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Identification of miscellaneous peptides from the skin secretion of the European edible frog, *Pelophylax kl. Esculentus*

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Abstract

The chemical compounds synthesised and secreted from the dermal glands of amphibian have diverse bioactivities that play key roles in the hosts' innate immune system and in causing diverse pharmacological effects in predators that may ingest the defensive skin secretions. As new biotechnological methods have developed, increasing numbers of novel peptides with novel activities have been discovered from this source of natural compounds. In this study, a number of defensive skin secretion peptide sequences were obtained from the European edible frog, *P. kl. esculentus*, using a 'shotgun' cloning technique developed previously within our laboratory. Some of these sequences have been previously reported but had either obtained from other species or were isolated using different methods. Two new skin peptides are described here for the first time. Esculentin-2c and Brevinin-2Tbe belong to the Esculentin-2 and Brevinin-2 families, respectively, and both are very similar to their respective analogues but with a few amino acid differences. Further, [Asn-3, Lys-6, Phe-13] 3-14 bombesin isolated previously from the skin of the marsh frog, *Rana ridibunda*, was identified here in the skin of *P. kl. esculentus*. Studies such as this can provide a rapid elucidation of peptide and corresponding DNA sequences from unstudied species of frogs and can rapidly provide a basis for related scientific studies such as those involved in systematic or the evolution of a large diverse gene family and usage by biomedical researchers as a source of potential novel drug leads or pharmacological agents.

Key words: Amphibian; Secretion; Mass Spectrometry; Peptide; Cloning

List of Abbreviation

P.kl. Esculentus	*Pelophylax kl. Esculentus*
MS	Mass spectrometry
RACE	Rapid Amplifiction of cDNA Ends
LCQ	Liquid chromatography quadrupole
ESI	Electrospray ionization

1. Introduction

Amphibian skin glands produce complex mixtures of bioactive compounds that have been used in traditional and folk medicines around the world for centuries. In modern times, it has become apparent that amphibians, whose living environments are full of various kinds of microorganisms, have developed a unique survival strategy for protecting themselves against potential pathogens. These highly-efficient host-defence compounds that are secreted from the skins of amphibians are attracting ever-increasing scientific attention, as it seems that they may be good templates for the development of new antibiotics designed to combat the emergence of pathogens that are resistant to conventional antibiotics. As many studies have shown, amphibian skin secretions not only produce potent broad-spectrum antimicrobial peptides, but also a number of peptides that have very similar structures and biological activities to mammalian neuropeptides and hormones, such as the bombesins, bradykinins and tachykinins [1-2]. In early research, hundreds or even thousands of amphibians were sacrificed to obtain enough material for biological and chemical analyses, though as modern methods and technologies have developed, this killing has become unnecessary. A good example of this advanced practice can be found in the mild electrical stimulation method that releases the secretions of the granular skin glands without damaging the host. This technique continues to play a key role in isolating and identifying the active peptides contained in skin secretions and in preservation of endangered species [1-3].

Members of the family Ranidae are widely distributed in Europe, Asia and North America, with an estimated 250 different species producing a large number of diverse antimicrobial peptides, more than 400 of which have been isolated so far [4-5]. Compared with the antimicrobial peptides isolated from other species that often contain a C-terminal amide, the peptides from ranid frog skin secretions are normally of 10-47 amino acid residues with a 6-9-membered cyclic loop region with a single disulfide bridge, called the Rana box, at the C-terminus [2, 6]. Based on the similarities of sequences between individual peptides, these can be classified into 13 peptide families comprising: brevinin-1s, brevinin-2s, esculentin-1s, esculentin-2s, japonicin-2s, nigrocin-2s, palustrin-1s, palustrin-2s, ranacyclins, ranatuerin-1s, ranatuerin-2s, and temporins. Generally, ranid frog antimicrobial peptides are cationic and adopt amphipathic α-helical structures in order to readily bind to bacterial cell membranes through which they induce cell lysis [7].

Esculentin-related peptides are regarded as the earliest characterised family and are the largest skin antimicrobial peptides, consisting of 46 amino acid residues, first isolated from the European edible frog, *P. kl. esculentus* [8-9]. After these reports, more families of peptides were described from this species, including bradykinins, brevinins and temporins. This edible frog species, *P. kl. esculentus*, which is a hybridogenetic

hybrid between *Rana ridibunda* and *Rana lessonae*, is a complex and special species for study, which not only represents a rich source for novel peptide discovery but also represents an important model for studying amphibian evolution [10].

