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Antibacterial peptides from pig intestine: Isolation of a mammalian cecropin

(chromatography/peptide sequencing/solid-phase synthesis/small intestine/vasoactive intestinal peptide)

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ABSTRACT **Pig small intestine was used as starting material for a batchwise isolation of a peptide fraction enriched in antibacterial activities against *Escherichia coli* (anti-Ec factor) and against *Bacillus megaterium* (anti-Bm factor). Separation and further purification were by different types of chromatography. Sequence analysis showed the anti-Bm factor to be apparently similar to vasoactive intestinal peptide. The anti-Ec factor was found to have a 31-residue sequence that was cecropin-like. It was named cecropin P1 and its structure was confirmed by solid-phase synthesis. Synthetic cecropin P1 with and without C-terminal amide was assayed on eight different bacteria. Mobility comparison between synthetic and natural cecropin P1 indicates that the natural peptide has a free C-terminal carboxyl group.**

The small intestine is an important endocrine organ, and a number of physiologically active peptides have been isolated from this tissue (1). During normal healthy conditions, the upper part of the small intestine contains few bacteria. Below the duodenum the concentration of bacteria progressively increases until the maximum, 10^{11} bacteria per g of bowel content, is reached in the large intestine. It is remarkable that such a mass of bacteria can coexist with a delicate host organ. Thus, one might predict that the intestine can produce antibacterial factors that regulate the bacterial concentration in the upper part of the intestine and protect the mucosa of the large intestine. Small basic peptides called cecropins play an important role in insect immunity (2) and structurally unrelated peptides called magainins (3) protect the amphibian skin from infections. Another group of antibacterial peptides, the defensins, was first isolated from mammalian granulocytes (4) and neutrophils (5) and recently also from insects (6, 7). In addition, bovine neutrophils have been found to contain bactenecins (8), another group of small basic peptides. Defensins and bactenecins contain one or more disulfide bridges, whereas cecropins and magainins are cysteine-free. We report here that the pig small intestine contains several potent antibacterial factors. The first two now purified and structurally analyzed were found to lack cysteine. One was apparently identical to vasoactive intestinal peptide (VIP); the other was cecropin-like. Part of this work was presented orally at the Federation of European Biochemical Societies meeting in Rome on July 4, 1989.

MATERIALS AND METHODS

Antibacterial Assays. Activity was based on an inhibition zone assay on thin agarose plates seeded with *Escherichia coli* D21 or *Bacillus megaterium* Bm11 (9). A standard curve was made from known amounts of synthetic cecropin A, and 1000 units was defined as an activity equal to that of 1 μg of cecropin A (10). A similar standard curve was made also for activity against *B. megaterium* Bm11 (anti-Bm factor) and the unit was defined in the same way as for anti-*E. coli* activity (anti-Ec factor). From the respective fractions in chromatograms, 200 μl was freeze-dried and redissolved in 10 μl of water, of which 3 μl was used for each assay.

Batchwise Purification of Antibacterial Factors. The starting material was a crude *c*oncentrate of *t*hermostable *i*ntestinal *p*olypeptides (CTIP) from the uppermost meter of the porcine small intestine, prepared essentially as described (11, 12). The amount of CTIP obtained (wet weight) was about 0.1% of the boiled intestinal tissue used. CTIP was dissolved at room temperature to 10% (wt/vol) in water. Two volumes of 95% ethanol were added and pH was brought to 7.5 with 0.3 M NaOH in 66% ethanol. A precipitate was formed and discarded. To the clear filtrate was added 1 volume of 95% ethanol precooled to −20°C. The suspension was kept at this temperature for 24 hr and the precipitate formed was removed by filtering. After removal of the ethanol, the peptides were precipitated at pH 3.5 by saturation with NaCl. The precipitate was dissolved in 0.2 M acetic acid and chromatographed on Sephadex G-25 fine equilibrated with this solvent. The elution pattern of the peptidal material resembled that shown in figure 2.1 of ref. 12. The eluate corresponding to fraction II of that figure was saturated with NaCl and the precipitate was collected (12). It was dissolved in water, adjusted to pH 4.0, and the peptides were reprecipitated with NaCl to saturation. The precipitate was extracted with methanol (50 ml/g) and the insoluble material was recovered by suction filtration and washed with ether. The material obtained after drying was about 0.6% of the CTIP taken for the preparation.

