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# Isolation of a Peptide Structurally Related to Mammalian Corticostatins from the Lamprey *Petromyzon marinus*

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**ABSTRACT.** Peptides in an extract of skin from the agnathan *Petromyzon marinus* (sea lamprey) were purified by reversed-phase high-performance liquid chromatography and characterized by Edman degradation. The primary structure of a cysteine- and arginine-rich peptide (termed lamprey corticostatin-related peptide [LCRP]) was established as Cys-Pro-Cys-Gly-Arg-Arg-Arg-Cys-Cys-Val-Arg-Gly-Leu-Asn-Val-Tyr-Cys-Cys-Phe. Mass spectrometry indicated that all cysteine residues are intramolecularly linked. This amino acid sequence shows structural similarity to rat corticostatin R4 and rabbit corticostatin R1. In particular, LCRP contains the polyarginine sequence at the N-terminus of the peptide that is believed to mediate both the inhibition of ACTH-stimulated steroidogenesis and the antimicrobial (defensin-like) actions of the corticostatins. COMP BIOCHEM PHYSIOL 114B, 133–137, 1996.

**KEY WORDS.** Sea lamprey, agnathan, skin, HPLC purification, corticostatin, defensin

## INTRODUCTION

The defensins are a family of arginine-rich peptides with a characteristic motif of six cysteine residues linked by three intramolecular disulfide bonds (reviewed in reference 13). Defensins are produced primarily by polymorphonucleated neutrophils and macrophages and have been isolated in multiple molecular forms from several mammalian species (human [22], rabbit [19], rat [1], mouse [17] and guinea pig [20]. Certain peptides of the defensin family are referred to as corticostatins because of their ability to bind specifically to the ACTH receptor of adrenocortical cells and inhibit ACTH-stimulated steroidogenesis (23,25). Other peptides of the family are cytotoxic toward a variety of prokaryotic and eukaryotic targets (hence the term defensins), exhibit anti-viral activity, are chemotactic for monocytes, inhibit protein kinase C, release histamine from mast cells and stimulate L-type $Ca^{2+}$ channels (reviewed in reference 13). Rat corticostatin R4 is the only member of the family yet characterized that displays potent corticostatic, cytotoxic, antimicrobial and $Ca^{2+}$ channel agonist activity (1,14). Corticostatins/defensins have not been isolated from non-mammalian vertebrates, but a 40-amino acid-residue peptide with six cysteine residues and some structural similarity to the mammalian defensins has been purified from the hemolymph of immunized larvae of the dipteran insect *Phormia terranovae* (11).

The success in identifying novel bioactive substances in amphibian skin (reviewed in reference 12) has led to systematic examination of extracts of skin from other classes of vertebrates. Particular emphasis has been placed on the isolation of peptides, lipids and alkaloids with antibiotic properties (reviewed in reference 15). The initial impetus for the present study was to examine an extract of skin of the sea lamprey *Petromyzon marinus* for the presence of peptides with a kinin-like ability to contract smooth muscle. It has been reported, for example, that a methanolic extract of the skin of the lamprey *Eudontomyzon danfordi vladykovi* possessed a bradykinin-like ability to contract the rat uterus and guinea pig intestine (7). The crude extract of *P. marinus* skin produced a slow contraction of the isolated frog rectum (K. Yano and J. M. Conlon, unpublished data), but we were unable to isolate any myotropic peptides in pure form. Our attempts, however, have led to the purification and characterization of a cysteine- and arginine-rich peptide with limited structural similarity to mammalian corticostatins/defensins that we term lamprey corticostatin-related peptide (LCRP).

## MATERIALS AND METHODS

### *Animals*

Adult female sea lampreys were captured during their final spawning migration from the ocean at the New Hampshire

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Fish and Game fish ladder on the Cocheco river in Dover, New Hampshire during the months of May and June. The animals were maintained in an artificial stream at the University of New Hampshire Anadromous Fish and Aquatic Invertebrate Research Laboratory. Lampreys were anesthetized with ethyl m-aminobenzoate methanesulfonate (MS 222) and killed by decapitation. Skin with some attached muscle was removed from five adult female specimens (body weight 180–220 g.) The tissue was immediately frozen on dry ice and stored at −20°C until time of extraction.

