THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 265, No. 31, Issue of November 5, pp. 18871-18874, 1990
Printed in U.S.A.
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Amino Acid Sequences of Two Proline-rich Bactenecins

ANTIMICROBIAL PEPTIDES OF BOVINE NEUTROPHILS*

(Received for publication, April 23, 1990)

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Bactenecins are highly cationic polypeptides of the large granules of bovine neutrophils, exerting *in vitro* a potent antimicrobial activity. Two bactenecins, with an approximate molecular weight of 7000 and 5000, called Bac7 and Bac5, are characterized by a high content of proline (>45%) and arginine (>23%) residues. Their complete amino acid sequences were determined by automated Edman degradation combined, in the case of Bac5, with plasma desorption mass spectrometry. Bac7 comprises 59 residues and includes three tandem repeats of a tetradecamer characterized by several Pro-Arg-Pro triplets spaced by single hydrophobic amino acids. Resolution of the primary structure of Bac5 required fragmentation with *N*-bromosuccinimide as well as digestion of the obtained C-terminal fragment with carboxypeptidases P and Y directly in the mass spectrometer. Bac5 comprises 42 amino acid residues with a repeated motif of Arg-Pro-Pro triplets also alternating with single apolar residues.

Neutrophils play a major role in host defense by their ability to engulf and then destroy invading microbes. Bacterial killing and virus inactivation in the phagolysosomes of neutrophils may be carried out by highly reactive oxygen derivatives and/or a variety of cytotoxic, cationic polypeptides (1–3). Some of these polypeptides have been extracted and purified from the granules, where they are normally stored, and their antimicrobial activity, amino acid composition, and sequence have been determined (4–9). From granule extracts of bovine neutrophils we have purified two proline-rich polypeptides with an apparent molecular weight of 7000 and 5000 (9). They have been called bactenecins and, in an abbreviated form, Bac7 and Bac5. Both peptides arise by proteolytic cleavage of inactive proforms, which are stored in the so-called large granules of the bovine neutrophils (10). *In vitro*, at concentrations of 10–50 μg/ml (2–10 μM) Bac7 and Bac5 efficiently kill *Salmonella typhimurium, Klebsiella pneumoniae,* and *Escherichia coli* and arrest the growth of *Enterobacter cloacae* and *Pseudomonas aeruginosa*. Their antibacterial effects are very likely due to an impairment of the function of the respiratory chain and of energy-dependent activities in the inner membrane of susceptible microorganisms.[1] Furthermore, Bac7 directly inactivates human herpes virus (7, 9).

In this paper we describe the determination of the complete amino acid sequences of Bac7 and Bac5, which were obtained by automatic Edman degradation, combined with plasma desorption mass spectrometry (PD-MS).[2] The uniqueness of the Bac7 sequence is the presence of three tandem repeats of a tetradecamer, in which Pro-Arg-Pro triplets are spaced by single hydrophobic amino acid residues. In Bac5 the repeated motif is an Arg-Pro-Pro triplet, which also alternates with single apolar residues.

MATERIALS AND METHODS

Materials—High performance liquid chromatography-grade acetonitrile was obtained from Riedel-de-Haen AG (Seelze, Federal Republic of Germany), trifluoroacetic acid from Janssen Chimica (Beerse, Belgium), and *N*-bromosuccinimide (NBS) from Fluka Chemie (Buchs, Switzerland). Sequencing grade carboxypeptidases P and Y were from Boehringer Mannheim. All the polyacrylamide gel electrophoresis reagents were purchased from Bio-Rad (Richmond, CA). Water was MilliQ™ grade (Millipore/Continental Water Systems, Bedford, MA).

Bactenecins—These were purified from extracts of granules of bovine neutrophils, using ion-exchange and reversed-phase high performance liquid chromatography as reported previously (9). Bactenecins homogeneity was evaluated by sodium dodecyl sulfate- and acid urea-polyacrylamide gel electrophoresis carried out as described (9). The purified polypeptides were stored in 0.1% trifluoroacetic acid at −80 °C.

Amino Acid Analysis—Peptides were hydrolyzed for 22, 48, and 72 h at 110 °C with 6 N HCl in evacuated sealed tubes. After removal of HCl by evaporation, amino acids were analyzed with an automatic amino acid analyzer (Carlo Erba, Milan, Italy).

