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Primary structure of glucagon from the gut of the common dogfish (*Scyliorhinus canicula*)

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The primary structure of glucagon isolated from the intestine of the common dogfish, *Scyliorhinus canicula*, was established as

5 10 15 20 25
H S E G T F T S D Y S K Y M D N R R A K D F V Q W L M N T.

The peptide shows four substitutions compared with human glucagon: Glu-3 for Gln, Met-14 for Leu, Asn-16 for Ser and Lys-20 for Gln. Glucagon represented the predominant molecular form of the glucagon-like immunoreactivity in the dogfish gut extracts demonstrating that the pathway of posttranslational processing of proglucagon in the gut of this fish differs markedly from the pathway in the mammalian gut.

Edman degradation; HPLC; Radioimmunoassay; Posttranslational processing; (Elasmobranch)

1. INTRODUCTION

Characterization studies of islet hormones from the lower vertebrates have concentrated upon the teleostean fishes. The segregation of the endocrine pancreas of certain of these fishes into 'principal islets' or Brockmann bodies has greatly facilitated purification of the peptides and the primary structure of glucagon has been determined for the anglerfish [1], catfish [2], Coho salmon [3], flounder [4] and daddy sculpin [5]. In the elasmobranchian class of fishes (sharks, rays and skates), however, the endocrine tissue is located, as in mammals, in islets distributed throughout the exocrine parenchyma but, unlike mammals, there is no topographical segregation into glucagon-rich and glucagon-poor islets [6]. The primary structure of glucagon from an elasmobranchian fish is known only for one species, the electric ray *Torpedo marmorata* [7].

Glucagon-like immunoreactivity, measured in radioimmunoassays with antisera raised against porcine glucagon, has been detected in the intestines of both teleostean and elasmobranchian fishes [7,8]. This material has not, however, been characterized and, up to the present time, glicentin [9] and its COOH-terminal fragment, oxyntomodulin [10] from the pig are the only intestinal glucagon-related peptides to have been isolated in pure form. In this investigation, the major molecular form of the glucagon-like immunoreactivity in the intestine of an elasmobranchian fish, the common dogfish (*Scyliorhinus canicula*), has been purified to homogeneity and identified as a 29-amino acid residue peptide homologous to mammalian pancreatic glucagon.

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2. MATERIALS AND METHODS

2.1. *Tissue extraction*

Intestinal tissue (317 g) from 5 adult dogfishes was homogenized at 4°C with 8 vols ethanol/0.7 M HCl (3:1, v/v) as previously described [11]. After centrifugation (1600 × *g*, 1 h) and removal of ethanol from the supernatant under reduced pressure, an aliquot of the extract (2 ml) was chromatographed at 4°C on an analytical gel filtration column (90 × 1.6 cm) of Sephadex G-50 fine (Pharmacia) equilibrated with 0.1 M ammonium acetate, pH 6.8. The column was eluted at a flow rate of 10 ml/h and 2.1 ml fractions were collected. Peptide material in the extract was concentrated using Sep-Pak C18 cartridges (Waters Associates) and bound material was eluted with acetonitrile/water/trifluoroacetic acid (80:19:1). The effluent was lyophilized.