### *Tissue extraction*

Tissue (142 g) was stirred with methanol (1500 ml) for 1 hr at 0°C. The supernatant was decanted and the tissue homogenized at 0°C in ethanol–0.7 M HCl (3:1, by volume; 1500 ml). The homogenate was stirred for 1 hr at 0°C and centrifuged (4000 ***g*** for 30 min). The methanolic and ethanolic extracts were combined, and the organic solvents were removed under reduced pressure. After further centrifugation (4000 ***g*** for 30 min), the extract was pumped onto 10 Sep-Pak $C_{18}$ cartridges (Waters Associates, Milford, MA) connected in series. Bound material was eluted with acetonitrile–water–trifluoroacetic acid (70:29.9:0.1, v/v/v) and lyophilized.

### *Purification of the peptide*

The extract was redissolved in 1% (v/v) trifluoroacetic acid–water (5 ml) and chromatographed on a 90 × 1.6-cm Sephadex G-25 column (Pharmacia, Uppsala, Sweden) equilibrated with 1 M acetic acid at a flow rate of 24 ml/hr. Absorbance was measured at 280 nm, and 2-ml fractions were collected. The fractions denoted by the bar in Fig. 1 were pooled (total volume 12 ml) and pumped at a flow rate of 2 ml/min onto a 25 × 1-cm Vydac 218TP510 ($C_{18}$) reversed-phase high-performance liquid chromatography (HPLC) column (Separations Group, Hesperia, CA) 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% over 10 min, held at this concentration for 30 min and raised to 49% over 60 min using linear gradients. Absorbance was measured at 214 and 280 nm, and fractions were collected by hand. The prominent peak designated LCRP in Fig. 2A was rechromatographed on a 0.46 × 25-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 was raised to 42% over 40 min using a linear gradient. The peptide was purified to apparent homogeneity by chromatography on a 0.46 × 25-cm Vydac 219TP54 phenyl column under the same elution condition used for the $C_4$ column.

### *Structural analysis*

The LCRP (~3 nmol) was reduced (dithiothreitol) and pyridylethylated (4-vinylpyridine) as previously described (5).

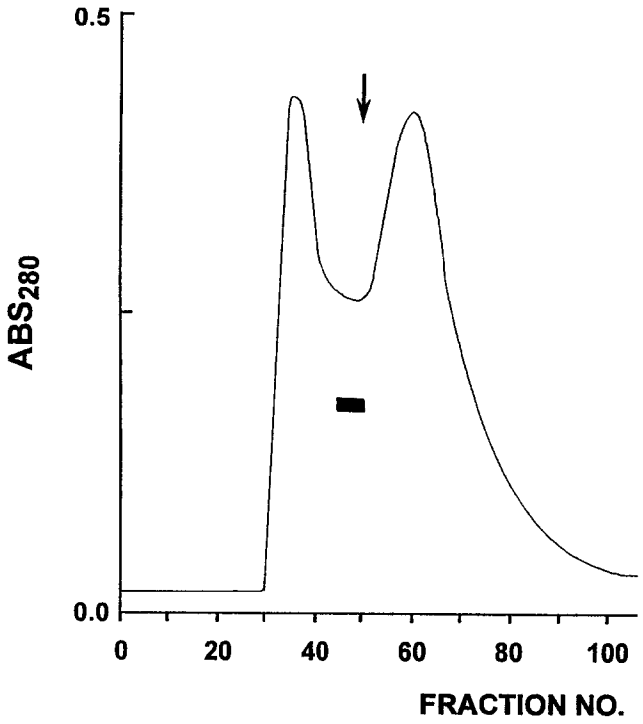


**FIG. 1. Purification by gel permeation chromatography on Sephadex G-25 of an extract of lamprey skin after partial purification on Sep-Pak cartridges. The fractions shown by the bar were pooled and subjected to further purification by reversed-phase HPLC. The arrow shows the elution volume of bradykinin.**

