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PURIFICATION AND PROPERTIES OF AN ANIONIC ZYMOGEN OF PHOSPHOLIPASE A FROM PORCINE PANCREAS

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SUMMARY

1. This paper describes the isolation and purification of an enzymically inactive precursor of porcine pancreatic phospholipase A (phosphatide acyl-hydrolase, EC 3 1 1 4).

2. The protein, which has a molecular weight of about 15 000, appears to consist of a single polypeptide chain, terminating at the NH_2 region in the amino acid sequence. Glu–Gly–Glu–Ile–Ser–Ser–Arg–Ala. , and having cystine as COOH-terminal amino acid.

3 The precursor molecule is activated by trypsin which splits the above –Arg–Ala– peptide bond, yielding active phospholipase A and the heptapeptide Glu–Gly–Glu–Ile–Ser–Ser–Arg

4 In this released peptide, as well as in the precursor molecule itself, the N-terminal glutamic acid residue has no free α-NH_2 group

5 Phospholipase A, isolated from autolysed pancreatic tissue, appears to be identical with the product obtained by trypsin activation of the pure precursor.

INTRODUCTION

In the foregoing paper[1] we described the isolation and purification of phospholipase A (phosphatide acyl-hydrolase, EC 3 1 1 4) from autolysed porcine pancreas In this study it was observed that freshly prepared homogenates of pig pancreas possess a low lipolytic activity and that a large increase in phospholipase A activity occurs during storage of the homogenate at room temperature Initially, these phenomena were attributed to a progressive solubilization of the enzyme caused by autolysis of the tissue However, it appeared that clear aqueous solutions of the crude enzyme also became gradually more active, even upon storage at 0° The process of activation could be greatly accelerated by the addition of small amounts of pure trypsin and appeared to be retarded in the presence of DFP The similar behaviour of many proteolytic enzymes, which occur in pancreas as zymogens, strongly suggested the existence in pancreas of an enzymically inactive precursor of phospholipase A In order to prove the presence of such a zymogen, the isolation of the protein was undertaken Preliminary experiments showed that the so-called prephospholipase A

in many respects behaves like the active enzyme, which might indicate that only small structural differences exist between the proteins Therefore, the purification procedure used for the active enzyme, and described in the previous paper, was applied with only minor modifications in the isolation of the precursor. The major problems in the isolation turned out to be the high susceptibility of the zymogen to proteolytic attack. Traces of trypsin rapidly converted the prephospholipase A into the active enzyme even at 0° and adequate protection of the zymogen could be obtained only in the presence of rather high concentrations of DFP.

EXPERIMENTAL

Most of the materials and methods used have been described in the preceding article[1].

Materials

Pronase was obtained from Calbiochem and shown by chromatography not to be contaminated with peptides or free amino acids.

Lyophilized samples of human and pig pancreatic juice were generously supplied by Professor G CLÉMENT (Laboratoire de Physiologie Animale et de la Nutrition, Dijon, France) and Dr L. SARDA (Institut de Chimie Biologique, Marseille, France)

Methods

The content of phospholipase A (= potential phospholipase A activity) in the various fractions (if necessary, dialysed to remove DFP) was measured after maximal conversion of the zymogen into phospholipase A by trypsin. The activity of the latter was determined by potentiometric titration as described in the foregoing paper[1]. Specific activity is given by the number of μequiv of alkali consumed per min per mg of protein in the standard assay procedure

Peptides were separated by high-voltage electrophoresis using pyridine–acetic acid and formic acid–acetic acid buffer solutions (pH 1.9–3.6), and by paper chromatography (Whatman 3 MM) in *n*-butanol–formic acid–water (70:9:2, v/v/v) Free amino acids were separated and identified by thin-layer chromatography on silicic acid and by paper chromatography in *n*-butanol–acetic acid–water (4·1 5, v/v/v, upper phase). Quantitative separation of the amino acids Glu, Ser and Gly was carried out on Whatman paper 3 MM, impregnated with borate buffer (pH 9.3) in the solvent system phenol–cresol (1:1, v/v).