2.2. *Purification of dogfish glucagon*

The intestinal extracts, after Sep-Pak concentration, were redissolved in 1% (v/v) trifluoroacetic acid (5 ml) and chromatographed at 4°C on a preparative gel filtration column (100 × 2.6 cm) of Sephadex G-50 fine equilibrated with 1 M acetic acid. The column was eluted at a flow rate of 40 ml/h and 6.6 ml fractions were collected. Fractions containing glucagon-like immunoreactivity were pooled, lyophilized and redissolved in 0.1% trifluoroacetic acid (1 ml). The sample was injected onto a semi-preparative Supelcosil LC-18-DB column (250 × 10 mm) maintained at 30°C and at a flow rate of 2 ml/min. The column was eluted with a linear gradient (volume 120 ml) formed from acetonitrile/water/trifluoroacetic acid (21.0:78.9:0.1) and acetonitrile/water/trifluoroacetic acid (49.0:50.9:0.1). Absorbance was monitored at 214 nm and 280 nm and 2 ml fractions were collected. The fraction with maximum glucagon-like immunoreactivity was lyophilized and redissolved in 0.1% trifluoroacetic acid (1 ml). The sample was injected onto a Supelcosil LC-3DP phenyl column (250 × 4.6 mm) maintained at 30°C and at a flow rate of 1.5 ml/min. The column was eluted with a linear gradient (15 ml) formed from 0.1% trifluoroacetic acid and acetonitrile/water/trifluoroacetic acid (17.5:82.4:0.1) followed by a linear gradient (60 ml) to acetonitrile/water/trifluoroacetic acid (45.5:54.4:0.1). The peak of dogfish glucagon was purified to homogeneity on a Supelcosil LC-18-DB column (250 × 4.6 mm) maintained at 30°C and at a flow rate of 1.5 ml/min. The column was eluted with a linear gradient (15 ml) formed from trifluoroacetic acid and acetonitrile/water/trifluoroacetic acid (28.0:71.9:0.1) followed by a linear gradient (90 ml) to acetonitrile/water/trifluoroacetic acid (45.5:54.4:0.1).

2.3. *Structural analysis*

Amino acid composition was determined on 1 nmol peptide using a Durrum D-500 automatic analyser. The primary structure of the peptide was determined by automated Edman degradation using an Applied Biosystems model 470A gas phase sequencer [12]. The detection limit for phenylthiohydantoin (PTH)-amino acids was 0.5 pmol. Approx. 2 nmol of peptide was used for the determination.

2.4. *Radioimmunoassay methods*

Glucagon-like immunoreactivity in fractions of chromatographic effluent was measured using an antibody directed against a site in the NH_2-terminal to central region of mammalian glucagon (probably residues 10–18) [13]. The immunochemical properties of dogfish glucagon were also studied using Unger antiserum 30K directed against a site in the COOH-terminal region of glucagon (probably residues 24–29) [14].

3. RESULTS

3.1. *Glucagon-like immunoreactivity in dogfish gut extracts*

Extracts of dogfish gut contained glucagon-like immunoreactivity equivalent to 31 pmol/g wet wt measured with an antiserum directed against the NH_2-terminal to central region of glucagon and 5 pmol/g measured with a COOH-terminally directed antiserum. These values must be taken as approximations, as the immunoreactivity in the extracts, measured with either antiserum, did not elute exactly in parallel with the porcine glucagon standard curve in radioimmunoassays. As shown in fig.1, the glucagon-like immunoreactivity in an aliquot of the gut extracts was eluted from an analytical gel filtration column as a major peak with an elution volume slightly less than that of

