Silefrin, a sodefrin-like pheromone in the abdominal gland of the sword-tailed newt, *Cynops ensicauda*

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Abstract Sodefrin-like female-attracting pheromone was purified from the abdominal glands of male sword-tailed newts, *Cynops ensicauda*, by gel-filtration chromatography and reversed-phase high-performance liquid chromatography. The final product comprises 10 amino acid residues with the sequence SILSKDAQLK which coincided with the sequence deduced from its precursor cDNA. This peptide was designated silefrin. The sequence of silefrin was different from that of sodefrin by two amino acid residues, with substitutions Leu for Pro and Gln for Leu at positions 3 and 8, respectively. Both native and synthetic silefrin exerted an equipotent activity in attracting conspecific females.

Key words: Sword-tailed newt; Female-attracting pheromone; Sodefrin homologue; Silefrin; Purification of silefrin; RIA for silefrin; *Cynops ensicauda*

1. Introduction

In urodeles, chemosignals are considered to contribute to sex recognition and courtship behavior [1,2]. Recently, a female-attracting pheromone was isolated from the abdominal glands of the male red-bellied newts (*Cynops pyrrhogaster*). The final product was revealed to be a decapeptide with the amino acid sequence SIPSKDALLK and was designated sodefrin. Sodefrin, however, did not show its effect on a congeneric species of female newt, the sword-tailed newt, *Cynops ensicauda*. On the other hand, *C. ensicauda* females responded to the aqueous extract of the abdominal glands from conspecific males, indicating that these two species of genus *Cynops* possess different female-attracting pheromones [3].

More recently, by use of a partial complementary DNA (cDNA) encoding sodefrin as a probe, a cDNA encoding the sodefrin precursor molecule was cloned from a cDNA library constructed from mRNAs of *C. pyrrhogaster* abdominal glands. Furthermore, a cDNA homologous with sodefrin precursor cDNA was also obtained from a cDNA library constructed from *C. ensicauda* abdominal gland mRNAs when a sodefrin precursor cDNA was used as a probe. This cDNA was revealed to encode a sodefrin-like sequence close to the 3′ end of the coding region [4].

This prompted us to isolate the putative sodefrin-like molecule from the abdominal glands of *C. ensicauda* and to test its female-attracting activity.

2. Materials and methods

2.1. Radioimmunoassay (RIA) of sodefrin-like peptide

Sodefrin-like peptide was synthesized (American Peptide, Sunnyvale, CA, USA) according to the amino acid sequence deduced from the nucleic acid sequence of a cDNA previously cloned [4]. For radioiodination, the sodefrin-like peptide with a tyrosine extension on its N-terminus (Tyr-sodefrin-like peptide) was synthesized (American Peptide). For antibody production, a sodefrin-like peptide that had been extended on its C-terminus with a cysteine residue (American Peptide) was coupled to keyhole limpet hemocyanin (Pierce, Rockford, IL, USA) with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester. An antiserum against the sodefrin-like peptide was generated in a rabbit by means of the lymph node injection technique, as described previously [5].

The radioiodination of Tyr-sodefrin-like peptide was carried out at room temperature according to the modified lactoperoxidase method described earlier [5]. The specific radioactivity of the radioligand, calculated by the method of Greenwood et al. [6], was about 80 μCi/μg. RIA was carried out by a double antibody method. The sodefrin-like peptide antibody finally diluted 1:2000 exhibited the ability to specifically bind 41.0% of the added radioligand in the absence of any unlabeled sodefrin-like peptide, when 100 μl of diluted antiserum and 100 μl of the label (about 20 000 cpm) were added to each incubation tube containing 300 μl of 1% bovine serum albumin (BSA)–phosphate-buffered saline (PBS) (0.01 M PB containing 0.14 M NaCl, 1% BSA and 0.1% NaN_3, pH 7.5). This dilution was used throughout the experiments. The RIA was performed in disposable polystyrene tubes (5×6 mm). The reference standard and test samples were serially diluted with 1% BSA–PBS and added to each assay tube containing 200 μl of 1% BSA–PBS in 100-μl volumes. Each preparation was assayed in duplicate. The antiserum was diluted 1:400 (final dilution 1:2000) with 0.05 M EDTA–PBS (0.01 M PB containing 0.14 M NaCl, 0.1% NaN_3 and 0.05 M EDTA, pH 7.5) supplemented with 1% normal rabbit serum. One hundred μl of the diluted antiserum and 100 μl of radioligand diluted with 1% BSA–PBS were added to each tube. All assay tubes were incubated for 16 h at room temperature. After incubation, the immune complexes were precipitated by the addition of 200 μl of goat anti-rabbit IgG serum diluted 1:100 with 0.05 M EDTA–PBS containing 3.2% polyethylene glycol 6000. After incubation for 4 h at room temperature, each tube was centrifuged at 4°C for 30 min at 3500 cpm and the supernatant was aspirated. The

