Corticotropin Like Intermediate Lobe Peptide in the Salmon Pituitary

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An intermediate lobe peptide, which are related to C-terminal region of corticotropin, ACTH (18-39) has been isolated from an alkaline extract of the pituitary gland of the salmon, *Oncorhynchus keta*, by salt precipitation, ion exchange chromatography, gel filtration and preparative paper electrophoresis. Sequence analysis of the peptide revealed the following primary structure: H-Arg-Pro-Val-Lys-Val-Tyr-Thr-Asn-Gly-Val-Glu-Glu-Gln-Ser-Ser-Glu-Gly-Phe-Pro-Ser-Glu-Met-OH. The amino acid sequence comparison suggested that the teleost peptide is much closer to the higher vertebrate, such as ostrich than the elasmobranch (dogfish).

It has been established in mammalian species that corticotropin (ACTH) and β -lipotropin (LPH) are derived from a common high molecular weight precursor¹⁾, and melanotropins (MSH) and endorphins (EP) are produced by further proteolytic processing of the precursor in pars intermedia^{2,3)}. In contrast with the striking development of the knowledge in mammalian species, only a little is known in lower vertebrates, i.e. ostrich ACTH⁴⁾, dogfish ACTH⁵⁾, CLIP⁶⁾, and MSHs^{6,7,8)}.

We have recently found that the intermediate hormones were predominant in the salmon, *Oncorhynchus keta*, while ACTH and β -LPH, secreted from pars distalis were undetectable. EP I⁹⁾, EP II¹⁰⁾, α -MSH¹¹⁾, β -MSH I¹¹⁾, and β -MSH II¹²⁾ were isolated and elucidated for their primary structures. In order to obtain structural information and thus better insight into the evolutionary history of the precursor, it is important to characterize the intermediate peptides in the teleost.

This communication describes the isolation and elucidation of the complete structure of a corticotropin like intermediate lobe peptide (CLIP) from the salmon.

Materials and Methods

Salmon pituitary glands were collected in Tsugaruishi river, Iwate, and stored at -80°C . CM-cellulose was purchased from Serva (Germany). Sephadex G 75 and G 100 from Pharmacia (Sweden). Trypsin, chymotrypsin and

carboxypeptidase A were obtained from Boehringer Mannheim (Germany). Fluorescein isothiocyanate and fluorescein thiohydantoin amino acids were prepared as described previously^{13,14)}. All chemicals used for the structural determination were sequential grade from Wako Junyaku and Tokyo Kasei (Japan). Polyamide thin-layer plates were obtained from Cheng Chin Trading Co. (Taiwan).

The pituitary extract was dissolved in water at pH 9.5 and then was submitted to salt fractionation with sodium chloride at pH 3, and ion-exchange chromatography using carboxymethyl cellulose¹⁵⁾. The corticotropin like peptide fraction was purified by gel filtration on Sephadex G 100, and finally by a preparative paper electrophoresis on Whatman 3MM. All fractions were examined by molecular weight and amino terminal residues. Molecular weight was estimated on a calibrated Sephadex G 75 column, using salmon EP I⁹⁾, EP II¹⁰⁾, β -MSH I¹¹⁾, α -MSH¹¹⁾, fin whale β -LPH¹⁶⁾ and fin whale ACTH¹⁷⁾ as standard peptides.

Amino acid analyses were performed by the method of SPACKMAN *et al.*¹⁸⁾, using a Hitachi 034 automatic analyzer. The peptide was digested by trypsin and chymotrypsin. The resulting peptides were fractionated by paper electrophoresis. Amino- and carboxylterminal residues were determined by the dansyl¹⁹⁾ and carboxypeptidase method²⁰⁾, respectively. Sequence analysis was performed by the fluorescein isothiocyanate method^{21,22)}, with direct identification on polyamide thin-layer

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Abbreviations used: ACTH, corticotropin; CLIP, corticotropin like intermediate lobe peptide; EP, endorphin; LPH, lipotropin; MSH, melanotropin.

plate²³⁾. The results were further confirmed by the dansyl-Edman method²⁴⁾.

