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Partial amino acid sequence of human pancreatic stone protein, a novel pancreatic secretory protein

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Pancreatic stone protein (PSP) is the major organic component of human pancreatic stones. With the use of monoclonal antibody immunoadsorbents, five immunoreactive forms (PSP-S) with close M_r values (14000–19000) were isolated from normal pancreatic juice. By CM-Trisacryl M chromatography the lowest-M_r form (PSP-S_1) was separated from the others and some of its molecular characteristics were investigated. The M_r of the PSP-S_1 polypeptide chain calculated from the amino acid composition was about 16100. The *N*-terminal sequences (40 residues) of PSP and PSP-S_1 are identical, which suggests that the peptide backbone is the same for both of these polypeptides. The PSP-S_1 sequence was determined up to residue 65 and was found to be different from all other known protein sequences.

INTRODUCTION

In previous papers we have described the isolation of an acidic glycoprotein from human pancreatic stones. This protein, referred to as the pancreatic stone protein (PSP), contains two or three phosphorylated residues (De Caro *et al.*, 1979, 1984). PSP is located in pancreatic acinar cells (Lechêne de la Porte *et al.*, 1984) and is secreted with the normal pancreatic secretion, probably accounting for up to 10% of the secretory proteins (Multigner *et al.*, 1985). PSP has been demonstrated, *in vitro*, to be an effective inhibitor of the formation and precipitation of crystals of $CaCO_3$ (Multigner *et al.*, 1983).

During purification of this protein from human pancreatic juice with the use of an immobilized monoclonal antibody against PSP (D4), we isolated several forms of PSP (PSP-S) with different M_r values (about 14000–19000) (Montalto *et al.*, 1985). On the other hand, immunoprecipitation with anti-PSP polyclonal antibodies of translation products of human pancreatic total polyadenylated RNA yielded a single polypeptide of M_r about 16000, which corresponds to the pre-PSP (Giorgi *et al.*, 1985). After Western blotting, the polyclonal antibodies against PSP recognize all forms, which suggests that all the PSP-S forms result from post-translational modifications such as glycosylation (Montalto *et al.*, 1985).

In the present paper, some molecular properties of PSP-S_1 and PSP, including partial amino acid sequence, are reported. A comparison is drawn between some of the molecular properties of the two polypeptides.

MATERIALS AND METHODS

Materials

Pure pancreatic juice from patients without any pancreatic disease was collected by endoscopic retrograde cannulation of the pancreas. Only samples devoid of free proteolytic activity were used (De Caro *et al.*, 1975). Pancreatic stones were obtained surgically from patients suffering from chronic calcifying pancreatitis. Phenylmethanesulphonyl fluoride and N^{α}-benzoylarginine were obtained from Sigma Chemical Co. 3-Phenylpropionic acid and CNBr were from Fluka.

Monoclonal antibodies

Monoclonal antibodies resulted from a fusion of X63 myeloma cells with splenocytes from BALB/c mice that had been immunized with PSP (Montalto *et al.*, 1985). Monoclonal antibodies from two positive hybridoma clones D4 and 2E7 were developed in mice ascites and subsequently purified on a Protein A–Sepharose column. Both antibodies were identified as IgG1. A 40 mg portion of each monoclonal antibody preparation was coupled to Affi-Gel 10 (Bio-Rad Laboratories), and the two immunoadsorbent columns were used to isolate immunoreactive forms of PSP from pancreatic juice and from pancreatic stones.

Purification of immunoreactive forms of PSP

For purification of the immunoreactive forms of PSP, the procedure described by Montalto *et al.* (1985) was modified as follows. To prevent any activation of proteolytic enzymes during chromatography, purification

Abbreviations used: PSP, pancreatic stone protein; PSP-S, secretory forms of PSP, PSP-S_1 being the lowest-M_r form and PSP-S_{2-5} representing several forms of higher M_r.

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of the immunoreactive forms was carried out in the presence of 3-phenylpropionate and benzoylarginine, known to be potent competitive inhibitors of pancreatic carboxypeptidases A and B; benzamidine (a competitive inhibitor of trypsin) and phenylmethanesulphonyl fluoride (a general inhibitor of serine proteinases) were also added.

