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Purification and Structural Determination of Insulins, Glucagons and Somatostatin from Stone Flounder, *Kareius bicoloratus*

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ABSTRACT—Insulin-I and -II were purified from stone flounder (*Kareius bicoloratus*), and their primary structures were determined. The amino acid sequences of insulin-I and -II from stone flounder were identical with those of barfin flounder (*Verasper moseri*) except for position 2 of both B-chains. Insulin-II of stone flounder had an extension of the two amino acid residues (Pyr-Ala), at the N-terminus of the B-chain. These structural characteristics of insulins from stone flounder support the idea (Andoh and Nagasawa, Zool. Sci. 15: 901, 1998) that insulin-I and -II of flounders arise from a single preproinsulin in each species by proteolytic cleavage at different sites of the signal peptide region, and suggest that this generation system of two molecular forms of insulin is not specific for barfin flounder. In the course of the purification of insulins, somatostatin-14 and two glucagons (glucagon-I and -II) were also purified from the extract of the Brockmann body. The amino acid sequence of somatostatin-14 of stone flounder was identical with those of mammals. Five amino acids were different between glucagon-I and -II of stone flounder. The amino acid sequences of both glucagons were highly conserved among several acanthopterygian and paracanthopterygian fish. These results suggest that their common ancestral species had both glucagons.

INTRODUCTION

Barfin flounder (*Verasper moseri*) contains two molecular forms of insulin in the Brockmann body. One is a normal type of insulin (insulin-I), while the other (insulin-II) has an extension of two amino acid residues (Pyr-Ala) at the N-terminus of B-chain (Andoh and Nagasawa, 1998). Andoh and Nagasawa (1998) proposed an idea that insulin-I and -II of barfin flounder arose from a single preproinsulin by proteolytic cleavage at different sites of the signal peptide region based on the results of amino acid sequence analyses of these insulins, nucleotide sequence analyses of preproinsulin gene from genomic and complementary DNA by polymerase chain reaction using several sets of primers, and Southern blot analysis of genomic DNA.

Multiple molecular forms of insulin have been purified from several species of fish. Insulins in these species appeared to be encoded by two distinct genes (bonito, Yamamoto *et al*., 1960; Kotaki *et al*., 1962; toadfish, Smith, 1966; paddlefish, Nguyen *et al*., 1994), or to be generated from a single proinsulin by proteolytic cleavage at different sites within the C-peptide region (ratfish, Conlon *et al*., 1989). Therefore, the manner generating two molecular forms of insulin in barfin flounder was novel.

In addition, two molecular forms of glucagon (glucagon-I and -II; paddlefish, Nguyen *et al*., 1994; eel, Conlon *et al*., 1988; anglerfish, Lund *et al*., 1983; Andrews *et al*., 1986; Nicholes *et al*., 1988; sculpin, Cutfield *et al*., 1986; Cutfield and Cutfield, 1993; tilapia, Nguyen *et al*., 1995), and peptides derived from prosomatostatin-I and -II (salmon, Plisetskaya *et al*.,1986; eel, Conlon *et al*., 1988; catfish, Oyama *et al*., 1980; Andrews and Dixon, 1981; sculpin, Conlon *et al*., 1987a; flounder, Conlon *et al*., 1987b) have also been purified. The existence of multiple molecular forms of glucagon and somatostatin has been reported only in fish unlike other vertebrates except for somatostatins of a frog (Vaudry *et al*., 1992).

In the present study, we examined whether stone flounder (*Kareius bicoloratus*) contains two molecular forms of insulin, especially insulin which contains Pyr, or not, by purification and amino acid sequence analyses. In addition, we describe purification and primary structures of glucagons and somatostatin which were purified from the side fraction during purification of insulins.

