Proteomic analysis of skin defensive factors of tree frog *Hyla simplex*

Supporting Information

Supplementary materials: detailed materials and methods

Figures S1A-N

Table S1

Supplementary materials (detail experimental methods)

Trypsin-inhibitory testing

The inhibition effects on the hydrolysis of synthetic chromogenic substrate (S-2238, H-D-Phe-Pip-Arg-pNA, Kabi Vitrum, Stockholm, Sweden) by trypsin were assayed in 50 mM Tris–HCl, pH 7.8 buffer at room temperature. The protease (final concentration 10 nM) and different amounts of the inhibitor (final concentrations ranging from 0.01 to 10 μM) were pre-incubated for 10 min at room temperature. The reaction was initiated by the addition of the substrate with a final concentration of 0.5 mM. The formation of *p*-nitroaniline was monitored continuously at 405 for 2 min. The inhibition constant K_i was determined according to the reported method [1].

Acute toxicity

The acute toxicities of peptides from the frog skin against several potential aggressors or predators were tested. All the animal experiments were reviewed and approved by the animal care and use committee of Kunming Institute of Zoology, Chinese Academy of Sciences.

(1) Insect toxicity

Laphygma exigua Hubner (n = 15, 0.25 to 0.35 g body weight (BW), 2.0-3.5 cm body length) was used in this test. The samples were dissolved in phosphate buffered saline (PBS, 0.137 M NaCl, 0.0027 M KCl, 0.010 M Na_2HPO_4, 0.002 M KH_2PO_4),

and intraperitoneally injected (i.p., 10 μl) into the worms with different concentration by a 50 μl syringe.

(2) Reptile toxicity

Enhydris plumbea (Common rice paddy snake) (n = 9, 40 to 50 g BW) was used in the test. Samples dissolved in the physiological saline (0.7% PS) were intraperitoneally injected (i.p.) into the snakes with different concentration by a 1 ml syringe.

(3) Bird toxicity

Coturnix coturnix (Common quail) (n = 10, 120-150 BW) was used in the test. Samples dissolved in 300 μl 0.9% PS were dropped into the nasal cavity with different concentration.

(4) Mammalian toxicity

Kunming mice (n = 10, 20-25 g BW) were intraperitoneally injected by different concentration of samples dissolved in 100 μl 0.9% PS.

For all the tested animals, the same volume of buffers (PBS, 0.7% PS or 0.9% PS) were used as blank control. Bovine serum albumin (BSA) with the same volume and concentration was administrated as a protein control. The tested animals were observed for 24 h after injection. Survival times were recorded and the LD50 value was calculated according to previous method [2].

Patch clamp recording on rat dorsal root ganglion neurons

Rat DRG neurons were acutely dissociated and maintained in a short-term

primary culture according to the procedures described by Xiao et al. [3]. Briefly, the rat dorsal root ganglia were removed quickly from the spinal cord, and then they were transferred into Dulbecco's modified Eagle's medium containing trypsin (0.3 g/l, type Ⅲ) and collagenase (0.7 g/l, type Ⅳ) to incubate at 34 ℃. After 22 min incubation, trypsin inhibitor (1.5 g/l, type Ⅱ-S) was added to terminate enzyme treatment. The DRG cells were transferred into 35-mm culture dishes (Corning, Sigma) containing 95% Dulbecco's modified Eagle medium, 5% newborn calf serum, hypoxanthine aminopterin thymidine supplement, and penicillin-streptomycin and then incubated in the CO_2 incubator (5% CO_2, 95% air, 37 ℃) for 1 - 4 h before the patch clamp experiment. Whole-cell patch clamp technique was used to recorded sodium currents from experimental cells. The bath solution is composed of (in mM) 35 NaCl, 2 KCl, 5 D-glucose, 1 $MgCl_2$, 1.5 $CaCl_2$, and 10 HEPES, pH 7.4; the pipette internal solution is composed of (in mM) 105 CsF, 35 NaCl, 10 HEPES, and 10 EGTA, pH 7.4. In order to allow adequate equilibration between the micropipette solution and the cell interior, the resting potential was held at -80 mV for at least 4 min after establishing the whole-cell recording configuration. Ionic currents were recorded on Aoxn 700B patch clamp amplifier (AXON, American). Linear capacitive and leakage currents were subtracted by the P/4 protocol. Experiments data were analyzed by the program of Clampfit10.0 (AXON, American) and Sigmaplot (Sigma).

Effects on isolated guinea pig ileum contraction

The effects of the tree frog skin compounds on isolated guinea pig ileum

contraction were tested according the methods previously described [4]. About 10 cm of the distal ileum of guinea pig of either sex (150–250 g body weight) was removed immediately after death and washed with Tyrode solution (137 mM NaCl, 2.7 mM KCl, 1.36 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.36 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, 5.04 mM D-glucose). The isolated ileum was cut into segments of 2 cm and mounted isotonically, under 1-g load, in a 5 ml muscle bath containing Tyrode solution maintained at 37°C and bubbled with air. Data were collected and analyzed by PcLab software package (Beijing Microsignalstar Technology Development Co. Ltd.).

HUVEC proliferation assay

HUVEC obtained from the Cell Bank of Kunming Institute of Zoology (Kunming, China). The cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 100 IU/ml of penicillin and 100 μg/ml of streptomycin in a humidified 5% CO_2 atmosphere at 37 °C. HUVEC was planted at 5×10^3 cells per well (180 μl) in 96-well plates. After attachment, Mili Q water (20 μl) was added to each well for the control, different concentrations (2, 10, 50, and 250 μg/ml) of peptide samples (20 μl) resolved in DMEM medium were added to the wells (five wells for each concentration). After 44 h incubation, 20 μl of MTT solution (5 mg/ml) was added to each well for another 4 h incubation at 37 °C. Cells were solubilized in 200 μl DMSO, and the absorbance at 570 nm was measured in a microplate spectrophotometer (Epoch, Bio-Tek Instruments, Inc., Winooski, VT, USA). The percentage of the proliferation rate was calculated for each concentration sample

group in comparison with control group according to the following formula:

Proliferation rate (%) = $(Ax - A_0) \times 100/A_0$

Where Ax and A_0 is the absorbance value at 570 nm of sample treated group and control group, respectively.

EGF or VEGF ELISA

HUVEC (2×10^5/well) was seeded in 12-well plates in DMEM medium containing 10% fetal bovine serum (FBS). After cell's adherent to the plates, the medium were replaced with 3% FBS DMEM medium containing different concentration of samples (2, 10, 50 and 250 μg/ml). After 24 h incubation, the concentration of EGF or VEGF in the supernatant was determined by a ELISA Kit (ExCell Biology Inc., Shanghai) according to the manufacture's instructions.

Western Blot Analysis

Phospho-ERK, ERK, phospho-JNK, JNK and phospho-AKT, AKT antibodies and IgG horseradish peroxidase conjugated secondary antibody were from Cell Signaling Technology (Beverly, MA, USA). β-actin mouse monoclonal antibody was purchased from Abcam (Hong Kong) Ltd.

HUVEC (2×10^5/well) was plated into 12-well culture plates in 2 ml of DMEM medium containing 10% FBS. After cell adherent, the medium was replaced by DMEM without serum for overnight incubation. Then cells were stimulated by adding different concentration (2, 10, 50 and 250 μg/ml) of samples for different time

intervals. Cells were washed twice with ice-cold phosphate-buffered saline (PBS), and then 150 μl of extraction lysis buffer (50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; 1 μg/ml each of aprotinin, leupeptin, and pepstatin; 1 mM sodium orthovanadate, and 1 mM NaF) was added to each well. After incubation for 30 min at 4°C, the cell lysate was centrifuged to remove debris, and stored at -70º C until further use. The protein concentration was determined using Bradford protein assay. Cellular protein (40 μg) from sample treated and control cell extracts was electroblotted onto a PVDF membrane following separation on a 10% SDS-polyacrylamide gel electrophoresis, respectively. The immunoblot was incubated with blocking solution (10 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 5% non-fat dry milk, and 0.1% Tween-20) at room temperature, followed by incubation overnight with a primary antibody (1:1000 dilution) for 2 h at 4°C. After three times of washing with Tween-20/Tris-buffered saline (TBST), blots were incubated with a 1:1000 dilution of horseradish peroxidase-conjugated secondary antibody for 1h at room temperature. Blots were again washed three times with TBST and then developed by ECL system (Tiangen Biotech, China).

