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PHOSPHOLIPASE A_2 ISOENZYME FROM PORCINE PANCREAS

PURIFICATION AND SOME PROPERTIES

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Summary

Porcine pancreas synthesizes a prephospholipase A_2 which occurs in a 5 : 95 ratio compared with the more abundant zymogen of the same enzyme* (phosphatide-acylhydrolase; EC 3.1.1.4).

These two prephospholipases could be well separated by CM-cellulose chromatography. Both the active and the zymogen form of the isoenzyme were isolated and purified.

The activation peptides of both prephospholipases appeared to be identical, while the active enzymes showed a few interesting differences. The most striking differences were the loss of one histidine and one methionine in the isoenzyme, corresponding to residues 24 and 27, respectively, in α-phospholipase A_2.

The positional and stereo specificity of both enzymes are the same, but the specific activity of the β-phospholipase A_2 is lower.

The molecular weight of the isoenzyme was estimated to be about 14 000, while the isoelectric points were 5.1 and 5.9 for the isoprecursor and active isoenzyme, respectively.

Introduction

Phospholipase A_2 (EC 3.1.1.4) catalyzes specifically the hydrolysis of fatty acid ester bonds at the 2-position of 1,2-diacyl-*sn*-phosphoglycerides [3]. This enzyme has been isolated from many sources, including snake venoms [4—7], bee venom [8] and pancreatic tissues of pig [1], ox [9,10], rat [11] and man [12,13]. Some rather common properties of all these phospholipases are heat stability, a relatively high number of disulfide bridges, a molecular

Abbreviation: Tos-PheCH_2Cl, L-(tosylamido-2-phenyl)ethyl chloromethyl ketone.

* In this paper the newly discovered zymogen will be denoted as iso- or β-prephospholipase A_2, whereas the previously isolated [1,2] more abundantly occurring precursor will be called the α-prephospholipase A_2. The same denotation will be used for the phospholipase A_2.

weight of approx. 14 000, and an absolute requirement of Ca^{2+} for enzymatic activity. Furthermore, it appears that phospholipases exist in pancreatic tissues as zymogens, which are converted to active enzymes via limited proteolysis by trypsin [2,10—12].

De Haas et al. [1,2] and Nieuwenhuizen et al. [14] have described the isolation and purification of α-phospholipase A_2 and an enzymatically inactive zymogen form from porcine pancreas. By applying milder procedures during the early stages of the purification, Nieuwenhuizen et al. [15] were able to isolate two different prephospholipases A_2*, both of which yielded after limited tryptic proteolysis the same active α-enzyme. In large scale purifications, starting from some 70 to 80 kg of fresh porcine pancreas, it appeared that there were ion-exchange column chromatography fractions with prephospholipase A_2 activity, that could not be attributed to the α-prephospholipase A_2. These fractions subsequently gave rise to the β-prephospholipase A_2.

The isoenzyme appeared to differ only slightly in amino acid composition from the more abundant α-phospholipase A_2; only one methionine and two histidine residues, instead of two and three, respectively, seeming the most important differences. In elucidating the mechanism of action of phospholipase A_2, we feel that the study of the isoenzyme can be very important in problems where the α-enzyme cannot be used, or not as easily as the β-enzyme.

In the present study we describe the isolation and purification of the β-prephospholipase A_2. In addition some properties of the isoenzyme are reported.

Materials and Methods

Fresh porcine pancreas was obtained from the local slaughterhouse. Unless otherwise mentioned, the material was kept at about 0—4° C throughout all following purification steps.

Ion-exchange chromatography was performed on DEAE- and CM-cellulose, obtained from Whatman (DE-52 and CM-52) and treated according to the instructions of the manufacturer. For adsorption chromatography hydroxylapatite (Biogel) was used.

