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Purification and Structural Characterization of Insulin From a Caecilian, *Typhlonectes natans* (Amphibia: Gymnophiona)

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CONLON, J. M., C. HILSCHER-CONKLIN AND S. K. BOYD. *Purification and structural characterization of insulin from a caecilian, Typhlonectes natans (Amphibia: Gymnophiona).* PEPTIDES **16**(8) 1385–1388, 1995.—Despite the important position of amphibia in phylogeny, efforts at the structural characterization of amphibian neurohormonal peptides have largely been confined to the Anurans (frogs and toads). Insulin was purified from an extract of the pancreas of the caecilian, *Typhlonectes natans*. The primary structure of the peptide was established as:

A-chain: Gly-Ile-Val-Glu-Lys[5]-Cys-Cys-Leu-Ser-Thr[10]-Cys-Ser-Leu-Tyr-Glu[15]-Leu-Glu-Ser-Tyr-Cys[20]-Asn

B-chain: Ile-Ala-Asn-Gln-His[5]-Leu-Cys-Gly-Ser-His[10]-Leu-Val-Glu-Ala-Leu[15]-Tyr-Leu-Val-Cys-Ala[20]-Asp-Arg-Gly-Phe-Phe[25]-Tyr-Thr-Pro-Lys-Ser[30]

This amino acid sequence contains several unusual substitutions (Gln → Lys at A5, His → Leu at A8, Gln → Glu at A15, and Gly → Ala at B20) that are not present in other amphibian insulins. The structure of insulin appears to be less well conserved among the different orders of amphibia, compared with reptiles and birds.

Insulin Amphibian Caecilian HPLC purification

CAECILIANS (order Gymnophiona), in comparison with the frogs and toads (order Anura) and salamanders (order Urodela), are the least well known of the living amphibians. Modern caecilians are classified in six families with a total of 34 genera (16). All species are restricted to the damp tropics and are limbless and burrowing in habit except for the typhlonectids, which are aquatic. The fossil record of the caecilians is very meager (13,17), with the result that the phylogenetic relationships between the different genera and between caecilians and other orders of amphibia are unclear (20). The geographical distribution of the families of caecilians does not conform with the present-day arrangement of the continents, suggesting that the origin of the families predates the break-up of Gondwanaland (6). Certain common anatomical features, such as the presence of pedicellate teeth, support the hypothesis that extant frogs, salamanders, and caecilians are derived from a common ancestor, but the argument that each of the modern orders evolved from a distinct group of Paleozoic amphibians cannot be rejected (6). It is believed, however, that the line of evolution leading to present-day caecilians diverged from that leading to frogs and salamanders at least 200 million years ago (10).

The amphibians, as representatives of the first terrestrial vertebrates, occupy a pivotal position in vertebrate phylogeny. Although numerous regulatory peptides have been isolated from skin and neuroendocrine tissues of frogs and have been well characterized [reviewed in (1)], our knowledge of the structure and biological activity of neurohormonal peptides from urodeles is very limited and from caecilians is nonexistent. This study describes the first isolation and determination of the amino acid sequence of an insulin from a caecilian, *Typhlonectes natans*, which is indigenous to the Amazon and Orinoco basins. The Typholonectidae are considered to have arisen relatively recently in South America from an ancestor belonging to the larger Caeciliaidae family (12).

METHOD

Tissue Extraction

Pancreata (n = 20, weight 1.0 g) were removed from adult specimens of *Typhlonectes natans* of both sexes (length 33–42 cm; weight 60–100 g) that had been anaesthetized by immersion in benzocaine solution (1:1000, w/v). Specimens were supplied by Wilson Pet Supply (Woodale, IL). The tissues were immediately frozen on dry ice and were stored at −80°C. Frozen tissue was homogenized at 4°C with ethanol/0.7 *M* HCl (3:1, v/v, 20 ml) as previously described (7). After centrifugation (1600 × g for 30 min), ethanol was removed from the supernatant under

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reduced pressure. The extract was pumped at a flow rate of 1 ml/min onto three Sep-Pak C-18 cartridges (Waters Associates) connected in series. Bound material was eluted with 70% (v/v) acetonitrile/water and lyophilized.

