

CURRENT STATUS OF PANCREATIC STONE PROTEIN

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ABSTRACT

Pancreatic stone protein (PSP) has been argued to play a crucial role in intraductal pancreatic stone formation in chronic pancreatitis. PSP was initially reported to inhibit calcium carbonate precipitation from human pancreatic juice and to be decreased in pancreatic secretions from patients with chronic pancreatitis. Recent clinical investigations have further demonstrated elevation of PSP in the serum and urine of patients with renal disease as well as pancreatic disease. However, the PSP reduction in pancreatic secretion in chronic pancreatitis remains controversial. Therefore, we review the current concept of PSP.

INTRODUCTION

Pancreatic stone protein (PSP) has been argued to play a crucial role in intraductal pancreatic stone formation in chronic pancreatitis. PSP was found as a major organic constituent of human pancreatic stones by Sarles' group.^{1,2)} Five immunoreactive forms of protein present in pancreatic juice were separated by SDS-gel electrophoresis (PSP S1-5, mol wt of 15,000 to 22,000).²⁻⁴⁾ PSP was reported to inhibit calcium carbonate precipitation from human pancreatic juice⁵⁾ and to be decreased in pancreatic secretions from patients with chronic pancreatitis, especially from those with pancreatic calcification.⁶⁻⁸⁾ Sarles et al. recently proposed that this family of molecules be renamed lithostatine S (the secretory form) and lithostatine H (the insoluble form resulting from hydrolysis by trypsin).⁹⁾ PSP is probably involved in pancreatic stone formation, but the details of its involvement remain unclear.

Recent clinical investigations have demonstrated that serum levels of PSP were elevated in patients with acute pancreatitis and pancreatic cancer, as well as in those with chronic pancreatitis.¹⁰⁾ Furthermore, a fibrillar protein (pancreatic thread protein, PTP)¹¹⁾ crossreacting with PSP has been found in the pyramidal cells in Alzheimer disease brain.¹²⁾ More recent investigation revealed that *reg*-protein,¹³⁾ PSP, and PTP are simply different names for a single protein deriving from the *reg* gene.¹⁴⁾

These observations are unexpected. Therefore, we extended the materials and subjects for measurement of PSP from patients with pancreatic diseases to those with extrapancreatic diseases, and studied the diagnostic and pathophysiological significance of PSP.¹⁵⁻²⁰⁾ We reviewed the current status of PSP in serum, urine, pancreatic juice, and pancreatic stone.

MEASUREMENT OF PSP

PSP concentration in pancreatic juice and serum has been determined by radial immunodiffusion,⁶⁾ fluorometric immunoassay,¹⁰⁾ radioimmunoassay,⁷⁾ and enzyme immunoassay (EIA).^{7,15,20,21)} Multigner *et al.* reported that substantially lower levels of PSP were observed in patients with chronic calcifying pancreatitis of different etiologies when compared with those in patients with other pancreatic diseases and controls.⁶⁾ On the other hand, studies by Schmiegel *et al.*¹⁰⁾ and us²¹⁾ showed no difference between the different pathologic groups. This discrepancy is partly due to the different specificities of antibodies used in the assay systems.⁷⁾ In these methods, polyclonal antibodies (raised against PSP S2-5)⁷⁾ and/or one monoclonal antibody (raised against PSP S1, purified from pancreatic stone and called D4-1-21, Immunotech, Marseille, France) were used.⁷⁾ The monoclonal antibody D4-1-21 recognizes the C-terminal part of PSP S1, and the polyclonal antibodies recognize the N-terminal portion of PSP S1.⁷⁾

We prepared monoclonal antibodies to PSP, characterized them in terms of binding affinity to PSP S1, and PSP S2-5, and designed a one-step sandwich enzyme immunoassay with similar reactivities toward PSP S1 and PSP S2-5.^{7,15,20)} Furthermore, our proposed method determines PSP in pancreatic juice after dissolving PSP precipitate with an acetate buffer to exclude the influence of PSP S1, precipitation caused by endogenous trypsin activation.^{20,21)}

