Pancreatic Stone Protein (Lithostathine), a Physiologically Relevant Pancreatic Calcium Carbonate Crystal Inhibitor?*

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Apart from digestive enzymes, pancreatic juice contains several proteins that are not directly involved in digestion. One of these, lithostathine, has been reported to exhibit calcite crystal inhibitor activity in vitro. As pancreatic juice is supersaturated with respect to calcium carbonate, it was hypothesized that lithostathine stabilizes pancreatic juice. Lithostathine is cleaved by trace amounts of trypsin, resulting in a C-terminal polypeptide and an N-terminal undecapeptide, which has been identified as the active site of lithostathine regarding crystal inhibition.

We produced rat lithostathine in a baculovirus expression system. In order to test its functional activity, the protein was purified using a nondenaturing multiprobe procedure. In the low micromolar range, recombinant polypeptide and an N-terminal undecapeptide, which has been identified as the active site of lithostathine regarding crystal inhibition.

The second product of trypsin cleavage, the N-terminal undecapeptide (called LS-N peptide hereafter), remains soluble. Bernard et al. (4) have shown that the N-terminal undecapeptide of human lithostathine is essential for its inhibitory activity and that the undecapeptide by itself at equimolar concentrations is about as active as the intact lithostathine, while the LS-C peptide is inactive.

Two different in vitro assays have been applied in order to determine functional properties of lithostathine. First, an approach was introduced whereby both nucleation and growth of calcium carbonate crystals was monitored (11). In a second assay, growth of calcium carbonate crystals was monitored (11). Early investigations into the function of lithostathine, which at that time was called pancreatic stone protein, exclusively used the first assay (5). When the crystal inhibitor activity of the LS-N peptide was reported, however, the second assay was introduced (4).

Neither the pathogenesis of pancreatic stones nor the roles of lithostathine and its trypsin cleavage products in the process of stone formation are fully understood. It is not known whether the formation of protein plugs and pancreatic stones within the duct system is of pathogenetic relevance in the development of chronic pancreatitis, but the obvious association of the two phenomena (plugs and stones) with the disease prompted us to investigate the physiological and pathophysiological roles of lithostathine.

Chronic pancreatitis in most cases is accompanied by stone formation within the pancreatic duct system (1). These stones primarily consist of calcium carbonate in the form of calcite (about 95% by weight) (2).

Based on in vitro assays, the pancreatic secretory protein lithostathine has been shown to have inhibitory activity against spontaneous calcium carbonate precipitation from highly supersaturated solutions (3) and against calcium carbonate crystal growth in metastable solutions (4). It has been suggested that the physiological role of lithostathine is to stabilize pancreatic juice (4), which theoretically is supersaturated with respect to calcium carbonate (2, 3).

Lithostathine is a highly soluble protein of 144 amino acids. It contains a trypsin-sensitive cleavage site (Arg11-Ile12) which is conserved in several species (5, 6). One of the trypsin cleavage products, the C-terminal peptide of 133 amino acids, called LS-C peptide hereafter, displays a much lower solubility and has a tendency to precipitate between pH 5 and 8 (7, 8). The LS-C peptide (also called lithostathine H, pancreatic stone protein PSP S1, or pancreatic thread protein) is the predominant component of the protein matrix of pancreatic stones (9).

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Two different in vitro assays have been applied in order to determine functional properties of lithostathine. First, an approach was introduced whereby both nucleation and growth of calcium carbonate crystals was observed (10). In a second assay, growth of calcium carbonate crystals was monitored (11).

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The abbreviations used are: LS-C peptide, C-terminal trypsin fragment of lithostathine; AcMNPFV, A. californica multiple nuclear polyhedrosis virus; BVEs, baculovirus expression system; HPLC, high performance liquid chromatography; LS, lithostathine; LS-N peptide, N-terminal trypsin fragment of lithostathine (11 amino acids); LSb/ AcMNPFV, recombinant baculovirus containing rat lithostathine cDNA; MWCO, molecular weight cutoff; PAGE, polyacrylamide gel electrophoresis; PSP, pancreatic stone protein; PSTI, pancreatic secretory trypsin inhibitor; S9, S. frugiperda cell line; Fmoc, N-(9-fluorenyl)-methoxycarbonyl.

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We have reported the high level secretation of rat lithostathine in a baculovirus expression system (BVES) (12). The use of a BVES allowed us to obtain rat lithostathine in the absence of pancreatic proteases. Polyclonal antibodies raised against baculovirus expressed rat lithostathine recognize rat lithostathine with high sensitivity and specificity. In order to test the functional activity of baculovirus expressed rat lithostathine and its two tryptic fragments, we have now purified the protein using a multi-step procedure. Here we report that purified rat lithostathine in vitro displays calcium carbonate crystal inhibitor activity. However, control proteins at similar concentrations appear to be equally active. Thus it is doubtful that the inhibitor activity of lithostathine is a specific functional property of this protein, and the question arises whether its inhibitory activity is of physiological relevance.

