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Cleavage of the Arg–Ile bond in the native polypeptide chain of human pancreatic stone protein

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The pancreatic stone protein (PSP) isolated from calculi (M_r 14000) and the 5 protein forms (PSP S1-5) detected in pancreatic juice (M_r 14000–19000) derive from the same source differing seemingly in their carbohydrate contents or/and in their polypeptidic chain lengths. This kind of protein would inhibit in vivo $CaCO_3$-crystal growth in pancreatic juice. PSP and PSP S1 N-terminal sequences are identical (NH_2Ile-). This report demonstrates that: (i) in PSP S2-5 the amino-terminal is blocked; (ii) the C-terminus is alike in every form; (iii) the single polypeptide chain of PSP S2-5 is converted into that of PSP S1 or PSP by the specific trypsin cleavage of the Arg–Ile bond.

Pancreatic stone protein; Pancreatic juice; Specific trypsin cleavage; End group analysis; Carboxypeptidase Y digestion; Carboxypeptidase P digestion; (Human pancreatic calculi)

1. INTRODUCTION

With the isolation of the pancreatic stone protein, PSP (M_r 14000) from calculi of patients suffering from chronic calcifying pancreatitis [1], it was possible to reveal by immunolocalization that the initial form of the protein is synthesized in pancreatic acinar cells and that it follows the same pathway as the digestive enzymes [2].

Using an immobilized monoclonal antibody against PSP, several forms of protein (PSP S1-5) with different M_r (14000–19000) were detected in pancreatic juice [3]. The differences in these protein forms might result from proteolytic cleavage(s) in the polypeptide chain and/or variations in carbohydrate contents. The role of this protein would be to inhibit in vivo the $CaCO_3$-crystal growth in pancreatic juice [4]. The smallest form, PSP S1, and the protein prepared from calculi, PSP, display the same electrophoretic migration in the presence of SDS [5]. In [5] it has been shown that Ile is the N-terminal amino acid in both proteins and that their N-terminal sequences are identical (the 40 and 65 first amino acids are known in PSP and PSP S1, respectively). Moreover, the same N-terminal sequence of the 45 first amino acids has been found in the protein called 'human pancreatic thread protein' by the authors [6] indicating that all these proteins derive from the same source. It is of great interest to proceed further with the structure comparison between PSP, PSP S1 and PSP S2-5 in order to understand the mechanism of formation of the modified forms. Here, the results on the determination of N- and C-terminal amino acid residues and the sequences of two homologous cystine pep-

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Abbreviations: TPCK, tosylphenylalanyl chloromethyl ketone; TLCK, tosyllysyl chloromethyl ketone; HPLC, high-pressure liquid chromatography

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tides resulting from the pepsin hydrolysis of PSP S1 and PSP S2-5 are compared. Limited proteolyses on PSP S2-5 are performed.

2. EXPERIMENTAL

Several methods have already been described: purification of PSP, PSP S1 and PSP S2-5 [5]; dansylation and amino acid analysis [7]. The peptide chromatography was performed on thin-layer cellulose (cell 400 Macherey Nagel) and developed in the solvent system butanol/pyridine/acetic acid/water (60:40:12:48, v/v, pH 4.8) according to the directions in [8]. Electrophoreses on thin-layer cellulose were conducted with buffer (pyridine/acetic acid/acetone/water, 20:40:150:790, pH 4.4) for 3 h at 300 V. The peptides were located using fluorescamine (0.01%). The cystine-containing peptides were revealed on aliquots with nitroprusside reagent [9]. The automated Edman degradation of peptide B10G1 (5 nmol) was carried out in a gas-phase sequencer (Applied Biosystems, model 470A). The PTH-amino acids were analyzed by HPLC on a Waters chromatograph using an ODS 5 μm Beckman column. The conditions for enzymatic digestions were as follows: protein (1.2–1.3 μmol) in 6 ml of 5% formic acid was incubated with pepsin (enzyme/substrate, 1:10, w/w) at 37°C. After 20 h, an equal amount of enzyme was added and the digestion was pursued 4 h more. The peptide A1 (100 nmol) in 0.1 ml 60 mM ammonium bicarbonate buffer (pH 8.5) was digested with TPCK-trypsin (enzyme/substrate, 1:20, w/w) for 30 min at 25°C. The peptide B1 (200 nmol) in 0.5 ml of 60 mM ammonium bicarbonate (pH 7.8) was digested with *S. aureus* V8 protease (enzyme/substrate, 1:40, w/w) for 18 h at 37°C. For C-terminal determination, 70 μM native or carboxymethylated reduced protein in 60 μl of 50 mM pyridine acetate buffer (pH 5.5), 6 M in urea, were incubated with 1:10 (w/w) carboxypeptidase P or Y (Boehringer Mannheim) for 3 h at 25°C and 20 h at 37°C respectively. The hydrolysates were directly investigated on an amino acid analyzer.

