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Research Article

Spleen antibacterial peptides: high levels of PR-39 and presence of two forms of NK-lysin

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Abstract. Antibacterial peptides were isolated from porcine spleen by acetic acid extraction, ion exchange chromatography and reverse-phase high-performance liquid chromatography. C-terminal ladder sequence analysis of a bioactive peptide with matrix-assisted laser desorption/ionization mass spectrometry after digestion with carboxypeptidases P and Y showed that it is identical to the antibacterial proline/arginine-rich intestinal peptide PR-39. It is present at high levels in granulocytes of the spleen, and peptides with C-terminal proline amide and internal adjacent Pro residues can be analyzed with this method. In addition, two forms of NK-lysin (NKL) were found. One, NKLi, is identical to that isolated from pig intestine, and the other, NKLbw, to a mature peptide deduced from a clone from a porcine bone marrow cDNA library.

Key words. Antibacterial peptide; PR-39; NK-lysin; MALDI mass spectrometry; carboxypeptidase Y; carboxypeptidase P; C-terminal ladder sequence analysis.

Introduction

Mammals have a battery of antibiotic peptides that protect them from bacterial invasion [1–3]. On a structural basis, antibacterial peptides can be divided into several groups [4]: linear peptides without Cys residues; peptides with an even number of intra-SS-linked Cys residues, and linear peptides with a high proportion of individual residue types, often Pro or Arg [5, 6]. Many antibiotic peptides are produced by circulating phagocytic or cytolytic cells, including the Pro/Arg-rich peptide PR-39 found in intestine and neutrophils [5, 6]. Also involved is a peptide with three intrachain disulfide bonds, NK-lysin (NKL), first isolated from intestinal extracts, but later found to be a component of the cytolytic machinery of T and natural killer (NK) cells [7]. Spleen, as much as intestine, is a secondary lymphoid organ and as such may contain a repertoire of antibacterial peptides from circulating cells. The present study was undertaken to isolate and characterize antibacterial peptides from spleen, using C-terminal ladder sequence analysis, which is particularly suited for peptide analysis [8]. In this analysis, carboxypeptidase Y and P (CPY and CPP) complement each other in releasing all amino acids, including Pro and C-terminally amidated residues at a similar rate [9–12].

We now show that porcine spleen is a rich source of antibacterial peptides and that C-terminal ladder sequence analysis using carboxypeptidases and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) is also well suited for characterization of Pro/Arg-rich peptides.

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Materials and methods

Materials. Carboxymethyl-cellulose CM 23 was from Whatman and DEAE-Sephadex from Pharmacia, while CPY and CPP, sequencing grade, were from Boehringer Mannheim.

Purification of peptides. Spleens were washed and cleaned in saline, cut into small pieces and frozen. One kilogram of spleens from 20 pigs was boiled for 10 min, cooled with ice and extracted in 10 vol of 3% acetic acid overnight. The extract was filtered and peptides were captured with alginic acid from the filtrate according to published procedures [7]. One-third of the material was applied to DEAE-Sephadex, eluted with 0.2 M acetic acid, and lyophilized. The dry material was dissolved in 22.5 mM sodium phosphate, pH 6.4, containing 0.5% thiodiglycol, and adsorbed to CM-cellulose. After washing with the buffer and with 0.2 M NaCl in the same buffer, a peptide fraction was eluted with 0.2 M HCl. It was submitted to reverse phase (RP)-high-performance liquid chromatography (HPLC) on Vydac C18 (22×250 mm; The Separation Group, Hesperia, CA) with a mobile phase of 0.1% trifluoroacetic acid (TFA)/water (eluent A) and 0.1% TFA/acetonitrile (eluent B). Peptides dissolved in eluent A were rechromatographed with a gradient of 10–20% B in 5 min and then 20–60% B in 40 min. Fractions 33–37 from the previous step were pooled and partially evaporated under a stream of nitrogen. The material was then chromatographed on a Resource RPC 1-ml column (Amersham Pharmacia Biotech) with the same mobile-phase system and the peptides were eluted with a gradient of 34–48% B in 20 min at 1 ml/min. Purity of isolated peptides was determined by capillary zone electrophoresis with a Beckman P/ACE 2000 [13].