**EXPERIMENTAL PROCEDURES**

**Expression of Rat Lithostathine in a BVES**

Suspension cultures of Spodoptera frugiperda (Sf900 II, Life Technologies, Inc.) to a density of about 2 × 10^6/ml were infected with a multiplicity of infection of 5 plaque-forming units/cell. The medium, which contained the secreted rat lithostathine (about 5–10 mg/liter of medium), was harvested 50–56 h postinfection.

The following protease inhibitors were added to the medium immediately before centrifugation: phenylmethylsulfonyl fluoride (Sigma), at a final concentration of 100 μg/ml; pepstatin A (Sigma), 1 μg/ml; leupeptin (Sigma), 0.5 μg/ml; and Foy 305 (ONO Pharmaceutical Co., Ltd., Osaka, Japan), 50 μg/ml. Pepstatin A, leupeptin, and Foy 305 were used at the same concentrations throughout all later procedures during purification of rat lithostathine.

**Purification of Baculovirus Expressed Lithostathine**

**Ultrafiltration**—The medium of LSRb/AcMNPV-infected Sf9 cultures was centrifuged at 4°C in two types of Centriprep tubes (purchased from Amicon, Beverly, MA). In order to remove soluble proteins with a molecular mass of more than 100 kDa, the medium was first centrifuged through tubes with a molecular weight cutoff of 100 kDa. The retentate was diluted to its original volume with phosphate-buffered saline at pH 7.4, containing three protease inhibitors (see above), and centrifuged once more. The pooled filtrate was then concentrated in Centriprep tubes with a MWCO of 10 kDa. The retentate contained the proteins with a molecular mass between 10 and 100 kDa. Rat lithostathine was concentrated about 30–50-fold.

**Dialysis**—Following ultrafiltration, the media concentrate, containing unknown salt and buffer concentrations, was dialyzed at 4°C against a 100-fold volume of 50 mM HEPES-Na (Sigma), pH 7.5, 10 mM NaCl in NaCl (chromatography buffer, three protease inhibitors added, see “Expression of Rat Lithostathine in a BVES”). Spectrapor dialysis tubing with a MWCO of 6–8 kDa was used for this purpose (purchased from Spectrum Medical Industries, Los Angeles, CA). The dialysate was exchanged once. Dialysis was completed after 24 h.

**Size Exclusion Column Chromatography**—The column chromatography equipment was set up in a cold room (4°C) and consisted of the following: an XK 16/100 column (Pharmacia Biotech Inc.) with Bio-Gel P-30 (medium-sized beads, from Bio-Rad) as column matrix, equilibrated with 50 mM HEPES-Na, pH 7.5, 10 mM NaCl (chromatography buffer); a peristaltic pump P-1, and a Redirac fraction collector (Pharmacia Biotech Inc.). The column bed volume was about 170 ml and the column height about 85 cm. Flow was 6 ml/h and fraction size 1.7 ml (1% of bed volume). Maximum sample loading volume was 5 ml (3% of bed volume).

The protease inhibitors (see “Expression of Rat Lithostathine in a BVES”) were used with all buffers. Six runs were performed with a total of 26.2 ml of media concentrate of 1640 ml of media from infected Sf9 cultures (representing about 3.3 × 10^6 cells).

**Anion Exchange Column Chromatography**—We used a 1.5/20 Econo column (Bio-Rad) with DEAE-Sephadex (Pharmacia) as column matrix, equilibrated at 4°C with chromatography buffer. The bed height was 14 cm and bed volume about 25 ml. A total of 22.5 ml sample (pooled fractions from size exclusion chromatography) was loaded in three runs. Samples were loaded at 18 ml/h. The column was then washed with 2 bed volumes of starting buffer (flow 18 ml/h). Proteins were eluted with a gradient of 10–300 mM NaCl, 50 mM HEPES-Na, pH 7.5 (flow 12 ml/h, 10 bed volumes, fraction size about 3.7 ml). Three protease inhibitors (see “Expression of Rat Lithostathine in a BVES”) were added to all buffers. The column was regenerated with 6 bed volumes of 1 M NaCl after each run.

**Pooling, Concentration, Dialysis, and Lyophilization of Purified Rat Lithostathine**—Anion exchange chromatography fractions which, based on silver stained SDS-PAGE, contained the highest concentrations of purified rat lithostathine were pooled (26.4 ml), concentrated to about 2.6 ml in Centriprep 10 devices, and dialyzed against a 100-fold volume of 20 mM NaHCO₃, (with three protease inhibitors; see “Expression of Rat Lithostathine in a BVES”), pH 8.7, at 4°C with two changes of the dialysate over about 40 h (dialysis tubing as under “Dialysis”). Final volume was 3.5 ml. This solution was aliquoted and lyophilized in a SpeedVac centrifuge (Savant Instruments Inc., Farmingdale, NY). Aliquots were stored at −70°C.