Here, we report the structures of several skin secretion peptides identified in *P. kl. esculentus* by use of "shotgun cloning" and LC/MS/MS fragmentation sequencing. Brevinin-1E and Brevinin-1Ra were previously reported from other closely related species of ranid, though this is the first time they have been found through 'shotgun' cloning. [Asn-3, Lys-6, Phe-13] 3-14-bombesin (NLGKQWAVGHFM) was identified by molecular mass fingerprinting of reverse phase HPLC fractions of skin secretion and its structure confirmed following LC/MS. Brevinin-2Tb and Esculentin-2b were obtained in previous studies of this species, however, some primary structural modifications in precursors were found here and this may arise through natural variation between individual specimens or discrete populations. This molecular natural selection provides a good basis for the diversity in chemical structure that may eventually lead to functional development and/or optimisation.

2. Materials and methods

2.1 Preparation of *P. kl. esculentus* skin secretion

Pelophylax kl. esculentus (n=30) obtained from a local herpetological supplier were all adults and secretion harvesting was performed in the field after which frogs were released. Gentle transdermal electrical stimulation (5V; 3ms pulses) for 30s was employed to collect skin secretions from the frog's dorsal skin. The stimulated secretions washed by deionised water from the skin were snap-frozen in liquid nitrogen and lyophilised, which was following stored at −20° C for further analysis.

2.2"Shotgun" cloning of *P. kl. esculentus* skin secretion-derived cDNA

Five milligrams of lyophilised skin secretion were dissolved in 1ml of cell lysis/mRNA protection buffer supplied by Dynal Biotec, UK. By the use of magnetic oligo-dT beads as described by the manufacturer (Dynal Biotec, UK), polyadenylated mRNA was isolated and subsequently subjected to 5′- and 3′-rapid amplification of cDNA ends (RACE) procedures to obtain full-length peptide precursor nucleic acid sequence data using a Switching Mechanism At 5' end of RNA Transcript (SMART) -RACE kit (Clontech, UK) essentially. Briefly, the 3′-RACE reactions employed four pairs of primers, OL-Signal(5′-CCCAAAGATGTTCACCTTGAAGAAA-3′)/NUP, RA-Signal(5′-ATGTTCACCATGAAGAAATC-3′)/RA-AS(5′-CTATCCCACATCAGGAGACTTTCC-3′), OL-Signal/C12-OAS(5′-GACATCTGTTGTGCATTCAGCTAA-3′) and OS-1(5′-GTTCACCATGAAGAAATCCCTGTTACT-3′)/NUP. These primers were designed to highly-conserved domains of the 5′-untranslated regions of previously

characterized peptide precursor cDNAs from ranid frogs. Based on a pGEM-T vector system (Promega Corporation), the gel purified 3′-RACE reactions were cloned and then sequenced by an ABI 3730 automated sequencer.

2.3 Identification and structural analysis of novel peptides

Five milligrams of lyophilised skin secretion were dissolved in 0.5 ml of 0.05/99.5 (v/v) trifluoroacetic acid (TFA)/water and centrifuged for clarification of microparticulate. A linear gradient formed from trifluoroacetic acid (TFA)/water; 0.1:99.9 (v/v), to trifluoroacetic acid (TFA)/water/acetonitrile; 0.1:19.9:80.0 (v/v/v) were pumped through a 1cm×25cm Jupiter 00G4052 semi-preparative C-5 reverse phase column (Phenomenex, UK) attached to a Cecil Adept Binary HPLC system (Adept Technology, Inc. USA) in 240 min at a flow rate of 1 ml/min for elution of collected supernatant. Samples (100µl) were removed from each fraction in triplicate, lyophilised and stored at −20°C prior to bioactivity assays. The fractions that exhibited specified activity were subjected to Matrix-Assisted Laser Desorption/ Ionization Time of Flight Mass Spectrometry (MALDI-TOF) MS analysis using a Perseptive Biosystems Voyager DE instrument (Framingham, MA, USA) in positive ion mode and α-cyano-4-hydroxycinnamic acid as matrix. Internal mass calibration of the instrument with peptide standards established the accuracy of mass determinations as ±0.01%.

2.4 Tandem mass spectrometry sequencing

20 µl of the diluted skin secretion fraction pumped directly onto an analytical HPLC column (Phenomenex C-18; 4.6 × 150 mm) connected to an LCQ Fleet ESI ion trap mass spectrometer (Thermo Fisher, San Jose, CA, USA) in the positive detection mode. The linear elution gradient was formed from 0.1/99.9 (v/v) trifluoroacetic acid (TFA)/water to trifluoroacetic acid (TFA)/water/acetonitrile; 0.1:19.9:80.0 (v/v/v) in 135 min at a flow rate 20 µl/min. Mass analysis was performed in a positive ion mode with acquired spectra in the range of m/z 500–2000 with N50% relative intensity during HPLC-MS. Parameters for electrospray ionization ion-trap mass spectrometry (ESI/MS) were: spray voltage +4.5 kV, drying gas temperature 320 °C, drying gas flow 200 µl/min, and maximum accumulation time – for the ion trap – 350 ms. The first mass analysis was performed in full scan mode, then peptide ions with N50% relative intensity were selected for fragmentation by collision induced dissociation (CID), to generate b and y ions that were detected in a second mass analysis. The instrument was controlled by Xcalibur software (Thermo, USA) and data analysis was performed using Proteome Discover 1.0 (Thermo, USA). Sequest™ algorithm was employed to compare the acquired fragment ion profiles with the theoretical fragment ions generated from a FASTA database.

