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Antibacterial and haemolytic peptides containing D-alloisoleucine from the skin of *Bombina variegata*

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A family of bombinin-related peptides is present in the skin of *Bombina variegata*. These peptides contain 27 residues with Gly as N-terminus and display antimicrobial activity. From sequence analysis of the cDNAs encoding for the corresponding peptide precursors, the presence of a novel 20-residue peptide with Ile as N-terminus was predicted. We have now purified a family of hydrophobic peptides named H1–H5, whose sequences correspond to the predicted peptide with some variability in positions 1, 2 and 8. In particular, H3–H5 contain a D-alloisoleucine residue in the second position. All these peptides display antibacterial and haemolytic activity.

Key words: D-alloisoleucine/D-amino acids/amphibian skin/antibacterial peptides/*Bombina variegata*/haemolysis

Introduction

It has been known for more than 20 years that the skin secretion of the European frog *Bombina variegata* possesses antibacterial and haemolytic activities. These were originally ascribed to a 24-residue peptide termed bombinin (Csordas and Michl, 1970). More recently, a peptide fraction was isolated from methanolic skin extracts of this frog that showed antibacterial but no haemolytic properties (Simmaco *et al.*, 1991). This fraction contained several peptides related to bombinin, all consisting of 27 amino acids, with different N-terminal and identical C-terminal sequences. A cDNA library prepared from skin of *B.variegata* was screened with synthetic oligonucleotides derived from the constant C-terminal region. Several clones were isolated which coded for the precursors of these different bombinin-like peptides (Simmaco *et al.*, 1991). From the sequence of the precursors, another peptide could be predicted with the N-terminal sequence Ile-Ile-Gly... and, depending on the processing reactions, the C-terminal sequence Leu-Leu or Leu-Leu-Lys-Lys-Ile-amide (see Figure 1). Similar results were obtained by analysing the skin secretion and cloned skin cDNAs of a related species, *B.orientalis* (Gibson *et al.*, 1991).

Here we report the isolation and characterization of several peptides closely related to the longer form of the peptide predicted from the sequence of the cloned cDNAs. The variants differ from each other by the presence of isoleucine or leucine in the first, leucine or methionine in the eighth, and, surprisingly, isoleucine or D-alloisoleucine in the second position. For these peptides we have chosen the name bombinin H as they are very hydrophobic and possess haemolytic activity.

Results

As already mentioned, from the sequence of the cloned cDNAs the existence of an additional peptide with the N-terminal sequence Ile-Ile-Gly... could be predicted (see Figure 1). Depending on whether processing of the precursor polypeptide at the pair of lysine residues takes place or not, this peptide should terminate either with leucine or with isoleucine-amide. These two peptides (A and B in Figure 1) were synthesized and used as markers to search for their natural counterparts. As expected, upon reverse-phase HPLC, these hydrophobic peptides elute late from the column. The elution conditions were optimized to improve the resolution of these peptides. The best results were obtained when isopropanol was also present in solvent B and a rather shallow gradient was chosen.

When crude skin secretion from *B.variegata* was fractionated under the same conditions, several peptides, termed H1–H5 (see Figure 2) were detected eluting in the same region as the synthetic peptide B. Amino acid analysis and automated Edman degradation demonstrated that these are different variants of the peptide B predicted from the sequence of the cloned cDNAs. Moreover, they all had a net charge of +3, determined by electrophoretic mobility, indicating that the COOH-end was amidated. Differences between the bombinins H1–H5 were observed at positions 1, 2 and 8 of the sequence (see Table I). The N-terminal residue was found to be isoleucine in bombinins H1 and H5, while in the other peptides both isoleucine and leucine were detected. At position 8 of bombinins H1 and H3, instead of leucine, as predicted from the cloned cDNAs, methionine was detected.

Amino acid analysis of the peptides H3, H4 and H5 yielded an unexpected result. Besides isoleucine, alloisoleucine (aIle) was detected in amounts corresponding to up to 0.8 mol per mol of peptide. This is far in excess of the trace amounts of aIle usually present in protein hydrolysates. As is well known, aIle can be readily separated from isoleucine upon ion-exchange chromatography.