**Preparation of Controls**—Chromatography buffer with three protease inhibitors (see “Expression of Rat Lithostathine in a BVES”) was treated the same way as the lithostathine containing fractions (see “Pooling, Concentration, Dialysis, and Lyophilization of Purified Rat Lithostathine”). This “no lithostathine control” was tested in the “crystal nucleation assay” (see below). Analogous treatment of two anion exchange chromatography fractions which were at least five fractions away from the lithostathine peak provided a “low lithostathine control,” which was also tested in the crystal nucleation assay.

**Modifications of These Procedures for Large Scale Purification**—Later on, when dealing with larger volumes of cell cultures, we switched from Centriprep tubes to a spiral cartridge system (SY100; MWCO 100 kDa; Grace Amicon, Danvers, MA) and stirred cells (model 8400) with YM10 membranes (MWCO 10 kDa; Grace Amicon).

Up to 2 liters of media supernatant could be applied to the reservoir of the spiral cartridge system. The solute was pumped through the spiral-wound membrane until the concentrate consisted of less than 100 ml (Amicon pump; setting 60: about 150 ml/min). The cartridge was washed with 200 ml of chromatography buffer (cf. “Size Exclusion Column Chromatography”) until the concentrate was again below 100 ml (setting 20). This wash procedure was repeated once, and the filtrate was collected and applied to a stainless steel reservoir (RS4, Grace Amicon) for concentration with YM10 membranes in two stirred cell devices (at 4°C).

Concentrates from 5 liters of media supernatant were combined, giving a volume of approximately 150 ml, and applied to a DAEASephaloc column (Pharmacia XK 26/200) with a matrix volume of 60 ml (chromatography was performed at 4°C). The column was washed extensively with more than 2 bed volumes of starting buffer. The initial gradient (10–300 mM NaCl, 50 mM HEPES, pH 7.5) was run at 0.5 ml/min for 300 min. A steeper gradient followed from 300 to 500 mM in 120 min. The fractions were evaluated by SDS-PAGE, and those containing relevant amounts of rat lithostathine were concentrated to a final volume of 9 ml in a Centriprep 10 device and then loaded onto a size exclusion chromatography column (Econo column 25/1200; Bio-Rad) with a P10 matrix (Bio-Rad) and a bed volume of 540 ml. Proteins were eluted at 9.5 ml/h. Fractions containing apparently pure rat lithostathine were combined and dialyzed extensively against 0.1 M NaHCO₃, pH 8.5, with protease inhibitors (three changes of buffer). The dialysate was centrifuged (1000 × g, 10 min, 4°C), the pellet discarded, and the supernatant lyophilized and stored at −70°C.

**Control Proteins**—Bovine trypsinogen, human serum albumin, and soybean trypsin inhibitor were purchased from Sigma. These control proteins were dissolved in MilliQ-purified water at 10 mg/ml and used only after an extensive solvent exchange against MilliQ-purified water by means of ultrafiltration through Centricron 10 devices (Amicon) at 4°C. Thus the initial buffers were dialyzed at least 10,000 times. Loss of protein checked by electrophoresis was negligible.

**Measurement of Protein Concentration**

Concentrations of baculovirus expressed rat lithostathine and of LS-C peptide were estimated on Coomassie Blue-stained SDS-polyacrylamide gels; band intensities were compared with various defined amounts of bovine serum albumin (Pierce). Concentration estimates for lithostathine were confirmed using amino acid analysis. The latter was performed on a Hewlett-Packard (Palo Alto, CA) analyzer using Amincoat software (Hewlett-Packard).
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Gel Electrophoresis and Immunodetection of Protein

SDS-PAGE and immunodetection of proteins on blots were performed as described elsewhere (12). Protein bands on polyacrylamide gels were visualized by staining with 0.1% Coomassie Blue R-250 (Bio-Rad) in 50% methanol plus 10% acetic acid or, alternatively, with silver nitrate (Sigma) (13). Low molecular weight markers (Bio-Rad) were used as protein size references.

