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# ISOLATION AND SEQUENCE DETERMINATION OF RAT ISLET AMYLOID POLYPEPTIDE

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SUMMARY: Rat islet amyloid polypeptide (IAPP) was isolated from the pancreata of normal rats by utilizing cross-reactivity of a radioimmunoassay system for human IAPP with rat IAPP. Rat IAPP was a 37-amino acid polypeptide with tyrosine amide at the C-terminus, as was the case with human IAPP. Amino acid sequences of rat and human IAPPs were 84% identical, and the most highly conserved sequences were found in the N- and C-terminal regions. Rat IAPP sequence was also 51% identical to those of $\alpha$ and $\beta$ rat calcitonin gene-related peptide sequences. 

Islet amyloid polypeptide (IAPP) (1), also called diabetes-associated peptide (DAP) (2) or amylin (3), is a 37-amino acid polypeptide which has been isolated from human pancreatic islet amyloid. Human IAPP has 46% amino acid sequence homology with calcitonin gene-related peptide (CGRP). IAPP has been demonstrated immunohistochemically in normal B cells and in islet amyloid (4-6). These findings suggest the possibility that IAPP may serve as a biologically active peptide related to B cell functions. To clarify the physiological significance of the peptide, isolation and sequence determination of rat IAPP should be performed. We have developed a highly sensitive radioimmunoassay (RIA) for human IAPP (7). The RIA was also able to detect approximately 1/200 of the immunoreactivity per wet tissue weight in rat pancreas as compared to human pancreas. By utilizing the cross-reactivity of an antiserum with rat IAPP, we isolated rat IAPP and determined its complete amino acid sequence.

## MATERIALS AND METHODS

Tissue extraction and isolation: The pancreata (33.4 g wet weight) were collected from 31 Sprague Dawley rats and heated at 95-100 °C for 10 min in 1M acetic acid containing 20mM HCl to inactivate proteases. The boiled tissue was homogenized by a Polytron for 10 min. The homogenate was centrifuged at 32,000 x g for 30 min. Two volumes of acetone were gradually added to the resulting supernatant and further stirred for 12 hr at 4 °C. After removing the

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precipitate by centrifugation at 24,000 x g for 30 min, the supernatant was evaporated by a rotary evaporator and filtered through a GF/B filter (Whatman). The sample was pumped up to an octadesylsilica column (90 ml, Chemcosorb LC- SORB SPW- C- ODS), washed with 1M acetic acid, and then eluted with a 60% acetonitrile ($CH_3CN$) solution containing 0.1% trifluoroacetic acid (TFA). The eluate was evaporated and loaded on an SP- Sephadex C- 25 column ($H^+$- form, 1.8 x 4 cm, Pharmacia), pre- equilibrated with 1M acetic acid. The column was washed with 1M acetic acid (SP- I fraction), then eluted successively with 2M pyridine (SP- II fraction) and 2M pyridine- acetate (pH 5.0) (SP- III fraction). The SP- III fraction was evaporated repeatedly to remove pyridine, then lyophilized. The dried material (80 mg) was subjected to gel filtration on a Sephadex G- 50 fine column (1.8 x 134 cm, Pharmacia), using 1M acetic acid as an elution solvent. An aliquot (16 $\mu$ l) of each fraction was submitted to the RIA for IAPP as described below. Fractions #46- 50 containing immunoreactive (ir)- IAPP were pooled and separated by ion exchange high- performance liquid- chromatography (HPLC) on a TSK gel CM- 2SW column (7.8 x 300 mm, Tosoh). Chromatographic conditions are presented in the legend of Fig. 2. The IAPP- immunoreactive fraction (fraction #25) was subjected to reverse- phase HPLC on a diphenyl column (4.6 x 250 mm, Vydac). The immunoreactive fraction (fraction #38) was further chromatographed on a Chemcosorb 3ODS- H column (4.6 x 75 mm, Chemco) to finally purify rat IAPP. The column effluents from HPLC were monitored by measuring absorbance at 210 nm and 280 nm, simultaneously. A portion comprising one- seventh of the purified rat IAPP was digested with 1 $\mu$ g of trypsin treated with L- 1- tosylamido- 2- phenylethyl- chloromethyl ketone (TPCK) in 0.1M Tris- HCl for 2 hr at 37 °C . The resulting digests were separated by reverse- phase HPLC on a Chemcosorb 3ODS- H column (2.1 x 75 mm, Chemco). Native rat IAPP and its tryptic peptides were submitted to amino acid and sequence analyses.