Purity was ascertained by starch and disc gel electrophoresis. The starch gel electrophoresis was performed on microscopic slides covered with 12% hydrolysed starch (Connaught) in 0.05 M acetate buffer, pH 5.5. Samples were subjected to 100 V, during 1.5—2.5 h. The strength of current was 10—15 mA per slide. Detection was achieved by a 1% solution of amido black in methanol/water/acetic acid (5 : 5 : 1, by vol). Disc gel electrophoresis was carried out on polyacrylamide gels (7.5% acrylamide, pH 8.5) based on the method described by Ornstein and Davies [16]. Conditions were 100 V, 1—1.5 h, 5 mA per tube. Fixing and staining was done during 30 min in 0.5% amido black in 7% acetic acid. Destaining occurred electrophoretically in 7% acetic acid during 15 h at about 1 mA per tube.

Phospholipase A_2 activity was assayed at 40° C and pH 8.0 in a pH-stat

* This has been proved to arise from elastase activity in the pancreas, by which the four N-terminal amino acids of the activation peptide of the α-prephospholipase A_2 are split off.

(Radiometer) using an aqueous emulsion of egg yolk as substrate, in the presence of sodium deoxycholate and Ca^{2+}, according to the method previously described in detail by de Haas et al. [1]. The content of prephospholipase A_2 was measured likewise after maximal conversion of the zymogen into phospholipase A_2 by trypsin. Specific activities are given by the uptake of μequiv of alkali per min and per mg of protein.

The molecular weight of the protein was determined by gel filtration on Sephadex G-75 (Pharmacia) according to the description of Whitaker [17] and by calculation based on the amino acid composition.

Measurement of the absorbance at 280 nm of an accurately weighed quantity of protein, dried during 24 h under high vacuum above P_2O_5, yielded a value of the molecular extinction.

The isoelectric points were determined by electrofocussing on an LKB column (LKB 8101), thermostated at 0°C, with the LKB ampholine solution, pH range 3—10. The anode solution was 1% H_2SO_4 and the cathode solution 12 g sucrose in 0.4 ml ethylenediamine and 40 ml water.

DL-1-Palmitoyl-2-oleoyl-lecithin* was used to determine the positional and stereo specificity of the isoenzyme, according to the method described by De Haas et al. [1]. This synthetic lecithin was prepared as described by Bonsen et al. [18].

Hexoses and pentoses were determined by the phenolsulphuric acid method of Dubois et al. [19], and hexosamines according to Marshal et al. [20].

Amino acid compositions were obtained on a Beckman Unichrom amino acid analyser according to the method described by Spackman et al. [21]. Proteins and peptides were hydrolysed during 24, 48 and 72 h at 110°C in evacuated sealed tubes containing 6 M HCl. Tryptophan content was determined by hydrolysis in the presence of thioglycolic acid as described by Matsubara et al. [22].

Isolation of tryptic peptides was achieved by chromatography on Sephadex G-15 or G-25 (Pharmacia), followed by high-voltage electrophoresis (3000 V; in pyridine/acetic acid/water (25 : 1 : 475, by vol.) and descending chromatography (solvent: butanol/acetic acid/water (4 : 1 : 5, by vol.)), both on Whatman-papers no. 1 and 3 MM.

Trypsin and Tos-PheCH_2 Cl-trypsin were obtained from Serva.

Results and Discussion

Purification procedure

In Table I are summarized the results of the purification procedures. The early steps corresponded to those previously described in detail by Nieuwenhuizen et al. [14], but adapted to about 75 kg of starting material.

Fresh porcine pancreas was successively defatted and homogenised in 0.1 M NaCl (3 volumes per 1 volume of pancreas). This crude homogenate was then brought to pH 4 with concentrated HCl, followed by heating in metal

* For the sake of simplicity, this name will be used. According to the tentative rules for lipid nomenclature (I.U.P.A.C.) the preferred name for this compound is 1-hexadecanoyl-2-9(*cis*)-octadecenoyl-*rac*-glycero-3-phosphorylcholine.