Limited Proteolysis with Trypsin

Three milligrams of purified recombinant rat lithosthine, dissolved in 1.5 ml of MilliQ-purified water, were incubated with 50 μl (5% w/w) of trypsin (from bovine pancreas, type XII, solubilized for an hour under a gentle stream of argon. The HPLC system was purchased from Kontron (Zürich, Switzerland). The undecapeptide was synthesized according to the FastMoc™ strategy using 2-[(S)-benzotriazol-1-yl]-1,1,3,3-tetramethyl ethylenurea hexafluorophosphate as the activating agent. The peptide was deprotected and cleaved from the resin by incubation in a mixture of 0.75 g of crystalline phenol, 0.25 ml of ethanedithiol, 0.5 ml of trifluoroacetic acid. The cleaved peptide was collected by precipitation in t-butyl methyl ether and subsequent centrifugation. After several t-butyl methyl ether washes the peptide was taken up in 10 ml of MilliQ-purified water and solubilized for an hour under a gentle stream of argon.

Mass Spectroscopy

Synthetic oligopeptides and the recombinant rat LS-N peptide were analyzed on a Perkin-Elmer Sciei API III mass analyzer (kindly operated by Dr. P. Hunziker).

The expected molecular mass of the rat LS-N peptide is 1226.4 Da and that of its human analog is 1252.5 Da (as calculated by GeneJockey software). This assay is referred to as crystal nucleation assay in this paper.

In order to obtain reliable measurements, the pH electrode was thoroughly rinsed in pure water over a period of about 10 min after each run (to remove the calcite precipitates on the pH electrode surface). After experiments involving proteins, the electrode was cleaned overnight using a special solution (Renovo N, Radiometer Copenhagen).

Inhibition of Calcium Carbonate Crystal Growth

An assay that determines crystal growth (11) was performed both at a volume of 20 ml (4) and adapted to 1 ml in 48-well flat bottom microtiter plates (Falcon, Becton Dickinson). This assay is referred to as “crystal growth assay” in this paper.

Seed crystals were prepared by dropwise addition of an equal volume of 0.2 M sodium bicarbonate to 0.2 M calcium chloride. To generate crystals from a volume of 0.5 liters, it took approximately 30 min to add the solutions together. After settling, the crystals were harvested by decanting the supernatant, centrifuged in a table top centrifuge (5 min at 900 × g; room temperature), and washed with chilled methanol (-20°C). To remove the methanol, centrifugation was repeated (5 min at 900 × g; 4°C); the slurry of crystals was then poured into a Petri dish and allowed to dry. For volumes of 20 ml, 5 mg of dry crystals were weighed out immediately prior to the experiment. The assay was started by addition of a metastable calcium carbonate solution containing 4.8 mM sodium bicarbonate and 0.27 mM calcium chloride (adjusted to pH 8.8 with a 200 mM stock solution of sodium carbonate). The solution was rapidly stirred at a constant setting of approximately 800 rpm.

For the 1-ml assay, the crystals were delivered to individual wells by taking up 25 mg in 100 ml of methanol from which aliquots of 1 ml were removed while vigorously stirring the slurry. The plates were allowed to dry in a chemical hood for at least 3 days. The assay was performed on a microplate shaker (Heidolph Titramax 100 rotational shaker, Bioblock Scientific, Switzerland) at setting 7, a shaking frequency sufficient...
ciently vigorous to homogeneously suspend the crystals in solution (approximately 700 rpm). One ml of the metastable calcium carbonate solution was added to each well containing 0.25 mg of calcite crystals. Lithostathine (stock solution 1.3 mg/ml, control proteins (10 mg/ml), or sodium phosphate (100-fold stock solutions) were added to the calcium carbonate solution just before the assay was started.

To monitor crystal growth, the decrease of soluble calcium was determined at defined time intervals (0, 20, 30, 60, and 120 min). To that end, 125-μl aliquots were removed at appropriate times and immediately centrifuged for 30 s in an Eppendorf centrifuge (15,800 × g, room temperature). The supernatant was transferred to a fresh tube until analysis. To be sure that during centrifugation precipitation or crystal growth did not proceed at an enhanced rate, test runs were performed whereby the samples taken from a 20-ml assay were either filtered (0.22 μm) to separate the crystals (4) or centrifuged as detailed. Both methods yielded identical results; the centrifugation method was preferred because it allowed the synchronous processing of several samples with small volumes.

The concentration of calcium in solution was determined with a commercially available kit which contains o-cresolphthalein-Complexon and ethanolamine buffer (calcium, MPR 2, Boehringer Mannheim, 1553593). To establish a linear relationship between calcium concentrations from 100 to 300 μM and the resulting optical density, the composition of the detection reagent had to be modified; the buffer and the chromophore reagent were premixed at equal volumes instead of 3:1. This reagent mix was added to a flat bottom 96-well microtiter plate (180 μl/well). Samples of 90 μl or standard solutions (108–270 μM calcium chloride, 4.8 mM sodium bicarbonate) were added, and the optical density at 570 nm was determined 5 min later with a microplate reader (MRX, Dynatec Produkte AG, Switzerland). The optical density of a 270 μM calcium standard solution was 1.3; day to day variations were negligible (±0.05 OD).