Radiant Heat Tail-Flick Test

Antinociception was assessed using the radiant heat tail-flick test (model YLS-12A, ZhengHua, China). Kunming male mice (20-25 g) were obtained from the Experimental Animal Center, Kunming Medical University. The sites (3 cm from the

tip) at mouse tails were marked with a pen and the light beam was focused on marked sites. Baseline latency of tail-flick for each mouse was determined and designated as the baseline latency. Untreated mice showing latency 3-5 s were selected for tail-flick test. The cutoff time is not more than 10 s to prevent tail damage. Mice were randomly assigned to each group (either experimental or control, n = 9). Sample dissolved in saline was intraperitoneally administered at a dose of 2.5 mg/kg body weight. Tail-flick latency was measured at 5, 15, 30 and 60 min after sample administration. Control groups received an injection of the same volume of saline. All experiments were approved by institutional animal use and care committees of Kunming Institute Zoology, Chinese Academy of Sciences.

Antimicrobial assays

Microorganisms used in antimicrobial assays including Gram-positive bacteria *Staphylococcus aureus* (ATCC2592) and *Bacillus cereus*, Gram-negative bacteria *Escherichia coli* (ATCC25922), *Bacillus dysenteriae and Psecdomonas aeruginosa* (ATCC27835), and fungus *Candida albicans* (ATCC2002) were obtained from Kunming Medical College. According to our previous methods [5], bacteria were first grown in Luria-Bertani (LB) broth to an absorbance of 0.8 at 600 nm. A 10 μl aliquot of the bacteria was added to 8 ml fresh LB broth with 0.7% agar and poured over a 90 mm Petri dish containing 25 ml LB broth with 1.5% agar. After the top agar hardened, a 20 μl aliquot of the test sample filtered on a 0.22 μm Millipore filter was

dropped onto the surface of the top agar and completely dried before being incubated overnight at 37 °C. If the sample examined had antimicrobial activity, a clear zone would be formed on the surface of the top agar representing inhibition of bacterial growth. Minimal inhibitory concentration (MIC) was determined in liquid LB medium by incubating the bacteria in LB broth with variable amounts of the sample tested. The MIC at which no visible growth occurred was recorded.