The detection of the peptides and amino acids was effected by the usual ninhydrin staining (collidine modification[2]), with the Sakaguchi reagent[3] and by exposure to chlorine gas, followed by spraying with KI–starch[4]

RESULTS

A. Purification procedure (see Table I)

1000 g of defatted porcine pancreas were rapidly ground in a mincer and further homogenized for 1 min in a Waring blendor in 3000 ml of ice-cold 0.15 M NaCl solution The homogenate was brought to pH 4 0 with conc HCl and heated at 70° for 3 min.

TABLE I

PURIFICATION OF PREPHOSPHOLIPASE A

1000 g of defatted pancreatic tissue were homogenized at 0° in 3000 ml of 0 15 M NaCl solution

Step	*Phospholipase A (units)*	*Prephospholipase A* (units)*	*Specific activity* of prephospholipase A*	*Yield (%)****
1 Crude homogenate	70 000–95 000	—**	—	—
2 Heat treatment at pH 4 0	55 000–75 000	—**	—	—
3 Removal of precipitated material and fat by centrifugation and filtration	40 000–50 000	500 000–600 000	12–13	100
4 0 35–0 60-Satd $(NH_4)_2SO_4$ precipitate	10 000–60 000	350 000–450 000	100–110	73
5 Dialysis and centrifugation	15 000–25 000	300 000–400 000	150–200	64
6 DEAE-cellulose chromatography (pH 7 3)	2 000– 3 000	200 000–300 000	600–700	46
7 CM-cellulose chromatography (pH 6 0)	1 000– 1 500	170 000–240 000	800–900	36

* Determined as phospholipase A after maximal activation of the zymogen with crystalline trypsin

** No reproducible figures could be obtained on account of endogenous trypsin inhibitors

*** These figures are mean values of several purification experiments.

The mixture was rapidly cooled to 0° and centrifuged at 4000 rev /min for 30 min in an IEC-International centrifuge at 0°. The slightly yellow supernatant fluid was freed from floating fat by filtration at 3° and brought to 0.35 S with solid $(NH_4)_2SO_4$ after

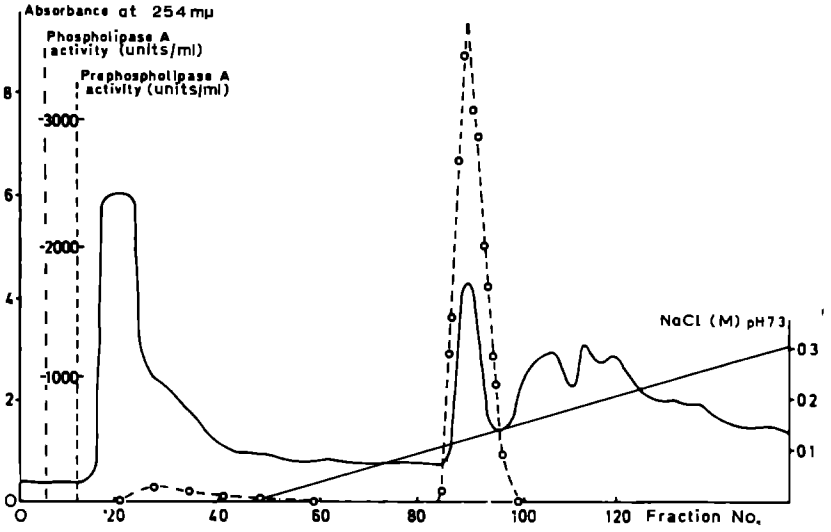


Fig 1 Elution pattern of prephospholipase A from a DEAE-cellulose column (55 cm × 3 5 cm) ———, absorbance at 254 mμ, ·— — —·—, phospholipase A units/ml, determined without trypsin activation, - - - - - -, prephospholipase A activity, determined after maximal activation with trypsin, as phospholipase A units/ml

