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The Activation, Purification and Properties of Rat Pancreatic Juice Phospholipase A^2

BO ARNESJÖ and ANDERS GRUBB

Division of Physiological Chemistry, Chemical Center, University of Lund P.O. Box 740, S-220 07 Lund 7, Sweden

Rat pancreatic juice prophospholipase is converted to active phospholipase A^2 (phosphatide acyl-hydrolase EC 3.1.1.4) when digested with trypsin in concentrations above the trypsin inhibitory activity of the juice. The released phospholipase A is progressively inactivated by the added trypsin and the tryptically activated proteolytic zymogens present in the juice. In the absence of calcium this potential phospolipase A degrading activity in the juice is completely destroyed by heating at 75°C. By the use of relatively high concentrations of trypsin and in the absence of calcium the prophospholipase that is not affected by the heat treatment is then quantitatively activated at 75°C.

By the quantitative activation of the cationic rat pancreatic juice prophospholipase and by the use of chromatographic procedures the enzyme was purified 150 – 200 times. The protein obtained was chromatographically and electrophoretically homogeneous.

Approximately 0.6 % (w/v) of the pancreatic juice proteins is free and activable phospholipase A^2.

The isolated, active enzyme, which is susceptible to the action of trypsin and which hydrolyses exclusively the β-fatty acid ester linkage of lecithin is inactive in the absence of calcium and bile salt. Optimum activities were found at pH 8 – 9, above 0.02 M $CaCl_2$ and when lecithin as substrate was solubilized with bile salts in concentrations giving micellar solutions. Substrate inhibition occurs at relatively low concentrations of lecithin. The protease inhibitor of Kunitz (TRASYLOL) inhibits the enzymic activity probably by interaction with the bile salt anions, with consequent interference with the solubilisation of the lecithin substrate.

The phospholipase A activity of the duodenal contents is largely derived from the pancreatic secretion. [1–11] In the pancreas and pancreatic juice, however, it is present mainly as an inactive precursor – prophospholipase.[12–14] When secreted into the duodenum this zymogen form of phospholipase A is activated by trypsin and perhaps also by other proteolytic enzymes. During this activation, part of the molecule is split off. [12] In its active form phospholipase A hydrolyses almost exclusively the fatty acid ester linkage at the 2-position of lecithin.[12–14]

In the present study we describe the quantitative activation of the prophospholipase in rat pancreatic juice, the tryptic degradation, and the purification of the active phospholipase A and also the substrate requirements for the purified activated enzyme.

MATERIALS AND METHODS

Materials. All reagents used were of reagent grade purity. The solvents were redistilled. Chloroform was stabilized with 1.5 % methanol.

Rat pancreatic juice was obtained from male Sprague-Dawley rats (AB Anticimex, Stockholm) by cannulation of the common duct and diversion of the bile flow. The juice was collected into beakers chilled with dry ice and subsequently lyophilized. The rats had free access to the laboratory pellets and to an aqueous solution containing 2 % (w/v) sucrose, 2 % (w/v) glucose, 0.59 % (w/v) $NaHCO_3$, 0.35 % (w/v) NaCl, 0.11 % (w/v) KCl, and 0.018 (w/v) $CaCl_2 \cdot 2H_2O$. On this regiment the rats could be kept in relatively good conditions in restraining cages for periods up to 1 – 3 weeks producing pancreatic juice of high enzymic activity. 1 ml juice gave approximately 25 mg of powder, which contained 65 – 80 % (w/v) protein.

Lecithin was purified by silicic acid column chromatography.[11] Additional precipitation of the lecithin with acetone and extraction of the precipitate with diethyl ether was performed when indicated in the text. The lecithin preparation was free of impurities as judged by thin layer chromatography.[11,15]

Lysolecithin was prepared from purified lecithin according to the method of Hanahan.[16] The remaining traces of snake venom phospholipase were inactivated by reflux of the extracted lysolecithin in an ethanol solution for 2 h.

Sodium taurocholate and sodium taurodeoxycholate were synthetized according to Norman [17] as modified by Hofman.[18] (Purity better than 98 % as indicated by thin layer chromatography.)

Crystalline trypsin (TRYPURE NOVO) was purchased from Novo AG, Copenhagen.

Protease inhibitor (TRASYLOL) was purchased from Bayer AG, Leverkusen. As pointed out by Werle and co-workers [19] this protease inhibitor is identical with the protease inhibitor of Kunitz.

Sephadex G 25 Fine (batch No. 2726) and G 50 Fine (batch No. 5556) were purchased form AB Pharmacia, Uppsala.

CM-cellulose (microgranular CM 52 pre-swollen) was obtained from Whatman.

