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# Clavanins, α-helical antimicrobial peptides from tunicate hemocytes

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**Abstract Hemocytes from the invertebrate *Styela clava*, a solitary tunicate, contained a family of four α-helical antimicrobial peptides that were purified, sequenced and named clavanins A, B, C and D. Each clavanin contained 23 amino acid residues and was C-terminally amidated. The tunicate peptides resembled magainins in size, primary sequence and antibacterial activity. Synthetic clavanin A was prepared and displayed comparable antimicrobial activity to magainins and cecropins. The presence of α-helical antimicrobial peptides in the hemocytes of a urochordate suggests that such peptides are primeval effectors of innate immunity in the vertebrate lineage.**

*Key words:* α-Helical; Antimicrobial peptide; Tunicate; Magainin

## 1. Introduction

Various antimicrobial peptides have been found in mammalian [1–3] and avian [4] leukocytes, amphibian skin [5,6], bovine lingual and respiratory tract epithelia [7,8] and the gastrointestinal and genitourinary cells of humans [9,10] and other mammals [11,12]. Given this widespread distribution, we sought homologues of these peptides in the hemocytes of tunicates, simple marine protochordates that are ancestral to early vertebrates [13]. Tunicate 'hemocytes' are phagocytic cells that correspond to the leukocytes of higher vertebrates [14]. This report describes four members of a 'new' family of antimicrobial peptides, clavanins. We derived the word clavanin from the Latin word *clava* (club), an ancient but still effective weapon of host defense.

## 2. Materials and methods

### 2.1. Tunicate hemocytes

Freshly harvested, live *Styela clava* were obtained from a local supplier (Marinus Biologicals, Long Beach, CA). Their tunics were bathed briefly in absolute ethanol, blotted dry and transected peribasally over a 50 ml test tube that contained 0.25 g of disodium EDTA. Hemolymph was collected dripwise through a Netwell polyester sieve with 74 μm apertures (Corning Costar, Cambridge, MA) to remove cellular aggregates. Processing 50 tunicates in a batch yielded approximately $2\times10^8$ hemocytes. These cells were centrifuged at $260\times g$ for 5 min at 4°C, resuspended in 50 ml of 0.34 M sucrose and recentrifuged as above. The recovered hemocytes were placed into ice-cold 5% acetic acid, briefly sonicated, stirred overnight at 4°C and clarified by centrifugation at $27000\times g$ for 30 min. The supernatant, which contained ≈15 mg protein/$2\times10^8$ hemocytes by BCA analysis, was recovered for further analysis.

### 2.2. Purification of antimicrobial peptides

Clavanins were identified and purified from these supernatants by a 3-stage procedure that began with ultrafiltration through a 10 kDa cutoff Amicon YM-10 membrane, followed by either gel permeation chromatography or preparative acid–urea polyacrylamide gel electrophoresis [15], followed by reverse-phase HPLC. The YM-10 filtrates contained ≈3.5 mg of protein/$2\times10^8$ hemocytes and were concentrated to 2 ml by vacuum centrifugation (Speed Vac, Savant Instruments, Hicksville, NY) before chromatography on a $1.2\times65$ cm Bio-Gel P-10 column that was eluted with 5% acetic acid. Because preparative acid–urea PAGE [15] was faster and afforded higher purity and recovery than gel chromatography, we used it increasingly as our studies progressed. Supernatants subjected to preparative PAGE were first lyophilized to dryness and resuspended in 2 ml of 5% acetic acid with 3 M urea and a trace of methyl green dye. Antimicrobial fractions were pooled as indicated, and purified by RP-HPLC a $4.6\times250$ mm Vydac C18 column, using various linear gradients of acetonitrile in 0.1% trifluoroacetic acid (TFA) or 0.13% heptaflurobutyric acid.

Throughout the purifications, fractions were lyophilized, concentrated and tested for antimicrobial activity against *Listeria monocytogenes* strain EGD by a radial diffusion technique [16]. The underlay gels contained 9 mM sodium phosphate, 1 mM sodium citrate buffer, 0.30 mg/ml of trypticase soy broth powder (BBL, Cockeysville, MD) and 1% agarose, and had a final pH of 6.5. The overlay gels (1% agarose and 60 mg/ml of trypticase soy broth powder, pH 7.3) were poured 3 h after adding the peptide samples to the underlay gel's sample wells.