3. RESULTS AND DISCUSSION

3.1. *N- and C-terminus determination*

Analyses were performed on the PSP S2-5 mixture since it has been impossible up to now to separate these protein forms. No N-terminal amino acid was found in carboxymethylated reduced PSP S2-5 using the dansyl method or automated Edman degradation. In the determination of C-terminal end of PSP, PSP S1 and PSP S2-5 by carboxypeptidases Y and P, the following results were obtained: (i) no amino acid was released from the proteins, even in presence of 6 M urea; (ii) the carboxymethylated reduced proteins, in 6 M urea, gave negative results with carboxypeptidase Y, whereas three amino acids (Lys, Asn and Phe) were freed with acid carboxypeptidase P (in the ratios of 1, 1 and 0.5 respectively) from the different forms of pancreatic stone protein. This last experiment shows that the C-terminal sequences of PSP, PSP S1 and PSP S2-5 are identical and suggests that the formation of PSP S1 would be the result of cleavage(s) in the N-terminal sequence of the native protein.

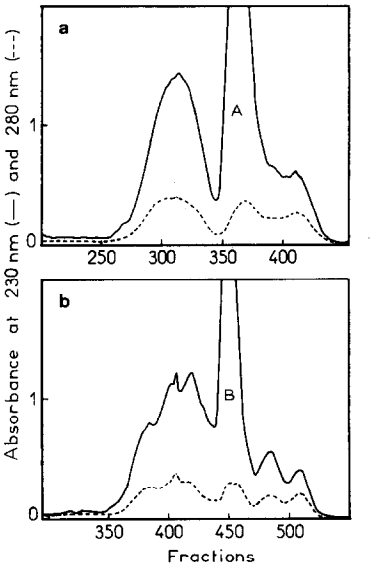

Fig.1. Gel filtration on Sephadex G-50 (superfine) of the peptic digestions of PSP S1 and PSP S2-5. Two or three connected columns were equilibrated and eluted with 1% acetic acid at 5°C. The effluents were monitored at 230 nm (——) and 280 nm (– – –). The cystine-positive fractions were revealed as indicated [9]. (a) The digest of PSP S1 (1.3 μmol) was applied to the column set (1.5 × 425 cm). Flow rate, 6.6 ml/h; 2.0-ml fraction. (b) The digest of PSP S2-5 (1.2 μmol) was applied to the column set (1.5 × 500 cm). Flow rate, 5.6 ml/h; 2.0-ml fraction.

Table 1

Various operations performed on fractions A and B in view of the identification of the cystine peptide they contain

A	B
Chromatography – electrophoresis ⟶ cystine-positive spot: A1	Filtration Sephadex G-25 – chromatography ⟶ cystine-positive spot: B1
Trypsin digest – chromatography ⟶ cystine-positive spot: A1T	*S. aureus* V8 protease digest – chromatography ⟶ cystine-positive spot: B1G
Oxidation – chromatography ⟶ A1T01 and A1T02	Oxidation – chromatography ⟶ B1G01 and B1G02

Table 2

Amino acid composition and sequences of peptides deriving from the fractions A and B (table 1)

Amino acid	A1		A1T01		A1T02		B1		B1G01		B1G02	
	a	b	a	b	a	b	a	b	a	b	a	b
Cys	1.5	(2)	0.8	(1)	1.0	(1)	1.5	(2)	1.2	(1)	0.9	(1)
Asx	1.2	(1)	1.0	(1)			0.9	(1)	0.1		0.8	(1)
Thr	0.7	(1)	0.9	(1)			1.3	(1)			1.0	(1)
Ser	1.8	(2)	0.8	(1)	0.6	(1)	2.2	(2)	1.0	(1)	1.0	(1)
Glx	1.0	(1)	1.0	(1)			1.0	(1)	1.0	(1)		
Pro	0.8	(1)	1.0	(1)			1.0	(1)	1.0	(1)		
Gly	1.5	(1)	1.0	(1)	0.3		1.6	(1)	0.2		1.3	(1)
Ala	1.4	(1)	0.9	(1)			1.2	(1)	0.1		1.1	(1)
Val												
Met												
Ile	1.0	(1)	0.8	(1)			1.0	(1)	1.0	(1)		
Leu												
Tyr	3.0	(3)	0.9	(1)	1.7	(2)	2.7	(3)			2.8	(3)
Phe												
His												
Lys												
Arg	1.3	(1)	0.9	(1)			2.2	(2)	1.0	(1)	1.0	(1)
Trp												
Total residues		(15)		(11)		(4)		(16)		(6)		(10)
N-terminal residue	Ile		Ile		Ser		n.d.		Arg		Gly	

A1 Ile Ser Cys Pro Glu Gly Thr Asn Ala Tyr Arg Ser Tyr Cys Tyr

B1G01 Arg Ile Ser Cys Pro Glu

B1 Arg Ile Ser Cys Pro Glu Gly Thr Asn Ala Tyr Arg Ser Tyr Cys Tyr

Values a are data of 24 h hydrolysis without corrections for losses; values b refer to the sequence of the characterized peptide. For A1 and B1 two hydrolyses were performed before and after performic acid oxidation