Methods. Protein was determined by the method of Lowry *et al.* [20] as modified by Eggstein and Kreutz [21] and phospholipid phosphorus according to Chen *et al.* [22]

Phospholipase A activity was assayed at 37°C by measuring the release of fatty acids during hydrolysis of purified lecithin. The substrate mixture contained lecithin dispersed by insonation in 0.025 M glycyl-glycine NaOH buffer containing bile salt and $CaCl_2$. Final volume of the incubation mixture 2 ml. The enzymic reaction was stopped by adding 2 ml ethanol:6 N H_2SO_4 40:1 and the released fatty acids extracted into 4 ml diethyl ether:heptane 1:1 by vigorous shaking. 2 ml of the upper phase was evaporated under a flow of nitrogen and redissolved in 3 ml absolute ethanol and titrated with standard NaOH using nile blue as indicator. The titration figures were related to a palmitic acid standard curve where fatty acid aliquots were subjected to the same extraction procedures as the incubation mixtures. Spontaneous hydrolysis of the lecithin never occurred in any of the different substrate mixtures. Controls for the content of fatty acids in the fractions to be tested were run by adding 2 ml ethanol:6 N H_2SO_4 40:1 before the substrate. Using this control method, virtually no free fatty acids were found in any of the fractions tested.

Chromatography on ion exchangers and on Sephadex was performed at 4°C and according to the instructions of the manufacturers. The absorbance at 280 mμ of the colum effluents was continuously registered with an LKB Uvicord II.

Agar gel and cellulose acetate electrophoresis were carried out in TRIS buffer, ionic strength 0.05, pH 8.5 – 9.5, on microscopic slides and Schleicher-Schüll strips, respectively. The samples were run for 40 min at 3.5 mA and 5 V/cm.

RESULTS AND DISCUSSION

Prophospholipase activation

Figs. 1 and 2 show the dependence of the rat pancreatic juice prophospholipase activation on the time of digestion with trypsin and on the amount of added trypsin. At 37°C, pH 7.6, and in the presence of 0.02 M $CaCl_2$ maximal activation of the prophospholipase present in 0.2 mg of juice powder is reached after 2.5 min with 10 – 20 μg of trypsin. Prolongation of the time of activation or increase of the added amounts of trypsin, however, causes a decrease of the measurable phospholipase A activity. Thus the addition to rat pancreatic juice of trypsin in amounts exceeding its trypsin inhibitory activity[13]

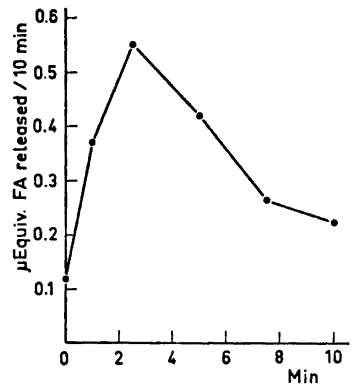

Fig. 1. Influence of preincubation time on the activation of prophospholipase at 37°C. 0.2 mg lyophilized rat pancreatic juice was preincubated at 37°C for different time intervals in 1 ml 0.05 M TRIS-HCl buffer, pH 7.6, 0.05 M in $CaCl_2$, containing 10 μg trypsin. The mixtures were then directly used for assay of phospholipase activity.

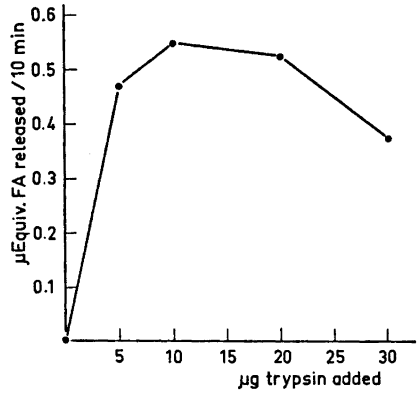

Fig. 2. Influence of the trypsin concentration on prophospholipase activation at 37°C. 0.2 mg lyophilized rat pancreatic juice was preincubated for 2.5 min at 37°C in 1 ml 0.05 M TRIS-HCl buffer, pH 7.6, 0.05 M with respect to $CaCl_2$, containing different amounts of trypsin. The mixtures were then directly used for assay of phospholipase activity.

not only causes activation but also inactivation of the phospholipase A activity. This inactivation, in turn, can be due either to direct tryptic attack or to indirect attack *via* tryptic activation of protease precursors present in the juice. To avoid this inactivating effect of added trypsin, the outstanding heat stability of the phospholipase A is used. Fig. 3 shows the dependence of prophospholipase activation on the time of preincubation and on the presence of calcium ions under the same conditions as described in Fig. 2 but when the preincubation at 37°C was stopped by heating at 75°C for 10 min. As demonstrated by the zero time value, activation of the prophospholipase can occur at 75°C. The inactivation of the activated enzyme is of lesser degree the shorter the tryptic digestion at 37°C is allowed to continue. In the presence of calcium ions almost total inactivation is found even with

