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PANCREATIC STONE PROTEIN, A PHOSPHOPROTEIN WHICH INHIBITS CALCIUM CARBONATE PRECIPITATION FROM HUMAN PANCREATIC JUICE

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SUMMARY : A soluble protein isolated from human pancreatic stones, and present in pancreatic secretion, strongly inhibits precipitation from supersaturated calcium carbonate solutions. Therefore, we suggest that this new secretory protein may act as an inhibitor of spontaneous calcium carbonate precipitation from supersaturated pancreatic juice.

INTRODUCTION : Pancreatic stones are found in pancreatic ducts of patients presenting with chronic calcifying pancreatitis (1,2). Such stones are composed of calcium carbonate ($CaCO_3$) crystallized as calcite (3). We have recently shown that human pancreatic stones contain a major soluble protein of Mr 13,500 (4). This pancreatic stone protein (PSP) is a calcium-binding protein (5), present in the zymogen granules of human pancreatic acinar cells (6) and has been found to be secreted in the pancreatic juice of normal subjects and stone formers (4).

There is substantial bicarbonate and calcium in the pancreatic juice of normal individuals (up to 140 and 4 mM respectively). This fact, associated with the low solubility of $CaCO_3$ (Ksp= 4.01×10^{-9} (mole/liter)^2) (7), induces supersaturation with respect to calcium and carbonate (1). Pancreatic juice, however, does not normally allow precipitation and crystallization of $CaCO_3$, except under pathological conditions. As reported in the case of human urine, we noted that the main question is not why stones can form but why stones do not form more generally (8).

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In the present study we report that PSP suppresses $CaCO_3$ nucleation and decreases the rate of crystal growth in vitro. Since PSP is a normal constituent of human pancreatic juice and different levels exist in normal subjects and stone formers, we suggest that this new secretory protein may act as an inhibitor of spontaneous $CaCO_3$ precipitation in vivo.

MATERIALS AND METHODS : PSP was isolated from human pancreatic stones as described elsewhere (4). Briefly, stones were ground to a powder with a mortar and pestle and demineralized by repeated extractions with 0.5 M EDTA, pH 8.0, at 4°C. After centrifugation the supernatant was exhaustively dialyzed against distilled water and freeze-dried. The protein content was routinely determined with the method of Lowry et al. (9). The homogeneity of PSP was verified with sodium dodecyl sulphate-polyacrylamide gel electrophoresis and with a double diffusion test against human pancreatic juice antiserum.

Proteins of human pancreatic juice were subjected to a gel filtration on Sephadex G 100 as previously described (10).

Human pancreatic carboxylic ester hydrolase (11), lipase (12), trypsinogen 1 (10), chymotrypsinogen A (13) and amylase (10) were purified as reported elsewhere. Human serum albumin and lactoferrin, phosvitin, heparin and chondroitin sulphate were purchased from Sigma Co. All other chemicals used were of reagent grade.

Inhibition of Calcium Carbonate precipitation : The effect of PSP on the rate of $CaCO_3$ precipitation was determined with supersaturated solutions prepared as described by Wheeler et al. (14). $CaCO_3$ formation was followed by recording the pH decrease of a solution containing 5 ml of 20 mM $NaHCO_3$, pH 8.7, and 100 µl of water (control) or PSP when 5 ml of 20 mM $CaCl_2$ was added. pH of the solutions were measured with a glass / calomel electrode pair. In addition, the reaction was followed by measuring absorbance at 570 nm with a double-beam spectrophotometer with temperature controlled cell compartiment and 1 cm pathlength quartz cuvettes were used. Crystal growth experiments were performed in a temperature controlled water jacket vessel. Solutions were stirred continously at a constant rate with Teflon-coated magnetic bars under a stream of N_2. The temperature was kept at 25 ± 0.1°C.

The precipitated crystals were studied by X-ray powder diffractometry by means of a 573 mm Debye-Scherrer camera with a Cu alpha energy source.

The decrease in free calcium was determined with a calcium ion electrode (Corning) and a calomel / ceramic junction reference electrode.

PSP was also tested in a $CaCO_3$ system in which ionic strength, pH, and electrolytes were modified according to physiological conditions containing (final concentrations) : 2 mM $CaCl_2$ and 60 mM $NaHCO_3$, 20 mM NaCl, 4 mM KCl, 1 mM NaH_2PO_4, 0.5 mM $MgCl_2$, pH 8.3, at 37°C.