RESULTS

The methanol-insoluble material was chromatographed on CM-Sepharose and S Sepharose fast flow, using volatile buffers (Fig. 1 *a* and *b*). These two steps together gave about a 200-fold purification of the antibacterial activity against *E. coli*, strain D21, and an overall recovery of about 20%. When an assay was performed also with *B. megaterium*, chromatography on S Sepharose separated two factors with different antibacterial activities (Fig. 1*b*). The peak of the anti-Bm factor was freeze-dried and purified to homogeneity by three consecutive runs on FPLC with a reversed-phase column (Fig. 1*c*). The specific activity of this material was about 2700 units/μg and the activity was trypsin sensitive. Repeated sequencing of the pure peptide showed it to be identical to VIP (13). The calculated molecular weight of VIP is 3326 and mass spectroscopy using a time-of-flight instrument gave for the anti-Bm factor a value of 3328. However, VIP purified by

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Abbreviation: VIP, vasoactive intestinal peptide.

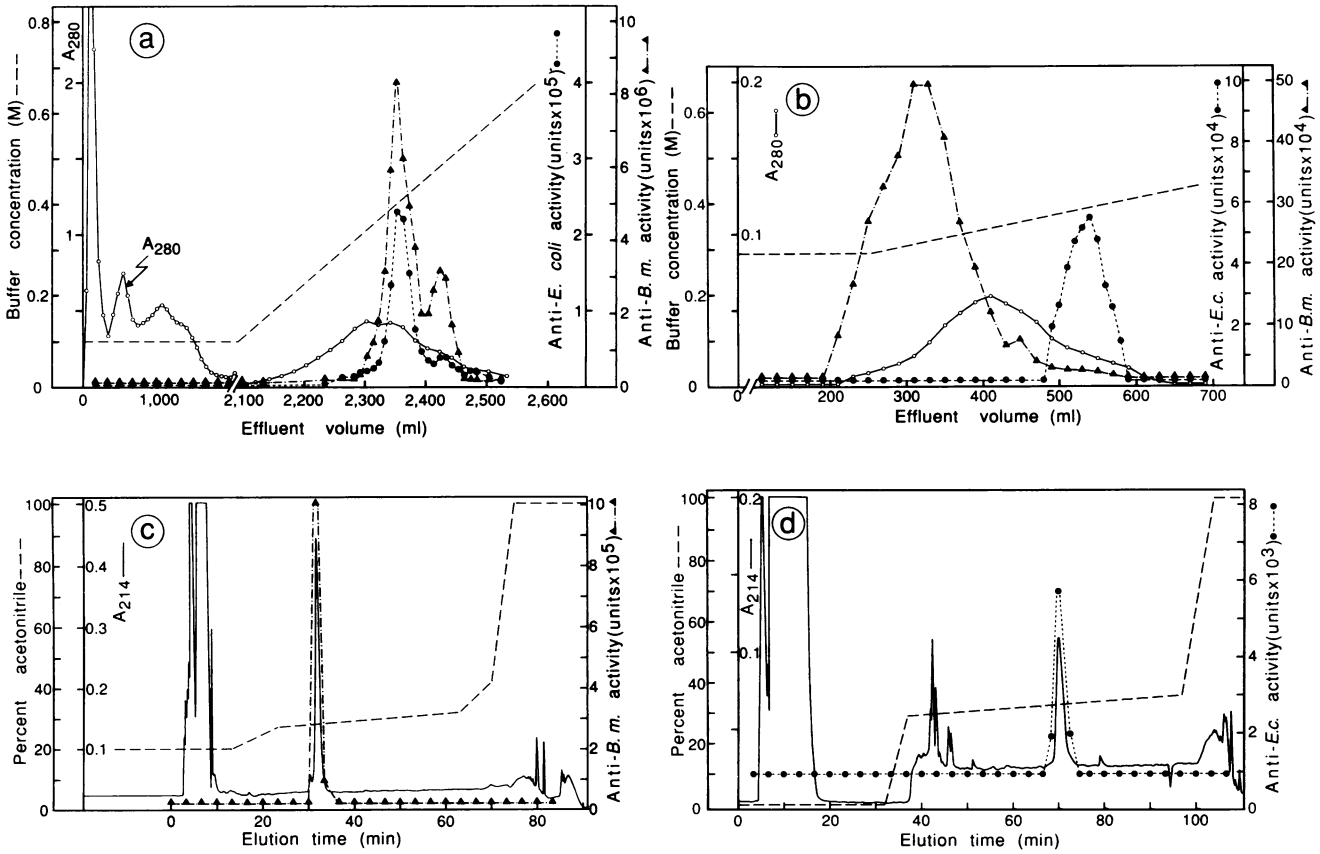

FIG. 1. Purification of anti-Bm factor and cecropin P1. The starting material was the dry methanol-insoluble peptide fraction isolated from pig intestine. One and one-half grams was dissolved in 80 ml of 0.1 M ammonium formate (pH 6.4) and treated batchwise with 30 ml of DEAE-Sepharose. The solution was applied to a 56-ml column with CM-Sepharose in equilibrium with 0.1 M ammonium formate (pH 6.4), and elution was first with 2100 ml of this solvent (*a*). It was followed by a gradient of 0.1–0.85 M ammonium acetate (pH 5.2) (300 plus 300 ml). From the fractions indicated, 200 μl was freeze-dried and redissolved in 10 μl of water, of which 3 μl was used for assay. The antibacterial activity was eluted at 0.38 M (*a*). The pooled peak fractions were diluted 1.3 times and applied to a 26-ml column with S Sepharose fast