ORGAN DISTRIBUTION OF PSP

Immunolocalization of PSP in the human digestive tract was reported by Lechene de la Porte *et al.*²²⁾ PSP was markedly present in the zymogen granules and condensing vacuoles of the normal pancreatic acinar cells. No PSP was specifically characterized in the hepatocytes, gastric mucosa, and enterocytes. However, a weak, but specific reaction was found in the secretory granules of Paneth cells. These results in tissue distribution of PSP are in good agreement with serum PSP levels in nonpancreatic diseases. Our preliminary measurements of serum PSP levels after total pancreatectomy suggest the extrapancreatic origin of PSP, because serum PSP was reduced, but did not fall below the lower normal limit. Gross *et al.*¹¹⁾ reported the tissue distribution of pancreatic thread protein (PTP), strongly suggested to be an identical protein to PSP.^{3,4,14)} Human pancreatic thread protein appeared to have remarkable specificity for the normal human pancreas, and was not detected in three pancreatic adenocarcinoma extracts or 100 sera from normal blood donors.¹¹⁾ Watanabe *et al.*¹⁴⁾ noticed that the amino acid sequence encoded by the human *reg* gene contained the sequence of PSP and PTP, and concluded that the three proteins are just different names for a single protein existing in several molecular forms, but deriving from the *reg* gene. They also examined the *reg* mRNA in various normal human tissue. The *reg* mRNA was detected in the pancreas at a high level and in the gastric mucosa and kidney at lower levels, but not in the liver, spleen, brain, thyroid gland, submandibular gland, esophageal mucosa, rectal mucosa, or lymphocytes.¹⁴⁾

PSP IN SERUM

Normal Range of Serum PSP

Serum PSP in controls (n=37) varied from 25.2 to 161.1 ng/mL with a mean of 78.6 ng/mL and 95% range (mean \pm 2SD=15.0 to 142.2 ng/mL) in our study.¹⁶⁾ Serum PSP levels of more than 142.2 ng/mL were considered abnormally high, and those less than 15.0 were considered abnormally low. Schmiegel *et al.*¹⁰⁾ set the cutoff line for normal serum PSP values, giving the

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95th percentile of the control group consisting of 68 normal blood donors, at 700 ng/ml. Their cutoff level was several times higher than ours.¹⁶⁾ The reason for the difference in the serum levels is unclear. It may be partly due to different affinities of antibodies used in the assay systems or to differences in the subjects studied.

Serum PSP in Pancreatic Diseases (Fig.1)

In our previous study¹⁶⁾ serum PSP in acute pancreatitis (mean \pm SD=1075.4 \pm 2849.1 ng/mL) was significantly higher than that of the controls (78.6 \pm 31.8 ng/mL, $p < 0.01$), chronic pancreatitis (156.8 \pm 82.8 ng/mL, $p < 0.05$), and pancreatic cancer (148.4 \pm 68.8 ng/mL, $p < 0.05$). There was no low PSP value in pancreatic diseases. Frequencies of abnormally high PSP values were 78.8% in acute pancreatitis, 43.8% in chronic pancreatitis, and 42.3% pancreatic cancer. Elevation of serum PSP tended to be greater in severe acute pancreatitis (3188.7 \pm 5975.4 ng/mL, $n=7$) than in milder acute pancreatitis (785.7 \pm 474.4 ng/mL, $n=29$), but not significantly. In chronic pancreatitis there was no significant difference in serum PSP levels between calcified (157.2 \pm 87.9 ng/mL, $n=14$) and noncalcified (156.5 \pm 81.2 ng/mL, $n=18$) pancreatitis. Location of tumor in pancreatic cancer did not affect serum PSP levels between head and body-tail cancer (140.6 \pm 62.0 ng/mL, $n=16$ vs 160.8 \pm 80.5 ng/mL, $n=10$, respectively).

In contrast to PSP in pancreatic secretions, serum PSP levels in pancreatic diseases were higher than those in controls, but the difference from controls reached statistical significance only in acute pancreatitis in our study.¹⁶⁾ Schmiegel et al.¹⁰⁾ also noted the serum PSP elevation in the majority of acute (80% of 20) and chronic (60% of 66) pancreatitis patients and in pancreatic cancer (36% of 25) patients. Their results of PSP levels of every pancreatic disease group differed significantly ($p < 0.001$) from those of normal blood donors.