3. Result

3.1 Molecular cloning of novel peptide precursor-encoding cDNA

Brevinin-1, Brevinin-2, Esculentin-1, Esculentin-2 and bombesin, these five different families of bioactive peptide precursors were repeatedly cloned from the cDNA library constructed from the skin secretion of *P. kl. esculentus* using the primers that were designed from previously characterised ranid frog peptide precursor cDNAs. The nucleotide of open-reading frames of the cloned precursor transcripts and its translated amino acid sequences are illustrated in Fig 1. The deduced single copies of mature peptide sequences located at the C-terminal regions were analysed using the Basic Local Alignment Search Tool (BLAST) program of the US National Centre for Biotechnology information (NCBI) on-line portal.

Brevinin-1Ra and Brevinin-1E were previously obtained from the skin secretion of the marsh frog, *Rana ridibundus*, by high-performance liquid chromatography/ tandem mass spectrometry (HPLC/MS/MS) analysis, though according to the disadvantages of MS/MS, Lys/Gln and Ile/Leu, were not resolved as they are too close or indeed identical in molecular masses. This is the first time these two precursor sequences have been confirmed using a molecular cloning method and their first identification in *P. kl. esculentus*. Brevinin-2Tbe, belonging to the Brevinin-2 subfamily, has a precursor that displays significantly structural similarity to Brevinin-2Tb (98%) and Brevinin-2Ei (94%). The mature peptide sequence of Esculentin-2c, which belongs to Esculentin-2 subfamily, displays 95% identify with Esculentin-2b, where there are only two amino acids differences among total 37 amino acids that are Lys13 and Met29 that take the place of Ala13 and Ile29 of Esculentin-2b. All mature peptide sequences obtained in this study are compared with the most similar peptides in the database in Fig 2. Esculentin-2c and Brevinin-2Tbe has become available in Genbank Nucleotide Sequence Database though the accession code KT437660 and KT437661.

3.2 Identification and structural analyses of [Asn-3, Lys-6, Phe-13] 3-14-bombesin in reverse phase HPLC fractions of *P. kl. esculentus* skin secretion

[Asn-3, Lys-6, Phe-13] 3-14-bombesin was identified in the reverse phase HPLC fractions based on its singly-charged and mono-isotopic molecular mass [M + H]1+ m/z of 1386.59 as determined by Matrix-Assisted Laser Desorption/ Ionization Time of Flight (MALDI-TOF) mass spectrometric analysis and confirmed by LCQ ESI MS full scan. The spectrum corresponding to the primary structure of [Asp-3, Lys-6, Phe-13] 3-14-bombesin (Fig 3) was produced by entrapment of the doubly-charged ion of this peptide by the ion trap of the LCQ Fleet mass spectrometer with further determined using MS/MS fragmentation.**4. Discussion**

In order to combat the increasing emergency of multiple drug-resistances in pathogenic bacteria all over world, scientists have been searching both chemical and natural product compound libraries for new lead compounds. The unique evolution of the amphibian host defence strategy not only provides a huge variety of bioactive peptides, but also their living environments and solutions to problems can supply clues for the development of possible therapeutics of medical or veterinary significance [11]. Amphibians are described as cold-blooded vertebrates covered by a skin that is rich in secretory glands [12-14] and it is these glands that manufacture, store and release the plethora of bioactive compounds. Members of the Ranidae ('true frogs') are such a good example that their skin secretions are constructed by a diverse range of bioactive compounds besides antimicrobial peptides. Generally, there are 10-20 unique peptides produced in one species which could have differences in sizes, sequences and spectrum of actions, etc., among frogs from different families, genera and species. Moreover, even members of the same species inhabiting different zones are able to create special peptides due to natural selection, such as Esculentin-2c that we report here. This phenomenon could explain why no two species have been found so far to produce the same antimicrobial peptides [2]. Such various peptides could be regarded as lead compounds as their potencies could be enhanced by chemical modification to promote the development of new drugs.

The European edible frog, *P. kl. esculentus*, is a hybrid originally produced between female *R.ridibunda* and male *R.lessonae*, whereas the lineages of *P. kl. esculentus* are maintained by mating females of P. kl. esculentus with males of *R.lessonae* [15]. Studies on the skin peptide precursor sequences of *P. kl. esculentus* by molecular cloning technology has indicated one common cDNA-encoding precursor structure, which has a highly conserved N-terminal preproregioin composed of a 22 residues long hydrophobic signal peptide, either intra- or inter-specifically, and an 16-25 residues acidic propiece that is followed by a typical prohormone processing signal Lys-Arg. Finally, a single copy of the mature peptide is encoded at the carboxyl terminus of the precursor sequence.