flow, equilibrated with 0.30 M ammonium acetate (pH 5.2). Elution was first with 230 ml of this solvent (*b*); this was followed by a gradient of 0.30–0.50 M ammonium acetate (300 plus 300 ml). The anti-Bm factor was eluted as a broad peak around 0.32 M and activity against *E. coli* was eluted at 0.40 M (*b*). The respective peak fractions were freeze-dried, dissolved in 0.1% trifluoroacetic acid (TFA), and further purified by FPLC with a PepRPC HR5/5 column (porous silica C_2/C_{18}) in equilibrium with 0.1% TFA. Solvent B was CH_3CN with 0.1% TFA. The anti-Bm factor was eluted with the following gradients: 0–14 min, 20% B; 14–24 min, 20–27% B; 24–64 min, 27–32% B. Peak activity was eluted by 28% B (*c*). The anti-Ec activity (cecropin P1) was purified by the following gradients: 0–5 min, 0–29% B; 5–65 min, 29–48% B. Peak activity was eluted at 33% B. This material was diluted three times and rerun on the FPLC (*d*). Elution was first with 0.1% TFA, until the base line was stable; elution was then with the following gradients: 0–5 min, 0–29% B; 5–65 min, 29–38% B. The active peak, eluted by 33% B, was freeze-dried and sequenced. All separation equipment was from Pharmacia LKB Biotechnology.

the established method showed <1% of the antibacterial activity of the anti-Bm factor. This would seem to suggest that the anti-Bm activity is due to an undetected minor component in VIP. If it is the case, then the specific activity of this material must be extraordinarily high.

Further purification of the anti-Ec factor was also by FPLC with a reversed-phase column. The final step was rechromatography on the same column (Fig. 1*d*). The last two steps were performed with about 1 mg and 5 μg of peptides, respectively. Recovery at this stage was often low, and we suspect losses due to irreversible adsorption. However, the specific activity after the last step was of the same order as for cecropin A from the cecropia moth (10).