TABLE I

PURIFICATION OF PREPHOSPHOLIPASE A_2

75 kg of defatted porcine pancreas were homogenised at 0°C in 0.1 M NaCl (NaCl solution: pancreatic tissue: 3 : 1, w/w)

Purification step	Activity in units × 10^{-6} *			Specific activity**			Yield (%)***		
	α-Precursor	β-Precursor	Total	α-Precursor	β-Precursor	Total	α-Precursor	β-Precursor	Total
1. Crude homogenate			—			—			—
2. Heat treatment			72			12			100
3. Salting out procedure			47			164			65
4. DEAE-cellulose (pH 7.3)			35			820			48
5. CM-cellulose (pH 6.0)	29	1.1		1300	700		40	1.5	
6. CM-cellulose (pH 5.6)		1.0			800			1.5	

* Expressed in units × 10^{-6} (1 unit = amount of enzyme that hydrolyses 1 μmol substrate per min. This means for step 1—4 potential (precursor) and direct (active form) units. Units are determined as phospholipase A_2 after maximal activation of the zymogen with trypsin).

** Expressed in μequiv acid released per min and per mg of protein. Protein concentration was determined from the molar extinction coefficient at 280 nm ($E^{1\%}_{280\,nm}$ = 13).

*** Yield in grams from 75 kg of pancreas is 22.5 and 1.2 for the α- and β-precursor, respectively.

buckets to 70°C for 3 min. The slurry was rapidly brought back to a temperature of about 4°C by cooling the metal buckets in an acetone/CO_2 mixture. After centrifugation for 10 min at 4000 rev./min in a WKF centrifuge G 50-K, the resultant supernatant was freed of remaining fat particles by addition of Hyflo Supercel (5 g per l supernatant) and filtration on a Büchner funnel. The filtrate was treated with $(NH_4)_2SO_4$ to 55 and 75% saturation, and the intermediate precipitate was collected after stirring during 1 h at 4°C, pH 7.0, by centrifuging 10 min at 4000 rev./min and 4°C. The precipitate was subsequently dissolved in a minimal amount of water and the trypsin inhibitor diisopropylphosphorofluoridate (DFP) was added to a final concentration of 10^{-2} M. After stirring at room temperature for 4 h, the solution was dialysed overnight at 4°C and then lyophilised. A crude chromatography on DEAE-cellulose, equilibrated with 0.005 M Tris · HCl buffer, pH 7.3, was attempted to bring about a separation of the proteins that are not bound by the ion exchanger at this pH from the proteins that can be eluted by 0.1 M NaCl. It appeared that only the fraction eluted by 0.1 M NaCl contained (pre)phospholipase A_2 activity. This fraction was then dialysed and lyophilised and next dissolved in water. DFP was added to a final concentration of 10^{-2} M and the solution was stirred for 1 h. After dialysing against 0.005 M acetate buffer, pH 6.0, the solution was chromatographed on a CM-cellulose column equilibrated with this dialysis buffer. Fig. 1 shows the elution pattern of this purification step. After combined recovery of the anionic, unabsorbed proteins, which contained no (pre)phospholipase A_2 activity, a linear salt gradient (0—0.4 M NaCl) was applied to the column. It is clear that β-prephospholipase A_2 activity (fraction II) was well separated from α-prephospholipase A_2 activity (fraction IV). Frac-