bringing the pH to 7.0 with conc NH_3. After centrifugation at 0° for 30 min at 4000 rev /min, the precipitate at 0–0 35 satn was discarded and the supernatant fluid was brought to 0.60 satn with solid $(NH_4)_2SO_4$. The crude prephospholipase A precipitate was isolated by centrifugation for 1 h at 4000 rev./min. The precipitate was dissolved in the minimal amount of water, immediately treated with 0.1 M solution of DFP in dry isopropanol (final DFP concentration 10^{-2} M) and stirred at room temperature for 4–6 h. The solution was subsequently dialysed overnight at 0° against 0.005 M Tris buffer (pH 7 3) A small precipitate, which usually formed during dialysis, was removed by high-speed centrifugation and the clear supernatant, after addition of DFP, was chromatographed on DEAE-cellulose at 4° The column was equilibrated against 0.005 M Tris buffer (pH 7.3) and eluted first with the same buffer and, after appearance of the peak of cationic proteins, the prephospholipase A was eluted by a linear salt gradient developed by the use of 0 3 M NaCl in the same Tris buffer (see Fig 1)

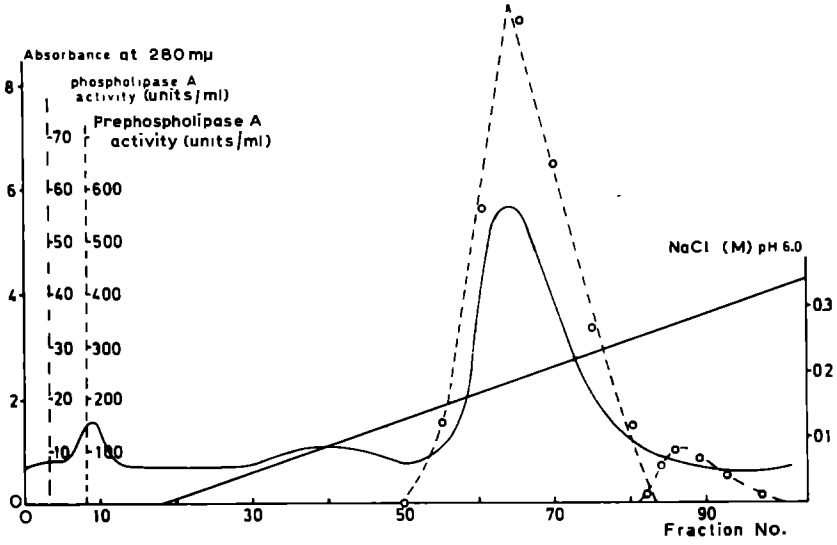
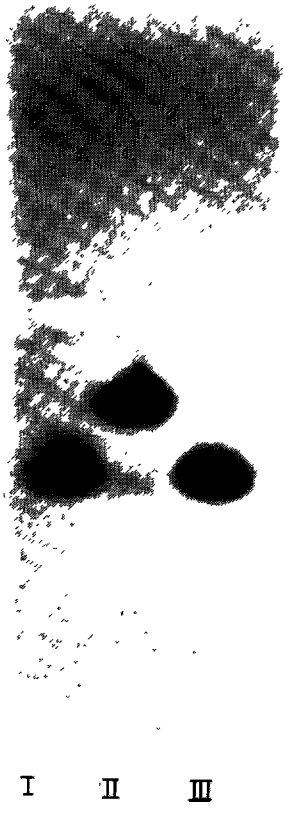


Fig 2 Elution pattern of prephospholipase A from a CM-cellulose column (55 cm × 3 5 cm) ———, absorbance at 280 mμ, — — · — —, phospholipase A units/ml, determined without trypsin activation, - - - -, prephospholipase A activity, determined after maximal activation with trypsin as phospholipase A units/ml