Amino acid analysis showed that the peptide was rich in lysine, serine, alanine, and isoleucine and low in or devoid of cysteine, histidine, tyrosine, phenylalanine, and methionine. Direct gas-phase sequence analysis with an Applied Biosystems instrument yielded the sequence of a 31-residue peptide. The calculated molecular weight assuming a free C-terminal carboxyl group was 3339 and mass spectroscopy gave a value of 3338. Sequence comparison with cecropin IA from the flesh fly *Sarcophaga peregrina* (14, 15) showed that 6 residues of the first 11 were identical, and a computer comparison, allowing three gaps, aligned the two peptides as shown in Fig. 2. Identical residues were found in 12 of 35 positions (4 residues marked as deleted). A similar comparison with cecropin B from the cecropia moth (16), allowing three gaps, gave 12 identical residues in 36 positions (8 residues marked as deleted). The GAP and the BESTFIT programs of the University of Wisconsin Genetics Computer Group gave the degree of similarity between the newly isolated peptide and the two cecropins ranging from 64% to 75%. Because of this high degree of resemblance with two insect cecropins, the new peptide was named cecropin P1 (P for porcine).

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P1: SWLSKTAKKLENSAK-KR---ISEGIAIAIQGGPR
    || |  || |       |   |  |  || |
IA: GWLKKIGKKIERVGQHTRDATI-QGLGIAQQAANVAATAR
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FIG. 2. Aligning of the sequences for porcine cecropin P1 and fly cecropin IA using the program BESTFIT of the University of Wisconsin Genetics Computer Group. Identical residues are indicated by a vertical bar. The single-letter amino acid code is used. Cecropin IA was found in a flesh fly, *S. peregrina* (14, 15), and in *Drosophila melanogaster* (D. Hultmark, personal communication).

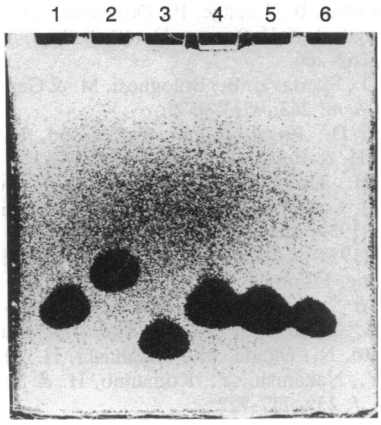

FIG. 3. Polyacrylamide gel electrophoresis of native cecropins at pH 4.3. Spots with antibacterial activity are visualized by a bacterial overlay as described (18). Lane 1, cecropin IA-NH_2; lane 2, cecropin IA-OH; lane 3, synthetic cecropin P1-NH_2; lane 4, natural cecropin P1; lane 5, synthetic cecropin P1-OH; lane 6, cecropin A from *Hyalophora*.

The sequence given in Fig. 2 was confirmed by degradation of cecropin P1 with staphylococcal Glu-specific endoprotease (Boehringer Mannheim), separation of the peptides by HPLC and sequence analysis, as well as mass spectroscopy (17). The structure was confirmed also by solid-phase synthesis of two peptides with the sequence given in Fig. 2, one having a free C-terminal carboxyl group, with the other being amidated. When the electrophoretic mobility at pH 4.3 of these peptides was compared to the natural cecropin P1, the latter was found to move as the synthetic peptide with a free C-terminal carboxyl group (Fig. 3). As references we included cecropin A from cecropia and cecropin IA from *Sarcophaga,* the latter with and without C-terminal amide (19). Thus, the natural peptide has most likely a free carboxyl group at the C terminus. This is contrary to what has been found previously for all insect cecropins (2), but it is in agreement with the magainins (3).

The two synthetic forms of cecropin P1 showed high activity against *E. coli* K-12 and against four clinical isolates of the same organism (Table 1). Also strains of *Salmonella* and *Acinetobacter* were highly sensitive to cecropin P1. Moderate or no activity was found against some Gram-positive bacteria, although *B. megaterium* was fairly sensitive. As earlier (19), the amidated form was somewhat more active against the Gram-positive bacteria tried. However, against *B. megaterium* the anti-Bm factor was 8–10 times more active than cecropin P1-NH_2.