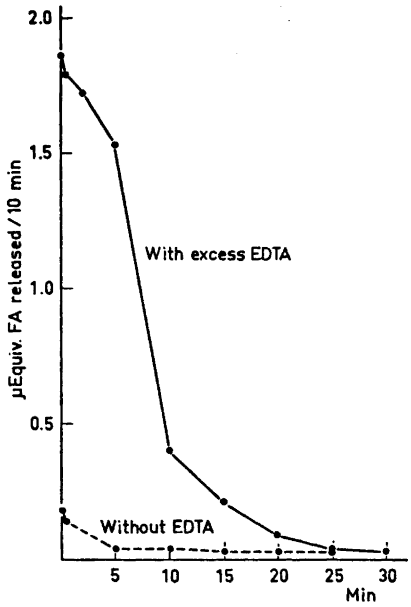

Fig. 3. Influence of heating after varying times of prophospholipase activation at 37°C in the presence or the absence of calcium ions. 1 mg lyophilized rat pancreatic juice was predigested at 37°C for different time intervals with 10 µg trypsin dissolved in 1 ml 0.05 M TRIS-HCl buffer, pH 7.6, either 0.05 M in $CaCl_2$ or 2 % in EDTA. The predigestion was stopped by transfer of the test tubes to a water-bath at 75°C for 10 min. Aliquots were taken for assay of phospholipase activity.

direct transfer to 75°C of the mixture made at 37°C. Judging from these results the optimal activation of the pancreatic juice prophospholipase is obtained by immediate transfer to 75°C of the activation mixture made at 37°C. During the short period of rise in temperature, however, not only the added trypsin but also the activated trypsinogen and perhaps also other trypsin activable proteinases of the juice can influence on the activation and inactivation of the phospholipase A. As the contents of trypsinogen and other protease precursors of the juice vary, the phospholipase activities formed under these conditions are not only a function of the prophospholipase content but also of the varying trypsin activable proteases. In order to obtain phospholipase activities independent of these varying endogenous proteinase concentrations it is thus necessary to eliminate their influence before the activation is performed. This can be achieved by heating the juice for 10 min at 75°C in the absence of calcium ions – a treatment not affecting the phospholipase A activity (see Enzyme stability). After addition of the activating trypsin the heating is then continued at 75°C for 10 min. In order to keep the temperature almost constant the activator solution (at 25°C) must be added in volumes not exceeding 5 % of the total volume. These procedures give highly active phospholipase A preparations, the activities of which do not diminish even with prolonged storage for a period of at least two weeks at 4°C. Fig. 4 shows that, under these conditions, the phospholipase A activity increases with increasing amounts of added trypsin and that at high concentrations of trypsin in the absence of calcium a plateau on the curve is formed indicating complete activation and virtually no inactivation of the phospholipase A. In this activation procedure the concentration of lyophilized rat pancreatic juice must

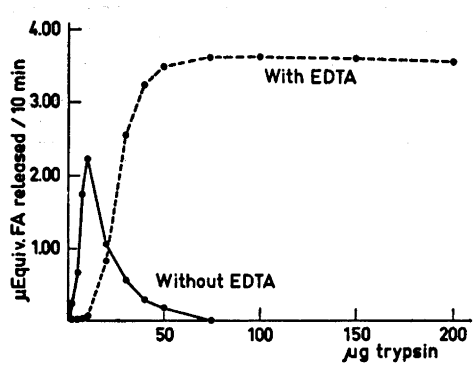

Fig. 4. Influence of trypsin on the activation at 75°C of the prophospholipase present in previously heated rat pancreatic juice. 1 mg lyophilized rat pancreatic juice dissolved in 0.75 ml distilled water was diluted to 0.95 ml with 0.1 M $CaCl_2$ or 10 % (w/v) EDTA. The mixtures were then heated at 75°C for 10 min. Directly afterwards varying amounts of trypsin dissolved in 0.05 ml 0.05 M TRIS-HCl buffer, pH 7.6, 0.02 M in $CaCl_2$, were added and the heating was allowed to continue at 75°C for another 10 min. After cooling to 4°C aliquots were taken for assay of phospholipase activity.