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Abbreviations: RIA, radioimmunoassay; HPLC, high-performance liquid chromatography; cDNA, complementary DNA; PBS, phosphate-buffered saline

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radioactivity in tubes containing the label and antiserum, but no unlabeled ligand, was designated 100% and the counts in other tubes were expressed as a fraction of this radioactivity. Statistics for linearity, parallelism, precision and potency estimation in the parallel line assay were computed according to the method of Bliss [7].

2.2. Purification and characterization of sodefrin-like molecule

The abdominal glands of the cloaca from 300 male sword-tailed newts (*C. ensicauda*) were homogenized in 300 ml of distilled water. The homogenate was centrifuged at 5000×*g* for 30 min at 4°C. The supernatant (2488 mg protein) was lyophilized and used as the starting material for purification of the sodefrin-like peptide. The starting material was processed in batches of 400 mg protein.

The lyophilized extract was dissolved in 0.15 M NH_4HCO_3 (pH 8.0). The fraction insoluble in the buffer was removed by centrifugation at 5000×*g* for 30 min and the supernatant was applied to a Sephadex G100 column (2.5×80 cm; Pharmacia, Sweden) equilibrated with the same solution. Each fraction was subjected to the RIA for the sodefrin-like peptide. Fractions containing a considerable amount of immunoreactive sodefrin-like peptide were pooled and lyophilized. The lyophilized sample obtained from gel-filtration chromatography was dissolved in 0.01% trifluoroacetic acid (TFA) and applied to an octadecyl silyl-silica cartridge (C_{18} Sep-Pak; Waters; Milford, MA, USA). The adsorbed substances were eluted with 80% methanol containing 0.01% TFA. The lyophilized C_{18}-adsorbed fraction was dissolved in 0.01% TFA and applied to an octadecyl silyl-silica column (ODS-A 120A, 5 μm, 4.6×250 mm; YMC Co., Japan) with a gradient of acetonitrile containing 0.01% TFA at a flow rate of 1 ml min^{-1}. The sodefrin-like peptide-rich fraction was lyophilized and applied to a phenyl column (Inertsil PH, 5 μm, 4×150 mm; Gas-Liquid Science Co., Tokyo, Japan) with a gradient of acetonitrile at a flow rate of 1 ml min^{-1} in the presence of 0.01% TFA. After the sodefrin-like peptide of each fraction had been measured by RIA, the appropriate fraction was lyophilized and further purified on a C_8 column (Superspher 60 RP-8, 4 μm, 4×125 mm; Cica-Merck, Tokyo, Japan) with a gradient of acetonitrile at a flow rate of 1 ml min^{-1} in the presence of 0.01% TFA. The amino acid sequence and molecular mass of native sodefrin-like peptide were determined by direct NH_2-terminal sequencing (Procise 492; Procise, CA, USA) and electrospray mass spectrometry (ESI-TOF/MS; Mariner, TX, USA), respectively.

2.3. Biological test

The biological test for female-attracting activity was performed according to the method described elsewhere [8]. Briefly, a plastic container (37 cm in diameter) was filled with 3000 ml of tap water and a female newt was put in a smaller cylinder of stainless steel mesh (15 cm in diameter) that had been placed in the center of the container. The container was divided into three sectors into which three sponge blocks with dimensions of 5.6×7.3×3.4 cm (one sponge block) were gently placed. One block contained the test substance dissolved in 100 ml water and the others contained tap water. Thirty seconds after the introduction of the sponge blocks, the inner cylinder was removed. The position of the snout of the test animal was observed, and the time spent by the snout in each sector was video-recorded for 10 min. In each series of tests, eight test females were used. The time spent by the snout of the test animals in each sector was analyzed statistically by Friedman's two-way analysis of variance, followed by the Wilcoxon matched-pairs signed-ranks test.