Table 1. Antibacterial activities given as lethal concentrations for two synthetic forms of cecropin P1

Organism and strain	P1-NH_2, μM	P1-OH, μM
E. coli		
K-12, D21	0.3	0.4
853/67, O149, K88	0.9	0.6
Bd2221/75, O8, K88	0.6	0.7
Bd4462/84, O101, K99	0.7	0.8
Bd4466/84, O64, K99	0.3	0.6
Salmonella typhimurium, LT2	0.9	1.7
Acinetobacter calcoaceticus, Ac11	0.2	0.5
Proteus vulgaris, Pv11	5.7	12
Pseudomonas aeruginosa, OT97	5.9	13
B. megaterium, Bm11	1.8	5.3
Streptococcus pyogenes	19	44
Staphylococcus aureus, Cowan I	>490	>520

The four last strains of *E. coli* were obtained from Olof Söderlind (SVA, Uppsala) and they are clinical isolates that are pathogenic to piglets. Söderlind's typing of O and K antigens is indicated after the strain numbers. Lethal concentration is the lowest concentration of cecropin that inhibits growth of the respective bacteria in thin agarose plates (9).

DISCUSSION

Fig. 4 shows the sequences for 10 different cecropins with the gaps introduced in Fig. 2 and an additional one after the conserved Trp-2 residue in all Lepidopteran cecropins. That this gap is justified was earlier shown by synthesis of analogs with and without the gap (19). The top three sequences in Fig. 4 are all of the D type, which differs somewhat from the others. Disregarding these three peptides, it can be seen that Lys-5 and the double Lys at positions 8 and 9 are conserved. Another highly conserved basic residue is Arg-18. The intron in the gene for cecropin B (21) is located between Ile-10 and Glu-11, and this site is conserved in six of the peptides, whereas it is Leu-Glu in the remaining four sequences. Since the codons for Ile and Leu can differ only in the first base, the splice site could in fact be conserved in all cecropins. Another important feature of the cecropin structure is the hinge region around Gly-25 and Pro-26. Here again, Fig. 4 shows that Gly-25 is conserved in 9 of 10 structures. Thus, despite the fact that the homology between the mammalian cecropin P1 and the insect cecropins is only around 33%, most of the features typical for the cecropins are indeed conserved.

Insects are known to lack lymphocytes and immunoglobulins, and the presence in insects of antibacterial factors like the cecropins, the attacins, and lysozyme was thought to be an alternative immune mechanism (2). However, the fact that defensins are found in mammals (4, 5) and in insects (6, 7) was

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                    1        10          20          30
Manduca     B-2      W--NPFKEL ERAGQRVRDA VTSAAPAVAT VGQAAAIAR
Hyalophora  D        W--NPFKEL EKVGQRVRDA VISAGPAVAT VAQATALAK
Antheraea   D        W--NPFKEL ERAGQRVRDA IISAGPAVAT VAQATALAK
Hyalophora  A       KW--KLFKKI EKVGQNIRDG IIKAGPAVAV VGQATQIAK
Bombyx      CM_IV   RW--KIFKKI EKVGQNIRDG IVKAGPAVAV VGQAATI
Bombyx      A       RW--KIFKKI EKMGRNIRDG IVKAGPAIEV LGSAKAI
Antheraea   B       KW--KIFKKI EKVGRNIRNG IIKAGPAVAV LGEAKAL
Hyalophora  B       KW--KVFKKI EKMGRNIRNG IVKAGPAIAV LGEAKAL
Sarcophaga  IA      GWLKKIGKKI ERVGQHTRDA TI-QGLGIAQ QAANVAATAR
Porcine     P1      SWLSKTAKKL ENSAK-KR-- -ISEGIAIAI QGGPR
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FIG. 4. Comparison of 10 known cecropin sequences. The *Manduca* sequence was from ref. 20, *Sarcophaga* was from ref. 14, porcine is given in the present paper, and back references to the others are found in a recent review (2). Bold letters indicate the conserved residues identical to the porcine cecropin P1 residues. The single-letter amino acid code is used.