Radioimmunoassay

Insulin-like immunoreactivity was measured by using an antiserum raised against pig insulin as described previously (11).

Purification of Caecilian Insulin

The lyophilized material was redissolved in 0.1% (v/v) trifluoroacetic acid/water (4 ml) and the extract was injected onto a (25 × 1 cm) Vydac 218TP510 C-18 reversed-phase HPLC column (Separations Group), equilibrated with 0.1% trifluoroacetic acid/water at a flow rate of 2 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 21% (v/v) over 10 min, held at this concentration for 30 min, and raised to 49% (v/v) over 60 min using linear gradients. Absorbance was measured at 214 and 280 nm, and individual peaks were collected by hand. The peak denoted by I in Fig. 1(A) was rechromatographed on a (25 × 0.46 cm) Vydac 214TP54 (C-4) column equilibrated with acetonitrile/water/trifluoroacetic acid (21.0:78.9:0.1, v/v/v) at a flow rate of 1.5 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 42% (v/v) over 40 min using a linear gradient. Caecilian insulin was purified to near homogeneity by a final chromatography on a (25 × 0.46 cm) Vydac 219TP54 (phenyl) column under the same conditions used for the C-4 column.

Peptide Characterization

Caecilian insulin (approximately 1 nmol) was incubated for 12 h at room temperature with dithiothreitol (2 mg) in 0.1 *M* Tris-HCl/6 *M* guanidine hydrochloride buffer, pH 7.5 (0.4 ml), under an atmosphere of argon. Cysteine residues were derivatized by addition of 4-vinylpyridine (3 μl) and the pyridylethylated A- and B-chains of insulin were separated on a (25 × 0.46 cm) Vydac C-4 column under the conditions used for the purification of intact insulin. Amino acid compositions were determined by precolumn derivatization with phenylisothiocyanate (5) using an Applied Biosystems model 420A derivatizer followed by reversed-phase HPLC using an Applied Biosystems model 130A separation system. The detection limit for phenylthiocarbamyl-coupled amino acids was 1 pmol. Hydrolysis (24 h at 110°C in 5.7 *M* HCl) of approximately 1 nmol of insulin was performed. The primary structures of the pyridylethylated A-chain and B-chain of insulin were determined by automated Edman degradation using an Applied Biosystems model 471A sequenator modified for on-line detection of phenylthiohydantoin (PTH)-coupled amino acids under gradient elution conditions. The detection limit for PTH-amino acids was 0.5 pmol.

RESULTS

The crude extract of the caecilian pancreas contained only trace amounts (<0.5 pmol/g tissue weight) of insulin-like immunoreactivity measured with an antiserum raised against pig insulin.

Purification of the Peptide

The elution profile on a semipreparative Vydac C-18 column of the extract, after partial purification on Sep-Pak cartridges, is shown in Fig. 1(A). The peak denoted by I contained material that inhibited slightly (approximately 5%) the binding of [3-[^{125}I]iodotyrosine-A14]human insulin to antibody in radioim-

