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The Phospholipase A_2 Activity of Human Small Intestinal Contents

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Studies are presented dealing with the properties, substrate requirements, kinetics and the behaviour during gel chromatography of the phospholipase A_2 present in human intestinal contents.

Phospholipase A_2 present in duodenal contents is completely stable when stored at 25°C in spite of the presence of high proteolytic activity. As judged by gel chromatography experiments this protection seems to be independent of partition of these enzymes between the emulsion or micellar phases and the water phase.

Sodium dodecyl sulfate in combination with bile salts and calcium highly stimulated the enzymic hydrolysis of egg lecithin. This made it possible to design a sensitive, accurate, automatic, titrimetric method for the assay of the phospholipase A_2 activity of intestinal contents.

The phospholipase A activity of the small intestinal contents is derived from the pancreatic secretion.[1–13] In the pancreas and pancreatic juice, however, it is present mainly as an inactive precursor, prophospholipase.[1–6,10,11] When secreted into the duodenum this enzymic precursor is actived by trypsin.[2–8,10,11] The active form of phospholipase A hydrolyses exclusively the fatty acid ester linkage at the 2-position of lecithin.[2–5,10,11] No other physiologically important enzyme activities capable of hydrolyzing the fatty acid ester bonds of lecithin are present in the intestinal lumen.[5,14]

Several methods for the measurement of phospholipase A activity in intestinal contents have been suggested.[5,8,9,13,15] All these methods have involved disadvantages by reason of the necessity of adding too high concentrations of intestinal contents. This has lead to uncontrolled detergent and free or esterfied fatty acid concentrations in the incubation mixture resulting in varying and relatively high blank values.

In the present study the main purpose was to study the properties, substrate requirements, kinetics, and the behaviour during gel chromatography of small intestinal phospholipase A_2. This was performed in order to design a sensitive and rapid routine method for the assay of the enzyme.

MATERIALS AND METHODS

All solvents and chemicals were of reagent grade purity. Chloroform was stabilized with 2 % ethanol.

Sodium taurodeoxycholate (NaTDC), sodium taurocholate, and sodium deoxycholate were synthesized and crystallized according to Norman [16] as modified by Hofman.[17] Purity better than 97 % as judged by thin layer chromatography of the three bile salts.[18]

Egg lecithin was prepared from fresh egg yolks as described by Hanahan.[19] Purity better than 97 % as judged by thin layer chromatography.[20] The lecithin was stored either in powder form in a nitrogen atmosphere at -20°C in the dark, or in a chloroform solution at -20°C in the dark. Several control experiments were run after further purification of lecithin.[3] No differences were observed.

Sodium dodecyl sulfate (NaDS), purity better than 99.8 % ,was purchased from AB Kebo, Stockholm, Sweden, and used without further purification.

Small intestinal contents were obtained from healthy male persons by duodenal or jejunal aspiration after oral (testmeal) or intravenous (pancreozymin) stimulation of the pancreatic secretion.

Sephadex G 100 was a product of Pharmacia Fine Chemicals, Uppsala, Sweden. The columns were packed according to standard procedures and the size of the columns are given in the text to the figures. Blue dextran 200 was used for indication of the void volumes of the columns and measured at 600 mμ in a spectrophotometer.

Phospholipase A activity was estimated in the following optimal way. A mixture 4.5 mmol/l in $CaCl_2$, 12.5 mmol/l in sodium taurodeoxycholate, and 2.5 mmol/l in sodium dodecyl sulfate containing 12 mg lecithin/ml was prepared. After vigorous shaking or sonication to dissolve the lecithin the solution was temperature-equilibrated at 60°C for 30 min. This mixture was used within 12 h. Two ml were transferred to an incubation flask kept at 60°C. During stirring the pH of the mixture was automatically kept at pH 7.5 with 0.01 mol/l NaOH, using a pH-stat (Radiometer, Copenhagen) with a TTT2 titrator connected to an ABU II Burette Unit with a 0.25 ml burette and a thermostatically controlled TTA31 titration assembly. The titration curves were recorded by means of a SBR2 Titrigraph. After measurement of the spontaneous hydrolysis rate (max. 0.05 μmol fatty acid released per min) for at least $2-3$ min, the enzyme source diluted to 0.2 ml was added. The automatic titration was then run at pH 7.5 for at least $3-5$ min after pH correction for the added enzyme source. A hydrolysis rate higher than 2.0 and lower than 0.1 μmol fatty acid released per min was not used in order to record activities directly proportional to the added amount of intestinal contents. These hydrolysis rate limits corresponded to $1:10-1:1000$ dilution of normal human small intestinal contents aspirated during digestion of a standard testmeal as previously described. Small intestinal contents having low phospholipase A activities were precipitated in 90 % ethanol, centrifuged and redissolved in water prior to the phospholipase A assay. Repeated assays of different concentrations of intestinal aspirates gave a mean coefficient of variation of $\pm$ 2.5 %.