Freeze-dried pancreatic juice (40 mg of protein) was resuspended under gentle stirring at 0 °C in phosphate-buffered saline (100 mM-NaCl/10 mM-KH_2PO_4/40 mM-Na_2HPO_4 buffer, pH 7.2) containing benzamidine (5 mM), phenylmethanesulphonyl fluoride (1 mM), benzoylarginine (0.5 mM) and 3-phenylpropionate (2 mM). After centrifugation (4000 *g* for 10 min), the clear supernatants were applied on to the immunoaffinity column (2.5 cm × 5 cm) containing 1.5 mg of monoclonal antibody/ml of gel and equilibrated with phosphate-buffered saline containing benzamidine (5 mM). The proteins adsorbed on the column were eluted with 0.2 M-glycine/HCl buffer, pH 2.8, and neutralized with 1 M-Tris/HCl buffer, pH 8.9. Protein fractions were concentrated in an ultrafiltration cell (Amicon) and dialysed overnight against phosphate-buffered saline at 4 °C. The two latter operations were carried out in the presence of the same concentrations of inhibitors. To improve purification the concentrated protein fractions were recycled on the same affinity column. The recovery of proteins from the second affinity column chromatography was about 75%.

Separation of PSP-S_1 from the PSP-S_{2-5} forms was carried out as follows. After their second elution from the immunoaffinity column, the proteins were maintained at pH 3.0, concentrated in an ultrafiltration cell and equilibrated on a Trisacryl GF05 column (2 cm × 43 cm) in 40 mM-sodium acetate buffer, pH 5.0. Proteins were then subjected to chromatography on a CM-Trisacryl M column (1.0 cm × 15 cm) (Fig. 2). Lastly, fractions containing PSP-S_1 and PSP-S_{2-5} forms were pooled, extensively dialysed against 1 mM-HCl at 4 °C, freeze-dried and stored at −30 °C.

PSP was also isolated from human pancreatic stones. The stones were ground to a powder and demineralized by repeated extractions in 0.5 M-disodium EDTA, pH 8.0, containing the four proteinase inhibitors (De Caro *et al.*, 1984). After centrifugation (4000 *g* for 30 min), the supernatants were dialysed exhaustively against distilled water and then freeze-dried. Freeze-dried extracts were dissolved in 1 mM-HCl, and just before loading on to the immunoaffinity column the pH was adjusted to 8.0 with 10 mM-Mes/NaOH buffer. After elution, PSP was finally dialysed against 1 mM-HCl at 4 °C and freeze-dried. The protein yield of repeated extractions was 0.1% of the initial powder weight, and the recovery of PSP from the immunoaffinity column chromatography was higher than 90%.

Amino acid analysis and *N*-terminal sequence determination

PSP and PSP-S_1 were reduced and *S*-carboxymethylated (Crestfield *et al.*, 1963). Amino acid analyses were carried out with a Beckman 120 C analyser after 6 M-HCl hydrolysis of the protein in evacuated sealed tubes at 110 °C for 24, 48 and 72 h. Cysteine was determined as carboxymethylcysteine and in certain experiments as cysteic acid (Moore, 1963). Tryptophan and γ-carboxyglutamic acid were determined after alkaline hydrolysis (Wilkinson *et al.*, 1976; Hauschka, 1977). The presence of free thiol groups was investigated with 5,5′-dithiobis-(2-nitrobenzoic acid) as described by Ellman (1959). Titration was carried out on 34 nmol of protein in the presence of 0.25% SDS. Human pancreatic lipase, which contains two free thiol groups per molecule, was used as a reference protein (De Caro *et al.*, 1981). The dansyl technique of Gray and Hartley, as described by Gray (1972) and as modified by Charles *et al.* (1974), was used to identify the *N*-terminal residues of proteins and peptides respectively.