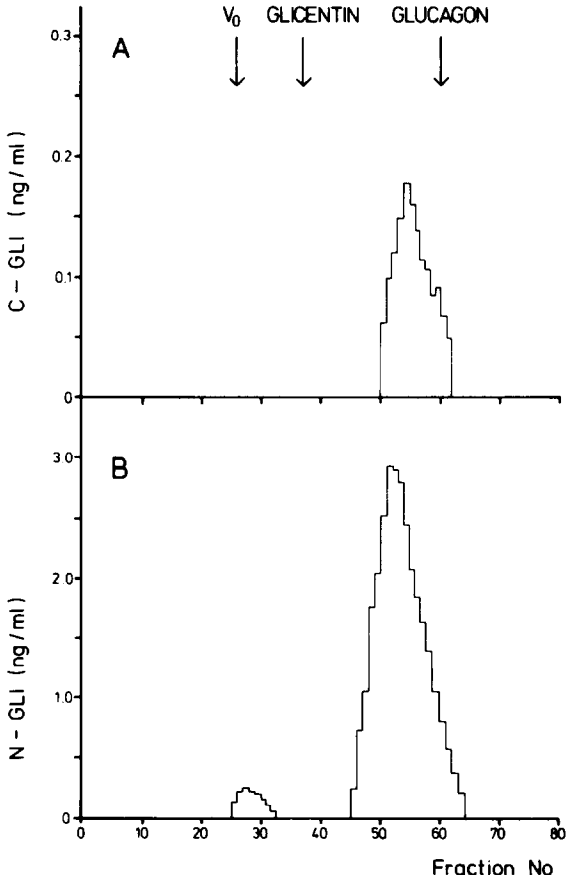

Fig.1. Elution profile on Sephadex G-50 of an extract of dogfish intestine. Glucagon-like immunoreactivity was measured (A) using an antiserum directed against the COOH-terminal region of glucagon (C-GLI) and (B) using an antiserum directed against the NH_2-terminal to central region of glucagon (N-GLI). The arrows show the void volume (V_0) and the elution positions of porcine glicentin and glucagon.

porcine glucagon. The component showed greater immunoreactivity with the NH_2-terminally than with the COOH-terminally directed antiserum. A minor component of higher molecular mass was immunoreactive towards the NH_2-terminal antiserum only.

3.2. *Purification of dogfish glucagon*

The glucagon-like immunoreactivity in the gut extracts, after concentration on Sep-Pak C18 cartridges, was eluted from a preparative gel filtration column as a broad major peak with K_{av} between 0.49 and 0.70. Fractions with maximum immunoreactivity were purified further by reverse-phase HPLC on a semi-preparative octadecyldimethylsilylsilica (C18) column (fig.2). Glucagon-like immunoreactivity was eluted from the column as a major peak with retention time between 45 and 46 min which corresponded with a prominent peak of UV-absorbance (peak B in fig.1). A second peak of glucagon-like immunoreactivity (peak A) was eluted from the column with retention time

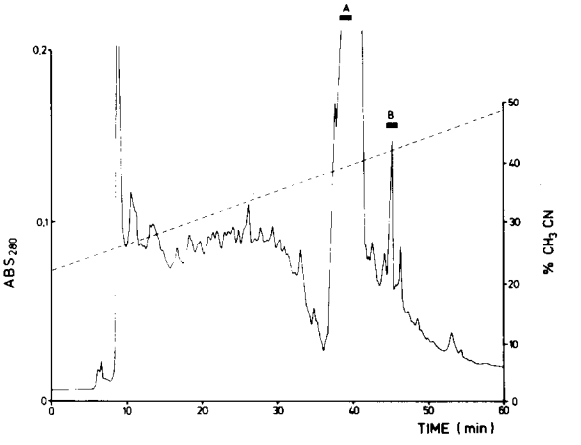

Fig.2. Reverse-phase HPLC on a Supelcosil LC-18-DB column of dogfish glucagon after partial purification by gel permeation chromatography. Glucagon-like immunoreactivity was measured in the fractions denoted A and B in the ratio 1:3. Peak B was purified further. (– – –) Concentration of acetonitrile in the eluting solvent.

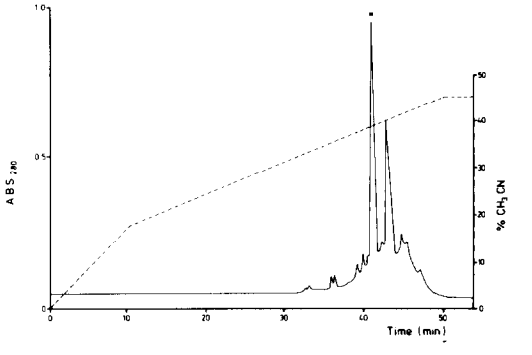

Fig.3. Reverse-phase HPLC on a Supelcosil LC-3-DP column of dogfish glucagon (peak B in fig.1). The peak denoted by the asterisk contained glucagon-like immunoreactivity. (– – –) Concentration of acetonitrile in the eluting solvent.

between 38 and 39 min and constituted approx. 30% of the immunoreactivity in peak B. Treatment of peak B material with hydrogen peroxide under the conditions described in [15] resulted in a change in retention time of the glucagon-like immunoreactivity to approx. 38 min suggesting that peak A may be an oxidized form of glucagon. The major component (peak B) was further purified by chromatography on a diphenylmethylsilylsilica column (fig.3). The prominent peak denoted by the asterisk displayed glucagon-like immunoreactivity. Dogfish glucagon was purified to homogeneity by chromatography on an analytical reverse-phase C18 column (fig.4). The final yield of purified material was approx. 3 nmol.