In the absence of proteins or peptides, the calcium concentration dropped to about 140 (130–150) μM within 2 h.

Samples contained various amounts of the following: baculovirus expressed rat lithostathine (0.3–3.6 μM), rat LS-N peptide (9 μM), rat LS-C peptide (0.6–2.4 μM), synthetic rat LS-N peptide (1.5–9 μM), synthetic human LS-N peptide (9–81 μM), bovine trypsinogen (3–9 μM), human serum albumin (3–9 μM), soybean trypsin inhibitor (9–81 μM), and controls with equivalent volumes of water.

Determination of Free Phosphorus in Pure Pancreatic Juice

A phosphorus determination kit was purchased from Boehringer Mannheim (124, 974). Free phosphorus was determined by first removing protein and lipid-bound phosphate; 10 μl of sample were added to 100 μl of 1.26 M trichloroacetic acid, incubated for 10 min at room temperature, and centrifuged at 15,800 × g for 10 min in an Eppendorf centrifuge. Ninety microliter of the supernatant were transferred to a 96-well microtiter plate, and 180 μl of the premixed developing reagent were added and incubated for 10 min. Standards prepared from a sodium phosphate solution were treated similarly. Optical density was read at 405 nm in a microplate reader.

RESULTS

Purification of Baculovirus Expressed Rat Lithostathine

About 50–56 h after infection of Sf9 cells with the recombinant baculovirus LSrB/AmNPV, the medium was separated from the cells by centrifugation. The recombinant protein was purified from the medium by a multi-step procedure.

Selective ultrafiltration resulted in a protein mixture enriched with low molecular mass proteins (10–100 kDa). Lithostathine was concentrated about 30–50-fold (Fig. 1). We used SDS-PAGE and Coomassie Blue staining to estimate the rat lithostathine yield after ultrafiltration. The loss of lithostathine seemed to be less than 20% in Centriprep 100 filtration and again less than 20% in Centriprep 10 concentration.

The proteins were then separated by size exclusion chromatography. A protein mixture highly enriched with rat lithostathine was eluted (Fig. 2). Positive fractions were pooled and separated by anion exchange column chromatography. Thus recombinant rat lithostathine was purified to apparent homogeneity (Fig. 3). The purified protein’s identity with rat lithostathine was confirmed by immunodetection (Fig. 4).

In a first series of small scale experiments, we had infected a total cell culture volume of 1640 ml, representing about 3.3 ×
10^9 cells. From this, the yield of purified lithostathine was 875 μg, i.e. 535 μg/liter of media or 265 μg/10^9 cells.

The original culture medium of infected cells contained about 5–10 mg of rat lithostathine/liter of media (12). Thus recovery following the purification procedure is about 5–10%. Later on we used larger cell culture volumes for infection and slightly modified purification procedures (cf. see “Modifications of These Procedures for Large Scale Purification”). Under these conditions, the yield was 0.8 mg/liter (about 10% recovery).

Inhibition of Crystallization

Baculovirus Expressed Rat Lithostathine Inhibits Calcium Carbonate Crystal Formation—Purified recombinant rat lithostathine was tested in a functional in vitro assay that measures nucleation and growth of calcium carbonate crystals (crystal nucleation assay).

A dose-dependent inhibition of crystal formation can be demonstrated by the addition of recombinant rat lithostathine; at a final concentration of 2.4 μM the pH remains above 8.0 for more than 15 min (Fig. 5a). The half-maximal effect is reached at about 0.6 μM.

As expected, addition of adequate amounts of the low lithostathine control fractions (cf. “Preparation of Controls”) caused reduced inhibitory activity, the no lithostathine control displayed no effect and neither did equivalent volumes of water (data not shown).

Calcium Carbonate Crystal Growth—In the crystal growth assay, recombinant rat lithostathine demonstrates a distinct inhibition of calcite crystal growth already at a concentration of 0.6 μM (Fig. 5b). This inhibition is clearly dose-dependent. The half-maximal effect is reached at about 0.8 μM. The three protease inhibitors present in the lithostathine preparation were inactive.

The Rat LS-C Peptide Inhibits Crystal Nucleation and Growth—The trypsin digest of recombinant rat lithostathine resulted in the two expected fragments. The LS-C peptide precipitated upon centrifugation and was thus quantitatively separated from the LS-N peptide that remained in the supernatant. The LS-C peptide was resuspended and tested in the crystal nucleation assay and in the crystal growth assay and showed a reduced but significant inhibitory activity in both (Fig. 6, a and b).