References

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Fig. S1A

Hylaserpin S2

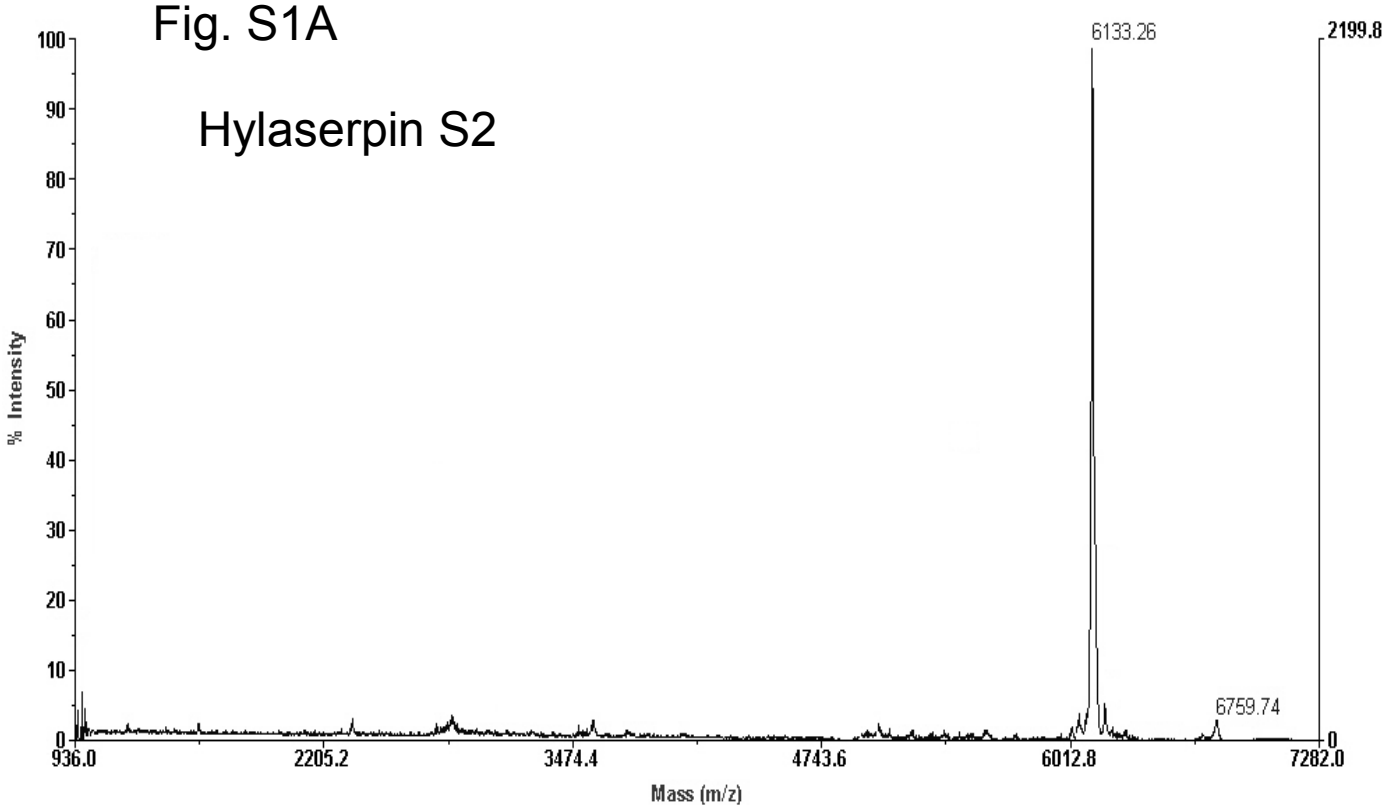

Fig. S1B

Analgesin a1

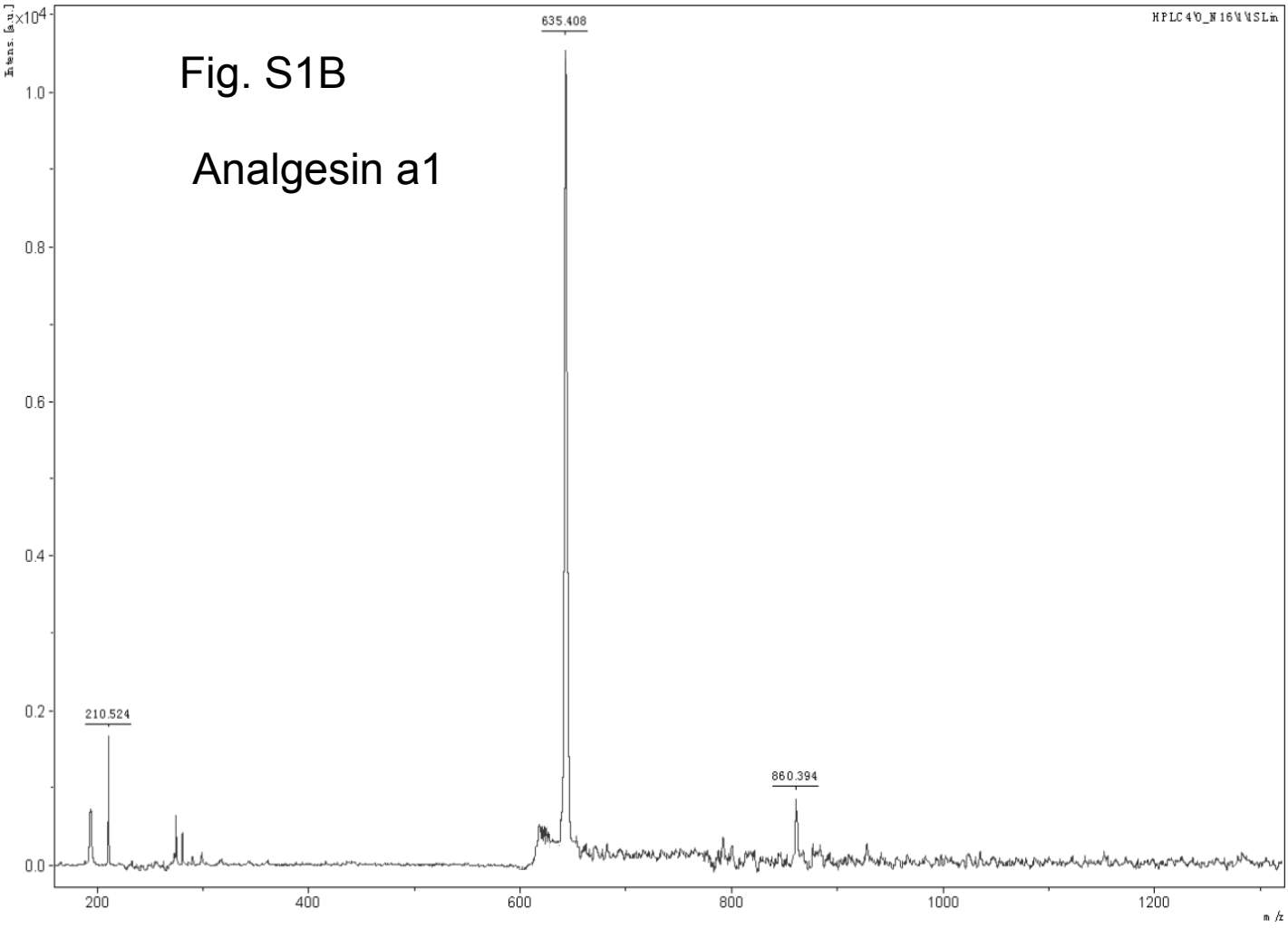