### 2.3. Peptide characterization

Purified clavanins were hydrolysed in vacuo in 5.7 N HCl for 20 h, derivatized with phenylisothiocyanate (PITC) and quantitated as phenylthiocarbamyl derivatives on a Picotag system (Waters Associates, Milford, MA). Amino acid sequences were determined by gas-phase Edman degradation with a Porton Model 2090 instrument, using purified 300 pmol samples of clavanins. Mass spectrometric analyses of native clavanins were done at the UCLA Center for Molecular and Medical Mass Spectrometry, using a ZAB-SE Fast atom bombardment instrument (VG Instruments, Manchester, UK).

Circular dichroism spectra were obtained on an AVIV model 62DS spectropolarimeter (AVIV Associates, Lakewood, NJ) at 23°C in a rectangular 0.5 mm path length cell that contained either 80% trifluoroethylene (TFE) or unilamellar ≈100 nm diameter liposomes in 10 mM phosphate buffer, pH 7.4. The liposomes, composed of a 3:1 molar ratio of egg phosphatidylglycerol and cardiolipin, resembled *S. aureus* membranes in composition [17] and were prepared by 'LiposoFast' extrusion (Avestin, Ottawa, Canada).

### 2.4. cDNA cloning and sequencing

Total RNA from tunicate pharyngeal tissues was isolated and purified using a total RNA Separator Kit (Clontech, Palo Alto, CA). First-strand cDNA synthesis and clavanin 3′ side cDNA amplification were carried out with a 3′ RACE Kit (Gibco BRL, Gaithersburg, MD). Total pharyngeal RNA (1 μg) and 10 μM adapter primer (1 μl) were used to obtain the first cDNA strand. A degenerate 30-base primer (5′-GTCGACTAGTCAYCAYGTIGGIAAYTTYGT-3′), that corresponded to amino acids 11–17 of clavanins A, B, C, and D (His–His–Val–Gly–Asn–Phe–Val) was synthesized. In the above notation, Y represents T or C and I represents inosine. The *Spe*I restriction site is underlined. PCR was performed in a total volume of 50 μl that contained: 1/10 vol. of cDNA, 10 pmol degenerate primer, 10 pmol AUAP primer, and 5 U of pfu DNA polymerase. The reaction was

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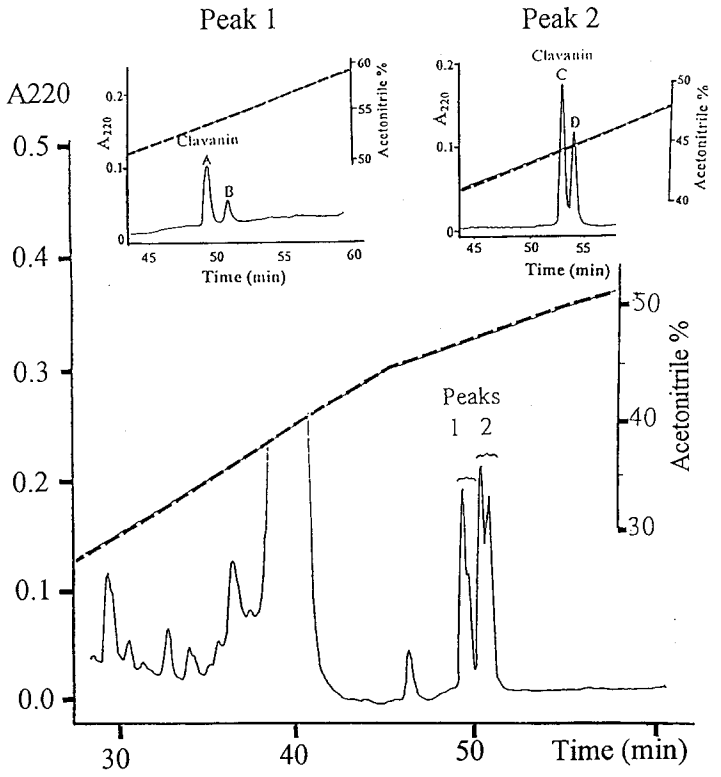