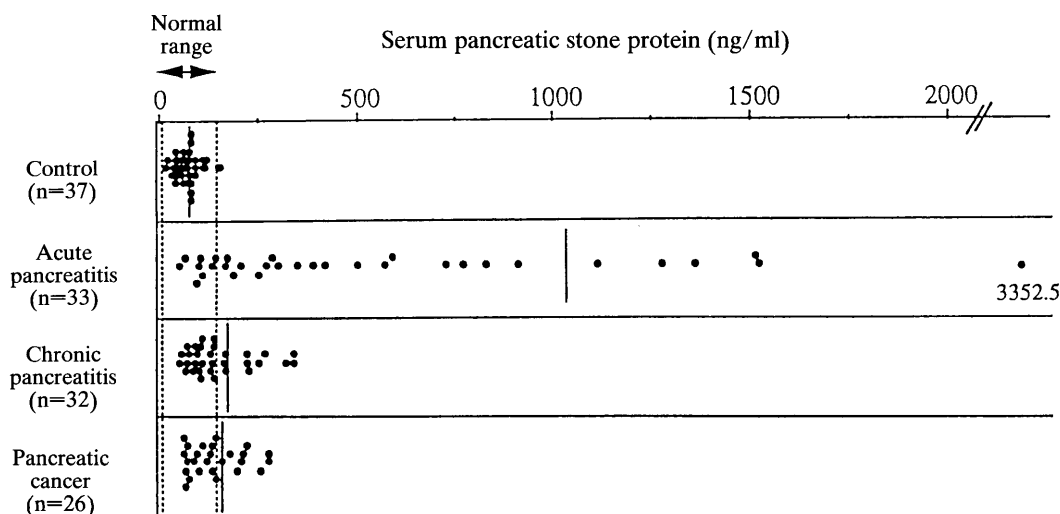


Fig. 1. Serum pancreatic stone protein in pancreatic diseases. Dotted lines indicate normal range of serum PSP obtained from controls, and vertical bar shows mean of the disease group.¹⁶⁾

Serum PSP in Nonpancreatic Diseases

Serum PSP levels in patients with chronic renal failure under hemodialysis (1796.0 ± 1492.9 ng/mL) were significantly ($p < 0.05$) higher than those in patients of all other disease groups, except acute pancreatitis, and all levels exceeded the upper normal limit of our previous study.¹⁶⁾ Serum PSP levels in other nonpancreatic diseases did not differ from that of the controls. The mean levels and frequencies of the abnormally high values of serum PSP were 134.7 ± 56.3 ng/mL and 33.3% in 15 patients with gastric cancer, 129.8 ± 148.5 ng/mL and 18.2% in 11 with gallstone, 170.5 ± 123.6 ng/mL and 38.5% in 13 patients with liver cirrhosis, 106.4 ± 46.2 ng/mL and 16.6% in 12 noninsulin-dependent diabetics, and 107.7 ± 64.9 ng/mL and 11.1% in 9 patients with peptic ulcer, respectively. There were no abnormally low serum PSP values in patients with nonpancreatic diseases, either.

There have been no comparable data on serum levels of PSP in nonpancreatic diseases. Chronic renal failure under hemodialysis revealed the highest PSP values in all disease groups, including acute pancreatitis. This suggests that serum PSP levels are influenced by renal diseases as well as pancreatic diseases.

Molecular Form of PSP in Serum

Cation exchange chromatography of patient sera on Mono S (HR 5/5), followed by the EIA of column fractions, was performed to characterize the molecule form of PSP.¹⁵⁾ An elution pattern for PSP of serum from a pancreatic cancer patient (serum PSP 2,340 ng/ml) is shown in Fig. 2A. Two peaks of immunoreactive PSP were observed. The major peak of immunoreactive PSP was eluted in a position corresponding to that of PSP S2-5. The minor peak of immunoreactive material was eluted at a position consistent with that of PSP S1. An elution pattern for PSP of serum from an acute pancreatitis patient (serum PSP 1,731 ng/ml) is shown in Fig. 2B.