The automated sequencing was performed with a Beckman 890 C sequencer or with a Socosi PS 100 instrument. The Socosi program was an adaptation of that described by Brauer *et al.* (1975), with 0.3 M- instead of 0.1 M-Quadrol. The Beckman program used was that indicated by the manufacturer (ref. 122 974), with the use of high speed for reagent delivery and 0.1 M- instead of 1 M-Quadrol. For each sequence, two runs were carried out. Phenylthiohydantoin derivatives were identified by h.p.l.c. (Bonicel *et al.*, 1981). Before cleavage with CNBr the free α- and ε-amino groups were blocked by reaction of the reduced and carboxymethylated protein with succinic anhydride. The sample was then dissolved in 70% (v/v) formic acid and incubated for 24 h at 4 °C under N_2 with an 180-fold excess of CNBr. After addition of an equal amount of reagent, incubation was continued for 24 h.

Gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed on 15% polyacrylamide slab gels also containing 8 M-urea (Laemmli, 1970). M_r measurements were performed on proteins after reduction and alkylation with dithiothreitol (0.1 M) and iodoacetamide (0.2 M) respectively. Electrophoresis was also performed in 10% polyacrylamide gel in a discontinuous buffer system at pH 5.0 (Reisfeld *et al.*, 1962). A 30 mM-NaCl/175 mM-β-alanine/acetate buffer was used for protein separation. The staining of protein bands was performed with Coomassie Blue R-250. Isoelectric focusing in a polyacrylamide slab gel (pH range 3–10) was performed in the presence of 8 M urea (De Caro *et al.*, 1984). The protein bands were located by using the procedure described by Scheele (1975).

Phosphorus determination and carbohydrate content

The phosphorus content of proteins was determined (Turner & Rouser, 1970) on proteins purified according to the procedure described above but in a 100 mM-NaCl/10 mM-Mes/NaOH buffer, pH 7.2, instead of phosphate-buffered saline. The presence of carbohydrates was investigated by periodate/Schiff staining on polyacrylamide gels after electrophoresis (Glossmann & Neville, 1971). Neutral sugars were determined by the phenol/H_2SO_4 method (Dubois *et al.*, 1956), with mannose as a standard.

RESULTS

Characterization of the PSP-S forms

Several molecular forms of PSP were purified from pancreatic juice by using the two monoclonal-antibody immunoadsorbent columns (Fig. 1*a*, lanes 1–4). The relative proportions of these immunoreactive forms

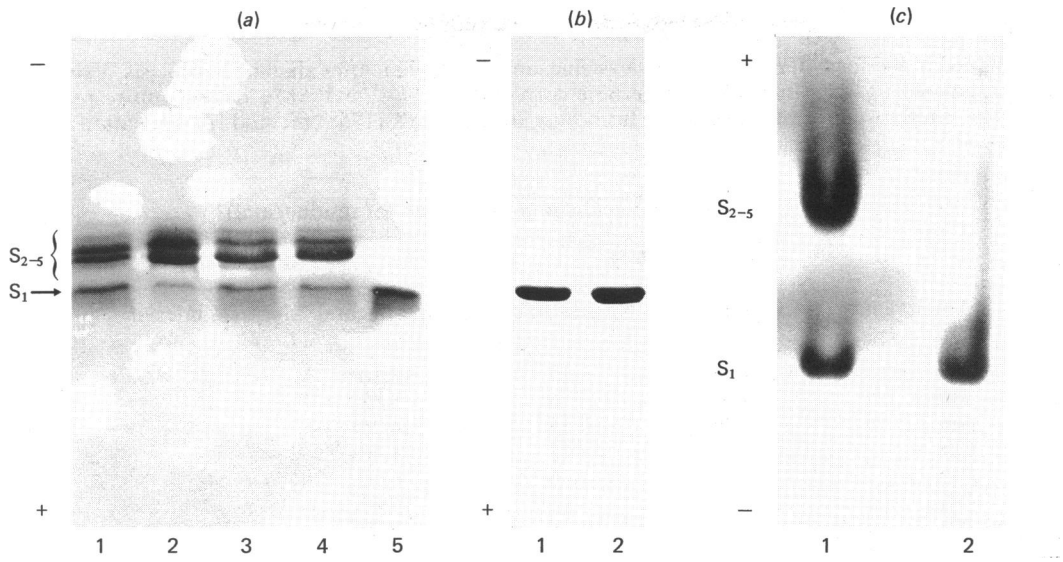

Fig. 1. Electrophoresis on polyacrylamide gels of PSP and of the immunoreactive forms of PSP isolated from pancreatic juice