Fig. S1C

Analgesin a2

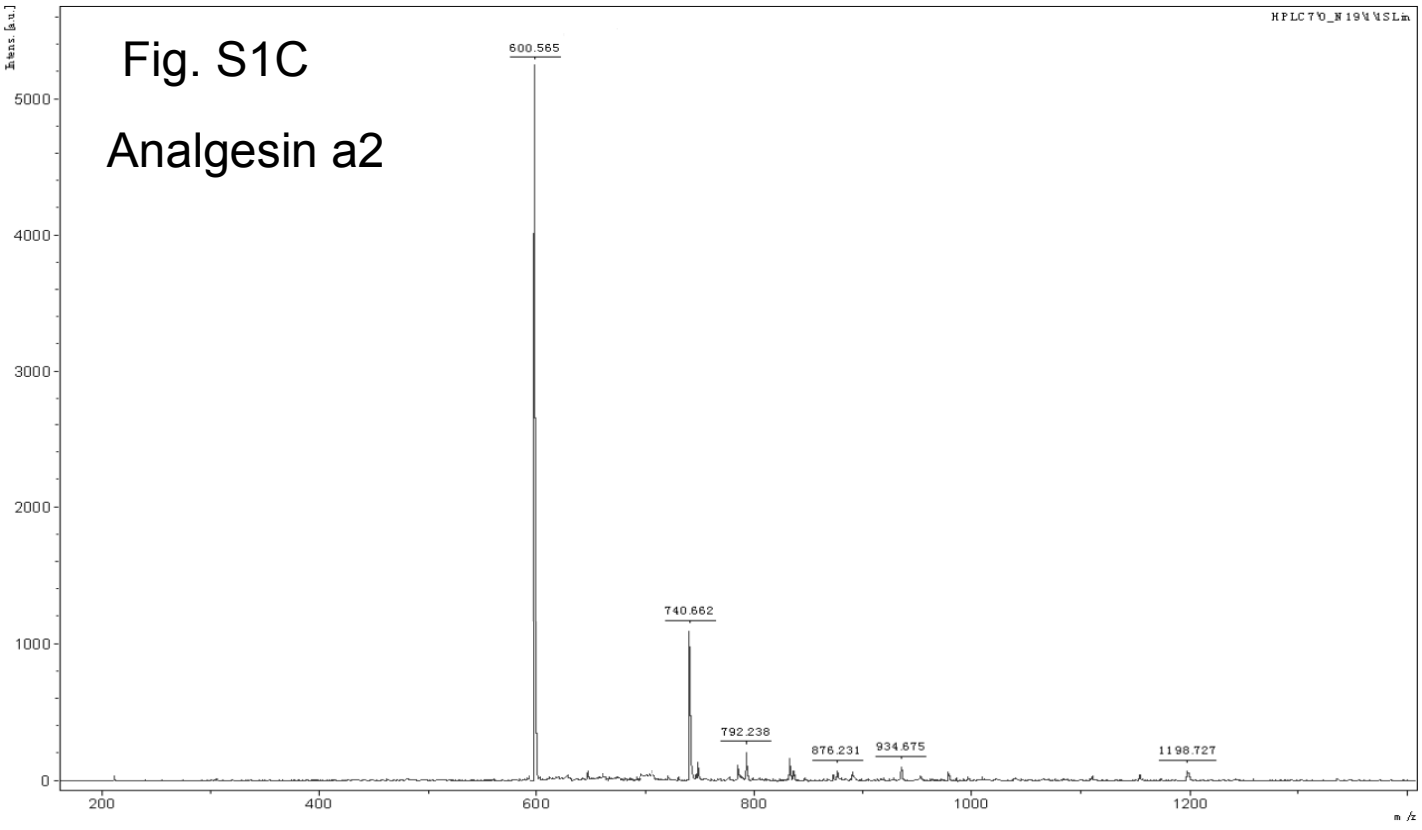

Fig. S1D

Tachykinin-H1

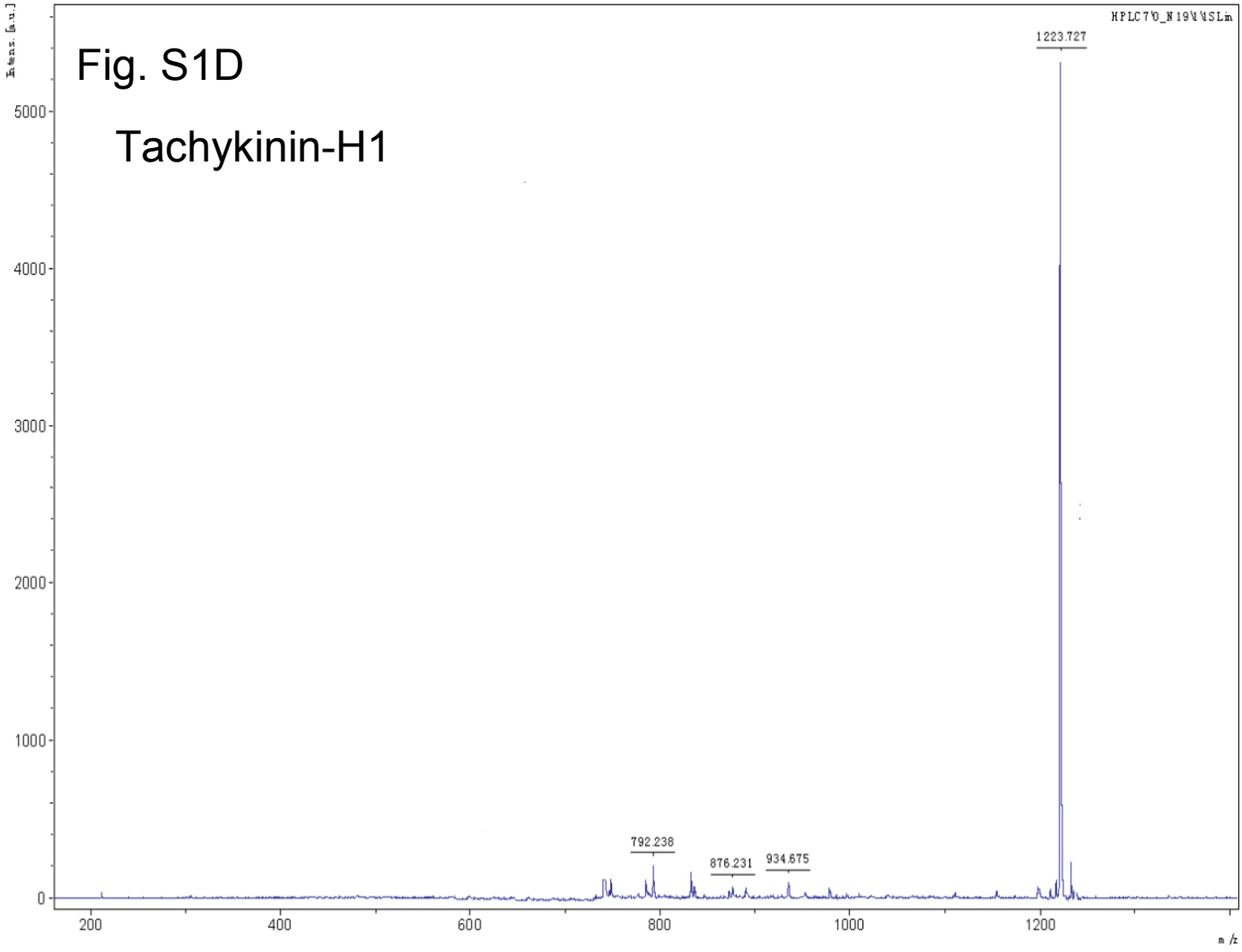

Fig. S1E

Anntoxin-S1

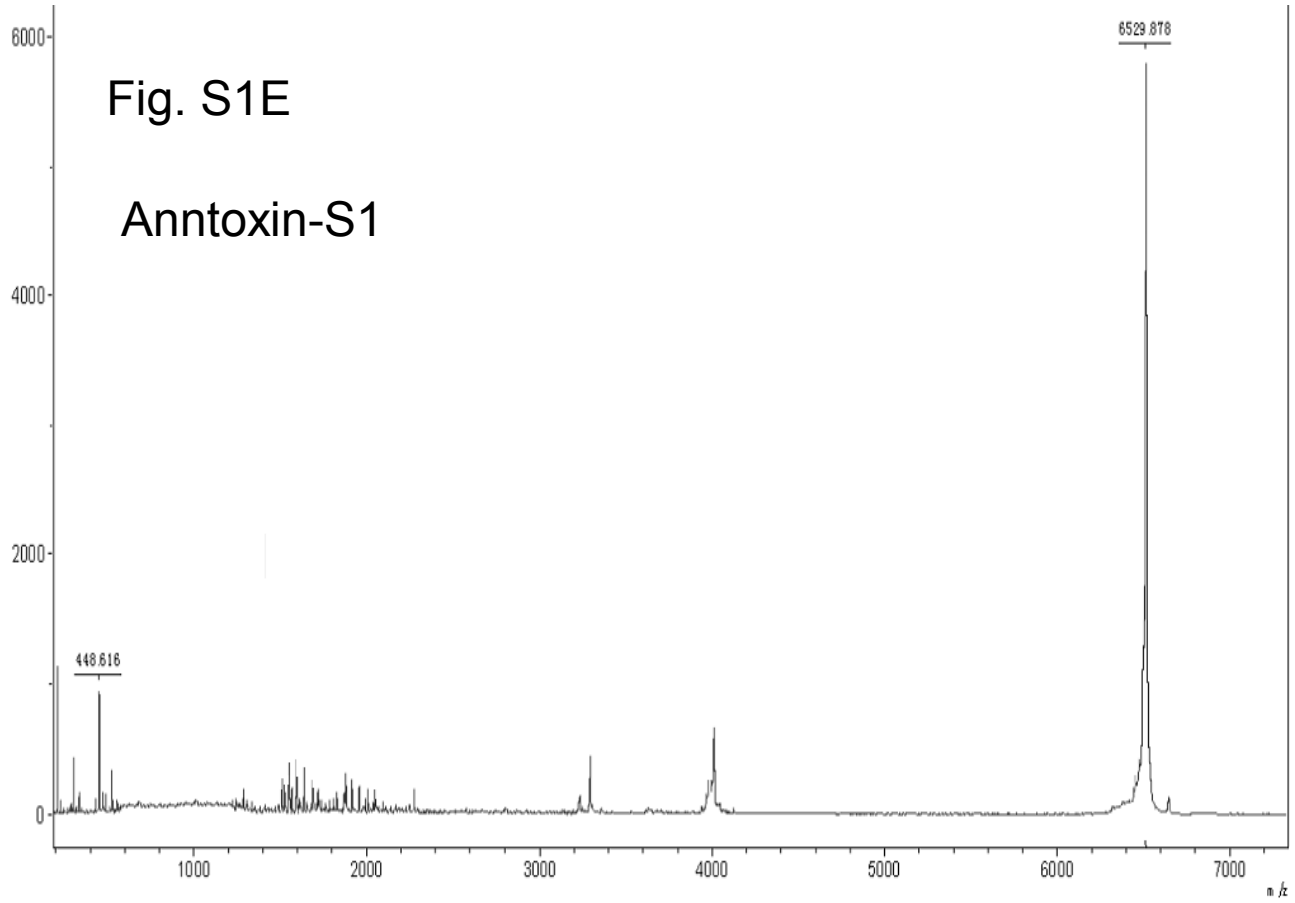