The effluent fractions containing zymogen were combined and sufficient 0 1 M DFP solution was added to give a final concentration of 10^{-2} M Concentration of the solution was effected by ultrafiltration *in vacuo* and the resulting solution was dialyzed against 0.005 M acetate buffer (pH 6 0) at 4° Rechromatography of the prephospholipase A was performed on a CM-cellulose column equilibrated against the above acetate buffer After appearance of a small break-through peak of anionic proteins, the zymogen was eluted by a linear NaCl gradient in 0.005 M acetate buffer between 0 and 0.4 M (see Fig. 2). The combined prephospholipase A fractions were exhaustively dialysed against distilled water and the zymogen was isolated by lyophilization from the DFP-treated solution.

B. Comments on the purification procedure

Requirement for DFP It is rather difficult to give exact and reproducible numbers on the enzymic activity in the various purification stages (Table I) The spreading in the relative amounts of active phospholipase A and of the zymogen is caused in the first place by differences in quality of the starting material Secondly, it appeared that the endogenous trypsin inhibitors, known to be present in pancreas[5], protect the zymogen against tryptic activation effectively only during the first purification stages After the $(NH_4)_2SO_4$ precipitation, solutions of the crude zymogen became highly labile and rapid conversion into phospholipase A took place, indicating the loss of a stabilising factor Therefore, all subsequent purification steps were performed in the presence of 10^{-2} M DFP*. Although under these conditions solutions of the impure precursor could be stored without any activation of the zymogen, it turned out that, during subsequent purification treatments, a progressive slow activation still took place Apparently trypsin (or another proteolytic enzyme also involved in the activation of the zymogen) is not completely irreversibly inactivated



I II III

Fig 3 Starch-gel electrophoretic pattern on a microscopic slide I, phospholipase A, II, prephospholipase A, III, prephospholipase A with a trace of trypsin

* This concentration gives no inhibition of phospholipase A activity

by DFP, making frequent additions of this reagent necessary Moreover, to obtain a zymogen preparation containing less than 1% of active phospholipase A, it is necessary to perform the column-chromatographic separations as quickly as possible at low temperatures

Column chromatography. Preliminary investigations into the electrophoretic behaviour of the zymogen indicated its isoelectric point to be about pH 6 5 Taking into account the isoelectric behaviour of phospholipase A (pH 7.4), the chromatography of the crude zymogen preparations on DEAE-cellulose was performed at low ionic strength at pH 7 3 As shown in Fig 1, an excellent separation could be effected between the active enzyme, which leaves the column immediately after the cationic proteins, and the prephospholipase A, which is eluted by a NaCl concentration of about 0 1 M The prephospholipase A obtained in this way still displayed a small phospholipase A activity (see Table I), which is produced mainly during the dialysis by spontaneous activation. A further drop in phospholipase A content of the zymogen could be effected by chromatography on CM-cellulose, as shown in Fig. 2

C. Properties of prephospholipase A

Electrophoretic analysis The purity of the isolated prephospholipase A was investigated by starch-gel and disc electrophoresis at various pH values. The protein, which revealed under these conditions only one band, became isoelectric at pH about 6 5 After trypsin activation the mobility of the prephospholipase A became identical with that of phospholipase A from autolysed pancreas[1] (see Fig 3)

Molecular weight determinations The molecular weight of the precursor was estimated by Sephadex gel filtration[6] and was calculated from the amino acid composition