(*a*) and (*b*) In the presence of SDS. (*a*) Lanes 1–4, different samples of PSP-S_{1-5} forms after elution from the immunoaffinity column; lane 5, PSP-S_1 (CM-Trisacryl column). (*b*) Lane 1, PSP (affinity column); lane 2, PSP-S_1 (CM-Trisacryl column). (*c*) In a non-dissociating system at pH 5.0. Lane 1, PSP-S_{1-5} (affinity column); lane 2, PSP-S_1 (CM-Trisacryl column).

estimated by scanning at 590 nm after SDS/polyacrylamide-gel electrophoresis gave 75–90% as the PSP-S_{2-5} forms, with the PSP-S_1 form accounting for the remainder. The separation of PSP-S_1 from the PSP-S_{2-5} forms was performed by CM-Trisacryl M chromatography (Fig. 2). Peak I contains the PSP-S_{2-5} forms, and peak II the PSP-S_1. They account for 60% and 20% of the load respectively. The electrophoretic mobilities on SDS/polyacrylamide-gel electrophoresis of PSP-S_1 and PSP are identical (Fig. 1*b*, lanes 1 and 2). The

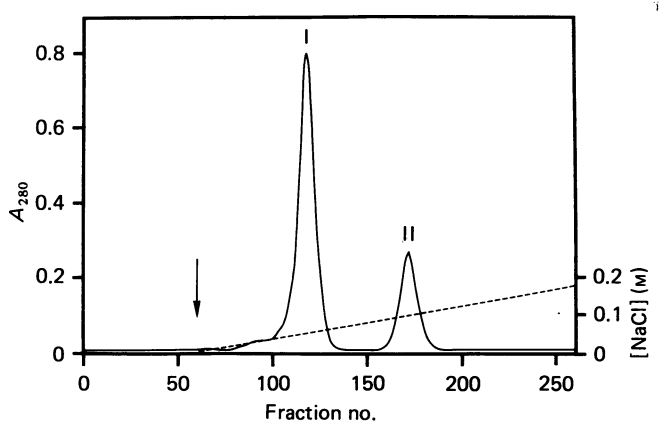

Fig. 2. CM-Trisacryl ion-exchange chromatography of proteins eluted from monoclonal-antibody immunoadsorbent columns

A 7.5 mg portion of protein in 50 ml of 40 mM-sodium acetate buffer, pH 5.0, was applied to a 1.0 cm × 15 cm column equilibrated with the same buffer. The column was eluted at a flow rate of 35 ml/h with a linear gradient of NaCl (0–0.175 M; 2 × 150 ml) (------). Fractions of volume 1.5 ml were collected. The PSP-S_{2-5} forms were eluted in peak I, and PSP-S_1 was eluted in peak II.

electrophoretic migration on non-dissociating polyacrylamide gel (Fig. 1*c*) showed a difference in charge between the PSP-S_1 and PSP-S_{2-5} forms. The latter were found to be less cationic than the PSP-S_1 form. This result reflects the fact that the PSP-S_{2-5} forms have a weaker cationic behaviour than PSP-S_1, as established by chromatography on a CM-Trisacryl M column. Moreover, the staining of the gels with the periodate/Schiff reagent demonstrated that the PSP-S_{2-5} forms are glycosylated, whereas PSP-S_1 was poorly stained under the same conditions (results not shown).