a first hint that antibacterial peptides may be a universal means for defense against bacterial infections. The present finding that cecropin P1 is produced in a mammal indicates that also the cecropins are likely to be widely distributed in the animal kingdom. In addition, known endocrine peptides may be found to be antibacterial in their natural or modified forms. It seems expedient if the protection of the intestine is based on small peptides, easily excreted and with a broad specificity. It is worth stressing that an antibacterial defense based on only RNA and protein synthesis is faster and easier to modulate than mechanisms depending also on cell proliferation.

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1. Mutt, V. (1986) *Chem. Scr.* **26B,** 191–207.
2. Boman, H. G. & Hultmark, D. (1987) *Annu. Rev. Microbiol.* **41,** 103–126.
3. Zasloff, M. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 5449–5453.
4. Selsted, M. E., Szklarek, D. & Lehrer, R. I. (1984) *Infect. Immun.* **45,** 150–154.
5. Selsted, M. E., Brown, D. M., DeLange, R. J., Harwig, S. S. L. & Lehrer, R. I. (1985) *J. Biol. Chem.* **260,** 4579–4584.
6. Matsuyama, K. & Natori, S. (1988) *J. Biol. Chem.* **263,** 17112–17116.
7. Lambert, J., Keppi, E., Dimarcq, J.-L., Wicker, C., Reichhart, J.-M., Dunbar, B., Lepage, P., Dorsselaer, A. V., Hoffmann, J., Fothergill, J. & Hoffmann, D. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 262–266.
8. Romeo, D., Skerlavaj, B., Bolognesi, M. & Gennaro, R. (1988) *J. Biol. Chem.* **263,** 9573–9575.
9. Hultmark, D., Engström, Å., Andersson, K., Steiner, H., Bennich, H. & Boman, H. G. (1983) *EMBO J.* **2,** 571–576.
10. Steiner, H., Hultmark, D., Engström, Å, Bennich, H. & Boman, H. G. (1981) *Nature (London)* **292,** 246–248.
11. Mutt, V. (1959) *Ark. Kemi* **15,** 69–74.
12. Mutt, V. (1978) in *Gut Hormones*, ed. Bloom, S.R. (Churchill Livingstone, Edinburg, U.K.) pp. 21–27.
13. Mutt, V. & Said, S. I. (1974) *Eur. J. Biochem.* **42,** 581–589.
14. Okada, M. & Natori, S. (1985) *J. Biol. Chem.* **260,** 7174–7177.
15. Matsumoto, N., Okada, M., Takahashi, H., Qu, X.-M., Nakajima, Y., Nakanishi, Y., Komanno, H. & Natori, S. (1986) *Biochem. J.* **239,** 717–722.
16. von Hofsten, P., Faye, I., Kockum, K., Lee, J.-Y., Xanthopoulos, K. G., Boman, I. A., Boman, H. G., Engström, Å, Andreau, D. & Merrifield, R. B. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 2240–2243.
17. Jeffery, J., Cederlund, E. & Jörnvall, H. (1984) *Eur. J. Biochem.* **140,** 7–16.
18. Hoffmann, D., Hultmark, D. & Boman, H. G. (1981) *Insect Biochem.* **11,** 537–548.
19. Li, Z.-q., Merrifield, R. B., Boman, I. A. & Boman, H.G. (1988) *FEBS Lett.* **231,** 299–302.
20. Dickinson, L., Russell, V. & Dunn, P. E. (1988) *J. Biol. Chem.* **263,** 19424–19429.
21. Xanthopoulos, K. G., Lee, J.-Y., Gan, R., Kockum, K., Faye, I. & Boman, H. G. (1988) *Eur. J. Biochem.* **172,** 371–376.