Compared with anti-bacterial mechanisms of conventional antibiotics that select intracellular targets and cellular processes such as DNA replication, protein and cell wall synthesis, the AMPs are able to rapidly disrupt the bacterial membranes directly with low selectivity making resistance evolution more unlikely. This fundamental mechanism of action supports AMPs as good candidates for new antibiotic drug development. Moreover, frogs from different species or subspecies produce various diverse antimicrobial peptides. Even the same species of frogs that live in different habitats or environments are capable of producing distinct repertoires of antimicrobial peptides to satisfy their special survival needs. The antimicrobial peptides modified by one or

several amino acids within their sequences could display differing sizes, net charges and hydrophobicity. Therefore, these analogues could exhibit differences in the spectrum of action and bioactivities to defend against the particular microbes that these species encounter. Esculentin-2c and Brevinin-2Tbe both have high similarity to the previously isolated antimicrobial peptides Esculentin-2b and Brevinin-2Tb, where just a few amino acid differences occur. These tiny changes inside peptide sequences, induced by either natural or artificial means, could create new or even higher potency antimicrobial peptides. For example, Brevinin-1BYa recently obtained from North American Foothill yellow-legged frogs, Rana boylii, belonging to the Brevinin-1 family, has broad-spectrum antibacterial and antifungal properties [16]. New research has discovered one portion of the residues of the full-length antimicrobial peptide sequences could also be the templates of new antibiotic development as they have potent abilities against many pathogens. Take Esculentin (1-21) for example, which is the N-terminal 1-21 region of the esculentin-1a isolated from *P. kl. esculentus*, exhibits the high antimicrobial activities against the most common mastitis-causing microbes in cattle [17,18].

Bombesin and its related homologues, which take part in the synthesis of neuropeptides and hormones and have widespread effects on the gastrointestinal tract and central nervous system' secretory functions, are widely distributed in the frog skin secretions, though its analogues obtained from *P. kl. esculentus* have been rarely reported before. [Asn-3, Lys-6, Phe-13] 3-14-bombesin, originally identified from the Marsh frog, *Rana ridibunda*, has an active core of eight amino acids at the C-terminus that is responsible for binding to receptors [19-20], and here, this peptide has been characterised in the skin secretion of *P. kl. esculentus* using an LC/MS technique. It was exciting to find another neuropeptide family, bombesin, represented in *P. kl. esculentus* skin in addition to bradykinin, that provides scientists with a better understanding of the bio-actions of the skin secretion of this species and an additional choice for neuropeptide study selection.

The skin secretions of *P. kl. esculentus* are a rich source of antimicrobial peptides that have high potency against bacteria including many pathogenic strains [16,21]. As more studies are performed on the isolation of peptides from amphibians, new peptides will be discovered and more additional bioactivities will be found that could supply great clues to improve therapeutic agents and drug development for human healthcare. Due to the development of molecular techniques, especially those that can provide comparisons of the nucleotide sequences of orthologous genes, new phylogenetic analysis of relationships between species has been made possible as an addition to the classic approach of using such aspects as the fossil record and morphological characteristics. Moreover, this improved understanding of amphibian evolutionary history should be more accurate, easier to understand and hence be more commonly accepted [22, 23].

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Conflict of Interest statement

The authors declare that they have no conflict of interest.

Ethical statement

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Data deposition footnote

The nucleotide sequence of the Brevinin-2Tbe and Esculentin-2c precursors from the skin secretion of the European edible frog, *Pelophylax kl. esculentus*, have been deposited in the EMBL Nucleotide Sequence Database under the accession code KT437661 and KT437660.

Legends to Figures

Fig 1. Nucleotide sequence and open reading frame amino acid translation of full length prepro cDNA from the skin secretion of the European edible frog Rana esculenta encoding brevinin-1E (a), brevinin-1Ra (b), brevinin-2Ec (c), Esculentin-2c (d), brevinin-Ei (e), brevinin-2Tbe (f) . The double underlined sequence is the putative signal peptide. The single underlined sequence is the sequence of the mature peptide and the stop codon is indicated by an asterisk.

Fig 2. (A) A comparison of the primary structures of the Brevinin-1 and -2 related peptides isolated from frogs of the *R.esculenta* complex with those of brevinin-1 and-2 from *R.brevipoda porsa*. (B) Comparison of the amino acid sequences of Esculentin-1 and -2 related peptides from skin secretions of *R.esculenta*. The disulphide bonds formed by the identical cysteine residues were underlined. Gaps (---) were introduced to maximise the identities. Amino acids common to all the peptides in each of the two subfamilies present in skin secretions of *R.esculenta* are in shadow.

Fig 3. (A) LCQ ESI Electrospray (LCQ) MS/MS spectrum of putative bombesin-related peptide. (B) Predicted b- and y-ion MS/MS fragment ion series (singly- and doubly- charged) of [Asn-3, Lys-6, Phe-13] 3-14-bombesin. Observed ions are indicated in black typeface.