Antibacterial assays. Activity was recorded by an inhibition zone assay on thin agarose plates seeded with *Escherichia coli* strain D21 or *Salmonella typhimurium* LT-2 [14]. A value of 1000 units was defined as an activity against *E. coli* D21 equal to that of 1 µg of cecropin A. The chromatographic fractions were lyophilized and samples of each fraction were redissolved at 5–50 µg/µl in 10–50 µl water, of which 3 µl was used for each determination.

NKL enzyme-linked immunosorbent assay. Peptide fractions and standard porcine NK-lysin were diluted or dissolved in 50 mM carbonate buffer, pH 9.6, and 50 µl duplicate samples were incubated at 4 °C overnight in microtiter plates (NUNC, maxisorp C96). The plates were blocked for 1 h at 37 °C with phosphate buffer, pH 7.5, 0.5 M NaCl and 1% bovine serum albumin (BSA) (sample buffer). As a primary antibody, 50 µl rabbit IgG-purified polyclonal NKL antibody [7] diluted 1:10,000 in sample buffer was added for 1 h at 37 °C and then detected by incubation with 50 µl premixed biotinylated goat anti-rabbit IgG (Sigma, B-7389, 1:20,000) and avidin-HRP (Dako, P347, 1:10,000) in sample buffer for 1 h at 37 °C. NKL immunoreactivity was detected by addition of 1,2 phenyldiamine