The derivatized peptide was purified by reverse-phase HPLC on a Vydac $C_4$ column under the conditions shown in Fig. 2B. The primary structure of the nonreduced and derivatized peptides (~2 nmol) were determined by automated Edman degradation using an Applied Biosystems (Foster City, CA) model 471A sequenator modified for online detection of phenylthiohydantoin-coupled amino acids (Pth-Xaa) under gradient elution conditions. Standard operating procedures were used, and the detection limit for Pth-Xaa was 1 pmol. Amino acid compositions were determined by precolumn derivatization with phenylisothiocyanate using an Applied Biosystems model 420A derivatizer and 130A separation system. Cysteine and tryptophan residues were not determined. The detection limit for phenylthiocarbamyl-labeled acids was 2 pmol. Hydrolysis (24 hr at 110°C in 5.7 M HCl) of ~500 pmol peptide was carried out. Mass spectrometry was performed at Novo Nordisk A/S (Bagsvaerd, Denmark) using an API III LC/MS/MS system (Sciex) as previously described (16). Approximately 100 pmol of peptide was used, and the accuracy of mass measurement was 0.02%.

## RESULTS

### *Purification of the peptide*

The elution profile on Sephadex G-25 of the tissue extract, after partial purification on Sep-Pak cartridges, is shown in Fig. 1. The fractions denoted by the bar, which had approxi-

mately the same elution volume as bradykinin, were pooled and chromatographed on a semipreparative Vydac $C_{18}$ column (Fig. 2A) The prominent peak designated LCRP was subjected to further purification. The peptide was separated from a later-eluting impurity on an analytical Vydac $C_4$ column (Fig. 2B) and purified to apparent homogeneity, as assessed by symmetrical peak shape, by chromatography on an analytical Vydac phenyl column (Fig. 2C). The final yield of pure material was ~7 nmol.

### *Structural characterization*

The primary structure of LCRP was determined by automated Edman degradation, and the results are shown in Table 1. The data indicated that the peptide contains 19 amino acid residues. During sequence analysis of the underivatized peptide, it was not possible to detect phenylthiohydantoin-coupled amino acids at cycles 1, 3, 8, 9, 17 and 18. However, after reduction of the intramolecular disulfide bridges in the molecule with dithiothreitol and derivatization of the −SH groups with 4-vinylpyridine, phenylthiohydantoin-coupled S-ethylcysteine was detected during these cycles. The results of amino acid analysis demonstrated that the underivatized peptide had the following composition (mol of residue/mol of peptide) : Asx 1.2 (1), Gly 1.9 (2), Arg 3.8 (4), Pro 1.1 (1), Tyr 1.0 (1), Val 1.9 (2), Leu 1.0 (1), Phe 0.9 (1). The values in parentheses show the number of residues predicted from the proposed structure. Agreement between the results of Edman degradation and amino acid composition analysis was good, demonstrating that the full sequence of the peptide had been obtained and that the peptide was >98% pure. The primary structure of LCRP was confirmed by electrospray mass spectrometry. The observed molecular mass of the peptide was 2200.7 ± 0.4 compared with a calculated mass of 2201.0 amu, assuming that all the cysteine residues are intramolecularly linked. There was insufficient peptide to determine the arrangement of the disulfide linkages in LCRP but by analogy with human defensin HNP-2 (21) and rat corticostatin R4 (4), the probable array of linkages is $Cys^1$-$Cys^{18}$, $Cys^3$-$Cys^9$ and $Cys^8$-$Cys^{17}$.

## DISCUSSION

This study has led to the identification of a novel 19-amino acid-residue peptide (LCRP) containing three intramolecular disulfide bridges. Isolation of the peptide was facilitated by the fact that its highly compact structure resulted in delayed elution from a Sephadex G-25 gel permeation column. The motif Cys-Xaa-Cys-$Xaa_4$-Cys-Cys-$Xaa_7$-Cys-Cys in LSP-1 was not present in any other peptide in the protein sequence database of the Protein Identification Resource (Washington, D.C.). However, the presence of several arginine residues in LCRP, in addition to six half cysteines, suggested that the peptide may be related to the mammalian