RESULTS AND DISCUSSION : As shown in Figure 1, the precipitating reaction (water control) can be divided into the following steps: (a) an initial reaction development in which pH and absor-

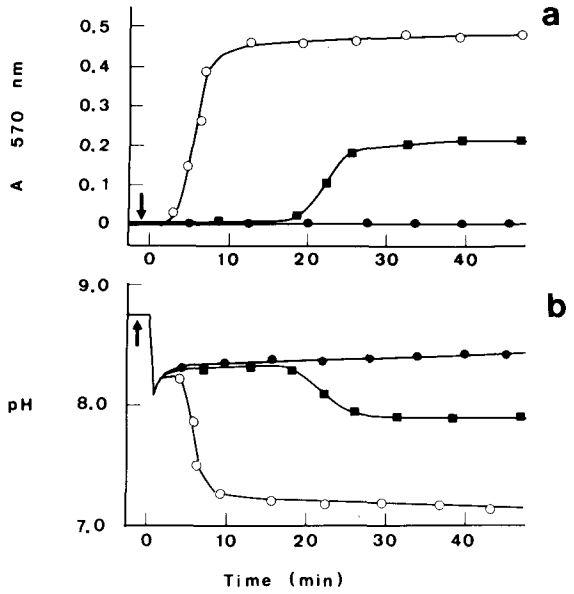

Fig. 1 Effect of PSP on $CaCO_3$ precipitation. (a), absorbance at 570 nm, and (b), pH variations as a function of time. The vertical arrows indicate the time of addition of 100 µl of water (○), 1.4 µg/ml PSP (■), 5.6 µg/ml PSP (●), before the addition of $CaCl_2$.

bance were relatively stable until nucleation occurs, (b) a second step in which pH decreased and precipitation could be observed, and (c) a final step where pH and absorbance remained stable. Under the conditions employed in this study, X-ray diffraction analysis showed that the crystallization rate involved only calcite formation.

When PSP at 1.4 µg/ml (final concentration) was added to $NaHCO_3$ prior to the addition of $CaCl_2$ (Fig.1), the initial stable period was greatly prolonged. Once nucleation had occurred, the rate of net precipitation or crystal growth was lower for reactions containing PSP than for controls. At a high PSP concentration (5.6 µg/ml), the pH decrease was delayed for several hours (Fig. 1), suggesting inhibition of nucleation.

A direct effect of PSP on crystal growth (Fig. 2) was demonstrated by the suppression of the pH decrease and of the increase in absorbance upon addition of PSP (5.6 µg/ml) after nucleation had occurred and crystal growth was in progress. Under the conditions reported here, this effect occurred at PSP concentrations estimated as 4×10^{-7} M or less.

In order to asses the specificity of the PSP effect, we tested several substances (Fig. 3). Highly purified human pancreatic