Fig. 1. RP-HPLC purification of clavanins A–D. Main figure: Clavanin-enriched fractions after preparative acid–urea–PAGE were pooled and subjected to RP-HPLC. The acetonitrile gradient is shown by an interrupted lin. Peak 1 contained a mixture of clavanins A and B, while peak 2 contained clavanins C and D. Inserts: The individual clavanins in peaks 1 and 2 were resolved by further RP-HPLC.

run for 35 cycles, with 1 min denaturation (94°C), 1 min annealing (48°C), and 2.5 min extension (72°C) per cycle. PCR products about 250 bp in size were cloned into pCRScript SK vector (Stratagene, La Jolla, CA). Sequencing was performed on the double-stranded plasmid by the fluorescein-labeled dideoxy nucleotide terminator method, and analyzed on an Applied Biosystems 373 DNA Sequencer (Perkin-Elmer, Palo Alto, CA).

*2.5. Bacteriologic studies*

Peptides were mixed with mid-logarithmic phase *E. coli* or *L. monocytogenes* in a sterile pH 6.5 solution that contained 10 mM sodium phosphate buffer and 0.3 mg/ml of trypticase soy broth powder. Such mixtures, typically in a final volume of 50–100 μl, were incubated in a 37°C shaking water bath and 10 μl aliquots were removed at intervals and directly or after dilution were plated with a Spiral plater [18] (Spiral Systems Instruments, Bethesda, MD). The resulting colonies were counted after an overnight incubation. Magainin 1 and cecropin P1 (both purchased from Bachem) were used as controls in these experiments.

*2.6. Synthetic clavanins*

Two forms of synthetic clavanin A, with a C-terminal carboxylic acid or amide, were synthesized by SynPep (Dublin, CA) using F-moc (9-fluoroenylmethyloxycarbonyl) chemistry. Their respective $mass_{av}$ values were: acid, 2667.2 (expected 2667.1) and amide, 2664.6 (expected 2666.1). These peptides were purified to virtual homogeneity by RP-HPLC.

## 3. Results

Fig. 1 shows the last few stages in the purification of clavanins A, B. C and D, accomplished by RP-HPLC. The primary sequences of these peptides are shown in Fig. 2. Each clavanin contained 23 residues, including an amidated C-terminal phenylalanine. Eighteen of the 23 residues (78.3%) were identical in every clavanin and have been bolded. Fig. 2 also indicates the consensus clavanin primary structure.

Clavanins A and B differed only by the conservative replacement of $lys_7$ (in A) by $arg_7$ in clavanin B. The measured mass of clavanin A, 2666.4, agreed with its calculated mass of 2667.1. Because C-terminal amidation is common in antimicrobial peptides, we prepared synthetic clavanin A with and without an amidated C-terminal phenylalanine. Although the calculated and measured masses of clavanin B were almost identical (2695.0 and 2694.8, respectively), native clavanins A and B migrated identically on acid–urea PAGE gels signifying that they had identical net charges (Fig. 3A). Native clavanin A migrated identically to the amidated peptide by acid–urea PAGE, (Fig. 3B). Taken together, these results indicated that native clavanins A and B were both amidated.

| | 1 | 6 | 11 | 16 | 21 |
|---|---|---|---|---|---|
| Clavanin A | VFQFL | GKIIH | HVGNF | VHGFS | HVF* |
| Clavanin B | VFQFL | GRIIH | HVGNF | VHGFS | HVF* |
| Clavanin C | VFHLL | GKIIH | HVGNF | VÝGFS | HVF* |
| Clavanin D | AFKLL | GRIIH | HVGNF | VÝGFS | HVF* |
| Consensus | □F△□L | G▲IIH | HVGNF | V△GFS | HVF* |
| Clavanin A | VFQFL | GKIIH | HVGNF | VHGFS | HVF* |
| Magainin 1 | GI | GKFLH | SAGKF | GKAFV | GEI |

Fig. 2. Primary sequences of clavanins A–D. The sequences are shown in standard single letter code. The following non-standard symbols have also been used: *, C-terminal amidation; Ý, methyltyrosine; □, a hydrophobic residue; △, a polar residue; ▲, a positively charged residue. In the consensus clavanin sequence, invariant residues have been bolded. Clavanin A is also aligned with magainin 1, with the identical residues bolded and the highly conservative substitutions underlined.