MATERIALS AND METHODS

Purification of insulin-I and -II, glucagon-II, and somatostatin

Insulin-I and -II, glucagon-II, and somatostatin were purified according to Andoh and Nagasawa (1997) except for fractionation of HPLC (Method A). Brockmann bodies (340 mg) on the gall bladder

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from stone flounder (N = 10) were extracted with ethanol/0.7 M HCl (3:1, v/v). After removal of ethanol by vacuum centrifugation, the extract was injected Sep-Pak cartridge C18 (Millipore, Milford, MA, USA). The eluate with 80% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid (TFA) was injected onto an ODS-120T analytical column (4.6 × 250 mm, Tosoh, Tokyo), and 500 μl fractions were collected. The fraction which was thought to contain insulins, glucagons and somatostatin was applied onto a μ RPC C2/C18 SC microbore column (2.1 × 100 mm, Pharmacia, Uppsala, Sweden). Fractions were collected peak by peak by monitoring absorbance at 280 nm and were subjected to mass spectral analysis. Reductive carboxymethylation and subsequent separation of putative A- and B-chains of insulins were also done according to Andoh and Nagasawa (1997) , and subjected to amino acid sequence analyses. A SMART system (Pharmacia) was used for the HPLC steps. The effluents and the gradient programs of HPLC steps were the same as those of Andoh and Nagasawa (1997).

Purification of insulin-I and -II , and glucagon-I and -II

Insulin-I and -II, and glucagon-I and -II were also purified by another method (Method B). The pancreatic hormones were extracted essentially according to Cutfield *et al.* (1986). The Brockmann bodies (160 mg, N = 6) were used for extraction. Precipitates after diethylether-ethanol precipitation were air-dried and dissolved in 3 M acetic acid. After centrifugation, the supernatant was injected onto an ODS-120T microbore column (2.0 × 150 mm, Tosoh). The elution conditions for HPLC were the same as those described in Andoh and Nagasawa (1998). Fractions were collected manually peak by peak by monitoring absorbance at 280 nm, and an aliquot of each fraction was subjected to mass spectral analysis. The fractions which showed a molecular weight nearly equal to that of barfin flounder insulin-II or mammalian glucagon were injected separately onto a μ RPC C2/C18 SC column. The materials were eluted at 30°C and a flow rate of 100 μl/min with the same solvents as the previous step of HPLC. After elution with 0.1% (v/v) TFA for 3 min, the concentration of acetonitrile in the eluting solvent was increased from 0 to 25% (v/v) over 5 min, to 40% (v/v) over 70 min and to 50% (v/v) over 10 min. The fractions containing putative glucagon-I were subjected to amino acid sequence analysis. In the case of putative insulin-II, amino acid sequence analysis was done after reductive carboxymethylation and the subsequent separation of A- and B-chains according to Andoh and Nagasawa (1997).

Digestion with pyroglutamate aminopeptidase

Digestion of S-carboxymethylated putative B-chain of insulin-II by *Pfu* pyroglutamate aminopeptidase (Takara, Otsu, Japan) and the subsequent separation of the digests were performed according to Andoh and Nagasawa (1998). The purified peptide was subjected to amino acid sequence and mass spectral analyses.

Estimation of peptide yields

The yields of hormones were estimated from the absorbances of mammalian hormones at the concentration of 1 mg/ml water for convenience. Absorbances used for insulin, glucagon, and somatostatin were 1.1 (porcine) at 276 nm, 2.3 (porcine) at 280 nm and 2.8 (human) at 280 nm, respectively.

Structural determination

Mass spectral analysis was carried out using Kompact MALDI I or MALDI III time-of-flight mass spectrometer (Shimadzu, Kyoto, Japan) with 2-(4-hydroxyphenylazo)benzoic acid as a matrix in the positive ion and linear modes. Automated Edman degradation was carried out on a protein sequencer, Applied Biosystems model 476A (Perkin Elmer, Norwalk, CT, USA) in the pulsed-liquid mode.

RESULTS

Purification of insulin-I and -II, glucagon-II, and somatostatin by Method A

Elution profile of the eluate from Sep-Pak cartridge by Method A from stone flounder Brockmann body on an ODS-120T analytical column is shown in Fig. 1. The fraction with the highest absorbance peak was collected and injected onto a μ RPC C2/C18 SC column for further purification (Fig. 2). The mass spectra of fractions at 63-64 min and 64-66 min were *m*/*z* 5778 and *m*/*z* 5959, respectively, suggesting that these fractions contained insulin-I and -II. The mass spectra of fractions at 51-52 min and 56-57 min were *m*/*z* 1652 and *m*/*z* 3503 which are close to the molecular weights of soma-