Lipase activity was measured by a scaled down version of the method described by Erlanson and Borgström.[21] Trypsin was assayed by automatic titration using TAME as the substrate.[22]

All values described in this paper represent the averages of three determinations.

RESULTS AND DISCUSSION

Properties, substrate requirements and kinetics of small intestinal content phospholipase A_2

Enzymic action of intestinal contents. The phospholipase A_2 activity of small intestinal aspirates stored for $3-4$ weeks at room temperature was found to be completely stable. This contrasts to the ready inactivation of purified active pancreatic juice phospholipase A_2 in the presence of trypsin at such concentrations as present in intestinal contents.[4] Thus the enzyme is

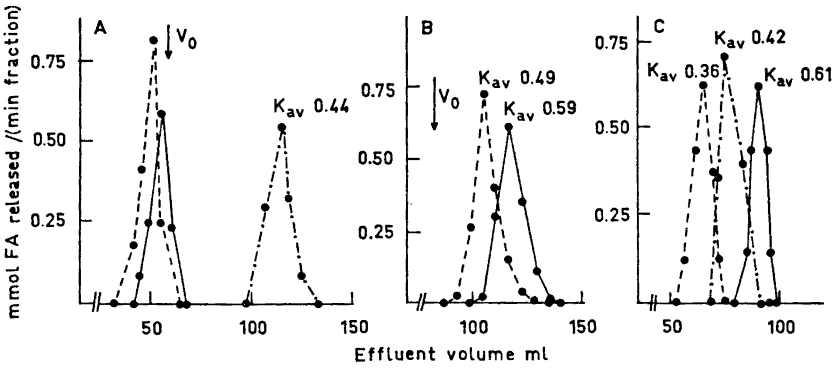

Fig. 1. One ml human intestinal contents was chromatographed on a Sephadex G 100 column (diameter 1.5 cm) equilibrated and eluted with 0.15 mol/l NaCl (A) and with 5.0 mmol/l sodium dodecylsulfate (B) or 6 mmol/l sodium taurodeoxycholate (C). Activity against micellar lecithin ——— , tributyrine – – – and TAME ·—·—·—· Flow rate approx, 6 ml/h.

protected against proteolytic inactivation in the intestinal lumen. This might depend either on the presence of bile salts [4] or on phase separation of the two enzymes or both. To test this, previously filtered intestinal contents were chromatographed on Sephadex G 100 equilibrated and eluted with 0.15 mol/l NaCl (Fig. 1A). Phospholipase A and lipase appeared in the void volume as previously observed for lipase by Erlanson and Borgström.[23] Trypsin was eluted at a K_{av} corresponding to its molecular weight. Accordingly both phospholipase A and lipase have a tendency to appear in fractions which under such circumstances contain the intestinal emulsion phase.[23] When chromatographed on Sephadex G 100 columns equilibrated and eluted with 5.0 mmol/l sodium dodecyl sulfate or 6 mmol/l NaTDC, however, phospholipase A, lipase, and trypsin were eluted at K_{av}'s corresponding to their molecular weights in previously tryptically digested human pancreatic juice [23] (Figs. 1B and 1C). In view of the results obtained in these latter experiments and in those by Erlanson and Borgström [23] phospholipase A and lipase in the presence of detergents were eluted in fractions which contained neither the emulsion nor the micellar phase of the intestinal contents. Therefore it seems that the protection of intestinal phospholipase A against tryptic inactivation is independent of phase separation.

Intestinal contents centrifuged at 55 000 g_{av} for 1 h exhibited exactly the same phospholipase A activity as before centrifugation. Ordinary filtration did not cause activity losses. It was completely precipitated in 90 % ethanol and quantitatively recovered after centrifugal sedimentation and consecutive solubilization of the sediment in water. In order to reduce the amount of base to be added for automatic pH correction when testing intestinal aspirates containing low phospholipase A activities but ordinary free or esterified fatty acid concentrations, a preceding alcohol precipitation is recommended.