2.4. Immunohistochemistry

Abdominal glands were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 h at 4°C. Following cryoprotection with 20% sucrose and embedment in OCT compound (Tissue-Tek, Miles, IN, USA), the fixed tissue was snap-frozen with liquid nitrogen. Frozen sections were cut at 10 μm with a Leitz 1720 Digital cryostat (Leitz, Germany) and placed on poly-L-lysine-coated slides. The sections were treated with 50 mM NH_4Cl in PBS for 20 min to quench free aldehyde groups and then washed with PBS. They were then incubated with 20% normal goat serum for 30 min before an overnight incubation with antiserum against sodefrin-like peptide diluted 1:1000 with 1% BSA–PBS or with antiserum absorbed with sodefrin-like peptide (10 μg/ml in 1:1000 dilution). After the sections had been washed with PBS, they were incubated with rhodamine-labeled affinity-purified goat antibody against rabbit IgG (Jackson Immunoresearch, PA, USA), washed with PBS and coverslipped with Perma Fluor (Lipshaw Immunon, PA, USA).

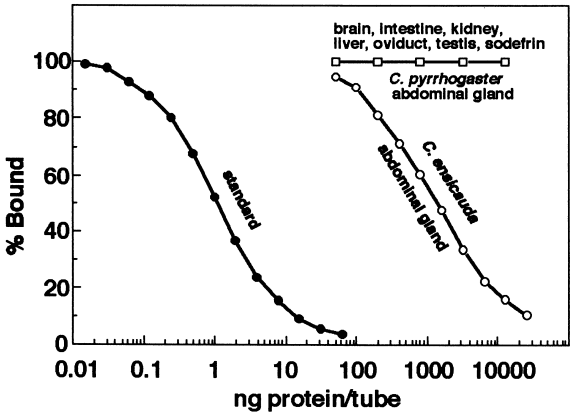

Fig. 1. Displacement of ^{125}I-sodefrin-like peptide with the aqueous extract of abdominal glands from *C. ensicauda* at various concentrations. The antiserum was used at a final dilution of 1:2000. Aqueous extracts of various *C. ensicauda* organs, as well as an extract of abdominal glands of *C. pyrrhogaster*, showed no cross-reaction. All points are averages of two determinations.

3. Results

3.1. Development of RIA for sodefrin-like peptide

The standard sodefrin-like peptide resulted in a log-dose inhibition of binding of ^{125}I-labeled Tyr-sodefrin-like peptide to its antiserum (Fig. 1). The sensitivity of the RIA was 34.3 ± 3.1 pg (mean of 10 assays ± S.E.M.) of sodefrin-like peptide standard per 100 μl assay buffer. Intraassay and interassay coefficients of variation were 2.72 and 3.83%, respectively. As shown in Fig. 1, the linear portion of the inhibition curve for the aqueous extract from the abdominal gland in the cloaca of *C. ensicauda* was parallel to the standard curve, whereas an extract from the abdominal gland of *C. pyrrhogaster* as well as synthetic sodefrin showed no cross-reactivity in this RIA system, nor did the aqueous extracts of the brain, intestine, kidney, liver, oviduct or testis prepared from *C. ensicauda*.

3.2. Purification of sodefrin-like peptide

The lyophilized extract of the abdominal glands (400 mg protein) dissolved in 0.15 M NH_4HCO_3 (pH 8.0) was fractionated by gel-filtration on a Sephadex G100 column. An aliquot of each fraction was used for the detection of sodefrin-like peptide. The sodefrin-like peptide (34.5 μg) emerged largely in fractions 92–110. These fractions were pooled and lyophilized. A small amount of immunoreactive sodefrin-like peptide (1.2 μg) was also detected in fractions 45–55. Lyophilized sample was applied to a C_{18} Sep-Pak cartridge, eluted with 80% methanol containing 0.01% TFA and lyophilized. The sample was then applied to reversed-phase high-performance liquid chromatography (HPLC) on ODS-A 120A. The immunoassayable peptide emerged in a peak fraction that eluted with a retention time of 12.40 min (Fig. 2A). This fraction was then subjected to reversed-phase HPLC on an Inertsil phenyl column. The peak fraction containing sodefrin-like peptide emerged as a peak with a retention time of 9.93 min (data not shown). This fraction was further purified by the final reversed-phase HPLC on Superspher 60 RP-8. As shown in Fig. 2B, the sodefrin-like peptide (27.5 μg of immunoassayable peptide) emerged as a single peak. Direct NH_2-terminal sequencing of this final product confirmed that it had