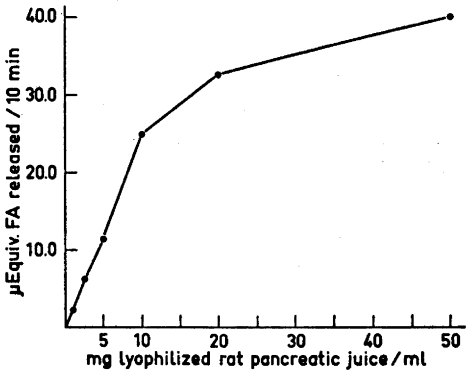

Fig. 5. Influence of pancreatic juice concentration on the activation of its content of prophospholipase. Different amounts of lyophilized rat pancreatic juice were dissolved in 0.75 ml distilled water and 0.2 ml 10 % EDTA and then heated at 75°C for 10 min. Immediately afterwards 50 µg trypsin/mg pancreatic juice was added, dissolved in 0.05 ml 0.05 M TRIS-HCl buffer, pH 7.6, 0.02 M in $CaCl_2$, and the heating was allowed to continue at 75°C for another 10 min. After rapid cooling to 4°C aliquots were taken for assay of phospholipase activity.

not exceed 10 mg/ml in order to get total activation of the prophospholipase present therein (Fig. 5). Also, as all other pancreatic juice enzymes capable of hydrolysing the fatty acid ester, linkages of lecithin are destroyed at 75°C,[13] this procedure can be used in the quantitative estimation of the prophospholipase A^2 activities of the rat pancreatic juice.

The autolytic release of free phospholipase A activity in ox and pig pancreas reported by Rimon and Shapiro[6] and De Haas *et al.*[10] indicates that during the period of autolysis trypsinogen and other proteinase zymogens are activated and then activate prophospholipase. At high concentrations of protein, however, this tryptic activation of prophospholipase is not total even at high temperatures (*vide supra*). Since trypsin and perhaps also other proteases are relatively heat stable in the presence of calcium they will inactivate the released and active phospholipase A during heating. These facts might explain the findings of Rimon and Shapiro[6] that phospholipase A activity autolytically released from ox pancreas can be totally inactivated by heat treatment in the presence of calcium and that afterwards intact prophospholipase can remain in the tissue.

De Haas *et al.*[10-12] found that during autolysis at room temperature porcine pancreatic homogenates release phospholipase A activity until a constant level is reached after 15 h. The subsequent heating at 70°C and at

high pH caused a considerable inactivation of the autolytically released phospholipase A. At pH 4, however, heating at 70°C for a relatively short time interval caused virtually no inactivation of the enzymic activity. These results can be due to the occurrence of an equilibrium during autolysis between proteolytic activation of prophospholipase and inactivation of the released phospholipase A, which is reached after 15 h, and to the fact that at pH 4 the proteolytic enzymes exhibit virtually no proteolytic activity. When subjecting pure porcine prophospholipase to the action of trypsin at 4°C the authors found a rectilinear relationship between phospholipase A release and the incubation time until complete activation was reached. No inactivation of the released phospholipase A by the added trypsin was reported even after prolonged incubation. This indicates that the phospholipase A inactivating effect found in pancreatic juice is not due to trypsin but to other trypsin activable proteolytic enzyme activities. In order to investigate these phenomena we decided to purify rat pancreatic juice phospholipase A.

Phospholipase purification

7.5 g lyophilized rat pancreatic juice was subdivided into 300 mg batches each of which was dissolved in 21 ml distilled water and 7.5 ml 10 % (w/v) EDTA. After heating at 75°C for 10 min, 15 mg trypsin in 1.5 ml 0.05 M TRIS-HCl buffer, pH 7.6, 0.02 M with respect to $CaCl_2$, was added and the heating continued for 30 – 60 min at 75°C. This period of time, longer than the 10 min described above, was required to achieve effective heat teatment of this large volume. The mixture was then rapidly cooled down to 4°C and after combination centrifuged at 4°C at 12 000 g_{av} for 10 min. The supernatant obtained was decanted and the sediment resuspended in distilled water and recentrifuged at 12 000 g_{av} for 10 min. The two combined supernatants were lyophilized, dissolved in 60 ml cold 0.04 M NH_4HCO_3 and centrifuged at 4°C at 4000 g_{av} for 10 min in order to remove the undissolved residue. The supernatant was subdivided into 3 parts each of which was chroma-

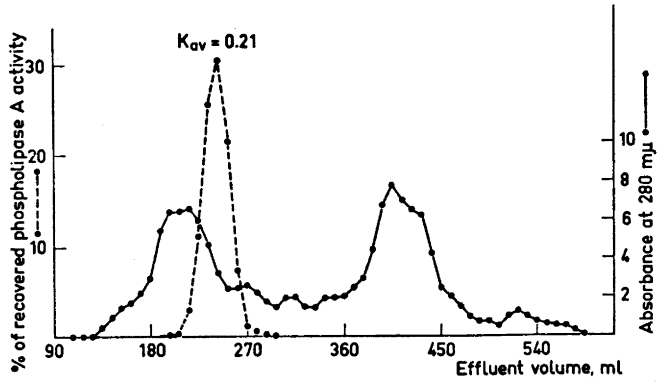

Fig. 6. Elution pattern of phospholipase A from a Sephadex G 50 column with 0.04 M NH_4HCO_3 at 4°C. Column dimensions 2.5 × 100 cm. Flow rate 9 ml/h.