Fig. S1F

Anntoxin-S2

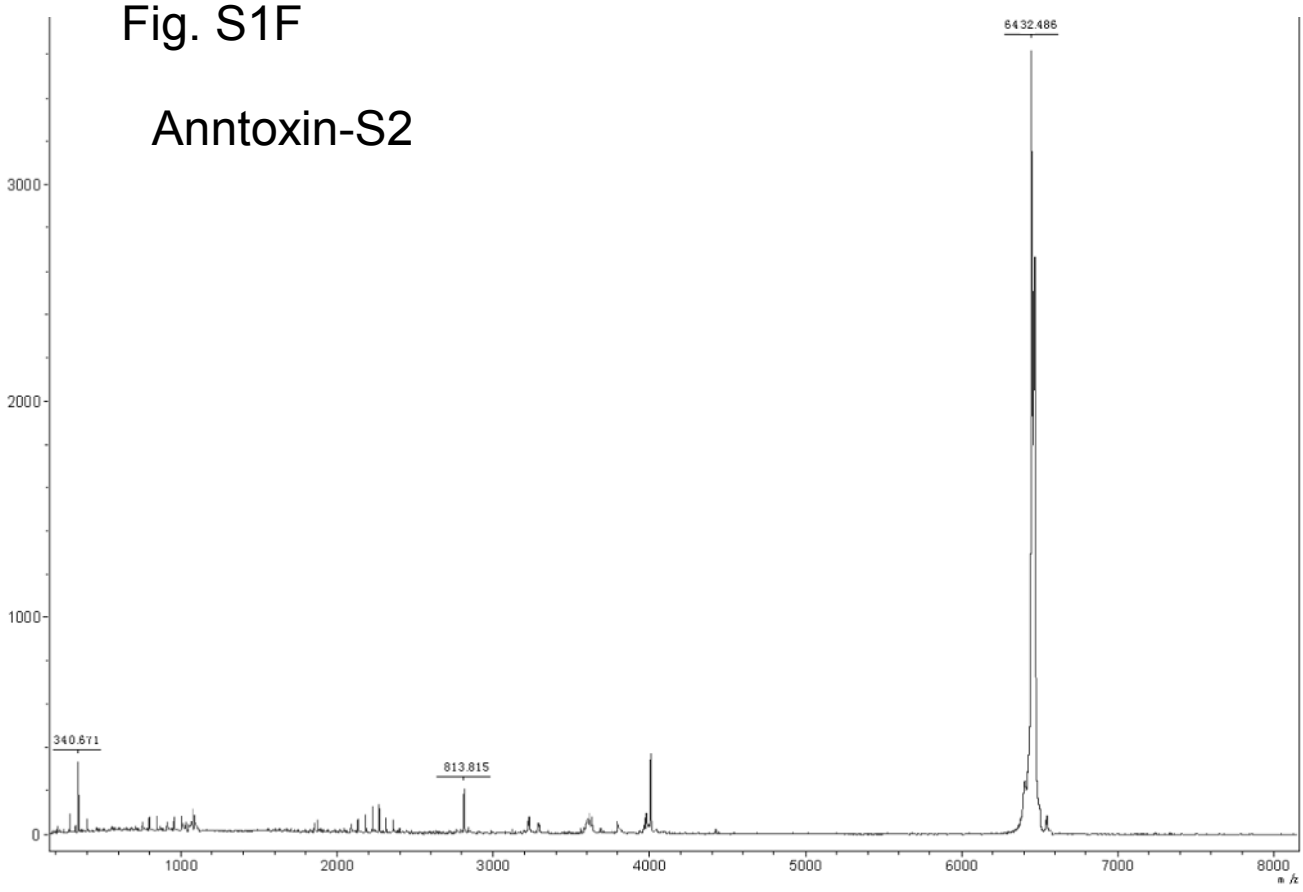

Fig. S1G

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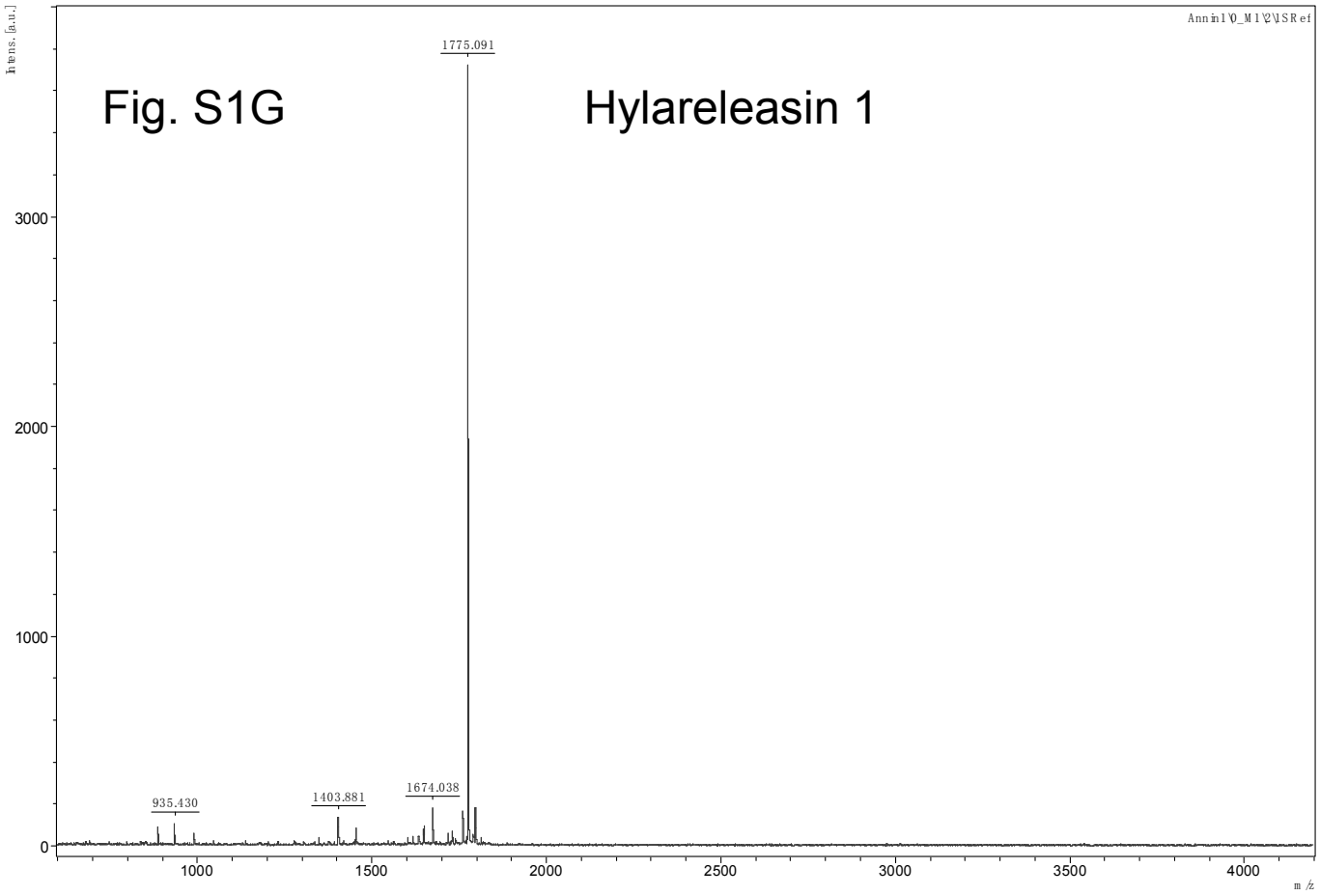