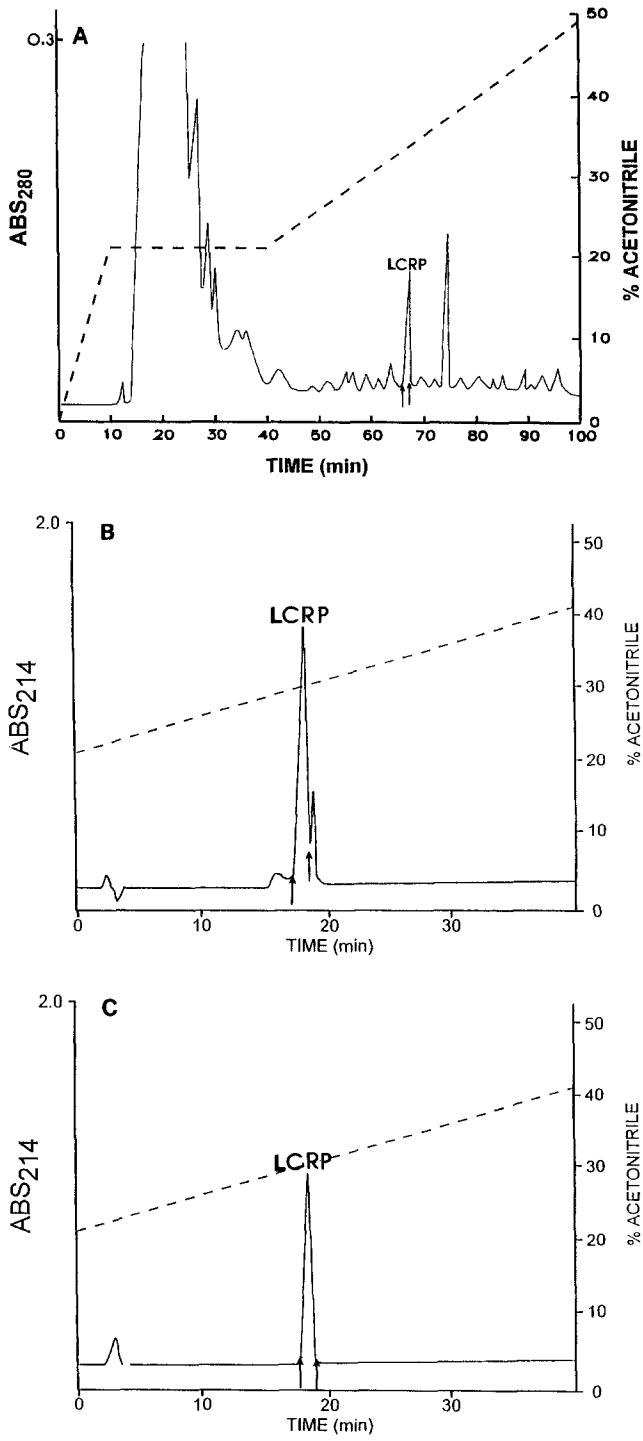


**FIG. 2. Purification by reversed-phase HPLC of LCRP on (A) Vydac $C_{18}$, (B) Vydac $C_4$ and (C) Vydac phenyl columns. The arrows show where peak collection began and ended, and the dashed line shows the concentration of acetonitrile in the eluting solvent. The peaks designated LCRP contain lamprey corticostatin-related peptide.**

**TABLE 1. Automated Edman degradation of lamprey corticostatin-related peptide**

| Cycle no. | Underivatized peptide | | Pyridylethylated peptide | |
|---|---|---|---|---|
| | Amino acid | Yield (pmol) | Amino acid | Yield (pmol) |
| 1 | ND | — | Cys | 975 |
| 2 | Pro | 939 | Pro | 558 |
| 3 | ND | — | Cys | 878 |
| 4 | Gly | 744 | Gly | 580 |
| 5 | Arg | 737 | Arg | 506 |
| 6 | Arg | 858 | Arg | 611 |
| 7 | Arg | 904 | Arg | 692 |
| 8 | ND | — | Cys | 809 |
| 9 | ND | — | Cys | 817 |
| 10 | Val | 626 | Val | 806 |
| 11 | Arg | 664 | Arg | 495 |
| 12 | Gly | 433 | Gly | 406 |
| 13 | Leu | 337 | Leu | 517 |
| 14 | Asn | 366 | Asn | 323 |
| 15 | Val | 225 | Val | 557 |
| 16 | Tyr | 211 | Tyr | 521 |
| 17 | ND | — | Cys | 333 |
| 18 | ND | — | Cys | 253 |
| 19 | Phe | 36 | Phe | 62 |

ND, no amino acid phenylthiohydantoin derivative detected.

defensins/corticostatins. The amino acid sequences of these peptides have been poorly conserved both between species and among the homologous peptides of a single species (1,13), but a comparison of the primary structure of LCRP with the structures of the known defensins shows that sequence identity is greatest with rat corticostatin R4 and rabbit corticostatin-I (Fig. 3). These peptides are the most potent of the mammalian corticostatins with respect to inhibition of ACTH-induced steroid release (4,13). Despite the fact that rabbit corticostatin-I and rat corticostatin R4 differ by 18 amino acid residues (Fig. 3); the corticostatic potency of the peptides differs by only 2-fold.