tographed at 4°C on a Sephadex G 50 column (2.5 × 100 cm) equilibrated and eluted with 0.04 M NH_4HCO_3. A typical elution pattern is shown in Fig. 6. The phospholipase A containing fractions from the three separate Sephadex separations were combined, lyophilized and then chromatographed at 4°C on a CM cellulose column (1 × 30 cm) previously equilibrated with 0.005 M phosphate buffer, pH 7. After appearance of the anionic peak the remaining proteins were eluted with a linear gradient, 0.02 – 0.13 M NaCl, in the same buffer. The elution pattern obtained is demonstrated in Fig. 7. All fractions

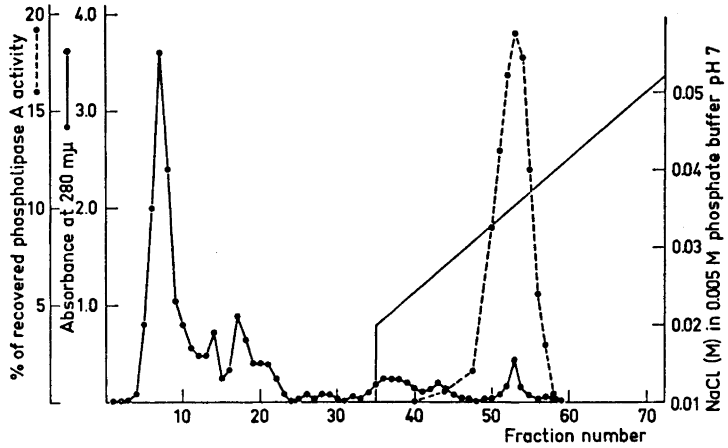

Fig. 7. Elution pattern of phospholipase A from a CM-cellulose column (1 × 30 cm) equilibrated at pH 7 with 0.005 M phosphate buffer.

containing phospholipase A were combined, lyophilized and then desalted by chromatography on a small (1 × 10 cm) Sephadex G 25 column using water as the eluent. The recovery from this column was not quantitative. When testing different methods for desalting of the obtained phospholipase A, however, this method gave better recoveries than either dialysis or ultrafiltration. All protein containing fractions were combined and lyophilized.

50 μg of protein obtained in this procedure revealed only one band on agar gel and cellulose acetate electrophoresis at pH 8.5 – 9.5. The purified rat pancreatic juice phospholipase A had a specific activity of 50 μequiv. fatty acid released/min/mg protein. Compared with the specific phospholipase A activity of totally activated juice, 0.25 – 0.35 μequiv., the enzyme has been purified 150 – 200 times. The yield in the procedure was 42% of the total active and activable phospholipase A of whole juice (Table 1). 0.5 – 0.7% of the rat pancreatic juice proteins is free or potential phospholipase A.

Initially lyophilized native rat pancreatic juice after desalting on Sephadex G 25 was chromatographed on DEAE-cellulose. Prophospholipase always appeared in the cationic break through fractions even at pH 9. During chromatography of desalted intact juice on CM cellulose at pH 8 the phospholipase A precursor was retained on the column. Thus rat pancreatic juice prophos-

Table 1. Purification of rat pancreatic juice phospholipase A. Pancreatic juice was produced by cannulation of the common duct and diversion of the bile flow in male Sprague-Dawley rats. The juice was collected into beakers chilled with dry ice and subsequently lyophilized. 7.5 g lyophilized rat pancreatic juice was used as the starting material.

Step	Total activity μequiv. FA released/min	Specific activity μequiv. FA released/min/mg protein	Yield %
1. Pancreatic juice	1248	0.256	–
2. Heat treatment and activation at pH 7.6 and at 75°C	1061	1.85	85
3. G 50 Sephadex and lyophilization	899	9.60	72
4. CM cellulose and lyophilization	811	48.60	64
5. Desalting on G 25 Sephadex and lyophilization	524	50.70	42

pholipase is a cationic protein, while the porcine prophospholipase has anionic properties.[12] In its activated form rat pancreatic phospholipase A seems to have approximately the same isoelectric point as the porcine enzyme, *i.e.* about 7.4 During chromatography of the intact pancreatic juice proteins on CM cellulose at pH 8 the active phospholipase A was found partly in the anionic and partly in the cationic fractions in which it was eluted at a lower ionic strength than the prophospholipase. As the free enzymic activity was spread all over the "early" cationic fractions and the recovery was found to be up to 10 times the applied activity the results indicate a high degree of activation of the prophospholipase while it was retained on the CM cellulose column. This activation was presumably due to the presence of traces of trypsin produced from trypsinogen after its separation from trypsin inhibitor(s) on the column.