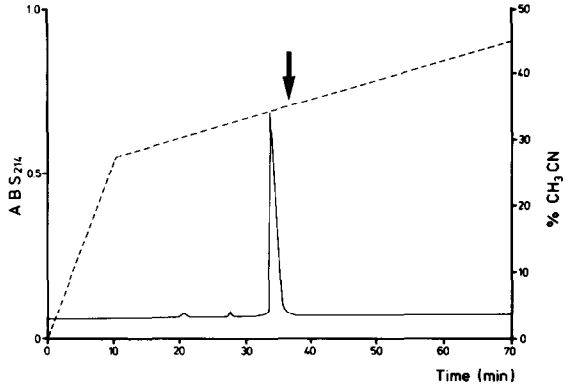

Fig.4. Purification to homogeneity of dogfish glucagon on a Supelcosil LC-18-DB column. The peptide was characterized by amino acid composition and Edman degradation. The arrow shows the retention time of porcine glucagon.

Table 1

Amino acid composition of dogfish glucagon compared with human glucagon

Residue	Dogfish	Human
Asx	4.65 (5)	4
Thr	2.90 (3)	3
Ser	3.79 (3)	4
Glx	2.45 (2)	3
Gly	1.29 (1)	1
Ala	1.20 (1)	1
Val	1.05 (1)	1
Met	1.85 (2)	1
Leu	0.96 (1)	2
Tyr	1.79 (2)	2
Phe	1.97 (2)	2
His	0.98 (1)	1
Lys	2.17 (2)	1
Arg	1.79 (2)	2

Tryptophan was not determined and the numbers in parentheses represent the values from the sequence determination

3.3. *Structural analysis*

The amino acid composition of dogfish glucagon (table 1) indicated a peptide of 28–29 residues that was structurally different from mammalian glucagon. The strong absorbance of the

Table 2

Automated Edman degradation of dogfish glucagon

Cycle no.	PTH-amino acid	Yield (pmol)
1	His	165
2	Ser	176
3	Glu	327
4	Gly	246
5	Thr	104
6	Phe	291
7	Thr	125
8	Ser	80
9	Asp	100
10	Tyr	177
11	Ser	44
12	Lys	101
13	Tyr	183
14	Met	138
15	Asp	101
16	Asn	159
17	Arg	86
18	Arg	102
19	Ala	113
20	Lys	66
21	Asp	73
22	Phe	136
23	Val	118
24	Gln	98
25	Trp	58
26	Leu	97
27	Met	87
28	Asn	12
29	Thr	23

The average repetitive yield was 95.4%

Table 3

A comparison of the sequences of glucagon from the dogfish (*Scyliorhinus canicula*), human, *Torpedo* and Coho salmon

	5					10					15					20					25				
Dogfish	H	S	E	G	T	F	T	S	D	Y	S	K	Y	M	D	N	R	R	A	K	D	F	V	Q	W	L	M	N	T
Human	-	-	Q	-	-	-	-	-	-	-	-	-	-	L	-	S	-	-	-	Q	-	-	-	-	-	-	-	-	-
Torpedo	-	-	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Salmon	-	-	-	-	-	-	S	N	-	-	-	-	-	Q	E	E	-	M	-	Q	-	-	-	-	-	-	-	-	S