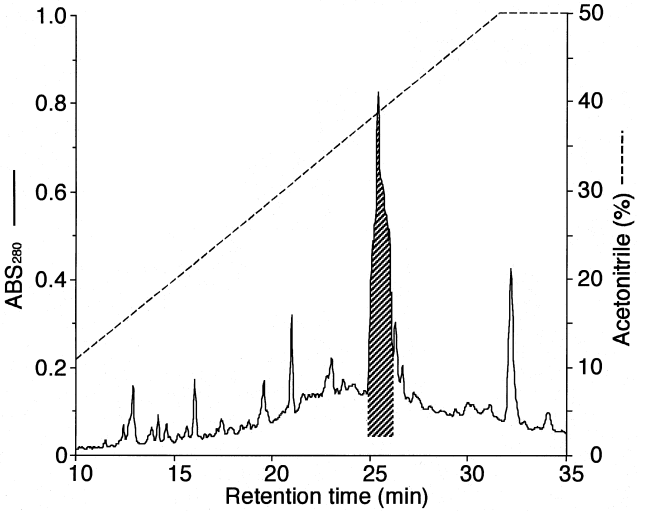

Fig. 1. Fractionation of an extract by Method A (see text) from stone flounder Brockmann body by HPLC. An ODS-120T analytical column was used and the shaded area was subjected to further purification.

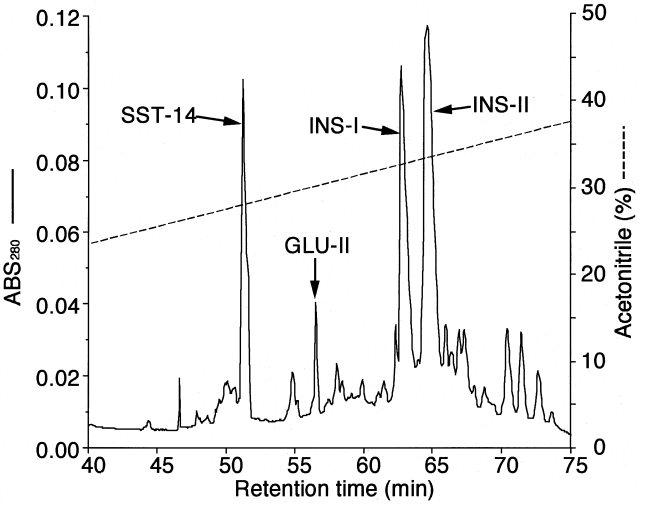

Fig. 2. Purification of pancreatic hormones fractionated by an ODS-120T analytical column. A μ RPC C2/C18 SC column was used. SST-14, GLU-II, INS-I and -II denote somatostatin-14, glucagon-II, insulin-I and -II, respectively.

tostatin-14 and glucagon of mammals. These were later found to be somatostatin-14 and glucagon-II by amino acid sequence analysis. The yields of insulin-I and -II, glucagon-II and somatostatin-14 were 14.4 nmol, 17.8 nmol, 0.9 nmol and 11.0 nmol, respectively.

Purification of insulin-I and -II, and glucagon-I and -II by Method B

The elution profile of 3 M acetic acid solution of the ethanol-diethylether precipitate of the Brockmann body extract on an ODS-120T microbore column is shown in Fig. 3. Mass spectra of fractions at 79-80 min and 81-82 min were *m*/*z* 5768 and *m*/*z* 5950, respectively, which were almost identical with those of putative insulin-I and -II purified by Method A. The insulin-II fraction was purified to homogeneity on a μ RPC C2/C18 SC column (profile not shown). Mass spectra of fractions at 73-74 min was *m*/*z* 3508, and that of fraction at 78-79 min on an ODS-120T microbore column were *m*/*z* 3552 and *m*/*z* 3862. Fraction at 78-79 min was further separated into two fractions by reversed phase HPLC on a μ RPC C2/C18 SC column (Fig. 4). In the mass spectra, prominent peaks were observed at *m*/*z* 3552 for the former fraction and at *m*/*z* 3862 for the latter fraction. The material in the former fraction was later found to be glucagon-I by amino acid sequence analysis. The yields of insulin-I and -II, and glucagon-I and -II by Method B were 15.2, 20.5, 3.4 and 9.9 nmol, respectively.