Substrate requirements and kinetics for pure active rat pancreatic phospholipase A

Incubation time. Fig. 8 A shows the time-activity curve for the hydrolysis of lecithin using optimal concentrations of lecithin, bile salt, and calcium. The reaction follows zero order kinetics until 50% of the substrate is converted. At the maximal concentrations of enzyme used in this study this usually does not occur within 10 min. As indicated by incubation with ^{3}H-β-acyl lecithin as the substrate only the labeled fatty acid is hydrolyzed.

pH. The dependence of the phospholipase A activity on the pH of the incubation medium is shown in Fig. 8 B. Lecithin solubilized with bile salt is hydrolyzed over a wide pH range, *i.e.* pH 7 – 10; optimum activity is found at pH 8 – 9.

Bile salts. Virtually no phospholipase A activity is found when bile salts are excluded from the incubation medium. As demonstrated in Fig. 8 D the

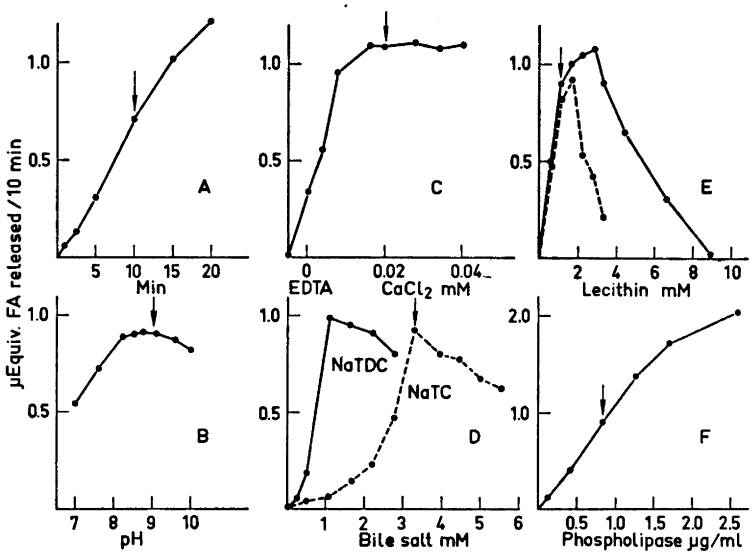

Fig. 8. The influence of A. incubation time, B. pH, C. calcium, D. bile salt, E. substrate concentration and F. enzyme concentration on the activity of pure rat pancreatic phospholipase A. When the influence of each particular parameter was tested the others were kept constant at the levels indicated by arrows in the separate figures. NaTDC = sodium taurodeoxycholate, NaTC = sodium taurocholate. In Fig. E the dotted line represents lecithin freshly prepared by silicic acid chromatography and then subjected to acetone precipitation and diethyl ether extraction while the other curve represents lecithin freshly purified by silicic acid column chromatography alone. All incubations were performed at 37°C in 0.025 M glycyl-glycine- NaOH buffer.

optimal concentrations of bile salts using 1.11 µmol lecithin/ml are found at 3.33 mM sodium taurocholate and 1.11 mM sodium taurodeoxycholate. These optimal bile salt concentrations correspond to the minimal concentrations at which the substrate mixture is converted to a water-clear solution as indicated by turbidity measurements at 600 mµ. Above these optimal concentrations of bile salts a slight inhibition is observed. These results indicate that rat pancreatic phospholipase A is almost exclusively active against micellar lecithin solubilized with bile salts in concentrations above the critical micellar concentration for the bile salt – lecithin mixtures. The solubilization of lecithin with α-acyl lysolecithin over a concentration range of 0 – 10 mM gave virtually no phospholipase A activity, although water-clear solutions were produced at around 0.03 – 0.04 mM lysolecithin. α-Acyl lysolecithin was shown not to inhibit the enzyme reaction in the presence of bile salt (*vide infra*). This indicates that the bile salts not only serve as solubilizers for the lecithin but also have a more specific effect on the interaction between the phospholipase A and the lecithin.

Calcium ions. Fig. 8 C shows the dependence of the phospholipase A activity on the concentration of $CaCl_2$ in the incubation medium. With the addition of an excess of EDTA the activity disappears completely even at

high concentrations of the enzyme. An increase of the calcium concentration above the EDTA concentration increases the enzymic activity until an optimum level is reached at a final concentration above 0.02 M free $CaCl_2$.