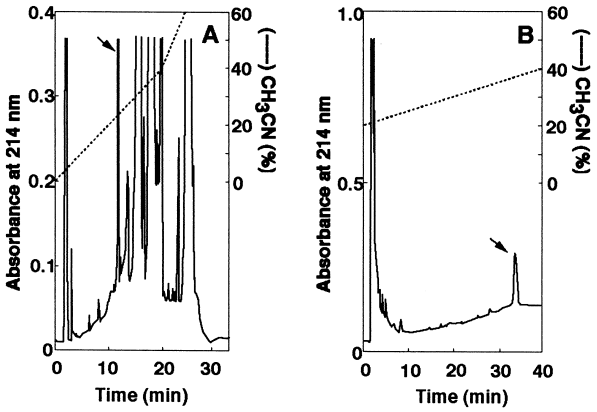

Fig. 2. Purification of sodefrin-like peptide by reversed-phase HPLC. A: The first step of purification by reversed-phase HPLC. Sodefrin-like peptide fraction obtained from the Sephadex G100 column was applied to a C_{18} Sep-Pak cartridge and eluted with 80% methanol containing 0.01% TFA. The lyophilized C_{18}-adsorbed fraction was dissolved in 0.01% TFA and applied to an ODS-A 120A (4.6×250 mm) column with a gradient of acetonitrile containing 0.01% TFA. Immunoassayable sodefrin-like peptide was detected in the peak fraction designated by the arrow. B: The final step of purification by reversed-phase HPLC. The fraction containing immunoreactive sodefrin-like peptide separated by reversed-phase HPLC on an Inertsil phenyl column was lyophilized and further purified on a Superspher 60 RP-8 (C_8) (4×125 mm) column with a gradient of acetonitrile containing 0.01% TFA. Immunoassayable sodefrin-like peptide was detected in the peak fraction designated by the arrow. Yield of the final product was 0.55 μg per gland.

the amino acid sequence of SILSKDAQLK, with a relative molecular mass of 1102.3 as estimated by electrospray mass spectrometry.

3.3. Female-attracting activity of native sodefrin-like peptide

The female-attracting activity of the native sodefrin-like peptide was tested on *C. ensicauda* females. The responses of female newts were dependent on the amount of native peptide (1–100 ng) absorbed in the sponge block. Synthetic sodefrin-like peptide also exhibited an equipotent activity, 10 ng being the minimum amount that exerted activity (Fig. 3).

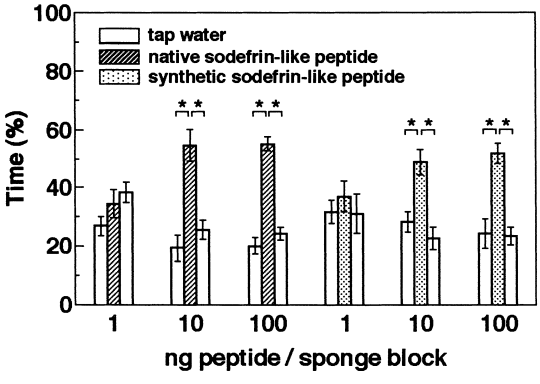

Fig. 3. Female-attracting effects of native or synthetic sodefrin-like peptide on females of *C. ensicauda*. Each sponge block contained tap water, or 1, 10 or 100 ng of native or synthetic sodefrin-like peptide. The preference tests were carried out as described in Section 2.3. Each column and vertical bar represent the mean of eight tests and S.E.M., respectively. $^*P < 0.01$.