The addition of different concentrations of human blood, serum, heparin, ε-amino caproic acid, tranexanic acid and of the reaction products in amounts corresponding to 50 % hydrolysis of the substrate did not alter the hydrolysis rate.

Influence of the volume of the incubation mixture. Routinely the assays of the phospholipase A_2 activity were run in 2.2 ml with vigorous stirring at constant temperature. After pH correction (50 – 100 μl 0.01 mol/l NaOH) the enzymic reaction was followed by the addition of 0.01 mol/l NaOH – with a maximal volume increase of 10 %. A dilution to 0.5 ml of an intestinal aspirate aliquot containing a suitable phospholipase A activity did, however, not change the constant hydrolysis rate. Therefore an increase of 22 % of the volume under the optimal conditions described above (see Materials and Methods) did not change the accuracy of the measurements.

Influence of incubation time on enzymic activity. Small intestinal aspirate aliquots exhibiting hydrolysis rates of 0.1 – 0.5 μmol fatty acid released per min gave a fatty acid release proportional to incubation time for at least 20 min, after pH correction of the whole incubation mixture. Routinely the enzymic activity was measured during 3 – 5 min and expressed as μmol fatty acid release per min. A hydrolysis of approximately 0.5 μmol fatty acid released corresponded to 5 – 10 % hydrolysis of the substrate. A spontaneous base catalyzed hydrolysis of the substrate mixture was observed especially above pH 8 and at high temperature (70 – 90°C). In all assays the proportion of the total hydrolysis rate dependent on this spontaneous hydrolysis was kept below one third of the enzymic hydrolysis and subtracted. When freshly prepared the pH of the substrate mixture was approximately 5. After storage a progressive decrease of the pH was found. Also, as the spontaneous hydrolysis rate increased with old mixtures only preparations made within 12 h were used.

Influence of pH on enzymic activity. The dependence of intestinal content phospholipase A on the pH of the incubation medium is shown in Fig. 2. A sharp optimum peak around pH 7.5 was found. Contrary to the findings by Borgström for lipase [24] and Vogel and Zieve for phospholipase A [13] no shift in the optimal pH was demonstrated when using different concentrations of bile salt. In previously tryptically digested human, pig, and rat pancreatic juice the phospholipase A has a rather broad pH optimum between pH 8 and 9 in the presence of bile salt.[4,10] This difference between pancreatic juice and intestinal content phospholipase A indicates an alteration of the physiochemical state of the enzyme when secreted into the duodenum. The details of this process cannot be judged at present.

Influence of calcium on enzymic activity. With the addition of an excess of EDTA no phospholipase A activity was found even with high concentrations of intestinal contents. An increase of the calcium concentration above the EDTA concentration increased the enzymic activity until an optimum level was reached at a final concentration above 3 mmol/l $CaCl_2$. At concentrations above 4.5 mmol/l $CaCl_2$ calcium was inhibitory (Fig. 3).

Influence of detergents on enzymic activity. In the absence of bile salt phospholipase A of small intestinal contents was inactive. This was demonstrated when testing phospholipase A containing fractions eluted with 0.15

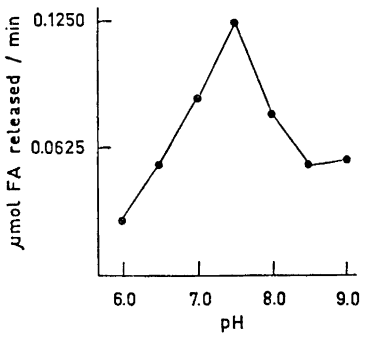

Fig. 2. Effect of pH on the rate of lecithin hydrolysis under optimal conditions (for details see text).

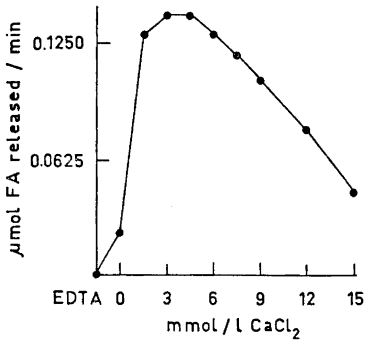

Fig. 3. Effect of calcium concentration on the rate of lecithin hydrolysis under optimal conditions (for details see text).