X-ray crystallography (9) and nuclear magnetic resonance spectroscopy (18,24) of several defensins have shown

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Rat        VTCTC RRTRC GFRER LSGAC GYRGR IYRLC CR

Rabbit     GI-A- --RF- PNS-- F--Y- RVN-A R-VR- -SRR

Human       A-Y- -IPA- IAG-- RY-T- I-Q-- LWAF- -

Lamprey      -P- G-R-- ***** ****- *V--L NVY*- -F
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**FIG. 3. A comparison of the amino acid sequences of rat corticostatin R4, rabbit corticostatin-I, human defensin HNP-1 and lamprey corticostatin-relate peptide LCRP. Deletions, denoted by *, are introduced into the sequence of LCRP to maximize structural similarity. – denotes residue identity.**

that the peptides contain an antiparallel $\beta$-sheet in a hairpin conformation that is stabilized by the disulfide bonds together with several loops. The mammalian defensins/corticostatins comprise 29–35 amino acid residues, and so structural similarity with LCRP is greatest if it is assumed that the region corresponding to residues (11–19), which comprises part of a loop region in human defensin HNP-1 (18), has been deleted. LCRP contains three arginine residues at the N-terminus of the peptide, and structure-activity studies have suggested that these residues are of major importance in mediating both the corticostatic and anti-microbial properties of the defensins. A binding study using rabbit corticostatin-I has indicated that the arginine residues at positions 6–8 of the peptide compete with the highly basic -$Gly^{14}$-$Lys^{15}$-$Lys^{16}$-$Arg^{17}$-$Arg^{18}$- region of ACTH for receptors on the adrenal cell (26). Similarly, the fact that the antimicrobial potency of arginine-rich defensins (rat corticostatin R4 and rabbit corticostatin-I) is 5–10-fold greater than the less cationic defensins (e.g., HNP-1) (13) supports the hypothesis that the polyarginine sequence is involved in the electrostatic binding of the defensin to the negatively charged head groups in the target cell membranes (9). Peptide LCRP also shares with corticostatin R4 the sequence Arg-Gly at the C-terminus of the molecule. These residues are part of a type I $\beta$ turn in the mammalian peptide (9).

The physiological role played by bioactive peptides in skin of Agnatha is unclear, but in the case of amphibia, there is strong evidence that they are an important component of the organism's survival strategy, protecting against ingestion by predators (2) and attack by microorganisms (6). Further studies are required to the test the microbicidal properties of LCRP against the range of bacteria and fungi that the sea lamprey is likely to encounter in its usual environment. It may be significant that the lamprey specimens were collected at a late stage in their upstream spawning migration at a time when they are particularly susceptible to fungal growth on the skin. The six half-cystine motif occurs in relatively small cardioactive peptide toxins from spiders (8), snails (10) and sea anemones (3). For example, the fish-hunting cone snail *Conus geographus* uses such toxins to paralyze its prey (10). Our preliminary data, however, do not indicate that LCRP is toxic toward fish. Intra-arterial injection of the peptide (~1 nmol) into an unanesthetized rainbow trout equipped with a pressure-sensing catheter in the dorsal aorta produced no behavioral response or change in blood pressure and heart rate (K. Olson and D. Duff, unpublished data).

Further studies must address the precise cellular localization of LCRP using an antiserum to the peptide in immunohistochemistry. The samples of lamprey skin used for extraction contained some attached muscle and so the possibility that LCRP is synthesized in macrophages and neutrophil-like granulocytes present in muscle as well as, or instead of, skin cannot be excluded. Similarly, as only female speci-

mens were used in this study, it is important to demonstrate that LCRP is also synthesized by male lampreys.

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