Substrate concentration. Rat pancreatic phospholipase A is inhibited by relatively low concentrations of its substrate lecithin (Fig. 8 E). This inhibition is observed at lower substrate concentrations when using fresher preparations of lecithin. After acetone precipitation and ether extraction the lecithin shows the most pronounced inhibition. This inhibitory effect has been noted by Vogel and Zieve.[1] An addition of fatty acids or lysolecithin to the incubation medium did not increase or diminish the inhibition when below the concentrations corresponding to at least 70 % hydrolysis of the β-fatty acid ester linkage of lecithin. Furthermore, the higher lecithin concentrations tested did not alter the isotropy of the substrate mixture. 1.11 μmol lecithin per ml has been chosen as the concentration routinely used in the assays. This substrate concentration is well above the K_1, relatively close to the optimal substrate concentration and has never shown any appreciable degree of inhibition even with very fresh lecithin preparations.

Enzyme concentration. Under the optimal conditions described above (0.02 M $CaCl_2$, 3.33 mM sodium taurocholate, pH 9, 37°C, 10 min incubation and using 1.11 mM lecithin) a rectilinear relationship exists between enzymic activity and the enzyme concentration within a range of $0-1.5$ μg/ml of rat pancreatic phospholipase A (Fig. 8 F). In order to measure small quantities of phospholipase A (< 0.125 μg/ml) the incubation time can be increased to $1-2$ h without any change in the linearity. The maximal enzymic activity suiting the rectilinear relationship corresponds to around 60 % hydrolysis of the β-fatty acid ester linkage of the lecithin.

Enzyme stability. Lyophilized rat pancreatic juice can be stored at -20°C for at least 6 months without any loss of its free or activable phospholipase A activity. Human pancreatic juice collected and lyophilized 14 years ago still exhibited a phospholipase A activity which was enhanced threefold when tryptically digested. In water solution, however, the activity of the rat pancreatic juice is rapidly lost as soon as the protease precursors have been spontaneously activated. This is usually observed after 15 to 20 h at 37°C and pH 7.6 and somewhat later at lower temperatures. The active purified phospholipase A is completely stable on storage both in lyophilized form and in solution at 4°C for at least 6 months. It resists heating to 75°C for 1 h in either water or 0.05 M TRIS-HCl buffer, pH 7.6 even in the presence of native rat pancreatic juice. The enzymic activity can be quantitatively recovered in the methanol-water phase during standard chloroform:methanol (2:1 v/v) lipid extraction. When incubated with trypsin at 37°C in 0.05 M TRIS-HCl buffer, pH 7.6, 0.05 M in $CaCl_2$, the activity of the pure phospholipase A as reduced as shown in Fig. 9. Thus trypsin is capable of inactivating rat pancreatic phospholipase A and therefore the tryptic activation of prophospholipase present in rat pancreatic juice must be carried out in a trypsinogen free medium during as short a time interval as possible in order to avoid inactivation of the phospholipase A. In the presence of bile salt, however, this inactivating effect of trypsin is reduced (Fig. 9). The finding that trypsin can inactivate rat pancreatic phospholipase A is contradictory to the results of De Haas *et al.* regarding the

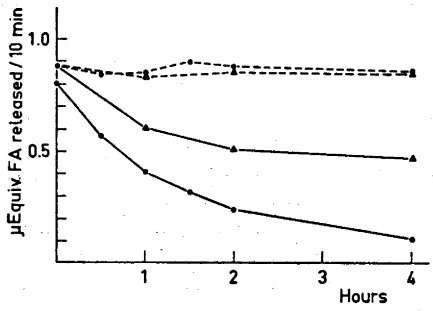

Fig. 9. The tryptic inactivation of rat pancreatic phospholipase A as a function of time. 8.0 µg/ml rat pancreatic phospholipase A was incubated at 37°C with 50 µg trypsin/ml in 0.04 M TRIS – HCl buffer, pH 7.6, 0.04 M in $CaCl_2$, with (▲) or without (●) 1.0 µmol/ml sodium taurocholate. After varying time intervals aliquots were taken for direct assay of phospholipase A activity. Controls for the phospholipase A activity were run in buffer or sodium taurodeoxycholate in bufter alone. Controls = dotted lines.