Cleavage of Bac5 with N-Bromosuccinimide— Bac5 was cleaved at the tyrosyl-peptide bond by treatment with NBS (11). The peptide (0.5 nmol) was incubated for 10 h at room temperature with 2 nmol of NBS in 200 μl of 50% acetic acid. The resulting fragments were separated by high performance liquid chromatography on a Nucleosil C_{18} column (Macherey-Nagel, Dueren, Federal Republic of Germany) using a linear 0–60% gradient of acetonitrile in 0.1% trifluoroacetic acid.

Sequence Analysis—For sequence analysis 100–500 pmol of Bac5 or Bac7 were subjected to automatic Edman degradation in a gas-phase sequencer constructed and operated as described (12). Sequencer cycles and repetitive yields were as follows: Bac7: 60, 96% (59 residues identified); Bac5: 30, 89% (25 residues identified); Bac5 C-terminal NBS fragment: 30, 88% (23 residues identified). On-line analysis of the phenylthiohydantoin amino acids was performed on a Hewlett-Packard 1090 liquid chromatograph (Hewlett-Packard, Palo Alto, CA) using a phenylthiohydantoin-C_{18} column (Applied Biosystems, Foster City, CA).

* This work was supported by grants from the Italian Ministry of University and Research, the Italian National Research Council (CNR, Progetto Finalizzato "Biotecnologia e Biostrumentazione"), and the Deutsche Forschungsgemeinshaft, Bonn, Federal Republic of Germany. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Skerlavaj, B., Gennaro, R., and Romeo, D., manuscript in preparation.

[2] The abbreviations used are: PD-MS, plasma desorption-mass spectrometry; NBS, *N*-bromosuccinimide.

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Mass Spectroscopy—PD-mass spectra were recorded with a Bio-Ion 20K time of flight spectrometer (Bio-Ion Nordic AB, Uppsala, Sweden) equipped with a 10-μCi ^{252}Cf primary source (13). Sample targets were prepared by electrospraying a nitrocellulose solution in acetone onto aluminized mylar foils of 1-cm diameter, as described previously (14). 100–500 pmol of peptide samples, dissolved in 0.5% trifluoroacetic acid, were loaded on the target with a microliter syringe; solution droplets (1–4 μl) were dried at room temperature for 1–2 min and targets introduced in the ion source.

In Situ Carboxypeptidase Digestion—Proteolytic digestions were carried out with the peptide adsorbed to the nitrocellulose target, by applying 2-μl solutions of carboxypeptidases P and Y (~1 mg/ml) in 50 mM ammonium acetate, pH 7. Reactions were carried out for 1 h and terminated by drying the sample target using the spin-drying technique.

Sequence Similarity Search—Sequence similarities between Bac5 or Bac7 and other known sequences were searched (December 1989) using the EMBL Data Bank.

RESULTS AND DISCUSSION

The results of the amino acid sequence analysis of Bac7 are shown in Fig. 1. Despite the high proportion of prolyl residues, which are difficult to cleave from the peptide chain and may therefore create overlap problems during Edman degradation, in three sequencer runs unambiguous and identical sequence information was obtained. The peptide comprises 59 amino acid residues and has a calculated mass of 6913 Da, which agrees well with the isotope-average mass of 6912 ± 2 Da obtained by PD-MS (data not shown) and with amino acid composition data (Table I).

Automated Edman degradation of Bac5 was inefficient and only 25 N-terminal residues were identified unambiguously (Fig. 2). Positive identification of further residues was prevented by an increasing overlap of signals from previous cycles, mainly due to incomplete degradation of the tandem prolyl residues. Therefore the peptide was cleaved at tyrosine 16 (Fig. 2) by treatment with NBS and the NBS fragment corresponding to the C-terminal portion was isolated by high performance liquid chromatography. Sequence analysis of this NBS fragment was liable to the same problems as mentioned above for complete Bac5 and only a partial sequence (23 residues) was obtained.

```
1                   5                  10
Arg - Arg - Ile - Arg - Pro - Arg - Pro - Pro - Arg - Leu - Pro - Arg - Pro - Arg -

15                  20                  25
Pro - Arg - Pro - Leu - Pro - Phe - Pro - Arg - Pro - Gly - Pro - Arg - Pro - Ile -

    30                  35                  40
Pro - Arg - Pro - Leu - Pro - Phe - Pro - Arg - Pro - Gly - Pro - Arg - Pro - Ile -

        45                  50                  55
Pro - Arg - Pro - Leu - Pro - Phe - Pro - Arg - Pro - Gly - Pro - Arg - Pro - Ile -

Pro - Arg - Pro
```