Fig. S1H

Hylareleasin 2

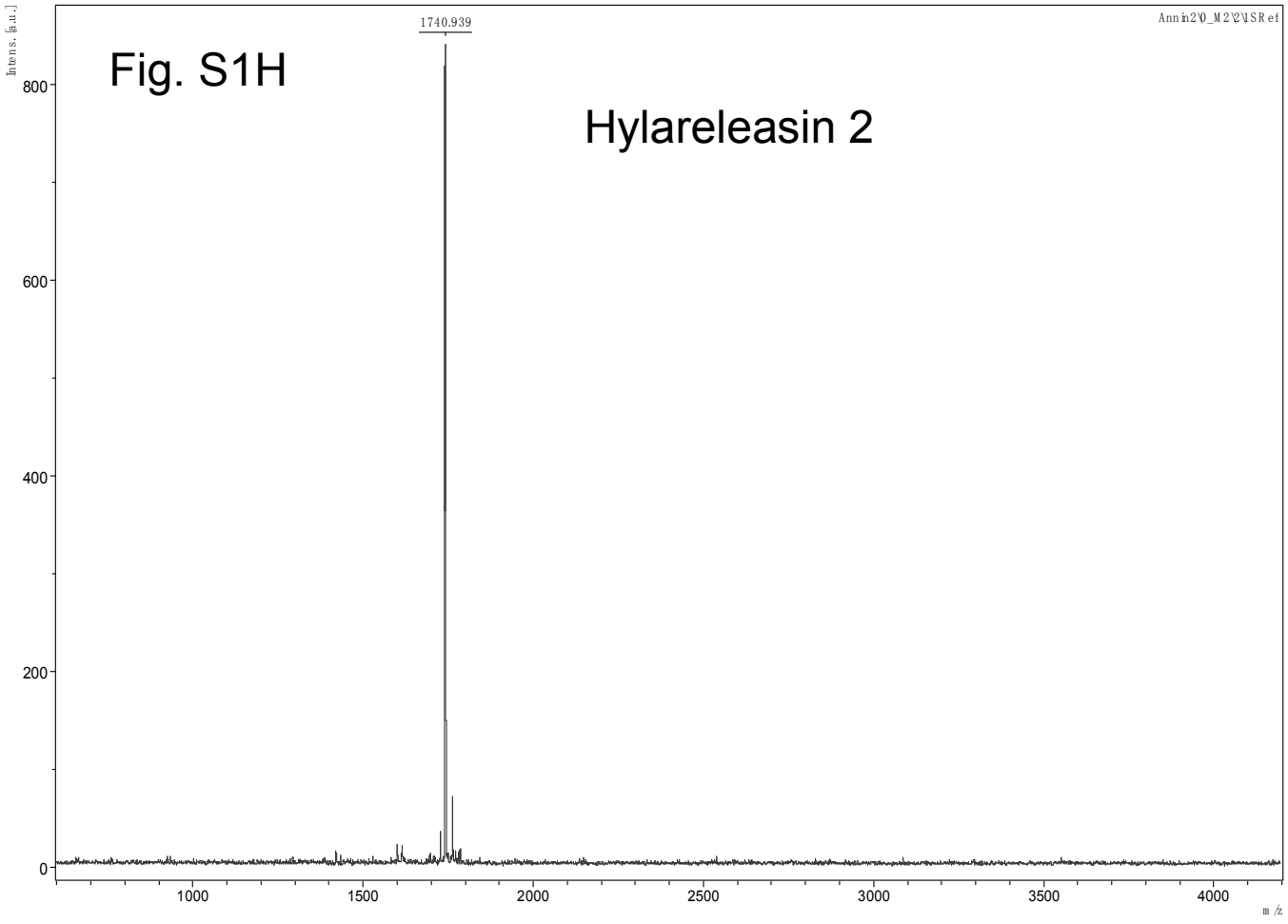


Fig. S1I

Hylareleasin 3

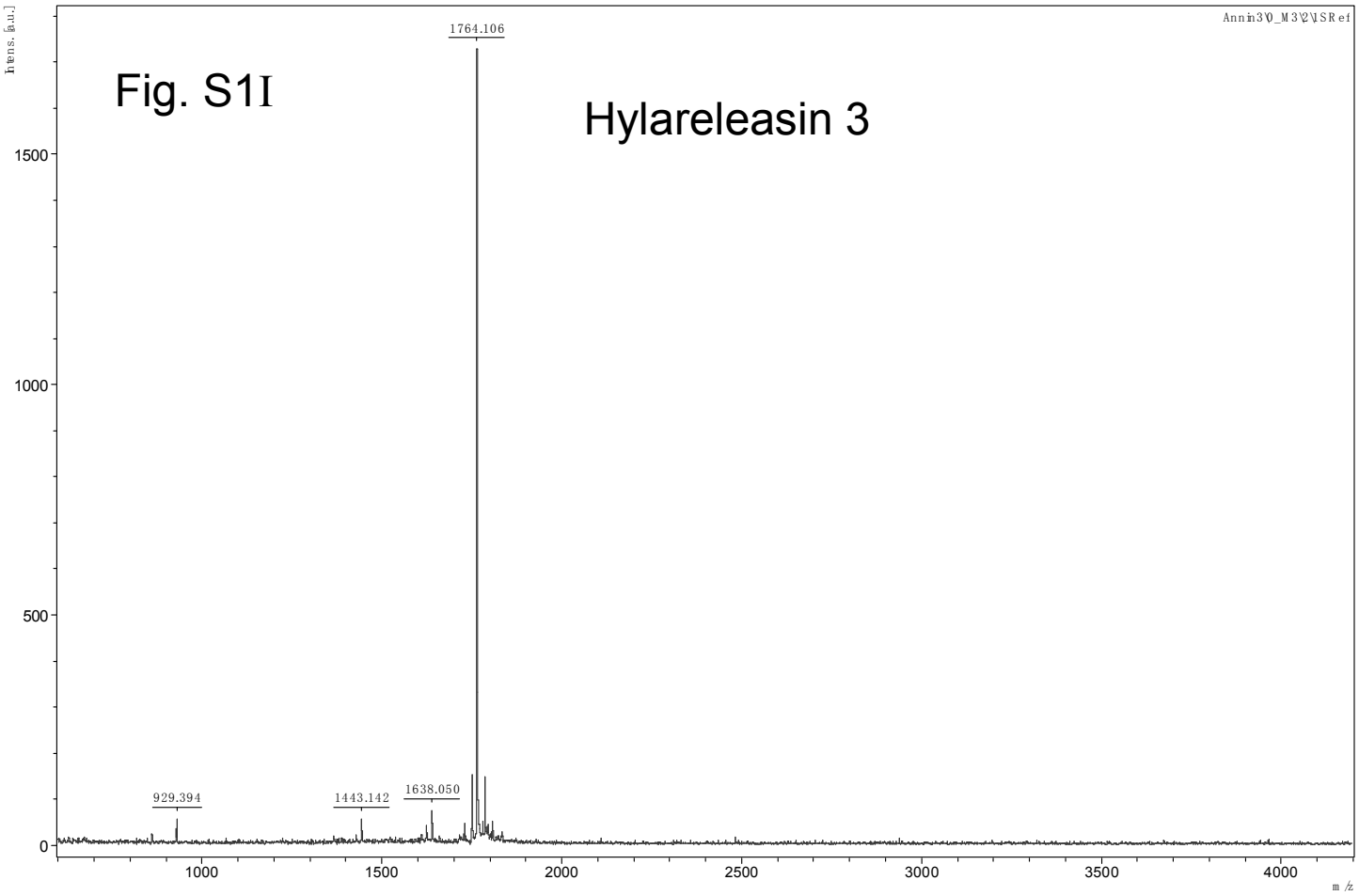
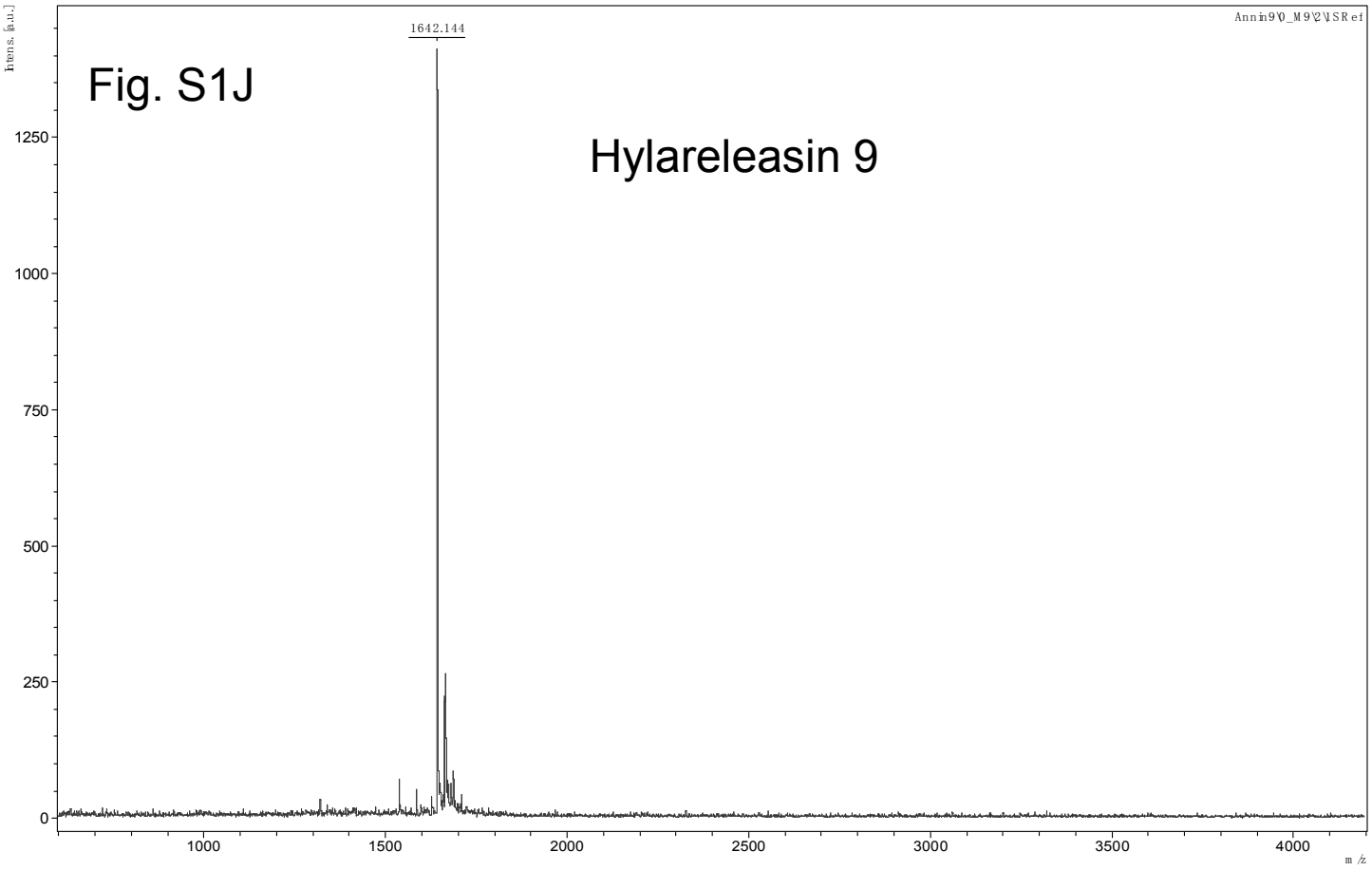