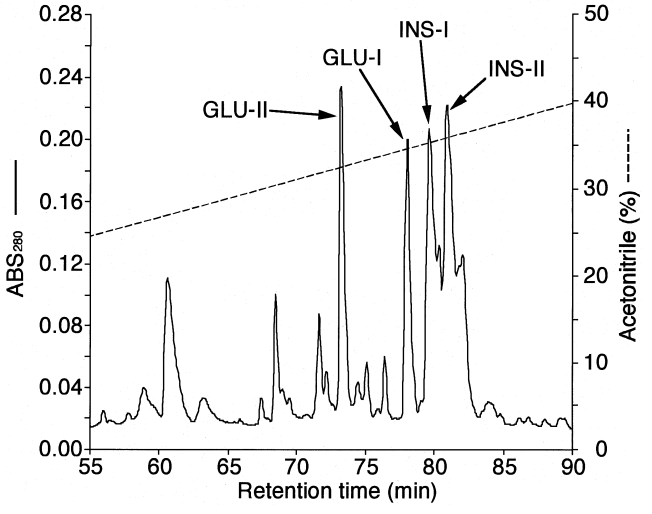

Fig. 3. Purification of pancreatic hormones prepared by Method B (see text) on an ODS-120T microbore column. GLU-I means glucagon-I, and other abbreviations are the same as those of Fig. 2.

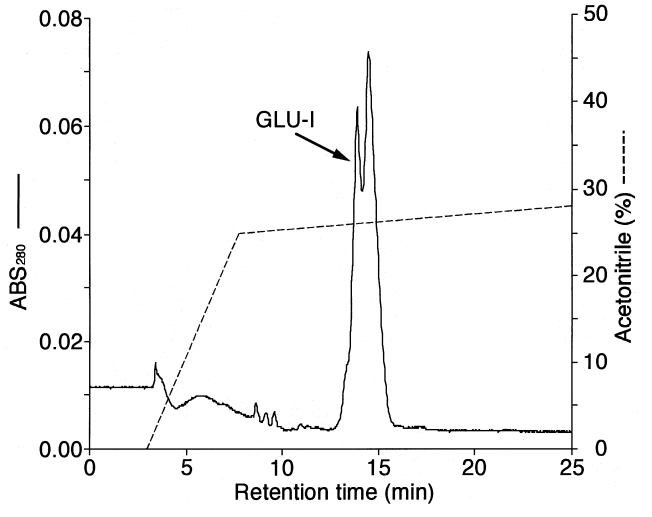

Fig. 4. Further purification of glucagon-I by HPLC on a μ RPC C2/C18 SC column.

Characterization of stone flounder insulin-I and -II

The amino acid sequences of insulin-I and -II purified by Method A and Method B, were analyzed after reductive carboxymethylation followed by separation of A- and B-chains. Both A-chains of stone flounder insulins consisted of 21 amino acid residues which were completely identical with those of barfin flounder and flounder (Fig. 5). The B-chain of stone flounder insulin-I consists of 30 residues and is identical with B-chain of flounder insulin (Fig. 5). The observed values for $(M + H)^+$ of A-chains of insulin-I and -II (*m*/*z* 2669 and *m*/*z* 2673, respectively) in the mass spectra were in good agreement with the theoretical values (*m*/*z* 2674). The observed values for $(M + H)^+$ of insulin-I B-chain (*m*/*z* 3442) also agreed well with the theoretical values (*m*/*z* 3447). These results es-