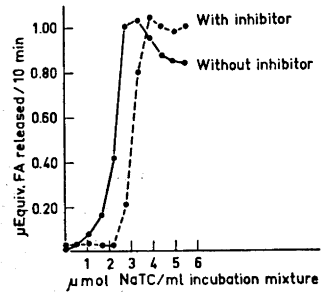

Fig. 10. The effect of bile salt on the inhibition of phospholipase A by the proteinase inhibitor of Kunitz. A suitable amount of rat pancreatic phospholipase A dissolved in 0.2 ml water was diluted to 1 ml with 0.05 M $CaCl_2$ and then equilibrated at 37°C for 10 min. The mixture was then incubated for 10 min at 37°C after addition of 1 ml 0.05 M glycylglycine NaOH buffer, pH 9, 2.22 mM in lecithin, 0.0025 mM in Kunitz inhibitor, and containing varying amounts of sodium taurocholate. The released fatty acids were titrated.

porcine enzyme.[12] (See prophospholipase activation). This contradiction, however, can be due to the fact that only low concentrations of trypsin are needed for activation of the pure phospholipase A precursor at 4°C. In addition, the results of Rimon and Shapiro [6] suggest that only phospholipase A activity towards lecithin and not towards other glycerophosphatides can be inactivated by trypsin and thus the use of whole egg yolks (De Haas *et al.*) instead of lecithin as the substrate can mask an inactivation occurring when the pure enzyme is subjected to the action of trypsin.

Phospholipase inhibition. Since phospholipase A in its active form may have a key position in the aetiology of acute pancreatitis and since the protease inhibitor of Kunitz has been tried as a remedy in this condition a possible interaction between phospholipase A and the inhibitor is of interest.[23–25] Addition of 0.01 µmol of the inhibitor to a substrate mixture 2.78 mM with respect to sodium taurocholate results in a complete inhibition of the phospholipase A activity exhibited by about 2 µg of the enzyme. This inhibition, however, is not observed when using bee venom as the source of phospholipase A. A preceding addition of trypsin or heparin to the inhibitor completely abolishes its phospholipase A inhibitory effect. The transition between zero and maximal inhibition occurs within a narrow range of the inhibitor concentrations (25 – 200 Units/incubation). This is not indicative of an ordinary enzyme inhibition. Fig. 10 illustrates an experiment where substrate solutions containing different concentrations of bile salt have been subjected to the action of 0.005 µmol Kunitz inhibitor. The inhibitor causes a parallel displace-

ment of the phospholipase A activity curve towards higher concentrations of bile salt. This shift corresponds to about 1 μmol sodium taurocholate/ml. Also as the inhibitor produces a change from isotropy to anisotropy (turbidity measurements at 600 mμ) of the substrate solution within the region of the parallel displacement of the curve the probable effect of the inhibitor is a derangement of the micellar state of the bile salt lecithin mixture. Bee venom displays a high phospholipase A activity in the absence of bile salts. Accordingly virtually no inhibition by the Kunitz inhibitor is observed.

The protease inhibitor of Kunitz is an extremely basic protein and is strongly positively charged at the pH of the substrate mixture. Therefore its effect on the micellar state of the lecithin might be due to an electrostatic interaction between the inhibitor and the bile salt anions. However, one molecule of the Kunitz inhibitor has the ability to disturb the action of around 200 bile salt anions and therefore the possible electrostatic interaction must be of a more complex type.

The optimal system for the measurement of total and activable phospholipase A^2 in rat pancreatic juice

Activation. 0 – 2.5 mg (0 – 1.9 mg protein; 0 – 0.1 ml intact juice) lyophilized rat pancreatic juice is diluted to 0.75 ml with distilled water. 0.2 ml 10% EDTA is added and the mixture heated at 75°C for 10 min. 100 μg trypsin in 0.05 ml 0.05 M TRIS-HCl buffer, pH 7.6, 0.02 M in $CaCl_2$, is then added and the heating at 75°C continued for another 10 min. After rapid cooling to 4°C 0.2 ml aliquots are taken for assay of phospholipase A. If only the originally present active phospholipase A^2 in the juice is to be estimated the same system can be used except that buffer alone is substituted for the trypsin solution.

Estimation of phospholipase A. The fraction to be tested (max. 0.2 ml) is diluted to 1 ml with 0.05 M $CaCl_2$ and then temperature equilibrated at 37°C for 10 min. 1 ml 0.05 M glycyl-glycine NaOH buffer, pH 9, 2.22 mM in ovolecithin and 6.66 mM in sodium taurocholate, is then added and the whole mixture incubated at 37°C for 10 min. The reaction is stopped and the released fatty acid determined as described under METHODS.

When comparing this procedure of heat treatment and activation at 75°C in the absence of calcium to the procedure for activation of pancreatic juice prophospholipase at 37°C using 10 – 20 μg trypsin/ml and 2.5 min preincubation time only small differences were found (for details see Fig. 1). This indicates that after tryptic digestion of rat pancreatic juice at 37°C in the presence of calcium and when acting on micellar lecithin-bile salt mixtures at pH 9 the hydrolytic activity is only to a very limited extent due to other enzymes than phospholipase A^2. In addition the results indicate that during the short period of tryptic digestion (2.5 min) at 37°C and in the presence of small quantities of trypsin (10 – 20 μg) negligible amounts of the phospholipase A is inactivated.

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