Table 1. Skin secretion peptides isolated from *Rana esculenta*

Name	Sequence	M.W.	**Ref.**
Brevinin-1E	FLPLLAGLAANFLPKIFCKITRKC	2676	**8**
Brevinin-1Ea	FLPAIFRMAAKVVPTIICSITKKC	2649	**8**
Brevinin-1Eb	VIPFVASVAAEMQHVYCAASRKC	2480	**8**
Brevinin-1Ecb	FLPLLAGLAANFFPKIFCKITRKC	2712	**8**
Brevinin-1Ra	VIPFVASVAAEMMQHVYCAASRRC	2640	**19, 20**
Brevinin-2E	GIMDTLKNLAKTAGKGALQSLLNKASCKLSGQC	3361	**8**
Brevinin-2Ea	GILDTLKNLAISAAKGAAQGLVNKASCKLSGQC	3242	**8**
Brevinin-2Eb	GILDTLKNLAKTAGKGALQGLVKMASCKLSGQC	3316	**8**
Brevinin-2Ec	GILLDKLKNFAKTAGKGVLQSLLNTASCKLSGQC	3519	**8**
Brevinin-2Ed	GILDSLKNLAKNAGQILLNKASCKLSGQC	2999	**8**
Brevinin-2Ef	GIMDTLKNLAKTAGKGALQSLVKMASCKLSGQC	3365	**25**
Brevinin-2Eg	GIMDTLKNLAKTAGKGALQSLLNHASCKLSGQC	3371	**25**
Brevinin-2Eh	GIMDTLKNLAKTAGKGALQSLLNHASCKLSKQC	3442	**25**
Brevinin-2Ei	GILDTLKNLAKTAGKGILKSLVNTASCKLSGQC	3309	**25**
Brevinin-2Ej	GIFLDKLKNFAKGVAQSLLNKASCKLSGQC	3181	**24**
Brevinin-2Tbe	GILDTLKNLAKTAGKGALQSLLNHASCKLSGQC	3354	**-**
CPRF-Ea	GLGSILGKILNVAGKVGKTIGKVADAVGNKE	3007	**24**
CPRF-Eb	GLGSFLKNAIKIAGKVGSTIGKVADAIGNKE	3055	**24**
CPRF-Ec	GLGSFFKNAIKIAGKVGSTIGKVADAIGNKE	3091	**24**
Esculentin-1	GIFSKFGRKKIKNLLISGLKNVGKEVGMDVVRTGIDIAG CKIKGEC	4884	**8**
Esculentin-1a	GIFSKLAGKKIKNLLISGLKNVGKEVGMDVVRTGIDIA GCKIKGEC	4799	**8**
Esculentin-1b	GIFSKLAGKKLKNLLISGLKNVGKEVGMDVVRTGIDIA GCKIKGEC	4802	**8**
Esculentin-1c	GIFSKLAGKKIKNLLISGLKNIGKEVGMDVVRTGIDIAG CKIKGEC	4813	**8**

Esculentin-2a	GILSLVKGVAKLAGKGLAKEGGKFGLELIACKIAKQC	3711	**8**
Esculentin-2b	GIFSLVKGAAKLAGKGLAKEGGKFGLELIACKIAKQC	3717	**8**
Esculentin-2c	GIFSLVKGAAKLLGKGLAKEGGKFGLELMACKIAKQC	3778	**-**
Ranacyclin E	SAPRGCWTKSYPPKPCK	1904	**26**
Temporin-1Ec	FLPVIAGLLSKLF	1417	**9,24**
Peptides A1	FLPAIAGILSQLF	1388	**9,24**
Peptides B9	FLPLIAGLLGKLF	1400	**9,24**
Temproin-1Ee	FLPVIAGVLSKLF	1402	**33**
Temporin-1Re	FLPGLLAGLL-NH_2	1012	**33**
[Asp3, Lys6, Phe13]3-14-bombesin	NLGKQWAVGHFM	1386	**20, 29, 32**
kunitzin-RE	AAKIILNPKFRCKAAFC	1893	**20,27,28**
Arg^0, Trp^5, Leu^8-bradykinin	RRPPGWSPLR	1221	**29, 30, 31, 32**

Fig 1.