Next, bombinin H3 which contained methionine in position 8 was cleaved with cyanogen bromide and the resulting fragments were separated by HPLC. Amino acid analysis of the two fragments clearly demonstrated that the alloisoleucine was present in the N-terminal part (data not shown). Bombinins H3 and H4 were then subjected to manual Edman degradation and after each step an aliquot was used for amino acid analysis. After the first step of degradation, the amino acid analysis of the two bombinins gave 0.8–0.9 mol of aIle and 1.0–1.1 mol of Ile, respectively, per mol of peptide. After the second step, 1.0

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Peptide A Ile-Ile-Gly-Pro-Val-Leu-Gly-Leu-Val-Gly-Ser-Ala-Leu-Gly-Gly-Leu-Leu

Peptide B Ile-Ile-Gly-Pro-Val-Leu-Gly-Leu-Val-Gly-Ser-Ala-Leu-Gly-Gly-Leu-Leu-Lys-Lys-Ile-NH_2

Fig. 1. Peptides predicted from the sequence of cDNAs encoding bombinin precursors (Simmaco *et al.*, 1991). Depending on the processing of these precursors in dermal glands, two peptides with different C-terminal sequences could be formed.

mol of Ile was detected per mol of both peptides, while aIle was absent. This experiment demonstrated that in both peptides aIle was present in the second position.

In view of the fact that opioid peptides from the skin of frogs belonging to the sub-family Phyllomedusinae contain a D-amino acid in position 2 (see Discussion), we decided to test whether these bombinins H contained L- or D-alloisoleucine. After total acid hydrolysis, a chiral amino acid analysis was performed as described previously (Scaloni *et al.*, 1991). Under these conditions, D-isoleucine and D-alloisoleucine co-elute, but they are well resolved from the corresponding L-isomers. This analysis demonstrated that in bombinins H3, H4 and H5, 0.8–0.9 mol of the D-isomer were present per mol of peptide. Chiral Edman degradation (Scaloni *et al.*, 1991) on bombinins H3 and H4 also demonstrated the presence of D-isoleucine or D-alloisoleucine in the second position (data not shown). The whole of these results clearly shows that these peptides contain one residue of D-alloisoleucine at position 2. Neither alloisoleucine nor a D-amino acid could be detected in the first two peaks corresponding to bombinins H1 and H2. This is in line with earlier observations with dermorphins and deltorphins (see Discussion) where it was found that the peptides containing one D-amino acid always eluted somewhat later upon HPLC than the corresponding L-isomers. Earlier experiments with these opioid peptides had also shown that those containing a D-amino acid as the second residue are not digested by aminopeptidases. As expected, bombinins H3–H5 were completely stable under conditions where bombinins H1 and H2 were partly digested by aminopeptidase M (data not shown).

From these results we conclude that bombinins H3, H4 and H5 each contain D-alloisoleucine as the second amino acid. The differences in the amino acid sequences of bombinins H1–H5 and their relative abundance in skin secretion of *B. variegata* are summarized in Table I.

Tests for biological activity

The antibacterial and haemolytic activities of the peptides from skin secretion of *B. variegata* were tested using an inhibition zone assay on agarose plate (Hultmark *et al.*, 1983). As shown in Table II, the three peptides tested, bombinins H1, H3 and H4, as well as the synthetic peptide B (see Figure 1) have similar antibacterial activities against *Escherichia coli* D21 and *Staphylococcus aureus* Cowan 1. The former is in the same range as that of magainin (Andreu *et al.*, 1992). The peptides also have some haemolytic activity, although this is at least 10 times lower than that of brevinin-1E or melittin (Simmaco *et al.*, 1993).

Discussion

The structure of the bombinin precursors deduced from the sequence of cloned cDNAs has suggested the presence of a hydrophobic peptide in skin secretion of *B. variegata*. Using this information, a family of such peptides differing in the

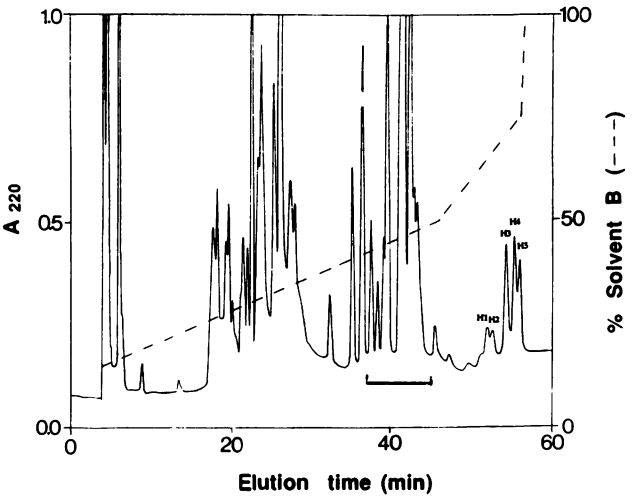

Fig. 2. Reverse-phase HPLC of crude skin secretion from *B. variegata*. Column, Aquapore RP300, 7.0 × 250 mm. Solvent system: solvent A, 0.2% (by vol) trifluoroacetic acid, solvent B, acetonitrile/isopropanol (4:1) in 0.2% trifluoroacetic acid; flow rate, 2.0 ml/min. A_{220}, absorbance at 220 nm. The horizontal bar indicates the elution position of bombinins (Simmaco *et al.*, 1991). Fractions marked H1–H5 were further analysed.

