chromatography. The various fractions were similar in their amino acid composition. These amino acid compositions were also similar to that of the intact vitelline envelope (Table I). These results seem to suggest that the vitelline envelope is composed of polypeptide units with possible repetitive sequences and these sequences are reflected in the lysis products. It should be noted that the high percentage of threonine and proline would tend to obscure small differences in composition that might exist in the lysis products.

Protein, Amino Sugar, and Neutral Sugar Content of the Lysis Products. The relative amounts of total amino acids, threonine, galactosamine, fucose, and galactose found in the intact vitelline envelope are kept in the lysis products (Table II). The molar ratio of threonine to galactosamine is about 1:1 for the various lysis products as it was in the intact envelope and the molar ratio of galactose to fucose varies between 1.3 and 1.8 for most of the lysis products, except DII where it is about 3.6:1.

These results suggest that the products of cleavage by the lysins and by dithiothreitol contain both neutral sugar, amino sugar, and amino acids. Thus the cleavage caused by the lysins or by dithiothreitol does not seem to uniquely involve either sugar to sugar cleavage or amino sugar to polypeptide cleavage. Moreover, the molar ratio of amino acids, amino sugar, and neutral sugars characteristic of the intact vitelline envelope is reflected in each of the products formed by the action of the lysins on the envelope.

Detection of New Functional Groups Resulting from the Lysin Action. Because dithiothreitol, a disulfide reducing reagent, dissolves the vitelline envelope, the possibility existed that lysins also react by enzymically breaking disulfide bonds. To check this possibility, the content of free sulfhydryl groups in the lysis products of lysin B was checked with the Ellman reagent. No free sulfhydryl groups could be detected in an assay sensitive to measure the conversion to free sulfhydryl groups of at least 15% of the total disulfide bonds in the original vitelline envelope.

No new free amino groups were detected in the lysis reaction of the vitelline envelope with either lysin A, B, or C by the ninhydrin assay which was capable of detecting at least 3% of the potential α-amino groups of the amino acids present in the vitelline envelope.

An assay for reducing sugar did not show an increase in reducing activity following lysis of the vitelline envelope with either lysins A, B, or C. The sensitivity of the assay was such that it could detect about 1% of the potential reducing sugar groups in the vitelline envelope. Thus our

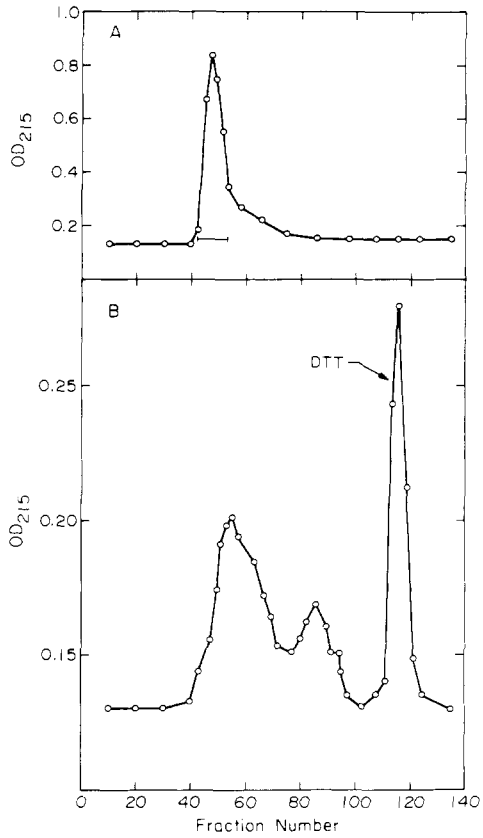

FIGURE 4: (A) Sepharose 6B chromatography of vitelline envelope fragment BI. The column (1.5 × 96 cm) was equilibrated with 0.01 M potassium phosphate buffer, pH 8.0, containing 0.5 M NaCl. Fraction volume was 1 ml. Solid bar denotes pooled fractions. (B) Pooled BI incubated with 0.001 M dithiothreitol and rerun on the same Sepharose 6B column.

assay for new sulfhydryl, amino, or reducing sugars which may appear following the action of lysins A, B, and C on the vitelline envelope were all negative, or in other words, the exact site and mode of action of the lysins on the envelope structure could not be detected by these methods.

Treatment of the Enzymatic Lysis Products with Dithiothreitol. Fraction BI emerges in the exclusion volume of a Sepharose 6B column (Figure 4A). After incubation of this fraction with 0.001 M dithiothreitol, it was rerun on the same Sepharose 6B column resulting in several new fractions of lower molecular weight than the original BI (Figure 4B). This result suggests the presence of disulfide bonds in BI. Similar degradation of AI and CI with dithiothreitol has been achieved. Fraction DI which was originally formed by the interaction of dithiothreitol with the vitelline envelope was not changed after reincubation with dithiothreitol, presumably since all its disulfide bonds were already reduced.

These results show that a fragment that was formed by the action of a lysin on the vitelline envelope could be further degraded by the disulfide reducing reagent dithiothreitol to form smaller fragments.

Discussion

This paper describes the products that are formed by lyzing the egg vitelline envelope of the giant keyhole limpet *Megathura crenulata* with either sperm lysins A, B, and C (Heller and Raftery, 1973) or dithiothreitol. Incubation of the vitelline envelope with any one of the lysins or with dithiothreitol results in complete envelope dissolution with formation of a highly viscous solution. On hydroxylapatite columns each mixture was separated into two fractions (I and II in Figure 3). Each fraction migrates as a separate band on cellulose acetate electrophoresis strips (Figure 2). The viscous solution suggests high molecular weight species and this is supported by the emergence of the lysis products in the exclusion volume of a Sepharose 6B column. The lysis products of the vitelline envelope of the sea snail *Tegula pfeifferi* have been reported to have a molecular weight of 5×10^6 daltons or more (Haino-Fukushima, 1974).