(a)

```
          M  F  T  M  K  K  S  M  L  L  L  F  F  L  G  T  I
    1 ATGTTCACCA TGAAGAAATC CATGTTACTC CTTTTCTTCC TTGGGACCAT
      TACAAGTGGT ACTTCTTTAG GTACAATGAG GAAAAGAAGG AACCCTGGTA
          N  L  S  L  F  E  E  E  R  D  A  D  E  E  E  R
   51 CAACTTATCT CTTTTTGAGG AAGAGAGAGA TGCCGATGAA GAAGAAAGAA
      GTTGAATAGA GAAAAACTCC TTCTCTCTCT ACGGCTACTT CTTCTTTCTT
          R  D  N  P  D  E  S  E  V  E  V  E  K  R  F  L  P
  101 GAGACAATCC AGATGAAAGT GAAGTTGAAG TGGAAAAACG ATTTCTTCCA
      CTCTGTTAGG TCTACTTTCA CTTCAACTTC ACCTTTTTGC TAAAGAAGGT
          L  L  A  G  L  A  A  N  F  L  P  K  I  F  C  K  I
  151 TTGTTGGCAG GTCTGGCTGC TAATTTCTTG CCGAAAATAT TTTGTAAAAT
      AACAACCGTC CAGACCGACG ATTAAAGAAC GGCTTTTATA AAACATTTTA
          T  R  K  C  *
  201 AACCAGAAAA TGTTGAAACT TTGGAATTGG AAATCATCTG ATGTGGAAAA
      TTGGTCTTTT ACAACTTTGA AACCTTAACC TTTAGTAGAC TACACCTTTT
  251 TCATTTAGCT AAATACACAT CAGATGTCTT ATAAAAAATA AAGATATTGC
      AGTAAATCGA TTTATGTGTA GTCTACAGAA TATTTTTTAT TTCTATAACG
  301 ATACAGAATA TAAAAAAAAA AAAAAAAAAA AAAAAAT
      TATGTCTTAT ATTTTTTTTT TTTTTTTTTT TTTTTA
```

(b)

```
          M  F  T  M  K  K  S  M  L  L  L  F  F  I  G  T  I
    1 ATGTTCACCA TGAAGAAATC CATGTTACTC CTTTTCTTTA TTGGGACCAT
      TACAAGTGGT ACTTCTTTAG GTACAATGAG GAAAAGAAAT AACCCTGGTA
          N  L  S  L  C  E  E  E  R  A  A  D  E  E  E  R
   51 CAACTTATCT CTCTGTGAGG AAGAGAGAGC TGCTGATGAG GAAGAAAGAA
      GTTGAATAGA GAGACACTCC TTCTCTCTCG ACGACTACTC CTTCTTTCTT
          R  D  D  Q  A  E  T  E  V  E  V  E  K  R  V  I  P
  101 GAGATGATCA AGCAGAAACA GAGGTTGAGG TGGAAAAACG AGTTATACCA
      CTCTACTAGT TCGTCTTTGT CTCCAACTCC ACCTTTTTGC TCAATATGGT
          F  V  A  S  V  A  A  E  M  M  Q  H  V  Y  C  A  A
  151 TTTGTGGCAA GTGTGGCTGC CGAAATGATG CAGCACGTGT ATTGTGCAGC
      AAACACCGTT CACACCGACG GCTTTACTAC GTCGTGCACA TAACACGTCG
          S  R  R  C  *
  201 TTCCAGAAGA TGTTAAATTA AATTGGAAAT CATCTGCTGT GGAAAATCAT
      AAGGTCTTCT ACAATTTAAT TTAACCTTTA GTAGACGACA CCTTTTAGTA
  251 TTAGCTAAAT GCTAAATGTC TTATAAAAAA ATAAAGTTGT TGCATACACT
      AATCGATTTA CGATTTACAG AATATTTTTT TATTTCAACA ACGTATGTGA
  301 GTTACAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAA
      CAATGTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTT
```

(c)

```
          M  F  T   M  K  K  S   L  L  L   L  F  F   L  G  T  I
    1  ATGTTCACCA TGAAGAAATC CCTGTTACTC CTTTTCTTTC TTGGGACCAT
       TACAAGTGGT ACTTCTTTAG GGACAATGAG GAAAAGAAAG AACCCTGGTA
         S  L  S   L  C  E   E  E  R  N   A  D  E   D  D  G
   51  CTCCTTATCT CTCTGTGAGG AAGAGAGAAA TGCTGATGAG GATGATGGGG
       GAGGAATAGA GAGACACTCC TTCTCTCTTT ACGACTACTC CTACTACCCC
       E  M  T  E   E  V  K   R  G  I   L  L  D  K   L  K  N
  101  AAATGACAGA GGAAGTAAAA AGAGGTATCC TCCTGGATAA GCTGAAGAAT
       TTTACTGTCT CCTTCATTTT TCTCCATAGG AGGACCTATT CGACTTCTTA
        F  A  K   T  A  G  K   G  V  L   Q  S  L   L  N  T  A
  151  TTTGCCAAGA CAGCAGGCAA AGGTGTGCTC CAGAGTCTGC TGAATACGGC
       AAACGGTTCT GTCGTCCGTT TCCACACGAG GTCTCAGACG ACTTATGCCG
         S  C  K   L  S  G   Q  C  *
  201  ATCTTGTAAA CTTTCTGGAC AATGTTAAAA CATGAATTGG AAGTCATTTG
       TAGAACATTT GAAAGACCTG TTACAATTTT GTACTTAACC TTCAGTAAAC
  251  ATGCAGAATA TCATTTAGCT AAATGCTAAA TGTCTGATAA AAAATAAAAA
       TACGTCTTAT AGTAAATCGA TTTACGATTT ACAGACTATT TTTTATTTTT
  301  GATCACACAA AAAAAAAAAA AAAAAAAAAA AAAAAAAA
       CTAGTGTGTT TTTTTTTTTT TTTTTTTTTT TTTTTTTT
```