Fig. S1J

Hylareleasin 9



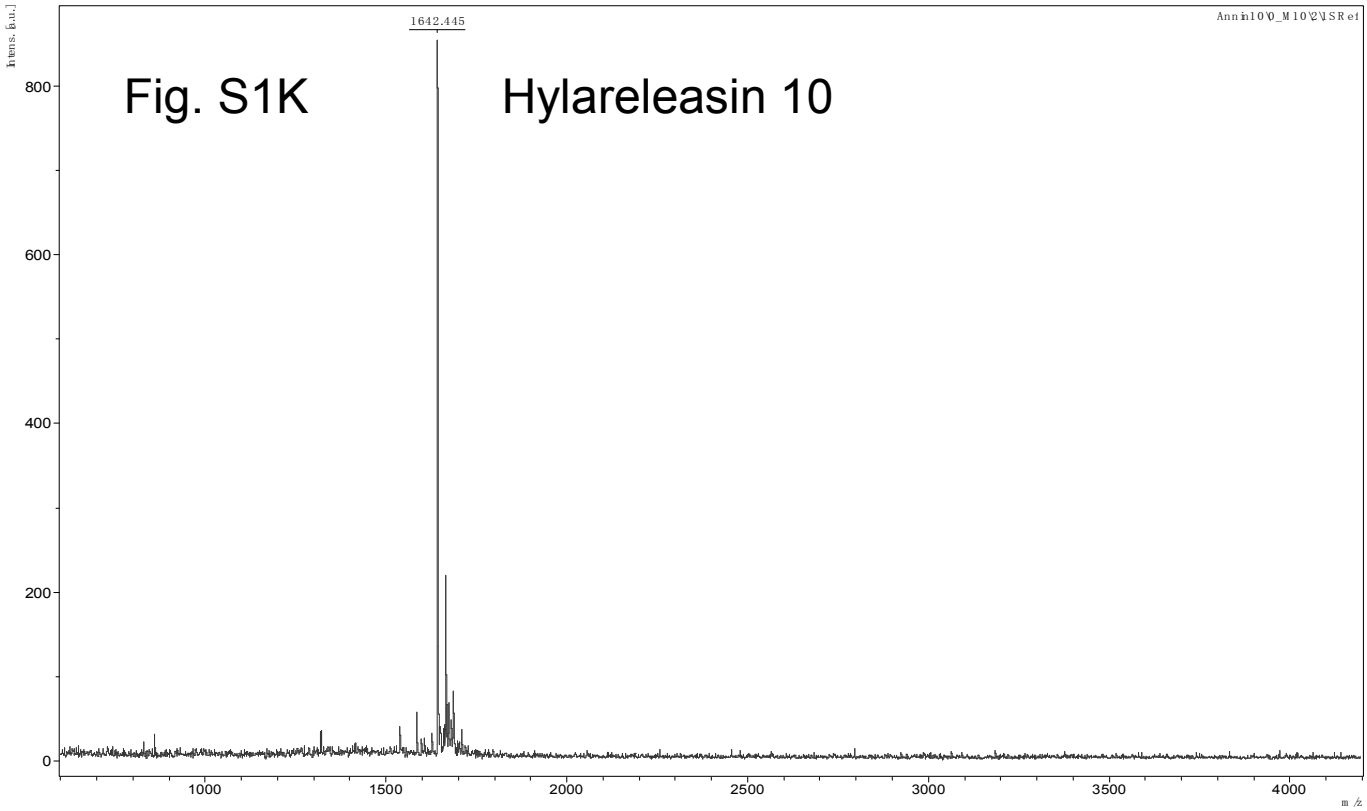


Fig. S1K
Hylareleasin 10
1642.445
Annin10\0_M10\2\1SRef
Intens. [a.u.]
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400
200
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2000
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3500
4000
m/z

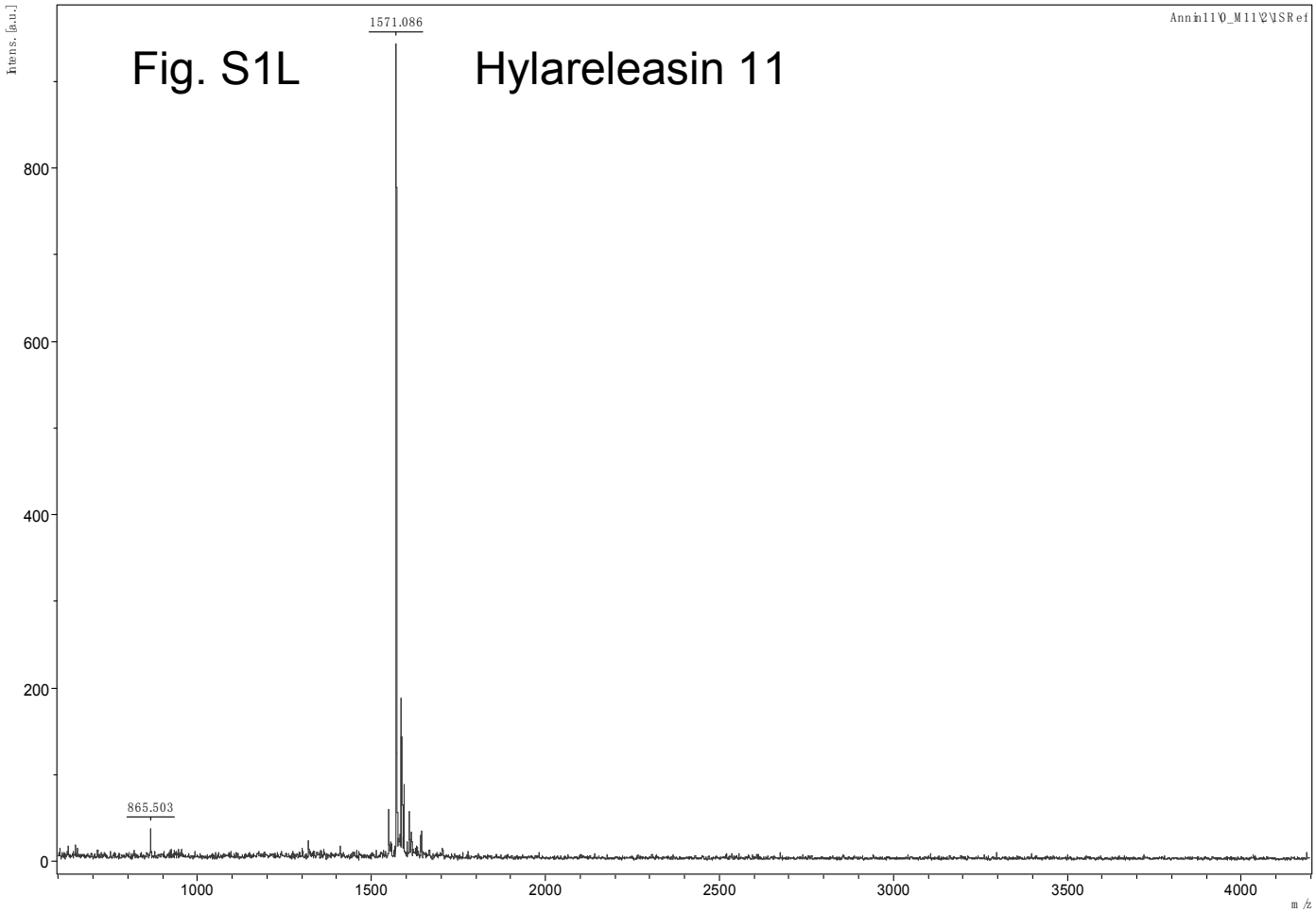
Fig. S1L
Hylareleasin 11
1571.086
865.503
Annin11\0_M11\2\1SRef
Intens. [a.u.]
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200
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2000
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3500
4000
m/z