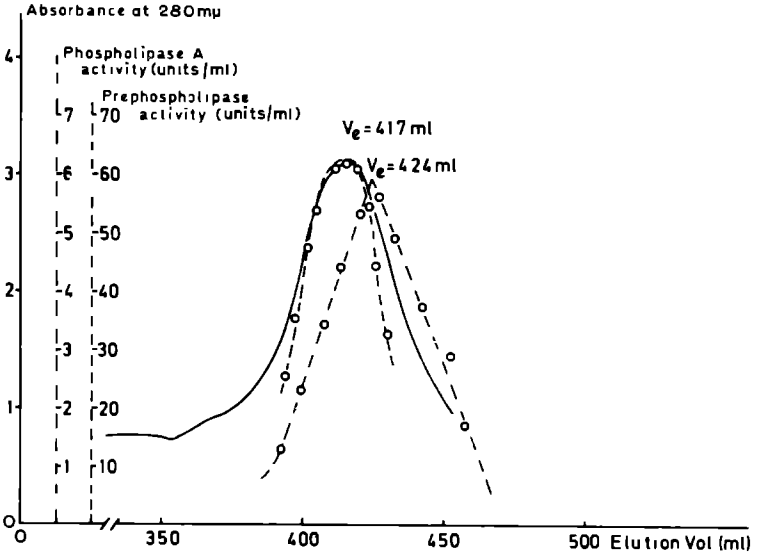


Fig 4 Elution pattern of a mixture of prephospholipase A and phospholipase A (9 1, w/w) from a Sephadex G-75 column (75 cm × 3 cm). ———, absorbance at 280 mμ, ·—·—·—·— —, phospholipase A units/ml determined without trypsin activation, - - - - -, prephospholipase A activity, determined after maximal activation with trypsin as phospholipase A units/ml

TABLE II

AMINO ACID COMPOSITION OF PREPHOSPHOLIPASE A

The results given are the average recoveries of two analyses after hydrolysis of the zymogen for 24 h and 48 h

Amino acid	*g residue per 100 g protein*	*Nearest integer per 14 000 g*	*Nearest integer × min mol wt*	*Integral number × mol wt of residue*
Asp*	17 62	23	15 024	2647.024
Thr	4 63	7	15 268	707.728
Ser	6 75	12	15 484	1044 936
Glu*	8 66	9	13 427	1162 026
Pro	4 21	6	16 408	582 684
Gly	2 93	7	13 642	399 364
Ala	4 08	8	14 000	568 624
Val	1 65	2	12 497	198 260
Cys	8 68	14	16 520	1444 016
Met	1 77	2	14 825	262 392
Ile	4 65	6	14 606	678 936
Leu	5 55	7	14 277	792 092
Tyr	8 40	8	15 543	1305 630
Phe	4 81	5	15 301	730 810
Lys	7 39	9	15 114	1153 548
His	2 97	3	13 859	411 420
Arg	5 23	5	14 933	780 940
Trp**	—	2	—	372 412
Total	99 98	135	14 749***	15242 572

* As no amide content has been determined, these values represent the sum of Asp + Asn and Glu + Gln.

** Determined spectrophotometrically according to the procedure of BENCZE AND SCHMID[17]

*** The average value

Using the calibration graph of the elution volumes of standard proteins from a Sephadex G-75 column, as given in the foregoing paper[1], it was found that a mixture of phospholipase A and the pure zymogen (1·9, w/w) could just be differentiated by measuring, at 280 mμ ultraviolet absorption and enzymatic activity after trypsin treatment (see Fig 4). The difference in elution volume of both proteins (7 ml) pointed to a difference in molecular weight of about 900 and the molecular weight value of the prephospholipase A was estimated to be 14 800 ± 500. This value is in agreement with the molecular weight calculated from the amino acid composition (Table II).

Amino acid composition and terminal amino acids The amino acid composition of the purified protein is given in Table II The presence of N-terminal amino acids was investigated by the dinitrophenylation techniques[7] as well as by the phenylisothiocyanate methods[8,9]. No terminal amino acid was released even after heat denaturation or performic acid oxidation[10] of the protein. The C-terminal amino acid of the precursor molecule turned out to be identical with that of the active enzyme No amino acids were released by carboxypeptidase A or B, but hydrazinolysis of the performic acid-oxidized protein, performed according to the method of BRAUNITZER[11] and AKABORI, OHNO AND SARITA[12], showed that cystine or cysteine occupies the C-

terminal position of prephospholipase A As titration with *p*-chloromercuribenzoate according to the procedures of BOYER[13] and BENESCH AND BENESCH[14] showed the absence of cysteine sulphydryl groups, we must conclude that the zymogen contains cystine as C-terminal amino acid From these results it seems probable that the zymogen is also made up of a single polypeptide chain cross-linked by 7 disulphide bridges and that during trypsin activation a small part of the polypeptide chain, situated in the N-terminal region of the precursor molecule, is split off