3.2. *Comparison of two homologous pepsin peptides from PSP S1 and PSP S2-5*

When PSP S1 was hydrolyzed by pepsin and the hydrolyzate filtered on Sephadex, the elution profile displayed a peak (peak A) sharply separated from the rest of the diagram (fig.1a). As will be demonstrated below, the major peptide purified from fractions A revealed to be the cystine pentadecapeptide representing the 15 first amino acids of PSP S1 [5]:

1 15
Ile Ser Cys Pro Glu Gly Thr Asn Ala Tyr Arg Ser Tyr Cys Tyr

Similar operations were tentatively performed on the PSP S2-5 mixture with a view to obtaining a homologous cystine peptide with a few amino acids preceding Ile. This would allow the determination of the nature of the bond cleaved in the formation of PSP S1. Fig.1b shows the Sephadex elution pattern of the pepsin hydrolysate of PSP S2-5. In the diagram, peak B is comparable to peak A (fig.1A) and its fractions were also cystine-positive. The different operations carried out on fractions A and B are given in table 1. The characterizations of most of the peptides mentioned in table 1 are indicated in table 2. In the case of A1, its amino acid composition and the fact that its N-terminal amino acid is an isoleucine provided sufficient support to conclude that it represents the sequence of the 15 first amino acids of PSP S1 [5]; since A1 was a cystine-containing peptide it is obvious that an S-S bridge connected Cys 3 to Cys 14. Moreover these data were confirmed by the study of the trypsin digest of A1. The cystine peptide A1T had the same amino acid composition as A1 and after oxidation two cysteic peptides were separated. A1T01 and A1T02 had the anticipated amino acid compositions. In the case of B1, its amino acid composition had an extra arginine compared to that of A1. After digestion with staphylococcal protease, the cystine peptide B1G obtained had the same composition as B1. After oxidation of B1G, two cysteic peptides B1G01 and B1G02 were separated. The automatic sequence performed on B1G01 definitely proved that the extra Arg immediately precedes Ile (table 2). It is concluded that the PSP S1 form arises from cleavage of the Arg–Ile bond in the native protein. Seemingly, the same process has given rise to the polypeptide chain of PSP, the protein found in pancreatic stones.

3.3. *Limited proteolysis of PSP S2-5 by trypsin and chymotrypsin*

The electrophoretic patterns (fig.2) showed that trypsin transformed PSP S2-5 into a major protein displaying a migration similar to that of PSP S1. It is interesting to note that this proteolysis was concomitant with the protein precipitation. In contrast, despite the great amount of enzyme involved, chymotrypsin did not cleave native PSP S2-5 efficiently. The determination of the N-terminal amino acid(s) in the digest confirmed the

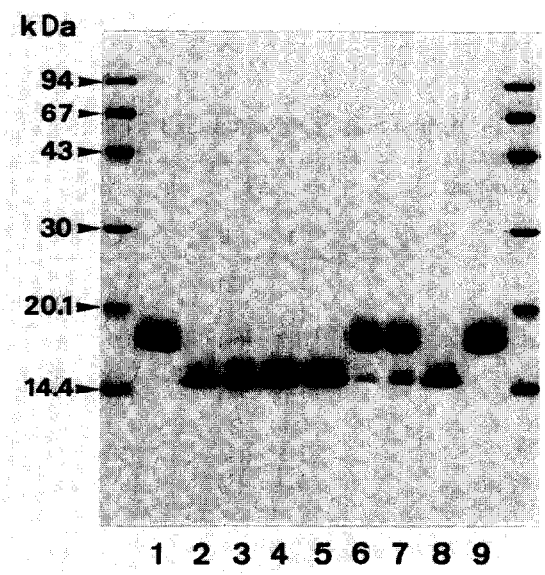

Fig.2. Limited proteolyses of PSP S2-5 by trypsin and chymotrypsin. Comparison of the electrophoretic patterns of the digests with those of PSP S1 and PSP S2-5. 15% polyacrylamide-SDS slab gel electrophoresis was carried out according to [10]. Before electrophoresis the protein samples were denatured, reduced and *S*-carboxymethylated. PSP S2-5 (lanes 1,9), PSP S1 (lanes 2,8). The proteolyses of PSP S2-5 (226 μM in 5 mM Tris-HCl buffer, pH 7.9, 40 mM in NaCl plus 20 mM in $CaCl_2$ in the case of trypsin) were performed for 1 h at 25°C with 0.1, 1 and 5% (w/w) TPCK-treated trypsin (lanes 3–5, respectively) and with 3 and 10% (w/w) TLCK-treated chymotrypsin (lanes 6,7). The reactions were arrested with 1 mM diisopropyl fluorophosphate for trypsin or 1 mM phenylmethylsulfonyl fluoride for chymotrypsin.

electrophoresis results. In the case of chymotrypsin, several poorly represented amino end groups were detected. In the case of trypsin, an important single N-terminal amino acid corresponding to Ile was found.

All the present results show that the Arg–Ile bond in PSP S2-5 is particularly susceptible to trypsin hydrolysis and suggest that the conversion of PSP S2-5 polypeptide chain into that of PSP S1 or PSP occurs in vivo by trypsin proteolysis.

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