Fig. S1M

Hylain 1

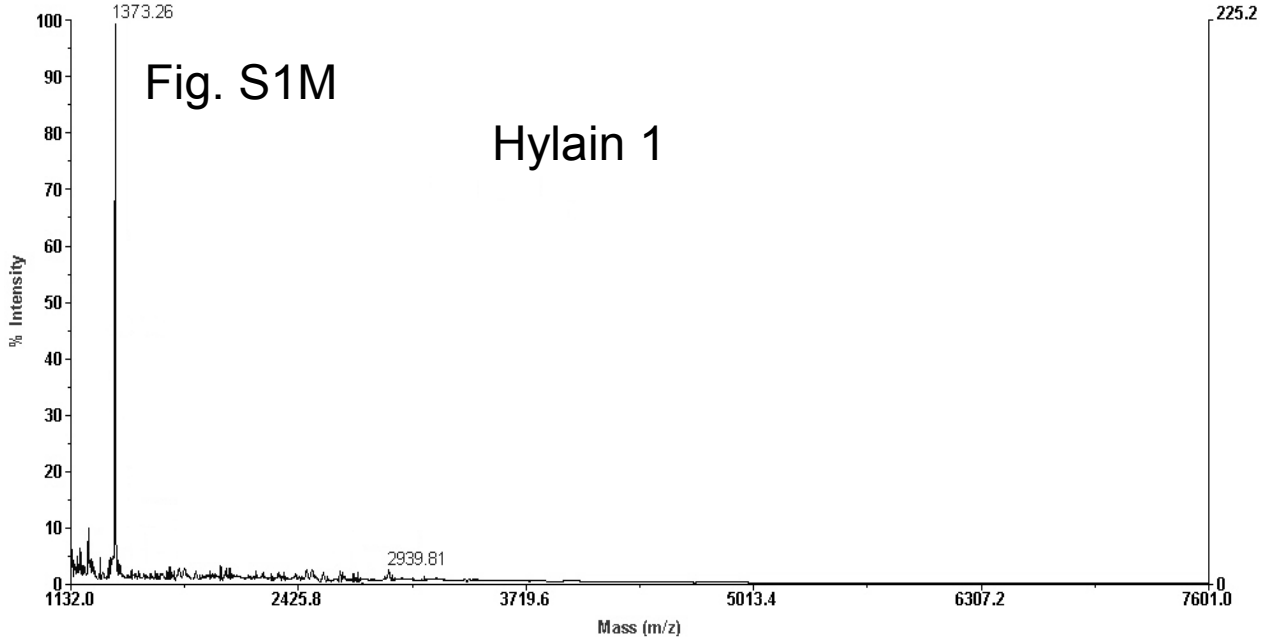

Fig. S1N

Hylain 2

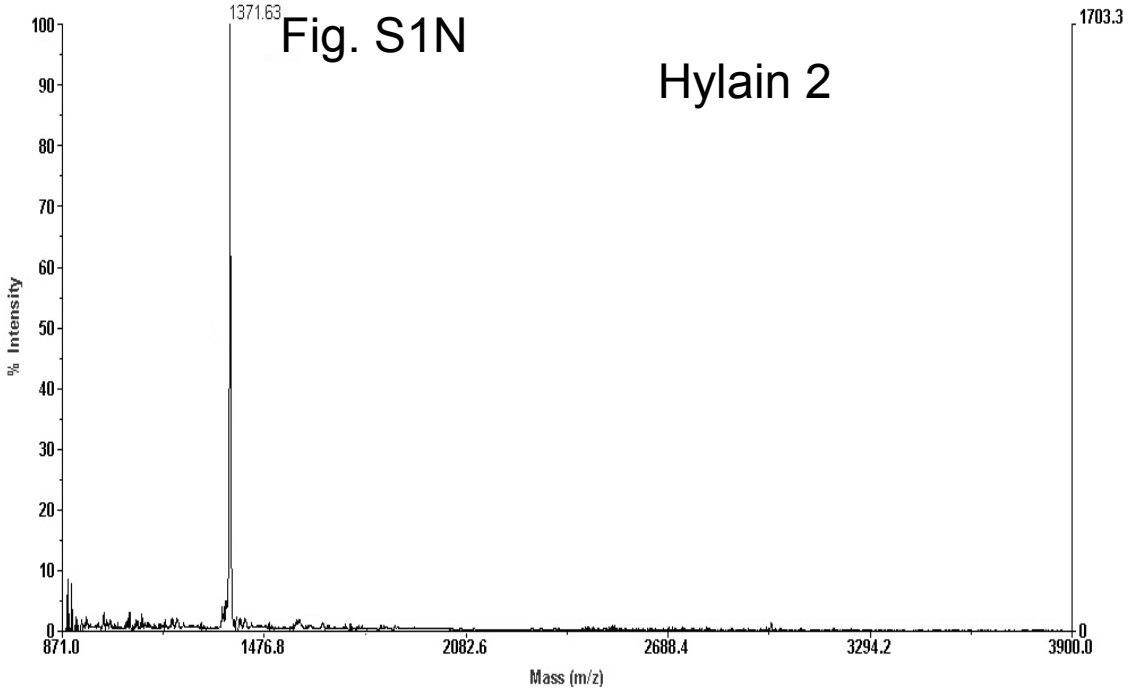

Table S1 Primers for cDNA cloning

Hylaserpin S1	5’-GG(A/T/C/G)CC(A/T/C/G)CA(T/C)GA(T/C) CA(T/C)GA(T/C)GA(T/C) CA-3’
Hylaserpin S2	5’-GA(A/G)GA(A/G)AG(C/T)TG(C/T)CC(A/T/C/G)CC(A/T/C/G)GG(A/T/C/G)G-3’
Tachykinin-H	5’- CC(A/T/C/G)CG(A/T/C/G)CC(A/T/C/G)GA(T/C)CA(A/G)TT(T/C)TA(T/C)-3’
Anntoxin-S1	5’-GC(A/T/C/G)TC(A/T/C/G)GA(T/C)TA(T/C)AG(A/G)TG(T/C)AA(T/C)TT-3’
Anntoxin-S2	5’- GC(A/T/C/G)GC(A/T/C/G)GC(A/T/C/G)GA(T/C)CA(T/C)AG(A/G)TG(T/C)G-3’
Hylareleasins	5’-GG(A/T/C/G)(C/T)T(A/T/C/G)(C/T)T(A/T/C/G)GA(T/C)CC(A/T/C/G)GT(A/T/C/G)AC(A/T/C/G)AA-3’
Hylain 1	5’-GG(A/T/C/G)AT(T/C/A)(C/T)T(A/T/C/G)GA(T/C)GC(A/T/C/G)AT(T/C/A)AA(A/G)G-3’
Hylain 2	5’-GG(A/T/C/G)AT(T/C/A)(C/T)T(A/T/C/G)GA(T/C)CC(A/T/C/G)TT(A/G)AA(A/G)G-3’