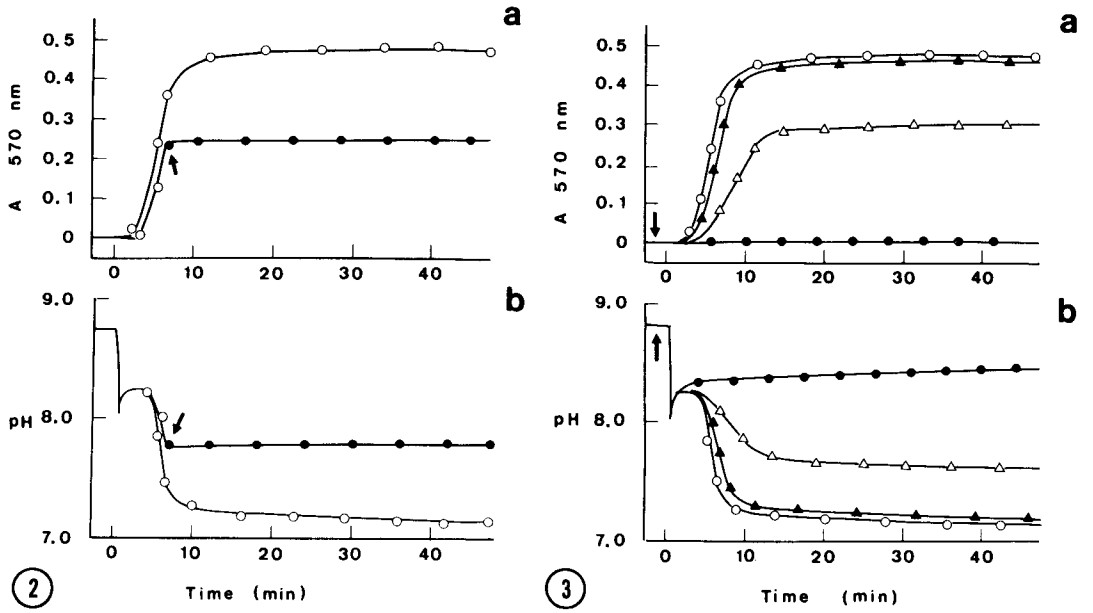

Fig. 2. Direct effect of PSP on $CaCO_3$ crystal growth. (a), absorbance at 570 nm , and (b), pH variations as a function of time. 100 µl of water (O) as in Fig.1. PSP (5.6 µg/ml, ●) was added when crystal growth was in progress (arrow).

Fig. 3. Comparative effects of several substances on $CaCO_3$ precipitation. (a), absorbance at 570 nm, and (b), pH variations as a function of time. The vertical arrows indicate the time of addition of 100 µl of water (O), 5.6 µg/ml PSP (●), 10 µg/ml albumin, lactoferrin, carboxylic ester hydrolase, lipase, trypsinogen 1, chymotrypsinogen A, or amylase (▲), 1.5×10^{-4} M EDTA, 0.6×10^{-4} M citrate, 15 µg/ml phosvitin, heparin or chondroitin sulphate (△).

secretory proteins, had no effect on both nucleation time and crystal growth. Similarly, human serum albumin and lactoferrin, present in elevated levels in the pancreatic juice of stone formers (15), had no effect. Glycosylaminoglycans which prevent the precipitation of calcium oxalate (16), had no effect on nucleation time but slightly decreased the rate of crystal growth. The same effect was observed with phosvitin, which inhibits calcium phosphate crystal growth, an with a calcium chelator such as EDTA, as previously reported (14). A similar result was obtained with trisodium citrate.

The inhibiting effect of PSP was not simply due to the removal of calcium, since at 1,000 times higher molar concentrations, a calcium chelator such as EDTA was less effective than PSP and since more than 98% of calcium in the medium was free at a PSP concentration of 5.6 µg/ml.

The results presented here demonstrate an inhibitory effect of PSP on crystal nucleation and growth in a $CaCO_3$ system. Such regulation by calcium-binding proteins has been demonstrated for a calcium phosphate system and has been proposed for components of human saliva (17) and bile (18). A regulatory effect for a carbonate mineralizing system has been recently described for the first time for a calcium-binding protein isolated from oyster shells (14).

The most striking feature of PSP is its high content of aspartic and glutamic acids, which represents 26.2 and 15.9 per cent, respectively of the total number of amino acids. In addition, 2 residues of O-phosphoserine and one of O-phosphothreonine were found (unpublished results). it has been suggested that phosphoproteins are potential regulatory agents for mineralization processes in tissue fluids (19). It is noteworthy that human salivary statherin, which inhibits calcium phosphate precipitation and therefore stabilizes supersaturated saliva (17), is, as PSP, an acid-rich peptide containing two O-phosphoserine residues.

When PSP was assayed in a $CaCO_3$ system in which ionic strength, pH and elctrolytes were modified according to physiological conditions, a similar inhibitory effect was observed but a larger quantity of PSP (15 µg/ml) was required (data not shown). When proteins of pancreatic juice were submitted to gel filtrations, the fractions containing PSP (detected by radial immunodiffusion) also inhibit $CaCO_3$ precipitation.

In conclusion, our results obtained in vitro support the belief that PSP acts in vivo as a stabilizer of pancreatic secretion. The presence of PSP in pancreatic stones and its role in stone development, however, remain to be explained. The calcium concentration is increased in the pancreatic juice of stone formers (20), leading to a higher supersaturation state (1). Measurements of PSP in pure pancreatic juice, performed by radial immunodiffusion, showed that patients with pancreatic stones have lower levels of PSP than patients without pancreatic disorders (21). These findings, together with the present study, suggest that PSP is less able to prevent stone formation under pathological conditions. Its presence in pancreatic stones may be reasonably explained by its affinity for $CaCO_3$ crystals. It is well known that inhibitor binding sites for calcium block the active sites of crystal growth, preventing further accretion of mineral, thus delaying precipitation (22, 23). The formation of a protein-crystal

complex may thus be attributed to a much higher affinity of the protein for the crystal than for free calcium.

Finally, since PSP, a novel pancreatic secretory protein without known enzymatic activity, represents a major component of human pancreatic secretion, the possibility cannot be excluded that it may be involved in other processes.

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