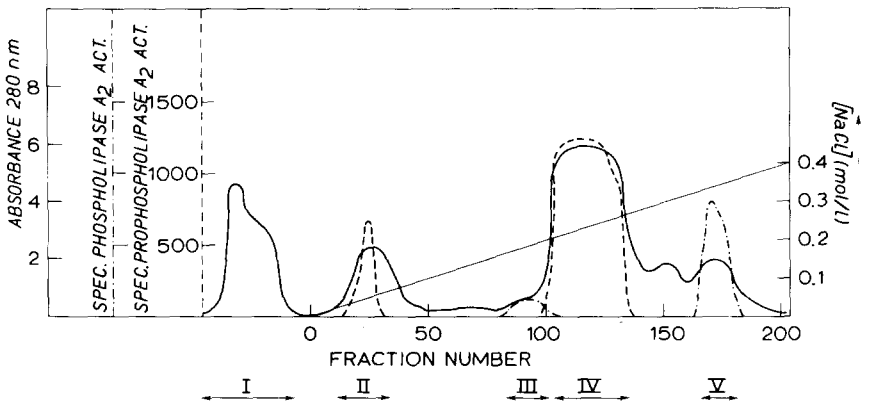

Fig. 1. Elution pattern of (pre)phospholipase A_2 from a CM-cellulose column at pH 6.0 (step 5, Table II). ———, absorbance at 280 nm; — · — ·, direct specific phospholipase A_2 activity (assayed in the egg yolk system without tryptic activation) in units/mg protein; - - - - - -, specific prephospholipase A_2 activity, determined after maximal activation with trypsin as phospholipase A_2 in units/mg protein. Elution buffer was 0.005 M sodium acetate/acetic acid, pH 6.0. Fraction I contains anionic proteins washed off from the column by the acetate buffer. A linear NaCl gradient resulted in the following fractions: II, β-prephospholipase A_2; III, β-phospholipase A_2; IV, α-prephospholipase A_2; V, α-phospholipase A_2.

tion V contained α-phospholipase A_2. A fraction of active isoenzyme (fraction III) eluted with the α-prephospholipase A_2, indicating that the trace of direct phospholipase A_2 activity that is always present (even immediately after purification) in samples of α-prephospholipase A_2, is due probably to isophospholipase A_2 rather than to active α-enzyme resulting from activation of the α-precursor. The purification of the isoprecursor to a homogeneous preparation was finally achieved by a second chromatography on CM-cellulose, this time at a more acidic pH (pH 5.6) and with a shallower gradient. The β-prephospholipase A_2 gave a single band on electrophoresis, at pH 5.5 on starch and at pH 8.5 on polyacrylamide. Conditions were such that the two zymogens were clearly separated (Fig. 2). In order to establish whether the isolated β-prephospholipase A_2 (fraction II) was the precursor of the directly active phospholipase A_2 in fraction IV, this active isoenzyme was also further purified. For that purpose the proteins of fraction III were incubated at pH 8.0 with 1% (w/w) trypsin in order to convert contaminating α-prephospholipase A_2 to the enzymatically active form. Chromatography on CM-cellulose achieved a separation of both phospholipases A_2 (fractions III_A and V_A; Fig. 3). Fraction III_A, not yet homogeneous, was chromatographed on DEAE-cellulose at pH 8.0 (0.005 M Tris; NaCl gradient 0—0.15 M). The isoenzyme was likewise purified to homogeneity by adsorption chromatography on hydroxylapatite with a linear phosphate buffer (0—0.3 M; pH 6.8) (Fig. 3b). Fraction III_B showed a constant specific activity across the peak and gave a single band on electrophoresis.

Activation of β-prephospholipase A_2

Activation of the isoprecursor could be achieved by incubating with 1% (w/w) trypsin at pH 8.0 and room temperature. The reaction mixture also contained Ca^{2+} (10^{-2} M) to protect the activated phospholipase A_2 against