RIA for rat IAPP: Rat IAPP contained in each fraction of the above chromatographies was detected by the aid of an RIA system for human IAPP (7). Antiserum #203- 5 used in this study was obtained after the fifth immunization with a synthetic C- terminal tetradecapeptide corresponding to the subsequence [24- 37] of human IAPP (7). Half- maximum inhibition of rat and human IAPPs was 5.6 pmol/tube and 29 fmol/tube, respectively, indicating that rat IAPP cross- reacted 0.52% with the antiserum. RIA procedures are reported elsewhere (7).

Sequence analysis: Amino acid analysis was carried out with an amino acid analyzer (Hitachi L- 8500). Each amino acid was measured quantitatively by the fluorescence- labeling method using o- phthalaldehyde in the presence of 2- mercaptoethanol. Cysteine residues were measured as cystine after acid hydrolysis. Sequence analysis was performed by a gas- phase sequenator (Model 470A, Applied Biosystems). The resulting PTH- amino acids were analyzed by reverse- phase HPLC (Model 120A, Applied Biosystems), linked with the sequenator. PTH- amino acids were measurable at concentrations as low as 0.5 pmol, and 25 pmol of a standard PTH- amino acid mixture (Pierce) was routinely used as a calibration mixture.

## RESULTS AND DISCUSSION

We conducted the isolation of rat IAPP by utilizing the cross- reactivity of an antiserum raised against human IAPP, since antiserum #203- 5 fortunately exhibited 0.52% cross- reactivity with rat IAPP.

IAPP immunoreactivity detected in rat pancreatic extracts was effectively limited to the fraction containing basic peptides of molecular weight less than 5,000 as follows. The extracts were first applied to a preparative C- 18 reverse- phase column. Peptides adsorbed on the column were subjected to batch- wise cation exchange chromatography on an SP- Sephadex C- 25 column. The SP- III fraction containing ir- IAPP thus separated was subjected to Sephadex G- 50 gel filtration. As shown in Fig. 1, the bulk of the ir- IAPP peak was eluted around fractions #48 and #49. Fractions #46- 50 were pooled, lyophilized, and then subjected to cation exchange HPLC. Figure 2 shows that almost all of the ir- IAPP emerged at fraction #25 with this ion exchange HPLC method. The fraction was further separated by reverse- phase HPLC