(d)

```
                M  K  K  S  L  L   L  L   F  F  I   G  T   I
    1  GTTCACCATG AAGAAATCCC TGTTACTCCT TTTCTTTATT GGGACCATCT
       CAAGTGGTAC TTCTTTAGGG ACAATGAGGA AAAGAAATAA CCCTGGTAGA
       S  L  S  L   C  Q  E   E  R  G   A  D  G  E   E  E  G
   51  CCTTATCTCT CTGTCAGGAA GAGAGAGGCG CCGATGGAGA AGAGGAAGGG
       GGAATAGAGA GACAGTCCTT CTCTCTCCGC GGCTACCTCT TCTCCTTCCC
        E  E  M   K  R  G  I   F  S  L   V  K  G   A  A  K  L
  101  GAAGAAATGA AAAGAGGTAT TTTCTCGCTA GTCAAAGGTG CAGCCAAGCT
       CTTCTTTACT TTTCTCCATA AAAGAGCGAT CAGTTTCCAC GTCGGTTCGA
         L  G  K   G  L  A   K  E  G  G   K  F  G   L  E  L
  151  ACTGGGCAAA GGTTTGGCCA AGGAAGGGGG CAAGTTTGGG CTGGAGCTTA
       TGACCCGTTT CCAAACCGGT TCCTTCCCCC GTTCAAACCC GACCTCGAAT
       M  A  C  K   I  A  K   Q  C  *
  201  TGGCTTGTAA AATTGCAAAA CAATGTTAAA TCTTCAATTG GAGGTCATCT
       ACCGAACATT TTAACGTTTT GTTACAATTT AGAAGTTAAC CTCCAGTAGA
  251  GATGTGGAAT ATCATTTAGC AAAATGCTAA TTGTCTAATA AAAAAAATAG
       CTACACCTTA TAGTAAATCG TTTTACGATT AACAGATTAT TTTTTTTATC
  301  CAATGTCACA AAAAAAAAAA AAAAAAAAAA AAAA
       GTTACAGTGT TTTTTTTTTT TTTTTTTTTT TTTT
```

(e)

```
          M  F  T   L  K  K  S   L  L  L   F  F  F   L  G  T  I
    1   ATGTTCACCT TGAAGAAATC CCTGTTACTC TTTTTCTTTC TTGGGACCAT
        TACAAGTGGA ACTTCTTTAG GGACAATGAG AAAAAGAAAG AACCCTGGTA
          S  L  S   L  C  Q   E  E  R  N   A  D  E   D  D  G
   51   CTCCTTATCT CTCTGTCAGG AAGAGAGAAA TGCTGATGAG GACGATGGGG
        GAGGAATAGA GAGACAGTCC TTCTCTCTTT ACGACTACTC CTGCTACCCC
        E  M  T  E   E  E  K   R  G  I   L  D  T  L   K  N  L
  101   AAATGACAGA GGAAGAAAAA AGAGGTATCC TGGATACGCT GAAGAATTTA
        TTTACTGTCT CCTTCTTTTT TCTCCATAGG ACCTATGCGA CTTCTTAAAT
         A  K  T   A  G  K  G   I  L  K   S  L  V   N  T  A  S
  151   GCCAAGACAG CAGGCAAAGG TATACTGAAG AGTCTGGTGA ATACGGCATC
        CGGTTCTGTC GTCCGTTTCC ATATGACTTC TCAGACCACT TATGCCGTAG
          C  K  L   S  G  Q   C  *
  201   TTGTAAACTT TCTGGACAAT GCTAAAACAT GAATTGGAAG TCATTTGATG
        AACATTTGAA AGACCTGTTA CGATTTTGTA CTTAACCTTC AGTAAACTAC
  251   CAGCATATCA TTTAGCTAAA TACTAAATGT CTGATAAAAA ATAAAAATAT
        GTCGTATAGT AAATCGATTT ATGATTTACA GACTATTTTT TATTTTTATA
  301   CACATGAAAA AAAAAAAAAA AAAAAAAAAA AAAA
        GTGTACTTTT TTTTTTTTTT TTTTTTTTTT TTTT
```