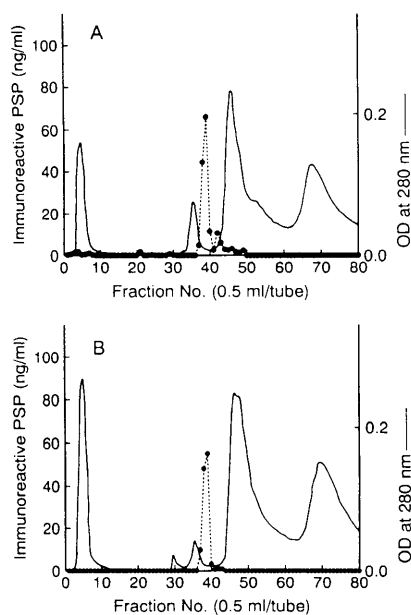


Fig. 2. Cation exchange (Mono S) chromatography of human serum. A: Pancreatic cancer (PSP value 2,340 ng/ml). B: Acute pancreatitis (PSP value 1,731 ng/ml). Closed circles and dotted line indicate amounts of immunoreactive PSP. Solid line means optical density (OD) at 280 nm.¹⁵⁾

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A single peak of PSP S2-5 was observed. Similar results were obtained in serum from one patient each with chronic pancreatitis (serum PSP 731 ng/ml), pancreatic cancer (serum PSP 1,836 ng/ml), and acute pancreatitis (serum PSP 3,250 ng/ml).

In sera from a chronic pancreatitis patient and acute pancreatitis patient, the immunoreactivity consists of only PSP S2-5, but in serum from a patient with pancreatic cancer, an immunoreactive fraction appears at the position corresponding to that of PSP S1 in addition to the PSP S2-5 fraction. The PSP S1 may indicate that PSP S2-5 was cleaved by proteinase. Therefore, the finding of PSP S1 in serum reflects the activation of proteinase in the pancreatic juice or pancreas. The mechanism of production of PSP S1 is not clear at present, and requires further study. Differential measurement of PSP S2-5 and PSP S1 may offer pathophysiological information; that is, elevation of PSP S1 in serum suggests activation of proteinases in the pancreatic juice or pancreas.

PSP IN URINE

Renal Lithostathine

The mRNA molecules for PSP were detected predominantly in the pancreas and at lower levels in the gastric mucosa and the kidney.¹⁴⁾ Verdier et al.²³⁾ recently reported the presence of lithostathine-like immunoreactivity (renal lithostathine) in the urine of healthy subjects and in renal stones. They suggested that the immunocytochemistry of the kidney sections indicated that the protein is localized in the cells of the proximal tubules and thick ascending limbs of the Henle's loops and that renal lithostathine inhibits CaCO₃ crystal growth in vitro.²³⁾ However, the concentrations and molecular form of renal lithostathine in human urine have not yet been determined. Therefore, we determined the immunoreactive PSP levels in human urine and studied its molecular forms.

When human urine was analyzed by Mono S cation exchange chromatography, one peak of immunoreactive PSP was detected. This peak was eluted at the position corresponding to that of PSP S2-5.¹⁷⁾ This suggests that urine PSP is similar to PSP S2-5 in ionic properties. However, Verdier et al.²³⁾ reported that renal lithostathine showed differences in molecular weight and immunoreactivity to antisera from the pancreatic protein, demonstrating that the proteins differed structurally by at least one epitope. We purified urine PSP from human urine and characterized some of its properties. The electrophoretic pattern of urine PSP was similar to that of PSP S2-5 moved more slowly. To clarify the reasons for this slower mobility, urine PSP was digested by trypsin, which split off glyco-undecapeptide from PSP S2-5. Then we analyzed the digested urine PSP for its N-terminal sequence (22 first amino acid) on SDS-PAGE and Mono S column chromatography. The N-terminal sequence (12 first amino acid) of urine PSP was the same as that of PSP S2-5.¹⁷⁾ These results suggest that urine PSP and PSP S2-5 have the same amino acid sequence and that the difference between the proteins is due to the variation in the carbohydrate side chain located at the fifth amino acid (threonine).²⁴⁾