The Rat LS-N Peptide Is Inactive—To test the hypothesis that the LS-N peptide contains the inhibitory activity, we purified the LS-N peptide from the supernatant of the tryptic digest of recombinant rat lithostathine using HPLC. Mass spectroscopy verified its expected size and it demonstrated the predicted composition by amino acid analysis. This peptide was tested in the crystal nucleation assay (Fig. 7a) and the crystal growth assay (Fig. 7b). It was virtually inactive in both assays when tested at a concentration of 9 μM.

The Synthetic Rat and Human LS-N Peptides—Synthesis of an analog of the rat LS-N peptide enabled us to apply higher concentrations. The synthetic rat LS-N peptide had been purified by HPLC and checked for correct composition by amino acid analysis and for correct size by mass spectroscopy. Hardly any effect was observed when this peptide was tested in the
without significant effect. In the crystal growth assay, concentrations up to 81 μM caused no inhibition either (Fig. 5b).

Inhibitory Activity of a Pancreatic Control Protein—In order to evaluate whether the crystal inhibition exhibited by lithostathine is a specific functional property of this protein only, we tested another pancreatic secretory protein in both assays: bovine trypsinogen displayed an inhibitory activity that is comparable to the one caused by lithostathine (Fig. 9, a and b, and Tables I and II).

Inhibitory Activity of Extrapancreatic Control Proteins—We furthermore tested two extrapancreatic proteins, human serum albumin and soybean trypsin inhibitor. Human serum albumin displayed a distinct and dose-dependent inhibitory effect in both assays, although somewhat weaker than recombinant rat lithostathine. Soybean trypsin inhibitor, on the other hand, was at least as active as lithostathine (Fig. 9, a and b, and Tables I and II).

Inhibitory Activity of Phosphate—When phosphate, another component of pancreatic juice, was tested in the same assays, a dose-dependent inhibitory activity was observed. Half-maximal inhibition was reached at about 15 mM phosphate in the crystal nucleation assay (Fig. 10a) and at about 6 mM in the crystal growth assay (Fig. 10b).

The phosphate concentration in rat pancreatic juice was determined (n = 3); under basal conditions it is about 800 μM.

DISCUSSION

We have expressed rat pancreatic lithostathine in the absence of pancreatic proteases in cells derived from ovaries of an unrelated species (BVES). We can exclude the presence of pancreas-specific contaminations that are a potential source of activity in pancreatic extracts, i.e. other proteins and peptides (12).

The high level of protein expression allowed us to purify significant quantities of rat lithostathine. We thus could test, in a rigorous fashion, the biological activities of the intact lithostathine molecule, purified to apparent homogeneity, and its proteolytic fragments, the N- and C-terminal peptides.

We used two different in vitro assays to assess the calcium carbonate crystal inhibitory activity of rat lithostathine. Our findings confirm an inhibitory effect of lithostathine in the low micromolar range in vitro, as reported for human lithostathine by Bernard et al. (4); both inhibition of crystal nucleation and inhibition of crystal growth could be demonstrated at concentrations that are about five times lower than the physiological concentration of lithostathine in pancreatic juice, which is approximately 10 μM (4). We have thus generated a recombinant rat lithostathine that is not only structurally and immunologically but also functionally equivalent to native rat lithostathine (14).

However, since other proteins, pancreatic and extrapancreatic ones, demonstrate such inhibitory activity in the same range of concentration, as we have shown in Fig. 9, a and b, and in Tables I and II, this is not a specific functional property of lithostathine. Nonspecific inhibition of calcite crystal growth was reported by Addadi and Weiner (15) who “found that growth of calcite is inhibited in a nonspecific manner at protein concentrations >0.5 μg/ml.” For a protein with a molecular mass of 25 kDa this limit would be 20 nM.

Pancreatic juice is a mixture of some 20 proteins, the total protein concentration being about 20–30 g/liter. At least two of these proteins (lithostathine and trypsinogen) might contribute to the crystal inhibitory capacity of the juice. Since extrapancreatic proteins, such as human serum albumin and soybean trypsin inhibitor, exhibited similar inhibitory activities, we have to assume that several of the 20 proteins in pancreatic juice contribute to the inhibition of calcite formation. The con-
centration of individual proteins in pancreatic juice may reach far higher than 10 μM; for trypsinogen 50 μM may be a good estimate, and for the combined concentration of proteins 0.5–1 mM seems not unlikely.