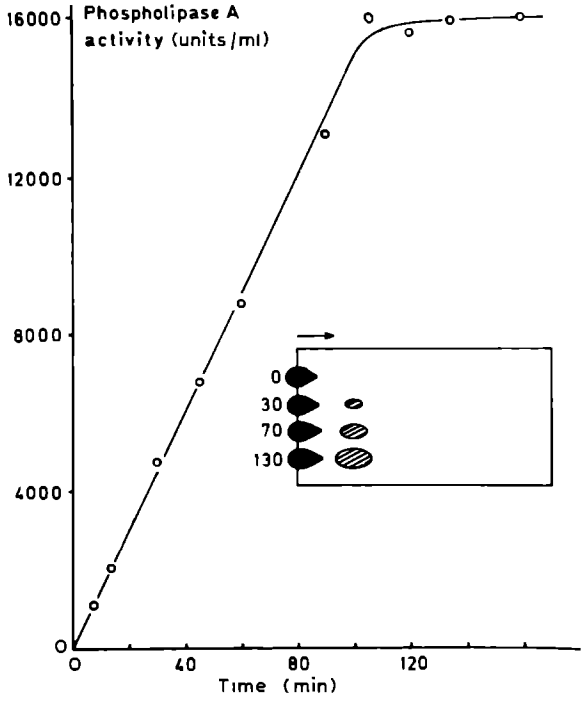


FIG 5 Rise in phospholipase A activity, during incubation of prephospholipase A with trypsin at 0° The inserted paper chromatogram shows the composition of the activation mixture with time of incubation in min

Conversion of prephospholipase A into the active enzyme and isolation of the activation peptide 300 mg of prephospholipase A dissolved in 15 ml of water were treated at 0° with 0 4 mg of crystalline trypsin. The pH of the incubation mixture was kept constant at pH 8 0 by using 0 1 M NaOH in the TTT-1 Radiometer titrator

As shown in Fig 5, a nearly linear rise in enzymic activity occurred during the first hour of incubation; after 80 min, 0 4 mg of trypsin was again added and the activation appeared to be complete after about 100 min reaction time Paper chromatography of the incubation mixture during activation revealed that the conversion of the zymogen into the active enzyme was accompanied by the formation of one peptide, which gave no reaction with ninhydrin and which reacted positively with the Sakaguchi reagent[3]. The trypsin activation reaction was stopped by the addition of DFP (final concentration 10^{-2} M) and separation of the phospholipase A and the