Characterization of PSP-S_1

Sequence determination. Before the determinations indicated below, PSP-S_1 was reduced and *S*-carboxymethylated. The amino acid composition (Table 1) was found to have the following characteristics. The main amino acids were aspartic acid/asparagine, glutamic acid/glutamine and serine. It is noteworthy that six tryptophan residues and only one methionine residue were present per molecule, and that γ-carboxyglutamic acid was absent. The presence of six half-cystine residues per molecule suggests the presence of three disulphide bridges, since no free thiol group was detected. The M_r calculated from the amino acid composition was 16112. Determination of the amino acid composition of PSP after 24 h hydrolysis gave identical values. A single *N*-terminal amino acid corresponding to isoleucine was found in PSP-S_1. Fig. 3 shows that the *N*-terminal sequences of PSP-S_1 and PSP are identical. Automated sequential degradation of PSP-S_1 gave data up to asparagine-38 except for residues 35, 36 and 37. The same experiment on PSP yielded the first 40 amino acid residues with the exception of residues 35 and 38. With the single methionine residue of the polypeptide chain (residue 34) it was possible to carry out the sequence farther. Before CNBr treatment, which, in principle, is liable to divide the molecule into two fragments, the

Table 1. Amino acid composition of human PSP-S_1 and PSP

Cysteine was determined in the form of carboxymethylcysteine, and tryptophan after alkaline hydrolysis. Values for serine and threonine were extrapolated to zero time (PSP-S_1) or were corrected for 17% and 7.6% destruction respectively after 24 h hydrolysis (PSP). The integer values were calculated according to Delaage's (1968) method. Abbreviation: Gla, γ-carboxyglutamic acid.

	Amino acid composition (mol of residue/mol)			
	PSP-S_1		PSP*	
Amino acid	Experimental	Nearest integer	Experimental	Nearest integer
Asx	16.89	17	18.10	18
Thr	5.83	6	5.58	6
Ser	14.71	15	14.25	14
Glx	13.24	13	11.40	11
Pro	6.38	6	6.10	6
Gly	10.96	11	14.40	14
Ala	7.65	8	7.30	7
Cys	5.68	6	5.83	6
Val	10.54	11	10.93	11
Met	0.99	1	1.02	1
Ile	4.19	4	3.76	4
Leu	8.32	8	8.50	8–9
Tyr	6.79	7	7.60	8
Phe	6.91	7	7.60	8
Lys	10.97	11	10.10	10
His	1.97	2	2.20	2
Arg	3.97	4	4.02	4
Trp	6.06	6	—	—
Gla	0	0		
Total		143		
M_r... 16112				

* Results for PSP obtained after 24 hydrolysis.

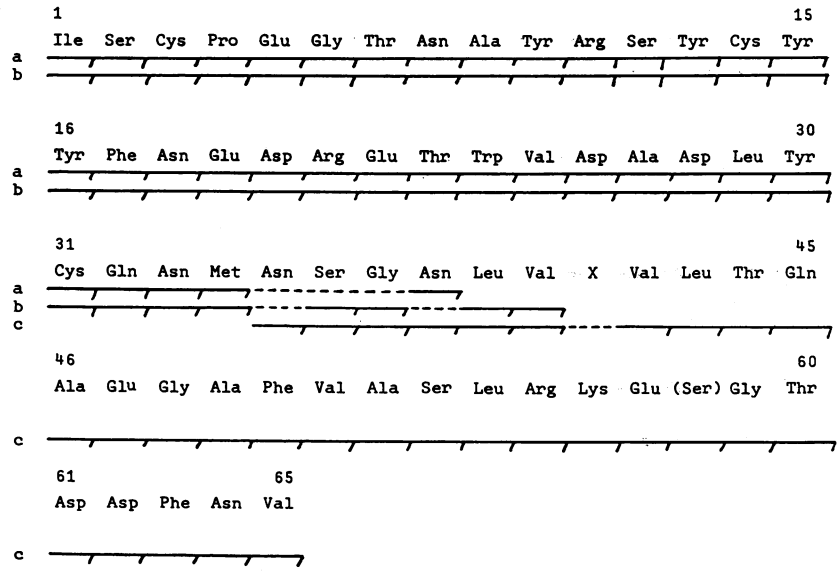

Fig. 3. Partial primary structure of PSP-S_1 and PSP

Automatic sequencing was performed (a) on PSP-S_1, (b) on PSP and (c) on PSP-S_1 after succinylation and CNBr treatment. X indicates unidentified residue; ---- indicates unidentified residue during the run under consideration; (Ser) indicates not clearly identified. In the experiments 1 mg of PSP-S_1, 2.8 mg of PSP and 1 mg of CNBr-cleavage peptides were sequenced with initial yields of 35, 25 and 20% respectively, and repetitive yields were 88–92% for each experiment. The phenylthiohydantoin derivative of succinyl-lysine was found between the glutamine and alanine phenylthiohydantoin peaks.