Table I. Amino acid differences among *B. variegata* peptides and their relative abundance

Peptide	Position in the sequence			Relative abundance (%)
	1	2	8	
H1	Ile	Ile	Met	9
H2	Leu 54% Ile 46%	Ile	Leu	4
H3	Ile 69% Leu 31%	D-aIle	Met	34
H4	Ile 58% Leu 42%	D-aIle	Leu	35
H5	Ile	D-aIle	Leu	18

Table II. Antibiotic and lytic activity of *B. variegata* peptides

Peptide	Lethal concentration (μM)		
	E. coli D21	*S. aureus* Cowan 1	Red blood cells
H1	3.8	2.1	ND
H3	3.7	2.4	15.7
H4	4.8	3.3	14.6
Peptide B (synthetic)	5.0	3.0	17.0
Bombinin[a]	3.0	14.0	>50

[a]Synthetic bombinin whose sequence was deduced from the cDNA of clone 42 (Simmaco *et al.*, 1991).

nature of the first, second and eighth residues could be isolated. In the cloned cDNAs, the genetic information for only one of these peptides containing Ile, Ile and Leu in the respective positions, was present. More recently, two additional clones were analysed which were found to contain a methionine codon in the eighth position (unpublished experiments). However, no clones encoding peptides with leucine as the N-terminal residue have been found. The reason for this could be that skin secretion was collected from *B. variegata* originating from Calabria, Italy, while the cDNA library was prepared from skin of frogs caught in lower Austria. Different alleles may be present in these local populations. All the peptides terminate with the sequence Leu-Lys-Lys-Ile-amide indicating that processing at the pair of lysine residues does not take place in the skin gland of *B. variegata*. It should be added that the synthetic peptide A (see Figure 1) which lacks the last three amino acids was devoid of antibacterial and lytic activity, as expected from the lack of positive charges (Cruciani *et al.*, 1988).

We have termed these peptides bombinin H as they are all hydrophobic and also have, as far as tested, some haemolytic activity. The bombinin-like peptides isolated previously from *B. variegata* (Simmaco *et al.*, 1991) as well as from *B. orientalis* (Gibson *et al*, 1991) are not haemolytic. Under our conditions, five peaks could be resolved and the peptides present in these peaks are referred to as bombinins H1−H5. From the sequence analysis it is clear that three of these peaks, H2−H4, still contain at least two peptides with either isoleucine or leucine as the N-terminal residue. These could not be resolved by reverse-phase HPLC.

These bombinins H apparently represent a new type of antibacterial peptide. In contrast to the bombinins and numerous peptides from skin secretion of various amphibia (Bevins and Zasloff, 1990; Boman, 1991), these cannot form an amphipathic helix. Bombinins H also do not show any similarity to other classes of antibacterial peptide isolated from diverse sources (Boman, 1991; Hoffman and Hetru, 1992; Hultmark, 1993; Simmaco *et al.*, 1993).

Of particular interest is the fact that some of these peptides contain D-alloisoleucine as the second amino acid. Interestingly, the presence of small quantities of D-alloisoleucine in hydrolysates of peptide fractions isolated from the skin of *B. variegata* was observed many years ago (Molzer, 1970). Since a normal codon for isoleucine was present in the cDNAs at those positions where D-alloisoleucine was found in the end-products, we conclude that this must be formed by a post-translational reaction whereby the configuration at the α-carbon atom is changed. In the course of this hypothetical reaction, L-isoleucine would be converted to D-alloisoleucine. As shown in Table I, the 'parent' peptides containing the normal amino acid are also present, albeit in smaller quantities. The reason for this could be 2-fold: the epimerization reaction is incomplete, or, after racemization, the peptides still containing the L-amino acid in position 2 are partly degraded, e.g. by aminopeptidases.

More than 10 years ago, dermorphin, the first peptide of animal origin containing a D-amino acid, was isolated from skin of a South American frog, *Phyllomedusa sauvagei* (Montecucchi *et al.*, 1981). This peptide with potent opioid activity was shown to have a D-alanine residue in the second position. Since then several additional dermorphins and deltorphins have been found in skin of different species of the sub-family Phyllomedusinae (Erspamer *et al.*, 1989; Kreil *et al.*, 1989; Mignogna *et al.*, 1992). They all contain a D-alanine, in one case a D-methionine residue, which is essential for biological activity. The present results show that peptides containing a D-amino acid may be more widespread among amphibians than was hitherto assumed. Moreover, they occur not only in opioid peptides but also in peptides with antibacterial activity. Contrary to the former, however, the presence of a D-amino acid is not essential for biological activity. The isomers containing L-isoleucine or D-alloisoleucine were found to have similar potency.