Comparison of PSP Levels in Paired Sera and Urines

Paired samples of serum and urine were obtained from four normal volunteers and nine patients in the intensive care unit (ICU). The concentrations of PSP in urine and sera are summarized in Table 1. The PSP values of the patients in the ICU were significantly ($p < 0.05$) higher than those of the normal subjects.¹⁷⁾ The concentration of PSP in urine was higher than that of each correspondingly paired serum (Table 1).¹⁷⁾ These data suggest that urine PSP is different from PSP S2-5 and is synthesized and/or modified in the kidney and possibly secreted in

part from the renal tubules into urine. Further study would be required to identify the sites of urine PSP synthesis or modification.

Table 1. PSP Concentration in Urine and Sera of Healthy Subjects and Patients in ICU¹⁷⁾

Subject	PSP concentration		
	Serum (ng/ml)	Urine (ng/ml)	Urine (mg/g CRN)
Healthy subject A	54	172	
B	30	40	
C	48	145	
D	67	123	
Cardiac insufficiency	718	3,890	6,660
Perforation of large intestine	1,170	13,600	29,900
Myocardial infraction	1,560	16,500	45,700
Carcinoma of the esophagus	385	4,380	6,640
Cancer of the pancreas	263	800	2,500
Acute cardiac insufficiency	2,910	38,200	27,400
Aneurysm of the abdominal aorta	2,100	36,400	36,400
Angina pectoris ¹⁾	526	7,300	29,700
Intestinal obstruction	1,260	11,000	33,100

Correlation with Other Factors

Random urine samples obtained from patients with various diseases were measured for PSP, N-acetylglucosaminidase (NAG), and amylase, and BUN and serum creatinine were determined for the correspondingly paired sera. Urine PSP values correlated with urine NAG ($r=0.354$, $n=111$) but not with urine amylase ($r=0.032$, $n=119$), BUN ($r=0.187$, $n=109$) and serum creatinine ($r=0.074$, $n=110$). The distribution of urine PSP values in the subjects with high NAG values (between 5 to 10 U/1 and up to U/1) showed a different pattern from that in the subjects with low NAG values (less than 5 U/1).¹⁷⁾

The correlation between urine PSP and NAG suggests a relationship between urine PSP and damage in the renal tubule. In contrast, insignificant correlation between urine PSP and BUN or serum creatinine indicates that most of the urine PSP is not regulated by glomerular filtration. In order to evaluate the clinical usefulness of this urine PSP assay, more assays dealing with samples from patients with renal diseases would be required.

PSP IN PANCREATIC JUICE

PSP Levels in Pancreatic Juice from Patients with and without Pancreatic Disease

Pancreatic stone protein (PSP) is secreted with the normal pancreatic secretion and probably accounts for up to 10% of the secretory protein. It is also an inhibitor of calcium carbonate crystal growth and possibly involved in stabilization of pancreatic juice.⁴⁻⁶⁾ Multinger *et al.*⁶⁾ reported that PSP concentrations quantitated by radial immunodiffusion with a polyclonal antiserum immunized with PSP isolated from pancreatic stones were markedly decreased in most pancreatic secretions derived from patients with chronic pancreatitis when compared with controls.

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They hypothesized that the decreased PSP levels observed could be a key factor in the growth of calcium carbonate crystals and stone formation during the course of chronic calcifying pancreatitis.⁶⁾ However, Schmiegel et al.¹⁰⁾ could not confirm significant differences of PSP concentrations determined in pancreatic secretions either from patients with chronic pancreatitis, pancreatic cancer, or nonpancreatic diseases. They used an antigen-inhibition fluorometric immunoassay (FIA) using a commercially available monoclonal antibody D4-1-21 from Immunotech, France. Provansal Cheylan et al.¹⁷⁾ also failed to find a significant difference in PSP concentrations in pancreatic juice between patients with chronic calcifying pancreatitis and other diagnostic groups when the PSP concentrations were measured by radioimmunoassay using the monoclonal antibodies to PSP extracted from pancreatic stone, D4-1-21 and 2E7. However, decreased PSP concentrations were found in chronic calcifying pancreatitis patients when compared with other diagnostic groups when a sandwich enzyme-linked immunosorbent assay (ELISA) using monospecific polyclonal antibodies to the secretory form of PSP (PSP S) were applied for PSP determination in pancreatic juice.⁷⁾