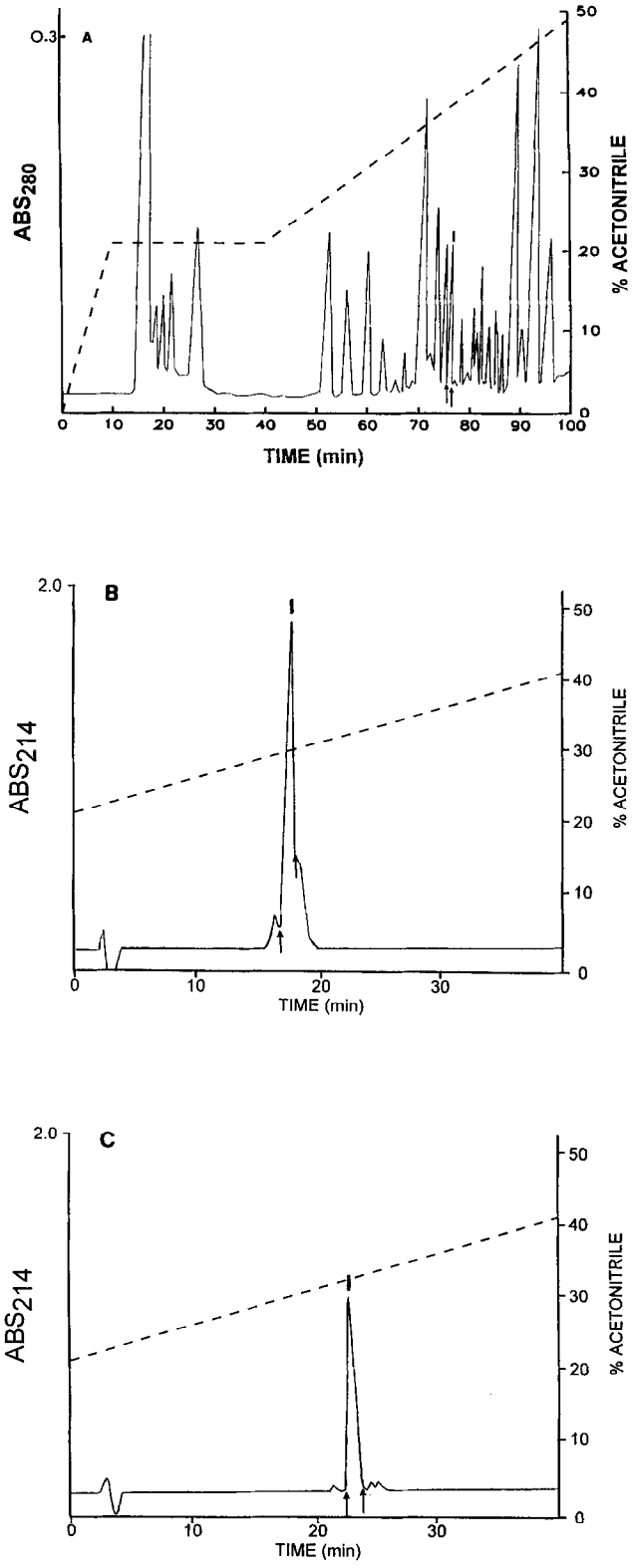

FIG. 1. Purification by reversed-phase HPLC of caecilian insulin on (A) semipreparative Vydac C-18, (B) analytical Vydac C-4, and (D) analytical Vydac phenyl columns. I denotes the peaks containing insulin-like immunoreactivity. The arrows show where peak collection began and ended and the dotted line shows the concentration of acetonitrile in the eluting solvent.

TABLE 1

AUTOMATED EDMAN DEGRADATION OF THE A-CHAIN AND B-CHAIN OF INSULIN FROM A CAECILIAN

Cycle No.	A-Chain	B-Chain
1	Gly (340)	Ile (630)
2	Ile (420)	Ala (562)
3	Val (390)	Asn (539)
4	Glu (298)	Gln (448)
5	Lys (307)	His (274)
6	Cys (224)	Leu (423)
7	Cys (235)	Cys (348)
8	Leu (271)	Gly (414)
9	Ser (36)	Ser (53)
10	Thr (70)	His (149)
11	Cys (136)	Leu (177)
12	Ser (26)	Val (153)
13	Leu (142)	Glu (112)
14	Tyr (96)	Ala (165)
15	Glu (79)	Leu (159)
16	Leu (98)	Tyr (144)
17	Glu (71)	Leu (148)
18	Ser (14)	Val (136)
19	Tyr (55)	Cys (98)
20	Cys (47)	Ala (106)
21	Asn (21)	Asp (82)
22		Arg (74)
23		Gly (93)
24		Phe (61)
25		Phe (79)
26		Tyr (49)
27		Thr (27)
28		Pro (39)
29		Lys (22)
30		Ser (8)