α-amino group of isoleucine-1 was blocked by succinylation (3-carboxypropionylation). After CNBr cleavage, a single *N*-terminal amino acid, asparagine, was found, and from the automated degradation the sequence was identified for 31 residues. The fact that the first amino acid residues of this sequence (2–6) were identical with the amino acid residues 36–40 of the PSP provided the proof that the new sequence, as expected, occupied positions 35–65 in the PSP-S_1 polypeptide chain. Apart from residue 41, which was not identified, and residue 58, which could not be clearly demonstrated, the sequence of the first 65 amino acid residues of PSP-S_1 was established without any ambiguity.

The first 57 residues from the *N*-terminal sequence were matched in 40-residue fragments and compared with all available sequences at the National Biomedical Research Foundation and GENPRO (Bolt, Beranek and Newman, Cambridge, MA, U.S.A.). No significant homology was found between PSP-S_1 and any of the available 3182 and 2204 sequences respectively as of May 1985.

Carbohydrate, phosphorus and isoelectric-point determinations. Determination of the neutral sugars of the PSP-S_1 form indicated a neutral sugar content of approx. 2 mol/mol of protein. On the other hand no trace of phosphorus could be detected with about 50 nmol of protein from four different samples. Isoelectric focusing of the polypeptide, which was performed in four different cases, showed only one band, with an isoelectric point of 6.25.

PSP-S_1 solubility

When the PSP-S_1 concentration reached 0.7–1 mg/ml the protein was insoluble between pH 5.0 and pH 9.0. In order to prevent any precipitation during the purification the PSP–S_1 concentration was maintained at around 0.1–0.2 mg/ml.

DISCUSSION

The choice of an immunological method for isolating pancreatic secretory forms of PSP has the advantage that it is simple, efficient and rapid. The monoclonal-antibody immunoadsorbent columns were used more than 100 times without any decrease in their adsorption capacity. To avoid any possible degradation by proteolytic enzymes, proteinase inhibitors were included in all our preparations. The use of the 2E7 monoclonal antibody directed against PSP from calculi confirms the previous finding with D4 monoclonal antibody that PSP in pancreatic juice takes several immunoreactive forms (Montalto *et al.*, 1985). The intense staining for sugars obtained with the PSP-S_{2-5} forms as compared with the mild staining of PSP–S_1 may indicate that the PSP-S_{2-5} forms have a higher sugar content. Differences in glycosylation of the latter may explain the observed heterogeneity.

The amino acid composition suggests that PSP-S_1 is composed of about 143 amino acid residues and that the M_r of the peptide moiety is about 16100. The migration of PSP-S_1 on polyacrylamide-gel electrophoresis in the presence of SDS gave a smaller size (M_r 14000). It is possible that, although the protein was denatured, particular features modified the electrophoretic migration. Elucidation of the total sequence of PSP-S_1 will perhaps clear up this point. The high aspartic acid/asparagine, glutamic acid/glutamine and serine content is noteworthy. The number of amide groups was not determined. Another point worth noting is the fact that the number of tryptophan residues per molecule (six) is particularly high compared with other proteins. The location of the three disulphide bridges per molecule remains to be investigated.