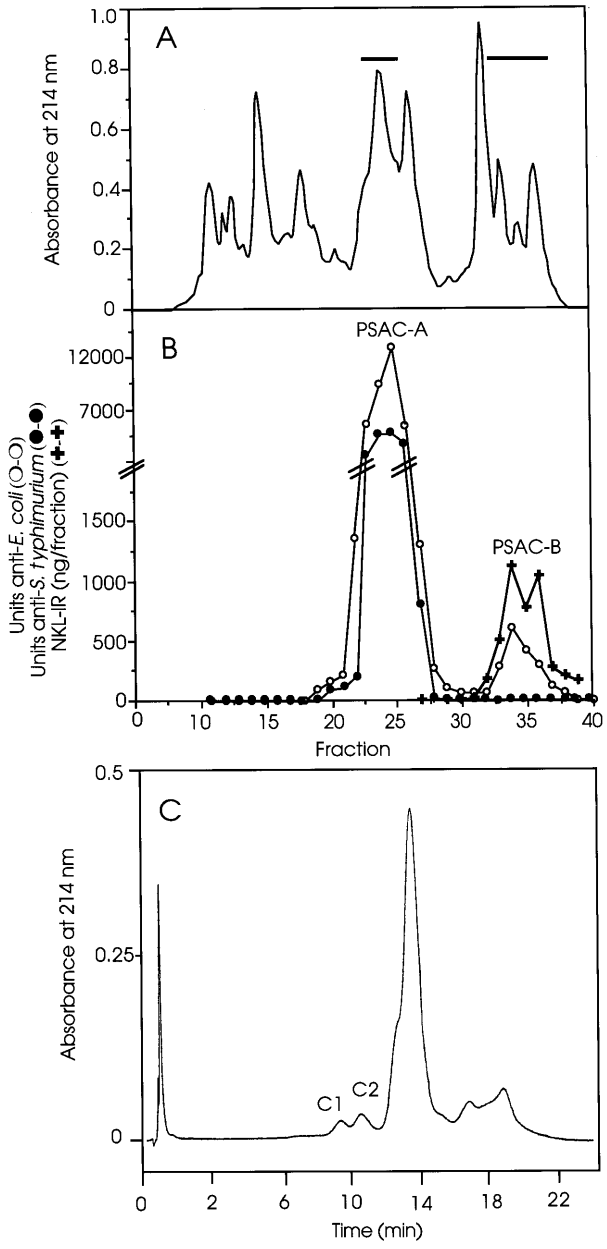

Figure 1. Purification of the spleen extract. (*A*) The material was loaded on Vydac C18 and eluted with a gradient of acetonitrile/0.1% TFA; the horizontal bars indicate the antibacterially bioactive fractions pooled for further analysis. (*B*) Antibacterial activity assay against *E. coli* (○-○) and *S. typhimurium* (●-●) was performed for each fraction. The NKL immunoreactivity (+-+) was determined by ELISA using polyclonal antibodies raised against purified NKL. (*C*) RP-HPLC of PSAC-B: pooled fractions 33–37 from (*A*) were analyzed on a column Resource RPC using a gradient of acetonitrile/0.1% TFA. Fractions C1 and C2 were then analyzed by MALDI-MS.

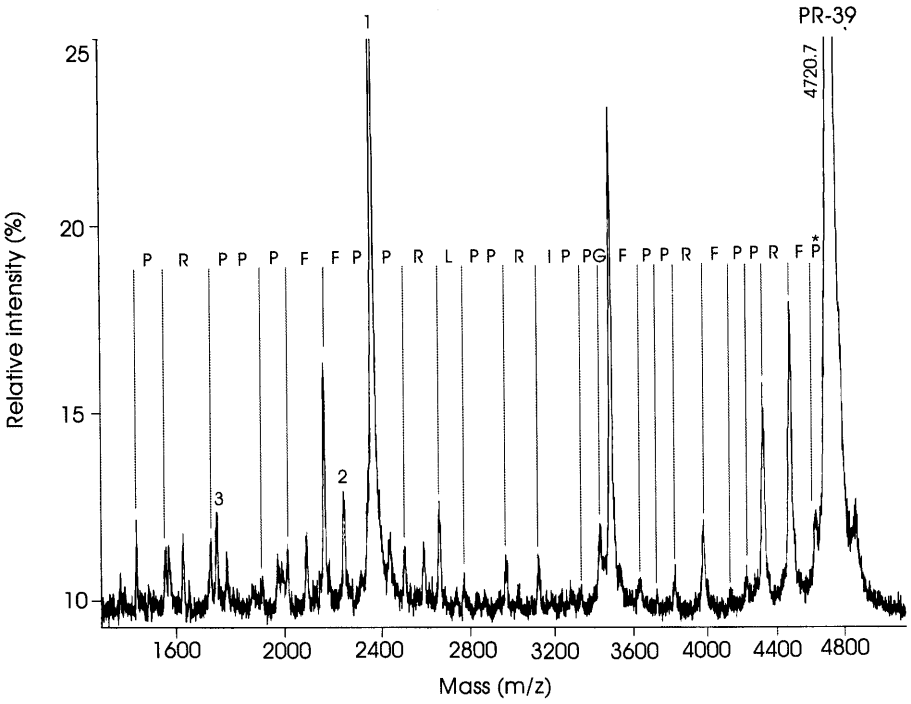

Figure 2. C-terminal ladder sequence analysis of PR-39 from porcine spleen. After 5 min incubation with CPY/CPP, the peptide digest was analyzed by MALDI-MS. P* indicates an amidated proline at the C-terminal position. 1, 2, and 3 are doubly-charged fragment ions.

(Dako, S2000) in citrate/phosphate/H_2O_2 and absorbance was recorded at 490 nm. After each incubation step, the plates were washed three times with phosphate/NaCl/BSA buffer containing 0.1% Tween 20.

Structural analysis. For C-terminal ladder sequence analysis, peptides were digested with a mixture of CPY and CPP and analyzed on a Finnigan Lasermat 2000 [8]. N-terminal sequence analysis was performed with an Applied Biosystems 477A instrument.