Cysteine was determined as its pyridylethylated derivative. Figures in parentheses are the yields of amino acid phenylthiohydantoins (pmol).

munoassay and was purified further. As shown in Fig. 1(B), the material was eluted from an analytical Vydac C-4 column as a well-defined major peak with a descending shoulder. The caecilian insulin was purified to apparent homogeneity, as assessed by a symmetrical peak shape, on an analytical Vydac phenyl column [Fig. 1(C)]. The final yield of purified insulin was approximately 3 nmol.

Structural Characterization

The results of automated Edman degradation are shown in Table 1. It was possible to assign without ambiguity phenylthiohydantoin derivatives of amino acids for 21 cycles of operation during sequence analysis of the A-chain and for 30 cycles in the case of the B-chain. The results of Edman degradation indicated that the caecilian insulin was > 98% pure. The results of amino acid analysis demonstrated that the caecilian insulin had the composition: Asx 3.0 (3), Glx 4.9 (5), Ser 4.8 (5), Gly 3.0 (3), His 1.9 (2), Arg 1.3 (1), Thr 2.0 (2), Ala 3.0 (3), Pro 1.3 (1), Tyr 3.1 (3), Val 2.4 (3), Ile 1.5 (2), Leu 6.6 (7), Phe 2.0 (2), Lys 2.2 (2) (mol residue/mol peptide). The values in parentheses show the number of residues predicted from the proposed structure. Agreement between the sequence analysis and amino acid composition data was good, demonstrating that the full sequence of the peptide had been obtained. The slightly low values for the amounts of valine and isoleucine are consistent with the presence of an Ile-Val bond in the molecule that is resistant to hydrolysis.

DISCUSSION

This article describes for the first time the purification and primary structure of an insulin from the pancreas of a caecilian, *T. natans*. As in the anurans, the pancreas of caecilians comprises a distinct elongate gland lying between the duodendum and stomach but, unlike the anurans, pancreatic juice is conveyed to the intestine through multiple pancreatic ducts (10). The isolation of appreciable quantities of insulin from the pancreatic extract (approx. 3 nmol/g tissue) indicates that the very low concentration of insulin-like immunoreactivity measured in the extract by radioimmunoassay was a consequence of the poor reactivity of the caecilian insulin towards an antibody raised against pig insulin. Previous studies (7,8) have shown that insulins with amino acid substitutions in the *N*-terminal region of the B-chain react poorly with this antiserum.

Although the amino acid sequence of insulin is known for at least 70 species, studies have focused upon mammals and teleost fish. Our knowledge of primary structures of amphibian insulins is confined to two closely related peptides isolated from the South African clawed toad *Xenopus laevis* (19) and to one peptide from the urodele, the three-toed amphiuma *Amphiuma tridactylum* (Cavanaugh and Conlon, unpublished data). Structures of the amphibian insulins are compared with those of known reptilian insulins [turtle *Psuedemys scripta* (7), rattlesnake *Crotalus atrox*

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                                A-Chain

Caecilian              GIVEK CCLST CSLYE LESYC N
Amphiuma            AR ----Q --HN- ---NQ --N-- -
Xenopus I              ----Q --H-- ---FQ ----- -
Xenopus II             ----Q --H-- ---FQ --N-- -
Turtle                 ----Q --HN- ----Q --N-- -
Rattlesnake            ----Q --EN- ----Q --N-- -
Colubrid snake         ----Q --EN- ----E --N-- -
Alligator              ----Q --HN- ----Q --N-- -
Human                  ----Q --TSI ----Q --N-- -