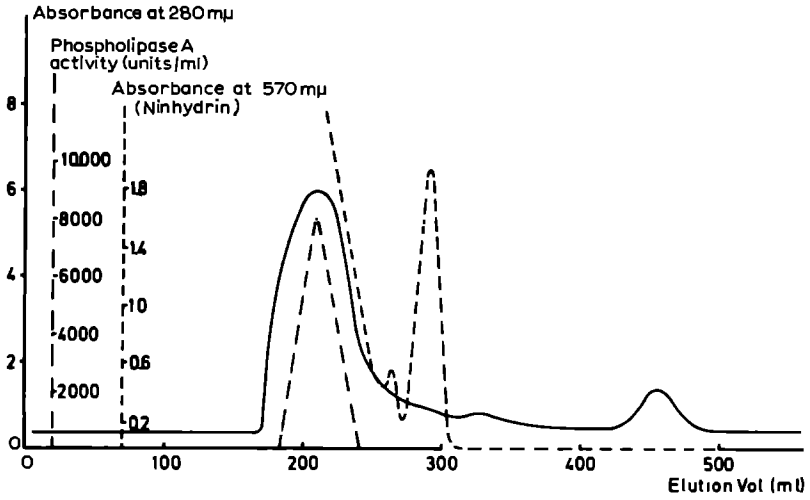


Fig. 6. Elution pattern of an incubation mixture of prephospholipase A and trypsin (750:1, w/w) from a Sephadex G-25 column (75 cm × 2.5 cm) ———, absorbance at 280 mμ, — — — —, phospholipase A units/ml, - - - - -, absorbance at 570 mμ measured after alkaline hydrolysis and ninhydrin reaction.

activation peptide formed was performed by gel filtration on a Sephadex G-25 column equilibrated with 1% acetic acid (v/v) (see Fig. 6). The enzymically active fractions were combined and after lyophilization we obtained 270 mg (95%) of electrophoretically pure phospholipase A with a specific activity of 800. Comparison of the protein with phospholipase A, isolated from autolysed pancreas[1], was performed by electrophoresis, N-terminal amino acid determination and MOORE AND STEIN analysis and both enzymes were shown to be identical. Comparison of the amino acid composition of the precursor and of the active phospholipase A (see Table II and Table II in ref. 1) showed both proteins to contain the same number of aromatic amino acids. As is clear from Fig. 6, the presence of the activation peptide in the eluate of the Sephadex column

TABLE III

AMINO ACID COMPOSITION OF THE ACTIVATION PEPTIDE

The results given are based on hydrolytic experiments of the peptide at 110° for 24 h and 48 h.

Amino acid	*g residue per 100 g of peptide*	*Nearest integer per 800 g*	*Nearest integer × min mol wt*	*Integral number × mol wt. of residue*
Ser	22.34	2	779.8	174.156
Glu	35.09	2	736.4	258.228
Gly	7.91	1	729.0	57.052
Ile	14.58	1	776.4	113.156
Arg	20.11	1	776.7	156.188
Total	100.03	7	760*	758.780

* The average value

could not be traced by ultraviolet absorption at 280 mμ, which also indicates the absence of aromatic amino acid in the activation peptide.

Therefore, the detection of this peptide was performed by the ninhydrin reaction after alkaline hydrolysis according to the procedure of MOORE AND STEIN[15] and by the technique of LOWRY *et al* [16]. The fractions containing the peptide were combined, lyophilized and subjected to a second separation on Sephadex G-25 in 1% acetic acid In this way the activation peptide was isolated in a yield of about 80% (12 mg) and shown by paper chromatography and paper electrophoresis to be homogeneous

Structural investigation of the activation peptide. The amino acid analysis shown in Table III reveals the activation peptide to be a heptapeptide composed of Ser_2, Glu_2, Gly, Ile, Arg, which is in agreement with the differences in molecular weight and amino acid composition between phospholipase A (see Table II and Table II in ref 1) and its precursor. Detection of the relatively small activation peptide on paper chromatograms by means of ninhydrin appeared to be impossible, which points to the absence of a free N-terminal amino acid Also end-group analysis according to the technique of Sanger[7] showed the absence of a free NH_2 group in the N-terminal amino acid of the peptide This result is in agreement with our idea that the activation peptide is split from the N-terminal region of the prephospholipase A molecule, which is itself also devoid of a free N-terminal amino acid. The presence of only one

TABLE IV

ANALYSIS AND MOST PROBABLE STRUCTURE OF FOUR PEPTIDES OBTAINED BY PRONASE DEGRADATION OF THE ACTIVATION PEPTIDE

10 mg of the heptapeptide, dissolved in 1 2 ml of 2% $NaHCO_3$ solution, were incubated for 1 h at 22° with 140 μg of pronase