Since the amino acid composition of PSP purified by classical chromatography procedures and published previously (De Caro *et al.*, 1984) is different from that of PSP-S_1 given in the present paper, it was important to re-investigate the PSP composition with a highly purified preparation (obtained with the use of monoclonal antibody immunoadsorbent). The results obtained show that the amino acid compositions of PSP and PSP-S_1 are identical (Table 1). The results on PSP and PSP-S_1 *N*-terminal sequences show that at least up to the first 40 amino acid residues the two polypeptide chains are identical. It is possible that the lengths of the two chains might not be exactly the same, possibly as the result of proteolysis. The sequence of PSP-S_1 is known at present up to residue 65 except for residue 41, which could not be identified, and residue 58, the determination of which was only tentative. From the sequential degradation of PSP-S_1, the first 38 amino acid residues were identified, but with three undetermined residues in positions 35, 36 and 37. By cleaving the single methionyl bond (34–35), the sequencing was continued from residue 35 to residue 65. The addition of the sequence determined after CNBr cleavage to the sequence 1–34 is not only logical on account of the specificity of CNBr but is also justified by the overlap (30–40) found with the sequence of PSP.

We reported earlier (De Caro *et al.*, 1984) that PSP isolated from calculi is a glycoprotein containing two or three phosphorylated residues per molecule; here we confirm the low neutral sugar content per PSP-S_1. However, PSP and PSP-S_1 are not identical molecules: no trace of phosphorus could be detected in PSP-S_1, and PSP-S_1 gives a single band on isoelectric focusing (pI 6.25), whereas PSP gives several bands (pI 5.1, 5.5 and 5.8) (De Caro *et al.*, 1984). These charge differences could be due either to a lack of phosphorus or to a deamidation reaction, which has been observed to result in a shift from negatively to positively charged proteins in bovine pancreatic stones (De Caro *et al.*, 1982).

Guy-Crotte *et al.* (1984) have reported the partial characterization of a protein present in human pancreatic juice, referred to as 'protein X'. The M_r, the amino acid composition and the *N*-terminal sequence of the first 15 residues of this protein do not differ significantly from those determined for PSP-S_1 and PSP. Moreover, those authors (Guy *et al.*, 1983; Guy-Crotte *et al.*, 1984) have found that the 'protein X' is immunologically identical with PSP. They have assumed that the 'protein X' and PSP could be a degradation product of trypsinogen 1. Our results indicate, however, that there is no similarity to be found when the partial sequence of PSP-S_1 is compared with the sequences of all other known proteins, in particular pancreatic secretory proteins such as trypsinogens. The hypothesis formulated by Figarella *et al.* (1984) and Guy-Crotte *et al.* (1984) thus appears to be incorrect.

During the present study a publication by Gross *et al.* (1985) gave the partial sequence of an unusual bovine pancreatic protein referred to as pancreatic thread

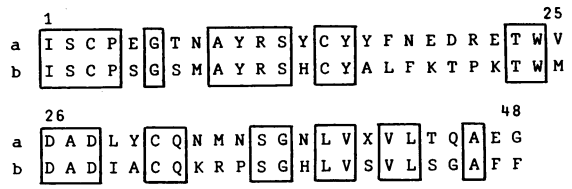

Fig. 4. Sequence comparison between human PSP-S_1 (a) and chain A of bovine pancreatic thread protein (b)

The standard IUPAC–IUB one-letter code for amino acids is used. Boxed letters indicate identical amino acids.

protein. This protein consists of two disulphide-linked polypeptide chains of different lengths (chain A, M_r 14000; chain B, M_r 3000). The chain A sequence was elucidated up to 48 residues, which exhibits 52% of homology with PSP-S_1 (Fig. 4). It is worthwhile to note that the bovine pancreatic thread protein and human PSP-S_1 possess eight and six tryptophan residues per molecule respectively and a homologous location of the three half-cystine residues present in the *N*-terminal sequences. Moreover, the two polypeptides present a similar solubility with respect to pH.

Further studies need to be undertaken to understand why only a single form, with M_r about 16000, is to be found in the stone while in pancreatic juice several molecular forms are present. It may be noted that using proteinase inhibitors we obtained only the 16000-M_r form (PSP) by demineralization of stones, as previously described without proteinase inhibitors.

We have shown previously that PSP is an inhibitor of $CaCO_3$ crystal growth. This inhibitory activity has been also found in the normal pancreatic juice (De Caro *et al.*, 1984), suggesting that these proteins could act *in vivo* as a stabilizer of supersaturated pancreatic secretion.

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