                                B-Chain

Caecilian           IANQH LCGSH LVEAL YLVCA DRGFF YTPKS
Amphiuma            -T--Y ----- ----- ----G ----- -S--
Xenopus I           LV--- ----- ----- ----G ----- -Y--V
Xenopus II          L---- ----- ----- ----G ----- -Y--I
Turtle              A---- ----- ----- ----G E---- -S--A
Rattlesnake         AP--R ----- ----- F-I-G E---- -S-R-
Colubrid snake      AP--R ----- ----- F-I-G E---- -S-RT
Alligator           A---R ----- --D-- ----G E---- -S--G
Human               FV--- ----- ----- ----G E---- ----T
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FIG. 2. A comparison of the primary structures of amphibian, repilian, and human insulins. (-) denotes residue identity.

(14), colubrid snake *Zoacys dhumnades* (21), and alligator *Alligator mississipiensis* (15)] and with human insulin in Fig. 2. The caecilian insulin contains 10 substitutions compared with amphiuma insulin and nine compared with both *Xenopus* insulins. However, one should not infer that the caecilians are more closely related to frogs than to salamanders because phylogenetic relationships based upon the amino acid sequences of insulin are frequently not in good agreement with those based upon classical morphological criteria ["molecular clocks that tell the wrong time" (3)], Indeed, a recent phylogenetic study comparing mitochondrial DNA sequences of the 12S and 16S rRNA genes has concluded the gymnophonians and the urodeles (as represented by the amphiuma) are sister taxa (12). The data in Fig. 2 indicate that the amino acid sequence of insulin has been less well conserved among the amphibia than among the reptiles. Insulin from phylogenetically ancient chelonian, the turtle, shows only three amino acid substitutions compared with insulin from a crocodilian, despite the fact that their lines of evolution diverged at least 300 million years ago. Similarly, a comparison of the primary structures of insulins from chicken, turkey, duck, and goose [reviewed in (19)] shows that the sequence of the hormone has been well conserved among the class Aves.

The unusual amino acid substitution Asn → Ser at A18 of the caecilian insulin is also found in *Xenopus* insulin I (19) and the glutamic acid residue at A15 is also found in insulin from a colubrid snake (20). However, *T. natans* insulin shows structural features not found in other amphibian or reptilian insulins (Fig. 2). In particular, the presence of a lysine at position A5, a residue on the surface of the insulin molecule that is considered to be a component of the receptor binding region (4), may be expected to significantly alter biological potency. Dogfish insulin, which contains a histidine residue at A5, is threefold less potent than bovine insulin in stimulating lipogenesis in rat fat cells (2). Similarly, it has been speculated that substitutions in the B17–B22 regions of hagfish insulin are responsible for its reduced biological activity (9) so that the novel substitution Gly → Ala at B20 of *T. natans* insulin may also affect potency. Along with residues B6, B10, B14, B17, A13, and A14, the glycine residue at B20 is also involved in the formation of zinc-containing hexamers (4). The B20 glycine residue has been conserved in all other vertebrate species studied to date except the hystricomorph rodents (18). The presence of a leucine residue at A8 in the caecilian insulin is unusual but has also been found in insulin from the bowfin *Amia calva* (8). *Xenopus* insulin and chicken insulin, which also contains a histidine residue at A8, have two- to threefold higher affinity than pig insulin in binding to the mammalian insulin receptor (19). Bowfin insulin, however, has 13-fold lower affinity in this system (8). The novel extension to the *N*-terminus of the A-chain of amphiuma insulin is not found in *T. natans*, suggesting that proinsulin is processed conventionally in this species.

Further studies with the extract of caecilian pancreas have led to the isolation of a peptide with structural similarity to mammalian glucagon-like peptide-1 (HADGTYTSDI^{10}SSYLEGQA-AK^{20}KFIDWLISME^{30}GRRLDG) (Conlon, unpublished data) but, inexplicably, attempts to identify glucagon and glucagon-like peptide-2 were unsuccessful.

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