FIG. 1. **Amino acid sequence of Bac7 obtained by automated Edman degradation.**

TABLE I

Amino acid composition of bactenecins

Values are mol-mol and the numbers in parentheses are from sequencing data.

Amino acid	Bac7	Bac5
Pro	28.9 (28)	20.1 (19)
Gly	2.6 (3)	
Ile	4.1 (4)	5.0 (5)
Leu	4.2 (4)	1.2 (1)
Tyr		0.8 (1)
Phe	3.3 (3)	6.1 (6)
Arg	16.3 (17)	8.8 (10)
(Total)	(59)	(42)

```
1                   5                  10                  15
Arg - Phe - Arg - Pro - Pro - Ile - Arg - Arg - Pro - Pro - Ile - Arg - Pro - Pro - Phe -

(1)                 20                  25  (10)                30
Tyr - Pro - Pro - Phe - Arg - Pro - Pro - Ile - Arg - Pro - Pro - Ile - Phe - Pro - Pro -

                35  (20)                40
Ile - Arg - Pro - Pro - Phe - Arg - Pro - Pro - Leu - Arg - Phe - Pro
```

FIG. 2. **Amino acid sequence of Bac5 obtained by a combination of Edman degradation and plasma desorption mass spectrometry.** Sequence numbers in parentheses refer to the amino acid sequence of the peptide fragment obtained by cleavage of Bac5 with *N*-bromosuccinimide (NBS fragment).

The complete sequence of Bac5 and of its C-terminal NBS fragment (residues 17–41) was thus established by determination of specific fragment ions using ^{252}Cf PD-MS. The usefulness of PD-MS for the accurate and sensitive moleular weight determination of polypeptides up to small proteins has been demonstrated in a number of recent bioanalytical applications (15, 16), while structurally significant fragment ions are normally limited and/or scarce. By contrast, the PD-mass spectra of Bac5 and its C-terminal NBS fragment (Fig. 3) show, in addition to abundant molecular ions, a consistent pattern of sequence-specific fragment ions from the terminal end (Fig. 2). These ions can be identified as carbenium fragments produced by cleavage at the Cα-CO (carbonyl) bonds along the peptide backbone. These fragment ions are detected with intensities of approximately 10% relative to the molecular ion intensity and are summarized in Table II for the Bac5 C-terminal peptide. The deduced amino acid sequence agrees well with the data obtained from Edman degradation (Fig. 2) and from amino acid analysis (Table I). An explanation for the unusual abundance of these fragments may be found in the unique structure of Bac5, which is highly rich in hydrophobic residues and proline. A similar series of sequence-specific fragment ions has been previously found in fast-atom bombardment MS of hydrophobic, proline-containing polypeptide antibiotics such as trichotoxins and paracelsin (17).

The C-terminal partial sequence of Bac5 was further ascertained by digesting the NBS fragment on the PD-MS target with carboxypeptidases Y and P. PD-mass spectra after digestion with the carboxypeptidases yielded molecular ions of only one truncated peptide with a mass of 2591 Da, corresponding to the loss of the four C-terminal amino acids, Leu-Arg-Phe-Pro (Fig. 4). This result can be explained by a very slow initial release of the C-terminal proline, followed by rapid digestion of the subsequent residues. The hydrolysis thus proceeds very fast and is slowed at the subsequent prolyl residue at position 38, while only little intermediate truncated peptides are produced.