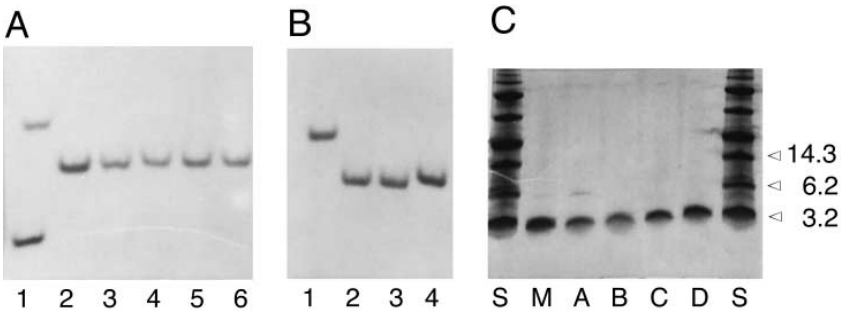


Fig. 3. A: Acid–urea PAGE. Lane 1 contained 1 μg each of defensins HNP-1 (above) and NP-1 (below), used as standards. Lane 2: A mixture of clavanins A–D, 0.5 μg each. Lanes 3–6: 1 μg of clavanins A, B, C and D, respectively. The gel was stained with Coomassie Blue. B: Acid–urea PAGE. Lane 1 contained 1 μg of human defensin HNP-1 as a standard. Lanes 2–4 contained 1 μg each of native clavanin A, synthetic clavanin A amide and synthetic clavanin A acid, respectively. The native form migrated alongside the amidated synthetic clavanin (C). SDS-PAGE. Both outside lanes (S) contained molecular weight standards. The remaining lanes contained 2 μg of a mixture of clavanins A–D (M), followed by 1 μg each of clavanins A,B,C and D (so labeled). The gel was silver-stained.

We could identify 19 of the 23 residues of clavanins C and D by direct peptide sequencing and identified the remaining residues ($tyr_{17}$, $his_{21}$, $val_{22}$, $phe_{23}$) by 3′ RACE-PCR cloning of a precursor whose (partial) sequence was HHVGNFVYGFSHVF(G). The full structures of the clavanin precursors will be described elsewhere (Zhao et al., unpublished). By FAB-MS, the calculated masses of clavanins C and D (2682.1 and 2673.0, respectively) were 14 mass units *less* than their measured values, suggesting that one residue had been methylated. Because we found no unmodified tyrosine in the amino acid analyses (data not shown) of these clavanins, we concluded that the methylated residue was $tyr_{17}$. The location of the tyrosine methyl group (α-, *o*- or N) in clavanins C and D remains to be determined. The presence and position of a glycine residue (*G*) in the clavanin C/D precursor [HHVGNFVYGFSHVF(*G*)] suggests that clavanins C and D are also amidated [19].

The presence of polar amino acids at relatively regular intervals along the clavanin backbone suggested that the peptides probably formed amphipathic helices. This conjecture