At present, nothing is known about the biosynthesis of these D-amino acids. Obviously, the primary product of translation must contain the corresponding L-amino acid which is then converted, partially or completely, to the D-isomer. It is noteworthy that in all cases known so far, the D-amino acid is found in the second position of the end-product. This applies not only to amphibian skin peptides, but also to two neuropeptides isolated from the brain of a snail (Kamatani *et al.*, 1989; Ohta *et al.*, 1991). In addition, the nature of the D-amino acid varies considerably; it is Ala, Met, and, as found recently (D.Barra, G.Mignogna, M.Simmaco, P.Pucci, C.Severini, G.Falconieri Erspamer, L.Negi and V.Erspamer, submitted), Leu for skin peptides of Phyllomedusinae, aIle for skin peptides from *B. variegata*, Phe and Asn for the snail peptides. Moreover, no regularity in the sequence of the neighbouring amino acid exists. This would indicate that the enzyme(s) catalysing these conversions from the L- to the D-isomer is not recognizing a particular sequence and a certain side chain, but rather a terminal amino group and the hydrogen linked to the α-carbon of the second residue.

Materials and methods

Collection and purification of skin secretion

The discharge of dermal glands of the frogs was initiated by mild electrical shocks (6 V, 4−5 × 1 s). The peptide-containing secretion was collected from the surface of the skin of six specimens of *B. variegata* by washing the dorsal region of each frog with 10 ml 0.05% (by vol) acetic acid. The combined solutions were lyophilized, yielding a white powder of ~250 mg. An aliquot (7.5 mg) was dissolved in 1 ml 10% (by vol) acetonitrile and fractionated by HPLC on a Beckman model 332 system using a reverse-phase column (Aquapore RP-300, 7 × 250 mm, Applied Biosystems) eluted with a 60 min gradient of 10−70% acetonitrile/isopropanol (4:1) in 0.2% (by vol) trifluoroacetic acid, at a flow rate of 2.0 ml/min. Elution of the peptides was monitored on a Beckman 165 spectrophotometer at 220 nm. Peak fractions were collected and lyophilized. A 1% aliquot of each peak was then used for assays of biological activity.

Antibacterial assays

The antibacterial activity was tested using an inhibition zone assay on agarose plates seeded with *E. coli* D21 or *S. aureus* Cowan I (Hultmark *et al.*, 1983). The test peptide (3 μl) was placed in small wells of thin agarose plates containing rich medium and ~2 × 10^5 bacterial cells. The plates were incubated overnight at 30°C. Inhibition zones were measured around wells and the lethal concentration (LC, the lowest concentration that inhibits growth) was calculated from the diameter of the zones obtained in serial dilutions of the test substance by using the formula given in Hultmark *et al.* (1983).

Haemolytic activity

The haemolytic activity was recorded by an adaptation of the antibacterial inhibiton zone assay on agarose plates with human red blood cells (Wade *et al.*, 1992). Sterile agarose plates contained 6 ml of medium with 1% agarose, 0.9% NaCl and human red blood cells which, after complete lysis in the same volume of distilled water, give 0.5 absorbance at 500 nm. The plates were incubated at 30°C for 24 h. Clear zones were measured and lytic concentration values calculated as above.

Structural analysis

N-terminal analysis of purified peptides was made after derivatization with dansyl chloride and HPLC separation (Simmaco *et al.*, 1990). Amino acid analyses were performed with a Pharmacia Alpha Plus 4151 analyser after vapour phase hydrolysis of the peptides (1–2 nmol) in 6 N HCl for 24–48 h. Peptide sequences were determined by automated Edman degradation with Applied Biosystems model 475A or 476A sequencers. To test for the presence of a D-amino acid, peptide hydrolysates were treated with a chiral reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine), and amino acid derivatives were separated by HPLC (Scaloni *et al.*, 1991). The chiral Edman degradation was performed as described previously (Scaloni *et al.*, 1991). The net charge of peptides was deduced from their electrophoretic mobility according to Offord (1966). For digestion with aminopeptidase M (Boehringer) peptides were incubated in Tris–HCl 10 mM, pH 7.5 at 37°C for 2 h.

Peptide synthesis

Peptides were prepared by solid-phase synthesis on a Pharmacia Biolynx automated peptide synthesizer according to fluoren-9-ylmethoxy carbonyl (Fmoc)-polyamide active ester chemistry (Atherton *et al.*, 1975). The products were purified by reverse-phase HPLC and the expected amino acid sequences were confirmed by automated Edman degradation.

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