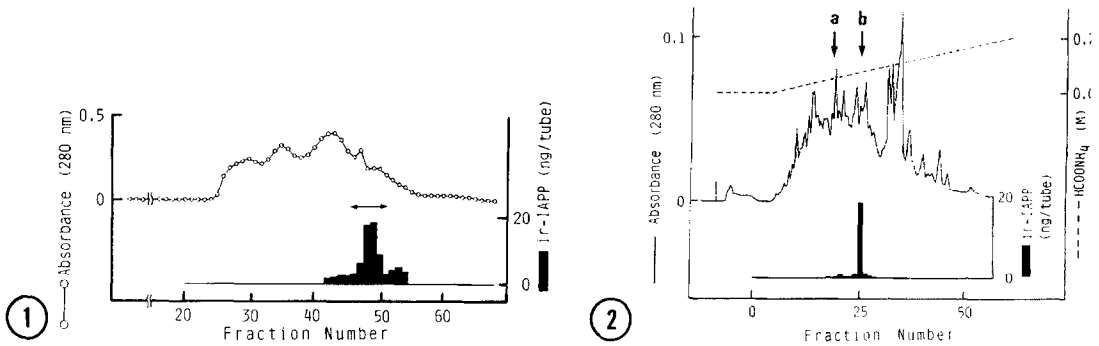


Fig. 1: Sephadex G-50 gel filtration of the SP-III fraction obtained from 33.4 g of rat pancreas.
Column: Sephadex G-50, fine (1.8 x 134 cm, Pharmacia). Flow rate: 8 ml/hr.
Fraction size: 5 ml/tube. Solvent: 1M acetic acid.
All fractions were monitored by the RIA for IAPP and the immunoreactivity observed is shown by the black bars.

Fig. 2: Ion exchange HPLC of ir-IAPP fraction from gel filtration.
Sample: Fractions #46-50 from Sephadex G-50 gel filtration (Fig. 1).
Column: TSK gel CM-2SW (7.8 x 300 mm, Tosoh). Flow rate: 2.0 ml/min.
Solvent system: (A) 0.01M $HCOONH_4$ (pH 6.6) : $CH_3CN$ = 90 : 10 (v/v).
(B) 0.75M $HCOONH_4$ (pH 6.6) : $CH_3CN$ = 90 : 10 (v/v).
Linear gradient from (A) to (B) for 90 min.
The immunoreactivity was detected at fraction #25.
Arrows indicate the elution positions of a) rat $\alpha$ CGRP and b) human CGRP, respectively.

on a diphenyl column as shown in Fig. 3. Rat IAPP was finally purified to a homogeneous state by reverse-phase HPLC on a C-18 column as shown in Fig. 4a.

Based on immunoreactivity and peak height with reverse-phase HPLC, the yield of isolated rat IAPP was estimated to be about 4.5 $\mu$ g. The amino acid composition of rat IAPP was as follows: Asp 5.85 (6), Thr 4.71 (5), Ser 2.96 (3), Glu 1.23 (1), Pro 2.94 (3), Gly 2.25 (2), Ala 3.18 (3), 1/2Cys 0.84 (2), Val 3.01 (3), Leu 4.00 (4), Tyr 1.06 (1), Phe 1.07 (1), Lys 1.13 (1), and Arg 2.05 (2). Numbers in parentheses represent the nearest integers. Rat IAPP was 37

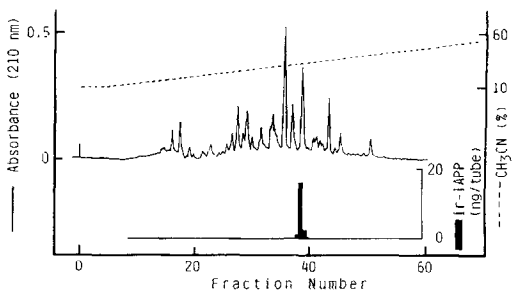


Fig. 3: Reverse-phase HPLC of ir-IAPP fraction from ion exchange HPLC.
Sample: Fraction #25 from ion exchange HPLC (Fig. 2).
Column: 219 TP 54 diphenyl (4.6 x 250 mm, Vydac). Flow rate: 1.0 ml/min.
Solvent system: (A) $H_2O$ : $CH_3CN$ : 10% TFA = 90 : 10 : 1 (v/v).
(B) $H_2O$ : $CH_3CN$ : 10% TFA = 40 : 60 : 1 (v/v).
Linear gradient from (A) to (B) for 80 min.
IAPP immunoreactivity was observed at fraction #38.