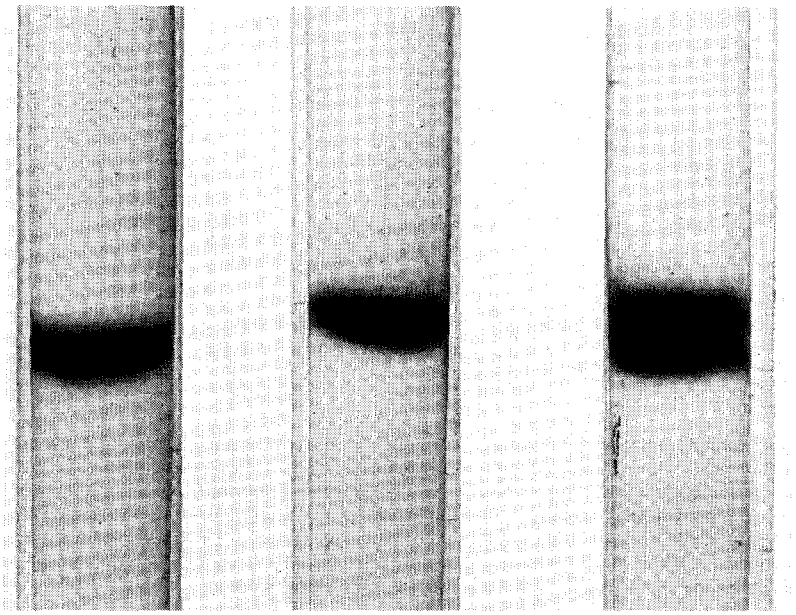

Fig. 2. Polyacrylamide gel electrophoresis of prephospholipases A_2 at pH 8.5. 100 V were applied for 1 h to 7.5% acrylamide gels (5 mA per gel). Fixing and staining was achieved by 0.5% amido black in 7% acetic acid; destaining was done electrophoretically at 1 mA per gel in 7% acetic acid. From left to right: 1, β-prephospholipase A_2; 2, α-prephospholipase A_2; 3, both prephospholipases A_2.

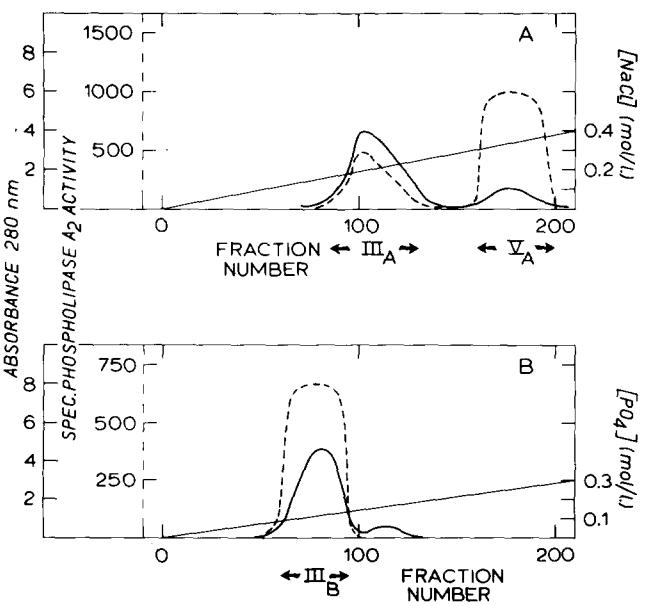

Fig. 3. Chromatography of fraction III (Fig. 1) after maximal tryptic activation. ———, absorbance at 280 nm; - - - - - -, specific phospholipase A_2 activity in units/mg protein. (a) Chromatography on CM-cellulose in 0.005 M sodium acetate/acetic acid buffer, pH 6.0, using a linear salt gradient of 0—0.4 M NaCl. Fraction III_A, β-phospholipase A_2 (not homogeneous); fraction V_A, α-phospholipase A_2 (homogeneous). (b) Chromatography of fraction III_A on hydroxylapatite in water, using a linear gradient of 0—0.3 M phosphate buffer, pH 6.8. Fraction III_B, β-phospholipase A_2 (homogeneous).

trypsin and to stabilize trypsin itself [23]. After attaining maximal activity, a fresh solution of DFP (1 g per 55 ml isopropanol) was added until the final DFP : protein ratio was about 5. This mixture was stirred for 1 h and then chromatographed on CM-cellulose. Elution with 0.005 M acetate buffer, pH 6.0, containing a linear gradient of NaCl (0—0.4 M) yielded two well-separated peaks of 206-nm absorbing material. The second of these peaks, which was eluted from the column at about 0.2 M NaCl, gave absorption at 280 nm and appeared to possess phospholipase A_2 activity. This fraction was dialysed and lyophilised. The first fraction was concentrated by lyophilisation, desalted on Sephadex G-25 in 10^{-3} M HCl, and again lyophilised. The dry powders, which represented a recovery of about 75%, were then hydrolysed in 6 M HCl for subsequent amino acid analysis. The results of these experiments are summarized in Table II. The first fraction contained a peptide, having the same amino acid composition as the activation peptide of the α-precursor. Moreover, both peptides showed the same migration behaviour on high-voltage paper electrophoresis and descending paper chromatography. On this basis we concluded that both peptides are identical. Furthermore, it appeared that the phospholipase A_2 arising from activation of β-prephospholipase A_2 possessed the same amino acid composition as β-phospholipase A_2 (fraction III_C; Fig. 3). Finally, the activated isoenzyme could not be separated electrophoretically from the isolated β-enzyme. These observations strongly suggest that the purified isophospholipase A_2 is indeed the active enzyme derived from the β-prephospholipase A_2.