Results

Purification of antibacterial fractions from pig spleen. Since most antibacterial peptides are known to have a positive net charge, we used a method to concentrate and separate basic peptides/proteins. Peptides were extracted and purified from porcine spleen as described in Materials and methods. The material was subjected to RP-HPLC using a 0.1% TFA/water/acetonitrile solvent system (fig. 1A). Each fraction was tested against *E. coli* and *S. typhimurium* (fig. 1B). Two antibacterial regions were found: pig spleen antibacterial components (PSAC)-A, eluting with 39% acetonitrile, and PSAC-B eluting with 47–52% acetonitrile. PSAC-A had a strong anti-*E. coli* and anti-*S. typhimurium* activity, while PSAC-B had only a mild anti-*E. coli* activity that spread over a number of fractions.

The yield of PSAC-A was high, approximately 4–5 mg recovered, 0.6–0.75 mg per spleen. PSAC-A was estimated to have 700 and 200 U/μg of anti-*E. coli* and anti-*S. typhimurium* activity, respectively. Purity of the sample was determined by capillary zone electrophoresis and the electropherogram revealed one major component.

PSAC-B, in addition to the anti-*E. coli* activity, showed high NKL immunoreactivity (fig. 1B). Fractions 33–37 were pooled and rerun on RP-HPLC using the same solvent system, but with a shallower gradient (fig. 1C). Two minor components (C1 and C2), both with anti-*E. coli* activity and NKL immunoreactivity, were efficiently separated from the bulk of UV-absorbing material (fig. 1C).

Structural analysis. PSAC-A was analyzed by MALDI-MS. The major component had a mass of 4720.3 ± 1.0 Da. It was then submitted to C-terminal ladder sequence analysis by digestion with a mixture of CPY and CPP [8]. The MALDI-MS spectrum (fig. 2) of the digest after 5 min incubation showed a C-terminal ladder sequence of 28 amino acids identical (table 1) to the amino acid sequence of PR-39 [5]. Identity to PR-39 is also confirmed by the mass value of 4720.3 ± 1.0 Da, which is in good agreement with the expected mass of 4719.7 Da. Furthermore, the activity correlates with that of PR-39 isolated from pig intestine [5].

Table 1. Sequence analysis of PR-39 from pig spleen. (The peptide was also submitted to N-terminal sequence analysis for 20 aa: RXRPRPPYLPXXRPPPFFXP.)

Residue from C-terminus		Observed mass	Calculated mass
0	$[M+H]^+$	4720.7[a]	4720.7
−1	Pro-NH_2	4624.9	4624.6
−2	Phe	4478.2	4477.4
−3	Arg	4320.7	4321.2
−4	Pro	4223.1	4224.1
−5	Pro	4129.1	4127.0
−6	Phe	3979.8	3979.8
−7	Arg	3823.8	3823.6
−8	Pro	3727.6	3726.5
−9	Pro	3633.8	3629.4
−10	Phe	3482.5	3482.2
−11	Gly	3427.8	3425.1
−12	Pro	3330.1	3327.6
−13	Pro	ND	3230.9
−14	Ile	3118.3	3117.7
−15	Arg	2961.9	2961.5
−16	Pro	ND	2864.4
−17	Pro	2766.0	2767.3
−18	Leu	2654.5	2654.1
−19	Arg	2498.2	2497.9
−20	Pro	ND	2400.8
−21	Pro	ND	2303.7
−22	Phe	2157.8	2156.5
−23	Phe	2010.4	2009.3
−24	Pro	1910.9	1912.1
−25	Pro	ND	1815.1
−26	Pro	1719.5	1718.0
−27	Arg	1562.6	1561.8
−28	Pro	1465.8	1464.7

ND, not detected. [a] Mass value used for internal calibration. ND, not detected.