Trace amounts of trypsin can cleave lithostathine, resulting in an N-terminal undecapeptide (LS-N peptide) and a C-terminal polypeptide (LS-C peptide). It is known that trypsinogen autoactivation takes place in the pancreas to a certain extent (16). Active trypsin is readily neutralized by the pancreatic secretory trypsin inhibitor (PSTI). Nevertheless, it might be assumed that minor amounts of lithostathine are cleaved within the pancreatic duct system. We tested the proteolytic fragments for crystal inhibitory activity. Of the two tryptic cleavage products only the LS-C peptide displayed such an inhibitory activity, which was, however, reduced in comparison to the intact molecule. The LS-C peptide easily forms precipitates and cannot be dissolved completely at the concentrations and pH used for the two in vitro assays. It was kept in suspension by vigorous stirring of the slurry, but the amount of peptide available for interaction with the calcite crystals is most probably reduced. The second tryptic fragment, the LS-N peptide, exhibited no inhibitory activity when tested at the same concentration range.

The discrepancy between our results and the findings published by Bernard et al. (4), who described an inhibitory activity of the human LS-N peptide, native or synthetic, almost as strong as the one of intact lithostathine, has no clear explanation. We synthesized an undecapeptide according to the known

**FIG. 7.** Neither the LS-N peptide generated by trypsin cleavage of recombinant rat lithostathine (●) nor the synthetic rat LS-N peptide (△) demonstrated inhibition in the crystal nucleation assay (a) or in the crystal growth assay (b) when tested at 9 μM. Control, ▲. Even at unphysiologically high concentrations the synthetic rat LS-N peptide did not significantly change the course of the pH (c); 10.5, ●, 21, □, 42 μM, ×; control, ▲. The decline of pH within the first 3 min was identical to the one observed in the control experiments, i.e. crystal nucleation was not influenced at all. Experiments were performed in duplicate or triplicate; mean values are shown.
sequence of the rat LS-N peptide (6). This undecapeptide, too, lacked inhibitory activity, although it was tested at concentrations up to five times higher than with the tryptic LS-N peptide, i.e., at least five times higher than the maximal expected physiological concentration.

We furthermore generated an undecapeptide with identical sequence to the human LS-N peptide, respecting the fact that the N terminus of lithostathine consists of pyroglutamine (17). When we tested it in the crystal nucleation assay, we did not observe any inhibitory effect, although we used concentrations up to 100 times higher than the concentration of recombinant rat lithostathine giving maximal, saturating inhibition of crystal nucleation.

To measure the activity of human LS-N peptide, however, Bernard and his collaborators (4) used the crystal growth assay only, which measures calcite accumulation onto preexisting...

**FIG. 8.** Neither in the crystal nucleation assay nor in the crystal growth assay did synthetic human LS-N peptide exhibit inhibitory activity. *a*, at a concentration of 243 μM (●), which is about 100 times higher than the concentration of human lithostathine giving a distinct inhibition, no relevant effect on the course of the pH was observed. 9 μM, ○; 81 μM, ×; control, ▲. Experiments were performed in duplicate or triplicate; mean values are shown. *b*, synthetic human LS-N peptide does not inhibit calcite crystal growth at concentrations up 81 μM (●); 9 (○), or 27 μM (■), control (■). Mean values (and standard deviation) are shown (n = 7).

**FIG. 9.** Three different control proteins caused a crystal inhibition comparable with the effect of lithostathine in the crystal nucleation assay. *a*, ●, 1 μM soybean trypsin inhibitor (n = 3); ●, 9 μM bovine trypsinogen (n = 4); ○, 9 μM human serum albumin (n = 2). So did 50 μM sodium phosphate (■). ▲, control (n = 29). Mean values are shown. With regard to the dose dependence of this activity see Table 1 or Fig. 10a. In the crystal growth assay (*b*), these proteins also displayed an unequivocal inhibitory effect; ●, 3 μM soybean trypsin inhibitor (n = 4); ○, 3 μM bovine trypsinogen (n = 2); ■, 9 μM human serum albumin (n = 6). ●, 10 μM sodium phosphate (n = 2) caused a saturating inhibition, too. ▲, control (n = 14). Mean values are shown. With regard to the dose dependence of this activity see Table II or Fig. 10b.
calcite seeds (11). Their results, with synthetic human LS-N peptide concentrations going up to 9 mM, indicated a significant inhibitory activity.

When we applied synthetic human LS-N peptide to the crystall growth assay, we again could not detect a significant inhibitory activity, although we used concentrations up to 81 mM. The differing results may be caused by the preparation and purification of the peptide; we have purified and analyzed our peptides by HPLC prior to testing. The amino acid composition as well as the molecular masses were as predicted. In addition, we carefully exchanged trifluoroacetic acid residuals by hydrochloric acid first and then removed excess hydrochloric acid by repeated drying and redissolution in water.

It is thus possible that the preparation used by Bernard et al. (4) was contaminated by acids that might have a strong influence on the in vitro assays.