TABLE II

AMINO ACID COMPOSITIONS

Amino acid	α-Precursor	Activation peptide of α-enzyme	α-Active enzyme	Iso-precursor	Activation peptide of iso-enzyme	Active iso-enzyme	CNBr fragment I (iso)	CNBr fragment II (iso)	Active site histidine peptide (iso)	Second histidine peptide (iso)
Asx	23	—	23	23	—	23	—	23	2	—
Thr	6	—	6	6	—	6	—	6	1	—
Ser	12	2	10	11	2	9	1	8	—	—
Glx	9	2	7	8	2	6	1	5	1	1
Pro	5	—	5	5	—	5	—	5	—	—
Gly	7	1	6	7	1	6	—	6	—	—
Ala	8	—	8	7	—	7	1	6	—	—
Cys (half)	12	—	12	12	—	12	—	12	2	—
Val	2	—	2	2	—	2	—	2	—	—
Met	2	—	2	1	—	1	1*	—	—	—
Ile	6	1	5	6	1	5	—	5	—	—
Leu	7	—	7	9	—	9	1	8	—	—
Tyr	8	—	8	8	—	8	—	8	1	—
Phe	5	—	5	5	—	5	1	4	—	—
Trp	1	—	1	1	—	1	1	—	—	—
Lys	9	—	9	9	—	9	—	9	—	1
His	3	—	3	2	—	2	—	2	1	1
Arg	5	1	4	5	1	4	1	3	1	—

* Determined as HoSer and HoSer-lacton.

We routinely isolate phospholipases as their inactive precursors. Since the pancreas contains only these zymogens, enzymatically active phospholipases A_2 (fractions III and V, Fig. 1) probably originate during the isolation procedures as a result of activation by traces of trypsin. In practice isolation of the precursors is easier, since they exhibit a lower affinity for endogenous lipids, and can be purified from impurities with similar isoelectric points, after activation by trypsin followed by ion-exchange chromatography.

Comparison of both phospholipases A_2

As seen in Table II, the amino acid composition of the isoenzyme was only slightly different from that of the α-enzyme. Besides differences in the number of serine, glutamine, alanine and leucine residues, the isoenzyme contains one residue less of methionine and histidine. In order to establish the sequential position of the missing methionine and histidine residues in the isoenzyme, in comparison with the already established (Fig. 4) sequence of the α-enzyme [24], the following experiments were performed.

CNBr cleavage. 50 mg isophospholipase A_2 was incubated under nitrogen and protected from light with 50 mg CNBr in 70% (v/v) trifluoroacetic acid [25] for 24 h at room temperature. The incubation mixture was then lyophilised and redissolved in 10^{-3} M HCl. Filtration on Sephadex G-25 in 10^{-3} M HCl yielded two peptides (with a recovery of 80%), suggesting that the sensitive methionine residue is positioned before the first disulfide bridge (Fig. 4). This