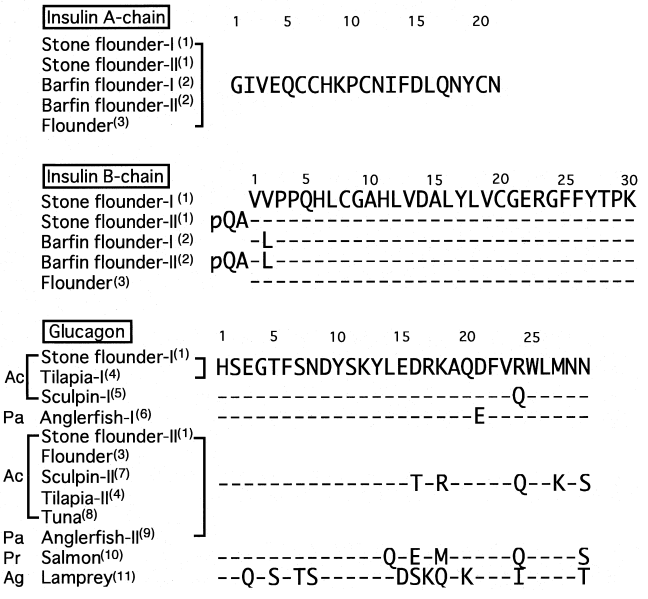

Fig. 5. Comparison of primary structures of stone flounder insulins and glucagons with those of several fish. Pyroglutamic acid residue was represented as pQ. Ac, Pa, Pr, and Ag indicate Acan-thopterygii, Paracanthopterygii, Protacanthopterygii, and Agnata, respectively. The sequences were cited from (1) the present work; (2) *Verasper moseri*, Andoh and Nagasawa (1998); (3) *Platichthys flesus*, Conlon *et al.* (1987b); (4) *Oreochromis nilotica*, Nguyen *et al.* (1995); (5) *Cottus scorpius*, Cutfield *et al.* (1986); (6) *Lophius americanus*, Nicholes *et al.* (1988); (7) *Cottus scorpius*, Cutfield and Cutfield (1993); (8) *Thunnus obesus*, Navarro *et al.* (1991); (9) *L. americanus*, Andrews *et al.* (1986); (10) *Oncorhynchus kistch*, Plisetskaya *et al.* (1986); (11) *Lampetra fluviatilis*, Conlon *et al.* (1995).

tablished their full sequences. On the other hand, significant amounts of phenylthiohydantoin amino acids were not obtained in automated Edman degradation of insulin-II B-chain. We tried to digest insulin-II B-chain with *Pfu* pyroglutamate aminopeptidase. The obtained sequence consisted of 31 amino acid residues. The difference in mass between undigested (*m*/*z* 3629) and digested material (*m*/*z* 3517) was 112, indicating that N-terminal amino acid residue of B-chain of stone flounder insulin-II was pyroglutamic acid residue (Pyr). From these results, it was established that insulin-II B-chain of the stone flounder consisted 32 amino acid residues, and had an extension of two amino acid residues (Pyr-Ala) at the N-terminus compared to insulin-I (Fig. 5). Val residues at position 2 of the stone flounder B-chains were substituted by Leu in barfin flounder insulin-I and -II.

Characterization of stone flounder glucagon-I and -II

Amino acid sequence analyses were carried out on glucagon-I purified by Method B and on glucagon-II by Method A. The amino acid sequences are shown in Fig. 5. The observed values of $(M + H)^+$ for glucagon-I and -II (*m*/*z* 3552 and *m*/*z* 3503, respectively) were consistent with the theoretical values (*m*/*z* 3552 and *m*/*z* 3508, respectively), establishing their full sequences. Both stone flounder glucagons consisted of 29 amino acid residues, and amino acid residue substitutions between these glucagons were observed at positions 16, 18, 24, 27 and 29.

One amino acid residue substitution was observed in glucagon-I among stone flounder, anglerfish and sculpin. Glucagon-I in stone flounder and tilapia were identical. On the other hand, stone flounder glucagon-II was identical with those of anglerfish, sculpin and tilapia, and also with glucagons of flounder and tuna.

Characterization of stone flounder somatostatin

Edman degradation of an intact peptide established the sequence of somatostatin on Ala-Gly-(Cys)-Lys-Asn^5-Phe-Phe-Trp-Lys-Thr^{10}-Phe-Thr-Ser-(Cys). The existence of two cysteine residues, which might form a disulfide bridge, was supported by mass spectral analysis. The observed value for $(M + H)^+$ (*m*/z 1652) was in good agreement with the theoretical value (*m*/*z* 1658). Somatostatin-II type molecule was not obtained.