peptide at 280 nm suggested that an additional tryptophan residue was probably present in the molecule. Unambiguous assignation of 29 amino acid residues was possible by automated Edman degradation (table 2). Although approx. 2 nmol of peptide (estimated by amino acid analysis) had been subjected to sequence analysis, the yields of PTH-amino acids were much lower than expected. This may have been a result of irreversible binding of the highly purified peptide to the walls of the polypeptide tube used for the final lyophilization step or may have arisen from a low initial yield in cycle 1 of the sequence determination. With the exception of a high relative amount of serine and glutamic acid, agreement between the amino acid composition data and the proposed sequence was good. High values for the amounts of serine and glutamic acid in peptides have been observed on previous occasions when relatively small quantities of material have been used for analysis and may represent carry-over from previous samples applied to the ion-exchange column of the automatic analyser. As antiserum 30K will bind only those glucagon-related peptides with a free COOH group at position Thr-29 [16] the immunohistochemical properties of dogfish glucagon indicate that the peptide is not extended beyond residue 29. The primary structure of dogfish glucagon is compared with glucagons from the human, *T. marmorata* (elasmobranch) and Coho salmon (teleost) in table 3.

4. DISCUSSION

The primary structure of proglucagon has been deduced from the nucleotide sequences of anglerfish [17], cow [18] and hamster [19] cDNAs and from DNAs prepared from human [20] and rat [21] genomic libraries. In the rat, preproglucagon mRNAs from pancreas and intestine are identical so that the diversity of the molecular forms of the proglucagon-derived gene products arises from different pathways of posttranslational processing of the primary transcript [22]. These pathways have recently been comprehensively reviewed [23,24]. In the pancreas of all species yet studied proglucagon is processed almost exclusively to glucagon but in the intestine of the rat [22] and pig [25] glucagon is virtually absent and its concentration in the human bowel is very low [26]. The present study has demonstrated, therefore, that the pathway of posttranslational processing of proglucagon in the dogfish gut follows a completely different pathway from that in the mammalian intestine. A high concentration of glucagon-like immunoreactivity measured with an antiserum directed against the COOH-terminal region of porcine glucagon has previously been found in extracts of the intestine of the elasmobranchian fish, *T. marmorata* [7]. Similarly, it has also been shown that the intestine of the amphibian, *Ambystoma mexicanum* (axolotl), contains predominantly a peptide of the same approximate molecular size as glucagon that cross-reacts strongly with COOH-terminally directed antisera [27]. Thus it may be speculated that the ability to regulate the processing of proglucagon differently in gut and pancreas may have arisen relatively late in evolution.

The primary structure of glucagon has been highly conserved between species. The amino acid sequence of the peptide is identical in all mammals yet studied, with the exception of the guinea pig [13]. In birds, substitutions are confined to Ser-28

for Asn in the chicken [28] and turkey [29] with the additional substitution Thr-16 for Ser in the duck [30]. Dogfish glucagon shows amino acid changes at four sites relative to human glucagon (table 3). Within the class elasmobranchii, the sequence of glucagon has been strongly conserved with only a single substitution Met-14 for Leu compared with the ray, *T. marmorata*. Similarly, the amino acid composition of glucagon from the elasmobranchian fish, spiny dogfish (*Squalus ancanthias*) resembled very closely that of the common dogfish (table 1) with two lysine and two methionine residues in the molecule [31]. Despite the similarities in trivial name, the line of evolution leading to the common dogfish (division Galeoidea) diverged from the line leading to the spiny dogfish (division Squaloidea) at least 159–200 million years ago. In contrast, dogfish glucagon show eight amino acid substitutions when compared with glucagon from the teleostean fish, the Coho salmon [3].

The high degree of conservation of the structure of dogfish glucagon in the NH_2-terminal to central region of the peptide is consistent with its strong reactivity towards an antiserum directed against this region of porcine glucagon. The reduced reactivity towards a COOH-terminally directed antiserum is, however, surprising in view of the fact that this region of the molecule is fully conserved. The presence of a lysine residue at position 20, however, may cause significant change in the conformation adopted by the hydrophobic COOH-terminal antigenic site.

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