Results

Isolation and Purification

Salmon pituitaries (30 g) were extracted with 100 ml of water, adjusted to pH 9.5 with $\text{Ca}(\text{OH})_2$ for 1 h at 4°C and with 70 ml of water for additional 1 h. The extract was acidified to pH 3 with 1 N HCl. The resulting precipitate was removed. Sodium chloride was added to the solution to a concentration of 40% saturation. The fraction (238 mg) obtained from the supernatant was submitted to ion-exchange chromatography of CM-cellulose column (1×25 cm), equilibrated with 0.01 M ammonium acetate, pH 4.6. The elution was performed with the same buffer and followed by a gradient elution of ammonium acetate as shown in Fig. 1. The fraction B (48 mg) was purified on Sephadex G 100 column (2.46×48 cm) in 0.05 M ammonium bicarbonate, pH 9.0 as shown in Fig. 2. The second fraction (18.3 mg) exhibited a molecular weight to be 2,700 daltons on

Table 1. Amino acid composition of salmon CLIP compared with ostrich and dogfish ACTH (18-39)

Amino acid	Salmon CLIP	ACTH (18-39)	
		Ostrich* ¹	Dogfish* ²
Lys	1.1 (1)	1	1
Arg	1.0 (1)	1	1
Asp	1.1 (1)	1	3
Thr	1.0 (1)	1	0
Ser	2.5 (3)	1	2
Glu	5.4 (5)	5	4
Pro	1.9 (2)	3	3
Gly	2.0 (2)	2	1
Val	2.6 (3)	3	2
Met	0.9 (1)	0	1
Ile	0.0 (0)	0	1
Leu	0.0 (0)	1	1
Tyr	0.9 (1)	1	1
Phe	1.0 (1)	2	1

*¹ Taken from Ref. 25, *² Taken from Ref. 5.

the calibrated Sephadex G 75 column as shown in Fig. 3. The amino acid composition of the peptide was similar to that of ostrich ACTH (18-39)⁴⁾. The final purification was performed

Fig. 1. Chromatography of a salmon CLIP concentrate on CM-cellulose. 238 mg of concentrate was placed on a column (1×25 cm) and eluted with ammonium acetate gradient as indicated in the figure. The eluate was collected in 2 ml fractions.

Fig. 2. Gel filtration of salmon CLIP fraction B in Fig. 1 on Sephadex G 100. 48 mg of the fraction was placed on a column (2.64×48 cm) and eluted with 0.05 M ammonium bicarbonate, pH 9.0. The eluate was collected in 3 ml fractions after 103 ml of forerunning.

Fig. 3. Molecular weight estimation of salmon CLIP on a Sephadex G 75 column (2.64×47 cm) with 0.05 M ammonium acetate, pH 7.0. Standard peptides: A; salmon α -MSH (11); B; salmon β -MSH I (11); C; salmon endorphin I (9); D; salmon endorphin II (10); E; fin whale ACTH (17); F; fin whale β -LPH (16).

with preparative paper electrophoresis on Whatman 3MM using pH 6.7 buffer (γ -collidine-acetic

acid-water, 17.8-6.2-1960, by volume) at 400 V for 3 h. After development of a guide strip with ninhydrin and Pauly reagents, five bands were obtained, as shown in Fig. 4, and were eluted with 0.1 N acetic acid. Two major bands, B (4 mg) and A (1 mg), exhibited identical amino acid compositions (Table 1). The A fraction could be a deamidated form of fraction B based on their electrophoretic mobilities.

Primary Structure

The structural studies were performed using fraction B. The peptide was digested with trypsin ($E/S=1/50$) and chymotrypsin ($E/S=1/400$) in 0.2 M ammonium acetate, pH 8.0 at 37°C for 6 h and 40 min, respectively. Each digest was fractionated on paper electrophoresis using the conditions as described above. Two peptides, one was basic and the other acidic, were isolated from both preparations. The amino acid compositions of these peptides are summarized in Table 2.

The amino acid sequence of intact peptide was determined up to the 14th residues, and the chymotryptic peptide (C-II) could be identified up to 16 residues by the fluorescein isothiocyanate me-

Fig. 4. Paper electrophoresis of salmon CLIP fraction II in Fig. 3. Collidine acetate, pH 6.7, 10 V/cm, 4 h. C, D and E were faint bands.