DISCUSSION

The present study demonstrated that stone flounder contained two molecular forms of insulin as barfin flounder (Andoh and Nagasawa, 1998), indicating that Pyr-B-chain of insulin is not specific to barfin flounder. Jorgensen (1960) found that flounder (*Pleuronectes flesus*, a synonymous species of *Platichthys flesus*) contained two electrophoretically distinct insulins (IIa and IIb). The mobilities of the A-chains of these molecules were identical, while that of the B-chain of IIb was greater than that of IIa, indicating that the structural difference between IIa and IIb was attributed to their B-chains. On the other hand, Conlon *et al*. (1987b) reported that the Brockmann body of flounder (*P. flesus*) contained insulin and a material with a weak insulin-like immunoreactivity by radioimmunoassay using an anti-mammalian insulin antiserum, which was eluted just after insulin on reversed phase HPLC. We also found that the Brockmann body of brown sole (*Limmanda herzensteini*) contained insulin-II-like material (Andoh and Nagasawa, unpublished data), which shows an insulin-like immunoreactivity in competitive enzyme immunoassay using an anti-bovine insulin antiserum developed by Andoh and Nagasawa (1997). These suggest that many species of flounders have two molecular forms of insulin.

Based on the analyses at both peptide and DNA levels, we proposed that insulin-I and -II of barfin flounder arose from a single preproinsulin by proteolytic cleavage at different sites of the signal peptide region (Andoh and Nagasawa, 1998). The structural difference between insulin-I and -II in stone flounder was like in barfin flounder.

In stone flounder, the yields of insulin-II was 1.2-1.4 fold higher than those of insulin-I by both methods (Figs. 2 and 3). In barfin flounder, the yield of insulin-II was two or three times higher than that of insulin-I by both Method A using Sep-Pak (Andoh and Nagasawa, 1998; in the first HPLC step) and Method B (Andoh and Nagasawa, unpublished data). These suggest that the ratio of insulin-I and -II in the Brockmann body is different between stone flounder and barfin flounder.

Glucagon-I was not obtained and the yield of glucagon-II was only 2.6 nmol/g tissue by Method A, while the yields of glucagon-I and -II were 24 and 62 nmol/g tissue, respectively, by Method B. Conlon *et al*. (1987b) purified glucagon (glucagon-II type molecule; 14 nmol/g tissue) by the method using Sep-Pak cartridges similar to Method A from flounder (*P. flesus*), but did not obtain a glucagon-I type molecule. These results indicate the difficulty in the purification of glucagons, especially glucagon-I, by Method A. On the other hand, somatostatin-14 was not obtained by Method B, somatostatin-14 may have been discarded in the centrifugation step after addition of NH_4OH or ethanol-diethylether precipitation.

Cutfield *et al*. (1986) and Cutfield and Cutfield (1993) showed that daddy sculpin (*Cottus scorpius*) contained two molecular forms of glucagon. The amino acid sequence of daddy sculpin glucagon-II was identical with that of anglerfish glucagon-II. There were four amino acid residue substitutions between daddy sculpin glucagon-I and -II. In this study, we showed that stone flounder glucagon-II was also identical with that of daddy sculpin and anglerfish, and that amino acid sequence homology between glucagon-I and -II of stone flounder is obviously lower than those among glucagon-I's of daddy sculpin, anglerfish, and stone flounder. These substitutions between glucagon-I and -II of stone flounder indicate that glucagon-I and -II are encoded by two different genes in these species, which suggests that these genes differentiated before the divergence of these species. Especially, anglerfish belongs to the superorder Paracanthopterygii, while the others belong to the superorder Acanthopterygii. These superorders are monophyletic groups which originated from super-

order Protacanthopterygii or its ancestral group (Greenwood *et al*., 1966; Nelson, 1994). These suggest that the species belonging to the superorder Acanthopterygii and Paracanthopterygii seem to contain two molecular forms of glucagon.

The amino acid sequence of somatostatin-14 from stone flounder was identical with those of mammals and several fish (e.g., Brazeau *et al*., 1973; Plisetskaya *et al*., 1986). This indicate that somatostatin-14 is conservative due to strong natural selection pressure through evolution.

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