mol/l NaCl from a Sephadex G 100 column where during chromatography the enzyme is separated by size from bile salts in micellar as well as molecular solution (Fig. 1A). Both sodium taurocholate (NaTC) and sodium taurodeoxycholate (NaTDC) stimulated the reaction. Much higher concentration of NaTC was needed and therefore NaTDC was routinely used. Sodium deoxycholate (NaDC) was found not to stimulate the enzymic activity due to the fact that at both acid and alkaline pH it is precipitated in a calcium chloride solution. This latter observation might explain the varying previous results regarding the necessity of calcium as a co-factor for phospholipase A catalysis.[4,8,9,12,13,15] As partly demonstrated in Fig. 4, NaTDC at concentrations above 5.0 mmol/l initiate lecithin fatty acid hydrolysis with optimal

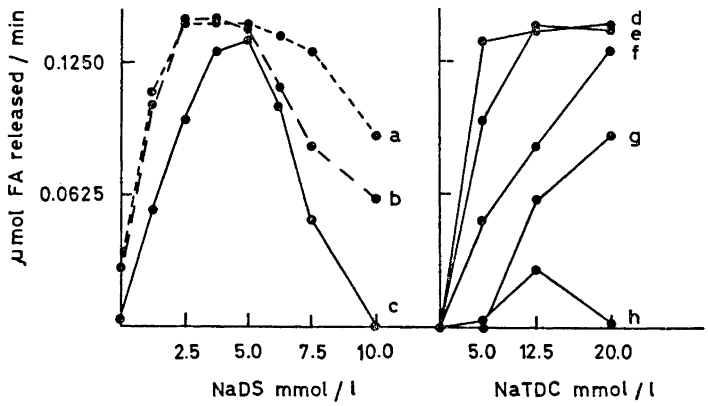

Fig. 4. Effect of varying relative concentrations of sodium taurodeoxycholate (NaTDC) and sodium dodecylsulfate (NaDS) on the rate of hydrolysis of lecithin (for details, see text). Concentrations of NaTDC (mmol/l): (a) 20.0; (b) 12.5; (c) 5.0. Concentrations of NaDS (mmol/l): (d) 5.0; (e) 2.5; (f) 7.5; (g) 100; (h) 0.0.

activity around 12.0 mmol/l NaTDC. At higher concentrations NaTDC is inhibitory. In contrast to tryptically activated phospholipase A from human and rat pancreatic juice the lowest concentration at which lecithin hydrolysis could be demonstrated did not correspond to the critical micellar concentration of the lecithin-bile salt mixture (turbidity measurements at 600 mμ).[4]

Although in concentrations high above the critical micellar concentration for its mixture with lecithin, sodium dodecyl sulfate (NaDS) *per se* had no stimulatory effect on the phospholipase A catalysis. In combination with NaTDC, however, the lecithin hydrolysis rate was stimulated between 5 and 50 times. This stimulation is demonstrated in Fig. 4. As can be seen there is maximal stimulation between 2.5 and 5.0 mmol/l NaDS in the incubation medium. This interaction between NaTDC and NaDS demonstrated in Fig. 4 might depend on several factors, such as that the combined detergents make the substrate more accessible for the enzyme or that NaDS alters the enzyme physicochemically, thus making it readily available for the lecithin substrate.

Influence of substrate concentration on the enzymic assays. In the presence of 4.5 mmol/l $CaCl_2$ and 12.5 mmol/l NaTDC and at 60°C optimal phospholipase A activity of small intestinal contents was obtained at 8 mg lecithin/ml (approx. 10 μmol/ml) (Fig. 5A). Higher concentrations of lecithin inhibited the reaction. At concentrations below the optimal an increase in the activity differences with increasing substrate concentration was observed. This resulted in a curvylinear Lineweaver-Burke representation at suboptimal substrate concentrations (Fig. 5B). The interpretation of such a substrate activation is difficult but might be explained either by a lecithin induced increase in enzymic solubilization or an increasing association of the enzyme with an increasing nonpolar phase of the mixed lecithin-bile salt micelles where the formation of enzyme-substrate complexes might take place.