We could not confirm a significant reduction of PSP in pancreatic secretions in either calcified (mean \pm SE=111 \pm 30 μ g/ml, 240 \pm μ g/mg protein, n=6) or noncalcified (305 \pm 133, 970 \pm 470, n=13) chronic pancreatitis when compared with controls (85 \pm 23, 340 \pm 160, n=16).²¹⁾ Our enzyme immunoassay for PSP has similar reactivities to PSP S1 and PSP S2-5. Furthermore, we measured PSP after dissolving insoluble PSP S1 (if present in pancreatic juice) with acetate buffer, pH 4.0.

Elution Pattern of PSP in Human Pancreatic and Duodenal Juices on HPLC

Human pancreatic juice was diluted and subjected to Mono S cation exchange chromatography. A single peak of immunoreactive PSP was usually eluted in a position corresponding to that of PSP S2-5 (Fig.3A).^{15,20)} When human pancreatic juice (2.0 ml) was analyzed immediately after addition of trypsin (640 μ l, 10 mg/ml), a peak of PSP S2-5 disappeared and a single peak of immunoreactive PSP was observed at a position consistent with that of PSP S1 (Fig.3B). After 1-h incubation, a single peak was observed again at the position of PSP S1 (Fig.3C). In a duodenal juice sample, a single peak of PSP S1 was observed, as in trypsin-added pancreatic juice (Fig.3D).²⁰⁾

Influence of Precipitated PSP S1 on PSP Assay

In a preliminary attempt to obtain precipitated PSP S1, we incubated pancreatic juice at 37 °C for 5 h, but no precipitate was detected. When pancreatic juice was incubated with high concentration of trypsin for 1 h, the PSP level tended to be underestimated because of precipitation of PSP S1. This underestimation can be prevented to a minimal level when the precipitate is solubilized in acidic pH and then PSP is assayed as in our previous studies.^{20,21)}

PSP IN PANCREATIC STONE

Protein analysis of intraductal precipitates and calculi is important to elucidate the mechanism of stone formation in chronic pancreatitis. We revealed immunoreactivities of PSP, lactoferrin, and human cationic trypsin in protein extracts of pancreatic stone from patients with chronic calcified pancreatitis (Table 2)²⁵⁾ (unpublished data). On immunostaining of pancreatic stone using an immunogold technique and scanning electron microscopy the immunoreactivity of PSP was observed diffusely in both the amorphous protein of the center of the stone and the concentric laminar layer of the periphery. The immunoreactivity of trypsin, on the other hand, was