Characteristic features of Bac7 sequence are: (*a*) the invariable presence of proline every second residue, from position 10 to the C terminus, and (*b*) the presence of three tandemly repeated tetradecamers in the region between residues 15 and 56, with the three C-terminal residues 57–59 matching the first three amino acids of the repeat. It would be interesting to know whether these repeats represent functional domains each encoded by individual 42-base pair segments derived by duplication of a primordial unit, as proposed for collagen genes (18, 19). Work in progress on Bac7 cloning should clarify this point. Tandemly repeated regions with abundance of prolyl residues are common to a variety of polypeptides. For instance, among the components of the proline-rich protein family, the polypeptide encoded by the mouse gene MP_2 contains 13 tandemly repeated tetradecamers with the prototype sequence PPPPGGPQPRPPQG (20). Further, two human genomic clones, also coding for proline-rich proteins,

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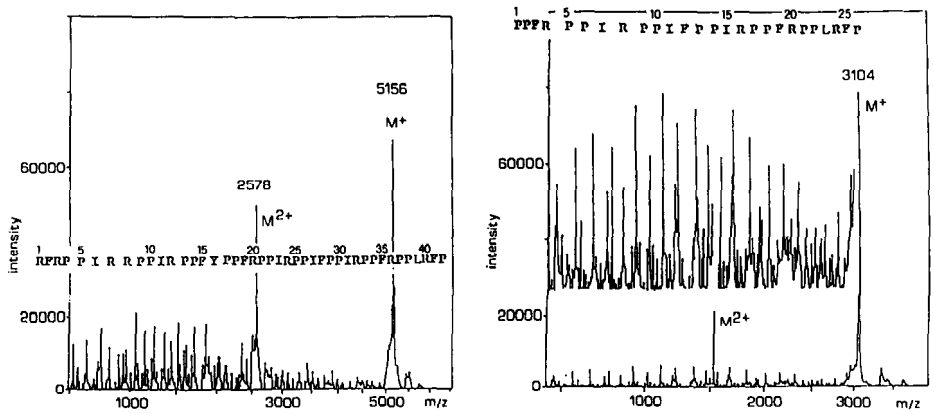

FIG. 3. **Plasma desorption mass spectrometry fragmentation pattern of Bac5 (*left*) and of its NBS fragment (*right*).**

TABLE II

PD-MS fragmentation pattern of the C-terminal peptide cleaved from Bac5 by N-bromosuccinimide

Amino acid residue	Molecular weight	Molecular weight of fragment	
		Calculated	by PD-MS
1. Pro	97		
2. Pro	97		
3. Phe	147		
4. Arg	156	497	
-CO	28	469	471
5. Pro	97	567	568
6. Pro	97	664	664
7. Ile	113	777	778
8. Arg	156	933	934
9. Pro	97	1030	1032
10. Pro	97	1127	1128
11. Ile	113	1241	1242
12. Phe	147	1388	1389
13. Pro	97	1485	1485
14. Pro	97	1582	1583
15. Ile	113	1695	1696
16. Arg	156	1852	1855
17. Pro	97	1949	1950
18. Pro	97	2046	2047
19. Phe	147	2193	2196
20. Arg	156	2349	2353
21. Pro	97	2447	2447
22. Pro	97	2544	2546
23. Leu	113	2657	2659
24. Arg	156	2813	2815
25. Phe	147	2960	2961
26. Pro	97	3058	3060
-COOH	45	3103	3104

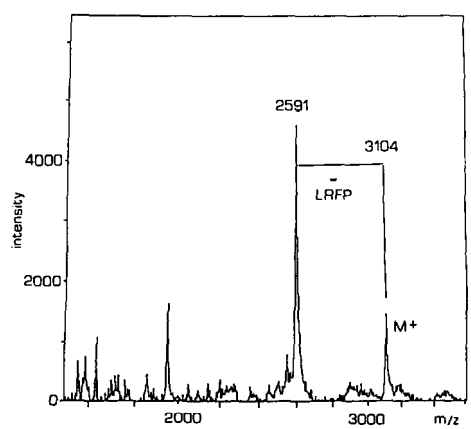

FIG. 4. **Plasma desorption mass spectrometry from *in situ* digestion of the NBS fragment of Bac5 with carboxypeptidases Y and P.**

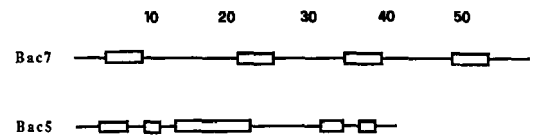

FIG. 5. **Predicted secondary structures of Bac7 and Bac5.** Predictions were carried out according to the algorithm of Garnier *et al.* (35) with decision constants taken as 0 (unbiased prediction). *Boxes*, turn/coil structures; *segments*, extended structures.

include regions comprised of several nearly identical tandem repeats of 21 amino acids (21).