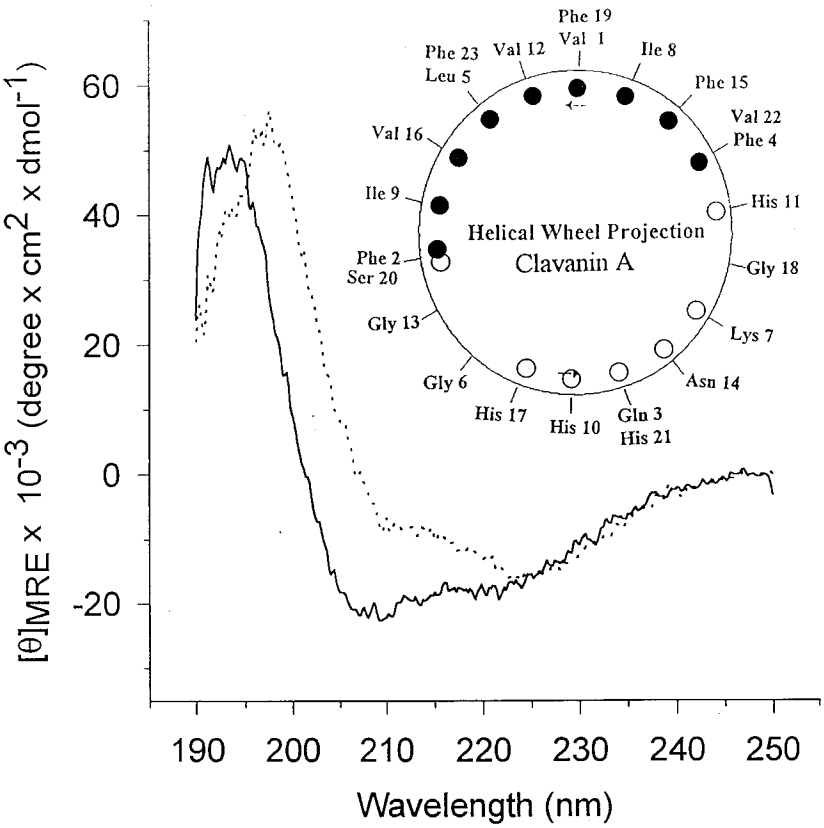


Fig. 4. Circular dichroism spectrum of synthetic clavanin A. The spectrum drawn with a solid line was obtained by placing 30 μg $ml^{-1}$ of peptide in a 4:1 mixture of trifluoroethanol (TFE) and 10 mM phosphate buffer, pH 7.4. The spectrum drawn with a dotted line was obtained by adding the peptide to a liposomal dispersion prepared from a 3:1 mixture of egg phosphatidylglycerol and cardiolipin in 10 mM phosphate buffer. The lipid/peptide ratio was 25:1. The insert shows a helical wheel projection of clavanin A. The incremental angle is 100°, the average hydrophobicity is 0.46 and the hydrophobic moment (μH) is 0.53. ●, Hydrophobic residues; ○, hydrophilic residues.

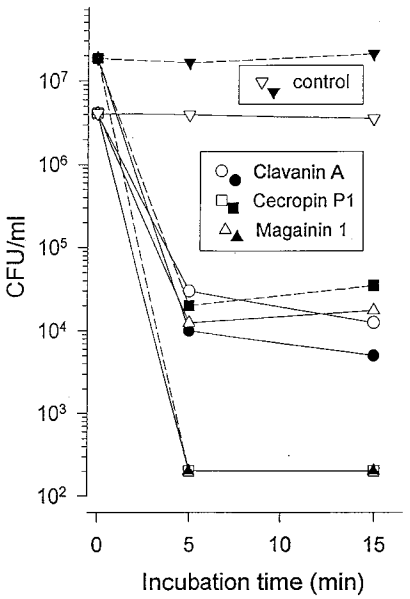


Fig. 5. Activity of α-helical peptides against *E. coli* and *L. monocytogenes*. Incubations were performed at 37°C with mid-logarithmic phase organisms. The medium contained 10 mM sodium phosphate buffer (pH 6.5) and 0.3 mg/ml trypticase soy broth. ●,■,▲: *L. monocytogenes* CFU/ml; ○,□,△: *E. coli*. CFU/ml. The controls (no added peptides) are represented by inverted triangles (▽,▼). All of the peptides were synthetic. They were tested at 3.5 μg/ml against *L. monocytogenes* and at 1.6 μg/ml against *E. coli*.

was supported by our measurements of circular dichroism (Fig. 4). In 80% trifluoroethanol, the CD spectrum of synthetic clavanin A (solid lines) contained a well-defined double minimum at –208 and 222 nm, characteristic of an α-helical conformation. In phosphate buffer containing a liposomal dispersion whose composition simulated *S. aureus* membranes (dotted lines), the minima at ≈210 and 224 nm also indicated substantial α-helical conformation.

A helical wheel projection of clavanin A demonstrated spatial segregation of its hydrophobic and charged residues (Fig. 4, insert), indicating the amphipathic nature of the clavanin α-helix. Magainins [20], cecropins [21] and many other antimicrobial peptides [22,23] also form amphipathic α-helices under conditions similar to those used in our experiments.

We compared the abilities of clavanin A, magainin 1 and cecropin P1 to kill mid-logarithmic phase *Listeria monocytogenes* and *E. coli* at pH 6.5 at 37°C (Fig. 5). Note that very low concentrations of clavanin A (and the other peptides) were rapidly bactericidal. After 5 min of incubation, 1.6 μg/ml of clavanin A reduced the number of viable *E. coli* ML-35p by more than 2 $\log_{10}$ and 3.5 μg/ml of clavanin A reduced the number of *L. monocytogenes* by more than 3 $\log_{10}$.