The similarity in amino acid, amino sugar, and neutral sugar composition between the various fractions and the intact vitelline envelope (Tables I and II) seems to suggest that the basic structure of the envelope has been preserved following lysis, namely polypeptide chains built to a large extent of closely spaced threonine residues that are linked to a carbohydrate moiety (Heller and Raftery, preceding article). There are, however, some indications of differences along the polypeptide chains as shown by the different chromatographic and electrophoretic behavior of fractions I and II of each of the lysis mixtures (Figures 2 and 3). These results could suggest the occurrence of regions of different structure not detected by amino acid and carbohydrate composition analysis. Moreover, fractions AII, BII, CII, and DII appear to possess different affinities toward Sepharose (as well as Sephadex and Bio-Gel) than fractions AI, BI, CI, and DI. The reason for the partial retardation of fractions AII, BII, CII, and DII on Sepharose, Sephadex, and Bio-Gel columns is not clear at the moment; a possible explanation is hydrogen bonding to the column material.

The lysis of the vitelline envelope by dithiothreitol, a disulfide reducing agent, is an indication that disulfide bonds are essential for the vitelline envelope structure. A similar view is held by Gusseck and Hedrick (1971) who concluded that disulfide bonds are required for the structural integrity of the amphibian jelly coat and by Inoue and Wolf (1974) who define a role for disulfide bonds in maintaining the structure of the mammalian zona pellucida. It was found that the vitelline envelope does not possess free sulfhydryl groups nor were any formed following the lysis by lysin B. Neither were new amino groups or reducing sugars found following lysis of the envelope with lysin B. It is possible, however, that the small number of products that are formed, each having a molecular weight of several millions, make it difficult to detect, for example, any free amino groups that might be formed by the action of a lysin acting as a protease on the vitelline envelope. It could be assumed, however, that if the lysins are proteases they must be highly specific, hydrolyzing only a few peptide bonds along the polypeptide chain. A less specific protease could be expected to cause the formation of many more and/or smaller products.

Such a mode of action of the lysins on the vitelline envelope, namely envelope dissolution by cleaving the polypeptide chain, can be supported by the fact that lysis product BI was further degraded by dithiothreitol to form smaller fragments (Figure 4B). In view of these findings, a model could be envisioned for the vitelline envelope of *Megathura crenulata*. According to this model, the vitelline envelope of the *M. crenulata* egg consists of polypeptide chains linked together by disulfide bridges. The polypeptide chains are built to a large extent of threonine residues linked to carbohydrate arms of various length and composition (Heller and Raftery, preceding article). Dithiothreitol dissolves the envelope by reducing the S–S bonds and thus causing the for-

mation of single polypeptides. The lysins in turn cleave the polypeptide chains causing formation of envelope fragments with intact S-S bonds. Those can be further reduced by dithiothreitol to form smaller fragments.

The exact site of lysin cleavage and the possible differences among the various lysis products await further studies.

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Reactivity of Fibrinogen and Fibrinopeptide A Containing Fibrinogen Fragments with Antisera to Fibrinopeptide A†

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ABSTRACT: Two antisera used in the radioimmunoassay for human fibrinopeptide A (FPA) which appear to have different immunochemical specificities have been tested for cross-reactivity with fibrinogen and with three fragments of fibrinogen which contain the FPA sequence. These fragments were the three-chain, NH_2-terminal disulfide knot (N-DSK) produced by CNBr cleavage of fibrinogen, the reduced, carboxymethyl Aα chain portion of the N-DSK, and fragment E produced by plasmin digestion of fibrinogen. One antiserum (R-2) showed high specificity for free FPA and less than 2% cross-reactivity with fibrinogen or the FPA-containing fragments. The other antiserum (R-33) possessed a much higher degree of cross-reactivity with the FPA-containing fragments. Synthetic and native fibrinopeptides were found to be indistinguishable in the assay system with either antiserum. As a result of these studies, an hypothesis has been developed concerning the nature of the antigenic determinants on FPA which favor measurement of free FPA and limit cross-reactivity with larger, FPA-containing peptides.

Thrombin cleaves the Aα chain of fibrinogen to release 2 mol of fibrinopeptide A (FPA[1]) per mole of fibrinogen to initiate fibrin aggregation (Blombäck and Vestermark, 1958; Blombäck and Yamashina, 1958). Thrombin also cleaves the Bβ chain to release fibrinopeptide B (FPB) at a slower rate than FPA (Blombäck and Vestermark, 1958; Bilezikian et al., 1975). A radioimmunoassay for FPA in plasma has been developed to serve as a quantitative index of thrombin action in vivo (Nossel et al., 1971), and this assay has been applied to measurement of FPA levels in normal plasma and in patients with thrombotic diseases (Nossel et al., 1974; Gerrits et al., 1974; Hardin et al., 1975). Since the validity of the assay as an index of thrombin action depends on the estimation of the free, circulating, 16-amino acid fibrinopeptide and not fibrinogen or other FPA-containing polypeptides such as those generated by plasmin, it is important to study the antisera used in the radioimmunoassay for cross-reactivity with fibrinogen and its degradation products. Two antisera that had sufficiently high titers of anti-FPA antibodies to be used in the routine plasma radioimmunoassay were studied. These antisera were also of special interest concerning immunochemical specificity, because they gave different values for FPA con-

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[1] Abbreviations used are: FPA, fibrinopeptide A; FPB, fibrinopeptide B; N-DSK, NH_2-terminal disulfide knot; PAGE, polyacrylamide gel electrophoresis; RCM, reduced, *S*-carboxymethylated; Pth, 3-phenyl-2-thiohydantoin.