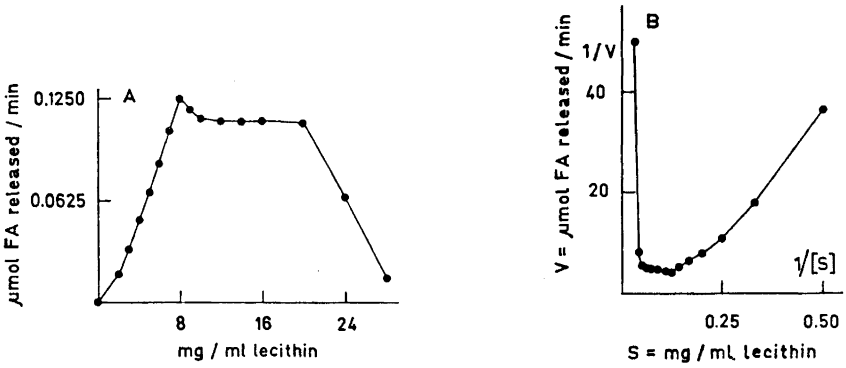

Fig. 5. Effect of lecithin concentration on the rate of lecithin hydrolysis at optimal concentration of sodium taurodeoxycholate. To the left: lecithin concentration *versus* hydrolysis rate. To the right: the corresponding Lineweaver-Burke representation (for details see text).

The addition of NaDS to the incubation mixture outlined in the preceding paragraph caused two modifications of the substrate concentration dependence.

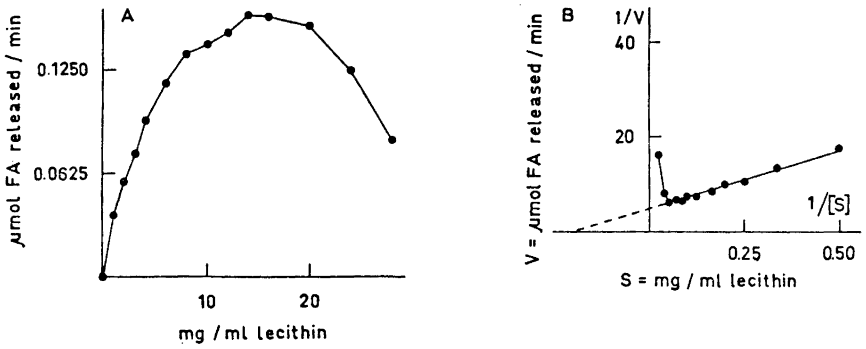

Fig. 6. Effect of lecithin concentration on the rate of lecithin hydrolysis at optimal concentration of sodium taurodeoxycholate and 5.0 mmol/l sodium dodecylsulfate. To the left: lecithin concentration *versus* hydrolysis rate. To the right: the corresponding Lineweaver-Burke representation(for details see text).

First, the optimal lecithin concentration was shifted towards higher values (Fig. 6A). Secondly, the activity difference at concentrations below the optimal decreased with increasing substrate concentrations and a straight line relationship in the Lineweaver-Burke diagram was obtained (Fig. 6B). These effects of NaDS might indicate that – in conformity with lecithin – it acts as a solubilizing agent for the phospholipase A in the water phase as well as in the micellar phase of the substrate mixture. Under these circumstances (12.5 mmol/l NaTDC, 4.5 mmol/l $CaCl_2$, 5.0 mmol/l NaDS and incubation at 60°C) the optimal lecithin concentration averaged 12 mg lecithin/ml and *Km* 5.8 mg/ml (approx. 7.25 µmol/l).

Influence of incubation temperature on enzymic reaction. Fig. 7 illustrates the effect of incubation temperature on the phospholipase A activity of small intestinal contents when tested under optimal conditions. The enzymic activity increased in an almost linear fashion with increasing temperature to 60°C above which a decreased activity was found. Routinely 60°C was used in the assays.

The fractions obtained in all the three types of gel chromatography described above (Figs. 1A, 1B, and 1C) were tested at 38°C and 60°C, respectively. At

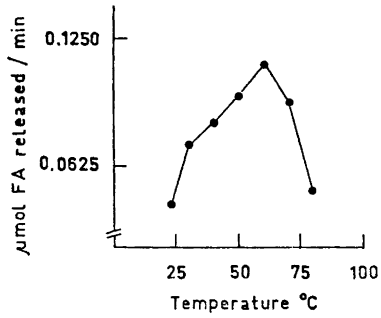

Fig. 7. Effect of incubation temperature on the rate of lecithin hydrolysis under optimal conditions (for details see text).

38°C no lecithin fatty acid release could be demonstrated in fractions other than those containing phospholipase A activity at 60°C. This result in combination with previous findings [5] indicates that the only lecithin fatty acid ester splitting enzyme activity in small intestinal contents is of the heat stable phospholipase A_2 type.

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