Fig. 5. Amino acid sequence determination of salmon CLIP. C: chymotryptic fragments, T: tryptic fragments, $\rightarrow$: Fluorescein isothiocyanate method, $\leftarrow$: Carboxypeptidase method, $\rightarrow\leftarrow$: Dansyl-Edman method (for three cycles without identification).

Table 2. Amino acid compositions of tryptic and chymotryptic fragments of salmon CLIP

Amino acid	Tryptic fragments		Chymotryptic fragments	
	T-1	T-2	C-1	C-2
Lys	1.0		1.0	
Arg	0.8		1.0	
Asp		1.0		1.0
Thr		0.8		0.8
Ser		2.5		2.7
Glu		5.1		5.1
Pro	1.2	0.8	1.3	1.0
Gly		1.9		2.0
Val	1.1	1.7	1.9	0.8
Met		0.8		0.8
Tyr		0.7	1.0	
Phe		0.9		1.0

thod^{21,22}). The sequence from residues 10 to 22 was further confirmed with C-II peptide by the dansyl-Edman method²⁴). The amino acid compositions of carboxypeptidase A digests of salmon CLIP as a function of time are presented in Table 3.

Based on these data, the complete amino acid sequence of salmon CLIP is proposed in Fig. 5.

Table 3. Kinetics of carboxypeptidase A digestion of salmon CLIP

Amino acid	30 min	18 h
(mole ratio)		
Met	1.0	1.0
Glu	0.0	0.7

Discussion

CLIP was isolated from porcine²⁵) and dogfish²⁶) pituitaries and the primary structures were identical to the respective ACTH (18-39). It has also been established that α -MSH, as well as CLIP is derived from ACTH, arising from the precursor by proteolytic cleavage in the pars intermedia²⁷). Similarly we can propose the salmon ACTH molecule with α -MSH, CLIP and a segment of ACTH (14-17) as shown in Fig. 6.

When compared with known structures of ACTH (18-39) from other species, seven amino acids for all species are conserved. Sixteen residues are invariant for salmon vs. ostich, 13-14 for salmon vs. mammals, and only 10 for salmon vs. dogfish. The comparison suggested that the teleost CLIP, as well as α -MSH¹¹) is much closer to the higher vertebrates than to the elasmobranch.

It is well known that the neurointermediate lobe of salmonoides form as much as two third the volume of the entire pituitary²⁸). This fact can explain why the intermediate hormones, such as EP, CLIP and MSH are predominant in the salmon pituitary, while ACTH and β -LPH, secreted from pars distalis were undetectable. We have found two different molecules of endorphin^{10,11}) and β -MSH^{12,13}) in the pituitary of the salmon. These findings strongly suggest that the salmon pituitary secretes two isomers of the precursor^{11,13}). The salmon CLIP also showed similar heterogeneity. Unlike the endorphins and β -

SALMON	:	(H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val)
DOGFISH	:	H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Met-Gly-Arg-Lys-Arg-
OSTRICH	:	H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Arg-Lys-Arg-
MAMMALS	:	H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-
		[α -MSH 5 10 13]
		[ACTH 5 10 15]
SALMON	:	Arg-Pro-Val-Lys-Val-Tyr-Thr-Asn-Gly-Val-Glu-Glu-Gln-Ser-Ser-Glu-Gly-Phe-Pro-Ser-Glu-Met-OH
DOGFISH	:	Arg-Pro-Ile-Lys-Val-Tyr-Pro-Asn-Ser-Phe-Glu-Asp-Glu-Ser-Val-Glu-Asn-Met-Gly-Pro-Glu-Leu-OH
OSTRICH	:	Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Val-Gln-Glu-Glu-Thr-Ser-Glu-Gly-Phe-Pro-Leu-Glu-Phe-OH
WHALE & HUMAN	:	Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe-OH
PORCINE	:	Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Leu-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe-OH
BOVINE & OVINE	:	Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Gln-Ala-Phe-Pro-Leu-Glu-Phe-OH
		[CLIP 5 10 15 20 22]
		[ACTH 20 25 30 35 39]

Fig. 6. Sequence comparison of salmon α -MSH and CLIP with ACTHs from various vertebrates. α -MSH=ACTH(1-13) and CLIP=ACTH(18-39).

MSHs, however, one of these may be a deamidated form generated during the isolation.

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