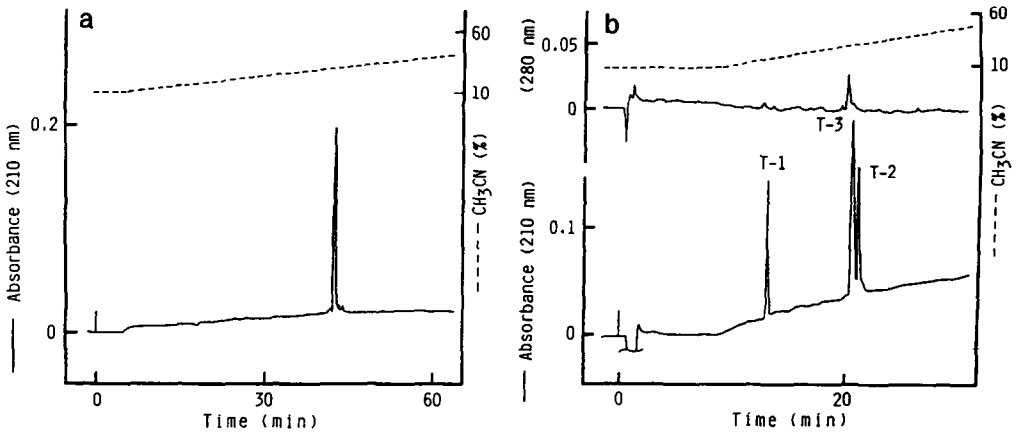


Fig. 4: (a) Final purification of rat IAPP by reverse-phase HPLC.
Sample: Fraction #38 from reverse-phase HPLC (Fig. 3).
Column: Chemcosorb 3ODS-H (4.6 x 75 mm, Chemco). Flow rate: 2.0 ml/min.
Solvent system: (A) $H_2O$ : $CH_3CN$ : 10% TFA = 90 : 10 : 1 (v/v).
(B) $H_2O$ : $CH_3CN$ : 10% TFA = 40 : 60 : 1 (v/v).
Linear gradient from (A) to (B) for 80 min.
(b) Reverse-phase HPLC of tryptic digests of the purified rat IAPP.
Sample: Tryptic digests of 1/7 portion of native rat IAPP.
Column: Chemcosorb 3ODS-H (2.1 x 75 mm, Chemco). Flow rate: 0.2 ml/min.
Solvent system: (A) $H_2O$ : $CH_3CN$ : 10% TFA = 100 : 0 : 1 (v/v).
(B) $H_2O$ : $CH_3CN$ : 10% TFA = 40 : 60 : 1 (v/v).
Linear gradient from (A) to (B) for 40 min.

amino acids long and contained two cysteine residues and one tyrosine, as was the case with human IAPP. Stepwise Edman degradation of rat IAPP (110 pmol) was performed by a gas-phase sequenator and the PTH-amino acids liberated were successfully identified up to the 29th step as shown in Fig. 5a. The sequence of the C-terminal region of rat IAPP was determined by analysis of its tryptic peptide. A portion comprising one-seventh of the purified rat IAPP was trypsinized and the resulting digests were separated by reverse-phase HPLC. Peptide T-3 was supposed to be a C-terminal fragment since it showed absorbance at 280 nm (Fig. 4b) and a tyrosine residue was not detected by the 29th step as shown in Fig. 5a. Peptide T-3 was submitted to the sequenator and its amino acid sequence was identified up to the C-terminus as shown in Fig. 5b. Tyrosine was detected at the C-terminus of peptide T-3.

Rat IAPP had high homology with rat CGRP, the C-terminus of which is amidated, suggesting that rat IAPP is also C-terminally amidated. Amidation of rat IAPP was clarified based on its net positive charge. The elution positions of rat IAPP and rat and human CGRPs by cation exchange HPLC are presented in Fig. 2. Net positive charges of rat $\alpha$-CGRP and human CGRP under chromatographic conditions (pH 6.6) were 2.5 and 4.5, respectively. Rat IAPP contained three basic amino acid residues and no acidic residue, nevertheless, its elution position was very close to that of human IAPP. This indicated that the net positive charge of rat