Amino acid composition	*N-terminal amino acid*	*C-terminal amino acid*	*Probable structure*
Glu, Gly	Glu*	Gly	Glu*–Gly
Ile, Ser_2, Arg	Ile	Arg	Ile–Ser–Ser–Arg
Glu_2, Gly	—	—	Glu*–Gly–Glu
Glu_2, Gly, Ile, Ser	—	—	Glu*–Gly–Glu–Ile–Ser

* The N-terminal amino acid Glu contains no free NH_2 group

arginine molecule in the peptide, combined with the well-known substrate requirement of trypsin, strongly suggests that this amino acid must occupy the C-terminal position in the heptapeptide This was proved by incubation of the peptide with the carboxypeptidases A and B Whereas no release of amino acids could be detected upon carboxypeptidase A incubation, the carboxypeptidase B was found to catalyze the release of arginine only To obtain further insight into the amino acid sequence of the C-terminal part of the activation peptide, the simultaneous action of both carboxypeptidases was investigated. A subsequent release of Arg, Ser and Ile was found and quantitation of the amino acids released was performed by the dinitrophenylation technique[7]. The ratio of DNP-Ser to DNP-Arg turned out to be 1.6–2 0, indicating that the C-terminal sequence of the heptapeptide must be –Ile–Ser–Ser–Arg.

Further fragmentation of the activation peptide was carried out by enzymic

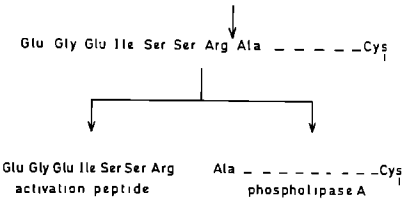


Fig 7 Trypsin-catalysed conversion of prephospholipase A into phospholipase A + activation peptide The N-terminal glutamic acid in the prephospholipase A as well as in the activation peptide has no free NH_2 group

hydrolysis with pronase (*Streptomyces griseus*) The peptides formed were separated by paper chromatography, eluted from the paper with 1% acetic acid and further investigated by MOORE AND STEIN analyses and end-group determinations The results, summarized in Table IV, gave as most probable structure of the heptapeptide the following sequence: Glu–Gly–Glu–Ile–Ser–Ser–Arg, in which the N-terminal glutamic acid has no free NH_2 group

DISCUSSION

As is clear from this and the preceding paper, porcine pancreas is a rich source of phospholipase A The major part of this enzyme, however, does not occur in the active form, but as an enzymically inactive precursor, apparently to avoid indiscriminate action of the enzyme on all cytoplasmic components of the cell This zymogen molecule is activated by trypsin, which specifically splits a very labile arginine–alanine linkage in the N-terminal region of the polypeptide chain, giving rise to the formation of active phospholipase A and a heptapeptide (Fig 7). Both the intact zymogen and the activation peptide contain N-terminal glutamic acid, which has no free α-NH_2 group. In the purification of the zymogen, the heat treatment at acidic pH might have converted the terminal glutamic acid into pyroglutamic acid The change in isoelectric point upon conversion of the zymogen into phospholipase A (from pH 6.5 to 7 4) indicates that the activation peptide must be acidic which could be demonstrated by paper electrophoresis These facts, however, are compatible also with the presence of a N-acylated glutamic acid residue

As regards the biological function of phospholipase A and its precursor in pancreas, one would expect, in accordance with the findings on proteolytic enzymes and their zymogens, that this enzyme, too, has a digestive task in the intestine and that the enzyme has to be transported in the juice as a harmless zymogen protein. It appeared, however, that human and pig pancreatic juice preparations investigated so far had a low content phospholipase A activity (*e.g.* lyophilized porcine juice was found to contain less than 2 5 units/mg of powder), but in both material we were also unable to detect the presence of significant quantities of prephospholipase A. As fresh native pancreatic juice from man and pig is difficult to obtain, it can not be precluded that damage of these proteins had occurred during the prolonged storage of the samples investigated A more detailed discussion on the function and metabolic role of this enzyme must await further studies

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