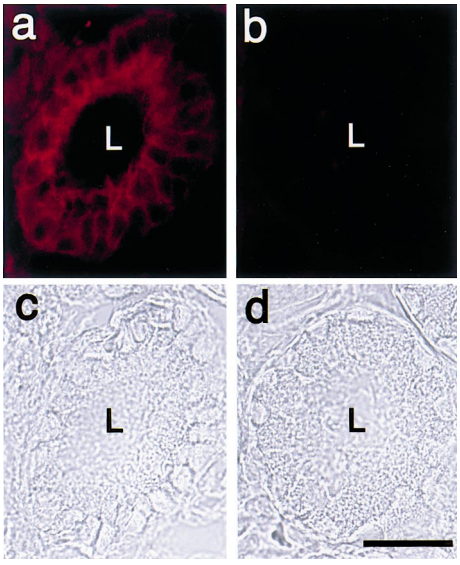

Fig. 4. Localization of sodefrin-like peptide immunoreactivity in the abdominal gland. Cryosections of abdominal gland were stained with antiserum against sodefrin-like peptide (a) or with the antiserum preadsorbed with synthetic sodefrin-like peptide (b). The antiserum stains the epithelial cells red in a punctate fashion. Panels (c) and (d) are bright-field views corresponding to sections (a) and (b), respectively, showing the morphology of the duct of the abdominal gland. L, lumen; bar, 50 μm.

3.4. Localization of sodefrin-like peptide in the abdominal gland

Localization of sodefrin-like peptide in the abdominal gland of *C. ensicauda* was examined by immunohistochemistry using its antiserum. An intense immunofluorescence was observed on the apical side of the epithelial cells in the cryosections incubated with the antiserum (Fig. 4a), whereas the antiserum preadsorbed with an excess of sodefrin-like peptide scarcely stained the epithelial cells (Fig. 4b).

4. Discussion

Since sodefrin was discovered as the first amphibian pheromone and as the first peptide pheromone in vertebrates, two other kinds of amphibian pheromones have been identified. One is a peptide pheromone, splendipherin, isolated from the skin glands of the male magnificent tree frog, *Litoria splendida*. This pheromone comprises 25 amino acids and exhibits a potent activity in attracting female partners [9]. The other is a proteinaceous pheromone (plethodontid receptivity factor) isolated from the submandibular gland of the male terrestrial salamander, *Plethodon jordani*. This 22-kDa protein increases female receptivity [10]. Thus, sodefrin-like peptide obtained in this experiment is the fourth pheromone ever identified in amphibians. The native peptide was revealed to be a variant form of sodefrin with two amino acid substitutions, Leu for Pro^3 and Gln for Leu^8 as expected from its precursor cDNA, which had been cloned previously [4]. We decided to call this peptide 'silefrin' (a combination of the first three N-terminal amino acids, SIL, and -efrin, derived from sodefrin). An aqueous extract of the abdominal glands of *C. pyrrhogaster* did not show any cross-reaction in the RIA for silefrin developed in this experiment. Likewise, the aqueous extract of abdominal glands of *C. ensicauda* showed no cross-reactivity in the RIA

system for sodefrin [5]. These facts clearly indicate that the abdominal glands of *C. pyrrhogaster* and *C. ensicauda* specifically secrete sodefrin and silefrin, respectively.

In the present experiment, silefrin was purified from fractions 92–110 having a molecular mass of less than 5000. However, the gel-filtration chromatographic profile also indicated the presence of immunoreactive silefrin in fractions 45–55 with a molecular mass of about 20 000. Judging from the value of its molecular mass, the latter seems to be a substance(s) related to the silefrin precursor. Preprosilefrin comprises 192 amino acid residues as deduced from its nucleotide sequence of cDNA [4].

The biological test revealed that the minimum effective amount of both native and synthetic silefrin absorbed in a sponge block placed in 3000 ml of water for attracting female sword-tailed newts was 10 ng. This is consistent with the value for sodefrin, the concentration neighboring the sponge block being estimated to be above 0.1 pM and below 1 pM [3]. Species specificity of silefrin and sodefrin has also been noted: silefrin attracts females of *C. ensicauda* but not those of *C. pyrrhogaster*, whereas sodefrin attracts females of *C. pyrrhogaster* but not those of *C. ensicauda* [4].

Frozen sections of abdominal gland stained with antiserum against sodefrin-like peptide by an immunofluorescence method showed that the apical region of the epithelial cells contained a greater abundance of the immunoreactive substance than the basal region of the epithelial cells, suggesting that the substance is to be discharged into the tubular lumen. We previously observed that immunoreactive sodefrin exists mainly in the apical region of the epithelium and occasionally in the lumen of the abdominal gland of *C. pyrrhogaster* [5]. Moreover, immunoelectron microscopic study revealed that sodefrin is located mainly in secretory granules [11].

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