Pancreatic juice contains a considerable amount of phosphate. According to Janowitz and Dreiling (18), the phosphate concentration in man is about 0.8 ± 0.4 mmol/kg H₂O, a range that we have confirmed with our own measurements.² We found basal rat pancreatic juice to contain phosphate at a concentration of about 0.8 mM. Tested in the two assays reported here, phosphate seems to be a powerful crystal inhibitor in vitro. It displays a strong inhibitory activity at concentrations about 20 times lower than its physiological concentration in pancreatic juice. Our data are in accord with earlier reports (19, 20). Reddy (20) has pointed out that crystal growth may be retarded by the presence of trace amounts of phosphate ions.

² Daniel Bimmler, Rolf Graf, George A. Scheele, and Thomas W. Frick, unpublished observations.

### TABLE I

Comparison of the crystal inhibitor activity of rat lithostathine (LS) and different control proteins tested in the “crystal nucleation assay”

| Protein conc. (µM) | LS  | HSA | TG  | STI |
|-------------------|-----|-----|-----|-----|
| 0                 | 7.46| 7.41| 7.39| 7.40|
| 0.1               |     |     |     | 7.59|
| 0.3               | 7.73| 7.41|     | 7.94|
| 0.6               | 7.89|     |     |     |
| 1                 |     | 7.59| 7.64| 8.16|
| 1.2               | 7.96|     |     |     |
| 2.4               |     | 8.10|     |     |
| 3                 | 7.63|     | 7.92|     |
| 9                 | 7.83|     | 8.06| 8.22|

### TABLE II

Comparison of the crystal inhibitor activity of rat lithostathine (LS) and different control proteins as assessed in the “crystal growth assay”

Mean values of the calcium concentration (mM) in the solution measured 120 min after adding a metastable calcium carbonate solution to calcite crystals; experiments performed at least in duplicate. Soybean trypsin inhibitor (STI) and bovine trypsinogen (TG) demonstrate an inhibitory effect comparable with lithostathine. Human serum albumin (HSA) exhibits the weakest effect. TG, bovine trypsinogen. Mean values shown, experiments performed at least in duplicate.

| Protein conc. (µM) | LS  | HSA | TG  | STI |
|-------------------|-----|-----|-----|-----|
| 0                 | 0.14| 0.13| 0.12| 0.12|
| 3                 | 0.17| 0.27| 0.28|     |
| 3.6               | 0.27|     |     |     |
| 9                 | 0.21| 0.25| 0.30|     |

Fig. 10. Crystal inhibitory activity of sodium phosphate in vitro. (a) In the crystal nucleation assay (○), sodium phosphate was added at 5 µM (○), 10 µM (●), 25 µM (▲), 50 µM (▲), and 100 µM (▲). Control (▲). A half-maximal effect was observed at about 15 µM sodium phosphate. Inhibition of calcite crystal growth by sodium phosphate (b); 1 µM (●), 5 µM (○), 10 µM (●), 25 µM (▲), 50 µM (▲), 100 µM (▲). Control (▲). A half-maximal effect was reached at about 6 µM sodium phosphate. Experiments were performed in duplicate; mean values are shown.
each. In rat pancreatic juice, total calcium is 1.8 mM, and free ionized calcium is certainly much lower. Bicarbonate secretion, which is dependent on the presence of secretin, varies from 25 to 70 mM in the basal versus the stimulated state of the rat pancreas (21). In the crystal growth assay, the concentrations of these ions are quite below the physiological values, and this solution is stable in the absence of preformed calcite crystals. Since the concentrations of bicarbonate and calcium are not representative for physiological conditions, a high degree of uncertainty remains when asserting functions of proteins such as lithostathine on the basis of these two physicochemical in vitro assays.

For many years, the interest into lithostathine has been focused on its presumed crystal inhibitor activity and onto its postulated active site, the LS-N peptide. Due to this, the LS-C peptide has received little attention, although its tendency to precipitate, forming small particles that serve as nuclei for calcite crystals and thus eventually favor the formation of stones in the pancreatic ducts.

While under normal circumstances pancreatic juice is stable and protected from intraductal homogeneous crystallization of calcium carbonate by an array of different proteins and ions, heterogeneous crystallization may be favored by the precipitated LS-C peptide.

We conclude that, when tested separately by in vitro assays, several organic and inorganic components of pancreatic juice (lithostathine, trypsinogen, phosphate) display calcium carbonate crystal inhibitory activity. Based on these results it seems improbable that lithostathine in vivo is “a key factor in the prevention of stone formation in pancreatic ducts” (23), a hypothesis that has been advanced for many years now (4, 24).

On the other hand, the tendency of the LS-C peptide to precipitate has been observed by several independent investigators, and its predominant presence in pancreatic stones has been reported repeatedly (25–28).

It is thus questionable whether the name “lithostathine” should be used further. We propose that until we know more about the protein’s function it should be called “pancreatic stone protein” again.

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