

## Isolation of human pancreastatin fragment containing the active sequence from a glucagonoma

K. Sekiya, M.A. Ghatei, N. Minamino\*, D. Bretherton-Watt, H. Matsuo\* and S.R. Bloom

*Department of Medicine, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London W12 0HS, England, and \*Department of Biochemistry, Miyazaki Medical College, Kiyotake, Miyazaki 889-16, Japan*

Received 11 December 1987

The primary structure of a human pancreastatin-like peptide was determined from a pancreatic glucagonoma. The 28-amino acid peptide was identified using a specific antibody raised against porcine pancreastatin 1–49 and showed a 75% sequence homology with porcine pancreastatin 22–49 and bovine chromogranin A 267–294. Several forms of pancreastatin-like immunoreactivity were found in human endocrine tumors of which the purified peptide was the smallest and contained the active sequence of pancreastatin.

Pancreastatin; Glucagonoma; Sequence analysis; (Human)

### 1. INTRODUCTION

Pancreastatin is a 49-amino acid C-terminal amidated peptide recently isolated from porcine pancreas by Tatemoto et al. [1], which has been shown to inhibit glucose-stimulated insulin release [1]. The reported bioactivity of this peptide suggests that its presence in man could have potential importance in normal and pathological states such as diabetes mellitus. A striking structural similarity between porcine pancreastatin and a part of bovine chromogranin A 267–294 has led to the suggestion that chromogranin A is a precursor protein of pancreastatin [2,3]. A sensitive RIA system has been developed using a specific antibody raised against porcine pancreastatin 1–49. Using this RIA, we detected PS-LI in a large number of human

neuroendocrine tumors. The high concentration of PS-LI in a pancreatic glucagonoma has enabled us to analyse and purify the human peptide.

### 2. MATERIALS AND METHODS

#### 2.1. Tumor

The pancreatic glucagonoma was obtained about 4 h postmortem from a 55-year-old female patient who had been definitively diagnosed by a combination of clinical and radiological features, a specific hormone assay which showed plasma glucagon levels >2000 pmol/l, and elevated blood sugar of 200–800 mg/dl. Immediately after removal, the tumor (about 200 g wet wt) was snap-frozen in liquid nitrogen and stored at –70°C for subsequent peptide extraction.

#### 2.2. Extraction

The tumor was homogenized and extracted in 550 ml of 0.5 M acetic acid with 0.1% DDT (Sigma, St Louis, USA) for 15 min at 100°C.

#### 2.3. Radioimmunoassay

Antisera were raised in adult rabbits immunised with synthetic porcine pancreastatin 1–49 amide (Peninsula, CA, USA) conjugated to BSA with glutaraldehyde [4]. One rabbit (YN4) produced a highly sensitive antiserum which was used for RIA at a final dilution of 1:80000. Synthetic Tyr<sup>0</sup>-pancreastatin 1–49 was iodinated by chloramine-T oxidation [5]. The iodinated product was purified by using HPLC. The assay was carried out in 0.8 ml of 60 mM phosphate buffer, pH 7.4, con-

*Correspondence address:* S.R. Bloom, Department of Medicine, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London W12 0HS, England

*Abbreviations:* PS-LI, pancreastatin-like immunoreactivity; DDT, Di-dithiothreitol; RIA, radioimmunoassay; BSA, bovine serum albumin; HPLC, high-pressure liquid chromatography; ACN, acetonitrile; TFA, trifluoroacetic acid; FPLC, fast-protein liquid chromatography

taining 10 mM EDTA, 8 mM sodium azide and 0.3% BSA. After addition of antibody and labelled peptide (300 count/10 s), the assay was incubated for 4 days at 4°C. The assay had 100% cross-reactivity with the pancreastatin fragment 33–49 and negligible cross-reactivity (<0.02%) with human insulin, glucagon, somatostatin, pancreatic polypeptide, arginine-vasopressin and gastrin.