The main feature of Bac5 is the presence of the repeated tetrapeptide Xaa-Pro-Pro-Yaa, where Xaa is always Arg, except at positions 16 (Tyr) and 28 (Phe), and Yaa is a large hydrophobic residue (Ile, Phe, or Leu).

Apart from their cationic character, the amino acid sequences of Bac7 and Bac5 do not resemble the reported sequences of antimicrobial polypeptides of human, rabbit, guinea pig, and rat neutrophils such as defensins and bactericidal/permeability increasing protein (22–26), nor the sequences of human eosinophil cationic protein (27) and major basic protein (28). The sequences of Bac7 and Bac5 are also heterologous with respect to other antimicrobial polypeptides such as magainins (29), purified from amphibia, or cecropins (30), attacins (31), and sarcotoxins (32), isolated from various insects. The only known antibacterial peptides rich in proline (33%) are a family of inducible peptides, known as apidecins, isolated from honeybees (33). These peptides are 18 residues in length, contain 6 prolyl and 3 arginyl residues, and, like Bac7 and Bac5, are active against Gram-negative bacteria.

The low number of component amino acids and the abundance of prolyl and arginyl residues make the structure of Bac7 and Bac5 definitely unusual. This was also confirmed after an extensive computer search for similar sequences. Both peptides show a 42–60% identity in 15–59 amino acid overlaps with a number of herpes, Epstein-Barr, or adenoviral proteins. However, this is mainly due to the abundance of prolyl residues in some regions of the latter proteins. Furthermore, Bac5 has a 62% (60%) identity in a 34 (30)-amino acid overlap with bovine (human) epidermal cytokeratin (type II), which contains a repeated Arg-Pro-Pro-Pro motif (34). By analogy, Bac7 also shows a 62% identity in a 42-amino acid overlap with the bovine cytokeratin. Finally, Bac7 exhibits a 47% identity in a 53-amino acid overlap with the mouse salivary proline-rich proteins, which contain in their repeated tetradecamer the PRPP sequence (20). To our knowledge these similarities do not appear functional and provide no clue for the elucidation of the potential mechanism of action of both bactenecins.

In contrast to the primary structure, the predicted (35)

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secondary structure of Bac5 and Bac7 shows a remarkable similarity. Both structures are built up from a series of turn/coil blocs interspersed among extended structures (Fig. 5). It can be conjectured that these peptides can adopt a grossly similar shape whereby identical or similar surface patterns may form. Beyond this general similarity there is a difference in the positioning of the strongly predicted turns. In Bac7, Arg is at position 3 of the turn (*X*-Pro-Arg-Pro), while in Bac5 Arg is at position 4 (Pro-Pro-*X*-Arg). It can be assumed that these two units belong to different, and probably non-standard, turn types.

Furthermore, initial model building experiments (not shown) reveal that both Bac5 and Bac7 can adopt conformations that allow asymmetrical clustering of polar and apolar amino acids. Similar amphiphilic surface patterns have been implicated in a wide variety of biologically active polypeptides whose function is related to lipid and cell membrane binding. Among the antimicrobial polypeptides, amphiphilic α-helices are formed by magainin 2 (36), melittin (37), and cecropin A (38) when exposed to hydrophobic environments such as phospholipid micelles or organic solvents. In this respect it is worth noting that both Bac7 and Bac5 permeabilize the outer membrane and the inner membrane of *E. coli,* as assessed by accessibility of normally impermeant substrates to periplasmic (β-lactamase) and cytoplasmic (β-galactosidase) enzymes.[1] Even though Bac7 and Bac5 are not expected to adopt an α-helical conformation because of the abundance of prolyl residues, the above considerations suggest that these polypeptides may adopt an amphiphilical structure on membrane surfaces.

Acknowledgments—We wish to thank Dr. S. Pongor for his valuable advice.

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J. Biol. Chem. 1990, 265:18871-18874.

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