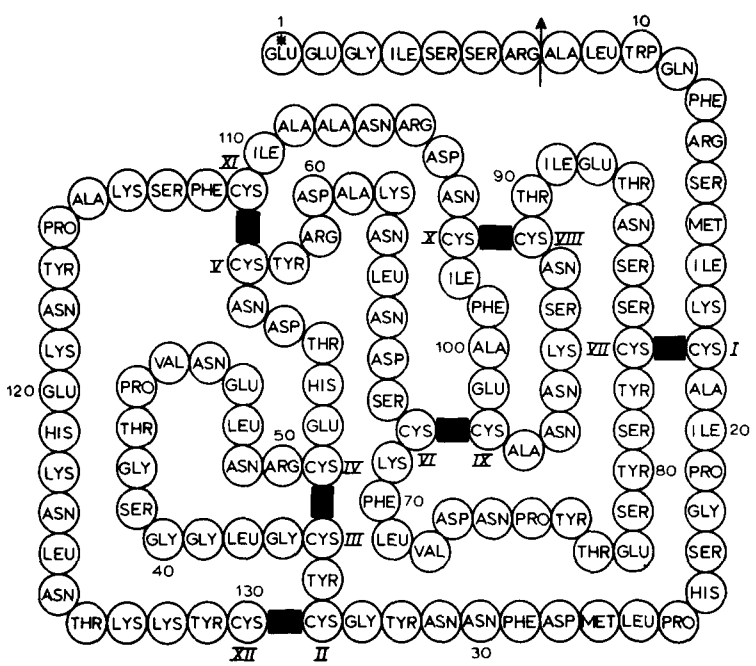

Fig. 4. Primary structure of porcine pancreatic α-prephospholipase A_2. *Glu (residue 1) stands for pyroglutamic acid. The arrow between residues Arg^7 and Ala^8 marks the place of attack by trypsin at the zymogen-enzyme conversion.

was confirmed by amino acid analysis of both fragments (Table II). The smaller fragment has an amino acid composition identical to the portion of the α-phospholipase A_2 Ala^8—Met^{15}.

A peptide map of a tryptic digest of S-sulfonated isoenzyme yielded only two histidine-positive Pauly spots. This observation furnishes further evidence, that the β-phospholipase A_2 contains only two histidine residues.

Isolation of histidine-containing peptides. Volwerk et al. [26] have presented evidence that residue His^{53} in α-phospholipase A_2 is part of the active site of the enzyme. To determine if this was also true for the isoenzyme, this protein was incubated with the radioactively labeled, active site-directed inhibitor *p*-bromo[^{14}C]phenacylbromide. After complete inactivation, disulfide bridges were cleaved by S-sulfonation, and the protein was then digested with 2% (w/w) Tos-PheCH_2Cl-trypsin at pH 8.0 and 37° C for three 6-h periods. The peptides were separated first on Sephadex G-15 in 10^{-3} M HCl and subsequently purified by high-voltage paper electrophoresis and descending paper chromatography. A single radioactively labeled peptide was isolated (recovery: 50%), which appeared to have the same amino acid composition as the active site His^{53}-containing peptide (Cys^{51}—Arg^{59}) of the α-enzyme. Both peptides also showed similar chromatographic and electrophoretic behaviour. We therefore conclude that both peptides are identical.

Tryptic digestion of intact β-phospholipase A_2 also yielded the tripeptide, Glu-His-Lys, indicating that residue His^{121} is also present in the isoenzyme.

It is now clear that, in comparison to the amino acid sequence of the α-enzyme, the missing methionine and histidine residues in the isoenzyme are Met^{27} and His^{24}.

Properties

The molecular weight of the β-phospholipase A_2 was determined by gel filtration on Sephadex G-75 in 0.75 M NaCl according to Whitaker [17]. The void volume of the column was determined with Dextran blue 2000, and a calibration curve was made using, as standard proteins, ribonuclease (mol. wt 14 000), porcine pancreatic α-phospholipase A_2 (mol. wt 14 000), α-chymotrypsin (mol. wt 22 500) and pepsin (mol. wt 35 500). The elution volume of the β-phospholipase A_2 corresponded to a molecular weight of 14 000 ±5%. The molecular weight calculated from the amino acid composition was 13 500.

The extinction coefficient $E_{1\,cm}^{1\,\%}$ of the β-enzyme at 280 nm was 13.

The isoenzyme was not a glycoprotein, since no more than 10—15 mol % carbohydrate could be detected.