#### 2.4. Purification

After centrifugation (8000 rpm) of the tissue extract for 30 min and filtration, the supernatant was adsorbed onto Sep-Pak C18 cartridge (Waters, USA) which had been previously equilibrated in aqueous 0.1% TFA. After removal of unadsorbed material by washing with water, the adsorbed peptides were eluted using 0.1% TFA in 60% ACN. The eluted sample was lyophilized and loaded onto a Sephadex G-50 superfine gel column (1.5 × 100 cm, Pharmacia, Sweden) and eluted with 0.1 M acetic acid at a flow rate 3.2 ml/h and 1.6 ml fractions were collected. The PS-LI peak of  $K_{av}$  0.40 (fig.1) was lyophilized, reconstituted with 0.1% TFA in water and subjected to reverse-phase FPLC on a PepRPC column (C18, Pharmacia) with a gradient from 0 to 45% solvent B (solvent A: 0.1% TFA in water; solvent B: 0.1% TFA in ACN) over 60 min at a flow rate of 1 ml/min, and 1 ml fractions were collected for subsequent assay of PS-LI. One major PS-LI peak was found at 20% ACN. The peak was reloaded twice on the same column with more shallow gradients 10–35% (2nd run) and 15–25% (3rd run) solvent B over 60 min. The PS-LI peak of the 3rd run was then subjected to FPLC ion-exchange system on a Mono Q column (Pharmacia) with a gradient from 0 to 100% solvent B [solvent A: 0.08 M Tris buffer, pH 9.2 (BDH Chemicals, England); solvent B: solvent A in 1 M NaCl] over 60 min at a flow rate of 0.5 ml/min (4th run), and 1 ml fractions were col-

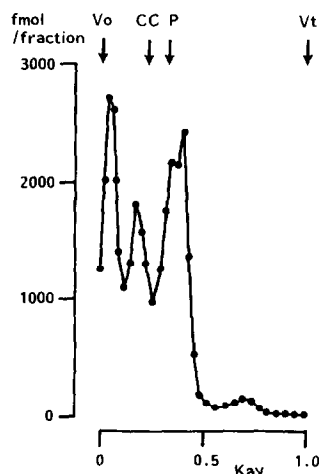


Fig.1. Sephadex G-50 gel permeation profile of PS-LI in extract of the tumor. The column was calibrated with dextran blue ( $V_0$ ), horse heart cytochrome *c* (CC), and  $\text{Na}^{125}\text{I}$  ( $V_t$ ) as molecular size markers. Arrow P indicates the eluted position of porcine pancreastatin. The concentrations of PS-LI are expressed as femtomole per fraction.

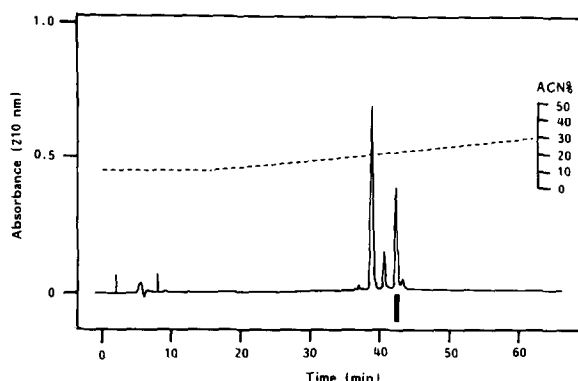


Fig.2. Final purification of human pancreastatin fragment from glucagonoma. Profile: 210 nm UV absorbance. Closed column: PS-LI. Sample: PS-LI fractions of FPLC 5th run at 36–37 min. Flow rate: 1.0 ml/min. Column: 219TP54 peptide & protein diphenyl column (4.6 × 250 mm, Vydac). Solvent:  $\text{H}_2\text{O}/\text{ACN}/10\%$  TFA = (A) 90:10:1, (B) 40:60:1 (v/v). Linear gradient elution from (A) to (B) for 120 min (---).

lected for subsequent assay. The peak of PS-LI was subjected again to FPLC on a PepRPC column under the same conditions as the 3rd run (5th run). The peptide was found to be only 40% pure and hence the lyophilized PS-LI material was further subjected to reverse-phase HPLC on a 219TP54 peptide & protein diphenyl column (4.6 × 250 mm, Vydac, CA, USA) at a flow rate of 1 ml/min with a linear gradient elution from 10 to 60% ACN in 0.1% TFA over 120 min at ambient temperature (the last run). Column effluents from HPLC were monitored by measuring absorbance at 210 nm and 280 nm.