The NKL-immunoreactive fractions, C1 and C2, were analyzed by MALDI-MS. Fraction C1 contained a component with a mass of 8923.0 ± 1.0 Da, in good agreement with the mass (8924.7 Da) of the NKL previously isolated from pig intestine (NKLi). Fraction C2 contains another component with a mass of 8827.1 ± 1 Da. Its primary structure was analyzed by Edman degradation for 19 cycles and found to be the same as that deduced for NKL derived from a bone marrow cDNA library (NKLbw) (table 2) but not previously obtained in peptide form. The mass value indicated that the mRNA product [7] had been processed to a 78-residue peptide in the same manner as NKLi (calculated mass value is 8828.7 Da, in good agreement with the experimental mass value of 8827.1 ± 1). Hence, C1 corresponds to NKLi and C2 to NKLbw, meaning that the fractions with minor antibacterial activity had also been identified.

Discussion

Several antibiotic peptides have been identified in mammals: α-defensin, β-defensin, cecropin P1, PR-39, protegrins, prophenin, and NKL [5, 7, 15–17]. All of these peptides have positive net charges and most are active against *E. coli*. Cecropin P1, PR-39, and protegrin-1 are also active against *S. typhimurium* [5, 15, 18]. Spleen contains heat-stable and acid-extractable antibacterial compounds. We have analyzed a fraction of basic peptides and found that PR-39, previously isolated from intestine [5] and granulocytes [6], can also be purified from spleen where it is the major cationic anti-*S. typhimurium* component. The amount of PR-39 in spleen, approximately 13.5 mg/kg wet tissue, clearly exceeds that found in the intestine. It is possible that PR-39 is the most abundant antibiotic peptide in porcine granulocytes, and these cells distribute the peptide to the spleen. PR-39 synthesis (mRNA transcript) occurs in young pig spleen up to 14 days of age, but in adult mammals only in the bone marrow and not in the circulating granulocytes [19]. PR-39 was initially described as an antibiotic molecule, but was later shown to be a multifunctional peptide, with chemoattractant and other cell stimulatory properties [6, 20]. Thus, the high levels now found indicate that it may have a function other than the antibacterial activity.

A variant form of PR-39 has been described in extracts from mammalian neutrophils [21], but the C-terminal sequence was not unambiguously identified, and raising the question whether there are different forms of PR-39. Pro-rich peptides, especially with repetitive proline residues, are sometimes difficult to analyze by Edman degradation, but using C-terminal ladder sequence analysis we have now identified a 28-residue region from the C terminus, showing that PR-39 in spleen is identical to PR-39 isolated from the intestine [5].

In addition, we have shown that the two closely related forms of NKL [5], one isolated from the intestine and the other only cloned from a bone marrow cDNA library, are both present in spleen and most likely originate from T/NK cells. NKL belongs to a family of saposin-like proteins [22]. Vertebrate peptides in this family are usually encoded by one gene. However, the protozoan parasite *Entamoeba histolytica* [23] and the model organism *Caenorhabditis elegans* [24] have saposin-like peptides encoded by separate genes. It seems reasonable that pig, as now shown, and therefore mammals in general, have

Table 2. N-terminal sequence analysis of NKL.

PSAC-C2	G	L/I	C	E	S	C	R	K	I	I	Q	K	L	E	D	M	V	G	→
NKLbw	G	L/I	C	E	S	C	R	K	I	I	Q	K	L	E	D	M	V	G	→
NKLi	G	Y/F	C	E	S	C	R	K	I	I	Q	K	L	E	D	M	V	G	→

Alignment between the N-terminal sequence of fraction C2 (obtained by 19 cycles of Edman degradation) and corresponding parts of NKLbw and NKLi. As shown, the peptide in fraction C2 is identical to NKLbw, previously not detected as a peptide, but different from NKLi in this segment at two positions.

at least two forms of NKL, possibly as allelic variants. The amount of peptide recovered was not sufficient for studies of membrane-lytic activity but the results show that the new form, NKLbw, is as potent as NKLi against *E. coli*.

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