Fig. 6 shows radial diffusion assays which compared the potency of native clavanins against *L. monocytogenes*, *E. coli* and *C. albicans*. In such assays, the X-intercepts of the plots indicate the minimal bactericidal concentrations. Note that clavanins C and D were approximately 3–5-fold more potent than clavanins A and B.

## 4. Discussion

We have shown that hemocytes of *Styela clava*, a solitary ascidian urochordate, contain clavanins, a family of broad-spectrum α-helical antimicrobial peptides. Figs. 2–5 indicate that clavanins show considerable structural [5] and functional [24] homology to magainin 1, a well-characterized antimicrobial peptide from the skin of *Xenopus laevis*.

Magainin 2 is identical in sequence to magainin 1 except for its $lys_{10}$ and $arg_{21}$ residues. Like clavanins, mature magainins contain 23 amino acids and are α-helical. Unlike clavainins, the magainins are not amidated. Although the 15 residues of clavanin A between $Leu^5$ and $Phe^{19}$ show 12 similarities (6 identical residues and 6 conservative substitutions) to the residues between $Ile^2$ and $Phe^{16}$ of magainin 1, magainins are lysine-rich peptides, whereas most of the positively charged residues of clavanins are histidines. Magainins are synthesized on a 303 residue prepropeptide that contains a single copy of magainin 1 and five copies of magainin 2 [25]. We will describe the structure of preproclavanins in a future communication.

Several other examples of convergent or conserved structur-

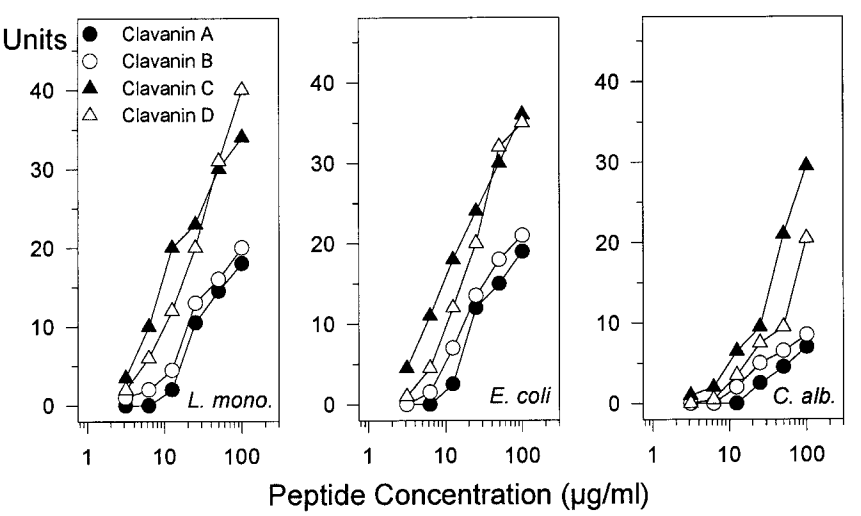


Fig. 6. Comparative activity of clavanins A, B, C and D. Serial 2-fold dilutions of native clavanins A–D were tested against *E. coli* ML-35p, *L. monocytogenes* EGD and *C. albicans* in radial diffusion assays. Zone diameters are expressed in units (10 U=1 mm). Note that clavanins C and D were about 3-fold more active than clavanins A and B.

al motifs in the antimicrobial polypeptides of 'higher' and 'lower' animals were recently reported. Amoebapore, a 77 residue cytotoxic and antimicrobial pore-forming peptide from *Entamoeba histolytica*, a parasitic protozoan, shows considerable homology to NK-lysin, a 78 residue antimicrobial peptide from porcine cytotoxic lymphocytes [26–28]. Similarly, potently antimicrobial protegrin peptides found in porcine neutrophils [29] show considerable resemblance to tachyplesin peptides found in horseshoe crab hemocytes [30]. Since endogenous antimicrobial peptides occur in insects [31] and plants [32], antimicrobial peptides may have assumed their position in the immune arsenal of living organisms even before multicellular organisms existed and before animals and plants diverged.

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