#### 2.5. Sequence analyses

Sequence analysis was performed mainly by a gas-phase sequencer (model 470A, Applied Biosystems) using about

Table 1

Amino acid composition of human pancreastatin fragment

| Amino acid |            |
|------------|------------|
| Ser        | 0.88 (1)   |
| Glu        | 11.29 (11) |
| Pro        | 1.91 (2)   |
| Gly        | 4.11 (4)   |
| Ala        | 1.07 (1)   |
| Val        | 1.77 (2)   |
| Met        | 0.78 (1)   |
| Leu        | 1.06 (1)   |
| Phe        | 0.92 (1)   |
| Lys        | 2.05 (2)   |
| His        | 0.96 (1)   |
| Arg        | 0.99 (1)   |
| Total      | (28)       |

Numbers in parentheses represent the nearest integers

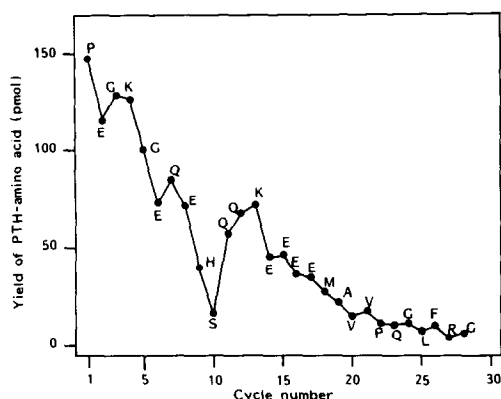


Fig. 3. Yield of PTH-amino acid liberated at each cycle of Edman degradation. One letter notation for amino acids was used.

270 pmol of the peptide [6]. The resulting PTH-amino acids were analyzed by reverse-phase HPLC (model 120A, Applied Biosystems) connected with a gas-phase sequencer. PTH-amino acids are measurable in quantities as low as 1 pmol. Amino acid analysis was carried out with Hitachi-835 amino acid analyzer, after acid hydrolysis of the peptide (~360 pmol) in 6 M HCl containing 0.1% phenol and 0.02% 2-mercaptoethanol at 110°C for 24 h. The C-terminal amino acid was determined by the method of Tatemoto and Mutt [7]; purified peptide was digested in 50 mM Hepes buffer (pH 7.55) with 1 µg of TPCK-trypsin at 37°C for 2 h and the glycine amide generated was identified as its dansyl (*N,N*-dimethylaminonaphthylsulfonyl) derivative on a polyamide sheet.

### 3. RESULTS

Fig. 1 shows the gel-permeation profile of the original tumor extract. Three major PS-LI peaks ( $K_{av}$  0.04, 0.18 and 0.40) were found, and the peak at  $K_{av}$  0.4 was used for purification because it was the nearest to porcine pancreastatin at  $K_{av}$  0.33. Fig. 2 shows the final HPLC purification profiles. A portion of the isolated peptide was hydrolyzed and analyzed for its amino acid composition. As summarized in table 1, this peptide consisted of 28 amino acid residues. Although 21 amino acids shorter than pig pancreastatin, this peptide has an amino acid composition similar to the C-terminal

half of porcine pancreastatin. Based on the amino acid analysis data, 2.10 nmol was purified from 200 g of human glucagonoma.

Amino acid sequence analysis was performed by a gas-phase sequencer and the PTH-amino acid liberated at each cycle of Edman degradation was successfully identified up to the C-terminal residue, as shown in fig. 3. C-terminal Gly-NH<sub>2</sub> was also clearly identified as its dansylated derivative by the method of Tatemoto and Mutt (not shown). Thus, the amino acid sequence of the peptide was established, as shown in fig. 4, and this peptide was found to correspond to the C-terminal subsequence 22–49 of porcine pancreastatin.

### 4. DISCUSSION

Human pancreastatin 1–28 amide differs in only seven amino acids from porcine pancreastatin 22–49 and bovine chromogranin A 267–294. Moreover, a single point mutation in each codon could have caused the change in each of the different amino acids between human pancreastatin and porcine pancreastatin 22–49, and 6 of the 7 amino acids in bovine chromogranin A 267–294. This is consistent with a common evolutionary origin of the genes for chromogranin A/pancreastatin of different species. The two larger molecular forms of PS-LI identified by gel chromatographic analysis are possibly large molecular mass precursor proteins, consistent with the hypothesis that pancreastatin is processed from a precursor such as chromogranin A.