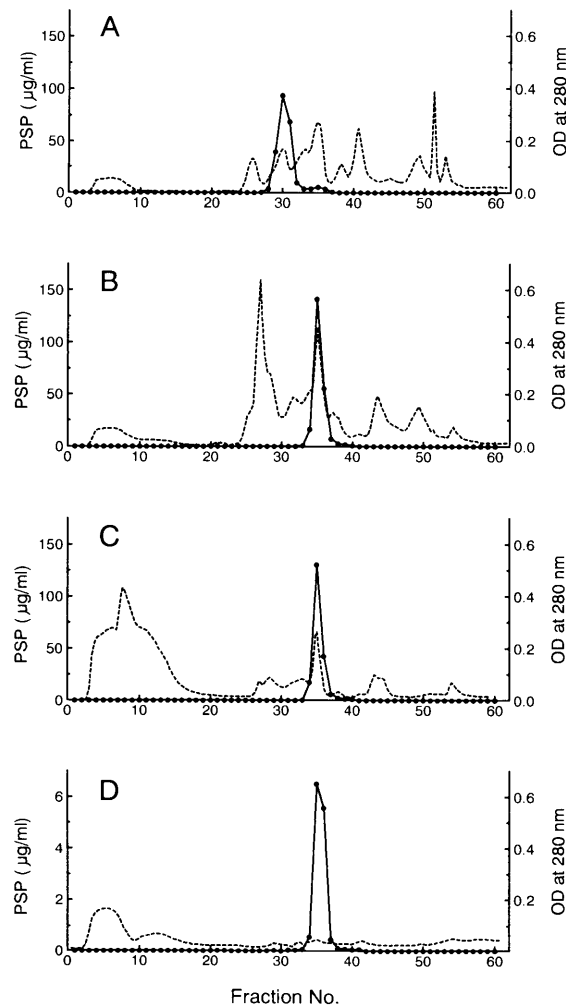


Fig. 3. Cation exchange (Mono S) chromatography of human pancreatic juice and duodenal juice. A: Pancreatic juice (PSP S2-5 content 116 μg). B: Pancreatic juice + trypsin (immediately after) (PSP S1 content 114 μg). C: Pancreatic juice + trypsin (after 1 h) (PSP S1 content 101 μg). D: Duodenal juice (PSP S1 content 7 μg). Closed circles and solid line indicate amounts of immunoreactive PSP. Dotted line indicates optical density (OD) at 280 nm. Each fraction was collected at 0.5 ml/tube.²⁰⁾

Table 2. Protein Content of Pancreatic Stone in Chronic Pancreatitis

Patient	Stone size	Protein ($\mu\text{g}/\text{mg}$ stone)	PSP ($\text{ng}/\mu\text{g}$ protein)	Lactoferrin ($\text{ng}/\mu\text{g}$ protein)	Trypsin ($\text{ng}/\mu\text{g}$ protein)
YA	small	29	419	1	12
SN	small	7	291	0	5
MU	mixed	6	105	0	42
HI	small	4	31	0	1
NM	small	4	10	0.1	22
KK	large	11	225	0	6
SU	large	9	92	3	10
KS	mixed	7	19	0	12

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detected more densely in the amorphous portion of the center than in the concentric laminar layer of the periphery.²⁸⁾ These results suggest that the presence of trypsinogen in pancreatic stone is not due to coprecipitation or adsorption of pancreatic enzymes but that trypsinogen is involved more closely in an initial step of intraductal precipitate formation than in a subsequent step of stone formation. Diffuse distribution of PSP is involved in both the initial step of intraductal precipitate formation and the subsequent step of stone formation in the pancreatic duct.

CONCLUSION

Our previous data suggest that major molecular forms of PSP are PSP S2-5 in the pancreatic secretions because only one peak of PSP was identified at the position corresponding to that of PSP S2-5 when PSP was analyzed on cation exchange (Mono S) chromatography.^{15,20)} Elution patterns of serum PSP usually demonstrate one peak consistent with that of PSP S2-5 when sera with elevated PSP were analyzed on the chromatography.¹⁵⁾ A few sera obtained from patients with pancreatic carcinoma or severe acute pancreatitis demonstrated a major peak of PSP S2-5 with a concurrent minor peak of PSP S1. Chromatopatterns of urine PSP showed a major peak of PSP S2-5 with or without a minor peak of PSP that was eluted at the position slower than that corresponding to that of PSP S1.¹⁷⁾ Therefore, our present assay using the monoclonal antibody recognizing PSP S1 and PSP S2-5 in a similar extent can be considered to reflect changes of PSP S2-5 in the pancreatic juices in chronic pancreatitis as well as in controls. However, on rare occasion of pancreatic cancer or severe acute pancreatitis, PSP S2-5 in the pancreatic juices can be converted, in part, to PSP S1 probably by intraductal premature activation of trypsinogen to trypsin. The same is also probably true of chronic pancreatitis, since intraductal activation of trypsinogen has been reported prior to recurrent attacks of pancreatitis.²⁶⁾ Therefore, even if the premature activation of trypsin occurs more frequently than we expect and soluble PSP S2-5 is subsequently converted, in part, to insoluble PSP S1, PSP levels in the pancreatic secretions, determined even by the assay specific to PSP S2-5, are probably not reduced in chronic pancreatitis. From our previous results the major or sole PSP constituent must be PSP S2-5, but not PSP S1 in pancreatic secretion, urine, and serum from subjects with usual forms of pancreatic diseases.^{15,20)}

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