(f)

```
          M  F  T   L  K  K  S   L  L  L   F  F  F   L  G  T  I
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        TACAAGTGGA ACTTCTTTAG GGACAATGAG AAAAAGAAAG AACCCTGGTA
          S  L  S   L  C  Q   E  E  R  N   A  D  E   D  D  G
   51   CTCCTTATCT CTCTGTCAGG AAGAGAGAAA TGCTGATGAG GACGATGGGG
        GAGGAATAGA GAGACAGTCC TTCTCTCTTT ACGACTACTC CTGCTACCCC
        E  M  T  E   E  E  K   R  G  I   L  D  T  L   K  N  L
  101   AAATGACAGA GGAAGAAAAA AGAGGTATCC TGGATACGCT GAAGAATTTA
        TTTACTGTCT CCTTCTTTTT TCTCCATAGG ACCTATGCGA CTTCTTAAAT
         A  K  T   A  G  K  G   A  L  Q   S  L  L   N  H  A  S
  151   GCCAAGACAG CAGGCAAAGG TGCGCTCCAG AGTCTGCTGA ATCATGCATC
        CGGTTCTGTC GTCCGTTTCC ACGCGAGGTC TCAGACGACT TAGTACGTAG
          C  K  L   S  G  Q   C  *
  201   TTGTAAACTT TCTGGACAAT GTTAAAACAT GAATTGGAAG TCATTTGATG
        AACATTTGAA AGACCTGTTA CAATTTTGTA CTTAACCTTC AGTAAACTAC
  251   CAGAATATCA TTTAGCTAAA TACTAAATGT CTGATAAAAA ATAAATAGAT
        GTCTTATAGT AAATCGATTT ATGATTTACA GACTATTTTT TATTTATCTA
  301   CAC
        GTG
```
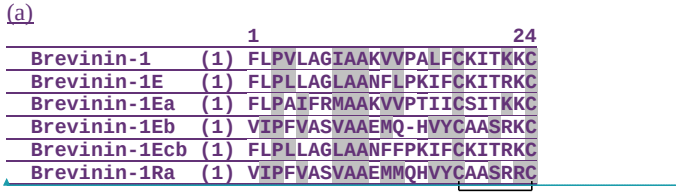
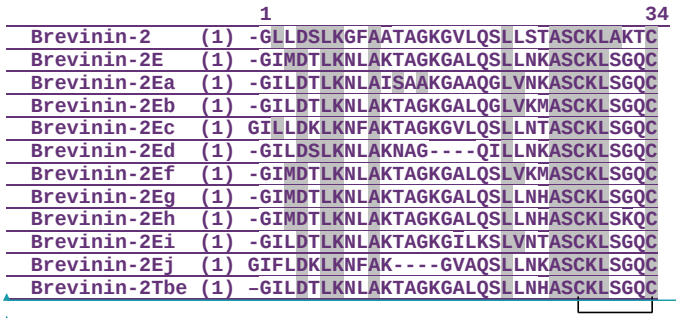

Fig 2.

(a)





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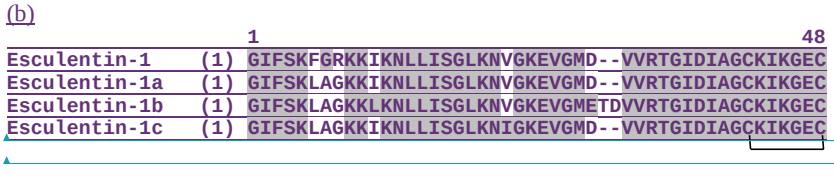

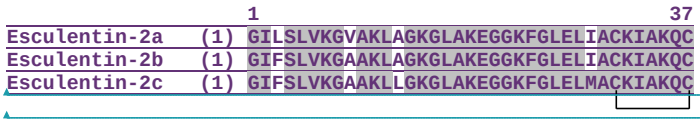

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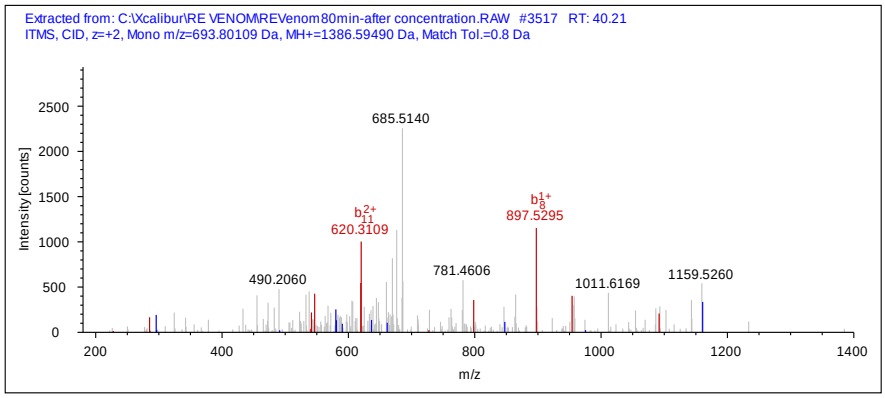
Fig 3.



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