The amino acid sequence of human pancreastatin is 21 amino acids shorter than porcine pancreastatin. For the human pancreastatin to be processed from a larger molecule would involve a split between two proline residues if the sequence of the large human form was similar to porcine pancreastatin. Such a processing is unusual, because X-Pro bond is usually resistant to proteolytic digestion [9]. Although we do not have any structural information of the N-terminal sequence

|      | 1 | 5 | 10 | 15 | 20 | 25 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |      |   |   |   |   |   |      |   |
|------|---|---|----|----|----|----|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|------|---|---|---|---|---|------|---|
| (a); | P | E | G  | K  | G  | E  | Q | E | H | S | Q | Q | K | E | E | E | M | A | V | V | P | Q | G | L | F | R | G | -NH2 |   |   |   |   |   |      |   |
| (b); | P | E | G  | K  | G  | A  | * | R | * | E | H | S | R | * | Q | E | * | E | E | E | T | * | A | G | * | A | P | Q    | G | L | F | R | G | -NH2 |   |
| (c); | P | E | G  | K  | G  | E  | W | * | A | * | H | S | R | * | Q | E | * | E | E | E | - | M | A | R | * | A | * | P    | Q | V | * | L | F | R    | G |

Fig. 4. Amino acid sequences of human pancreastatin fragment (a), porcine pancreastatin 22–49 (b) and bovine chromogranin A 267–294 (c). (\*) Different amino acid from human pancreastatin. One letter notation for amino acids was used.

in complete human pancreastatin, X residue of X-Pro structure might be aspartic acid, since Asp-Pro bond is easily cleaved in acetic acid solution at high temperature. On the other hand, the C-terminal heptapeptide amide, which is essential to exert biological activity [10], is completely conserved in both human and porcine pancreastatin. In recent years, chromogranin A has emerged as a potential universal marker for neuroendocrine tissues and tumors [11–15]. If human chromogranin A is a precursor protein of pancreastatin, neuroendocrine tumors could produce pancreastatin and thus possibly cause hyperglycemia. Further investigations are needed to clarify the biological role of this new peptide.

## REFERENCES

- [1] Tatemoto, K., Efendic, S., Mutt, V., Makk, G. and Feistner, G.J. (1986) *Nature* 324, 476–478.
- [2] Eiden, L.E. (1987) *Nature* 325, 301.
- [3] Huttner, W.B. and Benedum, U.M. (1987) *Nature* 325, 305.
- [4] Avrameas, S. and Ternynck, T. (1969) *Immunochemistry* 6, 53–59.
- [5] Hunter, W.M. and Greenwood, F.C. (1962) *Nature* 194, 495–497.
- [6] Minamino, N., Kangawa, K. and Matsuo, H. (1985) *Biochem. Biophys. Res. Commun.* 130, 1078–1085.
- [7] Tatemoto, K. and Mutt, V. (1978) *Proc. Natl. Acad. Sci. USA* 75, 4115–4119.
- [8] Gross, E. (1967) *Methods Enzymol.* 11, 238–255.
- [9] Schwartz, T.W. (1986) *FEBS Lett.* 200, 1–10.
- [10] Narita, K., Matsuo, H. and Nakajima, T. (1975) in: *Protein Sequence Determination* (Needleman, S.B. ed.) pp.30–130, Springer, Berlin.
- [11] Cohn, D.V., Elting, J.J., Frick, M. and Elde, R. (1984) *Endocrinology* 114, 1963–1974.
- [12] O'Connor, D.T. (1983) *Regul. Pep.* 6, 263–280.
- [13] O'Connor, D.T., Burton, D. and Deftos, L.J. (1983) *Life Sci.* 33, 1657–1664.
- [14] Wilson, B.S. and Lloyd, R.V.C. (1984) *Am. J. Pathol.* 115, 458–468.
- [15] Rindi, G., Buffa, R., Sessa, F., Tortora, O. and Salcia, E. (1986) *Histochem.* 85, 19–28.