To determine the isoelectric points of the phospholipases A_2 and their zymogens 10 mg of protein, containing a mixture of zymogen and active enzyme, was loaded on an electrofocusing column. A potential of 600 V was applied to the column for 60 h and the current never exceeded 2.5 mA. The pH and the absorbance at 280 nm were measured in 60 equal column fractions. Measurement of the enzymatic activity in the egg yolk assay allowed the identification of the active enzymes (Fig. 5). The isoelectric points were 5.1 and 5.9 for the β-precursor and the active isoenzyme, respectively, and 5.6 and 6.3 for the α-precursor and the active α-enzyme, respectively.

When determining the positional and stereo specificity of the β-phospho-

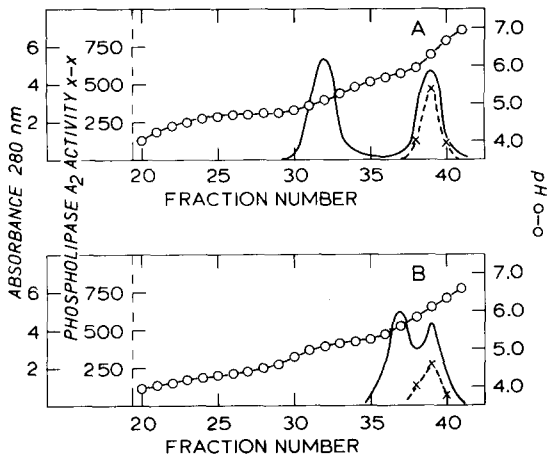

Fig. 5. Isoelectric focusing of (pre)phospholipases A_2 from porcine pancreas. The focusing was carried out during 60 h at 600 V in an LKB 8101 column using the LKB ampholine solution, pH range 3—10. Maximum current was 2.5 mA. The column was loaded with about 10 mg of protein. (a) Isoelectric focusing of β-prephospholipase A_2 and α-phospholipase A_2. (b) Isoelectric focusing of α-prephospholipase A_2 and β-phospholipase A_2.

lipase A_2 by incubation at pH 7.2 of 1 mg of the enzyme with 10 mg of DL-1-palmitoyl-2-oleoyl-lecithin in the presence of 10 mg sodium deoxycholate and 3.3 mg $CaCl_2$ (final volume: 1.1 ml), according to the method of De Haas et al. [1], it appeared that oleic acid was the only fatty acid released from DL-1-palmitoyl-2-oleoyl-lecithin; all palmitic acid remained in the lysolecithin fraction. It was thus concluded that the β-phospholipase A_2 acts stereo specifically on the 2-position of L-lecithins.

Yamaguchi et al. [27] have also described the presence of two thermostable phospholipases in porcine pancreas. Although there was no conclusive evidence that these enzymes were phospholipases A_2, it might be that they correspond to the enzymes we have isolated. The isoelectric points they have measured were nearly identical to those reported in the present investigation. However, there is a remarkable difference in the relative distribution of the two isoenzymes. While we found a ratio of about 5 : 95, Yamaguchi et al. [27] reported a ratio of about 40 : 60. Furthermore, we were successful in separating our prephospholipases on polyacrylamide gel electrophoresis.

Tsao et al. [28] have isolated multiple forms of phospholipase A_2 from commercial porcine pancreatin, that differ significantly in amino acid composition from our enzymes. With another assay system, which we did not try, they showed that some of their enzyme activity went undetected when the conventional egg yolk emulsion was used as substrate. However, they did not report the isolation of zymogen forms of the phospholipases, because in the source used, these forms would most likely not be present, due to activation by proteolytic enzymes. It cannot be ruled out that proteolysis was not limited to activation of precursors; the active enzymes may also have been attacked.

In the elucidation of the mechanism of action of phospholipase A_2, the isoenzyme, whose isolation is now reported, could be of great importance primarily because its amino acid composition contains only one methionine

and two histidine residues. In future reports we will present a comparison of the kinetics of both enzymes and detailed information about the function of the unique methionine residue in the isoenzyme.

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