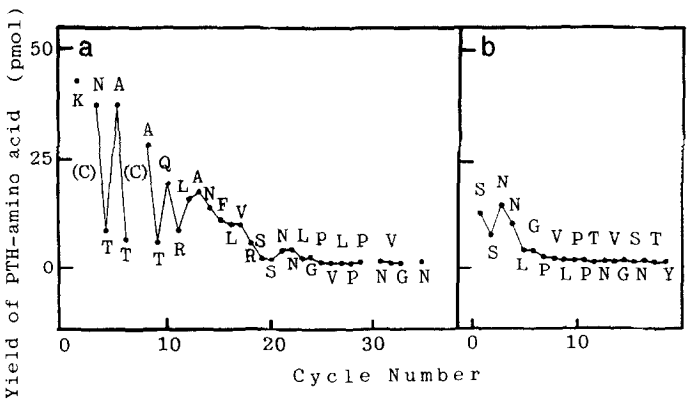


Fig. 5: Yield of PTH-amino acid at each cycle of Edman degradation.
Sample (a): Native rat IAPP. (b): Tryptic peptide T-3 of rat IAPP (Fig. 4b).
(The one-letter amino acid notation is used).

IAPP was 4, resulting from amidation of its C-terminus. A recent sequence analysis on cDNA encoding a precursor for rat IAPP has shown that the C-terminal tyrosine of rat IAPP is followed by Gly-Lys-Arg (8). The present study revealed that this Gly-Lys-Arg sequence was a site for proteolytic processing and subsequent amidation of the preceding tyrosine residue. Amidation of IAPP, in a manner similar to CGRPs and other biologically active peptides, is apparently important for its physiological function.

Amino acid sequences of rat and human IAPPs are presented in Fig. 6. Rat IAPP sequence was 84% identical to human IAPP sequence, and the most highly conserved sequences were found in the N- and C- terminal regions. A synthetic peptide corresponding to the subsequence [20- 29] of human IAPP has been found to form a twisted $\beta$- pleated sheet conformation characteristic of amyloid fibrils (9). This region of rat IAPP differs from human IAPP considerably, which may explain the lack of amyloid fibril formation in rat pancreas. Rat IAPP sequence was 51% identical to $\alpha$- and $\beta$- rat CGRP sequences. The sequences of rat

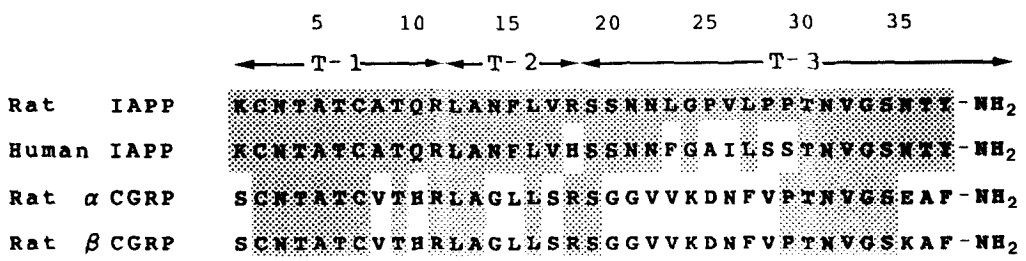


Fig. 6: Complete amino acid sequence of rat and human IAPPs and rat CGRPs.
T: tryptic peptide in Fig. 4b.

IAPP and CGRPs were most highly conserved in the N- and C- terminal regions, including residues [2-7] which putatively form the disulfide loop.

The present study revealed the presence of rat IAPP in the pancreas of normal rats. Rat IAPP is processed from a 70- amino acid precursor at Lys-Arg proteolytic cleavage sites (8). No appreciably IAPP- immunoreactive peaks with molecular weight greater than 5,000 were detected in gel filtration fractions, indicating that the 37- amino acid peptide exists in the pancreas as a mature form. Using antiserum #203-5, we have immunohistochemically determined that IAPP occurs in B cells of rat pancreas, to be published elsewhere. IAPP has been demonstrated to co- localize with insulin in secretory granules (10). These findings indicate that IAPP may be co- released with insulin from B cells in response to physiological stimulation or may regulate the synthesis and/or secretion of insulin. Identification and sequence determination of rat IAPP may help to clarify its physiological significance *in vivo*.

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